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Research Article

Preclinical Safety and Toxicological Evaluation of Mucuna Protein (80%) Via Acute and Sub-Acute Oral Toxicity Studies

Srilakshmi Aluri[1]*, Anzar CA[1], Joseph MV[1], Faizal Mohamed[2], Bineesh Eranimose[1] and Vadiraj Bharadwaj[1]

[1]*Research and Development Centre, Ingex Botanicals Limited Nelamangala, Bangalore, 562123, India*

[2]*Bio-Actives Japan Corporation, BAJ Building Toshima-KU, 171-0052, Japan*

***Corresponding Author:** Srilakshmi Aluri, Research and Development Centre, Ingex Botanicals Limited Nelamangala, Bangalore, 562123, India.

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Abstract

Mucuna pruriens (L.) DC., (Velvet bean), is a legume known for its high protein content and the neuroprotective compound notably L-DOPA. Despite their potential nutraceutical applications and traditional uses, thorough toxicological studies on protein concentrates still remain scarce. This study evaluated the acute and sub-acute oral toxicity of Mucuna Protein 80% following OECD guidelines in Wistar rats. No mortality and adverse effects were observed in single dose acute toxicity (2000 mg/kg) placing the protein in unclassified or category 5 under the Global Harmonized system. No significant behavioral or clinical toxicity signs were demonstrated at Sub acute repeated dosing (1000 and 2000 mg/kg) over 28 days. Quantification of anti-nutrient factors in Mucuna protein were also carried out and was found to be only in negligible amounts. Biochemical, Hematological and histopathological analysis indicated safety at the dosages tested and only minor dose-dependent increase in mean corpuscular hemoglobin at 2000 mg/kg and minor increase in basophil at 1000 mg/kg without any signs of kidney and liver toxicity. Organ weights remain constant and reveal normal structure. Overall study presented No Observed Adverse Effect Level (NOAEL) exceeding 2000 mg/kg. This supports the safety of Mucuna protein 80% for utilization in functional foods and nutraceuticals and establishes a scientific basis for its safe commercialization in accordance with regulatory standards.

Keywords: Acute Toxicity; *Mucuna pruriens*; Nutraceuticals; Phytochemicals; Sub-Acute Toxicity

Abbreviations

L-DOPA: L-3,4-dihydroxyphenylalanine; OECD: Organization for Economic Co-operation and Development; NOAEL: No Observed Adverse Effect Level; $LD_{50:}$ Lethal Dose, 50%; FRLHT: Foundation for Revitalisation of Local Health Traditions; HPLC: High Performance Liquid Chromatography; CCSEA: Committee for Control and Supervision of Experiments on Animals; EDTA: Ethylenediaminetetraacetic acid; RBC: Red Blood Cells; WBC: White Blood Cells; MCH: Mean Corpuscular Hemoglobin; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Concentration; RDW-CV: Red Cell Distribution Width; AST: Aspartate Transaminase; ALKP: Alkaline Phosphatase; ALT: Alanine Aminotransferase; ANOVA: Analysis of Variance; BLQ: Below the Limit of Quantification; GHS: Globally Harmonized System; CMC: Carboxymethylcellulose; VLDL: Very-Low-Density Lipoprotein; HDL: High-Density Lipoprotein

Introduction

Mucuna pruriens (L.) DC., commonly known as velvet bean, is a high-yielding underutilized leguminous crop recognized for its nutritional value and its ability to tolerate environmental stresses, ensuring its availability throughout the year [1]. Being native to tropical regions of Africa and Asia, particularly China and India, it holds an important place in Ayurveda, where it is valued for promoting neurological health and overall well-being [2,3]. The seeds are rich in bioactive compound, particularly L-DOPA, a precursor to Dopamine that plays a critical role in managing Parkinson's disease [4,5].

Mucuna is cultivated as a food crop as it contains approximately 27% of protein and is also rich in minerals and it's a main dish of tribal groups of Nigeria [6]. Indian Tribals like Mundari, Kanikkas and Dravidian groups also consume mature seeds. Tribals follow special processing methods like draining and boiling for prolonged time to reduce the toxicity [46]. The physiological attributes of *M. pruriens* are mainly due to its secondary metabolites, which exhibit neuroprotective, anti-neoplastic, anti-diabetic, anti-epileptic, antioxidant, aphrodisiac, cognitive, anti-inflammatory, antivenom, anti-helminthic, antimicrobial, analgesic activity, and metabolic regulatory effects [6-8]. Safety evaluation is necessary for *Mucuna* as the seeds contain anti-nutritional factors such as L-DOPA (7%-10%), phytic acid, trypsin inhibitors, alkaloids, tannins, and saponins [9,10]. Despite widespread belief in the safety of medicinal plants, studies have also revealed their potential toxic, mutagenic, and carcinogenic effects [11,12]. Believing the assumption that botanical products are safe is challenging and there is a need for rigorous toxicity testing of all herbal formulations [3,13,14].

Historically, it has been used for treating neurological disorders, including "Kampavata," akin to Parkinson's disease [3]. Beyond neurological uses, in Indian and West African medicine, seeds are also traditionally used against snakebites, aphrodisiacs, and as uterine stimulants [10]. Recent research reported effects, such as reducing amyloid-β accumulation and improving memory in the case of Alzheimer's disease models [10]. Agronomically, *M. pruriens* is a drought-tolerant climbing legume that grows well in acidic and low-nutrient soils but is sensitive to cold and slow-growing under wet, cool conditions [15,16]. It typically completes its life cycle in 160–165 days, with flowering between 60 and 70 days, extending up to 210–240 days in Northern plains [17,18]. The protein content (20–30%) of seeds makes it attractive as a vegan protein source with a growing demand for sustainable, hypoallergenic alternatives [19,20]. This demand aligns with global trends favoring legume proteins like soy and pea for their nutritional and environmental benefits, boosting interest in novel sources such as *Mucuna* protein [21].

Animal studies are critical for evaluating safety of substances by determining key parameters such as the median lethal dose (LD_{50}) and NOAEL. This preclinical data underscores the risk assessment and regulatory decision-making for product safety [8]. Scarce safety data exists for crude extracts and L-DOPA, but limited information is only available on concentrated protein isolates. The present study evaluates the acute and sub-acute oral toxicity of Mucuna Protein 80% in Wistar rats.

Materials and Methods

Plant Collection and Identification

Mucuna pruriens (L.) DC (Fabaceae) was collected and taxonomically authenticated for the current study by Foundation for Revitalisation of Local Health Traditions (FRLHT) with Ref. No. FRLHT/RD R/Authen/2025/03/01. The matured seeds were purchased from Adnan Traders, Rajkot, Gujarat, India, under Batch No. BH25R0013 with a Collection Number. 6764. The authenticated material was thoroughly cleaned, dried, and processed under appropriate conditions for the current studies.

Quantification of anti nutrient factors

L-DOPA quantification carried out by reverse phase HPLC with UV detection [46]. Hydrocyanic acid by picrate colorimetric method (A_{510}) following acid/enzymatic hydrolysis [48]. and phytic acid by Wade reagent spectrophotometric assay (A_{500}) following acid/enzymatic hydrolysis [47]. Tannins by vanillin-HCL assay and saponins by vanillin-sulfuric acid spectrophotometric methods [48,49].

Experimental animals

Albino Wistar rats were used in the present study to analyses the acute and sub-acute toxicity studies of Mucuna Protein 80% with Centre CCSEA approval (No. 889/PO/Re/S/05/CCSEA). Animals were provided with commercial feed and water ad libitum and acclimated for one week, housed under standard environmental conditions. All toxicological studies were performed in accordance with standard OECD guidelines.

Experimental design

Acute oral toxicity studies

Acute oral toxicity of Mucuna Protein 80% evaluated as per OECD Test Guideline 420 (Fixed Dose Procedure) using five healthy nulliparous, non-pregnant adult female Albino Wistar rats. A single dose of 2000 mg/kg body weight was administered orally and the Individual rats were observed for signs of toxicity or mortality initially during the first 30 minutes, periodically over 24 hours with special attention during the first 4 hours, and then daily for 14 days. Behavioral and autonomic responses were monitored along with specific signs such as tremors, salivation, lethargy, diarrhea, convulsions, sleep, and coma to assess the acute toxicity profile and classify them according to the Globally Harmonized System for acute toxicity [22].

Sub-acute toxicity studies

The sub-acute oral toxicity study of Mucuna Protein 80% was carried out as per OECD Test Guideline 407 (Repeated Dose Study) with minor modifications at two different dosages (1000 mg/kg and 2000 mg/kg body weight) for a period of 28 days. Hazard identification, risk assessment, dose-response relationships, and determination of the No-Observed Adverse Effect Level (NOAEL) were evaluated [23].

Observation parameters

Clinical observation

Daily clinical observations were systematically conducted at a consistent time after drug administration. Physical, neurological, and behavioral changes including posture and movement were monitored. Additionally, mortality and morbidity assessment were carried out twice daily followed by weekly bodyweight measurements and daily feed and water consumption were noted using standard measuring devices [23].

Laboratory investigations

Blood samples were collected from the anesthetized animals on 29th day of study via retro-orbital plexus into EDTA tubes for hematology and plain tubes (without anticoagulants) for serum biochemistry.

Hematological analysis

Leukocyte counts, platelet count, hemoglobin, erythrocyte count (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and red cell distribution width (RDW-CV) were analyzed using an automated hematology analyzer (YUMIZEN H500) [24].

Biochemical analysis

Serum was separated from blood samples by centrifugation (3500 rpm, 10 min) and quantified for biochemical parameters including aspartate transaminase (AST), alkaline phosphatase (ALKP), alanine aminotransferase (ALT), serum albumin, globulin, glucose, total bilirubin, cholesterol, protein, triglycerides, urea, uric acid, creatinine, and electrolytes (calcium, chloride, phosphorus, potassium, sodium,) using a VITROS 5.0/F auto analyzer and standard methods [25].

Histopathological analysis

Animals were sacrificed on the 29th day of study by overdose anesthesia and dissected. Organs (liver, heart, kidney, spleen, testis, and ovary) were excised, weighed and fixed (10% formal saline solution). Paraffin-embedded sections (5 µm thickness) were stained with hematoxylin and eosin for histopathology [26,27].

Statistical analysis

Results were expressed as mean ± SEM. Statistical analyses of the study were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test in GraphPad Prism v8.0. (p-value < 0.05 was considered significant) [28].

Results and Discussion

Quantification of anti-nutrient factors in Mucuna 80% protein

The analysis of Mucuna protein revealed the presence anti nutrient factors at negligible levels/ below quantifiable limits. This outcome can be attributed to the optimized extraction and purification process employed which effectively remove non proteinaceous constituents while retaining the essential amino acid profiles. The negligible levels of antinutrients in the 80% protein fraction indicates a high degree of safety and digestibility making it suitable for use in Nutraceuticals, functional foods and dietary supplements. Previous studies have reported the raw Mucuna seeds contain significant level of L-Dopa (3-7%) which can pose safety concerns; however, the current process demonstrated an effective removal of L-Dopa (0.04%), Hydrocyanic acid (BLQ), Phytic acid (0.02%), saponins (1%), tannins (0.5%) during protein isolation [9]. These findings are in agreement with earlier reports suggesting that thermal or enzymatic treatment substantially reduces or eliminate anti nutritional components in Mucuna seeds derivatives [46]. The present study thus confirms that developed Mucuna protein 80% is free from anti-nutrient interference offering an improved nutritional and safety profiles over Raw or minimally processed seed flours.

Acute oral toxicity effect of the Mucuna Protein 80% on Single dosing study

The acute oral toxicity was evaluated by administering a single limit dose of 2000 mg/kg and various parameters were monitored over a period of 14-days at different time intervals (2 hr, 1 day, 7-day, 14 day). The initial mean body weight of the animals was 187.00 ± 3.06 g, which increased by the end of the study to 193.70 ± 4.10 g, with a weight gain (6.67 ± 2.03 g Body weight gain), indicating no detrimental effect on general health. No mortality or toxic symptoms were observed throughout the observation period; this classifies it as non-toxic according to OECD guideline 423 [29].

Comprehensive behavioral and somatomotor examinations were carried out, including observations of fur and skin condition, eye appearance, respiratory function, urination, feces consistency, locomotor activity, and grip strength, all of which remained within normal limits. Additionally, the animals exhibited no signs of salivation, diarrhea, sedation, loss of righting reflex, writhing, itching, straub phenomenon, piloerection, convulsions, tremors, or coma. These observations were consistent with previous studies on *M. pruriens* seed extracts, which reported lack of mortality or significant toxic effects at doses up to 2000 mg/kg in laboratory rodent models [30].

These results demonstrated that a single oral dose of Mucuna Protein 80% (2000 mg/kg) is safely tolerated without causing any acute toxicity in the tested animals. Based on the absence of adverse effects and zero mortality throughout the study the median lethal dose (LD_{50}) of Mucuna Protein 80% is estimated to be greater than 2000 mg/kg. According to the Globally Harmonized System (GHS) of classification, the sample is categorized as either category 5 or unclassified for acute toxicity (LD_{50} > 2000 mg/kg), which signifies low acute oral toxicity. The median lethal dose (LD_{50}) of Mucuna Protein 80% is therefore estimated to be greater than 2000 mg/kg. This safety assessment was in consistent with earlier findings on *M. pruriens* extracts in the literature, where LD_{50} values exceed this limit [31].

Sub-acute Oral Toxicity effect of Mucuna Protein 80% on Body weight (Repeated dosing-28 days) study

The daily administration of Mucuna protein for about 28 days notably improves the body weight. Body weight was significantly ($P < 0.05$) higher on the 28th day in the male rats receiving 2000 mg/kg protein than normal control rats. Body weight gain was significantly ($P < 0.01$) higher in the 2000 mg/kg treatment group than the normal control group. (Table 3). Similar findings have been reported in previous reports, where improvement is seen in the body weight and physiological performance which indicate safety of Mucuna protein [32].

Table 1: Experimental Design of Acute Oral Toxicity Studies.

Animals used	Female Albino Wistar rats [5]
Age and Body Weight	8-12 weeks old; 180-220 g
Drug/Dose	Mucuna Protein 80% (2000 mg/kg b.w.p.o)
Duration of study	14 days

Table 2: Experimental Design of Sub-acute Toxicity Studies.

Animals model	Albino Wistar rats [Male 15 + Female 15]
Age and Body Weight	8-12 weeks old; 180-220 g
Drug/Dose	Mucuna Protein 80% (1000 mg/kg b.w.p.o and 2000 mg/kg b.w.p.o)
Duration of study	28 days
Animal Grouping	
Group I	10 rats, Normal control (received 0.5% CMC at the dose of 1 ml/100 gm, p. o. for 28 days)
Group II	10 rats, Received Mucuna Protein 80% (1000 mg/kg, p. o. for 28 days)
Group III	10 rats, Received Mucuna Protein 80% (2000 mg/kg, p. o. for 28 days)

Table 3: Effect of Mucuna Protein 80% on Body Weight Changes in Sub-acute Oral Toxicity Studies.

Sl. No.	Groups	Sex	Bodyweight (in g)					
			Base line	Day 7	Day 14	Day 21	Day 28	Changes in body weight gain
1.	Group-I (Control - 0.5% CMC)	Male (n = 5)	217.3 ± 1.86	221.7 ± 1.67	232.3 ± 2.85	240.7 ± 1.76	247.7 ± 5.88	(+) 30.33 ± 1.76
		Female (n = 5)	185.7 ± 5.93	191.0 ± 6.08	202.3 ± 4.84	215.7 ± 2.96	221.3 ± 3.28	(+) 35.67 ± 3.38
		Both (n = 10)	201.5 ± 7.61	206.3 ± 7.42	217.3 ± 7.16	228.2 ± 5.80	234.5 ± 6.08	(+) 33.0 ± 2.08
2.	Group-II (Mucuna Protein - 1000 mg/kg p. o.)	Male (n = 5)	214.3 ± 3.18	218.7 ± 3.48	227.7 ± 3.28	242.3 ± 4.18	251.7 ± 4.84	(+) 37.33 ± 2.60
		Female (n = 5)	187.0 ± 6.56	192.3 ± 6.33	202.3 ± 5.90	217.0 ± 3.61	227.0 ± 2.08	(+) 40.0 ± 6.66
		Both (n = 10)	200.7 ± 6.93	205.5 ± 6.72	215.0 ± 6.42	229.7 ± 6.18	239.3 ± 6.00	(+) 38.67 ± 3.25
3.	Group-III (Mucuna Protein - 2000 mg/kg p. o.)	Male (n = 5)	210.0 ± 3.46	216.7 ± 2.19	229.7 ± 1.20	253.0 ± 2.65	263.0 ± 2.65*	(+) 53.00 ± 3.61**
		Female (n = 5)	187.0 ± 5.69	192.0 ± 5.57	214.0 ± 2.65	219.7 ± 2.85	231.7 ± 1.45	(+) 44.67 ± 7.12
		Both (n = 10)	198.5 ± 5.94	204.3 ± 6.13	221.8 ± 3.74	236.3 ± 7.65	247.3 ± 7.14	(+) 48.83 ± 4.03**

Data expressed as mean ± SEM, with (+) indicate body weight gain. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests. with statistical significance: *** P < 0.001, ** - P < 0.01, *- P < 0.05.

Effect of Mucuna Protein 80% on observational and behavioral parameters in the Sub-acute Oral Toxicity (Repeated dosing-28 days) study

The repeated dosing sub-acute toxicity study of Mucuna Protein 80% was conducted on three groups of animals, including a control group (Group-I) and two treatment groups receiving Group-II (1000 mg/kg) and Group-III (2000 mg/kg) doses respectively, observed over 28 days. Across all groups and throughout the study period, there were no adverse observations related to fur and skin condition, eye abnormalities such as exophthalmos or lacrimation, respiration, urination, feces consistency, or sleep patterns, all of which remained normal (N). This result indicates good tolerability and safety of Mucuna Protein supported by similar toxicity studies [33].

Parameters such as sedation, loss of righting reflex, itching, and convulsion/tremor/coma were consistently absent in all animals from control and treatment groups during the entire dosing period. Importantly, there was no mortality recorded in any group at any time point. Overall, the study indicated that repeated oral administration of Mucuna Protein 80% up to 2000 mg/kg did not induce any observable toxic signs or mortality in the tested animals during the sub-acute toxicity assessment which is consistent with previous studies [33].

Effect of Mucuna Protein 80% on Feed intake and Water intake in the Sub-acute Oral Toxicity (Repeated dosing-28 days) study

The repeated 28 days daily dosing of Mucuna Protein 80% sample at the dose levels of 1000 mg/kg and 2000 mg/kg does not significantly influence the feed and water intake when compared to the normal control group. Therefore, the repeated daily dosing of Mucuna Protein 80% does not influence the regular diet and water intake (Table 4). This normal diet and hydration patterns are critical for both nutrient absorption and energy homeostasis, which aligns with safety and lack of negative health impact [34].

Table 4: Effect of Mucuna Protein 80% on Feed and water Intake in Repeated dosing Studies.

S. No.	Group	Sex	Feed intake (g/day/animal)				Water intake (ml/day/animal)			
			1st Week	2nd Week	3rd Week	4th Week	1st Week	2nd Week	3rd Week	4th Week
1.	Group-I (Control)	Male	10.35 ± 0.78	10.31 ± 1.00	11.65 ± 1.00	11.15 ± 0.75	13.43 ± 0.84	13.19 ± 0.82	14.44 ± 0.93	12.34 ± 0.84
		Female	9.22 ± 0.57	10.10 ± 0.65	10.68 ± 0.95	11.74 ± 0.88	12.42 ± 0.67	12.50 ± 0.43	12.45 ± 0.72	12.09 ± 0.42
		Both	9.79 ± 1.02	10.21 ± 0.76	11.17 ± 0.97	11.45 ± 0.80	12.93 ± 0.73	12.86 ± 0.65	13.45 ± 0.88	12.22 ± 0.60
2.	Group-II (Mucuna Protein -1000 mg/kg)	Male	10.84 ± 0.66	10.34 ± 1.00	11.68 ± 0.97	11.25 ± 1.02	14.12 ± 0.86	15.33 ± 0.78	15.60 ± 2.12	14.45 ± 0.22
		Female	9.79 ± 0.85	9.87 ± 1.02	10.39 ± 1.00	11.02 ± 0.73	12.62 ± 0.68	12.07 ± 1.00	13.65 ± 0.88	12.67 ± 0.54
		Both	10.32 ± 1.12	10.11 ± 0.78	11.04 ± 0.98	11.14 ± 0.85	13.37 ± 0.74	13.70 ± 0.87	14.62 ± 1.55	13.56 ± 0.33
3.	Group-III (Mucuna Protein -2000 mg/kg)	Male	10.13 ± 0.98	10.13 ± 0.79	12.05 ± 0.98	12.20 ± 0.62	12.84 ± 0.59	16.04 ± 0.33	15.04 ± 0.43	13.87 ± 0.72
		Female	9.63 ± 1.32	9.65 ± 0.87	10.64 ± 0.42	11.22 ± 0.80	13.02 ± 0.42	11.34 ± 0.74	13.24 ± 0.62	13.32 ± 0.61
		Both	9.88 ± 0.79	9.89 ± 0.82	11.35 ± 0.67	11.71 ± 0.77	12.93 ± 0.54	13.69 ± 0.58	14.14 ± 0.51	13.60 ± 0.70

Effect of Mucuna Protein 80% on Hematological Parameters in Repeated dosing Studies

The repeated administration of 1000 mg/kg and 2000 mg/kg Mucuna protein for 28 days does not significantly alter the RBC, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and Red Cell Distribution Width (RDW-CV) when compared to the normal control. At 1000 mg/kg MCHC was significantly increased ($P < 0.05$), indicating more hemoglobin than normal possibly due to vitamin B12 deficiency or liver dysfunction and absence of erythrocyte toxicity or anemia

[35]. No significant alterations were observed in the WBC, lymphocytes, neutrophils, eosinophil, monocytes, and platelet count but a significant ($P < 0.05$) increase in basophils were observed in 1000 mg/kg dosing. Alteration in the Basophil was observed in earlier studies [45]. This increase may be due to some allergic or inflammatory conditions since basophils are mediators of such immune responses but not seemed to be pathological (Table 5) [36,45].

Effect of Mucuna Protein-80% on Blood Glucose and Lipid Profile in Repeated dosing Studies

Repeated 28- day dosing of Mucuna protein-80% does not significantly alter blood glucose, triglycerides, VLDL, or HDL levels in either sex. However, serum total cholesterol was significantly ($P < 0.05$) increased in male rats administered with 2000 mg/kg Mucuna protein than normal control, potentially suggesting altered liver function (Table 6).

Table 5: Effect of Mucuna Protein 80% on Hematological Parameters in Repeated dosing Studies.

Parameter	Sex	Group-I (Control-0.5%CMC)	Group-II (Mucuna Protein- 1000 mg/kg)	Group-III (Mucuna Protein - 2000 mg/kg)
RBC ($x10^6/mm^3$)	Male	8.08 ± 0.12	8.34 ± 0.15	8.19 ± 0.12
	Female	6.67 ± 0.72	7.08 ± 0.82	7.11 ± 0.59
	Both	7.38 ± 0.45	7.71 ± 0.47	7.65 ± 0.36
Hemoglobin (%)	Male	12.17 ± 1.19	14.00 ± 0.25	11.80 ± 0.53
	Female	11.30 ± 1.29	12.60 ± 1.25	13.43 ± 0.22
	Both	11.73 ± 0.81	13.30 ± 0.65	12.02 ± 0.38
Hematocrit (%)	Male	42.97 ± 3.31	50.00 ± 1.04	43.17 ± 2.32
	Female	40.53 ± 4.98	41.93 ± 4.05	45.33 ± 3.84
	Both	41.75 ± 2.73	45.97 ± 2.60	44.25 ± 2.07
MCV (fL)	Male	58.27 ± 1.55	59.97 ± 0.87	55.13 ± 1.62
	Female	56.53 ± 1.35	58.27 ± 2.36	55.33 ± 2.33
	Both	57.40 ± 1.00	59.12 ± 1.19	55.23 ± 1.27
MCH (pg)	Male	16.43 ± 0.23	17.87 ± 0.32	16.57 ± 0.72
	Female	17.07 ± 0.53	17.87 ± 0.32	16.80 ± 0.42
	Both	16.75 ± 0.30	17.87 ± 0.20*	16.68 ± 0.38
MCHC (g/dL)	Male	28.23 ± 0.88	28.00 ± 0.21	29.43 ± 1.11
	Female	29.97 ± 0.55	30.30 ± 0.38	28.67 ± 1.20
	Both	29.10 ± 0.61	29.20 ± 0.55	29.10 ± 0.76
RDW-CV (%)	Male	11.70 ± 0.26	10.97 ± 0.59	11.13 ± 0.47
	Female	9.57 ± 0.20	10.57 ± 0.38	10.03 ± 0.61
	Both	10.63 ± 0.50	10.77 ± 0.32	10.58 ± 0.42
WBC (/µL)	Male	10013 ± 824.50	10473 ± 262.1	9623 ± 374.40
	Female	9257 ± 683.50	8820 ± 105.50	8603 ± 853.00
	Both	9635 ± 508.00	9647 ± 610.80	9113 ± 475.00
Neutrophils %	Male	23.37 ± 3.82	21.03 ± 1.84	23.03 ± 2.11
	Female	19.60 ± 2.32	28.64 ± 9.63	20.40 ± 2.36
	Both	21.48 ± 2.17	24.84 ± 5.96	21.72 ± 1.53

Lymphocytes %	Male	69.93 ± 4.07	76.13 ± 5.37	68.37 ± 3.15
	Female	71.67 ± 5.93	51.40 ± 15.05	65.73 ± 3.00
	Both	70.80 ± 3.24	63.77 ± 9.04	67.05 ± 2.02
Monocytes %	Male	1.83 ± 0.26	2.20 ± 0.72	2.57 ± 0.75
	Female	1.43 ± 0.56	2.13 ± 0.77	2.33 ± 0.88
	Both	1.63 ± 0.29	2.17 ± 0.47	2.45 ± 0.52
Eosinophils %	Male	2.30 ± 0.76	1.40 ± 0.40	1.77 ± 0.32
	Female	1.00 ± 0.29	1.17 ± 0.12	1.53 ± 0.58
	Both	1.65 ± 0.47	1.28 ± 0.20	1.65 ± 0.30
Basophils %	Male	1.13 ± 0.03	2.67 ± 1.14	1.60 ± 0.40
	Female	1.23 ± 0.15	3.93 ± 1.34	1.23 ± 0.32
	Both	1.18 ± 0.08	3.30 ± 0.83*	1.42 ± 0.22
Platelets ($x10^5/mm^3$)	Male	9.15 ± 1.49	7.82 ± 0.57	7.62 ± 0.93
	Female	7.85 ± 1.62	7.49 ± 1.87	8.12 ± 0.69
	Both	8.50 ± 1.03	7.66 ± 0.88	7.87 ± 0.53

Data expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests, with statistical significance: *** P < 0.001, ** - P < 0.01, *- P < 0.05.

Table 6: Effect of Mucuna Protein-80% on Blood Glucose and Lipid Profile in Repeated dosing Studies.

Parameter	Sex	Group-I (Control- 0.5%CMC)	Group-II (Mucuna Protein-1000 mg/kg)	Group-III (Mucuna Protein - 2000 mg/kg)
Blood Glucose (mg/dL)	Male	89.67 ± 4.33	85.67 ± 4.36	88.67 ± 3.53
	Female	89.33 ± 2.03	88.00 ± 2.52	83.67 ± 4.26
	Both	89.50 ± 2.14	86.50 ± 2.35	86.17 ± 2.71
Total Cholesterol (mg/dL)	Male	60.00 ± 2.65	76.33 ± 2.25	103.00 ± 9.85*
	Female	75.00 ± 2.65	70.33 ± 7.80	76.00 ± 6.08
	Both	67.50 ± 3.75	73.33 ± 9.76	89.50 ± 7.95
TG (mg/dL)	Male	114.00 ± 6.81	162.00 ± 23.86	114.70 ± 5.46
	Female	162.70 ± 35.35	132.70 ± 16.01	127.00 ± 8.72
	Both	138.30 ± 19.43	147.30 ± 14.43	120.80 ± 5.36
HDL (mg/dL)	Male	34.33 ± 0.88	35.33 ± 2.40	36.00 ± 1.53
	Female	31.00 ± 1.00	34.00 ± 1.53	31.33 ± 2.60
	Both	32.67 ± 0.95	34.67 ± 1.31	33.67 ± 1.71
VLDL (mg/dL)	Male	22.80 ± 1.36	32.40 ± 4.77	22.93 ± 1.09
	Female	32.53 ± 7.07	26.53 ± 3.20	25.40 ± 1.74
	Both	27.67 ± 3.89	29.47 ± 2.89	24.17 ± 1.07

Data expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests, with statistical significance: *** P < 0.001, ** - P < 0.01, *- P < 0.05.

Effect of Mucuna Protein-80% on Liver Function test in Repeated dosing Studies

Treatment with Mucuna Protein showed no significant changes in the serum total bilirubin, total protein, globulin, AST, ALKP, and ALT levels compared to normal control. A significant ($P < 0.05$) elevation of serum albumin level was observed in the female rats receiving 2000 mg/kg, might be due to excessive protein intake or certain inflammatory conditions. But it does not significantly alter the Albumin/ Globulin ratio (Table 7) indicating that these doses do not cause hepatocellular damage or impair liver function [37].

Table 7: Effect of Mucuna Protein 80% on Liver function test in Repeated dosing Studies.

Parameter	Sex	Group-I (Control-0.5% CMC)	Group-II (Mucuna Protein- 1000 mg/kg)	Group-III (Mucuna Protein –2000 mg/kg)
Total Bilirubin (mg/dL)	Male	0.43 ± 0.09	0.47 ± 0.07	0.67 ± 0.09
	Female	1.30 ± 0.35	0.93 ± 0.09	0.60 ± 0.06
	Both	0.87 ± 0.03	0.70 ± 0.02	0.63 ± 0.05
Total Protein (mg/dL)	Male	7.47 ± 0.12	7.50 ± 0.12	7.70 ± 0.21
	Female	7.83 ± 0.43	8.53 ± 0.63	7.53 ± 0.44
	Both	7.65 ± 0.20	8.02 ± 0.37	7.62 ± 0.22
Albumin (mg/dL)	Male	4.30 ± 0.12	4.40 ± 0.15	5.43 ± 1.69
	Female	4.67 ± 0.41	4.73 ± 0.56	7.20 ± 0.69*
	Both	4.48 ± 0.21	4.57 ± 0.27	6.32 ± 0.67*
Globulin (mg/dL)	Male	3.25 ± 0.36	3.20 ± 0.23	3.42 ± 0.52
	Female	3.18 ± 0.31	3.14 ± 0.30	5.20 ± 0.24
	Both	3.22 ± 0.84	3.17 ± 0.69	4.31 ± 0.22
A/G Ratio	Male	1.32 ± 0.10	1.38 ± 0.02	1.59 ± 0.07
	Female	1.47 ± 0.07	1.51 ± 0.01	1.39 ± 0.10
	Both	1.39 ± 0.04	1.44 ± 0.10	1.47 ± 0.09
AST (U/L)	Male	157.00 ± 10.81	172.00 ± 11.02	163.64 ± 8.39
	Female	160.70 ± 10.00	167.60 ± 9.12	171.20 ± 11.23
	Both	155.35 ± 11.03	169.00 ± 9.27	167.42 ± 8.77
ALT (U/L)	Male	84.72 ± 7.20	94.30 ± 2.82	94.11 ± 3.22
	Female	86.03 ± 9.34	89.75 ± 8.13	87.32 ± 6.07
	Both	85.36 ± 1.49	92.03 ± 8.59	90.72 ± 6.43
ALKP (U/L)	Male	294.30 ± 17.27	239.70 ± 22.98	239.30 ± 56.64
	Female	276.00 ± 30.81	221.70 ± 13.78	282.70 ± 43.59
	Both	285.20 ± 16.32	230.70 ± 17.66	261.00 ± 33.40

Data expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests, with statistical significance: *** $P < 0.001$, ** - $P < 0.01$, *- $P < 0.05$.

Effect of Mucuna Protein 80% on Kidney function test in Repeated dosing Studies

The urea and creatinine are important biomarkers of renal toxicity and their increase is the evidence of Kidney damage [44]. After repeated dosing of Mucuna Protein (1000 mg/kg and 2000 mg/kg) no alteration was observed in serum urea, creatinine, and uric acid levels indicating that the sub-acute repeated dosing of Mucuna protein does not affect the kidney function. This observation aligns with previous study demonstrating lack of nephrotoxic potential of *M. pruriens* possibly owing to their antioxidant and anti-inflammatory constituents (Table 8) [37].

Table 8: Effect of Mucuna Protein 80% on Kidney function test in Repeated dosing Studies.

Parameter	Sex	Group-I (Control-0.5%CMC)	Group-II (Mucuna Protein- 1000 mg/kg)	Group-III (Mucuna Protein - 2000 mg/kg)
Urea (mg/dL)	Male	42.33 ± 0.88	43.00 ± 6.81	44.67 ± 1.20
	Female	39.00 ± 4.04	46.00 ± 3.46	41.67 ± 4.18
	Both	40.67 ± 1.99	44.50 ± 3.48	43.17 ± 2.06
Creatinine (mg/dL)	Male	0.47 ± 0.07	0.47 ± 0.03	0.40 ± 0.06
	Female	0.43 ± 0.03	0.57 ± 0.03	0.33 ± 0.07
	Both	0.45 ± 0.03	0.52 ± 0.03	0.37 ± 0.04
Uric acid (mg/dL)	Male	2.60 ± 0.03	1.83 ± 0.04	2.33 ± 0.07
	Female	2.07 ± 0.53	1.90 ± 0.09	2.30 ± 0.80
	Both	2.34 ± 0.04	1.87 ± 0.03	2.32 ± 0.15

Data expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests, with statistical significance: *** $P < 0.001$, ** - $P < 0.01$, *- $P < 0.05$.

Effect of Mucuna Protein 80% on Serum electrolytes levels in Repeated dosing Studies

Serum electrolytes (calcium, sodium, potassium, phosphorus, chloride) remained unchanged after 28 days continuous dosing of Mucuna protein 80% compared to controls indicating no negative impact on electrolyte balance. This result was similar to previously known safety profile of *M. pruriens* reports (Table 9) [38].

Effect of Mucuna Protein 80% on relative organ weight in Repeated dosing Studies

No significant changes in relative organ weights were noted after 28 days of continued Mucuna protein dosing, indicating lack of inflammation or organ enlargement. These findings suggest that Mucuna protein administration of this dosage is safe at the organ level which also aligns with previous reports [39] (Table 10).

Table 9: Effect of Mucuna Protein 80% on Serum electrolytes level in Repeated dosing Studies.

Parameter	Sex	Group-I (Control-0.5%CMC)	Group-II (Mucuna Protein-1000 mg/kg)	Group-III (Mucuna Protein - 2000 mg/kg)
Calcium (mmoL/L)	Male	9.53 ± 0.09	9.63 ± 0.07	9.43 ± 0.26
	Female	9.37 ± 0.12	9.20 ± 0.25	9.53 ± 0.27
	Both	9.45 ± 0.08	9.42 ± 0.15	9.48 ± 0.17
Phosphorus (mmoL/L)	Male	7.17 ± 0.23	6.83 ± 0.40	6.30 ± 0.30
	Female	6.18 ± 0.08	5.92 ± 0.24	7.50 ± 0.06
	Both	6.68 ± 0.75	6.38 ± 0.53	6.90 ± 0.23
Sodium (mmoL/L)	Male	143.70 ± 2.33	129.00 ± 16.56	144.70 ± 1.45
	Female	138.00 ± 2.00	147.00 ± 2.52	143.00 ± 2.08
	Both	140.80 ± 1.87	138.00 ± 8.51	143.80 ± 1.20
Potassium (mmoL/L)	Male	11.07 ± 1.02	11.03 ± 0.46	12.30 ± 0.35
	Female	13.80 ± 0.90	13.30 ± 0.40	12.50 ± 0.76
	Both	12.43 ± 0.86	12.17 ± 0.58	12.40 ± 0.38
Chloride (mmoL/L)	Male	97.00 ± 2.08	114.30 ± 14.33	104.30 ± 3.93
	Female	98.00 ± 4.00	107.00 ± 1.53	105.70 ± 4.81
	Both	97.50 ± 2.03	110.70 ± 6.65	105.00 ± 2.79

Data expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests, with statistical significance: *** P < 0.001, ** - P < 0.01, *- P < 0.05.

Table 10: Effect of Mucuna Protein 80% on relative organ weight in Repeated dosing Studies.

S.No.	Group	Relative organ weight (g/100g b. w.)					
		Liver	Heart	Kidney	Spleen	Ovary	Testis
1.	Group-I (Control-0.5%CMC)	1.09 ± 0.20	0.35 ± 0.05	0.78 ± 0.01	0.38 ± 0.01	0.08 ± 0.01	1.17 ± 0.01
2.	Group-II (Mucuna Protein-1000 mg/kg)	1.04 ± 0.68	0.32 ± 0.04	0.70 ± 0.03	0.40 ± 0.01	0.07 ± 0.01	1.19 ± 0.01
3.	Group-III (Mucuna Protein-2000 mg/kg)	1.05 ± **0.90**	0.35 ± 0.02	0.74 ± 0.07	0.39 ±0.01	0.08 ± 0.01	1.20 ±0.02

Data expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison tests, with statistical significance: *** P < 0.001, ** - P < 0.01, *- P < 0.05.

Effect of Mucuna Protein 80% on Histopathological analysis of various organs in Repeated dosing Studies

Liver

Liver tissue histopathology studies of all treatments showed normal hepatocytes, sinusoids, and portal triad across all treatments, indicating no histological damage or inflammation and this result is supported by earlier studies reporting preserved liver architecture and absence of necrosis or degeneration following Mucuna administration, considering it as safe (Figure 1) [37].

Stomach

Histopathological examination of stomach tissues revealed normal mucosa, submucosa, muscularis propria, and intact villi across all treatment groups indicating no gastric tissue damage or inflammation and aligns with earlier studies demonstrating the gastroprotective effects of *M. pruriens* extracts [40] (Figure 2).

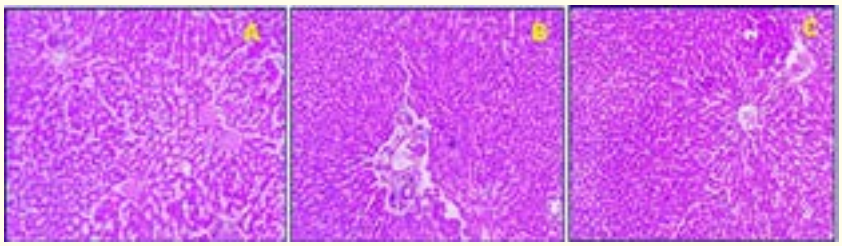

Figure 1: Histopathological images of liver section of the rat in repeated dose toxicity study at 10 X magnification, A) Control, B) Mucuna protein (1000 mg/kg), C) Mucuna protein (2000 mg/kg).

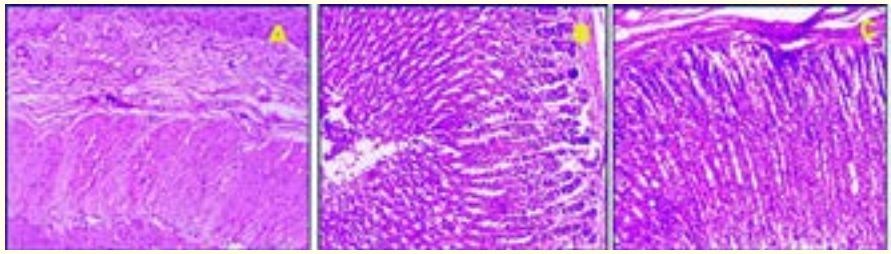

Figure 2: Histopathological images of stomach section of the rat in repeated dose toxicity study at 10 X magnification, A) Control, B) Mucuna protein (1000 mg/kg), C) Mucuna protein (2000 mg/kg).

Heart

Histopathology of heart tissue showed normal myocardial myocytes with prominent elongated nucleus and intact coronary artery, aorta, pulmonary vessels, and valves in all treatments which suggest no myocardial damage or vascular abnormalities supported by previous study reports on cardioprotective effects on experimental models of cardiac injury [41] (Figure 3).

Spleen

Histopathological evaluation of spleen tissues in all the treatments showed normal histological features of spleen with an external covering fibrous capsule with red pulp composed of RBC and white pulp of lymphoid cells. No pathological changes were observed in Cords of billroth and splenic vessels (Figure 4). The results indicated absence of inflammation or structural damage and the results are consistent with prior findings that *M. pruriens* do not induce spleen toxicity or immune tissue abnormalities [42].

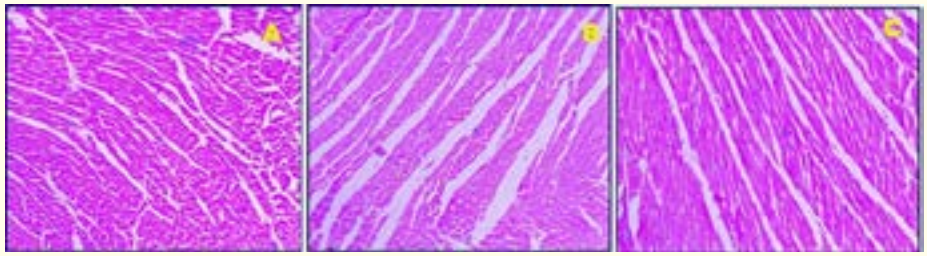

Figure 3: Histopathological images of heart section of the rat in repeated dose toxicity study at 10 X magnification, A) Control, B) Mucuna protein (1000 mg/kg), C) Mucuna protein (2000 mg/kg).

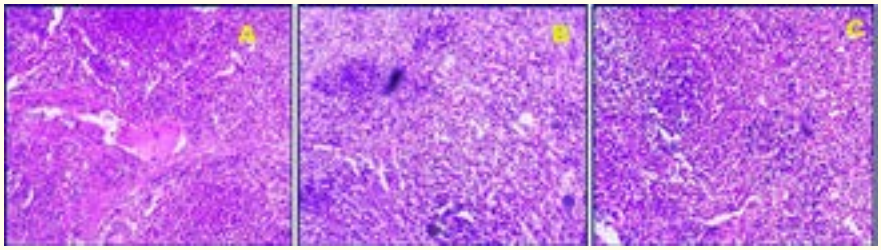

Figure 4: Histopathological images of spleen section of the rat in repeated dose toxicity study at 10 X magnification, A) Control, B) Mucuna protein (1000 mg/kg), C) Mucuna protein (2000 mg/kg).

Lungs

The histopathological studies of lung tissues showed normal histological features of lungs including normal lung parenchyma, alveoli, and bronchioles indicating no pulmonary tissue damage or inflammation, supporting earlier study on respiratory safety profile of *M. pruriens* [33] (Figure 5).

Kidney

The histopathological studies of right kidney tissue in all the treatments showed normal histological features of the kidney. The kidney section consists of cortex and medulla which is composed of glomerulus and tubules appearing normal with normal appearing blood vessels (Figure 6).

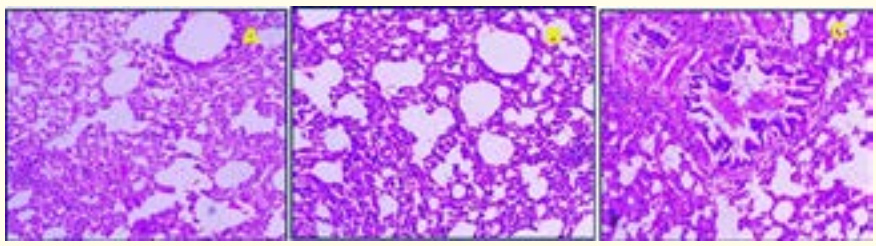

Figure 5: Histopathological images of lung section of the rat in repeated dose toxicity study at 10 X magnification, A) Control, B) Mucuna protein (1000 mg/kg), C) Mucuna protein (2000 mg/kg).

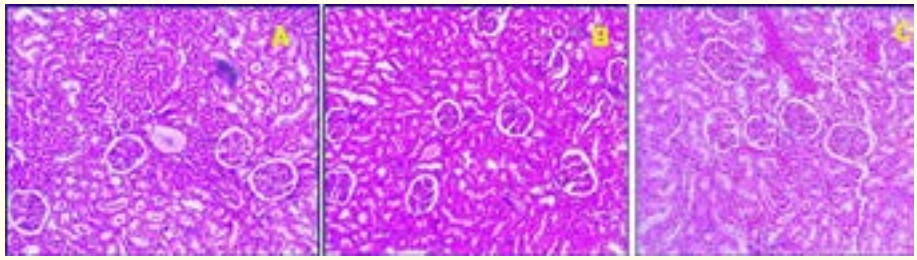

Figure 6: Histopathological images of kidney section of the rat in repeated dose toxicity study at 10 X magnification, A) Control, B) Mucuna protein (1000 mg/kg), C) Mucuna protein (2000 mg/kg).

Ovary

The histopathological studies of ovarian tissues showed normal histomorphology features of the ovary including normal ovarian stroma, developing follicles and carpus luteum indicating lack of kidney damage or inflammation. The findings were similar to a previous histology study which demonstrated renal safety and nephroprotective effects of *M. pruriens* [37] (Figure 7).

Testis

The histopathological examination of testis showed normal testicular features with normal histology of seminiferous tubules and spermatic cord (Figure 8). This finding indicates that this dosage does not alter testicular structure and the observations were consistent with earlier reports on beneficial effects of *M. pruriens* on testicular health and testicular protection against oxidative stress damage [43,45].

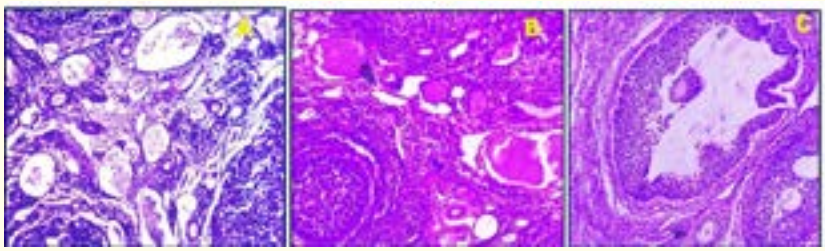

Figure 7: Histopathological images of ovary section of the rat in repeated dose toxicity study at 10 X magnification, A) Control, B) Mucuna protein (1000 mg/kg), C) Mucuna protein (2000 mg/kg).

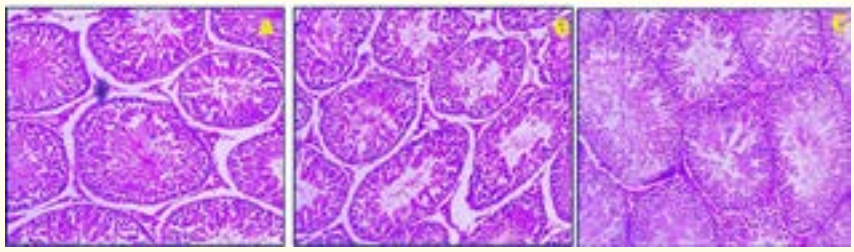

Figure 8: Histopathological images of testis section of the rat in repeated dose toxicity study at 10 X magnification, A) Control, B) Mucuna protein (1000 mg/kg), C) Mucuna protein (2000 mg/kg).

Conclusion

The present study demonstrates that Mucuna protein 80% is safe for oral administration in Wistar rats at doses up to 2000 mg/kg. No mortality or clinical signs of toxicity were observed, categorizing the protein as category 5 or unclassified according to the Globally Harmonized system. Hematological and biochemical analyses also supported safety with only minor dose-dependent changes. Overall, the study establishes a No Observed Adverse Effect Level (NOAEL) up to a limit of 2000 mg/kg/day, supporting its safety application in functional foods and nutraceuticals. Beyond toxicological studies, the present study also provides a robust scientific and regulatory value enabling safe commercialization of Mucuna Protein. The findings also promote sustainable utilization of leguminous plants advancing natural protein alternatives and development of safe, plant derived nutraceuticals. The lack of human clinical trials limits the determination of recommended daily intake levels, emphasizing the need for future human studies to enhance translational relevance.

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Conflict of Interest

The author(s) declared potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Srilakshmi Aluri, Anzar CA, Joseph MV, Bineesh Eranimose, Vadiraj Bharadwaj are employees of Ingex Botanicals Ltd.

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