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Involvement of Nitric Oxide in the Adaptogenic Effect of *Bacopa monniera* (Brahmi)

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ABSTRACT

Bacopa monniera (BM) is an Ayurvedic medicine, used for centuries as nootropoic, anxiolytic, antidepressant, analgesic, antipyretic, antiepileptic agent and adaptogen. Adaptogens are drugs that promote non-specific resistance of the body and recognized as useful anti-stress agents. Although, the adaptogenic effect of BM is well documented, its mechanism is still not well defined. Stress is known to increase nitric oxide (NO) level in brain tissues, moreover, BM reported to inhibit iNOS expression *in vitro*. Hence, the present investigation was designed to evaluate the involvement of NO in adaptogenic effect of BM. Mice were exposed to overnight audiogenic stress from day 1 to 10. Various drugs treatments were given before the exposure on each day. Effects of stress were assessed interms of anxiety by social interaction and depression by forced swim test. Results showed that exposure to audiogenic stress significantly induced anxiety and depression on day 1 and 4, whereas, no difference after day 7 and 10, indicated adaptation to stress. Administration of BM (40 and 80 mg/kg, *p.o.*) attenuated the effect of audiogenic stress and facilitated the adaptation to stress. L-arginine impaired the adaptation to stress. In addition, concomitant administration of BM and L-NAME or 7-NI produced synergistic effect in audiogenic stress mice. Moreover, BM attenuated the effects of L-arginine. Further, BM administration significantly decreased audiogenic stress-induced increased NOx levels in cortex, hypothalamus and hippocampus. Thus, BM has significant adaptogenic activity and this effect is probably medicated through nitrergic system.

Keywords: Audiogenic stress, anxiety, depression, adaptation to stress.

INTRODUCTION

Stress is a biological response perceived on physical or psychological or environmental stimulus. Though it is an unusual situation of altered physiological homeostasis, which elicits a physiological response involving both peripheral and central systems causing distress, such changes support the body to sustain the life in a given situation. [1]

These changes are intensely felt in the initial period of exposure, continue over a long period, and then subside, due to either acquaintance with changed homeostasis or lack of response to stressor, or both. This phenomenon of gradual declined in the stress response is called as adaptation. Lazarev, introduced a concept of adaptogen and then many plants have been studied for their adaptogenic effect and rejuvenating properties from the Ayurvedic system. [2-3]

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Bacopa monniera (Linn) (family: Scrophulariaceae) commonly known as Brahmi, have been used almost from 3000 years by Ayurvedic medical practitioners in India and is classified as a medhyarasayana, a drug used to improve memory and intellect. *Bacopa monniera* (BM) reported to possess anxiolytic, antidepressant and memory enhancing activity. [4-8] Several clinical studies have confirmed the beneficial actions of BM [9] and the pharmacological actions are mainly attributed to the saponin compounds present in the alcoholic extract of the plant. Rai and coworkers reported that BM has potent adaptogenic activity. [10] It attenuates stress induced alteration in plasma corticosterone and levels of monoamines like NA, 5-HT and DA in cortex and hippocampus regions of the brain. [11] It is also claimed to be useful in the treatment of cardiac, respiratory and neuropharmacological disorders like insomnia, insanity, depression, psychosis, epilepsy and stress. [9] Moreover, Pandareesh and Anand, reported that *bacopa monniera* extract pretreatment attenuates up-regulation of iNOS on exposure to sodium nitroprusside, a NO donor and also down-regulates the expression of iNOS. [12]

Stress has been involved in the pathogenesis of a diverse variety of diseases, like depression and anxiety, immunosuppression, endocrine disorders, memory impairment, peptic ulcer, hypertension and ulcerative colitis, etc. [13] Stress conditions augment nitric oxide synthase (NOS) expression in the brain, which suggests a role of nitric oxide (NO) in regulation of the hypothalamic-pituitary-adrenal (HPA) activity. [14-15] It has been also suggested that treatment with NOS inhibitors and reduction in NO levels can induce anxiolytic-and antidepressant-like effects. [16-17] Blockade of NO synthesis significantly impaired ACTH release in response to a mild electroshock and water avoidance stress, which cause rapid activation of the HPA axis. [18]

Bacopa monniera extract shown to be inhibits expression of iNOS. [12] It is used as anti-stress herbs. [9] Previous studies have shown that NO involves in stress effects. [15] However, no attempt was done so far to study the effect of BM on audiogenic stress-induced behavioral changes or changes in NO levels in brain. Hence, the present study is undertaken to evaluate the effect of BM on audiogenic stress-induced behavioral changes and possible involvement of NO.

MATERIALS AND METHODS

Animals

Adult male albino Swiss mice were born and reared in the animal house of the Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur from a stock originally purchased from the National Institute of Nutrition, Hyderabad, India. Mice (24–30 g) were group housed (4 per cage) in opaque polypropylene cages (28 × 21 × 14 cm) and maintained at 25±2°C under 12.12 h light/dark cycles (07.00–19.00 h) with free access to rodent chow (Trimurti Feeds, Nagpur, India) and water. The studies were approved by the Institutional Animal Ethics Committee, constituted for the purpose of control and supervision of experimental animals by the Ministry of Environment and Forests, Government of India, New Delhi, India. At the beginning of all studies, mice were naive to drug treatment and experimentation. Each experimental group comprised of 6-9 mice. Testing was carried out in a counterbalanced order with respect to the treatment conditions.

Stress procedure

Exposure to audiogenic stress was carried out as described earlier with some modifications. [19-20] Audiogenic stress was induced in mice by subjecting to broadband white noise at 100 dB intensity, produced by a white noise generator, amplified by an amplifier connected to a loud speaker fixed 30 cm above the animal cage. The apparatus was designed with the help of Department of U.S.I.C., R.T.M. Nagpur University, Nagpur. A sound level meter was used to measure the intensity of the noise. Mice were exposed to the audiogenic stress for 12 h (19.00-07.00) in bioacoustic chamber. Animals had free access to food and water during stress exposure.

Drugs and treatments

Plant extract of *Bacopa monniera* (BM, extract contained approximately 20% w/w of the active ingredients (bacosides A and B) by HPLC test method) was a gift sample by herbal manufacturer M/s. Natural Remedies Private Limited (Bangalore, India), the shelf-life of this extract was 2 years. L-Arginine hydrochloride, and L-N$^{\omega}$-nitro arginine methyl ester (L-NAME), 7-Nitroindazole (7-NI) were purchased from Sigma Aldrich, USA, sodium nitroprusside (Rankem, New Delhi.). BM was suspended in 0.3% carboxymethyl cellulose in distilled water. Fluoxetine hydrochloride was a gift from Reliance Laboratories Ltd., India, dissolved in 0.9% saline solution and diazepam (Calmpose, Ranbaxy, India) was dissolved in few drops of tween 80 (0.5%) and diluted with saline. 7-NI was dissolved in DMSO: saline in 1:10 ratios. L-arginine hydrochloride and L-NAME were dissolved in 0.9% saline solution and final volume made in artificial cerebrospinal fluid (aCSF) having composition 0.2 M NaCl, 0.02 M NaH_2CO_3, 2 mM KCl, 0.5 mM KH2PO4, 1.2 mM $CaCl_2$, 1.8 mM $MgCl_2$, 0.5 mM Na_2SO_4, and 5.8 mM D-glucose. All other chemicals were of analytical grade. All drugs solutions were freshly prepared. The doses of drugs are expressed in terms of their free bases.

Intracerebroventricular (i.c.v.) injection

Intracerebroventricular cannulation was carried out as described earlier. [21] In brief, mouse was anesthetized with ketamine and xylazine combination (100 and 5 mg/kg respectively, i.m.) and stainless steel cannula (Becton Dickinson, India, 24 gauge) was stereotaxically implanted with coordinates from Paxinos and Franklin [AP −0.82 mm; ML +1.5 mm and DV +2.0 mm; related to bregma]. A guide cannula was secured to the skull

using mounting screws and dental cement (Dental Products of India, Mumbai). A stainless steel dummy cannula was used to occlude the guide cannula when not in use. The animals were then allowed to recover for a week under antimicrobial cover of cefotaxim (50 mg/kg/day, *s.c.*), during which they were habituated to the experimental protocols to minimize nonspecific stress. Drug/vehicle solution (2µl in 1 min) was injected in to the right lateral ventricle with the help of Hamilton microliter syringe (Hamilton, Nevada, USA) connected to an internal cannula (31 gauges) by polyethylene tube. After injection, the syringe and the connected tubing was left in place for another 1 min before being slowly withdrawn to avoid backflow. At the end of all i.c.v. experiments, a dilute India ink was injected (2µl, i.c.v.) and animals were euthanized by pentobarbitone overdose. Only data from animals showed uniform distribution of ink into lateral ventricles were used for statistical analysis. Less than 10% of the mice were eliminated from results because of inaccurate cannula placement or injection leakage.

The *per se* activity of BM, L-arginine, L-NAME and 7-NI was tested by administering the BM (40, 80 and 120 mg/kg, *p.o.*), L-arginine (5, 10, 20 ηg/mouse, i.c.v.) or L-NAME (25, 50, 75 µg/mouse, i.c.v.) or 7-NI (0.1, 1, 10 ηmol/mouse, i.c.v.) to the mice. The doses selected for this experiment are based on earlier reports (Rai *et al*., 2003; Sheikh *et al*., 2007) [10-11] and preliminary observations carried out in the laboratory. Sixty minutes after *p.o*., and 10 min after i.c.v., administration animals were subjected to the social interaction or forced swim or locomotor test. The methods employed for assessing anxiety-and depression-like behavior have been described in detail under following section. Separate groups of mice were employed for each of the above groups.

To test the adaptogenic effect of BM using audiogenic stress-induced behavioral changes, BM (40 and 80 mg/kg, *p.o.*), L-arginine (5 ηg/mouse, i.c.v.) or L-NAME (25 µg/mouse, i.c.v.) or 7-NI (0.1 ηmol/mouse, i.c.v.) was administered to mice from day 1 to 10. An individual mouse was subjected to overnight audiogenic stress (19.00-07.00, from day 1 to 10), and after overnight exposure to audiogenic stress, individual mouse was subjected to social interaction test, forced swim test, or locomotor activity test on day 1, 4, 7, or 10 (08.00).

To test the hypothesis that adaptogenic effect of BM mediated through NO, BM (40 mg/kg, *p.o.*) was administered in L-arginine (5 ηg/mouse, i.c.v.) pretreated mice and after 60 min of p.o., administration individual mouse was subjected to social interaction test, forced swim test, or locomotor activity test.

In another set of experiment, we investigated the synergistic effect of concomitant administration of BM (40 mg/kg, *p.o.*) with L-NAME (25µg/mouse, i.c.v.) or 7-NI (0.1 ηmol/mouse, i.c.v.) and after 60 min of *p.o.*, administration individual mouse was subjected to social interaction test, forced swim test, or locomotor activity test.

To investigate the effect of BM on NOx levels, in another experiment after the above treatments mice were sacrificed and brain tissues were isolated to investigate NOx levels in cortex, hypothalamus and hippocampus, areas mostly related to anxiety and depression.

Social interaction test

Social interaction test was carried out as described earlier. [21] On days 1 and 2, each mouse was acclimatized for 5 min to a neutral cage (opaque plastic box: 34×22×19 cm). On day 3 (test day), a unfamiliar drug treated mouse was placed together with a unfamiliar untreated mouse in neutral cage, and the social interaction of a drug treated mouse was assessed for 5 min by recording the total time spent by an animal in the activities such as sniffing, adjacent lying, following, crawling under/over partner, and mutual grooming, etc. After each test the fecal matter from the cage was removed and the cage was cleaned with damp cotton soaked with alcohol (70% v/v). The observer was unaware about treatment identity. Increase in interaction time is considered as an anxiolytic effect.

Forced swim test

Forced swim test was carried out by a method described earlier. [21] Mouse was placed for 6 min in a glass cylinder (height: 35 cm; diameter: 17 cm) filled with water (25±1°C) up to 25 cm. Water depth was adjusted so that mouse can swim or float without touching hind limbs or tail to the bottom. As suggested by Porsolt, duration of immobility in the last 4 min was decided on the basis of cumulated time period during which the mouse was either immobile or made simple movements to keep its head above water. Decrease in immobility time is considered as antidepressant effect.

Locomotor activity

Locomotor activity was assessed in actophotometer (VJ Instruments, Karanja (Lad), Washim, India), having a diameter of 40 cm, equipped with three infrared beam cells pair, located on the walls of the circular arena and connected to digital counter. Locomotor activity was expressed as total number of counts of beams interrupted in 30 min.

Isolation of area specific brain tissues (Cortex, hypothalamus and hippocampus)

Following decapitation, the brains were carefully removed and dissected rapidly over the ice-cooled slab into the cortex, hypothalamus and hippocampus as per Glowinski and Iversen. [22] Each region was identified according to the mouse brain atlas of Paxinos and Franklin. [23]

Brain nitrates and nitrites (NOx) assay

Nitrate was reduced to nitrite with the help of copper-cadmium (Cu-Cd) alloy fillings. [24] In brief, brain tissues were homogenized in 1-ml distilled water and centrifuged at 10000× g for 15 min at 4ºC. The 0.4 ml of homogenates/standard nitrate was treated with 150 mg

Cu-Cd fillings in a clean eppendorf and was intermittently shaken for 1 h, after that centrifuged for 10 min, at 4000 rpm. 10.0µl of sample was injected in acid-iodide bath (18 ml distilled water + 2ml of 1M sulfuric acid + 20 mg of potassium or sodium iodide) and the corresponding change in current (pA) was recorded by NO-measuring system. NO-measuring system consists of amino series of NO amperometric sensors, which are covered with membranes that are selectively permeable to NO. Basic principle of the method is, "an electric potential is applied to the sensor's sensing element which forces NO to lose electrons to the sensing element. This result in an electric current and the magnitude of the electric current is proportional to the amount of nitric oxide diffused through the membrane, which is dependent on the concentration of NO in the sample". Brain supernatant protein was estimated by Lowry's method. [25] The data were expressed as ηM NOx/mg of protein.

Data analysis

Data was analyzed using one-way or two-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni post hoc test. The results are expressed as mean±SEM of 5-7 observations per group. $P<0.05$ was considered statistically significant in all the cases.

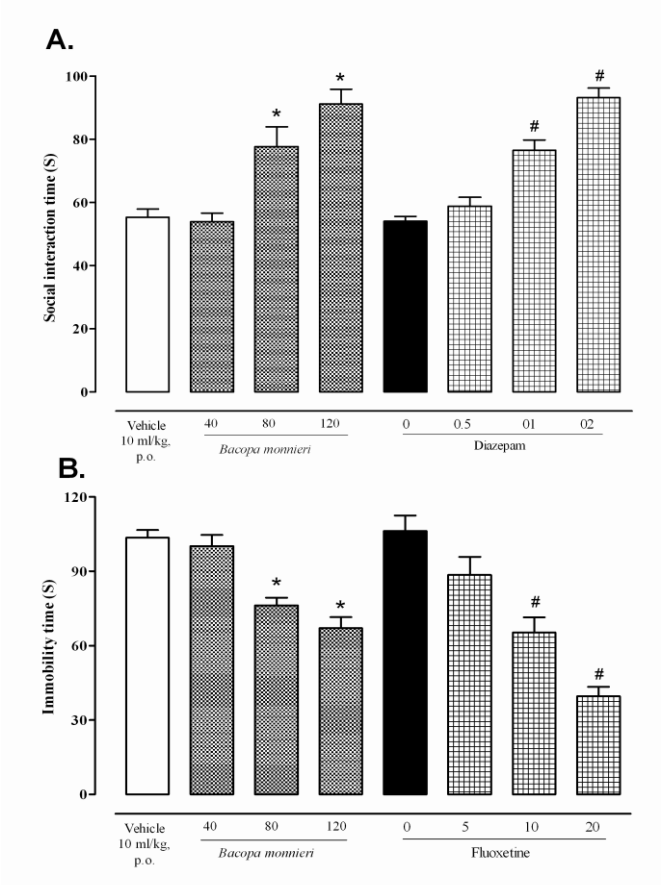

Fig. 1: Dose dependent effect of BM in social interaction test (A) forced swim test (B). Mice were treated with BM (40, 80, 120 mg/kg, *p.o.*) or diazepam (0.5, 1, 2 mg/kg, *i.p.*) or fluoxetine (5, 10, 20 mg/kg, *i.p.*) and after 30 min of *i.p.* and 60 min of per oral administration individual mouse was subjected to either social interaction test or forced swim test. Each bar represent separate group of animals [Mean±SEM (n = 5-7)]. **P*<0.01, #*P*<0.01 vs. their respective controls group (One-way ANOVA followed by Dunnett's test).

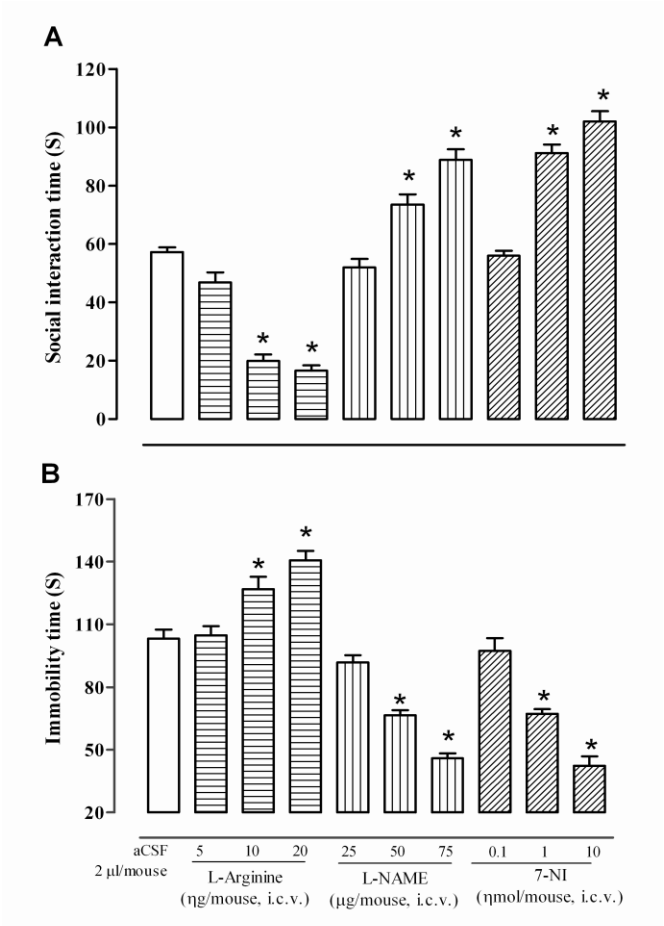

Fig. 2: Dose dependent effect of L-arginine, L-NAME and 7-NI in social interaction test (A) forced swim test (B). Mice were treated with L-arginine (5, 10, 20 ηg/mouse, i.c.v.) or L-NAME (25, 50, 75µg/mouse, i.c.v.) or 7-NI (0.1, 1, 10 ηmol/mouse, i.c.v.) and after 10 min of i.c.v. administration individual mouse was subjected to either social interaction test or forced swim test. Each bar represent separate group of animals [Mean±SEM (n = 5-7)]. **P*<0.01, vs. control group (One-way ANOVA followed by Dunnett's test).

RESULTS

Influence of BM, L-arginine, L-NAME, 7-NI

Social interaction test

Figure 1A and 2A illustrates the effect of BM and nitrergic modulators on the duration of social interaction time. One-way ANOVA revealed that there were significant differences between treatment groups [BM: $F(3,23)=17.34$, $P<0.0001$; L-arginine: $F(3,29)=72.67$, $P<0.0001$; L-NAME: $F(3,26)=33.29$, $P<0.0001$; 7-NI: $F(3,25)=88.74$, $P<0.0001$]. Post-hoc analysis showed that the BM (80 and 120 mg/kg), L-arginine (10 and 20 ηg/mouse), L-NAME (50 and 75µg/mouse) and 7-NI (1 and 10 ηmol/mouse) treated groups were significantly different from the vehicle treated group ($p<0.05$). BM, L-NAME and 7-NI significantly increased the time spent in social interaction indicating anxiolytic effect, whereas, L-arginine significantly decreased time spent in social interaction indicating anxiogenic effect. The effect of BM was comparable to that of diazepam, standard anxiolytic.

Forced swim test

Figure 1B and 2B illustrates the effect of BM and nitrergic modulators on the duration of immobility

time in FST model. One-way ANOVA revealed that there were significant differences between treatment groups [BM: F(3,23)=21.12, *P*<0.0001; L-arginine: F(3,29)=14.25, *P*<0.0001; L-NAME: F(3,26)=56.23, *P*<0.0001; 7-NI: F(3,25)=39.65, *P*<0.0001]. Post-hoc analysis showed that the BM (80 and 120 mg/kg), L-arginine (10 and 20 ηg/mouse), L-NAME (50 and 75µg/mouse) and 7-NI (1 and 10 ηmol/mouse) treated groups were significantly different from the vehicle treated group (p<0.05). BM, L-NAME and 7-NI significantly decreased the duration of immobility time indicating antidepressant effects, whereas, L-arginine significantly increased immobility time indicating depressant effect. The effect of BM was comparable to that of fluoxetine, a standard antidepressant.

Locomotor activity test

Figure 3 illustrates the effect of BM on the locomotor counts in actophotometer. One-way ANOVA revealed that there were no significant differences between treatment groups [F(3,23)=0.6023, *P*=0.6211].

Influence of BM treatment on L-arginine treated mice

Social interaction test

Figure 4A illustrates the effect of BM on the effect of L-arginine treatment on the duration of social interaction time. One-way ANOVA revealed that there were significant differences between treatment groups [F(4,33)=22.71, *P*<0.0001]. Post-hoc analysis showed that administration of BM (80 and 120 mg/kg) to L-arginine pretreated mice significantly attenuated L-arginine induced decreased social interaction time (p<0.05).

Forced swim test

Figure 4B illustrates the effect of BM on the effect of L-arginine treatment on the immobility time. One-way ANOVA revealed that there were significant differences between treatment groups [F(4,33)=5.870, *P*<0.0001]. Post-hoc analysis showed that administration of BM (80 and 120 mg/kg) to L-arginine pretreated mice significantly attenuated L-arginine induced increased immobility time (p<0.05).

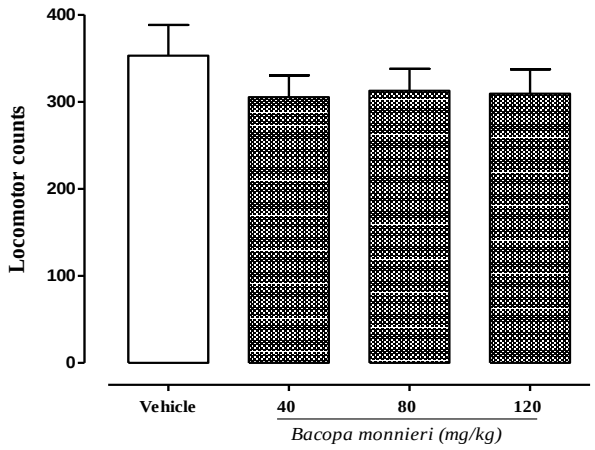

Fig. 3: Dose dependent effect of BM on locomotor counts. Mice were treated with BM (40, 80, 120 mg/kg, p.o.) and 60 min of per oral administration individual mouse was subjected to actophotometer. Each bar represent separate group of animals [Mean±SEM (n = 5-7)]. (One-way ANOVA followed by Dunnett's test).

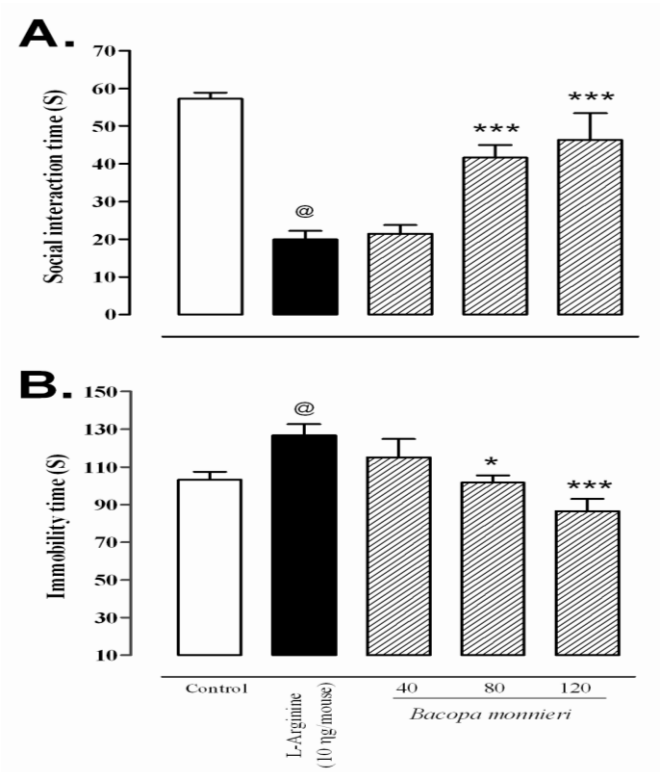

Fig. 4: Influence of BM on the effect of L-arginine in the social interaction test (A) and forced swim test (B). Mice were treated with BM (40, 80, 120 mg/kg, *p.o.*) and 20 min thereafter L-arginine (10 ηg/mouse, i.c.v.) was administered. 10 min after L-arginine administration; an individual mouse was subjected to either social interaction or forced swim test. Each bar represent separate group of animals [Mean±SEM (n = 5-7)]. @*P*<0.01 vs. vehicle treated group and **P*<0.05, ****P*<0.01 vs. L-arginine treated group (One-way ANOVA followed by Dunnett's test).

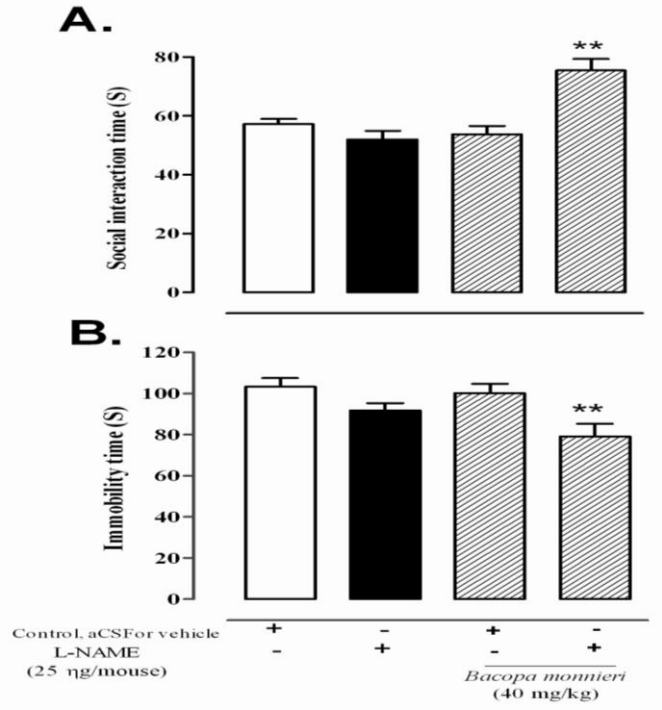

Fig. 5: Influence of sub-effective dose of BM with sub-effective dose of L-NAME in social interaction test (A) and forced swim test (B). Mice were treated with sub-effective dose of BM (40 mg/kg, *p.o.*) with sub-effective dose of L-NAME (25µg/mouse, i.c.v.), 10 minutes after last administration, an individual mouse was subjected to either social interaction or forced swim test. Each bar represent separate group of animals [Mean±SEM (n = 5-7)]. ***P*<0.05 vs. Controls group (One-way ANOVA followed by Dunnett's test).

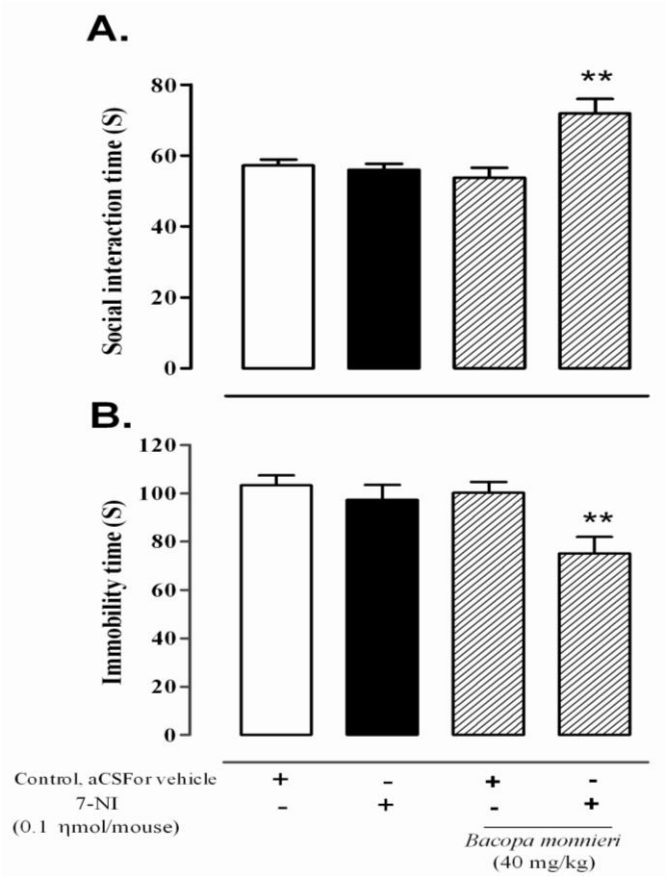

Fig. 6: Influence of sub-effective dose of BM with sub-effective dose of 7-NI in social interaction test (A) forced swim test (B). Mice were treated with sub-effective dose of BM (40 mg/kg, *p.o.*) with sub-effective dose of 7-NI (0.1 ηmol/mouse, i.c.v.), 10 minutes after last administration, an individual mouse was subjected to either social interaction or forced swim test. Each bar represent separate group of animals [Mean±SEM (n = 5-7)]. ***P*<0.05 vs. Controls group (One-way ANOVA followed by Dunnett's test).

Locomotor activity test

One-way ANOVA revealed that there were no significant differences between treatment groups [F(4,33)=0.1667, *P*=0.9533].

Influence of BM treatment on L-NAME treated mice

Social interaction test

Figure 5A illustrates the effect of BM on the effect of L-NAME treatment on the duration of social interaction time. One-way ANOVA revealed that there were significant differences between treatment groups [F(3,25)=14.31, *P*<0.0001]. Post-hoc analysis showed that administration sub-effective dose of BM (40 mg/kg) with sub-effective dose of L-NAME (25µg/mouse) significantly increased social interaction time (p<0.05), indicated synergistic effect.

Forced swim test

Figure 5B illustrates the effect of BM on the effect of L-NAME treatment on the immobility time. One-way ANOVA revealed that there were significant differences between treatment groups [F(3,27)=5.301, *P*=0.0060]. Post-hoc analysis showed that administration sub-effective dose of BM (40 mg/kg) with sub-effective dose of L-NAME (25µg/mouse) significantly decreased immobility time (p<0.05), indicated synergistic effect.

Locomotor activity test

One-way ANOVA revealed that there were no significant differences between treatment groups [F(3,25)=0.1614, *P*=0.9210].

Influence of BM treatment on 7-NI treated mice

Social interaction test

Figure 6A illustrates the effect of BM on the effect of 7-NI treatment on the duration of social interaction time. One-way ANOVA revealed that there were significant differences between treatment groups [F(3,25)=9.743, *P*=0.0003]. Post-hoc analysis showed that administration sub-effective dose of BM (40 mg/kg) with sub-effective dose of 7-NI (0.1 ηmol/mouse) significantly increased social interaction time (p<0.05), indicated synergistic effect.

Forced swim test

Figure 6B illustrates the effect of BM on the effect of 7-NI treatment on the immobility time. One-way ANOVA revealed that there were significant differences between treatment groups [F(3,25)=5.186, *P*=0.0066]. Post-hoc analysis showed that administration sub-effective dose of BM (40 mg/kg) with sub-effective dose of 7-NI (0.1 ηmol/mouse) significantly decreased immobility time (p<0.05), indicated synergistic effect.

Locomotor activity test

One-way ANOVA revealed that there were no significant differences between treatment groups [F(3,25)=0.4856, *P*=0.6961].

Influence of audiogenic stress and its adaptation

Social interaction test

Figure 7A exhibits mean time spent in social interaction in social interaction test between non stress and audiogenic stress group. Two-way RM ANOVA indicated that audiogenic stress had significant influence on time spent in social interaction [Time: F (3, 30) = 11.83, *P*<0.0001 and Treatment: F (1, 30) = 41.58, *P*<0.0001]. Further, post hoc test revealed that audiogenic stress significantly decreased social interaction time on day 1 and 4 (*P*<0.01), whereas, didn't had influence on day 7 and 10.

Forced swim test

Figure 7B exhibits mean immobility time in forced swim test between non stress and audiogenic stress group. Two-way RM ANOVA indicated that audiogenic stress had significant influence on immobility time [Time: F (3, 30) = 7.973, *P*=0.006 and Treatment: F (1, 30) = 90.06, *P*< 0.0001]. Further, post hoc test revealed that audiogenic stress significantly increased immobility time on day 1, 4 and 7 (*P*<0.01), whereas, didn't had influence on day 10.

Locomotor activity

Figure 8 exhibits mean locomotor counts of non stress and ausiogenic stress animals. Two-way RM ANOVA indicated that audiogenic stress had no significant influence on total locomotor counts [Time: F (3, 30) = 0.6216, *P*=0.6066 and Treatment: F (1, 30) = 0.8095, *P*=0.3894].

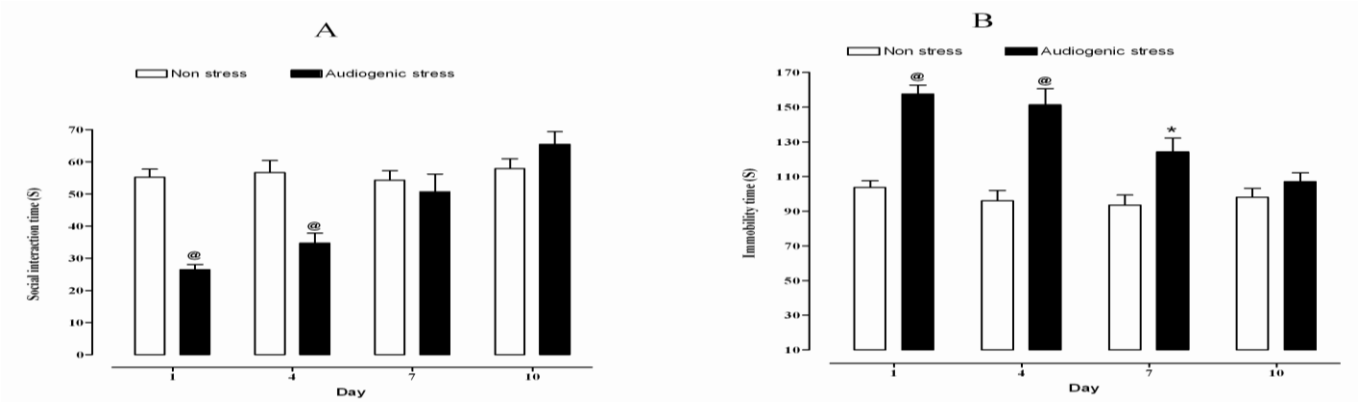

Fig. 7: Influence of audiogenic stress in social interaction test (A) and forced swim test (B). Mice were exposed to audiogenic stress for 12 h (20.00-08.00) from day 1-10, thereafter individual mouse was subjected to either social interaction or forced swim test to assess level of anxiety or depression on day 1st, 4th, 7th and 10th. Each bar represent separate group of animals [Mean±SEM (n=5-7)]. @*P*<0.01, **P*<0.05 vs non stress control group (Two-way RM ANOVA followed by Bonferroni post tests).

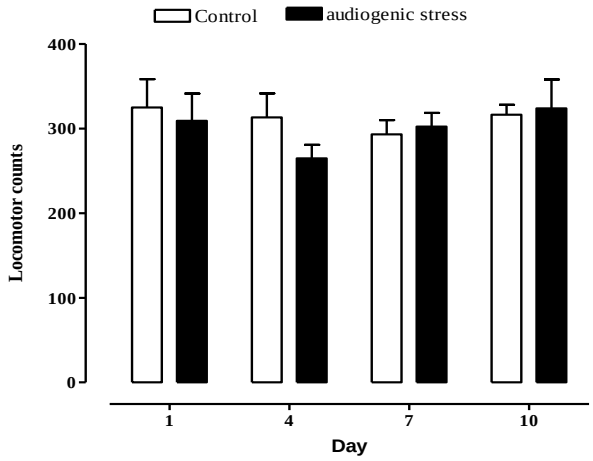

Fig. 8: Influence of audiogenic stress on locomor counts. Mice were exposed to audiogenic stress for 12 h (20.00-08.00) from day 1-10, thereafter individual mouse was subjected actophotometer on day 1st, 4th, 7th and 10th. Each bar represent separate group of animals [Mean±SEM (n=5-7)]. (Two-way RM ANOVA followed by Bonferroni post tests).

Influence of BM, L-arginine, L-NAME and 7-NI on audiogenic stress

Social interaction test

Figure 9A exhibits the effect of BM, L-arginine, L-NAME and 7-NI on social interaction in audiogenic stress exposed mice. Two-way RM ANOVA revealed that there were significant difference between treatments groups [Time: F (3, 105) = 73.69, *P*<0.0001 and Treatment: F (6, 105) = 20.72, P<0.0001]. post hoc test revealed that administration of BM (40 mg/kg) on day 7, BM (80 mg/kg) on day 4 and 7, and 7-NI on day 4 significantly increased social interaction time in audiogenic stress mice as compared to audiogenic stress vehicle treated group (*p*<0.05), indicated facilitation of adaptation to stress, whereas, L-NAME had no significant influence on social interaction time. Administration of L-arginine significantly increased social interaction time, indicated impairment of adaptation to stress.

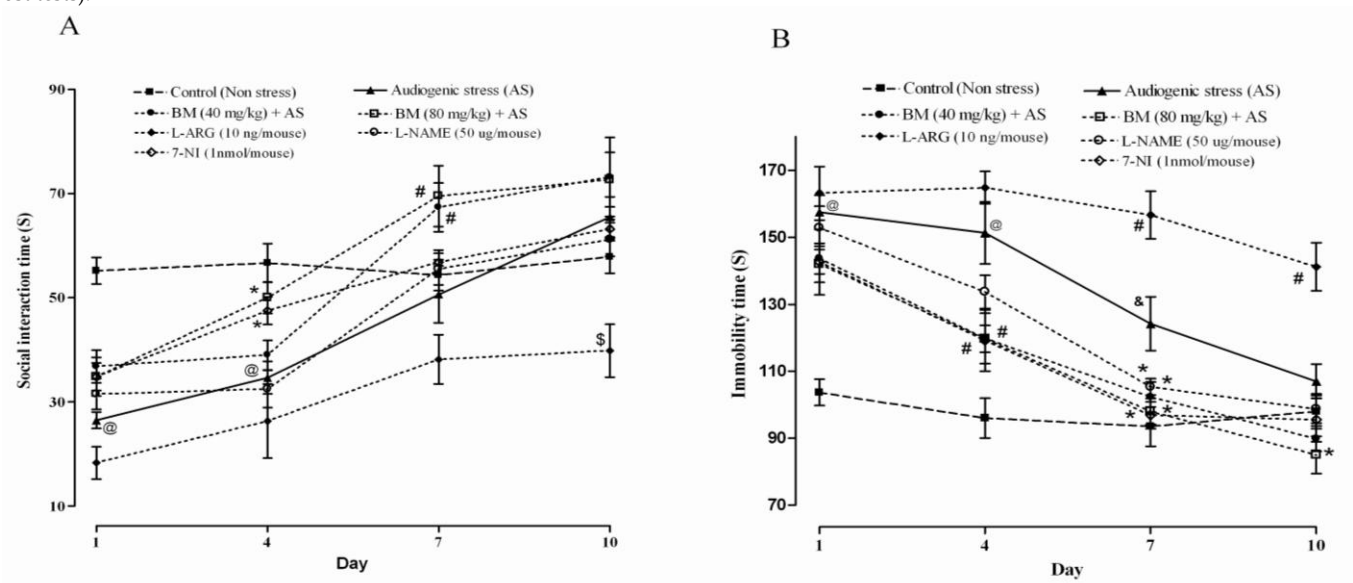

Fig. 9: Influence of BM, L-arginine, L-NAME and 7-NI on adaptation to audiogenic stress assessed by social interaction test (A) and forced swim test (B). Mice were treated with BM (40 and 80 mg/kg, *p.o.*) or L-arginine (10 ηg/mouse, i.c.v.) or L-NAME (50 µg/mouse, i.c.v.) or 7-NI (1 ηmol/mouse, i.c.v.) and after 10 min of i.c.v., 60 min of p.o. administration, individual mouse was exposed to audiogenic stress for 12 h (20.00–08.00) from day 1 to 10. Thereafter, individual mouse was subjected actophotometer on day 1st, 4th, 7th and 10th. Each bar represent separate group of animals [Mean±SEM (n=5-7)]. @*P*<0.01, **P*<0.05 vs. non stress control group, #*P*<0.01, $*P*<0.01, **P*< 0.05 vs. AS group (Two-way RM ANOVA followed by Bonferroni post tests). AS: Audiogenic stress exposed mice.

Forced swim test

Figure 9B exhibits the effect of BM, L-arginine, L-NAME and 7-NI on immobility time in audiogenic stress exposed mice. Two-way RM ANOVA revealed that there were significant difference between treatments groups [Time: F (3, 105) = 69.78, P<0.0001 and Treatment: F (6, 105) = 33.33, P<0.0001]. post hoc test revealed that administration of BM (40 mg/kg) on day 7, BM (80 mg/kg) on day 4 and 7, L-NAME and 7-NI on day 7 significantly decreased immobility time in audiogenic stress mice as compared to audiogenic stress vehicle treated group (p<0.05), indicated facilitation of adaptation to stress. Administration of L-arginine significantly decreased immobility time, indicated impairment of adaptation to stress.

Locomotor activity

Two-way RM ANOVA revealed that various treatments had no significant difference between locomotor counts [Time: F (3, 105) = 0.5230, P=0.9216 and Treatment: F (6, 60) = 1.194, P=0.2582].

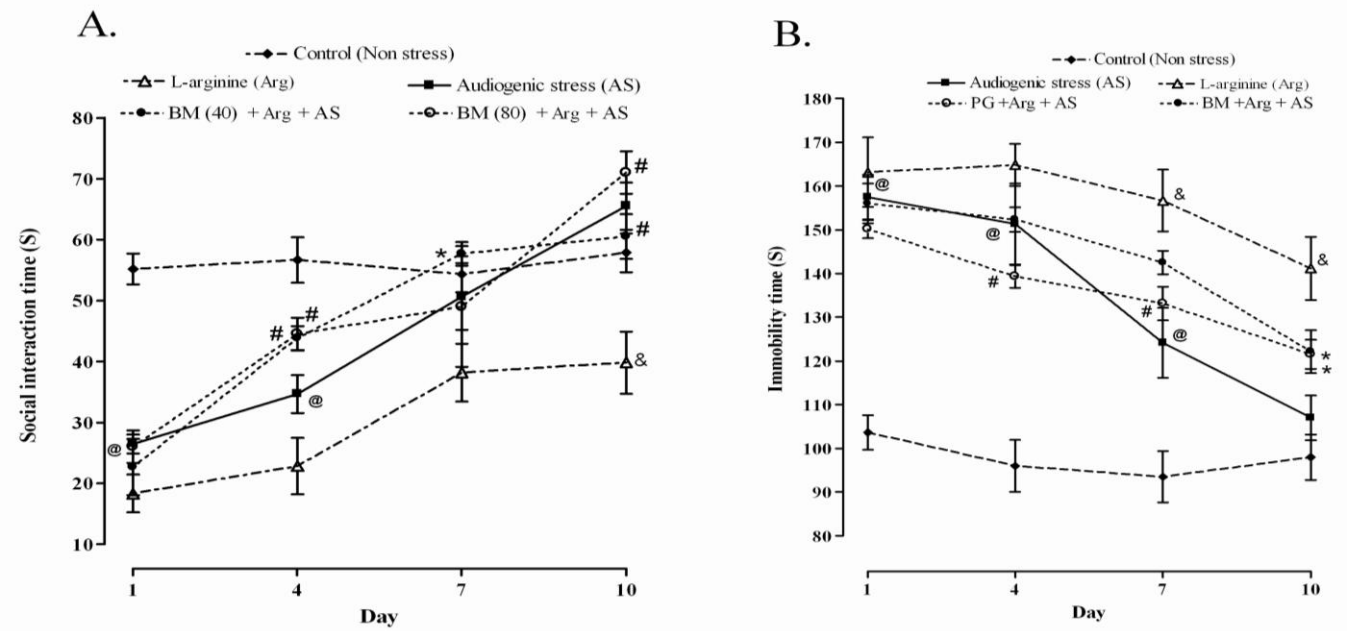

Fig. 10: Influence of BM on L-arginine pretreatment to audiogenic stress exposed mice assessed by social interaction test (A) and forced swim test (B). Mice were treated with BM (40 and 80 mg/kg, *p.o.*) and 20 min thereafter L-arginine (10 ηg/mouse, i.c.v.) was administered, after 10 min of last administration, mice were exposed to audiogenic stress for 12 h (20.00–08.00) from day 1 to 10. Thereafter, individual mouse was subjected either social interaction or forced swim test on day 1st, 4th, 7th and 10th. Each bar represent separate group of animals [Mean±SEM (n=5-7)]. @P<0.01, *P<0.05 vs. non stress control group, $$P$<0.01 &$P$<0.01 vs. AS group and #$P$<0.01, *$P$< 0.05 vs. L-arginine treated group (Two-way RM ANOVA followed by Bonferroni post tests). AS: Audiogenic stress exposed mice, Arg: L-arginine.

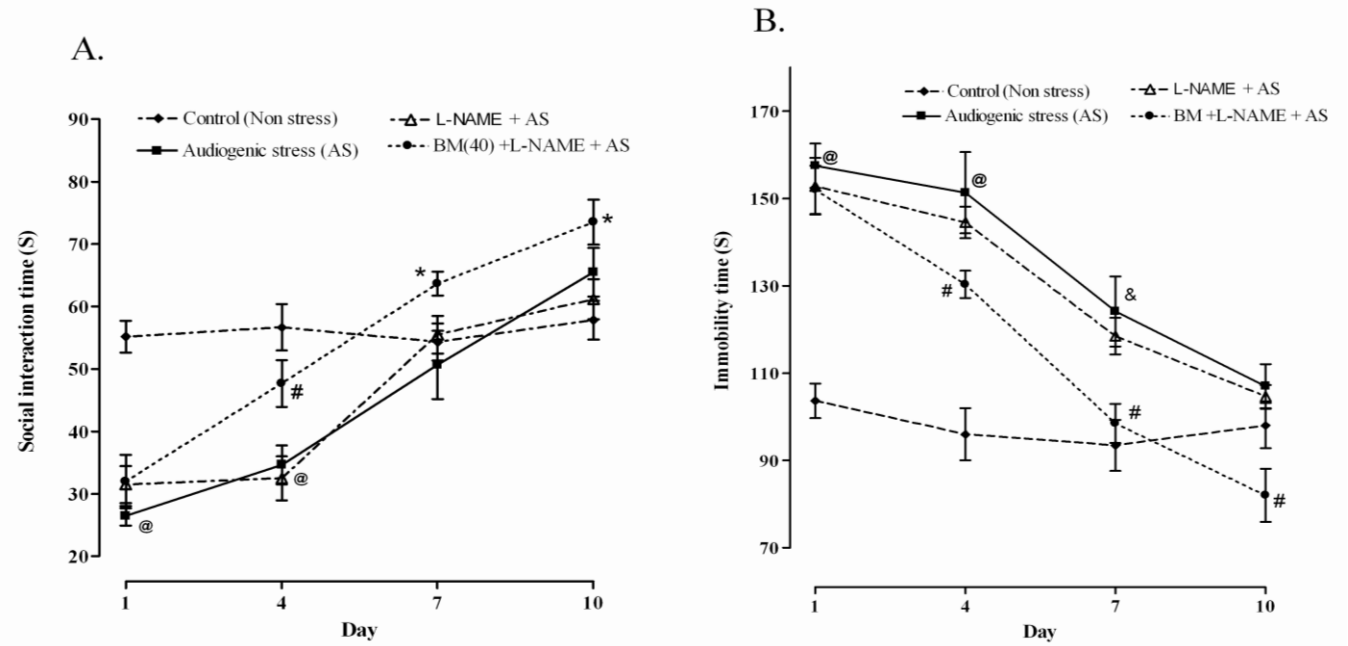

Fig. 11: Influence of concomitant administration of sub-effective doses of BM and L-NAME to audiogenic stress exposed mice assessed by social interaction test (A) and forced swim test (B). Mice were treated with BM (40 mg/kg, *p.o.*) and 20 min thereafter L-NAME (25µg/mouse, i.c.v.) was administered, after 10 min of last administration; mice were exposed to audiogenic stress for 12 h (20.00–08.00) from day 1 to 10. Thereafter, individual mouse was subjected either social interaction or forced swim test on day 1st, 4th, 7th and 10th. Each bar represent separate group of animals [Mean±SEM (n=5-7)]. @P<0.01, &P<0.05 vs. non stress control group, #P<0.01, *P< 0.05 vs. L-NAME treated group (Two-way RM ANOVA followed by Bonferroni post tests). AS: Audiogenic stress exposed mice.

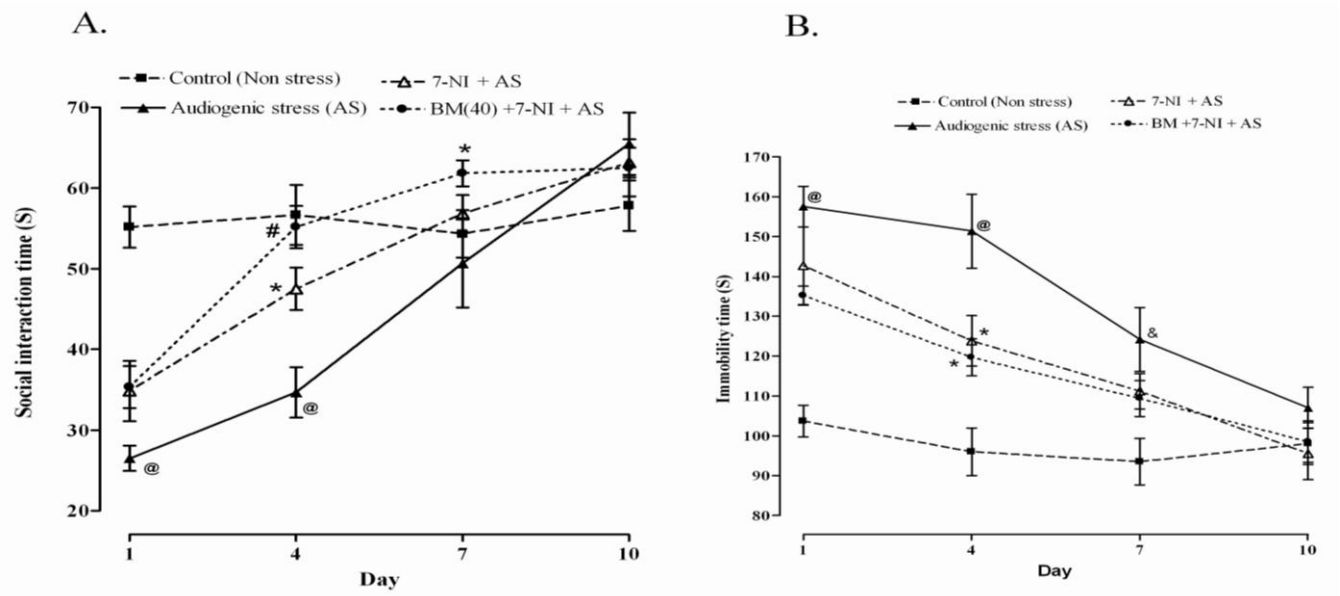

Fig. 12: Influence of concomitant administration of sub-effective doses of BM and 7-NI to audiogenic stress exposed mice assessed by social interaction test (A) and forced swim test (B). Mice were treated with BM (40 mg/kg, *p.o.*) and 20 min thereafter 7-NI (1 ηmol/mouse, i.c.v.) was administered, after 10 min of last administration, mice were exposed to audiogenic stress for 12 h (20.00–08.00) from day 1 to 10. Thereafter, individual mouse was subjected either social interaction or forced swim test on day 1st, 4th, 7th and 10th. Each bar represent separate group of animals [Mean±SEM (n=5-7)]. @*P*<0.01, &*P*<0.05 vs. non stress control group, #*P*<0.01, **P*< 0.05 vs. AS group (Two-way RM ANOVA followed by Bonferroni post tests). AS: Audiogenic stress exposed mice.

Influence of BM on L-arginine treatment in audiogenic stress mice

Social interaction test

Figure 10A exhibits the mean time spent in social interaction after administration of BM to L-arginine treated audiogenic stress exposed mice. Two-way RM ANOVA revealed that there were significant difference between treatments groups [Time: F (3, 75) = 47.63, *P*<0.0001 and Treatment: F (4, 75) = 19.16, *P*<0.0001]. post hoc test revealed that administration of BM (40 mg/kg) on day 4, 7 and 10 and BM (80 mg/kg) on day 4 and 10 significantly attenuated L-arginine induced decreased social interaction time in audiogenic stress exposed mice (*p*<0.05).

Forced swim test

Figure 10B exhibits the mean immobility time after administration of BM to L-arginine treated audiogenic stress exposed mice. Two-way RM ANOVA revealed that there were significant difference between treatments groups [Time: F (3, 75) = 23.89, *P*<0.0001 and Treatment: F (4, 75) = 83.92, P<0.0001]. post hoc test revealed that administration of BM (40 mg/kg) on day 10 and BM (80 mg/kg) on day 4, 7 and 10 significantly attenuated L-arginine induced increased immobility time in audiogenic stress exposed mice (*p*<0.05).

Influence of BM & L-NAME treatment in audiogenic stress mice

Social interaction test

Figure 11A exhibits the mean time spent in social interaction after administration of BM & L-NAME in audiogenic stress exposed mice. Two-way RM ANOVA revealed that there were significant difference between treatments groups [Time: F (3, 60) = 49.06, *P*<0.0001 and Treatment: F (3, 60) = 21.08, *P*<0.0001]. post hoc test revealed that administration of sub-effective doses of BM with L-NAME significantly increased social interaction time in audiogenic stress exposed mice as compared to their individual effect (*p*<0.05).

Forced swim test

Figure 11B exhibits the mean immobility time after administration after administration of BM & L-NAME in audiogenic stress exposed mice. Two-way RM ANOVA revealed that there were significant difference between treatments groups [Time: F (3, 60) = 47.25, *P*<0.0001 and Treatment: F (3, 60) = 50.82, *P*<0.0001]. post hoc test revealed that administration of sub-effective doses of BM with L-NAME significantly increased immobility time in audiogenic stress exposed mice as compared to their individual effect (*p*<0.05).

Influence of BM & 7-NI treatment in audiogenic stress mice

Social interaction test

Figure 12A exhibits the mean time spent in social interaction after administration of BM & 7-NI in audiogenic stress exposed mice. Two-way RM ANOVA revealed that there were significant difference between treatments groups [Time: F (3, 60) = 41.50, *P*<0.0001 and Treatment: F (3, 60) = 14.60, *P*<0.0001]. post hoc test revealed that administration of sub-effective doses of BM with 7-NI significantly increased social interaction time in audiogenic stress exposed mice as compared to their individual effect (*p*<0.05).

Forced swim test

Figure 12B exhibits the mean immobility time after administration after administration of BM & 7-NI in audiogenic stress exposed mice. Two-way RM ANOVA revealed that there were significant difference between treatments groups [Time: F (3, 60) = 26.87, *P*<0.0001 and Treatment: F (3, 60) = 21.92, *P*<0.0001]. post hoc test revealed that administration of sub-effective doses of

BM with 7-NI significantly increased immobility time in audiogenic stress exposed mice as compared to their individual effect (p<0.05).

***Per se* influence on NOx levels**

Table 1 exhibited NOx level in cortex, hypothalamus and hippocampus after treatment with BM and nitrergic modulators in mice. One-way ANOVA revealed that BM treatment had significant influence on NOx level in hypothalamus [hypothalamus: F(3,19)= 5.422, P=0.0019] and no significant in cortex and hippocampus [cortex: F(3, 19)=0.7603, P=0.5326; hippocampus: F(3, 19)=0.7604, P=0.5326]. Further, post hoc test revealed that BM (120 mg/kg) significantly decreased NOx levels in hypothalamus (P<0.05).

One-way ANOVA indicated that L-arginine, L-NAME and 7-NI had significant influence on NOx level [cortex: F(9, 49)= 119.8, P<0.0001; hypothalamus: (9, 49)= 259.4, P<0.0001; hippocampus: F(9, 49)=351.0, P<0.0001]. Further, post hoc test revealed that L-arginine (10 and 20 ηg/mouse) significantly increased NOx level in cortex, hypothalamus and hippocampus (P<0.05), whereas, L-NAME (50 and 75µg/mouse) and 7-NI (0.1 and 1 ηg/mouse) significantly decreased NOx level in cortex, hypothalamus and hippocampus (P<0.05).

Influence of audiogenic stress and BM on NOx levels

Table 2 exhibited effect of BM and nitrergic modulators on NOx level in cortex, hypothalamus and hippocampus in audiogenic stress animals. Two-way NRM ANOVA indicated that audiogenic stress, BM, L-arginine, L-NAME and 7-NI had significant influence on NOx level in cortex: [Treatment: F(6, 84) = 564.9, P<0.0001; Time: F(2, 84) = 429.9, P<0.0001]; hypothalamus: [Treatment: F(6, 84)=1386, P<0.0001; Time: F(2, 84)=1047, P<0.0001]; hippocampus: [Treatment: F(6, 84)=673.5, P<0.0001; Time: F(2, 84)=961.7, P<0.0001]. Further, post hoc test indicated that audiogenic stress significantly increased NOx level in cortex on day 4, hypothalamus on day 4, 7 and 10 and hippocampus on day 4 as compared to non stress group (P<0.05). Treatment with BM, L-NAME and 7-NI significantly decreased audiogenic stress-induced increased NOx level in cortex, hypothalamus and hippocampus on day 4. Administration of L-arginine significantly increased audiogenic stress-induced increased NOx level in cortex, hypothalamus and hippocampus on all day.

In another set of experiment, administration of BM to L-arginine pretreated stress exposed mice showed significant decreased in L-arginine-induced increased NOx level in cortex, hypothalamus and hippocampus on day 4, 7 and 10 (P<0.05), whereas, concomitant administration of BM with L-NAME significantly decreased NOx level in cortex on day 4, hypothalamus and hippocampus on day 4, 7 and 10 as compared to L-NAME treatment in stressed animals (P<0.05). In addition, concomitant administration of BM with 7-NI significantly decreased NOx levels (P<0.05) in hypothalamus on day 4 and 10, and hippocampus on day 7 and 10 as compared to 7-NI treatment in stressed animals (Table 3).

DISCUSSION

The results of the present study revealed that BM *per se* dose dependently exhibited anxiolytic-and antidepressant-like effects. These results are well in accordance with earlier reports. [5-6] In addition, L-NAME and 7-NI dose dependently exhibited anxiolytic-and anti-depressant-like effects, whereas, L-arginine produced just opposite effects. Moreover, administration of BM to L-arginine pretreated mice significantly decreased L-arginine induced anxiety-and depression-like effects. It has been also found that concomitant administration of sub-effective dose of BM with sub-effective dose of L-NAME or 7-NI significantly decreased anxiety-and depression-like effects. These effects of BM focused towards the involvement of nitric oxide in the anxiolytic-and antidepressant-like effects.

All the living beings are susceptible to variety of stressful situation and which can elicit by different factors like, environmental, social, or pathological conditions occurring during the life and determine changes in the nervous and endocrine systems.

Table 1: Dose dependent effect of BM, L-arginine, L-NAME and 7-NI on NOx level in cortex, hypothalamus and hippocampus. Each value represent separate group of animals [Mean±SEM (n=5 or 6). *P<0.05, $^{\$}P$<0.05 vs. respective control group (One-way ANOVA followed by Dunnett's test).

Treatment	Brain NOx level (ηM/mg of protein)		
	Cortex	Hypothalamus	Hippocampus
Control	2.15±0.10	2.96±0.19	1.63±0.09
BM (40 mg/kg)	2.26±0.15	2.67±0.22	1.64±0.22
BM (80 mg/kg)	2.08±0.30	2.77±0.34	1.60±0.15
BM (120 mg/kg)	2.36±0.34	2.34±0.22*	1.78±0.30
Control	1.88±0.07	3.11±0.17	1.48±0.11
L-arginine (5 ηg/mouse)	2.00±0.07	3.05±0.19	1.56±0.06
L-arginine (10 ηg/mouse)	2.46±0.05$^{\$}$	3.92±0.12$^{\$}$	2.67±0.06$^{\$}$
L-arginine (20 ηg/mouse)	3.15±0.24$^{\$}$	5.53±0.10$^{\$}$	4.09±0.10$^{\$}$
L-NAME (25µg/mouse)	2.00±0.10	2.97±0.32	1.77±0.12
L-NAME (50µg/mouse)	1.64±0.03	2.18±0.13$^{\$}$	1.34±0.06
L-NAME (75µg/mouse)	1.56±0.06$^{\$}$	1.75±0.09$^{\$}$	1.09±0.17$^{\$}$
7-NI (0.1 ηg/mouse)	1.96±0.09	3.10±0.10	1.50±0.14
7-NI (1 ηg/mouse)	1.63±0.16$^{\$}$	2.49±0.11$^{\$}$	1.35±0.09
7-NI (10 ηg/mouse)	1.16±0.08$^{\$}$	1.52±0.13$^{\$}$	0.81±0.09$^{\$}$

Table 2: Influence of BM, L-arginine, L-NAME and 7-NI on NOx level in cortex, hypothalamus and hippocampus in audiogenic stress exposed mice. Each value represent separate group of animals [Mean±SEM (n=5 or 6). #*P*<0.05 vs. non stress group and **P*<0.05, vs. stress group (Two-way NRM ANOVA followed by Bonferroni post tests).

Groups	Days	Brain NOx level (ηM/mg of protein)		
		Cortex	Hypothalamus	Hippocampus
Non stress	4	1.97±0.03	3.18±0.03	3.00±0.12
	7	1.82±0.09	3.03±0.10	2.82±0.12
	10	1.92±0.10	3.00±0.06	2.56±0.11
Stress	4	4.62±0.31#	7.23±0.31#	5.61±0.13#
	7	2.10±0.23	4.12±0.06#	2.93±0.16
	10	1.85±0.15	3.24±0.02#	2.52±0.14
BM (40 mg/kg)	4	3.11±0.25*	5.23±0.15*	5.47±0.35
	7	2.13±0.10	3.56±0.15*	2.86±0.20
	10	1.88±0.10	3.22±0.40	2.45±0.12
BM (80 mg/kg)	4	2.92±0.11*	4.23±0.14*	3.56±0.21*
	7	2.04±0.09	3.12±0.10*	2.28±0.20*
	10	2.14±0.10	3.22±0.16	2.16±0.15
L-arginine	4	5.77±0.13*	8.41±0.09*	6.60±0.14*
	7	4.31±0.38*	6.30±0.16*	5.05±0.20*
	10	4.22±0.23*	6.23±0.06*	5.45±0.10*
L-NAME	4	2.65±0.02*	3.59±0.08*	3.34±0.05*
	7	1.98±0.01	3.12±0.03*	2.98±0.03
	10	1.96±0.06	2.92±0.04*	2.25±0.07*
7-NI	4	2.14±0.09*	3.47±0.03*	3.24±0.06*
	7	1.98±0.22*	3.35±0.10*	2.99±0.01
	10	1.75±0.07	3.24±0.09	2.38±0.13

Table 3: Influence of BM on L-arginine or L-NAME or 7-NI pretreated audiogenic stress exposed mice. Each value represent separate group of animals [Mean±SEM (n=5 or 6). #*P*<0.05 vs. stress group, **P*<0.05 vs. L-arginine treated group, $ *P*<0.05 vs. L-NAME treated group, ^*P*<0.05 vs. 7-NI treated group (Two-way NRM ANOVA followed by Bonferroni post tests).

Groups	Days	Brain NOx level (ηM/mg of protein)		
		Cortex	Hypothalamus	Hippocampus
Stress	4	4.62±0.31	7.23±0.31	5.61±0.13
	7	2.10±0.23	4.12±0.06	2.93±0.16
	10	1.85±0.15	3.24±0.02	2.52±0.14
L-arginine	4	5.77±0.13#	8.41±0.09#	6.60±0.14#
	7	4.31±0.38#	6.30±0.16#	5.05±0.20#
	10	4.22±0.23#	6.23±0.06#	5.45±0.10#
BM (40 mg/kg) + L-arginine	4	3.61±0.22*	6.22±0.19*	4.20±0.10*
	7	2.00±0.09*	5.10±0.12*	3.15±0.04*
	10	2.10±0.20*	4.05±0.10*	2.70±0.31*
BM (80 mg/kg) + L-arginine	4	2.76±0.10*	5.03±0.30*	4.38±0.15*
	7	2.38±0.20*	5.26±0.10*	3.56±0.10*
	10	2.98±0.14*	5.55±0.12	4.10±0.20*
L-NAME	4	2.65±0.02#	3.59±0.08#	3.34±0.05#
	7	1.98±0.01	3.12±0.03#	2.98±0.03
	10	1.96±0.06	2.92±0.04#	2.25±0.07#
BM (40 mg/kg) + L-NAME	4	2.30±0.02$	3.10±0.02$	3.10±0.06$
	7	2.00±0.15	3.02±0.11$	2.69±0.10$
	10	2.00±0.10	2.88±0.12$	2.20±0.06$
7-NI	4	2.14±0.09#	3.47±0.03#	3.24±0.06#
	7	1.98±0.22#	3.35±0.10#	2.99±0.01
	10	1.75±0.07	3.24±0.09	2.38±0.13
BM (40 mg/kg) + 7-NI	4	2.07±0.06^	3.70±0.10^	3.10±0.10^
	7	1.78±0.20^	3.24±0.07^	2.96±0.06
	10	1.65±0.05^	3.00±0.10^	2.10±0.14^

Literature documented and focused on stress-induced alterations of behavioral and biochemical effects in these systems. [26-28] Normally such stress-induced changes are adaptive in nature until and unless such changes caused diseased conditions. Adaptation to stress decreases the stress-induced changes and altered metabolism. The desire to control the coping mechanism has led to emergence of science of adaptation and focusing to find the mechanism of adaptation that may help in the modification of responses. Exposure to audiogenic stress produced behavioral changes which revealed by the increased anxiety and depression in the mice. Further, continuous exposure of mice to audiogenic stress for 7 days produced adaptation which revealed by the normalizing the social interaction time in social interaction test and immobility time in forced swimming test. Moreover, administration of BM prior to the exposure to the audiogenic stress significantly decreased not only anxiety-and depression-like effects on day 4 and 7 but also decreased time required for adaptation. Further, administration of L-arginine significantly increased anxiety-and depression-like effects and produced impairment of adaption to stress.

L-NAME and 7-NI produced just opposite effects to that of the L-arginine. In addition, concomitant administration of BM with L-NAME or 7-NI produced facilitation of adaption to stress.

BM reported to possess anxiolytic activity and improve memory retention in Alzheimer's disease; [5-6] the observed behavioral changes are associated with changes in levels of monoamines NA, DA and 5-HT in cortex and hippocampus regions of brain. [11, 29] But, the nitrergic basis for the observed behavioral changes was not established. In our study, exposure to audiogenic stress significantly increased NOx levels and after adaption to stress on day 7 showed normalization of NOx levels. Stress reported to increase NOx levels in various brain regions. [30] Treatment with BM decreased NOx levels on day 4 and 7, where we also found decreased anxiety and depression, further supports that decreased nitric oxide level produces anxiolytic-and antidepressant-like effects. Our results are in accordance with studies that have shown that NOS inhibitors and reduction of NO levels in different brain areas can induce anti-stress or anxiolytic- or antidepressant-like effects, thus implicating NO in the neurobiology of stress. [16, 30-31] Pandareesh and Anand, reported that BM down regulated the expression of iNOS. [12] In addition, combination of sub-effective doses of BM with L-NAME or 7-NI produced synergistic effect, further supports inhibitory role BM on NOS.

Noteworthy, the reversal of effect of BM by the pretreatment with L-arginine, a NO precursor, further indicates the involvement of NO in the effects of BM. Nitric oxide is involved in the regulation of neurotransmission in nervous system. [32-33] Neuronal nitric oxide synthase (nNOS) is most highly expressed in cell populations of the hypothalamus. [15, 34] A major source of NO at the median eminence might be endothelial in origin rather than neuronal. Under *in vivo* conditions both neuronal and endothelial NOS are involved in the NOS-inhibitor induced impairment in ACTH and corticosterone secretion. [35] Increased NO levels during stress exposure are reported to induce apoptosis [36] and NO is reported to involved in adaptation. [30] It is reported that NO at low levels induces brain derived necrosis factor [37] and inhibit apoptosis [38] thus facilitated the process of adaptation.

Stress management is not an easily accomplished task (see http://www.isma.org.uk). The benzodiazepine anxiolytics, despite having significant anti-stress activity against acute models of stress, have not proved effective against stress-induced adverse effects on immunity, behaviour, cognition, peptic ulcer and hypertension. [13] Furthermore, these drugs have liability of dependence and produces adverse effects. [39] In such situation, adaptogens appears to be stress-protective and facilitate the adaptation.

Therefore, considering the effects of BM shown herein, it is feasible to suggest that the adaptogenic effect of BM might be related to its inhibitory role on nitric oxide shown by decreased NOx levels in cortex, hypothalamus and hippocampus. Results of the present study supports the anti-stress activity of BM reported previously (Chowdhuri *et al*., 2002). Further, studies are to confirm this possible adaptogenic mechanism of BM.

In conclusion, the results of the present study revealed that BM exerted adaptogenic activity assessed by social interaction and forced swim test. In addition, adaptogenic effect was shown to be dependent on its interaction with nitric oxide. Moreover, BM decreased NOx levels in cortex, hypothalamus and hippocampus. Altogether, the results indicate that *Bacopa monniera* may produce adaptogenic effect through nitric oxide. This is preliminary data from our laboratory, further experiments are necessary to clarify the effects of *Bacopa monniera*, and evaluate its benefits in stress management strategies.

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