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Research Article

"UP REGULATING MRNA EXPRESSION OF CLOCK AND MTHFR GENE AND DECREASED LEVEL OF PLASMA HOMOCYSTEINE INVOLVED IN THE ANTIDEPRESSANT EFFECT OF POLYHEBRAL FORMULATION IN A RAT FST MODEL"

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ABSTRACT

Polyherbal formulation (PF) is an Indian herbal medicine containing four herbal drugs - Nyctanthes arbortristis (65 mg/kg), Hippophae salicifolia(50 mg/kg), Ocimum tenuiflorum (45 mg/kg) and Reinwardtia indica (40 mg/kg) . Previous pharmacological studies have shown that four herbs in PH have antagonistic effects on mRNA level of COMT and MAO A & B . Furthermore, oleanceae, elaeagnaceae, lamiaceae and linaceae from the four herbs in PF may provide protective effect in depression. Highly expressed mRNA level of CLOCK& MTHFR gene, leads to decreased level of plasma homocysteine in an individual,thus increasing receptor concentration of neurotransmitter in neurons. However, antidepressant effect has not been reported before and its mechanism has not been fully clarified. The present study aims to investigate the antidepressant potential of ethanol extract of PF and its effect on mRNA level of CLOCK& MTHFR gene. Albino rats models of depression including forced swim test (FST) and tail suspension test (TST) were used to evaluate the effects of the ethanol extract of PF. A possible mechanism was explored in the tests of antagonism of COMT and MAO-A & B in rats in the previous study. The force swim stress-(FSS-) induced depressive rats were applied in exploring the mechanisms of PF treatment as an antidepressant. Daily oral administration of PF for 28 days significantly alleviated the FSS-induced depressive symptoms. In addition, effect of PF on mRNA level of CLOCK& MTHFR gene were determined by using RT-PCR in the frontal, hippocampus and hypothalamus brain region of rats and also investigated the level of hcy by ELISA, the expressions of those molecular bio-markers relating to depression in rat brains were altered by the treatment of PF. These PF-regulated the mRNA levels of Clock and MTHFR and maintained the level of plasma homocysteine (hcy). In previous study, oral administration of the ethanol extract of PF (200, 400, 800mg/kg) or Sertraline (10mg/kg) significantly reduced the duration of immobility in FST and TST. However, the effect was dose-dependent. The results suggested that the anti-depressant- action of PF might be mediated by an increase in level of mRNA of Clock and MTHFR in different areas of rat's brain and decreases the level of plasma hcy in blood. The rats treated with ethanol extract of PF (200, 400, 800mg/kg) or Sertraline (10mg/kg), which acted agonistic on mRNA level of Clock and MTHFR gene in the frontal cortex, hippocampus and hypothalamus , moreover the maximal up regulation of Clock and MTHFR mRNA levels by PF was obtained at a dose of 200 mg/kg in FSS-exposed rats. Thus, PF could serve as alternative medicine for depressive patients. These results indicate that the ethanol extract of PF produced antidepressant-like effect and the possible mechanism. Being a poly herbal formulation, the observed activity profile may be attributed to one or more bioactive principles present in the components of this formulation. Out of these two preclinical study it is concluded that the PF is effective and safe antidepressant drug.

INTRODUCTION

Depression is the most burdensome diseases of society and associated with miserable symptom and sign. Depressed persons feel sad, anxious, empty, hopelessness, worried worthless, guilty, irritable, restless etc. Depression is clinically characterized by other symptoms that reflect alternation in cognitive, psychomotor, biological, motivational, behavioral and emotional processes. Appetite loss or overeating, attempt to suicide, insomnia or excessive sleeping, fatigue all are significantly involved with depressed patients. Depression mainly occurs due to certain life events, side effect of some drugs, including genetic factors etc. It is associated with significant morbidity, mortality and disability [1,2]. Although, if these clinical symptoms and signs persist for more than two weeks, it may cause real suffering and also interfere with the

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business and pleasure of daily life. In everyday conversation people say they are depressed when they are feeling unhappy, down, blue or sad, hopeless. Therefore, depression affects the quality of life of many people, is a major cause of suicidal death and also considered as a significant risk factor for cardiovascular diseases [3,4]. It is most common mental disorder having a high prevalence rate in women due to additional stresses of work, home responsibilities, caring for children, aging parents, poverty, and abuse relationship strains than men. This rate may be linked to biological life cycle, psychosocial and hormonal factors that a women experience [5].

It is a neurological condition with a complex biological pattern in etiology. Moreover, environmental stress modulate subsequent vulnerability to depression. Earlier adversity seems to induce heightened reactivity to stress through several possible mechanisms. This increased reactivity results in an enhancement of biological stress-response mechanisms. Regulators of this system, particularly signal transduction pathways involving in the regulation of key genes in this system. This system is potentially vulnerable to ROS and indirectly, to the effects of cytokines. Ultimately, some of these effects may be controlled by chemical modification of DNA, specifically, methylation of promoters or other gene regions [6].

On the basis of previous study, it has been found that multiple genetic factors are involved in the development of depression, but number of genes involved are still unknown. This is hard to believe that only one gene can cause depression in such a vast population, But cumulative effect of genes acting together may cause a person to become vulnerable to depression. It has been found that, several candidate genes such as clock, Methylene-TetraHydroFolate Reductase (MTHFR), are involved as monoamine oxidase (MAO) and catechol O-methyltransferase (COMT), as they have suggested repeatedly to be implicated in DD [7,8]. Clock gene directly affect addictive behaviours like locomotion sensitization and reward, based on previous experiments [9] and encode the protein, which plays a central role in the regulation of circadian rhythms. This gene also encodes a transcription factor of helix-loop-[helix (bHLH) family that contains DNA binding histone acetyltransferase activity. Numerous studies have identified hundreds of genes in the human brain that are likely involved in important daily rhythmic events, including the sleep/wake cycle and metabolism. The link between depression and abnormal sleep has been known before. The brain cells is thought to possess a molecular 'clock,' which keeps ticking in multiple brain regions. The genes that are involved in the time-keeping process is known as “clock gene” and the disruption of this process leads to major or clinical depression [10]. Therefore, clock gene is an essential regulator of circadian rhythms [10,11]

Another gene, MTHFR is also an important gene which is associated with number of neuropsychiatric disorders including psychiatric disorders like schizophrenia (SZ), bipolar disorder (BPD) and unipolar depressive disorder (UDD) etc [12]. MTHFR is a crucial enzyme involved with folate-mediated pathway which is one-carbon metabolism and essential requirement for the biosynthesis of purine and thymidylate 48. The methylation of both DNA and amino acids are involved in the synthesis of neurotransmitters and regulation of brain function.

Mutation in the MTHFR gene (down regulation or decrease mRNA expression) reduces several enzymatic activities including decrease in the level of L-methylfolate which declines accessibility of methyl groups, thus elevates homocysteine levels. Low production of L-methylfolate give rise to a lack of monoamine while low availability of methyl group have epigenetic effects on the expression of many genes [13-14]. Reduced methylation associates with aberrant building of cell membranes and has damaging effects on myelin structure, leading to impaired neurotransmission [15].

Hypercysteinemia modulate the activity of N methyl-D-aspartate receptors (NMDAR), that have properties in the long-term potentiality means learning and memory. The action of homocysteine on NMDAR may impair learning and memory and can even be excitotoxic, found in cell death [16].

Therefore, pharmacotherapy of this disorder has evolved over centuries. A number of antidepressant drugs such as - sertraline, imipramine, dothiepin and clomipramine etc are frequently used to treat DD, These drugs target brain region including frontal cortex, hippocampus and hypothalamus and restores the level of the neurotransmitter, [17-18] in depressive patients. However, only restricted patients respond to these drugs; however, it is now clear that a large percentage of patients suffering from DD may not benefit from antidepressant drugs treatment [19].

Numerous evidences from family studies indicate that individual responses to specific antidepressant treatments varies due to genetic variation [20-22]. Pharmacogenetics holds the potential to genetically predict who will and will not benefit from antidepressant drugs [23-25].So, there is an urgent need for researchers to investigate more effective antidepressant therapies without or with less adverse effects.

Indian herbal medicines are most commonly used modalities of alternative medicine therapy. A scientific research has suggested that herbal medicines is promising in managing mild to moderate depression [26-27]. Ancient medicinal books and literature have reported that a number of single and polyherbal formulations (PF) were used to prevent or relieve mental depression among the population and PF have more efficacy than single plant [28]. Because PF generally provide synergistic effect and also minimize the unfavourable effects of other synthetic drugs [29].

N.arbor-tristis have serotonergic properties and its leaves have numerous bioactive chemicals, including oleanolic acid, nyctanthic acid and iridoid glycosides (arborsides A, B, C) which could interact with 5-HT1A, act on CNS to improve and decline the depressed mood [30-32].*H. salicifolia* is a nutrient effluent plant, having anti inflammatory activity, it normalize the level of homocysteine through folic acid receptor-mediated diagnosis (FRD); control the neuronal aging from the oxidative damage due to the presence of cerebroside, flavoind, folic acid, 1-O-hexadecanolenin and qurecetine and omega-7 [33-35].*O. tenuiflorum* act as a COX-2 inhibitor and anacetyl

cholinesterase inhibitor due to the presences of alpha terpinene & eugenol and increases the level of neurotransmitters and may Regulate the synaptic signal in the brain. [36-37]

R. indica contains active biomolcules terpenoids, glycosides and saponins, which could [1] potentially help in the management of hyperglycemia and also involved in neuro-protection and act as a anti-coagulation in brain [38]. However, PF had affirmative effect in the management and treatment of mood disorder like depression. Although these herbs have been used frequently for number of pharmacological effects and has a strong potent psychoneuro pharmacologically active compound and is subjected for antidepressant activity [39-40].

Our pervious studies has reported that the polyherbal formulation consisting of *Nyctanthes arbortristis (N. arbortristis), Hippophae salicifolia (H. salicifolia), Ocimum tenuiflorum (O.tenuiflorum), Reinwardtia indica (R. indica)* are used to treat the mental diseases having symptoms of depression.

Clock and MTHFR gene serve as promising candidates in translational studies of antidepressant action. Still, there are limited literatures on the effect of PF as antidepressant treatment on the expression of gene. However, none of the literatures had examined about the influence of PF antidepressant treatment on the folate metabolism enzyme and clock gene. Therefore, the current study attempts on evaluating this anti-depressant activity of polyherbal formulation on the expression of MTHFR and clock gene in frontal cortex, hippocampus and Hypothalamus regions of brain in force swim rat, quantified the mRNA levels of the MTHFR and clock in 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

In vivo study

The experiments were performed on healthy albino Wistar rats (AW, N=36) (150-180gm (8weeks) of both sex procured from Animal Central House, Institute of Medical Science, Banaras Hindu University, Varanasi, India. The rats were kept in plastic cages with paddy husk as bedding in the animal house with a regulated ambient temperature of 23 ± 2ºC, a relative humidity of 30–70% with a 12 hr/12 hr light dark cycle and had free access of food with water ad libitum. The experiments were carried out between 10.00 am to 5.00 pm and animals were] allowed to acclimatize to the laboratory conditions for 7 days prior to dosing. The water used was always double distilled. The animals were used only once for each experiment.

Collection, identification & hydro alcoholic extraction of PF

PF *Nyctanthes arbor-tristis (N. arbortristis) Ocimum tenuiflorum (O. tenuiflorum), and Reinwardtia indica (R. indica)* were collected from forests of India. Hippophae salicifolia (H. salicifolia) were collected from Himachal Pradesh (Leh & Laddakh). These four plants, belonging to different families were used in this present study. These were identified and authenticated by Dr. R.C. Satish Kumar, M.D.(Ayu) M.B.A (H.M.), Interdisciplinary Institute of Indian System of [21]Medicine (IIISM), Sri Ramaswami Memorial University (SRM) University, Kattankulathur Chennai, India. Voucher specimens of the plants have been deposited (Accession No.: SH-2010, LH-2008, SH-2008 & SH-2008) in the herbarium for further reference. 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 invaccuom 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.

Drugs and Chemicals

Sertraline hydrochloride (SER), a SSRI antidepressant, was gifted from Pharmaceutical Company, Badi, Punjab and was used as standard drug for antidepressant effect. Trizol (Cat. No-15596026 from Invitrogen), Primers set (table-1) were purchased from Imperial life science. cDNA synthesis kit (Applied Biosystems™, Catalog no.4368814- Foster City,CA,USA) and Syber green mixture ABI 200rxn qPCR Kit was 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 purchased from Invitrogen. Ribo lock RNase Inhibitor (Applied Biosystems) (40U/uL) (Cat.No.E00381).

Homocysteine (Cata.No.KA1242) (ELISA Kit from Novous biologicals a biotechne brand Taiwan and performed by Benesphera ELISA microplate Reader E21 and washer W21).

Optimum drug dose determination:- The optimum dose of polyherbal formulation was determined on the basis oral administration of different doses of each ingredient extract for a period of 3 months. The effect of different doses of extract of the plants containing quantified active molecules significantly reduced lipofuscin content (sugars and metals, including mercury, aluminum, iron, copper and zinc) and increased the acetylcholineconcentration in a dose dependent manner. The dose is fixed up considering the level of the test substance which is effective and safe also.

Forced swim stress (FSS)

Forced swim was used to induce stress in rats and has shown the alterations in biogenic homocysteine and gene expression. The method for stress was similar to FST below [41-42]. All the groups of rat were subjected to swimming test except group I. Rats were subjected to FST 1 h after the last treatment. The effects of drug administration were examined on the total duration of immobility of the rats during the FST. Rats were submitted to 5 min forced swim sessions on days 28 between 11.00AM to12.00PM.

Grouping and Drug Administration

The 36 AW rats were equally and randomly assigned in to six groups, namely,

Group I: 0.5% Carboxyl Methyl cellulose (CMC) plus unstressed (CMC+unstressed), Group II: Water plus Stressed (FST + Vehicle), Group III: Sertraline hydrochloride(FST+SER) (10 mg·kg−1, i.p.) plus stressed and

Group VI and IV: PF (polyherbal formulation) plus stressed (200, 400 and 800 mg·kg−1 p.o., respectively) (FST+200mg/kg, FST+400mg/kg,FST+800mg/kg) and drugs were administrated once daily for 28 consecutive days to animals. Both the antidepressant drug solutions were freshly prepared each morning. Rats were administered with SER and PF, respectively, 30 min and 1 h before the test swim session. SER was administered intraperitoneally in a volume equivalent to 2 ml/kg. PF were each given orally by gavage. Doses were calculated as mg/kg of base and determined as described previously and all drug were dissolved in 0.5% CMC [43].

Total RNA preparation

Total RNA from brain tissues (frontal cortex, hippocampus and hypothalamus) was isolated withTRIZOL reagent according to the manufacturer's protocol (Invitrogen Life Technologies, Cartsbad, 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 manufacturer. RNA concentration was determined using 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 were checked by electrophoresis of 2 lg 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 2lg total RNA and 2 µl (50 lM) 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 by Millipore water 3.2 µl were mixed well. The mixtures were incubated at 25 ºC for 10 min, 37 ºC for 60min, 37ºC for 60 min, 85 ºC for 5minand de-naturation held on 4 ºC.

Complimentary DNA (cDNA) conformation

To ensure the cDNA synthesised1 µl total cDNA were subjected to PCR amplification in a volume of 25µl containing 1 lM each of sense (5'TGTCACCAACTGGGACGATA3') (5'GGGGTGTTGAAGGTCTCAAA 3'0), 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 95ºC 5 min, followed by 30 cycles of 94 ºC 1 min,60ºC 1 min, and 72ºC 1min. The presence of a 150 bp PCR products 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 lM), and 3.5µl DEPC water. The general PCR condition profile was as follows: polymerase activation at 95 ºC for 10 min, followed by 40 cycles of denaturing at 95 ºC for 15 s, annealing at 60 ºC for 30 s, and extension at 72 ºC for 60 s. After amplification, a melting curve was acquired to determine the optimal PCR conditions by heating the PCR products at 20 ºC/s to 95 ºC , then cooling at 20 ºC to 60 ºC. 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 dimmers. Each sample was run on the plate as a single reaction and a standard curve was generated on each plate.

Quantification and normalization

Comparative curve method was used for the quantification of the mRNA levels (ABI PRISM Sequence Detection System). For each unknown sample the comparative amount was calculated using linear regression analysis from their respective curves. The expression levels of clock and MTHFR 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 comparisons 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.

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)	T_a (°C)
Beta Actine	NM_031144.3	sense antisense	TGTCACCAACTGGGACGATA GGGGTGTTGAAGGTCTCAAA	165	50
Clock	NM_021856.2	sense antisense	GAACTTGGCGTTGAGGAG TCT GTG ATC GAA CCT TTC CAG TGC	150	52.3
MTHFR	XM_006239414	sense antisense	ACCTGCACAGAGCCAAGC CAGTGGTCACCTACAGGGTCT	73	59.1

RESULT AND DISCUSSION

The effect of PF and Sertraline on the level of homocysteine (Hcy)

The effect of PF and Sertraline on the level of homocysteine (Hcy), Various studies suggest that higher plasma homocysteine (hcy) is a sensitive marker of functional deficiency of either folic acid or vitamin B12. Many types of stress may impair the metabolism of hcy which causes hyperhomocysteinemia (HHcy) that raised the neurotoxic effects in the pathogenesis of depression [44]. Previous reports suggest that stress inhibit synaptic transmission at the neuromuscular junction, targeting primarily the motor nerve terminals. However, Hhcy indicates a failure of methylation of hcy to methionine due to shortage in supply of methyl groups

from methyl folate, leading to depression. Folate deficiency associate with significant fall in SAM and effect the mood [45].

Forced swim stress resulted in significant (p 0.001) increase in Hcy level in rats when compared to vehicle-treated unstressed rat. PF (200,400 and 800 mg/kgbw/day) and Sertraline (10mg/kg) per se administered for 28 consecutive days significantly reduced Hcy level in rat ($p < 0.001$, $p < 0.01$,$p < 0.01$and $p<0.05$ respectively) when compared with those of respective group I. However, the lowest dose (200 mg/kgbw/day) of PF showed significant decrease in Hcy level in rats when compared with their respective control groups (Table.2). Various results have shown that evaluated homocysteine has been linked to depression [46,14]. This result correlated with other studies carried out by Kumari *et al*.,(2016) who have reported that stress suspension rats treated with polyherbal formulation (same herbal mixture), showed significant improvement in Hcy [47].

Table 2 Effects of hydro alcoholic extract of PF at 200,400 and 800 mg/kgbw/day, Sertraline at 10 mg/kgbw/day on level of homocysteine (C) in the rat.

Group	Homocysteine (µ mol/)
I	3.9±0.27
II	9.1±0.29
IV	5.3±0.18
V	5.6±0.22
V	6.4±0.24
VI	6.4±0.13
Group comparison	
Group I vs II	<0.001
Group I vs III	<0.01
Group I vs IV	<0.01
Group I vs V	<0.01
Group I vs VI	<0.05
Group II vs I	<0.001
Group II vs III	<0.01
Group II vs IV	<0.001
Group II vs V	<0.01
Group II vs VI	<0.05

Values are presented as MEAN±SD. for statistical significance *p < 0.05, **p < 0.01, ***p < 0.001; groups were compared with group I and II, p value shown in the table.

Effects of Polyherbal formulation on the expression of genes-Clock and MTHFR

This study aims to use FST in isolation to induce depression in rat model and to exhibit specific stress features such as behavioral reflecting, immobility, sadness, anxiety, disturbances, 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 it also shows affected genetic and neuro-biochemical patterns. After 28 days induction of stress in vehicle group, rats exhibit all symptoms of depression and reduction in the interest level. At the same time, rats treated with PF could reverse this change and signify that this depression model was reliable. Here, we have provided the evidence to support the antidepressant role of PF in FST rat model system. The effect of PF in the FST treated rats showed that the level of mRNA of Clock and MTHFR were increased, the regulation of clock and folate metabolism factors, could lead to the improved behaviour of FST-treated rats. However, the molecular targets of PF in various regions of brain like frontal cortex, hippocampus and hypothalamus have not been revealed. However, these herbal combinations also contain numerous amounts of bioactive chemicals.

Compared to the SER group, SER (Selective serotonin reuptake inhibitor) is one of the first-generation Tricyclic antidepressant drugs. PF showed similar effects and implied that it might exert anti-depression by modulating serotoninergic, dopaminergic and nor-adrenalinergic neuronal systems. In order to explore the mechanism of PF in the regulation of neurotransmitters, the mRNA expression levels of related proteins were evaluated. Numerous neurotransmitters exert their action on the brain by synaptic signal transduction and has different pathways of synthesis, serotonin has different pathway whereas dopamine and nor epinephrine share the same biosynthetic pathway. The enzymes responsible for the synthesis of neurotransmitters and the brain receptors were increased in response to PF in FST model of rats. Based on these results, it can be said that PF may regulate the neurotransmitter system by synthesis, storage, up regulating the receptors and restoring the catabolic enzyme and other gene. Pivac *et al*., (2011) and Liao *et al*.,(2013) has supported our finding - chronic stress exposure caused low level of Clock & MTHFR gene in the brain of depressed rats and antidepressant drug treatment could decrease and increase them respectively,[48-49]. Although up-regulation of mRNA Clock & MTHFR could improve the condition depression [50].

However, SER could upregulate the levels of Clock and MTHFR and decrease plasma hcy level. PF follows slightly different action of mechanism for its antidepressant properties and normalize the level of mRNAof Clock and MTHFR.

Effect of PF and SER on Clock mRNA levels in the frontal cortex, hippocampus and hypothalamus

Repeated administration (for 28 days) of SER and PF significantly increased Clock mRNA level in all the brain areas (frontal cortex, hippocampus and hypothalamus) whereas in Group II rats, low level of Clock mRNA was expressed in various regions of rat's brain (fig- 1). Overall, SER affected the expression of clock gene at frontal cortex, hippocampus and hypothamulus regions of brain. After PF, Clock mRNA was up-regulated with statistically significant differences ($P < 0.05$)in the brain areas including frontal cortex, hippocampus and hypothalamus. This result recommended that PF plays an important role in the up regulation of clock mRNA expression in all the regions of brain and Group IV, treated with PF at 200mg/kgbw/day works excellently in recovering the condition via maximum up regulating the expression of clock gene in frontal cortex and hypothalamus. These results are correlated with other studies carried out by Christiansen *et al*., (2016) who have reported altered expression pattern of m-RNA of Clock genes in the depressed rats [51].

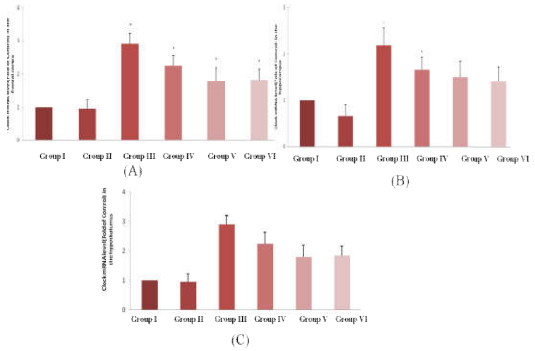

Figure 1 PF up regulate the mRNA expression of Clock gene in different brain regions in the groups A-Frontal Cortex;B-Hippocampus;C-Hypothalumus

Effect of PF and SER on MTHFR mRNA levels in the frontal cortex, hippocampus and hypothalamus

Transcriptional analyses of MTHFR mRNA levels in all the areas of brain showed a more significant down regulation in FSS plus vehicle control of group II than in group I. SER and PF significantly increased MTHFR mRNA level in all the areas of brain (frontal cortex, hippocampus and hypothalamus) (fig-2) MTHFR mRNA was up-regulated with statistically significant differences ($P < 0.001$) in frontal cortex and hypothalamus after the PF and SER treatment and only markedly increases in expression of hippocampal MTHFR mRNA was seen. This result recommends that PF plays an important role in up regulation of MTHFR mRNA expression in all the regions of brain and PF with 200mg/kg dose can excellently help in recovery via maximum up regulated expression of MTHFR gene. Our finding correlated with other studies carried out by Devlin *et al*.,(2010) Rinki *et al*.,(2013) who have reported MTHFR gene is involved in depression [52-53]. Yang *et al*., (2017) have also supported MTHFR gene mRNA expression associated with depression[54].

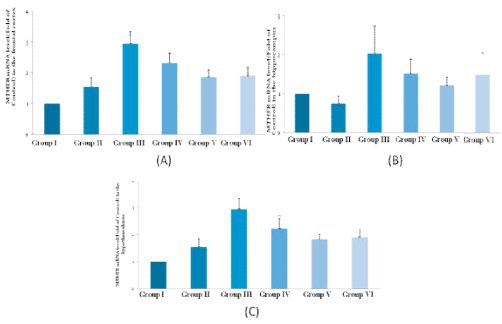

Figure 2 PF up regulate the mRNA expression of MTHFR in different brain regions in the groups A-Frontal Cortex;B-Hippocampus;C-Hypothalumus.

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 healthy food supplement. More importantly, this valuable herbal formula has no toxic effect on long time intake [55].

CONCLUSION

Based on the results obtained out of present study it is concluded that PF has antidepressant activity; its mechanism is supposed to suppress the level of plasma hcy in stressed rat. This decreasing effect of PF restart the re-methylation and increased the concentration of neurotransmitter. Simultaneously, the anti-depressive action of PF, accounted by increasing the expression of Clock and MTHFR gene in the brain. Since, PF is a combination of several active compounds, which has multi-targeted activity, exerted marked beneficial effects on biomarkers associated with depression. PF with 200 mg/kg treatment have shown the significant improvement and altered the expression of clock and MTHFR gene. Thus, it is concluded that PF is a potent antidepressant agents and can be utilized for human consumption. This drug is effective and safe and can be given for longer period to human beings, suffering for depression. Consequently, PF could serve as alternative medicine and health food supplement for depressive patients

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Conflict of Interest

There is no any conflict of interest between authors.

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