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Original Research Article

Zebra fish behavioral assessment by using *Calycophyllum Spruceanum* bark methanolic extract

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ABSTRACT

In neurobehavioral research, animal model has played a crucial role in yielding experimental data as well as, in the development of new insights. Researchers are always trying to develop novel animal models to understand fundamental features of physiological, behavioral and psychological disorders. Zebra fish (Danio rerio) is now considered globally as a new and useful model in biomedical research. The objective of the present study was to assess behavioral changes in adult zebra fish after acute exposure to different pharmacological and herbal compound. Adult zebra fish of 4-5-months-old were exposed to different concentrations of unknown test drug with the standard for 5 min. The test was conducted separately for each drug concentration as well as control. Behavioral activity parameters viz. novel tank test and light and dark transition test were recorded for each animal during the exposure period. Results: Zebra fish exposed to Test drug (Calycophyllum Spruceanum) showed change in behavioral activity with significant to the standard drug caffeine. In view of the above findings, these results suggested that exposure of adult zebra fish with drug produce the expected changes in the behavior like stress and anxiety; therefore, adult zebra fish can be used an alternative approach for the assessment of new chemical entities for their effect on behavioral activity.
Dark Transition Test.

1. Introduction

The majority of safety issues concern with central nervous system (CNS) stimulant/depressant activity of new chemical entities (NCEs) has been evaluated using locomotor activity parameter in rodents. During drug development, effect on locomotor activity is an undesirable property and is typically detected only in later stage of preclinical safety studies conducted on higher vertebrates. The need for detection of these effects is essential at early stages of drug discovery. Over the last decade, zebra fish (*Danio rerio*) one of the unique vertebrate models has rose to prominence for assessing drugs in vivo with respect to a wide range of toxicological and safety pharmacological evaluation because of fully sequenced genome and highly conserved genetic pathway between zebra fish and human. Recent research has revealed the emergence of zebra fish as a model for neurobehavioral studies since zebra fish larvae display learning, sleep, drug addiction, locomotor behavior, and other neurobehavioral phenotype that can be related to those seen in human. The overall organization of the zebra fish brain is similar to other vertebrates and the blood brain barrier is functional at 10 days post-fertilization (d.p.f.). Zebra fish takes up hydrophilic substance easily from the water through the gills and from entire body surface which makes easy to develop CNS models in zebra fish. In this respect, recent studies identified the potential of the larvae zebra fish as well as adult zebra fish for their locomotor

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behavior.[1]

2. Materials and Methods

2.1. Experimental animals[2]

Around 4-5-month old, stock of adult zebra fish (Danio rerio) of either sex was purchased from a local vendor (Hyderabad, India) and acclimatized for at least 1 month before starting the study. For all experiments zebra fish were housed in a 40-L tank, filled with deionized water maintained at 28- 30°C and pH = 7.0-8.0 with constant filtration and aeration. Water conditioning and environmental quality was maintained according to the aquarium system use and care manual and The Zebra fish Book.[26] A dark-light cycle of 12-h light:12-h dark (on: 8:00 am; off: 20:00 pm) was maintained. hardness (75–200 mg/L CaCO3), ammonia, dissolved oxygen (7.8 mg/L at 28.0 °C), salinity (0.25-0.75 ppt) and conductivity was monitored on regular basis to ensure good water quality for housing zebra fish [27]. Utmost care was taken to ensure that all the animals were treated humanely. Zebrafish were fed twice daily with tetra bits adult zebrafish diet (Spectrum Brands Company, Germany), and the diet was supplemented with live artemia. Behavioral testing of drug effects took place during the light phase between 10:00 am and 3:00 pm.

2.2. Chemicals used

Caffeine (1,100 μM)

2.3. Plant material collection

The barks of the plant, Calycophyllum Spruceanum were collected and authentication was done by botanist Dr. Madhava Chetty, Assistant Professor, Department of Botany in S.V. University, Tirupati.

2.4. Preparation of extracts

The bark of a fresh plant was collected and the adhered dirt was removed. The leaves were removed and cleaned with distilled water, blotted and dried by the shade instead of sun light. The shade-dried material was powdered using a commercial mixer. This powder was further sieved to get a fine powder and was utilized for solvent extraction. Around 100 g of the powder was kept for the Soxhlet extraction using 1000 ml methanol. This particular cycle was repeated again and again, for hours to a few days, till the color of the solvent faded away in the siphon of the Soxhlet. The extract was then concentrated under reduced pressure and stored in a refrigerator before further use. At the end of the process of the hot extraction, the extract was filtered by filter paper. The extract was then placed in desiccators to get rid of remaining moisture, if present, and lastly, was preserved in air tight containers at 4o C in a refrigerator.[3]

3. Experimental Procedure

3.1. Behavioral test[4–6]

Behavioral tests were conducted between 10:00 AM to 3:00 PM. Before experimentation, zebra fish were transferred in their home cages from the animal unit to the experimental room one hour before each test session. After the habituation period in the laboratory the zebra fish were subjected to the test. In each study the zebra fish were randomly selected (n=8) and divided into groups; Group-I placebo control and group-II drug (caffeine 1, 100 μM) treated. The drug was prepared immediately before use and administered through dissolving in water. Individual zebra fish was transferred from its home tank to a 250 ml beaker filled with 200 ml de-chlorinated water (control) or 200 ml de-chlorinated drug treated water. Each subject was randomly assigned to a treatment group. One animal at a time from particular group was immersed in a solution containing the drug/vehicle for 30 min prior to behavioral observation. Experiments were conducted 30 minutes after vehicle/drug administration to the respective group. All the apparatus was cleaned thoroughly before and after each trial to remove any trace of odor. The experiment was done in a sound attenuated room and each six-minute test sessions was recorded via an overhead video camera which was used to analyze the behavior later. After six minutes the zebra fish was removed from the test tank. A number of tests session were conducted to observe the behavior of zebra fish. All behavioral recordings were carried out with an observer not aware of the treatment and behavioral end points of the zebra fish.[7]

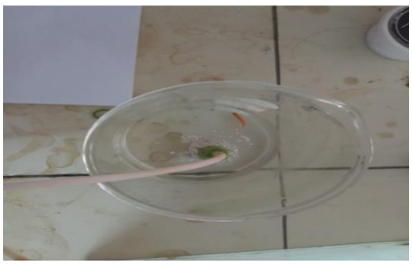


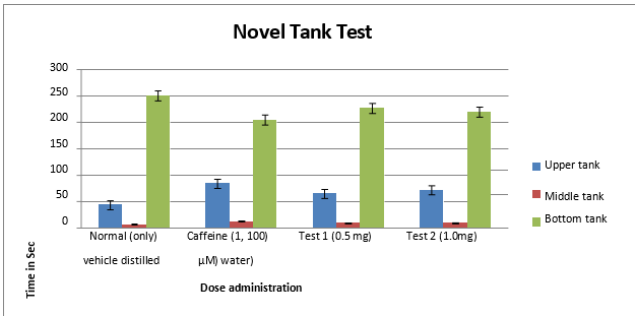
Fig. 1: Performing behavioral testin using caffeine standard drug.

Fig. 2: Performing behavioral test in using caffeine standard drug.

4. Result

Table 1: Results showing the CNS responsein seconds by zebra fish after drug administration.

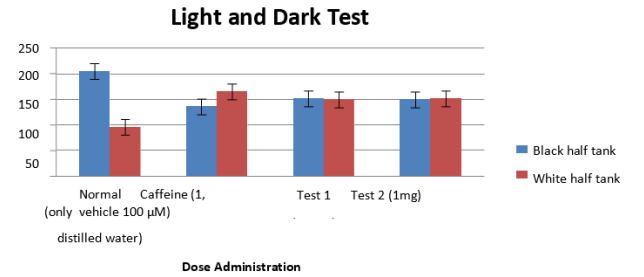
Drug/Dose	Novel Tank Test (Sec)		
Tank no.	Upper tank	Middle tank	Bottom tank
Normal (only vehicle distilled water)	44±0.82	7±1.41	249±1.41
Caffeine (1, 100 μM)	84±1.15	12±2.71	204±2.45
Test 1 (0.5 mg)	65±2.94	9.25±2.63	225.75±4.79
Test 2 (1.0mg)	71.75±3.59	9.75±1.71	218.5±2.52



Graph 1: Graph showing the CNS response in seconds by zebra fish after drug administration.

Table 2: Results showing the CNS response in seconds by zebra fish after drug administration

Light/Dark Transition Test		
Drug/Dose	Black half tank	White half tank
Normal (only vehicle distilled water)	205±0.00	95±0.71
Caffeine (1, 100 μM)	135±0.71	165±2.12
Test 1 (0.5mg)	151±0.71	149±1.41
Test 2 (1mg)	149±1.41	151±1.41



Graph 2: Graph showing the CNS response in seconds by zebra fish after drug administration

5. Discussion

The effect on CNS behavior (convulsion or locomotor activity) in adult as well as larval zebra fish has been extensively studied using narcoleptics or alcohol. The present study provides the preliminary evidence that the adult zebra fish may be an excellent tool for early stage pharmacological and/or safety investigation of the new chemical entities for their effects on locomotor behavior and the possible involvement of different receptors and mechanism. To prove this, we investigated the effect on locomotor activity of different category of established psychomotor stimulants such as caffeine (inhibitor of adenosine A1/A2A receptor and phosphodiesterases); scopolamine (centrally acting nonselective muscarinic receptor antagonist with both psychostimulant and depressant properties depending upon dose); and psychomotor depressants, that is, clonidine ($\alpha 2$ agonist), diazepam (benzodiazepine), pentobarbitone (barbiturates), ramelteon (MT1/MT2 blocker), chlorpromazine (antipsychotic), venlafaxine and imipramine (antidepressant), and rasagiline (anti-Parkinson) on adult zebra fish. As per our knowledge no extensive study has been conducted on zebra fish using these compounds. To validate this model each fish was exposed into different drug concentrations for 15 min. The reason behind selecting the experiment duration of 15 min is on the basis of study conducted on the effect of alcohol in zebra fish. In this study, brain level of alcohol was estimated after 15 min of exposure and it was found to be 90% of the tank alcohol concentration.

By assuming the same achieved concentration with these drugs we exposed each zebra fish up to 15 min. Using zebra fish in the standard procedure, it is observed that out of 11 compounds tested, nine compounds decrease locomotor behavior as anticipated, while caffeine showed biphasic response and scopolamine did not show any changes in locomotor activity. Clonidine, a known $\alpha 2$ agonist, inhibits noradrenergic activity by acting on presynaptic auto-receptor known to decrease locomotor activity in small animals. The findings of our study are

in line with above reported studies which support the involvement of noradrenergic pathway in zebrafish. The effect of pentobarbitone, diazepam, and ramelteon (all are known sedatives) on locomotor behavior of zebrafish was evaluated. Benzodiazepines and barbiturates are known to cause sleep promoting effect to the loss of consciousness in humans and other mammals. The inhibitory effect of conventional hypnotic agents (pentobarbitone and diazepam) are due to activation of GABAA receptors is the major mechanism of depressant/sedative action which potentiate the rest behavior in zebra fish as we found in our study. It has been already reported that the brain of adult zebra fish has higher expression of MT1 receptor than MT2. Ramelteon is a selective melatonin receptor (MT1 and MT2) agonist promotes the effect of melatonin on sleep. Our results of ramelteon in zebra fish showed significant reduction in locomotor activity confirms its action more towards through MT1 receptor.[8] To the best of our knowledge, it's a first kind of study on the effect of locomotion behavior of adult zebra fish using a selective melatonin receptor agonist. Chlorpromazine a typical antipsychotic drug is known to produce extrapyramidal motor effects associated with blockade of dopamine receptors (D2) in the basal ganglia. Preclinically however, one of the most consistent effects observed with the acute administration of chlorpromazine is suppression of voluntary behavior including motor. Our results are also in agreement with previous findings in rodents showed significant decrease in locomotor behavior supports the involvement of dopaminergic pathway in zebra fish. Rasagiline is reported as a selective irreversible monoamine oxidase (MAO)-B inhibitor at low dose and inhibits MAO-A at higher dose. It has anti-Parkinson and neuroprotective action.[9] observed that concomitant irreversible blockade of both MAO-A and -B, long exposure/chronic treatment, and accumulation of extracellular monoaminesmay be necessary to induce locomotor activation. Selective inhibition of MAO-B does not cause significant changes in CNS steady state level of norepinephrine (NE), dopamine (DA) and serotonin (5-hydroxytryptamine (5-HT)). However, it has been anticipated that the used dose of rasagiline in this experiment caused unexpected decrease in locomotor activity in zebrafish.[7] High dose of rasagiline may be causing blockade of MAO-A, which is responsible for most of the adverse effects of MAO-A inhibitors or less systemic exposure failed to induce proper response to increase DA and 5-HT level which are required for hyperactivity. Another class of compounds venlafaxine and imipramine, known antidepressants, has sedation and decreased psychomotor activity as the most common side effects of these drugs. Alterations in monoamine (DA, 5-HT, and NE) level are likely to disturb both psychomotor activity and mood. Previous studies have shown that acute and chronic administration of norepinephrine transporter (NET) inhibitors and serotonin transport protein (SERT) inhibitor alone, decrease motor activity in rodents. Imipramine is a tricyclic nonselective NE transporter (NET) and serotonin transporter (SERT) inhibitor, while venlafaxine is a selective NET and SERT inhibitor and a moderate inhibitor of DA reuptake that increases the level of NA and serotonin in rat hippocampus and exhibits decrease in locomotor activity of zebrafish after acute exposure up to 15 min as expected. Among all the tested drugs, scopolamine was the only one which did not show significant effect on locomotion as reported in rodents. Loss of inhibition of mesopontine cholinergic neurons via muscarinic receptors is one mechanism by which scopolamine could increase locomotion in rodents. In our experiment, nonsignificant increase in total mobility time and decrease in immobility time was observed with no changes in distance traveled and speed of zebrafish compared to control suggesting the probability of low systemic exposure of scopolamine that failed to inactivate muscarinic receptors properly. In this study exposure of zebrafish to nonselective A1 and A2A receptor antagonist caffeine showed a biphasic response pattern similar to reported in rodents. Significant decrease in locomotor activity was observed at higher dose which was probably due to A1 receptor blockage and increased locomotor activity found at low doses was probably due to A2A receptor blockage. Taken together, the data presented here describes that adult zebrafish is sensitive enough to different classes of compounds already known for their effect on locomotor behavior and opens further possibilities to explore the utility of adult zebrafish as a model for initial pharmacological and safety pharmacology evaluation of NCEs.

Our results confirm that when a zebra fish is presented to an unfamiliar environment it shows robust anxiety-like behavioral responses, which were ensured by using the novel tank diving test, open field test and light/dark transition. Psychoactive drugs like diazepam reverted anxiety-like behavioral responses, due to high sensitivity, the behavioral and physiological endpoints of the zebra fish can be manipulated. Due to this high sensitivity and manipulation, the novel tank paradigm possesses a great potential for use in the screening of novel compounds of possible therapeutic value. behavioral endpoints such as thigmotaxis (staying closed to the walls)[10]decreased exploration and freezing found in mice or rats which indicate anxiogenic behavior is now applied to zebra fish model of anxiety. Centre and periphery ratio of rodents in open field test and top: bottom ratio in novel tank test is similar kind of behavioral endpoints. Studies have indicated that similar environmental conditions cause anxiety like behavior both in rodents as well as in zebra fish. Fish can also form special memories just like the rodents and use them to guide themselves and establish safe zones in novel environment. A similarity has also been found

between the hyper arousal behavior found in rodents in dangerous situations and erratic movements shown by Zebra fish insignificant decrease in locomotor activity was observed at higher dose, which was probably due to A1 receptor blockage and increased locomotor activity found at low doses was probably due to A2A receptor blockage. Taken together, the data presented here describes that adult zebrafish is sensitive enough to different classes of compounds already known for their effect on locomotor behavior and opens further possibilities to explore the utility of adult zebrafish as a model for initial pharmacological and safety pharmacology evaluation of NCEs. Zebra fish was infected with S. aureus pre-incubated with grape seed extract. There was a significant decrease in the inflammatory response and mortality of zebra fish infected with S. aureus.[11]

Studies have indicated that similar environmental conditions cause anxiety like behavior both in rodents as well as in zebra fish. Fish can also form special memories just like the rodents and use them to guide themselves and establish safe zones in novel environment. A similarity has also been found between the hyper arousal behavior found in rodents in dangerous situations and erratic movements shown by Zebra fish in novel tank. Thus, zebra fish could be considered as useful animal model for the study of anxiety and screening of new drugs on the basis of comparative study of behavioral endpoints of zebra fish. The light dark preference test used for rodents is also a useful paradigm for investigating anxiety like behavior in Zebrafish. Studies show that anxiolytic drugs increase the exploratory behavior and time spent in white compartment while anxiogenic drugs cause the opposite effect. The anxiolytic effect of diazepam was observed in the present study too.[12] Open field activity test in zebra fish is extensively used in pharmacological studies. Swimming in the center of an open field suggests an anxiolytic like behavior. α- Fluoro methyl histidine shows an anxiolytic effect by increasing swimming time in the center and similar effect was observed in the present study. Exposure to chronic fluoxetine and acute ethanol reduces the erratic movements in open field. The result of the present study is critical for the validation of Zebra fish as a model of anxiety. There are several characteristics which make Zebra fish an important test subject which could prove useful in gaining a greater understanding of neuropharmacological mechanisms in mammals and facilitate behavior-based drug discovery. Since zebra fish have robust physiological responses and quantifiable behavioral and neuropathological phenotypes analogous to humans.[13] Several beneficial properties make zebra fish a promising alternative to mammalian model. Low maintenance cost and rapid life cycle of zebra fish makes easy to maintain in large number of fish in small area which is important for large scale behavioral studies. Zebra fish readily acclimatizes to new environments, is constantly active and very little disturbed by the presence of observers. These qualities make zebra fish an excellent species choice for behavioral study. Zebra fish has similarity in basic organization of brain components to that of humans which make it useful in the study of brain disorders. The zebra fish has been used in the study of neurodegenerative diseases such as Parkinson's disease, Huntington's and Alzheimer's diseases.[14]

Reported the use of zebra fish model and PC-12 cell line for evaluating the neuroprotective effect of ethanol extract of the fruit of Alpinia oxyphylla. The basic and complex brain phenomena as well as endocrine mechanisms of zebra fish and mammals are substantially homologous. Just like humans, zebra fish employs cortisol as the primary stress response hormone unlike corticosterone by rodents. The hypothalamus pituitary inter renal (HPI) axis of zebra fish is homologous to the hypothalamus pituitary adrenal (HPA) axis of humans. Cortisol is the primary stress hormone in both species. Zebra fish model enables greater insight into neural mechanism associated with anxiety related disorders since it possesses all the classical vertebrates' neurotransmitters and its neuroendocrine system yields robust cortisol responses to stress. Much like rodents, zebra fish has the ability to learn through classical conditioning. It also offers an alternative and efficient mode of drug delivery via the gills.[15] Zebra fish can serve, therefore, as an inexpensive and potentially high throughput model for behavioral phenotyping and psychopharmacological screening of medicinal plants for drug development.

6. Conclusion

Zebra fish can serve, therefore, as an inexpensive and potentially high throughput model for behavioral and psychopharmacological screening of medicinal plants for drug development. In view of the above findings, these results suggested that exposure of adult zebrafish with *Calycophyllum Spruceanum* Bark Methanolic Extract produce the expected changes in the behavior like stress and anxiety; therefore, adult zebrafish can be used an alternative approach for the assessment of new chemical entities for their effect on behavioral activity.

7. Source of Funding

None.

8. Conflict of Interest

None.

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