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Acute and Sub-Acute Toxicity Evaluation of Ethanolic *Halimeda gracilis* Extract (Kadarpassi Chooranam): Advancing Preclinical Safety Data for Marine-Based Therapeutics in Wistar Rats

Mushraf Syed[1], R. Jayanthi[2], Bernaitis L[3], Danie George[4], Manimekalai K[5] , Sabari Anandh J V[6*]

[1]Assistant Professor, Department of Pharmacology, American University of Antigua, College of Medicine, University Park, Jabberwock Beach Road, P.O.Box 1451, Coolidge, Antigua.

[2] Associate Professor, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed-to-be-University), Puducherry, India.

[3] Assistant Professor, Department of Microbiology, Kanyakumari Medical Mission Research Centre and hospital (St.Joseph University), Muttom- 629202, Kanyakumari (D.t), India.

[4]Assistant Professor, Department of Rachana Shareera, Vaidya Ratnam Ayurveda College, Thrissur- 680322, Kerala

[5]Professor and Head, Department of Pharmacology, MGMCRI, Sri Balaji Vidyapeeth (Deemed-to-be-University), Puducherry-607403, India.

[6*]Assistant Professor, Department of Pharmacology, St. Peter's Medical College, Hospital and Research Institute, Hosur-635130, Krishnagiri District, India.

* Corresponding authors

[5a*] Dr. Sabari Anandh J V, Assistant Professor, Department of Pharmacology, St. Peter's Medical College, Hospital and Research Institute, Hosur- 635130, Krishnagiri District, India.

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KEYWORDS

Halimeda gracilis, marine green algae, ethanolic extract, acute toxicity, repeated dose toxicity.

ABSTRACT:

Background:

Marine-derived algae are recognised as a promising source of compounds with medicinal properties and therapeutic potential. However, the limited toxicological data available restrict their translational applications. *Halimeda gracilis*, a green alga traditionally utilised in Siddha medicine as kadarpassi chooranam, has demonstrated diverse pharmacological activities. This study aimed to evaluate the acute and sub-acute oral toxicity of the ethanolic extract of *Halimeda gracilis* (Kadarpassi Chooranam) in Wistar rats, providing foundational safety data to support its potential pharmaceutical applications.

Methods:

Acute toxicity was assessed following OECD Test Guideline 423 at a single dose of 2000 mg/kg of Ethanolic Extract of Kadarpassi Chooranam (EEKPC). Sub-acute toxicity (28 days) was evaluated following OECD Guideline 407 using daily doses of 250, 500, and 1000 mg/kg. Hematological, biochemical, gross pathological, and histopathological parameters were evaluated.

Results:

No mortality or treatment-related abnormalities were observed at any dose of EEKPC. The LD50 exceeded 2000 mg/kg, and the NOAEL was 1000 mg/kg/day. All physiological, biochemical, and histopathological findings were within normal limits.

Conclusion:

These findings indicate that the EEKPC is well-tolerated under experimental conditions, supporting

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its safe use as a potential phytopharmaceutical or nutraceutical ingredient. Further chronic toxicity and mechanistic studies are warranted to confirm its long-term safety.

1. Introduction

Seaweeds, commonly referred to as marine algae, are photosynthetic organisms that inhabit diverse marine environments. Based on pigment composition, they are classified into Chlorophyta, Phaeophyta, and Rhodophyta [1,2,3]. These algae contribute significantly to marine ecology by providing habitat and primary productivity [4]. Their renewable nature supports applications across various industries, including food, agriculture, cosmetics, and pharmaceuticals [1,5]. Seaweed contains diverse bioactive compounds, including polysaccharides, polyphenols, and vitamins, which impart various therapeutic effects [5].

Green algae (Chlorophyta) are prominent photosynthetic eukaryotes found in marine, freshwater, and terrestrial habitats [6,7]. Their polysaccharide-rich structure contributes to antiviral, antibacterial, cytotoxic, and immunomodulatory [8,9]. Halimeda gracilis contains phenolics, flavonoids, and alkaloids responsible for its antioxidant, anti-inflammatory, and anticancer activities. In Siddha medicine, it forms the basis of Kadarpassi Chooranam [10,11].

This study evaluates the safety of the ethanolic extract of Halimeda gracilis (kadarpassi chooranam) through acute and repeated-dose toxicity studies in Wistar rats to establish its safety for therapeutic applications.

Materials and Methods:

Collection and Preparation of Kadarpasi Chooranam:

Fresh samples of the marine green alga *Halimeda gracilis* were collected from the coastal region of Rameswaram, Tamil Nadu, India. Species identification was confirmed by a marine biologist, and herbarium specimens were prepared for repository documentation (J Agarah, 1887). Samples were washed with seawater to remove epiphytes and then rinsed with tap water to eliminate salt and debris. The cleaned algae were shade-dried, and Kadarpassi Chooranam was prepared following the Siddha Pharmacopeia guidelines [12].

Preparation of Ethanolic Extract:

The ethanolic extract of Kadarpassi Chooranam (EEKPC) was prepared by loading 40 g of powdered sample into a Soxhlet apparatus and extracting with ethanol for 40 hours. The extract was concentrated at 45°C using a water bath and stored at 4°C until further analysis [13].

Ethical Clearance and IAEC Approvals:

All procedures received approval from the Institutional Animal Ethics Committee of MGMCRI. Acute toxicity study approval: 02/IAEC/MG/10/2020-II. Repeated dose toxicity approval: 08/IAEC/MG/10/2020-II.

Animals:

Wistar rats were procured from BIOGEN Breeding Center, Bengaluru, and maintained under standard laboratory conditions with controlled temperature (26 ± 2°C) and humidity (45–55%). They received standard pellet diet and water ad libitum.

Acute toxicity study:

Acute oral toxicity was evaluated according to OECD Guideline 423 [14.15]. Female Wistar rats (170–200 g) were divided into two groups (n = 3/group). After overnight fasting, EEKPC was administered orally at a dose of 2000 mg/kg body weight. Animals were observed at multiple intervals during the first 4 hours and daily thereafter for 14 days. Body weights were recorded before dosing and at weekly intervals. A gross pathological examination was performed at the study termination.

Repeated oral dose toxicity study:

The repeated dose toxicity study was performed in accordance with OECD guideline 407[14.15]. After completing the acute oral toxicity assessment, Wistar albino rats of both sexes (n = 40; 20 males and 20 females) were assigned to four groups, each comprised of ten animals (five males and five females per group). Group I served as the control, while Groups II, III, and IV received oral doses of EEKPC at 250 mg/kg, 500

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mg/kg, and 1000 mg/kg body weight, respectively (see Table 1).

The 28-day repeated dose study followed OECD guideline 407[14.15]. Forty rats (20 males, 20 females) were allocated into four groups: control, 250 mg/kg, 500 mg/kg, and 1000 mg/kg EEKPC. Additional satellite groups (n = 10 each) were included for extended observation. EEKPC was administered orally once daily. Animals were monitored for clinical signs, behavior, and toxicity. At study completion, animals were fasted overnight, euthanized, and subjected to necropsy. Major organs were harvested for histopathological examination Table 1.

Hematological analysis:

Blood collected in EDTA tubes was analyzed for complete blood count parameters using an automated veterinary hematology analyzer.

Biochemical analysis:

Serum samples were analyzed for glucose, bilirubin, creatinine, cholesterol, albumin, globulin, SGOT, SGPT, alkaline phosphatase, and A/G ratio.

Histopathological Examination:

Euthanized animals' organs liver, heart, kidneys, pancreas, spleen, and reproductive organs (uterus in females and testes in males)—were excised for examinationwere fixed in 10% neutral buffered formalin, processed using standard histological procedures, sectioned, stained, and examined microscopically for cellular alterations.

Table 1: Experimental design for repeated oral dose toxicity study of EEKPC

Study	Test Drug	Groups	Dose(mg/kg)	Species/ Strain	No. Of Animals and Sex	Total Animals
Repeated Dose Toxicity Study	EEKPC	Control	Sterile water 1ml/100g	Wistar Rats	5 male + 5 females	10
		Low Dose	250mg/kg	Wistar Rats	5 male + 5 females	10
		Medium Dose	500mg/kg	Wistar Rats	5 male + 5 females	10
		High Dose	100 mg/kg	Wistar Rats	5 male + 5 females	10
Satellite Group						
Control Group			Sterile Water1ml/100g	Wistar Rats	5 male + 5 females	10
High Dose of EEKPC			1000mg/kg	Wistar Rats	5 male + 5 females	10
Total number of animals						60

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Table 2: Observation of behavioral activity in acute oral toxicity

Groups	Day 0	Day 7	Day 14
Test 1	Nil	Nil	Nil
Test 2	Nil	Nil	Nil

Table 3: Biochemical parameters for *Kadarpassi Chooranam* in ethanol extract

		Male				Female			
S. No.	**Biochemical parameters**	**control group**	**KPC 250mg/kg**	**KPC 500mg/kg**	**KPC 1000mg/kg**	**control group**	**KPC 250mg/kg**	**KPC 500mg/kg**	**KPC 1000mg/kg**
1	Bilirubin	0.26±0.025	0.306±0.013	0.332±0.029	0.242±0.063	0.28±0.057	0.292±0.025	0.324±0.011	0.226±0.011
2	Serum creatinine	0.67±0.228	0.58±0.084	0.416±0.034	0.44±0.055	0.65±0.218	0.54±0.114	0.426±0.03	0.44±0.055
3	SGOT	24.5±2.5	25.6±2.881	29.2±3.701	21±1.768	21.9±4.037	25.2±3.633	27.2±1.789	16.7±2.465
4	SGPT	26±2.5	26.4±3.05	29±3.24	22.2±1.304	26.9±4.588	28.6±2.702	32±1.00	22.4±2.408
5	ALP	70.48±0.841	71.8±2.049	74.8±1.643	73.6±1.517	70.05±1.067	72±1.871	75.2±2.168	73.8±1.643
6	Blood urea	16.6±0.418	17±1.581	16.4±2.302	17.1±0.742	17.4±1.387	19±1.414	16.6±1.817	16.8±0.837
7	Total Cholesterol	94.2±3.347	94.6±2.408	92.6±2.302	93.2±1.924	96.2±3.768	94.8±2.168	93±3.082	92.6±1.673

Table 4: Biochemical Parameters for *Kadarpassi Chooranam* - satellite group

	Male			Female	
S. No.	**Biochemical Parameters**	**control group**	**KPC 1000mg/kg**	**control group**	**KPC 1000mg/kg**
1	Bilirubin	0.268±0.04	0.234±0.04	0.264±0.042	0.23±0.015
2	Serum creatinine	0.574±0.147	0.44±0.055	0.59±0.114	0.44±0.055
3	SGOT	24.6±3.05	19.38±2.465	24.2±2.049	16.16±1.128
4	SGPT	26.2±3.114	23.14±1.885	27.6±2.966	21.64±0.868
5	ALP	70.75±0.304	73.8±1.817	69.77±1.111	73.8±1.924
6	Blood urea	16.67±0.597	17.06±0.904	17.07±0.983	17.1±0.742
7	Total Cholesterol	94±1.871	94.2±1.483	93.6±1.673	92.4±1.817

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Table 1: Hematological parameters for EEKPC

		Male				Female			
S. No.	**Haematological parameters**	**Control group**	**KPC 250mg/kg**	**KPC 500mg/kg**	**KPC 1000mg/kg**	**Control group**	**KPC 250mg/kg**	**KPC 500mg/kg**	**KPC 1000mg/kg**
1	Total WBC count	13.290±1.072	12.960±1.143	12.55±2.546	11.881±1.597	13.801±1.181	13.08±1.528	10.33±1.852	11.505±1.698
2	Neutrophils	22.4±2.881	19.2±1.924	26.4±2.705	24±2.915	20.2±1.924	19.2±1.924	25.8±3.347	24.2±2.95
3	Lymphocytes	76.6±2.408	80±2	72.6±2.702	74.2±3.194	78.6±1.949	79.8±2.588	73.2±3.114	74.6±2.074
4	Mixed cells (MXD)	1±0.707	0.8±0.837	1±0.707	1.8±1.095	1.2±0.837	0.8±0.837	1±1	1.2±1.304
5	Haemoglobin	11.76±0.586	12.4±0.274	13.58±0.963	14.1±0.962	11.76±0.635	12.38±0.363	12.54±0.207	12.17±0.479
6	Total RBC count	7.94±0.495	7.936±0.553	8.58±1.06	7.81±1.024	7.89±0.39	7.684±0.356	7.5±0.809	8.36±0.336
7	PCV	42.584±3.131	44.04±1.898	46.96±3.129	40.3±2.636	44.028±2.382	42±2.345	41.98±1.04	42.97±0.881
8	Platelets count	5.986±0.293	6.982±0.538	7.264±1.356	9.02±2.722	5.97±0.273	7.664±0.34	8.416±0.481	9.18±0.517

Table 6: Hematological parameters for *Kadarpassi Chooranam* - satellite group

	Male			Female	
S. No.	**Haematological parameters**	**control group**	**KPC 1000mg/kg**	**control group**	**KPC 1000mg/kg**
1	Total WBC count	13.33±8.69	11.99±1.037	13.54±1.44	11.187±1.899
2	Neutrophils	22±1.871	23.6±2.881	21.2±1.643	24.2±2.683
3	Lymphocytes	77±2	74.8±3.271	78±1.871	75±2.25
4	Mixed cells (MXD)	1±0.707	1.6±1.14	0.8±0.447	0.8±0.447
5	Haemoglobin	12±0.324	12.99±0.808	11.62±0.342	11.96±0.352
6	Total RBC count	8.16±0.23	8.18±0.432	7.64±0.152	7.8±0.316
7	PCV	43.876±2.126	42.16±0.416	41.85±2.114	42.5±0.797
8	Platelets count	5.92±0.327	9.134±2.187	6.04±0.27	8.82±0.327

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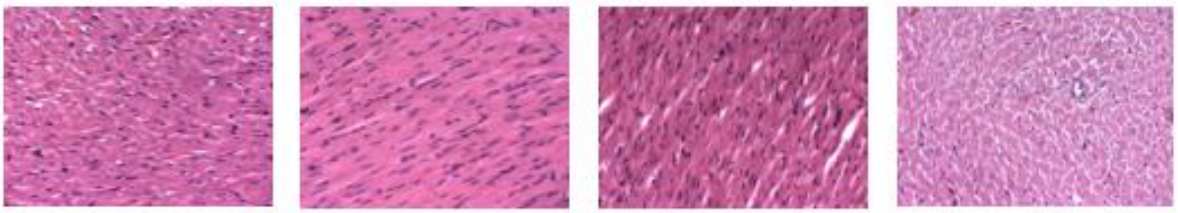
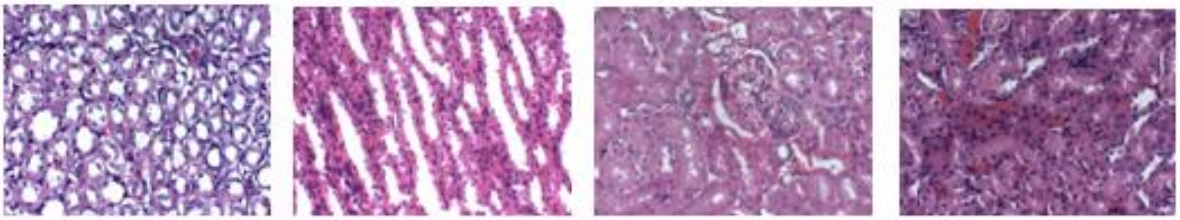
Histopathological observation:

Heart:



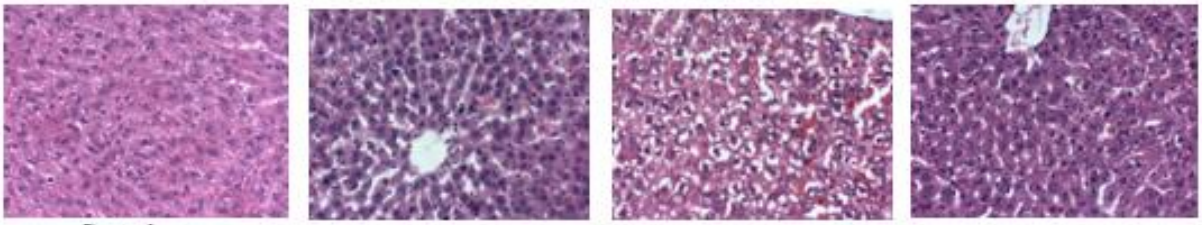
Control group | KPC-250mg/kg | KPC-500mg/kg | KPC-1000mg/kg

Kidney:



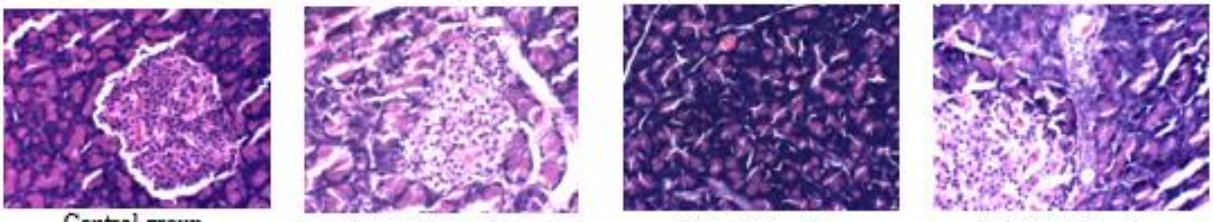
Control group | KPC-250mg/kg | KPC-500mg/kg | KPC-1000mg/kg

Liver:



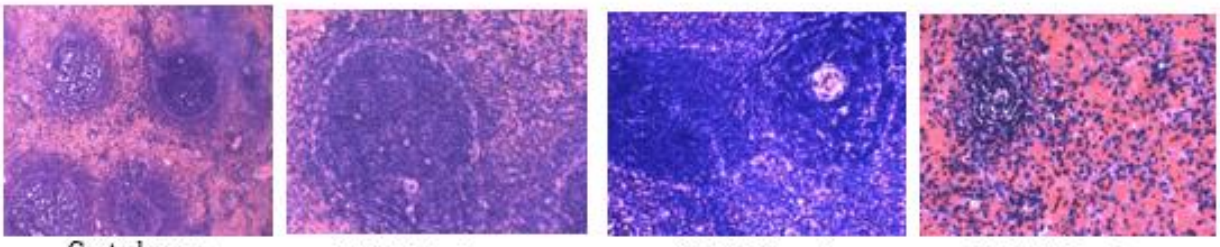
Control group | KPC-250mg/kg | KPC-500mg/kg | KPC-1000mg/kg

Pancreas:



Control group | KPC-250mg/kg | KPC-500mg/kg | KPC-1000mg/kg

Spleen:



Control group | KPC-250mg/kg | KPC-500mg/kg | KPC-1000mg/kg

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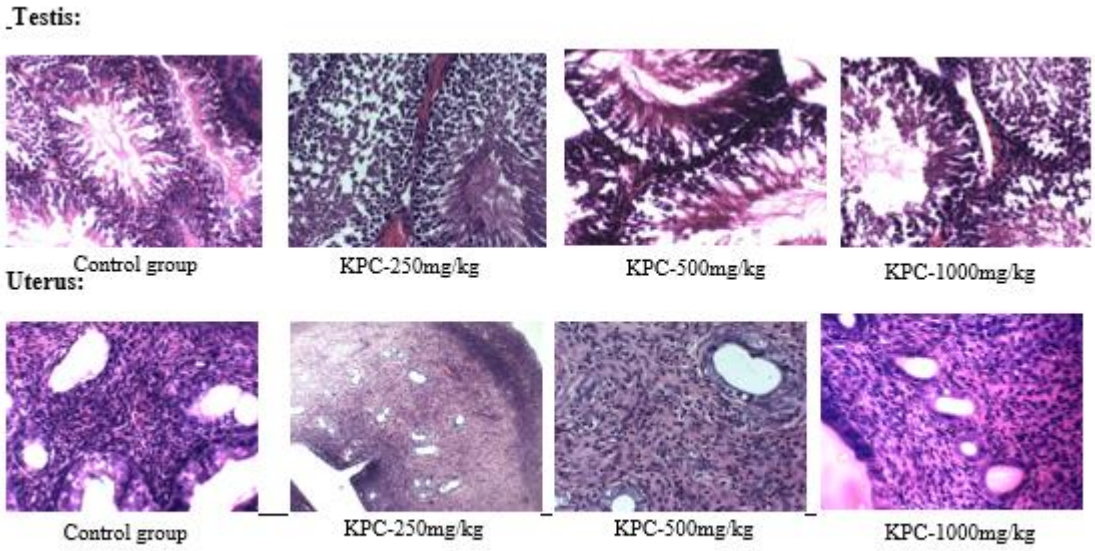

Figure 1: Repeated dose toxicity in Ethanol extract of *Kadarpassi Chooranam*: Histopathological changes

Results:

Acute Oral Toxicity of EEKPC:

All animals remained healthy with no signs of toxicity during the 14-day observation period. No mortality or behavioral abnormalities were recorded (see Table 2).

Repeated oral dose toxicity of EEKPC:

Animals treated with 250, 500, and 1000 mg/kg showed no treatment-related abnormalities. Hematological, biochemical (Tables 3-6), and histopathological (Figure 1) parameters were comparable to those of the controls. The LD_{50} was found to exceed 2000 mg/kg, with the No-Observed-Adverse-Effect Level (NOAEL) determined to be 1000 mg/kg.

Liver, heart, kidneys, pancreas, spleen, testes, and uterus organ sections in EEKPC-treated rats showed preserved tissue structure without inflammatory or degenerative changes (Figure 1).

Discussion:

This study assessed the safety profile of EEKPC through acute and repeated-dose toxicity studies. Acute toxicity testing at 2000 mg/kg revealed no mortality or toxicity, indicating an LD50 greater than 2000 mg/kg.

In recent decades, there has been a resurgence of interest in natural medicines across the world, largely driven by the perception that "natural" implies "safe." Indeed, many traditional systems prescribe the use of plant-based formulations, which have been used for centuries without overt reports of toxicity when prepared properly. However, such historical use does not necessarily guarantee safety, particularly when modern extraction methods, doses, or adulterations are introduced. More rigorous toxicity assessment is therefore crucial to support the safe development of phytomedicines.

Safety concerns in herbal medicines generally arise not only from inherent phytochemical toxicity, but also from adulteration, contamination (heavy metals, pesticides, microbial toxins), species misidentification, and interactions with other drugs[16].For example, the presence of heavy metals like lead, cadmium, or arsenic in herbal formulations has led to reported adverse effects[17-19]. Moreover, some traditional preparations have been found to be adulterated with conventional pharmaceuticals, thereby complicating the toxicity profile [19-21]. A review of herbal toxicity also highlights the need for combining in vitro, in vivo, and "omics" approaches to detect subtle or chronic toxicities [18,19].

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Many recent articles emphasize the importance of rigorous safety and quality-control in phytopharmacology, focusing on standardization, reproducibility, and mechanistic toxicology (e.g. articles in recent volumes highlight safety, efficacy, and mechanism of action studies in herbal research). Such contributions reinforce that for herbal extracts to be accepted broadly, they must meet modern standards of toxicological evidence, not rely solely on traditional claims [22,19].

Against this backdrop, our study initiated directly with acute (OECD 423) and subacute (OECD 407) oral toxicity investigations of the EEKPC. In the study results, the absence of mortality, stable body weights, and no significant alterations in hematological, biochemical, or histopathological parameters suggest that when the extract is prepared following standard protocols (consistent with classical literature) and administered within tested dose ranges, it appears non-toxic in Wistar rats. These findings lend empirical support to the dictum in many traditional systems that herbal medicines (when processed by correct procedures) are safe, but only within defined dose limits and under controlled conditions.

However, it is worth noting that our study covers only up to 28 days. Long-term (chronic) toxicity, reproductive toxicity, genotoxicity, and carcinogenicity remain to be explored. Some phytochemicals, despite being safe in short-term studies, may show cumulative or latent toxicity over extended exposure. Also, our safety profile applies strictly to the standardized extract (EEKPC) we used; deviations in method, ecological source of biomass, seasonal variation, or extraction solvent could alter safety [23,20]. Thus, in line with standards increasingly emphasized in *Phytomedicine* and allied journals, stringent quality control (botanical authentication, chemical fingerprinting, contamination screening) and stepwise toxicological evaluation (including chronic/omics-based methods) will be essential before translational application [17-19].

Acute oral toxicity:

An acute oral toxicity study employing OECD test guideline 423 (Acute Toxic Class Method) was carried out in a sequential, stepwise fashion. Three animals per step were dosed with EEKPC at 2000 mg/kg body weight using a gastric intubation needle. Throughout the 14-day observation period, no morbidity, mortality, or abnormal clinical signs were detected, and gross pathological evaluations revealed no adverse findings. These results indicated that the LD_{50} of EEKPC exceeded 2000 mg/kg body weight and qualified it for category 5 under the GHS classification.

Repeated dose toxicity:

A 28-day repeated-dose toxicity study was performed in Wistar rats following the acute oral toxicity assessment. This study aimed to assess the toxicological impact of EEKPC on a wide range of organ systems and physiological parameters with repeated, short-term exposure. The information generated will be instrumental in designing future studies, such as sub-chronic and chronic toxicity investigations. The main objectives were to (i) identify adverse effects that occur only after prolonged exposure, (ii) determine the target organs most susceptible to toxicity, and (iii) establish the dosage levels at which such effects emerge[24]. The study aimed to provide information on toxic effects, identify potential target organs, assess effects on physiological functions, and evaluate changes in hematology, clinical chemistry, pathology, and histopathology. The persistence or reversibility of effects can be seen by obtaining information from the recovery group after withdrawal of treatment (Handbook of Non-clinical Safety Testing, TDR/WHO) [25].

Three dose levels of EEKPC were chosen and fixed as low, mid, and high doses, respectively, for the main group, which consisted of five animals of each sex per group. These dose levels were 250, 500, and 1000mg/kg body weight/day. A second satellite group of ten animals (five of each sex) was utilized for the control and high-dose groups to observe the reversibility, persistence, and delayed toxic effects. The satellite group was followed for an additional 14 days post-treatment.

The EEKPC was given orally to the treatment groups (low, mid, high, and satellite high dose) for 28 days. Mortality, morbidity, clinical signs of toxicity, and functional observations showed changes during the experimental period. Also, blood parameters such as hematology and serum biochemistry were analyzed at the end of treatment and recovery. Gross pathology and organ weight changes were observed in both main and satellite group animals. A histopathological examination of control and high-dose animals was performed.

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No mortality or morbidity related to the test item was observed, and clinical signs in the treated main and satellite groups were comparable to those in the control group.

There were no differences in haematological parameters [RBC, total and differential WBC, PCV (packed cell volume), and platelets] or biochemical parameters (total cholesterol, total bilirubin, blood urea, serum creatinine, alanine transaminase, alkaline phosphatase, SGOT, and SGPT) in test-item-treated animals.

Histopathological examination revealed no EEKPC-related lesions or abnormalities in animals receiving the high dose compared to controls. Throughout the post-treatment observation period, no significant changes were noted in the satellite group, and no evidence of delayed toxicity was observed. (Figure 1)

Under the specified experimental conditions, EEKPC was well tolerated at doses up to 1000 mg/kg body weight per day following oral administration for 28 consecutive days in Wistar albino rats. Thus, the NOAEL of EEKPC is 1000mg/kg/day in both male and female Wistar albino rats.

Moreover, the observed stability in the body weights of the animals throughout the observation period supports the hypothesis that EEKPC does not exert any detrimental impact on metabolic functions at the tested dosage. These outcomes are especially significant as they contribute to the body of evidence supporting the clinical use of traditional remedies derived from marine organisms.

In the context of repeated-dose toxicity assessments, further investigation into the long-term safety and potential accumulation of bioactive compounds in the body is warranted. It is essential to evaluate the effects of prolonged exposure to determine any chronic toxicity or potential adverse health implications. Future studies should focus on biochemical and histopathological evaluations to provide a more comprehensive understanding of the extract's safety over extended periods.

In conclusion, the acute toxicity findings EEKPC demonstrated excellent safety in both acute and repeated-dose toxicity studies. No mortality or biochemical, hematological, or histopathological alterations were observed up to 1000 mg/kg/day. The LD50 exceeds 2000 mg/kg, and the NOAEL is 1000 mg/kg/day, promising a safety profile with potential implications for therapeutic applications. However, further detailed studies, including long-term toxicity assessments and mechanistic investigations of the bioactive compounds, are essential to fully validate their safety and therapeutic efficacy. The fusion of traditional knowledge with contemporary scientific research holds significant potential for the development of innovative phytopharmaceuticals derived from marine resources.

Declarations:

Ethical Approval: All animal experiments were conducted in accordance with CPCSEA guidelines and approved by the Institutional Animal Ethics Committee (IAEC), Mahatma Gandhi Medical College and Research Institute, Puducherry (Acute Toxicity: Ref. 02/IAEC/MG/10/2020–II; Repeated Dose Toxicity: Ref. 08/IAEC/MG/10/2020–II).

Consent for publication: Not applicable

Availability of data and materials: Not applicable

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions

SM: Led manuscript drafting, data interpretation, and critical revision for scientific accuracy and clarity. Coordinated correspondence and finalized the version to be submitted.

RJ: Assisted in biochemical and haematological analysis, interpretation of results, and critical revision of the manuscript for important intellectual content.

BL: Contributed to the conception and design of the study, acquisition of animal data, and supervised the acute and sub-acute toxicity experiments. Participated in data analysis and interpretation.

DG: Contributed to the anatomical and histopathological interpretation of organ sections and reviewed the manuscript for intellectual depth and scientific accuracy.

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MK: Contributed to critical manuscript review. Facilitated standardization and preparation of *Halimeda gracilis* extract. Provided overall project supervision.

SAJV: Contributed to study conception, design, and critical manuscript review. Provided overall project supervision and approved the definitive version for submission.

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