Original Article

Effect of *Withania somnifera* on Expression of Selected Genes in Hippocampus of Male Wistar Rats Subjected to Chronic Unpredictable Mild Stress

Abstract

Background: Depression affects millions globally, with existing treatments having many side effects. *Withania somnifera* (WS) shows potential as an antidepressant and neuroprotective agent, possibly by influencing brain-derived neurotrophic factor (BDNF)-related pathways. **Aim:** This study evaluated the effect of WS alone and in combination with fluoxetine on neuritin, NARP, and BDNF Exon-III gene expression in the hippocampus of male Wistar rats subjected to chronic unpredictable mild stress (CUMS). **Materials and Methods:** Thirty male Wistar rats were divided into five groups (n = 6 each): normal group (NG), disease control (DC), standard treatment (ST), WS, and combination group of fluoxetine and WS (FW). Depression was induced using CUMS, except in the NG. The sucrose preference test confirmed depression at the end of 3rd week and assessed treatment effects at the end of 7th week. Gene expression in the hippocampus was analyzed through real-time PCR at the end of 7th week. **Results:** After 7 weeks, the ST, WS, and FW groups showed a significant increase in sucrose preference compared to the DC group. The ST and FW groups showed significant upregulation of all three genes selected in the present study. Comparison between NG and FW groups showed no significant difference in gene expression. **Conclusion:** This study highlights the antidepressant effects of WS by demonstrating its effect on BDNF-associated gene expression. Fluoxetine combined with WS demonstrated additive effects which proves an adjuvant role of WS in the treatment of depression. Further studies involving human subjects are essential to validate the antidepressant effects of WS and its additive effects with fluoxetine.

Keywords: *Brain-derived neurotrophic factor exon-III, depression, fluoxetine, gene expression, NARP, neuritin, Withania somnifera*

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Introduction

Depression is a prevalent mental disorder that affects a significant portion of the global population and approximately 5% of adults worldwide. Around 280 million people around the globe suffer from depression.[1] In India, it is estimated that 57 million people are affected by depression.[2] It is a disorder of major public health importance, in terms of its prevalence, mental suffering, and dysfunction as well as morbidity and economic burden.[3] The current treatment modalities for depression include tricyclic antidepressants, selective serotonin reuptake inhibitors, selective noradrenaline reuptake inhibitors, and atypical antidepressants. These drugs have various adverse effects such as sedation, weight gain, cardiac arrhythmias, gastrointestinal dysfunction, and sexual dysfunction. Discontinuation of antidepressants can cause withdrawal symptoms such as dizziness, nausea, fatigue, anxiety, instability of gait, and insomnia.[4,5]

Ashwagandha, also known scientifically as *Withania somnifera* (WS) (L.), is predominantly grown in various Asian countries such as India, Afghanistan, Baluchistan, and Sind, and also in with regions in the Mediterranean. It has been found to possess antidepressant properties, along with various medicinal benefits such as anxiety relief and antioxidant effects.[6] In addition, it is believed to offer protection against neurodegenerative disorders such as Alzheimer's and Parkinson's disease.[7] The use of WS as a potential adjuvant in depression has been shown in mice as well as rats.[8,9] However, its effect on the production of brain-derived neurotropic factor (BDNF) has not been studied so far.

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BDNF is a crucial element in mediating the effects of antidepressants, acting as a link between the drugs and the neuroplastic changes that alleviate depressive symptoms.[10] It is essential for neuronal maintenance and growth. It plays an important part in synaptic plasticity, which strengthens or weakens over time in response to activity or experience.[11] Reduced levels of BDNF have been linked to an increased occurrence of depressive symptoms.

Fluoxetine, a standard medication used for chronic depression, selectively enhances gene expression in specific brain regions associated with BDNF-induced long-term potentiation (LTP). It also leads to the upregulation of specific genes, namely, NARP, neuritin, and BDNF exon-III, in the brain. These genes are associated with BDNF-LTP and the production of different proteins that aid neuronal growth, maintenance, and plasticity. Upregulation of these genes can help restore BDNF levels, indicating a potential role for BDNF in the pathophysiology and treatment of depression This suggests a link between treatment of depression and the molecular mechanisms underlying synaptic plasticity.[12,13]

To date, no research has tested the effect of WS on the expression of the genes mentioned above. Therefore, this study aimed to evaluate the effect of *Withania Somnifera* on the expression of selected genes in the hippocampus of male Wistar rats subjected to chronic unpredictable mild stress (CUMS).

Materials and Methods

The present study was an interventional study conducted in a medical college for a duration of 7 weeks. Adult healthy male Wistar rats weighing 200 ± 20 g were obtained from the Central Animal House of the Medical College. The rats were acclimatized to 12:12 h light–dark cycle for 7 days before starting of the experiment. They were maintained at constant temperature (22°C–25°C). They were fed standard chow pellets with water *ad libitum*. The animals were housed in groups of six in polypropylene cages.

The following chemicals and drugs were used which were procured from the following agencies:

- The hydro-alcoholic extract of WS root required for this experiment was obtained from Natural Remedies (Bangalore, Karnataka) as a free sample and fluoxetine was purchased from the pharmacy of the hospital attached to the medical college
- RNeasy Mini Kit [Cat No. 74104] – (JJ Biotech, Bengaluru, Karnataka)
- cDNA Kit [Cat No. RR037A]- (Juniper Lifesciences, Bengaluru, Karnataka)
- Sybr green kit [Cat No. RR820A] – (Juniper Lifesciences, Bengaluru, Karnataka)
- Sample Protector for RNA/DNA [Takara – CatLog No. 9750]- (Juniper Lifesciences, Bengaluru, Karnataka)
- Primers for the selected genes-Bioserve Biotechnologies India Pvt Ltd., (Hyderabad, Telangana).

The experiment was conducted according to Committee for Control and Supervision of Experiments on Animals guidelines and was approved by the Institutional Animal Ethics Committee, letter no 17/2 dated June 26, 22.

For induction of CUMS, 30 rats were divided into 5 groups of 6 animals each. All the groups except the normal group (NG) were exposed to CUMS to induce depression for 7 weeks. Dose has been selected as per previous studies.[14,15] The grouping of animals and the doses of drugs administered are detailed in Table 1. The study protocol is detailed in Table 2.

The model for depression was established by administering CUMS for a period of 7 weeks. Eight different stressors stated below were given randomly.[16,17] The experimental schedule for the CUMS procedure is detailed in Table 3.

To prevent habituation and ensure the unpredictability of the stressors, all the stressors were randomly scheduled over a period of 1 week and repeated throughout the 7-week experiment.

During this test, rats were given 1% sucrose solution for 24 h and then both sucrose solution and fresh water were made available to rats for another 24 h. After depriving the rats of drinking for 23 h, the rats were given both 1% sucrose solution and fresh water for 1 h again.[18,19]

Sucrose preference will be calculated as: Sucrose Preference (%) = (Sucrose Intake [ml]/[Sucrose Intake (ml) + Water Intake (ml)]) × 100%.

CUMS induces anhedonia in rats, a state equivalent to clinical depression in humans. Anhedonia is evaluated by measuring the consumption of sucrose water relative to normal water. Typically, healthy rats show a preference for sweetened sucrose water over regular water, while in the state of anhedonia, there is a reduction in sucrose water consumption and an increase in preference for regular water compared to normal rats.

Table 1: Study groups and drugs administered

Group	Treatment	Drug and dose	Route	Duration
Group 1	NG	Distilled water - 0.5 mL	Oral	7 weeks
Group 2	DC	Distilled water - 0.5 mL	Oral	7 weeks
Group 3	ST	Fluoxetine - 5 mg/kg	Oral	Week 3–7
Group 4	Treatment with WS	WS (root extract) - 50 mg/kg	Oral	Week 3–7
Group 5	Treatment with FW	Fluoxetine - 5 mg/kg + WS (root extract) - 50 mg/kg	Oral	Week 3–7

NG: Normal group; DC: Disease control; ST: Standard treatment; WS: *Withania somnifera*; FW: Fluoxetine and WS

Following the sucrose preference test (SPT) at 7th week, animals were euthanized using thiopentone sodium administered intraperitoneally (90–120 mg/kg) followed by decapitation. The entire brain was dissected out of the skull and further dissection for the hippocampus was carried out on a cold plate.[20,21] The isolated hippocampus was immediately immersed in triazole reagent contained in an Eppendorf tube and stored at −80°C for the study of gene expression analysis.

The following parameters were assessed:

- SPT at the end of 3rd week and 7th week
- Gene expression of neuritin, NARP, and BDNF Exon – III genes in the hippocampus at the end of 7th week.

All data were expressed as the mean ± standard error of the mean. The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. P <0.05 was considered statistically significant.[22] Data were analyzed using GraphPad Prism software – version 10.2 (GraphPad software, 3rd order polynomial, San Diego, California, USA).

Table 2: Study protocol

Experimental step	Timeline
CUMS	From week 1 to 3
SPT (screening for the establishment of depression)	Beginning of week 4
CUMS (animals with established depression) + treatment	Week 4–7
SPT	End of week 7
Sacrificing of animals	End of 7th week by euthanasia followed by isolation of brain tissue for gene expression study

CUMS: Chronic unpredictable mild stress; SPT: Sucrose preference test

Results

The results of the study are presented in the form of tables and bar graphs. Table 4 presents the results of the SPT and gene expression analysis in male Wistar rats subjected to CUMS-induced depression and treated with different drugs.

Results of Sucrose preference test at the end of 3rd week

SPT was done at the end of 3rd week.

One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the sucrose preference of the disease control (DC) group, standard treatment (ST) group, WS group, and combination group (Fluoxetine and WS [FW]) with NG.

There was a statistically significant decrease in sucrose preference (P < 0.0001, Tukey's multiple comparisons test) seen in DC group, ST group, WS, and combination (FW) group compared to NG [Table 4 and Graph 1].

Gene expression

Neuritin, NARP, and BDNF Exon- III gene expression levels were measured by real-time PCR at the end of treatment from the hippocampus tissue of depressed rats.

One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the neuritin, NARP, and BDNF Exon- III gene expression levels of ST group, WS group and combination group (FW) with DC group and NG.

Table 3: Experimental schedule for the chronic unpredictable mild stress procedure

Weeks	Days						
	Day 1 (h)	Day 2 (h)	Day 3 (h)	Day 4 (h)	Day 5 (h)	Day 6 (h)	Day 7 (h)
Week 1	Food and water deprivation (24)	Light and dark succession - every 2 h for 10 h	Exposure to empty bottle (2)	Overnight illumination (12)	Space reduction (12)	Damp sawdust in cage (24)	45° cage tilt (12)
Week 2	White noise exposure (12)	Space reduction (12)	Damp sawdust in cage (24)	45° cage tilt (12)	Food and water deprivation (24)	Overnight illumination (12)	Exposure to empty bottle (2)
Week 3	Overnight illumination (12)	Space reduction (12)	Food and water deprivation (24)	White noise exposure (12)	Exposure to empty bottle (2)	Light and dark succession - every 2 h for 10 h	Damp sawdust in cage (24)
Week 4	Damp sawdust in cage (24)	45° cage tilt (12)	Light and dark succession - every 2 h for 10 h	White noise exposure (12)	Overnight illumination (12)	Food and water deprivation (24)	Space reduction (12)
Week 5	Light and dark succession - every 2 h for 10 h	Space reduction (12)	Food and water deprivation (24)	45° cage tilt (12)	Damp sawdust in cage (24)	Space reduction (12)	Overnight illumination (12)
Week 6	Overnight illumination (12)	White noise exposure (12)	45° cage tilt (12)	Damp sawdust in cage (24)	Space reduction (12)	Light and dark succession - every 2 h for 10 h	Food and water deprivation (24)
Week 7	Damp sawdust in cage (24)	45° cage tilt (12)	Food and water deprivation (24)	Space reduction (12)	Overnight illumination (12)	Exposure to empty bottle (2)	White noise exposure (12)

Neuritin gene expression

There was statistically significant increase in neuratin gene expression was seen in ST group ($P < 0.05$) and in combination group (FW) ($P < 0.001$) compared to DC group [Table 4 and Graph 2].

NARP expression

There was a statistically significant increase in NARP gene expression seen in ST group ($P < 0.05$) and in combination group (FW) ($P < 0.01$) compared to DC group [Table 4 and Graph 3].

Brain-derived neurotrophic factor exon – III expression

There was a statistically significant increase in BDNF Exon– III gene expression seen in ST group ($P < 0.05$) and in combination group (FW) ($P < 0.01$) compared to DC group [Table 4 and Graph 4].

Results of sucrose preference test at the end of 7th week

There was a statistically significant increase in sucrose preference seen in ST group WS group and in combination group (FW) ($P < 0.0001$, Tukey's multiple comparisons test) compared to DC group [Table 4 and Graph 5].

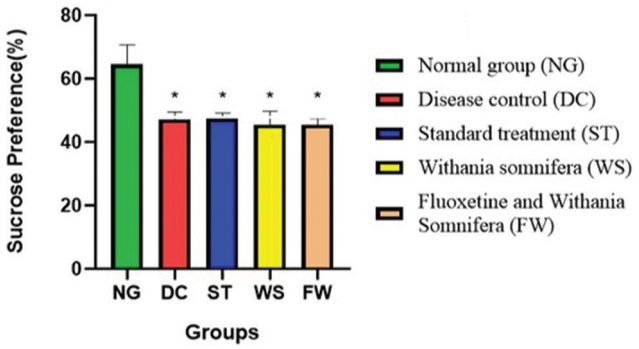
Discussion

The present study was conducted to evaluate the effect of *Withania Somnifera* (WS) root extract on gene expression of selected genes in the hippocampal tissue of male Wistar rats using CUMS as a model of depression.

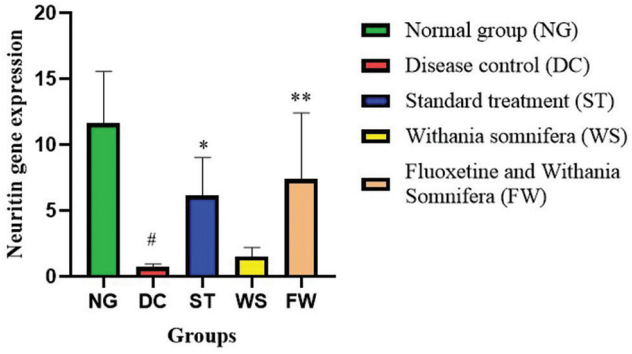
WS is commonly known as Ashwagandha. Its roots, leaves, and extracts are used as a source of antidepressants. The bioactive compounds in WS, such as with anolides, contribute to its neuroprotective, anti-inflammatory, and adaptogenic properties.[23]

The literature reviewed suggests that no study has been done so far to investigate the effect of WS on the gene expression of the genes selected in the present study.

BDNF is a widely studied neurotrophin that plays a significant role in the survival and development of neurons and its deficiency contributes to the development of major depressive disorder. Reduced levels of BDNF have been linked to an increased occurrence of depressive symptoms. Antidepressants can help restore BDNF levels, indicating a potential role for BDNF in the pathophysiology and treatment of depression.



Graph 1: Effect of chronic unpredictable mild stress on sucrose preference test at end of 3rd week. Data expressed as mean ± standard error of mean ($n = 6$). Data analyzed by one-way analysis of variance followed by Tukey's multiple comparison test. *$P < 0.0001$ when compared to the normal group



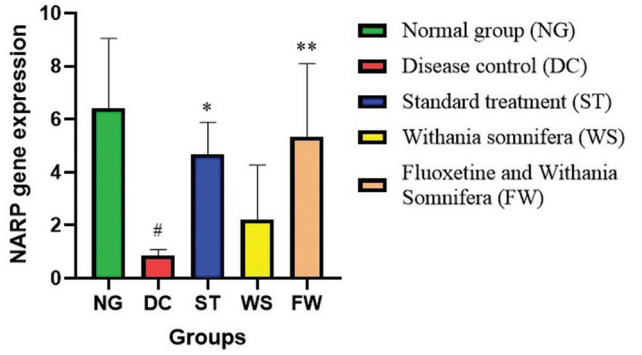
Graph 2: Effect of various drugs on gene expression of neuritin in hippocampus of rats. Data expressed as mean ± standard error of mean ($n = 6$). Data analyzed by One-way analysis of variance followed by Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.001$ compared to the disease control group; #$P < 0.0001$ compared to normal group

Table 4: Effect of various drugs on sucrose preference test and gene expression in male Wistar rats subjected to chronic unpredictable mild stress

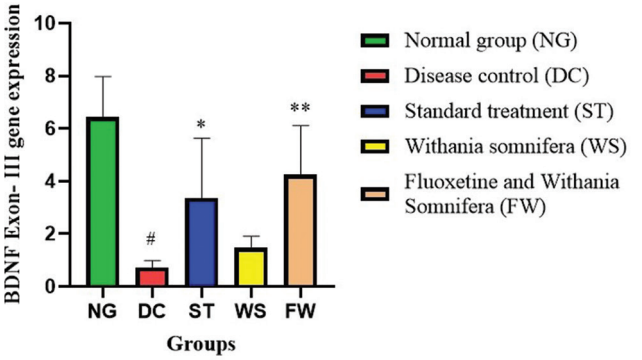
Groups	NG	DC	ST	WS	FW	*F* (4,25)	ANOVA (*P*)
SPT (3rd week)	64.48±2.541	47.12±0.9611#	47.45±0.6886#	45.54±1.692#	45.55±0.7567#	29.24	<0.0001
Neuritin gene expression	11.62±1.605	0.7244±0.09828****	6.122±1.183*	1.521±0.2783	7.389±2.05***	12.11	<0.0001
NARP gene expression	6.403±1.082	0.8526±0.09432****	4.67±0.4985*	2.22±0.837	5.331±1.133**	7.697	<0.001
BDNF exon-III gene expression	6.435±0.6311	0.7125±0.1126****	3.366±0.9238*	1.465±0.1812	4.248±0.7602**	13.85	<0.0001
SPT (7th week)	75.50±2.205	23.57±1.587	64.59±2.235##	59.40±3.005##	68.10±1.998##	80.66	<0.0001

**P*<0.05; ***P*<0.01; ****P*<0.001 compared to the DC group; *****P*<0.0001 when compared to NG; #*P*<0.0001 when compared to NG; ##*P*<0.0001 when compared with DC group. Data expressed as mean±SEM (*n*=6). Data analyzed by one-way ANOVA followed by Tukey's multiple comparison test. SEM: Standard error of mean; NG: Normal group; DC: Disease control; ST: Standard treatment; WS: *Withania somnifera*; FW: Fluoxetine and WS; ANOVA: Analysis of variance; BDNF: Brain-derived neurotrophic factor; SPT: Sucrose preference test

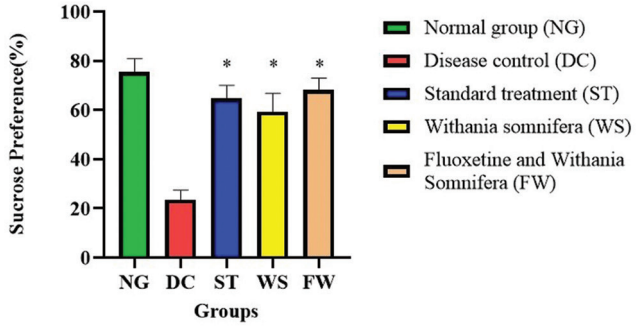
In this study, CUMS model was used to establish depression. This model causes depression by continuously overwhelming the brain's stress response systems, which leads to ongoing disturbances in the reward pathways causing anhedonia.[24,25]



Graph 3: Effect of various drugs on gene expression of NARP in hippocampus of rats. Data expressed as mean ± standard error of mean (*n* = 6). Data analysed by One-way analysis of variance followed by Tukey's multiple comparison test. **P* < 0.05, ***P* < 0.01compared to the disease control group; #P < 0.0001 when compared to normal group



Graph 4: Effect of various drugs on gene expression brain-derived neurotrophic factor Exon– III in hippocampus of rats. Data are expressed as mean ± standard error of mean (*n* = 6). Data analysed by One-way analysis of variance followed by Tukey's multiple comparison test. **P* < 0.05, ***P* < 0.01compared to the disease control group; #*P* < 0.0001 when compared to normal group



Graph 5: Effect of various drugs on sucrose preference at the end of 7th week. Data expressed as mean ± standard error of mean (*n* = 6). Data analysed by One-way analysis of variance followed by Tukey's multiple comparison test. **P* < 0.0001 when compared with disease control group

Symptoms observed in acute models can sometimes overlap with anxiety symptoms, leading to confusion among investigators in differentiating between depression- and anxiety-related behaviors. These acute models provide only specific endpoints that may not fully capture the complexity of depressive symptoms and behaviors.[26,27] Hence, a chronic model of depression was used in this study.

To check for the establishment of depression, SPT was done. It is widely acknowledged as an objective measure to assess the establishment of depression and the effectiveness of antidepressants in experimental animals.[28] This test measures anhedonia, a significant sign of depression, by measuring an animal's preference for sucrose solution over plain water. A decrease in preference for the sucrose solution suggests anhedonia and thus establishment of depression. Our study also demonstrated that the CUMS model successfully induced anhedonia in rats, evidenced by a significant reduction in sucrose preference in the depressed rats compared to normal controls at the end of 3 weeks.

Effect on neuritin gene expression

In our assessment of neuritin gene expression, we observed that there was a statistically significant upregulation of neuritin gene expression in the ST group and the combination group (FW) compared to DC group.

The comparison between the NG and combination group (FW) group showed no statistically significant difference in neuritin gene expression, indicating that the combination group (FW) group effectively brought neuritin gene expression levels to near normal.

Effect on NARP gene expression

In our analysis of NARP gene expression, we observed that there was a statistically significant upregulation of NARP gene expression in the ST group and the combination group (FW) when compared with DC group.

The comparison between the NG and combination group (FW) group showed no statistically significant difference in NARP gene expression, indicating that the combination group (FW) group effectively brought NARP gene expression levels to near normal.

Effect on brain-derived neurotrophic factor exon-III gene expression

In our assessment of BDNF Exon-III gene expression, there was a statistically significant upregulation of BDNF Exon-III gene expression in the ST group and in the combination group (FW) compared to DC group.

The comparison between the NG and combination group (FW) group showed no statistically significant difference in BDNF Exon- III gene expression, indicating that the combination group (FW) group effectively brought BDNF Exon- III gene expression levels to near normal.

There was an upregulation in the expression of neuritin, NARP, and BDNF Exon-III gene seen with WS group, but it was not statistically significant.

The results of our investigation are consistent with earlier research. A study was conducted by Dwivedi on rats to examine the effect of fluoxetine on gene expression associated with BDNF. According to their findings, fluoxetine caused an increase in the expression of genes related to the production of BDNF.[29]

A study conducted by Molteni *et al*. on rats to investigate the effect of fluoxetine on BDNF mRNA levels in the hippocampus found that the injection of fluoxetine increased the expression of mRNA coding for BDNF.[30]

Effect on sucrose preference test

At the end of 7 weeks, there was an increase in sucrose preference seen in ST group, WS group, and the combination (FW) group which was statistically significant compared to the DC group. The comparison between the NG and combination group (FW) group showed negligible difference of sucrose preference, indicating that the combination group (FW) group effectively brought the sucrose preference of rats to near normal. This showcases the antidepressant effect of WS and its combination with fluoxetine.

The findings of a study conducted by KrishnaRaju *et al*. supported the use of WS in the treatment of depression, highlighting its anti-inflammatory and antioxidant properties, which contribute significantly to its therapeutic potential. It was found that WS decreases pro-inflammatory cytokines and oxidative stress indicators, both of which are commonly elevated among people suffering from depression.[31]

Furthermore, a study conducted by Jain *et al*. demonstrated that WS is neuroprotective in the hippocampus by lowering the amount of degenerating hippocampal cells. This study discovered that WS improves neuronal survival and reduces apoptosis in the hippocampus, which is crucial for mood regulation and cognitive function.[32] The neuroprotective effects of WS are due to its capacity to boost antioxidant defenses and regulate the expression of neurotrophic factors, which promote neuronal growth and functioning.[33] Consequently, WS acts as an antidepressant by enhancing BDNF levels, mitigating oxidative stress, reducing the apoptosis of hippocampal cells, and increasing neuroplasticity.

The findings of the present study have found that WS has antidepressant effects when used in combination with fluoxetine and the combination upregulates the genes that encode for BDNF.

Strengths

- This is the only study so far which has evaluated the effect of *Withnia somnifera* at the genetic level to understand the underlying mechanism of its anti-depressant action
- It is also the only study which has investigated the interaction between fluoxetine and *Withnia somnifera* at the genetic level.

Limitations of study

The study did not address the effect of test drugs on other well-known markers for depression, namely, cortisol, glutamate, monoamines, and GABA levels.

Conclusion

This study has improved our understanding of WS's antidepressant action, by investigating its influence on the gene expression of neuritin, NARP, and BDNF Exon-III which are required for the production of BDNF. Furthermore, it was found that the effect of administration of fluoxetine and *Withnia somnifera* in combination had an additive effect when used for the treatment of depression. However, further studies involving human subjects are essential to validate the antidepressant effects of WS and its additive effects with fluoxetine.

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Ethical statement

The study was approved by the institutional Ethics Committee of KLE Academy of Higher Education and Research, Deemed to be University, Jawaharlal Nehru Medical College, Belagavi. Approval No 17/2.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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