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AMELIORATION OF HEPATIC DAMAGE AND GENETIC TOXICITY IN CCL4-INDUCED RATS BY *JATROPHA CURCAS* EXTRACT

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ABSTRACT

The current study is conducted to evaluate the enzymatic and non-enzymatic antioxidant activity of *Jatropha curcas* leaves methanolic extract. Additionally, evaluation the changes in gene expression and DNA damage in female rat liver after intraperitoneally injection of carbon tetrachloride (CCl_4) as well as demonstration the possible therapeutic effects of *J. curcas* extract in comparison with the reference hepatoprotective drug; silymarin were studied. The present results declare that, injection of rats with CCl_4 twice a week for six consecutive weeks induced hepatic toxicity. However, treatment of intoxicated rats with 250 mg/kg body weight daily for one month with methanolic extract of *J. curcas* showed enhancement in the glutathione (GSH), catalase (CAT), glutathione peroxidase (GPx) and malondialdehyde (MDA) levels with percentages of improvement reached to 28.34, 12.78, 47.88 and 51.65%, respectively. In addition, the results indicated that treatment of female rats with CCl_4 significantly increased the DNA damage in liver cells and significantly induced alterations of the genes expression encoding antioxidant enzymes; superoxide dismutase (SOD), CAT and GPx as well as HSP70 proteins in liver tissues as compared to control rats. While, treatment with *J. curcas* extract as compared to silymarin decreased significantly the rate of the DNA damage and suppressed the alterations in the gene expression of rats induced by CCl_4. Thus, the present results strongly suggeste that, *J. curcas* extract has markedly ameliorative role associated with CCl_4-induced $_{geno\ and}$ hepatotoxicity in rats.

KEYWORDS: ***Jatropha curcas,*** **Antioxidant enzymes, Gene expression, DNA damage**

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INTRODUCTION

Liver is the key organ of metabolism and excretion. It is often exposed to a variety of xenobiotics and therapeutic agents and until today, people have not yet found an actual curative therapeutic agent for liver disorder [1]. Liver disorders are one of the serious health problems through the world. Despite remarkable advances in the field of modern medicine, hepatic diseases remain a major public health problem, thus the search for new effective medicines without side effects is still ongoing [2]. Natural remedies from traditional plants are seen as effective and safe alternative treatments for hepatotoxicity [3,4]. Free radicals are reactive molecules in the body. It causes various kinds of degenerative diseases such as cancer, aging, arthritis, ischemia and liver disorders. Degenerative diseases can be managed with antioxidant properties of plant. Several studies showed that hepatoprotective effects of medicinal plants are associated with phytoextracts/phytocompounds rich in natural antioxidants [5]. Many phytochemicals are strong antioxidants, effective antimicrobials; possess substantial anticarcinogenic and antimutagenic properties [6]. As well as they are also active in reducing high blood pressure [7]. Carbon tetrachloride (CCl_4) has been used in animal model to induce liver damage similar to that of acute viral hepatitis in human patients [8]. The principle causes of carbon tetrachloride in inducing the hepatic damage are lipid peroxidation, decreased activities of antioxidant enzymes and generation of free radicals [9]. Also, this component (CCl_4) is a commonly used model for screening of the anti-hepatotoxic and/or hepatoprotective activities of the drugs [10]. Different parts of *Jatropha curcas* (physics nut) plant, including the leaves, oil, sap, stem, roots and bark have numerous health benefits such as; skin injections, antidote for snake bites, leprosy and rheumatism, treatment of tooth ache and muscular pains, pile, fever, jaundice, gonorrhea, constipation, heart burn and as purgative as well as contraceptive principle [11]. Therefore, the present study is designed to evaluate the ameliorative role and therapeutic effect of *J. curcas* extract against CCl_4-induced hepato- and genotoxicity in female rats, through measuring, antioxidant enzymes (SOD, CAT and GPx), non-enzymatic antioxidant; GSH as well as an oxidative stress marker; MDA. Beside, oxidative stress related genes (HSPs) expression in rat liver was determined to provide the protective mechanism of *J. curcas* on hepatic cells.

MATERIALS AND METHODS

(i) Collection and preparation of J. curcas leaves

(ii) Fresh leaves of *J. curcas* were collected from the farm of Aromatic and Medicinal Plant Department, Agriculture Research Centre (ARC), Egypt. The plant was kindly authenticated by Mrs Treas Labib, Herbarium section, El-Orman Botanical Garden, Giza, Egypt. The leaves were washed with tap water, then with distilled water to remove dust and dirt. Leaves were air dried under shade, then grinded and homogenized to coarse powder finally stored in opaque screw tight jars until use

(iii) Crude methanolic extract preparation

About 300 g of powdered leaves of *J. curcas* were extracted by cold maceration in methanol on shaker (Heidolph). After complete extraction the extract was filtered by using Whatman grade No. 4 filter paper and Buchner. The filtrate was concentrated using Rotary evaporator (Heidolph) at 40°C under vacuum and stored in refrigerator (4°C) till biological assay and chemical analysis.

(iv) Chemicals and reagents

All chemicals in the present study are of analytical grade, products of Sigma, Merck and Aldrich. All kits were the products of Biosystems (Alcobendas, Madrid, Spain), Sigma Chemical Company (St. Louis, MO, USA), Biodiagnostic Company (Cairo, Egypt).

(v) Biological experiment

1. Experimental animals

Fifty female albino rats (130-150 g), were obtained from the Animal House, National Research Centre (NRC), Egypt. All animals were kept in controlled environment of air and temperature (26-29°C) with access of water and diet. Anesthetic procedures and handling with animals complied with the ethical guidelines of Medical Ethical Committee of National Research Centre in Egypt.

2. Experimental design

Fifty female albino rats were used in this study. Animals were divided into five groups of ten rats each as follows: **Group 1:** was served as normal control rats; **Group 2:** was served as normal control rats treated with *J. curcas* extract; **Groups 3**: was considered as CCl_4-intoxicated rats; **Group 4:** was considered as CCl_4-intoxicated rats orally administered with crude methanolic extract of *J. curcas* daily for 30 days. **Group 5**: was considered as CCl_4-intoxicated rats orally administered with silymarin daily for 30 days.

3. Doses and route of administration

Administration regimen of CCl_4 was twice a week for six consecutive weeks. CCl_4 (0.5 ml/kg) was suspended in olive oil (1:9 v/v) and injected intraperitoneally [12]. Leaves extracts of *J. curcus* was administrated orally at a dose of 250 mg/kg daily for one month [13]. Silymarin; a reference herbal drug was orally administered at a dose of 50 mg/kg, daily for one month [14]. Normal control group received orally 0.5 ml normal physiological saline and intraperitoneally 0.5 ml olive oil. Normal rats (G 2) were treated orally with the same dose of *J. curcas* extract.

4. Sample preparations

After 30 days of treatments, rats were fasted overnight (12-14 hours), anesthetized by diethyl ether, rats of each group were sacrificed, liver was removed immediately. Liver in each exponential group weighed and homogenized in 5-10 volumes of appropriate medium using electrical homogenizer, centrifuged at 3000 rpm for 15 min, the supernatants (10%) were collected and placed in Eppendorff tubes then stored at -80°C and used for determination of antioxidant and oxidative stress biomarkers (GSH, CAT, GPx and MDA).

5. Biochemical examination

All antioxidant and oxidative stress biomarkers (GSH, CAT, GPx and MDA) were determined in liver tissue homogenate. GSH as non-enzymatic antioxidant was determined according to the method of Beutler et al. [15] While, CAT enzyme activity was determined in liver homogenate according to the method of Monhanty et al. [16]. Moreover, GPx enzyme activity was determined in liver homogenate according to the method described by Kageyama [17]. In addition, liver MDA level was estimated according to the method of Satoh [18].

Calculation:

$$\% \text{ Change} = \frac{\text{Mean of control} - \text{Mean of treated}}{\text{Mean of control}} \times 100$$

$$\% \text{ of improvement} = \frac{\text{Mean of treated} - \text{Mean of disease}}{\text{Mean of control}} \times 100$$

6. Comet Assay

Liver tissues were homogenized in a density gradient of Gradisol L (Aqua Medica, Lodz, Poland). A freshly prepared suspension of cells in 0.75% low melting point agarose (Sigma Chemicals) dissolved in phosphate buffer saline (PBS; Sigma chemicals) was cast onto microscope slides precoated with 0.5% normal melting agrose. The cells were then lysed for 1h at 4°C in a buffer

consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100 and 10 mM Tris, pH 10. After the lysis, DNA was allowed to unwind for 40 min in electrophoretic solution consisting of 300 mM NaOH, 1mM EDTA, pH>13. Electrophoresis was conducted at 4°C for 30 min at electric field strength 0.73 V/cm (30mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 ug/ml ethidium bromide (Sigma Chemicals) and covered with cover slips. The slides were examined at 200 x magnification fluorescence microscope (Nikon Tokyo, Japan) connected to a COHU 4910 video camera (Cohu, Inc., SanDiego, CA, USA) equipped with a UV vilter block consisting an excitation filter (359 nm) and barrier filter (461nm) and connected to a personal computer-based image analysis system, Lucia-Comet v. 4.51. Fifty images were randomly selected from each sample and the comet tial DNA was measured [19]. Endogenous DNA damage was measured as the mean comet tail DNA of liver tissues of several rat groups. The number of cells scored for each animal was 100 [20].

7. Gene expression analysis

7.1. RNA extraction

Total RNA was isolated from 100 mg of liver tissues of female rats by the standard TRIzol extraction method (Invitrogen, Paisley, UK) and recovered in 100 µl molecular biology grade water. In order to remove any possible genomic DNA contamination, the total RNA samples were pre-treated using DNA-free™ DNase removal reagents kit (Ambion, Austin, TX, USA) following the manufacturer's protocol.

7.2. Reverse transcription

The complete Poly(A)+ RNA samples were reverse transcribed into cDNA in a total volume of 20 µl using 1 µl oligo(dT) primer. The composition of the reaction mixture, termed as master mix (MM), consisted of 50 mM MgCl2, 10x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; Perkin-Elmer), 10 mM of each dNTP (Amersham, Brunswick, Germany), and 50 µM of oligo(dT) primer. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through polymerase chain reaction (PCR).

7.3. Quantitative Real Time-PCR

The first strand cDNA from different samples was used as templates for RT-PCR with a pair of specific. The sequences of specific primer and product sizes are listed in Table 1. β-actin was used as a housekeeping gene for normalizing mRNA levels of the target genes [21]. PCR reactions were set up in 25 µl reaction mixtures containing 12.5 µl 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µl 0.2 µM sense primers, 0.5 µl 0.2 µM antisense primer, 6.5 µl distilled water, and 5 µl of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each qRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers**.**Each experiment included a distilled water control. The quantitative values of RT-PCR (qRT-PCR) of antioxidant enzymes genes (SOD1, CAT, GPx) and heat shock protein-70 gene (HSP70) were normalized on the bases ß-actin expression (Table 1). At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

7.4. Calculation of Gene Expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula found in the manufacturer's instruction pamphlet:

$Ef = 10^{-1/slope}$

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the

$2^{-\Delta\Delta CT}$ method if Ef for the target (OC, COL and ACP5) and the reference primers (β-Actin) as follows:

$\Delta C_{T(test)} = C_{T(target, test)} - C_{T(reference, test)}$,

$\Delta CT(calibrator) = C_{T(target, calibrator)} - C_{T(reference, calibrator)}$,

$\Delta\Delta CT = \Delta C_{T(Test)} - \Delta C_{T(calibrator)}$.

The relative expression was calculated by $2^{-\Delta\Delta CT}$.

8. Statistical Analysis

Biochemical results were subjected to one-way analysis of variance ANOVA and the significance of the differences between means was tested using co –state computer program, where the significance is at $P \leq 0.05$. While , all data of gene expression were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System followed by Scheffé-test to assess significant differences between groups [22]. The values are expressed as mean±SEM. All statements of significance were based on probability of $P \leq 0.05$.

Table 1
Primers and reaction parameters in RT-PCR

Target cDNA	Primer name	Primer sequence (5′–3′)	PCR product size (bp)
β-Actin	F	CAC GTG GGC CGC TCT AGG CAC CAA	189
	R	CTC TTT GAT GTC ACG CAC GAT TTC	
SOD1	F	ACA CAA GGC TGT ACC ACT GC	103
	R	CCA CAT TGC CCA GGT CTC C	
CAT	F	TGC CGT CCG ATT CTC CAC AG	115
	R	TCC CAC GAG GTC CCA GTT AC	
GPx	F	GTC CAC CGT GTA TGC CTT CTC C	218
	R	TCT CCT GAT GTC CGA ACT GAT TGC	
HSP70	F	ATC TCC TGG CTG GAC TCT AAC A	241
	R	CAC CCA TCT GTC TCC TAG ATC A	

¥: SOD= Super oxide Dismutase , CAT= Catalase, GPX= Glutathione peroxidase, HSP70= Heat Shock Protein -70 gene

RESULTS

1. Effect of *J. curcas* methanolic extract on enzymatic and non-enzymatic antioxidant

Table (2) showed the manipulation of GSH, CAT, GPx and MDA levels in liver tissue homogenates of control and different treated rats. It can be easily noticed that, GSH and GPx levels were significantly reduced in CCl_4-intoxicated with percentages 32.93 and 53.90%, respectively. While, CAT enzyme activity was insignificantly changed in CCl_4-intoxicated rats as compared to normal control group. MDA level was significantly increased in CCl_4-intoxicated rats with percentage increase reached to 58.60%. Treatment of intoxicated rats with crude methanolic extract of *J. curcas* as well as silymarin reference drug showed insignificant change in GSH, GPx and MDA levels as compared to normal control rats. From the present results it could be concluded that, the highest shortage in GSH and GPx levels as well as the highest increase in MDA level were observed in CCl_4-intoxicated. However, treatment of intoxicated rats with silymarin drug declared the better ameliorative percentage in GSH level (29.99%) than crude methanolic extract (28.34%). While the highest percentage of improvement in GPx enzyme activity and MDA level was obtained throughout treating of intoxicated rats with methanolic extract (47.88 and 51.65%) followed by silymarin drug (34.03 and 46.00%).

2. DNA damage

The proportion of DNA damage significantly increased in female rats treated with CCl_4 as compared to the control and silymarin groups. This damage of DNA decreased by using *J. curcas* extract and silymarin as protective agents. DNA damage was significantly reduced using *J. curcas* extract and silymarin in rats injected with CCl_4 and treated with *J. curcas* or silymarin. *J. curcas*

extract and silymarin greatly ameliorated the genetic materials, and gave the low proportion of DNA damage (14.8 and 13.6%, respectively) in respect to CCl_4 treatment (23.6%) (Table 3).

3. Effect on expression of stress-related genes in liver

Quantitative RT-PCR experiments were conducted to verify the expression of antioxidant and stress related genes induced in rat liver as a result of CCl_4 treatment. The mRNA expression of SOD1, CAT, GPx, and HSP70 were measured by Real-time quantitative PCR (Figures 1-4). The results showed that the expression of all of the antioxidant and stress related genes in CCl_4 treated rats were reduced significantly compared with the control group and silymarin treatment (Figures 1-3), except for the expression of HSP70 (Figure 3), which increased significantly ($P < 0.05$). Compared to the CCl_4 group, the expressions of SOD1, CAT, GPx mRNA were increased significantly in the groups of silymarin or *J. curcas* treated CCl_4-intoxicated rats, whereas mRNA expression of HSP70 decreased significantly ($P < 0.05$).

Table 2

Effect of crude methanolic extract of J. curcas supplementation on non-enzymatic antioxidant and oxidative stress in normal, CCl_4-intoxicated rats and different therapeutic groups

Groups	Parameters	GSH	CAT	GPx	MDA
Normal control	Mean± S.D.	539.75±55.08 [b]	315.19±59.12 [a]	6.14 ±0.08 [a]	2959.32 ±352.24 [b]
Normal control rats treated with *J. curcas*	Mean± S.D. % Change to control	565.00±23.54 [b] 4.68	324.11±23.02 [a] 2.830	6.21 ±0.36 [a] 1.140	3087.57±233.17 [b] 4.33
CCl_4-intoxicated rats	Mean ±S.D. % Change to control	361.98 ±34.53 [c] 32.93	310.00 ±26.45 [a] 1.64	2.83 ±0.12 [b] 53.90	4693.70 ±274.20 [a] 58.60
Intoxicated rats treated with *J. curcas* methanolic extract	Mean ±S.D. % Change to control % of improvement	514.21 ±47.61 [b] 4.73 28.34	350.31 ±15.15 [a] 11.14 12.78	5.77 ±0.60 [a] 6.02 47.88	3164.98±699.82 [b] 6.94 51.65
Intoxicated rats treated with silymarin	Mean ±S.D. % Change to control % of improvement	523.87 ±47.79 [b] 2.94 29.99	350.29 ±13.91 [a] 11.13 12.78	4.92 ±0.47 [a] 19.86 34.03	3332.29±396.33 [b] 12.60 46.00

-All values are means±SD of 10 rats in each group
-Data were analyzed using analysis of variance (ANOVA) combined with co-state computer program, where unshared letter is significant at p≤0.05

Table 3

Visual score of DNA damage in female rats treated with CCl_4 and J. curcas or silymarin extracts using comet assay

Treatment	Number of animals	No. of cells		Class[¥] (Range)				DNA damaged cells (%)
		Analyzed(*)	Total comes	0	1	2	3	
Control	5	500	33	467	22	11	0	6.6
J. Curcas	5	500	34	466	23	11	0	6.8
CCl_4	5	500	118	382	32	42	44	23.6
CCl_4 +*J. curcas*	5	500	74	426	27	31	16	14.8
CCl_4 + silymarin	5	500	68	432	24	29	15	13.6

[¥]: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. (*): No of cells analyzed were 100 per an animal.

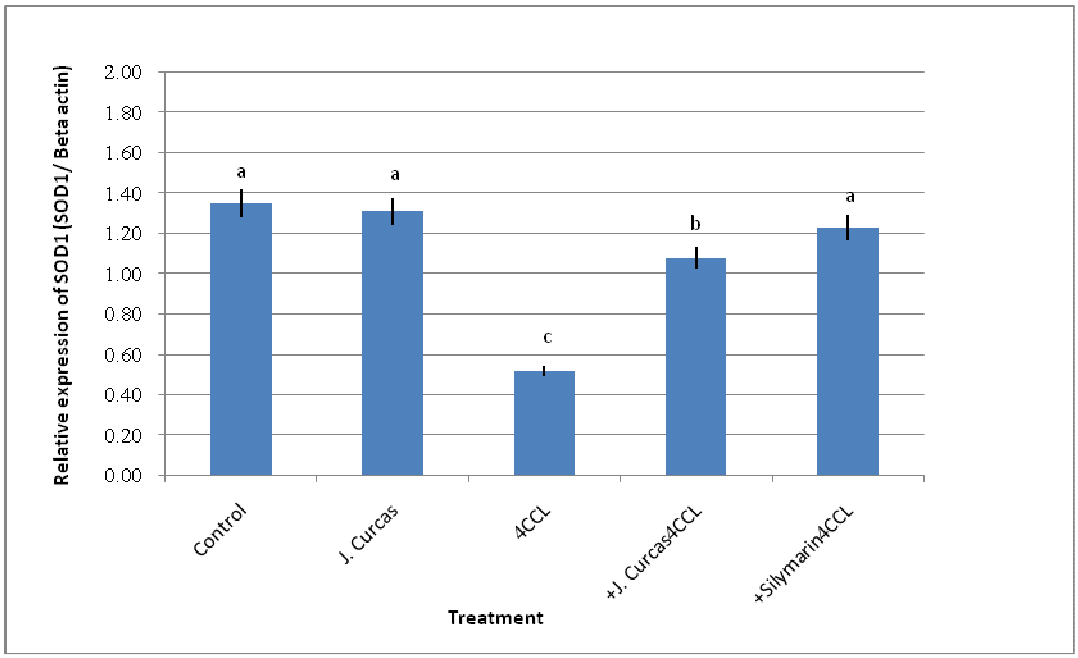

Figure 1

Quantitative RT-PCR confirmation of SOD1 gene in liver tissues of female rats injected with CCl4 and treated with J. curcas or silymarin extracts. ^{a,b,c} ***columns with different letters differ significantly (P ≤0.05).***

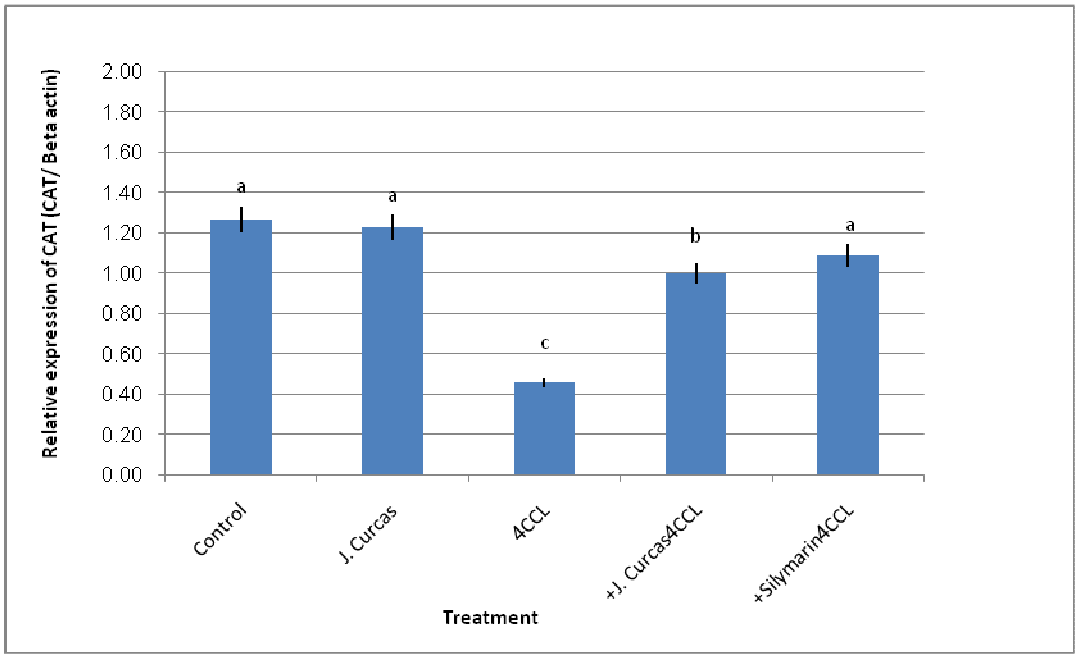

Figure 2

Quantitative RT-PCR confirmation of CAT gene in liver tissues of female rats injected with CCL4 and treated with J. curcas or silymarin extracts. ^{a,b,c} ***columns with different letters differ significantly (P ≤0.05).***

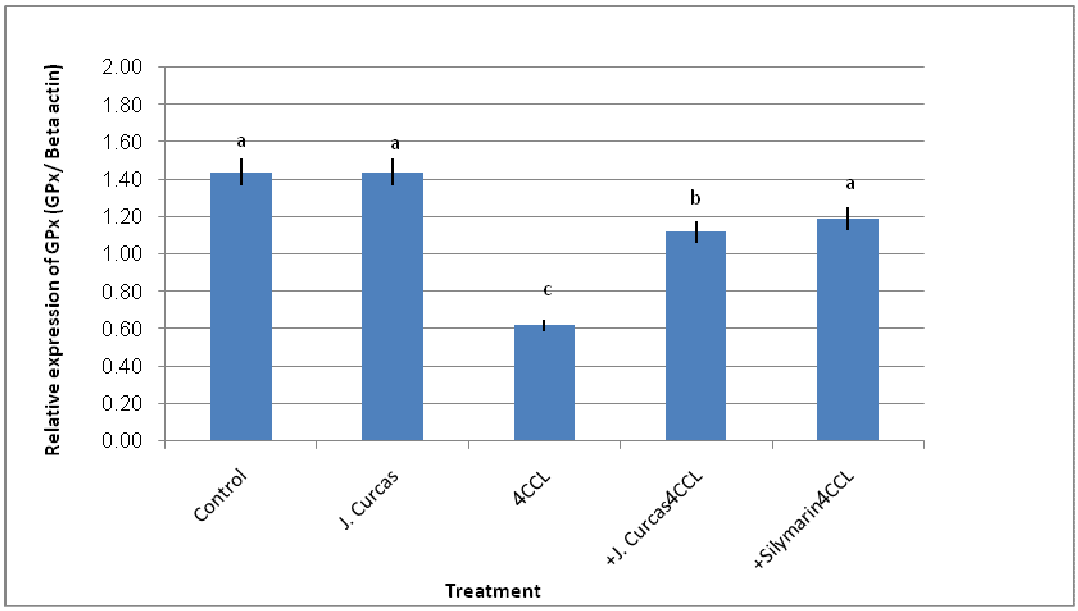

Figure 3

Quantitative RT-PCR confirmation of GPx gene in liver tissues of female rats injected with CCL4 and treated with with J. curcas or silymarin extracts. [a,b,c] columns with different letters differ significantly (P ≤0.05).

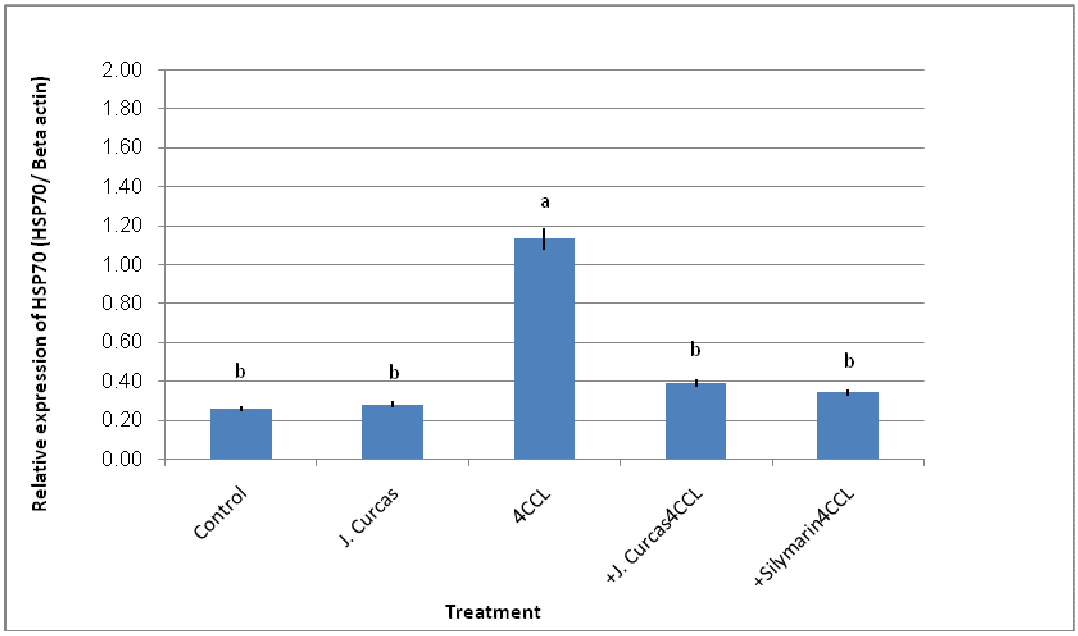

Figure 4

Quantitative RT-PCR confirmation of HSP70 gene in liver tissues of female rats injected with CCL4 and treated with J. curcas or silymarin extracts. [a,b,c] columns with different letters differ significantly (P ≤0.05).

DISCUSSION

In living systems, liver is considered to be highly sensitive to toxic agents. Oxidative stress can be resulted from the increased production of free radicals with/or a marked reduction of antioxidant defenses and this case of oxidative stress leads to oxidative damage of membranes and tissue injury [23]. The antioxidant defense systems are represented in two ways either enzymatic, including SOD, CAT, glutathione-S-transferase (GST), glutathione reductase and glucose-6-phosphate dehydrogenase, or non-enzymatic, such as vitamins C and E as well as thiols, especially the

reduced glutathione molecule [24]. It is known that, GSH enzyme is involved together with GPx in the protection of the organism against reactive oxygen species. GSH has many important functions in the cell as it directly participates in the scavenging of free radicals: hydrogen peroxide, superoxide anion and hydroxyl radicals [25]. Catalase is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity, is found in the red cells and in the liver [26]. Also, it decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals [27]. The non-enzymic antioxidant, glutathione is considered as one of the most abundant tripeptides present in the liver. Its functions are mainly concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals, and maintenance of membrane protein thiols as well as substrate for glutathione peroxidase and GST [28]. Therefore, 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. According to the present results, GSH and GPx enzyme activities were significantly reduced in CCl4-induced toxicity in rats with percentage decrease 32.93 and 53.90%, as compared to a normal control group. These results are agreed with the results obtained by Sahreen et al. [29]. Where, CCl4-induced in rats depletes the hepatic GSH contents. This may be depended on the finding of Palanivel et al. [30]. The authors observed reduction in the GSH level associated with an enhanced lipid peroxidation in CCl4 treated rats. However, not only lipid peroxides are noxious to the living organism, but also some of their stable breakdown products such as MDA have been recognized to cause some cell alterations by modifying protein structures [31]. The reaction between MDA and the primary amino groups of proteins forms schiff base compounds, which give rise to intra and intermolecular linkages that in turn can lead to polymerization and inactivation of enzymes. In addition, MDA reactivity towards amino groups can result in inhibition of DNA and RNA protein synthesis [32]. Evidence suggested that various enzymatic and non enzymatic systems have been developed by the cell to attenuate ROS [33]. Although, when a condition of oxidative stress establishes, the defense capacities against ROS becomes insufficient. Therefore, ROS affects the antioxidant defense mechanisms, reduces the intracellular concentration of GSH, decrease the activity of SOD and enhances lipid peroxidation [34]. It was recorded that SOD enzyme activity was inhibited in CCl4 treated rats that might be due to inactivation of the antioxidative enzymes [35]. This may cause an increased accumulation of superoxide radicals, which could further stimulate lipid peroxidation [36]. In addition, the inhibition in GSH concentration can affect redox equilibrium leading to enhanced lipid peroxidation processes [36]. Treatment with either leaves or stems extract of *Rumex dentatus* (Polygonaceae) normalized the antioxidant levels through their high content of flavonoids and anthraquinones as they have the ability to scavenge free radicals [35].

A number of studies have revealed that GSH conjugates play a major role in eliminating the CCl4-induced toxic metabolites which are the main cause of liver injuries [28] Moreover, CCl4 induced inhibition in GSH-Px that may probably be due to its inactivation during the catalytic cycle [37]. *J. curcas* methanolic extract possessed potent free radical scavenging activity, and it is regarded as a potential source of natural antioxidants that may be a good candidate for pharmaceutical plant based products [38]. Moreover, the phytochemical contents and biological activities of the methanolic extract from different parts of *J. curcas* (leaves, stem bark, root and latex) were conducted by Oskoueian et al. [39], who indicated the occurrence of phenolics, flavonoids and saponins compounds in methanolic extract. The authors added that, both of methanolic and hot water extracts showed antioxidant and antimicrobial activities against Gram negative and Gram positive bacteria. Furthermore, the hepatoprotective studies showed that plants have active ingredients that are capable of free radical scavenging in living systems [40]. Phenolic compounds are promising bioactive secondary metabolites playing an important role in detoxification of free radicals [41]. Igbinosa et al. [38] mentioned that, *J. curcas* is a potential source of natural antioxidants such as; phenol, flavonoids, flavonols and proanthocyanidin and may be a good candidate for pharmaceutical plant based products. It is well documented that the compounds quercetin, rutin (flavonoids), Vitamins C and E are strong antioxidants [42].Thus, the ameliorative effect of *J. curcas* may be relied on the basis of; the methanolic extract may ameliorate the levels of H_2O_2 and O_2^-, consequently restoring enzyme activity and also induce the de novo synthesis of the antioxidant enzymes [37]. Also, Silymarin as an antioxidant flavonoid

complex derived from the herb milk thistle (*Silybum marianum*), has the ability to attenuate free radicals elevation, chelates metal ions, inhibits lipid peroxidation and prevents liver glutathione depletion [43]. The current study revealed that treatment of female rats with CCl_4 significantly increased the DNA damage in liver cells and significant induction of the gene expression alterations of genes encoding antioxidant enzymes (SOD1, CAT and GPx) and HSP70 in liver tissues compared with the control group. This study proved that CCl_4 treatment has a mutagenic effect on genomic material of female rats. Our findings on DNA damage are in agreement with that reported by Abdou et al. [10], who found that administrations of CCl_4 to rats caused significant increase of DNA damage as compared to normal control. The DNA damage can originate from the direct modification of DNA by chemical agents or their metabolites; from the processes of DNA excision repair, replication and recombination; or from the process of apoptosis [44].

Following administration, CCl_4 is activated by cytochrome P_{450} system to form trichloromethyl (CCl_3) radical. This radical binds to cellular molecules (nucleic acids, proteins and lipids) thereby impairing crucial cellular processes such as lipid metabolism, with the potential outcome of fatty degeneration, while the reaction between trichloromethyl (CCl_3) radical and DNA is thought to function as initiator of heaptic cancer [45]. This radical can also react with oxygen to form the trichloromethylpheroxy (CCl_3OO) radical, a highly reactive species. This compound initiates the chain reaction of lipid peroxidation, culminating in destruction of polyunsaturated fatty acids, especially those associated with phospholipids [45]. This leads to alteration of permeabilities of mitochondrial, endoplasmic reticulum and plasma membranes, resulting in the loss of cellular calcium sequestration and disruption of calcium, homeostasis with subsequent cell damage [46]. Oxidative stress induced by oxygen-derived species can produce a multiplicity of modifications in DNA including base and sugar lesions, strand breaks, DNA protein, cross-links and base-free sites. If left un-repaired, oxidative DNA damage can lead to detrimental biological consequences in organisms, including cell death, mutations and transformation of cells to malignant cells [10]. Previous studies proved that changes in genomic DNA could reflect DNA alterations from single base changes (point mutations) to complex chromosomal rearrangements [47]. So, the DNA damage formation causes genomic instability including gene expression of animal genes [48]. The current results indicated that mRNA expression in liver tissues of CCl_4 group was significantly lower in the antioxidant enzymes (SOD1, CAT and GPx) and higher in HSP70 compared with those in normal control. Similarly, CCl_4 has also been reported to activate and increase the expression of tumor necrosis factor- α (TNF- α) nitric oxide (NO) and transforming growth factors (TGF)-α and-β in the cell, processes that appear to direct the cell primarily toward self-destruction or fibrosis. TNF-α pushes the cell toward apoptosis [49], whereas, the TGFs appear to direct toward fibrosis [46]. Antioxidants are closely related to their biofunctionalities, such as the reduction of cellular abnormalities like DNA damage, mutagenesis, carcinogenesis and which is also associated with free radical propagation in biological systems [50].

There is however no report yet of the effect of *J. curcas* on antioxidant/ oxidative stress related gene expression (such as SOD1, CAT and GPx/ HSPs) under chronic CCl_4 toxicity. However, Sundari et al [51] showed that several extracts of *J. curcas* were capable of scavenging hydroxyl and may have a stronger hydroxyl radical scavenging activity . SOD1, CAT and GPx are known to prevent the harmful effects of free radicals by over-expression of their mRNAs and reduce the formation of the reactive metabolites induced by mutagenic drugs or CCl_4. On the other hand, heat Shock Proteins (HSPs) are synthesized from stress genes under stress response conditions. In general, the major HSPs are expressed at low levels and functional as molecular chaperones [52]. Under stress conditions, the newly synthesized stress proteins play an essential role in maintaining cellular homeostasis by assisting in the correct folding of nascent and stress-accumulated misfolded proteins [53]. It is clear that stress induced by mutagenic drugs or heavy metals cause expression of heat shock proteins [54]. The HSP70 is one of the most abundantly induced proteins under a variety of stress conditions and the most extensive oxidative stress marker at present [55]. In the same line with these findings, our study revealed that the expression of HSP70 was over-expressed in CCl_4 group and reduced significantly by treatment with *J. curcas* extract. Moreover, the present study reveal that *J. curcas* and silymarin extracts decreased significantly the rate of the DNA damage in female rats induced by CCl_4 treatment. The present

results indicate that both the extracts of *J. curcas* and silymarin had a prominent effect on hydroxyl radical/and or super oxide scavenging. These results are in agreement with those of Sundari et al. [51], who reported that *J. curcas* fractions decreased the DNA damage in human peripheral blood lymphocytes exposed to UVB-irradiation. Also, Katiyar et al. [56] reported that silymarin protects epidermal keratinocytes from ultraviolet radiation-induced apoptosis and DNA damage by nucleotide excision repair mechanism. In addition, our recent study revealed that detoxified *J. curcas* seed meal decreased the DNA fragmentation induced by benzene exposure in male rats [57].

It has been found that, flavonoids, coumarins and other polyphenols present in the leaf, stem bark and root extracts of *J. curcas* are well known for their strong antioxidant properties which play important roles in protecting cells and organs from oxidative damage [58]. In addition, the presence of steroids and terpenoids in *J. curcas* plant play an important role in free radical scavenging activity [59]. Previous report showed that high concentrations of the methanol extract of *J. curcas* have been reported to be more effective in quenching free radicals in the system [60].

It has been established that plant extracts and their active compounds enhances DNA repair mechanism and inhibits DNA strand breaks in radiation exposed cells [61]. Antioxidant potential of *J. curcas* has been proposed for its anti-genotoxic potential. Several investigators have demonstrated that natural antioxidants scavenge ROS and protect cellular DNA against agents-induced oxidative damage [62]. Hence, it can be postulated that *J. curcas*, by virtue of its free radical scavenging capacity and DNA repairing capacity restore DNA damage induced by CCl_4. The DNA damage induced by CCl_4 is reduced by *J. curcas*, extracts as measured by the decrease DNA fragmentation and gene expression alterations. The antioxidant activity of the *J. curcas* is attributed to the hydrogen donating ability of phenolic compounds present in the extracts [63].

The present study demonstrate that treatment of female rats with silymarin extract decreased the DNA damage and alterations in the expression of antioxidant (SOD1, CAT and GPx) as well as stress related gene (HSP70) induced by CCl_4 in female rats. Cells undergo apoptosis because of irreparable DNA damage. If this damage can be repaired, cells may avoid apoptosis as well as cells may avoid abnormal deregulation, proliferation or replication of damaged DNA containing cells, and thus malignancy can be inhibited [56]. Our data show that silymarin prevents CCl_4 induced apoptosis due to DNA damage in liver cells, and this prevention may be due to repairing of DNA damaged induced by CCl_4. Silymarin is used as a standard drug in various experimental and clinical studies due to its proven hepatoprotective effects [64]. This ability of silymarin may be due to its free radical scavenger activity [19]. In an earlier report, the combination of silymarin with Phyllanthus amarus extract offered significant hepatoprotection against CCl_4 induced liver damage [65]. This was also confirmed by findings from present study.

CONCLUSION

In conclusion, the present study prove that *J. curcas* and silymarin are able to significantly alleviate the oxidative stress induced by CCl_4 in rats. These results revealed that *J. curcas* and silymarin have therapeutic effect in curing some health problems associated with toxication status of CCl_4 treatment in female rats.

Conflict of Interest

Conflict of Interest declared none.

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