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Acute and Sub-Chronic Toxicity of the Herbal Remedy Loi-Hoa-Vien-Tot-Bung (LHVTB) in Mice

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ABSTRACT

This study evaluated the preclinical safety of the herbal remedy Loi-hoa-vien-tot-bung (LHVTB) by assessing its acute and sub-chronic oral toxicity in mice. In the acute toxicity test, seven groups of *Mus musculus* (ten per group) received a single oral dose of LHVTB from 0 to 15 g/kg body weight. In the sub-chronic evaluation, mice received daily oral doses of 0.47 g/kg (the therapeutic equivalent dose) and 1.41 g/kg for 28 consecutive days. Monitored parameters included signs of toxicity, body weight changes, and hematological and biochemical markers. Following the study, vital organs (liver, kidneys, spleen) were subjected to gross macroscopic and detailed histopathological examinations to detect any abnormalities. The acute study revealed no mortality or signs of systemic toxicity up to the 15 g/kg limit dose. In the sub-chronic study, continuous administration of LHVTB at both dose levels did not significantly alter the general condition, body weight gain, or key hematological and biochemical profiles compared to the control group. Furthermore, macroscopic and microscopic examinations of the liver, kidneys, and spleen revealed no treatment-related pathological changes. Collectively, these findings demonstrate that the LHVTB capsule exhibits a safety profile, with no evidence of acute or 28-day sub-chronic toxicity in mice at the tested doses.

Keywords: Loi-Hoa-Vien-Tot-Bung, Acute Toxicity, Subchronic Toxicity, Peptic Ulcer Disease.

Introduction

Peptic ulcer disease (PUD) is a significant global health issue, typified by a high prevalence and a substantial negative impact on quality of life, characterized by sores/injuries in the lining of the stomach or small intestine. The global annual incidence of PUD is estimated to be between 0.1% and 0.3%, with a lifetime prevalence of 5-10%[1,2]. The pathogenesis of PUD is primarily understood as an imbalance between aggressive factors (e.g., gastric acid, pepsin) and protective mechanisms of the gastric mucosa. Primary causes include infection with *Helicobacter pylori* bacteria and the long-term use of non-steroidal anti-inflammatory drugs (NSAIDs)[3]. Current conventional treatments, including proton pump inhibitors (PPIs) and H_2-receptor antagonists, are effective but can be associated with various adverse effects, including nausea, constipation, and headaches, particularly with long-term use[3–6]. This has led to a growing interest in traditional herbal medicines as alternative or complementary therapies, aimed not only at reducing adverse effects but also at improving the inflammatory condition at the lining of the stomach or small intestine.

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In Vietnam, traditional medicine offers a rich repository of herbal remedies for PUD, often formulated based on centuries of knowledge and experience[7–9]. Loi-hoa-vien-tot-bung (LHVTB) is a polyherbal formulation from the Loi Hoa Duong pharmacy, traditionally used for the treatment of PUD. Its formulation, comprising 14 medicinal herbs, is based on the pharmacological principles of neutralizing acid, protecting the gastric mucosa, and reducing inflammation[10–13]. The current study aimed to systematically evaluate the acute and sub-chronic oral toxicity of the LHVTB capsule, a traditional Vietnamese herbal remedy used for peptic ulcer disease. Despite its empirical use, comprehensive scientific validation of its safety profile was previously lacking.

Materials and Methods

Collection and preparation of the Herbal extract of LHVTB

LHVTB capsules were purchased from TPP-FRANCE Pharmaceutical Joint Stock Company, Vietnam (Lot No. 01/TCSP/ĐP-TPP). Each capsule contained 500 mg of a dried mixed extract derived from 14 medicinal herbs (Table 1). The powder capsule was suspended in distilled water to form a homogenous solution for administration to the experimental animals.

Animals

Healthy, adult *Mus musculus* mice (6-8 weeks old, weighing 20 ± 2 g) of both sexes were procured from the Pasteur Institute (Ho Chi Minh City, Vietnam) and the Institute of Vaccines and Medical Biologicals (Nha Trang, Vietnam). The animals were housed in polypropylene cages under standard laboratory conditions (temperature: 25 ± 2°C; humidity: 55-60%; 12-hour light/dark cycle). They were provided with standard pellet chow and water *ad libitum*. The animals were acclimatized to the laboratory environment for at least one week before the commencement of the experiments.

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Table 1: Ingredients of the Loi-hoa-vien-tot-bung capsule

No.	Medicinal materials	Content
1	*Os Sepiae*	133.3 mg
2	*Radix et Rhizoma Glycyrrhizae*	33.3 mg
3	*Cortex Magnoliae officinalis*	433.3 mg
4	*Radix Angelica dahuricae*	200.0 mg
5	*Herba Pogostemonis*	400.0 mg
6	*Fructus Amomi*	333.3 mg
7	*Rhizoma Atractylodis*	333.3 mg
8	*Folium Ampelopsis cantoniensis*	400.0 mg
9	*Tuber Corydalis*	100.0 mg
10	*Rhizoma Zingiberis*	166.6 mg
11	*Rhizoma Bletilla*	53.3 mg
12	*Fructus Chaenomelis*	200.0 mg
13	*Rhizoma Coptidis*	83.3 mg
14	*Rhizoma Curcumae longae*	66.7 mg

Procedure

Acute Oral Toxicity Study

The acute oral toxicity study was performed according to the OECD guidelines[14] and following a previous study[15]. The animals were fasted for 16 hours before the administration of the extract suspension. The mice (n=10 per group, 10 males) were randomly divided into seven groups. A single oral dose of LHVTB suspension was administered by gavage at increasing doses of 0.47, 0.94, 1.88, 3.76, 7.52, and 15 g/kg body weight to the first six groups. The 7th group (the control group) received an equivalent volume of distilled water. The animals were observed continuously for the first 4 hours and then daily for 14 days for any signs of toxicity, abnormal behavior, or mortality. The median lethal dose (LD_{50}) was calculated.

Sub-chronic Oral Toxicity Study (28-day)

Based on the results of the acute toxicity study, a 28-day sub-chronic toxicity study was conducted following previous methods [16,17].

The mice (n=8 per group, 4 males and 4 females) were randomly assigned to three groups, including the control group, which received distilled water. The low-dose group received LHVTB at 0.47 g/kg/day (therapeutic equivalent dose), and the high-dose group received LHVTB at 1.41 g/kg/day (3x therapeutic dose). The test substance was administered orally once daily for 28 consecutive days. General health, toxic signs such as alterations in motor activity or feeding behavior, changes to the skin, fur, pupils, or mucous membranes, adverse respiratory, circulatory, or neurological manifestations, and alterations in excretory patterns and body weight were recorded weekly.

Hematology and serum biochemistry

On day 29, after overnight fasting, all animals were euthanized in strict accordance with the AVMA Guidelines for the Euthanasia of Animals (2020)[18], and blood samples (1.08 – 1.32 mL per animal) were collected via cardiac puncture under light carbon dioxide (CO_2) anesthesia into EDTA-coated and plain tubes. Vital organs, including the liver, kidneys, and spleen, were then excised, weighed, and inspected for any gross pathological changes. The organs were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin. Sections of 5 µm thickness were prepared and stained with hematoxylin and eosin (H&E) for microscopic and histopathological examination. Hematological parameters, including red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), hematocrit (HCT), and platelet count, were analyzed. Biochemical parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total bilirubin, and creatinine, were measured using standard diagnostic kits.

Ethics approval

All experimental procedures involving animals received approval from the Biomedical Research Ethical Committee of Can Tho University of Medicine and Pharmacy (Approval No. 25.003.HV.CTUMP/PCT-HDDD, April 2025). The study was performed in strict compliance with national guidelines for the care and use of laboratory animals[19].

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 20.0. The differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

The results of the acute oral toxicity study are presented in Table 2. Following a single oral administration of LHVTB at doses ranging from 0.47 to 15 g/kg, no mortality was observed in any of the treated groups. The zero-mortality rate was consistent across all observation time points, including at 24 hours, 72 hours, and up to the end of the 7-day observation period. Furthermore, no clinical signs of toxicity or abnormal behavioral changes were noted in any of the mice throughout the study. Consequently, the median lethal dose (LD_{50}) of LHVTB was determined to be greater than 15 g/kg body weight.

Table 2: Mortality rates observed following a single oral administration of Loi-hoa-vien-tot-bung in mice

Groups	Number of mice	Dose (g/kg)	Concentration (g/mL)	Administered volume (mL/animal)	Mortality (%)		
					at 24h	at 72 h	at 7 days
Group 1	10	0.47	0.031	0.3	0	0	0
Group 2	10	0.94	0.063	0.3	0	0	0
Group 3	10	1.88	0.125	0.3	0	0	0
Group 4	10	3.76	0.251	0.3	0	0	0
Group 5	10	7.52	0.501	0.3	0	0	0
Group 6	10	15.00	1.000	0.3	0	0	0
Control group	10	0	0	0.3	0	0	0

Mice (n=10 per group) were administered a single oral dose of LHVTB and observed for 7 days. No mortality or signs of toxicity were recorded at any dose level.

During the 28-day experimental period, general behavior and clinical signs were monitored daily. These included alterations in motor activity or feeding behavior, changes to the skin, fur, pupils, or mucous membranes, adverse respiratory, circulatory, or neurological manifestations, and alterations in excretory patterns. No mortality or treatment-related signs of toxicity were observed in either the control or the LHVTB extract-treated groups. All mice exhibited normal physical activity, food and water consumption, and appearance, including smooth fur and healthy skin and mucous membranes. Fecal consistency remained normal for all groups. At the conclusion of the study, there were no statistically significant differences in the mean body weights between the groups. The final average body weight for the control group was 30.75 ± 0.39 g, compared to 30.8 ± 0.35 g for the low-dose group and 30.88 ± 0.41 g for the high-dose group. This indicates that sub-chronic oral administration of LHVTB extract did not induce any significant changes in body weight relative to the control group. The administration of LHVTB at low-dose and high-dose for 28 days had no significant impact ($p > 0.05$) on the WBC, RBC, hematocrit, MCV, or platelet count, as shown in Table 3. There were no statistically significant differences ($p > 0.05$) observed between the control and LHVTB-treated groups (low and high dose) for any of the parameters evaluated. This indicates that LHVTB did not adversely affect the red blood cells, the white blood cells, and the platelet count in mice under the tested conditions. Following 28 days of treatment, biochemical

analysis revealed no statistically significant differences (p>0.05) in any of the measured parameters between the LHVTB-treated groups (low and high dose) and the control group. Key indicators of liver function, such as AST, ALT, albumin, and bilirubin, as well as the kidney function marker creatinine, remained stable. These results suggest that the administration of LHVTB over the study period is safe and does not induce toxicity in the liver or kidneys, nor does it alter the basic biochemical profile in mice, as indicated in Table 4. After the intervention, the vital organs, including the liver, kidneys, and spleen, were examined microscopically for any signs of damage, as shown in Figure 1. The analysis showed that the tissue and cell structures of the organs from the LHVTB-treated group were normal and indistinguishable from those of the healthy, untreated group. This confirms that the LHVTB is safe and does not cause harm to these internal organs.

Table 3: Changes in hematological parameters in mice after 28 days of treatment with Loi-hoa-vien-tot-bung (mean ± SD)

Indicators	Control group	Loi-hoa-vien-tot-bung groups	
		Low dose	High dose
RBC (10^{12}/L)	7.94 ± 1.51	7.50 ± 1.4	7.85 ± 0.72
Hemoglobin (g/dL)	117.13 ± 19.85	116.88 ± 8.87	124.38 ± 10.08
Hematocrit (%)	0.39 ± 0.07	0.37 ± 0.06	0.41 ± 0.03
MCV (fL)	49.14 ± 1.65	49.15 ± 1.45	51.36 ± 2.53
WBC (G/L)	10.24 ± 1.46	9.58 ± 3.52	7.58 ± 1.33
Neutrophil (G/L)	0.80 ± 0.52	1.25 ± 0.60	1.08 ±0.22
Lymphocytes (G/L)	8.83 ± 1.04	7.64 ± 2.93	6.55 ± 1.21
Platelets (G/L)	595.50 ± 475. 68	638.13 ± 375.36	492.13 ± 183.53

There were no significant differences between the groups (p>0.05)

Table 4: Changes in biochemical parameters in mice after 28 days of treatment with Loi-hoa-vien-tot-bung (mean ± SD)

Indicators	Control group	LHVTB groups	
		Low dose	*High dose*
AST (UI/L)	155.50 ±75.71	218.50 ±114.03	207.25 ± 48.12
ALT (UI/L)	66.13 ± 32.71	97.00 ± 26.19	87.88 ± 17.90
Total albumin (g/dL)	36.80 ± 5.93	44.63 ± 9.73	37.50 ± 1.77
Total bilirubin (µmol/L)	7.81 ± 5.76	5.96 ± 3.71	3.13 ± 0.65
Total cholesterol (mmol/L)	3.08 ± 0.40	3.30 ± 0.84	2.48 ± 0.54
Creatinine (µmol/L)	95.12 ± 39.95	101.00 ± 9.53	89.25 ±13.94

There were no significant differences between the groups (p>0.05)

The use of traditional herbal medicine to treat various health conditions is a practice spanning centuries, and its popularity has surged in recent years as a preferred alternative to conventional medicine[20]. The World Health Organization (WHO) estimates that up to 80% of the population in developing countries relies on these remedies[21]. However, this widespread use often contrasts with a lack of rigorous scientific data on the toxicity and side effects of many medicinal plants. The potential for toxicity is a concept well-recognized within traditional medicine systems. Eastern Traditional Medicine, for example, has a long-standing pharmacopoeia that includes herbs with known potency and toxicity, such as *Radix aconiti*, *Heliotropium indicum* L., and *Semen Strychnos nux-vomica* and *Croton tiglium*[22-25]. Historically, the safe application of these materials has relied on two critical principles: meticulous processing methods designed to mitigate toxicity, and strict control over dosage and duration of administration to ensure therapeutic benefits outweigh potential harm. This traditional understanding highlights the imperative for modern scientific validation. Therefore, conducting systematic acute and sub-chronic toxicity studies is essential, not only for novel natural products but especially for established herbal remedies that have a long history of empirical use. Such research provides the robust safety evidence necessary to support their safe and effective integration into contemporary healthcare.

The acute oral toxicity study revealed no mortality or overt signs of toxicity in *Mus musculus* mice following single oral administration of LHVTB at doses up to 15 g/kg. This finding is consistent with the OECD Test Guideline 420 for Fixed Dose Procedure and 423 for Acute Toxic Class Method, where the absence of mortality at the highest tested dose (15 g/kg, which is significantly higher than 5 g/kg) classifies the substance as "virtually non-toxic" or "low toxicity" (OECD Toxicity Class 5: LD_{50} > 5000 mg/kg)[14,26,27] . The administration of high doses (up to 33 times the anticipated human therapeutic dose) did not induce any abnormal behavior or clinical signs, further supporting the low acute toxicity of LHVTB. This high safety margin is crucial for a product intended for long-term use.

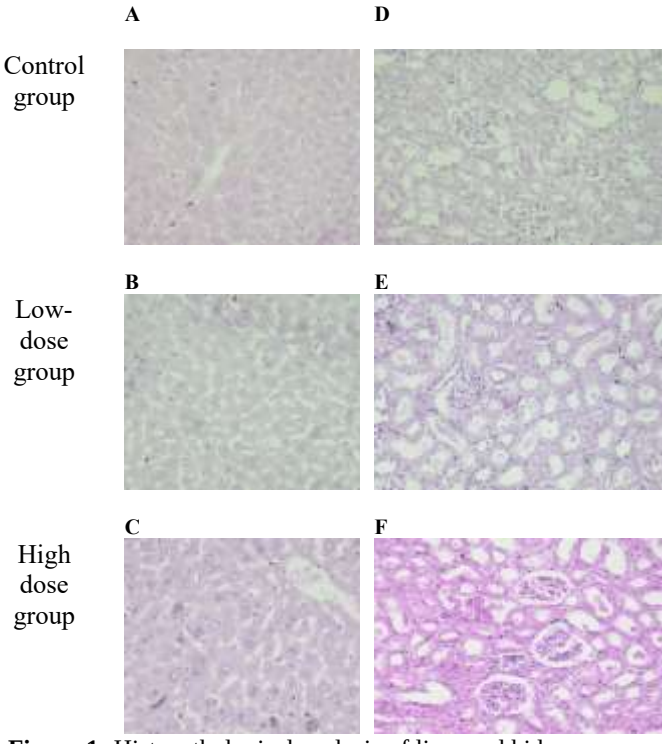

Figure 1: Histopathological analysis of liver and kidney tissues after 28-day oral administration of LHVTB. Representative microscopic images of liver sections from (A) the control group, (B) the low-dose LHVTB group (0.47 g/kg), and (C) the high-dose LHVTB group (1.41 g/kg). Representative images of kidney sections from (D) the control group, (E) the low-dose LHVTB group, and (F) the high-dose LHVTB group. All sections were stained with Hematoxylin and Eosin (H&E). Original magnification: 400x.

In the sub-chronic oral toxicity of LHVTB, body weight is a sensitive indicator of nutritional status, metabolism, and systemic toxicity of a test substance in experimental animals[27]. In this 28-day sub-chronic study, LHVTB administration did not significantly affect the body weight gain of mice compared to the control group. The observed weight gain (approximately 11g over 28 days) was within the normal physiological growth range for *Mus musculus* mice aged 3-6 weeks[28,29]. Consistent and stable weight gain across all groups suggests that LHVTB is unlikely to cause adverse effects such as anorexia, gastrointestinal disorders, metabolic disturbances, or target organ

damage. This is consistent with OECD guidelines, which emphasize that a body weight loss exceeding 10% in repeated-dose toxicity studies warrants particular attention[27]. Similar findings have been reported for *Cassipourea flanaganii* extraction[30], where no significant body weight changes were observed in treated mice, indicating their safety regarding metabolic and digestive processes.

Furthermore, the evaluation of hematological parameters shows that the hematopoietic system is highly susceptible to toxic compounds, making these parameters reliable indicators of physiological and pathological status[31]. Our study demonstrated that 28-day oral administration of LHVTB at both tested doses did not induce any statistically significant changes in red blood cell count (RBC), mean corpuscular volume (MCV), hemoglobin (Hb), or hematocrit (HCT) compared to the control. All values remained within the normal physiological reference ranges for healthy *Mus musculus* mice[32]. This suggests that LHVTB does not negatively impact erythropoiesis, red blood cell maturation, or overall oxygen-carrying capacity. The stability of MCV further indicates that the red blood cells produced are of normal size, ruling out microcytic or macrocytic anemia, which are often associated with iron/folic acid/vitamin B12 metabolic disorders or micronutrient deficiencies[33].

Furthermore, white blood cell (WBC) count, neutrophils (NEU), lymphocytes (LYM), and platelet count (PLT) also remained within physiological limits[34]. This stability indicates that LHVTB did not significantly affect the innate or adaptive immune system, nor did it induce chronic inflammatory responses or cell-mediated immune suppression, which are common risks associated with long-term use of toxic substances. The neutrophil-to-lymphocyte ratio (NLR), a sensitive biomarker for hematological toxicity and inflammatory responses[35], also showed no significant changes. The absence of altered platelet counts (Table 3) suggests no impact on coagulation or thrombocyte function, ruling out potential risks of hemorrhage or thrombosis[36]. These findings are consistent with previous 28-day sub-acute toxicity studies on natural herbal extracts, such as *Erodium guttatum*[30] and *Cyperus rotundus*[37], which also reported no significant hematological abnormalities, further reinforcing the hematological safety of LHVTB for prolonged use.

On the liver function parameters, the liver plays a central role in drug biotransformation and detoxification, and its function is often evaluated by serum biochemical enzymes[38]. In this study, serum levels of AST, ALT, albumin, total bilirubin, and cholesterol in the LHVTB-treated groups were not significantly different from the control group (Table 4). Although some numerical variations were noted, they remained within physiological reference ranges[12]. This indicates that LHVTB did not cause significant hepatic cell damage, disrupt protein or lipid metabolism, and implies a favorable hepatotoxic profile even with prolonged use. The non-significant increase in AST and ALT levels, consistent with studies on *Magnolia officinalis*[39], indirectly suggests the hepatoprotective potential of the herbal components within LHVTB. Several ingredients in LHVTB are known for their liver-protective effects. *Magnolia officinalis*, with its active compounds magnolol and honokiol, reduces hepatocyte damage and exhibits antioxidant and anti-inflammatory properties[40,41]. *Radix Glycyrrhizae*, containing glycyrrhizin, stabilizes hepatocyte membranes, reduces AST and ALT levels[42], and protects the liver, as demonstrated in previous toxicity studies[43]. Additionally, *Curcuma longa* (turmeric), containing curcumin, enhances endogenous glutathione and inhibits pro-inflammatory cytokines, contributing to liver protection and stabilization of liver cell membranes[44,45]. The stable albumin levels further indicate undisturbed protein synthesis by the liver[46]. The total bilirubin levels, crucial for assessing hemoglobin metabolism and bile excretion, showed a slight, non-significant decrease in the high-dose group, remaining within physiological limits. This suggests that LHVTB will not cause cholestasis or negatively affect bilirubin metabolism and excretion by the liver. These observations align with studies on *Magnolia officinalis*, which show antioxidant and hepatoprotective activities in both acute and sub-chronic liver injury models[40]. Curcumin also aids in the conjugation of free bilirubin for easier excretion[47]. Collectively, these findings provide strong evidence that LHVTB did not induce significant hepatotoxicity at the tested doses.

Moreover, the kidneys are vital excretory organs, and their function is a critical assessment point in toxicity studies. Creatinine, a stable protein component in blood, is a reliable indicator of renal excretory capacity[48]. Although creatinine levels in all groups were slightly higher than the general reference range for *Mus musculus* mice (0.2–0.8 mg/dL), there were no statistically significant differences between LHVTB-treated groups and the control group (Table 4). This suggests that LHVTB at therapeutic and threefold higher doses did not cause overt nephrotoxicity or impair glomerular filtration. However, the relatively large standard deviation in the control group might reflect inter-individual biological variability or extraneous factors during sample processing. While our current results do not indicate acute kidney injury, the slightly elevated creatinine levels, even if non-significant, warrant further investigation. This includes comprehensive renal histopathology and urinalysis to completely rule out any subtle effects on kidney function in future studies.

Considering the gross and histopathological findings of isolated organs, the absence of significant and microscopic changes in the liver, kidneys, and spleen further supports the non-toxic nature of LHVTB at the administered doses. The overall normal appearance and cellular integrity of these vital organs, coupled with stable biochemical and hematological parameters, corroborate the safety findings. Also, minor observations, such as hydropic degeneration or vascular congestion, were not widespread or severe enough to be considered treatment-related pathological lesions, particularly when compared to findings in control groups or histological data. This comprehensive pathological assessment reinforces the conclusion that LHVTB did not induce significant organ damage following 28-day oral administration.

Conclusion

In conclusion, the acute oral administration of Loi-hoa-vien-tot-bung at doses up to 15 g/kg body weight did not result in any mortality or signs of toxicity, indicating an LD_{50} higher than 15 g/kg and classifying the formulation as virtually non-toxic according to OECD guidelines. In addition, the 28-day repeated oral administration of Loi-hoa-vien-tot-bung at therapeutic and threefold higher doses did not induce significant sub-chronic toxicity. This was evidenced by the absence of adverse effects on general condition, body weight gain, hematological parameters, liver function, kidney function, and the presence of no gross or histopathological abnormalities in the liver, kidneys, or spleen. Based on the findings of this study, it is recommended that future investigations may be required to expand the toxicological profile of Loi-hoa-vien-tot-bung capsule.

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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