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Chrysin Attenuates Chronic Unpredictable Mild Stress Induced Changes in Behavior, Inflammation and Improves Adrenergic, Serotonergic Function: An *In-vivo* and Biochemical Study

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Abstract

Chrysin (5,7-dihydroxyflavone) is a flavonoid with a vast number of pharmacological properties because of its antioxidant potential. Chronic stress is one of the predominant etiological factors which evoke molecular alterations in the brain leading to the development of depressive disorder. In the present study, we investigated the effect of Chrysin on Chronic Unpredictable Mild Stress (CUMS) induced alterations in behavior, noradrenergic as well as serotonergic function, and inflammation in brain. Randomly, mice were divided into four groups of six animals in each group. On 28th day after assessing behavioral parameters, brain biochemical markers were assessed. From the results, it is concluded that the chrysin protects the brain cells from CUMS induced molecular changes by attenuation of inflammation and oxidative stress.

1. Introduction

Depression is a mental disorder that has high incidence, high recurrence, and high self-mutilation and suicide rates[1]. Clinically, depression is characterized by persistent depressed mood, mental retardation, loss of interest, and other symptoms[2]. According to the World Health Organization, an estimated 350 million people suffer from depression around the world, and more than 8,00,000 people commit suicide each year[3]. Currently, the effective remission rate of antidepressants ranges from 60% to 80%, and the rate of favorable prognosis is approximately

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30%[4]. The antidepressant drugs used in the clinic today have heterogeneity in the therapeutic response, side effects and cost. Furthermore, treatment of depression with conventional antidepressant drugs provides a complete remission in 70% of the treated individuals[5]. Therefore, there is a need to develop new treatment strategies that can significantly improve outcomes. Traditional or natural medicine is one of the most important sources for new antidepressants and the study of the antidepressant-like effects of herbs is an increasing interest[6].

Emerging evidence suggests that the pathological process of depression involves oxido nitrosative stress and the neuroinflammatory cascade[7], as well as deficiencies in cell proliferation, aberrant cytokine production, and disordered neuroplasticity[8]. Interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α), affect neurotransmission, neuroendocrine function, and behavior[9]. Inflammatory cytokines activate excitotoxic cellular cascades, resulting in apoptosis in the hippocampus and cortex, which are associated with depression-like behaviors[10]. CUMS animal model validly and reliably induces depression-like behavior and physiological effects that mimic neuropsychiatric disorders[11].

Chrysin (5,7-dihydroxyflavone) is a flavonoid, found in honey and many plant[12]. Chrysin has a vast number of pharmacological properties such as anti-inflammatory activity by blocking histamine release and proinflammatory cytokine expression, anti-asthmatic activity, anticancer activity, prevents metastatic progression in breast cancer cells, improves post-ischaemic functional recovery acts as cardioprotective, prevents osteoporosis by activation of Estrogen Receptor (ER)/mitogen activated protein kinase (MAPK)[13-15]. Chrysin protects neurons from cell death and enhances neurological function.[15] We hypothesize that chrysin influences oxidative stress and inflammatory cytokines in rodents subjected to CUMS and the present study was designed to explore the antidepressant-like effects of Chrysin using CUMS model.

2. Materials and Methods

2.1 Chemicals

Chrysin was procured from Sigma-Aldrich and all the reagents used were of analytical grade and were procured from Elite Scientific, Mumbai, India.

2.2 Animals

Adult healthy Swiss Albino mice of either sex (20–30g) were used. The animals were acclimatized to the laboratory conditions for a period of seven days i.e. room temperature (27±3°C), relative humidity (65±10%), and 12h light/dark cycle. All animals were fed with rodent- pellet diet and water was allowed *adlibitum*. CUMS procedure was carried out according to the method described bySchweizer[16], (2009) with minor modifications. The stressors used were, food deprivation for 6 hrs, water deprivation for 6 hrs, empty water bottle for 3hrs, soiled cage for 24hrs, cage tilt at 45 degrees for 24hrs, forced swim in cold water for 5 minutes, tail suspension for 5 minutes. These stress methods were randomly applied each day for 28 days (4 weeks) consecutively, such that each mice receives only one stress in a day, the same stress method was not continuously applied so as to ensure that the mice would be unable to anticipate the next type of stress applied. Study protocol was approved by Institutional Animal Ethics Committee (Approval No.: 1529/PO/A/11/CPCSEA/IAEC3/PRO-07/2014–2015).

2.3 Experimental Protocol

The animals were randomly divided into 6 groups; each group consists of 6 animals. Group 1: Control group – received 2% gum acacia for 28 days orally by gavage.
Group 2: Depression control – CUMS and received 2% gum acacia for 28 days orally by gavage.
Group 3: Standard control group – CUMS and received Imipramine 10 mg/kg orally by gavage in 2% gum acacia for 28 days.
Group 4: Test group - CUMS and received Chrysin 50mg/kg orally by gavage in 2% gum acacia for 28 days.

The behavioral parameters of all animals were evaluated by Forced Swim Test (FST), Tail Suspension Test (TST), Locomotor activity on 0th day and 28th day. In addition Sucrose Preference Test (SPT) was conducted to evaluate anhedonia, which is the core symptom of depression. All the animals were then sacrificed and brain 5-Hydroxy tryptophan (5-HT), Norepinephrine

(NE), Malondialdehyde (MDA), Glutathione (GSH), Myeloperoxidase (MPO), Interleukin (IL) -1 ß, IL-6 and Cyclooxygenase-2 (COX-2) levels were measured.

2.4 Behavioural Studies

2.4.1 Forced Swim Test (FST)

FST was proposed as a model for antidepressant activity by Porsolt[17].

2.4.2 Tail Suspension Test (TST)

The total duration of immobility by tail suspension was measured according to the method of Steru[18].

2.4.3 Locomotor Activity

The locomotor activity was measured using Actophotometer. It consists of cage which has 30 X 30 X 30 cm, and at the bottom six lights and photocells were placed in the outer periphery of the bottom in such a way that a single mouse blocks only one beam. Photocell is activated when the rays of light falls in photocells, the beam of light is interrupted as and when animal crosses the light beam, the number of interruptions were recorded for a period of 5 minutes[19].

2.4.4 Sucrose Preference Test (SPT)

The SPT was conducted according to the method of Willner[20]. The SPT was conducted at 7:00 am on 0th day and 28th day. The consumed volume of sucrose solution and water was recorded after 2 h and Sucrose Preference Ratio (SPR) was calculated according to the following equation:

SPR = Sucrose intake (g)/[Sucrose intake (g) + Water intake (g)] ×100%.

2.5 Biochemical Estimations

2.5.1 Brain Homogenate Preparation

Mice were sacrificed after 28 days by cervical dislocation and brain was isolated on ice and homogenized.

2.5.2 Lipid Peroxidation and Reduced Glutathione

5% brain homogenate was prepared in50mM phosphate buffer (pH7.0) containing0.1mM EDTA. Homogenate was centrifuged at 10,000 RPM for 10min at 0°C in cold centrifuge and the resulting supernatant was used for the estimation of MDA and GSH.

MDA levels were determined as per the method of Ohkawa at room temperature by measuring absorbance at 532nm using (ELICO, 171) Spectrophotometer. The values were expressed in n moles of MDA formed for mg protein/hr/min[21].

Reduced glutathione was measured according to the method of Ellman, at room temperature and absorbance was measured at 412 nm using a (ELICO, 171) UV-Visible Spectrophotometer[22].

2.5.3 Estimation of Myeloperoxidase

The activity of myeloperoxidase was assessed using the modified method of Mullane *et al.*, 1995. The absorbance was measured at 460 nm for 3 min at an interval of 60 Sec. One unit of enzyme activity is defined as the amount of MPO that causes a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as units/gm of tissue[23].

2.5.4 Estimation of Nor-Epinephrine and Serotonin

Tissue samples were homogenised in ice cold butanol to give final concentration of 50mg/ml. Homogenates were centrifuged at 800xg for 15min at 4^{0}C. Residue was discarded and to the supernatant 2.5ml distilled water and 2.5ml of n-heptane were added. Contents were thoroughly mixed and centrifuged at 100xg for 5min. Aqueous phase was separated and to this 200mg of alumina was added followed by 1.5ml of 2M sodium acetate and the pH was adjusted to 8.0 using 1N sodium hydroxide. Alumina was washed twice with 2ml of distilled water, tubes were vortexed and centrifuged at 1000xg for 5min. Supernatant was discarded and the walls of tube were blotted with strips of filter paper. Monoamines were isolated by shaking the alumina with 2ml of2N acetic acid, tubes were again centrifuged at 100xg for 5min. 1ml of supernatant was used for NE, whereas 1.5ml was used for the estimation of 5-HT[24].

To 1ml of supernatant, 100µl of EDTA was added pH was adjusted to 6.3 and to this 100 µl of Iodine was added and mixed thoroughly. Samples were allowed to stand at room temperature for 2min and to this 200µl of alkaline sulphite solution was added and contents were

shaken well and allowed to stand at room temperature for 2min, pH was adjusted to 5.4 with 5N acetic acid. The samples were heated in a boiling water bath for 2min. The tubes were cooled and fluorescence of NE was read with excitation and emission wavelength of 385 and 485nm respectively with slit width of 10/10nm.

To 1.5ml of supernatant, 100µl of cysteine, 1.5ml of hydrochloric acid and 100µl of O-Phthalidaldehyde (OPA) were added and tubes were kept at room temp for 20min. 100µl of sodium metaperiodate was added and the tubes were heated at 80^{0}C in boiling water bath for 20min. Samples were cooled and the fluorescence of serotonin was read in a Spectrofluorometer with excitation and emission wavelength of 360 and 470nm respectively with slit width of 20/10nm.

2.6 Estimation of Inflammatory Markers

The inflammatory markers, IL-1β, IL-6, TNF-αand COX-2 levels were estimated in brain by using ELISA kits. The concentration of the cytokines in 100 µl samples was determined according to the manufacturer's protocol and all samples and standards were assayed in duplicate.

2.7 Statistical Analysis

Data were expressed as mean ± SEM. Difference in mean values between groups was analyzed by one–way analysis of variance (ANOVA)followed by Dunnett's test in order to detect inter-group differences. A value of P<0.05 was considered as statistically significant.

3. Results

3.1 Results of Behavioural Parameters

3.1.1 Effect of Chrysin on immobility time in Tail Suspension Test

There was a significant increase in the immobility time (P<0.001) in stressed mice when compared to control mice. On 28th day Chrysin at a dose of 50 mg/kg significantly reversed depression induced increase in immobility time (P<0.001) as compared to depression control group (Table 1).

3.1.2 Effect of Chrysin on Immobility Time, Climbing Time and Swimming Time in FST

There was a significant increase in the immobility time (P<0.001) in stressed mice when compared to control mice. Imipramine at a dose of 10 mg/kg showed significantly lesser immobility time (P<0.001) when compared to depression control group. Chrysin (50mg/kg) significantly attenuated CUMS induced increase in immobility time (P<0.001) as compared to depression control group on day 28 (Table 2). There was a significant reduction in climbing time and swimming time (P<0.001) of the depression control mice when compared to normal control. Imipramine (10 mg/kg) showed significant increase in climbing time and swimming time (P<0.001) as compared to depression control group. On 28th day, Chrysin significantly restored CUMS induced decrease in climbing time and swimming time (P<0.001) when compared to depression control group (Table 2).

Table 1. Effect of Chrysin on immobility time in Tail Suspension Test

Groups	Immobility time (sec)	
	0th day	**28th day**
Normal control	78±6.5	76±6.0
Depression control	80±7.3	223±13.5***
Imipramine (10 mg/kg)	81±5.9	77±6.1^{+++}
Chrysin (50 mg/kg)	83±6.6	71±6.2^{+++}

Values are expressed as mean ± SEM of 6 animals,

*** P<0.001 vs. Normal control group, $^{+++}$ P<0.001 vs. Depression control group

Table 2. Effect of Chrysin on immobility time in Forced Swim Test

Groups	Immobility time (Sec)		Climbing time (Sec)		Swimming Time (Sec)	
	0th Day	28th day	0th day	28th day	0th day	28th day
Normal control	98±10.6	83±9.7	59±4.3	81±5.5	83±6.9	76±6.1
Depression control	104±11.2	171±13.5***	61±3.6	32±1.7***	75±5.7	37±2.1***
Imipramine (10 mg/kg)	101±9.8	54±4.3+++	63±4.1	107±9.9+++	76±7.1	79±6.3+++
Chrysin (50 mg/kg)	103±11.4	53±4.5+++	59±5.4	163±14.7+++	78±6.7	54±1.9++

Values are expressed as mean ± SEM of 6 animals,

*** P<0.001 vs. Normal control group, +++ P<0.001 vs. Depression control group

3.1.3 Effect of Chrysin on Locomotor Activity and Sucrose preference Test

Imipramine and Chrysin significantly restored locomotor count and improved sucrose preference (P<0.001) when compared to depression control (Table 3).

3.2 Neurotransmitter Estimations

3.2.1 Effect of Chrysin on Nor-epinephrine and Serotonin Levels

Imipramine (10 mg/kg) and chrysin (50 mg/kg) significantly

Table 3. Effect of Chrysin on locomotor activity and sucrose preference test

Groups	Locomotor count		Percentage sucrose preference	
	0th Day	28th Day	0th Day	28th Day
Normal control	125±13.1	120±12.2	30±1.9	27±2.1
Depression control	118 ±13.8	23±2.1***	28±1.3	9±1.4***
Imipramine (10 mg/kg)	120±13.3	108±11.5+++	31±2.4	30±2.7+++
Chrysin (50 mg/kg)	121±10.4	120±11.6+++	31±2.6	35±1.9+++

Values are expressed as mean ± SEM of 6 animals,

*** P<0.001 vs. Normal control group, +++ P<0.001 vs. Depression control group

Table 4. Effect of Chrysin on brain Nor-epinephrine and Serotonin levels

Groups	Norepinephrine levels (nM/mg protein)	Serotonin levels (nM/mg protein)
Normal control	7.1±1.07	8.4±1.37
Depression control	2.7±0.6***	3.1±1.03***
Imipramine (10 mg/kg)	6.3±1.8+++	5.9±1.6+++
Chrysin (50 mg/kg)	6.5±2.11+++	7.7±2.2+++

Values are expressed as mean ± SEM of 6 animals,

*** P<0.001 vs. Normal control group, +++ P<0.001 vs. Depression control group

restored (P<0.001) the brain norepinephrine and seroton in levels (P<0.001) as compared to depression control group (Table 4).

3.3 Antioxidant and Anti-inflammatory Levels

3.3.1 Effect of Chrysin on Brain Lipid Peroxides, Reduced Glutathione and Myeloperoxidase Levels

Imipramine (10 mg/kg) significantly (P<0.001) reversed depression induced increase in the brain lipid peroxides (MDA) and Myelopexoxidase (MPO) levels as compared to depression control group. Chrysin (P<0.001) at a dose of 50 mg/kg significantly reversed depression induced increase in brain lipid peroxide levels and myeloperoxidase levels. Similarly, Imipramine (P<0.001) and Chrysin (P<0.001) significantly restored depression induced decrease in reduced glutathione levels as compared to depression control. In Chrysin treated animals, reduced glutathione levels reached normal values indicating complete recovery (Table 5).

3.4 Effect of Chrsyin on IL-6, IL-1β, TNF-α and COX-2 Levels

Imipramine and Chrysin significantly (P<0.01) reversed CUMS induced increase in the levels of IL-6, IL-1β, TNF-α and COX-2 as compared to depression control group (Table 6).

4. Discussion

The CUMS model is an authentic animal model of depression with high potential, as it mimics the core

Table 5. Effect of Chrysin on brain Malondialdehyde, Reduced Glutathione and Myeloperoxidase levels

Groups	Malondialdehyde (nM/mg protein/hr)	Reduced glutathione (nM/mg protein)	Myeloperoxidase (units/gm)
Normal control	2.3±0.71	58.5±5.3	3.7±0.8
Depression control	6.4±2.5***	18.7±1.56***	8.7±2.6***
Imipramine (10 mg/kg)	3.7±1.2^{+++}	53.6±4.5^{+++}	4.7±2.6^{+++}
Chrysin (50 mg/kg)	2.7±0.9^{+++}	59.7±4.1^{+++}	4.1±0.74^{+++}

Values are expressed as mean ± SEM of 6 animals,

*** P<0.001 Vs Normal control, $^{+++}$ P<0.001 vs. Depression control

Table 6. Effect of Chrsyin on Interleukin-6, Interleukin-1β, Tumour necrosis factor-α and Cyclooxygenase-2

Groups	IL-6 (ng/mg protein)	IL-1β (ng/mg protein)	TNF-α (ng/mg protein)	COX-2 (ng/mg protein)
Normal control	0.56±0.11	0.69±0.18	0.46±0.17	0.72±0.15
Depression control	3.01±0.65***	2.93±0.43***	2.19±0.34**	2.86±0.62***
Imipramine (10 mg/kg)	1.77±0.42^{++}	1.84±0.53^{++}	1.98±0.53*	1.64±0.38^{++}
Chrysin (50 mg/kg)	1.16±0.29^{+++}	0.94±0.37^{+++}	1.67±0.45^{++}	1.21±0.56^{+++}

Values are expressed as mean ± SEM of 6 animals,

*** P<0.001 vs. Normal control group, $^{++}$ P<0.01, $^{+++}$ P<0.001 vs. Depression control group

symptoms of depression by exposing animals to diverse kind of stress every day[25]. Hence, we selected it to explore the antidepressant-like effects of Chrysin. In the present study, we assessed the antidepressant-like effect of Chrysin and also investigated the possible mechanism. Our results revealed that mice exposed to CUMS for 28 consecutive days, demonstrated a remarkable decrease in the sucrose intake, locomotion, and increase in immobility time, which are regarded as induction of depressive like behavior in mice. In the present study, CUMS resulted in depression-like behavior associated with inflammation as assessed by increased levels of TNF-α, IL-1β, IL-6 and COX-2. CUMS animals also exhibited oxidative stress as observed by increased levels of MDA and decreased levels of GSH with concurrent decrease in serotonin, nor-epinephrine levels.

The FST and TST are well established screening paradigms, most frequently used to determine depression/anti-depressant-like behaviour in rodents after exposure to various stressors. The data of this investigation showed that mice subjected to chronic stress exhibited increased duration of immobility in FST and TST. This is in agreement with previous reports that rodents exposed to chronic stress exhibited increase duration of immobility in FST[26]. Studies reported that reduction in the immobility duration in TST is an indicative of anti-depressant-like effect[27]. Administration of the Chrysin significantly reversed the increase in duration of immobility in stressed mice, indicating the anti-depressant-like action. Our results are in agreement with previous results that treatment with Quercetin and other phytochemicals like curcumin, piperine, L-menthone significantly reduced the duration of immobility in stressed mice[28–30]. Locomotor activity is assessed to distinguish antidepressants from psychostimulants to prevent false positive results[31]. The present data on locomotor count is in agreement with previous findings that CUMS subjected mice showed decrease in locomotor count as compared to normal control mice[32]. In our study, CUMS mice exhibited decreased locomotor count which indicated reduced exploration and apathy. Treatment with Chrysin and Imipramine showed significant ameliorative effect on locomotor count in CUMS mice. Decreased sucrose preference, a form of anhedonia, is considered as an indicator for depressive-like behaviour[11]. The Sucrose preference decreases over weeks of CUMS exposure but can be restored to normal levels by chronic treatment with antidepressant drugs[33]. In the present study, we found that treatment with Chrysin and Imipramine significantly restored sucrose preference index and effectively attenuated the depressive-like behavior of CUMS-exposed mice.

Clinical depression and animal models of depression are accompanied by oxidative and nitrosative stress, which is evidenced by increased plasma levels of peroxides, lipid peroxidation indicated by increased levels of malondialdehyde and xanthine oxidase[34]. Increased lipid peroxidation (LPO) and decreased antioxidant enzyme levels in depressed patients are reported and preclinical studies suggested that antioxidants have antidepressant properties[35-38]. The increased level of LPO observed in depressed (control) mice, indicating an excessive formation of free radicals. Earlier studies showed that, malondialdehyde (MDA) a bio transformed output of lipid peroxidation was found in greater amount in biomatrix of depressive patients as compared to control subjects[39]. Studies evidenced that flavonoids like hesperidin, apigenin, quercetin reduced the lipid peroxidation and improved GSH activities in the brain of stressed mice[40]. Treatment with Chrysin significantly reversed CUMS induced increase in the brain lipid peroxide levels by restoring GSH levels.

Myeloperoxidase is an enzyme produced by activated leukocytes during the innate immune response which catalyzes the formation of reactive oxygen species. It is a surrogate marker of inflammation and pro-oxidative processes in patients with depression[41], increased mRNA expression of the MPO gene was observed in patients with depression[42]. Pro-inflammatory markers increased in depressed patients[43]. In fact depression like behaviors can be induced in the laboratory animals by administration of interferon (IFN)-α, a powerful inflammatory cytokine, shown to produce depression like symptoms in patients treated for hepatitis C[43,44]. The increased oxidative stress in depressive states leads to increased expression of NF-kβ which leads to increased levels of various pro-inflammatory cytokines and neuronal inflammation followed by neuronal death[43]. Cytokines and other pro-inflammatory mediators, including IL-6, IL-1β, and TNF-α, interact with every pathologic process involved in mood regulation[9].

Elevated pro-inflammatory cytokines was reportedasabiomarkerofdepression[45]. Three pro-inflammatory cytokines, IL-6, TNF-α and IL-1β are implicated in the pathophysiology of depressive illness[46]. In the present study, the results showed higher levels of TNF-α, IL-1β and IL-6 in depression control mice. Imipramine markedly reversed depression induced increase in TNF-α, IL-1β and IL-6 levels in the brain of CUMS mice. Treatment with Chrysin effectively reversed the elevated levels of TNF-α,IL-1β and IL-6. Cyclooxygenase-2 (COX-2) is the key enzyme for the production of a series of inflammatory cytokines. COX-2 and its downstream inflammatory product PGs are significantly increased in this process to trigger an inflammatory cascade reaction[48]. It was reported that apigenin, a flavonoid significantly reduced the MPO, TNF-α, IL-1β, IL-6 and COX-2 levels in brains of CUMS rats[49]. Our results demonstrated that Chrysin reversed CUMS induced increase in TNF-α, IL-1β, IL-6 and COX-2 levels, indicating anti-inflammatory activity of Chrysin.

It was reported that the decrease of 5-HT, DA and NE levels is a key pathological sign of depression onset[50]. In the present study, neurotransmitters 5-HT and nor-epinephrine were assessed in brain of mice and the level of 5-HT and NE was decreased in CUMS exposed mice. In the present study, we didn't measure the expression of SERT. However, 5-HT levels were decreased with concomitant increase of TNF-α, IL-6 in CUMS mice. Chronic stress exposure reduced the amounts of monoamine neurotransmitters 5-HT and NE in the brains of animals exposed to CUMS[29,51]. This study is in line with the previous studies, where the levels of monoamine neurotransmitter levels were up regulated by the application of antidepressant drugs[52].

5. Conclusion

CUMS resulted in depression-like behavior due to inflammation induced decreased serotonin and norepinephrine in the brain of mice. Chrysin produced significant anti-depressant like activity in comparison with Imipramine, which is a standard drug used in the treatment of depression. Chrysin showed similar or equivalent effect with that of Imipramine in both behavioural and neurochemical estimations. The activity of Chrysin is by reversing the inflammation and oxidative stress restored serotonergic and nor-adrenergic neurons and thus serotonin and nor-adrenaline levels. This can be due to amelioration of inflammation and oxidative stress thereby preventing the injury to serotonergic and noradrenergic neurons, which may restore serotonin and nor-epinephrine levels.

6. Conflicts of Interest

The authors declare that they have no conflicts of interest.

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