Original Article

Assessment Of Toxicity And Safety Margin Of Phenylalanine And Its Metabolites Through In-Vivo And In-Vitro Model

Samrat Bose[1*], Ayan Goswami[1], Shirsendu Mandal[1], Rajesh Khan[1], Himangshu Sekhar Maji[2]

[1]Division of Pharmacology, Guru Nanak Institute of Pharmaceutical Science and Technology,157/F, Nilgunj Rd, Sahid Colony, Panihati, Kolkata, West Bengal 700114
[2] Division of Pharmaceutical Technology, JIS UNIVERSITY, 81, Nilgunj Rd, Panjavilla, Jagarata Pally, Deshpriya Nagar, Agarpara, Kolkata, West Bengal 700109

***Corresponding author**
Mr. Samrat Bose Assistant Professor Guru Nanak Institute of Pharmaceutical Science and Technology
Mail id: samratbose009@gmail.com , https://orcid.org/0000-0002-6484-3598
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Abstract

Phenylalanine (PA), an emergent essential amino acid, is a building block of protein, which propagates significant metabolites to assure the compatibility of the body. During derangement of metabolism of PA or genetic mutation, which alters tyrosine synthesis, leads to phenylketonuria (PKU). Genetic similarities of Danio rario with humans allow the comparison of metabolic pathways in fish model.

Objective:
The study is to evaluate the toxicity and safety margin of Phenylalanine, and one of its metabolites, sodium phenylpyruvate (SPP). Toxicological assessment LC50 study(OECD TG 236), developmental toxicity study (total body length, head size, caudalfin length of embryo), and hepatoxicity study (SGPT, SGOT, Histopathology, i.e., sinusoidal dilation, cytolysis, karyolysis, cell-to-cell adhesion, vacuolization, and degenerated hepatocytes) has been performed on Danio rario model. For phenylalanine, LC50 values are, 1949, 829, 213, 133, 92.68 µg/ml, for phenylpyruvate, LC50 values are, 224.9, 169.82, 105.15, 97.05, 79.79 µg/ml. For developmental study, PA (0.5 mg/ml, 0.75mg/ml, 1mg/ml) and SPP (0.25mg/ml,0.40mg/ml, 0.60mg/ml) concentrations are considered. For hepatotoxicity study on adult zebrafish groups (n=6), treated with PA and SPP with concentrations of 10mg/kg, 20mg/kg, and 40mg/kg separately.

Results:
After performing the experiment, it has been observed that for PA at its 1mg dose showed significant difference for head length/width and vertical/longitudinal caudal fin length. But for SPP, total body lengthand longitudinal caudal fin length showed significant different at 0.6 mg dose. The SGPT-SGOT test results for hepatotoxicity shows that SPP is more toxic than PA. Histopathological study of hepatocytes of Danio rario shows more positive results for SPP treatment than PA at dose dependent manner. Therefore, it can be concluded thata higher concetration of SPP can cause potential toxicity and health hazards.

Keywords: Danio rario, Embryotoxicity, phenylalanine, phenylpyruvate, hepatotoxicity, histopathology, developmental toxicity, amino acids

Introduction

Amino acids are molecules that it can be combine to form of proteins, Amino acids and proteins are the building blocks of life. When proteins are digested or broken in our body, then the amino acids are left. The human body can be uses amino acids to make protein to help the body. Essential amino acids, also known as indispensable amino acids, are amino acids that humans and others vertebrates cannot be synthesize from the metabolic intermediates.These amino acids must be supplied from an exogenous diet because the human body lacks the metabolic Pathway required to synthesize these amino acids. [1, 2]. In nutrition, amino acids are classified as either essential or non-essential. These classifications resulted from the early studies on human nutrition, which it can be show that specific amino acids were required for growth or nitrogen balance even when there is an adequate amount of alternative amino acids.[3]. Although variations are possible depending on the metabolic state of an individual, thegeneral held through is that the there are 9 essential amino acids, including valine, tryptophan, threonine, isoleucine, methionine, and mnemonic PVT TIM Hall is commonly use device to remember these amino acids are includes the first letter of all the essential amino acids. In term of nutrition, the nine essential amino acids are obtainable by a single complete protein. By definition, contains all the essential amino acids. Complete proteins usually derived from animal-based sources of nutrition. [4, 5]. Nonessential means that our body does not can produces the amino acid, if we don'tget it from food, we only eat. Nonessential amino acids are including: alanine, arginine, asparagine, aspartic acids, cystenine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine.[6]

Objectives

1. To procurement of zebrafish
2. Acclimatization
3. To separate male and female fish
4. To prepare them for breeding

Materials and methods

Separated Breeding chambers, Micro pipette (T-1000, T-100, T-10), Commercial Foods, SGPT KIT, SGOT KIT

Figure 1. Materials used in the research purpose

Methods

Zebrafish maintenance

1. Zebrafish are collected from the market and acclimatized in our labenvironment for 7 days.
2. After collection the fish and separated fishes according to male and female.
3. Provided live food and microplate and aeration.
4. Zebrafish are kept in a circulating system that continuously filters and aerates the system water to maintain the water quality which is required for healthy aquatic environment.
5. The circulating system filters must be checked and changed regularly to ensuretheir proper function. These filters should be changed regularly to ensure properand clean water supply to all the fish tank.
6. The pH of the system water should be checked daily and maintained between 6.8-7.5
7. Ideal temperature is 28°C with a light and dark cycle of 14:10 h.
8. Fishes are divided into different experimental groups.

LC_{50} Study of test compound

1. Zebra fishes Should be maintained under observed the Standardlaboratory Conditions.
2. Breeding process will start in a breeding chamber by taking male &female in the ratio of 2:3.
3. Proper connect aeration and temperature should be maintained for24hrs.
4. After 24hrs fish are separated and embryos are collected.
5. Preparation of different concentration of test compound from stocksolution.
6. Two control and 7 different concentration of test solution (350µg/ml,250µg/ml. 150µg/ml, 90µg/ml, 60µg/ml, 50µg/ml) for SPP (350µg/ml, 250µg/ml, 150µg/ml, 100µg/ml, 90µg/ml,60µg/ml,50µg/ml) for LPA [7].
7. After preparing different concentrated solution, 20 embryos put in toeach Petri plate by 1000 micro litter micropipette.
8. After we can observe the percentage mortality and defects (Tail bend,neck bend) of embryos after 24 hrs, 72hrs, 96hrs, and 120 hrs.

LC50 Study of Phenylalanine on Embryo

Figure 2. **LC50 Study of Phenylalanine on Embryo.** 5 Days Observation at 24 hrs Interval i.e., 24 hrs,48 hrs,72 hrs,96 hrs,120 hrs

LC50 Study of Phenylpyruvate on Embryo

Figure 3. **LC50 Study of Phenylpyruvate on Embryo.** 5 Days Observation at 24 hrs Interval i.e., 24 hrs,48 hrs,72 hrs,96 hrs,120 hrs

Anesthesia

At first, removed the number of experimental fish from the aquarium. Use a net to transfer the fish into anaesthetics solution. The observed the fish gradually swim, spread the pectoral fins horizontally,gasp, and have operculum movements within 1 min. As the tomes goes on, observed the fish lay on the bottom of the case andfinally stop swimming and the surgical plan of anaesthesia will reach. When the fish stops to gasp, and the operculum movements are very slow. Atthis point, fish is ready for blood collection or dissection. Then placed on petri dish and water soaked using filter to gently dry of thebody surface.

Study of hepatotoxicity

Weighing of wild types pf zebrafish approaches 3.4-5 gm

Dissection of zebrafish

A male zebrafish will be dissected first, followed by a female fish. Before, beginning the dissection, anesthetize a fish in 0.2% ethyl 3 amino benzoate and then euthanize it by incubation in ice water for 15 minutes. Begin by lightly patting the fish dry on a paper and placing it on a filter paper or dissection mat. Externally, zebrafish have a single dorsal, caudal and anal fins and paired pectoral and pelvic fins. Snip and skin on the belly of the fish just anterior to the anal fin. Cut the belly skin and underlying muscle along the belly from the anal fine to the Carefully removed thefish skin and underlying muscle from the side of the fish, So, many internal organs are visible now [8, 9].

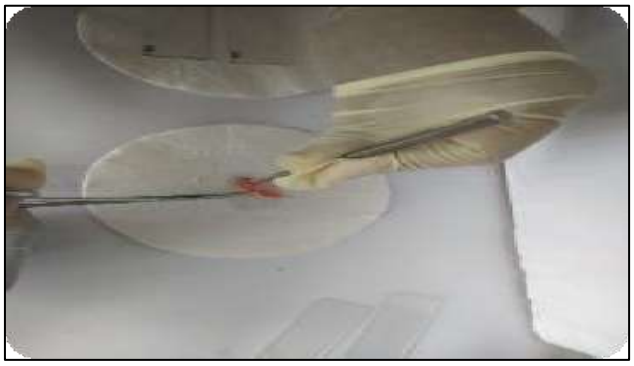
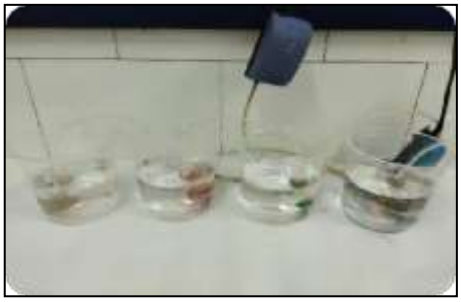


Figure 4. Dissection of zebrafish

Phenylalanine Induced in zebrafish liver (SGPT/SGOT TEST)

First group of zebrafish received phenylalanine at the dose of 10mg, 20mg,40mg for 3to 4 days, Dose was given in intraperitoneal (IP) infusion. After it was fed orally by the live food and microplate at the same concentration of dose 10mg,20mg,40mg. after dissection, homogenization of liver take place in cooling centrifuge and last observation done by UV Spectrometer [10].

Figure 5. Different concentration of phenylalanine 10mg, 20mg, 40mg



To study the liver function, the transaminase enzymes (SGPT and SGOT) were measured in the respective groups of liver. For assaying the hepatoprotective action of phenylalanine and nonspecific host response parameter like morphological alteration. Enzymes release and intracellular killing capacity of peritoneal macrophages were studied from the respective group of liver. Weighting of different concentration of Test drug LPA and SPP by using DhonaBalance

Sodium Phenyl pyruvate Induced in Zebrafish liver (SGPT/SGOT TEST)

2nd group of zebrafish received phenylpyruvate at dose of 10mg, 20mg, 40mg, for 3to 4 days, Dose was given in Intraperitoneal (IP) infusion. After it was fed by live food and microplate at the same concentration of dose is 10mg,20mg,40mg. after dissection, homogenization of liver take place in cooling centrifuge and last observation done by UV spectrometer.

Figure 6. Different concentration of Sodiumphenyl pyruvate 10mg, 20mg, 40mg

To study the liver function, the transaminase enzymes (SGPT/SGOT) were measure inthe respective group of liver, for assaying the hepatoprotective action of Phenylpyruvate.

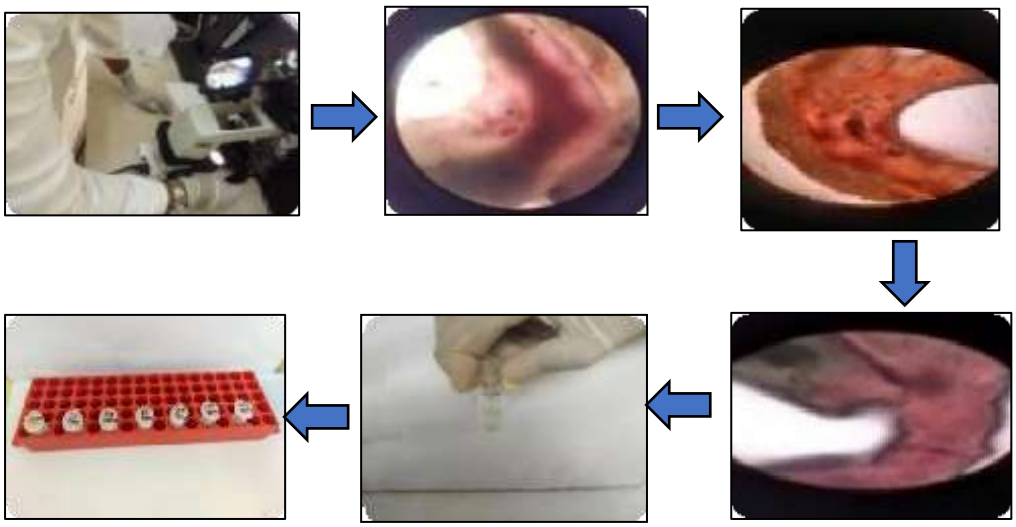


Figure 7. Zebrafish liver observation under microscope

ImageJ software used for the Image processing

Developmental Toxicity study through Image J software

ImageJ is a public domain Java image processing and analysis program inspired by NIH Image for the Macintosh. It runs, either as an onlineapplet or as a downloadable application, on any computer with a Java 1.5 or later virtual machine. Downloadable distributions are available for Windows, Mac OS X, and Linux. It can display, edit, analyze, process, save and print 8–bit, 16–bit and 32– bit images. It can read many image formats including TIFF, GIF, JPEG, BMP, DICOM, FITS, and 'raw'. It supports 'stacks' (and hyperstacks), a series of images that share a single window. It is multithreaded, so time-consuming operations such as image file reading can be performed in parallel with other operations1. It can calculatethe area and pixel value statistics of user-defined selections. It can measure distances and angles. It can create density histograms and line profile plots. It supports standard image processing functions such as contrast manipulation, sharpening, smoothing, edge detection, and median filtering. It does geometric transformations such as scaling, rotation, and flips. The image can be zoomed up to 32:1 and down to 1:32. Allanalysis and processing functions are available at any magnification factor. The program supports any number of windows (images) simultaneously, limited only by available memory. [11, 12].

Prior Considerations for Using ImageJ Software with Minimum Errors

All of the captured images should be with the same resolution and clarity. The magnification of all the specimens should be the same to avoid sizedissimilation. There should not be any editing or reframing of the files/images as it mayaffect the comparability. Any comparable scale of similar resolution to the images is required for settingthe scale in ImageJ software.

Measurement of full body length of embryo using Image J software

Figure 8. Length measurement of Embryo

1. Open the image in ImageJ and draw a straight line over the image as required
2. Goto ‘Analyzes’ and choose ‘Measure’
3. The measurement table will be popped upwhere the length in mm is shown

Measurement of fin length of embryo using Image J

Open the image in ImageJ and draw a straight line carefully to measure thelongitudinal and vertical length of the fin.

Measurement of the head length of embryo using ImageJ:

Figure 9. Fin length measurement

Open an image in ImageJ and draw a straight line on the head of the embryo which is tensed to measure, and get the result by going to ‘analyze’ and select to ‘measure’. The result will show the length and width of head respectively.

Measurement of the head length of embryo using Image J

Figure 10. Head length of embryo calculation

Study of Histopathology of Zebrafish liver

The hepatocytes are primary cells of the zebrafish liver like in superior mammals. These cells act in the processing of protein, Carbohydrate, lipids and vitamins, and detoxication and xenobiotic compounds, the hepatocytes are accumulable to synthesize the bile, which it can be transported through the bile ducts, Blood vessels are frequent and abundant in the liver. A common tissues alteration can be found in the hepatocytes is cytoplasm vacuolization due to a decrease glycogen stores and lipid accumulation. Which it could be due to the action of toxin agents. This process is believed to hamper the normal function of the liver. However, the decrease of glycogen stores should not be considered alone since due to the small size and high agility of zebrafish, zebrafish has an accelerated metabolism which it can consume the glycogen if the animal is active enough. Changes in the nuclei morphology, such as vacuolization atrophy, are often observed when function alterations occur in the hepatocytes and can precede pyknosis (reduction and rounding of the nuclei that precedes apoptosis) and cellulardegeneration. On the other hand, hypertrophy of the nuclei indicates intense metabolicactivity in the hepatocytes. Which can be caused by exposure to the toxin agents. When the hepatocytes degenerate, a relative reduction in the frequency of nuclei isalso observed, which it can be believed to be defence mechanism. We observed histopathological factors of zebrafish liver under the microscope and findings six parameters indication by symbolic representation [13]. **Sinusoidal dilatation: Enlargement of hepatic capillaries**

1. Cytolysis: Lysis of hepatic cell
2. Karyolysis: In the nuclei of necrotic hepatocytes, there were several inflammatory lesions on the surface of the liver.
3. Cell to cell adhesion decrement: one cell interacts with another cell at thatsame position.
4. Vacuolization: The formation of vacuoles like structure, within or adjacent tocells.
5. Degenerated hepatocytes: Formally degeneration of hepatocytes, is a form ofliver parenchymal cell. Cell is death.

The Histopathological findings parameter has been observed at Control and different drug concentration like 10mg/kg, 20mg/kg and 40mg/kg.

Table 1. Histopathological observation

Histopathological findings	**Symbolic Representat**	**Control**	**Treated with Phenylalanine**	**Treated with PS**

	ion		10 mg	20 mg	40 mg	10 mg	20 mg	40 mg
Sinusoidal dilatation	Arrow	-	+	+	+	++	+++	+++
Cytolysis	Asterisks	-	-	-	-	+	++	+++
Karyolysis	Small arrow	-	-	-	-	+	++	++
Cell-to-cell adhesion decrement	Double arrow	-	-	-	+	++	++	+++
Vacuolization	Arrowheads	-	-	-	-	+	++	+++
Degenerated hepatocytes	Circle	-	-	-	-	+	++	+++
(-) none; (+) mild; (++) moderate; (+++) severe								

The sinusoidal dilatation has been shows mild cases (+) at 10mg/kg, 20mg/kg, 40mg/kg different concentration of drug of PA. and others factors has been shows negative control (-) at different concentration. For SPP the sinusoidal dilatation has been shown moderate case (++) at the concentration of 10mg/kg, and shown the severe case (+++) at the concentration of 20 mg/kg and 40mg/kg. and cytolysis has been shown mild case (+) at 10mg/kg concentration and moderate case (++) at 20mg/kg and severe case (+++) at 40mg/kg. Karyolysis shows mild cases (+) at 10mg/kg concentration and moderate case (++) at 20mg/kg concentration and severe case (+++) at 40mg/kg concentration. Cell to cell adhesion decrement has been shown moderate case (++) at 10mg/kg concentration, shows moderate case (++) 20mg/kg concentration and shows severe case (+++) at the concentration of 40mg/kg. Vacuolization has been shows mild case (+) at 10mg/kg concentration and shows moderate case (++) at 20mg/kg, and shows severe case (+++) at 40mg /kg. Degenerated hepatocytes have been shows mild case (+) at 10mg/kg, shows moderate case (++) 20mg/kg and shows severe case at (+++) at 40mg/kg. Finally, Histopathological slides in comprising with the control, no such different has been observed except Sinusoidal Dilatation and Cell-to-cell adhesion decrement in case of Phenylalanine at 40mg/kg but for phenylpyruvate. Cellular deformities were observed in hepatocytes i.e., Sinusoidal dilatation, Cytolysis, Karyolysis, Cell-to-cell adhesion decrement, Vacuolization, Degenerated hepatocytes.

Results and discussion

Experimental data of LD50 study of Phenylalanine

Table 2. LD_{50} of Phenylalanine

PHENYLALANINE	24 hrs		48 hrs		72 hrs		96 hrs		120 hrs	
	No of Embryo	% Survival	No of Embryo alive	% Survival	No of Embryo alive	% Survival	No of Embryo alive	% Survival	No of Embryo alive	% Survival

	alive									
50 µg/ml	10	100	10	100	8	80	8	80	8	80
60 µg/ml	10	100	9	90	8	80	6	60	6	60
100 µg/ml	10	100	9	90	8	80	7	70	6	60
150 µg/ml	9	90	8	80	6	60	5	50	3	30
250 µg/ml	9	90	8	80	5	50	3	30	1	10
350 µg/ml	8	80	7	70	3	30	2	20	0	0

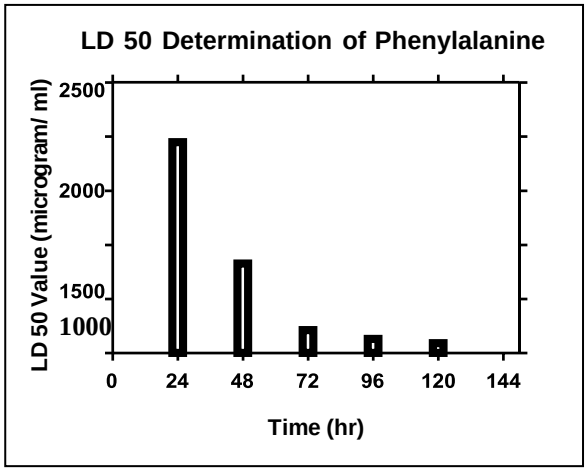
Table 3. LD_{50} of sodium phenyl pyruvate

SODIUM PHENYL PYRUVATE	24 hrs		48 hrs		72 hrs		96 hrs		120 hrs	
	No of embryo alive	% survival	No of embryo alive	% survival	No of embryo alive	% survival	No of embryo alive	% survival	No of embryo alive	% survival
50 µg/ml	10	100	9	90	9	90	9	90	8	80
60 µg/ml	10	100	9	90	7	70	7	70	6	60
100 µg/ml	8	80	7	70	7	70	6	60	5	50
150 µg/ml	6	60	5	50	3	30	2	20	1	10
250 µg/ml	5	50	3	30	1	10	0	0	0	0
350 µg/ml	3	30	2	20	0	0	0	0	0	0

Statistical analysis

Statistical analysis of Phenyl-alanine

Phenylalanine	
Time(Hrs)	LD 50 Value (µg/ml)
24	1949
48	829
72	213
96	133
120	92.68



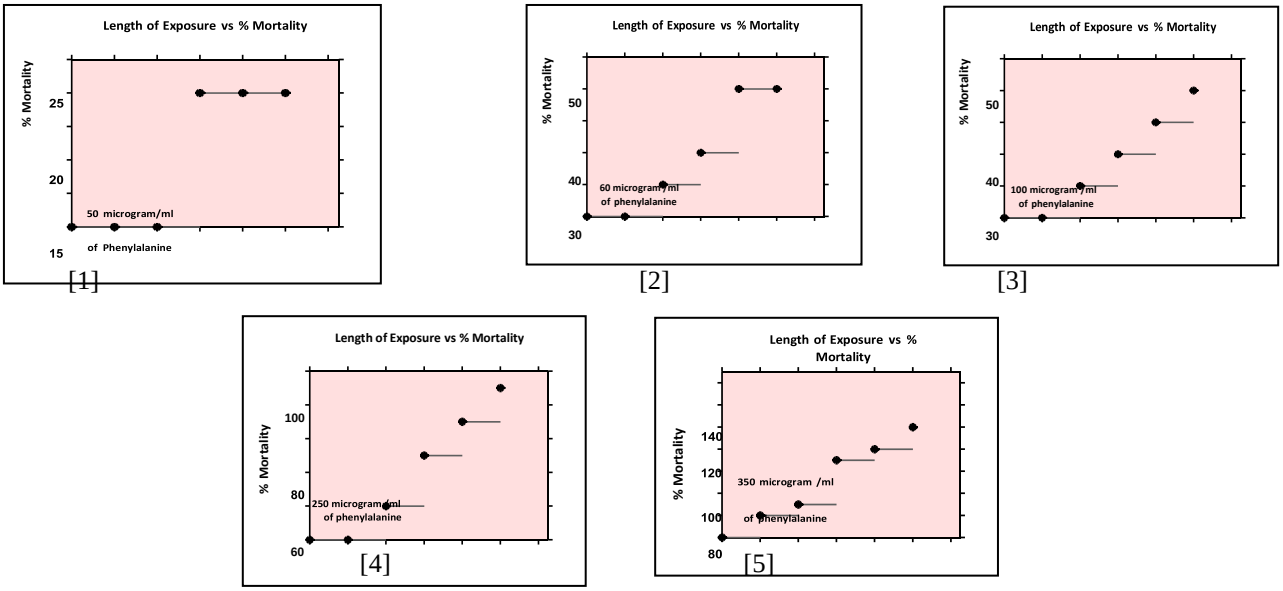
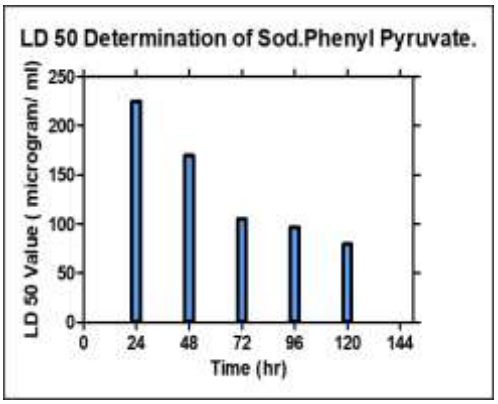
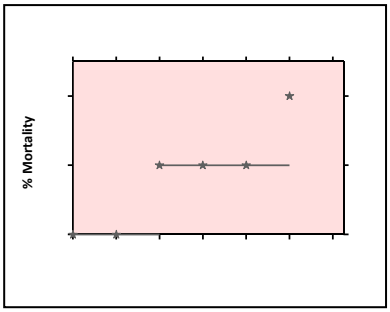


Figure 11. Exposure time and death rate due to Phenylalanine toxicity

Statistical Analysis of Phenyl-pyruvate

Sodium Phenylpyruvate	
Time (Hrs)	LD 50 Value (µg/ml)
24	224.9
48	169.82
72	105.15
96	97.05
120	79.79





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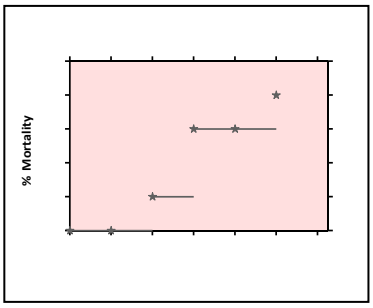

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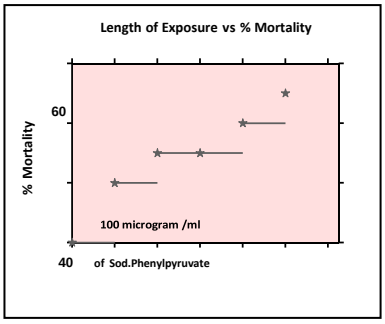

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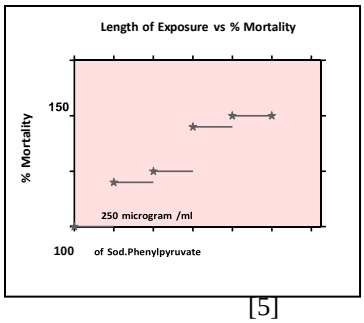

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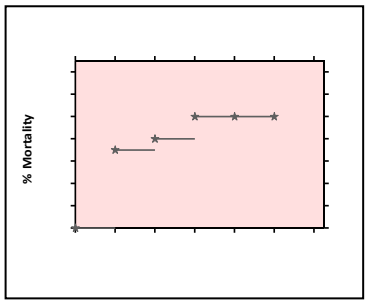

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Figure 12. Exposure time and death rate due to Sodium Phenylpyruvate

Experimental Data of Phenylalanine of Developmental Toxicity Test on (BodyLength, Head, and Fin)

Table 4. Developmental Toxicity Test on body length

PHENYLALANINE Body length			
Fish No.	0.5 mg	0.75 mg	1 mg
	in mm	in mm	in mm
1	1.819	2.174	2.152
2	2.724	2.087	2.62
3	2.395	2.07	2.345
4	1.914	2.229	2.756
5	2.025	2.19	2.277
6	2.166	2.651	2.204
Average	2.173833333	2.23	2.392333333

Table 5. Developmental Toxicity Test on fin

PHENYLALANINE Fin

Fish No.	0.5 mg		0.75 mg		1 mg	
	Longitudinal	Vertical	Longitudinal	Vertical	Longitudinal	Vertical
1	0.242	0.289	0.373	0.309	0.296	0.412
2	0.249	0.393	0.295	0.493	0.342	0.538
3	0.26	0.37	0.316	0.475	0.33	0.505
4	0.298	0.379	0.343	0.387	0.377	0.456
5	0.29	0.286	0.307	0.367	0.48	0.416
6	0.23	0.347	0.371	0.348	0.348	0.347
Average	0.2615	0.344	0.334166667	0.3965	0.362166667	0.445666667

Table 6. Developmental Toxicity Test on head

Experimental Data of Sodium Pyruvate of Developmental Toxicity on (BodyLength, Head, Fin)

Table 7. Developmental Toxicity Test on head

PHENYLALANINE **Head**						
Fish No.	0.5 mg		0.75 mg		1 mg	
	Length	Width	Length	Width	Length	Width
1	0.893	0.63	0.995	0.994	1.413	1.05
2	0.877	0.819	1.327	1.226	1.431	1.138
3	1.247	1.025	1.252	0.916	1.451	1.222
4	0.979	0.956	1.326	0.948	1.424	1.191
5	0.548	0.46	1.209	0.907	1.146	1.023
6	1.121	0.883	1.04	0.727	1.261	1.024
Average	0.9441666667	0.7955	1.1915	0.953	1.354333333	1.108

SPP Head						
Fish No.	0.25 mg		0.40 mg		0.60 mg	
	Length	Width	Length	Width	Length	Width
1	0.79	0.583	1.019	0.793	0.539	0.344
2	0.659	0.437	1.047	0.672	0.364	0.151
3	0.884	0.619	0.842	0.699	0.714	0.546
4	0.768	0.564	0.615	0.476	0.635	0.446
5	1.104	0.925	0.65	0.437	0.826	0.503
6	0.786	0.531	0.624	0.454	0.861	0.551
Average	0.831833333	0.609833333	0.7995	0.5885	0.6565	0.4235

Table 9. Developmental Toxicity Test on body length

SPP Body length			
Fish No.	0.25 mg	0.40 mg	0.60 mg
	in mm	in mm	in mm
1	2.245	1.638	1.79
2	1.969	1.802	1.637
3	2.064	1.326	1.308
4	2.307	1.848	1.962
5	2.117	1.422	1.784
6	1.968	1.935	1.6
Average	2.111666667	1.661833333	1.680166667

SPP Fin						
Fish No.	0.25 mg		0.40 mg		0.60 mg	
	Longitudinal	Veretical	Longitudinal	Veretical	Longitudinal	Veretical
1	0.223	0.208	0.22	0.233	0.11	0.183
2	0.255	0.228	0.261	0.231	0.108	0.177
3	0.26	0.247	0.196	0.235	0.12	0.196
4	0.2	0.251	0.206	0.245	0.16	0.252
5	0.265	0.207	0.217	0.273	0.18	0.229
6	0.249	0.231	0.214	0.1256	0.1	0.191
Average	0.242	0.228666667	0.219	0.223766667	0.129666667	0.204666667

Table 9. Developmental Toxicity Test on fin

Statistical analysis of phenylalanine and sodium pyruvate of developmental toxicity

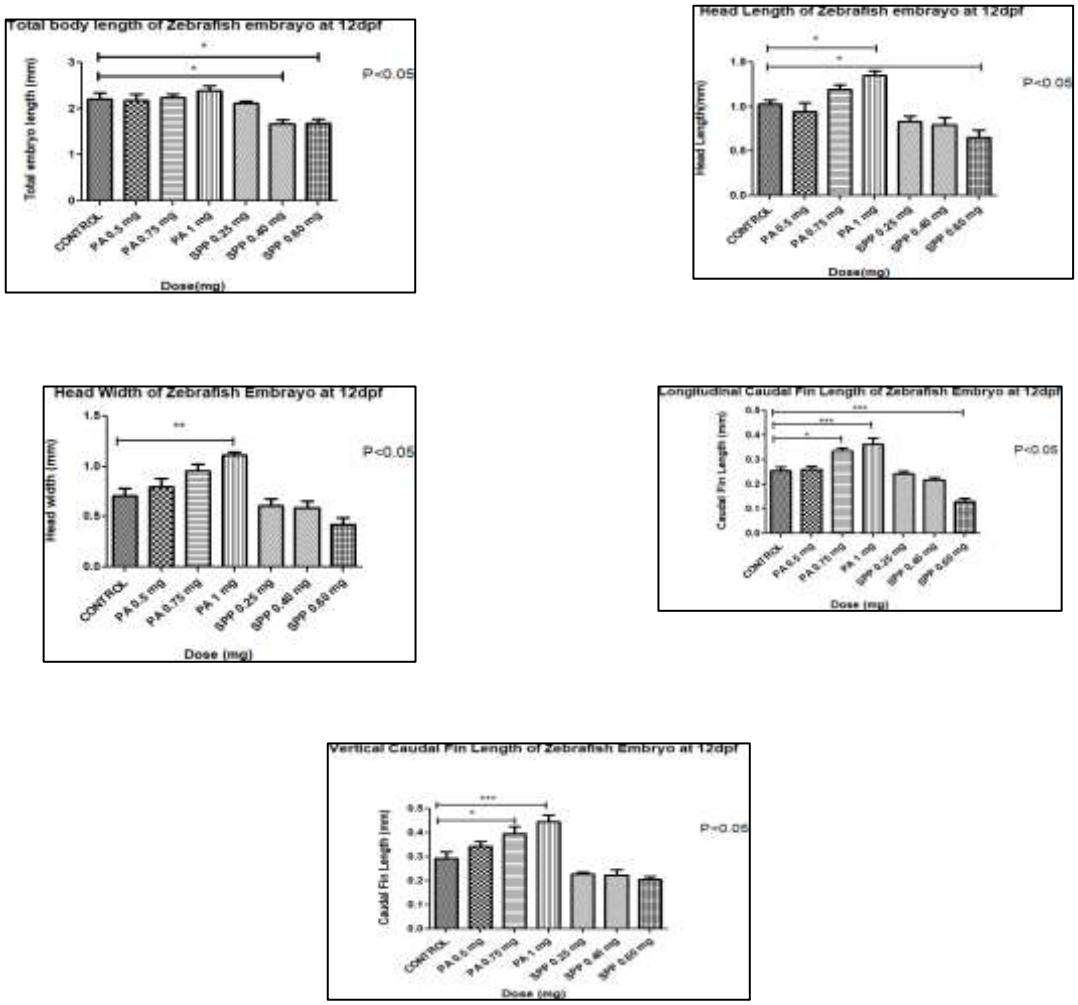
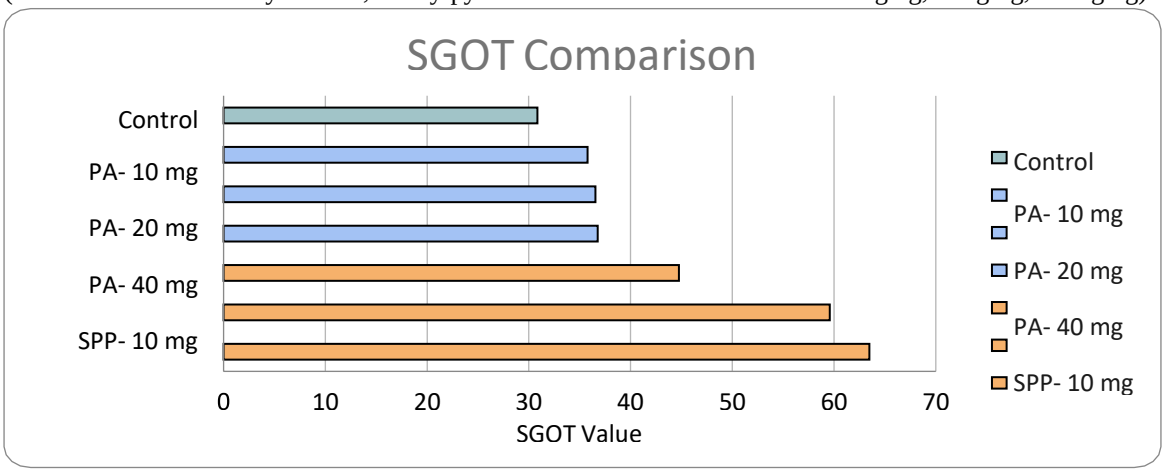


Figure 13. Statistical analysis of phenylalanine and sodium pyruvate of developmentaltoxicity

Biochemical Estimation of Hepatic Enzyme on Adult Zebrafish of SGPT SGOT:

(After induction of Phenylalanine, Phenylpyruvate at different concentration 10mg/kg,20mg/kg, 40 mg/kg)



Biochemical Estimation Hepatic Enzyme on Adult Zebrafish of SGPT SGOT. (After induction of Phenylalanine, Phenylpyruvate at different concentration 10mg/kg,20mn/kg, 40 mg/kg)

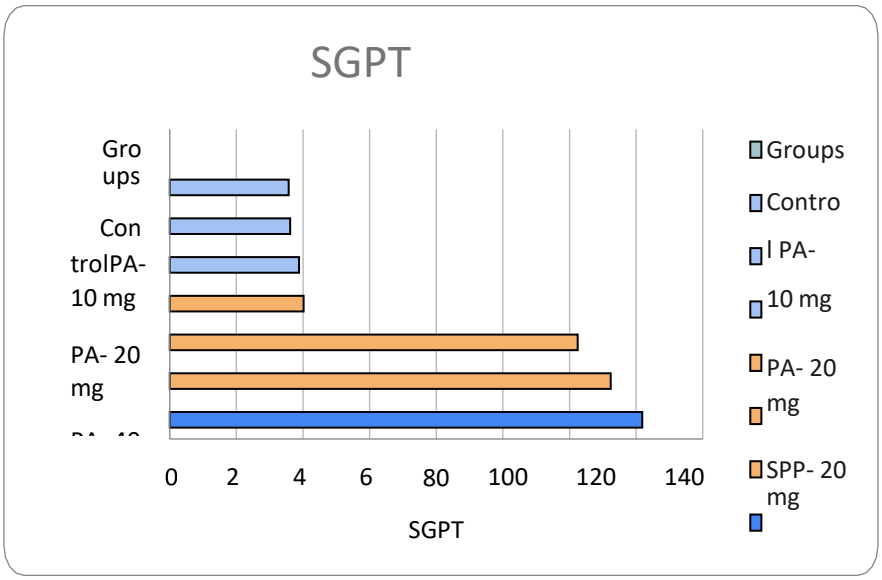


Figure 15. SGPT profile

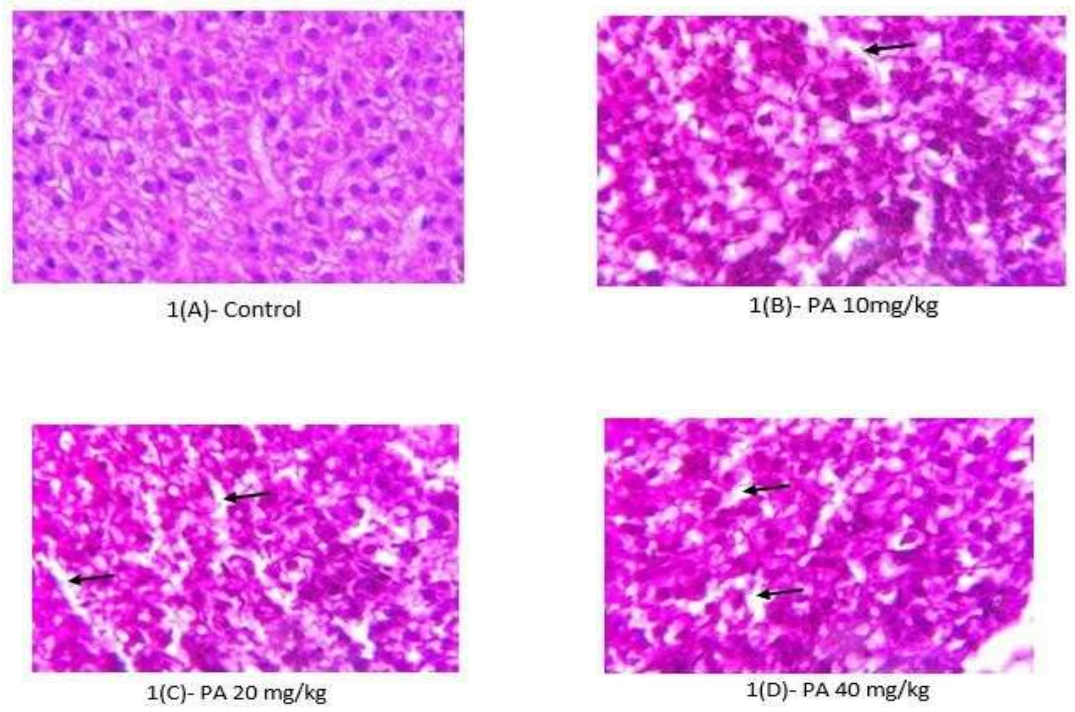


Figure 16. Histopathology of hepatocytes of control group of zebrafish versus sodium phenylpyruvate in 10mg/kg, 20mn/kg, 40 mg/kg induced zebrafish (1000x magnification)

Table 10. Histopathological data treated with Phenylalanine

Histopathological Findings	Symbolic representation	Control	Treated with Phenylalanine		
			10 mg/kg	20 mg/kg	40 mg/kg
Sinusoidal dilatation	Arrow	-	+	+	+
Cytolysis	Asterisks	-	-	-	-
Karyolysis	Small arrow	-	-	-	-
Cell-to-cell adhesion decrement	Double arrow	-	-	-	+
Vacuolization	Arrowheads	-	-	-	-
Degenerated hepatocytes	Circle	-	-	-	-
(-)none; (+)mild; (++)moderate; (+++)severe					

Histopathology of hepatocytes of control group of zebrafish versus sodium phenylpyruvate 10mg/kg, 20mn/kg, 40 mg/kg induced zebrafish (1000x magnification)

Table 11. Histopathological Findings treated with PSS data

Histopathological Findings	Symbolic representation	Control	Treated with PSS		
			10 mg/kg	**20 mg/kg**	**40 mg/kg**
Sinusoidal dilatation	Arrow	-	++	+++	+++
Cytolysis	Asterisks	-	+	++	+++
Karyolysis	Small arrow	-	+	++	++
Cell-to-cell adhesion decrement	Double arrow	-	++	++	+++
Vacuolization	Arrowheads	-	+	++	+++
Degenerated hepatocytes	Circle	-	+	++	+++
(-) none; (+) mild; (++) moderate; (+++) severe					

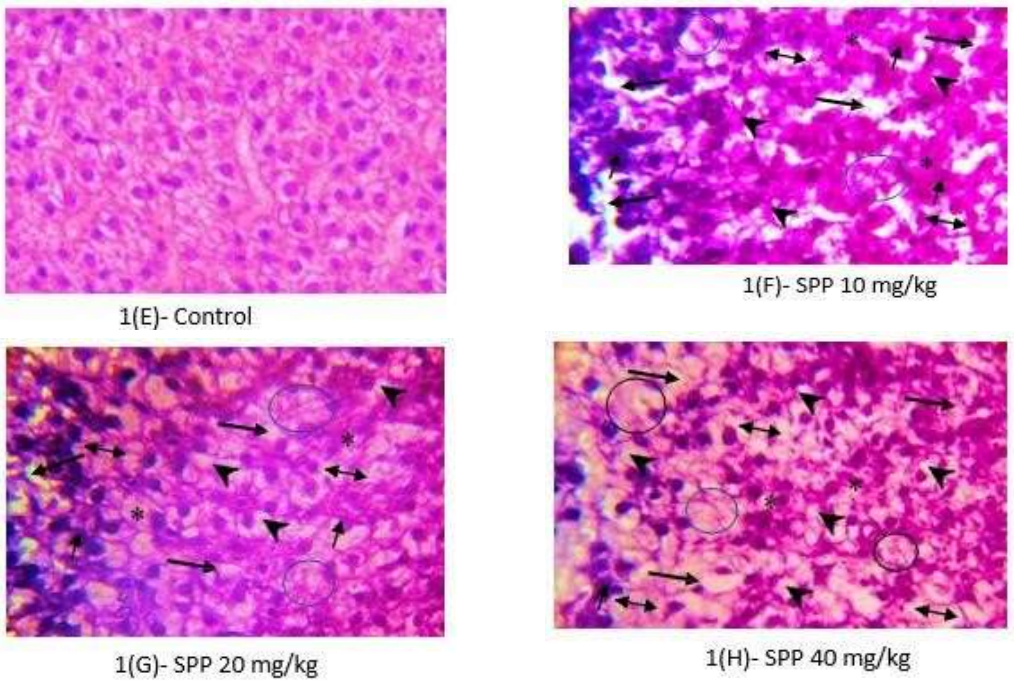


Figure 17. In-Vivo study of toxicity

Conclusion

According to OECD guideline (236) the LC50 study has been done on phenylalanine and sodium phenylpyruvate. The LC50 values of phenylalanine at 24 hrs, 48hrs, 72 hrs, 96hrs and 120hrs is 1949,829,213,133,92.6 microgram/ml and for sodium phenylpyruvate the values are 224,169,105,97,79.79 microgram/ml. During developmental toxicity study three different concentration of phenylalanine sodium pyruvate has been charged to the healthy embryo. At day 12 it is observed that sodium pyruvate cause growth retardation considering 4 parameters i.e., head length, head width, total body length and caudal fin length . In case of phenylalanine at its initial two concentration no such effect observed only at its highest concentration some abnormalities are observed i.e., ., head length, head width, and caudal fin length Which is significant different than the control. After that drug has been charge through
i.p route at three different concentration i.e.,10,mg/kg,20mg/kg,40mg/kg and incubated for 72hrs for the biochemical estimation of SGPT and SGOT .For Phenylalanine no significant change observed but for phenylpyruvate. Both SGPT& SGOT values become much higher than the normal. Finally, Histopathological slides in comprising with the control, no such different has been observed except Sinusoidal Dilatation and Cell-to-cell adhesion decrement in case of Phenylalanine at 40mg/kg but for phenylpyruvate. Cellular deformities were observed in hepatocytes i.e., Sinusoidal dilatation, Cytolysis, Karyolysis, Cell-to-cell adhesion decrement, Vacuolization, Degenerated hepatocytes.

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Conflict of Interest

No conflict of Interest

Funding

Not applicable

Data consent

All data are revealed

References

1. Aristoy MC, Toldrá F. Essential amino acids. InHandbook of seafood and seafood products analysis 2009 Nov 24 (pp. 305-326). CRC Press.
2. Volpi E, Kobayashi H, Sheffield-Moore M, Mittendorfer B, Wolfe RR. Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. The American journal of clinical nutrition. 2003 Aug 1;78(2):250-8.
3. Choi BH, Coloff JL. The diverse functions of non-essential amino acids in cancer. Cancers. 2019 May 15;11(5):675.
4. Wu G, Wu Z, Dai Z, Yang Y, Wang W, Liu C, Wang B, Wang J, Yin Y. Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. Amino acids. 2013 Apr;44(4):1107-13.
5. Hou Y, Wu G. Nutritionally essential amino acids. Advances in Nutrition. 2018 Nov 1;9(6):849-51.
6. Gullino P, Winitz M, Birnbaum SM, Cornfield J, Otey MC, Greenstein JP. Studies on the metabolism of amino acids and related compounds in vivo. I. Toxicity of essential amino acids, individually and in mixtures, and the protective effect of L-arginine. Archives of Biochemistry and Biophysics. 1956 Oct 1;64(2):319-32.
7. Gullino P, Winitz M, Birnbaum SM, Cornfield J, Otey MC, Greenstein JP. The toxicity of individual essential amino acids and their diastereomers in rats and the effect on blood sugar levels. Archives of Biochemistry. 1955;58:253-5.
8. Yudkoff M, Daikhin Y, Nissim I, Horyn O, Luhovyy B, Lazarow A, Nissim I. Brain amino acid requirements and toxicity: the example of leucine. The Journal of nutrition. 2005 Jun 1;135(6):1531S-8S.
9. Teng M, Zhu W, Wang D, Qi S, Wang Y, Yan J, Dong K, Zheng M, Wang C. Metabolomics and transcriptomics reveal the toxicity of difenoconazole to the early life stages of zebrafish (Danio rerio). Aquatic Toxicology. 2018 Jan 1;194:112-20.
10. Tanguay RL, Abnet CC, Heideman W, Peterson RE. Cloning and characterization of the zebrafish (Danio rerio) aryl hydrocarbon receptor. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression. 1999 Jan 18;1444(1):35-48.
11. Santoso F, Sampurna BP, Lai YH, Liang ST, Hao E, Chen JR, Hsiao CD. Development of a simple ImageJ-based method for dynamic blood flow tracking in zebrafish embryos and its application in drug toxicity evaluation. Inventions. 2019 Nov 1;4(4):65.
12. Zhang Y, Li S, Li J, Han L, He Q, Wang R, Wang X, Liu K. Developmental toxicity induced by PM2. 5 through endoplasmic reticulum stress and autophagy pathway in zebrafish embryos. Chemosphere. 2018 Apr 1;197:611-21.
13. Driessen M, Kienhuis AS, Pennings JL, Pronk TE, van de Brandhof EJ, Roodbergen M, Spaink HP, van de Water B, van der Ven L. Exploring the zebrafish embryo as an alternative model for the evaluation of liver toxicity by histopathology and expression profiling. Archives of toxicology. 2013 May;87(5):807-23.