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Acute Toxicity Assessment and Behavioral Responses Induced by Kandhamal haladi in Adult Zebrafish (*Danio rerio*)

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Abstract: Turmeric has been used traditionally for its antimicrobial activity. Turmeric is used as a spice, food preservative, and coloring material in India. It has been used in Ayurveda for various diseases. So we decided to evaluate the toxicity effect of Kandhamal haladi, commonly use in the food industry, cosmetics, and pharmacology. The adult zebrafish (*Danio rerio*) model was used to evaluate the median lethal concentration (LC_{50}). In the present study, acute toxic effects and behavioral alterations induced by exposure of the freshwater fish *Danio rerio* (zebrafish) to Kandhamal haladi was reported. Seven healthy specimens of the average size (3-4 cm in length and 1.0±0.78 g in weight) in each group were exposed to different concentrations of Kandhamal haladi for 24 h, 48 h, 72 h, and 96 h along with naïve and control group in a static system. After 96 h exposure at a water temperature of 28.5ºC, their abnormal swimming behavioral patterns and abnormal ventilatory (respiratory) function, including hyperactivity, hypoactivity, and gulping, were observed. Alterations in behavioral patterns were well noticeable during the period of the experiment. The 96 h LC_{50} value of Kandhamal haladi to *Danio rerio* was found to be 173.516 µM with lower and upper confidential limits (95%) as 152.146 µM and 200.072 µM respectively by using IBM SPSS statistics 25 software and 173.780 µM using Microsoft Office Excel 2007 based on Finney's probit analysis statistical method. Mortality and behavioral changes were increased with increasing concentration of the compound within 24 to 96 hours. In addition to dose and dose-time dependent increase in the mortality rate, anxiety signs in the form of behavioral changes were observed in response to different test concentrations. Further researches are recommended to study the processes by which this chemical affects physiology and histology of fish and their accumulation in fish tissues. Our findings suggest that Kandhamal haladi can be used to fight against different fish diseases because of its low toxic effect on fishes, and supplementation of haladi could be recommended in aquaculture through a feed to prevent disease impact.

Keywords: acute toxicity; behavioral changes; geographical indication (GI) tag; Kandhamal haladi (KH); median lethal concentration (LC_{50}); Finney's probit analysis; adult zebra fish.

1. Introduction

Turmeric derives its name from the Latin word "*terra merita*," meaning meritorious earth, implying to the color of ground turmeric, which resembling a golden pigment. It was in 1753 that the genus *Curcuma* was established by Linnaeus in his Species *Plantarum* [1]. This

was based on a plant observed by Hermann in what was then known as Ceylon (now Sri Lanka). The generic epithet is derived from the Arabic word *karkum*, meaning yellow, referring to the yellow color of the rhizome, and *Curcuma* is the Latinized version [2-3]. *Curcuma* was described early (1678–1693) by Van Rheede in *Hortus Indicus Malabaricus* [4]. He recorded two species of *Curcuma* under the local names "*Kua*" and "*Manjella Kua*," which were later identified as *Curcuma zedoaria* Rosc. and *Curcuma longa* L., respectively [5]. "*Manjella Kua*" was selected as lectotype of *Curcuma longa* L. by Burtt (1977) [5]. He further reinstated the name of *Curcuma longa* L. and *Curcuma domestica* Val. as its synonym belongs to the family Zingiberaceae. The genus *Curcuma* consists of about 110 species distributed chiefly in South and Southeast Asia. Hooker (1879) confirmed 27 species of *Curcuma* in British India (The Flora of British India) [6]. Turmeric (*Curcuma longa* L.) is a rhizomatous herbaceous perennial plant of the ginger family and used in India for thousands of years as a part of food preparation and many major Ayurvedic and Siddha drugs [7-8]. It was first used as a dye and later for its medicinal properties and is reported that its extracts have antifungal and antibacterial properties [9]. Turmeric is under evaluation for its potential effect on Alzheimer's disease and diabetes [10-11]. Turmeric or Haladi is widely used in Ayurveda and Siddha formulations for treating various diseases like biliary disorders, anorexia, cough, diabetes, wounds, hepatic disorders, rheumatism, and sinusitis [12]. *Ichthyophthirius multifiliis* is a ciliated parasite that elicits great economic losses in aquaculture [13]. Curcumin has the potential to be a safe and effective therapeutics for controlling ichthyophthiriasis in aquaculture [13]. Turmeric supplementation in the food of *Labeo rohita* (Linn.) reduced bacterial pathogenicity of *Aeromonas veronii* [14]. Mycobacteriosis is a common disease of laboratory zebrafish as well as wild and captive fishes worldwide [15-16]. Mycobacteriosis is a significant and commonly identified disease in zebrafish research facility [17]. Mycobacterium species have long been recognized as a significant source of morbidity and mortality in finfish aquaculture, as well as in wild finfishes. Mycobacteria infecting fishes also include zoonotic pathogens that can cause protracted illness, especially in immune-compromised individuals [18]. Mycobacteriosis also affects birds throughout the world, and the species pathogenic to birds are considered ubiquitous environmental saprophytes [19]. Turmeric has antimicrobial and anti-protozoal activity. Mycobacteriosis may be prevented and cured by turmeric. It contains many phytochemicals like curcumin, bis-methoxy-curcumin, d-methoxy-curcumin, curcuminol, curcumol, eugenol, terta-hydro-curcumin, tri-ethyl-curcumin, turmerine, turmerols, volatile oils (turmerone, atlantone, and zingiberene), sugars, proteins, and resins [20-21]. The pharmacological activity of turmeric has been attributed mainly to curcuminoids [22]. In our present study, acute toxicity bioassay and behavioral studies were carried out in a static system to determine the LC_{50} values for 96 hours.

1.1. Geographical indication status in India.

The protection of geographical indications (GIs) has, over the years, emerged as one of the most contentious intellectual property rights issues in the realm of the World Trade Organization (WTO) [23]. In 2019 two products from the state of Odisha got GI tag out of which one is Kandhamal haladi (KH) by intellectual property India. Kandhamal haladi (KH) got geographical indication number-610 been placed under class-30.

1.2. Kandhamal haladi (KH).

In India, turmeric is known as “haladi”. Kandhamal haladi or Kandhamal turmeric is a variety of turmeric indigenous to southern Odisha. Kandhamal is a district in the state of Odisha and is famed for its turmeric. The agricultural product stands out for its healing properties. The local variety of turmeric grown from ancient times is having 2 – 3% curcumin (Figure 1), 12-15% of oleoresin [Demethoxycurcumin (Figure 2), and Bisdemethoxycurcumin (Figure 3)], and 5.3% of volatile oil [24]. Kandhamal haladi has more oleoresin and volatile oil contents compared to other turmeric varieties [24]. It gives a strong aroma and has a high medicinal value.

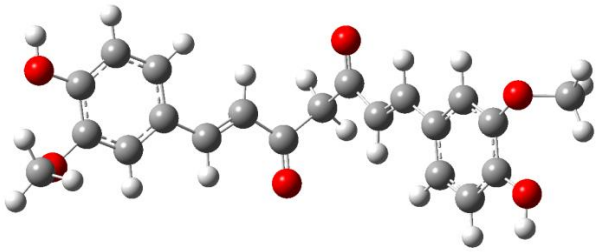

Figure 1. Structure of curcumin using GaussView 4.1.2.

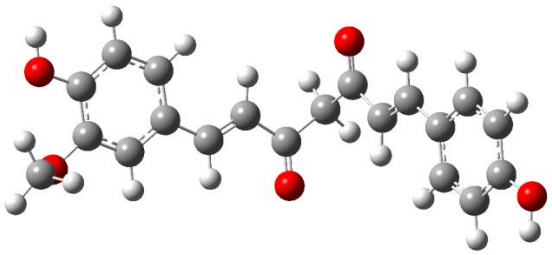

Figure 2. Structure of Demethoxycurcumin using GaussView 4.1.2.

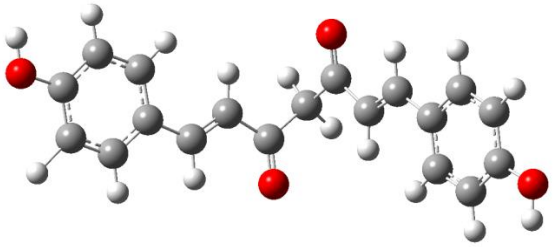

Figure 3. Structure of Bisdemethoxycurcumin using GaussView 4.1.2.

1.3. Zebrafish (Danio rerio) as a toxicity model.

The zebrafish (*Danio rerio*) is a small tropical fish, member of the minnow family Cyprinidae of the order Cypriniformes. Hamilton (1822) described *Cyprinus rerio* from Gangetic provinces in the book “An account of the fishes found in the river Ganges and its branches”, together with the other ten species [25]. The name Danio is derived from the Hindi word Dhan which means paddy [26]. It is a popular aquarium fish, its trade name zebra danio in India (and thus often called a "tropical fish" although both tropical and subtropical). Globally zebrafish known as “leopard danio” and vernacular name for zebrafish in Odia language is “poncho geraldi” [27]. *Danio rerio* is distributed throughout South and Southeast Asia. The natural occurrence of the species is predominantly related to the Ganges and Brahmaputra river basin localized in north-eastern India, Bangladesh, and Nepal [28-29]. In the early 1970's, scientists of the University of Oregon determined the zebrafish as a vertebrate model for

development and genetics. Dr George Streisinger (1927-1984) is considered the founding father of zebrafish research [30]. Together with Dr Charles Kimmel and other researchers of the University of Oregon's Institute of Molecular Biology, he published several studies on zebrafish development and genetics [31]. Nowadays, hundreds of research centers worldwide use zebrafish in fundamental and applied research. In the last decade, zebrafish have become an important model organism in the field of biomedical, eco-toxicological, and genetics. The intricate features of zebrafish render this fish a novel vertebrate model among the organisms. Consequently, the zebrafish has become a popular model organism also for clarification of the roles of specific genes and signaling pathways during the development [32-33]. An increasing interest emerged in the field of toxicology and ecotoxicology as a non-mammalian vertebrate organism in risk assessment and regulation. Zebrafish share physiological, morphological, and histological similarities with mammals and have been recognized as valuable models for evaluating drug candidates for toxicity and safety liabilities [34]. The zebrafish has its advantages compared to the traditional *in vivo* model like the mouse in that multiple organs can be observed; pharmacodynamic, pharmacokinetic, and metabolite activity can be evaluated; low cost; short life cycle; less amounts of a test compound; and high output [35]. Zebrafish model system has been successfully used in studying developmental toxicity, teratogenicity, cardiovascular toxicity, liver toxicity, behavioral toxicity, kidney toxicity, and a series of evaluation assays [36]. But what boosted the scientific community to recognize zebrafish as leading vertebrate organism in the above-mentioned field, has been the complete sequencing of its genome [37]. The zebrafish (*Danio rerio*) is an important vertebrate model organism in scientific research. As a model biological system, the zebrafish possesses numerous advantages for researchers. However, detailed studies on the pharmacological activity and potency of Kandhamal haladi in its unrefined natural state are scanty. This study gives the gross effects of natural state Kandhamal haladi on adult zebrafish.

1.4. Median lethal concentration (LC_{50}).

Toxicology can be defined as that branch of science that deals with poisons and a poison can be defined as any substance that causes a harmful effect when administered, either by accident or design, to a living organism [38]. Median lethal concentration (LC_{50}) is the concentration of a test chemical that is estimated to be lethal to 50% of the test organisms within the test duration [39]. In environmental studies, it can also mean the concentration of a chemical in water. According to the Organisation for Economic Cooperation and Development [39] guidelines for the testing of chemicals, a traditional experiment involves groups of animals exposed to a specific concentration (ccohs). However, in this case, both exposure time and concentrations are important. Research studies described the highest doses at which no toxic effects were observed, and at lowest doses, toxic or adverse effects were observed. The terms refer to the actual doses used in experimental animal studies are NOEL (no observed effect level) and LOEL (lowest observed effect level). For many chemicals and effects, there will be a dose below which no effect or response is observed. This is called the threshold dose. This concept is of significance because it implies that a no observed effect level (NOEL) can be determined and that this value can be used to determine the safe intake for food additives such as turmeric and contaminants such as pesticides. Alternatives that are occasionally used in place of LC_{50} are the LC_{25} and LC_{75} , which refer to the lethal concentration that kills 25% and 75% of test subjects, respectively. There are some criteria for aquatic environment toxicity by GHS (Globally Harmonized System of Classification and Labelling of Chemicals) [40] (Table

1). Toxicants enter into the body through ingestion, skin, and inhalation. It is absorbed into the bloodstream and distribution throughout the body tissues and organs and affects body metabolism [38] (Fig 4).

Table 1. GHS (Globally Harmonized System of Classification and Labelling of Chemicals) criteria for aquatic environment toxicity [40].

GHS (Globally Harmonized System of Classification and Labelling of Chemicals) criteria
Hazardous to the aquatic environment
Category 1 (Very toxic to aquatic life) 96 hr LC_{50} (fish) ≤ 1 mg/L
Category 2 (Toxic to aquatic life) 96 hr LC_{50} (fish) >1 ≤ 10 mg/L
Category 3 (Harmful to aquatic life) 96 hr LC_{50} (fish) >10 ≤ 100 mg/L

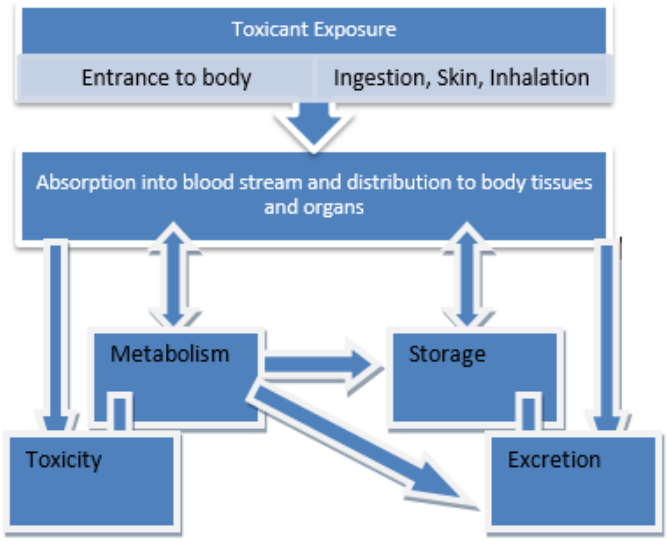

Figure 4. Fate and effect of toxicants on the living system [38].

2. Materials and Methods

2.1. Chemicals and reagents.

The imperative chemicals and standard reagents used in the experiments were purchased from Sigma-Aldrich unless otherwise mentioned.

2.2. Experimental animals.

Adult zebrafish (short-fin, 6–8 month-old, 3-4 cm in length, ~50:50 male: female ratio and weigh about 1.0±0.78g) were collected from ornamental fish hatchery of the Central Institute of Freshwater Aquaculture (CIFA), Odisha, India and were maintained in a 50-L aquarium at 28.5 °C. Tanks were filled with filtered water, and the pH of the system water was checked daily and was maintained between 6.8 – 7.5. When necessary, sodium bicarbonate was used to increase the pH. The salinity and hardness of water were maintained between 0 to 0.1 ppt and 100 mg/L $CaCO_3$, respectively. They were left for acclimatization for 9 days (48 hours settling-in + 7 days acclimatization) and feed twice per diem with packed fish food collected from a pet store, and dead fish were immediately removed to avoid possible putrefaction with the deterioration of water quality. Illumination was provided by ceiling-mounted light tubes on a 14:10 h (day: night) cycle, consistent with the zebrafish standard of care. The zebrafish were divided into three experimental groups such as naive (without any supplementation), control

(supplemented with the vehicle, dimethyl sulfoxide), and Kandhamal haladi (supplemented with Kandhamal turmeric). Seven zebrafish per group were taken in triplicates and maintained with different doses (20 µM to 300 µM) of Kandhamal haladi or Kandhamal turmeric in 2-L capacity rectangular aerated glass containers. No food was provided to the fishes during the assessment. The fishes were exposed to the test chemical, i.e., Kandhamal turmeric, for a period of 96 hours, under static condition. Fishes were considered dead when there was no visible movement (e.g., no opercular motion), and touching the caudal peduncle produced no reaction. Mortalities and visible abnormalities related to appearance and behavior were recorded. The concentrations to kill 50% of the fish (LC_{50}) are determined [41-43, 39]. The acute toxic effect of zebrafish within 96 h was determined as LC_{50} and then subjected to probit analyses by Finney's method using Microsoft Office Excel 2007 and using the IBM SPSS Statistics version 25 software to estimate LC_1 to LC_{99} values (Figure 5). Results with $p<0.05$ were considered to be statistically significant. The LC_{50} value is obtained by using the regression equation arithmetically and also by graphical interpolation by taking logarithms of the Kandhamal haladi concentration versus probit value of percentage mortality.

2.3. Dose standardization of Kandhamal haladi (KH).

A waterborne acute toxicity test for turmeric was carried out to determine the dose-response curve [39]. Kandhamal haladi (KH) was dissolved in 0.1% dimethyl sulfoxide (DMSO) [44]. Then the Kandhamal turmeric was supplemented at different concentrations of 20 µM, 40 µM, 60 µM, 80 µM, 100 µM, 120 µM, 140 µM, 160 µM, 180 µM, 200 µM, 220 µM, 240 µM, 260 µM, 280 µM, and 300 µM for waterborne administration for a period of 96 hours.

3. Results and Discussion

The mortality of the adult zebrafish for Kandhamal haladi doses 20 µM, 40 µM, 60 µM, 80 µM, 100 µM, 120 µM, 140 µM, 160 µM, 180 µM, 200 µM, 220 µM, 240 µM, 260 µM, 280 µM, and 300 µM were examined for a duration of 96 h (Table 4). No naïve fish died during the acclimatization before exposure, and no control fish died during acute toxicity tests. Adult zebrafish exposed during the period 24 h to 96 h had significantly increased in mortality with increasing concentration. There were significant differences in the number of dead adult zebrafish between the duration of 24 h to 96 h in each group. There was a 100% mortality above 400 µM concentration within the 96 h dose for all fishes. Median lethal concentrations of 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 99% test are presented in Table 5. Because mortality data are collected for each exposure concentration in a toxicity test for durations of 96 hours, data can be plotted in the straight line of best fit is then drawn through the points (Figure 5 and Figure 6). Behavioral change was for a general adaptation of organisms towards environmental stress. Behavioral changes presented in Supplementary Table 2 (Supplementary data) records the behavioral responses, including hyperactivity (Figure 7), hypoactivity (Figure 8), and gulping (Figure 9) with a standard error of the mean (SEM). Within the naïve and control group, no significant changes in behavioral responses were detected. In the case of Kandhamal haladi exposure, behavior changed with exposure time and concentration. Higher concentrations resulted in a greater degree of the behavioral responses, which may be due to the toxic effect exerted by the Kandhamal haladi.

3.1. Statistical analysis.

Significant differences ($P < 0.05$) were observed in the LC_{50} mean values for the zebra fish to each concentration of Kandhamal haladi (Table 4). The logarithmic value of the concentration was obtained by interpolation from the linear correlation between probits and log (c). The LC_{50} values in 96 h time intervals were determined by probit analysis (Supplementary Data, Supplementary Table 3), with a confident limit of 5 % level [45]. The LC_{50} value (with 95% confidence limits), the correlation between mortality against concentrations were obtained. Different concentrations of Kandhamal haladi showed a different percentage of mortality (Figure 5 and Figure 6). It was detected only at the concentration of 20 µM no mortality was observed at 96 h, whereas at 400 µM showed 100% mortality of the animal. Computation of median lethal concentration by probit analysis using IBM SPSS Statistics 25 and Microsoft Office Excel 2007 showed LC_{50} value at 173.516 µM and 173.780 µM, respectively. The LC_{50} values were highly significant, $p<0.05$ (Table 6 and Table 7). The values are also plotted as a graph, and the results of correlation analysis using Microsoft Office Excel 2007 showed that % mortality (Y-variable) against concentrations of Kandhamal haladi (X-variable) was highly positive correlation r= +0.986 (Figure 6). Probit transformed response graph plotted by using IBM SPSS Statistic 25 also showed a highly positive correlation r= +0.926 (Figure 5). It was observed that at the concentration above 400 µM showed 100% mortality. Tables 6 and 7 depicted the parameter estimates of probit analysis and chi-square test for the acute toxicity of Kandhamal haladi to *Danio rerio*. The behavior of the animal showed a drastic alteration in Kandhamal haladi-treated zebrafish when compared to control group and naïve group, where at the beginning of the treatment, all the fishes showed spontaneous swimming activity, and it gradually decreased to become lethargic (Figure 7 and Figure 8). Irregular, erratic, abnormal swimming movements, reactive to stimulus, schooling behavior, and change in body pigmentation became more apparent with an increase in the duration of exposure at all test concentrations. Too much behavioral changes (cough, mucus secretion, irregular ventilation, and yawn) at higher concentration might be due to demonstration of the disturbances in the physiological mechanism which is supposed to initiate, maintain and terminate the behavior [46]. It may reduce the supply of oxygen and causing immediate fish death. With increasing exposure, their opercular movements became least and died with mouth opened. It was concluded that Kandhamal haladi is low toxic and does not have a significant effect on the behavior in low doses, which has less toxic to the health of adult zebrafish, whereas curcumin had moderate acute toxicity in embryo zebrafish assay [35].

Table 2. Clinical signs and symptoms observed in adult zebrafish [39].

Clinical sign	Definition	Synonyms
LOSS OF EQUILIBRIUM (sub-categories below)		
Abnormal horizontal orientation	Loss of balance displaying as abnormal horizontal orientation/posture in the water column	Keeling lost righting reflex
Abnormal vertical orientation	Head-up or head-down posture	
Loss of buoyancy control	Floating at the surface or sinking to the bottom	
ABNORMAL SWIMMING BEHAVIOUR (sub-categories below)		
Hypoactivity	Decrease in spontaneous activity	Torpid, apathy, lethargy, weak, immobility, inactivity, ceased swimming, quiescent
Hyperactivity	Increase in spontaneous activity	Erratic swimming, skittering

Corkscrew swimming	Rotation around a long axis; erratic movements, often in Bursts	Rolling, spiraling, spiral swimming, tumbling, circling movements
Convulsions	Abnormal involuntary and uncontrolled contraction of muscles	Seizures, twitching, muscle spasms, shaking, shuddering, vibration
Tetany	Rigid body musculature (intermittent or permanent)	Paralysis
Irritated skin behaviors		Flashing, scraping, rubbing
Abnormal surface distribution/behavior	Abnormal depth selection, close to water/air interface	Jumping, surfacing; on/at/near/just below surface/top
Abnormal bottom distribution/behavior	Abnormal depth selection, close to the base of the tank	Diving, sounding; lying on/ orientation to / collecting at / near / just above bottom
Over-reactive to stimulus	Flight (startle) or avoidance response to: visual (hand passing over the top of the tank, light beam), tactile (touch) or vibration (tank rapped lightly) stimulus	Hyperexcitability; hyperactivity after stimulus/threat
Under-reactive to stimulus		Not responsive to external stimulation; inactivity after stimulus/ threat
Loss of schooling / shoaling behavior	Individual fish show loss of aggregating and social interactions	Isolation, social isolation
Dense schooling / shoaling behavior	Increase in the clumped association of fish	Crowding
ABNORMAL VENTILATORY (RESPIRATORY) FUNCTION (sub-categories below)		
Hyperventilation	Increased frequency of opercular ventilator movements, with possible open mouth and extended operculate	Rapid/strong respiratory rate/ function. Heavy gill movements, strong ventilation, strongly extended gills, abnormal opercular activity, operculate spread apart, mouth open
Hypoventilation	Decreased frequency of (and possibly shallow) opercular Ventilator movements	Reduced/laboured/weak/slow respiration/respiratory action/ventilation
Irregular ventilation	Irregular opercular ventilator movements	Sporadic / spasmodic respiration / gill movement
Coughing	Fast reflex expansion of mouth and operculate not at water surface assumed to clear ventilatory channels	Gasping, abnormal opercular activity, yawn
Gulping	Mouth (and opercular) movements at the water surface, resulting in the intake of water and air	Piping
Head shaking	Rapid lateral head movements	
ABNORMAL SKIN PIGMENTATION (sub-categories below)		
Darkened		Changed / increased / dark(ened) color / pigmentation / melanistic markings
Lightened		Pallor, pale/changed/weak pigmentation
Mottled		Discolored patches
OTHER VISIBLE (APPEARANCE & BEHAVIOUR) ABNORMALITIES (sub-categories below)		
Exophthalmia	Swelling within the orbital socket(s) resulting in bulging of one or both eyes	Exophthalmos, exophthalmos, popeye, protruding eyeball
Edema	Abdominal swelling due to the accumulation of fluid. May cause protruding scales and/or fissure in the abdominal wall	Distended/swollen/bloated abdomen/gut area; dropsy
Hemorrhage	Petechiae (pinhead-sized spots) and/or hematoma (area of blood) due to intradermal or sub-mucus bleeding	

Mucus secretion	Excess mucus production	Mucus build-up (close pay attention to eyes); increased secretion (mucus on the skin or in water); mucus loss
Fecal (anal) casts	String of feces hanging from the anus or on the tank floor	
Aggression and/or cannibalism		Aggression, direct attack, the domination of choice tank locations, pick at or eat bodies of dead fish

Table 3. Finney's table for the transformation of percentages of mortality to probit values (Finney, 1952).

%	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09
%	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Table 4. Mortality table for Kandhamal haladi with probit value.

Conc. (µM)	**log 10 (conc.)**	**Zebrafish (N=21)**	**No of death after 96 hr**	**Zebrafish death (%)**	**Probit value**
Naive	-	21	0	0	-
Control	-	21	0	0	-
20	1.30103	21	0	0	0
40	1.60206	21	2	10	3.72
60	1.778151	21	3	14	3.92
80	1.90309	21	3	14	3.92
100	2	21	5	24	4.29
120	2.079181	21	6	29	4.45
140	2.146128	21	8	38	4.69
160	2.20412	21	9	43	4.82
180	2.255273	21	9	43	4.82
200	2.30103	21	12	57	5.18
220	2.342423	21	12	57	5.18
240	2.380211	21	14	67	5.44
260	2.414973	21	15	71	5.55
280	2.447158	21	15	71	5.55
300	2.477121	21	18	86	6.08

Table 5. LC_{1-99} values and associated 95% confidence limit for the zebrafish that experienced mortality when exposed to Kandhamal haladi.

95% Confidence Limits for Concentration (µM)			
Lethal concentration	Estimate	Lower Bound	Upper Bound
LC_1	24.313	12.760	36.060
LC_{10}	58.771	40.786	74.132

LC_{20}	85.224	65.911	101.355
LC_{30}	111.416	92.157	128.390
LC_{40}	140.085	120.770	159.670
LC_{50}	173.516	152.146	200.072
LC_{60}	214.925	187.445	256.355
LC_{70}	270.229	230.363	339.959
LC_{80}	353.277	289.790	478.641
LC_{90}	512.292	394.605	776.634
LC_{95}	696.316	507.231	1162.760
LC_{99}	1238.325	808.966	2489.412

Table 6. Parameter estimates of 96h LC_{50} probit analysis for Kandhamal haladi.

a. PROBIT model: PROBIT (p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm).

Parameter		Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PROBIT[a]	Concentration (µM)	2.726	0.347	7.847	0.000	2.045	3.406
	Intercept	-6.104	0.769	-7.932	0.000	-6.873	-5.334

Table 7. Chi-square tests of 96h LC_{50} probit analysis for Kandhamal haladi.

		Chi-Square	df[b]	Sig.
PROBIT	Pearson Goodness-of-Fit Test	4.931	13	.977[a]

a. Since the significance level is greater than .150, no heterogeneity factor is used in the calculation of confidence limits.

b. Statistics based on individual cases differ from statistics based on aggregated cases.

Table 8. 96h LC_{50} values for freshwater zebrafish after exposure to Kandhamal haladi by using two methods.

Methods	96h LC_{50} value (µM)	Regression equation
Finney's probit analysis using MS Office Excel 2007	173.780	y = 0.290x - 4.828 R^2 = 0.986
Finney's probit analysis using IBM SPSS statistics 25	173.516	y=-5.77+2.58*x R^2 linear= 0.926

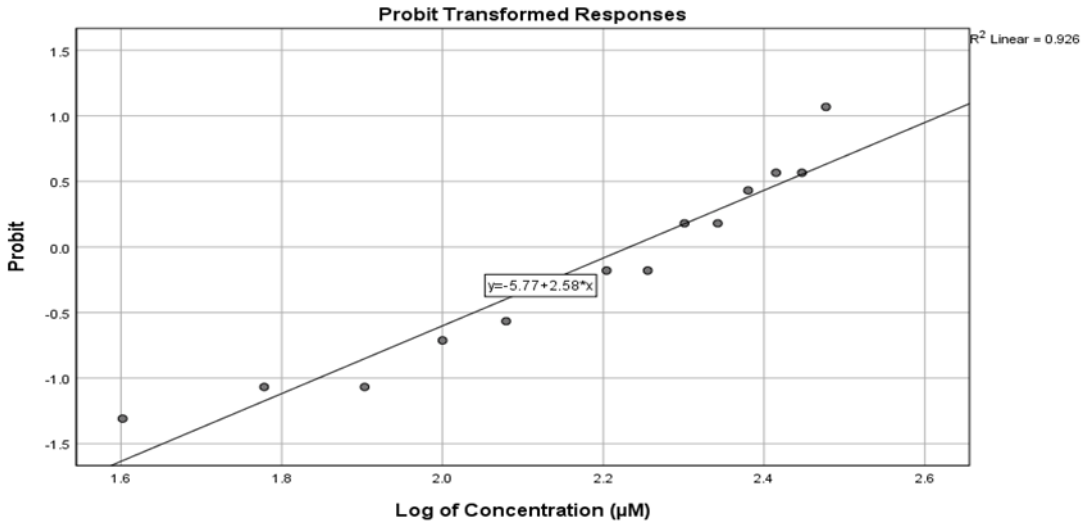

Figure 5. The graph showing linear curve between probit mortality of fish against log concentration in *Danio rerio* on exposure to Kandhamal haladi using IBM SPSS Statistics 25.

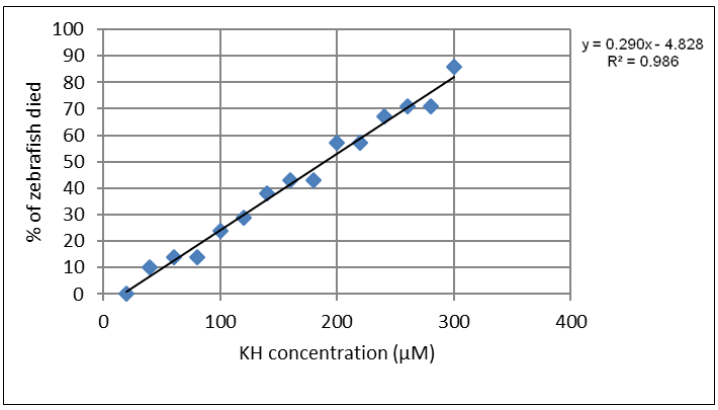

Figure 6. The graph showing the linear curve between the mortality percentage of fish against log concentration in *Danio rerio* on exposure to Kandhamal haladi using MS Office Excel-2007.

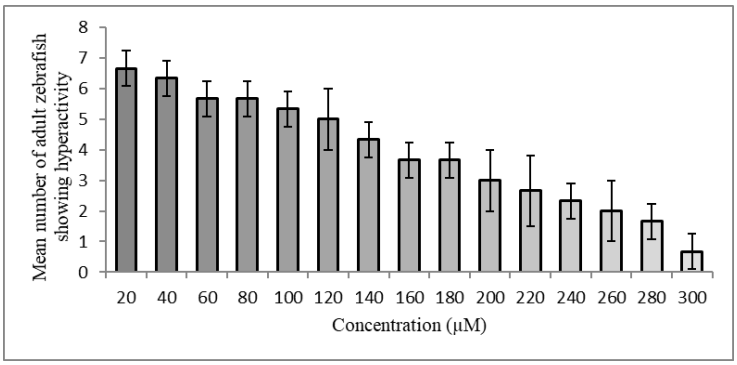

Figure 7. Number of adult zebrafish showing hypersensitivity exposed to Kandhamal haladi for 96hrs. **Values are represented as Mean±SEM (n=7).**

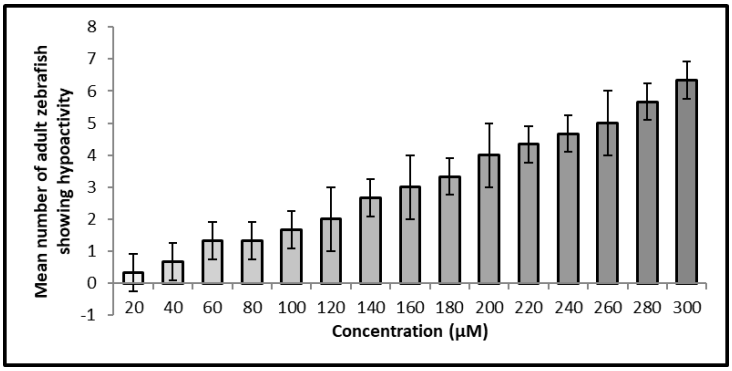

Figure 8. Number of adult zebrafish showing hyposensitivity exposed to Kandhamal haladi for 96hrs. **Values are represented as Mean±SEM (n=7).**

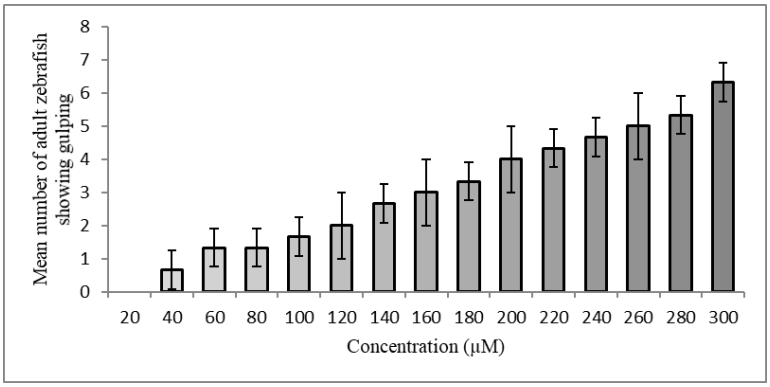

Figure 9. Number of adult zebrafish showing gulping behavior exposed to Kandhamal haladi for 96hrs. **Values are represented as Mean±SEM (n=7).**

4. Conclusions

From the results of this experiment, it was found that there was a positive relationship between the mortality and concentration levels; when the concentration level increased, the mortality rate also increased. However, there was a negative relationship between the mortality time and concentration level; when the concentration level increased, the mortality time decreased. Also, behavioral changes increased with increased concentration of Kandhamal haladi. We applied two statistical methods of data evaluation for acute toxicity assessment. Our results were similar in the two methods used. Our findings suggest that Kandhamal haladi can be used to fight against different fish diseases because of its low toxic effect on fishes, and supplementation of haladi could be recommended in aquaculture through the feed to prevent disease impact.

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Conflicts of Interest

The authors declare no conflict of interest.

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