Exploring the Antidepressant Effects of Luteolin in Mice Model of Depression

Master of Science in Healthcare Biotechnology

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A thesis submitted in partial fulfillment of the requirement for the degree of

Masters of Science in Healthcare Biotechnology

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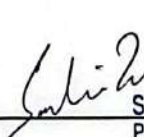
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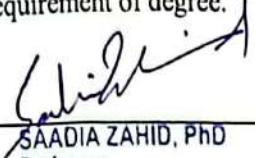
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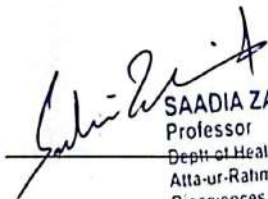
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"I dedicate this thesis to my parents, whose unwavering support, encouragement, and love have been the driving forces behind my academic journey."

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5-HT	5-hydroxytryptamine
ATF6	Activating Transcription Factor 6
ACTH	Adrenocorticotrophic Hormone
AD	Alzheimer's Disease
ADRD	Alzheimer's Disease Related Dementia
ANOVA	Analysis Of Variance
BDNF	Brain Derived Neurotrophic Factor
BrdU	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
CNS	Central Nervous System
CMS	Chronic Mild Stress
CRS	Chronic Restraint Stress
CUMS	Chronic Unpredictable Mild Stress
CUS	Chronic Unpredictable Stress
cDNA	Complementary DNA
CA3	Cornu Ammonis 3
CRH	Corticotrophin Releasing Hormone
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
DSS	Dextran Sulphate Sodium
DA	Dopamine
DCX	Doublecortin

ER	Endoplasmic Reticulum
FXT	Fluoxetine
H & E	Hematoxylin & Eosin
HPA	Hypothalamic Pituitary Adrenal Axis
IRB	Institutional Review Board
IL-10	Interleukin 10
IL-6	Interleukin 6
i.p	Intraperitoneal
LOD	Late Onset Depression
LH	Learned Helplessness
LPS	Lipopolysaccharide
LUT	Luteolin
mRNA	Messenger RNA
μ	Micro
MDD	Mild Depressive Disorder
MAOIs	Monoamine Oxidase Inhibitors
MAOs	Monoamine Oxidases
NeuN	Neuronal Nuclear Protein
NE	Norepinephrine
NSFT	Novelty Suppressed Feeding Test
PFA	Paraformaldehyde
PD	Parkinson's Disease
PBS	Phosphate Buffered Saline

PTSD	Post Traumatic Stress Disorder
RT-PCR	Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
SCO	Scopolamine
SNRIs	Selective norepinephrine Reuptake Inhibitors
SSRIs	Selective Serotonin Reuptake Inhibitors
SPS	Single Prolonged Stress
SEM	Standard Error of Mean
STZ	Streptozotocin
SPT	Sucrose Preference Test
SST	Sucrose Splash Test
TST	Tail Suspension Test
TCAs	Tricyclic Antidepressants
TNF-α	Tumor Necrosis Factor Alpha
UPR	Unfolded Protein Response
UCMS	Unpredictable Chronic Mild Stress
WHO	World Health Organization

ABSTRACT

Depression is a complex and heterogenous mental health condition which affects the overall well-being of an individual by influencing thoughts, emotions, sleep, behavior, and physical health. Reports from the world health organization (WHO) have affirmed that the global impact of depression is substantially increasing to the level that it is anticipated that by 2020, it will become the primary cause of disease worldwide. Currently, the treatment of depression involves selective serotonin reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors, monoamine oxidase inhibitors, tricyclic and tetracyclic antidepressants. These synthetic antidepressants, although widely prescribed, have significant limitations since they focus exclusively on one aspect of pathogenesis, resulting in the majority of them exhibiting a slow-acting nature. Moreover, they have multiple side effects that include insomnia, nausea, weight changes and sexual dysfunction. In contrast, natural plant-based flavonoid compounds having lesser side effects are known to produce anti-depressant like effects by targeting multiple pathways simultaneously. The study explored the effect of intraperitoneal administration of 10mg/kg Luteolin (LUT) on nine weeks of unpredictable chronic mild stress (UCMS) mouse model of depression in comparison with Fluoxetine (FXT). The mice were assigned randomly to six distinct study groups, with each group consisting of six mice (n=6). Coat state was examined each week as an indicator of stress. The UCMS group showed significantly altered coat state from week 4 (1.50 ± 0.183) ($p<0.001$) till week 9 (1.583 ± 0.154) ($p<0.0001$). The chronic LUT treatment showed significant ($p<0.01$) improvement in the coat state in the ninth week of UCMS (0.583 ± 0.154). Behavioral tests were carried out to evaluate the impact of UCMS and LUT on anhedonia, anxiety, short-term spatial memory, helplessness, and behavioral

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despair through SST, NSFT, Y-maze test, TST, and FST. The LUT treated UCMS group indicated significantly (p<0.0001) reduced anhedonia by increasing the grooming time (185±9.473 seconds) and decreasing the latency to initiate grooming behavior (p<0.001) (2±0.4472 seconds). The present study however showed no significant impairment in anxiety and short-term spatial memory in UCMS in comparison to control group. The results in TST indicated significant reduction (p<0.0001) in immobility time in UCMS+LUT treated group when compared to UCMS (98±9.2 seconds). The latency to immobility was significantly (p<0.05) increased in FST after LUT treatment (29±3.5 seconds) while the immobility time (p<0.0001) (20±5.3) and number of immobile episodes (p<0.001) (8.8±1.5) were significantly reduced when compared to UCMS group. Histopathological assessment using hematoxylin and eosin staining showed significant (p<0.0001) reduction in neuronal cells in the hippocampal dentate gyrus area in UCMS-treated group (13±0.865), however the LUT treated UCMS group significantly (p<0.0001) restored this neuronal damage (25.00±1.683). For investigating the role of LUT on neurogenesis and endoplasmic reticulum (ER) stress, the expression levels of NeuN and ATF6 were examined through real time quantitative PCR. The LUT treated UCMS group (1.0±0.030) showed significant (p<0.01) improvement in NeuN expression in comparison to UCMS group (0.38±0.051). The ATF6 levels were significantly (p<0.001) decreased after LUT administration in UCMS group (3.3±0.018) indicating its potential role in neurogenesis by inhibiting the ER stress.

The results of the study strongly indicated the potential antidepressant effects of LUT by reducing anhedonia and improving helplessness and behavioral despair through behavioral

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analysis in UCMS model of depression. Meanwhile, it also exhibits neuroprotective role by significantly improving neurogenesis along with enhancing the NeuN expression in the hippocampus. The study also suggests its potential involvement in alleviating the observed ER stress in depression. Hence, LUT can serve as an effective treatment strategy for depression.

INTRODUCTION

Depression or major depressive disorder (MDD) is marked by a fusion of both physiological and psychological symptoms that impact the severity and prognosis of the illness (Lorenzo *et al*., 2023). It is a persistent, recurring, and serious mental health condition associated with elevated mortality rates and concurrent medical conditions (Gold *et al*., 2015). Moreover, it stands out as one of the prevalent mental disorders experienced throughout various stages of life (Lorenzo *et al*., 2023). Its occurrence differs among diverse populations, and recent data indicated a 10.4% prevalence within the past 12 months and a lifetime prevalence of 20.6% (Hasin *et al*., 2018). MDD, being one of the most prevalent conditions, significantly restricts psychological functioning and reduces the overall life quality or standard of living (Malhi & Mann, 2018). Some of the major symptoms of depression include anxiety, sleep disturbances, insomnia, anhedonia, dysfunctional sexual response, feeling sad or low emotional state, apathy, pain in muscles or joints, headache, fatigue, and limitation in daily and occupational activities (Paykel *et al*., 1995; Opdyke *et al*., 1995; Nierenberg *et al*., 1999). These symptoms elevated the likelihood of both impaired functioning and an increased risk of suicide (Trivedi *et al*., 2008). Depression not only induces significant mental distress but also disrupts essential biological processes that govern metabolism, inflammation, involuntary responses, coagulation, neuroendocrine system, sleep and appetite (LeDoux, 1995; Gold *et al.*, 2015). These disruptions eventually lead to various diseases like osteoporosis, coronary artery disease, and many other metabolic disorders (Gold *et al*., 2015). MDD also presents a significant risk of developing conditions like Alzheimer's disease and related dementia (ADRD) thus reflecting adverse health outcomes (Lorenzo *et al*., 2023).

The use of animal models in investigating the pathways or mechanisms of human illnesses and to identify new therapeutic drugs has long been in practice (Markov & Novosadova, 2022). In neuroscience, animal models play a crucial role particularly due to the difficulty in imagining the widespread of invasive procedures for studying the human brain (Markov & Novosadova, 2022). Researchers face considerable challenges in modeling depression in animals because of the heterogenous nature of depression (Van Loo *et al*., 2012). Moreover, several symptoms exhibited by depressed patients cannot be effectively modeled and assessed in animals. Furthermore, the underlying pathophysiological mechanisms related to the onset of depression are not completely comprehended (Markov & Novosadova, 2022). Nevertheless, it is now acknowledged that in humans stress elevates the risk of depression, leading to the frequent use of stress models in animal research. In 1892, R. Katz first proposed the chronic stress model of depression (Katz, 1982) while in 1987, changes in this model were made by P. Willner who included more naturalistic stressful factors in the existing model applied specifically in an unpredictable manner, thus naming it unpredictable chronic mild stress (UCMS) model (Willner *et al*, 1987). It is known to result in a number of behavioral, biochemical, neurobiological and physiological changes (Broekkamp, 1997). Th primary outcome of UCMS is anhedonia where one is unable to feel any pleasure. UCMS is reported to be the best model as it represents the most effective effort to replicate the human condition in animal models as it signifies the optimal endeavor to emulate the human condition in animal models despite it being highly sensitive to even minor alterations in design (Markov & Novosadova, 2022). Besides that, the reproducibility of UCMS is considered to be in question as the primary effects of UCMS are not successfully replicated by all laboratories (Markov & Novosadova, 2022).

The understanding of the pathophysiology of MDD has advanced significantly, however, one mechanism is not sufficient to adequately elucidate all facets of the condition (Kamran *et al*., 2022). Some of the diverse pathophysiological mechanisms in MDD involve biogenic amine hypothesis, neurotrophic hypothesis, cytokine or inflammatory hypothesis and endocrine hypothesis (Thompson *et al*., 2015). The endoplasmic reticulum (ER) stress response is said to have a significant role in the development of psychiatric diseases, including depression as it is a cellular mechanism through which cells guard themselves against various harmful insults that lead to the unfolding of proteins in the ER. ATF6 functions as a central regulator and activator of various other ER stress response elements (ERSEs) indicating its importance in unfolded protein (UPR) response (Doroudger *et al*., 2009).

Although MDD is a condition that can be treated, the effectiveness of treatments varies among individuals, with some responding positively and others not experiencing the same level of benefit (He *et al*., 2019). For the better management of MDD, both pharmacological and non-pharmacological interventions like psychotherapy, transcranial magnetic stimulation and electroconvulsive therapy are usually employed (Li *et al*., 2021; Okbay *et al*., 2016). Psychotherapy that encompasses both cognitive and interpersonal therapy is known to alleviate symptoms and enhance the overall quality of life for individuals experiencing MDD (Kolovos *et al*., 2016).

The most frequently prescribed antidepressants approved by the FDA for managing MDD include a diverse range of classes such as MAOIs, TCAs, SSRIS, SNRIs, and many other receptor agonists or antagonists (Bains & Abdijadid., 2020). The major problem with these conventional treatment options is that they specifically target only one aspect of

pathogenesis and hence are only effective in 50% of the total cases of MDD (Menard *et al*., 2016). This underscores the reality that MDD diagnosis relies entirely on observable behavioral symptoms. As a result, the therapeutic choices for MDD are not tailored to the specific pathology of the disorder (Kamran *et al*., 2022).

In contrast to synthetic medications, numerous plants contain flavonoids that exhibit the potential to act multiple molecular targets (Pannu *et al*., 2021). Flavonoids, being the most naturally occurring polyphenolic compounds, are known for their pharmacological properties (Harbone & Williams, 2000). Numerous studies in animal models of rodents depicted the antidepressant potential of specific flavonoids due to their ability to reverse depressive behaviors (Pannu *et al*., 2021). Luteolin, a natural flavone, is known to reduce inflammation, oxidative stress and exhibits cardioprotective effects (Achour *et al*., 2021; Wang *et al*., 2016). The neuroprotective role of Luteolin in Alzheimer's disease has already been reported where it reflected both its anti-oxidative and anti-inflammatory effects (Sawmiller *et al*., 2014; Wang *et al*., 2016). Despite the positive and promising benefits of Luteolin, its exact mechanism of action as an antidepressant still needs to be explored.

Aim and Objectives

The present study aimed to explore the antidepressant effects of LUT, natural plant-based flavone, on behavioral despair and neurogenesis in comparison to the standard drug, FXT, an SSRI, in the UCMS depression model.

Objectives of the study include:

- Exploring the antidepressant effects of LUT on anhedonia and behavioral despair displayed by the UCMS mice model via behavioral tests.

- Examining the effect of LUT and FXT treatment on neurodegeneration in the hippocampal region of mice brain.

- Investigating the effect of LUT on the mRNA expression of NeuN and ATF6.

LITERATURE REVIEW

2.1 Major Depressive Disorder

Major Depressive Disorder (MDD) is one of the most prevalent mental health conditions characterized by increased morbidity and mortality. The World Health Organization estimates that approximately 10% to 15% of the overall population will encounter clinical depression at some point in their lives (Tsuang *et al.*, 2002). According to WHO, around 5% of men and 9% of women are expected to experience a depressive disorder each year (Kessler *et al.*, 2005). It has been reported by WHO that around 350 million people are currently suffering from depression worldwide. A survey carried out in 17 countries concluded that 1 out of 17 people encountered at least a single depressive episode over a period of one year (Kessler & Ustun, 2008). The altered mood state in depression is known to be experienced by everyone at some point in their lifetime. Depression, however, is also characterized by biobehavioral or clinical syndrome referred to as major depressive disorder (Fava & Kendler, 2000). In United States, the prevalence of MDD is around 16.2% (Kessler *et al.*, 2003). MDD is ranked as the third highest contributor to the global disease burden and by 2030, it is expected to be the primary cause (Keller *et al.*, 1992).

The physical signs in depression are expressed with some level of consistency. These indicators include slowed movements, reduced expression, and gestures. Some of the major signs exhibited by individuals suffering from major depression include fatigue, self-preoccupation, reduced alertness or attentiveness, boredom along with a considerable lack of interest in their surrounding environment (Blacker & Clare, 1997). Anxiety is one of the key and significant features of the emotional state often expressed as pronounced levels of restlessness and agitation. The clinical signs of depression include muscle tension, hands

wringing, frequent episodes of crying and misery. In addition to this, physiological symptoms like increased heart rate, dry mouth or tongue, sweaty hands, cold skin, dilated pupils, tremors and altered blood pressure are also indicated in patients of mild depressive disorder (Gupta, 2009). The risk of suicide in depression ranges between 2.2% (in less severe cases) and 8.6% (in severe cases) (Bostwick *et al*., 2000). In US, suicide mark as one of the 10 major leading cause of death contributing to more than 30,000 deaths in a year (Kalpan *et al*., 1988).

2.2 Incidence of Depression

Women are recognized to experience depression more frequently than men (Woody *et al*., 2017). In US, women are known to experience about twice as much depression as in men over the past 30 years with a ratio of about 3:1 (Agostino *et al*., 2021). Consistently across all adult age groups, women were reported to have a two to three times higher likelihood of experiencing of depression in comparison to men (Wiseman *et al*., 2015). Women of childbearing age are particularly susceptible, with 10–20% of them experiencing postpartum depression (Kessler & Ustun, 2008). The occurrence of symptoms related to depression was most pronounced among females in the 18–24 age group. Females aged 40 and older who underwent twin pregnancies reported significantly elevated PDS rates in comparison to both younger women carrying twins and mothers aged 40 and above with singleton pregnancies (Bradshaw *et al*., 2021).

2.3 Etiological or Risk Factors of Depression

The neuropathology associated with major depressive disorder has various explanations, but different brain abnormalities are known to be linked with MDD. It is commonly characterized as a dysfunction or disruption in neurotransmission or the brain circuits for regulating mood, pleasure, reward etc (Krishana & Nestler, 2008). It has been proposed that these malfunctions may be attributed partially to genetic predisposition, longer periods of stress, sudden adverse incidents, or injury to tissues (Palazidou, 2012). Despite the significant number of potential risk factors for major depressive disorder (MDD) identified by psychiatric epidemiology, a challenge has been the inability to distinguish between association and causation (Rojas *et al*., 2020). Gender, stressful conditions, adverse childhood experiences and specific personality traits are four notable risk factors that have consistently been linked with MDD (Gerke *et al*., 2018). Thus, an intricate interaction between genetic and environmental factors is thought to be the major etiological factor in MDD (Filatova *et al*., 2021). The genes specifically implicated in the functional regulation of neurotransmitters are believed to be functionally abnormal in individuals with MDD (Berton & Nestler, 2006).

2.3.1 Genetic or Hereditary Factors

Depression is a complex disease influenced not only by environment but also by genetic factors, as revealed by many genetic and twin studies (Su *et al*., 2019). The heritability of psychiatric disorders varies, ranging between medium to high (GrotZinger *et al*., 2022). However, the extent to which variation in genes is specific to a particular disorder or shared among different disorders remains unclear (Costanzo *et al*., 2021). Numerous studies

carried out on twins indicated a moderately heritable component of about 37% for depression, with estimates ranging between 26% and 49% (Huider *et al.*, 2021). Considering the moderate genetic influence of major depressive disorder, recognizing clinical subtypes with increased heritability would be valuable (Heim & Binder, 2012). The latest genome-wide association studies (GWASs) on depression have successfully identified over 80 replicated loci which serve as a foundation for investigating their significance associated with depression (Ormel *et al.*, 2019).

2.3.2 Environmental Factors

The development, progression, and pathology of MDD are likely influenced by a multitude of factors, with life experiences being the most extensively studied. The risk factors of depression include social stress, social isolation, bullying, financial problems, death of a loved one or pregnancy but is not specifically limited to these life changes and events. Mental health is known to be greatly affected during COVID-19 pandemic. A report clearly showed a notable rise in depressive symptoms during the time of COVID-19 (Chang *et al.*, 2021). The consumption of alcohol may contribute to this increase (Dogan-Sander *et al.*, 2021). Furthermore, the use of specific medications like alpha-interferon, isotretinoin etc., is associated with an elevated likelihood of developing MDD (Kridin & Ludwig, 2023).

2.4 Impact of Depression on Life Events

The impact of depression on individuals varies with the intensity or seriousness of the condition, substantially impacting the physical well-being and overall quality of life. This results in negative effects on physical functioning, hindering the effective fulfillment of

social and family roles (Rao *et al*., 2020). Unfortunately, approximately 80% of depressive

individuals experience some form of disruption in their day-to-day functioning (Pratt &

Brody, 2008). Data collected during COVID-19 pandemic indicated that about 39.5% of

people showed elevated levels of depressive symptoms due to disturbances in well-being

activities (McMahon *et al*., 2022). Depression has also been recognized to induce a variety

of other physical illnesses e.g., diabetes (Wu *et al*., 2020), hypertension (Lim *et al*., 2021),

arthritis (Fakra & Marotti, 2021), respiratory disorders (Aldabayan *et al*., 2022),

cardiovascular disorders (Shao *et al*., 2020) etc.

The overall influence or the disease burden is typically assessed through factors such as

financial cost, morbidity, mortality, and particularly the resulting disability (Dugdale &

Healy, 2014). Depression has a substantial global impact on the total disease burden,

encompassing disability and associated costs for therapy and utilization of health services.

Regarding the overall years lost due to disability, depression stands out as the foremost

contributor to global disability (Murray, 2022). As per the WHO Global Burden of Disease

Survey, projections indicated that, as of 2020, depression is anticipated to hold the second

position in terms of the magnitude of disability experienced by individuals (Lancu *et al*.,

2020).

The financial consequences of depression are quite huge and noteworthy. The projected

global societal expense associated with mental health over the next two decades is

estimated at 16 trillion US dollars, with WHO figures indicating an escalating global

financial strain. Depression is expected to transition from the fourth most costly disease in

2000 to the second most costly worldwide by 2020 (Tomlinson, 2020). In the United States,

the yearly expenses of depression, encompassing both productivity declines and heightened medical costs, exceeds 83 billion dollars (Greenberg *et al*., 2003). In the United Kingdom, depression incurs an annual economic cost of nearly £11 billion while it costs about AU$14.9 billion to Australian economy about annually (Jorm *et al*., 2006).

2.5 Pathophysiology of Depression

2.5.1 Neurotrophic Hypothesis

Various risk factors associated with depressive episodes usually undergo considerable changes over the course of the illness. The initial phase of depression is triggered by significant psychosocial stressors thus considered to be typically reactive. On the other hand, the subsequent episodes are usually triggered by minor stressors that occur spontaneously and are increasingly endogenous (Kessing *et al*., 2004). The duration of depression is usually attributed to decreased hippocampal volume along with other brain regions (Chen *et al*., 2020). This implies that depression if left untreated could lead to a significant reduction in hippocampus eventually resulting in increased sensitivity to stress (Weismann *et al*., 2020) and likelihood of experiencing a recurrence (Schoenfeld *et al*., 2017). The different mechanisms proposed specifically to explain reduction in brain volume associated with depression includes neurotoxic effects of glucocorticoids along with glutamatergic activity, reduced neurotrophic factors as well as neurogenesis (Dean & Keshavan, 2017). Solid evidence to support these mechanisms is still lacking because of the absence of imaging tools for direct in-vivo examination of neurotoxic and neurotrophic processes (Martinowich *et al*., 2007).

The neurotrophic associated depression hypothesis relies significantly on the relationship between diminished levels of brain-derived neurotrophic factor (BDNF) and a heightened occurrence of depression, depressive symptoms, loss of neuronal cells, and neuronal atrophy (Skaper, 2018). Moreover, the reinstatement of BDNF function is connected to the administration of antidepressants (Deyama et al., 2023). BDNF is of particular interest, as preclinical studies demonstrate associations between stress-induced depressive behaviors, reduction in levels of BDNF in the hippocampus, and increased BDNF expression after treatment with an antidepressant (Mondal & Fatima, 2018). However, there are multiple studies revealing contradictory results in experimental studies thus questioning BDNF quantification in blood serum or plasma for better diagnosis and prognosis of depression (Groves, 2007). The effect of rapid acting antidepressants on BDNF is of increasing interest of researchers in more recent investigations (Duman *et al*., 2021; Wilkinson *et al*., 2018). In the brain, specifically the hypothalamus and hippocampus, pro-BDNF is formed which further synthesizes BDNF (Chao, 2003). The pro-BDNF then splits into the mature form of BDNF and the associated pro-peptide. The disruption in the equilibrium between pro-BDNF and mature BDNF is believed to result in both neuronal degeneration and behavioral impairment (Aloe et al., 2002; Caétren, 2004; Lessmann et al., 2003).

2.5.2 Synaptic Transmission Hypothesis

The nerves in the brain communicate with each other through chemical transmission (Jekely, 2021). Learning and plasticity in the CNS are attributed to pre and post synaptic events (Kania *et al*., 2017). In synaptic transmission various steps are involved, first the neurotransmitters are synthesized, stored in secretory vesicles, and then released in synapse

between pre and post synaptic neurons in response to calcium (Yan & Rein, 2022; Brigitta, 2002). The neurotransmitter then binds to a specific receptor on postsynaptic neuron therefore resulting in reduced synaptic transmission or binds to auto-receptors in presynaptic neuron thus regulating the synthesis and release of the neurotransmitter. The termination of synaptic transmission also occurs because of the reuptake of neurotransmitter in the pre-synapse (Wang & Dudko, 2021). The termination of the action of neurotransmitters as well as the induction of various cellular responses are significantly important in synaptic transmission (Wang et al., 2021; Duman *et al*., 1997). A series of transmembrane signaling reactions, usually coupled with G proteins (guanine nucleotide binding proteins), are initiated upon binding of neurotransmitters to their post synaptic receptors. These signaling cascade ultimately result in survival of neurons as well as synaptic potentiation (Vose *et al*., 2017). The dysregulation at any of the steps in this synaptic transmission could potentially contribute to MDD. The mode of action of many antidepressants is based on this synaptic transmission (Duman *et al*., 2019).

2.5.3 Biogenic Amine or Monoamine Hypothesis

About 30 years ago, one of the most important hypotheses of depression was proposed which stated that the deficiency of monoaminergic transmitters norepinephrine (NE), 5-hydroxy tryptamine (5-HT) or dopamine (DA) are potential contributors to depression. Reserpine being an antihypertensive drug has shown significantly reduce the levels of these neurotransmitters thus leading to MDD (Schildkraut, 2006; Coppen, 1967). Taking into account the source of noradrenergic, serotonergic, and dopaminergic neurons in the brain and their extensive projections into various brain regions, it becomes evident that

monoaminergic systems have a crucial role in numerous behavioral indications e.g., mood, alertness, laziness, motivation, agitation etc (Rostami *et al*., 2023; Ago *et al*., 2023). Disruptions in the production, retention, or discharge of neurotransmitters, as well as alterations in the responsiveness of their receptors or functions at the subcellular messenger level, may contribute to abnormal functioning and the resulting behavioral consequences associated with depression (Goulty et al., 2023; Jacob & Nienborg, 2018).

2.5.4 Hypothalamic-Pituitary-Adrenal (HPA) Axis Hypothesis

The potential involvement of the HPA axis in the mammalian response to stress has long been known (Varghese & Brown, 2001). Consequently, changes in HPA axis observed during depressive illness are thought to signify the impact of stress and contribute to the expression of symptoms related to depression (Sharma *et al*., 2020). The release of several hormones from hypothalamus e.g., corticotropin-releasing hormone (CRH), leads to the production of adrenocorticotrophic hormone (ACTH) from the pituitary gland in case of depressed state (Sukhareva, 2021). Consequently, this leads to an elevated glucocorticoids secretion from the adrenal cortex (Walker & Spencer, 2018). Besides BDNF and other neurogenesis markers, the overall loss of brain volume in depression is also known to be linked with the toxicity of glucocorticoids and glutamatergic neurons (Sharma *et al*., 2020).

2.5.5 Inflammation Hypothesis

Various researchers over the past two decades have identified that the development as well as the functional disruptions in MDD are associated with neuroinflammation. Numerous studies revealed that depression is more common in individuals suffering from either

autoimmune or infectious diseases (Jeon & Kim, 2017). Furthermore, even those without MDD history may display symptoms related to depression when exposed to cytokines and the use of antidepressants has been shown to alleviate this distress (Miller & Raison, 2016). Elevated levels of pro-inflammatory cytokines along their associated receptors have been documented in MDD patients (Kop *et al*., 2002). The levels of TNF-α, IL-6 and IL-10 are found to be notably elevated in MDD patients as opposed to the control subjects (Mao *et al*., 2018). Treatment with antidepressants resulted in remarkable reduction in the expression of these inflammation-mediated cytokines thus depicting their possible involvement in underlying mechanisms of depression (Haroon *et al*., 2018).

2.5.6 Circadian Rhythm Hypothesis

Depression and its association with sleep disturbances and daytime fatigue has been reported (Mirchandaney *et al*., 2023). The patients with depression exhibit compromised sleep-wake regulation resulting in circadian rhythm (Reynolds *et al*., 2020). Certain symptoms of depression like mood, psychomotor activity and positive or negative memories may display various variations throughout the day (Johnson et al., 2017). In studies with young and healthy individuals, even mild alterations in circadian rhythm timing have demonstrated specific effects on subsequent mood (Fang et al., 2019). Different ways to manipulate circadian rhythms like light therapy are known to work for depressed individuals (Wang *et al*., 2015). The influence of antidepressants on sleep-wake cycle, affecting behavioral conduct, physiological processes, and hormonal regulation, collectively contributes to the biological basis of circadian rhythm hypothesis (Bovy *et al*.,

2022; Yasugaki *et al*., 2023). Depression is thus known to be linked to circadian abnormalities. Depression is thus known to be linked to circadian abnormalities.

2.5.7 Mitochondrial and Endoplasmic Reticulum (ER) Stress Hypothesis

On a cellular level, several studies have established a relation between mitochondrial function and the capacity to manage severe environmental stress (Picard *et al*., 2018). There is evidence of dysfunctional mitochondrial function in individuals with depression (Manji *et al*., 2012). Moreover, there is an association between mitochondrial genetic variants and post-traumatic stress disorder (PTSD) (Flaquer *et al*., 2015). In elevated anxiety situations, alterations in mitochondrial activity, energy metabolism as well as redox reactions have been observed (Filiou & Sandi, 2019). Significant evidence suggested that increased anxiety levels are associated with altered or disrupted mitochondrial function. Nevertheless, the function of other crucial cellular organelles that regulate responses to alterations in the environment, such as the endoplasmic reticulum (ER), are not fully investigated in the neuropathology of depression (Walter & Ron, 2011). ER is crucial in maintaining the balance of proteins within the cellular environment, serving as a primary hub for synthesizing essential proteins along with their proper folding, and maturation, constituting a minimum of about 30% of the protein content (Sossin & Costa-Mattioli, 2019). The synthesis of protein is crucial for the storage of memories and serves as a significant pathway that regulates synaptic activity (Park & Kaang, 2019). Any disruptions can lead to an overload of the ER with improperly folded proteins, resulting in a condition termed "ER stress" (Raven *et al*., 2021). When faced with ER stress, cells activate a set of signaling pathways collectively known as unfolded protein response or UPR (Wang &

Kaufman, 2016). The stimulation of the UPR results in the upregulation of numerous genes, enhancing the overall quality as well as the capacity of protein folding within ER (Read & Schroder, 2021). One of the transmembrane proteins of endoplasmic reticulum is ATF6 which is made up of 670 amino acids (Timberlake & Dwivedi, 2019). During ER stress, ATF6 undergoes cleavage, and its N terminal fragment translocate into the nucleus where it interacts with various target genes e.g., ER stress response elements (ERSEs) thereby resulting in transcription of numerous genes associated with ER-mediated stress response (Kowalczyk *et al*., 2021). This response also adjusts protein translation and the overall secretory capacity (Timberlake *et al*., 2018). ATF6 is reported to cleave the expression of nearly 400 genes many of which are known for their protective role (Hetz & Saxena, 2017). Extensive research spanning the last two decades has focused on investigating the connection between the UPR and neurological disorders, evidence supporting the UPR involvement in the physiological processes of CNS has only recently become available (Ziel & Scheper, 2020).

2.4.8 Metabolic Disorders Hypothesis

Individuals with metabolic disorders are known to undergo depression at some stage in their life. The contribution of metabolomics in the pathology and pathophysiology of depression in both the animal models and clinical practice has been observed. A study revealed about 23 differentially expressed metabolites in MDD patients, 5 of which serve as potential biomarkers as they were capable of accurately distinguishing MDD subjects. These key metabolites include some lipid or protein complexes, molecules involved in

energy and lipid metabolism as well as some amino acids. These findings enhance the

capability to differentiate between individuals with depression and those who are healthy.
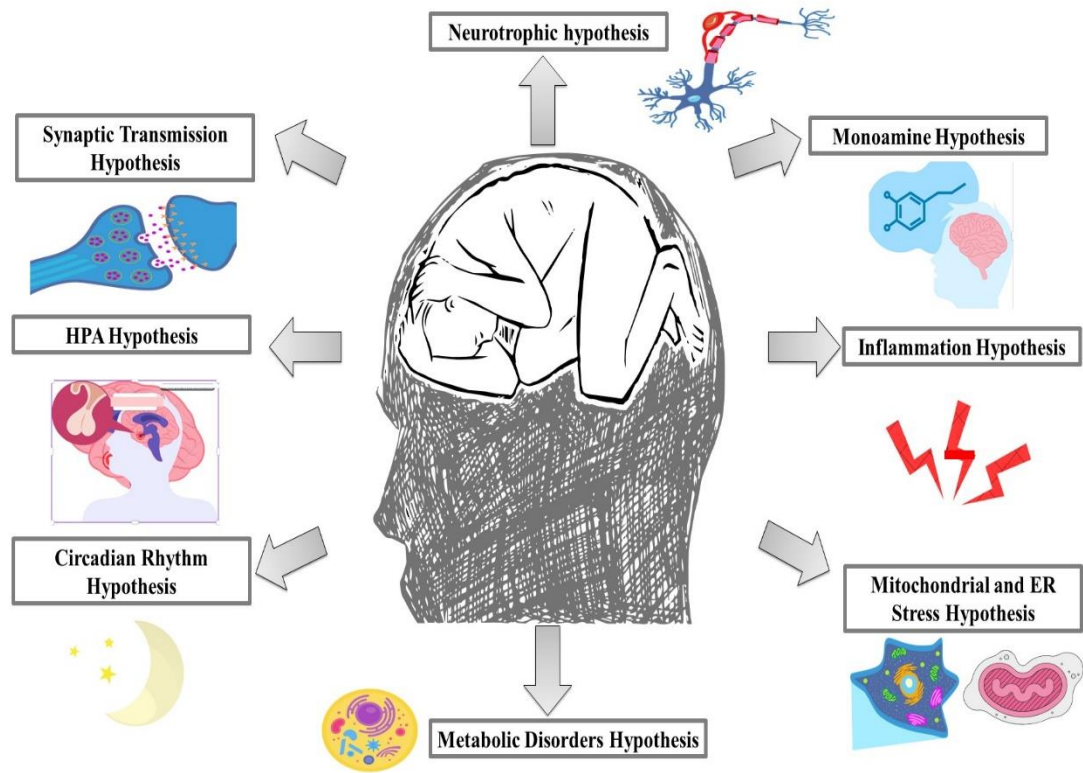
Figure 2.1. Pathophysiological Hypothesis of Depression

2.6 Animal Models of Depression

There are various animal models for studying MDD, which can be broadly categorized into those responsible for inducing acute and chronic forms of depression. Olfactory bulbectomy (Neil & Moore, 2003), forced swim (Porsolt *et al*., 1997) and tail suspension models (Steru *et al*., 1985) comprised the acute models whereas chronic mild stress (CMS) (Willner, 1997), learned helplessness (Weiss *et al*., 1975), corticosterone (Zhao *et al*., 2008) or LPS induction (Conner *et al*., 2009), unpredictable chronic mild stress models are typically used for chronic induction of depression. The acute models of depression are swiftly induced within one or two sessions and can be treated by short-term or sub-chronic utilization of antidepressant drugs (Kitada *et al*., 1981). Chronic depression models are considered to have greater validity as compared to acute models in reflecting human depression since depressed patients only exhibit a response to chronic treatment.

2.6.1 Learned Helplessness Model

For the development of learned helplessness (LH) model of depression, the rays or mice are subjected to a period of stress-exposure, during which they experience inescapable stressors such as electrical foot-shocks in varied sessions (Landgraf *et al*., 2015). The behavior or performance of the animals weas then evaluated in an active avoidance test after the completion of the stress-exposure session. The animals are restricted to a specific region in a shuttle box chamber usually at one of its corners administered with foot-shocks for the active avoidance test. The animal, however, is given the chance to actively avoid the foot-shocks. Animals that have been previously subjected to unavoidable stress exhibit diminished capacity to escape in this model (Duman, 2010). However, various forms of

antidepressant treatment can restore the reduced ability to escape (Martin *et al*., 1990). The

model also demonstrates strong validity in predicting the effectiveness of various

antidepressants (Anismon & Matheson, 2005). Animals exhibiting helplessness also

display several features that bear similarity to human depression, including reduced motor

movement, reduced weight, disrupted sleep patterns, diminished determination, and

elevated levels of stress hormones (Maier, 1984).

2.6.2 Corticosterone Induced Model

The chronic administration of corticosterone is a thoroughly validated model known to

induce symptoms associated with depression (Dieterich *et al*., 2019). It is usually observed

that patients with increased glucocorticoid levels develop depressive symptoms related to

cognitive and psychiatric dysfunction (Brown *et al*., 2004). The circulating levels of

glucocorticoids are known to increase after repeated corticosterone injections (Johnson *et

al*., 2006). In addition, repeated corticosterone injections of 20 mg/kg for 1, 3 and 5 weeks

resulted in higher immobile durations in FST and TST which is associated with time in a

direct manner (Zhao *et al*., 2008). The corticosterone injections of 21 days resulted in

dysregulation of circadian rhythm, elevated oxidative stress and inflammatory response

thus leading to depression (Ma et al., 2018).

2.6.3 Lipopolysaccharide Induced Model

LPS induced depression model display considerable variations and is frequently employed

to investigate the inflammation associated depression mechanisms as well as role of

different therapeutic drugs (Yin *et al*., 2023). LPS works by elevating the expression of

various inflammation-mediated markers or cytokines (Cavaillon, 2018). These inflammatory cytokines eventually lead to neuroinflammation in the brain (Yan *et al*., 2021). Chronic LPS administration also increased the time spent immobile in FST and TST thus representing clear symptoms related to depression (Lu *et al*., 2021; Huang *et al*., 2023).

2.6.4 Unpredictable Chronic Mild Stress Model

In 1982, The UCMS model has been used for a long time to induce depression. For studying depression, UCMS model has widely been recognized as the most efficient and valuable rodent model (Antoniuk *et al*., 2019). In UCMS model, mice are subjected to a series of minor intensity stressors at unpredictable intervals for a period of nine weeks to establish different phenotypes belonging to physical, physiological, biological, and behavioral origin (Nollet *et al*., 2013). Two stressors are usually given in a day and are randomized on a weekly basis to develop unpredictability for the mouse model. Some of the stressors include restraint stress, social stress, no sawdust, wet sawdust, cage tilt, rat feces, alteration in the 12h light/dark cycle etc (Nollet *et al*., 2013). The coat state and weight of the mice are usually quantified to assess the physical changes during model development. The depressive symptoms induced by UCMS reflect apathy and anhedonia in humans (Corrigan, 2023). These conditions are reported to be reduced after the chronic treatment of various antidepressant drugs. Indeed, the UCMS model more closely resembles the chronic as well as the multidimensional nature of clinical depression. The chronic and unpredictable stressors applied in this model contribute to its ability to capture aspects of the complexity seen in human depressive conditions (Mineur *et al*., 2006).

2.7 Conventional Treatment Options for MDD

2.7.1 Monoamine Oxidase Inhibitors (MAOIs)

MAOIs were among the initial antidepressant drugs in 1950s (Suchting *et al*., 2021). They are efficient for treating atypical depression, anxiety related disorders, panic attacks, and for bipolar disorders in some cases (Menkes *et al*., 2016; Stein *et al*., 2004). The four MAOIs approved by FDA include selegiline, isocarboxazid, tranylcypromine and phenelzine. MAOIs as the name suggest work by blocking the action of monoamine oxidases which serve as catalysts for oxidative deamination of monoamines, encompassing various neurotransmitters and catecholamines (Kulbe, 2019). MAO-A and MAO-B are isoenzymes of MAOs, and they can catalyze neurotransmitters including serotonin, histamine as well as catecholamines like epinephrine, nor-epinephrine, dopamine etc (Edmondson & Binda, 2018). The inhibition of monoamine oxidases results in the subsequent increase in the concentration of neurotransmitters and catecholamines. The antidepressant effect is thus produced by the accumulation of serotonin, dopamine, epinephrine etc. in the synapse in CNS (Lacovino *et al*., 2018).

Monoamine oxidase inhibitors (MAOIs) exert a potent hypotensive effect, resulting in dizziness in nearly half of their users (Edinoff *et al*., 2022). This requires careful monitoring especially in elderly patients (Poelgeest *et al*., 2021). A hypertensive crisis referred to as the "cheese reaction" is a severe and fatal side effect associated with MAOIs. This occurs when individuals taking MAOIs are exposed to tyramine, a sympathomimetic amine commonly found in cheese (Edinoff *et al*., 2022). Other potentially fatal side effects include serotonin syndrome that can be triggered by the concurrent use of MAOIs with other antidepressants or serotonergic agents, discontinuation syndrome, sexual

dysfunction, hyper neuromuscular activity, increased heart rate, tremors etc. Thus, it is not totally safe to take MAIOs for treatment of MDD (Wang *et al*., 2016; Boyer & Shannon, 2005; Haddad, 2001).

2.7.2 Tricyclic Antidepressants (TCAs)

In 1959, for the very first time TCAs were introduced as a therapeutic option for MDD (Dopheide, 2006). Their chemical structure comprised of three rings hence the name tricyclic. They operate by blocking the reuptake of some of the inhibitors like serotonin and norepinephrine which are involved in the regulation of mood, attention, and pain. Imipramine was the first FDA approved TCA available and it led to the development of other TCAs like doxepin and many others (Stokes *et al*., 2020). While tricyclic antidepressants (TCAs) show comparable effectiveness to SSRIs in managing major depressive disorder (MDD), they however induce more pronounced detrimental effects because of their anticholinergic effects and reduced overdose threshold (Anderson, 2000; Thorstrand, 1976)). Because of these considerations, TCAs are generally not regarded as the initial choice for treating major depressive disorder (MDD), despite their established high efficacy in addressing severe or treatment-resistant depression (Wolff *et al*., 2013).

2.7.3 Serotonin/ Nor-Epinephrine Re-Uptake Inhibitors (SNRIs)

SNRIs are a better therapeutic choice for clinical depression as they produce their effect by inhibiting the pre-synaptic transmission of both serotonin and norepinephrine, major neuro-amines of MDD (Lambert & Bourin, 1997). The increased amount of both serotonin and norepinephrine at presynaptic terminals results in increased stimulation of postsynaptic

receptors and additional postsynaptic neuronal stimulation (Fanelli *et al*., 2021). They represent a multifaceted category of medications with a broad range of medical applications. While the distinct medical applications may vary among different SNRIs, they are the primary choice for treating conditions like anxiety, depression, fibromyalgia (Fanelli *et al*., 2021). SNRIs intake results in less severe side effects like increased anxiety levels, insomnia, headaches etc. They are reported to induce more nausea, insomnia as well as high blood pressure when compared to SSRIs (Stewart *et al*., 1993).

2.7.4 Selective Serotonin Reuptake Inhibitors (SSRIs)

The significance of changes in the sensitivity of receptors in the underlying mechanisms of depression has been known, according to which researchers developed SSRIs that function by blocking the reuptake of serotonin and hence are sensitive to serotonin receptors (Feighner, 1999). Immediately after the administration of an SSRI, the serotonin reuptake pump gets blocked resulting in a continuous elevation in serotonin levels in the somatodendritic region and not where it is presumably required i.e., axon terminals (Feighner, 1999). This explains the slow therapeutic action of SSRIs. The first SSRI made available was zimelidine (Montomery et al., 1981) which was discontinued in markets due to observed neurotoxicity and immunogenicity in patients (Feighner, 1999). The systematic research on zimelidine paved the way for a new generation of antidepressants, the SSRIs. SSRIs have since emerged as the most extensively prescribed class of antidepressants worldwide (Yuan *et al*., 2020). SSRIs demonstrate a broad range of clinical applications and have been prescribed in numerous psychiatric disorders (Serretti, 2018). The acute and long-term administration of SSRIs demonstrated them to be well tolerated exhibiting safety

in cases of overdose and low likelihood of inducing seizures (Wang *et al.*, 2018). However, some have reported that they lose their efficacy if continued for longer periods of time (Murphy *et al.*, 2021). This is because the chronic intake of SSRIs results in de-sensitization of auto-receptors in somatodendritc region consequently resulting in inhibition of serotonergic neurotransmission (Commons & Linnros, 2019).

2.7.4.1 Fluoxetine

Fluoxetine (FXT) was the first SSRI approved by FDA in the year 1987. It is available under the brand name "Prozac" and manufactured by Elli Lily & Co (Szoke-Kovacks *et al.*, 2020). They sell FXT in 10, 20 and 40 mg tablets. A 20 mg/5ml oral syrup of FXT is also available (Davey *et al.*, 2019). FXT is known to efficiently penetrate CNS and the volume of distribution as well as the half-life are also high (Alboni *et al.*, 2017). It is metabolized by enzymes of cytochrome P450 resulting in several metabolites (Mikov *et al.*, 2017).

It has been revealed that FXT administration resulted in 1.5-4 fold increase in extracellular concentrations of serotonin in different regions of brain (Stucky & Johnson, 2022). In relevance to that, a concurrent decrease in the overall synthesis and release of serotonin has also been observed (Wong *et al.*, 1975). This demonstrates that FXT administration exhibits a feedback mechanism thus reducing the overall serotonin turnover. FXT administration resulted in reduced aggressiveness, decreased compulsive behaviors, and increased analgesic response (Fakhoury, 2016). In animal models of depression e.g., in learned helplessness and social isolation models, FXT administration remarkably lowers the time spent immobile in FST and TST thus reducing behavioral despair (Qian et al.,

2023). On the other hand, several side effects following the FXT administration have been observed usually at 20-80 mg doses (Wernicke, 2004). Sexual and erectile dysfunction have predominately been reported (Moses & Javanbakht, 2023). It is also known to exhibit discontinuation syndrome increasing the risk of suicide (Fava & Cosci, 2019). Aggressiveness and violent acts have been demonstrated in patients taking FXT (Sharma et al., 2016) along with serotonin syndrome, platelet aggregation, increased risk of bleeding as well as hyponatremia (Li *et al*., 2020; Yeung et al., 2013). The mild side effects typically associated with FXT include weight loss, diarrhea, nausea, headache, drowsiness etc. (Pinna *et al*., 2006).

Although FXT is the primary choice for treating depression, it should be prescribed for cases of moderate to severe depression due to potential side effects. Despite the potential adverse effects, FXT may still be considered the most effective treatment currently available for depressive disorders. Additionally, it serves as a standard comparator in research studies, against which other antidepressant drugs are evaluated. Indeed, this also underscores the ongoing necessity for the development and introduction of improved and safer treatment options for depression.
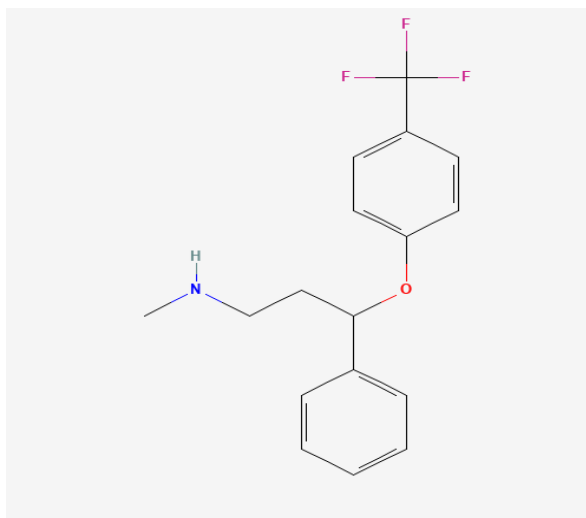
Figure 2.2. Structure of Fluoxetine retrieved from PubChem.

2.7.5 Flavones as Antidepressants

The chemical structure of flavones consists of a 2-pheny.4-H chromen-4-one skeleton exhibiting the potential to improve health. The linkages present in flavonoids comprise of C-C or C-O-C due to which they exist in different polymeric forms (Guan & Liu, 2016). Natural products containing two or more flavonoid units are widespread, exhibiting broad physiological activities, low toxicity, and minimal side effects. Phytochemicals i.e., flavonoids are present in a range of fruits and vegetables, contributing to color, flavor, and aroma, while also providing multiple nutrients and health benefits Harbone & Williams, 2000; Karakaya & EL, 1999). In animal studies, numerous flavonoids have demonstrated anti-inflammatory, antioxidant as well as antidepressant properties (Nogueira *et al*., 2015; Mai *et al*., 2015; Ahmed *et al*., 2015).

2.7.5.1 Luteolin

Luteolin (3′,4′,5,7-Tetrahydroxyflavone), a flavone characterized by its yellow crystalline appearance is abundant in various medicinal herbs and plants, is reported to exhibit a wide range of pharmacological properties (Kim *et al*., 2000; Peters *et al*., 1986). From a chemical perspective, it possesses a C6-C3-C6 structure comprising a total of three rings of which two have benzene and the remaining one has oxygen, featuring a double bond between carbon number 2 and 3 (Bravo, 1998; Lin *et al*., 2008). The -OH groups as well as 2-3 double bonds in its structure are predominantly associated with its pharmacological actions (Lin *et al*., 2008). It occurs naturally in glycosylated form and can be found in various fruits and vegetables like *Brassica oleracea* i.e., (broccoli), *Thymus vulgaris* i.e., thyme, *Origanum vulgare* i.e., oregano, *Capsicum annuum* i.e., peppers, *Rosmarinus officinalis* i.e., rosemary, *Punica granatum* i.e., pomegranate and many others (Lopez-Lazaro, 2009; Shimoi *et al*., 1998). About 36-25 million years old fossils of Ulmaceae species are found to have glycosides of luteolin. It has been reported that luteolin or its glycosidic forms are present in over 350 plant species (Lopez-Lazaro, 2009).   The anticancer, anti-inflammatory, antioxidative and analgesic properties of luteolin are reported, however, there is a lack of scientific research regarding its neuroprotective effects (Seelinger *et al*., 2008; Aziz et *al*., 2018; Xio et al., 2016; Nabavi *et al*., 2014). About 36-25 million years old fossils of Ulmaceae species are found to have glycosides of luteolin. It has been reported that luteolin or its glycosidic forms are present in over 350 plant species (Lopez-Lazaro, 2009).
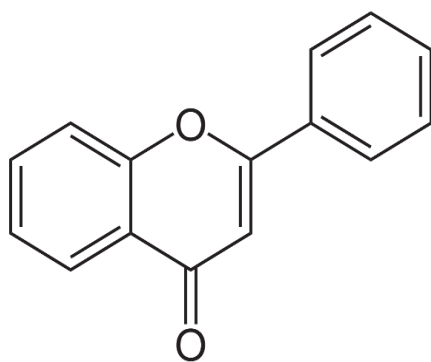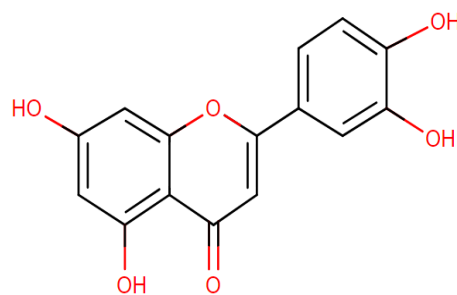
(A)   (B)

Figure 2.3. Structure of Flavanoids (A) & Structure of Luteolin (B) retrieved from DrugBank.

MATERIALS AND METHODS

3.1 Chemicals and Biochemicals

Luteolin, 3′,4′,5,7-Tetrahydroxyflavone (catalogue number: 491-70-3, Lot number: C13830607, molecular weight: 286.24) was purchased from Shangahi Macklin Biochemical Co., Ltd. Fluoxetine (fluoxetine hydrochloride) manufactured by the company Lilly and sold under the brand name Prozac was purchased from local pharmacy in Islamabad, Pakistan. Primer sequences required for quantitative real time expression analysis were ordered from Oligo^{TM} and all other chemicals for RNA extraction and cDNA synthesis were purchased from ThermoFisher Scientific, USA.

3.2 Animals

A total of 36 male BALB/c mice aged between 6-8 weeks and weighing be 30-40g were procured from and housed in laboratory animal facility of ASAB, NUST and given a period of 1 week to adapt to the environmental conditions i.e., standard room temperature (25 ± 2ºC), humidity ($50\pm5\%$), 12h light/dark cycle, standard diet and *ad libitum* access to water.

3.3. Ethical Statement

The study was conducted under the laws and regulations of all ethical committees i.e., Institute of Laboratory Animal Research, Division on Earth and Life Sciences and National Institute of Health, USA. The study was approved by the Institutional Review Board (IRB) of ASAB, NUST under the IRB No. 01-2023-ASAB-01/01.

3.4 Establishment of UCMS Depression Model

The UCMS protocol employed was in accordance with the methodology outlined by Nollet et al (Nollet et al., 2013) and was adjusted to suit the environment within the animal facility. Modifications were made to enhance the ethological significance of stress by reducing its intensity. This experimental protocol involved exposing mice to a variety of randomly scheduled, mild environmental stressors. Mice were subjected to a total of ten stressors i.e., no bedding for 3 hours (Frisbee *et al*., 2015), 45° cage tilt with bedding for 3 hours (Frisbee *et al*., 2015), cold swim at 5°C for 2 minutes (Liang *et al*., 2016), substitution of bedding with water at 1 cm depth for 4 hours (Frisbee *et al*., 2015), exposure to predator sounds for 15 minutes (Mutlu *et al*., 2013), restraint stress for 3 hours (Lee *et al*., 2021), damp bedding (Farooq *et al*., 2018) for 3 hours, introduction of rat droppings in mouse cages (Farooq *et al*., 2018), 45° cage tilt without bedding (Isingrini *et al*., 2010) and overnight illumination (Liang *et al*., 2016) for five days across a nine-week period. The administration of these diverse stressors was randomized weekly to enhance unpredictability. To uphold ethical standards, the stress procedure excluded food or water deprivation. Two stressors were given to each mouse daily, one of which was given during the day and the second one was given in the afternoon after a delay of about 30 minutes during which the mice were allowed to destress. The mice were moved to their clean cages and kept in their standard conditions after finishing the daily stress session. The UCMS schedule followed during nine weeks is shown in Table 3.1.

Table 3.1. Schedule of UCMS followed for 9 weeks/ 5 days per week.

	MONDAY		TUESDAY		WEDNESDAY		THURSDAY		FRIDAY	
WEEK 1	No bedding	Cage tilt with bedding	Cold swim	Substitution of bedding with water	Restraint	Predator sounds	Addition of Rat Droppings	Damp Sawdust	Cage tilt without bedding	Constant illumination
WEEK 2	Predator sounds	Cold swim	Damp Sawdust	Cage tilt without bedding	Addition of Rat droppings	No bedding	Constant illumination	Substitution of bedding with water	Restraint	Cage tilt with bedding
WEEK 3	Damp Sawdust	Restraint	No bedding	Addition of Rat droppings	Cage tilt with bedding	Constant illumination	Cage tilt without bedding	Cold swim	Predator sounds	Substitution of bedding with water
WEEK 4	Cage tilt without bedding	Constant Illumination	Substitution of bedding with water	Predator sounds	Cold swim	Damp sawdust	Restraint	Cage tilt with bedding	No bedding	Addition of Rat droppings
WEEK 5	Substitution of bedding with water	Addition of Rat droppings	Cage tilt with bedding	Restraint	Constant Illumination	Cage tilt without bedding	Predator sounds	No bedding	Damp sawdust	Cold swim
WEEK 6	Damp Sawdust	No bedding	Predator sounds	Addition of Rat droppings	Substitution of bedding with water	Cold swim	Cage tilt without bedding	Constant illumination	Cage tilt with bedding	Restraint
WEEK 7	Cage tilt without bedding	Predator sounds	Cage tilt with bedding	Constant illumination	No bedding	Addition of Rat droppings	Restraint	Substitution of bedding with water	Cold swim	Damp sawdust

WEEK 8	Substitution of bedding with water	Restraint	Damp Sawdust	Addition of Rat droppings	Cage tilt with bedding	Predator sounds	No Bedding	Cold swim	Constant illumination	Cage tilt without bedding
WEEK 9	Cage tilt with bedding	Addition of Rat droppings	Restraint	Cold swim	Constant illumination	Damp Sawdust	Cage tilt without bedding	Predator sounds	No Bedding	Substitution of bedding with water

3.5 Evaluation of Coat State

The coat state condition of mice was examined each week as an indicator of depressive-like behavior induced by UCMS as it serves as a pharmacologically validated measure for this motive (Farooq *et al*., 2012; Hache *et al*., 2012; Santarelli *et al*., 2003). This assessment involved scoring seven different body parts i.e., head, fore paws, neck, hind paws, back, tail, and ventral coat of each mouse after which the cumulative score for each mouse was determined. A score of 0 is assigned to each body region if it is in good condition, characterized by smooth and glossy fur without any tousled or spiked areas. A 0.5 coat score is allotted if the zone is in a moderately bad state, with slightly fluffy fur and some spiky patches. A score of 1 is assigned if the zone is in a bad state, marked by dirty, unkempt fur that is fluffy over most of the body with minor discoloration. The overall score was determined by summing up the scores assigned to each body part (Nollet., 2021).

3.6 Experimental Design

The study consisted of a total of 36 mice (n_t=36) which were divided randomly into six groups, each comprising 6 animals each i.e., n=6. Group I consisted of control mice that were not exposed to stress and were treated with saline (0.9% NaCl). Group II included unstressed mice receiving FXT (20mg/kg in 0.9% NaCl at 10ml/kg body weight), while Group III consisted of non-stress exposed mice which received LUT (10mg/kg in 0.9% NaCl at 10ml/kg body weight). Groups IV, V, and VI consisted of mice exposed to various stressors over a period of nine weeks. Group IV received saline treatment, Group V received FXT (20 mg/kg in 0.9% NaCl at 10ml/kg body weight), and Group VI was given LUT (10mg/kg in 0.9% NaCl at 10ml/kg body weight). Saline, FXT, and LUT were

administered via intraperitoneal injection during the last three weeks of UCMS i.e., week

7 to week 9 (Table 3.2). After week 9, behavioral tests were carried out to evaluate the

outcome of respective treatment options. After behavioral analysis, mice were dissected,

and their hippocampus was isolated and further subjected to histological assessment

(Figure 3.1, 3.2). This was followed by genomic assessment through quantitative real time

PCR.

Table 3.2. Experimental Groups for UCMS Induced Depression Model.

Sr. No.	Experimental Groups	Total number of animals (n)	Treatment (Week 7 – 9)	Injection Volume
1.	Control	6	Saline	10ml/kg
2.	Fluoxetine	6	Fluoxetine (20mg/kg)	10ml/kg
3.	Luteolin	6	Luteolin (10mg/kg)	10ml/kg
4.	UCMS	6	Saline	10ml/kg
5.	UCMS+ Fluoxetine	6	Fluoxetine (20mg/kg)	10ml/kg
6.	UCMS+ Luteolin	6	Luteolin (10mg/kg)	10ml/kg

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                                                          ┌─────────────────────┐
                                                          │  Sucrose Splash Test │
                                                          └─────────────────────┘
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                          │ Treatment (7-9 weeks) │        │ Novelty Suppressed  │
                          └──────────────────────┘        │   Feeding Test      │
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   UCMS Model (9 weeks)   │  Behavioral Analysis  │        │     Y MazeTest      │
                          └──────────────────────┘        └─────────────────────┘
                          ┌──────────────────────┐        ┌─────────────────────┐
                          │ Dissections and Sample│        │ Tail Suspension Test│
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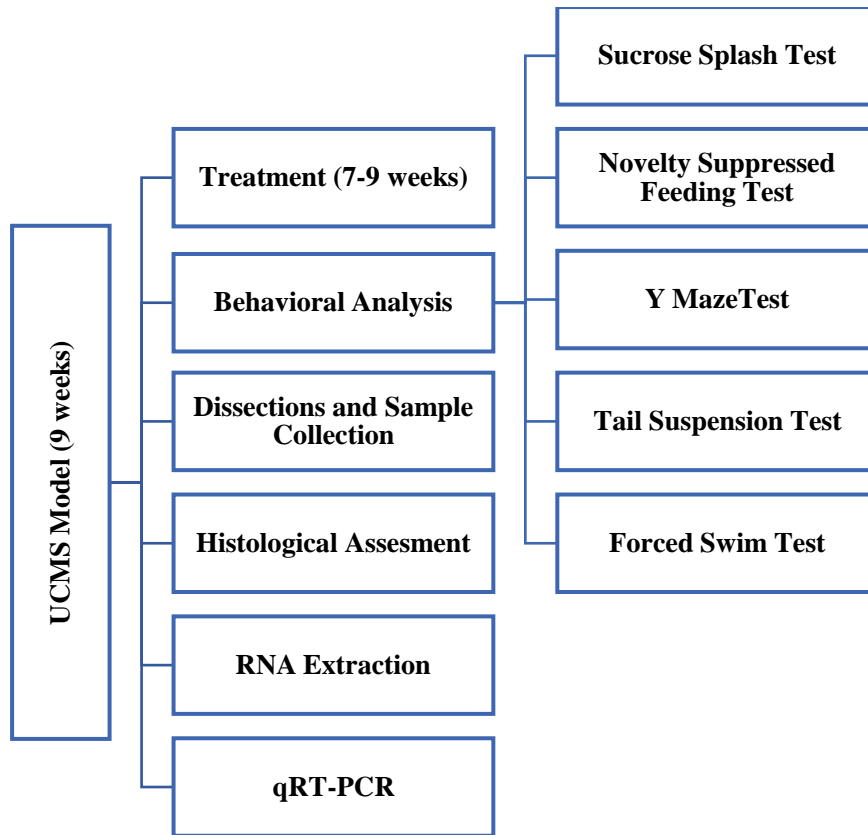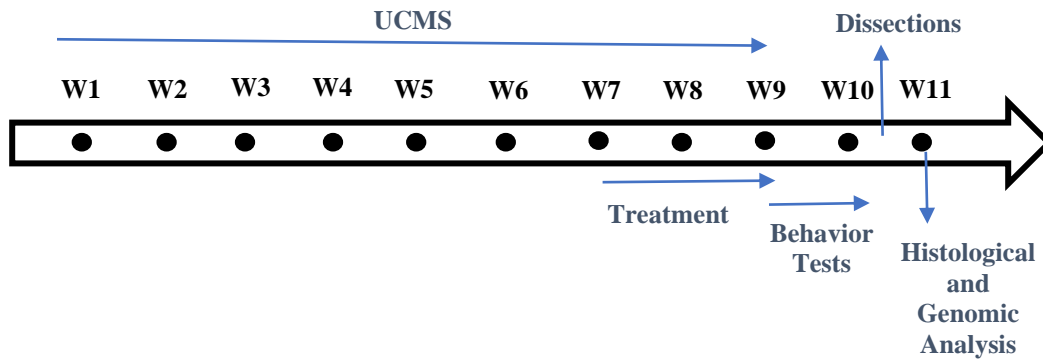
Fig 3.1. Experimental Design.

Fig 3.2. Study Design.

3.7 Behavioral Analysis

Five behavioral tests were carried out to evaluate the antidepressant effect of LUT; Sucrose Splash Test, Novelty Suppressed Feeding Test (NSFT), Y Maze Test, Tail Suspension Test (TST) and Forced Swim Test (FST). Behavioral tests were conducted in accordance with behavioral battery, wherein each successive test exhibited increased invasiveness. A 24-hour recovery period was observed between each test to eliminate potential carryover effects stemming from the handling or testing procedures (Puścian *et al*., 2014; Wolf *et al*., 2016). Mice of the male gender were chosen to mitigate the impact of the estrous cycle on anxiety and cognitive behaviors, which are typically exhibited by females (ter Horst *et al*., 2012. Animals were shifted to the experimental room about 30 minutes before the start of each behavioral test to acclimatize to the testing environment.

3.7.1. Sucrose Splash Test

The sucrose splash test (SST) reveals grooming behavior which is a kind of motivational behavior associated with some depression symptoms e.g., apathetic behavior. A 10% sucrose solution was prepared from D-sucrose (Lot number: 10A0391127D), purchased from phytotechnology laboratories. It was then sprayed approximately 4-5 times to the back fur of a mouse within its home cage. The mouse's coat gets dirtied because of the viscous nature of sucrose prompting the initiation of grooming behavior. Grooming activity was assessed by observing actions such as licking, biting, or scratching of the fur, undertaken to remove the applied solution. After applying sucrose solution, the total grooming time, latency to the first grooming episode, and the number of total grooming episodes were recorded over a 5-minute period serving as an indicator of self-care and

motivational behavior. These motivational behaviors are often used to assess mice's response and adaptation to stress, with changes in grooming patterns considered indicative of alterations in mood and emotional well-being (Zou *et al*., 2015).

3.7.2 Novelty Suppressed Feeding Test

The novelty suppressed feeding test was conducted in an open field box (40 cm x 40 cm x 40 cm). Before initiating the test, mice underwent a 24-hour food deprivation period. In the testing process, a solitary pellet of standard chow was positioned on a white paper at the center of the box, and an animal was placed in one of the corners. The time taken to visibly chew the pellet was recorded during a 10-minute period (Blasco-Serra *et al*., 2017). This testing scenario induced a conflicting motivation by pitting the instinct to consume the food pellet against the fear of entering the arena. This approach is quite successful in revealing the outcome of chronic antidepressant treatment in unstressed mice (Santarelli *et al*., 2003).

3.7.3 Y Maze Test

The Y-maze test was employed to evaluate spatial short-term memory. The apparatus comprised three arms, each situated at a 120° angle from its neighboring counterpart. These arms were assigned as the start arm, other arm, and novel arm. In the habituation phase, the mouse was initially kept in the start arm and permitted to explore both the start arm and the other arm. In the first trial, the novel arm was blocked by a wooden plank and left unblocked during the second trial. The maze was positioned in a tranquil room, kept at a temperature of 25 ± 2 °C. Visual cues were deliberately positioned within the field of vision

of the test mice in all three arms. The initial trial extended for a duration of 10 minutes, during which the subject was allowed to freely explore the Y-maze. Following a10-minute inter-trial interval, the subject was reintroduced to the maze for an additional 5 minutes with the novel arm unblocked. The data was analyzed in terms of the percentage (%) of spontaneous alternations (SA) (Conrad *et al*., 1996).

Percentage SA= No. of SA / total no. of alternations - 2 x 100

3.7.4 Tail Suspension Test

TST is a behavioral assessment for mice that proves valuable in screening potential antidepressant drugs and evaluating other interventions anticipated to impact behaviors associated with depression (Can *et al*., 2012). An adhesive tape was used to suspend the mice through its tail at approximately 50cm from the ground surface. The experiment extended for a duration of 6 minutes and the time each mouse remained immobile was measured during the final 4 minutes. Mice were categorized as immobile solely when they hung passively without displaying any movement (Cheng *et al*., 2022).

3.7.5 Forced Swim Test

FST is a behavioral evaluation employed in rodents to assess the effectiveness of antidepressant drugs, appraise the efficacy of new compounds in treating depression, and explore experimental interventions designed to induce or prevent depressive-like states (Can *et al*., 2012). Mice were placed in a plastic jar of height 30 cm and diameter 20 cm filled with water up to 12 cm and were forced to swim for a period of 6 minutes. The last 4 minutes were evaluated to determine the latency to immobility, total immobility time and

total number of immobile episodes. Mice were classified as immobile if their heads were observed floating on the water's surface or if their limbs showed no signs of movement in the water (Cheng *et al*., 2022).

3.8 Statistical Analysis

The data underwent analysis through GraphPad prism 8.0.1. For examination of the statistical significance in behavioral analysis, one-way ANOVA was used while two-way ANOVA was employed to analyze the coat state of mice over the duration of UCMS. For the determination of group-to-group differences Bonferroni's multiple comparison test was used. The error bars were depicted as Mean ± SEM, with statistical significance noted for p-values less than 0.05.

3.9 Brain Dissections

All animals were subjected to anesthesia (chloroform) and euthanized for the retrieval of brain samples. The heads of the animals were extended forward, and a cut was made posterior to the ears using surgical scissors. An incision was then made upward along the sagittal suture, which was peeled off to extract the entire brain without causing damage. The harvested brain was promptly transferred to a cold metal tray where pre-chilled 1X phosphate buffer saline (PBS) was first used to clear off excess blood. The cerebellum and olfactory bulb were removed after which the cortical halves of cerebral cortex were opened along the mid-section for the extraction of hippocampus. The hippocampal samples were immediately shifted to pre-chilled, labelled Eppendorf tubes and stored at -80ºC.

3.10 Histological Assessment

3.10.1 Tissue Fixation and Slide Preparation

For histopathological assessment, ice chilled PBS was used to wash whole brain samples which were then immediately transferred to 50 ml falcon tubes containing 4% paraformaldehyde (PFA) solution and kept at a temperature of 4 °C for 24 hours for tissue fixation. The tissue samples were then dehydrated using three different concentrations of isopropanol i.e., 75%, 95% and 100% respectively for 1 hour. This was followed by 4 hours of xylene incubation, after which the samples were subjected to paraffin embedding by using molten paraffin. The tissue samples were then placed at 60 °C in an incubator for 4 hours. They were then allowed to solidify at 4 °C prior to cutting.

3.10.2 Hematoxylin and Eosin Staining

H & E staining was done for microscopic examination of tissues in order to identify pathological differences. The process involved de-paraffinizing 5μm tissue sections from control, diseased, and treated groups, followed by an 8-minute incubation in Mayer's hematoxylin solution. After a 10-minute wash in warm water, the sections were immersed in 95% ethanol and counterstained with eosin for 30 seconds. Subsequently, the prepared tissue slides were observed using an OPTIKA B-150 LED microscope at 4X, 10X, and 40X resolutions. OPTIKA Vision Lite 2.1 software was used for capturing images. The cell counting was done using ImageJ software.

3.11 Gene Expression Analysis

3.11.1 mRNA Extraction

The RNA extraction was done using the Tri-reagent protocol. First, the tissue samples were homogenized in 1ml TRIzol using an Ultrasonic Processor UP400S (Hielscher Ultrasound Technology) and then kept in incubator at 4°C for 15 minutes. After adding 200 µl of chloroform, each sample underwent shaking for a duration of 15 seconds and incubated for an additional 10 minutes at 4°C. The refrigerated centrifuge was set to 12,000 rpm at 4°C, and the sample was centrifuged for 15 minutes. The colorless upper aqueous phase obtained post-centrifugation was gently removed without disrupting or mixing the layers and transferred to new autoclaved tubes. Next, 500µl of isopropyl alcohol was added to the samples, and they were incubated for 10 minutes. Another centrifugation step at 12,000 rpm for 10 minutes was performed to precipitate the RNA. The RNA formed a pellet on either the side or base of the microcentrifuge tube. The supernatant obtained after centrifugation was disposed of. 1000 µl of chilled absolute ethanol was used to wash the pellet, followed by a final 5 minutes of centrifugation step at 7500 rpm at 4°C. The ethanol was gently discarded after washing to prevent dislodging of the pellet. After air-drying the pellet, 30µl DEPC water was added, and it was kept at -80°C until further use.

3.11.2 RNA Quality Measurement

Nanodrop was used to assess the RNA concentration in each sample, with the A 260/280 ratio serving as an indicator of the extracted RNA quality. The observed 260/280 ratios ranged between 1.8-2.0. A ratio less than1.8 indicates the existence of contaminants.

3.11.3 cDNA Synthesis

RNA quantification was conducted using the Biophotometer plus (Eppendorf, Germany).

For reverse transcription, 3µg of RNA was used in each reaction mixture. The reaction

mixture comprised 2µl of 10mM dNTPs, 4µl of 5X RT buffer, 1µl of 10mM oligo dT

(previously heated at 55 °C for 5 minutes), and 1µl of RevertAID enzyme. The total

reaction volume was modified to 25 µl by incorporating nuclease-free water into the

reaction mixture. The thermocycling profile for cDNA synthesis is shown in Figure 3.3.
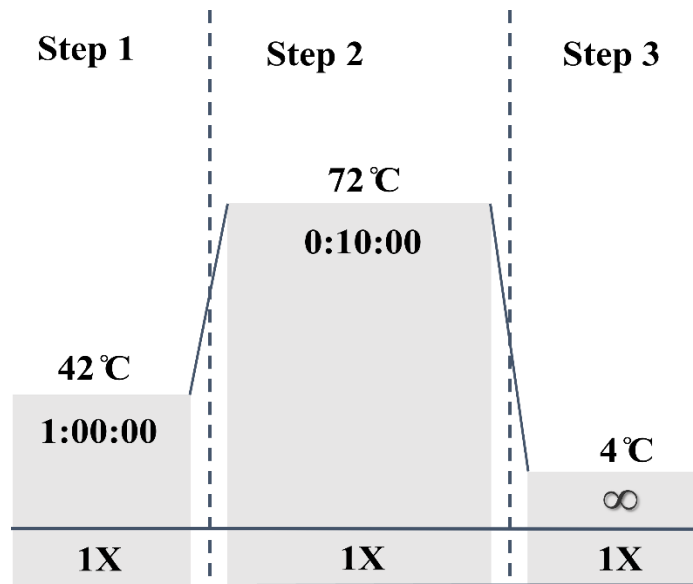
Figure 3.3. Thermocycling Profile for cDNA Synthesis.

3.11.4 Real Time PCR

The hippocampal expressions of ATF6 and NeuN were evaluated using quantitative real-time PCR. The reaction mixture consisted of 1µl each of forward and reverse primers, 4µl of SYBR green dye, and 1µl of cDNA template. Nuclease-free water was incorporated to adjust the reaction volume to 20µl. Each of the samples were run in duplicates. The thermocycling conditions for all three genes consisted of a 5 minute of pre-incubation step at 95°C, followed by three step amplification at 95 °C for 30 seconds, 60 °C for 1 minute and 72 °C for 30 seconds. The total cycles for each of the three steps of extension were kept at 35. The primer sequences for β-actin, ATF6 and Neun were shown in Table 3.3 whereas the profile for expression analysis of all three genes was shown in Figure 3.4. To analyze the quality of PCR product, the dissociation curve was utilized. All values were standardized based on the values of β-Actin. The data of relative gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method (Ma *et al*., 2016).

Table 3.3. Primer Sequences for qRT-PCR.

Gene	Primer	Sequences
β-actin	Forward	5'-GACGGCCAAGTCATCACTATT-3'
	Reverse	5'-CCACAGGATTCCATACCCAAGA-3'
ATF6	Forward	5'-TCCCTTGGGAGTCAGACCTAT-3'
	Reverse	5'-GCTGAGTTCAAGAACACGAGTC-3'
NeuN	Forward	5'-GGCAATGGTGGGACTCAAAA-3'
	Reverse	5'-GGGACCCGCTCCTTCAAC-3'

Step 1	Step 1	Step 2	Step 3

95.0°C

05: 00

95.0°C

00: 30

72.0°C

00: 30

60.0°C

01: 00

Preincubation
x 1

3 Step Amplification
x 35
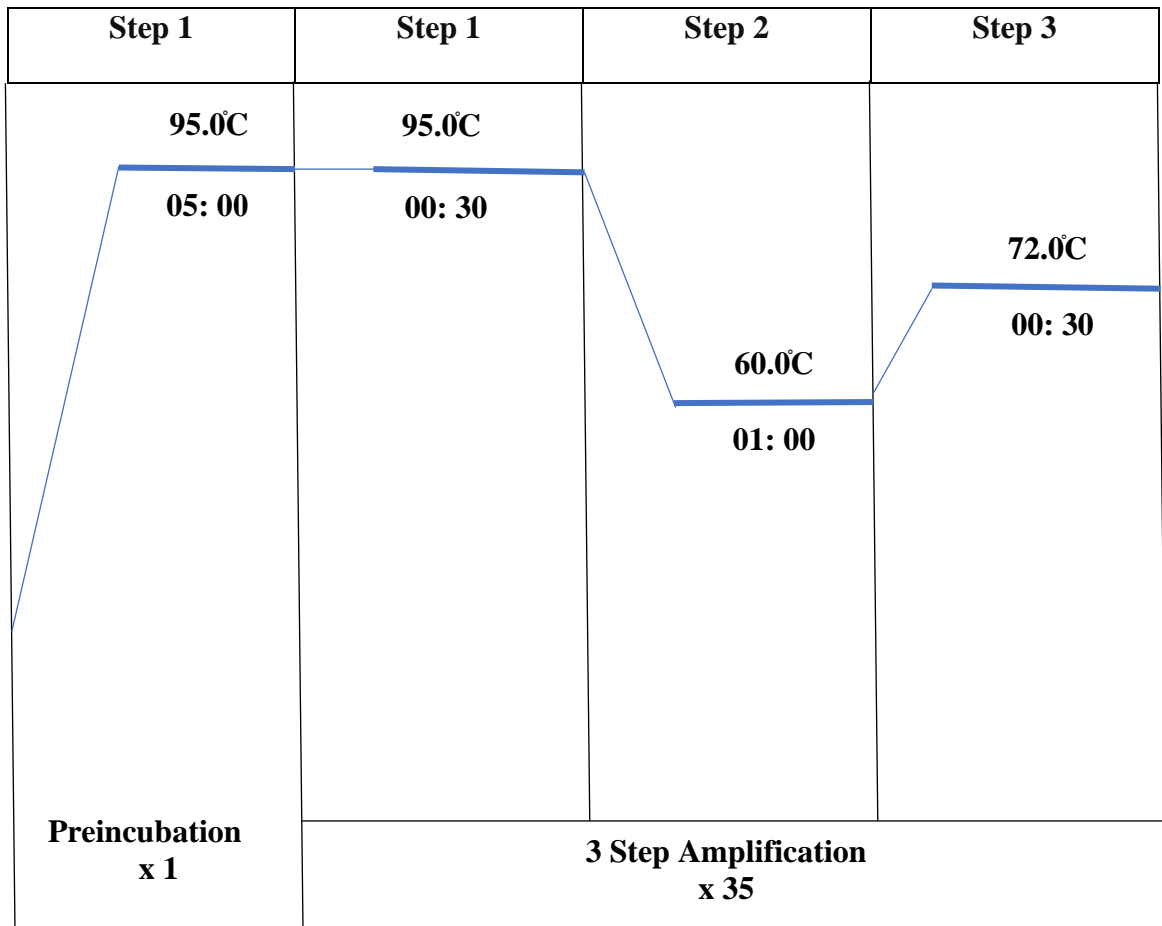
Fig 3.4. Real Time qPCR profile for gene expression of β-actin, NeuN and ATF-6.

RESULTS

4.1 Behavioral Assessment

4.1.1 Effect of UCMS and Luteolin on Coat Deterioration

The condition of the coat was assessed weekly, and the obtained values were subjected to comparison between six experimental groups and over the 9 weeks duration of UCMS. Two-way ANOVA revealed that UCMS had no impact on the coat state till the first 4 weeks. However, UCMS significantly deteriorated coat state from week 4 (1.500±0.183) (p<0.001) till week 9 (1.583±0.154) (p<0.0001) when compared to control group (0.167±0.105) (Figure 4.1). The FXT treated UCMS group (0.750±0.112) (p<0.05) and the LUT treated UCMS group (0.583±0.154) (p<0.01) significantly enhanced the condition of the mice's coat in the last week of UCMS (Figure 4.1). This indicated that LUT exhibits better antidepressant potential in comparison to the standard drug i.e., FXT.

Fig 4.1. Effect of Luteolin on coat state in non-UCMS and UCMS induced mice. Data was analyzed in GraphPad prism 8.0.1 by using two-way ANOVA and expressed as Mean ± SEM. The symbols *, # and + are used to show significance between Saline vs UCMS, UCMS vs Fluoxetine and UCMS vs Luteolin respectively. #=p<0.05, ++=p<0.01, ***=p<0.001, ****=p<0.0001.

4.1.2 Effect of UCMS and Luteolin on Anhedonia

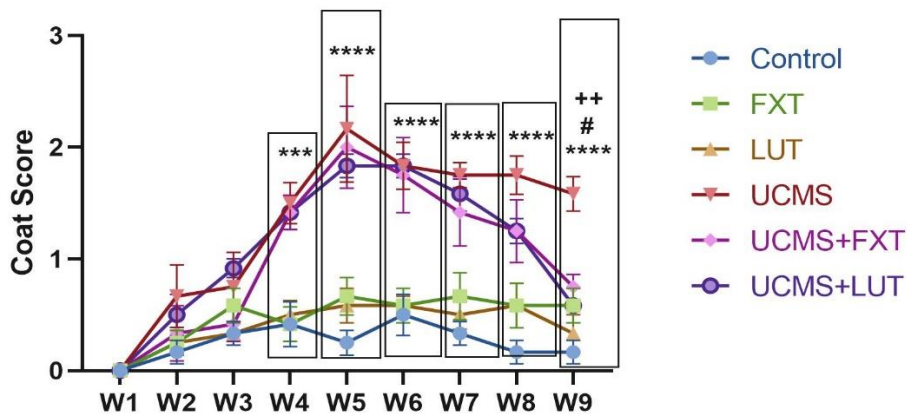The SST was conducted to examine the depressive-like behaviors particularly anhedonia in mice. Anhedonia was quantified by examining the latency of mice to initiate first grooming behavior, total time spent on grooming and total grooming episodes. The longer the delay in initiating grooming, the greater the indication of depression or anhedonia. Similarly, a shorter grooming behavior duration indicated a higher level of depression. The correlation between the total grooming episodes in SST and depressive symptoms is observed in a way that an increase or decrease in the frequency of grooming episodes may correspond to heightened or reduced depressive symptoms, respectively.

The latency before first grooming behavior was significantly ($p<0.0001$) higher in UCMS (9 ± 2.017 seconds) group in comparison to control group (2 ± 0.5164 seconds) as shown in Figure 4.2 A. The LUT treatment (2 ± 0.4472 seconds) showed better anti-anhedonia effect in contrast to the FXT treated group ($p<0.005$) (3.5 ± 0.8851 seconds) by significantly ($p<0.001$) reducing the latency to grooming in comparison to the UCMS group as shown in Figure 4.2 A.

The total time spent on grooming was significantly ($p<0.0001$) lower in UCMS (85.33 ± 18.61 seconds) when compared to the control group (153 ± 13.2 seconds ($p<0.0001$). The UCMS+LUT group (185 ± 9.473 seconds) showed significant ($p<0.0001$) improvement in total grooming time in comparison to the UCMS group which was better than the effect shown after FXT administration (161 ± 10.99 seconds) ($p<0.01$) as shown in fig 4.2 B. These results clearly indicated that luteolin could act as an antidepressant drug potentially by improving anhedonia like depressive symptoms.

The grooming frequency or the grooming episodes were also significantly (p<0.0001) lower in UCMS (12±1.77 seconds) in comparison to the control group (23.67±2.140 seconds). The UCMS+LUT (16.67±0.4944 seconds) group increased the total number of grooming episodes in comparison to the UCMS group (Figure 4.2 C). This was comparable to the FXT treated group (15.83±1.195 seconds) which also non-significantly enhanced the number of grooming episodes (Figure 4.2 C). This could be because the mice in both the treated groups dedicated more time to individual grooming episodes, resulting in a reduced overall frequency of grooming episodes.

(A)

(B)

(C)
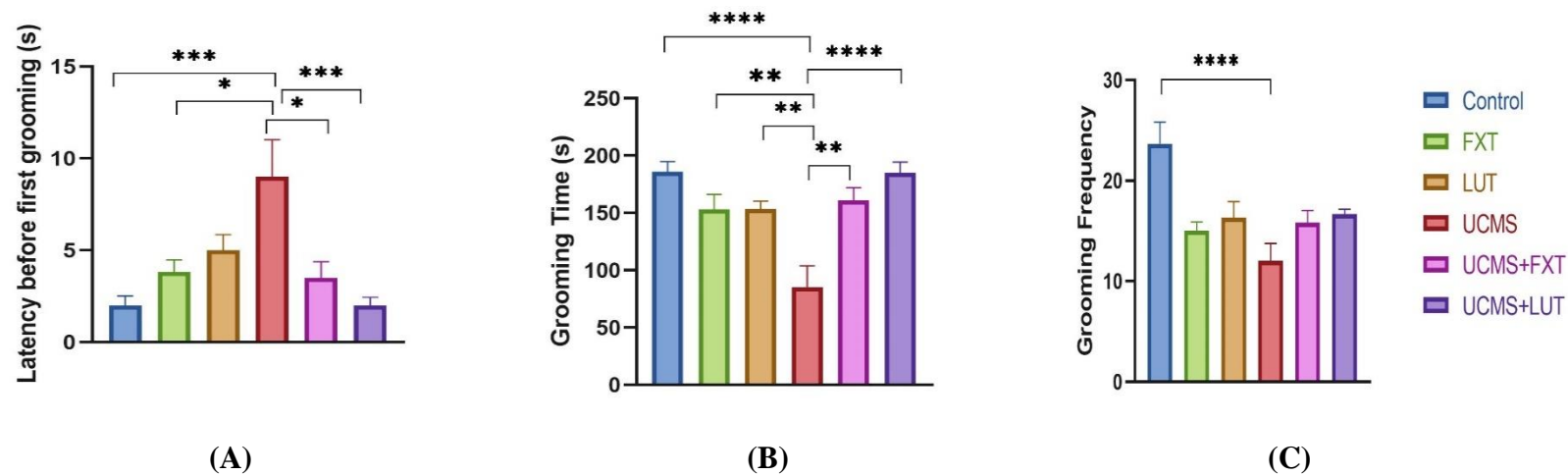
Fig 4.2. Effect of UCMS and LUT on Anhedonia; Latency before first grooming (A), Time spent on grooming (B) and Grooming frequency (C) in sucrose splash test. Data was analyzed through GraphPad Prism 8.0.1 by using one-ways ANOVA and expressed as Mean ± SEM for calculation of the difference between groups. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

4.1.3 Effect of UCMS and Luteolin on Anxiety

NSFT was conducted to identify behaviors associated with depression and anxiety by quantifying the time it took for the mice to exhibit feeding behavior in response to a novel environment. An increase in the latency to consume food in an unfamiliar setting is a potential indicator of anxiety.

The UCMS group (185±65.8) showed increased latency to feed in comparison to control group (89±15.24). The UCMS+ LUT (67.33±9.824) reduced the time taken by mice to eat the pellet of food showing the better anxiolytic effect of LUT in contrast to FXT treatment (88±25.57). Although, the results were non-significant, a clear trend was evident (Figure 4.3).
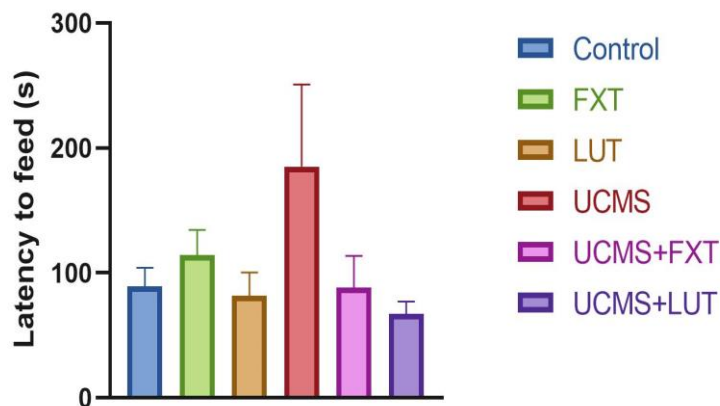
Fig 4.3. Effect of UCMS and LUT on anxiety; Latency to eat in novelty suppressed feeding test. Data was analyzed in Graphpad Prism 8.0.1 by using one-way ANOVA and expressed as Mean ± SEM for calculation of the difference between groups.

4.1.4 Effect of UCMS and Luteolin on Short-Term Spatial Memory

Short-term spatial memory was evaluated in Y maze by measuring two parameters i.e., spontaneous alternations and time spent in novel arm. Mice display spontaneous alternations by sequentially entering all three arms of Y maze due of their tendency for exploration. A non-significant difference between spontaneous alternations in UCMS (76.78±3.271) and control group (88.5±5.315) was observed indicating slight deterioration of spatial short-term memory in UCMS (Figure 4.4 A). The UCMS+LUT (84.60±4.046) non-significantly enhanced the percentage of spontaneous alternations which was comparable to the UCMS+FXT (82.23±4.721) group. (Figure 4.4 A).

Moreover, the total time spent in novel arm is lower in UCMS (86.33±1.892) when compared to control group (112.7±6.081) but the results were non-significant (Figure 4.4 B). Both the UCMS+FXT (89.83±8.585) and the UCMS+LUT (110.7±7.388) non-significantly improved the time spent in novel arm but the Mean ± SEM values indicated that the LUT administration enhanced the exploratory behavior of mice in contrast to the FXT treated group (Figure 4.4 B).
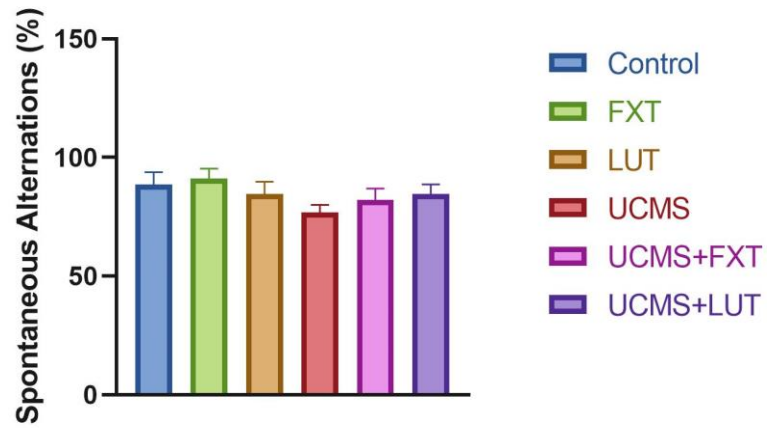
(A)

(**B**)

Fig 4.4 Effect of UCMS and LUT on spatial memory; Percentage of spontaneous alternations in Y maze test (A) and Time spent in novel arm in Y maze test (B). Data was analyzed in Graphpad Prism 8.0.1 by using one-way ANOVA and expressed as Mean ± SEM for calculation of the difference between groups.

4.1.5 Effect of UCMS and Luteolin on Helplessness and Behavioral Despair

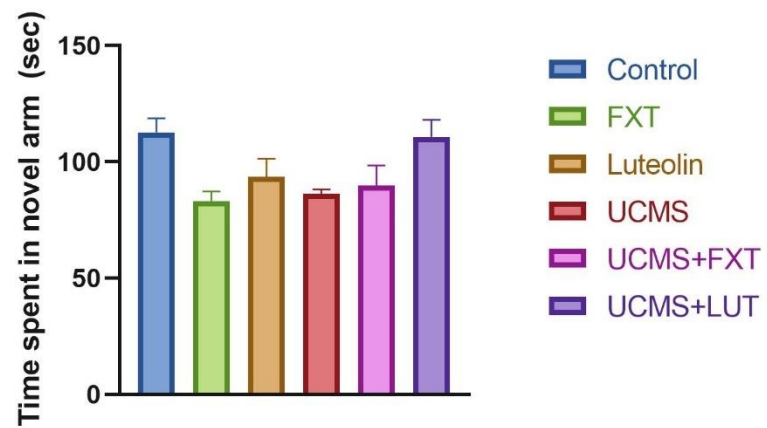TST was conducted to measure helplessness and despair in mice. The mice after being suspended by their tails exhibited a state of immobility. This immobility is associated with a sense of despair or hopelessness.

In TST, the total time spent immobile by mice in the UCMS group (188±8.6) was significantly (p<0.001) longer when compared to the control group (127±16 seconds). The FXT-treated control group (134±5.5) increased the immobility in mice which was opposite to the effect of LUT-treated control group (102±4.2) reflecting the positive impact of LUT on helplessness and despair. LUT (10mg/kg) administration (98±9.2 seconds) resulted in significantly (p<0.0001) reduced immobility time and it was comparable to that of the positive drug i.e., FXT (118±6 seconds) (p<0.0001) (Figure 4.5). The decreased immobility time by LUT indicated active coping strategy or reduced despair like behavior.

FST was performed to examine depressive-like behavior in UCMS mice and potential antidepressant effects of LUT. The parameters assessed in FST included latency to immobility, total immobility time and total immobile episodes.

Latency to immobility refers to the amount of time mice took to first exhibit immobility in the forced swim situation. A shorter latency to immobility is associated with increased susceptibility to stress or depressive behaviors. The latency to immobility was significantly (p<0.0001) shorter in UCMS (4.7±1.3 seconds) versus the control group (47±6.6 seconds) indicating that the depressed mice failed to struggle in FST and became immobile earlier than the control mice. The UMCS+FXT group (46±6.8 seconds) significantly (p<0.0001) improved the latency to immobility. Likewise, the UCMS+LUT group (29±3.5 seconds)

also showed to significantly (p<0.05) enhance the latency to immobility thus exhibiting clear antidepressant properties (Figure 4.6 A).

In FST, the total time a mouse spends immobile during the 4-minute duration is considered a key parameter for identifying antidepressant effects. A longer immobility time indicates a higher level of despair or a lack of motivation to escape the aversive situation. The immobility was significantly (p<0.0001) higher in UCMS mice (124±10) in comparison to control mice (11±2.4). This reflected a state of despair and helplessness in UCMS mice. The time spent immobile was significantly reduced in FXT-treated group (39±4.2) as well as LUT-treated group (20±5.3) (p<0.0001) (Figure 4.6 B).

The total immobile episodes in FST refers to instances when mice transition from activity to immobility and back to activity again. An increased number of immobile episodes indicates that the mouse is intermittently giving up and then reinitiating its response. The number of immobile episodes were significantly (p<0.0001) higher in UCMS group (20±1.8) when compared to control group (5.7±1.1). This is indicative of lesser coping strategies in depressed mice. The UCMS+FXT group (12±1.1) significantly (p<0.05) lessen the total number of immobile episodes and the UCMS+LUT group (8.8±1.5) also significantly (p<0.001) lower the number of immobile episodes however the Mean ± SEM values revealed that LUT could be a better antidepressant option in comparison to FXT when it comes to enhancing the coping as well as escape strategies in mice (Figure 4.6 C).
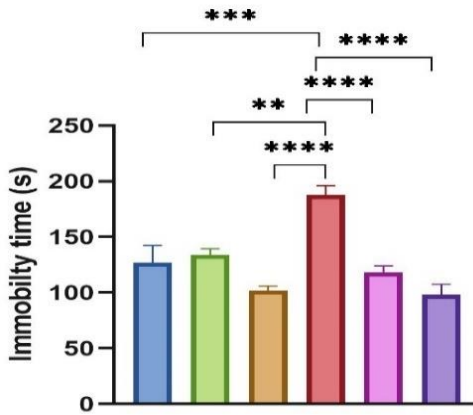
(A)

4.5. Effect of UCMS and LUT on helplessness and behavioral despair; Immobility time in TST. Data was analyzed in GraphPad Prism 8.0.1 by using one-way ANOVA and expressed as Mean $\pm$ SEM for calculation of the difference between groups. *=p<0.05, **=P<0.01, ***=p<0.001, ****=p<0.0001.

(A)   (B)   (C)
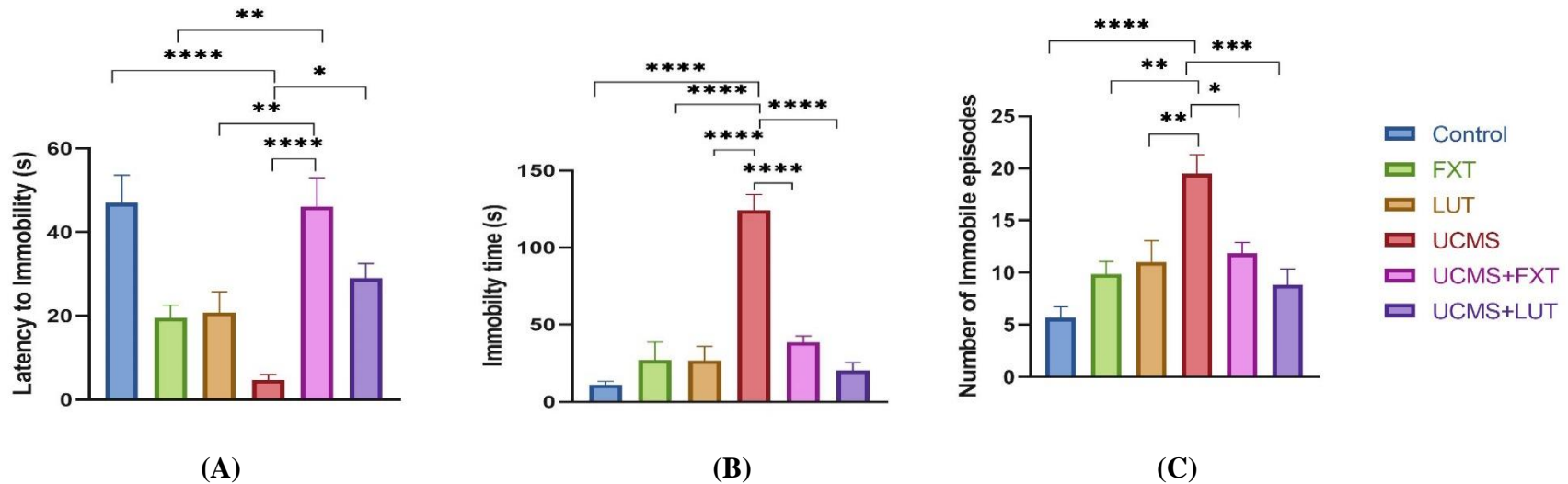
4.6. Effect of UCMS and LUT on helplessness and behavioral despair; Latency to immobility in FST (A), Immobility time in FST (B), and Number of immobile episodes (C). Data was analyzed in GraphPad Prism 8.0.1 by using one-way ANOVA and expressed as Mean ± SEM for calculation of the difference between groups. *=p<0.05, **=P<0.01, ***=p<0.001, ****=p<0.0001.

4.2 Histological Assessment

Histopathological analysis for all study groups was done using hematoxylin and eosin staining to determine morphological alterations within the hippocampus of mice. Cells were densely packed in control group, whereas the UCMS group exhibited noticeable cell dispersion. Treatment with both FXT and LUT in the UCMS groups resulted in the restoration of cells, appearing tightly packed, as evident under 40X magnification.

The number of cells in UCMS mice (13±0.865) is significantly lower than the control group (22±0.4082) ($p < 0.01$) clearly depicting neuronal loss in diseased group. The number of neuronal cells in FXT-treated control group is significantly reduced when compared to control ($p < 0.001$) as well as LUT-treated control group (25.75±1.702) ($p < 0.0001$). Both the UCMS+FXT (21.75±1.436) ($p < 0.01$) and UCMS+LUT (25.00±1.683) significantly increase the number of neuronal cells ($p < 0.0001$). These results showed that LUT restored the neuronal cells in the DG region of hippocampus thus indicating its neuroprotective effect.
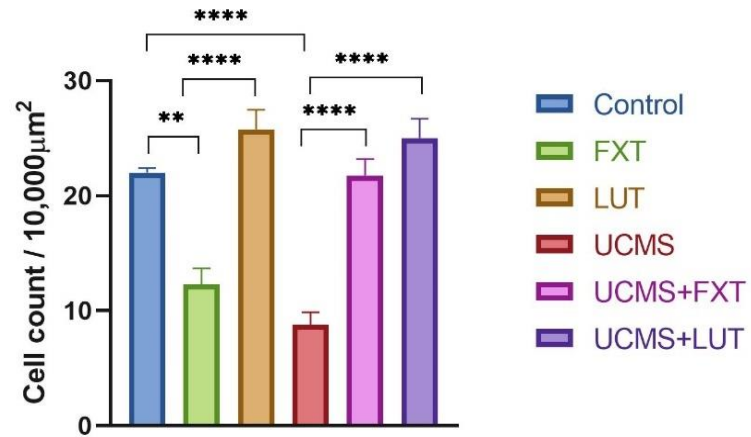
Fig 4.7. Effect of UCMS and LUT on cell count in dentate gyrus region of hippocampus. Data was analyzed in GraphPad Prism 8.0.1 by using one way ANOVA and expressed as Mean $\pm$ SEM for calculation of the difference between groups. **=P<0.01, ****=p<0.0001.
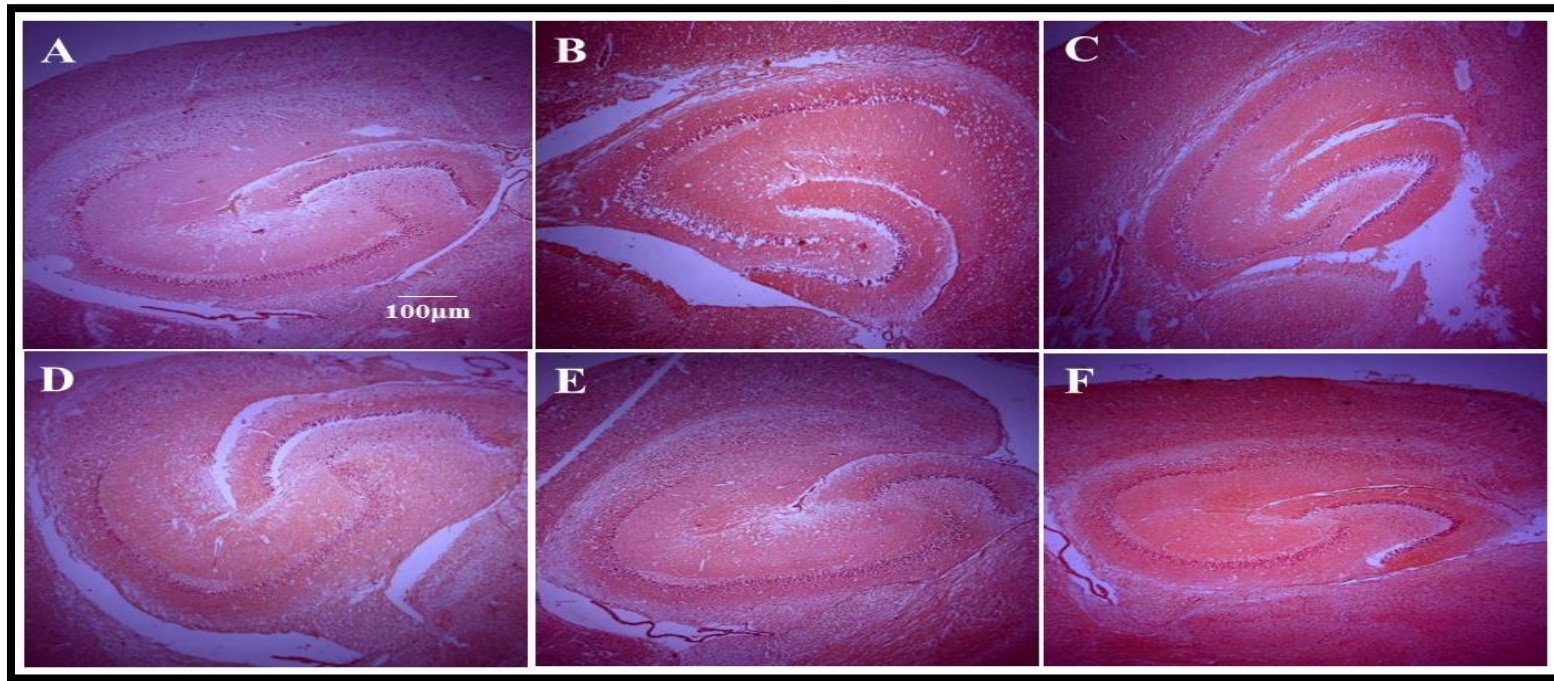
Fig 4.8. H & E-stained hippocampal dentate gyrus. (A) Control, (B) FXT (C) LUT, (D) UCMS, (E) UCMS+FXT, (F) UCMS at 4X

magnification.
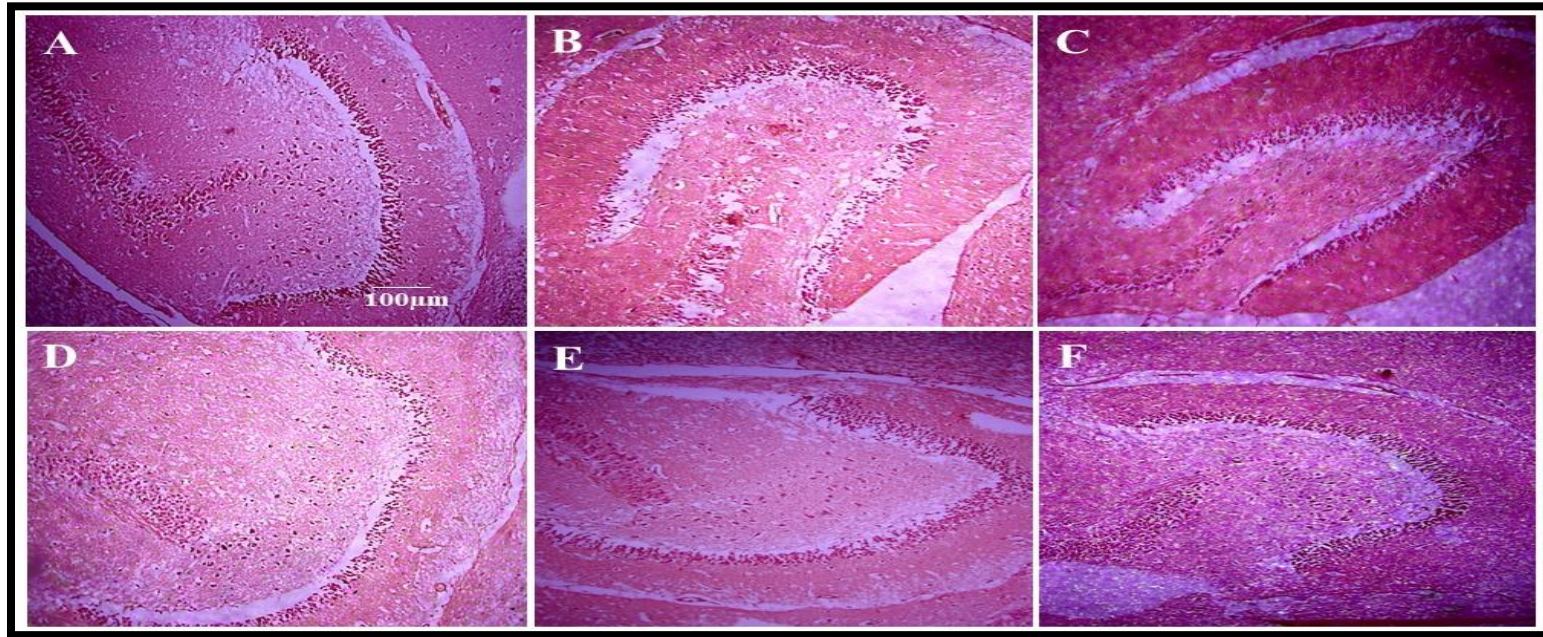
Fig 4.9. H & E-stained hippocampal dentate gyrus. (A) Control, (B) FXT (C) LUT, (D) UCMS, (E) UCMS+FXT, (F) UCMS+LUT

at 10X magnification.
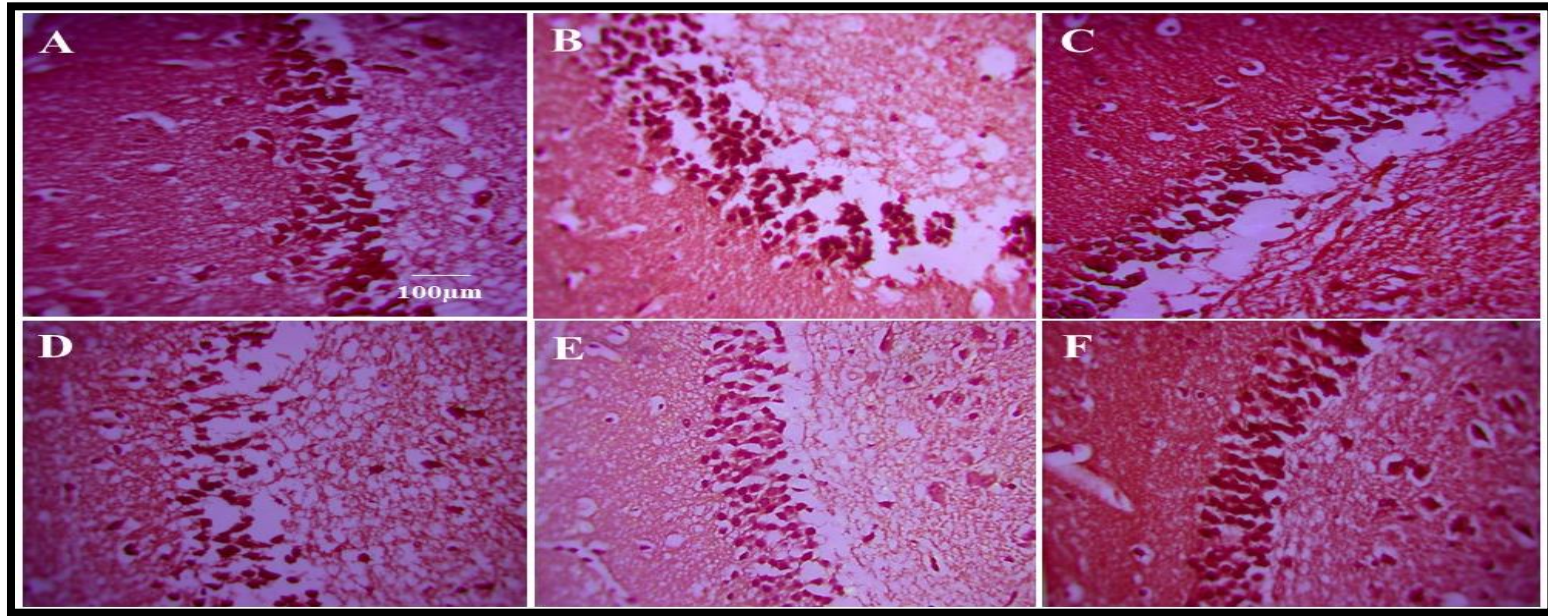
Fig 4.10. H & E-stained hippocampal dentate gyrus. (A) Control, (B) FXT (C) LUT, (D) UCMS, (E) UCMS+FXT, (F) UCMS+LUT

at 40X magnification.

4.3 Genomic Assessment

4.3.1 Effect of UCMS and Luteolin on NeuN Expression

The effect of the study groups on mRNA expression of NeuN in hippocampus was identified using quantitative real-time PCR. UCMS (0.38 ± 0.051) induced a significant ($p<0.01$) reduction in NeuN levels in comparison to control group (1.0 ± 0.0078). The FXT-treated control group (0.64 ± 0.083) non-significantly decreased the NeuN levels while the LUT-treated control group (0.90 ± 0.010) non-significantly enhanced its expression. The FXT treated UCMS group (1.4 ± 0.092) significantly ($p<0.001$) improved the NeuN expression. The UCMS+LUT group (1.0 ± 0.030) significantly elevated the expression of NeuN in hippocampus and brought it closer to control group.
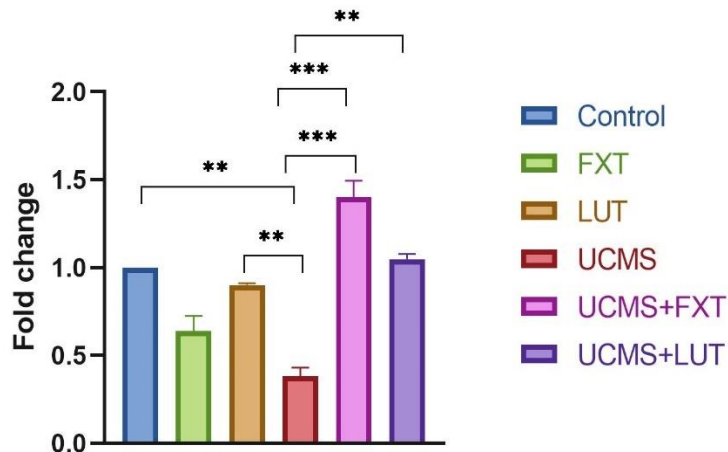
Fig 4.11. Effect of UCMS and LUT on NeuN expression. Data was analyzed in GraphPad Prism 8.0.1 by using one way ANOVA and expressed as Mean $\pm$ SEM for calculation of the difference between groups. *=$p<0.05$, **=$P<0.01$, ***=$p<0.001$.

4.3.2 Effect of UCMS and Luteolin on ATF6 Expression

The ATF6 levels were significantly (p<0.0001) higher in UCMS (5.2±0.090) than control

group (1.0±0.070). The treatment with LUT (3.3±0.018) significantly reduced (p<0.001)

ATF6 levels when compared to UCMS group (5.2±0.090). The UCMS+FXT group

(0.32±0.042) also showed significant reduction in the ATF6 expression in comparison to

UCMS (p<0.0001) and UCMS+LUT group (p<0.0001). The alone FXT treated group

(2±0.1) increased the ATF6 levels while the ATF6 expression in alone LUT treated group

(1±0.08) is comparable to the control group. The reduced ATF6 expression in UCMS+LUT

and UCMS+FXT group depicted their potential role in lowering ER stress.
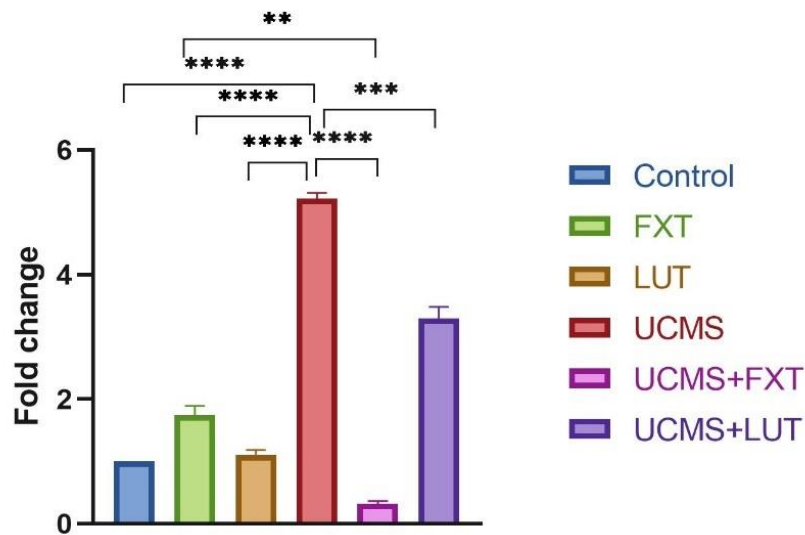
Fig 4.12. Effect of UCMS and LUT on ATF6 expression. Data was analyzed in GraphPad Prism

8.0.1 by using one way ANOVA and expressed as Mean ± SEM for calculation of the difference

between groups. **=P<0.01, ***=p<0.001, ****=p<0.0001.

DISCUSSION

Depression is a widespread mood disorder marked by persistent emotions of sorrow, diminished interest, and changes in autonomic functions, such as variations in appetite, sleep patterns, and energy levels. The study is designed to evaluate the antidepressant effects of a natural plant-based compound, LUT, on UCMS model of depression. Based on the neurogenic hypothesis of depression, the impaired neurogenesis in the adult hippocampus contributes to depression (Baptista & Andrade, 2018), the study explored the effect of UCMS and LUT on the hippocampus DG area. The study also investigated the expression levels of NeuN to further elucidate the neuroprotective role of luteolin. ER stress has been recognized to play a role in the pathophysiology of depression (Mao *et al*., 2019) hence the expression levels of ATF6 as one of the regulatory transcription factors in ER stress were examined to determine the effect of LUT on ER stress in the hippocampus of UCMS exposed BALB/c mice.

For the validation of the UCMS model, the coat score, a maker for UCMS induced depressive behaviors was evaluated over a period of 9 weeks. A significant decline in the condition of the coat was observed in UCMS mice from week 4 till the last week of UCMS. It is reported that the coat deterioration in 4-weeks UCMS procedure is both gender and strain sensitive and is shown to significantly deteriorate in three different strains of male mice i.e., C57BL/6J, DBA/2J and BALB/cJ (Mineur *et al*., 2006). Other studies also reported significant deterioration which is indicative of depressive symptoms in mice (Ibarguen-Vargas *et al*., 2008; Farooq *et al*., 2012; Culig *et al*., 2017). The current study showed a significant improvement in the coat state during the 9th week of FXT treatment. This was consistent with the results of Isingrini *et al* (Isingrini *et al*., 2010). The

administration of LUT in current study also alleviated depression by significantly lowering the coat score thus improving the coat state of mice. Although the impact of LUT on the UCMS exposed mice has not been examined before, reports revealed that it attenuates CUMS-induced depressive symptoms by displaying significantly increased sucrose preference in sucrose preference test (SPT) observed in the C57BL/6J mice (Xie *et al*., 2023).

Anhedonia, one of the clinical features of depression involves impairment in the ability to experience pleasure (Rizvi *et al*., 2016). The present study demonstrated significant anhedonia in UCMS mice as indicated by greater latency to initiate grooming behavior, reduced grooming time and lesser grooming episodes or frequency observed in SST. Burstein & Doron, 2018 reported an increased anhedonia in a 4-week UCMS model of pre-adolescent outbred male mice in sucrose preference test (Burstein & Doron, 2018). It is also evident in mice subjected to both social isolation and environmental stressors by measuring the latency to bite in cookie test which is significantly increased in UCMS-induced male iBax mice in comparison to control or treated group (Eliwa *et al*., 2021). A 4-week UCMS model of ICR outbred male mice also demonstrated anhedonia by measuring sucrose/ ethanol preference (Burstein *et al*., 2017). FXT, an SSRI, was used as positive control in this study. The study showed that the chronic FXT administration resulted in significantly reduced anhedonia indicating improved motivation in UCMS-induced mice. Interestingly, in comparison, LUT exerts similar effects depicting significant improvement in motivation and self-care behaviors. It was also reported earlier that luteolin treatment showed significant increase in sucrose intake in SPT in a dose dependent manner in noise-induced depression model of male Kunming mice (Cheng *et al*., 2022).

The current study also assessed the anxiolytic effects of LUT in comparison to FXT in UCMS-induced mice via NSFT. The results showed that both FXT and LUT reduced anxiety in mice. A trend of anxiety like behavior was observed in UCMS group however the results were non-significant. Although chronic stress profoundly influences the physical conditions of all animals without any exceptions, but some might display anhedonia while others might show alterations in other symptoms related to depression like reduced sucrose preference, reduced body weight, impaired locomotor activity or anxiety (Markov & Novosadova, 2022). In contrast, a study showed that 4-weeks of C57BL/6 mice UCMS model showed higher latency in NSFT than the controls thus depicting higher anxiety. However, the same study also showed that the chronic restraint stress (CRS) model had no significant difference in the latency to eat versus the control group, but the total food consumed was higher in CRS mice than UCMS mice thus indicating that appetite could potentially be a confounding factor in this test and not all stress models lead to depressive symptoms like anxiety (Zhu *et al*., 2014). However, a 9-week UCMS model showed significantly higher latency in BALB/c mice in comparison to the control group (Ibarguen-Vargas *et al*., 2008), but it could be because the UCMS protocol involved individual isolation of mice which might play a significant role in developing anxiety like behaviors. FXT is reported to have potential side effects e.g., nervousness and anxiety (Wernicke, 2004). A study indicated that prenatal FXT treatment in rats leads to higher latency in NSFT and hence higher anxiety levels in comparison to control group (Olivier *et al*., 2011). Likewise, the current study indicated that FXT administration in healthy subjects increased anxiety levels while the UCMS+FXT-treated group reflected no significant impact in improving the latency to eat and hence the overall anxiety. Other

SSRIs, like duloxetine and desvenlafaxine are reported to significantly decrease the latency to eat in NSFT after 1mg/kg reserpine administration for 3 days in mice (Blasco-Sera *et al.*, 2017). The anxiolytic properties of LUT were examined in the acute colitis model induced through dextran sulphate sodium (DSS) where LUT demonstrated significantly reduced latency to eat in NSFT after 15 days intraperitoneal administration of 15 mg/kg LUT in male mice (Gadotti & Zamponi, 2019). Intragastric LUT administration at 25mg/kg dosage for 6 weeks significantly reduced anxiety in 6-weeks CUMS-induced model of LOD rats in open field test (Wu *et al.*, 2023). 14-days LUT treatment in SPS exposed rats showed significant anxiolytic effects by increasing the total time spent and total number of entries in EPM (Sur & Lee, 2022). The present study also indicated a decrease in anxiety after chronic LUT administration, however more studies are required to decipher the underlying molecular mechanism and mode of action associated with anxiolytic effects of LUT.

The study also quantified the impact of UCMS and LUT treatment on short term spatial memory through the Y maze test. Although, the percentage of spontaneous alternations and the total time spent in novel arm were lesser in UCMS-induced mice in comparison to control group, no significant alteration in memory was observed. This finding is in accordance with Pothion *et al* where no significant alteration of memory was observed in CBA/H and C57BL/6 mice while the spontaneous alteration was reduced in DBA/2 mice. This study revealed that the UCMS model is sensitive to the strain of mice (Pothion *et al.*, 2004). In another study, 30 days of CUMS-induced rats showed a non-significant reduction in spontaneous alternations (Foyet *et al.*, 2017). The FXT and LUT treatment in the present study indicated no significant impact on short term spatial memory which could be

attributed to small sample size. Sass & Wortwein reported significant impaired memory in FXT-treated rats in morris water maze test (Sass & Wortwein, 2012). FXT is reported to delay impairments in the spatial memory along with the reference and working memory in mice exhibiting AD (Chao *et al*., 2021). A study demonstrated that FXT exposure during the juvenile stage led to a persistent impairment in spatial memory in male subjects (Flores-Ramirez *et al*., 2019). Exposure to fluoxetine during gestation resulted in deficits in novel object recognition memory and remote memory retention independent of the hippocampal involvement (Morago-Amaro *et al*., 2021). LUT is reported to improve memory in STZ-induced AD model of rats (Wang et al., 2016). LUT administration also alleviated cognitive dysfunction by suppressing neuroinflammation (Yao *et al*., 2018). The role of FXT and LUT on memory still needs to be explored in depression.

UCMS-induced depression leads to behavioral despair and helplessness which is quantified by measuring the time spent immobile in TST and FST. The results clearly indicated a significant reduction in immobility in both FST and TST, thus displaying behaviors consistent with a significant state of behavioral despair. The latency to immobility as well as the total immobile episodes in FST also validated the presence of behavioral despair in UCMS-induced mice. A study showed that both LPS and UCMS induction for 4 weeks resulted in significantly higher immobility time in male C57BL/6J adult mice (Zhao *et al*., 2019). The FST and TST immobile time was also significantly elevated in 6 weeks of UCMS-treated male ICR mice (Tao *et al*., 2016). The FXT treatment (10 mg/kg) of 21 days is reported to reverse this behavioral despair in both TST and FST by significantly decreasing the immobile time in corticosterone-induced depression model of mice (Herbet *et al*., 2021). The FXT treatment is also reported to significantly decrease immobile time

in FST and TST in a dose dependent manner (Kulkarni & Dhir, 2007). These results were consistent with the results of the current study where FXT treatment significantly reduced the immobile time in TST and FST, respectively. The administration of LUT in present study indicated significant lowering of the immobile time along with significant decrease in the latency to immobility and number of immobile episodes in TST and FST respectively. Thus, LUT potentially alleviated depressive behaviors through improved ability to deal with stressful situations as well as displaying more active and adaptive behaviors. These results were in accordance with a study that showed significant reduction in immobility time in noise induced depression model (Cheng et al., 2022).

The reduction in hippocampal volume and impaired neurogenesis in hippocampus are reported in MDD (Fang *et al*., 2018). The present study also demonstrated dispersed cells in UCMS as compared to compact organization in control group. The FXT-treated UCMS group showed significant improvement in the cell count which is in accordance with the results of other studies. A significant improvement in neurogenesis or cell proliferation was evident post FXT administration in UCMS-induced model (Khemissi *et al*., 2014; Zavvari *et al., 2020*; Patricio *et al*., 2021). In addition, a study on Parkinson's disease showed significant increase in Ki-67 positive cells post FXT treatment (Mendonca *et al*., 2022). Similarly, FXT treatment in adult mice also showed hippocampal neurogenesis by significantly elevating the number of BrdU cells (Hill *et al*., 2015). The current study demonstrated neuroprotective role of LUT as it promotes hippocampal neurogenesis or proliferation of cells in the DG area of hippocampus. A similar role of LUT was evident in other rodent models of depression. LUT treatment alleviated impaired neurogenesis in late

onset depression (LOD) rats by significantly increasing the BrdU/ doublecortin (DCX) and Ki-67/Nestin positive cells and BDNF levels (Yoo *et al*., 2013; Li *et al*., 2022).

For further insight into the neuroprotective effects of LUT, gene expression of NeuN was also assessed.   The analysis showed a significant decrease in hippocampal NeuN expression in the UCMS-induced mice. The FXT-treated UCMS group showed significant improvement in NeuN expression in the present study. While LUT treatment post UCMS also ameliorates the expression of NeuN. Zhou *et al* reported a similar effect of LUT in the Ts65Dn induced down syndrome model (Zhou *et al*., 2019).

The understanding of the origin as well as the fundamental neurobiological mechanisms of depression are crucial for exploring new therapeutics for the management and prevention of the disease. ER stress plays an important role in contributing to depression (Mao et al., 2019) contributing to neuronal loss (Kubera *et al*., 2011) and reduced neurogenesis (Malberg *et al*., 2000). The postmortem studies of subjects who died of suicide reported higher levels of ER stress associated proteins in prefrontal cortex (Oyadomari *et al*., 2002). Increased levels of ER stress related proteins in the DG and other regions of hippocampus indicated the presence of significant depression in corticosterone-induced mouse model (Gourley *et al*., 2008; Shim et al., 2009). These observations imply that the pathogenesis of depression may involve neuronal cell death and ER stress. The study further explored the effect of UCMS, FXT and LUT on ATF6 expression, one of the regulatory genes involved in UPR and is known to exhibit both pro-apoptotic as well as cell survival properties. A significant elevation in hippocampal expression of ATF6, observed in the present study in UCMS mice reveals the association of ER stress with depression. It is postulated that increased ATF6 expression might have contributed to the reduced neuronal

count observed in UCMS mice in the present study. Increased expression of ATF6 in the prefrontal cortex of MDD subjects is evident in studies (Yoshino *et al*., 2020). Similarly, in learned helpless model of rats significantly increased hippocampal expression of ATF6 was observed (Timberlake & Dwivedi, 2016). A marked reduction in ATF6 expression by FXT and LUT administration in the present study indicates a possible reduction in ER stress, that might have contributed to higher hippocampal neuronal cell count. Previous reports on FXT showed its inhibitory effect on ER stress in PD rats (Peng *et al*., 2018) and involvement in ER stress-mediated apoptosis as reported in human glial cell lines (Khin *et al*., 2020). Likewise, LUT exhibits hepatocytes protection against tunicamycin-induced ER stress in hepatocytes and SH-SY5Y cells (Ishisaka *et al*., 2011; Jegal et al., 2020). However, further studies to identify the mRNA and protein expression of other upstream and downstream genes involved in the UPR signaling are required. This is essential to establish a connection between the decreased ATF6 expression and increased neuronal cells in the hippocampus.

CONCLUSIONS AND FUTURE PROSPECTS

The study showed antidepressant potential of LUT in UCMS model of mice which is believed to be much more reflective of the chronic and unpredictable nature of stress in human life compared to other stress models. LUT, being a plant-based compound is thought to have lesser side effects in comparison to conventional SSRIs and is recognized for its potential health benefits. The current study through behavioral assessment indicated the antidepressant potential of LUT through alleviation of anhedonia, anxiety, helplessness, and behavioral despair. The present findings revealed several potential neuroprotective mechanisms of LUT like promotion of neurogenesis via regulation of NeuN and mitigating the ER stress through ATF6 regulation. However, further studies are needed to establish a connection between neuronal cell death in hippocampus and increased ATF-6 expression in UPR signaling. Moreover, investigating the impact of LUT on additional pathogenic pathways associated with depression is essential to explore its potential as a multi-target agent.

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Exploring the Antidepressant Like Effects of Luteolin in Mice Model of Depression

ABSTRACT

Depression is a complex and heterogeneous mental health condition which affects the overall well-being of an individual by influencing thoughts, emotions, sleep, behavior, and physical health. Reports from the world health organization (WHO) have affirmed that the global impact of depression is substantially increasing to the level that it is anticipated that by 2020, it will become the primary cause of disease worldwide. Currently, the treatment of depression involves selective serotonin reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors, monoamine oxidase inhibitors, tricyclic and tetracyclic antidepressants. These synthetic antidepressants, although widely prescribed, have significant limitations since they focus exclusively on one aspect of pathogenesis, resulting in the majority of them exhibiting a slow-acting nature. Moreover, they have multiple side effects that include insomnia, nausea, weight changes and sexual dysfunction. In contrast, natural plant-based flavonoid compounds having lesser side effects are known to produce anti-depressant like effects by targeting multiple pathways simultaneously. The study explored the effect of intraperitoneal administration of 10mg/kg Luteolin (LUT) on nine weeks of unpredictable chronic mild stress (UCMS) mouse model of depression in comparison with Fluoxetine (FXT). The mice were assigned randomly to six distinct study groups, with each group consisting of six mice (n=6). Coat state was examined each week as an indicator of stress. The UCMS group showed significantly altered coat state from week 4 (1.50±0.183) (p<0.001) till week 9 (1.583±0.154) (p<0.0001). The chronic LUT

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