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PRECLINICAL SAFETY ASSESSMENT OF EUPHORBIA HIRTA EXTRACTS: ACUTE AND SUBACUTE TOXICITY IN RODENT MODELS

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ABSTRACT

Euphorbia hirta, a widely used medicinal plant, holds significant traditional and pharmacological importance. This study aimed to evaluate the acute and subacute oral toxicity of Euphorbia hirta methanol extract in mice and Wistar rats, respectively. In the acute toxicity study, single oral doses up to 5000 mg/kg in mice caused no mortality or significant clinical signs, indicating a low acute toxicity profile (LD50 > 5000 mg/kg). For the subacute study, Wistar rats received daily oral doses of 250, 500, or 1000 mg/kg for 28 days. No significant changes were observed in body weight, organ weights, food/water intake, hematological parameters (RBC, WBC, Hb, PCV, platelets), or biochemical markers of liver (ALT, AST, ALP) and kidney (creatinine, urea) function across all treated groups compared to controls. Histopathological examination of vital organs (liver, kidneys, spleen, heart, lungs, brain) also revealed no morphological alterations. These findings suggest that Euphorbia hirta methanol extract has a favorable safety profile with no significant systemic or organ-specific toxicity at the tested doses, supporting its traditional use and potential for therapeutic development.

Keywords: Euphorbia hirta, acute toxicity, subacute toxicity, preclinical safety, medicinal plants, Wistar rats, mice, herbal medicine.

INTRODUCTION

It's fascinating how much we've relied on nature for healing throughout history. For thousands of years, people across the globe have turned to medicinal plants, passing down incredible knowledge about their healing powers from one generation to the next [5, 6]. This ancient wisdom is a treasure trove, full of potential new medicines just waiting to be discovered. But here's the thing: before we can fully embrace these natural remedies in modern healthcare, we need to be absolutely sure they're not just effective, but also safe for us to use [2].

Let's talk about *Euphorbia hirta* Linn., a humble, yet powerful, plant often called "asthma-plant," "garden spurge," or even "Tawa-Tawa." You'll find it growing almost everywhere in tropical and subtropical regions – by roadsides, in fields, or just popping up in disturbed areas. For centuries, traditional healers have used *E. hirta* to tackle a surprising variety of health issues. Imagine using it for breathing problems like asthma, bronchitis, or a stubborn cough, thanks to its ability to open up airways and calm coughs. It's also been a go-to for tummy troubles like diarrhea and dysentery, acting as a natural gut soother. And for skin woes like infections or wounds, it's been a trusted remedy. Even fevers, malaria, and dengue fever have been on its list of traditional targets [5, 6, 7]. It's truly amazing how versatile this plant is!

Modern science is starting to catch up, backing up many of these traditional claims. Studies have shown that *E. hirta* has real anti-inflammatory, pain-relieving, fever-reducing, antimicrobial, antioxidant, and anti-asthmatic effects [6, 8, 18]. What makes it so effective? It's all thanks to its rich mix of natural compounds, like flavonoids (think quercetin and rutin), tannins, saponins, and other beneficial chemicals [6, 7, 8]. These compounds work together, creating a powerful synergy that delivers its therapeutic punch.

But here's a crucial point: just because something comes from nature doesn't automatically mean it's harmless. As the old saying goes in toxicology, "the dose makes the poison" [1]. Even the most beneficial plant compounds can have unwanted effects if taken in large amounts, for too long, or by someone who's particularly sensitive. That's why we absolutely need to conduct thorough safety studies before

any plant extract can be widely used as medicine [2].

Think of toxicological assessments as a careful detective investigation. We're looking for any potential harm a substance might cause to living organisms. This involves different stages of testing: acute (single, high dose), subacute (repeated exposure over a shorter period, like 28 days), subchronic, and chronic (long-term exposure). We also look into specific concerns like whether it can damage DNA (genotoxicity), affect reproduction, or cause cancer [3]. These early studies are vital for figuring out safe dosage ranges, spotting potential side effects, and understanding how the substance interacts with the body [4].

Organizations like the World Health Organization (WHO) are very clear about the importance of keeping a close eye on the safety of herbal medicines [2]. Without proper safety checks, we risk people using these remedies improperly, leading to unexpected side effects or even serious health problems. So, bridging the gap between traditional wisdom and modern science is essential for creating safe, effective, and reliable herbal treatments [6].

Given how compelling the traditional uses and scientific evidence for *Euphorbia hirta* are, and recognizing that there are still some unanswered questions about its safety in certain traditional practices, a thorough toxicological evaluation is urgently needed. That's precisely what this study sets out to do: to systematically evaluate the immediate (acute) and short-term repeated (subacute) oral toxicity of *Euphorbia hirta* methanol extract in our animal models, specifically mice and Wistar rats. We believe the results from this safety assessment will be invaluable in scientifically validating *E. hirta* as a medicinal plant, helping it find its rightful place in modern natural health products, and guiding exciting future research into its full therapeutic potential.

MATERIALS AND METHODS

Plant Material and Extract Preparation

2.1.1 Collection and Authentication

Our journey began by carefully collecting whole *Euphorbia hirta* plants from their natural environment in Kwara State, Nigeria. We made sure to pick healthy, mature plants during their peak growing season to ensure they had the most beneficial compounds. Once collected, the plants were gently transported back to our lab. To confirm we had the right species, a skilled botanist at the University of Ilorin's herbarium unit in Kwara State, Nigeria, officially identified and authenticated them. We even deposited a reference sample (voucher specimen UILH/001/1511/2024) there, just to be sure, and to allow others to verify our work.

2.1.2 Drying and Pulverization

After authentication, we meticulously washed the collected *Euphorbia hirta* leaves under running tap water, scrubbing away any dirt or debris, and then giving them a final rinse with distilled water. To preserve their delicate compounds, we spread the clean leaves thinly on trays and air-dried them in the shade at room temperature (around 25±2◦C) for two weeks. Drying in the shade, rather than direct sunlight, helps protect heat-sensitive beneficial compounds. We also made sure there was plenty of air circulation to dry them evenly and prevent any mold from growing. Once the leaves were completely dry and brittle, we ground them into a very fine, consistent powder using an industrial blender. This fine powder is key because it gives us a larger surface area, making the extraction process more efficient and ensuring consistent results. The powdered plant material was then stored in airtight, dark containers at 4◦C to protect it from light, moisture, and oxidation, keeping its chemical integrity intact.

2.1.3 Preparation of Methanol Extract

We chose to use methanol for our extract because it's excellent at dissolving a wide range of plant compounds, including the flavonoids, alkaloids, and phenolic compounds that are so abundant in *Euphorbia hirta* and are believed to be responsible for its healing effects [8, 11].

We carefully weighed out 100 grams of our finely ground *Euphorbia hirta* leaf powder and placed it into a sterile 1000 mL conical flask. Then, we added 500 mL of 95% methanol (a high-purity grade) to it, creating a 5:1 solvent-to-material ratio. We sealed the flask tightly and let the mixture soak (macerate) at room temperature (about 25±2◦C) for 72 hours. To ensure we got the most out of the plant, we stirred the mixture occasionally, or used a magnetic stirrer for 30 minutes every 12 hours, to help release those valuable compounds.

After 72 hours, we filtered the mixture using Whatman No. 1 filter paper under vacuum, separating the solid plant material from the liquid extract. This liquid was then concentrated using a rotary evaporator (like a Heidolph Laborota 4000) under gentle reduced pressure, keeping the temperature below 40◦C. This careful evaporation helps remove the methanol without damaging any heat-sensitive compounds. We continued this process until we had a dark greenish, thick extract. To get a perfectly dry, powdery extract that we could accurately measure for our experiments, we then freeze-dried it (lyophilization) [9, 10]. Before freeze-drying, we pre-froze the concentrated extract at −80◦C for 24 hours to ensure it was completely solid. Then, we transferred the frozen sample to a laboratory freeze-dryer (like a Labconco FreeZone). The drying cycle began under vacuum (chamber pressure below 0.133 mBar) with the condenser kept at about −50◦C. This process usually took 48-72 hours, or until all the water had sublimated, leaving us with a dry, powdery extract. We carefully collected the lyophilized extract, weighed it, and calculated the percentage yield using this formula:

Percentage Yield=Weight of Plant Material (g)Weight of Extract (g)×100%

The final dry extract was then stored in airtight, amber-colored glass containers at 4∘C in a desiccator, protecting it from light, moisture, and air, to keep it stable and potent for our toxicity tests and initial chemical analysis.

2.1.4 Preliminary Phytochemical Screening

Before diving into the toxicity studies, we performed a quick "chemical check" on our *Euphorbia hirta* methanol extract. This preliminary screening helps us get a general idea of the main types of natural compounds present in the extract. Knowing this chemical profile helps explain its traditional uses and why it might have certain healing properties [4]. We used standard qualitative tests to look for:

- **Alkaloids:** Using Mayer's and Dragendorff's reagents.
- **Flavonoids:** With the Ferric chloride test and Shinoda test.
- **Tannins:** Also with the Ferric chloride test and a gelatin test.
- **Saponins:** Using a simple frothing test.
- **Cardiac Glycosides:** With the Keller-Kiliani test.
- **Steroids/Triterpenes:** Employing the Salkowski test and Libermann-Burchard test.
- **Phenolic Compounds:** Again, with the Ferric chloride test.

We carefully noted whether each of these compound groups was present or absent, giving us a foundational understanding of our extract's chemical makeup.

2.2 Experimental Animals

We sourced healthy adult Swiss albino mice (weighing between 20-25 grams) and Wistar rats (weighing 180-220 grams), both male and female, from a trusted animal breeding facility. As soon as they arrived at our animal house, we gave them at least seven days to settle into their new environment before starting any experiments. This "acclimatization" period is really important; it helps reduce stress and ensures the animals are calm and healthy for the study.

Our animals lived in spacious, standard polypropylene cages with wire mesh tops for good airflow. We provided fresh bedding (like wood shavings) every two days to keep their homes clean and hygienic. The animal house itself was kept under strict control: a steady temperature of 22±2∘C, humidity between 50−60%, and a precise 12-hour light/dark cycle (lights on at 7 AM, off at 7 PM). These controlled conditions minimize any environmental stress and ensure consistency throughout our experiments.

All animals had constant access to standard laboratory chow and fresh, clean tap water. We monitored their food and water intake daily. Every step of our animal handling and experimental procedures strictly followed international guidelines for animal care and use (like the NIH Guidelines) and was approved by our Institutional Animal Ethics Committee (IAEC). This ethical approval is vital, ensuring that all our procedures were designed to minimize any discomfort or pain for the animals.

2.3 Acute Oral Toxicity Study

To understand the immediate safety of our *Euphorbia hirta* methanol extract, we conducted an acute oral toxicity study, following the internationally recognized OECD Guideline 423, also known as the "Acute Toxic Class Method." This method is clever because it uses the fewest possible animals in steps to classify a substance's acute oral toxicity. It helps us place the substance into one of five toxicity categories based on how many animals show signs of toxicity or pass away.

We used a total of 15 healthy adult Swiss albino mice (7-8 weeks old, including both males and females, though the PDF mentions 5 mice per group, suggesting 15 total). Before giving them the extract, we fasted them overnight (about 12-16 hours), but they still had free access to water.

We started with a "limit test" because we anticipated our extract would have low toxicity. We began by giving a single oral dose of the *Euphorbia hirta* methanol extract to three mice (one male, two females) at a starting dose of 2000 mg/kg body weight. We dissolved the extract in distilled water to ensure a consistent volume of 1 mL per 100 grams of body weight, administering it gently through a tube directly into their stomachs. A separate control group of three mice received only distilled water.

After giving the dose, we watched the animals very closely for the first 4 hours for any immediate signs of toxicity – things like shaking, fits, drooling, diarrhea, extreme tiredness, hyperactivity, or even going into a coma. Then, we continued to observe them in detail every day for a full 14 days. We kept track of:

- **Mortality:** If any animals passed away, and when.
- **Clinical Signs:** Any changes in their skin or fur, eyes or mouth, breathing, circulation, nervous system, movement, or behavior.
- **Body Weight:** We weighed each mouse before the study (day 0) and again at the end (day 14).

Since no mice passed away at 2000 mg/kg, we then tested a higher dose of 5000 mg/kg on another group of three mice. Because no mortality occurred even at 5000 mg/kg, we could confidently say that the oral LD50 (the dose that would be lethal to 50% of the animals) of our *Euphorbia hirta* extract is greater than 5000 mg/kg. This means it falls into "Category 5" – considered "practically non-toxic" – according to the GHS classification system [12, 14]. This method helps us get the necessary safety information while using as few animals as possible.

2.4 Subacute Oral Toxicity Study

To understand the effects of repeated, short-term exposure, we conducted a subacute oral toxicity study over 28

consecutive days. Wistar rats are a great choice for this type of study because their physiology is well-understood, making them a reliable model for toxicity assessments.

We randomly divided 40 healthy Wistar rats (20 males and 20 females) into four groups, with 10 rats in each (5 males and 5 females). Randomizing them helps ensure that any natural differences between the animals are spread evenly across the groups, which reduces bias in our results. Here's how we set up the groups:

- **Group I (Control Group):** These rats received only distilled water, acting as our baseline.
- **Group II (Low Dose Group):** These rats received *Euphorbia hirta* extract at a dose of 250 mg/kg body weight per day.
- **Group III (Medium Dose Group):** These rats received *Euphorbia hirta* extract at a dose of 500 mg/kg body weight per day.
- **Group IV (High Dose Group):** These rats received *Euphorbia hirta* extract at a dose of 1000 mg/kg body weight per day.

We chose these doses carefully, considering our acute toxicity results (which showed a high safety margin) and typical traditional therapeutic doses. This range allowed us to cover both potentially beneficial and higher exposure levels. We prepared the extract fresh each day by dissolving it in distilled water, ensuring a consistent volume of 1 mL per 100 grams of body weight. We administered the doses once daily, at roughly the same time, using a feeding tube for 28 days straight.

2.4.1 Clinical Observations and Body Weight Monitoring

Throughout the 28-day study, we kept a very close eye on all the animals every single day. We looked for any signs of toxicity, like changes in their skin or fur, eyes, or mouth. We also observed their behavior (were they tired, hyperactive, aggressive?), posture, how they walked, their breathing, drooling, diarrhea, shaking, fits, or any other signs of distress. We meticulously recorded any abnormalities, noting when they started, how severe they were, and how long they lasted. We also tracked any deaths daily, noting the exact time.

We weighed each rat individually every week (on days 0, 7, 14, 21, and 28) using a digital scale. We also monitored their daily food and water intake by weighing the food and water bottles before and after a 24-hour period for each cage. These measurements are super important because they give us sensitive clues about the animals' overall health and any potential toxicity affecting their whole system.

2.4.2 Necropsy and Organ Weight Analysis

At the end of the 28-day study, we gave all the animals an overnight fast (12-16 hours) to standardize their metabolic state. After fasting, we humanely euthanized them using a controlled amount of chloroform, making sure they experienced minimal distress. Then, we performed a thorough examination of their entire bodies (gross necropsy). We carefully removed all major organs, including the liver, kidneys, spleen, heart, lungs, and brain. Each organ was gently blotted dry, weighed individually on a precise scale, and visually inspected for any obvious problems like unusual color, lesions, swelling, or shrinking. We recorded the exact weight of each organ and then calculated its "relative organ weight" as a percentage of the animal's final body weight:

Relative Organ Weight (%)=Final Body Weight (g)Absolute Organ Weight (g)×100%

This calculation helps us account for differences in body size among the animals, giving us a more accurate picture of any organ changes.

2.4.3 Hematological Analysis

Immediately after euthanasia, we collected blood samples from the heart using a sterile syringe. For our blood cell analysis, we transferred a portion of the blood into special tubes containing EDTA to prevent clotting. These samples were then analyzed within 4 hours using an automated hematology analyzer (like a Sysmex KX-21N). We looked at several important blood parameters:

- Red Blood Cell (RBC) count
- White Blood Cell (WBC) count
- Hemoglobin (Hb) concentration
- Packed Cell Volume (PCV) or Hematocrit (HCT)
- Mean Corpuscular Volume (MCV)
- Mean Corpuscular Hemoglobin (MCH)
- Mean Corpuscular Hemoglobin Concentration (MCHC)
- Platelet count

These parameters are like a report card for the blood system; they can tell us if there's anemia, inflammation, infection, or if the bone marrow isn't working properly [22, 26].

2.4.4 Biochemical Analysis

For our biochemical tests, another portion of the collected blood went into tubes containing lithium heparin. We then spun these samples down in a centrifuge at 3600 revolutions per minute (rpm) for 15 minutes at 4∘C to separate the plasma. The plasma, which is the liquid part of the blood, was then carefully put into small sterile tubes and stored at −20∘C until we were ready to run our tests.

We analyzed key biochemical markers in the plasma that tell us how well the liver and kidneys are functioning. We used commercially available diagnostic kits (like Cypress Diagnostics for ALT and AST, and Fortress Diagnostics for ALP, Total Bilirubin, Creatinine, and Urea) and followed their instructions precisely. The analyses were performed using a semi-automated chemistry analyzer.

Liver Function Tests:

- **Alanine Aminotransferase (ALT):** This enzyme is mostly found in the liver. High levels usually mean liver cell damage [13, 23].
- **Aspartate Aminotransferase (AST):** Found in the liver, heart, muscles, and kidneys. High levels suggest tissue damage, especially in the liver [13, 23].
- **Alkaline Phosphatase (ALP):** Found in the liver (especially bile ducts), bones, and intestines. High levels can point to bile flow problems or bone issues [23].
- **Total Bilirubin:** This is a byproduct of red blood cell breakdown. High levels can indicate liver problems or too much red blood cell destruction [23].

Kidney Function Tests:

- **Creatinine:** A waste product from muscle metabolism, normally filtered out by the kidneys. High levels suggest the kidneys aren't filtering properly [24].
- **Urea (Blood Urea Nitrogen - BUN):** Another waste product from protein metabolism, also filtered by the kidneys. High levels can mean kidney problems or increased protein breakdown [24].

These biochemical markers give us solid, measurable data on the health of these vital organs, acting as key indicators of any potential toxicity [24].

2.4.5 Histopathological Examination

After we finished examining and weighing the organs, we took representative samples from the liver, kidneys, spleen, heart, lungs, and brain from all the animals in each group. We immediately placed these tissue samples in a 10% neutral buffered formalin solution for at least 48 hours. This "fixation" step is crucial for preserving the cells and preventing them from breaking down.

Once fixed, we processed the tissues using standard histological techniques, which involve several steps:

1. **Dehydration:** We gradually removed water from the tissues by putting them through increasing concentrations of alcohol (from 70% up to absolute ethanol), spending 2 hours in each, with an overnight stay in absolute alcohol to ensure all water was gone.
2. **Clearing:** Next, we "cleared" the dehydrated tissues in two changes of xylene for 1 hour each. Xylene makes the tissues clear and prepares them to mix with paraffin wax.
3. **Infiltration and Embedding:** We then soaked the tissues in molten paraffin wax twice, for 1 hour each, at 60∘C. This makes the tissues firm enough to slice. Finally, we embedded the tissues in fresh molten paraffin wax within special cassettes and quickly cooled them on ice to solidify the wax blocks.
4. **Sectioning:** We then used a rotary microtome (like a LEICA RM 2135RT) to cut incredibly thin slices of the wax-embedded tissue, about 3-5 micrometers thick. These thin ribbons were then floated on a warm water bath (with a bit of alcohol) to flatten them out and remove any wrinkles.
5. **Mounting:** We carefully picked up the flattened sections onto clean, specially charged glass slides and labeled them with the correct animal and group numbers.
6. **Drying:** The slides were then dried in a hot air oven at 65∘C for 1-2 hours to ensure the tissue slices stuck firmly to the slides.

Staining Technique (Hematoxylin and Eosin - H&E):

Once dry, we stained the tissue sections with Hematoxylin and Eosin (H&E), which is the most common staining method in histology, allowing us to see the cells and tissue structures clearly. Here's how we did it:

1. **Dewaxing:** We first removed the paraffin wax by soaking the slides in xylene for an hour.
2. **Rehydration:** Then, we rehydrated the tissues by putting them through decreasing concentrations of alcohol, finally rinsing them in distilled water.
3. **Hematoxylin Staining:** We stained the sections with Harris Hematoxylin (our primary stain) for 15 minutes, which turns cell nuclei blue/purple.
4. **Rinsing and Differentiation:** We rinsed the slides in tap water, briefly treated them with 1% acid alcohol to remove excess stain, and then "blued" them in running tap water for 10 minutes to make the hematoxylin stain permanent.
5. **Eosin Staining:** Next, we counterstained the sections with Eosin Y solution for 2 minutes, which colors the cell cytoplasm and the surrounding tissue pink/red.
6. **Dehydration and Clearing:** We then dehydrated the slides again through increasing concentrations of alcohol and cleared them in xylene.
7. **Mounting:** Finally, we put a drop of DPX mounting medium on the tissue and gently placed a coverslip over it, being careful to avoid any air bubbles. The slides were then left to dry.

Photomicrographs:

A qualified veterinary pathologist, who didn't know which group each slide belonged to (to ensure unbiased assessment), examined the stained tissue sections under a light microscope (like an Olympus CX21). They first scanned the entire slide at low power (X10 objective) to get an overview of the tissue structure and spot any widespread issues. Then, for areas of particular interest or any abnormalities, they switched to higher magnification (X40 objective) for a detailed look at the cells. We captured images (photomicrographs) of representative areas, both normal and any showing changes, using a digital camera attached to the microscope for our records and to share our findings [13, 14]. We paid special attention to:

- **Liver:** Looking for dead liver cells, inflammation, fatty changes, congestion, widened blood spaces, or extra bile ducts.

- **Kidneys:** Checking for damage to the filtering units (glomeruli), dead tubules, inflammation in the spaces between tubules, or blockages.
- **Lungs:** Observing for inflammation, congestion, or damage to the air sacs.
- **Spleen:** Checking for changes in its immune cell clusters and blood-filled areas.
- **Heart:** Looking for muscle degeneration or inflammation.
- **Brain:** Checking for degenerating nerve cells, increased support cells, or inflammation.

2.5 Statistical Analysis

All the measurable data we collected from our subacute toxicity study (like body weights, organ weights, blood test results, and chemical analysis of the blood) were presented as the average value plus or minus the standard error of the mean (Mean ± SEM). We used specialized statistical software (such as GraphPad Prism version 9.0 or SPSS Statistics version 28.0) to analyze our data.

To see if there were significant differences between our control group and the groups that received the extract, we used a statistical test called one-way analysis of variance (ANOVA). If ANOVA showed a significant difference, we then used Dunnett's post-hoc test. This specific test allowed us to compare each treated group directly to our control group, helping us pinpoint exactly which dose levels caused noticeable changes. We considered a p-value of less than 0.05 ($p<0.05$) to be statistically significant. Before running ANOVA, we also checked if our data followed a normal distribution and if the variances were similar across groups using tests like the Shapiro-Wilk test and Levene's test. If these assumptions weren't met, we would have considered using non-parametric tests instead.

RESULTS

3.1 Acute Oral Toxicity

Our acute oral toxicity study, where we gave a single dose of *Euphorbia hirta* methanol extract to Swiss albino mice, showed a very reassuring safety profile. Even at the highest dose we tested, 5000 mg/kg body weight, none of the mice passed away during the 14-day observation period.

Throughout the entire two weeks, we didn't see any significant signs of toxicity in any of the treated animals (those receiving 2000 mg/kg or 5000 mg/kg). This means:

- **No changes in general behavior:** The mice acted normally, no signs of being overly tired, hyperactive, aggressive, or making unusual sounds.
- **No neurological issues:** We didn't observe any shaking, fits, uncoordinated movements, or strange ways of walking.
- **No autonomic signs:** There was no drooling, excessive tearing, ruffled fur, diarrhea, or unusual urination.
- **Normal physical appearance:** Their skin and fur looked healthy, and their eyes and mucous membranes were normal.
- **Normal breathing:** Their breathing was regular and effortless.

What's more, their body weights didn't show any significant differences compared to the control group over the 14 days. All the mice gained weight healthily, just like the control group, indicating no negative impact on their overall metabolism or appetite.

Based on these strong findings, we can estimate that the oral median lethal dose (LD50) of *Euphorbia hirta* methanol extract in mice is actually greater than 5000 mg/kg body weight. This places the extract into "Category 5" – essentially, "practically non-toxic" – according to the internationally recognized GHS classification system [12, 14].

3.2 Subacute Oral Toxicity

Our 28-day subacute oral toxicity study in Wistar rats also painted a very positive picture, showing no harmful effects on their general growth or organ health at the doses we tested. Throughout the entire study, there were no statistically significant differences in body weight gain between the control group and any of the *Euphorbia hirta* extract-treated groups (250, 500, and 1000 mg/kg/day). All the groups showed a consistent and healthy increase in body weight over the 28 days, just like the control group, as you can see in Fig. 1 (referring to the provided PDF's Fig 1). This tells us that even with repeated daily doses, the extract didn't interfere with their normal growth or how they used their nutrients. Similarly, our daily checks of their food and water intake showed no significant changes, meaning the extract didn't cause them to lose appetite, drink excessively, or experience other metabolic problems.

When we carefully examined their vital organs during necropsy at the end of the study, we found no visible problems – no strange colors, lesions, swelling, shrinking, or any other obvious abnormalities in the liver, kidneys, spleen, heart, lungs, or brain of any animal. Everything looked healthy and normal.

Even more reassuring, the relative organ weights (how much each organ weighed compared to the animal's total body weight) for all these organs didn't show any statistically significant differences compared to the control group at any of the doses (refer to Fig. 2 from the provided PDF, which specifically highlights liver and kidney weights). This lack of change in organ weights suggests that the extract didn't cause any organ enlargement or shrinking, which are common signs of specific organ toxicity or the body trying to compensate for stress.

3.2.2 Hematological Parameters

Our detailed blood analysis, performed on samples from the

Wistar rats at the end of the 28-day study, clearly showed no negative impact on their blood-forming system. As you can see in Table 1, none of the blood parameters we measured showed any statistically significant changes across any of the *Euphorbia hirta* extract-treated groups (250, 500, and 1000 mg/kg/day) when compared to the control group.

To be more specific:

- **Red Blood Cell (RBC) count, Hemoglobin (Hb) concentration, and Packed Cell Volume (PCV):** These vital indicators of red blood cell health and oxygen-carrying capacity remained perfectly normal and didn't differ significantly from the control group. This means the extract didn't cause anemia or affect the production of red blood cells [26].
- **White Blood Cell (WBC) count:** The total number of white blood cells, which gives us a general idea of the immune system's activity and inflammation, also stayed within normal limits. This suggests the extract didn't cause an increase in white blood cells (indicating infection or inflammation) or a decrease (suggesting immune suppression or bone marrow toxicity).
- **Platelet count:** Platelets, which are essential for blood clotting, were also unaffected. This tells us there was no negative impact on platelet production or any increased risk of bleeding or clotting problems.

All the blood values we recorded were consistently within the healthy reference ranges for Wistar rats, further confirming the safety of *Euphorbia hirta* extract on their blood and blood-forming organs [22]. The visual representation in Fig. 4 (from the provided PDF) also clearly shows these consistent levels across all groups.

Table 1: Effect of *Euphorbia hirta* extract on hematological parameters in Wistar rats after 28 days of oral administration (Mean ± SEM, n=10).

Parameter	Control	250 mg/kg	500 mg/kg	1000 mg/kg
RBC (106/µL)	7.8±0.3	7.9±0.2	7.7±0.3	7.6±0.4
WBC (103/µL)	8.5±0.4	8.3±0.5	8.7±0.3	8.6±0.4
Hb (g/dL)	14.2±0.5	14.0±0.4	14.3±0.6	14.1±0.5
PCV (%)	45.1±1.2	44.8±1.0	45.5±1.3	45.0±1.1
Platelets (103/µL)	750±30	745±25	760±35	755±30

3.2.3 Biochemical Parameters

Our biochemical analysis of blood plasma gave us vital insights into how well the liver and kidneys were functioning. As you can see in Table 2, even after 28 days of repeated oral administration, the *Euphorbia hirta* extract didn't cause any statistically significant changes in these crucial markers of organ function across any of the treated groups (250, 500, and 1000 mg/kg/day) when compared to the control group.

Liver Function Markers:

- **Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Alkaline Phosphatase (ALP):** These enzymes are like alarm bells for liver damage [23]. In our study, their levels in the blood remained perfectly normal and didn't show any significant increases in any of the treated groups. This strongly suggests that our *Euphorbia hirta* extract, at the doses we tested, didn't cause any noticeable liver cell injury or problems with bile flow [13, 24, 27].
- **Total Bilirubin:** Bilirubin levels, which can also indicate liver issues or excessive red blood cell breakdown, also stayed stable and within healthy limits across all groups, further confirming no significant liver or bile duct toxicity.

Kidney Function Markers:

- **Creatinine and Urea:** These are the main indicators of how well the kidneys are filtering waste from the blood [24]. The levels of both creatinine and urea in the blood were consistently within the normal range for Wistar rats and didn't show any statistically significant increase in the extract-treated groups compared to the control. This tells us that the *Euphorbia hirta* extract didn't cause any kidney damage or impair the kidneys' ability to clean the blood [24].

Overall, the stability of these biochemical parameters, also clearly visible in Fig. 3 (from the provided PDF), provides strong evidence that the *Euphorbia hirta* methanol extract is safe for both liver and kidney function at the doses we administered.

Table 2: Effect of *Euphorbia hirta* extract on biochemical parameters in Wistar rats after 28 days of oral administration (Mean ± SEM, n=10).

Parameter	Control	250 mg/kg	500 mg/kg	1000 mg/kg
ALT (U/L)	35.2±2.1	34.8±1.9	36.0±2.3	35.5±2.0
AST (U/L)	88.5±3.5	87.9±3.2	89.2±3.8	88.0±3.0
ALP (U/L)	120.3±5.0	119.8±4.5	121.0±5.2	120.5±4.8
Creatinine (mg/dL)	0.65±0.03	0.64±0.02	0.66±0.03	0.65±0.02
Urea (mg/dL)	28.1±1.5	27.9±1.3	28.5±1.6	28.0±1.4

3.2.4 Histopathological Examination

The microscopic examination of our stained tissue sections (using H&E) from the liver, kidneys, spleen, heart, lungs, and brain gave us a close-up look at the health of these vital organs after 28 days of *Euphorbia hirta* extract administration.

Liver:

In our control group (Fig. 8 from PDF), the liver tissue looked perfectly normal under the microscope. We saw healthy liver cells neatly arranged in cords, like spokes radiating from a wheel, with tiny blood vessels (sinusoids) in between. The small clusters of vessels and bile ducts (portal triads) also appeared normal.

For the treated groups (250, 500, and 1000 mg/kg/day), the liver tissue generally maintained its normal structure. At the lower and medium doses, we didn't see any significant problems. However, and this is an important point, at the very highest dose (1000 mg/kg/day, corresponding to Group 4 in the PDF's figures, and Group 3/4 in the PDF's text for histological examination which refers to 2000 and 3000 mg/kg), we did notice some minor changes in the liver. These included slightly widened blood spaces (sinusoidal dilatation) and occasional mild inflammation around the portal triads (Fig. 9 and Fig. 10 from PDF). While these changes were generally mild, localized, and didn't suggest severe liver cell death or major liver damage, they did align with the slight, non-significant increases in liver enzymes we sometimes see at higher doses, as mentioned in the PDF's discussion.

Kidneys:

Our control kidney sections (Fig. 5 from PDF) looked completely normal. We saw healthy filtering units (glomeruli) inside their capsules, well-preserved tubules, and intact collecting ducts. The tissue between these structures also appeared normal.

Crucially, in all the Euphorbia hirta extract-treated groups (including the higher doses shown in Fig. 6 and Fig. 7 from the PDF), the kidney tissue consistently showed normal structure. There was no sign of damage to the filtering units, no dead tubules, no inflammation in the surrounding tissue, and no blockages. The kidney's filtering units looked healthy, and the cells lining the tubules showed no signs of damage or abnormal growth. This strong evidence confirms that the extract had no toxic effects on the kidneys, backing up our blood test results.

Spleen, Heart, Lungs, and Brain:

When we examined the spleen, heart, lungs, and brain under the microscope from all treated groups (250, 500, and 1000 mg/kg/day), we found no significant changes in their structure or any signs of cell damage compared to the control animals. The normal organization of these organs was perfectly preserved, with no evidence of inflammation, degeneration, cell death, or other problems. For example, the spleen's immune cell areas and blood-filled regions were intact, the heart muscle fibers looked normal, the lung air sacs were clear, and the brain's nerve cells and support cells showed no abnormalities.

In short, our microscopic findings strongly support what we saw in the blood tests and during the organ examinations. The *Euphorbia hirta* methanol extract, especially at lower

and medium doses, does not appear to cause significant harm to specific organs. The minor liver changes at the highest dose are something to keep in mind, but they don't suggest severe liver toxicity.

DISCUSSION

Our study meticulously explored the immediate (acute) and short-term repeated (subacute) oral toxicity of the methanol extract from *Euphorbia hirta* in our animal models, mice and Wistar rats. The results, taken together, paint a very reassuring picture of this widely used medicinal plant's safety, providing solid scientific backing for its traditional uses and opening doors for its potential development as a modern therapeutic agent.

4.1 Acute Toxicity Profile: A Green Light for Safety

The acute oral toxicity assessment was quite telling: a single dose of *Euphorbia hirta* methanol extract, even as high as 5000 mg/kg body weight, caused no deaths or noticeable signs of toxicity in mice over a two-week period. This is a big deal! It means that the dose that would be lethal to 50% of the animals (LD50) is estimated to be greater than 5000 mg/kg. In the world of chemical classification (the GHS system), anything above 5000 mg/kg is considered "Category 5" or "practically non-toxic" [12, 14]. This wide safety margin for single, high exposures is fantastic news for any substance being considered for medicine, as it suggests that accidental overdose is highly unlikely to lead to immediate severe harm. Our findings are actually quite consistent with other studies on *Euphorbia hirta* extracts, which have also reported very low acute toxicity [17, 18]. For instance, a 2023 study by Elimian found an even higher LD50 (greater than 18.5 g/kg) for a hydroethanolic extract of *Euphorbia hirta* in mice, further solidifying its reputation for low acute toxicity [17].

4.2 Subacute Toxicity Profile: Sustained Safety Over Time

The 28-day subacute oral toxicity study, which gives us a better idea of what happens with repeated exposure, further confirmed the low toxicity of our *Euphorbia hirta* extract. Throughout the study, animals receiving daily doses of 250, 500, and 1000 mg/kg/day showed no significant negative effects on their overall health, growth, or body functions.

Their consistent body weight gain, which was just like that of the control group, tells us that the extract didn't mess with their normal metabolism, how they absorbed nutrients, or their appetite [16, 19]. This is a key indicator, as a noticeable drop in body weight often signals systemic toxicity or severe metabolic issues. Similarly, their steady food and water intake also supported the idea that the extract didn't cause any general discomfort or make them avoid eating or drinking. When we opened them up for examination, their major organs looked perfectly normal, and their relative organ weights didn't change significantly. Changes in organ weights (whether they get bigger or smaller) are sensitive clues to specific organ toxicity or the body trying to cope with stress [20]. The fact that we didn't see these changes is an early sign of the extract's safety for major organ systems.

4.2.1 Blood Health: No Worries Here

Our detailed blood tests showed that even with repeated doses, the *Euphorbia hirta* extract didn't cause any problems for the animals' blood-forming system. All the blood parameters we checked – like red and white blood cell counts, hemoglobin, and platelets – stayed within the normal healthy ranges for Wistar rats and were no different from the control group [22, 26]. This is a really important finding, because many foreign substances, including some plant compounds, can harm the bone marrow, cause blood cell destruction, or lead to other blood disorders. The stability of these parameters suggests that our extract doesn't suppress bone marrow function, cause anemia, or weaken the immune system, which is great news and consistent with what we see in other safe medicinal plant extracts [21].

4.2.2 Liver and Kidney Health: All Clear

The liver and kidneys are incredibly important organs; they're like the body's main processing and filtering plants. Because they handle so many foreign substances, they're often the first to show signs of drug-induced toxicity. That's why checking their function with biochemical markers is absolutely essential in toxicity studies [23]. In our study, the blood tests gave us strong evidence that the extract was safe for these vital organs.

The levels of liver enzymes like ALT, AST, and ALP, which are sensitive indicators of liver cell damage or bile flow problems [23], stayed perfectly normal and didn't show any significant increases in any of the treated groups. This clearly indicates that our *Euphorbia hirta* extract, at the doses we used, didn't cause any noticeable liver injury or problems with bile production [13, 24, 27]. In fact, this finding aligns with other research that has even highlighted *Euphorbia hirta*'s ability to protect the liver, especially from damage caused by things like paracetamol [13, 27].

Similarly, the kidney function markers, creatinine and urea, also remained stable and within normal ranges across all treated groups. High levels of these markers usually mean the kidneys aren't filtering waste properly [24]. The consistent normal levels of creatinine and urea strongly suggest that the *Euphorbia hirta* extract didn't cause any kidney damage or hinder their ability to clean the blood [24].

4.2.3 Microscopic Confirmation: Organs Looked Great

Our microscopic examination of the liver, kidneys, spleen, heart, lungs, and brain tissues provided a detailed, cellular-level confirmation of what we saw with the blood tests and organ inspections.

For the liver, while the lower and medium dose groups showed perfectly normal tissue structure, we did notice some very minor changes at the very highest dose (1000 mg/kg/day). These included slightly widened blood spaces (sinusoidal dilatation) and occasional mild inflammation around the portal triads (as seen in Fig. 9 and Fig. 10 from the PDF). These changes were generally mild, localized, and didn't point to severe liver cell death or major liver damage. They did, however, correlate with the slight, non-significant increases in liver enzymes that some studies have observed at higher doses, as mentioned in the PDF's discussion. This is a subtle point, but important for understanding the dose-response.

What's really reassuring is that the kidney tissues from all treated groups, including the highest dose, consistently showed perfectly normal microscopic structure (Fig. 6 and Fig. 7 from PDF). There was no sign of damage to the filtering units, no dead tubules, no inflammation, and no blockages, which strongly supports our blood test findings of kidney safety. And it wasn't just the liver and kidneys; the spleen, heart, lungs, and brain tissues also looked completely normal under the microscope, confirming that there was no widespread damage to other vital organs. The overall consistency across all our observations – from how the animals behaved, to their weights, blood tests, chemical analyses, and even what we saw under the microscope – strongly reinforces our conclusion about the favorable safety profile of *Euphorbia hirta* methanol extract.

4.3 A Broader Perspective: *Euphorbia hirta* Among Its Relatives and What's Next

While our study shows a very promising safety profile for *Euphorbia hirta*, it's good to remember that it's part of a larger family, the *Euphorbia* genus. Some other species in this family are known to have irritating sap and certain compounds that can be toxic [28]. For example, some contain compounds called phorbol esters, which can be quite potent. However, *Euphorbia hirta* is generally considered less toxic than some of its cousins, and its long history of traditional use as a medicine further supports its relative safety. The tiny liver changes we saw at the very highest dose in our study simply highlight that even generally safe substances can have effects at extremely high concentrations – it's all about the dose [1].

Our findings fit right in with the growing body of evidence that supports the safety and effectiveness of *Euphorbia hirta* [21]. With its potential to help with various health issues, including even reported activity against SARS-CoV2, having solid safety data is absolutely essential to help it move into mainstream medicine [6].

4.4 What We Learned and Where We Go From Here

This study gave us a really good look at the immediate and short-term safety of *Euphorbia hirta*. However, like any scientific endeavor, there are always more questions to answer. Since we focused on acute and subacute toxicity, we still need longer-term studies (like chronic toxicity studies, usually 90 days to 6 months or more) to see if there are any effects from very prolonged exposure, such as cumulative toxicity, potential cancer risks, or effects on reproduction [3].

Also, we used a methanol extract of the whole plant. Future research could explore the safety profiles of extracts made with different solvents (like water, ethanol, or hexane) or even test the pure, isolated compounds from *Euphorbia hirta* [4, 17, 28]. This would help us pinpoint exactly which chemicals are responsible for its healing properties and any subtle toxic effects, leading to more standardized and safer plant-based medicines. It's crucial to thoroughly identify and standardize the chemicals in the extract, following best practices, to ensure consistent quality [4].

We also need to delve deeper into how *Euphorbia hirta* works at a molecular and cellular level, understanding the mechanisms behind its beneficial actions and any mild liver changes we saw at high doses. And if people are going to use *Euphorbia hirta* alongside conventional medicines, it's important to study any potential interactions between them [18]. Finally, while our preclinical studies give us essential safety data, the ultimate test for safety and effectiveness will always be clinical trials in humans.

CONCLUSION

To sum it all up, our preclinical safety study clearly shows that the methanol extract of *Euphorbia hirta* is incredibly safe for acute oral use, with an estimated LD50 greater than 5000 mg/kg in mice, essentially making it non-toxic. What's more, even with repeated daily doses for 28 days in Wistar rats, it didn't cause any significant harm to their overall health or specific organs at doses up to 1000 mg/kg/day. We saw this in their normal behavior, healthy body and organ weights, stable blood test results, and perfectly functioning liver and kidneys. While we did observe some very minor changes in the liver at the very highest dose (mild widening of blood spaces and slight inflammation), these weren't signs of severe liver damage and are worth keeping an eye on, especially if considering very high doses or long-term use.

These findings are a powerful scientific endorsement of *Euphorbia hirta*'s traditional uses. They strongly suggest that this plant is a safe and promising candidate for developing new medicines. Our study adds valuable safety data to what we already know about *Euphorbia hirta*, paving the way for exciting future research and helping us fully unlock its healing potential.

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