Archives of Razi Institute, Vol. 76, No. 6 (2021) 1777-1786

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DOI: 10.22092/ARI.2021.356239.1812

Original Article

Enzymatic Effectiveness of Alcoholic and Aqueous Extract of *Salvia Officinalis* in Mice Poisoned with Tetrachloride

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Received 6 December 2021; Accepted 13 October 2021

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Abstract

Regarding the antioxidant, anti-inflammatory, and antibacterial effects of *Salvia officnalis* (*S. officinalis*) extracts and the use of medicinal herbs as an alternative to chemical drugs, this study aimed to evaluate the enzymatic changes and reduction of hepatocyte damage in mice poisoned with carbon tetrachloride (CCl4) after treatment with aqueous and alcoholic extract of *Salvia officnalis*. A total of 40 adult male mice were divided into eight groups including six experimental, one negative, and one positive control group, which were exposed to CCl4 at the concentration of 2.3 mg/kg. The active compounds in the alcoholic and aqueous extracts of *S. officinalis* were obtained using high-performance liquid chromatography. Subsequently, *S. officinalis* extract in 100, 200, and 300 mg /kg doses were fed orally to mice for six days. The enzymes (GST, ALP, ALT, AST, and MDA) were determined in mice serum. The study results showed that enzyme activity was significantly decreased in the group treated with *S. officinalis* extract, and the concentration of 300mg/kg proved to be most effective. In addition, it was indicated that the alcoholic extract had a higher effect than the aqueous extract, which might be due to the greater amount of active compounds in the alcoholic extract. The improving effects of *S. officinalis* can be attributed to the bioactive components with antioxidant properties that inhibit the damaging effects of free radicals, chemical drugs, and tissue damage.

Keywords: Alcoholic extract, Aqueous extract, CCl4, Enzymatic effective, *Salvia officnalis*

1. Introduction

Salvia officinalis is a small perennial herbaceous plant from the Labiatae family, with branches that rise about 30 cm above the ground and green spots that become dark red with age. *S. officinalis* was originally cultivated in the Middle East and the Mediterranean region. This plant is one of the oldest and most famous plants in ancient and modern medicine. Today, the cultivation of this plant is common throughout the world (1).

One of the therapeutic effects of *S. officinalis* includes antioxidant effects, and antioxidants (containing flavonoids and phenolic acids) are important compounds of the volatile oil (Thujone) (2, 3). *S. officinalis* is used as an antiseptic and aromatic agent in the treatment of infections, as well as muscle cramps and mild indigestion (such as heartburn and bloating), excessive sweating, age-related cognitive disorders, and throat and skin inflammation (4, 5).

Comparison between the effects of aqueous and alcoholic extract of *S.officinalis* as an anti-inflammatory agent showed that the alcoholic extract of *S. officinalis* had a higher efficacy against *Streptococcus mutans* than aqueous extract. Moreover, the toxicity test results of alcoholic extract of *S. officinalis* revealed that it is a non-toxic compound (4, 6).

The liver plays an important role in protecting the body against the toxicity of chemical compounds. Regarding the increase in the environmental pollutants

following the development of the chemical industry and the production of drugs and pesticides that are considered harmful to human health, liver toxicity has become a common phenomenon due to such mechanisms as necrosis and fatty liver (7). The increase in the activity of liver enzymes can occur as a result of an increase in the synthesis processes in the cell or as a response to the growth processes occurring in the cell (8). Alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase (ALT) are concentrated in the liver and kidneys and are used to determine the extent of injury to hepatocytes (8). On the other hand, antioxidants have been shown to increase the activity of the glutathione S transferase (GST) enzyme through one of the most important detoxication processes that remove the toxic metabolites of some carcinogens and mutagens (8, 9).

Malondialdehyde (MDA) is the final product of the oxidation of polyunsaturated fatty acid and has a high level of toxicity. Moreover, it inhibits antioxidant enzymes and acts as a tumor initiator and pro-cancer agent (10). The MDA production creates free radicals in the process of lipid oxidation which results in damage to the unsaturated fatty acids in the cell membrane. That usually occurs within the process of acid oxidation in the cell (11).

This study aimed to evaluate the effect of the alcoholic and aqueous extract of *S. officinalis* on AST, ALT, GST, and MAD in the serum of male laboratory mice poisoned with CCl4.

2. Materials and Methods

2.1. Preparation of the Alcoholic and Aqueous Extracts of *S. officinalis*

Alcoholic extract of *S. officinalis* leaves was obtained using ethyl alcohol 70% and soxhlet extraction machine and dried using a rotary evaporator at 50° C. Aqueous extract was obtained using distilled water and following the above-mentioned technique. Wavelengths were determined using a UV-visible spectrophotometer to detect alcoholic and aqueous extracts at the wavelength of 278,356 nm and 305, respectively. The active compounds in the alcoholic and aqueous extract of *S. officinalis* were determined using the high-performance liquid chromatography (HPLC) Shimadzu A6 type, and the column type ODS CR18 (12). Mobile phase A was composed of anionic water+phosphoric acid at the rate of 1000:1 (v/gm), and mobile phase B was composed of acetonitrile+phosphoric acid at the rate of 1000:1 (v/gm). The plan for mixing mobile phase solutions in the separation process during its passage through the separation column consisted of (20min%, (15 30%, 25min) - 100%, (45min0) % B=0 and the average speed of the mobile phase. 1ml / 1min.

2.2. Approximate Qualitative Chemical Analysis of *S. officinalis*

Chemical qualitative analysis was performed and some active chemical substances in *S. officinalis* were detected based on the method mentioned in the studies conducted by Fu, Zheng (12), and Timm (13).

1) Detection of anthraquinones: 2 ml of chloroform CHCl3 were added to 1 ml of *S. officinalis* extract in a test tube. Shaking and filtration were performed subsequently. The filtered mixture was shaken again after the addition of an equal amount of ammonia solution 10%. The appearance of bright pink color, followed by a vortex mixer indicates the presence of anthraquinones.

2) Detection of flavonoids: In a test tube, an ammonia solution was added to the *S. officinalis* extract in a ratio of 1: 5, followed by the addition of 1 ml of concentrated sulfuric acid (H_2SO_4). The yellow color appeared, and the indication of the presence of the flavonoids disappeared afterward.

3) Detection of glycosides: 5 ml of *S. officinalis* extract was added to 2 ml of acetic ice, followed by the addition of one drop of ferric chloride solution ($FeCl_3$) and 1 ml of concentrated H_2SO_4. A brown ring formed on the inner face, indicating the presence of glycosides.

4) Detection of Phenols: 5 ml of plant extract was placed in a test tube to which a few drops of a $FeCl_3$ solution (5.0%) were added. The appearance of a dark

green color indicated the presence of phenolic compounds.

5) Detection of steroids: 2 ml of anhydrous acetate was added to 5.0 ml of plant extract, and 2 ml of H_2SO_4 was added afterward. The color changed from violet to blue or green, indicating the presence of steroids.

6) Detection of tannins: 5 ml of distilled water was added to 1 ml of plant extract and transferred to a boiling water bath. The mixture was then cooled with the gradual addition of a few drops of a ferric sulfate solution (1.0%) until a greenish-brown or bluish-black color appeared, indicating the presence of tannins.

7) Detection of Resins: 5 ml of hexane (C_5H_{10}) was added to 1.0 g of plant powder, followed by the addition of the same amount of copper acetate solution. The mixture was then shaken well and left aside until the layers separated and a green color appeared, indicating the presence of resins.

2.3. Animals Used

The current study was conducted on male mice (Balb/c) aged 8-10 weeks, at the weight range of 25-35 g. The mice were obtained from the animal house of the College of Veterinary Medicine, University of Tikrit, Tikrit, Iraq.

2.3.1. Experiment Design

The mice were randomly distributed into control and experimental groups. A total of 40 adult male mice were divided into eight groups, including a negative and a positive control groups and six experimental groups (three groups for each of the aqueous and alcoholic extracts) as follows:

First Group: Included five mice as negative controls that were not exposed to CCl4.

Second group: Included five mice as positive controls that were orally exposed to CCl4 (2.3 mg/kg) on the first day.

Third group: Included five mice that were orally exposed to CCl4 (2.3 mg/kg) on the first day, and 100 mg/kg of the aqueous extract solution was given to them after 6 h. The solution was given once a day for six weeks.

Fourth group: Included five mice that were orally exposed to CCl4 (2.3 mg/kg) on the first day, and 200 mg/kg of the aqueous extract was given to them after 6 h. The solution was given once a day for six weeks.

Fifth group: Included five mice that were orally exposed to CCl4 (2.3 mg/kg) on the first day, and 300 mg/kg of the aqueous extract was given to them after 6 h. The solution was given once a day for six weeks.

Sixth group: Included five mice that were orally exposed to CCl4 (2.3 mg/kg) on the first day, and 100 mg/kg of an alcoholic extract solution was given to them after 6 h. The solution was given once a day for six weeks.

Seventh group: Included five mice that were orally exposed to CCl4 (2.3 mg/kg) on the first day, and then 200 mg/kg of an alcoholic extract solution was given to them after 6 six h. The solution was given once a day for six weeks.

Eighth group: Included five mice that were orally exposed to CCl4 (2.3 mg/kg) on the first day, and then 300 mg/kg of an alcoholic extract solution was given to them after six h. The solution was given once a day for six days.

2.4. Collection of Blood Samples

The blood samples were obtained from the corner of mice's eyes using a capillary tube implanted in the orbital sinus (13). Subsequently, 2 ml of blood was placed in anticoagulant-free tubes. The serum was then separated by centrifugation at 3000 rpm for one min and stored in a freezer at -20 °C until the required analysis was performed subsequently (9).

2.5. Serum Biochemical Tests

Changes in AST and ALT enzymes were assessed using a commercial analysis kit from the French company Biomerieux according to the Reitman and Frankel colorimetric method (1957) (13). Changes in ALP enzyme were assessed using the same commercial analysis kit and Liang, Yuan (14) colorimetric method.

The GST enzyme was analyzed using the Biolabo kit (France) following the method used by Iniaghe, Malomo (15).

2.6. Estimation of Malondialdehyde Serum Level in Animals

Measurement of MAD, a by-product of fat oxidation, was performed using the colorimetric method (which depends on the interaction between the atoruric acid and MAD), following the method used by Elkhalafawy, Lasheen (16).

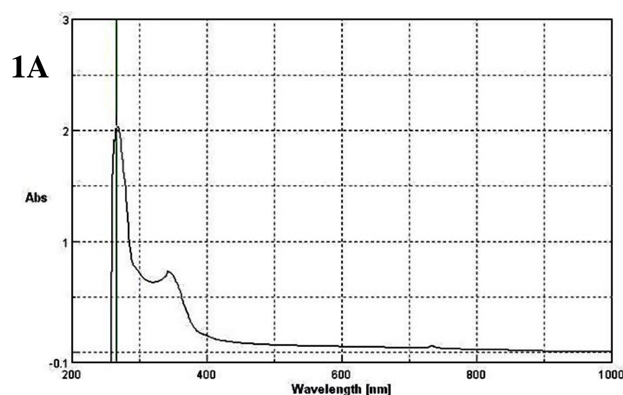
2.7. Statistical Analysis

The results obtained were analyzed using SAS 2001 program to find the significant differences between the groups. The Least Significant difference analysis was used at a probability level $P \leq 0.05$ to determine differences in treatment levels.

3. Results and Discussion

3.1. Measurement of the UV Spectra

The separation of aqueous and alcoholic extracts of *S. officinalis* leaves was confirmed using spectroscopy at the wavelength of 278, 356 nm, and 305 nm for alcoholic and aqueous extracts, respectively. The results are presented in figure 1 (A and B).



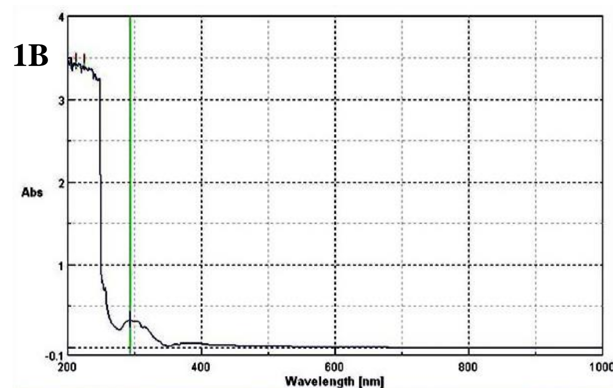


Figure 1. 1A. A UV-visible spectrum of alcoholic extract of *S. officinalis* leaves. **1B.** A UV-visible spectrum of aqueous extract of *S. officinalis* leaves

3.2. High-Performance Liquid Chromatography Analysis

Figure 2 shows the separation process of the components of the alcoholic extract of *S. officinalis* leaves. The first compound appeared at the retention time of 3.2 min, and other active compounds appeared at retention times of 7.6, 7.9, and 8.5 min using an HPLC device (14).

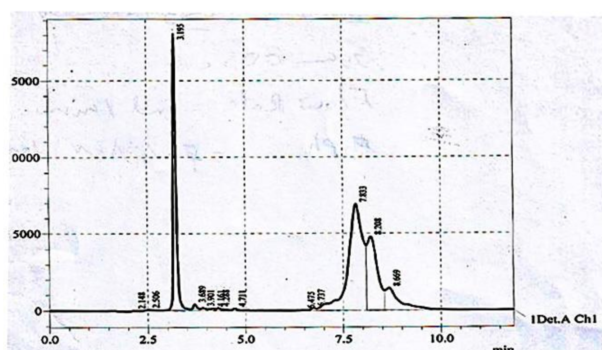


Figure 2. Separation process of the alcoholic extract of *S. officinalis* leaves using HPLC

3.3. Chemical Detection of the Active Substances of the *S. officinalis* Extract

The presence of flavonoids, saponins, steroids, tannins, terpenes, and alkaloids was confirmed through the chemical assessment of the active substances of the *S. officinalis* extract (Table 1). Tannins contain some phenolic compounds, such as gallic acid and tannic acid, which can break down enzymes involved in amino acid production and are essential for increasing cell division. Saponins contain saponin glycosides with hydroxyl groups that can dissolve the lipid layer present in the cell walls. This in turn affects the selectivity of the cell wall which facilitates the entry and exit of substances through the cell wall (15).

Elkhalafawy, Lasheen (16) showed that other secondary compounds with medicinal and physiological activity have anti-inflammatory, anti-

allergic, anti-viral, anti-cancerous, and anti-oxidants activities that repel free radicals.

Table 1. Chemical analysis of the active substances of *S. officinalis* leaves extract

No.	Materials	Results
1	Anthraquinones	+
2	Flavonoids	+
3	Phenols	+
4	Saponins	+
5	Steroids	+
6	Tannins	+
7	Resins	+

3.4. GST Enzyme Activity

The effects of the aqueous and alcoholic extract of *S. officinalis* on GST enzyme in the blood serum of mice poisoned with carbon tetrachloride (3.2 mg /kg) are presented in figure 3 and tables 2-3.

Based on the obtained results, the alcoholic extract had a higher effect than the aqueous extract of *S. officinalis*. The reason is that the alcoholic extract contained more active compounds, such as flavonoids and terpenes. The findings indicated that the level of GST activity decreased in the group treated with 100 mg/kg of the alcoholic and aqueous extract of *S. officinalis*, compared with the positive control group (the values were 64.85 and 66.92U/dHb, respectively). Moreover, a significant decrease in the activity of the GST enzyme was observed at a concentration of 200 mg/kg of both the alcoholic and aqueous extracts (the values were 58.76 and 61.37U/dHb, respectively). The effectiveness of alcoholic and aqueous extract at the concentration of 300 mg/kg was confirmed as well (the values were 53.19 and 57.09U/dHb, respectively).

According to the obtained results, the enzyme's activity decreased, compared to the positive control group that was given CCl_4. The reason was that the effectiveness of the GST enzyme increased with the administration of CCl_4. Moreover, in the treatment groups, the mice body contained antioxidants which help improve the body's immunity (17).

Based on the above results, the alcoholic extract of *S. officinalis* could increase the deoxidizing substance and reduce free radicals. This in turn decreased the effect of the main substance on the function of the GST enzyme. Therefore, the effect of the equal forms of GST transition coenzyme on controlling cell proliferation was observed in all hepatocellular carcinoma cells (18).

Many researchers have reported the effect of different types of cancers (e.g., esophageal, intestinal, and stomach cancers) on the activity of the GST enzyme. Therefore, it is necessary to maintain the balance of the GST enzyme and preserve the contents or components of the GST enzyme.

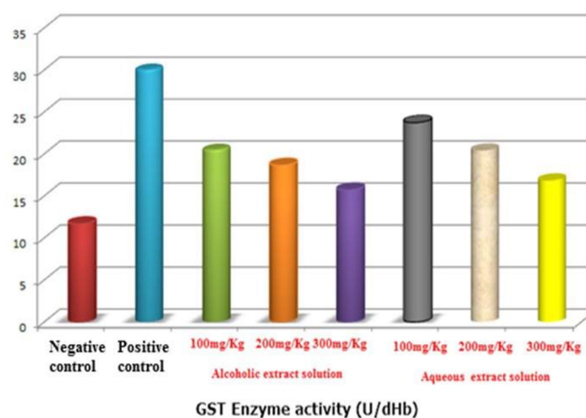


Figure 3. Effect of the aqueous and alcoholic extract of *S. officinalis* on GST enzyme

Table 2. Effect of alcoholic extract of *S. officinalis* on levels of biochemical parameters in mice poisoned with CCl_4 ($P\leq0.05$)

Parameter	Negative control	Positive control	Alcoholic extract		
			100mg/Kg	200mg/Kg	300mg/Kg
ALP	$45.63^{a}\pm3.67$	$69.32^{a}\pm4.01$	$64.85^{b}\pm4.67$	$58.76^{b}\pm3.78$	53.19a±3.91
MDA	$11.68^{c}\pm1.27$	$30.04^{b}\pm2.12$	$20.51^{a}\pm2.01$	$18.65^{a}\pm2.22$	$15.71^{b}\pm1.97$
GST	$0.98^{c}\pm0.07$	$2.2^{c}\pm0.09$	$1.93^{b}\pm0.05$	$1.67^{a}\pm0.04$	$1.42^{a}\pm0.04$
ALT	$31.45^{c}\pm4.17$	$44.67^{b}\pm3.99$	$40.92^{b}\pm3.51$	$37.56^{a}\pm3.13$	$34.78^{a}\pm3.22$
AST	$39.06^{a}\pm4.78$	$46.31^{a}\pm3.56$	$42.12^{c}\pm2.89$	$39.91^{a}\pm1.98$	$36.23^{b}\pm4.01$

Data as mean±SE (n=5).

Table 3. Effect of aqueous extract of *S. officnalis* on levels of biochemical parameters in mice poisoned with CCl_4 ($P\leq0.05$)

Parameter	Negative control	Positive control	Aqueous extract		
			100mg/Kg	200mg/Kg	300mg/Kg
ALP	$45.63^{a}\pm3.67$	$69.32^{a}\pm4.01$	$66.92^{c}\pm4.16$	$61.37^{b}\pm3.52$	$57.09^{c}\pm2.97$
MDA	$11.68^{c}\pm1.27$	$30.04^{b}\pm2.12$	$23.73^{b}\pm2.41$	$20.41^{c}\pm1.93$	$16.84^{a}\pm1.82$
GST	$0.98^{c}\pm0.07$	$2.2^{c}\pm0.09$	$2.04^{c}\pm0.11$	1.84b±0.07	$1.59^{b}\pm0.06$
ALT	$31.45^{c}\pm4.17$	$44.67^{b}\pm3.99$	$42.26^{a}\pm3.97$	$40.07^{b}\pm4.01$	$36.56^{b}\pm4.36$
AST	$39.06^{a}\pm4.78$	$46.31^{a}\pm3.56$	$44.61^{c}\pm2.96$	$40.81^{c}\pm3.09$	$38.32^{a}\pm2.87$

Data as mean±SE (n=5).

3.5. Activity of Alkaline Phosphatase Enzyme

The ALP enzyme has been found in different tissues and body organs, including the intestine, bone marrow, liver, and kidneys. Enzyme activity varies according to the changes in the pH, temperature, amount of acidity, and the concentration of the main substances in the presence of activators or inhibitors in the reaction medium. The effect of the alcoholic and aqueous extract of *S. officinalis* at three concentrations of the ALP enzyme in the blood of laboratory mice poisoned with CCl_4 was compared with that in the negative and positive control (Figure 4 and Tables 2 and 3). The ALP enzyme activity decreased in the group treated with 100 mg/kg of the alcoholic and aqueous extract, compared with the group poisoned with CCl_4 (65.80U/mg and 66.71U/mg, respectively) ($P\leq0.05$). More decrease was observed in groups treated with 100 and 200 mg/kg of alcoholic and aqueous extracts. The activity of the ALP enzyme in the concentration of 300 mg/kg of the alcoholic or aqueous extract was determined at 50.90 U/mg and 54.06 U/mg, respectively.

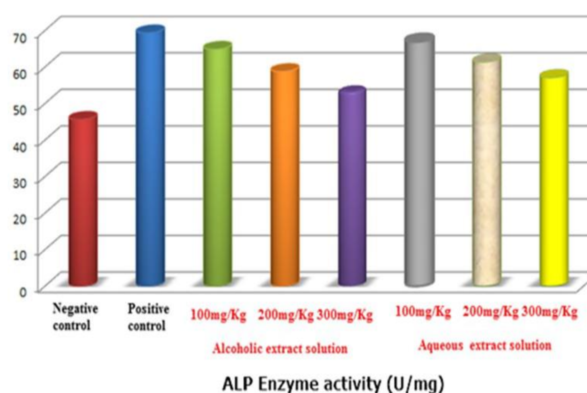


Figure 4. Effect of the aqueous and alcoholic extract of *S. officinalis* on ALP enzyme

Based on the obtained results, the alcoholic and aqueous extract of *S. officinalis* had a significant effect on the activity of the ALP enzyme in the blood of the laboratory mice poisoned with CCl_4, compared to the control group. This indicated that the cells had an antioxidant substance before CCl_4 was given as a stimulant. *S. officinalis* contains effective compounds that prevent enzymes from damaging the function and structure of proteins. It also inhibits the oxidation process of cellular contents, including nucleic acids, proteins, and lipids (19).

3.6. Aspartate Aminotransferase Enzyme Activity

The AST (EC 2.6) enzyme has been detected in high concentrations in many tissues, such as the heart, liver, and kidneys, and in lower concentrations in other tissues, such as muscles, red blood cells, and other body organs (20).

Based on the evidence, the concentration of AST enzyme in tissues increases following the damage to different body organs. The amount of enzyme increased in the tissues of different organs, including heart or liver in such diseases as hepatitis, severe anemia, mononucleosis, hereditary hemorrhage, multiple tumors, postoperative condition, acute burns, primary muscle disease, and exposure to very dangerous pollutants, such as benzene (C_6H_6) and carbon tetrachloride (CC_{14}) (20).

figure 5 and tables 2 and 3 show the effect of aqueous and alcoholic extract of *S. officinalis* on AST serum enzyme in mice poisoned with CC_{14} (3.2 mg/kg). The results showed that the alcoholic extract had a higher effect than the aqueous extract. The reason was that the alcoholic extract contained more active compounds, such as flavonoids and terpenes. The results indicated a lower level of AST enzyme activity in the group treated with 100 mg/kg of the alcoholic and aqueous extract. The level of enzyme in the serum of mice was determined at 39.91 and 40.817 U/L, respectively. Moreover, the effectiveness of alcoholic and aqueous extracts at the concentration of 300 mg/kg was determined at 36.23 U/L and 38.32U/L, respectively.

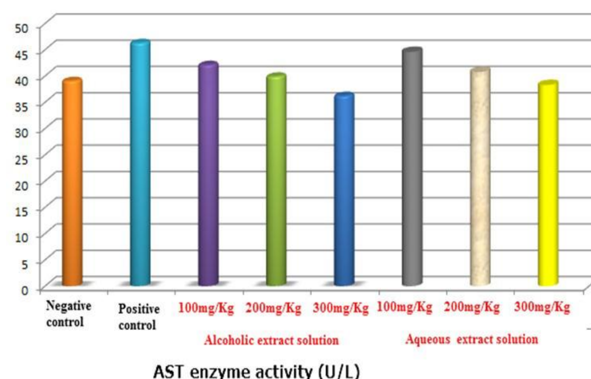


Figure 5. Effect of the aqueous and alcoholic extract of *S. officinalis* on the AST enzyme

The AST enzyme level increased with the administration of CCl_4 to the positive control group, which may indicate the severity of damage to the liver or other organs associated with CCl_4 (21).

It is evident from the above results that the alcoholic extract of *S. officinalis* can reduce injury or damage to the liver or organs that may cause an increase in the value of AST.

3.7. Activity of ALT Enzyme

The ALT is the second aminotransferase enzyme formerly known as Glutamate pyruvate transaminase. This enzyme has been found in high quantities in the liver, and in smaller quantities in the heart, skeletal muscle, and other organs. The ALT is elevated in liver diseases, myocardial infarction, muscle diseases, bile duct disorder, and when the body is exposed to toxic substances (20). The activity of the ALT enzyme increases significantly in pathological conditions in the liver tissue which is affected by an increase in cell permeability due to the abundance of enzymes in the cytoplasm (20).

Figure 6 and tables 2 and 3 show the effect of the aqueous and alcoholic extract of *S. officinalis* in serum AST of mice poisoned with carbon tetrachloride (3.2 m/kg). It was observed that the alcoholic extract had a higher effect than the aqueous extract. The results indicate the decreased activity of ALT enzyme in the group treated with 100 mg/kg of alcoholic and aqueous extract of *S. officinalis*, compared with a control group (the values were 40.92 and 42.267U/L, respectively). More decrease was observed at the activity of ALT

enzyme at a concentration of 200 mg/kg of both the alcoholic and aqueous extracts (the values were 37.56 and 40.077U/L, respectively). The results indicated the effectiveness of the alcoholic and aqueous extract at a concentration of 300 mg/kg (the values were 34.78U/L and 36.56 U/L, respectively).

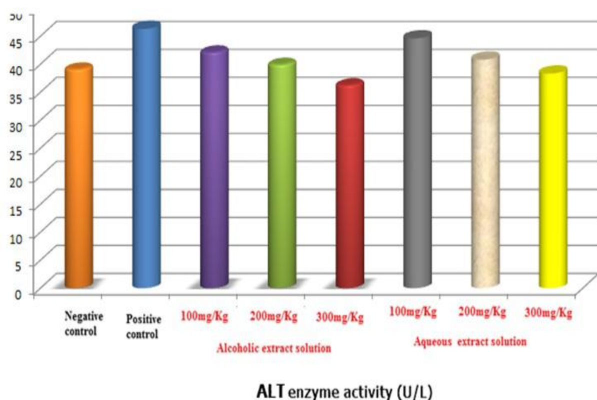


Figure 6. Effect of the aqueous and alcoholic extract of *S. officinalis* on the ALT enzyme

The ALT enzyme level elevated with the administration of serum carbon tetrachloride to the positive control group. This may indicate the severity of damage to the liver or other organs caused by CCl_4 (21). According to the results of this study, the alcoholic extract of *S. officinalis* can reduce injury or damage to the liver or organs that may increase the value of ALT.

3.8. Level of Malondialdehyde Activity

The study results showed the effect of aqueous and alcoholic extract of *S. officinalis* at three different concentrations on the level of MDA in the serum of laboratory mice poisoned with CCl_4 (at a concentration of 3.2 mg/ kg for one day). The results in the group of the experiment were significantly different from those obtained from the negative and positive control groups. A decrease in the MDA and the level of lipid peroxidation was observed in the group treated with 100 mg/kg of *S. officinalis* extract, compared with the negative control group (Figure 7 and Tables 2 and 3). The decrease in MDA was higher in the group treated with 200 mg/kg of extract (whether aqueous or alcoholic), compared to the group treated with 100 mg/kg of the extract. This result suggests the effectiveness of the extract.

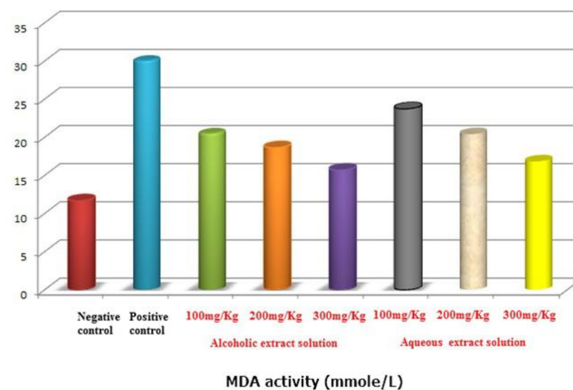


Figure 7. Effect of the aqueous and alcoholic extract of *S. officinalis* on the MDA activity

A significant decrease was observed in the group treated with 300 mg/kg of the extract, compared to the previous two groups (group 3 & 4). This indicates the effect of increased concentration of the alcoholic or aqueous extract of *S. officinalis* on the MDA activity and effectiveness of lipid peroxidation (22). The alcoholic or aqueous extract of *S. officinalis* was effective after CCl4 toxicity, indicating that the cells contained antioxidant flavonoids. Treatment of laboratory animals with alcoholic or aqueous extracts of *S. officinalis* leads to a decrease in the level of MDA. This confirms the antioxidant effect of *S. officinalis* extract and the fact that it contains active substances that are highly effective in reducing free radicals by a significant rate (23).

The results of the study showed that a high concentration of the alcoholic or aqueous extract of *S. officnalis* had high antioxidant effects. Moreover, it was revealed that the alcoholic extract had a higher effect, compared to the aqueous extract. The reason was that

the alcoholic extract contained more active compounds, including flavonoids and terpenes, as well as main active substances for controlling the effectiveness of all the studied enzymes in the blood serum of laboratory animals after the administration of CCl_4.

Authors' Contribution

Study concept and design: R. K.
Acquisition of data: N. H. A.
Analysis and interpretation of data: D. A. I.
Drafting of the manuscript: R. K.
Critical revision of the manuscript for important intellectual content: R. K.
Statistical analysis: N. H. A.
Administrative, technical, and material support: N. H. A.

Ethics

The present study was approved by the Ethics Committee of the Tikrit University, Tikrit, Iraq under the project number (No. 2020-7845-1458).

Conflict of Interest

The authors declare that they have no conflict of interest.

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