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Article

Assessment of active constituents and total flavonoids of *Ammi majus* plant extracts on immunological and kidney protective activities in mice

Farah T.O.Al-Jumaili[1*], Maysaa Hamid[1], Ashwaq S. Abed[2]
[1]College of Biotechnology, AL-Nahrain University, Iraq.
[2] Biotechnology Research Center, Al-Nahrain University, Iraq.
*Correspondence: biotech.farah@yahoo.com, Orchid: 0000-0003-2840-9521
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Abstract: *The Ammi majus* (Kella) plant is frequently used to cure various health issues. This study aimed to investigate the most important phytochemical compounds of Kella seeds and evaluate the effect of its aqueous and alcoholic extracts on CCl_4-induced nephrotoxicity in mice using a count of white blood cells (WBC) test and some biochemical markers for renal functions besides the histopathological study of kidney tissue. According to secondary metabolite analysis, the seeds contained tannins, alkaloids, saponins, flavonoids, and polyphenols at varying levels depending on the solvent utilized. Regarding total flavonoids, the alcoholic extract showed the highest concentration (193.2±25.7 mg/g) compared to the aqueous extract (176.3±19.2 mg/g). The WBC count test in the *in vivo* study showed an improved immune system by increasing the number of WBCs in mice treated with plant extracts and reducing the toxic effects of the CCl_4 compound compared with a negative control group. In the positive control group (treated mice with CCl_4), the biochemical analysis and histological study of the kidney sections revealed that CCl_4 causes nephrotoxicity through increasing urea and creatinine concentrations in the blood and lowering total protein content, as well as the formation of necrotic tissue in the kidney sections.
In contrast, these markers decreased in groups of mice treated with plant extracts, particularly alcoholic extract. In conclusion, the *A. majus* plant can improve kidney function during CCl_4 doses. Consequently, it could be a promising treatment for nephrotoxicity caused by certain drugs.

Keywords: *Ammi majus*; kidney markers; nephrotoxicity.

Introduction

Traditional medicine systems take a holistic approach to treating disease conditions by considering mind-body-physiology. For example, Ayurvedic philosophy recommends delivering "a group of phytochemical compounds" that can act on multiple disease targets and the immune pathways to provide adaptogenic and immunomodulatory effects [1]. The widespread use of synthetic medications and chemicals has resulted in several human health problems, including renal damage and numerous disorders. Ammi majus plant (Kella) belongs to the Apiaceae (Umbelliferae) family and is a wild medicinal herb, widely distributed in the Egyptian region, containing important bioactive compounds (such as coumarins, flavonoids, and essential oils) in all parts [2],

which has been used for centuries to treat a variety of diseases as a diuretic, immune-stimulatory, antioxidant, treatment of diabetes, vertigo, spastic bronchitis, abdominal cramps, and kidney stones[3, 4]. However, the main phytochemicals of Ammi plant species are derivatives of furanochromone and coumarins, which recorded more than 80 different compounds such as khellin and visnagin (the major compounds), khellinin, khellinone, khellol, visnadine, visnaginone, ammoidin, and others [5]. he therapy of kidney illnesses is frequently coupled with the use of khellin and visnagin; they have been demonstrated to protect renal epithelial cells against oxalate and Ca-oxalate monohydrate crystal injury, as well as to inhibit oxalate synthesis associated with hyperoxaluria by raising urine pH and concentration of citrate [6]. Several methods and different solvents using heated water [7], methanol [8], and ethanol [9] were tested to prepare Ammi plant extract to evaluate their phytochemicals and pharmaceutical activities. Carbon tetrachloride (CCl_4) is an organic chemical compound extensively used in vitro and in vivo investigations as a hepatotoxicity, nephrotoxicity, and carcinogenesis model to assess the effects of various plant extracts or drugs[10, 11]. The kidney is abundant tissue in mitochondria, which allows it to produce the "reactive oxygen species ROS" from NADPH oxidases and the mitochondrial respiratory chain. It is a particularly susceptible organ to oxidative stress damage [12].

The current project investigated the effects of different A. majus extracts on CCl_4-induced kidney damage and nephron-protection in mice.

Materials and Methods

Plant material

The dried seeds of the Ammi majus plant were collected from Baghdad's local market in December 2020 and were previously identified by the Iraqi Center of Herbs. After removing the impurities, the seeds were homogenized to a fine powder using the electric mill and stored in airtight bottles.

Plant extraction

Aqueous extraction

In a clean flask, 10 g powdered plant seeds were mixed with 100 ml sterilized distilled water and placed in a shaker incubator for 24h at 37°C. The mixture was then filtered through gauze into a glass tube, centrifuged at 500 rpm for 10 min, and the upper phase was filtered through Whitman No.1 filter papers before being distributed in sterilized Petri dishes. The aqueous extract was kept in Oven at 60°C until it dried up [13]. The dry scales of the extract were stored in a sterile sealed tube at -20°C until use.

Alcoholic extraction

Twenty g of seeds were extracted with 250 ml of methanol (70%) using a Soxhlet extraction device for 6 h at 55°C, [14] with some modifications. The extract was then filtered and dried with a rotary evaporator before being stored at 4°C until use.

Determination of the active phytochemicals in seeds of the Ammi majus plant

According to [15], the qualitative phytochemicals investigations of Ammi majus seeds were carried out as follows:

Detection of Tannins

A few drops of a 1% Lead acetate solution were added to each extract. The presence of tannins is indicated by forming a gelatinous or white precipitate.

Detection of Glycosides

One ml of each seed extract was added to 2 ml of Benedict reagent, which was then placed in a boiling water bath for 5 min and cooled. The red deposit revealed the presence of polysaccharides.

Detection of alkaloids (Dragangroff test)

Solution A comprises 60 mg Bismuth sub-nitrate dissolved in 0.2 ml HCl, whereas solution B has 600 mg Potassium iodide K.I. diluted in 1 ml D.W. The solutions A and B were combined and added to the plant aqueous extract; the presence of alkaloids was determined by forming an orange-to-brown color.

Detection of Saponins

The detection method will be done by vigorously shaking the plant extract solutions. Saponins will be detected by the appearance of foam at the top of the extract.

Detection of Flavonoids

The presence of flavonoids was confirmed by mixing a small amount of Sodium hydroxide (NaOH) solution with a small amount of plant extract solutions, allowing it to produce a bright yellow color.

Detection of Polyphenolic Compounds

A few drops of 3% Ferric chloride ($FeCl_3$) solution were added to each seed extract. The appearance of a brown precipitate after the reaction indicates the presence of polyphenolic compounds.

Determination of Total Flavonoids

The total flavonoid content in the plant extract was evaluated using the $AlCl_3$ colorimetric method, as illustrated in [16], with some modifications. The standard solution of Rutin was prepared in methanol at concentrations (2.5, 5, 10, 20, 40, and 80) µg/ml. Briefly, (3.2 mg) of each plant extract was dissolved in 5 ml of 50% methanol and 1 ml of 5% ($NaNO_3$) solution. After 6 min, 1 ml of 10% ($AlCl_3$) solution was added, and the mixture was left for 5 min before adding 10 ml of 10% NaOH solution. With distilled water (D.W.), the mixture was brought up to 50 ml with vigorous shaking. Immediately, the absorbance was measured at 510 nm.

Plant tested doses

The doses utilized in this study were based on 10% of the LD50 (20 mg/kg) as determined by [17]. The aqueous plant extract was dissolved in sterilized distilled water to prepare the treatment doses. In contrast, the alcoholic plant extract was dissolved in a few drops of Dimethyl sulfoxide (DMSO) and then diluted with appropriate volumes of sterilized distilled water. Carbon tetrachloride (CCl_4) was dissolved in virgin Olive oil and used for nephrotoxicity induction.

In vivo assay

For induction of acute toxicity, 24 adult male Swiss albino mice (body weight 22-28 g) were used. These mice were purchased from the "General Company of Veterinary Medicine" in Baghdad, Iraq, and kept in a well-ventilated room with customized pellets and a water diet. A single dose of 0.1 ml of each dose was given intraperitoneally for 7 days. Finally, on day 8, animals were slaughtered for laboratory testing. The animals were divided into six groups according to the Table 1 criteria.

Group 1 (-ve control)	**Four mice were treated with D.W.**
Group 2 (+ve control)	**Four mice were treated with CCl_4 at 20 mg/kg.**
Group 3	**Four mice were treated with *A. majus* aqueous extract at 20mg/kg.**
Group 4	**Four mice were treated with *A. majus* alcoholic extract at 20mg/kg**
Group 5	**Four mice were treated with *A. majus* aqueous extract + CCl_4 at 20 mg/kg of each treatment.**
Group 6	**Four mice were treated with *A. majus* alcoholic extract+ CCl_4 at 20 mg/kg of each treatment**

Table 1. Animals groups.

White blood cell (WBC) count

At week 8, the blood samples were taken from mice. The number of WBCs was calculated using the method described by [18]

Assessment of Kidney Biochemical Analysis

After the dosing period, Kidney protective actions were assessed using specific parameters, including evaluation of urea, creatinine, and total serum protein enzymes in the serum, along with histological investigation of kidney tissue. All tested mice were euthanized under the influence of diethyl ether through exsanguination. Blood was drawn from a cardiac puncture and left to clot for 15 minutes at room temperature in an Eppendorf tube. Centrifugation at 3000 rpm for 10 minutes was used to separate the serum [19]. In addition, mice kidneys were removed and preserved in Formalin 10% for histological analysis.

Urea

The activity of the urea enzyme was measured according to [20] in mice serum. The urea concentration (unit/l) was estimated using a commercial kit's standard curve (BioMerieux Company).

Creatinine

A colorimetric reaction assay was conducted to determine creatinine concentration (unit/l) depending on the standard curve of the commercial kit (BioMerieux Company), using the method of [21].

Total Serum Protein

The total serum protein (unit/l) was adopted according to the method of [22], using a commercial kit made by Biosystem Company.

Renal histopathological study

Preparation of histopathological sections was carried out according to [23]. Their kidneys were chopped into small pieces (2×2×2) mm before being pre-fixed in 2.5% Glutaraldehyde diluted in phosphate buffer (PBS, pH 7.4). After that, the specimens were washed several times in the same buffer and placed in PBS for 12 h before being post-fixed in 1% Osmium tetroxide for 1 h. The fixed tissues were dehydrated in ascending ethanol concentrations (50, 70, 90, and 99) % for 2 h at each concentration and then cleared in Xylene for 2 h. The samples were first immersed in paraffin xylene (1:1) for 30 min at 57-58 ºC, then in paraffin alone for 2 h at 60-70 ºC, before being embedded in pure paraffin wax (melting temperature 57-58 ºC), then allowed to solidify at room temperature. Ultimately, the tissue samples were sectioned using a rotary microtome (5µm) and then stained via hematoxylin and eosin dyes [24]. The specimens were examined and photographed under a light microscope.

Statistical analysis

Statistical analysis was carried out using a one-way analysis of variance. Data were represented as mean ± standard error at $P \leq 0.05$.

Results

Phytochemical screening and total flavonoids

Table 2 displays various phytochemical compounds detected in aqueous and alcoholic extracts from the Ammi majus plant seeds. The aqueous extract showed many tannins and polyphenol compounds, while the alcoholic extract appeared to have many total alkaloids and flavonoid compounds. Both extracts contained the same quantity of saponins, and no glycoside compounds were detected.

Regarding the concentration of phenolic compounds in both A. majus extracts, Table (3) shows that the alcoholic extract had the highest concentration of total flavonoids (193.2±25.7 mg/g) over the aqueous extract (176.3±19.2 mg/g).

Test name	Aqueous extract	Alcoholic extract
Tannins	++ve	+ve
Glycoside	Trace	-ve
Alkaloids	+ve	++ve
Saponins	++ve	++ve
Flavonoids	+ve	++ve
Polyphenols	++ve	+ve

Table 2. Investigation of main bioactive phytochemical compounds and total flavonoids of *Ammi majus seed* extracts.

Concentration (mg/g)	Aqueous extract	Alcoholic extract
Total Flavonoids	176.3±19.2	193.2±25.7

Table 3. Concentrations of total flavonoid compounds in Ammi majus seed extracts.

White Blood Cells (WBC) count

Table (4) shows the results of the total WBC count in the mice studied. Treating mice with CCl_4 in the positive control group decreased the WBC count (3.5±9.15 cells/cu.mm) compared to other treatments. While healing mice with plant extracts (aqueous or alcoholic) showed a reduction in the effectiveness of CCl_4 by increasing WBC count (5.3±0.01, and 6.8±0.13) cells/cu.mm, respectively. It should be mentioned that treating healthy mice with A. majus extracts showed an increase in the WBC count compared to the negative control. It is worth noticing that treatment with alcoholic extract performed the highest increase in WBC count (7.5±0.27 cells/cu.mm) compared to other treatments.

WBC count (cells/cu.mm)					
Group 1 (–ve control)	**Group 2** (+ve control) CCl_4	**Group 3** Aqueous extract	**Group 4** Alcoholic extract	**Group 5** Aqueous extract+ CCl_4	**Group 6** Alcoholic extract+ CCl_4
6.1±0.03	3.5±0.15	6.9±0.12	7.5±0.27	5.3±0.01	6.8±0.13

Table 4. Effect of aqueous and alcoholic extract of Ammi majus seeds on the white blood cells WBC count (cells/cu.mm) of mice in the study groups.

Acute toxicity of CCl_4 has been reported to increase WBCs, means of lymphocytes, granulocytes, and monocytes, as well as lower RBC levels in the blood in animal studies [27]. Additionally, [28] found that highly toxic substances or high stress cause damage to the bone marrow, which in turn reduces the production of blood cells, including leukocyte cells.

According to the presented data, different extracts of the A. majus plant showed diverse phytochemical ingredients, resulting in varied therapeutic actions depending on the extraction type.

Kidney Biochemical Analysis

Injection mice with CCl_4 substantially elevated serum urea and creatinine levels (87±11.3 and 2.7±0.2) unit/l compared with a negative control group (32.7±3.6 and 0.8±0.01) unit/l, respectively. Conversely, the total protein was considerably decreased in CCl_4-treated mice (2.5±0.9 unit/l) compared with a negative control group (5.8±1.3 unit/l) (Table 5). The treatment of mice with the alcoholic extract of A. majus plant interacted with CCl_4 reduced the toxic effect of CCl_4 by lowering urea values and creatinine and raising the rate of total protein (66.1±8.1, 1.7±0.10, 4.3±1.2) unit/l, respectively. However, compared to other treatments, the combination of the aqueous extract of plant with CCl_4 reduced the toxicity of CCl_4 but slower than the combination of the alcoholic extract with CCl_4.

Groups	Urea (unit/l)	Creatinin (unit/l)	Total proteins (unit/l)
Group 1: (-ve control)	32.7±3.6	0.8±0.01	5.8±1.3
Group 2: (+ve control), CCl_4	87±11.3	2.7±0.2	2.5±0.9
Group 3: Aqueous extract of *A. majus*	44.5±5.3	1.1±0.02	6.1±1.7
Group 4: Alcoholic extract of *A. majus*	38.7±3.9	0.9±0.01	5.7±1.31
Group 5: Aqueous extract + CCl_4	75.2±9.4	2.1±0.11	3.9±1.1
Group 6: Alcoholic extract + CCl_4	66.1±8.1	1.7±0.10	4.3±1.2

Table 5. Effect of aqueous and alcoholic extract of Ammi majus on the values of kidney function enzymes of mice in the study groups.

Renal Histopathological Study

The histopathology of mice kidneys in negative control revealed typical cortical and medullary structures, including glomeruli and renal tubules, as shown in Figure (1, A). In CCl_4-treated mice, the histological section showing apparent glomerular and tubular damages confirmed injury in renal structure (Figure 1, B). Besides this, no abnormalities were noted in the kidneys of the experimental groups treated with aqueous and alcoholic extracts of the Ammi majus plant, even combined with CCl_4, as shown in Figure (1, C-F).

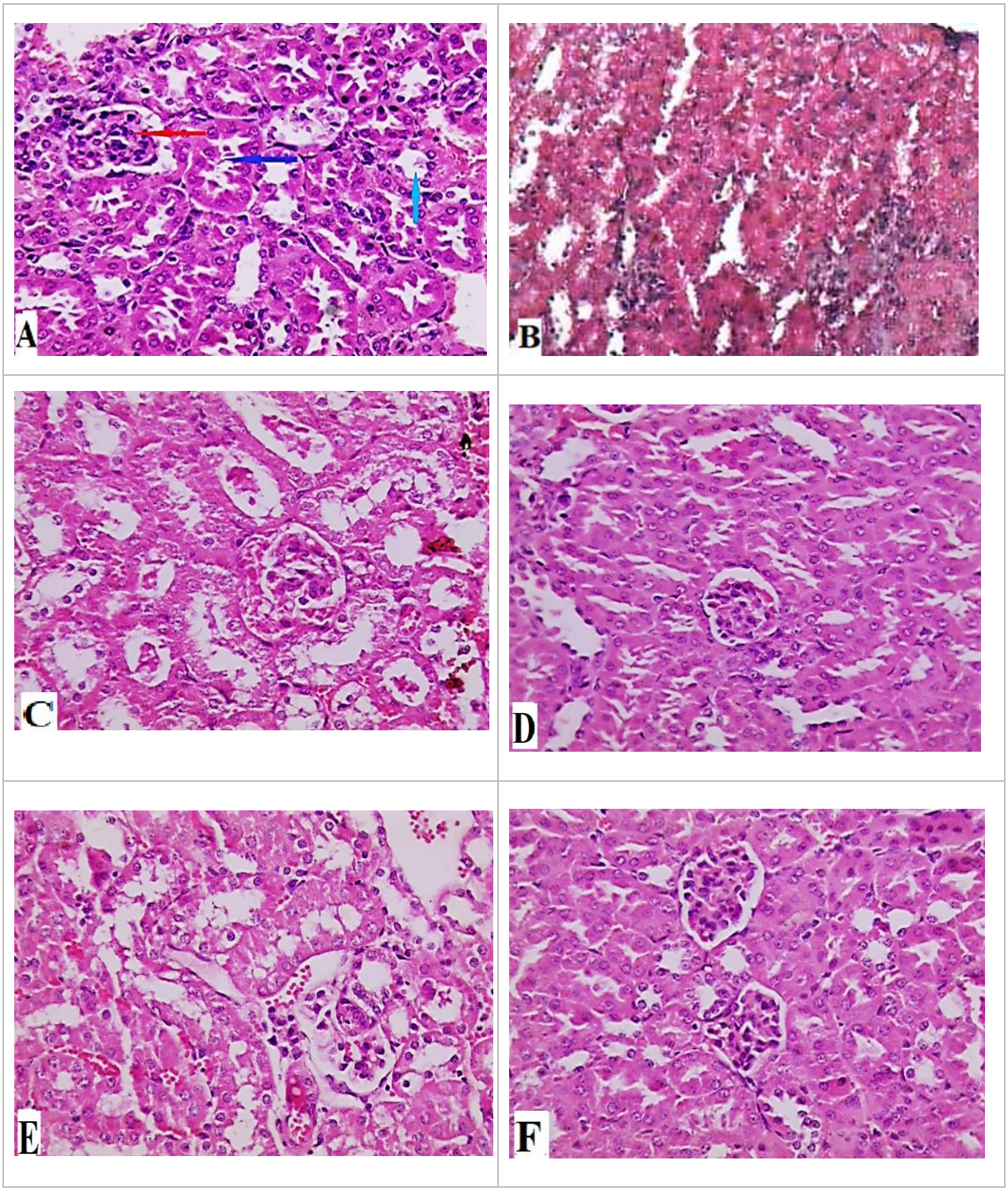

Figure1. Sections of kidney tissues in mice groups treated with distilled water, -ve control (A), CCl_4 +ve control (B), aqueous extract (C), alcoholic extract (D), aqueous extract + CCl_4 (E), Alcoholic extract + CCl_4 (F), are illustrated (H&E, 40×).

Discussion

Several factors influence the yield of Ammi majus compounds and their biological activity, including the kind of solvent, extraction process, extraction time, temperature, and physical features of sample [25]. The polarity of the solvent and solubility in the extraction affect the quality and quantity of the plant's active compounds [2]. High concentrations of coumarins and flavonoids had been detected in Ammi majus [3] and Ammi visnaga [26], which are responsible for their biological activities [9].

The higher content of alkaloids and flavonoids in alcoholic extract than in aqueous extract could explain its effectiveness in boosting experimental animals' immune systems by increasing their WBC count, as these phytochemicals are scientifically known for their effective properties as antioxidants [29] and treat various tumors and cancers [30]

Many investigations revealed that ingestion of CCl_4 causes the generation of free radicals in various organs, including blood, liver, kidneys, and other organs [11]. After ingesting the animals with CCl_4, it was found that the quantity of CCl_4 is distributed relatively equally in the kidneys than in the liver [31] because the kidneys have a high binding affinity for CCl_4 [32]. Also, kidney function is negatively affected by CCl_4, which raises blood urea nitrogen, creatine kinase, creatinine, and lactate dehydrogenase. Therefore, an increase in these parameters is often associated with nephrotoxicity [27]. The primary toxicity of CCl_4 is due to its release of free radicals, often Trichloromethyl radical ($^{\bullet}CCl_3$) and trichloromethyl peroxyl radical ($^{\bullet}CCl_3O_2$). Consequently, these radicals will bind to intracellular proteins, lipids of the cell membrane, and DNA, resulting in lipid peroxidation, protein denaturation, DNA oxidative and damage, consequently all of which contribute to cell death [27,33]

In contrast, combined A. majus extracts with CCl_4 effectively reduced the elevations in urea and creatinine concentrations as well as increased total proteins; this could be due to the antioxidant properties of phytochemicals in A. majus, as well as the high levels of polyphenols and flavonoids found in its extracts, which scavenge free radicals and protect kidneys. Phytochemicals are the most potent free radical scavengers and are regarded as the best antioxidants found in plants [34]. Bioactive constituents, like phenolic compounds, have antioxidant properties as break-down reactions of lipid oxidation chain via providing H^+ to the active free radicals through their phenolic -OH groups [29]. The presented results agreed with the results of the antioxidative activities of Ammi majus [2] and Ammi Vinegar [26].

However**,** these sections show mild to moderate degenerative glomeruli and renal tubule changes. The significant damage to the renal structure, including glomerular and tubular destruction, was induced by CCl_4. These effects are expected due to CCl_4-producing reactive radicals that cause membrane phospholipid peroxidation [33]. The produced free radicals will either cause peroxidative damage by attaching to DNA and proteins or target "polyenoic fatty acids" in cell membranes, resulting in secondary lipid radicals. As a result, the lipid peroxidation process is started [11]. Treatment of mice with A. majus extracts (aqueous or alcoholic) reduced the histological changes caused by CCl_4, this influence which could be due to its antiradical or antioxidant properties [2]. Intriguingly, the current study demonstrated that the A. majus plant is rich in phytochemicals [3] and a good source of natural antioxidants, including flavonoids, coumarins [35], and khellins [36], associated with lipid peroxidation. Moreover, polar and semi-polar solvents effectively extract active compounds from the A. majus plant.

Conclusion

Our findings show that Ammi majus seeds extractions contain important phytochemical components, particularly alcoholic extract, compared to aqueous extract. However, the results varied according to the type of solvent. All parameters related to white blood cell count and Kidney biochemical functions revealed that CCl_4 caused significant damage to renal tissue in mice. Treating mice with A. majus seeds extracts (especially the alcoholic extract) enhanced the blood immune system and reduced kidney damage caused by the CCl_4

compound. In conclusion, A. majus can be considered a medicinally important plant for its potential healing properties in treating kidney problems and many other diseases. However, it should be used in appropriate doses and quantities to avoid side effects.

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