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EVALUATION OF ACUTE AND SUBACUTE ORAL TOXICITIES OF AQUEOUS SEED EXTRACT OF *PIMPINELLA ANISUM* L. IN MALE MICE

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Abstract

Description of the subject: *Pimpinella anisum* L. is widely used in traditional medicine. Hence, it is necessary to determine its toxic effect on liver, since it is a highly susceptible organ to tissue injury.
Objective: To investigate the toxic potential of the aqueous seed extract of *Pimpinella anisum* L., according to OECD guidelines.
Methods: In the acute oral toxicity, the aqueous extract was given to mice at doses ranging from 175 to 5000 mg/kg bw. In the subacute toxicity, repeated doses of 250, 500 and 1000 mg/kg bw were administered orally to mice for 28 days. At the end of experiment, the animals were sacrificed for biochemical and histological liver assessments.
Results: In acute and subacute toxicity studies, administered doses to mice did not cause death or toxic signs and no significant behavioral and body weight changes were observed, except for drowsiness and decreased motor activity, which were observed in mice treated at 1000 mg/kg bw. The serum biochemical parameters showed changes in extract-treated groups. Furthermore, the histological examination of the liver revealed observable cellular damage in extract-treated groups.
Conclusion: These results indicated that the aqueous seed extract of *Pimpinella anisum* L. may not have any single dose toxicity. But, at repeated high doses, the liver cellular structure can be negatively affected.
Keywords: *Pimpinella anisum* L.; aqueous extract; toxicity; biochemical parameters; histological examination.

ÉVALUATION DES TOXICITÉS ORALES AIGUË ET SUBAIGUË DE L'EXTRAIT AQUEUX DES GRAINES DE *PIMPINELLA ANISUM* L. CHEZ LES SOURIS MÂLES

Résumé

Description du projet : *Pimpinella anisum* L. est largement utilisée dans la médecine traditionnelle. Il est donc nécessaire de déterminer son effet toxique sur le foie, car c'est un organe très sensible aux lésions tissulaires.
Objectif : Etudier le potentiel toxique de l'extrait aqueux des graines de *Pimpinella anisum* L. selon les lignes directrices de l'OCDE.
Méthodes : Dans la toxicité orale aiguë, l'extrait aqueux a été administré aux souris à des doses allant de 175 à 5000 mg/kg pc. Dans la toxicité subaiguë, des doses répétées de 250, 500 et 1000 mg/kg pc ont été administrées par voie orale aux souris pendant 28 jours. A la fin de l'expérience, les animaux ont été sacrifiés pour des évaluations biochimiques et histologiques du foie.
Résultats : Dans les études de toxicité aiguë et subaiguë, les doses administrées n'ont pas provoqué de mort ni de signes toxiques et aucun changement significatif du comportement et du poids corporel n'a été observé, à l'exception de la somnolence et de la diminution de l'activité motrice qui ont été observées chez les souris traitées à 1000 mg/kg pc. Les paramètres biochimiques sériques ont montré des changements chez les groupes traités. De plus, l'examen histologique du foie a révélé des dommages cellulaires observables chez les groupes traités.
Conclusion : Ces résultats ont indiqué que l'extrait aqueux des graines de *Pimpinella anisum* L. pourrait ne présenter aucune toxicité à dose unique. Mais, aux doses élevées répétées, la structure cellulaire du foie peut être affectée négativement.
Mots clés : *Pimpinella anisum* L.; extrait aqueux; toxicité; paramètres biochimiques; examen histologique.

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INTRODUCTION

Medicinal plants have long been used for the treatment of certain diseases, they are the backbone of traditional medicine. Traditional medicine refers to any ancient and culturally based health care practice differing from scientific medicine and is largely transmitted orally by communities of different cultures [1]. For centuries, in Algeria as in all countries of the Maghreb, medicinal and aromatic plants are used mainly in rural areas by the elderly who are still experiencing some herbal tea recipes [2]. Although Algeria is one of the riches Arab countries with 3164 plant species [3]. However, although medicinal plants have beneficial properties to human health, they are not without risk of intoxication. The review of Meagan Thompson et *al*. [4] highlighted various plants that are hepatotoxic. Liver is the principal site for metabolism and excretion in body [5]. During evaluation of liver toxicity caused by various compounds, the degree of steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis are often used as toxicity markers [6]. Our study focuses on Aniseed (*Pimpinella anisum* L.), plant of the Apiaceae family [7]. This spice and medicinal plant is one of the most ancient crops cultivated in the eastern Mediterranean Region, western Asia, Middle East, Mexico, Egypt, and Spain [8]. As medicinal plant, *Pimpinella anisum* L. has been used as anti-oxidant, antispasmodic, anti-microbial, digestive stimulant, and galactogogue [9]. Several researchers have pointed out the potential toxicity, as well as the risks associated with the use of certain species of plants [10]. Hence, our study was aimed to determine the toxicity effects of aqueous seed extract of *Pimpinella anisum* L. by performing acute and subacute oral toxicities on experimental male mice.

MATERIAL AND METHODS

1. Collection of plant material

Pimpinella anisum L. seed were collected from Oued Chaaba region of Batna, in the North-East of Algeria and their botanical identity was confirmed at National High School of Agronomy, Algiers, Algeria.

2. Preparation of aqueous seed extract

For preparing the aqueous extract, 250 g of powdered seeds were macerated in 2000 ml of distilled water for 24 hours under agitation at room temperature. The macerate was filtered and concentrated under reduced pressure at 60°C by means of the rotary evaporator (Stuart RE300). The resultant concentrate was dried at 40°C for 48 hours. The extract representing a percentage yield of 22.04% was stored at 4°C.

3. Animal conditions

The animal model used for conduction acute and subacute oral toxicities was Swiss albino male mice weighing 19-25 g. They were bred in the animal house of Antibiotical Company SAIDAL (Medea, Algeria). The mice were kept under ambient temperature (25°C±2), relative humidity 60±10% and natural photoperiod of 12 hours light/dark cycle. Animals were allowed free access to standard mice diet and water.

4. Acute oral toxicity study

The acute oral toxicity study of the aqueous seed extract of *Pimpinella anisum* L. was performed according to the Organization for Economic Cooperation and Development (OECD) guideline 425 for the Testing of Chemicals [11], where limit test dose of 5000 mg/kg bw was used. All the animals were fasted for 16 hours prior the experiment but had free access to water. A total of 25 male mice were divided into five groups. One group received distilled water and served as control group, while the other four groups considered as treated groups received separately oral doses of 175, 550, 1750, and 5000 mg/kg bw of the extract. The animals were carefully observed during the first 4 hours after the administration of aqueous seed extract. Thereafter, the animals were observed daily for a period of 14 days for recording the clinical signs, behavior change, mortality, and body weight change.

5. Subacute oral toxicity study

The experimental protocol was performed according to OECD guideline 407 for the Testing of Chemicals [12]. Three doses of 250, 500 and 1000 mg/kg bw of the aqueous seed extract were orally administered respectively to three groups each consisting of 5 male mice. The control group received only vehicle. During the experimental period, the mice received drinking water and food *ad libitum*. The animals of all groups were weighed weekly and observed twice daily during a 28-day period for mortality and any abnormal clinical signs.

6. Blood sampling and biochemical study

On 29th day, the overnight fasted mice were anaesthetized with diethyl ether inhalation. Blood samples were taken by cardiac puncture and collected into tubes without anticoagulant for the assessment of liver injury.

Measurement of ALP (alkaline phosphatase), ALT (alanine aminotransferase), AST (aspartate aminotransferase) enzymes and serum level of total bilirubin have been performed using Automated Biochemistry Analyzer (Random Access Clinical Analyzer, PICTUS 200).

7. Histological examination

After mice dissection, the liver was removed, washed with physiological saline, weighed to calculate the relative liver weight and rapidly immersed in 10% neutral buffered formalin for histological study. The tissues were dehydrated with graded series of ethanol (70%, 80%, 90%, 95% and 100%), cleared in xylene, and finally embedded in paraffin wax [13]. Tissue sections of 4-5 µm in thickness were prepared using a rotary microtome (CUT 5062, SLEE) and stained with hematoxylin and eosin for light microscopic analysis.

8. Statistical analysis

All values were expressed as the mean ± standard error of mean (S.E.M). Differences between control and experimental groups were determined by One-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test using Statistical Package for the Social Sciences (SPSS, version 26.0). A value $p<0.05$ was considered as statistically significant.

RESULTS

1. Acute oral toxicity study

During the 14 days after administration of the aqueous seed extract of *Pimpinella anisum* L. at doses of 175, 550, 1750, and 5000 mg/kg bw in mice, no deaths were recorded (Table 1).

Table 1: Mortality of mice according to administered doses.

Control group [a]		Test groups [b]							
		175 mg/kg		550 mg/kg		1750 mg/kg		5000 mg/kg	
4 h	14 days	4 h	14 days	4 h	14 days	4 h	14 days	4 h	14 days
0/5 [c]	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

a: treatment without aqueous extract; b: treatment with aqueous extract; c: number of dead mice/number of mice used

Furthermore, no toxic signs were observed in all groups. The mice did not display significant changes on the appearance and the general behaviors (Table 2).

Moreover, during the study period, the aqueous extract treatments did not cause any significant changes ($p>0.05$) in body weights of treated groups compared to the control group (Table 3).

Table 2: General appearance and behavioral changes for control group and test groups.

Observation	Control group [a]		Test groups [b]							
			175 mg/kg		550 mg/kg		1750 mg/kg		5000 mg/kg	
	4 h	14 days	4 h	14 days	4 h	14 days	4 h	14 days	4 h	14 days
Skin and fur	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Convulsions	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Breathing abnormalities	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Lethargy	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Drowsiness	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Increased motor activity	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Decreased motor activity	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Restlessness	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Itching	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

a: treatment without aqueous extract; b: treatment with aqueous extract

Table 3: Effects of the aqueous seed extract of *Pimpinella anisum* L. doses on mice body weight.

Body weight (g)	Control group [a]	Test groups [b]							
		175 mg/kg	*p* value	550 mg/kg	*p* value	1750 mg/kg	*p* value	5000 mg/kg	*p* value
0 day	23 ±0.71	22.4±0.81	0.966^{ns}	21.4±1.03	0.513^{ns}	21±0.89	0.326^{ns}	22.4±0.87	0.966^{ns}
7th day	26.6±0.93	26±0.63	0.965^{ns}	25.2±0.86	0.613^{ns}	24.6±0.98	0.317^{ns}	26.2±0.86	0.992^{ns}
14th day	30.2±0.92	29.8±0.66	0.990^{ns}	29.2±0.86	0.806^{ns}	28.8±0.8	0.580^{ns}	30.2±0.86	1.000^{ns}

Values are presented as mean ± S.E.M; N=5. ns: non-significant value ($p>0.05$) compared to the control group with statistical analysis by one-way ANOVA followed by Dunnett's post-hoc test. a: treatment without aqueous extract; b: treatment with aqueous extract.

2. Subacute oral toxicity study

The effects of 28-day repeated doses of the aqueous seed extract of *Pimpinella anisum* L. was determined based on the toxicity indicators such as: mortality, general behaviors, body weight, relative liver weight, biochemical analysis, and histological examination.

2.1. Clinical observations, body weight, and relative liver weight

Overall, no mortality was recorded in all groups throughout the 28 days of the experiment. No clinical toxicity signs were observed in the experimental mice, except for the mice treated with the extract at a dose of 1000 mg/kg bw where some changes in general behaviors, such as decreased motor activity and drowsiness, were seen (Table 4).

Table 4: Effects of subacute dose of the aqueous seed extract of *Pimpinella anisum* L. on mice general appearance and behavioral changes.

Observation	Control group [a]	Test groups [b]		
		250 mg/kg	500 mg/kg	1000 mg/kg
Skin and fur	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent
Convulsions	Absent	Absent	Absent	Absent
Breathing abnormalities	Absent	Absent	Absent	Absent
Lethargy	Absent	Absent	Absent	Absent
Drowsiness	Absent	Absent	Absent	Present
Increased motor activity	Absent	Absent	Absent	Absent
Decreased motor activity	Absent	Absent	Absent	Present
Restlessness	Absent	Absent	Absent	Absent
Itching	Absent	Absent	Absent	Absent

a: treatment without aqueous extract; b: treatment with aqueous extract

Changes in body and relative liver weights of all groups throughout the treatment period are presented in Tables 5 and 6, respectively. No statistically significant differences (p>0.05) in body weight were found in extract-treated groups compared to the control group. Furthermore, the measurements of absolute and relative liver weights showed a slight reduction at a dose of 1000 mg/kg bw compared to the control group. Nevertheless, the difference was not statistically significant (p>0.05).

Table 5: Body weight changes in mice after 28 days of treatment.

Parameter	Week	Control group [a]	Test groups [b]					
			250 mg/kg	*p* value	500 mg/kg	*p* value	1000 mg/kg	*p* value
Weekly body Weight (g)	1st	26±1.3	24.8±0.97	0.773^{ns}	26.4±1.07	0.987^{ns}	25.2±0.92	0.914^{ns}
	2nd	30.4±1.17	28.8±0.97	0.631^{ns}	30±1.18	0.989^{ns}	29.6±1.17	0.923^{ns}
	3rd	34.6±1.08	32.6±0.81	0.401^{ns}	32.8±1.24	0.481^{ns}	33.6±0.93	0.832^{ns}
	4th	36.4±1.03	34.4±0.93	0.318^{ns}	34±0.89	0.195^{ns}	33.4±0.81	0.086^{ns}

Values are presented as mean ± S.E.M; N=5. ns: non-significant value (p>0.05) compared to the control group with statistical analysis by one-way ANOVA followed by Dunnett's post-hoc test. a: treatment without aqueous extract; b: treatment with aqueous extract.

Table 6: Relative liver weight changes in mice after 28 days of treatment.

Parameters	Control group [a]	Test groups [b]					
		250 mg/kg	*p* value	500 mg/kg	*p* value	1000 mg/kg	*p* value
Absolute liver weight (g)	2.21±0.12	2.14±0.1	0.942^{ns}	2.05±0.08	0.564^{ns}	1.92±0.07	0.136^{ns}
Relative liver weight (g/100 g bw)	6.04±0.18	6.22±0.18	0.765^{ns}	6.04±0.13	1.000^{ns}	5.75±0.12	0.442^{ns}

Values are presented as mean ± S.E.M; N=5. ns: non-significant value (p>0.05) compared to the control group with statistical analysis by one-way ANOVA followed by Dunnett's post-hoc test. a: treatment without aqueous extract; b: treatment with aqueous extract.

2.2. Biochemical parameters

Table 7 shows the serum levels of ALP, ALT, AST and total bilirubin in mice receiving the aqueous seed extract of *Pimpinella anisum* L. at doses of 250, 500 and 1000 mg/kg bw. The obtained data revealed that serum ALT and AST levels increased significantly (p<0.05) following the repeated administration of the aqueous seed extract. Also, the serum ALP level increased significantly p <0.05) at a dose of 1000 mg/kg bw of the aqueous seed extract comparing with control group. In addition, there was significant difference (p<0.05) in serum total bilirubin level between extract-treated group at 1000 mg/kg bw and the control group.

Table 7: Effects of subacute dose of the aqueous seed extract of *Pimpinella anisum* L. on liver function test.

Biochemical parameters	Control group [a]	Test groups [b]					
		250 mg/kg	*p* value	500 mg/kg	*p* value	1000 mg/kg	*p* value
Alkaline phosphatase (U/l)	111±0.89	111.2±1.16	1.000^{ns}	113.2±0.73	0.947^{ns}	205.6±6.92	0.000*
Alanine aminotransferase (U/l)	30±1.14	87.6 ±2.84	0.000*	229.8±3.57	0.000*	494.8±2.99	0.000*
Aspartate aminotransferase (U/l)	80±2.00	103.6±2.11	0.000*	146 ±2.04	0.000*	273.2±3.39	0.000*
Total bilirubin (mg/dl)	0.41±0.04	0.44±0.03	0.959^{ns}	0.52 ±0.01	0.186^{ns}	2.03 ±0.05	0.000*

Values are presented as mean ± S.E.M; N=5. ns: non-significant value (p>0.05); *: significant value (p<0.05) compared to the control group with statistical analysis by one-way ANOVA followed by Dunnett's post-hoc test. a: treatment without aqueous extract; b: treatment with aqueous extract.

2.3. Histological parameters

The macroscopic examination of the liver for gross pathological lesions has demonstrated the absence of any gross morphological abnormalities in all experimental mice. However, the microscopic examination showed remarkable histopathological changes in the liver of extract-treated groups compared with that of the control group after repeated administration of the aqueous seed extract of *Pimpinella anisum* L. during 28 days (Fig. 1).

Light microscopic analysis of the liver sections of control group showed a normal histological structure (Fig. 1a). Mice treated with the aqueous seed extract at a dose of 250 mg/kg bw showed cytoplasmic alteration of hepatocytes and slight sinusoidal dilation (Fig. 1b). As for the liver sections of mice treated at 500 mg/kg bw, the microscopic examination exhibited signs of inflammation, mild fibrosis, marked dilation of sinusoids and degeneration of hypatocytes (Fig. 1c). Moreover, degeneration and necrosis of hepatocytes, mild fibrosis, signs of inflammation and cholestasis were seen in extract-treated mice at a dose of 1000 mg/kg bw (Fig. 1d).

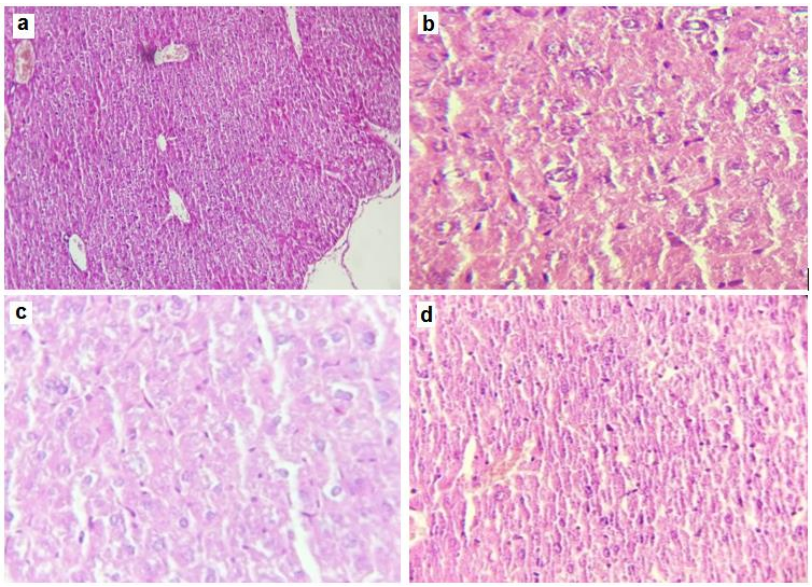

Figure 1: Light micrographs of mice liver sections (400×, H&E)

(a) Microphotograph of liver section of the control group showing hepatic parenchyma composed of central vein, portal area, and hepatocytes enclosed with sinusoid capillary and arranged as cords of cells; (b) Microphotograph of liver section of extract-treated group at a dose of 250 mg/kg bw showing hypertrophy of hepatocytes with cytoplasmic vacuolation, and dilation of sinusoids; (c) Microphotograph of liver section of extract-treated group at a dose of 500 mg/kg bw showing inflammatory cell infiltrate, fibroplastic cells, dilation of sinusoids and degeneration of hepatocytes; (d) Microphotograph of liver section of the extract-treated group at a dose of 1000 mg/kg bw showing necrosis and degeneration of hepatocytes, fibroplastic cells, inflammatory cell infiltrate and slight accumulation of brown interstitial pigment representing sign of cholestasis.

DISCUSSION

Medicinal herbs are usually self-prescribed by the consumers and there is a lack of control and review in terms of dose, manner, and frequency of administration [14]. Thus, to specify the toxic and adverse effect of each herbal medicine is a vital base to ensure the safe use of herbal medicine [15]. Therefore, Toxicity results from animals will be crucial in definitively judging the safety of medicinal plants.

In traditional medicine, plant extracts are prepared as aqueous suspensions, for example, infusions, decoctions, and poultices [13]. A practical way to characterize the toxicity of a substance is to determine its lethal dose 50 (LD_{50}). This dose makes it possible to identify the symptoms of intoxication. It is often used as a starting point for toxicity studies.

This present study focused on the seed of *Pimpinella anisum* L. which is extensively used in traditional medicine. Based on the acute oral toxicity results in mice, it was estimated that the aqueous seed extract of *Pimpinella anisum* L. does not cause acute toxicity effects. This can be attributed to sub-sufficient absorption of the extract in the gastro intestinal tract, or a high first-pass metabolism rate in the liver, by which toxic components would have been converted to their harmless derivatives [16]. Accordingly, the LD_{50} value was estimated to be greater than 5000 mg/kg bw which can mean that the aqueous seed extract of *Pimpinella anisum* L. could be assigned as a class 5 drug [17]. Regulatory agencies generally agree that once a compound reaches a dose of 5000 mg/kg bw by the oral route it can be considered "Generally Regarded As Safe (GRAS)" [18]. Moreover, if a high dose (e.g., 5000 mg/kg bw) is found to be survivable, no further acute testing will be conducted [19].

Subacute toxicity tests are conducted to evaluate the toxicity of the chemical after repeated administration and also to help in establishing doses to be used in subsequent sub-chronic and chronic toxicity studies [20, 21]. The first signs of toxicity caused by drugs and chemicals are evaluated by changes in general behaviors and body weight which are considered as one of the critical parameters [22]. In the present study, the results showed that treatment with the aqueous seed extract at a dose of 1000 mg/kg bw produced decrease in motor activity and drowsiness which suggests a reduction in the excitability of the central nervous system [23].

These data suggest that at repeated high-dose, the aqueous seed extract of *Pimpinella anisum* L. causes a sedative effect. Otherwise, these symptoms may be due to the organ dysfunction. In addition, the results obtained showed a weight gain in all groups after 28 days of study. However, mice treated with the aqueous seed extract at a dose of 1000 mg/kg bw experienced a slight reduction in the body weight gain compared to the control group. This indicates that the administration of aqueous seed extract of *Pimpinella anisum* L. at high-dose has negligible level of toxicity on the growth of mice. Scientific evidence confirmed that increases or decreases in the body weight are accompanied with accumulation of fats and physiological adaptation responses to the plant extracts rather than to the toxic effects of chemicals or drugs that lead to decrease appetite and, hence, lower caloric intake by the animal [24].

Furthermore, organ weight changes are sensitive indicators of toxicity, effects on enzymes, physiologic disturbances, and target organ injury caused by the substance under test [25]. The liver is the largest solid organ, the largest gland and one of the most vital organs that functions as a centre for metabolism of nutrients and excretion of waste metabolites [26], and is the major site of xenobiotic metabolism and transformation [27]. More than a thousand drugs and herbal remedies have been reported to cause a variety of different liver disorders [28]. Almança et *al*. [29] reported that after some exposure to potentially toxic substances, there will be a slight reduction in internal organ weights. In the present study, there were no significant differences ($p>0.05$) in relative liver weights between control and extract-treated groups, but the reduction in relative liver weight observed in extract-treated mice at a dose of 1000 mg/kg bw compared to the control group indicates the toxic potential of the aqueous seed extract of *Pimpinella anisum* L. at high doses.

The application of blood serum or plasma enzymes as marker to measure organ damage, cell damage, enzyme induction, activation or inhibition of enzymes becoming very common in toxicity studies [30]. The biochemical indices monitored in the liver are useful markers for assessment of the state of the liver, describing its functionality, cellular integrity and its link with the biliary tract [31].

In this present study, blood tests performed to assess liver health included liver enzymes levels (alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP]), and serum total bilirubin level.

ALP, a marker enzyme for plasma and endoplasmic reticulum, is often employed to assess the integrity of plasma membrane [32]. ALP is not specific to the liver, it is also produced in bone, intestine and placenta [33]. In the present study, the elevation serum content of ALP caused by repeated dose of 1000 mg/kg bw of the aqueous seed extract of *Pimpinella anisum* L. may be attributed to hepatic dysfunction [34]. The liver enzymes AST and ALT reflect hepatocyte cell death [34]. However, ALT is the more specific marker of hepatocyte injury as AST can be also elevated in the state of cardiac arrest or muscle injury [36, 37]. Those enzymes are release into blood from the cytosol and subcellular organelles of hepatocytes once liver is injured [30]. In the present study, the serum levels of AST and ALT enzymes increased significantly ($p<0.05$), this means that the liver function was affected. But, the serum AST levels in the extract-treated mice at a dose of 500 and 1000 mg/kg bw were less than that of ALT. Lawrence [38] reported that most causes of hepatocellular injury are associated with an AST that is lower than the ALT. In addition, significant increase in serum ALT level at a dose of 1000 mg/kg bw indicates direct hypatocyte damage or inflammation [39].

Bilirubin is produced by the breakdown of heme, a component mostly derived from the haemoglobin of red blood cells or from other hemoproteins, such as myoglobin, cytochromes, and catalase. It was generally considered as a useless metabolite, with little physiological function, and can be toxic at very high levels [40]. Besides this, serum bilirubin concentration reflects the ability of hepatocytes to take up, conjugate and secrete bilirubin, so it is a functional marker rather than a marker of cellular integrity as reflected by serum transaminase levels. In the absence of biliary obstruction, total bilirubin is an insensitive measure of liver function. This means that by the time total bilirubin elevates, substantial hepatic injury has occurred [41]. Furthermore, the degree of increase in serum bilirubin values has prognostic significance in chronic liver injuries, but not in acute injuries [42]. In this finding, the total bilirubin level in all experimental mice was within the normal range except in extract-treated mice at a dose of 1000 mg/kg bw where the serum level was mildly elevated which indicates that there were no any chronic liver injury due to the administration of aqueous seed extract of *Pimpinella anisum* L. for 28 days.

Besides that, the liver enzyme pattern characterized by R ratio (ALT/ALP) between 2 and 5 means the presence of mixed-type injury [43]. In the present study, prominent elevations in both ALP and ALT at a dose of 1000 mg/kg bw yielded R ratio equal to 2.41, indicating the presence of hepatocellular and cholestatic injury caused by high-dose of the aqueous seed extract of *Pimpinella anisum* L. in experimental mice.

Apart from the blood analysis, histopathological analysis provides supportive evidence for biochemical assessments [14]. The functional studies in toxicology should be coupled with the appropriate histological studies, because morphological studies are useful especially during the anatomical localization of action of toxin [44]. Macroscopic and microscopic assessments of pathological alteration in the organs of treated animals are the basis of safety assessment [45].

It is well known that the central role of liver in drug metabolism predisposes it to toxic injury [46]. Hence, hepatic pathology is central to many toxicological pathology studies [47]. In the present study, intake of the aqueous seed extract of *Pimpinella anisum* L. did not cause morphological abnormalities on liver of experimental mice, but it induced histological changes according to the extract dose.

Changes in liver architecture indicate toxicity and adverse effect on liver. The hepatotoxic effect may be due to anyone or more phytochemicals present in the extract [31]. Plants can contain pharmacologically useful and active compounds but they can also contain toxic substances. Accumulated metabolites apparently have the property of capturing free radicals, a process which may lead to liver toxicity, damage to the hepatocyte's mitochondria and difficulty in metabolizing [14].

In this present study, doses of 250 and 500 mg/kg bw of *Pimpinella anisum* L. aqueous seed extract in mice showed abnormalities in liver tissues. The microscopic examination of hematoxylin and eosin stained liver sections showed that after 28 days of treatment, the aqueous seed extract caused structural alteration indicated by less organized hepatocytes.

Also, the cytoplasm of most hepatocytes was filled with vacuoles and the walls of the sinusoids were dilated. The hepatocytes are the main functional cells of the liver. A compromise in the integrity of the hepatocytes could lead to improper functioning of the liver [48]. Due to a central role in metabolism, hepatocytes are targeted in disorders of nutritional excess [49]. Loss of normal structure of hepatocytes shows cytoplasmic alteration. Because of disturbance of the cell membrane integrity, accumulation of intracytoplasmic fluid may occur. This causes vacuolation and swelling of cells. This change may be a precursor to hepatocyte necrosis [47]. Sinusoidal dilation, on the other hand, has been suggested to represent the early stages of hyperplasia of the sinusoidal lining cells [50].
Moreover, histopathological assessment of liver tissues at a dose of 1000 mg/kg bw in this present study showed necrosis and degenerative changes in numerous hepatocytes indicated by diminution of cells when compared to control. This cellular damage may be due to the interference of the inactivation of enzymatic proteins with normal cellular metabolism [41]. Furthermore, hepatocellular injury accompanied by inflammatory infiltrate is the most common histological abnormality where cholestasis is rarely the predominant lesion, as it is often accompanied by hepatocellular injury producing a mixed pattern [51]. In this study, inflammatory infiltrate and cholestasis were observed. Infiltration of different inflammatory cells is typically a response to parenchymal cell death with causes ranging from infectious agents, exposure to toxicants, generation of toxic metabolites and tissue anoxia [46]. Besides, cholestasis is defined as an impairment of bile secretion and flow followed by a lack of bile in intestine and accumulation of potentially toxic bile acids in the liver and the systemic circulation. Cholestasis results in intrahepatic retention of cytotoxic bile acids which can thus lead to liver injury or liver fibrosis [52].

CONCLUSION

The results of the present study suggest that the orally administered aqueous seed extract of *Pimpinella anisum* L. is safe up to 5000 mg/kg bw. However, the prolonged use of the extract at high doses has deleterious effects on liver, thereby inducing a mixed cholestatic-hepatocellular liver injury and therefore, its prolonged consumption in high doses should be avoided.

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