

Original article:

Subchronic Toxicity Testing of Ethanol Extract of Red Betel Vine (*Piper crocatum*) Leaves in DDY Strain Mice

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Abstract

Background: The red betel vine (*Piper crocatum*) is a plant that has potential as a medicinal plant. The use of medicinal plants should be tested for safety. Acute toxicity testing has been performed with safe results for consumption, but there is no known long-term effect on various organs. **Objective:** To determine whether an ethanol extract of the red betel vine could be used safely on different parts of DDY mice. **Methods:** The treatment of ethanol extract was performed orally every day by using a feeding tube with different doses in mice within 3 months. Those were divided into several groups, each consisting of 5 male and 5 female mice. The dosages used consisted of 50 mg/kgBW, 100 mg/kgBW, 200 mg/kgBW, and 400 mg/kgBW, with aquadest and solvent as controls. The treatment was done every day for 3 months. At the end of the study, the mice were terminated, and their organs were collected, which included the spleen, liver, kidney, heart, lungs, brain, testes, and ovaries. **Results:** The results of this study show no significant difference compared to controls in all organs. There are liver changes from cloudy swelling degeneration and reversible hydropic degeneration, but the effect is reversible. **Conclusion:** To conclude, long-term use of the red betel vine as a medicinal plant can be recommended for healthy people but not for people with a history of hepatic disorders.

Keywords: Ethanol extract, red betel leaf, toxicity

International Journal of Human and Health Sciences Vol. 08 No. 01 January'24
DOI: <http://dx.doi.org/10.31344/ijhhs.v8i1.623>

Introduction

Currently, there is a tendency for people to use herbal medicine to treat the disease. Some people want to go back to nature. One of the medicinal plants believed to have a lot of usefulness is the red betel vine. The red betel vine (*Piper crocatum*) is one of the Indonesian medicinal plants that has the potential to be further developed. The red betel vine is a creeper plant, very similar

in growth habits to the green betel. The stem is round, purplish green, has no flowers, and besides, it tastes very bitter. Heart-shaped leaves, cordate at base, acuminate at apex, with a smooth margin. The length of the leaves can reach 15-20 cm. The upper surface (adaxial) is green with silver shades, while the lower surface (abaxial) is burgundy.

The red betel vine is known to contain flavonoids, alkaloids, tannins, and essential oils. Several

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studies have reported the ability of the red betel vine to be antibacterial and antiseptic, antimycobacterial, anti-diabetic, anti-inflammatory, anti-proliferative, anti-oxidant, and others¹⁻⁶. Regularly, all medicines, whether they are synthetic or of plant origin, should fulfill the basic requirements of being safe and effective. The purpose of toxicity testing is to generate information or data about the toxicity of a material in animal tests⁷. Toxicity research in experimental animals is the main source of data for toxicological evaluation because it can reveal a range of effects due to exposure to toxicity in various doses for various exposures⁸.

Toxicity tests are grouped into short-term (acute) and long-term tests. The acute toxicity test was conducted to obtain information about the symptoms of poisoning, the cause of death, the sequence of death processes, and the lethal dose (LD) of a material. Long-term toxicity tests gather data on specific toxic effects like teratogenic, mutagenic, carcinogenic, and dependent effects. In previous studies, researchers have determined that the ethanol extract of red betel vine leaf has an LD₅₀ of 18 g/KgBW, indicating its safety for consumption. Similarly, the volatile oil of red betel vine leaves has an LD₅₀ of 9.51 g/KgBW⁹. In some applications, such as anti-diabetic, antimycobacterium requires prolonged use. Therefore, we need to know the safety of using red betel vine in the long term. This study aims to determine the safety of red betel vine leaf extract through toxicity tests.

The oral sub-chronic toxicity test looks for toxic effects caused by oral medication given repeatedly to test animals but not for more than 10% of

their lifespan. The oral sub-chronic toxicity test involves giving multiple doses of a test substance to different groups of animals for 28 or 90 days, with additional satellite groups if needed to observe any delayed or reversible effects^{10,11}.

Methods

This experimental research uses 60 male and female mice with Deutsch Democratic Yokohama (DDY) strain aged 8-10 weeks with a body weight 25-30 grams. The test animals were obtained from Animal Experiment Service Unit IV, The Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada, Indonesia.

Before the research, the mice were adapted to the cage to be used with standard feed for 3-7 days. Mice were divided into six groups, each consisting of 5 males and five females. Parameters observed in sub-chronic toxicity tests included weight, physiological conditions, blood test results (SGOT, SGPT, urea, and creatinine), and histopathologic examination (liver, kidney, stomach, lien, heart, lung, brain, and genital organs). Group I was given a dose of 50 mg/kgBW, group II was given a dose of 100 mg/kgBW; group III was given a dose of 200 mg/kgBW, group IV was given a dose of 400 mg/kgBW, group V as control was given distilled water and group VI as controls with a solvent (DMSO). The treatment of ethanol extract was given per-oral every day for 90 days. Every week, the mice were weighed, and their physiological condition was observed. At the end of the treatment, mice blood samples were collected from the orbital vein by anesthetized.

Furthermore, the mice were terminated, and the organs of the heart, lungs, stomach, kidneys, liver, and spleen were collected.

Organs were inserted in a container containing 10% formalin buffer. Samples examined at the research laboratory of the Universitas Islam Indonesia made histopathological slides. The histopathologic examination was performed by trimming longitudinally in the center to prepare the preparatory block. One slice was made in the preparation slice and then observed in the whole field



Figure 1. Red betel vine plant and leaves. Red betel vine leaves, the upper green leaves with silver hue, the bottom dark red leaves.²

of view with 40x, 100x, and 400x magnification. The examination and photograph were taken with an Optilab camera and a light microscope (The Olympus CX21). Then the results were analyzed.

Observations on the liver were made with 400x magnification in five different fields of view. Each field is calculated using 100 cells, including calculation of normal liver cell count, turbid degeneration, hydropic degeneration, and necrosis, and then assessed liver cell damage based on scoring histopathology Manja Roenigk. The number of cells counted is then multiplied by the value of each level of change. The minimum value if all cells are normal hepatocytes is 100, and the maximum value if the total liver cell is damaged is 400 – assessment of liver cell damage based on scoring histopathology Manja Roenigk.

The images in the cerebral cortex include the number of pyramidal neurons, the size of the pyramidal neurons, and the thickness of the cerebral cortex. The number of pyramidal neurons was observed in three fields of view between the external pyramidal and internal pyramidal layers with 40x magnification. The size of a pyramidal neuron by measuring the diameter of each of the three largest pyramidal neurons (μm) with a total

of 3 viewing fields with 40x magnification. Cortex cerebri measured its thickness with μm size in 3 fields of view between molecular and multi-formic layers with 4x magnification. The results then analyzed, including the average number of pyramidal neurons, the size of pyramidal neurons, and the thickness of the cerebral cortex.

Results

The results showed that there was no change in behavior patterns in male mice and female mice. Mice behavior patterns are still within normal limits. Provision of red betel vine leaf ethanol extract for 90 days also did not cause weight loss of mice at doses of 50, 200, 400 mg/kgBW and control. Interestingly, only the test group of animals administered with the concentration of red betel leaf ethanol extract at a dosage of 100 mg/kg body weight exhibited a decrease in body weight (Table 1). From the table 2, it can be observed that the results of the One-Way ANOVA test on all blood parameters, namely SGOT, SGPT, urea and creatinine, did not show significant differences compared to control group ($P>0.05$). The results of the anatomical pathology examination are shown in figures 2 & 3.

Table 1. Sub-Chronic Toxicity Study: Effects of Red Betel Vine Leaf Ethanol Extract on Mice

Group	Dose (mg/kgBW)	Mice (M/F)	Parameters Observed	Treatment	Duration	Results
I	50	5M/5F	Weight, Physiology, Blood (SGOT, SGPT, Urea, Creatinine), Histopathology (Liver, Kidney, Stomach, Lien, Heart, Lung, Brain, Genital Organs)	Ethanol Extract (Per-oral)	90 days (Every week)	No change, no weight loss
II	100	5M/5F			90 days (Every week)	Sustained weight loss
III	200	5M/5F			90 days (Every week)	No change, no weight loss
IV	400	5M/5F			90 days (Every week)	No change, no weight loss
V (Control)		5M/5F		Distilled Water (Per-oral)	90 days (Every week)	No change, no weight loss

Table 2. Results of One-Way ANOVA Test

Inter-Group Serum Parameters	P value
SGOT	0.523
SGPT	0.205
Urea	0.607
Creatinine	0.085

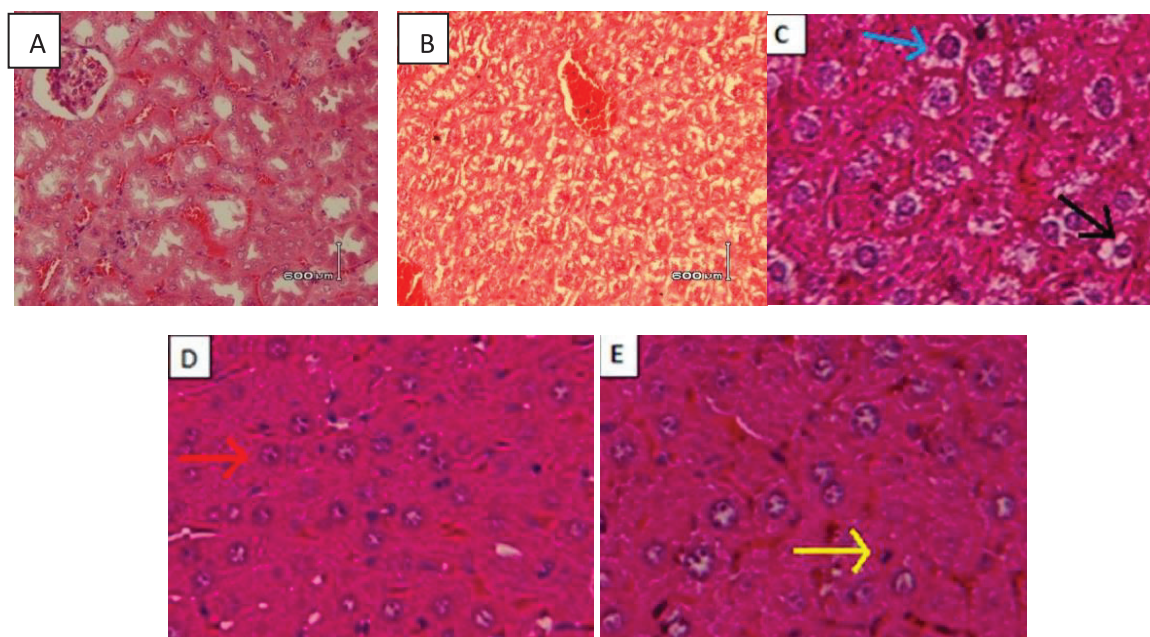


Figure 2. A) Normal renal image. B) Liver with cloudy swelling degeneration. C) Liver with hydropic degeneration. D & E) Normal limits liver image.

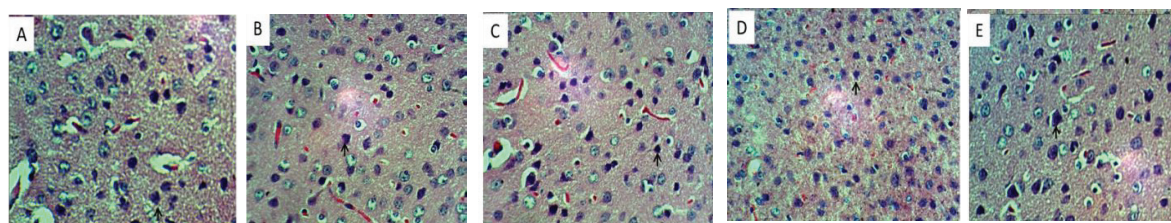


Figure 3. Pyramidal neuron of cerebri cortex of mice ddy strain. (Hematoxylin & Eosin Staining, 40× magnification). A) Dose Of 50 Mg/Kgbw; B) Dose Of 100 Mg/Kgbw; C) Dose Of 200 Mg/Bw D) Dose Of 400 Mg/Bw; E) Control (Aquadres).

Discussion

Blood test results are Serum Glutamic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT), urea, and creatinine. All groups of both treatment and control had elevated levels of blood parameters but no known cause. Thus, repeated administration of ethanol extract on sub-chronic toxicity test did not significantly influence the blood parameters of SGOT, SGPT, urea, and creatinine of mice in each group. There was no significant difference between control and treatment ($p > 0.05$). This study is in accordance with previous studies, which stated that giving red betel leaves did not affect the activity of the mice's SGOT and SGPT with diabetes mellitus, and the extract was not toxic⁸.

In heart histopathology, heart damage is assessed by the presence or absence of degeneration or vacuolization of the heart muscle and cellular

elevation between heart muscles. Assessment of whether heart muscle necrosis occurs, infiltration of lymphocyte cells between the heart muscle or inflammation, and whether fibrosis or extension of the fibrous connective tissue between the heart muscle occurs. The results showed no difference between treatment and control group ($p > 0.05$)¹².

In lung examination of mice, lung damage was assessed by the extent of damage to congestion, inflammation around bronchioles, interstitial inflammation or alveolar septa, and alveolar wall thickening and fibrosis¹³. In the renal histopathological examination, there are several changes that occurs in all groups of test animals, both treated and controlled groups. Some of the mice had hydropic degeneration included in the control group. Hydropic degeneration occurs in the control group. Hydropic degeneration is illustrated by clear vacuoles in the renal tubular

epithelium in the absence of other morphological changes associated with degeneration. Abnormal or pathological hydropic degeneration can be found anywhere along the tubules, but this disorder is more common in the proximal contralateral tubules¹⁴. In this study, there is no statistically significant difference between the renal condition of the control group and the treatment group ($p > 0.05$).

The splenic examination is done by observing whether there is a decrease in the number of white follicles or pulp and the size of the white pulp, whether there are red pulp changes such as congestion and increased cell activity in the red pulp, and whether apoptosis, necrosis, and fibrosis occurs. The results showed no significant difference between the treatment and control groups ($p > 0.05$).

In the hepatic examination, the observation includes the presence or absence of swelling and ballooning view of hepatocytes, the presence or absence of hepatic fat in the form of vacuolization of hepatocytes, and the presence or absence of necrosis images of eosinophilic (pink) cytoplasm—assessment of liver cell damage based on scoring histopathology Manja Roenigk. The results showed that in Group II (100 mg/kgBW), there was a change of cloudy swelling degeneration and reversible hydropic degeneration. However, for other doses were still within normal limits. Furthermore, it is still not known precisely the cause of change at a dose of 100 mg/kgBW. The liver is the main organ that functions in the metabolism and elimination of foreign substances. The process of detoxification that occurs in the liver causes continuous exposure to the stress of the cells in the liver. Therefore, the liver becomes the target organ for drug toxicity¹⁵.

In the gastric examination, the examination was performed on a microscope by observing five different fields to determine the presence or absence of desquamation, erosion, ulceration, inflammation, degeneration, and necrosis. *Piper crocatum* has various metabolites that might influence the reported potential therapeutic activities with different preparation-application techniques, uses, and duration³.

In the brain examination, the observations included the number and size of pyramidal neurons and the thickness of the cerebral cortex. There is a significant difference in the number of pyramidal neurons ($p < 0.05$). There were significant

differences in cortex thickness ($p < 0.05$), but the transformation is a positive change¹⁶. Red betel contains alkaloids, flavonoids, saponins, tannins, polyphenolic compounds, and essential oils. Flavonoid substances have neuroprotective ability to the brain through protein kinase modulation and signal kinase lipid kinase cascade, protein kinase C and mitogen activated protein (MAP) kinase that cause changes in gene expression and caspase activity that inhibit induced nerve cell damage by oxidative stress. In addition, flavonoids improve endothelial function and peripheral blood flow, thus increasing cerebral blood flow (CBF). These changes trigger angiogenesis, the growth of new nerve cells in the hippocampus and changes in the morphology of neurons that play a role in maintaining optimal function of neurons¹⁷.

In the examination of male genital organs performed by observing the histopathology of the testis in the presence or absence of damage to the seminiferous tubules of inflammation, degeneration and necrosis. Whereas in females, by observing ovarian histopathologic view with the presence or absence of damage to the ovaries in the form of inflammation, degeneration, and necrosis. There was no necrosis and inflammation in all groups of mice in both treatment and control; there was cell degeneration in each group of both treatment and control. Degeneration is reversible, and it is possibly not caused by the effect of ethanol extract of red betel vine leaf because $p > 0.05$. Therefore, there was no significant difference between treatment and control for both male and female mice ($p > 0.05$).

All natural and chemical ingredients that enter the body through the mouth will undergo a gastrointestinal absorption process. One of the gastrointestinal organs that has the function of absorption is the stomach. The stomach becomes the initial exposure to substances that enter the body orally. The liver has an essential function in the body's metabolism. The circulation of drugs and toxic substances is also neutralized and secreted by the liver via the bile. Therefore, it can be understood the direct influence of compounds that enter the body orally in the stomach and liver. The positive effects of this study on the brain and the result obtained in the dose of group II (100mg/kgBW) should be investigated further in future studies.

Conclusion

In general, red betel vine leaf ethanol extract is safe for long-term (sub-chronic) consumption; however, it is recommended that usage should be discontinued immediately after the end of medication.

Conflict of interest: None declared.

Funding statement: We are grateful for the financial support of this research funded by the Research Grant Program, Faculty of Medicine,

Universitas Islam Indonesia

Ethical clearance: The research was approved by the ethical committee of the Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta, Indonesia (Ref: 04/Ka.Kom.Et/70/KE/VIII/2015).

Authors' contribution: All authors were involved equally in study design, data collection, literature review, data analysis, manuscript writing, revision and finalizing.

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