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Original Research Article

Acute and Sub-acute Toxicity Studies of Sweetsop Starch in Female Sprague-Dawley Rats

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ABSTRACT

Using natural super disintegrants to develop fast-dissolving tablets (FDTs) is gaining attention. We aimed to extract the starch from the pulp of *Annona squamosa. L* (sweetsop) fruit and explore its potential as a super-disintegrant in designing FDTs. This study evaluated the oral acute and sub-acute toxicity of the sweetsop starch. In the acute toxicity assay, sweetsop starch was administered orally to female rats at 2000 mg/kg per body weight. In the sub-acute toxicity study, sweetsop starch was administered orally to female rats at 10, 100, and 1000 mg/kg doses. In the acute toxicity study, the starch did not produce any behavioural changes, signs of toxicity, or mortality at 2000 mg/kg in female rats. Also, in the sub-chronic toxicity (28-days) study, sweetsop starch did not produce any signs of toxicity or changes in behavioural parameters up to 2000 mg/kg in the experimental animals. The starch did not significantly change haematological, biochemical, or histopathological parameters in the treated rats. The oral acute and sub-acute toxicity evaluation results in female rats revealed that the starch extracted from the pulp of Sweetsop does not produce any significant toxic effects in the experimental animals. Therefore, sweetsop starch can be considered safe in the pharmaceutical industry.

***Keywords*:** *Annona squamosa*, Oral acute toxicity, Sweetsop starch.

Introduction

Owing to its multiple advantages, the oral route is considered the most preferred route among all the routes of drug administration. It offers several advantages such as cost-effectiveness, high patient compliance, convenience, non-invasiveness (painless), least sterility constraints, compactness, flexibility in designing the dosage form, ease of production, ease of drug withdrawal in case of overdosing, least requirement for training on administration, possibility for self-medication, possibility for prolonged drug action, and possibility for controlling rate and extent of drug release.[1] Further, tablets are the most preferred and popular oral dosage forms. However, administration of tablets is difficult for patients who have difficulty swallowing. For patients who are pediatric or geriatric, those with dysphagia, those with neurological, developmental, or mental disorders, or those suffering from nausea or low fluid intake, tablets may not be the best option.[2] To overcome the difficulty in administering normal tablets in all these conditions, the pharmaceutical research community has invented an innovative class of tablets called fast-dissolving tablets (FDTs), which rapidly dissolve in saliva without requiring water, providing an expedient and patient-friendly option for people who have difficulty swallowing.[3,4] Disintegrants are one of the main excipients of tablet formulation, which facilitate the disintegration of tablets. However, the disintegrants that facilitate a quicker disintegration in smaller quantities are regarded as super disintegrants.[5] In neoteric years, there has been a growing interest in using natural super disintegrants for FDTs.[5,6]

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Starch is a natural biodegradable polymer (polysaccharide) used as an essential substance in food, textile, pharma, and several other industries. It can be extracted from staple foods such as potato, rice, maize, wheat, etc.[7] Due to unique features such as inert nature, availability at low cost, etc., starch is used as a binder, diluent, and disintegrant in tablet formulations.[8]

Annona squamosa L. is a small tropical tree belonging to the *Annonaceae* family. The fruits of this plant are edible and well-known as sugar apples or sweetsops.[9] The starch content of the fruits has been reported to be 19-25%.[9] Our current research aims to extract the starch from sweetsop fruit pulp, explore its potential as a super-disintegrant in designing FDTs, and evaluate its acute and sub-chronic toxicity effects in female rats.

Materials and Methods

Extraction of sweetsop starch

Mature custard apples (Sweet apples or Sweetsop) were obtained from the local market in Bhadrachalam, Telangana. Fruits were washed thoroughly with water, and the skin was peeled off. Then, the seeds inside the pulp were removed, and the pulp was then chopped into small pieces and blended in distilled water in a 1:3 ratio. The blending continued until a thick and homogenous paste was formed. The homogenate was then filtered using a cheesecloth or a fine mesh sieve to remove larger debris. The filtrate obtained was filtered through a cheesecloth or a fine mesh sieve, and the resultant solution was allowed to settle. The supernatant was removed, and the slurry was mixed with distilled water, stirred gently, and then allowed to settle down. This step was repeated 2-3 times. The pH of the extract was adjusted to 7. Then, the slurry was allowed to dry in an oven at 40°C till the complete moisture was removed. Then, the dried starch was powdered with mortar and pestle to break the lumps.

Experimental animals

Female Sprague-Dawley (SD) rats at age 8-9 weeks weighing 180 – 220 g were selected for this study. The rats were housed in standard conditions suggested by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The animals were kept in an environment with a 50-70% relative humidity, 22°C ± 03°C

temperature, and 12 hours/12 hours light/dark cycle. The animals were fed with standard (commercially available) rodent pelleted food *ad libitum* and had unlimited access to water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) (Approval number: AKRGCP/Pheuc/2021-3). All the experiments were performed in compliance with the recommendations given by CPCSEA.

Toxicity Testing

Toxicity testing is an essential regulatory obligation to test a new compound used in pharmaceutical formulation. This preclinical toxicity testing on biological systems uncovers the potential toxic effects of the test compound(s). The studies provide information on species-specific, organ-specific, and dose-dependent toxic effects of an investigational product.[10, 11, 12]

Oral acute toxicity study

The oral acute toxicity evaluation was performed as per the recommendations given in Organization for Economic Co-operation and Development (OECD) guideline 423.[13] Various literature reported that the LD_{50} value of the starch isolated from staple foods such as potato, rice, and maize was found to exceed 2000 mg/kg. Therefore, we performed the limit test with the dose of 2000 mg/kg for our oral acute toxicity testing with reference to the OECD 423 guidelines. In this test, 6 young nulliparous and nonpregnant adult female rats, aged 8-9 weeks and weighing 180 – 220 g, were selected. It is of note that literature on LD_{50} studies shows that gender-specific sensitivity is very low, and studies that showed gender-specific differences revealed that female animals are slightly more sensitive than males. Therefore, to minimise the number of animals and identify the toxic effects more certainly, this study utilised female rats only. The female rats selected were weighed and subjected to overnight fasting but had unlimited access to water. Subsequently, the animals were administered orally with the starch extracted from sweetsop pulp using a gavage at a 2000 mg/kg dose. The fasting continued until 4 hours after dosing. The animals were observed for any signs and symptoms of toxicity per the recommendations of the OECD guideline 423. The animals were observed very keenly during the 1st four hours after dosing, and after that, animals were observed once every 30 min during the 1st day. Subsequently, the animals were monitored once daily until 14 days. The animals were closely observed for any changes in their fur, skin, eyes, mucous membranes, and functions related to respiration, circulation, the autonomic and central nervous systems, and somatomotor abilities. Particular focus was placed on detecting tremors, seizures, secretion of saliva from the mouth, dysentery (diarrhoea), sluggishness, abnormal sleep patterns, and coma.

Oral sub-acute toxicity study

The oral sub-acute (repeated dose 28-day) toxicity evaluation was performed following the recommendations provided in the OECD guideline 407.[14] For this study, female rats aged 8-9 weeks and weighing 180 – 220 g were selected. The female rats selected were nulliparous and nonpregnant. A total of 40 female rats were grouped into 4 groups, each comprising 10 rats. Group 1 serves as the control group and receives normal saline orally for 28 days. Groups II, III, and IV belonged to treatment groups and received the sweetsop orally at 10 mg/kg, 100 mg/kg, and 1000 mg/kg, respectively. All the rats were subjected to daily observation for any signs and symptoms of toxicity.

General clinical observations

Animals were carefully observed for general clinical signs once daily. All the animals were observed for mortality and morbidity twice daily. Animals were observed for clinical signs such as changes in skin, fur, eyes, or mucous membranes, the incidence of discharges and excretions, lacrimation, pupil size, respiratory pattern, changes in gait, posture, response to handling, presence of convulsions, stereotypic behaviour, and bizarre behaviour.

Body weight, food, and water consumption

The body weight of each rat was recorded once weekly before treatment. Also, animals' food and water intake was recorded weekly.

Blood collection and isolation of serum

On day 27 of treatment, the rats were fasted, and on day 28, the animals were anesthetised using isoflurane. The blood was collected into sterile tubes containing anticoagulants using the retroorbital bleeding technique. Then, the blood samples were centrifuged for 15 min at 6000 rpm. The resultant supernatant serum was isolated and stored at –20°C for further investigation.

Hematological evaluation

Blood samples collected were utilised for haematological tests. The haematological parameters evaluated include red blood cells (RBC), haemoglobin (Hb), hematocrit (HCT), mean corpuscle volume (MCV), mean corpuscle hematocrit (MCHT), erythrocyte sedimentation rate (ESR), platelets, white blood cells (WBC), lymphocyte, monocyte, neutrophil, and eosinophil.

Biochemical evaluation

The serum isolated from the blood samples was used to estimate the biochemical parameters. The biochemical parameters estimated include alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, creatinine, albumin, total serum protein (protein), urea, cholesterol, triglycerides.

Hormonal evaluation

The serum isolated from the blood samples was used to estimate the hormones. The hormones estimated include triiodothyronine (T3), thyroxine (T4), and thyroid-stimulating hormone (TSH).

Determination of organ weights

All the animals were euthanised by using cervical dislocation, and then an autopsy was done. All the organs were isolated carefully, washed with normal saline, wiped with tissue paper, and scrutinised for any visible signs of toxicity. The weight of the organs was recorded carefully.

Histopathological examinations

The preparation of organ tissue section staining was done as described by Namoju *et al*. 2022.[15] In brief, the tissue samples of the organs were subjected to the following steps in the same sequential order: i) fixing in formalin (10%), ii) soaking in phosphate buffer. iii) dehydration with ethyl alcohol (70%, 80%, 90%, and 100%), iv) clearing alcohol by soaking xylene, v) embedding in paraffin, vi) sectioning using a microtome (5µm), vii) mounting sections on glass slides, viii) drying the mounted sections, ix) deparaffinisation using xylene, x) rehydration using ethanol (100%, 95%, and 70%), xi) staining with hematoxylin & eosin (H&E), and xii) microscopic examination for histological changes.

Statistical analysis

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

Results and Discussion

The administration of sweetsop starch at a dose level of 2000 mg/kg did not produce any signs or symptoms of toxicity (Table 1). No morbidity and mortality were observed. The clinical symptoms, such as external appearance, behaviour, and daily routine activities, were normal. There were no abnormalities in body weight gain. The mean body weight of the rats on Day 1, Day 7, and Day 14 were found to be 208.5 ± 5.25 g, 229.2 ± 4.18 g, and 259.8 ± 5.82 g. No colour change in fur, skin, eyes,

or mucous membranes was observed. All the rats were found to have normal posture and respiration throughout the study. The animals showed no signs of diuresis, diarrhea, lethargy, ataxia, abnormal gait, tremors, convulsions, lacrimation, salivation, coma, abnormal sleep, abnormal motor activity, or behavioural abnormality. Histopathological evaluations did not reveal any anomalies in rats of all the study groups. The results of acute toxicity testing suggest that the LD_{50} value of the starch extracted from the pulp of sweetsop fruit would be much greater than 2000 mg/kg. Therefore, for the Globally Harmonized System (GHS) developed by the United Nations for international standardisation of hazard classification and communication, sweetsop starch can be considered a substance with low health hazard potential.[16] Similarly, in the oral sub-acute toxicity study, any alteration in vitals, general signs, or development of abnormality in the behaviour and function of the organs in the body can be attributed to toxic insults. When a substance with toxic potential enters the body, it interacts with the host body at molecular, cellular, organs, or systems and can cause adverse outcomes.[17] Administration of sweetsop starch for 28 days did not cause any abnormalities in clinical symptoms (Table 2). The high dose (1000 mg/kg) employed in the study also did not cause any toxic effects similar to that of the control group. No mortality and morbidity were observed in all the study groups. Skin colour, fur colour, eye colour, and mucous membrane colour of animals were not changed. Animals did not show any discharges (excretions) and lacrimation. All the animals showed normal pupil size, respiration, gait, and posture. Furthermore, no convulsions, tremors, stereotypic behaviour, or bizarre behaviour was observed in the study groups' rats. This indicates the non-toxic nature of sweetsop starch at the doses used in this 28-day sub-acute toxicity testing. Therefore, sweetsop starch can be considered a safe substance with low health hazard potential and can be used in the pharmaceutical industry.

Table 1: Effect of sweetsop starch at 2000 mg/kg on body weight, morbidity, and mortality in oral acute toxicity study

Parameter	Incidence
Morbidity	0/6
Mortality	0/6
Colour change in fur	0/6
Colour change in skin	0/6
Colour change in eyes	0/6
Colour change in mucus membranes	0/6
Abnormal posture	0/6
Abnormal respiration	0/6
Diuresis	0/6
Diarrhea	0/6
Lethargy	0/6
Ataxia	0/6
Abnormal gait	0/6
Tremors	0/6
Convulsions	0/6
Lacrimation	0/6
Salivation	0/6
Coma	0/6
Abnormal sleep	0/6
Abnormal behavior	0/6

(n=6).

It is well reported that toxic assault may lead to a reduction in body weight or weight gain and organ weight.[18] Therefore, a reduction in body weight can be considered an indicator of toxicity. However, interestingly, in this sub-acute study, we observed a slight increase in the body weight of the rats receiving sweetsop starch for 28 days (Figure 1). However, this weight gain is not statistically significant. This might be due to extra caloric intake in the form of starch. Also, the treatment groups did not differ in the food and water intake. This indicates that sweetsop starch does not negatively affect food and water intake in female rats (Figures 2 and 3). The hematopoietic system is believed to be highly vulnerable to toxic assaults, and therefore, upon exposure to toxic substances, several alterations occur in haematological parameters. Thus, the hematopoietic system plays a vital role in indicating healthy physiological and pathological states. Hence, evaluating haematological parameters is essential to understanding and interpreting the outcomes of exposure to a toxicant.[19] The haematological parameters such as RBC count, Hb, Haematocrit (HCT), - mean corpuscle volume (MCV), mean corpuscle hematocrit (MCHC), Erythrocyte sedimentation rate (ESR), platelet count, WBC count, lymphocytes (%), monocytes (%), neutrophils (%), and eosinophils (%) were found to have no significant difference among all the study groups (Table 3). This indicates that sweetsop starch has no adverse effects on haematological parameters and its non-toxic nature. Determining various biochemical parameters (such as enzymes, biomolecules, and chemical substances that have crucial roles) has become a critical means of identifying the toxic potential of chemical or physical agents or pathological conditions. Variations in biochemical parameters are observed when there is damage at the cellular or tissue level.[20] In our sub-acute toxicity study, there was no significant difference in the biochemical parameters such as aspartate aminotransferase (ALT), alanine transaminase (AST), alkaline phosphatase (ALP), bilirubin, creatinine, albumin, total serum protein, urea, cholesterol, and triglycerides among the study groups (Table 4). This indicates that sweetsop starch has no adverse effect on biochemical parameters.

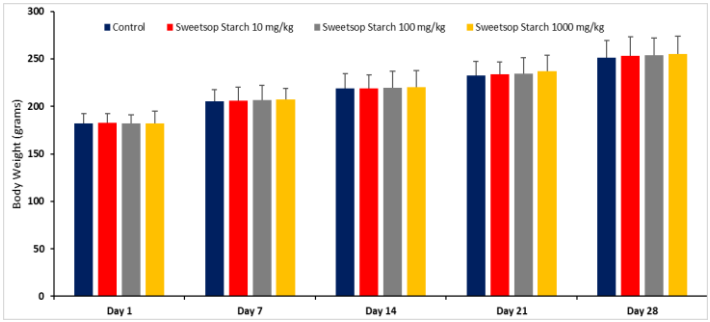

Figure 1: Effect of 28-day treatment with sweetsop starch on body weight of the female rats. All the values are expressed as mean ± SEM (n=10).

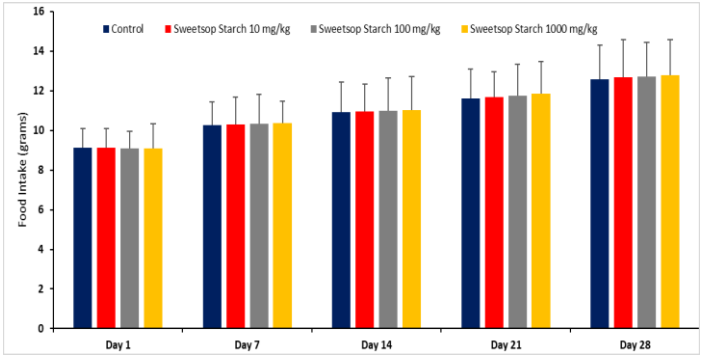

Figure 2: Effect of 28-day treatment with sweetsop starch on food intake of the female rats. All the values are expressed as mean ± SEM (n=10).

It is well known that, due to the toxicity, variations may happen in the production of hormones or perturbations in the biological pathways

involving the hormones. Therefore, the evaluation of hormones also reveals the toxic potential of chemical or physical agents or pathological conditions.[17] In our sub-acute toxicity testing, administration of sweetsop starch did not cause any significant aberrations in the hormonal levels (Table 4). This was visible as all the study groups were found to have similar thyroid-stimulating hormone triiodothyronine (T3), thyroxine (T4), and thyroid-stimulating hormone (TSH) levels. The reduction in the internal organ size and weight negatively impacts growth. Therefore, a change in organ weight serves as an indicator of toxicity.[21] In the present study, the relative weights of the organs such as the brain, kidney, heart, liver, lungs, spleen, ovary, and uterus showed no significant difference (Table 5). These results showed that sweetsop starch does not affect the growth and histology of internal organs, indicating that the sweetsop starch is safe for use in drug formulation. Aberrations in the microscopic histological structure of tissues or organs are used as the first step to interpret the potentially toxic effects of an investigational agent.[22] Evaluation of histopathological changes gives valuable information about the mechanism, extent, and characteristics of toxicity and target organ toxicity. In the sub-acute toxicity testing, no histological aberrations were observed. The liver, intestine, and kidney histology showed no anomalies in all the study groups (Figure 4), indicating the non-toxic nature of sweetsop starch.

Table 2: Effect of sweetsop starch on general clinical signs and symptoms in female rats in oral sub-acute toxicity study

Parameter	Incidence			
	Control	Sweetsop Starch		
		10 mg/kg	100 mg/kg	1000 mg/kg
Morbidity	0/10	0/10	0/10	0/10
Mortality	0/10	0/10	0/10	0/10
Color change in fur	0/10	0/10	0/10	0/10
Color change in skin	0/10	0/10	0/10	0/10
Color change in eyes	0/10	0/10	0/10	0/10
Color change in mucus membranes	0/10	0/10	0/10	0/10
Abnormal posture	0/10	0/10	0/10	0/10
Abnormal respiration	0/10	0/10	0/10	0/10
Diuresis	0/10	0/10	0/10	0/10
Diarrhea	0/10	0/10	0/10	0/10
Lethargy	0/10	0/10	0/10	0/10
Ataxia	0/10	0/10	0/10	0/10
Abnormal gait	0/10	0/10	0/10	0/10
Tremors	0/10	0/10	0/10	0/10
Convulsions	0/10	0/10	0/10	0/10
Lacrimation	0/10	0/10	0/10	0/10
Salivation	0/10	0/10	0/10	0/10
Coma	0/10	0/10	0/10	0/10
Abnormal sleep	0/10	0/10	0/10	0/10
Stereotypic behavior	0/10	0/10	0/10	0/10
Bizarre behavior	0/10	0/10	0/10	0/10

(n=10).

Table 3: Effect of sweetsop starch on haematological parameters in female rats in oral sub-acute toxicity study (n=10).

Parameter	Control	Sweetsop Starch		
		10 mg/kg	100 mg/kg	1000 mg/kg
RBC ($x10^6$)/µl	07.82 ± 1.89	07.79 ± 1.64	07.78 ± 1.02	07.80 ± 1.46
Hb (g/dl)	16.23 ± 1.67	15.85 ± 1.32	16.18 ± 1.48	15.93 ± 1.11
HCT (%)	51.81 ± 3.67	52.11 ± 3.67	51.08 ± 3.70	51.90 ± 3.69
MCV (fL)	65.86 ± 3.81	65.24 ± 3.89	65.56 ± 3.21	64.98 ± 3.72
MCHC (%)	30.67 ± 2.34	30.88 ± 2.22	30.54 ± 2.38	30.24 ± 2.11
ESR (mm/hr)	04.51 ± 0.78	04.35 ± 0.65	04.62 ± 0.66	04.38 ± 0.64
Platelets ($x10^6$)/µl	1257 ± 163	1242 ± 178	1236 ± 193	1230 ± 173
WBC ($x10^6$)/µl	07.56 ± 0.73	07.45 ± 0.44	07.48 ± 0.62	07.51 ± 0.59
Lymphocytes (%)	85.67 ± 6.34	83.17 ± 6.94	84.56 ± 6.59	84.74 ± 6.82
Monocytes (%)	00.16 ± 0.001	00.17 ± 0.001	00.17 ± 0.001	00.16 ± 0.001
Neutrophils (%)	11.87 ± 1.78	11.67 ± 1.84	11.72 ± 1.64	11.59 ± 1.58
Eosinophils (%)	02.30 ± 0.32	02.28 ± 0.34	02.33 ± 0.33	02.29 ± 0.28

Table 4: Effect of sweetsop starch on biochemical parameters in female rats in oral sub-acute toxicity study

Parameter	Control	Sweetsop Starch		
		10 mg/kg	100 mg/kg	1000 mg/kg
ALT U/L	24.32 ± 2.45	25.11± 2.67	25.00 ± 2.76	24.94 ± 2.13
AST U/L	116 ± 8.34	119 ± 7.45	117 ± 8.88	120 ± 9.65
ALP U/L	155 ± 12.34	161 ± 12.89	158 ± 10.66	154 ± 11.76
Bilirubin mg/ml	2.43 ± 0.67	2.54 ± 0.53	2.53 ± 0.61	2.51 ± 0.58
Albumin mg/dl	4.34 ± 0.67	4.56 ± 0.62	4.47 ± 0.71	4.36 ± 0.71
Urea mg/dl	28.92 ± 3.45	26.89 ± 4.44	27.32 ± 3.66	28.12 ± 3.82
BUN mg/dl	16.56 ± 1.39	16.56 ± 1.39	16.56 ± 1.39	16.56 ± 1.39
Creatinine mg/dl	0.79 ± 0.07	0.81 ± 0.06	0.81 ± 0.05	0.82 ± 0.04
Protein mg/dl	6.66 ± 0.89	6.56 ± 0.73	6.55 ± 0.74	6.56 ± 0.72
Glucose mg/dl	96.12 ± 6.76	96.12 ± 6.66	95.19 ± 6.45	96.23 ± 6.47
Cholesterol mMol/L	54.73 ± 5.14	55.12 ± 5.12	56.01 ± 5.28	55.22 ± 5.18
Triglycerides mMol/L	74.31 ± 4.76	74.63 ± 4.13	74.32 ± 4.31	74.65 ± 4.68
TSH ng/mL	4.58 ± 0.82	4.61 ± 0.83	4.57 ± 0.69	4.65 ± 0.75
T3 ng/mL	1.62 ± 0.21	1.65 ± 0.11	1.65 ± 0.17	1.63 ± 0.16
T4 ng/ mL	57.92 ± 6.56	58.11 ± 6.44	57.68 ± 6.62	57.98 ± 6.71

All the values were expressed as mean ± SEM (n=10).

Table 5: Effect of sweetsop starch on organ weights in female rats in oral sub-acute toxicity study

Parameter	Control (mg/kg)	Sweetsop Starch		
		10 mg/kg	100 mg/kg	1000 mg/kg
Brain	0.69 ± 0.18	0.67 ± 0.16	0.68 ± 0.16	0.68 ± 0.17
Kidney	0.68 ± 0. 14	0.67 ± 0. 16	0.68 ± 0. 12	0.67 ± 0. 12
Heart	3.01 ± 0.78	3.03 ± 0.82	3.02 ± 0.69	3.03 ± 0.72
Liver	2.86 ± 0.76	2.81 ± 0.72	2.79 ± 0.72	2.80 ± 0.69
Lungs	0.49 ± 0.12	0.47 ± 0.13	0.48 ± 0.13	0.48 ± 0.15
Spleen	0.16 ± 0.01	0.16 ± 0.02	0.15 ± 0.03	0.15 ± 0.03
Ovary	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.01
Uterus	0.26 ± 0.04	0.25 ± 0.06	0.28 ± 0.02	0.25 ± 0.03

All the values were expressed as mean ± SEM (n=10).

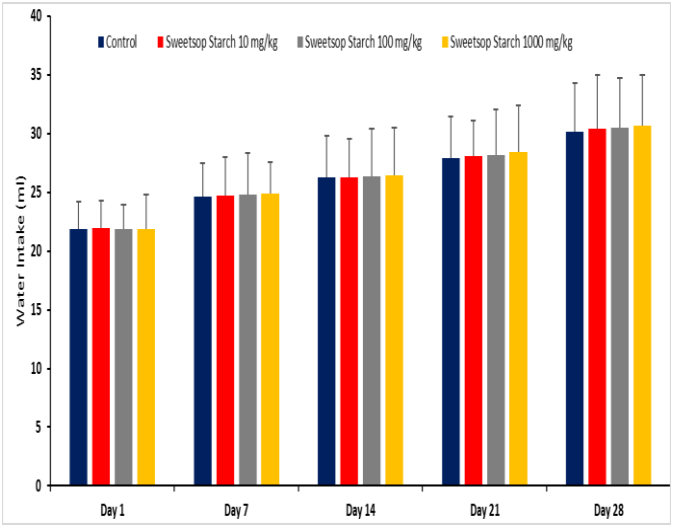

Figure 3: Effect of 28-day treatment with sweetsop starch on water intake of the female rats. All the values are expressed as mean ± SEM (n=10).

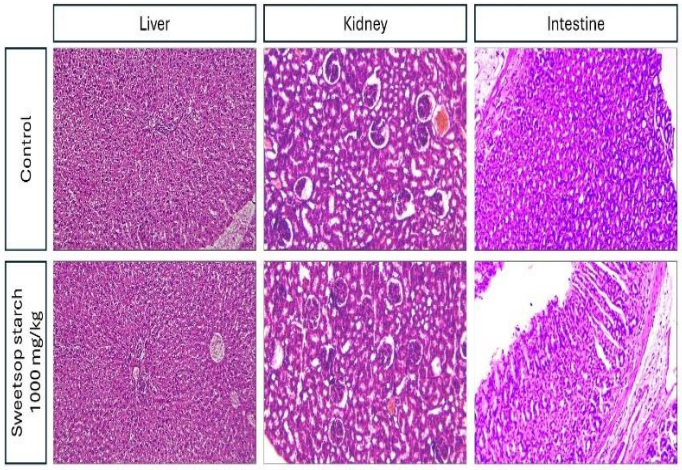

Figure 4: Effect of 28-day treatment with sweetsop starch on histology of liver, kidney, and intestine of the female rats. All the values are expressed as mean ± SEM (n=10).

Conclusion

The oral acute toxicity evaluation in rats revealed that the starch extracted from the pulp of sweetsop at a dose of 2000 mg/kg does not produce any signs and symptoms of toxicity. Therefore, it can be considered as a substance with low health hazard potential. Further, the 28-day oral sub-acute toxicity evaluation results demonstrated that the starch isolated from the pulp of sweetsop at a maximum dose of 1000 mg/kg does not produce any significant toxic effects in rats. Therefore, the results of this study advocate that the starch extracted from the pulp of sweetsop is safe to 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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