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Original Research Article

Assessment of the Acute and Chronic Toxicity Studies of Ethanol Extract of *Blumea balsamifera* (L.) DC. Leaves on Murine Models

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ABSTRACT

Blumea balsamifera (L.) DC. (Asteraceae) has been a traditional therapeutic method for centuries, yet limited research has addressed its potential toxicity. This study aims to evaluate the *in vivo* toxicity of ethanol extract from *Blumea balsamifera* leaves (EEBB) through acute and sub-chronic toxicity tests in mice. In the acute test, single doses of EEBB (1000, 3000, and 5000 mg/kg) were orally administered and monitored for 14 days, extended to 42 days (satellite treatments). Sub-chronic toxicity involved EEBB doses (100, 300, and 5000 mg/kg) administered for 90 days, with further monitoring up to day 118 (satellite treatments). Results, EEBB contained various phytochemical compounds, including alkaloids, flavonoids, tannins, and terpenoids, etc. No clinical abnormalities were observed in both acute and sub-chronic toxicity experiments. However, a significant increase in body weight and weight gain was consistently noted in the experiments ($p < 0.05$), and food and water consumption were affected upon EEBB exposure ($p < 0.05$). After an extended observation period (42 and 118 days), alterations in hematological parameters ($p > 0.05$), biochemical evaluations ($p > 0.05$), urine composition ($p > 0.05$), relative organ weights ($p > 0.05$), and tissue structure in the heart, liver, and kidneys of satellite treatments with EEBB returned to normal. Despite initial observations of some transient adverse effects, the body appeared to adapt over time, resulting in no significant long-term changes ($p < 0.05$). These findings suggest the safety and potential benefits of the *B. balsamifera* leaf extract in mitigating potential adverse effects associated with its use.

***Keywords*:** Acute toxicity, subchronic toxicity, mouse models, safety assessment, oral dosing

Introduction

In recent decades, plants have emerged as a valuable resource for the global field of medicine, serving in the research and development of widely used therapeutic drugs.[1] Most chemical compounds of plant origin exhibit biological activity, and these natural products, traditionally passed down through folk knowledge, have provided a robust foundation for the treatment of numerous diseases. This is substantiated and supported by credible scientific pieces of evidence.[2] Nonetheless, the collection of information or evidence related to the potential toxicity of medicinal plants for human consumers poses a significant challenge. This issue is complicated by numerous studies that have demonstrated that not all herbal remedies guarantee safety. They can induce harmful effects or adverse reactions in the organisms that use them. Furthermore, the identification of active compounds and the analysis of the side effects of herbal medicines are exceedingly complex.[3] Hence, the assessment of the toxicological effects of any herbal extract intended for use in animals or humans is an indispensable component in the evaluation of potential adverse effects.

Blumea balsamifera (L.) DC. (Asteraceae) has been employed as a traditional therapeutic method for millennia and boasts a rich historical tradition in several Southeast Asian countries such as China, Malaysia, Thailand, Vietnam, and the Philippines.

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Species within the Blumea genus are widely distributed across tropical regions of Asia, Africa, and the Pacific, with the highest diversity observed in the tropical Asian region. In recent decades, researchers have concentrated their efforts on studying the species within the Blumea genus that are used in components of the Ayurvedic traditional medical system in India and traditional beverages known as "Loloh".[4] In China, *B. balsamifera* is utilized as a fragrance due to its high essential oil content. Its entire plant or leaves are employed in traditional Chinese medicine to treat various conditions, including scabies, dermatitis, beriberi, back pain, menstrual pain, arthritis, skin injuries, and even in the formulation of insect repellents.[5] In Thailand, dried leaves of *B. balsamifera* can be used to make medicinal cigarettes to alleviate symptoms of sinusitis, abdominal pain, and cough, and can even be combined with other plants to create postpartum herbal baths for women. In the Philippines, *B. balsamifera* serves as a traditional remedy for kidney stones, common colds, and diuretic purposes. Furthermore, in other Asian countries such as Malaysia, *B. balsamifera* is also incorporated into Ayurvedic traditional medicine systems.[4]

While there have been some studies related to the pharmacological effects of *B. balsamifera*, the safety of ethanol extract from *Blumea balsamifera* leaves (EEBB) in vivo remains unconfirmed. Based on the traditional use of *B. balsamifera*, this study was conducted to comprehensively assess the acute and subchronic toxicity of EEBB on an animal model. The results of this research will provide a foundation for utilizing this extract in research and applications for the treatment of arthritis, pain relief, and cancer prevention.

Materials and Methods

Collection of plant material

The leaves of the *Blumea balsamifera* L. (DC.) plant were collected in Cam My district, Dong Nai province, Vietnam (Coordinates: 10°49'26.8"N 107°12'10.8"E), in April 2023. The plant specimen, labeled as BB110423VST, is currently held at the Plant Biotechnology

Laboratory of the Institute of Biotechnology and Food Technology, Ho Chi Minh City University of Industry, Vietnam. *B. balsamifera* leaves were thoroughly washed with distilled water, air-dried at room temperature, further dried in a Memmert (Germany) drying cabinet at 60°C, and then ground into a fine powder. The powder is stored in moisture-resistant bags for use in subsequent experiments.

Preparation extraction
Approximately 250 grams of leaf powder were soaked in 800 mL of 96% ethanol for 72 hours. Subsequently, this mixture was filtered through the Whatman No. 4 filter paper. The residue was then re-extracted under the same conditions until the extracting solvent became colorless. The resulting solution after filtration was further concentrated using a rotary evaporator R-II (BUCHI, Switzerland) at a speed of 40 revolutions per minute and a temperature of 65°C. The obtained extract solution (referred to as EEBB) was stored at 4°C until further detailed analysis was conducted.

Qualitative phytochemical analysis of ethanol extract B. balsamifera leaves
The ethanol extract from *B. balsamifera* leaves was used to quantitatively analyze essential plant chemical compounds, which include alkaloids, phenols, flavonoids, saponins, tannins, terpenoids, cardiac glycosides, steroids, phlobatanins, and anthraquinones. The experiments followed standard procedures established and described in the study by Nhung and Quoc.[6]

Preparation and treatment of experimental animals
Female Swiss albino mice, with an average weight of approximately 30 ± 1 grams, were utilized, and they were obtained from the Pasteur Institute of Ho Chi Minh City, Vietnam. The mice were housed in the animal facility at the East Agriculture and Food Company, Ho Chi Minh City, at a room temperature of 28 ± 2°C and a relative humidity of (50 ± 10%), following a 12-hour light/dark cycle. The cages were made of glass (dimensions: length × width × height = 60 × 30 × 30 cm), with six mice in each cage, and equipped with a stainless steel hanger attached to the cage wall for food and water. The mice were provided with standard rodent pellets and water. Before the experiments, the mice were acclimatized to the laboratory environment for 7 days. Throughout the study, we adhered to ethical principles related to animal care, as outlined in the Basel Declaration on Animal Research and complied with the Livestock Law in Vietnam (No. 32/2018/QH14).[7,8] Our experimental procedures followed the Guidelines for preclinical and clinical trials of oriental medicines and herbal medicines (Decision 141/QD-K2ĐT) in 2015 in Vietnam.[9] The utilization of laboratory animals adhered to the National Ethical Guidelines for Medical Research issued by the Ministry of Health of Vietnam.[10] All experimental animals were cared for and handled by trained personnel, adhering to ethical principles related to animal research, as regulated by the Ethics Committee for Animal Research at the Ho Chi Minh City University of Industry, Vietnam.

Acute toxicity testing
The acute toxicity tests were carried out following the OECD in 2021 with test no. 423 (OECD - Organisation for Economic Cooperation and Development) for oral acute toxicity testing.[11] Thirty-six healthy female Swiss albino mice (30 ± 1 g) were randomly divided into six treatments, each consisting of six mice. The mice's body weights were determined before the experiment. Each animal in the $EEBB_{1000}$, $EEBB_{3000}$, and $EEBB_{5000}$ treatments was administered a single dose of EEBB at 1000, 3000, and 5000 mg/kg body weight, respectively, and observed for 14 days. In the control treatment, animals received an equivalent volume of distilled water. The mice in the satellite control treatment and satellite $EEBB_{5000}$ treatment were given distilled water and EEBB at 5000 mg/kg (a single dose), respectively, and monitored for 42 days. Immediately after the extract administration, the mice were individually observed for 30 minutes. Subsequently, they were observed for clinical signs (changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, and central nervous system activity, behavior patterns such as tremors, convulsions, salivation, diarrhea, lethargy, and coma), pathological changes, and mortality rate within 24 hours on the first day, followed by daily observations for 14 and 42 days. The body weight of the mice was recorded before EEBB administration and weekly after treatment. The mice were euthanized at the end of the study by exposure to CO_2.[12] Relative organ weights, histopathology, hematological and biochemical parameters, and urine volume were collected, recorded, analyzed, and evaluated.

Sub-chronic toxicity study (90-day repeated dose)
The sub-chronic toxicity test with repeated doses for 90 days was conducted following OECD in 2018 with test no. 408.[13] Thirty-six healthy female Swiss albino mice weighing 30 ± 1 g were used for the subchronic toxicity evaluation. All mice were randomly allocated into six treatments, each consisting of six mice. The control treatment received distilled water at the same time. The $EEBB_{100}$, $EEBB_{300}$, and $EEBB_{500}$ treatments were administered corresponding doses of EEBB at 100, 300, and 500 mg/kg, respectively, continuously for 90 days. The mice in the satellite control treatment and satellite $EEBB_{500}$ treatment were given distilled water and EEBB at 500 mg/kg, respectively, for 90 continuous days, followed by 28 days of rest without treatment. All animals were daily observed for responses, behaviors, clinical signs, and mortality until the end of the experiment. Body weight, food, and water consumption, as well as urine volume, were recorded weekly. At the end of the study, the mice were euthanized by exposure to CO_2.[12] The organs (heart, liver, and kidney) were excised and carefully dissected, weighed, and macroscopically examined. Relative organ weights, hematological and biochemical analysis, urine analysis, and histopathological examination of the organs were conducted.

Clinical observations
Observations regarding mortality rates, changes in appearance, expressions, and behaviors (including phenomena such as trembling, convulsions, urination, and diarrhea), clinical signs (including alterations in skin and fur, eyes and mucous membranes, respiratory, circulatory, and nervous system activities), as well as any injuries or pathological symptoms, were conducted on each mouse within 30 minutes after the administration of the substances and continuously monitored for 24 hours. Subsequently, the mice were observed daily for 14 and 90 days. The satellite treatments were further observed for an additional 28 days without further treatment.

Body weight assessment
The body weight of all animals was measured using an electronic scale (Sartorius, Germany) and data was recorded on day 0 before their exposure to EEBB. Throughout the experiment, the body weight of the mice was measured weekly. The percentage weight gain (WG%) was calculated using the following formula:[12]

$$\text{WG (\%)} = \frac{\text{Final body weight (g)} - \text{Initial body weight (g)}}{\text{Initial body weight (g)}} \times 100$$

Food and water consumption
The data regarding food and water intake were recorded before being provided to the mice. All remaining food and water were collected and weighed at the end of each day. The daily food and water intake was calculated using the following formula:[12]
Food consumption (g) = Initial food consumption (g) - Remaining food consumption (g)
Water intake (mL) = Initial water intake (mL) - Remaining water intake (mL)

Hematological and biochemical parameters
Hematological and biochemical analyses were conducted on all surviving mice at the end of the experiment. Blood was collected via retro-orbital puncture. A blood sample was transferred to EDTA-containing tubes. Hematological analysis was performed using an automated veterinary hematology analyzer (VET 2800, Mindray - Brazil) to assess red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), platelet count (PLT), and white blood cell count (WBC). The remaining blood samples were transferred to non-anticoagulant tubes and allowed to stand at room temperature for 45 minutes, after which they were centrifuged at 3500 revolutions per minute for 10 minutes. Serum from each sample was collected and

stored in frozen tubes at -80°C until further analysis. Total protein (PT), glucose (GLU), triglycerides (TRI), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), uric acid (UA), blood urea nitrogen (BUN), and creatinine (CRE) were evaluated using the LabMax Progress device (Labtest - Brazil) and Lab test equipment.

Assessment of relative organ weight

On the final day of the experiment, all mice were humanely euthanized by exposure to CO_2 gas.[14] Subsequently, the animals were dissected, and a macroscopic examination of internal organs such as the heart, liver, and kidneys was conducted. These organs were observed comprehensively to detect any abnormalities and assess the presence of lesions. Following this, the internal organs were cleaned of excess fat and weighed to determine their absolute weights. The relative organ weight (ROW) was calculated using the formula:[12]

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

Assessment of morphology and histopathology of organs

Following the surgery, the heart, liver, and kidneys were collected and subjected to morphological and color analysis. Subsequently, they were fixed in a 10% formalin solution, embedded in paraffin, and sectioned into thin slices with a thickness of 5 µm, followed by salt removal, hydration, and staining with hematoxylin and eosin (H&E). Histological analysis was performed using a light microscope (Olympus BX53, Japan) with a magnification level set at 200×.

Urinalysis

Every weekend during the experiment, each mouse was individually placed in a glass cage. In this case, the mice experienced overnight fasting but were still provided with water. Urine samples from each treatment of animals were simultaneously collected for the assessment of specific gravity, pH, ketone, urobilinogen, occult blood, as well as the concentration of ions including Na^+, K^+, and Cl^-. These parameters were determined using an automated urine analyzer (MSLUA10, China).

Statistical analysis

The results are presented as mean values ± standard deviation. Differences between the treatments were determined using one-way analysis of variance (ANOVA) followed by Fisher's LSD test, utilizing Stagraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, Virginia, USA), with the statistical significance threshold set at $p < 0.05$.

Results and Discussion

Qualitative phytochemical analysis

After conducting a preliminary analysis of the plant chemical composition of EEBB, we identified the presence of compounds such as alkaloids, phenols, flavonoids, saponins, tannins, terpenoids, cardiac glycosides, steroids, while the absence of phlobatanins and anthraquinones (Table 1).

The assessment of the toxicity of medicinal plants is a crucial step in verifying their toxicity and ensuring the safety of potential herbal remedies. Although *B. balsamifera* has been widely used in the traditional treatment of various diseases, there is currently a scarcity of experimental data regarding its safety. This study was conducted to evaluate the acute and sub-chronic toxicity of *B. balsamifera* in an animal model. Phytochemical compounds are often recognized for their preventive and therapeutic effects on various diseases. However, the application of many of these compounds in disease treatment is frequently limited by poor absorption and potential toxicity.[15] The extract of *B. balsamifera*, containing various phytochemical compounds, has been used in traditional medicine. Alkaloids can affect the nervous system and provide pain relief, phenols have antioxidative, antibacterial, and anti-inflammatory effects, flavonoids protect cells from oxidative damage, saponins reduce cholesterol, tannins, terpenoids, and steroids exhibit anti-inflammatory, antibacterial, and immune-regulatory properties.[16] Nevertheless, incorrect use and dosage can lead to adverse effects, and in some cases, toxicity. Alkaloids, for example, may lead to addiction and have negative effects on the nervous system, phenols and flavonoids may negatively affect digestion, saponins and tannins may interfere with nutrient absorption, and terpenoids may be toxic to the liver and nervous system.[17] EEBB may contain chemical toxicity due to the presence of phytochemical constituents in the extract, and adverse effects may occur if the extract is not used properly and in excessive quantities beyond regulated limits. Therefore, the assessment of EEBB toxicity before pharmacological research and clinical applications is necessary and mandatory. Identifying plant-derived chemical compounds in EEBB also contributes to toxicity assessment, exposure duration control, determining potential extract effects, and understanding the impact of bioactive phytochemical compounds in EEBB for their appropriate and regulated usage.[12]

Clinical assessment

In the acute toxicity test, the use of ethanol extract from *B. balsamifera* leaves at doses of 1000, 3000, and 5000 mg/kg in female Swiss albino mice for 14 and 42 days (satellite treatments) did not result in any abnormal behaviors or alterations in food consumption. Morphological observations did not reveal any changes in the skin, fur, eye membranes, behavioral patterns, trembling, salivary secretion, diarrhea, respiratory activity, circulatory system, or nervous system activity in the mice. No instances of mortality were recorded at the tested doses, and the body weight of the mice remained unaffected. Furthermore, no abnormal signs were observed in the overall activity of mice using the highest dose of 5000 mg/kg.

For the sub-chronic experiment, the administration of ethanol extract derived from the leaves of *B. balsamifera* at different doses (100, 300, and 500 mg/kg) to mice over 90 days, followed by an additional 28-day observational phase (within the satellite treatments) without any further treatment, yielded results indicating the complete absence of any anomalous signs related to either behavior or physical condition. All subjects displayed normal responses to handling, and no changes were observed in terms of salivation, fur appearance, eye color, sleep patterns, salivary secretion, bowel movements, respiration, circulatory parameters, or nervous system activity. There were no deviations from the norm regarding motor function, convulsions, or tremors amongst the experimental mice. Importantly, all mice survived until the culmination of the experiment.

Body weight changes

Table 2 and Figure 1 illustrate the changes in body weight and weight gain observed in mice during the acute toxicity test. The subjects were exposed to both the control treatment and various doses of EEBB over 14 and 28 days (during satellite treatments). Both body weight and the percentage of weight gain exhibited a progressive increase from the initial day, reaching statistical significance after 7 days and 14 days within both the control treatment and the EEBB treatments at dosages of 1000, 3000, and 5000 mg/kg ($p < 0.05$). Notably, a substantial difference was observed in both body weight and weight gain between the treatments receiving EEBB treatments and the control treatment ($p < 0.05$).

Table 1: Preliminary qualitative phytochemical analysis of the ethanol extract of *B. balsamifera* leaves

Compounds	EEBB	Compounds	EEBB
Alkaloids	+	Terpenoids	+
Flavonoids	+	Steroids	+
Saponins	+	Cardiac glycosides	+
Phenols	+	Phlobatanins	-
Tannins	+	Anthraquinones	-

(+) Present in EEBB; (-) Not present in EEBB

Table 2: The weekly changes in body weight during the acute toxicity study

Days/ Treatments	Treatments for the acute toxicity study				Satellite treatments for the acute toxicity study	
	Control treatment	$EEBB_{1000}$ treatment	$EEBB_{3000}$ treatment	$EEBB_{5000}$ treatment	Satellite control treatment	Satellite $EEBB_{5000}$ treatment
"0"	29.86 ± 0.06^{a}	30.14 ± 0.03^{b}	29.91 ± 0.04^{a}	30.25 ± 0.03^{c}	30.22 ± 0.05^{c}	30.14 ± 0.03^{b}
7 days	30.29 ± 0.03^{a}	30.52 ± 0.04^{c}	30.31 ± 0.05^{a}	30.62 ± 0.07^{d}	30.52 ± 0.05^{c}	30.44 ± 0.04^{b}
14 days	30.91 ± 0.03^{a}	31.17 ± 0.03^{c}	30.92 ± 0.06^{a}	31.23 ± 0.07^{d}	31.15 ± 0.03^{c}	31.07 ± 0.02^{b}
42 days	-	-	-	-	39.79 ± 0.04^{a}	40.19 ± 0.04^{a}

Values are expressed as Mean ± SD, letters (a, b, and c) represent the difference between treatments ($p < 0.05$).

However, by day 42, the body weight and percentage of weight gain in the satellite $EEBB_{5000}$ treatment did not differ significantly from those in the satellite control treatment ($p > 0.05$).

The data presented in Table 3 and Figure 2 demonstrate substantial alterations in the body weight and weight gain of the mice involved in the sub-chronic toxicity test. Across all treatment regimens, encompassing both the control and EEBB treatments over 90 days, extending to 118 days in the satellite treatment, there was a consistent upward trend in body weight during the treatment and observation periods. The body weight and the percentage of weight gain consistently exhibited noteworthy increases commencing from day 0, with statistically significant disparities emerging after 30, 60, and 90 days in both the control treatment and EEBB treatments at doses of 100, 300, and 500 mg/kg ($p < 0.05$). Significantly, substantial differences in both body weight and weight gain were evident between the EEBB treatments and control treatment ($p < 0.05$). Nevertheless, on the 118th day, the body weight and the percentage of weight gain in the satellite $EEBB_{500}$ treatment did not display substantial alterations compared to the satellite control treatment ($p > 0.05$).

The rate of body weight gain in mice after exposure to *B. balsamifera* leaf extract is used as an indicator to assess the overall health status of the animals. Body weight gain is also related to the accumulation of muscle, fat, and fluids in the body.[18] The conversion of food into energy and substances in the body leads to metabolic activity and body development, facilitating growth.[19] In the extraction of *B. balsamifera* leaves, the presence of saponin, a plant compound, has been identified. Saponin enhances cell permeability, stimulates the active transport of mucosal cells in the intestine, creating favorable conditions for nutrient absorption. Saponin further promotes the protein synthesis process while inhibiting protein breakdown. Moreover, it increases the absorption of vitamins and minerals in the intestine. Polyphenols, a type of biologically active compound, affect the composition of the gut microbiota and lipid metabolism in animal bodies. Furthermore, the gut microbiota converts polyphenols into biologically active molecules, enhancing the biological absorption of lipids.[20] In this study, body weight and the percentage of weight gain consistently demonstrated significant increases in both experimental models throughout the experiment. This suggests that EEBB has a positive effect on body weight and weight gain in the animals participating in the experiment. This may imply that EEBB has the potential to positively influence weight gain or body growth, possibly as a result of the effects of the components or chemical compounds present in the extract.

Food and water intake

Table 4 presents a comprehensive overview of the dietary patterns observed in mice exposed to different doses of EEBB during acute toxicity tests. Among these observations, the treatment subjected to the highest EEBB dosage (5000 mg/kg) exhibited a minor decrease in food consumption. Nevertheless, it is noteworthy that this change did not reach statistical significance when compared to the control treatment ($p > 0.05$). Remarkably, after a 14-day before EEBB treatment (3000 mg/kg), a significant surge in food consumption was observed in the subjects, in stark contrast to the control treatment ($p < 0.05$). In the satellite control treatments, food consumption remained relatively unaltered and exhibited no statistically significant variance ($p > 0.05$). By the 42nd day, the food consumption in the satellite treatments had regressed to levels akin to the control treatment ($p > 0.05$). Water intake displayed a diminishing trend throughout the study (Table 4, $p < 0.05$). Within the first 14 days of observation, the EEBB showcased a significant decrease in water consumption compared to the control treatment ($p < 0.05$). However, the satellite treatments, which were monitored for an extended 42-day period, experienced a noteworthy escalation in water consumption relative to the EEBB treatments ($p < 0.05$), ultimately aligning with the levels seen in the control treatment ($p > 0.05$).

Table 5 provides an in-depth examination of the dietary patterns and water intake behaviors exhibited by both the satellite treatments and the subjects involved in the sub-chronic toxicity tests. The findings reveal a significant reduction in both food and water intake within the EEBB treatment (100, 300, and 500 mg/kg) compared to the control treatment ($p < 0.05$).

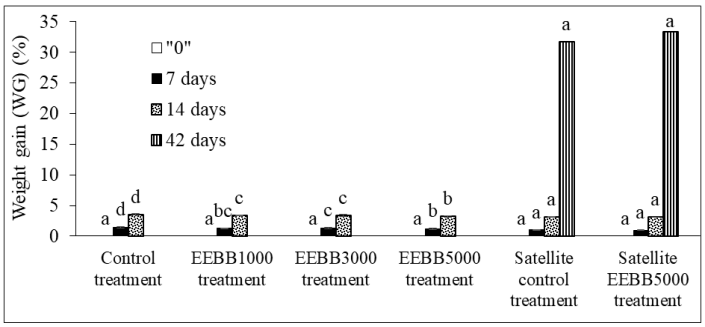

Figure 1: The influence of *B. balsamifera* leaf extract on the percentage of body weight gain in mice in the acute toxicity study is presented. Values are expressed as Mean ± SD, with letters (a, b, c, and d) indicating significant differences between treatments ($p < 0.05$).

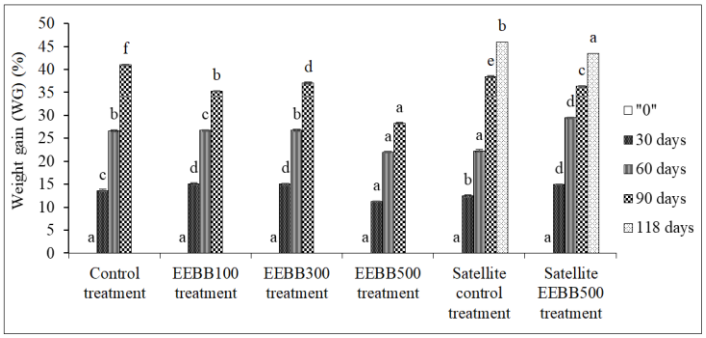

Figure 2: The influence of *B. balsamifera* leaf extract on the percentage of weight gain in mice during the sub-chronic toxicity study (90-day repeated dose) is presented. The values are expressed as Mean ± SD, and letters (a, b, c, d, e and f) denote significant differences between the treatments ($p < 0.05$).

Table 3: The weekly body weight fluctuations in the sub-chronic toxicity study (90-day repeated dose study)

Days/ Treatments	Treatments for the sub-chronic toxicity study				Satellite treatments for the sub-chronic toxicity study	
	Control treatment	$EEBB_{100}$ treatment	$EEBB_{300}$ treatment	$EEBB_{500}$ treatment	Satellite control treatment	Satellite $EEBB_{500}$ treatment
"0"	30.27 ± 0.05^{b}	29.75 ± 0.04^{ab}	29.56 ± 0.04^{a}	30.14 ± 0.07^{c}	29.85 ± 0.06^{b}	30.22 ± 0.07^{c}
30 days	34.39 ± 0.06^{d}	34.24 ± 0.06^{c}	33.99 ± 0.08^{b}	33.52 ± 0.08^{a}	33.55 ± 0.04^{a}	34.73 ± 0.06^{e}
60 days	38.35 ± 0.08^{b}	37.71 ± 0.07^{ab}	37.45 ± 0.06^{ab}	36.77 ± 0.05^{a}	36.49 ± 0.05^{a}	39.12 ± 0.08^{d}
90 days	42.67 ± 0.04^{e}	40.25 ± 0.05^{b}	40.51 ± 0.06^{c}	38.66 ± 0.04^{a}	41.33 ± 0.05^{d}	41.19 ± 0.04^{d}
118 days					43.55 ± 0.04^{b}	43.38 ± 0.05^{a}

Values are expressed as Mean ± SD, letters (a, b, c, d, and e) represent the difference between treatments ($p < 0.05$).

Table 4: Changes in food and water intake in the acute toxicity study

Days/ Treatments	Treatments for the acute toxicity study				Satellite treatments for the acute toxicity study	
	Control treatment	$EEBB_{1000}$ treatment	$EEBB_{3000}$ treatment	$EEBB_{5000}$ treatment	Satellite control treatmen	Satellite $EEBB_{5000}$ treatment
Total food consumption (g)	5.87 ± 0.05^{ab}	5.93 ± 0.04^{b}	5.85 ± 0.05^{a}	5.81 ± 0.08^{a}	5.88 ± 0.05^{a}	5.89 ± 0.04^{a}
Total water intake (mL)	7.74 ± 0.06^{d}	7.51 ± 0.08^{c}	7.33 ± 0.06^{b}	6.92 ± 0.06^{a}	7.75 ± 0.05^{a}	7.76 ± 0.04^{a}

Values are expressed as Mean ± SD, letters (a, b, c, and d) represent the difference between treatments ($p < 0.05$).

Table 5: The weekly changes in food and water intake in the sub-chronic toxicity study (90-day repeated dose study)

Parameters	Treatments for the sub-chronic toxicity study				Satellite treatments for the sub-chronic toxicity study	
	Control treatment	$EEBB_{100}$ treatment	$EEBB_{300}$ treatment	$EEBB_{500}$ treatment	Satellite control treatmen	Satellite $EEBB_{500}$ treatment
Total food consumption (g)	8.11 ± 0.08^{b}	8.03 ± 0.05^{a}	8.09 ± 0.07^{ab}	8.05 ± 0.04^{ab}	12.57 ± 0.07^{a}	12.73 ± 0.06^{a}
Total water intake (mL)	10.95 ± 0.05^{c}	10.63 ± 0.06^{b}	10.23 ± 0.07^{b}	9.95 ± 0.03^{a}	9.91 ± 0.07^{a}	10.02 ± 0.06^{a}

Values are expressed as Mean ± SD, letters (a, b, and c) represent the difference between treatments ($p < 0.05$).

Notably, the satellite treatments exhibited no significant deviations in terms of food and water intake when comparing the satellite control treatment with the satellite $EEBB_{500}$ treatment ($p > 0.05$).

The amount of food and water consumed by experimental animals is used to assess overall health, metabolic processes, energy balance, and the accumulation of substances within the body.[18] Food and water intake is regulated through a complex biological mechanism to ensure long-term body weight stability. Feelings of hunger and thirst play a crucial role in body weight regulation. The glycosides present in EEBB have stimulated appetite by increasing ATP production in the hypothalamic nerve cells, leading to the stimulation of the feeding center and enhancing feeding reflexes in animals.[21] Increased food and water consumption has contributed to the body weight gain in experimental mice.[18] The results regarding food and water intake in this study have shown that EEBB significantly affects the consumption of these substances and becomes pronounced after a specified period. EEBB can influence the dietary habits of mice, and this effect is evident in both acute and sub-chronic experiments. However, in both experimental models, the satellite treatment treatments did not exhibit significant changes in food consumption compared to the satellite control treatments. Nevertheless, in the acute toxicity test, water consumption increased significantly in the satellite treatment after a period of discontinuation of EEBB use.

Hematological and blood biochemistry analysis

Table 6 is dedicated to the presentation of hematological and biochemical analysis results concerning the acute toxicity study of EEBB in mice. Upon oral administration of EEBB at doses of 1000, 3000, and 5000 mg/kg, there was a noteworthy escalation ($p < 0.05$) in key hematological parameters, such as RBC, HGB, HCT, WBC, and PLT, as well as liver function parameters, including GLU and ALT, and kidney function parameters, such as BUN and CRE, in the $EEBB_{1000\text{-}5000}$ treatments when compared to the control treatment ($p < 0.05$). Nevertheless, the data reveals the absence of significant disparities ($p > 0.05$) in the hematological and biochemical analysis results between the satellite $EEBB_{5000}$ treatment treatment and the satellite control treatment after a 42-day observation period.

In Table 7, an extensive analysis of hematological and biochemical parameters in the sub-chronic toxicity study, involving a 90-day repeated dose of EEBB, is presented. The findings exhibit significant variations ($p < 0.05$) in these parameters among treatments. Notable distinctions are observed in the values of RBC, WBC, PLT, PT, GLU, AST, and ALT in the mice subjected to EEBB doses ranging from 100 to 500 mg/kg, in contrast to the control treatment ($p < 0.05$). All treatments supplemented with various EEBB doses showcased a significant increase in RBC, WBC, PLT, PT, GLU, AST, and ALT values when compared to the control treatment ($p < 0.05$). Interestingly, parameters associated with kidney function, including sodium, potassium, chloride, urea, creatinine, BUN, and uric acid, remained relatively stable in the test treatment, not exhibiting significant changes when compared to the control treatment ($p < 0.05$). Conversely, parameters linked to liver function, such as total protein, albumin, triglycerides, ALT, and AST, exhibited considerable alterations compared to the control treatment ($p < 0.05$). Intriguingly, no significant deviations were observed in hematological parameters, kidney parameters, or liver parameters within the satellite $EEBB_{500}$ treatment following an extensive 118-day observation period ($p > 0.05$).

Hematopoiesis is the process of differentiation of stem cells into mature blood cells. Alterations in the hematopoietic system provide a better understanding of the risk of toxicity in animals. Terpenoids and glycosides can modulate the immune system and possess antibacterial, anti-inflammatory, and antioxidant properties. Polyphenols in EEBB enhance gut health through the Akt/mTOR pathway and microRNA. Liver and kidney functions can be assessed by analyzing serum biochemical parameters.[18]ALT and AST reflect the integrity, while total protein describes the liver cell function. AST and ALT are produced by liver cells, and any toxic or foreign substance entering the liver increases the serum levels of AST and ALT. Elevated serum AST and ALT levels are indicative of liver toxicity, while a decrease suggests inhibition of liver enzymes.[18] Alkaloids and flavonoids are bioactive compounds that protect the liver by stimulating RNA polymerase enzyme activity, enhancing DNA synthesis, and regenerating damaged liver cells due to toxicity. Flavonoids exhibit a wide range of pharmacological activities, including antioxidant, anti-inflammatory,

and anti-apoptotic properties. Several polyphenolic compounds are effective in mitigating harmful effects on the kidneys.[22] In the current study, the administration of EEBB exerted a substantial impact on hematological and biochemical parameters. EEBB was found to induce alterations in hematological parameters and assess liver function in mice, with pronounced effects evident at doses administered in both acute and sub-chronic studies. However, kidney function remained relatively stable in both cases. Interestingly, the data revealed no significant differences ($p > 0.05$) in the hematological and biochemical analysis results between the satellite EEBB treatment and the satellite control treatment after 42 and 118 days of observation.

Relative organ weight

Figure 3 presents the outcomes of the relative organ weights (ROW) concerning the heart, liver, and kidneys in the acute toxicity investigation. During the initial 14-day period of the trial, noteworthy distinctions emerged in the ROW of the heart, liver, and kidneys within the $EEBB_{1000\text{-}5000}$ treatment as compared to the control treatment ($p < 0.05$). Nevertheless, both the $EEBB_{1000\text{-}5000}$ treatment and control treatment experienced a marked reduction in the ROW of these organs ($p < 0.05$), and after 118 days of monitoring, there were no significant discrepancies in ROW between the $EEBB_{1000\text{-}5000}$ treatment and the control treatment ($p < 0.05$).

Figure 4 displays the relative organ weights in both the sub-chronic toxicity and satellite treatments. The relative weights of the heart, liver, and kidneys exhibited a marginal increase within the $EEBB_{100\text{-}500}$ treatments when compared to the control treatment ($p < 0.05$). Evaluation of the satellite treatment treatments revealed that there were no statistically significant deviations in the relative organ weights between the satellite $EEBB_{500}$ treatment and satellite control treatment ($p > 0.05$).

When studying the toxicity of plant extracts, changes in the relative organ weights (ROW) of vital organs play a critical role as a sensitive indicator of potential toxicity, physiological disturbances, and organ damage within the body.[22] Recent studies have demonstrated that bioactive molecules, especially phenolic compounds, exhibit antioxidant effects against organ toxicity. Phenolic compounds present in plant extracts have the ability to safeguard and counteract liver and kidney toxicity by improving oxidative stress conditions, inhibiting inflammation, and apoptosis processes.[23] Polyphenols regulate immune responses and suppress the synthesis of pro-inflammatory cytokines and gene expression. They deactivate NF-κB, modulate protein kinase activation pathways, and prevent the expression of TLR receptors and inflammatory genes.[24] In both acute and sub-chronic toxicity experiments, there was a slight yet statistically significant increase in the ROW of the heart, liver, and kidneys among the EEBB treatments compared to the control treatment ($p < 0.05$). This suggests a positive impact of EEBB at various dosage levels. However, the use of EEBB did not result in long-term effects on the ROW of these vital organs and the stability of organ weights was maintained even after the cessation of EEBB treatment.

Histopathological analysis

The examination of the macroscopic characteristics of the heart, liver, and kidneys in the acute toxicity test animals treated with EEBB did not reveal any anomalies in terms of color or structure when compared to the corresponding organs in the control treatment (Figure 5A). The anatomical morphology of the heart, liver, and kidneys in the control treatment, $EEBB_{5000}$ treatment, and satellite $EEBB_{5000}$ treatment all displayed favorable attributes. These organs exhibited a consistent deep red hue, uniform structure, smooth surfaces, soft tissue texture, and elasticity. No indications of swelling, indentations, or the presence of hemorrhagic spots were discerned. These findings suggest that there were no significant impairments or substantial morphological alterations in these vital organs subsequent to EEBB treatment. Histological examination of the liver from the control treatment revealed intact Kupffer cells and liver cells (Figure 5Bd). Similar normal features were also observed in the liver sections of animals from the satellite treatment receiving the highest dose (satellite $EEBB_{5000}$) (Figure 5Bf). Conversely, animals treated with EEBB at 5000 mg/kg showed alterations in liver cells and Kupffer cells (Figure 5Be), indicating that the liver was undergoing stress due to the extract's effects, and the hepatic immune system was reacting to the extract's influence. Regarding the histological features of the kidneys, renal corpuscles and Bowman's capsules were normal in the control treatment (Figure 5Bg) and the satellite $EEBB_{5000}$ treatment (Figure 5Bi). In contrast, kidney sections from animals exposed to EEBB (5000 mg/kg) exhibited significant changes in the form of an expansion of the renal space and the formation of nonuniform Bowman's capsules (Figure 5Bh). This suggests that EEBB may affect the overall function of the kidneys. The adjustments to the space and structure in the satellite $EEBB_{5000}$ treatment contribute to maintaining water and electrolyte balance in the body. Histological analysis of the heart did not reveal any pronounced changes in the control treatment (Figure 5Ba) and the satellite $EEBB_{5000}$ treatment (Figure 5Bc), with all showing normal structures in the cardiac muscle, connective tissue, and nucleus. There was, however, dilation and alterations in muscle fiber structure in the cardiac muscle of the $EEBB_{5000}$ treatment (Figure 5Bb). This indicates that EEBB stimulated heart activity to enhance blood circulation.

The evaluation of the anatomical morphology of the heart, liver, and kidneys in the sub-chronic toxicity test participants revealed no deviations in terms of color or structure when compared to the corresponding organs in the control treatment (refer to Figure 6A). Of notable importance, the anatomical characteristics of the heart, liver, and kidneys in the control treatment, $EEBB_{500}$ treatment, and satellite $EEBB_{500}$ treatment all exhibited positive attributes.

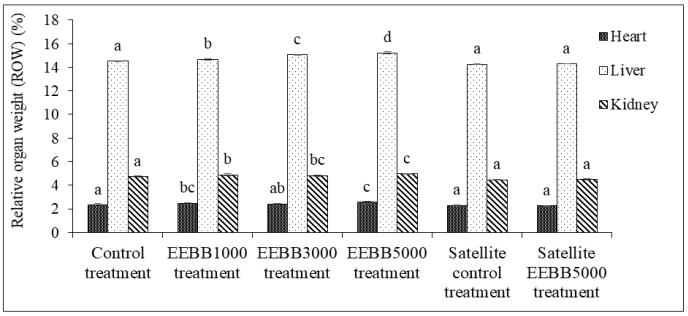

Figure 3: The effect of *B. balsamifera* leaf extract on the relative organ weight in mice in the acute toxicity study is depicted. The values are expressed as Mean ± SD, with letters (a, b, c, and d) denoting significant differences between treatments ($p < 0.05$).

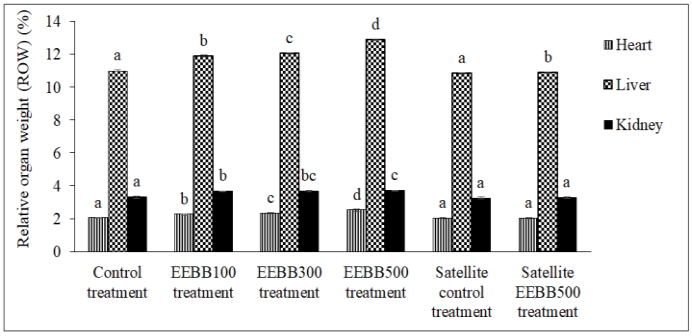

Figure 4: The impact of *B. balsamifera* leaf extract on the relative organs weight of mice in the sub-chronic (90-day repeated dose) toxicity study. Values are expressed as Mean ± SD, letters (a, b, c, and d) represent the difference between treatments ($p < 0.05$).

Table 6: Changes in hematological and blood biochemistry analysis in the acute toxicity study

Days/ Treatments	Treatments for the acute toxicity study				Satellite treatments for the acute toxicity study	
	Control treatment	$EEBB_{1000}$ treatment	$EEBB_{3000}$ treatment	$EEBB_{5000}$ treatment	Satellite control treatmen	Satellite $EEBB_{5000}$ treatment
RBC ($\times10^6$ cells/mm^3)	8.42 ± 0.08^a	8.51 ± 0.09^{ab}	8.59 ± 0.07^{bc}	8.68 ± 0.07^c	8.44 ± 0.07^a	8.43 ± 0.08^a
HGB (g/dL)	13.98 ± 0.04^b	13.89 ± 0.07^a	14.09 ± 0.07^c	14.17 ± 0.06^d	13.99 ± 0.05^a	14.02 ± 0.05^a
HCT (%)	0.45 ± 0.06^a	0.54 ± 0.07^b	0.63 ± 0.06^c	0.71 ± 0.07^d	0.47 ± 0.04^a	0.49 ± 0.02^a
WBC ($\times10^3$ cells/mm^3)	3.53 ± 0.06^a	3.69 ± 0.08^b	3.85 ± 0.05^c	3.99 ± 0.07^c	3.55 ± 0.04^a	3.58 ± 0.04^a
PLT ($\times10^3$ cells/mm^3)	665.77 ± 15.72^a	748.05 ± 14.59^c	731.58 ± 13.13^{bc}	719.03 ± 18.12^b	672.43 ± 6.14^a	679.09 ± 5.12^a
PT (g/dL)	4.64 ± 0.07^a	5.13 ± 0.06^c	4.87 ± 0.05^b	5.34 ± 0.05^d	4.68 ± 0.04^a	4.73 ± 0.05^a
GLU (mg/dL)	5.59 ± 0.06^a	5.98 ± 0.08^d	5.87 ± 0.06^c	5.71 ± 0.08^b	5.63 ± 0.03^a	5.67 ± 0.05^a
TRI (mg/dL)	121.37 ± 20.71^a	117.09 ± 16.31^a	121.69 ± 17.52^a	152.68 ± 93.91^a	120.26 ± 9.37^a	123.37 ± 8.15^a
AST (U/L)	91.05 ± 1.97^a	98.16 ± 1.91^b	94.29 ± 4.93^{ab}	103.71 ± 3.13^c	92.24 ± 1.1^a	93.32 ± 0.9^a
ALT (U/L)	59.43 ± 2.26^a	64.78 ± 1.92^d	61.21 ± 2.37^b	62.99 ± 2.16^c	60.47 ± 1.22^a	60.76 ± 1.22^a
ALP (U/L)	122.34 ± 10.82^a	118.78 ± 9.76^a	126.01 ± 8.52^a	129.68 ± 8.82^a	123.39 ± 7.19^a	124.42 ± 4.98^a
UA (mg/dL)	12.91 ± 0.93^a	13.68 ± 0.64^a	13.17 ± 0.63^a	13.42 ± 0.68^a	12.98 ± 0.83^a	13.04 ± 0.75^a
BUN (mg/dL)	12.46 ± 0.98^a	12.71 ± 1.02^b	13.21 ± 0.92^d	12.96 ± 0.85^c	12.49 ± 0.94^a	12.57 ± 0.85^a
CRE (mg/dL)	0.39 ± 0.02^a	0.42 ± 0.01^b	0.43 ± 0.02^b	0.41 ± 0.02^{ab}	0.39 ± 0.03^a	0.4 ± 0.02^a

Values are expressed as Mean ± SD, letters (a, b, c, and d) represent the difference between treatments ($p < 0.05$). Note: Red blood cells (RBC), Hemoglobin (HGB), Hematocrit (HCT), White blood cells (WBC), Platelet (PLT), Total protein (PT), Glucose (GLU), Triglyceride (TRI), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Uric acid (UA), Blood urea nitrogen (BUN), Creatinine (CRE).

These organs manifested a consistent deep red shade, even structural features, smooth external surfaces, soft tissue composition, and elasticity. No signs of enlargement, depressions, or the presence of hemorrhagic lesions were evident. Consequently, it can be inferred that the EEBB treatment process did not induce any notable harm or significant morphological modifications in these critical organs. Histological assessment of the heart displayed no noticeable changes in the control treatment (Figure 6Ba) and the satellite $EEBB_{500}$ treatment (Figure 6Bc), with all specimens exhibiting standard structures in the cardiac muscle, connective tissue, and nucleus. However, in the cardiac muscle of the $EEBB_{500}$ treatment (Figure 6Bb), there was evidence of expansion and alterations in muscle fiber structure. These findings imply that EEBB serves to stimulate cardiac activity, leading to enhanced blood circulation. In the liver, histological examination of the control treatment revealed unaltered Kupffer cells and liver cells (Figure 6Bd). Comparable normal characteristics were also observed in liver sections from the satellite treatment receiving the highest dose (satellite $EEBB_{500}$) (Figure 6Bf). Conversely, animals treated with EEBB at 500 mg/kg exhibited changes in liver cells and Kupffer cells (Figure 6Be), indicating that the liver was experiencing stress as a result of the extract's influence, and the hepatic immune system was responding to this impact. Concerning the histological features of the kidneys, renal corpuscles and Bowman's capsules displayed normalcy in the control treatment (Figure 6Bg) and the satellite $EEBB_{500}$ treatment (Figure 6Bi). Conversely, kidney sections from animals exposed to EEBB (500 mg/kg) showcased significant changes, including the expansion of renal space and the formation of irregular Bowman's capsules (Figure 6Bh). This implies that EEBB could affect the overall kidney function. The structural adjustments to the renal space in the satellite $EEBB_{500}$ treatment play a role in maintaining the body's water and electrolyte balance.

The heart, liver, and kidneys function in coordination to maintain endocrine balance, regulate blood pressure, and ensure the proper handling of nutrients and waste products. The heart pumps blood throughout the body, delivering oxygen and nutrients to all cells and tissues. The liver assists in balancing blood sugar and detoxifying the body. The kidneys remove waste substances and excess products from the blood, control water balance, and electrolyte levels in the body.[25] Plant extracts can stimulate heart activity, increasing heart rate or blood flow. Extracts can also cause the expansion of renal spaces, altering kidney structure.

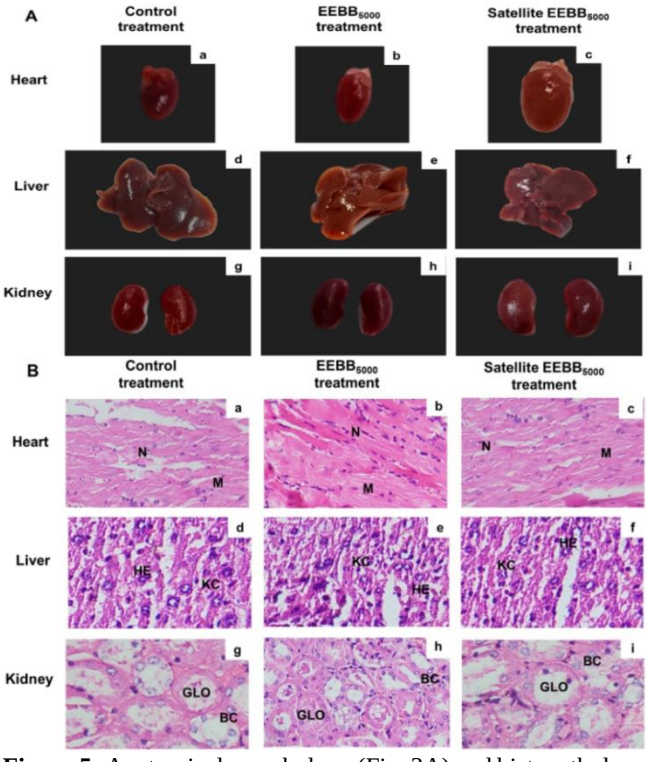

Figure 5: Anatomical morphology (Fig. 3A) and histopathology (Fig. 3B) of representative mouse internal organs in the study of the acute toxicity of EEBB. Magnification 200× (hematoxylin-eosin stain). N, nucleus; M, myocardium; GLO, glomerulus; BC, Bowman's capsule; HE, Hepatocytes; KC, Kupffer cells.

Table 7: Changes in hematological and blood biochemistry analysis in the sub-chronic toxicity study (90-day repeated dose study)

	Treatments for the sub-chronic toxicity study	Satellite treatments for the sub-chronic toxicity study

Parameters	Control treatment	$EEBB_{100}$ treatment	$EEBB_{300}$ treatment	$EEBB_{500}$ treatment	Satellite control treatmen	Satellite$EEBB_{500}$ treatment
RBC ($\times10^6$ cells/mm^3)	8.24 ± 0.05^a	8.59 ± 0.06^b	8.81 ± 0.08^c	9.01 ± 0.08^d	8.31 ± 0.08^a	8.39 ± 0.07^a
HGB (g/dL)	13.32 ± 0.07^a	13.61 ± 0.08^b	13.94 ± 0.07^b	9.95 ± 0.03^a	13.41 ± 0.06^a	13.49 ± 0.08^a
HCT (%)	0.39 ± 0.05^a	0.55 ± 0.03^b	0.73 ± 0.06^c	0.89 ± 0.06^d	0.41 ± 0.08^a	0.44 ± 0.08^a
WBC ($\times10^3$ cells/mm^3)	3.34 ± 0.07^a	3.51 ± 0.07^b	3.69 ± 0.06^c	3.84 ± 0.05^d	3.42 ± 0.08^a	3.46 ± 0.04^a
PLT ($\times10^3$ cells/mm^3)	683.49 ± 17.56^a	767.97 ± 19.14^c	794.76 ± 18.81^d	742.78 ± 15.89^b	690.39 ± 13.64^a	697.44 ± 11.86^a
PT (g/dL)	5.03 ± 0.06^a	5.28 ± 0.06^b	5.53 ± 0.06^c	5.79 ± 0.07^d	5.13 ± 0.06^a	5.21 ± 0.07^a
GLU (mg/dL)	6.25 ± 0.03^a	6.38 ± 0.07^b	6.56 ± 0.06^c	6.75 ± 0.07^d	6.28 ± 0.05^a	6.34 ± 0.05^a
TRI (mg/dL)	136.53 ± 15.95^a	141.63 ± 14.77^a	149.82 ± 13.02^a	145.72 ± 15.62^a	138.26 ± 10.58^a	140.63 ± 10.36^a
AST (U/L)	99.55 ± 2.99^a	109.51 ± 3.38^c	114.48 ± 2.54^d	104.53 ± 2.33^b	100.55 ± 2.56^a	101.54 ± 2.29^a
ALT (U/L)	68.64 ± 2.69^a	72.07 ± 1.97^b	78.94 ± 1.87^d	75.51 ± 2.13^c	69.33 ± 2.26^a	70.01 ± 1.45^a
ALP (U/L)	134.88 ± 9.21^a	138.93 ± 7.77^{ab}	147.02 ± 7.33^{ab}	142.97 ± 7.08^b	136.23 ± 5.85^a	137.59 ± 3.34^a
UA (mg/dL)	11.97 ± 0.71^a	12.21 ± 0.71^a	12.44 ± 0.73^a	12.69 ± 0.64^a	12.09 ± 0.47^a	12.21 ± 0.54^a
BUN (mg/dL)	10.31 ± 0.77^a	11.41 ± 0.86^b	11.45 ± 0.84^b	11.35 ± 0.72^b	10.41 ± 0.96^a	10.48 ± 1.01^a
CRE (mg/dL)	0.31 ± 0.02^a	0.36 ± 0.02^c	0.33 ± 0.01^{ab}	0.34 ± 0.01^{bc}	0.31 ± 0.02^a	0.32 ± 0.01^a

Values are expressed as Mean ± SD, letters (a, b, c, and d) represent the difference between treatments ($p < 0.05$). Note: Red blood cells (RBC), Hemoglobin (HGB), Hematocrit (HCT), White blood cells (WBC), Platelet (PLT), Total protein (PT), Glucose (GLU), Triglyceride (TRI), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Uric acid (UA), Blood urea nitrogen (BUN), Creatinine (CRE).

Additionally, some plant chemicals have the capacity to modify liver cell structure, causing liver stress and stimulating the hepatic immune system to respond to the extract's influence.[26] In the present study, the effects of EEBB at doses of 5000 and 500 mg/kg showed signs of dilation and alterations in heart muscle fiber structure, caused changes in liver cells and Kupffer cells, and led to significant alterations in the renal corpuscles and Bowman's capsules over a 14 days and 90 days observation period. This suggests that under the influence of EEBB, the heart is stimulated to enhance its activity, improving blood circulation. The liver undergoes stress, and the hepatic immune system reacts to the extract. The kidneys may be affected in their overall function. However, after 42 days and 118 days of observation, tissue structure in the satellite $EEBB_{5000}$ treatments and satellite $EEBB_{500}$ treatment returned to a normal state, indicating that EEBB maintained the normal characteristics of heart tissue. The liver did not experience severe issues, and the hepatic immune system did not react excessively to the extract. Furthermore, EEBB did not cause significant changes in kidney function or affect the kidney's ability to maintain water and electrolyte balance in the body.

Urinalysis

In the acute toxicity experiment, there were significant differences in specific gravity, pH, ketone, urobilinogen, Na^+, K^+, and Cl^- levels between the treatments with EEBB (1000, 3000, and 5000 mg/kg) and the control treatments over the 14-day observation period ($p < 0.05$, Table 8). Continuing observations and monitoring in the satellite $EEBB_{5000}$ treatment and satellite control treatment for 42 days, the results in Table 8 showed no significant differences in the study parameters related to the mouse urine composition between the satellite $EEBB_{5000}$ treatment and the satellite control treatment ($p > 0.05$).

In the sub-chronic toxicity experiment, there were no abnormal changes in specific gravity, pH, ketone, urobilinogen, Na^+, K^+, and Cl^- levels in the urine of the satellite $EEBB_{500}$ treatment and satellite treatment after 118 days of treatment and observation ($p > 0.05$, Table 9). However, at day 90 of the treatment process, these parameters exhibited significant alterations, indicating a significant difference between the treatments treated with EEBB (100, 300, and 500 mg/kg) and the control treatment ($p < 0.05$, Table 9).

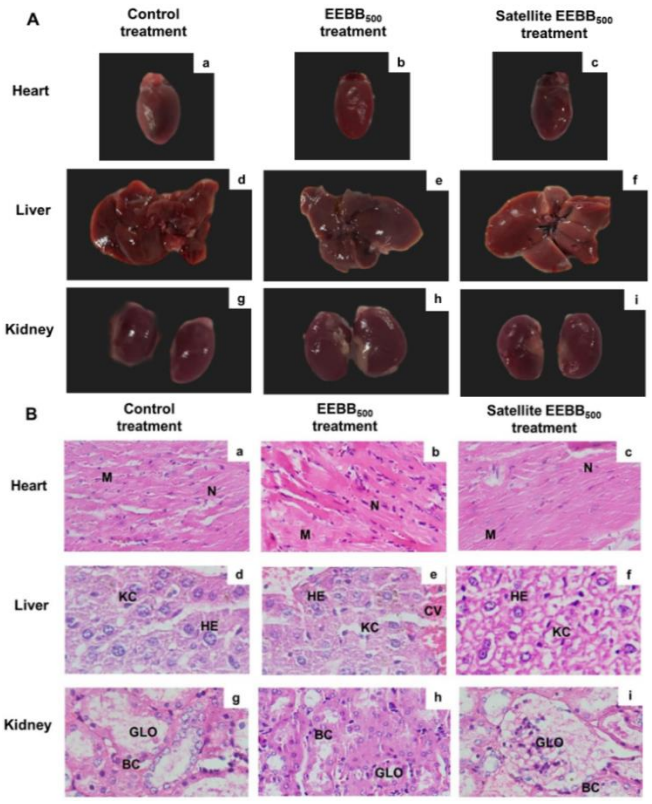

Figure 6: Anatomical morphology (Fig. 6A) and histopathology (Fig. 6B) of representative mouse internal organs in the the sub-chronic (90-day repeated dose) toxicity of EEBB. Magnification 200× (hematoxylin-eosin stain). N, nucleus; M, myocardium; GLO, glomerulus; BC, Bowman's capsule; HE, Hepatocytes; CV, Central vein; KC, Kupffer cells.

Table 8: Urinalysis changes in the acute toxicity study

Parameters	Treatments for the acute toxicity study				Satellite treatments for the acute toxicity study	
	Control treatment	$EEBB_{100}$ treatment	$EEBB_{300}$ treatment	$EEBB_{500}$ treatment	Satellite control treatmen	Satellite $EEBB_{500}$ treatment

Specific gravity	1.014 ± 0.001[a]	1.021 ± 0.001[c]	1.017 ± 0.002[b]	1.024 ± 0.002[d]	1.015 ± 0.001[a]	1.015 ± 0.001[a]
pH	7.31 ± 0.01[a]	7.38 ± 0.02[b]	7.46 ± 0.01[c]	7.53 ± 0.01[d]	7.32 ± 0.02[a]	7.33 ± 0.01[a]
Ketone (mg/dL)	0.82 ± 0.02[a]	0.86 ± 0.01[b]	0.89 ± 0.01[c]	0.94 ± 0.01[d]	0.83 ± 0.02[a]	0.84 ± 0.02[a]
Urobilinogen (mmol/L)	29.02 ± 0.01[a]	32.41 ± 0.02[b]	30.33 ± 0.02[c]	31.79 ± 0.02[d]	29.17 ± 0.08[a]	29.33 ± 0.01[a]
Occult gravity (cells/mL)	Absent	Absent	Absent	Absent	Absent	Absent
Na^+ (mmol/L)	67.95 ± 0.01[a]	73.39 ± 0.02[c]	70.67 ± 0.02[b]	76.11 ± 0.02[d]	68.29 ± 0.01[a]	68.35 ± 0.01[a]
K^+ (mmol/L)	42.15 ± 0.02[a]	43.84 ± 0.01[b]	45.52 ± 0.03[c]	47.21 ± 0.01[d]	42.64 ± 0.01[a]	42.86 ± 0.01[a]
Cl^- (mmol/L)	67.88 ± 0.01[a]	73.31 ± 0.01[c]	76.03 ± 0.01[d]	70.59 ± 0.01[b]	67.91 ± 0.01[a]	68.02 ± 0.01[a]

Values are expressed as Mean ± SD, letters (a, b, c, and d) represent the difference between treatments ($p < 0.05$).

Table 9: Urinalysis changes in the sub-chronic toxicity study (90-day repeated dose)

Parameters	Treatments for the sub-chronic toxicity study				Satellite treatments for the sub-chronic toxicity study	
	Control treatment	$EEBB_{100}$ treatment	$EEBB_{300}$ treatment	$EEBB_{500}$ treatment	Satellite control treatmen	Satellite $EEBB_{500}$ treatment
Specific gravity	1.013 ± 0.001[a]	1.024 ± 0.001[b]	1.033 ± 0.002[c]	1.042 ± 0.001[d]	1.015 ± 0.001[a]	1.016 ± 0.001[a]
pH	7.43 ± 0.02[a]	7.58 ± 0.01[b]	7.51 ± 0.02[c]	7.65 ± 0.01[d]	7.44 ± 0.01[a]	7.45 ± 0.02[a]
Ketone (mg/dL)	0.74 ± 0.02[a]	0.78 ± 0.01[b]	0.85 ± 0.02[c]	0.81 ± 0.01[d]	0.75 ± 0.01[a]	0.76 ± 0.01[a]
Urobilinogen (mmol/L)	32.75 ± 0.02[a]	34.06 ± 0.03[b]	35.37 ± 0.02[c]	36.68 ± 0.01[d]	32.88 ± 0.01[a]	32.97 ± 0.01[a]
Occult gravity (cells/mL)	Absent	Absent	Absent	Absent	Absent	Absent
Na^+ (mmol/L)	63.44 ± 0.02[a]	68.51 ± 0.02[c]	65.98 ± 0.01[b]	71.05 ± 0.02[b]	63.59 ± 0.01[a]	63.84 ± 0.01[a]
K^+ (mmol/L)	46.47 ± 0.02[a]	47.86 ± 0.01[b]	51.58 ± 0.01[c]	49.72 ± 0.01[d]	46.73 ± 0.01[a]	46.93 ± 0.01[a]
Cl^- (mmol/L)	74.83 ± 0.01[a]	72.82 ± 0.02[b]	80.82 ± 0.01[c]	83.81 ± 0.01[a]	74.96 ± 0.01[a]	75.11 ± 0.01[a]

Values are expressed as Mean ± SD, letters (a, b, c, and d) represent the difference between treatments ($p < 0.05$).

In clinical studies, the assessment of urine composition plays a significant role in diagnosing and detecting metabolic disturbances. Changes in urine parameters reflect the body's metabolic disorders. Prolonged exposure of the animal body to toxic substances (xenobiotics) severely affects the nervous, immune, endocrine, cardiovascular, pulmonary, musculoskeletal, liver, and kidney systems.[27] Therefore, the liver and kidneys become the primary detoxification organs for the body. The detoxification process in the body is complex, as toxins are metabolized and eliminated through the liver and kidneys via processes such as hydrolysis, oxidation-reduction, and excretion. Eventually, these toxic substances are excreted through urine.[28] In the current study, the results of two toxicity tests showed significant differences in the study parameters between the EEBB treatments and the control treatment. In the sub-chronic toxicity test, after 14 days of observation, the EEBB treatments (1000, 3000, and 5000 mg/kg) exhibited significant changes in specific gravity, pH, ketones, urobilinogen, Na^+, K^+, and Cl^- when compared to the control treatment. In the sub-acute toxicity test, after 90 days of treatment, the EEBB treatments (100, 300, and 500 mg/kg) again showed significant changes in the study parameters compared to the control treatment. This indicates that EEBB significantly affects these study parameters during treatment. However, the results of both toxicity tests showed that after an extended period of observation (42 days in the sub-chronic toxicity test and 118 days in the sub-acute toxicity test), there were no significant abnormal changes in the study parameters in the urine composition between the EEBB (5000 or 500) satellite treatment and the satellite control treatment. This suggests that, despite some initial adverse effects observed in the sub-chronic and sub-acute toxicity tests, the body had adapted, and the treatment process did not lead to significant changes in the study parameters in urine composition.

Conclusion

In conclusion, the ethanol extract from *Blumea balsamifera* leaves is rich in various phytochemical compounds. The study's acute and sub-chronic toxicity tests revealed no clinically abnormal signs. However, significant changes in body weight and weight gain, as well as altered food and water consumption, were observed during the experiments. Extended observations showed a return to normal in hematological and biochemical parameters, urine composition, relative organ weight, and tissue structure in the satellite treatment with EEBB. Although transient adverse effects were initially noted, the body adapted over time, resulting in no significant long-term changes. These findings indicate the safety and potential benefits of *Blumea balsamifera* leaf extract in mitigating associated adverse effects.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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