EFFECT OF *WITHANIA SOMNIFERA* ON SELECTED GENE EXPRESSION IN BRAIN TISSUE OF MALE WISTAR RATS SUBJECTED TO CHRONIC STRESS INDUCED DEPRESSION.

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ABBRIVATIONS

5-HT-T	:	5-Hydroxy tryptamine transporter protein
APA	:	American Psychiatric Association
BDNF	:	Brain-derived neurotrophic factor
CNS	:	Central nervous system
CREB	:	Cyclic-AMP response element binding protein
CRH	:	Corticotropin-Releasing Hormone
CRP	:	C-Reactive protein
CUMS	:	Chronic unpredictable mild stress
DA	:	Dopamine
DALY	:	Disability adjusted life years
DC	:	Disease control group
DNA	:	Deoxyribonucleic Acid
FST	:	Forced swim test
FW	:	Combination of Fluoxetine and Withania somnifera group
GABA	:	Gamma aminobutyric acid
HP	:	Hippocampus
HPA	:	Hypothalamic–pituitary–adrenal axis
HYP	:	Hypothalamus

IL-1β	:	Interleukin-1 beta
IL-6	:	Interleukin-6
LTP	:	Long term potentiation
MAOI	:	Monoamine Oxidase Inhibitor
MDD	:	Major depressive disorder
NAc	:	Nucleus accumbens
NaSSA	:	Noradrenergic and Specific Serotonergic Antidepressant
NE	:	Norepinephrine
NDRI	:	Norepinephrine-Dopamine Reuptake Inhibitor
NG	:	Normal group
NGF	:	Nerve Growth Factor
PCR	:	Polymerase Chain Reaction
RIMA	:	Reversible Inhibitor of Monoamine Oxidase A
RNA	:	Ribonucleic Acid
SARI	:	Serotonin Antagonist and Reuptake Inhibitor
SNRI	:	Serotonin-Norepinephrine Reuptake Inhibitor
SSRI	:	Selective Serotonin Reuptake Inhibitor
SPT	:	Sucrose Preference Test
ST	:	Standard treatment group
TCA	:	Tricyclic Antidepressant

TNF-α	:	Tumor Necrosis Factor Alpha
TST	:	Tail Suspension Test
VTG	:	Ventral tegmental areas

ABSTRACT

Introduction and Objective :-

The present study was designed to evaluate the effect of *Withania somnifera* (WS) alone and in combination with Fluoxetine on gene expression of Neuritin, NARP & BDNF Exon – III gene in hippocampus of male Wistar rats using chronic unpredictable mild stress (CUMS) as a model of depression.

Methodology :-

The study included 30 male Wistar rats divided into 5 groups of 6 rats in each group. The groups were Normal Group (NG), Disease Control (DC) group, Standard Treatment (ST) group, *Withania somnifera* (WS) group and combination of Fluoxetine and *Withania somnifera* (FW) group. Depression was induced in all groups by using chronic unpredictable mild stress (CUMS) except the normal group. Sucrose preference test (SPT) was done at the end of third week to test for establishment of depression. At the end of seventh week, SPT was again done to check for reduction of depression in all treatment groups. Lastly all rats were euthanized and hippocampus tissue was used for gene expression analysis. Real – time PCR was used to analyse the gene expression.

Results :-

The Standard Treatment (ST) group, and combination of Fluoxetine and *Withania somnifera* (FW) group showed statistically significant upregulation of all three genes namely, Neuritin, NARP and BDNF Exon -III selected in present study. The comparison between the Normal group (NG) & combination group (FW) group showed negligible difference between gene expression of all the three genes. At the end of the seven weeks, it was found that there was a statistically significant increase in sucrose preference seen in Standard treatment (ST),

Withania somnifera (WS) group and combination group (FW) group. However, a negligible difference was found between found between sucrose preference of Normal group (NG) & combination group (FW) group.

Conclusion :-

In conclusion, this study has improved our understanding of *Withania somnifera's* anti-depressant action, by investigating its influence on the gene expression of Neuritin, NARP and BDNF Exon -III which are required for 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 treatment of depression.

Key words: Depression, *Withania somnifera*, Fluoxetine, Gene expression, Neuritin, NARP, BDNF Exon -III.

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INTRODUCTION

Depression is a prevalent psychological disorder that affects a substantial portion of the total world population. Approximately 280 individuals worldwide are suffering from depression.[1] An estimated 57 million people in India are suffering from depression.[2] This condition is of significant importance to public health due to its high prevalence, impact on mental well-being, morbidity, and economic burden.[3] The medication that is currently being used to treat depression have number of adverse effects, including drowsiness, weight gain, cardiac arrhythmias, gastrointestinal malfunction, sexual dysfunction, and other undesirable side effects. Additionally, discontinuing antidepressant medication might result in withdrawal symptoms such as light-headedness, nausea, fatigue, anxiety, instability of gait, and insomnia.[4,5]

Ashwagandha, which is also known as *Withania somnifera (*L.), is a xerophytic plant that resembles wood and is typically found in the Mediterranean and Asian regions. In Ayurvedic medicine, it has been extensively utilized as an adaptogen that helps in reducing stress, anxiety, and improving overall mental wellbeing.

It has been found to possess antidepressant properties, along with various other medicinal benefits.[6,7] Furthermore, it is considered to provide protection against neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease that are prevalent today.[8] Various research on both mice and rats, have demonstrated the potential of *Withania somnifera* as an adjuvant drug in the treatment of depression.[9,10]

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.[11]

It is essential for neuronal maintenance, and growth. It plays an important part in synaptic plasticity, which strengthen or weakens over time in response to activity or experience.[12] 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, viz NARP, Neuritin, and BDNF exon-III, in the brain. These genes are associated with BDNF-LTP and production of different proteins that aid neuronal growth, maintenance and plasticity. This suggests a link between treatment of depression and the molecular mechanisms underlying synaptic plasticity.[13,14] To date, no research has tested the effect of *Withania somnifera* on the expression of genes mentioned above.

Therefore, this study aimed to evaluate the effect of *Withania Somnifera* on expression of Neuritin, NARP, BDNF Exon – III when given alone or in combination with Fluoxetine in the hippocampus, of male Wistar rats using chronic unpredictable mild stress (CUMS) as a model of depression.

OBJECTIVES

The objectives of the study were as follows:

Primary Objective –

- To determine the effect of *Withania somnifera* on gene expression of Neuritin, NARP & BDNF Exon – III in hippocampus of male Wistar rats using chronic unpredictable mild stress (CUMS) as a model of depression.

Secondary Objective –

- To determine the effect of *Withania somnifera* in combination with Fluoxetine on gene expression of Neuritin, NARP & BDNF Exon – III in hippocampus of male Wistar rats using chronic unpredictable mild stress (CUMS) as a model of depression.

REVIEW OF LITERATURE

3.1.1 Introduction and Epidemiology

Mental health is one of the most important aspects of the overall health of an individual. Throughout ancient times, mental illnesses have afflicted people. They are characterized by abnormalities in thought, feeling, mood, or the highest integrative components of behavior, such forming social bonds or making plans for the future activities.[15] A psychiatric condition, often known as a mental disorder, is a psychological pattern or aberration that is typically linked with suffering or incapacity and is not regarded to be part of a person's normal cultural development.[16]

According to a survey undertaken by the World Health Organization (WHO), more than a third of people in most countries, report issues that match criteria for diagnosis of one or more of the prevalent mental disorders at some point in their lives. Five distinct illnesses caused over 15 million DALYs each, with mental and behavioural problems contributing to 7.4% of DALYs. The most common causes were major depressive disorder (2.5%), anxiety disorders (1.1%), drug abuse disorders (0.8%), alcohol abuse disorders (0.7%), and schizophrenia (0.6%).[17]

Depression is one of the most common psychiatric disorders. Approximately 280 individuals worldwide are affected by depression. In India it is estimated that 57 million people are affected by depression.[1,2]

The World Mental Health Survey, which was done in 17 countries, indicated that one out of every 20 persons had experienced depression in the preceding year.[18] In terms of the significance to public health, depression ranks third in the globe, accounting for 4.3% of all deaths from disease. If current trends continue, they will be the primary cause of sickness burden by 2030.[19]

People have recorded instances of depression since antiquity. Many ancient texts, including the Old Testament, contain descriptions of what are today known as mental disorders. The Greek physician Hippocrates first used the words "mania" and "melancholia" to describe emotional and mental disturbances in around 400 BC. In approximately 30 AD, the Roman physician Celsus provided a description of melancholia, which he derived from the Greek words melan (meaning black) and chole (meaning bile), referring to a state of depression induced by an excess of black bile.[20]

3.1.2: DEPRESSION:-

Depression is a state of low mode and aversion to activity that can affect an individual's thoughts, feelings, behaviour and sense of well-being. [21] It can also be defined as the presence of somatic and cognitive changes that significantly affect an individual's capacity to function, combined with melancholy, empty and irritable mood.

Depression may manifest as a single episode or as recurrent episodes. The course may be somewhat protracted up to 2 years or longer in those with the single-episode form. The prognosis for recovery from an acute episode of depression is good for most patients with major depressive disorder. Three out of four patients with MDD experience recurrences throughout their life, with varying degrees of residual symptoms between episodes.[20]

The DSM-5-TR, The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision[22], states that in order to diagnose patient MDD, the following criteria must be met:-

A. Five (or more) of the following symptoms must be present during the same 2-week period and represent a change from previous functioning: at least one of the symptoms is either

(1) Depressed mood or

(2) Loss of interest or pleasure.

1. Depressed mood most of the day, nearly daily, as indicated by either subjective report or observation made by others.
2. Markedly reduced interest or pleasure in all, activities most of the day, nearly daily.
3. Psychomotor agitation / retardation nearly every day.

4. Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month).
5. Insomnia or hypersomnia nearly daily.
6. Feeling of worthlessness or excessive or inappropriate guilt nearly daily (not merely self-reproach or guilt about being sick).
7. Diminished ability to think or concentrate, or indecisiveness, nearly every day.
8. Fatigue or loss of energy nearly every day.
9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

B. The symptoms cause clinically significant distress or impairment of social, occupational, or other important areas of functioning.

C. The episode is not attributable to the physiological effects of a substance or to another medical condition.

Note: - Criteria A-C represents a major depressive disorder.

D. The occurrence of the major depressive episode is not better explained by specified or unspecified schizophrenia spectrum and other psychotic disorders.

E. There has never been a manic or hypomanic episode.

3.1.3: Classification of Depressive disorders: -

The American Psychiatric Association (APA) uses the DSM-5-TR[22] to classify Major Depressive Disorders (MDDs).

Table 1: **The criteria for diagnosis, along with their corresponding codes and descriptions**

Disorder	DSM-5-TR Code	Description
Major Depressive Disorder		
-**Single Episode**	F32.x	One episode of major depressive disorder.
- **Recurrent Episode**	F33.x	Repeated episodes of major depression.
Persistent Depressive Disorder (Dysthymia)	F34.1	Chronic depressive symptoms lasting at least two years, including persistent low mood and other symptoms like low energy and poor concentration.
Disruptive Mood Dysregulation Disorder	F34.8	Severe temper outbursts and persistent irritability that occur frequently and are inconsistent with developmental level, lasting at least a year.
Premenstrual Dysphoric Disorder	F32.81	Significant mood swings, irritability, and anxiety before menstruation, improving after it starts.
Substance/Medication-Induced Depressive Disorder	F10.14 - F19.14, F10.94 - F19.94	Depressive symptoms caused by substance use or withdrawal.

Depressive Disorder Due to Another Medical Condition	F06.31, F06.32	Depressive symptoms resulting from another medical condition.
Other Specified Depressive Disorder	F32.8, F33.8	Depressive symptoms causing distress but not meeting criteria for any specific depressive disorder.
Unspecified Depressive Disorder	F32.9, F33.9	Depressive symptoms causing distress without enough information for a specific diagnosis.

3.2: Pathophysiology of depression

The monoamine hypothesis postulated that depression resulted from a reduction in the quantity or function of monoamine neurotransmitters in particular of the brain. In the past ten years, there has been growing evidence that the pathophysiology of depression involves not just the monoamine deficit but also endocrine and neurotrophic factors. Even though depression affects a significant portion of the population, little is known about its pathogenesis.[23]

There are multiple explanations for this disparity. Compared to other organs, it remains exceedingly difficult to identify neurological changes in the brain. There are two main ways for recording abnormal brain circuit activity: post-mortem investigations and neuroimaging methods. Post-mortem investigations involve studying the brain after death, whereas neuroimaging methods use indirect indications of neuronal activation to uncover abnormalities in brain activity. Secondly, most cases of depression are idiopathic. Genuine depression genes, which can be used to generate disease models in mice, have not yet been identified.[24]

3.2.1: Neural circuitry of depression.[24-26]

The pathophysiology of depression has been linked to several brain areas. The following diagram can help to illustrate the following **Figure-1.**

(a) Hedonic and reward deficits associated with depression are mediated by the Nucleus Accumbens (NAc). This is supported by stimulation of the NAc32 or the subgenual cingulate cortex[27],which improve depression in patients with treatment-resistant depression. It is believed that this effect results from either depolarization blocking or stimulation of crossing axonal fibres, which modifies specific areas' activity.

(b) Elevated activity-dependent release of Brain Derived Neurotrophic Factor (BDNF) within mesolimbic dopamine circuit (dopamine – producing ventral tegmental areas - VTG to dopamine sensitive NAc) mediates the susceptibility to social stress through activation of transcription factor cyclic-AMP response element binding protein (CREB) by phosphorylation.[28]

(c) Studies on neuroimaging clearly link the processing of emotional cues like "frightful faces" to the amygdala.

(d) Stress causes increase in cortisol concentration which reduces the concentration of neurotrophins like BDNF, which regulates the level of neurogenesis and the complexity of neuronal activity in the hippocampus (HP). It also lowers CREB activity.

(e) Metabolic hormones such as ghrelin and leptin, which are produced peripherally in addition to cortisol, causes mood shifts through their actions on the hypothalamus (HYP) and other limbic areas.[29]

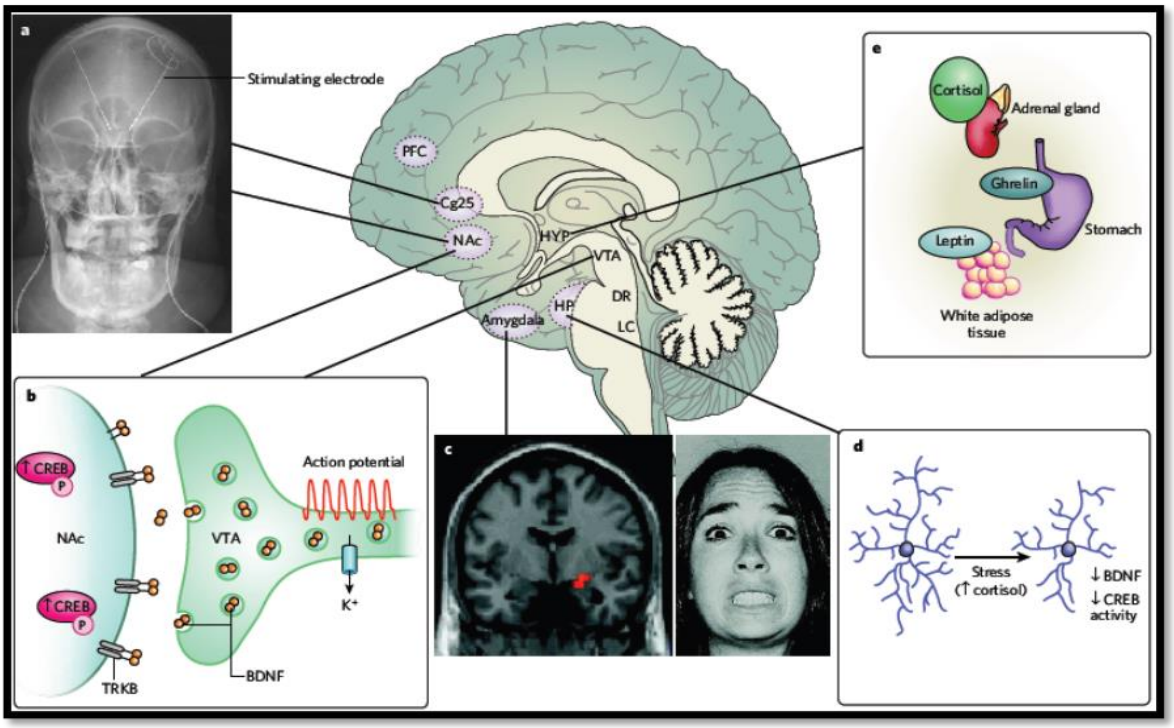


Figure 1: Neural circuitry of depression.[24]

3.2.2: Various proposed theory of depression: -

1. **The Monoamine theory.**

The monoamine hypothesis was first proposed by Joseph J. Schildkraut in 1965.[30] According to this theory, there is a quantitative or functional deficiency of monoamines like serotonin, norepinephrine, and dopamine in certain areas of the cortex and limbic system.[15,31]The lines of evidence in support of this theory are elaborated further. It was observed that iproniazid, a monoamine oxidase inhibitor, caused euphoric mood in patients on treatment. Conversely, reserpine, which is known to cause depletion of monoamines, caused depression in some patients.

Genetic studies also lend weight to the monoamine hypothesis. People who are homozygous for the short allele of the polymorphic promoter region of the serotonin transporter gene are more prone to get major depression and suicidal behavior in response to stress. But the most important evidence comes from the fact that all the

current antidepressants have their action due to their ability to enhance the availability of these monoamines at the synapses in various regions of the brain. These drugs either decrease the neuronal uptake of monoamines (like the SSRIs, which inhibit serotonin reuptake thereby increasing synaptic availability) or inhibit the degradation of the monoamines, like monoamine oxidase inhibitors.

Although the monoamine-based agents are potent antidepressants, and alterations in central monoamine function contribute to their antidepressant action, the cause of depression is far from being a simple deficiency of these central monoamines. Monoamine oxidase inhibitors and SSRIs produce an immediate increase in monoamine transmission, whereas their mood-enhancing properties require weeks of treatment. Conversely, experimental depletion of monoamines can produce a mild reduction in mood in non-medicated depressed patients, but such manipulations do not affect mood in healthy controls.

Researchers have come up with newer theories of depression, to overcome shortcomings of monoamine hypothesis.

2. The neurotrophic hypothesis:

The central nervous system (CNS) development and function are significantly impacted by the neurotrophins, a family of related, secreted peptides. Through interactions with their transmembrane glycoprotein receptors, neurotrophins control neurite development and neuronal survival. The neural plasticity is significantly influenced by these neurotrophins.[32] Neurotrophins are classified into numerous families, the most significant of which is the nerve growth factor (NGF) and includes brain-derived neurotrophic factor (BDNF).[15,33]Effective antidepressant medications promote neurogenesis and synaptic connections in cortical areas like the hippocampus, which is linked to depression and the loss of neurotrophic support. It is believed that BDNF affects the growth and survival of neurons by activating tyrosine kinase

receptor B in glia and neurons. The hippocampus, anterior cingulate gyrus, and medial frontal cortex all experience atrophic alterations and volume loss as a result of decreased BDNF levels brought on by stress and pain.[34,35]

This loss of volume in the aforementioned areas of the brain has also been found in post-mortem studies of depressed patients. Even imaging studies of depressed patients report a reduction in the volume of the hippocampus. The magnitude of the reduction has been directly related to the length of the illness.[15] Further, direct BDNF infusion into mice' hippocampal, midbrain, and prefrontal cortex has demonstrated effects similar to those of an antidepressant.

Human studies also support the neurotrophic hypothesis of depression, as it has been found that BDNF levels in the serum and cerebrospinal fluid are decreased in patients with depression.[33]

3. **Neuroimmune mechanism.**

Cytokines and other immune factors have also been found to play a key role in modulating brain development and neuronal plasticity. Increasing evidence indicates that pro-inflammatory cytokines, including IL-1β, TNF-α, and IL-6, as well as a disruption in immune mediators such as acute phase response protein, C-reactive protein (CRP), nitric oxide, and glucocorticoids, play a role in the development of depression and associated symptoms such as anorexia, sleep disturbance, fatigue, and cognitive impairment.[24] Recent preclinical research suggests that inhibiting the signalling of pro-inflammatory cytokines can lead to antidepressant benefits. Mice that have had specific sections of the gene responsible for producing IL-6 or the TNF-α receptors removed, exhibit behavioural characteristics similar to those seen in individuals being treated for depression.[36] A centrally administered antagonist of the IL-1β receptor reversed the behavioural and antineurogenic effects of chronic stress.

Roughly 30% of individuals treated with recombinant interferons develop depression as a side effect of treatment.[37]

4. Neuroendocrine factors in depression.

Numerous hormonal irregularities are known to be linked to depression. Abnormalities in the hypothalamo-pituitary-adrenal (HPA) axis in patients with Major Depressive Disorder (MDD) are among the most well-established of these findings. Additionally, chronically high levels of cortisol and corticotropin-releasing hormone (CRH) are linked to MDD. Similar to the symptoms of MDD, exogenous glucocorticoids and endogenous cortisol increase are linked to mood disorders and cognitive impairments.[15,33] Glucocorticoids can cause atrophic alterations in hippocampus. This might be an excess of causative factor in the decrease volume of the hippocampus observed in depression.[38] Patients with depression have also been reported to have thyroid dysfunction. It has been observed that up to 25% of depressed patients have abnormal thyroid function.[39,40]

These include a blunting of response of thyrotropin to thyrotropin-releasing hormone and elevations in circulating thyroxin during depressed states. Clinical hypothyroidism often presents with depressive symptoms, which resolve with thyroid hormone supplementation. Thyroid hormones are also commonly used in conjunction with standard antidepressants to augment therapeutic effects of the latter.

Lastly, there is evidence linking sex steroids to the aetiology of depression. Postpartum and postmenopausal estrogenic insufficiency states are regarded to be major contributors to depression in certain women.[41] Similar to this, testosterone shortage in men can occasionally be linked to symptoms of depression. Hormone replacement therapy in hypogonadal men and women may be associated with an improvement in mood and depressive symptoms.[15]

5. Epigenetic theory

Environmental experiences can modify gene activity without altering the DNA sequence through a process known as epigenetic modifications. This might be the reason for the contradictory findings from studies on genetic associations with depression.
Two primary chromatin-modifying pathways have been the subject of epigenetic research in depression studies.

The first mechanism appears to be important in understanding how maternal behaviour influences adult emotional processing: DNA methylation of cytosine. For instance, compared to adult rats whose mothers display high rates of maternal behaviour, adult offspring of mothers who display low rates of maternal behaviour show higher levels of anxiety and lower expression of glucocorticoid receptors in the hippocampus. Increased methylation of the glucocorticoid receptor gene promoter efficiently mediates this decreased production of glucocorticoid receptors.

The second process is histone acetylation, which is associated with transcriptional activation and decondensed chromatin. This modification appears to be a key substrate for the action of antidepressants.[42,43]

6. Role of Substance P, Gamma Amino Butyric Acid (GABA), Glutamate and Enkephalins

Neurokinin antagonists (substance P antagonists) have previously been demonstrated to have antidepressant properties. Depression can be caused by a lack of gamma amino butyric acid (GABA) and neuroactive peptides (particularly vasopressin and endogenous opiates). Overactivity of acetylcholine, corticotrophin releasing factor, and glutamate is hypothesized to cause depression.[15,44]

3.2.3: Convergence of Various Theories of Depression

The various hypotheses on the pathophysiology of depression mentioned earlier are not mutually exclusive. There is a lot of evidence that suggests the neurotrophic, neuroendocrine, and monoamine systems are convergent. For instance, abnormalities in the HPA axis and steroid hormone levels may contribute to the suppression of BDNF gene transcription. The hippocampus, which contains a high density of glucocorticoid receptors, is significantly affected by these factors, as the binding of glucocorticoids influences HPA axis function.[15,38]

3.3: Risk factors for development of depression.

3.3.1 Genetic factors: -

Family, twin, and adoption studies have shown that both severe depression and bipolar disease are substantially heritable even though no specific abnormalities in genes influencing neurotransmitter or hormone production or release have been identified. Stress and genes are supposed to affect the degree and extent of neuronal processes, new neuron creation, and neuronal repair in severe depression. People with variances in the proximal 5' regulatory protein of the gene encoding the 5-Hydroxy tryptamine transporter protein (5HT-T) have been shown to suffer from notable depression in stressful surroundings.[16]

3.3.2 Psychological elements:

- Social and family contacts get disturbed.
- Pessimism and poor self-esteem.
- Gender: women are more prone than men to have emotional problems (related to social, occupational roles, biological and psychological changes).
- Socioeconomic status — a poor socioeconomic level appears to be linked to an increased occurrence of mood disorders.

3.3.3 Disease risk variables:

Many diseases, especially those with a long and severe course and/or result, are regularly associated with depression in different degrees.[45]

3.4: Pharmacotherapy of depression

Table 2: Pharmacotherapy of depression.[5,46-48]

Classification of Drugs	Mechanism of Action	Adverse Effects
I. Monoamine Oxidase Inhibitors (MAOI)		
Nonselective irreversible monoamine oxidase inhibitors. • Isocarboxazid • Phenelzine • Tranylcypromine	Inhibit deamination of norepinephrine (NE), serotonin (5-HT) and dopamine (DA) resulting in increased levels of NE, 5-HT, and DA.	Agitation, hallucination, mania, peripheral neuropathy. Hypertensive crisis on consumption of food containing tyramine
2. Selective monoamine oxidase B (MAO-B) inhibitor • Selegiline	Acts in high doses and exerts antidepressant actions.	Postural hypotension, nausea, confusion, psychosis. Is converted into amphetamine - may cause insomnia.
3. Reversible inhibitors of MAO-A (RIMA's) • Moclobemide • Clorgyline	Reversibly inhibits MAO-A selectively. Shorter duration of action.	Nausea, dizziness, headache, insomnia, rarely liver damage. No hypertensive crisis on ingestion of food containing tyramine.
II. Tricyclic Antidepressants (TCA)		
1. NE + 5-HT reuptake inhibitors • Amitriptyline • Imipramine • Trimipramine • Doxepine • Dothiepin • Clomipramine	Inhibit reuptake of biogenic amines NE and 5-HT into their respective neurons and thus potentiate them.	Due to blockade of α1 adrenergic receptors → orthostatic hypotension, dizziness. Blockade of muscarinic cholinergic receptors → dry mouth, blurred vision, urinary retention, and constipation. Blockade of H1 (histamine) receptors causes → sedation, weight gain. Also block Na+ (Sodium) channels in the heart and brain → cardiac arrhythmias and seizures.

2. Predominantly NE reuptake inhibitors • Protriptyline • Maprotiline • Amoxapine	There may be lesser or no blockade of dopamine reuptake. They differ in their selectivity and potency for different amines.	
III. Selective Serotonin Reuptake Inhibitors (SSRI's)		
- Fluoxetine - Fluvoxamine - Paroxetine - Sertraline - Citalopram	Selectively inhibit the reuptake of 5-HT and increases its levels in the synapses.	Actions on 5-HT2A, 5-HT2C receptors in the limbic cortex → nervousness, anorexia, agitation, panic attacks. Actions on 5-HT2A receptors in the basal ganglia → akathisia, psychomotor retardation, mild Parkinsonism, dystonic movements. Actions on 5-HT2A receptors in the brainstem → myoclonus and insomnia. Actions on 5-HT2A receptors in the spinal cord → sexual dysfunction. Action on 5-HT3 receptors in brainstem or hypothalamus → nausea and vomiting. Action on 5-HT3 receptors in gastro-intestinal tract → cramps and diarrhoea.
IV. Atypical Antidepressants		
1. Norepinephrine reuptake inhibitors (NRI) • Reboxetine • Tomoxetine	Selectively inhibit reuptake of norepinephrine (NE). Increase in the NE levels in the pathway from locus coeruleus to frontal cortex is responsible for antidepressant action. It improves fatigue, apathy and psychomotor	Stimulation of β1 receptors in cerebellum and peripheral nervous system → tremors. Stimulation of NE receptors in: - limbic system → agitation - brain stem cardiovascular centres → increases the blood pressure - heart → alteration of heart rate. Stimulation of sympathetic nervous system can cause

	retardation by increasing NE levels in the pathway from locus coeruleus to limbic cortex.	reduction in the parasympathetic tone and can cause dry mouth, constipation, urinary retention.
2. Norepinephrine and dopamine reuptake inhibitor (NDRI) • Bupropion	Inhibit reuptake of NE and dopamine.	Long term effects unknown Can cause agitation, dry mouth, nausea, reinforcement and abuse.
3. Dual serotonin and norepinephrine reuptake inhibitor (SNRI) • Venlafaxine • Duloxetine	Inhibits the reuptake of NE and 5-HT selectively. No interaction with cholinergic, histamine and adrenergic receptors.	Hypertension, impotence, anxiety, nausea, sweating.
4.Noradrenergic and specific serotonergic antidepressant (Na SSA) • Mirtazapine	Antagonizes α2 action and increases NA, 5-HT release. No action on monoamine transporter. Has antagonist action at 5-HT2A, 5-HT2C, 5-HT3 (no nausea, vomiting, sexual dysfunction) and histamine (H1) receptors.	Sedation, weight gain.
Dual 5-HT_2 receptor antagonist/serotonin reuptake inhibitor (SARIs) • Trazadone • Nefazodone	By inhibiting the transporter, it increases 5-HT level at synapses. Antagonizes 5-HT2A (no sexual dysfunction, anxiety, insomnia). Inhibits histamine (H1) receptors.	Sedation, priapism

3.5: Screening methods for antidepressant activity:

3.5.1 Animal models for screening antidepressant activity are given in table below: -

Table 3: Animal models for screening antidepressant activity.[49-51]

SR.No	Model	Advantage	Disadvantage
1.	**Catalepsy Antagonism**	The test can be considered as specific for central stimulants allowing the possibility to distinguish between antidepressants and central stimulants of the amphetamine type.	Common laboratory animals cannot be used.
2.	**Forced swim test (FST)**	Sensitive to antidepressants Easy to perform High reproducibility	Sensitive to acute treatment. Validity for MAOIs is uncertain. Risk of hypothermia.
3.	**Modified FST**	Sensitive to antidepressants. Easy to perform.	Sensitive to acute treatment. Validity for MAOIs uncertain. Risk of hypothermia. Not applicable in rats.
4.	**Tail Suspension Test (TST) in mice**	Sensitive to antidepressants. Easy to perform. High reproducibility.	Applicable only in certain mouse strains.
5.	**Intracranial self-stimulation**	Measures affective state and motivation. Responds to chronic antidepressants.	Further validation required in models of depression.
6.			Time consuming and its specificity questionable.

	Learned Helplessness in Rats	Can be regarded as an additional measure for antidepressant activity in addition to other tests	The major drawback of the model is that most of the depression-like symptomatology does not persist beyond 2–3 days following cessation of the uncontrollable shock.
7.	**Muricide Behavior in Rats**	A selective inhibition of mouse-killing behaviour in rats by antidepressants. The test can be used to evaluate antidepressants such as tricyclics and MAO inhibitors.	The mouse-killing behaviour is inhibited not only by antidepressants but also by central stimulants like d-amphetamine, some antihistamines and some cholinergic drugs
8.	**Behavioural Changes After Neonatal Clomipramine Treatment**	Might correlate well with childhood depression. Studied by many researchers.	Specificity of the procedure to evaluate potential antidepressant compounds remains to be established.
9.	**Chronic Stress Model of Depression**	Simulates in animals the symptom of anhedonia, a major feature of depression.	Time consuming and observations may vary subjectively.
10.	**Novelty-Induced Hypophagia Test**	Chronic, but not acute antidepressant treatment alters behaviour	Time consuming
11.	**Reduction of Submissive Behaviour**	Submissive behaviour for one subject can be objectively measured.	Time consuming
12.	**Elevated plus maze (Anxiolytics Screening)**	Easy to perform, Reliable measures of anxiolytic activity. Does not required any sophisticated instrument.	Time consuming

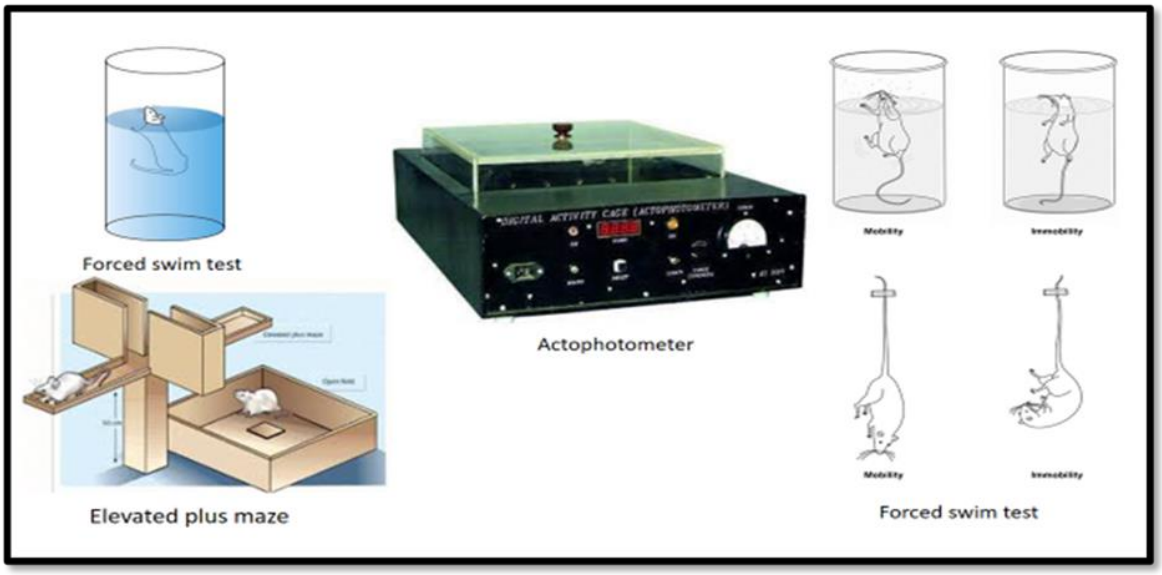


Figure 2: **Commonly used screening methods for antidepressant activity-I**

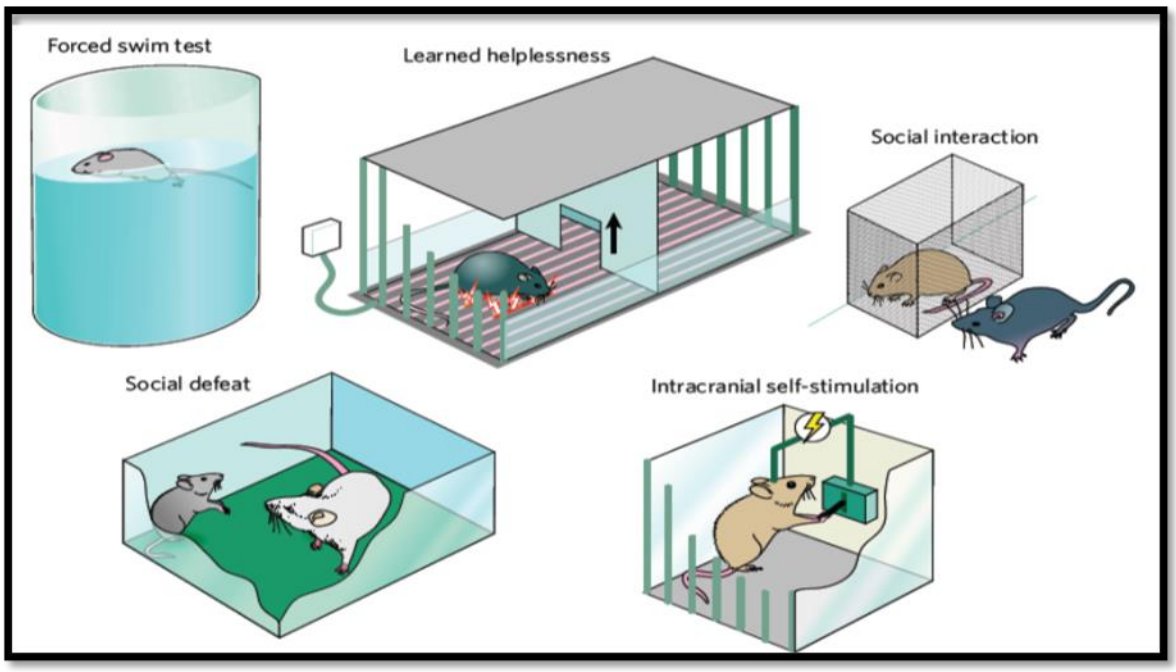


Figure 3: **Commonly used screening methods for antidepressant activity -II**

3.5.2: Chronic Unpredictable Mild Stress model.[52-54]

The CUMS or CMS (chronic unpredictable mild stress or chronic mild stress) depression paradigm was used in the current study. The CUMS model was developed over 20 years ago as an animal model of depression. According to this paradigm, animals develop anhedonia through prolonged exposure to a series of mild but unanticipated stresses. This model has been used since its inception in several studies examining the neurological traits associated with depression.

• After the CUMS technique, animals would have a condition of decreased reward salience, which is comparable to the anhedonia observed in major depressive disorder.

• In addition to being a helpful tool for examining new systems that may be disrupted in depression.

The chronic mild stress paradigm: -

(1) Elicits a variety of neurobiological changes that are similar to those seen in depressive disorders

(2) Aids in the development of new targets for the treatment of depression in major depressive disorder.

Chronic Unpredictable Mild Stress (CUMS) induced anhedonia in rats can be 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.[55]

3.6: DRUGS USED IN PRESNET STUDY

Withania somnifera (L)

Ashwagandha, also known as *Withania somnifera* (L.), is a wood-like xerophytic plant that thrives primarily in Mediterranean and Asian regions. In Ayurvedic medicine, *Withania somnifera* has been extensively utilized as an adaptogen. It works on the neuroendocrine system, which helps to normalize physiological function.[56] It has been proven to help in the alleviation of anxiety and depression symptoms. It also offers protection against various neurological disorders.[6,8]Studies in animals have shown that *Withania somnifera* leads to increase of BDNF production in the brain.[57] Studies suggest that *Withania somnifera* exerts a regulatory effect on neurotransmitters such as serotonin, dopamine, and norepinephrine, whose dysregulation is known to cause depression.[7]

Mechanism of action

Antidepressant effects of *Withania somnifera* are supported by various mechanisms as follows: -

1) In animal studies, *Withania somnifera* has been shown to regulate levels of neurotransmitters such as dopamine, serotonin, and gamma-aminobutyric acid (GABA) which is one of the mechanism by which it reduces depression.[58]
2) BDNF plays a crucial role in neuronal growth, survival, and synaptic plasticity, contributing to mood regulation and cognitive function. Research has demonstrated that *Withania somnifera* elevates brain-derived neurotrophic factor (BDNF) levels.[59,60]

3) *Withania somnifera* also possesses anti-inflammatory and antioxidant properties because of its bioactive compounds like such as withanolides and flavonoids. They help reduce inflammation and oxidative stress in the brain which cause depression.[61]

3.7: Role of selected genes [62-66]

Effects and Upregulation of Neuritin, NARP, and BDNF Exon III by Antidepressants

3.7.1: Neuritin

- Its function is to modulate synaptic activity and plasticity and also to stabilizes synaptic connections which are important for long-term potentiation (LTP) and memory formation.
- Antidepressants like SSRIs, SNRIs, and tricyclic antidepressants have been shown to increase Neuritin expression.

3.7.2: NARP (Neuronal Activity-Regulated Pentraxin)

- NARP is essential for clustering AMPA receptors at excitatory synapses, which boosts synaptic transmission and plasticity. This function is vital for learning and memory processes, particularly in the hippocampus.
- Evidence suggests chronic antidepressant treatment can potentially upregulation NARP gene expression

3.7.3: BDNF Exon III (Brain-Derived Neurotrophic Factor Exon III)

- It is essential for neuronal growth, survival, and function.
- It is critical for neuronal growth survival, learning, and memory and it also regulates mood and cognitive functions.
- Chronic treatment with SSRIs, SNRIs, and tricyclic antidepressants increases BDNF mRNA levels, including transcripts containing exon III.
- This upregulation promotes neurogenesis, synaptic plasticity, and neuronal survival, contributing to antidepressant effects.

METHODOLOGY

Adult healthy male Wistar rats weighing 200± 20 grams were obtained from Central animal house of the institution. The rats were acclimatized to 12:12 hour light – dark cycle for 7 days, prior to starting of experiment. They were maintained at constant room temperature (22^{o}-25^{o}) and on chow pellet (Amrut Brand) with water ad libitum. The animals were housed in groups in polypropylene cages with 6 animal per cage.

The study was approved by the Institutional Animal Ethics Committee (IAEC) Letter no 17/2 dated 26/6/2022 attached in Annexure – 1. The study was conducted as per guidelines of Committee for Control and Supervision of Experiments on Animals (CCSEA).

For chronic unpredictable mild stress (CUMS) 30 rats were divided into 5 groups with six animals in each group. Details about the grouping and dose mentioned in Table 4

Drugs used in present study.

1. Tablet Fluoxetine

It was procured from the pharmacy of the hospital attached to the medical college.

***2. Withania Somnifera* Root extract (60% hydro-alcoholic extract)**

It was procured from Natural Remedies, Bangalore as a free sample.

3. Thiopentone sodium

It was procured form Anaesthesia department of medical college.

All the drugs were given after dissolving in distilled water and were administered orally.

Reagents and Kits:

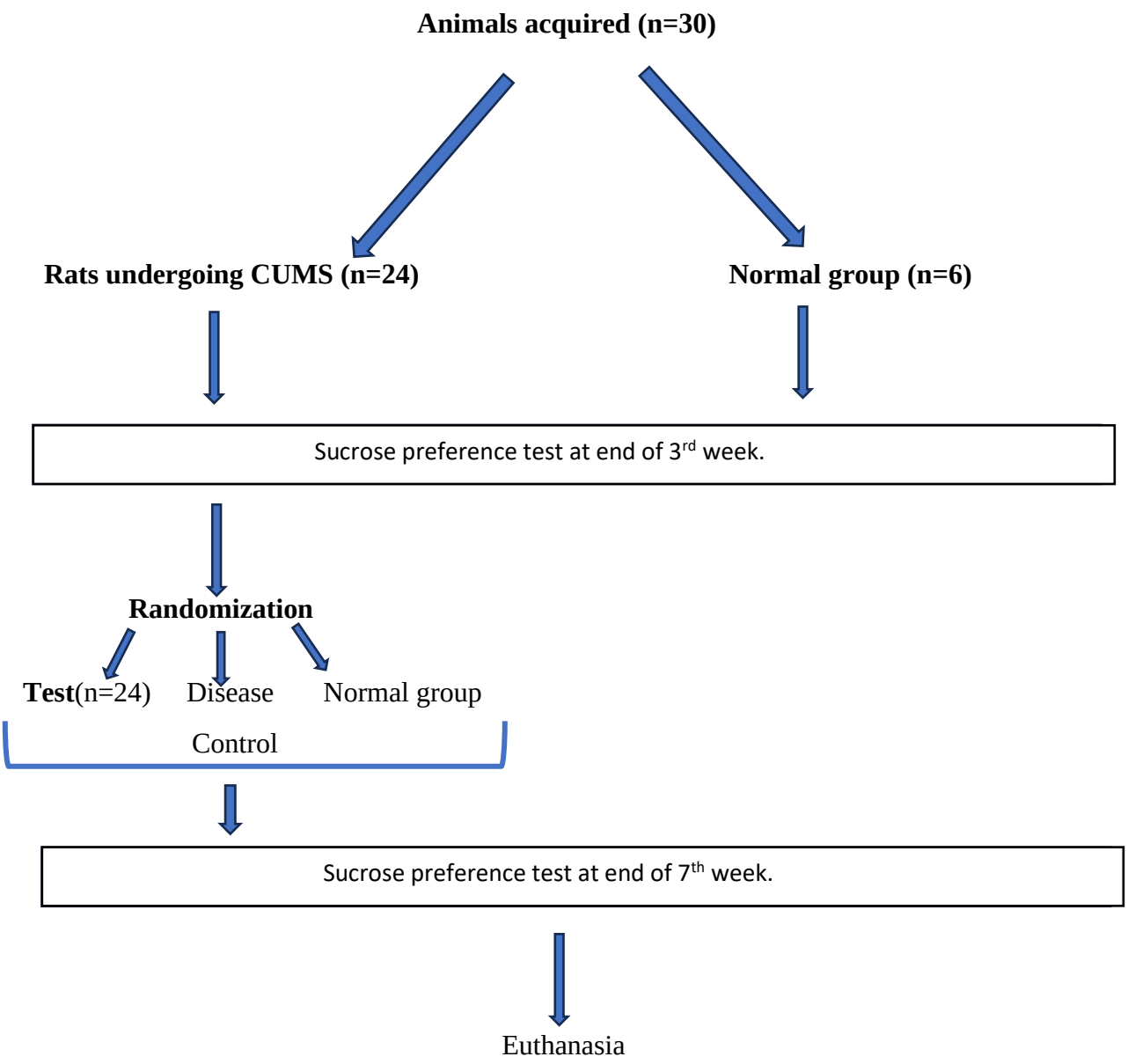
- RNeasy Mini Kit (Cat No. 74104) - JJ Biotech, Bangalore.
- cDNA Kit (Cat No. RR037A) Juniper Lifesciences, Bangalore.
- Sybr green kit (Cat No. RR820A) Juniper Lifesciences, Bangalore.
- Sample Protector for RNA/DNA (Takara – CatLog No. 9750) Juniper Lifesciences, Bangalore.
- Primers for the selected genes were procured from Bioserve biotechnologies India Pvt Ltd, Hyderabad.

Table 4: Study groups along with drug administered.

Groups	Treatment	Dose	Route	Duration
Group 1	Normal group (NG)	Distilled water 0.5ml	Oral	7 weeks
Group 2	Disease control (DC)	Distilled water 0.5ml	Oral	7 weeks
Group 3	Standard treatment (ST)	Fluoxetine 5mg/kg	Oral	Week 3 to 7
Group 4	*Withania Somnifera (WS)*	*Withania Somnifera* (Root extract) 50mg/kg	Oral	Week 3 to 7
Group 5	Fluoxetine and *Withania Somnifera* (FW)	Fluoxetine 5mg/kg + *Withania Somnifera* (Root extract) 50mg/kg	Oral	Week 3 to 7

Dose has been selected as per previous studies.[67-68]

STUDY PROTOCOL



Experimental Induction of Chronic unpredictable mild stress.

The model for depression was established by administering chronic unpredictable mild stress (CUMS) for a period of seven weeks. Eight different stressors stated below were given randomly.[52,69-71]

Table 5: **Experimental Schedule for the Chronic Unpredictable Mild Stress Procedure**

W/D	Day1	Day2	Day3	Day4	Day5	Day6	Day 7
Week 1	Food and Water Deprivation - 24 hours	Light and dark succession -Every 2 hours for 10 hours	Exposure to empty bottle - 2 hours	Overnight illumination - 12 hours	Space reduction -12 hours	Damp saw dust in cage -24 hours	45^o cage tilt -12 hours
Week 2	White noise exposure - 12 hours	Space reduction - 12 hours	Damp saw dust in cage -24 hours	45^o cage tilt -12 hours	Food and Water Deprivation - 24 hours	Overnight illumination - 12 hours	Exposure to empty bottle 2 hours
Week 3	Overnight illumination - 12 hours	Space reduction - 12 hours	Food and Water Deprivation - 24 hours	White noise exposure – 12 hours	Exposure to empty bottle - 2 hours	Light and dark succession -Every 2 hours for 10 hours	Damp saw dust in cage - 24 hours
Week 4	Damp saw dust in cage - 24 hours	45^o cage tilt – 12 hours	Light and dark succession -Every 2 hours for 10 hours	White noise exposure – 12 hours	Overnight illumination - 12 hours	Food and Water Deprivation - 24 hours	Space reduction -12 hours
Week 5	Light and dark succession - Every 2 hours for 10 hours	Space reduction -12 hours	Food and Water Deprivation - 24 hours	45^o cage tilt – 12 hours	Damp saw dust in cage -24 hours	Space reduction -12 hours	Overnight illumination - 12 hours
Week 6	Overnight illumination - 12 hours	White noise exposure – 12 hours	45^o cage tilt – 12 hours	Damp saw dust in cage - 24 hours	Space reduction -12 hours	Light and dark succession -Every 2 hours for 10 hours	Food and Water Deprivation - 24 hours
Week 7	Damp saw dust in cage - 24 hours	45^o cage tilt – 12 hours	Food and Water Deprivation - 24 hrs	Space reduction -12 hours	Overnight illumination - 12 hours	Exposure to empty bottle - 2 hours	White noise exposure – 12 hours

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

SUCROSE PREFERENCE TEST (SPT)[72-75]

During this test, rats were given 1% sucrose solution for 24 hours.

Then both sucrose solution and fresh water were made available to rats for another 24 hours.

After depriving the rats of drinking for 23 hours, the rats were given both 1% sucrose solution and fresh water for 1 hour again.

Sucrose preference will be calculated as = Sucrose intake (ml)/ [sucrose intake(ml)/water intake(ml)] x100%

Principle:

Chronic Unpredictable Mild Stress (CUMS) causes anhedonia in rats, which is a condition comparable to clinical depression. Anhedonia is assessed by quantifying the intake of sucrose water compared to regular water. Usually, healthy rats demonstrate a preference for sweetened sucrose water over regular water. But rats in the state of anhedonia tend to consume less sucrose water and more normal water.[76]

Following the sucrose preference test at seventh week, animals were euthanized using Thiopentone sodium administered intraperitoneally at a dose of 120 mg/kg followed by decapitation. The entire brain was dissected out of the skull and further dissection for hippocampus was carried out on a cold plate.[77-79]The isolated hippocampus was immediately

immersed in triazole reagent contained in an Eppendorf tube and stored at -80^{o}C for gene expression analysis study.

Figure 4: Sucrose preference test.

Gene Expression Analysis

RNA extraction:

RNA was extracted from tissue specimen by using The RNeasy Mini Kit The stepwise protocol is as follows:

Tissue samples were processed for RNA extraction using the RNeasy Mini Kit.

Here is the step-by-step protocol:

1. A maximum of 20 milligrams of tissue was utilized. In 350µl of Buffer RLT, the tissue was broken down and the resulting lysate was mixed thoroughly. Centrifuged at full speed for 3 minutes, the lysate was removed. Carefully removing the supernatant with a pipette allowed it to be utilized in step 2.
2. The lysate was thoroughly mixed with 1L of 70% ethanol using a pipette. Step 3 was immediately implemented.
3. A RNeasy Mini spin column, which was put in a 2 ml collection tube, was used to transfer up to 700 µl of the sample, which included any precipitate. The column was centrifuged at ≥8000 x g for 15 seconds with the lid closed. We threw out the flow-through.
4. The RNeasy spin column was supplemented with 700 µl of Buffer RW1. The column was centrifuged at ≥8000 x g for 15 seconds with the lid closed. We threw out the flow-through.
5. Half a millilitre of RPE Buffer was added to the RNeasy spin column. The column was centrifuged at ≥8000 x g for 15 seconds with the lid closed. Its flow-through was thrown out.
6. Half a millilitre of RPE Buffer was added to the RNeasy spin column. The column was centrifuged at ≥8000 x g for 2 minutes with the lid closed.

7. In a fresh 1.5 ml collection tube, the RNeasy spin column was inserted. Directly onto the spin column membrane, 30 µl of water that was devoid of RNase was introduced. To extract the RNA, the column was spun at ≥8000 x g for 1 minute with the lid closed.

cDNA Conversion

cDNA conversion was performed using the PrimeScript RT Reagent Kit.

The reaction mixture was prepared on ice, and the volume recommendations used per reaction are as follows:

Reagent	Volume	Final Concentration
5X PrimeScript Buffer (for Real Time)	2 µl	1X
PrimeScript RT Enzyme Mix I	0.5 µl	
Oligo dT Primer (50 µM)	0.5 µl	25 pmol
RNase-Free dH2O	1.5 µl	
RNA Template	5 µl	
RNA Template	5 µl	
Total	10 µl	

The tube was briefly centrifuged, and then incubated under the following conditions:

1. **Reverse transcription**: 37°C for 15 minutes
2. **Inactivation of reverse transcriptase with heat treatment**: 85°C for 5 seconds
3. **Storage**: 4°C

cDNA samples were stored at -20°C until further use in PCR.

Real-Time PCR

Target Genes: Neuritin, NARP, BDNF Exon -III

Primers for these genes were obtained from Bioserve Biotechnologies India Pvt Ltd, Hyderabad.

Primer Sequences [80-82]

β-actin primers:[83]

- **Forward primer**: - GCCCTGGCACCCAGCACAAT
- **Reverse primer:** - GGAGGGGCCGGACTCGTCAT

Neuritin Primer:

- **Forward Primer:** GGGACTTAAGTTGAACGGCA
- **Reverse Primer:** ACCCAGCTTGAGCAAACAGT

NARP Primer:

- **Forward Primer:** GGCAAGATCAAGAAGACGTTG
- **Reverse Primer:** TCCAGGTGATGCAGATATGGT

BDNF Exon -III:

- **Forward Primer:** TGCGAGTATTACCTCCGCCAT

- **Reverse Primer:** AGGATGGTCATCACTCTTCTC

Real-time PCR was carried out.

Procedure

1. A plate layout was prepared in the RealPlex software before the preparation of the master mix.

2. The master mix was prepared on ice.

The tubes were briefly centrifuged and then placed in the RealPlex Master Cycler (Eppendorf, Hamburg, Germany).

PCR Conditions

Initial Denaturation: 95°C for 30 seconds

Cycling (40 cycles):

- 95°C for 20 seconds
- 60°C for 30 seconds
- 72°C for 30 seconds

Melting Curve (Dissociation Curve): 60°C to 95°Cover for 20 minutes

cDNA samples were stored at -20°C until further use in PCR.

All the reactions were run in duplicates. Positive reaction was detected by accumulation of a fluorescent signal in the form of an amplification plot obtained in the Realplex software. Gene expression was calculated as fold change in increase/decrease in gene expression.

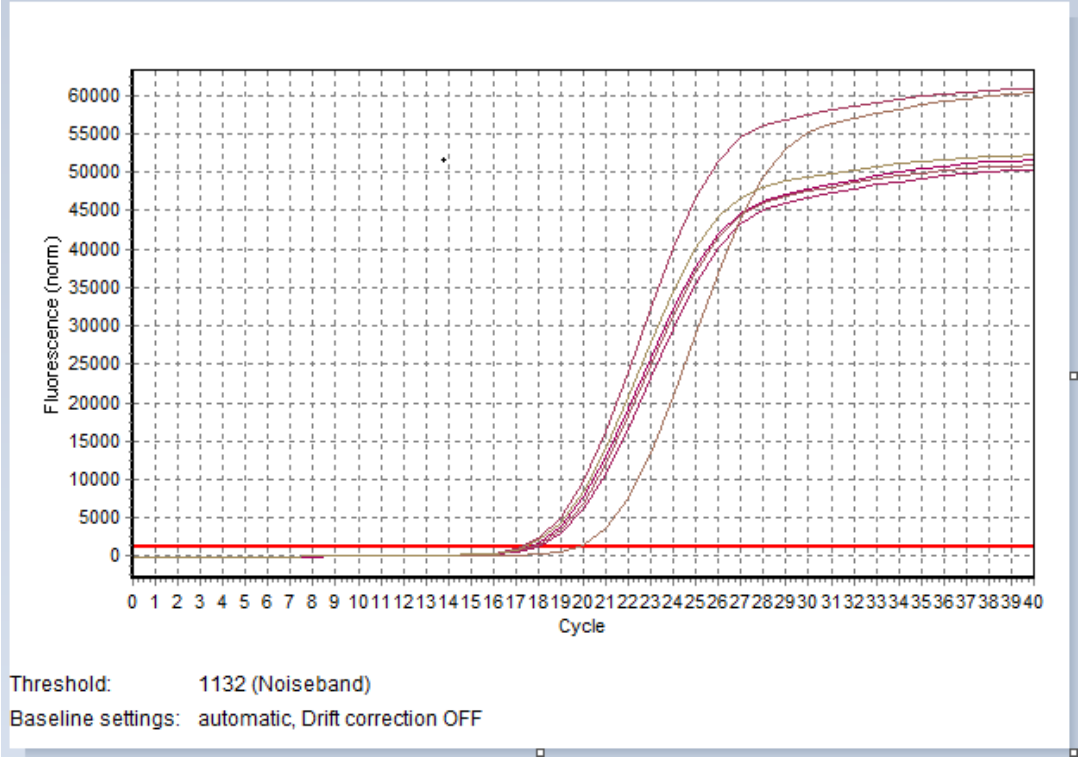


Figure 5: RT PCR Amplification plot

Data processing and analysis / statistical analysis:

All data was expressed as the Mean ± standard error of mean (S.E.M). The data was analysed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. $p < 0.05$ was considered statistically significant. Data analysis was conducted using Graph Pad Prism software – version 10.2 (Graph Pad software, 3rd order polynomial, San Diego, California, USA)

RESULTS

Results of Sucrose Preference Test

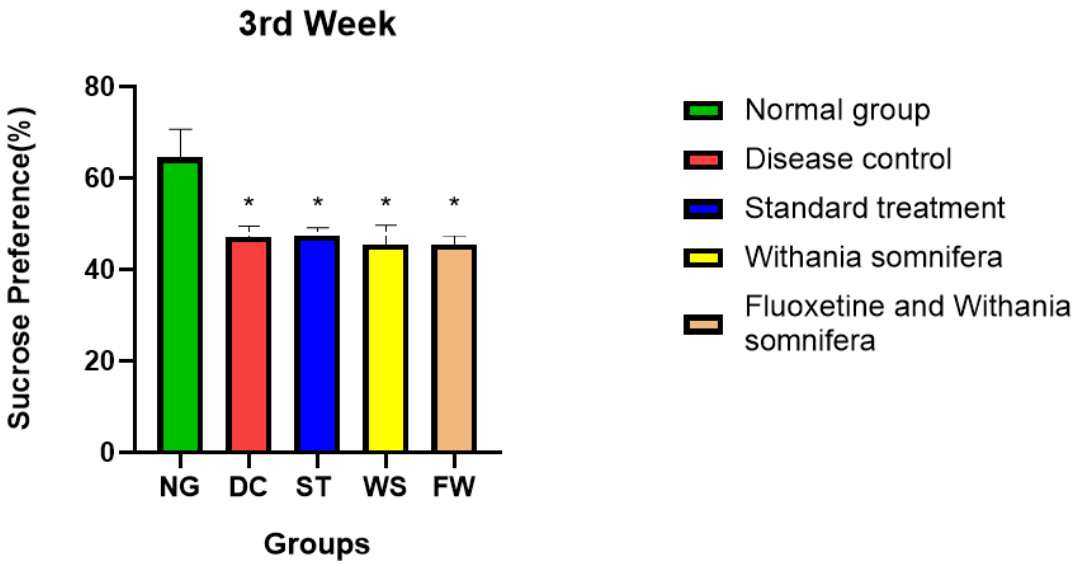
- Sucrose Preference Test was done at the end of 3rd week.
- One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare sucrose preference of Disease Control group, Standard Treatment group, *Withania somnifera* group and combination of Fluoxetine and *Withania somnifera* with Normal Group. [Table 6, Graph1]

Table 6: Effect of Chronic unpredictable mild stress (CUMS) on Sucrose Preference Test (SPT) at the end of 3rd week.

Groups	**Normal Group (NG)**	**Disease Control (DC)**	**Standard Treatment (ST)**	***Withania somnifera* (WS)**	**Fluoxetine and *Withania somnifera* (FW)**	**ANOVA p value**
Sucrose Preference in percentage. (Mean ± S.E.M)	64.48 ± 2.541	47.12 ± 0.9611 *	47.45 ± 0.6886 *	45.54 ± 1.692 *	45.55 ± 0.7567 *	< 0.0001

Data expressed as Mean± S.E.M (n=6). Data analyzed by One way ANOVA followed by Tukey's multiple comparison test. *p < 0.0001 when compared to normal group.

Graph 1: Effect of Chronic unpredictable mild stress (CUMS) on Sucrose Preference Test (SPT) at end of 3rd week.



Data expressed as Mean± S.E.M (n=6). Data analyzed by One way ANOVA followed by Tukey's multiple comparison test. *p < 0.0001 when compared to normal group.

Gene expression: -

Neuritin gene expression: -

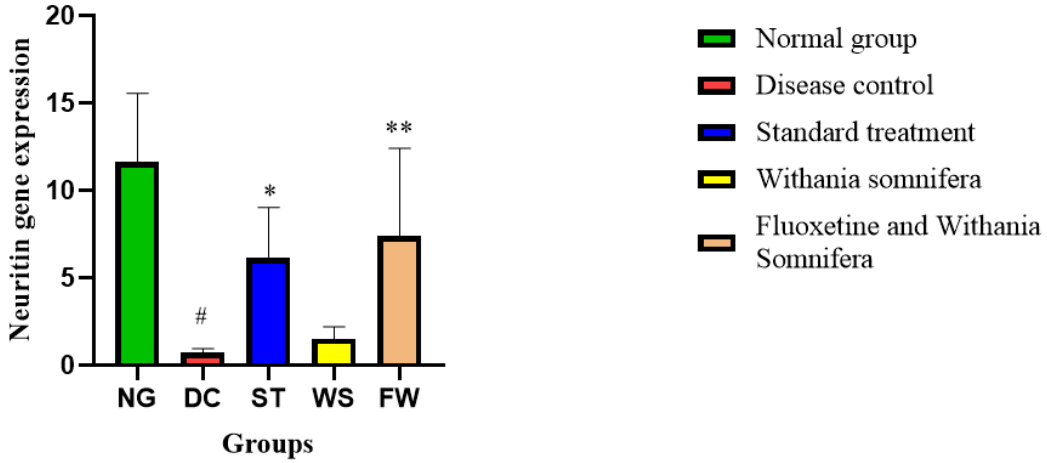
- Neuritin gene expression levels were measured by Real-time PCR at the end of treatment from the hippocampus tissue of depressed rats.
- One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the Neuritin gene expression levels of Standard Treatment group, *Withania somnifera* group and combination of Fluoxetine and *Withania somnifera* with Normal Group and Disease Control group.[Table 7, Graph 2]

Table 7: Effect of various drugs on Neuritin gene expression in hippocampus of rats.

Groups	**Normal Group (NG)**	**Disease Control (DC)**	**Standard Treatment (ST)**	***Withania somnifera* (WS)**	**Fluoxetine and *Withania somnifera* (FW)**	**ANOVA p value**
Gene expression (Mean± S.E.M)	11.62 ± 1.605	0.7244 ± 0.09828 #	6.122 ± 1.183 *	1.521 ± 0.2783	7.389 ± 2.05 **	< 0.0001

Data expressed as Mean ± S.E.M (n=6). Data analysed by One way ANOVA 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.

Graph 2: Effect of various drugs on Neuritin gene expression in hippocampus of rats.



Data expressed as Mean ± S.E.M (n=6). Data analysed by One way ANOVA 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.

NARP expression-

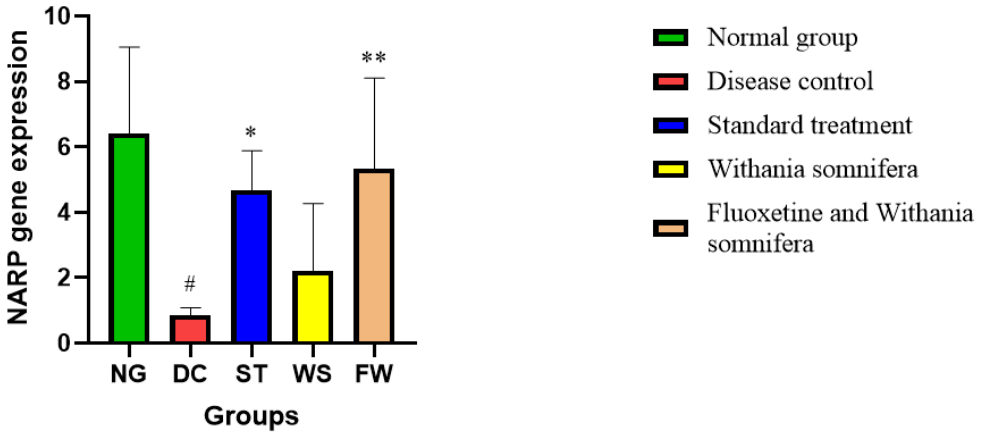
- NARP gene expression levels were measured by Real-time PCR at the end of treatment from the hippocampus tissue of depressed rats.
- One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the NARP gene expression levels of Standard Treatment group, *Withania somnifera* group and combination of Fluoxetine and *Withania somnifera* with Normal Group and Disease Control group. [Table 8, Graph 3]

Table 8: Effect of various drugs on NARP gene expression in hippocampus of rats.

Groups	**Normal Group (NG)**	**Disease Control (DC)**	**Standard Treatment (ST)**	***Withania somnifera* (WS)**	**Fluoxetine and *Withania somnifera* (FW)**	**ANOVA p value**
Gene expression (Mean ± S.E.M)	6.403± 1.082	0.8526± 0.09432 #	4.67± 0.4985 *	2.22 ± 0.837	5.331 ± 1.133 **	< 0.001

Data expressed as mean ± S.E.M (n=6). Data analysed by One way ANOVA 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 3: Effect of various drugs on NARP gene expression in hippocampus of rats.



Data expressed as Mean ± S.E.M (n=6). Data analysed by One way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$compared to the disease control group; # $p < 0.0001$ when compared to normal group.

BDNF EXON – III expression-

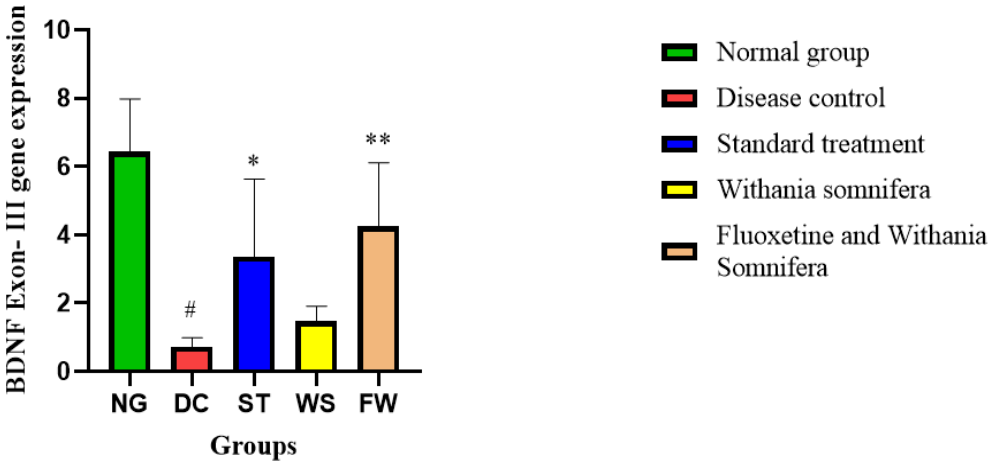
- BDNF Exon-III gene expression levels were measured by Real-time PCR at the end of treatment from the hippocampus tissue of depressed rats.
- One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare the BDNF Exon- III gene expression levels of Standard Treatment group, *Withania somnifera* group and combination of Fluoxetine and *Withania somnifera* with Normal Group and Disease Control group. [Table 9, Graph 4]

Table 9: Effect of various drugs on BDNF Exon – III gene expression in hippocampus of rats.

Groups	**Normal Group (NG)**	**Disease Control (DC)**	**Standard Treatment (ST)**	***Withania somnifera* (WS)**	**Fluoxetine and *Withania somnifera* (FW)**	**ANOVA** **p value**
Gene expression (Mean± S.E.M)	6.435 ± 0.6311	0.7125 ± 0.1126 #	3.366 ± 0.9238 *	1.465 ± 0.1812	4.248 ± 0.7602 **	< 0.0001

Data are expressed as Mean ± S.E.M (n=6). Data analysed by One way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$compared to the disease control group; # $p< 0.0001$ when compared to normal group.

Graph 4: Effect of various drugs on BDNF Exon – III gene expression in hippocampus of rats.



Data are expressed as Mean ± S.E.M (n=6). Data analysed by One way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$compared to the disease control group; # $p< 0.0001$ when compared to normal group.

Sucrose preference test at the end of 7th week.

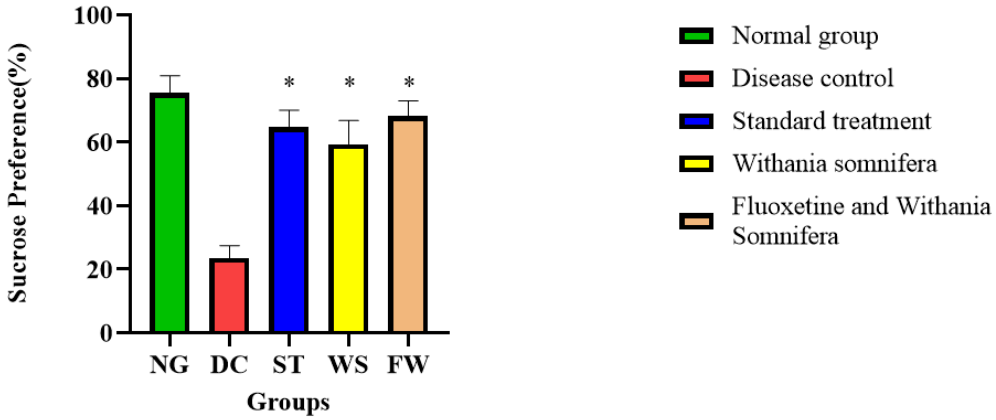
- Sucrose Preference Test was done at the end of 7th week.
- One-way ANOVA followed by Tukey's multiple comparisons test was performed to compare sucrose preference of Standard Treatment group, *Withania somnifera* group and combination of Fluoxetine and *Withania somnifera* with Normal Group and Disease control group.[Table 10, Graph 5]

Table 10: Effect of various drugs on sucrose preference at the end of 7th week.

Groups	**Normal Group (NG)**	**Disease Control (DC)**	**Standard Treatment (ST)**	***Withania somnifera* (WS)**	**Fluoxetine and *Withania somnifera* (FW)**	**ANOVA** **p value**
Sucrose Preference in percentage. (Mean ± S.E.M)	75.50 ± 2.205	23.57 ± 1.587	64.59 ± 2.235 *	59.40 ± 3.005 *	68.10 ± 1.998 *	< 0.0001

Data expressed as Mean± S.E.M (n=6). Data analysed by One way ANOVA followed by Tukey's multiple comparison test. * $p < 0.0001$ when compared with disease control group

Graph 5: Effect of various drugs on sucrose preference at the end of 7th week.



Data expressed as Mean± S.E.M (n=6). Data analysed by One way ANOVA followed by Tukey's multiple comparison test. * $p < 0.0001$ when compared with disease control group.

DISCUSSION

Depression, an increasingly prevalent mental health disorder, is progressively impacting communities globally, with its frequency and consequences increasing each year. The progress in comprehending and managing depression has undergone substantial advances. However, the worldwide impact of this condition continues to increase, demanding continuous attempts in research, treatment, and awareness.

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 chronic unpredictable mild stress (CUMS) as a model of depression. The secondary objective was to the study the interaction between *Withania Somnifera* and Fluoxetine at the genetic level by administering them in combination.

Withania somnifera, commonly known as Ashwagandha, whose roots, leaves and extracts are used as a source of antidepressant in several model system. The bioactive compounds in *Withania somnifera*, such as withanolides, contribute to its neuroprotective, anti-inflammatory, and adaptogenic properties.[84]

It also possesses antidepressant property; the underlying pathways are not well understood. The literature reviewed suggests that no study has been done so far to investigate the effect of *Withania somnifera* on gene expression of the genes selected in the present study.

Brain-derived neurotrophic factor (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 depression disorder (MDD). 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. Hence the present study was planned to investigate the effect of *Withania Somnifera* on expression of genes encoding BDNF, namely Neuritin, NARP and BDNF Exon III.

In this study, Chronic Unpredictable Mild Stress (CUMS) model was used to establish depression. It involves exposing animals to various stress factors like food and water deprivation, exposure to empty bottle, damp sawdust in cage, light and dark succession, space reduction, 45°cage tilt, overnight illumination, white noise exposure in an unpredictable manner. This exposure causes depression by continuously overwhelming the brain's stress response systems, which leads to ongoing disturbances in the reward pathways causing anhedonia. Moreover, it disrupts the control of sleep and homeostasis, leading to notable changes in mood and behaviours that are characteristic of depressive disorders.[85-88]

Symptoms observed in acute models can sometimes overlap with anxiety symptoms, leading to confusion amongst investigators in differentiating between depression and anxiety-related behaviours. These acute models provide only specific endpoints that may not fully capture the complexity of depressive symptoms and behaviours.[89,90] Hence, chronic model of depression was used in this study.

To check for establishment of depression, sucrose preference test was done. It is widely acknowledged as an objective measure to assess the establishment of depression and the effectiveness of antidepressants in experimental animals. 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. In the study by Fernandez JW et al., the SPT was effectively used to assess postpartum depression in rats, replicating the outcomes seen in other models of

depression. Our study also demonstrated that the chronic unpredictable mild stress (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 three weeks.

Effect on Neuritin expression: -

In our assessment for Neuritin gene, we observed that there was statistically significant upregulation in Neuritin gene expression in the Standard Treatment (ST) group and the combination group (FW) compared to Disease Control (DC) group.

The comparison between the Normal group (NG) & combination group (FW) group showed negligible difference of Neuritin gene expression, indicating that the combination group (FW) group effectively brought Neuritin gene expression levels to near normal.

Effect on NARP expression: -

In our analysis of NARP gene expression, we observed that there was a statistically significant upregulation seen in Standard Treatment (ST) group & combination group (FW) when compared with Disease Control (DC) group.

The comparison between the Normal group (NG) & combination group (FW) group showed negligible difference of NARP gene expression, indicating that the combination group (FW) group effectively brought NARP gene expression levels to near normal.

Effect on BDNF Exon- III expression: -

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

The comparison between the Normal group (NG) & combination group (FW) group showed negligible difference of 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 expression of Neuritin, NARP, and BDNF Exon -III gene seen with Withania somnifera (WS) group, but it was not statistically significant.

The results of our investigation are consistent with earlier research undertaken by Dwivedi Y et al. and Molteni R et al. Dwivedi Y et al. conducted a study on rats to examine the effect of Fluoxetine on gene expression associated with brain-derived neurotrophic factor (BDNF). According to their findings, Fluoxetine caused an increase in the expression of genes related to the production of BDNF.[91]

A study conducted by Molteni R 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.[92]

Effect on sucrose preference test: - At the end of seven weeks, there was an increase in sucrose preference seen in Standard Treatment group (ST), *Withania somnifera*(WS) group & the combination (FW) group which was statistically significant compared to the Disease Control (DC) group. The comparison between the Normal group (NG) & 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 *Withania somnifera* and its combination Fluoxetine.

The findings of a study conducted by KrishnaRaju AV et al. supported the use of *Withania somnifera* in the treatment of depression, highlighting its anti-inflammatory and antioxidant properties, which contribute significantly to its therapeutic potential. It was

found that *Withania somnifera* decreases pro-inflammatory cytokines and oxidative stress indicators, both of which are commonly elevated among people suffering from depression.[93]

Furthermore, a study conducted by Jain et al. demonstrated that *Withania somnifera* is neuroprotective in the hippocampus by lowering the amount of degenerating hippocampal cells. This study discovered that *Withania somnifera* improves neuronal survival and reduces apoptosis in the hippocampus, which is crucial for mood regulation and cognitive function.[94]The neuroprotective effects of *Withania somnifera* are due to its capacity to boost antioxidant defences and regulate the expression of neurotrophic factors, which promote neuronal growth and functioning.[95]

The findings of the present study have found that *Withania somnifera* 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 in order 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 drug on other well-known markers for depression viz cortisol, glutamate, monoamines and GABA levels.

CONCLUSION

In conclusion, this study has improved our understanding of *Withania somnifera's* antidepressant action, by investigating its influence on the gene expression of Neuritin, NARP and BDNF Exon -III which are required for 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 treatment of depression.

SUMMARY

The present study was designed to evaluate the effect of *Withania somnifera* (WS) alone and in combination with Fluoxetine on gene expression of Neuritin, NARP & BDNF Exon – III genes in hippocampus of male Wistar rats using chronic unpredictable mild stress (CUMS) as a model of depression.

The study included 30 male Wistar rats divided into 5 groups of 6 rats in each. The groups were Normal Group (NG), Disease Control (DC) group, Standard Treatment (ST) group, *Withania somnifera* (WS) group and combination of Fluoxetine and *Withania somnifera* (FW) group. Depression was induced in all groups by using chronic unpredictable mild stress (CUMS) except in the normal group. Sucrose preference test (SPT) was done at the end of third week to test for establishment of depression. At the end of seventh week, SPT was again done to check for reduction of depression in all treatment groups. Lastly all rats were euthanized and the hippocampal tissue was used for gene expression analysis which was performed using Real – time PCR.

We found that the Standard Treatment (ST) group, and the combination group showed statistically significant upregulation of all three genes namely, Neuritin, NARP and BDNF Exon -III. The comparison between the Normal group (NG) & combination group (FW) group showed negligible difference of gene expression of all three genes , indicating that the combination group (FW) group effectively brought gene expression levels of all three genes to near normal.

At the end of the seventh week there was also a statistically significant increase in sucrose preference seen in all treatment groups compared to Disease control (DC) group . However, the comparison between the Normal group (NG) & 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. According to the findings of the study, combination of Fluoxetine and *Withania somnifera* (FW) is a viable choice as it had an additive effect when used in the treatment of depression.

This study has improved our understanding of the mechanism of action as an antidepressant of *Withania somnifera*, by investigating its influence on the gene expression of Neuritin, NARP and BDNF Exon -III which are required for production of BDNF.

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ANNEXURE – I

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
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JNMC,Belagavi

Dr. Vishwanatha Swamy
AHM
Link Nominee CPCSEA.
Dept of Pharmacology &
Toxicology
KLE's Coll Of Pharmacy,
Hubballi

CERTIFICATE

This is to certify that the M.D/ M.D.S/ Ph.D/ Research project Entitled " Effect of Withania somnifera on selected Gene expression in brain tissue of Male Wistar Rats subjected to chronic stress induced depression.'

Submitted by PG Pharmacology, JNMC.

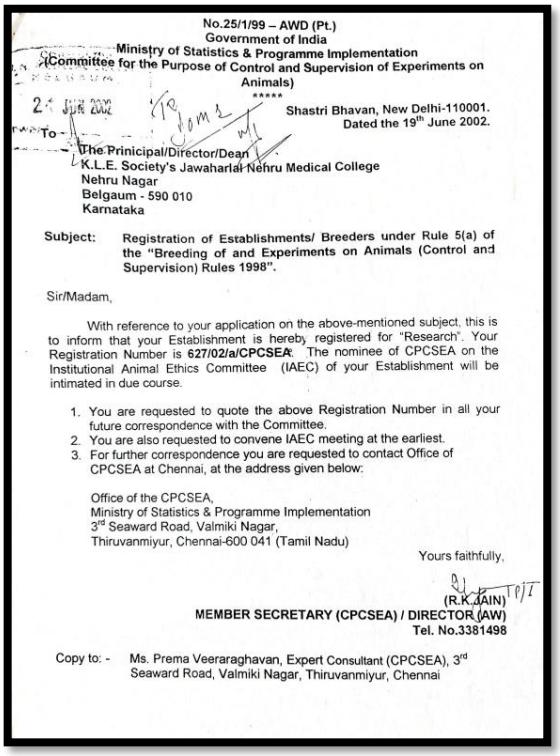
Has been approved by the Institutional Animal Ethical Committee Meeting held on 25-6-22 vide Resolution No. 17/2.

For sanction of 30 Male Wistar Rats

Main Nominee CPCSEA
IAEC-JNMC, Belagavi.
Signature and Name
CPCSEA-Main Nominee

Member Secretary
IAEC-JNMC, Belagavi.
Signature and Name
Chairman/Mem.Secretary

ANNEXURE-II



No.25/1/99 – AWD (Pt.)
Government of India
Ministry of Statistics & Programme Implementation
(Committee for the Purpose of Control and Supervision of Experiments on Animals)
*****
Shastri Bhavan, New Delhi-110001.
Dated the 19th June 2002.

To -
The Principal/Director/Dean
K.L.E. Society's Jawaharlal Nehru Medical College
Nehru Nagar
Belgaum - 590 010
Karnataka

Subject: **Registration of Establishments/ Breeders under Rule 5(a) of the "Breeding of and Experiments on Animals (Control and Supervision) Rules 1998".**

Sir/Madam,

With reference to your application on the above-mentioned subject, this is to inform that your Establishment is hereby registered for "Research". Your Registration Number is **627/02/a/CPCSEA**. The nominee of CPCSEA on the Institutional Animal Ethics Committee (IAEC) of your Establishment will be intimated in due course.

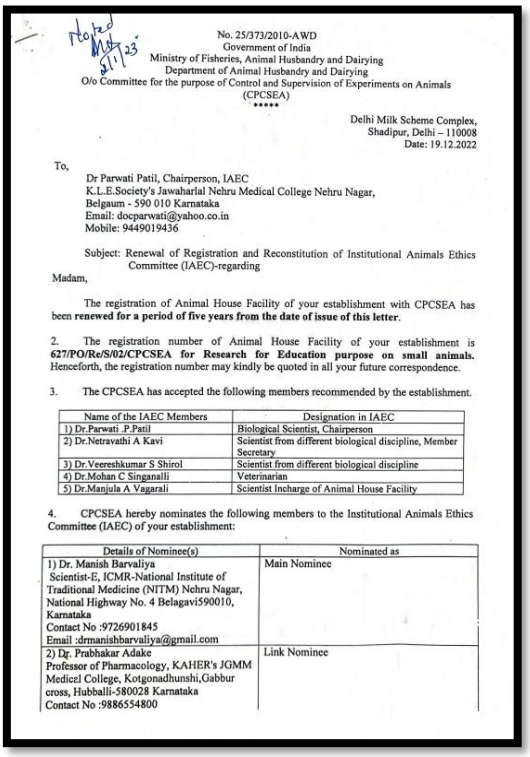
1. You are requested to quote the above Registration Number in all your future correspondence with the Committee.
2. You are also requested to convene IAEC meeting at the earliest.
3. For further correspondence you are requested to contact Office of CPCSEA at Chennai, at the address given below:

Office of the CPCSEA,
Ministry of Statistics & Programme Implementation
3rd Seaward Road, Valmiki Nagar,
Thiruvanmiyur, Chennai-600 041 (Tamil Nadu)

Yours faithfully,

(R.K. JAIN)
MEMBER SECRETARY (CPCSEA) / DIRECTOR (AW)
Tel. No.3381498

Copy to: - Ms. Prema Veeraraghavan, Expert Consultant (CPCSEA), 3rd Seaward Road, Valmiki Nagar, Thiruvanmiyur, Chennai



No. 25/373/2010-AWD
Government of India
Ministry of Fisheries, Animal Husbandry and Dairying
Department of Animal Husbandry and Dairying
O/o Committee for the purpose of Control and Supervision of Experiments on Animals
(CPCSEA)
*****

Delhi Milk Scheme Complex,
Shadipur, Delhi – 110008
Date: 19.12.2022

To,

Dr Parwati Patil, Chairperson, IAEC
K.L.E.Society's Jawaharlal Nehru Medical College Nehru Nagar,
Belgaum - 590 010 Karnataka
Email: docparwati@yahoo.co.in
Mobile: 9449019436

Subject: Renewal of Registration and Reconstitution of Institutional Animals Ethics Committee (IAEC)-regarding

Madam,

The registration of Animal House Facility of your establishment with CPCSEA has been **renewed for a period of five years from the date of issue of this letter.**

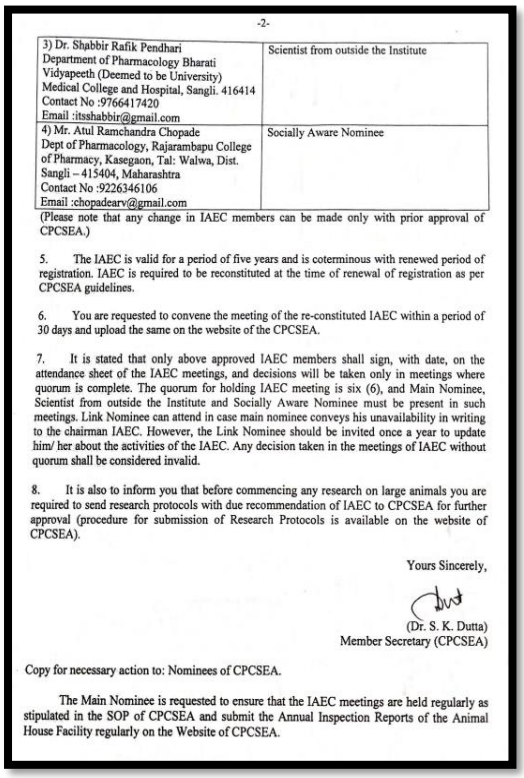
2. The registration number of Animal House Facility of your establishment is **627/PO/Re/S/02/CPCSEA for Research for Education purpose on small animals.** Henceforth, the registration number may kindly be quoted in all your future correspondence.

3. The CPCSEA has accepted the following members recommended by the establishment.

Name of the IAEC Members	Designation in IAEC
1) Dr.Parwati .P.Patil	Biological Scientist, Chairperson
2) Dr.Netravathi A Kavi	Scientist from different biological discipline, Member Secretary
3) Dr.Veereshkumar S Shirol	Scientist from different biological discipline
4) Dr.Mohan C Singanalli	Veterinarian
5) Dr.Manjula A Vagarali	Scientist Incharge of Animal House Facility

4. CPCSEA hereby nominates the following members to the Institutional Animals Ethics Committee (IAEC) of your establishment:

Details of Nominee(s)	Nominated as
1) Dr. Manish Barvaliya Scientist-E, ICMR-National Institute of Traditional Medicine (NITM) Nehru Nagar, National Highway No. 4 Belagavi590010, Karnataka Contact No :9726901845 Email :drmanishbarvaliya@gmail.com	Main Nominee
2) Dr. Prabhakar Adake Professor of Pharmacology, KAHER's JGMM Medicel College, Kotgonadhunshi,Gabbur cross, Hubballi-580028 Karnataka Contact No :9886554800	Link Nominee



-2-

3) Dr. Shabbir Rafik Pendhari Department of Pharmacology Bharati Vidyapeeth (Deemed to be University) Medical College and Hospital, Sangli. 416414 Contact No :9766417420 Email :itsshabbir@gmail.com	Scientist from outside the Institute
4) Mr. Atul Ramchandra Chopade Dept of Pharmacology, Rajarambapu College of Pharmacy, Kasegaon, Tal: Walwa, Dist. Sangli – 415404, Maharashtra Contact No :9226346106 Email :chopadearv@gmail.com	Socially Aware Nominee

(Please note that any change in IAEC members can be made only with prior approval of CPCSEA.)

5. The IAEC is valid for a period of five years and is coterminous with renewed period of registration. IAEC is required to be reconstituted at the time of renewal of registration as per CPCSEA guidelines.

6. You are requested to convene the meeting of the re-constituted IAEC within a period of 30 days and upload the same on the website of the CPCSEA.

7. It is stated that only above approved IAEC members shall sign, with date, on the attendance sheet of the IAEC meetings, and decisions will be taken only in meetings where quorum is complete. The quorum for holding IAEC meeting is six (6), and Main Nominee, Scientist from outside the Institute and Socially Aware Nominee must be present in such meetings. Link Nominee can attend in case main nominee conveys his unavailability in writing to the chairman IAEC. However, the Link Nominee should be invited once a year to update him/ her about the activities of the IAEC. Any decision taken in the meetings of IAEC without quorum shall be considered invalid.

8. It is also to inform you that before commencing any research on large animals you are required to send research protocols with due recommendation of IAEC to CPCSEA for further approval (procedure for submission of Research Protocols is available on the website of CPCSEA).

Yours Sincerely,

(Dr. S. K. Dutta)
Member Secretary (CPCSEA)

Copy for necessary action to: Nominees of CPCSEA.

The Main Nominee is requested to ensure that the IAEC meetings are held regularly as stipulated in the SOP of CPCSEA and submit the Annual Inspection Reports of the Animal House Facility regularly on the Website of CPCSEA.