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Preclinical Safety Evaluation of Potentised Ethanolic Dilutions of *Bryonia alba*: An Acute and Subacute Oral Toxicity Study in Wistar Rats

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Abstract

Bryonia alba (B. alba) potentised ethanolic dilutions used in the homoeopathic system of medicine have potential immunomodulatory and antioxidant properties effective against viral infections, evidenced by clinical reports. However, data on its safety are unavailable, which has given impetus to carry out acute and subacute toxicity evaluations of ethanolic dilutions that have been homoeopathically potentised, particularly *B. alba* at 6C, 30C, and 200C. In the acute oral toxicity study, a 2000 µL/kg dose of *B. alba* potentised dilutions was administered to Wistar albino rats, and their behaviour and mortality were monitored for 14 days. In the subacute study, the animals were administered a daily dose of 200 µL/kg for 28 days and monitored for clinical signs and changes in body weight, followed by the evaluation of biochemical, haematological, organ weight, and histopathological parameters. In the acute oral toxicity study, no mortality was reported in the rats throughout the study, indicating that a single dose of 2000 µL/kg of *B. alba* is safe. Compared to the normal control group, no changes in the aforementioned parameters were found during the 28-day sub-acute investigation at a dose of 200 µL/kg/day. Based on these findings, no observed adverse effect level of *B. alba* - 6C, 30C, and 200C potentised dilutions was established as greater than 200 µL/kg/day. These findings support the use of potentised *B. alba* dilutions in future pharmacological and clinical research, showing that they are safe under the conditions evaluated.

Major Findings: The study demonstrated that potentised ethanolic dilutions of *B. alba* (6C, 30C, and 200C) showed no toxicity in Wistar rats at doses up to 2000 µL/kg (acute) and 200 µL/kg/day (subacute). No mortality, behavioral changes, or significant alterations in biochemical, haematological, or histopathological parameters were observed, indicating a favorable safety profile.

Keywords: *Bryonia*, Homoeopathy, Immunomodulatory, OECD, Preclinical, Safety

1. Introduction

Bryonia alba Linn. (*B. alba)* is a plant species of the *Cucurbitaceae* family, found primarily in Europe and Western Asia, with a long history of use in folk medicine[1]. Historically, traditional medicine has utilised *B. alba* roots to treat rheumatoid arthritis, inflammation, cough, pneumonia, and oedema[2]. These roots are also

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recognised for their diuretic and laxative effects, which aid in reducing oedema and alleviating joint pain[3]. Additionally, *B. alba* and its extracts/compounds have shown potential in areas such as immunomodulatory[2] anti-inflammatory[4], antinociceptive[4], and antioxidant[4-6] activities based on reported studies. Phytochemical studies have identified various compounds in *B. alba,* such as polyhydroxy unsaturated fatty acids, tannins, saponarin, isovitexin, vitexin, lutonarin, cucurbitacin, essential oils, wax, carbohydrates, and amino acids. Additionally, *B. alba* contains alkaloid bryonicine glycosides, 22-deoxocucurbitosides A and B, 22-deoxocucurbitosides D, 23,24-dihydrocucurbitacin D, triterpenoids, cucurbitacin L, and flavonoids such as isoorientin, saponarin, isovitexin, vitexin, and 5, 7, 4'-trihydroxy flavone 8-C-glucopyranoside[7].

Bryonia alba is also popular as a homoeopathic remedy listed in the essential drug list of India[8], and is commonly used to alleviate symptoms such as dry, itchy coughs, headaches, constipation, painful coughs, joint sprains, and stomach aches[9]. Initially, the roots of *B. alba* were powdered and macerated with 90% ethanol for 5 days[10]. The ethanolic solution obtained was further serially diluted with dispensing alcohol and succussions with each dilution to yield potentized dilutions of *B. alba.* These dilutions were labelled with the letter "C," which stands for the centesimal scale. A 1:100 ratio was associated with each dilution step in this method, meaning that one part of the medicinal solution was diluted in 99 parts of the solvent. 6C, for instance, denoted a solution that has undergone six successive 1:100 dilutions, while 30C and 200C indicate far higher levels of serial dilution, which is frequently employed in homoeopathy to create ultra-diluted formulations that are thought to potentise and maintain therapeutic efficacy[11]. Ethanolic dilutions of *B. alba* were reported to be effective against influenza in 1920 during the Spanish flu[2], chikungunya fever[2], and even COVID-19 (30C and 200C)[3,12]. These findings support its use as a treatment for viral infections and highlight its immunomodulatory and antioxidative properties. However, despite the extensive use of *B. alba* as a homoeopathic medicine, data substantiating its safety in acute overdoses and repeated usage are not available. Considering that alcohol can be toxic at moderate to high doses, it is essential to conduct preclinical investigations to test the safety of *B. alba* ethanolic potentised dilutions. Thus, the current investigation aimed to examine the acute and subacute oral toxicity of 6C, 30C and 200C potentised dilutions of *B. alba* in experimental rats according to OECD guidelines with slight modifications. These dilutions were assessed for their safety, as they are most frequently used and listed in the national list of essential Ayush medicines[13].

2. Materials and Methods

2.1 Homoeopathic Medicine

6C, 30C, and 200C potentised dilutions of *B. alba* (Batch No. 0204) and dispensing alcohol at 90% (Batch No. 8345) manufactured by Hahnemann Publishing Co. Pvt. Ltd., Kolkata, a GMP-certified company, were used in this experiment.

2.2 Preparation of Stock Solution

Stock solutions of vehicle control, 6C, 30C, and 200C potencies of *B. alba* were prepared by diluting 1ml of vehicle/ potentised dilution with 9ml of double-distilled water for administration to the experimental animals[11].

2.3 Experimental Animals

A week before the experiments, healthy Wistar albino rats of both sexes were obtained from an in-house small animal house facility. Three rats per cage, in polycarbonate cages, were kept under conventional conditions with a 12-hour light-dark cycle, 23 ± 2°C ambient temperature, and 55–65% relative humidity. Animals were given a standard pellet diet procured from VRK Industries (Maharashtra, India) and water *ad libitum*. Corn cobs, used as bedding material, were procured from Sparconn Life Sciences. The Institutional Animal Ethics Committee of Dr. Anjali Chatterji, Regional Research Institute for Homoeopathy, approved this study under proposal no. DACRRIH/CPCSEA/IAEC/2021/006 and the use and care of experimental animals followed the Committee for Control and Supervision of Experiments on Animals (CCSEA) recommendations. Before starting the study, the veterinarian conducted health examinations of the animals, and only healthy animals were used in the experiments. The entire investigation adhered to Animal Research Reporting of In-Vivo Experiments (ARRIVE) guidelines to maintain the integrity of animal-based scientific research.

2.4 Coding of the Test Drugs

To ensure blinding of the researchers, *B. alba* potentised dilutions and the vehicle was coded, and decoding was performed after analysis of the results.

2.5 Acute Oral Toxicity Study

In this study, the acute oral toxicity of *B. alba* was examined in female rats using the OECD 423 guideline with minor changes[14]. The experiment involved 30 rats with weights ranging from 150 to 200gm, randomly divided into five groups, each with six animals. In this study, the acute oral toxicity of *B. alba* was examined in female rats using the OECD 423 guideline with minor changes.

In Step 1, the animals were fasted overnight, and all interventions were administered at a single dose of 2000 µL/kg body weight. After administration, food was withheld for 3-4 hours. If no mortality was observed in the treated animals, another set of three animals received interventions at the same dose to confirm the results (Step 2). However, if there was any mortality related to the test substance, additional testing at the next lower dose level (1000 µL/kg) in two steps might be necessary. The process is illustrated in Figure 1.

The rats were observed hourly for mortality and clinical signs of toxicity, including physical changes (fur, eye, and urine colour), behavioural changes (alertness, vocalisation, tremors, twitches, convulsions, sedation, sleep, catatonia, ataxia), respiratory patterns (apnoea, dyspnoea), reflexes, posture (body and limb positions), and autonomic effects (discoloured faeces, urination, lacrimation, and piloerection). Changes in body weight were measured weekly. After 14 days, all animals from both steps were sacrificed by carbon dioxide (CO_2) euthanasia, exsanguination, and a gross necropsy investigation was conducted[15].

2.6 Sub-Acute Oral Toxicity Study

A subacute oral toxicity study was carried out using Wistar albino rats of both sexes following Organisation for Economic Cooperation and Development (OECD) guideline 407 [16]. A total of 100 rats (50 males and 50 females) with body weights ranging from 150 to 200 g were used. The rats were first randomly split into five groups according to sex (n=10, five males and five females per group). Group 1 (Normal Control) received distilled water, and Group 2 (Vehicle Control) was administered 90% dispensing alcohol, each at a dose volume of 200 µL/kg body weight/day via oral gavage. Groups 3, 4, and 5 were treated with *B. alba* at potentized 6C, 30C, and 200C dilutions, respectively, at the same dose volume. The dose volume of 200 µL/kg/day was chosen based on our acute oral toxicity study (Section 2.5), which showed no mortality or toxicity symptoms up to a dose of 2000 µL/kg. Accordingly, 1/10th of this highest non-lethal dose was chosen to ensure safe and consistent exposure over the 28-day treatment period as per OECD 407. Additionally, five recovery groups comprising 10 rats each (five males and five females) were included for the Normal Control, Vehicle Control, and each of the three *B. alba* treatment groups. Distilled water was administered to Group 1R (Normal Control Recovery) and 90% dispensing alcohol to Group 2R (Vehicle Control Recovery) via oral gavage at a dose volume of 200 µL/kg/day. Groups 3R, 4R, and 5R were treated with potentised *B. alba* 6C, 30C, and 200C dilutions, respectively, at the same dose volume for 28 days. These groups received their respective treatments for 28 days, followed by an additional 14-day recovery period to observe if there was any reversal, delayed, or persistent occurrence of toxic effects.

During the treatment phase, the rats were monitored daily for general clinical signs, preferably 1 hour after administration, to capture the peak time of the anticipated effects after dosing. Physical and behavioural observations such as appendages, posture, reflexes, sensory responses, and respiratory and autonomic effects were recorded daily. Additional observations included mortality, body weight (once per week), and feed consumption (once per week). On the 29th and 43rd days, respectively, haematological and clinical biochemical parameters were also measured, including glucose, albumin, total proteins, alkaline phosphatase, renal function tests, hepatic function tests, lipid parameters, Ca^{++}, Na^{+}, K+, and Cl^{-}.

All surviving rats were euthanised on day 29 (control and treated groups) and day 43 (recovery groups) by CO_2 asphyxiation, followed by exsanguination for gross pathology examination. Macroscopic and histopathological analyses of tissues and organs were performed for all groups[17]. The percentage change in body weight and relative organ weight was calculated using the following equations:

$$\%Body\ Weight\ Change = \frac{Day\ 1 - Day\ 14}{Day\ 1} \times 100 \ldots. (1)$$

$$\%\,\mathrm{Re}lative\ Organ\ Weight = \frac{Absolute\ Organ\ Weight}{Body\ weight\ at\ sacrifice} \times 100 \ldots. (2)$$

2.7 Blood Collection and Serum Separation

Before blood collection, the rats were fasted overnight and placed under a cocktail of xylazine (10 mg/kg) and

ketamine (80 mg/kg) i.p. Blood was collected from the retro-orbital sinus into potassium Ethylenediamine Tetraacetic Acid (EDTA) tubes for haematology analysis and clot activator tubes for clinical biochemistry analysis. Haematological analysis was performed using a veterinary haematology analyser (VH 50, Genrui Biotech Pvt. Ltd., China). The serum was separated from the blood samples for clinical biochemistry analysis by centrifuging them for 10 minutes at 25°C at 5000 rpm. The serum was then analysed using commercially available biochemistry kits (Transasia Pvt. Ltd., India) and a fully automated clinical chemistry analyser (EM 360, Transasia Pvt. Ltd., India).

2.8 Gross Necropsy and Histopathology

After completing the study, the rats were humanely euthanised using CO_2 and a thorough gross necropsy was conducted to record any observations. The brain, heart, thymus, lungs, liver, kidneys, adrenal glands, testes, ovaries, spleen, and uterus were among the organs that were meticulously removed, along with any attached adipose tissues. Relative organ weights were also documented. The collected tissues were stored in 10% phosphate-buffered neutral formalin and then processed following standard procedures to prepare sections. Haematoxylin-eosin staining was used to stain the slides, and histopathological examination was performed by a veterinary pathologist using an upright microscope (BX 43, Olympus, Japan).

2.9 Statistical Analysis

SPSS V26 software was used to examine the data using one-way analysis of variance (ANOVA) and Tukey's post hoc test. The findings are presented as the mean ± Standard Error of the Mean (SEM). A statistically significant difference from the control group was defined as a p-value of less than 0.05.

3. Results

3.1. Acute Oral Toxicity Study

All the animals in steps 01 and 02, which received a single dose of *B. alba* in its potentised dilutions of 6C, 30C, and 200C, as well as the vehicle control (90% alcohol) and normal control (distilled water), did not experience any mortality or morbidity. However, one animal each from the *B. alba*-6C group and the vehicle control group exhibited transient ataxia, which resolved within one hour. Additionally, mild abnormalities in limb positioning were observed in two animals from the same groups, which recovered spontaneously within two hours. No clinically significant toxicological symptoms or indications were observed in the animals treated with 30C and 200C potentised dilutions. All treated animals appeared normal and did not exhibit any additional symptoms of clinical toxicity. The effects could be related to alcohol rather than the medicine since the symptoms were also present in the vehicle control group.

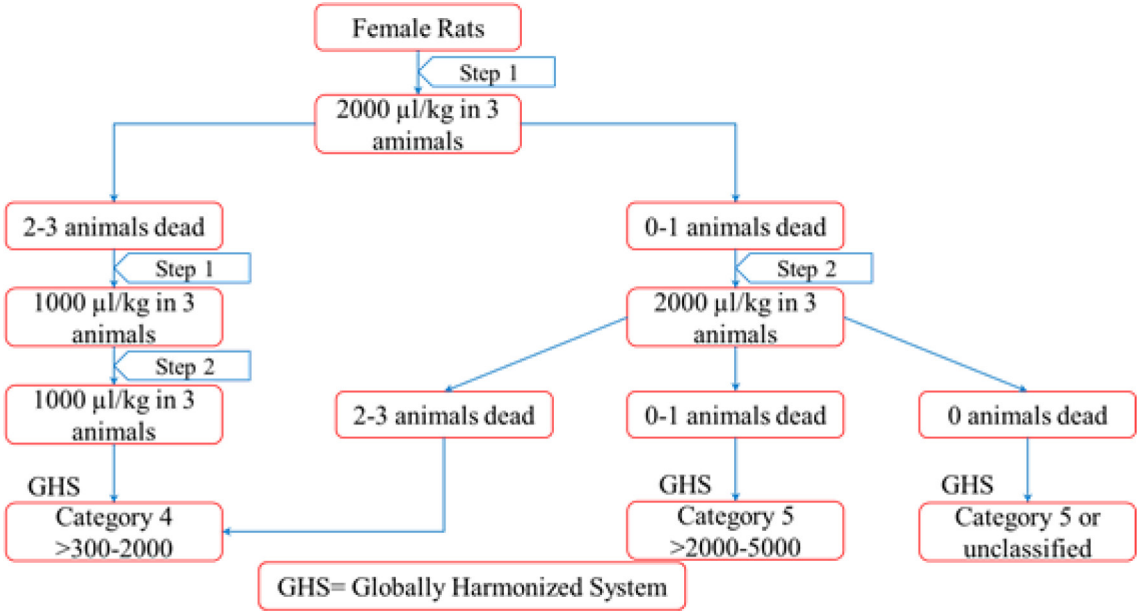


Figure 1. Stepwise procedure of oral acute toxicity study.

Moreover, the gross necropsy evaluation of the treated animals in steps 01 and 02 revealed normal external body surfaces, orifices (including nasal, vaginal, urethral, and anal), and cavities (such as abdominal and thoracic). As there were no mortalities or abnormal gross pathological changes in the treated animals, the collected tissues were not examined microscopically. Based on these findings, it can be inferred that the LD_{50} of potentized dilutions of *B. alba* is greater than 2000 µL/kg, and it is assigned to category 5 or unclassified according to the Globally Harmonised System (GHS) classification.

3.2. Sub-Acute Oral Toxicity Study

3.2.1. General Clinical Signs and Mortality

Throughout the study, the rats in all groups were carefully monitored for signs of toxicity for two hours following *B. alba* administration. The rats in both the *B. alba-treated* and control groups showed no abnormal behaviour during extensive clinical observations, which included open field, home cage, handling, and sensory observations. No animal mortality occurred during the study, including the recovery phase, demonstrating that *B. alba* in its 6C, 30C, and 200C potencies is safe when administered repeatedly for 28 days.

3.2.2. Body Weight and Feed Intake

In the subacute toxicity study, there were no notable changes in body weight and feed intake in male or female animals treated with *B. alba* and dispensing alcohol for 28 days, in comparison to the normal control group. This trend remained consistent during the recovery phase in this study. No statistically significant differences were found among the groups (Figures 2A – 2D).

3.2.3. Biochemical and Haematological Analysis

The analysis of biochemical markers revealed no significant differences in serum enzyme levels between male and female rats treated with *B. alba* dilutions and their respective normal control groups (as shown in Tables 1 and 2). Furthermore, assessment of haematological parameters showed no notable treatment-related changes in rats administered *B. alba* dilutions or dispensing alcohol when compared to their corresponding normal controls (as indicated in Table 3).

3.2.4. Gross Necropsy and Histopathological Evaluations

In the subacute toxicity study, gross necropsy and histopathological examinations were conducted on days 29 and 43. Results from these examinations showed no noticeable changes in the animals' external surface or internal organs in the *B. alba* groups compared to their respective normal control groups. Moreover, no treatment-related alterations in organ weight ratios were observed in the euthanised animals on days 29 and 43, expressed as a percentage of body weight (relative organ weight) and statistically compared to their respective normal control groups (Summarised in Table 4). Additionally, when the organs were histologically examined, no pathologically significant abnormalities were found in either sex of the treated animals, in contrast to the normal control group (as shown in Figures 3 and 4).

4. Discussion

The current study sought to evaluate the safety of the homoeopathic medicine *B. alba* in its 6C, 30C, and 200C potentised dilutions through acute and subacute oral toxicity tests. Acute oral toxicity data are essential for classifying compounds based on toxicity severity and for guiding risk assessments for human health. Subacute toxicity tests aim to evaluate the negative effects of repeated drug use, particularly oral administration, which is common in clinical practice[17].

The acute oral toxicity study involved administering 2000 µL/kg of 6C, 30C, and 200C *B. alba* in Step 01 to three female rats from each group, with no reported deaths. A confirmatory dose of 2000 µL/kg (Step 02) was subsequently administered to another three female rats from each group, and no mortality was reported. With the study endpoint reached, further testing was discontinued. During the study, animals treated with *B. alba* 6C and the vehicle control exhibited non-persistent ataxia (poor coordination, jittery walking, and stumbling), but all animals recovered within an hour. Minor changes in limb position were also observed with 6C and vehicle control, but subsided after two hours. No other signs of toxicity were observed, such as diarrhoea, hair loss, behavioural abnormalities, or moribundity in treated animals. Various health indicators, including skin, fur, eyes, mucous membranes, salivation, and sleeping patterns, appeared normal in both the control and *B. alba* treated groups. In addition, there were no treatment-related effects on body weight, feed, water intake, or gross necropsies. The results indicate that *B. alba* 6C, 30C, and 200C potentised dilutions are safe up to a single dose of 2000 µL/kg.

Table 1. Effect of *B. alba* during sub-acute oral toxicity research on biochemical parameters (lipid and liver parameters) of male rats

Parameters	Groups									
	Day 29					Day 43				
	Control	Vehicle Control	*B. alba*-6C	*B. alba*-30C	*B. alba* -200C	Control (Recovery)	Vehicle Control (Recovery)	*B. alba* -6C (Recovery)	*B. alba* -30C (Recovery)	*B. alba* -200C (Recovery)
Glucose(mg/dL)	83.68±2.85	83.58±3.91	85.40±5.43	76.42±3.48	90.90±3.23	78.54±3.87	77.42±4.71	79.26±4.53	77.02±2.91	78.82±4.49
TC (mg/dL)	75.60±11.83	78.00±5.30	77.00±8.04	77.00±4.62	73.20±1.98	71.40±3.47	74.40±5.77	72.80±3.06	73.20±7.41	74.80±3.99
Tgl (mg/dL)	90.52±8.03	112.10±14.38	116.00±17.47	114.12±13.74	123.92±17.03	129.34±6.08	125.10±12.48	130.68±7.91	120.78±3.46	124.10±11.84
HDL (mg/dL)	41.30±3.44	40.08±0.91	41.72±2.06	41.64±2.62	39.62±1.23	40.40±1.26	42.68±1.49	42.18±1.88	40.62±1.34	42.14±2.43
SGOT(U/L)	142.72±9.65	130.80±11.02	135.60±12.69	133.30±7.89	153.24±5.76	131.40±8.88	134.52±8.81	138.88±7.81	142.16±8.98	122.64±9.23
SGPT (U/L)	45.88±6.04	41.18±2.78	35.14±2.95	43.04±1.61	38.58±4.54	31.04±3.50	35.92±4.61	40.80±3.59	36.74±5.05	38.20±3.68
ALP (U/L)	302.60±22.43	262.00±32.60	291.40±20.31	318.40±22.98	283.60±20.18	230.00±25.23	244.20±42.12	274.60±34.74	263.00±32.76	229.40±36.01
Albumin(g/dL)	2.54±0.06	2.57±0.03	2.59±0.09	2.61±0.06	2.54±0.04	2.94±0.08	2.99±0.10	2.81±0.10	2.91±0.16	3.00±0.11
TP(g/dL)	6.74±0.14	6.74±0.12	6.52±0.16	6.68±0.21	6.43±0.08	7.01±0.13	6.93±0.11	6.92±0.13	7.01±0.08	6.14±0.73
TB (mg/dL)	0.12±0.01	0.11±0.01	0.13±0.01	0.11±0.01	0.11±0.00	0.10±0.01	0.10±0.00	0.11±0.01	0.11±0.01	0.11±0.01
DB (mg/dL)	0.10±0.01	0.09±0.00	0.10±0.01	0.07±0.01	0.09±0.01	0.07±0.01	0.08±0.01	0.09±0.01	0.09±0.01	0.09±0.01

Values expressed as Mean ± SEM. There were no statistically significant differences.

Table 2. Effect of *B. alba* during sub-acute oral toxicity research on biochemical parameters (renal parameters and serum electrolytes) of male rats

Parameters	Groups									
	Day 29					Day 43				
	Control	Vehicle Control	*B. alba*-6C	*B. alba*-30C	*B. alba* -200C	Control (Recovery)	Vehicle Control (Recovery)	*B. alba* -6C (Recovery)	*B. alba* -30C (Recovery)	*B. alba* -200C (Recovery)
Urea (mg/dL)	26.34±3.24	26.32±2.63	22.88±2.45	23.12±2.89	23.74±2.03	25.74±3.71	27.36±2.86	28.58±1.21	28.22±3.89	26.58±2.83
SC (mg/dL)	0.86±0.04	0.91±0.05	0.94±0.06	0.88±0.05	0.99±0.04	0.89±0.09	0.86±0.14	0.91±0.04	0.94±0.08	0.86±0.07
Calcium (mg/dL)	11.42±0.29	11.26±0.31	11.76±0.22	11.32±0.34	11.68±0.40	11.10±0.30	11.06±0.30	11.22±0.09	11.32±0.37	11.04±0.16
Sodium (mmol/L)	144.68±1.40	142.72±0.47	143.56±1.03	144.52±0.69	144.34±0.94	143.84±0.57	144.10±0.93	144.90±0.67	145.18±0.86	144.24±0.81
Potassium(mmol/L)	4.14±0.19	4.12±0.10	4.50±0.27	4.54±0.18	4.11±0.15	3.98±0.13	4.10±0.14	3.93±0.13	4.11±0.13	4.19±0.20
Chloride (mmol/L)	102.22±0.40	102.40±0.28	103.58±0.57	102.76±0.51	102.58±0.35	104.12±0.39	103.70±0.36	103.10±0.69	103.46±0.49	104.38±0.62

Values expressed as Mean ± SEM. There were no statistically significant differences.

Table 3. Effect of *B. alba* during sub-acute oral toxicity research on biochemical parameters (lipid and liver parameters) of female rats

Parameters	Groups									
	Day 29					Day 43				
	Control	Vehicle control	*B. alba*-6C	*B. alba*-30C	*B. alba* -200C	Control (Recovery)	Vehicle control (Recovery)	*B. alba* -6C (Recovery)	*B. alba* -30C (Recovery)	*B. alba* -200C (Recovery)
Glucose(mg/dL)	73.26±2.93	76.24±7.75	81.70±4.44	70.80±5.27	80.08±6.41	73.82±2.68	74.00±4.11	70.36±2.48	78.36±4.24	72.50±3.38
TC (mg/dL)	91.40±8.68	89.60±5.20	91.60±7.12	90.20±7.49	91.20±5.09	74.60±1.69	78.80±5.42	84.40±10.78	76.60±3.91	79.36±7.75
Tgl (mg/dL)	89.16±5.29	93.06±3.29	93.02±7.48	83.20±5.04	88.80±3.34	88.42±5.40	93.22±3.91	92.86±2.70	97.12±5.07	92.22±7.42
HDL (mg/dL)	43.32±2.83	44.00±3.32	44.26±2.17	43.10±2.56	43.30±2.49	41.00±1.18	41.54±1.59	41.04±1.22	40.32±0.93	43.46±3.05
SGOT(U/L)	136.12±16.80	130.40±11.58	130.66±6.12	134.48±6.78	138.12±11.92	123.76±5.78	130.40±11.58	125.88±5.68	125.50±5.04	135.08±19.42
SGPT (U/L)	32.30±5.09	30.42±3.32	33.38±2.53	33.00±4.00	29.46±2.28	28.24±4.07	28.48±2.19	27.42±1.74	26.88±4.75	27.40±2.92
ALP (U/L)	148.60±8.18	184.00±17.85	175.40±19.69	182.20±16.55	153.40±15.23	183.00±18.90	184.00±17.85	187.80±17.21	184.20±22.78	183.40±17.70
Albumin(g/dL)	3.07±0.09	2.92±0.10	3.02±0.14	3.05±0.10	3.07±0.07	3.06±3.06	2.94±0.13	3.04±0.07	2.98±0.12	3.02±0.12
TP(g/dL)	7.12±0.14	7.02±0.07	6.96±0.13	7.15±0.13	7.15±0.14	7.18±0.12	7.08±0.10	7.06±0.27	7.15±0.13	7.30±0.21
TB (mg/dL)	0.12±0.01	0.12±0.02	0.12±0.01	0.10±0.01	0.12±0.01	0.11±0.01	0.11±0.01	0.12±0.01	0.14±0.03	0.12±0.02
DB (mg/dL)	0.11±0.01	0.10±0.01	0.10±0.01	0.09±0.00	0.09±0.01	0.08±0.01	0.08±0.01	0.09±0.01	0.09±0.03	0.07±0.02

Values expressed as Mean ± SEM. There were no statistically significant differences.

Table 4. Effect of *B. alba* during sub-acute oral toxicity research on biochemical parameters (renal parameters and serum electrolytes) of female rats

Parameters	Groups									
	Day 29					Day 43				
	Control	Vehicle control	*B. alba*-6C	*B. alba*-30C	*B. alba* -200C	Control (Recovery)	Vehicle control (Recovery)	*B. alba* -6C (Recovery)	*B. alba* -30C (Recovery)	*B. alba* -200C (Recovery)
Urea (mg/dL)	37.84±1.60	38.10±2.19	37.50±2.82	35.00±2.62	37.62±2.59	31.44±1.08	32.38±1.50	33.96±1.97	35.86±2.31	31.64±1.39
SC (mg/dL)	0.72±0.07	0.75±0.06	0.78±0.04	0.70±0.05	0.70±0.07	0.67±0.07	0.73±0.11	0.68±0.10	0.74±0.12	00.69±0.08
Calcium (mg/dL)	11.22±0.27	11.60±0.25	11.00±0.19	10.92±0.19	11.14±0.28	10.78±0.11	10.76±0.08	10.80±0.22	10.80±0.13	10.76±0.18
Sodium (mmol/L)	144.30±0.39	144.48±0.40	144.36±0.47	144.60±0.38	144.54±0.51	144.38±0.33	144.50±0.45	144.56±0.60	144.20±0.54	144.36±0.79
Potassium(mmol/L)	3.91±0.25	4.08±0.23	4.40±0.22	4.16±0.24	4.37±0.28	4.19±0.24	4.08±0.18	4.10±0.12	4.15±0.18	4.29±0.20
Chloride (mmol/L)	104.37±0.59	104.92±0.60	104.36±0.40	103.88±0.58	104.24±0.43	103.76±0.33	103.88±0.31	103.62±0.43	103.94±0.54	103.90±0.23

Values expressed as Mean ± SEM. There were no statistically significant differences.

Table 5. Effect of *B. alba* during sub-acute oral toxicity research on the haematological parameters of rats

	Groups									
	Day 29					Day 43				
Parameters	**Control**	**Vehicle control**	***B. alba*-6C**	***B. alba*-30C**	***B. alba* -200C**	**Control (Recovery)**	**Vehicle control (Recovery)**	***B. alba* -6C (Recovery)**	***B. alba* -30C (Recovery)**	***B. alba* -200C (Recovery)**
	Male Rats									
HGB (g/dL	14.62±0.45	14.26±0.31	14.32±0.14	15.06±0.41	14.42±0.33	14.86±0.13	14.84±0.30	14.97±0.17	14.46±0.31	15.04±0.33
RBC (10^6/µL)	7.60±0.37	7.14±0.25	7.65±0.34	7.76±0.19	7.47±0.14	8.11±0.17	7.48±0.25	7.89±0.11	7.79±0.21	8.03±0.17
WBC (10^3/µL)	6.85±1.07	8.46±0.93	8.44±0.76	9.28±0.73	8.00±0.64	7.85±0.54	7.43±0.34	8.41±0.46	8.17±0.62	7.71±0.81
MCV (fL)	61.86±0.48	61.98±0.69	62.58±1.27	61.94±0.83	61.52±0.52	57.50±1.05	57.66±1.08	57.88±0.80	57.02±0.81	58.14±0.91
MC (pg)	19.68±0.48	19.16±0.63	20.14±0.46	19.44±0.21	19.54±0.27	18.96±0.39	18.88±0.30	18.94±0.21	18.54±0.28	18.94±0.19
MCC (g/dL)	32.40±0.46	31.76±0.19	31.98±0.23	31.66±0.27	31.78±0.17	32.50±0.19	33.38±0.48	32.76±0.20	32.54±0.19	32.50±0.26
PLT (10^3/µL)	775.40±31.20	712.00±28.99	740.80±37.76	672.40±45.91	727.80±10.60	772.20±66.03	758.20±28.65	734.40±47.58	709.00±51.32	753.80±50.25
	Female Rats									
HGB (g/dL	14.78±0.15	15.10±0.17	14.84±0.16	14.66±0.20	14.40±0.25	14.70±0.21	14.70±0.34	14.54±0.31	14.48±0.26	14.48±0.35
RBC (10^6/µL)	7.20±0.10	7.38±0.15	7.46±0.10	7.32±0.16	7.12±0.11	7.45±0.10	7.60±0.13	7.50±0.20	7.33±0.12	7.28±0.15
WBC (10^3/µL)	9.27±0.87	9.60±0.82	8.84±0.27	8.18±1.11	9.01±0.59	6.03±0.58	6.59±0.29	6.17±0.60	6.03±0.63	6.54±0.39
MCV (fL)	64.42±0.56	64.82±0.94	63.03±0.56	63.34±0.94	64.56±1.13	66.54±0.90	65.28±1.86	65.94±1.01	67.10±0.89	67.96±0.44
MC (pg)	20.10±0.29	20.52±0.49	19.90±0.19	20.06±0.22	20.24±0.40	19.64±0.27	19.33±0.34	20.16±0.83	19.70±0.20	19.90±0.11
MCC (g/dL)	31.20±0.16	31.32±0.28	31.68±0.08	32.14±0.62	31.36±0.26	29.58±0.73	29.63±0.39	29.84±0.31	29.26±0.35	29.12±0.23
PLT (10^3/µL)	731.60±67.22	682.00±18.88	705.40±23.62	713.20±25.03	769.40±49.80	776.40±28.35	735.75±23.01	729.60±23.83	707.40±68.93	721.40±27.69

Values are expressed as mean + S.E.M.; no statistically significant variations were observed in haematological markers.

Table 6. Effect of *B. alba* during sub-acute oral toxicity research on relative organ weights of rats

	Groups									
	Control (Recovery)	**Vehicle Control (Recovery)**	***B. alba* -6C (Recovery)**	***B. alba* -30C (Recovery)**	***B. alba* -200C (Recovery)**	**Control (Recovery)**	**Vehicle Control (Recovery)**	***B. alba* -6C (Recovery)**	***B. alba* -30C (Recovery)**	***B. alba* -200C (Recovery)**
Brain	0.62 ± 0.01	0.61 ± 0.01	0.61 ± 0.03	0.62 ± 0.01	0.61 ± 0.01	0.61 ± 0.01	0.62 ± 0.02	0.62 ± 0.02	0.62 ± 0.01	0.62 ± 0.01
Thymus	0.17 ± 0.01	0.16 ± 0.00	0.17 ± 0.00	0.17 ± 0.00	0.16 ± 0.00	0.17 ± 0.01	0.16 ± 0.00	0.17 ± 0.00	0.18 ± 0.00	0.18 ± 0.00
Heart	0.42 ± 0.01	0.43 ± 0.00	0.43 ± 0.01	0.42 ± 0.01	0.42 ± 0.00	0.43 ± 0.01	0.42 ± 0.01	0.42 ± 0.01	0.43 ± 0.01	0.43 ± 0.01
Liver	4.16 ± 0.03	4.16 ± 0.03	4.20 ± 0.32	4.15 ± 0.09	4.14 ± 0.03	4.17 ± 0.10	4.16 ± 0.06	4.17 ± 0.02	4.17 ± 0.03	4.17 ± 0.03
Lungs	0.79 ± 0.04	0.80 ± 0.02	0.79 ± 0.03	0.79 ± 0.02	0.80 ± 0.02	0.84 ± 0.02	0.83 ± 0.01	0.84 ± 0.01	0.84 ± 0.02	0.84 ± 0.02
Spleen	0.32 ± 0.01	0.34 ± 0.02	0.34 ± 0.03	0.34 ± 0.01	0.33 ± 0.01	0.36 ± 0.01	0.37 ± 0.02	0.36 ± 0.00	0.36 ± 0.01	0.36 ± 0.01
Kidneys	0.92 ± 0.02	0.92 ± 0.03	0.93 ± 0.03	0.93 ± 0.01	0.92 ± 0.01	0.87 ± 0.01	0.88 ± 0.02	0.88 ± 0.01	0.88 ± 0.02	0.88 ± 0.02
Adrenal	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Testes	0.85 ± 0.03	0.87 ± 0.02	0.88 ± 0.02	0.84 ± 0.03	0.84 ± 0.02	0.85 ± 0.01	0.84 ± 0.01	0.84 ± 0.02	0.84 ± 0.01	0.84 ± 0.01
	Female Rats									
Brain	0.78 ± 0.01	0.79 ± 0.02	0.77 ± 0.02	0.78 ± 0.01	0.79 ± 0.01	0.80 ± 0.01	0.79 ± 0.01	0.80 ± 0.02	0.81 ± 0.01	0.80 ± 0.00
Thymus	0.18 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.00	0.20 ± 0.00	0.21 ± 0.01	0.21 ± 0.00	0.21 ± 0.01	0.20 ± 0.00	0.20 ± 0.01
Heart	0.43 ± 0.02	0.42 ± 0.02	0.42 ± 0.01	0.43 ± 0.01	0.44 ± 0.00	0.42 ± 0.01	0.43 ± 0.01	0.43 ± 0.01	0.43 ± 0.01	0.43 ± 0.01
Liver	4.66 ± 0.07	4.67 ± 0.11	4.69 ± 0.10	4.65 ± 0.11	4.68 ± 0.05	4.71 ± 0.07	4.71 ± 0.08	4.69 ± 0.05	4.72 ± 0.03	4.72 ± 0.07
Lungs	0.94 ± 0.03	0.96 ± 0.02	0.95 ± 0.02	0.94 ± 0.01	0.95 ± 0.01	0.95 ± 0.01	0.95 ± 0.02	0.93 ± 0.03	0.95 ± 0.03	0.94 ± 0.01
Spleen	0.33 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.31 ± 0.00	0.32 ± 0.01	0.32 ± 0.00	0.32 ± 0.01	0.33 ± 0.00	0.32 ± 0.01	0.32 ± 0.00
Kidneys	0.84 ± 0.02	0.83 ± 0.01	0.84 ± 0.02	0.85 ± 0.01	0.84 ± 0.01	0.83 ± 0.02	0.86 ± 0.01	0.86 ± 0.01	0.85 ± 0.01	0.86 ± 0.01
Adrenal	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
Ovaries	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
Uterus	0.29 ± 0.02	0.31 ± 0.01	0.29 ± 0.01	0.29 ± 0.00	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.28 ± 0.00	0.29 ± 0.01	0.29 ± 0.01

Values expressed as mean + S.E.M.; no statistically significant variations were observed in relative organ weights.

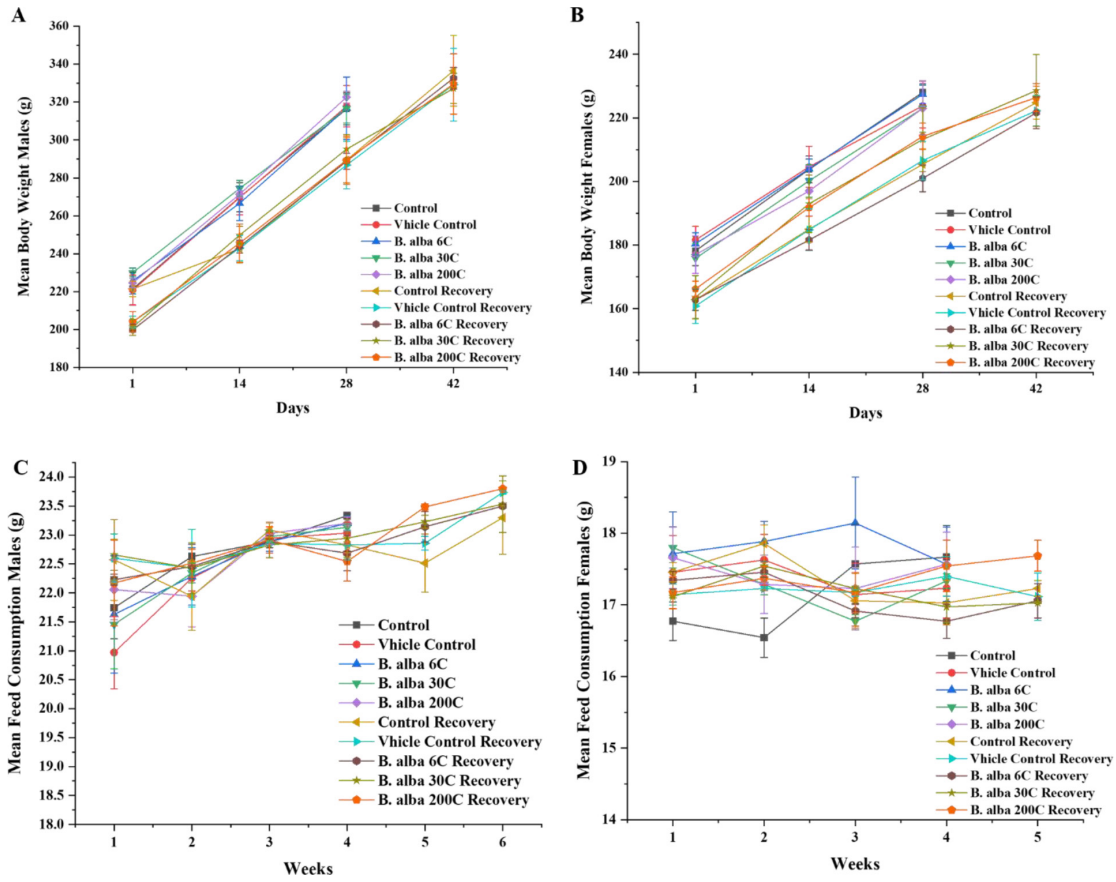


Figure 2. Mean body weight and feed consumption of male (A, C) and female (B, D) rats orally administered with *B. alba* for 28 days.

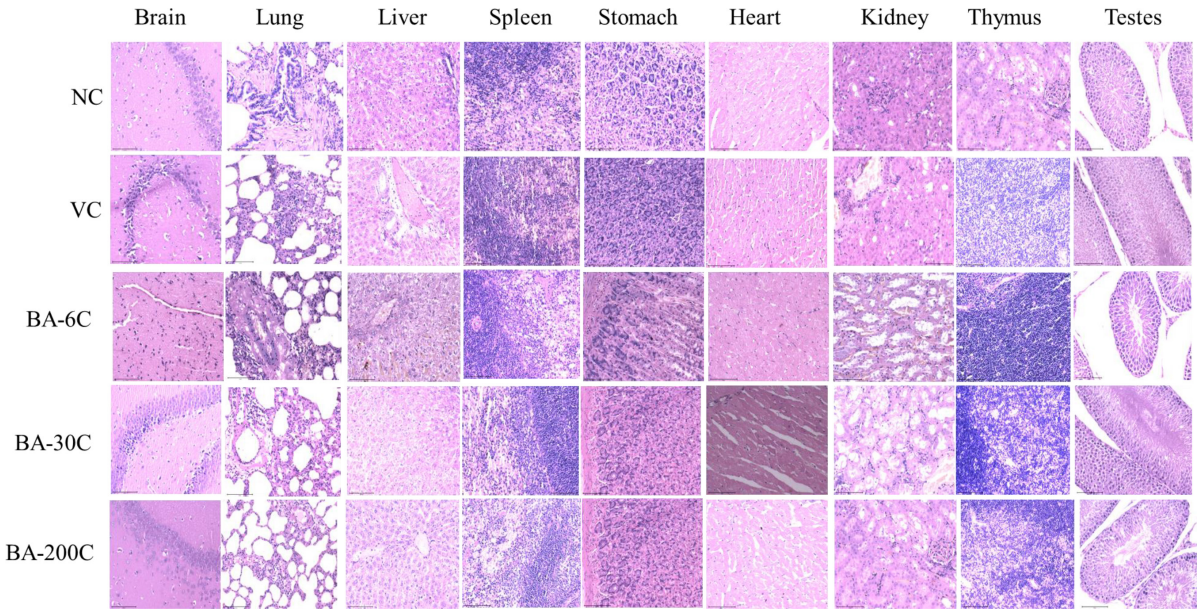


Figure 3. Photomicrographs of organ sections (brain, lung, liver, spleen, stomach, heart, kidney, thymus, and testes) from male rats in control and *B. alba*-treated groups following 28 days of oral administration (H&E staining, 40× magnification). No significant histopathological alterations were observed in the architecture of these organs in the treated groups compared to the normal control group. NC=Normal Control, VC=Vehicle Control, BA=*Bryonia alba*, C= Centesimal.

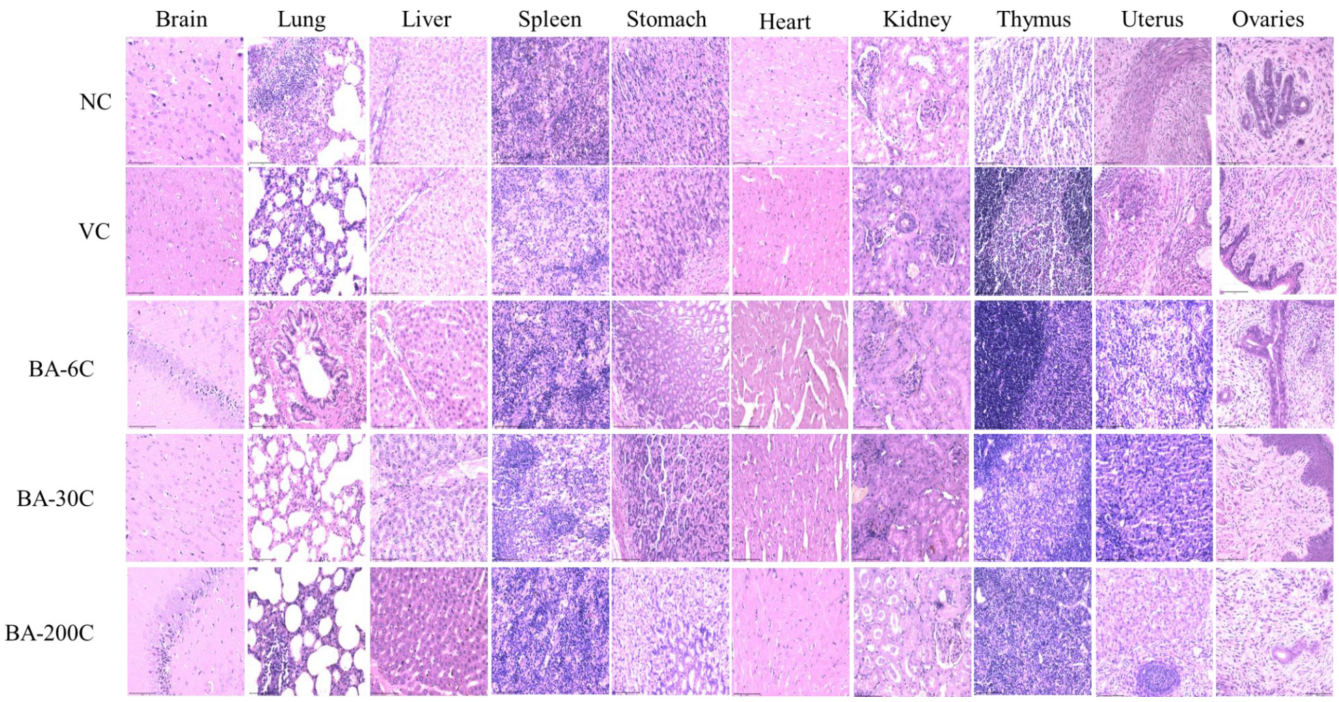


Figure 4. Photomicrographs of organ sections (brain, lung, liver, spleen, stomach, heart, kidney, thymus, uterus and ovaries) from female rats in control and *B. alba*-treated groups following 28 days of oral administration (H&E staining, 40× magnification). No significant histopathological alterations were observed in the architecture of these organs in the treated groups compared to the normal control group. NC=Normal Control, VC=Vehicle Control, BA=*Bryonia alba*, C= Centesimal.

Furthermore, the long-term safety of 6C, 30C, and 200C potentised dilutions of *B. alba* were evaluated in Wistar albino rats by administering them over 28 days. Additionally, recovery groups were set up to monitor the reversibility of any observed or potentially delayed toxic effects for an additional 14 days after administration for 28 days. Notably, there was no mortality in either the main or recovery groups due to *B. alba* or dispensing alcohol (90%) treatment during the experiment. The condition of the animal's skin, fur, eyes, mucous membranes, salivation, and sleeping habits exhibited no abnormal differences between *B. alba* and the normal control groups[17].

The study also examined the impact of *B. alba* treatment on animal body weight and relative organ weight. The results showed no substantial differences in body weight between the *B. alba* and normal control groups. Similarly, when examining the relative organ weights, including the brain, heart, liver, kidneys, spleen, thymus, lungs, and reproductive organs such as testes, ovaries, and uterus, there were no significant changes in any of these organs in the animals subjected to the investigation. This indicated that *B. alba* treatment did not cause any damage or adverse effects on the organs. Furthermore, proper nutritional intake is essential for assessing the treatment's effectiveness and safety[18]. *B. alba* treatment did not adversely affect the physiological status of the animals because the amount of food consumed did not differ significantly from that of the normal control group.

Biochemical data play a critical role in assessing drug toxicity, particularly concerning kidney and liver functions, which are vital for an organism's survival. Kidney function is often evaluated using blood creatinine levels because the kidneys remove various medications and metabolites, making them susceptible to preclinical toxicity[19,20]. Additionally, transaminases, such as SGOT and SGPT, serve as valuable markers for evaluating liver health and predicting potential pharmaceutical toxicity. Increased serum bilirubin levels, produced as a by-product of haemoglobin breakdown in the liver, spleen, and bone marrow, may indicate issues like haemolysis, liver injury, or hepatic duct obstruction[21]. *B. alba* treatments in this study did not have a major impact on hepatic or renal biochemical parameters, suggesting its safety upon repeated administration.

Furthermore, evaluating lipid profiles is crucial for the overall risk assessment of metabolic and cardiovascular illnesses[22]. The study also examined triglyceride, total cholesterol, and High Density Lipoprotein (HDL) levels in rats treated with *B. alba* and the control group. The results indicated that the serum lipid levels remained within the typical clinical range throughout the study. Additionally, blood glucose monitoring looks for patterns in variations in blood glucose (sugar) levels brought on by diet, activity, medication, and/or medical disorders such as diabetes that are linked to blood glucose fluctuations. Abnormal blood sugar levels can result in immediate and/or long-term, life-threatening problems[23]. The results revealed that continuous usage of *B. alba* did not alter glucose levels during the study.

Electrolytes are electrically charged molecules that are essential for various bodily functions, including acid-base balance, muscle contractions, nerve impulses, and hydration regulation[24]. This study assessed sodium, potassium, chloride, and calcium serum levels to understand the impact of administering *B. alba*. No significant changes in the electrolyte levels were observed, further supporting the safety of the treatment.

In line with biochemical evaluations, the haematological system has higher clinical relevance for toxicity; hence, blood tests are essential for risk assessment[15]. The haematological parameters of the present study did not reveal any alarming findings because all the alterations were within the typical anticipated range for the Wistar albino rats used in this study and did not statistically differ from the normal control group.

Assessing organ histopathological abnormalities is critical for determining the safety of the analysed samples. Haematological and biochemical analyses may not always reveal changes in the internal cell structure of organs, which can be detected through histological examinations[11,15]. In the sub-acute toxicity study, gross necropsy examination was conducted on days 29 (main groups) and 43 (recovery groups), and no abnormal alterations were observed in the animals' external surfaces or internal organs. In addition, histological analysis of the organs revealed no pathologically significant variations compared to the control group. Thus, the study findings suggest that *B. alba* (6C, 30C, and 200C) did not exhibit substantial toxicity or adverse effects in the tested animals.

5. Conclusion

B. alba, in 6C, 30C, and 200C potentised dilutions, was safe when administered as a single oral dose of up to 2000 µL/kg body weight to female Wistar rats. *B. alba* administration for 28 days had no detrimental effects on the body weight of animals, feed consumption, or haematological, biochemical, and histological parameters. The NOAEL of *B. alba* in 6C, 30C, and 200C potentised dilutions was found to be greater than 200 µL/kg/day, according to the results. These results support the safety of potentized *B. alba* dilutions and provide a basis for their potential application in future pharmacological and clinical investigations.

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7. Ethical Statement

The Institutional Animal Ethics Committee (IAEC) approved the study under Proposal No. DACRRIH/CPCSEA/IAEC/2021/006 and the use and care of experimental animals followed the CCSEA recommendations.

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