Advances in Bioresearch
Adv. Biores., Vol 12 (3) May 2021: 59-75
©2021 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.12.3.5975

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ORIGINAL ARTICLE

Stress Management with a Combination of Bioactive Extracts in Swiss albino mice

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ABSTRACT
The present study was designed to explore the antidepressant potential of combination of Ocimum sanctum and Bacopa monera at different doses in unstressed and stressed mice. Swiss male albino mice were subjected to chronic unpredictable mild stress daily for 42 days to induce depressive-like behaviour. Ocimum sanctum (25, 50, 100 mg/kg p.o), Bacopa monera (30, 60, 120 mg/kg p.o) and Fluoxetine (10 mg/kg, i.p) were administered for 6 weeks to separate groups of unstressed and stressed mice. Open field test, Elevated plus maze, Morris water maze test were used to evaluate antidepressant effect of the drugs. Different doses of combination of Ocimum sanctum and Bacopa monera and fluoxetine significantly decreased immobility period of unstressed mice in MWM, improving spontaneous locomotor activity as compared to their respective controls. The combination OS and BM, significantly inhibited brain MAO-A activity, decreased plasma nitrite, brain malondialdehyde and increased catalase, SOD, reduced glutathione levels and decreased plasma corticosterone level of unstressed and stressed mice. The present study suggested that the combination of OS + BM (25 + 30 mg/kg) was found significant effective possibly through by increasing spontaneous locomotor activity, improving spatial learning and memory, causing inhibition of MAO-A enzyme, decreasing plasma corticosterone levels, elevating antioxidant status, decreasing lipid peroxidation, and nitrite levels in brain. Thus, combination of OS + BM (25 + 30 mg/kg) may be explored further for management of mood disorder depression.
Keywords: *Chronic unpredictable mild stress; Ocimum sanctum; Bacopa monera; Depression; mood disorder.*

Received 17.02.2021 Revised 22.04.2021 Accepted 03.05.2021

How to cite this article:
V M Bhalchim, P A Pansare, R R Doke, K R Rode , S R Sainani. Stress Management with a Combination of Bioactive Extracts in Swiss albino mice. Adv. Biores. Vol 12 [3] May 2021. 59-75

INTRODUCTION

Depression a common chronic psychiatric disorders[1] characterized by persistent negative mood, thoughts, loss of interest, anhedonia, feeling of worthlessness, sleep disturbances and 14 suicidal tendencies and cognitive dysfunction [2], Over 322 million people globally wherein India is leading country with 57 million population 'living with depression. Many other factors like psychological, social, environmental and genetic can result in depression [3]. It is thought that hypothalamic-pituitary-adrenal (HPA) axis hyperactivity and monoamine deficiency are to be involved in pathogenesis of depression [4,5]. The monoaminergic theory explains the depletion of monoamine neurotransmitters like serotonin, dopamine, noradrenaline [6]in the hippocampus, limbic system and frontal cortex and also increased oxidative stress and nitrosative damage[7] is responsible for depression. Some emerging evidence suggested that prolonged exposure to stress leads to memory impairment [8] as the hippocampus is sensitive to chronic stress [9]. Depression induced by stress causes inhibition of hippocampal neurogenesis leading to hippocampal atrophy [10].BDNF a neurotrophic factor responsible for neurite growth, differentiation and survival of neurons in the central nervous system[11]. Some studies explored that stress leads to disturbance in synapse morphology, reducing neurogenesis, and neurotransmission in the hippocampus[12]. Although the underlying pathophysiology of depression is not clearly understood yet but some preclinical and clinical evidences suggested there decrease in the level of two vita neurotransmitters, serotonin (5-HT) and Norepinephrine (NE) [13, 14] that are involved in cognitive

processes in central nervous system (CNS) [15]. MAO is a pivotal enzyme that is associated with metabolism of these monoamines. Decreased levels of 5-HT and NE increases oxidative and nitrosative stress found in patients with major depression [16]. Some studies explored that nitric oxide plays a prominent role in pathogenesis of major depression. Stressful conditions in mice significantly found to increase plasma nitrite levels, an index of nitric oxide production.

In depression, there is hypersecretion of corticotropin releasing hormone and impairment in responsiveness towards glucocorticoids[17]in almost 80% depressive patients. When chronic stress is subjected to mice it exhibits the same hyperactivity of hypothalamic pituitary axis [18]. Stress occurs due to day to day life events. Stress plays a crucial role in the development of human depression [19]. Laboratory animals when subjected to CUMS exhibit depressive symptoms resembling human depression [20, 21]. CUMS induced increases brain oxidative stress thus considered as a pivotal factor for neurotoxicity and neuronal death [22, 23]. Currently available antidepressants mainly act on central nervous system (i.e serotonergic and noradrenergic synaptic neurotransmission) [24] The most commonly prescribed are the Selective serotonin reuptake inhibitors (paroxetine, fluoxetine, citalopram, escitalopram, sertraline) and Selective noradrenaline reuptake inhibitors (reboxetine and desipramine)[25]. Other available drugs are Tricyclic antidepressants (imipramine, doxepin) and MAO inhibitors (moclobemide, clorgyline). Although these drugs are effective to minimise the depressive episodes, a significant proportion of depressive patients do not show any improvement until 2-3 weeks after start of treatment. Moreover, one-third of patients do not respond to treatment[26]. Additionally these antidepressants are associated with variety of side effects including sedation, anticholinergic effects (dried mouth, blurred vision, urinary retention, constipation, etc.), seizures, impotence, postural hypotension, anxiety, dizziness, respiratory problems, weight gain, cheese reaction (in case of MAO inhibitors), cardiac dysrhythmias, insomnia agitation and drowsiness[25]. Therefore it has become a need to replace these antidepressants drugs and thus find an alternative and explore plants and their bioactive phytoconstituents exhibiting antidepressant activity.

Several ayurvedic herbal drugs have been proven to improve various disorders. Among one of those are *Ocimum sanctum* (Tulsi) and *Bacopa monera* (Brahmi), which have been individually proven to possess a variety of pharmacological activity. Interestingly according to literature *Ocimum sanctum* have been reported possess anticarcinogenic [27], antidiabetic [28], anti-inflammatory [29], antioxidative [29], antibacterial[30], antistress [31], and antiulcer properties whereas *Bacopa monera* possess mental stress, psychiatric disorders, anxiety disorders, obsessive compulsive disorders, hysteria, epilepsy and insomnia and memory loss[32]. Since both these drugs have been proven individually to exhibit antistress activity. Thus the present study was designed to explore potential antidepressant activity of combination of *Ocimum sanctum* and *Bacopa monera* with varying dose combination in chronic unpredictable mild stress in mice.

MATERIAL AND METHODS

Experimental Animals

The experiments were carried out on naïve Swiss albino mice (Crystal Biological Solutions, India) weighing 25-30 g at the beginning of the experiments. Female mice were not used as estrogens have been reported to possess antidepressant activity [33]. The Animals were housed separately in groups of 10 per cage (polycarbonate cage size: 29 cm ×22 cm ×14 cm) maintained under standard laboratory conditions (12hr light/dark cycle, room temperature 22±5^{0}C, relative humidity 50-70%) with free access to food and water and were kept for acclimatization for at least 7 days. Different mice were used for each drug treatment. The experimental protocol was approved by Institutional Animal Ethics Committee and animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India Registration no. (198/PO/Re/S/2000/CPCSEA) of Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune-410018.

Drugs and Chemicals

Following herbal extracts of both *Ocimum sanctum* and *Bacopa monera* (Phyto life sciences pvt ltd. Ahmedabad, India with certificate of analysis (COA) and Fluoxetine (Sigma Aldrich) were used in the present study.

Vehicles

Both *Ocimum sanctum* and *Bacopa monera* were dissolved in distilled water and Fluoxetine hydrochloride dissolved in saline as per required.

Selection of doses

The doses of *Ocimum sanctum* (25, 50, 100 mg/kg) [34] and *Bacopa monera* (30, 60, 120 mg/kg) [35] were selected on the basis of literature. Drug solutions were freshly prepared on each day of experiments.

Chronic unpredictable mild stress

Different stress methods were performed on mice to induce depression as reported in earlier studies [1, 36, 38]. The mice were daily subjected to different stresses over a 6 weeks period once in day during 9:00 a.m – 6:00 p.m. Daily routine paradigm was prepared and stresses were performed accordingly as mentioned in table 1. Drugs were administered prior 30 mins before the stress paradigm.

	Mon	Tue	Wed	°CThur	Fri	Sat	Sun
Week 1	F	E	C	B	T	O	S
Week 2	W	S	O	T	C	E	B
Week 3	E	C	B	F	S	T	O
Week 4	O	W	S	T	E	B	C
Week 5	C	B	W	O	T	S	E
Week 6	S	O	E	C	B	T	F

F: Food deprivation (10-12 hrs); W: Water deprivation (10-12 hrs); E: Exposure to empty cage (24 hrs); O: Foreign object (24 hrs); C: Cold swimming (5 °C) fir 1 min; S: Forced swimming (37 °C); B: Wet bedding (10- 12 hrs); T: Cage tilting at 45°C for 7 hrs.

Experimental Protocol

The animals were randomly divided into 12 different groups having 10 animals each.

Group-1: Normal; Group-2:Stressed (CUMS)group; Group-3: Fluoxetine (10 mg/kg; i.p); Group-4: OS (25 mg/kg); Group-5: OS (50 mg/kg); Group-6: OS (100 mg/kg); Group-7: BM (30 mg/kg); Group-8: BM (60 mg/kg); Group-9: BM (120 mg/kg); Group-10: OS + BM (12.5 + 15 mg/kg); Group-11: OS + BM (25 + 30 mg/kg); Group-12: OS + BM (50 + 60 mg/kg).

Behavioural parameters

Open field test

The open field test was performed to evaluate the locomotor activity as per procedure described in previous studies [39]. The locomotor activity of the mice was measured in a locomotor monitoring cage (30×30×40 cm, VJ instruments Pvt. Ltd., Washim) for 5 min. The immobility time and total distance travelled by mice was evaluated. The entire session was video recorded attached to a computerized system. Apparatus was cleaned with 70% alcohol between every animal tested.

Elevated plus maze

The anxiety and depressive behavior in rodents was evaluated as per described procedure in previous studies [39]. The EPM apparatus consists of a central platform with two opposing open arms (31.5 × 8 cm) and two closed arms (31.5 × 8 cm) originated. Arms of the EPM were elevated to the height of 70 cm from the ground. Mice were placed into an open field box (31.5×31.5×40 cm) for 5 min, and then placed on the central platform such that their head faced an open arm and were allowed to explore EPM for 5 min. The percentage of open arm entries of mice was recorded. Apparatus was cleaned with 70% alcohol between every animal tested.

Morris water maze test

The spatial learning and memory was evaluated via MWM as previously reported [40]. The MWM computer-aided controlling system consisted of a black circular pool (150 cm diameter and 60 cm height) which was divided into four quadrants and filled with water (23°C) to a depth of 50 cm and a camera hanged over it. A black platform (10 cm in diameter) was fixed in the center of one quadrant and submerged 2 cm below the water surface. The acquisition tests were performed twice daily from different starting positions for three consecutive days. Mice able to escape onto the platform within 90 s were allowed to stay on it for 10 s, and then were warmed in the home cage. However, if they failed to find the platform within 90 s, they were kept on the platform for 15 s to help remember the platform location. The average escape latency time of mice was measured on the fourth day and was analyzed by video tracking system [41].

Biochemical estimations

After subjecting unstressed and stressed mice to MWM on the 42nd day and 1 h after drug administration on the 43rd day, blood (0.5–0.8 ml) was withdrawn from the retro-orbital plexus of mice. Blood was centrifuged at 2500 rpm for 10 mins and plasma was separated which was used to analyse corticosterone

levels in plasma. After collecting blood samples, mice were sacrificed by decapitation, and their brains were isolated. Briefly, 10% w/v homogenate of the brain was prepared in ice cold homogenizing solution (Tris- sucrose buffer, 0.25 M, pH 7.4) for 1 minute on ice using a tight- fitting Teflon pestle attached to a homogenizer (Remi Scientific, India). Homogenization was performed with 10-12 strokes set to 600-1,000 rpm at 40 c. The homogenate was then inspected, if intact tissue was still evident the homogenization was repeated. Resulting homogenates were centrifuged at 1000xg for 10 min at 40°C to obtain the nuclear pellet. Mitochondria were obtained by centrifuging the post-nuclear supernatant at 10,000 x g for 20 min at 40 c to obtain the mitochondrial pellet and cytosolic fraction. The pellet was washed three times with an ice-cold mannitol-sucrose-HEPES buffer (pH 7.4) to get intact mitochondria re-suspended in the same buffer. The resuspended mitochondria pellets were stored at -70°C and were used to analyse MAO-A levels whereas the nitrite level, antioxidants levels, MDA levels were measured by using brain homogenate [36].

Estimations of blood plasma cortisol level

The cortisol level in the blood plasma was estimated according to the method described by Bartos and Pesez[42]. To 50 µl of sample in ethanol, 25 µl of 0.10% solution of p-nitroso- N,N-dimethylaniline in ethanol was added and the tubes were immersed in ice water for 5 min, and then 25 µl of 0.10 N sodium hydroxide was added. The tubes were plugged with cotton-wool, and were let to stand at 0 to 8^0 C for 5 h, protected against light. To the above solution, 100 µl of buffer pH 9.8, 250 µl of 0.10% solution of phenol in ethanol and 25 µl of 1.0% aqueous solution of potassium ferricyanide were added. The tubes were kept in a water bath at 20 ± 280 C for 10 min. The absorbance was read at 650 nm using a microplate reader (Biotek, Mumbai).

Estimation of protein content

Protein content in the homogenate was determined using the dye binding method of Bradford, where in bovine serum albumin (BSA) was used as a standard. Briefly, 20 µl of brain homogenate was added to 1 ml of Bradford Reagent (Sigma Aldrich) and incubated at 37°C for 15 min. The absorbance was measured at 595 nm with the help of a UV spectrophotometer (Shimadzu-1800).

Estimation of MDA assay level

Malondialdehyde (MDA) is one of the major degradative products of lipid peroxidation and serves as a marker for oxidative stress. The extent of lipid peroxidation in the brain was determined quantitatively by performing the spectrophotometric method as described earlier [43]. Briefly the method describes, 0.2ml tissue homogenate was added to 0.2ml of 8.1% SDS, 1.5ml of 20% acetic acid solution adjusted to pH 3.5 with NAOH and 1.5 ml of 0.8% aqueous solution of TBA, the final mixture volume was adjusted to 4.0 ml with distilled water, and then heated at 950C for 60 min in a water bath. After cooling, 1ml of distilled water and 5.0ml of the mixture of n-butanol and pyridine (15:1, v/v) were added to the above reaction mixture and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm using a UV-spectrophotometer. Lipid peroxide level (LPO) was expressed in terms of nmol of MDA/mg of protein [44].

Estimation of Nitrite level

The levels of the nitric oxide (NO) derivative nitrite were determined in the rat hippocampus with the Griess reagent. The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide was determined by a colorimetric assay with Greiss reagent (0.1% N-(1- naphthyl) ethylene diamine dihydrochloride, 1% sulphanilamide and 5% phosphoric acid) (Green et al., 1982). Equal volumes of the supernatant and Greiss reagent were mixed and incubated for 10 min at room temperature in the dark. The absorbance was measured at 540 nm using a flexible monochromator-based multi-mode microplate reader (Synergy HT, Bio-Tek). The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve [45].

Estimation of Catalase activity

The catalase activity was measured in the hippocampus and prefrontal cortex according to procedure mentioned previously[46]. The disappearance of H2O2 is measured with little modifications. Briefly, assay mixture consists of 0.3 M H2O2 in 50 mM Potassium phosphate buffer (pH 7) followed by 0.1 ml of tissue homogenate (as described in 2.5). The rate of decrease in the absorbance at 240 nm was recorded for 2 min. The results were expressed as units of CAT activity/ mg of protein.

Estimation of SOD activity

Superoxide dismutase (SOD) activity in the brain homogenate was assayed by monitoring its ability to scavenge superoxide radicals generated by auto-oxidation of pyrogallol in the alkaline medium as per previously described method [47, 48] with minor modification. Briefly, each 3 ml reaction mixture contained 2.8 ml of Potassium phosphate buffer (0.1M, pH 7.4), 0.1ml tissue homogenate and 0.1 ml pyrogallol solution (2.6 mM in 10 mM HCl). The rate of increase in the absorbance at 325 nm was

recorded for a period 5 min with 30sec interval. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 ml of the assay mixture.

Estimation of Reduced Glutathione activity

Reduced glutathione (GSH) level was measured according to method described earlier [49]. Briefly, 1.0 ml of post mitochondrial supernatant (10%) was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4-80 C for at least 1 h and then subjected to centrifugation at 1200 rpm for 15 min at 4 8C. The assay mixture contained 0.1 ml of supernatant, 2.7 ml of phosphate buffer (0.1 M, pH 7.4), and 0.2 ml of 5,5-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm, and GSH levels were calculated using molar extinction coefficient of 1.36 _ 104M_1 cm_1) and expressed as micromole per milligram protein.

Estimation of MAO-A activity

The mitochondrial brain fraction was used to measure MAO-A levels as mentioned in procedure previously[50, 51]. The mitochondrial fraction of brain was washed twice with about 100 ml of sucrose–Tris–EDTA buffer and suspended in 9 volumes of cold sodium phosphate buffer (10 mM, pH 7.4, containing 320 mM sucrose) and mingled well at 4-8°C for 20 min. The mixture was then centrifuged at 15,000 rpm for 30 min at 0-8°C and the pellets were resuspended in a cold sodium phosphate buffer. Then, 2.75 ml of sodium phosphate buffer (100 mM, pH 7.4) and 100 ml of 4 mM 5-hydroxytryptamine were mixed in a quartz cuvette which was placed in UV–visible spectrophotometer 2203 (Systronics, Ahmedabad). This was followed by the addition of 150 ml of the solution of mitochondrial fraction to initiate the enzymatic reaction and the change in absorbance was recorded at wavelength of 280 nm for 5 min against the blank containing sodium phosphate buffer and 5-HT.

Statistical analysis

Data of all the results were represented as mean ± SEM. The analysis of all the studies were done with the help of analysis of variance (one way ANOVA) comparison test. For behavioral test and biochemical analysis different comparisons were done.

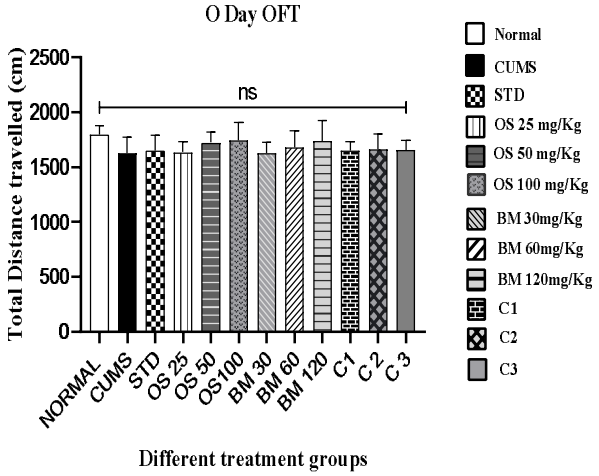
RESULTS

Behavioral parameters

Open field test

The OFT was conducted on day 0, day 21 and day 42 and shows the effect of CUMS and all treatment in mice on the locomotor activity in the open field. In the given test,the total distance travelled and immobility time was measured. On the day 0 there was no significant change in any parameter measured, namely total distance travelled and duration of immobility time in all the treatment groups in comparison with CUMS treated groups (Fig 1A, 2A).

Total distance travelled



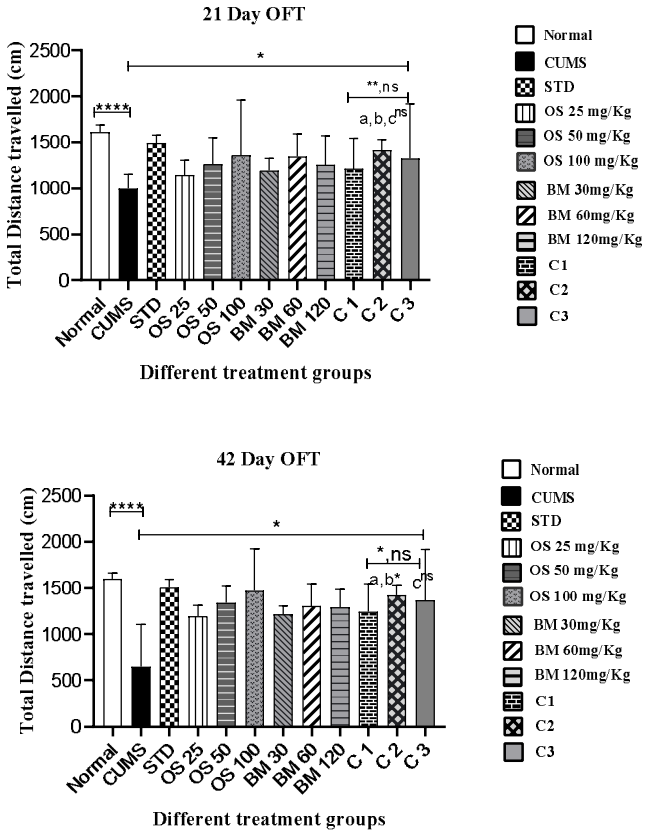
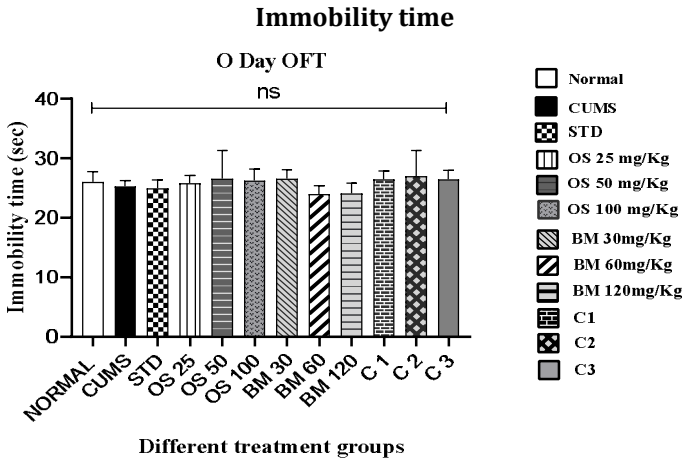


Fig 1: Effect of combinational therapy on locomotor activity. Values are expressed in Mean and SEM .a P<0.0001 as compared to stressed (CUMS) group; bP<0.05 as compared to Fluoxetine treated group; c P<0.05 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

On the day 21st the CUMS treated mice showed significant decreased in locomotor activity by crossing less number of lines as compared to normal control (P<0.0001) and all treatment groups (P<0.05) wherein OS + BM (25 + 30 mg/kg)showed significant (P<0.01) improvement in total locomotor activity by increased the number of lines crossed as compared to CUMS treatment group (Fig. 1B). Similarly the immobility was increased in stressed mice as compared to normal group (P<0.001) and all treatment groups (P<0.01) wherein OS + BM (25 + 30 mg/kg) showed significant (P<0.01) decrease in immobility time as compared to CUMS treatment group (Fig. 2B).



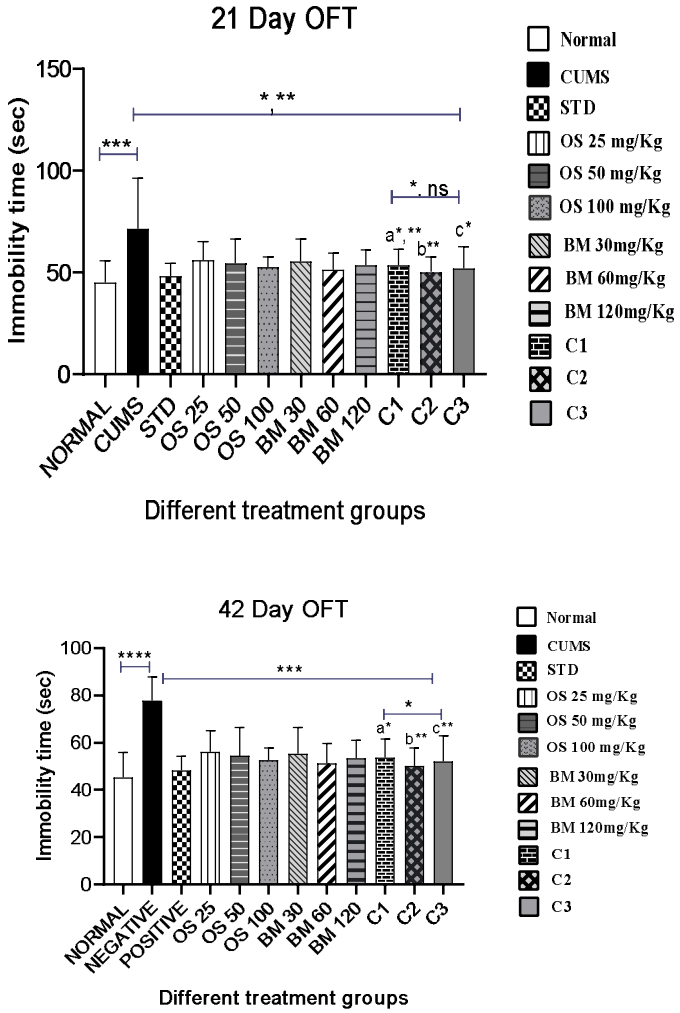


Fig 2: Effect of combinational therapy on immobility time. Values are expressed in Mean and SEM. a P<0.0001 as compared to stressed (CUMS) group; bP<0.001 as compared to Fluoxetine treated group; c P<0.01 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

On the day 42nd the CUMS treated mice showed significant decreased in locomotor activity by crossing less number of lines as compared to normal control (P<0.001) and all treatment groups wherein OS + BM (25 + 30 mg/kg) doses showed significant (P<0.05) improvement in total locomotor activity by increased the number of lines crossed as compared to CUMS treatment group (Fig. 1C). Similarly the immobility was increased in stressed mice as compared to normal group (P<0.0001) and all treatment groups (P<0.001) wherein OS + BM (25 + 30 mg/kg) showed significant (P<0.05) decrease in immobility time as compared to CUMS treatment group (P<0.05) (Fig. 2C).

Elevated plus maze

The EPM was conducted on day 0, day 21, day 42 and shows the effect of CUMS and treatment in mice on the open arm entries in Elevated Plus Maze. In the given test, the total number of open arm entries was measured. On day 0 there was no significant change in total number of open arm entries when all treatment groups compared with CUMS treated groups (Fig 3A).

On the day 21st the CUMS treated mice showed significant decreased in open arm entries as compared to normal control (P<0.0001) whereas OS (25, 50, 100 mg/kg), BM (30, 60, 120 mg/kg), and combination of both at different doses (12.5 + 15 mg/kg; 25 + 30 mg/kg; 50 + 60 mg/kg) treated mice showed significant (P<0.05) increase in open arm entries as compared to CUMS treatment group (Fig 3B).

On the day 42nd the CUMS treated mice showed significant decreased in open arm entries as compared to normal control (P<0.0001) whereas OS (25, 50, 100 mg/kg), BM (30, 60, 120 mg/kg), and combination of both at different doses (12.5 + 15 mg/kg; 25 + 30 mg/kg; 50 + 60 mg/kg) treated mice showed significant (P<0.05) increase in open arm entries as compared to CUMS treatment group (Fig 3C).

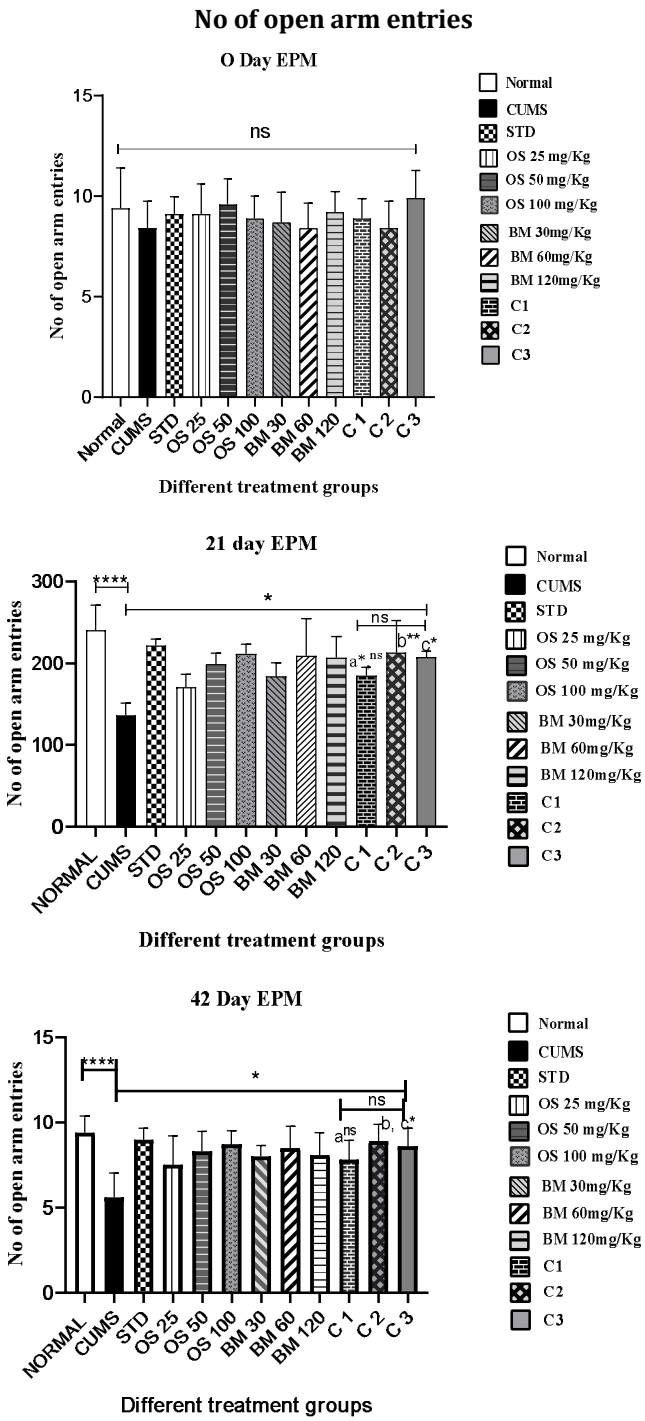


Fig 3: Effect of combinational therapy on number of open arm entries. Values are expressed in Mean and SEM. a P<0.0001 as compared to stressed (CUMS) group; bP<0.05 as compared to Fluoxetine treated group; c P<0.05 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

Morris water maze

The MWM was conducted on day 0, day 21, day 42 and shows the effect of CUMS and treatment in mice for the escape latency time in Morris water maze. In the given test,

The escape latency time taken by mice was measured. On the day 0 there was no significant change in total number of open arm entries when all treatment groups compared with CUMS treated groups (Fig 4A).

On the day 21st the CUMS treated mice showed significant higher escape latency time as compared to normal control (P<0.001) whereas OS (25, 50, 100 mg/kg), BM (30, 60, 120 mg/kg), and combination of both at different doses (12.5 + 15 mg/kg; 25 + 30 mg/kg; 50 + 60 mg/kg) treated mice showed significant (P<0.001) lesser escape latency time as compared to CUMS treatment group (Fig 4B).
On the day 42nd the CUMS treated mice showed significant higher escape latency time as compared to normal control (P<0.001) whereas OS (25, 50, 100 mg/kg), BM (30, 60, 120 mg/kg), and combination of both at different doses (12.5 + 15 mg/kg; 25 + 30 mg/kg; 50 + 60 mg/kg) treated mice showed significant (P<0.001) lesser escape latency time as compared to CUMS treatment group (Fig 4C).

Escape latency time

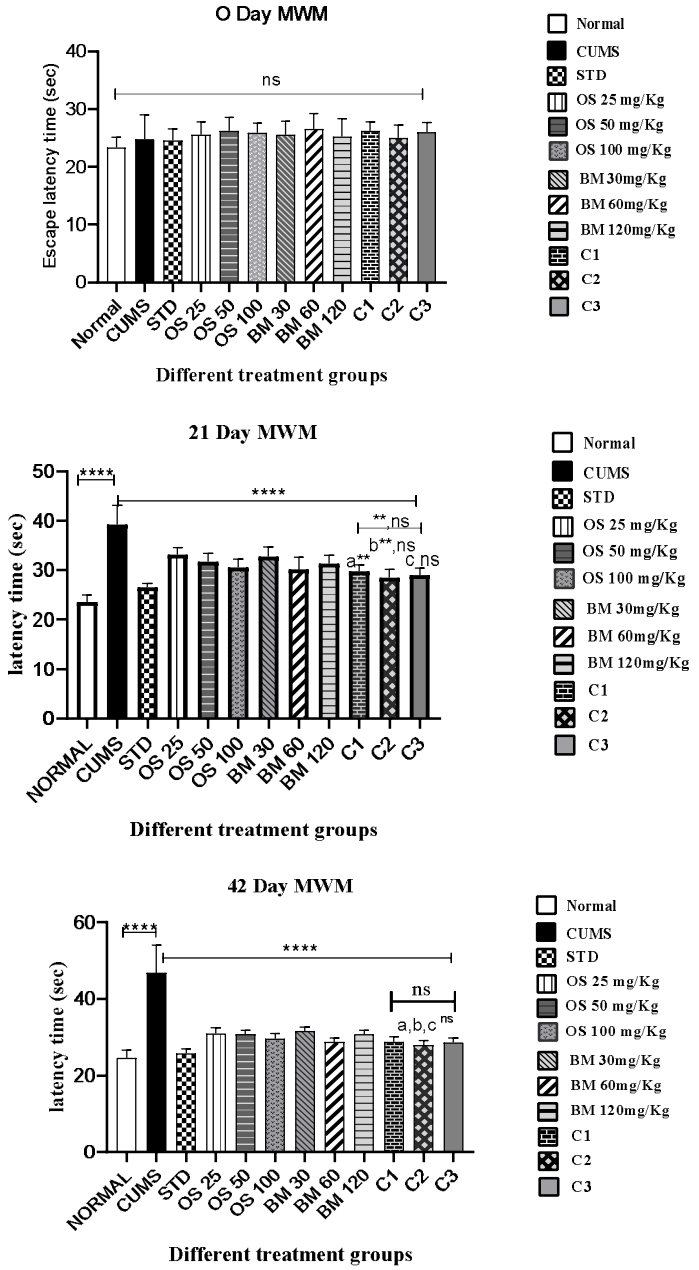


Fig 4: Effect of combinational therapy on escape latency time. Values are expressed in Mean and SEM. a P<0.0001 as compared to stressed (CUMS) group; bP<0.0001 as compared to Fluoxetine treated group; c P<0.05 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

Biochemical parameters

Effect of combinational treatment on blood plasma cortisol level

After 42 days treatment the CUMS treated mice showed significant increase in plasma cortisol level as compared to normal control mice (P<0.0001) (Fig 5) whereas different treatment groups (P<0.05)

showed significant decrease in plasma cortisol level as compared to CUMS treatment mice. Combinational groups showed significant results at different doses in comparison with standard groups (P<0.001) where OS + BM (25 + 30 mg/kg) was more effective than other two combinations. Similarly, combinational groups showed significant decrease in cortisol level on comparison with individual OS and BM at different doses where OS + BM (12.5 + 15 mg/kg) (P<0.001) compared with OS 25 mg/kg and BM 30 mg/kg, OS + BM (25 + 30 mg/kg) (P<0.0001, P<0.001) compared with OS 50 mg/kg and BM 60 mg/kg, OS + BM (50 + 60 mg/kg) (P<0.0001, P<0.01) compared with OS 100 mg/kg and BM 120mg/kg.

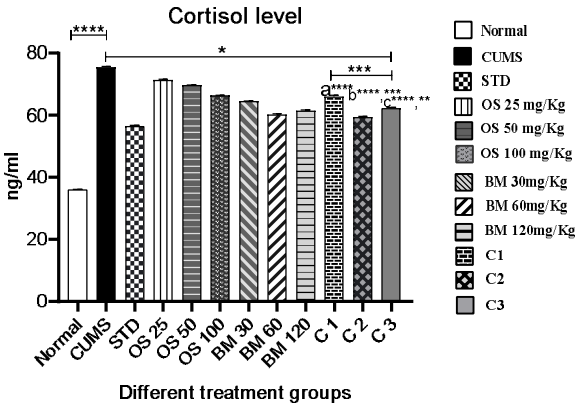


Fig 5: Effect of combinational therapy on cortisol level. Values are expressed in Mean and SEM. a P<0.0001 as compared to stressed (CUMS) group; bP<0.05 as compared to Fluoxetine treated group; c P<0.0001 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

Effect of combinational treatment on MDA level

After completion of 42 days treatment the MDA level in CUMS treated mice showed significant increase in MDA level as compared to normal control mice (P<0.0001) (Fig 6) whereas different treatment groups (P<0.0001) showed significant decrease in MDA level as compared to CUMS treatment mice. Combinational groups (P<0.0001) showed significant results at different doses in comparison with standard groups where OS + BM (25 + 30 mg/kg) was more effective than other two combinations. Similarly, combinational groups showed significant decrease in MDA level on comparison with individual OS and BM at different doses where OS + BM (12.5 + 15 mg/kg) (P<0.001) compared with OS 25 mg/kg and BM 30 mg/kg, OS + BM (25 + 30 mg/kg) (P<0.01) compared with OS 50 mg/kg and BM 60 mg/kg, OS + BM (50 + 60 mg/kg) (P<0.001) compared with OS 100 mg/kg and BM 120mg/kg.

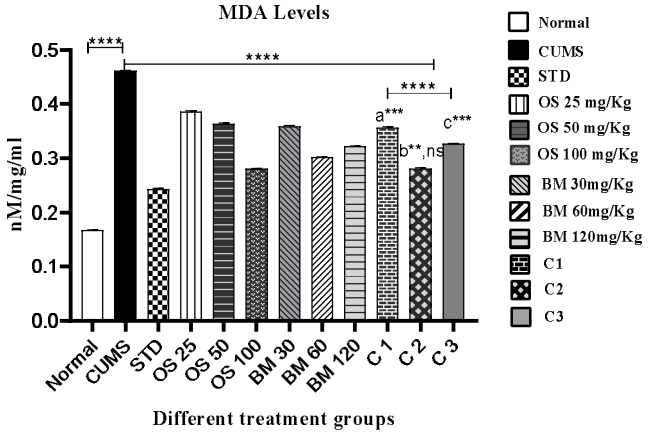


Fig 6: Effect of combinational therapy on MDA level. Values are expressed in Mean and SEM. aP<0.0001 as compared to stressed (CUMS) group; bP<0.001 as compared to Fluoxetine treated group; c P<0.001 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

Combinational treatment effect on nitrite level

The nitrite level after 42 days found to show a significant increase in CUMS treated mice (P<0.0001) as compared to normal control mice (Fig 7) whereas different treatment groups (P<0.01) showed significant decrease in nitrite level as compared to CUMS treatment mice. No significant results were found on comparison of combination groups with standard groups. Similarly, combinational groups showed significant decrease in MDA level on comparison with individual OS and BM at different doses where OS + BM (12.5 + 15 mg/kg) (P<0.0001)compared with OS 25 mg/kg and BM 30 mg/kg, OS + BM (25 + 30 mg/kg) (P<0.0001) compared with OS 50 mg/kg and BM 60 mg/kg, OS + BM (50 + 60 mg/kg) (P<0.01, P<0.0001) compared with OS 100 mg/kg and BM 120mg/kg.

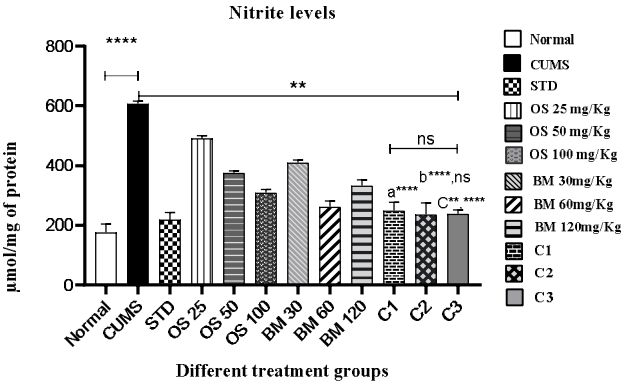


Fig 7: Effect of combinational therapy on Nitrite level. Values are expressed in Mean and SEM. a P<0.0001 as compared to stressed (CUMS) group; bP<0.01 as compared to Fluoxetine treated group; c P<0.0001 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

Effect of combinational treatment on Catalase activity

After 42 days treatment decrease in catalase activity was observed in CUMS treated mice (P<0.0001) as compared with normal control mice (Fig 8) whereas different treatment groups (P<0.001) showed significant increase in catalase level as compared to CUMS treatment mice. Significant increase (P<0.01) results were found on comparison of combination groups with standard groups. Similarly, combinational groups showed significant decrease in MDA level on comparison with individual OS and BM at different doses where OS + BM (12.5 + 15 mg/kg) (P<0.05, P<0.01) compared with OS 25 mg/kg and BM 30 mg/kg, OS + BM (25 + 30 mg/kg) (P<0.05, ns) compared with OS 50 mg/kg and BM 60 mg/kg, OS + BM (50 + 60 mg/kg) (P<0.05) compared with OS 100 mg/kg and BM 120mg/kg.

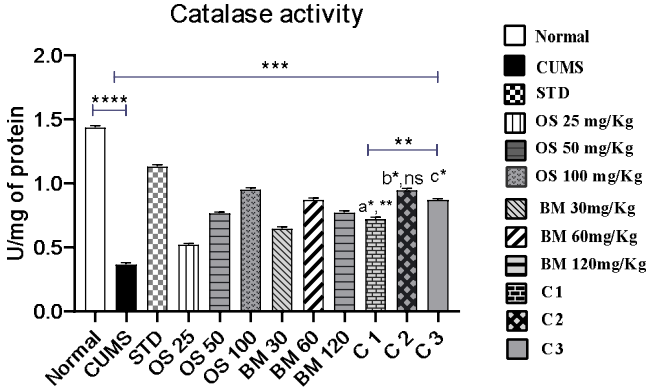


Fig 8: Effect of combinational therapy on catalase activity. Values are expressed in Mean and SEM. a P<0.0001 as compared to stressed (CUMS) group; bP<0.001 as compared to Fluoxetine treated group; c P<0.01 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple
comparisons).

Effect of combinational treatment on Superoxide dismutase activity

After 42 days treatment decrease in SOD activity was observed in CUMS treated mice (P<0.0001) as compared with normal control mice (Fig 9) whereas different treatment groups (P<0.0001) showed

significant increase in SOD level as compared to CUMS treatment mice. No significant results were found in comparison of combination groups with standard groups. Similarly, combinational groups showed significant decrease in MDA level on comparison with individual OS and BM at different doses where OS + BM (12.5 + 15 mg/kg) (P<0.0001) compared with OS 25 mg/kg and BM 30 mg/kg, OS + BM (25 + 30 mg/kg) (ns) compared with OS 50 mg/kg and BM 60 mg/kg, OS + BM (50 + 60 mg/kg) (ns) compared with OS 100 mg/kg and BM 120mg/kg.

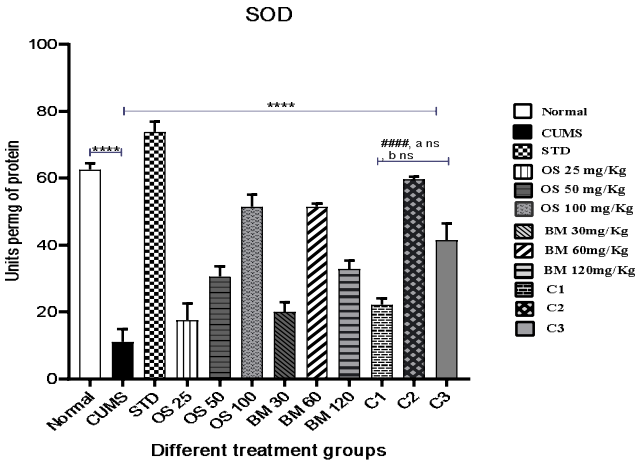


Fig 9: Effect of combinational therapy on Superoxide dismutase activity. Values are expressed in Mean and SEM. a P<0.0001 as compared to stressed (CUMS) group; bP<0.0001 as compared to Fluoxetine treated group; c P<0.05 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

Effect of combinational treatment on Reduced glutathione level

At the end of 42 days the glutathione level in CUMS treated mice showed significant decrease as compared to normal control mice (Fig 10) whereas different treatment groups showed significant increase glutathione level as compared to CUMS treatment mice.Combinational groups showed significant increased glutathione level at different doses on comparison with standard group where OS + BM (25 + 30 mg/kg) showed no significant result with standard group. Similarly, combinational groups showed significant increase in glutathione level on comparison with individual OS and BM at different doses where OS + BM (12.5 + 15 mg/kg) compared with OS 25 mg/kg and BM 30 mg/kg, OS + BM (25 + 30 mg/kg) compared with OS 50 mg/kg and BM 60 mg/kg, OS + BM (50 + 60 mg/kg) compared with OS 100 mg/kg and BM 120mg/kg.

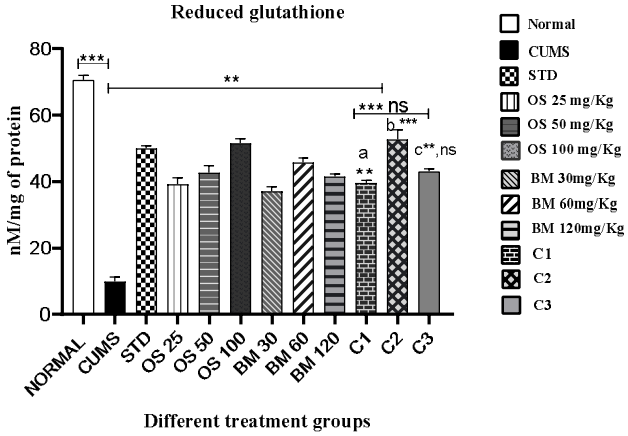


Fig 10: Effect of combinational therapy on reduced glutathione level. Values are expressed in Mean and SEM. a P<0.001 as compared to stressed (CUMS) group; bP<.0.01 as compared to Fluoxetine treated group; c P<0.001 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

Effect of combinational treatment on MAO-A enzyme level

At the end of 42 days treatment the MAO-A level in CUMS treated mice showed significant increase as compared to normal control mice (Fig 11) whereas different treatment groups showed significant decrease in MAO-A level as compared to CUMS treatment mice. Combinational groups showed significant decreased MAO-A level at different doses in comparison with standard groups where OS + BM (25 + 30 mg/kg) showed no significant result with standard group. Similarly, combinational groups showed significant decrease in MAO-A level on comparison with individual OS and BM at different doses where OS + BM (12.5 + 15 mg/kg) compared with OS 25 mg/kg and BM 30 mg/kg, OS + BM (25 + 30 mg/kg) compared with OS 50 mg/kg and BM 60 mg/kg, OS + BM (50 + 60 mg/kg) compared with OS 100 mg/kg and BM 120mg/kg.

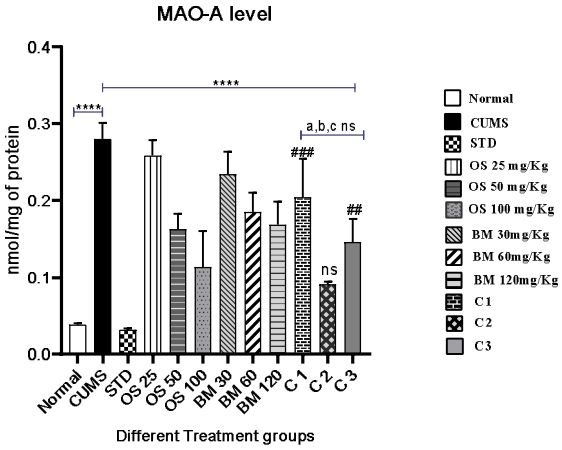


Fig 11: Effect of combinational therapy on MOA-A level. Values are expressed in Mean and SEM. a P<0.0001 as compared to stressed (CUMS) group; bP<0.0001 as compared to Fluoxetine treated group; c P<0.01 as compared to individual doses of respective herb + CUMS group (repeated measures one-way ANOVA followed by Sidak's test for multiple comparisons).

DISCUSSION

Although from past few years herbals drugs and their bioactive phytoconstituents have been extensively explored for showing promising effects for betterment of health [51, 52, 53]. They are used as alternative therapeutic agents and thus are well effective in management of several central nervous system disorders including mood disorder (depression) [54]. The incidence of depression is rising and has increased the number of patients suffering from low mood, interest loss, slow thought and cognitive deficit. In our daily social life, the most important factor arises in the development and acceleration of depression is chronic stress [55]. Some mounting evidence suggested that cognitive and emotional biases are involved in the development and maintenance of depression, especially in response to stress. To produce a good depression model, the chronic unpredicted mild stress (CUMS)procedures used in laboratory research keep animals exposed to different kinds of stress every day. This model is able to mimic the same behavioural and physiological symptoms as of clinical human depression well [56]. Therefore, the CUMS is the one of the most promising rodent models for depression and we chose it to evaluate the herbal drug combination for their antidepressant activity.

In the present study, we found that CUMS induced depressive behaviours such as anhedonia and anxiety via open field test, elevated plus maze test. Along with depression-like behaviour, mice when subjected to prolonged CUMS exposure consistently led to deterioration of spatial learning abilities, reference memory and working memory via Morris water maze test.

The combination of *Ocimum sanctum* and *Bacopa monera* used have been previously reported for its toxicity, efficacy and therapeutics purpose. The *in vivo* toxicity study of combination was found to be safe, no symptoms of toxicity. The phytoconstituents present in Ocimum sanctum majorly contain 70 % eugenol which possess antioxidant property whereas *Bacopa monera* majorly composed of Bacosides possessing to inhibit MAO-A enzyme.

In our behavioural parameters, 6 weeks CUMS procedure led to depressive symptoms via open field test where spontaneous locomotor activity by rodents was observed as previously reported. The spontaneous locomotor activity was decreased in the mice exposed to CUMS in the OFT on the other hand the spontaneous locomotor activity was increased in the *Ocimum sanctum*[57], *Bacopa Monnieri*[10,34], and

combinational groups at different doses as consistent with previous reports. Similarly the anxiety and depressive behaviour in mice was evaluated by placing mice in EPM as per earlier studies. The CUMS showed decreased in number of open arm entries as compared to other treatment mice groups, showing preference to the open arms was in line according to previous studies reports. On the other hand CUMS also impaired cognitive memory which was evaluated by placing mice in MWM, one of the methods to evaluate spatial memory. Similar results were obtained according to previous reports where cognitive impairment was observed in the stressed group. Our behavioural results suggested that there were improvement in the performance of mice of treatment groups at graded doses wherein the locomotor activity, anxiety and depressive behaviour, spatial learning memory was found to improve thereby decreasing the depressive symptoms.

Glucocorticoid is involved in regulating neuronal survival, neuronal excitability, neurogenesis and memory acquisition. Previous evidences suggested that high levels of glucocorticoid may contribute to the development and manifestation of depressive symptoms by activating the glucocorticoid receptor (GR) and thus leading to dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis activity [4, 16]. Besides, glucocorticoid is found to impair cognitive functions mediated by the frontal cortex and memory retrieval mediated by hippocampus [17]. Plasma glucocorticoid, an indicator of depression in laboratory animals. It was observed that CUMS induced an increased elevated glucocorticoid level in mice, which was in line according to the previous studies and proved the validation of the CUMS model. However, combination of OS and BM at graded dose treatment significantly decreased the elevated glucocorticoid in CUMS mice, thus results were inline according to previous studies indicating the alleviation of depression severity and cognitive deficits [17].

The monoamine hypothesis has been focused by researchers showing deep involvement in pathophysiology of depression leading to decreased monoamines levels serotonin (5-HT), dopamine (DA) and norepinephrine(NE) [13]. Recent evidence proved that 5-HT and DA are the important neurotransmitters involved in the cognitive functions, and thus modulation of 5-HT, DA and their receptors might counteract the associated cognitive deficits in depression. In our present study the levels of monoamines were reduced due to exposure to CUMS as compared to normal mice, on administration of combination of OS and BM at graded doses significantly increased the monoamines levels in hippocampus or frontal cortex. The data obtained was significantly consistent with the previous research and suggested that the antidepressant effect of OS and BM combination might be partially due to the modulation of three major monoamine neurotransmitters 5-HT, DA and NE.

Mounting evidence supports that neuroplasticity hypothesis , which suggested BDNF involvement in many behavioural and molecular mechanisms of antidepressant though its function is to regulate synaptic plasticity, neuronal circuit formation, and neuronal survival [10]. BDNF are majorly found in the hippocampus and respond to vulnerable mood change and stress responses like glucocorticoid toxicity. Earlier reports suggested that CUMS leads to depletion in the BDNF cells population in response to glucocorticoid [17]. Moreover, increases of BDNF have been shown to attenuate depression-related changes. In this study, we found that BDNF protein expression in the hippocampus was reduced due to chronic CUMS exposure in mice, administration of OS and BM combination led to increased in BDNF cells in hippocampus. Thus increased BDNF population due to combination showed significant improvement in behavioural paradigm as an antidepressant effect in CUMS model.

Oxygen free radicals play a crucial role in major depression. CUMS caused marked oxidative stress, robust increase in the levels of ROS, hydroperoxides, lipid peroxidation, nitrite concentration in *in- vivo* models. Similarly, in our present study the exposure of chronic CUMS for 42 days resulted in marked increase in nitric oxide levels and lipid peroxidation whereas treatment with combination of OS and BM at different doses combination were significantly decreased the elevated levels of lipid peroxidation and nitrite level in hippocampus similar to earlier reports [21].

Chronic exposure of CUMS generates oxidative stress on brain cells particularly due to overproduction of reactive oxygen species (ROS) thereby declining antioxidant level in the brain [58]. The oxidative damage majorly indicated by increased MDA level, a product of lipid peroxidation aggravates symptoms of depression. Meanwhile antioxidant enzymes like SOD, CAT, GSH play defensive roles against ROS. In our present study, administration of combination of OS and BM showed a reversal effect by increasing SOD, CAT, GSH level in the hippocampus induced by CUMS procedure significantly. Significant results were observed thus the combination was effective in elevating the antioxidant levels. Thus data obtained suggested that the antioxidant activity of OS and BM combination at different doses have a potential relationship with its antidepressant effects.

MAO-A a crucial enzyme involved majorly in depression. Increased MAO-A levels mainly causes metabolism of 5-HT and NE thus leading to mood and behaviour changes. CUMS mainly elevates MAO-A

levels in stressed mice in comparison with unstressed mice. OS and BM (25 + 30 mg/kg) was effective to decrease the MAO-A levels in unstressed mice compared with stressed mice.

CONCLUSION

Based on the results obtained, the present study suggested that the combination of OS + BM (25 + 30 mg/kg) was found significant effective in reducing depressive symptoms in unstressed mice in comparison with stressed mice, possibly through by increasing spontaneous locomotor activity, improving spatial learning and memory, causing inhibition of MAO-A enzyme, decreasing plasma corticosterone levels, elevating antioxidant status, decreasing lipid peroxidation, and nitrite levels in brain. Further, decrease of plasma corticosterone levels might also be responsible for antidepressant-like activity of *Ocimum sanctum* and *Bacopa monera* in mice subjected to CUMS. Thus, combination of OS + BM (25 + 30 mg/kg) may be explored further for management of mood disorder depression.

ACKNOWLEDGEMENTS

I am thankful to Dr. D. Y. Patil institute of Pharmaceutical sciences and research, pimpri, Pune 411015.

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