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SHORT COMMUNICATION

Assessment of Anxiolytic Activity of Brahmi (*Bacopa monnieri*) in Zebrafish Model System

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Abstract

The increasing prevalence of anxiety and stress-related disorders has made it a leading contributor to the global health burden. The present treatment options have severe side effects and show remission on discontinuation of the medication. Hence, there is an urgent need to explore safer alternative treatments for long-term usage with minimum toxicity. The medicinal plant *Brahmi* (*Bacopa monnieri*) has been used in Indian traditional medicine as a neural tonic for centuries. The present study aimed to study the toxicity and anxiolytic activity of *Brahmi* using the zebrafish model system. The toxicity assays determined the minimum effective concentration of *Brahmi* to be 0.01%. In addition, behavioral assays such as thigmotaxis and scototaxis and endocrine assays such as the measurement of cortisol levels in stressed zebrafish larvae were performed. Zebrafish embryos exposed to 0.2% *Brahmi* up to seven days post fertilization (dpf) did not show any developmental toxicity. Behavioral and endocrine assays were performed on 5dpf zebrafish larvae treated with 0.01% *Brahmi* extract. Our studies show that *Brahmi* significantly reduced thigmotaxis (wall hugging) and scototaxis behavior in zebrafish larvae exposed to osmotic stress as compared to untreated stressed larvae. Stress activates the hypothalamic-pituitary-interrenal axis and stimulates the release of cortisol in zebrafish larvae. Whole body cortisol assay has shown that *Brahmi* significantly reduced the stress-induced release of cortisol in zebrafish larvae. Our studies report that *Brahmi* mitigates the stress response in zebrafish larvae and has minimum toxicity. This suggests that *Brahmi* may be a safe option for long term management of stress.

Keywords: Cortisol, HPI Axis, Scototaxis, Stress, Thigmotaxis

1. Introduction

Anxiety disorders and depression are becoming leading contributors to the global health burden. COVID-19 pandemic has further exacerbated the scale and burden of mental health problems with an increase of 25% globally with women and young people being hit the worst[1,2]. Anxiety disorders are comorbid and are often diagnosed together with depression, mood disorders, bipolar disorders, and substance abuse[3], which can lead to significant impairment of general well-being and impact normal day-to-day functioning. The first line of drugs for these disorders is selective serotonin-norepinephrine reuptake inhibitors. Other treatment options include tricyclic antidepressants, mixed antidepressants (e.g., mirtazapine), antipsychotics, antihistamines (e.g., hydroxyzine), alpha- and beta-adrenergic medications (e.g., propranolol, clonidine), and GABAergic medications (benzodiazepines, pregabalin, and gabapentin)[4,5]. Most of these drugs are addictive, have severe side effects such as sedation, a decrease in cognitive ability, and hence are not recommended for long-term use[6]. A high relapse rate is observed in patients when treatment is stopped. An increased risk for suicidal ideation (not suicides) in children and adolescents has been reported in cases treated with antidepressants such as escitalopram, citalopram, paroxetine, sertraline, mirtazapine, and venlafaxine[7]. Popular anxiolytic drugs like Alprazolam have also been

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seen to cause adverse side effects including insomnia, poor coordination, slurred speech, muscle weakness, memory problems, etc[8]. An increased mortality rate has also been associated with the use of anxiolytic drugs[9].

To circumvent the concerning issues with psychiatric drugs, alternative therapeutic options such as phytochemicals are being explored extensively for their efficacy and non-addictiveness. Phytochemicals derived from traditional herbs are investigated for their therapeutic potential, pharmacokinetics and identification of bioactive compounds. They are of special interest due to their low cost, low toxicity and minimum secondary side effects[10]. *Brahmi* (*Bacopa monnieri*), a plant belonging to the family Scrophulariaceae, has been used in the Indian traditional medicine system of Ayurveda as a neural tonic and memory enhancer for centuries. The active chemicals isolated from *Brahmi* predominantly include compounds like dammarane-type triterpenoid saponins called bacosides[11]. *Brahmi* extracts with bacosides has been reported to show anxiolytic activity in animals[12]. *Brahmi* has been shown to have GABAergic activity and also modulates the activities of Hsp70, P450 and superoxide dismutase (SOD) in the brain and thereby protecting the brain from the deleterious effect of stress[13–15]. *Brahmi* extract effectively normalizes stress-induced plasma corticosterone levels, and monoamines such as 5HT, Norepinephrine, and dopamine in the rat brain[16]. The present study aims to further explore the anxiolytic therapeutic potential of *Brahmi* using the zebrafish model system.

Zebrafish (*Danio rerio*) larvae are rapidly gaining popularity as translational neuroscience and behavioral research model. Zebrafish is a vertebrate and shares 70% genetic homology[17], and most of its organ systems and bioprocesses are similar to human systems. In addition, the zebrafish model provides many practical advantages such as external fertilization, high fecundity, optical transparency, small size, low-cost easy maintenance, and the ability to perform high throughput assays. Zebrafish embryo toxicity assays enable high throughput *in vivo* drug toxicology evaluation in a much shorter time frame of seven days as compared to other vertebrates or mammalian assays[18].

Zebrafish have been shown to exhibit a wide range of complex behaviors similar to humans including anxiety, depression, learning and memory, avoidance, and defense[19]. A repertoire of behaviors associated with different developmental stages of zebrafish has been characterized and has proven to be useful in modeling various neurological disorders[20]. While many behaviors can be observed and quantitated manually by using specifically created set-ups and mazes, advances in IT-based methodology and video-tracking software tools have enabled automated quantification for the study of complex behavior in zebrafish.

Anxiety and stress involve emotion based behavioral responses which are conserved across vertebrate species. Behaviors such as Visual Motor Responses (VMR), change in locomotion and swimming pattern, and freezing has been well studied. Zebrafish larvae have a natural preference for light and show scototaxis (preference for dark or avoidance of bright lighting) behavior under stress. Hence scototaxis (dark/light) assays serve as a measurable anxiety index in zebrafish larvae and have been used to assess anxiolytic/anxiogenic drugs. Thigmotaxis is another measurable behavioral response to stress. Thigmotaxis is the wall-hugging behavior where the zebrafish larvae show the tendency to stay near the wall of multi-well plates or Petri dishes and avoid the central clear areas, when under stress. Both scototaxis and thigmotaxis behaviors can be studied in zebrafish larvae as early as 5dpf[20].

An organism responds to a variety of stresses with a combination of neural, endocrine and autonomic processes that prepares it to cope with adverse stress-causing events. In humans, stress induces various biological responses, including the release of catecholamines, and Hypothalamus-Pituitary-Adrenal (HPA) axis activation. In response to stress, the HPA axis is stimulated and Corticotrophin-Releasing Hormone (CRH) is released from the hypothalamus, which then activates the corticotrophs in the pituitary gland to secrete Adrenocorticotropic Hormone (ACTH). The ACTH stimulates the adrenal glands to synthesize and release the stress hormone cortisol. Under chronic stress, blood cortisol levels are much increased. Cortisol elicits various physiological responses including neuroendocrine, autonomic, metabolic and immuno-modulatory responses which contribute to increased risk in the development of many diseases[21].

Zebrafish is gaining relevance for modeling stress responses *in vivo*[22]. Zebrafish possess an evolutionarily conserved stress endocrine Hypothalamic-Pituitary-Interrenal (HPI) axis that is structurally and functionally homologous to the mammalian HPA axis[23]. In zebrafish, stress activates the HPI axis to trigger the hypothalamus

to initiate CRH/ACTH cascade-stimulated synthesis and release of cortisol by the interrenal tissues. Notably, zebrafish, like humans, use cortisol as their principal stress response hormone (rather than corticosterone, as do rats), making them an excellent animal model for the study of human stress physiology[19].

The objective of our study was to assess the anxiolytic properties of *Brahmi* using a zebrafish model system by performing behavioral and endocrine assays.

2. Materials and Methods

2.1 Zebrafish Maintenance, Breeding, and Egg Collection

Wild type ASWT zebrafish (*Danio rerio*) was obtained from CSIR-Institute of Genomics and Integrative Biology, Mathura Road, New Delhi. Adult fish were maintained at 28 ± 2°C, in a light controlled room (14 hours light and 10 hours dark cycle). Male and female fish were housed separately in a Recirculating Aquatic Eco-System (PENTAIR B262-2-B) in 1.5 litres and 3 litres tanks with an optimum density of approximately 10 fish per litre. Fishes were fed a combination diet of live artemia and adult dry feed (TetraMin ~ 0.05g/10 fishes). While handling the fish, utmost care was taken to minimize animal suffering.

Breeding tanks with male and female fish were set up in the evening, a day before, for egg collection. A plastic separator was placed in the middle of the breeding tank to separate male and female fish. The fish were allowed to mate, and spawn and fertilised eggs were collected the next morning. The fertilisation rate was found to be > 90%. Fertilised eggs were transferred into Petri plates containing embryo water (0.06 g ocean sea salt/litre of RO water) and grown in the incubator at 28 °C[24].

2.2 Brahmi Toxicity Assay

Embryos at 1000 cell stage or 3.5 hours post-fertilization (hpf) were transferred into 6-well experimental plates with a density of 25 embryos/8ml/well. Four replicates, each for test samples and control were set up for the study (n=100 embryos per treatment). For all experiments, Embryo Water (EW) was used as a control. Embryos were exposed to and grown in the different concentrations of *Brahmi* extract (0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.2% and 0.5%) up to 7dpf. *Brahmi* extract was obtained from Basic Ayurveda, Ghaziabad, Uttar Pradesh, India (Basic Ayurveda's *BRAHMI* Ras (Juice)), First press virgin juice™. *Brahmi* extract had no added preservatives and alcohol. All the treatment studies with zebrafish embryos were carried out till 7 dpf. After every 24 hours, respective solutions were changed (all solutions were brought to 28 °C before embryo transfers) and the dead embryos, if any, were removed. The embryos were visualized and photographed using an EVOS-inverted microscope. The embryos were monitored up to 7 dpf for various parameters like survival rate, hatching rate, and other morphological and developmental anomalies were scored.

2.3 Stress Response Assays

2.3.1 Thigmotaxis Behavioral Assay

Thigmotaxis behavioral assay was performed as per the standard protocol by Schnörr *et al.*,[25] with slight modification. Zebrafish embryos were treated with 0.01% *Brahmi* (minimum effective concentration) at the 1000 cell stage and were grown in an incubator at 28 °C. At 5dpf *Brahmi* treated and control, larvae were transferred to a 12-well plate with a density of 10 larvae/well and acclimatized in white light (12-well plate kept over a laptop screen as in Figure 1. Post acclimatization, the zebrafish larvae were exposed to osmotic stress (200mM NaCl) for 10 minutes[26] and their behavior was tracked using video recording with a 12MP digital camera using the 'Lapse It' App. The behavioral response of the stressed vs control larvae was observed in picture frames taken every 30 seconds by the 'Free Studio' app. The preference of zebrafish larvae to cling to the walls of the petri-dish is indicative of an anxiety state. The larvae in the center of the well were counted; the larvae hugging the walls of the wells were ignored. The experiment was repeated five times. Statistical analysis was done using a two-tailed paired Students T-test using the graph pad prism software.

2.3.2 Scototaxis (Dark/Light Preference) Behavioral Assay

For performing the scototaxis assay, black paper was pasted on 12 well plates such that each half of the well was exposed to light and the other half remained dark as in Figure 1. The protocol of Bai *et al.,* 2016 was followed[27]. Zebrafish embryos were treated with 0.01% *Brahmi* (minimum effective concentration) at the 1000 cell stage and were grown in an incubator at 28°C. At 5dpf *Brahmi* treated and control, larvae were transferred to a 12-well plate with a density of 10 larvae/ well and acclimatized to white light and later exposed to osmotic stress (200mM

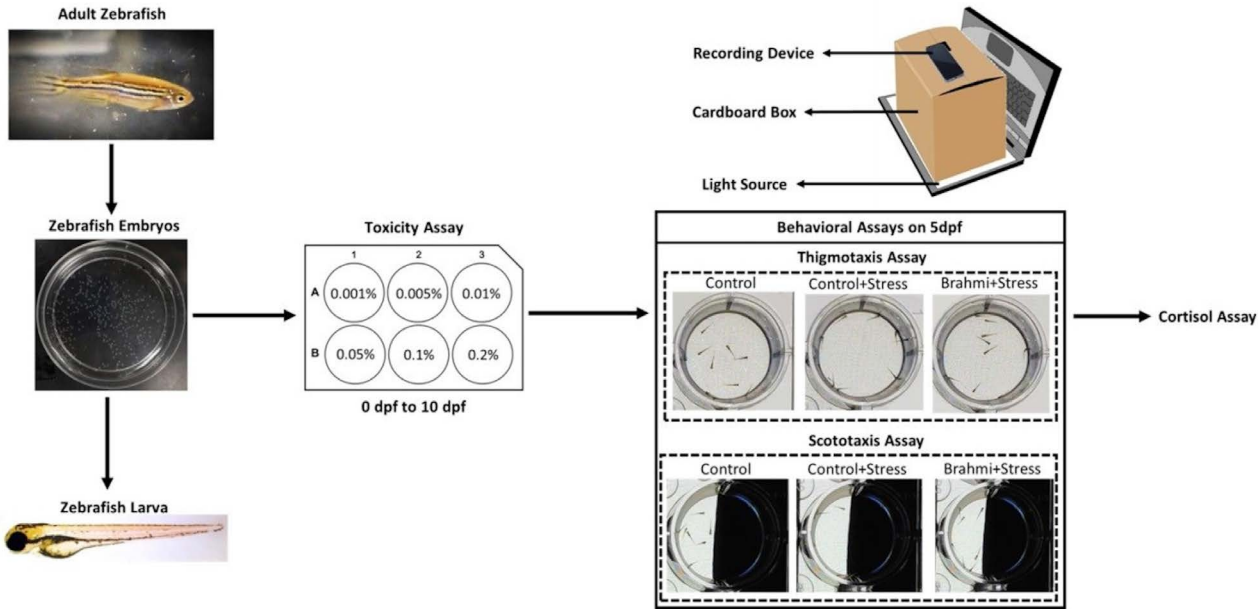

Figure 1. Graphical representation of anxiety behavior assay in zebrafish larvae. 5 day old larvae (dpf: days post fertilization) are subjected to osmotic stress and thigmotaxis and scototaxis assays are performed to study the behavior of the stressed larvae in the presence of *Brahmi*.

NaCl) for 10 minutes. The behavior of the stressed vs control larvae and their response to light vs. dark was tracked using Video recording with a 12MP digital camera using the 'Lapse It' App. The behavioral response of the stressed vs. control larvae were observed in picture frames taken every 30 seconds by the 'Free Studio' app. The larvae in the lighted half of the well were counted. The experiment was repeated five times. Statistical analysis was done using a two-tailed paired Students T-test with the graph-pad prism software.

2.3.3 Cortisol Extraction and ELISA

Whole zebrafish larvae extracts were used for cortisol estimation using the method of Yeh *et al.*, 2013 with slight modification[28]. After stress assays, larvae (control and *Brahmi* treated) were immobilized in ice-cold water and a group of 25 larvae of each treatment set were collected in 1.5 ml Eppendorf tubes. Excess water was removed, and the samples were frozen at -20 °C for long term use. For cortisol extraction, the tubes containing larvae were thawed over ice and 100 µl H_2O was added to the samples. The samples (larvae) were homogenized for 20 seconds using a pellet mixer. 900 µl ethyl acetate (Sigma) was added to each tube, and vortexed for 30 seconds (sec) at maximum speed. The solvent and aqueous phases were separated by centrifuging the tubes for 5 minutes at 3000x g at 4 °C. The aqueous layer was frozen in an ethanol/dry-ice bath to be discarded later. The solvent layer containing cortisol was decanted into a new 1.5 ml tube. The solvent was evaporated at 37 °C in an incubator. The extracted cortisol was dissolved in 50µl sample-buffer (0.2% Bovine Serum Albumin (BSA) in 1X Phosphate Buffer Saline (PBS). This may be frozen at -20 °C till further use or can be straightaway processed for ELISA. ELISA for estimating cortisol was performed using a competitive binding assay kit (DBC-Diagnostics Biochem Canada Inc., Can-C-270). The samples were spinned for 5 sec using a vortex mixer and 20µL of the sample was taken for ELISA. The experiment was repeated three times. Statistical analysis was done using a two-tailed paired Student's T-test with the graph pad prism software.

3. Results

3.1 Brahmi Toxicity Assays

Zebrafish embryos exposed to different concentrations of *Brahmi* extract (0.001%, 0.005%, 0.01%, 0.05%, 0.1%, and 0.2%) did not show any significant developmental toxicity. Embryos showed up to seventy percent (70%) survival rate on 0.2% *Brahmi* extract exposure up to 7dpf. Embryos exposed to 0.01% of *Brahmi* showed no development toxicity and had a survival rate of eighty

-two percent (82%) (Figure 2A). Thus, all the behavioral assays were conducted at 0.01% (the minimum effective concentration).

Hatching rate, a developmental index, was observed in embryos treated with different concentrations of *Brahmi* extract (0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.2%). No significant change in the hatching rate was observed in *Brahmi* extract treated embryos at all concentrations. Ninety-eight percent (98%) of the *Brahmi* treated and control embryos hatched by 5dpf (Figure 2B).

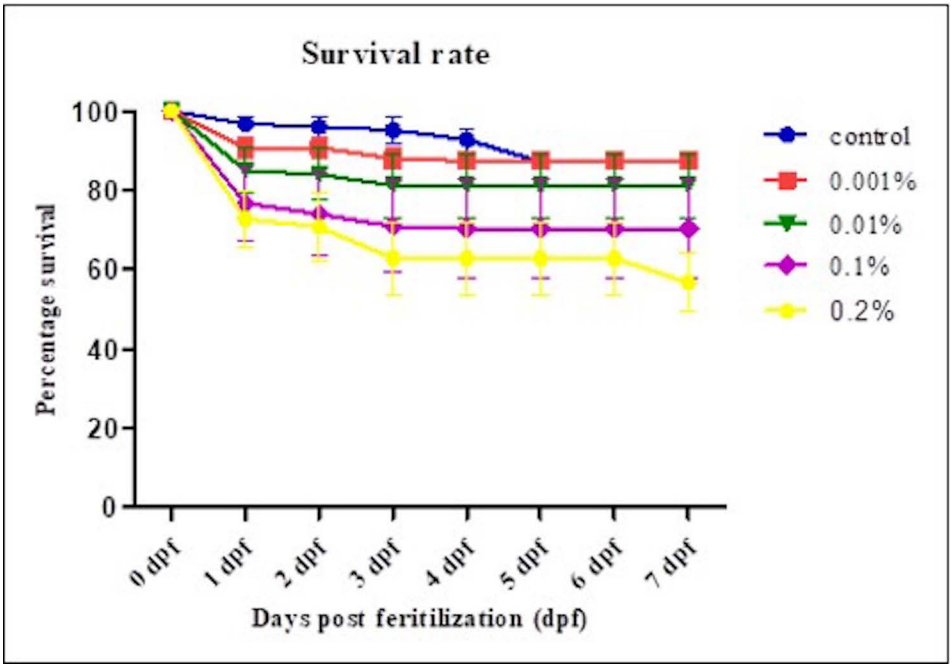

Figure 2A. The survival rate of zebrafish embryos (n = 100) treated with varied concentrations of *Brahmi*. The data presented is for four replicates (n = 25 embryos). Mean + SEM calculated by Graph pad Prism 5.0.

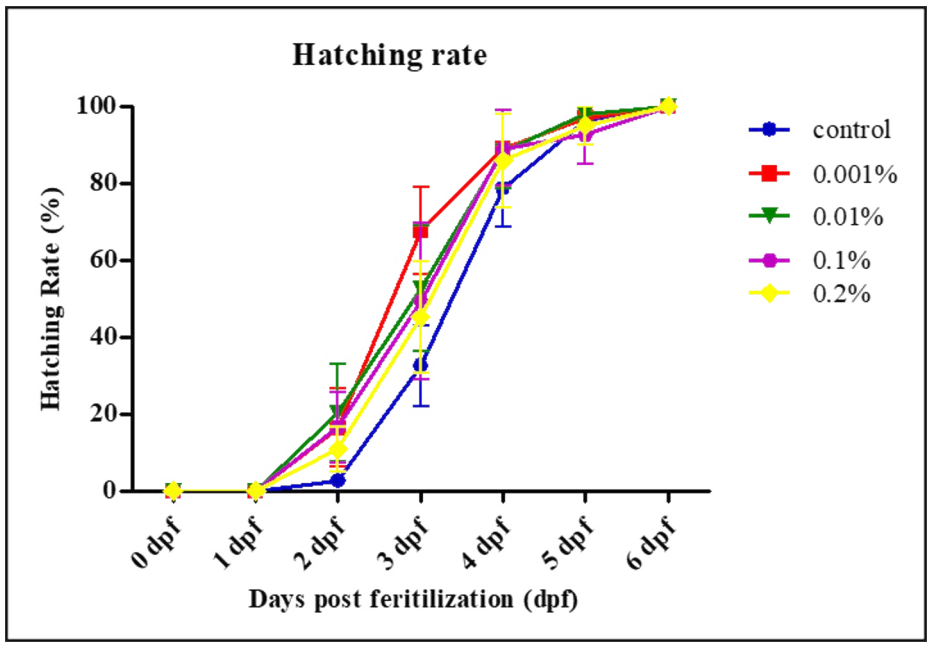

Figure 2B. Hatching rate of zebrafish embryos (n = 100) treated with varied concentrations of *Brahmi*. The data presented is for four replicates (n = 25 embryos). Mean + SEM calculated by Graph pad Prism 5.0.

3.2 Thigmotaxis Response

The 5dpf zebrafish larvae showed significant thigmotaxis (wall-hugging) behavior on exposure to osmotic stress (200mM NaCl). A significant drop in the number of larvae in the center of the well was observed when exposed to osmotic stress as compared to control larvae (Figure 3). Statistical analysis using a two-tailed paired Student's T-test showed that over a 10-minute period there was a ~2.5fold decrease in the number of larvae that moved to the centre of the well when exposed to osmotic stress as compared to the unstressed control larvae. *Brahmi* treated (0.01%) zebrafish larvae when exposed to osmotic stress did not show thigmotaxis/ stress response. During the 10-minute period of osmotic stress exposure, the number of larvae in the centre of the well was comparable to non-stressed control larvae. This anxiolytic response of stressed larvae upon *Brahmi* treatment was maximally observed between 4.5 - 6.5 minute osmotic stress exposure time interval. During the time interval from 4.5 - 6.5 minutes, 60-70% of the *Brahmi* treated, osmotically stressed larvae moved to the centre of the well as compared to only 20-30 % untreated osmotically stressed control larvae.

3.3 Scototaxis Response

The 5dpf zebrafish larvae showed a marked preference for dark compartments on exposure to osmotic stress (200mM NaCl), as compared to the control. Statistical analysis using two-tailed paired Student's T-test using the graph pad prism software showed that over a 10 minute period there was a ~2 fold decrease in the number of osmotically stressed larvae in the light-exposed compartment of the well compared to control (Figure 4). *Brahmi* treated zebrafish larvae when exposed to osmotic stress did not show any increase in preference for the dark compartment. *Brahmi* treated osmotically stressed larvae showed a ~2 fold increase in the number of larvae in the light-exposed compartment of the well as compared to osmotically stressed control untreated larvae (Figure 4). This anxiolytic response of *Brahmi* treatment was maximally observed between 5.0 - 6.5 minute time interval of the osmotic stress exposure. In this time interval about 65-75 % of *Brahmi* treated stress exposed larvae were observed in the light-exposed compartment of the well as compared to 30-40 % of untreated stressed larvae which were observed in the light exposed compartment.

3.4 Cortisol Release

Stress activates the zebrafish HPI axis to stimulate the hypothalamus to initiate the CRH/ACTH cascade which results in the increased synthesis and release of cortisol by the inter-renal tissue. Measurement of whole-body cortisol levels of control (unstressed and untreated) larvae, osmotically stressed and osmotically stressed

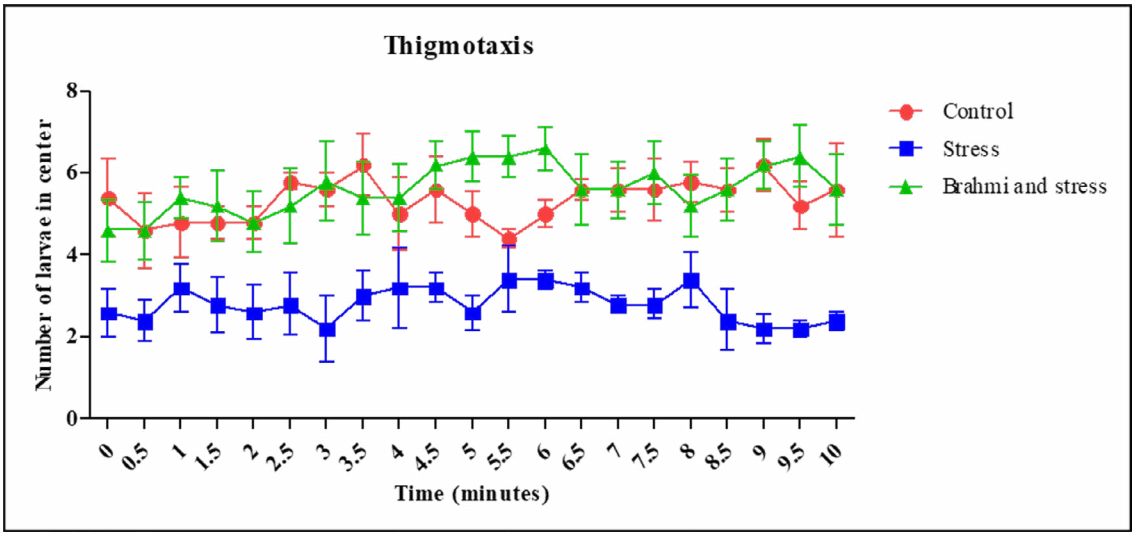

Figure 3. Thigmotaxis (wall-hugging behavior) assay of control, stressed untreated, and *Brahmi* treated zebrafish larvae: 10 larvae each of control, osmotically stressed with 200mM NaCl untreated and *Brahmi* treated were studied for the time period of 10 minutes using Video recording with 12MP digital camera using 'Lapse It' App. The behavioral response of the larvae was observed in pictures frames taken every 30 seconds by the 'Free Studio' app and the number of larvae present in the center of the well were noted. The experiment was repeated five times and mean + SEM is calculated.

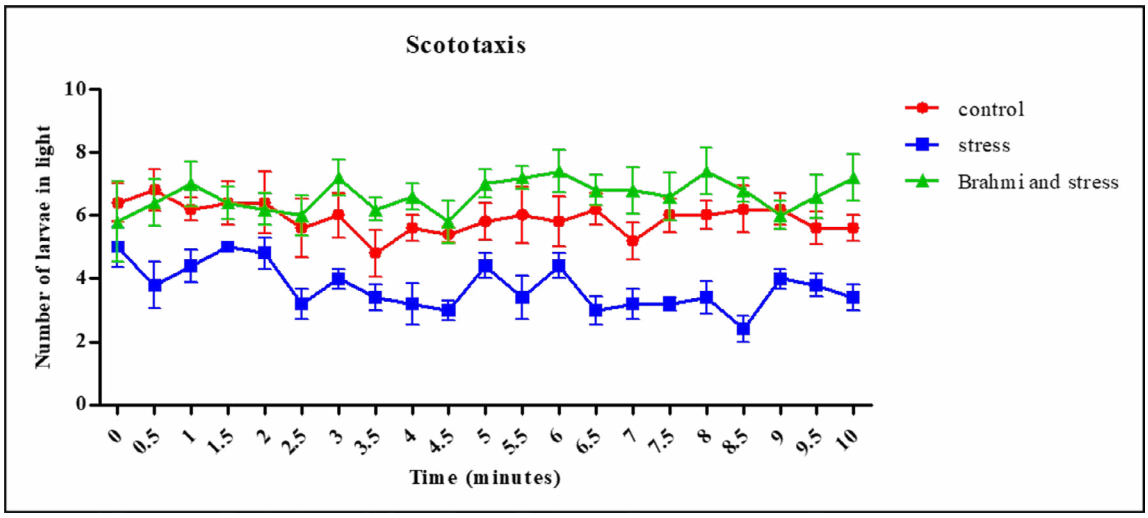

Figure 4. Scototaxis (dark/ light assay)) assay of control, stressed untreated, and *Brahmi* treated zebrafish larvae: 10 larvae each of control, osmotically stressed with 200mM NaCl untreated and *Brahmi* treated were studied for the time period of 10 minutes using Video recording with 12MP digital camera using 'Lapse It' App. The behavioral response of the larvae was observed in pictures frames taken every 30 seconds by the 'Free Studio' app and the number of larvae present in the lighted half of the well was noted. The experiment was repeated five times and mean + SEM is calculated.

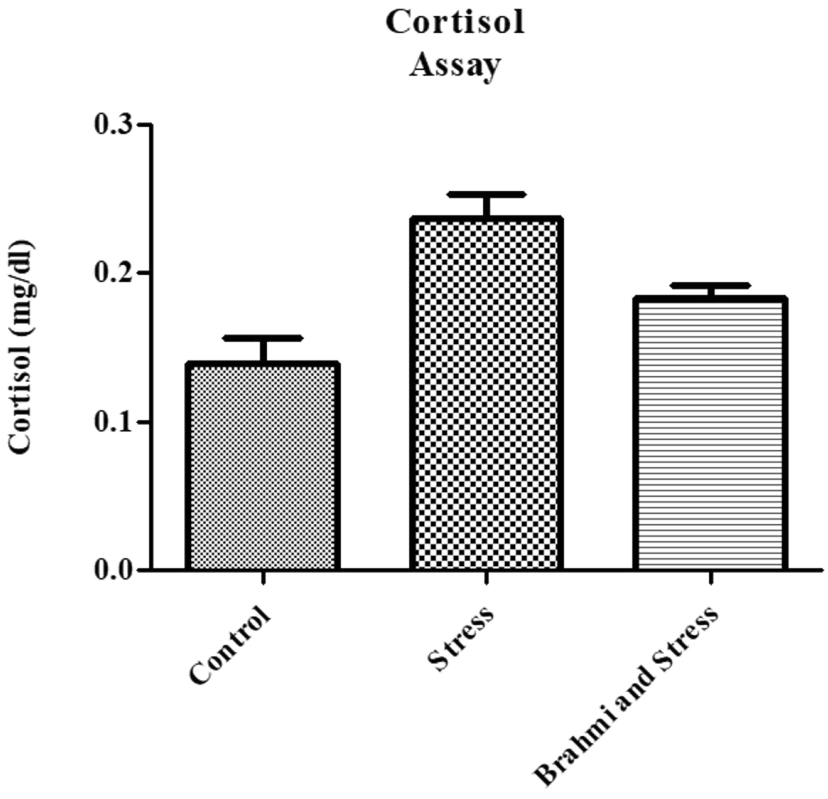

Figure 5. Cortisol assay of whole body extract of control, untreated and *Brahmi* treated osmotically stressed 5dpf zebrafish larvae. Cortisol assay of 25 larvae extract each of control, treated and *Brahmi* treated osmotically stressed was performed using competitive ELISA. The experiment was repeated three times and mean + SEM was calculated.

Brahmi treated larvae was done by ELISA. Zebrafish larvae exposed to 10 minutes of osmotic stress treatment showed a 1.8 fold increase in cortisol levels as compared to non-stressed control larvae (Figure 5). The effect of stress was reduced in the osmotically stressed *Brahmi* treated larvae as the levels of cortisol were 1.2 fold less as compared to osmotically stressed control larvae. *Brahmi* treatment inhibits the release of cortisol in stressed zebrafish larvae.

4. Discussion

Our studies showed that zebrafish embryos treated with up to 0.2% *Brahmi* extract did not show any significant developmental toxicity. No phenotypic and morphological developmental deformities were observed in zebrafish embryos treated with up to 0.2% *Brahmi* extract till 7 dpf. Embryos treated with 0.2% *Brahmi* showed ~70% survival rate even after 7dpf. *Brahmi* did not have any significant effect on the hatching rate of embryos when compared with untreated control embryos. These results are in alignment with toxicity studies carried out to assess the effects of *Brahmi* in other animals and humans. The acute toxicity studies in mice at different dose levels of *Brahmi* resulted in zero mortality and no observed abnormal behavior[29]. Another study in rats showed that a single oral administration of *Brahmi* extract at the concentration of 5,000 mg/kg did not cause any serious undesirable behavioral or health impact. No significant change in hematological, biochemical, and histopathological parameters were observed even after 270 days of treatment of up to 1,500 mg/kg *Brahmi* in rats[30]. No significant toxic effects of *Brahmi* have been reported in humans till date. A four weeks study of 100 mg - 200 mg oral treatment per day did not show any hematological, or biochemical abnormalities[31]. No significant changes in acute human cognitive effects were observed in a study involving a single dose administration of 300 mg *Brahmi* extract with evaluations done at 2 hr. post-administration[32]. Our studies on zebrafish and other studies using other animal systems provide evidence that *Brahmi* treatment has minimum toxic effects both in acute dosage and long-term chronic treatment, hence can be used for long term treatment option with minimum side effects.

The anxiolytic activity of *Brahmi* extract was studied in zebrafish larvae at 5dpf using behavioral assays such as thigmotaxis and scototaxis. Thigmotaxis assay in zebrafish larvae is a well validated assay to assess anxiolytic or anxiogenic activity of drugs and chemicals[33] under osmotic stress zebrafish larvae show significant thigmotaxis activity as compared to control larvae. However, our studies show that the thigmotaxis activity in osmotically stressed larvae was reduced in the presence of *Brahmi*. In fact, during the 5- 6 minute time interval of stress exposure, reverse thigmotaxis was observed with a 10% increase in number of larvae in the centre of the well as compared to the control. This suggests that *Brahmi* exerts anxiolytic effects on stressed zebrafish larvae in a thigmotaxis assay.

The scototaxis assay allows to assess anxiety based on the light preference of zebrafish larvae. Unstressed larvae prefer to stay in light whereas a stress condition directs them towards darker areas. Our studies showed a 20-25 % decrease in the number of osmotically stressed larvae in the light-exposed area when compared to control larvae. However, the osmotically stressed larvae when treated with *Brahmi* tend to stay in light (25-30 % increase) compared to untreated stressed larvae. During 5- 6.5 minute time intervals of stress exposure, 10% more larvae as compared to control were observed in the light-exposed area of the well.

These two behavioral assays with 5dpf zebrafish larvae show that 0.01% *Brahmi* treatment at 1000 cell stage zebrafish embryos do not show stress response when exposed to osmotic stress. *Brahmi* treated zebrafish larvae in fact show reverse thigmotaxis and increased preference for light when compared to untreated stressed larvae and even compared to control non-stressed larvae. Hence *Brahmi* shows significant anxiolytic activity in zebrafish larvae.

Stress response in zebrafish is mediated by Hypothalamic-Pituitary-Inter-Renal (HPI) axis which is analogous to Hypothalamic-Pituitary-Adrenal (HPA) axis in humans[34]. As in humans, stress induced increase in plasma cortisol levels has also been observed in zebrafish. Our studies have shown ~2 fold increase in whole body cortisol levels in larval zebrafish in response to 10 minute exposure to osmotic stress as compared to control. In *Brahmi* treated zebrafish larvae, whole body cortisol levels were significantly reduced as compared to untreated stressed larvae. Hence *Brahmi* inhibited the activation of stress-induced HPI axis thereby, inhibiting the release of cortisol.

5. Conclusions

The present study confirms that *Brahmi* has very low toxicity as no developmental toxicity was observed in zebrafish embryos up to 7 days of 0.2% exposure to *Brahmi* extract. *Brahmi* exhibited significant anxiolytic activity in zebrafish larvae as shown by both behavioral and endocrine studies in response to osmotic stress.

6. Acknowledgments

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