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Acute and Sub Acute Toxicity Study of Ethanolic Extract of *Plakobranchus ocellatus* on Female Wistar Rats

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ABSTRACT

The sea slugs Plakobranchus ocellatus (Sacoglossa, Gastropoda) possess naturally photo synthetically active chloroplasts from ingested algae (functional kleptoplasts) in the epithelial cells of its digestive gland that is retained up to 10 months.). The present study aimed to evaluate the safety use of the acute toxicity of the crude ethanolic extract of Plakobranchus ocellatus that was evaluated by administration of the extract by intra peritoneal route to the female wistar rats. A single extract dose of 500, 1000, 1500, 2000, 2500, 3000 and 3500 mg/kg body weight) respectively and observed for 14 days. For sub-acute toxicity studiesthe most effective extracts of nudibranchwhich showed minimum toxic effect for the development of medicine was weighed and titrated slowly by using 2% Acacia gum till a uniform suspension was formed. On 28th day, the treated animals were sacrificed and blood samples were collected by orbital sinus. The organs such as liver, spleen, and kidney were processed to study, hematological and histopathological changes.

Key words: *Plakobranchus ocellatus (Sacoglossa, Gastropoda), acute and subacute toxicity Study.*

INTRODUCTION

Nudibranch are often regarded as shell-less marine opisthobranch which belongs to the class gastropod and phylum Mollusca, these are usually noted for their extraordinary colours and striking forms. Nudibranch means naked gills, in some species of this group a protective flap is present mostly on the dorsal part or along the side as these species tend to bury into sand or mud. Nudibranchia is the largest group in Opisthobranchia with more than 3500 described species so far. The gills can be used in identification of the species. Nudibranchs are found virtually at all depths of sea from Antarctica to the tropics. They dwell at all depth but reach their greatest size and variation in warm, shallow waters and most of them spend their adult life at the bottom of the sea. The opisthobranchs molluscs possess a rich variety of secondary metabolites and great biomedical potential that represent the most intensively studied group of molluscs in natural product chemistry.

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As this marine molluscs represents a very interesting source of marine bioactive molecules, the present study was carried out to prospect the bioactive potential of *Plakobranchus ocellatus* which was obtained from Andaman waters.

MATERIALS AND METHODS

Sampling and identification

Plakobranchus ocellatus was collected during low tide by handpicking method from Burmanella (Lat:11˚30.998'N and Long: 92˚44.100'E)of South Andaman. The samples were kept in sterile seawater in containers and transported to the research laboratory. The specimen was identified based on the morphological characters and available literatures[1,2,3,4,5,6,7,8,9,10].

Preparation of ethanolic extracts

The specimens of *Plakobranchus ocellatus* were cut into small pieces using sterile scissors and homogenized in a mortar and pestle by following aseptic techniques. The homogenized samples were extracted with ethanol at room temperature for 48 hrs. The extracts were centrifuged at 27˚C at 10000 rpm for 10 min and the supernatant was collected and concentrated under vacuum in a rotary evaporator (Buchi) at 40˚C.

Toxicity Studies

Acute toxicity studies

Female wistar rats were used for acute toxicity study. Ten animals were kept fasting for overnight providing only water, after which the animal extracts were administered [i.p] at the dose of (500, 1000, 1500, 2000, 2500, 3000 and 3500 mg/kg body weight) respectively and observed for 14 days.(Mohamed Sathak A.J. College of Pharmacy, Tamil Nadu Institutional **Ethical committee No: 991/C/06/CPCSEA**). If the mortality was observed in six out of nine animals, then the dose administered was assigned as toxic dose. If mortality was observed in three animals, then the same dose was repeated again to confirm the toxic dose. One tenth of the maximum dose of the extract tested for acute toxicity was selected for evaluation of sub-acute toxicity analysis.

Sub-acute toxicity studies

The most effective extracts of nudibranch which showed minimum toxic effect for the development of medicine was weighed and titrated slowly by using 2% Acacia gum till a uniform suspension was formed. Twelve wistar rats were selected (6 males and 6 females) and grouped as below [Table 1]. Food and water were given *ad libitum*. Animals were treated [*i.p*] with the chosen extract for 28 days (Group II). Control was maintained with the administration of 2% acacia gum (Group I). Animals were treated intraperitonealy [*i.p*] with DMSO [40mg/kg]. On 28th day, the treated animals were sacrificed and blood samples were collected by orbital sinus. The organs such as liver, spleen, and kidney were processed to study, hematological and histopathological changes.

Table 1: Group specification

Group	Animals		Name of the animal species	Drug dose
	Male	***Female***		
I	3	3	Acacia suspension	2%
II	3	3	Nudibranch	250 mg.kg^{-1}

On day 28th of the dosing period, all the animals were anesthetized by chloroform anaesthesia. Different organs *viz.*, the liver, spleen and kidney were carefully dissected out and weighed by using digital electronic balance (absolute organ weight). The relative organ weight was calculated as follows:

$$\text{Relative organ weight (g)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}}$$

The blood samples were drawn by orbital sinus and further used for the analysis of various haematological and histological changes. The results are expressed as means ± standard error of the mean. To determine the gross pathology and microscopic examination, the dead animals were weighed and necropsied soon after death and macroscopic examination of the organs were carried out. Tissue biopsies from the liver, kidney and spleen were fixed in 10% normal saline. Histopathological sections were taken by using microtome and stained with haematoxylin and eosin for microscopic examination.

RESULTS

Plakobranchus ocellatus

KINGDOM- ANIMALIA

PHYLUM- MOLLUSCA

CLASS- GASTROPODA

SUBCLASS- HETEROBRANCHIA

INFRACLASS- OPISTHOBRANCHIA

ORDER- NUDIBRANCHIA

FAMILY: PLAKOBRANCHIDAE

GENUS: *PLAKOBRANCHUS*

SPECIES: *OCELLATUS*

Description: *P. ocellatus* measured 4-6 cm long. It is one of the commonly seen nudibranch in the shallow waters of Andaman Islands. This species was reported from India four decades ago (VirabhadraRao, 1961). It is mostly seen half buried in the sandy area or coral reef of intertidal area. Parapodia is present in this species which fold over the backside of the animal. The green ridges is packed full of microscopic chloroplast, which appears as a green patch in the mantle that line inside of the parapodia. The rhinopore is creamy white in colour.

Distribution: Widespread throughout the tropical indo west pacific. India: gulf of Kutch, widespread in Andaman Islands.

TOXICITY ANALYSIS

Acute toxicity analysis

The LD_{50} value of the nudibranch sample is 2500 mg.kg-1 and it is slightly toxic. At the dose level of 2500 mg.kg-1 four animals were dead. The animal showed irritability, rigidity and abnormal secretion. The LD_{50} value of the nudibranch sample seems to be 2500 mg.kg-1 as per the Hodge and Sterner scale [Table:2]

Table 2: Effect of different concentrations of *P. ocellatus* on the percentage mortality of rats

Group	Dose (mg.kg^{-1})	No. of animals dead (N=10)	% of Dead animals
I	500	0	0
II	1000	0	0
III	1500	0	0
IV	2000	2	20
V	2500	4	40
VI	3000	7	70
VII	3500	8	80

During the fourth week of treatment, no mortality was observed. The animals initially showed jumping, hyperactivity and later laboured respiration. No adverse clinical manifestations like diarrhoea, haematuria, restlessness and impaired movement were observed in the experimental animals during the dosing period [Table 3-5]

Table 3: Effect of *P. ocellatus* (2000 mg.kg-1) on behavioural and clinical characteristics of treated mice

Time (h)	0.5	1	2	4	5	
Dose mg/kg	2000					
Hyperactivity	o	o	+	o	o	Simulation
Bioerection	o	o	o	o	o	
Twitching	o	o	o	o	o	
Rigidity	o	o	o	o	+	
Irritability	o	o	o	+	‡	
Jumping	+	o	o	o	o	
Clonic Convulsion	o	o	o	o	o	
Tonicconvulsion	o	o	o	o	o	
Ptosis	o	o	o	o	o	Depression
Sleep(loss of RR)	o	o	o	o	o	
Sedation	o	o	o	o	o	
Loss of Tractions	o	o	o	o	o	
Lost of pinna reflex	o	o	o	o	o	
Loss of Pl Reflex	o	o	o	o	o	
Catatonia	o	o	o	o	o	
Ataxia	o	o	o	o	o	
Loss of muscle tone	o	o	o	o	o	
Analgesia	o	o	o	o	o	
Loss of traction	o	o	o	o	o	
Sturaub tail	o	o	o	o	o	
Labored response	o	o	o	+	‡	Autonomic effect
Cynosis	o	o	o	o	o	
Blanching	o	o	o	o	o	
Reddening	o	o	o	o	o	
Abnormal secretion	o	o	o	o	‡	
Remarks/Mortality	o	o	o	o	6/10	

Table 4: Effect of *P. ocellatus* (2500 mg.kg-1) on behavioural and clinical characteristics of treated mice

5	4	2	1	0.5	Time (h)	
2500					Dose mg/kg	
o	‡	‡	‡	o	Hyperactivity	Simulation
o	o	o	o	o	Bioerection	
o	o	o	o	o	Twitching	
‡	o	o	o	o	Rigidity	
o	+	o	o	o	Irritability	
o	o	o	o	+	Jumping	
o	o	o	o	o	Clonic Convulsion	
o	o	o	o	o	Tonicconvulsion	
o	o	o	o	o	Ptosis	Depression
o	o	o	o	o	Sleep(loss of RR)	
o	o	o	o	o	Sedation	
o	o	o	o	o	Loss of Tractions	
o	o	o	o	o	Lost of pinna reflex	
o	o	o	o	o	Loss of Pl Reflex	
o	o	o	o	o	Catatonia	
o	o	o	o	o	Ataxia	
o	o	o	o	o	Loss of muscle tone	
o	o	o	o	o	Analgesia	
o	o	o	o	o	Loss of traction	
o	o	o	o	o	Sturaub tail	
‡	‡	o	o	o	Labored response	Autonomic effect
o	o	o	o	o	Cynosis	
o	o	o	o	o	Blanching	
o	o	o	o	o	Reddening	
‡	‡	o	o	o	Abnormal secretion	
8/10	o	o	o	o	Remarks/Mortality	

Table 5: Effect of *P. ocellatus* (3500 mg.kg^{-1}) on behavioural and clinical characteristics of treated mice

5	4	2	1	0.5	Time (h)	
		3500			Dose mg/kg	
o	‡	‡	‡	o	Hyperactivity	Simulation
o	o	o	o	o	Bioerection	
o	o	o	o	o	Twitching	
‡	o	o	o	o	Rigidity	
o	‡	‡	‡	o	Irritability	
o	o	o	‡	‡	Jumping	
o	o	o	o	o	Clonic Convulsion	
o	o	o	o	o	Toniconvulsion	
o	o	o	o	o	Ptosis	Depression
o	o	o	o	o	Sleep(loss of RR)	
o	o	o	o	o	Sedation	
o	o	o	o	o	Loss of Tractions	
o	o	o	o	o	Lost of pinna reflex	
o	o	o	o	o	Loss of Pl Reflex	
o	o	o	o	o	Catatonia	
o	o	o	o	o	Ataxia	
o	o	o	o	o	Loss of muscle tone	
o	o	o	o	o	Analgesia	
o	o	o	o	o	Loss of traction	
o	o	o	o	o	Sturaub tail	Autonomic effect
o	‡	‡	o	o	Labored response	
‡	o	o	o	o	Cynosis	
o	o	o	o	o	Blanching	
o	o	o	o	o	Reddening	
‡	‡	‡	o	o	Abnormal secretion	
10/10	o	o	o	o	Remarks/Mortality	

Sub-Acute Toxicity Analysis:

The sub-acute toxicity studies revealed that, no distinct clinical signs were observed in the rat at the dose levels tested. There were no changes in the properties of stool, urine and eye colour of all the animals. No mortality was observed during the dosing period, the rat were well tolerated in the given dose level.

From day 0 to day 28, there were variable changes in the body weight of rat. The control rat gained weight throughout the duration of the treatment. The rat treated with the halophytic extract of nudibranch group gained weight in a similar lesion as the control rat. But, these changes in the body weight of treated rat were not significant when compared to the control group [Table 6]. It is also evident that, the rate of food consumption is in coincidence with the body weight in both control and treated rat groups [Table 7].

Table 6: Effect of *P. ocellatus* on the average weekly weight of experimental rat

Name of the animal extract	Dose	Average body weight (g)				
		Day- $_0$	Day- $_7$	Day- $_{14}$	Day- $_{21}$	Day-$_{28}$
Control	2ml of 2% Acacia gum	207 ± 2.5	214 ± 2.3	221 ± 2.0	228 ± 2.0	235 ± 2.05
P.ocellatus	200mg.kg^{-1}	218 ± 2.6	221 ± 2.1	226 ± 1.83	229 ± 1.87	234 ± 2.0

Table 7 : Effect of *P. ocellatus* on the average food consumption of experimental rat

Name of the animal extract	Dose	Average food consumption (g)			
		Day- $_7$	Day- $_{14}$	Day- $_{21}$	Day-$_{28}$
Control	2ml of 2% Acacia gum	142.64±1.53	143.12±1.05	141.12±1.0	142.68±1.08
P.ocellatus	200mg.kg^{-1}	138.25±1.08	139.64±0.95	142.42±1.08	141.34±0.9

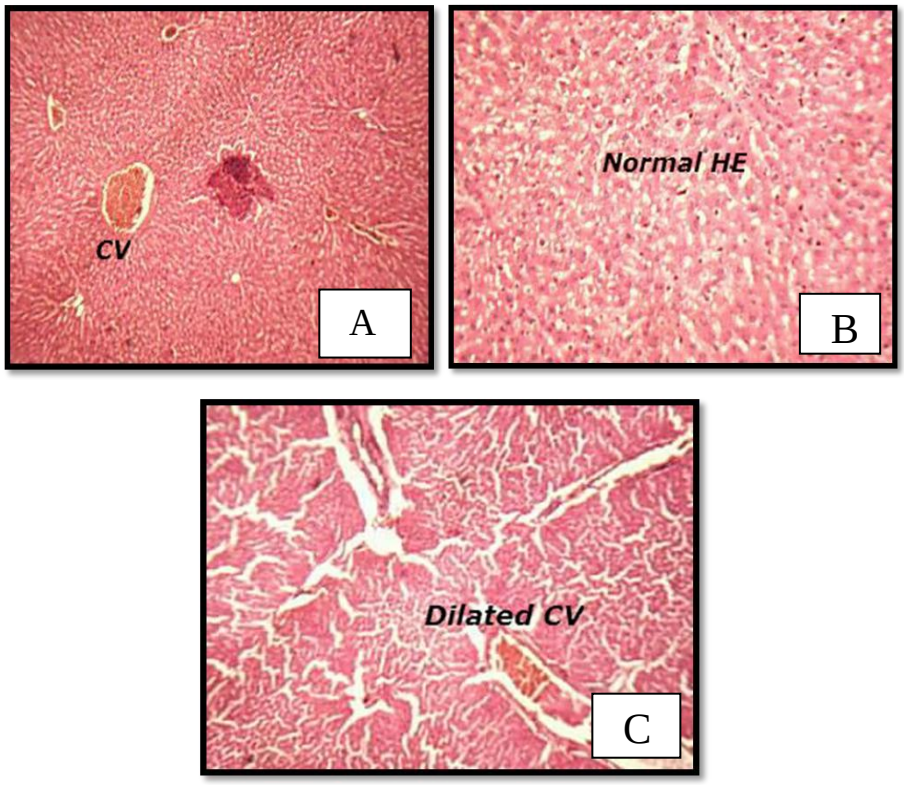
The effect of nudibranch extract on the elite organs weight of rat reveals that, there is no marked difference was observed in liver; spleen and kidney analysed by the present study in both the control and treated groups. [Table 8]

Table 8: Effect of *P. ocellatus* on the weight of the elite organs of experimental rat

Group	Weight of the Organs (mg.100g^{-1} body weight)		
	Liver	Spleen	Kidney
Control	3.94 ± 0.26	0.46 ± 0.08	0.46 ± 0.04
P.ocellatus	3.92 ± 0.07	0.59 ± 0.12	0.78 ± 0.06

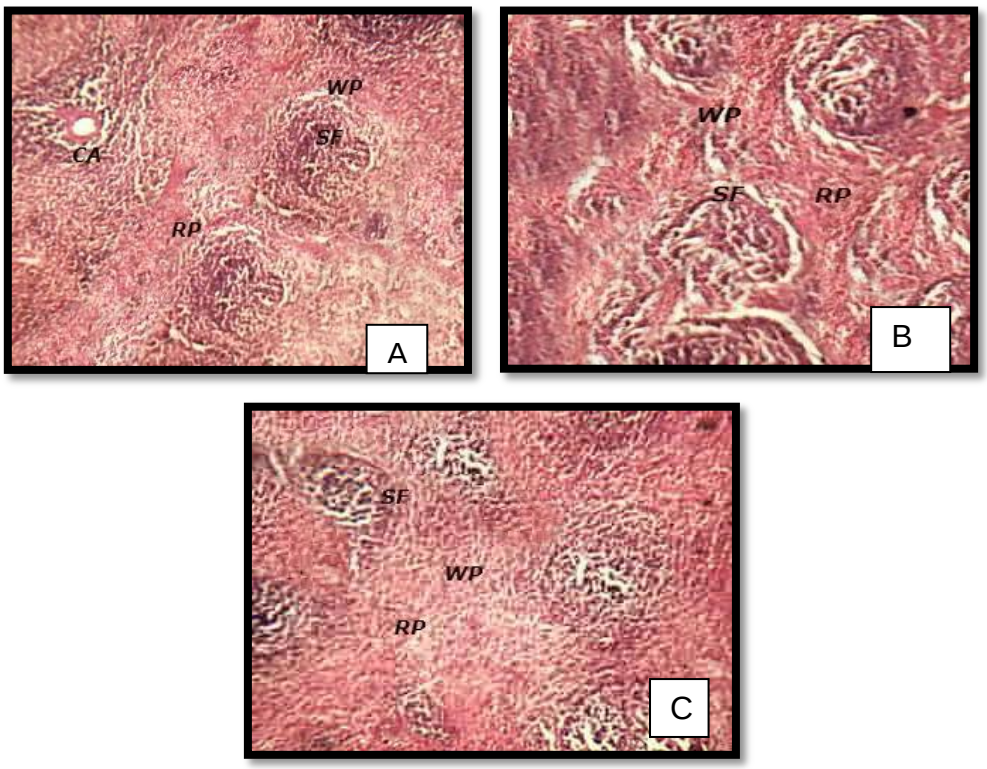
The histopathological studies revealed that, the extracts from nudibranch treated in wistar rats in general showed no changes like hydrophic changes, congestion in central vein, congestion in sinusoidal spaces in the elite organs when compared to control rats. However, it is negligible. They appear to be normal as control. Moreover the animals treated with DMASO showed signs of focal necrosis, thickening of blood vessels and inflammation in the elite organs [Figs 1-3]

Fig. 1: Histopathological studies in liver of rats treated with *P. ocellatus* extract



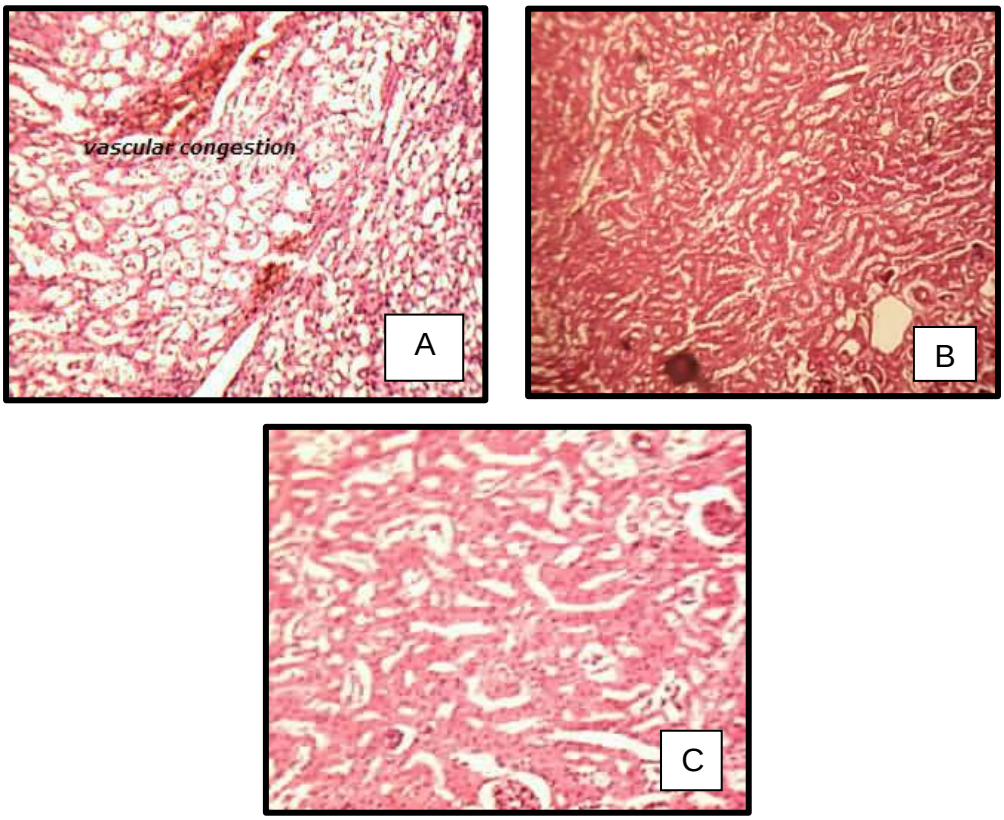
(A) Animals treated with control showing normal liver with central vein [CV]
(B) Animals treated with nudibranch extract [200mg/kg] showing normal liver with normal hepatocytes [HE].
(C) Animals treated with DMASO showing dilated CV with lymphocytes infiltration.

Fig. 2: Histopathological studies in spleen of rats treated with *P. ocellatus* extract



(A) Animals treated with control showing normal spleen with red pulp[RP], white pulp[WP], sinusoidal follicles[SF] and central artery[CA]
(B) Animals treated with DMASO showing aggregation of sinusoidal follicles [A-SF]
(C) Animals treated with *P. ocellatus* extract [200mg/kg] showing normal spleen with RP, WP, SF.

Fig. 3: Histopathological studies in kidney of rats treated with *P. ocellatus* extract



(A) Animals treated with control showing normal kidney with glomeruli [GL]
(B) Animals treated with DMSO showing congested vascular spaces with maximum lymphocytes infiltration
(C) Animals treated with *P. ocellatus* extract [200mg/kg] showing normal kidney with glomeruli [GL].

CONCLUSION

The purpose of this study was to look at the toxicity profile of the *P. ocellatus* extract. A 28- day study is considered for sub-acute study, which is well accepted for eliciting any toxicity on long-term feeding. It gives valuable information on the cumulative toxicity of a substance on target organs or physiological and metabolic effects of the compound at low dose on prolonged exposure. A wide variety of adverse effects can be detected from subacute toxicity studies. The result from such studies can provide information, which will aid in selecting dose level. The long term safety level of a compound can be predicted from acute or shorter than subacute studies. Acutely nontoxic compounds may be toxic on prolonged exposure even at low dose levels due to cumulation, changes in enzyme level and disruption of physiological and biochemical homeostasis. Subacute studies are generally carried out in few days to three months.

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