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Phytochemical composition and toxicity assessment of *Ammi majus* L.

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ABSTRACT

Objective: To assess the acute and subacute toxicity as well as the phytochemical composition of two extracts and three fractions of *Ammi majus* L.

Methods: The aqueous extracts were prepared separately by maceration for 48 h and by infusion for 1 h, while the fractions were prepared by the Soxhlet extractor, successively employing cyclohexane, ethyl acetate, and ethanol. The acute toxicity study was carried out in accordance with the OECD N°423 guideline at a single dose (2 000 mg/kg) in mice for 14 days. The subacute toxicity study was performed by a daily oral administration of 250 mg/kg 2 for 10 d and 100 mg/kg doses for 28 d. Phytochemical screening was performed using staining and precipitation reactions, while the chemical characterization of some analytes was detected by HPLC-MS/MS analysis.

Results: In the acute toxicity study, no signs of toxicity such as convulsion, salivation, diarrhea, sleep and coma were observed during 30 minutes and 14 days, so the lethal dose was higher than 2 000 mg/kg for each extract and fraction. The subacute toxicity results showed that at a dose of 250 mg/kg, 61.10% of the animals died and the rest developed morbidity. On the other hand, at a dose of 100 mg/kg, all the animals were still alive after 28 days, with no morbidity and the biochemical parameters were normal with no abnormalities in the liver, kidneys and pancreas. Phytochemical screening indicated the presence of flavonoids, tannins, coumarins, and free quinones and the absence of alkaloids and anthocyanins.

Conclusions: The extracts and fractions of *Ammi majus* L. are not

Significance

The use of plants in traditional medicine is still empirical, with the doses and the duration of treatment, for instance, not being clearly defined. This study revealed the safety of different extracts and fractions of *Ammi majus* L. in mice by the *in vivo* study of acute toxicity (at 2 000 mg/kg) and subacute toxicity (at 100 mg/kg) as well as characterized and quantified some phytochemicals. To suggest safe dose levels for clinical studies, further studies on other animals as well as prolonged duration are needed.

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toxic in the short and long term with a varied chemical composition. Toxicological tests on animals other than rodents and in the long term (more than 28 days) are needed to further confirm the safety of *Ammi majus* extracts.

KEYWORDS: *Ammi majus* L.; Phytochemical composition; Acute toxicity; Subacute toxicity; Flavonoids

1. Introduction

Due to their richness in secondary metabolites, medicinal and aromatic herbs have been widely used by the world's population as the basis for medical treatments[1]. Secondary metabolites contain several potential biological properties, which form the scientific basis of the introduction of herbs in folk medicine. They have been described as antiviral antibiotics and allow good absorption of UV radiation, and it has been shown that some herbs possess estrogenic properties and interactions with animal fertility[2]. They have been used throughout human history as spices, condiments, pigments, and pharmaceuticals. Terpenes, polyphenols, and alkaloids are the most important classes of secondary metabolites[3]. Statistically, more than 80% of the population of developing countries relies on natural plant-based medicines[4]. The higher acceptance of this traditional medicine might contribute to its accessibility, affordability, and historical experimental basis[5]. As a rule, effective drugs lead to undesirable effects. However, herbal drugs are generally considered to be safe and effective agents, which leads people to come back to them[6].

The use of plants in traditional medicine is still empirical. For instance, the doses and the duration of treatment are not clearly defined[7]. There is a lack of scientific and experimental toxicity studies that could provide a safe and effective profile for practical application in local communities[8]. For the safe and effective application of medicinal plants, it is necessary to evaluate their degree of safety, to ensure constant and adequate quality and effectiveness[9]. Many studies have shown that the extracts of certain medicinal plants are toxic at certain doses and in the long term, which makes their traditional use risky[10,11].

Ammi majus L. (*A. majus*), commonly called by the Moroccan population as Atrilal, trillane, Belala, Rjel l'aghrabe, or Ich Omla[12,13], belongs to the family of Apiaceae (umbelliferae). *A. majus* L. is native to the Middle East and is largely distributed in Western Asia, Europe and the Mediterranean, including Northern Africa, as well as Western Africa. *A. majus* L. is widely cultured in India and other tropical countries due to its beneficial value[14].

In folk medicine, the Moroccan population uses *A. majus* L. in decoction, infusion, or cataplasm as an anti-dermatitis drug, a diuretic, and for the treatment of vitiligo[15]. The mixture of honey with the seeds of *A. majus* L. and the roots of *Anacyclus pyrethrum* is also used for the treatment of vitiligo[16], and it has been cited as being employed to treat cardiovascular diseases[17]. The fruit is largely used by the Egyptian population for the treatment of leukoderma, and as a diuretic, emmenagogue, and blood purifier[18]. It is also used against kidney stones and urinary tract infections[19,20], while the Iranian population uses *A. majus* against psoriasis and vitiligo, and the Chinese population uses it as a diuretic and carminative, and to treat angina pectoris and asthma[21,22].

Considering the abovementioned benefits of *A. majus*, the main goal of this study is to characterize certain phytochemicals and measure the level of safety of the aqueous extracts and the organic fractions of *A. majus* by studying the acute and subacute toxicity.

2. Materials and methods

2.1. Chemical reagents

All chemical reagents were of analytical grade. Ethanol, ethyl acetate, cyclohexane, iron chloride, sodium hydroxide, Dragendorff's reagent, Mayer's reagent, hydrochloric acid, sulfuric acid, chloroform, and methanol were obtained from Sigma Aldrich.

2.2. Collection and identification of the plant

Seeds of *A. majus* were harvested at the time of its fruiting in the region of Rabat, Morocco (coordinates: 33°58'06''N, 6°49'04''W) in May 2019. Botanical identification was performed at the Scientific Institute of Rabat and a sample was deposited at the herbarium under the code RAB111737. *A. majus* L. seeds were dried at room temperature in the laboratory (in Rabat), for 2 weeks, away from sun and humidity.

2.3. Preparation of the extracts

2.3.1. Maceration and infusion

Crushed and dried seeds of *A. majus* (34 g) were macerated (E_1) and infused (E_2) separately with 650 mL of distilled water for 48 h and 1 h, respectively, with intermittent shaking. The extracts were filtered through Whatman filter paper No. 1 (0.45 μm), and then the water was removed under vacuum on a rotary evaporator (BUCHI RE-111 Rotavapor W/461 Water bath) under 40 ℃ to constant dry. The aqueous extracts were lyophilized and stored at 4 ℃ for further analysis.

2.3.2. Soxhlet fractionation

The organic fractions were prepared using a Soxhlet apparatus with cyclohexane (F_1), ethyl acetate (F_2), and ethanol (F_3), successively. At the end of each extraction, the fractions were dried using a rotatory evaporator at 40 ℃. Finally, the dried organic fractions were transferred into screw-capped amber vials and preserved at 4 ℃ for further analysis.

2.4. Animals

Female Swiss albino mice (20-30 g) were provided by the animal house of the Faculty of Medicine and Pharmacy, Mohammed V University, Rabat, Morocco. Animals were reared at (22 ± 2)℃ with 14 h of light and 12 h of darkness, with free access to food and water.

2.5. Phytochemical screening

2.5.1. Determination of flavonoids

Flavonoids were detected by the "Cyanidin" reaction. Briefly, 2 mL of each extract and fraction were added with a few drops of concentrated HCl and small quantities of magnesium. An orange-to-red coloration appears in the case of the presence of flavonoids.

2.5.2. Determination of tannins

The presence of tannins was detected by adding 3 mL of each extract and fraction with a few drops of the $FeCl_3$ aqueous solution (10%) (*m/v*). A positive test is revealed by the appearance of a blue-black or blue-green coloration.

2.5.3. Determination of anthocyanins

To each 1 mL of a given extract and fraction, we add 1 mL of concentrated sulfuric acid (H_2SO_4) and 1 mL of ammonium hydroxide (NH_4OH). Red coloration in an acidic medium and a purplish-blue coloration in a basic medium indicates the presence of anthocyanins.

2.5.4. Determination of coumarins

We added 0.5 mL of NH_4OH (25%) to 2 mL of each extract and fraction. Under the UV lamp at 366 nm, an intense fluorescent light indicates the presence of coumarins.

2.5.5. Determination of free quinones

Free quinones were detected by adding a few drops of NaOH (10%) (*m/v*) to 3 mL of each extract and fraction. When the color turns yellow, red, or purple, it indicates the presence of free quinones.

2.5.6. Detection of terpenoids

Terpenoids were detected by adding 0.3 mL of chloroform to 3 mL of each extract and fraction, followed by the addition of 1.2 mL of concentrated H_2SO_4. The formation of a brownish-red or purple ring at the contact zone generally reveals the presence of terpenoids.

2.5.7. Detection of alkaloids

Alkaloids were detected on the basis of precipitation reactions with Bouchardat, Mayer, and Dragendorff's reagent. To 3 mL of each extract and fraction were added 1 mL of each of the reagents (Mayer; Dragendorff; Bouchardat), and then the solution was allowed to stand for 10 min. A positive test is revealed by the appearance of an orange precipitate with Dragendorff's reagent, a yellowish-white precipitate with Mayer's reagent, and a brown precipitate with Bouchardat's reagent.

2.5.8. Detection of saponins

The presence of saponins in our extracts was detected by the foaming test. Briefly, 5 mg of each extract and fraction was diluted in 5 mL of distilled water or dimethyl sulfoxide, introduced into a test tube, and then shaken vigorously for about 15 s. The formation of a stable foam (greater than 1 cm in height), persisting for 15 min indicates the abundant presence of saponins.

2.6. Quantification of bioactive compounds of A. majus by the HPLC–MS/MS system

The extracts and fractions of *A. majus* were solubilized in a suitable solvent and filtered by a 0.2 µm filter before HPLC-MS/MS analysis. A Shimadzu-Nexera ultra-high performance liquid chromatography (UHPLC) coupled to a tandem mass spectrometer was used to quantify 56 phytocompounds. The reverse phase UHPLC was equipped with an automatic sampling probe (model SIL-30AC), binary pumps (model LC-30AD), a column oven (model CTO-10ASvp), and a degasser (model DGU- 20A3R). Chromatographic conditions were adapted to obtain optimal separation of 56 compounds and to overcome suppression effects. Different columns like Agilent Poroshell 120 EC-C18 (150 mm×2.1 mm, 2.7 µm) and RP-C18 Inertsil ODS-4 (100 mm×2.1 mm, 2 µm), different mobile phases (B) namely methanol and acetonitrile, different mobile phases additives such as acetic acid, formic acid, ammonium acetate, and ammonium formate, different column temperatures such as 25℃, 30℃, 35℃, and 40℃ were experimented and applied until the optimal conditions were reached. Therefore, the chromatographic separation was performed on an Agilent Poroshell 120 EC-C18 model reverse phase chromatographic column (150 mm×2.1 mm, 2.7 µm). The temperature of the column was set at 40℃. The elution gradient consisted of eluent A (5 mM ammonium formate+0.1% formic acid+water) and eluent B (5 mM ammonium formate+formic acid+0.1% methanol). The following gradient elution profile was used: 20%-100% B (0-25 min), 100% B (25-35 min), and 20% B (35-45 min). In addition, the solvent flow rate was set to 0.5 mL/min and the injection volume was adjusted to 5 µL.

Detection was performed in negative and positive modes using a Shimadzu LCMS-8040 tandem mass spectrometer equipped with an electrospray ionization (ESI) source. LC-ESI-MS/MS results were acquired and processed by LabSolutions software (Shimadzu). The multiple reaction monitoring (MRM) modes were employed for the quantification of phytocompounds. The detection and selective quantification of phytochemicals were performed by the MRM method based on the screening of specified phytochemical precursor ionic transitions to fragments. In order to generate an optimal phytochemical fragmentation and a maximum transmission of the desired product ions the collision energies have been optimized. The MS operating parameters were applied as follows: drying gas

flow rate (N_2), 15 L/min; nebulizing gas flow rate (N_2), 3 L/min; DL temperature, 250 ℃; thermal block temperature, 400 ℃, and interface temperature, 350 ℃.

2.7. Acute toxicity

The assessment of toxicity of a substance for therapeutic use is undeniably at the forefront of the drug discovery process. Short-term toxicity assessment is usually the first test model studied *in vivo* during preclinical drug development.

The acute oral toxicity assessment for each *A. majus* seed extract and fraction was performed using the guidelines established by the Organization for Economic, Cooperation, and Development (OECD 423). For each extract and fraction, three non-pregnant and nulliparous female mice weighing between 20 and 30 g were fasted for 4 h, with free access to water, each mouse was placed separately and individually in sterile polypropylene cages, then each *A. majus* seed extract and fraction was administered orally using an esophageal probe at 2 000 mg/kg. The dose of extracts and fractions has been chosen by the guide according to the toxic potential of the substances. After the administration of the extracts, the animals were observed for 30 min, and 14 d. The variations in body weight, mortality as well as clinical signs such as convulsion, salivation, diarrhea, lethargy sleep, and coma were noted and recorded. The design of the acute oral toxicity assessment is shown in Figure 1.

2.8. Subacute toxicity

The study of subacute oral toxicity over 28 days was conducted according to OECD Test Guideline No. 407. For this purpose, sixty-six female mice were divided into eleven groups of six mice each and placed individually and separately in sterile polypropylene cages as follows: the first group served as the control group which did not receive any treatment, while the other groups received daily E_1, E_2, F_1, F_2, and F_3 at 250 mg/kg and 100 mg/kg. The mice were weighed weekly to record any changes in weight and abnormalities were observed. On day 29 of the experiment, the mice were fasted for 4 h, then anesthetized to collect blood, and euthanized using a surgical blade to remove vital organs such as the spleen, lung, liver, kidneys, and pancreas. The design of the subacute toxicity assessment is shown in Figure 1.

2.9. Histopathological analysis

Vital organs including the kidneys, liver, and pancreas were collected, weighed, and fixed in 10% *v/v* neutral buffered formaldehyde solution for histopathological examination. These vital organs were dehydrated, clarified, and infiltrated with paraffin, and the formed sections had a thickness of 2 µm. The collected tissues were stained with hematoxylin-eosin before histopathological examination.

2.10. Biochemical analysis

The blood samples were collected in ordinary blood collection tubes (lithium heparin), then centrifuged at 4 000 rpm for 10 min to obtain plasma for determination of urea (UR), creatinine (CR), total cholesterol (TC), alanine aminotransferase (ALT), aspartate aminotransferase (AST), very low-density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL) and

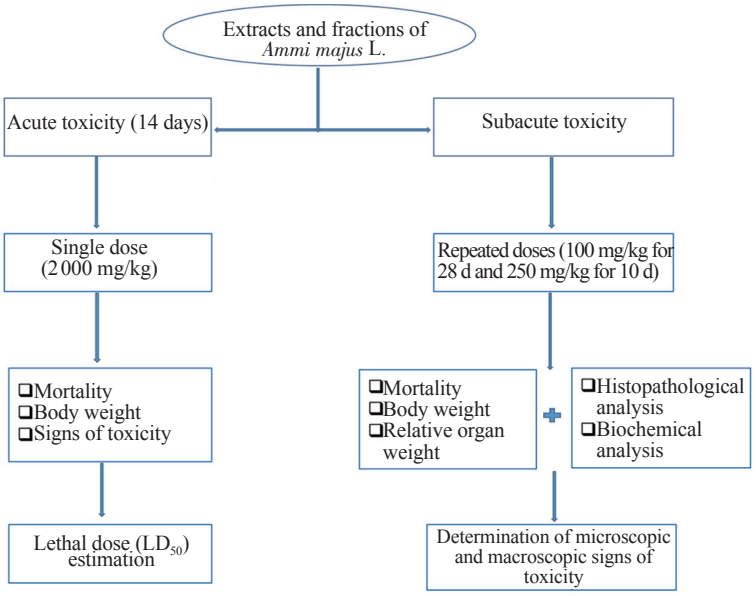

Figure 1. Design of acute and subacute toxicity study of extracts and fractions of *Ammi majus* L.

total proteins (TP). These analyses were performed using specific kits for each parameter.

2.11. Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). Differences were compared by employing Tukey's *post hoc* test at $P < 0.05$.

2.12. Ethical statement

All experimental procedures have been done in accordance with the "Principles of Laboratory Animal Care" and conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" of the National Academy of Sciences and approved by the National Institutes of Health. All efforts were made to minimize the number of animals required for the experiments and animal suffering and ethics approval was obtained from the Central Animal Facility and Laboratory of Toxicology-Pharmacology, Faculty of Medicine and Pharmacy, Mohammed V University of Rabat, Morocco without a special code assigned.

3. Results

3.1. Phytochemical screening

The results showed the presence of flavonoids, tannins, coumarins, and free quinones, and the absence of alkaloids and anthocyanins in all extracts and fractions. In addition, terpenoids were present in all the organic fractions, while being absent in the two aqueous extracts. Saponins were detected only in the ethanolic fraction (F_3) but absent in the other fractions and the aqueous extracts.

3.2. Bioactive compounds in the extracts and fractions of A. majus L.

The results of chemical composition are presented in Table 1. As shown in Table 1, the ethanolic fraction showed the highest content of total compounds analyzed (10.807 mg/g extract), while the cyclohexane fraction had the lowest content (0.028 mg/g extract). More precisely, the ethanolic fraction revealed the highest content of quinic acid (9.436 mg/g extract), followed by the aqueous extract (6.775 mg/g extract). Indeed, aconitic acid was only detected in the aqueous extracts with a small amount in the macerated extract. Gallic acid, protocatechuic acid, and gentisic acid were detected in the polar extracts. In addition, chlorogenic acid was only detected in the aqueous extract (0.929 mg/g extract), the ethyl acetate fraction (0.025 mg/g extract), and the ethanolic fraction (0.189 mg/g extract). Then, protocatechuic aldehyde was detected in ethyl acetate and ethanol fractions with the values of 0.017 and 0.007 mg/g extract, respectively. The macerated aqueous extract and ethyl acetate fraction showed moderate amounts of 4-OH benzoic acid with 0.161 and 1.128 mg/g extract, respectively. Vanilic acid and syringic acid were detected in the macerated aqueous extract with 0.210 and 0.241 mg/g extract, respectively. Vanilin was only present in the macerated aqueous extract and the ethyl acetate fraction with a small amount. Syringic aldehyde was detected in the ethyl acetate fraction only. Piceid was revealed in the macerated aqueous extract and the ethyl acetate fraction with 0.105 and 0.157 mg/g extract, respectively. *P*-coumaric acid and salicylic acid were detected in the aqueous extracts, ethyl acetate, and ethanol fractions, however, they were absent in the cyclohexane fraction. Ferulic acid was detected in the macerated aqueous extract (0.375 mg/g extract), the ethyl acetate fraction (0.387 mg/g extract), and the infused aqueous extract (0.056 mg/g extract). On the other hand, it was not detected in the cyclohexane and ethanolic fractions. The HPLC-MS results are shown in Figure 2.

3.3. Acute toxicity test

The acute toxicity of the fractions and extracts of the wild species *A. majus* was assessed. The results of the fractions and extracts at a dose (2000 mg/kg) showed no clinical signs of toxicity such as convulsion, salivation, diarrhea, sleep and coma (observation during the first 30 minutes and during the 14 d of the study). All animals tested lived without mortality and morbidity and their behavior remained normal during 14 d of observation. According to OECD No.423, it is not permitted to increase the dose above 2000 mg/kg for animal protection reasons, except in justified cases. These results mean that the lethal dose (LD_{50}) was higher than 2000 mg/kg. During this period, the mean body weight of each group did not change significantly and especially after 10 d (Figure 3), therefore, from these results and based on EOCD No. 423 guidelines, the extracts and fractions of *A. majus* were considered non-toxic for single oral administration at 2000 mg/kg.

3.4. Subacute toxicity test

3.4.1. Body weight of the animals

As shown in Table 2, no significant variation in body weight was observed in the treated groups compared to the control group, but the groups treated with the cyclohexanic, ethyl acetate, and ethanolic fractions showed a slight decrease in body weight during the second week, after this week these groups recovered their weights, during the treatment period. The treated groups and the control group underwent a gain in body weight (Table 2). This shows that the extracts and fractions studied can be non-toxic, since no severe weight loss of the animals was observed. At the dose of 250 mg/

kg, 61.10% of the animals treated for 10 d died and the rest of the animals developed morbid forms such as hair loss, inability to move, and severe decrease in body weight, which implies that this dose was toxic; therefore, we stopped the study on this dose.

Table 1. Compounds identified from the extracts and fractions of *Ammi majus* L.

Analyte	RT	M.I. (*m/z*)	F.I. (*m/z*)	Mol formula	E_1 (mg/g extract)	E_2 (mg/g extract)	F_1 (mg/g extract)	F_2 (mg/g extract)	F_3 (mg/g extract)
Quinic acid	3.0	190.8	93	$C_7H_{12}O_6$	2.108	6.775	N.D	0.118	9.436
Aconitic acid	4.0	172.8	129	$C_6H_6O_6$	0.153	0.040	N.D	N.D	N.D
Gallic acid	4.4	168.8	79	$C_4H_4O_4$	0.319	0.035	N.D	0.015	0.018
Protocatechuic acid	6.8	152.8	108	$C_7H_6O_5$	0.430	0.477	N.D	0.314	0.128
Gentisic acid	8.3	152.8	109	$C_7H_6O_4$	0.069	0.063	N.D	0.042	0.030
Chlorogenic acid	8.4	353.0	85	$C_{15}H_{14}O_6$	N.D	0.929	N.D	0.025	0.189
Protocatechuic aldehyde	8.5	137.2	92	$C_7H_6O_4$	N.D	N.D	N.D	0.017	0.007
4-OH Benzoic acid	10.5	137,2	65	$C_{22}H_{18}O_{11}$	0.161	N.D	N.D	1.128	N.D
Vanilic acid	11.8	166.8	108	$C_{12}H_{16}N_2O_4$	0.210	N.D	N.D	N.D	N.D
Caffeic acid	12.1	179.0	134	$C_{15}H_{14}O_6$	0.037	0.038	N.D	0.212	0.059
Syringic acid	12.6	196.8	166.9	$C_8H_8O_4$	0.241	N.D	N.D	N.D	N.D
Vanillin	13.9	153.1	125	$C_9H_8O_4$	0.067	N.D	N.D	0.119	N.D
Syringic aldehyde	14.6	181.0	151.1	$C_9H_{10}O_5$	N.D	N.D	N.D	0.028	N.D
Piceid	17.2	391.0	135.0/106.9	$C_{21}H_{20}O_9$	0.105	N.D	N.D	0.157	N.D
p-Coumaric acid	17.8	163.0	93	$C_{22}H_{18}O_{10}$	0.267	0.298	N.D	1.923	0.370
Ferulic acid	18.8	192.8	149	$C_9H_8O_3$	0.375	0.056	N.D	0.387	N.D
Salicylic acid	21.8	137.2	65	$C_{11}H_{12}O_5$	0.022	0.043	N.D	0.043	0.038
Cynaroside	23.7	447.0	284	$C_9H_6O_2$	N.D	0.043	N.D	N.D	N.D
Miquelianin	24.1	477.0	150.9	$C_7H_6O_3$	N.D	0.009	N.D	N.D	N.D
Isoquercitrin	25.6	463.0	271	$C_{27}H_{36}O_{19}$	0.229	0.035	N.D	0.015	0.085
Quercitrin	29.8	447.0	301	$C_{21}H_{20}O_{10}$	N.D	0.109	N.D	0.020	0.017
Astragalin	30.4	447.0	255	$C_{21}H_{20}O_{11}$	0.325	0.018	N.D	0.048	0.095
Quercetin	35.7	301.0	272.9	$C_{15}H_{10}O_7$	0.266	N.D	N.D	0.034	0.025
Naringenin	35.9	301.0	119	$C_{15}H_{12}O_5$	0.003	N.D	N.D	0.039	N.D
Luteolin	36.7	269.0	151.0/175.0	$C_{15}H_{10}O_6$	N.D	N.D	N.D	0.008	N.D
Kaempferol	37.9	284.8	239	$C_{15}H_{10}O_6$	0.231	N.D	N.D	0.027	0.010
Acacetin	40.7	283.0	239	$C_{16}H_{12}O_5$	0.028	0.027	0.028	0.034	0.030
Total	-	-	-	-	5.646	8.995	0.028	4.753	10.807

N.D: not detected; E_1: aqueous macerated extract; E_2: aqueous infused extract; F_1: cyclohexanic fraction; F_2: ethyl acetate fraction, F_3: ethanolic fraction, RT: retention time, M.I.: molecular ions of the standard analytes, F.I.: fragment ions.

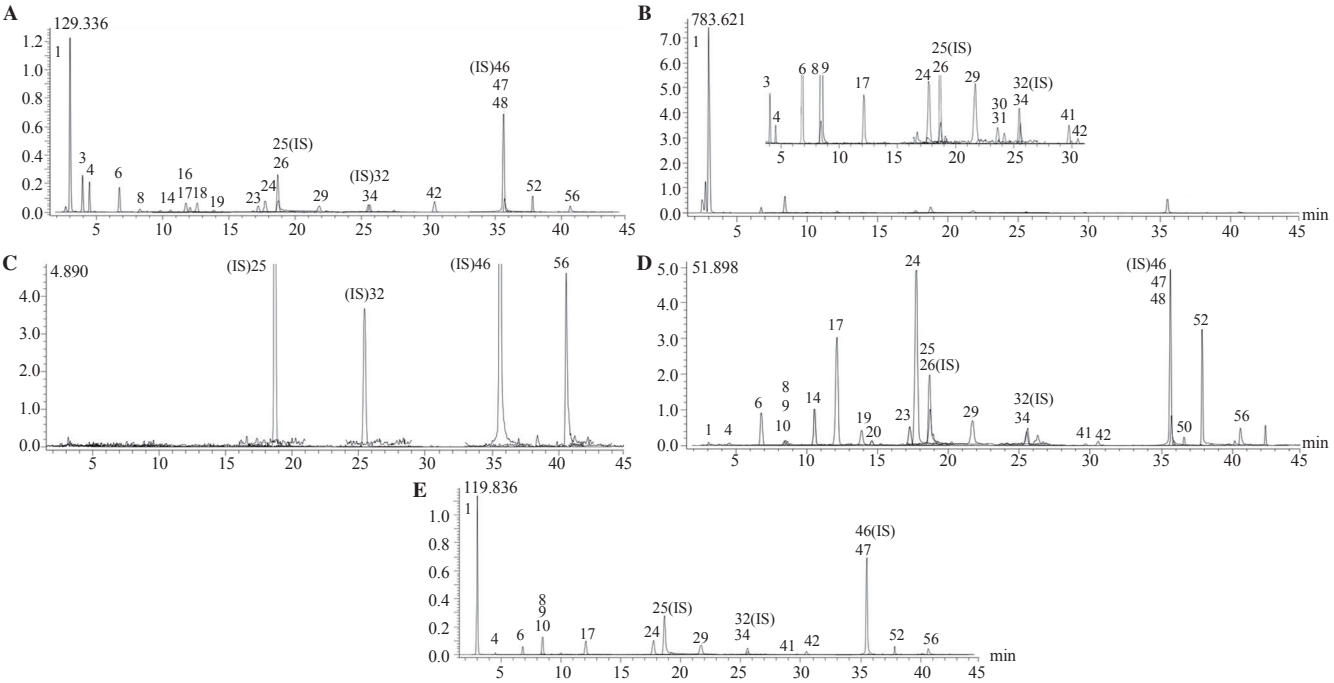

Figure 2. Peaks of the macerated aqueous extract (A), the infused aqueous extract (B), the cyclohexanic fraction (C), the ethyl acetate fraction (D), and the ethanolic fraction (E) by HPLC-MS/MS analysis.

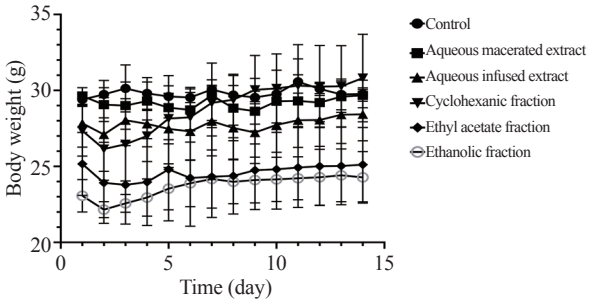

Figure 3. Body weight of the animals treated with the extracts and fractions of *Ammi majus* at 2 000 mg/kg during the acute toxicity study.

3.4.2. Relative weights of the organs

For more information on the toxicological effects of the extracts and fractions of *A. majus* L. in the subacute toxicity study, the weight of some vital organs including the liver, kidneys, lung, spleen, and pancreas was determined.

To evaluate the effect of the extracts and fractions on vital organs, we made comparisons between all treated groups and the control group (Table 3). The relative weight of vital organs (the liver, kidney, spleen, lung, pancreas) in all treated groups was not significantly different ($P < 0.05$) from the relative weight of the same vital organs in the control group, which means that the administration of the fractions and extracts does not constitute any risk for the development of these vital organs.

3.4.3. Biochemical analysis

The evaluation of biochemical parameters in the control and treatment groups is presented in Table 4. The ALT level was increased significantly ($P < 0.05$) in the groups treated with the aqueous macerated and infused extracts as well as cyclohexanic and ethanolic fractions, while the ALT level in the group treated with the ethyl acetate fraction was not significantly different ($P > 0.05$) from that in the control group. The ethyl acetate and the ethanolic fractions showed no significant change in AST level compared to the control group. However, the level of AST was significantly different ($P < 0.05$) in the groups treated with the macerated and infused aqueous extracts, as well as the cyclohexanic fraction compared to the control group. The protein level was increased significantly ($P < 0.05$) in the groups treated with the infused water extract, the cyclohexanic fraction and the ethyl acetate fraction, while it was decreased in the groups treated with the ethanolic fraction compared to the control group. On the other hand, no significant difference was observed in the group treated with the macerated aqueous extract compared to the control group.

In addition, we found that the levels of urea and creatinine in the groups treated with the extracts and fractions at 100 mg/kg were not significantly different ($P < 0.05$) from the control group, indicating no toxic effects on the kidneys.

The level of TC in the groups treated with the macerated aqueous extract and the infused aqueous extract was increased significantly ($P < 0.05$) compared to the control group, while it was decreased in the group treated with the ethyl acetate and ethanolic fractions. Similarly, the LDL level was increased significantly ($P < 0.05$) in the groups treated with the macerated aqueous extract, the infused aqueous extract, and the cyclohexanic fraction, compared to the control group, while it was decreased in the group treated with the ethyl acetate fraction. The group treated with the ethanolic fraction showed no change in LDL level. The level of VLDL was significantly different ($P < 0.05$) in the group treated with the ethanolic fraction compared to the control, while in other groups, no significant difference was noticed. Additionally, no significant difference ($P < 0.05$) in HDL was observed in all the groups treated with the fractions and extracts of *A. majus* L. in comparison with the control.

3.4.4. Histopathological analysis

All groups treated with the extracts and fractions at 100 mg/kg showed no severe abnormalities in the kidneys, liver, and pancreas, such as inflammation of the glomerulus, interstitial inflammation, or lobular atrophy, except two mice of the group treated with the macerated aqueous extract which showed vascular congestion in the kidneys. In addition, in a mouse in the group treated with the

Table 2. Body weight of the groups treated with the extracts and fractions of *Ammi majus* at 100 mg/kg during the subacute toxicity study (g).

Time	Control	Aqueous macerated extract	Aqueous infused extract	Cyclohexanic fraction	Ethyl acetate fraction	Ethanolic fraction
Week 1	25.75±1.18	28.40±1.34	30.09±3.01	26.25±2.98	27.38±1.14	26.32±2.86
Week 2	26.43±1.34	28.90±0.62	30.22±2.90	25.25±4.42	26.25±2.87	25.40±1.67
Week 3	27.43±1.12	30.00±2.70	29.20±2.94	30.10±0.70	27.11±0.20	30.00±2.82
Week 4	27.43±0.56	30.75±2.06	31.05±2.12	30.50±2.96	29.50±1.73	30.25±2.62

Values are expressed as mean ± SD (n=6) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

Table 3. Relative weights of vital organs in the treatment and control groups during the subacute toxicity study.

Organs	Control	Aqueous macerated extract	Aqueous infused extract	Cyclohexanic fraction	Ethyl acetate fraction	Ethanolic fraction
Liver	1.63±0.09	1.64±0.20	1.48±0.32	1.52±0.08	1.37±0.18	1.53±0.20
Kidneys	0.37±0.04	0.37±0.11	0.33±0.07	0.30±0.05	0.34±0.15	0.36±0.08
Lung	0.32±0.09	0.29±0.07	0.36±0.06	0.33±0.20	0.31±0.06	0.33±0.03
Spleen	0.12±0.01	0.13±0.05	0.19±0.05	0.15±0.03	0.14±0.01	0.13±0.07
Pancreas	0.09±0.06	0.11±0.01	0.09±0.04	0.13±0.06	0.11±0.01	0.13±0.02

Values are expressed as mean ± SD (n=6) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

ethyl acetate fraction, peri-canal inflammation was observed in the pancreas (Figure 4).

4. Discussion

A. majus L. is characterized by its furanocoumarins and is widely used in folk medicine to treat some chronic diseases, mainly vitiligo. Clinically, *A. majus* L. has already been tested on patients suffering from this disease, but in toxicology, this plant is not yet valued. In order to present a toxicological study of *A. majus*, we attempted to evaluate the acute and subacute toxicity of 2 extracts and 3 fractions of this species in mice. Mice are one of the most commonly used mammals in preclinical research ranging from pharmacology to safety assessments, as they are genetically very similar to humans, and in addition, the lifespan of a mouse is roughly equivalent to 30 years in humans. The use of females only in this study is justified by the OECD guideline that the female mouse is more sensitive than the male. The adverse effects of a drug or substance are accompanied by a change in body weight[23]. Changes in body weight could be due to organic damage induced by the plant extract tested[24]. For this reason, the body weights for both the acute and subacute toxicity studies were noted. The results of the acute toxicity show no mortality, no morbidity, and no signs of toxicity, and the body weight of the animals remained in a normal state. Therefore, according to the classification of Duan & Liang[25], we conclude that the extracts and fractions of *A. majus* at 2 000 mg/kg are not toxic at a single dose.

During 10 d of administration, 61.10% of the animals died after consuming the extracts and fractions of *A. majus* at the dose of 250

Table 4. Biochemical markers of the control group and the groups treated with the fractions and extracts of *Ammi majus* at 100 mg/kg.

Parameter	Control	Aqueous macerated extract	Aqueous infused extract	Cyclohexanic fraction	Ethyl acetate fraction	Ethanolic fraction
AST (U/dL)	48.40±11.03	46.53±9.15*	50.20±7.47*	46.06±6.58*	48.64±4.52	47.51±8.12
ALT (U/dL)	103.20±8.70	108.33±7.55*	104.79±6.15*	106.47±5.46*	103.37±8.24	105.88±9.65*
CR (mg/dL)	1.06±0.30	1.11±0.19	1.22±0.50	1.33±0.33	1.11±0.19	1.66±0.33
UR (g/dL)	0.59±0.07	0.61±0.11	0.66±0.10	0.54±0.06	0.53±0.03	0.57±0.04
TP (g/dL)	62.88±0.22	63.29±0.63	68.10±1.39*	68.31±10.56*	68.80±6.54*	55.49±10.48*
TC (mg/dL)	127.60±8.70	131.34±10.58*	129.12±26.23*	128.10±3.99	123.12±3.34*	122.09±3.90*
HDL (mg/dL)	7.36±0.06	7.97±1.25	7.40±0.89	7.33±0.54	7.76±1.61	6.54±0.89
LDL (mg/dL)	60.15±1.01	62.59±4.68*	63.63±11.61*	64.33±1.77*	54.52±1.73*	60.78±1.73
VLDL (mg/dL)	26.26±0.15	27.26±2.11	26.98±5.24	28.02±0.79	26.62±0.66	28.41±0.78*

Values are expressed as mean ± SD (n=6) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. *Significantly different from the control group at $P < 0.05$. AST: aspartate aminotransferase, ALT: alanine aminotransferase, CR: creatinine, UR: urea, TP: total protein, TC: total cholesterol, HDL: high density lipoprotein, LDL: low density lipoprotein, VLDL: very low-density lipoprotein.

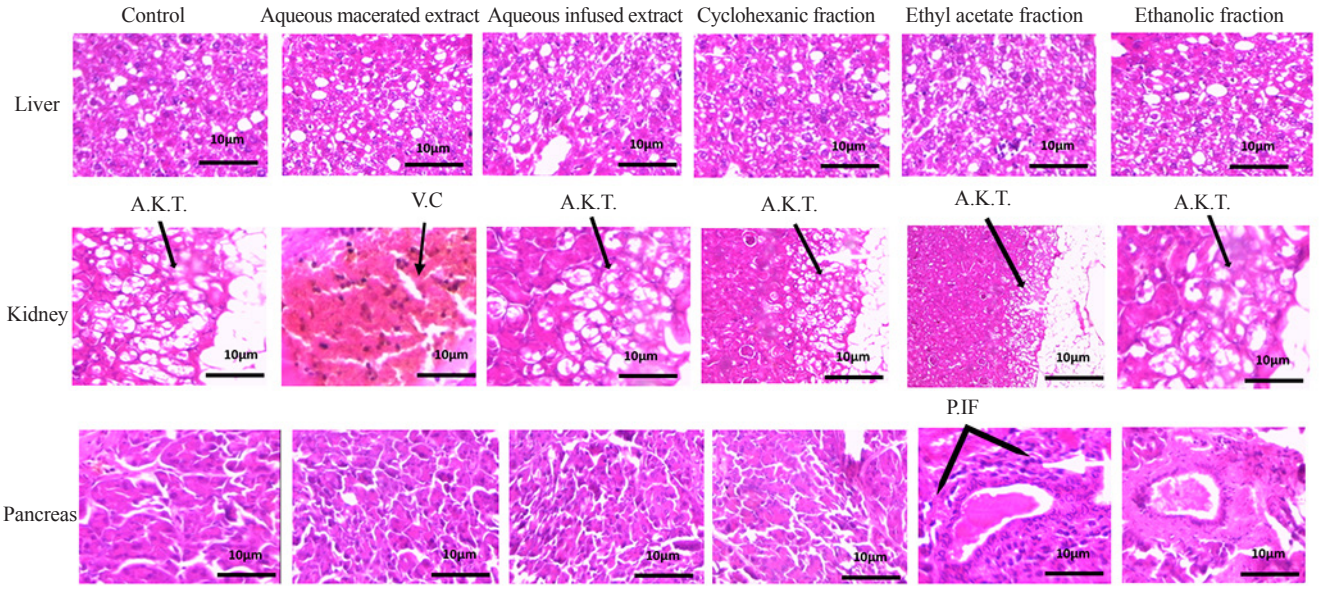

Figure 4. Results of histopathological analysis of the liver, kidneys, and pancreas after oral administration of *Ammi majus* for 28 days (H&E, magnification: 100×). There are no significant pathological lesions observed in vital organs of the treatment groups except peri-canal inflammation (P.IF) in the pancreas of the group treated with the ethyl acetate fraction, as well as vascular congestion (V.C) in the kidneys of the group treated with the macerated aqueous extract. A.K.T.: aspect of the renal tubes.

mg/kg, while the rest had morbid forms with a severe drop in body weight, which shows that the dose is toxic. As a result, the study was stopped at this dose. At the dose of 100 mg/kg, no mortality or morbidity was observed and no severe decrease in body weight was noted in any of the treatment groups during 28 d of the study. Similarly, changes in relative organ weight are often associated with organ hypertrophy (congestion, edema) or volume reduction (necrosis, atrophy) caused by toxic substances[26]. A study by Aboryag *et al.*[27] revealed that kidney weight was altered due to morphological abnormalities like tubular hypertrophy. Since the relative weight of the organs was not significantly different ($P > 0.05$) in the groups treated with the extracts and fractions at the dose of 100 mg/kg compared to the control group, it can be concluded that the organs studied do not show any toxic effect due to the repeated consumption of the extracts and fractions of *A. majus*.

The liver and kidneys are the organs most exposed to toxic substances, as they mainly remove the toxins from the blood toward the feces and urine. The gross appearance of these organs and their weight at autopsy provide important measures for assessing the adverse effects of medicinal plants[28]. We tried to evaluate the levels of AST and ALT because they are important parameters that increase in the blood when the liver is damaged or injured[29]. Indeed, high levels of these enzymes are linked to hepatitis, liver necrosis, and liver toxicity. This makes these enzymes useful in the diagnosis of liver diseases. AST and ALT levels were significantly different in some treated groups compared to the control group. The work of Dashti *et al*.[30], and Abubakar *et al.*[31] showed that the range of ALT in the control group was between 40.75 and 50.34 U/dL and AST was between 103.20 and 129.26 U/dL, which are different from the results found in the present study. In addition, the level of protein in the blood is a determinant of liver toxicity. Due to exposure to a substance, the activity of the liver can be determined by protein synthesis, and by examining the aberrant levels of protein in the blood to see if the hepatocytes have been destroyed[32]. Based on the work of Hsu *et al.*[33] and Li *et al.*[34], the protein level range in the control group is 41.36-67.84 g/dL, which shows that the protein in the groups with the extracts and fractions of *A. majus* is in a normal state. Based on the levels of the abovementioned parameters as well as the histopathological results, it can be said that the liver function in the treatment groups is not damaged and is in a normal state.

Similarly, UR and CR are the most essential parameters involved in kidney function that damage the glomeruli of the kidneys due to their elevated level in the blood. Their levels were not significantly different in all treatment groups compared to the control group. Moreover, the microscopic observation of the kidneys showed no abnormality compared to the control group, which implies that the kidneys are in a normal state.

Lipoproteins (HDL, LDL, VLDL) are protein particles that carry fats, such as cholesterol and triglycerides. Dyslipidemia is known by the decrease of lipoprotein (HDL), hyperlipidemia, and an increase of LDL in the blood, which makes the β-cells of the pancreas unable to produce insulin. Therefore, there is a risk of developing type 2 diabetes[35–37]. Even if TC in the groups treated with the aqueous extracts as well as ethyl acetate and ethanolic fractions was significantly different from the control group, these values are higher compared to the work of Hsu *et al.*[33] and Shakibaie *et al.*[38] with TC ranging from 55.17 to 114.60 mg/dL. The range of LDL levels is 39.3-81.44 mg/dL[39,40], which shows that LDL is in a normal state, like HDL and VLDL. Based on biochemical analysis of lipoproteins (HDL, LDL, VLDL) and TC as well as microscopic observation, it can be concluded that the pancreas is in a normal state.

Although this study showed the safety of the extracts and fractions of *A. majus* L. at 100 mg/kg, this is valid for a repeated dose for 28 d in rodents, therefore, a repeated dose toxicity evaluation must be performed for 90 d (subchronic toxicity) and for more than 180 d (chronic toxicity) to demonstrate the safety of the 100 mg/kg dose as well as other studies must be performed on other animal subjects (dogs, pigs).

Phytochemical screening was performed on *A. majus* extracts and fractions to highlight the different families of secondary metabolites. This screening is based on staining or precipitation reactions, with the presence of such a family marked by staining or precipitation. The extracts and fractions of *A. majus* contain flavonoids, tannins, coumarins, and free quinones, while they are free of anthocyanins and alkaloids. The results are in agreement with the work of Abdul-Jalil *et al.*[41] where the presence of flavonoids in different extracts of *A. majus* was found. Harsahay *et al.*[42] and Bartnik *et al*.[43] showed the presence of coumarins in different extracts of *A. majus* seeds, which also confirms the results found in the present study. Mohammed and El-Sharkawy[44] isolated two alkaloids from the methanolic extract of *A. majus*. However, alkaloids are absent in our extracts, which may be ascribed to the climate, the soil, or the edaphic conditions. Since our plant was harvested in Rabat (Morocco) and Mohammed and El-Sharkawy[44] harvested their plant from the Delta region (Egypt), we can say that the parameters already mentioned may have affected the production of alkaloids in *A. majus* harvested in Morocco.

Analysis of the extracts and fractions of *A. majus* L by HPLC-MS/MS showed the detected compounds. Quercetin and kaempferol were detected in the macerated aqueous extract as well as ethyl acetate and ethanoic fractions. These results are in agreement with the results of Abdul-Jalil *et al.*[41], who showed that the seeds of this plant contain 0.036% of quercetin and 0.045% of kaempferol. In addition, ellagic acid is not detected in all extracts and fractions of *A. majus*. However, Nazik *et al.*[45] detected this analyte in the aqueous fractions of this plant, which may be due to the region of harvest (soil and climate may influence the elaboration of phytocompounds) as well as the extraction technique.

Although this study allowed the identification and quantification of many bioactive compounds, this is still insufficient. For full quantification and identification of compounds from *A. majus* extracts and fractions, further chromatographic and spectroscopic

studies must be conducted.

In conclusion, the phytochemical screening and identification by HPLC-MS/MS allowed us to highlight some phytochemical compounds contained in the extracts and fractions of *A. majus* L. The results of the acute toxicity study showed that the extracts and fractions investigated were not toxic at 2 000 mg/kg with a single dose, while the results of the subacute toxicity showed that the biochemical parameters were in the normal state although some parameters presented a slight increase or decrease. The histopathological analysis showed that the harvested organs did not undergo any abnormalities, except in some mice where we noticed some abnormalities in the kidneys and pancreas. However, this does not mean that the fractions and extracts are toxic at the dose of 100 mg/kg, because these abnormalities were observed in a minority of mice. For future studies, we will investigate chronic toxicity at different doses and evaluate the antidiabetic activity *in vivo*.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

AD and HB were responsible for conceptualization, as well as project administration. NS, FZB, GZ, and MAY conducted investigations. AB, KWG, THS, and LCM also contributed to the drafting and critical revision of the manuscript. KWG, THS, and AB were responsible for validation, and LR, AZ, and KWG contributed to visualization. OE was responsible for writing the original draft, while MA, AZ, LR, AB, KWG, THS, and LCM contributed to the review and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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