NEUROBEHAVIOURAL AND BIOCHEMICAL EVIDENCE OF ANTIDEPRESSANT ACTIVITY OF TROXERUTIN IN MICE

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CERTIFICATION

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DEDICATION

I dedicate this work to glory of God Almighty for His faithfulness and awesomeness.

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ABSTRACT

Depression is a debilitating neurological illness characterised by behavioural and neurochemical changes. Conventional antidepressants have poor efficacy, serious side effects and primarily target monoaminergic systems, whereas, there is involvement of neuroinflammation, necessitating newer antidepressants. Troxerutin, a bioflavonoid has been reported to have beneficial antidepressant and anti-inflammatory potentials; however, its mechanism is not fully elucidated. This study was designed to elucidate the antidepressant activity and underlying molecular mechanisms of troxerutin in Swiss mice.

Troxerutin antidepressant activity was evaluated in 140 mice. In the acute study, 50 mice were used for Tail Suspension Test (TST) and Forced Swim Test (FST). Animals in each test were assigned into five treatment groups (n=5): distilled water (10 mL/kg), troxerutin (10, 20, 40 mg/kg) and imipramine (25mg/kg), respectively and their duration of immobility was measured 30 minutes after treatment. In the chronic studies, 90 mice were used in three experimental models: Chronic Unpredictable Mild Stress (CUMS), lipopolysaccharide (LPS), and Reserpine-induced Depression (RID). Animals were assigned into six treatment groups (n=5): distilled water (10mL/kg) naive, distilled water (10mL/kg) negative control, troxerutin (10, 20, 40 mg/kg) and imipramine (25mg/kg) respectively. Animals in CUMS, LPS or RID were exposed to stressors for 14 days (CUMS), single dose LPS (0.83mg/kg) after 7 days treatment, and reserpine (0.5mg/kg, daily) for seven days, respectively except for naïve group. The animals were subjected to behavioural assessment: Sucrose Preference Test (SPT), and Y-Maze in CUMS, Novelty Suppressed Feeding (NSF) in LPS, Splash Test in RID. Brain samples were collected for biochemical and histological analysis. In CUMS and LPS, the concentration of BDNF,CREB, MAPKs, corticosterone, TNF-α, IL-6, serotonin, norepinephrine, and dopamine were measured by ELISA and the levels of GSH, malondialdehyde, SOD, and nitrite were measured by spectrophotometry. In RID samples, concentrations of BDNF, CREB, serotonin, norepinephrine, and dopamine were measured. Histology assessment of prefrontal cortex and hippocampus was done by H&E staining for CUMS, and immunohistochemistry staining for iNOS in LPS model. Data were analysed using ANOVA at $\alpha_{0.05}$.

Troxerutin significantly reduced immobility period in TST and FST. In chronic models, troxerutin reversed behavioural deficits compared to negative controls in SPT (75.40±3.38, 74.04±3.10, 75.76±1.62 vs 38.26±5.02%), Y-Maze (76.20±2.28, 75.40±1.03, 77.20±1.42 vs 45.20±1.31%), NSF (101.4±6.99, 100±3.06, 99.6±7.03 vs 216±4.94 sec), and Splash Test (100.6±3.01, 99.20±3.89, 100.6±3.25 vs 32.00±3.01). Troxerutin increased BDNF (184.2±1.47, 185.6±1.42, 190.0±0.50 vs 161.8±2.44pg/mL), MAPKs (2304±117.2, 2279±9.657, 2416±19.94 vs 1778±24.36pg/mL), CREB (15.97±0.60, 16.02±0.58, 17.86±1.35 vs 11.65±0.26ng/mL). Troxerutin also increased serotonin, norepinephrine and dopamine levels, but decreased corticosterone, TNF-α, and IL-6 in CUMS. Similar results were obtained with LPS and RID. Troxerutin compared to negative control increased GSH (0.626±0.02, 0.670±0.03, 0.668±0.03 vs 0.258±0.05µmol/g), SOD (4.90±0.21, 5.08±0.36,5.74±0.31 vs 2.44±0.27U/mg) but reduced nitrite (36.00±4.89, 31.60±4.99, 32.00±5.09 vs 76.00±9.69µg/ml),MDA (3.60±0.74, 3.60±0.24, 3.20±0.20 vs 8.60±0.24nmol/mg) in CUMS, and the same pattern was observed in LPS. Also, troxerutin increased neuronal density in CUMS and decreased iNOS expression in LPS.

Troxerutin demonstrated antidepressant-like effect in mice by reversing neurobehavioural deficits in depression via inhibition of neuroinflammatory markers, modulation of oxidative stress, and up-regulation of monoaminergic neurotransmitters, and neurotrophic factors.

Keywords: Troxerutin, Bioflavonoid, Antidepressant, Neuroinflammation
Word count: 473

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LIST OF ABBREVIATIONS

BDNF	Brain-Derived Neurotrophic Factor
CREB	Cyclic-AMP Response Element Binding Protein
CUMS	Chronic Unpredictable Mild Stress
DALY	Disability Adjusted Life Years
ELISA	Enzyme Linked Immunoassay
FST	Forced Swim Test
GSH	Glutathione
IL-6	Interleukin-6
IMI	Imipramine
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
MAPKs	Mitogen Activated Protein Kinases
MDA	Malondialdehyde
NFKB	Nuclear Factor Kappa-B
NE	Norepinephrine
OFT	Open Field Test
PWS	Protracted Withdrawal Syndrome
5-HT	5-Hydroxytryptamine
SOD	Superoxide Dismutase
SPT	Sucrose Preference Test
TBA	Thiobarbituric Acid
TNF-α	Tumor Necrotic Factor-α
TCA	Trichloroacetic Acid
TST	Tail Suspension Test
TXN	Troxerutin
YLD	Year Lost to Disability

CHAPTER ONE
INTRODUCTION

1.1 Background

Depression as chronic disease is a stress-related debilitating and long-lasting illness characterised by diverse neurological symptoms which negatively affect thought, behavioural disposition, cognition and sense of well-being. The disease can occur at any age, it can sometimes start from childhood to late life and without effective therapeutic intervention the prognosis is often severe (Bernaras *et al*., 2019). The overwhelming burden of the disease has made depression a major public health challenge being identified globally as a primary cause of the disability burden ascribed to Disability Adjusted Life Years (DALY) and it has remained a leading cause of Year Lost to Disability (YLD) since year 2000 (James *et al*., 2018). Globally, depression alone is responsible for about 3.8% of disease burden worldwide, affecting about 280 million of world's population and it has been projected to rise, thereby becoming a main cause of incapacitation, reduced productivity, impairment of function and increased mortality (WHO, 2021). Generally, depression is a very common condition affecting all age groups and lifetime prevalence of depressive episodes is up to 20.6% in an individual (Hasin *et al*., 2018).

Clinically, major depressive disorder primarily presents as a combination of three major symptoms which are seen as low mood, loss of interest and low energy. However, these clinical presentations of depressive episodes are not limited to a specific condition, it often equally present as an attending co-morbidity in almost all chronic illnesses (Gin & John, 2018). In Nigeria for example, depressive co-morbidity and its correlates accounts for up to 15% of patients accessing primary care and the characteristic depressive behaviour exhibited by patients often worsen the treatment outcome (Adewuya *et al.*, 2021).

Therefore, the characteristic distinction in major depression is the involvement of other symptoms such as mood swing, anxiety, learning and memory deficit, helplessness, feeling worthless and guilty, irritable, altered arousal and alertness, aches and pains or restless, appetite loss or overeating, insomnia or excessive sleeping, and suicidal behaviour, thus explaining the heterogeneity of depressive disorder (Vares *et al*., 2015). Behavioural responses to depressive disorder are essentially disruptive as depression alters cognitive functions making the individual engage in anti-social behaviours that threatens wellbeing and survival.

The complexity of clinical symptoms of depression has further compounded the many myths and unclear circumstances surrounding the causes and development of the illness. However, hypothetical speculations have put forward various theories on the biological basis of depression (Sanz *et al*, 2017). Review of the identified causes of depression suggests that the disease is an intricate heterogeneous disorder of diverse aaetiology involving multiple interactions between brain systems and neuromodulatory pathways. First, the study of the pathophysiology of depression implicated dysfunction in monoamine neurotransmitter circuits as the primary basis of depression (Hasler, 2010), while the observed atypical anomalous response of the hypothalamic-pituitary-adrenal (HPA) axis has equally been implicated to contribute significantly to stress mal-adaptation underlying depression which suggests stressful life events as primary aetiological factors resulting in monoaminergic deficit (Keller *et al*., 2017). In a study of the mechanistic interplay, stressful life event triggers several neural circuits which include the locus coeruleus region of hypothalamus and the autonomic noradrenergic centres in the brainstem resulting in various stress responses across the CNS, and maladaptive neuroplastic changes of these brain centers culminating in depressive episode (Nuria *et al*., 2019). Perhaps these neuroplastic changes result in neuronal loss which further exacerbate the stress mal-adaptation by direct alteration of the neuroendocrine and neuroimmune signalling systems, thus implicating the involvement of neuroimmune and neuroinflammatory system in aaetiology of depression (Lee & Giuliani, 2019). Consequently, several agents have been developed as antidepressants based on their

established activity in the modulation of the altered neural pathways implicated in depression and their ability to attenuate the neurological deficits.

Despite the profoundly negative effects of depressive disorder on public health, there is limited progress in the discovery of therapeutically effective drugs (Simone & Nicolas, 2019). Conventional antidepressants basically act via a single or multiple mechanisms that involves the monoaminergic neurotransmitters; that is, Dopaminergic Noradrenergic and/or Serotonergic, but they are unsatisfactory because of the limited therapeutic significance and associated diverse side effects (Al-Harbi, 2012). For example, the tricyclic antidepressants exhibit characteristic delay in the onset of their antidepressant effect, with a lag phase of about 3-4 weeks (Machado-Vieira *et al*., 2010). Furthermore, alterations in synaptic plasticity from neurobiological maladaptive mechanisms in hippocampus requires denovo synthesis of proteins for neurogenesis and this could account for the latency in the therapeutic activity of conventional antidepressants (Thakker-Varia *et al*., 2014). Newer agents targeting the monoamine pathways and other neurochemical signalling that could enhance upregulation of neurotrophic factors and promote neurogenesis have been hypothesized to provide better outcome in depressive disease management. One of the factors identified to be responsible is the fact that serotonergic neurosystem circuitously interacts with neurotrophic factors signalling, mainly Brain Derived Neurotrophic Factor (BDNF), creating essential homeostatic feedback loop vital for regulation of neurogenesis,synaptogenesis, and neuroprotection in adult brain (Yu & Chen, 2011). Beyond the monoaminergic are other neurotransmitter systems and recent studies have implicated the glutamatergic pathway as cogent mechanism underlying rapid antidepressant activities (Mathews *et al*., 2012). Several agents targeting the glutamatergic system have shown promising activities, for example, an antagonist used as general anaesthesia is Ketamine which at its subanaesthetic dose acts on the glutamatergic system through modulation of the mTor signalling pathway to exhibit rapid-onset antidepressant activity (Matveychuk *et al.,* 2020).

A major limitation in treatment of depressive disorder are the accompanying adverse effects of the conventional antidepressants which include insomnia, weight gain, dry mouth, constipation, inability to concentrate and sexual dysfunction (Santarsieri &

Schwartz, 2015). The complications from these side effects has led to low compliance with no adherence of up to 52.29% and the refusal to take antidepressants in appropriate doses among patients result in high rate of therapeutic failure (Nirmal *et al*., 2020). Additionally, the cost of the available antidepressant drugs poses economical challenges for depressed patients, thus contributes significantly to the residual symptoms after treatment and high rate relapse seen in approximately 60% of treated depressed patients (Pim *et al*., 2020). According to report on world mental health, in developing countries the estimated population of depressed patients who received no treatment averages between 76% to 85% and the leading contributing factor for this identified barrier to accessing care is lack of resources (Kessler *et al*., 2009).

Results from randomised controlled clinical trials have suggested that isolated compounds of plant origin may have fewer side effects being a natural product and equally be a cheaper alternative, thereby improving compliance and therapeutic outcome (Haller *et al*., 2019). Historically, among various cultures when people are bereaved or depressed, concoctions tincture are specially prepared and given to depressed victims to alleviate their mood and help cope with the situation (González *et al*., 2020). This is a popular practice among the Japanese and a commonly used plant for such concoction is *Sophora Japonica* (Inoue *et al*., 2017). Hence, focus on natural compounds is very likely to provide the breakthrough for discovery of compounds with activities beyond the monoamines modulation that offers better therapeutic efficacy and fewer side effects. Technically, the plant, *Sophora Japonica*, is a viable candidate for potential source for novel compounds with beneficial and positive therapeutic antidepressant activities with possible less toxic side effects. Troxerutin (TXN), commonly denoted Vitamin P4 is an active plant-based bioflavonoid compound isolated from the plant *Sophora japonica* and it has been postulated to be a potential novel antidepressant candidate owing to its identified anti-inflammatory and anti-apototic properties that could mitigate the stress induced neurodegenerative alteration underlying depression (Geetha *et al*., 2017).

The associative symptoms of depression are diverse and unpredictable, however, in humans the diagnosis of depression utilises the use of symptomatic criteria listed on the

diagnostic Manual tool for assessment of metal health status (Tolentino *et al*., 2018). In the search for investigational new drug that could serve as a novel antidepressant, where possible, the human symptoms are mimicked as endophenotypic symptoms in mice measured with experimental procedures and such validated standard tests are herewith listed on table 1.1.

Table 1.1: Depressive symptoms and correlating endophenotypes in mice

Assessment Symptoms in Humans	**Correlating paradigms in Mice**	**Experimental Model**
Decreased interest and/or loss of pleasure	Anhedonia	Sucrose Preference
	Behavioural despair	Forced swim Tail suspension
Weight gain or loss	Stress-induced weight loss or gain	Weighed on scale
Psychomotor retardation	Alterations in locomotion	Open Field Test
Fatigue or loss of energy	Reduced homecage activity	Alterations of homecage activity: Grooming Rearing
Diminished thinking ability or attention deficit	Working and spatial memory deficit	Y maze
	Anxiety-related behaviour	Novelty-induced hypophagia

1.2 Statement of Research Problem

Depression has remained a leading cause of disease burden globally and the incidence rate has continued to rise, with up to 49.86% increase over the last decade (Liu *et al*., 2020). Therefore, depression remains a major public health issue.

A more serious challenge with depression is the high rate of therapeutic failures (Crowe *et al*., 2023). The treatment of depression principally depends on use of antidepressant class of drugs whose target is chiefly the monoaminergic neurotransmitters system (Racagni & Popoli, 2010). However, recent findings elucidating the involvement of other numerous neuronal signalling pathways have shown the therapeutic limitations seen in these currently available antidepressants.

The characteristic delay in the antidepressant effect and the attending side effects has equally made treatment of depression with these conventional antidepressants very insufficient (Brendan *et al*., 2023). This accounts for the high rate of noncompliance of the patients to their treatment regimen thereby causing a negative treatment outcome.

Another major concern with use of conventional antidepressants is the protracted withdrawal syndrome (PWS) that follows after stopping the medications. The withdrawal experience often results in a rebound phenomenal that presents with a more severe correlate of the primary disorder (Cosci & Chouinard, 2020). The lack of sustained suppression of depressive symptoms after withdrawal, whether steeped or abrupt, withdrawal results in rebound depressive symptoms (Michael *et al*., 2020). The PWS is present in almost all classes of antidepressant; the rate among commonly used antidepressants is illustrated in Figure 1.1 below.

This unavailability of therapeutically efficient medications in management of depression has created a significant gap in medical practices that has made the identification of novel antidepressants targeting multiple pathways a high priority.

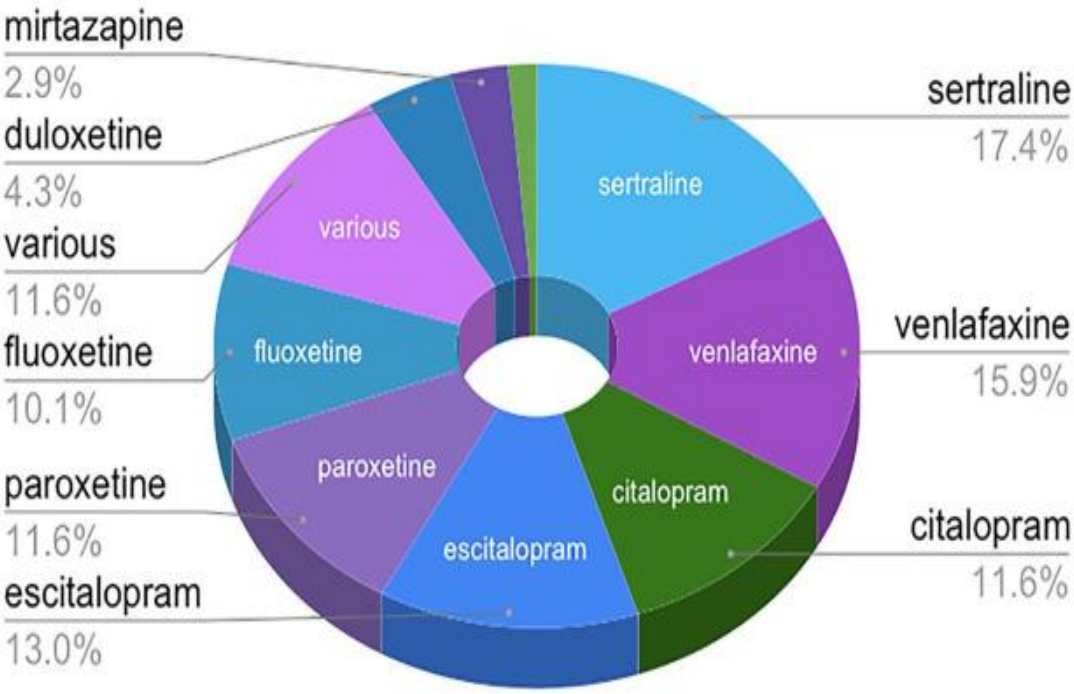

Figure 1.1: Rate of Protracted Withdrawal Syndrome (PWS) with conventional antidepressants (Michael *et al*., 2020)

In summary, novel antidepressants having early onset activity and reduced side effects with increased therapeutic efficacy is still lacking and the discovery of such is a main concern while putting in perspective the global burden of major depressive disorder (Jorm *et al*., 2017).

1.3 Justification of the Study

Naturally occurring compounds including flavonoids offer robust biological activities with low side effects and are tissues protective in many cases (Pribac *et al*., 2015). Bioflavonoids are plant derivatives occurring naturally and are of wide varieties depending on their source and biochemical structure. They have been shown to exert beneficial biological and therapeutic activities, while some groups can cross into brain and modulate central nervous system (CNS) by mediating neuroprotective signals for neuronal survival against stress induced injury (Panche *et al*., 2016). They may act by suppression of pro-inflammatory neuronal cells (microglia and astrocytes) activation and/or by upregulating synaptic plasticity, promoting memory and cognitive function (Jang & Johnson, 2010). Generally, much of their antidepressant activities has been ascribed to the amelioration of oxidative stress (Lucian *et al*., 2017). Vitamins are common nutrient source and like other vitamins, Vitamin P4 is of plant origin (Xin *et al*., 2018). As stated above, Troxerutin being a flavonoid has been found to have documented valuable pharmacological profile which includes antioxidant, anticancer, antidiabetic and antiinflammatory activities (Zahra *et al*., 2021). Troxerutin is also structurally related to the naturally occurring bioflavonoid, rutin, which exerts antidepressant effect via glutamatergic pathway other than the monoaminergic system and its neuroprotective effects are devoid of cytotoxic side effects (Maryam *et al.*, 2020). Troxerutin has been shown to exert neuromodulatory activities in the brain through its ability to freely traverse into the brain (Mintz *et al*., 2019). Thus, it is hypothesized that Troxerutin being a bioflavonoids will possess effective neuroprotective and antidepressant activity with limited toxicity.

1.4 Aim of Study

This study aims to elucidate possible molecular mechanism of antidepressant activities of Troxerutin using behavioural, biochemical and histological evidences.

1.5 Specific Objectives of the Study

- Determination of the acute toxicity (LD_{50}) of Troxerutin.
- Evaluation of the antidepressant activities of Troxerutin in acute model.
- Determine the protracted effect of Troxerutin on depression.
- To evaluate the antidepressant activities of Troxerutin in chronic model.
- To evaluate the antidepressant activities of Troxerutin in neuroinflammatory model of depression using LPS.
- Measure the effect of Troxerutin on altered behavioural activities including learning and memory in depression.
- Assess the neuroprotective properties of Troxerutin by histological and biochemical evaluation.
- Identify activities of Troxerutin on the key molecular targets, monoaminergic system and neurotrophic factors, implicated in pathophysiology of depression.

1.6 Significance of Study

A cited report of the World Health Organisation identified global use of plant-based remedies as the primary form of healthcare, reported to be about 80%, while the rate in low-income developing countries was estimated to be as high as 95% and it is sometimes the only accessible source of primary healthcare to the poor in those countries (Tugume & Nyakoojo, 2020). Troxerutin being a natural bioflavonoid compound of plant source would have beneficial pharmacology profile that would be economical. Troxerutin as a potential and promising candidates for novel antidepressant with possible early therapeutic onset, substantially satisfactory response rates and functionally neuroprotective properties will significantly reduce the burden of depressive disorders thereby reducing the associated DALY and enabling quality living. As a natural compound the compliance among patients is also likely to be very high.

CHAPTER TWO

LITERATURE REVIEW

2.1 Basic Features of Neurobehavioural Disorders

Neurobehavioural disorders result in functional disabilities with increasing direct and indirect cost on the global economy, reaching billions of dollars annually (Hackman *et al*., 2010). First and foremost, behavioural changes are critical signs of alteration in Central Nervous System (CNS) functions. The changes can be in cognitive functions, intellectual functions such as memory impairment or emotional as seen in mood change, and could result from accompanying neural lesions, brain atrophy or structural changes (Robert *et al*., 2015). Beneath these functional changes in the CNS are alterations in its neuronal circuitry (Schmidt *et al*., 2012).

The CNS functions are modulated through interplay of multiple neuronal pathways and neurotransmitter systems. In mood disorder, the dysfunction of the neurocircuitry projections in specific brain regions; the pre-frontal cortex, the striatum, the hippocampus as depicted in Figure 2.1 below are markedly affected and the alteration of biological functions of the released neurotransmitter systems formed the basis of neurobehavioural disorders, particularly depression (Price & Drevets, 2010).

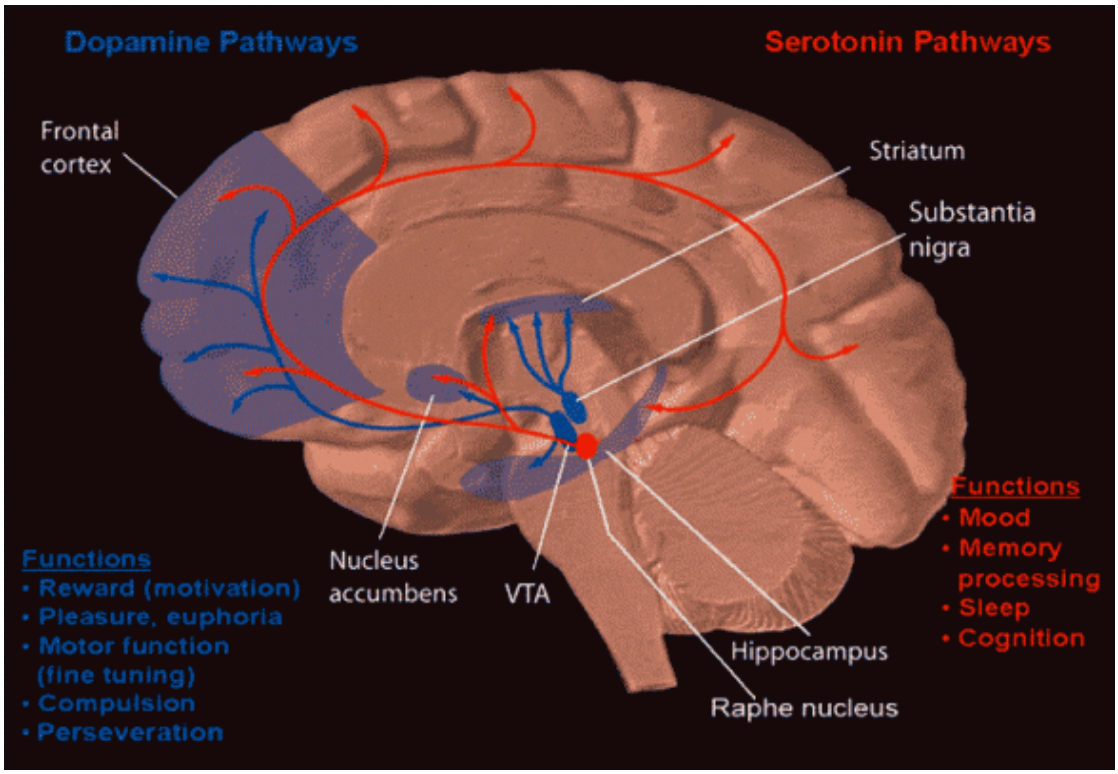

Figure 2.1: Monoamine Neurotransmitter system pathway and Mood Disorder

(Chandler *et al*., 2013)

Many studies have evidently shown that treatments with modulators of specific neurotransmitter pathways within the medial prefrontal cortex (mPFC) exhibit an effective therapeutic effect in neurobehavioural deficit (Leuchter *et al*., 2012).

2.2 Depression

Depression and its pharmacological intervention has been a critical subject of scientific debates since the Aristotelian era of medicine many millennia ago. Depression remained a fundamental topic in the Hippocratic medicine era thousands of years ago and it was named *melancholia* (excess black bile theory). Today, depression has continued to be a critical issue in modern medical practice (Tipton, 2021). Many studies have addressed the pathophysiological basis of this disease; some have dedicated research towards identifying effective antidepressant drugs while others focused on mechanistic study of these drugs and how to modify them for improved therapeutic outcomes. These various studies have all contributed tremendously to the advances in pharmacotherapy of depression and it is essential to highlight significant areas of these studies in order to identify and mitigate gaps in effective development of novel antidepressant drug.

Depression is a very common neurobehavioural disorder that impairs daily life and it is often characterised by persistently low mood or disinterestedness in pleasurable activities, termed anhedonia. There are many possible causes which are either biological or environmental or combination of both. Currently, the pathophysiology of depression is not fully understood resulting in persistent limitation in the treatment rationale (Nestler & Hyman, 2010). The World Health Organisation report on mental disorder approximated about 450 million people globally to be affected by at least one form of mental or behavioural disorder, whereas, in low income countries it affects up to 15% of adults (Ferrari *et al*., 2013). When the global disease burden of depression was at 12.3%, while considering the slow progress in finding effective therapeutic agent, it was hypothesized that the global disease burden will possibly rise to 15% by 2020, and the hypothesis was true as depression becomes the second leading cause of impaired wellbeing worldwide (WHO, 2020). In year 2000 depression was rated among the top eleven cause of Disability Adjusted Life Years (DALY) globally and the report of year 2012 showed depression to be among the top nine with an increase from 64 million to 77 million (2.2% to 2.7%) and

remained the leading cause of YLD since year 2000 (WHO, 2018) implying that the projection was valid as we have seen it today. Due to the rapid increase in disease burden and the slow progress in finding effective antidepressant agents, it is very important to highlight the pathological mechanism underlying depression and the possible therapeutic targets in order to mitigate further increase of the disease burden.

Depression cannot be classified as a homogeneous disorder but a multifarious disease of possibly different forms with diverse aetiology. The varying symptoms further implicated interplay of multiple neural pathways which may be independently or collectively associated. The recent understanding of the pathophysiology of depression has substantially transitioned beyond the initial excess of black bile theory of Galen, and now modern science highlights involvement of biological and environmental interactions (Nemeroff, 2020).

In summary, alterations in multiple neurobiological systems have been identified to lead to depression and these processes are metabolic, inflammatory, and stress-response pathways (Iwata *et al*., 2012). Although individual variations due to the genetic polymorphisms are exhibited by different subgroups of depressed patients, however, the depressed brain shows several common characteristic pathophysiological features that serve as hallmark of depressed state (Thomas & Duman, 2017). There are usually significant neural activity differences between brains of healthy individuals and that of patients with major depressive disorder (MDD).

2.2.1 Aetiology of Depression

No singular independent cause has been attributed to the aetiology of depression but underlying biological factors and environmental factors are the major precipitating aetiology. Biological factors include genetic, neurological, hormonal, immunological, and neuroendocrinological variations. Age and sex-related processes also contributes to biological variation observed among patients with respect to onset of depression (Verduijn *et al*., 2015). The commonest environmental factor responsible for initiation of depressive behaviour is stressful life events. Although the same stressor may not trigger depression in

another individual, hence, these personal vulnerabilities further explains the genetic correlates for aetiology of depression. In experimental studies the biological models of depression chiefly utilizes face-validity stress paradigms for both adaptogenic and neurobehavioural endophenotypes of depression.

Generally, stress mechanism remains the most important etiological factor for major depressive disorder. Furthermore, environmental factor as the etiological basis of depression has adopted the stress-induced depression theory in which the environmental trigger itself has been identified as a stressor, thereby, corroborating the fact that stress is the major cause of depression. The stress response in itself is a physiological process by which the body effectively handles stress process resulting from stressors which are ever present part of life. A failure of the response process is the maladaptive pathology that often results in failure to cope with stressor, triggering depressive symptoms. Alternatively, excessive stress response sequel to activation of physiological process can also result in depressive disorder. The stress response is mediated by hormones, neurotransmitters, cytokines and growth factors that are released.

2.2.2 Theory of Depression: Neurochemical Imbalance

Generally, the hypothesis of neurochemical imbalance theory of depression stemmed from the observation of reduced activity, melancholia, in depressed patients and the theory was very popular amongst scientist in the early 1960s and the chemicals of focus were monoamine neurotransmitters; norepinephrine, dopamine and serotonin, leading to development of tricyclic antidepressants (Massart *et al*., 2012). The hypothesis thus based its theory on decreased monoamine function in the brain as the primary cause of depression. This was evidenced when out of providence the drugs Iproniazid and Imipramine that were being used as non-psychiatric agents showed potent disease modifying effects in depressed patients by enhancing central serotonin or norepinephrine transmission. Further focus on the monoamines have led to development of diverse classes of conventional antidepressants, for example, specific agents targeting serotonergic system led to the development of the more classical antidepressants, the selective serotonin reuptake inhibitors (SSRIs) that are commonly used now (Francesc, 2013). Although

many evidences were put forward but the theory was substantiated by the observation from these mood altering agents where their administration produces a consonant modulation of the level of monoamine neurotransmitter in the brain. Apart from these agents that elevate the monoamines levels leading to elevated mood, the contrary was true for the antihypertensive drug, reserpine, which when administered depletes the monoamine presynaptic storage and causes patient to have the typical low mood synonymous to a depressed state. Characteristically, in animal studies serotonin levels were significantly reduced following administration of Reserpine, further suggesting that depression results from imbalance in the neurotransmitters level (Kyzar *et al*., 2013).

The projections of neurochemical pathways in the forebrain are directly responsible for behavioural and cognitive functions mediated by this brain region. Prominent amongst these pathways are the monoamine projections which originated from the midbrain and brainstem; nuclei; the dopaminergic and serotonergic projections formed from ventral tegmental and dorsal raphe respectively, while noradrenergic formed from locus coeruleus (Chandler *et al*., 2013). Structural magnetic resonance imaging (MRI) of post-mortem brains have shown reduced activities in these brain areas of depressed patient relatively to normal subjects (Kempton *et al*., 2011).

In practice, despite demonstrated potency of conventional drugs targeting the monoaminergic pathways the observed deficit in patients' response suggests that the aetiology of depression is far beyond simple imbalance in the central monoamine function, other contributing factors such as gene-related susceptibility are likely to be involved (Chávez-Castillo *et al*., 2019). Furthermore, studies that evaluated mood changes in both healthy and depressed patients showed that depletion of monoamines only resulted in altered mood among unhealthy patients and no changes observed in healthy patients (Gonda *et al*., 2021). It is thus obvious that the pathologic basis of depression is beyond the monoaminergic system despite being the fundamental basis. It is also noteworthy that the reserpine-induced depressive state was only seen in a subset of patients, suggesting involvement of other mechanisms apart from depletion of the monoamines. The GABAergic and the glutamatergic neurotransmitter system also exhibit characteristics

alteration in their functional neurochemical activity within the pathway in several brain regions, all of which deregulate the brain neurotrophic factors expression (Kaltenboeck & Harmer, 2018).

2.2.3 Role of Neurotrophic Factors

Hypothesis for depression involving deficiency of neurotrophins stemmed from the observed diminish in hippocampal volume and neuronal loss that correlates to impaired neuroplasticity due to deficient neurotrophic factors expression in subsets of depressed patients (Hayley & Anisman, 2013). Primarily, neurotrophic factors are fundamental in enabling neuroplasticity for promoting neuronal growth and neuronal survival, however, dysfunctional neurotrophic signalling limits neuroplasticity and underlie the pathophysiology of major depression. These neurotrophic factors are also essential for protein synthesis necessary for neuronal growth, proliferation and survival, therefore, the delayed onset of therapeutic activities commonly seen in conventional antidepressants has been shown to be consistent with the neuroplasticity theory as the underlying cause (Levy *et al*., 2018). Exposure to either acute or chronic stress results in neuronal damage and neuronal atrophy but the neurotrophic factors maintain neurons to ensure survival or stimulate neurogenesis for ameliorating neuronal deficits. Common neurotrophic factors mediating neurogenesis in limbic system includes Brain-Derived Neurotrophic Factor (BDNF), Vascular Endothelium Growth Factor (VEGF-B).Although the VEGF in experimental studies of potential therapeutic targets yielded noteworthy results, the BDNF target has been clinically accepted as a more specific and pharmacologically active target to develop innovative antidepressant agents (Cristy, 2017).

Brain Derived Neurotrophic Factor is extensively expressed in adult limbic system which modulates several cognitive functions and stress coping responses. Studies show that stress-related event reduces the BDNF expression and functions in the hippocampus, resulting in depressive disorder that is reversible by long-term administration of antidepressants with an analogous increase in BDNF-mediated signalling (Eero & Lisa, 2021). Additionally, the stress-induced neurotoxicity is characterized not only by hippocampal atrophy from low BDNF concentration but also accompanied by abnormally

elevated level of cortisol and novel antidepressants at molecular level have been found to reverse the hippocampal neuronal loss while increasing the BDNF concentration (Bora *et al*., 2012). Similar results from a post-mortem study equally reported reductions in BDNF levels and downregulated expression of its receptors in forebrain of depressed patients (Castren & Rantamaki, 2010). Another post-mortem study showed that inhibition of Eukaryotic Elongation Factor-2 kinase leads to subsequent increase in translation, thus substantiating the role of increased BDNF concentration in reversal of depressive endophenotypes and has been proposed to be the underlying mechanism of the rapid onset of activity effects seen in ketamine and other similar agents with rapid onset of antidepressant activity (Monteggia *et al*., 2013). In the treatment of resistant depression (TRD) Ketamine exhibited rapid antidepressant activity which was ascribed to the modulation of BDNF receptors, upregulation of mediators, and ability to rapidly modify synaptic functions. It has been postulated that novel compounds with potential efficacy similar to ketamine may equally act via these markers and such paradigm proffers pathway for efficacious drug development in treatment of depression. Furthermore, compounds that inhibit serotonergic receptors, 5-HT2A/2C receptor equally stimulate the BDNF mRNA expression in the hippocampus, thereby reversing the stress-induced neuronal loss with a characteristic rapid onset of activity (Zhang & Stackman, 2015).

There are multiple pathways involved in the synthesis/stimulation and neuromodulatory activities on neurotrophins that are potential targets for novel rapid acting antidepressants. Notably, the first target is the normalizing of the neurotrophic factors level as index metric of reversed stress-induced changes in depressive disorders. Besides the reduced BDNF level, inhibition of the Glycogen synthase kinase-3beta (GSK-3ß) signalling pathway with resultant ß-catenin reduction were significant findings in prefrontal cortex of post-mortem depressed patients (Beurel *et al*., 2015). Evidently, findings from several previous studies showed that some antidepressants and mood stabilizing drugs like valproate and antipsychotics directly and/or indirectly interacts with the GSK-3ß signalling and modulates ß-catenin expression in their mechanism of action. Mechanistically, novel compounds at molecular level may induce antidepressant or anxiolytic activities through GSK-3ß inhibition, resulting upregulation of cortical ß-catenin level (Xinguo *et al*., 2013).

Scientific evidences showed that in nearest future neurotrophic modulatory pathway will ultimately represent one of the most innovative target mechanisms for effective treatment of neurobehavioural diseases (Pilar-Cuéllar *et al*., 2013).

Recent studies on pathophysiology of depression at cellular and molecular levels have implicated the pivotal role of Mitogen-Activated Protein Kinases (MAPKs) in pathogenesis and treatment outcome of depression. Findings from studies showed significant downregulation of the ERK signalling in depressed patients and novel antidepressants are likely to produce their effects partly via normalizing the MAPKs levels and their effect on the neurotrophic factors due to interplay between the ERK signalling and neurotrophic factors-mediated signalling(Wang & Mao, 2019).. This is exemplified in a cascade in the limbic system where BDNF release produces immediate upstream ERK response and the ERK activation downstream produced transcription factor, cAMP Response Element-Binding protein (CREB), in stress-induced depression (Yao *et al*., 2021). Moreover, long term administration of conventional antidepressants eventually produces similar neurochemical cascade of activities described above. Thus, pathophysiology of depression involves disruption of ERK signalling in different parts of the brain regions, and this mechanism is responsible for the failed neuroadaptations critical for enduring depression-inducing conditions (Wang & Mao, 2019). Molecular studies showed ERK is coupled in a cascade to diverse neurotransmitter receptors in the CNS, majorly the monoamine receptors and neurotrophic factors receptor. Downstream to the receptor is the stress-induced coupling of the extracellular MAPK cascade, an interaction that leads to reduction of TrkB phosphorylation vis-à-vis a decrease in ERK phosphorylation (Yan *et al*., 2016). These evidences illustrated in Figure 2.2 below further support the theory that vulnerability to stress-induced depression directly correlates to a reduced BDNF level, hence, BDNF targeting could serve as a molecular biomarker representing novel antidepressant activity. This BDNF theory further explains the gene-environment involvement in the pathological basis of depression.

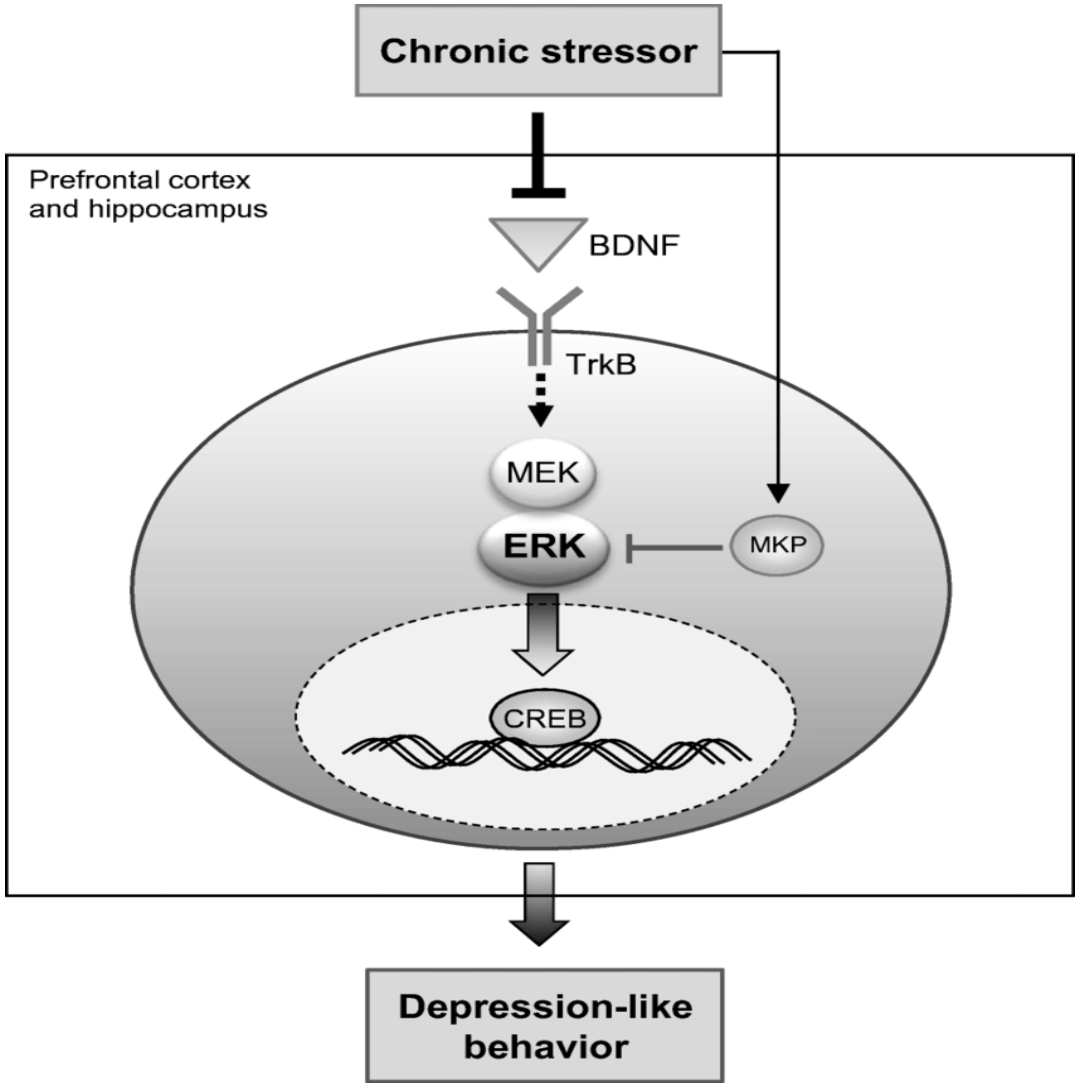

Figure 2.2: Involvement of BDNF-ERK-CREB signalling in stress-induced depression (Wang & Mao, 2019).

Clinical evidences suggested higher vulnerability to stress-induced depression among patients with BDNF polymorphism (Val66/Met) a genetic aberration resulting in decreased release of BDNF, thus, further substantiatescardinal role of neurotrophic factors in depression (Youssef *et al*., 2018). Basically, anatomic decrease in hippocampal neuronal density in depressed patients correlates biologically to the observed reduction in neurotrophic factor release and function in multiple brain regions (Miranda *et al*., 2019).

Many studies further substantiated that chronic stressors are a major cause of depression mediated by altered BDNF expression with downstream dysregulation of ERK signal-transduction pathway (Li *et al*., 2017). Chronic stress directly activate extracellular MKP, a subclass of ERK, increasing the MKP activity directly inhibits the BDNF-ERK-CREB signalling cascade that causes reduced activation of the transcription factors. Consequently, a lowered transcription causes decreased gene expression, especially the genes required for stress coping behaviours (Guan *et al*., 2013).

2.2.4 Role of Neuroinflammatory, Neuroendocrine and Neuroimmune Interactions

Inflammatory process mediates onset of depressive disorder and it has been dubbed the macrophage hypothesis due to products of macrophage released in response to inflammation, a proposition suggested by Smith, (1991), and this hypothesis remained valid till date as several recent clinical studies showed consistent significant elevation in serum glucocorticoid concentrations in depressed patients (Islam *et al*., 2018). Chronic administration of glucocorticoids induces some depression-like symptoms in rodents, thus, dysregulation of HPA-axis is critical in pathophysiology of depression (Anacker *et al*., 2011). Relatively to anatomic functions and neurotrophic activities, excessive activation of glucocorticoid receptor inhibits neuronal proliferation rates in subgranular zone (SGZ) and produce atrophic changes in other brain areas (Saaltink & Vreugdenhil, 2014). The excess glococorticoid release could further explain the volumentric reductions seen in brain subregions of post-mortem depressed patients.

Neuroimmune malactivation is a central maladaptive mechanism involved in pathophysiology of depression. Clinical evidences further proved inflammatory

hypothesis of depression valid due to the observed abnormally elevated levels of inflammation mediating cytokines in depressed patients (Osimo *et al*., 2020). A significant number of depressive symptoms have been explained by the neueroendocrine and neuroimmune interactions. For example, oxidative stress-induced elevation of pro-inflammatory cytokines mediates sickness behaviour and other associated symptoms, while the neuroendocrine through the stress hormones (cortisol, corticotropin-releasing-hormone, and adrenocorticotropic hormone) is linked to negative cognitive symptoms of depression (de Souza-Talarico *et al*., 2011). Furthermore, in experimental model of chronic stress-induced depression, physical stress induces depressive behaviour via increased pro-inflammatory cytokines release which further inhibit neuropeptides function and neurotransmitter release (Farooq *et al*., 2017).

To establish the neurohumoral involvement in depression, administration of Lipopolysaccharide (LPS), bacterial endotoxin induces depression-like symptoms. Exposure of rodents to LPS in behavioural models activates immune chemotactic response and triggers release of proinflammatory cytokines, for example, interleukins (Sangaran *et al*., 2021). It also initiates the release of Nitric Oxide, free radicals and myriads inflammatory mediators that activate EPK nuclear factor signal transduction pathways and downregulates corresponding transcription factor, CREB (Noworyta-Sokołowska *et al*., 2013). Overwhelming activation of neurohumoral system translates to uncontrollable inflammatory reactions marked by reduced superoxide dismutase (SOD) activity and elevated malondialdehyde level, which forms active mechanisms of LPS-induced depression (Amal *et al*., 2018). Induction of depression by LPS has also been associated with glutamatergic pathway through the activity of quinolinic acid on the NMDA receptor (Ester *et al*., 2019). Agonism activates indoleamine 2,3-dioxygenase (IDO), an inflammatory product of macrophages that are not naturally found in significant quantity in the CNS, and their release depletes serotonin concentration by degrading tryptophan, the precursor of serotonin. Thus, inflammatory overstimulation can cause serotonergic deactivation via kynurenine pathway and consequently explain the interplay of other mechanisms involved in monoamine theory of depression and evidently, antidepressant drugs have been seen to interfere with neuroinflammatory processes (Walker *et al*., 2013).

Also, treatment resistant depression is associated with excessively high levels of interleukins released in uncontrolled inflammatory response (Osimo *et al*., 2020).

Another neuroinflammatory mediator playing critical role in pathophysiology of depressive behaviour highlighted above is putative neurotransmitter, nitric oxide (NO), an intracellular gaseous molecule involved in regulation of neuroimmune system (Peng *et al*., 2012). The production of NO is dependent on an enzyme, nitric oxide synthase (NOS) which exists in three isoforms that are genetically unrelated. The first isoform is neuronal nitric oxide synthase (nNOS), while second is endothelial nitric oxide synthase (eNOS), and third is inducible nitric oxide synthase (iNOS), all which are respectively identified in neurons, endothelial cells, and lastly iNOS that is synthesized in response to inflammatory stimulation, as in LPS-induced depression (Zhou & Zhu, 2009). Although these enzymes are widely distributed in brain where immunohistochemical analysis have identified regions like hippocampus and cerebral cortex to contain localized cells that are NOS-positive, however, under normal physiological conditions, iNOS are not present in the brain, they have no physiological role but as the name connotes, they are induced in inflammation as an important mediator (Garry *et al*., 2015). Hence, iNOS is primarily the identified isoform that plays major role in depressive behaviour triggered in the unpredictable stress model and the LPS-induced model and various studies have suggested the involvement of the enzyme as fundamental in depressive disorder and serves as a viable target for antidepressant therapeutics. As illustrated in Figure 2.3 below, interplay between environmental stress, neurotrophic factors and immune system mediates the immunoinflammatory signalling underlying the pathogenic mechanism of MDD; therefore, novel agents targeting the inflammatory pathways are likely to show better efficacy compared to the conventional drugs (Schmidt *et al*., 2016).

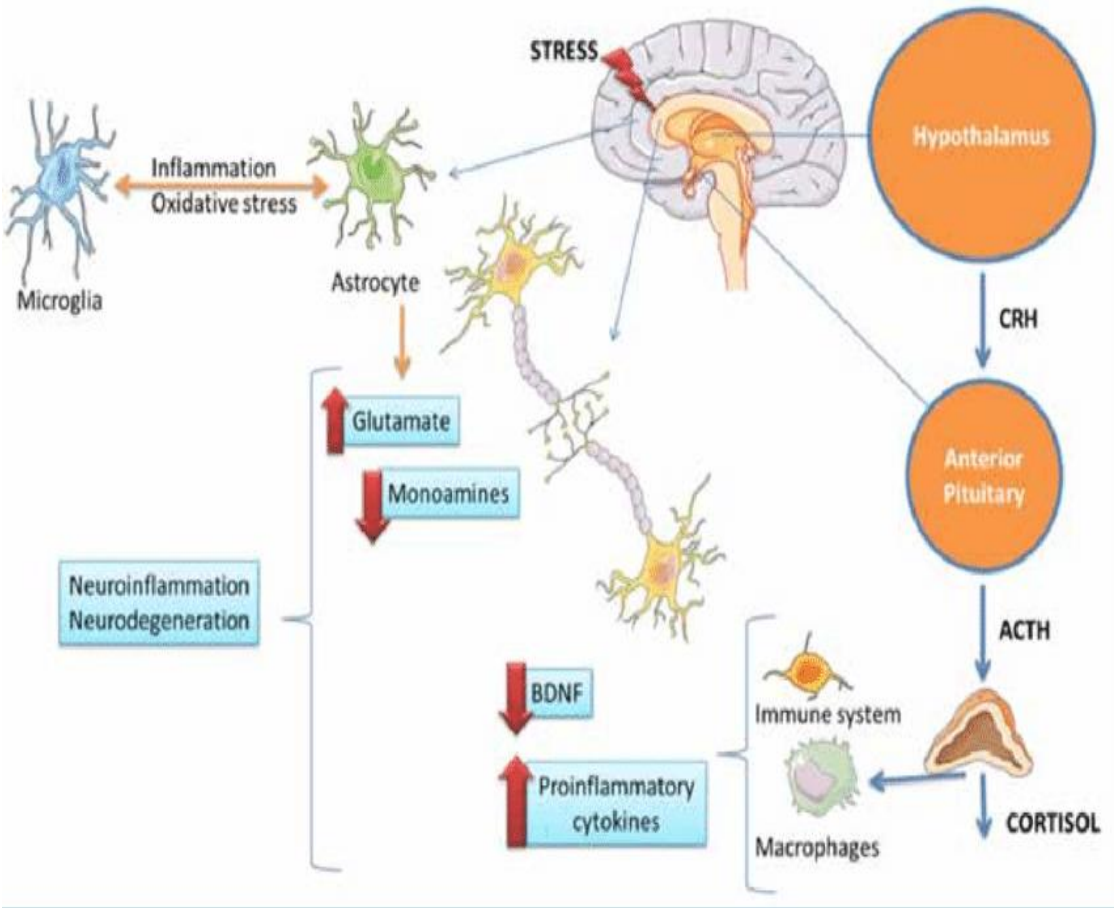

Figure 2.3: Multisystems interplay in pathophysiology of depression (Sahin *et al*., 2016)

2.3 Structural Changes in Depression

Recent studies demonstrate that exposure to either environmental or biogenic stress in both rodents and patients having MDD can result in structural alterations in the brain. The common structural features include reduced neuronal processes, functions and density. As illustrated in Figure 2.4 below, the structural changes correlate with reduced activity areas in the brain. The reversal of this structural loss was observed following treatment with antidepressants (Andrade & Rao, 2010).

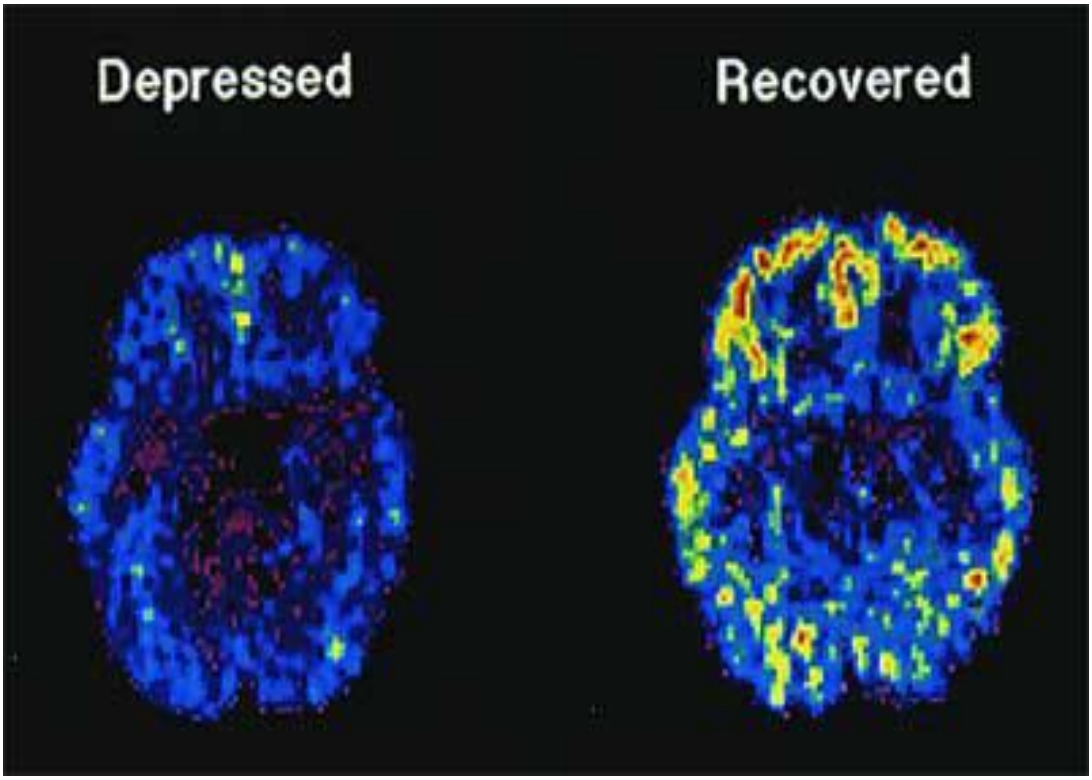

Figure 2.4: PET imaging of neuronal activity in depressed and recovered brain

(Yasuhisa *et al*., 2016)

Repeated stress stimulates excess glutamate release and subsequent neurotoxicity represented by reduced neuronal volume, lower number of cellular matter and shortened apical dendrites (Cooper *et al*., 2021). Brain imaging delineate structural differences between brains of healthy individuals and that of patients with MDD, where structural atrophy was observed in the brain subregion of the latter, with a correlating reduced activity compared to non-depressed patients as shown in Figure 2.5 below.

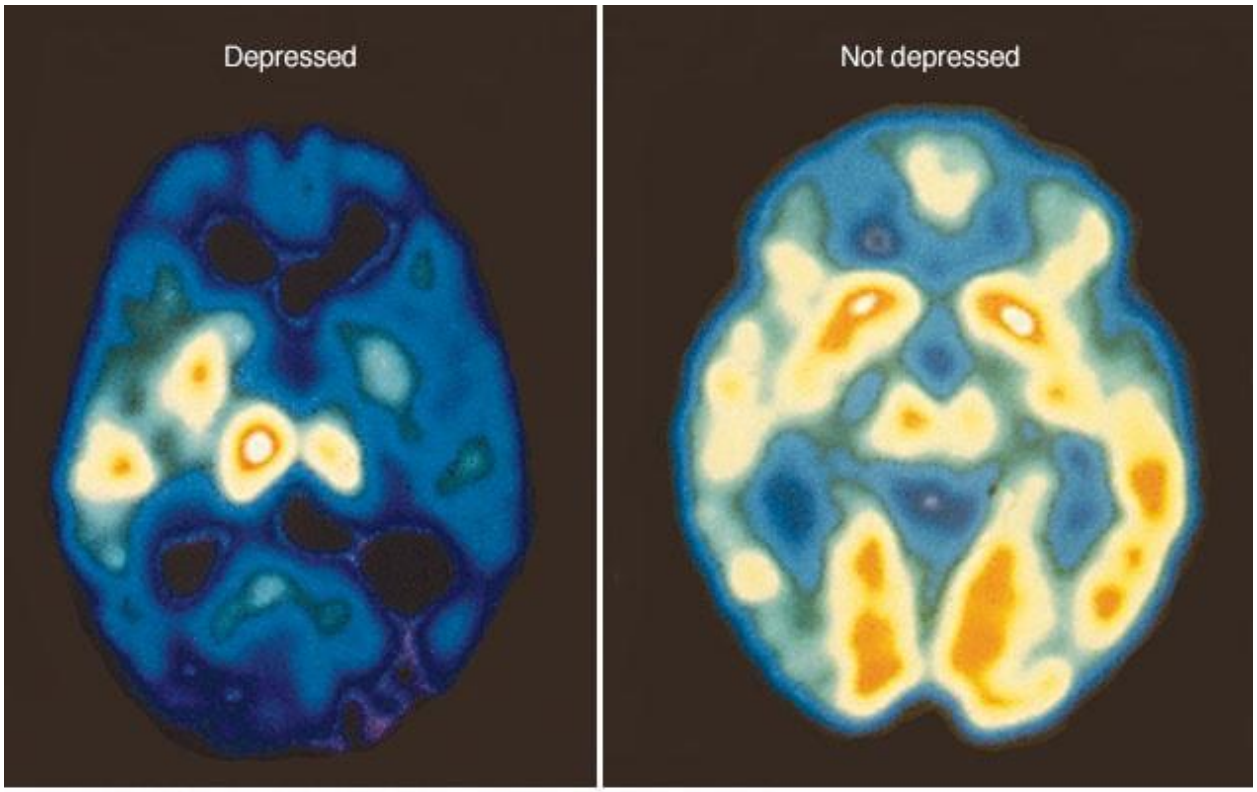

Figure 2.5: Scan image of brain activities in depressed and non-depressed brains

(Source: Mayo Foundation for Education and Research, 2020)

The key areas that have been implicated include atrophy in the CA3 pyramidal cells, the dentate gyrus, interneurones and the grey matter. Histological examination of these regions is characterized by a reduced neuronal density and cellular volume. In the cortex for example, the volumentric and cellular loss corresponds with every patient showing cognitive deficiency symptoms (Zuo *et al*., 2018). Evidently, the structural damages from the apoptotic processes can be prevented or reversed by long-term use of conventional antidepressants (Songjun *et al*., 2021).

Typically, excess glutamate in synaptic junctions is removed by glia cells, thus, fewer neurotransmitters are available at the post-synaptic neurones. However, in stress-induced glia loss there is excess extracellular glutamate. Consequently, the excess glutamate binds extrasynaptic receptors leading to sustained phosphorylation of dendritic Eukaryotic Elongation Factor 2 (eEF2). This phosphorylation directly inhibits BDNF synthesis causing deficient neurotrophic factors that could have complemented synaptic growth, hence, further atrophic loss on neuronal mass (Verpelli *et al*., 2010).

It has been established that mTOR complex 1, a primer of the mTOR signalling pathway promotes the mRNA translation necessary for new dendrite formation. However, under stressful conditions Regulated in Development and DNA damage Response 1 (REDD1) is activated thereby blocking mTORC1. Histological examination of post-mortem brain of depressed patients shows synaptic loss, axonal damage and absence of spines on many dendrites (Pastor *et al*., 2017).

Experimentally, depressive behaviours can be induced in rodents using chronic and unpredictable stressors. The observed structural changes from such studies are marked with gross loss of synaptic function markedly seen in postsynaptic neurons (Figure 2.6) (Moench & Wellman, 2014). In summary, during controllable stress, the stressful event initiates neurogenesis; however, when the stress becomes an overwhelming and uncontrollable stress, the nerve terminals will become shrunken and eventual neuronal loss persists in multiple brain regions (Snyder *et al*., 2011).

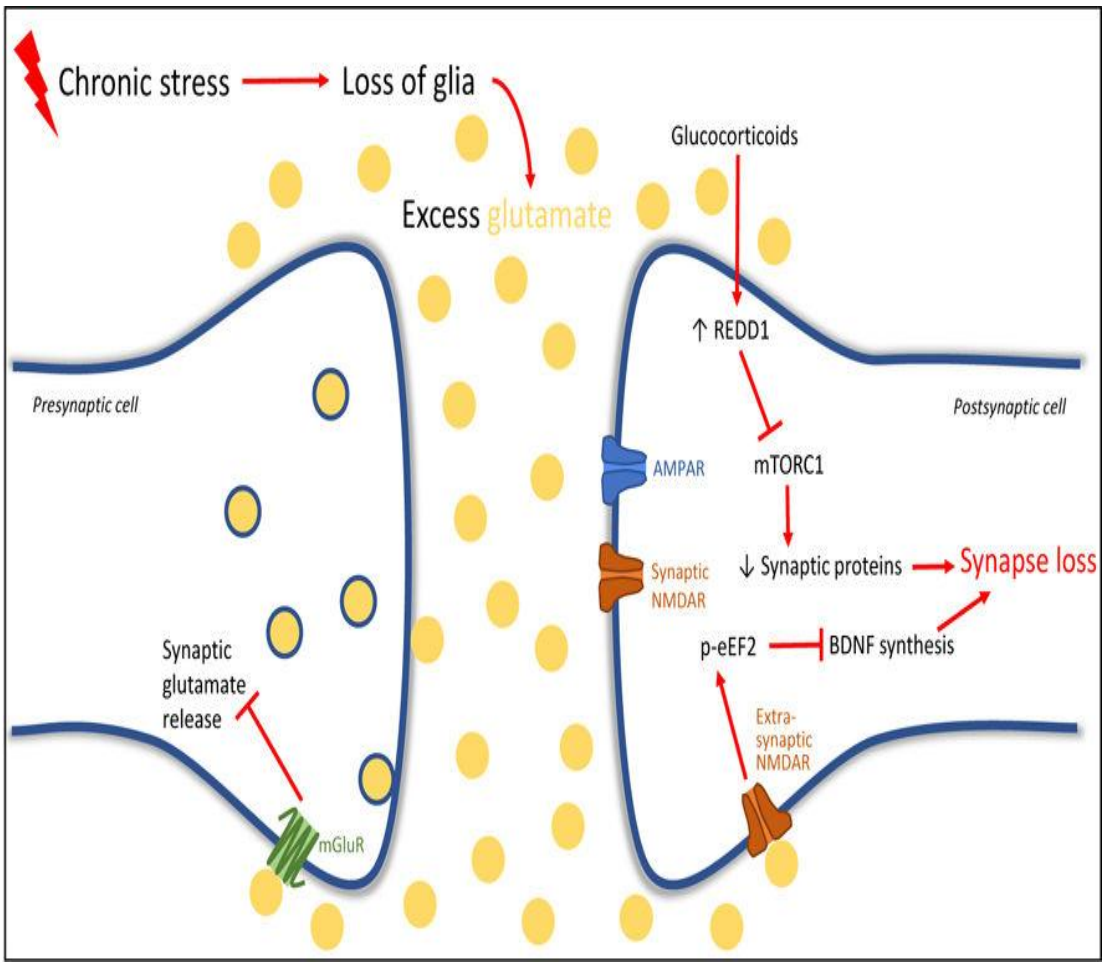

Figure 2.6: Mechanism of Post-synaptic axonal loss (Pittenger & Duman, 2008)

2.4 Organization of CNS and Drug Interactions

The neurons form functional units of the CNS and their aggregation through a highly condensed and interconnected synaptic network forms signalling pathway. The interconnections are sites for drug interaction and modulation. Thus, the network determines the biological activities of the brain region where they are condensed, and this could be localized or general in the elicited diverse biological functions (NIDA, 2017). The organization of the CNS involves the pre-synaptic neurons interacting with the post synaptic via the synaptic cleft and drugs acting in the CNS can elicit biological interaction at any site on any of these three functional components of the CNS. Each of these components have specific binding sites with receptors required for activating signalling pathways and binding these sites could either promote or block the cellular function. Generally, activation of the pre-synaptic receptors initiates the neurotransmitter synthesis in the cell body, storage or transport down the axons and released at the terminals into the synaptic cleft. Reuptake into terminals for storage or forward movement to bind post-synaptic receptors or enzymatic degradation is the possible fates within the cleft. From synthesis to post-synaptic activation, drugs acting in the CNS interact with one or more of the steps for therapeutic efficacy (Zhang & Sulzer, 2012). These interactions form the signalling pathways involved in response to rapid-acting antidepressants and the convetional agents.

The effects of a CNS active drug are variable, either direct or indirect acting but generally classified into additive, depressant or stimulant. Direct acting drugs are specific in terms of mechanism of action or binding site, that is, they directly interact with specific receptor or signalling pathway. However the indirect acting has affinity for multiple sites and they require multiple mechanisms for them to elicit direct interaction with the signalling pathway, hence, their interaction is non-specific. The efficacy of a non-selective CNS active drug depends on the extent of its off-target effects, explaining the higher potency of specific direct acting drugs. For example, the conventional antidepressant class, tricyclic antidepressants which have activity at the noradrenergic, dopaminergic and serotonergic sites would likely exhibit limited efficacy due to interaction of these receptors with other

neurosystems controlling respiratory and cardiovascular functions that would be seen as major side effects of these antidepressants (Moraczewski & Aedma, 2022).

2.5 Therapeutics of Depression

Many compounds with diverse site of actions in the CNS have been proposed and the currently approved drugs targeting these multiple sites are still limited in therapeutic outcome due to the attending adverse effects and low therapeutic efficacy. Contributing to this therapeutic failure is the variability in the individual response and degree of susceptibility. Usually the therapeutic effects of these antidepressants are not seen immediately when treatment is initiated, rather it takes about an average of three and half weeks (Machado-Vieira *et al*., 2010). The endpoint of therapeutic intervention in depression involves synaptogenesis, neurogenesis and/or neuroprotection, all of which requires initiation of protein synthesis via mRNA transcription which with conventional antidepressants takes weeks to initiate or implement, thus explaining the characteristic delay in onset of therapeutic effect (Gerd *et al*., 2018).

Another limitation in the therapeutics of depression is on the subject of compliance where a very low group of patients indicated to have complied religiously with their dosing regimen. The complaints among those who failed to comply were hinged on the adverse effects that interfere with daily life such as the inability to drive a car, excessive dry mouth, inability to empty bowel and majorly reduced sexual performance among male patients (Kikuchi *et al*., 2011). Additionally, relapse and residual symptoms frequently seen in patients paying out of pocket was ascribed to high cost of these synthetic drugs that further creates economic stress on the patients. Hitherto, innovative antidepressants that mitigate both the challenges observed concerning compliance and early onset of therapeutic effects is still far from being discovered or available for clinical use. Therefore, new drugs through identification of promising lead compounds are the focus presently in therapeutics of depression. Currently, antidepressants targeting monoamine neurotransmitters remained the first line approach in pharmacological treatment of depression but more specific and direct approaches are required for better intervention

(Peter *et al*., 2015). There are different classes of currently available antidepressants but the commonest ones are describe in table 2.1 below.

Beyond the conventional antidepressants, TCAs, MAO inhibitors, SSRIs and SNRIs, there are preclinical findings suggesting other potential targets. These include the Serotonin Receptors modulators (5HT1 A agonist, 5HT2B antagonist, 5HT2 C antagonist, 5HT3 antagonist), Adrenergic Receptor modulator (Alpha 2 A adrenoceptor agonist), Dopaminergic Receptor modulator (Dopamine D2 agonist, Dopamine D3 agonist), VMAT2 inhibitor, TAAR1 agonist, Glutamatergic Receptor modulators (NMDA open channel blockers, GluN2B antagonist, Glycine agonist, MGluR2 Negative Allosteric Modulator, MGluR5 Negative Allosteric Modulator, AMPAkines), Neuroplasticity Modulator (Neurogenesis stimulator NSI-189), TrkB agonist, Cholinergic modulators (Muscarinic antagonist, A4B2 Nicotinic antagonist, Muscarinic M3 antagonist, A7 Nicotinic agonist), Opioid receptor modulators (Kappa opioid antagonist, Nociceptin antagonist), HPA-axis modulators (CRF1 receptor antagonists, Neurokinin 1 receptor antagonist, Neurokinin 2 receptor antagonist), Neuropeptide targets (Vasopressin 1B receptor antagonists, Orexin receptor antagonists), Neuroinflammatory modulators (TNF alpha antibody, KMO antagonist, COX-2 inhibitor, P2x7 antagonist), Presynaptic intracellular amines modulators (traceamine associated receptor 1 agonist), Cannabinoid modulator (fatty acid amide hydrolase inhibitor, CB1 receptor inverse agonist), Chromatin modulator (Histone deacetylase 2 inhibitors), however, none of these has shown successful outcome in clinical trials.

Table 2.1: Common classes of antidepressants

S/N	Class	Mechanism
1	Tricyclic antidepressants (TCAs) e.g Imipramine	non-specifically blocks reuptake of monoamine neurotransmitters
2	Selective Serotonin-reuptake Inhibitors e.g Citalopram	specifically blocks the reuptake of serotonin at pre-synaptic terminals
3	Monoamine Oxidase-B Inhibitors e.g Rasagiline	Inhibits the enzymatic degradation of monoamine neurotransmitters
4	Reversible inhibitor of monoamine oxidase A e.g Moclobemide	Reversible inhibition of the enzymatic degradation of monoamine neurotransmitters
5	Serotonin-norepinephrine reuptake Inhibitor e.g Venlafaxine	Blocks reuptake similar to TCAs

(Yohn *et al*, 2017)

2.6 Novel Rapid-acting Antidepressants

Ketamine is glutamatergic modulator solely used clinically as an anaesthetic agent, but recent findings have suggested its rapid antidepressant activity in rodents following single subanaesthetic dose, however, due to the unwanted side effects it is not clinically indicated for depression (Thu & Alain, 2019). Notwithstanding, elucidating the mechanism of antidepressant activity of ketamine can provide more insight in understanding of pathophysiology of depression and discovery of more efficacious compounds with rapid onset of action. Over the years, several biological compounds have been proposed to potentially have rapid-acting antidepressants property, with some showing similar efficacy to that of ketamine and they all share a common downstream mechanism ascribed to their activity as illustrated in Figure 2.7, which is enhanced synaptogenesis and sustained plasticity (Duman, 2018).

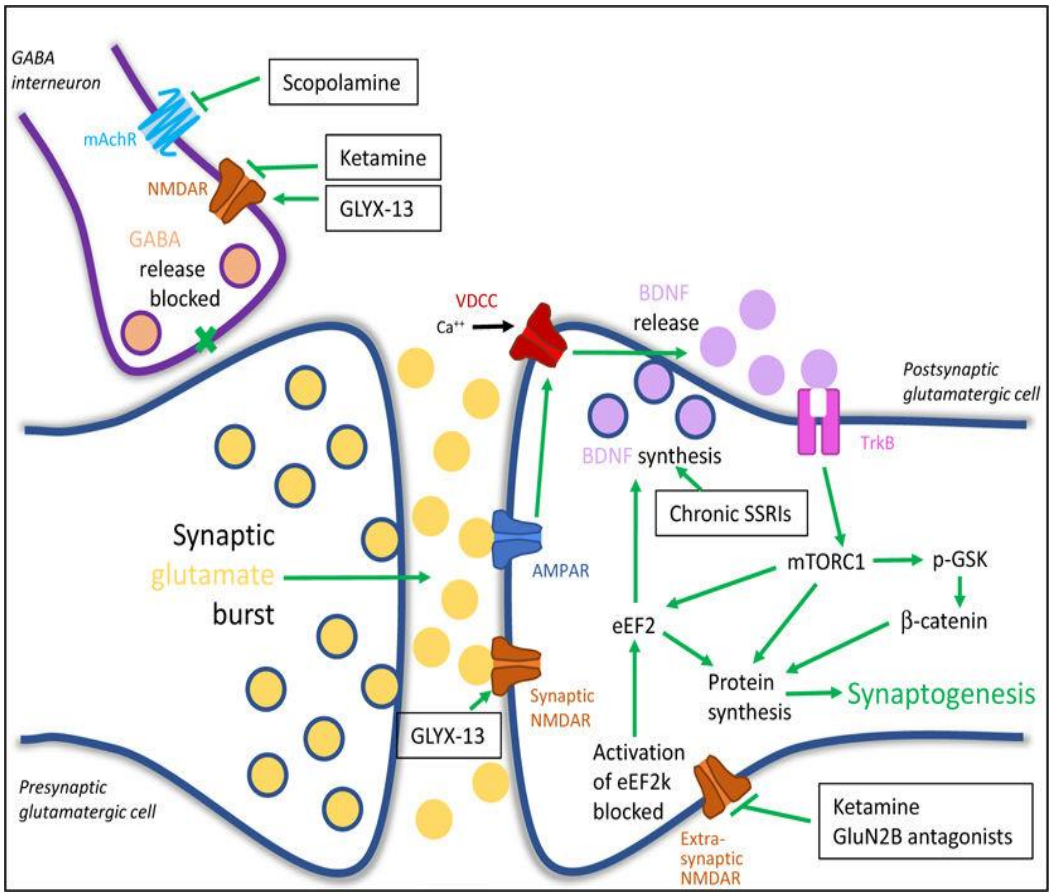

Figure 2.7: Signalling pathways involved in response to rapid-acting antidepressants (Thomas & Duman, 2017)

Similar to ketamine is GLYX-13, a partial allosteric modulator that also acts on N-Methyl D-Aspartate (NMDA) receptor but at a different binding site. This tetrapeptide agent shows a rapid antidepressant effect and adverse effects are mild compared to ketamine probably because of its characteristic allosteric property and specific site binding profile. GLYX-13 also upregulates neurotrophic factors through the activation of mTORC1 signalling thereby enhancing synaptogenesis in the cortex (Po-Sheng *et al*., 2020). Apart from binding the glycine-site, GLYX-13 may interact with glutamate signalling directly or indirectly at postsynaptic NMDA receptors or on GABAergic neurones respectively to initiate synaptogenesis. Clinical trials are being conducted to assess overall effectiveness of GLYX-13 since it has been proposed as candidate drug with higher tolerability response (Jeffrey *et al*., 2013).

Another compound proposed for repositioning as rapid antidepressant agent is the antimuscarinic drug, Scopolamine, a drug primarily used to prevent nausea associated with motion sickness. This non-selective anticholinergic agent was claimed to exert rapid therapeutic activity onset in depressed individuals at a very low dose of 4μg/kg (Navarria *et al*., 2015). The mechanism underlying its rapid antidepressant activity is similar to Ketamine, it also interact with mTORC1 signalling and promotes formation of new neuronal dendrites spine within medial Prefrontal Cortex (mPFC) (Jaffe *et al*., 2013). Review of mechanism elucidated that the activity of Scopolamine at muscarinic receptors in interneurones in the pyramidal cells is somewhat similar to that of Ketamine on the NMDA receptors. Probing further, it was experimentally deduced using viral-mediated knockdown protocol, the target specificity mediating antidepressant activity of Scopolamine identified only M1-AChRs on GABAergic interneurones as only target required for antidepressant effect (Wohleb *et al*., 2016).

Furthermore, the drugs with rapid acting antidepressant properties likely share similar molecular responses in common even with conventional antidepressants targeting monoaminergic systems, as described by the loss of antidepressant activity in BDNF restricted knockout mice treated with ketamine or fluoxetine, thus explaining similar molecular downstream mechanism shared by both Ketamine and conventional

antidepressants, but this molecular process activation in conventional antidepressants is possibly indirect and less efficient causing delayed onset of antidepressant effect (Kevin *et al*., 2012). Further experimental evidences showed that one key difference determining treatment response after drug administration is that the rapid-acting agents unlike the conventional antidepressant not only increases the BDNF gene expression common to both but further enhances release of the synthesized BDNF after single dose administration in rapid acting agents (Lepack *et al*., 2014).

The mechanism of BDNF release occurs by the stimulation of AMPA receptors through the glutamatergic system. The GABAergic system acts as the prime inhibitory pathway of the brain and both Ketamine and Scopolamine has been found to act via glutamatergic receptors and cholinergic receptors respectively to disinhibit the firing of GABA interneurones thereby blocking the release of GABA neurotransmitter and consequently causing transitory surge in glutamate. The surge of glutamate depolarizes the postsynaptic neuronal cell and stimulates release of intracellular calcium via opened voltage-gated calcium channels and subsequent release of BDNF (Colla *et al*., 2021). Generally, neurogenesis is the hallmark of successful antidepressant response and rapid-acting antidepressants exhibits protracted effect after single dose probably due to immediate release of neurotrophic factors whereas the protracted effect is absent in conventional antidepressants (Woelfer *et al*., 2019)

2.7 CNS Active Plants as Sources for Novel Antidepressants

Ethnopharmacological studies have revealed the importance of plant-based medicine as indispensable alternative for therapeutic drug development and this growing area of research takes its root form various traditional and folkloric herbal systems. Use of these plants in experimental models significantly demonstrated those that they have the ability to attenuate CNS functions. For example, *Treculia africana* was shown to possess sedative (CNS depression) properties in experimental models of mice using neurobehavioural models of drug discovery (Aderibigbe & Agboola, 2010). The specific mechanism underlying its activities was also put forward to strongly validate that the plant is centrally acting via recognized receptor signalling (Aderibigbe *et al*., 2010). Leaves extract of

Morus alba showed significant dose-dependent effect on dopaminergic receptors which suggests the plant's antipsychotic potential (Halemani *et al*., 2017).

In the CNS, these plants can have different effects at varying doses, for example, *Morus alba* at certain dose exhibits significant antidepressant property and at higher doses showed a marked sedative effect characterized by alterations in muscle strength, cage activity and perception of pain (Sattayasai *et al*., 2008). Methanolic extracts of the peel and seed of *Nephelium longan* fruits showed dose dependent CNS depressant in open field and hole cross tests in rats and antinociceptive activities in the acetic acid-induced writhing model (Ripa *et al*., 2011). The ethanolic concentrate of *Morus mesozygia* leaves reduced the time duration of immobility in acute model of depression, showing significant antidepressant activity similar to Imipramine, a tricyclic antidepressant and the antidepressant activity was elucidated to involve modulation of monoaminergic system (Abimbola *et al*., 2020). Most of these plants having antidepressant properties have had their characteristic efficacy attributed to bioactive agents present in them, particularly the flavonoid components (Jeanette & Brijesh, 2018).

2.8 Flavonoids and Oxidative Stress

Flavonoids are directly associated with human nutritional elements, hence, the need to evaluate structural and functional relationship of flavonoids with human health. This influenced array of researches that have shown flavonoids to be important bioagents that are of immense potential therapeutic values. Consideration of the conventional antidepressants and their associated serious side effects has obligated focus of research towards novel natural products as potentially active antidepressants. The principally identified classes of natural products that have clinically shown robust antidepressant activities are the flavonoids. A typical example is the flavonoid isolated from *Hypericum perforatum* (St John's Wart) which when compared to placebo intake showed significant activities that is similar to that of currently available Monoamine Oxidase inhibitors (MAOI) and its side effects were negligible compared to other conventional antidepressant drugs (Jat, 2013). It has been postulated that flavonoids have rich antioxidant properties which for those acting in the CNS can prevent neuronal loss induced by oxidative stress.

Recent studies expounding the possible role of flavonoids as therapeutically active antidepressants have evidently identified that these compounds attenuates several processes involved in the pathophysiology of depression, but significantly due to attenuation of oxidative stress. In a study, the compound quercetin, a natural bioflavonoid was shown to reverse CUMS-induced depressive behaviour in rodents by activating antioxidative processes, modifying the dysregulated inflammatory responses and restoration of the stress-induced down-regulation of BDNF (Zhong-Xuan *et al*., 2021). Similarly, naturally occurring compounds that are flavonoids like Rutin, Chrysin, Nobiletin, Hesperidin, Naringenin, Hyperoside, Vitexin and Astilbin were all shown in various studies to exhibit characteristic antidepressant properties through attenuation of oxidative stress-induced depressive behaviour (Hritcu *et al*., 2017). Generally, these natural compounds like 3,5,6,7,8,3′,4′-heptamethoxyflavone acts via multiple mechanisms of action in the CNS to demonstrate their antidepressant activity either by suppression of oxidative-nitrosative stress thereby increasing the BDNF levels in the hippocampus, preserving neural function or inducing hypocammpal neuroprotection, restoring the brain levels of monoamines or up-regulation of monoaminergic neurotransmitters (Sawamoto *et al*., 2017).

While having activity at specific targets, some of these flavonoids have been found to possess synergistic properties of enhancing antidepressant activities of the conventional antidepressants through activation of the multiple targets for a robust antidepressant activity. For example, hesperidin, a natural occurring flavonoid extracted from orange peel and naringenin another flavonoid isolated from *Mentha aquatica*, both enhance the pharmacokinetics profile of rasagiline (Ravindrababu *et al*., 2016).

Although not all flavonoids shows the characteristic antioxidant activity, however, common with the studied ones is that these flavoniods are often isolated as the major bioactive compounds from medicinal plants with well documented use for many years in traditional medicine. In the Chinese folkloric medicine, the *Herba epimedii* plant was widely documented as commonly used herb for centuries in treating various conditions including depression and recently an active bioflavonoid, Icariin, isolated from the plant has been shown to have antidepressant-like activities by attenuating oxidative stress related damage (Ying *et al*., 2005). Likewise the compound, Hyperoside, which was

isolated from the leaves of the plant *Apocynum venetum*, as documented in Chinese folklore, this plant leaves decoction was widely used in treating various mental illness, and the compound has been found to exhibit antidepressant-like activities (León *et al*., 2018). Another bioactive flavonoid with clinically validated antidepressant properties is Kaempferitrin, the compound is isolated from *Justicia spicigera*, a plant used for many centuries by the native Americans for treating multiple disorders (Cassani *et al*., 2014). Across Asia, a popular plant, *Sophora japonica*, was widely used for many centuries as documented in the Chinese and European pharmacopeia as medicinal herb for treatment of dizziness or fatigue and achieving mental alertness. These plants belongs to the new genus, *Styphnolobium*, and it has been found to contain over 153 metabolites of various classes of which Troxerutin is a major bioactive flavonoid found in the plant (He *et al*., 2016). Similar compounds isolated from *Sophora japonica* include rutin, quercetin, isorhamnetin, isorhamnetin, genistein and kaempferol, all of which has been proposed to exhibit some levels of antidepressant activities (Abd-Alla *et al*., 2021). Previous studies explicated antioxidant profile of the flavonoid components of *Sophora japonica* in *in-vitro* models, the antioxidant property was very significant (Chen & Hsieh, 2010). In another study it was reported that polysaccharides of *Sophora japonica* exhibits characteristic antioxidant property by scavenging hydroxyl and superoxide anion (Liyan *et al*., 2019).

Further studies showed that the isolates, quercetin and rutin exhibits significant antioxidative properties but quercetin showed a better activity when compared to other isolates of the *Sophora japonica* plant (Karim *et al*., 2011). Generally, CNS-active flavonoids can traverse blood-brain-barrier to mediate pharmacological action protecting neurons against stress-induced injury through suppression of microglia and astrocytes activity. Thus, it is herewith hypothesized that Troxerutin being a flavonoid, will possess strong neuroprotective and antioxidant property. The folkloric use of the sources of Troxerutin in different cultures, such as coffee and tea in the West, pagoda concoction in the East even till date makes Troxerutin a potential candidate for novel antidepressant agent with early therapeutic onset, substantially satisfactory response rates and functionally neuroprotective activity.

2.9 Troxerutin

Troxerutin is a bioflavonoid rutin isolated from the tree, *Sophora japonica*. There are other plants that contain Troxerutin, in fact it has also been found in trace in tea plant, coffee, fruits and common vegetables (Niranjan *et al*., 2016). Troxerutin is chemically denoted as vitamin P4 with a nomenclature of 3',4',7'-Tris[O-(2-hydroxyethyl)]rutin. Documentaries of European Pharmacopoeia has reported that troxerutin contains mainly tris(hydroxyethyl)rutin while other trace components (mono, tetra and bis hydoxyethyl derivatives) are less than 20% (Bianchi *et al*., 2017). As depicted in figure 2.8 below, the chemical structure of troxerutin is consistent with that of other flavonoids belonging to the class flavonols, example of which are quercetin, kaempferol, myricetin, and fisetin. They form a glycone moiety which structurally has heterocyclic ring-linked dual benzene side chains attached to hydrocarbon skeleton.

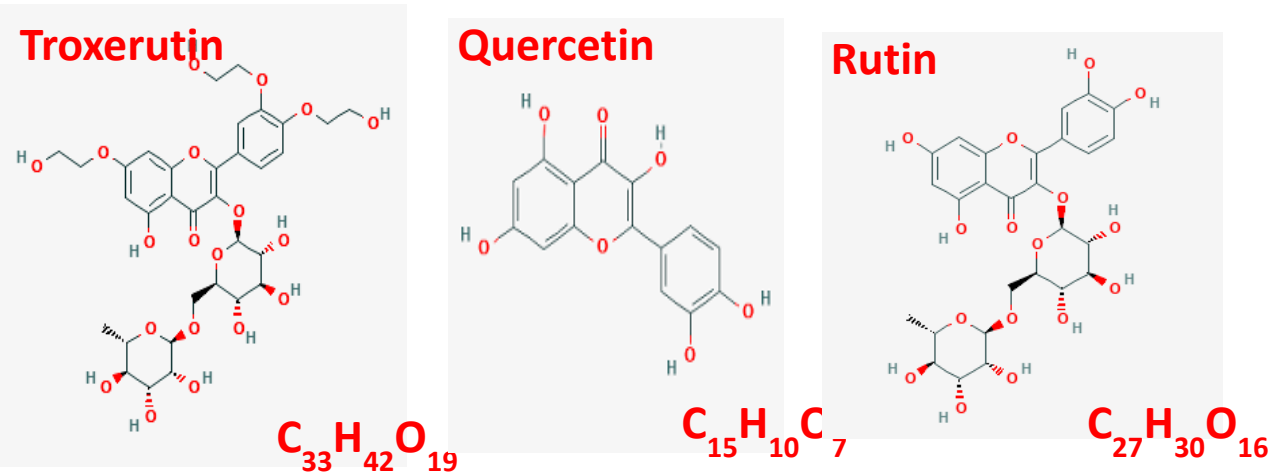

Figure 2.8: Biochemical structures of Troxerutin, Quercetin, and Rutin (Hongfei *et al*., 2021)

This primary structural skeleton has been found to be responsible for the common biological activities of the flavonoids, these accounts for their efficacy in scavenging reactive oxygen species, the peroxyl, superoxide, and peroxynitrite radicals (Shashank & Abhay, 2013). It is noteworthy that level of oxidation and pattern of substitution of the rings and side chains structural patterns are responsible for variations in their diverse biological activities. For example, catechol in primary benzene ring promotes inhibition of lipid peroxidation and enhanced affinity for scavenging reactive carbonyl compounds (Katarzyna & Izabela, 2021). Generally, the pharmacokinetics and therapeutic efficacy of Troxerutin is relative to its structural aromatic configuration, presence and position of hydroxyl side chains and other functional groups, hence, troxerutin is large with a chemical formula, $C_{33}H_{42}O_{19}$. For example, Troxerutin possesses glycolyl and glyceryl side chains (figure 2.9), which in some conventional drugs are responsible for their pharmacological activities, like muscle relaxant activity of the drug mephenesin, the bronchodilator effect of dyphylline, the analgesic-anti-inflammatory effect of both glafenin and etofenamate.

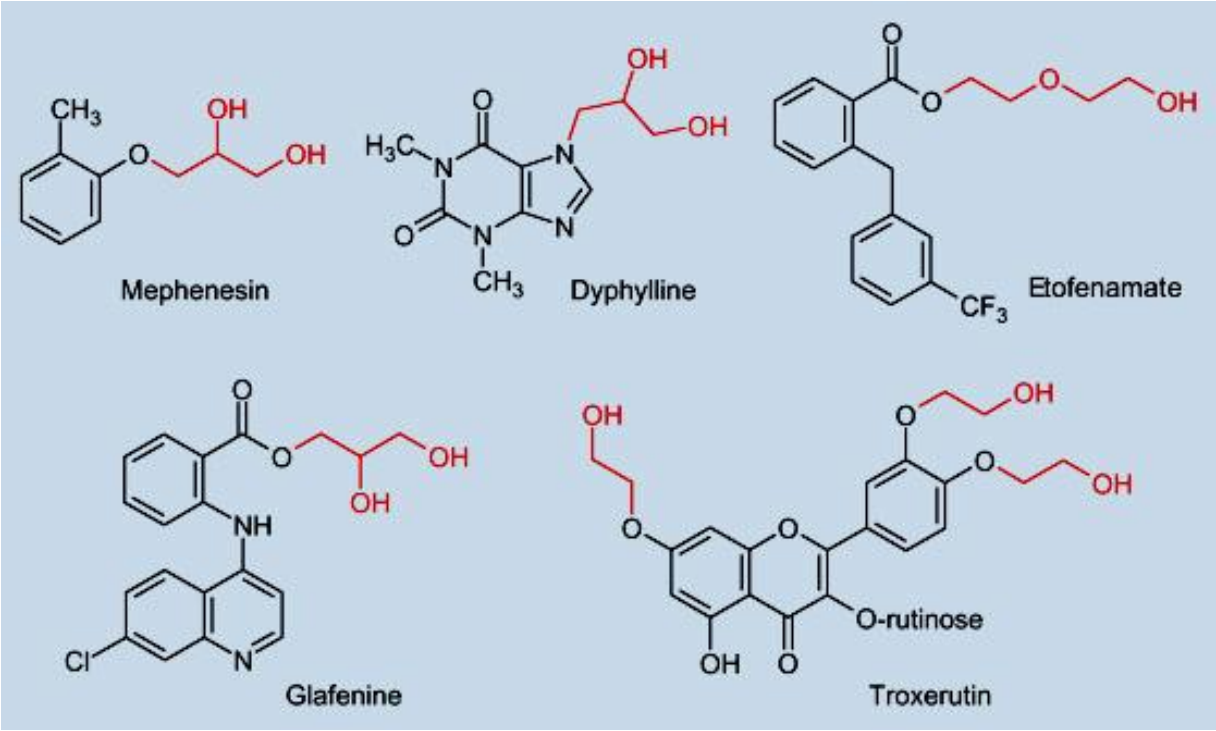

Figure 2.9: Structural elucidation showing presence of glycolyl and glyceryl side chains (Hongfei *et al*., 2021)

Literatures have shown several studies highlighting the biological activities of Troxerutin resulting from its elucidated unique structural functions, which were summarily antioxidant, anti-inflammatory and antithrombic activities. These activities further explain the tissue protective effect of troxerutin in various experimental studies. Complementing its neuroprotective effects, in the CNS, Troxerutin in high cholesterol diet-induced diabetes reverses insulin resistance through scavenging reactive oxygen species and prevents associated retinopathy (Lu *et al*., 2011). Furthermore, its tissue protective properties enhances its use as beneficial cofactor in management of clinical conditions requiring cytotoxic medications or drugs with short therapeutic window. For example, coumarin is used in management of chronic venous insufficiency, although coumarin is hepatotoxic, Troxerutin is used as hepatoprotective agent in preparation of coumarin for use (Adam *et al*., 2005). Troxerutin has been found to protect cells against DNA damages induced by radiation and promote cellular repair, and to a large extent it has also been suggested to have good safety profile, even in the elderly patients and pregnant women (Ranjith *et al*., 2013). The DNA repair property has allowed use of Troxerutin as adjunct in radiotherapy to protect normal tissues, reduce severity of injury and promote recovery. In ischemic injury, Troxerutin also preserves the cardiac tissues and improves perfusion by its antioxidant and anti-inflammatory activities on coronary endothelium (Geetha *et al*., 2014). Ability of Troxerutin to easily pass throughthe blood-brain-barrier suggests its possible neuromodulatory activity, which could probably alleviate cognitive deficits and other symptoms associated with several neurodegenerative diseases including depressive disorder, hence, the need for this study.

CHAPTER THREE
MATERIALS AND METHODS

All investigational procedures were designed and done following standard protocols and the reporting guidelines of Animal in Research: Reporting *In-Vivo* Experiments (ARRIVE) was adopted for documenting experimental outcomes involving use of animals (McGrath *et al*., 2010).

3.1 Preparation of Troxerutin

Pharmaceutical grade Troxerutin (Batch number: FTF201112) was supplied from Xi'an Biological Technology, China. The chemical properties as provided in the certificate of analysis were given as follows:

The compound is hygroscopic and in powder form having a crystalline texture.

The color is yellow with light green tint.

The compound dissolves freely in water and is partially soluble in ethanol.

Melting point- 181ºC

The compound was stored in a cool dry place as instructed by manufacturer and the stock solution was prepared by taking required weight for volume of distilled water and dose volume was calculated according to animal weight, and administered intraperitonealy in all the experimental protocols.

3.2 Animal Use in the Experiment

The animals used in the study were male Swiss mice, the average weight was between 20 to 25 g, and for every experimental procedure, different set of mice were used. All animals were housed under standard condition and allowed to acclimatize before subjection to experimental protocols. Animals were provided access to food and water

freely except in stress paradigms that involves mild deprivation according to the protocol. The use of animals in the protocols was according to National Research Council, (2011) standard guidelines for animal use in biomedical research. Ethical approval was obtained from ACUREC with file number UI/ACUREC/22-034

3.3 Drugs and Chemicals

Troxerutin (Xi'an Biological Technology, China), Lipopolysaccharide (Sigma-Aldrich, St.Louis, MO, USA), Reserpine (Pfizer Inc., New York, NY, USA), Imipramine (Shanghai Zhongxi Pharmaceutical Co., Ltd. Shanghai, China), Serotonin ELISA kits (Biobase Inc., China), Brain Derived Neurotrophic Factor (BDNF) ELISA kits (Biobase Inc., China), Dopamine ELISA kits (Biobase Inc., China), Norepinephrine ELISA kits (Biobase Inc., China), Corticosterone ELISA kits (Biobase Inc., China), TNF-α ELISA kits (Biobase Inc., China), IL-6 ELISA kits (Biobase Inc., China), MAPKs ELISA kits (Biobase Inc., China), CREB ELISA kits (Biobase Inc., China), CuSO4. 5H2O (BDH Chemicals, England), Trichloroacetic acid (TCA) (Sigma-Aldrich, St.Louis, MO, USA), Thiobarbituric acid (TBA) (Sigma-Aldrich, St.Louis, MO, USA), Immunohistochemical mouse IgG antibody (Elabscience, USA), Chromogen diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, St.Louis, MO, USA).

3.4 Toxicity Test

Toxicity assessment of Troxerutin in this study was evaluated using the reviewed Lorke acute toxicity test protocol by Chinedu *et al*. (2013). The experiment was done in two phases; phase one was an initial dose finding procedure where three groups consisting of three mice in each group to make nine animals in total were used. Each group of animals were administered different doses of Troxerutin, group 1, 2 and 3 received 10, 100 and 1000 mg/kg intraperitonealy (i.p) respectively. Following respective treatments, all treated animals were observed for 24 hours. Behavioural changes and the mortality was monitored within this period. This was followed by the second phase of the experiment where animals were distributed into another three treatment groups consisting of one mouse each and the respective treatment was as follows; group 1, 2 and 3 received 1,600, 2,900 and 5,000 mg/kg, respectively. The animals were then monitored from the time of treatment for the next 24 hours for signs of behavioural changes as well as mortality.

After the 24 hours of observation, the Median Lethal Dose which is the index of acute toxicity was determined by calculating geometric mean, taking the values of highest dose without mortality and lowest dose that causes mortality as the mean variables. The formula for calculation is, $LD_{50}=\sqrt{(D_0 \times D_{100})}$, where LD_{50} is Median Lethal Dose, $D_0$is Highest dose without mortality and $D_{100}$is Lowest dose where mortality occurs.

3.5 Dose Selection

Doses of Troxerutin were selected empirically, starting with a low dose for achieving potency to where significant activities were observable, then graded geometrically to higher tolerable doses. The dose of vehicle, distilled water (0.2 mL/20 g), and that of standard control drug, Imipramine (25 mg/kg), was selected as stated in literature of previous studies and the standard clinically recommended dosage (Adeoluwa *et al*., 2015).

3.6 Antidepressant Study: Tail Suspension Test (TST)

Tail suspension test was first proposed by Steru *et al*. (1985) as a viable tool for measuring antidepressant-like activities, it utilizes the symptomatology of behavioural despair as its endpoint in determining experimental outcome. In this study, 25 animals were divided into five (n = 5) treatment groups as follows: group A 10 ml/kg distilled H_2O, groups B, C and D received 10, 20 and 40 mg/kg troxerutin respectively, and group E was treated with 25 mg/kg Imipiramine. After administration of the respective treatments, animals were introduced into the experimental procedure after 30 minutes post-treatment, with the whole procedure lasting 6 minutes for each animal. In the procedure animals were poised by tail at a height of about 15 cm above the ground and duration of immobility was measured in last 4 minutes of the experiment. Measurement was done manually by an independent observer using a stopwatch and the period of immobility was accounted as when animal remained motionless or mere swinging without any limb movement.

3.7 Antidepressant Study: The Forced Swimming Test (FST)

When subjected to inescapable stress, rodents tend to exhibit characteristic despair behaviour which can be modelled to investigate predictive validity of antidepressants and the FST designed as inescapable stress model by Porsolt *et al*. (1977) was also used to

evaluate antidepressant activity of Troxerutin in acute model in this study. Twenty-five animals were divided into five (n = 5) treatment groups as follows: group A 10 ml/kg distilled H_2O, groups B, C and D received 10, 20 and 40 mg/kg troxerutin respectively, and group E was treated with 25 mg/kg Imipiramine. In the experiment, animals were allowed to swim in water at room temperature inside a transparent plexigas cylinder having a dimension of 40 by 18 centimeters. The water level was filled up to about 15 centimeter depth and each animal was introduced 30 minutes post-treatment, allowed to swim for 6 minutes and measure of behavioural despair was taken as duration of immobility within the last 4 minutes of swimming which was measured manually with a stopwatch by an independent observer. Criteria for immobility were absence of movement of any form except those necessary to keep afloat.

3.8 Measurement of Protracted Effect in TST and FST

This procedure was followed as described by Ma *et al*. (2013), which correlates release of neurotrophic factors to post-treatment efficacy of up to 8 days seen in single dose of rapidly acting antidepressants but absent in conventional antidepressants whose post-treatment effect only lasts for few hours. The animals in the acute study above were again subjected to FST and TST as previously described; 24 hours, 72 hours, and 7 days after the initially administered treatment to measure protracted effect of single dose of Troxerutin. The measure of behavioural despair was recorded manually with a stopwatch by an independent observer. The studies were carried out with the same time frame between 7:00 am and 12:00 am daily in order to ensure there is no alteration in behaviour due to change in biological rhythm.

3.9 Locomotor Activity Study

Enhanced locomotor activity may precipitate false positive outcomes in the acute antidepressant study; hence, locomotor activity of treated mice in OFT test was done using a model described by Akanmu *et al.,* (2011) to determine specificity of antidepressant-like activity of Troxerutin. The open field consists of an activity cage having three opaque sides and one other side made of transparent Plexiglas to form a box shape with dimensions of 28 cm by 28 cm by 28 cm. the floor of the box was painted into grid lines

with a black paint to form 16 squares with each having a dimension of 7 cm by 7 cm. In the experiment, 25 animals were divided into five (n = 5) treatment groups as follows: group A 10 ml/kg distilled H_2O, groups B, C and D received 10, 20 and 40 mg/kg troxerutin respectively, and group E was treated with 25 mg/kg Imipiramine. Each animal was individually introduced to the centre of activity box 30 minutes after treatment and were allowed to explore freely for 5 minutes during which number of squares completely crossed with all four paws were counted and recorded by an independent observer.

3.10 Chronic Unpredictable Mild Stress (CUMS)

Stress theory of depression is an extensively accepted phenomenon for describing the primary aetiology and CUMS model is an acceptable face validity model that utilizes different stressors to induce depressive behaviour in mice. In this study, mice were subjected to 14-day unpredictable stress paradigm using protocol described by Kumar *et al*. (2011). Animals were randomly distributed to 6 treatment groups of 5 mice each. The groups were as follows: Group 1- which was vehicle control that was not subjected to CUMS, Group 2- was vehicle treated control subjected to CUMS, Group 3- subjected to CUMS but treated with Troxerutin (10mg/kg), Group 4- subjected to CUMS but treated with Troxerutin (20mg/kg), Group 5- subjected to CUMS but treated with Troxerutin (40mg/kg), Group 6- subjected to CUMS but treated with Imipramine (25mg/kg). The group 1 vehicle treated mice were the only non-stressed group while the other groups were subjected to random stressors of different modalities for 14 consecutive days as presented on Table 3.4. All the animals were treated daily with the corresponding dosage as highlighted above and treatment was administered one hour prior daily subjection to stress. After the 14-day stress exposure and treatment, behavioural tests which were Sucrose Preference Test and FST were done for elucidating reversal of CUMS-induced depressive-like phenotypes, Novelty-induced rearing (NIR) and grooming (NIG) for measuring behaviour preservation and Y-Maze Test for measuring reversal of CUMS-induced cognitive disturbances.

Table 3.1: The daily schedule of stressors

Day	Stressor
Day 1	Water deprivation with access to empty bottles for 6 hours
Day 2	Exposure to Wet bedding for 12 hours
Day 3	Alteration of circadian rhythm by overnight illumination
Day 4	Food deprivation and exposure to empty food container for 6 hours
Day 5	Tilted housing at 45 degrees for 7 hours
Day 6	Cold water (4^{o}C) swimming for 20 seconds
Day 7	Inescapable immobilization for 15 minutes
Day 8	Alteration of circadian rhythm by overnight illumination
Day 9	Inescapable immobilization for 15 minutes
Day 10	Cold water (4^{o}C) swimming for 20 seconds
Day 11	Tilted housing at 45 degrees for 7 hours
Day 12	Water deprivation with access to empty bottles for 6 hours
Day 13	Exposure to Wet bedding for 12 hours
Day 14	Food deprivation and exposure to empty food container for 6 hours

(Kumar *et al*., 2011)

3.10.1 Sucrose Preference Test (SPT)

The test determines anhedonia, a symptom commonly seen as disinterestedness and loss of pleasure in human depression. The experimental protocol was done as described by Mohamed *et al*. (2016). It comprises of the pre-cums phase and the post-cums test phase. The pre-cums involved the training phase which was carried out after 1 week of animals' acclimatization. In the training course the animals were fasted and were only given 1% (w/v) sucrose solution. The pre-CUMS test was done 72 hours after by fasting the animals for 23 hours and on the 24th hour the baseline evaluation was done by housing individual animal in a cage containing two pre-weighed bottles A and B respectively containing water and sucrose solution. The bottles were removed after 1 hour and the amount consumed was calculated by weighing the bottles and determining the lost volume as consumption. The same test procedure was repeated on day 15 after CUMS to determine the post-cums alternation in sucrose preference.

The calculation was done using the formula:

$$\boldsymbol{Sucrose\ preference} = \frac{sucrose\ solution\ intake(g)}{Sucrose\ solution\ intake\ (g) + \ water\ intake\ (g)}\ X\ 100$$

Equation 3.1

3.10.2 The Forced Swimming Test (FST)

After the one hour of completion of SPT on the day 15, the animals in CUMS were subjected to FST as described in section 3.7 above.

3.10.3 Y-Maze Test

Behavioural evaluation using Y-maze is a paradigm described by Akanmu *et al.* (2021) as a tool for assessment of spatial working memory using the principle of spontaneous alternation. Maze of wooden stand at 15cm height and the stand supports its three arms that were spaced at 120 degrees to give a Y-shape orientation. The dimension of each arm is 41 cm in length and 5 cm in width. They are labelled A, B and C respectively and they converged at a centre connecting to the floor stand. Spontaneous memory alternation

performance of mice in the CUMS protocol was assessed by introducing the animals individually at the arm labelled A, and sequence of entry was recorded during this period of 5 minutes. When the whole body of the animal enters an arm, with or without the tail area, this was taken as an entry and that arm was recorded. A consecutive entry into the three arms in direct triple order is taken as an alternation and the total number of alternations observed in 5 minutes was taken as the actual alternation. The overall alternation behaviour of the animals was reported in percentage using the formula:

$$\boldsymbol{Alternation\ behavior}(\%) = \frac{Actual\ alternation}{Possible\ alternation}\ X\ 100$$

Equation 3.2

3.10.4 Novelty-induced Rearing and Grooming

Alteration of innate behavioural profiles is a core depressive symptom in animals subjected to CUMS protocol and this was individually evaluated for rearing and grooming behaviour in an activity cage as described by Aderibigbe *et al.* (2010). The rearing behaviour of the animals was measured as vertical activity when standing hind limbs and the fore limbs lifted in the air or against the cage walls, while grooming behaviour was counted as number of times animal engaged in face washing or body cleaning behaviour with its paw or with mouth. Each animal in CUMS was assessed individually and frequency of rearing and grooming activity was counted over a period of 5 minutes by an independent observer and recorded.

3.10.5 Locomotor Activity (CUMS)

The animals from CUMS were further subjected to OFT to rule out possible unspecific locomotor interference induced by Troxerutin in CUMS which was evaluated using a model designed by Akanmu *et al.* (2011) as described in section 3.9 above.

3.11 Lipopolysaccharide-induced Depression

Lipopolysaccharide, an endotoxin of bacterial origin, at non-lethal dose induces depressive symptoms in mice and this experimental model represents the neuroinflammatory pathogenesis of depression. In this study the dose of LPS, 0.83mg/kg, i.p, used and the experimental protocol was selected as illustrated by a previous study

(Sulakhiya *et al*., 2016). Animals were assigned into 6 treatment groups of 5 mice each. The treatment groups were as follows; Group 1- was given vehicle only, Group 2- was given vehicle and LPS, Group 3- which received Troxerutin (10 mg/kg) and LPS, Group 4- was given Troxerutin (20 mg/kg) and LPS, Group 5- was given Troxerutin (40 mg/kg) and LPS, Group 6- received Imipramine (25 mg/kg) and LPS. The respective treatment was done for 6 consecutive days without administration of LPS and animals were fasted (acute food deprivation) on the 6th day till the morning of the 7th day, equalling a 24 hours fasting. On the 7th day, LPS was dissolved in distilled water and was administrated 1 hour after treatment with vehicle, Troxerutin or imipramine as indicated. The neurobehavioural tests, Novelty Suppressed Feeding and FST, were then assessed. All test animals were subjected to locomotor activity test to rule out unspecific effects of interventions.

3.11.1 Novelty Suppressed Feeding (NSF)

Hyponeophagia is a common depressive behaviour and the assessment was here done using the method of Samuels and Hen (2011). In the NSF, after the 24 hours acute food deprivation and LPS challenge, animals were singly introduced at corner end in open field which has a normal chow diet pellet dropped in the center; the arena has a dimension of 40 cm x 40 cm. The lag time taken for each mouse to have a first bite of the chow within a 5 minutes test period was recorded as latency to feed. Animals were returned into their respective home cages which contained chow diet pellet feed of known weight and each animal was housed individually. The amount of feed consumed by the animal in 5 minutes was determined and recorded.

3.11.2 Forced Swimming Test (FST)

After the completion of NSF 24 hours post LPS exposure the animals were subjected to FST as described in section 3.7 above.

3.11.3 Locomotor Activity (LPS)

The animals from the LPS study above were further subjected to OFT to rule out possible unspecific locomotor interference induced by Troxerutin, which was evaluated using a model designed by Akanmu *et al.* (2011) as described in section 3.9 above.

3.12 Reserpine-Induced Depression (RID) Study

Reserpine is an antihypertensive drug that depletes monoamine storage thereby precipitating depressive behaviour. The modified method described by Park*et al*. (2018) was used to assess the antidepressant-like activity of Troxerutin on the monoaminergic system. Animals were randomly assigned to 6 treatment groups with 5 mice each, where, Group 1 administered vehicle only, Group 2 was given vehicle and reserpine, Group 3 received Troxerutin (10mg/kg) and reserpine, Group 4 received Troxerutin (20mg/kg) and reserpine, Group 5 received Troxerutin (40mg/kg) and reserpine, and group 6 received Imipramine (25mg/kg) and reserpine, dose of reserpine used was 0.5mg/kg, i.p. After acclimatizing, the animals received treatment for 7 days and the reserpine was given 1 hour after respective pretreatment. The behavioural tests; Splash test and TST were conducted after treatment on the 7th day. All animals were subjected to locomotor activity test to rule out unspecific effects of interventions.

3.12.1 Splash Test

The animals exposed to the reserpine-induced depression paradigm were subjected to Splash test protocol as previously illustrated by Mélanie *et al*. (2020) which in principle states that depressed animals exhibits reduced self-care. The animals were introduced individually in an activity cage with a dimension of 45 cm × 25 cm × 25 cm having opaque sides and a transparent side for observation of animals' grooming behaviour. At the start of the procedure, a viscous 10% Sucrose solution was prepared and splashed on the back side of the animals in the activity cage. The viscosity of the solution triggers enhanced grooming response which was measured over a 5 minutes period and recorded as index of self-care and motivation. After each test round the activity cage treated with ethanol and dried before concecutive procedure.

3.12.2 Tail Suspension Test

The TST protocol was done as previously described in section 3.6 above. After administration of the respective treatments on the day 7 of reserpine-induced depression, animals were introduced into the experimental procedure after 30 minutes post-treatment, with the whole procedure lasting 6 minutes for each animal. The measurement was done

manually by an independent observer using a stopwatch and the period of immobility was accounted as when animal remained motionless or mere swinging without any limb movement.

3.12.3 Locomotor Activity

The animals from the RID study above were further subjected to OFT which was evaluated using a model designed by Akanmu *et al.* (2011) as described in section 3.9 above.

3.13 Reserpine Antagonism in Mice Model

Blockade of monoamine reuptake at presynaptic neurons and acute emptying of the vesicles into the synaptic cleft following administration of reserpine 2.5 mg/kg, i.p. induces severe hypothermia, ptosis, and diarrhoea which in principle are a hallmark of depressive state that can be reversed by monoaminergic modulating agents. The study protocol was as designed by Bourin *et al.* (1983). Animals were randomly sorted into 5 treatment groups; where Group 1 received distilled water (0.2 ml/20 g, i.p.), while Groups 2 to 4 were given Troxerutin, 10, 20 and 40 mg/kg, i.p. respectively and Group 5 was given Imipramine 25 mg/kg, i.p, while reserpine 2.5 mg/kg, i.p. was equally given 10 minutes after to all the animals in the various groups. To measure the induced hypothermia, the rectal temperatures were measured with a digital thermometer at every hour over a period of 4 hours. The baseline values taken at 0 hour, that is, before drug administration were used as reference index for changes in temperature. The induced ptosis was simultaneously measured as a degree of eye closure on a scale of 0 to 4, with eyes fully opened as 0, one-quarter closed as 1, half closed as 2, three-quarter closed as 3 and fully closed as 4. Also, the degree of diarrhoea was graded by the integrity of the animal droppings.

3.14 Sample Preparation for Biochemical and Enzyme Assays

On completion of behavioural assessments, collection of blood samples from the orbital sinus of animals in the respective groups was done according to the method of Parasuraman *et al.*, (2010). After which the animals from respective groups were then

sacrificed by cervical dislocation, their craniums were opened to harvest the brains which was rinsed with ice-cold 1.15% KCl, and parched with tissue paper. The brains were weighed individually and recorded, after which they were individually placed in specimen bottle containing ice-cold phosphate buffer solution adjusted to a 7.4 pH. The brains were minced and then homogenized completely. Homogenates were afterwards centrifuged in a 4°C cold centrifuge for 10 minutes at rpm of 10,000g. The resulting supernatant from the centrifuged homogenate was decanted as the mitochondrial fraction (PMF) into Eppendorf tubes and this supernatant was used in the respective biochemical analysis: in CUMS and LPS, the levels of Glutathione (GSH), Malondialdehyde (MDA), Superoxide Dismutase (SOD), and Nitrite were measured by spectrophotometry and the concentration of TNF-α, IL-6, INF-γ, MAPKs, Corticosterone, Serotonin, Noradrenaline, Dopamine, BDNF and CREB were measured by ELISA. In RID samples, concentrations of BDNF, CREB, 5-HT, NE, and DA were measured by ELISA.

3.14.1 Estimation of protein in test samples

Using the Biuret method as described by Gornal *et al*. (1949), the procedures was carried out by first preparing the Biuret reagent which was done by dissolving 3g of CuSO4.5H2O in 500ml of 0.2M NaOH, and 5g potassium iodide added, then made up to 1 litre by adding 0.2M NaOH. After preparing the Biuret reagent, the resulting test samples from section 3.14 above were diluted 100 times with distilled water and 1ml of the diluted sample was taken and added to 3ml of Biuret reagent in triplicate. Mixture was incubated at room temperature for 30 minutes and absorbance read at 540nm.

3.14.2 Estimation of Glutathione (GSH) Concentration

In principle, sulfhydryl compounds react with Ellman's reagent to form a characteristic yellow product that corresponds to its concentration and this can be measured using colorimetric method. However, for GSH, the sample solution requires treatment with an acid to form precipitates so as to eliminate possible protein cysteine thiol group interference with the chromophoric outcome. Since larger percentage of GSH exists in reduced form, hence the concentrations of reduced GSH in the test sample supernatant aliquot above was determined with bioassay protocol of Moron *et al*. (1979). In this protocol, the acid used for deproteinization was trichloroacetic acid. First, a 20%

trichloroacetic acid (TCA) solution was prepared and 0.4 ml was taken and added to 0.4 ml of brain tissue supernatant aliquot. Resulting mixture was centrifuged for 20 minutes at rpm of 10,000g using a cold centrifuge at 4^{o}C. After centrifuging the mixture, 0.25 ml of supernatant was added to 2 ml DTNB reagent and 0.75 ml of phosphate buffer. To determine the concentration of reduced GSH, absorbance of final volume was read against blank with spectrophotometer at a 412nm wavelength within 30 minutes of color development. The result indicates reduced GSH concentration in each sample, and unit was in micromoles per gram.

3.14.3 Determination of Lipid Peroxidation

Products of lipid peroxidation are thiobarbuturic acid reactive substances (TBARS) and their measurement directly correlates to degree of peroxidation. In principle, malondialdehyde reacts with TBA and forms a characteristic pink complex whose optical density corresponds to MDA concentration, and this can be measured using colorimetric method. In the protocol for determination of lipid peroxidation, the bioassay method of Ohkawa *et al.* (1979) was used. As described in the protocol, a mixture was formed by adding 0.5 ml distilled water, 1.0 ml 10% TCA and 0.5 ml of brain supernatant aliquot, which was centrifuged for 10 minutes at a rpm of 3,000g. The supernatant was collected and 0.9 ml was added to 0.1 ml TBA. Resulting mixture was heated at 80ºC for 40 minutes in water bath after which it was cooled to room temperature. To determine absorbance, the cooled final volume was read against blank at a 532 nm wavelength. The extinction coefficient E_{532nm} used for quantifying TBARS was 1.56×10^{5} M^{-1} cm^{-1}. The tissue protein was quantified as in section 3.14.1 above and the MDA content of the samples was expressed in nanomole of MDA per milligram protein.

$$MDA = \frac{\text{Absorbance} \times \text{Mixture Volume}}{\text{E532nm} \times \text{Sample Volume} \times \text{Protein (mg)}}$$

Equation 3.3

3.14.4 Determination of Nitrite Levels

The nitrites estimation in tissue sample was based on the principle that nitrite forms colored azo dye complexes when reacted with primary aromatic amines in acidic solution,

and the colorimetric absorbance of the diazonium salt colored complexes corresponds to the measure of nitrite levels. In this study, the bioassay method of Green *et al*. (1982) was followed for estimation of Nitrite levels. First, the aromatic amine in acidic solution was prepared by adding 1% (w/v) sulfanilamide in 5% aqueous m-phosphoric acid with 0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride, refrigerated at 0^0C for 1 hour. After the one hour refrigeration, equal volume 0.5 ml of acidic solution and brain supernatant aliquot were mixed. The resulting solution was incubated in dark for 10 minutes at room temperature and absorbance was measure with a spectrophotometer at wavelength of 540 nm.

3.14.5 Superoxide Dismutase (SOD) Activity

In principle, oxidation of epinephrine generates Superoxide ($O_2^{\bullet-}$) radical and are converted to adrenochrome, a colored product, however this can be inhibited by superoxide dismutase at pH 10.2, hence, the produced adenochrome corresponds to the yielded Superoxide ($O_2^{\bullet-}$) from which the activity of SOD can be deduced colorimetrically. Using the Misra and Fridovich (1972) method, the brain supernatant aliquot was diluted to 1 in 10 dilutions by taking 1ml of each sample and mixing with 9ml of distilled water. In another beaker, 0.3mM adrenaline was freshly prepare for the SOD reaction. From the diluted sample, 0.2ml was collected into the cuvette and the sample was equilibrated by adding 2.5ml of 0.05M carbonate buffer (pH 10.2). The cuvette was placed in the spectrophotometer and to initiate the enzymatic reaction, 0.3ml adrenaline was added. Absorbance was read with a spectrophotometer at 480nm wavelength against blank containing 3ml final volume, with continuous monitoring of absorbance increase over 150 seconds for every 30 seconds change, calculated with the formula:

$$\text{Absorbance increase per minute } = \frac{A3 - A0}{2.5}$$

Equation 3.4

$$\%\ \mathbf{inhibition} = \mathbf{100} - \left(\frac{\mathbf{Increase\ in\ absorbance\ for\ substrate}}{\mathbf{Increase\ in\ absorbance\ for\ blank}} \times \mathbf{100}\right)$$

Equation 3.5

3.14.6 Evaluation of Serum Corticosterone Concentrations

The collected blood samples were centrifuged and the serum aliquoted. Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**80, 40, 20, 10, 5, 0 ng/mL**) was set in duplicate in the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of the dilution solution was added to each testing sample well, and 10µl of the blood serum samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, stand for 30s after which the plate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of the provided Chromogen Substrate A was added to all wells including the blank well, another 50µl of the provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of the Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance was measured with a microplate reader at 450nm within 15min after adding the Stop Solution.

3.14.7 Acetylcholinesterase (AChE) Activity

The method of quantifying the enzymatic activity of AChE used in this study was as described by Ellman *et al*. (1961) which was based on the principle that acetylcholine hydrolysis acetylcholine iodide to two main products, acetic acid and thiocholine which when reacted with DTNB forms a characteristic yellow colored complex whose optical density corresponds tolevel of the enzymatic activity and this can be measured using

colorimetrically. In the procedure, a 100µl of aliquots of brain tissue homogenates was diluted with 650µL Phosphate buffer and then added to 100 mL DTNB and the substrate, 25µL of acetylthiocholine iodide (75 mM). A yellow color was formed and absorbance was measured at 412 nm. The absorbance readings were taken at every 1 minute interval over a period of 5 minutes. Acetylcholinesterase activity was expressed in nanomole of acetylthiocholine iodide hydrolyzed per mg of protein per minute. Using Biurets method, protein concentration was assessed as previously described in section 3.14.1 above.

3.14.8 Determination of Noradrenaline (NE) concentrations

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**8, 4, 2, 1, 0.5, 0 ng/mL**) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of dilution solution was added to testing sample well, and 10µl of the brain tissue homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, allowed stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of the provided Chromogen Substrate A was added to all wells including the blank well, another 50µl of the provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance was measured at 450nm within 15min after adding the Stop Solution.

3.14.9 Determination of 5-Hydroxytryptamine (5-HT) concentrations

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**240, 120, 60, 30, 15, 0 ng/mL**) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of dilution solution was added to each testing sample well, and 10µl of the brain tissue homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, allowed stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of the provided Chromogen Substrate A was added to all the wells including the blank well, another 50µl of provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of the Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance was measured at 450nm within 15min after adding the Stop Solution.

3.14.10 Determination of Dopamine (DA) concentrations

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**120, 60, 30, 15, 7.5, 0 pg/mL**) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of dilution solution was added to testing sample well, and 10µl of the brain tissue

homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, allowed to stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of provided Chromogen Substrate A was added to all the wells including the blank well, another 50µl of provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of the Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance was measured at 450nm within 15min after adding the Stop Solution.

3.14.11 Determination of BDNF Concentrations

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**800, 400, 200, 100, 50, 0 pg/mL**) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl dilution solution was added to testing sample well, and 10µl of the brain tissue homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-

dried. The diluted wash buffer was then added to the entire well, allowed stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of provided Chromogen Substrate A was added to all the wells including the blank well, another 50µl of the provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance measured at 450nm within 15min after adding the Stop Solution.

3.14.12 Estimation of CREB Levels

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**48, 24, 12, 6, 3, 0 ng/mL**) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of dilution solution was added to testing sample well, and 10µl of the brain tissue homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, allowed to stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of the provided Chromogen Substrate A was added to all the wells including the blank well, another 50µl of provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of the Stop Solution was taken and pipette into all the wells respectively. The blank well

was taken as zero and absorbance was measured at 450nm within 15min after adding the Stop Solution.

3.14.13 Determination of Mitogen-Activated Protein Kinases (MAPKs) Concentrations

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**8000, 4000, 2000, 1000, 500, 0 pg/mL**) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of dilution solution was added to testing sample well, and 10µl of the brain tissue homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, allowed stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of the provided Chromogen Substrate A was added to all the wells including the blank well, another 50µl of provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of the Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance was measured at 450nm within 15min after adding the Stop Solution.

3.14.14 Determination of Interferon γ (IFN-γ) Concentrations

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**800, 400, 200, 100, 50, 0**

pg/mL) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of dilution solution was added to testing sample well, and 10µl of the brain tissue homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, allowed to stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of the provided Chromogen Substrate A was added to all the wells including the blank well, another 50µl of provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of the Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance was measured at 450nm within 15min after adding the Stop Solution.

3.14.15 Measurement of Interleukin 6 (IL-6) Levels

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**120, 60, 30, 15, 7.5, 0 pg/mL**) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of dilution solution was added to testing sample well, and 10µl of the brain tissue homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at

temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, allowed stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of the provided Chromogen Substrate A was added to all the wells including the blank well, another 50µl of provided Chromogen Substrate B was similarly added to each well including the blank well, and the plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of the Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance was measured at 450nm within 15min after adding the Stop Solution.

3.14.16 Determination of Tumor Necrosis Factor - α (TNF-α) Concentrations

Using commercially available ELISA kits (Biobase Inc., China), the standard wells in a 96-well plate were set and 50µl of each standard solution (**640, 320, 160, 80, 40, 0 pg/mL**) was added in duplicate to the standard wells respectively. The first two wells of the 96-well plate were used as the blank wells where no test sample or the HRP-Conjugate reagent was added. On the microplate, the testing sample wells were set in duplicate and 40µl of dilution solution was added to testing sample well, and 10µl of the brain tissue homogenate samples were added to make a 5-fold sample final dilution and the plate was gently swirled to allow mixing of the samples and the reagents. Afterwards, 100µl of the enzyme, HRP-Conjugate reagent was pipette to all the testing sample wells and standard wells. All the wells were cover sealed and the plate was placed in an incubator set at temperature of 37℃ for 1 hour duration. The supplied wash solution was separately prepared for use by diluting it in distilled water to a 20-fold dilution. The incubated plate was removed from the incubator, the solutions were discarded and the plate was swing-dried. The diluted wash buffer was then added to the entire well, allowed stand for 30s after which the pate was drained. The wash process was repeated for 5 times and the plate was again dried by patting on tissue paper. After drying, 50µl of the provided Chromogen Substrate A was added to all the wells including the blank well, another 50µl of provided Chromogen Substrate B was similarly added to each well including the blank well, and the

plate was placed in dark enclosure for 15 minutes at 37℃ using an incubator. Then 50µl of the Stop Solution was taken and pipette into all the wells respectively. The blank well was taken as zero and absorbance was measured at 450nm within 15min after adding the Stop Solution.

3.15 Histology and Immunohistochemistry Analysis

Immediately after the behavioural studies mice from the CUMS and LPS chronic study paradigms were deeply anaesthetized and sacrificed. Each animal was administered 50mg/kg pentobarbital sodium intraperitoneally and were subsequently administered 4% paraformaldehyde in 0.01 M phosphate buffer via transcardiac perfusion in order to achieve in-situ tissue fixation. The brains were excised and postfixed in buffered formalin until sectioning for study.

3.15.1 Histological Assessment – H&E Staining

The postfixed brains were processed for histological findings in hippocampus and the prefrontal cortex. Multiple serial sections of brain were collected by cutting coronal brain sections at 25 mm using a microtome. Then the collected serial sections were further subjected to slide preparation and mounting process which sequentially includes dehydration, clearing, infiltration, embedding, sectioning and lastly staining with H&E dye. All of the prepared slides were properly labelled for identification matching treatment groups and were viewed with Olympus binocular microscope at x400 magnification for morphological architecture and structural integrity of the hippocampal and cortical brain regions. Photomicroraphs of each section under view was taken with the aid of a digital camera and the images were carefully assessed with ImageJ software for structural alterations. Furthermore, the neuronal densities of cells in these brain regions were assessed by manually counting the total viable and functional cells.

3.15.2 Immunohistochemical studies

Immunohistochemistry staining was carried out by fixing the excised brains individually for 48 hours in 4% Paraformaldehyde solution after which serial coronal sections were obtained by cutting the brains serially into multiple sections of 5 µm thickness. Tissue

blocks were prepared as free-floating slices by processes including paraffin embedding, deparaffinising, rehydrating and pretreatment with hydrogen peroxidase.

3.15.2.1 NF-κB Expression Immunohistochemistry

The protocol was as described by Kim *et al*. (2016). The paraffin embedded sections from the procedure highlighted above in section 3.15.2 were incubated in antibody diluent solution that contains anti-NF-κB antibody (1:200 dilutions) for 60 minutes at room temperature. After incubation with secondary antibody, placed in the chromogen substrate, 3,30-diaminobenzidine (DAB), and incubated for 1 – 3 minutes. The free-floating sections were then rinsed with 0.1 mol phosphate buffer and counterstained with Mayer's hematoxylin. For each test animal, three independent sections were prepared. Using a binocular microscope with a digital camera for capturing the positively stained expressions on micrographs, the NF-κB-positive nuclei were counted manually at x400 magnifications. The field was viewed at an area of 500 by 500 mm^2 in both hemispheres of the brain and mean value for the three sections were calculated as NF-κB expression activity.

3.15.2.2 iNOS Expression Immunohistochemistry

The protocol was as described by Kim *et al*. (2016). The paraffin embedded sections from the procedure highlighted above in section 3.15.2 were incubated in antibody diluent solution that contains anti-iNOS antibody (1:1,000 dilutions) for 60 minutes. After incubation with secondary antibody, sections were placed in the chromogen substrate, 3,30-diaminobenzidine (DAB), and incubated for 1 – 3 minutes. The free-floating sections were then rinsed with 0.1 mol phosphate buffer and counterstained with Mayer's hematoxylin. For each test animal, three independent sections were prepared. Using a binocular microscope with a digital camera for capturing the positively stained expressions on micrographs, the iNOS-positive nuclei were counted manually at x400 magnifications. The field was viewed at an area of 500 by 500 mm^2 in both hemispheres of the brain and mean value for the three sections for each animal was calculated as iNOS expression activity.

3.16 Data Analysis

The recorded data from all experimental procedures above were analysed using GraphPad Prism Biostatistics Software Version 4. The mean of data results within groups were calculated and presented as mean ± standard error of mean (SEM), and these mean values were used to analyse significance across groups statistically with the method of "One Way Analysis of Variance" (ANOVA) and Tukey's post hoc test. Level of significance for all tests was set at 95% confidence interval, that is, $p < 0.05$.

CHAPTER FOUR

RESULTS

4.1 Acute Toxicity of Troxerutin

In acute toxicity model, treatment with Troxerutin at all administered doses in the paradigm produced no mortality among the treated animals, and no sign of toxicity or behavioural change was observed within the 24 hours post-treatment at highest dose of 5,000 mg/kg, i.p. body weight using the Lorke's method.

4.2 Effect of Troxerutin (TXN) on TST

In the TST, a significant difference [$F(5, 24) = 46.65$, $P < 0.001$] in immobility period was observed across treatment groups of Troxerutin and Imipramine compared to the vehicle-treated controls, result graphically presented in Figure 4.1 below.

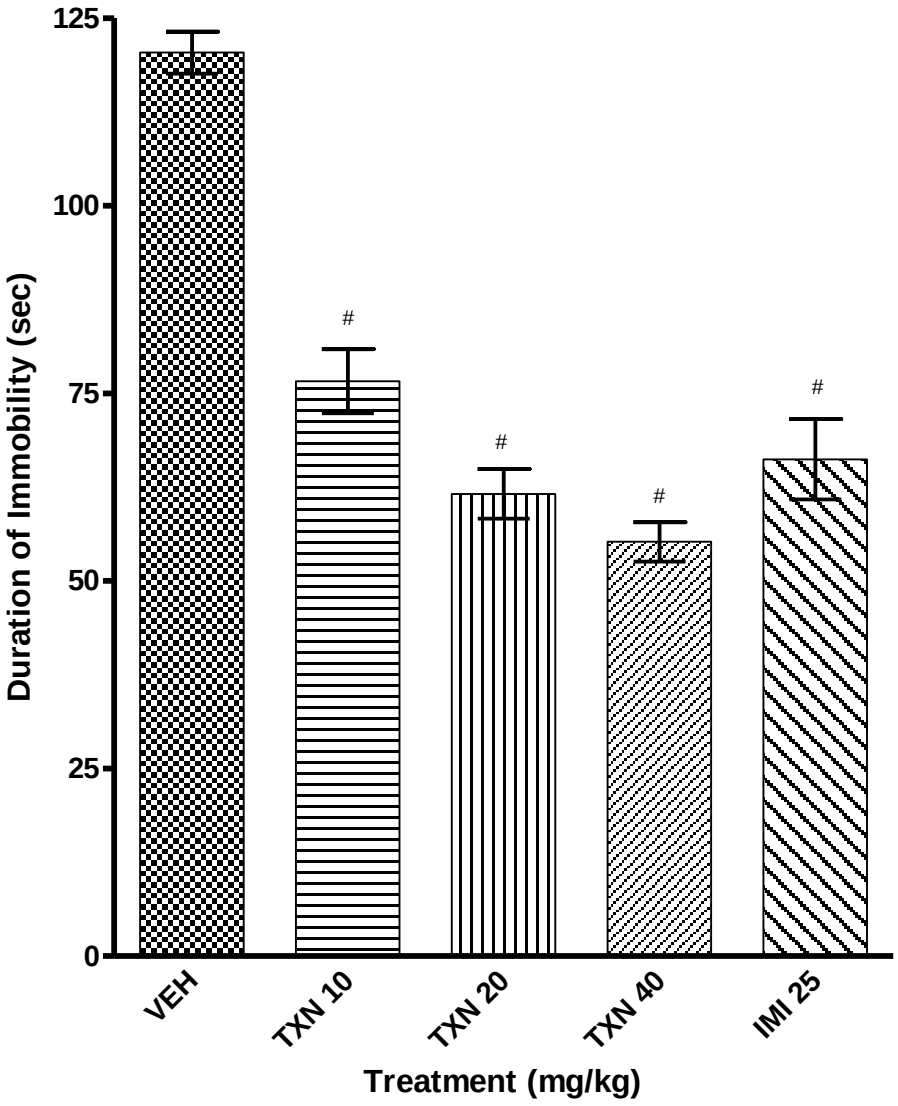

Figure 4.1: Effect of Troxerutin on TST in mice.

#P < 0.001 compared to vehicle. (VEH- Vehicle, TXN- Troxerutin, IMI- Imipramine)

4.3 Effect of Troxerutin (TXN) on FST in Mice

Duration of immobility was significantly different [$F(5, 24) = 22.69$, $P < 0.001$] in Troxerutin and Imipramine treated groups compared to vehicle as graphically depicted in Figure 4.2 below.

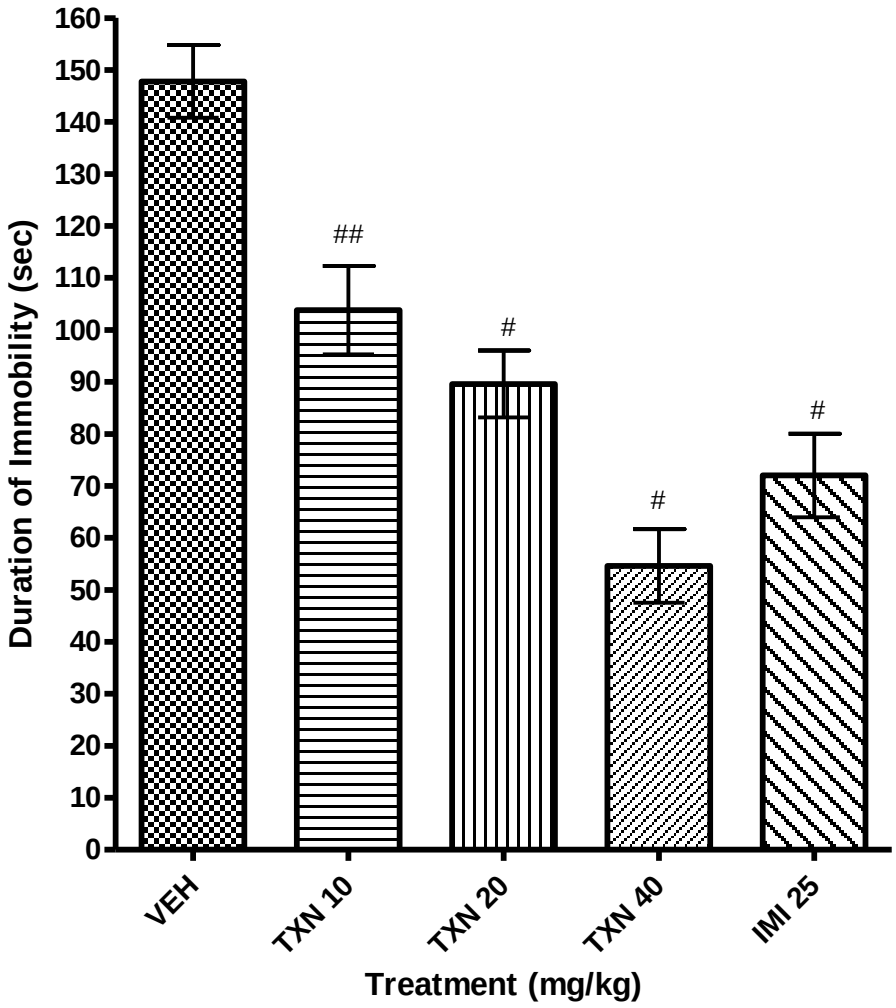

Figure 4.2: Effect of Troxerutin on FST in mice.

#P < 0.01 compared to vehicle, ##P < 0.001 compared to vehicle. (VEH- Vehicle, TXN- Troxerutin, IMI- Imipramine)

4.4 Effect of Troxerutin (TXN) on Locomotor Activity in OFT

There was no significant difference in the locomotor activities exhibited by the Troxerutin-treated and Imipramine-treated groups compared to the vehicle group, Figure 4.3 below.

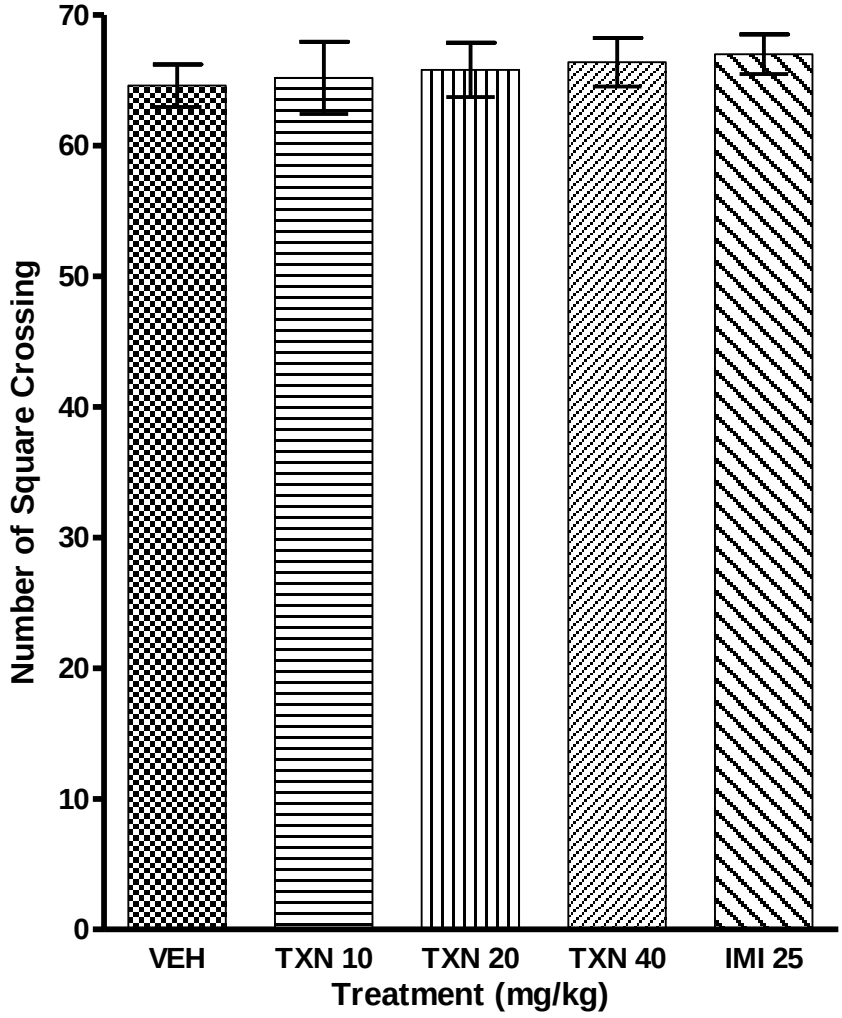

Figure 4.3: Locomotor activity in open field.

(VEH- Vehicle, TXN- Troxerutin, IMI- Imipramine)

4.5 Protracted Activity of Troxerutin (TXN) on FST and TST

The results of protracted activity of single dose of Troxerutin on FST and TST were presented below on Table 4.1 and 4.2 respectively.

In the FST three days post-treatment, the duration of immobility was significantly different [$F(5, 24) = 15.07$, $P < 0.0001$] in Troxerutin-treated groups compared to vehicle-treated control. On day 7 post-treatment, Troxerutin (20 and 40 mg/kg) showed significant difference [$F(5, 24) = 6.138$, $P < 0.0001$] compared to controls.

In the TST three days post-treatment, the duration of immobility was significantly different [$F(5, 24) = 35.21$, $P < 0.0001$] in Troxerutin-treated groups compared to vehicle-treated control. On day 7 post-treatment, Troxerutin (20 and 40 mg/kg) showed significant difference [$F(5, 24) = 10.72$, $P < 0.0001$] compared to controls.

Table 4.1: Protracted Effect of single dose Troxerutin on FST in mice

Treatment (i.p)	Day 3 – Immobility (sec)	Day 7 – Immobility (sec)
VEHICLE	141.4±4.60	142.6±6.23
TROXERUTIN 10	115.2±5.49*	142.4±13.61
TROXERUTIN 20	101.4±5.82***	106.2±5.43*
TROXERUTIN 40	94.4±3.89***	100.2±7.27*
IMIPRAMINE 25	124.4±6.59	136±6.33

*P < 0.05 compared to vehicle control, ***P < 0.001 compared to vehicle control

Table 4.2: Protracted effect of single dose Troxerutin on TST in mice

Treatment	Day 3 – Immobility (sec)	Day 7 – Immobility (sec)
VEHICLE	121.6±4.80	125.2±4.40
TROXERUTIN 10	97.8±5.24*	119.0±3.60
TROXERUTIN 20	79.2±3.45***	96.0±3.05***
TROXERUTIN 40	58.8±2.74***	97.0±4.82***
IMIPRAMINE 25	107.8±3.99	117.0.4±4.44

*P < 0.01 compared to vehicle control, ***P < 0.001 compared to vehicle control

4.6 Effect of Troxerutin on SPT in CUMS in mice

In the pre-test baseline study, percentage of sucrose preference showed no significant difference, $p > 0.05$, however, after 14-day exposure to stress, there was significant difference in sucrose consumption in vehicle-treated animals compared to unstressed group and its baseline pre-test performance, [$F(5, 24) = 25.31$, $P<0.001$ (Figure 4.4)].

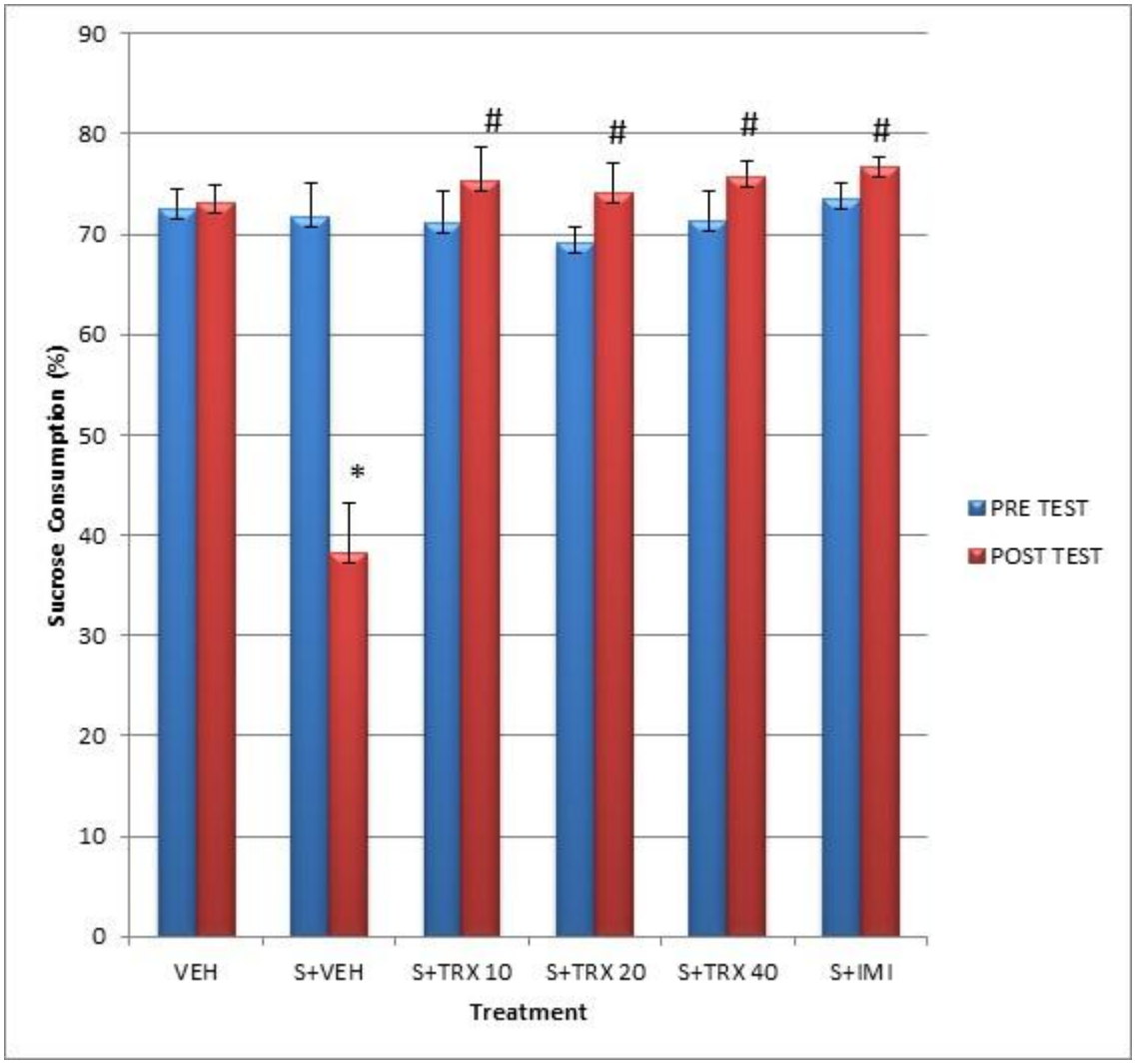

Figure 4.4: Effect of Troxerutin on Sucrose preference in mice.

*P < 0.001 compared to unstressed group. #P < 0.001 in comparison with vehicle stressed group. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.7 Effect of Troxerutin on FST in CUMS

The vehicle control animals exposed to stressors in the CUMS paradigm exhibits increased immobility compared to the non-stressed controls. Result showed significant difference [F (6, 29) = 68.47] in immobility time of troxerutin-treated and imipramine-treated groups compared to this stressed vehiclecontrols, represented on Figure 4.5.

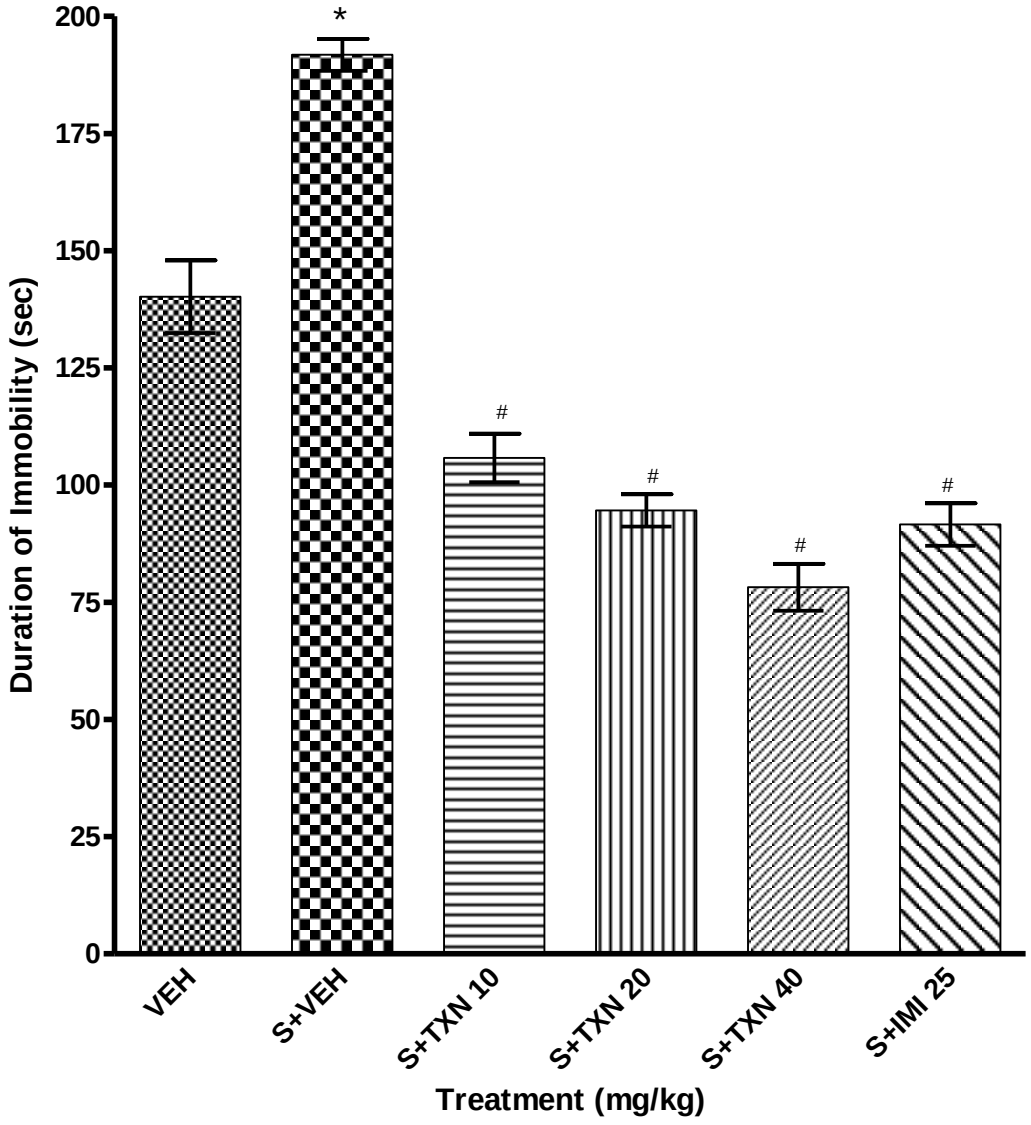

Figure 4.5: Immobility time in FST post-CUMS.

*P < 0.001 in comparison to non-stressed vehicle control. #P < 0.001 compared to vehicle-treated stressed group. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.8 Effect of Troxerutin on Y-maze activity in CUMS

Alteration in memory function by exposure to CUMS significantly differs in stressed vehicle controls as compared to other groupsin Y-maze, $F(6, 29) = 55.59$, $p<0.001$, Figure 4.6.

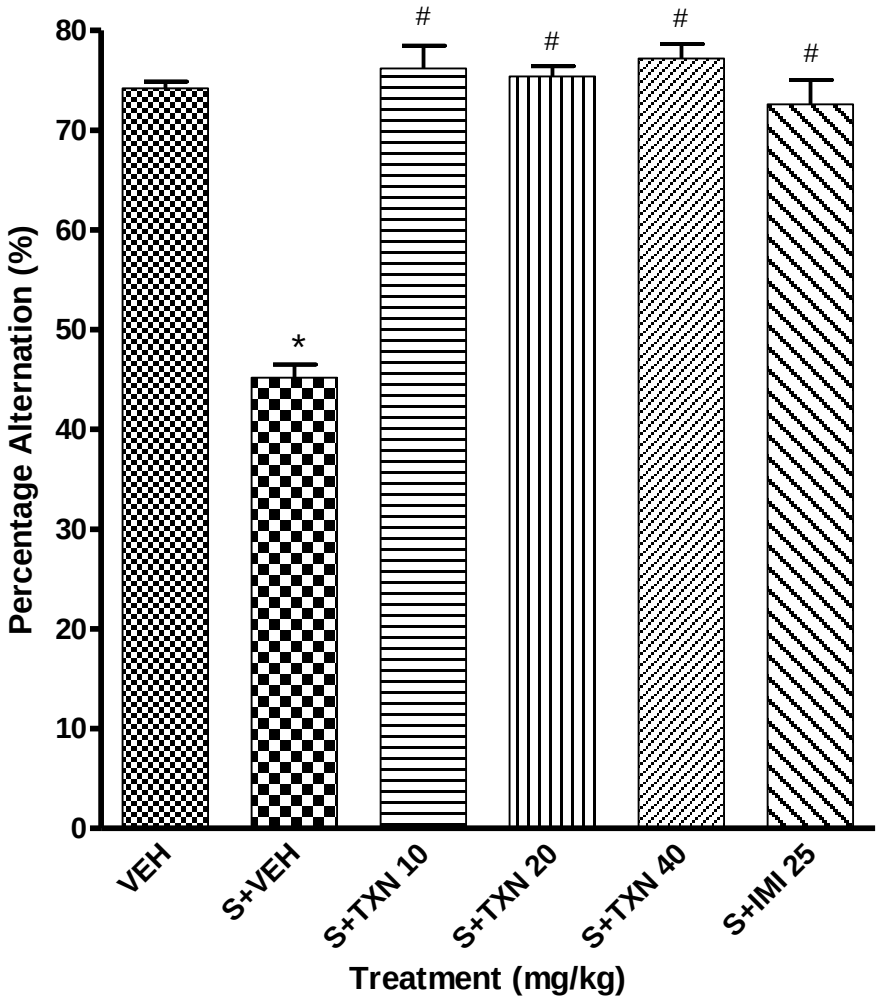

Figure4.6: Effect of Troxerutin on memory in Y maze.

*P < 0.001 compared to vehicle non-stressed controls. #P< 0.001 compared to vehicle-treated stressed group. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.9 Effect of Troxerutin on Rearing Activity in CUMS

The vertical movement activities of vehicle-treated stressed animals were impaired compared to non-stressed group, $P< 0.001$. Troxerutin and imipramie-treated animals showed significant reversal of this stress-induced impairment, [$F (6, 29) = 55.59$, $p < 0.001$], Figure 4.7.

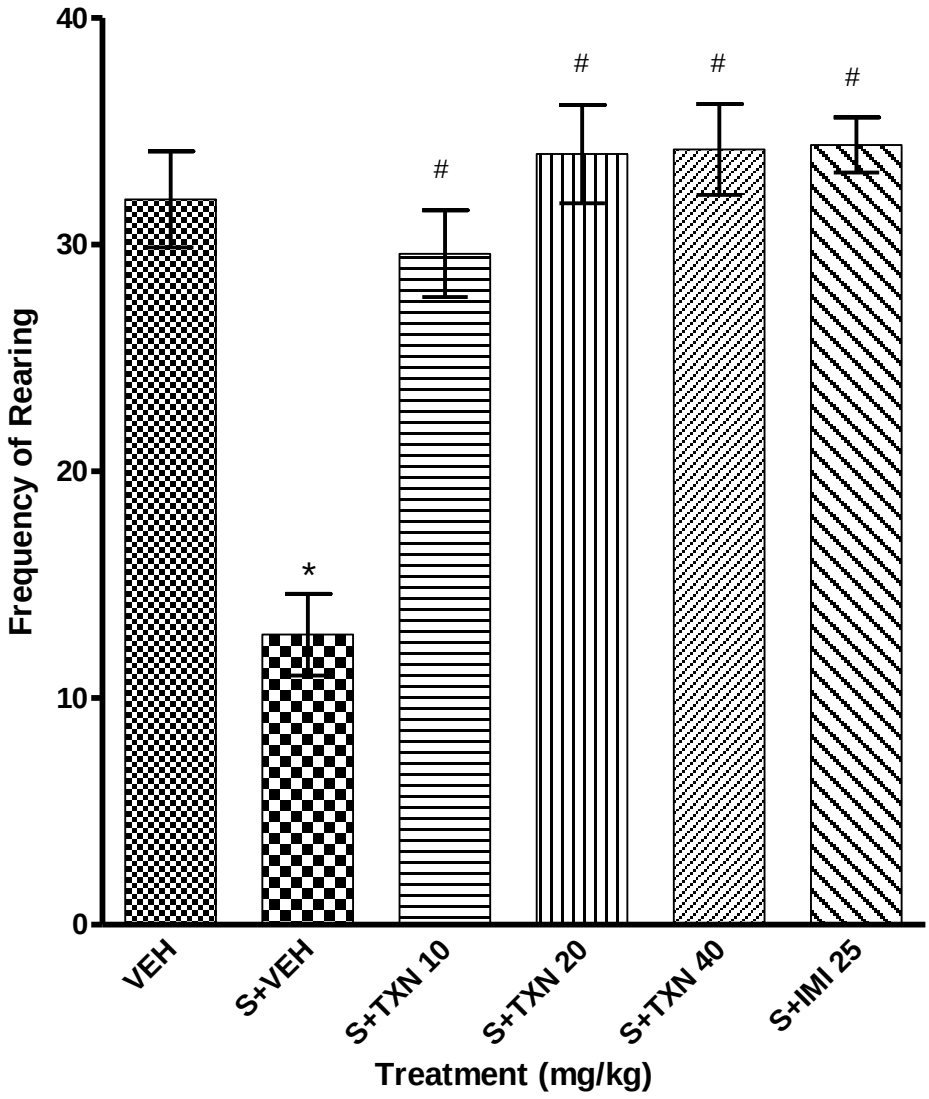

Figure4.7: Effect of Troxerutin on rearing activities in mice subjected to CUMS.

*P < 0.001 compared to non-stressed vehicle-treated. #P < 0.001 compared to stressed vehicle-treated. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.10 Effect of Troxerutin on Grooming Activityin mice subjected to CUMS

Comparing the vehicle treatment groups, the frequency of grooming activities in stressed animals were significantly reduced in comparison with non-stressed groups, $P < 0.001$. The Troxerutin and Imipramine treatment group showed significant difference in frequency of grooming compared to stressed vehicle control, $F(6, 29) = 8.324$, $P < 0.001$, Figure 4.8.

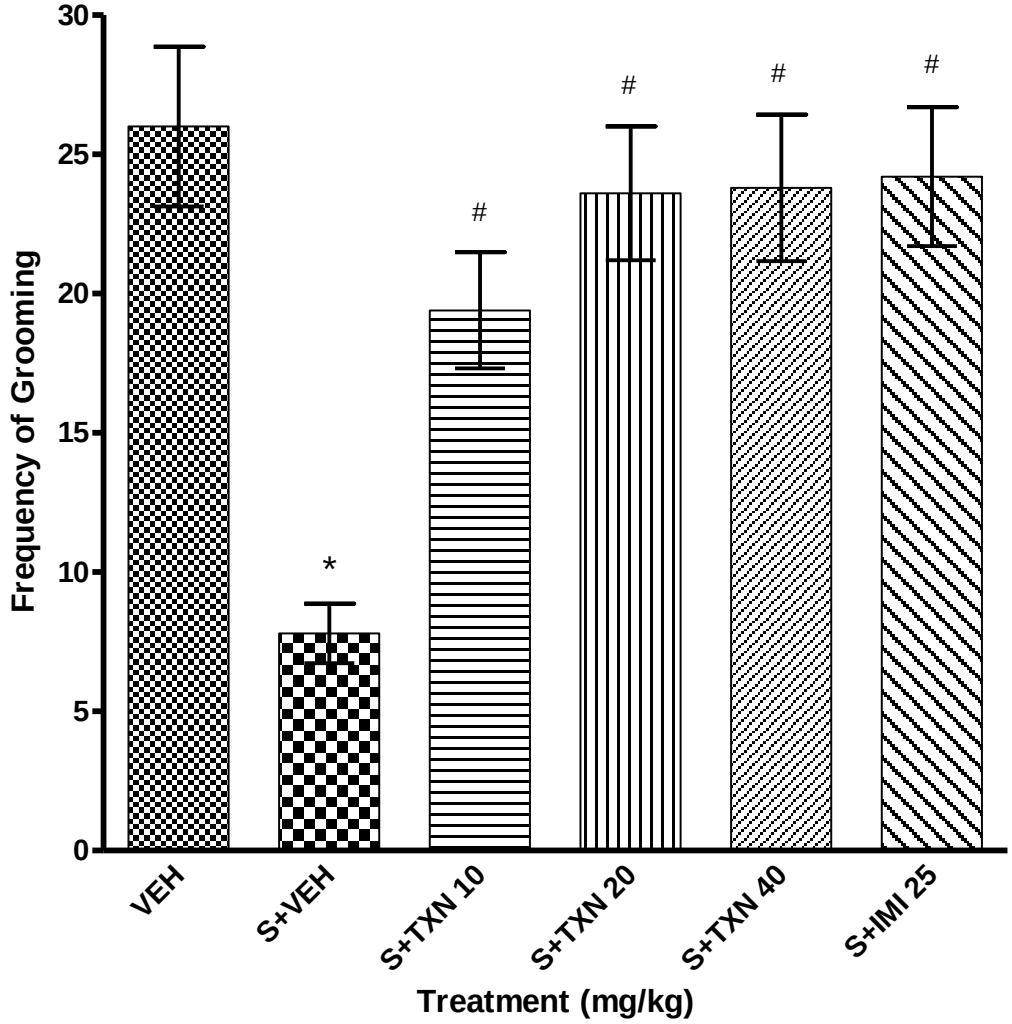

Figure4.8: Effect of Troxerutin on grooming activities in mice subjected to CUMS.

*P < 0.001 compared to non-stressed vehicle. #P < 0.001 compared to stressedvehicle. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.11 Effect of Troxerutin (TXN) on Locomotor Activity in CUMS

Troxerutin and Imipramine showed no significant difference in number of squares crossed compared to the non-stressed vehicle control in open field, $p>0.05$, Figure 4.9.

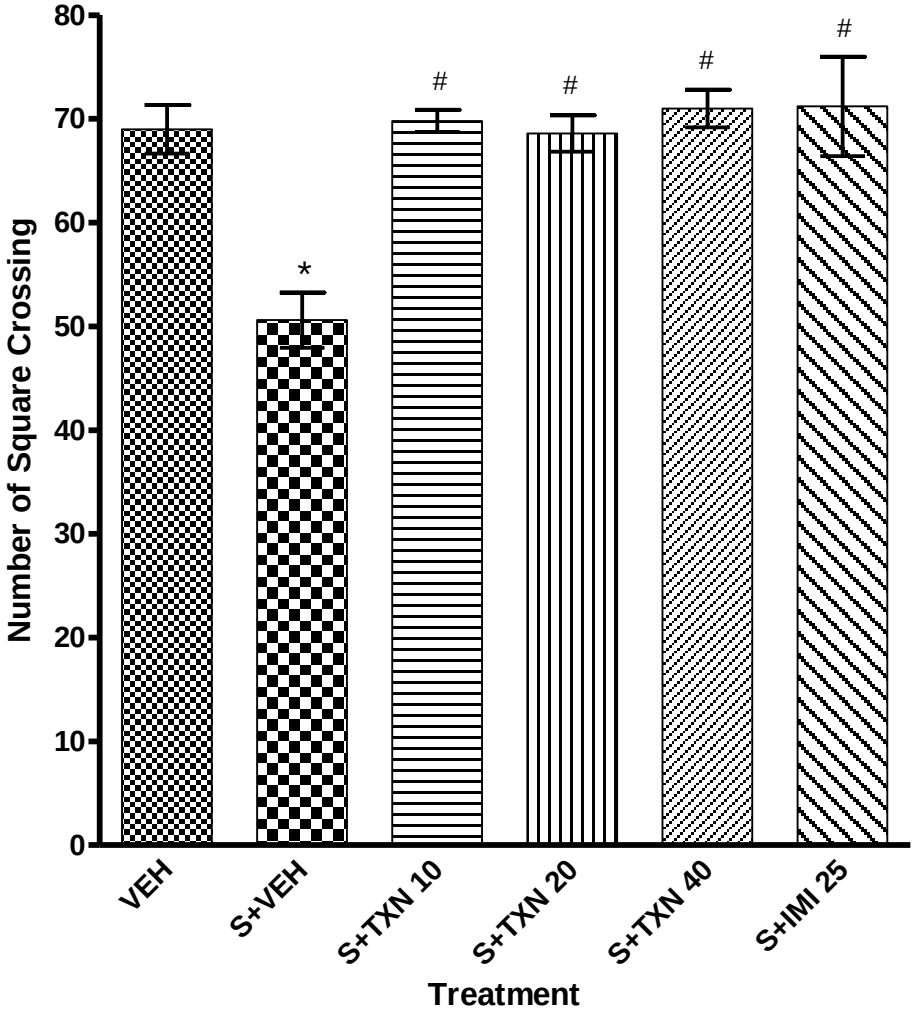

Figure 4.9: Effect of Troxerutin (TXN) on locomotor activity in CUMS.

F (6, 29) = 8.832. *P < 0.001 compared to non-stressed vehicle. #P < 0.001 compared to stressed vehicle.(VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.12 Effect of Troxerutin on brain reduced Glutathione levels in CUMS

Levels of reduced glutathione showed significant difference comparatively between stressed vehicle and unstressed groups, $P< 0.001$, and the treatment with Troxerutin and Imipramine also showed significant difference compared to stressed vehicle, $F (5, 24) = 20.07.$, $P <0.001$, Figure 4.10.

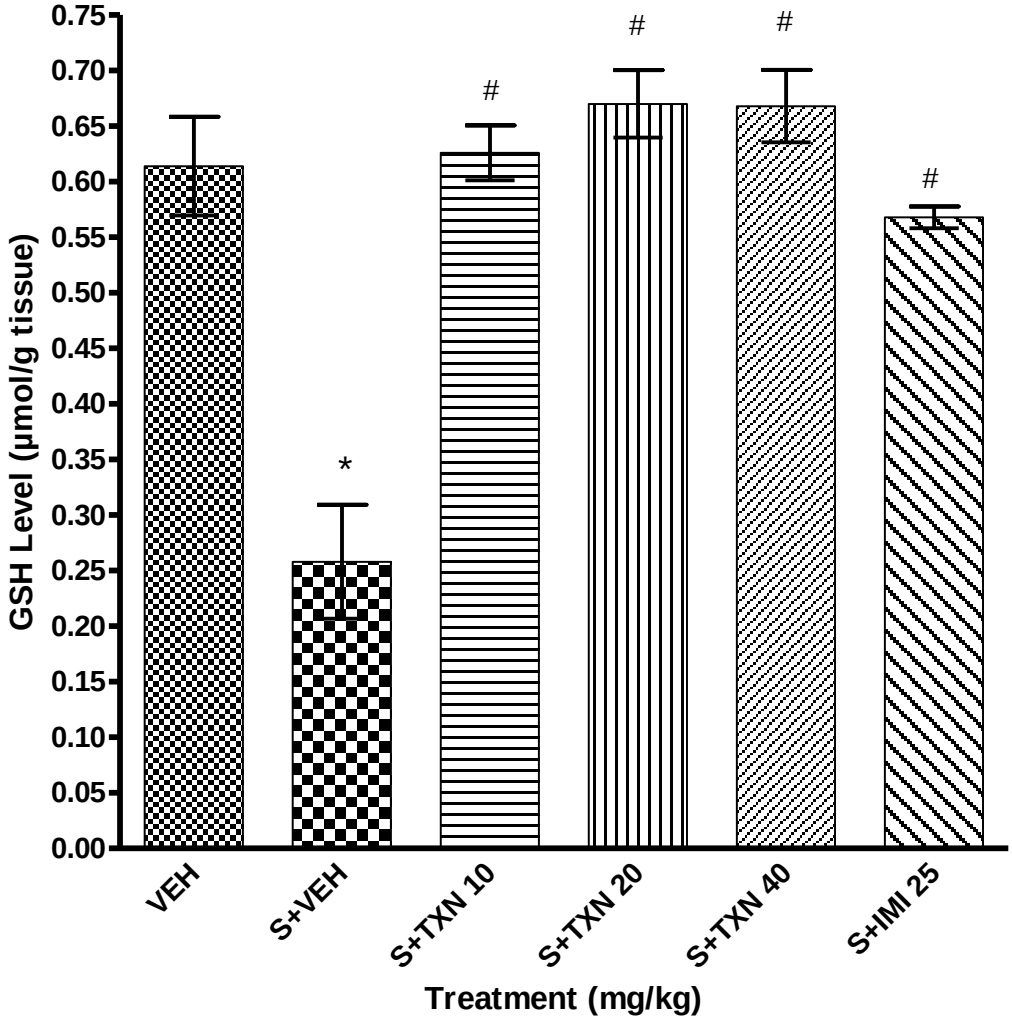

Figure 4.10: Effect of Troxerutin on GSH Levels in mice subjected to CUMS.

*P < 0.001 compared to non-stressed vehicle group. #P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.13 Effect of Troxerutin on Malondiadehyde levels in CUMS

The animals subjected to stress in the CUMS paradigm showed significant difference in Malondialdehyde levels when compared to unstressed groups, $P < 0.001$. The levels of MDA in animals treated with Troxerutin or Imipramine showed significant difference as compared to vehicle treated animals subjected to stress, $F(6, 29) = 19.68$, $P < 0.001$, Figure 4.11.

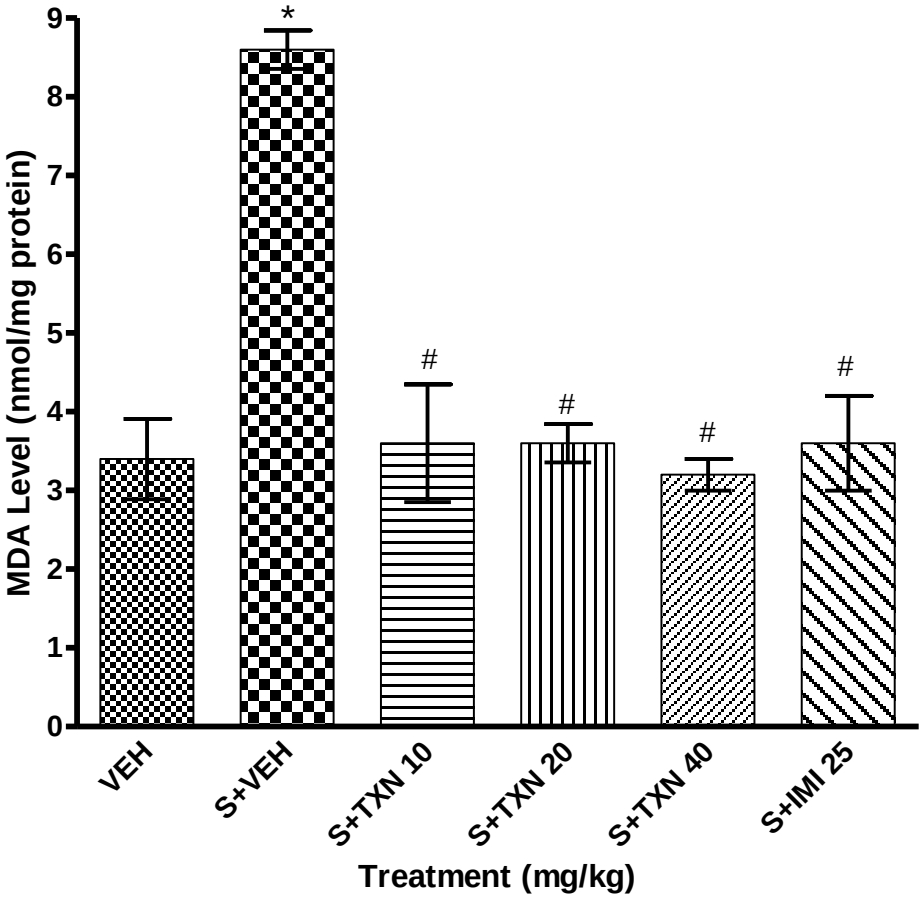

Figure 4.11: Effect of Troxerutin on MDA Levels in mice subjected to CUMS.

*P < 0.001 compared to non-stressed control. #P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.14 Effect of Troxerutin on Nitrite Levels in CUMS

The animals subjected to stress in the CUMS paradigm showed a significant difference in Nitrite levels when compared to unstressed groups, $P< 0.001$. The levels of Nitrite in animals treated with Troxerutin or Imipramine showed significant difference compared to vehicle-treated animals subjected to stress, $P< 0.001$, Figure 4.12.

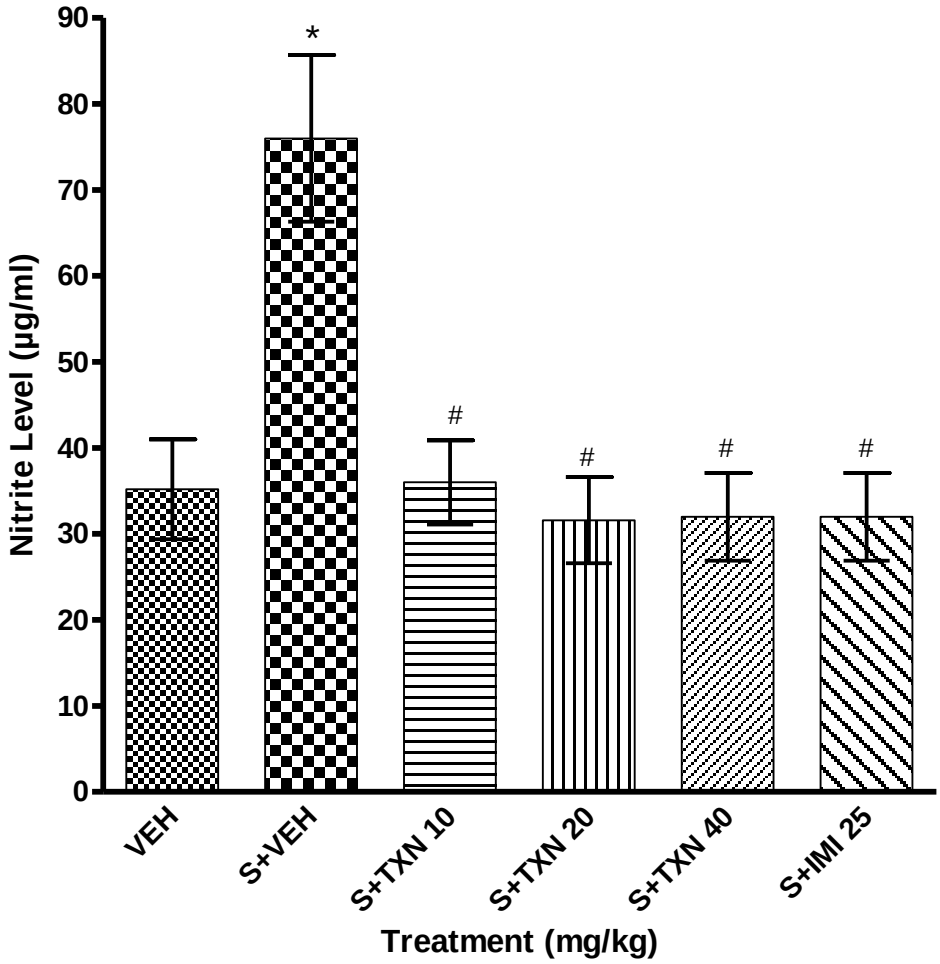

Figure 4.12: Effect of Troxerutin on Nitrite Levels in mice subjected to CUMS.

F (6, 29) = 8.037. *P < 0.001 compared to non-stressed control. #P < 0.001 compared to vehicle stressed control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.15 Effect of Troxerutin on SOD levels in CUMS

The animals subjected to stress in the CUMS paradigm showed significant difference in SOD levels when compared to the unstressed groups, $p < 0.01$. The levels of SOD in animals treated with Troxerutin or Imipramine showed significant difference compared to vehicle treated animals subjected to stress, $P< 0.001$, Figure 4.13.

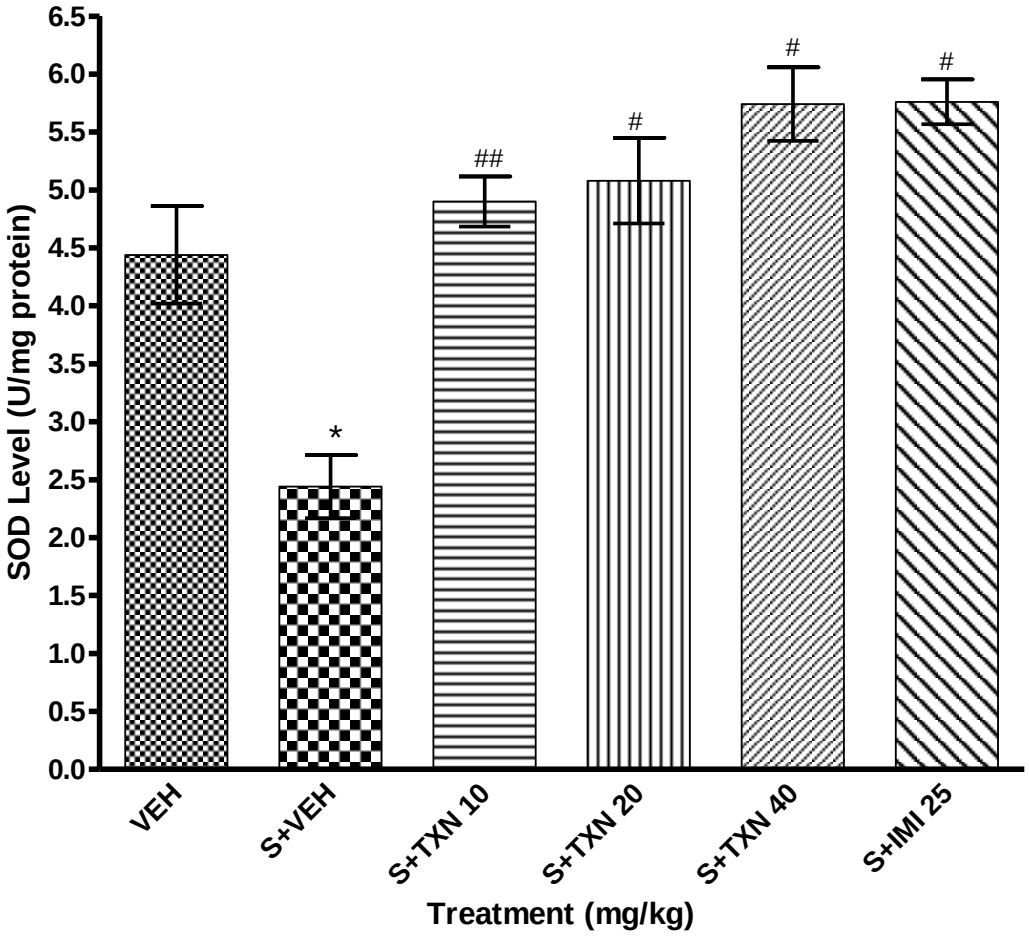

Figure 4.13: Effect of Troxerutin on SOD Levels in mice subjected to CUMS.

F (6, 29) = 15.89. *P < 0.01 compared to non-stressed control. #P < 0.001 compared to vehicle stressed control. ##P < 0.01 compared to vehicle stressed control

(VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.16 Effect of Troxerutin on serum Corticosterone levels in CUMS

In CUMS paradigm the stressed controls showed significant difference in Corticosterone levels when compared to the unstressed groups, $P< 0.01$. The levels of Corticosterone in animals treated with Troxerutin or Imipramine showed significant difference compared to vehicle-treated animals subjected to stress, $p < 0.001$, Figure 4.14.

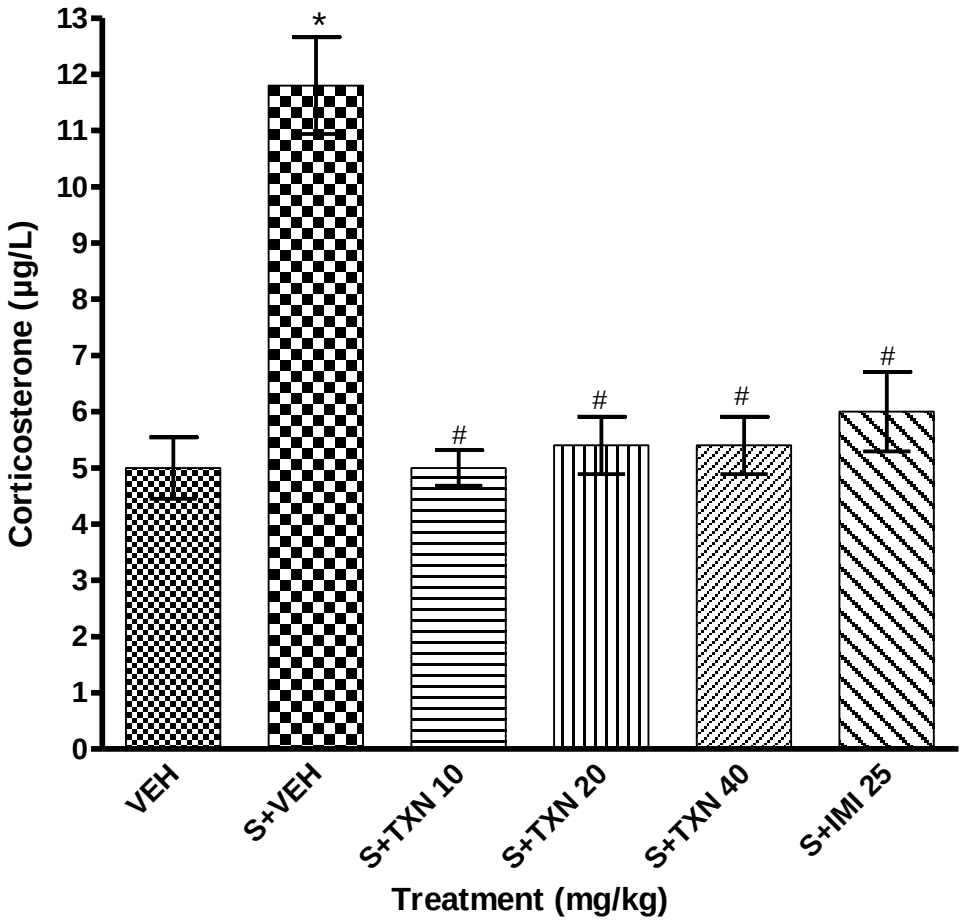

Figure 4.14: Effect of Troxerutin on Corticosterone Levels in mice subjected to CUMS.

F (6, 29) = 19.57. *P < 0.001 compared to non-stressed control. #P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.17 Effect of Troxerutin on brain TNF-α Levels in CUMS

The animals subjected to stress in CUMS paradigm showed significant difference in TNF-α levels when compared to the non-stressed group, $P< 0.01$. The levels of TNF-α in animals treated with Troxerutin or Imipramine showed significant difference compared to vehicle-treated animals subjected to stress, $p < 0.01$, Figure 4.15.

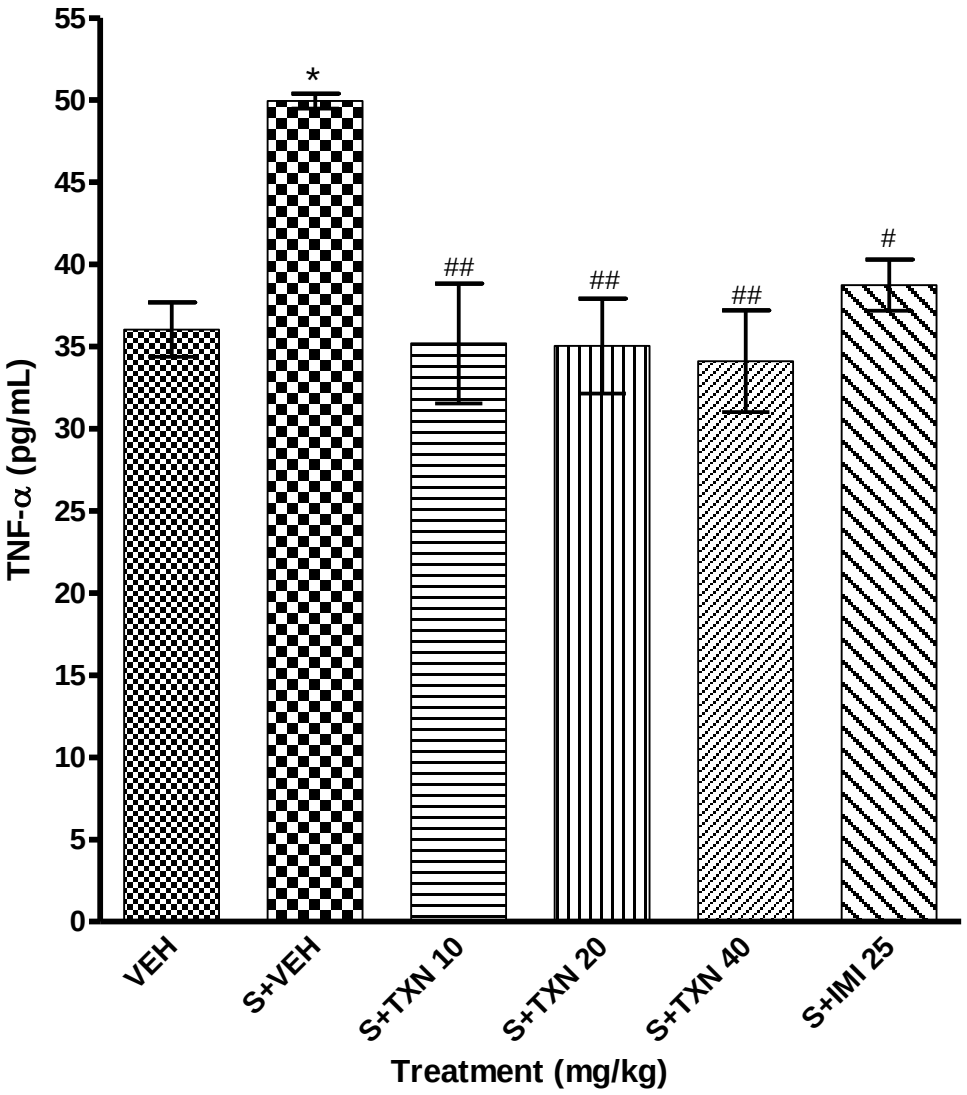

Figure 4.15: Effect of Troxerutin on TNF-α Levels in mice subjected to CUMS.

F (6, 29) = 5.86. *P < 0.01 compared to unstressed group. #P < 0.05 compared to stressed vehicle control. ##P < 0.01 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.18 Effect of Troxerutin on Interferon-γ level in CUMS

The animals subjected to stress in the CUMS paradigm showed a significant difference in Interferon-γ levels when compared to unstressed groups, $p < 0.01$. The levels of Interferon-γ in animals treated with Troxerutin or Imipramine showed significant difference compared to the vehicle treated animals subjected to stress, $p < 0.01$, Figure 4.16.

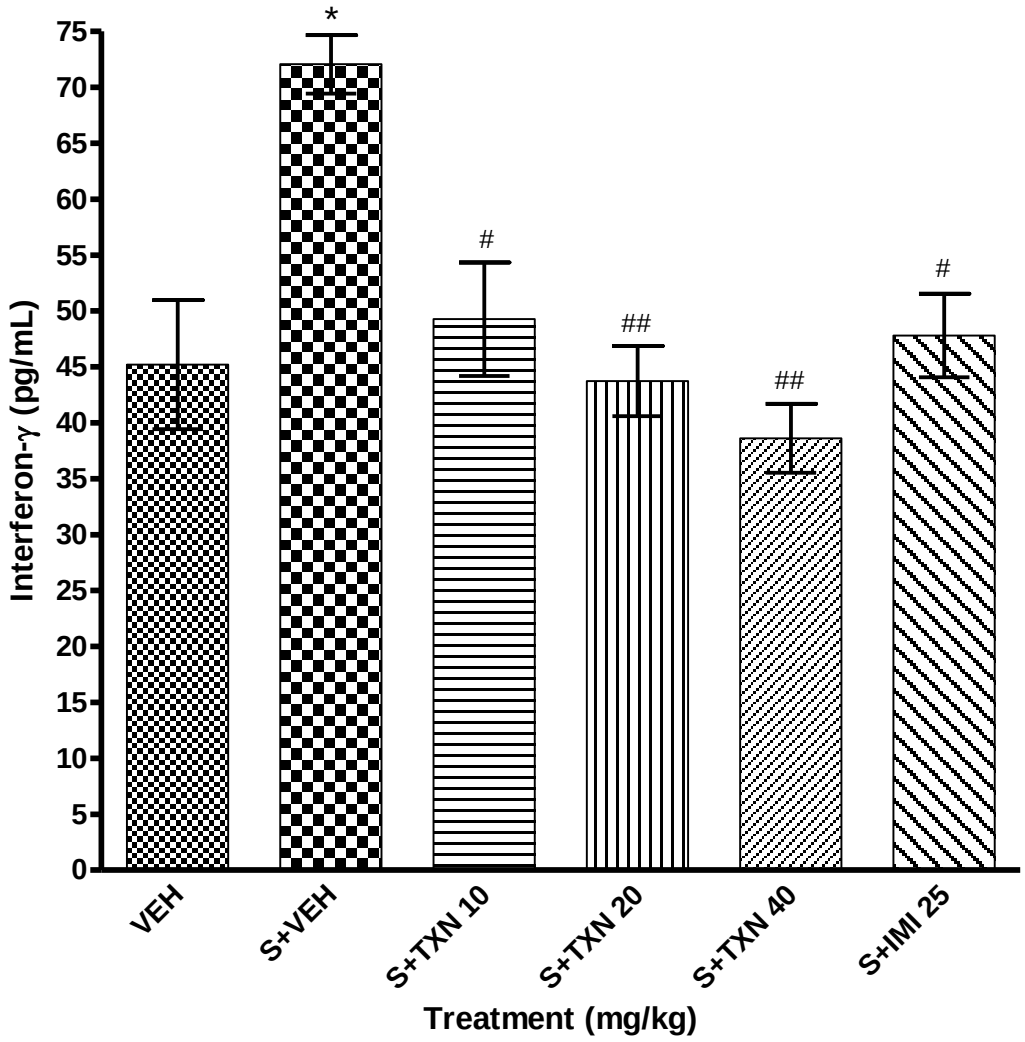

Figure 4.16: Effect of Troxerutin on Interferon-γ Levels in mice subjected to CUMS. F (6, 29) = 8.256. *P < 0.01 compared to unstressed control. #P < 0.01 compared to stressed vehicle control. ##P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.19 Effect of Troxerutin on brain Interleukin 6 (IL-6) level in Mice subjected to CUMS

The animals subjected to stress in the CUMS paradigm showed significant difference in IL-6 levels when compared to the unstressed groups, $p < 0.001$. The levels of IL-6 in animals treated with Troxerutin or Imipramine showed significant difference as compared to vehicle treated animals subjected to stress, $p < 0.05$, Figure 4.17.

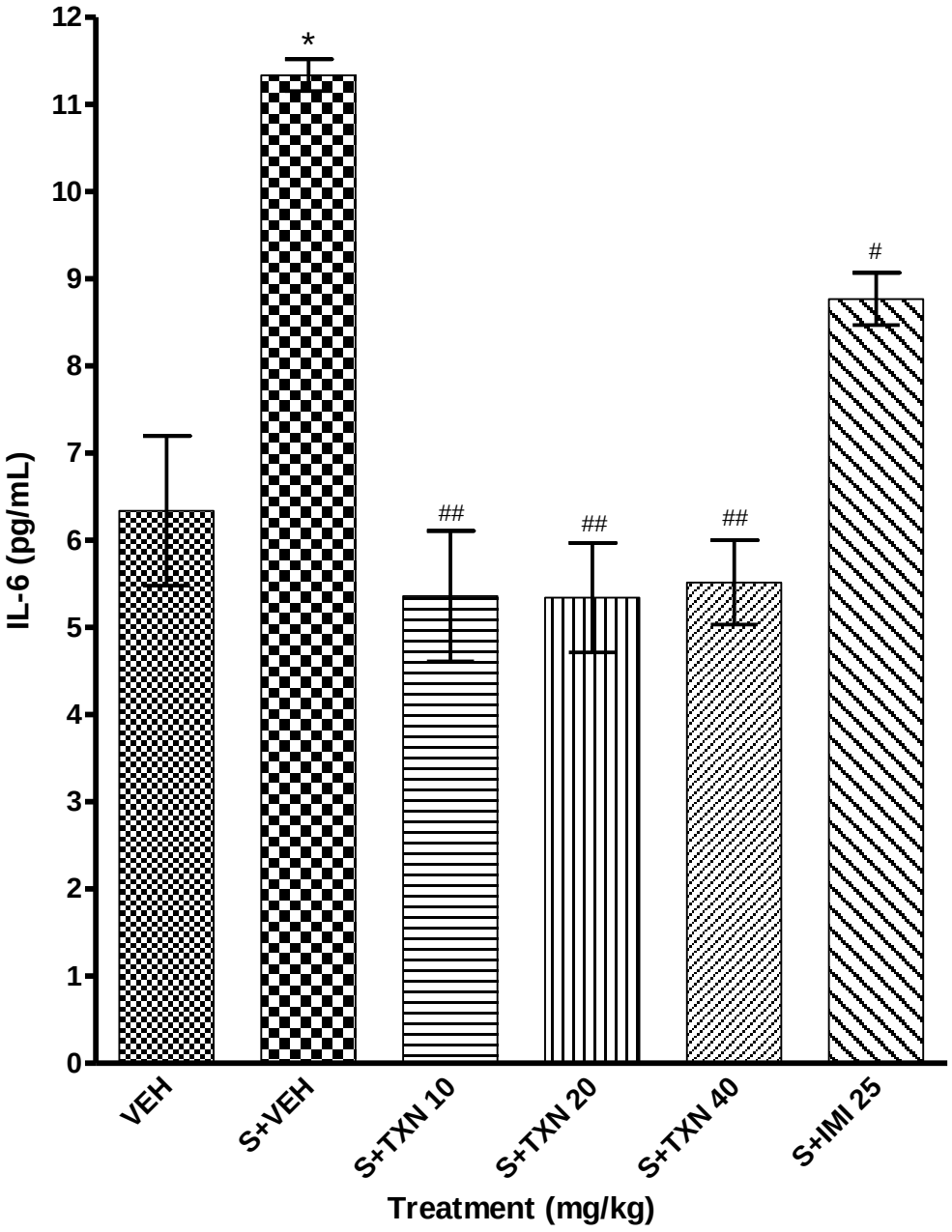

Figure 4.17: Effect of Troxerutin on IL-6 Levels in mice subjected to CUMS.

F (6, 29) = 17.50.*P < 0.01 compared to unstressed control. #P < 0.01 compared to stressed vehicle control. ##P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.20 Effect of Troxerutin on brain Mitogen-activated Protein Kinases (MAPKs) concentrations in CUMS

The animals subjected to stress in the CUMS paradigm showed significant difference in MAPKs levels when compared to unstressed groups, $p < 0.01$. The levels of MAPKs in animals treated with Troxerutin or Imipramine showed significant difference compared to vehicle treated animals subjected to stress, $p < 0.01$, Figure 4.18.

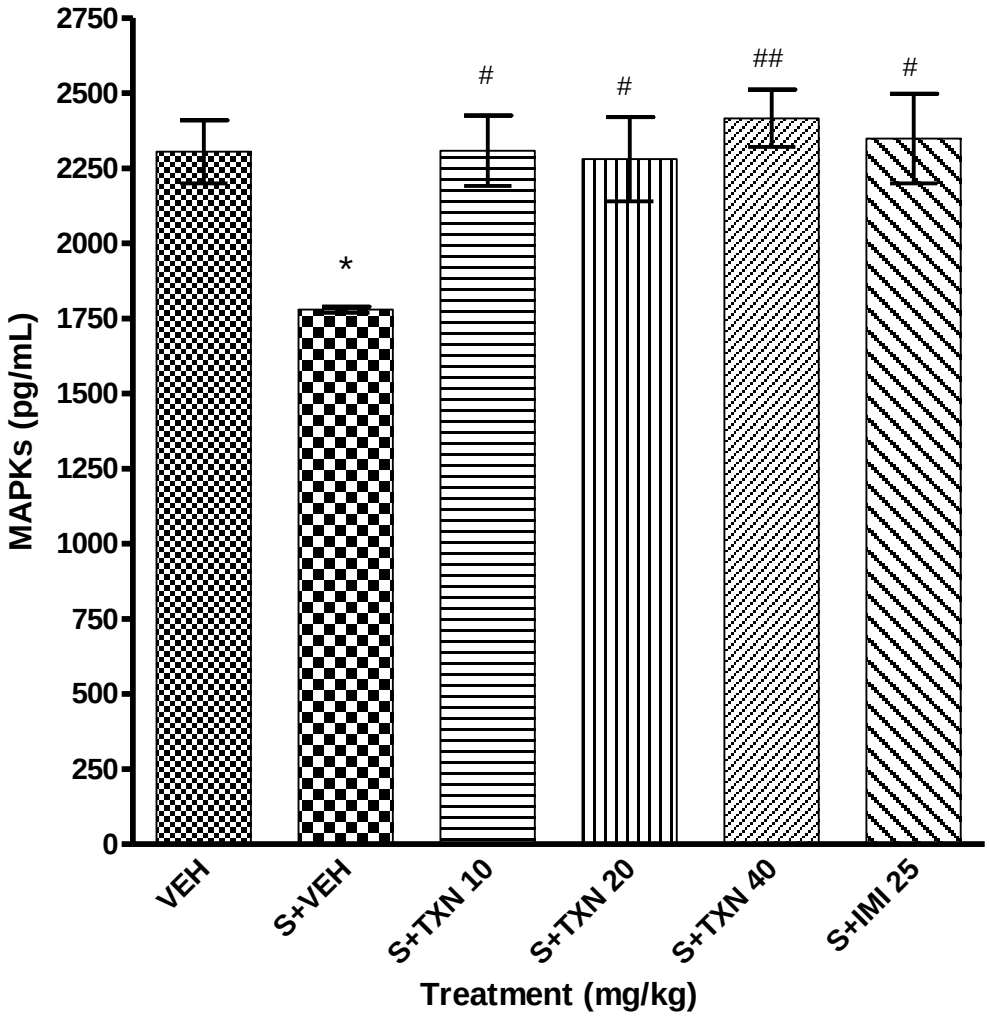

Figure 4.18: Effect of Troxerutin on MAPKs Levels in CUMS.

F (6, 29) = 16.05.*P < 0.01 compared to unstressed control. #P < 0.01 compared to stressed vehiclecontrol. ##P < 0.001 compared to stressed vehicle control.

(VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.21 Effect of Troxerutin on brain Acetylcholine Esterase (AChE) activity in CUMS

The animals subjected to stress in CUMS paradigm showed significant difference in AChE activity when compared to the unstressed groups, $p < 0.001$. The activity of AChE in animals treated with Troxerutin or Imipramine showed significant difference compared to the vehicle treated animals subjected to stress, $p < 0.001$, Figure 4.19.

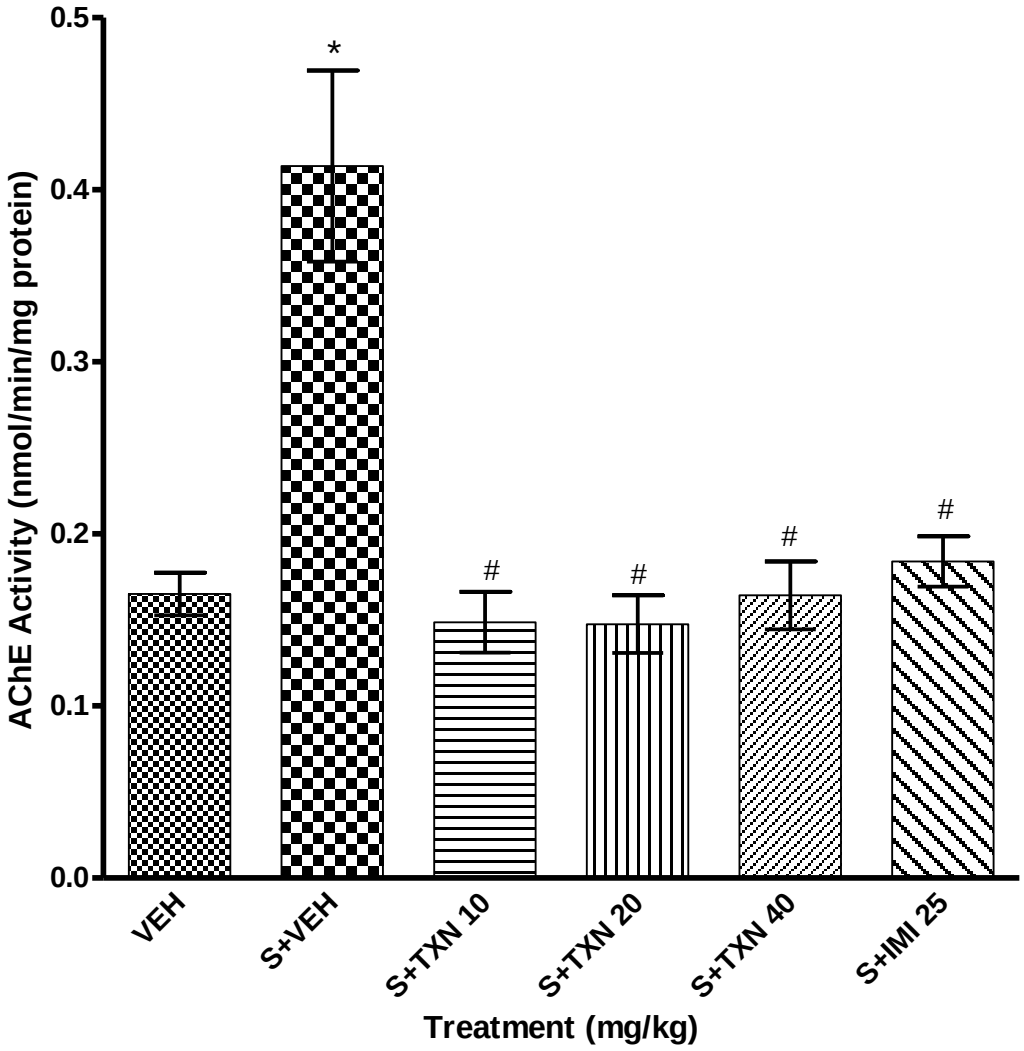

Figure 4.19: Effect of Troxerutin on AChE activity in CUMS.

F (6, 29) = 14.55. *P < 0.001 compared to non-sressed control. #P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine).

4.22 Effect of Troxerutin on Noradrenaline (NA) concentrations in CUMS

The animals subjected to stress in the CUMS paradigm showed significant difference in NA levels when compared to unstressed groups, $p < 0.05$. The levels of NA in animals treated with Troxerutin or Imipramine showed significant difference as compared to the vehicle treated animals subjected to stress, $p < 0.05$, Figure 4.20.

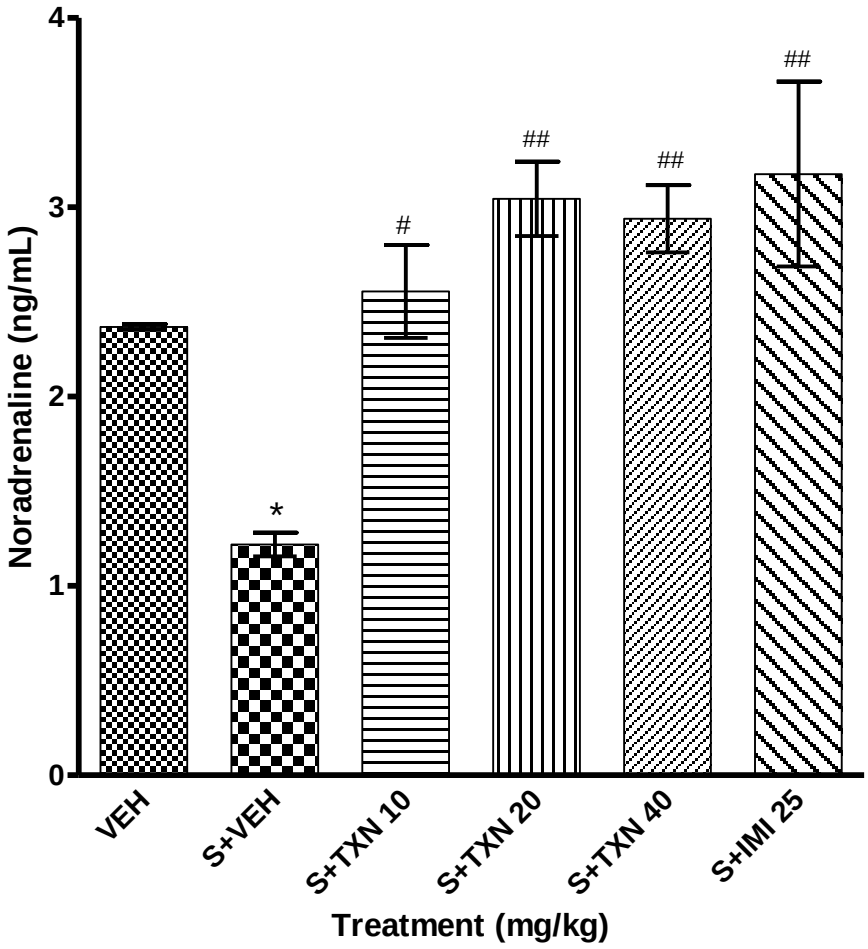

Figure 4.20: Effect of Troxerutin on NA Levels in CUMS.

F (6, 29) = 8.34. *P < 0.05 compared to unstressed control. #P < 0.05 compared to stressed vehicle control. ##P < 0.001 compared to stressed vehicle control.

(VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.23 Effect of Troxerutin on 5-Hydroxytryptamine (5-HT) concentrations in CUMS

The animals subjected to stress in the CUMS paradigm showed a significant difference in serotonin levels when compared to unstressed control, $p < 0.01$. The levels of 5-HT in animals treated with Troxerutin or Imipramine showed significant difference compared to the vehicle treated animals subjected to stress, $p < 0.01$, Figure 4.21.

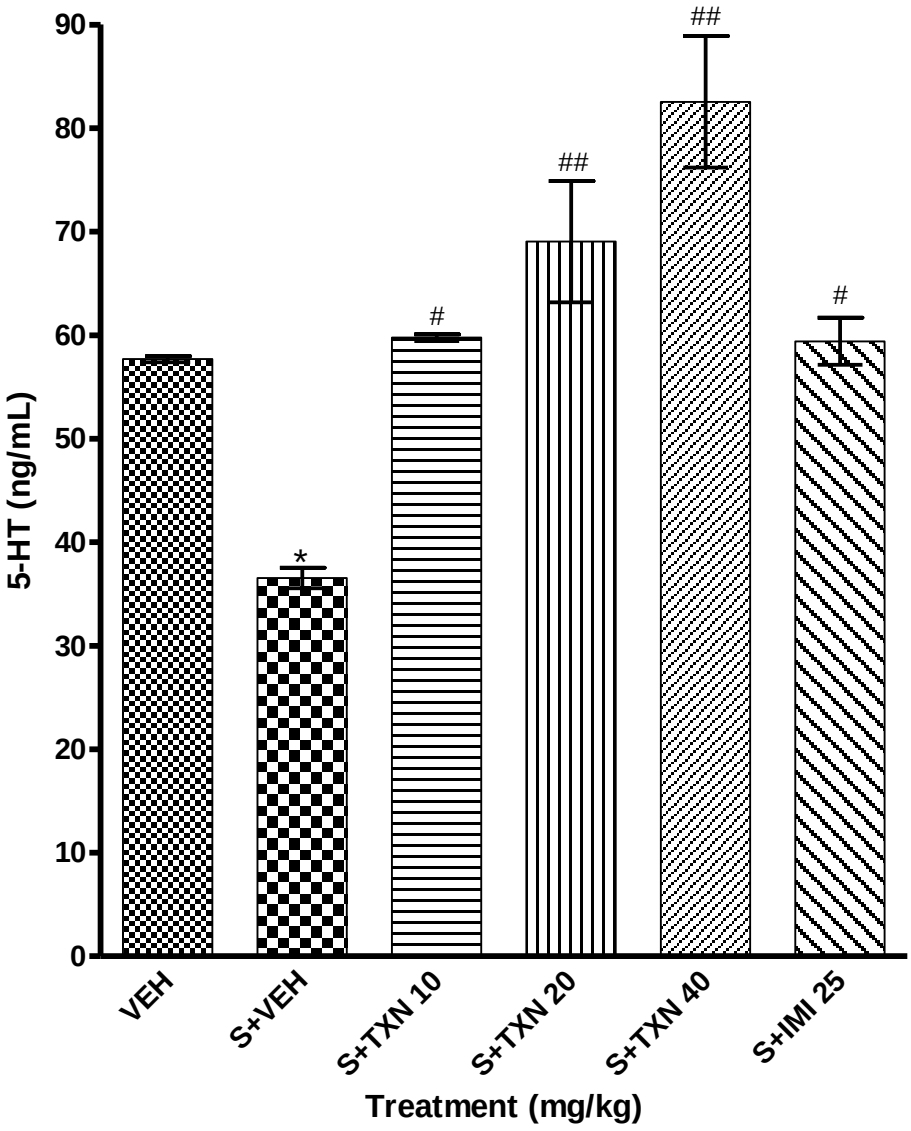

Figure 4.21: Effect of Troxerutin on Serotonin Levels in mice subjected to CUMS.
F (6, 29) = 16.89. *P < 0.01 compared to unstressed control. #P < 0.01 compared to stressed vehicle control. ##P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.24 Effect of Troxerutin on Dopamine (DA) concentrations in CUMS

The animals subjected to stress in the CUMS paradigm showed significant difference in DA levels when compared to unstressed groups, $p < 0.001$. The levels of DA in animals treated with Troxerutin or Imipramine showed significant difference compared tovehicle treated animals subjected to stress, $p < 0.001$, Figure 4.22.

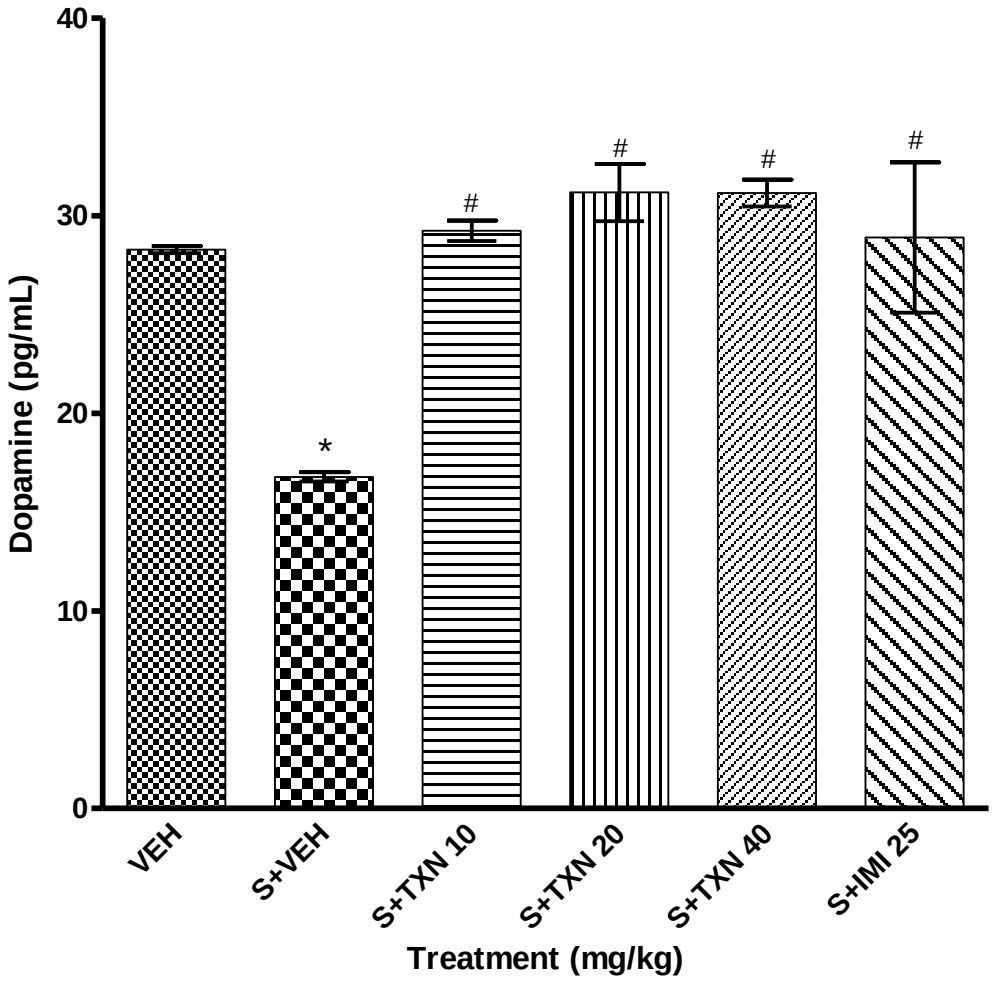

Figure 4.22: Effect of Troxerutin on DA Levels in mice subjected to CUMS.

F (6, 29) = 10.15. *P < 0.01 compared to unstressed control. #P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.25 Effect of Troxerutin on BDNF Concentrations in Mice subjected to CUMS

The animals subjected to stress in the CUMS paradigm showed significant difference in BDNF levels when compared to unstressed groups, $p < 0.01$. The levels of BDNF in animals treated with Troxerutin or Imipramine showed significant difference compared to vehicle treated animals subjected to stress, $p < 0.001$, Figure 4.23.

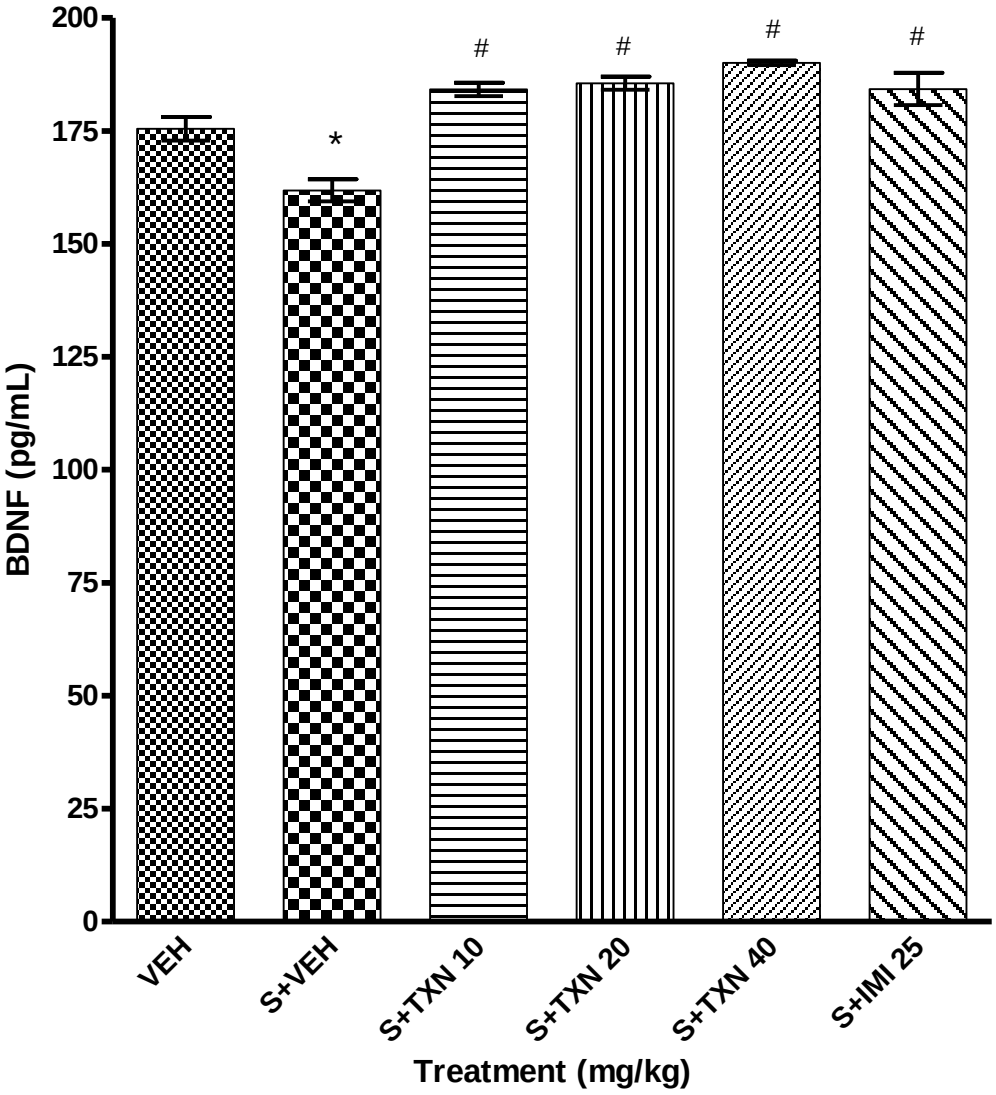

Figure 4.23: Effect of Troxerutin on BDNF Levels in mice subjected to CUMS.

F (6, 29) = 20.64. *P < 0.01 compared to unstressed control. #P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.26 Effect of Troxerutin on CREB Levels in Mice subjected to CUMS

The animals subjected to stress in the CUMS paradigm showed significant difference in CREB levels when compared to unstressed groups, $p < 0.01$. The levels of CREB in animals treated with Troxerutin or Imipramine showed significant difference compared to the vehicle treated animals subjected to stress, $p < 0.01$, Figure 4.24.

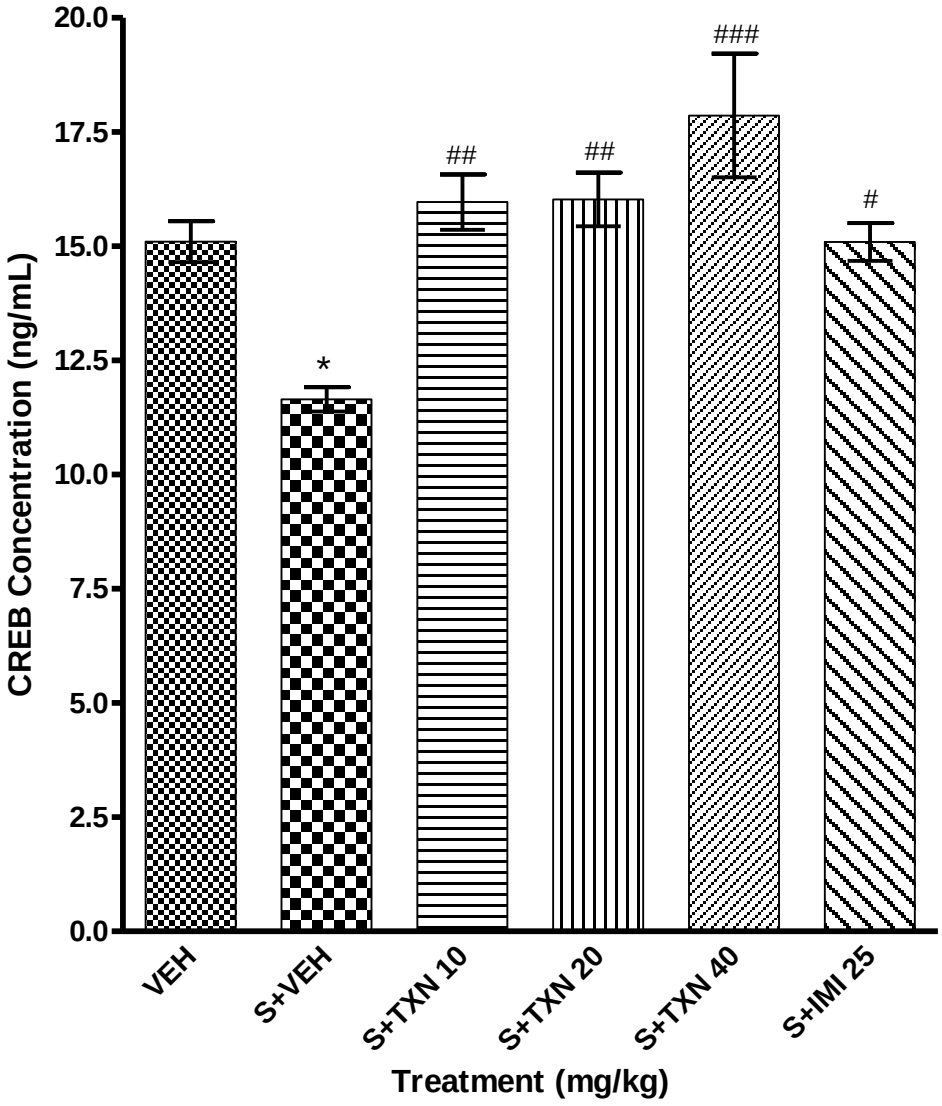

Figure 4.24: Effect of Troxerutin on CREB Levels in mice subjected to CUMS.

F (6, 29) = 8.40. *P < 0.01 compared to unstressed control. #P < 0.05 compared to stressed vehiclecontrol. ##P < 0.01 compared to stressed vehicle control. ###P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.27 Histological Assessment (H&E Staining) of Prefrontal Cortex of Mice in CUMS

The vehicle treated animals subjected to stress in the CUMS paradigm showed significant difference in neuronal density compared to Troxerutin groups, $p < 0.001$ (Figure 4.25). The histological examination shows preserved brain structure in animals treated with Troxerutin or Imipramine compared to vehicle stress controls with multiple foci neuronal lesions and vacuolated cells, Plate 4.1.

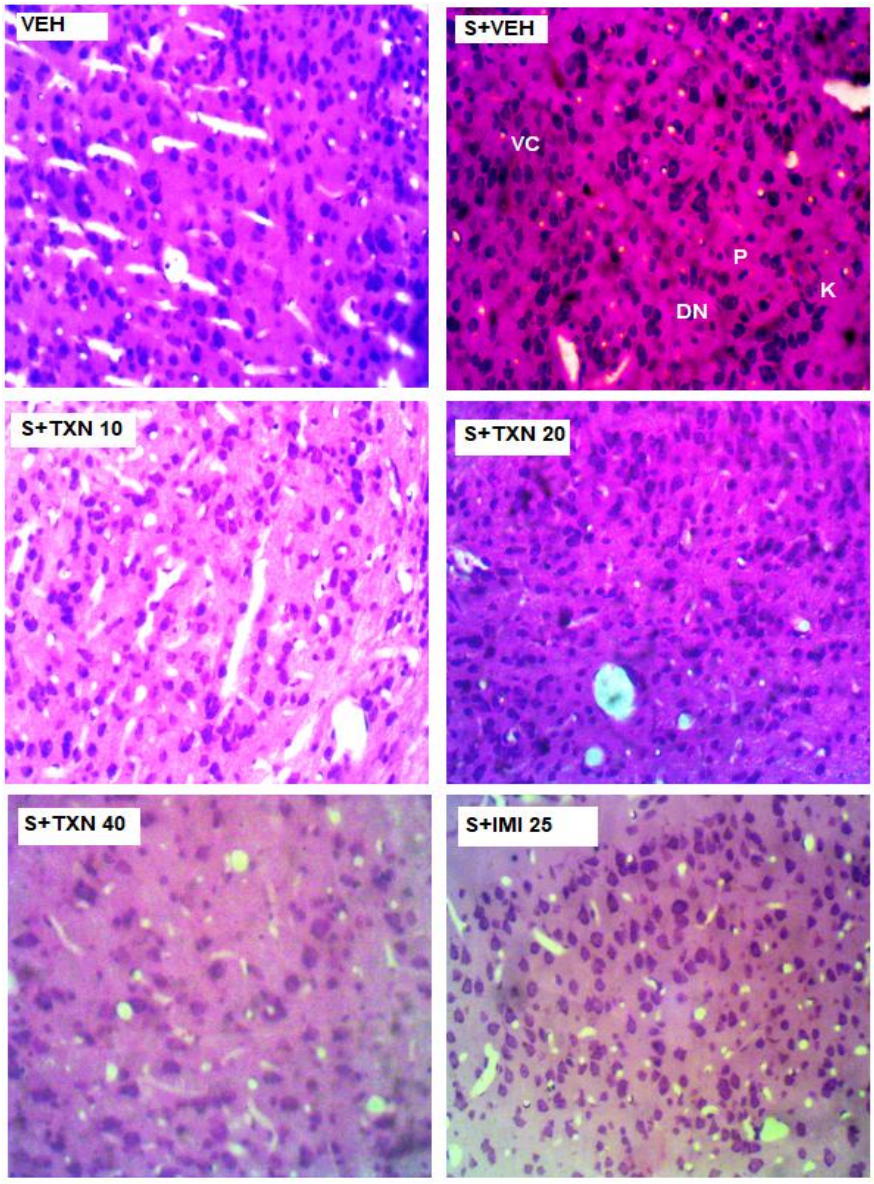

Plate 4.1: Photomicrographs of Layer 2/3 area of prefrontal cortex showing surviving neurons in mice subjected to CUMS (x400 magnification).

Neuronal density is shown in Figure 4.25 below. DN-degenerating neurons, P-pyknosis, VC-vacoulated cytoplasm, K-karyopyknosis. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

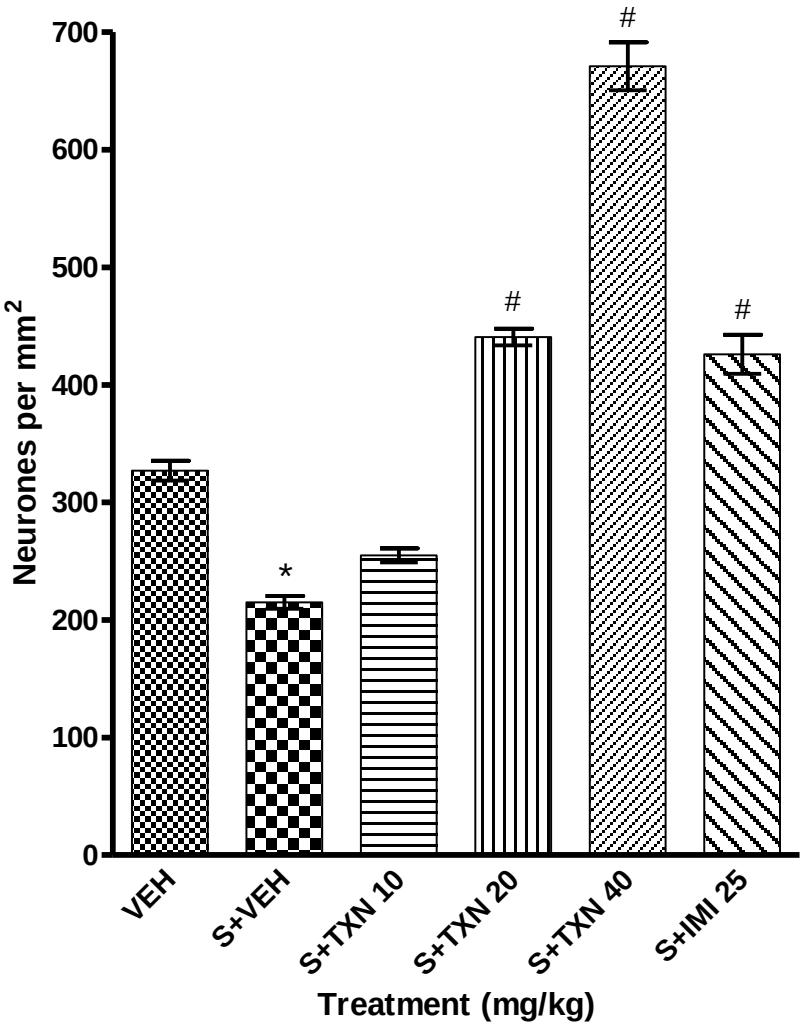

Figure 4.25: Neuronal density in prefrontal cortex of mice in CUMS.

*P < 0.001 compared to unstressed control. #P < 0.001 compared to stressed vehicle treated control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.28 Histological Assessment (H&E Staining) of the Hippocampus of Mice in CUMS

The vehicle treated animals subjected to stress in the CUMS paradigm showed significant difference in neuronal density compared to Troxerutin groups, $p < 0.001$ (Figure 4.26). The histological examination shows preserved brain structure in animals treated with Troxerutin or Imipramine compared to vehicle stress controls with multiple foci neuronal lesions and vacuolated cells, Plate 4.2

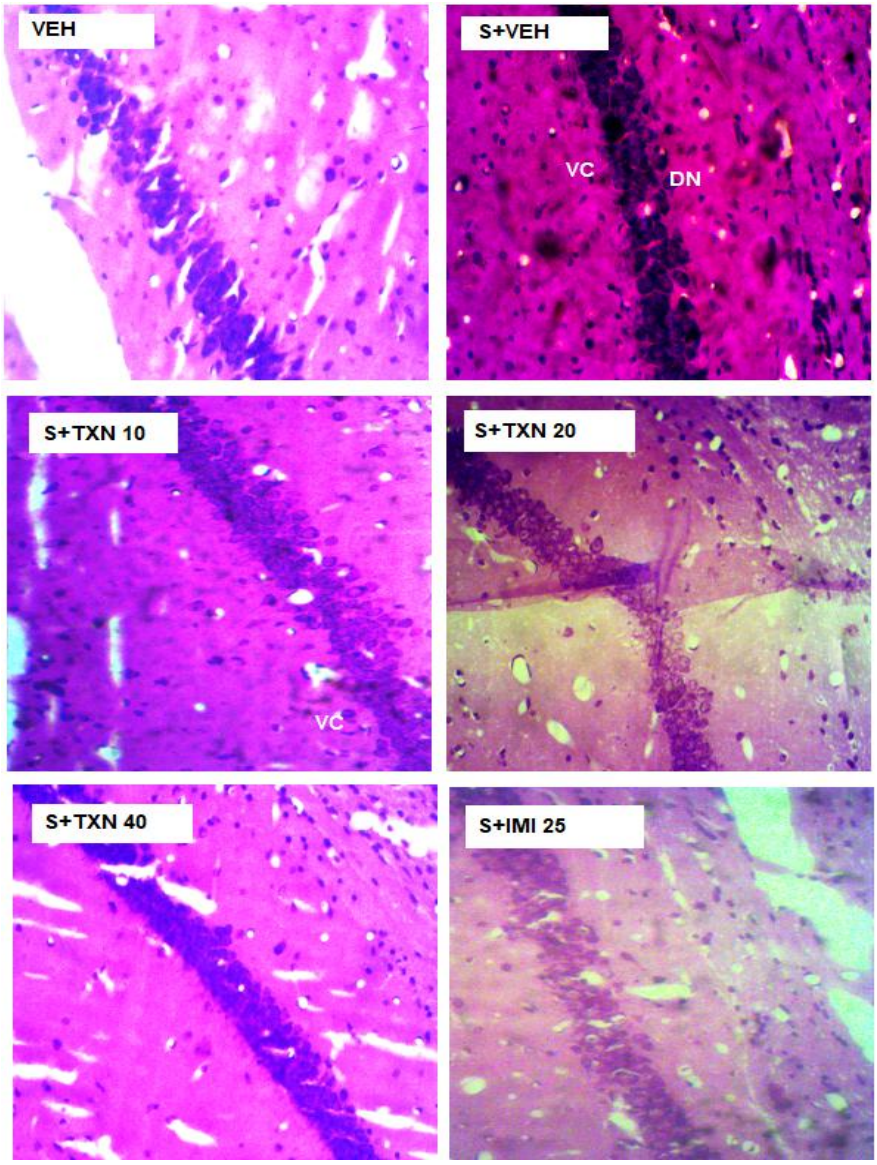

Plate 4.2: Photomicrographs of cornus ammonis region of hippocampus showing surviving neurons in CUMS (x400 magnification).

Neuronal density is shown in Figure 4.26 below. DN-degenerating neurons, VC-vacoulated cytoplasm. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

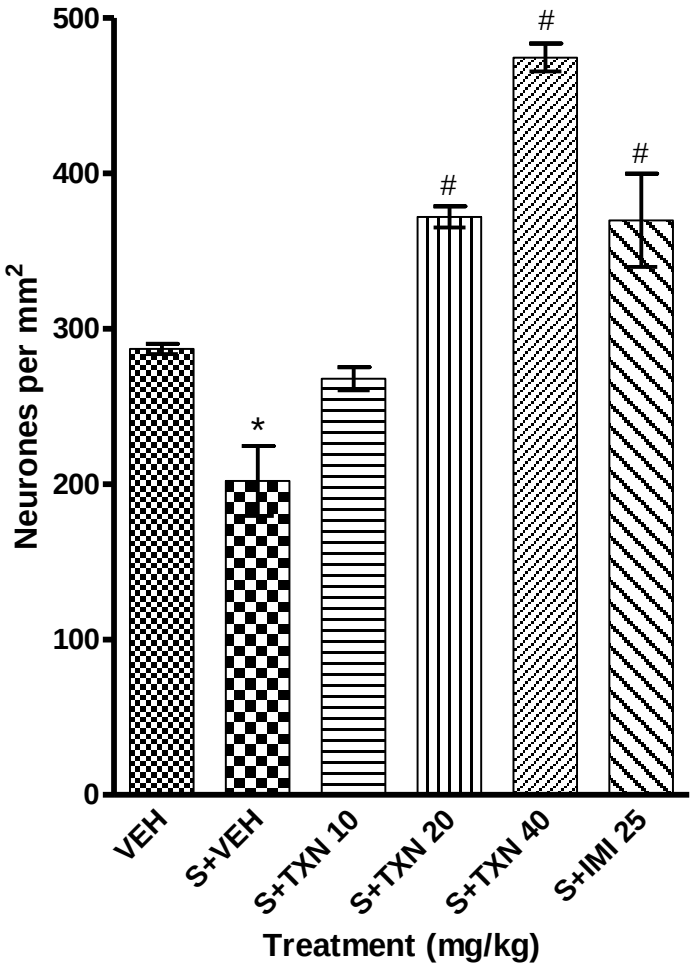

Figure 4.26: Neuronal density in hippocampus of mice subjected to CUMS.

*P < 0.001 compared to unstressed control. #P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.29 Immunohistochemistry of NF-κB Expression in Prefrontal Cortex of mice subjected to CUMS

The vehicle treated animals subjected to stress in the CUMS paradigm showed significant difference in density of NF-κB positive neurons compared to Troxerutin groups, $p < 0.001$ (Figure 4.27). The histological examination shows preserved brain structure in animals treated with Troxerutin or Imipramine compared to vehicle stress controls with multiple foci NF-κB positive neurons, Plate 4.3.

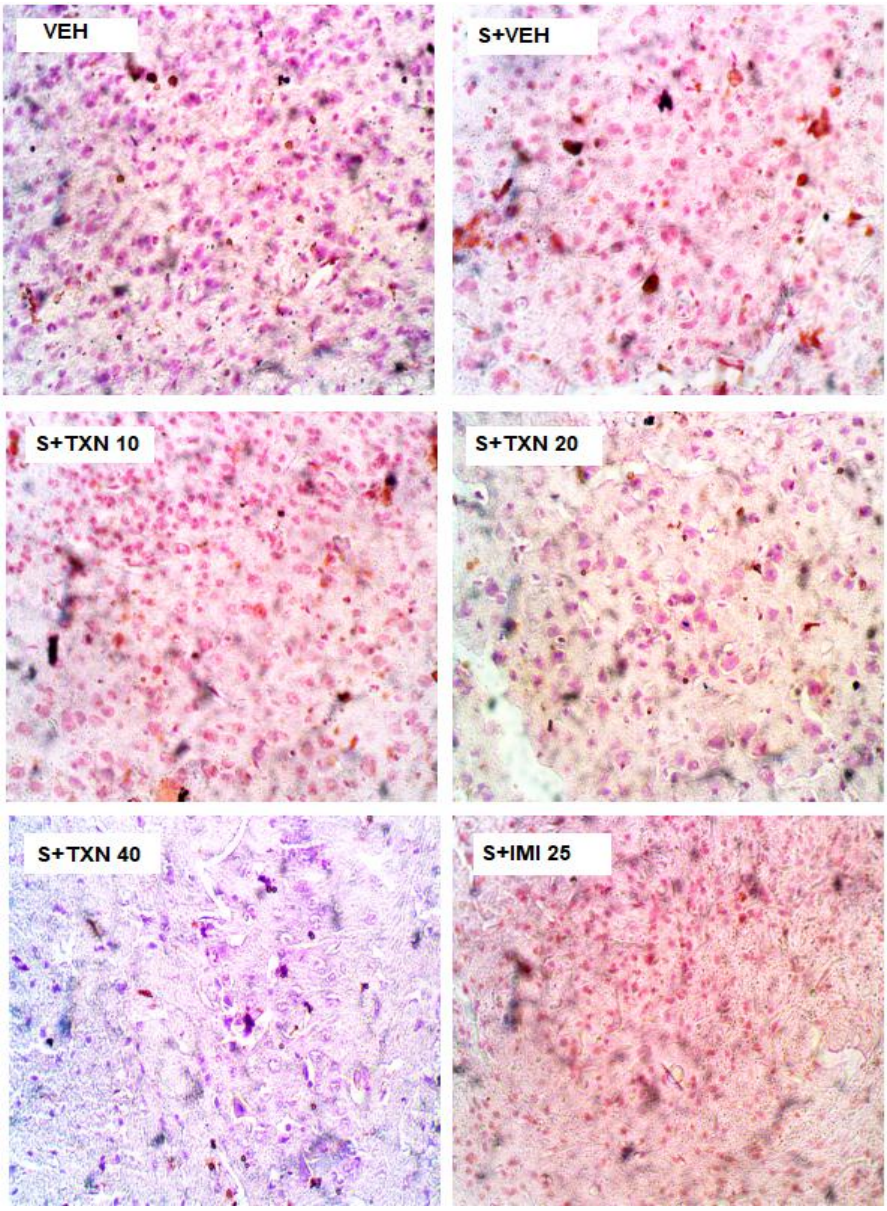

Plate 4.3: Photomicrographs of Layer 2/3 region of prefrontal cortex showing positive NF-kB stained cells in mice subjected to CUMS (x400 magnification).

Neuronal density is shown in Figure 4.27 below. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

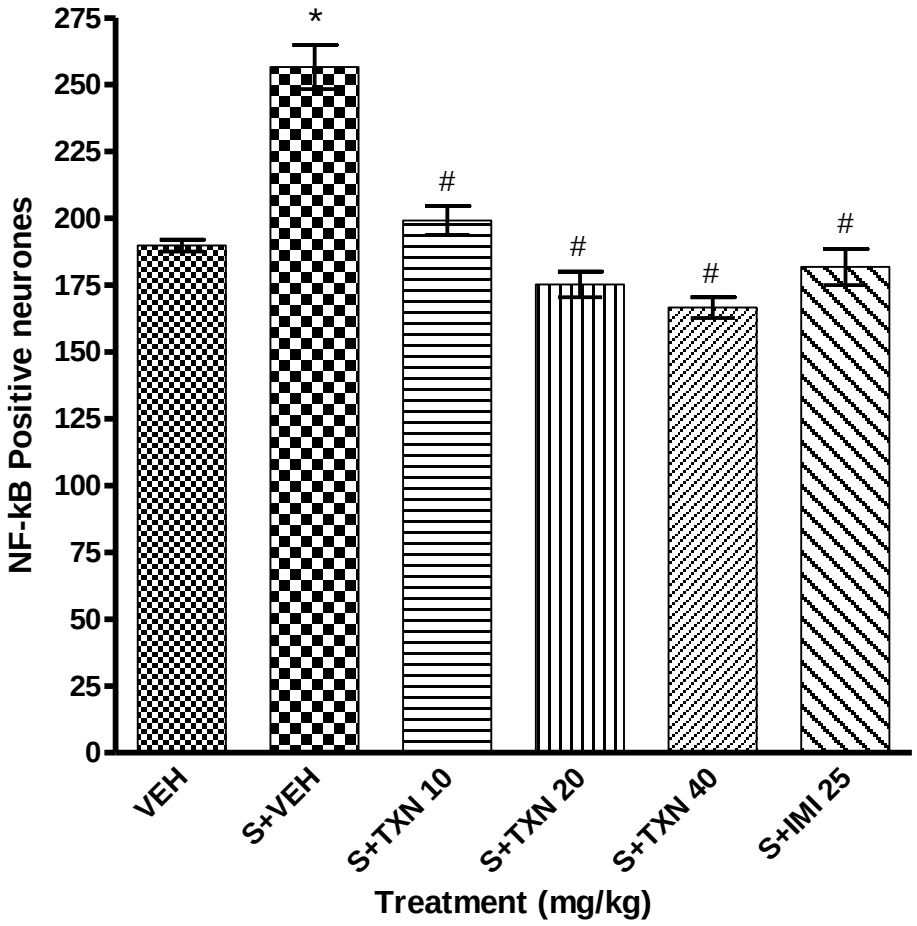

Figure 4.27: Neuronal density of NF-kB cells in prefrontal cortex of mice subjected to CUMS.

*P < 0.001 compared to unstressed control. #P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.30 Immunohistochemistry of NF-κB Expression in Hippocampus of mice subjected to CUMS

The vehicle treated animals subjected to stress in the CUMS paradigm showed significant difference in density of NF-κB positive neurons compared to Troxerutin groups, $p < 0.01$ (Figure 4.28). The histological examination shows preserved brain structure in animals treated with Troxerutin or Imipramine compared to vehicle stress controls with multiple foci NF-κB positive neurons, Plate 4.4.

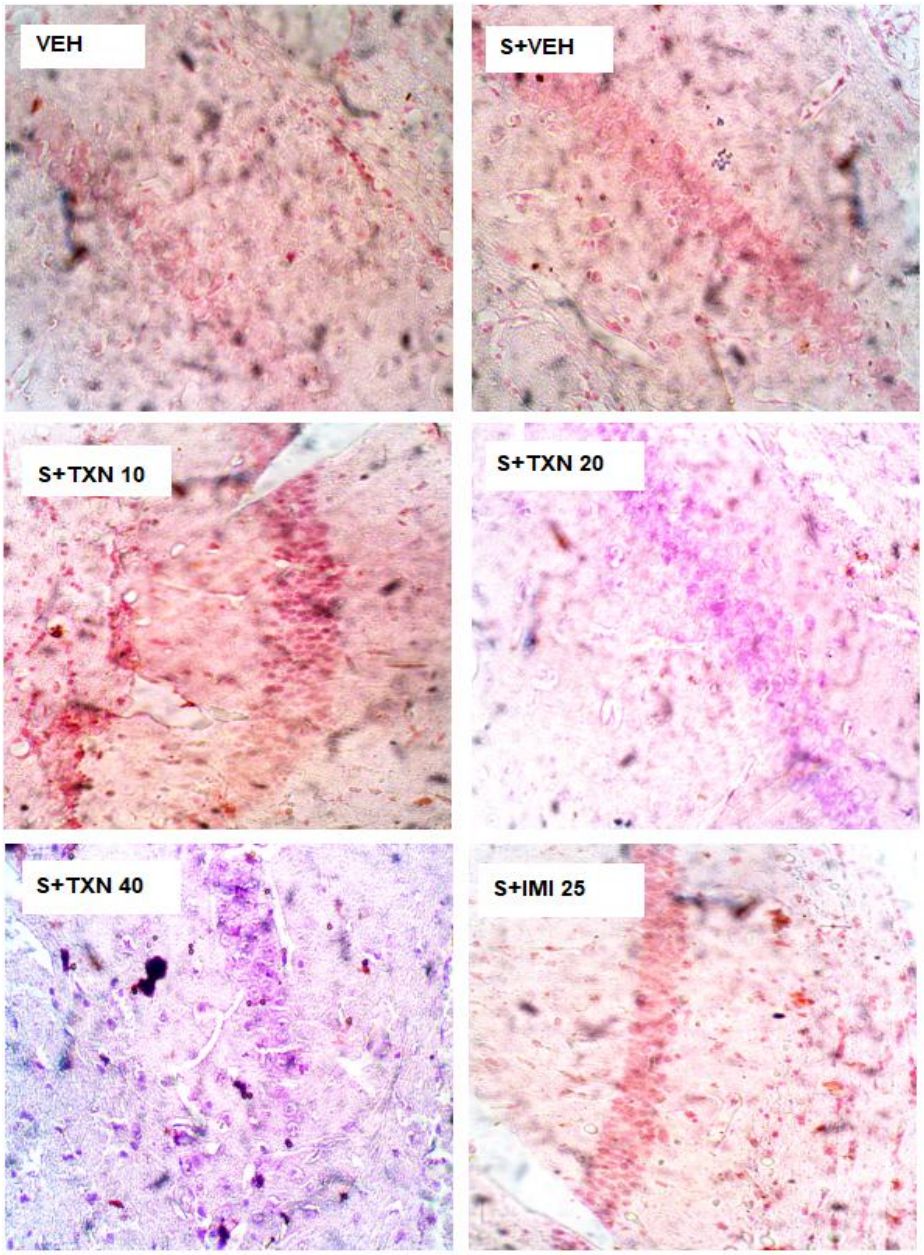

Plate 4.4: Photomicrographs of cornus ammonis region of Hippocampus showing positive NF-kB stained cells in mice subjected to CUMS (x400 magnification).

Neuronal density is shown in Figure 4.28 below. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

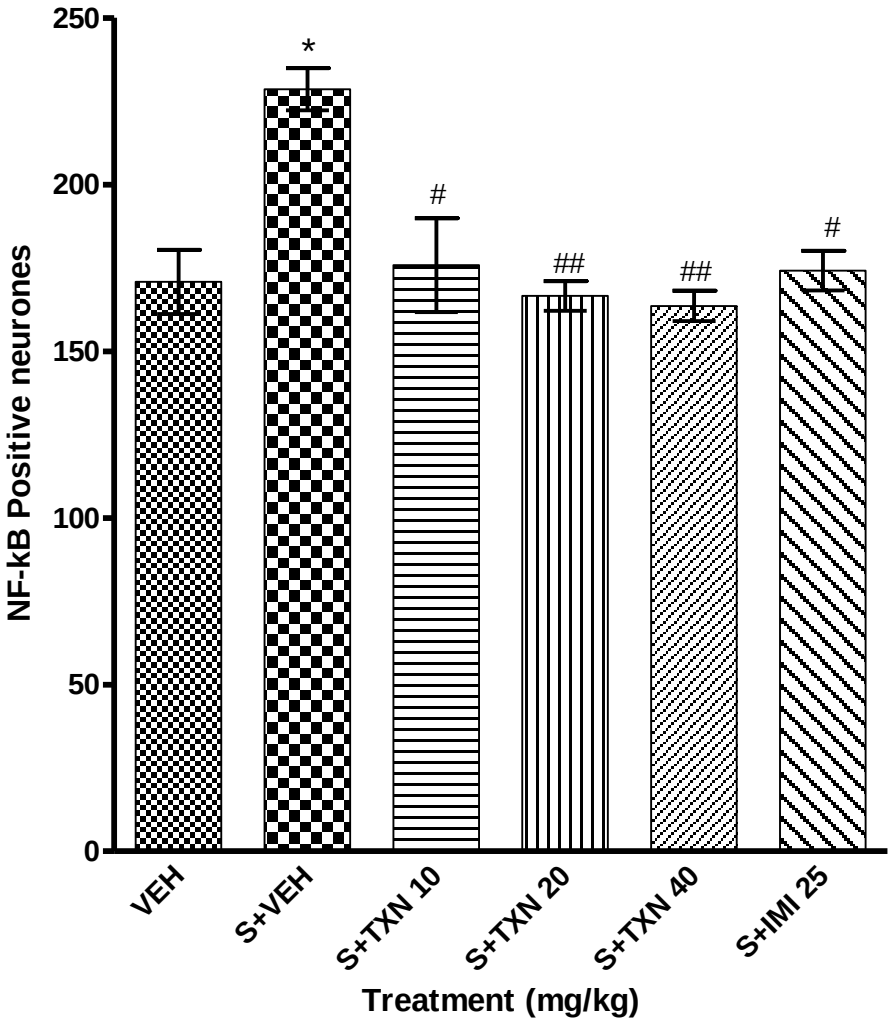

Figure 4.28: Neuronal density of NF-kB positive cells in hippocampus of mice in CUMS.

*P < 0.001 compared to unstressed control. #P < 0.01, ##P < 0.001 compared to stressed vehicle control. (VEH- Vehicle, S-stress, TXN- Troxerutin, IMI- Imipramine)

4.31 Effect of Troxerutin (TXN) on Novelty Suppressed Feeding in Mice Exposed to LPS

The results of latency to feed and the feed consumption were presented on Table 4.3 and Table 4.4 respectively. Exposure to LPS significantly increased latency to feed in vehicle-treated mice compared to LPS-free group, however, the latency was significantly reduced [$F(5, 24) = 51.71$, $p = 0.4848$] by Troxerutin (10, 20 and 40 mg/kg, i.p) and Imipramine (25mg/kg, i.p), $p<0.001$. The feed consumption was significantly reduced in vehicle-treated mice exposed to LPS compared to LPS-free group, however, the consumption was significantly increased [$F(6, 29) = 3.80$, $p = 0.011$] by Troxerutin and Imipramine, $p<0.001$.

Table 4.3: Latency to Feed in Novelty Suppressed Feeding

Treatment	Latency to Feed (sec)
VEH	99.4±5.99
LPS+VEH	216±4.94*
LPS+TXN (10 mg/kg)	101.4±6.99#
LPS+TXN (20 mg/kg)	100±3.06#
LPS+TXN (40 mg/kg)	99.6±7.03#
LPS+IMI (25 mg/kg)	106.4±9.32#

*P < 0.001 compared to vehicle-treated LPS-free group. #P < 0.001 compared to vehicle-treated LPS-exposed group

Table 4.4: Consumption in Novelty Suppressed Feeding

Treatment	Consumption (g)
VEH	1.24±0.1691
LPS+VEH	0.32±0.037*
LPS+TXN (10 mg/kg)	1.14±0.1833#
LPS+TXN (20 mg/kg)	1.2±0.1871#
LPS+TXN (40 mg/kg)	1.14±0.2337#
LPS+IMI (25 mg/kg)	1.28±0.2354#

*P< 0.05 compared to vehicle-treated LPS-free group. #P < 0.05 compared to vehicle-treated LPS-exposed group.

4.32 Effect of Troxerutin on FST in mice Exposed to LPS

The vehicle-treated LPS-exposed animals in the LPS-induced depression paradigm exhibits characteristic increase in immobility period compared to the control that was not exposed to LPS, whereas the troxerutin-treated and imipramine-treated animals significantly reduced immobility compared to this LPS-exposed vehicle-treated controls, $P < 0.001$, represented on Figure 4.29.

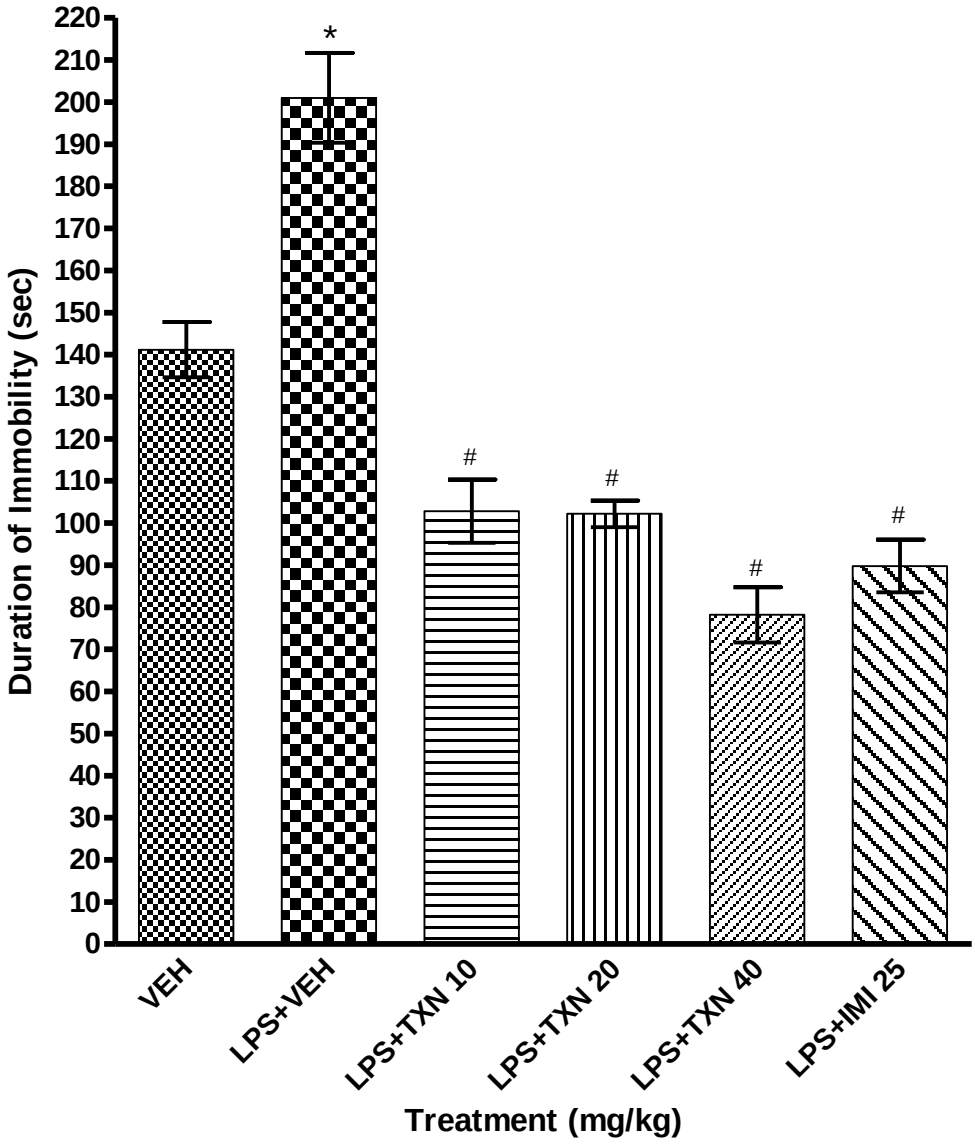

Figure 4.29: Effect of Troxerutin on immobility time in FST post-LPS exposure.

F (6, 29) = 40.43. *P < 0.001 compared to vehicle-treated LPS-free group. #P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.33 Effect of Troxerutin (TXN) on Locomotor Activity in OFT in LPS

Vehicle-treated mice exposed to LPS exhibited characteristic reduction in locomotor activity, $p<0.001$ compared to LPS-free control ands was reversed by Troxerutin and Imipramine, $F(6, 29) = 15.82$. The reversal was not different from the LPS-free control animals in open field, $p > 0.05$, Figure 4.30.

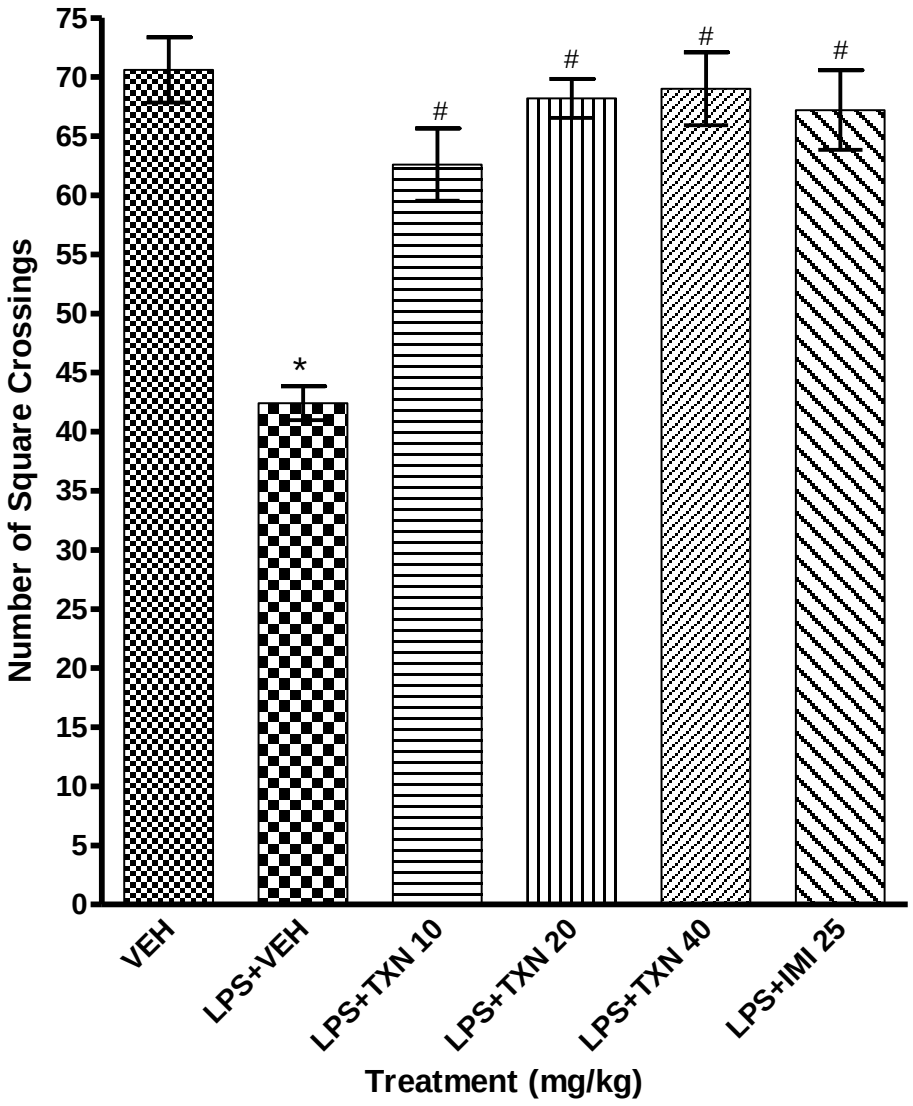

Figure 4.30: Effect of Troxerutin (TXN) on locomotor activity of mice exposed to LPS in open field.

*P < 0.001 compared to vehicle-treated LPS-free group. #P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.34 Effect of Troxerutin on reduced Glutathione levels in Mice Exposed to LPS

The levels of reduced glutathione showed significant difference comparatively between the vehicle-treated group that received LPS and the group that did not, $p < 0.001$, and the treatment with Troxerutin and Imipramine also showed significant difference compared to vehicle-treated LPS-exposed animals, $p<0.001$, Figure 4.31.

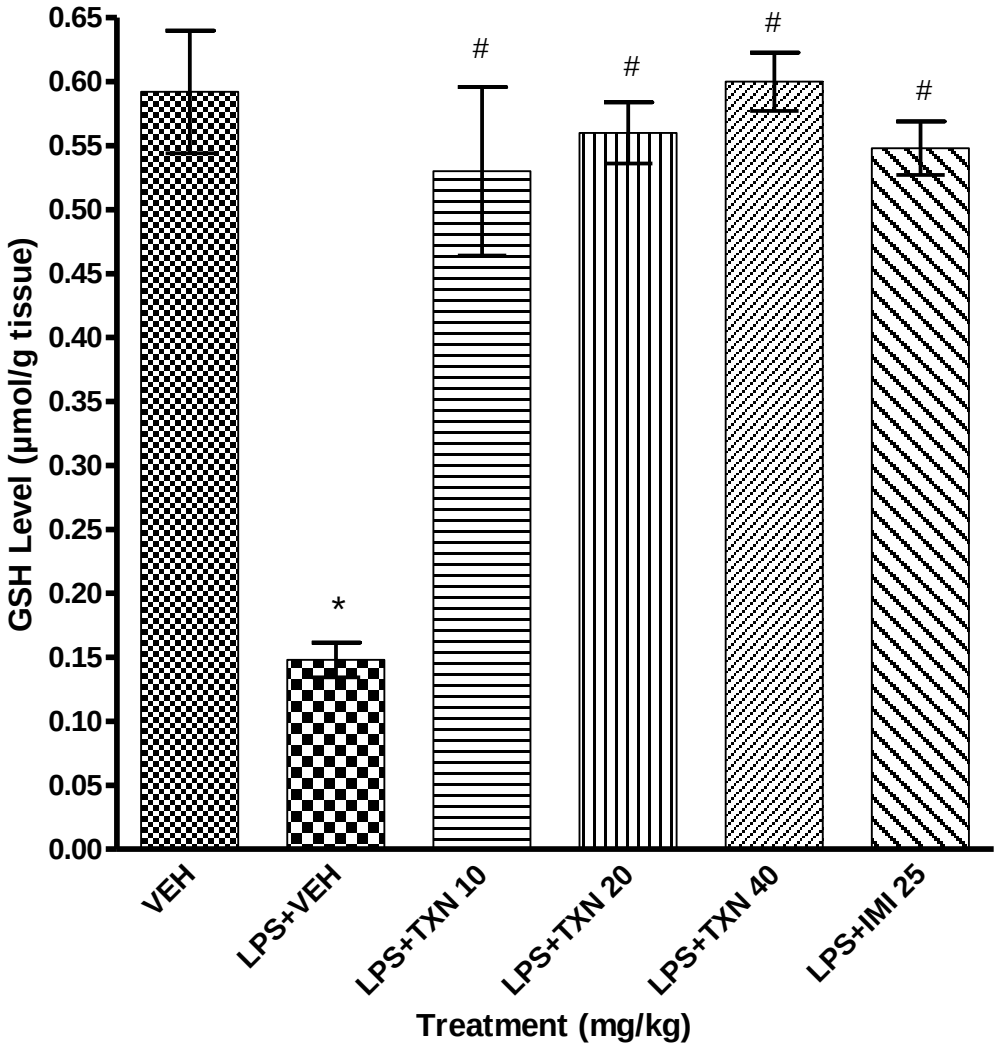

Figure 4.31: Effect of Troxerutin on GSH Levels in mice exposed to LPS.

F (6, 29) = 21.45. *P < 0.001 compared to vehicle-treated LPS-free group. #P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.35 Effect of Troxerutin on Malondialdehyde levels in LPS

In the vehicle-treated groups, LPS-exposed mice in the LPS-induced depression paradigm showed significant difference in Malondialdehyde levels compared to the LPS-free control, $P< 0.001$. The levels of Malondialdehyde in LPS-exposed animals treated with Troxerutin or Imipramine showed significant difference compared to vehicle-treated animals exposed to LPS, $p < 0.001$, Figure 4.32.

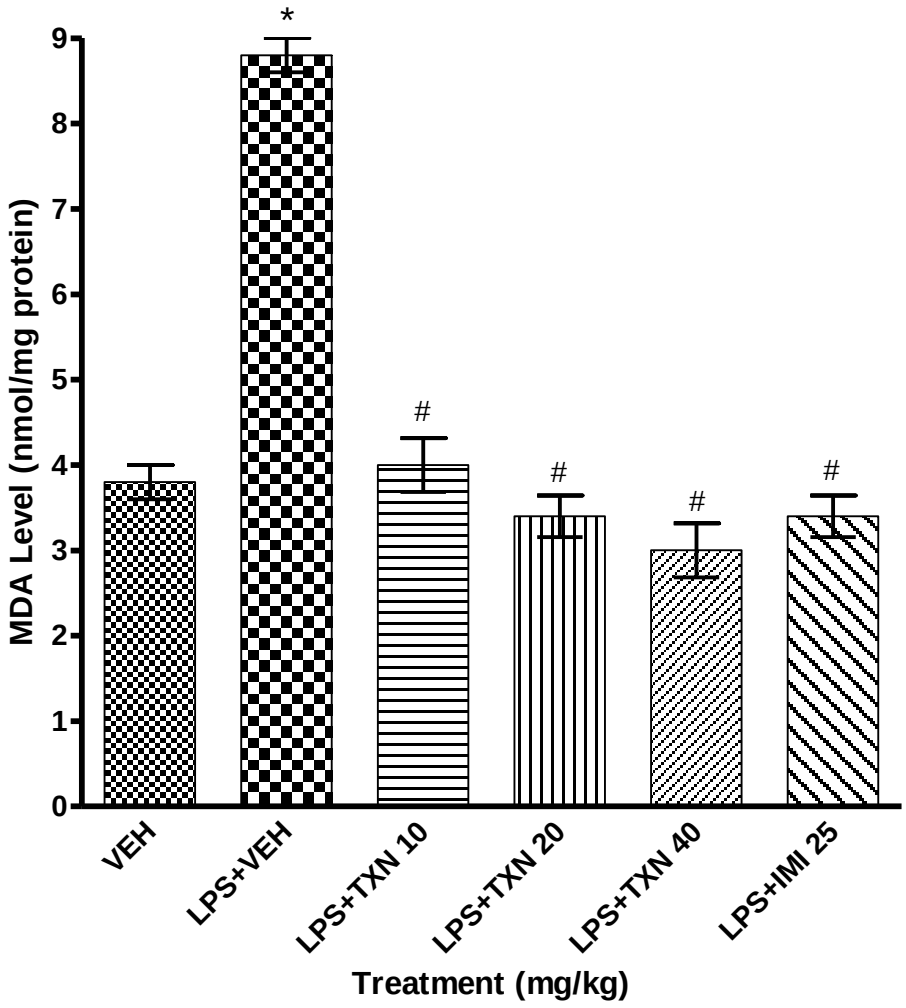

Figure 4.32: Effect of Troxerutin on MDA Levels in mice exposed to LPS.

F (6, 29) = 71.52. *P<0.001 compared to vehicle-treated LPS-free group. #P<0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.36 Effect of Troxerutin on Nitrite Levels in LPS

In the LPS-induced depression paradigm, there was significant difference in Nitrite levels in the vehicle-treated groups, animals exposed to LPS and LPS-free group, $p < 0.001$. The levels of Nitrite in LPS-exposed Troxerutin or Imipramine group showed significant difference compared to vehicle treated animals exposed to LPS, $p < 0.001$, Figure 4.33.

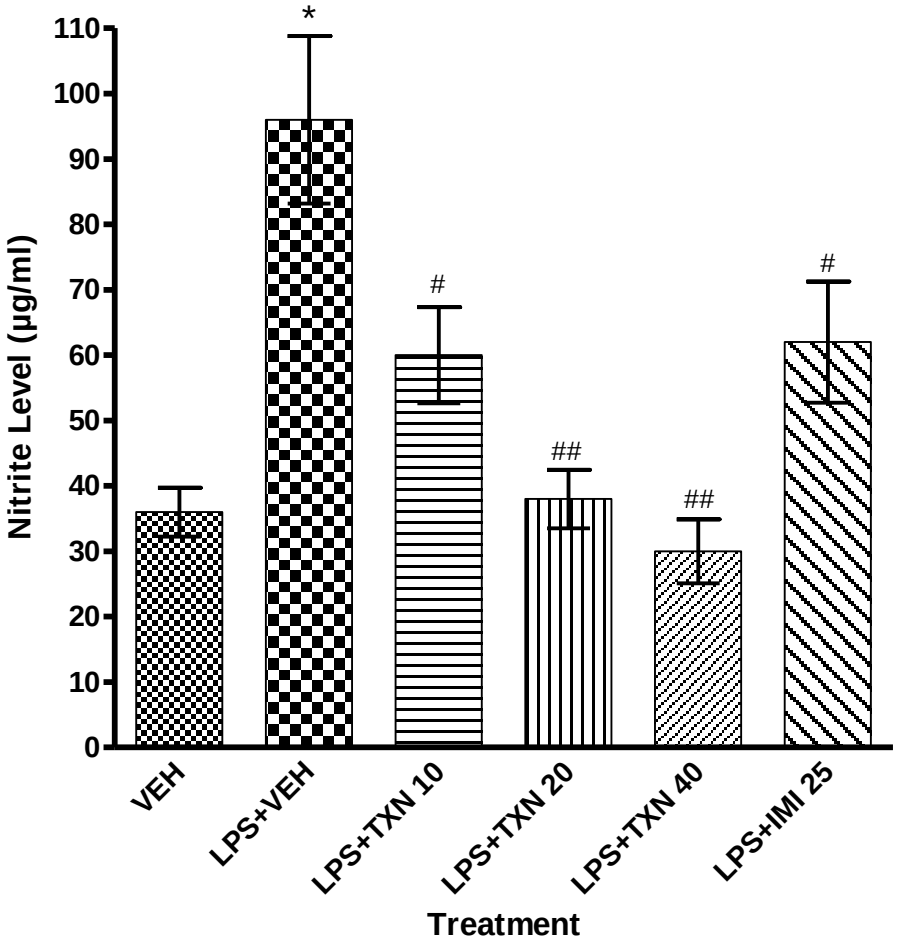

Figure 4.33: Effect of Troxerutin on Nitrite Levels in mice exposed to LPS.

F (6, 29) = 10.01. *P < 0.001 compared to LPS-free group. #P < 0.05,##P < 0.001 compared to vehicle LPS-exposed group. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN-Troxerutin, IMI- Imipramine)

4.37 Effect of Troxerutin on SOD levels in LPS

In the vehicle-treated groups, LPS-exposed group in the LPS-induced depression paradigm showed significant difference in SOD levels compared to LPS-free group, $p < 0.001$. The levels of SOD in LPS-exposed Troxerutin or Imipramine group showed significant difference compared to vehicle control exposed to LPS, $p < 0.001$, Figure 4.34.

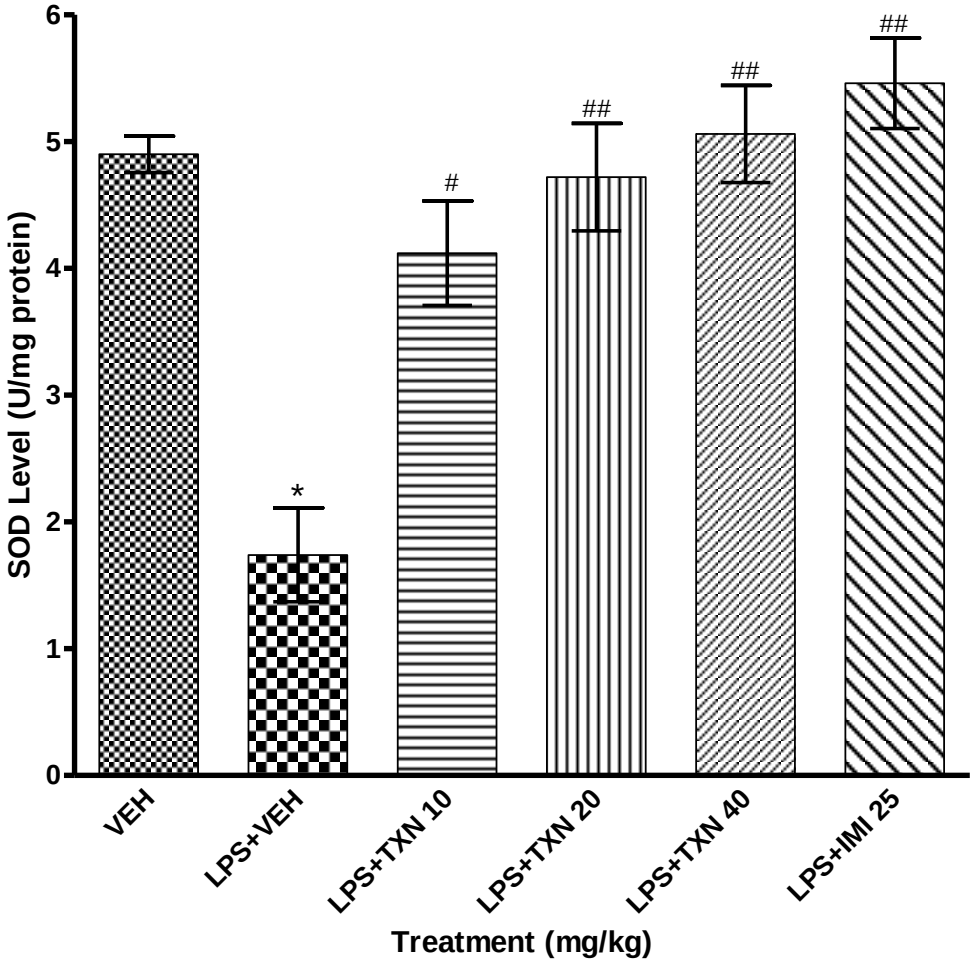

Figure 4.34: Effect of Troxerutin on SOD Levels in mice subjected to LPS.

F (6, 29) = 13.86. *P < 0.001 compared to vehicle-treated LPS-free group. #P < 0.01 compared to vehicle-treated LPS-exposed group. ##P < 0.001 compared to vehicle-treated LPS-exposed group (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI-Imipramine)

4.38 Effect of Troxerutin on serum Corticosterone levels in LPS

In the vehicle-treated groups, the animals exposed to LPS in the LPS-induced depression paradigm showed a significant difference in Corticosterone levels compared to LPS-free group, $p < 0.001$. The levels of Corticosterone in LPS-exposed in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated cotrols exposed to LPS, $p < 0.001$, Figure 4.35.

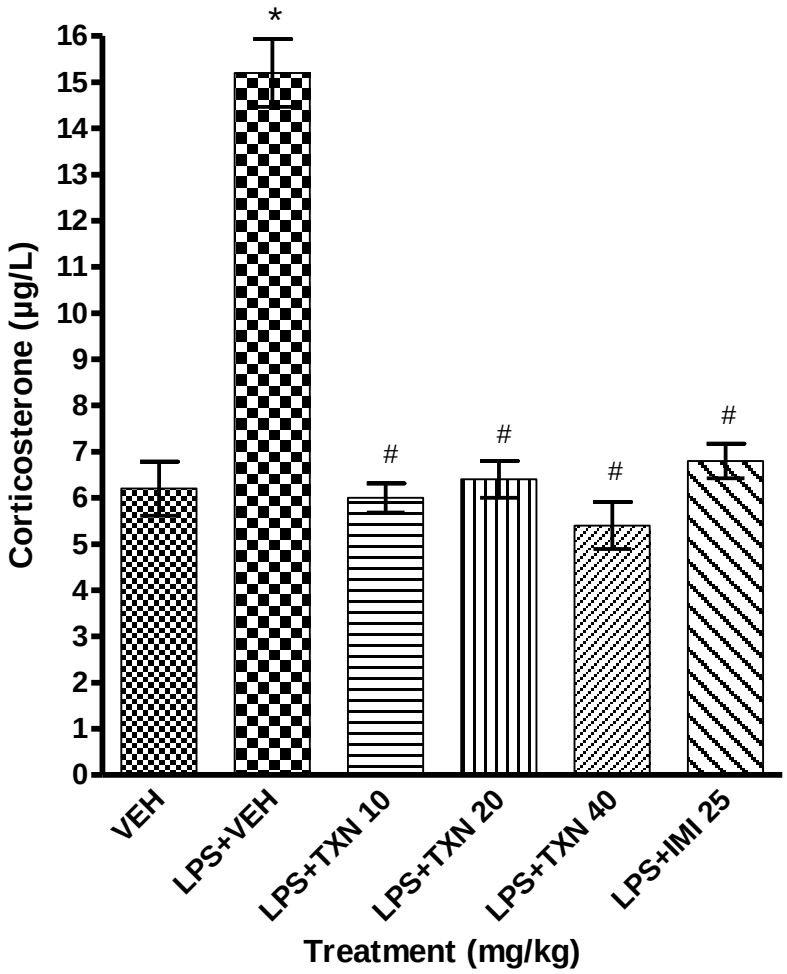

Figure 4.35: Effect of Troxerutin on Corticosterone Levels in mice exposed to LPS.
F (6, 29) = 53.90. *P < 0.001 compared to vehicle-treated LPS-free group. #P < 0.001 compared to vehicle-treated LPS-exposed group (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.39 Effect of Troxerutin on brain TNF-α Levels in LPS

In the vehicle-treated groups, controls exposed to LPS in the LPS-induced depression paradigm showed significant difference in TNF-α level compared to LPS-free group, $p < 0.001$. The levels of TNF-α in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated controls exposed to LPS, $p < 0.001$, Figure 4.36.

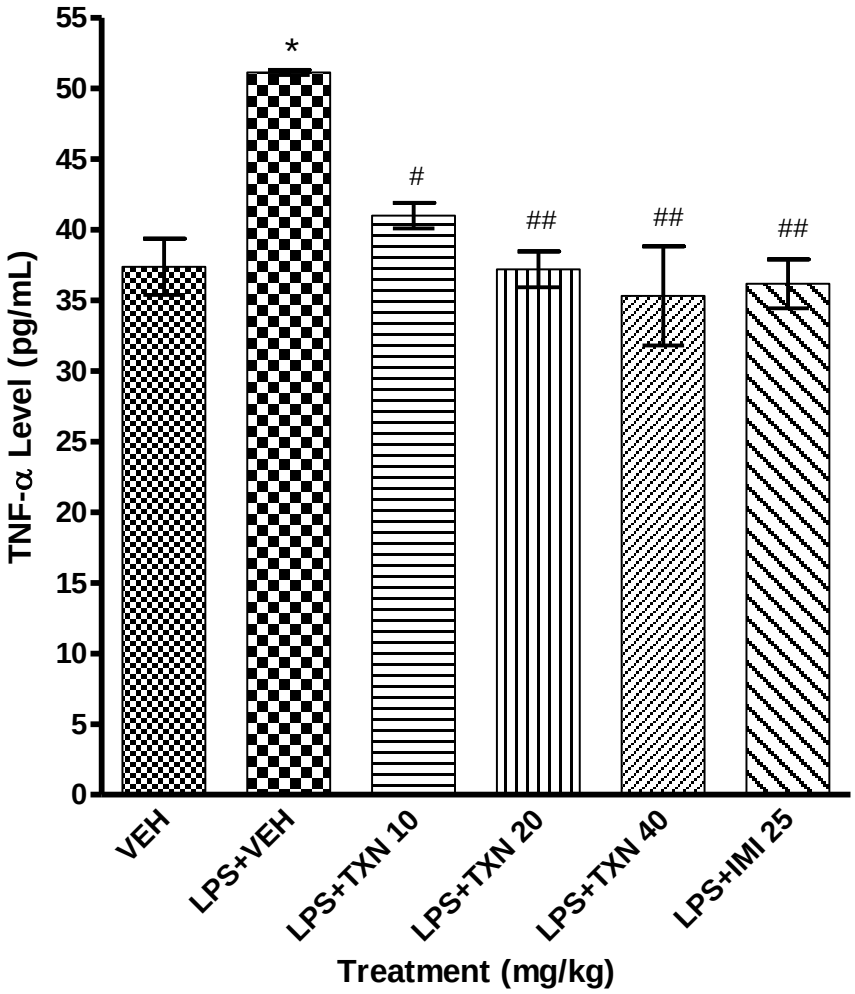

Figure 4.36: Effect of Troxerutin on TNF-α Levels in mice exposed to LPS.

F (6, 29) = 9.74. *P < 0.001 compared to vehicle-treated LPS-free group, #P < 0.05 compared to vehicle-treated LPS-exposed group, ##P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI-Imipramine)

4.40 Effect of Troxerutin on brain Interferon-γ level in LPS

In the vehicle-treated groups, animals exposed to LPS in the LPS-induced depression paradigm showed significant difference in IFN-γ level compared to LPS-free group, $p < 0.001$. The levels of IFN-γ in Troxerutin or Imipramine groups showed significant difference compared to the vehicle treated controls exposed to LPS, $p < 0.001$, Figure 4.37.

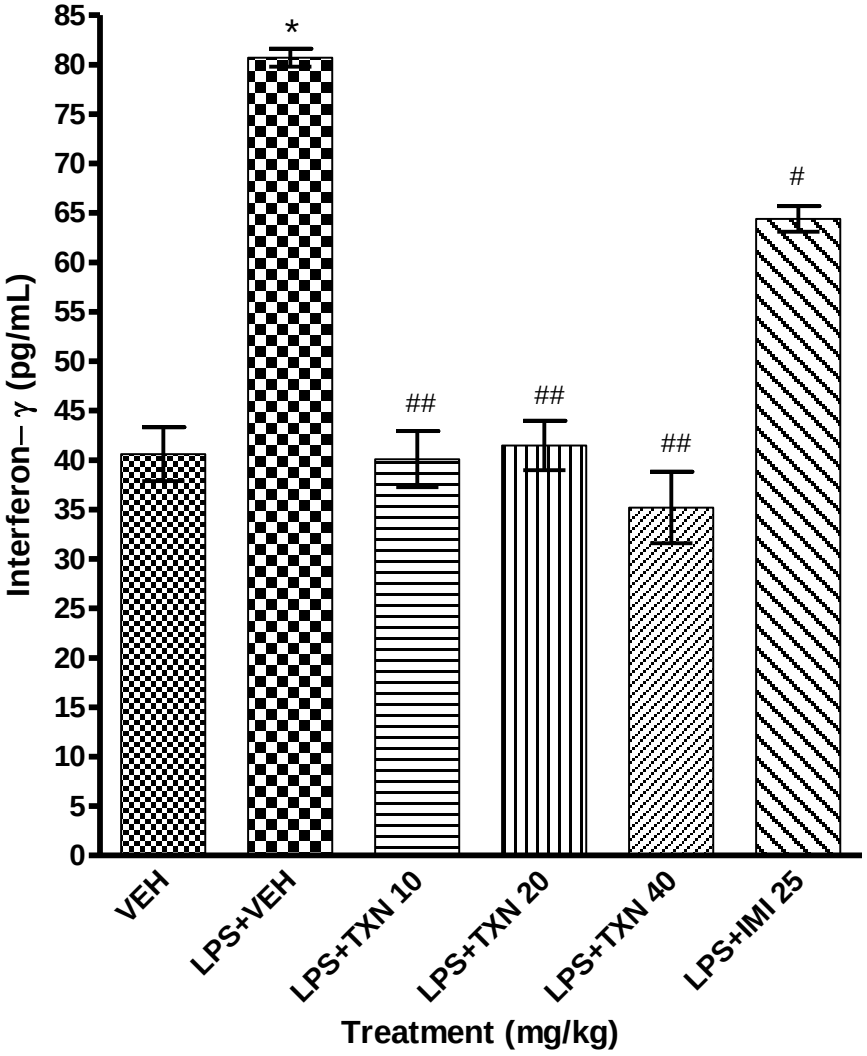

Figure 4.37: Effect of Troxerutin on Interferon-γ Levels in mice exposed to LPS.

F (6, 29) = 52.52. *P < 0.001 compared to vehicle-treated LPS-free group, #P < 0.01 compared to vehicle-treated LPS-exposed group, ##P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI-Imipramine)

4.41 Effect of Troxerutin on brain Interleukin 6 (IL-6) level in LPS

In the vehicle-treated groups, animals exposed to LPS in the LPS-induced depression paradigm showed significant difference in IL-6 level compared to LPS-free group, $p < 0.001$. The levels of IL-6 in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated controls exposed to LPS, $p < 0.001$, Figure 4.38.

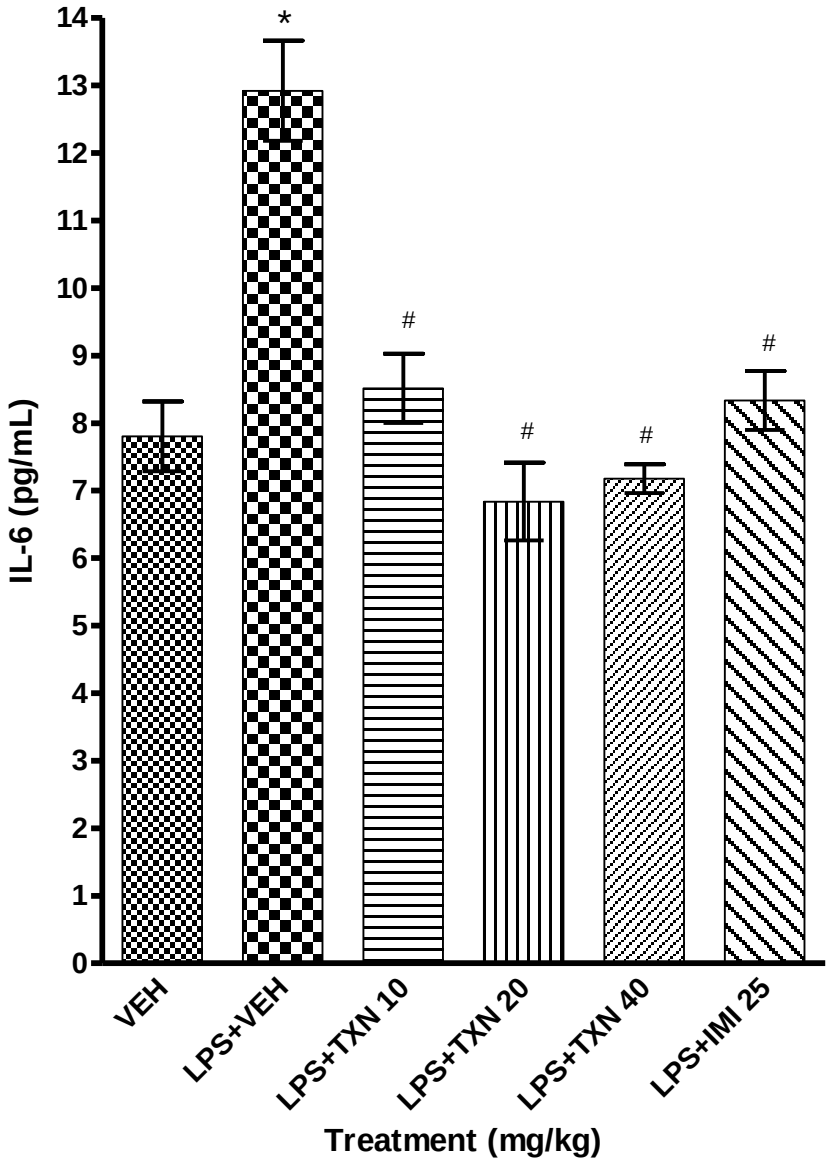

Figure 4.38: Effect of Troxerutin on IL-6 Levels in mice exposed to LPS.

F (6, 29) = 17.84. *P < 0.001 compared to vehicle-treated LPS-free group, #P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.42 Effect of Troxerutin on brain MAPKs concentrations in Mice exposed to LPS

In vehicle-treated groups, mice exposed to LPS in the LPS-induced depression paradigm showed significant difference in MAPKs level when compared to LPS-free group, $p < 0.01$. The levels of MAPKs in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated controls exposed to LPS, $p < 0.001$, Figure 4.39.

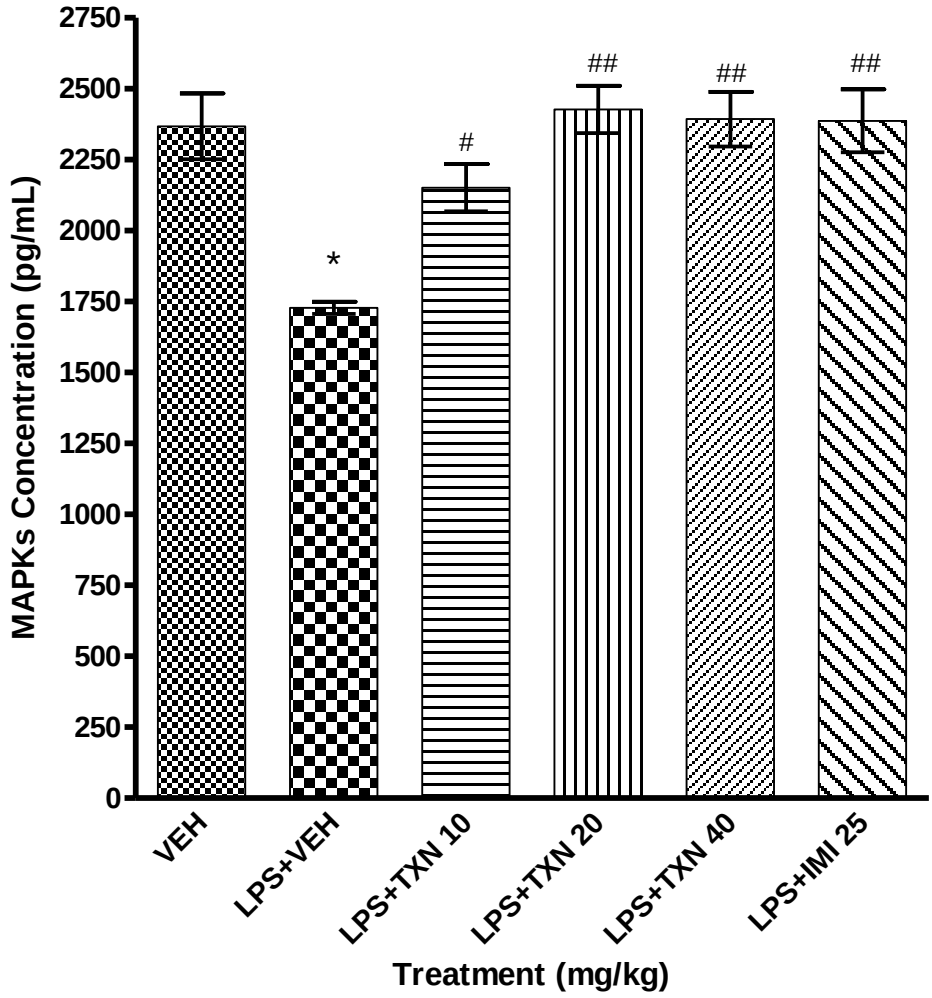

Figure 4.39: Effect of Troxerutin on MAPKs Levels in mice exposed to LPS.

F (6, 29) = 8.39. *P < 0.01 compared to vehicle-treated LPS-free group, #P < 0.01 compared to vehicle-treated LPS-exposed group, ##P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI-Imipramine)

4.43 Effect of Troxerutin on Noradrenaline (NA) concentrations in LPS

In the vehicle-treated groups, mice exposed to LPS in the LPS-induced depression paradigm showed significant difference in NA level compared to LPS-free group, $p < 0.01$. The levels of NE in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated controls exposed to LPS, $p < 0.001$, Figure 4.40.

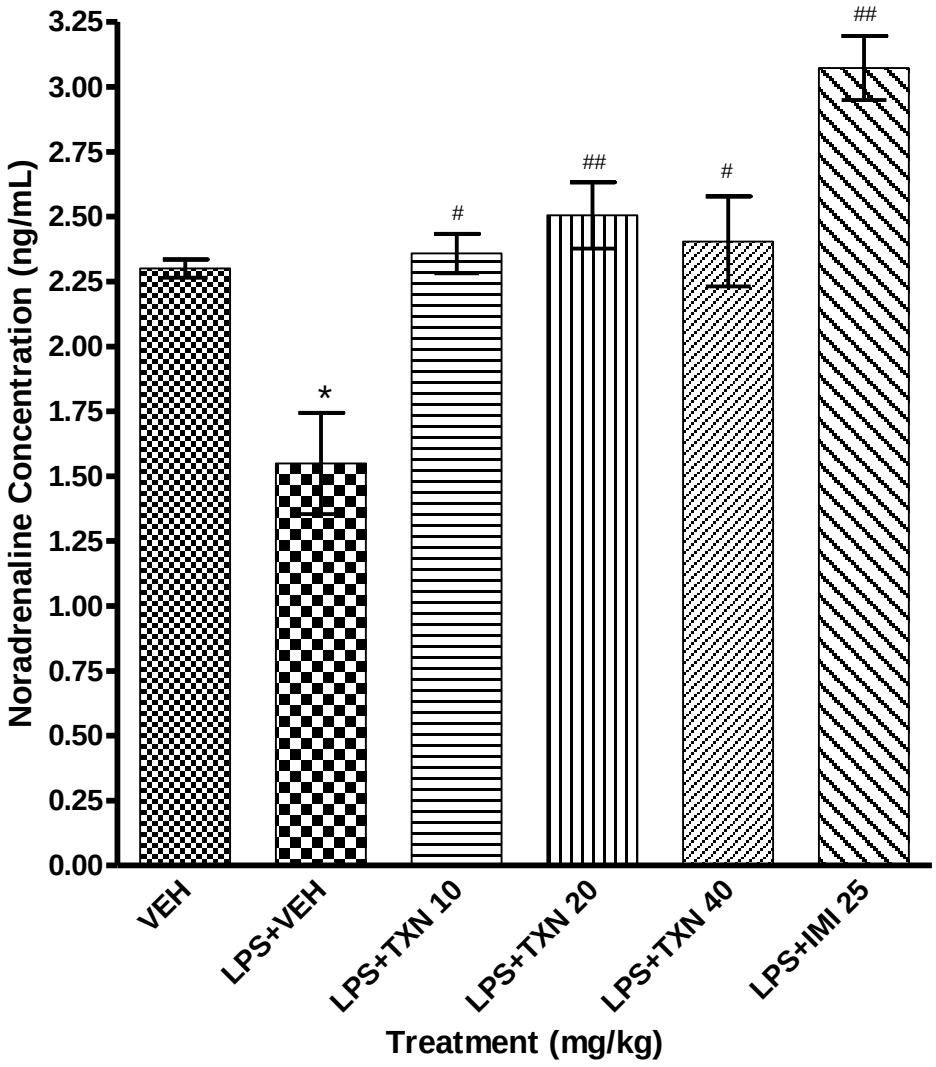

Figure 4.40: Effect of Troxerutin on NA Levels in mice exposed to LPS.

F (6, 29) = 13.43.*P < 0.01 compared to vehicle-treated LPS-free group, #P < 0.01 compared to vehicle-treated LPS-exposed group, ##P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI-Imipramine)

4.44 Effect of Troxerutin on Serotonin (5-HT) concentrations in Mice exposed to LPS

In the vehicle-treated groups, mice exposed to LPS in the LPS-induced depression paradigm showed significant difference in 5-HT level when compared to LPS-free group, $p < 0.01$. The levels of 5-HT in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated group exposed to LPS, $p < 0.001$, Figure 4.41.

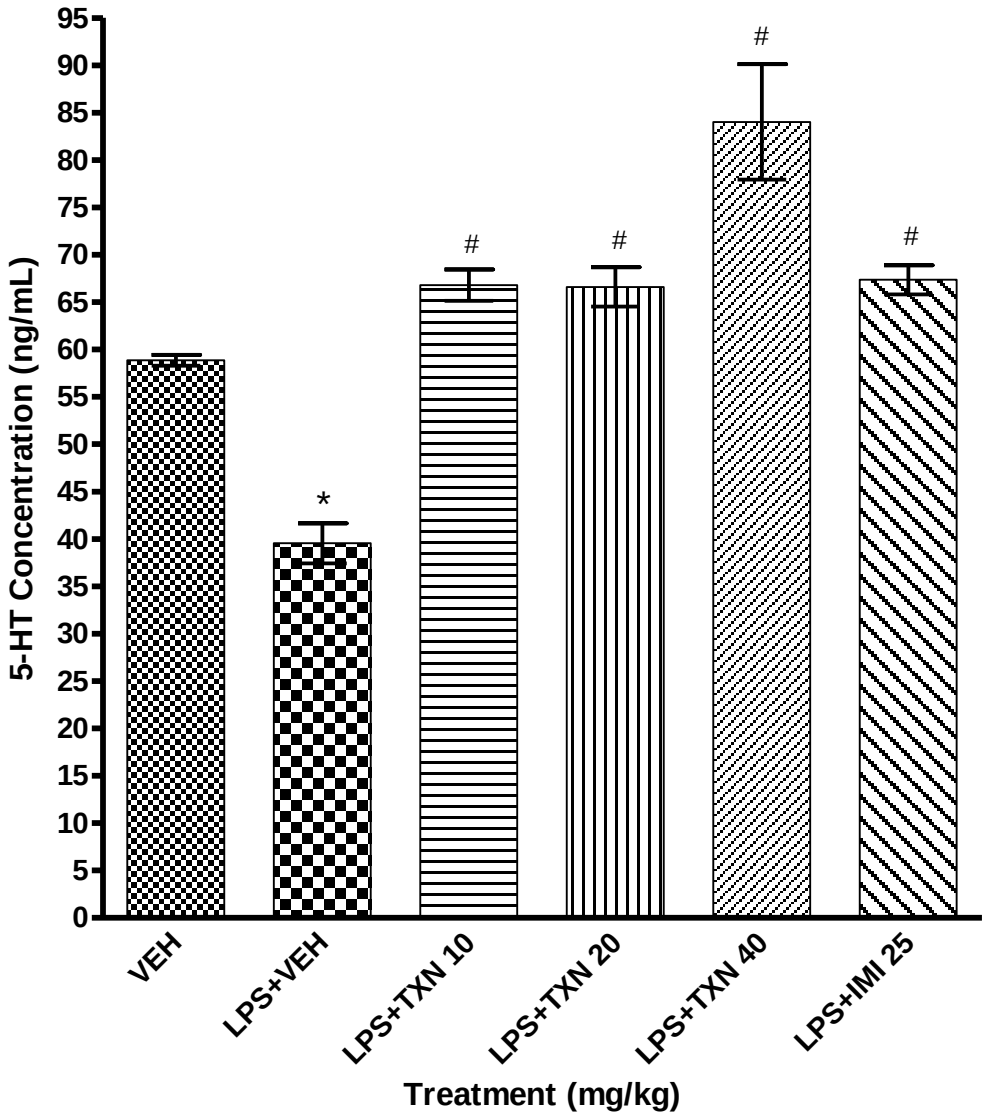

Figure 4.41: Effect of Troxerutin on 5-HT Levels in mice exposed to LPS.

F (6, 29) = 24.56. *P < 0.01 compared to vehicle-treated LPS-free group, #P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.45 Effect of Troxerutin on Dopamine (DA) concentrations in Mice exposed to LPS

In the vehicle-treated groups, mice exposed to LPS in the LPS-induced depression paradigm showed significant difference in DA level compared to LPS-free group, $p < 0.05$. The levels of DA in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated controls exposed to LPS, $p < 0.05$, Figure 4.42.

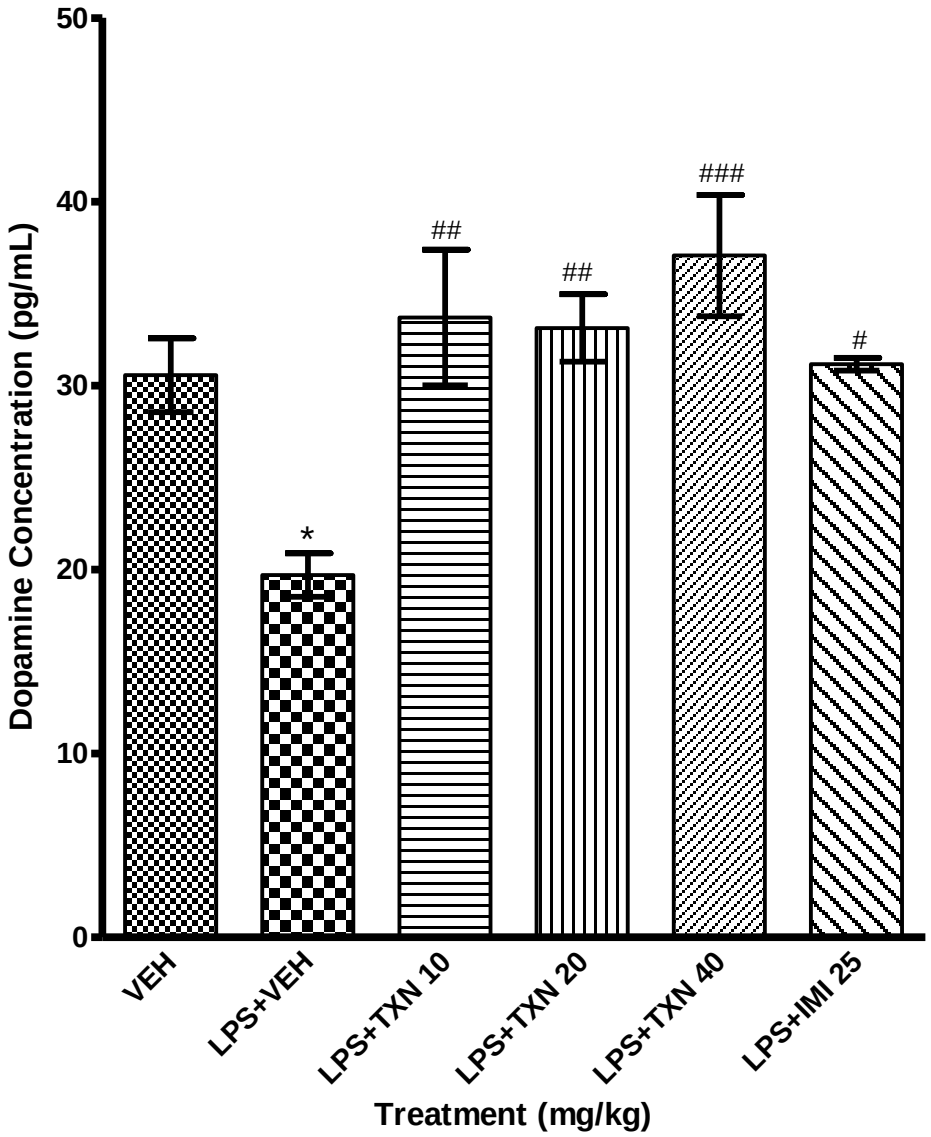

Figure 4.42: Effect of Troxerutin on DA Levels in mice exposed to LPS.

F (6, 29) = 6.35. *P < 0.001 compared to vehicle-treated LPS-free group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to vehicle-treated LPS-exposed controls. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.46 Effect of Troxerutin on BDNF Concentrations in Mice exposed to LPS

In the vehicle-treated groups, mice exposed to LPS in the LPS-induced depression paradigm showed significant difference in BDNF level compared to LPS-free group, $p < 0.001$. The levels of BDNF in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated controls exposed to LPS, $p < 0.001$, Figure 4.43.

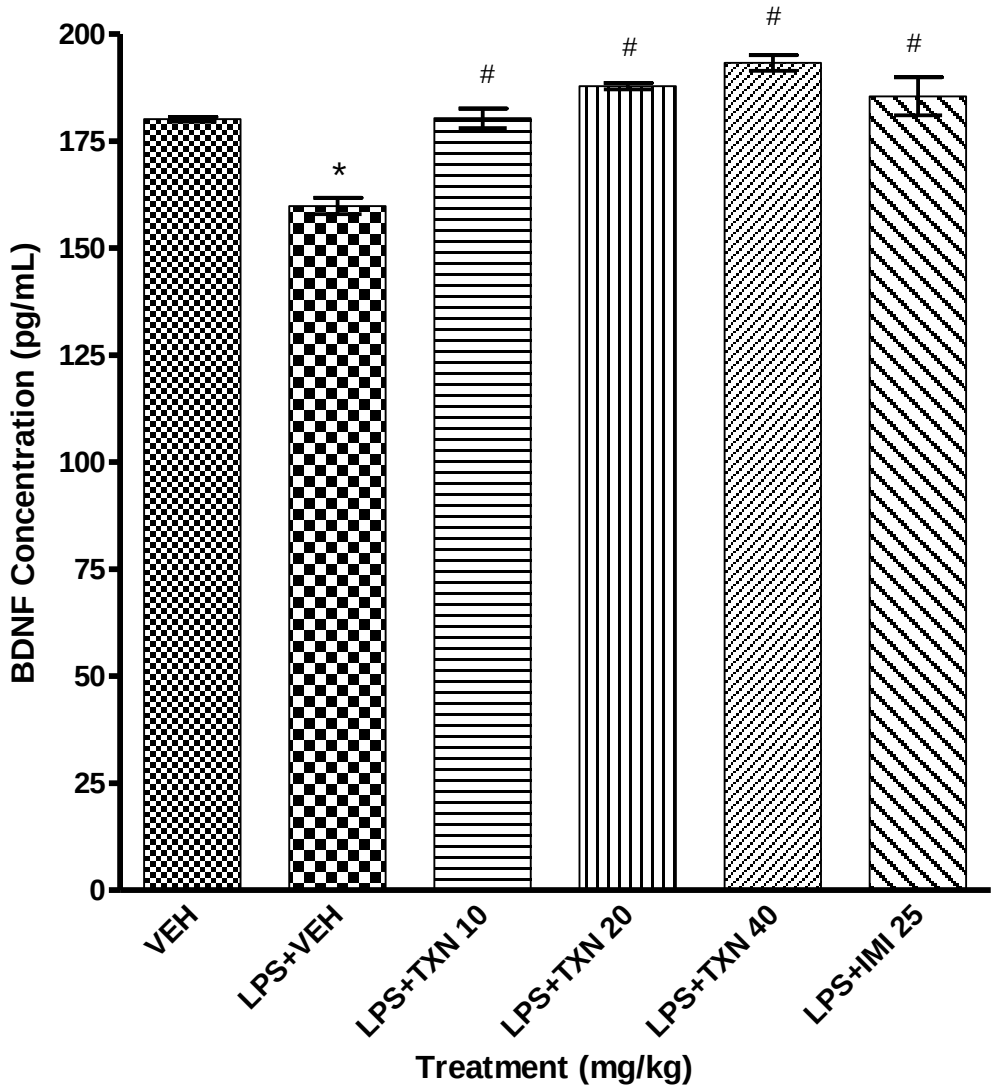

Figure 4.43: Effect of Troxerutin on BDNF Levels in mice exposed to LPS.

F (6, 29) = 24.38. *P < 0.001 compared to vehicle-treated LPS-free group, #P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.47 Effect of Troxerutin on CREB Levels in Mice exposed to LPS

In the vehicle-treated groups, mice exposed to LPS in the LPS-induced depression paradigm showed significant difference in CREB level compared to LPS-free group, $p < 0.01$. The levels of CREB in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated controls exposed to LPS, $p < 0.01$, Figure 4.44.

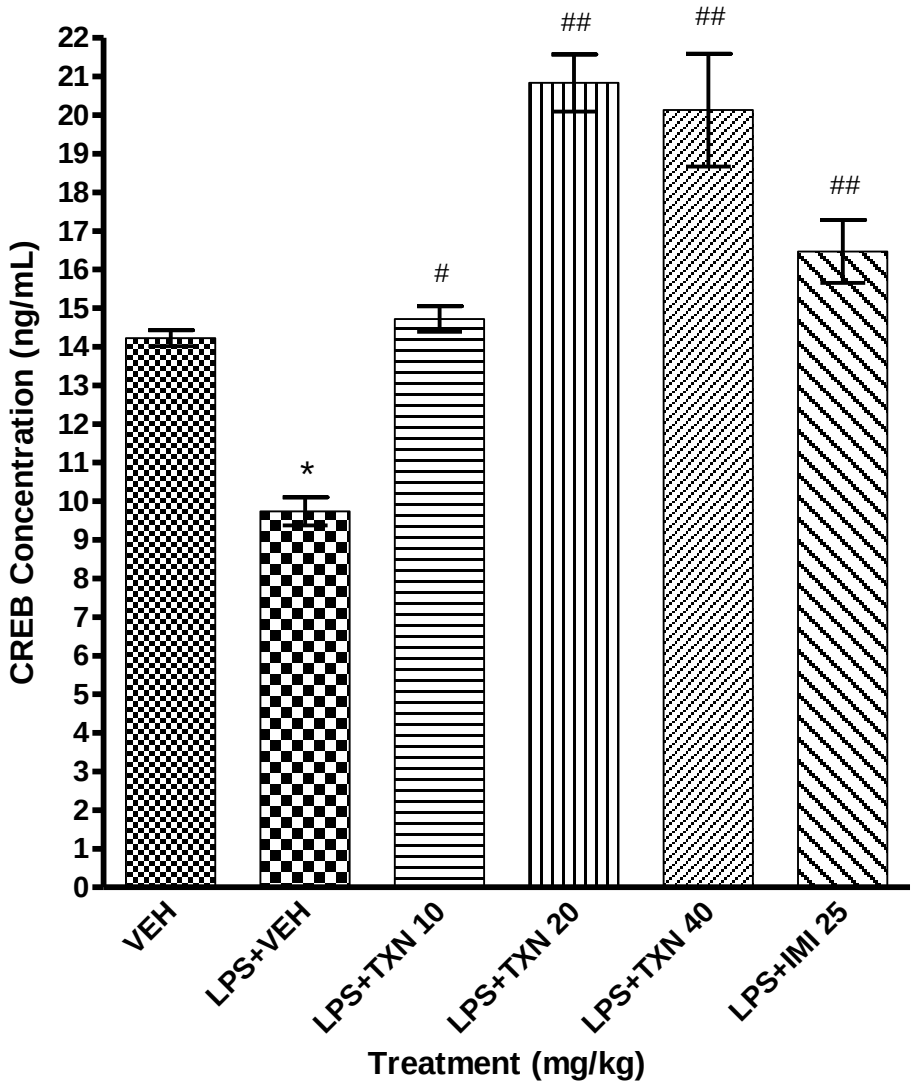

Figure 4.44: Effect of Troxerutin on CREB Levels in mice exposed to LPS.

F (6, 29) = 27.98. *P < 0.01 compared to vehicle-treated LPS-free group, #P < 0.01,##P < 0.001compared to vehicle-treated LPS-exposed controls. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.48 Immunohistochemistry of NF-κB Expression in Prefrontal Cortex of mice exposed to LPS

The vehicle treated animals exposed to LPS paradigm showed significant difference in density of NF-κB positive neurons compared to Troxerutin groups, $p < 0.01$ (Figure 4.45). The histological examination shows preserved brain structure in animals treated with Troxerutin or Imipramine compared to vehicle-treatedLPS controls with multiple foci NF-κB positive neurons, Plate 4.5.

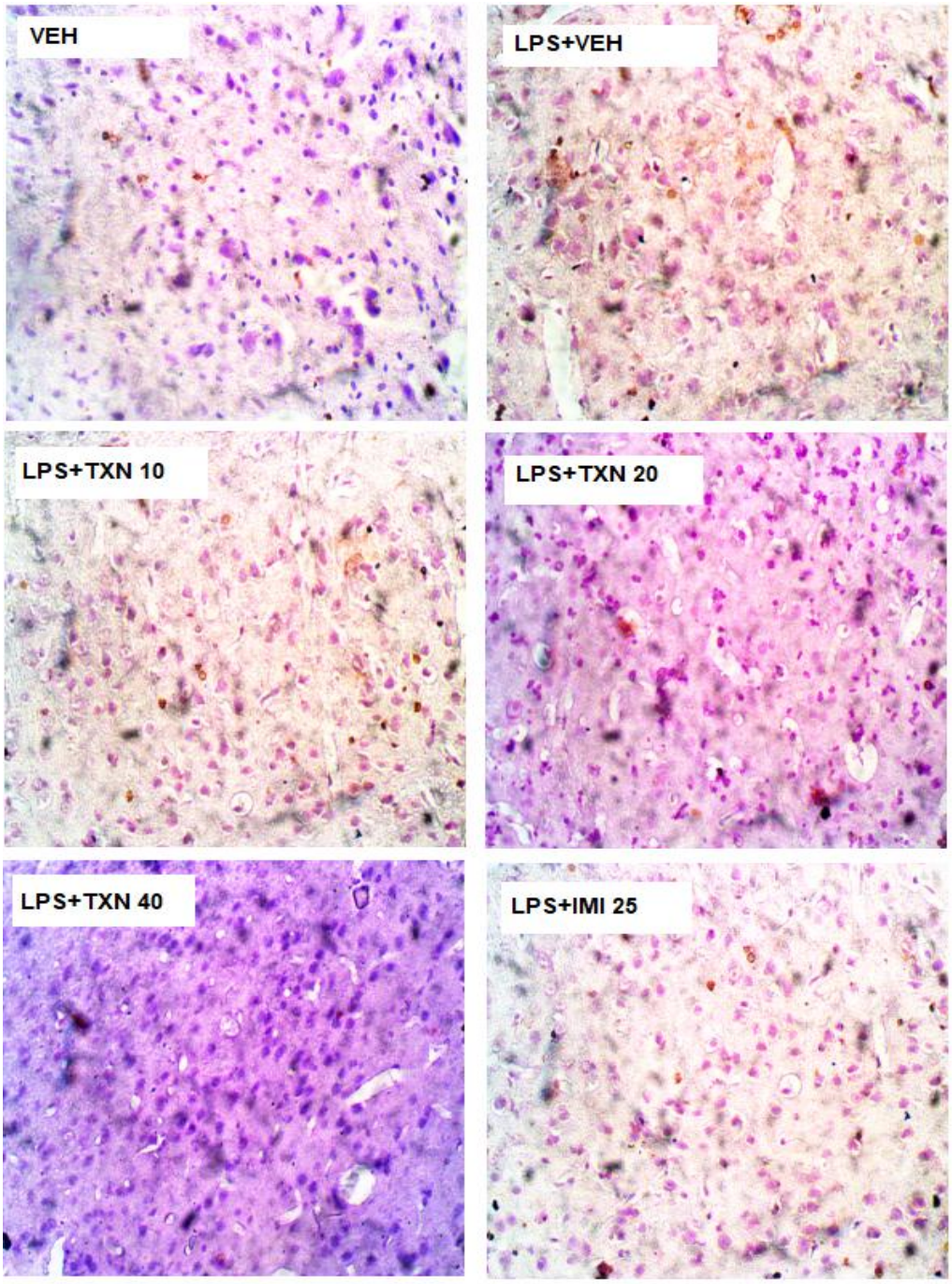

Plate 4.5: Photomicrographs of Layer 2/3 region of Prefrontal Cortex showing positive NF-kB stained cells in mice subjected to LPS (x400 magnification).

Neuronal density is shown in Figure 4.45 below. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

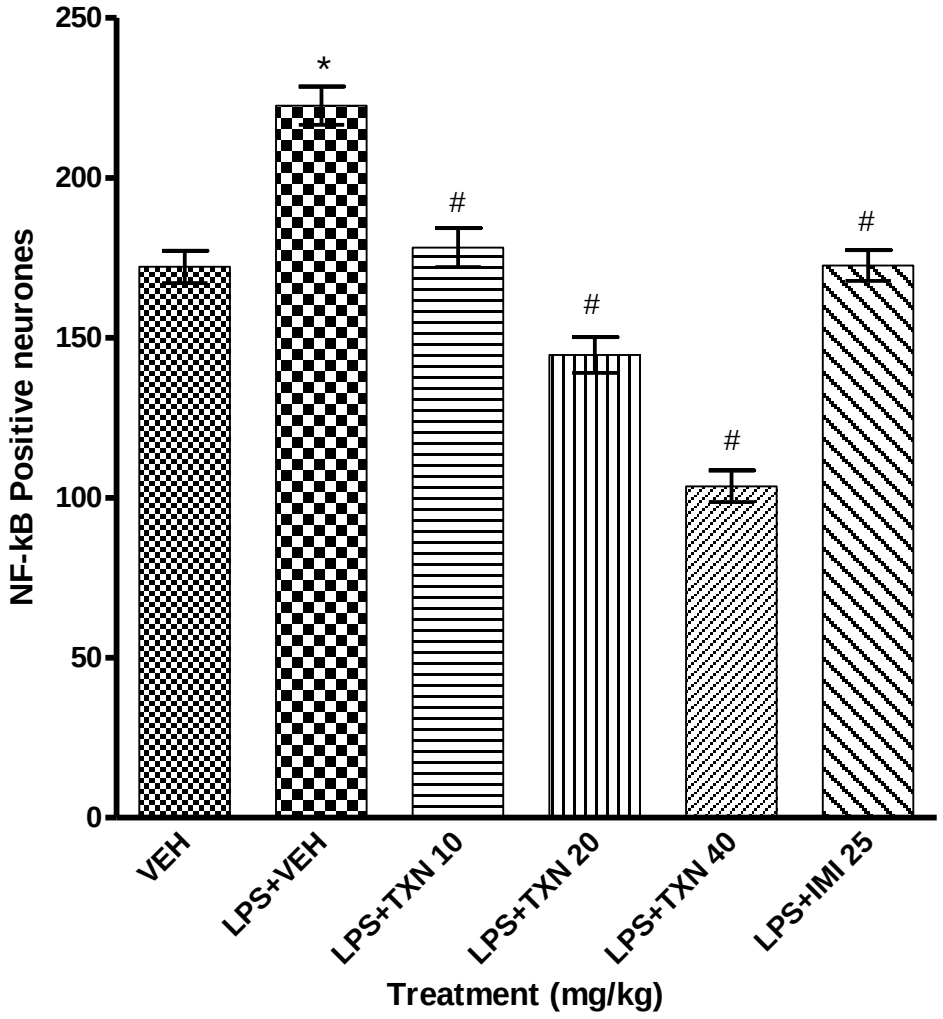

Figure 4.45: Neuronal density of NF-kB positive cells in prefrontal cortex of mice exposed to LPS.

*P < 0.001 compared to vehicle-treated LPS-free group, #P < 0.001 compared to vehicle-treated LPS-exposed controls. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN-Troxerutin, IMI- Imipramine)

4.49 Immunohistochemistry of NF-κB Expression in Hippocampus of mice exposed to LPS

The vehicle treated animals exposed to LPS paradigm showed significant difference in density of NF-κB positive neurons compared to Troxerutin groups, $p < 0.01$ (Figure 4.46). The histological examination shows preserved brain structure in animals treated with Troxerutin or Imipramine compared to vehicle-treatedLPS controls with multiple foci NF-κB positive neurons, Plate 4.6.

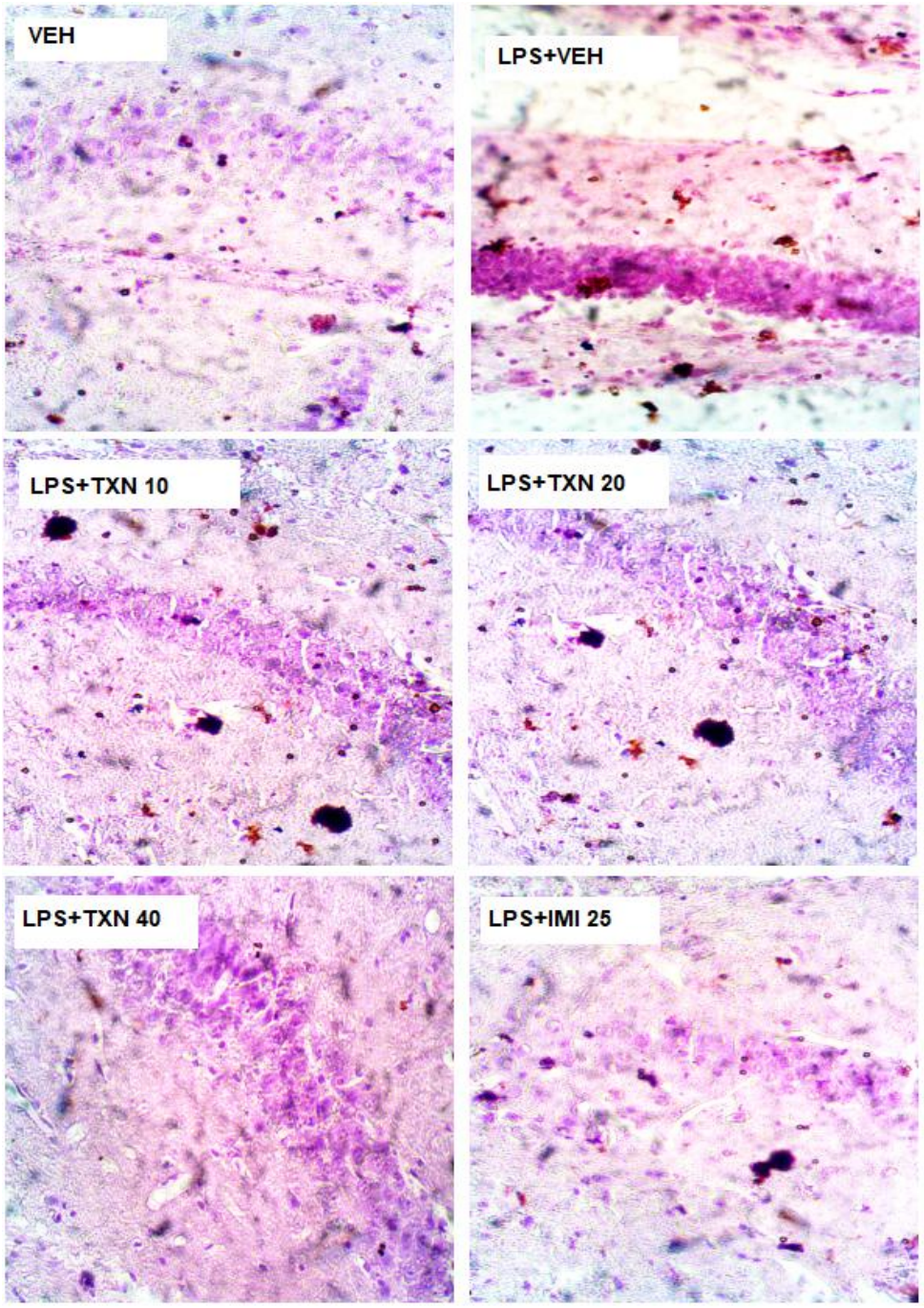

Plate 4.6: Photomicrographs of cornus ammonis region of Hippocampus showing positive NF-kB stained cells in mice subjected to LPS (x400 magnification).

Neuronal density is shown in Figure 4.46 below. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

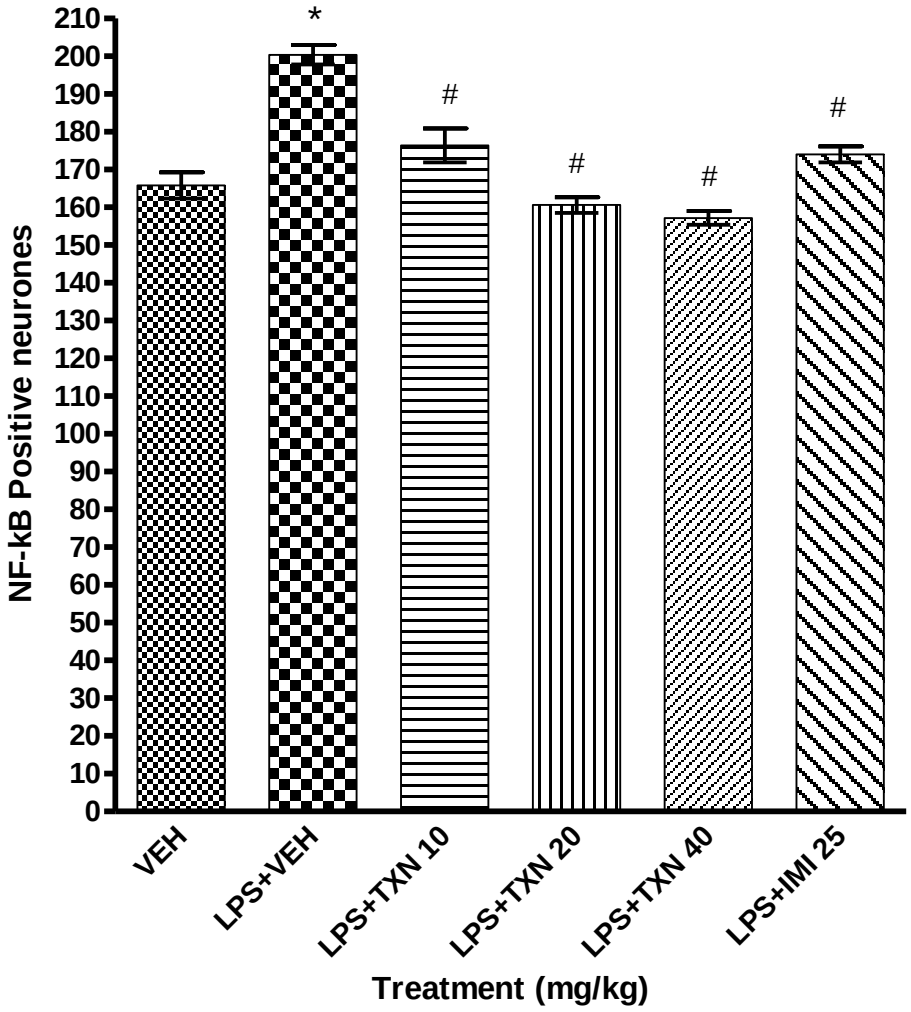

Figure 4.46: Neuronal density of NF-kB positive cells in hippocampus of mice exposed to LPS.

*P < 0.001 compared to vehicle-treated LPS-free group, #P < 0.001 compared to vehicle-treated LPS-exposed control. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.50 Immunohistochemistry of iNOS Expression in Prefrontal Cortex of mice exposed to LPS

The vehicle treated animals exposed to LPS paradigm showed significant difference in density of iNOS positive neurons compared to Troxerutin groups, $p < 0.001$ (Figure 4.47). The histological examination shows preserved brain structure in animals treated with Troxerutin or Imipramine compared to vehicle-treatedLPS controls with multiple foci iNOS positive neurons, Plate 4.7.

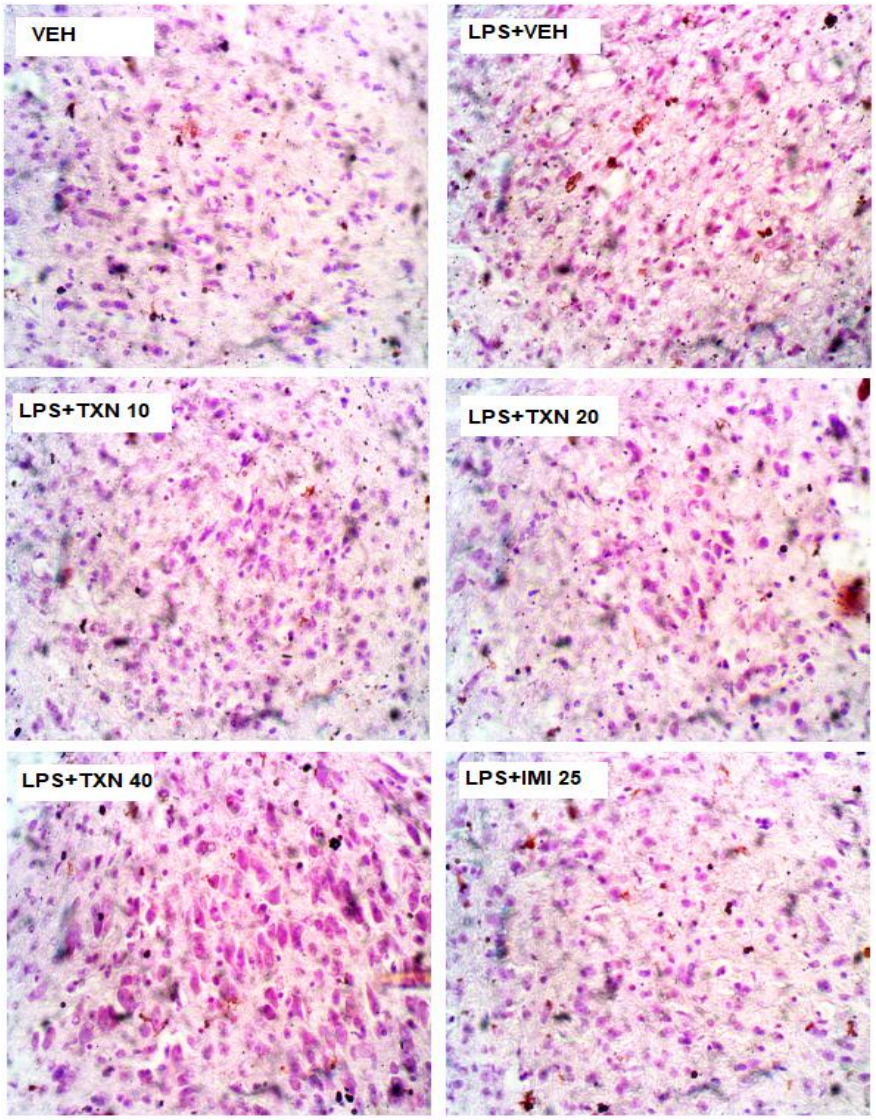

Plate 4.7: Photomicrographs of Layer 2/3 region of prefrontal cortex showing positive iNOS stained cells in mice subjected to LPS (x400 magnification).

Neuronal density is shown in Figure 4.47 below. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

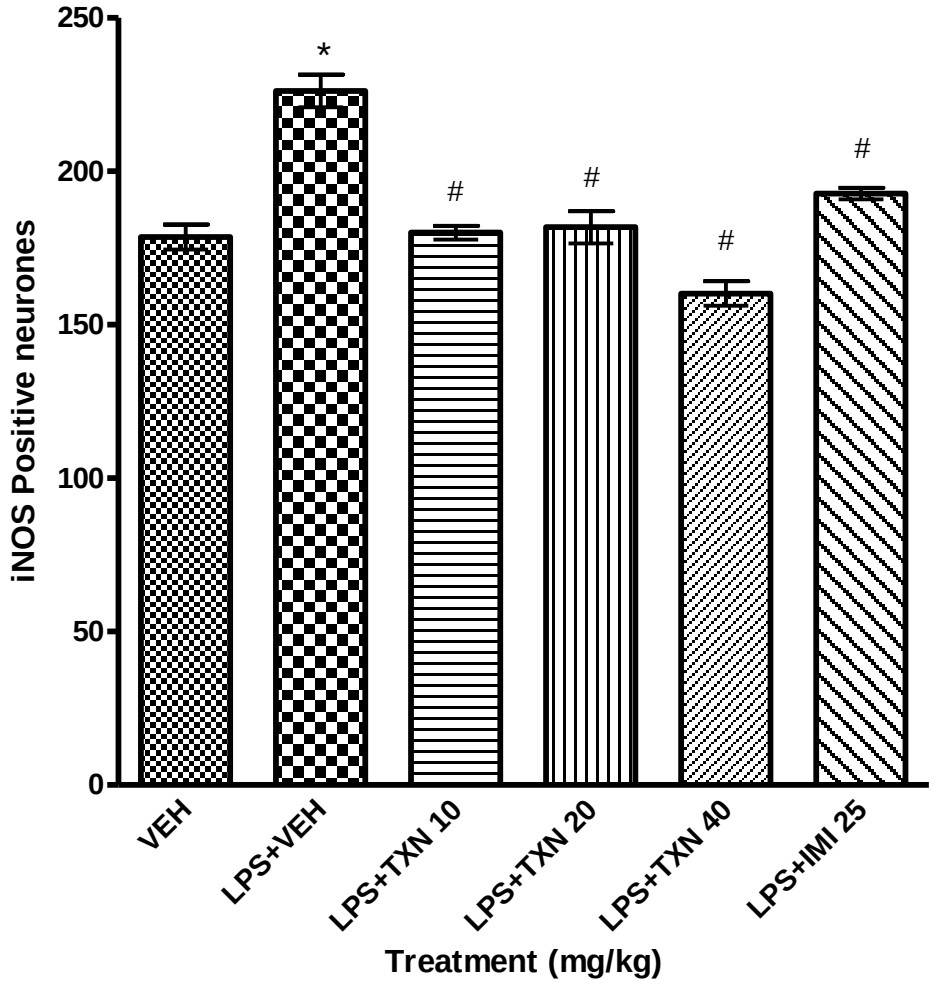

Figure 4.47: Neuronal density of iNOS positive cells in prefrontal cortex of mice exposed to LPS.

*P < 0.001 compared to vehicle-treated LPS-free group, #P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.51 Immunohistochemistry of iNOS Expression in Hippocampus of mice exposed to LPS

The vehicle treated animals exposed to LPS paradigm showed significant difference in density of iNOS positive neurons compared to Troxerutin groups, $p < 0.001$ (Figure 4.48). The histological examination shows preserved brain structure in animals treated with Troxerutin or Imipramine compared to vehicle-treatedLPS controls with multiple foci iNOS positive neurons, Plate 4.8.

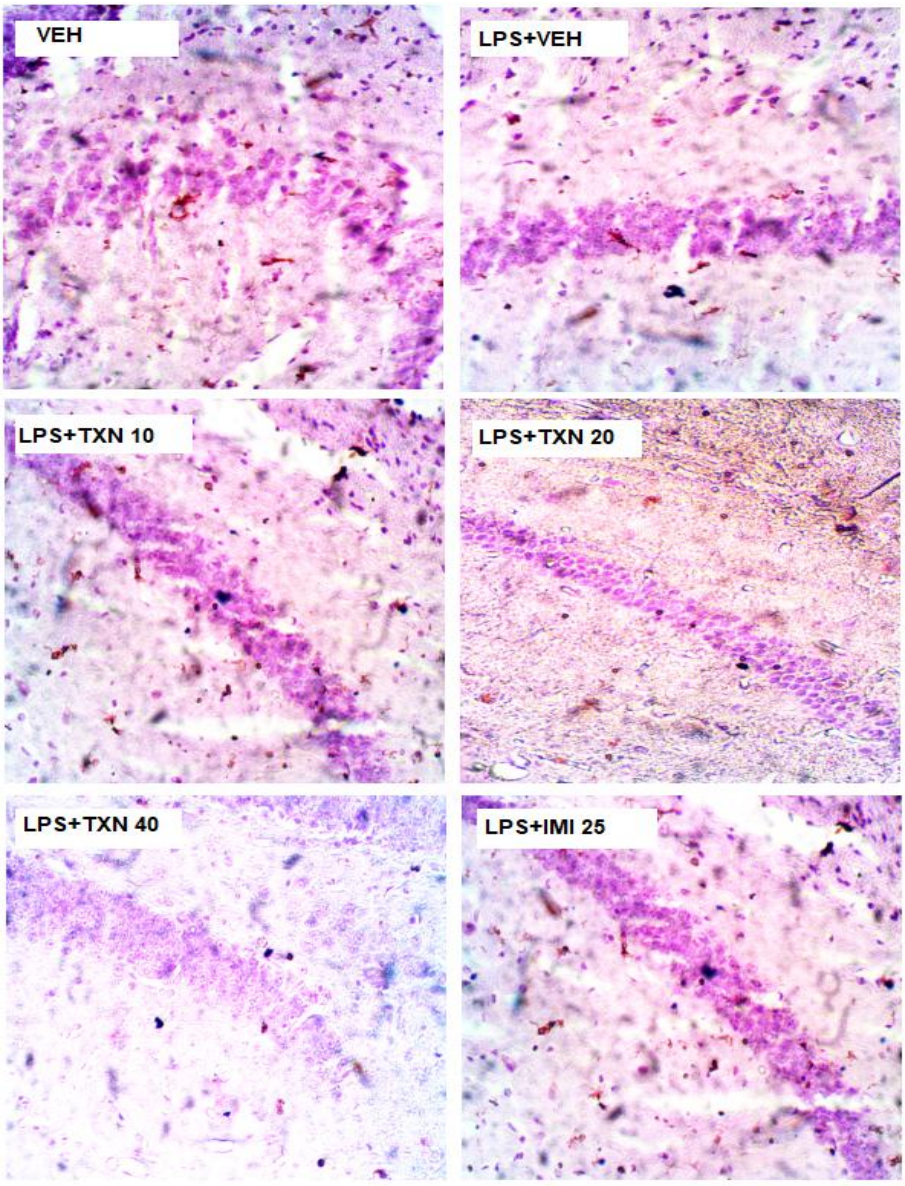

Plate 4.8: Photomicrographs of cornus ammonis region of Hippocampus showing positive iNOS stained cells in mice subjected to LPS (x400 magnification).

Neuronal density is shown in Figure 4.48 below. (VEH- Vehicle, LPS- Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

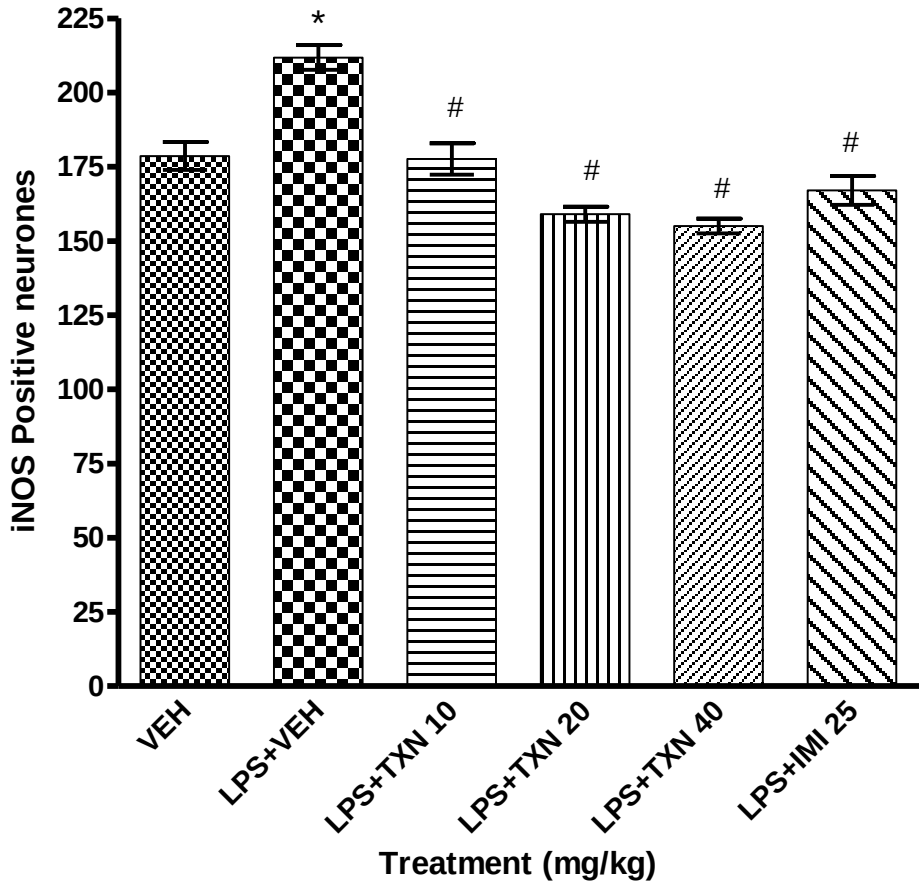

Figure 4.48: Neuronal density of iNOS positive cells in hippocampus of mice exposed to LPS.

*P < 0.001 compared to vehicle-treated LPS-free group, #P < 0.001 compared to vehicle-treated LPS-exposed group.(VEH- Vehicle, LPS-Lipopolysaccharide, TXN- Troxerutin, IMI- Imipramine)

4.52 Effect of Troxerutin on TST in Reserpine-Induced Depression (RID)

The vehicle-treated animals exposed to Reserpine in the reserpine-induced depression paradigm exhibits significant increase in immobility period compared to control group that was not exposed to reserpine, $P < 0.05$.There was significant difference, $P < 0.001$ in immobility duration in troxerutin-treated and imipramine-treated groups compared to reserpine-exposed vehicle-treated controls, as presented on Figure 4.49.

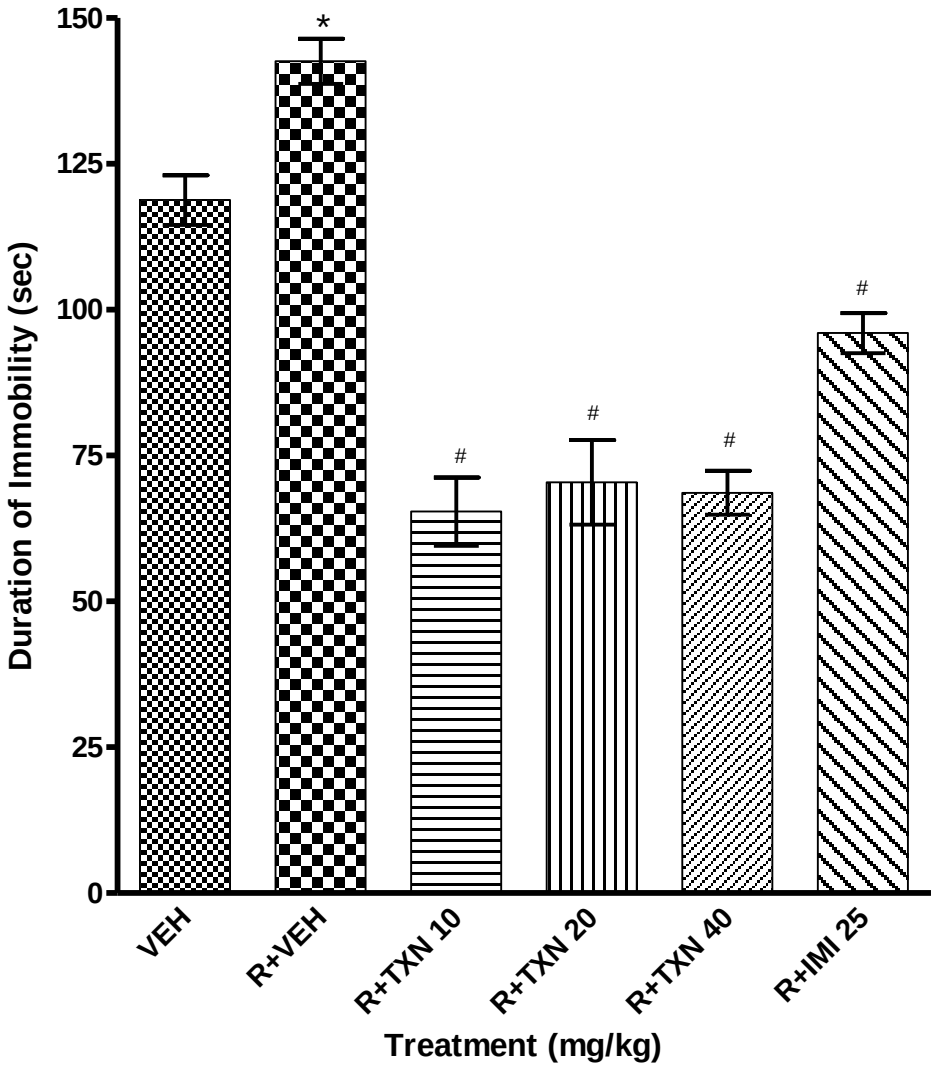

Figure 4.49: Effect of Troxerutin on immobility time in TST post-Reserpine exposure.

F (6, 29) = 41.13. *P < 0.05 compared to vehicle-treated reserpine-free group. #P < 0.001 compared to vehicle-treated reserpine-exposed group. (VEH- Vehicle, R-Reserpine, TXN-Troxerutin, IMI- Imipramine)

4.53 Effect of Troxerutin on grooming activity in splash test in mice subjected to Reserpine-Induced Depression (RID)

Comparing the vehicle treatment groups, the frequency of grooming activities in reserpine-exposed animals were significantly different compared to unexposed group, $P < 0.001$. The Troxerutin and Imipramine treatment group showed significant difference in frequency of grooming compared to vehicle-treated reserpine-exposed group, $P < 0.001$, Figure 4.50.

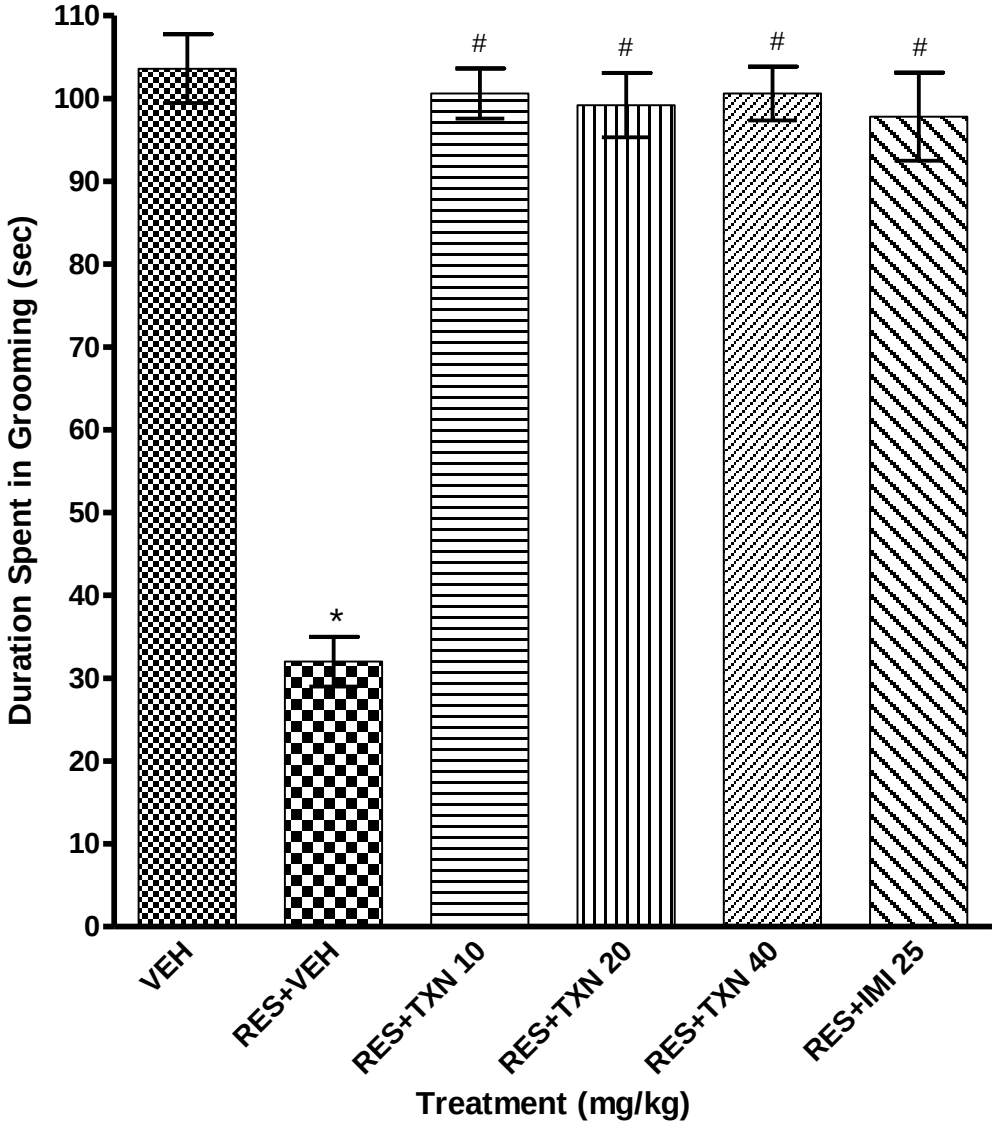

Figure 4.50: Effect of Troxerutin on grooming activities in mice subjected splash test. F (6, 29) = 52.48. *P < 0.001 compared to vehicle-treated reserpine-free group. #P < 0.001 compared to vehicle-treated reserpine-exposed group. (VEH- Vehicle, RES-Reserpine, TXN- Troxerutin, IMI- Imipramine)

4.54 Effect of Troxerutin (TXN) on Locomotor Activity in Mice Exposed to Reserpine

Troxerutin treatment at doses 10, 20 and 40 mg/kg showed no significant difference in number of squares crossed compared to vehicle-treated Reserpine-free control animals in open field, $p > 0.05$, Figure 4.51.

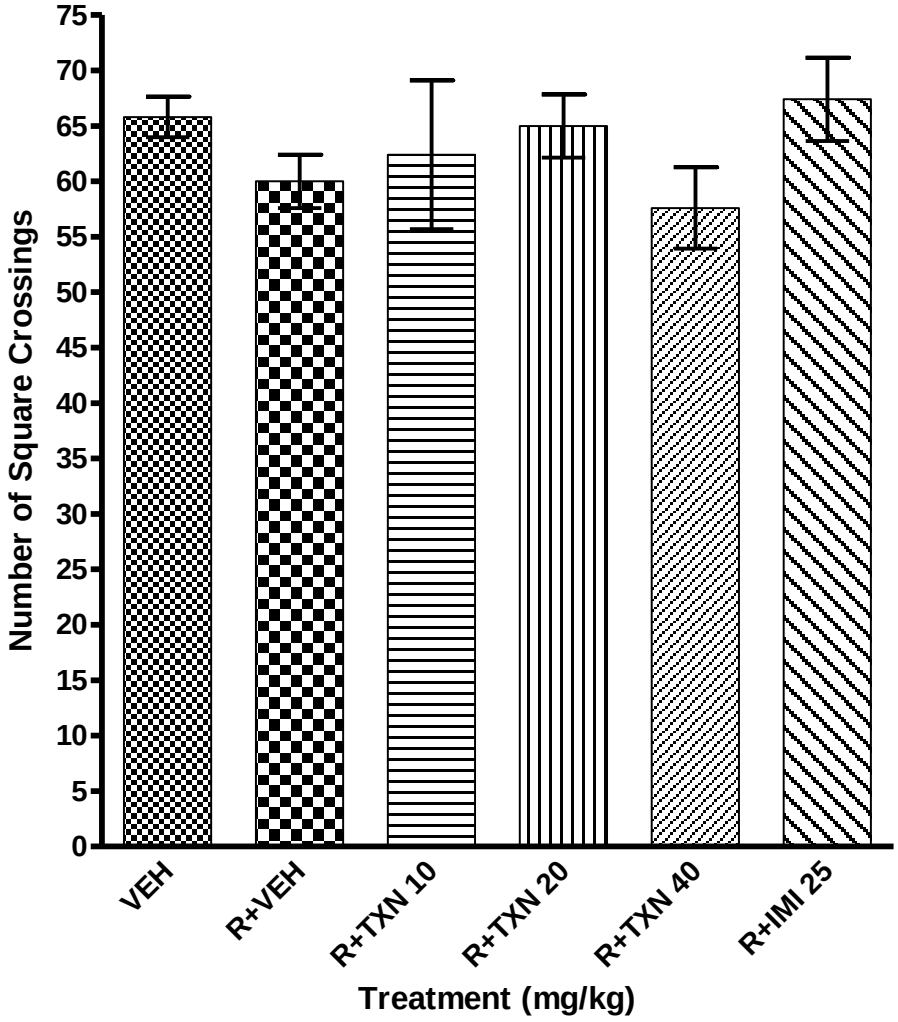

Figure 4.51: Effect of Troxerutin (TXN) on locomotor activity of mice exposed to Reserpine in open field.

(VEH- Vehicle, R-Reserpine, TXN- Troxerutin, IMI- Imipramine)

4.55 Effect of Troxerutin onNoradrenaline (NA) concentrations in Mice exposed to Reserpine

In the vehicle-treated groups, the animals exposed to Reserpine in the Reserpine-induced depression paradigm showed a significant difference in NA level compared to Reserpine-free group, $p < 0.01$. Levels of NA in Reserpine-exposed animals treated with Troxerutin or Imipramine showed significant difference compared to vehicle treated animals exposed to LPS, $p < 0.001$, Figure 4.52.

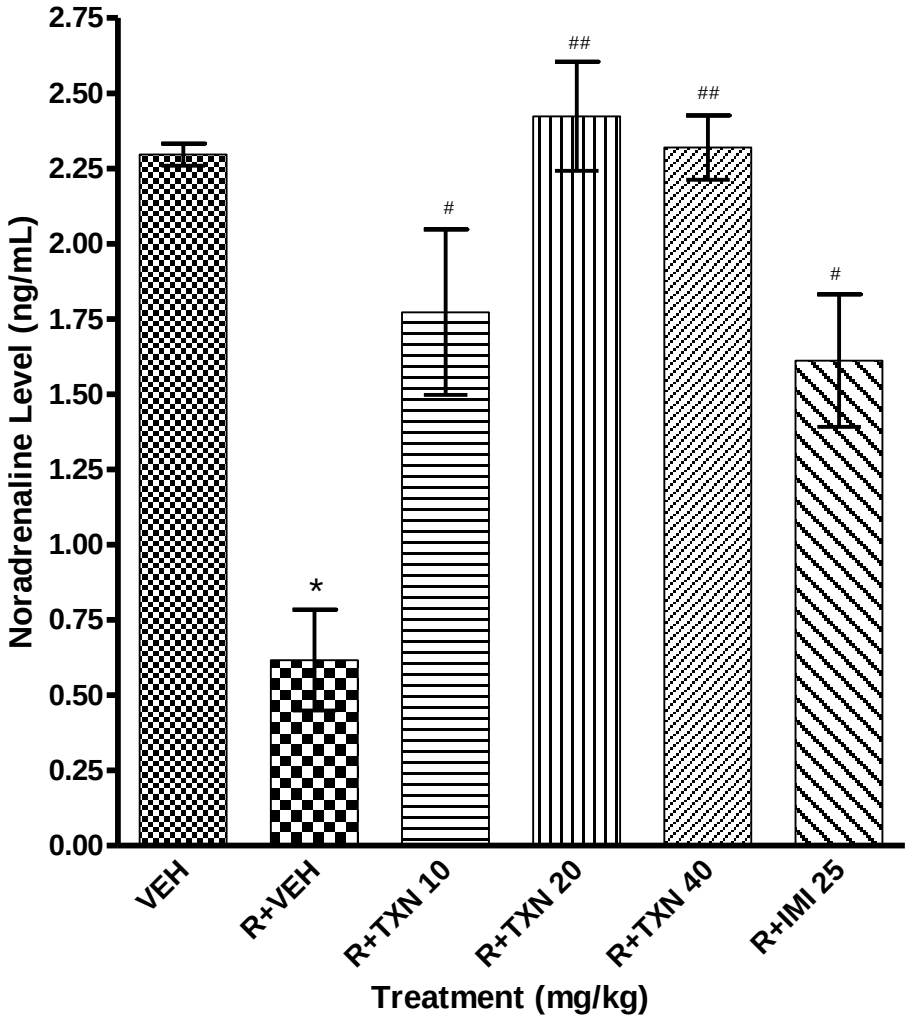

Figure 4.52: Effect of Troxerutin on NA Levels in mice exposed to Reserpine.

F (6, 29) = 14.16. *P < 0.001compared to vehicle-treated Reserpine-free group, #P < 0.01, ##P < 0.001 compared to vehicle-treated LPS-exposed group. (VEH- Vehicle, R- Reserpine, TXN- Troxerutin, IMI- Imipramine)

4.56 Effect of Troxerutin on5-Hydroxytryptamine (5-HT) concentrations in RID

In the vehicle-treated groups, mice exposed to Reserpine in the Reserpine-induced depression paradigm showed significant reduction in 5-HT level compared to Reserpine-free group, $p < 0.01$. The levels of 5-HT in Troxerutin or Imipramine groups showed significant increase compared to vehicle treated animals exposed to Reserpine, $p < 0.001$, Figure 4.53.

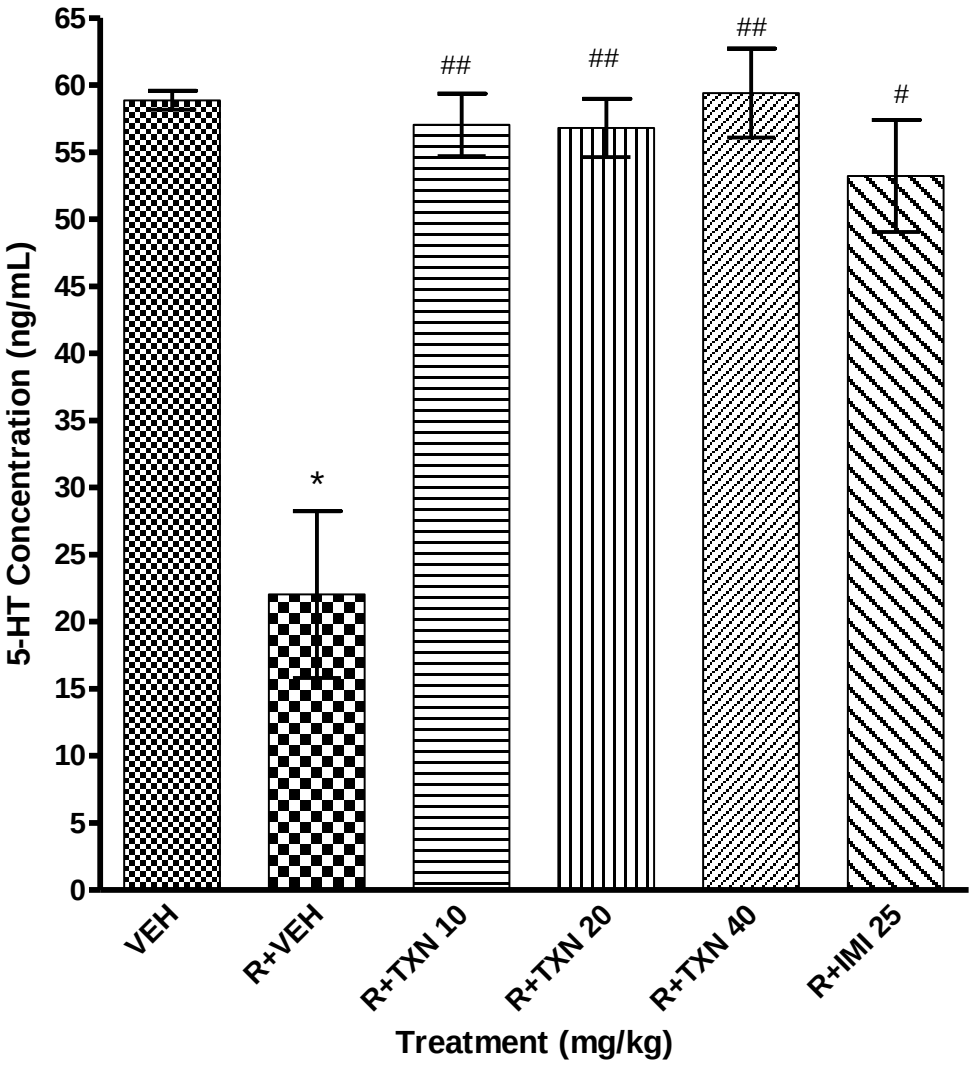

Figure 4.53: Effect of Troxerutin on 5-HT Levels in mice exposed to Reserpine.

F (6, 29) = 14.95. *P < 0.001 compared to vehicle-treated Reserpine-free group, #P < 0.01, ##P < 0.001 compared to vehicle-treated Reserpine-exposed group. (VEH- Vehicle, R-Reserpine, TXN- Troxerutin, IMI- Imipramine)

4.57 Effect of Troxerutin on Dopamine (DA) concentrations in Mice exposed to Reserpine

In the vehicle-treated groups, the animals exposed to Reserpine in the Reserpine-induced depression paradigm showed significant reduction in DA level compared to Reserpine-free group, $p < 0.05$. The levels of DA in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated group exposed to Reserpine, $p < 0.05$, Figure 4.54.

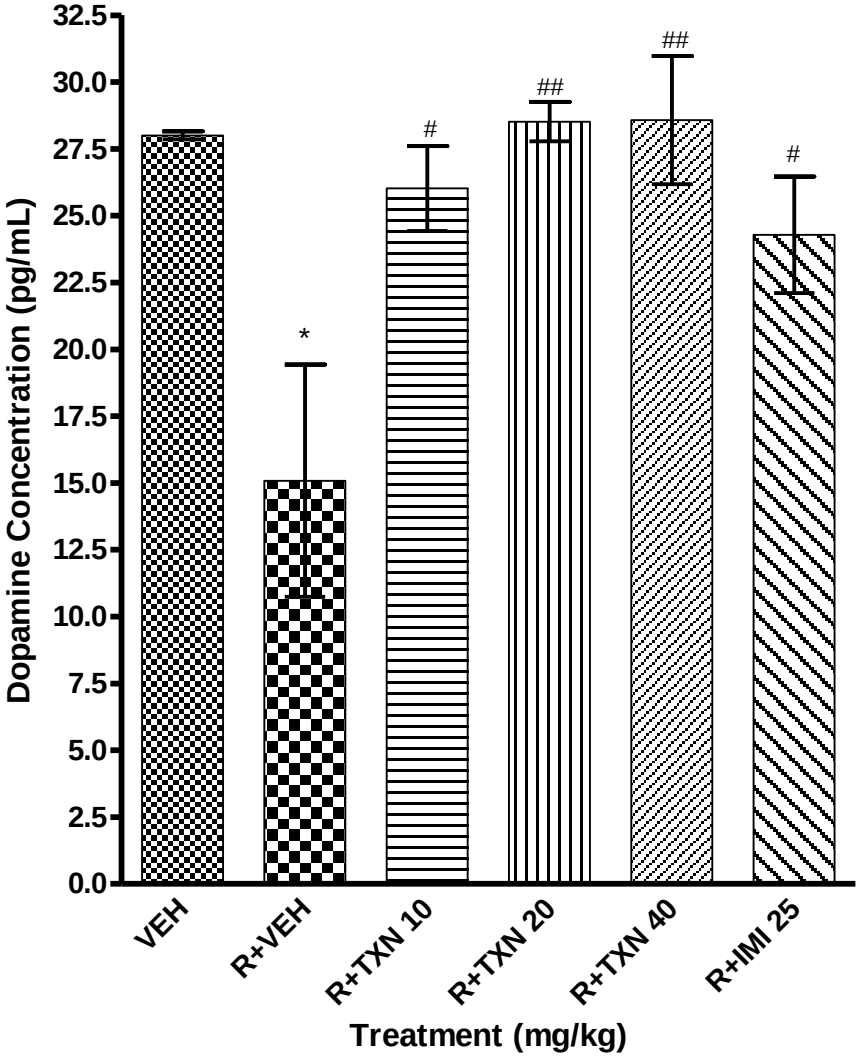

Figure 4.54: Effect of Troxerutin on DA Levels in mice exposed to Reserpine.

F (6, 29) = 4.95. *P < 0.01 compared to vehicle-treated Reserpine-free group, #P < 0.05,##P < 0.01 compared to vehicle-treated Reserpine-exposed group. (VEH- Vehicle, R- Reserpine, TXN- Troxerutin, IMI- Imipramine)

4.58 Effect of Troxerutin on BDNF Concentrations in RID

In the vehicle-treated groups, mice exposed to Reserpine in the Reserpine-induced depression paradigm showed significant difference in BDNF level compared to Reserpine-free group, $p < 0.001$. The levels of BDNF in Troxerutin or Imipramine groups showed significant increase compared to vehicle treated group exposed to Reserpine, $p < 0.001$, Figure 4.55.

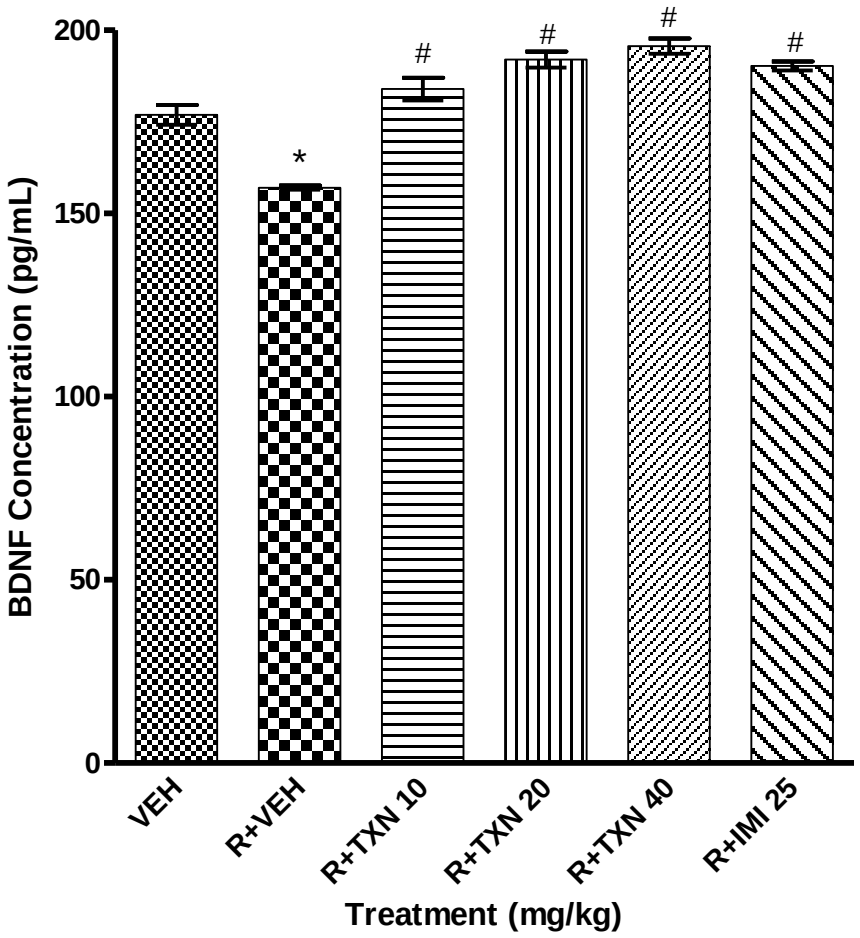

Figure 4.55: Effect of Troxerutin on BDNF Levels in mice exposed to Reserpine.

F (6, 29) = 43.02. *P < 0.001 compared to vehicle-treated Reserpine-free group, #P < 0.001 compared to vehicle-treated Reserpine-exposed group. (VEH- Vehicle, R- Reserpine, TXN- Troxerutin, IMI- Imipramine)

4.59 Effect of Troxerutin on CREB Levels in RID

In the vehicle-treated groups, mice exposed to Reserpine in the Reserpine-induced depression paradigm showed significant difference in CREB level compared to Reserpine-free group, $p < 0.01$. The levels of CREB in Troxerutin or Imipramine groups showed significant difference compared to vehicle treated controls exposed to Reserpine, $p < 0.01$, Figure 4.56.

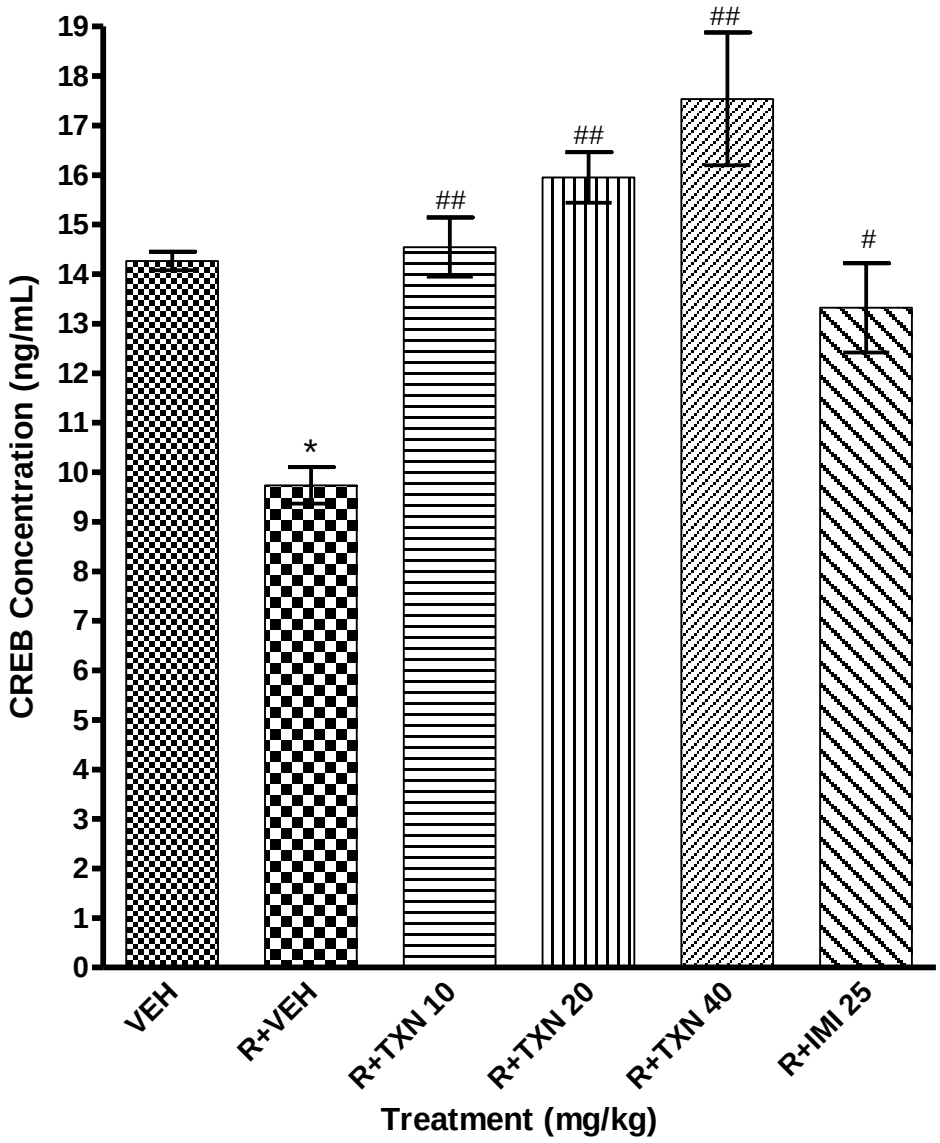

Figure 4.56: Effect of Troxerutin on CREB Levels in mice exposed to Reserpine.

F (6, 29) = 7.87. *P < 0.01 compared to vehicle-treated Reserpine-free group, #P < 0.01,##P < 0.001 compared to vehicle-treated Reserpine-exposed group. (VEH- Vehicle, R-Reserpine, TXN- Troxerutin, IMI- Imipramine)

4.60 Effect of Troxerutin on Reserpine Antagonism

As shown in Table 4.5, 4.6 and 4.7; respectively, treatment with Troxerutin and Imipramine showed significant difference in the rectal temperature, score of ptosis and degree of diarrhoea in vehicle-treated animals given reserpine (2.5 mg/kg i.p). The induced hypothermia was reversed by Troxerutin and Imipramine in the latter 2 hours of the study while they both reversed the induced ptosis and diarrhoea throughout the study period.

Table 4.5:Effect of Troxerutin on Reserpine-induced hypothermia

Treatment	0HR	1HR	2HRS	3HRS	4HRS
VEH	36.8±0.23	35.9±0.14	34.5±0.12	32.8±0.46	32.5±0.14
TXN (10 mg/kg)	36.9±0.24	37.1±0.29	37.1±0.29*	36.2±0.20*	36.3±0.18*
TXN (20 mg/kg)	36.9±0.25	37.1±0.15	36.9±0.15*	36.9±0.25*	36.9±0.24*
TXN (40 mg/kg)	36.8±0.21	36.7±0.24	36.6±0.24*	36.6±0.25*	36.8±0.21*
IMI (25 mg/kg)	36.8±0.18	36.7±0.24	36.6±0.24*	36.5±0.28*	36.6±0.25*

*P < 0.01 compared to vehicle control

Table 4.6:Effect of Troxerutin on Reserpine-induced ptosis

Treatment	1HR	2HRS	3HRS	4HRS
VEH	3.2±0.4	3.4±0.3	3.6±0.3	3.0±0.5
TXN (10 mg/kg)	1.0±0.3*	1.6±0.3*	1.0±0.0*	0.6±0.3*
TXN (20 mg/kg)	0.8±0.4*	1.0±0.3*	0.8±0.2*	0.8±0.4*
TXN (40 mg/kg)	0.6±0.3*	0.8±0.2*	0.8±0.2*	0.8±0.3*
IMI (25 mg/kg)	1.0±0.0*	1.0±0.0*	1.0±0.0*	1.0±0.3*

*$P < 0.01$ compared to vehicle control

Table 4.7:Effect of Troxerutin on Reserpine-induced diarrhoea

Treatment	1HR	2HRS	3HRS	4HRS
VEH	3.6±0.4	4.2±0.6	5.4±0.7	6.0±0.7
TXN (10 mg/kg)	1.6±0.2*	1.8±0.4*	1.0±0.5*	0.8±0.2*
TXN (20 mg/kg)	0.8±0.4*	1.2±0.2*	1.0±0.5*	1.0±0.3*
TXN (40 mg/kg)	1.0±0.3*	1.2±0.4*	0.6±0.3*	0.8±0.2*
IMI (25 mg/kg)	1.0±0.3*	1.2±0.2*	1.6±0.3*	1.6±0.3*

*$P < 0.01$ compared to vehicle control

CHAPTER FIVE

DISCUSSION

5.1 Acute toxicity (LD_{50}) of Troxerutin

In preclinical drug discovery studies, acute toxicity has remained a vital tool for probing pharmacological safety profile of novel compounds. Generally, natural compounds of plant origin, predominantly bioflavonoids are considered to be tolerable and classified as low risk substances (Ortiz-Andrade *et al*., 2020). However, other studies have shown such flavonoids to elicit significant adverse effects, therefore, evaluation of acute toxicity of any compound cannot be overemphasized (Sean *et al*., 2016). In this study, the phase two of the experimental protocol where the highest dose (5,000 mg/kg) was administered did not record any mortality among the animals, suggesting the acute lethal dose (LD_{50}) of Troxerutin to be high. According to the standard guidelines, compounds with LD_{50} greater than 2000 mg/kg are considered low risk substances (OECD, 2011) and the study result shows that Troxerutin is a low risk compound. In reference to summation of Aderibigbe *et al*., (2010) that the safety profile of a drug evaluated in acute toxicity model is directly proportional to its acute lethal dose, that is, the higher the LD_{50}, the less toxic the compound is assumed to be, hence, Troxerutin can be considered to have a high safety profile from the outcome of this study.

5.2 Antidepressant activities of Troxerutin in acute model

Neurobehavioural assessment studies are paradigms devised to mimic or elicit endophenotypic correlations of a specific neurological disorder such that the characteristic behavioural changes can be measured experimentally. In depressive disorder, the FST and TST are designed as endpoint models for measuring behavioural despair, a major symptomatic deficit in depression. In this study, Troxerutin at all administered doses

significantly reduced immobility duration in TST and the activity was comparable to Imipramine. Similarly, in FST, all doses of Troxerution reduced immobility time significantly with the animals showing resilience after several unsuccessful attempts to escape unlike the vehicle-treated controls that remained docile for longer period, signifying depressive behaviour. In drug discovery, FST and TST model were designed as acute paradigms for quick identification of novel antidepressants after acute administration and these models were sufficient as the behavioural despair seen as prolonged period of immobility in animals were reversed by almost all agents with antidepressant property (Krishnan & Nestler, 2011). The results in the acute model provided facile evidences of antidepressant potential of Troxerutin which was similar to that of standard control antidepressant drug used in the study. Furthermore, lower doses of Troxerutin significantly reduced immobility time in both TST and FST, and this suggests Troxerutin to have better potency and at its higher dose it could be more efficacious.

5.3 Protracted effect of Troxerutin on depression

Certain compounds have been observed to produce long-lasting antidepressant effect after a single dose administration. A predictor of rapid antidepressant is sustained activity which has been observed in agents like Ketamine (Kenji, 2020). This is a desired property that mitigates the untoward therapeutic delay that often discourages compliance in conventional antidepressants. In the study of protracted activities in TST and FST, a single dose administration of Troxerutin significantly decreased immobility time 72 hours post-treatment, however, the effect was diminished in the standard drug. By the day 7 post-treatment, the antidepressant effect was still observable in Troxerutin-treated animals except for those who received lower doses and it was totally lost in animals treated with Imipramine, a conventional tricyclic antidepressant. This long-lasting activity is thus dose dependent and it was evident in animals treated with Troxerutin. As shown in previous studies, the uniqueness of drug like Ketamine as antidepressant is the ability to elicit long-lasting effect after single dose administration, and this was ascribed to their ability to rapidly activate molecular signalling implicated in antidepressant mechanism (Pochwat *et al*., 2018). Therefore, it was surmised that Troxerutin from the findings of its significant

protracted effect activates the central molecular processes involved in antidepressant modulation.

5.4 Antidepressant activities of Troxerutin in chronic model on behavioural, molecular and histological markers

In the CUMS-induced depression model the vehicle-treated animals after 14-day exposure to stress exhibited marked depressive behaviour characterized by alteration of rearing and grooming behaviour, prolonged immobility in FST, anhedonic behaviour in SPT, and cognitive decline in maze exploration. Significantly, all these neurobehavioural deficits were reversed in Troxerutin-treated animals. Rearing and Grooming is an important adaptive behaviour that is elicited specifically by rodents in stressful depressive conditions (Akanmu *et al.,* 2007). In this study, Troxerutin induced stress adaptation or coping behaviour in form of increased rearing and grooming behaviour. There were no significant difference in frequency of rearing and grooming between unstressed controls and the animals treated with Troxerutin, or the standard drug, Imipramine. In the FST after subjection to CUMS, Troxerutin at all doses reduced immobility period and the effect was significant in comparison to vehicle-treated stressed animals. The findings of this study showed that vehicle treated mice subjected to chronic stress exhibited characteristic prolongation of immobility in FST, thus showed depression-like behaviour, which was reversed in animals treated with Troxerutin. The sucrose preference were similar among all groups pre-exposure to CUMS, however, after subjection to CUMS the induced anhedonia was reversed by treatment with Troxerutin. There were no differences in sucrose preference across the unstressed group, the troxerutin-treated group and the Imipramine-treated group. However, the vehicle-treated stressed group classically showed marked alteration in sucrose preference showing that the CUMS model satisfactorily mimicked depressive status, and this was reversed by treatment with Troxerutin. Previous studies have established the reversal of CUMS-induced behavioural deficits in FST and SPT as primary neurobehavioural contrivance for face-validity of antidepressant activity (Autry *et al*., 2011). It was evident in this study that Troxerutin possesses robust antidepressant activity as it was able to reverse the observed behavioural deficits elicited by long-term unpredictable stress exposure. Accompanying stress mal-adaptation is the usual cognitive decline presenting as memory alteration. The Y-maze explores working

memory ability in animals which is often impaired in behavioural despair. This connotes that depressed animals would perform poorly while being tested in the Y-maze. This study showed reduced percentage alternation in stressed animals but this reduction was reversed in animals treated with Troxerutin. Despite the exposure to unpredictable stressors, the animals treated with Troxerutin showed increased percentage alternation with correlates to higher memory function compared to vehicle-treated stressed animals. The activities of the cholinergic system have been identified to significantly contribute to central memory functions and cognition (Pepeu & Giovannini, 2010).

The effect of Troxerutin on memory function was further elucidated by evaluating the brain AChE activity, which was reversed to normal in Troxerutin treated animals but significantly elevated in vehicle-treated stressed controls. Precipitating neurobehavioural symptoms of depression are biochemical changes exemplified as disarray of their normal homeostatic status.

First of this are the antioxidative biomarkers involved in the attenuation of reactive oxygen species (ROS), their clearance or production control. Excessive ROS production underlies oxidative damage that affects neuronal macromolecules (lipids, proteins, DNA etc.) and resulting neuronal damage and depression. These biochemicals have been used as biomarkers of depressive state because their quantitative level in the brain or serum correlates with biological status and they generally return to normal levels following successful antidepressants treatment (Bilici *et al*., 2001). The results of this study shows derailed endogenous antioxidant status when comparing the unstressed control to the vehicle-treated stressed controls. The MDA level (a measure of lipid peroxidation) was abnormally elevated in stressed negative controls, and likewise the nitrite levels, and conversely, the levels of endogenous antioxidants, SOD and GSH were abnormally reduced significantly. The chronic administration of Troxerutin reverses these changes and returned the endogenous antioxidants to normal levels. In the Troxerutin-treated groups there was significant decrease in lipid peroxidation compared to controls and the GSH levels were markedly increased. Thus, Troxerutin showed a significant antioxidant activity which could be concluded as one of its active antidepressant mechanisms. Another marker of oxidative stress is the corticosterone, which has been found to be abnormally elevated in stress-induced depressive state. In the aetiology of depression, the

HPA-axis is vital to the therapeutic outcome and this is a common pathway required primarily for antidepressant activity.Administration of Troxerutin in the CUMS model significantly reduced serum corticosterone levels, explaining the role of Troxerutin in HPA-axis modulation.

In coping with stress, cytokines are mildly released, however prolonged exposure to stress as modelled in CUMS-induced depression results in cytokines overproduction and uncontrolled inflammatory response leading to neuronal apoptosis. Evaluation of TNF-α, INF-γ and IL-6 levels in brain of animals in the study showed abnormally elevated levels in vehicle-treated stressed mice, but their levels were reversed to normal in Troxerutin-treated animals. Also in immunohistochemistry analysis, the expression of NF-kB was significantly reduced by treatment of animals with Troxerutin. The inhibition of neuroinflammatory markers plays vital role in neuroprotective mechanisms by preventing activation of neurodegenerative mediators and it preserves normal biologic functions despite the stressful exposures. Evidently, Troxerutin shows neuroprotective activity by attenuating cytokines inflammatory responses. Similarly, the levels of MAPKs were significantly increased by treatment with Troxerutin. MAPKs is the chief signalling pathway involved in induction and regulatory mechanism of inflammatory response. Excessive activation of MAPKs cascade reduces the levels and results in dysregulated inflammatory response with overproduction of pro-inflammatory cytokines and chemokines, especially the IL-6, which eventually causes stress mal-adaptation (Klemm *et al*., 2017). In this study the abnormally reduced MAPKs level induced by unpredictable stress was reversed with Troxerutin treatment.

A major hallmark of depression is the neurochemical depletion characterized by decreased level of neurotrophic factors and monoamine neurotransmitters. In the present study the levels of the neurotransmitters serotonin, noradrenaline and dopamine were elevated by administration of Troxerutin, however, their levels were significantly lowered in vehicle-treated stressed controls indicating depressive monoaminergic deficiency. One functional mechanism attributed to antidepressant activity of a bioactive flavonoid is the modulation of the monoaminergic system where they stimulate increase in noradrenaline, dopamine

and essentially, serotonin level in the brain (Yan *et al*., 2015). Significantly, serotonin was markedly increased in brains of animals treated with Troxerutin compared to the control animals, and serotonin has been implicated as the critical neurotransmitter in depressive conditions. Hence, the antidepressant activity of Troxerutin is valid in reversal of neurochemical decline seen in depression. The levels of the neurotrophic factor, BDNF, and the transcription factor, CREB, in the animals subjected to CUMS were significantly lower than normal controls; however, this was reversed by treatment with Troxerutin. The data from this study shows elevated BDNF and CREB in animals treated with Troxerutin, suggesting the antidepressant efficacy of Troxerutin. Neurotrophic factor mediates synaptic plasticity, neurogenesis, and synaptogenesis all of which are essential molecular mechanisms preventing neurones structural and functional impairment that could result in depression. Studies have shown that antidepressant increases the activities of neurotrophic factors and their transcription factors as well. BDNF is a fundamental neurotrophic factor and its transcription factor, CREB, is equally essential for synaptogenesis and neurogenesis by promoting neuronal survival. To support the evidences from the BDNF and CREB quantification, the histological analysis showed high neuronal density in animals treated with Troxerutin. The numbers of surviving neurons were significantly higher in animals treated with Troxerutin at doses 20 and 40 mg/kg compared to the negative controls in both the hippocampus and the prefrontal cortex.

5.5 Antidepressant activities of Troxerutin in neuroinflammatory model on behavioural, molecular and histological markers

LPS-induced depression is a valid model of depression given that administration of LPS initiates inflammatory processes that cause increased neuroinflammation mediated by hypersecretion of proinflammatory cytokines and subsequent neurobehavioural deficits exhibited as depressive disorder. In assessing the neurobehavioural deficits induced by LPS administration, the vehicle-treated animals exposed to LPS in this study shows prolonged duration of immobility when subjected to FST. The troxerutin-treated groups when subjected to FST post exposure to LPS exhibited reduced duration of immobility and the significant difference when compared to the vehicle-treated group that was exposed to LPS showed the antidepressant-like activity of Troxerutin. This effect was

observed in all doses of Troxerutin and the standard drug, Imipramine. Several studies have shown that agents with antidepressant properties reduces the duration of immobility in animals subjected to FST following exposure to LPS and an example is fluoxetine (Ohgi *et al*., 2013). Additionally, the NSF is a major neurobehavioural assessment in LPS-induced depression; it mimics the sickling endophenotype of human depression, loss of appetite and disinterestedness. In this study, evaluation of NSF in LPS exposed animals showed increased latency to feed and very small consumption in the vehicle-treated animals compared to animals that were not exposed to LPS. Treatment with Troxerutin at all doses reverses this deficit by reducing the latency to feed and consuming more feed similar to the Imipramine group. This reduced latency and increased consumption among the Troxerutin-treated animals are evidences of the antidepressant property of Troxerutin. Following the neurobehavioural assessment was the neuroinflammation evaluation where assessment was made for brain antioxidant biomarkers, neuroinflammatory and neuroimmune biomarkers. First was evaluation of MDA, Nitrite, SOD and GSH activity in the brains of the animals in the study. The level of MDA and Nitrite was abnormally elevated while the SOD and GSH levels were significantly reduced in the vehicle-treated animals exposed to LPS. Animals treated with Troxerutin showed a characteristic reversal of these biochemicals derangement despite LPS exposure. There was reduction in MDA and Nitrite levels, and an increase in the SOD activity and GSH levels in the brains of animals that receives Troxerutin. Elevation of endogenous antioxidants is a marker of positive and controlled response to stressors and it is evident in the reduced lipid peroxidation and the decreased the formed nitrite metabolites. Another marker of oxidative damage is the serum corticosterone level. Administration of Troxerutin in the LPS-induced model significantly reduced serum corticosterone levels, explaining the role of Troxerutin of regulation of the HPA-axis. In the aetiology of depression, the role of the HPA-axis is vital to the therapeutic outcome and this is a common pathway required primarily for antidepressant activity. Secondly, the levels of released proinflammatory cytokines were evaluated and the administration of Troxerutin at all doses significantly reduced the levels of brain TNF-α, IL-6, and INF-γ concentration. Also in immunohistochemistry analysis, the expression of NF-kB and iNOS in the prefrontal cortex and the hippocampus was significantly reduced by treatment of animals with

Troxerutin. Nitric oxide is an important neuromodulatory retrograde neurotransmitter whose function is regulated under pathologic conditions by the three NOS isoforms and of the three isoforms, iNOS has been found to be chief mediator in conditions initiating immune responses like the LPS-induced oxidative stress. Therefore amelioration of iNOS expression is a desirable effect in investigating antidepressant activities and Troxerutin demonstrated significant reduction of expression of iNOS in both the prefrontal cortex and hippocampus of animals exposed to LPS. The ability of Troxerutin to attenuate behavioural response in LPS-induced overwhelming oxidative stress was further substantiated by the result of its effect on brain MAPKs which serve as regulator of inflammatory factors activation. The observed decrease in MAPKs concentration in brains of vehicle-treated mice exposed to LPS was reversed in the Troxerutin-treated groups, and the Troxerutin activity was similar to that of Imipramine. Study data shows that the levels of MAPKs were significantly increased by treatment with Troxerutin at all doses. All these neuroinflammatory and neuroimmune attenuation by Troxerutin clearly supports the antidepressant effect of Troxerutin but not completely considering the importance of neurotrophic factors and monoamine neurotransmitters. In the assessment of neurotrophic impact of Troxerutin, the levels of brain BDNF and CREB were significantly increased in both Troxerutin-treated and Imipramine-treated groups. Similarly, there is complete reversal of reduction in serotonin, noradrenaline and dopamine levels in Troxerutin-treated animals. Serotonin was markedly elevated and significantly increases in a dose-dependent fashion among the various Troxerutin groups. A similar observation has been reported in related bioflavonoid, quercetin, where administration at 40 mg/kg reverses behavioural decline of animals in FST and the BDNF expression was upregulated in animals exposed to LPS (Fang *et al*., 2020). Normally, LPS exposure induces increased kynurenine metabolites release and eventual breakdown of tryptophan, a precursor of serotonin. Therefore, the exposure to LPS reduces monoamine levels which are complimentary to the onset of depression (Veit *et al*., 2021). However, this study data shows that administration of Troxerutin prevents onset of depression in LPS-exposed animals, thereby suggesting that Troxerutin possesses viable antidepressant property.

5.6 Antidepressant activities of Troxerutin on Monoaminergic system

To further predict Troxerutin attenuation of neuroamine depletion resulting in depression, the reserpine-induced depression model was employed and the results shows that Troxerutin at all doses reverses the behavioural and neurotransmitter deficit induced by the paradigm. In the TST, which was used as predictive validity assessment test, Troxerutin reduces immobility duration significantly at all doses and its activity was similar to Imipramine-treated animals. Also, in the splash test, the grooming activity was enhanced by treatment with Troxerutin; treatment shows increase in grooming behaviour. While assessing the neurobehavioural evidences, the molecular evidences also supports the findings as Troxerutin reverses the induced depletion of monoamine neurotransmitters. The data showed increase in serotonin, noradrenaline and dopamine levels in Troxerutin-treated animals. In another model of reserpine antagonism where administration of reserpine (2.5 mg/kg, i.p) causes vesicular emptying and consequent depletion of monoamine store resulting in quantifiable depressive symptoms: hypothermia, diarrhoea and ptosis (Bourin *et al*., 1983). This study results shows that prior treatment with Troxerutin prevented the clinical observations of hypothermia, diarrhoea and ptosis induced by reserpine. This outcome is indicative that Troxerutin can promote rapid synthesis of monoamine neurotransmitters, a major requirement for rapid acting antidepressants.

Depressive state is characterized by deficiencies in behavioural and cognitive functions that are often associated with neuronal lesions in multiple brain regions resulting in atrophy in areas, commonly the prefrontal cortex and the hippocampus (Robert *et al*., 2014). In this study, the chronic models classically mimicked depressive state in the animals subjected to the paradigm but vehicle-treated. In the CUMS study for example, this group compared to the normal control, showed prolonged immobility time in FST, reduced sucrose consumption, decreased grooming and decreased alternation in Y-maze, all of which are suggestive of marked depressed state. The histological findings also showed reduced neuronal density with numerous cells having vacuolated cytoplasm, a feature of neuronal atrophy in these animals. However, the administration of Troxerutin reversed all these deficiencies, and generally, agents with antidepressant activity have been shown to reverse the depressive state in rodents via up-regulation of neurotrophic

factors (Hayley & Anisman, 2013). The enzyme-linked quantitative analysis of BDNF levels, primary neurotrophic factor linked with depression, in the three chronic models showed upregulation of BDNF levels in the brain of Troxerutin-treated animals, thus, explaining the observed neurogenesis and reversed depressive state in these animals.

Furthermore, the BDNF-ERK-CREB signalling cascade responsible for regulation of synaptic proteins such as scaffold proteins essential in maintaining synaptic plasticity or G protein-coupled receptors fundamental in post-synaptic transduction have been shown to be dysregulated by exposure to chronic stress (Guan *et al*., 2013). This abnormality in ERK pathway is predictive of depressed state and treatment with antidepressant reverses this dysregulation (Wang & Mao, 2019). Chronic administration of Troxerutin showed elevated levels of BDNF and transcription factor, CREB, and MAPKs, suggesting its regulatory effect on the ERK pathway. Studies have shown that all components of MAPK cascade are localized in the postsynaptic neurons (Mao & Wang, 2016). Hence, activity of Troxerutin on post-synaptic microdomain suggests its stimulatory effect both on synthesis and release of neurotrophic factors. The BDNF release produces immediate upstream ERK response whose activation consequently reverses stress-induced depression (Yao *et al*., 2021). Significantly, this release of BDNF is pivotal in rapid onset of antidepressant activity (Zhang & Stackman, 2015).

The neurotrophic modulatory pathway represents novel molecular target for antidepressant activity (Pilar-Cuéllar *et al*., 2013) and the results of this study have shown that Troxerutin acts via this target mechanism to elicit its antidepressant activities. Also, inflammatory mediators activate the signal transduction pathways and downregulates the corresponding transcription factor, CREB (Noworyta-Sokołowska *et al*., 2013). In this study, Troxerutin acts by regulating the inflammatory cytokines and their levels in mice brain were regularized similar to the normal controls. Basically, antidepressant drugs have been identified to regulate neuroinflammatory processes (Walker *et al*., 2013).

To rule out false positive neurobehavioural activities, the effect of Troxerutin on locomotor activity in the open field was evaluated in all the experimental models in this

study and there were no observed alterations induced by Troxerutin in all respective paradigms. When compared to the respective vehicle-treated naïve animals, there was no significant difference in locomotor activity of troxerutin-treated animals. A previous study suggested that Troxerutin improves cognitive functions in open field without alteration in locomotor activity (Ashfaq *et al*., 2023). The open field tests results of this study were similar, showing that Troxerutin does not alter locomotor activity.

CHAPTER SIX

SUMMARY, CONCLUSIONAND RECOMMENDATIONS

6.1 Summary

In summary, considering the safety profile of Troxerutin and its proven antidepressant-like activity in this study depicted in Fig 6.1 below, it is safe to posit Troxerutin as candidate for clinical antidepressant study for investigational new drug with robust antidepressant potentials.

Collectively, this study of provides neurobehavioural, biochemical, molecular and histological evidences that strongly supports the antidepressant activity of Troxerutin. The elucidated outcome in all antidepressant neurobehavioural assessment showed similar results even under different models.

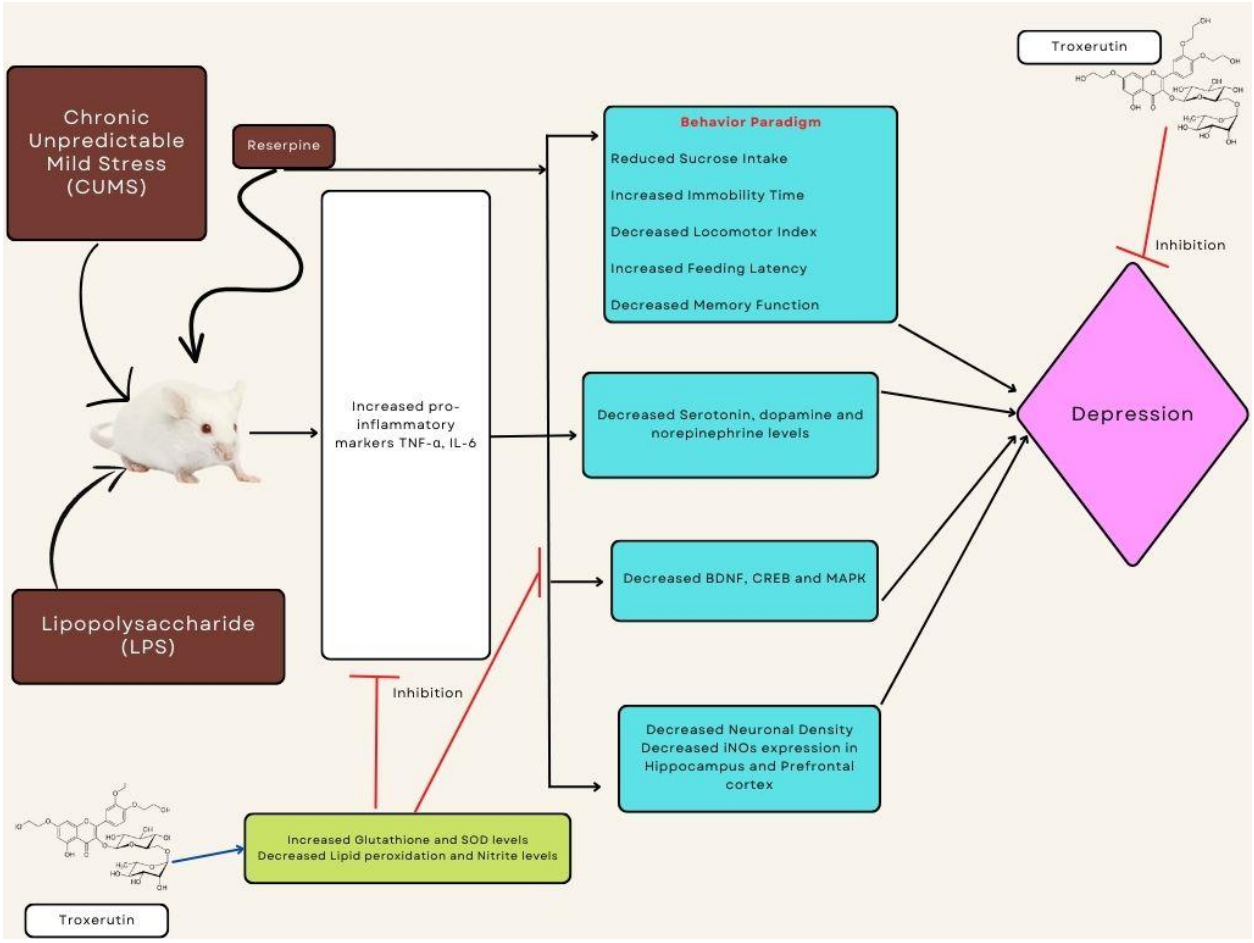

Figure 6.1: Overview of antidepressant activity of Troxerutin in this study.

6.2 Conclusion

This study showed that administration of Troxerutin could normalize neurobehavioural alterations in depressive disorders, reverse the attending neuroanatomical structural changes and regulate neurobiochemical and neurocytokines imbalances elicited at molecular level. These findings evidently confirm the antidepressant effects of Troxerutin through neurobehavioural, neurohumoral and neurobiochemical mechanisms.Therefore, primary findings concluded that Troxerutin could improve the depressive-like symptoms induced by both external and internal oxidative and nitrosative stress. Hence, Troxerutin has a valid antidepressant property which is protracted and potentially rapid in onset, and this antidepressant activity is mediated by regulating monoaminergic system, attenuating meuroinflammatory neuroprotective mechanisms and promoting synaptogenesis.

6.3 Recommendation

The outstanding result from this study impels that Troxerutin evidently has significant antidepressant activity and this should be harnessed by integrating the compound into clinical trial for investigational new drug.

6.4 Contributions to knowledge

This study of antidepressant activity of Troxerutin in mice is novel and the findings showed that Troxerutin being a novel compound can attenuate depressive endophenotypes in mice. This outcome has in many ways contributed to the knowledge of pharmacological properties of this naturally occurring compound, Troxerutin, by showing that it has a very high safety profile and it is therapeutically active at low doses.

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