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Research Article

STUDIES ON SENSITIVITY OF ZEBRAFISH AS A MODEL FOR PARKINSON'S DISEASE: COMPARISON WITH MICE MODEL

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ABSTRACT

Objective: The objective of the study is to evaluate the antiparkinsonian activity of hydroalcoholic extract of the seeds of *Canavalia gladiata* (HECG) in zebrafish and Swiss albino mice.

Materials and Methods: Catalepsy was induced in zebrafish by exposing them to haloperidol solution. Treatment groups were exposed to bromocriptine and HECG, 30 min before haloperidol exposure at the dose of 2, 5, and 10 µg/mL. Latency to travel from one fixed point to another, time spent near the bottom of the tank, and complete cataleptic time were evaluated to assess behavioral changes. In mice, catalepsy was induced using haloperidol (1 mg/kg i.p.). Treatment groups received bromocriptine (2.5 mg/kg) and HECG at the dose of (100, 200, and 300 mg/kg) orally. Bar test for catalepsy, motor coordination test by rotarod, and locomotor activity by actophotometer were carried out to assess behavioral changes.

Results: Bromocriptine and HECG-treated groups showed significant difference in behavioral parameters as compared to haloperidol control group in both the experimental models.

Conclusion: *Canavalia gladiata* seeds exhibited significant antiparkinsonian activity in haloperidol mouse model and zebrafish. Zebrafish can be used with ease and effectiveness for initial screening of drugs before subjecting them to rodent testing.

Keywords: *Canavalia gladiata*, Catalepsy, Haloperidol, Zebrafish.

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease of the central nervous system which mainly affects the motor system. It is also known as slowly progressive neurodegenerative disease where there is a loss of dopaminergic neurons projecting from *substantia nigra* pars compacta toward neostriatum leading to the imbalance between dopamine and acetylcholine. In addition to this, proteins called Lewy bodies accumulate in dopaminergic neurons [1,2].

Some of the toxins such as rotenone, paraquat, and MPTP which are known to produce PD such as symptoms in mammals also cause dopaminergic loss in zebrafish. Antipsychotics such as haloperidol which act by temporary blockade of dopaminergic neurons are known to produce cataleptic movements in zebrafish, which leads to aberrant swimming patterns (upside down, circular, and arrow-like swimming toward bottom) [3,4].

Zebrafish is becoming an attractive model organism for understanding biology and developing therapeutics, because as a vertebrate, it shares considerable similarity with mammals in both genetic compositions and tissue/organ structures and yet remains accessible to high throughput phenotype-based genetic and small-molecule compound screening [5]. Zebrafish models have significantly contributed to our understanding of vertebrate development and more recently human disease. The growing number of genetic tools available in research on zebrafish has resulted in the identification of many more genes involved in developmental and disease processes [6]. Phytomedicines are well tolerated, with fewer side effects; in contrast, synthetic drugs can be highly effective, their usage is often hampered by severe side effects [7]. Hence, in this research, we studied the antiparkinsonian activity of hydroalcoholic extract of the seeds of *Canavalia gladiata* (HECG) (test drug) and bromocriptine (standard drug) in zebrafish, which was then compared with their effect on mice to determine the utility of zebrafish as an animal model for PD.

MATERIALS AND METHODS

Plant material

Fresh plant specimen of *Canavalia gladiata* was collected and authenticated by Dr. Praveen Kale, Botanist at St. Xavier's College, Mumbai - 400 001, and specimen was submitted to the Department of Pharmacology, Oriental College of Pharmacy (OCP), Sanpada, Navi Mumbai.

Extraction

The seeds were cleaned, dried, and powdered. The dried powder was extracted with a mixture of 90% v/v ethanol (50%) and distilled water (50%) in a 250 ml Soxhlet at 60°C. The solvent obtained was evaporated to remove excess of the solvent, concentrated, and then used for the study. The yield was observed to be 18% w/w.

Preliminary phytochemical screening

The phytochemical test of the hydroalcoholic HECG was performed for the presence of various phytoconstituents (carbohydrates, proteins, steroids, glycosides, saponins, flavonoids, alkaloids, phenolic compounds, and tannins) using standard procedures [8,9].

Animals

Zebrafish

Zebrafishes were procured from Vikrant Aquaculture, Mumbai. Adult wild-type AB strains of zebrafish (3–5 cm) of both the sexes (4–6 months old) were used. The fishes were habituated to the laboratory conditions for at least 14 days and housed in a tank filled with unchlorinated aquarium water and continuous aeration. Density of five fishes per liter was maintained. Animals were kept on 14:10 h

light/dark cycle and were fed twice a day with aquarium food (blood worms).

Swiss albino mice

Swiss albino mice procured from Anjuman-I-Islam's Kalsekar Technical Campus, New Panvel, were used for the study. They were acclimatized in the animal house of Oriental College of Pharmacy. Animals were fed standard diet, and water was given *ad libitum*. 12:12 h light/dark cycle was maintained. All animal protocols were approved by IAEC of Oriental College of Pharmacy.

Drugs and chemicals

Hydroalcoholic Hydro alcoholic extract of the seeds of *Canavalia gladiata* (100 mg/kg, 200 mg/kg, and 300 mg/kg).

Standard drug: Bromocriptine (Inga Pharma Pvt. Ltd., Mumbai).

Drug to induce catalepsy: Haloperidol (injection Serenace) (RPG Life Sciences, India).

Sodium carboxymethyl cellulose (Loba Chemie, Mumbai).

Dimethyl sulfoxide (DMSO) (Encore, Mumbai).

***In vivo* behavioral studies**

Haloperidol-induced catalepsy in zebrafish [10]

Fishes were divided into eight groups (n=6), namely, vehicle control, haloperidol control, bromocriptine (2, 5, and 10 µg/mL), and HECG-treated group (2, 5, and 10 µg/mL). Behavioral study was done during day phase, between 10:00 am and 5:00 pm.

Each fish from bromocriptine and HECG-treated groups were individually exposed to the solution of respective drugs at the concentrations of 2, 5, and 10 µg/mL in a 300-mL beaker for 30 min. Once this exposure was given, then fishes were transferred to another beaker containing fresh aquarium water, where they were kept for 15 min, then fishes from all treatment groups were individually transferred to fresh 300-mL beaker containing 9-µg haloperidol solution, where they were kept for another 30 min. After haloperidol exposure, fishes were shifted to the examination tank to evaluate various cataleptic parameters, where they were habituated for 5 min. Examination tank was filled with fresh aerated aquarium water. It consists of a 5-L tank with number of vertical lines drawn on one of the faces of tank at the

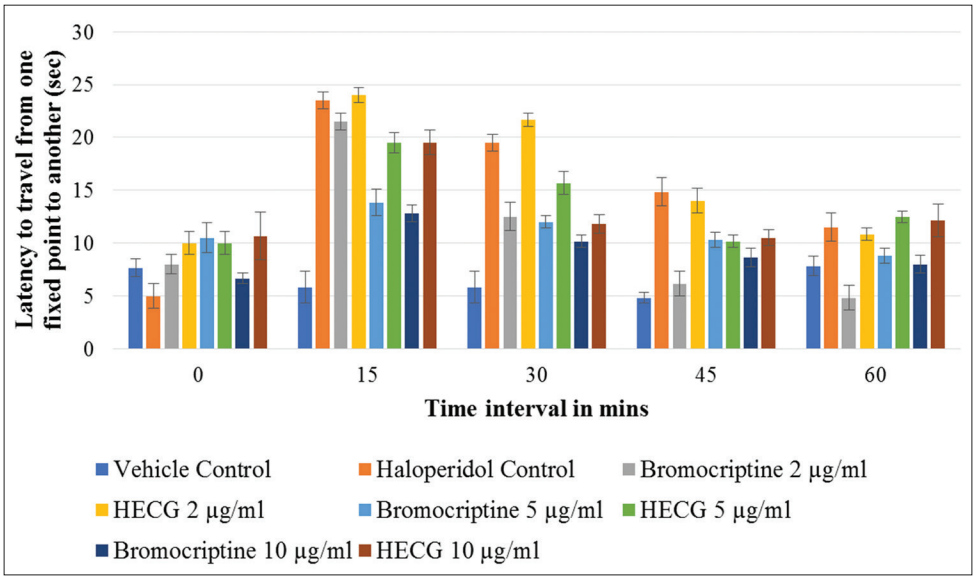

Fig. 1: Effect of bromocriptine and HECG on latency to travel from one fixed point to another (s)

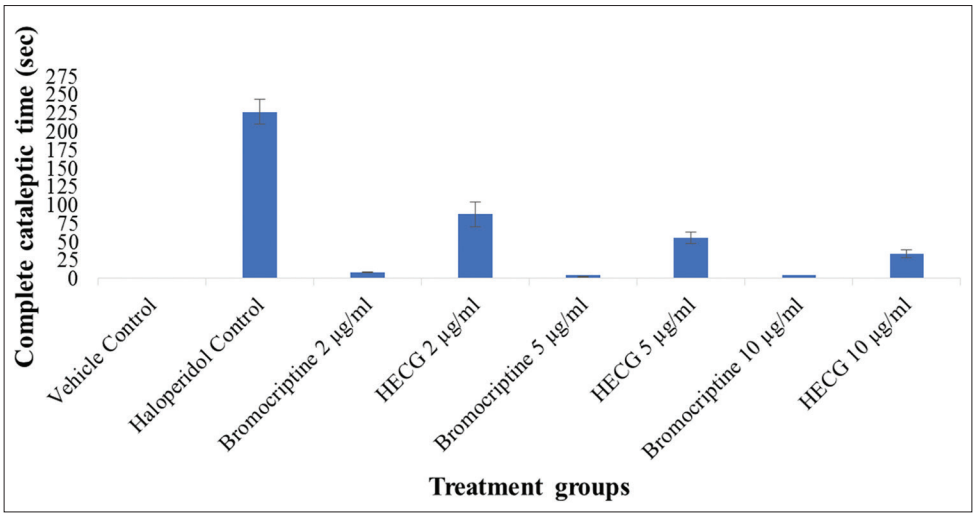

Fig. 2: Effect of bromocriptine and HECG on complete cataleptic time (s)

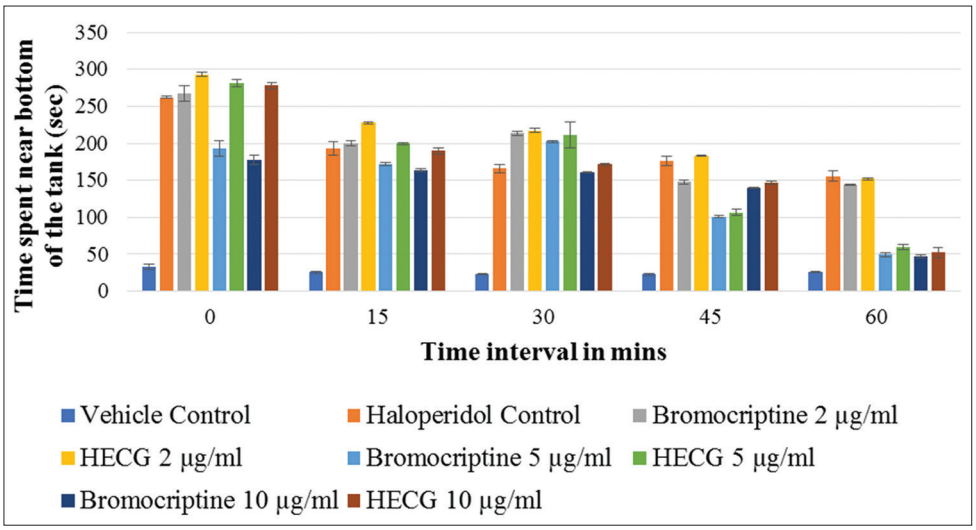

Fig. 3: Effect of bromocriptine and HECG on time spent near the bottom of the tank (s)

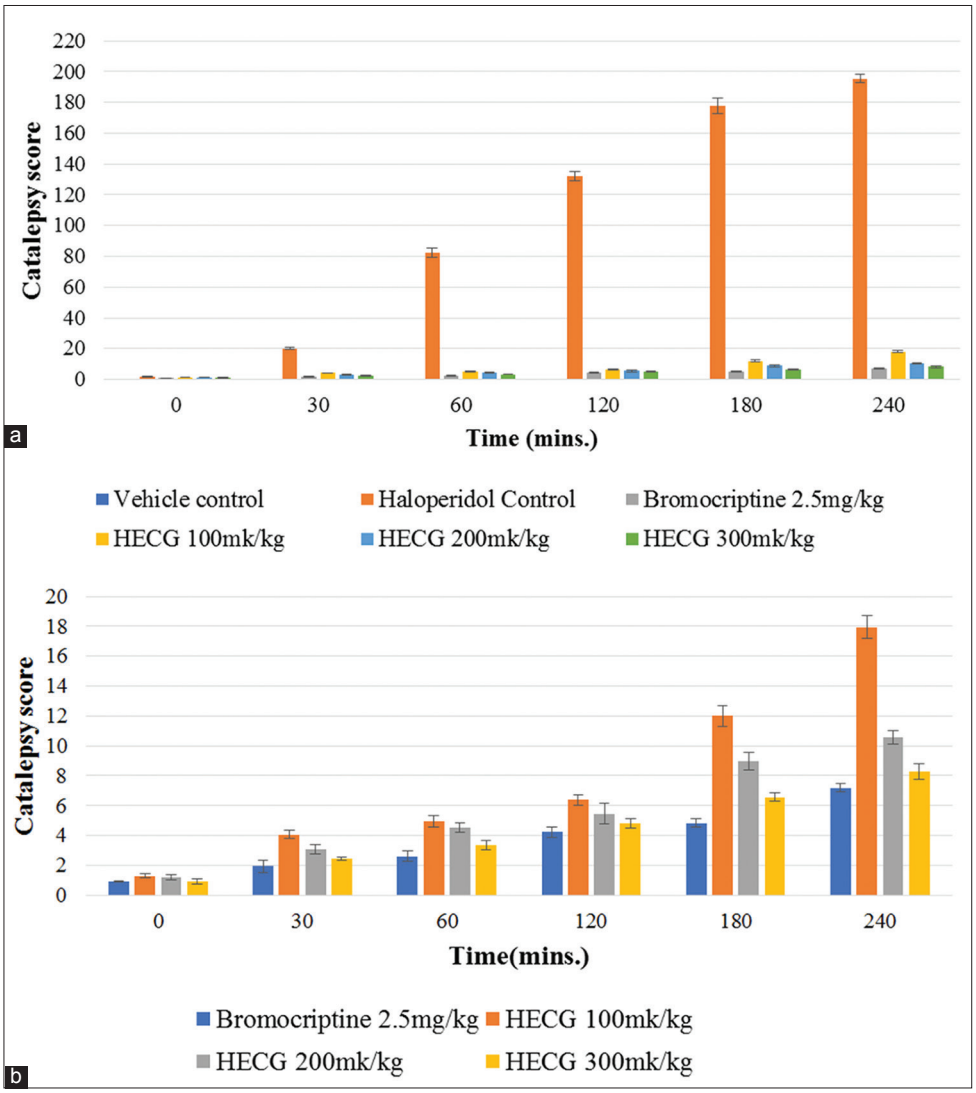

Fig. 4: (a) Effect of bromocriptine and HECG on catalepsy in bar test (b) Effect of bromocriptine and HECG on catalepsy in bar test (Graph of only standard and test)

spacing of 5 cm and with one horizontal line which divides the water-filled portion of the tank into two equal halves. These vertical lines were used to calculate the speed of fish by measuring the time taken by fish to travel from first vertical line to last, and horizontal line will give idea

about the time spend in the upper and lower half of the tank by the fish. Behavioral study was done for 1 h, and parameters were measured at 15-min time interval, namely, 0, 15, 30, 45, and 60 min. Since after 1 hr all fish recovered from the effect of haloperidol, examination time was standardized to 1 hr. Recording was done for 5 min at every time interval, and average readings during 5 min were calculated for every individual fish at each time point.

Following behavioral parameters in zebrafish were evaluated:

Latency to travel from one fixed point to another

In this time taken by the fish to travel from first vertical line to last was calculated. This gives an idea about the speed of fish.

Complete cataleptic time

Time for which the fish remained completely cataleptic during 1 h examination period.

Time spent near the bottom of the tank

Time spent below the horizontal line drawn on the examination tank was measured at the different time intervals. This gave an idea about the anxious behavior of the fish under study.

Haloperidol-induced catalepsy in mice

Mice were randomly divided into six groups (n=6), namely, vehicle control (vehicle treated), haloperidol control, bromocriptine, and HECG-treated group (low dose, intermediate dose, and high dose).

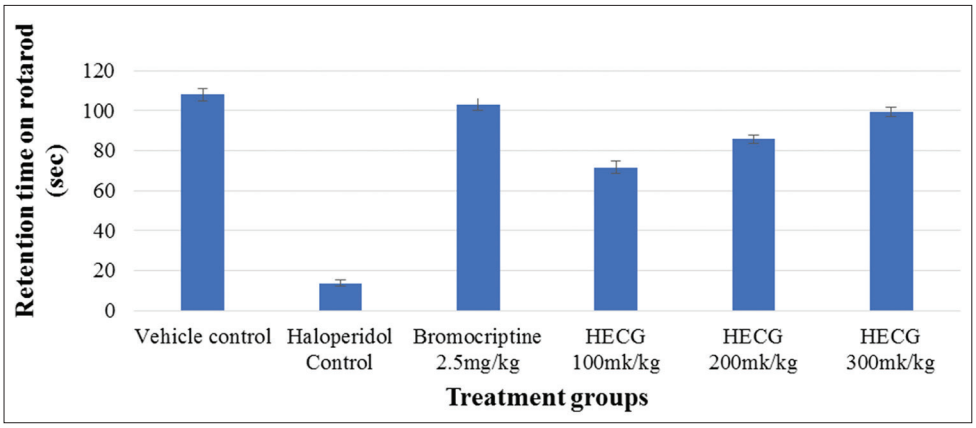

Fig. 5: Effect of bromocriptine and HECG on motor co-ordination test using rotarod

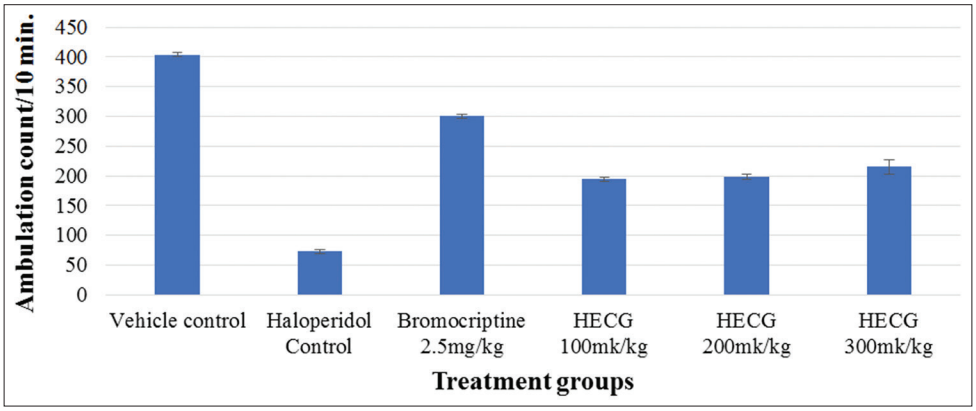

Fig. 6: Effect of bromocriptine and HECG on locomotor activity using actophotometer

Table 1: Effect of bromocriptine and HECG on latency to travel from one fixed point to another (s)

Treatment groups	Latency to travel from one particular point to another in sec Mean±SEM				
	Time interval in min				
	0	15	30	45	60
Vehicle control	7.66±0.84	5.83±1.49	5.83±1.53	4.83±0.47	7.83±0.94
Haloperidol control	5±1.15	23.5±0.76***	19.5±0.76***	14.83±1.35***	11.5±1.33
Bromocriptine 2 µg/ml	8±0.93	21.5±0.76	12.5±1.33*	6.16±1.19***	4.83±1.19**
HECG 2 µg/ml	10±1.06**	24±0.73	21.66±0.61	14±1.18	10.83±0.60
Bromocriptine 5 µg/ml	10.5±1.40**	13.83±1.24**	12±0.57*	10.33±0.71	8.83±0.70
HECG 5 µg/ml	10±1.06**	19.5±0.99	15.66±1.08	10.16±0.60	12.5±0.56
Bromocriptine 10 µg/ml	6.66±0.49	12.83±0.79***	10.16±0.60***	8.66±0.88**	8±0.85
HECG 10 µg/ml	10.66±2.27**	19.5±1.17	11.83±0.87*	10.5±0.76	12.16±1.53

All values are expressed in Mean±SEM in sec (n=6). Haloperidol control group was found to significantly (***p<0.001) decrease the latency to travel from one fixed point to another as compared to vehicle control group. Treatment groups were found to significantly (***p<0.001, **p<0.01, and *p<0.05) increase the latency to travel from one fixed point to another as compared to haloperidol control group. SEM: Standard error of the mean

Table 2: Effect of bromocriptine and HECG on complete cataleptic time (s)

Treatment groups	Complete cataleptic time in sec (Mean±SEM)
Vehicle control	0.00±0.00
Haloperidol control	226.5±17.05***
Bromocriptine 2 µg/ml	8.33±0.88**
HECG 2 µg/ml	87±16.39
Bromocriptine 5 µg/ml	2±0.36***
HECG 5 µg/ml	54.66±7.94
Bromocriptine 10 µg/ml	0.83±0.19***
HECG 10 µg/ml	33.16±5.20*

All values are expressed in Mean±SEM in sec (n=6). Haloperidol control group was found to significantly (***p<0.001) increase the complete cataleptic time as compared to vehicle control group. Treatment groups were found to significantly (***p<0.001, **p<0.01, and *p<0.05) decrease the complete cataleptic time as compared to haloperidol control group. SEM: Standard error of the mean

Bromocriptine and HECG were administered orally as a suspension in sodium carboxymethyl cellulose (0.5%). 1 h after the drug administration, the animals were challenged with haloperidol 1 mg/kg intraperitoneal (i.p.) administration, and catalepsy was evaluated using standard bar test at 0 (before haloperidol administration), 30, 60, 120, 180, and 240-min time interval. Locomotor activity was evaluated using actophotometer, and motor coordination test was done by rotarod test.

Bar test [11]

Bar test was used to measure the catalepsy. In the bar test, front paw of the animals was placed on a horizontal bar located 3 cm and 7 cm above and parallel to the base alternately. The time at which the animal removes its paw from the bar was noted.

Catalepsy scoring was given as follows:

Step I: The mice were taken out of the home cage and placed on a table. If the mice failed to move when touched or pushed gently on the back, a score of 0.5 was assigned.

Step II: The front paws of the mice were placed alternately on a 3-cm high block. If the mice failed to correct the posture within 15 s, a score of 0.5 for each paw was added to the score of step I.

Step III: The front paws of the mice were placed alternately on a 7-cm high block, if the mice failed to correct the posture within 15 s, a score of 1 for each paw was added to the scores of steps I and II.

Motor coordination test (rotarod test) [12]

Motor coordination test was conducted using a rotarod apparatus. The animals were placed on rotating rod before the treatment, and the mice stayed on the rod without falling for 120 s were selected for the study. The time mice take for falling from the rotating rod was noted before and after the treatment.

Test for locomotor activity (actophotometer)

This test provides an index of basal locomotor activity in a familiar environment [13]. Actophotometer consists of cage which has six lights and six photocells, which are placed in the outer periphery of the bottom in such a way that single mice block only one beam at a time. A Photocell gets activated when the rays of light falls on photocells, the beam of light is interrupted as and when animal crosses the light beam. The number of cut interruptions were recorded for 10 minutes [14].

Statistical analysis

The data were analyzed with InStat statistical software. The results are expressed as the mean±standard error of the mean (SEM) for each group. Statistical differences were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's t-test. Results were considered to be statistically significant at ***p<0.001, **p<0.01, and *p<0.05.

RESULTS

Phytochemical analysis

The phytochemical analysis of extract revealed that the hydroalcoholic HECG shows the presence of carbohydrates, proteins, saponins, flavonoids, alkaloids, phenolic compounds, and tannins.

Phytochemicals	Observations
Carbohydrates	+
Proteins	+
Steroids	-
Glycosides	-
Saponins	+
Flavonoids	+
Alkaloids	+
Phenolic compounds	+
Tannins	+

+: Present, -: Absent

Catalepsy in zebrafish

Latency to travel from one fixed point to another

Haloperidol significantly decreased the latency to travel from one fixed point to another as compared to vehicle control group. As shown in Fig. 1. It can be observed from Table 1 that groups treated with bromocriptine and HECG significantly recovered latency to travel from one point to another, as compared to haloperidol control group.

Complete cataleptic time

Haloperidol control group significantly increased the complete cataleptic time in zebrafish. As shown in Fig. 2. It can be observed from Table 2 that Bromocriptine and HECG produced drastic and statistically significant reduction in complete cataleptic time as compared to haloperidol control group.

Time spent near the bottom of the tank

Haloperidol control group was significantly different from vehicle control. As shown in Fig. 3. It can be observed from Table 3 that Bromocriptine and HECG exhibited significant reversal of the anxious behavior as compared to haloperidol control group.

Catalepsy in mice

Bar test

It can be observed from Table 3 that Bromocriptine and HECG exhibited significant reversal of the anxious behavior as compared to haloperidol control group.

Motor coordination test

Spontaneous motor activity was significantly decreased in haloperidol-treated group as compared to control group. HECG at given doses significantly increased the locomotor activity as compared to haloperidol control group. As shown in Fig. 5. Retention time on rotarod was significantly decreased in the haloperidol-treated group as compared with the control group and it was significantly dose-dependently improved with HECG treatment. As shown in Table 5.

Test for locomotor activity

From Fig. 6 and Table 6 it can be observed that Reduction in locomotion induced by haloperidol was successfully regained by bromocriptine and HECG.

DISCUSSION

Haloperidol-induced catalepsy in zebrafish

Catalepsy was induced in zebrafish using standardized dose of haloperidol (9 µg) by giving them direct exposure in a beaker. It was observed that the effect of haloperidol lasts for an hour in a fish, and hence, the study duration of 1 h after haloperidol exposure

Table 3: Effect of bromocriptine and HECG on time spent near the bottom of the tank (s)

Treatment groups	Time spent near the bottom of the tank in sec Mean±SEM				
	Time interval in min				
	0	15	30	45	60
Vehicle control	33.5±3.67	25.66±1.08	23.83±0.70	22.66±1.70	26.16±0.94
Haloperidol control	262.33±1.52**	193.16±8.78**	165.83±5.57	176.33±6.72***	155.83±6.77***
Bromocriptine 2 µg/ml	267.5±10.70	199.66±3.62	213.83±3.10**	147.66±3.28	144±1.21
HECG 2 µg/ml	292.83±2.88*	227.5±1.17*	217.16±2.99**	183.33±1.25	152.16±1.35
Bromocriptine 5 µg/ml	193.16±10.93	171.83±2.41	202.66±1.60	100.66±1.52***	49.66±2.66**
HECG 5 µg/ml	281.83±5.04	199.5±1.33	211.16±17.29*	106.66±3.98***	59.33±3.63*
Bromocriptine 10 µg/ml	177.83±6.75	163.66±1.54*	161±1.06	140±0.73*	46.83±1.77***
HECG 10 µg/ml	278.16±4.42	189.66±4.12	171.83±1.57	147.16±2.31	52.5±7.17**

All values are expressed in Mean±SEM in sec (n=6). Haloperidol control group was found to significantly (***p<0.001 and **p<0.01) increase the time spent near the bottom of the tank as compared to vehicle control group. Treatment groups were found to significantly (***p<0.001, **p<0.01, and *p<0.05) decrease the time spent near the bottom of the tank as compared to haloperidol control group. SEM: Standard error of the mean

Table 4: Effect of bromocriptine and HECG on catalepsy in bar test

Time interval in min	Mean±SEM (cataleptic score)					
	Vehicle control	Haloperidol control	Bromocriptine 2.5 mg/kg	HECG 100 mg/kg	HECG 200 mg/kg	HECG 300 mg/kg
0	0.00±0.00	1.92±0.28***	0.92±0.04*	1.28±0.14	1.21±0.17	0.92±0.14**
30	0.00±0.00	1.95±0.64***	1.95±0.41**	4.07±0.30	3.07±0.34*	2.44±0.12**
60	0.00±0.00	82.25±3.24***	2.61±0.33***	4.95±0.39	4.53±0.34	3.36±0.29**
120	0.00±0.00	132.14±3.28***	4.23±0.35***	6.38±0.37	5.46±0.70*	4.80±0.31**
180	0.00±0.00	177.67±5.17***	4.84±0.26***	12±0.70	8.97±0.57*	6.57±0.26**
240	0.00±0.00	195.57±2.58***	7.18±0.28***	17.94±0.76	10.55±0.46*	8.25±0.52**

All values are expressed in Mean±SEM of cataleptic score (n=6). Haloperidol control group was found to significantly (***p<0.001) increase the cataleptic score as compared to vehicle control group. Treatment groups were found to significantly (***p<0.001, **p<0.01, and *p<0.05) decrease the cataleptic score as compared to haloperidol control group. SEM: Standard error of the mean

Table 5: Effect of bromocriptine and HECG on motor coordination test using rotarod

Treatment groups	Time of stay on rotarod (s) (Mean±SEM)
Vehicle control	108±3.15
Haloperidol control	13.83±1.74***
Bromocriptine 2.5 mg/kg	103.16±3.17***
HECG 100 mg/kg	71.66±3.01
HECG 200 mg/kg	85.83±2.15*
HECG 300 mg/kg	99.5±2.48***

All values are expressed in Mean±SEM (n=6). Haloperidol control group was found to significantly (***p<0.001) decrease time of stay on rotarod as compared to vehicle control group. Treatment groups were found to significantly (***p<0.001 and *p<0.05) increase time of stay on rotarod as compared to haloperidol control group. SEM: Standard error of the mean

Table 6: Effect of bromocriptine and HECG on locomotor activity using actophotometer

Treatment groups	Ambulation count/10 min (Mean±SEM)
Vehicle control	403.66±3.47
Haloperidol control	72.5±2.81***
Bromocriptine 2.5 mg/kg	300±3.48***
HECG 100 mg/kg	194.33±3.15
HECG 200 mg/kg	198.5±4.81
HECG 300 mg/kg	215.33±12.51*

All values are expressed in Mean±SEM of ambulation counts (n=6). Haloperidol control group was found to significantly (***p<0.001) decrease ambulation counts as compared to vehicle control group. Treatment groups were found to significantly (***p<0.001 and *p<0.05) increase ambulation counts as compared to haloperidol control group. SEM: Standard error of the mean

was standardized and kept constant throughout the study period. Bromocriptine and HECG were used to compare the efficiency of fish model with respect to mice model. Bromocriptine is insoluble in water; hence, it was first solubilized in 0.5% DMSO containing water (well below the permitted limit of DMSO in fish) and then given as exposure to the fish. Both the drugs, bromocriptine and HECG, were tried arbitrarily at different concentrations, namely, 2, 5, 10 µg/mL. All drugs including haloperidol and treatment drugs were given in the form of exposure since it directly goes into systemic circulation of fish through gills.

Catalepsy was successfully induced in fish by haloperidol. During induction of catalepsy, fish started showing aberrant swimming patterns such as upside down, circular swimming, and arrow-like swimming, and finally, the state of complete catalepsy was achieved.

Various behavioral parameters studied in fish

Latency to travel from one fixed point to another

Catalepsy diminishes the speed of fishes due to the rigidity of muscular movements. In the present study, after induction of catalepsy, rigidity of fins and muscular movements were observed due to which there was a difficulty in swimming experienced by the fishes. Thus, they took more time to travel from one particular point of the tank to another. Keeping this into consideration, time taken by the fish to travel from first vertical line of the examination tank to last line was calculated and recorded at every time interval during the study period.

Complete cataleptic time

Time for which the fish was in completely firm/immovable state was used as an index of locomotor activity in fishes.

Time spent near the bottom of the tank

It is a well-known fact that zebrafish swim near the surface of the water. When they are transferred to a new environment (examination tank), they initially spend more time near the bottom, and after some time, they come toward surface of the tank, this is attributed toward their exploratory and most often due to their anxiety. Thus, the time spent near the bottom of the tank gives an idea about the extent of anxiety of

fish. Hence, it was calculated by measuring the time spent by the fish below the horizontal line drawn on the examination tank.

Haloperidol-induced catalepsy in mice

Catalepsy (rigidity in movements), akinesia (slowing of movement), tremors, and memory loss are some of the major symptoms of PD. Among these, catalepsy is one of the major symptoms that makes the life of PD patient uncomfortable.

Bromocriptine is a well-known dopamine (D2) receptor agonist and is commonly used to improve the symptoms related to rigidity. Hence, this drug was used as standard in the present study to compare the efficiency of both the models (zebrafish and mice).

Catalepsy was induced in mice by i.p. administration of haloperidol (1 mg/kg). This cataleptic behavior induced by haloperidol and protective effect of standard (bromocriptine) and HECG used was evaluated using bar test, rotarod apparatus, and actophotometer.

Bar test

This test gives the idea about the extent of catalepsy induced in an animal. In the present study, bromocriptine and HECG reversed the effects of haloperidol in bar test in dose-dependent manner.

Motor coordination test by rotarod

Imbalance is one of the symptoms of PD, to evaluate it this test was carried out. The test consists of a rotating rod on which the animal balances.

Haloperidol-treated mice, subjected to the rotarod test, exhibited a significant loss of muscular coordination and it could be due to loss of muscular strength.

HECG prevented the motor impairment in a dose-dependent manner which was altered by haloperidol. It indicates that HECG may have active constituents with CNS-stimulant activity.

Locomotor activity by actophotometer

Due to the catalepsy, movement restrictions or sometimes freezing of the movements is exhibited by PD patient. Hence, the drug which improves the locomotor activity can modify the condition of PD patient.

The results indicated that haloperidol caused significant decreased locomotor counts in actophotometer. Daily treatment with HECG significantly reversed the decrease in locomotor activity as assessed on day 7 in a dose-dependent manner.

Haloperidol model in mice is well established and very commonly used one. Zebrafishes have the tendency to swim from one side of the tank to another and near the surface of water. However, these behaviors were completely abolished by haloperidol. Fishes started swimming erratically, i.e., upside down, arrow-like or in circles, and spending more time near the bottom of the tank due to anxiety. All these erratic behaviors were reversed by bromocriptine and HECG. Diminished swimming speed due to haloperidol was also recovered by bromocriptine and HECG. This suggests that the dopaminergic system of zebrafish works like mammalian dopaminergic system. Hence, it can be the perfect model organism for PD research.

CONCLUSION

In conclusion, we can say that zebrafish may become efficacious tool for high throughput screening for many diseases. They can be used with ease and effectiveness for initial screening of various drugs before subjecting them to rodent testing. Thus, saving number of rodents and also it assures 3R's of pharmacological testing.

Canavalia gladiata exhibited significant antiparkinsonian activity in haloperidol mouse model and zebrafish. It appears to be the most promising plant due to its L-DOPA content and potential antioxidant activity. The predictable mode of action of this plant may be due to increased synthesis of dopamine from L-DOPA and decreased lipid peroxidation due to the presence of flavonoids and polyphenols. These findings provide evidence for its use as antiparkinsonian medication, including prevention of PD, improvement of PD symptoms. Further studies are required to investigate the phytoconstituents responsible for the activity and also to establish the exact mode of action.

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AUTHOR'S CONTRIBUTIONS

Saniya Pathan designed the experimental study and carried out the analysis. Mr. Imtiyaz Ansari contributed in preparing the manuscript and revision. Both the authors have read and approved the final manuscript.

CONFLICTS OF INTERESTS

The authors have none to declare.

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