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Study of the Effects of Empagliflozin on the Model of Chronic Depression and Brain Toll-Like Receptors (TLRs) Gene Expression in Male Rats

A thesis

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بِسْـــمِ اللَّـهِ الرَّحْمَـــٰنِ الرَّحِيـــمِ

(الَّذِينَ آمَنُوا وَتَطْمَئِنُّ قُلُوبُهُم بِذِكْرِ اللَّهِ ۗ أَلَا بِذِكْرِ اللَّهِ تَطْمَئِنُّ الْقُلُوبُ)

صدق الله العظيم

سورة الرعد, الآية (28)

DEDICATION

To my dear mother and father...

To my beloved brothers and sisters...

To my beloved wife...

To my beloved kids, Helen and Humam...

With all love, cordiality and sincerity, I am pleased to present this work to you as part of my gratitude for your supportive role, and I hope to be a source of pride for you.

Hatem.

Supervision certificate

We certify that this thesis entitled **" Study the effects of Empagliflozin on model of chronic depression and brain Toll like Receptors (TLRs) gene expression in male rats"** was prepared under our supervision in the department of pharmacology as a partial fulfillment for the master degree of Pharmacology and Toxicology .

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Summary

Depressive disorders are among the leading causes of global disease burden. Side effects, delayed onset of action, and toxicity are important issues in the present therapies of depression. There is evidence that antidepressants are only modestly helpful in treating depression.

Consequently, a new medications for the treatment of depression are essential. Empagliflozin(EMP) is an oral antihyperglycemic. It is a pleiotropic compound with anti-inflammatory and antioxidant properties.

The aims of this study were to evaluate the antidepressant effect of empagliflozin in male rats and the effect of stress on TLR4 gene expression, pro-inflammatory cytokines, and oxidative stress. To our knowledge, this is the first study conducted to evaluate the antidepressant effect of empagliflozin in brain of male rats.

The animals were divided into five groups each group with 10 rats. For the induction of chronic stress, the Katz method was used with some modifications. The animals in G1 did not treated or stressed, for 24 days, each animal in G2, G3, G4, and G5 underwent stress, Each animal in G2 received 0.5 ml of D.W orally for 14 days starting on tenth day of chronic unpredictable stress (CUS) without any treatment, Each animal in G3 received fluoxetine (10 mg/kg/day+ CUS), and each animal in G4 and G5 was administered (EMP 10 mg/kg/day and 20 mg/kg/day+ CUS, respectively) orally for 14 days starting on tenth day of CUS.

Each animal was weighted and the sucrose preference test(SPT) and the forced swimming test (FST) were performed on days 0, 10, and 25 .The animals were decapitated on the 25th day of experiment, and the brain tissue was prepared for biochemical and gene expression measurements.

There was a significant ($p < 0.05$) decrease in mean weight in G2, G3, G4, and G5 on day 10 as compared to day 0. On day 25 there was a significant decrease in

weight in G2, G4, and G5 as compared to day 0 . In G3,G4 and G5 the means of SPI significantly increased ($p< 0.05$) on day 25 as compared to day 10. On day 25 the means of SPI in G3,G4 and G5 significantly increased ($p< 0.05$) compared to G2. Furthermore, the mean of SPI in G2, G3,G4 and G5 significantly decreased ($p< 0.05$) on day 10, as compared to day 0. In G2, G3,G4 and G5 the means of immobility time in FST significantly increased ($p< 0.05$) on day 10, as compared to day 0.

In G3,G4 and G5 the means of immobility time significantly decreased ($p< 0.05$) on day 25 as compared to day 10.On day 25 the means of immobility time in G3,G4 and G5 significantly decreased ($p< 0.05$) as compared to G2. The TLR-4 gene expression, in G2 there was a significant increased ($p< 0.05$) in the mean of TLR-4 gene fold changes as compared to G1,whereas in G3,G4 and G5 there was a significant decreased ($p< 0.05$) in the mean of TLR-4 gene fold changes compared to G2. A significant ($P<0.05$) increased in IL-6 and TNF-α means concentration in G2 as compared to G1.

In G3 and G5 there was a significant ($P<0.05$) decrease in IL-6 and TNF-α mean concentration compared to G2. In term of antioxidant enzymes, there was a significant ($p<0.05$) increased in the mean of antioxidant enzymes activity in G3,G4,and G5 as compared to G2 .

In conclusion, empagliflozin ameliorate depressive-like behavior induced by the CUS model in male rats. Also, it has anti-inflammatory and anti-oxidant effects in brain of male rats.

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Abbreviations	Meaning
3-HA	3-Hydroxy-Anthralinic acid
3-HK	3-Hydroxy-Kynurenine
5-HT	5-Hydroxy Tryptamine
5-HT2C	5-Hydroxy Tryptamine2 C subunit
ACC	Anterior Cingulate Cortex
ACTH	Adreno-Cortico-Tropic Hormone
APA	American Psychiatric Association
ATP	Adenosine Tri-Phosphate
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
cAMP	Cyclic Adenosine Mono-Phosphate
CAT	Catalase
COVID-19	Coronavirus Disease 2019
CRF	Corticotrophin-Releasing Factor
CRP	C-Reactive Protein
CSF	Cerebro-Spinal Fluid
CUS	Chronic Unpredictable Stress
CuZnSOD/SOD1	Copper and Zinc Superoxide Dismutase
DA	Dopamine
DAMPs	Damage-Associated Molecular Patterns
DNA	Deoxyribo-Nucleic Acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
EC SOD/SOD3	Extracellular Copper and Zinc Superoxide Dismutase
eEF2	Eukaryotic Elongation Factor 2
EPM	Empagliflozin
FDA	Food and Drug Administration
FST	Forced Swimming Test
GABA	Gamma-Amino Butyric Acid
GAPDH	Glycer-Aldehyde-3-Phosphate Dehydrogenase

GPX	GSH Peroxidase
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
HKGs	House-Keeping Genes
HMGB1	High Mobility Group Box 1 protein
HPA	Hypothalamic-Pituitary-Adrenal
HSPs	Heat Shock Proteins
ICD-10	International Classification of Diseases 10th Revision
IDO	Indoleamine 2,3-Dioxygenase
IFN –α	Interferon- alpha
IFN-γ	Interferon gamma
IKK	IκB Kinase
IL-1	Interleukin-1
IL-12	Interleukin-12
IL-18	Interleukin-18
IL-1β	Interleukin-1β
IL-3	Interleukin-3
IL-6	Intereukin-6
IRAK4	Interleukin 1 Receptor Associated Kinase 4
KYN	Kynurenine
KYNA	kynurenic Acid
LPS	Lipopolysaccharide
LTP	long-Term Potentiation
MAOIs	Monoamine Oxidase Inhibitors
MAPK	Mitogen-Activated Protein Kinase
MDA	Malondialdehyde
MDD	Major Depression Disorder
MnSOD/SOD2	Manganese Superoxide Dismutase
mTOR	Rapamycin
MyD88	Myeloid Differentiation primary response 88

NF-κB	Nuclear transcriptional Factor κB
NMDA	N-Methyl-D-Aspartate
OS	Oxidative Stress
PAMPs	Pathogen-Associated Molecular Patterns
PPD	Post-Partum Depression
PRRs	Pattern Recognition Receptors
qPCR	Quantitative Polymerase Chain Reaction
QUIN	Quinolinic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
S100B	S100 calcium-binding protein B
SAD	Seasonal Affective Disorder
SGLT2	Sodium-Glucose co-Transporter 2
SNRI	Serotonin-Norepinephrine Reuptake Inhibittors
SOD	Superoxide Dismutase
SPT	Sucrose Preference Test
SSRIs	Selective- Serotonin Reuptake Inhibitors
TAB	TAK1-Associated Binding
TAK1	Transforming growth factor-β-Activated Kinase 1
TCAs	Tricyclic Antidepressants
TIRAP	TIR Domain-Containing Adaptor Protein
TLR4	Toll-Like Receptor 4
TLRs	Toll-like Receptors
TNF-α	Tumor Necrosis Factor alpha
TPH	Tryptophan Hydroxylase
TRAF6	TNF Receptor-Associated Factor 6
UK	United Kingdom
WHO	World Health Organization

Chapter One
Introduction
And
Literature Review

1.1. Introduction

Depression is a common mental disorder, it involves a depressed mood or loss of pleasure or interest in activities for long periods of time, it can happen at any age of life and it is costly for society (WHO 2023). According to studies, it is a troublesome condition that harms educational pathways, job performance, and other aspects of life. Depression would be the leading source of discomfort among all diseases by 2030 (Zhang *et al*., 2017).

Age, gender, physical health, childhood trauma, and separated or divorced marital status are risk factors for major depressive disorder in human (Kamran *et al*., 2022).

A combination of symptoms including low mood, poor energy or weariness, and anhedonia, comprise the clinical symptoms of this disorder. Other symptoms are frequently present as well including autonomic and gastrointestinal problems, sleep and psychomotor problems, guilt emotions, poor self-esteem, and suicidal thoughts (Kennedy, 2022).

Depression is a complicated disorder with various types and likely several causes, rather than a single, uniform condition. The exact mechanism(s) through which depression develops remain unknown (Kamran *et al*.,2022).

The monoamine hypothesis was the first theory of depression; later, other hypotheses were developed, such as the hypothesis of stress-induced depression, the hypothesis of cytokines, the neuroplasticity hypothesis, and the hypothesis of Gamma-Aminobutyric Acid (GABA) and glutamate (Jesulola *et al*., 2018).

None of these theories can exhaustively explain all the processes and symptoms of depressive disorders (Filatova *et al*., 2021). The modulation of Toll-like receptors (TLRs) is an additional key mechanism that may elucidate the pathophysiology of

depression (Hung *et al*., 2014). TLRs are a group of pattern recognition receptors with a distinct molecular signature of microorganisms known as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Pascual *et al*.,2021). The stimulation of these receptors by their corresponding PAMPs or DAMPs may lead to pro-inflammatory cytokine release and the induction of innate and acquired immune responses (Behzadi *et al*., 2021). Stress results in the release of DAMPs, which bind to toll-like receptors and cause inflammation, which has an essential role in depression's pathogenesis (Serna-Rodríguez *et al*., 2022).

There are two primary therapy choices for depression management: psychotherapy and pharmacotherapy. Several recommendations suggest medication or a combination of psychotherapy and medications for treating moderate-to-severe depression (Nabila *et al*., 2022).

Different medication categories are used for treating depressive disorders (Brigitta, 2022). Selective serotonin reuptake inhibitors, tricyclic antidepressants , serotonin-norepinephrine reuptake inhibitors, monoamine oxidase inhibitors, norepinephrine reuptake inhibitors with serotonin receptor antagonism.

Fluoxetine is one of the most common first-line antidepressants, and it relieves depressive symptoms by increase serotonin concentration in brain (Pourhamzeh *et al*., 2022).

Side effects, delayed onset of action, and toxicity are important issues in the present therapy for depression (Nitzan *et al*., 2022). There is evidence that antidepressants are only modestly helpful in treating depression (Cipriani *et al*., 2018). Moreover, only 41% of depressed people responded well to treatment (Furukawa *et al*., 2021). Consequently, a new target and new medications for the treatment of depression are essential.

Empagliflozin (EMP) was the final SGLT2 inhibitor authorized by the Food and Drug Administration (FDA). It was authorized by the "European Medicines Agency" in May 2014 and the FDA in August 2014. It is an oral antihyperglycemic drug that reduces glucose levels by inhibiting insulin-independent renal glucose reabsorption in the proximal tubules (Chadeve,2020). It is a pleiotropic compound with anti-inflammatory and antioxidant effects (Winiarska *et al*., 2021). Additionally, there is evidence empagliflozin reduces lung fibrosis-related inflammation and oxidative stress (Kabel *et al*., 2020).

Through attenuation of Toll-like receptor-4(TLR4) activity, empagliflozin has a renoprotective effect (Ashrafi *et al*., 2020). Empagliflozin decreases inflammation in the kidneys, liver, and heart in insulin resistance and diabetic animal models (Maayah *et al*., 2021;Ahmed *et al*., 2021). These evidence suggests that the therapeutic advantages of empagliflozin are independent of antidiabetic-related effects. Given its anti-inflammatory and antioxidant properties, empagliflozin may be beneficial for treating depression.

1.2. Aims of the study:-

Evaluate the potential antidepressant effects of empagliflozin.

This will be achieved by the following objectives:

1. Establish a model of chronic unpredictable depression in male rats.

2. Determine the association between stress and TLR4 gene expression, pro-inflammatory cytokines and oxidative stress in the brain of male rats.

3. Study the effects of empagliflozin on TLR-4 gene expression, pro-inflammatory cytokines, and oxidative stress.

Literature review

1.3. Depression

According to the World Health Organization (WHO), depression is a mental illness with clinical features such as despair, a lack of passion, apathy, decreased appetite, feelings of shame, poor self-esteem, disrupted sleep, feelings of weariness, and difficulty focusing (WHO 2023). Over 350 million individuals of all ages worldwide have depression. As stated by the World Mental Health Study taken in 17 countries, 1 in 17 people experienced at least one episode of depression in the past 12 months (Moreno -Agostino *et al*., 2021). In addition, the illness remission rate is often below 50% (Kamran *et al*., 2022).

Depression often begins in adolescence or early adulthood and affects women more frequently than men, with 10–20% of women of reproductive age suffering from postpartum depression (Shitu *et al*., 2019).

The estimated annual cost of lost production due to depression ranges from $30,1 billion to $51,5 billion, demonstrating the negative impact of depression on economy (Greenberg *et al*., 2021). The financial burden of depression per year in the United States, including lost productivity and higher medical expenditures, is $83 billion, which is more than the cost of the war in Afghanistan.

Depression in the UK costs the economy almost £11 billion annually in missed wages, demands on the healthcare system, and medical prescriptions. The cost of depression-related impairment to the Australian economy each year is $14.9 billion, while the cost of treating depression in Australia is over $600 million (Annells *et al*., 2016).

1.3.1. Epidemiology of Depression

Depression is a very common psychological disorder. Its lifetime frequency is roughly 12%, and the occurrence rate among women is about double that of men (Pedersen *et al* ., 2014). This variation has been attributed to hormonal changes, childbearing effects. Each year, over 700,000 people die by suicide . For those aged between 15 and 29, the fourth most common reason for death is suicide (Evans-Lacko *et al*., 2018).

Globally, 34% of 12-19 year-old teenagers are at risk of depression (Shorey *et al*., 2022).Depression was reported by 28.4% of older people worldwide . Depression is more prevalent in those who lack close social interactions, are divorced, separated, or are bereaved (Kim & Lee, 2021).

There has been no variation in the frequency of depression across races and socioeconomic backgrounds . It is more widespread in rural settings than in urban ones . Depression risk factors include age, female sex, family status, medical conditions,, and childhood shock. Depression is also one of the most prevalent comorbidities associated with a variety of chronic medical conditions, including cancer , cardiovascular disease , diabetes mellitus , inflammatory, and neurological problems (Guo *et al*., 2018).

1.3.2. The Etiology of Depression

Biological, genetic, environmental, and psychological variables are hypothesized to contribute to the etiology of depressive disorder (Alshaya ., 2022). Recent concepts imply that it is mostly associated with increasingly complex brain circuits and neuroregulatory mechanisms, leading to neurotransmitter system

disruptions. GABA as well as glutamate and glycine have been shown to participate in depression's pathophysiology (Pedersen *et al* ., 2014).

Furthermore, increased thyroid and growth hormones have been linked to the development of mood disorders (Duval *et al*., 2021). Early exposure to excessive stress may have significant impacts on neuroendocrine and behavioral responses that might change the development of the cerebral cortex and result in serious depression in adulthood (Juruena *et al*., 2020).

Alcohol use may contribute to etiology of depression (Evans *et al*., 2021). Certain drugs, including interferon- alpha (IFN –α) , isotretinoin, and rimonabant, also raised the likelihood of acquiring depression (Fallah & Rademaker, 2022).

Studies including families, adoption, and twins imply that genes contribute to depression susceptibility, and according to genetic studies, monozygotic twins have an extremely high concordance chance of developing depressive episodes (Shadrina *et al*., 2018).

1.3.3 Risk factors for Depression

1.3.3.1. Environmental Factors

Numerous factors may contribute to the onset of depressive episodes, but life events have been examined the most (Flouri *et al*., 2018). A diagnosis of a severe medical condition, the death of a loved one, the birth of a child, financial difficulties, bullying, social exclusion, and delivery are just a few examples of risk factors connected to life events.

The COVID-19 pandemic and the ensuing limitations have had a significant negative influence on psychological health (Benke *et al*., 2020; Rehman *et al*., 2022). As shown by studies, the COVID-19 pandemic significantly increased the symptoms

of depression (Fioravanti *et al*., 2022; Ravens-Sieberer *et al*., 2022; Nogueira *et al*., 2022).

1.3.3.2. Genetic Factors

Psychiatric diseases can be moderately to highly heritable; however, it is unknown to what extent genetic variation is unique to each condition or shared by all of them and according to twin studies and other comparable research, the heritability of Major Depression Disorder (MDD) is modest (37%) but can range from 26% to 49% .Recurrent depression was first shown to be a more heritable variant of the condition (Kendall *et al*., 2021).. Females have a far greater heritability rate than male (Zhao *et al*., 2020).

1.3.4. Types of Depression

1.3.4.1 Major Depression Disorder(MDD)

This type of depression is often characterized by dysphoric mood and loss of motivation, as well as physical body changes including weight gain or loss, increased or decreased hunger, disturbed sleep patterns, and chronic lethargy. In addition to a lack of focus and clarity of thinking, disruptions in cognitive and executive functioning manifest as a preoccupation with death and suicide. Most of these symptoms are often present almost every day, resulting in significant pain and impairments in social and work functioning (Pedersen *et al* ., 2014).

1.3.4.2. Dysthymic Disorder

Alternatively known as “persistent depression disorder”, for at least 2 years in adults and 12 months in children and adolescents, most of the day is spent in a state of depression or sadness. Due to brief remissions, the majority of patients do not

match all of the characteristics for major depressive disorder. Patients may not always fulfill all of the diagnostic criteria for MDD (Fekadu *et al*., 2017).

1.3.4.3. Melancholic Depression

There is a near-total lack of the ability to experience pleasure. Psychomotor lethargy and early morning mood worsening are also seen in this type of depression. Those who have more extreme forms of depression or psychotic depression are more likely to exhibit this type of depression (Benazzi, 2022).

1.3.4.4. Seasonal Affective Disorder (SAD)

This form of depression is supposed to recur each autumn or early winter. The SAD depression is marked by despair, shame, and unworthiness, as well as emotional instability-symptoms that are often prevalent in other depressive diseases. Patients also demonstrate a considerable increase in appetite and a need for high-carbohydrate meals, both of which contribute to weight gain (Praschak-Rieder & Willeit, 2022).

1.3.4.5. Postpartum Depression (PPD)

This depicts a diverse range of depressive symptoms that mothers experience. Before or after giving birth, these symptoms could appear. As a result, they are both considered "peripartum" events. According to the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) , prenatal mood changes, signs of anxiety, and "baby blues" increase the likelihood of postpartum depression (Liu *et al*., 2022).

1.3.4.6. Psychotic depression

It is a serious form of depression that is exhibiting psychotic symptoms. It is frequently viewed as a mixed state that cannot be distinguished from either psychosis or depression. Psychotic characteristics like hallucinations or delusions are among the symptoms. In addition to its intensity, psychotic depression is linked to a protracted course, a subpar response to treatment options, and a greater recurrence rate (Dubovsky *et al*., 2021).

1.3.5. Hypothesis of Depression

1.3.5.1. The monoamine hypothesis

Based on the monoamine hypothesis, the symptoms of depression are caused by changed levels of 5-hydroxytryptamine (5-HT), noradrenaline (NA), and/or dopamine (DA) (Boku *et al*. 2018).

Multiple evidence supporting the idea that antidepressant treatments enhance the neurotransmission of these neurotransmitters served as the foundation for this concept (Delcourte *et al*., 2021). Numerous evidence indicate that catecholamine neurotransmitters are not the only chemicals contributing to the pathogenesis of depressive disorders (Filatova *et al*.,2021).

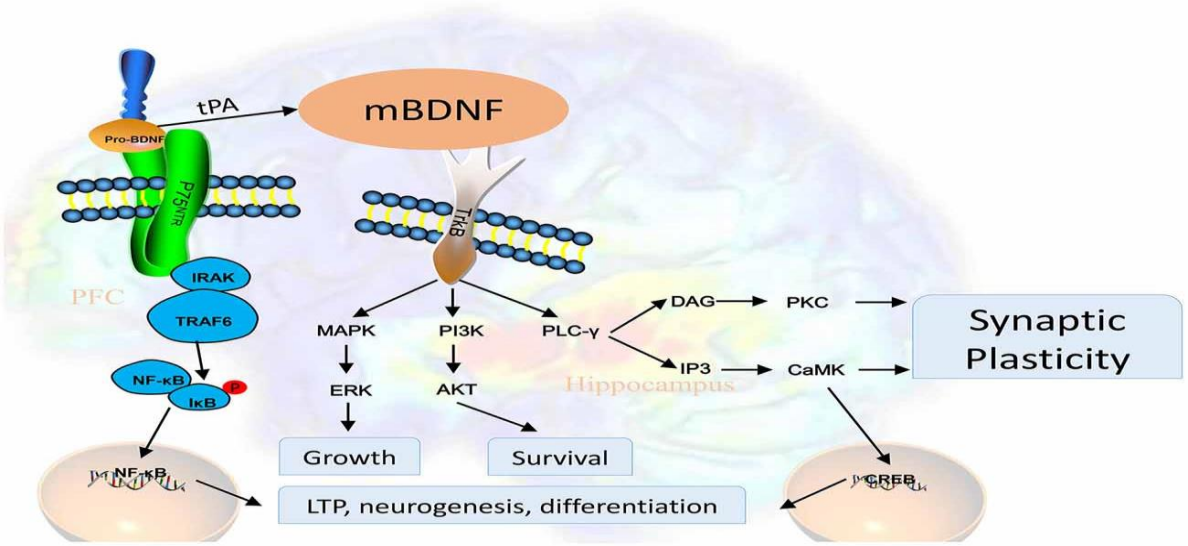
1.3.5.2. Neurotrophic hypothesis of Depression

This hypothesis adopts the role of brain-derived neurotrophic factor (BDNF) to encourages neuronal survival, neuroplasticity, and synaptogenesis (Yang *et al*., 2020)(Fig.1.1). Lowered concentrations of BDNF are related to decreased synaptic plasticity and neuronal atrophy, whereas high concentrations are related to neuronal survival and differentiation (Carniel & da Rocha, 2021). Low neuroplasticity has been associated with depression (Levy *et al*., 2018).

The antidepressant treatment stimulates many levels of neuroplasticity and boosts BDNF expression, resulting in the development of antidepressant effects (Rana *et al*., 2021; Zanos & Gould, 2018).

Certain studies show low BDNF levels in depressed individuals and increased BDNF levels after antidepressant treatment, particularly with SSRIs and ketamine (Arosio *et al*., 2021; Mosiolek *et al*., 2021).

Consequently, it is suggested that BDNF may serve as a biomarker for depression (Rana *et al*., 2021) .



tPA, tissue plasminogen activator; brain-derived neurotrophic factor, ;phosphoinositide 3-kinase (PI3K), AKT, protein kinase B; phospholipase C-γ (PLC-γ), BDNF, IP3, inositol trisphosphate 3; CaMK, calmodulin-dependent protein kinase ;, PKC, protein kinase C ; IRAK, interleukin-1 receptor-associated kinase; TRAF6, tumor necrosis factor receptor-associated factor 6; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; MAPK, mitogen-activated protein kinase; , ERK, extracellular signal-regulated kinases; Trk, activates tyrosine kinase; CREB, cAMP response element-binding protein.

Figure 1.1: Signaling pathways of BDNF (Yang *et al*., 2020).

1.3.5.3. The Cytokine Hypothesis

Proinflammatory cytokines have been shown to have neurotoxic effects, which may be associated with depression (Chen *et al*., 2020). Chronic stress, a crucial contribution to the etiology of depression, is a primary mechanism through which systemic inflammation may result in the augmentation of neuroinflammation (Hayley *et al*., 2021).

Chronic stress alters Blood Brain Barrier (BBB) permeability and the generation of pro-inflammatory cytokines, that may be carried over the BBB by cytokine-specific BBB transporters (Kim & Won ., 2017). It alters astrocyte function via pathways involving prostacyclin, Nitric Oxide and Reactive Oxygen Species (ROS) (Varatharaj & Galea, 2017). It is also raises sympathetic nervous system activity, which in turn elevates the number of peripheral monocytes that may enter the brain and drive microglial activation (Kaufmann *et al*., 2017).

Activated microglia secrete pro-inflammatory cytokines such as IL-1 and Tumor Necrosis Alfa TNF-α , both of which are toxic to the brain (Won & Kim, 2020). When proinflammatory cytokines stimulate the Hypothalamic-Pituitary-Adrenal Axis (HPA axis), cortisol is produced (Milligan *et al*.,2021). Long-term exposure to cortisol may influence inflammatory regulation by decreasing the peripheral immune system's responsiveness to anti-inflammatory feedback (Chen *et al*., 2021).

Blood levels of Interleukine-6 (IL-6), TNF-α , and C-reactive protein (CRP) were significantly higher in individuals with depression compared to healthy subjects in numerous studies of peripheral inflammatory indicators (Osimo *et al*., 2020).

The kynurenine (KYN) pathway is also linked to the neuroinflammatory state (Fig.1.2). L-tryptophan is converted to serotonin by tryptophan hydroxylase (TPH), while proinflammatory cytokines promote the KYN pathway, which converts L-

tryptophan to KYN through indoleamine 2,3-dioxygenase (IDO), a distinct mechanism for tryptophan metabolism (Troubat *et al*., 2021).The enzyme kynurenine 3-monooxygenase then converts kynurenine into the neurotoxic chemicals 3-hydroxy-kynurenine (3-HK), 3-hydroxy-anthralin acid (3-HA), and quinolinic acid (3-QA). KYN is also converted by kynurenine aminotransferases into neuroprotective kynurenic acid (KYNA) (Duda *et al*., 2019).

These product of KYN pathway lead to neurotoxic effects through induce oxidative stress and glutamate-mediated excitotoxicity (Kim & Won, 2017; Troubat *et al*., 2021).

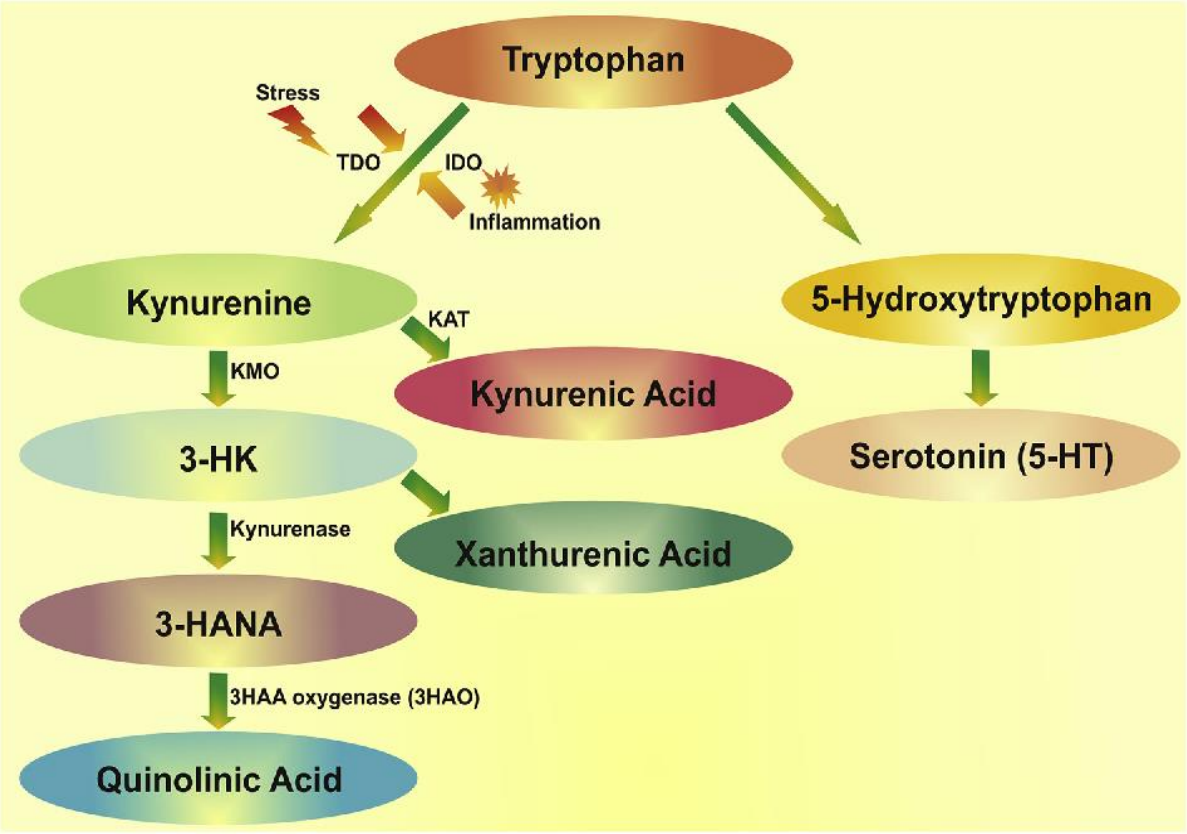


Figure.1.2 : kynurenine pathway (Parrott *et al* .,2016)

Abbreviation: Indoleamine-2,3-dioxygenase (IDO); tryptophan-2,3-dioxygenase (TDO); kynurenine-3-monooxygenase (KMO); kynurenine aminotransferase (KAT); 3-hydroxykynurenine (3-HK); 3-hydroxyanthranilic acid (3-HANA).

1.3.5.4.The endocrine hypothesis

The appropriate activation and cessation of HPA axis activity promotes survival and improves physiological and behavioral stress responses ,while dysregulation lead to depression, anxiety, and post-traumatic stress disorder (Kinlein *et al*., 2022).

According to studies, cortisol levels are higher in depressed patients (Choi *et al*., 2022;Choi *et al*., 2018). In a systematic review, melancholic and psychotic depression showed the most pronounced rise in baseline cortisol levels, although atypical depression did not (Nandam *et al*., 2020).

It's interesting to note that both mania and major depressive disorder can be precipitated by Cushing's syndrome or protracted corticosteroid medication (Santos *et al*., 2021). Higher evening cortisol levels indicate a higher likelihood of long-lasting depression episodes .Increased cortisol secretion may play a role by inducing tryptophan 2,3-dioxygenase, the main metabolising enzyme of tryptophan (Anacker *et al*., 2022)..

1.3.5.5. The Glutamate Hypothesis of Depression

The main excitatory neurotransmitter in the brain is glutamate, an amino acid (McGrath *et al*., 2022). Over the last few decades, there has been a growing understanding of the roles the glutamatergic system plays in the pathogenesis and treatment of mood disorders in general and depressive disorders in particular (Alexander *et al*., 2021).

Although glutamate is present throughout the brain, excessive glutamate release has been linked to brain damage brought on by excitotoxicity . Preclinical evidence of the antidepressant effects of NMDA antagonists supports the hypothesis that the glutamatergic system is involved in mood disorders (Borbély *et al*., 2022).

Additionally, studies that showed changes in the levels of glutamate (both peripherally and centrally) in people with mood disorders supported this involvement (Fullana *et al*., 2019). Ketamine have been shown to have strong antidepressant and anxiolytic effects in recent clinical and preclinical research through modulation glutamate receptor (Zanos & Gould, 2018).

1.3.5.6. Oxidative Stress Hypothesis

Oxidative stress is characterized by an imbalance between Reactive oxygen species (ROS) production and cellular antioxidant capability. Reactive nitrogen species (RNS), ROS, and carbon- and sulfur-centered radicals are all produced as byproducts of oxidative phosphorylation, which is the main source of Adenosine triphosphate (ATP) in cells (Lindqvist *et al*., 2017). Because it consumes a lot of oxygen and has a high concentration of oxidizable lipids, the brain is more vulnerable to harm from elevated ROS than other organs of the body (Schieber & Chandel, 2014). Increased ROS generation may result from this higher oxygen demand (Belleau *et al*., 2019).

The pathophysiology and development of MDD have been linked to elevated ROS by various mechanisms such as tissue damage, inflammation , neurodegeneration , autoimmune mechanisms generated by tissue damage and apoptosis (Mellon, 2016).

1.3.6. Diagnosis of depression

There are currently no particular biomarkers that may be employed in clinical settings or for academic research to support the diagnosis of depression (Kamran *et al*.,2022).In order to diagnosis the depression , multidimensional approach is used that is based on a psychiatric examination using The Diagnostic and

Statistical Manual of Mental Disorders-5 (DSM-5) and the International Classification of Diseases diagnostic criteria-10 (ICD-10) (Carter, 2014).

Depression-related symptoms must satisfy the DSM-5 criteria in order to be identified as such: persons must have feelings of sadness, low mood, and loss of interest in their typical activities that have continued for at least two weeks. These changes must also be accompanied by at least five other common symptoms of depression, including: modification of appetite, weight gain or loss, insomnia, low energy, hopelessness, an inability to focus, Suicidal thoughts .

Under ICD-10, the patient must exhibit two of the first three symptoms (depressed mood, lack of interest in daily activities, and decreased energy) as well as at least two of the following seven symptoms; modification of appetite, weight gain or loss, insomnia, low energy, hopelessness, an inability to focus, Suicidal thoughts . symptoms must have been present for at least two weeks. In both ICD–10 and DSM–IV the symptoms must result in impairment of functioning that increases with the episode severity. No medical problem or drug usage must be the cause of the symptoms, must rule out any other mental health conditions that may potentially cause depression but are not severe depressive disorders(Kennedy ,2022).

1.3.7. Treatment of depression

Although depression is a curable illness, not everyone responds to therapy. Depression is treated with medication and non-medication therapies, including psychotherapy and electroconvulsive therapy (ECT) (He *et al*., 2021; Li *et al*., 2021) Consequently, psychotherapy alone or along with antidepressant medication is often recommended for the treatment of depression.

1.3.7.1 Pharmacological treatment

1.3.7.1.1 Selective Serotonin Reuptake Inhibitors(SSRIs):

These drugs act by inhibiting the reuptake of 5-HT into presynaptic neurons resulting in increased its concentration at synapses. Although SSRIs enhance serotonin levels in the synapses, there is a delayed start of action that can last for two to three weeks (Edinoff *et al*.,2021).

The Food and Drug Administration (FDA) has approved the following SSRIs to treat depression: fluoxetine, citalopram, escitalopram, paroxetine, and sertraline (Edinoff *et al*.,2021).

SSRIs are the first-choice treatment for depressed individuals of all ages due to their high tolerance and broad-spectrum effectiveness. Patients did, however, suffer side effects such as gynecomastia, excitement, diarrhea, sleeplessness
(Ji *et al*., 2019).

1.3.7.1.2. Monoamine Oxidase Inhibitors (MAOIs) :

Monoamine Oxidase Inhibitors increase monoamines concentration at synapses by prevent monoamine oxidases A and B from oxidizing serotonin (5-HT), norepinephrine, and dopamine .

Iproniazid was the first MAOI used to treat depression. Phenelzine and isocarboxazid, in addition to tranylcypromine, are further first-generation MAOIs that are still utilized for Treatment-Resistant Depression combined with another antidepressant , hypertension is an adverse effect of these medications (Aree, 2020).

1.3.7.1.3. Tricyclic Antidepressants

Tricyclic antidepressants (TCAs) inhibit reuptake transporters of monoamines, causing an excess of 5-HT and norepinephrine in the presynaptic cleft, and block 5-HT and norepinephrine transporter presynaptic receptors to increase their activity. TCAs also have a number of negative side effects due to their competitive antagonistic actions on postsynaptic H-receptors, α-receptors , and μ- receptors (Elias *et al*., 2022).

Among the first generation of TCAs created were imipramine, clomipramine, and doxepin. Compared to imipramine, clomipramine is more effective at treating depression. Doxepin improves sleep in depressed patients (Wichniak *et al*., 2017) and is approved by the FDA to treat insomnia (Sateia *et al*., 2017).

Nortriptyline is commonly prescribed to people with major depressive disorder and ischemic heart disease because it reduces platelet activation (Elias *et al*., 2022). Sadly, TCAs may have substantial side effects. The antagonistic activation of H-receptors, α-receptors, and μ- receptors resulted in varying degrees of adverse consequences, including drowsiness and cardiac abnormalities, including irregular cardiac conduction, arrhythmia, and myocardial infarction (Elias *et al*., 2022).

1.3.7.1.4. Serotonin-Norepinephrine Reuptake Inhibitors(SNRIs):

These drugs act by inhibiting the reuptake of serotonin and norepinephrine, two important neurotransmitters implicated in depression, Venlafaxine, the first SNRI, was authorized by the FDA and sold in the United States as a first-line antidepressant (Mekonnen *et al*., 2020).

Desvenlafaxine, duloxetine, levomilnacipran, milnacipran, also authorized for treatment of depression. SNRIs have side effects including drowsiness, constipation,

sleeplessness, and perhaps the disruption of metabolic pathways connected to hyperglycemia (Mekonnen *et al*., 2020).

1.3.7.2. Psychotherapies

Psychotherapies are the second-line treatment for depression after antidepressants, and both are successful. Cognitive behavioral therapy is the most extensively researched form of psychotherapy for depression. however, many other forms, including interpersonal, psychodynamic, problem-solving, behavioral activation (Cuijpers *et al*., 2021). Only 41% of people with depression benefit from psychotherapy (Furukawa *et al*., 2021).

1.4. Oxidative stress

Oxidative stress (OS) is a physiological condition that happens in the body. oxidative stress may be defined as an imbalance between pro-oxidants and antioxidants in the body ,Several enzyme systems such as the superoxide dismutase (SOD), catalase (CAT) and glutathione(GSH) involved in vivo redox homeostasis keep normal intracellular ROS levels low (Liguori *et al*.,2018).

A free radical is any atom or molecule with one or more solitary electrons in its outer layer. Several metabolic processes produce free radicals as a physiological byproduct (Gulcin, 2020).

Some of free radicals are kept in a regulated state because they help the organism perform critical processes; on the other hand, free radicals exist in their natural state and interact with diverse tissue components (Liguori *et al*.,2018). The oxygen-containing radicals are the most significant free radicals generated by metabolic processes (ROS) (Phaniendra *et al*.,2015). The superoxide dismutase and catalase are antioxidant enzymes that eliminate free radicals such as peroxide and hydrogen peroxide and have antioxidant properties (Salehpour *et al*.,2019).

In terms of antioxidant enzyme performance in depressed individuals, it appears that superoxide dismutase is raised or lowered in response to high superoxide concentrations (Bhatt *et al*., 2020) . More particular, SOD appears to be enhanced in red blood cells while decreasing in serum and plasma (Kotzaeroglou& Tsamesidis,2022). The same appears to be true for the enzyme catalase (Bhatt, 2014).

1.4.1. Superoxide dismutase

Superoxide dismutases (SODs) are a group of metalloenzymes. These proteins catalyze the dismutation of superoxide anion free radical (O2-) into molecular oxygen and hydrogen peroxide (H_2O_2) (Younus ,2018).

Superoxide dismutase is regarded as the initial line of defense against rising OS. (Mittal *et al*., 2014). Based on the metal cofactors present in the active sites, SODs can be classified into four distinct groups: Copper-Zinc-SOD (Cu, Zn-SOD), Iron SOD (Fe-SOD), Manganese SOD (Mn-SOD), and Nickel SOD.The different forms of SODs are unequally distributed throughout all biological kingdoms and are located in different subcellular compartments (Younus ,2018). According to many studies, SOD activity is either enhanced or lowered during the depression (Liu *et al*., 2017).Unipolar depression is connected to a high level of OS when antioxidant levels—like SOD—are low and Malondialdehyde (MDA) levels are high (Bajpai, 2014). Antidepressant therapy restores the elevated levels of SODs to normal. Patients' postmortem imaging brain tissues, in particular the prefrontal cortex, showed increased SOD activity. According to a clinical investigation, individuals with acute-phase MDD had significantly higher blood levels of SOD and CAT activity than those without the condition(Caruso *et al*., 2020).

1.4.2. Catalase enzyme

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen which catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS)(Rai, 2019). Catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water and oxygen each second (Muraro, 2019).

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Jena *et al*.,2022). It contains four iron-containing heme groups that allow the enzyme to react with hydrogen peroxide (Hansberg *et al*.,2022). Catalase levels in the brain tissues of stressed mice were shown to be lower as a result of chronic unexpected mild stress (Bhatt, 2014). In acute episodes of depression, catalase concentration has been found to increase in several clinical trials (Camkurt, 2017).

The idea that OS is elevated in depression is thus supported by increased catalase activity. Even in the absence of an altered OS, catalase overexpression enhances learning and memory and lessens anxiety symptoms, and antidepressant therapy raises the levels of this antioxidant enzyme in depressed individuals (Muraro, 2019).

1.5. Interleukine-6(IL-6)

Interleukine-6 is a soluble mediator with a pleiotropic effect on inflammation, immune response, and hematopoiesis. IL-6 is made up of 212 amino acids, including a 28-amino-acid signal peptide, and its gene has been mapped to chromosome 7p21. Although the core protein is ∼20 kDa, glycosylation accounts for the size of 21–26 kDa of natural IL-6 (Zhang *et al*.,2017). Many researches shows that IL-6 plays an important role in the pathophysiology of depression and is the most consistently elevated cytokine in MDD patients' blood samples. A longitudinal study discovered

that children with higher circulating levels of IL-6 at age 9 had a 10% higher risk of developing MDD by age 18 compared to children with lower circulating levels of IL-6 or the general population, providing the first encouraging evidence for the role of IL-6 in the occurrence of depression. Another evidence for potential role of IL-6 in depression is that peripheral levels of IL-6 were found to be positively correlated with symptom severity in patients not respond to anti-depressant treatment (Khandaker et al., 2014).

In animal researches, mice that shown depression symptoms like anhedonia were found with raised circulating levels of pro-inflammatory cytokines such as IL-6 (Haapakoski et al.,2015). Moreover, in another investigation on male Wistar rats, blood levels of pro-inflammatory cytokines such as IL-6 were shown to be greater in acute and restraint stress rats than in non-stressed (Roohi et al.,2021).

Cytokines can reduce serotonin production by activating the enzyme indoleamine 2,3 dioxygenase (IDO), which converts the precursor of serotonin (tryptophan) to kynurenine (KYN) rather than metabolizing tryptophan to serotonin, resulting in serotonin depletion that connected to depression (Zhang *et al.*,2017).

Moreover, cytokines can influence serotonin signaling by increasing monoamine transporter expression and function (Ślusarczyk *et al*., 2015). Previous study found that IL-6 directly regulates serotonin transporter (SERT) levels and so modulates serotonin reuptake (Kong *et al*.,2015).

1.6. Tumor Necrosis Factor Alfa(TNF-α)

TNF-α is a multifunctional cytokine that is involved in a variety of physiological and pathological processes (Kalliolias &Ivashkiv, 2016). It was first discovered for its potential to cause tumor cell necrosis, but it has since been linked to a wide range of biological activities (Sedger & McDermott,2014). TNF-α is

primarily generated and released by macrophages and via numerous cell types capable of generating it including glial cells and neurons in the brain (Probert, 2015).

TNF-α is produced and localizes in the cell membrane until it is proteolytically cleaved by the TNF—converting enzyme, releasing the soluble form of the protein. The transmembrane and soluble forms of the protein are both physiologically active, attaching to and activating TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (Jang *et al*.,2021). Although TNFR1 and TNFR2 share several roles (e.g., development of immune defense systems, induction of inflammation, and encouragement of cell proliferation and survival), they also exhibit unique and sometimes opposing, biological activities (Menard *et al*., 2017). Principally, TNFR1 is associated with pathological processes such as inflammation, apoptosis, and necrosis, while TNFR2 is largely tied to physiological responses such as host defense, tissue repair, and regeneration (Medler & Wajant ,2019).

TNF-α is important in many physiological processes, notably those involving the central nervous system .TNF-α, for example, has a direct influence on neuronal function and survival in the brain, regulating neurotransmitter production and release, managing synaptic transmission, and contributing to myelin creation and maintenance (Cheng *et al*.,2018). A large body of evidence supports the role of TNF-α in depression . Microarray mRNA studies demonstrate increased expression of transemembraneTNF-α in the prefrontal cortex of suicide victims (Brymer *et al*., 2019). In fact, higher suicidal ideation itself is associated with an increased cytokine profile, including elevated TNF-α (Cheng *et al*.,2018), and high circulating levels of TNF-α are found in peripheral tissues of suicide victims (Rani *et al*.,2022). Several lines of evidence support the efficacy of TNF-α inhibitors in the treatment of depression. Patients with rheumatoid arthritis and plaque psoriasis taking prescribed etanercept, which is a TNF-α antagonist, reported significant reductions in depressive symptoms (Benedetti *et al*.,2022).

1.7. Toll-like receptors (TLRs)

Toll-like receptors are pattern-matching receptors that activate innate immune responses in response to the detection of certain molecules. Ten unique TLRs have so far been discovered in humans, whereas 13 have been discovered in rodents (Anwar *et al*., 2019).

By localizing at the plasma membrane or in endosomes, these receptors facilitate the activation of appropriate immune responses against several pathogens, including bacteria, viruses, and fungi. TLRs are capable of detecting a wide range of molecules, including Pathogen-associated molecular pattern molecules (PAMPs), and Damage-associated molecular patterns (DAMPs); these increase susceptibility to depression (Slavich & Irwin, 2014).

Each TLR have ligand-specific PAMPs such as Bacterial tri-acylated proteins (TLR1/6), bacterial di-acylated lipoproteins (TLRs 2/6), bacterial flagellin (TLR-5), bacterial lipoteichoic acid (TLR-2), and Gram-negative bacterial lipopolysaccharide (TLR-4). TLR3, 7, 8, and 9 are endosomal-associated TLRs that recognize double and single stranded viral RNA.

It was recently proposed that TLR10 recognizes double-stranded Ribonucleic acid (dsRNA) and human immunodeficiency virus HIV-1 protein (Henrick *et al*., 2019). TLRs are expressed not only in the immune cells of the periphery but also in the cells of the central nervous system such as microglia, astrocytes, neurons, and oligodendrocytes. These cells could be involved in altering mental moods and might have a direct impact on brain activity, which would help explain depression (Figueroa-Hall *et al*., 2020).

1.7.1. Role of TLR4 in Depression

There are growing evidence that toll-like receptors, particularly TLR4, are important in the pathophysiology of major depressive illness (Zhang *et al*.,2020). The downstream signaling of TLR4 leads to the production of pro-inflammatory cytokines, such as TNF-α and IL-6 in response to exogenous pathogen-associated molecular patterns like Lipopolysaccharide (LPS) (Liu *et al*., 2014).

Stress resulting from physical, physiological, and psychological factors produces and releases DAMPs (Serna-Rodríguez *et al*., 2022). One of the DAMPs in psychiatry that has received the most research is S100 calcium-binding protein B (S100B). This small (10 kDa) protein is one of the S100 family of Ca2+-binding proteins. S100b is a product of astrocytes. An investigations has shown a correlation between depressive-like behavior and elevated hippocampus S100b expression after chronic stress (Hao *et al*., 2020). In the absence of stress, Heat Shock Proteins (HSPs), which act as chaperones and support the production and assembling of proteins, are ubiquitously produced intracellular proteins. However, through binding to the TLR2 and TLR4, during necrosis or stress release of these proteins into the extracellular matrix provokes an inflammatory process and increases the release of inflammatory cytokines (Borges *et al*., 2016).

One of the recognized HSPs, inducible HSP70, which controls inflammatory responses to stress, has been linked to mental conditions including depression (Abautret-Daly *et al*.,2018). Numerous studies using animal depression models observed substantial alteration in HSP70 expression following acute stress exposure (Benatti *et al*.,2018). The abundant nuclear protein high mobility group box 1 protein (HMGB1), which has been associated with various inflammatory illnesses, is present in the majority of cell types (Musumeci *et al*., 2014). In a healthy body, HMGB1 is

mostly found in the nucleus, where it controls gene transcription and serves as a Deoxyribonucleic acid (DNA) chaperone (Musumeci *et al*., 2014).

1.7.2. Signals of TLR4

The downstream signaling of TLR4 depend on the recruitment and activation of molecular machinery that confers plasticity to the system. This machinery is necessary for initiation of two-distinct signaling cascades: the myeloid differentiation protein 88 (MyD88) or the TIR-domain containing adapter inducing IFNβ (TRIF)-dependent signaling pathways. These two pathways are distinguished by the adapter molecules recruited to each receptor

The activation of TLR4 by PAMPs, such as LPS, or DAMPs, such as HMGB1and HSPs, can activate two pathways: the myeloid differentiation factor 88 dependent pathway (MyD88-dependent pathway) and the MyD88-independent pathway (Li & Wu 2021). Following LPS stimulation, interleukin 1-associated kinases-1 and 4 (IRAK1 and IRAK4) are recruited to the MyD88-dependant pathway (A) and interact with tumor necrosis factor associated factor 6 (TRAF6) proteins.

TRAF6 recruits transforming growth factor-β-activated kinase-1 (TAK1) and TAK1-binding proteins (TABs) which activate the Nuclear factor kappa (NF-κB) and/or mitogen-activated protein kinases (MAPK).

In resting states, NF-κB is sequestered in the cytosol by inhibitory IkB kinases (IKKα and IKKβ) (Yu *et al*.,2023). Phosphorylation of the IKK complexes by TAK1 results in their proteasomal degradation and liberation of NF-κB which subsequently translocates from the cytosol to the nucleus where it can induce gene expression (Chen *et al*.,2020). Concurrently, TAK1 activates the MAPK pathway resulting in the phosphorylation and activation of the transcription factor activator protein 1 (AP-1) which translocates to the nucleus and binds to DNA.

Additionally, the LPS/TLR4 MyD88-independent signaling pathway involves the activation of TIR-domain-containing adapter-inducing interferon-β (TRIF) (B) and signaling to TANK-binding kinase (TBK1), IKK and interferon regulatory factor 3(IRF3). This pathway results in interferon-related cytokines, and can potentiate NF-κB gene transcription. Ultimately, gene transcription leads to the production of IL-1β, IL-6, IL-8, TNF-α, TNF-β, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2(COX-2) (Figueroa-Hall *et al*.,2020) (Fig 1.3).

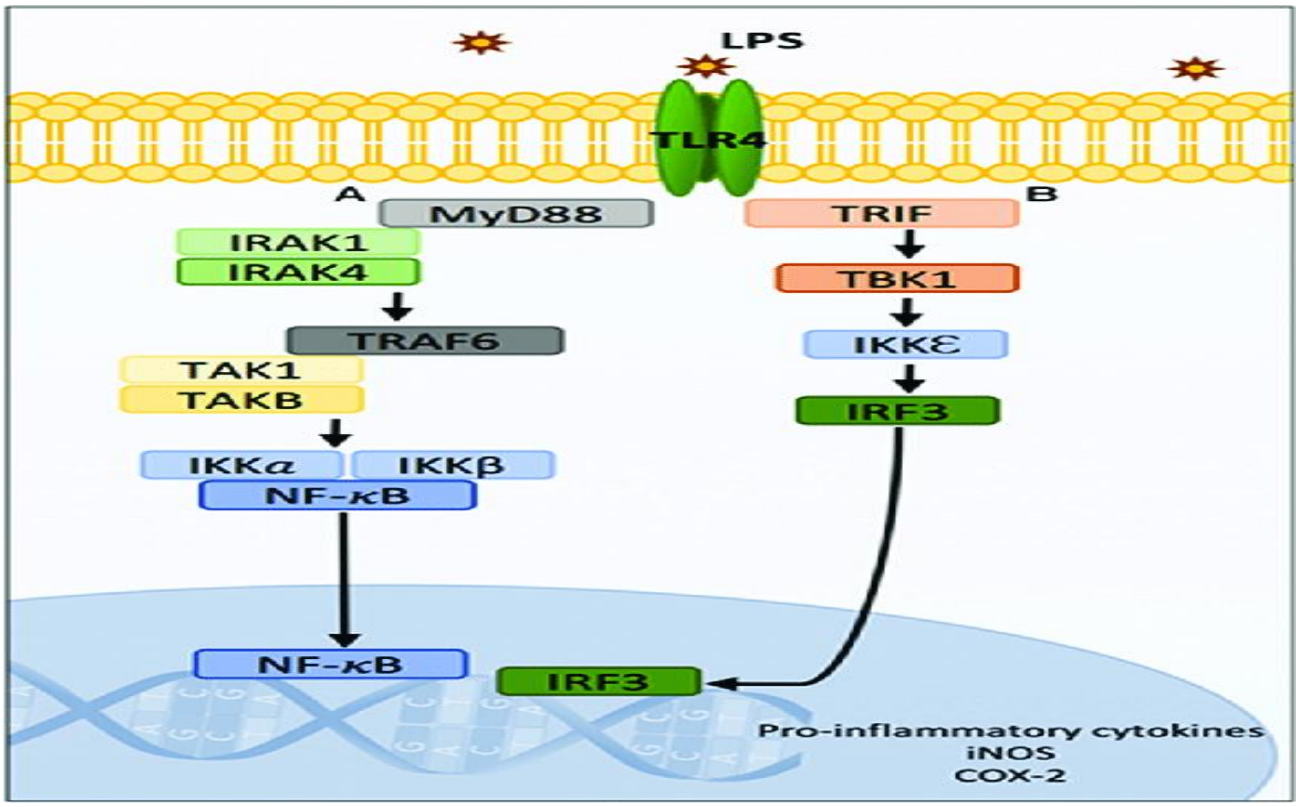


Figure 1.3: Signaling of TLRS in depression (Li *et al*.,2021).

Abbreviations: LPS, Lipopolysaccharide; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response gene 88; IRAK1 and IRAK4, interleukin 1-associated kinases-1 and 4; TRAF6, tumor necrosis factor associated factor 6; TAK1, transforming growth factor-β-activated kinase-1; TABs, TAK1-binding proteins; MAPK, mitogen-activated protein kinases; IKKα and IKKβ, inhibitory IkB kinases; AP-1, activation of the transcription factor activator protein 1; TRIF, TIR-domain-containing adapter-inducing interferon-β; TBK1, TANK-binding kinase; IRF3, IKK, and interferon regulatory factor 3; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2.

1.7.3.Expression Pattern and Functionality of TLR4 Across Species

In the human central nervous system (CNS), TLR4 is expressed by two types of non-neuronal supportive cells: the CNS residential macrophages or microglia and the macroglial cells such as astrocytes . In microglia, TLR4 protein is localized within intracellular vesicles and is undetectable on the cell surface (only ~15% of microglia cells express TLR4). Astrocytes and oligodendrocytes also slightly express TLR4. In astrocytes, TLR4 is exclusively localized on the cell surface and is not detectable within intracellular vesicles. This difference in the TLR4 subcellular localization may be linked to the different phagocytic and antigen processing properties of microglia and astrocytes. Development of multiple sclerosis lesions in humans is associated with higher levels of TLR4 expression. The TLR4 mRNA level increases in a human brain endothelial cell line following oxidative stress (Vaure & Liu, 2014).

Primary rat brain endothelial cells slightly express the TLR4 mRNA under basal conditions . Rat brain expresses TLR4 and its expression pattern evolves during the brain development. The number of TLR4-positive cells is considerably lower in P3 and P5 (preterm human equivalents) rat brains than in P7, P9, or P14 (human early childhood equivalent) brains. Inflammatory events often associate with oxidative stress, which in turn affects TLR expression. As in human, oxidative stress induces TLR4 mRNA synthesis in rat cerebral endothelial cells (Vaure & Liu, 2014).

Rat microglia clearly expresses TLR4 and CD14. Similar to mice but in contrast to humans, neither astrocyte nor oligodendrocyte precursors express TLR4 in rats. Thus, microglia appears to be the major cell type in the rat CNS able to transduce LPS signals. LPS-treated rat microglia from the forebrain induces oligodendrocyte death. LPS does not affect the TLR4 expression in cultured glial cells but LPS-activated microglia produce a wide variety of reactive oxygen species and cytokines. Similar to the mouse, this could explain the microglia-mediated injury to oligodendroglial precursors following LPS exposure since rat oligodendroglial precursors are vulnerable to such mediators (Vaure & Liu, 2014).

1.8. Empagliflozin

Empagliflozin is a strong and specific inhibitor of sodium-glucose cotransporter 2 (SGLT2). Since August 2014, the FDA has approved empagliflozin for the treatment of type II diabetes mellitus (T2DM) (Moon *et al*., 2022). Approximately 90% of renal glucose reabsorption in the proximal tubule of the nephron is mediated by SGLT2 (Ramani *et al*.,2022). It is lipid-soluble and cross the blood-brain barrier

(Tahara *et al*.,2016). Recent research indicates that empagliflozin has powerful anti-inflammatory properties (Lee *et al*., 2021).

Furthermore, a significant reduction in mitochondrial ROS levels, IL-6 and NF-kB expression, and an increase in SOD1(SOD bind to copper and zinc) were observed (Uthman *et al*., 2022). This gives evidence that empagliflozin therapy has anti-inflammatory and antioxidant characteristics (Canet *et al*., 2021). By blocking the TLR4/NF-kB signaling pathway, empagliflozin produced renoprotective, antioxidative, and antiapoptotic effects and reduced inflammation (Al-Wakeel *et al*., 2022). Empagliflozin significantly reduced the levels of inflammatory markers such as TNF-α , TLR4, IL-6, and IL-18 (Maayah *et al*., 2021).

In human proximal tubular cells, SGLT2i inhibit the formation of ROS and the expression of pro-inflammatory markers (including TLR4, NF-kB, and IL-6) caused by high glucose level (Pirklbauer, 2021). Empagliflozin has been found to lower the formation of ROS and increase antioxidant defense systems (Gokbulut *et al*., 2022).

1.8.1. Chemical structure of Empagliflozin

Formula($C_{23}H_{27}ClO_7$) . It is A C-glycosyl compound consisting of a β-glucosyl residue having a (4-chloro-3-{4-[(3S)-tetrahydrofuran-3-yloxy]benzyl}phenyl group at the anomeric center (Figure 1.4).

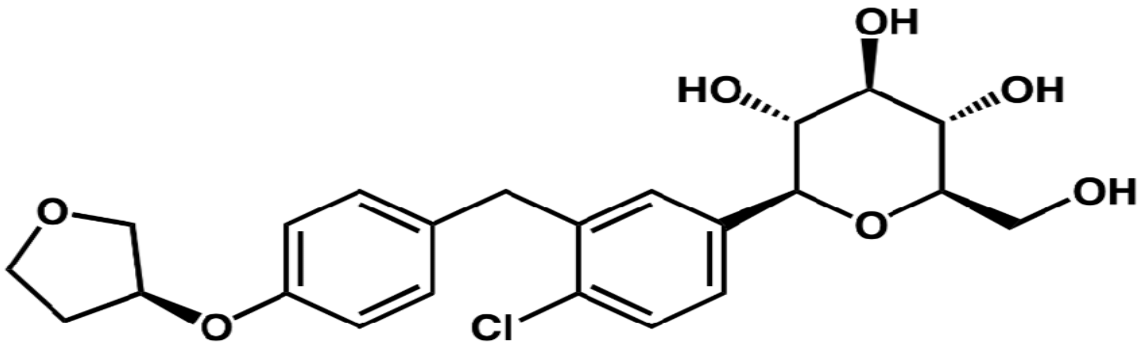


Figure 1.4:Structure of empagliflozin (Moon *et al*.,2022).

1.8.2. Mechanism of action of Empagliflozin

Empagliflozin increases urine excretion of glucose and decreases renal reabsorption of glucose via inhibiting SGLT2 present in the proximal tubules of the kidneys. The medication has a glucose-lowering effect without the help of insulin.

Empagliflozin at doses 10 mg and 25 mg increase urine glucose excretion by around 64 grams daily and 78 grams daily in diabetic patients, respectively. Additionally, empagliflozin has been linked to decreased blood pressure and weight loss without raising heart rate (Heise *et al*., 2016) (Figure 1.5).

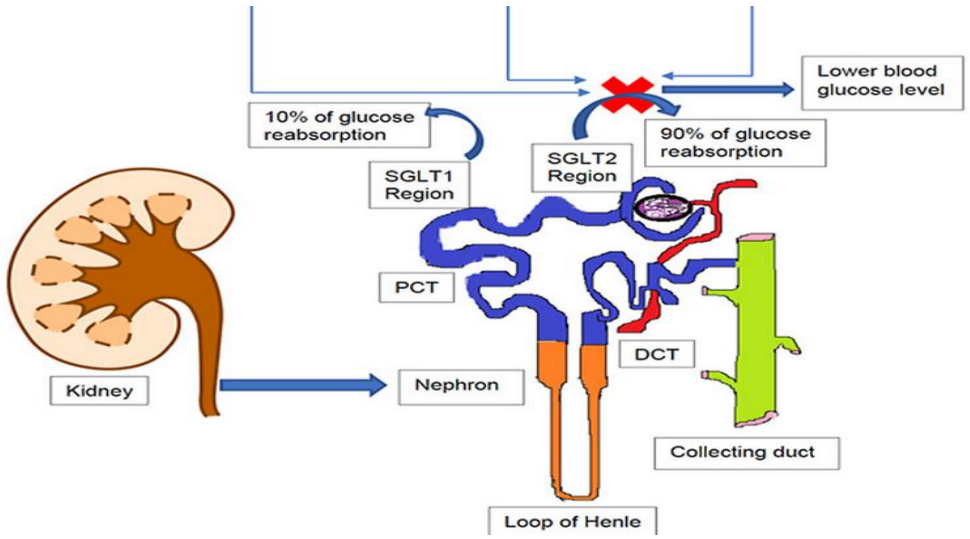


Figure 1.5: Mechanism of action of empagliflozin (Ramani *et al*.,2022).

1.8.3. Side effects of Empagliflozin

Based on their mode of action, SGLT2 inhibitors produce a variety of side effects, including urinary tract infections, vaginal infections, and diabetic ketoacidosis. In clinical studies empagliflozin was typically well tolerated (Kaku *et al*.,2022).

1.9. Fluoxetine

Fluoxetine is a selective serotonin reuptake inhibitor of the second generation SSRI. It acts as an antidepressant and the FDA approved its use for other conditions including obsessive-compulsive disorder, panic disorder, bulimia, premenstrual dysphoric disorder, and bipolar depression (Shelton, 2022).

Fluoxetine has unapproved applications for treating social anxiety disorder, adult post-traumatic stress disorder, Raynaud's phenomenon, and Autism (Zanardi *et al*., 2022). The oral bioavailability of fluoxetine is 90% this allows the medication and its active metabolite, norfluoxetine, to be transported to the brain (Wenthur et al., 2014).

1.9.1. Chemistry and structure of Fluoxetine

Formula ($C_{17}H_{18}F_3NO$)

It is An N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine that has an R configuration (Figure 1.6) .

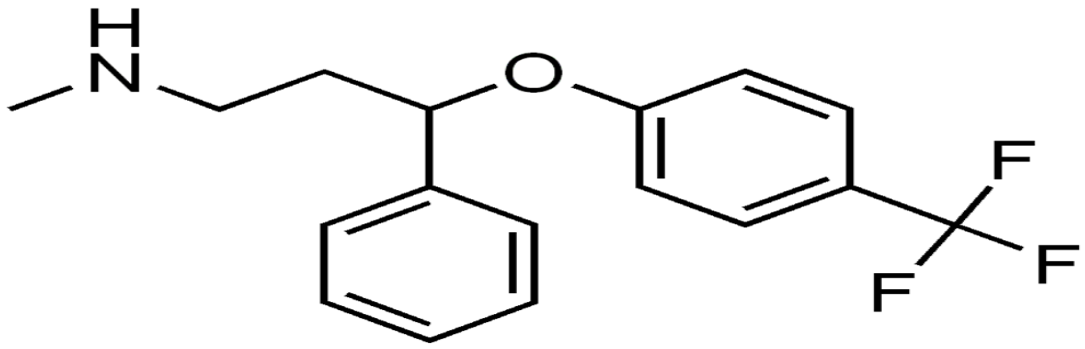


Figure 1.6: Structure of fluoxetine (Shelton,2022).

1.9.2. Mechanism of Action of Fluoxetine

Fluoxetine exerts its effects by blocking the reuptake of serotonin into presynaptic serotonin neurons by blocking the reuptake transporter protein located in the presynaptic terminal. As a result, levels of 5-HT increase across the brain . Fluoxetine also has mild activity at the 5HT2A and 5HT2C receptors.

Fluoxetine has minimal activity on noradrenergic reuptake. Due to its reuptake of serotonin, fluoxetine produces an activating effect, and due to its long half-life, the initial antidepressant effect emerges within 2 to 4 weeks (Efinger *et al*., 2021).

1.9.3. Side Effects of Fluoxetine

Most common side effects reported by adults include insomnia, nausea, diarrhea, anorexia, dry mouth, headache, drowsiness, anxiety, nervousness, yawning, decreased libido, decreased arousal (seen as decreased lubrication in women and decreased erectile function in men), bruising, bleeding (rarely), hyperhidrosis, suicidal ideation and behavior (especially in teenagers), weight gain/loss, decreased orgasm (anorgasmia and ejaculation latency), muscle weakness, tremors (Mullen,2018)

1.10. Animal Models of Depression

1.10.1. Chronic Unpredictable Stress (CUS)

The chronic unpredictable stress paradigm is the animal model most widely utilized (He *et al*., 2020). Willner et al. created a model of chronic mild stress (CMS) in which alternating types of mild stressors are applied (Willner, 2017). The chronic unexpected stress created a depression-like phenotype with favorable facial, structural, and predictive validity (Becker *et al*., 2021).

The chronic unpredictable stress paradigm causes a variety of physiological alteration in rodents, including sleep disruption, a decline in sexual, aggressive, and exploratory behavior, a drop in locomotor activity and weight, stimulation of HPA axis with enhanced expression of CRH (corticotropin-releasing hormone) in the hypothalamus, ACTH (adrenocorticotropic hormone)/corticosterone hypersecretion and adrenal hypertrophy (He *et al*., 2020). The chronic unpredictable stress produces aberrant regulation of the HPA axis, alterations in the serotonergic, noradrenergic, and dopaminergic systems, and a decrease in hippocampus BDNF levels (Becker *et al*., 2021).

Typically, to produce chronic unpredictable stress, animals are randomly subjected for three weeks to a range of long-term inescapable stressors, including walking on ice, tube restraint, tail suspension, day and night reversal, tail clipping, and water or food restriction (Lezak *et al*., 2022). Afterward, behavioral features are evaluated. Anhedonia and despaired are the most prominent symptoms seen by animals exposed to CUS (Planchez *et al*.,2019). Chronic antidepressant therapy increase sucrose preference in Sucrose Preference Test (SPT) and extended periods of immobility in the Forced Swimming Test (FST) (Sequeira-Cordero *et al*., 2019).

1.10.2. Behavioral tests

1.10.2.1. Sucrose Preference Test (SPT)

Anhedonia, or a diminished ability to sense enjoyment, is frequently assessed in animals using the sucrose preference test (Markov,2022). An effective depression model with anhedonia is shown by a reduction in the sucrose uptake ratio of an experimental animal. It is commonly acknowledged that the SPT is the best-suited behavioral test for the chronic mild stress paradigm (Hao *et al*., 2019). The two primary internal characteristics that characterize an exceedingly severe depressive state in psychopathology are anhedonia and pressure sensitivity.

It also symbolizes a state of exhaustion or a lack of energy brought on by depressive stress, which antidepressants can treat (He *et al*., 2020). An animal is considered depressed if its sucrose intake is decreased. This test has the following benefits : (1) It is the simplest test for anhedonia in animals with depression; (2) it causes less animal suffering; and (3) it is appropriate for use in investigations on the comorbidity of depression (Hao *et al*.,2019).

1.10.2.2. Forced Swimming Test (FST)

The FST is based on the concept that when an animal is placed in a container filled with water, it will first try to escape but gradually show immobility which might be interpreted as a sign of behavioral despair. For a variety of reasons, the FST model is widely used in animal research (Gorman-Sandler *et al*.,2021). First, it involves subjecting the animals to stress, which has been linked to a propensity for depression. Additionally, depression is sometimes seen as an inability to deal with stress. Second, it has been demonstrated that taking antidepressants pharmacologically before the test can minimize immobility in the FST. It is frequently used to screen for new compounds with antidepressant properties (Harada *et al*.,2023). Additionally, it has been demonstrated that the FST exhibits some of the same characteristics as human depression, including alterations in food intake, irregular sleep patterns, and medication withdrawal-induced anhedonia. This is also the rationale for the use of this test to assess depressive-like behavior in mutant mice, together with an alteration in baseline immobility (Hao *et al*., 2019).

1.11. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Regardless of a cell's unique purpose within a tissue or organism, housekeeping genes are necessary for the upkeep of fundamental cellular processes that are necessary for a cell to survive. Therefore, it is anticipated that they will be produced in every cell of an organism under normal circumstances, regardless of the

type of tissue, developmental stage, cell cycle phase, or external signal. In methods like qRT-PCR, Western blotting, RNA blotting, and others, HKGs are frequently employed as reference genes to normalize the expression of target genes (Jiang *et al*., 2020).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a critical regulatory enzyme that catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate during glycolysis and is one of the most often utilized housekeeping genes in gene research. Housekeeping genes including GAPDH, frequently utilized as reference genes because their expression levels stay reasonably steady in response to any treatment (Jiang *et al*., 2020).

Chapter Two
Materials and Methods

2.1.Materials

2.1.1.Animals

Fifty male rats were purchased from a private animal house in Babylon province. They weighed (245-300 g). The rats were kept at room temperature with a 14/10-hour light-dark cycle and without limitations, access to food and water in the College of Medicine/University of Babylon's Animal House

2.1.2.Instruments and Equipment

The instruments and equipment utilized in the study with their suppliers are listed below (Table 2.1).

Table 2.1: List of instruments and equipment.

No.	**Instrument / equipment**	**Company / Country**
1	ELISA reader	CYAN /Belgium
2	Eppendorf tubes	Sigma(England)
3	Exispin vortex centrifuge	Bioneer/ Korea
4	High speed Cold Centrifuge	Eppendorf/ Germany
5	Hot plat stirrer	Labtech /Korea
6	Incubator	Memmert (Germany)
7	Micropipettes (different volumes)	Eppendorf / Germany
8	Miniopticon Real Time PCR	Bio-Rad/ USA
9	Nanodrop	Thermo Scientific/ UK
10	Refrigerator	Concord/ lebanon
11	Sensitive balance	Sartorius (Germany)
12	Spectrophotometer	CYAN /Belgium
13	Thermocycler apparatus	Bioneer/ Korea
14	Vortex	CYAN/ Belgium
15	Water bath	PolyScience (USA)

2.1.3.Kits of RNA Extraction

The kits utilized in this study, together with their manufacturers was listed in the following table (Table 2.2).

Table 2.2 :The kits of RNA extraction, DNase I enzyme, M-MLV Reverse Transcriptase and GoTaq® qPCR Master Mix used in the study.

No.	Kit	Company	Country
1	**Total RNA Extraction Kit AccuZol™**	**Bioneer**	**Korea**
	Trizol reagent 100ml		
2	**DNase I enzyme kit**	**Promega**	**USA**
	DNase I enzyme		
	10x buffer		
	Free nuclease water		
	Stop reaction		
3	**M-MLV Reverse Transcriptase kit**	**Bioneer**	**Korea**
	M-MLV Reverse Transcriptase (10,000U)		
	5X M-MLV RTase reaction buffer		
	dNTP		
	100mM DTT		
	RNase Inhibitor		
4	**GoTaq® qPCR Master Mix**	**Promega**	**USA**
	qPCR Master Mix, 2X SYBER green dye, Taq DNA polymerase dNTPs (dATP, dCTP, dGTP, dTTP) and 10X buffer		
	DEPC water		

2.1.4. Primers:

National Center for Biotechnology Information (NCBI)-Gene Bank database and the online Primer 3 design program were used to create the primers for the target gene (TRL-4) and the housekeeping gene (GAPDH). These primers were provided by (Bioneer company, Korea) as following table (Table 2.3):

Table 2.3: Primers of TLR-4 and housekeeping gene.

Primer	Sequence (5'-3')		Product Size	NCBI Reference
TLR4 gene	F	**TCCGCTGGTTGCAGAAAATG**	84bp	NM_019178.2
	R	AGGCAGGAAAGGAACAATGC		
GAPDH gene	F	AGTTCAACGGCACAGTCAAG	99bp	NM_017008.4
	R	CCCATTTGATGTTAGCGGGA		

2.1.5. Chemicals :

The chemical substances employed in this study are listed in the following table:

Table 2.4 : A list of chemicals and their sources that used in the study.

No.	Chemicals	Company	Country
1	Chloroform	Labort	India
2	DEPC water	Bioneer	Korea
3	Isopropanol	Labort	India
4	Absolute Ethanol	Labort	India
5	RNase free water	Bioneer	Korea

2.1.6.Drugs

Empagliflozin 25 mg tablets from (Boehringer company, Germany) were used in this experiment. Empagliflozin was prepared as suspension. Fluoxetine suspension (FLUNIL), 20mg/5ml manufactured by (INTAS company, India) used in this experiment. Based on previous study in rodent models demonstrating anti-inflammatory and anti-oxidative properties, the doses of empagliflozin (10 mg/kg and 20 mg/kg per day) were used in the current study (Heimke *et al*., 2022).

2.2.Methods

2.2.1.Study design

This study was an experimental animal study in which 50 male albino rats were used, five groups of 10 rats have randomly divided into 10 cage, each cage has five rats. The study has begun on September 1, 2022, until February 1, 2023, in the College of Medicine/University of Babylon's Animal House.

2.2.2. Ethical Approval

This study was approved by the committee of publication ethics at College of Medicine, University of Babylon, Iraq. Under the reference No.4-1 on 6-7-2022.

2.2.3.Chronic unpredictable stress (CUS)

For the induction of chronic stress, the Katz method was used with some modifications. The protocol was used as prescribed by previous study (Jasib *et al*.,2022) .The stressors were used in the CUS protocol include the following (Picture 2.1):

1-Wet bedding (300 ml of water was added to sawdust bedding and mixed).

2-Cage tilting (the top of the cage tilted upwards by 45 degrees, containing the food and water).

3- Loaded cage (10 rats in one cage)

4- Light/dark cycle inversion.

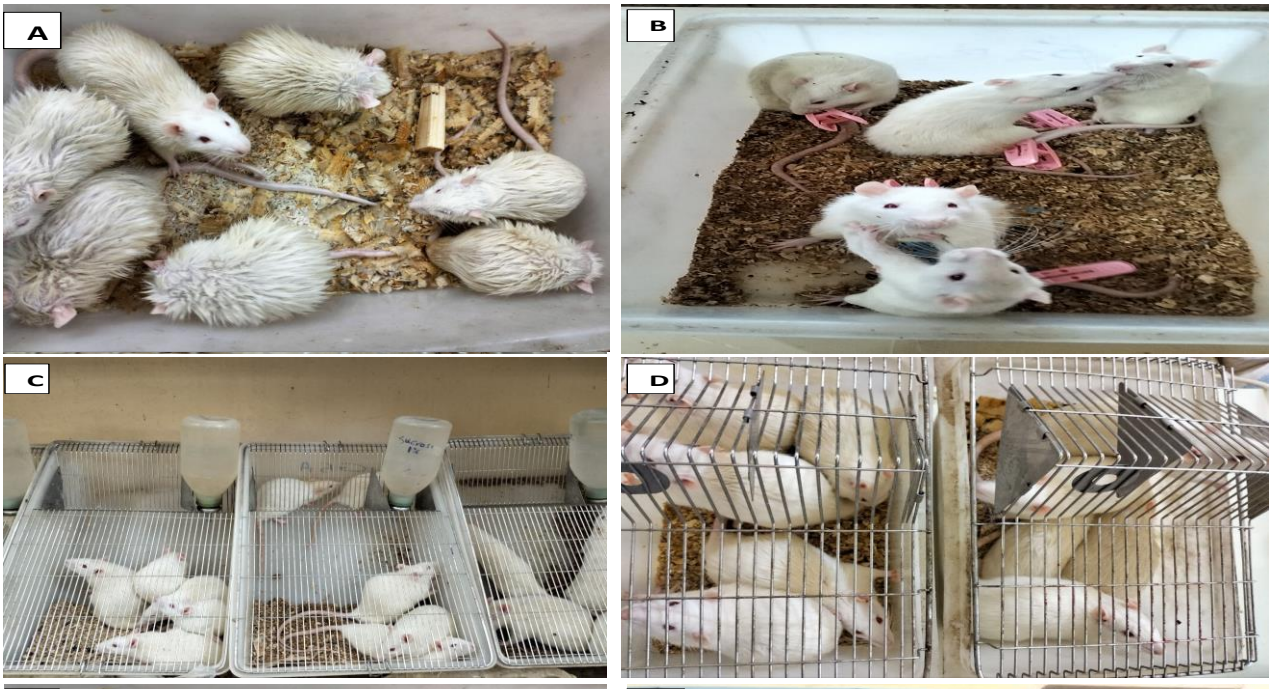
5-Five rats in a tiny cage are restrained and placed in an immobile posture.

Each stressor was presented to the animal three to four times during the experiment. To examine depressive-like behavior in rats using the CUS model, the sucrose preference test and the forced swimming test were performed. These tests were performed on day 0 as a baseline, on day 10 to assess depressive-like behavior, and on day 25 to evaluate the anti-depressive effects of a particular medicine.

Table 2.5: Chronic Unpredictable Stress protocol.

Day	CUS protocol
1	15 minute forced swimming, 12 hrs crowded cage
2	12 hrs cage tilting(45°),1hr restraint
3	Reversal of the light/dark cycle
4	12 hrs wet bedding,12 hrs crowded cage
5	24 hrs food deprivation, 1 hr restraint
6	2 miutes Tail pinch,12 hrs water deprivation
7	12 hrs cage tilting(45°),1hr restraint

8	15minutes forced swimming,12 hrs crowded cage
9	Reversal of the light/dark cycle
10	12 hrs wet bedding,12 hrs crowded cage
11	24 hrs food deprivation, 1 hr restraint
12	Reversal of the light/dark cycle
13	12 hrs wet bedding, 12 hrs crowded cage
14	24 hrs food deprivation, 1 hr restraint
15	2 minutesTail pinch,12 hrs water deprivation
16	12 hrs cage tilting(45°),1h rrestraint
17	15 minute forced swimming, 12 hrs crowded cage
18	24 hrs food deprivation, 1 hr restraint
19	Reversal of the light/dark cycle
20	12 hrs wet bedding, 12 hrs crowded cage
21	2 minutesTail pinch,12 hrs water deprivation
22	12 hrs food and water deprivation
23	12 hrs cage tilting(45°),1hr restraint
24	Reversal of the light/dark cycle

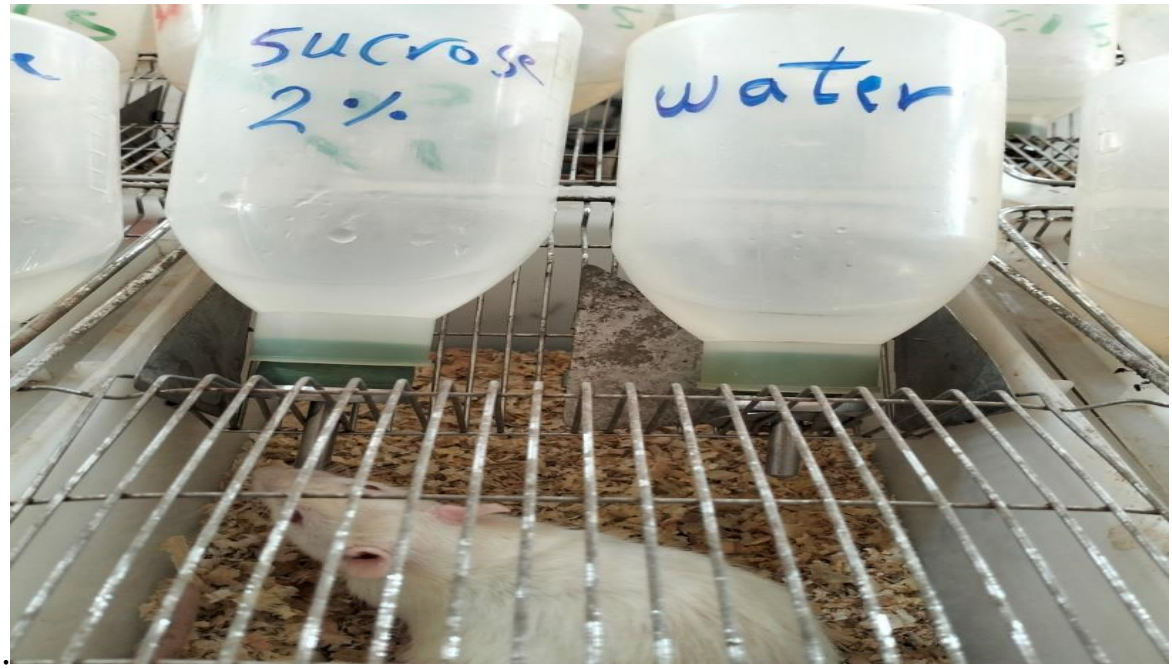


Picture 2.1: Chronic Unpredictable Stress Protocol(CUS):A) Wet bedding. B)Restraint .C) Gage tilting. D) Crowded cage .

2.2.4.Sucrose Preference Test(SPT)

The sucrose preference test was used to assess anhedonia in rats as one of the fundamental symptoms of depression (Markov,2022). Rats were initially trained to drink 2% sucrose solution for 3 consecutive days in two bottles for a cage without water access. Then one of these bottles was replaced with a bottle of tap water for the following 24 hours.

The rats then spend the next 24 hours without food or drink. After this period of deprivation, rats were subjected to SPT for one hour as follows: each rat was removed from its cage and placed in another one that was supplied with two bottles, one of which contained 2% sucrose solution and the other one, tap water with change the position of the two bottles each 30 min. The following formula was used to calculate SPI: sucrose preference (%) = sucrose intake/(sucrose intake +water intake) ×100%.



Picture.2.2:Sucrose Preference Test.

2.2.5. Forced Swimming Test (FST)

This behavioral test was employed to evaluate the despair behavior of depression (Belovicova *et al*.,2017). In the FST, rats were subjected to an unavoidable swimming circumstance in which they must swim in a water-filled cylindrical container (20cm*70 cm dimensions) until they stop struggling after a time of vigorous activity.

As previously documented in numerous research (Muhammad *et al*.,2021), the rats were trained to swim for 15 minutes, and then the test was conducted the next day but with a 5-min time limit. Water was replaced and the cylinder was completely washed following each animal test. To prevent hypothermia, the animals were then dried and kept warm using heating lamps. The duration of each animal's total immobility was determined after the test was recorded. The animal looks to be floating during the whole time it is immobile, stops moving, and only makes little

movements to maintain its head above the water's surface was measured as immobility time picture (2.4).

Picture 2.3:Forced Swimming Test.

2.3. General procedure of experiment

1- For each rats were performed behavioral test, including SPT and FST, on days 0, 10, and 25.

2- The rats in G1 were not exposed to CUS and not get any treatment for 24 days.

3- The rats in G2 exposed to CUS for 24 days and received 0.5 ml of distilled water through oral gavage for fourteen days starting on the tenth day of CUS without any treatment.

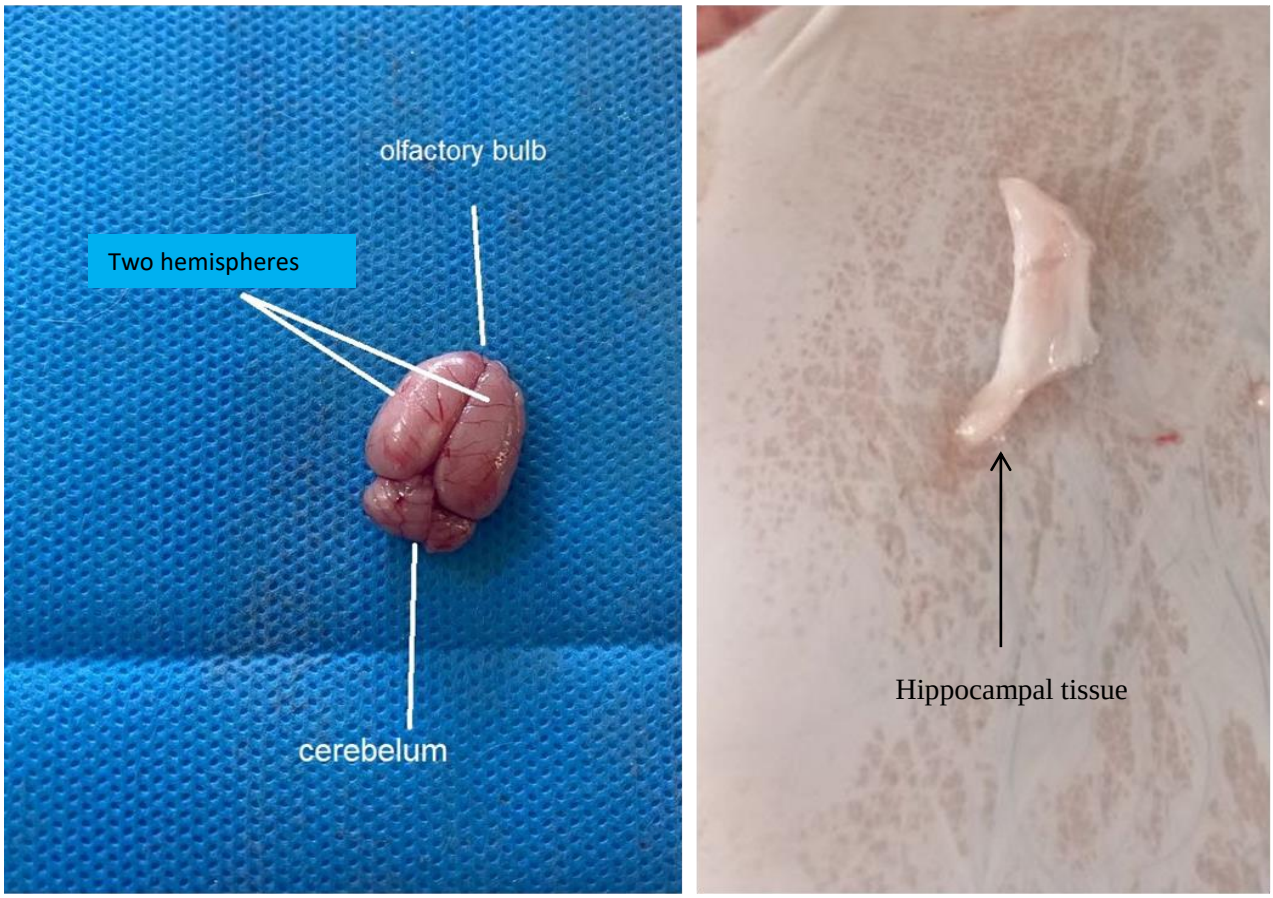
4. The rats in G3 exposed to CUS for 24 days and received fluoxetine treatment (10 mg/kg/day) orally every day for fourteen days starting on day 10 of CUS.

5. The rats in G4 and G5 exposed to CUS for 24 days and received an oral gavage dosage of empagliflozin 10 mg/kg/day and 20 mg/kg/day respectively for fourteen days starting on day 10 of CUS.

2.4. Rat brain decapitation and processing of tissue samples

The animals were decapitated on the 25th day, 24 hours after the last dose of treatment. After dissecting the skull from the posterior foramen magnum, the brains were extracted. The cerebellum and olfactory bulbs were removed, and the brain was carefully removed from the skull. The hippocampus, which has a banana- like shape was removed carefully. The cortex was held while rolling up the hippocampus using two spatulas.

The isolated hippocampus was directly kept in Trizol reagent and transferred to the laboratory for RNA extraction and measurement of gene expression for TLR4 and GAPDH. The remaining part of the brain tissue was washed with phosphate buffer saline (PBS) to remove excess blood and weighed before homogenization. It was homogenized in phosphate buffer saline (1g of total brain tissue was homogenized in 10 mL of PBS with a glass homogenizer on ice) and used for IL-6,TNF-α,SOD, and CAT measurements.



Picture 2.4: Brain and Hippocampal tissue of male rat.

2.5. Measurement of Superoxide Dismutase Activity

2.5.1.Procedure of SOD measurement:

Superoxide dismutase was measured according to SOD kit (SolarBio, China) protocol.

1- Homogenized 1 g of rat brain tissue with 10 mL of extraction reagent on an ice bath, and then centrifuged at 8000 ×g for 10 minutes at 4℃ to remove insoluble materials, and place the supernatant on ice before testing.

2. A spectrophotometer was preheated for 30 minutes and set on 560 nm wavelength, then zero with distilled water.

3. Reagent I, Reagent II, and Reagent V were kept in the water bath for more than 5 minutes at 37 °C, then the reagents were added as in the following (Table 2.6):

Table 2.6: Addition the reagents of SOD kit

Reagent(µl)	Test tube(T)	Control tube(C)	Blank tube(B1)	Blank tube(B2)
Sample	90	90	-	-
Reagent I	240	240	240	240
Reagent II	6	-	6	-
Reagent III	180	180	180	180
Distilled water	480	486	570	576
Reagent v	30	30	30	30

4. The mixture was mixed properly and incubated at room temperature for 30 minutes. After that, the absorbance was measured for each tube at 560 nm .

5.Calculations:

$\Delta AT=AT-AC$, $\Delta AB=AB1-AB2$.

$P =[\Delta AB -\Delta AT]\div\Delta AB\times 100\%$

SOD (U/g mass)$=11.4\times P\div 49$

[AT =Absorbance of test, AC= Absorbance of control, AB1= Absorbance of Blank 1].

[AB2= Absorbance of Blank2, P= Inhibition percentage] .

2.6. Measurement of Catalase Activity.

2.6.1.Procedure of CAT measurement

Catalase was measured according to CAT kit (SolarBio, China) protocol.

1- The working solution of reagent was prepared just before use by adding 100 µl of Reagent II to 20 mL of Reagent I and mix thoroughly .

2- 1 g of rat brain tissue was homogenized with 10 mL of extraction reagent on an ice bath, and then centrifuge at 8000 ×g for 10 minutes at 4℃ to remove insoluble materials, and place the supernatant on ice before testing.

3- A spectrophotometer was preheated for 30 minutes and set on 240 nm wavelength, and adjusted with distilled water.

4- 1 mL of CAT working reagent and 35 µl of sample was added in a glass cuvette, and mix for 5 seconds. Then, immediately detect the absorbance at 240 nm at the initial time (A1) and the absorbance after reaction for 1 minute (A2).

5- Calculations

$\Delta A=A1-A2$, CAT (U/g mass) = $678*\Delta A$. [A1= Absorbance at initial time. A2= Absorbance after reaction for 1 minute.]

2.7. Measurement of IL-6

2.7.1. Reagent Preparation of Rat IL-6 ELISA Kit

According to the protocol of the kit (Rat Interleukin ELISA Kit, BT LAB, Shanghai Korain, kit No. E0135Ra) all reagents were brought at room temperature before use. To make a 24ng/L standard stock solution, combine 120µl of the standard (48ng/L) with 120µl of standard diluent. Before producing dilutions, let the standard

sit for 15 minutes with mild agitation. Duplicate standard points were created by serially diluting the standard stock solution (24ng/L) 1:2 with standard diluent to get solutions of 12ng/L, 6ng/L, 3ng/L, and 1.5ng/L/L. The dilution of standard solutions was prepared as follows (Table 2.7):

Table 2.7: Reagent Preparation of Rat IL-6 ELISA Kit.

Concentrations	No. of standard	Solution mixture
24ng/L	Standard No.5	120µl Original standard + 120ul Standard diluent
12ng/L	Standard No.4	120µl Standard No.5 + 120ul Standard diluent
6ng/L	Standard No.3	120µl Standard No.4 + 120ul Standard diluent
3ng/L	Standard No.2	120µl Standard No.3 + 120ul Standard diluent
1.5ng/L	Standard No.1	120µl Standard No.2 + 120ul Standard diluent

After that, prepared a wash buffer by diluting 20 ml of a concentrated wash buffer solution with 500 ml of distilled water.

2.7.2. Procedure of measurement of IL-6

1. Prepared all reagents, standard solutions, and samples as instructed. The experiment is conducted at a room-temperature environment.
2. A 50 µl of the standard was added into a standard well.
3. A 40µl of the sample was added to sample wells and then added 10µl Rat IL6 antibody to sample wells, then added 50µl streptavidin-HRP to sample wells and standard wells .Mixed well and covered the plate with a sealer and incubated for 60 minutes at 37°C.

4-The sealer was removed and the plate washed 5 times with wash buffer, the wells were soaked with 300µl wash buffer for 1 minute for each wash. Placed paper towels on the plate to dry it.

5- A 50µl (substrate solution A) was added to each well and then added 50µl (substrate solution B) to each well. The plate covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

6. A 50µl Stop Solution was added to each well, the blue color was changed into yellow immediately.

7- Determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution .

8- To determine the concentration of IL-6 a standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph and then determine the concentration of each sample, these calculations were performed with computer-based curve-fitting software, and the best-fit line was determined by regression analysis.

2.8. Measurement of TNF-α

2.8.1. Reagent Preparation of Rat TNF-α ELISA Kit

According to the protocol of the kit (Rat Interleukin ELISA Kit, BT LAB, Shanghai Korain, kit No. E0764Ra) All reagents were brought at room temperature before use. To make a 640ng/L standard stock solution, 120µl of the standard (1280ng/L) was reconstituted with 120µl of standard diluent. Before creating dilutions, the standard was allowed to sit for 15 minutes with mild agitation.

Duplicate standard points were created by serially diluting the standard stock solution (640ng/L) 1:2 with standard diluent to provide 320ng/L, 160ng/L, 80ng/L,

and 40ng/L solutions. The following dilutions were proposed for standard solutions (Table 2.8):

Table 2.8: Reagent Preparation of Rat TNF-α ELISA Kit.

Concentrations	No. of standard	Solution mixture
640ng/L	Standard No.5	120µlOriginal standard +120µlStandard diluent
320ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluent
160ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
80ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
40ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

After that, prepared a wash buffer by diluting 20 ml of a concentrated wash buffer solution with 500 ml of distilled water.

2.8.2. Procedure of measurement of TNF-α

1. All reagents, standard solutions, and samples were prepared as instructed. The experiment is conducted in a room-temperature environment.

2. A 50 µl of the standard was added into a standard well.

3.A 40µl sample was added to sample wells and then added 10µl Rat TNF-α antibody to sample wells, then added 50µl streptavidin-HRP to sample wells and standard wells. Mixed well and covered the plate with a sealer. Incubated 60 minutes at 37°C.

4.The sealer was removed and washed the plate 5 times with wash buffer, the wells were soaked with 300µl wash buffer for 1 minute for each wash. The plate was blotted onto paper towels to dry.

5. A 50µl (substrate solution A) was added to each well and then added 50µl (substrate solution B) to each well. The plate covered with a new sealer and incubated for 10 minutes at 37°C in the dark.

6.A 50µl Stop Solution was added to each well, the blue color changed into yellow immediately.

7. Determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution .

8. To determine the concentration of TNF-α a standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. and then determine the concentration of each sample, these calculations were performed with computer-based curve-fitting software, and the best-fit line was determined by regression analysis.

2.9. Total RNA extraction:

Total RNA were extracted from hippocampal tissue samples by using (TRIzol® reagent kit) and done according to company instructions as following steps:

1- 100 mg hippocampal tissue sample was homogenized in 1 ml of (TRIzol® reagent).

2- 200µl chloroform was added to each tube and shaken vigorously for 1 minute .

3- The mixture was incubated on ice for 5 minutes. Then centrifuged at 12000 rpm, 4C°, for 15 minutes.

4- 500 µl of Supernatant was transferred into a new eppendorf tube, and 500µl isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and

incubated at -20 C° for 10 minutes. Then, centrifuged at 12,000 rpm , 4C° for 10 minutes. The supernatant was removed.

5- 1ml of (80% Ethanol) was added and mixed by vortex again. Then, centrifuge at 12000 rpm, 4C° for 5 minutes. The supernatant was removed

6- The RNA pellet was dried by inverting the tubes on tissue papers for 10 minutes.

7- 100µl of free nuclease water was added to dissolve the RNA pellet, and incubating for 10 minutes at 60 C° with taping the tubes during incubation Then, the extracted RNA sample was kept at -20.

2.10.Estimate of the RNA concentration and purity:

The Nanodrop spectrophotometer (THERMO.USA) was used to measure and assess the purity of the extracted total RNA.

The absorbance in a spectrophotometer at wavelengths of 260 and 280 nm on the same Nanodrop machine was measured as follows:

1- The Nanodrop program was opened, and the relevant program (Nucleic acid, RNA) was chosen.

2-With a lense paper, the pedestals were cleaned many times.

3- 2 µl of free nuclease water was then carefully pipetted and placed on the lower measurement pedestal to blank out the Nanodrop.

4- Following pedestal cleanup, 1 µl of yield RNA is pipetted for analysis.

2.11. DNase-I Treatment:

The extracted RNA was treated with DNase- I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using (**the DNase-I enzyme kit**) and done according to the method described by Promega company, USA instructions as follows (Table 2.9):

Table 2.9: DNase-I Treatment

Mix	Volume
Total RNA 100ng/µl	10µl
DNase I enzyme	1µl
10X buffer	4µl
DEPC water	5µl
Total	20µl

After that, the mixture was incubated for an additional 30 minutes at 37°C. A 1 µl stop reaction was then added, and the mixture was allowed to settle at 65 °C for 10 minutes to suppress the DNase-I enzyme.

2.12. cDNA synthesis:

The TLR-4 and GAPDH genes' cDNAs were produced from DNase-I treated RNA samples using the **M-MLV Reverse Transcriptase kit** as the manufacturer's instructions, as shown in the following tables (Table 2.10; Table 2.11):

Table 2.10: Step 1 cDNA synthesis

RT master mix	Volume
Total RNA 100ng/µl	8µl
Random Hexamer primer	1µl
DEPC water	1µl
Total	10µl

Following a 10-minute denature period at 65°C, the RNA and primer were promptly cooled on ice.

Table 2.11: Step 2 cDNA synthesis

RT master mix	Volume
Step 1 RT master mix	10µl
M-MLV RTase (200u)	1µl
5X M-MLV RTase reaction buffer	4µl
100mM DTT	2µl
dNTP	2µl
RNase inhibitor	1µl
Total	20µl

The tubes were then introduced into the vortex and spun down momentarily. RNA was converted into cDNA using the thermocycler conditions listed below (Table 2.12):

Table 2.12: Step 3 thermocycler conditions

Step	Temperature	Time
cDNA synthesis (RT step)	42 °C	1 hour
Heat inactivation	95 °C	5 minutes

2.13. Quantitative Real-Time PCR

To assess gene expression levels, quantitative real-time polymerase chain reaction qPCR has been widely employed (Rao *et al*.,2013).

Relative quantification assesses changes in gene expression relative to a reference gene. The 2ΔΔCT method was used to measure the gene expression. The steps of this process, which were carried out by the directions of the Promega company (USA), are as follows:

1- qPCR Master mix preparation

qPCR master mix was prepared by using **GoTaq® qPCR Master Mix kit** based on SYBER green dye detection of target and GAPDH gene amplification in Real-Time PCR system and include the follow: (Table 2.13).

Table 2.13: qPCR Master mix preparation

qPCR master mix	volume
cDNA template (100ng)	5µL
Forward primer(10pmol)	1 µL
Reverse primer (10pmol)	1 µL
qPCR Master Mix	12.5 µL
DEPC water	5.5 µL
Total	25 µL

After that, these qPCR master mix component that mentioned above placed in qPCR plate strip tubes and mixed by Exispin vortex centrifuge for 3 minutes, then placed in Miniopticon Real-Time PCR system.

2- qPCR Thermocycler conditions

The qPCR plate was loaded, and the following thermocycler conditions were as follows: (Table.2.14):

Table 2.14: Conditions for qPCR thermocycler.

qPCR step	**Temperature**	**Time**	**Repeat cycle**
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	40
Annealing	60 °C	30 sec	
Extension Detection(scan)	72°C	1 min	

2.14. Data analysis of qRT-PCR:

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) (The ΔCT Method Using a reference gene) that described by (Rao *et al*.,2013) as following equation:

ΔCT (Test) = CT (target gene, test) – CT (HKG gene, test)

ΔCT (Control) = CT (target gene, control) – CT (HKG gene, control)

ΔΔCT= ΔCT (Test)– ΔCT (Control)

Fold change (target / HKG) = $\mathbf{2^{-(\Delta\Delta CT)}}$.

2.15. Statistical analysis:

The statistical package of social science (SPSSv 25) statistics for Windows®7 was used to conduct the statistical analysis. Test of normality distribution and test of homogeneity is done, one-way ANOVA, followed by Tukey test were used in the statistical analysis. In the case of abnormality distribution the Kruskal–Wallis one-way analysis of variance was used.

The outcomes were presented as the mean plus the standard error of the mean (SEM). The p-value less than 0.05, considered statistically significant.

Chapter Three
Results

3.1.The weight

There was a significant ($p < 0.05$) decrease in mean weight in G2, G3, G4, and G5 on day 10 as compared to day 0. On day 25 there was a significant ($p < 0.05$) decrease in mean weight in G2, G4, and G5 as compared to day 0 and no significant change in mean weight in G1 at day 10 and day 25 as compared to day 0 . In G3 there was no significant difference in mean weight on day 25 as compared to day 0 (Table 3.1; Figure.3.1).

Table 3.1: The mean of weight ± SEM in all rat groups on days 0,10, and 25.

Days	G1	G2	G3	G4	G5
Day 0	268.5 ±6.28	274±9.45	272.5±6.80	271±6.04	268±4.42
Day 10	268 ±5.38	239.5±3.45*	243.5±4.34*	236.5±5.91*	239.5±6.80*
Day 25	267±5.87	239.5±5.02*	258±5.01	242.5±8.47*	240.5±5.02*

* Significantly decreased compared to day 0. ($p < 0.05$)

G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10 mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group).

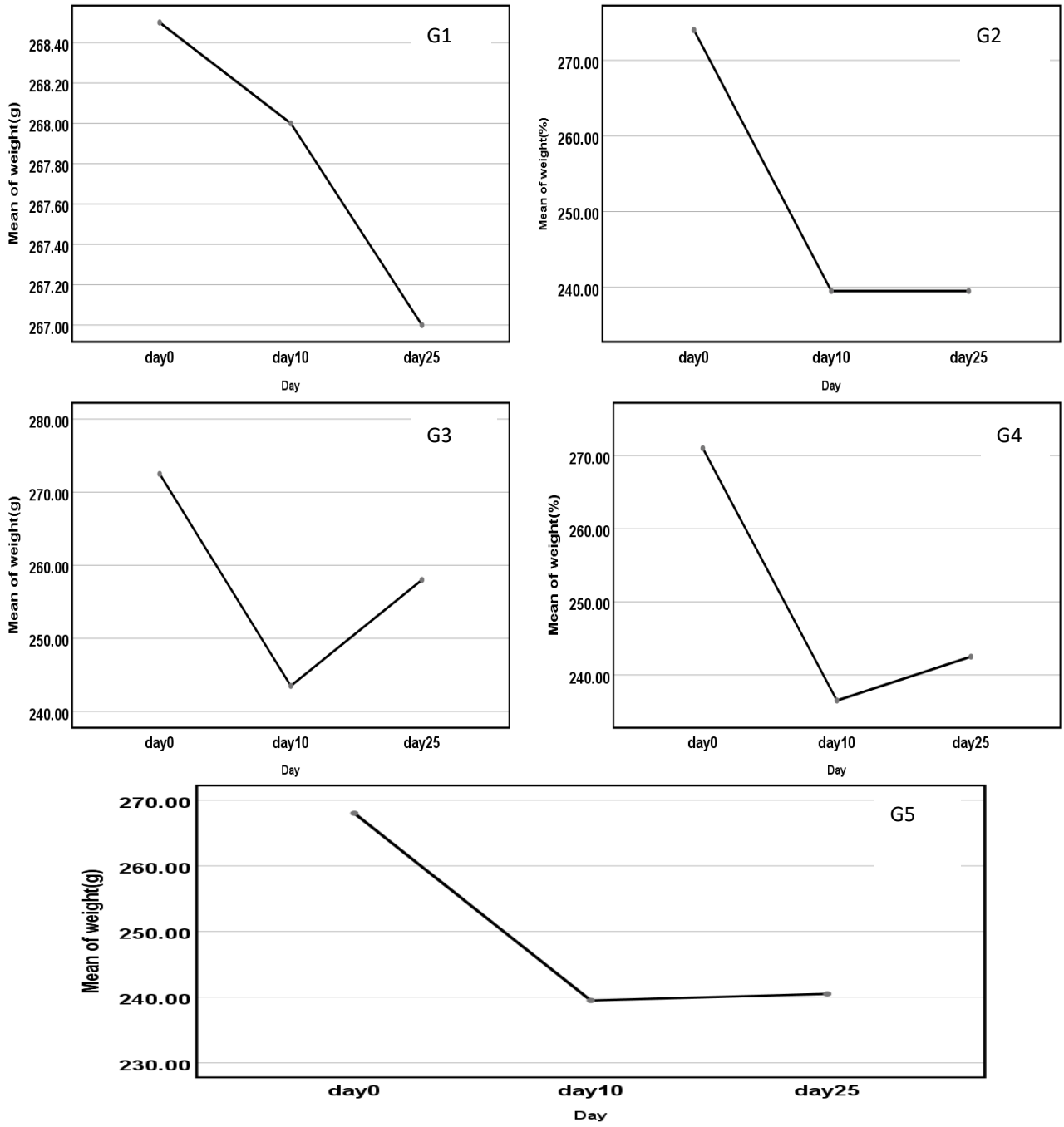


Figure.3.1:Comparison between the mean of weight ± SEM in all rat groups on days 0,10, and 25.

G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10 mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group).

3.2.Behavioral tests

3.2.1. The sucrose preference test

The mean of sucrose intake on days 10 and 25 as compared to day 0 in the G1(untreated and unexposed to CUS) were not significantly different while in rats that were untreated and exposed to CUS (G2), the mean of sucrose intake on days 10 and 25 significantly decreased ($p < 0.05$) in contrast to day 0(Table 3.2 ; Figure. 3.2).

Furthermore, the mean of sucrose intake on day 10 significantly ($p < 0.05$) decreased ($p < 0.05$) compared to day 0 in G3 (treated with 10 mg /kg/day fluoxetine for 14 days +CUS),G4 (treated with 10 mg/kg/day empagliflozin +CUS),G5 (treated with 20 mg/kg/day empagliflozin +CUS). On day 25, there was a significant ($p < 0.05$) increase in SPI compared to day10 in G3, G4, and G5 (Table 3.2 ; Figure. 3.2)

Table 3.2: The mean of sucrose preference index ± SEM in all rat groups on days 0,10, and 25.

SPT(%)	G1	G2	G3	G4	G5
Day 0	70.07 ±1.81	68.35±2.12	74.5±1.32	70.17± 1.36	73.5±1.19
Day 10	72.2 ± 2.24	61±1.22*	64.9±1.54*	62± 1.04*	65.3±1.34*
Day 25	69.3±1.22	58.4± 1.40*	72.1±1.74**	68.3±1.57**	70.1±1.69**

*Significantly decreased compared to day 0.

** Significantly increased compared to day 10.

SPT(Sucrose preference test).

G1(control group , untreated and unexposed to CUS),G2(untreated and exposed to CUS), G3 (treated with 10 mg /kg fluoxetine+ CUS for 14 days), G4 (treated with 10 mg/kg empagliflozin+ CUS, G5 (treated with 20 mg/kg empagliflozin+ CUS). (No.=10 rats in each group). ($p < 0.05$)

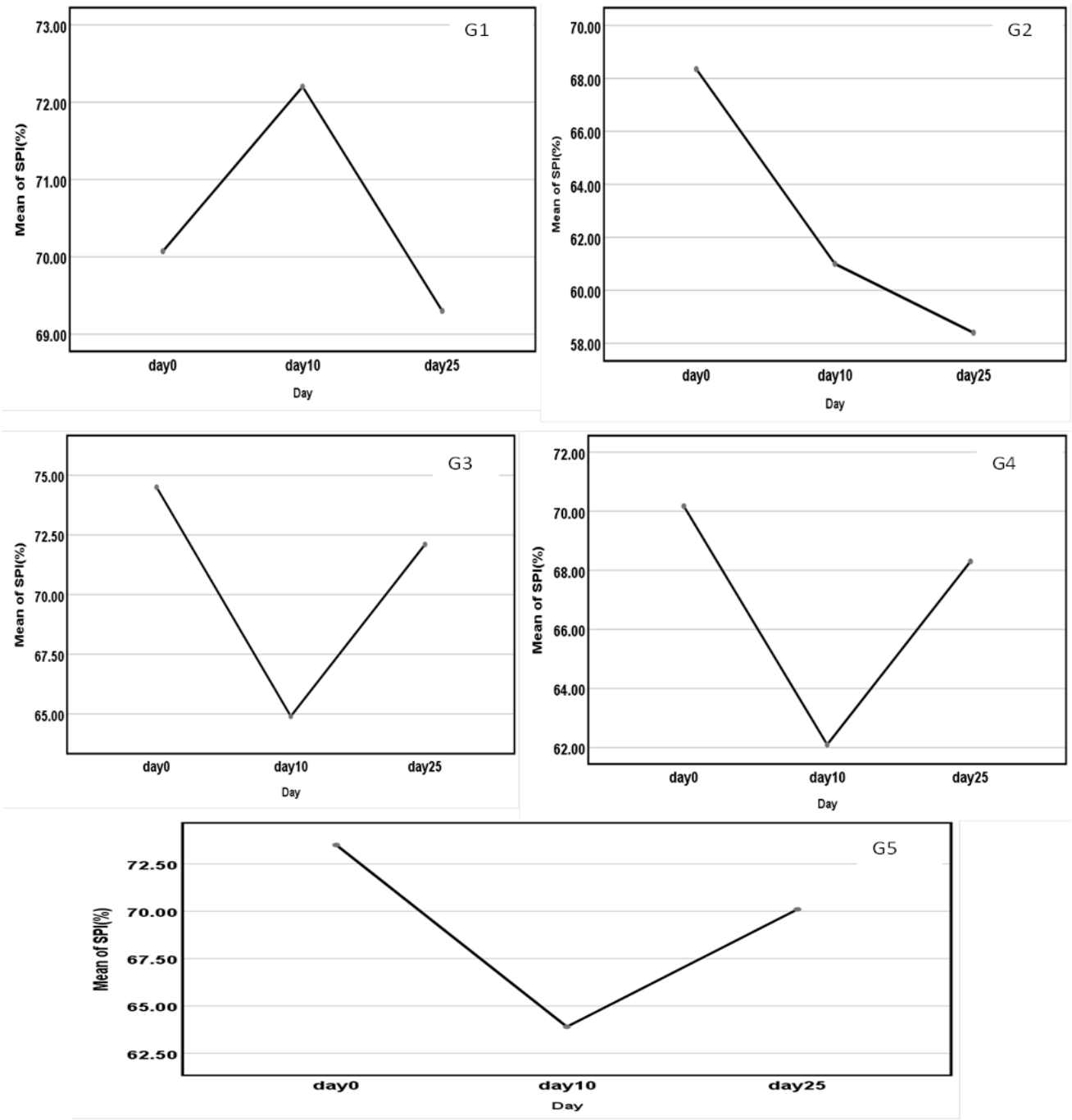


Figure 3.2: Comparison between the mean of sucrose preference index ± SEM in all rat groups on days 0,10, and 25.

G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10 mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group).

3.2.2. The Forced Swimming Test

There were no significant differences in the mean of immobility time on days 10 and 25 in comparison to day 0 in G1 that was untreated and unexposed to CUS), while in rats that were untreated and exposed to CUS (G2), the mean of immobility time on days 10 and 25 significantly increased ($p < 0.05$) as compared to day 0 (Table 3.3 and Figure. 3.3).

Furthermore, the mean of immobility time significantly increased on day 10 ($p < 0.05$) in contrast to day 0 in G3 (administered 10 mg /kg/day fluoxetine for 14 days+ CUS), G4 (administered 10 mg/kg/day empagliflozin+ CUS), G5 (administered 20 mg/kg/day empagliflozin+ CUS) (Table 3.3 and Figure. 3.3).

On day 25 there is a significant ($p < 0.05$) decrease in immobility time compared to (day 10) in G3, G4, and G5 (Table 3.3 and Figure. 3.3).

Table 3.3: The mean of immobility time ± SEM in all rat groups on days 0,10, and 25

FST(sec)	G1	G2	G3	G4	G5
Day 0	42.7±1.11	43±1.95	41.5±1.66	46.2± 1.55	39.7±1.23
Day 10	44.6±1.88	70.3±1.83*	70±1.93*	71.9±1.21*	66.3±1.86*
Day 25	47±1.27	73.8±2.11*	46.6±1.57**	49.2± 0.84**	43.8±1.31**

* increased significant compared to day 0

** decreased significant compared to day 10

FST(Forced swimming test). G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10 mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group). ($p < 0.05$)

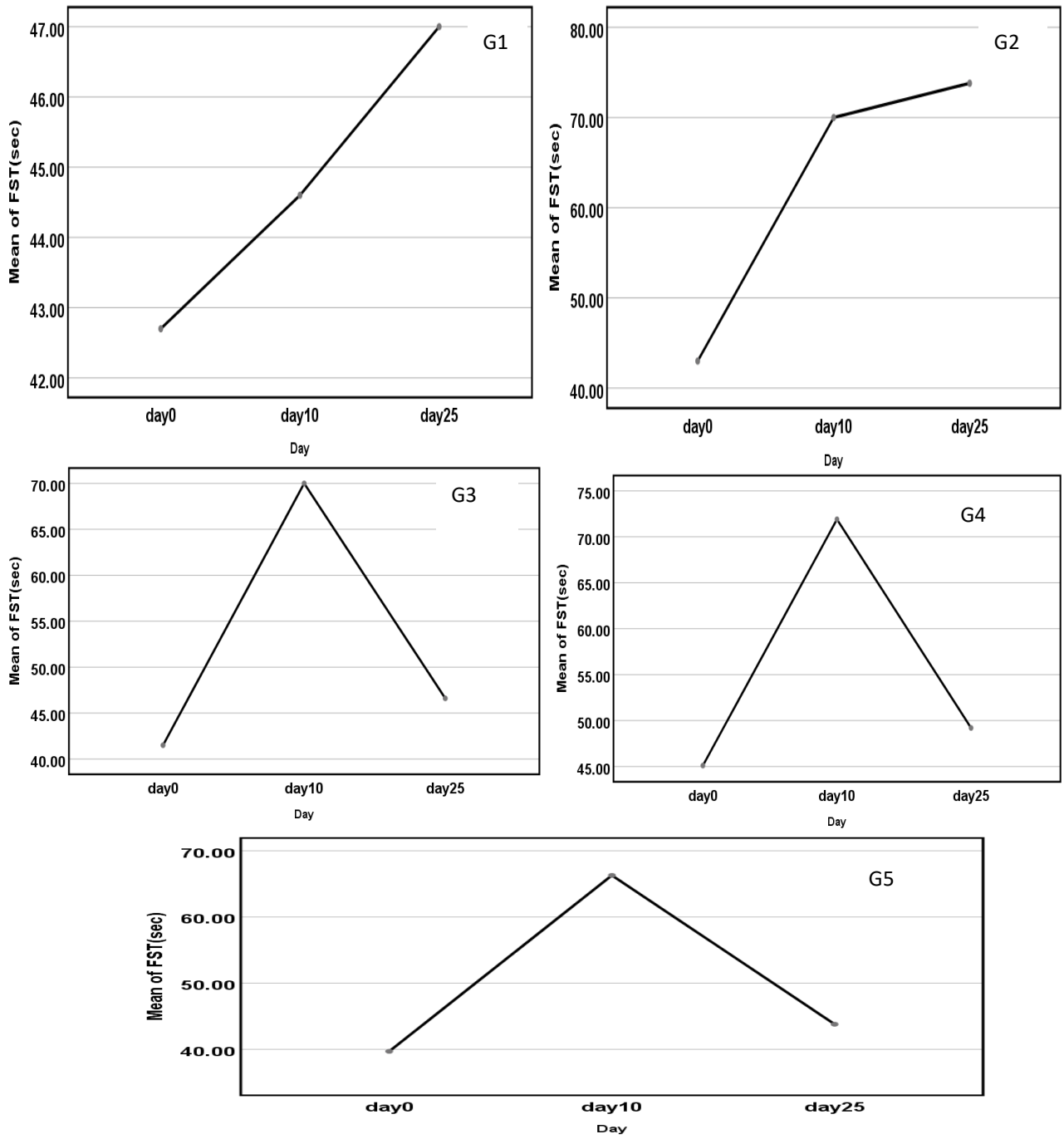


Figure 3.3: Comparison between the mean of immobility time ± SEM in all rat groups on days 0,10, and 25

G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10 mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group).

3.3. Comparison of the mean of weight on day 25:

On day 25, there was a significant ($p<0.05$) decrease in the mean of weight in G2, G4, and G5 compared to G1 and no difference in weight between G3 and G1 on day 25 (Table.3.4; Figure.3.4).

3.4. Comparison of the mean of SPI on day 25:

On day 25, there was a significant ($p<0.05$) decrease in mean of sucrose intake in G2 compared to G1, and a significant increase in mean of sucrose intake in G3, G4, and G5 compared to G2 (Table.3.4; Figure 3.5).

3.5. Comparison of the mean of immobility time on day 25:

On day 25, there was a significant ($p<0.05$) increase in mean of immobility time in G2 compare to G1, and significant ($p<0.05$) decrease in mean of immobility time in G3,G4 and G5 compared to G2 (Table.3.4;Figure .3.6).

Table.3.4: Comparison of the Mean ± SEM of SPI, weight, and immobility time, among rat groups on day 25.

Groups	G1	G2	G3	G4	G5
Mean of Weight on day 25	267± 5.87	239.5±5.02$^{\alpha}$	258±5.01	242.5±8.47$^{\alpha}$	240.5±5.02$^{\alpha}$
Mean of SPI on day 25	69.3±1.22	58.4±1.40$^{\alpha}$	72.1±1.74***	68.3±1.57***	70.1±0.99***
Mean of immobility time on day 25	47±1.27	73.8±2.11*	46.6±1.57 **	49.2±0.84 **	43.8±1.31 **

*significantly increased compared to G1,** significantly decreased compared to G2, $^{\alpha}$ significantly decreased compared to G1,*** significantly increased compared to G2. ($p<0.05$)

G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10 mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group).

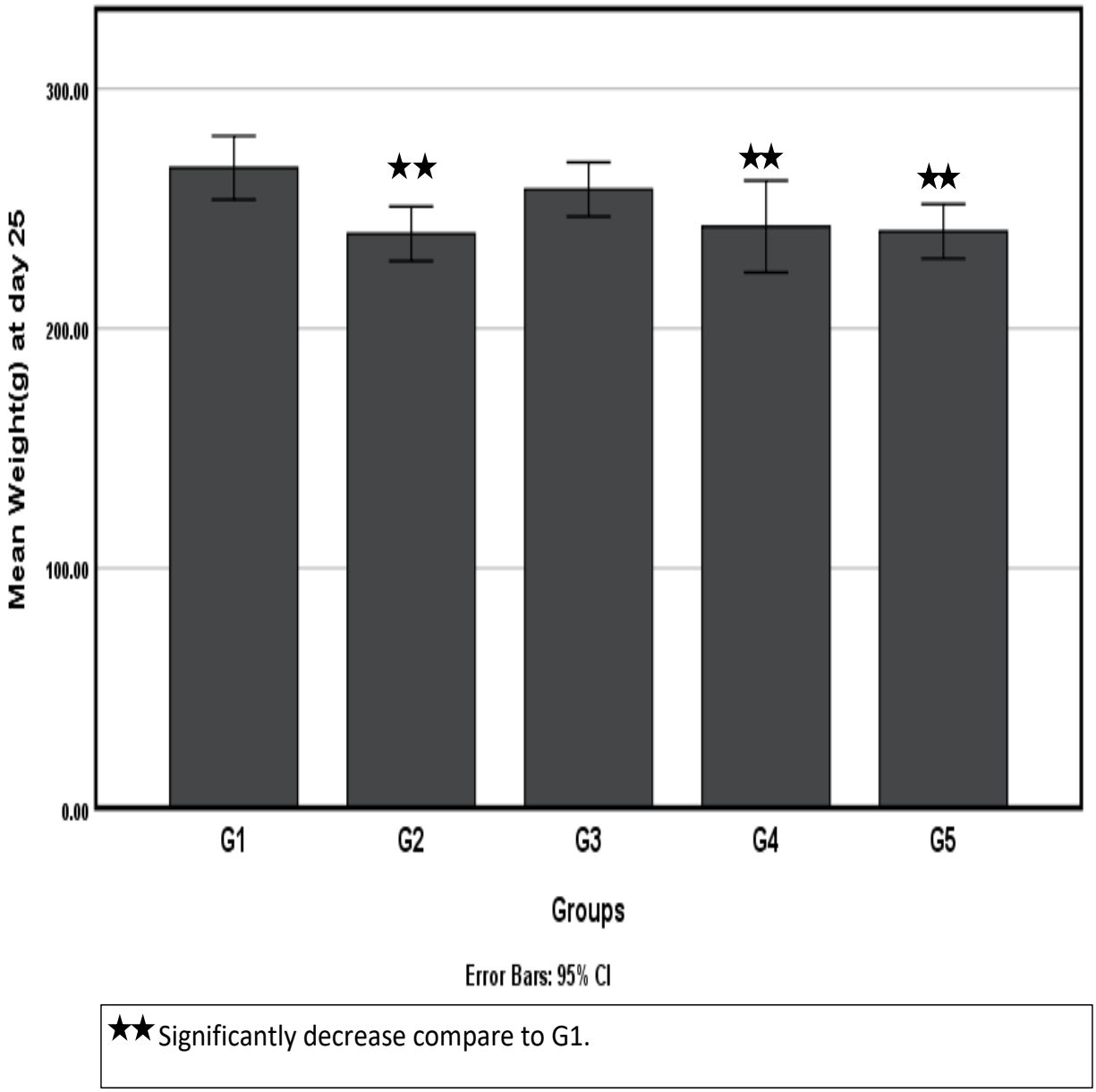


Figure.3.4: Comparison between the mean of weight ± SEM in all rats groups on day 25.

G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group). ($p<0.05$)

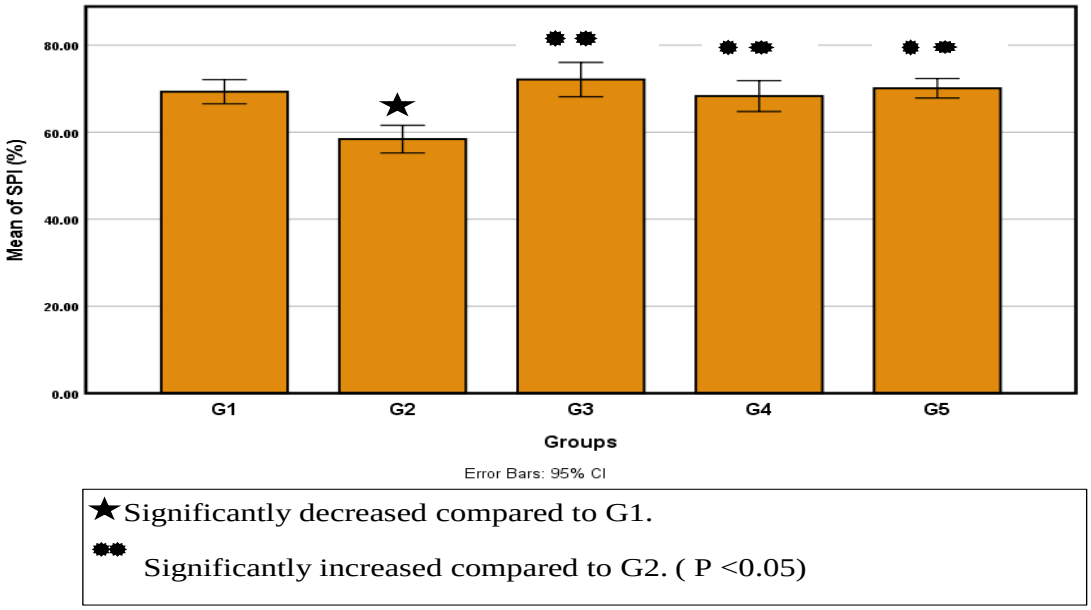


Figure.3.5: Comparison between the mean of SPI ± SEM in all rats groups on day 25.

G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group).

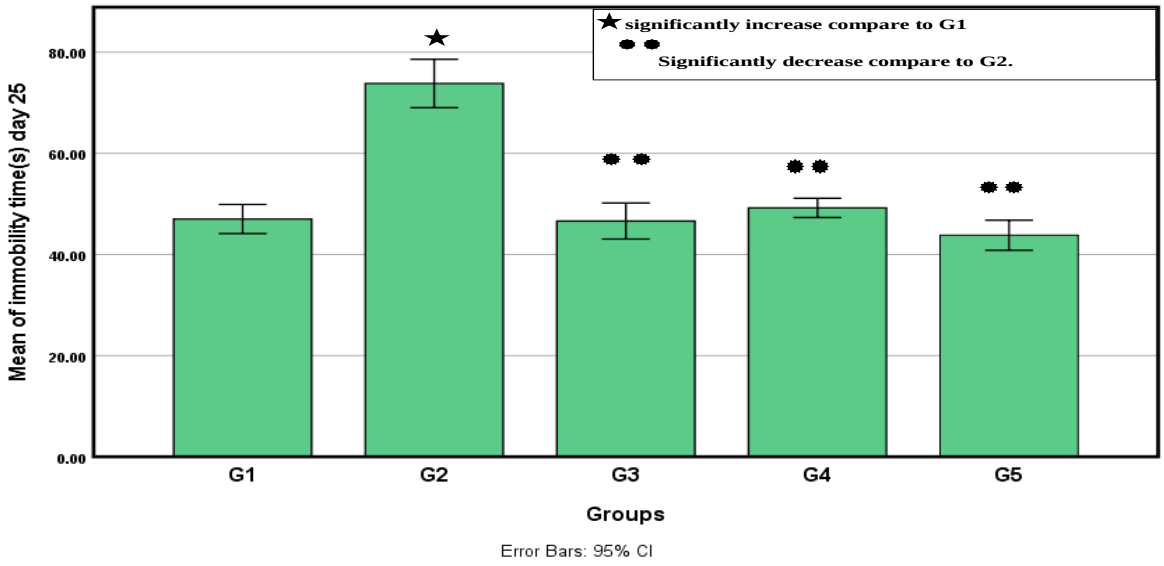


Figure.3.6: Comparison between the mean of immobility time ± SEM in all rat groups on day 25.

G1(control group , untreated and unexposed to CUS), G2(untreated and exposed to CUS), G3(treated with 10 mg /kg/day fluoxetine for 14 days), G4 (treated with 10mg/kg/day EMP, G5 (treated with 20 mg/kg/day EMP).(No.=10 rats in each group).

3.6. Antioxidant enzymes

There was a significant ($p<0.05$) decrease in the mean of SOD concentration in rats exposed to chronic unpredictable stress(G2) compared to rats in the normal group (G1) on day 25 (Table 3.5; Figure. 3.7).

Furthermore, there were a significant ($p<0.05$) increase in the mean of SOD concentration in groups treated with fluoxetine 10 mg/kg/day (G3), empagliflozin 10 mg/kg/day(G4), and empagliflozin 20 mg/kg/day(G5) compared to G2 on day 25 (Table 3.5; Figure. 3.7). Also, there was a significant($p<0.05$) increase in the mean of CAT concentration in G3, G4 and G5 compared to G2 on day 25 (Table 3.5; Figure. 3.7).

Table 3.5: The mean of SOD and CAT concentration ± SEM in all rats groups on day 25.

Groups	G1	G2	G3	G4	G5
SOD	20.24±0.68	14.26±0.79*	18.94±0.79**	17.51±0.55**	17.84±0.76**
CAT	24.27±0.97	11.40±0.89*	16.79± 0.94**	18.20±0.81**	21.99±0.77**

* Significantly decreased compared to G1.

** Significantly increased compared to G2.

G1(untreated and unexposed to CUS),G2(untreated and exposed to CUS), G3(treated with 10 mg /kg fluoxetine+ CUS for 14 days), G4 (treated with 10 mg/kg empagliflozin+ CUS, G5 (treated with 20 mg/kg empagliflozin+ CUS). (No.=10 rats in each group). ($P<0.05$)

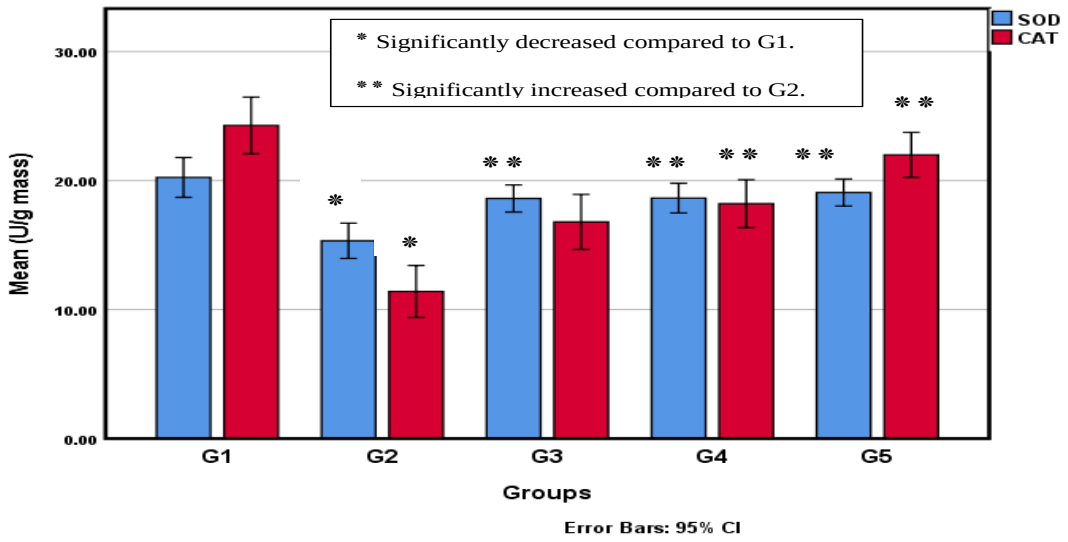


Figure 3.7:Comparison between the mean of SOD and CAT concentration ± SEM in all rats groups on day 25.

G1(untreated and unexposed to CUS),G2(untreated and exposed to CUS), G3(treated with 10 mg /kg /day fluoxetine+ CUS for 14 days), G4 (treated with 10 mg/kg/day/day empagliflozin+ CUS, G5 (treated with 20 mg/kg empagliflozin+ CUS). (No.=10 rats in each group).

3.7. IL-6 and TNF-α on day 25 :

A significant (P<0.05) increased in IL-6 mean concentration in G2 and G4 compared to G1. In G3 and G5 there was a significant (P<0.05) decrease in IL-6 mean concentration compared to G2. With regard to TNF-α, A significant (P<0.05) increase in TNF-α mean concentration in G2 compared to G1.

There were significant (P<0.05) decreases in TNF-α mean concentration in G3, G4,and G5 compared to G2 (Table.3.6; Figure.3.9).

Table.3.6: The mean of IL-6 and TNF-α concentrations ± SEM in all rats groups on day 25.

Groups	G1	G2	G3	G4	G5
IL-6	43±0.41	62.46±1.39*	36.81±1.01 **	63.80±0.48*	35.26±0.74 **
TNF-α	102.92±0.29	166.18±1.76*	90.08± 1.58**	107.00±0.64**	97.29± 1.17**

*Significant increase compared to G1. ** Significant decrease compared to G2.

G1(untreated and unexposed to CUS),G2(untreated and exposed to CUS), G3(treated with 10 mg /kg fluoxetine+ CUS for 14 days), G4 (treated with 10 mg/kg empagliflozin+ CUS, G5 (treated with 20 mg/kg empagliflozin+ CUS). (No.=10 rats in each group). (P<0.05)

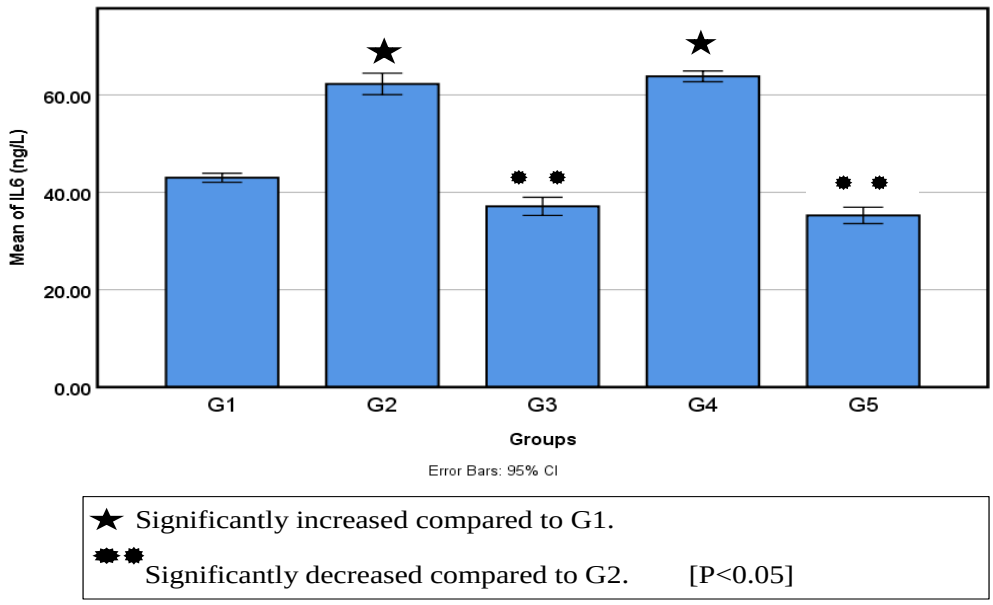


Figure.3.8:Comparison between the mean of IL-6 concentrations ± SEM of IL-6 concentrations in all rats groups on day 25.

G1(untreated and unexposed to CUS),G2(untreated and exposed to CUS), G3(treated with 10 mg /kg fluoxetine+ CUS for 14 days), G4 (treated with 10 mg/kg empagliflozin+ CUS, G5 (treated with 20 mg/kg empagliflozin+ CUS). (No.=10 rats in each group).

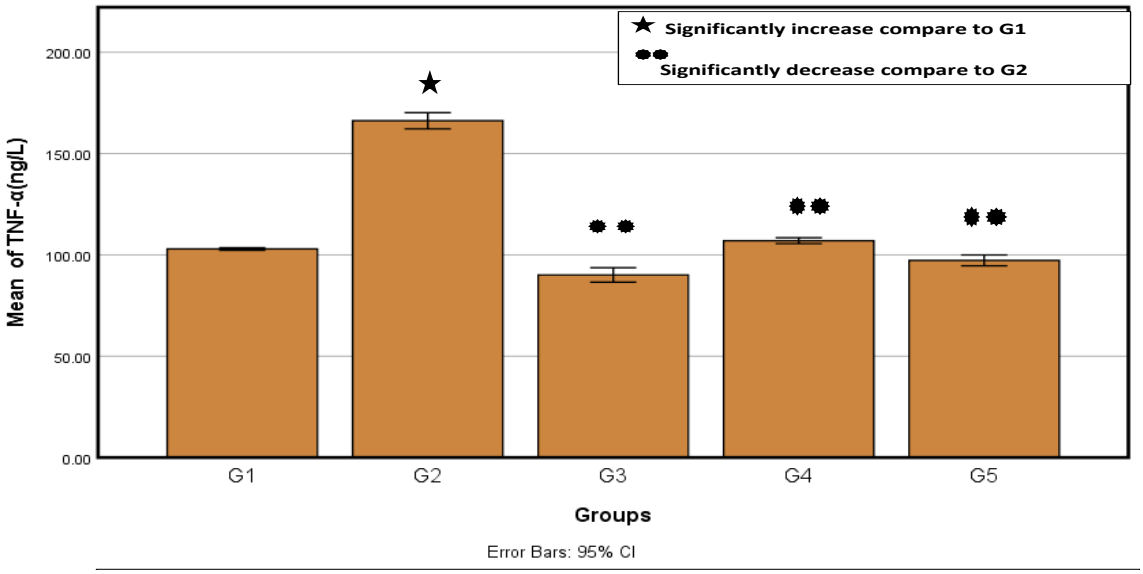


Figure.3.9: Comparison between the mean of TNF-α concentrations ± SEM in all rats groups on day 25.

G1(control,untreated and unexposed to CUS),G2(untreated and exposed to CUS),G3(treated with 10 mg /kg fluoxetine+ CUS for 14 days),G4 (treated with 10 mg/kg empagliflozin+ CUS, G5 (treated with 20 mg/kg empagliflozin+ CUS). (No.=10 rats in each group). (P<0.05)

3.8. TLR-4 gene expression:

There was a significant increased ($p< 0.05$) in the mean of TLR-4 gene fold changes in rats that were exposed to chronic unpredictable stress for 24 days (G2) compared to rats in G1. In rats being under CUS and treated with 10 mg/kg/day for fourteen days (G3) a significant ($p< 0.05$) decrease in mean of gene expression of TLR-4 compared to G2.

Rats that were exposed to CUS and given empagliflozin 10 mg/kg/day for fourteen days periods (G4) show a significant ($p< 0.05$) decrease in the mean of TLR-4 gene fold changes. In group 5 in which rats were exposed to CUS and treated with 20 mg/kg/day for fourteen days a significant ($p< 0.05$)decrease in the mean of TLR-4 gene fold changes. Also there were significant decrease ($p< 0.05$) in mean of TLR-4 gene fold in G5 compare to G3 (Table 3.7; Figure. 3.10).

The Ct values refers to the amount of cycles required for the fluorescent signal to exceed background levels. Generally, the more target DNA that is in the sample, the faster its amplification will be and thus, the fewer cycles required before the fluorescence signal crosses the background threshold (lower Ct value). Conversely, if there are low amounts of target DNA, it will require more cycles before the fluorescence can cross the background threshold (higher Ct value). G2 (untreated and exposed to CUS) show fewer cycles that reflex high amount of TLR-4 gene.

G3 (treated with 10 mg /kg/day fluoxetine+ CUS for 14 days), G4 (treated with 10 mg/kg/day empagliflozin+ CUS), G5(treated with 20 mg/kg/day empagliflozin+ CUS) show the higher Ct value compared to G2, that indicate low TLR-4 levels in these groups (Figure.3.11). With regard to housekeeping gene all groups show the same (Ct value) of GAPDH amplification (Figure.3.12).

Table 3.7:The mean of TLR-4 fold changes ± SEM in all rats groups on day 25

Groups	G1	G2	G3	G4	G5
Mean of TLR-4 fold changes	2.32 ± 0.10	5.17± 0.21*	2.79 ± 0.14**	2.34 ±0.16**	1.38 ±0.12 ** α

*Significantly increase compare to G1,** Significantly decrease compare to G2, α Significantly decrease compare to G3.

G1(untreated and unexposed to CUS),G2(untreated and exposed to CUS), G3(treated with 10 mg /kg fluoxetine+ CUS for 14 days), G4 (treated with 10 mg/kg empagliflozin+ CUS, G5 (treated with 20 mg/kg Empagliflozin+ CUS). (No.=10 rats in each group). ($p< 0.05$).

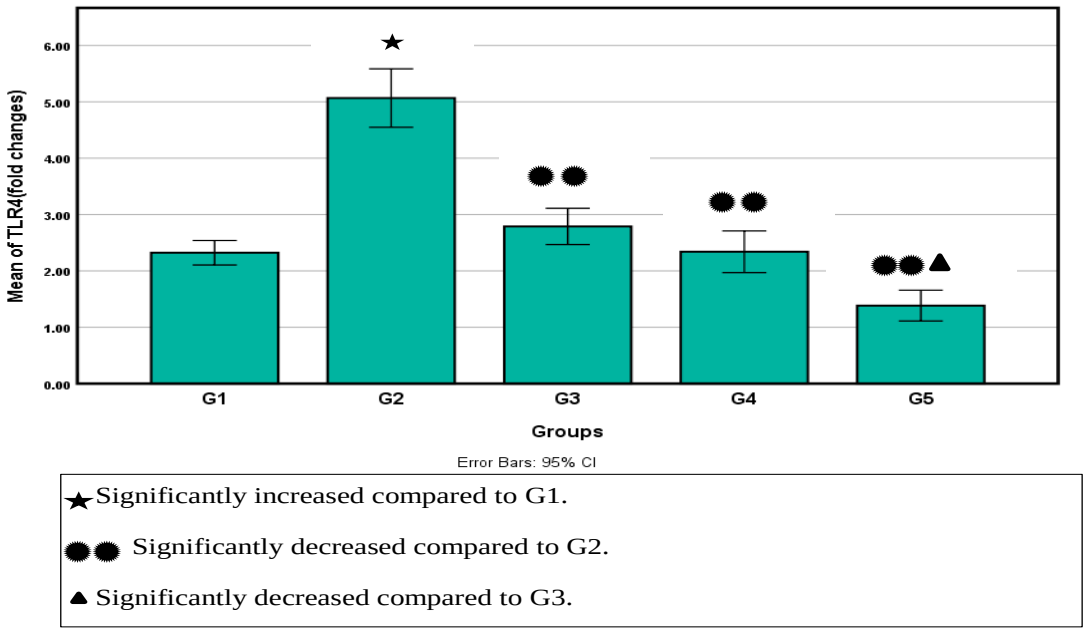


Figure 3.10: Comparison between the mean of TLR-4 fold changes ± SEM in all rats groups on day 25. G1(untreated and unexposed to CUS),G2(untreated and exposed to CUS), G3(treated with 10 mg /kg fluoxetine+ CUS for 14 days), G4 (treated with 10 mg/kg empagliflozin+ CUS, G5 (treated with 20 mg/kg empagliflozin+ CUS). (No.=10 rats in each group).

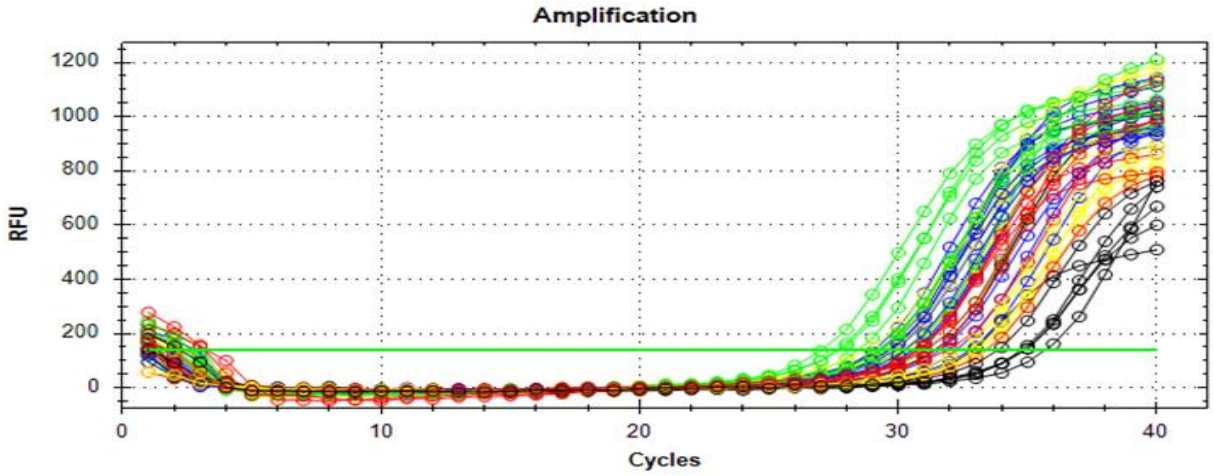


Figure 3.11: The Real Time PCR amplification plots of TLT4 gene expression in rats groups on day 25. Yellow blot (G1, untreated and unexposed to CUS), Green blot (G2,untreated and exposed to CUS), Blue blot (G3,treated with 10 mg /kg/day fluoxetine+ CUS for 14 days), Red blot (G4, (treated with 10 mg/kg/day empagliflozin+ CUS, Black blot (G5, treated with 20 mg/kg/day empagliflozin+ CUS). (No.=10 rats in each group).

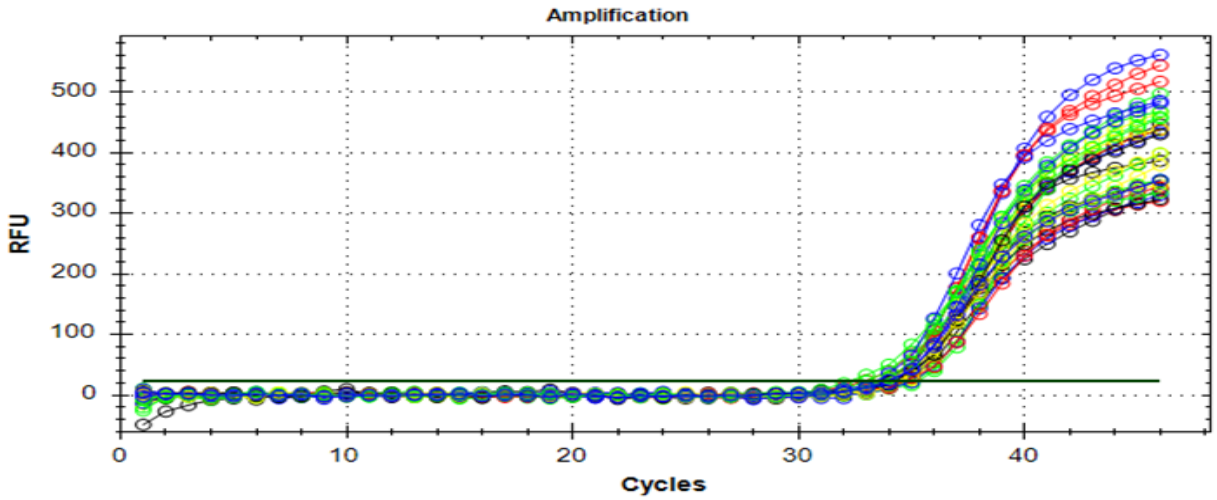


Figure 3.12: The Real Time PCR amplification plots of housekeeping GAPDH gene expression in rats groups on day 25. Yellow blot (G1, untreated and unexposed to CUS), blot (G2,untreated and exposed to CUS), Red blot (G3,treated with 10 mg /kg/day fluoxetine+ CUS for 14 days), Green blot (G4, (treated with 10 mg/kg/day empagliflozin+ CUS, Black blot (G5, treated with 20 mg/kg/day empagliflozin+ CUS). (No.=10 rats in each group).

3.9. Correlation among TLR4, IL-6, TNF-α, SOD, CAT, Immobility time, Sucrose index, and weight:

TLR4 has a strong positive correlation with IL-6, TNF-α, and immobility time and a strong negative correlation with SOD, CAT, and Sucrose index.There was a strong positive correlation between IL-6, TNF-α, and immobility time, while there was a strong negative correlation between IL-6, SOD, CAT, Weight, and Sucrose Index.

Furthermore, there was a strong positive correlation between TNF-α, TLR4, and immobility time. Also a strong negative correlation between TNF-α, SOD, and CAT (Table 3.8).There was no correlation between TNF-α and weight and a strong negative correlation between TNF-α and Sucrose index (Table 3.8).

A strong positive correlation between SOD, CAT, and Sucrose index, while no correlation between SOD and weight, and there was a strong negative correlation between SOD and immobility time .

Catalase has a strong negative correlation with immobility time, No correlation among weight, immobility time, and Sucrose index. A strong negative correlation is present between immobility time and the Sucrose index (Table 3.8)

Table 3.8: Correlation among TLR4, IL-6, TNF-α, SOD,CAT, Immobility time, Sucrose index ,and weight

		TLR4	IL-6	TNF-α	SOD	CAT	Weight	Immobility Time	SPI
TLR4	Pearson Correlation		.703	.912	-.655	-.559	-.264	.837	-.663
	Sig.		.001	<.001	<.001	<.001	.064	<.001	<.001
IL-6	Pearson Correlation			.657	-.469	-.359	-.340	.516	-.510
	Sig.			<.001	.010	.016	<.001	<.001	<.001
TNF-α	Pearson Correlation				-.653	-.325	-.222	.865	-.747
	Sig.				<.001	.021	.121	<.001	<.001
SOD	Pearson Correlation					.477	.150	-.611	.446
	Sig.					<.001	.298	<.001	.001
CAT	Pearson Correlation						.021	-.534	.187
	Sig.						.883	<.001	.193
Weight	Pearson Correlation							-.151	.128
	Sig.							.295	.377
Immobility Time	Pearson Correlation								-.652
	Sig.								<.001

Pearson Correlation (0.3 $\rightarrow$ 1) strong positive correlation

(0 $\rightarrow$ 0.3) weak positive correlation

(0 $\rightarrow$ ($^{-}$0.3)) weak negative correlation

(($^{-}$0.3) $\rightarrow$ $^{-}$1) strong negative correlation, Sig.(p <0.05).

Chapter Four
Discussion

4.1.Depression

It is one of the most prevalent psychiatric disorders and has an impact on cognitive abilities, overall health, and quality of life.

Many hypotheses have attempted to explain the pathogenesis of depression. The first of these hypotheses is the monoamine hypothesis, which assumes that a defect in the synthesis, storage, or release of some neurotransmitters such as norepinephrine and serotonin leads to development the symptoms of depression (Brigitta, 2022). This hypothesis was based on some evidence, as some antidepressant drugs lead to an increase in the concentration of these neurotransmitters in some areas of the brain (Siddiqi *et al*.,2022).

After that, hypotheses were adopted that tried to explain the cause of depression. Some of them explained the cause of depression by a defect in the plasticity of the neurons (He *et al*.,2022), and others mentioned that an increase in oxidative stress leads to the death of neurons and their loss of ability to perform their function, which results in the development of depression symptoms (Behl *et al*.,2022), and each of the preceding hypotheses has evidence that supporting it.

This study concentrates on the role of inflammatory processes, oxidative stress and the impact of cytokines on the pathogenesis of depression. Many experimental and clinical studies have shown the clear role of cytokines in the development and severity of depression symptoms (Wang & Chen, 2017; Chen *et al*., 2021).

Most of the current depression medications improve the concentration of neurotransmitters in the brain, but there are many problems associated with these treatments, so not all patients respond to them (Papp *et al*.,2022).

The adverse effects of antidepressant lead to serious complications in some patients, and some patients might cease to take them (Tomlinson *et al*.,2022). Therefore, attention was directed towards empagliflozin because some studies have found that it may attenuate some inflammatory processes and counteract oxidative stress, in addition to its good safety profile (Abed *et al*.,2021).

To our knowledge, this study is the first to look at the antidepressant effects of empagliflozin in male rats exposed to CUS and examine the empagliflozin impact on TLR-4 gene expression in the hippocampal tissue, the production of pro-inflammatory cytokines (IL-6, TNF-α) and effect on antioxidant enzymes (SOD, CAT). Findings from this study may encourage future studies on the impact of empagliflozin on mental disorders.

4.2. The changes in the weight

Weight loss becomes evident ten days after the application of the chronic unpredictable stress protocol in all groups compared to the control group (Table 3.1). A decrease in body weight in the rats that were exposed to CUS suggested the advancement of depressive-like behavior since weight changes are regarded as one of the signs of depression (Liang *et al*., 2022).This reduction in weight may be attributed to decrease appetite during depression period (Cosgrove *et al*.,2020).

This result agrees with other studies that found that rats exposed to chronic unpredictable stress showed a weight reduction (Amiresmaeili *et al*., 2018;Sequeira-Cordero *et al*., 2019;Mohseni-Moghaddam *et al*., 2022).

Treatment with 10 mg/kg/day of fluoxetine on day 25 resulted in a marginal weight gain that was not statistically different from the mean of weight on day 10 (Table 3.1). The impact of fluoxetine on appetite suppression and glucose metabolism

may be to blame for the unimprovement in the weight. A thorough investigation reveals that fluoxetine reduces body weight in humans by influencing lipid and glucose metabolism (Zhang *et al*.,2022). According to another comprehensive study, fluoxetine helps people lose weight by stifling their hunger (Melendez-Mierd, 2022). This result was consistent with findings from other research (Khedr *et al*., 2018).

The rats that were treated with EMP (10 and 20 mg/kg/day) showed decreased in weight after 14 days of treatment (Table 3.1). These outcomes may be explained by the fact that EMP inhibits the sodium-glucose co-transporter, which reduces renal glucose absorption and causes calorie loss.

Furthermore, according to study by Mironova & Hanjieva-Darlenska, EMP lowers leptin, a key hormone in weight control (Mironova & Hanjieva-Darlenska, 2021). These findings are consistent with those of other trials where EMP promoted weight loss (Malnská *et al*., 2022).

4.3. Chronic Unpredictable Stress and Behavioral tests

The average sucrose consumption was decreased on day 10 by a statistically significant percentage in the rats to which the stress protocol was applied compared to day 0 (Table 3.3). This reflects the lack of desire for pleasure, which is one of the most important forms of depression (Markov,2022).

The forced swimming test sessions showed that the rats that were exposed to chronic unpredictable stress for 10 consecutive days suffered from despair, which was concluded from an increase in the immobility time during the swimming test in these rats compared to the results of day 0 (Table 3.2). Non-depressed rats have the propensity to flee the tank and keep swimming, but depressed rats exhibit a

diminished drive to survive, which manifests in an increase in the immobility period (Gorman-Sandler & Hollis, 2021).

Numerous studies have found that prolonged stress promotes depressive symptoms (Markov, 2022), includes anhedonia (lack of desire to pleasure) and despair or hopelessness, which has been shown to emerge in rats following CUS (Fang *et al*., 2021), the sucrose preference test and the forced swimming test both assess these characteristics (Wang *et al*., 2021).

These findings are consistent with prior research showing that rats subjected to chronic stress consume fewer sweet solutions (Goudarzi *et al*., 2020; Strekalova, 2022) and show signs of despair (Malyshev *et al*., 2022; Shemirani *et al*., 2022).

Stress induces depressive symptoms through many mechanisms involving impairments in serotonin neurotransmission, reduction of hippocampal neurogenesis, disruption of the hypothalamic-pituitary-adrenal axis, increased oxidative stress, reduction in the plasticity of neurons, synaptic plasticity, and induction of inflammation in neurons through increased proinflammatory cytokine production (Hao *et al*., 2019).

On a biochemical level, persistent stress would result in increased glutamate release in the prefrontal cortex and the hippocampus (Lee *et al*., 2022).The main cell responsible for removing glutamate from the brain is called a glial cell, and stress would decrease glial cell proliferation and increase apoptosis, which is why the hippocampus shrinks in MDD (Khan *et al*., 2020).

When compared to rats exposed only to stress, the results of 14 days of treatment with EMP (20 and 10 mg/kg/day) with the continuation of chronic stress revealed a significant improvement in the percentage of sucrose consumption as well as a decrease in immobility time in these rats, which is similar to the results seen in the

group of rats given fluoxetine (10 mg/kg/day) for 14 days with the continuation of chronic stress (Table 3.2;Table 3.3). The results also did not differ from the results that appeared in the control group, and this is an indication of the improvement of behavioral characteristics in these rats.

Fluoxetine is a proven treatment for depression, and some animal studies have shown that it increases sucrose consumption and increase swimming time (Fang *et al*., 2022). Neuroinflammation is a key factor that interacts with the three neurobiological correlates of major depressive disorder: depletion of brain serotonin, dysregulation of the hypothalamus–pituitary–adrenal axis and alteration of the neuroplasticity these changes lead to development of depression (Troubat *et al.*,2021) ,therefore the ability of empagliflozin to reduce these behavioral symptoms of depression may be due to its anti-inflammatory and antioxidant properties as found in this study, which leads to decrease the neuroinflammation.

4.4. Antioxidant enzymes (SOD, CAT)

On day 25, superoxide dismutase and catalase enzyme activity decreased significantly in the rat group that exposed to CUS (G2) compared to the normal rat group (G1) (Table 3.5). This can be explained by the fact that chronic stress leads to rise in the level of free radicals and the fact that antioxidant enzymes are the first defense against oxidative stress; most of these enzymes are consumed, and therefore their concentration decreases, and this, in turn, leads to oxidative stress (Kotzaeroglou & Tsamesidis, 2022).

One of the hypotheses that explain the pathology of depression is the stress hypothesis, in which an imbalance between free radicals and antioxidant enzymes leads to oxidative stress, which involves the development of symptoms of depression through a set of mechanisms (Salim, 2017).

When the capacity of the antioxidant response system to neutralize ROS is exceeded, significant protein oxidation and lipid peroxidation take place (He *et al*., 2017).

Increased ROS generation and worn-out antioxidant defenses activate pro-inflammatory signaling, which damages vital macromolecules and results in cellular death. The brain is more susceptible to oxidative stress due to its higher oxygen use, higher lipid content, and lesser antioxidant defense (Bhatt *et al*., 2020).

This result agree with findings from earlier investigation (Kaur et al., 2020;Jasib *et al*., 2022). On the other side, superoxide dismutase and catalase levels are significantly enhanced in rat groups treated with 10 mg/kg/day EMP+ (CUMS), G5 (treated with 20 mg/kg/day EMP+ (CUMS), and 10 mg/kg fluoxetine plus CUS for 14 days compared to the group exposed to CUS (G2) only (Table 3.5).

This finding is consistent with several studies (Shakerinasab *et al*., 2022; Oshima *et al*., 2022, Gohari *et al*., 2022) that found EMP restored or increased antioxidant levels in a variety of tissues (Botros *et al*., 2022), and fluoxetine improved antioxidant enzyme concentrations (Ekeanyanwu *et al*., 2021).

These findings support previous findings that oxidative stress is increased in depression and may be decreased by antidepressant medication, indicating that antidepressant neuroprotection is mediated through an antioxidant defense mechanism (Liao *et al*.,2020).

In a rat model, SGLT2 was linked to a considerable improvement in brain mitochondrial function, including reduced ROS generation, swelling of the mitochondria, and depolarization of the mitochondrial membrane (Sa-Nguanmoo *et al*., 2017).

Empagliflozin dramatically increased glutathione reductase and catalase leukocyte expression in T2DM patients while concurrently reducing pro-oxidative myeloperoxidase (Iannantuoni *et al*., 2019). According to these findings, the antioxidant capabilities of EMP can reduce oxidative stress and hence improve depressive-like behavior.

4.5. IL-6 and TNF-α

The rats that were exposed to CUS only (G2) have higher levels of pro-inflammatory cytokines (IL-6 and TNF-α) than the control group (G1) (Table.3.6). This is because prolonged stress enhances TLR-4 gene expression and numerous DAMPs are generated in response to stress (Bueno *et al*., 2016). When these DAMPs attach to TLR-4, they raise the level of NF-kB, which serves as a transcriptional factor and leads to increased production as well as secretion of TNF-α and IL-6.

This is compatible with the previous finding that the immune system is activated as a result of stress, and proinflammatory cytokines are produced more often (Koo & Wohleb, 2021).

It is widely known that inflammation and inflammatory markers contribute to the development of MDD and can induce depression through many mechanisms (Maeng & Hong, 2019). Cytokines can cause hyperactivity and loss of negative feedback of the HPA axis, with consequential elevations in the levels of corticotropin releasing hormone(CRH) and cortisol.

Pro-inflammatory cytokines (particularly IL-6 and TNF-α) are indicated to prevent the entry of the cortisol–glucocorticoid–receptor (GR) complex into the neuronal nucleus and inhibit its binding to DNA (Lorkiewicz & Waszkiewicz, 2021). This cytokine-induced GR resistance to cortisol results in glucocorticoid resistance

leading to steroid insensitivity and cortisol hypersecretion. Under conditions of chronic stress, persistent CRH and cortisol hypersecretion will lead to adverse effects on immune function and on emotional stability, including the features seen in MDD (Sasaki *et al*., 2019).

Pro-inflammatory cytokines are indicated to inhibit neurogenesis through the direct induction of neuronal death via free radical generation and excitotoxic mechanisms, and indirectly via the pathophysiological effects of hypercortisolaemia. Persistent hypercortisolemia can act directly via voltage-gated ion channels promoting calcium entry into neurons, and resulting in neuronal destruction (Makhija & Karunakaran,2013).

Pro-inflammatory cytokines can alter the mechanisms of synthesis and reuptake of key neurotransmitters in clinical depression, namely serotonin (5-HT). The degradation of tryptophan, a precursor for 5-HT, through the kynurenine pathway has important neuropsychiatric indications (Ogyu *et al*.,2018).Whereas only a small proportion of tryptophan is used for 5-HT synthesis, over 95% is degraded in the hepatic system via tryptophan dioxygenase (TDO), generating the end product nicotinamide adenine dinucleotide (NAD (Jenkins *et al*.,2016).

The extrahepatic degradation of tryptophan can also occur through indoleamine 2,3 dioxygenase (IDO), an enzyme highly inducible by pro-inflammatory cytokines, including IFN-γ, IL-1, and TNF-α (Roohi *et al*., 2021). IDO activates the kynurenine pathway, which redirects dietary tryptophan away from 5-HT production, generating kynurenine (KYN) and other metabolites, including kynurenic acid (KA), 3-hydroxy-kynurenine (3-HK), and quinolinic acid (QA).

The reduction in 5-HT biosynthesis and the subsequent central 5-HT deficiency may potentiate the onset of depressive symptoms. Increased levels of IL-1β and TNF-

α activate the mitogen-activated protein kinase (MAPK) pathway, which enhances the activity of cell membrane transporters and the reuptake of the neurotransmitters 5-HT, dopamine, and norepinephrine. Induction of the IDO and MAPK pathways through pro-inflammatory cytokines leads to a depletion of synaptic 5-HT through reduced synthesis and increased reuptake, respectively (Grygiel-Górniak *et al*.,2019).

Furthermore, by increasing the expression and functionality of monoamine transporters, cytokines can modify serotonin signaling, these transporters have been shown to re-uptake serotonin, influence the plasticity of neurons and hippocampus neurogenesis, and disrupt the neurotrophic factor signaling cascade (Yao *et al*., 2020; Koo & Wohleb, 2021).

Pro-inflammatory cytokines such as IL-6 and TNF-α were found to be more prevalent in depressed people (Maeng & Hong, 2019; Obermanns *et al*., 2021).

Additionally, infliximab, a TNF-α inhibitor, was injected under conditions of ongoing mild stress. This resulted in higher sucrose intake during the sucrose preference test, and a significantly shorter time spent immobile during the FST (Uzzan & Azab, 2021).

The first idea contends that higher concentrations of activated 5HT-receptors found in immune cells dynamically regulate the production of pro- and anti-inflammatory cytokines, and that this may be affected by an increase in serotonin availability following antidepressant therapy (Szaach *et al*.,2019).

The second idea postulated that antidepressants might reduce cytokine levels by boosting the production of cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate leads to the activation of protein kinase A which in turn leads to an increase in cAMP-responsive element binding protein (CREB) that have the effect of reducing the release of inflammatory cytokines (Keil *et al*.,2016).

Antidepressants also treat synaptic abnormalities and reduce IL-1, IL-6, and TNF-α levels (Garca-Garca *et al*., 2022); Juszczyk *et al*., 2022).

According to (Zhang *et al*. 2017), there is growing evidence that IL-6 is one of the cytokines that is consistently raised in blood samples taken from MDD patients and plays an important part in the development of depression (Haapakoski *et al*., 2015).

An important finding in this study showed that levels of TNF-α in the brain tissue decrease significantly in rats treated with 10 mg/kg/day fluoxetine+ (CUMS), 10 mg/kg/day EMP+ (CUMS), and 20 mg/kg/day EMP+ (CUMS) for 14 days compared to the stressed group (Table.3.6).

This may explain the improvement in behavioral symptoms in this groups since there is a positive strong correlation found between these cytokines and behavioral characteristics in this study. The same results appeared concerning IL-6 except that 10 mg/kg/day show no decrease in IL-6 levels.

This is compatible with studies show anti-inflammatory effects of EMP (Abdelzaher *et al*.,2023). Empagliflozin reduces IL-6 and TNF-α levels in a variety of tissues, including the kidney and lung (Abdelhamid *et al*.,2020; Pirklbauer,2021;Heimke *et al*., 2022; Wang *et al*., 2022), also it lowers IL-6 and TNF-gene expression in brain microglia (Pawlos *et al*., 2021). Fluoxetine has anti-inflammatory characteristics, owing to a decrease in IL-6 and TNF-α levels (Mojiri-Forushani *et al*.,2022). A meta-analysis of fluoxetine-treated patients found low levels of these cytokines (Almeida *et al*., 2020; Garca-Garca *et al*.,2022), and other research has shown that fluoxetine reduces IL-6 and TNF-α levels in depression-modeling rats (Lu *et al*., 2017).

Fluoxetine controls several intricate inflammatory pathways to lessen inflammation in neurons, including inflammasomes, TLR4, peroxisome proliferator-

activated receptor gamma, and nuclear factor kappa-light-chain-enhancer of activated B cells (Dionisie *et al*., 2021).

4.6.TLR-4 gene expression:

Toll-like receptor-4 is found in many different types of cells, such as macrophages, microglia, astrocytes, and neurons, and it regulates the adrenal response to stress and inflammatory stimuli as well as the cerebral response to stress (Zhao *et al*., 2017).

Activation of the TLR-4 likely contributes to the pathophysiology of stress-evoked depression (Guo *et al*., 2019), since a wide range of endogenous molecules that are generally kept intracellularly and released in response to physical and psychological stress, such as heat shock proteins and the high mobility group box 1 (HMGB1) protein (Schaefer, 2014; Borges *et al*., 2016), act as ligands for the TLR-4 and increase its gene expression that lead to increased levels of NF-kB and activator protein-1,which subsequently promote the synthesis and release of inflammatory cytokines (Zhou *et al*., 2020).

Some depressive symptoms are linked to TLR-4 expression (Wang *et al*., 2020). This does not contradict what was found in this study, where signs of depression appeared in addition to the elevation of the TLR-4 gene expression in hippocampal tissue rats brain that were exposed to chronic unpredictable stress in G2 for a period of 24 days compared to the rats in the control group (G1) (Table 3.7), and it was found that there was a strong positive correlation between the gene expression of TLR-4 and the signs of depression (Table 3.8).

This outcome is in line with previous findings indicating that stress-exposed animals had greater levels of TLR4 mRNA in their prefrontal cortex and

hippocampus (Amiresmaeili *et al*., 2018; Hussein *et al*., 2021). Previous study have found that TLR-4 gene expression in the mouse brain increases in response to stress (Wang Y *et al*., 2018).

On the other side, a reduction in TLR-4 gene expression was shown in groups treated with EMP 10 and 20 mg/kg/day for 24 days (Table 3.7). The ability of EMP to inhibit the gene expression of TLR-4 was found in studies conducted on rats, where it was found that EMP at a dose of 10 mg/kg/day reduces the gene expression of TLR-4 in kidney tissues and protects them from stress (Ashrafi Jigheh et al., 2020; Ahmed et al.,2021). Inhibiting TLR-4 gene expression significantly reduced depression-like behavior and improved depression symptoms as found in some studies (Medina-Rodriguez *et al*., 2020; Bijani *et al*., 2022; Wei *et al*., 2022). This is consistent with what was found in this study, where the rats treated with EMP showed a clear improvement in the sucrose intake and increase period of swimming.

In group 3, in which rats were treated with fluoxetine 10 mg/kg/day, the gene expression of TLR-4 also decreased (Table 3.7), and this is compatible with other studies (Amiresmaeili *et al*., 2018; Mojiri-Forushani *et al*., 2022). In this study, EMP at a dose of 20 mg/kg/day reduced TLR-4 gene expression in hippocampal tissue more than fluoxetine at 10 mg/kg/day (Table 3.7).

An important finding reached in this study is the ability of EMP to attenuate the depressive-like behavior in the CUS model in male rats through anti-inflammatory properties by inhibiting gene expression of TLR-4 and decreasing levels of pro-inflammatory cytokines in addition to anti-oxidant properties, so it may be a promising treatment for depression.

Chapter Five
Conclusions
And
Recommendations

Conclusions:

1-Empagliflozin ameliorate depressive-like behavior induced by the CUS model in male rats .

2- Empagliflozin was increased SPI in SPT and decreased immobility time in FST.

3- Empagliflozin was decreased levels of IL-6 and TNF-α in brain of male rats.

4- Empagliflozin increased levels of antioxidant enzymes (SOD, CAT) in brain of male rats.

5- Empagliflozin was decreased gene expression of TLR-4 in hippocampal tissue.

6-There are no significant differences between fluoxetine and empagliflozin in the effect on the behavioral tests or anti-inflammatory effects in male rats.

7- The gene expression of TLR-4 in hippocampal tissue is decreased by empagliflozin at the dose of 20mg/kg greater than fluoxetine 10mg/kg in male rats.

8- TLR4 has a strong positive correlation with IL-6, TNF-α, and immobility time and a strong negative correlation with SOD, CAT, and Sucrose index

Recommendations:

1-Further studies need to study the effect of empagliflozin on others theories of depression.

2- Further studies need to study the effect of empagliflozin on other toll-like receptors that are involved in the pathogenesis of depression such as TLR-2.

3- Further studies need to study the efficacy of empagliflozin in combination with antidepressants such as fluoxetine.

4- Clinical studies need to improve the antidepressant effect of empagliflozin in humans and the safety of the doses used in this study.

References

References

- Abautret-Daly, Á., Dempsey, E., Parra-Blanco, A., Medina, C., & Harkin, A. (2018). Gut–brain actions underlying comorbid anxiety and depression associated with inflammatory bowel disease. Acta neuropsychiatrica, 30(5), 275-296.
- Abdelhamid, A. M., Elsheakh, A. R., Abdelaziz, R. R., & Suddek, G. M. (2020). Empagliflozin ameliorates ethanol-induced liver injury by modulating NF-κB/Nrf-2/PPAR-γ interplay in mice. Life Sciences, 256, 117908-117937.
- Abdelzaher, W. Y., De Waard, M., Abdelmonaem, A. A., Ali, D. M., El-Tahawy, N. F. G., Rifaai, R. A., ... & Abdel-Aziz, A. M. (2023). Empagliflozin Protects against Haloperidol Experimentally-Induced Ovarian Toxicity. Pharmaceuticals, 16(2), 168-180.
- Abed, F. N., Abbas, E. C., Al-Khalidi, H. A., AlMudhafar, A. M., & Hadi, N. R. (2021). Anti-inflammatory and antioxidant effect of Empagliflozin on cerebral ischemia/reperfusion injury in rat model. Eur J Mol Clin Med, 7(1), 4324-4334.
- Ahmed, A. S., Mona, M. M., Abdel-Kareem, M. A., & Elsisy, R. A. (2021). SGLT2 inhibitor empagliflozin monotherapy alleviates renal oxidative stress in albino Wistar diabetic rats after myocardial infarction induction. Biomedicine & Pharmacotherapy, 139, 111624-111635.
- Alexander, L., Jelen, L. A., Mehta, M. A., & Young, A. H. (2021). The anterior cingulate cortex as a key locus of ketamine's antidepressant action. Neuroscience & Biobehavioral Reviews, 127, 531-554.
- Almeida, I. B., Gomes, I. A., Shanmugam, S., de Moura, T. R., Magalhães, L. S., de Aquino, L. A. G., ... & Santos, M. R. V. (2020).

Inflammatory modulation of fluoxetine use in patients with depression: a systematic review and meta-analysis. Cytokine, 131, 155100-155106.

- Alshaya, D. S. (2022). Genetic and epigenetic factors associated with depression: an updated overview. Saudi Journal of Biological Sciences, 29,103311,1-11.
- Al-Wakeel, D. E., El-Kashef, D. H., & Nader, M. A. (2022). Renoprotective effect of empagliflozin in cafeteria diet-induced insulin resistance in rats: Modulation of HMGB-1/TLR-4/NF-κB axis. Life Sciences,301, 120633-120645.
- Amiresmaeili, A., Roohollahi, S., Mostafavi, A., & Askari, N. (2018). Effects of oregano essential oil on brain TLR4 and TLR2 gene expression and depressive-like behavior in a rat model. Research in Pharmaceutical Sciences, 13(2), 130–141
- Anacker, C., O'Donnell, K. J., & Meaney, M. J. (2022). Early life adversity and the epigenetic programming of hypothalamic-pituitary-adrenal function. Dialogues in clinical neuroscience, 16:3, 321-333.
- Annells, S., Kho, K., & Bridge, P. (2016). Meditate don't medicate: How medical imaging evidence supports the role of meditation in the treatment of depression. Radiography, 22(1), e54-e58.
- Anwar, M. A., Shah, M., Kim, J., & Choi, S. (2019). Recent clinical trends in Toll-like receptor targeting therapeutics. Medicinal research reviews, 39(3), 1053-1090.
- Aree, T. (2020). Supramolecular complexes of β-cyclodextrin with clomipramine and doxepin: Effect of the ring substituent and component of drugs on their inclusion topologies and structural flexibilities. Pharmaceuticals, 13(10), 278-290.

- Arosio, B., Guerini, F. R., Voshaar, R. C. O., & Aprahamian, I. (2021). Blood brain-derived neurotrophic factor (BDNF) and major depression: do we have a translational perspective?. Frontiers in behavioral neuroscience, 15, 626906,1-14.
- Ashrafi Jigheh, Z., Ghorbani Haghjo, A., Argani, H., & Sanajou, D. (2020). Sodium-glucose co-transporters and diabetic nephropathy: Is there a link with toll-like receptors?. Clinical and Experimental Pharmacology and Physiology, 47(6), 919-926.
- Bajpai, A., Verma, A. K., Srivastava, M., & Srivastava, R. (2014). Oxidative stress and major depression. Journal of clinical and diagnostic research: JCDR, 8(12), CC04-CC07.
- Becker, M., Pinhasov, A., & Ornoy, A. (2021). Animal models of depression: what can they teach us about the human disease?. Diagnostics, 11(1), 123-154.
- Behl, T., Makkar, R., Sehgal, A., Singh, S., Sharma, N., Zengin, G., ... & Brisc, C. (2021). Current trends in neurodegeneration: Cross talks between oxidative stress, cell death, and inflammation. International Journal of Molecular Sciences, 22(14), 7432-7451.
- Behzadi, P., García-Perdomo, H. A., & Karpiński, T. M. (2021). Toll-like receptors: general molecular and structural biology. Journal of Immunology Research, 2021,1-21.
- Belleau, E. L., Treadway, M. T., & Pizzagalli, D. A. (2019). The impact of stress and major depressive disorder on hippocampal and medial prefrontal cortex morphology. Biological psychiatry, 85(6), 443-453.

- Belovicova, K., Bogi, E., Csatlosova, K., & Dubovicky, M. (2017). Animal tests for anxiety-like and depression-like behavior in rats. Interdisciplinary toxicology, 10(1), 40-44.
- Benatti, C., Alboni, S., Blom, J. M., Mendlewicz, J., Tascedda, F., & Brunello, N. (2018). Molecular changes associated with escitalopram response in a stress-based model of depression. Psychoneuroendocrinology, 87, 74-82.
- Benazzi, F. (2022). Various forms of depression. Dialogues in clinical neuroscience,8(2),151-161.
- Benedetti, F., Zanardi, R., & Mazza, M. G. (2022). Antidepressant psychopharmacology: is inflammation a future target?. International clinical psychopharmacology, 37(3), 79-81.
- Benke, C., Autenrieth, L. K., Asselmann, E., & Pané-Farré, C. A. (2020). Lockdown, quarantine measures, and social distancing: Associations with depression, anxiety and distress at the beginning of the COVID-19 pandemic among adults from Germany. Psychiatry research, 293, 113462,1-10.
- Bhatt, S., Nagappa, A. N., & Patil, C. R. (2020). Role of oxidative stress in depression. Drug discovery today, 25(7), 1270–1276.
- Bijani, S., Dizaji, R., Sharafi, A., & Hosseini, M. J. (2022). Neuroprotective effect of apigenin on depressive-like behavior: mechanistic approach. Neurochemical Research, 47(3), 644-655.
- Boku, S., Nakagawa, S., Toda, H., & Hishimoto, A. (2018). Neural basis of major depressive disorder: beyond monoamine hypothesis. Psychiatry and clinical neurosciences, 72(1), 3-12.

- Borbély, É., Simon, M., Fuchs, E., Wiborg, O., Czéh, B., & Helyes, Z. (2022). Novel drug developmental strategies for treatment-resistant depression. British Journal of Pharmacology, 179(6), 1146-1186.
- Borges, T. J., Lang, B. J., Lopes, R. L., & Bonorino, C. (2016). Modulation of alloimmunity by heat shock proteins. Frontiers in Immunology, 7, 303-309.
- Botros, S. R., Matouk, A. I., Anter, A., Khalifa, M. M., & Heeba, G. H. (2022). Protective effect of empagliflozin on gentamicin-induced acute renal injury via regulation of SIRT1/NF-κB signaling pathway. Environmental Toxicology and Pharmacology, 94, 103907-103920.
- Brigitta, B. (2022). Pathophysiology of depression and mechanisms of treatment. Dialogues in clinical neuroscience,4(1),7-20.
- Brymer, K. J., Romay-Tallon, R., Allen, J., Caruncho, H. J., & Kalynchuk, L. E. (2019). Exploring the potential antidepressant mechanisms of TNFα antagonists. Frontiers in Neuroscience, 13, 98(1-9 pages).
- Bueno, B. G., Caso, J. R., Madrigal, J. L. M., & Leza, J. C. (2016). Innate immune receptor Toll-like receptor 4 signalling in neuropsychiatric diseases. Neuroscience & Biobehavioral Reviews, 64, 134-147.
- Camkurt, M.A. et al. (2017) Evaluation of malondialdehyde, superoxide dismutase and catalase activity in fetal cord blood of depressed mothers. Clin. Psychopharmacol. Neurosci. 15, 35–39.
- Canet, F., Iannantuoni, F., Marañon, A. M. D., Díaz-Pozo, P., López-Domènech, S., Vezza, T., ... & Víctor, V. M. (2021). Does Empagliflozin

Modulate Leukocyte–Endothelium Interactions, Oxidative Stress, and Inflammation in Type 2 Diabetes?. Antioxidants, 10(8), 1228-1238.

- Carniel, B. P., & da Rocha, N. S. (2021). Brain-derived neurotrophic factor (BDNF) and inflammatory markers: Perspectives for the management of depression. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 108, 110151,1-8.
- Carter, M. J. (2014). Diagnostic and statistical manual of mental disorders. Therapeutic recreation journal, 48(3), 275-277.
- Caruso, G., Grasso, M., Fidilio, A., Tascedda, F., Drago, F., & Caraci, F. (2020). Antioxidant properties of second-generation antipsychotics: Focus on microglia. Pharmaceuticals, 13(12), 457-477.
- Chadeve, A.(2020).A review on pharmacological and therapeutic effects of empagliflozin in patients with type 2 diabetes mellitus. Asian Journal of Pharmaceutical and Clinical Research, vol. 13(5) , 16-21
- Chen, H., Ma, Y., Chen, M., Chen, J., & Chen, J. (2021). Safflower extract improves depression in mice by inhibiting the TLR4-NLRP3 inflammation signaling pathway. Ann. Palliat. Med, 10, 8015-8023.
- Chen, L., Wang, X., Zhang, Y., Zhong, H., Wang, C., Gao, P., & Li, B. (2021). Daidzein Alleviates Hypothalamic-Pituitary-Adrenal Axis Hyperactivity, Ameliorates Depression-Like Behavior, and Partly Rectifies Circulating Cytokine Imbalance in Two Rodent Models of Depression. Frontiers in Behavioral Neuroscience, 15,1-12.
- Chen, L., Zheng, L., Chen, P., & Liang, G. (2020). Myeloid differentiation primary response protein 88 (MyD88): the central hub of TLR/IL-1R signaling. Journal of Medicinal Chemistry, 63(22), 13316,1-13.

- Chen, M. H., Kao, Z. K., Chang, W. C., Tu, P. C., Hsu, J. W., Huang, K. L., ... & Bai, Y. M. (2020). Increased proinflammatory cytokines, executive dysfunction, and reduced gray matter volumes in first-episode bipolar disorder and major depressive disorder. Journal of affective disorders, 274, 825-831.
- Cheng, Y., Desse, S., Martinez, A., Worthen, R. J., Jope, R. S., & Beurel, E. (2018). TNFα disrupts blood brain barrier integrity to maintain prolonged depressive-like behavior in mice. Brain, behavior, and immunity, 69, 556-567.
- Choi, K. W., Na, E. J., Fava, M., Mischoulon, D., Cho, H., & Jeon, H. J. (2018). Increased adrenocorticotropic hormone (ACTH) levels predict severity of depression after six months of follow-up in outpatients with major depressive disorder. Psychiatry research, 270, 246-252.
- Choi, W., Kang, H. J., Kim, J. W., Kim, H. K., Kang, H. C., Lee, J. Y., ... & Kim, J. M. (2022). Associations of serum cortisol levels, stress perception, and stressful events with suicidal behaviors in patients with depressive disorders. Journal of affective disorders, 297, 602-609.
- Cipriani, A., Furukawa, T. A., Salanti, G., Chaimani, A., Atkinson, L. Z., Ogawa, Y., ... & Geddes, J. R. (2018). Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. Focus, 16(4), 420-429.
- Cosgrove, K. T., Burrows, K., Avery, J. A., Kerr, K. L., DeVille, D. C., Aupperle, R. L., ... & Simmons, W. K. (2020). Appetite change profiles in depression exhibit differential relationships between systemic

inflammation and activity in reward and interoceptive neurocircuitry. Brain, behavior, and immunity, 83, 163-171.

- Cuijpers, P., Quero, S., Noma, H., Ciharova, M., Miguel, C., Karyotaki, E., ... & Furukawa, T. A. (2021). Psychotherapies for depression: a network meta-analysis covering efficacy, acceptability and long-term outcomes of all main treatment types. World Psychiatry, 20(2), 283-293.
- Delcourte, S., Etievant, A., & Haddjeri, N. (2021). Role of central serotonin and noradrenaline interactions in the antidepressants' action: Electrophysiological and neurochemical evidence. Progress in brain research, 259, 7-81.
- Dionisie, V., Filip, G. A., Manea, M. C., Manea, M., & Riga, S. (2021). The anti-inflammatory role of SSRI and SNRI in the treatment of depression: a review of human and rodent research studies. Inflammopharmacology, 29, 75-90.
- Dubovsky, S. L., Ghosh, B. M., Serotte, J. C., & Cranwell, V. (2021). Psychotic depression: Diagnosis, differential diagnosis, and treatment. Psychotherapy and Psychosomatics, 90(3), 160-177.
- Duda, W., Curzytek, K., Kubera, M., Connor, T. J., Fagan, E. M., Basta-Kaim, A., ... & Lasoń, W. (2019). Interaction of the immune-inflammatory and the kynurenine pathways in rats resistant to antidepressant treatment in model of depression. International Immunopharmacology, 73, 527-538.
- Duval, F., Mokrani, M. C., Erb, A., Danila, V., Lopera, F. G., Foucher, J. R., & Jeanjean, L. C. (2021). Thyroid axis activity and dopamine function in depression. Psychoneuroendocrinology, 128, 105219,1-7.

- Edinoff, A. N., Akuly, H. A., Hanna, T. A., Ochoa, C. O., Patti, S. J., Ghaffar, Y. A., ... & Kaye, A. M. (2021). Selective serotonin reuptake inhibitors and adverse effects: a narrative review. Neurology International, 13(3), 387-401.
- Efinger, V., Müller, W. E., & Friedland, K. (2021). Antidepressants: Pharmacology and Biochemistry. In NeuroPsychopharmacotherapy, Cham: Springer International Publishing . 1109-1134.
- Ekeanyanwu, R. C., Nkwocha, C. C., & Ekeanyanwu, C. L. (2021). Behavioural and biochemical indications of the antidepressant activities of essential oils from Monodora myristica (Gaertn) seed and Xylopia aethiopica (Dunal) fruit in rats. IBRO Neuroscience Reports, 10, 66-74.
- Elias, E., Zhang, A. Y., & Manners, M. T. (2022). Novel Pharmacological Approaches to the Treatment of Depression. Life, 12(2), 196-227.
- Evans, S., Alkan, E., Bhangoo, J. K., Tenenbaum, H., & Ng-Knight, T. (2021). Effects of the COVID-19 lockdown on mental health, wellbeing, sleep, and alcohol use in a UK student sample. Psychiatry research, 298, 113819,1-5.
- Evans-Lacko, S., Aguilar-Gaxiola, S., Al-Hamzawi, A., Alonso, J., Benjet, C., Bruffaerts, R., ... & Thornicroft, G. (2018). Socio-economic variations in the mental health treatment gap for people with anxiety, mood, and substance use disorders: results from the WHO World Mental Health (WMH) surveys. Psychological medicine, 48(9), 1560-1571.
- Fallah, H., & Rademaker, M. (2022). Isotretinoin for acne vulgaris–an update on adverse effects and laboratory monitoring. Journal of Dermatological Treatment, 1-11.

- Fang, X., Jiang, S., Wang, J., Bai, Y., Kim, C. S., Blake, D., ... & Lu, X. Y. (2021). Chronic unpredictable stress induces depression-related behaviors by suppressing AgRP neuron activity. Molecular psychiatry, 26(6), 2299-2315.
- Fang, Y., Ding, X., Zhang, Y., Cai, L., Ge, Y., Ma, K., ... & Hu, G. (2022). Fluoxetine inhibited the activation of A1 reactive astrocyte in a mouse model of major depressive disorder through astrocytic 5-HT2BR/β-arrestin2 pathway. Journal of neuroinflammation, 19(1), 1-20
- Farooq, R. K., Asghar, K., Kanwal, S., & Zulqernain, A. (2017). Role of inflammatory cytokines in depression: Focus on interleukin-1β. Biomedical reports, 6(1), 15-20.
- Fekadu, N., Shibeshi, W., & Engidawork, E. (2017). Major depressive disorder: pathophysiology and clinical management. J Depress Anxiety, 6(1), 255-257.
- Figueroa-Hall, L. K., Paulus, M. P., & Savitz, J. (2020). Toll-like receptor signaling in depression. Psychoneuroendocrinology, 121, 10484,1-14.
- Filatova, E. V., Shadrina, M. I., & Slominsky, P. A. (2021). Major depression: one brain, one disease, one set of intertwined processes. Cells, 10(6), 1283-1312.
- Fioravanti, G., Benucci, S. B., Prostamo, A., Banchi, V., & Casale, S. (2022). Effects of the COVID-19 pandemic on psychological health in a sample of Italian adults: A three-wave longitudinal study. Psychiatry Research, 315, 114705,1-9.

- Flouri, E., Narayanan, M. K., & Nærde, A. (2018). Stressful life events and depressive symptoms in mothers and fathers of young children. Journal of Affective Disorders, 230, 22-27.
- Fullana, M. N., Ruiz-Bronchal, E., Ferrés-Coy, A., Juárez-Escoto, E., Artigas, F., & Bortolozzi, A. (2019). Regionally selective knockdown of astroglial glutamate transporters in infralimbic cortex induces a depressive phenotype in mice. Glia, 67(6), 1122-1137.
- Furukawa, T. A., Shinohara, K., Sahker, E., Karyotaki, E., Miguel, C., Ciharova, M., ... & Cuijpers, P. (2021). Initial treatment choices to achieve sustained response in major depression: a systematic review and network meta-analysis. World Psychiatry, 20(3), 387-396.
- Furukawa, T. A., Suganuma, A., Ostinelli, E. G., Andersson, G., Beevers, C. G., Shumake, J., ... & Cuijpers, P. (2021). Dismantling, optimising, and personalising internet cognitive behavioural therapy for depression: a systematic review and component network meta-analysis using individual participant data. The Lancet Psychiatry, 8(6), 500-511.
- García-García, M. L., Tovilla-Zárate, C. A., Villar-Soto, M., Juárez-Rojop, I. E., González-Castro, T. B., Genis-Mendoza, A. D., ... & Martinez-Magaña, J. J. (2022). Fluoxetine modulates the pro-inflammatory process of IL-6, IL-1β and TNF-α levels in individuals with depression: A systematic review and meta-analysis. Psychiatry Research, 307, 114317-114327.
- Gohari, S., Reshadmanesh, T., Khodabandehloo, H., Karbalaee-Hasani, A., Ahangar, H., Arsang-Jang, S., ... & Gohari, S. (2022). The effect of EMPAgliflozin on markers of inflammation in patients with concomitant type 2 diabetes mellitus and Coronary ARtery Disease: the EMPA-

CARD randomized controlled trial. Diabetology & Metabolic Syndrome, 14(1), 1-13.

- Gokbulut, P., Kuskonmaz, S. M., Koc, G., Onder, C. E., Yumusak, N., Erel, O., ... & Culha, C. (2022). Evaluation of the Effects of Empagliflozin on Acute Lung Injury in Rat Intestinal Ischemia-Reperfusion Model. Journal of Endocrinological Investigation,106, 1-10.
- Gorman-Sandler, E., & Hollis, F. (2021). The forced swim test: Giving up on behavioral despair (Commentary on Molendijk & de Kloet, 2021). Eur. J. Neurosci, 6(15270), 10-1111.
- Goudarzi, M., Nahavandi, A., Mehrabi, S., Eslami, M., Shahbazi, A., & Barati, M. (2020). Valproic acid administration exerts protective effects against stress-related anhedonia in rats. Journal of Chemical Neuroanatomy, 105, 101768-101776.s
- Greenberg, P. E., Fournier, A. A., Sisitsky, T., Simes, M., Berman, R., Koenigsberg, S. H., & Kessler, R. C. (2021). The economic burden of adults with major depressive disorder in the United States (2010 and 2018). Pharmacoeconomics, 39(6), 653-665.
- Grygiel-Górniak, B., Limphaibool, N., & Puszczewicz, M. (2019). Cytokine secretion and the risk of depression development in patients with connective tissue diseases. Psychiatry and Clinical Neurosciences, 73(6), 302-316.
- Gulcin, İ. (2020). Antioxidants and antioxidant methods: An updated overview. Archives of toxicology, 94(3), 651-715.
- Guo, L. T., Wang, S. Q., Su, J., Xu, L. X., Ji, Z. Y., Zhang, R. Y., ... & Ma, S. P. (2019). Baicalin ameliorates neuroinflammation-induced depressive-like behavior through inhibition of toll-like receptor 4

expression via the PI3K/AKT/FoxO1 pathway. Journal of neuroinflammation, 16(1), 1-21.

- Guo, Q., Bai, X., & Feng, N. (2018). Social participation and depressive symptoms among Chinese older adults: A study on rural–urban differences. Journal of affective disorders, 239, 124-130.
- Haapakoski, R., Mathieu, J., Ebmeier, K. P., Alenius, H., & Kivimäki, M. (2015). Cumulative meta-analysis of interleukins 6 and 1β, tumour necrosis factor α and C-reactive protein in patients with major depressive disorder. Brain, behavior, and immunity, 49, 206-215.
- Hansberg, W., Nava-Ramírez, T., Rangel-Silva, P., Díaz-Vilchis, A., & Mendoza-Oliva, A. (2022). Large-size subunit catalases are chimeric proteins: a H2O2 selecting domain with catalase activity fused to a Hsp31-derived domain conferring protein stability and chaperone activity. Antioxidants, 11(5), 979 (16 pages).
- Hao, T., Du, X., Yang, S., Zhang, Y., & Liang, F. (2020). Astrocytes-induced neuronal inhibition contributes to depressive-like behaviors during chronic stress. Life sciences, 258, 118099,1-58.
- Hao, Y., Ge, H., Sun, M., & Gao, Y. (2019). Selecting an appropriate animal model of depression. International journal of molecular sciences, 20(19), 4827-4833.
- Harada, H., Mori, M., Murata, Y., Kawanabe, S., Terada, K., Matsumoto, T., ... & Enjoji, M. (2023). Dynamic Changes of Behavioral Despair, HPA Axis Activity, and Hippocampal Neurogenesis in Male Rats Induced by Social Defeat Stress. Journal of Integrative Neuroscience, 22(2), 43-54.

- Hayley, S., Hakim, A. M., & Albert, P. R. (2021). Depression, dementia and immune dysregulation. Brain, 144(3), 746-760.
- He, L. W., Zeng, L., Tian, N., Li, Y., He, T., Tan, D. M., ... & Tan, Y. (2020). Optimization of food deprivation and sucrose preference test in SD rat model undergoing chronic unpredictable mild stress. Animal models and experimental medicine, 3(1), 69-78.
- He, L., He, T., Farrar, S., Ji, L., Liu, T., & Ma, X. (2017). Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. Cellular Physiology and Biochemistry, 44(2), 532-553.
- He, P., Liu, X., Wen, J., & Zhang, Y. (2021). Major clinical advances of depression: Now and future. In E3S Web of Conferences (Vol. 292, p. 03102-03109). EDP Sciences.
- He, T., Wu, Z., Zhang, X., Liu, H., Wang, Y., Jiang, R., ... & Yang, C. (2022). A Bibliometric Analysis of Research on the Role of BDNF in Depression and Treatment. Biomolecules, 12(10), 1464-1477.
- Heimke, M., Lenz, F., Rickert, U., Lucius, R., & Cossais, F. (2022). Anti-Inflammatory Properties of the SGLT2 Inhibitor Empagliflozin in Activated Primary Microglia. Cells, 11(19), 3107-3123.
- Heise, T., Jordan, J., Wanner, C., Heer, M., Macha, S., Mattheus, M., ... & Broedl, U. C. (2016). Pharmacodynamic effects of single and multiple doses of empagliflozin in patients with type 2 diabetes. Clinical Therapeutics, 38(10), 2265-2276.
- Henrick, B. M., Yao, X. D., Zahoor, M. A., Abimiku, A. L., Osawe, S., & Rosenthal, K. L. (2019). TLR10 senses HIV-1 proteins and significantly enhances HIV-1 infection. Frontiers in immunology, 10, 482-496.

- Hu, C., Luo, Y., Wang, H., Kuang, S., Liang, G., Yang, Y., ... & Yang, J. (2017). Re-evaluation of the interrelationships among the behavioral tests in rats exposed to chronic unpredictable mild stress. PLoS One, 12(9), e0185129.
- Hung, Y. Y., Kang, H. Y., Huang, K. W., & Huang, T. L. (2014). Association between toll-like receptors expression and major depressive disorder. Psychiatry research, 220(1-2), 283-286.
- Hussein, A. J., Selman, S. M., & Abdul-razzaq, L. (2021). Effects of Withania Somnifera on Model of Chronic Stress and Brain Toll-Like Receptors 2and 4 Gene Expression in Male Rats. Systematic Reviews in Pharmacy, 12(6), 1932-1939.
- Iannantuoni, F., M. de Marañon, A., Diaz-Morales, N., Falcon, R., Bañuls, C., Abad-Jimenez, Z., ... & Rovira-Llopis, S. (2019). The SGLT2 inhibitor empagliflozin ameliorates the inflammatory profile in type 2 diabetic patients and promotes an antioxidant response in leukocytes. Journal of clinical medicine, 8(11), 1814-1825.
- Jang, D. I., Lee, A. H., Shin, H. Y., Song, H. R., Park, J. H., Kang, T. B., ... & Yang, S. H. (2021). The role of tumor necrosis factor alpha (TNF-α) in autoimmune disease and current TNF-α inhibitors in therapeutics. International journal of molecular sciences, 22(5), 2719-2735.
- Jasib Habeeb, Y., Mohammed Selman, S., & Jeafer Mehrath, A. (2022). Study of the Effects of Cordia myxa Fruit Extract on Induced Animal Model of Depression in Male Rats. Archives of Razi Institute, 77(4), 1511-1519.
- Jena, A. B., Rath, S., Subudhi, U., & Dandapat, J. (2022). Molecular interaction of benzo-a-pyrene inhibits the catalytic activity of catalase:

Insights from biophysical and computational studies. Journal of Molecular Structure, 1265, 133494,1-13.

- Jenkins, T. A., Nguyen, J. C., Polglaze, K. E., & Bertrand, P. P. (2016). Influence of tryptophan and serotonin on mood and cognition with a possible role of the gut-brain axis. Nutrients, 8(1), 56.
- Jesulola, E., Micalos, P., & Baguley, I. J. (2018). Understanding the pathophysiology of depression: From monoamines to the neurogenesis hypothesis model-are we there yet?. Behavioural brain research, 341, 79-90.
- Ji, O. S., Karalis, P. G., Daas, M., & Condon, J. (2019). Gynecomastia Associated With Citalopram. The Primary Care Companion for CNS Disorders, 21(6), 27311(9 pages).
- Jiang, T., Dai, S., Yi, Y., Liu, Y., Zhang, S., Luo, M., ... & Xu, D. (2020). The combination of HPRT and GAPDH is the best compound reference genes in the fetal rat hippocampus. Developmental Neurobiology, 80(7-8), 229-238.
- Juruena, M. F., Eror, F., Cleare, A. J., & Young, A. H. (2020). The role of early life stress in HPA axis and anxiety. Anxiety Disorders, Advances in Experimental Medicine and Biology, vol 1191, 141-153.
- Juszczyk, G., Mikulska, J., Kasperek, K., Pietrzak, D., Mrozek, W., & Herbet, M. (2021). Chronic stress and oxidative stress as common factors of the pathogenesis of depression and Alzheimer's disease: The role of antioxidants in prevention and treatment. Antioxidants, 10(9), 1439-1470.
- Kabel, A. M., Estfanous, R. S., & Alrobaian, M. M. (2020). Targeting oxidative stress, proinflammatory cytokines, apoptosis and toll like

receptor 4 by empagliflozin to ameliorate bleomycin-induced lung fibrosis. Respiratory physiology & neurobiology, 273, 103316,1-33.

- Kaku, K., Yamamoto, K., Fukushima, Y., Lliev, H., & Yasui, A. (2022). Safety and effectiveness of empagliflozin in Japanese patients with type 2 diabetes: final results of a 3-year post-marketing surveillance study. Expert Opinion on Drug Safety ,21(10), 1315-1328.
- Kalliolias, G. D., & Ivashkiv, L. B. (2016). TNF biology, pathogenic mechanisms and emerging therapeutic strategies. Nature reviews rheumatology, 12(1), 49-62.
- Kamran, M., Bibi, F., ur. Rehman, A., & Morris, D. W. (2022). Major Depressive Disorder: Existing Hypotheses about Pathophysiological Mechanisms and New Genetic Findings. Genes, 13(4), 646-665.
- Kaufmann, F. N., Costa, A. P., Ghisleni, G., Diaz, A. P., Rodrigues, A. L. S., Peluffo, H., & Kaster, M. P. (2017). NLRP3 inflammasome-driven pathways in depression: clinical and preclinical findings. Brain, behavior, and immunity, 64, 367-383.
- Kaur, D., Sasmal, D., Grewal, A. K., & Singh, T. G. (2020). Antidepressant-Like Effect of Essential Oil of Cymbopogon Flexuosus in A Chronic Unpredictable Mild Stress-Induced Depression Model In Mice. Plant Archives, 20(1), 3849-3854.
- Keil, M. F., Briassoulis, G., Stratakis, C. A., & Wu, T. J. (2016). Protein kinase A and anxiety-related behaviors: a mini-review. Frontiers in endocrinology, 7, 83(1-8).
- Kendall, K. M., Van Assche, E., Andlauer, T. F. M., Choi, K. W., Luykx, J. J., Schulte, E. C., & Lu, Y. (2021). The genetic basis of major depression. Psychological Medicine, 51(13), 2217-2230.

- Kennedy, S. H. (2022). Core symptoms of major depressive disorder: relevance to diagnosis and treatment. Dialogues in clinical neuroscience, 10:3, 271-277.
- Khan, A. R., Geiger, L., Wiborg, O., & Czéh, B. (2020). Stress-induced morphological, cellular and molecular changes in the brain—lessons learned from the chronic mild stress model of depression. Cells, 9(4), 1026(26pages) .
- Khandaker, G. M., Pearson, R. M., Zammit, S., Lewis, G., & Jones, P. B. (2014). Association of serum interleukin 6 and C-reactive protein in childhood with depression and psychosis in young adult life: a population-based longitudinal study. JAMA psychiatry, 71(10), 1121-1128.
- Khedr, S. A., Elmelgy, A. A., El-Kharashi, O. A., Abd-Alkhalek, H. A., Louka, M. L., Sallam, H. A., & Aboul-Fotouh, S. (2018). Metformin potentiates cognitive and antidepressant effects of fluoxetine in rats exposed to chronic restraint stress and high fat diet: potential involvement of hippocampal c-Jun repression. Naunyn-Schmiedeberg's Archives of Pharmacology, 391, 407-422.
- Kim, Y. J., & Lee, S. A. (2021). The relationship of lifestyle factors with the prevalence of major depressive disorder by ecological factors. Psychiatry investigation, 18(4), 340-347.
- Kim, Y. K., & Won, E. (2017). The influence of stress on neuroinflammation and alterations in brain structure and function in major depressive disorder. Behavioural brain research, 329, 6-11.
- Kinlein, S. A., Wallace, N. K., Savenkova, M. I., & Karatsoreos, I. N. (2022). Chronic hypothalamic-pituitary-adrenal axis disruption alters

glutamate homeostasis and neural responses to stress in male C57Bl6/N mice. Neurobiology of Stress, 100466,1-10.

- Kong, E., Sucic, S., Monje, F. J., Reisinger, S. N., Savalli, G., Diao, W., ... & Pollak, D. D. (2015). STAT3 controls IL6-dependent regulation of serotonin transporter function and depression-like behavior. Scientific reports, 5(1), 9009(1-8).
- Koo, J. W., & Wohleb, E. S. (2021). How stress shapes neuroimmune function: Implications for the neurobiology of psychiatric disorders. Biological psychiatry, 90(2), 74-84.
- Kotzaeroglou, A., & Tsamesidis, I. (2022). The Role of Equilibrium between Free Radicals and Antioxidants in Depression and Bipolar Disorder. Medicines, 9(11), 57.
- Kotzaeroglou, A., & Tsamesidis, I. (2022). The Role of Equilibrium between Free Radicals and Antioxidants in Depression and Bipolar Disorder. Medicines, 9(11), 57-62.
- Lee, M. T., Peng, W. H., Kan, H. W., Wu, C. C., Wang, D. W., & Ho, Y. C. (2022). Neurobiology of depression: chronic stress alters the glutamatergic system in the brain—focusing on AMPA receptor. Biomedicines, 10(5), 1005,15 pages.
- Lee, N., Heo, Y. J., Choi, S. E., Jeon, J. Y., Han, S. J., Kim, D. J., ... & Kim, H. J. (2021). Anti-inflammatory effects of empagliflozin and gemigliptin on LPS-stimulated macrophage via the IKK/NF-κB, MKK7/JNK, and JAK2/STAT1 signalling pathways. Journal of Immunology Research, 2021, vol. 2021, Article ID 9944880, 11 pages.
- Levy, M. J., Boulle, F., Steinbusch, H. W., van den Hove, D. L., Kenis, G., & Lanfumey, L. (2018). Neurotrophic factors and neuroplasticity

pathways in the pathophysiology and treatment of depression. Psychopharmacology, 235(8), 2195-2220.

- Lezak, K. R., Missig, G., & Carlezon Jr, W. A. (2022). Behavioral methods to study anxiety in rodents. Dialogues in clinical neuroscience, 19(2), 181-191.
- Li, D., & Wu, M. (2021). Pattern recognition receptors in health and diseases. Signal transduction and targeted therapy, 6(1), 291-316.
- Li, H., Liu, S., Han, J., Li, S., Gao, X., Wang, M., ... & Jin, T. (2021). Role of toll-like receptors in neuroimmune diseases: therapeutic targets and problems. Frontiers in Immunology, 12 ,777606,1-19.
- Li, Z., Ruan, M., Chen, J., & Fang, Y. (2021). Major depressive disorder: advances in neuroscience research and translational applications. Neuroscience bulletin, 37(6), 863-880.
- Liang, X., Tang, J., Qi, Y. Q., Luo, Y. M., Yang, C. M., Dou, X. Y., ... & Tang, Y. (2022). Exercise more efficiently regulates the maturation of newborn neurons and synaptic plasticity than fluoxetine in a CUS-induced depression mouse model. Experimental Neurology, 354, 114103.
- Liao, D., Lv, C., Cao, L., Yao, D., Wu, Y., Long, M., ... & Jiang, P. (2020). Curcumin attenuates chronic unpredictable mild stress-induced depressive-like behaviors via restoring changes in oxidative stress and the activation of Nrf2 signaling pathway in rats. Oxidative Medicine and Cellular Longevity, 2020, , 9268083, 1-11.
- Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., ... & Abete, P. (2018). Oxidative stress, aging, and diseases. Clinical interventions in aging,13, 757-772.

- Lindqvist, D., Dhabhar, F. S., James, S. J., Hough, C. M., Jain, F. A., Bersani, F. S., ... & Mellon, S. H. (2017). Oxidative stress, inflammation and treatment response in major depression. Psychoneuroendocrinology, 76, 197-205.
- Liu, B., Liu, J., Wang, M., Zhang, Y., & Li, L. (2017). From serotonin to neuroplasticity: evolvement of theories for major depressive disorder. Frontiers in cellular neuroscience, 11, 305-314.
- Liu, J., Buisman-Pijlman, F., & Hutchinson, M. R. (2014). Toll-like receptor 4: Innate immune regulator of neuroimmune and neuroendocrine interactions in stress and major depressive disorder. Frontiers in Neuroscience, 8, 309-314.
- Liu, X., Wang, S., & Wang, G. (2022). Prevalence and risk factors of postpartum depression in women: a systematic review and meta-analysis. Journal of Clinical Nursing, 31(19-20), 2665-2677.
- Lorkiewicz, P., & Waszkiewicz, N. (2021). Biomarkers of post-COVID depression. Journal of Clinical medicine, 10(18), 4142,1-22.
- Lu, Y., Ho, C. S., Liu, X., Chua, A. N., Wang, W., McIntyre, R. S., & Ho, R. C. (2017). Chronic administration of fluoxetine and pro-inflammatory cytokine change in a rat model of depression. PloS one, 12(10), e0186700.
- Maayah, Z. H., Ferdaoussi, M., Takahara, S., Soni, S., & Dyck, J. R. (2021). Empagliflozin suppresses inflammation and protects against acute septic renal injury. Inflammopharmacology, 29(1), 269-279.
- Maeng, S. H., & Hong, H. (2019). Inflammation as the potential basis in depression. International Neurourology Journal, 23(Suppl 2), S63-71.

- Makhija, K., & Karunakaran, S. (2013). The role of inflammatory cytokines on the aetiopathogenesis of depression. Australian & New Zealand Journal of Psychiatry, 47(9), 828-839.
- Malínská, H., Hüttl, M., Marková, I., Miklánková, D., Hojná, S., Papoušek, F., ... & Vaněčková, I. (2022). Beneficial effects of empagliflozin are mediated by reduced renal inflammation and oxidative stress in spontaneously hypertensive rats expressing human c-reactive protein. Biomedicines, 10(9), 2066-2075.
- Malyshev, A. V., Sukhanova, I. A., Ushakova, V. M., Zorkina, Y. A., Abramova, O. V., Morozova, A. Y., ... & Haile, C. N. (2022). Peptide LCGA-17 attenuates behavioral and neurochemical deficits in rodent models of PTSD and depression. Pharmaceuticals, 15(4), 462-482.

- Markov, D. D. (2022). Sucrose preference test as a measure of anhedonic behavior in a chronic unpredictable mild stress model of depression: Outstanding issues. Brain Sciences, 12(10), 1287
    - Markov, D. D. (2022). Sucrose preference test as a measure of anhedonic behavior in a chronic unpredictable mild stress model of depression: Outstanding issues. Brain Sciences, 12(10), 1287-1310.
    - McGrath, T., Baskerville, R., Rogero, M., & Castell, L. (2022). Emerging Evidence for the Widespread Role of Glutamatergic Dysfunction in Neuropsychiatric Diseases. Nutrients, 14(5), 917-944.
    - Medina-Rodriguez, E. M., Rice, K. C., Beurel, E., & Jope, R. S. (2020). (+)-Naloxone blocks Toll-like receptor 4 to ameliorate deleterious effects of stress on male mouse behaviors. Brain, behavior, and immunity, 90, 226-234.

- Medler, J., & Wajant, H. (2019). Tumor necrosis factor receptor-2 (TNFR2): an overview of an emerging drug target. Expert opinion on therapeutic targets, 23(4), 295-307.
- Mekonnen, A. D., Mills, A. A., Wilhite, A. L., & Hoffman, T. K. (2020). Desvenlafaxine-associated hyperglycemia: A case report and literature review. Mental Health Clinician, 10(3), 85-89.
- Meléndez-Mierd, Y. R. C. G. (2022). Use of Fluoxetine to Reduce Weight in Adults with Overweight or Obesity: Abridged Republication of the Cochrane Systematic Review, Obesity Facts, 15(4), 473-486.
- Mellon, S. H., Wolkowitz, O. M., Schonemann, M. D., Epel, E. S., Rosser, R., Burke, H. B., ... & Cole, S. W. (2016). Alterations in leukocyte transcriptional control pathway activity associated with major depressive disorder and antidepressant treatment. Translational psychiatry, 6(5), e821-e821.
- Menard, C., Pfau, M. L., Hodes, G. E., Kana, V., Wang, V. X., Bouchard, S., ... & Russo, S. J. (2017). Social stress induces neurovascular pathology promoting depression. Nature neuroscience, 20, 1752-1760.
- Milligan Armstrong, A., Porter, T., Quek, H., White, A., Haynes, J., Jackaman, C., ... & Groth, D. (2021). Chronic stress and A lzheimer's disease: the interplay between the hypothalamic–pituitary–adrenal axis, genetics and microglia. Biological Reviews, 96(5), 2209-2228
- Mironova, D., & Hanjieva-Darlenska, T. (2021). Effect of metformin and empagliflozin on adiponectin and leptin levels in rat model of hypo-and hyperthyroidism. Biotechnology & Biotechnological Equipment, 35(1), 208-213.

- Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. Antioxidants & redox signaling, 20(7), 1126-1167.
- Mohseni-Moghaddam, P., Dogani, M., Hatami, M., Roohollahi, S., Amiresmaeli, A., & Askari, N. (2022). A behavioral and molecular study; ameliorated anxiety-like behavior and cognitive dysfunction in a rat model of chronic unpredictable stress treated with oregano extract. Brain and Behavior, 12(8), e2727(1-12).
- Mojiri-Forushani, H., Khajehali, E., Adelipour, M., & Mohammadi, A. (2022). Inhibitory effects of fluoxetine on the secretion of inflammatory mediators and JAK/STAT3 and JNK/TLR4 gene expression. Molecular Biology Reports, 1-11.
- Mojiri-Forushani, H., Khajehali, E., Adelipour, M., & Mohammadi, A. (2022). Inhibitory effects of fluoxetine on the secretion of inflammatory mediators and JAK/STAT3 and JNK/TLR4 gene expression. Molecular Biology Reports, 1-11.
- Moon, J. S., Kim, N. H., Na, J. O., Cho, J. H., Jeong, I. K., Lee, S. H., ... & Won, K. C. (2022). Safety and Effectiveness of Empagliflozin in Korean Patients with Type 2 Diabetes Mellitus: Results from a Nationwide Post-Marketing Surveillance. Korean Diabetes Journal, Diabetes Metab J. 2023,47(1),82-91.
- Moreno-Agostino, D., Wu, Y. T., Daskalopoulou, C., Hasan, M. T., Huisman, M., & Prina, M. (2021). Global trends in the prevalence and incidence of depression: a systematic review and meta-analysis. Journal of Affective Disorders, 281, 235-243.

- Mosiołek, A., Mosiołek, J., Jakima, S., Pięta, A., & Szulc, A. (2021). Effects of antidepressant treatment on neurotrophic factors (BDNF and IGF-1) in patients with major depressive disorder (MDD). Journal of Clinical Medicine, 10(15), 3377,1-17.
- Muhammad, R. N., Ahmed, L. A., Abdul Salam, R. M., Ahmed, K. A., & Attia, A. S. (2021). Crosstalk among NLRP3 inflammasome, ET BR signaling, and miRNAs in stress-induced depression-like behavior: a modulatory role for SGLT2 inhibitors. Neurotherapeutics, 1-18.
- Mullen, S (2018) . Major depressive disorder in children and adolescents. Ment Health Clin. 2018 Nov;8(6):275-283.
- Muraro, C. et al. (2019) Major depressive disorder and oxidative stress: in silico investigation of fluoxetine activity against ROS. Appl. Sci. 9, 3631-3646.
- Musumeci, D., Roviello, G. N., & Montesarchio, D. (2014). An overview on HMGB1 inhibitors as potential therapeutic agents in HMGB1-related pathologies. Pharmacology & therapeutics, 141(3), 347-357.
- Nabila, R. A. A. (2022). The advantages of non-invasive brain stimulation for patients with major depressive. Journal of Psychiatry Psychology and Behavioral Research, 3(1), 25-28.
- Nandam, L. S., Brazel, M., Zhou, M., & Jhaveri, D. J. (2020). Cortisol and major depressive disorder—translating findings from humans to animal models and back. Frontiers in psychiatry, 10, 974(15 pages).
- Nitzan, K., Parker, M. O., Shamir, A., Chen, G., & Doron, R. (2022). The burden of stress and depression–new insight into faster and efficient treatment. Frontiers in Behavioral Neuroscience, 16, 998307.

- Nogueira, J., Gerardo, B., Silva, A. R., Pinto, P., Barbosa, R., Soares, S., ... & Freitas, S. (2022). Effects of restraining measures due to COVID-19: Pre-and post-lockdown cognitive status and mental health. Current Psychology, 41(10), 7383-7392.
- Obermanns, J., Krawczyk, E., Juckel, G., & Emons, B. (2021). Analysis of cytokine levels, T regulatory cells and serotonin content in patients with depression. European Journal of Neuroscience, 53(10), 3476-3489.
- Ogyu, K., Kubo, K., Noda, Y., Iwata, Y., Tsugawa, S., Omura, Y., ... & Nakajima, S. (2018). Kynurenine pathway in depression: A systematic review and meta-analysis. Neuroscience & Biobehavioral Reviews, 90, 16-25.
- Oshima, H., Miki, T., Kuno, A., Mizuno, M., Sato, T., Tanno, M., ... & Miura, T. (2019). Empagliflozin, an SGLT2 inhibitor, reduced the mortality rate after acute myocardial infarction with modification of cardiac metabolomes and antioxidants in diabetic rats. Journal of Pharmacology and Experimental Therapeutics, 368(3), 524-534.
- Osimo, E. F., Pillinger, T., Rodriguez, I. M., Khandaker, G. M., Pariante, C. M., & Howes, O. D. (2020). Inflammatory markers in depression: a meta-analysis of mean differences and variability in 5,166 patients and 5,083 controls. Brain, behavior, and immunity, 87, 901-909.
- Papp, M., Cubała, W. J., Swiecicki, L., Newman-Tancredi, A., & Willner, P. (2022). Perspectives for therapy of treatment-resistant depression. British Journal of Pharmacology, 179(17), 4181-4200.
- Parrott, J. M., Redus, L., Santana-Coelho, D., Morales, J., Gao, X., & O'connor, J. C. (2016). Neurotoxic kynurenine metabolism is increased

in the dorsal hippocampus and drives distinct depressive behaviors during inflammation. Translational psychiatry, 6(10), e918-e918.

- Pascual, M., Calvo-Rodriguez, M., Núñez, L., Villalobos, C., Ureña, J., & Guerri, C. (2021). Toll-like receptors in neuroinflammation, neurodegeneration, and alcohol-induced brain damage. IUBMB life, 73(7), 900-915.
- Pawlos, A., Broncel, M., Woźniak, E., & Gorzelak-Pabiś, P. (2021). Neuroprotective effect of SGLT2 inhibitors. Molecules, 26(23), 7213-7229.
- Pedersen, C. B., Mors, O., Bertelsen, A., Waltoft, B. L., Agerbo, E., McGrath, J. J., ... & Eaton, W. W. (2014). A comprehensive nationwide study of the incidence rate and lifetime risk for treated mental disorders. JAMA psychiatry, 71(5), 573-581.
- Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: properties, sources, targets, and their implication in various diseases. Indian journal of clinical biochemistry, 30, 11-26.
- Pirklbauer, M. (2021). Anti-inflammatory potential of Empagliflozin. Inflammopharmacology, 29(2), 573-576.
- Planchez, B., Surget, A., & Belzung, C. (2019). Animal models of major depression: drawbacks and challenges. Journal of Neural Transmission, 126, 1383-1408.
- Pourhamzeh, M., Moravej, F. G., Arabi, M., Shahriari, E., Mehrabi, S., Ward, R., ... & Joghataei, M. T. (2022). The roles of serotonin in neuropsychiatric disorders. Cellular and Molecular Neurobiology, 42(6), 1671-1692

- Praschak-Rieder, N., & Willeit, M. (2022). Treatment of seasonal affective disorders. Dialogues in clinical neuroscience,5(4), 389-398.
- Probert, L. (2015). TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. Neuroscience, 302, 2-22.
- Rai, A. et al. (2019) Catechin ameliorates depressive symptoms in Sprague Dawley rats subjected to chronic unpredictable mild stress by decreasing oxidative stress. Biomed. Rep. 11, 79–84.
- Ramani, J., Shah, H., Vyas, V. K., & Sharma, M. (2022). A review on the medicinal chemistry of sodium glucose co-transporter 2 inhibitors (SGLT2-I): Update from 2010 to present. European Journal of Medicinal Chemistry Reports,6,100074(pp.1-15).
- Rana, T., Behl, T., Sehgal, A., Srivastava, P., & Bungau, S. (2021). Unfolding the Role of BDNF as a Biomarker for Treatment of Depression. Journal of Molecular Neuroscience, 71(10), 2008-2021.
- Rani, T., Behl, T., Sharma, N., Makeen, H. A., Albratty, M., Alhazmi, H. A., ... & Bungau, S. G. (2022). Exploring the role of biologics in depression. Cellular Signalling, 98, 110409,1-10.
- Rao, X., Huang, X., Zhou, Z., & Lin, X. (2016). An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostatistics, bioinformatics and biomathematics, 3(3), 71–85.
- Ravens-Sieberer, U., Kaman, A., Erhart, M., Devine, J., Schlack, R., & Otto, C. (2022). Impact of the COVID-19 pandemic on quality of life and mental health in children and adolescents in Germany. European child & adolescent psychiatry, 31(6), 879-889.

- Rehman, U., Yıldırım, M., & Shahnawaz, M. G. (2022). A longitudinal study of depression, anxiety, and stress among Indians during COVID-19 pandemic. Psychology, Health & Medicine, 1-9.
- Roohi, E., Jaafari, N., & Hashemian, F. (2021). On inflammatory hypothesis of depression: what is the role of IL-6 in the middle of the chaos?. Journal of neuroinflammation, 18(1), 1-15.
- Salehpour, F., Farajdokht, F., Cassano, P., Sadigh-Eteghad, S., Erfani, M., Hamblin, M. R., ... & Mahmoudi, J. (2019). Near-infrared photobiomodulation combined with coenzyme Q10 for depression in a mouse model of restraint stress: reduction in oxidative stress, neuroinflammation, and apoptosis. Brain research bulletin, 144, 213-222.
- Salim, S. (2017). Oxidative stress and the central nervous system. Journal of Pharmacology and Experimental Therapeutics, 360(1), 201-205.
- Sa-Nguanmoo, P., Tanajak, P., Kerdphoo, S., Jaiwongkam, T., Pratchayasakul, W., Chattipakorn, N., & Chattipakorn, S. C. (2017). SGLT2-inhibitor and DPP-4 inhibitor improve brain function via attenuating mitochondrial dysfunction, insulin resistance, inflammation, and apoptosis in HFD-induced obese rats. Toxicology and applied pharmacology, 333, 43-50.
- Santos, A., Webb, S. M., & Resmini, E. (2021). Psychological complications of Cushing's syndrome. Current Opinion in Endocrinology, Diabetes and Obesity, 28(3), 325-329.
- Sasaki, K., Othman, M. B., Ferdousi, F., Yoshida, M., Watanabe, M., Tominaga, K., & Isoda, H. (2019). Modulation of the neurotransmitter

systems through the anti-inflammatory and antidepressant-like effects of squalene from Aurantiochytrium sp. Plos one, 14(6), e0218923.

- Sateia, M. J., Buysse, D. J., Krystal, A. D., Neubauer, D. N., & Heald, J. L. (2017). Clinical practice guideline for the pharmacologic treatment of chronic insomnia in adults: an American Academy of Sleep Medicine clinical practice guideline. Journal of Clinical Sleep Medicine, 13(2), 307-349.
- Schaefer, L. (2014). Complexity of danger: the diverse nature of damage-associated molecular patterns. Journal of Biological Chemistry, 289(51), 35237-35245.
- Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. Current biology, 24(10), R453-R462.
- Sedger, L. M., & McDermott, M. F. (2014). TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants–past, present and future. Cytokine & growth factor reviews, 25(4), 453-472.
- Sequeira-Cordero, A., Salas-Bastos, A., Fornaguera, J., & Brenes, J. C. (2019). Behavioural characterisation of chronic unpredictable stress based on ethologically relevant paradigms in rats. Scientific reports, 9(1), 1-21.
- Serna-Rodríguez, M. F., Bernal-Vega, S., Camacho-Morales, A., & Pérez-Maya, A. A. (2022). The role of damage associated molecular pattern molecules (DAMPs) and permeability of the blood-brain barrier in depression and neuroinflammation. Journal of Neuroimmunology, 371, 577951.

- Serna-Rodríguez, M. F., Bernal-Vega, S., Camacho-Morales, A., & Pérez-Maya, A. A. (2022). The role of damage associated molecular pattern molecules (DAMPs) and permeability of the blood-brain barrier in depression and neuroinflammation. Journal of Neuroimmunology, 371,577951.
- Shadrina, M., Bondarenko, E. A., & Slominsky, P. A. (2018). Genetics factors in major depression disease. Frontiers in psychiatry, 9, 334-342.
- Shakerinasab, N., Azizi, M., Mansourian, M., Sadeghi, H., Salaminia, S., Abbasi, R., ... & Doustimotlagh, A. H. (2022). Empagliflozin Exhibits Hepatoprotective Effects Against Bile Duct Ligation-induced Liver Injury in Rats: A Combined Molecular Docking Approach to In Vivo Studies. Current Pharmaceutical Design, 28(40), 3313-3323.
- Shelton, R. C. (2022). Selective Serotonin Reuptake Inhibitors and Related Antidepressants. The American Psychiatric Association Publishing Textbook of Mood Disorders, 239.
- Shemirani, S. B., Khalili, M. A. N., Mahdavi, S. M., & Chahardehi, A. M. (2022). The effect of mirtazapine on reducing chronic stress in male rats. International Clinical Neuroscience Journal, 9, e21-e21.
- Shitu, S., Geda, B., & Dheresa, M. (2019). Postpartum depression and associated factors among mothers who gave birth in the last twelve months in Ankesha district, Awi zone, North West Ethiopia. BMC pregnancy and childbirth, 19(1), 1-9.
- Shorey, S., Ng, E. D., & Wong, C. H. (2022). Global prevalence of depression and elevated depressive symptoms among adolescents: A systematic review and meta-analysis. British Journal of Clinical Psychology, 61(2), 287-305.

- Siddiqi, N. J., de Lourdes Pereira, M., & Sharma, B. (2022). Neuroanatomical, biochemical, and functional modifications in brain induced by treatment with antidepressants. Molecular Neurobiology, 59(6), 3564-3584.
- Slavich, G. M., & Irwin, M. R. (2014). From stress to inflammation and major depressive disorder: a social signal transduction theory of depression. Psychological bulletin, 140(3), 774-786
- Ślusarczyk, J., Trojan, E., Głombik, K., Budziszewska, B., Kubera, M., Lasoń, W., ... & Basta-Kaim, A. (2015). Prenatal stress is a vulnerability factor for altered morphology and biological activity of microglia cells. Frontiers in cellular neuroscience, 9,82,1-12.
- Strekalova, T. (2022). How the sucrose preference succeeds or fails as a measurement of anhedonia. In Psychiatric Vulnerability, Mood, and Anxiety Disorders: Tests and Models in Mice and Rats ,vol (190), 89-102.
- Szałach, Ł. P., Lisowska, K. A., & Cubała, W. J. (2019). The influence of antidepressants on the immune system. Archivum immunologiae et therapiae experimentalis, 67, 143-151.
- Tahara, A., Takasu, T., Yokono, M., Imamura, M., & Kurosaki, E. (2016). Characterization and comparison of sodium-glucose cotransporter 2 inhibitors in pharmacokinetics, pharmacodynamics, and pharmacologic effects. Journal of pharmacological sciences, 130(3), 159-169.
- Tomlinson, A., Efthimiou, O., Boaden, K., New, E., Mather, S., Salanti, G., ... & Cipriani, A. (2019). Side effect profile and comparative tolerability of 21 antidepressants in the acute treatment of major

depression in adults: protocol for a network meta-analysis. BMJ Ment Health, 22(2), 61-66.

- Troubat, R., Barone, P., Leman, S., Desmidt, T., Cressant, A., Atanasova, B., Brizard, B., El Hage, W., Surget, A., Belzung, C., & Camus, V. (2021). Neuroinflammation and depression: A review. The European journal of neuroscience, 53(1), 151–171.
- Uthman, L., Li, X., Baartscheer, A., Schumacher, C. A., Baumgart, P., Hermanides, J., ... & Weber, N. C. (2022). Empagliflozin reduces oxidative stress through inhibition of the novel inflammation/NHE/[Na+] c/ROS-pathway in human endothelial cells. Biomedicine & Pharmacotherapy, 146, 112515,6 pages.
- Uzzan, S., & Azab, A. N. (2021). Anti-TNF-α compounds as a treatment for depression. Molecules, 26(8), 2368-2377.
- Varatharaj, A., & Galea, I. (2017). The blood-brain barrier in systemic inflammation. Brain, behavior, and immunity, 60, 1-12.
- Wang, J., Yang, C., Liu, Z., Li, X., Liu, M., Wang, Y., ... & Sun, N. (2020). Association of the TLR4 gene with depressive symptoms and antidepressant efficacy in major depressive disorder. Neuroscience Letters, 736, 135292(1-22).
- Wang, L., & Chen, J. (2017). Progress in studies on TLR4 signaling pathway and major depressive disorder. Zhong nan da xue xue bao. Yi xue ban= Journal of Central South University. Medical Sciences, 42(6), 725-729.
- Wang, Q., Ju, F., Li, J., Liu, T., Zuo, Y., Abbott, G. W., & Hu, Z. (2022). Empagliflozin protects against renal ischemia/reperfusion injury in mice. Scientific Reports, 12(1), 19323(1-15).

- Wang, S., Leri, F., & Rizvi, S. J. (2021). Anhedonia as a central factor in depression: Neural mechanisms revealed from preclinical to clinical evidence. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 110, 110289(1-18).
- Wang, Y., Xu, J., Liu, Y., Li, Z., & Li, X. (2018). TLR4-NF-κB signal involved in depressive-like behaviors and cytokine expression of frontal cortex and hippocampus in stressed C57BL/6 and ob/ob mice. Neural Plasticity, 2018, 7254016(1-12).
- Wei, Y., Hu, Y., Qi, K., Li, Y., Chen, J., & Wang, R. (2022). Dihydromyricetin improves LPS-induced sickness and depressive-like behaviors in mice by inhibiting the TLR4/Akt/HIF1a/NLRP3 pathway. Behavioural Brain Research, 423, 113775.
- Wenthur, C. J., Bennett, M. R., & Lindsley, C. W. (2014). Classics in chemical neuroscience: fluoxetine (Prozac). ACS chemical neuroscience, 5(1), 14-23.
- Wichniak, A., Wierzbicka, A., Walęcka, M., & Jernajczyk, W. (2017). Effects of antidepressants on sleep. Current psychiatry reports, 19(9), 1-7.
- Willner, P. (2017). Reliability of the chronic mild stress model of depression: A user survey. Neurobiology of stress, 6, 68-77.
- Winiarska, A., Knysak, M., Nabrdalik, K., Gumprecht, J., & Stompór, T. (2021). Inflammation and oxidative stress in diabetic kidney disease: the targets for SGLT2 inhibitors and GLP-1 receptor agonists. International Journal of Molecular Sciences, 22(19), 10822(pp.1-38).
- Won, E., & Kim, Y. K. (2020). Neuroinflammation-associated alterations of the brain as potential neural biomarkers in anxiety

disorders. International Journal of Molecular Sciences, 21(18), 6546,1-19.

- World Health Organization.2023. Global health estimates. Depressive disorder (depression). Availble at https://www.who.int/news-room/fact-sheets/detail/depression . Accessed at first of February 2023 .
- Yang, T., Nie, Z., Shu, H., Kuang, Y., Chen, X., Cheng, J., ... & Liu, H. (2020). The role of BDNF on neural plasticity in depression. Frontiers in cellular neuroscience, 14, 82-94.
- Yao, Y., Ju, P., Liu, H., Wu, X., Niu, Z., Zhu, Y., ... & Fang, Y. (2020). Ifenprodil rapidly ameliorates depressive-like behaviors, activates mTOR signaling and modulates proinflammatory cytokines in the hippocampus of CUMS rats. Psychopharmacology, 237, 1421-1433.
- Younus H. (2018). Therapeutic potentials of superoxide dismutase. International journal of health sciences, 12(3), 88–93.
- Yu, F., Chen, J., Lin, J., Zhong, Z., Lu, Y., Zeng, X., & Lei, X. (2023). TLR4 involved in immune response against Vibrio Parahaemolyticus by MyD88-dependent pathway in Crassostrea hongkongensis. Fish & Shellfish Immunology,134,108591.
- Zanardi, R., Spangaro, M., Attanasio, F., Sapienza, J., Martini, F., Fregna, L., ... & Colombo, C. (2022). 11.1 Antidepressants. Fundamentals of Psychiatry for Health Care Professionals, 333.
- Zanos, P., & Gould, T. (2018). Mechanisms of ketamine action as an antidepressant. Molecular psychiatry, 23(4), 801-811.
- Zhang, J. C., Yao, W., Dong, C., Yang, C., Ren, Q., Ma, M., & Hashimoto, K. (2017). Blockade of interleukin-6 receptor in the periphery promotes rapid and sustained antidepressant actions: a possible

role of gut–microbiota–brain axis. Translational psychiatry, 7(5), e1138-e1138.

- Zhang, J., Zhang, N., Lei, J., Jing, B., Li, M., Tian, H., ... & Li, X. (2022). Fluoxetine shows neuroprotective effects against LPS-induced neuroinflammation via the Notch signaling pathway. International Immunopharmacology, 113, 109417(pp.1-12).
- Zhang, K., Lin, W., Zhang, J., Zhao, Y., Wang, X., & Zhao, M. (2020). Effect of Toll-like receptor 4 on depressive-like behaviors induced by chronic social defeat stress. Brain and behavior, 10(3), e01525,1-10.
- Zhao, D., Xu, X., Pan, L., Zhu, W., Fu, X., Guo, L., ... & Wang, J. (2017). Pharmacologic activation of cholinergic alpha7 nicotinic receptors mitigates depressive-like behavior in a mouse model of chronic stress. Journal of neuroinflammation, 14, 1-15.
- Zhao, L., Han, G., Zhao, Y., Jin, Y., Ge, T., Yang, W., ... & Li, B. (2020). Gender differences in depression: evidence from genetics. Frontiers in genetics, 11, 562316,1-15.
- Zhou, Z., Hou, J., Mo, Y., Ren, M., Yang, G., Qu, Z., & Hu, Y. (2020). Geniposidic acid ameliorates spatial learning and memory deficits and alleviates neuroinflammation via inhibiting HMGB-1 and downregulating TLR4/2 signaling pathway in APP/PS1 mice. European Journal of Pharmacology, 869, 172857,1-12.

تعد اضطرابات الاكتئاب من بين الأسباب الرئيسية لعبء المرض العالمي. كما ان الآثار الجانبية وتأخر بدء العمل والسمية من القضايا المهمة في العلاجات الحالية للاكتئاب و هنالك أدلة على أن مضادات الاكتئاب لا تفيد إلا بشكل متواضع في علاج الاكتئاب وبالتالي ، فإن ايجاد ادوية جديدة لعلاج الاكتئاب امر ضروري. الإمباغليفلوزين هو علاج لارتفاع سكر الدم ولدية اثار جانبية بسيطة مقارنة بالتأثيرات السلبية للعلاجات المضادة للاكتئاب. هدفت هذه الدراسة إلى تقييم التأثير المضاد للاكتئاب لعقار إمباغليفلوزين في ذكور الجرذان وتأثير الاجهاد على التعبير الجيني لمستقبلات TLR4 ، السايتوكينات المسببة للالتهابات ، والإجهاد التأكسدي. على حد علمنا ، هذه الدراسة هي أول دراسة لتقييم التأثير المضاد للاكتئاب لعقار إمباغليفلوزين في دماغ ذكور الجرذان.

لتحريض الاجهاد المزمن ، تم استخدام طريقة كاتز(Katz) مع بعض التعديلات. لم تتم معالجة الحيوانات في المجموعة الاولى أو تعريضها للإجهاد ، لمدة 24 يومًا ، كل حيوان في المجموعة الثانية و المجموعة الثالثة و المجموعة الرابعة و المجموعة الخامسة تعرض للإجهاد ، تلقى كل حيوان في المجموعة الثاني(٠,٥) مل من الماء المقطر عن طريق الفم لمدة ١٤ يومًا بدءًا من اليوم العاشر من الإجهاد المزمن غير المتوقع مع استمرار تطبيق الاجهاد المزمن على تلك الجرذان دون أي علاج ، تلقى كل حيوان في المجموعة الثالثة(فلوكستين (10 مجم / كجم / يوم + اجهاد مزمن غير متوقع) ، وتم إعطاء كل حيوان في المجموعة الرابعه والمجموعة الخامسة (١٠) مجم / كجم / يوم و(20) مجم / كجم / يوم امباغليفوزين + الاجهاد المزمن ، على التوالي)عن طريق الفم لمدة ١٤ يومًا بدءًا من اليوم العاشر من تطبيق الاجهاد المزمن.

تم وزن كل حيوان وتم إجراء اختبار تفضيل السكروز واختبار السباحة القسري في الأيام ٠ و ١٠ و ٢٥ بعد انتهاء مدة التجربة تم استخلاص الدماغ في اليوم الخامس والعشرين من التجربة ، وتم تحضير أنسجة الدماغ لإجراء التحاليل للسايتوكينات والانزيمات المضادة للأكسدة بواسطة مقياس الممتز المناعي المرتبط بالإنزيم وقياس التعبير الجيني بواسطة تفاعل البلمرة المتسلسل.

كان هناك انخفاض معنوي ($p<0.05$) في متوسط الوزن في المجموعة الثانية والمجموعة الثالثة و المجموعة الرابعة و المجموعة الخامسة في اليوم العاشر مقارنة باليوم الاول. في اليوم الخامس والعشرون كان هناك انخفاض معنوي في الوزن في

المجاميع الثانية و الرابعة و الخامسة مقارنة اليوم الاول. في المجاميع الثالثة و الرابعة و الخامسة ، ازداد متوسط تفضيل السكروز بشكل ملحوظ ($p<0.05$) في اليوم 25 مقارنة باليوم 10. في اليوم 25 زاد متوسط تفضيل السكروز في المجموعة الثالثة و الرابعة و الخامسة بشكل ملحوظ ($p<0.05$) مقارنة بالمجموعة الثانية. علاوة على ذلك ، انخفض متوسط تفضيل السكروز في المجموعة الثانية و الثالثة و الرابعة و الخامسة بشكل ملحوظ ($p<0.05$) في اليوم العاشر ، مقارنة باليوم(٠). في المجاميع الثانية و الثالثة و الرابعة و الخامسة ، ازدادت بشكل ملحوظ متوسط وقت عدم الحركة($p<0.05$) في اليوم العاشر ، مقارنة باليوم (٠).

في المجاميع الثالثة و الرابعة و الخامسة ، انخفض متوسط زمن عدم الحركة بشكل ملحوظ ($p<0.05$) في اليوم الخامس والعشرون. مقارنة باليوم العاشر في اليوم الخامس والعشرون ، انخفض متوسط زمن عدم الحركة في المجاميع الثالثة و الرابعة و الخامسة بشكل ملحوظ ($p<0.05$) مقارنة إلى المجموعة الثانية باليوم الخامس والعشرين. التعبير الجيني لمستقبلات TLR-4 ، في المجموعة الثانية كانت هناك زيادة مهمة احصائيا ($p<0.05$) في متوسط تغييرات طيات الجين TLR-4 مقارنة بالمجموعة الاولى ، بينما في المجاميع الثالثة و الرابعة و الخامسة كان هناك انخفاض كبير ($p<0.05$) في متوسط تغيرات طية الجين TLR-4 مقارنة بالمجموعة الثانية. كما كانت هنالك زيادة ملحوظة ($p<0.05$) في تراكيز IL-6 و TNF-α في المجموعة الثانية مقارنة بـ المجموعة الاولى. في المجموعة الثالثة و الخامسة كان هناك انخفاض معنوي ($p<0.05$) في متوسط تراكيز IL-6 و TNF-α مقارنة بـ المجموعة الثانية. فيما يخص الأنزيمات المضادة للأكسدة ، كانت هنالك زيادة معنوية في متوسط نشاط إنزيمات مضادات الأكسدة في المجموعة الثالثة و الرابعة و الخامسة مقارنة بالمجموعة الثانية.

توصلت هذه الدراسة الى ان الامباغليفلوزين يحسن اعراض الاكتئاب الناجمة عن نموذج الاجهاد المزمن في ذكور الجرذان. كما انه يثبط التعبير الجيني لمستقبلات TLR-4 ويقلل من انتاج السايتوكينات المسببة للالتهاب ويزيد من فعالية الانزيمات المضادة للأكسدة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل / كلية الطب

دراسة تأثير عقار إمباغليفلوزين على نموذج الاكتئاب المزمن والتعبير الجيني لمستقبلات (TLRs) في ذكور الجرذان

رسالة

مقدمة الى مجلس كلية الطب/ جامعة بابل

كجزء من متطلبات نيل درجة الماجستير في الادوية/ الادوية والسموم

من قبل

حـاتـم كـريـم مـجـول كـاظـم

بكالوريوس صيدلة - كلية الصيدلة - جامعة بابل (٢٠١٣ - ٢٠١٤)

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