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ASSESSMENT OF TOXIC EFFECTS OF *Hedyotis capitellata* WALL. LEAVES ETHANOL EXTRACT VIA BIOLOGICAL ASSAYS IN MICE (*Mus musculus*)

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Abstract

Hedyotis capitellata Wall. is a traditional plant known for its various significant pharmacological effects, such as gastric protection, pain relief, anti-inflammatory properties, etc. Despite its wide usage in traditional medicine, no scientific studies have been published on its toxicity. The current study evaluated the potential toxicity of ethanol leaf extract of *H. capitellata* (EtHC) through acute and sub-chronic oral administration in mice following guidelines 423 and 408 of the OECD for chemical testing. To assess the acute toxicity of EtHC, single doses of 1000, 3000, and 5000 mg/kg body weight were administered to mice and continuously observed for 14 days. Additionally, daily doses of 100, 300, and 500 mg/kg body weight were also given to mice for 90 days to investigate sub-chronic toxicity. At the end of the study, blood, urine, and vital organs were collected for haematological, biochemical, urine analysis, and histopathological studies. The results demonstrated no cases of mortality or signs of toxicity observed in the acute toxicity test. Throughout the 90-day study period, all tested extract doses exhibited no harmful effects on the organs. Haematological and biochemical parameters in the acute toxicity (5000 mg/kg) and subchronic toxicity (500 mg/kg) groups (e.g., red blood cell count: $6.59 \pm 0.41 \times 10^6$ cells/mm^3, aspartate aminotransferase (AST) level: 16.62 ± 0.31 U/L, alanine aminotransferase (ALT) level: 18.15 ± 0.58 U/L, urine (pH 7.05 ± 0.09, specific gravity 1.17 ± 0.11) did not significantly differ from the control group ($p > 0.05$). However, individual differences were observed in the white blood cell count ($EtHC_{5000}$ group) and glucose level ($EtHC_{500}$ group) compared to the control group ($p < 0.05$). These changes were considered individual variations and not biologically significant. Histopathological examination of the organs also revealed no abnormalities in the extract-treated group. This study demonstrates that the ethanol leaf extract of *H. capitellata* is non-toxic and could be considered for treating various diseases, such as pain relief, anti-inflammatory effects, gastric ulcer treatment, etc.

INTRODUCTION

Traditional medicinal plants are often advertised as natural and harmless. These claims are based on the use of herbal medicine in disease treatment over many centuries [1]. However, some medicinal plants must be used cautiously as they may cause adverse reactions, particularly in cases of high dosage or prolonged use or when combined with conventional drugs. In response to community health concerns, this study evaluates the toxic effects of *Hedyotis capitellata* Wall. and addresses the demands encouraged by various health and scientific organizations [2].

In Vietnam, the use of medicinal plants is an important and essential part of traditional healthcare systems. However, the use of certain folk herbal remedies still needs to be well-regulated. Therefore, there is a risk of

misidentification, interaction, and inappropriate use of these herbs when used simultaneously with other drugs. *Hedyotis capitellata* Wall. (Rubiaceae family) is a traditional medicinal plant in Vietnam, often growing wild in mountainous provinces such as Cao Bang, Lang Son, Bac Kan, Thai Nguyen, Ha Giang, Ha Tay, Khanh Hoa, Kom Tum, Lam Dong, and Dong Nai. *H. capitellata* is a herbaceous climbing plant with a cylindrical stem, covered with fine hairs, divided into many nodes, with opposite oval or egg-shaped leaves,and white or yellow flowers growing in clusters. Its capsule fruit contains many small seeds inside. In folk medicine, the leaves and stems of *H. capitellata* are used for their cooling, detoxifying, anti-inflammatory, diuretic, and acid-neutralizing properties. Therefore, *H. capitellata* is used as an herbal remedy to alleviate symptoms of stomach pain, support the treatment of gastric inflammation, improve acid reflux conditions, and heal ulcers [3].

Although Vietnamese people have been exposed to *H. capitellata* and specifically used it to treat conditions such as stomach ulcers, mouth and throat sores, skin ulcers, and wounds caused [3], there is still no specific information in scientific reports regarding its toxicity profile, except for some reports on its phytochemical constituents [4], anti-inflammatory and anticancer activities [5]. Therefore, the current study was conducted to evaluate the acute and sub-acute toxicity of *H. capitellata* leaf extract in mice.

MATERIALS AND METHODS

Materials Preparation

Fresh leaves of *H. capitellata* were harvested in Cam My district, Dong Nai province (Vietnam), in September 2022 (Figure 1A). The specimen evidence was sent to the Plant Biotechnology Laboratory, Institute of Biotechnology and Food Technology, Ho Chi Minh City University of Industry, Vietnam, with a reference number (HC270922VST) for future reference. The upper part of the leaves was separated, washed, cut into small pieces, and dried in the shade for seven days until the moisture content reached about 10-12% (Figure 1B). The dried leaves were then ground into powder (<0.3 mm) and stored in a moisture-proof container for further studies (Figure 1C).

Figure 1. Ethanol extraction of *H. capitellata* leaves (A. Fresh leaves; B. Dried leaves; C. Dried leaf powder; D. EtHC).

Extract Preparation

The *H. capitellata* leaf powder was soaked in ethanol (96%) with materials to solvent ratio of 25/80 (g/mL) for 72 h at room temperature. The resulting extract was filtered through Whatman filter paper (No. 4) and concentrated using a rotary evaporator at 65°C for one h. The total soluble solid (TSS) of the extract was tested by hand refractometer (TSS: 18.3°Brix). The obtained extract (called EtHC) was stored in a refrigerator at 4°C until used for further experiments (Figure 1D).

Phytochemical Screening of Extract

The standard analytical techniques were used to determine the chemical groups or compounds in aqueous extracts from various plant species. These qualitative tests are based on colour-forming or precipitating reactions with the presence of specific chemical compounds [6]. Standard qualitative methods were used to analyze the ethanol extract from *H. capitellata* leaves below [7-9].

Qualitative Screening for Carbohydrates

2 mL of EtHC is prepared in a test tube, then two drops of Molisch reagent (adding naphthol in 95% ethanol) are added, followed by slowly adding concentrated H_2SO_4 without strong mixing. A purple ring appears at the interface between the acid and test layers, confirming the presence of carbohydrates.

Qualitative Screening for Tannins and Phlobatannins

Most tannins, a group of phenolic compounds commonly found in plants, are soluble in water. Phlobatannin is considered a new class of tannin condensed isomers. 2 mL of 5% ferric chloride solution is added to 1 mL of EtHC. The appearance of a dark blue or black colour confirms the

presence of tannins. Add a few drops of dilute HCl (1%) to 1 mL of EtHC. The red precipitate confirms the presence of phlobatannins.

Qualitative Screening for Saponins

2 mL of EtHC and 2 mL of distilled water are shaken for 15 min in a graduated cylinder. A thick layer of foam (1 cm) is a positive reaction to the presence of saponins.

Qualitative Screening for Polyphenols

Prepare 2 mL of distilled water, then add a few drops of 10% ferric chloride to 1 mL of EtHC. The formation of a blue or green colour indicates the presence of polyphenols.

Qualitative Screening for Flavonoids and Phenolic Flavonoids

Mix 2 mL of EtHC with 1 mL of 2N sodium hydroxide. A yellow colour indicates the presence of flavonoids. Mix 1 mL of EtHC with 2 mL of 10% lead acetate solution, and the brown precipitate indicates a positive reaction, confirming the presence of phenolic flavonoids in the extract.

Qualitative Screening for Alkaloids

1 mL of EtHC is mixed with 2 mL of concentrated HCl. Then a few drops of Mayer's reagent (mercury chloride and potassium iodide solution in water) are added. A green or white precipitate indicates the presence of alkaloids.

Qualitative Screening for Proteins and Amino Acids

3 mL of EtHC is mixed with three drops of 5% lead acetate solution and the resulting solution is heated. A purple or blue colour indicates a positive reaction, confirming the presence of amino acids. 3 mL of EtHC, 3 mL of 4% sodium hydroxide solution, and a few drops of 1% copper sulfate are added to create a purple solution, indicating the presence of proteins.

Experimental Animals

Healthy Swiss albino mice (*Mus musculus*), 7-8 weeks old, weighing between 30 to 32 g, were purchased from the Pasteur Institute of Ho Chi Minh City. The mice were housed in glass cages (30 × 60 × 30 cm = 54 cm^3) with a cover, and the bedding was made of odour-free wood chips treated with the biological product EM (Effective Microorganisms) and replaced every 3 days. The mouse cages (6 mice/cage) were placed in the Animal House of the Biology Experiment Garden, Biotechnology - Food Technology Institute, Ho Chi Minh City University of Industry, at room temperature (30 ± 2°C) and a 12/12 h light/dark cycle. Before and during the study, the mice were fed with rodent chow and drank clean water treated with an RO filtration system. Before any experimental procedures were performed, the mice were acclimatized to the laboratory conditions for 15 days [10]. The process of dividing the experimental animals into groups was randomized. The animals were divided into groups based on body weight so that the variation in body weight did not exceed ± 25% of the average body weight. The care and implementation of experimental procedures on animals strictly adhered to the Guidelines for Care and Use of Laboratory Animals [11], in accordance with laws, regulations, and global standards for animals used in research [12] and the Helsinki Declaration (2008) on ethical principles for animal experimentation [13]. The study was approved by the Ethics Committee of the Council for Scientific Research and Technology Development of the Ho Chi Minh City University of Industry, Vietnam (code 119/HĐ-ĐHCN).

Acute Toxicity Test

The acute oral toxicity test of *H. capitellata* leaf extract was conducted following the OECD Guideline 423 for acute oral toxicity testing (2002) [14], with minor modifications. The number of animals used in this study adhered to the OECD guidance principles (2001) [15]. The animals were randomly divided into four groups (n = 6), a control group receiving distilled water, and test groups ($EtHC_{1000}$, $EtHC_{3000}$, and $EtHC_{5000}$) receiving a single dose of EtHC at 1000, 3000, and 5000 mg/kg, respectively. Before the experiment, all mice were weighed, marked for identification, and fasted overnight but allowed access to water. After extract administration, fasted mice were observed for an additional 4 h and monitored continuously for 1 h and intermittently for 4 h, followed by once every 12 h and continuously for the next 14 days. The following signs of mouse reaction and behaviour were observed throughout the study: changes in fur, skin, eyes, mucous membranes, and autonomic activity such as piloerection, changes in pupil size, tearing, and abnormal respiration; changes in gait and posture were also monitored, along with stereotypic activities such as excessive grooming, circling, etc. During the study period, clinical observations of mortality rate, behaviour, and abnormal reactions were recorded daily. Additionally, body weight, food consumption, and water intake were measured weekly until the end of the experiment. At the end of the experiment, haematological and biochemical parameters, relative organ weights, urine analysis, and histopathological examination were performed to evaluate acute toxicity.

Sub-chronic Toxicity Study

Chronic toxicity evaluation of repeated EtHC doses over 90 days was conducted using the method described in OECD

Guideline 408 for 90-day repeated dose oral toxicity in rodents [16] with minor modifications. Test mice were randomly divided into four groups (n = 6). The first test groups ($EtHC_{100}$, $EtHC_{300}$, and $EtHC_{500}$) were administered EtHC doses of 100, 300, and 500 mg/kg, respectively, for 90 days. The control group (concurrent control) received a regular distilled water dose. Animals were observed daily during the experiment to detect mortality rates and abnormal clinical signs. Body weight and the amount of food and water consumed were also measured weekly until the end of the experiment. On the last day of the experiment, a total of available blood from each animal was collected via the retro-orbital sinus puncture method [17] and analyzed for haematology and serum biochemistry. Urine was also collected and analyzed for composition. Following anaesthesia with 9 mg/kg ketamine 10% and 10 mg/kg xylazine 2% [18], animals were humanely euthanized due to decreased circulation volume. Relative organ weight analysis and histopathological evaluation were performed to conclude the sub-chronic toxicity effects of EtHC.

Clinical Observations and Survival Rates

The animals were monitored twice daily for mortality rate and any abnormal reactions or behaviours. Clinical observations included changes in fur, skin, eyes, mucous membranes, and autonomic activity such as piloerection, changes in pupil size, tear flow, and abnormal breathing patterns. Changes in gait and posture were also monitored, along with stereotypic behaviours such as excessive grooming, repetitive circling, etc. The observation period was one week before administering the experimental drug until the scheduled necropsy.

Body Weight

The individual body weight of all animals was recorded regularly by electronic scale (Sartorius, Germany). Body weight was recorded on the first day before administering EtHC (day 0). After administering EtHC, the body weight of the mice was measured on the 7 and 14th day (acute toxicity test), and on the 30, 60, and 90th day (sub-chronic toxicity test). The percentage of weight gain (WG %) was calculated using the following formula:

$$\text{Weight gain (\%)} = \frac{\text{Final body weight (g)} - \text{Initial body weight (g)}}{\text{Initial body weight (g)}} x\ 100 \quad [19]$$

Food and Water Intake Consumption

The amount of food and water was weighed and recorded prior to administration to the mice. Any remaining food and water were collected and weighed at the end of each day. The daily consumption of food and water was calculated using the formula:

Food consumption (g) = Initial food consumption (g) - Remaining food consumption (g)

Water intake (mL) = Initial water intake (mL) - Remaining water intake (mL)

The amount of food and water consumed was recorded daily. The average weekly consumption was calculated after each week by averaging the daily consumption [20].

Hematological and Biochemical Study

At the end of the experiment, the blood samples were collected by the retro-orbital sinus puncture method. The blood was divided into two groups of tubes, half containing ethylenediamine tetra-acetic acid (EDTA) anticoagulant and the other half without anticoagulant. The EDTA-containing tubes were used to determine haematological parameters, including white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), hematocrit (HCT), and platelet count (PLT), using an automated haematology analyzer Nihon Kohden MEK-9100 (Japan). The non-anticoagulated blood samples were allowed to clot at room temperature, and the serum was obtained by centrifugation (3000 rpm for 10 min). Subsequently, biochemical analyses were performed on the serum to quantify aspartate aminotransferase (AST) and alanine aminotransferase (ALT), total protein, glucose to evaluate liver damage, and creatinine and urea to evaluate kidney damage using a fully automated biochemical analyzer CA-400 (Japan).

Relative Weight of the Organs

All animals were fasted overnight before being subjected to dissection. The animals were anaesthetized using a combination of 10% ketamine and 2% xylazine and sacrificed by inhalation of CO_2 gas. Macroscopic examination of the thoracic and abdominal cavities, skull, pelvis, and important organs was performed. Important organs such as the liver, kidneys, and stomach were removed from the body and rinsed with 10% neutral buffered formalin. Macroscopic examination of these organs was also conducted to detect any lesions or other abnormal signs. An Entris Sartorius analytical balance (Germany) was used to measure the absolute weight of the liver, kidneys, and stomach. The relative weight of each organ (ROW%) was calculated using the formula:

$$ROW(\%) = \frac{\text{Absolute visceral weight (g)}}{\text{Body weight on the day of surgery (g)}} \times 100 \quad [21]$$

Histopathology Study

After anaesthesia and dissection, the abdominal cavity of the mouse was surgically opened and vital organs (liver, kidney, and stomach) were separated from the body, washed with physiological saline, blotted dry with Whatman No.1 filter paper, and examined for visible injuries. The organs were then weighed and preserved in 10% formaldehyde. Subsequently, each organ was sectioned, embedded in paraffin wax, and stained with hematoxylin and eosin (H&E). Paraffin sections (5 µm) were cut and mounted on glass slides. Further processing involved removing paraffin from the tissue sections on the slides with ethanol and xylene. Optical microscopy images of the stained tissue sections were generated using a digital microscope (Olympus®) at 200× magnification.

Urinalysis

In the final week of acute and subchronic toxicity testing, fresh urine from the animals (they had been fasted overnight but still had free access to water) in each group was collected simultaneously for evaluation. The specific gravity, pH, protein, glucose, red blood cells, white blood cells, Na^+, and K^+ levels in the urine were assessed using the Clinitek Status automated urine analyzer, Erlangen (Germany).

Statistical Analysis

The experimental results are presented in the form of $\bar{X} \pm$ SD. The data were statistically analyzed using ANOVA. Tukey's post-hoc test was performed using Statgraphics Centurion XVIX statistical software. The significance level used to test the differences between treatments was set at $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical Analysis of EtHC

Preliminary chemical analysis of *H. capitellata* leaf ethanol extract revealed the presence of carbohydrates, tannins, phlobatanins, polyphenols, flavonoids, phenolic flavonoids, alkaloids, and terpenoids. There is no presence of proteins, amino acids, steroids, saponins, and glycosides.

Phenolic compounds have been recognized for their numerous health benefits. Various pharmacological activities such as antioxidant, anticancer, and antibacterial effects have been attributed to them [22]. Flavonoids are a potential source of antioxidants. They have the ability to reduce free radicals and reactive oxygen species. The shielding effect of flavonoids in the extract leads to their free radical scavenging, antioxidant, and anti-inflammatory properties [23]. The pain-relieving, anti-inflammatory, and antipyretic effects have been observed with flavonoids, tannins, alkaloids, and saponins through the donation of hydrogens generated by antioxidant reactions that produce peroxyl radicals. These compounds are widely shown to target organs involved in pain perception through the modulation of opioidergic mechanisms [24]. EtHC exhibits both pharmacological properties and toxicity due to the presence of biologically active molecules. EtHC inherently possesses chemical toxicity due to its plant constituents and may be associated with adverse effects if not used properly and in sufficient quantities. Therefore, upon discovering bioactive compounds present in EtHC, toxicity evaluation becomes a crucial and mandatory step to be performed before pharmacological screening and clinical applications. The benefits of toxicity assessment of EtHC in animal models include controlled exposure time, examination of different tissues for potential hazards, and determination of the effects on the biologically active molecules present in EtHC [25].

Behavioural Responses and General Appearance

Acute Toxicity Evaluation of EtHC in Mice

Evaluation of acute toxicity of EtHC in mice: No adverse effects or mortality were observed in mice treated with single doses of EtHC at 1000, 3000, and 5000 mg/kg. No significant differences in behavioural responses and general appearance (Table 1) were noted in mice treated with EtHC compared to the control group. The body weight of the mice slightly increased during the experiment but remained within the normal range. Based on these data, the median lethal dose (LD_{50}) of EtHC is > 5000 mg/kg.

Sub-chronic Toxicity Evaluation of EtHC in Mice

Repeated doses of EtHC at 100, 300, and 500 mg/kg for 90 days did not cause any adverse effects on the skin, hair, eyes, mucous membranes, secretions, excretions, or self-regulatory activity during the entire study period (Table 1). No deaths were observed and there was no difference compared to the control group.

The evaluation of behavioural and reactive effects is an essential component of in vivo screening for the toxicity of extract compounds in mice, including monitoring general health, sensory function, motor ability, outward appearance, etc. [26]. Changes in reaction and behaviour are used as preliminary indicators to assess toxicity. Motor function is a complex behaviour influenced by various brain systems (such as the dopaminergic system in the cerebral and cerebellar regions) as well as peripheral abnormalities (muscular factors). Movement is necessary for many complex behavioural tasks, and changes in motor activity are relevant in assessing behavioural effects and reactions in *in vivo* toxicity screening of extract compounds [26]. Emotions and anxiety refer to the "psychological, physiological, and behavioural states caused by actual or

potential threats to the health and survival of animals", characterized by the increase arousal, activation of the autonomic and endocrine systems, and involving a shift in behaviour from ongoing behavior (exploration, foraging) to escape behaviour (fleeing) or other defensive behaviours. Animals employ these changes to cope with unfavourable or unexpected situations [27]. In this study, no cases of mortality, altered motor function, behaviour, emotions, or outward appearance were observed in the groups treated with EtHC. According to the OECD criteria for the classification of chemicals and chemical mixtures in the Globally Harmonized System (GHS), substances with LD_{50} values > 2000-5000 mg/kg are classified as category 5 of the GHS [28]. Therefore, with an LD_{50} value > 5000 mg/kg for EtHC via oral administration, it is considered relatively safe.

Table 1. Effect of EtHC on behavioural responses and general appearance in mice

Behaviors and reactions	Acute toxicity test				Sub-chronic toxicity test			
	Control group	$EtHC_{1000}$	$EtHC_{3000}$	$EtHC_{5000}$	Control group	$EtHC_{100}$	$EtHC_{300}$	$EtHC_{500}$
Jitter	Nil	Nil	Nil	Light	Nil	Nil	Nil	Light
Quiver	Nil	Light	Nil	Light	Nil	Nil	Light	Light
Move	Flexible	Flexible	Flexible	Flexible	Flexible	Flexible	Flexible	Flexible
Hair	Soft and silky	Soft and silky	Soft and silky	Soft and silky	Soft and silky	Soft and silky	Soft and silky	Soft and silky
Writhing	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Saliva	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Dead	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

Body Weight and Consumption of Food and Water

Changes in food intake, water consumption, and body weight have been used as indicators of the general health status of experimental animals. Food intake is regulated through a complex biological mechanism that ensures relatively stable body weight over time [29]. The food and water consumption of mice in all groups were not significantly affected during the treatment and recovery period (Table 2). The food and water consumption of the group treated with EtHC in both experiments did not differ significantly from the control group ($p > 0.05$). However, the water intake of mice increased in the 5000 mg/kg group (acute toxicity test) (4.95 ± 0.11 mL/day) compared to the control group (4.67 ± 0.1 mL/day) ($p < 0.05$), and the food intake also increased in the group treated with 500 mg/kg EtHC (sub-chronic toxicity test) (5.95 ± 0.11 g/day) compared to the control group (5.56 ± 0.11 g/day) ($p < 0.05$). Overall, food and water consumption changes did not exhibit a dose-response relationship with EtHC. The plant component of EtHC contains polyphenols, which can improve appetite, leading to enhanced weight control. Some polyphenols from plants can regulate different metabolic pathways and activate the AMPK (protein adenosine 5'-monophosphate) pathway, which is beneficial for lipid breakdown. The polyphenols in EtHC may enhance metabolism by regulating the activity of AMPK [30]. This report agrees with previous studies showing that the leaf extract of Nauclea latifolia (family Rubiaceae) improved appetite, increased food and water consumption, and increased body weight [31].

Table 2. Food and water intake in acute and sub-chronic toxicity tests of EtHC

Acute toxicity test				
Parameters	Control group	$EtHC_{1000}$	$EtHC_{3000}$	$EtHC_{5000}$
Food consumption (g/day)	5.15 ± 0.31	5.31 ± 0.51	5.27 ± 0.68	5.22 ± 0.59
Water consumption (mL/day)	4.67 ± 0.1	4.73 ± 0,28	4.82 ± 0,27	4.95 ± 0.11*
Sub-chronic toxicity test				
Parameters	Control group	$EtHC_{100}$	$EtHC_{300}$	$EtHC_{500}$
Food consumption (g/day)	5.56 ± 0.11	5.61 ± 0.27	5.77 ± 0.44	5.95 ± 0.11*
Water consumption (mL/day)	5.02 ± 0.54	5.08 ± 0.42	5.16 ± 0.44	5.29 ± 0.34

Values are expressed as Mean ± SD (n = 6). Values that are significantly different versus the corresponding control group (*$p < 0.05$) were marked with an asterisk.

There were no statistically significant differences observed in body weight and weekly body weight gain between the EtHC-treated groups and the control group in both acute and subchronic toxicity experiments (2.35 ± 0.23% and 11.31 ± 0.14%, respectively, compared to 3.6 ± 0.14% and 12.22 ± 0.13% in the control group, respectively) ($p > 0.05$) (Figure 2 and 3). The increase in body weight of mice after consuming EtHC could be influenced by the presence of saponins in the extract. Saponins are converted into sapogenin aglycones (steroids or triterpenes) [32], which stimulate the feeding centres in the brain, altering neurotransmitter release and affecting food intake, such as agents influencing the central serotonergic or dopaminergic systems, increasing appetite [33]. However, the changes in mouse weight during the experimental period fall within the weight range of normal Swiss albino mice (31-33g), according to the statistics of Webster et al. [34]. This indicates that EtHC is non-toxic and does not affect mouse growth. The changes in body weight are within the range of normal data accumulated in the author's laboratory, indicating that these changes are not related to EtHC treatment and have no clinical significance.

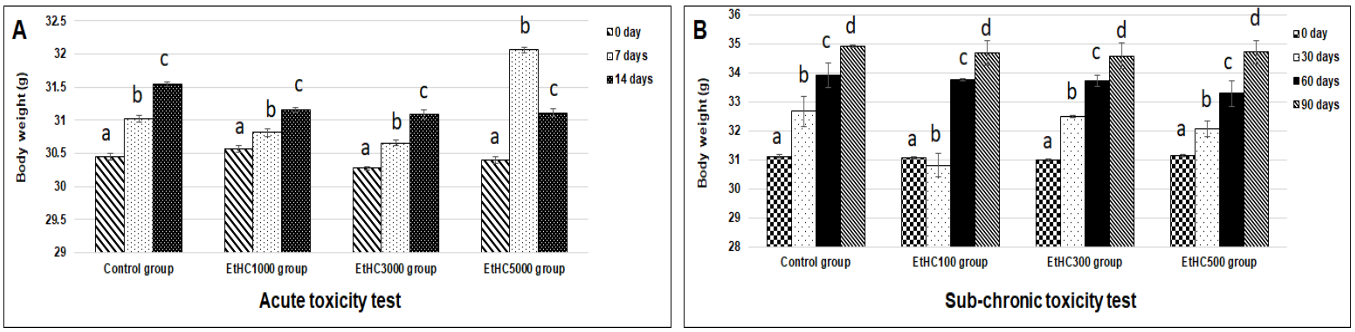

Figure 2. Effect of EtHC on body weight in Swiss albino mice in acute and sub-chronic toxicity testing (A. Body weight in acute toxicity test; B. Body weight in sub-chronic toxicity test). Values are expressed as Mean ± SD (n = 6), and letters (a, b, c, d) indicate significant differences ($p < 0.05$) at different survey time points within the same group.

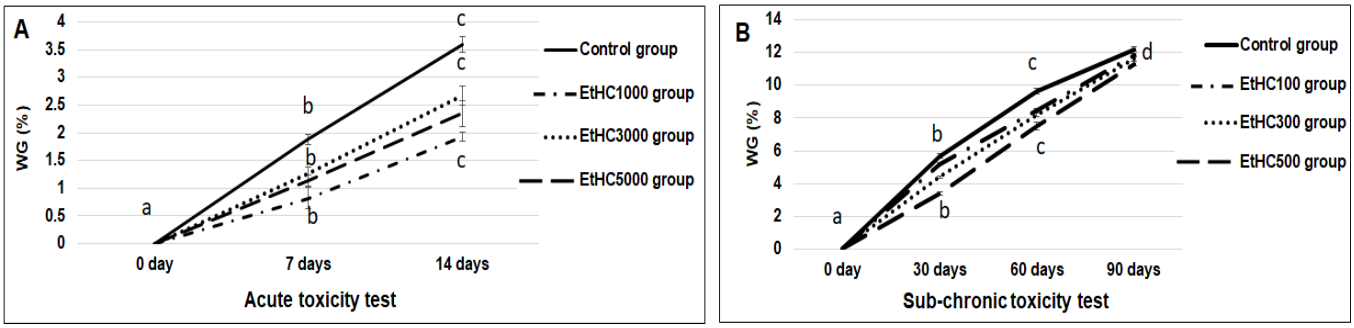

Figure 3. Weekly body weight gain of control group mice and those treated with EtHC in the acute and sub-acute toxicity study (A. Weekly body weight gain in acute toxicity test; B. Weekly body weight gain in sub-chronic toxicity test). Values are expressed as Mean ± SD (n = 6), and letters (a, b, c, d) indicate significant differences ($p < 0.05$) at different survey time points within the same group.

Hematology and Biochemistry

Changes in the hematopoietic system are highly predictive for toxicity studies in animals [29]. In this study, mice treated with EtHC and monitored for 14 and 90 days showed no significant changes in any blood parameters. Hematological parameters were analyzed and found to be within the normal range, with no significant differences observed between the control and EtHC-treated groups ($p > 0.05$) (Table 3). There were no significant differences in RBCs, HGB, HCT, and PLT parameters in both acute and subchronic toxicity experiments ($p > 0.05$). However, the WBC count in the groups treated with 5000 and 500 mg/kg EtHC (3.82 ± 0.11 and 3.99 ± 0.05 × 10^3 cells/mm^3, respectively) showed a slight increase and a significant difference compared to the control group (3.67 ± 0.12 and 3.88 ± 0.09 × 10^3 cells/mm^3, respectively). The slight increase in WBC count suggests that the use of EtHC may elicit a response due to the effects of active compounds (phenolic compounds, saponins, tannins, etc.) present in the extract [35]. However, the haematological levels still fall within the normal range for Swiss albino mice, as reported

by Reste et al. [33]. Biochemical analysis of all EtHC-treated groups (100, 300, and 500 mg/kg) compared to the control group revealed no statistically significant differences ($p > 0.05$) (Table 4). Changes in total protein, ALT, AST, and ALP parameters were within the normal range ($p > 0.05$), except for a slight increase in glucose levels in the $EtHC_{500}$ group (61.76 ± 0.06 mg/dL) compared to the control group (61.07 ± 0.07 mg/dL) ($p < 0.05$).

Table 3. Effect of EtHC on haematological parameters in Swiss albino mice in acute and sub-chronic toxicity testing

Acute toxicity test				
Parameters	Control group	$EtHC_{1000}$	$EtHC_{3000}$	$EtHC_{5000}$
RBCs ($\times 10^6$ cells/mm^3)	6.48 ± 0.37	6.54 ± 0.42	6.51 ± 0.39	6.59 ± 0,41
HGB (g/dL)	12.87 ± 0.28	12.94 ± 0.39	13.08± 0.52	12.99 ± 0.32
HCT (%)	0.38 ± 0.25	0.45 ± 0.3	0.41 ± 0.28	0.47 ± 0.26
WBCs ($\times 10^3$ cells/mm^3)	3.67 ± 0.12	3.75 ± 0.37	3.71 ± 0.28	3.82 ± 0.11*
PLT (x10^3 cells /mm^3)	628.27 ± 97.32	632.01 ± 93.42	636.89 ± 87.54	638.89 ± 94.07
Sub-chronic toxicity test				
Parameters	Control group	$EtHC_{100}$	$EtHC_{300}$	$EtHC_{500}$
RBCs ($\times 10^6$ cells/mm^3)	7.66 ± 0.32	7.71 ± 0,37	7.78 ± 0,46	7.75 ± 0.44
HGB (g/dL)	13.49 ± 0.34	13.78 ± 0.34	13.64 ± 0.24	13.59 ± 0.32
HCT (%)	0.46 ± 0.29	0.52 ± 0.35	0.49 ± 0.34	0.55 ± 0.33
WBCs ($\times 10^3$ cells/mm^3)	3.88 ± 0.09	3.94 ± 0.35	3.91 ± 0.27	3.99 ± 0.05*
PLTs ($\times 10^3$ cells /mm^3)	649.18 ± 82.21	664.91 ± 95.75	653.13 ± 89.9	658.56 ± 90.51

Values are expressed as Mean ± SD (n = 6). Values that are significantly different versus the corresponding control group (*$p < 0.05$) were marked with an asterisk. Note: RBCs (Red Blood Cells), HGB (Hemoglobin), HCT (Hematocrit), WBCs (White Blood Cells), PLTs (Platelets Count).

Table 4. Effect of EtHC on biochemical parameters in Swiss albino mice in acute and sub-chronic toxicity testing

Acute toxicity test				
Parameters	Control group	$EtHC_{1000}$	$EtHC_{3000}$	$EtHC_{5000}$
TP (g/dL)	6.89 ± 0.21	7.05 ± 0.38	6.96 ± 0.31	7.09 ± 0.42
Glucose (mg/dL)	61.15 ± 0.41	61.26 ± 0.37	61.31 ± 0.29	61.22 ± 0.29
AST (U/L)	16.55 ± 0.51	16.69 ± 0.31	16.49 ± 0.38	16.62 ± 0.31
ALT (U/L)	16.98 ± 0.47	17.29 ± 0.42	17.12 ± 0.43	17.37 ± 0.36
ALP (U/L)	120.25 ± 10.66	117.12 ± 16.31	124.27 ± 15.02	121.91 ± 13.02
Sub-chronic toxicity test				
Parameters	Control group	$EtHC_{100}$	$EtHC_{300}$	$EtHC_{500}$
TP (g/dL)	7.11 ± 0.59	7.24 ± 0.25	7.18 ± 0.35	7.25 ± 0.24
Glucose (mg/dL)	61.07 ± 0.07	61.19 ± 0.31	61.24 ± 0.29	61.76 ± 0.06*
AST (U/L)	16.89 ± 0.34	17.14 ± 0.35	17.21 ± 0.47	16.94 ± 0.59
ALT (U/L)	18.08 ± 0.41	18.33 ± 0.49	18.27 ± 0.47	18.15 ± 0.58
ALP (U/L)	126.53 ± 15.06	130.27 ± 18.06	129.79 ± 18.14	127.16 ± 15.5

Values are expressed as Mean ± SD (n = 6). Values that are significantly different versus the corresponding control group (*$p < 0.05$) were marked with an asterisk. Note: TP (Total Protein), AST (Aspartate aminotransferase), ALT (Alanine aminotransferase), ALP (Alkaline phosphatase).

Hematological and biochemical data play a crucial role in determining the toxicity of drugs. Analyzing blood parameters is appropriate for risk assessment as haematological systems have a higher predictive value for toxicity [36]. In the current study, the haematological data indicated that EtHC did not affect hematopoiesis as no significant differences were observed between the toxicity test group and the control group. Liver and kidney function

were assessed by analyzing serum biochemistry, which is important for toxicity evaluation. Elevated liver enzyme levels indicate hepatocellular toxicity, while decreased levels may indicate enzyme inhibition. Reduced total protein levels in serum indicate decreased synthesis function [29]. In this study, no significant changes were observed in the concentrations of AST, ALT, TP, and glucose between the control and toxicity test groups. Our extract did not induce significant changes in creatinine and urea concentrations in the groups treated with EtHC compared to the control group.

Urinalysis

Urinalysis data serve as indicators of liver and kidney function. Urinalysis is a valuable tool for evaluating the toxic effects of experimental compounds on the liver and kidneys. The pathological condition of the kidneys can be assessed through urinalysis data. However, these parameters can be subject to fluctuations due to factors such as stress or environmental changes, including urinary retention or contamination [37]. At the conclusion of the experimental phase, specifically on days 14 and 90 of the testing period, urinalysis was conducted, revealing no significant differences in specific weight, pH, Na^+, and K^+ parameters between the treatment groups and their respective control groups ($p > 0.05$). Glucose was not detected, and only a few red and white blood cells were observed in the urine (Table 5). This study found no abnormal indications in the urinalysis parameters of the EtHC-treated groups, indicating the safety of the tested dose of the plant extract.

Table 5. Effect of EtHC on urine composition of Swiss albino mice in acute and sub-chronic toxicity testing

Acute toxicity test				
Parameters	Control group	$EtHC_{1000}$	$EtHC_{3000}$	$EtHC_{5000}$
Specific weight	1.22 ± 0.11	1.18 ± 0.12	1.16 ± 0.11	1.17 ± 0.11
pH	7.03 ± 0.06	6.97 ± 0.05	6.95 ± 0.06	7.05 ± 0.09
Na^+ (ppm)	541.37 ± 48.69	549.64 ± 44.86	552.76 ± 41.32	546.84 ± 46.91
K^+ (ppm)	41.33 ± 0.06	41.43 ± 0.31	41.51 ± 0.34	41.76 ± 0.04*
Glucose (mg/dl)	Nil	Nil	Nil	Nil
Protein (mg/dl)	Absent	Absent	Absent	Absent
Red blood cells (cells/µL)	Absent	Absent	Absent	Absent
White blood cells (cells/µL)	Absent	Absent	Absent	Absent
Sub-chronic toxicity test				
Parameters	Control group	$EtHC_{100}$	$EtHC_{300}$	$EtHC_{500}$
Specific weight	1.16 ± 0.11	1.19 ± 0.11	1.15 ± 0.13	1.17 ± 0.13
pH	7.04 ± 0.07	7.02 ± 0.06	6.96 ± 0.07	6.94 ± 0.08
Na^+ (ppm)	552.24 ± 42.55	557.35 ± 40.45	561.49 ± 47.86	563.78 ± 46.25
K^+ (ppm)	41.53 ± 0.28	44.58 ± 0.31	44.62 ± 0.37	44.69 ± 0.38
Glucose (mg/dl)	Nil	Nil	Nil	Nil
Protein (mg/dl)	Absent	Absent	Absent	Absent
Red blood cells (cells/µL)	Absent	Absent	Absent	Absent
White blood cells (cells/µL)	Absent	Absent	Absent	Absent

Values are expressed as Mean ± SD (n = 6). Values that are significantly different versus the corresponding control group (*$p < 0.05$) were marked with an asterisk.

Relative Organ Weight

In toxicity studies, relative organ weight changes are sensitive indicators of toxicity, affecting enzymes, disrupting physiology, and causing target organ damage. An increase in organ weight indicates hypertrophy, while a decrease indicates target organ necrosis. Although relative organ weight provides useful signals of drug toxicity effects, these data must be interpreted in conjunction with gross pathology, clinical pathology, and histopathological findings [37]. The results of the current study showed no significant differences ($p > 0.05$) in relative organ weights of the liver, kidney, and stomach between the experimental groups and the control group (Table 6, Figure 4). This indicates that EtHC did not cause any harmful effects on the liver, kidneys, and stomach. The histopathological findings in Figure 6 also support these conclusions.

Table 6. Effect of EtHC on organ weight of Swiss albino mice in acute and sub-chronic toxicity testing

Acute toxicity test				
Organs	Control group	$EtHC_{1000}$	$EtHC_{1000}$	$EtHC_{1000}$
Stomach	0.17 ± 0.04	0.18 ± 0.04	0.19 ± 0.04	0.18 ± 0.04
Liver	1.15 ± 0.03	1.17 ± 0.07	1.16 ± 0.06	1.18 ± 0.03
Kidney	0.26 ± 0.03	0.25 ± 0.03	0.27 ± 0.02	0.26 ± 0.02
Sub-chronic toxicity test				
Organs	Control group	$EtHC_{1000}$	$EtHC_{1000}$	$EtHC_{1000}$
Stomach	0.21 ± 0.04	0.22 ± 0.04	0.21 ± 0.04	0.23 ± 0.05
Liver	1.21 ± 0.04	1.19 ± 0.03	1.22 ± 0.04	1.21 ± 0.03
Kidney	0.29 ± 0.03	0.28 ± 0.04	0.27 ± 0.03	0.29 ± 0.03

Values are expressed as Mean ± SD (n = 6). Values significantly different from the corresponding control group ($p < 0.05$) were marked with an asterisk.

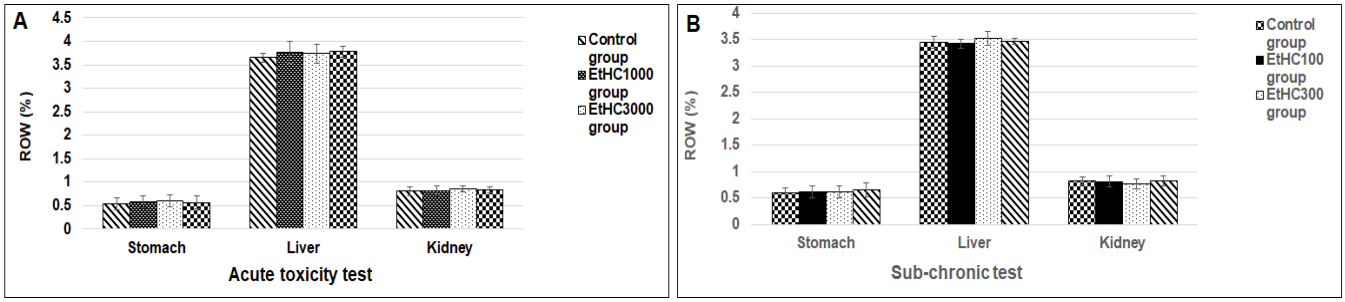

Figure 4. Relative organ weights were in the control and EtHC-treated groups in the acute and subchronic toxicity study (A. Relative organ weights in acute toxicity test; B. Relative organ weights in sub-chronic toxicity test). Values are presented as a percentage of mean ± SD, n = 6.

Macrostructure and Organ Histopathology

Observation of the major internal organs (liver, kidney, and stomach) of mice in the test and control groups showed that the organs were not damaged, and the structural images were within normal limits (Figure 5). There were no unusual color or external shape expressions of the liver (Figures 5B and 5C) and kidney (Figure 5F and 5G). The stomach mucosa was pale pink and showed no abnormality in structure (Figure 5I and 5K). There was no difference in the macroscopic structure of mice's liver, kidney, and stomach tissues in the test groups compared to the corresponding control group (Figure 5A, 5E, and 5H).

The results of histological observations of the organs showed no signs of abnormality in the liver, kidney, and stomach tissues' structural components related to the different dose levels of the test samples compared to the control (Figure 6). In the liver tissue, the hepatocytes were stained with clear nuclei with purple, and the extra-cellular components were stained pink. The hepatocyte structure was uniform, with a central vein, portal triad, and normal connective tissue. No differences were observed between the test groups (Figure 6B and 6C) and the control group (Figure 6A). In the kidney tissue, the glomeruli (capillaries, arterioles, and Bowman's capsule), tubules, renal pelvis, and interstitium were all normal, without any signs of damage. The renal cells were uniformly stained, with nuclei stained purple and extra-cellular components stained pink. No damage was observed in the test groups (Figure 6F and 6G), and no differences were observed compared to the control group (Figure 6E).

In the stomach, the mucosa layer consists of regular columnar epithelium with surface mucous cells (chief cells and parietal cells) secreting alkaline, viscous mucus that adheres tightly to the cell surface, forming gastric pits that penetrate into the surface epithelial layer and communicate with gastric glands. The gastric glands open at the base of the gastric pits. The submucosa layer comprises deep connective tissue containing blood vessels, capillaries, lymphocytes, and surrounding gastric glands. The muscularis mucosae layer consists of a thin smooth muscle layer, with circular fibers on the inner side and longitudinal fibers on the outer side, separating the submucosal layer from the underlying mucosa. The histological structure of the stomach was similar in the experimental group (Figure 6I and 6K) and the control group (Figure 6H).

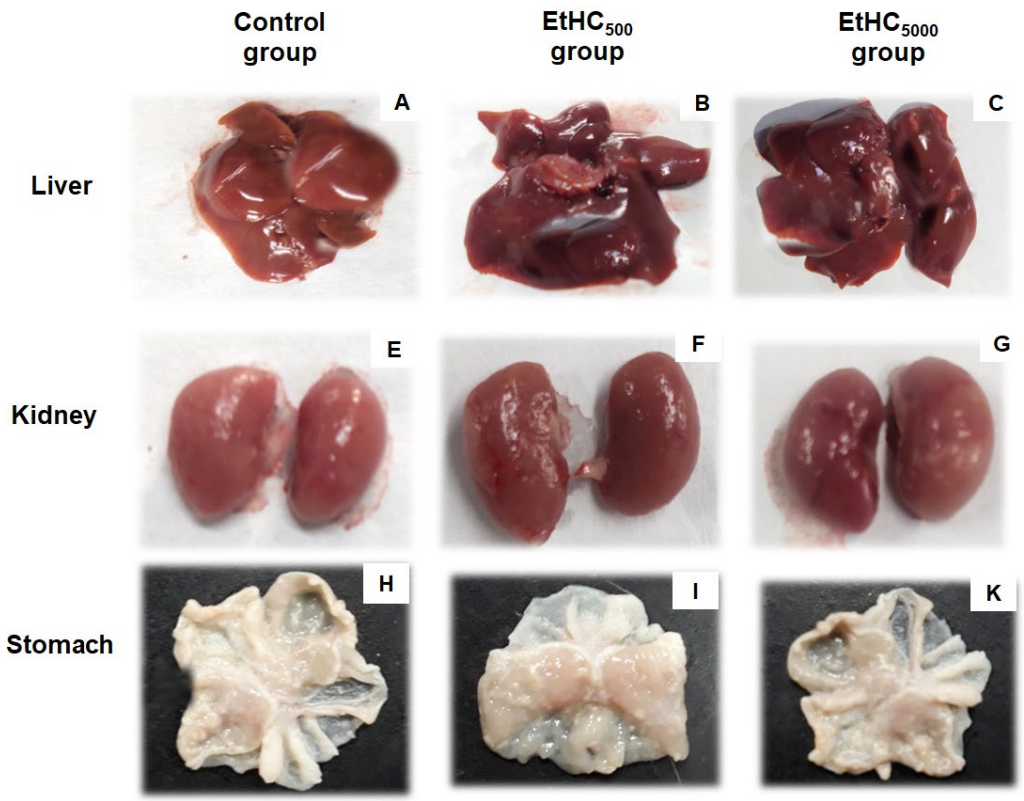

Figure 5. Macroscopic structure of liver, kidney, and stomach of mice in EtHC toxicity test.

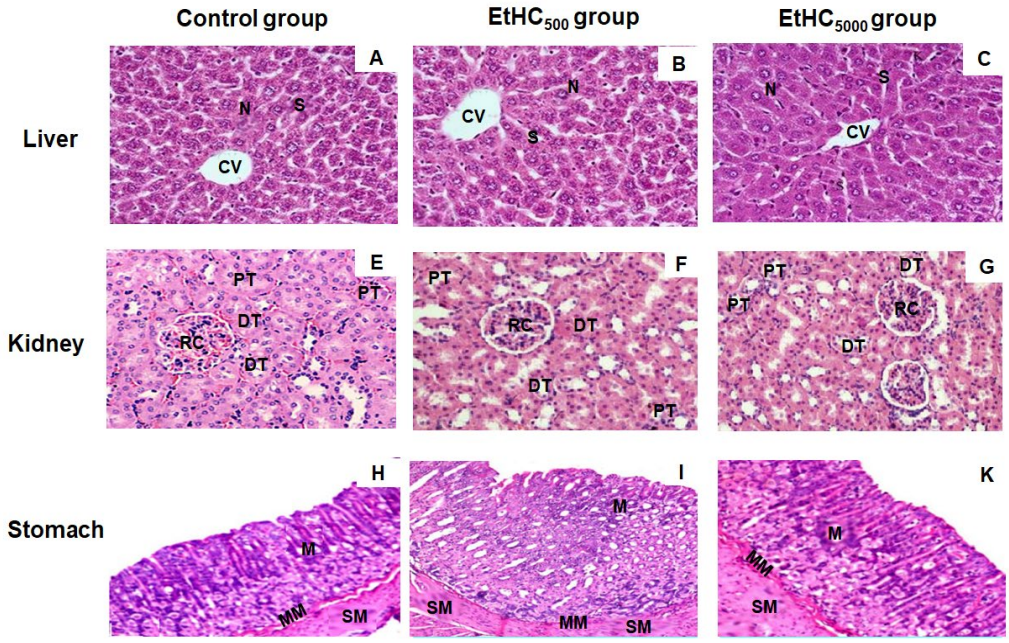

Figure 6. Histopathology of liver, kidney, and stomach (H&E staining, magnification 200) of mice in EtHC toxicity test. Liver tissue annotation: central vein (CV), hepatic sinusoid (S), and prominent nucleus (N). Renal tissue annotation: renal corpuscle (RC), proximal tubule (PT), and distal tubule (DT). Stomach tissue annotation: gastric mucosa (M), submucosa (SM), and muscular mucosa layer (MM).

CONCLUSION

The safety and efficacy of ethanol leaf extract of *H. capitellata* (EtHC) were confirmed through acute and sub-chronic oral toxicity evaluations following OECD guidelines 423 and 408, respectively. The animals' behavior, reactions, and experimental parameters were consistently monitored and stable throughout the 14-day acute toxicity and 90-day sub-chronic toxicity studies ($p < 0.05$), demonstrating the safety of EtHC in the test animals. With test doses of up to 5000 mg/kg EtHC (acute toxicity) and 500 mg/kg EtHC (sub-chronic toxicity), the interaction of the components within EtHC exhibited a wide safety margin in the experimental animals. The current study has demonstrated that the ethanol leaf extract of *H. capitellata* holds potential as a component for herbal products.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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