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Research Article

Assessment of Behavioural Patterns and Liver Histopathology in Zebrafish *(Dania rerio)* Exposed to *Viruddha Kshira Samyogas* – An Experimental Study

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Abstract:

Introduction: *Viruddhahara* explained in Ayurvedic classics holds significant influence in health and behaviour. This study aims to bridge the gap between traditional Ayurvedic wisdom and contemporary scientific investigation by examining the behavioural patterns and liver histopathology of zebrafish exposed to *Viruddha Kshira samyogas*

Materials and Methods: For this Experimental study, zebrafish *(Dania rerio)* were subjected to different *Viruddha Kshira* combinations, *kshira* alone, and standard fish feed for a period of 45 days.

Significance and Justification for using Zebra Fish as an Animal model

Zebra fishes are tropical fresh-water fish in the minnow family. The name "Zebra fish" comes from the horizontal blue stripes on either side of their bodies[1]. Although humans may appear to be extremely different than zebra fish, it has similar genetic structure to humans as they share 70 percent of genes with us. Further 84 percent of genes known to be associated with human disease have a zebrafish counterpart.[2] As a vertebrate, the zebrafish has the same major organs and tissues as humans. The twin attributes of zebrafish as a model system are the ability to apply efficient invertebrate-style genetics to vertebrate-specific questions, and the optical clarity of embryos and larvae, which allow easy visualization of developmental processes.[3]

The other advantages of zebrafish over classical vertebrate models are high fecundity, external fertilization, ease of genetic manipulation, and transparency through early adulthood that enables powerful imaging modalities.

Justification for comparing the phenomenon of digestion between zebrafish and Humans:

The gastrointestinal system of zebra fish is highly homologous to that of mammals, containing a liver, pancreas, gall bladder, and a linearly segmented intestinal track with absorptive and secretory functions. The intestinal epithelium displays proximal-distal functional specification and contains many of same epithelial cell lineages found in mammals including absorptive enterocytes, goblet cells, and enteroendocrine cells. Enterocytes have a basolateral nucleus and form tight junctions, apical microvilli, and an intestinal brush border.

Results and Discussion: The findings revealed that all groups fed with *Viruddha Kshira* combinations experienced abnormal eating patterns and swimming behaviours compared to the control group fed with fish feed. Notably, the group fed with *kshira* and *Matsya* exhibited the most severe outcomes, with mortality observed in all fishes followed by the group fed with *kshira* and *lavana.* The observed alterations in weight further emphasized the influence of dietary factors on zebrafish growth and health, with *Viruddha Kshira* combinations leading to weight loss. Furthermore, histopathological analysis indicated varying degrees of liver pathology among the groups, with the combination of *kshira* and *lavana* showing the most severe impact on liver health.

Conclusion: This study highlights the multifaceted impact of *viruddha kshira samyogas* on zebrafish behaviour, health, and liver histopathology which evidently implies the hazards of consuming *viruddha aahara* combinations in humans in this fast-moving fashion world who consider these frequently used food combinations as immaterial and indifferent.

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INTRODUCTION:

Nutritional practices play a significant role in shaping the health and behaviour of human beings which is recognised across various cultures and traditions. In *Ayurveda, Aahara* is considered paramount for sustaining life. Dietary choices and overall nutritional intake play pivotal role in health, affecting oxidative stress, genetic expression, and cellular health. Optimal health is achieved through a balanced diet along with healthy eating habits. The *Ahara vidhi* outlined in *Ayurveda* not only includes the different food items, their compatibility and inherent qualities of the food items, but also the etiquettes and practices to be followed during food intake. The concept of *Viruddhahara,* or incompatible food combinations is deemed crucial in Ayurvedic classics. It is highly relevant even during modern times as it disrupts the homeostasis of *doshas*, potentially leading to adverse health effects. Among the various *Viruddha* combinations, *Viruddha Kshira samyogas* have garnered attention for their purported impact on health. Despite its historical significance, scientific exploration into the effects of *viruddhahara* remain sparse. The impact of *viruddhahara* on the complex biological systems remain largely unexplored.

In recent years, zebrafish (*Danio rerio*) have emerged as a valuable model organism for studying various physiological and behavioural responses to environmental stimuli due to their genetic similarity to humans[4]. The remarkable adaptability and complex behavioural repertoire exhibited by zebra fish, make them suitable models for researching the effects of diet on behaviour. Behavioural studies in zebrafish typically encompass a diverse array of activities, including feeding, swimming, social interactions, aggression, mating, and responses to environmental stimuli[5,6,7]. Changes in the behavioural patterns of zebra fish when exposed to various factors provides insights into the effects of environmental factors, genetic variations, and dietary interventions.

This experimental study aims to bridge the gap between traditional Ayurvedic wisdom and contemporary scientific investigation by examining the behavioural patterns and liver histopathology of zebrafish exposed to *Viruddha Kshira samyogas*. As liver plays a vital role in metabolism, and maintaining homeostasis within the body, it is particularly susceptible to the effects of dietary factors[8]. Understanding the impact of *Viruddha Kshira* combinations on zebrafish behaviour and the influence on liver histopathology offer valuable insights into the broader implications of *viruddhahara* on human health.

Materials and Methods:

Preparation of *Viruddha Kshira* Combinations:

Combinations of *kshira* with *Lavana, Kadali, maasha, matsya* and *dadima* was selected for the study. *Viruddha Kshira* combinations were prepared by mixing equal proportions of milk with each ingredient *(lavana, kadali, maasha, matsya* and *dadima)* separately. Pellets of *Kshira* were also prepared.

Zebrafish Husbandry:

12 weeks old zebrafish (*Danio rerio*) were acquired and acclimated to laboratory conditions. Zebrafish were housed in standard laboratory tanks with temperature maintained between 28°C and pH between 7.0-7.5. Appropriate filtration systems were fitted to the tanks to maintain water quality. Fish were kept under a 14:10 light-dark cycle for 2 weeks.

Experimental Design:

Tanks were divided into groups based on the type of diet fed i.e. different *Viruddha Kshira samyogas, kshira* alone, and standard fish feed (control). Zebrafish were randomly assigned to experimental tanks with male and female in the ratio 2:1. These groups were fed with different *viruddha* combinations, *kshira* alone and fish feed for a period of 3 months.

Behavioural Assay:

Behavioural patterns, including eating, swimming, and social behaviour were observed twice a day for a period of 45 days. Eating behaviour was assessed by quantifying the amount of food consumed and the feeding frequency. Fast eating was considered the normal eating behaviour in zebra fishes. Slow eating or not eating is considered abnormal. Swimming behaviour was evaluated based on parameters such as hyperactive swimming characterised by excessively fast and frenetic movements, hypoactive swimming displayed by reduced activity or sluggish movements, lateral swimming, cross swimming, erratic or uncoordinated swimming, vertical swimming, bouncing or bobbing, swimming at the bottom of the tank and spiral swimming. Free swimming, schooling, thigmotaxis, occasional circling and turning are considered normal swimming pattern exhibited by zebra fish. Immobility or paralysis was also noted. Social behaviour was monitored to assess changes in social dynamics within experimental groups. Shoaling, group cohesions are normal behaviour of zebrafish while social withdrawal exhibited by isolation, escalated aggressive interactions such as prolonged chasing, fin nipping etc are considered abnormal. The weight of the fishes was measured before and after the experiment and a comparison was made.

Procedure of Histopathological Examination:

Zebrafish liver samples were collected from fishes fed with each *Viruddha Kshira* combinations (*kshira* with *lavana, kadali, maasha, matsya,* and *dadima*), group fed with *kshira* alone and control group fed with standard fish feed. Samples were collected following 45 days exposure period. Fixative solution

was prepared by combining 3 ml of 100% Ethanol, 1000 µl of 10% formalin and 200 µl of Acetic acid. Zebrafish liver samples were immersed in the Dietrich's fixative solution immediately after collection. Following fixation, samples were dehydrated through a series of graded ethanol solutions. Dehydrated samples were cleared using xylene to remove any remaining water and render tissues transparent. Subsequently the cleared samples were infiltrated and embedded in paraffin wax. Embedded samples were sectioned and the sections were mounted onto glass slides and allowed to dry completely. Prepared slides were subjected to Haematoxylin and Eosin (H&E) staining. Stained slides were examined under a light microscope at various magnifications to assess histopathological changes in zebrafish liver tissue.

Observations:

Behavioural Assay:

a) Eating Behaviour: (Chart No.1)

In the group fed with fish feed, all the fishes (both male and female) had normal eating with no slow eating episodes. In the group given *kshira* alone, the mean observation with fast eating was 36.25 days out of 45 days and on 8 days slow eating was observed.

In the group fed with *kshira* and *lavana*, 3 male fishes were found dead on 27th day, 30th day and 32nd day. Both male and female fishes exhibited 62% instances of slow eating, respectively.

In the cohort provided with *Kshira* and *Kadali,* one male fish coagulated on the 40th day, with only one fish refraining from eating on that particular day, aligning with the observed coagulation. Slow eating was noted on 15 days of observation out of 45 days in male fishes. Among female fishes fed with *Kshira* and *Kadali*, slow eating was observed on 21 days.

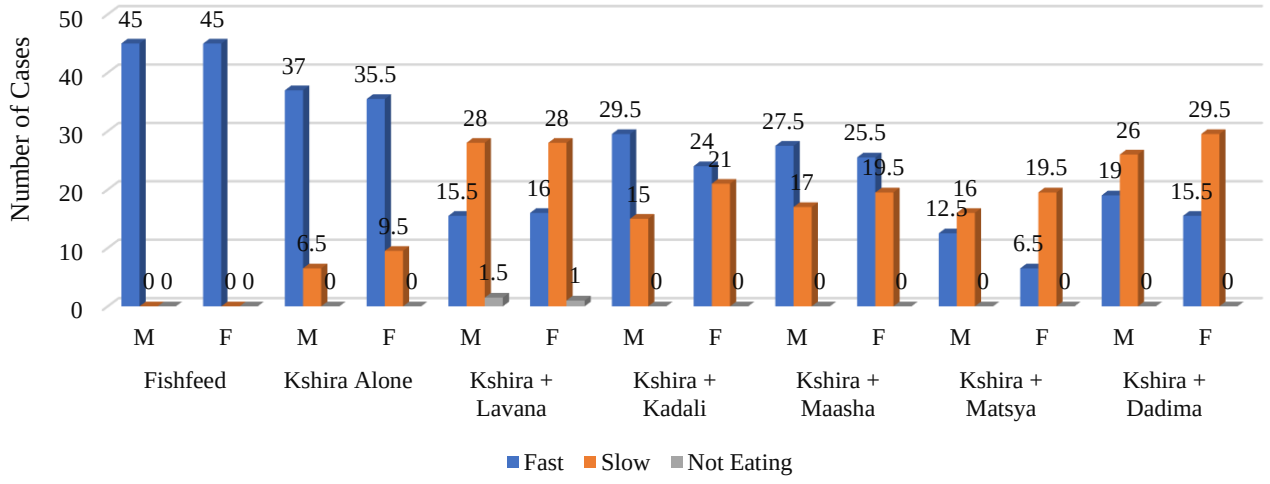
In the group given *kshira* and *maasha*, slow eating was observed on 18.25 days and normal fast eating on remaining days.

In the group fed with *kshira* with *matsya,* 1 fish was found dead on 17th day while all the male fishes coagulated by 30th day. Male fish exhibited cannibalism. In case of female fishes, all fishes coagulated on 27th day. Slow eating was observed frequently in both male and female fishes with 86% slow eating episodes before all fishes coagulated.

Within the group fed with *Kshira* and *Dadima*, no mortalities were recorded. However, 26 and 29.5 instances of slow eating were observed in male and female fishes, respectively.

In terms of eating behaviour, only the group fed with fish feed exhibited normal behaviour, while all other groups displayed abnormal behaviours. The abnormal eating pattern in the group fed with *kshira* alone was significantly less when compared to the groups fed with *viruddha kshira samyogas*. Among the *Viruddha* combination groups, the one fed with *Kshira* and *matsya* showed the most severe outcomes, with mortality observed in all fishes in both male and female groups. Following this, the group fed with *Kshira* and *Lavana* exhibited adverse effects. Instances of slow eating or not eating coincided with the coagulation or mortality of the fishes.

Chart No.1: Eating Pattern of Adult Zebra Fishes Exposed to *Viruddha Kshira Samyogas*



Swimming Behaviour: (Chart No.2)

In the group fed with fish feed, all observations of swimming pattern were normal behaviour. In the group fed with *kshira* pellets alone, except for 2 instances of slightly aggressive behaviour in male fish and 1 instance of aggressive behaviour in female fish, rest all observations were normal. In the group fed with *kshira* and *lavana*, the fishes exhibited abnormal swimming patterns. Among the abnormal swimming behaviour, cross swimming and swimming at the bottom were exhibited frequently accounting for 10 and 13 instances respectively in male fishes and 15 and 14 instances in female fishes. Followed by which, chasing (6 instances in male, 3 instances in female) and lateral swimming (4 instances in male and 2 instances in female) was also noted. Aggressive behaviour was noted in 4 and 3 observations in male and female fishes while slight aggressive swimming was noted in 1 and 2 instances among

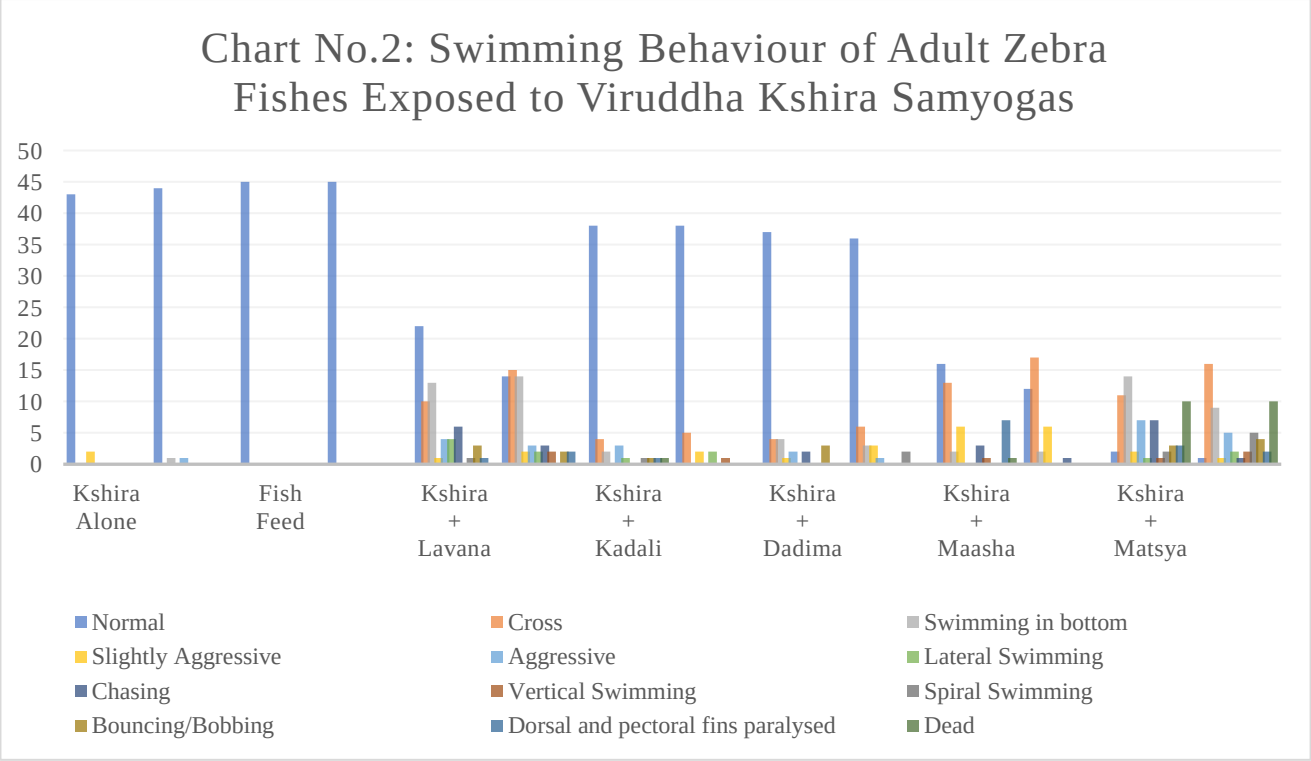
male and female fishes fed with *kshira* and *lavana.* Vertical swimming was noted only in female fishes fed with *kshira* and *lavana* while spiral swimming was observed in male fishes only. Bouncing and bobbing was exhibited by fishes fed with *Kshira* and *lavana* (3 instances in male, 2 instances in female). Dorsal and pectoral fin paralysis was observed in 1 male fish and 2 female fishes fed with *kshira* and *lavana.*

In the group fed with *kshira* and *kadali,* both male and female fishes exhibited 38 instances of normal swimming behaviour on observation. Cross swimming was the most common abnormal pattern exhibited by fishes fed with *kshira* and *kadali* showing (4 in male, 5 instances in female fishes). 2 male fishes were found swimming at the bottom and 3 instances of aggressive swimming and 1 instance of lateral swimming was noted in male fishes. Female fishes exhibited lateral swimming and vertical swimming in 2 and 1 instance respectively. Male fishes exhibited bouncing and spiral swimming. 1 male fish fed with *kshira* and *kadali* was found dead. Dorsal and pectoral fin paralysis was observed in that.

In the group fed with *kshira* and *maasha,* normal swimming behaviour was exhibited by both male and female fishes in 16 and 12 instances respectively. Similar to other groups, cross swimming was the most common pattern observed (13 Male, 17 Female) followed by aggressive swimming (6 instances in each male and female). Male fishes were found to chase other fishes in 3 instances of observation. Swimming at the bottom was observed both in male and female fishes in 2 instances. Dorsal and pectoral fin paralysis was observed in 4 male fishes fed with *kshira* and *maasha*. 1 male fish was dead in the group.

The group fed with *kshira* and *matsya* exhibited the most abnormal swimming pattern and all the male and female fishes coagulated in this group. Cross swimming and swimming at the bottom were commonly observed with 11 and 14 instances in male fishes and 16 and 9 fishes in female. Aggressive swimming was exhibited by both male and female fishes fed with *kshira* and *matsya* (7, 5 instances respectively). Male fishes exhibited chasing frequently (7 instances) while only one instance of chasing was observed in female fishes. Spiral swimming (2,5 instances), Bouncing (3,4 instances), vertical swimming (1,2 instances) was other abnormal swimming behaviour exhibited by male and female fishes fed with *kshira* and *matsya*. 3 male fishes had dorsal and pectoral fin paralysis while 2 female fishes had paralysis.

In the group fed with *kshira* and *dadima*, normal swimming was observed in 37 and 36 instances in male and female fishes respectively. Cross swimming and swimming at the bottom were observed in 4 instances each in male fishes and 6 and 3 instances respectively in female fishes. Aggressive swimming was observed in male fishes compared to female fishes which we found to chase other fishes also. Spiral swimming was observed in female fishes while bouncing was seen in male fishes.



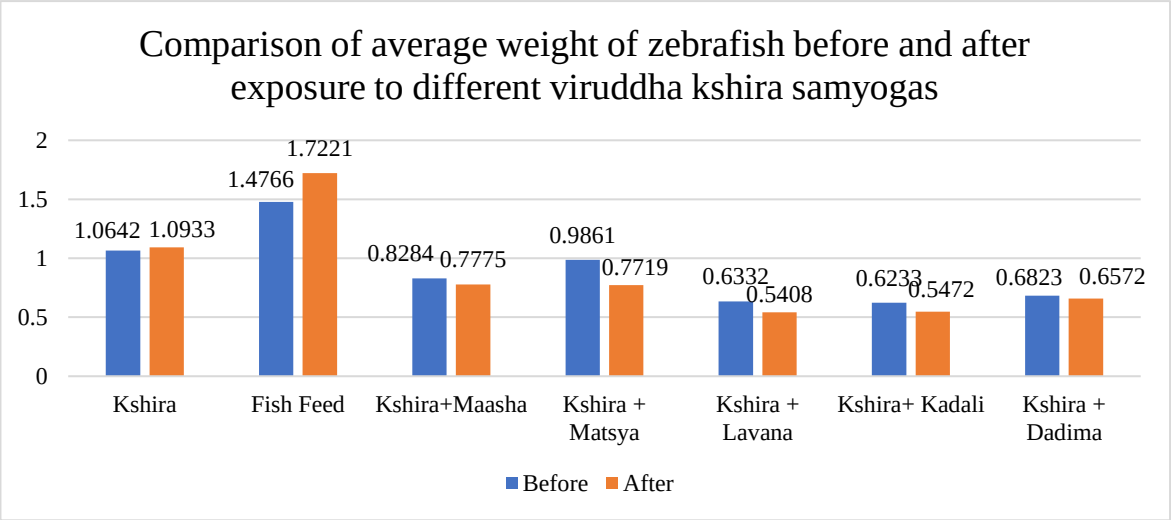
Social Behaviour:

The social behaviour in the groups fed with fish feed and *kshira* alone doesn't show any abnormal social behaviour. In the groups fed with different *viruddha kshira samyogas*, few episodes of aggressive behaviour were noted. Prolonged chasing was observed in the groups fed with *kshira* and *lavana,* *kshira* and *matsya* and *kshira* and *maasha*. Social isolation was seen in fishes in the group fed with *kshira* and *dadima.*

BodyWeight:

The average weight of zebrafish was compared before and after exposure to different *viruddha samyogas*. In the group fed with

fish feed, average weight was 1.47g and there was 14.25% increase in the body weight after experimentation. In the group fed with *kshira* alone, the average body weight increased from 1.0642 to 1.0933 with 2.66% gain. Loss in average body weight was observed in all groups fed with *viruddha kshira samyogas* (Chart. No.3). The group *kshira* with *matsya* had highest loss in average body weight (27.75%) followed by *kshira* with *lavana* (17.09%). The loss in body weight in the groups fed with *kshira* and *kadali, kshira* with *maasha* and *kshira* with *dadima* are 13.91%, 6.55% and 3.81%.



Histopathological Examination:

Histopathologic examination of liver cells of zebrafishes fed with normal fish feed showed no abnormality (Fig.No.1). Congestion and mild multifocal hepatocellular degeneration were observed in histopathological examination of liver cells of zebra fishes fed with *kshira* alone (Fig.No.2).

Liver cells of zebrafishes fed with *kshira* with *lavana* (Fig.No.3) had worst histopathologic appearance with necrotic cells and moderate multifocal vacuolar degeneration of hepatocytes followed by the liver cells of zebrafishes fed with *kshira* with *dadima* (Fig.No.4) with few necrotic cells and mild multifocal degeneration of hepatocytes. In *kshira* with *kadali* (Fig.No.5) and *kshira* with *maasha* (Fig.No.6) groups, congestion and mild to moderate multifocal vacuolar degeneration of hepatocytes was noted. Mild hepatocellular degeneration was observed in histopathological examination of liver cells of zebrafishes fed *kshira* with *Matsya* (Fig.No.7).

Fig.No.1: Normal histopathological study of liver cells of zebrafish fed with normal fish food.

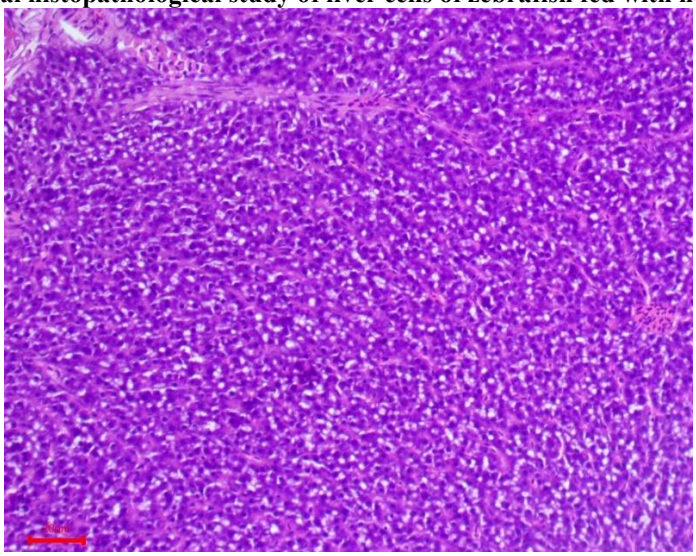


Fig.No.2: Histopathological changes seen in zebra fish liver cells treated with *kshira* alone showing congestion, mildly degenerated hepatocytes

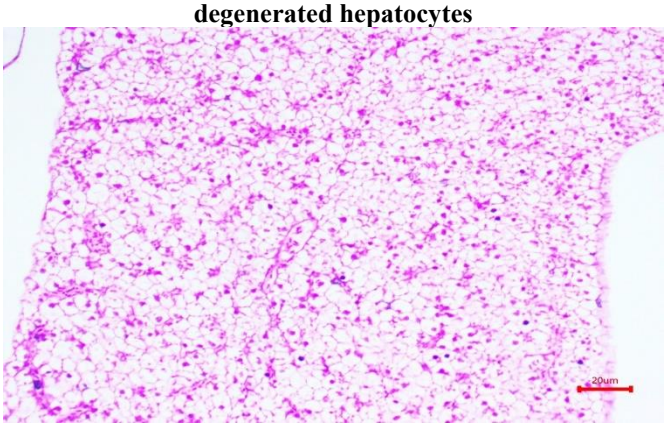


Fig.No.3. Histopathological changes seen in zebra fish liver cells treated with *kshira* and *lavana* showing necrotic cells and moderate multifocal vacuolar degeneration

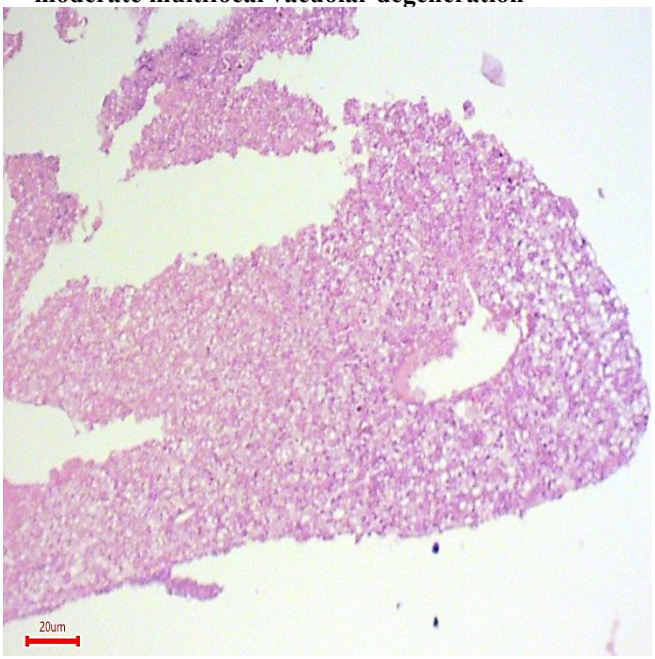


Fig.No.4: Histopathological changes seen in zebra fish liver cells treated with *kshira* and *dadima* showing few necrotic cells and mild multifocal degeneration of liver cells.

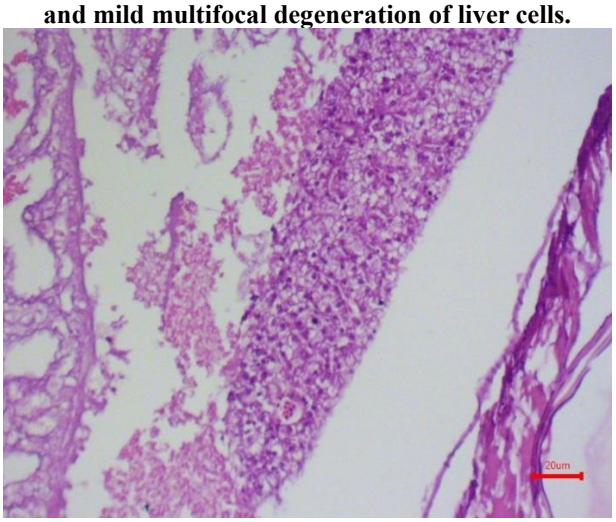


Fig.No.5: Histopathological changes seen in zebra fish liver cells treated with *kshira* and *kadali* showing congestion and mild to moderate multifocal vacuolar degeneration of hepatocytes

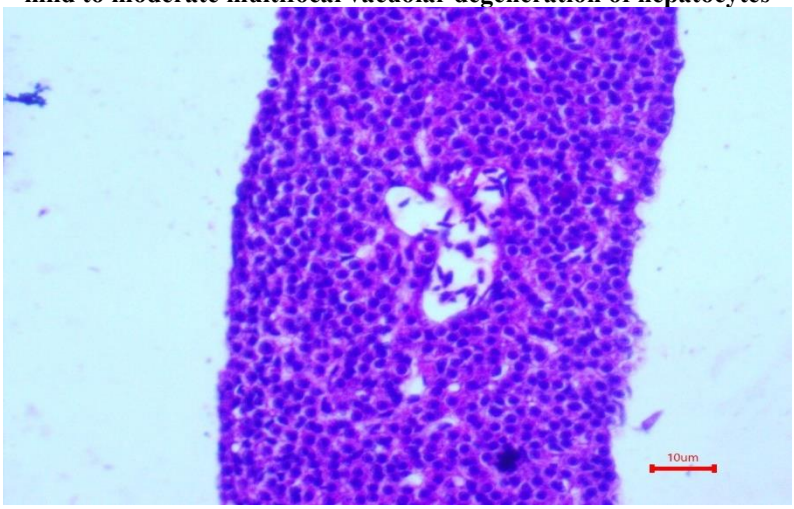


Fig.No.6: Histopathological changes seen in zebra fish liver cells treated with *kshira* and *maasha* showing congestion and mild to moderate multifocal vacuolar degeneration of hepatocytes

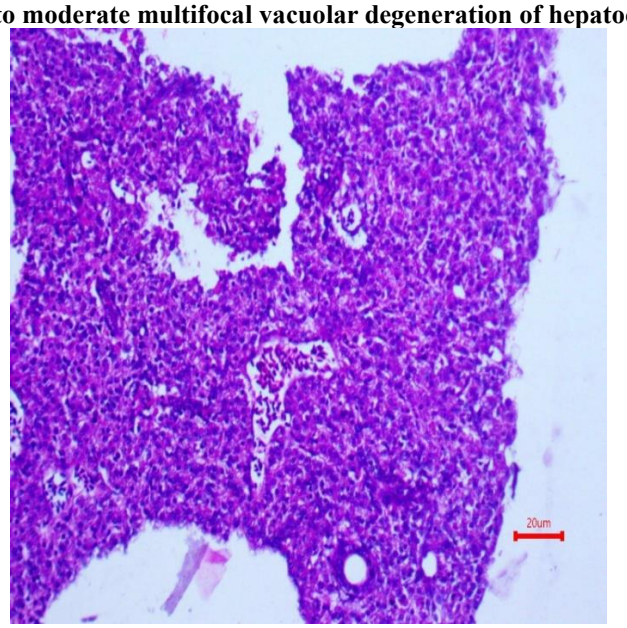
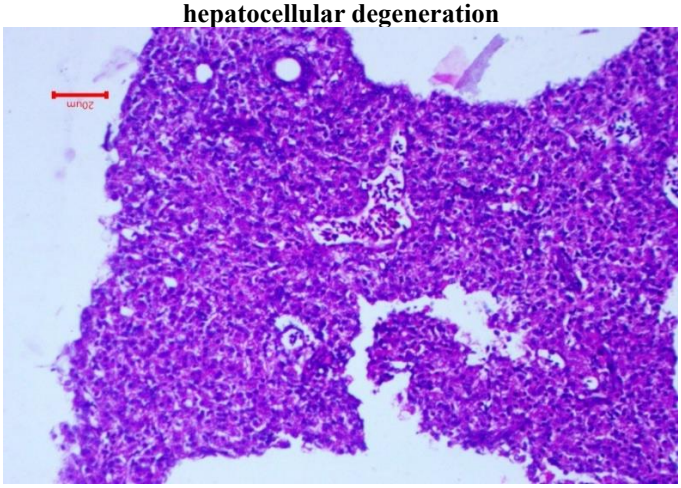


Fig.No.7: Histopathological changes seen in zebra fish liver cells treated with *kshira* and *matsya* showing mild hepatocellular degeneration



Discussion:

Zebrafish is increasingly used in behavioural assays to understand the toxicity of drugs. A wide range of behaviour assays testing simple reflexes, pavlovian techniques, visual discrimination, fear conditioning, spatial orientation which includes both appetitive and aversive techniques were conducted to study stress response in zebrafish[9,10,11.] The direct interference of neurotransmission, changes in intracellular signalling, disruption of the musculoskeletal system, and altered growth and development can be analysed by observing behavioural changes. Oliveria et al in their study observed adult zebra fishes as social animals which exhibits shoaling and schooling[12]. Bailey et al analysed the functional homology between zebrafish and mammalian response to drugs[13]. Jones L.J. et al in their study observed conspecific directed aggression in adult zebrafishes[14]. They observed the visually driven tendency to stay in the chamber that provides visual access to conspecifics develops by 3 weeks old fishes which is in concordance with the development of neural systems. Matteo zarantoniello et al studied the physiological and behavioural response of zebra fish to insect-based diet[15]. Reif D et al opined that studying behavioural patterns of zebrafish can give insights into possible functional outcomes of chemical toxicity, drug efficacy, probable mode of action etc[16].

In the present study, the behavioural pattern observed in zebrafishes fed with different *viruddha kshira* combinations gave insights into possible functional outcome of *viruddha kshira samyogas.* Both male and female fishes were fed with each combination of *viruddha, kshira* alone and fish feed.

With regard to eating behaviour, normally Zebrafish exhibit a diurnal feeding pattern characterized by regular feeding episodes throughout the day. They are opportunistic feeders, actively foraging for food particles suspended in the water column or present on the substrate. Zebrafish often exhibit probing movements like mouth flicks and use a combination of visual and chemical cues to locate food. Abnormal eating behaviour in zebrafish can manifest in various forms. Overeating, undereating, erratic eating behaviours are exhibited by zebra fish when exposed to environmental stress. Chemical toxicity may interfere with sensory perception or may disrupt metabolic processes, resulting in abnormal feeding patterns. Eating behaviour alterations may occur as a result of pharmacological interactions or physiological responses to the administered substances.

Zebrafish exhibits decreased feeding activity, disruption in normal feeding pattern due to stress induced changes in circadian rhythms or hormonal regulation[17,18]. Hyperactive feeding responses, competition for food resources is observed in stress induced aggression. De marco et al observed temporary suppression of feeding behaviour as response to stressors[19]. Slow eating episodes observed in fishes fed with different *viruddha samyogas* of *kshira* can be attributed to stress. Stress responses in zebrafish are mediated by complex neuroendocrine pathways involving the release of stress hormones and dysregulation of neuropeptide signalling pathways[20,21]. Stress induced alterations in sensory perception of zebrafish, immunomodulation and inflammatory responses may impair food recognition and negatively impact feeding behaviour[22].

The findings of the present study highlight the significant impact of *viruddha kshira samyogas* on the feeding behavior of zebrafish. The group fed with normal fish feed demonstrated normal eating behaviour, indicating that it is appropriate and well-tolerated diet for zebrafish. In contrast, groups fed with various *viruddha* combinations of *kshira (Lavana, Kadali, Maasha, Matsya, Dadima)* exhibited abnormal eating patterns. The group of zebrafishes fed with *kshira* alone showed relatively less abnormal eating episodes compared to groups fed with *viruddha kshira samyogas*. Among the groups of zebrafishes fed with different *viruddha samyogas*, the one fed with *kshira* and *Matsya* exhibited the most severe outcomes, with mortality observed in all fishes in both male and female groups. Also, the male fishes exhibited cannibalism and coagulation of both male and female fishes in the group suggest potential toxic effects of kshira with matsya combination. Cannibalism among male zebrafish fed with *kshira* and *matsya* suggests heightened aggression or territorial behavior, possibly exacerbated by nutritional deficiencies or aversive reactions to the *kshira* and *matsya* combination. Similarly, the group fed with *kshira* and *lavana* exhibited adverse effects, with mortality observed in male fishes. This indicates a significant detrimental effect on the survival of zebrafish in this group. Slow eating episodes were frequent (62%) in both male and female fishes fed with *kshira* and *lavana*, suggesting the combination of *kshira* and *lavana* is not well tolerated by zebrafish. The presence of *lavana* with *kshira* might have disrupted the osmoregulation leading to physiological stress resulting in adverse effects on metabolism and organ function.

In the group fed with *kshira* and *kadali*, one male fish coagulated on the 40th day, coinciding with a day when only one fish refrained from eating. The relatively high frequency of slow eating episodes indicates potential dietary aversion or digestive discomfort. High complex carbohydrates and fiber in *kadali* and the milk proteins together results in difficulty in digestion and hence reduced appetite and gastrointestinal disturbances. This is exhibited by zebra fishes as slow eating episodes. In the group fed with *kshira* and *maasha*, slow eating episodes were observed. The phytic acids and tannins in *maasha* may interact with milk protein and affect the digestion and absorption of nutrients.

The group fed with *kshira* and *dadima* exhibited instances of slow eating in both male and female fishes while there was no mortality in the group suggesting a milder negative impact on zebrafish health. Tannins present in *dadima* have been known to bind with milk proteins forming insoluble complexes which interferes with digestion of milk proteins. Interaction of tannins and milk protein may exaggerate the allergic reactions also. The interactions could also bring about dysbiosis in the gut[23]. The observed correlation between abnormal eating patterns and adverse outcomes underscores the importance of *ahara samyogas*.

Swimming behaviour:

In the current study, different *viruddha kshira samyogas* were evaluated for their effects on swimming patterns in zebrafish, with notable variations observed among the experimental groups. The group fed with fish feed exhibited normal swimming behaviour. This serves as a standard for comparison

with other experimental groups. The group of zebrafishes fed with *kshira* alone exhibited mostly normal swimming pattern except for few instances of slightly aggressive behaviour. This suggests that *kshira* alone may not significantly affect swimming behaviour in zebrafish, indicating a relatively benign impact on the overall health.

In the group fed with *kshira* and *lavana* combination, a range of abnormal swimming behaviours was observed including cross swimming, swimming at the bottom, chasing, lateral swimming, aggressive behaviour, vertical swimming, spiral swimming, bouncing, and dorsal and pectoral fin paralysis. Osmotic stress due to salt can cause physiological disturbances exhibited as swimming at the bottom, lateral swimming, or vertical swimming. Electrolyte imbalance also affects nerve function and muscle coordination. This explains the observed dorsal and pectoral fin paralysis in some fishes fed with *kshira* and *lavana*. Cross swimming, being the most common abnormal pattern can be attributed to potential disruption in their motor coordination or balance linked to neurological deficits or alterations in sensory perception caused by the diet. Swimming at the bottom suggests hypoactive behaviour or muscle weakness. Also changes in buoyancy or energy levels due to *viruddha* combination can result in bottom swimming.

In the group fed with *kshira* and *kadali,* both male and female zebrafish exhibited 38 instances of normal swimming behavior indicating that the diet did not severely impair their basic locomotor functions as compared to other *viruddha* groups. Cross swimming was the most common abnormal pattern observed. Instances of aggressive swimming in male fishes could be indicative of elevated stress levels. Alterations in hormone regulation or neurotransmitter activity can also bring about altered swimming behaviour in zebrafish. The occurrence of lateral swimming in female fishes and vertical swimming in one instance in the group fed with *kshira* and *kadali* suggests disturbances in their orientation or buoyancy control triggered by the *viruddha* combination. Neurological and muscular abnormalities induced by *kshira* and *kadali* combination could also have led to bouncing or spiral swimming. Dorsal and pectoral fin paralysis in a male fish is the most concerning finding indicating severe neuromuscular dysfunction which likely compromised the fish's ability to swim and maintain buoyancy.

In the group fed with *kshira* and *maasha*, there was increased instances of abnormal swimming pattern exhibited by zebrafishes of both genders. Cross swimming, swimming at bottom in few instances and chasing and aggressive swimming at another instances suggest stress induced by the *viruddha* combination. Dorsal and pectoral fin paralysis was observed in the group with mortality of a male fish implying potential neuromuscular deficits.

The group of zebrafishes fed with *kshira* and *matsya* displayed the most pronounced abnormal swimming patterns compared to other groups. Cross swimming and swimming at the bottom were commonly observed in both male and female fish, suggesting disruptions in motor coordination or buoyancy control. Increased aggressive and chasing behaviour exhibited by both male and female fishes suggest potential stress and social conflict among the fishes. Other observed abnormal swimming behaviours like spiral swimming, bouncing, and vertical swimming indicates disturbances in swimming coordination or neuromuscular function induced by *kshira* and *matsya* combination. Fin paralysis and mortality of all the fishes in the group suggests potential aggregation behaviour induced by the *viruddha* combination of *kshira* and *matsya.*

In the group fed with *kshira* and *dadima*, overall, the findings suggest that the *kshira* and *dadima viruddha* combination affected swimming behaviors differently between male and female zebrafish. While both genders exhibited normal swimming patterns, there were notable differences in the frequency and type of abnormal behaviours observed. Aggressive swimming behaviour was noted especially in male fishes compared to female fishes suggesting hyperactivity.

Wayne Barnaby et al in their study found that drug induced blockade of gamma-aminobutyric acid (GABA) receptors causes hyperactive swimming in zebrafishes[24]. Ruiter et al in their study discovered that cadmium exposure in sublethal concentrations resulted in lengthened bottom swimming during a novel tank test and ascribed bottom swimming and other antioxidant system changes to suspected alteration in DNA methyltransferase activity[25]. Changes in neurotransmitter function or neuronal activity could disrupt normal swimming patterns and lead to erratic or aggressive behaviour in zebrafish. Excessive movements of zebrafish also led to decreased glucose and acetaldehyde metabolite contents and increased amino acid amounts, which further proved the shortage of energy-supplying substances[26].

Social Behaviour:

Exposure to unwanted stimuli can result in avoidance behaviour in zebrafish leading to social isolation. Many a times, in addition to visual stimuli, olfactory and tactile stimuli also play a role in the social behavioural pattern of zebrafishes[27].

Body Weight:

Zebrafish fed with fish feed exhibited a modest increase in average body weight amounting to 14.25%. Zebrafish fed with *kshira* alone showed a lesser increase in average body weight (2.66%) compared to the fish feed group. However, all groups fed with *viruddha kshira samyogas* experienced decrease in average body weight. The group fed with combination of *kshira* and *matsya* exhibited the highest loss in average body weight (27.75%) indicating nutritional deficiencies that hindered growth. The group fed with *kshira* and *lavana viruddha* combination also experienced a significant loss in average body weight (17.09%). The groups fed with *kshira* and *kadali*, *kshira* and *maasha,* and *kshira* and *dadima* also experienced varying degrees of weight loss (13.91%, 6.55%, and 3.81% respectively). The noted alterations in weight highlight the importance of dietary factor in influencing zebrafish growth and health. Nutritional imbalances or toxic effects from *viruddha kshira samyogas* may have contributed to weight loss in these groups.

Histopathological examination reveals tissue alterations thereby help in detect deleterious effects of xenobiotic agents[28]. Previously several toxicity studies have been conducted on different organs of zebra fish like intestines, kidney, gills, muscles, gonads, pharynx, liver etc[29,30]. For histopathological study, scanning electron microscopy and transmission electron

microscopy should be performed after H& E staining or paraffin staining to examine the ultrastructural changes in the sinusoids. In the present study, liver cells of zebra fish were taken considering the metabolism. Congestion and multifocal vacuolar degeneration of liver cells are histopathological findings suggesting pathological changes within the liver tissue due to *viruddha kshira* combinations. No abnormal histological changes were noted in the liver cells in the group fed with normal fish feed. The group fed with *kshira* alone showed congestion and mild multifocal hepatocellular degeneration. Varying degrees of liver pathology including congestion, vacuolar degeneration and necrosis were observed in the groups fed with different *viruddha* combinations. In the present study, histopathological examination of liver cells of zebra fishes revealed the group fed with *kshira* with *lavana* had worst histopathologic appearance with necrotic cells and moderate multifocal vacuolar degeneration of hepatocytes indicating a more severe impact on liver health compared to other combinations. *Kshira* and *dadima* combination also showed necrosis, congestion and mild multifocal vacuolar degeneration of hepatocytes, indicating a more severe impact compared to other combinations except for *kshira* and *lavana. Kshira* combined with *kadali, maasha, matsya*, and *dadima* led to congestion and mild to moderate multifocal vacuolar degeneration of hepatocytes, suggesting that these combinations also have deleterious effects although the severity varies.

Liver congestion indicates that there is an excessive accumulation of blood within the liver tissue, which can impair its function and lead to further pathological changes. Cytoplasmic vacuolar degeneration, the most common observed histopathology indicates cellular injury and damage. Congestion and multifocal vacuolar degeneration observed together in liver histopathology of zebrafish fed with different *viruddha* combinations suggests that there is ongoing damage and stress to the liver tissue.

Conclusion:

The findings of the current study underscore the importance of *ahara vidhi* explained in *Ayurveda* in maintaining optimal behavioural and physiological functioning. Slow eating or not eating episodes, observed in conjunction with abnormal swimming patterns and mortality events in Zebra Fishes, highlight the multifaceted impact of incompatible food combinations on overall health and behaviour in Humans. Histopathological changes in the liver cells indicate toxicity of *viruddha kshira samyogas*. By understanding the intricate behavioural changes induced by *viruddhahara*, we pave the way for future research endeavours aimed at optimizing dietary practices.

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