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Protective Effect of *Conocarpus erectus* Extracts on CCl_4-Induced Chronic Liver Injury in Mice

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Abstract: Plants still an excellent source of new therapeutic agents owing to their versatile applications. As a folk medicine *Conocarpus erectus* L. (family Combretaceae) was reported to be used for healing from many diseases. The four defatted methanol extracts of *C. erectus* different parts (leaves, stems, fruits and flowers) showed high antioxidant and hepatoprotective activity monitored by two *in vitro* antioxidant methods; phosphomolybdenum and reducing power activity and *in vivo* test. The antioxidant activity of the four extracts are attributed to the presence of phenolic compounds as major components in these extracts. CCl_4 increased significantly the levels of ALT (P<0.001), blood urea (P <0.01) and without any differences in total proteins, albumins, globulins and A/G ratio compared to normal mice. Treatment of toxicated mice with different parts of defatted methanol extracts of *Conocarpus erectus* in a dose of 500 mg/kg for two weeks decreased significantly (P <0.5 and P <0.01) the levels of ALT and without any difference in blood urea, total proteins, albumins, globulins and A/G ratio. In conclusion, this plant may have great relevance in the prevention and therapies of diseases in which oxidants or free radicals are implicated after more *in vivo* studies for understanding their mechanism of action as antioxidant.

Key words: Hepatoprotective · Antioxidant · Conocarpus Erectus · CCl_4

INTRODUCTION

Liver is one of the largest organs in human body and it is the key organ of metabolism and extraction. It regulates many metabolism functions. Therefore, any hepatic injury is associated with distortion of these metabolic functions [1].

Most biological molecules have more than one function. In particular, many molecules have the ability to directly/indirectly scavenge free radicals and thus act as antioxidants in living organisms. The increased levels of these molecules during oxidative stress seem to be a biological response that may protect cells from oxidation, in synergy with other antioxidant defense systems [2, 3].

It is widely known that oxidative stress produces a series of reactive intermediates, such as H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$, lipid peroxides, $NO^{\cdot}$ and so on, that may damage the cell directly through their chemical action or may indirectly produce injury by activating a multitude of other different mechanisms. Both these mechanisms are able to disrupt the cell through the activation of inflammatory processes or through apoptosis [4].

The host reaction involves reactive oxygen species (ROS), which induce the antioxidant defense process of the organ and may initiate a fibrogenesis cascade in the liver. Therefore, the free radicals may be a major component of the disease and the liver damage increase with increasing of (ROS) production. In addition, ROS production is associated with the inflammatory response and depends on balance between the opposing mechanisms, which can either terminate the oxidative process or lead to increased generation of potentially harmful, long-lived oxidants [5, 6].

It is evident that the antioxidants are useful in decreasing or preventing the deleterious consequences of the oxidative stress. Due to the antioxidant and free radical scavenging activity of melatonin, it showed high protective activity against the pathological changes associated with CCl_4 [5].

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Treatment options for common liver diseases such as cirrhosis, fatty liver and chronic hepatitis are problematic. The effectiveness of treatments such as interferon, colchicine, penicillamine and corticosteroids are inconsistent at best and the incidence of side effects profound. All too often, the treatment is worse than the disease. Conservative physicians often counsel watchful waiting for many of their patients, waiting in fact for the time when the disease has progressed to the point that warrants the use of heroic measures. Physicians and patients are in need of effective therapeutic agents with a low incidence of side effects. Plants potentially constitute such a group.

Many plants were subjected by several investigators to evaluate their hepatoprotective activity further more numerous botanicals were used traditionally by herbalists for the preventation and treatment of liver disease in which it confirmed by clinical research in this century further more numerous medicinal plants and their formulation are used for liver disorders in ethnomedical practice as well as traditional medicine in Chinese [7].

Several hundred plants have been examined for use in a wide variety of liver disorders. The latter category of plants include: *Silybum marianum* (milk thistle), *Picrorhiza kurroa* (kutkin), *Curcuma longa* (turmeric), *Camellia sinensis* (green tea), *Chelidonium majus* (greater celandine), *Glycyrrhiza glabra* (licorice) and *Allium sativa* (garlic) showed remarkable liver protective effects [8].

Silymarin has been reported to protect liver cells from a wide variety of toxins, including acetaminophen, ethanol, carbon tetra chloride and D-galactosamine [9, 10]. Silymarin has also been found to protect liver cells from ischemic injury [11], radiation [12], iron toxicity [13] and viral hepatitis [14].

Carbon tetrachloride (CCl_4), a hepatotoxin, has been used extensively to induce liver injury in various animal models for decades [15]. Liver injuries induced by CCl_4 are the best-characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of antihepatotoxic and/or hepatoprotective activities of drugs [16]. The experimentally induced cirrhotic response by CCl_4 in rats and mice has been shown to be similar to human cirrhosis of the liver [17]. CCl_4 causes mitochondrial stress, which activates signaling cascades involving the activation of caspases, resulting in apoptosis or necrosis. It is known recently that mitochondria in cells not only provide ATP by oxidative phosphorylation but also play many other roles, such as modulation of intracellular Ca^{2+} homeostasis, pH control and induction of apoptotic and excitotoxic cell death [16, 18].

Conocarpus erectus L. (family Combretaceae) is a mangrove species commonly called button wood growing on shorelines in tropical and subtropical regions around the world. As a folk medicine, it was reported to be astringent, styptic and tonic preventing anemia, catarrh, conjunctivitis, diabetes, diarrhea, fever, gonorrhea, headache, hemorrhage, orchitis, prickly heat, swellings and syphilis [19, 20].

The present study was designed to evaluate the hepatoprotective and antioxidant activity of *Conocarpus erectus* extracts for scavenging the reactive oxygen species (ROS) and reduced the oxidative damage in the liver of mice induced with CCl_4.

MATERIALS AND METHODS

Chemicals: All chemicals used for extraction of plant were analytical grade and were obtained from Sigma/Aldrich Chemical Company, St. Louis MO, USA.

Preparation of Defatted Methanol Extracts: Two hundred grams of finely *Conocarpus erectus* plant powder for each plant part; leaves, fruits, stems and flowers; prepared in previous work [21] were soaked in 1000 ml methanol for one week at room temperature with shaking day by day followed by filtration and again extraction for four times. The organic solvent was removed using rotatory evaporator under vacuum affording known weight of each crude methanol extract. The methanolic crude extracts were defatted by washing several times with petroleum ether (60-80°C). The defatted crude methanol extracts were ready for bioassay.

In vitro antioxidant activity: Two different chemical methods were used for the evaluation of the antioxidant activity of defatted crude methanol extracts phosphomolybdenum method and reducing power assay. These assays were performed as described by Abdel-Hameed, [22].

Determination of the total antioxidant capacity by phosphomolybdenum method: Three hundred µml of each sample solution and ascorbic acid (100 µg/ml) were combined with 3 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). A typical blank solution containing 3 ml of reagent solution and an appropriate volume of the same solvent

was used for the sample. All tubes were capped and incubated in a boiling-water bath at 95°C for 90 min. After the samples were cooled to room temperature, the absorbance of the solution of each sample was measured at 695 nm against the blank using a UV/Vis spectrophotometer. The experiment was performed in triplicates. The antioxidant activity was expressed as the number of equivalents of ascorbic acid.

Reducing Power Assay: Two ml of each sample and ascorbic acid in methanol (200 µg/ml) were mixed with 2 ml of sodium phosphate buffer (0.2M, pH 6.6) and 2 ml of 1% $K_3Fe(CN)_6$ were incubated at 50°C for 20 min. After adding 2 ml of trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant solution (2 ml) was taken out and immediately mixed with 2 ml of methanol and 0.5 ml of 0.1 % ferric chloride. After incubation for 10 min, the absorbance against the blank was determined at 700 nm. Triplicates were made for each tested sample and ascorbic acid. The increase in absorbance of the reaction mixture indicates an increased reduction power. The reducing power activity was expressed as the number of equivalents of ascorbic acid.

Estimation of the Total Phenolic, Flavonoid and Taninn Contents: In this study, the total phenolic, flavonoid and tannin contents of the four defatted methanol extracts were measured according to the methods described by Abdel-Hameed, [22].

The total phenolic content of plant extracts was determined using Folin-Ciocalteu's reagent (FCR). Hundred µl of each sample solution (100 µg/ml) and also 100 µl of gallic acid (100 µg/ml) were mixed with 500 µl of the FCR and 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 hrs. Then the absorbance at 765 nm was determined against a blank that contained all reagents without the samples or the gallic acid at the same conditions. All determinations were carried out in triplicates. The total phenolic content was expressed as the number of equivalents of gallic acid (GAE).

The flavonoids content was determined by aluminium chloride method using rutin as a reference compound. Hundred µl of each sample solution (1 mg/ml) was mixed with 100 µl of 2% aluminum trichloride in ethanol and a drop of acetic acid and then diluted with ethanol to 5 ml. The absorption at 415 nm was read after 40 min. Blank was prepared from all reagents without the samples. The absorption of the standard rutin solution (100 µg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates. The amount of flavonoids in plant extracts in rutin equivalents (RE) was calculated by the following formula:

$$X = (A - m_o)/(A_o - m)$$

where X is the flavonoid content, mg/mg plant extract in RE, A is the absorption of plant extract solution, A_o is the absorption of the standard rutin solution, m is the weight of plant extract (mg) and m_o is the weight of rutin in the solution (mg).

The total content of tannins adsorbed by casein was determined using FCR. About 10 ml (100 µg/ml) of each fraction (solution 1, S1) was mixed with 100 mg of casein with shaking for two hours (adsorption of tannins) and then filtered (solution 2, S2). The total phenolic contents for both solutions S1 and S2 using Folin-Ciocalteu's method as described before. The difference between absorbance of S1 and S2 correspond to concentration casein-adsorbed tannins in sample. All determinations were carried out in triplicates. The total casein-adsorbed tannins are expressed as the number of equivalents of gallic acid (GAE).

Experimental Animals: Laboratory outbred Swiss albino mice (CD_1), weighing 20±2g were used, they were obtained from the Schistosome Biology Supply Center (SBPC), Theodor Bilharz Research Institute (TBRI) Giza, Egypt.

Acute Toxicity: A group of 36 adult normal male CD-1 Swiss albino mice weighing 20±2 g were used for each one of four defatted methanol plant extract to study the acute toxicity. It was subdivided into six subgroups each of six mice. All subgroups were treated orally with rising doses of 500, 1000, 2000, 3000, 4000 and 5000 mg/kg of each defatted methanol plant extract. Mortality rates were recorded 24 hrs post treatment.

Experimental Animal Groups: A group of 48 adult normal male CD-1 Swiss albino mice weighing 20±2 g were used. Animals were classified according to the following groups for defatted methanol plant extracts. Each group consists of eight mice.

Group 1: Normal untreated control.

Group 2: Toxicated group with CCl_4 intraperitonial (i.p.).

Group 3: Toxicated group with CCl_4 (i.p.) then treated orally with defatted methanol extract of leaves.

Group 4: Toxicated group with CCl_4 (i.p.) then treated orally with defatted methanol extract of fruits.

Group 5: Toxicated group with CCl_4 (i.p.)then treated orally with defatted methanol extract of flowers.

Group 6: Toxicated group with CCl_4 (i.p.) then treated orally with defatted methanol extract of stems.

Mice toxicated with CCl_4 solution 99% (diluted 1:10 in olive oil) in a dose of 3ml/kg body weight three times/ week for two weeks and daily treated orally with defatted methanol plant extracts of *Conocarpus erectus* in dose of 500 mg/kg for two weeks. All animals were sacrificed 48 hrs post treatment; mice were sacrificed by rapid decapitation. After sacrifice, blood samples were collected and sera were separated by centrifugation at 3000 r.p.m. for 15 min to be used for the estimation of parameters related to liver function tests (alanine aminotransferase (ALT), Total proteins, albumin, globulins and A/G ratio).

Conventional Liver Function Tests: Determination of serum alanine aminotransferase (ALT), serum Total proteins, serum Albumin, Globulins and A/G ratio using Kits according to the previous methods [23-25].

Kidney Function Test: Determination of serum blood urea using Kits according to the previous method [26].

Statistical Analysis: Data were analyzed using version 13.0 of the SPSS software package (SPSS Inc., Chicago, IL) and the results were expressed as means ± SEM. Statistical analysis of results was carried out using Students t-test [27]. The degree of significance (probability p-value) was obtained from the corresponding tables. All determinations in tables 2 and 3 were carried out in triplicates and the values are mean ± standard deviation.

RESULTS

In Vitro Antioxidant Activity: Table (1) showed that the methanol extract of fruits has the higher antioxidant capacity (630.1±5.79 mg ascorbic acid equivalent /g extract) than the other three extracts; flowers, stems and leaves with 579.5±7.58, 570.7±4.37 and 376.3±2.19 mg ascorbic acid equivalent/g extract respectively. On the other hand the data of reducing power activity in Table (1) showed that the methanol extract of fruits has the highest reducing power activity (530.3±26.24 mg ascorbic acid equivalent /g extract) followed by the three other methanol extracts of flowers, stems and leaves with 479.8±8.75, 479.8±17.50 and 393.9±15.15 mg ascorbic acid equivalent /g extract, respectively.

Total Phenolic, Flavonoid and Tannin Contents: Table (2) showed that the methanol extracts of fruits and stems have high phenolic contents equivalent to 581.1±9.01 and 433.9±6.88 mg/g GAE respectively whereas the methanol extracts of flowers and leaves have moderate phenolic contents (236.78±14.35 and 216.09±14.35 mg/g GAE respectively).

The estimation of total flavonoids in the four methanol extracts (Table 2) revealed that the extract of leaves has the highest total flavonoid contents equivalent to 27.0±1.34 mg/g RE followed by the three other methanol extracts of fruits; flowers and stems (19.3±0.66; 11.6±0.33 and 6.5±0.83 mg/g RE respectively. The estimation of total casein-adsorbed tannin content of the four methanol extracts by FCR method (Table 2) revealed that the two methanol extracts of stems and fruits have the highest contents equivalent to 158.62±6.89 and 151.72±13.79 mg/g GAE respectively whereas the other two methanol extracts; flowers and leaves; have lower contents (119.54±7.96 and 68.97±6.89 mg/g GAE, respectively).

Acute Toxicity and Determination of Ld_{50}: No dead animals (0 % mortality) were observed 24 hours post treatment with rising doses of different defatted methanol plant extracts starting from 500 mg/kg to 5000 mg/kg.

$LD_{50} > 5000$ mg/kg

Hepatoprotective Effect: CCl_4 increased significantly ($P<0.001$) the levels of ALT, blood urea ($P<0.01$) and without any differences in total proteins, albumins, globulins and A/G ratio compared to normal mice. Treatment of toxicated mice with defatted methanol extracts of *Conocarpus erectus* different parts in a dose of 500 mg/kg for two weeks decreased significantly ($p<0.5$ and $P<0.01$) the levels of ALT and insignificantly decrease in blood urea. No differences in total proteins, albumins, globulins and A/G ratio were recorded when compared to control group (Table 3).

Table 1: Total antioxidant capacity and reducing power activity of defatted methanol extract of *Conocarpus erectus* different parts

Plant part	Total antioxidant capacity [mg equivalent to ascorbic acid /g extract][a]	Reducing power activity [mg equivalent to ascorbic acid /g extract][b]
Leaves	376.3±2.19	393.9±15.15
Stems	570.7±4.37	479.8±17.50
Flowers	579.5±7.58	479.8±8.75
Fruits	630.1±5.79	530.3±26.24

Values of total antioxidant capacity and reducing power activity expressed as mean of triplicate determinations ± standard deviation

[a]Antioxidant capacity monitored by the phosphomolybdenum method expressed by mg equivalent to ascorbic acid /g extract

[b]Reducing power activity expressed by mg equivalent to ascorbic acid /g extract

Table 2: Total phenolic, flavonoid and tannin compounds of defatted methanol extract of *Conocarpus erectus* different parts

Plant part	Total phenols (mg gallic acid equivalents/g plant extract)[a]	Total flavonoids (mg rutin equivalents/g plant extract)[b]	Total tannins (mg gallic acid equivalents/g plant extract)[c]
Leaves	216.09±14.35	27.0±1.34	68.97±6.89
Stems	433.9±6.88	6.5±0.83	158.62±6.89
Flowers	236.78±14.35	11.6±0.33	119.54±7.96
Fruits	581.1±9.01	19.3±0.66	151.72±13.79

[a]Total phenols expressed by mg equivalent to gallic acid /g extract

[b]Total flavonoids expressed by mg equivalent to rutin /g extract

[c]Total tannins expressed by mg equivalent to gallic acid /g extract

Table 3: Effect of *Conocarpus erectus* defatted methanol extracts on ALT, Total protein, Albumin, Globulins, A/G ratio and Blood Urea in mice toxicated with CCl_4

Animal groups	ALT (U/L)	Total protein (g/dL)	Albumin (g/dL)	Globulins (g/dL)	A/G ratio (g/dL)	Blood Urea (mg/dL)
Normal N=8	10.8±1.4	6.5±0.2	3.6±0.2	2.9±0.1	1.2±0.1	24.4±1.0
Control (Ccl_4) N=8	20.9±1.0###	6.0±0.2	3.0±0.2	3.0±0.1	1.0±0.1	31.1±1.8##
Leaves extract N=8	15.7±1.4*	6.4±0.3	3.3±0.1	3.0±0.3	1.1±0.1	28.9±1.3
Stem extract N=8	16.7±1.0*	6.4±0.2	3.3±0.1	3.1±0.1	1.1±0.1	27.6±2.1
Flowers extract N=8	14.2±1.1**	6.4±0.3	3.4±0.2	3.0±0.2	1.1±0.1	26.8±4.7
Fruits extract N=8	16.6±1.6*	6.2±0.3	3.2±0.1	3.0±0.2	1.1±0.1	27.1±2.7

Significance differences versus normal

* Significance differences versus infected control

N = number of animals in each group

DISCUSSION

The liver is considered the main organ responsible for the synthesis, uptake and degradation of a number of biological materials in blood including proteins and enzymes. Although the liver has strong regeneration ability, when cellular loss exceeds a certain threshold, the insufficient functions cause hepatic failure, leading to liver disease. Overdose of drug or ischemia/reperfusion induces necrotic and apoptotic cell death of hepatocytes and non-parenchymal liver cells [28].

It is known that acute and chronic hepatic injury, which cause high morbidity and mortality world-wide, are induced by a disparate range of pathophysiological conditions. Liver intoxication has increased as a result of exposure to high levels of environmental toxins [29]. This is because the liver has an important role in detoxification [30]. Although the pathogenesis of acute cirrhosis is not fully understood, it is clear that reactive oxygen species (ROS) have a key function in pathological changes in the liver [31, 32]. In fact, studies of causative factors involving liver injury have been

performed for a number of years now and it is well known that ROS are heavily involved in the cause and progression of hepatic damage [31, 32]. Biological membranes are particularly susceptible to ROS effects. The peroxidation of unsaturated fatty acids in biological membranes produces: a decrease in fluidity, then loss of function, disruption of integrity and finally, cell death [31, 32].

CCl_4 is a classical hepatotoxicant that causes rapid liver damage progressing from steatosis to centrilobular necrosis. Long-term administration of CCl_4 causes chronic liver injury and is a widely accepted model to produce hepatic fibrosis [9]. Hepatic damage induced by CCl_4 resulted in an increase in serum ALT concentration [5, 6, 33].

Serum enzymes of intracellular hepatic origin, including ALT are used in the evaluation of hepatic disease and their assays are considered helpful in screening the liver damage [34]. The elevation of concentrations of serum enzymes such as ALT is generally regarded as one of the sensitive markers of hepatic damage [8]. Elevation of serum ALT activity reflects either chronic or acute active liver damage. Acute inflammatory hepatocellular disorders result in extremely high serum transaminases levels [35].

In the present study, it was found that CCl_4 caused significant elevations in the level of serum ALT. The increase in the serum enzymatic levels recorded for ALT are likely the result of the damaged hepatic cells and/or impaired permeability of cell membrane due to the toxic effects of CCl_4. This finding is in agreement with Wang *et al.* [36] and Poojari *et al.* [37] who reported that CCl_4 induced increase in biochemical marker enzyme ALT.

Ccl_4-induced acute liver injury may be initiated by the $^{\bullet}CCl_3$ radical, which is formed by a metabolic enzyme (cytochrome P450) and could induce peroxidation of the unsaturated fatty acids of cell membrane and lead to membrane injury and leakage of enzyme such as ALT [38]. In fact, ALT are the most sensitive indicator of liver injury, with the extent of hepatic damage assessed by the serum level of enzymes released from cytoplasm. It has been demonstrated that ALT enzyme is one of the indices of the degree of cell membrane damage [39].

In recent years, many researchers have examined the effects of plants used traditionally by indigenous healers and herbalists to support liver function and treat diseases of the liver. In most cases, research has confirmed traditional experience and wisdom by discovering the mechanisms and modes of action of these plants as well as reaffirming the therapeutic effectiveness of certain plants or plant extracts in clinical studies [7].

Studies on hepatotoxicity induced by CCl_4 indicated that hepatic damage can probably be prevented by some herbal extracts [40,41]. Herbs are also known to play a vital role in the management of various hepatic disorders [41, 42]. Hepatoprotective studies by Mahmoud *et al.* [8] showed that plants have active ingredients that are capable of free radical scavenging in living organisms.

In the present work, Treatment of toxicated mice with defatted methanol extracts of *Conocarpus erectus* different parts in a dose of 500 mg/kg for two weeks decreased significantly the levels of ALT and insignificantly decrease in blood urea. Also, no differences in total proteins, albumins, globulins and A/G ratio were recorded when compared to control group. Previous work by Abdel-Hameed *et al.* [21] revealed that the four defatted methanol extracts of *C. erectus* different parts (leaves, stems, fruits and flowers) showed high free radical scavenging activity toward DPPH radical with SC_{50} between 6.47-9.4µg/ml. There are many methods; chemical and biochemical used to assay *in vitro* the antioxidant properties of plant extracts. In this work and to confirm the antioxidant properties of the four extracts, phosphomolybdenum and reducing power activity methods were used. It has been reported that the two methods were successfully used as significant indicators to antioxidant activity of many plant extracts [22, 43-45].

In this work, the results showed that the four extracts have high antioxidant activity. From results obtained in Tables 2 and 3, the antioxidant activity of the four extracts based on phosphomolybdenum and reducing power methods are attributed to the presence of phenolic compounds as major components in these extracts. Cao *et al.* [46] reported that, Plant phenolics such as flavonoids, phenolic acids and tannins found in vegetables, fruits or medicinal plants constitute one of the major groups of compounds acting as primary antioxidant or free radical terminators.

CONCLUSION

The results of this study provide evidence that the four defatted methanol extracts of *Conocarpus erectus* different parts; leaves, stems, flowers and fruits; have

high antioxidant and hepatoprotective activity. The phenolic compounds are the major components of these extracts and the antioxidant properties were attributed to them. Therefore, this plant may have great relevance in the prevention and therapies of diseases in which oxidants or free radicals are implicated after more *in vivo* studies for understanding their mechanism of action as antioxidant.

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