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Original Research Article

Acute and Sub-Chronic Toxicity Studies of Aqueous Ethanol Leaf Extract of *Vitex negundo* L in Experimental Animals

Tu T.T. Nguyen[1]*, Phuong T. Do[1], Lan T. N. Nguyen[2], Anh T.V. Pham[3]

[1]*Traditional Medicine Faculty, Hanoi Medical University, Hanoi, Vietnam*
[2]*Department of Internal Medicine, Hanoi Medical University, Hanoi, Vietnam*
[3]*Department of Pharmacology, Hanoi Medical University, Hanoi, Vietnam*

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ABSTRACT

Vitex negundo L is a medicinal plant with many notable therapeutic effects. While the beneficial effects of *Vitex negundo* have been widely described, evidence of this herb's safety, such as acute and sub-chronic toxicity effects, remains scarce. This study aimed to evaluate the safety of this herb through acute and sub-chronic toxicity evaluation of aqueous ethanol extracts of its leaves in experimental animals. The acute toxicity study of aqueous ethanol leaf extract of *Vitex negundo* was performed in mature *Swiss* mice. The sub-chronic toxicity study was conducted in rabbits following the World Health Organization's guidance on evaluating the safety and efficacy of herbal medicines. In the acute toxicity study, the *Vitex negundo* leaf extract at 620.25 g/kg/day - the highest tolerable dose in mice, yielded no oral toxicity. In the sub-chronic toxicity study, *Vitex negundo* was administered orally to 2 groups of rabbits at doses of 3.2 g/kg/day and 9.6 g/kg/day, respectively, for eight weeks. The results showed no sign of general toxicity. There were no changes in growth or hematological parameters, liver and kidney functions, and no effect in the macromorphology and micromorphology of the treated animals' livers and kidneys.

***Keywords*:** *Vitex negundo* L, herbal extract, acute toxicity, sub-chronic toxicity.

Introduction

Vietnam is located in the tropics and is home to numerous species of medicinal plants. The government and the Ministry of Health often encourage the study of medicinal plants used for healing because this aims to enhance the supply of good medicine for the community in terms of efficacy, safety, and availability. The genus *Vitex* has about 150 species worldwide, of which there are 15 species in Vietnam. The tree is distributed primarily in the low mountains and midlands and sometimes in the North and the South plains.

Vitex negundo has a bitter taste, aroma, and warmth. It clears heat, is antipyretic, invigorates blood, expels dampness, and stimulates digestion. *Vitex negundo* leaf, when steamed, can treat colds, fever, headaches, stuffy noses, and coughs. In contrast, the decoction of *Vitex negundo* leaf can treat rheumatism, paralysis, joint pain, sciatica, dysmenorrhea, and amenorrhea.[1,2] The published chemical studies on *Vitex negundo* showed a broad spectrum of compounds, such as iridoid glycosides, quinic acid derivatives, flavonoids, and lignans.[3,4] Pharmacological studies have also been conducted on this medicinal plant's analgesic, anti-inflammatory, antifungal, antibacterial, cough reduction, expectorant and antipyretic properties.[5,6]

Despite *Vitex negundo's* benefits, evidence of this herb's safety in acute and sub-chronic toxicity remains scarce. The toxicity profile is essential before considering a plant extract's pharmacological activities and applications.

*Corresponding author. E mail: thanhtu@hmu.edu.vn
Tel: +(84) 902031192

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To leverage such a valuable medicinal plant in Vietnam, we conducted this study to evaluate the acute and sub-chronic toxicity of aqueous ethanol extracts of *Vitex negundo* leaf in experimental animals.

Materials and Methods

Plant material

The *Vitex negundo* leaves were collected in the Socson district of Hanoi, Vietnam, on May 5, 2015. The sample was taxonomically authenticated at the Vietnam Institute of Ecology and Biological Resources. The voucher specimen (HMU-VN2018-01) was deposited in the same institute.

Extract preparation for animal testing

Plant materials (5 kg) were crushed into small pieces and extracted with 70% aqueous ethanol three times (solvent/material ratio, 6/1). The combined extract was then distilled under reduced pressure at 55-60°C to two-thirds of the original volume and stirred for 24 hours. After that, the extract was filtered to remove settled resin and distilled to remove the organic solvent completely to obtain a concentrated extract weighing 500g. The final extract was stored under refrigerated conditions until further use.

Animals

Ethical Approval

The Ethical Council of Hanoi Medical University, Vietnam, approved the research protocols for this study with approval number 168/HDDDDHYHN.

This study used healthy mature *Swiss* mice (18-22 g) and mature New Zealand white rabbits (1.8-2.5 kg). The animals had free access to food and water and were maintained under standard laboratory conditions for one week before the study.

Acute toxicity study

According to traditional usages, the *Vitex negundo* dosage was about 40 g of dried leaves/day, equivalent to approximately 0.8 g/kg of a person weighing 50 kg.

In the acute toxicity study, the *Swiss* mice were divided randomly into 5 groups of ten animals. They were fasted overnight before the experiment and were given oral doses of crude ethanol leaf extract of *Vitex negundo* as indicated below:

i) Group 1: 124.05 g/kg
ii) Group 2: 248.10 g/kg
iii) Group 3: 372.15 g/kg
iv) Group 4: 496.20 g/kg
v) Group 5: 620.25 g/kg

After treatment, the general conditions of the mice were monitored for signs of intoxication, and mortality in each group was recorded for 72 hours post-administration. A linear graph was generated to determine the median lethal dose (LD_{50}). The mice's general conditions (activity, eating, and excretion) were routinely monitored in each group for seven days post-extract administration.

Sub-chronic toxicity study

The sub-chronic toxicity study was performed according to the World Health Organization's guidance on evaluating the safety and efficacy of herbal medicines. A total of 30 rabbits were randomly divided into three groups; each had 10 rabbits, and each rabbit was kept in a separate cage. Rabbits were given *Vitex negundo* extract or distilled water daily in the morning for eight consecutive weeks. The control group 1 was given distilled water at a dose of 2 mL/kg/day. The dose administered to treatment group 2 was 3.2 g/kg/day (equivalent to 0.8 g/kg/day, human dose). Treatment group 3 was given 9.6 g/kg/day (3 times the dose to group 2 animals).

i) Group 1: Control group (distilled water 2mL/kg/day)
ii) Group 2: *Vitex negundo* extract 3.2 g/kg/day
iii) Group 3: *Vitex negundo* extract 9.6 g/kg/day

Before and during treatments, the rabbits were evaluated for general conditions, body weight, and hematopoietic function based on erythrocyte counts, average red blood cell volume, hemoglobin, hematocrit, white blood cell counts, and platelet counts. In addition, the hepatic function was assessed by quantifying the following enzymes and metabolites in the blood: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, total albumin, and total cholesterol; renal function was also evaluated by quantifying serum creatinine levels. The hematological parameters were determined using blood-testing solutions ABX Minidil LMG of ABX Diagnostics on Vet abcTM Animal Blood Counter. The biochemical parameters were determined using kits for testing enzymes and metabolites in blood from Hospitex Diagnostics (Italy) and DIALAB GmbH (Austria) on the Screen Master Machine of Hospitex Diagnostics (Italy).[7] All monitored parameters were assessed before administration (T_0), four weeks (T_4), and eight weeks (T_8) after administration.

Histopathology of rabbits was evaluated after eight weeks of *Vitex negundo* administration, and the whole organs (livers and kidneys) from the rabbits were rapidly removed from them and fixed in 10% neutral-buffered formalin. 5 µm thick sections from fixed and paraffin-embedded samples were processed. The sections were stained with hematoxylin and eosin (H&E) and checked for histopathological assessment.[8] A light microscope was used to detect the effects of the extract on the liver and kidney structures. The microstructure of the liver and kidney from at least 30% of rabbits in each group was randomly checked.[9]

Statistical analysis

Data were presented as mean ± standard deviation (mean ± SD). ANOVA test (for normally distributed data) and Kruskal-Wallis test (for data not normally distributed) were performed to test for statistical significance using the SPSS program (version 18. SPSS Inc., USA). The differences were statistically significant with $p < 0.05$.

Results and Discussion

Acute toxicity of Vitex negundo extract in mice

Mice were treated with Vitex negundo extract at different doses, ranging from 124.05 g/kg body weight to 620.25 g/kg body weight, in a volume of 0.25 mL/10 g, three times daily.

After oral administration of *Vitex negundo* extract, all groups had no deaths within 72 hours. Animals in groups 1-3 (dose range of 124.05 - 372.15 g/kg bw) experienced normal eating and movement and had dry stools. However, in groups 4-5 (dose range of 496.20 - 620.25g/kg), some mice had diarrhea within 24 hours post-extract administration but returned to normal stool after that (Table 1).

Herbal medicines have played an important role in treating various diseases for centuries.[10] Assessing and evaluating the toxic profile is a vital step before the screening of pharmacological activities and applications of an herbal extract. According to the General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine of the World Health Organization, all drugs and chemicals derived from medicinal herbs must be evaluated in animals through acute and sub-chronic toxicity studies before being tested on humans.[11] While available literature has investigated the pharmacological profile and beneficial effects of *Vitex negundo*, evidence about the toxicity of this medicinal plant remains scarce.[12–14] Therefore, the current study focused on assessing the acute toxicity of *Vitex negundo* extract in mice and its sub-chronic toxicity in rabbits.

Acute toxicity refers to adverse effects after a single dose of a substance is administered orally or via the dermal route. Studies on acute toxicity serve to determine the dose resulting in mortality, often characterized by lethal dose (LD_{50}) or serious toxicological events after single or multiple administrations.[9] Examining acute toxicity thus provides basic information for subsequent research of herbal extract applications. Using LD_{50} as an indicator, the degree of substance toxicity can be classified into six levels, namely extremely toxic ($LD_{50} < 1$ mg/kg), highly toxic (LD_{50} at 1 - 50 mg/kg), moderate toxicity (LD_{50} at 50 - 500 mg/kg), slightly toxic (LD_{50} at 500–5000 mg/kg), practically nontoxic (LD_{50} at 5000-15,000 mg/kg) and relatively harmless ($LD_{50} > 15,000$ mg/kg).[15] In this study, mice were given the most concentrated *Vitex negundo* extract, with a maximum volume of 0.25 mL/10 g per mouse and a maximum of 3 times in 24 hours. The dose is equivalent to 620.25 g/kg, which is over 64 times higher than the normal human dose (calculating extrapolation coefficient in mice 10). No mortality was recorded during the 7-day follow-up at this oral dose, and LD_{50} was undetermined. As defined by WHO, *Vitex negundo* extract was safe at the dose(s) given in this experiment. Therefore, *Vitex negundo* extract can be considered safe for human use within these doses.

Table 1: Mice mortality after seven days of oral administration Vitex negundo

Group number	Number of mice	Drinking volume (ml/kg)	*Vitex negundo* dose (g/kg)	Number of dead/live animals after 7 days	Abnormal signs
1	10	15	124.05	0/10	0/10
2	10	30	248.10	0/10	0/10
3	10	45	372.15	0/10	0/10
4	10	60	496.20	0/10	Diarrhea 5/10
5	10	75	620.25	0/10	Diarrhea 8/10

Table 2: Effects of long-term administration of *Vitex negundo* extract on rabbits' body weights.

Time point	Weight (kg)		
	Control group	**Group 1**	**Group 2**
T_0	2.00 ± 0.14	2.04 ± 0.14	1.93 ± 0.14
T_4	2.33 ± 0.12*	2.43 ± 0.17*	2.25 ± 0.22*
T_8	2.46 ± 0.14*	2.63 ± 0.25*	2.48 ± 0.16*

**p<0.05*

Sub-chronic toxicity of Vitex negundo extract in rabbits

The effect of *Vitex negundo* on rabbits' general conditions and body weight during long-term administration.

During the 8-week treatment period, rabbits in 3 groups were normal and agile, with bright eyes, silky fur, normal appetite, and dry stool. Table 2 showed that during the treatment period, while the body weight of each group increased significantly over time, no significant difference was observed between the control and treated groups.

Regarding the assessment of sub-chronic toxicity, repeated-dose toxicity tests were performed to investigate the general conditions (body weight, appearance, and behavior) and the potential toxic effects on the animals' haematology, biochemical parameters, and histopathology. The treated animals showed no symptoms of sub-chronic toxicity, and no digestive problems were detected. Deaths and clinical signs of local or systemic toxicity were not observed. Animal behaviors were documented in terms of general health and clinical signs of toxicity; however, no abnormalities were observed. Similarly, there were no significant differences in the body weight of the treated and control groups. In general, changes in body weight, appearance, or behavior of an animal following the administration of drugs indicate the adverse effects of the compounds.[16] Results of this study suggest that the extract did not negatively influence the appetite or induce any unwanted impact on the growth of the animals.

According to the World Health Organization, hematological parameters are highly sensitive and considered reliable indicators in testing the toxicity of herbal extract.[11] Blood is vitally important and closely related to every part and organ of the body. Pathologically, values of haematological parameters cannot only be influenced by abnormal events in every organ but also reflect the condition of the haematopoietic organ.[17] The blood components will vary if the drug affects the blood-forming organs. Therefore, the experimental animals' red blood cell count, haemoglobin content, haematocrit, white blood cell count, white blood cell formula, and platelet count were determined. After four weeks and eight weeks of treatment with *Vitex negundo* extract, the above haematological indicators of animals in both treatment and control groups were not significantly different from before using the extract (pre-and post-testing, $p > 0.05$). All parameters were within the normal reference range of the species.[18] Thus, *Vitex negundo* extract did not indicate toxicity on haematopoietic and other related organs.

Effects of Vitex negundo on haematological parameters.

The haematopoietic system is one of the most sensitive targets of poisonous chemicals, and haematological parameters play a vital role in assessing physiological and pathological status in preclinical and clinical studies. Furthermore, when the data are collected and analyzed from animal studies, any changes in the haematological system have a higher predictive value for human toxicity.[11] The analysis of haematological parameters, including red blood cell count, hemoglobin concentration, white blood cell count, and platelet count in rabbits treated with *Vitex negundo* extract of 3.2 g/kg/day and 9.6 g/kg/day did not significantly differ from those of the control group (Figure 1). In addition, the haematological parameters in both treated groups at week 4 and week 8 were not significantly different from before taking the extract ($p > 0.05$).

Assessing the effects of Vitex negundo extract on liver function

The liver function of the treated rabbits was assessed by aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity. Figure 1 demonstrated that after 4 and 8 weeks of *Vitex negundo* extract administration, both treated groups indicated no significant alteration compared to the control rabbits. Additionally, no significant effects were observed in the albumin and total cholesterol levels in the treated rabbits ($p > 0.05$).

Assessing the effects of Vitex negundo on kidney function

Furthermore, the kidney function test showed no significant change in the creatinine levels of both treated groups compared to the control after 4 and 8 weeks of extract administration (Figure 1).

The hepatic and renal functions of the treated rabbits were also evaluated. To assess the extent of hepatocellular damage, serum levels of liver-derived enzymes, namely AST (aspartate aminotransferase) and ALT (alanine aminotransferase), are usually quantified. Elevated levels of these enzymes are often associated with drug toxicity due to hepatocellular damage and may lead to liver metabolic dysfunction.[19,20]

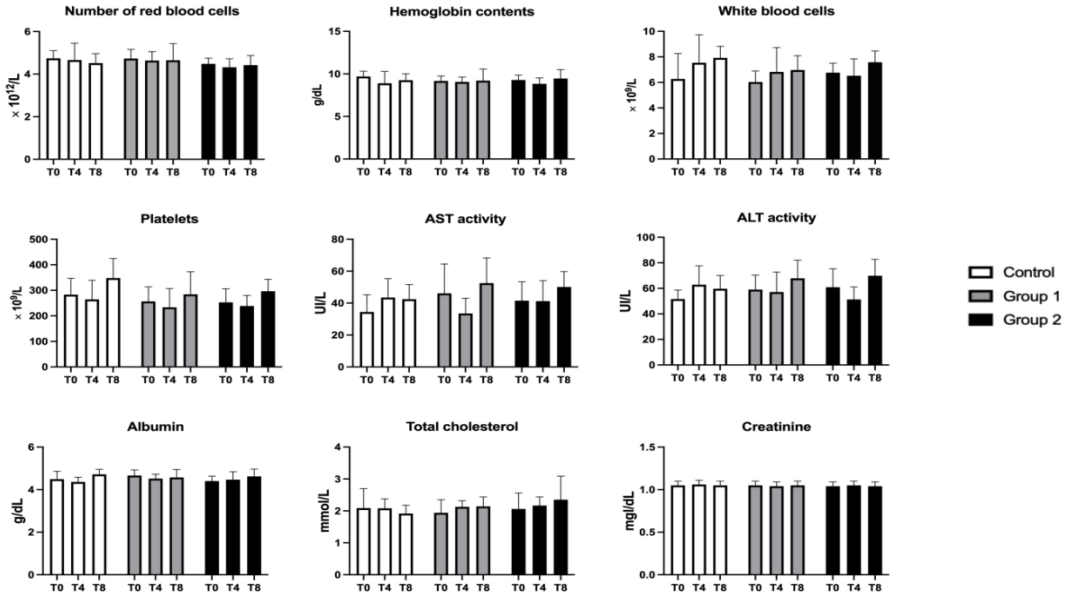

Figure 1: Effects of *Vitex negundo* extract on red blood cells count, haemoglobin contents, white blood cells count, platelets, AST activity, ALT activity, blood albumin, total cholesterol and creatinine of rabbits before administration (T_0), four weeks (T_4), and 8 weeks (T_8) after administration.

In the current study, after eight weeks of taking the extract, the activity of ALT and AST and other indicators of liver function (albumin and total blood cholesterol) in both test groups were within the normal limits and showed no significant increase. The kidneys are the body's excretory organs, and renal parenchyma cells are highly vulnerable to endogenous and exogenous substances.[21] When a compound is introduced into the body, it can induce toxicity and damage the kidneys, affecting kidney function. Assessment of renal function after taking a drug is usually achieved by a quantitative blood creatinine test. Creatinine is the most stable protein in the blood, almost independent of diet or physiological changes, but only dependent on the ability of the kidneys to excrete it. When the glomeruli are damaged, blood creatinine levels rise earlier than urea. Blood creatinine is a more reliable and essential indicator than blood urea, which is currently used to assess and monitor kidney function.[21,22] The creatinine concentration in the treated animals after administering *Vitex negundo* extract did not change significantly compared to the control group before and after taking the extract ($p > 0.05$). These data indicated the safety of *Vitex negundo* extract concerning liver and kidney functions.

Histopathological results

Histopathological changes were assessed after 8 weeks of treatment. In all experimental rabbits (both control and 2 treatment groups), no macroscopic changes were observed in the heart, lungs, liver, spleen, pancreas, kidney, and digestive system. The normal histology of rabbit livers was observed in both the control and test groups. However, mild degeneration was found in some sites (Figure 2). Histology of the kidney sections of the rabbits also showed normal patterns without any damage and degeneration (Figure 3).

Macroscopic and microscopic histological assessment of the liver and kidney is a mandatory criterion when evaluating sub-chronic toxicity. Furthermore, microscopic examination is the gold standard for assessing the damage to the two major organs responsible for drug metabolism and elimination.[11] In all the test rabbits, no pathological changes in the liver and kidneys were observed. This result was consistent with the previously discussed biochemistry of hepatic and renal function data. Our findings were also supported by previous *in vitro* and *in vivo* studies in rabbits, which demonstrated the hepatoprotective activity of *Vitex negundo* leaf extract against drug-induced hepatotoxicity.[23–25]

Our results align with available knowledge on the toxicological characteristics of *Vitex negundo* extract. However, the doses used in this study were considerably higher than in other studies. In a toxicity study of methanol leaf extract of *Vitex negundo* in mice, an extract dose of about 2000 mg/kg induced no sign of lethargy, jerk, convulsion, and mortality, while the sub-chronic toxicity investigation reported that a dose of 400 mg/kg was safe for mice.[26] A study in India also described the Acute and sub-chronic dermal toxicity of *Vitex negundo* essential oil. The research also revealed that behavioral, biochemical, haematological, and histopathological abnormalities were absent from animals treated with 250 and 1000 mg/kg/day *Vitex negundo* oil.[27] Toxicological effects of butanol extract of mature fresh leaves of *Vitex negundo* were also evaluated. The results of that study were consistent with the studies mentioned earlier, with no impairment or lesion detected.[28,29] The difference in dose among studies may be attributable to the different vehicles, concentrations of extracts, and initial concentration. However, it is noteworthy that mortality was not recorded in experimental animals; thus, LD_{50} was undetermined in all studies.

To the best of our knowledge, the current study is among a handful of research revealing the toxicity profile of *Vitex negundo* leaf extract. Nevertheless, the results of this study should be used in light of some limitations. While the acute and sub-chronic toxicity of *Vitex negundo* extract on such vital organs as the liver and kidneys were assessed, we could not examine its potential effects on other vital structures, such as the nervous, reproductive, or immune systems, due to resource constraints. The duration of this study was also shorter than the typical subchronic toxicity studies recommended in the regulatory guidelines (8 weeks vs. 90 days). It is, therefore, necessary to consider that more extended exposure periods may also give different results.

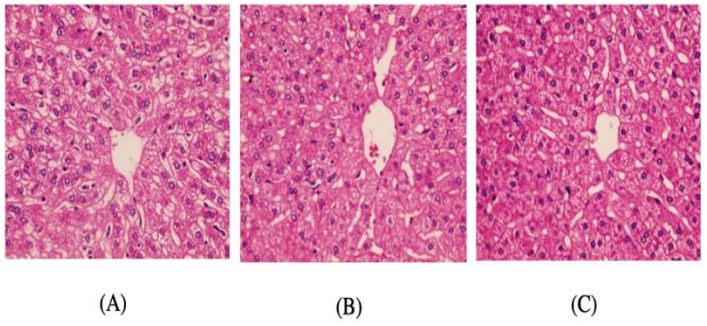

Figure 2: Histopathological images of representative rabbit livers from the vehicle (A), 3.2 g/kg *Vitex negundo* (B), and 9.6 g/kg *Vitex negundo* (C) groups after 8 weeks (HE × 400).

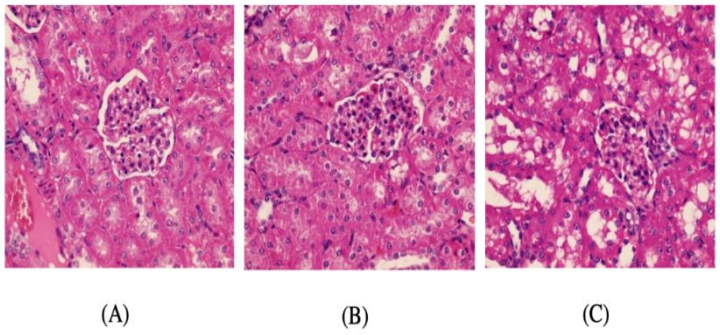

Figure 3: Histopathological images of representative rat kidneys from the vehicle (A), 3.2 g/kg *Vitex negundo* (B), and 9.6 g/kg *Vitex negundo* (C) groups after 8 weeks (HE × 400).

Conclusion

In conclusion, the administration of *Vitex negundo* extract in the acute toxicity study revealed that the extract is safe, with no deaths and no symptoms of toxicity observed. Similarly, in the sub-chronic toxicity study, after 8 weeks of extract treatment, even at doses three times higher, there were no detrimental effects on the body weight, haematological and biochemical parameters, as well as macromorphology and micromorphology of the livers and kidneys of the treated animals compared to the control group ($p > 0.05$). Hence, this study concludes that the ethanol extract of *Vitex negundo* could be used as an herbal drug for the treatment of various diseases for which it is indicated at the experimental doses reported in this study.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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