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Evaluation of developmental toxicity of Unani herbal formulation *Habbe Sara* in zebrafish (*Danio rerio*)

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ABSTRACT: *Habbe Sara* is an herbal formulation used in the Unani system of medicine to treat convulsions in adults and children. It is believed to strengthen the brain and nervous system, reducing the frequency of fits. In this study, the effects of various concentrations of *Habbe Sara* ranging from 50 to 400 ppm on in zebrafish were examined. The study found that increasing concentrations of *Habbe Sara* resulted in higher mortality rates with an LC_{50} value of 251.18 ppm. In addition, its higher concentrations showed delayed growth, decreased in heartbeat rate, reduced hatchability, and various developmental defects in the treated embryos. Therefore, caution should be exercised when administering high doses of *Habbe Sara* to pregnant women.

KEYWORDS: Teratogenic effect; Habbe Sara; developmental defect; zebrafish; toxicity

1. INTRODUCTION

Anti-convulsant medications are commonly used to treat epileptic seizures, but certain drugs such as topiramate, valproate, and carbamazepine have been associated with an increased risk of teratogenic effects in pregnant women [1–3]. These effects can lead to organ malformations, delayed embryogenesis, developmental defects, as well as cognitive and behavioral impairments. Therefore, it is crucial for women undergoing anti-convulsant drug treatment to consult their physicians and thoroughly discuss the potential risks and benefits before deciding whether to continue the treatment.

Habbe Sara is an unani herbal formulation manufactured by Rex (U&A) Remedies Pvt Ltd. It is used in the Unani system of medicine to address liver disorders, febrile convulsions, and epileptic fits. It contains extracts from three plants and herbs: *Aloe vera* extract (Sibr), *Boswellia serrata* extract (Kundur), and *Castoreum gland* extract (Jund Bedastar) [4]. Like conventional anti-convulsant drugs, *Habbe Sara* may also carry a risk of developmental toxicity and teratogenic effects. Therefore, it is essential to investigate these potential effects to ensure its safe use as an anti-epileptic drug in pregnant women.

The hypotheses under examination in this study involve the study of potential for developmental toxicity and teratogenic effects. It is postulated that exposing zebrafish embryos to *Habbe Sara* may lead to evident developmental toxicity, characterized by delayed organogenesis, structural abnormalities, malformations, altered embryonic development, and the possibility of cognitive or behavioral impairments. Thus, this research aims to contribute valuable insights into the safety profile of *Habbe Sara*, especially regarding its potential impact on embryonic development. Such insights are crucial for determining the suitability of this herbal formulation for use as an anti-epileptic drug in pregnant women.

Zebrafish have become a valuable model for pharmacological screening and developmental toxicity studies due to their rapid organogenesis, significant genetic conservation, and similarity to humans and other vertebrates [5–7]. Additionally, their small size, optical transparency, easy maintenance, high productivity, and breeding efficiency make them advantageous over other animal models [8–11]. Compared to cell-based assays, which are alternative models for drug testing, the zebrafish model can significantly reduce costs, expedite drug discovery, and provide more accurate results [11,12]. By utilizing the benefits of

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the zebrafish model, we have planned to conduct a comprehensive investigation into the developmental toxicity and teratogenic potential of *Habbe Sara* in zebrafish embryos.

2. RESULTS AND DISCUSSIONS

The qualitative phytochemical investigation was conducted to examine the chemical constituents of *Habbe Sara*. The tests results presented in Table 1 revealed the presence of glycosides, flavonoids, tannins, and phenolic compounds in the formulation.

Table 1. The result of preliminary phytochemical screening of herbal formulation *Habbe Sara*

Class of chemical constituents	Result
Carbohydrate	-
Protein	-
Amino acid	-
Fats and oil	-
Volatile oil	-
Glycoside	+
Steroids	-
Flavonoids	+
Phenolic	+
Tannins	+
Alkaloids	-
Acidic compound	-
Organic acids	-

+ / - sign indicates presence/ absence of respective class of phytoconstituents

Assessing zebrafish embryonic mortality or death serves as a direct method for evaluating general toxicity [13]. Investigation into the impact of a pharmaceutical on zebrafish embryonic mortality aids in comprehending the potential risks and benefits associated with therapeutic interventions. This study employed the LC_{50} value as an indicator to assess the degree of mortality induced by the test drug. The LC_{50} value was determined using the probit method (refer to Figure 1). Notably, exposure to *Habbe Sara* at concentrations of 300 and 400 ppm resulted in the highest mortality among zebrafish embryos, with an LC_{50} value of 251.18 ppm. Higher LC_{50} values imply lower drug toxicity, signifying that a greater drug concentration is necessary to cause 50% mortality in the test organisms [14,15].

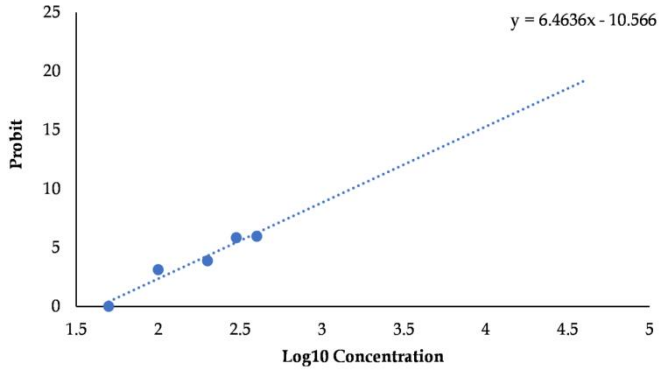


Figure 1. The Log_{10} Concentration vs probit of herbal formulation *Habbe Sara*.

Muscular contractions in zebrafish embryos occur spontaneously, typically emerging around 18–19 hours post-fertilization (hpf). These contractions are believed to be initiated by early activity in the embryonic central nervous system [16,17]. The quantification of embryonic movements per minute was conducted at 24 hpf in zebrafish embryos (see Figure 2). Notably, exposure to *Habbe Sara* resulted in a dose-dependent reduction in the number of embryonic movements. However, this decrease in embryonic

movement was found to be statistically significant ($p < 0.01$) only at a concentration of 400 ppm of *Habbe Sara*. During the early stages of embryo growth, these movements are attributed to primary motor neurons [16,17). The dose-dependent reduction in embryonic movement observed with *Habbe Sara* may be linked to its impact on the early development of the nervous and musculoskeletal systems, potentially causing alterations or impairments in the embryo's movement patterns.

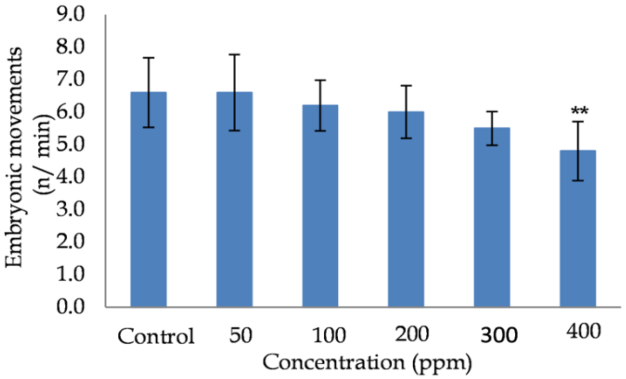


Figure 2: Effect of *Habbe Sara* on embryonic movement in zebrafish embryos.
Values were represented as mean ± SD (n = 10 embryos); *p < 0.05, *p < 0.01 as compared to control.

The investigation into the impact of *Habbe Sara* on the heartbeat of zebrafish embryos offers valuable insights into the pharmacological properties and safety assessment of this test drug. Understanding how a drug affects cardiac function and development is crucial for assessing its potential therapeutic applications and potential risks. The modulation of heartbeat serves as a sensitive biomarker of drug-induced changes in the cardiovascular system, reflecting the compound's influence on this vital physiological process [18]. There was no statistically significant difference was observed in the heartbeat of zebrafish embryos at the 0 to 100 ppm concentration of *Habbe Sara*. The *Habbe Sara* at 200 to 400 ppm concentration revealed statistically significant ($p<0.01$) decreased in the heartbeat rate in zebrafish embryos (see Figure 3). Various drug can alter the heartbeat, either by accelerating or slowing it down which indicate the impact of drug-induced changes in the cardiovascular system [19].

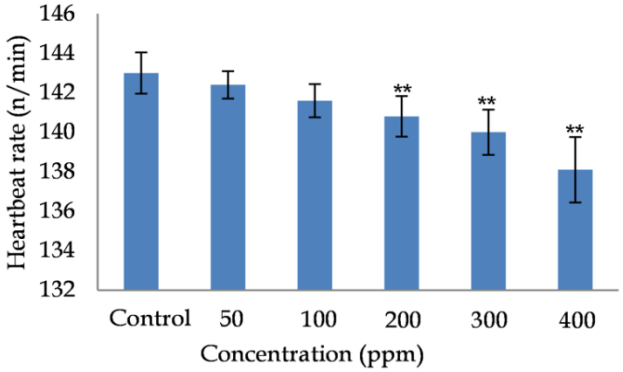


Figure 3: Effect of *Habbe Sara* on Heartbeat rate in zebrafish embryos.
Values were represented as mean ± SD (n = 10 embryos); *p < 0.05, *p < 0.01 as compared to control.

Hatching represents a critical phase characterized by intricate molecular signaling and coordinated enzymatic breakdown of the chorion. In our investigation, the impact of *Habbe Sara* on hatchability was observed to be dose-dependent, as presented in Table 2. Hatching initiation occurred at 48 hpf in zebrafish embryos under control conditions and with *Habbe Sara* concentrations of 50 and 100 ppm. Notably, concentrations ranging from 200 to 400 ppm of *Habbe Sara* did not result in any hatching at 48 hpf. By 72 hpf, the maximum number of embryos hatched in the control group (20/20) and *Habbe Sara* concentrations of 50 ppm (18/20), 100 ppm (18/20), and 200 ppm (16/20). However, no hatching was observed in zebrafish embryos exposed to 300 ppm and 400 ppm concentrations of *Habbe Sara* even after 72 hpf. After 96 hpf, only a few embryos hatched in the 300 ppm (4/20) and 400 ppm (2/20) concentrations of *Habbe Sara*.

Table 2. Quantitative assessment of developmental effects of *Habbe Sara* in zebrafish embryos

Parameters		Drug concentration (ppm)					
	n	Control	50	100	200	300	400
24 hpf							
Number of embryos	n	20	20	20	20	20	20
Coagulated/dead	n	0	0	0	0	0	3
Lack of tail detachment	n	0	0	0	1	1	3
Lack of eye development	n	0	0	1	1	1	3
Head malformation	n	0	0	0	0	0	3
Lack of somite formation	n	0	0	0	0	0	3
Sum of affected	n	0	0	1	1	2	3
Sum of survived	n	20	20	20	20	20	17
48 hpf							
Number of embryos	n	20	20	20	20	20	17
Coagulated/dead	n	0	0	0	0	3	5
Lack of tail detachment	n	0	0	0	0	0	0
Lack of eye development	n	0	0	0	0	0	2
Head malformation	n	0	0	0	0	0	2
Sum of embryos hatched	n	12	10	4	0	0	0
Yolk sac edema	n	0	1	0	0	0	2
Sum of affected	n	0	1	0	0	0	5
Sum of survived	n	20	20	20	20	17	12
72 hpf							
Number of embryos	n	20	20	20	20	17	12
Coagulated/dead	n	0	0	0	0	6	8
Lack of eye development	n	0	0	0	0	0	0
Head malformation	n	0	0	0	0	0	0
Sum of embryos hatched	n	20	18	18	16	0	0
Yolk sac edema	n	0	1	1	0	0	3
Sum of affected	n	0	1	1	0	6	8
Sum of survived	n	20	20	20	20	11	4
96 hpf							
Number of embryos	n	20	20	20	20	11	4
Coagulated/dead	n	0	0	2	3	7	1
Lack of eye development	n	0	0	0	0	1	0
Head malformation	n	0	0	0	0	1	0
Sum of embryos hatched	n	20	20	20	17	4	2
Yolk sac edema	n	0	0	1	2	6	2
Abnormal head-trunk angle	n	0	0	0	0	1	1
Sum of affected	n	0	1	2	5	9	4
Sum of survived	n	20	20	18	17	4	3

n= Represent number of embryos; hpf: hours post fertilization; ppm: part per million

The evaluation of *Habbe Sara* impact on total body length provided valuable insights into its influence on fundamental processes in embryonic development. Our study utilized ImageJ software for precise measurements and quantitative assessment of alterations in total body length at 96 hpf in zebrafish embryos. The results revealed a statistically significant decrease in total body length ($p < 0.01$) when

zebrafish embryos were exposed to concentrations of *Habbe Sara* ranging from 100 to 400 ppm, as depicted in Figure 4.

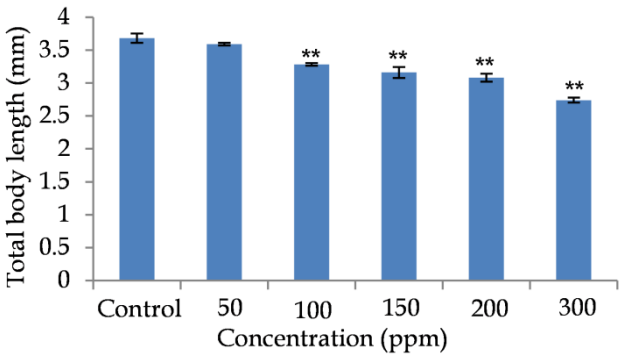


Figure 4: Effect of *Habbe Sara* on Total body length in zebrafish embryos.
Values were represented as mean ± SD (n = 10 embryos); *p < 0.05, *p < 0.01 as compared to control.

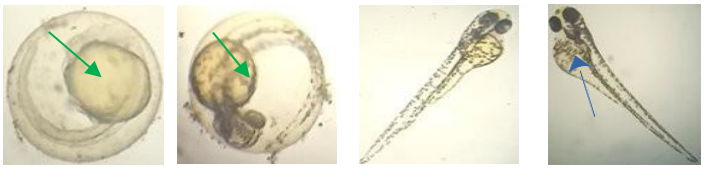
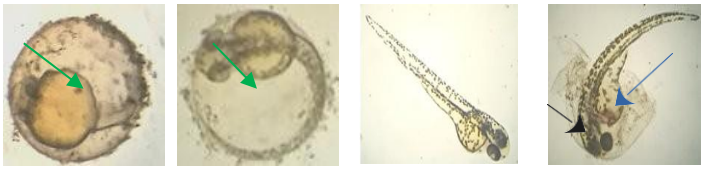
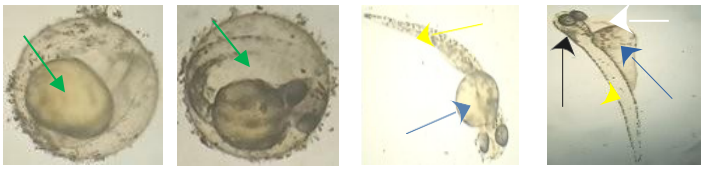
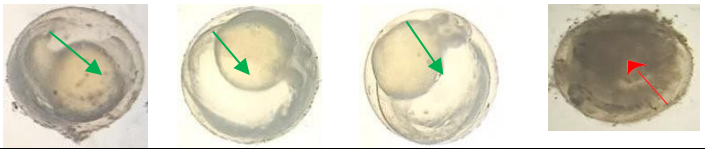
Delayed growth in zebrafish embryos serves as a crucial indicator of the adverse effects of a test drug on embryonic development. Our study disclosed a notable slowdown in embryonic growth at concentrations ranging from 200 to 400 ppm of *Habbe Sara*, as indicated by the green arrow in Table 3. The examination of a test drug's impact on hatchability, embryonic growth, and total body length in zebrafish embryos is essential for comprehending its effects on developmental toxicity [20–22]. Our findings indicate that *Habbe Sara* not only leads to decreased hatchability and total body length but also induces a dose-dependent delay in embryonic growth. This delay could be attributed to disruptions in crucial molecular pathways governing cell division, differentiation, and tissue development. A thorough investigation into the specific cellular and molecular mechanisms affected by *Habbe Sara* is imperative for a comprehensive understanding of its developmental toxicity. Consistent results across various studies imply a shared mechanism through which certain drugs may adversely influence embryonic growth and hatchability [15,23]. It is important to note that such similarities with established literature provide a basis for comparative analysis and suggests that *Habbe Sara* at higher concentrations might indeed hinder embryonic growth.

In the concentration range from 0 to 100 ppm, the embryos displayed typical morphological development, including the formation of eyes and head, a well-developed yolk sac, complete detachment of tails, and an absence of coagulation. These observations indicate that at lower concentrations, *Habbe Sara* does not negatively affect the early stages of zebrafish embryo development. Nevertheless, with an increase in the concentration of *Habbe Sara* to the range of 200 to 400 ppm, adverse effects became increasingly evident, as documented in both Table 2 and Table 3.

Our findings indicate that concentrations of *Habbe Sara* at 200 ppm and above are associated with the presence of an abnormal head-trunk angle, as indicated by the black arrow in Table 3. This observation is significant as it provides insights into the potential developmental impacts of this substance on embryonic morphology. The head-trunk angle serves as a critical parameter in evaluating the proper axial development of vertebrates, including zebrafish embryos [24]. The abnormal head-trunk angle noted at concentrations of 200 ppm and beyond suggests a disruption in the normal patterning and alignment of the embryonic axis. This deviation implies that *Habbe Sara* may interfere with fundamental processes governing neural tube formation, and axial elongation during early embryonic development.

Habbe Sara, particularly at concentrations of 100 ppm and above, induces yolk sac edema in zebrafish embryos, as indicated by the blue arrow in Table 3. This occurrence signifies a important impact on embryonic development. Yolk sac edema is a well-recognized indicator of physiological stress and disruptions in embryonic homeostasis. Similar to human embryos, zebrafish embryos possess a protruding yolk sac that serves as a reservoir of proteins, lipids, and micronutrients, supporting metabolic functions and growth until the initiation of external feeding in zebrafish [25]. Edema in this structure may indicate a multifaceted impact of *Habbe Sara* on critical physiological processes."

Table 3. The representative images of different developmental effect of *Habbe Sara* in zebrafish embryos.

***Habbe sara* Concentration (ppm)**	**24hpf**	**48hpf**	**72hpf**	**96hpf**
0				
50				
100				
200				
300				
400				

Yolk sac edema (blue arrow) observed at 96 hours post-fertilization (hpf) in the concentration range of 100 to 300 ppm; Pericardial edema (white arrow) observed at 96 hpf in the 300 ppm concentration of *Habbe Sara*; Embryo coagulation (red arrow) observed at 96 hpf in the 400 ppm concentration of *Habbe Sara*; Abnormal head-trunk angle (black arrow) indicated at 96 hpf in the concentration range of 200-300 ppm of *Habbe Sara*; Delayed embryogenesis (green arrow) observed from 24 to 96 hpf in the concentration range of 100-400 ppm of *Habbe Sara*; Partial scoliosis (yellow arrow) observed at 72 hpf in the 300 ppm concentration and at 96 hpf in the concentration range of 200-300 ppm of *Habbe Sara*.

The observed pericardial edema at 300 ppm concentration of *Habbe Sara* is a notable discovery that underscores the concentration-dependent effects of this substance on embryonic development, as denoted by the white arrow in Table 3. The pericardium serves to surround and safeguard the developing heart, and the presence of edema in this region suggests potential interference with cardiovascular development and disruption of embryonic osmoregulation [26].

Zebrafish provide unique advantages in modeling human scoliosis due to their spine's morphology and structure closely resembling that of humans, along with a significant genetic similarity. Scoliosis, characterized by a lateral curvature of the spine exceeding 10°, was examined in our study, revealing that *Habbe Sara* at a concentration of 300 ppm induces partial scoliosis, as denoted by the yellow arrow in Table 3. Existing scientific literature suggests a potential association between scoliosis and neuromuscular

abnormalities [27]. The observation of partial scoliosis in our study implies a concentration-dependent impact on the axial skeleton and associated musculature.

The occurrence of embryo coagulation, indicated by the red arrow in Table 3, is observed at a concentration of 400 ppm of *Habbe Sara* at 96 hpf in zebrafish embryos. This finding is significant, suggesting a concentration-dependent adverse effect on embryonic development and integrity. The coagulation of embryos may involve interference with the normal development of the circulatory system. At 400 ppm, *Habbe Sara* could potentially disrupt blood vessel formation or impair the proper functioning of the cardiovascular system, resulting in coagulation within the embryos. An in-depth exploration of the specific effects of *Habbe Sara* on vascular development, blood flow patterns, and the expression of genes associated with circulatory function can provide valuable insights into the underlying causes of embryonic coagulation.

This study is the first investigation of the effect of *Habbe Sara* on developmental defect in zebrafish embryos. Our findings reveal that exposure to *Habbe Sara*, an herbal formulation, at higher concentrations (200-400 ppm) severely disrupts multiple aspects of zebrafish embryonic development. The exposure resulted in severe morphological defects across several body systems, including delayed embryogenesis, coagulation of embryos, yolk sac and pericardial edema, and scoliosis. These concentration ranges, although higher than levels likely to be clinically achieved, provide insight into the biological activity and developmental toxicity risk of complex herbal preparations. Earlier investigations of other test drugs revealed similar toxicological effects in developing embryos [8,15,28,29].

The observed range of defects is likely linked to different active ingredients present in *Habbe Sara*, with each component targeting specific molecular pathways that regulate tissue and organismal morphogenesis. Conducting further studies specifically centered on isolated active compounds would enhance our understanding and help distinguish between distinct and shared toxicity mechanisms.

3. CONCLUSION

Our investigation revealed a direct relationship between the concentration of *Habbe Sara* and the severity of developmental toxicity. As the concentration increased, we observed a corresponding increase in developmental deffects on the exposed embryos. These effects included delayed growth, reduced hatchability, and higher mortality rates, all of which followed a dose-dependent pattern.

Furthermore, at higher concentrations of *Habbe Sara*, we observed additional defects such as decreased heartbeat rate and total body length, malformation of the eye and head, abnormal head-trunk angle, yolk sac edema, and coagulation of embryos.

Our findings clearly demonstrate that the concentration of *Habbe Sara* plays a crucial role in determining the extent of developmental toxicity in zebrafish embryos. The results establish a significant link between the concentration of *Habbe Sara* and the observed developmental toxicity endpoints.

However, to gain a comprehensive understanding of the underlying mechanisms behind *Habbe Sara*-induced developmental toxicity, further research is essential. Such knowledge will provide valuable insights into the potential risks associated with the use of this formulation and help to develop appropriate safety measures for its use in the pregnant women.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Drug

The formulation of *Habbe Sara* was procured from unani medical store, Mumbai, Maharashtra, India. It was triturated, dissolved in distilled water to make different concentrations for conducting this study.

4.1.2 Zebrafish embryos collection and maintenance

The adult zebrafish which show characteristics described by Wixon (2000) were housed in aquariums with fish water [10]. The fish were properly cared for and bred in accordance with standard procedures [30]. The research proposal underwent approval from the Institutional Animal Ethical Committee of the campus, with the assigned protocol approval number AIKTC/SoP/IAEC/2022/01.

4.2 Methods

4.2.1 Preliminary Phytochemical Screening

To identify and characterize the various phytochemical constituents present in *Habbe Sara*, a qualitative phytochemical investigation was conducted using standard methods [31]. The screening aimed to detect the presence of a range of compounds, including carbohydrates, proteins, fats and oils, alkaloids, amino acids, flavonoids, glycosides, phenolics, tannins, and steroids. Through this analysis, we sought to gain insights into the diverse chemical components that make up *Habbe Sara*.

4.2.2 Zebrafish developmental toxicity

Zebrafish embryo toxicity test of different concentrations of *Habbe Sara* was conducted by following OECD test guideline No. 236, 2013 [32]. The procedures used in this study were adopted with a few modifications of methods studied earlier [15,28,33]. After conducting an initial range finding, five concentrations of *Habbe Sara* (50, 100, 200, 300, and 400 ppm) were selected as the final exposure concentrations. The fertilized embryos were selected 4 hours post fertilization (hpf) and expose to selected concentrations of *Habbe Sara* in 24 well microplates and incubated at 26.0°C ± 1.0°C. The assessment of developmental toxicity was conducted by observing various parameters of lethality for every 24 hours post-post fertilization (hpf) to the test drug till 96 hpf. The malformed images of larvae and embryos were captured by using a digital microscope (Labomed) to assess different parameters of the teratogenic effects of the drug [14,34].

4.2.3 Determination of LC_{50}

The LC_{50} of the test formulation was calculated by recording total mortality after 96 hpf to the selected concentrations of *Habbe Sara*. The zebrafish embryos were considered dead when there was no heartbeat or coagulation of eggs observed under a digital microscope. The LC_{50} was investigated by using probit analysis.

4.2.4 Hatchability

Embryos were considered hatched when the entire larvae move out of the chorion. The number of hatched embryos was measured at 24, 48, 72, and 96 hpf.

4.2.5 Heartbeat rate

The number of heart-beats was recorded after 48 hpf by recording live video under a Labomed digital microscope for 15 seconds. To determine the heart-beat per minute, the number of heartbeats for a duration of 15 seconds were multiply by four [35,36].

4.2.6 Total body length

For measuring total body length, larvae after 96 hpf were anesthetized, and images were captured. The total body length was calculated by using open-source ImageJ software.

4.2.7 Embryonic movement

The number of embryonic movements per minutes after 24 hpf in zebrafish embryos were recorded by capturing video footage under digital microscope at 4x objective lens. The video footages were analyzed manually to measure number of spontaneous movements such as tail flicking, body twitching etc.

4.3 Statistical analysis

The statistical data was analyzed by ANOVA followed by a post-hoc test and descriptive statistics.

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Author contributions: Concept – A.S.; Design – M.F., S.S., A.S.; Supervision – A.S., S.S.; Resources – S.S., A.S.; Materials – A.C., S.S., M.F., A.W., I.S.; Data Collection and/or Processing – K.K., M.F., A.C., I.S., A.N.; Analysis and/or Interpretation – A.W., M.F., A.N., K.K., A.S.; Literature Search – A.C., I.S., A.W., M.F., A.N, K.K.; Writing – I.S., S.S., A.S.; Critical Reviews – S.S., A.S., I.S., A.N., A.C., A.W., S.S., M.F., K.K.

Conflict of interest statement: The authors declare that they have no conflicts of interest.

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