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Achyranthes aspera ameliorates stress induced depression in mice by regulating neuroinflammatory cytokines

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ABSTRACT

Background and aim: *Achyranthes aspera* Linn. (*A. aspera*) (family: Amaranthaceae) is highly recognized in ethnomedicine and traditional systems of Indian medicine as a nervine restorative for several psychiatric disorders. Study presented here was designed to appraise the antidepressant-like effects of *A. aspera* in murine model of chronic unpredictable mild stress (CUMS) induced depression.

Experimental procedures-: Rodents were exposed to different stressor in unpredictive manner during CUMS protocol once a day for 4 weeks. Mice were intraperitoneally injected with *A. aspera* extract (2.5, 5 and 10 mg/kg) or fluoxetine (10 mg/kg) or betaine (20 mg/kg) once daily during day 15–28 of the CUMS protocol. Sucrose preference, motivation and self-care, immobility latency and plasma corticosterone were evaluated after 24 h of last stressor. After behavioral assessments TNF-α, Il-6 and BDNF immunocontent was determined in hippocampus and prefrontal cortex.

Results and conclusion: *A. aspera* extract as well as betaine improved sucrose preference, increased grooming frequency and latency in splash test and ameliorated depression-like condition in CUMS mice in Porsolt test. *A. aspera* treatment decreased the elevated plasma corticosterone and reversed the effect of CUMS on TNF-α, Il-6 and BDNF immunocontent in mice. The results of the present study suggest *A. aspera* as a promising indigenous medicine for stress associated neurobehavioral and comorbid complications.

1. Introduction

Depression is a universal psychiatric condition characterized by defeated interest, motivation and anhedonia with frequent suicidal tendencies.[1] Chronic stress exposure leads to and prominently involved in instigation of clinical depression and mood disorders.[2] Repeated stress exposure results in hypothalamic pituitary adrenal (HPA) axis hyperactivity and increases the individual's susceptibility to many psychiatric and neurological complications. HPA axis hyperactivation characterized by increased levels of corticosteroids and its normalization may be a critical strategy for therapeutic interventions and mechanism of antidepressant agents. In addition, several reports proposed that neuroinflammation is the key pathological factor involved in pathogenesis of depression.[3] Psychological and physical stressors can activate immune and inflammation processes,[4] through release of inflammatory cytokines like IL-6, and TNF-α.[5] Several antidepressant drugs reversed the depression through reduction in inflammatory markers and corticosterone levels.[6] The neurotrophic theory of depression propose that brain derived neurotrophic factor (BDNF) is critically associated the etiopathophysiology of depressive comorbidities.[7] Chronic stress exposure leads to decreased BDNF expression in the brain limbic system which can be restored following antidepressant therapy.[8]

Current pharmacological approaches to the management of

Abbreviations: A. aspera, *Achyranthes aspera*; CUMS, Chronic Unpredictable Mild Stress; TNF-α, Tumor Necrosis Factor- Alpha; Il-6, Interleukin-6; BDNF, Brain Derived Neurotrophic Factor; HPA, Hypothalamic Pituitary Adrenal; SPT, Sucrose Preference Test; OFT, Open Field Test; FST, Forced Swim Test.

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depression and mood disorders are yet to exhibit desirable results in clinical practice. Although numerous classes of drugs that are available for the treatment of depression, significant number of patients (40%) do not respond well to the therapy. Moreover, most of the antidepressant drugs are associated with common side effects like agitation, nausea, headache, sleeplessness or drowsiness and sexual problems. Thus, there is a great need of new therapeutic strategies for the treatment of depression. The emerging clinical cases have shown increasing interests among health practitioners and patients in botanical based medicine due to their high safety profile.[9–11] Phytomedicine are traditionally used by human for the treatment of chronic disorders of brain and central nervous system.[12] *Achyranthes aspera* Linn. (*A. aspera*) (family-Amaranthaceae) has been widely used traditional and alternative systems of medicine practiced in countries like India, Pakistan, Sri Lanka, Philippines, Kenya etc.[13] *A.aspera* Linn. (Family: Amaranthaceae) is commonly known as prickly chaff flower. *Achyranthes*-a genus of herbs or subshrubs includes about 21 species which are distributed in tropical and subtropical regions. The roots of *A. aspera* are reported to contain alkaloids i.e. betaine trimethyl glycine), achyranthine (1-methyl-3-pyrrolidinecarboxylic acid); saponins (dammarane saponins) i.e. achyranthoside D, ginsenoside RO, sulphachyranthoside D, achyranthoside B; Saponins (oleanolic acid glycosides) i.e. zingibroside R1, bidentatoside, bidentatoside II, momordinIb; steroids i.e. beta-ecdysone, 20,26-dihydroxyecdysone, stachysterone D; beta-D-fructofuranoside with niacin, ascorbic acid, behenic acid and other fatty acids and esters,[14,15] essential oil and terpenoids etc.[16]

Indian communities have been traditionally using *A. aspera* as a brain tonic for the treatment of neurological and psychiatric disorders.[17–19] Some indigenous communities in Northern and central India consume *A. aspera* roots as well as whole plant powder to cure epilepsy.[20] Recent experimental studies also showed beneficial effect of *A. aspera* in epilepsy,[21] depression,[22] anxiety,[23] nootropic,[24] and cerebroprotective[13] activities. It also exhibits antioxidant[25] and anti-inflammatory potential.[26]

Although Indian communities have been traditionally using *A. aspera*, the behavioral and biochemical evidences are not available to prove its therapeutic application in the treatment of depression. As the natural products are one of the best source of new drugs owing to their phytochemical diversity and pleiotropic pharmacological effects, it is worthwhile to investigate the antidepressant like effect of the *A. aspera* extract and its possible mechanism of action. We hypothesized that *A. aspera* may exhibit antidepressant activity against chronic stress induced depression in mice due to its favorable neuropharmacological profile. In the present study we analyzed the effect of repeated *A. aspera* administration on chronic stress associated changes in mice sucrose preference test, self-care and motivational behaviours in splash test, duration of immobility in forced swim despair test, body mass, plasma corticosterone levels and hippocampal and prefrontal cortex (PFC) immunocontent of BDNF and inflammatory markers (IL-6 and TNF-α).

2. Material and methods

2.1. Plant material and extract preparation

The roots of *A.aspera* (Family: Amaranthaceae) were collected in the month of November and authenticated with Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India (voucher specimen no. 1012). The roots were washed with tap water, shade dried at room temperature and finely powdered (passed through mesh sieve # 40) using a grinder. Further, the powdered roots (200 g) of *A. aspera* were macerated with methanol (2000 ml) for 3 days with intermittent shaking. The menstruum was collected in total 9 equal batches with constant time interval. It was filtered, combined and the solvent was evaporated using rotary vacuum evaporator (Buchi, Switzerland) under reduced pressure at 60 °C. Further, the concentrated extract was lyophilized and stored in a refrigerator. The percentage yield of pale yellow powdered extract was found to be 7.856 w/w (dry weight basis of the powdered roots).

2.2. Drugs and chemicals

Betaine (98.00%), corticosterone (98.5%) and HPLC grade methanol (99.8%) were procured from Sigma-Aldrich (St. Louis, MO, USA). BDNF (MBS355435), TNF–α (MBS825075) and IL-6 (MBS2508516) ELISA kits procured from Mybiosource (San Diego, USA). Fluoxetine was received as a gift sample from SUN pharmaceuticals (Baroda, India). *n*-hexane, dichloromethane (DCM) and ether were procured from S.D. Fine Chemicals (Mumbai, India). HPLC grade water has been obtained from Millipore filtration assembly (Millipore, USA). All chemicals used in the present study were of analytical grade.

2.3. Standardization of plant extract

The methanolic extract of *A. aspera* was subjected to preliminary phytochemical screening by different qualitative chemical tests for detection of both the primary and secondary plant metabolites.[27,28] For this purpose, the methanolic extract of *A. aspera* roots was used in few mg quantities for different chemical tests. In some of the chemical tests, dried extract was directly treated with the chemical reagents and in other tests, it is either suspended in water, sodium bicarbonate or first acidified with dilute HCl solution. The chemical tests were performed by using Molish's reagent and Fehling's solution A and B (carbohydrates and glycosides), Liebermann-Burchard reagent (steroids), foam test (saponins), $FeCl_3$ reagent (tannins and phenolics), Shinoda test (flavonoids), Dragendorff's reagent (alkaloids), ninhydrin solution (amino acids) and Sudan red III solution (volatile oils). Following preliminary phytochemical screening for the phytoconstituents, the methanolic extract of *A. aspera* was defatted with *n*-hexane in a separating funnel and the aqueous fraction was evaporated under reduced pressure to concentrate using rotary vacuum evaporator. Betaine in *A. aspera* extract was confirmed with spraying of Dragendorff's reagent, 10% ethanolicsulphuric acid followed by drying at 110 °C for 5 min of high-performance thin layer chromatography (HPTLC) plate (Silica gel G60 F254, Merck, Germany) eluted with methanol: water (85:15 v/v).

Betaine content in *A. aspera* extract was determined by validated HPTLC method as per earlier reports with appropriate and minor modifications.[21,29] Methanolic stock solutions of aqueous extract (10 mg/ml) and betaine (1 mg/ml) were used for HPTLC performed using aluminium backed precoated HPTLC plate (silica gel G60 F254, Merck, Germany) developed using methanol: water (85:15, v/v) and Camag-HPTLC system (Camag, Switzerland). Chromatographic detection was done by reading the absorbance at 520 nm of the dried plates (110 °C for 5 min) prior sprayed with Dragendorff's reagent, 10% ethanolic sulphuric acid. Betaine quantification was done from the calibration curve obtained by validated method.

2.4. Subjects

Adult male Swiss Albino mice (25–30 g) group housed in polypropylene cages maintained at temperature (25 ± 2 °C) and relative humidity (60 ± 5%) were used. Mice had *ad libitum* access to food and water with the exception during specific experimental

protocols. Identical environmental and handling conditions were executed for testing control, stressed and treatment groups. Animals were transferred into fresh cages on a prefixed day once a week and water bottles were replaced with new one three times per week. All the experiments were approved and performed out as per the guidelines of Institutional Animal Ethical Committee at Smt. Kishoritai Bhoyar College of Pharmacy, Kamptee, Nagpur (853/IAEC/2018–19/14) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests; Government of India; New Delhi.

2.5. Chronic unpredictable mild stress (CUMS)

We followed the CUMS procedure in mice as per the available reports[30] and standardized at our laboratory.[31] The CUMS procedure continued up to 4 weeks and include mild stressors on weekly basis. Animal were randomly exposed to different stresses such as tail pinch, overnight lighting, shaker stress, electric foot shock, restrained stress, overnight soiled cage and tilted cage (Table S1) once a week after which mice were transferred to home cages. After 15 days of CUMS exposure, separately grouped animals were injected (i.p.) with *A. aspera* extract (2.5, 5 and 10 mg/kg) or fluoxetine (10 mg/kg) or betaine (20 mg/kg) or saline (1 ml/kg) daily between 09:00 to 10:00 a.m. Control group of unstressed mice received saline (1 ml/kg, i.p.) and were handled daily without any exposure to stressor. All the behavioral tests, except sucrose preference test (SPT), were conducted after 24 h of last stressor to circumvent the sudden effect of stressor as well as treatment. Sucrose intake and preference was observed on day 0, 14, 21 and 28 of the protocol whereas body weights were noted on daily basis.

2.6. Sucrose preference test (SPT)

Basic Method for sucrose preference test was adapted from the earlier reports with minor modifications.[32,33] The test was conducted on days 0, 14, 21 and 28 during the chronic stress protocol. Seventy-two hours before the initiation of the test session each mouse was trained to drink sucrose solution (1% w/v) and they were later presented with two bottles, one containing sucrose solution and other containing tap water (100 ml each). In the middle of the test the two bottles were randomly changed in order to minimize side preference while drinking. Drinking of sucrose solution was observed for 3 h to elude the error of consumption of very little amount of sucrose in 1 h. The bottles were weighed to determine consumption of sucrose solution and tap water. The sucrose preference was calculated as sucrose preference (%) = [(Sucrose consumption)/(Water consumption + sucrose consumption)] X 100.

2.7. Splash test

The splash test was performed to assess the cleaning behavior described as grooming of fur by licking and scratching on application of sucrose solution (10%) onto mouse dorsal coat. The sucrose solution defiles the fur and persuades the self-care behavior in animals. The splash was conducted for 5 min to record the grooming latency and frequency. Depressive behavior was characterized by increased time of instigation of grooming action and reduction in the time spent in grooming.[34]

2.8. Forced swim test (FST)

The method of FST was executed as described earlier.[35] Mice were introduced individually in glass cylinders (height: 25 cm × diameter: 10 cm) containing 10 cm of water at 25 ± 1 °C and forced to swim for a 6 min test session. A pretest session of 5 min was conducted 24 h before test to minimize the swim stress effects on FST behavior. The immobility time was measured by trained observer blind to the treatment. A mouse was considered immobile when it remained floating motionless inwater except making any necessary movement to keep its head above water.

2.9. Open field test (OFT)

The possible intervention of locomotor effects of the drug and its association with immobility was evaluated by OFT conducted 30 min after FST. Open field was circular (diameter - 84 cm, height - 30 cm) drawn with three concentric circles (14, 28 and 42 cm radius) and radiating lines drawn from the center to divide into 36 sections by. Illumination was maintained identical as that of FST. Number of sectional crossings (ambulations) and number of rearings were recorded for 5 min.

2.10. Estimation of corticosterone, BDNF, TNF–α and IL-6

After behavioral studies, blood sample was collected from the retro-orbital plexus of the mice from all the treatment groups. Corticosterone levels were estimated according to procedure as described earlier.[36] Immediately after behavioral assessments and blood withdrawal, mice were euthanized by decapitation. The mouse brains were dissected on ice to separate hippocampus and PFC and cleaned to remove extraneous tissues. Tissues were separately placed in microcentrifuge tubes and flushed with liquid nitrogen before homogenization at −80 °C with complete extraction lysis buffer (Sigma-aldrich, USA). BDNF (MBS355435), TNF–α (MBS825075) and IL-6 (MBS2508516) in hippocampus and PFC was estimated using commercially available ELISA kits from Mybiosource, CT, USA.

2.11. Statistical analysis

The results of biochemical estimations were analyzed by one-way ANOVA whereas splash test, FST, OFT data was analyzed by two-way ANNOVA was applied to assess the effect of sucrose preference and body weights. The data from sucrose preference test and body weight changes were analyzed on three variables viz. Stress, day and treatment by three-way ANOVA. Post hoc mean was compared by Bonferroni's test. All recorded observations were expressed as mean ± standard error of mean (SEM) and differences with $P < 0.05$ were considered significant. All statistical analyses were performed using Graph Pad Prism software (8.0).

3. Results

3.1. Phytochemical screening and HPTLC standardization of *A. aspera* root extract

Observations of qualitative chemical tests of *A. aspera* methanolic extract revealed the presence of saponins, phenolics, steroids, alkaloids, carbohydrates, flavonoids, glycosides, and amino acids (Table S2). As shown in the HPTLC chromatograms of *A. aspera* root methanolic extract (50 μg/band) (Fig. 1A) and the standard betaine (4 μg/band) (Fig. 1B), betaine was eluted at Rf value 0.55 ± 0.01 without adjacent peak/spot of interfering impurity, with methanol-water (85: 15, v/v) mobile phase. The relationship between peak area and betaine concentration was linear in the range of 2–10 μg/band with regression equation (correlation coefficient) as $y = 3056.6x + 2571.93$ ($r^2 = 0.9991$) and without significant difference in the intercepts and slopes of standard curves ($P \geq 0.05$) (Table 1). Betaine content in the methanolic extract of

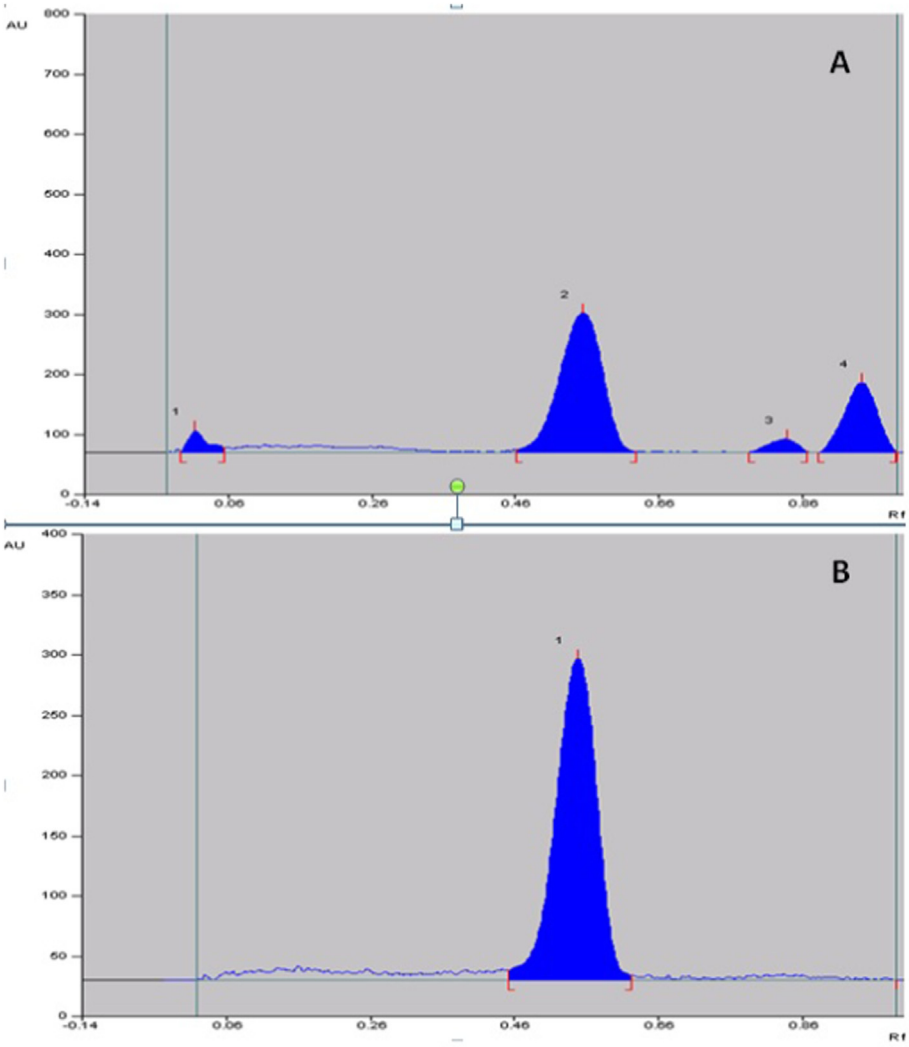

Fig. 1. HPTLC chromatogram of (A) *A. aspera* extract and(B) betaine read at 520 nm.

Table 1
The linear regression data for the calibration curves and validation parameters of betaine ($n = 3$) of the methanolic extract of *A. aspera* roots.

Parameters	Betaine
Linearity range (μg spot^{-1})	2–10
Regression equation	y = 3056.6x+2571.93
Correlation coefficient (r^2)	0.9991 ± 0.0003
Slope ± SD	3056.6 ± 18.02
Intercept ± SD	2571.93 ± 89.91
R_f	0.55 ± 0.01
Precision (repeatability)	
Intra-say (*%RSD*)	0.58–0.74
Inter-day (*%RSD*)	0.39–0.71
Accuracy (%)	99.10
LOD (μg)	0.097
LOQ (μg)	0.2941

A. aspera roots was 9.39 ± 1.65% w/w (Fig. 1). The peak of betaine was well separated from the other constituents of methanolic extract. Intra-day and inter-day precision study and the percent relative standard deviation (% RSD) for intra-day/inter-day precisions (n = 6) of betaine were 0.58–0.74/0.39 to 0.71 suggesting the acceptable precision for HPTLC method. The recovery for betaine was found to be 99.23%. The LOD, (S/N = 3) and LOQ, (S/N = 10) were 0.097 and 0.2941 μg/band. The supplementary data for validation can be found in Table S3, S4 and S5.

3.2. *A. aspera inhibits in stress induced anhedonia*

As shown in Fig. 2, prolong and repeated CUMS exposure of animal significantly declined the sucrose preference as compared against non-stressed animals to under same conditions. Sucrose preference in CUMS animals on day 14 ($P < 0.001$), 21 ($P < 0.001$) and 28 ($P < 0.001$) was significantly higher as compared to control non-stressed mice. A three way ANOVA indicated that administrations of *A. aspera* extract [5 as well as 10 mg/kg] or fluoxetine (10 mg/kg) or betaine (20 mg/kg) improved the sucrose preference in CUMS animals [$F_{CUMS\ X\ Time\ X\ Treatment}$ (15, 260) = 2.54, $P < 0.01$; $F_{CUMS\ X\ Time}$ (3, 260) = 165.89, $P < 0.001$; $F_{CUMS\ X\ Treatment}$ (5, 260) = 8.18, $P < 0.001$; $_{Time\ X\ Treatment}$ (15, 260) = 1.71, $P > 0.05$; F_{CUMS} (1, 260) = 1354.33, $P < 0.001$; F_{Time} (3, 260) = 10.41, $P < 0.001$; $F_{Treatment}$ (5, 260) = 7.42, $P < 0.001$] (Fig. 2B). Post hoc mean comparisons revealed that sucrose preference in *A. aspera* extract [5 mg/kg - $P < 0.05$; 10 mg/kg – $P < 0.001$) or fluoxetine (10 mg/kg – $P < 0.001$) or betaine (20 mg/kg – $P < 0.01$) treated CUMS mice was different from saline injected CUMS mice on day 28. Further, administrations of fluoxetine (10 mg/kg) as well as betaine (20 mg/

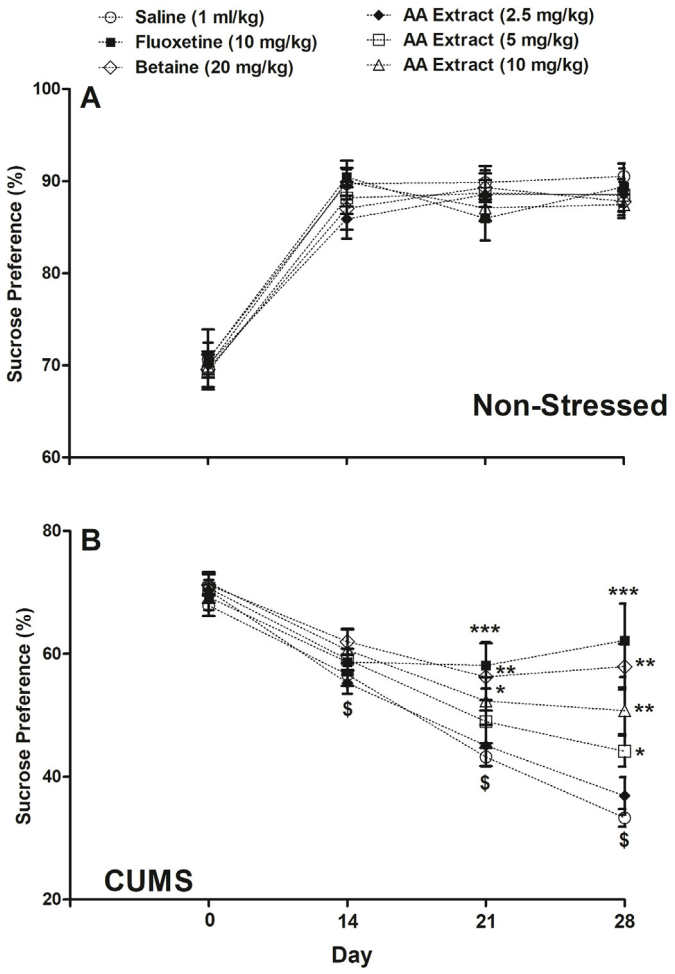

Fig. 2. Sucrose preference in *A. aspera* extract and fluoxetine treated mice. (A) Non-stressed or (B) CUMS mice were injected (i.p.) with saline (1 ml/kg) or *A. aspera* extract (2.5–10 mg/kg) or fluoxetine (10 mg/kg) from 15th day onwards and sucrose preference was monitored on day 0, 14, 21 and 28. Each data point indicate mean sucrose preference (%) ± SEM (n = 6–7). $- P < 0.05vs. vehicle treated control non-stressed animals; *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle treated control CUMS mice.

kg) to CUMS animals also improved the sucrose preference and different from saline injected CUMS mice on day 21 (P < 0.001, P < 0.01) and 28 (P < 0.001, P < 0.01) (respectively). However, *A. aspera* extract (2.5 mg/kg) was ineffective. Sucrose preference in *A. aspera* extract or fluoxetine treated non-stressed mice was not different from saline injected non-stressed animals (Fig. 2A).

3.3. A. aspera improves self-care in splash test

Twenty-eight days exposure of animal to CUMS affected the self-care behavior in mice as reflected from decreased grooming latency (P < 0.001) (Fig. 3A) and number of grooming attempts (P < 0.01) (Fig. 3B). Two way ANOVA revealed that *A. aspera* extract [5 mg/kg (P < 0.05 and P < 0.001) and 10 mg/kg (P < 0.01 and P < 0.001)] and fluoxetine (10 mg/kg) (P < 0.001 and P < 0.001) administration for 14 days improved self-care as revealed by significant difference in the grooming latency [$F_{CUMS \times Treatment}^{3,45} = 8.92$, P < 0.001; $F_{CUMS}^{1,45} = 99.58$, P < 0.001; $F_{Treatment}^{3,45} = 4.25$, P < 0.05] (Fig. 3A) and licking frequency [$F_{CUMS \times Treatment}^{3,45} = 4.97$, P < 0.05; $F_{CUMS}^{1,45} = 1.52$, P > 0.05; $F_{Treatment}^{3,45} = 3.58$, P < 0.05](Fig. 3B) from vehicle treated CUMS control animals. Similarly, fluoxetine showed significantly decreased grooming latency [$F_{CUMS \times Treatment}^{1,21} = 31.61$, P < 0.001; $F_{CUMS}^{1,21} = 76.23$, P < 0.001; $F_{Treatment}^{1,21} = 20.57$, P < 0.01] and increased grooming frequency [$F_{CUMS \times Treatment}^{1,21} = 11.41$, P < 0.01; $F_{CUMS}^{1,21} = 0.06$, P > 0.05; $F_{Treatment}^{1,21} = 23.30$, P < 0.01] in CUMS mice. CUMS mice administered with betaine (20 mg/kg) also exhibited significantly decreased grooming latency [$F_{CUMS \times Treatment}^{1,23} = 24.90$, P < 0.001; $F_{CUMS}^{1,23} = 100.61$, P < 0.001; $F_{Treatment}^{1,23} = 11.48$, P < 0.01] and increased grooming frequency [$F_{CUMS \times Treatment}^{1,23} = 7.95$, P < 0.01; $F_{CUMS}^{1,23} = 0.37$, P > 0.05; $F_{Treatment}^{1,23} = 2.56$, P < 0.05]. *A. aspera* extract (2.5, 5 and 10 mg/kg) and fluoxetine (10 mg/kg) treatment did not alter in number of grooming attempts and grooming duration in non-stressed animals tested in splash test.

3.4. A. aspera decreased the immobility time in CUMS exposed mice in FST

As illustrated in Fig. 4, depression-like behavior as revealed by increased immobility duration as compared to non-stressed animals was observed in CUMS mice (P < 0.001). A two way ANOVA with post hoc Bonferrroni's mean comparisons between groups revealed that 14 day treatment of *A. aspera* extract (5 and 10 mg/kg) decreased immobility in CUMS mice by 27% (P < 0.05) and 32% (P < 0.05) (respectively) as compared to control CUMS animals [$F_{CUMS \times Treatment}^{3,45} = 3.09$, P < 0.05; $F_{CUMS}^{1,45} = 19.66$, P > 0.001; $F_{Treatment}^{3,45} = 2.66$, P > 0.05]. Fluoxetine (10 mg/kg) treatment also exhibited antidepressant-like effect in separate group of CUMS mice as revealed by decreased immobility duration by 24% (P < 0.01) in CUMS animals [$F_{CUMS \times Treatment}^{1,21} = 7.27$, P < 0.05; $F_{CUMS}^{1,21} = 28.23$, P < 0.001; $F_{Treatment}^{1,21} = 7.11$, P < 0.05]. Administration of betaine (20 mg/kg) to CUMS mice also decreased immobility time [$F_{CUMS \times Treatment}^{1,23} = 15.31$, P < 0.01; $F_{CUMS}^{1,23} = 86.68$, P < 0.001; $F_{Treatment}^{1,23} = 16.47$, P < 0.01].

3.5. Locomotor effects of A. aspera on open field test

As shown in Fig. 5, significant changes in the locomotor activity were observed in CUMS mice. CUMS exposure of animals for 4 weeks decreased the number of ambulations (P < 0.01) (Fig. 5A) and rearings (P < 0.01) (Fig. 5B) in saline treated CUMS exposed animals when compared against vehicle treated unstressed control mice. A two way ANOVA indicated that treatment of *A. aspera* extract (2.5–10 mg/kg) significantly enhanced the decreased ambulations [$F_{CUMS \times Treatment}^{3,45} = 1.34$, P > 0.05; $F_{CUMS}^{1,45} = 13.42$, P < 0.01; $F_{Treatment}^{3,45} = 0.54$, P > 0.05] (Fig. 5A) and rearings [$F_{CUMS \times Treatment}^{3,45} = 0.77$, P > 0.05; $F_{CUMS}^{1,45} = 13.82$, P < 0.01; $F_{Treatment}^{3,45} = 1.57$, P > 0.05] (Fig. 5B) in CUMS mice. Likewise administration of fluoxetine (10 mg/kg) reversed the effect of CUMS induced decreased ambulations [$F_{CUMS \times Treatment}^{1,21} = 2.05$, P > 0.05; $F_{CUMS}^{1,21} = 7.66$, P < 0.05; $F_{Treatment}^{1,21} = 3.72$, P > 0.05] and rearings [$F_{CUMS \times Treatment}^{1,21} = 2.65$, P > 0.05; $F_{CUMS}^{1,21} = 8.57$, P < 0.01; $F_{Treatment}^{1,21} = 4.25$, P > 0.05]. Betaine (20 mg/kg) administration reversed the effect of CUMS on ambulations [$F_{CUMS \times Treatment}^{1,23} = 2.75$, P > 0.05; $F_{CUMS}^{1,23} = 9.21$, P < 0.05; $F_{Treatment}^{1,23} = 23.97$, P < 0.01] when compared against saline injected CUMS control animals. Post hoc Bonferroni's mean comparisons revealed the significant difference in ambulations (P < 0.05 and P < 0.01, respectively) and rearings (P < 0.05and P < 0.05, respectively) between *A. aspera* extract (10 mg/kg) or fluoxetine (10 mg/kg) and saline treated CUMS mice. Betaine (20 mg/kg) treatment significantly (P < 0.05) enhanced the ambulations as compared to saline injected CUMS animals. However, the mean rearings in betaine (20 mg/kg) treated mice were not significantly different from control group. The difference in the number of ambulations and rearings between *A. aspera* extract (2.5

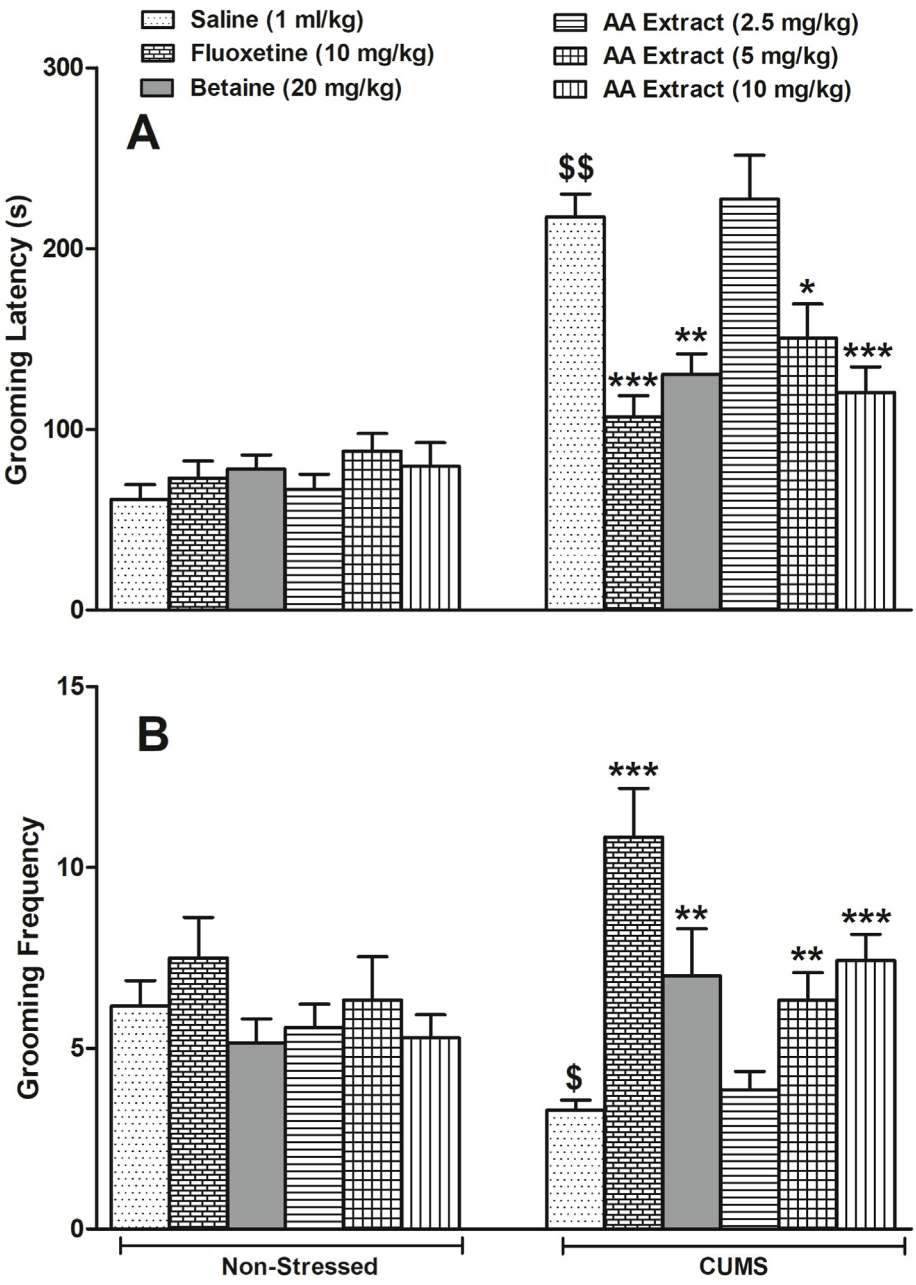

Fig. 3. Motivational behaviors (A) grooming latency (s) and (B) grooming frequency studied in splash test following *A. aspera* extract or fluoxetine treatment to nonstressed and CUMS mice. Each bar indicates mean grooming frequency/grooming latency (s) ± SEM (n = 6–7). $- P < 0.01, $$- P < 0.001 vs. vehicle treated control non-stressed animals; *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle treated control CUMS mice.

and 5 mg/kg) and saline treated CUMS exposed mice was not statistically significant. Moreover, administrations of *A. aspera* extract (2.5, 5 and 10 mg/kg) or fluoxetine (10 mg/kg) or betaine (20 mg/kg) to unstressed control failed to alter the basal locomotor activity.

3.6. Effect of A. aspera on body weight in CUMS mice

Body weight of the individual mice was supervised every day and presented on weekdays as variations in body weight were not significantly different on daily basis. Exposure of animals to CUMS reduced body weights (Fig. 6). Significant variation in the body weight between CUMS and non-stressed mice was evident on day 14 ($P < 0.001$), 21 ($P < 0.001$) and 28 ($P < 0.001$). Repeated treatment of CUMS mice with *A. aspera* extract (2.5, 5 and 10 mg/kg) and fluoxetine (10 mg/kg) as well as betaine (20 mg/kg) from day 15–28 produced significant effect on change in body weight [$F_{CUMS\ X\ Time\ X\ Treatment}(15, 260) = 1.90$, $P < 0.05$; $F_{CUMS\ X\ Time}(3, 260) = 142.85$, $P < 0.001$; $F_{CUMS\ X\ Treatment}(5, 260) = 7.82$, $P < 0.001$; $F_{Time\ X\ Treatment}(15, 260) = 1.72$, $P < 0.05$; $F_{CUMS}(1, 260) = 1151.44$, $P < 0.001$; $F_{Time}(3, 260) = 269.15$, $P < 0.001$; $F_{Treatment}(5, 260) = 4.05$, $P < 0.01$] (Fig. 6). Animals from *A. aspera* extract [5 mg/kg ($P < 0.05$) and 10 mg/kg ($P < 0.01$) but not 2.5 mg/kg], fluoxetine (10 mg/kg) ($P < 0.001$) and betaine ($P < 0.01$) revealed significantly different % body weight change as compared to control CUMS animals.

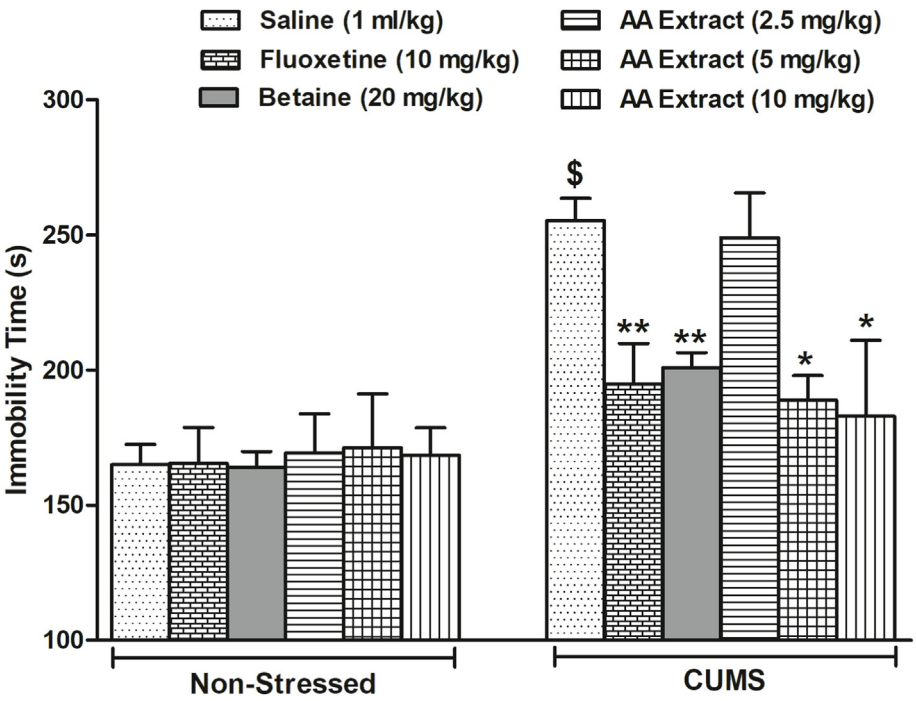

Fig. 4. Immobility duration effects following *A. aspera* extract or fluoxetine injection in non-stressed and CUMS mice studied in FST. Each bar indicates mean immobility time (s) ± SEM. $- P < 0.001 vs vehicle treated control non-stressed animals; *P < 0.05, **P < 0.001 vs vehicle treated control CUMS mice.

3.7. Effect of A. aspera on plasma corticosterone levels in CUMS mice

CUMS exposure increased corticosterone levels in plasma on day 28 ($t = 6.81$, $df = 11$, $P < 0.001$) (Fig. 7). *A. aspera* extract (2.5–10 mg/kg) [$F^{4,32} = 11.49$, $P < 0.001$], fluoxetine (10 mg/kg) [$F^{2,18} = 31.90$, $P < 0.001$] as well as betaine (20 mg/kg) [$F^{4,32} = 18.93$, $P < 0.001$] injections to CUMS animals decreased plasma corticosterone levels. Post hoc analysis suggested that plasma corticosterone in *A. aspera* extract [5 mg/kg ($P < 0.05$), 10 mg/kg ($P < 0.05$)], fluoxetine (10 mg/kg) ($P < 0.01$) and betaine (20 mg/kg) ($P < 0.05$) treated mice were significantly different from vehicle control CUMS animals.

3.8. Effect of A. aspera on PFC and hippocampus BDNF, TNF-α and IL-6 immunocontent

Exposure of mice for 28 days to CUMS procedures produced significant reduction in BDNF ($t = 3.81$, $df = 11$, $P < 0.01$ and $t = 4.16$, $df = 11$, $P < 0.01$) (Fig. 8A and D) and elevation TNF-α ($t = 7.93$, $df = 11$, $P < 0.001$ and $t = 5.63$, $df = 11$, $P < 0.01$) (Fig. 8B and E) and IL-6 ($t = 4.53$, $df = 11$, $P < 0.001$ and $t = 5.58$, $df = 11$, $P < 0.001$) (Fig. 8C and F) immunocontent within hippocampus and PFC (respectively). Administration of *A. aspera* extract (2.5, 5 and 10 mg/kg) from day 15 onward to CUMS mice increased the immunocontent of BDNF [$F(4, 32) = 7.31$, $P < 0.001$ and $F(4, 32) = 4.35$, $P < 0.01$] (Fig. 8A and D) and reduced the immunocontent of TNF-α [$F(4, 32) = 8.39$, $P < 0.001$ and $F^{4,32} = 8.38$, $P < 0.001$] (Fig. 8B and E) and IL-6 [$F(4, 32) = 3.55$, $P < 0.05$ and $F^{4,32} = 3.89$, $P < 0.05$] (Fig. 8C and F) immunocontent in hippocampus and PFC respectively. Similarly, treatment of fluoxetine (10 mg/kg) to CUMS animals from day 15–28 also augmented BDNF [$F(2, 18) = 11.05$, $P < 0.001$ and $F^{2,18} = 10.28$, $P < 0.01$] (Fig. 8A and D) and attenuated TNF-α [$F(2, 18) = 13.98$, $P < 0.001$ and $F^{2,18} = 17.28$, $P < 0.001$] (Fig. 8B and E) and IL-6 [$F(2, 18) = 11.75$, $P < 0.001$ and $F^{2,18} = 14.23$, $P < 0.001$] (Fig. 8C and F) immunocontent in hippocampus and PFC respectively. One way ANOVA indicated the significant effect of betaine (20 mg/kg) treatment to CUMS mice on BDNF [$F(2, 19) = 5.43$, $P < 0.05$ and $F(2, 19) = 8.78$, $P < 0.01$] (Fig. 8A and D), TNF-α [$F(2, 19) = 40.71$, $P < 0.001$ and $F^{2,19} = 13.78$, $P < 0.001$] (Fig. 8B and E) and IL-6 [$F(2, 19) = 8.49$, $P < 0.01$ and $F^{2,19} = 10.25$, $P < 0.01$] (Fig. 8C and F) immunocontent in hippocampus and PFC, respectively.

Post hoc mean analysis by Bonferroni's test indicated that BDNF ($P < 0.05$ and $P < 0.05$, respectively), TNF-α ($P < 0.05$ and $P < 0.05$, respectively) and IL-6 ($P < 0.05$ and $P < 0.05$, respectively) immunocontent in hippocampus and PFC (respectively) were significantly different in *A. aspera* extract (10 mg/kg) treated CUMS mice as compared to CUMS control. Likewise, hippocampal and PFC immunocontent of BDNF ($P < 0.01$ and $P < 0.05$, respectively), TNF-α ($P < 0.01$ and $P < 0.01$, respectively) and IL-6 ($P < 0.05$ and $P < 0.05$, respectively) were also significantly altered in fluoxetine (10 mg/kg) injected mice when compared against vehicle injected CUMS animals. The mean values of BDNF immunocontent in hippocampus ($P < 0.05$) and PFC ($P < 0.05$) of animals injected with betaine (20 mg/kg) was significantly higher as compared to saline control group. Whereas mean TNF-α ($P < 0.01$ and $P < 0.05$) and IL-6 ($P < 0.05$ and $P < 0.05$) in betaine (20 mg/kg) mice hippocampus and PFC (respectively) was significantly lower as compared to control CUMS mice. However, BDNF, TNF-α and IL-6 immunocontent in hippocampus and PFC were not significantly different in *A. aspera* extract (2.5 and 5 mg/kg) treated CUMS mice from vehicle treated CUMS control animals.

4. Discussion

The present study was planned to validate the antidepressant potential of *A. aspera* in animal studies as number of Indian communities have been traditionally using *A. aspera* as a brain tonic for the treatment of neurological and psychiatric disorders. *A. aspera* extract reversed the CUMS induced anhedonia in SPT, diminished

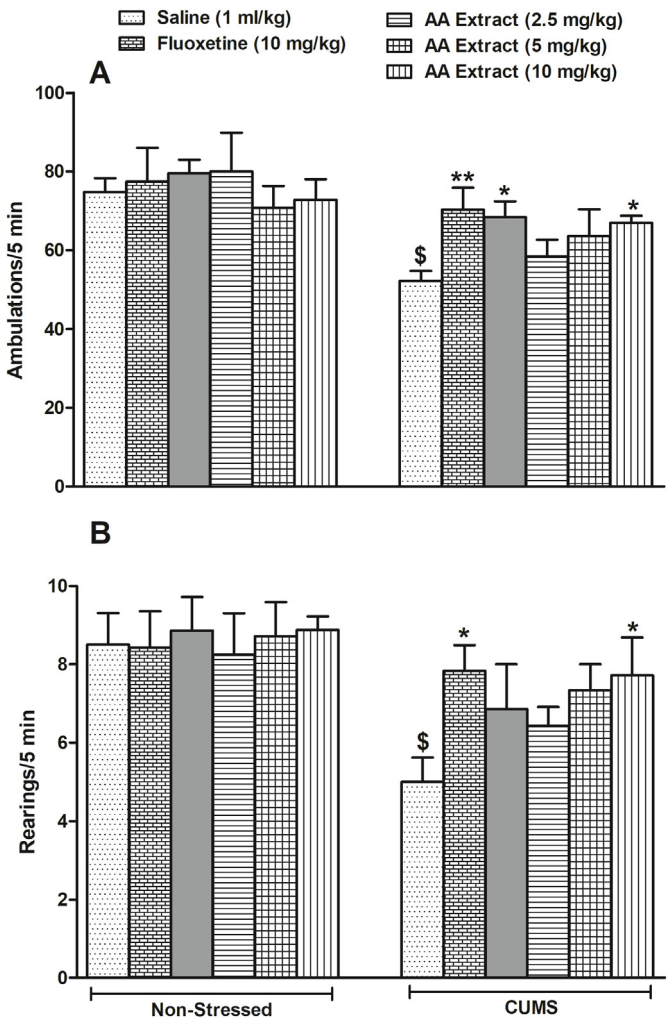

Fig. 5. Open field behaviors (A) ambulations and (B) rearings in OFT following *A. aspera* extract or fluoxetine treatment in nonstressed and CUMS mice. Each bar indicates mean locomotor counts (5 min) ± SEM (n = 6–7). $-P < 0.01vsvehicle treated control non-stressed animals; *P < 0.05, **P < 0.01 vs vehicle treated control CUMS mice.

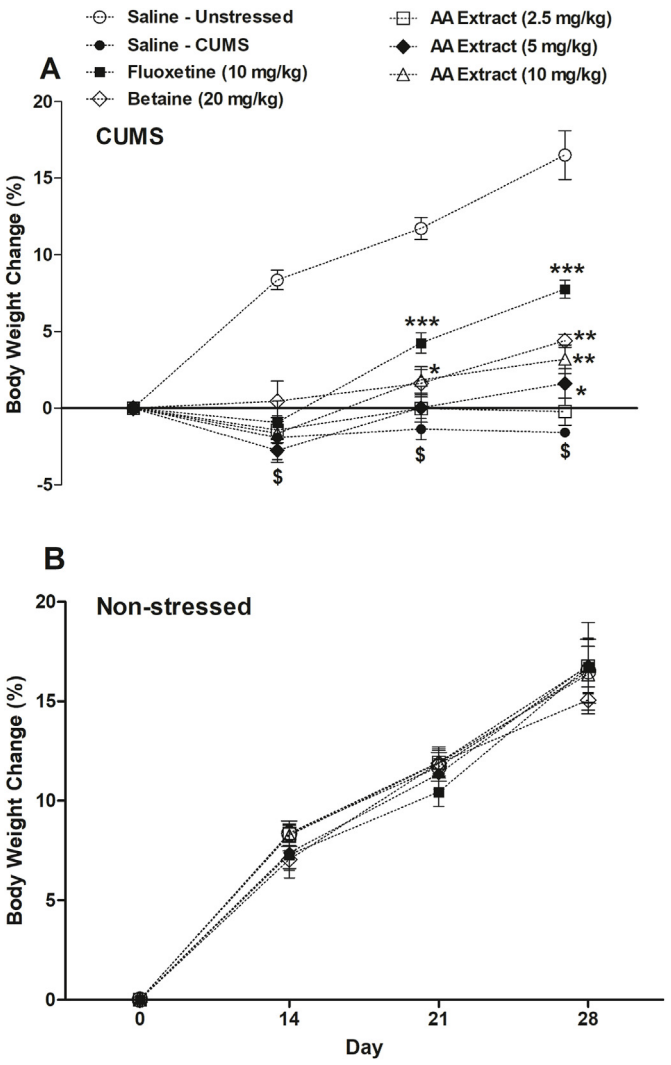

Fig. 6. Body weigh changes following *A. aspera* extract or fluoxetine treatment in (A) CUMS and (B) non-stressed mice. Each point indicates mean body weight change (%) ± SEM. $P < 0.001 vs vehicle treated control non-stressed animals; *P < 0.05, **P < 0.01, and ***P < 0.001vsvehicle treated control CUMS mice.

self-care and motivational behavior in splash test and increased immobility time in FST. Further, it normalized the cytokines and BDNF within hippocampus and prefrontal cortex altered by CUMS exposure.

CUMS procedure is directly linked with dysregulation of HPA-axis leading to number of psychiatric disorders including depression. In our procedure, we adopted the methodology to expose the animals with mild stressors and in unpredictable manner.[36,37] In the CUMS model, due to an obvious relation of anhedonia with reduced consumption as well as preference for sucrose suggest generalized diminished sensitivity to reward and have been standardized at our laboratory.[31] We found that CUMS exposure leads to decreased sucrose preference, grooming latency and frequency, and increased immobility duration. SPT was calculated as a fraction of intake of sucrose solution to the total fluid intake (sucrose + water). Collectively these behavioral abnormalities may be correlated with anhedonia, diminished self-care and motivation, apathy and despair or helplessness exhibiting core symptoms of clinical depression. These behavioral abnormalities were associated with enhanced plasma corticosterone levels in CUMS exposed animals. These results confirmed that CUMS exposure leads to depression like phenotype. However, it is not clear whether stressed animals immobile for a longer time in the FST because they developed a depressive-like phenotype or a locomotor deficit. The OFT was designed to assess the locomotor activity of rodents, which was used as a behavioral endpoint.[38] Animals displayed decreased locomotor activity in OFT after CUMS,[39] which indicates the loss of exploration and interest, two instinctive activities of normal animals in a novel environment.[40] It is important to note that, anhedonia, the major endpoint of CUMS model,[41] is associated with other analogues of depressive symptoms, such as increased floating duration in FST and decreased exploration of novelty in OFT. We observed that chronic administration of *A. aspera extract* to CUMS exposed animals, not only corrected behavioral abnormalities but also normalized the plasma corticosterone levels. This clearly suggest that antidepressant like effect of *A. aspera* might be associated with stabilization of HPA-axis dysregulated by CUMS exposure in mice. Thus, *A. aspera* treatment might be associated with maintenance of brain homeostasis as adaptation response to chronic stress. It is important to note that several clinically available antidepressant drugs produce their pharmacological action through regulation of HPA-axis and plasma cortisol levels in

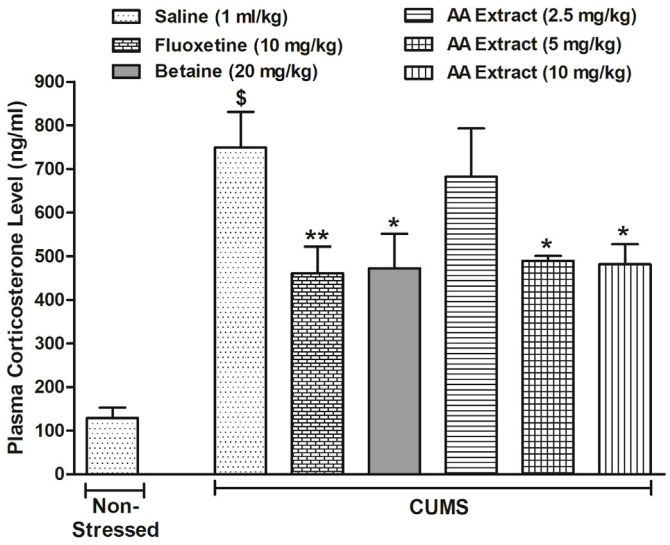

Fig. 7. Plasma corticosterone levels on day 28 following *A. aspera* extract and fluoxetine treatment in CUMS mice. **After OFT, blood samples were collected from the retro-orbital plexus of the animals and separated plasma was evaluated for corticosterone levels.** Each bar indicates mean plasma corticosterone (ng/ml) ± SEM (n = 6–7). $- P < 0.001 vs vehicle treated control non-stressed animals; *P < 0.05, **P < 0.01 vs. vehicle treated control CUMS mice.

addition to their direct effect on brain neurotransmission system.[6] However, orbital blood withdrawal is known to induce acute stress in the experimental animals, thus could have influenced the corticosterone analysis. Hence, there is need of more refined protocol to minimize the acute stress induced by orbital blood withdrawal. Although, we could not analyze neurotransmitter levels, earlier studies predicted antidepressant like effect of A. *aspera through* modulation of central neurotransmitter system like dopamine or serotonin.[24]

Emotional challenges like stress exposure for chronic period may alter the body weight of animals.[42] In the present study, CUMS exposure significantly decreased the body weights as compared to vehicle treated nonstressed animals. *A. aspera* treatment mitigated the body weight reduction induced by CUMS protocol. Thus, *A. aspera* can also be advantageous for stress related feeding disorders and efficient for the treatment of loss of body weight associated with stress. However, body weight alterations can be considered as a bewildering feature of sucrose consumption which could produce to false results in CUMS procedures.

Neuroinflammation plays important role in pathogenesis and progression of psychiatric disorders.[3] Psychological and physical stressors can activate immune system leading to inflammatory cytokines release (TNF-α, IL-6 etc.) in some brain areas[4] and are associated with depression like illness.[5,43] In present investigation, our results demonstrated that CUMS significantly augmented the hippocampal and PFC expression of inflammatory cytokines IL-6, and TNF-α in mice, which is consistent with a previous study.[5] Chronic *A. aspera* treatment significantly attenuated the immunocontent of IL-6, and TNF- α in the PFC and hippocampus of mice. We may recall that *A. aspera* exhibits anti-inflammatory effects in rodents.[26] We proposed that inhibition of cytokine release by *A. aspera* might be associated with its antidepressant like effect in CUMS induced depression.

Responsiveness of brain to intrinsic and extrinsic triggers including stress and depression alters the neural plasticity in multiple encephalic regions like hippocampus and PFC. However, its dysregulation, by continuous stress exposure can be associated with inception and progression of depression.[43] The role of neurotrophins such as BDNF in the pathophysiology of depression is fairly acknowledged.[44,45] Atrophy of limbic structures, including the hippocampus and prefrontal cortex observed in depressed subjects is associated with reduced BDNF expression.[44] Importantly, it is worth to note that several drugs exerts their antidepressant effect by increasing BDNF levels within hippocampal and PFC.[46–48] The results observed in the present investigation support this notion. Chronic *A. aspera* treatment restores BDNF level in hippocampus and PFC reduced by chronic stress exposure. Thus, the anti-depressant like effect of *A. aspera* observed herein might be attributed to enhanced neuroplasticity by up regulation of BDNF within hippocampus and PFC. However, more studies are required to analyze its effect on other encephalic regions like amygdala and interconnecting signaling pathways like NMDA, glutamate and glucocorticoid. Overall, this study provides strong experimental evidences for the neuropharmacological application of *A. aspera* in several parts of India and supported by earlier observation demonstrating its effect in cognitive impairment, epilepsy, anxiety, and cerebral ischemia.[13,20,23,24] However, it needs to be established how modulation of BDNF by *A. aspera* leads to inhibitions of neuroinflammatory cascade to provide beneficial effect in depression like phenomenon.

We have designed additional protocols for examining the antidepressant-like effect of betaine, an active and important constituent of *A. aspera*. We found that administration of betaine inhibited the CUMS induced anhedonia in SPT, diminished self-care and motivational behavior in splash test and increased immobility time in FST. Further, it normalized the cytokines and BDNF within hippocampus and prefrontal cortex altered by CUMS exposure. Betaine, a most abundantly found water soluble alkaloid in *A. aspera*, it is a quaternary ammonium compound containing a carboxyl group, known as trimethylglycine, Pharmacologically it is known to dilate blood vessels, lower blood pressure and heart rate, and increases the rate and amplitude of respiration with spasmolytic, diuretic, purgative, hepatoprotective effects in rats with musculotropic activity.[49] Recent reports on betaine suggest it's potential *anti*-neuroinflammatory role as it suppresses the pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, Interleukin 6 (IL-6), Interleukin-1 β (IL-1β), cyclooxygenase-2 (COX-2), and nuclear factor-κB (NF-κB) with oxidative stress.[50] Moreover, it shows anti-inflammatory and anti-arthritic activities.[51,52] It is also recognized as a novel psychotherapeutic for schizophrenia,[53] Alzheimer's and Parkinson's diseases[54] and ameliorates memory dysfunctioning in the experimental rats by its influence on the GABAergic neuronal system.[55] In view of its neuropharmacological profile and inhibitory influence on inflammatory cytokines, it is reasonable to assume that antidepressant like effect of *A. aspera* extract might be contributed (in principle) by betaine. The results further strengthen the importance of present study in clinical context and strongly support further investigations of betaine in depressive illness and mood disorders.

5. Conclusion

Current study demonstrated the beneficial effect of *A. aspera* extract and its active constituent, betaine in CUMS induced depression in mice. *A. aspera* extract as well as betaine reversed the CUMS induced anhedonia, diminished self-care, stabilized the body weight reduction, and inhibited corticosterone plasma levels elevation in mice; and normalized the cytokines and BDNF within hippocampus and prefrontal cortex altered by CUMS exposure. The study validated the use of *A. aspera* for stress associated depression and related complications and strongly support further investigations of betaine in clinical context.

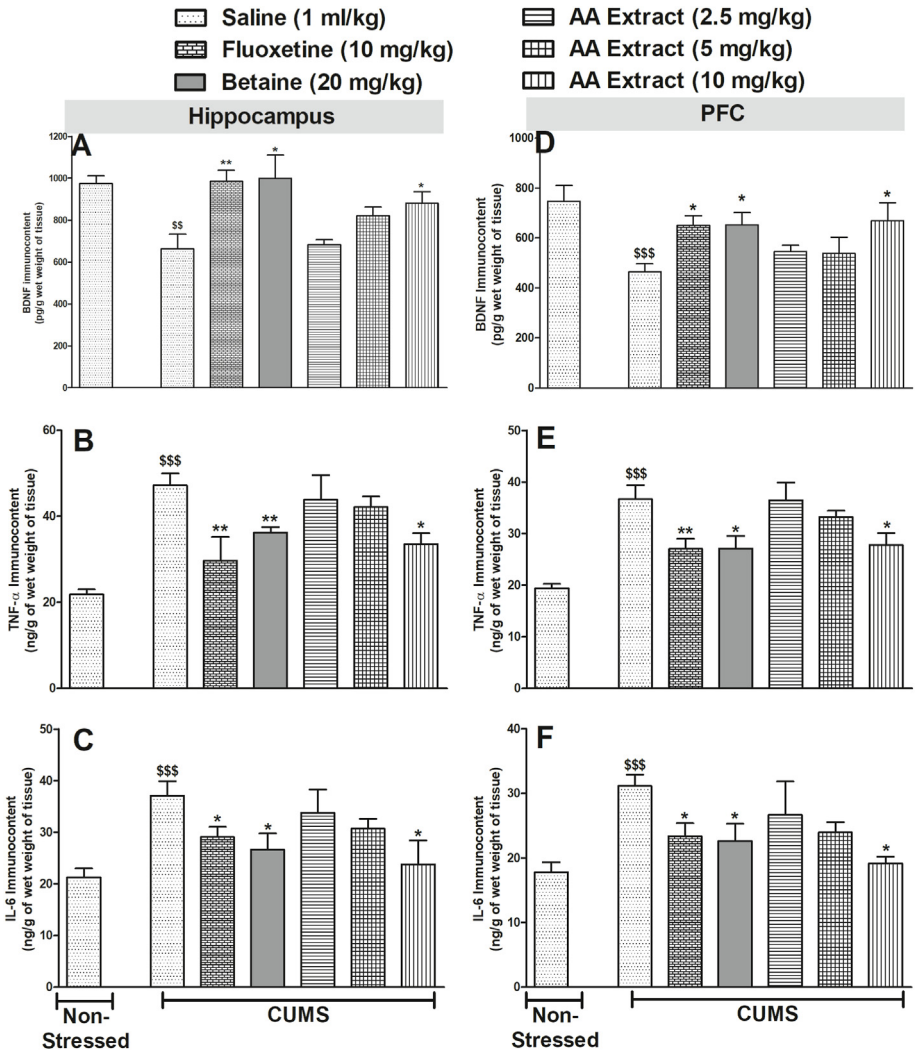

Fig. 8. BDNF (A and D), TNF-α (B and E) and IL-6 (C and F) immunocontent in hippocampus (A, B, C) and PFC (D, E, F) following *A. aspera* extract and fluoxetine treatment in CUMS mice. Each bar indicates mean BDNF, TNF-α and IL-6 immunocontent (ng/g wet weight of tissue) ± SEM (n = 6–7). $$P < 0.01, $$$P < 0.001 vs vehicle treated control non-stressed animals; *P < 0.05, **P < 0.01 vs vehicle treated control CUMS mice.

Declaration of competing interest

Authors declare that there are no actual or potential conflicts of interests including any financial, personal or other relationships.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2022.06.001.

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