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SAFETY STUDY OF CARBOXYMETHYLATED BASELLA ALBA MUCILAGE: A SUBCHRONIC ORAL TOXICITY EVALUATION IN WISTAR ALBINO RATS

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ABSTRACT

Background: The evaluation of toxicity is of paramount importance in the screening of a new compound. *Basella alba* mucilage possesses a versatile excipient property that can be innovated with its chemical modification to get the functionalized mucilage. A few pharmacological activities of *Basella alba* mucilage have also been reported earlier, but its toxicity study in rats has yet to be discussed. **Aim:** The study aims to assess the in vivo toxicity of carboxymethylated *Basella alba* mucilage in Wistar albino rats for 28 days. **Material and Methods:** In the current investigation, Carboxymethylated *Basella alba* mucilage is taken, and its subchronic toxicity study is carried out in forty-eight healthy rats (twenty-four male rats and 24 female rats), divided into four groups containing six rats of each sex. All the biochemical and hematological parameters and histopathological investigation were estimated for all the animal groups. **Result:** The subchronic toxicity study reveals that the modified mucilage is safe for all doses (20mg/kg body weight, 40mg/kg body weight, 80 mg/kg body weight). The study showed no significant difference in the dose group's behavioral toxicity, nephrotoxicity, and hepatotoxicity compared to the control. All the hematological and biochemical parameters lay in the normal range. The histopathological examination of treatment groups showed no abnormality or lesion in the tissue samples of internal organs. **Conclusion:** The study confirms the safety of Carboxymethylated *Basella alba* mucilage for use in pharmaceutical formulations.

INTRODUCTION

The rapid advancement of drug delivery demands the exploration of polysaccharides in innovative forms to be the most promising candidate with the desired functionalities. Polysaccharides are hemocompatible and interact well with the living cells [1]. They are effectively used in the pharmaceutical industry but show limited use due to their poor stability, surface characteristics, and low solubility. Therefore, they can be

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chemically modified or coupled with another polymer to overcome the challenges in the field of drug delivery [2]. *Basella alba*, from the family Basellaceae, contains mucilage in most parts [3]. Basella mucilage mainly contains galactose monosaccharide [4]. *Basella alba* mucilage, a natural polysaccharide, has established itself as an excipient in pharmaceutical dosage form with versatile properties but needs modification to improve its characteristics [5]. Therefore, carboxymethylation of the mucilage can be done to replace the hydroxyl group with the carboxymethyl group. The present research uses functionalized *Basella alba* mucilage to study their safety in Wistar albino rats. Natural polymers are non-toxic, but since they have been functionalized, it is of great concern to evaluate their safety before using them to prepare pharmaceutical formulations. Deshmane et al. (2023) conducted an oral toxicity study of functionalized tamarind for safe use in drug delivery systems. They have sulfonated and thiolated pure tamarind to get the derivatives. The rat toxicity study showed that the modified tamarind was safe to use compared to the native tamarind [6]. Kaur et al. (2020) modified chitosan to chitosan-catechol conjugate to improve its mucoadhesive properties and assessed its acute oral toxicity in mice, which was considered safe [7]. In another research work by Kausar et al. (2021), an acute oral toxicity study of modified *Artemisia vulgaris* seed mucilage was done in rats. The mucilage was blended with acrylamide, showing no toxic effects [8]. Sumaira et al. (2021) grafted quince seed mucilage with acrylamide and studied its acute toxicity in mice; the mucilage-based network was found safe orally [9]. Pasha et al. (2021) extracted and modified date palm mucilage to prepare nanoparticles, checked its acute oral toxicity in mice and found it safe [10]. Therefore, numerous toxicity assessments of functionalized polymers have been reported in the literature. Still, there is no report on the sub-chronic toxicity study of *Basella alba* mucilage in rats. The present work investigates the safety of carboxymethylated *Basella alba* mucilage by carrying out the sub-chronic oral toxicity study of 28 days in albino rats. The hematological investigation, examination of biochemical parameters, and histopathological studies were done to investigate the safety of the modified mucilage.

MATERIALS AND METHODS

Materials

The plant *Basella alba* was obtained from the Sodepur market. Its stem and fruits were cleaned and dried under shade. Botanical Survey of India, Kolkata, identified the plant with voucher no. MC0105. All the analytical grade reagents were used as received. Freshly prepared double-distilled water was used.

Isolation and chemical modification of mucilage

Mucilage was extracted from the plant's stem and fruits by precipitation [11]. The carboxymethylation process is achieved through a sodium hydroxide- chloroacetic acid system in which the –CH2COO—- group replaces the hydroxyl group on sugar residue. The process followed the method given by Chowdhury et al. [12].

Experimental animals

Male and female Wistar rats 6-8 weeks old of 120-150 g body weight were purchased from M/S Chakraborty Enterprises, Kolkata, and were kept in polycarbonate-made cages. Six rats of the same sex were housed in cages with husk bedding at 20 to 24°C and relative humidity of 40 to 70%, maintaining 12 hours of dark and light cycles. They were acclimatized seven days before the experiment and were on a regular laboratory diet with water ad libitum. A veterinary examination of the rats was done before acclimatization, and the experiment started. The experimental protocol followed schedule Y requirements of the Drug and Cosmetics Act OECD Principles of Good Laboratory Practices. All the experimental protocols and procedures were reviewed and approved by the Institutional Animal Ethics Committee, TAAB Biostudy Services, Kolkata (Registration no.1938/P.O./Rc/S/17/CPCSEA). The ethical guidelines were strictly maintained throughout the experiment per the competent authority guidelines.

Experimental Design

The subchronic toxicity of carboxymethylated Basella alba mucilage was studied in four groups consisting of six rats of the same sex. The experimental protocol followed OECD test guideline 407: repeated dose 28 days oral toxicity study with minor modifications [13]. 24 male and 24 female healthy rats were kept in cages according to sex and marked in the fur by picric acid. The female rats were not pregnant and nulliparous.

Group I (Control): Received saline solution orally (0.9%w/v)

Group II: Administered 20 mg/kg body weight of Carboxymethylated mucilage (low dose) orally [14,15]

Group III: Administered 40 mg/kg body weight of Carboxymethylated mucilage (medium dose) orally [14,15]

Group IV: Administered 80 mg/kg body weight of Carboxymethylated mucilage (high dose) orally

The formulation prepared from Carboxymethylated *Basella alba* mucilage will have a human dose of 350 mg/day, equivalent to 5.83mg/kg bw. The following calculation converted the middle dose: 7×5.83 mg/kg bw = 40.81 mg/kg bw ~ 40 mg/kg bw. The low and high doses were calculated as ½ and 2 folds of the middle dose, respectively. The dose was selected based on the dose level guidelines of OECD 407. The Carboxymethylated Basella alba mucilage was dissolved in 2 ml of drinking water, and the solution was administered to the rats using oral gavage. The doses were administered orally once a day at similar times each day for 28 days [14,15].

Observation

During the experimental period, the rats were observed for behavioral changes in the morning and afternoon of each day for 28 days. Mainly, motor activity and anxiety in rats were checked and noted for any changes. The rest/ sleep timings were checked, and their scratching activity was monitored. The clinical signs were checked continuously up to 6-8 hours every day after administration of the dose and recorded carefully as a score sheet. Several signs were checked, like changes in the fur, eyes, skin, mucous membrane, secretions, and excretion. Changes in posture, gait, and repetitive movements were noted.

The rats were observed twice daily for mortality, once a week for food consumption, and once for body weight measurement. After administering the doses for 28 days, the animals were fasted overnight for hematological and biochemical investigation [16]. The blood sample was withdrawn from the orbital sinus and anticoagulated with heparin.

Hematological study

As per CPCSEA guidelines, 10% of rats' total circulating blood volume can be withdrawn every two to four weeks. The total blood volume of a rat is estimated to be approximately 8% of its body weight. Therefore, ether anesthesia withdrew a small blood volume (<1 ml) from the retro-orbital venous plexus. At first, ether was applied topically to the eye, and the animal was held with a thumb and forefinger to distort the skin around the eye. A capillary was inserted into the medial canthus of the eye at a 30° angle with slight thumb pressure to collect the blood. The capillary tube was removed, and the region was wiped with sterile cotton. Gentle finger pressure was applied to stop bleeding in some rats. The freshly collected blood sample mixed with heparin was used to examine hematological parameters using the Medonic M series cell counter (Model- M32). The hematological parameters studied were Hemoglobin (Hb), Reticulocyte (Rt), Hematocrit (HCT), Platelets, Neutrophils (N), Lymphocytes (L), Monocytes (M), Eosinophils (E), Red Blood Corpuscles (RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), White Blood Corpuscles (WBC) [17].

Biochemical study

The blood sample collected was centrifuged at 2000 rpm for 20 min to separate serum. The obtained serum was used to examine biochemical parameters using a Microlab-300 semi-auto analyzer. The biochemical parameters studied were Total Serum Protein [18], Blood Urea Nitrogen (BUN) [19], Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT) [20], Serum Alkaline Phosphatase (SAP), Blood Sugar, Creatinine, Total Bilirubin.

Necropsy

Cervical dislocation is the preferred method for euthanasia for rats weighing less than 200g, as per CPSCEA. On day 29, the cervical dislocation method was followed to sacrifice the animals as per CPCSEA guidelines. At first, the rat was restrained on a flat surface, and the base of the tail was grasped with one hand, and a pen was placed against the back of the neck at the base of the skull to bring dislocation. A quick forward-down push with the pen restraining the head with a backward pull at a 30° angle by the hand holding the tail separated the cervical tissues. During the necropsy procedure, the body organs, external surfaces, and cavities were checked without any alteration of tissue or organs. The abdominal and chest cavities were opened, and the organs of the liver, kidney, and heart were removed and immersed in a fixative solution. Necropsy of animals of all groups was done, and the liver, kidney, and heart weights were taken [21].

Histopathology study

The histopathology study investigated the changes between the normal tissue components and the tissue components in pathological conditions. Identification and confirmation of the safety of the test compound are made by standard hematoxylin-eosin staining procedure and its analysis under light microscopy.

The histopathology study was carried out using the following steps [22]:

Tissue collection and fixation

The tissue samples were collected from group I (control) and IV (high dose) rats. The tissue collected from the kidney, heart, liver, lungs, stomach, and small intestine was washed with phosphate buffer solution and immediately preserved in 10% formalin solution for 4 days to enable fixation. After fixation, the tissues were washed with running water and then distilled.

Tissue processing

After fixation, the tissues were processed to prepare thin microscopic sections following the steps:

i. Dehydration

The tissues need to be dehydrated to infiltrate with paraffin. Therefore, they were transferred through an ascending ascending-graded alcoholic solution. The tissues were dipped in 50% alcohol (1 h), 70% alcohol (overnight), 90% alcohol (2 h), and absolute alcohol (1 h).

ii. Clearing

The dehydrant present in the tissue is cleaned with xylene by dipping the tissues in xylene for 15 minutes. This process makes the tissues transparent by increasing their refractive index.

iii. Embedding

Paraffin was taken in a porcelain dish and melted at 58-60°C. The tissues were taken out from xylene and dipped in molten paraffin for 2 h, which was maintained at 60°C to embed the tissues in paraffin. The embedding process ensures the proper alignment and orientation of tissue in the paraffin block.

iv. Block preparation

Molten paraffin was poured into a paper boat, and the tissues were correctly oriented in the depression's center with forceps. The formation of air bubbles is prevented and removed during the settling of paraffin to form a paraffin block

v. Section Cutting

Serial sections of 3-5µm paraffin ribbon were obtained from a rotary microtome by sectioning the paraffin block. A small ribbon section was placed in a glass slide coated with egg albumin and gently stretched with forceps. The glass slide was placed in a hot air oven for 15 min to allow the section to adhere to the slide.

vi. Staining with hematoxylin and eosin

The slides were dipped in xylene, absolute alcohol, 90% alcohol, 70% alcohol, and water for 10 min in each liquid. Hematoxylin was used to stain the section for 3-5 min. Excess hematoxylin was removed in acid alcohol, and excess stain was removed by placing the slide under running water for 10 minutes. The slides were then dipped in 90% alcohol and 70% alcohol for 10 min. Then, staining the slides was done with eosin for 2 min. The excess stain was removed by washing with 90% alcohol. The slides were kept in absolute alcohol for 10 minutes, in xylene for 5 minutes, and finally mounted in Dibutylphthalate Polystyrene Xylene (DPX).

vii. Evaluation

The stained tissue section was observed under a microscope with 40X magnification to investigate any adverse effects of the carboxymethylated *Basella alba* mucilage

Statistical analysis

The data from the experiments were depicted as mean± S.D. One-way analysis of variance (ANOVA) with Graph Pad Prism 9 software was used to perform the statistical analysis of the significant differences between control and treatment groups. The statistically considerable value was considered at the p-value less than 0.5.

RESULT

Clinical observations

During the twenty-eight days dosing period, the intoxicating sign was absent in all four groups of animals, and no abnormality was observed, as shown in Table 1. No mortality was recorded throughout the dosing period in any dose group of animals.

Table 1: *Effect of carboxymethylated Basella alba mucilage on clinical signs and symptoms*

Group	Animal no	Dose (mg/kg)	Observed sign	Mortality
Sex: Male				
I	1-6	0 (Control)	NIL	NIL
II	13-18	20	NIL	NIL
III	25-30	40	NIL	NIL
IV	37-42	80	NIL	NIL
Sex: Female				
I	7-12	0 (Control)	NIL	NIL
II	19-24	20	NIL	NIL
III	31-36	40	NIL	NIL
IV	43-48	80	NIL	NIL

Determination of body weight

The mean body weight of all animal groups was taken and compared with the control group. No reduction in weight was observed for any group, and the results of the dose group were comparable with those of the control group. Figures 1(A) and

1(B) show the normal weight gain of male and female rats of all groups, respectively.

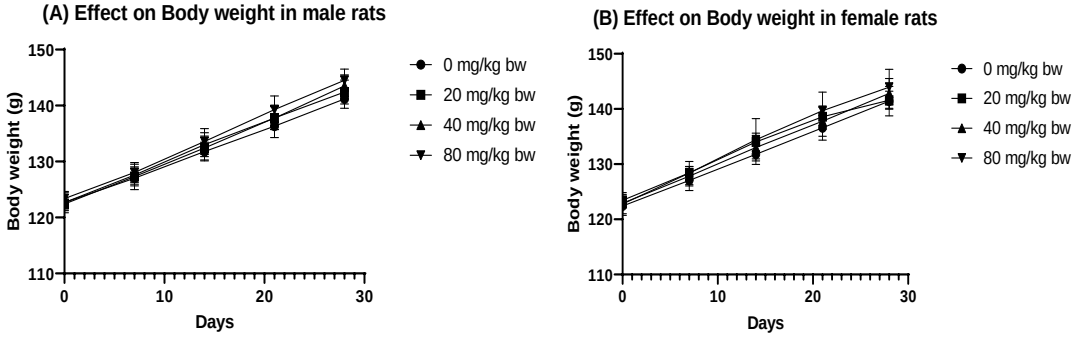


Figure 1: Effect of Carboxymethylated *Basella alba* mucilage on body weight of (A) male (B) female rats (graph plotted from data expressed as mean±SD, (n = 6), *p < 0.05 vs. control)**.**

Determination of Food Consumption

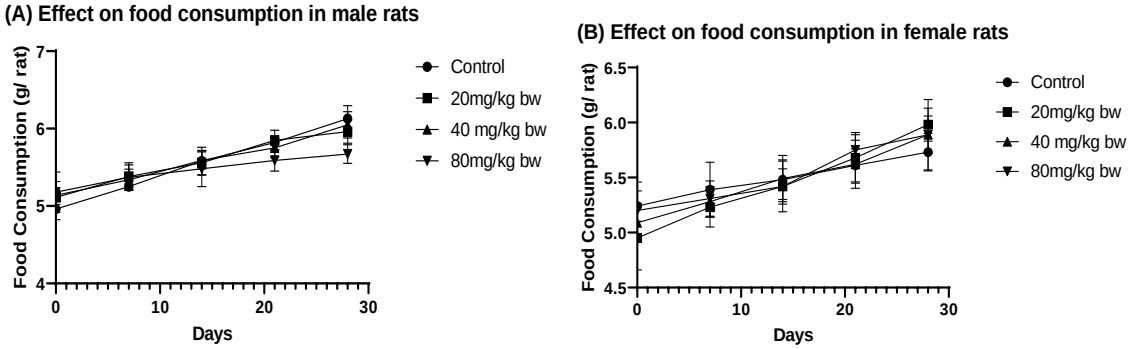
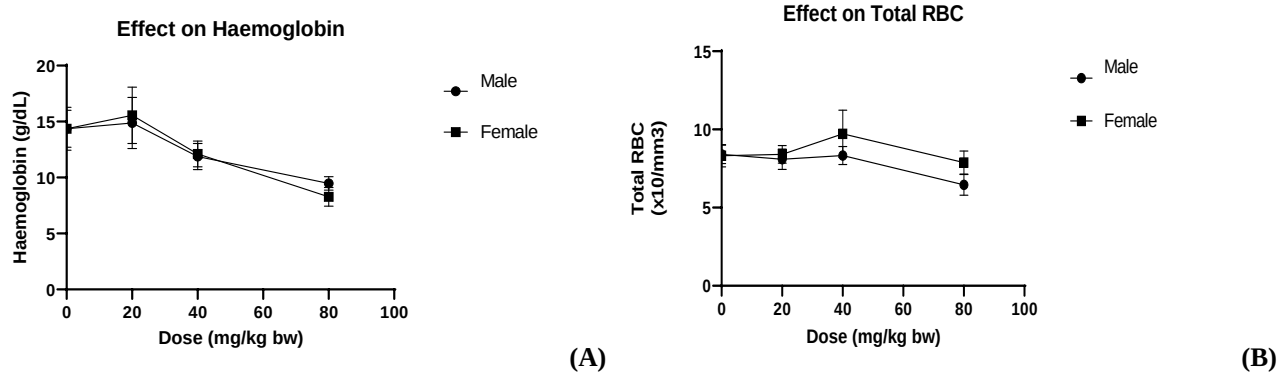


Figure 2: Effect of Carboxymethylated *Basella alba* mucilage on Food consumption (A) male (B) female rat (graph plotted from data expressed as mean±SD, (n=6), *p< 0.05 vs. control)

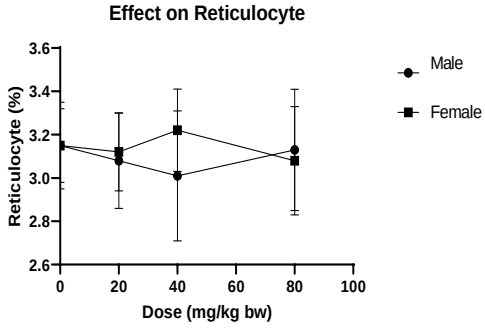
All the rats from all groups consumed food regularly and were satisfactory. There was no abnormality or any reduced food consumption in any group. The amount of food consumption does not show any statistical difference with the control group as shown in figure 2(A) and 2(B) for male and female rats respectively.

Hematological analysis

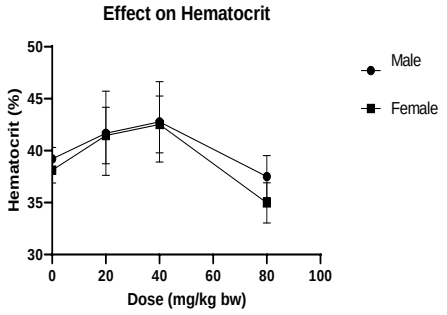
The hematological parameters of the low, middle, and high dose group of animals were compared with the control group as shown in figure 3 (A-K) for male and female rats respectively. Although the hematological parameters showed a slightly decreased value in the case of group IV animals but was not of concern as the values were within the normal range. The decrease may be due to less food and water intake No significant difference was observed between the control and treatment groups, suggesting no adverse reaction occurred due to the administration of the modified *Basella alba* mucilage.



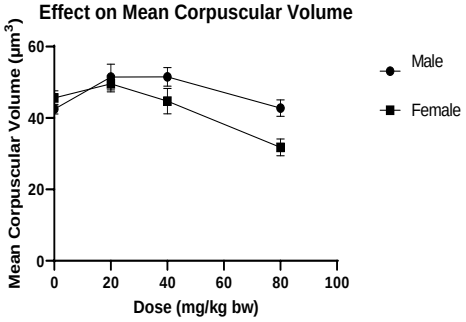
(A) **(B)**



(C)



(D)



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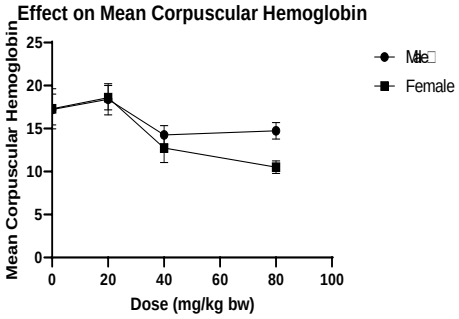
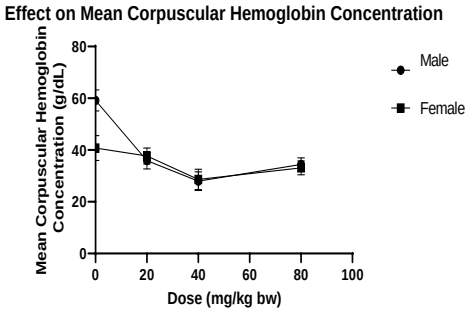


(F)



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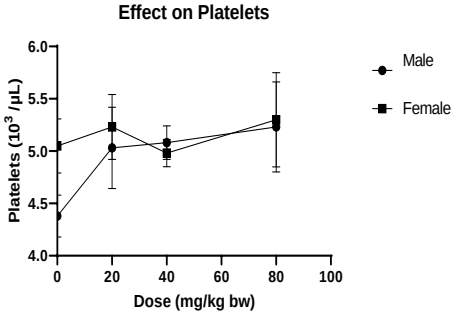

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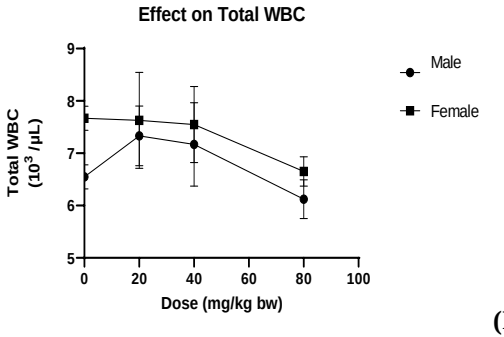
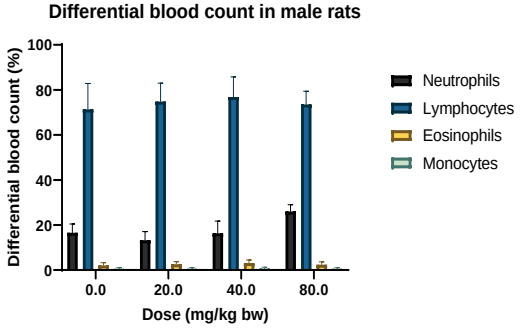
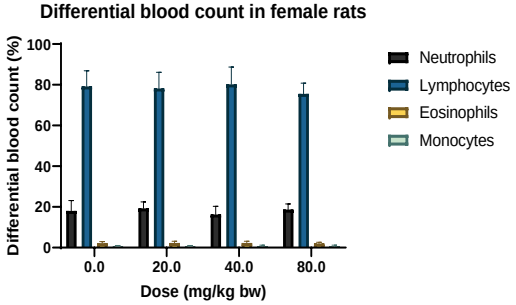


(I)



(J)



(K)

Figure 3: Effect of Carboxymethylated Basella alba mucilage on Haematological parameters (A) Hb (B) RBC (C) Rt (D) HCT (E) MCV (F) MCH (G) MCHC (H) Platelets (I) WBC (J) Differential count in male rats (K) Differential count in female rats (graph plotted from data expressed as mean±SD, (n = 6), $p < 0.05$ vs. control).

Biochemical analysis

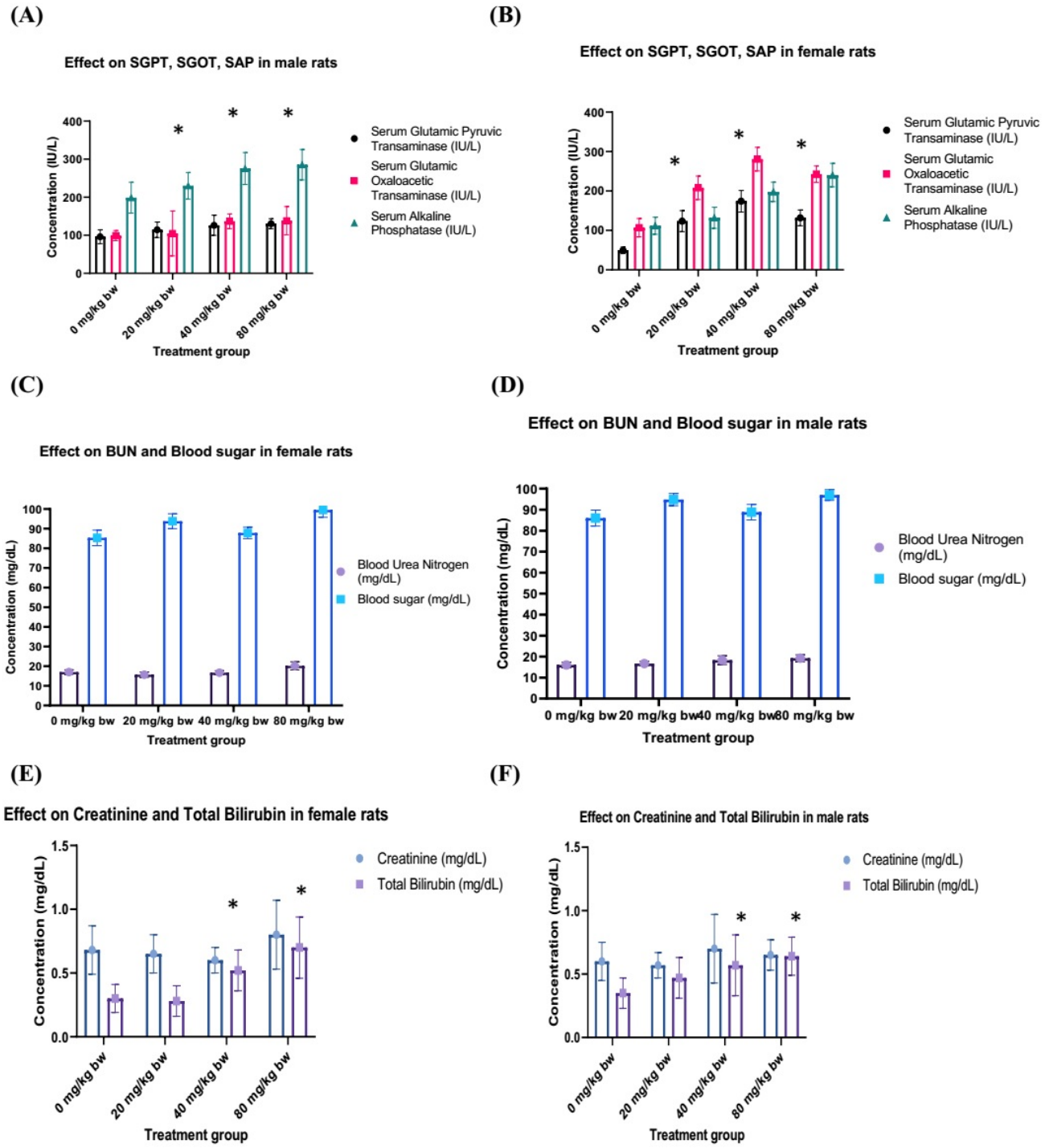


Figure 4: Effect of Carboxymethylated Basella alba mucilage on Biochemical parameters (A-F) (graph plotted from data expressed as mean±SD, (n = 6), $p < 0.05$ vs. control**)**

After 28 days of sub-chronic oral toxicity, the whole blood sample was withdrawn from the retro-orbital plexus of each rat. All the parameters of biochemical analysis were found to be within permissible limits; however, the SGPT, SGOT, and SAP levels were slightly increased in dose groups compared to the control, as shown in Figure 4 (A-F) in male rats. Since the values were within the permissible limit, it does not have any clinical significance. There was no other significant difference between the control and the dose groups, suggesting that the carboxymethylated mucilage has no adverse effect on the biochemical parameters.

Organ weight

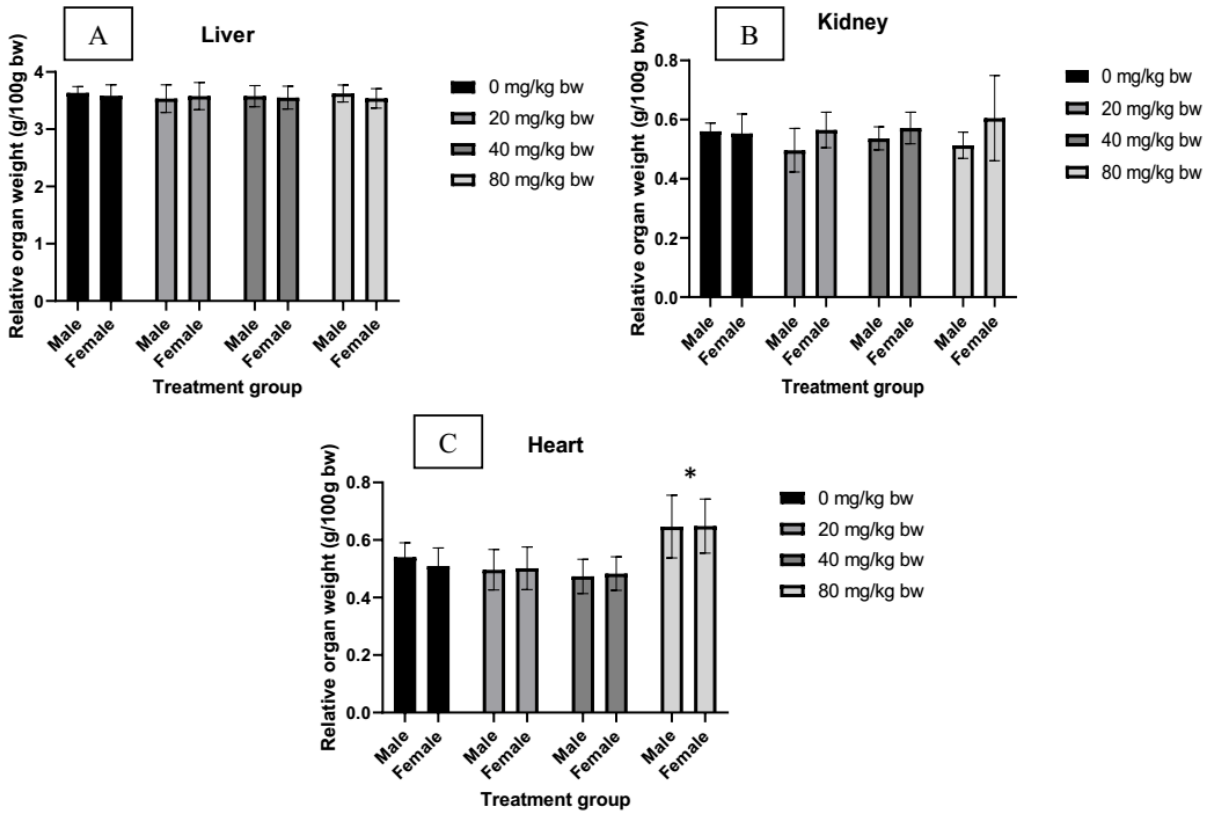


Figure 5: Relative organ weight of control and treatment group of animals (A) Liver (B) Kidney (C) Heart (graph plotted from data expressed as mean±SD, (n = 6), $p < 0.05$ vs. control).

The data shows the normal organ weight of both the control and dose groups of animals. The relative weight of kidneys in the treatment group's female rats was slightly higher than in the male rats. The relative weight of the heart of group IV animals was somewhat higher than that of other groups of animals. However, these differences were not dependent on dose. No significant difference in relative organ weight between the control and dose groups, as shown in Figure 5.

Histopathological Analysis

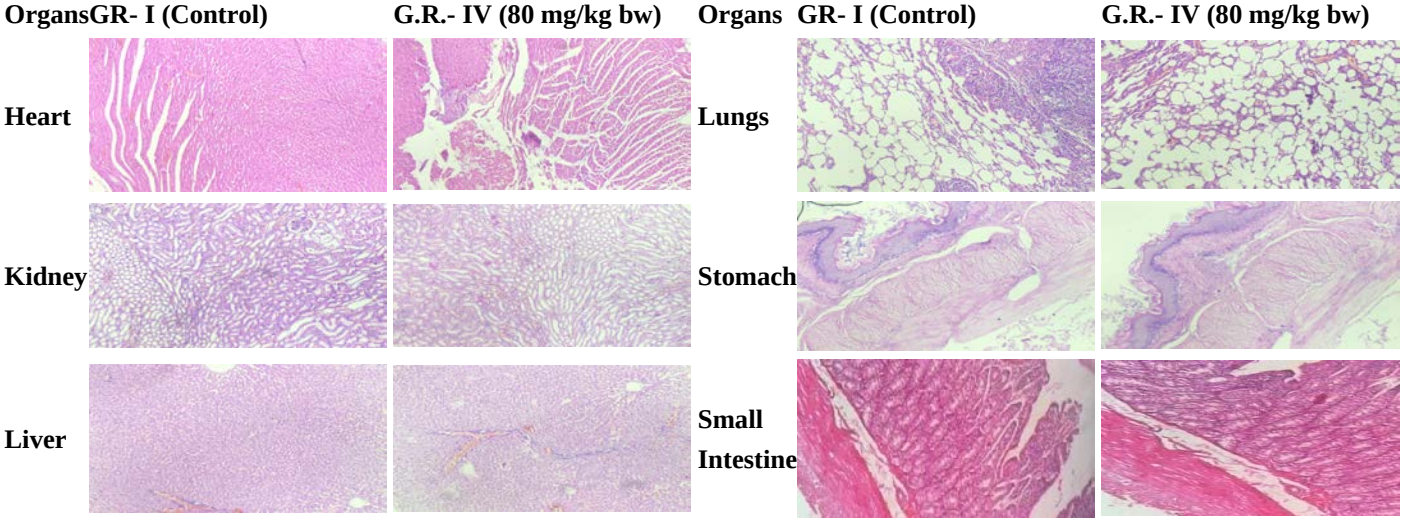


Figure 6: *Histopathology of internal organs of rats in subchronic toxicity study of carboxymethylated Basella alba mucilage (40X magnification)*

The histopathology of internal organs of the control group was found to be similar to group IV (high dose group), and no significant difference was observed. The tissue sections of the liver, kidney, heart, lungs, stomach, and small intestine in the control group and high-dose group (Group IV) were observed critically, and no abnormality was detected as shown in figure 6.

DISCUSSION

This is the first report of toxicity study of carboxymethylated *Basella alba* mucilage in rats. The sub-chronic toxicity model helps to know about the long-term adverse effects; therefore, this can be used to establish appropriate criteria for the safe use of mucilage in humans. Hematological parameters are very reliable in assessing the toxicity of a substance [23]. All the hematological parameters were in the acceptable range, so the tiny differences in the result were not clinically significant. Biochemical analysis effectively evaluates the physiological body functions [24]. All the biochemical parameters were in the range and showed minor variations. Body weight and internal organ weight also help to assess toxicity [25]. The results were within the normal range for albino rats, and the differences were insignificant [26].

The histopathology section of the heart shows normal heart tissue consisting of ventricles, atrium, intraventricular septum, pericardium, and endocardium in both control and group IV animals. Signs or symptoms of any pathological process were not observed. The histopathology results of the heart agreed with the study by Zahkouk et al. [27]. The kidney section of both control and group IV rats shows normal kidneys comprising glomeruli, renal tubule, interstitial tissues, blood vessels, and renal pyramid. The histopathology of the kidney section is comparable with the study by Safitri et al. [28]. The occurrence of any pathological lesion was not visible. The liver section of the control and high-dose group shows formation of hepatic lobules due to the arrangement of hepatocytes around hepatic venules in cords. The portal tract and sinusoidal space are within normal limits. No Pathological lesion or any abnormality was not observed. The result of the histopathological analysis of the liver is in agreement with the histopathological profile of rats studied by Safitri et al. [28]. Section of the lungs shows normal lung tissue containing various divisions of the respiratory tract, alveolar duct, interstitial tissue, alveoli, and blood vessels in both control and high-dose groups. Any abnormality, neoplastic process or granuloma was not detected. The section of the lungs is comparable with the histopathology of normal lung tissue analyzed by Dhouib et al. [29]. The stomach section shows histology of a normal stomach, oesophagus consisting of the mucosal layer, submucosa, muscular propria, and serosa. Any incidence of ulcer or pathological lesion was not observed. Section of the small intestine shows normal intestinal tissue, serosa, muscularis, submucosa, and mucosa without any lesion, inflammation, or abnormality in both the control group and high dose group of rats. The histopathology of the stomach and small intestine were similar to the histopathology results of normal rats, as stated by Maynard and Downes [30]. However, sub chronic toxicity or chronic toxicity studies for 90 days or more can be carried out to support the result observed in the current study.

CONCLUSION

The sub-chronic (28 days) toxicity study of carboxymethylated *Basella alba* mucilage in Wistar albino rats did not show any sign of toxicity or caused mortality in neither male nor female rats, suggesting that the functionalized mucilage is well accepted by the rats. All the hematological, biochemical parameters were within the normal range suggesting the non toxic property of mucilage. The food consumption, body weight examination and behavioural assessment of the rats were found to be normal. Therefore, the carboxymethylated mucilage is safe to use and can be used to formulate advanced pharmaceutical formulations. It can be used to explore the newer aspects of polysaccharides, where the tailored mucilage can be used to deliver the drug intelligently from the formulation. New novel targeted formulations employing naturapolyceutics can be prepared from the tailored polysaccharide. The study can also be a source of information for further research on the modified mucilage in humans. Therefore, the safety of the tailored mucilage can encourage the exploration of multifunctional properties of the carboxymethylated *Basella alba* mucilage with respect to the native polysaccharides to establish as pharmaceutical excipients in the pharmaceutical industry. This will further help to commercialize the underutilized natural polysaccharides and replace synthetic bio-incompatible polymers.

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FINANCIAL ASSISTANCE
Nil

CONFLICT OF INTEREST
The authors declare no conflict of interest

AUTHOR CONTRIBUTION
Moumita Chowdhury carried out the study design, experimental work, data collection, interpretation of results, and manuscript preparation. Pintu Kumar De reviewed the results and approved the final version of the manuscript. All authors read and approved the final manuscript.

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