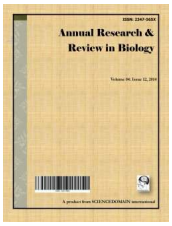

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Effects of Acute and Sub-acute Oral Toxicity Studies of Ethanol Extract of *Tanacetum parthenium* (L) Sch. Bip. Aerial Parts in Mice and Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EER and EJAF designed the study. Author JATJ performed the statistical analysis. Author JLAA wrote the protocol. Author OHC wrote the first draft of the manuscript. Authors OHC and CAC managed the analyses of the study. Author HJG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Many herbal products could have serious side effects. This study aim is to evaluate the *in vivo* acute and sub-acute oral toxicity of ethanol extract of *Tanacetum parthenium* (L) Sch. Bip. in mice and rats.
Study Design: In acute study: a total of 10 female Balb/c mice (20-25 g) were divided into 2 groups (n=5) namely control and tested group which received ethanol extract of *T. parthenium* orally at

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dose of 2000 mg/kg body weight for 14 days via oral gavage meanwhile in sub-acute study a total of 30 female albino rats (140-160 g) were divided into 3 groups (n=10) namely control and tested groups which received an oral dose of ethanol extract of *T. parthenium* at doses of 500 and 1000 mg/kg of body weight respectively for 28 days via oral gavage.

Place and Duration of Study: Laboratory of Pharmacology and Toxicology, Faculty of Health Sciences, Universidad Nacional San Cristobal de Huamanga, Ayacucho, Peru.

Methodology: Phytochemical screening was assessed by using chemical reactives. The oral acute toxicity was developed in rats and mice, according to the Organization of Economic Co-operation and Development (OECD) guidelines. Blood samples were collected at the end of experiment for evaluation of haematology and serum biochemistry. In addition, liver was collected for histopathological examination. The toxicity of the extract was evaluated by observing and evaluating the changes of haematology, serum biochemistry parameters and also histopathological changes of liver.

Results: Phytochemical study confirmed flavonoids, phenolic compounds, triterpenes and steroid, alkaloids and saponins in ethanol extract of *T. parthenium*. In both acute and sub-acute oral toxicity studies, there was no mortality as a sign of toxicity observed during the period of experiment. Haematology and serum biochemistry parameters did not show any significant changes compared with control group. Similarly, histopathological examination of liver revealed steatosis and inflammatory cells in liver.

Conclusion: Results indicated that the oral administration of *T. parthenium* at various doses had toxic effects on the liver tissues in the sub-acute toxicity study.

Keywords: Tanacetum parthenium; acute toxicity; sub-acute toxicity; haematology; histopathology.

ABBREVIATIONS

T. parthenium: Tanacetum parthenium; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.

1. INTRODUCTION

Toxicity studies in medicinal plants are necessary for evaluating safety and assess its intake in human beings [1]. It is known the introduction to the commercial market of a great number of new natural products, although the majority of these compounds have contributed to improve the quality of life, they are related with determined risks for its toxicity [2]. Medicinal plants contain active ingredients generally responsible of its therapeutic properties but also are attributed intoxications and serious side effects [3].

In the world, herbal medicines are getting considerable interest. According to the last reports of the World Health Organization (WHO), herbal medicines are used for prevention and treatment of many diseases by 80% of people in developing countries. In these countries, the public interest in herbal prescriptions has also recently increased [4].

The use of animals in pharmacological experiments commonly called preclinical studies is the first step to develop natural products for human use [5]. Currently, guidelines from institutions such as Organization of Economic Cooperation and Development (OECD), International Conference on Harmonisation (ICH), European Medicines Agency (EMA) and Food and Drug Administration (FDA) are used to evaluate different toxicity studies considering ethical principles as 3R (reduce, refinement, reuse) [6].

Medicinal plants are the best sources of natural medicine in the world [7]. *Tanacetum parthenium* (L) Sch. Bip. (Family: Asteraceae) known as “santa maría” in Peru is a perennial and aromatic plant. It is used traditionally for the treatment of fever, headache, rheumatoid arthritis, bites of insect, infertility and problems with the menstruation [8]. Various studies were carried out in order to isolate phytochemicals such as parthenolides, canin, artecanin, santamarin, luteolin, tanetin, apigenin, 6-hydroxy-flavanols, camphor, borneol, germacrene, polyacetylenes, pyrethrin, melatonin and tannins, otherwise, the active metabolite is parthenolide which is the sesquiterpene lactone most abundant as phytochemical marker [9].

The ethanol extract of *Tanacetum parthenium* possesses therapeutic potential endorsed scientifically that makes possible the employment of this plant in diverse conditions. Many authors have validated different pharmacological effects like anti-inflammatory, analgesic, antimicrobial, anti-leishmanial, anti-platelet, anticancer and anti-pyretic [10]. However, the existent data about the toxicity of *T. parthenium* is limited, studies of acute and sub-acute toxicity in experimental animals do indispensable to evaluate its toxic potential.

The main objective was to evaluate the acute and sub-acute toxicity of ethanol extract of *Tanacetum parthenium* (L) Sch. Bip. in rodents.

2. MATERIALS AND METHODS

2.1 Chemicals

Chemicals were of analytical grade and purchased from Merck (Lima, Peru).

2.2 Animals

Female Balb/C mice with weight of 20–25 g were used in the acute toxicity test. For the subacute toxicity, albino Holtzman rats weighing 140–160 g were used. Animals were purchased from the National Institute of Health (NIH-Peru), Lima, Peru. All animals were housed under standard laboratory conditions [(22± 2) ºC] with access to balanced food and water *ad libitum*. Experimental protocols were carried out and following the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NHI-Peru).

2.3 Plant Material

Aerial parts of *T. parthenium* were collected, in December, 2015, from Huamanga, province and department of Ayacucho, Peru. A voucher specimen identified as 17-USM-2015 was deposited at the National Herbarium of Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru.

2.4 Extraction of Plant Material

1.0 Kg of collected samples were dried at room temperature, at the Laboratory of Pharmacology and Toxicology, Faculty of Health Sciences, Universidad Nacional San Cristobal de Huamanga, the powder material was exhaustively macerated with 96% ethanol (3 L) for 7 days. The extract was filtered and evaporated by using a rotavap with 80 rpm and 40℃.

2.5 Phytochemical Screening

Ethanol extract was evaluated by using specific chemical reactives for each secondary metabolite such as phenolic compounds, flavonoids, quinone, triterpenes, flavonoids, tannins, saponins, steroids and alkaloids [11].

2.5.1 Test for phenolic compounds

Ferric chloride ($FeCl_3$) test: A few drops of $FeCl_3$ solution were added to extract solution. A dark green or blue-black indicated the presence of phenolic compounds.

2.5.2 Test for tannins

Gelatin test: A few drops of 2% gelatin and 1% sodium Chloride solutions separately were added to extract solution in water. A white precipitate indicated the presence of tannins.

2.5.3 Detection of flavonoids

Shinoda test: If a red color is observed once magnesium leads and hydrochloride acid concentrate are added to the test tube, it indicates flavonoids are present.

2.5.4 Detection of alkaloids

1% Hydrochloric acid solution was used to dissolve the ethanolic extract, which was filtered, and these were used for three types of Alkaloid reactions.

Dragendorff's test: A red precipitate was observed, which proved the presence of Alkaloids.

Mayer's test: White precipitation was evidenced, which is typical for Alkaloids.

Wagner's test: A reddish brown color appeared once the filtrates were treated with iodine in Potassium Iodide solution (Wagner's reagent), hence proving Alkaloid presence.

2.5.5 Detection of saponins

Froth test: Distilled water was used to dilute the ethanolic extract. A froth appears when the test tube was vigorously shaken for more than one minute, thus proving the presence of Saponins.

2.5.6 Detection of triterpenes and steroids

Lieberman's test: Dichloromethane was used to dissolve the ethanol extract and this solution was then filtered. A few drops of concentrated H_2SO_4 and acetic anhydride were added to the filtrate, the solution was shaken and then kept still. Triterpenes and steroids were indicated by a red color in the intermediate layer.

2.5.7 Detection of quinone

Bornträger's test: Chloroform was used to dissolve the ethanol extract and the solution was then filtered. A few drops of 10% sodium hydroxide were added to the filtrate; the solution was shaken and then kept still. Quinones were indicated by a red color in the upper layer.

2.5.8 Detection of aminoacids

Ninhydrin test: A few drops of concentrated 1% ninhydrin were added to 2 mL of extract solution dissolved in water. A violet color appears once it is heated.

2.6 Acute Toxicity Study

Ethanol extract of *T. parthenium* was evaluated according to Organization for Economic Cooperation and Development (OECD) guideline 423, where the limit test dose of 2000 mg/kg was used as protocol. Balb/c mice (25–30 g) of female sex were kept at overnight fasting before to test, with access to water *ad libitum*. Mice were randomized into two groups, each comprising 5 animals. One group served as a negative control (1% tween 80;10 mL/Kg) and the other was considered as tested group, which received orally *T. parthenium* (dissolved in tween 1%) extract at dose of 2000 mg/kg. At the beginning of the evaluation, the body weight (b.w.) of each animal was determined. Mice were observed for any toxic sign for 24 h after the treatment. Next, mice were observed for a period of 14 days. Behavioral changes and other parameters such as changes of body weight, food intake, death, motor activity, tremor, diarrhea, changes in eye and skin colors, etc. [12]

2.7 Sub-acute Toxicity Study

The oral sub-acute toxicity was tested according to OECD guideline 407 [13]. Adult healthy albino rats (150–160 g) of female sex were divided into 3 groups of 10 animals each and were placed under standard conditions; furthermore, a balanced diet for rodents was administered to avoid any alterations in regard to its body weight. Group I was assigned as negative control and the other two groups received *T. parthenium* extract at a dose of 500 and 1000 mg/kg of body weight respectively for 28 consecutive days.

At the end of the study, the blood samples were collected into test tube to determine biochemical and hematological parameters. The animals were sacrificed by an anesthesia (thiopental; 100 mg/Kg) after an overnight fasting (8 h). Next, liver was separated, processed, and embedded into paraffin blocks. Sections were cut at 5 µm thickness and stained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (Olympus BX51) to observe for structural changes and inflammatory cells, and confirmed by an experienced histopathologist [14].

2.8 Statistical Analysis

Data were expressed as mean ± SEM. They were analyzed by SPSS v. 24 program for Windows. Data were analyzed by One-way ANOVA followed by Tukey's multiple comparison test. P values less than 0.05 were considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Determination of Phytochemical Constituents

Phytochemical analysis is a qualitative test which indicates the presence of groups of compounds in a sample by using formation of a precipitate or a color change. The extract of *T. parthenium* indicated various classes of secondary metabolites such as tannins, phenolic compounds and flavonoids; however, quinone was negative in according to Table 1.

3.2 Acute Toxicity Study

The acute toxic effect of the ethanolic extract was determined in according to OECD guideline 423, where the limit test dose of 2000 mg/kg was used. No sings related to toxic symptom or mortality was observed after oral administration of the tested plant extract. The general behavioral was observed first for a short period (4 h) followed by long period (14 days), did not revealed any important changes in behavior, breathing, skin effects, water consumption, impairment in food intake and temperature. Hence, lethal dose 50 (LD_{50}) of the extract was considered be more than 2000 mg/kg. Although,

there were sign of irritation, and drowsiness after the administration of *T. parthenium* extract in some animals into group, compared to control group (tween 80, 1%). Table 2 shows the results for acute toxicity study after the oral administration of the extract compared with normal group. Fig. 1 reveals the weight variations of the ethanol extract of *Tanacetum parthenium* aerial parts for 14 days, where no changes were presented at the end of the experiment.

3.3 Sub-acute Toxicity Study

Tables 3, 4 and Fig. 2 show the results of the sub-acute toxic test of *T. parthenium* extract and they were determined in according to OECD guideline 407. All animals treated with the extract at a dose of 500 and 1000 mg/kg daily survived until the end of the experiment. No clinical toxicity signs were evidenced in the treated groups compared to the control group. There were no weight variations between the treated groups as shown in Fig. 2. In regard to hematological and biochemical parameters, the results were not significant such as hematocrit (P= 0.545), hemoglobin (P= 0.112) and red blood cells (P = 0.331), glucose (P = 0.096), cholesterol (P= 0.213), urea (P = 0.670), creatinine (P = 0.210) compared with control group.

In Fig. 3, Histological changes in liver were observed in groups treated with the ethanol extract of *T. parthenium* at doses of 500 and 1000 mg/Kg. Steatosis and inflammatory cells were the main findings reported in liver tissues of rats.

3.4 Discussion

According to the World Health Organization 80% of the population in the world uses the medicinal plants as an alternative treatment [15]. The uses of plants as a source of nature therapy in primary health care have become very popular in developing countries. Currently, medicinal plants play an important role in the management various diseases and have been preferred by researchers as complementary alternative medicine [16].

Table 1. Phytochemical constituents of the ethanolic extract of *T. parthenium aerial parts*

Constituents	Test	Result
Alkaloids	Mayer	+
	Dragendorff	+
	Wagner	+
Flavonoid	Shinoda	+
Quinone	Bornträger	-
Phenols compounds	Ferric chloride	+
Saponins	frothing	+
Tannins	Gelatin	+
Terpenes and steroids	Liebermann–Burchard	+

(+) Positive, (-) Negative

Table 2. General behavior of the mice to the dose of 2000 mg/kg with extract of *Tanacetum parthenium*

General Behavior	Control group (1% tween 80)	Treatment 2000 mg/kg b.w.
Decrease of motor activity	Not present	2/5
Increase of motor activity	Not present	Not present
Loss of reflections or straightening	Not present	2/5
Change in the skin	Not present	1/5
Erection of the tail	Not present	2/5
Piloerection	Not present	Not present
Drowsiness	Not present	Not present
Diarrhea	Not present	Not present
Aggressive	Not present	Not present
Afraid	Not present	2/5
Death	Alive	Alive
Weight variation	Not present	Not present

Mice were observed daily for signs of toxicity for 14 days

Table 3. Biochemical parameters in rats treated with ethanol extract of *Tanacetum parthenium* for 28 days

Biochemical parameter	Control (1% tween 80)	Treatment 500 mg/kg b.w	Treatment 1000 mg/kg b.w.
Glucose (mg/dL)	102.06 ± 1.0	98.66 ± 7.2	104.28 ± 8.0
Cholesterol (mg/dL)	75.12 ± 5.0	84.92 ± 6.2	75.12 ± 5.0
Urea (mg/dL)	18.60 ± 0.3	18.72 ± 0.4	19.23 ± 0.4
Creatinine (mg/dL)	0.55 ± 0.02	0.60 ± 0.01	0.55 ± 0.01
AST (IU/L)	8.00 ±0.3	10.62 ±1.0*	11.10 ± 0.5*
ALT (IU/L)	7.80 ± 0.7	9.94 ± 0.2*	10.44 ± 0.7*
ALP (IU /L)	178.6 ± 2.9	175.88 ± 3.5*	179.6 ± 3.1

Values are expressed as mean ± standard deviation (n = 10). Analysis of variance (P = 0.024). ALT, alanine aminotransferase; AST, Aspartate aminotransferase; ALP, alkaline phosphatase.
** Tukey test (P < 0.001) versus Control Group*

Table 4. Hematological parameters in rats treated with ethanol extract of *Tanacetum parthenium* for 28 days

Hematological parameter	Control group (1% tween 80)	Treatment 500 mg/kg b.w.	Treatment 1000 mg/kg b.w.
Hematocrit (%)	50.4 ± 0.92	47.2 ± 1.77	49.6 ± 2.62
Hemoglobin (g/dL)	16.6 ± 0.31	15.6 ± 0.56	16.35 ± 0.86
Red blood cells (10^6 cell/mm^3)	8.06 ± 0.39	8.4 ± 0.25	8.2 ± 0.33
Leucocytes (10^6 cell/mm^3)	9.7 ± 0.25	8.58 ± 0.12*	9.12 ± 0.23*
Neutrophils (%)	21 ± 0.71	21.2 ± 0.37	20.0 ± 0.84
Lymphocytes (%)	73.4 ± 0.68	72.6 ± 0.68	72.8 ± 1.11
Monocytes (%)	2.6 ± 0.50	3.0 ± 0.32*	3.8 ± 0.49*
Eosinophils (%)	2.2 ± 0.49	2.4 ± 0.40*	3.0 ± 0.45*
Basophiles (%)	0.8 ± 0.20	0.7 ± 0.20	0.4 ± 0.24*

Values are expressed as mean ± standard deviation (n = 10). Analysis of variance (P = 0.044).
** Tukey test (P < 0.001) versus Control Group*

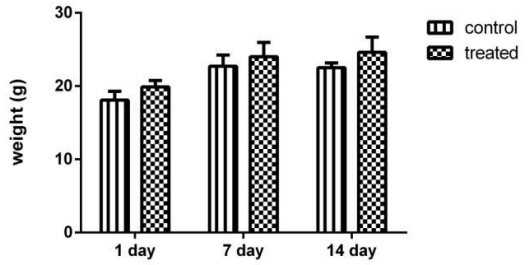

Fig. 1. Weight variations in the acute toxicity test of *Tanacetum parthenium* administered in mice

However, there is a lack of validated scientific studies on the side effects of these treatments. The oral acute toxicity of the extract indicated no major changes in behavior and death were observed in all groups. However, sedation, erection of the tail and drowsiness were confirmed in treated group with 2000 mg/kg b.w [Table 2] and [Fig. 1]. Furthermore, the LD_{50} is considered to be more than 2000 mg/kg. Any pharmaceutical drug or compound with the oral LD_{50} higher than 2000 mg/kg could be considered safe and low toxic [17]. This suggests that the ethanolic extract of *T. parthenium* is practically non-toxic in single dose by oral administration.

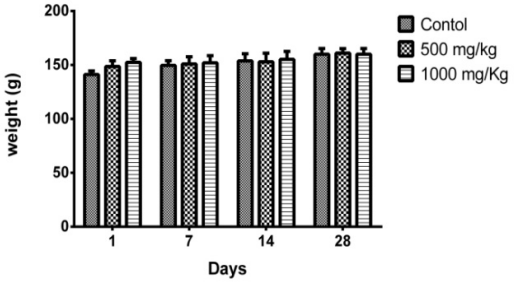

Fig. 2. Weight variations in the sub-acute toxicity test of *Tanacetum parthenium* administered in rats for 28 days

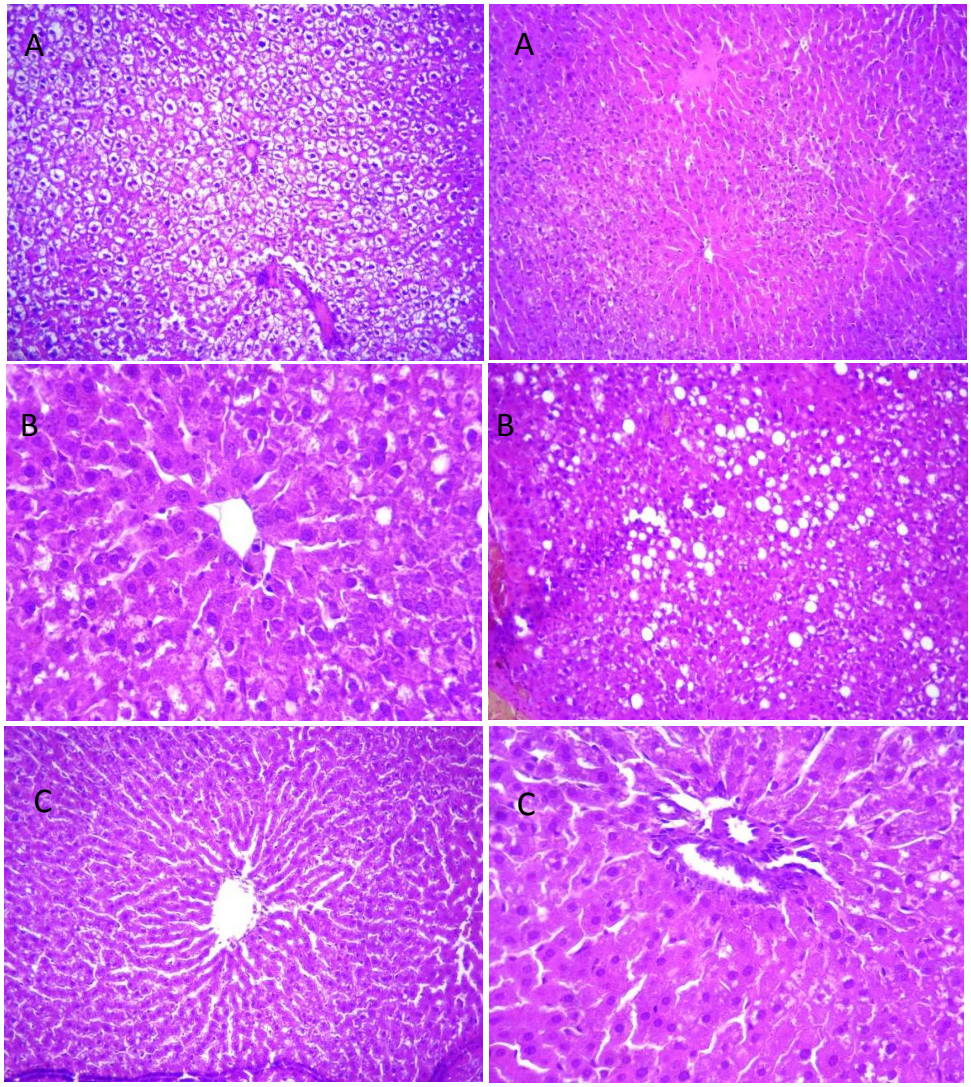

Fig. 3. Hematoxylin and eosin staining of livers from control and experimental animals (400×)
(A) Control Group: Normal liver. (B) Extract 500 mg/kg: micro-vesicular steatosis 10-20% of extension, congestion in centrilobular vein, portal space with slight infiltrated inflammatory cells (C) Extract 1000 mg/kg: steatosis macro and micro-vesicular <5%, congestion in centrilobular vein, centrilobular necrosis and portal space with slight infiltrated inflammatory cells

Plants could be used in chronical diseases like cancer, hypertension, diabetes or hyperlipidemia and in this case multiple dose have to be used, to avoid these serious side effects a subacute toxicity study can be confirmed by using analysis of weight variations, histological studies, hematological and biochemical parameters. A sub-acute toxicity study was therefore carried out with doses of 500 and 1000 mg/kg of extract as per OECD guideline [18]. Scientific evidences confirmed that increases or decreases in the body weights are accompanied with accumulation of fats and physiological adaptation responses to the plant extracts rather than to the toxic effects of chemicals that lead to decrease appetite and, hence, lower caloric intake by the animal [19].

After 28 days of daily treatment some parameters showed no significance ($P > 0.05$) when compared to control group. The bone marrow is responsible for the production of the blood cell and some phytochemicals isolated from plant have affected red blood cell levels [20]. Hence, the tested plant extract may not have harmful effects on bone marrow function

and justify the fact that at all doses of *T. parthenium* does not induce anemia, making it safe. On the other hand, estimation of serum biochemical parameters in treated animals showed significance ($P < 0.001$) compared to control group such as transaminases enzyme (AST and ALT) were observed positive significant in extract treated animal for 500 and 1000 mg/kg extract as compared to respective control group. Many studies have confirmed that elevated serum levels of hepatic enzymes, transaminases (AST and ALT) are not a directly linked for liver injury but increase levels are responsible to cause inflammation, cellular leakage and damage of cell membrane in hepatocytes [21].

The main target organ for isolated compound and plant extract is liver where exposed to the foreign substances being absorbed in intestines and metabolized to other compounds which may or may not be hepatotoxic to the animals [22]. Therefore, the increase in liver hepatic enzyme (AST and ALT) after administration of the ethanolic extract could be due to certain phytochemical compound such as alkaloids, triterpenes and tannins that might have toxic potential on liver with increasing dose and result liver injury [23]. In our histological examination slight significant changes were observed compared to control group, these findings are correlated to biochemical parameters in AST and ALT values. Micro-vesicular steatosis, congestion in centrilobular vein, portal space with slight infiltrated inflammatory cells were confirmed at dose of 500 mg/kg and 1000 mg/Kg. Other researches with extract of plants showed similar histopathological changes in liver [24,25]. These abnormalities of liver tissues could be due to the presence of secondary metabolites such as alkaloids, tannins, saponins, flavonoids and triterpenes [26]. There are two main biological components that are associated with liver damage, alkaloids, and flavonoids, which are frequent constituents of commonly used medicinal plants. The interaction with the different cytochrome P-450 isoforms, inflammatory, and oxidative activities seem to be the main damage pathway involved in the liver [27,28]. This may lead to steatosis and necrosis in hepatocytes.

The limitations of this study include the lack of assessment of the active ingredient. Although the exact damage of *T. parthenium* to other organs was not studied, it is possible to understand potential side effects with our findings in biochemical, hematological parameters and histopathology. However, is possible that new studies should be tested using chronical models of toxicity.

4. CONCLUSION

This study revealed very important findings on the acute and subacute toxicity profile of the ethanolic extract of *T. parthenium* that should be very useful for any clinical study of *T. parthenium*. These results confirmed that the use of this plant is safe for human consumption at doses less to 500 mg/Kg.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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