

Chronic Polyherbal Antidepressant Treatment Down-Regulates COMT, MAO-A and MAO-B Gene Expression in the Frontal Cortex, Hippocampus and Hypothalamus of Rat

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Running title- Anti-depressant activity of a polyherbal formulation and mRNA expression of catabolic gene

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Abstract

Polyherbal formulation (PF) is an Indian herbal decoction which contains *Nyctanthes arbortristis*, *Hippophae salicifolia*, *Ocimum tenuiflorum* and *Reinwardtia indica*. PF has been used to treat stress related psychiatric disease with the symptoms of depression and forgetfulness in ancient India until today. However, the mechanism of its antidepressant action is still unknown. Here, the force swims stress-(FSS-) induced depressive rats were applied in exploring the action mechanisms of PF treatment. Daily oral and intra-peritoneal administration of PF for 28 days significantly alleviated the FSS-induced depressive symptoms. In addition, the expressions of those molecular bio-markers relating to depression in rat brains were altered by the treatment of PF. This PF regulated the mRNA level of MAO-A, MAO-B and COMT. The results suggested that the anti-depressant-like action of PF might be mediated by an increased of neurotransmitters and decreased the expression of MAO-A, MAO-B and COMT in the various areas of the brain including the frontal cortex, hippocampus and hypothalamus. In addition, the statistically significant ($P < 0.05$) down regulation of MAO-A, MAO-B and COMT mRNA levels by PF was obtained at a dose of 200 mg/kg in the frontal cortex, hippocampus and hypothalamus of FSS-exposed rats. Thus, PF could serve as alternative medicine for depressive patients.

Keywords: Depression; Antidepressant; *Nyctanthes arbortristis*; *Hippophae salicifolia*; *Ocimum tenuiflorum*; *Reinwardtia indica*; Forced Swimming Stress; MAO-A, MAO-B; COMT

Introduction

Currently, most of individuals are suffering from a depression, an emotional disease that is characterized by low mood, body discomfort, loss of interest or pleasure, feelings of guilt or low self-worth, appetite, low energy, poor concentration and sleep disorders [1]. In clinical expression, depression affects patients' daily life and social function [2]. According World Health Organization (WHO) depression will be the second highest psychiatric disease to threaten human's health and increase the economic burden by 2020 [3]. So, depression is a most common psychiatric disorder having an incidence more than 15% and women are more affected (25%) by depression [4].

A number of antidepressant drug such as- sertraline, imipramine, dothiepin and clomipramine etc., are used for the treatment of depression [5]. These drugs are targeted to restoring the decreased level of the neurotransmitter in the brain of depressive patients. However, only restricted patients are responding to these drugs, but drugs are associated with possible side effects [6]. Therefore, these drugs may achieve their therapeutic effects by the normalization of the abnormal dispersion of biogenic amine in the brains' region, including

frontal cortex, hippocampus and hypothalamus [7-8]. On the other hand, serial adaptive process of brain cells to the repeated administrations of antidepressant drugs improved the level of neurotransmitter [8].

Traditional Indian medicine has offered a possible psychotherapy for the treatment of depression. In ancient medicinal books and literature have reported that a number of single and polyherbal formulations (PF) are used for treating neuropsychiatric disorders [9]. PF generally provide synergistic effect and also minimize the unfavorable effects of other drugs [10]. This study has been first time reported with these herbal combination-*Nyctanthes arbortristis* (*N. arbortristis*), *Hippophae salicifolia* (*H. salicifolia*), *Ocimum tenuiflorum* (*O. tenuiflorum*) and *Reinwardtia indica* (*R. indica*) used to treat the diseases having symptoms of depression. Because of *N. arbor-tristis* possess serotonergic properties and its leaves have numerous bioactive chemicals, including oleanolic acid, nyctanthic acid and iridoid glycosides (arborsides A, B, C) with potential health benefits [11,12]. *H. salicifolia* is a nutrient effluent plant, having anti-oxidant properties and anti-inflammatory due to the presence of cerebroside, flavonoid, folic acid, 1-O-hexadecanolenin and quercetin [13].

O. tenuiflorum possesses cholinergic properties and contains eugenol, which has reported acetylcholinesterase inhibitory activity [14,15]. *R. indica* contains terpenoids, glycosides and saponins, which could potentially help in the management of hyperglycemia [16]. Although these herbs have been used frequently, but the mechanism of this PF for anti-depression is still unknown, which therefore hinders the further clinical application of this herbal formula.

The four individual herbs of PF are known to affect our nervous system and frequently applied in the treatment of anti-depression. The pharmacological effects of bioactive molecules deriving from these four herbs for anti-depression have been reported. Oleanolic and nyctanthic acid from the leaves of *N.arbortristis*, could be interacting with 5-HT_{1A}, act on CNS improvement and decline the depressed mood [11,17]. From the seed & fruit of *H.salicifolia*, folic acid, flavonoids and omega-7 normalize the level of homocysteine through folic acid receptor-mediated diagnosis (FRD); control the neuronal aging from the oxidative damage and act as anti-inflammatory effect [18-19]. This PF may also act as COX-2 inhibitor and an acetyl cholinesterase inhibitor due to the presences of alpha-terpinene & eugenol from *O.tenuiflorum* [14,20], increases the level of neurotransmitters and may regulated the synaptic signal in the brain. The total saponins, terpenoids and glycosides derived from root of *R. indica*, are the main chemicals for the neuro-protection and anti-coagulation in brain [16,21]. However, PF had positive effect to depression; we could come to the next step to analyze the mechanism.

It is assumed that synchronized expression of genes involved in the biosynthesis and the function of neurotransmitters may underlie the molecular basis of the therapeutic effects of chronic antidepressant treatment. Chronic treatment of antidepressant drugs can lead to alter the expression of genes, involved in the synthesis of neurotransmitter like tyrosine hydroxylase [22], serotonin receptors [23], aromatic L-amino

acid decarboxylase [24] and dopamine receptors [25]. The catabolic enzymes are involved in keeping the dynamic balance of neurotransmitters in the brains' areas including the frontal cortex, hippocampus and hypothalamus. Several neurotransmitters, including serotonin (5-HT), norepinephrine (NE) and dopamine (DA) in the brain are degraded by the catabolic enzyme such as monoamine oxidase (MAO) and catechol O-methyltransferase (COMT) through the oxidative deamination [26].

MAO (EC 1.4.3.4) is a mitochondrial outer membrane-bound flavo enzyme and plays a critical role in the regulation of normal central nervous system activity (CNS). The two subtypes of MAO, termed MAOA and MAOB, share roughly 70% amino acid identity, according to the deduced amino acid sequences from various species whereas these enzymes have unique substrate & inhibitor specificities [8]. Both are encoded by two separate but related genes at the same chromosome region Xp11.2-11.4 [27]. COMT gene is located at chromosome 22q11, catalyzes the transfer of a methyl group to the catecholamine neurotransmitters such as dopamine, nor epinephrine. It has two isoforms, soluble and membrane-bound, which are generated by alternative splicing from a single copy [28].

As we know currently, humans often experience a variety of stressful and unpleasant conditions; leading to chronic stress affects behaviors and cognitive function, and predisposes an individual to anxiety, anhedonia and depression [29]. These impairments become more affected in the expression of genes in the brain and antidepressant treatment [30].

Therefore, both serve as a promising candidate in translational studies of antidepressant action. Still, there are limited literatures, reporting the effect of chronic PF as antidepressant treatment on the catabolic enzymes and expression of gene. However, none of literatures examining the influences of chronic PF antidepressant treatment on the catabolic enzymes of neurotransmitters. So, here, we were applying forced swim stress (FSS) induced depressive rat models to search the action of PF as anti-depression and quantified the mRNA levels of the MAOA, MAOB and COMT in the various brain tissues (frontal cortex, hippocampus and hypothalamus) of rats, by using reverse-transcription coupled with real-time quantitative PCR, which is a sensitive and accurate method with a large dynamic range to quantify the gene expression.

Materials and Methods

Chemicals

Sertraline hydrochloride (SER), a SSRI antidepressant, was gifted from Pharmaceutical Company, Badi, Punjab. Trizol (Cat. No- 15596026 from Invitrogen), Primers set (Table-1) were purchase from Imperial life science. cDNA synthesis kit and Syber green mixture ABI 200rxn qPCR Kit were purchased from Applied Biosystems (Foster City, CA, USA). Diethylpyrocarbonate (DEPC), Agrose (500g), 10M dNTP mix 100µl with Mg⁺⁺ buffer, Taq brazilian origin (500U) and Tri

buffer (TAB) 500g were purchase from Invitrogen. Ribo lock RNase Inhibitor (Applied Biosystems) (40U/uL) (Cat.No.E00381). Other chemicals like Isopropanol, chloroform and ethanol used were of analytical grade from standard companies.

Collection, identification & hydro alcoholic extraction of PF

The plants were collected from Indian forests, except fruits & leaves of *H. salicifolia* were collected from Himachal Pradesh (Leh & Laddakh). The voucher specimens of these plants have been deposited as like Accession No.: SH-2010, LH-2008, SH-2008 & SH-2008 in the herbarium for further reference, respectively. The leaves (*N. Arbortristis*), fruits & seeds (*H. salicifolia*), whole plant (*O. tenuiflorum*) and roots (*R. indica*) were identified by Professor N. K. Dubey (Department of Botany, Faculty of Science, Banaras Hindu University, and Varanasi, India).

The shade dried plants parts were used for the preparation of hydro alcoholic extraction. Powdered plant materials (500-700g) were extracted with 70% ethanol by a cold extraction process. The extract was then concentrated in-vacuo and the yield percent were calculated as like-*N. arbortristis* (0.91-2.11%), *O.tenuiflorum* (0.84-0.98%), *H.slacifolia*(1.22-1.27) and *R.indica* (0.98-2.03%) and all extracts were stored at 4 °C until use.

In vivo study

Healthy albino Wistar rats (AW) weighing 150-200gm were used at the beginning of experiments. The animals were housed in a temperature (23 ± 2°C) and (30–70%) humidity-controlled environment with a 12-h light/dark cycle. Rats had free access to food and water. All experimental procedures were approved by the Ethical Committee on Animal Experiments of the Institute of Medical Science, Banaras Hindu University (reference number; ECR/526/Inst/UP/2014Dt. 31.1.14). The experiments were carried out between 10.00 AM to 5.00 PM and rats were allowed to adapt to the laboratory circumstances for 7 days prior to dosing.

Forces swim stress (FSS)

The rats (n=36) were randomly assigned to six groups of six individuals: control, FSS plus vehicle, FSS plus SER (10 mg/kg), FSS plus PF (200 mg/kg), FSS plus PF (400 mg/kg) and FSS plus PF (800 mg/kg).The control animals were given with 0.5% carboxyl methyl cellulose (CMC). For another five groups, the animals were treated simultaneously with force swim stress (FSS). PF and SER were orally and intra peritoneal respectively, administrated daily at 30 min before the stress test for 28 days treatment. Doses were calculated as mg/kg of base and determined as described previously (Watanabe et al., 2002), and all drugs were dissolved in 0.5% CMC. Both the antidepressant drug solutions were freshly prepared each morning.

Forced swimming was used to induce stress in rats and has shown the alterations in gene expression [30].This method was similar to forces swim test [31-32]. All rats, except control were subjected to a swimming test, 30 min after the last treatment. Rats were submitted to 5 min forced swim sessions on days 28 between 11.00AM to 12.00AM. Rats were sacrificed by cervical dislocation at 29th days. Immediately, the whole brain of each rat was removed and chilled in an ice-cold saline solution. Dissected various brain areas (frontal cerebral cortex, hippocampus and hypothalamus) on cold plate were immediately stored in liquid nitrogen at -80°C until assay.

Total RNA preparation

Total RNA from brain tissues (frontal cortex, hippocampus and hypothalamus) was isolated with TRIZOL reagent according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA) and RNA dissolved in DEPC water.

DNase digestion

Aliquots of total RNA were digested with RNase-Free DNase (Invitrogen) to remove trace contaminated genomic DNA according to the protocol from the manufacturer. RNA concentration was determined using a spectrophotometer, the stock concentration of RNA ranged from 2lg/ll. The RNA purity was measured by determining the optical density (OD) of the RNA extracts at 260 nm and 280 nm.

RNA quality assurance

The integrity of 28S and 18S rRNA was checked by electrophoresis of 2 µg total RNA in 1.5% agarose gel and in a running buffer containing TAB.

Reverse transcription

Reverse transcription was performed using applied bio system cDNA synthesis kit. A 20µl reaction mixtures containing 2 µg total RNA and 2 µl (50 IM) random hexamers (Applied Biosystems), 10µl reaction buffer, 25x dNTP 0.8 µl, multiscribe RT 1 µl and RNase Inhibitor (Applied Biosystems) 1 µl and volume makeup of Millipore water 3.2 µl were mixed well. The mixtures were incubated at 25 °C for 10 min, 37 °C for 60min, 37°C for 60 min, 85 °C for 5min and de-naturation held on 4 °C.

Complementary DNA (cDNA) conformation

To ensure the cDNA synthesized 1µl total cDNA were subjected to PCR amplification in a volume of 25µl containing sense and antisense primers (5'TGTCACCAACTGGGACGATA3') (5'GGGGTGTGAAGGTCTCAA3'), dNTP (0.5µl), 10X PCR buffer (2.5µl), 1.5 mM MgCl₂, 0.1% vol/vol (0.75µl) and 1.25µl Taq polymerase. PCR conditions were as follows: initial denaturation at 95°C 5 min, followed by 30 cycles of 94 °C 1 min, 60°C 1 min, and 72°C 1 min. The presence of a 150 bp PCR product of rat beta actine gene indicates presence of cDNA.

Real-time quantitative PCR conditions

Real-time quantitative PCR was performed using ABI Sequence Detection System in combination with continuous SYBR Green detection (Applied Biosystems). Real-time PCR was performed in a 10 µl reaction volume containing 1µl cDNA, 5µl SYBR Green PCR Master Mix, 1µl each of sense and antisense primers (10 IM), and 3.5µl DEPC water. The general PCR condition profile was as follows: polymerase activation at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s. After amplification, a melting curve was acquired to determine the optimal PCR conditions by heating the PCR products at 20 °C/s to 95 °C, then cooling at 20 °C to 60 °C. The characteristics of the primers for these genes are given in Table-1. Following each run a melt curve was obtained to confirm the amplification specificity and the absence of primer dimers. Each sample was run at the plate as a single reaction and a standard curve were generated on each plate.

Quantification and normalization

Comparative curve method was used for the quantification of the mRNA levels (ABI PRISM Sequence Detection System).

Table 1: Set of Primer sequences with ascension number, PCR products size and annealing temperature for each gene used in real-time quantitative PCR.

Genes	GenBank		Primer Sequences (5'-3')	Size (bps)	Tm (°C)
Beta Actine	NM_031144.3	Sense antisense	TGTCACCAACTGGGACGATA GGGGTGTGAAGGTCTCAAA	165	50
Mao-A	NM_033653.1	Sense antisense	GCCAGGAACGGAAATTTGTA TCTCAGGTGGAAGCTCTGGT	145	50
Mao-B	NM_013198.1	Sense antisense	TGGGAAGATTCCAGAGGATG GCTGACAAGATGGTGGTCAA	231	50
COMT	NM_012531.2	Sense antisense	ATCTTCACGGGGTTTCAGTG GAGCTGCTGGGGACAGTGAG	150	50

Results

Effect of PF and SER on MAO- A mRNA levels in the frontal cortex, hippocampus and hypothalamus

Figure 1 shows the effect of PF and SER treatment on MAO-A mRNA levels in the frontal cortex, hippocampus and hypothalamus of FSS-exposed rats. FSS significantly increased MAO-A mRNA levels in the frontal cortex (fig-1A), hippocampus (1B) and hypothalamus (fig-1C) of rats as compared to the controls. We observed that MAO-A mRNA was highly expressed in the hippocampus region of the brain as compared to the frontal cortex and hypothalamus, after the exposure of FSS. PF treatment at daily doses of 200,400 and 800 mg/kg significantly decreased MAO-A mRNA levels in the frontal cortex, hippocampus and hypothalamus of FSS-exposed rats, as compared to the vehicle controls. SER treatment (10 mg/kg) also significantly decreased the level of MAO-A mRNA.

For each unknown sample the comparative amount was calculated using linear regression analysis from their respective curves.

The expression levels of MAOA, MAOB and COMT mRNA were normalized by the housekeeping genes i.e., Beta actine mRNA. All experiments were performed in triplet.

Statistical analysis

Data were expressed as the Mean±SD. Differences of gene expression among multiple group comparisons were first assessed by one-way analysis of variance (ANOVA) followed by Dunnet post-hoc comparison test with control animals.

Significant differences were defined as those with a p value smaller than 0.05. All the calculations were implemented using Statistical Package for the Social Sciences (SPSS) for windows version 16.0.

The maximal down regulation of MAO- A mRNA levels by PF was obtained at a dose of 200 mg/kg in the frontal cortex, hippocampus and hypothalamus of FSS-exposed rats.

Effect of PF and SER on MAO- B mRNA levels in the frontal cortex, hippocampus and hypothalamus

MAO-B mRNA expression in the frontal cortex and hypothalamus of FSS⁺ vehicle increased significantly in comparison with that in the control group with statistically significant differences (P < 0.05), while MAO-B mRNA expression in hippocampus of FSS⁺ vehicle with increased highly significant differences (P< 0.001). These results indicated that PF and SER decreased the expression of MAO-B in the frontal cortex, hippocampus and hypothalamus tissue (Figure 2A-C).

The results showed that PF and SER were down regulated MAO-B mRNA expression in hippocampus with statistically highly significant differences (p < 0.001) when compared to

the frontal cortex and hypothalamus region of the brain. This result suggested that after 28 days treatment at doses of 200mg/kg PF highly down-regulate MAO-B expression in the stressed rats and PF can improve this situation.

Effect of PF and SER on Catechol-O-methyltransferase (COMT) mRNA levels in the frontal cortex, hippocampus and hypothalamus

The level of mRNA encoding COMT was markedly expressed in the various regions of the brain-Frontal cortex and hypothalamus in FSS plus vehicle group while COMT mRNA, with statistically significant differences ($P < 0.001$) expressed in the hippocampus area of brain in the same group. SER could decreased the mRNA levels of COMT with statistically significant difference ($P < 0.05$) in the frontal cortex and hippocampus while in hypothalamus only reduced gene expression of COMT in the stressed rats when compared with control. As like this, the PF treatment in these dosages -200mg/kg, 400mg/kg and 800mg/kg were markedly down regulated the mRNA levels of COMT in the frontal cortex and hypothalamus region, whereas PF was reduced with high significant difference ($p < 0.001$) in the hippocampus tissue of stressed rats (Figure 3 A,B,C). This result recommended that PF plays an important in down regulation of COMT mRNA expression in all regions of brain and PF treatment with 200mg/kg dose can excellently recovered the condition via maximum down regulated the expression of COMT.

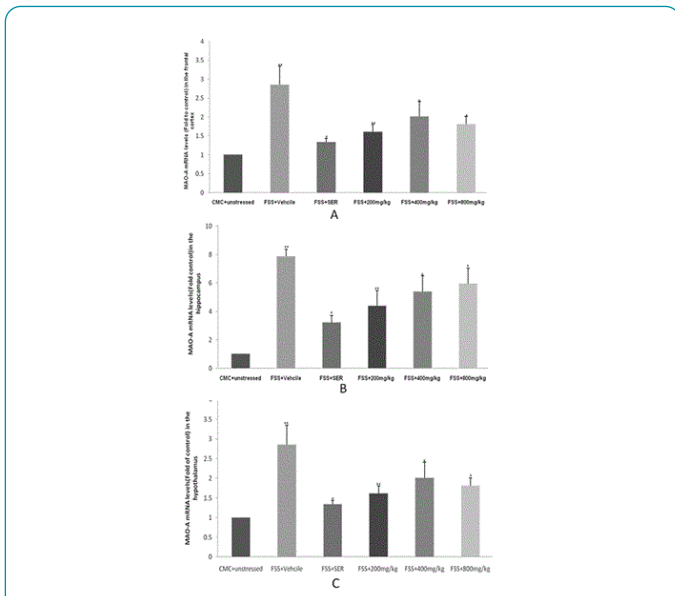


Figure 1: The effect of PF decreases the mRNA expression of MAO-A in frontal cortex (A) hippocampus (B) and hypothalamus (C) in the following groups (= 6 per group): PF, control, and model. ** < 0.01, as compared with control group.

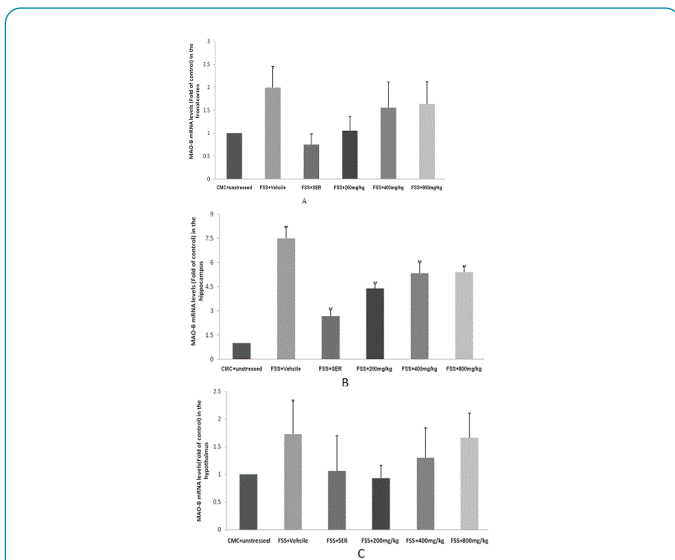


Figure 2: The effect of PF decreases the mRNA expression of MAO-B in frontal cortex (A) hippocampus (B) and hypothalamus (C) in the following groups (= 6 per group): PF, control, and model. ** < 0.01, as compared with control group.

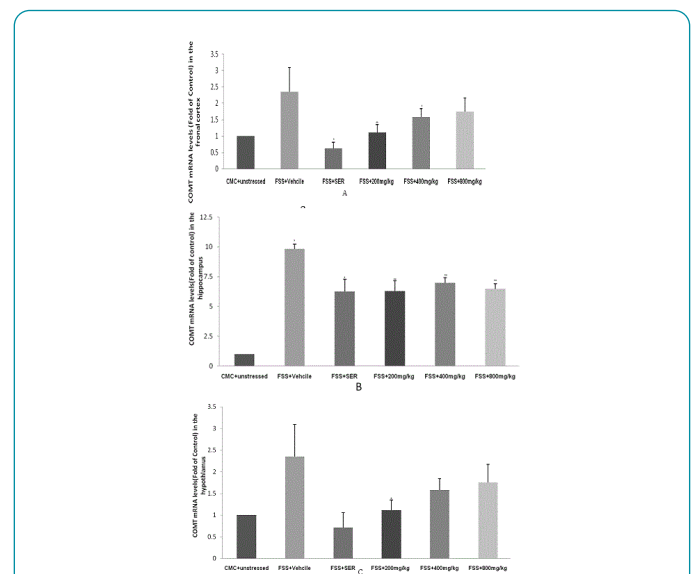


Figure 3: The effect of PF decreases the mRNA expression of COMT in frontal cortex (A) hippocampus (B) and hypothalamus (C) in the following groups (= 6 per group): PF, control, and model. ** < 0.01, as compared with control group.

Discussion

The present study aims to use FSS or FST with isolation to ascertain depression model and exhibit specific characteristic stress features such as behavioral reflecting, immobility, sadness, anxiety, disturbances in, or components of, higher-order behavioral dimensions which are changeable and unpredictable. These are closely related to the mechanism of occurrence and development of depression in human and also

affected the genetic and neuro-biochemical patterns [30,31-33].

In this study, after 28 days of induction of stress, in vehicle group, rats exhibit all symptoms of depression or explore interests reduced; at the same time as PF group rats could reverse this change and indicated this depression model was reliable. Here, we provided evidence to support the anti-depression role of PF in FSS rat model system. The intake of PF in the FSS-treated rats could result that the level of mRNA of MAO-A, MAO-B and COMT were decreased and under this scenario, the regulation of these catabolic factors could lead to a result of the improved behavior of those FSS-treated rats. However, the molecular targets of PF in the various regions of brains -frontal cortex, hippocampus and hypothalamus have not been revealed, in particular this herbal combination is containing the numerical amount of bioactive chemicals.

Compared to the SER group, SER (Selective serotonin reuptake inhibitor) is one of the first-generation tricyclic anti-depression drugs. PF showed similar effects and implied that it might exert anti-depression actions by modulating serotonergic, dopaminergic and nor-adrenalinergic neuronal systems. In order to explore the mechanism of the PF antidepressant drug in the neurotransmitter regulation, the mRNA expression levels of related proteins were evaluated. As we know that numerous neurotransmitter exert in the brain for the action of synaptic signal transduction and has a different pathway of own synthesis, including- serotonin has a different pathway whereas dopamine and nor epinephrine, they share the same biosynthesis pathway. In the treatment of PF in those FSS-treated rats, the enzymes responding for the synthesis of neurotransmitters and it's the brain receptors were increased. Based on the results, PF might regulate the neurotransmitter systems by acting on synthesis, storage, up regulating the receptors and restore the catabolic enzyme.

As available report these catabolic genes- MAO-A, MAO-B and COMT involved in the development of depression and degradation of biogenic amine neurotransmitters in the brain [8]. Liao et al., and Pivac et al., [34-35] has supported our finding - chronic stress exposure caused the higher level of MAO-A, MAO-B and COMT in the brains of rat and giving anti-depression drug treatment could decrease them. Although, low mRNA expression of MAO-A and MAO-B predicts higher self-reported happiness and control symptoms of psychiatric disorder [36]. Other studies demonstrated that associations between the expression of COMT and depression disorder as well as antidepressant treatment responses and down regulation of COMT gene improved the depression condition [35,37]. However, SER could up regulate the levels of neurotransmitters and down regulate its catabolic enzyme as like this mechanism, PF were followed the slightly different action of mechanism for its antidepressant properties and normalized the level of mRNA of MAO-A, MAO-B and COMT.

Based on our current study, PF is containing several numerical amount of bioactive chemical which might have multi-targets in the various areas of brain frontal cortex, hippocampus and hypothalamus for anti-depression. These findings are in line with previous studies. Thus, PF could be a

valuable herbal formula for the treatment of antidepressant, either as a form of health food supplement. More importantly, this valuable herbal formula has no any toxicity effect of chronic intake [38].

Conclusion

The treatment of PF could alleviate the depression-like symptoms in FSS-induced rats. The anti-depressive action of PF might be accounted by modulating the neurotransmitter system and altering the expression of catabolic factors in the brain. This herbal formula is being chemically standardized. PF with 200 mg/kg treatment have shown the significant improvement and altered the expression of catabolic gene. Therefore, PF could serve as alternative medicine and health food supplement for depressive patients. Since PF is a combination of several active biomolecules, the identification of active ingredients here will be further evaluated and in response to PF suggesting that treatment × gene interactions may improve cell function profiles.

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Conflict of Interest

There is no any conflict of interest between authors.

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