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Acute Toxicity Screening of Aqueous Extract Derived from *Stevia rebaudiana*, *Musa acuminata* and *Solanum lycopersicum*

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Abstract

Oxidative stress plays a critical role in the progression of various chronic diseases, and plant-derived antioxidants are increasingly explored for their therapeutic potential. *Stevia rebaudiana*, *Musa acuminata* (banana), and *Solanum lycopersicum* (tomato) are rich in bioactive compounds with notable antioxidant properties. The present study aimed to evaluate the acute oral toxicity of a tricomponent aqueous extract formulation (Extract B) composed of Stevia, banana, and tomato in a 7:3:3 ratio using female Albino Wistar rats, following OECD guideline 423. A single oral dose of 2000 mg/kg body weight was administered, and animals were monitored for 14 days for behavioural changes, clinical signs of toxicity, mortality, body weight, and food intake. Blood samples were collected for haematological and biochemical analysis, and vital organs were examined for gross morphological and histopathological changes. Data were expressed as mean ± SEM and statistically analysed using an unpaired statistical test analyzed with $p < 0.05$ considered statistically significant. One mortality was recorded in the extract-treated group during Step 2, whereas the remaining animals survived until the end of the 14-day observation period without marked clinical signs of toxicity. Body weight and food consumption remained stable across the groups. Haematological and biochemical parameters, including glucose, urea, creatinine, alkaline phosphatase, and aspartate aminotransferase, were within normal physiological limits. Histopathological examination of liver, kidney, spleen, and brain tissues revealed no abnormalities or toxicity-related changes. The results suggest that the aqueous formulation of these three plant components did not produce consistent acute systemic toxicity in surviving rats at 2000 mg/kg. It did not produce consistent acute toxicity in surviving animals; however, one mortality warrants cautious interpretation.

Major Findings: This study provides essential preclinical safety data supporting the future use of this polyherbal extract in managing oxidative stress-related conditions and warrants further pharmacological investigation.

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1. Introduction

Free radicals and Reactive Oxygen Species (ROS) are major contributors to oxidative stress, which is implicated in the pathogenesis of various chronic diseases, including cancer, diabetes, cardiovascular disorders, and neurodegenerative conditions[1,2]. These reactive species are generated endogenously through metabolic processes or exogenously via exposure to pollutants, radiation, and xenobiotics[3]. The human body maintains a balance between oxidants and antioxidants through an intricate network of enzymatic and non-enzymatic defence mechanisms[4]. However, when the production of ROS exceeds the capacity of antioxidant defences, oxidative damage to lipids, proteins, and DNA occurs, contributing to the onset and progression of several pathological conditions[5]. This imbalance, known as oxidative stress, has emerged as a critical therapeutic target.

To mitigate oxidative damage, increasing attention has been directed toward the use of dietary or supplemental antioxidants[6]. While synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been widely used, concerns about their long-term safety have prompted a shift toward natural sources[7]. Medicinal plants and commonly consumed fruits are rich in bioactive compounds, such as flavonoids[8], phenolic acids[9], glycosides, alkaloids, and terpenoids known for their potent free radical scavenging activity and minimal side effects. Their ability to modulate oxidative stress, inflammation, and cellular signalling makes them valuable in the prevention and management of chronic diseases[10].

Among these, *Stevia rebaudiana*, *Musa acuminata* (banana) and *Solanum lycopersicum* (tomato) have garnered scientific interest due to their diverse phytochemical profiles and nutritional value[11,12]. *Stevia rebaudiana* is a natural sweetener that contains steviol glycosides, compounds with antioxidant and anti-inflammatory properties, and has been reported to have an oral LD_{50} as high as 17 g/kg in rats, indicating a wide safety margin[13]. *Musa acuminata*, especially in its ripe and unripe forms, is a source of dopamine[14] catecholamines, flavonoids, and dietary fibre, with previous studies suggesting its safety at doses above 5000 mg/kg. *Solanum lycopersicum* (tomato) is a widely consumed vegetable known for its high content of lycopene, quercetin, kaempferol, and hydroxycinnamic acid derivatives[15] antioxidants that contribute to cardiovascular and anticancer effects[16]. Tomato-based diets have shown excellent tolerability and protective roles in oxidative stress models[17,18].

Considering the complementary and potentially synergistic antioxidant actions of these plants, a tricomponent polyherbal formulation was developed using aqueous extracts of Stevia, Banana, and Tomato[19]. The formulation aimed to enhance therapeutic potential through additive or synergistic effects while ensuring biocompatibility and safety. Water was chosen as the extraction solvent to preserve hydrophilic phytoconstituents and to mimic traditional usage practices, thereby improving translational relevance[19].

Plant-derived antioxidants may protect cells by scavenging free radicals, reducing lipid peroxidation, chelating metal ions and supporting endogenous antioxidant defence. However, antioxidant-rich extracts may also show dose-dependent toxicity or pro-oxidant effects when used at high concentrations or for prolonged periods. Therefore, although *Stevia rebaudiana*, *Solanum lycopersicum* and *Musa acuminata* contain phenolics, flavonoids, steviol glycosides, lycopene, dopamine and catecholamines with possible antioxidant benefits, their safety must be confirmed before further pharmacological use in oxidative stress-related disorders.

Before advancing to pharmacological, therapeutic, or clinical studies, it is essential to establish the toxicological safety profile of the formulation. Acute toxicity studies serve as a foundational step in determining the appropriate dose range, possible adverse effects, and target organ toxicity.

The above literature encouraged us to fortify the water-soluble ingredients of stevia leaves, banana and tomato fruits for enhancing the antioxidant potential of the formulation[20] which was further screened for acute toxicity in Albino Wistar rats as per OECD 423 guidelines.

Therefore, the present investigation was designed to evaluate the acute oral toxicity of the aqueous tricomponent extract (Stevia: Banana: Tomato in a 7:3:3 ratio) in nulliparous, non-pregnant female Albino Wistar rats. The study was conducted in accordance with OECD guideline 423, which provides a structured

approach for estimating the toxicity of substances. The findings of this study will provide critical preclinical safety data to support the further development and therapeutic application of this formulation for oxidative stress-associated disorders.

2 Methodology

2.1 Plant Materials Extract Preparation and Animal Species

Fresh leaves of *Stevia rebaudiana* and fruits of *Musa acuminata* (banana) and *Solanum lycopersicum* (tomato) were procured from the local market.

The plant materials were washed, shade-dried, and coarsely powdered. Aqueous extracts were prepared using either Soxhlet extraction or microwave-assisted extraction techniques, depending on the material. Specifically, ripe and unripe banana and tomato powders were extracted separately. Based on a factorial design, tricomponent formulations were prepared (7:3:3) for stevia: tomato: banana. Extract, exhibiting superior antioxidant and phenolic properties, was selected for acute toxicity assessment.

Nulliparous, non-pregnant[21] female[22] Albino Wistar rats[23] of 8-12 weeks of age were used as per OECD 423 guidelines. Albino Wistar rats (4-5 weeks old) procured from Central Drug Research Institute, Lucknow, India (Reg. No. 34/GO/Re-SL/Bi-S/99/CPCSEA) and studied at Hygia Institute of Pharmaceutical Education & Research, Lucknow, India (Reg. No. 1088/Re/S/07/CPCSEA) as per the approved protocol: HIPER/IAEC/43/20/08.

2.2 Acute Toxicity Study

Step 1: Extract Dosing of 2000 mg/kg in Three Rats

Groundwork for control and test groups: We housed Albino Wistar rats at the temperature of 22°C (± 3°C), relative humidity of 50-60 %, with a limit of minimum 30% and a maximum of 70%, except during room cleaning. Artificial lighting was maintained 12 hours dark and light cycle (6 am - 6 pm). For feeding, an orthodox laboratory diet was provided with an unrestricted supply of drinking water (*Ad libitum*). Female rats were caged separately until they attained the age of 7-8 weeks. Out of these nulliparous and non-pregnant rats, six rats were selected randomly and marked for identification.

Control group: Three rats were kept in separate cages for 7 days before dose administration to let them acclimatise to the laboratory conditions. Thus, test rats at the start of step 1 dosing were 8-9 weeks old and subjected to the oral administration of the extract at the start of step 1 of the acute toxicity study.

Test group: Three rats were kept in separate cages for 7 days before the dose administration to allow them time for acclimatisation to the laboratory conditions. Therefore, control rats at the start of step 1 dosing were also 8 - 9 weeks old and had age coordination with the test group. The control rats[24] fed with 1% carboxymethyl cellulose in the current study.

Preparation of test doses: A uniform volume of the extract was used in both steps of the study by altering the concentration of the dosing preparation. Administered volume of liquid at a time should not exceed one mL per 100 g of rat body weight (aqueous solutions may be two mL per 100 g of rat body weight). Thereby, a dose volume of one mL per 100 g of rat body weight was employed for preparing the aqueous solutions of the doses shortly (5-15 minutes) before administration.

Dose administration: Rats were fasted by withholding food but not water overnight[25]. After fasting, the animals were weighed and the extract administered to rats in a single oral dose by gavage using an intubation cannula. After the dose administration, food was withheld for 3.5 hours. solution[26].

Observation parameters Signs of toxicity were observed individually frequently on day 1 and thereafter daily for 14 days. We noted the food intake from the quantity of food supplied and the amount remaining after 24 hours. The individual weights of animals were measured 30 minutes before extract administration and daily thereafter for 14 days. At the termination of the step 1 experimental period, rats were sacrificed by anaesthesia using sodium phenobarbitone. The morphology of organs was studied for gross pathology. Liver, heart, brain, kidneys, stomach and spleen[27] were removed, cleaned with saline, and weighed[28]. Each rat was dissected immediately, blood was collected in a test tube containing EDTA via inferior vena cava puncture and subjected to haematological and biochemical examinations. After Hematoxylin and Eosin (H and E) staining, the spleen, liver, kidney and brain were observed microscopically.

Step 2: Extract Dosing of 2000 mg/kg Body Weight in Three Additional Rats

Nulliparous and non-pregnant female rats that attained the age of 10-11 weeks were kept in separate cages for 7 days before step 2 dosing to allow for acclimatisation

to the laboratory conditions. This also allowed a further delay of 7 days for survival confirmation of the previously dosed animals (step 1). After the confirmation of the absence of drug-related mortality / moribund status of the studied animals, further dosing of three animals with the same dose, i.e. 2000 mg/kg, was repeated. Six rats (3 test and 3 control) whose weight was in the range within ± 20 % of the average weight of any previously dosed animals were randomly selected for the step 2 study and marked for identification. Each animal, at the initiation of its step 2 dosing, was 11-12 weeks old. The procedure used for dose preparation, dose administration and monitoring signs of toxicity was identical to step 1 of the acute toxicity study (OECD 423 guidelines). Morphological, biochemical and microscopic studies in dead and surviving animals were similar to step 1 of the study.

2.3 Statistical Analysis

Data were expressed as mean ± SEM for each group (n = 3). Statistical significance between control and test groups was evaluated by an unpaired statistical test using GraphPad Prism 8.4.3.686. Significance was defined as $p > 0.05$ (non-significant), $p \leq 0.05$ (significant), $p \leq 0.01$ (very significant), and $p \leq 0.001$ (highly significant).

3. Results

Triple component extract consisting of stevia extract: tomato extract: banana extract in a ratio of 7:3:3 (B) was screened for acute toxicity in Albino Wistar rats.

3.1 Clinical Signs and Mortality

Throughout the 14-day observation period following the administration of a single oral dose of 2000 mg/kg of Extract B, no mortality or morbidity was recorded in any of the animals in Step 1, as per OECD 423 protocol. The animals exhibited no significant behavioural abnormalities, such as tremors, convulsions, salivation, diarrhoea, lethargy, coma, or postural defects. Coat condition, activity level, and responsiveness to external stimuli remained normal in all test and control groups. These findings indicate that the tricomponent aqueous extract formulation is well tolerated at the tested dose.

3.2 Body Weight and Food Consumption

Body weight was monitored daily and showed a steady and progressive increase in all groups over the 14-day study duration. There was no statistically significant difference ($p > 0.05$) in body weight gain between the control and extract-treated groups (Table 1). Food consumption remained consistent, and no episodes of anorexia or reduced intake were noted. The relative food consumption (g of food per g of body weight) also did not vary significantly between the groups, further suggesting no adverse effect of the extract on appetite or nutrient assimilation.

3.3 Organ Weights and Gross Morphology

Post-mortem examination revealed no gross pathological lesions in the excised organs. Organs such as the liver, kidneys, heart, spleen, brain, and stomach appeared normal in size, shape, and colour. Relative organ weights (g organ/kg body weight) were comparable between the treated and control groups, with no statistically significant deviations. The absence of hypertrophy, atrophy, or oedema in any of the organs supports the non-toxic nature of the extract at the administered dose.

3.4 Hematological Parameters

- Haematological assessments were performed to evaluate the impact of the extract on blood components. The following findings were observed:
- Haemoglobin (Hb), Red Blood Cell (RBC) count, and hematocrit levels were within physiological limits, indicating no induction of anaemia.
- White Blood Cell (WBC) counts showed no leukocytosis or leukopenia.
- Differential leukocyte counts, including neutrophils, lymphocytes, monocytes, eosinophils, and basophils, were normal and comparable to the control group.

The lack of significant haematological deviations suggests that Extract B does not adversely affect hematopoiesis or immune status.

3.5 Biochemical Parameters

- Serum biochemistry was evaluated to assess the functional integrity of major organs, particularly the liver and kidneys. The key findings include:
- Glucose levels remained stable, indicating no hypoglycemic or hyperglycemic effect.
- Urea and creatinine values were within normal reference ranges, suggesting preserved renal function.
- Alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels did not show significant

Table 1. Body weight of rats (g) during the acute toxicity study at a dose of 2000 mg/kg

Step 1				Signs of toxicity
Observation time	**Control**	**Test**	**p-value (significance level)**	**None**
30 minutes before the dose	164.03 ± 0.69	168.13 ± 2.67	0.2109 (Non-significant)	None
24 hours	165.23 ± 0.84	169.90 ± 2.87	0.1941 (Non-significant)	None
Day 2	166.77 ± 0.80	170.87 ± 3.68	0.3378 (Non-significant)	None
Day 3	168.17 ± 0.20	172.57 ± 3.73	0.3045 (Non-significant)	None
Day 4	169.40 ± 0.61	174.43 ± 3.44	0.2228 (Non-significant)	None
Day 5	170.83 ± 0.20	176.10 ± 3.46	0.2037 (Non-significant)	None
Day 6	172.50 ± 0.06	178.00 ± 2.76	0.1174 (Non-significant)	None
Day 7	173.80 ± 0.40	179.13 ± 2.52	0.1047 (Non-significant)	None
Day 8	175.97 ± 0.43	180.97 ± 2.19	0.0883 (Non-significant)	None
Day 9	177.50 ± 0.80	182.60 ± 1.71	0.0540 (Non-significant)	None
Day 10	180.33 ± 0.86	183.80 ± 1.30	0.0906 (Non-significant)	None
Day 11	182.47 ± 1.11	185.77 ± 1.46	0.1461 (Non-significant)	None
Day 12	183.87 ± 0.81	187.23 ± 1.84	0.1695 (Non-significant)	None
Day 13	185.23 ± 0.80	188.70 ± 2.24	0.2183 (Non-significant)	None
Day 14	186.43 ± 1.13	190.07 ± 1.96	0.1846 (Non-significant)	None
Step 2				
Observation time	**Control**	**Test**	**p-value (significance level)**	None
30 minutes before the dose	182.00 ± 0.67	185.60 ± 2.06	0.1724 (Non-significant)	None
24 hours	182.80 ± 0.93	187.33 ± 1.91	0.0997 (Non-significant)	None
Day 2	185.17 ± 0.62	188.53 ± 1.90	0.1669 (Non-significant)	None
Day 3	186.90 ± 0.53	189.80 ± 2.40	0.3035 (Non-significant)	None
Day 4	188.83 ± 0.30	192.03 ± 2.37	0.2513 (Non-significant)	None
Day 5	182.00 ± 0.67	193.73 ± 2.16	0.2602 (Non-significant)	None
Day 6	193.50 ± 0.51	195.83 ± 2.54	0.4189 (Non-significant)	None
Day 7	195.80 ± 0.99	198.30 ± 2.57	0.4154 (Non-significant)	None
Day 8	197.93 ± 0.75	200.67 ± 2.66	0.3787 (Non-significant)	None
Day 9	199.27 ± 0.82	202.67 ± 2.04	0.1972 (Non-significant)	None
Day 10	201.07 ± 0.62	204.77 ± 1.75	0.1174 (Non-significant)	None
Day 11	202.67 ± 0.50	206.63 ± 2.03	0.1311 (Non-significant)	None
Day 12	204.20 ± 1.01	208.60 ± 2.16	0.1392 (Non-significant)	None
Day 13	206.20 ± 0.85	210.03 ± 1.96	0.1481 (Non-significant)	One mortality
Day 14	208.20 ± 0.68	214.15 ± 1.05	0.0149 (Significant)	None, n = 2

elevation, indicating an absence of hepatocellular damage or biliary obstruction.

These results reinforce the biochemical safety of the extract formulation.

The extract did not provoke changes in behaviour, eye, postural abnormalities, breathing, skin, defecation, yellowing or loss of hair in both steps of the study. Excessive weight gain (Figures 1 and 2), salivation, tremors, convulsions, diarrhoea, lethargy, sleep and coma were absent.

Food consumption (Table 2) during step 1 in the test group was in the range of (7.67 - 8.00 g) and control (7.83 - 8.90 g). The second step followed a series of tests and control group as (8.90 - 9.73 g) and (9.37 - 10.45

g), respectively. The relative food consumption (g/g of rat body weight) range of the test and control group was (4.28 - 4.85) and (4.67 - 4.78), respectively, during step 1. Subsequently, step 2 witnessed the test group and control group in a series of (4.55 - 4.97) and (4.87 - 5.17), respectively (Table 3). Morphological (Tables 4 and 5), biochemical (Table 6) parameters were subjected to statistical analysis.

Table 2. Food consumption (g) during the acute toxicity study at a dose of 2000 mg/kg

Step 1			
Period	**Control**	**Test**	**p-value (significance level)**
Day 1	7.83 ± 0.15	7.83 ± 0.20	>0.9999 (Non-significant)
Day 2	7.97 ± 0.12	7.90 ± 0.26	0.8298 (Non-significant)
Day 3	7.90 ± 0.10	8.07 ± 0.27	0.5970 (Non-significant)
Day 4	7.67 ± 0.13	8.13 ± 0.28	0.2120 (Non-significant)
Day 5	7.80 ± 0.10	8.20 ± 0.12	0.0589 (Non-significant)
Day 6	7.93 ± 0.23	8.27 ± 0.17	0.3097 (Non-significant)
Day 7	7.63 ± 0.39	8.40 ± 0.15	0.1432 (Non-significant)
Day 8	7.77 ± 0.22	8.50 ± 0.15	0.0514 (Non-significant)
Day 9	7.97 ± 0.38	8.37 ± 0.19	0.4018 (Non-significant)
Day 10	7.87 ± 0.37	8.57 ± 0.20	0.1733 (Non-significant)
Day 11	7.93 ± 0.24	8.70 ± 0.21	0.0735 (Non-significant)
Day 12	7.93 ± 0.32	8.77 ± 0.22	0.0969 (Non-significant)
Day 13	7.80 ± 0.56	8.77 ± 0.12	0.1649 (Non-significant)
Day 14	8.00 ± 0.53	8.90 ± 0.12	0.1719 (Non-significant)
Step 2			
Period	**Control**	**Test**	**p-value (significance level)**
Day 1	9.07 ± 0.03	9.43 ± 0.20	0.1489 (Non-significant)
Day 2	9.13 ± 0.07	9.50 ± 0.15	0.0927 (Non-significant)
Day 3	9.13 ± 0.12	9.63 ± 0.18	0.0792 (Non-significant)
Day 4	9.13 ± 0.15	9.37 ± 0.27	0.4851 (Non-significant)
Day 5	9.17 ± 0.20	9.77 ± 0.24	0.1290 (Non-significant)
Day 6	8.90 ± 0.36	9.77 ± 0.38	0.1754 (Non-significant)
Day 7	9.30 ± 0.21	9.87 ± 0.33	0.2228 (Non-significant)
Day 8	9.27 ± 0.27	9.97 ± 0.28	0.1506 (Non-significant)
Day 9	9.07 ± 0.37	10.10 ± 0.30	0.0963 (Non-significant)
Day 10	9.23 ± 0.35	10.20 ± 0.30	0.1051 (Non-significant)
Day 11	9.53 ± 0.34	10.30 ± 0.35	0.1910 (Non-significant)
Day 12	9.60 ± 0.45	10.33 ± 0.33	0.2589 (Non-significant)
Day 13	9.67 ± 0.47	10.33 ± 0.27	0.2826 (Non-significant)
Day 14	9.73 ± 0.49	10.45 ± 0.35	0.3726 (Non-significant)

Table 3. Relative food consumption (g/g) during the acute toxicity study at a dose of 2000 mg/kg

Step 1			
Period	**Control**	**Test**	**p-value (significance level)**
Day 1	4.85 ± 0.05	4.70 ± 0.14	0.3632 (Non-significant)
Day 2	4.78 ± 0.04	4.75 ± 0.13	0.8374 (Non-significant)
Day 3	4.60 ± 0.06	4.76 ± 0.15	0.3682 (Non-significant)
Day 4	4.64 ± 0.06	4.75 ± 0.06	0.2464 (Non-significant)
Day 5	4.68 ± 0.12	4.74 ± 0.10	0.7377 (Non-significant)
Day 6	4.47 ± 0.23	4.77 ± 0.11	0.2929 (Non-significant)
Day 7	4.50 ± 0.12	4.78 ± 0.07	0.1295 (Non-significant)
Day 8	4.59 ± 0.23	4.67 ± 0.04	0.7327 (Non-significant)
Day 9	4.47 ± 0.21	4.73 ± 0.06	0.3033 (Non-significant)
Day 10	4.47 ± 0.14	4.76 ± 0.08	0.1571 (Non-significant)
Day 11	4.40 ± 0.19	4.77 ± 0.11	0.1643 (Non-significant)
Day 12	4.28 ± 0.31	4.72 ± 0.05	0.2316 (Non-significant)
Day 13	4.35 ± 0.29	4.75 ± 0.05	0.2471 (Non-significant)
Day 14	4.35 ± 0.27	4.75 ± 0.08	0.2321 (Non-significant)
Step 2			
Period	**Control**	**Test**	**p-value (significance level)**
Day 1	4.80 ± 0.04	5.17 ± 0.15	0.1197 (Non-significant)
Day 2	4.97 ± 0.01	5.04 ± 0.16	0.6303 (Non-significant)
Day 3	4.93 ± 0.03	5.04 ± 0.13	0.4483 (Non-significant)
Day 4	4.89 ± 0.06	5.08 ± 0.16	0.3106 (Non-significant)
Day 5	4.84 ± 0.07	4.87 ± 0.14	0.8097 (Non-significant)
Day 6	4.80 ± 0.11	5.04 ± 0.17	0.3080 (Non-significant)
Day 7	4.60 ± 0.17	4.99 ± 0.25	0.2703 (Non-significant)
Day 8	4.75 ± 0.08	4.98 ± 0.22	0.3768 (Non-significant)
Day 9	4.68 ± 0.12	4.97 ± 0.20	0.2841 (Non-significant)
Day 10	4.55 ± 0.17	4.98 ± 0.19	0.1569 (Non-significant)
Day 11	4.59 ± 0.16	4.98 ± 0.18	0.1774 (Non-significant)
Day 12	4.70 ± 0.16	4.99 ± 0.21	0.3393 (Non-significant)
Day 13	4.70 ± 0.20	4.97 ± 0.19	0.4054 (Non-significant)
Day 14	4.69 ± 0.22	4.92 ± 0.16	0.4341 (Non-significant)

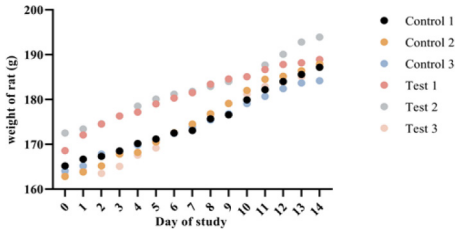


Figure 1. Increase in rat weight during step 1 of the study.

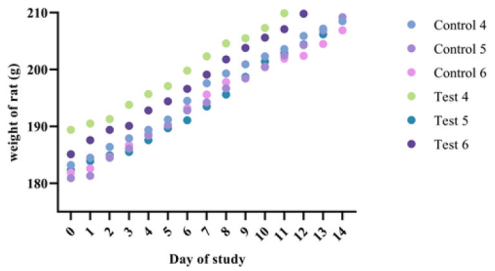


Figure 2 Increase in rat weight during step 2 of the study.

4 Discussion

Consumption of high-calorie foods is a major contributor to metabolic disorders[29]. As a succour, the World Health Organisation promotes a reduction in sugar intake[30]. This assists people in moving toward a state of the finest health[31]. And a low-calorie diet has a retrogressive effect on diabetes and obesity[32]. The reported antioxidant activity[33,34] and the low-calorie profile of stevia leaves

Table 4. Consequence of extract treatment on the change in body weight

Acute toxicity study Initial		Body weight (g)			
		Final	Change		
Step 1	Control 1	165.2	187.2	+ 22.0	22.4 ± 1.46
	Control 2	162.8	187.9	+ 25.1	
	Control 3	164.1	184.2	+ 20.1	
	Test 1	168.6	188.9	+ 20.3	21.93 ± 1.13 (p = 0.8126, Non-significant)
	Test 2	172.5	193.9	+ 21.4	
	Test 3	163.3	187.4	+ 24.1	
Step 2	Control 4	183.2	208.5	+ 25.3	24.63 ± 1.04
	Control 5	180.9	206.9	+ 26.0	
	Control 6	181.9	204.5	+ 22.6	
	Test 4	189.4	215.2	+ 25.8	25.9 ± 1.19 (p = 0.4661, Non-significant)
	Test 5	182.3	206.2 (day 13)	+ 23.9 (day 13)	
	Test 6	185.1	213.1	+ 28.0	

Table 5. Effect of extract on relative organ weights (g/kg of body weight)

Step 1			
Organ	**Control**	**Test**	**p-value (significance level)**
Brain	0.87 ± 0.01	0.78 ± 0.04	0.0860 (Non-significant)
Heart	0.35 ± 0.01	0.29 ± 0.02	0.0488 (Significant)
Liver	4.51 ± 0.07	4.44 ± 0.04	0.4050 (Non-significant)
Spleen	0.27 ± 0.01	0.29 ± 0.01	0.1210 (Non-significant)
Left Kidney	0.88 ± 0.88	0.88 ± 0.03	0.9458 (Non-significant)
Right Kidney	0.83 ± 0.04	0.81 ± 0.04	0.6705 (Non-significant)
Stomach	0.59 ± 0.03	0.60 ± 0.04	0.8406 (Non-significant)
Step 2			
Organ	**Control**	**Test**	**p-value (significance level)**
Brain	0.89 ± 0.03	0.77 ± 0.07	0.1777 (Non-significant)
Heart	0.34 ± 0.03	0.28 ± 0.02	0.1342 (Non-significant)
Liver	4.48 ± 0.11	4.39 ± 0.11	0.6072 (Non-significant)
Spleen	0.28 ± 0.01	0.30 ± 0.01	0.2507 (Non-significant)
Left Kidney	0.87 ± 0.04	0.86 ± 0.03	0.8891 (Non-significant)
Right Kidney	0.79 ± 0.02	0.80 ± 0.04	0.7669 (Non-significant)
Stomach	0.58 ± 0.02	0.60 ± 0.03	0.7430 (Non-significant)

encouraged us for its addition to fruit extracts. We hoped for further enhancement of antioxidant activity with an additional benefit of the low-calorie load of the developed extract. The significant *in vitro* results encouraged us to screen the toxicity profile of the extract in the biological system. Stevia extract has an oral LD50 of 17 g/kg body weight[35]. Tomato, as well as banana, has an LD50 of more than 5000 mg/kg of rat body weight[36]. Thus, stevia, banana and tomato have considerably low toxicity and a 2000 mg/kg dose as per the rat body weight was selected for the acute toxicity study.

During Step 1, no mortality or moribund condition was observed in any animal treated with the extract at 2000 mg/kg. However, in Step 2, one mortality was recorded in the extract-treated group on Day 13. The remaining animals survived until the end of the 14-day observation period and did not show marked behavioural abnormalities. Therefore, the extract did not produce consistent mortality at 2000 mg/kg, but the occurrence of one death indicates that the formulation should be interpreted with caution and requires further dose-dependent toxicity evaluation.

Body weight, food consumption and food consumption relative to rat body weight between control and test groups were comparable. Organ weight and the morphology of extract treated group groups showed non-significant changes as compared to the control group. Likewise, haematological and biochemical parameters indicated the absence of hematotoxicity as well as hepatotoxicity due to the non-significance between the control and test groups.

Although isolated statistically significant differences were observed for Step 2 Day 14 body weight, Step 1 relative heart weight and Step 2 urea level, these changes were not accompanied by consistent clinical signs, gross pathological changes, haematological abnormalities or histopathological evidence of organ injury. Therefore, these isolated findings should be interpreted cautiously and may not indicate a consistent toxicity pattern, particularly considering the small group size. Microscopic screening of the spleen, liver, kidney, and brain confirmed the absence of structural modification. Hematoxylin and Eosin-stained sections showed normal histology of the brain, showing grey and white matter of the cerebral cortex, and normal red and white pulp of the spleen. Normal histology of the liver is revealed through the central vein and hepatic lobules along with the portal triad. The cortical part of the kidney showed normally appearing glomeruli along with the cross-section of the proximal and distal arms of collecting tubules. Thus, we found a similar safety profile for the current tomato formulation as reported previously by Phachonpai[36]. The outcomes of the current study support a same opinion as Saad[37] that stevia products are safe and do not cause any health problems.

We classified the extract in category 5 (LD50 > 2000-5000 mg/kg body weight) as per the globally harmonised classification system, and the LD50 cut-off as 2500 mg/kg of body weight. Customarily, the extract dose used for biological screening in animals is $1/10^{th}$ of the observed acute toxicity test or extrapolated from the human dose. The reference body surface area of a rat (0.02 m^2) is multiplied by the maximum dose tested during the acute toxicity study[38]; here, 0.02 × 2000 = 40 mg/kg. Therefore, biological profiling may be executed in the range of 40-200 mg/kg of rat body weight.

The present findings agree that plant-derived/polyherbal preparations showed no major acute toxicity when assessed through mortality, behaviour, body weight, organ weight, haematological, biochemical and histopathological parameters[39,40]. The safety profile may be related to antioxidant phytoconstituents such as phenolics and flavonoids, which reduce oxidative stress through free-radical scavenging, metal chelation and modulation of oxidative pathways[41,42]. Future studies should include detailed phytochemical standardisation using HPTLC, FTIR or related fingerprinting methods to improve reproducibility and quality control of the formulation[43]. No mortality was observed in Step 1; however, one mortality occurred in the extract-treated group during Step 2. The surviving animals showed no marked behavioural abnormality, body-weight suppression, biochemical disturbance or histopathological alteration. Therefore, the extract did not show a consistent pattern of acute systemic toxicity at 2000 mg/kg, but the single mortality warrants cautious interpretation. Further dose ranging, repeated dose toxicity and detailed phytochemical standardisation studies are recommended before pharmacological or translational development. These observations are consistent with previous reports showing low acute toxicity of plant-derived antioxidant preparations. The presence of phenolics and flavonoids may support cellular protection by reducing oxidative stress, scavenging reactive oxygen species and limiting lipid peroxidation. However, these mechanistic links require

confirmation through dedicated antioxidant, molecular and repeated-dose toxicity studies.

5. Conclusions

The plethora of biologically active constituents and satisfactory preclinical evidence of safety paves the path for further phytochemical and pharmacological screening for the development of a prophylactic/therapeutic option in the management of free radical-mediated disorders. The current toxicity Study provides a strong foundation for establishing *in vitro* observations *in vivo,* initially in animal models and further continued as human studies.

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Author Contributions

Shom Prakash Kushwaha: Experimental work; Pavan Kumar, Bidhyut Kumar Dubey, Arun Kumar and Kuldeep Singh: Drafting of research findings; Syed Misbahul Hasan, Muhammad Arif, Pushpendra Soni, Abdul Hafeez, Ajay Kumar Shukla and Karuna Shanker Shukla: Proofreading and improving the draft of the article; Sujeet Kumar Gupta and Sushil Kumar: Supervision of work. All authors have read and agreed to the published version of the manuscript.

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