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THE PREVENTIVE ROLE OF *Thymus vulgaris* ALCOHOLIC LEAF EXTRACT AGAINST CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY IN MALE ALBINO RABBITS

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Abstract

This study aimed to evaluate the preventive role of *Thymus vulgaris* alcoholic leaves extract against carbon tetrachloride (CCl_4) hepatotoxicity in male albino rabbits. Thirty rabbits were randomly divided into 5 groups (6 rabbits/group). They were dosed daily for 21 days as follows: The 1st group was given 1.5 ml olive oil/kg of body weight (BW) orally days/week as a negative control. The 2nd group was treated with 3 ml orally (1 CCl_4:1 olive oil) as a singular dose every 3 days/week a control. The 3rd, 4th and 5th groups were treated with 3 ml CCl_4/kg BW, and after 30 min of the ingestion, these groups were treated once with *Thymus vulgaris* alcoholic (200, 400, 600 mg/kg) daily, respectively. The results showed that treatment with CCl_4 caused a significant decrease in the level of protein, albumin, globulin and high-density lipoprotein-cholesterol, but a significant increase in the level of bilirubin, cholesterol, triglycerides, very low-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase in serum of positive group as compared with the negative group. A significant decrease in the level of the glutathione, increase in the level of malondialdehyde and protein were observed in liver tissue, which indicates the ability of CCl_4 to induce hepatotoxicity. The results showed that the rabbits that dosed with *Thymus vulgaris* extract caused an increase in some of the liver function test and lipid profile. It is concluded that the *Thymus vulgaris* possesses liver protective activity against CCl_4 induced hepatotoxicity in rabbits.

INTRODUCTION

The liver is one of the largest internal organs in the body. It is involved with almost all the biochemical metabolic pathways for nutrient supply, energy provision, growth, reproduction, and fighting against diseases [1]. The major functions of the liver are proteins, lipids and carbohydrate metabolisms, detoxification, secretion of bile, and storage of vitamins and iron [2]. As a major organ of metabolism and excretion, the liver is constantly given the task of detoxifying microorganisms, environmental pollutants and chemotherapeutic agents [3]. Therefore, the liver is highly affected primarily by toxic substances such as CCl_4 through different mechanisms [4]. The CCl_4 induced hepatotoxicity is widely used to study of hepatoprotective impacts of plant extracts and drugs [5].

At present, there is an increasing interest in medicinal herbs or plants in most countries all over the world. There are many plants and herbal formulations that claim to have hepatoprotective activities [6]. Of those plants, the Thyme (*Thymus vulgaris*) is the only cultivated type of *Thymus* genus, which contains 300 species that are grown widely. *Thymus vulgaris* belongs to the family Lamiaceae [7]. Thyme is used for different medical purposes. Antitussive, expectorant and spasmolytic impacts are considered to be the main pharmacological properties of thyme, and because of that it is used for diseases of the upper respiratory tract, alone or mixed with other herbs [8].

Thymus vulgaris is a herb that is characterized by a natural source of antioxidant [9], and it contains a high percentage of essential oils such as thymol and carvacrol [10], and it contains essential antioxidants such as phenols, flavonoids, saponins, resins, gums and tannins [11]. On the other hands, Vetvicka and Vetvickoua [12] revealed that the limited impacts of the thyme derived essential oils as immunomodulator, anti-inflammatory and liver protection.

The current study aimed to assess the hepatoprotective activity of *Thymus vulgaris* alcoholic leaves extract against CCl_4 induced hepatotoxicity in male rabbits. The rabbits used in this study had similar metabolism to human beings.

MATERIALS AND METHODS

Plant Material

The plant material used in the current study was the leaves of *Thymus vulgaris*. The leaves were obtained from the local markets at Mosul city, Iraq. One kg of these leaves were weighed, washed with tap water to remove dust, and dried for 10 days at room temperature in shade. The dried leaves were ground by the electric grinder to obtain the dry powder. This powder was put in polyethylene bags.

Preparation of Thyme Alcoholic Leaves Extract

The alcoholic extract of *Thymus vulgaris* was prepared using the Harborne method [13]. 50 grams of dried powdered leaves were dissolved in 100 ml (70% v/v) ethanol. The flask containing the mixture was put on a magnetic stirrer for 10 min at 40 °C, and the mixture was left for 24 h at 24 ± 28°C in a dark place. Then, the mixture was filtered, and this process was repeated more than 3 times, and the filtrate was evaporated by rotary vacuum at low temperature (40°C) to obtain crude extract. The residual extracts were lyophilized, and the powder was kept at - 20°C until used [7].

Two grams extract was dissolved in 3 ml ethanol alcoholic (95% v/v), and the volume was supplemented with distilled water to obtain extract at 200 mg/ml. Then, other concentrations were prepared and kept in the refrigerator until used.

Chemicals

Carbon tetrachloride [CCl_4] (98.8% purity) was used in this study. It was obtained from Merck, Germany. Since CCl_4 is a hepatotoxic agent, it induces hepatitis in the animals.

Animals Used and Prepared

In this study, thirty adult male New Zealand white rabbits (*Oryctolagus cuniculus*) were obtained from the College of Veterinary Medicine, Mosul University, Mosul City. Their age ranges were 8 to 10 months, weighing from 1250 to 1500 g. They were placed in aluminum cages under constant conditions of room temperature (24±2 °C) and lighting (14 hours light/10 hours dark), and then subjected to a probation period of two weeks for the rabbits to acclimatize to the place. Rabbits were supplied a tap water *ad libitum* and standard forage diet until the end of the experiment.

Experimental Design

Thirty adult male rabbits were divided randomly into five groups (6 rabbits per group with approximately similar weights), placed in separate cages and were dosed and treated daily for three successive weeks as follows:

1. The first group was served as a negative control group (untreated group): This group includes rabbits that were given olive oil. A singular dose (1.5 ml/kg of BW) was given orally every 3 days/week by a gavage tube [14].
2. The second group was served as a positive control group: A dose of CCl_4 in olive oil (1:1) (3 ml/kg of BW) was given as a singular dose every 3 days/week for 21 days [14].
3. Third group (T1): A dose of CCl_4 in olive oil (1:1) (3 ml/kg BW) was given as a singular dose every 3 days/week for 21 days, and after 30 minutes of the ingestion, this group was treated with of *Thymus vulgaris* alcoholic extract (200 mg/kg of BW) daily once for 21 days [14].
4. Fourth group (T2): A dose of CCl_4 in olive oil (1:1) (3 ml/kg BW) was given orally as a singular dose every 3 days/ week for 21 days, and after 30 minutes of the ingestion, this group was treated with of *Thymus vulgaris* alcoholic extract of (400 mg/kg BW) daily once for 21 days.
5. Fifth group (T3): A dose of CCl_4 of in olive oil (1:1) (3 ml/kg BW) was given orally as a singular dose every 3 days/week for 21 days, and after 30 minutes of the ingestion, this group was treated with of *Thymus vulgaris* alcoholic extract of (600 mg/kg BW) daily once for 21 days.

Collection and Preservation of Samples and Tissues

At the end of the experiment (21 days), all rabbits were fasted for 14 h (overnight). Then, the blood samples were collected by heart stab and further separated to obtain serum for biochemical testing. Rabbits were then killed by dislocation of their necks after collected the blood samples. The liver of these rabbits were removed after dissection and put it in ice-cold normal saline.

Three grams of liver homogeny was prepared in 3 ml of phosphate buffer pH 7.4 by using an ultrasonic tissue homogenizer. The resulting homogenate was centrifuged at 4000 rpm for 15 min at 4 °C [15]. Then the resultant

supernatant was taken to estimate the concentration of glutathione (GSH) by using Ellman's reagent method [16], malondialdehyde (MDA) by using the thiobarbituric acid (TBA) method [17], and amount of protein by using the Lowry method [18].

Serum Biochemical Parameters Determination

The blood serum was used to estimate the concentration of several biochemical parameters using the methods described in Table 1. The estimation of the concentration of biochemical parameters that include lipids (cholesterol, triglycerides, high-density lipoprotein-cholesterol HDL-c) and liver functions test (protein, albumin, total bilirubin, ALP, AST, ALT) were carried out using kits obtained from English Randox Company, Franch Biolabo Company and German Biocon Company. They were estimated in serum using available kits based on the spectrophotometric methods according to the manuals supplied.

Globulin was estimated according to the following equation [19]:

$$\text{Protein} - \text{Albumin}.$$

Very low-density lipoprotein-cholesterol VLDL-c was estimated according to the following equation [20]:

$$\text{VLDL-c (mg/dl)} = \frac{Triglycerides}{5}$$

Low-density lipoprotein-cholesterol LDL-c was estimated according to the following equation [20]:

$$\text{LDL-c (mg/dl)} = \text{Cholesterol-} (\text{HDL-c} + \text{VLDL-c}).$$

The atherogenic index was estimated according to the following equation [21]:

$$\text{Atherogenic index} = \frac{\text{Cholesterol}}{HDL-c}$$

Table 1: The methods used for estimation of biochemical parameters.

Measured biochemical parameters	Method used	Reference
Protein concentration	Biuret method	Gornall *et al.* [22]
Albumin concentration	Bromocresol green method	Doumans *et al*. [23]
Total Bilirubin concentration	Colorimetric method	Jendrassik & Grof [24]
Cholesterol concentration	Enzymatic method	Allain *et al*. [25]
Triglycerides concentration	Enzymatic method	Fossati & Preencipe [26]
HDL- C concentration	Enzymatic method	Sugiuchi *et al*. [27]
ALP activity (Alanine aminotransferase)	Colorimetric method	Kind & King [28]
AST activity (Aspartate aminotransferase)	Colorimetric method	Reithman & Frankel [29]
ALT activity (Alkaline phosphatase)	Colorimetric method	Reithman & Frankel [29]

Statistical Analysis

The data were analyzed using the Complete Randomized Design (CRD.), Duncan Multiple Range Test. The results were taken to test for differences between groups. The differences were considered significant if $P \leq 0.05$. The Statistical Software used was IBM ® SPSS Statistics Version 23.

RESULTS AND DISCUSSION

Effect of the Treatment on Liver Functions and Oxidative Stress Status of Liver Tissue

Table 2 shows the changes in protein, albumin, globulin and total bilirubin concentration in experimental groups. The results showed a significant decrease in concentration of each of protein, albumin, and globulin. In contrast, the total bilirubin concentration showed a significant increase in serum of male albino rabbits treated with CCl_4 of 3 ml/ kg BW. A single dose was given for 3 days/week for 21 days (Positive control) as compared with the negative the

Table 2: The protective effect of *Thymus vulgaris* alcoholic leaves extract on liver function tests (liver biochemical indices) in CCl_4 induced hepatotoxicity in male albino rabbits.

Parameters / Treatment groups	Mean ∓ SD*						
	Protein concentration (g/dl)	**Albumin concentration(g/dl)**	**Globulin concentration (g/dl)**	**Total Bilirubin concentration (mg/dl)**	**ALP Activity (U/L)**	**AST Activity (U /L)**	**ALT Activity (U /L)**
Negative control group	8.29±0.19^{a}	4.38±0.17^{b}	3.90±0.38^{a}	0.19±0.05^{b}	24.47±0.60^{c}	15.36±0.73^{d}	26.36±0.22^{d}
Positive control group (CCl_4 3ml /kg BW.)	5.36±0.11^{d}	3.18±0.04^{d}	2.18±0.67^{d}	0.24±0.01^{a}	43.23±1.36^{a}	41.47±0.17^{a}	72.36±0.67^{a}
CCl_4 + *T. vulgaris* (200 mg/kg BW.)	6.35±0.22^{c}	3.87±0.69^{c}	2.48±0.16^{b}	0.22±0.06^{b}	37.23±0.71^{b}	32.40±0.58^{b}	49.26±0.75^{b}
CCl_4 + *T. vulgaris* (400 mg/kg BW.)	7.47±0.13^{b}	4.09±0.02^{b}	3.38±0.12^{c}	0.21±0.08^{b}	23.50±1.10^{c}	26.90±2.27^{c}	38.19±0.41^{c}
CCl_4 + *T. vulgaris* (600 mg/kg BW.)	8.81±0.11^{a}	5.17±0.04^{a}	3.64±0.75^{a}	0.19±0.01^{b}	22.16±0.73^{c}	19.48±0.80^{d}	27.57±0.80^{d}

*Values represent Mean ∓ SD of three replicate (n=6 rabbits/ group).

Numbers are followed vertically by various letters indicate a significant difference at the level of probability ($P \leq 0.05$) and correct reverse according Duncant test between groups

negative group. However, when rabbits were dosed with *Thymus vulgaris* alcoholic extract at different concentration (200, 400, 600 mg/kg BW) concomitantly with CCl_4 for 21 days resulted in a significant increase in the concentration of each protein, albumin and globulin followed by a significant decrease in total bilirubin concentration compared to the positive group. The results in Table 2 are consistent with the results of [5,6,30,31] for the treatment of animals (rats or rabbits) with different CCl_4 concentrations, and the extraction of different plants for different periods.

The liver is the largest vital internal organ in our body that is responsible for the detoxification of drugs and toxic chemicals. Therefore, it is the target organ for all toxic chemicals. Many chemicals found in the ecosystem are toxic and required precise identification of their potential risks to both human and animal health. Among different chemicals that cause organ injury, CCl4 was found to be the most toxic [14]. The toxicity of CCl_4 to the liver is largely due to its active metabolite [6].

Diminution of protein, albumin and globulin concentrations caused by CCl_4 is another indication of liver damage. *Thymus vulgaris* alcoholic leaves extract increased the concentration of serum protein towards the normal value of each, which indicates liver activity. Stimulating protein synthesis is the contributory hepatoprotective mechanism, which speeds up the regeneration and production of liver cells [32].

When liver cells are damaged the ALP, AST and ALT of liver cells will be released into serum. Therefore, the activity of these enzymes should serve as hepatotoxicity indices [7]. Based on the biochemical indices (Table 2), the treatment of CCl_4 dosed rabbits resulted in a significant increase in serum ALP, AST and ALT activity relative to the negative group. Meanwhile, the different *Thymus vulgaris* extract concentrations, each one alone concomitantly with CCl_4 causes a difference in increase and decrease in ALP activity as compared with the positive group, but the activities of AST and ALT were shown descending increase in these activities of enzymes as compared with positive group. The results of these marker enzymes' activities in our study were consistent with the results of several studies using a CCl_4 model that caused liver damage [6,30,31,33,34]. But when treatment of rabbits with *Thymus vulgris* extract, the activities of these marker enzymes were almost normal or only slightly elevated indicating protection against liver damage. ALP activity is linked to hepatocyte function.

In this study, hepatotoxicity induced by CCl_4 was reflected by a marked rise in ALP, ALT and AST activities. A significant increase of ALP, AST and ALT activities in serum after dosing with CCl_4 indicates a loss of the functional and structural integrity of the liver and release of these enzymes from liver cells to the circulatory system.

The results in Table 3 show a significant decrease in the concentration of glutathione GSH, while showing a significant increase in the concentration of each malondialdehyde MDA and protein in liver tissue respectively of male rabbits treated with a single dose of CCl_4 of (3 ml/kg BW.) for 3 days/week for 21 days (positive group) compared with the negative group. But when rabbits were treated with *Thymus vulgaris* each one alone of (200,400 mg/kg BW.) concomitantly with CCl_4 causes a significant decrease in GSH concentration in liver tissue as compared with the positive control group. But when treated with (600 mg/kg B.W of *T. vulgaris*) concomitantly with CCl_4 has led to return the level of GSH concentration to its natural value. And accompanied by a significant descending increase in concentration of malondialdehyde. But a significant decrease in the concentration of protein in all groups of treated with different concentration *Thymus vulgaris* concomitantly with CCl_4 compared with positive group.

CCl_4 was induced hepatotoxicity by metabolic activation or was commonly used to induce liver damage because it was metabolized by cytochrome P-450 in hepatocytes, generating a highly reactive carbon-centered trichloromethyl free radical (CCl_3), in which combination with cellular proteins and lipid in the presence of O_2 induces lipid peroxidation, and thus causes liver fibrosis [5,31,35,36,37].

Lipid peroxidation is the main sign of oxidative toxicity caused by the induction of oxidative degradation of cellular membrane lipids rich in polyunsaturated fatty acids forming MDA. Thus, it leads to an increase in MDA concentration, which is one of the end products of lipid peroxidation [38]. The positive group dosing with CCl_4 alone showed a maximum increase in the concentration of MDA approximately two-fold compared with the negative group. This is an indication of hepatotoxicity causing a change in function and structure of cellular membranes [31,39,40] resulting in the production of many free radicals [41].

These results showed that the treatment with *Thymus vulgaris* extracts protected against the loss of these antioxidants (albumin, bilirubin, GSH) activities, which is known to serve biological functions, including cell protection from oxidative stress status by free radicals [42]. Glutathione, a non - enzymatic antioxidant, is the first line of defense to protect cells against oxidative stress, which contains sulfhydryl group that is associated with different types of free radicals [43].

Effect of Treatment on Lipid Profile

The results showed in Table 4 show the effect of CCl_4, *Thymus vulgaris* extract and their combination on lipid profile. After 21 days of CCl_4 treatment with 3 ml/kg BW, there was a significant increase in concentration cholesterol, triglycerides, very low-density lipoprotein-cholesterol VLDL-c, low-density lipoprotein–cholesterol LDL-c and atherogenic index compared with the negative group. In contrast, HDL-c showed a significant decrease compared with the negative group. But treatment with *Thymus*

Table 3: The protective effect of *Thymus vulgaris* alcoholic leaves extract on oxidative stress status and protein concentration of liver tissue in CCl_4 induced hepatotoxicity in male albino rabbits.

Parameters / Treatment Groups	Mean ∓ SD*		
	GSH concentration (µmol/g wet tissue)	**MDA concentration (nmol/ g wet tissue)**	**Protein concentration (mg/g wet tissue)**
Negative group	7.271±0.15[a]	375.93±10.56[e]	55.510±0.39[b]
Positive group (CCl_4 3ml /kg BW.)	5.510±0.10[d]	727.06±5.06[a]	74.766±1.32[a]
CCl_4 + *T. vulgaris* (200 mg/kg BW.)	6.106±0.04[b]	539.23±6.26[b]	25.37±0.91[e]
CCl_4 + *T. vulgaris* (400 mg/kg BW.)	6.615±0.17[c]	447.80±3.03[c]	28.043±0.71[d]
CCl_4 + *T. vulgaris* (600 mg/kg BW.)	7.280±0.12[a]	409.93±4.78[d]	3.333±0.60[c]

* Values represent Mean ± SD of three replicate (n=6 rabbits/group).
Numbers are followed vertically by various letters indicate a significant difference at the level of probability ($P \leq 0.05$) and correct reverse according Duncan test between groups.

vulgaris alcoholic extract at 200, 400, 600 mg /kg BW concomitantly with CCl_4, respectively for 21 days showed a significant decrease in the concentration of cholesterol, triglycerides, very low-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol and atherogenic index compared with positive group. However, the concentration of high-density lipoprotein-cholesterol HDL-c showed an increase in this parameter as compared with the positive group.

The results in this study are identical with the results of many studies using a modal of CCl_4 [5,33,44,45,46,47]. The changes in the concentration of the lipid profile could be attributed to the defect of liver functions. The CCl_4-treated rabbit was found to increase cholesterol and triglycerides resulting in an increase in triglyceride measured VLDL-c concentration, of which 55% of VLDL-c is triglycerides [48]. The increase in triglycerides and VLDL-c could be attributed to the decrease in lipoprotein lipase in lipid tissue [15], which catalyzes the conversion of triglycerides to fatty acid. And the increase in cholesterol could be attributed to the inhibition of 3-hydroxy-3-methylglutarate reductase which is responsible for the regulation of concentration of cholesterol in the blood causing its accumulation and the increased concentration [49]. The increase in LDL-c may result from the effect of CCl_4, which inhibits the production of Apo-protein B-100 from the liver resulting in the production of LDL-c, which has a small size and rich in cholesterol and had difficult metabolism than other lipids. The changes in concentration of these lipoproteins could be due to the dissociation caused by CCl_4 as a result of the formation of free radicals and also to the increase in oxidative stress status [50].

Liver diseases lead to changes in lipoproteins levels, increase in VLDL-c, LDL-c, and decrease in HDL-c in serum as a result of liver damage causing biochemical changes affecting the transport of lipoprotein from the liver [51]. Saraswat *et al*. [52] had confirmed that the decrease in the HDL-c after treatment with CCl_4 results from the inhibition of production of protein, and the differences in phospholipids metabolism, which indicates the abnormal lipoprotein level in the serum. The treatment with *Andrographis paniculata* plant extract results in a better level of lipoprotein in serum, which indicates the effect of this extract in the protection of liver protein production and phospholipids.

When rabbits were treated with different concentrations of *Thymus vulgaris* extract concomitantly with CCl_4, the treatment causes an increase in HDL-c and a decrease in cholesterol, triglycerides, VLDL-c and LDL-c levels due to the presence of active *Thymus* components. The GSH defense system is accelerated and inhibited free radicals synthesis causing the return of the cell to its normal state [53]. Assmann & Gotto [54] had also pointed to the increase in HDL-c was regarded as a pointer for the correct metabolic activities, because it acts as a carrier for cholesterol transport to the liver, where it is degraded and excreted out. Guifraz *et al.*[55] found that the mice injected with CCl_4 and *Taraxacum officinale* extract caused the conversion of VLDL-c, LDL-c and HDL-c to the normal levels, and this was due to the high level of flavonoids and phenolic compounds and their effect on antioxidants and free radicals in mice liver.

CONCLUSION

It was concluded from this study that it seems likely that the dosage of rabbits with (200, 400, 600 mg/kg BW) of *Thymus vulgaris* alcoholic extract for 21 days of treatment caused improvement and enhancement of liver function and possesses liver protective activity against CCl_4 hepatotoxicity in rabbits.

Table 4: The protective effect of *Thymus vulgaris* alcoholic leaves extract on serum lipid profile in CCl_4 induced hepatotoxicity in male albino rabbits.

Parameters / Treatment Groups	Mean ∓ SD*					
	Cholesterol concentration (mg/dl)	**Triglycerides concentration (mg/dl)**	**VLDL-c concentration (mg/dl)**	**LDL-c concentration (mg/dl)**	**HDL-c concentration (mg/dl)**	**Atherogenie index**
Negative group	186.30±2.81^{b}	149.60±0.52^{b}	29.92±0.10^{b}	121.48±3.45^{b}	34.56±1.01^{b}	5.39±0.24^{b}
Positive group (CCl_4 3ml/kg BW.)	234.90±3.43^{a}	205.50±2.36^{a}	41.09±0.47^{a}	176.47±3.39^{a}	17.30±0.49^{d}	13.58±0.78^{a}
CCl_4 + *T. vulgaris* (200 mg/kg BW.)	150.20±1.32^{c}	119.40±0.66^{d}	23.88±0.13^{e}	104.98±1.27^{b}	21.36±0.40^{c}	7.03±0.15^{b}
CCl_4 + *T. vulgaris* (400 mg/kg BW.)	111.80±5.26^{d}	134.60±2.08^{c}	26.92±0.41^{d}	46.64±4.77^{c}	38.23±0.81^{b}	2.92±0.07^{c}
CCl_4 + *T. vulgaris* (600 mg/kg BW.)	97.60±1.09^{e}	141.50±2.53^{e}	28.31±0.50^{c}	23.62±3.80^{d}	45.63±2.82^{a}	2.14±1.09^{d}

*Values represent Mean ± SD of three replicate (n=6 rabbits/ group).

Numbers are followed vertically by various letters indicate a significant difference at the level of probability ($P \leq 0.05$) and correct reverse according Duncant test between groups.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this manuscript.

ANIMAL ETHICS

The animals used in this study were treated with compassion while conducting all the transactions, in terms of providing abundant food and water, health care, and the manner of handling the animal gently during dosing, according to the animal ethics followed in international research.

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