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Acute and Repeated Dose 28-Day Oral Toxicity Studies of Phlorotannin Rich Fraction of *Sargassum tenerrimum*, A Marine Brown Algae

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Abstract

Sargassum tenerrimum is a marine brown algae rich in phlorotannins, a class of marine polyphenols with significant biological activities. The present study aimed to prepare a phlorotannin-rich fraction from *S. tenerrimum* (PST) and evaluate its acute and subacute oral toxicity in Wistar albino rats according to the procedures and methods of the OECD test guidelines for acute and repeated dose 28-day oral toxicity studies. *S. tenerrimum* powder was extracted with ethanol and further fractionated with ethylacetate, 1-butanol, and water. The ethyl acetate fraction was found to have the highest total phlorotannin concentration and was evaluated for its safety. In the acute oral toxicity study, a single dose of PST at 2000 mg/kg body weight did not result in any treatment-related clinical symptoms of toxicity or mortality. Therefore, the median lethal dose (LD50) of PST was identified as greater than 2000 mg/kg. The subacute oral toxicity investigation at 200, 400, and 800 mg/kg doses administered for 28 days with a 14-day recovery period revealed no treatment-related adverse clinical symptoms or mortality/morbidity. The treated animals exhibited normal weight gain, feed intake and did not result in clinically significant toxicity as measured by clinical blood chemistry and hematological markers. Gross and histological examinations of selected tissues did not reveal any notable adverse alterations associated with the intervention. Under the study's findings, the LD50 for PST was determined to be >2000 mg/kg b.wt. and No Observed Adverse Effect Level (NOAEL) to be 800 mg/kg rat b.wt.

Keywords: Acute Toxicity, Phlorotannins, Polyphenols, Safety, *Sargassum tenerrimum,* Sub-acute Toxicity

1. Introduction

Marine polyphenols are distinct secondary metabolites of algae that are not found in terrestrial flora and have been identified as potential biomedical candidates[1] Among marine algae, brown marine algae or brown seaweeds are well-known sources of polyphenols. The marine polyphenols are mainly composed of phloroglucinol units, i.e., phlorotannins, with a special structural makeup[2]. It's no coincidence that people in Southeast Asia, Japan, and coastal Western European countries frequently consume marine brown algae, which is high in polyphenols[3]. The *Sargassum* genus has been extensively studied among brown algae and is widespread in tropical and temperate oceans. The rich diversity of the genus *Sargassum* speaks of an under-explored reservoir of their biological activities with significant pharmaceutical use[4]. Amidst the *Sargassum* genus, *Sargassum tenerrimum* is found across the tropics and predominantly distributed on the shores of India, Pakistan, Japan, China, and East Asia[5]. *Sargassum tenerrimum* shows

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anticancer, anti-inflammatory, antioxidant, antifungal, antibacterial, antiviral, and antipredatory activities evident from the published preclinical reports[4-7]. These reports indicate brown algae as a novel candidate for bioprospection and drug development to better treat hard-to-cure ailments such as cancer, various inflammatory metabolic and neurodegenerative disorders, infectious diseases, etc. Marine polyphenols, i.e., phlorotannins, are likely to play a significant role in the actions of *S. tenerrimum*, considering their inherent existence and biomedical potential.

Although it has been considered that medicines derived from natural sources are safe, most medications can have side effects. Establishing a safe dose range, target organ toxicity, and therapeutic window are necessary to guarantee the security of using natural remedies[8]. Despite a few published reports on the efficacy of *Sargassum tenerrimum*, there is no information in the scientific literature about the extraction of phlorotannins from *Sargassum tenerrimum* and the safety profile of this seaweed or its extracts/fractions in acute overdosage and repeated usage. Toxicity tests are required for the development of new drugs as well as their long-term use. The impact of a single dose applied for two weeks is evaluated using an acute oral toxicity test. This test also determines a preliminary dosage range and the median lethal dose (LD_{50}). Repeated-dose toxicity tests must be conducted to assess the no observed adverse effect level (NOAEL) and determine the most adversely impacted organs[9]. The current investigation was therefore planned to be carried out in a phased manner. Phase 1 of the study involved the preparation of an ethanolic extract followed by fractionization using different solvents. The phlorotannin-rich solvent fraction was identified by estimating the total phlorotannin content of the crude and solvent fractions. The second phase was intended to investigate the phlorotannin-rich solvent fraction's acute oral toxicity using the OECD 423 guideline and a repeated dose 28-day oral toxicity study using the OECD 407 guideline in Wistar albino rats. It is anticipated that the present study will aid researchers in choosing safe doses for further preclinical efficacy studies of phlorotannin derivatives of *Sargassum tenerrimum*.

2. Materials and Methods

2.1 *Sargassum tenerrimum* Sampling and Chemicals

Fresh algae *Sargassum tenerrimum* was collected in November 2021 from the Mandapam region (09 17.417 0N; 079 08.558 0E), located on the southeast coast of India. The algae sample was washed with fresh water to remove debris, epiphytes, and adhering sand particles and then transported in sealed bags. The herbarium sheets were deposited in the Center for Medicinal Plants Research in Homoeopathy, Emerald, Tamil Nadu, and identified as *Sargassum tenerrimum J. Agardh,* and the accession number is given as 003/2021. In the laboratory, seaweed was preserved in a formaldehyde acetic acid alcohol (FAA) solution for microscopical study. For other studies, it was cleaned with tap water to eliminate any presence of salt, shade dried, and ground into a powder to pass through a 40-mesh sieve, then stored at room temperature for future use. Phloroglucinol and 2,4-dimethoxybenzaldehyde (DMBA) were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., India. All other chemicals and reagents used in this study were of analytical grade.

2.2 Extraction and Fractionization of *Sargassum tenerrimum* Ethanolic Extract

The conditions used for extraction were adapted from those proposed by Yajing Li[10] with some modifications. Powdered and shade-dried seaweed (1 kg) was extracted with n-hexane (600ml X 2), at room temperature for 24 hrs to remove lipid pigments. The residue was left in the desiccator for 2 hrs to draw out all the remaining n-hexane. Then, residue (100 g) was extracted twice with 30% ethanol (2 X 500mL) at room temperature for 03 hrs under constant agitation in a rotary shaker (Remi) with a speed of 125 rpm. The combined filtrate was concentrated *in vacuo* at 40°C using a rotary flash evaporator to about 100 ml. Next, the combined extract was sequentially partitioned three times with ethyl acetate (1:1, v/v) and 1-butanol (1:1, v/v) to fractionate polar and non-polar compounds. The fractions were concentrated in a rotary flash evaporator and yielded ethyl acetate, 1-butanol fractions, and aqueous residue. All the fractions were freeze-dried until further analysis at 4°C.

2.3 Estimation of Total Phlorotannin Content (TPC) of Crude Extract and Fractions

Phlorotannins (1,3,5-trihydroxy benzene), a polymer made of phloroglucinol, are thought to represent the majority of the polyphenolics in brown algae. Hence, in the study, phloroglucinol was used as the standard. Total Phlorotannin Content (TPC) in the ethyl acetate (EtOAC), 1-butanol and water fractions were determined according

to the 2,4-dimethoxybenzaldehyde (DMBA) colorimetric method, as previously described by Amarante, *et al.,* with slight modifications[11]. The working reagent was prepared by mixing equal volumes of DMBA (2g/100 ml solution) and hydrochloric acid (6%, v/v), prepared in glacial acetic acid and kept at room temperature. Then, 50 µL of each extract / fraction was mixed with 250 µL of working reagent at room temperature for one hour. A UV-Visible spectrometer was used to measure the absorbance at 515 nm (PerkinElmer). The TPC was expressed as mg phloroglucinol equivalent/g dry weight extract. The ethyl acetate portion was determined to have the highest TPC.

2.4 Experimental Animals

Wistar albino rats of both sexes (6-8 weeks, 150-200 g; both sex) bred in the in-house small animal house facility of Dr. Anjali Chatterji Regional Research Institute for Homeopathy, Kolkata was used in the study. Animals were housed (3 rats per cage) in polycarbonate cages under standard conditions (25 ± 2 °C, 60-70 % humidity) and maintained on a 12h light and dark cycle with free access to food and water. Autoclaved corn cob (Sparconn Life Sciences Pvt. Ltd., Karnataka) was used as bedding material, and feed was procured from Vishnu Traders, Roorkee. Animals were acclimatized to laboratory conditions for 7 days before the start of the experiment. All experimental procedures were carried out between 09:00 and 15:00 hrs. The experimental protocols were approved by the Institutional Animal Ethical Committee (DACRRIH/CPCSEA/IAEC/2022/02) of Dr. Anjali Chatterji Regional Research Institute for Homeopathy, Kolkata. Experiments were conducted under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) on the use and care of experimental animals. Before dosing, the animals were subjected to health checkups by the veterinarian based on the parameters of cage-side observation, skin and coat, posture and gait, respirations, disease symptoms, external orifices, behaviour, and body surfaces. Only healthy animals were involved in the study.

2.5 Experimental Design

2.5.1 Acute Oral Toxicity Study

An acute oral toxicity study was carried out per the OECD 423 guideline with slight modifications. In total, 12 female Wistar albino rats were divided into two groups, i.e., the control received 0.5% Carboxymethyl cellulose (CMC), and the test control received a phlorotannin-rich fraction of *Sargassum tenerrimum* (PST). 0.5% CMC was administered at a dose volume of 10 ml/kg, and PST was given at a dose of 2000 mg/kg at a dose volume of 10 ml/kg suspended in 0.5% CMC. The test was carried out in two steps. In step 1, before dosing, rats were fasted overnight and administered respective interventions in a single dose. After administration, food was withheld for a further 3-4 hrs. If no mortality was observed in the administered animals, then the extract was dosed in another set of three animals at 2000 mg/kg to confirm the same, i.e., step 2. If test substance-related mortality is produced, further testing at the next lower level may need to be carried out, i.e., 1000mg/kg in two steps. The procedure is depicted in Figure 01. During the experiment, the animals were observed for detailed clinical signs at the 0^{th} minute (prior dosing), 30^{th} (±5 min) minute, 1hr (±10 min), 2hr (±10 min), 3hr (±10 min), 4hr (±10 min), and 24hr (±10 min) after the dose administration on day 01 in step 01 and step 02. The observations included general behaviour, respiratory pattern, reflexes, motor activities, autonomic effects, skin and fur texture changes. Careful inspections of animals ensued twice daily during the study period to observe the mortalities if any. Body weight was noted once a week. At the end of the 14^{th} day, all the surviving animals of step 01 and step 02 were sacrificed by CO_2 euthanasia, dissected, and a gross necropsy examination was carried out and reported.

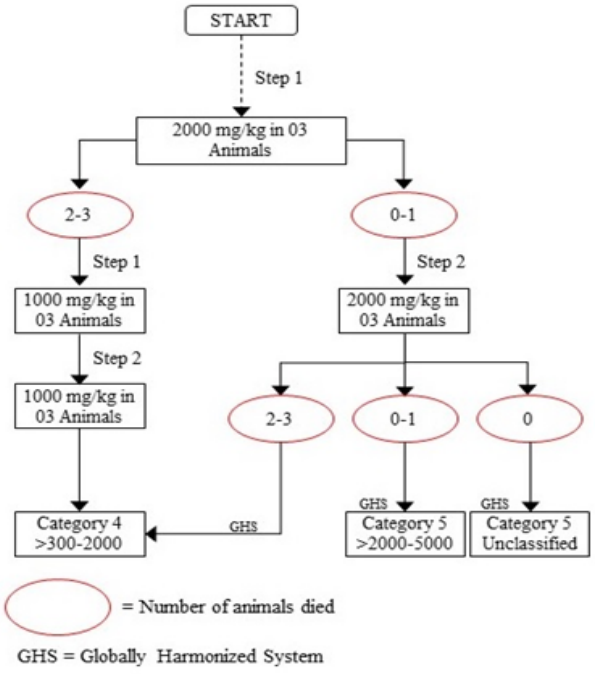


Figure 1. Steps involved in the acute oral toxicity study of a phlorotannin-rich fraction of *Sargassum tenerrimum.*

2.5.2 Repeated Dose 28-Day Oral Toxicity Study

A repeated dose 28 days oral toxicity study was conducted according to OECD guideline 407 with slight modifications. 40 Wistar albino rats (20 males and 20 females) were randomly allotted to four groups of 5 male and 5 female rats per group: G I-G IV (main groups). G I, G II, G III, and G IV were administered 0.5% CMC, 200, 400, and 800 mg/kg of PST, respectively. Additional two satellite groups, G IR and G IVR (recovery groups), for control (0.5% CMC) and high dose (800 mg/kg) having 5 male and 5 female rats per group, were given respective treatment for 28 days and after that, monitored for a further period (recovery period) of 14 days without treatment to determine whether toxic effects were reversible, persistent, or manifested later. The dosing volume of 10 mL/kg for all dose levels was constantly maintained based on the animal's weekly body weight. All treatments were administered orally once daily for 28 days.

2.5.2.1 Mortality/morbidity and Clinical Signs of Toxicity

All animals were observed twice daily for mortality and morbidity throughout the study. During the treatment phase, general clinical signs were examined once daily, preferably 1 hour after dosing, to account for the peak period of predicted effects following dosing. Detailed clinical observations include home cage observations, handling observations, open field observations, and sensory observations. In homecage observations, posture, vocalization, and palpebral closure were observed in rats. Handling observations included animal appearance, ease of removal, ease of handling, lacrimation, salivation, piloerection, urination, and defecation. On the other hand, open-field observations included gait, stereotype behaviour, arousal, abnormal behaviour, grooming, and clonic-tonic movements. Sensory observations involved approach response, touch response, auditory response, air righting reflex, and surface righting reflex.

2.5.2.2 Body Weight and Feed Consumption

All animals' body weights were measured on the day of administration, the first day of treatment before dosing, and then every week following that. The quantity of feed consumed by the control and different treatment groups was recorded at the commencement of treatment and weekly after that until the scheduled sacrifice.

2.5.2.3 Clinical Pathology

Clinical biochemistry and haematological investigations were performed on day 29 (control and treated groups) and 43 (reversal groups). All the rats were subjected to overnight fasting, and blood samples were withdrawn from the retro-orbital sinus under ketamine plus xylazine anaesthesia. Blood was obtained for haematology in tubes containing potassium EDTA and for clinical biochemistry in clot activator tubes. The serum for clinical chemistry analysis was separated by centrifugation of blood samples approximately at 5000 rpm for 10 minutes at 25°C. Hematological estimations were done by using a Veterinary hematology analyzer. The parameters evaluated included haemoglobin (Hb), total erythrocyte count (RBC), total leukocyte count (WBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), platelet count (PLT), Serum biochemical analysis was carried out using Fully automated biochemistry analyzer (EM 360, Transasia Pvt. Ltd). The parameters estimated were total protein, albumin, Serum Glutamate Oxaloacetate Transferase (SGOT), Serum Glutamate Pyruvate Transferase (SGPT), alkaline phosphatase, total bilirubin, serum creatinine, urea, glucose, Total Cholesterol (TC), triglyceride (TGL), Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), calcium, sodium, potassium, and chloride.

2.5.4.4 Gross Pathology and Histopathology

At the end of the study, overnight fasted rats were weighed and euthanized using carbon dioxide (CO_2) asphyxiation. The euthanized rats were subjected to a detailed gross necropsy, and the findings were recorded. The brain, thymus, heart, liver, lungs, adrenals, kidneys, spleen, testes/ovaries, and uterus were isolated, adherent adipose tissues from the organs were trimmed off, and absolute, relative organ weights were recorded. All the tissues were preserved in 10% neutral buffered formalin. These tissues were dehydrated in graded alcohol, inserted in paraffin, and cut into 4-5 µm thick sections using a rotatory microtome (Leica, India). The sections of the slide were stained with hematoxylin-eosin, and photomicroscopic analysis was performed using a Nikon Ni-Ti-2-U upright trinocular microscope with a 40X objective.

2.6 Statistical Analysis

Statistical analyses were performed using IBM SPSS (Version 26) software. The data were represented as mean

± SD (standard deviation). All statistical analyses were performed using two-tailed tests considering p values less than 5% ($p < 0.05$) as statistically significant. Data were subjected to the Shapiro-Wilk normality test to check the normal distribution of data. The numerical data, then related to body weight, feed consumption, relative organ weight, haematology, and clinical biochemistry data, were compared between the control group and any treatment group by one-way ANOVA, followed by Dunnett's post hoc test for each parameter separately.

3. Results

3.1 Estimation of Total Phlorotannin Content (TPC) of Crude Extract and Solvent Fractions of *Sargassum tenerrimum*

The extraction yield of the ethanolic crude extract of *S. tenerrimum* was approximately 21.4% w/w, and fractions generated from the ethanolic extract after solvent partitioning, namely, the aqueous, ethyl acetate, and 1-butanol, had a yield of 28.4%, 19.9%, and 5.1% respectively. The TPC in the ethyl acetate fraction (60.1 ± 1.9 mg PGE/g extract) was higher than that of the crude extract, thus confirming its additional purity. At the same time, aqueous (7.2 ± 3.3 mg PGE/g extract) and 1-butanol (1.8 ± 0.9 mg PGE/g extract) portions were poorer in TPC. Therefore, in the current study, the phlorotannin-rich fraction of the ethyl acetate was further examined for its safety in Wistar albino rats.

Yield is expressed as %w/w of dry algae (for crude extract) or crude extract (for fractions). PGE—phloroglucinol equivalents; the samples were analyzed in triplicate, and data were expressed as mean ± standard deviation.

3.2 Acute Oral Toxicity Study

During the study, no abnormal clinical signs, symptoms of toxicity, mortality, or moribundity were observed in animals administered with PST at a 2000 mg/kg dose in steps 1 and 2 (Table 2), and the animals were normal. No, statistically significant changes in absolute body weight were observed in animals treated with PST compared to the control group (Table 3). No abnormalities were found during the evaluation of the external surface of the body, orifices (anal, urethral, vaginal, and nasal), and cavities (thoracic and abdominal cavities) of the PST-treated animals. Histopathology was not carried out because the gross pathological examination revealed no anomalies.

3.3 Repeated Dose 28-Day Oral Toxicity Study

3.3.1 Mortality/morbidity and clinical signs

No mortality/morbidity and clinical signs of toxicity were observed in animals during the treatment period of 28 days and the post-dosing recovery period of 14 days (Table 04). Rats from the PST-treated and control groups did not show any abnormalities during detailed clinical observations, which included home cage observations, handling observations, open field observations, and sensory observations. Rats from all the groups showed normal appearance, gait, arousal, behaviour, approach response, touch response, auditory response, air righting reflex, and surface righting reflex.

3.3.2 Body Weight Gain and Feed Consumption

Animals in the control and PST-treated groups (200, 400, and 800 mg/kg) gained weight normally during the 28-day treatment period and following the recovery period of

Table 1. Extraction mass yield and total phlorotannin content of *S. tenerrimum* crude extract and fractions

Fraction	Yield (%)	Total Phlorotannin Content (mg PGE/g Extract)
Ethanolic crude extract	21.4	16 ± 2.3
1-butanol	5.1	1.8 ± 0.9
Ethyl acetate	19.9	60.1 ± 1.9
Aqueous	28.4	7.2 ± 3.3

Table 2. Summary of detailed clinical signs of a phlorotannin-rich fraction of *Sargassum tenerrimum* during oral acute toxicity study at 2000mg/kg dose

Observations	0 min	30 min	1 hr	2 hr	4hr	24 hr	14th day
Physical Observations							
Skin	N	N	N	N	N	N	N
Fur	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N
Urine	N	N	N	N	N	N	N
Behavioral Observations							
Alertness - Exploratory Activity	N	N	N	N	N	N	N
Grooming	N	N	N	N	N	N	N
Vocalization	NO	NO	NO	NO	NO	NO	NO
Tremors	NO	NO	NO	NO	NO	NO	NO
Sedation	NO	NO	NO	NO	NO	NO	NO
Ataxia	NO	NO	NO	NO	NO	NO	NO
Posture							
Surface Righting Reflex	N	N	N	N	N	N	N
Body Position	N	N	N	N	N	N	N
Limb Position	N	N	N	N	N	N	N
Sensory Responses							
Touch Response	N	N	N	N	N	N	N
Approach Response	N	N	N	N	N	N	N
Reflexes							
Pinna	N	N	N	N	N	N	N
Corneal	N	N	N	N	N	N	N
Respiratory Effects							
Apnoea	NO	NO	NO	NO	NO	NO	NO
Dyspnoea	NO	NO	NO	NO	NO	NO	NO
Autonomic Effects							
Discoloured faeces	NO	NO	NO	NO	NO	NO	NO
Lacrimation	NO	NO	NO	NO	NO	NO	NO
Salivation	N	N	N	N	N	N	N
Piloerection	NO	NO	NO	NO	NO	NO	NO
Diarrhoea	NO	NO	NO	NO	NO	NO	NO

N = Normal; NO = No abnormality Observed

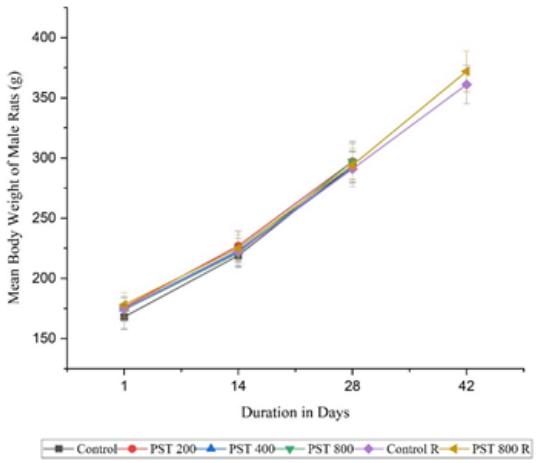


Figure 2. Group mean body weight (Mean ± SD) of male rats orally administered with a phlorotannin-rich fraction of *Sargassum tenerrimum* for 28 days (n = 5), R = Recovery.

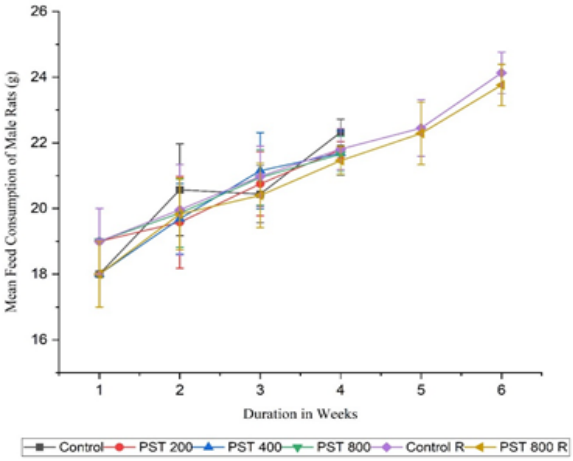


Figure 4. Group mean feed consumption (Mean ± SD) of male rats orally administered with a phlorotannin-rich fraction of *Sargassum tenerrimum* for 28 days (n = 5), R = Recovery.

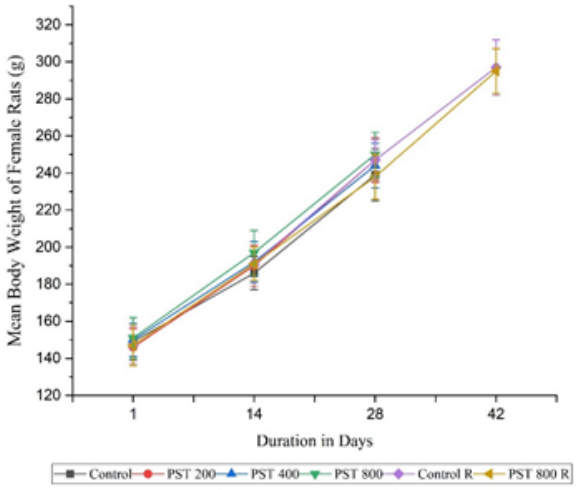


Figure 3. Group mean body weight (Mean ± SD) of female rats orally administered with Phlorotannin-rich fraction of *Sargassum tenerrimum* for 28 days (n = 5), R = Recovery.

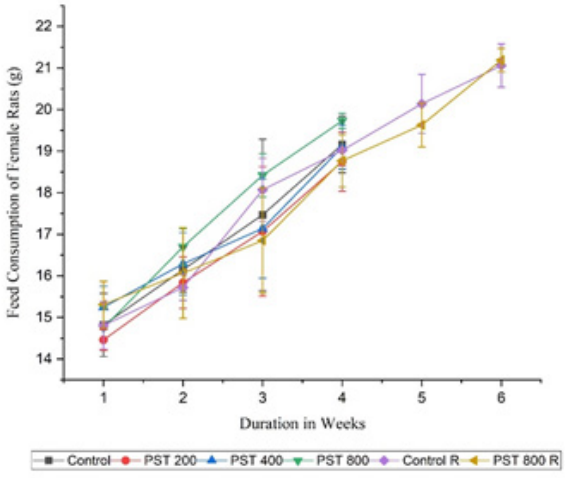


Figure 5. Group mean feed consumption (Mean ± SD) of female rats orally administered with a phlorotannin-rich fraction of *Sargassum tenerrimum* for 28 days (n = 5), R = Recovery.

Table 3. Body weight (g) during acute oral toxicity study of a phlorotannin-rich fraction of *Sargassum tenerrimum* at 2000mg/kg dose (n = 6)

Treatment	Body weight (g)		
	Day 01	Day 07	Day 14
Control (0.5% CMC)	169.43 ± 5.13	190.68 ± 5.82	214.62 ± 7.75
Phlorotannin-rich fraction of *Sargassum tenerrimum*	168.72 ± 7.20	186.23 ± 4.86	205.82 ± 5.05

Values are expressed as mean ± standard deviation (n = 06) at 5% level of significance (* = P < 0.05). Statistical evaluation was carried out using one-way ANOVA followed by Dunnett's post hoc test. No statistically significant changes were found.

Table 4. Mortality in rats treated with a phlorotannin-rich fraction of *Sargassum tenerrimum* during repeated dose 28-day oral toxicity study (n = 5)

Group	1 day	≤ 1 week	≤ 2 week	≤ 3 week	≤ 4 week	≤ 5 week	≤ 6 week	Final Mortality
Male								
Control	0	0	0	0	0	NA	NA	0/5
PST 200 mg/kg	0	0	0	0	0	NA	NA	0/5
PST 400 mg/kg	0	0	0	0	0	NA	NA	0/5
PST 800 mg/kg	0	0	0	0	0	NA	NA	0/5
Control-R	0	0	0	0	0	0	0	0/5
PST 800 mg/kg-R	0	0	0	0	0	0	0	0/5
Female								
Control	0	0	0	0	0	NA	NA	0/5
PST 200 mg/kg	0	0	0	0	0	NA	NA	0/5
PST 400 mg/kg	0	0	0	0	0	NA	NA	0/5
PST 800 mg/kg	0	0	0	0	0	NA	NA	0/5
Control-R	0	0	0	0	0	0	0	0/5
PST 800 mg/kg-R	0	0	0	0	0	0	0	0/5

C Final mortality = dead animals/total animal number; NA = Not Applicable; R = Recovery groups

14 days (Figures 2 and 3). Concerning feed consumption, no difference was observed in the control, all treated dose groups, and reversal dose groups at weekly intervals (Figures 4 and 5).

3.4 Clinical Pathology

After 28 days of administration of PST, at 400 mg/kg and 800 mg/kg b.wt. doses, a non-significant decrease in

Table 5. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on biochemical parameters in male rats during repeated dose 28-day oral toxicity study

Parameter	Groups					
	Control	**PST- 200 mg/kg**	**PST- 400 mg/kg**	**PST- 800 mg/kg**	**Control (Recovery)**	**PST- 800 mg/kg(Recovery)**
Glucose (mg/dL)	87 ± 14.14	85.2 ± 8.48	80.8 ± 12.02	78.8 ± 12.72	94.2 ± 5.65	88.2 ± 6.36
TC (mg/dL)	84 ± 9.89	81.2 ± 6.36	77.2 ± 3.44	75.4 ± 6.02	89.8 ± 5.65	84 ± 4.24
Tgl (mg/dL)	78.6 ± 8.48	74.4 ± 9.89	71.8 ± 5.31	69.2 ± 7.07	80.6 ± 13.43	78 ± 11.31
HDL (mg/dL)	36.4 ± 3.54	38.8 ± 3.54	39.6 ± 7.78	41.6 ± 4.24	37.8 ± 3.54	40.2 ± 5.66
LDL (mg/dl)	32.8 ± 5.65	29.8 ± 4.95	28 ± 3.53	25.8 ± 3.53	34.8 ± 3.62	30.4 ± 1.41
TP (g/dL)	6.38 ± 0.48	6.11 ± 0.24	6.17 ± 0.02	6.45 ± 0.51	6.26 ± 0.55	6.32 ± 0.64

Albumin (g/dL)	3.87 ± 0.17	3.81 ± 0.21	3.79 ± 0.28	3.95 ± 0.11	3.76 ± 0.37	3.83 ± 0.41
SGOT (U/L)	151.2 ± 23.33	145.6 ± 7.07	149.2 ± 12.02	143.2 ± 7.77	147 ± 8.48	150.8 ± 10.60
SGPT (U/L)	66.0 ± 7.07	62.4 ± 9.19	58.6 ± 10.60	63.0 ± 12.72	60.2 ± 7.07	56.4 ± 4.95
ALP (U/L)	167 ± 7.07	161.4 ± 9.19	158.4 ± 9.89	165 ± 16.26	151.4 ± 16.26	156.2 ± 11.31
TB (mg/dL)	0.090 ± 0.009	0.088 ± 0.005	0.097 ± 0.004	0.094 ± 0.009	0.094 ± 0.013	0.101 ± 0.001
Urea (mg/dL)	40.4 ± 7.07	43 ± 4.62	41.8 ± 2.82	37 ± 4.95	35.4 ± 2.82	33 ± 4.24
SC (mg/dL)	0.576 ± 0.021	0.574 ± 0.049	0.562 ± 0.120	0.528 ± 0.014	0.470 ± 0.02	0.488 ± 0.035
Sodium (mmol/L)	153.79 ± 2.34	149.72 ± 6.44	153.69 ± 2.47	157.63 ± 2.62	160.28 ± 2.65	156.74 ± 2.17
Potassium (mmol/L)	4.84 ± 0.56	4.60 ± 0.48	5.00 ± 0.24	4.96 ± 0.26	4.96 ± 0.26	4.88 ± 0.03
Chloride (mmol/L)	101.38 ± 1.49	102.42 ± 2.56	105.12 ± 3.53	98.26 ± 1.88	96.76 ± 2.05	101.83 ± 1.30
Calcium (mg/dL)	12.14 ± 0.55	11.74 ± 0.41	12.03 ± 1.87	11.92 ± 1.17	11.40 ± 1.14	± 2.24

Values are expressed as mean ± standard deviation. N = 5. TP = Total protein; SGOT = Serum Glutamate Oxaloacetate Transferase; SGPT = Serum Glutamate Pyruvate Transferase; ALP = Alkaline phosphatase; TB = Total Bilirubin; SC = Serum Creatinine; TC = Total Cholesterol; Tgl = Triglyceride; LDL = Low-density lipoprotein; HDL = High-density lipoprotein. There were no statistically significant differences in the biochemical parameters.

Table 6. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on biochemical parameters in female rats during repeated dose 28-day oral toxicity study

Parameter	Groups					
	Control	PST- 200 mg/kg	PST- 400 mg/kg	PST- 800 mg/kg	Control (Recovery)	PST- 800 mg/kg(Recovery)
Glucose (mg/dL)	79.0 ± 7.07	75.0 ± 8.48	73.0 ± 5.65	71.4 ± 7.07	86.0 ± 3.53	83.0 ± 9.89
TC (mg/dL)	75 ± 8.48	72.8 ± 6.36	70.6 ± 3.53	66.8 ± 7.77	82.6 ± 12.02	80.4 ± 7.07
Tgl (mg/dL)	64.4 ± 9.89	59.8 ± 9.89	58.0 ± 14.84	56.8 ± 7.07	69.2 ± 7.86	67.6 ± 9.89
HDL (mg/dL)	37.2 ± 7.07	37.4 ± 3.53	36.2 ± 7.07	39.6 ± 4.95	36.8 ± 6.36	37.8 ± 5.65
LDL (mg/dl)	29.34 ± 7.47	24.32 ± 6.28	23.98 ± 4.24	23.64 ± 8.05	31.68 ± 8.49	30.95 ± 11.81
TP (g/dL)	6.54 ± 0.24	6.11 ± 0.50	6.31 ± 0.46	6.52 ± 0.33	6.38 ± 0.07	6.57 ± 0.56
Albumin (g/dL)	3.71 ± 0.27	3.62 ± 0.14	3.69 ± 0.20	3.63 ± 0.48	3.64 ± 0.30	3.74 ± 0.33
SGOT (U/L)	136.6 ± 6.36	131.4 ± 26.87	126.4 ± 14.84	140.4 ± 19.79	124.0 ± 20.5	131.6 ± 7.07

SGPT (U/L)	57.4 ± 7.07	51.4 ± 8.48	53.4 ± 3.53	50.2 ± 6.36	46.6 ± 9.89	52.4 ± 7.43
ALP (U/L)	130.2 ± 7.77	134 ± 10.6	129.6 ±19.79	126 ± 14.84	125.2 ± 7.25	119.2 ± 9.89
TB (mg/dL)	0.104 ± 0.006	0.097 ± 0.011	0.113 ± 0.006	0.103 ± 0.028	0.113 ± 0.017	0.122 ± 0.007
Urea (mg/dL)	44.8 ± 4.24	47.4 ± 7.07	43.0 ± 7.07	40.0 ± 4.72	41.0 ± 4.95	36.4 ± 4.65
SC (mg/dL)	0.576 ± 0.021	0.574 ± 0.049	0.562 ± 0.12	0.528 ± 0.014	0.470 ± 0.02	0.488 ± 0.035
Sodium (mmol/L)	138.15 ±8.5	134.83 ± 10.86	142.31 ± 11.87	147.34 ± 6.68	152.37 ± 9.24	159.30 ± 7.07
Potassium (mmol/L)	4.37 ± 0.12	4.22 ± 0.01	4.01 ± 0.26	4.42 ± 0.24	3.92 ± 0.29	4.10 ± 0.36
Chloride (mmol/L)	100.40 ± 1.20	101.19 ± 6.14	98.70 ± 1.2	103.99 ± 2.13	103.57 ± 2.85	99.45 ± 2.04
Calcium (mg/dL)	10.32 ± 0.7	10.8 ± 2.2	10.24 ± 0.91	11.37 ± 0.92	9.85 ± 0.23	10.77 ± 0.25

Values are expressed as mean ± standard deviation. n = 5. TP = Total protein; SGOT = Serum Glutamate Oxaloacetate Transferase; SGPT = Serum Glutamate Pyruvate Transferase; ALP = Alkaline phosphatase; TB = Total Bilirubin; SC = Serum Creatinine; TC = Total Cholesterol; Tgl = Triglyceride; LDL = Low-Density Lipoprotein; HDL = High-Density Lipoprotein. There were no statistically significant differences in the biochemical parameter.

Table 7. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on Haematological parameters in male rats during repeated dose 28-day oral toxicity study

Parameter	Control	PST (200 mg/kg)	PST (400 mg/kg)	PST (800 mg/kg)	Control (Recovery)	PST- 800 mg/kg (Recovery)
HGB (g/dL	14.20 ± 0.90	14.50 ± 0.32	14.43 ± 0.28	14.50 ± 0.25	14.89 ± 0.89	14.46 ± 0.98
RBC (10^6/µL)	9.02± 0.96	8.84 ± 0.27	8.75 ± 0.32	9.1 ± 0.18	9.14 ± 0.8	8.93 ± 0.54
WBC (10^3/µL)	8.1 ± 0.24	8.23 ± 0.43	8.02 ± 0.30	8.54 ± 0.29	8.34 ± 0.36	8.7 ± 0.39
MCV (fL)	55.6 ± 3.50	54.75 ± 3.48	56.40 ± 2.45	57.92 ± 2.70	54.20 ± 4.3	57.25 ± 2.48
MCH (pg)	20.3 ± 2.27	19.50 ± 3.46	20.01 ± 3.30	19.89 ± 2.33	21.25 ± 2.72	20.32 ± 3.58
MCHC (g/dL)	37.3 ± 3.18	36.20 ± 2.7	35.68 ± 3.00	36.72 ± 3.56	38.56 ± 3.57	35.57 ± 2.39
PLT (10^3/µL)	775.30 ± 66.5	760.90 ± 85.56	767.80 ± 24.54	769.10 ± 34.38	769.54 ± 62.46	762.60 ± 84.30

Values are expressed as mean ± standard deviation. n=05. HGB: Haemoglobin concentration; RBC: Red Blood Cell; WBC: White Blood Cell; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration; PLT: Platelet Count. There were no statistically significant differences in the hematological parameters.

blood glucose and lipid levels (except HDL) was observed. However, after the drug administration was stopped and the levels were checked after the recovery period, the levels were comparable to control animals. The other biochemical parameters, i.e., liver profile, renal profile, calcium, and electrolytes, were comparable to the control group after

Table 8. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on haematological parameters in male rats during repeated dose 28-day oral toxicity study

Parameter	Control	PST (200 mg/kg)	PST (400 mg/kg)	PST (800 mg/kg)	Control (Recovery)	PST- 800 mg/kg(Recovery)
HGB (g/dL	12.91 ± 0.32	12.02 ± 0.70	12.7 ± 0.46	13 ± 0.73	13.2 ± 0.45	12.95 ± 0.87
RBC (10^6/µL)	7.5 ± 0.6	7.50 ± 0.92	7.60 ± 0.89	7.85 ± 0.45	7.95 ± 0.78	7.5 ± 0.48
WBC (10^3/µL)	8.12 ± 0.3	8.20 ± 0.34	8.48 ± 0.4	8.45 ± 0.4	8.46 ± 0.54	8.35 ± 0.55
MCV (fL)	53.9 ± 3.45	55.93 ± 2.75	52.67 ± 3.5	51.80 ± 3.42	52.70 ± 3.69	52.35 ± 2.9
MCH (pg)	9.7 ± 2.2	18.38 ± 2.34	20.3 ± 2.9	19.70 ± 2.7	19.95 ± 3.05	19.60 ± 2.8
MCHC (g/dL)	35.61 ± 3.56	34.63 ± 2.87	35.28 ± 3.22	34.16 ± 3.05	37.2 ± 3.87	35.2 ± 3.7
PLT (10^3/µL)	746.2 ± 35.4	739.8 ± 40.2	741.6 ± 26.24	718.8 ± 34.45	757.35 ± 36.25	748.2 ± 35.2

Values are expressed as mean ± standard deviation. n = 5. HGB: Haemoglobin concentration; RBC: Red Blood Cell; WBC: White Blood Cell; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration; PLT: Platelet Count. There were no statistically significant differences in the hematological parameters.

receiving a daily dose of PST for 28 days. (Tables 5 and 6). During the study period, there were no significant PST treatment-related changes in the haematological parameters of the animals compared to the control group (Tables 7 and 8).

3.5 Gross Pathology and Histopathology

The administration of the PST did not result in any noticeable morphological or hemorrhagic alterations in the organs as observed under the microscope. There were

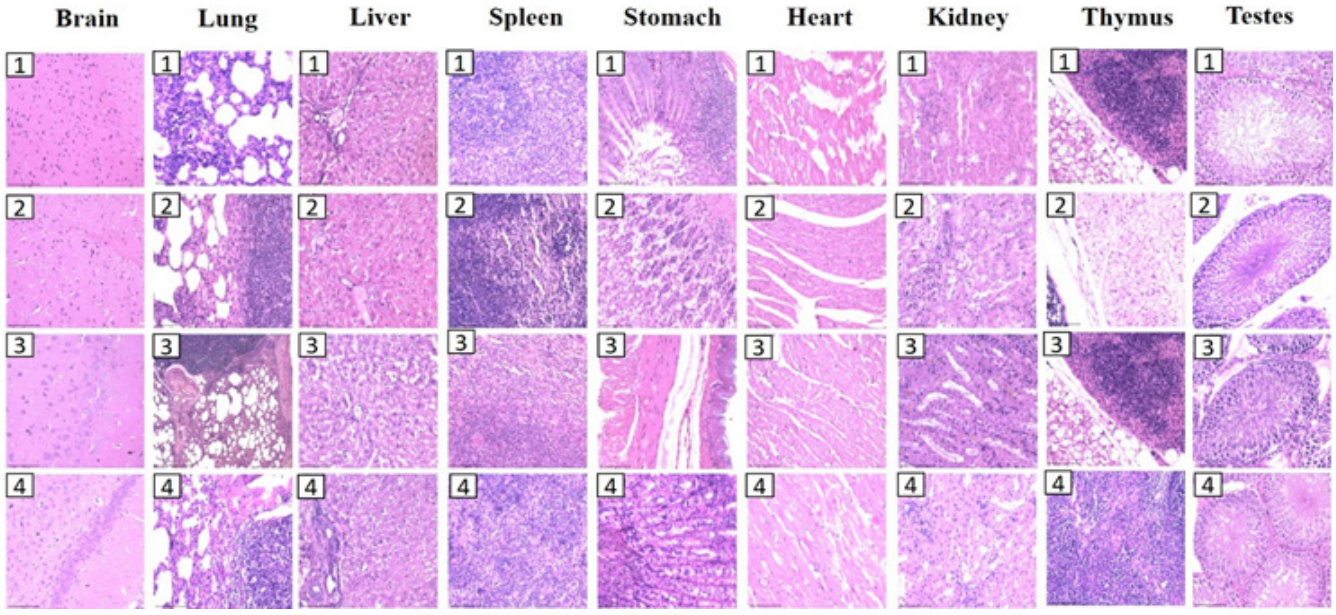


Figure 6. Photomicrographs of sections of the brain, lung, liver, spleen, stomach, heart, kidney, thymus, and testes of male rats treated with Phlorotannin-rich fraction of *Sargassum tenerrimum* for 28 days. Subindices 1, 2, 3 and 4 refer to the control group, 200, 400 and 800 mg/kg/day, respectively. H-E staining, 40X.

Table 9. Effect of a phlorotannin-rich fraction of *Sargassum tenerrimum* on absolute organ weights and relative organ weights (mean ± SD) of female rats during repeated dose 28-day oral toxicity study

	Control	PST- 200 mg/kg	PST- 400 mg/kg	PST- 800 mg/kg	Control (Recovery)	PST- 800 mg/kg (Recovery)
Necropsy Body weight	238.8 ± 13.95	247 ± 12.02	244 ± 11.81	250.4 ± 12.14	296.6 ± 14.76	295 ± 11.73
Brain						
Absolute	1.75 ± 0.04	1.75 ± 0.03	1.76 ± 0.04	1.75 ± 0.04	1.84 ± 0.02	1.96 ± 0.03
Relative	0.73 ± 0.05	0.71 ± 0.03	0.72 ± 0.03	0.70 ± 0.03	0.62 ± 0.03	0.66 ± 0.02
Thymus						
Absolute	0.15 ± 0.02	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.19 ± 0.02	0.19 ± 0.01
Relative	0.06 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.00
Heart						
Absolute	0.94 ± 0.05	0.96 ± 0.09	0.99 ± 0.10	0.99 ± 0.18	1.29 ± 0.07	1.21 ± 0.10
Relative	0.39 ± 0.02	0.39 ± 0.02	0.41 ± 0.02	0.39 ± 0.06	0.44 ± 0.01	0.41 ± 0.03
Liver						
Absolute	7.01 ± 0.39	7.08 ± 0.65	7.11 ± 0.42	7.12 ± 0.29	10.40 ± 1.50	9.81 ± 0.91
Relative	2.94 ± 0.06	2.86 ± 0.16	2.91 ± 0.09	2.85 ± 0.08	3.50 ± 0.37	3.32 ± 0.19
Lungs						
Absolute	1.30 ± 0.13	1.40 ± 0.07	1.29 ± 0.15	1.42 ± 0.10	1.63 ± 0.04	1.56 ± 0.14
Relative	0.54 ± 0.03	0.57 ± 0.01	0.53 ± 0.04	0.57 ± 0.02	0.55 ± 0.01	0.53 ± 0.04
Spleen						
Absolute	0.90 ± 0.07	0.93 ± 0.05	0.96 ± 0.06	0.98 ± 0.14	1.24 ± 0.07	1.15 ± 0.13
Relative	0.38 ± 0.01	0.38 ± 0.02	0.39 ± 0.02	0.39 ± 0.05	0.42 ± 0.00	0.39 ± 0.03
Kidneys						
Absolute	1.81 ± 0.11	1.84 ± 0.15	1.86 ± 0.09	1.86 ± 0.15	2.36 ± 0.10	2.41 ± 0.16
Relative	0.76 ± 0.03	0.75 ± 0.02	0.76 ± 0.01	0.74 ± 0.05	0.80 ± 0.02	0.82 ± 0.04
Ovaries						
Absolute	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.19 ± 0.00	0.19 ± 0.01
Relative	0.06 ± 0.00	0.06 ± 0.00	0.06 ±0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
Uterus						
Absolute	0.47 ± 0.07	0.46 ± 0.04	0.44 ± 0.03	0.46 ± 0.03	0.56 ± 0.02	0.53 ± 0.09
Relative	0.20 ± 0.02	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.00	0.19 ± 0.00	0.18 ± 0.02

Values are expressed as mean ± standard deviation. n = 5. There were no statistically significant differences in the absolute and relative organ weights of rats.

also no statistically significant differences in male and female rats' absolute and relative organ weights (Table 9). Histopathological examination of the different organs demonstrated no pathological features in either the control or the PST-treated groups in both sexes (Figures 6 and 7).

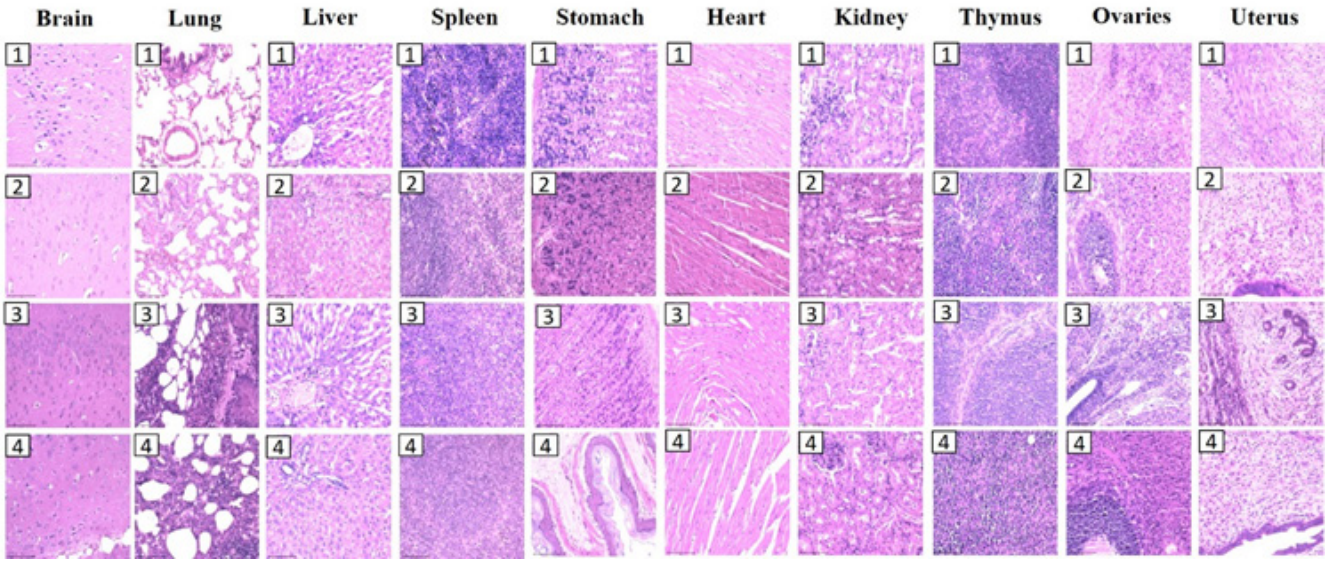


Figure 7. Photomicrographs of sections of brain, lung, liver, spleen, stomach, heart, kidney, thymus, ovaries, and uterus of female rats treated with Phlorotannin-rich fraction of *Sargassum tenerrimum* for 28 days. Subindices 1, 2, 3 and 4 refer to the control group, 20, 400 and 800 mg/kg/day, respectively. H-E staining, 40X.

4. Discussion

Marine brown seaweeds are highly valuable biologically and a well-known source of phlorotannins, which are structurally distinct polyphenol metabolites[12]. *Sargassum tenerrimum* is an under-explored brown alga that has the potential to be developed as a functional ingredient in pharmaceutical formulations. In the present study, shade-dried powder of *S. tenerrimum* was extracted with ethanol and further fractionated with ethyl acetate, 1-butanol, and water. The ethyl acetate fraction of *S. tenerrimum* showed significantly greater total phlorotannin content than those in 1-butanol and aqueous fractions (2.04 mg GAE/g). Phlorotannin extraction from the seaweed's ethyl acetate fraction may be commercially viable based on the yield and phlorotannin concentration of the fraction. The absence of literature data concerning the levels of phlorotannin content in crude/purified fractions of *S. tenerrimum* hinders a direct comparison with our results. In light of the enrichment of phlorotannins in the ethyl acetate solvent fraction, it was further examined for its safety using acute and repeated dose toxicity studies in Wistar albino rats.

Acute oral toxicity is regarded as the first step in determining the safety of the test material because it gives information on potential health risks from acute exposure. It is clear from the acute oral toxicity test findings that a single acute dose of PST given to Wistar albino rats at a dose of 2000 mg/kg could not result in any mortality and was, therefore, determined to be safe. According to the Globally Harmonized System (GHS) classification system, PST was assigned to Category 5 or Unclassified status as the LD_{50} was found to be greater than 2000 mg/kg rat body weight[13].

Repeated-dose toxicity studies are carried out to assess the potential detrimental effects that may result from repeated exposure to the test material over an extended period of time. Additionally, it offers details on the potential for cumulative effects and an estimate of the dose at which the test material is continuously exposed without showing any adverse effects[14]. Repeated oral administration of PST at dose levels of 200, 400 and 800 mg/kg did not show any mortality or morbidity throughout the dosing period of 28 days and recovery period of 14 days. Any drug with a toxic effect causes disrupted physiological homeostasis, which is visible or observable by phenotypic alterations or animal behaviors[15]. PST-treated rats' normal physiological responses were validated by extensive clinical observations, including home cage observations, handling observations, open field observations, and sensory observations. These findings were consistent with those of the rats in the control group.

In growing animals, the inability to maintain or reach the expected body weight is a sign of abnormality. Body weight is also an objective sign of animal stress and a measure of pain and discomfort in experimental animals[16]. Male and female animals from the PST-treated groups and recovery groups

showed normal body weight gain compared with the control group, indicating their wellness during PST treatment. Feed consumption also did not alter significantly during the study period in PST-treated group animals compared to control animals. The faecal colour of animals administered with PST was greenish-like, presumably derived from orally administered PST.

Twenty-eight days of administration of PST at 400 mg/kg and 800 mg/kg body weight doses caused a non-significant decrease in fasting blood glucose and lipids (except HDL). However, after the recovery period, the fasting blood glucose and lipid (except HDL) levels improved and were comparable to those of the control group. In animals administered PST at 200 mg/kg, the levels of the above parameters were comparable to control animals. Levels of hepatic biomarkers, i.e., SGOT, SGPT, ALP, total protein, albumin, total bilirubin levels and renal biomarkers, i.e., serum creatinine, were found to be comparable in PST-treated animals compared to control group animals during the study. The haematological parameters were within the normal range in male and female animals administered with PST during the study. At the end of the study, gross pathological observations revealed no abnormality, and the relative organ weights of the brain, lung, liver, spleen, stomach, kidney, thymus, spleen, heart, testes/ovary, and uterus were comparable to the control groups. Histopathological examination of PST-treated rats revealed no alterations in the brain, liver, lungs, kidney, heart, stomach, testes/ovary, or uterus compared to the control group rats, indicating that PST has no adverse effects on these tissues. Based on this study's results, repeated PST administration at 200 mg/kg, 400 mg/kg and 800 mg/kg p.o. was found safe in Wistar albino rats. Therefore, it may be concluded that there is no hazardous property of PST at the doses of 200 mg/kg, 400 mg/kg and 800 mg/kg p.o., and that the same doses were found safe after 28 days of continuous daily administration.

5. Conclusion

The ethyl acetate fraction of *Sargassum tenerrimum* was found to have the highest total phlorotannin content among the fractions. Hence, it was identified as the phlorotannin-rich fraction and further investigated for its safety in Wistar albino rats. The acute oral toxicity study results indicate that the LD_{50} of PST is greater than 2000 mg/kg body weight, as it is devoid of any biologically relevant toxicity after single oral administration in female Wistar albino rats. After 28 days of repeated administration of PST, no signs of significant toxicity were observed at all the tested dose levels, i.e., 200, 400 and 800 mg/kg body weight in male and female rats. Hence, the No Observed Adverse Effect Level (NOAEL) for PST in 28 days of repeated oral toxicity study was found to be 800 mg/kg body weight. Further analytical studies characterising the phlorotannins in the ethyl acetate fraction of *S. tenerrimum* are warranted. These findings can be the basis for further preclinical development of phlorotannin-derived compounds from S. *tenerrimum* and their screening as novel functional foods, feeds, and pharmaceuticals.

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