

Investigating Developmental Toxicity of Phenylalanine and Phenylpyruvate: Insights from the *Danio rerio* Embryo Model

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ABSTRACT

Phenylalanine (PA) as Essential amino acids required from the outer side the body for normal physiological function and development. Different scientific published literature is indicating the pathophysiological role of PA, specifically in PKU and responsible for Neurotoxicity, Birth Defects, Developmental Delays, Microcephaly, Seizures, Musty odor and Eczema. The metabolic pathway of PA also creates concern about the responsibility towards its metabolites Phenylpyruvate (PP). Therefore this current study will investigate the role of PA & PP about its developmental effect (Eye Diameter, Head Length, Head Width, Yolk Sac Area, Pericardial Area, Caudal Fin Length, Total Body Length, Heartbeat Rate, Axial Angle, and Circulation) on Zebra fish Embryo. The entire study has been done on *Danio rerio* embryo considering PA concentration 0.3 µg/ml, 0.5 µg/ml, 1 µg/ml and PP concentration 0.3 µg/ml, 0.5 µg/ml, 0.7 µg/ml. Finally it has been observed PA does not have major effect in the selected concentration except Total Body Length but PP has shown significant developmental effect in most of the parameters.

KEYWORDS: Developmental toxicity, Phenylalanine, Phenylpyruvate, zebrafish, embryo model, Teratogenic effects.

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INTRODUCTION

Introduction: Day by day in increase uses of dietary supplements which contain amino acids (free form) become a focal point in nutritional science and public health [1]. When the common people the users of nutritional supplements seek to improve their health through this product, naturally it creates a need to understand the safety profiles and potential risks [2-3]. Total 4/5th of the world population's dependent partially of fully on folk medicine, indigenous medicine, and ethnomedicine, showcasing its importance in larger healthcare system [5-7]. the connections between traditional medicine and modern dietary supplements support the questions of efficacy and safety, when it comes to amino acids that are often considered as safe and professed health benefits [8][2]. On other side, lysine in the diet with a higher level disrupts the quality of protein, as non-essential amino acids Glycine will show it toxic effect with higher concentration present in diet [9-11]. The on-going regulation at different countries fail to provide sufficient mechanisms for monitoring dosing and make certain the quality and purity of the products [12][3]. "High-risk people new born, toddlers, young adults, 1st trimester and 3rd trimester of pregnancy, nursing women, are particularly at risk due to their specific physiological needs and the lack of particular safety evaluations leads to their circumstances [3][13-14].

This study will use *Danio rerio* "embryos" for evaluating the toxicity, teratogenic effects (if any) of specific amino acids—particularly L-Phenylalanine and Phenylpyruvate. The *Danio rerio* model is increasingly recognized for its relevance in toxicological research due to the genetic analogy to humans and its limpid embryos that permit for live observation of growth processes. [15-17]. Individuals, differences in body physiology, heightened nutritional demands, or existing health problems can make them more susceptible to harm, particularly when amino acids are consumed in uncontrolled amount [18-19].

1.1 Potential Risks for Vulnerable Populations

1.1.1 Children: Pregnant Women: Athletes: are venerable for interaction between different supplement consumed parallel which could lead to adverse effects [20-24][13]. **Individuals with Specific Health Conditions:** Person with PKU may build-up toxic content inside his body just because he cannot metabolize phenylalanine effectively. [3] [25-26].

1.2 Regulatory and Safety Concerns: WHO has mentioned the need of improved guideline to assure the safety and as well as efficacy of nutritional supplements Furthermore, many studies have excluded the vulnerable groups from initial safety evaluations, which potentially limits the uses of clinical data for the final guideline [3] [12-13].

1.3 Regulatory Frameworks: The specific Dietary Supplement Health and Education Act (DSHEA) implemented by FDA at U.S. This official guideline allows manufacturers to market amino acid supplements without pre-market approval, believing

instead on post-market surveillance. [12][27].

OBJECTIVE:

To perform the Developmental Toxicity of Phenylalanine and Phenylpyruvate on *Danio rerio* Embryo Model.

LITERATURE REVIEW:

WHO recognizes indigenous medicine as a crucial healthcare resource in many developing nations, where it often act as the basic source of health care alongside food supplements.[4] Approximately 80% of the global population utilizes traditional medicine, with significant reliance observed in various regions, particularly in Africa and Asia[28-30].

Lack of Clinical Safety Information

3.2.1 Insufficient Testing: There is insufficient data and report of the both animal and human studies to address the issue on critical outcome after the use of supplemental amino acids. [31-33] **No Established Upper Limits:** The concern authority or experts often struggled to identify safe upper intake levels when amino acids are being used as an ingredient consumed through dietary proteins. This lack of understanding requires the need for comprehensive safety evaluations, specifically as many consumers do not know the risk associated with uncontrolled consumption of amino acid supplementation[31][34]. **Vulnerable Populations:** Certain groups, i.e., athletes, paediatric age group, toddlers, , 1st to 3rd trimester of pregnancy, and individuals with specific biochemical condition, may be at higher risk for undesired effects from amino acid supplementation. However, these populations are often overlooked from initial safety studies (during clinical trials), which limits the understanding of potential risks [31-32]

3.2 Absence of Regulatory Tools

Regulatory Challenges: It is clear and evident that the regulation to control the uses of amino acid use in different dietary supplements and food products is inadequate. Existing regulation of different countries is insufficient which can lead to unsafe consumption practices [31][34].

Quality Control Issues: There is urgent need for improved regulatory standards to ensure that amino acid supplements assure safety and quality criteria..[34-35] **International Harmonization:** Divergent policies and regulations across different regions create challenges for the global harmonization of amino acid supplementation standards [31-34] Various countries with millions of population like China and Indonesia, including Thailand is lacking regulatory policies governing food uses of amino acids which clearly denotes the safety concern of AA (amino acids).[35]Country like Japan, Korea, Singapore located in Asia and some Latin American countries do not have regulatory structure to monitor and strict control of amino acid use as food supplements.[36]At uncontrolled **consumptions of histidine (>24 g/d)**, shows several adverse effect like **reduction in serum zinc level and cognitive weakness** along with that a **safer upper level has not been confirmed**[37-38]

3.4 Relevance of Zebrafish Model:

A Systematic Review" by Jayasinghe and Jayawardena supports the compliance of the zebrafish model with the 3Rs principle of animal welfare—"Replacement", "Reduction", and "Refinement". [39-41] The rise of zebrafish as a toxicological model over the last twenty years exemplifies convergence and persistence due to several key factors like **Convergence**. [42-43].**Persistence** [43-44]A popular study performed by Caballero and Candiracci, highlights the utility of zebrafish as a model for cardiotoxicity studies, despite the bodily differences between zebrafish and mammals hearts. [45-48]

3.5 During Phenylketonuria: Whether The Phenylalanine The Sole Cause Of Brain Dysfunction? Or Other Factors Are Contributing?

The incorporation of phenylalanine into the brain is facilitated by the high plasma phenylalanine concentrations, but the entry of other big neutral AAs is inhibited [49-50]. A lack of some major neutral AAs in the brain, in addition to phenylalanine's neurotoxicity, may significantly impact cognitive performance in phenylketonuria.[51] However, this disruption may not be due to excessive phenylalanine levels but rather to a deficiency of big neutral AAs.[52]

Effects of Elevated Phenylalanine Concentrations in the Brain

According to Schindeler et al. (2007), supplementing with large neutral AAs (LNAA) for a brief time improved cognitive abilities without lowering phenylalanine levels in the brain [53]. Additionally, it has been demonstrated that phenylalanine inhibits the role of human tyrosine and an enzyme tryptophan hydroxylase when applied in *Escherichia coli* [53]. However, there is ongoing discussion regarding how high cerebral phenylalanine concentrations may affect was shown that upper level of phenylalanine reduced the activity of Hydroxymethylglutaryl-CoA reductase, impairing the synthesis of cholesterol [49]. The *In vivo* observation of low energy metabolism in PKU suffering subjects utilizing positron emission tomography may be related to decreased pyruvate kinase activity [54].

3.6 Phenylalanine Containing Common Food

As an essential amino acid Phenylalanine has been found in various food items i.e., Meat \Poultry product Dairy , Soy products Nuts seed , Whole grain Oats, wheat Quinoa. commonly consumed by the people [59-60].

3.7 Aspartame Containing Common Food and Food Supplement

Aspartame an Artificial Sweeteners being used in different item like Beverages (7Up Ten, Crystal Light, Diet 7Up, Diet 7Up Cherry, Diet Coke, Mints/Gum (Breath Savers Peppermint, Doublemint Gum, Citrucel Sugar Free Orange Fiber Powder, Metamucil Smooth Texture Orange Sugar Free Fiber Powder) etc [61-62].

The global regulations not directly supervising the uses of Phenylalanine in different food item, rather controlling the uses of **ASPARTAME**. The reason behind this regulation is

- As Artificial Sweeteners 'Aspartame' is common.
- Aspartame after metabolism converted in to Phenylalanine (50% of its original amount). Thus more focus on Aspartame.

US-FDA is has set no upper limit for the consumption of Phenylalanine, only for the PKU compromised patient limits has been fixed which is 120–360 $\mu\text{mol/L}$ (children) and 120–600 $\mu\text{mol/L}$ (adults) [55]. Therefore Setting of an upper limit of Phenylalanine consumption is very important for the healthy people. The upper limits for different countries are same but the population-specific differences in the PAH gene is significant among Indians, Chinese, Americans, and Africans [56-58]. Therefore consumption limit must be set as the metabolic pathway has been controlled by the PAH gene and other enzymes are variable amongst Indians, Chinese, Americans, and Africans. The Phenylalanine reported to produce several pathophysiological conditions like [64] Neurotoxicity, Birth Defects, Developmental Delays, Microcephaly, Seizures Musty odor Eczema, But the permissible limit for the Phenylalanine has been set only to prevent **NEUROTOXICITY/NEUROCOGNITIVE**. A scientific reference supporting the statement that phenylpyruvate levels are significantly elevated in the blood and urine of untreated PKU patients, and that although exact concentration ranges are not universally stated [63]. Therefore the role of L-Phenylalanine and Phenylpyruvate for the specific outcome like Growth Failure, Birth Defects, and Microcephaly is a concern as well as the allowable limit of L-Phenylalanine in food supplement is also another concern.

METHODOLOGY

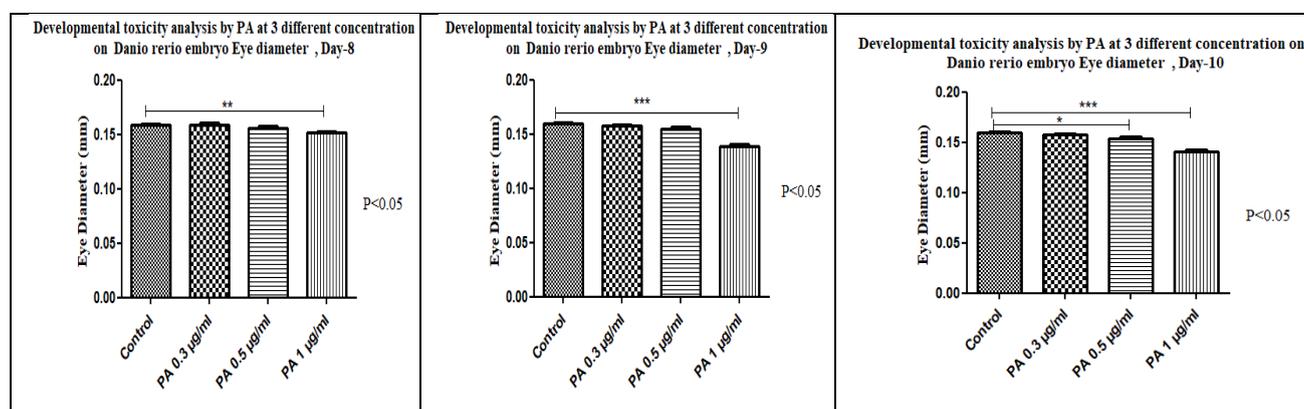
4.1 Procurement and Authentication of *Danio rerio*: Adult zebrafish (*Danio rerio*) were procured from the local fish market in Kolkata. The specimens were authenticated by the Department of Fisheries Resource Management, West Bengal University of Animal and Fishery Sciences, which confirmed the species identity as *Danio rerio* (Hamilton, 1822), commonly known as the zebrafish or zebra danio. Taxonomic classification provided was: Kingdom: *Animalia*; Phylum: *Chordata*; Class: *Actinopterygii*; Order: *Cypriniformes*; Family: *Cyprinidae*; Subfamily: *Danioninae*; Genus: *Danio*; Species: *Danio rerio*.

4.2 Acclimatization and Breeding of Fish: All fish were acclimatized in laboratory aquaria for two weeks under optimal conditions. Zebrafish, known for their thermal tolerance between 10.6°C and 41.7°C, were maintained at 28 ± 1°C, the temperature optimal for growth and breeding. To minimize stress, tanks were enriched with artificial plants and maintained under a 14:10 h light:dark cycle with continuous aeration. Prior to breeding, male and female fish were segregated into separate tanks for 1–2 weeks to ensure proper gonadal maturation. Laid embryos were collected with a fine strainer, rinsed with system water, and transferred to Petri dishes containing embryo medium (E3 solution).

4.3 Developmental Toxicity Assay: Developmental toxicity studies were conducted according to OECD Test Guideline 236 (Fish Embryo Acute Toxicity Test). Healthy fertilized embryos at 24 hours post-fertilization were exposed to different concentrations of L-phenylalanine (PA) and sodium phenylpyruvate (PP). Six nominal concentrations—50, 60, 100, 150, 250, and 350 $\mu\text{g/mL}$ —were used initially for LC₅₀ determination across 24–120 h exposure periods. For detailed developmental evaluation, optimized sublethal concentrations were selected: PA (0.3, 0.5, and 1 $\mu\text{g/mL}$) and PP (0.3, 0.5, and 0.7 $\mu\text{g/mL}$). Exposure lasted ten days, during which embryos were maintained in 24-well plates (one per well) under standard conditions. **Ten developmental parameters**—Eye Diameter, Head Length, Head Width, Yolk Sac Area, Pericardial Area, Caudal Fin Length, Total Body Length, Heartbeat Rate, Axial Angle, and Circulation—were monitored daily at 24-hour intervals under a compound microscope.

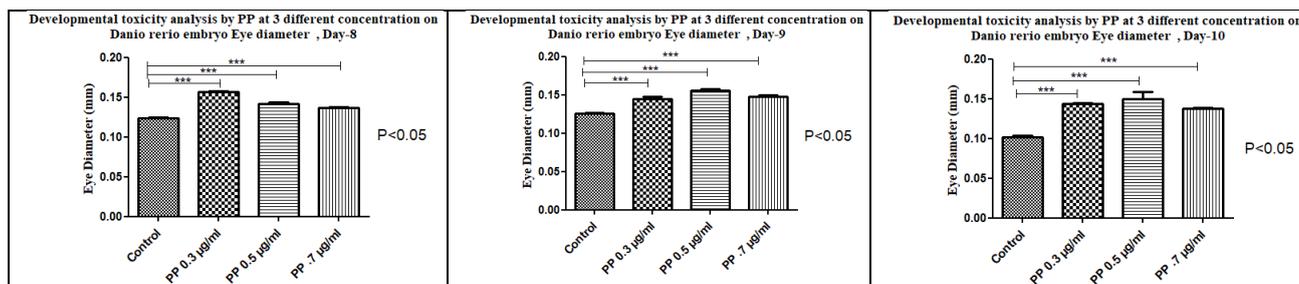
4.4 Image Processing and Analysis: Morphometric measurements were quantified using ImageJ software (NIH, USA).

5. Results & Discussion: Through daily monitoring, it was observed that both PA and PP at low concentrations mostly allowed normal development, but significant differences appeared as concentrations and exposure time increased. The LC₅₀ values for phenylalanine dropped from 1949 $\mu\text{g/mL}$ at 24 hours to 92.68 $\mu\text{g/mL}$ at 120 hours; for phenylpyruvate, the LC₅₀s were markedly lower, falling from 224.9 $\mu\text{g/mL}$ at 24 hours to just 79.79 $\mu\text{g/mL}$ at 120 hours, highlighting more potent toxicity for PP.



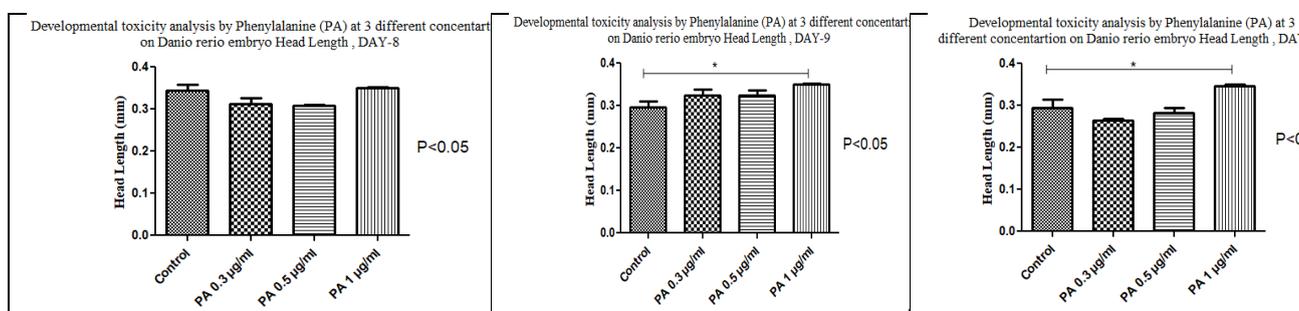
EYE DIAMETER (mm)			
Day	0.3 µg/ml	0.5 µg/ml	1 µg/ml
Day-1	ns	ns	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	ns
Day-6	ns	ns	ns
Day-7	ns	ns	ns
Day-8	ns	ns	**
Day-9	ns	ns	***
Day-10	ns	*	***

Fig: 01 After conducting the development toxicity experiment focusing on the parameter “EYE DIAMETER” in *Danio rerio* it was noted that initially, no deformities were observed, indicating a low level of significance. However, from the days 9 and 10 onward, highly significant difference was observed at the higher concentration (1µg/ml) of the test drug Phenylalanine subsequently and on the day 10 slightly significant difference was observed on the concentration 0.5 µg/ml of the test drug



EYE DIAMETER (mm)			
Day	0.3 µg/ml	0.5 µg/ml	0.7 µg/ml
Day-1	ns	ns	ns
Day-2	*	*	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	***
Day-6	ns	ns	***
Day-7	***	***	***
Day-8	***	***	***
Day-9	***	***	***
Day-10	***	***	***

Fig:02 After performing the developmental toxicity experiment considering the parameter i.e., “Eye diameter” on *Danio rerio*, it has been observed that at initial days there is no such deformity observed thus the level of significance is very less but later on specifically from day 5 & 6, at highest concentration (0.7µg/ml) of test drug i.e.,PP there is a significance difference (highly) observed. Finally on day 7, day 8, day 9 & day 10, significant difference observed in all 3 concentration(0.3 µg/ml, 0.5µg/ml & 0.7µg/ml on test drugs.



Head Length (mm)			
DAY	0.3µg/ml	0.5µg/ml	1µg/ml
Day-1	ns	ns	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	ns
Day-6	ns	ns	ns
Day-7	ns	ns	ns
Day-8	ns	ns	ns
Day-9	ns	ns	*
Day-10	ns	ns	*

Fig: 03 After conducting the development toxicity experiment focusing on the parameter “HEAD LENGTH” in *Danio rerio* it was noted that initially, no deformities were observed, indicating a low level of significance. However, from the days 9 and 10 onward, slightly significant difference was observed at the higher concentration (1µg/ml) of the test drug Phenylalanine subsequently.

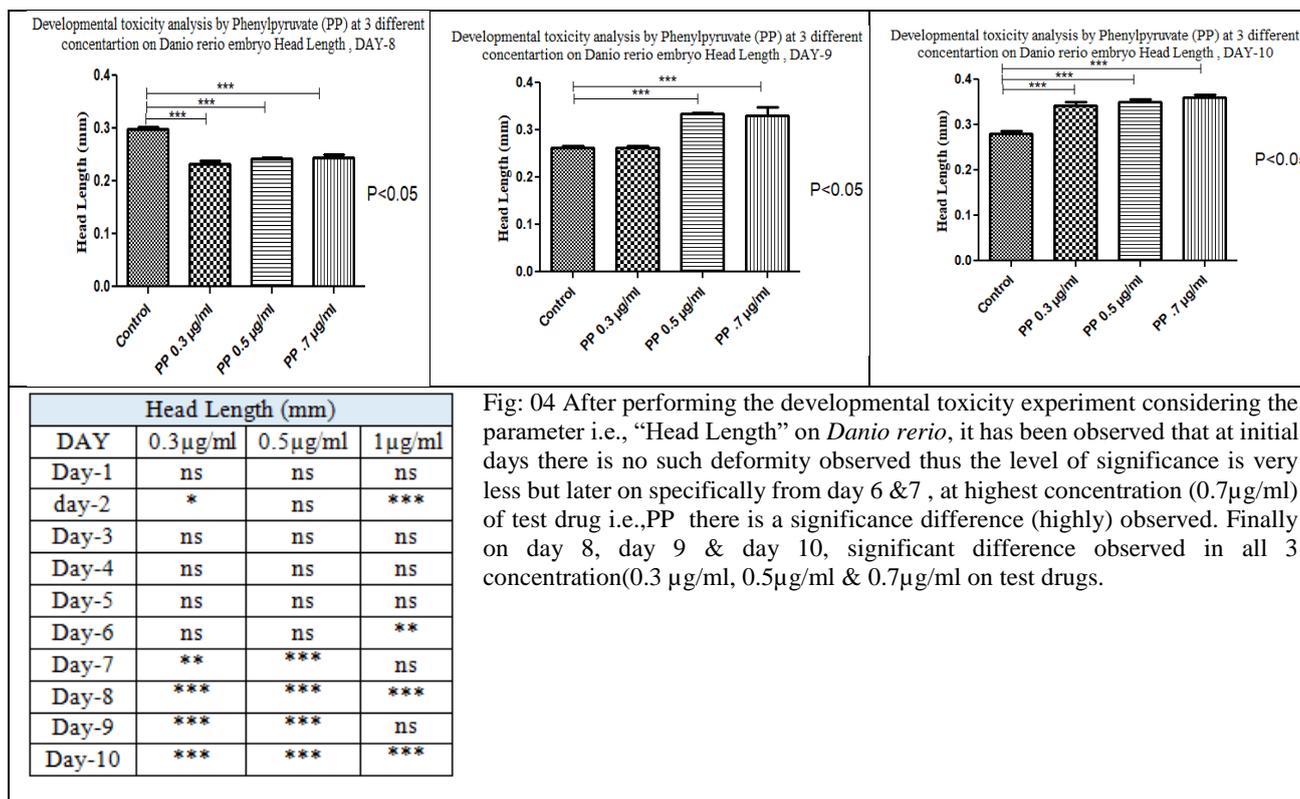


Fig: 04 After performing the developmental toxicity experiment considering the parameter i.e., “Head Length” on *Danio rerio*, it has been observed that at initial days there is no such deformity observed thus the level of significance is very less but later on specifically from day 6 & 7, at highest concentration (0.7µg/ml) of test drug i.e.,PP there is a significance difference (highly) observed. Finally on day 8, day 9 & day 10, significant difference observed in all 3 concentration(0.3 µg/ml, 0.5µg/ml & 0.7µg/ml on test drugs.

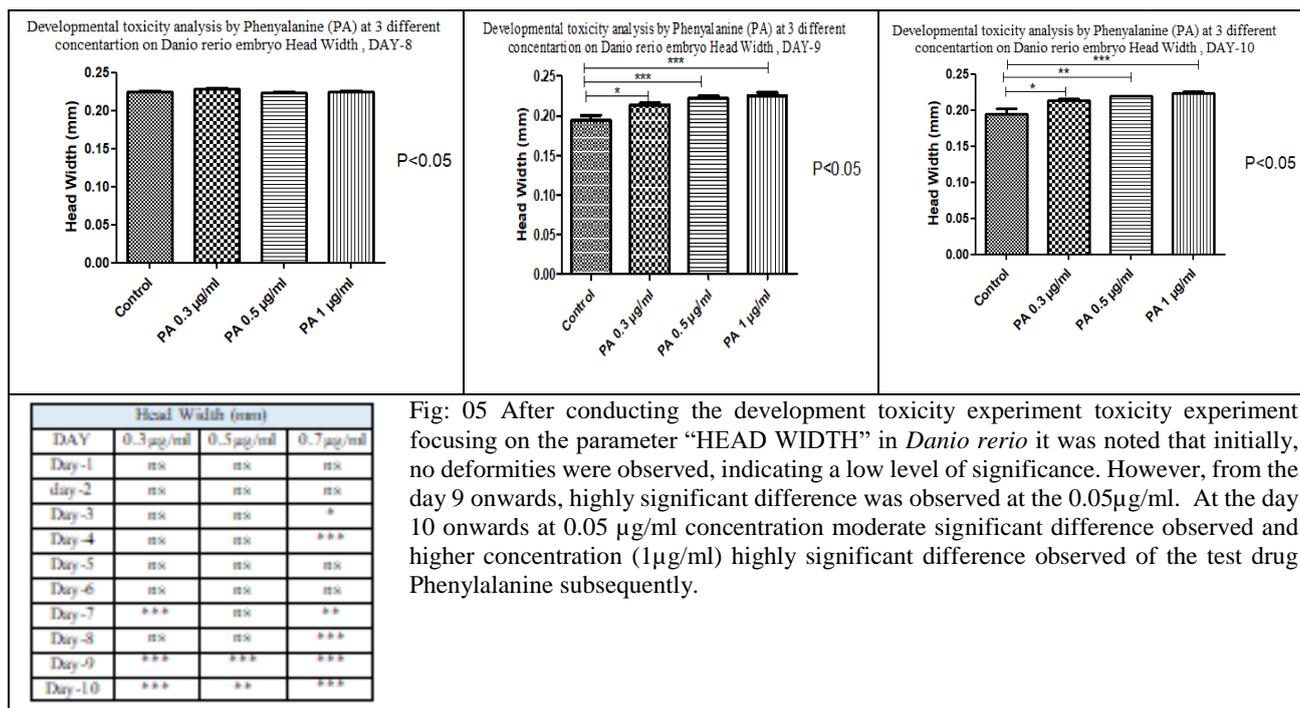
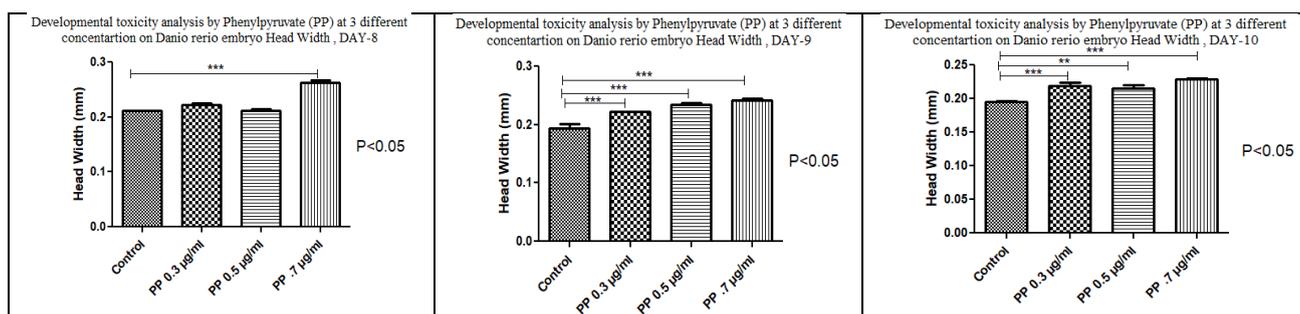


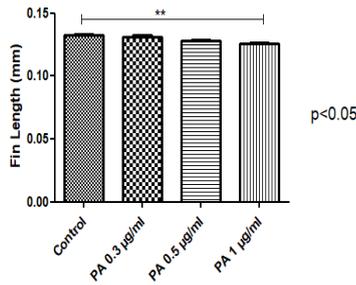
Fig: 05 After conducting the development toxicity experiment focusing on the parameter “HEAD WIDTH” in *Danio rerio* it was noted that initially, no deformities were observed, indicating a low level of significance. However, from the day 9 onwards, highly significant difference was observed at the 0.05µg/ml. At the day 10 onwards at 0.05 µg/ml concentration moderate significant difference observed and higher concentration (1µg/ml) highly significant difference observed of the test drug Phenylalanine subsequently.



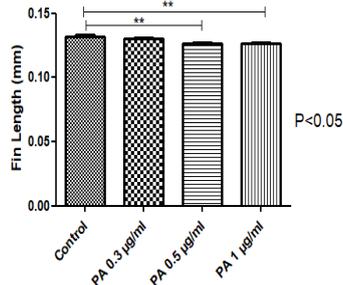
Head Width (mm)			
DAY	0.3 µg/ml	0.5 µg/ml	0.7 µg/ml
Day-1	ns	ns	ns
Day-2	ns	ns	ns
Day-3	ns	ns	*
Day-4	ns	ns	***
Day-5	ns	ns	ns
Day-6	ns	ns	ns
Day-7	***	ns	**
Day-8	ns	ns	***
Day-9	***	***	***
Day-10	***	**	***

Fig: O6 After performing the developmental toxicity experiment considering the parameter i.e., “Head Width” on *Danio rerio*, it has been observed that at initial days there is no such deformity observed thus the level of significance is very less but later on specifically from day 3, day 4, day 5, day 6, at highest concentration (0.7 µg/ml) of test drug i.e., PP there is a significance difference observed. Finally on day 8, day 9 & day 10, significant difference observed in all 3 concentration (0.3 µg/ml, 0.5 µg/ml & 0.7 µg/ml) on test drugs.

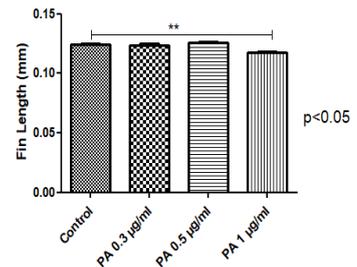
Developmental toxicity analysis by Phenylalanine (PA) at 3 different concentration on *Danio rerio* embryo Fin Length, DAY-8



Developmental toxicity analysis by Phenylalanine (PA) at 3 different concentration on *Danio rerio* embryo Fin Length, DAY-9



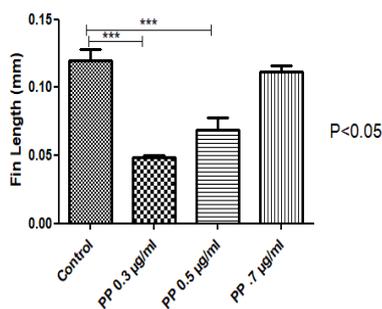
Developmental toxicity analysis by Phenylalanine (PA) at 3 different concentration on *Danio rerio* embryo Fin Length, DAY-10



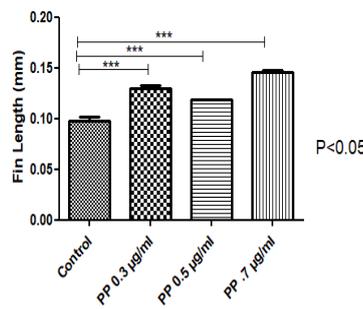
Fin Length (mm)			
DAY	0.3 µg/ml	0.5 µg/ml	1 µg/ml
Day-1	ns	ns	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	ns
Day-6	ns	ns	ns
Day-7	ns	ns	ns
Day-8	ns	ns	**
Day-9	ns	**	**
Day-10	ns	ns	**

Fig: O7 After conducting the development toxicity experiment focusing on the parameter “FIN LENGTH” in *Danio rerio* it was noted that initially, no deformities were observed, indicating a low level of significance. However, from the day 8 onwards, moderate significant difference was observed at the higher concentration (1 µg/ml). At the day 9 onwards at 0.5 µg/ml concentration moderate significant difference observed and higher concentration (1 µg/ml) moderate significant difference observed. At the day 10 onwards at the higher concentration (1 µg/ml) highly significant difference observed of the test drug Phenylalanine subsequently

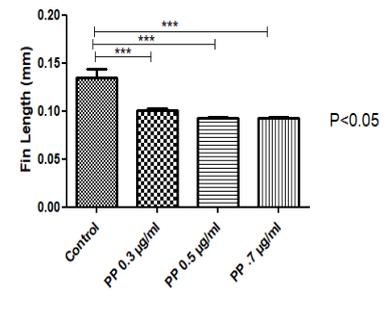
Developmental toxicity analysis by Phenylpyruvate (PP) at 3 different concentration on *Danio rerio* embryo Fin Length, DAY-8



Developmental toxicity analysis by Phenylpyruvate (PP) at 3 different concentration on *Danio rerio* embryo Fin Length, DAY-9

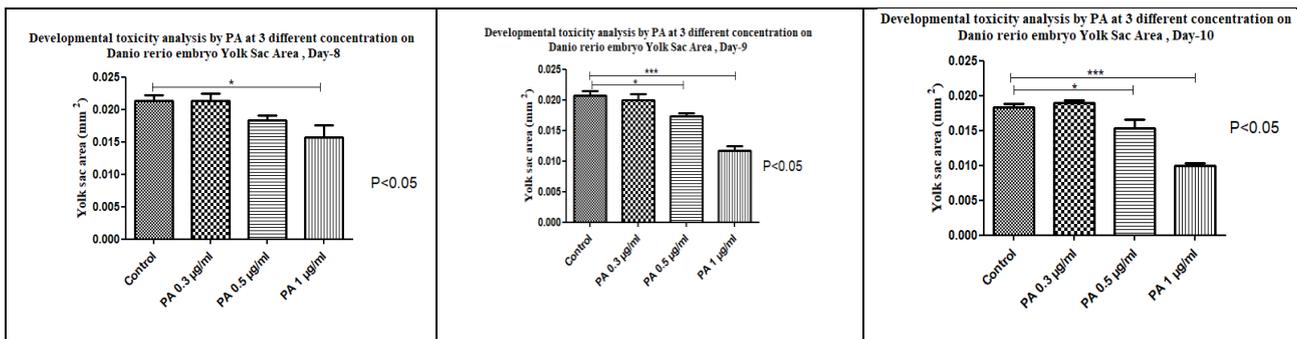


Developmental toxicity analysis by Phenylpyruvate (PP) at 3 different concentration on *Danio rerio* embryo Fin Length, DAY-10



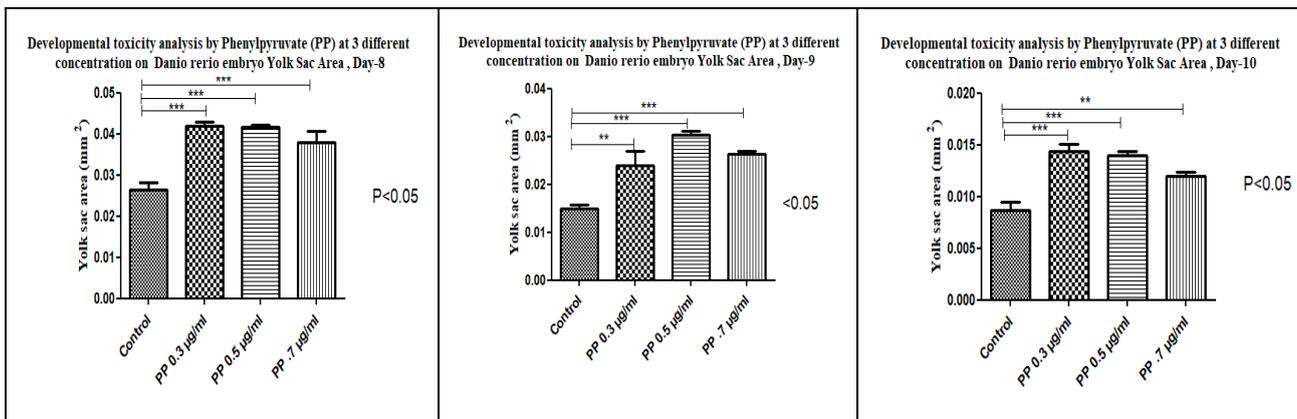
Fin Length (mm)			
DAY	0.3µg/ml	0.5µg/ml	0.7µg/ml
Day-1	ns	*	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	**	*	ns
Day-6	ns	ns	***
Day-7	ns	ns	***
Day-8	***	***	ns
Day-9	***	***	***
Day-10	***	***	***

Fig: 08 After performing the developmental toxicity experiment considering the parameter i.e., “Fin Length” on *Daneorerio* , it has been observed that at initial days there is no such deformity observed thus the level of significance is very less but later on specifically from day 6 & 7 , at highest concentration (0.7µg/ml) of test drug i.e.,PP there is a significance difference observed. After that on day 8 & day 9,day 10 significant difference observed on all 3 concentration (0.3 µg/ml, 0.5µg/ml & 0.7µg/ml on test drugs



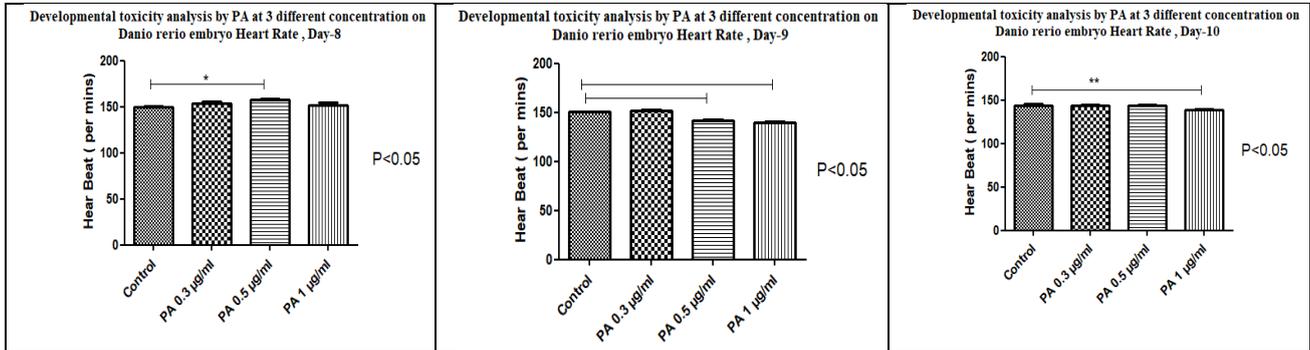
Yolk Sac Area (mm ²)			
Day	0.3 µg/ml	0.5 µg/ml	1 µg/ml
Day-1	ns	ns	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	ns
Day-6	ns	ns	**
Day-7	ns	ns	ns
Day-8	ns	ns	*
Day-9	ns	*	***
Day-10	ns	*	***

Fig: 09 After conducting the development toxicity experiment toxicity experiment focusing on the parameter “YOLKSAC AREA” in *Danio rerio* it was noted that initially, no deformities were observed, indicating a low level of significance. However, from the day 6 moderate significant difference was observed at the higher concentration (1µg/ml). At the day 8 onwards, slightly significant difference was observed at the higher concentration (1µg/ml). At the day 9 onwards at 0.5 µg/ml concentration slightly significant difference observed and higher concentration (1µg/ml) highly significant difference observed. At the day 10 onwards at 0.5 µg/ml concentration slightly significant difference observed and at the higher concentration (1 µg/ml) highly significant difference observed of the test drug Phenylalanine subsequently.



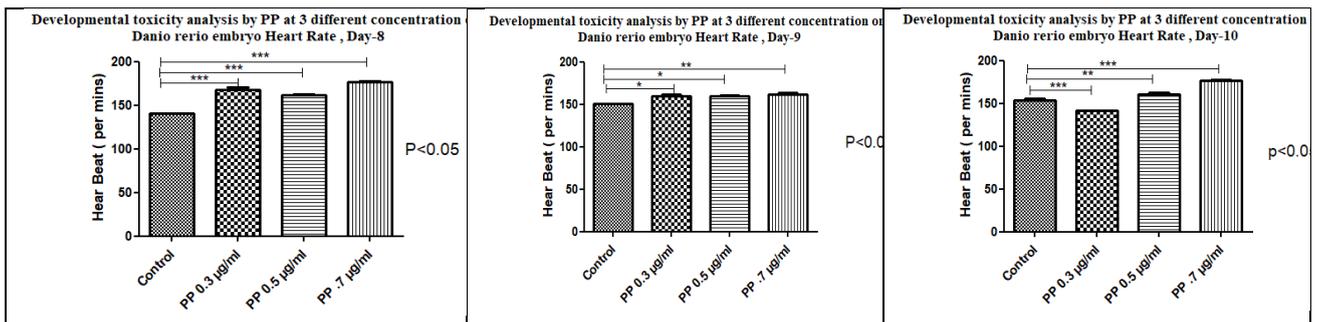
Yolk Sac Area			
Day	0.3 µg/ml	0.5 µg/ml	0.7 µg/ml
Day 1	ns	ns	ns
Day 2	ns	ns	**
Day 3	ns	ns	*
Day 4	**	***	ns
Day 5	***	**	ns
Day 6	***	***	***
Day 7	***	***	***
Day 8	***	***	***
Day 9	**	***	***
Day 10	***	***	**

Fig: 10 After performing the developmental toxicity experiment considering the parameter i.e., “Yolk Sac Area” on *Danio rerio*, it has been observed that at initial days there is no such deformity observed thus the level of significance is very less but later on specifically from day 4 & 5, at concentration 0.3 µg/ml & 0.5 µg/ml of test drug i.e., PP there is a significance difference observed. Finally on day 6, day 7, day 8, day 9 & day 10, significant difference observed in all 3 concentration (0.3 µg/ml, 0.5 µg/ml & 0.7 µg/ml on test drugs



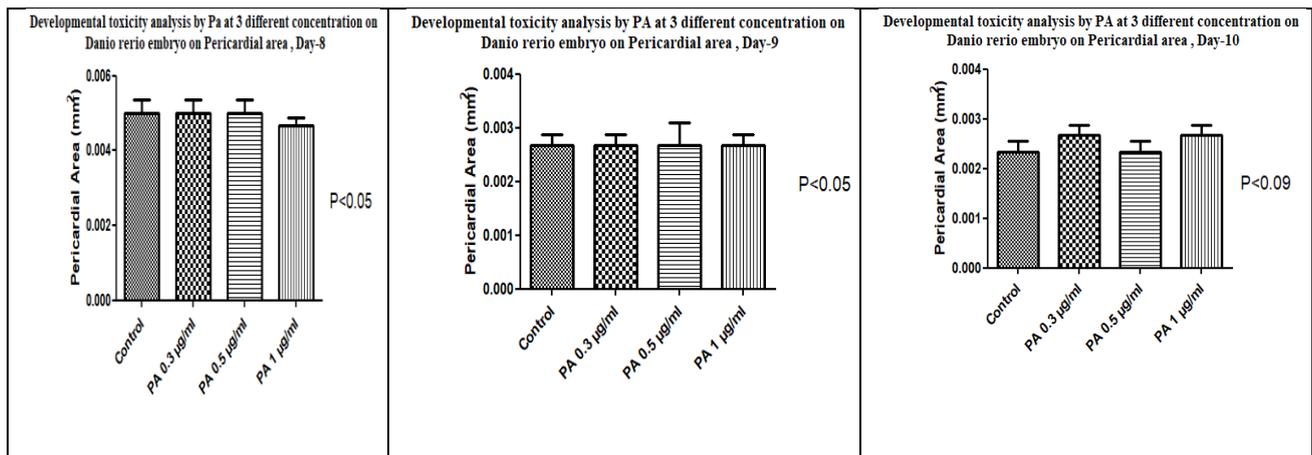
Heart Rate_PA (per min)			
DAY	0.3µg/ml	0.5µg/ml	1 µg/ml
Day-1	ns	ns	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	ns
Day-6	ns	ns	ns
Day-7	ns	ns	*
Day-8	ns	*	ns
Day-9	ns	***	***
Day-10	ns	ns	**

Fig: 11 After conducting the development toxicity experiment focusing on the parameter “HEART BEAT” in *Danio rerio* it was noted that initially, no deformities were observed, indicating a low level of significance. However, from the day 7 slightly significant difference was observed at the higher concentration (1µg/ml). At the day 8 onwards, slightly significant difference was observed at 0.5 µg/ml concentration. At the day 9 onwards at 0.5 µg/ml concentration highly significant difference observed and higher concentration (1µg/ml) also highly significant difference observed. At the day 10 onwards at the higher concentration (1 µg/ml) highly significant difference observed of the test drug Phenylalanine subsequently.



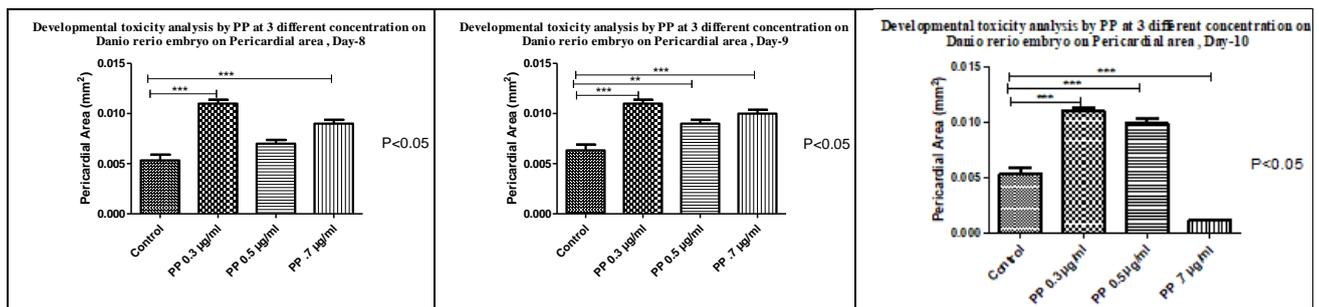
Heart Rate_PP (per min)			
DAY	0.3µg/ml	0.5µg/ml	0.7µg/ml
Day-1	ns	**	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	ns
Day-6	ns	ns	ns
Day-7	ns	*	*
Day-8	***	***	***
Day-9	*	*	**
Day-10	***	**	***

Fig: 12 After performing the developmental toxicity experiment considering the parameter i.e., “Heartbeat” on *Danio rerio*, it has been observed that at initial days there is no such deformity observed thus the level of significance is very less but later on specifically from day 7 at concentration 0.5 µg/ml & 0.7 µg/ml of test drug i.e., PP there is a significance difference observed. Finally on day 9 & day 10, significant difference observed in all 3 concentration (0.3 µg/ml, 0.5 µg/ml & 0.7 µg/ml on test drugs



Pericardial Area_PA (mm ²)			
DAY	0.3µg/ml	0.5µg/ml	0.7µg/ml
Day-1	ns	ns	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	ns
Day-6	ns	ns	ns
Day-7	ns	ns	ns
Day-8	ns	ns	ns
Day-9	ns	ns	ns
Day-10	ns	ns	ns

Fig: 13 After conducting the development toxicity experiment focusing on the parameter “Pericardial Area” in *Danio rerio*, it was noted that initially no deformities were observed, from Day 1 (72 hpf) to Day 10 (288 hpf).



Pericardial Area_PP (mm ²)			
DAY	0.3µg/ml	0.5µg/ml	0.7µg/ml
Day-1	ns	ns	ns
Day-2	ns	ns	ns
Day-3	ns	ns	ns
Day-4	ns	ns	ns
Day-5	ns	ns	ns
Day-6	**	ns	**
Day-7	***	ns	***
Day-8	***	ns	***
Day-9	***	**	***
Day-10	***	***	***

Fig : 14 After performing the developmental toxicity experiment considering the parameter i.e., “Pericardial Area” on *Danio rerio*, it has been observed that at initial days there is no such deformity observed thus the level of significance is very less but later on specifically from day 6, 7,8, at concentration 0.3 µg/ml & 0.7µg/ml of test drug i.e.,PP there is a significance difference observed. Finally on day 9 & day 10, significant difference observed in all 3 concentration (0.3 µg/ml, 0.5µg/ml & 0.7µg/ml on test drugs).

Physiological disruptions were also evident. **Heartbeat**, which maintained a steady 150–160 beats per minute in low-dose and control groups, fell sharply to around 138 beats/minute under maximum PA and PP exposure by day 10. Similarly, **Total Body Length** and **Pericardial Area** measurements showed contraction at high doses: the total body length, which should reach about 1.17 mm, was reduced at the highest concentrations. Notably, **Spinal Axis Angle** (used to detect skeletal deformations) was unchanged in controls and lower doses, but deviated significantly for PA and especially for PP at high concentrations—pointing towards a dose-dependent risk of malformations as exposure increases.

CONCLUSION:

The present study demonstrates that both phenylalanine (PA) and its metabolic derivative, sodium phenylpyruvate (PP), exert concentration- and time-dependent developmental toxicity in zebrafish embryos. Through continuous monitoring of morphological and physiological endpoints, a progressive decline in LC50 values was noted with increasing exposure duration, emphasizing cumulative toxicity. The reduction of LC50 for PA from 1949 µg/mL at 24 hours to 92.68 µg/mL at 120 hours, and for PP from 224.9 µg/mL to 79.79 µg/mL over the same period, underscores a pronounced toxic potential, particularly for PP. Morphometric parameters reinforced these findings, showing marked alterations in organ development. Notable reductions in eye diameter, fin length, and yolk sac area were observed in a dose-dependent manner, accompanied by disrupted head morphology. These changes indicate that elevated concentrations of PA and PP can interfere with tissue differentiation and nutrient utilization during embryogenesis. The decline in yolk sac area suggests impaired nutrient mobilization, while variations in head dimensions indicate altered craniofacial morphogenesis.

Physiological parameters such as heartbeat rate, total body length, and pericardial area revealed further evidence of functional impairment. The significant decrease in cardiac rhythm from 150–160 bpm in normal and low-dose groups to around 138 bpm at peak exposure indicates cardiac stress and developmental arrest. Additionally, the increased spinal axis deviation at higher doses signifies a direct teratogenic effect, with PP producing more severe skeletal anomalies than PA.

Overall, these findings reveal that both PA and PP interfere with the normal growth and organogenesis of zebrafish embryos, with sodium phenylpyruvate displaying substantially higher embryotoxicity. This highlights potential risks associated with excessive intake of phenylalanine or its precursors, particularly through aspartame-containing products and unregulated dietary supplements. Therefore, controlled consumption and stringent monitoring of such compounds are imperative to prevent metabolic and developmental toxicity.

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