Toxicological Profile of Pomegranate (Punica Granatum) Peel Extract and Histopathological Assessment in Zebrafish (Danio rerio): An In-Vivo Study

Renu Agarwal[1], Chandrasekar Shobana[2], Durairaj Rohini[3], Boopathy Usharani[4*], Suganthi V[5]

[1]Research Scholar, Dept of Biochemistry, School of Life Sciences, VISTAS, Pallavaram. Vice Principal, Former Dean & Head, AssistantProf., PG Dept. of Food Science Nutrition & Dietetics, Shrimathi Devkunvar Nanalal Bhatt Vaishnav College for Women, Affiliated to University of Madras, Chennai, Tamilnadu, India

[2,3,4]Associate Prof., Dept of Biochemistry,School of Life Sciences VISTAS, Pallavaram, Chennai, Tamilnadu, India; E-mail: raniushab1@gmail.com

[5]Associate Prof. & HOD, Dept. of Home Science, Nutrition, FSM and Dietetics, Anna Adarsh College for Women, Anna Nagar, Chennai, Tamilnadu, India

Abstract: Background: Fruit – by - product includes peels, seeds, leaves, residual pulp, stems and discarded pieces from a variety of sources. Pomegranate (Punica granatum) peel is a good source of bioactive compounds, antioxidants, nutraceuticals, and functional properties and there is a healthy trend towards by-product utilization and value addition. However, its toxicityand its adverse effects were not intensively studied. Objective: This study aimed to examine the In-vivo toxicity of Pomegranate Peel Powder (PPP) extract and histopathological assessment using zebrafish (Danio rerio). Methods: Decoction (Aqueous) by Soxhlet method was used for extraction from Pomegranate Peel Powder (PPP). Dense extract was used to study toxicity level and it was assessed using Dose Dependent Toxicity Assessment (DDTA) with Zebrafish. The mature Zebrafish were divided into eight groups (A, B, C, D, E, F, G, andcontrol) based on their average body weight, with eight fishes in each tank. Fish groups (8 fishes/concentration) were treated to different doses and concentrations of Pomegranate PeelPowder (PPP). Fish mortality was monitored and recorded after 24, 48, 72, and 96 hours. After acute toxicity analysis, H & E staining was performed to analyse the zebrafish brain. Atleast 3 fish from each group were taken and analysed for histopathological scoring. Result: At 24 hr and 96 hr exposure periods, the lethal dosage, to kill 50% of test fishes, was 800 mg/L.Fish treated with 200 mg/L dosage had a score grade of 1 and showed no toxic pathological changes when compared to 400 and 800 mg/L doses because they considerably had decreasedpathological scores of neuronal damages, which was equivalent to the control group. Zebrafishtreated with 12.5 – 200 mg/L showed no toxic effect in the brain of fish, which was comparablewith the control. Conclusion: The current study showed that the No-Observed Adverse Effect Level (NOAEL) is evaluated to be 200 mg/L dosage. Thus, PPP has less toxicity, and its use is suggested with potential applications against diseases.

Key Words - Pomegranate Peel Powder, Toxicity, Histopathology, Zebrafish

1. Introduction

Various parts of the Pomegranate tree and fruit are used for therapeutic and culinary applications. Punica granatum L. has a long history to treat several conditions including diarrhoea, ulcers, aphthae, haemorrhage, and respiratory complications [1]. People use pomegranate for high blood pressure, athletic performance, heart disease, diabetes, and many other conditions, but there is no good scientificevidence to support most of these uses. Pomegranate Peel Powder (PPP) has diverse pharmacological functions such as antioxidant [2].

PPP possesses strong antibacterial,antioxidant and anti-tyrosinase activities. Therefore, the PPP could be exploited as a potentialsource of natural antimicrobial and antioxidant agents [3]. They are also usedto treat parasitic and microbial infections. For example, the use of 250 µ g/mL pomegranate peel extract (PPE) was most effective to inhibit antibiotic resistant strains of Salmonella typhimurium and Staphylococcus aureus in meat surfaces [4].

There are various kinds of traditional medicines derived from plants and have studied in much chemical content and efficacy in them. But there are still many plants whose toxicity levels are not yet known, so it needs to be further investigated to determine safety ingredient [5]. Toxicity tests are needed to assess the safety of the drug, or ingredients used as supplements or food [6]. It isalso to protect the community from potentially harmful effects.

One of the methods used to test the toxicity is to use the Zebrafish (Danio rerio). This method is easy to work with, cheap, short time detection, and accountable [7]. The toxicity test in red pomegranate is not widely known. The root, stem,or peel of pomegranate is possibly unsafe when taken by mouth in large amounts. So, based onthis background, the researcher is interested in doing a red pomegranate toxicity test on zebrafish (Danio rerio). This

study aims to determine the toxic effects of PPP on Zebrafish (Danio rerio) and to determine LC 50 value of Zebrafish (Danio rerio) after supplying of PPP.

1.1 Objectives

This study aimed to examine the In-vivo toxicity and histopathological assessment of Pomegranate Peel Powder (PPP) extract using zebrafish (Danio rerio). The purpose of the studywas to assess the histological alterations in the adult zebrafish (Danio rerio L.) brain after long-term exposure to Pomegranate Peel Powder (PPP) *in eight groups (A, B, C, D, E, F, G, and control)* at concentrations of 12.5, 25, 50, 100, 200, 400, 800 and tank water mg/L for 24, 48, 72, and 96 hours.

2 Material and Methods

2.1 Plant Extraction

The Punica grantum L. peel used in the current study was collected from local market

of Chennai city, Tamilnadu, India. To prepare PPP, pomegranates were manually peeled, shadedried and powdered by a mixer grinder.

2.2 Extraction – Decoction Method

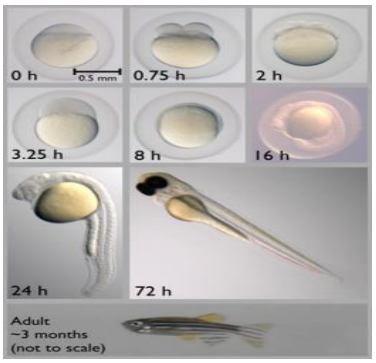
50 grams of Pomegranate Peel Powder (PPP) was added to 500 mL of 80% methanol for 10 days in a Soxhlet extraction apparatus. After extraction the solvent was removed, by means of a rotary evaporator, yielding the extracted compound. The stock peel extract was keptin sterile containers at 4°C until use. The dense extract is then filtered before being utilized orfurther processing.

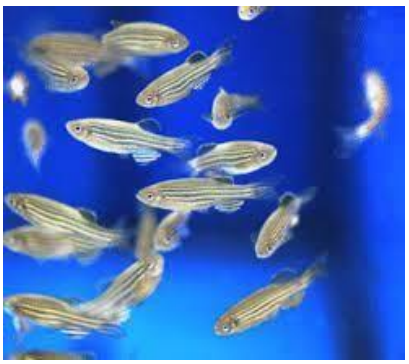
Table –1 Extraction Procedure

Steps (Extraction Yield- 71.26%)			
I	**II**	**III**	**IV**
Wash, clean andpeel off fre Pomegranate 	Drying of Pomegranatepeel (Shade 	Fine powdered 	Decoction (Aqueous) - Extraction 

2.3 Selection of Animal

Genetic, pharmacological, and behavioural investigations with Zebrafish (*Danio rerio*) are becoming increasingly popular. Zebrafish are used as an animal model for severalhuman illnesses since they are vertebrates and share 70–80% of our genetic makeup. Zebrafish has a high fertility, low care expenses, transparent embryos, and rapid development as advantages.





(Source: Wikipedia and Max-Planck-Gesellschaft)

Zebrafish (Danio rerio)

2.4 Zebrafish Maintenance

About 100 mature, mixed-gender Zebrafish of the AB strain from a nearby aquariumsupply shop were procured. Every fish was kept in a circulating 3 L system tank that regularly aerates and filters the system water (dechlorinated water) to preserve the water quality for a healthy aquatic habitat. The illumination was set to 14:10 hours (light: dark), and the tank's temperature was kept between 26-28.5 °C. They were provided with a nutritionally adequate standard laboratory diet. Live brine prawns (Artemia sp.) and Tetra- Min flake meal were mixed and given to them 1-2 times per day. The pH of the system water was measured daily and kept between 6.8 and 7.5, and the salinity of the water shouldbe between 0.5-2 ppt. Fish tanks were cleaned on a regular basis.

2.5 Experimental Design

2.5.1 Toxicological Study

Grouping

The mature Zebrafish were divided into eight groups (A, B, C, D, E, F, G, and control) based on their average body weight, with eight fishes in each tank. The fish in eachgroup varied in weight from 0.15 to 3 g prior to treatment, all the fish in each group were acclimated in a 3 L circulating tank for 10 days. A consistent mixed diet (brine prawns andTetra-Min flake meal given 1-2 times daily) was supplied to all the fish in the groups.

Table-2 Weight of The Zebrafish in each group

	WEIGHT OF THE INDIVIDUAL FISHES IN EACH GROUP (in grams)							
Group No. Of Fish	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Control
1	0.21	0.46	0.21	0.62	1.04	0.68	2.01	
2	0.38	0.26	0.21	0.30	0.60	0.58	0.86	
3	0.32	0.30	0.40	0.21	1.60	0.99	0.83	
4	0.25	0.32	0.56	0.17	0.75	0.63	3.05	
5	0.26	0.27	0.32	1.90	0.15	0.90	0.98	
6	0.45	0.29	0.54	0.133	0.99	0.98	0.78	
7	0.43	0.28	0.69	0.38	0.81	0.97	0.56	
8	0.98	0.56	0.25	0.44	0..45	0.40	1.72	

According to OECD standards 203, aqueous extract of Pomegranate peel powder was tested for acute toxicity

in zebrafish (*Danio rerio*) for 96 hours. For 96 hours, the before mentioned fish groups (8 fishes/concentration) were treated to different doses and concentrations of Pomegranate peel powder. Using the median lethal concentration (LC50), the approach for calculating the acute toxicity test that is most frequently accepted. The amountof test sample in water needed to kill 50% of a test batch of fish during a specific exposure period is known as the LD50. Both before (48 hours prior to treatment) and throughout the experiment, the fish were not fed. Prior to and following the experiment, the mean average weight of fish in each group will be measured. The fatal concentration (LC50) in *Danio rerio* was established using seven different concentrations of Pomegranate peel powder extract and one control (normal system water). 3 L of dechlorinated water was used to dissolve each concentration.

Table – 3 Concentration of Aqueous Extract of Pomegranate Peel Powder (PPP) todetermine Acute Toxicity in Zebrafish (*Danio rerio*)

Groups	Concentration of Extract (mg/L)
A	12.5
B	25
C	50
D	100
E	200
F	400
G	800
Control	System water

In a 3 L tank with 96 hours of exposure, about eight healthy fish were exposed to variousconcentrations. A constant temperature of 25°C and a pH of 7.0 were maintained for both the treatment and control groups. Fish mortality was monitored and recorded after 24, 48, 72, and96 hours. It is thought that a fish is dead if there is no visible operculum movement and no response when the caudal peduncle is touched. To avoid spreading illness, deceased fish were removed from the tank once mortalities were reported. The fish in each group were moved to new tanks with their appropriate amounts of aqueous extract of pomegranate peel powder after 24 hours. After 96 hours of exposure, the cumulative mortalities and 96-hour lethal concentrations (LC50) values for each test sample were determined.

2.5.2- Histopathological Sampling and Biochemical

2.5.2.1 Analysis Procedure

After acute toxicity analysis, fish were sacrificed using a higher dose of MS-222. The

head of the fish was cut open to remove the brain. The extracted brain was fixed in fixative andtissues were processed to prepare paraffin wax blocks. H & E staining was performed to analysethe zebrafish brain. The H & E-stained slides were observed under a microscope and microscopic lesions were recorded. At least 3 fish from each group were taken and analysed for histopathological scoring. Histological alterations were scored as (-) no histopathology; (+) histopathology in <25% of the field, (++) histopathology in > 75% of the field, and (+++) histopathology in all fields.

2.5.2.2 Ethical Considerations

All experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), on 22nd July 2023, at Centre for Laboratory animal Technology and Research, Sathyabama Institute of Science and Technology, Chennai., prior to the initiationof the experiment and the laboratory animals were taken care of according to the committee for the purpose of control and supervision of experiments on animals (CPCSEA) regulations.

3. Results

3.1 Toxicological Assessment -

Figure - 1 Acute toxicity analysis (Labels: Left to Right 12.5, 25, 50, 100, 200, 400,800mg/L and control tank)

Table - 4: Number of live fishes in each Concentration at different time interval and itsLC50

Concentration (mg/L)	No. of live fishes													IC 50
	0th	8th	16th	24th	32nd	40 th	48th	56th	64th	72nd	80 th	88 th	96th	
A (12.5)	8	8	8	8	8	8	8	8	8	8	8	8	8	-
B (25)	8	8	8	8	8	8	8	8	8	8	8	8	8	-
C (50)	8	8	8	8	8	8	8	8	8	8	8	8	8	-
D (100)	8	8	8	8	8	8	8	8	8	8	8	8	8	-
E (200)	8	8	8	8	8	8	8	8	8	8	8	8	8	-
F (400)	8	8	7	7	7	7	6	6	6	6	6	6	6	-
G (800)	8	7	6	6	5	5	4	3	3	2	0	0	0	48th
Control (normal dechlorinated water)	8	8	8	8	8	8	8	8	8	8	8	8	8	-

Table -5: Percentage of live fishes in each concentration at time interval

Group	Concentration (mg/L)	Live fishes at different time interval (in %)			
		24th Hour	48th Hour	72nd Hour	96th Hour
A	12.5	100	100	100	100
B	25	100	100	100	100
C	50	100	100	100	100
D	100	100	100	100	100
E	200	100	100	100	100
F	400	87.5	75	75	75
G	800	75	50	25	0

Table 6: Mortality of fishes in each concentration at time interval

Group	Concentration (mg/L)	Mortality of fishes at different time interval (in %)			
		24th Hour	48th Hour	72nd Hour	96th Hour
A	12.5	0	0	0	0
B	25	0	0	0	0
C	50	0	0	0	0
D	100	0	0	0	0
E	200	0	0	0	0
F	400	12.5	25	25	25
G	800	25	50	75	100

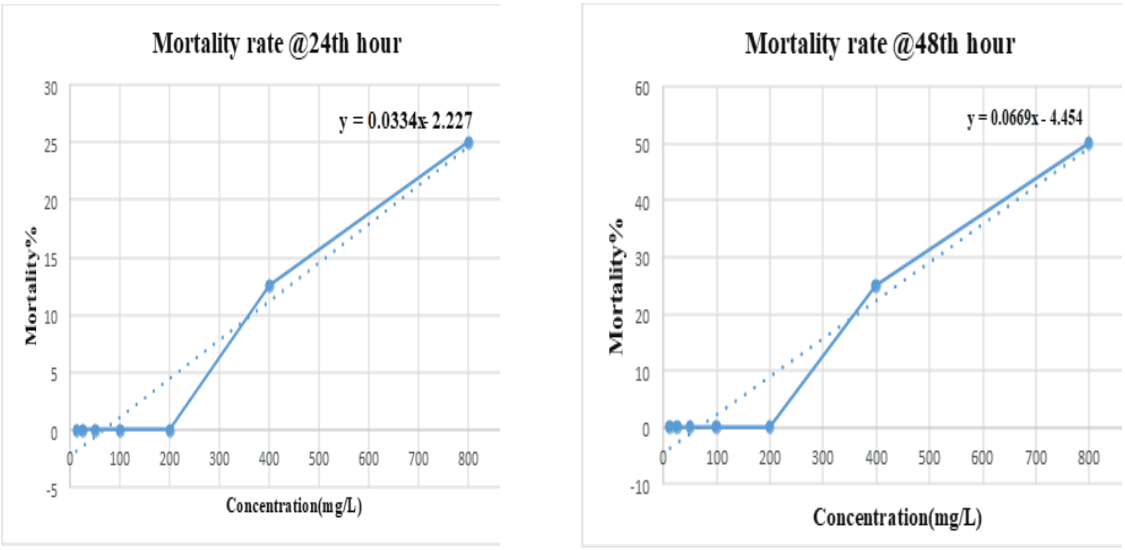


Figure 2 Graphical Representation of Mortality rate @24th and @48th hour

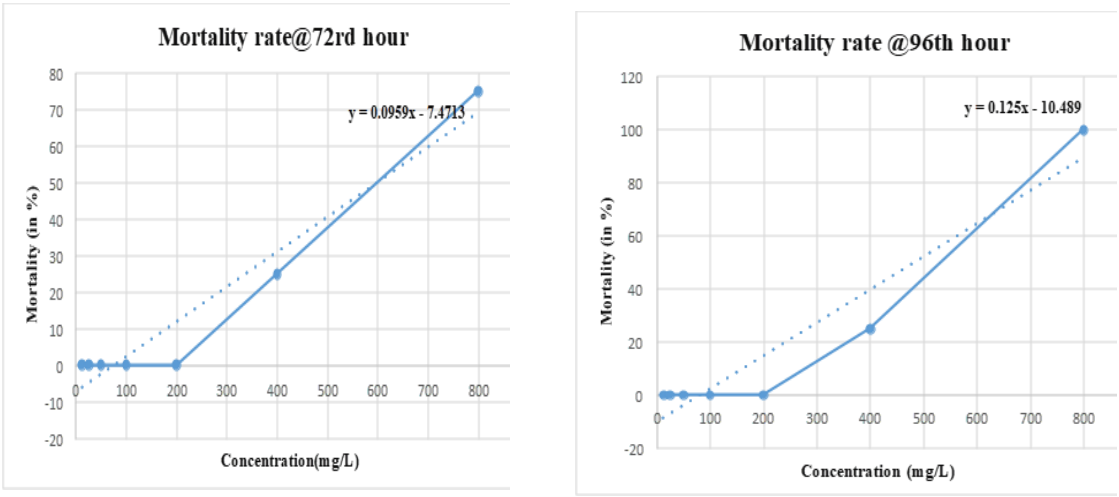


Figure 3 Graphical Representation of Mortality rate @72nd and @96th hour

The aqueous extract of pomegranate peel powder was found to be toxic, as evidenced by the 100% fatality rate. The LC_{50} values of different concentrations of pomegranate peel powder extract were 1563.6, 813.96, 599.28, and 483.91 mg/L for 24, 48, 72, and 96 hours, respectively. Concentration-dependent change in the mortality of fishes exposed to different

Table -7: LC_{50} value

TIME (Hours)	LC50 value (mg/L)
24	1563.6
48	813.96
72	599.28
96	483.91

concentration of pomegranate peel powder were clearly observed at 24, 48, 72, 96 hours. The pomegranate peel powder was found to be lethal at the highest concentration of 800 mg/L within 24 hours of exposure to the sample. As the exposure duration rose from 24 to 96 hours,the death rate increased dose-dependently and decreased time-dependently, and the median fatal concentration decreased. At 200 mg/L of pomegranate peel powder extract, no mortality was found. At 24 hr and 96 hr exposure periods, the lethal dosage to kill 50% of test fishes was800 mg/L. During the trial period, there was no mortality in the control group.

3.2 Histopathological Assessment

Table -8 Pathological scoring of H & E-stained zebrafish brain

S. No.	Groups	Pathological Scoring
1	Control	-
2	800 mg/L	+++
3	400 mg/L	++
4	200 mg/L	+
5	100 mg/L	-
6	50 mg/L	-
7	25 mg/L	-
8	12.5 mg/L	-

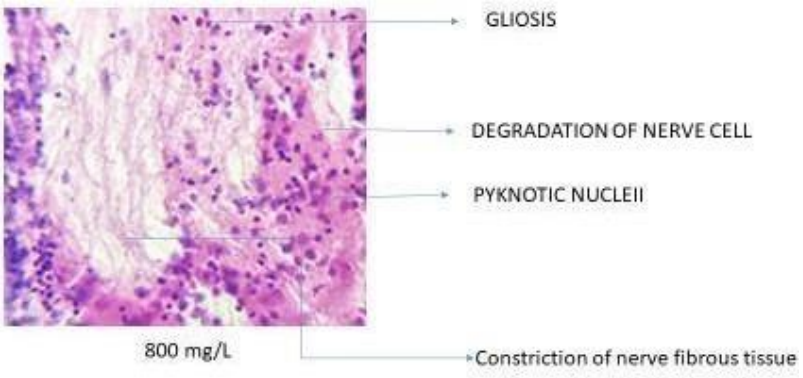


Figure -4 Pathological reference of 800 mg/L treated zebrafish brain

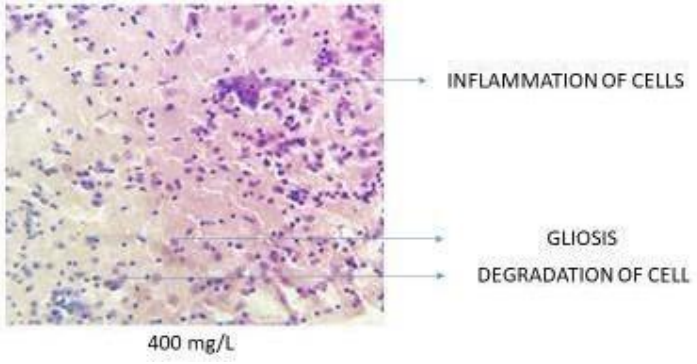


Figure-5 Pathological reference of 400 mg/L treated zebrafish brain

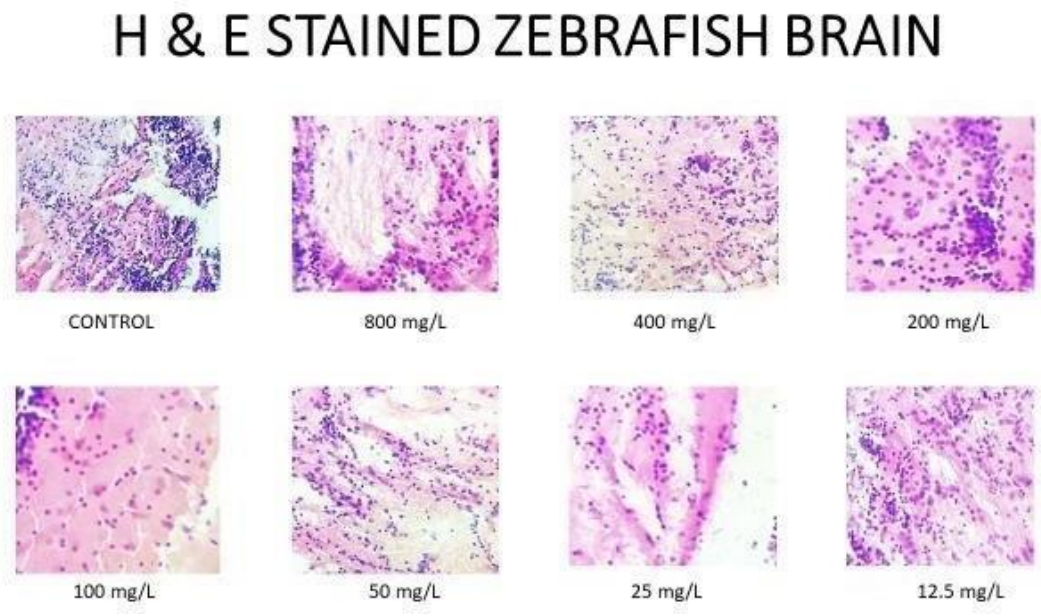


Figure-6 Haematoxylin and Eosin staining of zebrafish brain

Histopathological assessments of the hippocampus and cortical regions of the brain were performed on all group (12.5 – 800 mg/L) zebrafish, and the mean pathology score was reported. The brain and hippocampus regions of control fish treated with system water appearnormal, with spherical neuronal bodies and circular, conspicuous nuclei, and are scored as 0. In the higher dosage group (800 mg/L), there was a substantial increase in the glial cell proliferation in several parts of the zebrafish brain, as well as inflammation and constriction ofneuronal cells, and therefore rated as 3. The brains of zebrafish treated with the 400 mg/L revealed gliosis which was lesser than the higher doses and moderate inflammation in the cortex, no constriction of neurons was visually seen in zebrafish brains indicating that 400 mg/L was less toxic compared to the higher dose, 800 mg/L. Fish treated with 200 mg/L dosagehad a score grade of 1 and showed no toxic pathological changes when compared to 400 and 800 mg/L doses because they considerably had decreased pathological scores of neuronal damage, which was equivalent to the control group. Zebrafish treated with 12.5 – 200 mg/L showed no toxic effect in the brain of fish, which was comparable with the control.

Conclusion

Pomegranate Peel Powder (PPP) has an acute toxic potential against Zebrafish, which is indicated by LC50 values<1000 mg / ml. LC50 value of Pomegranate Peel Powder (PPP) is 200 mg / ml. It was concluded that PPP has the potential to harm the nervous system. PPP exposure at 200, 400 and 800 mg/L concentrations for 90 days produce mild to moderate pathological changes in the cortex The results allowed us to have a baseline for more in-depth studies on efficacy and safety of Pomegranate Peel Powder (PPP) for a possible therapeutically application for the public health.

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