Toxicity and Hepatic Effect of *Piper betel* (Betel Vine) Leaf Crude Extract on Wistar Rat

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ABSTRACT

Piper betel, commonly known as Betel vine, holds a prominent place in traditional medicine, particularly in Asia, for its wide range of health benefits. With this, the study investigated the toxicity and potential hepatic effects of *a crude extract of Piper betel leaves in* Wistar rats. It utilized an experimental design. Using the up-and-down method, the result indicated that the median lethal dose of the extract was greater than 2000 mg/kg. This finding similarly suggests that the crude leaf extract demonstrates low acute toxicity, by the absence of mortality and the lack of observable physical or behavioral changes even at a dose of 2000 mg/kg. These results suggest that the extract possesses a wide safety margin and may be considered relatively non-toxic within the tested dosage range. The liver enzyme tests revealed no significant hepatotoxic effects in any dose group, based on liver enzyme levels and bilirubin concentrations; the extract did not induce significant hepatotoxicity at low, medium, or high doses. This indicates that the extract is considered safe at the tested doses and potentially suitable for further pharmacological development. Findings from the histopathological analysis indicate that *Piper betel* extract may provide protective effects against alcohol-induced hepatic injury. Hence, the positive results of this study contribute to understanding the plant's safety profile and promote responsible application in both traditional and modern medicine.

Keywords: alcohol-induced liver disease, ethanolic extract, hepatotoxicity, median lethal dose (LD50), toxicity level

INTRODUCTION

Background of the Study

The increasing reliance on plant-based remedies in traditional medicine highlights the need for careful scientific validation of their safety profiles. *Piper betel*, commonly known as betel vine, is widely used in ethnomedicine for its antimicrobial, antioxidant, and anti-inflammatory properties (Dubey et al., 2023; Singh et al., 2023). However, the hepatotoxic or hepatoprotective potential of its crude leaf extract remains underexplored, particularly in controlled experimental settings. This study applied the principle of evidence-based toxicology, integrating traditional knowledge with established scientific procedures to evaluate the biological and toxicological effects of natural products on vital organs, such as the liver. Scientific procedures require the use of a standard model animal, such as the Wistar rat, due to its physiological similarities to humans and well-documented responses to hepatotoxic substances.

While the Betel vine is widely utilized in traditional medicine for its various health-promoting effects, comprehensive studies on its safety profile, especially its potential toxicity and effects on liver function, are endorsed for further investigation. Furthermore, there is a lack of in-depth toxicological studies using *in vivo* models, which are essential for understanding how betel vine's compounds interact with mammalian liver tissues. Limited information is available on its hepatotoxic effects, which raises concerns about potential adverse impacts that might compromise liver health. Thus, this study aimed to investigate the toxicity and hepatic effects of *a crude extract of P. betel leaf in* Wistar rats. By focusing on *in vivo* hepatic

assessments, the research sought to determine the effects that P. betel may exert on the liver, an important organ, at varying dosages.

Statement of the Objectives

The primary objective of this study was to investigate the toxicity and potential hepatic effects of *Piper betel* leaf extract on Wistar rats. The research was conducted from January 2025 to May 2025.

Specifically, this study intended to achieve the following objectives:

1. To establish the median lethal dose (LD50) of the Betel vine extract.
2. To establish the toxicity level of the Betel vine extract.
3. To determine the potential therapeutic effect of the Betel vine *crude* leaf extract (CLE).

METHODOLOGY

Research Design

This study employed an experimental design using an animal model to assess the antioxidant and immunomodulatory potential of *Piper betel* L. crude leaf extract. Healthy adult male Wistar rats were used in in vivo controlled experiments to assess the extract's physiological effects. The research design involved multiple treatment groups, including control and experimental groups, with varying treatment amounts. This method enabled assessment of dose-dependent effects.

Study Site and Sample Collection

Piper betel leaves were collected from the mountains of Barangay Dagupan, Quezon, Nueva Vizcaya, Philippines. The leaves were collected and then air-dried for two weeks before the commencement of experimentation. The experimental procedures were carried out at the Philippine Institute of Traditional and Alternative Health Care (PITAHC), located in Dalan Na Pagayaya, Regional Center, Carig Sur, Tuguegarao City, Cagayan, and at the Center for Natural Sciences Laboratory of Saint Mary's University.

Data Gathering Procedure

Fresh *P. betel* leaves were washed, air-dried in the shade for 5–10 days, cut into smaller pieces, and soaked in 95% ethanol at room temperature (28–30°C) for 48 hours with occasional stirring. The mixture was filtered using Whatman No. 1 filter paper, and the filtrate was concentrated using a rotary evaporator, following the modified method of Karunanithi et al. (2022). The toxicity test followed the Up-and-Down (Staircase) method, in which one rat per day received a single dose of P. betel extract at 700 mg/kg, with subsequent doses adjusted by ±150 mg/kg based on survival until 4–6 reversals were observed. Toxicity signs were recorded for each rat over 24 hours. To induce hepatotoxicity, healthy adult rats were given 40% ethanol via oral gavage for 7 days to mimic alcohol-induced liver disease, taking into account rodents' rapid ethanol metabolism. The rats were then treated with varying doses of *P. betel* extract for another 7 days. Five groups (3 rats each) were assigned: low, medium, and high-dose extract groups (based on LD_{50} results), a saline control group, and an ethanol-only control group. After treatment, liver function tests were performed to measure serum levels of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), and Total Bilirubin. The values obtained were evaluated to establish the potential therapeutic effect of the leaf extract on alcohol liver damage (ALD). For the histopathological analysis, liver tissues (2–3 mm) were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Sections (2–8 μm) were cut using a microtome, deparaffinized, and stained with hematoxylin and eosin (H&E) for microscopic examination. This procedure was adapted from Isdadiyanto et al. (2022).

Treatment of Data

Determination of LD50

After 4–6 reversals — where a reversal is defined as a change in outcome from survival to death or vice versa — the LD_{50} is calculated using the following formula:

$$LD_{50} = X_0 + (d \times \sum k)/n$$

X_0 = lowest dose at which a reversal occurred
d = dose increment
k = score for each rat (+1 for survival, -1 for death)
n = total number of animals

Given that no deaths occurred at the maximum dose tested, the exact median lethal dose (LD_{50}) cannot be calculated. This suggests that *P. betel* leaf crude extract exhibits a high margin of safety in acute oral administration.

Liver Enzyme Test

The absorbance values of both the standard provided with the assay kit and the samples were substituted into the formula supplied by the manufacturer to calculate the activity.

The ALT enzyme activity was determined by using the formula:
$ALT\ Activity = absorbance\ of\ sample absorbance\ of\ standard\ x\ (98) Value\ of\ standard$

The AST enzyme activity was determined by using the formula:
$AST = Absorbance\ of\ sample Absorbance\ of\ standard\ x\ Value\ of\ standard$

The ALP enzyme activity was determined by using the formula:
$AST = Absorbance\ of\ sample Absorbance\ of\ standard\ x\ (100) Value\ of\ standard$

Total bilirubin levels were determined by using the formula:
$Total\ Bilirubin = Absorbance\ of\ sample Absorbance\ of\ standard\ x\ 4\ Value\ of\ Calibrator$

The results obtained from the liver enzyme tests before and after the treatment with PBL were analyzed using the chart below.

Table 1

Serum Chemistry Reference Range (Rats)

Analyte	Normal Values
AST	34-109 u/l
ALT	13-56 u/l
ALP	95-611 u/l
Total bilirubin	0.2-0.7 mg/dl

Histopathological Analysis

Liver tissues (2–3 mm) were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in graded ethanol (35–100%), cleared in xylene, and embedded in paraffin wax. Thin sections were cut using a microtome, deparaffinized, rehydrated, and stained with hematoxylin and eosin. The stained sections were then dehydrated, cleared in xylene, and mounted with a coverslip for microscopic examination, following a modified method from

Isdadiyanto et al. (2022). The procedures for histopathological analysis were limited to the preparation of liver tissue slides for examination. The prepared slides consisted of liver tissues obtained from ALD-induced rats and treated rats.

Ethical Consideration

The study was submitted for ethics review to Saint Mary's University Research Ethics Board (SMUREB) with address and contact information at 2nd Floor, Rev. John Van Bauwel Hall, SMU Main Campus, Ponce Street, Don Mariano Marcos; Bayombong, 3700 Nueva Vizcaya, Philippines (email: reb@smu.edu.ph; cellphone: 09177053041). The reference number is SMUREB code 0854

Animal research permit is sought from the Bureau of Animal Industry at Visaya Avenue, Diliman, Quezon City, through the Institutional Animal Care and Use Committee of the Philippine Institute of Traditional Health Care (PITAHC)-Cagayan Valley Herbal Processing Plant at Carig Sur, Tuguegarao City, Cagayan.

RESULTS AND DISCUSSIONS

Section 1. Determination of LD50

Table 2 shows the rats' responses to different doses of the leaf crude extract, which serves as the basis for establishing the LD50 for the test organisms.

Table 2

Response of Rats to Varying Doses of P. betel Extract

Rat	Dose (mg/kg)	Response
1	700 (mg/kg)	Alive
2	850 (mg/kg)	Alive
3	1000 (mg/kg)	Alive
4	1200 (mg/kg)	Alive
5	1500 (mg/kg)	Alive
6	1700 (mg/kg)	Alive
7	1900 (mg/kg)	Alive
8	2000 (mg/kg)	Alive

Table 2 shows that no mortality was recorded in any of the rats having treatment at any of the tested dosages. Furthermore, no visible symptoms of toxicity were seen within 24 hours of administration. There were no physical symptoms such as tremors, salivation, or changes in breathing, or behavioral abnormalities such as lethargy or convulsions. These results show that the LD50 is above 2000 mg/kg.

The study conducted by Arawwawala et al. (2011) on *P. betel* grown in Sri Lanka investigated the safety profile of ethanolic extract of *P. betel* leaves on cold ethanolic extract (CEE) and hot water extract (HWE) in rats. The extracts were administered orally at high doses (1,500 mg/kg/day) in sub-chronic 14-day and chronic 42-day toxicity studies. No treatment-related deaths or morbidity were observed, indicating a high safety margin. Additionally, rats treated with both CEE and HWE maintained normal food and water intake, body weight gain, feces consistency, and urine color, suggesting no adverse effects at these dosages. In another study on the Evaluation of antianxiety properties of *P. betel* L. leaf extracts on Swiss albino mice by Nayak et al. (2021), a single dose of 2000mg/kg of *P. betel* ethanolic extract (PBEE) and *P. betel* aqueous extract (PBAE) was given orally to five female Swiss albino

mice. After being observed for the first 30 minutes after dosing, hourly for the first 4 hours, 24 hours, and daily for the next 14 days. The highest doses of PBEE and PBAE in mice (2000 mg/kg) did not cause behavioral changes or mortality, suggesting that the LD50 for each extract was greater than 2000 mg/kg.

Given that all rats survived at doses up to 2000 mg/kg, the median lethal dose (LD_{50}), which is the dose required to cause death in 50% of the test population, was not reached within the tested dose range. Therefore, the median lethal dose (LD_{50}) of *P. betel* leaf crude extract under the conditions of this study is greater than 2000 mg/kg.

Section 2. Determination of the toxicity level of the *P. betel* extract.

The preceding table (Table 2) provides data on the toxicity level of the Betel vine crude leaf extract. As shown in the table, the crude extract of *P. betel* was well tolerated and did not produce acute toxic effects at doses up to 2000 mg/kg. The survival of rats at a dose of 2000 mg/kg strongly indicates that the crude leaf extract has low acute toxicity.

The study by Sanubol et al. (2016) assessed the toxicity of essential oils and crude extracts from nine Piper species to human cells, including HeLa cells from cervical cancer and normal leukocytes. With values ranging from 0.023 μg/ml *(P. semiimmersum*) to 0.059 μg/ml (*P. betloides*), the essential oils showed lower IC50 values, indicating higher toxicity. In contrast to crude extracts, which had LD50 values above 5000 mg/kg and were classified as slightly hazardous (Class III) by WHO standards, these essential oils demonstrated greater toxicity to both normal leukocytes and HeLa cells. With LD50 values above 5000 mg/kg in animal models, the results indicate low acute oral toxicity and that crude extracts of *P. betel* and related species are not harmful to normal human cells, even at high doses. An acute toxicity study by Murwanti et al. (2023) administered doses up to 5000 mg/kg body weight without causing any toxic symptoms or death. Clinical signs, body weight, organ weights, hematology, clinical chemistry, urinalysis, gross pathology, and histopathology all showed no significant difference between the treated and control groups. Organ weights, such as the heart, lungs, liver, and kidneys, showed no significant variation. Clinical chemistry parameters (ALT, AST, glucose, cholesterol, urea, and creatinine) remained stable, except for a minor, non-toxicologically significant decrease in total protein at the maximum dose.

The Betel vine crude leaf extract exhibits low acute toxicity across a wide range of doses, as evidenced by the absence of mortality and physical or behavioral changes, even at 2000 mg/kg. These results suggest that the extract has a high margin of safety.

Section 3. Hepatic Effects of the *Piper betel* Crude Leaf Extract (CLE)

This section presents the hepatic effects of the leaf crude extract as shown by the result of the liver enzyme tests before and after the treatment of the ALD-induced rats with *P. betel* crude leaf extract (CLE).

Table 3
Liver Function Test Results Before and After Treatment

Group	± ALT(u/l)		± ALP(u/l)		± AST(u/l)		± Total bilirubin (mg/dl)	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
I	77.28	44.46	122.04	90.23	35.17	33.2	0.75	1.26
II	107.62	44	121.96	66.1	42.6	27.32	1.03	0.60
III	99.14	47	149.30	115	44.7	35.1	1.90	0.72
IV	95.6	47	122.9	63.14	47.8	30	1.78	0.58

V	102.84	47	127.7	89	47.2	29	1.84	0.73

Table 3 shows the results of liver function tests conducted on ALD-induced rats. It summarizes the mean values of liver function markers after one week of treatment with various concentrations of the extract following alcohol-induced liver damage. The table presented also summarizes the values of liver function markers following one week of treatment with different concentrations of the extract after being induced with liver damage using alcohol. The values are expressed as mean. It reveals that all groups showed ALT levels within the normal range. However, total bilirubin, AST, and ALP provided more distinct indicators of liver function improvement or ongoing damage.

Zahr et al. (2010) observed that a single 4-day binge ethanol exposure had no significant effect on serum, liver, or brain cytokines, whereas Mahdi et al. (2023) emphasized that prolonged or high-dose alcohol consumption is required to elicit significant changes in liver biomarkers such as ALT, AST, ALP, and bilirubin. This suggests that short-term exposure may trigger compensatory and antioxidant responses in the liver, temporarily mitigating cellular damage (Wang et al., 2015). Evidence from various rat models also supports the dose-dependent nature of hepatoprotective and hepatotoxic effects. Ayenew and Wasihun (2023) observed that a medium dose of treatment (200 mg/kg) significantly improved liver enzymes, comparable to silymarin. Similarly, Huang et al. (2017) noted that medium doses reduced ALP and total bilirubin, but significant AST reduction occurred only at the highest dose. In the study by Yan et al. (2015), a high dose (500 mg/kg) of total flavonoids from Abelmoschus manihot was also found to result in the most significant reduction in ALP and bilirubin, effectively alleviating cholestatic liver injury.

Hence, under the parameters of this study, the medium and high doses of *P. betel* extract showed optimal effects with normal Alanine aminotransferase (ALT) levels. In contrast, a medium dose showed an optimal effect on bilirubin levels. The high dose of *P. betel* extract showed optimal effects on Alkaline phosphatase (ALP) and Aspartate aminotransferase (AST) levels. Low-dose and untreated rats showed some benefits, but this may be associated with either insufficient or excessive stress response.

Microscopic Examination of Liver Sections Post-Alcohol and *Piper betel Crude* Leaf Extract (CLE) Treatment

This section includes representative photomicrographs of liver tissues to demonstrate histopathological findings across treatment groups. Figure 3 shows the histology of the rat liver in all groups under 100x magnification. Hematoxylin and Eosin staining of the liver sections of **(a)** induced with 40% ethanol, **(b)** low dose (700 mg/kg), **(c)** medium dose (1500 mg/kg), **(d)** high dose (2000 mg/kg), **(e)** treated with saline, and (**f)** with no treatment at all.

Figure 1

Histology of Rat Liver

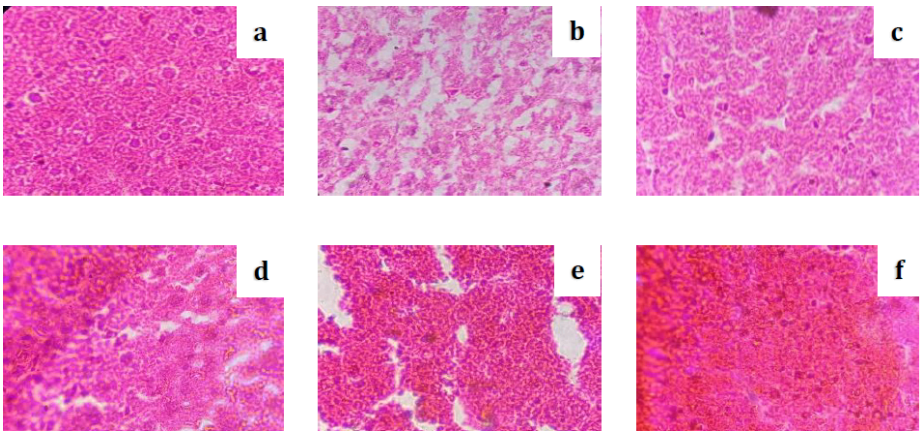

Figure 2

Reference figure for the healthy rat liver

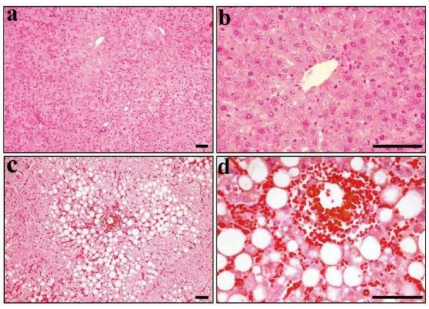

Figure 2. Light microscopy of liver tissue in different groups. A-b: In controls, normal liver architecture was seen; c-d: After alcohol treatment, severe liver damage was noted. The 6-week ethanol treatment resulted in marked steatosis and hemorrhage in the liver tissue of rats. (H&E, scale bar, 50 μm) Aktas et al. (2011)

Figure 2 shows the histology of the rat liver that exhibits an extreme shade of pink coloration in H&E-stained liver sections. The coloration is specifically evident in (a), as well as in (e) and (f), representing alcohol-induced liver damage in the saline-treated and untreated groups. In contrast, the liver tissue in (b), (c), and (d), which corresponds to the low, medium, and high dose groups treated with P. betel crude extract, closely resembles the normal histology seen in Figures 4 (a) and (b), as the reference guide. This observation suggests that *P. betel* extract exerts a protective effect against alcohol-induced liver damage.

According to Ali et al. (2017), the extreme pink coloration in H&E-stained liver sections results from eosin's increased binding to denatured or aggregated intracellular proteins, indicating hepatocellular injury. Normally, hepatocytes exhibit a balanced pink hue due to eosin binding to proteins in their native state. However, upon exposure to toxic agents, ischemia, or chemical insults, protein structures are disrupted, exposing additional eosin-binding sites. This leads to intensified staining, which correlates with areas of necrosis and pathological damage. Studies using different models have provided additional evidence that extreme pink coloration is associated with severe tissue damage. A study by Almansour et al. (2017) found that histological examination of rat liver tissues revealed regions of hepatocyte necrosis and cellular swelling that were bright pink. The extreme pink color in these cases was attributed to the

accumulation of denatured proteins in the cytoplasm, indicating compromised cellular integrity due to nanoparticle toxicity.

Hence, the different shades of pink/red from eosin may correspond to protein content and structural features. Additionally, the white spaces in the tissue typically indicate areas where cells are missing or destroyed. The similarity in staining between the liver sections of extract-treated rats and those of the normal reference group suggests that *P. betel* extract may exert a protective effect against alcohol-induced liver damage.

CONCLUSIONS AND RECOMMENDATIONS

Conclusion

This study investigated the toxicity and potential hepatic effects of *Piper betel* leaf extract on Wistar rats. Results showed that the median lethal dose (LD50) of the *Piper betel* crude leaf extract is higher than 2000 mg/kg. This means that the substance has a wide safety margin, implying further that normal or elevated levels of exposure are unlikely to pose a significant health risk. With the established LD50, the crude leaf extract has a low acute toxicity. This implies that the plant in its crude extract form is in the lowest hazard category or even excluded. The significant drop in ALT, AST, ALP, and Total Bilirubin levels in the rats after administration of Piper betel CLE at different doses indicates the plant's hepatotherapeutic effect. This means that the plant can restore normal liver function after damage. Hence, this study strengthens the scientific basis for the inclusion of *Piper betel* in evidence-based herbal therapies. The findings support its safe use and contribute to its responsible application in traditional and modern medicine.

Recommendations

1. Further research on Piper betel is recommended to determine its full pharmacological potential. This includes assessing the hepatoprotective, antioxidant, anti-inflammatory, immunomodulatory, and anticancer properties in vivo and in vitro, as well as isolating bioactive compounds and analyzing their mechanisms of action.

2. The ethanol administration period should be extended; a longer induction phase allows for the formation of more consistent and clinically relevant hepatic lesions, as well as liver function test results.

3. The administration period of *P. betel* crude extract should be longer than the duration of ALD induction to allow time for potential hepatic effects to manifest or reverse ethanol-induced liver damage.

4. To further establish its safety profile and ascertain the upper threshold of tolerance, it is advised to evaluate the toxicity of *P. betel* leaf crude extract in Wistar rats at doses greater than 2000 mg/kg.

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