

Research Article**Reversal of neuro-inflammation and oxidative stress by polyherbal formulation in an animal model of chronic unpredictable mild stress**Krishna M. Shah^{1*}, Vandana Mody², Sunita S. Goswami¹¹Department of Pharmacology, L. M. College of Pharmacy, Ahmadabad, Gujarat 380009 India²Phyto Department, Cadila Pharmaceuticals Limited, Ahmedabad, Gujarat, 382225 India

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Abstract

Background: Chronic unpredictable mild stress (CUMS) provokes behavioral alterations, oxidative-stress and neuroinflammation. CUMS mimics the clinical aspects of anxiety and depression in human beings. Existing antidepressant drugs possess slow onset of action, low response rates and problem of drug resistance. Therefore, there is a scope of alternative therapy for the treatment of neuropsychological illnesses. **Objectives:** The present study is aimed to assess the efficacy of polyherbal formulation in CUMS model using mice and to explore the possible mechanism for the same. **Materials and Methods:** Mice were subjected to a series of stressful events for a period of 28 days. Drug treatments were given for a period of 28 days after the induction of disease. Parameters studied included behavioural aspects, sucrose preference test, brain neurotransmitters (5-HT, nor-adrenaline and dopamine) levels, serum pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6), corticosterone, quinolinic acid and oxidative markers. **Results:** Treatment with polyherbal formulation (400 & 800 mg/kg) significantly ameliorated behavioral deficits in several tests like forced swim test, tail suspension test, photoactometer and elevated plus maze model. It also reduced ($p < 0.001$) anhedonia using sucrose preference test. Significant up regulation of serotonin and other neurotransmitters along with reduction in oxidative stress was observed in treated animals. Further, polyherbal formulation also significantly attenuated the stress-induced increase in serum levels of TNF- α , IL-1 β , IL-6, corticosterone and quinolinic acid. **Conclusion:** Our data suggest that this formulation enhances the neuroprotective effects against CUMS-induced oxidative stress, neuroinflammation and behavioral deficits.

Keywords: Antidepressant, neuro-inflammation, oxidative-stress, neurotransmitters

Introduction

Depression is a mental disorder, which encompasses emotion, cognition, and physical symptoms with considerable morbidity and mortality. In India, the lifetime prevalence of anxiety and depression is 24.4% and 18.5%, respectively, and co-morbidity of anxiety with depression is high about 87% (Sahoo and Khes, 2010). Stress is any transformation in environmental conditions that disturb the normal physiological equilibrium and linked to various neurological illnesses. Literature supports the role of inflammation and immune system deregulation in pathophysiology of depression (Raison et al. 2006; Leonard

2010). Clinical evidence suggested that antidepressant drugs are effective in treating depressive episodes, but less efficacious in recurrent depression and in preventing relapse (Fava and Offidani, 2011).

Traditional pharmacognosy isolates single active principles which may be self-defeating because overall biological effects rely on synergistic interactions between plant components. Poly herbal formulation (PHF) possesses some advantages such as to reduction in dose, convenience, and ease of administration (Modak et al. 2007; Little 2009; Bhushan et al. 2014). The multitarget responses of herbal drugs are proven to be beneficial in chronic conditions and so forth, and also in restoring the health status (Morphy et al. 2004). So, there is a scope for the development of such treatment which works not only behavioural defects of the depression and anxiety but also useful for removal of toxins from brain and produces calming effect. Tensnil Syrup, a

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PHF (Poly Herbal Formulation), contains extracts of garmarogor, devdaru, shankhvali, pitapapado, brahmi, jatamansi, nagarmoth, kadu, tagar, himaj, draksha, ashwagandha. In the light of above, the aim of the present study includes studying reversal of neuro-inflammation and oxidative stress by polyherbal formulation in an animal model of chronic unpredictable mild stress.

Materials and methods

Drugs and chemicals

Tensnil Syrup was supplied by the manufacturer, Cadila Pharmaceutical Private Limited. Fluoxetine was provided by pharmACE laboratory, Ahmedabad. All other chemicals were of analytical grade.

Animals

The experiment was performed according to the guidelines of the animal ethics committee and approved by Institutional Animal Ethics Committee (IAEC) (LMCP/COLOGY/16/09). Thirty male swiss albino mice weighting 30–40 g were obtained. After 2 weeks of acclimatization, the mice were randomly divided into five groups (6 mice in each): control, chronic unpredictable mice stress (CUMS) exposed, CUMS + Fluoxetine 20 mg/kg, CUMS + PHF 400 mg/kg, CUMS + PHF 800 mg/kg.

Chronic unpredictable mild stress:

Mice were exposed to a random pattern of mild stressors (Murua et al. 1991) daily for 28 days which scheduled for period of 1 week and repeated throughout experiment. Stressors included cage tilting at 45°, cold swimming, tail pinch, housing in mild damp saw dust, wet saw dust, overnight illumination, and food and water deprivation. The whole experiment lasted for 8 weeks (56 days). Behavior tests including forced swim test, tail suspension test, locomotor activity using photoactometer, elevated plus maze and sucrose preference test were performed at the end of every week. Blood samples were collected from the retro orbital at the end of the experiment for the estimation of serum proinflammatory cytokines (TNF- α , IL-1 β and IL-6), corticosterone, quinolinic acid and levels of oxidative and antioxidant enzymes. Then, the mice were killed by decapitation. The skull was opened, and the brain was dissected out on an ice plate for analysis of brain neurotransmitters namely 5-hydroxy tryptamine, noradrenaline, and dopamine.

Behavioural tests:

Forced swim test (FST):

The mice were taken to a separate room and were immediately placed in a cylinder (45 cm high, 20 cm diameter) filled to 30 cm depth and maintained at $25 \pm 1^\circ\text{C}$ for the duration of 5 minutes (Porsolt et al., 1978).

Tail suspension test (TST):

The mice were hung 25 cm above the floor by the tip of the tail.

The immobility time was counted during a test period of 6 min (prior 1 min to adapt and recorded the last 5 min) (Steru et al., 1985).

Locomotor activity:

Each mouse was placed in infrared light-sensitive photocells using a digital photoactometer. The mice was observed for a period of 5 min and the values were expressed as counts per 5 min (Mishra and Kumar, 2014).

Elevated plus maze (EPM)

EPM consisted of two open arms (30 \times 5 cm), two enclosed arms (30 \times 5 cm), and a connecting central platform (5 \times 5 cm) and elevated 38.5 cm above the ground. At the beginning, each mouse was placed in the central zone, facing one of the close arms. An arm entry was defined as a mouse having entered an arm of the maze with all four legs (Lister, 1987).

Sucrose preference test (SPT)

Mice were trained to drink 1% sucrose solution before starting of CMS procedure for 1 hour. Each group provided simultaneously with both sucrose (1%) solution and water (Dhingra and Valecha, 2014).

Proinflammatory cytokines estimation

ELISA kits for TNF- α , IL-6, IL-1 β (Krishgen Biosystem, CA, USA) were used for the estimation of proinflammatory cytokines.

Brain neurotransmitters analysis

The estimation of serotonin, noradrenaline and dopamine in mice brain was carried out according to the fluorometric method. Brain tissue sample was homogenized in 10 volumes of cold acidified N-butanol using a glass homogenizer. Duplicate internal standard tubes were carried in parallel with the brains homogenates. The monoamines were assayed in the aqueous phase. Excitation and emission wavelengths of 295 and 355 nm, respectively, were used for measurement of serotonin. Noradrenaline fluorescence was measured at excitation and emission wavelengths of 380 and 480 nm, while dopamine fluorescence was measured at excitation and emission wavelengths of 320 and 375 nm in the same sample (Schlumpf et al., 1974).

Adrenal gland weight

The animals were sacrificed using CO₂ chamber. The Adrenal gland was dissected out from animal and washed using saline (Pesarico et al., 2016).

Serum corticosterone measurement

Estimation of corticosterone was done by spectrophotometer (Katyare and Pandya, 2005).

Serum quinolinic acid estimation

Serum quinolinic acid estimation was performed according to the method of Junichi, Masahiko and Akihito using fluorometric method (Odo et al., 2009).

Oxido-nitrosative stress parameters

The lipid peroxidation was measured according to method of Wills. The amount of MDA was measured by reacting it with thiobarbituric acid and measured at 532 nm (Wills, 1966). The reduced glutathione was measured as per the method of Ellman. The serum was precipitated with 4% sulfosalicylic acid and

supernatant give yellow color with 5, 5'-dithiobis-(2-nitrobenzoic) acid (DTNB). It was measured immediately at 412 nm (Ellman, 1959).

Results

Forced Swim Test (FST)

Exposure to CUMS for 4 weeks resulted in depressive-like behavior as it significantly increased the duration of immobility time of the FST (normal control; 111.83 ± 6.72 , disease control; 173.83 ± 4.14 , $p < 0.001$). Treatment with Fluoxetine (reference standard, 20 mg/kg) and PHF (400

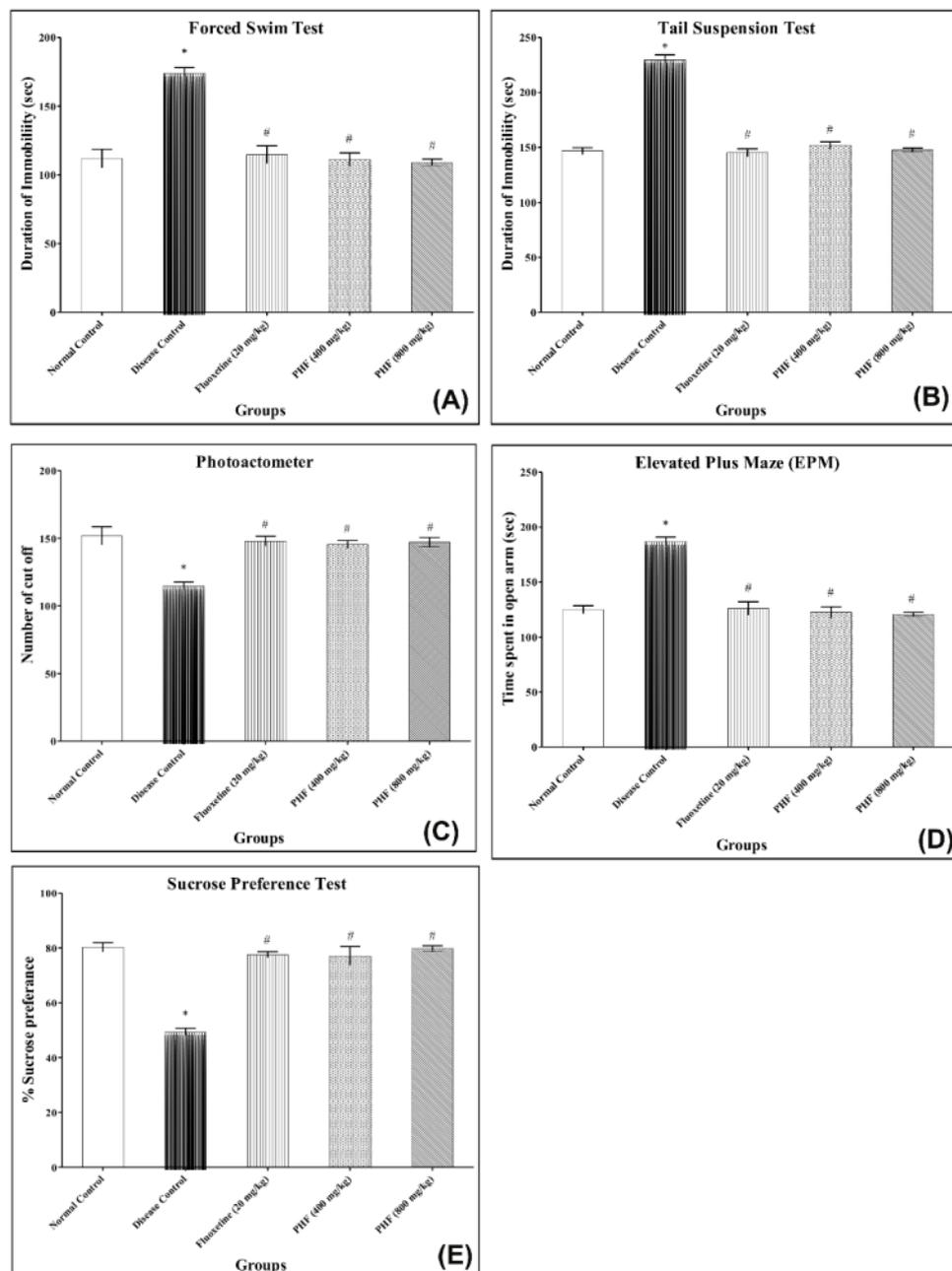


Figure 1. Results of different behavioural tests (A) Forced Swim Test; (B) Tail Suspension Test; (C) Locomotor activity; (D) Elevated Plus Maze; (E) % Sucrose preference. One-way ANOVA Followed by Tuckey's multiple comparison tests. * $p < 0.001$ when compared with the normal control group; # $p < 0.001$ when compared with the disease control group.

mg/kg & 800 mg/kg) after 4th week, significantly reduced the immobility time in comparison to the disease control group (disease control; 173.83 ± 4.14 , Fluoxetine; 114.67 ± 6.44 , $p < 0.001$, PHF (400 mg/kg); 111.00 ± 4.87 , $p < 0.001$, PHF (800 mg/kg); 109.17 ± 2.36 , $p < 0.001$) (Figure 1).

Tail Suspension Test (TST)

The duration of immobility was measured in the TST to evaluate the stress related despairing status in mice. The duration of immobility of CUMS group was significantly longer than that of control group ($P < 0.001$). After drugs treatment, the immobility time of fluoxetine and PHF groups were significantly reduced as compared to disease group ($P < 0.001$, Fig. 2), suggesting that PHF (400 & 800 mg/kg) could reverse despairing status in the CUMS-induced mice (Figure 1).

Locomotor activity

The locomotor activity observed using photoactometer was

significantly ($p < 0.001$) reduced in the disease control group treated with CUMS. Fluoxetine and PHF (400 & 800 mg/kg) treated groups were compared with CUMS-induced disease control group, showed significant ($p < 0.001$) increase into number of cut off (Figure 1).

Elevated Plus Maze (EPM)

CUMS-induced an anxiogenic effect in diseased group and significantly ($P < 0.001$) increased the time spent in open arm in plus maze. Both the treatments including fluoxetine and PHF significantly ($p < 0.001$) reversed the time spent in open arm when compared with the disease control group (Figure 1).

Sucrose Preference Test (SPT)

There was no significant difference in sucrose preference (%) among all the groups in the baseline test. Exposure of the mice to stress for 28 successive days significantly

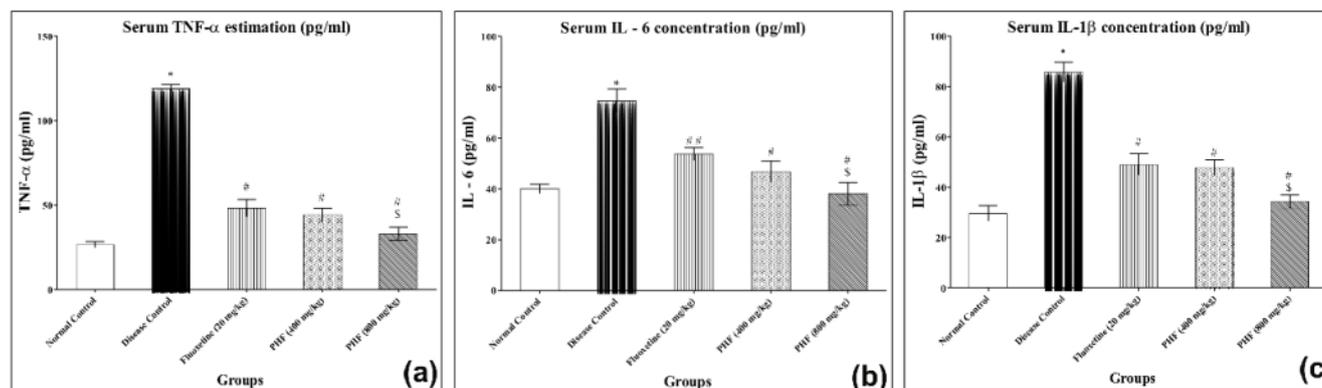


Figure 2. Level of Pro-inflammatory cytokines: (a) Serum TNF – α estimation (b) Serum IL-6 concentration (c) Serum IL - 1β concentration. One-way ANOVA Followed by Tuckey's multiple comparison tests. * $p < 0.001$ when compared with normal control group; ** $p < 0.01$ when compared with normal control group # $p < 0.001$ when compared with the disease control group; \$ $p < 0.05$ when compared with the standard (fluoxetine (20 mg/kg)) group.

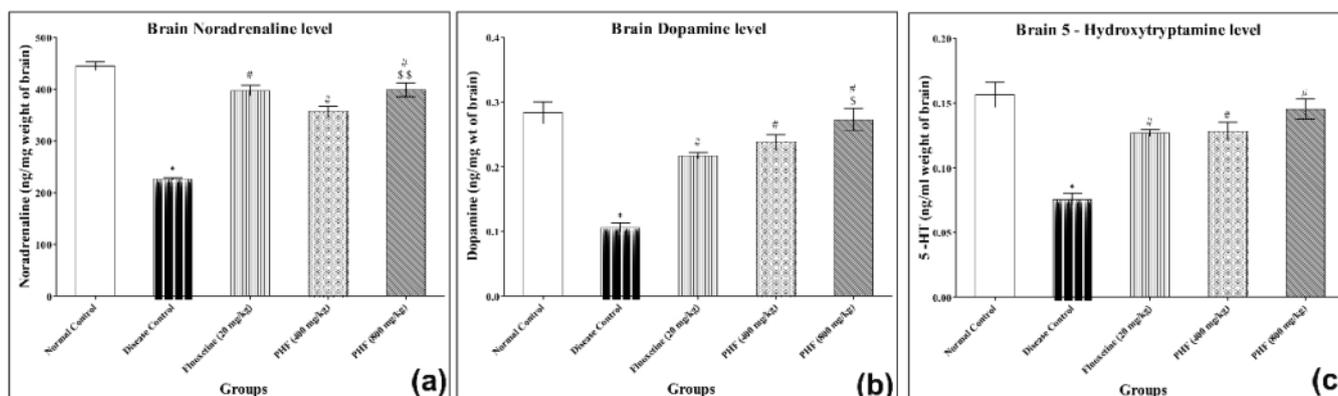


Figure 3. Levels of Brain neurotransmitters (a) Brain Noradrenaline level (b) Brain Dopamine level (c) Brain 5 – HT level. One-way ANOVA Followed by Tuckey's multiple comparison tests. * $p < 0.001$ when compared with normal control group; # $p < 0.001$ when compared with the disease control group; \$ $p < 0.05$ when compared with the standard group.

decreased sucrose preference (%) in stressed mice as compared to control group. Reduced sucrose preference (%) in stressed mice was significantly restored by the administration of fluoxetine (20 mg/kg) or PHF (400 & 800 mg/kg) for 28 successive days (Figure 2).

Pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β)

CUMS animals showed significant ($p < 0.001$) elevation in the levels of neuroinflammation markers, TNF- α , IL-6, IL-1 as compared to the disease group. PHF (400 & 800 mg/kg) treatment significantly ($p < 0.001$) attenuated the increased levels of TNF- α , IL-6, IL-1 when compared with the CUMS-induced disease control group (Figure 2). Further, comparison between PHF 800 mg/kg treated group and fluoxetine (20 mg/kg), PHF treated group significantly ($p < 0.05$) lowered TNF- α , IL-6, IL-1 β levels (Figure 2).

Levels of brain neurotransmitters

All three neurotransmitters namely noradrenaline, dopamine and 5-hydroxy tryptamine were significantly ($p < 0.001$) reduced in

the disease control group as compared to normal control. After treatments for 28 days with fluoxetine and PHF (400 & 800 mg/kg) showed significantly ($p < 0.001$) reversal effect, i.e. increased in the levels of all three neurotransmitters. Also treatment with 800 mg/kg PHF showed significant rise into levels of noradrenaline ($p < 0.01$) and dopamine ($p < 0.05$) when compared with the standard (Figure 3).

Serum concentration of corticosterone and quinolinic acid

CUMS-induced significant increase in the levels of serum corticosterone and quinolinic acid were observed in the disease control group as compared to the normal control group. Treatment with fluoxetine 20 mg/kg and PHF-400 & 800 mg/kg showed significantly reduced levels of both the markers. Moreover, serum concentration of quinolinic acid in PHF-800 mg/kg treated animals significantly ($p < 0.05$) reduced as compared to the standard treatment with fluoxetine (Figure 4).

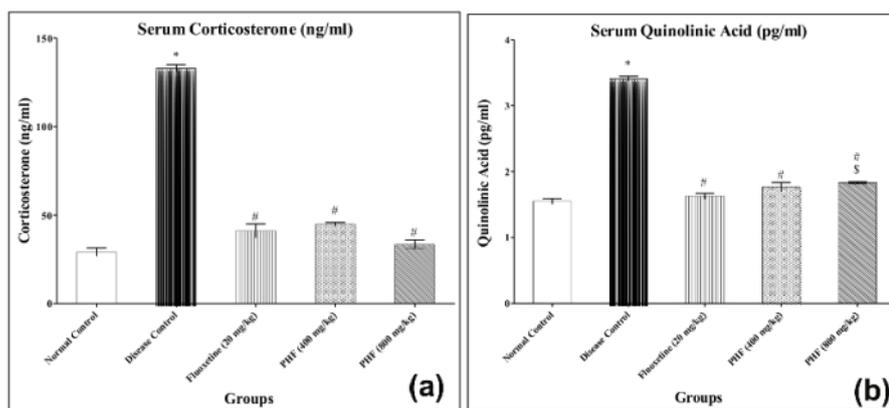


Figure 4. (a) Levels of Corticosterone; (b) Levels of quinolinic acid. One-way ANOVA Followed by Tuckey's multiple comparison tests. * $p < 0.001$ when compared with the normal control group; # $p < 0.001$ when compared with the disease control group; \$ $p < 0.05$ when compared with the standard group.

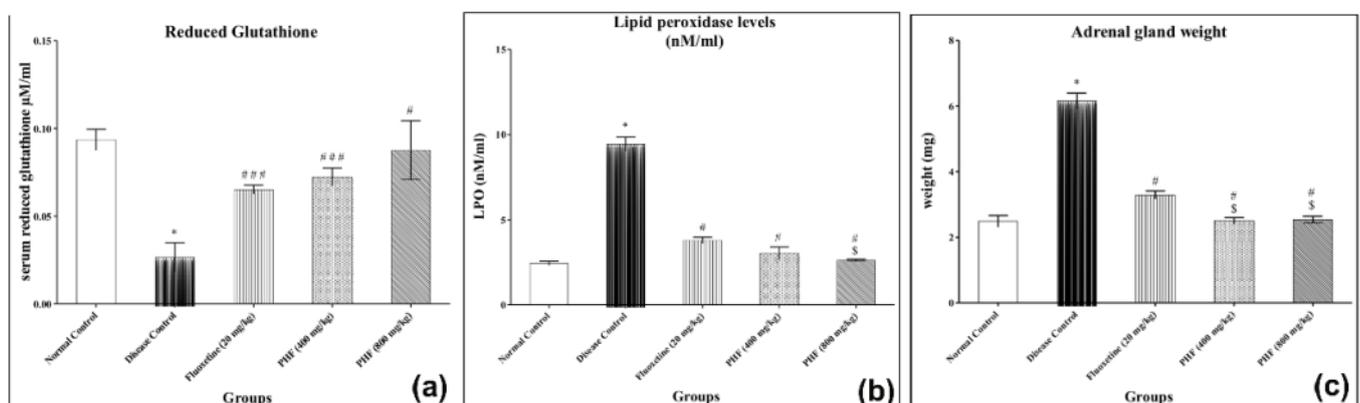


Figure 5. (a) Level of reduced glutathione; (b) Level of lipid peroxidase; (c) Adrenal gland weight. One-way ANOVA Followed by Tuckey's multiple comparison tests. * $p < 0.001$ when compared with normal control group; # $p < 0.001$ when compared with the disease control group; ### $p < 0.05$ when compared with the disease control group; \$ $p < 0.05$ when compared with the standard group.

Oxido-nitrosative stress parameters

CUMS-induced significant rise in oxidative stress was observed in the disease control group when compared with the normal group. Treatments with fluoxetine and PHF-400 & 800 mg/kg significantly ($p < 0.05$, 0.05 and 0.001) ameliorated the level of reduced glutathione as to that of disease control group respectively. A higher level of lipid peroxidase was observed in the disease control group. Poly herbal formulation significantly ($p < 0.05$) attenuated the lipid peroxidase level as compared to fluoxetine treated animals (Figure 5).

Adrenal gland weight

Statistical analysis of the relative adrenal gland weight revealed a significant main effect of CUMS. As shown in figure 5, CUMS increased ($p < 0.001$) the relative weight of adrenal gland when compared with that of controlled mice. PHF at both doses, was significantly ($p < 0.001$) effective against the increase of the relative adrenal gland weight induced by CUMS (Figure 5).

Discussion

Major depression and anxiety disorders are associated with functional and morphological alterations in brain, along with the symptoms reflecting both cognitive dysfunction and anxiety. Clinical studies have shown that stressful life experiences are important etiological factors in the development and maintenance of depression and affective disorders (Kendler et al., 1995, Kessler, 1997).

The present study is aimed to assess the antidepressant-like effect of PHF on mice exposed to CUMS and to explore the underlying mechanism of this effect. CUMS for 28 days significantly activated HPA axis, which is manifested by elevated levels of proinflammatory cytokines, chemokines, and adhesion molecules in the periphery and central nervous system and causes generation of reactive oxidative stress markers (Dunbar et al., 1992, Hestad et al., 2003, Raison et al., 2006, Maes et al., 2009).

In this study, exposure to CUMS-induced a depressive status in mice as it resulted in increased immobility time in the FST and TST. The forced swim test has been used to detect helpless behavior as measured by immobility time in the chronic mild stress model in mice. Our data showed that stressed mice exhibited a significant prolongation of immobility time in the FST/TST over the end of the last week of CUMS, compared to control and this is also supported by an earlier study (Chhillar and Dhingra, 2013). PHF (400 & 800 mg/kg) treatment significantly reduced the duration of immobility in the FST/TST, implying that the poly herbal formulation reversed the depression-like symptoms of CUMS mice, thus showed significant antidepressant-like effect.

Anxiety is thought to be a negative emotion caused by many kinds of stress. In this field, the EPM task has become one of the

most popular animal paradigms used in our study. In this test, the anxiety-like behavior (i.e., decreased time spent in the open arms) is potentiated by prior exposure to a variety of stressors (Korte and De Boer, 2003), as confirmed by our data. Our study has demonstrated that, orally administered polyherbal formulation, decreased the anxiety-like effects of stress in mice after the CUMS protocol. Locomotor activity is considered as an index of alertness and a decrease in it is indicative of anxiety-like activity. The effect of stress on locomotor activity is still controversial (Mao et al. 2010, Liu et al., 2012). However, both the doses of PHF under the study have shown anti-anxiety like activity.

SPT signifies the anhedonia-like behavioral change, a core symptom of depressive disorder (Jindal et al., 2013). With this test, a reduced consumption of sucrose solutions reflecting a decrease in responsiveness to rewards is interpreted as indicating anhedonia. In our study, mice exposed to CUMS consumed less sucrose solution as compared to control group, while treatment with PHF significantly reversed this behavioral change, revealing antidepressant-like effect. Taken together, the behavioral results indicated that, PHF treatment might exert antidepressant-like effects in the CUMS-induced mice.

Depression is accompanied by changing immune function and initiation of the inflammatory response in the central and peripheral system (Dinan, 2009). In animals, variety of stressors increases the concentrations of proinflammatory cytokines, including TNF- α , IL-6 and IL-1 β (Madrigal et al., 2002, O'Connor et al., 2003) that is also observed in our model. Repeated administration with the fluoxetine and poly herbal formulation prevented the increase in the levels of pro-inflammatory cytokines levels caused by CUMS in the mouse serum. Moreover, decreased levels of cytokines with the treatment of poly herbal formulation was found significant as compared to standard treatment. These data suggest that the antidepressant-like effect of formulation may be mediated by reducing the levels of stress-induced pro-inflammatory cytokines signaling pathway.

It has been reported that the hypothalamic-pituitary-adrenal (HPA) axis could be activated by an inflammatory cytokines (Turnbull and Rivier 1995, Dunn, 2000) which leads to abnormally high glucocorticoid (corticosterone in rodents or cortisol in primates) levels in blood (Pan et al., 2006) thereby plays an important role in the pathophysiology of depression (Pariante and Lightman, 2008, Bosch et al., 2012). Cortisol is known to regulate neuronal survival, neuronal excitability, neurogenesis and memory acquisition. Elevated levels of cortisol may thus contribute to the manifestation of depressive symptoms by impairing these brain functions (Sousa et al., 2008).

Literature suggested that chronic antidepressant treatment in rodents reduced HPA activity (Mason and Pariante, 2006). CUMS-induced hyperactivity of HPA axis led to increase in plasma corticosterone levels and increase adrenal gland weight, which is supported by observations from other studies (Swaab et al., 2005). Treatment with PHF reduced CUMS-induced hyperactivity of HPA axis in mice, as indicated by significant reduction of plasma corticosterone levels and adrenal gland weights in stressed mice.

Once cytokine signals reach the brain, they have the capacity to influence the synthesis, release, and reuptake of mood-relevant neurotransmitters including the monoamines (Miller, 2009). The breakdown of TRP (tryptophan) is believed to contribute to reduced serotonin availability (Schwarcz and Pellicciari, 2002). Cytokines also have been shown to influence the synthesis of DA (dopamine). Activation of microglia is associated with increased NO (nitric oxide) production (Zielasek and Hartung, 1996), suggesting that cytokine influences on BH4 (tetrahydrobiopterine) via NO may be a common mechanism by which cytokines reduce DA availability in relevant brain regions (Kitagami et al., 2003). Consistent with these findings, our results showed a decrease in noradrenaline, dopamine, and 5-hydroxytryptamine levels into CUMS-induced mice. However, PHF treatment restored the concentrations of these neurotransmitters, suggesting that the amelioration of depressive behaviours after PHF treatment as relevant to an increase their concentration in the brain.

In addition, Kynurenic acid inhibits the release of glutamate, while QUIN (quinolinic acid) promotes glutamate release through activation of N-methyl-D-aspartate (NMDA) receptors (Dantzer, 2017). QUIN also activates and/or kills astrocytes and this amplifies the inflammatory response in the brain. Thus, the relative induction of QUIN may determine the effects of cytokines on the CNS and remains an important area for investigation. Our results also implied that QUIN level in the serum raised in the CUMS-induced mice, while the antidepressant-like effects of PHF was accompanied with the decrease of QUIN in the serum. Furthermore, this formulation seems to exert more pronounced antidepressant-like effect compared to fluoxetine.

Another major mechanism of QUIN neurotoxicity is through lipid peroxidation in combination with glutamate release may contribute to CNS excitotoxicity (Rios and Santamaria, 1991, Santamaria et al., 2001). Studies also have indicated that QUIN forms a complex with iron, and electron transfer from this complex to oxygen results in the formation of reactive oxygen species which then mediate lipid peroxidation (Goda et al., 1996). Oxygen free radicals can accumulate in brain and have a potent role in neurodegeneration associated with depression (Serrano and Klann, 2004). Oxidative stress is therefore known

to be one of the primary causes for neuronal dysfunction and depression (Manji and Duman, 2001). In our study, we found a significant increase in oxidative damage as indicated by increased lipid peroxidation, and depletion of reduced glutathione levels thereby strengthening the hypothesis of oxidative stress-induced depressive illness. Treatment with PHF for four successive weeks significantly reversed these parameters. Thus, polyherbal formulation showed significant anti-oxidant activity in mice.

In conclusion, the present study demonstrated that PHF treatment could significantly mitigate behavioural deficits, elicited by CUMS. Significant up regulation of serotonin and other neurotransmitters along with the reduction in oxidative stress was observed with this polyherbal formulation. Further, treatment also significantly attenuated the stress-induced increase in serum levels of TNF- α , IL-1 β , IL-6, corticosterone, quinolinic acid. This study provides new insight into the anti-depressant effects of this polyherbal formulation with multiple targets of depression. So, this may be novel therapeutic strategies for depression.

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Conflicts of interest

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