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Original Research Article

Acute and Sub-Acute Toxicity Studies of Soursop Starch in Swiss mice and Wistar Rats

Vasudeva Reddy. Matta[1] and Santosh Kumar. Rada*[1]

[1] *Department of Pharmaceutics GITAM School of Pharmacy, GITAM (Deemed to be University), Rushikonda, Visakhapatnam, Andhra Pradesh - 530045, India.*
**Corresponding Author:*
Santosh Kumar Rada
Department of Pharmaceutics GITAM School of Pharmacy, GITAM (Deemed to be University), Rushikonda, Visakhapatnam, Andhra Pradesh - 530045, India.

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ABSTRACT

The recent utilization of natural superdisintegrants in the development of fast-dissolving tablets (FDTs) is becoming increasingly common. The starch from the *Annona muricata* (soursop) fruit was isolated and assessed for its potential as a new natural superdisintegrant for formulating FDTs. The acute and sub-acute toxicity of the isolated soursop starch and the dosage level in the final formulation were investigated. In the acute toxicity study, Swiss mice were given orally the maximum dose of 2000 mg/kg of soursop starch. In the sub-acute toxicity study, 50, 300, 1000 and 2000 mg/kg doses were administered to Wistar rats for 28 days. The results showed that soursop starch did not induce any behavioural changes, signs of toxicity, or mortality at the 2000 mg/kg dose in mice during the acute toxicity study. Similarly, in the 28-day oral toxicity study, soursop starch did not cause any toxicity or alterations in behavioural parameters up to the 2000 mg/kg dose in Wistar rats. Also, no significant changes were observed in haematological, biochemical, or histopathological parameters in rats administered soursop starch. Overall, the findings from the oral acute and sub-acute toxicity assessments in mice and Wistar rats at the maximum dose of 2000mg/kg reveal that the starch derived from soursop pulp is relatively safe to use for consumption.

***Keywords*:** *Annona muricata*, Oral acute toxicity, Oral sub-acute toxicity, Soursop starch (SSS)

Introduction

A novel type of tablet known as a fast-dissolving tablet (FDT) has been developed to address the challenges of oral administration.[1] FDTs dissolve rapidly in saliva without needing water, providing a convenient and patient-friendly option for individuals who struggle with swallowing tablets. Disintegrants play a crucial role in tablet formulation by aiding in disintegration, with superdisintegrants promoting faster disintegration in smaller quantities. In recent years, there has been a growing interest in utilizing natural superdisintegrants in formulating FDTs.

Starch, a naturally occurring biodegradable polymer (polysaccharide), holds significant importance across various industries, including food, textiles, pharmaceuticals, and more. It can be extracted from everyday stable food like potatoes, rice, maize, and wheat. Starch's unique properties, such as its inert nature and affordability, make it a valuable ingredient in tablet formulations, where it serves as a binder, diluent, and disintegrant. *Annona muricata*, a tropical tree belonging to the Annonaceae family, produces edible fruits commonly known as soursop. This plant has medicinal values like anti-cancer, anti-bacterial, anti-diabetic and anti-ulcer activities.[2,3]

*****Corresponding author. E mail: srada@gitam.edu
Tel: +91 7680009722

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These fruits contain a starch content of about 30 %.[4] The study focused on extracting starch from soursop fruit pulp and investigating its potential as a new natural superdisintegrant in developing fast-dissolving tablets (FDTs). The acute and sub-acute toxicity studies of the starch were assessed.

Materials and Methods

Collection and identification

The soursop fruits were obtained from Raiytu Bazar fruit market located at MVP colony Visakhapatnam, Andrapradesh, India on September 09, 2023. The collected fruits were authenticated by taxonomist Dr K. Madhava Cheety (plant taxonomist-IAAT:337) affiliated to Sri Venkateswara University (Department of Botany) Tirupati, Andrapradesh dated September 11, 2023 and deposited at the institution's herbarium with the voucher specimen number as 0117.

Processing of soursop starch

The fruits were washed thoroughly to remove the external matter and then peeled, followed by the removal of seeds from the fruits. The pulp was cut into 5-6 cm cubes, immediately rinsed in sodium sulphite solution (an antioxidant)[5] and dried at 50^0 C. These dried chips were converted to flour by using the ball mill. The known weight (500 g) of the flour was dispersed in five times its weight of distilled water for about 120 minutes (min). The dispersion was sieved with a muslin cloth. The residue was washed with water until the wash water was clean.[4]

The starch milk (filtrate) was centrifuged at 5000 rpm (rotations per minute) for 30 min, and the supernatant was decanted. The resulting starch sediment, which contained a thin yellowish mucous layer, was dispersed in a solution of 0.3% sodium hydroxide (w/v) and repeatedly washed (3-4 times) with the same until a clean white starch was obtained. The clean starch was dispersed in distilled water and washed repeatedly until the washing water was neutral to litmus (pH 7). Then, the dried starch was powdered and stored in an airtight container.[4]

Experimental animals
Swiss mice and Wistar rats were used for the acute and sub-acute toxicity studies, respectively. These animals (either sex) were of the age of 8-9 weeks, weighing about 130-150 g (either sex rats) and 20-25 g (male mice). These animals were quarantined as per the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). [6] Rats were housed in 6 cages, whereas mice were housed individually. Polycarbonate cages lined with heat-treated hardwood chips and covered with polyester filter sheets were used for both species.[7] The area temperature was maintained at 20-25^{0}C with relative humidity from 50 ± 10 %. Also, the animals were exposed to 12 hours of light and 12 hours of dark conditions and were given standard food, with free access to water. [8,9] The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) (Approval number: 10/IAEC/VCOP/2024). All the experiments were performed in compliance with the recommendations given by CPCSEA.

Toxicity Testing
Acute Toxicity-Oral administration
The study was designed with slight modifications as per the OECD (The Organization for Economic Co-operation and Development) guideline (423) for testing chemicals with acute oral toxicity.[10] The Swiss male mice weighing about 20-25 g (age 8-9 weeks) were selected for the study. A total of 20 mice (10 of control and 10 of test) were used to evaluate the acute toxicity of 2000 mg/kg body weight of prepared soursop starch (a new superdisintegrant). As per the available literature, it is clear that the Lethal Dose (LD_{50}) of natural starches was more than 2000 mg/kg. Hence, the study was performed at 2000 mg/kg body weight. [11] The mice were weighed, grouped and fasted overnight (12 hours) before the start of the study while the water was accessible for all the animals. After 12 hours of fasting, the prepared soursop starch (2000mg/kg) was given to the test group while the control group was administered water. The mice were continuously observed for the first 4 hours while the fasting was continuous during this period.[12]
The animals were further monitored for any abrupt changes or deaths for 24 hours. After 24 hours, the animals were monitored for a week. Further, these were monitored for 14 days for 24 hours (once daily). The animals were closely observed for all the unusual effects like changes in the eye, skin, mucus membrane, motor activity, respiration, seizures, tremors, diarrhea and coma.[13]

Sub-Acute Toxicity-Oral administration
The sub-acute toxicity studies were performed as per the OECD guideline 407.[14]. The Wistar rats (both sexes) of 8-9 weeks' age weighing about 130-150 g were selected. A total of 30 rats were grouped into five groups. Each group comprised of six rats. Group I the control group; the others were test groups at different doses. Groups II, III, IV and V were administered the oral doses of 50 mg/kg, 300 mg/kg, 1000mg/kg and 2000mg/kg, respectively. The rats were fasted for 12 hours before dosing with the soursop starch and observed for unusual signs and toxic symptoms for 28 days (daily once observation)
The animals were observed for clinical signs every day. The animals were observed twice daily for mortality and morbidity and all the general clinical signs such as unusual behavior, change in skin, eyes, mucous membrane, pupil size, respiratory patterns, ANS and CNS behaviors.

Variation of Body weight:
The body weight of each animal was recorded before treatment (Day 0) and weekly once after that (W1, W2, W3 & W4) until Day 28.

Determination of organ weights
After the successful completion of the Day 28 study, the animals were humanely sacrificed using inhalation of 5-8 % Isoflurane anaesthesia, and all the major organs were isolated, carefully washed with 0.9 % normal saline, wiped with tissue paper, and examined for signs of toxicity. Also, their weights were recorded. [15,16,17]

Blood collection and isolation of serum
In the 28-day study period, on day 27, the animals were kept fasting, and after day 28, they were weighed and sacrificed, and blood was collected using the retroorbital bleeding technique. These blood samples were collected in sterile tubes with the anticoagulant; the samples were centrifuged for 10 minutes at 6000 rpm. The serum (supernatant) was collected and stored at -20^{0}C for biochemical testing.[18]

Haematological evaluation
The animal blood samples were evaluated for complete blood picture like haemoglobin (Hb), white blood cells (WBC), red blood cells (RBC), erythrocyte sedimentation rate (ESR), Differential count Percentage (neutrophil, lymphocyte, eosinophil, monocyte, and basophils)

Biochemical evaluation
The collected blood samples were subjected to centrifugation, and serum was used to estimate biochemical parameters. To perform the biochemical analysis, used the commercially available kits from Biochrome Scientific, Hyderabad, India and followed the process provided in the manual. Each test has a defined standard concentration. The test preparation has been extrapolated to the calibrated values. The parameters estimated were Liver glycogen, SGPT (Serum Glutamic-Pyruvic Transaminase), SGOT (Serum Glutamic-Oxaloacetic Transaminase), ALP (alkaline phosphatase), Bilirubin blood urea and Serum Creatinine.[19,20,21]

Histopathological examinations
Histopathological assessments were conducted on the liver, kidney, and heart tissues of the experimental rats. After 28 days, all rats were humanely euthanized using inhalation of 5-8 % Isoflurane anaesthesia. The organs liver, kidney and heart were surgically removed, washed with saline solution, and preserved in a 10 % buffered formalin solution. The tissue samples were dehydrated in a graded sequence of ethanol (70-99%), rinsed in toluene, and wrapped in paraffin after fixing. Thin sections, approximately 5 μm in thickness, were prepared using a Rotary Microtome, mounted on glass slides, and stained with Hematoxylin and Eosin to observe histopathological alterations. A macroscopic examination of the liver, kidney, and heart was performed to identify any gross pathological changes (lesions) resulting from exposure to the test sample, and these findings were compared against those of the control group of the toxicity assessment. [19,22, 23,24]

Statistical analysis
The results were expressed as the mean ± standard error of the mean (SEM), and the data were analyzed using one-way analysis of variance (ANOVA) and followed by Dunnett's post-hoc tests. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using Graph Pad PRISM-5 software.

Results and Discussion

Acute toxicity assessment
The administration of soursop starch at a dose of 2000 mg/kg did not result in any signs or symptoms of toxicity. There were no observed cases of morbidity or mortality. Clinical evaluations indicated that the external appearance, behaviour, and daily activities of the mice remained normal (Table 1). Furthermore, no abnormalities in body weight gain were noted. No changes in fur colour, skin condition, eye appearance, or mucous membranes were detected. Throughout the study, all mice maintained normal posture and respiration. There were no indications of diuresis, diarrhoea, lethargy, ataxia, abnormal gait, tremors, convulsions, lacrimation, salivation, coma, unusual sleep patterns, or behavioural irregularities. The findings from the acute toxicity assessments suggest that the LD_{50} of the starch extracted from soursop fruit pulp is likely significantly higher than 2000 mg/kg.[25]

Consequently, according to the Globally Harmonized System (GHS) established by the United Nations for the international standardization of hazard classification and communication, soursop starch can be classified as a substance with a low potential for health hazards.

Table 1: Effect of soursop starch at 2000 mg/kg on general signs and symptoms in oral acute toxicity (n=10).

Parameter	Incidence
Morbidity	0/10
Mortality	0/10
Stereotypy	0/10
Abnormal gait	0/10
Abnormal posture	0/10
Abnormal respiration	0/10
Abnormal sleep	0/10
Ataxia	0/10
Color change in eyes	0/10
Color change in fur	0/10
Color change in mucus membranes	0/10
Color change in skin	0/10
Coma	0/10
Convulsions	0/10
Diarrhea	0/10
Diuresis	0/10
Lacrimation	0/10
Lethargy	0/10
Salivation	0/10
Tremors	0/10

Oral sub-acute toxicity assessment

General clinical observations

Any changes in vital signs, general health, or the emergence of abnormal behaviours and organ functions can be due to toxicity. When a potentially toxic substance is introduced into the body, it interacts with the host at various levels, molecular, cellular, or systemic, leading to adverse effects. In this study, the administration of soursop starch over 28 days did not result in any clinical abnormalities (Table 2). Even at the higher dose of 2000 mg/kg, no toxic effects were observed, similar to the control group. There were no instances of morbidity or mortality across all study groups. The animals exhibited no changes in skin colour, fur colour, eye colour, or mucous membrane appearance.

Additionally, there were no signs of discharge or lacrimation. All groups of animals displayed normal pupil size, respiration, gait, and posture. Furthermore, no convulsions, tremors, repetitive behaviours, or unusual actions were noted in any of the rats. These findings strongly suggest that soursop starch is non-toxic at the doses tested during this 28-day oral sub-acute toxicity evaluation. Therefore, soursop starch can be regarded as a safe substance with a low potential for health hazards, making it suitable for use in the pharmaceutical industry.

Body mass, food intake, and water intake

Toxicity insults have a direct relationship with weight loss, weight growth, and decreased organ weight. As such, a decrease in body weight may serve as a toxicity signal. Interestingly, nonetheless, we saw a marginal increase in the body weight of rats given soursop starch for 28 days in our sub-acute investigation. However, there is no statistically significant increase in weight (Table 3, Figure 1). This could be the result of consuming more starch than necessary. Moreover, there was no variation in the amount of food and drink consumed by either treatment group. It is evident that soursop starch has no detrimental effects on the amount of food and water that rats consume.

Table 2: Effect of soursop starch on general signs and symptoms in oral sub-acute toxicity (n=6).

Parameter	Incidence				
	Control	Soursop Starch			
		50 mg/kg	300 mg/kg	1000 mg/kg	2000 mg/kg
Morbidity	0/6	0/6	0/6	0/6	0/6
Mortality	0/6	0/6	0/6	0/6	0/6
Color change in fur	0/6	0/6	0/6	0/6	0/6
Color change in skin	0/6	0/6	0/6	0/6	0/6
Color change in eyes	0/6	0/6	0/6	0/6	0/6
Color change in mucus membranes	0/6	0/6	0/6	0/6	0/6
Abnormal posture	0/6	0/6	0/6	0/6	0/6
Abnormal respiration	0/6	0/6	0/6	0/6	0/6
Diuresis	0/6	0/6	0/6	0/6	0/6
Diarrhea	0/6	0/6	0/6	0/6	0/6
Lethargy	0/6	0/6	0/6	0/6	0/6
Ataxia	0/6	0/6	0/6	0/6	0/6
Abnormal gait	0/6	0/6	0/6	0/6	0/6
Tremors	0/6	0/6	0/6	0/6	0/6
Convulsions	0/6	0/6	0/6	0/6	0/6
Lacrimation	0/6	0/6	0/6	0/6	0/6
Salivation	0/6	0/6	0/6	0/6	0/6

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Coma	0/6	0/6	0/6	0/6	0/6
Sleep disturbances	0/6	0/6	0/6	0/6	0/6
Stereotypic behavior	0/6	0/6	0/6	0/6	0/6
Bizarre behavior	0/6	0/6	0/6	0/6	0/6

Table 3: Effect of soursop starch on body weights of rats in oral sub-acute toxicity (n=6)

Group	Body Weight in grams (Mean ± SEM)				
	Initial	1st week	2nd week	3rd week	4th week
I (Control)	130 ± 0.1	138 ± 0.1	145 ± 0.1	148 ± 0.5	148 ± 0.5
II (50 mg/kg)	132 ± 0.2^{ns}	139± 0.11^{ns}	143 ± 0.1^{ns}	149 ± 0.1^{ns}	153 ± 0.4^{ns}
III (300 mg/kg)	133 ± 0.12^{ns}	140 ± 0.4^{ns}	145 ± 0.1^{ns}	145 ± 0.2^{ns}	150 ± 0.3^{ns}
IV(1000 mg/kg)	134 ± 1.2^{ns}	138 ± 0.3^{ns}	144 ± 0.2^{ns}	148 ± 0.3^{ns}	154 ± 0.2^{ns}
V (2000 mg/kg)	135 ± 0.2^{ns}	141 ± 0.1^{ns}	144 ± 0.3^{ns}	150 ± 0.5^{ns}	153± 0.1^{ns}

ns=non-significant SEM= standard error of the mean

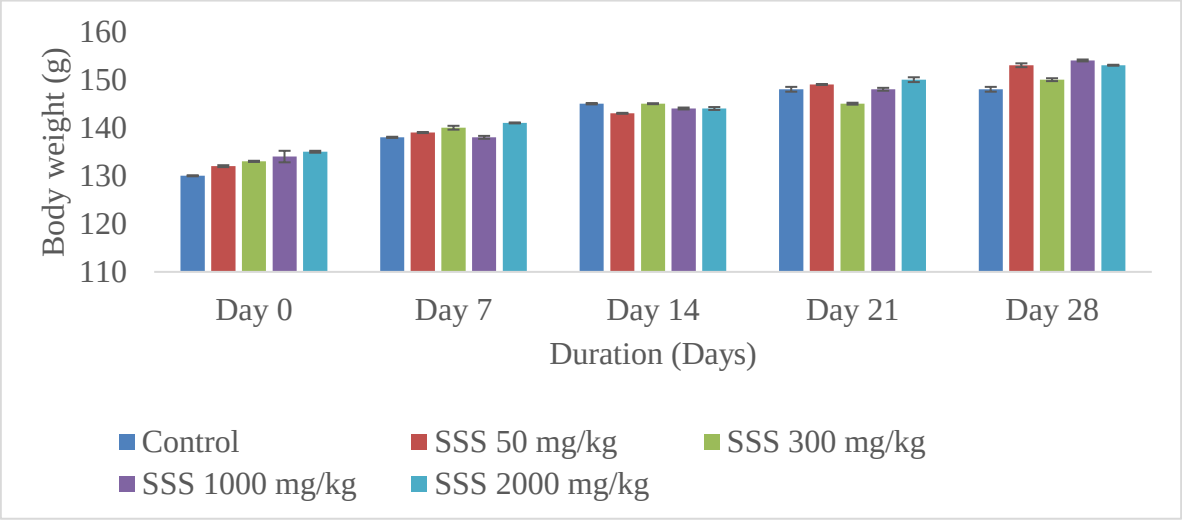

Figure 1: Effect of 28-day treatment with soursop starch on body weight of the rats. All the values are expressed as mean ± SEM (standard error of the mean), (n=6).

Haematological assessment

Since the hematopoietic system is thought to be extremely susceptible to toxicity, exposure to toxic chemicals causes a number of changes in haematological parameters. As a result, the hematopoietic system is essential for evaluating the effect of toxicity. For this reason, assessing haematological parameters is crucial to comprehending and interpreting the effects of toxicant exposure. The Haematological parameters, including the Hb, ESR, RBC count, WBC count, neutrophils, lymphocytes, eosinophils, monocytes, and basophils, are reported in Table 4. All study groups showed no statistically significant differences in the haematological parameters. This demonstrates that soursop starch is non-toxic and has no detrimental effects on haematological parameters.

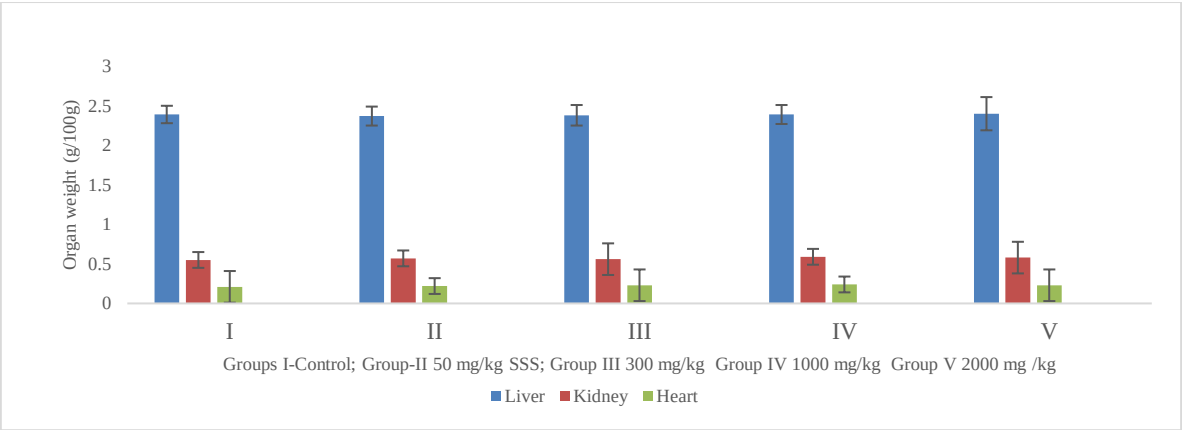

Figure 2: Effect of 28-day treatment with soursop starch on organ body weight. All the values are expressed as mean ± SEM (standard error of the mean), (n=6)

Biochemical analysis

The identification of pathological states or the potentially harmful nature of chemical or physical agents can be achieved through the determination of numerous biochemical parameters, such as enzymes, biomolecules, and chemical compounds that play a key role. This is because a change in these biochemical parameters occurs when there is damage to cells or tissues. The biochemical indicators, including ALP, bilirubin, SGPT, SGOT, liver glycogen, blood urea and serum creatinine, are reported in Table 5. These, results did not significantly differ between the groups in the sub-acute toxicity investigation. This suggests that the starch from soursop does not adversely affect any biochemical parameters. These findings demonstrate that the soursop starch is non-toxic.

Table 4: Effect of soursop starch on haematological parameters in rats in oral sub-acute toxicity (n=6).

Parameters	Groups				
	I (Control)	II (50 mg/kg)	III (300 mg/kg)	IV (1000 mg/kg)	V (2000 mg/kg)
ESR(mm/h)	6.12 ± 0.1	6.18 ± 0.2^{s}	6.19 ± 0.24^{ns}	6.21 ± 0.15^{ns}	6.25 ± 0.2^{ns}
Hb (%)	10.16±0.1	10.18±0.2^{ns}	10.21±0.1^{ns}	10.28±0.2^{ns}	10.29±0.1^{ns}
RBC	6.11±1.2	6.12±1.1^{ns}	6.18±1.2^{ns}	6.19±1.1^{ns}	6.21±1.2^{ns}
WBC	7.01±1.2	7.11±1.1^{ns}	7.31±1.5^{ns}	7.44±1.5^{ns}	7.54±1.6^{ns}
N	66.28±1.1	67.31±1.2^{ns}	66.56±1.1^{ns}	66.48±1.3^{ns}	68.84±1.2^{ns}
L	28.84±1.2	28.74±1.3^{ns}	28.88±1.1^{ns}	28.32±1.3^{ns}	28.48±1.1^{ns}
E	1.1±1.1	1.2±0.1^{ns}	1.3±0.1^{ns}	1.2±0.1^{ns}	1.1±0.1^{ns}
M	2.48±1.0	2.37±1.1^{ns}	2.48±1.2^{ns}	2.41±1.3^{ns}	2.45±1.1^{ns}
B	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0

N= Neutrophils, L= Lymphocytes, E= Eosinophils, M= monocytes B=Basophils; ns=non-significant. All the values are expressed as mean ± SEM (standard error of the mean)

Table 5: Effect of soursop starch on biochemical parameters in rats in oral sub-acute toxicity (n=6)

Parameters	Groups				
	I (Control)	II (50 mg/kg)	III (300 mg/kg)	IV (1000 mg/kg)	V (2000 mg/kg)
Liver glycogen (mg%)	125.1 ± 1.1	124.5 ± 1.1^{ns}	126.7 ± 1.2^{ns}	128.1 ± 1.3^{ns}	129.4 ± 1.1^{ns}
ALP (IU/L)	207.01 ± 1.1	207.14 ± 1.2^{ns}	207.57 ± 1.2^{ns}	208.12 ± 1.3^{ns}	208.15± 1.7^{ns}
SGOT (IU/L)	114.11 ± 1.1	114.77 ± 1.2^{ns}	114.66 ± 1.1^{ns}	114.41 ± 1.2^{ns}	114.74 ± 1.0^{ns}
SGPT (IU/L)	42.44 ± 1.0	42.28 ± 1.1^{ns}	43.01 ± 1.0^{ns}	43.12 ± 1.1^{ns}	43.74 ± 1.2^{ns}
Bilirubin (mg/dL)	0.69±0.1	0.68±0.1^{ns}	0.65±0.2^{ns}	0.67±0.3^{ns}	0.70±0.3^{ns}
Blood urea (mg%)	38.22 ± 0.2	38.34 ± 0.1^{ns}	38.74 ± 0.2^{ns}	38.77 ± 0.1^{ns}	38.35 ± 0.2^{ns}
Serum creatinine (mg/dL)	0.68 ± 0.1	0.69 ± 0.1^{ns}	0.71 ± 0.4^{ns}	0.72 ± 0.3^{ns}	0.69 ± 0.1^{ns}

ns=non-significant; All the values are expressed as mean ± SEM (standard error of the mean)
ALP = alkaline phosphatase; SGOT= Serum Glutamic-Oxaloacetic Transaminase; SGPT= Serum Glutamic-Pyruvic Transaminase

Determination of organ weights

The reduction in the internal organ size and weight signifies a negative impact on growth. Therefore, a change in organ weight serves as an indicator of toxicity. The organ weights of the sub-acute study are reported in Table 6 and Figure 2. The relative weights of the organs, such as liver, kidney and heart, were found to have no significant difference. This indicates that soursop starch does not affect the growth and histology of internal organs, thereby suggesting that soursop starch is safe to use.

Histopathological examinations

Aberrations in the microscopic histological structure of tissues or organs are used as the first step to interpret the potential toxic effects**.** Evaluation of histopathological changes gives valuable information about the mechanism, extent, and characteristics of toxicity, as well as target organ toxicity. In the sub-acute toxicity testing, no histological aberrations were observed. The histology of the liver, kidney, and heart was found to have no anomalies (Figure 3) in any of the study groups. This suggests the non-toxic nature of soursop starch.

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Table 6: Effect of soursop starch on organ weights in rats in oral sub-acute toxicity (n=6)

Group	Organ weight (g per 100 g body weight), mean ± SEM		
	Liver	Kidney	Heart
I (Control)	2.39 ± 0.11	0.55 ± 0.1	0.21 ± 0.2
II (50 mg/kg)	2.37 ± 0.12^{ns}	0.57± 0.1^{ns}	0.22 ± 0.1^{ns}
III (300 mg/kg)	2.38 ± 0.13^{ns}	0.56 ± 0.2^{ns}	0.23 ± 0.2^{ns}
IV (1000 mg/kg)	2.39 ± 0.12^{ns}	0.59 ± 0.1^{ns}	0.24 ± 0.1^{ns}
V (2000 mg/kg)	2.40 ± 0.21^{ns}	0.58 ± 0.2^{ns}	0.23 ± 0.2^{ns}

ns=non-significant; SEM=standard error of the mean

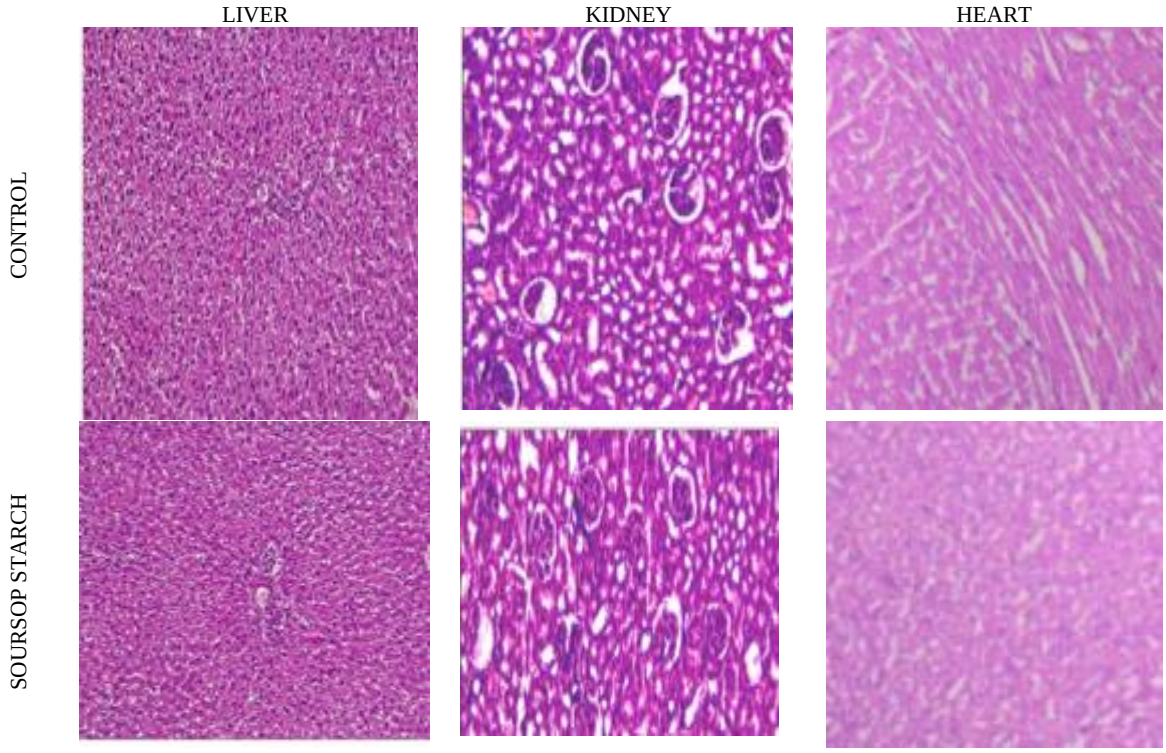

Figure 3: Effect of 28-day treatment with soursop starch on histology of liver, kidney, and heart of the rats. No changes were found in the organ tissues.

Conclusion

The oral acute toxicity evaluation in rats revealed that the starch isolated from the pulp of soursop at a dose of 2000 mg/kg does not produce any signs and symptoms of toxicity, and therefore, it can be considered a substance with low health hazard potential. Further, the results of the 28-day oral sub-acute toxicity evaluation demonstrated that the starch isolated from the pulp of soursop at a maximum dose of 2000 mg/kg does not produce any major toxic effects in rats. Therefore, the results of this study suggest that the starch isolated from the pulp of soursop is safe for use.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

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