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Neurobehavioral assessments of Novel Pyrimidine derivative on experimental Zebra Fish

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A B S T R A C T

The present research is to explore the anti-anxiety property of novel synthesized pyrimidine derivative in zebra fish. We have investigated the anti-anxiety property of synthesized drug in different anti-anxiety model, trapezoid, light and dark and open field. LC_{50} value for drug was found as 5.5 mg/l. The dose are selected as in different concentration 1.1mg/l and 0.5mg/l. The Dive tank model was showing that fishes in test drug 1.1mg/L prefer to stay on the upper part of the trapezoidal tank, as well as the entry duration of the fish on the upper and middle part is also maximum as compare to lower part. Unfamiliar aquatic white/black maze environment model was showing that fishes in test drug 1.1mg/L prefer to stay in black zone and they are showing longer freezing time than other two groups. Open field treatment model was showing that fishes in test drug 1.1mg/L prefer to stay in inner zone and they are showing longer freezing time than other two groups. The overall result shows that the drug has anti-anxiety and sedation activity. Sedation activity is more prominent than anti-anxiety property.

KEYWORDS:

Neurobehavioral Study, Zebrafish, Pyrimidine derivative, anti-anxiety, Sedation, anti-anxiety Model, Cortisol Estimation.

1. INTRODUCTION

Neurobehavioral disorders are composed of Variety of behavioral disability which is associated with brain disease like stroke, multiple sclerosis, dementia, ischemia neuro-oncological conditions and transient as well as permanent brain impairments like metabolic and toxic encephalopathy's."Neurobehavioral" refers to the type of behavioral problems that are associated with brain disorders. It is a term commonly used to describe the significant behavioral problems frequently seen after traumatic brain injury [1].

The zebrafish, binominal name is *Danio rerio* is a tropical freshwater fish belonging to the Cyprinidae family of the order Cypriniformes. The zebrafish (*Danio rerio*) is rapidly becoming a popular model organism in pharmacogenetics and neuropharmacology..

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Both larval and adult zebrafish are currently used to increase our understanding of brain function, dysfunction, and their genetic and pharmacological modulationIt is an important and widely used vertebrate model organism in scientific research, and was the first vertebrate to be cloned. Numerous behavioural tests illustrate how various common neurobehavioral disorders can be modeled or studied in zebrafish [2]. Zebrafish can be used in

Research as an alternative to mammalian species because it is Genetic similarity to humans, Easier to house and care for than rodents, Impact of any genetic mutation or drug treatment is easy to see, Lots of offspring, Easier to introduce genetic changes. It has been successfully utilized in developmental biology, a discipline that often employs molecular biology and genetics methods. The zebrafish genome is well characterized and its sequencing has just been completed by the UK Sanger Institute, which has further increased interest in this fish as a model organism in neuroscience and pharmacology [3, 4]. Zebrafish (*Danio rerio*) have served as animal models in behavioural neuroscience and behaviour genetics for the past three decades. Recent research has demonstrated the suitability of adult zebrafish to

model some aspects of complex behaviour. Studies of reward behaviour, learning, memory, aggression, anxiety and sleep strongly suggest that conserved regulatory processes underlie behaviour in zebrafish and mammals [5, 6].

The substituted Pyrimidine derivatives were found to possess wide range of pharmacological activities of clinical importance in the view of their extensive occurrence in nature and low toxicity. Pyrimidine derivatives were associated with vast pharmacological activities such as. Anti-inflammatory [7-9], analgesic [7, 9, 10], anti-bacterial [7, 11, 12], anti-HIV [13], anti-pyretic [10], neurotropic [14], anti-cancer [15-17], anti-microbial [18] and anti-epileptic [19-, 21] etc. activities. In this research we describe different protocol to assess complex behaviors of zebrafish such as sedation and stress after introducing novel pyrimidine derivative sensitivity to light, dryness, grittiness sensation, itchy and scratchy feeling, watering of eyes, and swelling of eyelid. The conjunctivitis caused due to bacteria known as bacterial conjunctivitis.

Heliotropium indicum(HI) linn (Family Boraginaceae) is a medicinal plant. It has various medicinal uses in the treatment of disease conditions such as abdominal pains, amenorrhoea, aysmenorrhoea, skin rashes, wounds, hypertension, ocular infections, convulsion and dizziness. Cold infusion of the leaves used as an enema stops abdominal pains; this preparation also removes cataract in the eye; the juice from the leaves is squeezed into the eye to stop dizziness; decoction of the whole plant is used to treat convulsion in children. Other medicinal uses of *Heliotropium indicum* linn comprises the use of juice of the leaves as an antiseptic and anti- inflammatory agent when applied to wounds, sores, boils, gum boils and pimples on the face. Boiled with castor oil, it is applied to sores from scorpion bites and also locally used in treating ophthalmic disorders like erysipelas and pharyngodynia[1,2]. Our aim of the project is to prepare and evaluate the eye gel from HI leaf extract to treat bacterial conjunctivitis.

2. MATERIAL AND METHODS

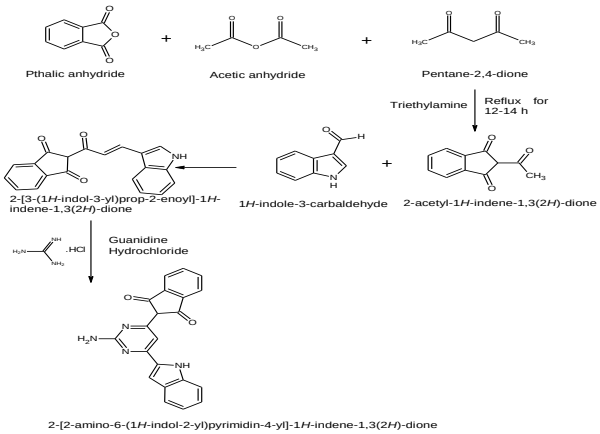
Reagent: Synthesized drug 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3 (2H)-dione was used for testing neurobehavioral study on zebra fish. Diazepam was used as standard drug and DMSO was used as solvent.

Animals housing: Total 250 adult (5-7 month-old) male and female zebrafish were obtained from a local commercial distributer (Maruthi Aquarium, Saptagiri, Bangalore). All fish were given at least 14 days to acclimate to the laboratory environment and housed in one group 40-L tank. The tanks were filled with filtered (facility) water and maintained room temperature and provided a 12-h cycle (light on at 6.00 h, light off at 18.00 h) according to the standards of zebrafish care. All fish used in this study were experimentally naïve and fed tropical flakes twice a day. Following behavioral testing, the animals were euthanized in ice cold water, and immediately dissected on ice for further analysis [24-27].

Synthesis of Pyrimidine derivative: 2-Acetyl-indane-1,3-dione was obtained by mixing pthalic anhydride (7.4 g, 0.05 M) and acetic anhydride (30.6 g, 0.3 M) with acetyl acetone (5 mL, 0.05 M). The mixture was heated under reflux until the pthalic anhydride was completely dissolved. Then it was cooled to 80˚ C and triethylamine (15mL, 0.15M) was added. After 12- 14 h, the reaction mixture was poured into a mixture of crushed ice (200 g) and concentrated HCL (50mL). The resulting precipitate was filtered and washed with water until neutral to litmus. The precipitate was dissolved in NaOH (500mL, 3% solution) and filtered again. The filtrate was acidified with HCL (1:1) to get the precipitate of 2-Acetyl-indane-1,3-dione. The product was then recrystalized from ethanol.

General scheme for the synthesis of Pyrimidine derivative:

Indole-3-carbaldehyde [0.15 g, 0.001 M] was taken in a conical flask, 2-Acetyl indane-1; 3-dione [0.2 g, 0.001 M] was added to follow by ethanol [8 mL]. Sodium hydroxide solution [10 mL, 70%] in water was added drop wise. The mixture was stirred for 25-30 minutes and was poured into crushed ice and kept overnight. Acidified with 5% concentrated HCl. The formed precipitate was filtered and washed twice with cold water. The resulting solid chalcone was collected and allowed air dry and was re-crystalised from ethanol. Chalcone was placed in a flat bottomed flask, fitted with a reflux condenser and stirred with Guanidine Hydrochloride and Ethanol. The mixture is been stirred at 80˚C-90˚C for 3-4 hours. The Solid separated filtered and recrystallized from methanol.



Acute Toxicity studies:

Acute Toxicity Study was done for dose calculation and to determine the toxic level of the synthesized product on the experimental zebrafish. The observation was done preferably for 96 h. 7 fishes was used for each test concentration including the control groups. Different concentrations in a geometric series with a factor preferably not more than 2.2 fishes will be considered dead if there will no visible movement.7 fishes were taken in each group and treated with 2.5mg, 3.5mg, 5.5mg and 12mg respectively to check the toxic dose from low range to high range of dose.

Drugs in different concentration was measured and mixed with 1% of DMSO as a solvent because drug was not soluble in water and then missed up with 1 L water. We choose DMSO as a solvent as the drug is soluble in DMSO and moreover it has not any toxic effect on fish [22, 23]. 7 fishes in each group were kept in different container containing different drug solution and were observed for 96 h. With this 4 different concentration control group and solvent control group (1% DMSO in water) were tested and confirmed that DMSO does not show any toxic effect and also it does not change any behavioral activity.

Behavioural testing:

Dive tank environment model on Zebrafish:

A novel dive tank will be designed in such a way that it will looks like trapezoidal structure where the sides of the trapezoids are lesser than as compared to upper section of the dive tank. The novel tank test, used to assess zebrafish anxiety and locomotion was a 2.5 L trapezoidal tank (15cm height × 28cm top × 23cm bottom ×7cmwidth) maximally filled with water (2 L) and divided into three equal virtual horizontal portions, by line marking the outside walls.

In Experiment 1, fish (n = 7 in each group) were pre-exposed to synthesized drug for 3 min to the novel tank filled with drug-treated water. Zebrafish behavior was recorded by trained observers, scoring the time spent in top(s), number of entries to the top, as well as the number and duration (s) of freezing bouts. Freezing was defined as a total absence of movement, except for the gills and eyes for 2 s or longer.

The tank will fix on a black counted top with exposed white back ground against its back wall to enhance contrast for video recording by using digital camera. All the fishes in the dive tank will observed and monitored for 6 mins [24-27].

Unfamiliar aquatic white/black maze environment model on zebrafish:

The animals will immerse into the main tank where it will be acclimatized for minimum period of 3 weeks before starting the experiment. Individual fish will net from the fish tank and will immerse in to the center of the section of the white/black plus maze for 6 min. All the animals for this experiment will divide into three groups each containing 7 animals. Based on the natural preference of zebrafish for dark environments, was a rectangular tank (15cm height×30cm length×16cm width) filled with water to 2 L, and divided into two equal vertical portions demarcated by black and white coloration. Fish (n = 7 in each group) were individually introduced into the black half (facing the wall), and video-recorded for 6min, scoring the latency to enter (s), time spent (s), average entry duration (s) and the number of entries to the white half (due to the dark background, zebrafish behavior in the black compartment was not assessed here) [24-27].

Open field treatment model on zebrafish:

Conceptually similar to rodent open field Test field model apparatus is constructed by using a square plastic tank which was divided in to 16 virtual squares (4×4). Among them 4 square were defined as inner zones as dotted lines another 12 square are defined as outer zone. The zebrafish will expose to the aquatic open field test tank for 6 min. Following a pre-treatment, the animals (n = 7 in each group) were individually placed in the center of the tank, and recorded for 6min. Calculate the time spent (s), distance travelled (m), and the number of visits to predefined central and peripheral zones.

The behavioral symptoms will monitor and recorded by using digital camera. The parameter which will observe is described as 1) Number of cross lines in to outer zone was observed at different time interval from starting time 1 min to time 6 min, 2) Number of cross lines to inner zone starting time 1 min to 6 min and 3) Total number of cross into inner zone[24-27].

Estimation of cortisol in various treatment model (whole body cortisol assay):

Immediately after testing, the animals were euthanized using ice cold water. Cortisol concentration in the plasma will be calculated based on the absorbance of standardized concentrations. Plasma samples were taken from fish used in Experiments 1, 2 and 3. Individual Zebrafish samples obtained from experimental and control group. Then the cortisol was estimated from the serum by ELISA method [25].

3. RESULTS AND DISCUSSION

Drug profile: 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione.

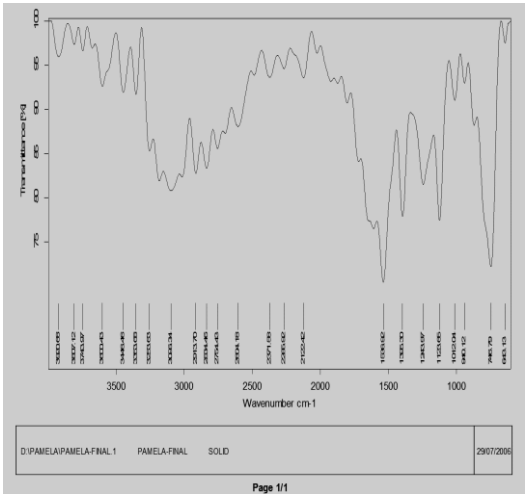
Table 1: Final step product details and physicochemical parameters

Mobile Phase: Ethyl acetate: Ethanol (2.5:0.5)

Molecular Formula	Molecular Weight	Melting Point (℃)	Percentage Yield (%)	R_f Value	Solubility	Colour
$C_{22}H_{15}N_3O_2$	353.37	281 – 284	75%	0.63	DMSO	Light Brown to Dark Brown

Identification and characterization:

The synthesized compound was identified and characterize by following method as follows: - Thin layer chromatography, Melting point and Infrared spectroscopy



Graph 1: FT-IR spectra of 2-[2-amino-6-(1*H*-indol-2-yl)pyrimidin-4-yl]-1*H*-indene 1,3(2*H*)-dione

Table 2: FT-IR spectral details of 2-[2-amino-6-(1*H*-indol-2-yl)pyrimidin-4-yl]-1*H*-indene-1,3(2*H*)-dione

Sl. No	Functional group	Wave number(cm^{-1})
2	C-N (aromatic stretch –amines)	1243.97
6	C-H (stretch-alkanes)	2913.70
7	=C-H (stretch-alkanes)	3096.34
8	N-H (stretch)	3253.63

The entire experimental results are discussed according to different zebrafish investigative model. Mortality rates at different doses at different interval of time are shown in table 3.

Acute toxicity study:

The results showed that no fishes died during the acclimatization period in control group and lower dose group during the treatment of 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione in zebrafish. In addition no mortality was found for control and lower group (table no 3). LC_{50} value for 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione was found as 5.5 mg/l. The LC_{50} values of drug were selected for further experimental therapeutic dose calculation. **LC_{50}** can be defined as the median lethal concentration in water which kills 50 per cent of test animals within a specific period of exposure. Dose selection for 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione: From the toxicity study we can conclude that 12 mg is the lethal concentration and it's a toxicdose and LC_{50} value is 5.5 mg. So depending upon the study report we took 1/5 and 1/10 of the LC_{50} value.

Table 3: % mortality of zebrafish during different time interval after the exposure to different concentration of 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione

Group	No of fish	Dose (mg/l)	Mortality (Number)				% mortality
			24 h	48 h	72 h	96 h	
1	7	2.5	0	0	0	0	0
2	7	3.5	0	0	0	0	0
3	7	5.5	3	0	0	0	43
4	7	12	7				100

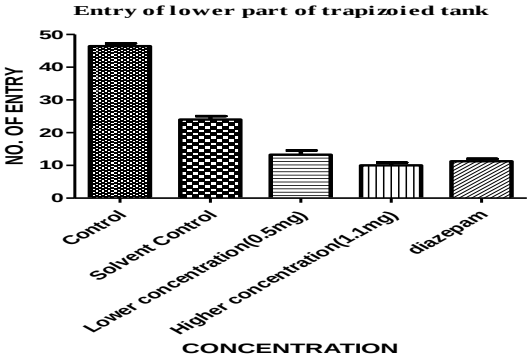
Dive tank environment model on Zebrafish:

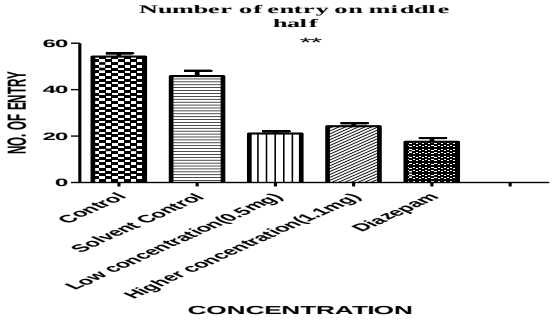
After study of various parameters [table no 4, Number of entry in lower part, Time spent in lower part (s), Time spent in middle half (s), Number of entry on middle half, Number of entry on 2/3 half, Time spent in 2/3 half (s)] in different groups of zebrafish we conclude that after giving the dose of 1.1mg/L of 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione fishes are mainly preferred middle and upper portion of the tank. Table no 4 represent the number of cross upper to two thirds from the lower zone by experimental zebrafish in different treatment groups. When we compared three groups (standard diazepam, test drug 1.1mg/L, test drug 0.5mg/L) it was showing that fishes in test drug 1.1mg/L prefer to stay on the upper part of the trapezoidal tank, as well as the entry duration of the fish on the upper and middle part is also maximum as compare to lower part. Which indicate the stress level of the fish of 1.1mg/L group is reduced.

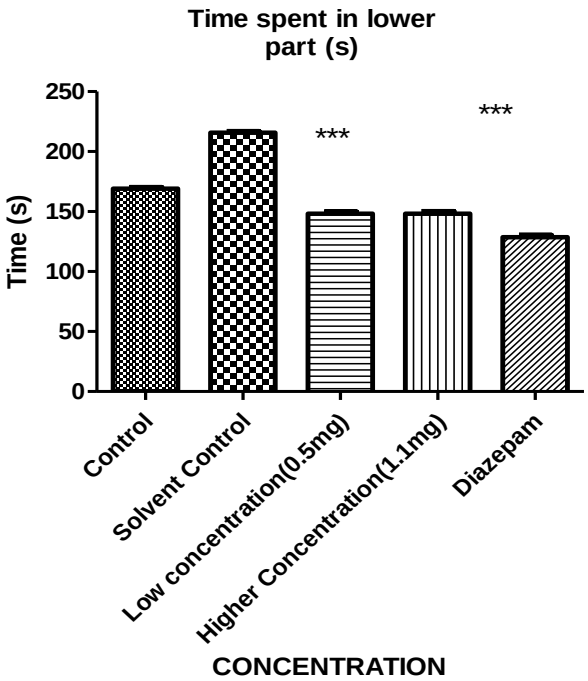
Table 4: Study of various behavioral parameters on Trapezoid tank

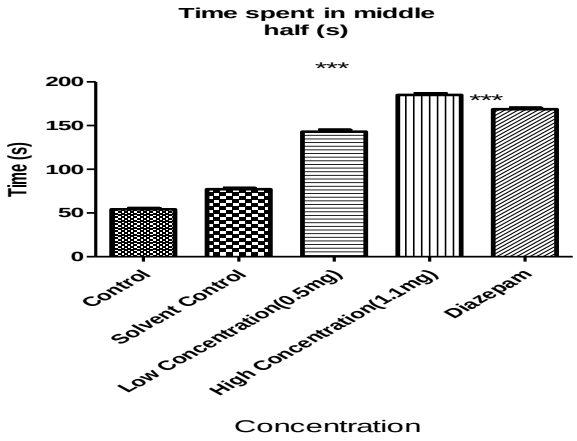
Group name (n : 7)	Treatment Treatment time: 6mins	Number of entry in lower part	Time spent in lower part (s)	Time spent in middle half (s)	Number of entry on middle half	Number of entry on 2/3 half	Time spent in 2/3 half (s)
Control	no treatment	46.43 ±0.84	169.0 ±1.25	54.29 ±1.3	124.4 ±1.8	25.29 ±1.08	61.57 ±1.13
Solvent control	1% DMSO solution	24 ±1.069	215.7 ±1.340	77.29 ±1.322	45.86 ±2.27	21.14 ±1.056	62.57 ±1.270
Low concentration	0.55mg + 1% DMSO	13.29 ±1.26	148.1*** ±1.72	143.0*** ±2.03	21.14 ±0.91	9.57 ±0.89	69.00* ±2.4
High concentration	1.1mg + 1% DMSO	10.00 ±0.87	148.3 ±1.94	185.1 ±1.73	24.29 ±1.30	15.86 ±1.43	55.29 ±1.78
Standard (Diazepam)	5mg +1% DMSO	11.29 ±0.74	128.6*** ±1.94	168.7*** ±1.53	17.57* ±1.60	5.85*** ±0.91	61.71* ±1.107

Results were expressed as Mean ± SEM, (n=7), ****P <0.05, **P<0.01, ***P < 0.001* compared to control and solvent control treated fishes.

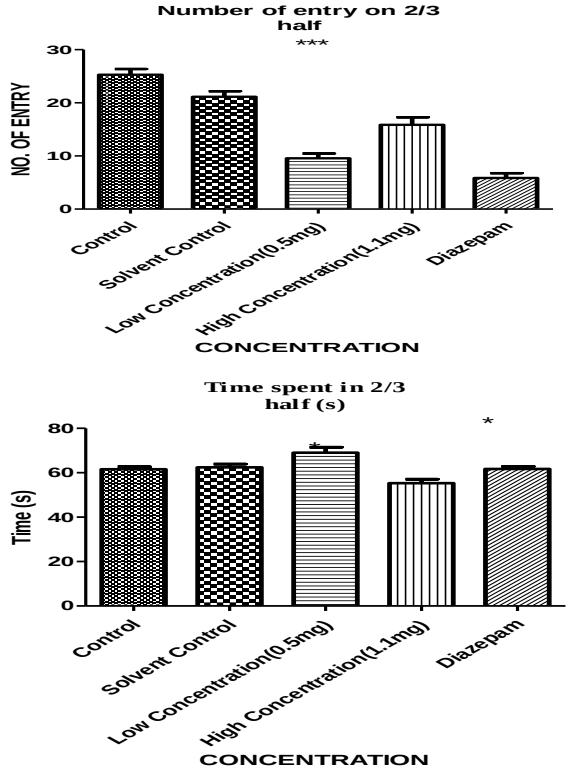








Group name (n = 7)	Treatment	Treatment time (min)	Time spent in white zone	Number of entry to white zone	Number of entry to black zone	Time spent in black zone
Control	No treatment	6	186.8±1.72	52.0±0.5	48.67±0.71	135.7±0.71
Solvent control	1% DMSO	6	256.00± 0.57	57.17±0.60	56.0±0.577	102.00±0.73
Low Dose	0.5 mg + 1% DMSO	6	55.33***± 1.44	22.17***± 0.47	19.50***± 0.42	300.2***±0.60
High Dose	1.1 mg + 1 % DMSO	6	15.17 ±0.70	7.0±0.57	7.5±0.42	339.3±0.66
Standard (Diazepam)	Diazepam + 1% DMSO	6	21.17***± 0.70	12.50***± 0.70	15.50***± 0.76	334.00***±0.55



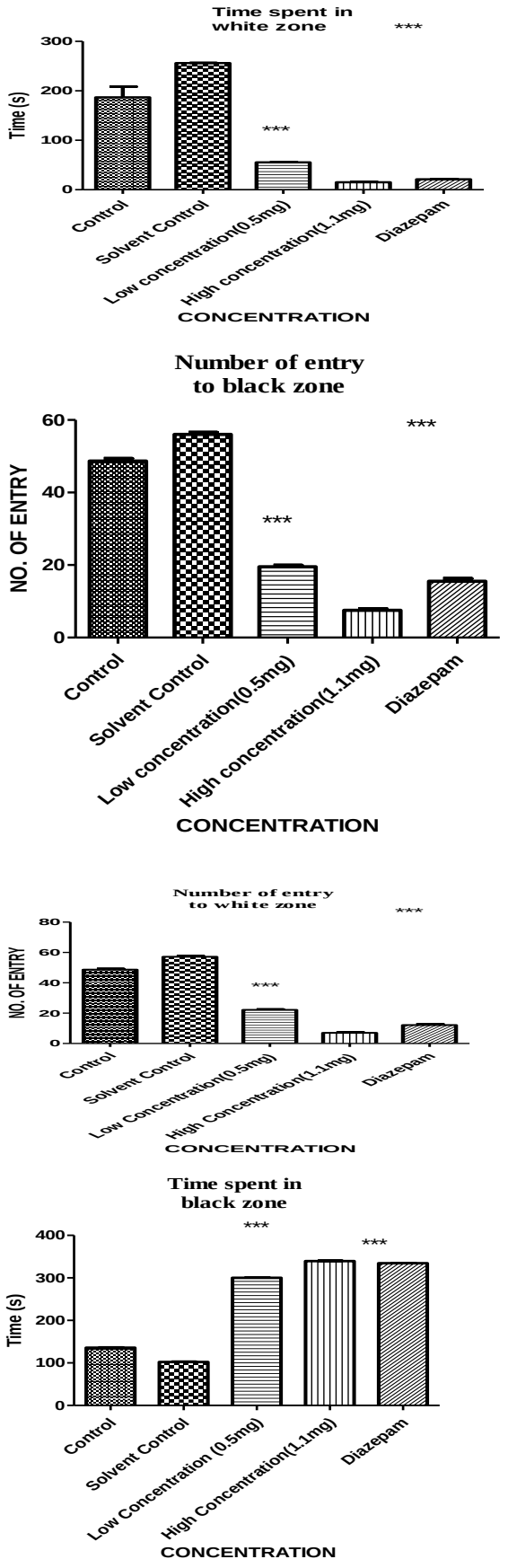
Graph 2: Behavioral effects of 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione (1.1mg/L and 0.5mg/L) on zebrafish tested in the novel tank test.

Unfamiliar aquatic white/black maze environment model on zebrafish: After study of various parameters [table no 5, Treatment time(min), Time spent in white zone, Number of entry to white zone, Number of entry to black zone and Time spent in black zone] in different groups of zebrafish we conclude that after giving the dose of 1.1mg/L of 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione fishes are mainly preferred black zone due to sedation.

Table 5: Study on various neurobehavioral parameters of zebrafish in Light/Dark Model

Results were expressed as Mean ± SEM, (n=7), ***P <0.05, **P<0.01, ***P < 0.001** compared to control and solvent control treated fishes.

When we compared three groups (standard diazepam, test drug 1.1mg/L and test drug 0.5mg/L) it was showing that fishes in test drug 1.1mg/L prefer to stay in black zone and they are showing longer freezing time than other two groups. Which indicate that this drug have sedation action on brain and also the stress level of the fish of 1.1mg/L group is reduced.



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Open field treatment model on zebrafish:

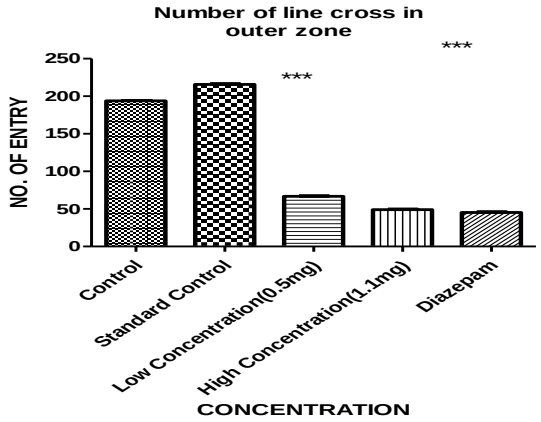
After study of various parameters [table no 6, Time spent in outer zone(s), Number of line cross in outer zone, Time spent in inner zone (s) and Number of line cross in inner zone] in different groups of zebrafish we conclude that after giving the dose of 1.1mg/L of 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione fishes are mainly preferred inner zone due to sedation.

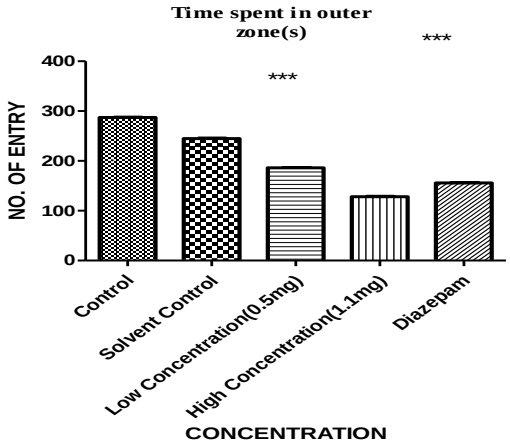
When we compared three groups (standard diazepam, test drug 1.1mg/L, test drug 0.5mg/L) it was showing that fishes in test drug 1.1mg/L prefer to stay in inner zone and they are showing longer freezing time than other two groups. Which indicate that this drug have sedation action on brain and also the stress level of the fish of 1.1mg/L group is reduced.

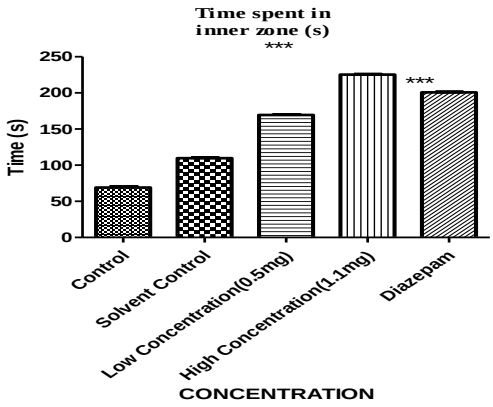
Table 6: Study of various neurobehavioral parameter on zebrafish in open field

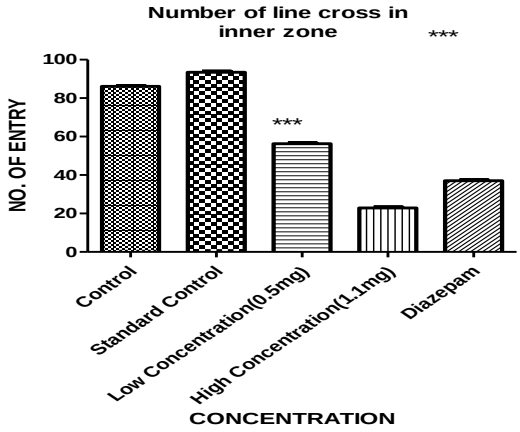
Results were expressed as Mean ± SEM, (n=7), ***P <0.05, **P<0.01, ***P < 0.001* compared to control and solvent control treated fishes

Group (n = 7)	Treatment	Treatment time (min)	Time spent in outer zone(s)	Number of line cross in outer zone	Time spent in inner zone (s)	Number of line cross in inner zone
Control	No treatment	6	250 ±0.57	193.9 ±0.34	69 ±1.5	86.14 ±0.34
Solvent control	1% DMSO solution	6	244 ±0.76	215.7 ±0.60	109.7 ±0.56	93 ±0.56
Low concentration	0.5 mg Drug in 1% DMSO	6	186.3*** ±0.42	66.71*** ±0.42	169.6*** ±0.48	56.29*** ±0.42
High Concentration	1.1 mg Drug in 1% DMSO	6	128.3 ±0.42	149.14 ±0.50	225.3 ±0.56	22.86 ±0.508
Standard (Diazepam)	Diazepam in 1% DMSO	6	155.9*** ±0.5	45.29*** ±0.56	200.7*** ±0.56	37.0*** ±0.48









Graph 4: Behavioral effects of 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione (1.1mg/L and 0.5mg/L) on zebrafish tested in the novel tank test. Results were expressed as Mean ± SEM, (n=7), ****P* <*0.05, ***P*<*0.01, ****P* < *0.001* compared to control and solvent control treated fishes.

All the statistical analysis were performed using statistical program Graph Pad. For each study treatment and standard group are compared using an Analysis of Variance (ANOVA).

Cortisol estimation: In cortisol estimation the result shows (table 5.5.1) that the cortisol level decreased in test group compared to the standard group. So we can conclude that this drug 2-[2-amino-6-(1-H-indol-2-yl) pyrimidine-4-yl]-1H-indane-1,3(2H)-dione have stress reducing activity on brain.

Table 7: Cortisol value determination table

GROUP	VALUE (µg/dL)
Control (No drug)	1.05
Standard (Diazepam)	0.66
Test	<0.5

Discussion:

The synthesized drug Pyrimidine Derivative should significant property of anti-anxiety and sedation activity. The drug at contraction level of 1.1mg/ l is very good in anti-anxiety and sedation activity. At 0.5 mg/l it is showing little bit less and comparable to diazepam. our study describe the stress reducing property and the sedation activity of the synthesized Pyrimidine Derivative. The result of Drive tank environment model shows the duration of time fish spent in the middle and bottom part of the tank is more than diazepam standard group and control group. The result can be described as the drug has potential stress reducing activity.

In the Unfamiliar aquatic white black maze environment model the test group fish preferred black zone rather than white zone and also time fish spent in black zone is more when it is compared with standard and control group. So it can be described as the drug have potential sedation as well as stress inducing activity that's why the fishes preferred black zone more than white zone. In the Open field treatment model the fishes of the test group drug preferred inner zone to stay and as well as time spent in inner zone is more as compared with control and standard group. The result can be described as the drug have potential stress reducing as well as sedation activity.

The cortisol level after the treatment of the synthesized drug showing less concentration as compared to diazepam treated zebra fish. This change of the cortisol concentration level can be described as the drug have potential stress reducing activity because cortisol level decreases when the

stress level is decreases.

The whole study is generating an important aspect of novel synthesized drug. The drug is pyrimidine based and has a good ant sedative activity in zebra fish.

Although the mechanism of action is not well established but the environmental features of the animal and the neuro cortisol levels in zebra fish body will surely open an area for future research.

CONCLUSION

Recent reviews have summarised that zebra fish is showing neurobehavioral activities on various drugs as zebra fish is important tool for molecular study. As pyrimidine is having various activities on brain so it was synthesized and use on the brain of zebra fish and it was showing sedation action. So this work was designed to find out the common structural features required to possess good neurobehavioral activity. More experiments work is needed to modify in pyrimidine derivative to find out the effective pharmacophores feature required for the drug to possess good sedation activity.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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