# ANTI-STRESS POTENTIALS AND THE MECHANISM OF ACTION OF MORIN HYDRATE IN SWISS MICE



BY

**OLONODE ELIZABETH TOYIN**

**Matriculation number 160394**

**B.Sc (Hons) Biochemistry (Ife), M.Sc Pharmacology (Ibadan)**



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# ABSTRACT

Stress, is an integral component of life that distorts body homeostasis. The available anti-stress drugs are often ineffective in combating its multiple effects. Adaptogens with antioxidant and neuroprotective effects are known to relieve stress. Morin hydrate (MH), a flavonoid from *Morus alba* with known antioxidant and neuroprotective properties has not been investigated for its anti-stress potential. The study was designed to investigate the anti-stress property of MH and its mechanisms of action in mice.

Eighty male Swiss mice (22.0 ± 2.5 g) were used for acute studies: Swimming Endurance Test (SET), Anoxic Tolerance Test (ATT) and Acute Restraint Stress (ARS). The SET and ATT consisted of 5 treatment groups each (n = 5): vehicle (normal saline, 10 mg/mL), MH (5, 10, 20 mg/kg) and adaptogen (ginseng, 25 mg/kg), administered intraperitoneally. Thirty minutes later, immobility time was measured in SET and convulsion latency in ATT. In ARS, thirty male mice were allotted into treatment groups I-VI (n = 5): vehicle (10 mg/mL), vehiclestress control (10 mg/mL), MH (5, 10, 20 mg/kg) and ginseng (25 mg/kg), and treated for 7 days prior to being restrained except group 1. Thereafter, mice were assessed for anxiety and depression in Elevated-Plus Maze (EPM) and Forced-Swim Test (FST), respectively. For chronic studies, ninety male mice were used in 3 models [Chronic-Restraint Stress (CRS), Paradoxical Sleep-deprivation (PSD) and Chronic-Unpredictable Stress (CUS)] and grouped, respectively as in ARS. In CRS, mice were pre-treated and restrained for 14 days, and thereafter assessed for anxiety and depression. Mice were sleep-deprived for 48 hours in PSD, and exposed to stressors for 14 days in CUS before testing for memory and anxiety behaviours using Y-maze and EPM, respectively. Brain glutathione (GSH), malondialdehyde, nitric-oxide and blood glucose were determined spectrophotometrically in ARS, CRS, PSD and CUS. Serum corticosterone, brain tumor necrosis factor-alpha (TNF-α) and interleukin-1beta were measured in CUS using ELISA. Brain nuclear factor-κB (NF-κB) and inducible nitric-oxide synthase (iNOS) expressions in CUS were measured using immunohistochemistry. Data were analysed using ANOVA at $\alpha_{0.05}$.

Morin hydrate (5, 10, 20 mg/kg) significantly reduced immobility (10.8 ± 0.20, 7.04 ± 0.77, 9.16 ± 0.59 s against 11.9 ± 0.25 s) in SET and prolonged convulsion latency in ATT (33.1 ± 1.26, 34.1 ± 2.40, 34.8 ± 1.06 s against 21.9 ± 1.15 s). The MH decreased anxiety, depression-like symptoms, malondialdehyde and nitric-oxide but increased GSH in ARS and CRS. The MH reversed memory

impairment (65.87 ± 3.59, 69.17 ± 6.51, 62.0 ± 5.12% against 50.87 ± 2.87%) and anxiety (64.75 ± 5.36, 57.75 ± 2.95, 61.00 ± 1.68 s against 45.0 ± 1.87 s) in PSD. Also, MH increased GSH (104.6 ± 8.50, 97.3 ± 6.51, 91.5 ± 7.70 µmol/g tissue against 50.22 ± 1.41 µmol/g tissue) but decreased malondialdehyde (7.57 ± 0.25, 4.50 ± 0.13, 3.16 ± 0.22 µmol/g tissue against 8.60 ± 0.14 µmol/g tissue) and nitric-oxide (163.0 ± 8.67, 121.3 ± 6.67, 124.7 ± 3.33 µmol/g tissue against 338.0 ± 16.77 µmol/g tissue). The MH increased GSH concentration in CUS and ameliorated CUS-induced increases in glucose, malondialdehyde and nitric oxide levels compared to controls. Morin hydrate reduced corticosterone (7.93 ± 0.19, 7.18 ± 0.21, 7.46 ± 0.20 ng/mL against 8.54 ± 0.14 ng/mL), TNF-α (49.19 ± 0.55, 47.60 ± 2.48, 35.22 ± 1.77 pg/mL against 92.37 ± 7.90 pg/mL), and interleukin-1beta (74.45 ± 2.18, 46.45 ± 2.71, 43.12 ± 1.55 pg/mL against 98.72 ± 4.03 pg/mL). Morin hydrate reduced iNOS and NF-κB protein levels in CUS.

Morin hydrate exhibited antistress potential *via* mechanisms related to inhibition of hypothalamic-pituitary-adrenal axis hyperactivation, oxidative stress and neuroinflammation.

Keywords: Morin hydrate, Anti-stress property, Neuroinflammation, Corticosterone, Antioxidant activity.

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## DEDICATION

To my Sustainer and Master Planner, GOD ALMIGHTY

## ACKNOWLEDGEMENTS

My profound gratitude goes to the Almighty God, the owner of my life, my pillar of strength, my master planner, who has helped me thus far, provided all my needs, sustained me, and gave me the courage to start and finish this program.

I'm grateful to my supervisor, Dr Adegbuyi O. Aderibigbe who forms a crucial part of my mentoring in the field of neuropharmacology. I am thankful to him for equipping me with the veritable skills of experimental data acquisition, interpretation and scientific reporting. Through his thorough and diligent supervision, I have acquired immense practical knowledge and skills of research methodology. I appreciate him also for his speed and readiness in going through my write up despite his tight schedule, and putting me through on how best to discuss my findings.

I appreciate the Head of department of Pharmacology and Therapeutics, University of Ibadan, Professor E.O Iwalewa who showed his Fatherly concern when I was lagging behind in my research work due to financial constraints. I thank all my Lecturers Prof. Catherine O. Falade, Prof. A. Sowunmi, Prof. A.S. Adeagbo, Prof. O.G. Ademowo, Prof. Grace O. Gbotosho, Prof. F. Fehintola, Dr S. Umukoro and Dr Aduragbenro D. A. Adedapo who all taught me, and gave me the background knowledge of pharmacology. I appreciate Dr Oyindamola A. Abiodun for her love and encouragement and her keen interest in my progress both academically and otherwise. I sincerely appreciate Dr A.M. Ajayi, Dr A.G. Bakre, Dr O.A. Adeoluwa and Dr. A.T. Eduviere who are in one way or the other pillars of the success of my research work. Your labour of love, sacrifices and encouragements when I was about giving up, will never be forgotten. I will never forget your willingness to help, even when it wasn't convenient, just for me to succeed. I am also grateful to other members of academic staff of the department: Prof. O.S. Fagbemi, Dr. S.O. Micheal, and Mr. J.O. Badejo. I sincerely appreciate the help of the technical staff of the department in persons of Mr. W.B. Adegoke, Mr. M.O. Olatunde, and Mr. O.P. Akintoye for their kind assistance and supply of needed reagents and equipment, and also for their moral support and encouragement throughout my research. I am grateful to Mrs. A.J. Oyagbil for her motherly concerns and encouragements, Mrs. A. Olayinka, Mrs. Oluyemisi T. Adeeko and Mr A.K. Liasu for their help, kindness and support.

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And to everyone who contributed in making my dream a reality, I say a big thank you!

Elizabeth T. Olonode

# CERTIFICATION

I certify that this work is an original research carried out by Miss. Elizabeth Toyin OLONODE in the Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Ibadan.

SUPERVISOR

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Dr A.O. Aderibigbe

Reader,

Department of Pharmacology and Therapeutics

Faculty of Basic Medical Sciences,

University of Ibadan,

Ibadan,

Nigeria.

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# CHAPTER ONE

# INTRODUCTION

## 1.1. GENERAL INTRODUCTION

In our day to day life, all living organisms are faced with stressful situations which are basically a response of the body and mind to homeostatic changes and in order to survive, undergoes some physiological, morphological, and biochemical modifications. Stress can be defined as a psychological condition arising from unpleasant life experiences which imposes a strain on the emotional and mental state of individuals. Stress can also be defined as the perception of being burdened with demands which surmounts an individual's coping capability (Dorcddula *et al.*, 2014). Thus, with proper understanding of the above definitions, stress can therefore be defined as an emotional, environmental or physiological condition which requires behavioral, physiologic, morphologic, and biochemical adjustment in order to cope or survive.

Stress could be productive (eustress), or harmful (distress). Too little stress could result in stagnancy and unfulfillment while excessive stress could be damaging to health. In general, a reasonable amount of stress is needed to challenge and motivate individuals in a productive manner. Eustress presents the opportunity for motivation and productivity, bringing about personal growth, satisfaction and health enhancing benefits. Through evolution, living systems have been able to maintain a balanced homeostatic condition through strings of physiological and at times behavioral responses; functions which may as well be permanently damaged or disturbed due to unpredictable, overwhelming and continous exposure to stressful conditions, especially those which occur during vulnerable periods in life (Tilbrook and Clarke, 2006). Numerous researches conducted in human volunteers as well as in animals suggested that exposure to stressful life experiences can present substantial risks for psychopathology, particularly in genetically susceptible individuals (Krugers *et al.*, 2010). Stress contributes crucially to pathogenesis and progression of anxiety, Post-traumatic stress disorders (PTSD), bipolar disorders, schizophrenia, depression, and pathological aging (Joshi *et al.*, 2012). Stress affect the aftermath of foetus development and cause behaviours which can be detrimental to health such as drug dependence, smoking, illicit substance use, and sleep loss, and associated

with the major physical causes of death including carcinomas, cardiovascular diseases, and stroke (Cohen *et al.*, 2007).

Walter Cannon, during the American Great Depression first described the body's remarkable resilience to stress or "accidents of existence" characterized by increases in the heart's workload as the "fight or flight" response. He described this reaction which he also termed hyper-arousal or the acute stress response as the reaction which triggers a general sympathetic outflow which prepares an animal to either fight back or flee. This fight or flight response which regulate stress responses in several organisms is taken to be the first phase of the general adaptation syndrome. In 1936, Hans Selye; an assistant Professor of endocrinology attempted to investigate the effect of an ovarian extract on rats. Whenever he attempted administering the extract to the rats by injection, he ended up releasing them, run after them to track them down again before eventually injecting them with the extract. In the wake of doing this for some months, he discovered that the animals had developed gastric ulcerations, adrenal hypertrophy, and shrunken immune tissues. He then carried out the same experiment on another set of animals but in this case, the rats were only injected with saline solution, and handling them the same way as the ovarian extract treated groups. He found out the animals showed the same symptoms and from his observation, he reasoned that perhaps the change noted in the animals was attributable to the traumatic experience of his handling and not as a result of the ovarian extract. In order to establish his findings, he performed the test during winter. Some animals were placed on roof tops while some were kept in the boiler room. Some underwent forceful exercises while surgical operation was performed on others. All these conditions were indicative of a stress response that would induce structural alterations in the body. Adrenal enlargement, thymus atrophy and peptic ulcers were observed in all the animals. He then adopted the word 'stress' from engineering to describe the phenomenon and made the observation that stress is a non-specific phenomenon but produces a group of homologous reactions to a wide variety of stressors, leading to diseases, in a way similar to that in which numerous ambiguous conditions can pressurize a bit of metal and cause it to break like glass (Selye, 1998). He also described the 'fight or flight' syndrome as a mechanism including three phases; the alarm, adaptation and exhaustion phase which he described to be as a result of adaptative responses in circumstances whereby an organism's option is to either fight back or flee in order to live or survive. During the alarm phase, a number of physiological changes are observed due to a rapid elevation in catheecholamine levels, a

delayed but persistent increase in pheripheral glucocorticoid levels and rapid increase in excitatory amino acid levels in several regions of the brain (Moghaddam, 1993). Although these changes are necessary in order to survive physical short term stress, they may provoke some adverse effects if they persist.

Selye's model was however subjected to criticism by some researchers particularly on his opinion that the stress response determiners are unspecific. The justification therefore is that his research focused on physical stress like pain and tempemture extremities. whereas majority of the most detrimental stresses encountered in reality are psychological and induced by an individuals' way of interpreting events. After several years of research, John Mason: a physician in the year 1968 defined three major psychological events which could trigger the stress response. But before achieving that, he conducted a trial using human subjects where he measured the stress hormone levels in a group of individuals before subjecting them to various conditions which in his own point of view are stressful (e.g., various stressful jobs, parachute jumping, air-traffic controllers) in order that each individual can explain the psychological features of any condition they find stressful after being exposed to such conditions. After exposure, he again measured the stress hormone levels. From his result , he observed and inferred that a situation has to be interpreted as being uncontrollable, novel, and unpredictable for it to trigger a stress response (Lupien *et al.*, 2007). Though these findings incited a public argument between the two Scientists, additional investigations established that the stress response determiners are very definite and as such can be predicted and measured. Literally, extreme or prolonged stressful exposure results in a novel physiological or biochemical balance which could be favourable or deleterious, resulting in several damages and diseased states as a result of a maladaptation or allostasis (McEwen, 1998).

Several stress research aim at drug development and establishment of methodologies capable of stimulating an organisms' inherent adaptive system in order to enable it survive severe or chronic stress conditions, while simultaneously maintaining to a greater degree, the potentiality for both physical and mental work (Panossian and Wikman, 2010). No drug has been stated in the modern pharmacopeia for the treatment of stress, although several drugs including diazepam, amphetamines, caffeine, and some anabolic steroids have been used by some individuals to fight stress (Hoffman, 2001). A person undergoing severe stress but with mild symptoms of anxiety

will most likely be prescribed a mild anxiolytic such as buspirone (Hoffman, 2001), but when anxiety level is pronounced, antidepressant drugs especially amitriptyline, venlafaxine and fluoxetine would be highly recommended. In some cases, these drugs can cause some adverse reactions such as increasing the risk of depression and anxiety.

Stressful stimuli activate and trigger the release of inflammatory mediators resulting in alterations in the oxidative/nitrosative pathways in the brain (Munhoz *et al.*, 2008). A variety of plants and natural compounds have been shown in several studies to exhibit antistress and adaptogenic activities owing to their neuroprotective potentials and ability to suppress the activation of the inflammatory pathway. Morin hydrate is a flavonoid first isolated from mulberry (*Morus alba* L) branches. It's been indicated to demonstrate antioxidant, antidepressant, anxiolytic, anti-inflammatory and neuroprotective activities (Campos-Esparza *et al.*, 2009). Thus, this research aim at evaluating the antistress potentials and the mechanism of action of morin hydrate in Swiss mice.

## 1.2. STUDY OBJECTIVES

1. To investigate the anti-stress activity of morin hydrate using different standard acute and chronic stress models
2. To explore the modulatory role of morin hydrate in stress-induced behavioral changes: anxiety, depression and memory impairment.
3. To determine the effects of morin hydrate on antioxidant and pro-oxidant status.
4. To investigate the outcome of morin hydrate treatment on stress-induced hyperglycemia and dyslipidemia
5. To assess the outcome of morin hydrate treatment on the hypothalamic-pituitary-adrenal (HPA) axis.
6. To examine and quantify the outcome of morin hydrate treatment on chronic stress-induced neurodegeneration.
    - Histological studies and cell count of the prefrontal cortex and CA3 hippocampal neurons in the chronic restraint stress model.

- Histological study and cell count of the hippocampal CA1 neurons in the sleep deprivation model.
- Histological studies and cell count of the hippocampal dentate gyrus and CA3 pyramidal neurons in the chronic unpredictable stress model.

7. To explore the effect of morin hydrate on chronic stress-induced neuroinflammation:
   - Interleukin-1 beta (IL-1β)
   - Tumor necrosis factor-alpha (TNF-α)
   - Inducible nitric oxide synthase (iNOS)
   - Nuclear factor-kappa B (NF-κB)

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1. STRESSORS

Persons, objects, situations or conditions which trigger the stress response system of an individual can be labeled as a stressor. There exist the likelihood of stressors overwhelming the resources available to be utilized for the stress response and thus, are generally recognized as threats to health. Stress can be categorized into two major types: internal/emotional stressor and external stressors.

2.1.1. Internal/emotional stressors: Thesestressors include anxiety, fear, and personality traits, and they affect a person more than the external stressors. For example, worrying about an examination is actually an internal stressor which may bring about anxiety which is evident by excessive perspiration and/or difficulty in sleeping. An individual's personality traits, especially if they are negative such as distrustfulness, apprehension, perfectionism, negativism and despair could also pose as internal stressors to such individuals (Sinccro, 2012).

2.1.2. External stressors: Whatever triggers stress from the surroundings or envionment is called an external stressor. These stressors could arise from the family such as family duties, responsibilities, financial challenges and interrelationships, social stressors that develop from one's place of work, school, and relationship with people and stressors that are associated with notable life changes (change stressors) such as finding a job, getting married, and childbearing. These change stressors are usually followed by decision stressors whereby the stress ensues from the necessity to make crucial decisions (Sinccro, 2012).

Other types of stressors which may fall under either internal or external stressors include:

1. Chemical stressors: Chemical stressors are either endogenous or exogenous. Endogenous chemical stressors are created within the body system resulting from improper diet or bad nutrition, while exogenous stressors are toxins from outside the body, they include chemicals like food additives, pollution, and drugs (Sincero, 2012).

2. Disease stressors: These are stressors that arise as a result of illnesses such as diet restrictions or being confined to bed and environmental stressors such as pollutants, noise, change of weather and congestion.

## 2.2. CATEGORIES OF STRESS

### 2.2.1. Acute stress

Acute stress is a reaction/response to a sudden danger which could either be realistic or perceived. Although the term 'stress' implies an unfavourable perception, acute or short term stress is what really produces enthusiasm, delight and fervor in our lives. It is a psychological condition which arises due to experiencing a frightening event or trauma. It is brought about by our daily life needs and burdens, making it the most experienced type of stress generally. For example, preparing for an exam is a circumstance that causes acute stress which can bring about some psychological and/or physiological symptoms such as tension, headaches, stomach upsets, and insomnia. The occurrence of acute stress is short-lived but if accumulation of this stress occurs, it may evoke some emotional and physical problems like anger, apprehension, fear, depression, headache, hypertension, stomach upset, etc. (Sincero, 2012).

### 2.2.2. Episodic/periodic stress

Episodic stress implies acute stress that occurs and is suffered repeatedly. It ceases from time to time unlike acute stress. Episodic or periodic stress is usually perceived by pessimists, worry warts, people who undergo stress to achieve the impracticable and outrageous goals they set for themselves. Episodic stress is also generally experienced by people who are aggressive, contentious and atimes uptight and bitter. This can evoke anxiety disorders, emotional traumas, cardiovascular diseases, prolonged duration of recurrent depression in addition to some physical problems as seen in acute stress (Sincero, 2012).

### 2.2.3. Chronic stress

Chronic stress is evoked through persistent or long-lived exposure to some stressful conditions such as traumas, protacted illnesses, relationship defect, etc. These stressful conditions seem to

be endless and the individual exposed to them feels there's no way out of the situation. Accumulation of these stresses as a result of prolonged exposure can be threatening to life and could lead to violence, suicidal thoughts and self-inflicted injury. Chronic stress results in definite physical symptoms, probably requiring medical treatment. Critical pathological diseases like cancer, diabetes, stroke, cardiac arrest and psychological disorders are connected to chronic stress (Sincero, 2012).

## 2.3. THE STRESS RESPONSE AND GENERAL ADAPTATION SYNDROME

The physiological process which controls or co-ordinates the biological responses to stressful stimuli is called the stress response (Esch *et al.*, 2002). When organisms are confronted by stressors, an array of physiological responses is evoked to nullify the potential threat while attempting to return the body to homeostasis. The General Adaptation Syndrome was first described by Hans Selye in 1936 as an inherent struggle to re-establish homeostasis in contempt of a stressful stimulus. It comprises of three distinct phases: alarm, resistance, and exhaustion (Pawar and Hugar, 2012).

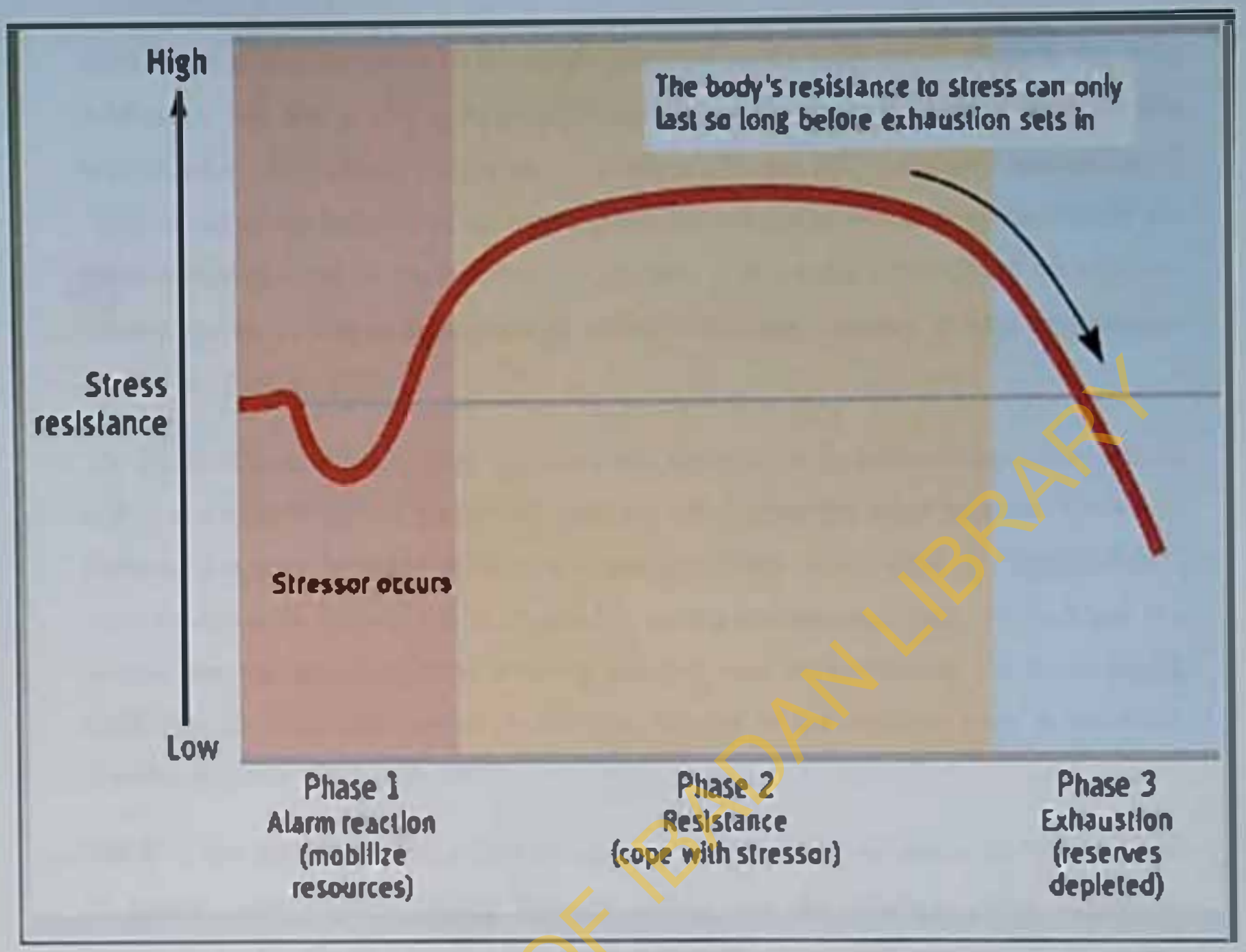


Figure 1: The general adaptation syndrome (Nichols, 2010).

The alarm phase, commonly called "fight-or flight" response is portrayed by shock and apprehension when an individual is confronted by a novel condition. This first phase comprises of a physiological reaction which forms one of the most basic innate survival instincts. Upon the mind's perception of a real or fictional stressor, the body acts via an autonomic nervous system (ANS) response which the cerebral cortex activates. The sympathetic nervous system of the ANS empowers the body to fight or flee via signalling for discharge of the various stress hormones while the parasympathetic nervous system retards all systems triggered by the stress response, counteracting the activities of the sympathetic branch. The response of the sympathetic system to stress includes chains of biochemical interactions between several body parts. The adrenal glands release adrenaline and noradrenaline upon stimulation by the hypothalamus when they perceive that additional energy is required to combat or flee from a stressor. If the stressor persists, the body adjusts to the resistance phase by attempting some means of coping or adapting, and this

result in the gradual depletion of the body's resources as the body cannot adapt to the stress indefinitely. This finally leads to the exhaustion phase, a stage where the body is no longer able to adapt, often called adrenal maladaptation (Pawar and Hugar, 2012). Adrenal maladaptation is characterized by the depletion of the body's resources and loss of normal body function. If this phase is prolonged and there is no proper intervention, it may cause exhaustion of the body and immune system as well as impairment of systemic functions, resulting in long term damage (Sonkar and Mishra, 2011).

The neural or Sympathetic-adrenal-medullary and endocrine or Hypothalamic-pituitary-adrenal (HPA) axis systems are two interwoven pathways which direct the stress response. The neural pathway is majorly activated neurally in response to stress, which leads to "fight-or-flight", while the endocrine pathway can be triggered by several mechanisms (Smith, 2012). These two systems are typically functioning within a sensitive state of equilibrium (i.e homeostasis), established to sustain the normal physiological balance of the organism even in extremely stressful situations (Esch *et al.*, 2002).

The HPA axis activation comes a little bit slower and it triggers the neurons of the hypothalamus to release corticotrophin-releasing hormone (CRH) into the pituitary portal circulation. Subsequently, the pituitary secretes into the blood stream adrenocorticotrophin (ACTH) which travels down to the adrenal glands and stimulates the stress hormones; cortisol (in humans) or corticosterone (in rodents) to be released (Lupien *et al.*, 2007). The third major event of the stress response is excitatory amino acid, glutamate release in substantial amounts into the extracellular space in various brain regions (Moghaddam, 1993). These major mediators of the stress response can trigger an acute phase response analogous to an inflammatory response evoked by an organism in response to acute injury or infections

The endocrine and metabolic functions of the HPA-axis make it an exceptionally complex system (Smith, 2012). A contributing factor to its complexity is the fact that glucocorticoids elicits diverse effects on target systems in the entire organism with the overall aim of increasing the availability of energy substrates in various body parts (e.g maintaining glucose supply in the brain), and allowing maximum adaptations to the fluctuating environmental demands. The HPA-axis is also activated by hormones, chemical messengers like interleukin-6 which increases cortisol release (Smith, 2012). Though the HPA axis activation may be considered as the

fundamental adaptive mechanism in response to change, its persistent and/or sustained activation can threaten the health of the organism. Glucocorticoids impairs growth and tissue repair, antagonizes insulin and cause a rise in blood pressure, thus resulting in the pathogenesis of diabetes, vascular disease and hypertension. Moreover, HPA axis activation inhibits immune functions, which if inhibited for a long time can be detrimeotal to the organism because it increases the risk of infection (Lupien *et al*., 2007).

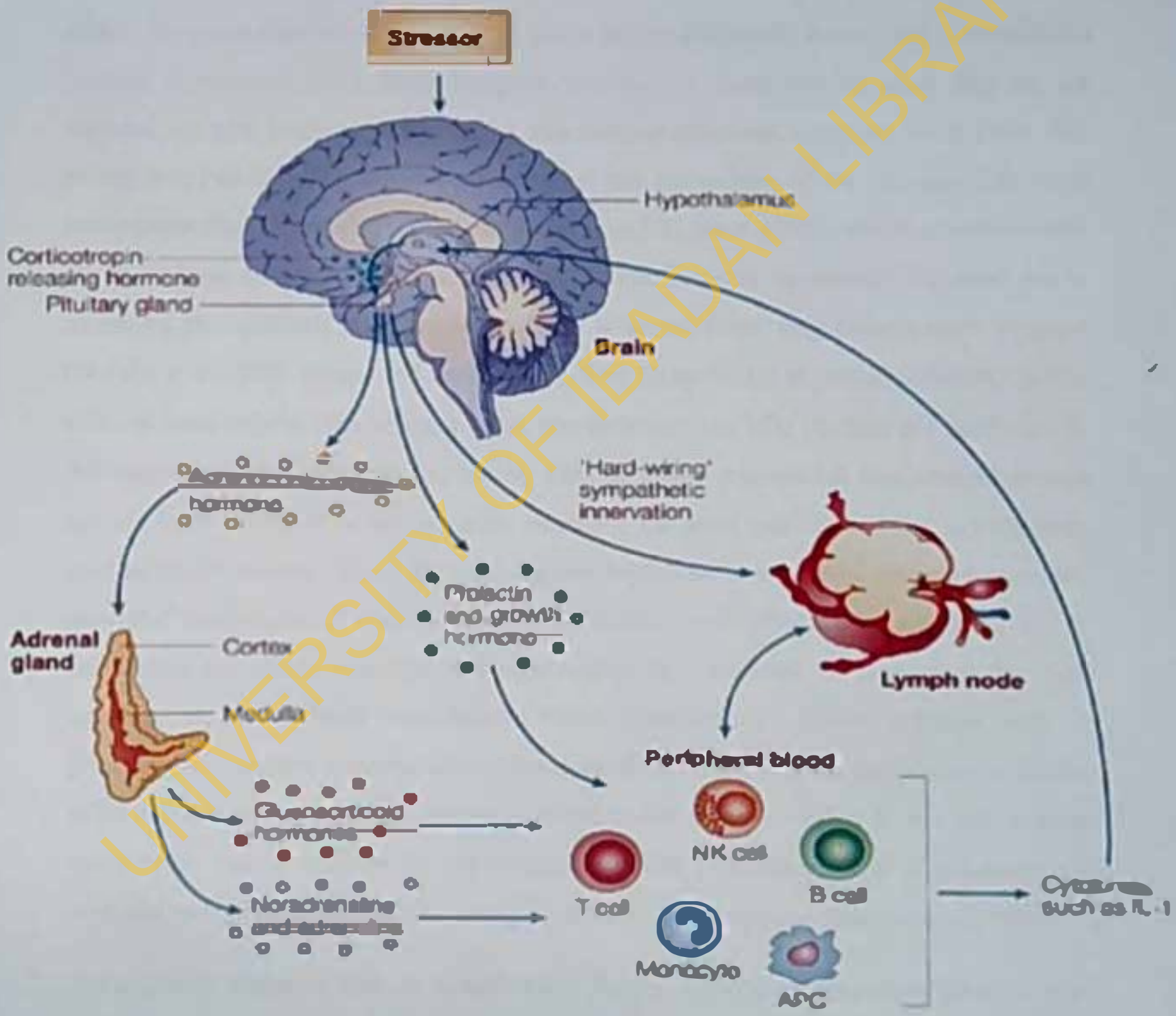


Figure 2: The HPA axis (Glaser and Kiecolt-Glaser, 2005).

## 2.4. MEDIATORS OF THE STRESS RESPONSE

### 2.4.1. Glucocorticoids.

The zona fasciculate of the adrenal cortex produces glucocorticoids which are steroid hormones and secreted in a rhythmic and periodic manner. The synthesis and secretion of these hormones, a classic endocrine response to stress is regulated by pituitary ACTH (Sapolsky *et al.*, 2000; Grippo and Scotti, 2013). In the mammalinn brain, glucocorticoids act primarily through two distinct intracellular receptors namely; mineralocorticoid (or type 1) receptors (MR) with high affinity for glucocorticoids and so at basal levels are predominantly bound, and glucocorticoid (or type 2) receptors (GR). These receptors bind to heat shock proteins when they are not occupied, but upon binding glucocorticoids. two identical monomers aggregate into a dimer, they release their heat shock protein and are conveyed into the nucleus, where they modulate genes transcription (Sorrells *et al.*, 2009). The GR have tenfold lower affinity and as a consequence, are under basal conditions partly occupied but as MR becomes increasingly saturated due to increasing glucocorticoid levels during moderate to severe stress, they become more occupied (Sorrells *et al.*, 2009; Grippo and Scotti, 2013). Both receptors are expressed differently across different brain regions, GRs having a higher concentration than MRs (Grippo and Scotti, 2013). Although stress could alter their distribution, MRs are greatly expressed in hippocampal neurons and are fairly expressed in the amygdala subnuclei, the locus coeruleus and the hypothalamic paraventricular nucleus. These are brain regions implicated in cognitive, neuroendocrine and emotional manipulation of stressful occurences (Munhoz *et al.*, 2006; Grippo and Scotti, 2013). Distribution and affinity variations in glucocorticoids have prompted the proposition that their signaling can have different transcriptional effects (Sorrells *et al.*, 2009). Different levels of glucocorticoid can have opposing effects. Basal and elevated stress levels can produce a specific effect which is mediated by high occupancy of MR, while an inverse effect is observed at either levels below normal mediated by low occupancy of MR or elevated levels of glucocorticoid mediated by high occupancy of GR, giving an inverse 'U' shaped curve (Sorrells *et al.*, 2009).

The actions of glucocorticoids are numerous and diverse, nevertheless, one of the principal roles of glucocorticoids is the regulation and modulation of the stress response following stressful exposure especially by triggering the HPA axis feedback inhibition mechanism (Grippo and Scotti, 2013). Glucocorticoids, together with catecholamines triggers the mobilization of energy

stores which is made available to the skeletal muscles with the purpose of energizing the organism to either fight back or escape (Grippo and Scotti, 2013) Simultaneously, glucocorticoids also suppress the activity of functions which are not immediately needed such as the immune system, as glucocorticoids released in response to stressful stimuli have been well documented for their anti-inflammatory and immonosupressive potentials (Sorrells *et al.*, 2009).

### 2.4.2. Monoamines

The expression of the monoamines noradrenaline, serotonin and dopamine in particular neurons increases soon after a stressful event and is either evoked directly by the brain circuits involved in interpretation of the stressful stimuli or indirectly via the adrenergic system activation (Goto *et al.*, 2007). Various kinds of stress produce pronounced elevations in noadrenergic functions in the brain. For instance, stress selectively increases monoamines turnover in the amygdala, prefrontal cortex, nucleus accumbens and hippocampus. Increase in norepinephrine turnover also occurs in the locus coeruleus, limbic regions, and the cerebral cortex (Joels and Baram, 2009). It has been shown that electric foot-shock, tail-pinch and restraint stresses elevate noradrenaline metabolism in the amygdala and hypothalamus (Ahmad *et al.*, 2012).

Monoamine release in response to stressful stimuli happens very rapidly and although there are region-specific differences, their actions hardly ever last through the duration of the stressful exposure. This is due to the fact that monoamines typically function via G protein-coupled receptors, which promptly activates effectors downstream and so, their rapid elevation is swiftly interpreted by the neurons expressing the receptors. Each behavioural facets of the stress response is modulated by each monoamine. For instance, elevated level of norepinephrine allegedly triggers a switch from information processing and attention to vigilance, offering a greater solution regardless of challenges (Aston-Jones and Cohen, 2005). Furthermore, dopamine released in the prefrontal cortex during mild stress was reported to improve evaluation of risks and decision making, while serotonin helps in curtailing anxiety following stressful exposure (Goto et al., 2007). Thus, collectively, monoamines foster significant behavioural schemes which enable the animal withstand and endure the early phase of stress (Joels and Baram, 2009).

### 2.1.3. Neuropeptides.

Several neuropeptides liberated in specific neurons during stress contributes to the stress response habitually through activating multiple receptors (Koob, 2008). These neuropeptides include vasopressin, corticotrophin releasing hormone (CRH), neuropeptide Y and substance P (Grippo and Scotti, 2013). Corticotrophin releasing hormone is liberated from the terminals of nerve axons in the hypothalamic median eminence in response to stress and perform their function on receptors in the pituitary. The peptide acts locally through two receptors; corticotrophin releasing hormone receptor (CRHR) 1 and 2 within seconds after its release, to exert neuromodulatory effects on target neurons. The stimulating activity of stress-induced CRH release is primarily mediated via CRHR1 (Gallagher *et al.*, 2008). Depending on the dose and specific region involved, occupation of CRH receptor affects neuronal firing patterns, gene expression and behavior (Gallagher *et al.*, 2008; Koob, 2008). For instance, reports show that CRH released during short-term stress in the amygdala central nucleus improves memory consolidation and moderate stress-induced CRH release from the interneurons of the hippocampus promotes long-term potentiation and improves memory. Conversely, CRH release in large amounts in the hippocampus during chronic stress triggers hyper excitability, seizures and cause hippocampal CA3 neuronal cell loss (Blank *et al.*, 2002; Roozendaal *et al.*, 2002; Chen *et al.*, 2008). Furthermore, CRH contributes to the alterations observed in the neurons of the pyramidal layer of the hippocampus caused by severe stress in both matured and developing brains (McEwen, 2007).

The interaction between vasopressin which acts on different types of neurons and hypothalamic CRH during stress promotes ACTH release from the pituitary. The excitatory activities of vasopressin in the amygdala possibly modulate stress-induced behavioural responses. Furthermore, vasopressin may also contribute to anxiety and emotional memory (Koob, 2008).

Substance P, an important element in pain perception is an 11 amino acid peptide widely distributed in the CNS as well as in the peripheral and enteric nervous systems (Brodesi *et al.*, 2003; Ebner and Singewald, 2006). It is implicated in diverse physiological and pathophysiological processes such as stress regulation, in addition to affective and anxiety-related behaviour (Ebner and Singewald, 2006). Substance P and its primary binding site, the neurokinin-1 (NK-1) receptor are widely distributed in brain tissue, mostly in areas that are

important for processing and responding to physiological and psychological stressors, and in the areas that control and regulate emotions (Singewald *et al*., 2008 ). It has been found in both autonomic afferents and in unmyelinated C-type sensory fibers and is involved in sensory pathways, including nociceptive signaling, within the dorsal horn of the spinal cord. It has been demonstrated that substance P perform an important function in the stress response and contributes to stress-induced inflammation based on its location in the peripheral nervous system and throughout most of the body (Grippo and Scotti, 2013). Substance P is elevated in the brain in reaction to diverse psychological stressors such as immobilization stress (Bradesi *et al*., 2003). Additionally, it interacts with the HPA axis, leading to increases in CRF and ACTH during psychological stressors (Grippo and Scotti, 2013).

Specialized neurons of the hypothalamus, brain stem and the limbic system expresses neuropeptide Y (NPY), accounting for its effect on stress coping, feeding and stress-induced behavioural alterations (Reichmann and Holzer, 2016). This peptide released from postganglionic sympathetic nerves stands out among other mediators as a result of its distinctive anxiolytic, neuroprotective and calming effects (Holzer *et al*., 2012; Reichmann and Holzer, 2016). It is expressed in distinct brain regions during various stressful conditions in both humans and animals but the magnitude and direction of its expression is greatly influenced by the nature and duration of the stress (Reichmann and Holzer, 2016). Neuropeptide Y is critical for stress adaptation as it counteracts the biological actions of stressful stimuli-induced CRH release, hence terminating the stress response (Holzer *et al*., 2012).

### 2.4.4. Glutamate

There are evidences that stress alters glutaminergic transmission in the prefrontal cortex and hippocampus which presents a vital process through which stress affects some aspects of psychological functions and contributes to structural abnormalities seen in depressed subjects (Popoli *et al*., 2012). Moreover, functional disorder of glutaminergic neurotransmission is a vital characteristic in psychological disorders and memory impairment (Moghaddam, 2003).

Genetic proof for glutamatergic pathway participation in regulating emotional behaviour was acquired through examining mice with genetically engineered NMDA receptor genes (Boyce-Rustay and Holmes, 2006). Several studies have revealed the mechanisms through which

glutamate neurotransmission is affected by stress and glucocorticoids. Acute stress through glucocorticoid receptors activation induces persistent potentiation of glutaminergic receptors trafficking along with transmission of excitatory synapses in the prefrontal cortex neurons, and to significantly facilitate performance on a behavioral task which encompasses prefrontal cortex-mediated working memory (Fontella *et al.*, 2004). Moreover, mice exposed to chronic immobilization stress demonstrated a rise in both basal glutamate and depolarization-dependent glutamate release from the synaptic terminals of hippocampal neurons, signifying an abnormality in the processes involved in termination of glutamate secretion (Yuen *et al.*, 2009).

### 2.4.5. Molecular chaperones

Generally called Heat Shock Proteins (HSPs), molecular chaperones are expressed when cells are exposed to adverse changes in their environment as well as other metabolic insults. With many of these stressors, cell survival is threatened. Studies have shown that these proteins provide protection against the life threatening effects of stressors by assisting in repairing damaged proteins, promoting their proper three-dimensional conformation and preventing them from forming aggregates (Panossian and Wikman, 2010). Thus, one function attributed to HSPs is to protect the cell from death in the presence of a stressor. For example, mammalian cells injected with antibodies specific to the most highly induced stress protein, HSP 70, quickly die following their exposure to a brief heat shock treatment. The major pharmacological activity of adaptogens is carried out by the 72kDa heat shock protein (Hsp 72). Its expression can be initiated by adaptogens in human microglia, where it functions to maintain the homeostasis of neuronal cells by acting as both a chaperone and a cytokine (Panossian *et al.*, 2010). It delivers antigens to antigen presenting cells (APC) through binding to antigenic peptides and stimulates the release of proinflammatory cytokines, thus enhancing immune function (Asea and Brown, 2008; Hecker and McGarvey, 2011).

### 2.4.6. Cytokines

At the initial phase of stress, up-regulated glucocorticoids together with catecholamines inhibit the secretion of proinflammatory cytokines like interferongamma (INF-γ), TNF-α, IL-1β and IL-6 directly or indirectly while promoting antiinflammatory cytokines secretion. At the prolonged stage, these cytokines are inhibited via negative feedback regulation (Tian *et al.*,

2014). Other studies carried out have shown that contrary to the fact that chronic stress suppresses proinflammatory cytokines release, it may also increase proinflammatory cytokines (Gouin *et al.*, 2012). For instance, some researchers by adopting a variety of paradigms have performed meta-analysis to more than 300 studies about chronic stress, and have found an increased production of IL-6 and INF-γ during the chronic stress, compared with the control groups (Segerstrom and Miller, 2004). These conclusions are quite inconsistent and have been suggested to be due to individual differences, type, duration and intensity of stressors, and detection methods (Sorrells *et al.*, 2009). Expantiating further on these inconsistencies, Tian *et al* (2014) considered that there are three serial stages in chronic stress upon which upregulation/downregulation of cytokines depends. They stated that at the early stage, chronic stress downregulates proinflammatory cytokines whilst upregulating anti-inflammatory cytokines. At the second stage, sustained stress may lead to HPA axis fatigue and glucocorticoid resistance; in which glucocorticoid receptors are downregulated and the sensitivity of the immune system to cortisol declines. Accordingly, the inflammatory pathways are activated, and the genes responsible for proinflammatory cytokine production are activated (Cohen *et al.*, 2012). Except the stressful stimuli is eliminated, the third stage proceed which is characterized by further increase in proinflammatory cytokines which to a certain level induce inflammatory response which may induce various diseases (Tian *et al.*, 2014).

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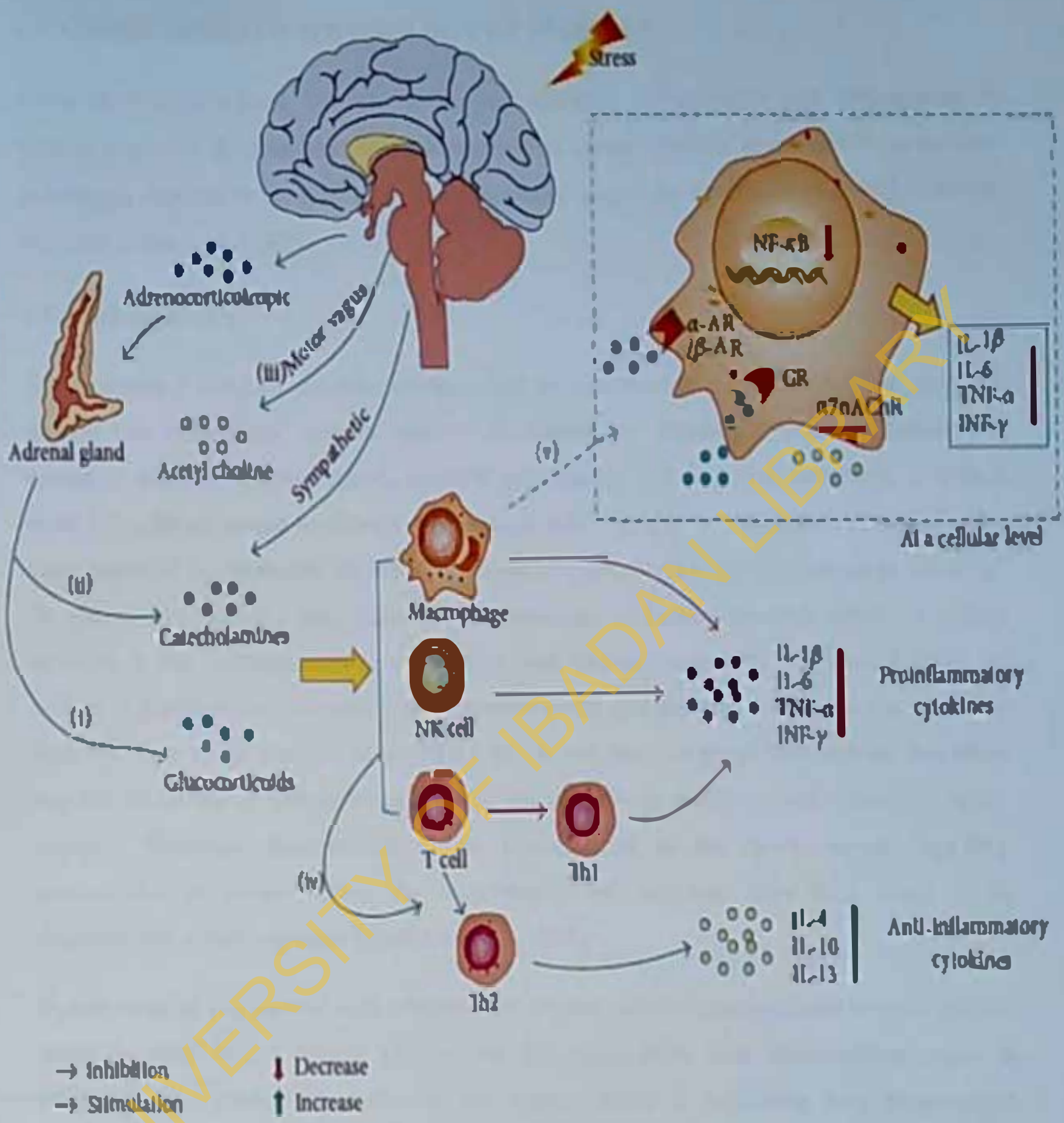


Figure 3: Prolonged stress-induced inflammatory pathway (Tian *et al*., 2014).

## 2.5. STRESS AND PATHOPHYSIOLOGY OF DISEASES

Stress performs a critical function in the predisposition, advancement and consequence of neurodegenerative diseases and mental illnesses. The extent at which stress contribute to these pathologies depends on the kind of stressor implicated and/or the timeframe of its effect on the organism (Esch *et al.*, 2002).

### 2.5.1. Schizophrenia

Schizophrenia is a mental disorder, characterized by a mixture of symptoms that are generally divided into three major clusters: positive (hallucinations, delusions), negative (anhedonia, emotional withdrawal, poor rapport, passivity and apathy) and cognitive symptoms. It affects about 0.5-1.0% of people worldwide, occurring roughly equally in both men and women. The exact causes of schizophrenia are not fully understood and till date, are still subject to debate as the illness is a complex one. Although the consensus of current research which is widely accepted is that, schizophrenia is a developmental disorder caused by a genetic liability or biological predisposition interacting with environmental and psychosocial stress; the so-called diathesis stress model (Douma *et al.*, 2011). Behavioral and biological data indicate that stress worsens symptoms of schizophrenia and that the diathesis is associated with excessive stress response. Moreover, abnormalities in the key elements of the stress cascade including abnormalities in cortisol levels, the hippocampus and cognition have been found to be characteristics of schizophrenia (Corcoran *et al.*, 2002).

Studies revealed that persons with schizophrenia express reduced glucocorticoid receptor mRNA levels in their frontal cortex. This proves that some HPA axis abnormalities occur in schizophrenia. Furthermore, evidences that chronic stress is correlated with hippocampal abnormalities as seen in schizophrenia exists (Corcoran *et al.*, 2002). Studies revealed that glucocorticoid elevation diminishes population of neuronal cells and dendrite arborisation, observations that have been reported in schizophrenia (Sapolsky, 2000). Thus, the pathophysiology of schizophrenia and some other chronic stress-induced mental illnesses are directly connected with an irregularity in the normal hippocampal neurons turnover (Arango *et al.*, 2001).

There are other mechanisms whereby stress contributes to the pathophysiology of schizophrenia. for example through the activation of dopaminergic transmission or through excitotoxic injury to vulnerable inhibitory hippocampal GABA-ergic interneurons. A study conducted in schizophrenic rats showed that animals with neonatal excitotoxic damage in the hippocampus show a very high level of mesolimbic dopamine release in response to stress. Also in healthy individuals, cortisol increases dopamine metabolism in the nucleus accumbens and raises the plasma level of homovanillic acid; a key product of dopamine metabolism (Posener *et al.*, 1999). Furthermore, *N*-methyl-D-aspartate receptor hypofunction has been proposed to decrease GABA transmission and consequently enhance glutamatergic excitotoxicity which contributes to schizophrenia (Deutsch *et al.*, 2001).

### 2.5.2. Depression

Though the molecular processes underlying the physiopathology of depression are not totally clear, studies have shown that acquired irregularities in the stress response pathway are significantly involved (Negmo *et al.*, 2000). Chronic exposure to stressful stimuli triggers excessive HPA axis activation and strong evidences signifying the contribution of HPA axis hyperactivity to the pathogenesis of mood disorders exists (Pariante and Miller, 2001). Depression is characterized by a decline in glucocorticoid receptors expression, followed by an altered feedback inhibition by endogenous glucocorticoids (Cotter and Pariante, 2002). Also, stress-induced structural changes such as dendritic arborization and reduced neuronal volume in certain brain areas like the hippocampus are clinically implicated in major depression (McEwen, 2000, Sapolsky, 2000).

Additionally, stress could initiate depression via the inflammatory pathway. The third stage of stress is characterized by further increase in proinflammatory cytokines which eventually trigger an inflammatory response (Tian *et al.*, 2014). These circulatory proinflammatory cytokines enters the brain through the weak region of blood-brain barrier or by their specific transport proteins on the brain endothelial cells or transmit the signals to the specific regions of the brain by the vagus nerve fibers (Raison *et al.*, 2006). In the CNS, the proinflammatory cytokines alter the metabolic processes of serotonin and dopamine. Suppression of secretion and reuptake block of these neurotransmitters is linked to the pathogenesis of major depression and offer insights into its therapy. Studies suggest that the major factor linking chronic stress and depression is

CRH overexpression. The corticotrophin releasing hormone of the paraventricular nuclei can be activated by proinflamatory cytokines which also up-regulate adrenocorticotrophin hormone (ACTH) and cortisol.

### 2.5.3. Anxiety

Anxiety is a natural affective response to threat or possible threat, but when the emotion is inappropraite, extreme and persistent, it is classified as pathological (Gross and Hen, 2004). Anxiety disorder is a major mental disorder which has adverse consequences on bodily functions, thereby disrupting the body's physiological balance (Ray *et al.*, 2003). It is a harsh emotional condition whereby fear perception is disparate to the type of threat. Anxiety is usually considered as long-term stress and both conditions are believed to promote the pathogenesis of each other. It's not clear why anxiety and stress seem to contribute to each other, though the onset of anxiety has been linked to the stress response system hyperactivity as well as hormone or neurotransmitter misfiring (Rachal *et al.*, 2001).

### 2.5.4. Posttraumatic stress disorder (PTSD)

Evidence suggests that severe traumatic events which are abnormal to human experiences can trigger PTSD (Maes *et al.*, 2001). Posttraumatic stress disorder results from exposure to extreme stressful conditions or traumatic events, with resultant response of fear, horror and helplessness . Posttraumatic stress disorder patients usually manifest three specific symptoms: re-experiencing the event (flashbacks, nightmares and images), evasion of aide memoire of incident, and hyper-arousal (sleeplessness, bad temper, distractions, and hyper-vigilance) for at least one month (North *et al.*, 1999). Traumatized individuals may come down with acute stress disorder during the first month following a stressful exposure. Though acute stress disorder doesn't always result in PTSD, it could elevate the risk of PTSD (Harvey and Bryant, 1998).

### 2.5.5. Alzheimer's disease

Stress alters brain integrity through the induction of memory loss and neuronal degeneration and this effect plays a significant role in the pathophysiological processes associated with Alzheimer's disease (McEwen, 1999; Lupien *et al.*, 2007). Acute or short-term stress enhances thinking while persistent and elevated levels of stress, arousal, and fear induce deficiency in

learning and loss of memory. Learning and memory deteriorate alongside increase in stress and arousal levels, in conformity with the typical inverse U-shaped curve (Garcia, 2001). One of the mechanisms through which stress exerts its influence on memory function is an elevation in corticosterone level (Wolf, 2008). Glucocorticoid receptors are densely populated in the prefrontal cortex and hippocampus; brain regions concerned with memory processing. The hippocampus is extremely prone to stressful stimuli (Perlman *et al.*, 2007). Stress-related corticosterone secretion suppresses hippocampal neuml activity, which is linked to learning and memory and cause hippocampal atrophy (Garcia, 2001). Thus, the pathogenesis of memory loss could be characterized by stress-induced HPA axis activation. This shows that glucocorticoids are able to modulate hippocampal synaptic plasticity as time pro gresses, resulting in dendritic structural changes.

Alzheimer's disease is a gradual irreversible disease occurring prevalently in the aged (about 60-65yrs but rapidly doubles every 5 years after) (Alzheimer's Association, 2011). It causes most of the dementia cases and is characterised by the development of neurofibrillary tangles and senile plaques; two proteins implicated in the progression which develops to gradual neurodegeneration and eventually death (Maccioni *et al.*, 2001). Oxidative stress was shown to participate majorly in the changes observed in normal signalling pathways of neuronal cells resulting in structural and physiological abnormalities and neurodegeneration. Hence, oxidative stress along with chronic inflammation may perhaps be linked with Alzheimer's disease. Proinflammatory cytokines released during prolong stress are also believed to participate in the neuronal degeneration seen in AD (Esch *et al.*, 2002). Research indicates that interleukin 6 (IL-6) expressions is related to social stress, and raised IL-6 levels were biochemically evaluated in brains of AD patients (Hull *et al.*, 1996). Also, IL-6 immunoreactivity was confirmed in AD plaques. Therefore, IL-6 expression may come before alterations of neuronal cells in Alzheimer's disease and chronic stress might significantly influence the pathophysiology underlying AD (Esch *et al.*, 2002).

### 2.5.6. Cardiovascular Disease

Elevated cardiovascular responsiveness to stress participates in the advancement of prospective cardiovascular diseases. Cardiovascular response to stress is mostly attributed to catecholamine hyperstimulation (Treiber *et al.*, 2003). These responses include mobilization of lipids, vascular

resistance, increased cardiac output and platelet aggregation. With repeated stress, the smooth muscles of the vessels begin to wear out, and glucose and the lipids mobilized into the systemic circulation by stress begins to deposit beneath the lining of blood vessels, causing thickening of the vessels (Sapolsky, 1998). This effect furthers the cause and development of atherosclerotic heart disease and high blood pressure (Esch *et al.*, 2002). In addition, adrenaline released during stress mobilizes stored fats which thicken vascular cells making them susceptible to clot. Blood vessels clog up and blood flow is reduced. This gives rise to artherosclerosis; a proven outcome of chronic stress in humans (Fauvel *et al.*, 2001).

Proinflammatory cytokines released during prolong stress are also believed to contribute to atherosclerotic plaque formation and cardiac irritability (Hansson, 2005). The atheroma is preceeded by an aggregation of lipid-laden cells, macrophages, and some T cells, beneath the endothelium. The activated macrophages induce the liberation of free radicals, chemokines, cytokines, and other inflammatory molecules which eventually cause inflammation and tissue damage, which promote the atherosclerotic plaque formation. Besides, the activated NF-κB induces cardiac hypertrophy (Hansson, 2005).

### 2.5.7. Diabetes

One of the major responses of the body to stress is the mobilization of fatty acids and glucose from their stores into the blood stream and in type 2 diabetes, excess circulatory glucose and fatty acids results in insulin receptor down-regulation, blockade of insulin production and insulin resistance (Sapolsky 1998). Stress also aggravates type 1 diabetes where the immune system invades the pancreatic cells, reducing their capacity to produce insulin. Excessive IL-6, TNF-α, and IL-1β production has been observed in people with diabetes (Van Greevenbroek *et al.*, 2013). Studies suggested that inflammatory cytokines, including TNF-α, could induce insulin-8 resistance (Dandona *et al.*, 2004) and accumulating data have shown that lack of TNF-α or inhibiting its receptor induces the increased sensitivity of insulin (Dandona *et al.*, 2004). Additionally, the beta cells of the pancreas, which secrete insulin, can be disordered by activated NF-κB, or damaged by iNOS, which is stimulated by the proinflammatory cytokines (Perreault and Marette, 2001).

## 2.6. ANIMAL MODELS OF STRESS

As with humans, many diverse stressors can induce neuroendocrine and behavioural changes in animals. These changes are commonly employed to mimic in animals the signs and symptoms that are indicative of particular psychiatric disorders in humans. The possibility of this is due to the fact that when similar strains of animals which are of the same age and sex are kept under a controlled environmental condition, they exhibit similar reactions to stressful conditions which are commensurate with the duration and severity of the stress. Therefore, stress protocols can be measured and regulated so as to get responses that are reproducible. In reality, stressful conditions differ from mild discomfort to trauma, and for this reason, similar varieties are needed in stress models. Animal stress models can be grouped practically into physical and psychological stress models (Golbidi *et al.*, 2015).

### 2.6.1. Animal model of physical stress

Physical stressors consist of thermal stress, restraint (or immobilization) stress etc.

#### 2.6.1.1. Thermal stress

Thermal stress can be applied either by forcing the animals (rats or mice) to swim or stand in cold water (15-20°C) with their heads above the water level for 15 minutes (Retana-Márquez *et al.*, 2003), or lowering the temperature to 4°C while shortening the swimming time to 5 minutes (Lee *et al.*, 2002). Exclusion criteria include animals that are unable swim or those that remained submerged in water. Immersion in cold water could be used to model acute as well as chronic stresses in animals. In the acute stress paradigm, 30 minutes post swimming time, the animals are sacrificed; while animals come in contact with the stressful stimuli for extended period of time (between 10-20 days) in the chronic stress model and thereafter, sacrificed an hour after the last stress exposure (Golbidi *et al.*, 2015). Acute thermal stress could be induced by a single exposure of animals to cold (4°C) for the duration of 15 or 30 minutes, while for chronic stress, the acute stress procedure is repeated for 7 – 10 days (Qui *et al.*, 2004). Another study reported the animals were maintained at -8°C for 4 hours in refrigerated compartment (Ferreira *et al.*, 2011).

This model has been modified by researchers to include the application of cold stress just to the sole of the feet in an effort to mimic everyday human life and to develop a more ethically

acceptable technique (Kanayama *et al.*, 1997). To apply cold stress locally, the floor of animal cages can be cushioned with ice in order to maintain a steady contact between the cold source and the animal's paws. This method has been reported to induce endothelial dysfunction, hypertension, proteinuria, hypercoagulation, decreased hepatic-renal circulation, and increased sympathetic system activity (Kanayama *et,al.*, 1997).

#### 2.6.1.2. Immobillzation stress

Immobillzation stress can be applied either by placing the animal in restrainer or by tying the limbs together before securing the animal on a plastic board using an adhesive tape (Kulkami and Juvekar, 2009). The time period of immobilization varies for both acute and chrome stress (Kulkami and Juvekar, 2009; Bathia *et al.*, 2011; Doreddula *et al.*, 2014). Immobilization portrays a stressful and inescapable life situation in which the physiologic response is hardly modulated by adaptation (Bathia *et al.*, 2011). Immobilization could also be combined with thermal stress and this is believed to amplify stress responses. The animals are placed, following restraining in a resupine posture, in a frosty environment (4-6 °C) for 2 hours (Krishnamurthy *et al.*, 2011) or immersed in very cold water for a period of 1 hour (Klenerová *et al.*, 2007). This immobilization in a cold environment triggers sympathetic system and HPA axis activation, consequently initiating stress ulcers in less than 3 hours (Golbidi *et al.*, 2015).

### 2.6.2. Animal model of psychological stress

#### 2.6.2.1. Noise induced stress

Environmental noise is a general problem which confronts people living in the urban areas. It induces stress and poses a risk to health (Kazi and Oommen, 2014). Prolonged exposure to noise pollution triggers recurrent sympathetic nervous system stimulation resulting in cardiovascular complications and a reduction in parasympathetic tone. Such occurences can alter a transient rise in blood pressure, converting it into a more persistent hypertension (Golbidi *et al.*, 2015). Noise stress induces memory and cognitive damage as experiments in rats indicated that it alters neurotransmitter levels in different brain sections and diminish dendrite growths (Naqvi *et al.*, 2012). Noise stress causes morphological transformations of the blood vessels as demonstrated by enhanced permeability of mesenteric circulation to albumin and

elevated degranulation of the mast cell. Manipulation of the diet by consuming additional amount of vitamin E has been shown to nullify these changes (Baldwin and Bell, 2007). Furthermore, noise pollution affects the respiratory organelles in the heart muscles and triggers the ROS generation, resulting in oxidative stress (Ostrander *et al.*, 2006).

### 2.6.2.2. Electrical shock stress

An alternative approach for psychological stress induction in animals is by exposing the animals to weak electrical currents. In this method, the animals are placed in specific cages with floors which are electrically charged to conduct electric current to the feet of the animals. Several procedures are being employed to induce different levels of stress. These procedures vary as regards recurrence, timeframe of exposure and intensity of electrical shock (Rostamkhani *et al.*, 2012). Animal's ability to predict or avoid the stressful stimuli can affect the extent of response (Nalivaiko *et al.*, 2010). The shock applied could be mild (0.5mA for 0.5s), 45 min after oral administration of drug (Bali *et al.*, 2013) or severe (multiple foot shocks of 2mA, 50Hz of 2ms duration, at 10s interval) 60 min after drug administration (Thakur *et al.*, 2015). It could also be acute, in which case animals are exposed to electric foot shock (1mA, 1Hz), for a duration of 10s every 60s for 1 day, or chronic where the animals were exposed for 15 and 30 days (Rostamkhani *et al.*, 2012). Unpredictable electrical shock was described to trigger cardiovaseular problems, and symptoms which mimic depression in humans such as body weight gain, withdrawal from social activity and reduced locomotor activity (Bobrovskaya *et al.*, 2013).

### 2.6.2.3. Behavioral despair stress

Behavioural despair stress depicts a life threatening and unavoidable circumstance which causes psychological stress that triggers the stress response. In this paradigm, animals are compelled to swim in a standardized tank full of water. There are diverse modifications of this test as regards the water temperature and the animal's swimming time. This procedure is being employed in screening for anti-depressant-like properties and stress-induced nociception (Castagne *et al.*, 2009). The time for which an animal remains inactive in water following repeated forced swimming sessions is reduced by antidepressants. However, many arguments have been raised regarding the relevance of the floating behaviour as some researchers have insinuated that this behaviour could be acquired in order to conserve energy for survival (Scharf and Schmidt, 2012).

The Forced swim test is depicted to affect cardiac function and promote artherosclerosis in normal diet-fed rats exposed to a stress protocol comprising of restraint and forced swim (Devoki *et al.*, 2013). The protocol increased blood cholesterol and triglycerides levels, reduced HDL levels and enhanced oxidative stress, effects which persisted even 20 weeks after termination of stress exposure.

### 2.6.2.4. Social defeat stress

Social defeat test is mostly used in rodents and is based on the principle that when the test animal is exposed to a dominant and aggressive counterpart for a known period of time, it mimics psychological stress in humans. To promote aggressive behavior, the dominant animal may be housed with a female prior to social defeat test to establish a sense of territorial authority and possession. As with other behavioral tests, there are some modifications in terms of frequency and length of exposure and measured responses (Golden *et al.*, 2011). Defeat stress reduces social interaction and enhances anxiety-originated behavior in the submissive animal. Similar to stress-induced psychopathology in human, the animal's behavioral disturbances respond to chronic treatment with antidepressant drugs. This resemblance in the chronology of response is considered strength of this test, which not only models depression-related psychological disorders but may also represent social phobia and post-traumatic stress disorders (Pollak *et al.*, 2010). In rats, the social defeat test has been shown to be associated with sympathetic over-activity and tachyarrhythmia, and induction of a pro-arrhythmogenic state in the myocardium (Carnevali *et al.*, 2013).

### 2.6.2.5. Neonatal-maternal separation stress

Maternal separation has been arguably proposed as the commonest paradigm of prolonged trauma (Smith 2012). It's a chronic behavioral stress paradigm that evaluates how psychological stresses experienced in early life can affect subsequent events in life. Normally for the first few weeks of their life, pups remain with their mothers till their weaning age of 21-30 days, but this test involves temporarily separating or isolating the pups for a set time period per day throughout the early postnatal life starting from day 2 and ending on day 14 (Kosten *et al.*, 2007). There are behavioural and structural outcomes to this protocol. Isolation stress triggers HPA axis activation and induces both functional and morphological alterations in different regions of the brain. This

method is employed in evaluating the impact of neonatal isolation on synaptic plasticity (Blaise *et al.*, 2008), adulthood addiction, response to psycho-stimulants (Kosten and Kehoe, 2005), nociceptive stimuli (Coutinho *et al.*, 2002), and exploratory activities (Becker *et al.*, 2007).

### 2.6.2.6. Changing light-dark cycle

Any disturbance in the sleep-wake cycle (circadian rhythm) of an organism can result in malfunction of the metabolic, hormonal, immunologic, and infact, the total physiological functions of the body (Hickie *et al.*, 2013). It is controlled by the melatonin-serotonin balance. Bright light increases brain serotonin levels while melatonin is produced in the dark by the pineal glands situated near the center of the brain. These pineal glands receive visual information via the hypothalamic suprachiasmatic nuclei (SCN) which functions in regulating daily fluctuations of the internal environment, regulating and aligning them to the changing day and night cycles (Hickie *et al.*, 2013). Stress can be induced in nocturnal animals by placing them in darkness all through the day and under bright light at night, thus disturbing their circadian rhythm. As the animals adjust to this new routine, they undergo acute stress accompanied by some neurological symptoms (Maquet, 2001).

### 2.6.2.7. Chronic unpredictable mild stress (CUMS)

This model utilizes a set of mild socio-environmental stressors which the animals are being exposed to once or twice per day for a period of seven (7) to fifty-four (54) days in order to mimic long term stress in humans (Buwalda *et al.*, 2005; Pandey *et al.*, 2010). Inspite of the existing discrepancies between protocols, stressors which are most commonly applied include restraint, wet beddings, food and/or water deprivation, cage tilting, solitary confinement, forced swimming, exposure to temperature changes, and reversal of the light-dark cycle (Munhoz *et al.*, 2006; Isingrini *et al.*, 2011). Behavioral consequences of this method includes lack of motivation towards gaining rewards and self care (grooming) behaviour, reduced consumption of sweet foods and drinks, and decreased responsiveness to acute stresses, which all are symptoms resembling anhedonia in human mood disorders (Isingrini *et al.*, 2011; Bayramgurler *et al.*, 2013). These symptoms can be reversed through chronic administration of compounds possessing antidepressant activity (Pollak *et al.*, 2008). Furthermore, HPA axis hyperactivity, adrenal glands hypertrophy and body weight decrease commensurate with major depression in

humans (Lucca *et al.*, 2008). This procedure also induces immunological and neurohumoral changes which are related to depressive disorders in humans for example, increase in the level of corticosterone, increased TNF-α and IL-β concentration and elevated reactive oxygen species activity (Grippo *et al.*, 2005). Chronic unpredictable mild stress also present some adverse cardiovascular outcomes in rats such as reduction in endothelial nitric oxide synthase (eNOS) expressions, elevation of blood lipids, enhanced sensitivity to the vasoconstricting actions of phenylephrine as well as enlargement of the innermost layer of the aorta (Bayramgurler *et al.*, 2013). Four weeks of CUMS also caused an elevation in the resting heart rate and decreased variations of heart rate in experimental animals (Golbidi *et al.*, 2015).

#### 2.6.2.8. Predatory stress

The predatory stress protocol entails exposing the experimental animals to their characteristic predators or their odours. For example, a mouse comes in contact with a cat or cat odour. This situation leads to acute stimulation of the sympathetic system and provokes the entire stress-associated endocrine and neurological responses (Marilia *et al.*, 2007). This model is widely used not only in the study of anxiety in animals, but for screening of substances for anxiolytic properties as well (Barros *et al.*, 2008). Predatory stress model is applicable in the study of post-traumatic stress disorders due to the fact that the test is characterized by unpredictability, uncontrollability and sufficient severity (El Hage *et al.*, 2006). Inspite of the fact that predatory stress is proposed to be an acute stress paradigm, long-term anxiogenic behaviours have been reported and this may be as a result of habituation of the prey to predator exposure (El Hage *et al.*, 2006; Jaggi *et al.*, 2011). Buspirone, diazepam, and substance P all mitigate fear-induced responses and enhanced exploratory behaviours (Barros *et al.*, 2008)

## 2.7. STRESS MANAGEMENT

Our environment is filled with stressful life events and under this condition, effective coping strategies are essential for survival. Poor stress management can lead to depression, loss of memory, lack of concentration, irritability, disruption of sleep and eating patterns, withdrawal or isolation problems and some other physical complaints such as chronic headaches, fatigue,

weight loss, etc. In order to achieve an effective stress management, a proper recognition of stress, the stressor, the manifestation, and its effect on one's well-being are required.

Fortunately, nature has provided us with herbal remedies that can help in that regard. Several plants and their isolated compounds have been found to possess adaptogenic and rejuvenating properties and each of these plants possess its own unique set of properties. Some of these plants include: *Eleutherococcus senticosus* (Song *et al.*, 2002, Wiegant *et al.*, 2009), *Gingko biloba*, *Panax ginseng* (Rai *et al.*, 2001), *Emblica officinalis*, *Ocimum sanctum* (Ravindran *et al.*, 2005), *Withania somnifera*, *Evolvulus alsinoides* (Siripurapu *et al.*, 2005), *Psidium guajava* (Lakshmi and Sudhakar, 2009), *Bacopa monnifera* (Rai *et al.*, 2003b) and *Curcuma longa* (Bhatia *et al.*, 2011).

### 2.7.1. Adaptogens

Many herbs and formulations have been recommended in traditional medicine to endow an individual with the ability to resist and/or endure the stress without effecting any change in the body's physiological functions. This drug-induced condition of 'non-specific' stress resilience is described as adaptogenic activity and the drugs which possess the ability to induce stress resilience are called adaptogens (Lakshmi and Sudhakar, 2009). Thus, adaptogens can be defined as medicinal plants which endow the body with the ability to resist stress while enhancing attention, performance and stamina (Panossian *et al.*, 2010).

Dr. Nikolai Lazarev, a Russian phyto-pharmacist while working on Eleuthero root originated the term 'adaptogen' in 1947. He described adaptogens as agents that confer the ability to withstand the detrimental effects of various stressors through the development of 'non-specific' resistance in an organism. This definition provides the idea that administration of an adaptogen enables an organism to pre-adjust in a way that enables it to be more suitably fit for responding when various demands are in the long run placed on it. Adaptogen initially refers to the ability of a plant to adapt to its surroundings. Plants fight stressors by drawing on information coded in their DNA (Guthrie, 2014). For instance a savannah plant is greatly specialised to germinate in an environment characterized by prolonged dry season by developing long tap roots to reach ground water, thick barks to withstand annual fires, trunks that serve as water reservoirs during the short rainy season and leaves that are shed during the prolonged season of drought to conserve water

Also a rainforest plant adapts by possessing wide leaves or climbing on other trees so as to access the sun rays. This survival instinct being an adaptogenic response to stress, gives an idea that plant's DNA can also achieve the resilience and adaptation in humans that it has been able to achieve in plants for years. Brekhman and Dardymov in the year 1968 formally defined adaptogens as innocuous agents which produces a nonspecific biological response which enhances the entire body's resistance to multiple forms of stress, and normalizes various bodily functions, irrespective of existing pathological conditions (Panossian and Wikman, 2010). Each adaptogen has its own unique set of properties due to varying active constituents. Some are calming while others are stimulating, some dial down a hyperactive immune system while others enhances immune response, but they all help the body function better under stress (Guthrie, 2014).

### 2.7.2. Molecular mechanisms underlying the actions of adaptogens

The protective actions of adaptogens against the perturbations induced by stress are related with their ability to regulate metabolic equilibrium by means of various mechanisms that are interrelated with the HPA axis and manipulation of principal stress response mediators. Some mediators implicated in homeostatic control at the cellular and organism levels include cortisol, glucocorticoid receptors, nitric oxide and Forkhead box O (FOXO). Glucocorticoid receptor regulates the secretion of cortisol, which is the stress hormone. Nitric oxide is an intracellular signalling molecule implicated in controlling stress-induced neurohormonal and immunological stimulations as well as in stress response regulation, while FOXO is the protein which regulates the synthesis of proteins associated with stress resilience and cell survival (Samuel *et al.*, 2008). Upon exposure to stressful conditions, a cascade of signaling proteins including the JNK 1 (Jun N-terminal protein kinase 1, also called stress-activated protein kinases; SAPKs) is activated. JNK triggers the formation of nitric oxide and elevates free radicals generation which in turn inhibits the formation of ATP which is needed for energy and for maintenance of the heat shock proteins, and suppresses glucocorticoid receptors, thereby disrupting the feedback inhibition of cortisol discharge, causing a rise in the circulatory cortisol level which subsequently inhibits the immune system and activates an anti-inflammatory response (Panossian and Wikman, 2010; Yamasaki *et al.*, 2012). The HSPs assists other proteins in their appropriate three-dimensional configuration, refolding of misfolded proteins and prevents their aggregation (Panossian *et al.*,

2010). The Hsp 72 performs a primary function of eliminating misfolded proteins or deficiently synthesized polypeptides from cells which would otherwise interrupt their normal cellular functions. Thus, Hsp 72 participates essentially in the maintenance of cellular homeostasis, protects the cell and enhances cell survival despite harmful cellular stress.

While carrying out an experimental study on animals, Panossian *et al.*, (2010) revealed that adaptogens triggers up-regulation of intracellular Hsp 72 expression both in presence and absence of stressful stimulus, which is then conveyed to the extracellular space where it stimulates the immune system of the host. An intracellular increase in the expression of Hsp 72 causes the inhibition of stress-triggered deleterious signal transduction cascades, for example JNK-induced apoptosis and enables the initiation of few of the beneficial functions of JNK including the activation of FOXO translocation into the nucleus and initiation of the synthesis of proteins which contributes to stress-resistance and increase survival. This increase in circulating Hsp72 was considered to be associated with an increase tolerance to adaptogen-induced stress observed in the study. Indeed, the formation and release of Hsp 72 is a well-known stress response mediator implicated in the reparation of proteins during physical load. They therefore hypothesized that adaptogens act by triggering self-defence mechanisms in the face of stressful stimuli which makes the organism less sensitive (or adapt) to the actual stress, thus acting like mild stressors (stress-mimetics), or low molecular weight "vaccines" (Panossian and Wikman, 2010).

### 2.7.3. Some major adaptogens

#### 2.7.3.1. *Eleutherococcus senticosus*

*Eleutherococcus senticosus* (commonly called Siberian ginseng) is a woody plant widely dispersed across Asia and Southeast Russia (Yan-Lin *et al.*, 2011). It is the first identified adaptogen and extensive research has been carried out to explore other pharmacological potentials of this herb. Siberian ginseng has been known in Chinese traditional medicine as an adaptogen with highly beneficial effects on the spleen and the kidney as it helps nourish and support both organs (Huang *et al.*, 2011a). The pharmacological effects of *Eleutherococcus senticosus* was first discovered in the late 50s and 60s, and ever since, several investigations using cultured cell lines and small laboratory animals have revealed its other beneficial effects

such as antioxidant, antimicrobial (Kim *et al.*, 2006), antidiabetic, anticancer, anti-inflammatory and antinociceptive (Jung *et al.*, 2003). *Eleutherococcus senticosus* is used as a nutritional supplement and as immune system booster. Additionally, it helps in the restoration and/or rejuvenation of normal biochemical, physiological or immunological functions which may have been damaged (Winston and Maines, 2007). Infusions from the stem bark and leaves reduced fatigue in rats and possess protective effects against behavioural and physiological alterations caused by stress. One of the major active constituents of *E. senticosus* Eleutheroside E, have been shown to contribute to majority of these effects (Huang *et al.*, 2011b). Eleutheroside E was found to prolong swimming time, inhibit the elevation of corticosterone and help the recovery of swimming stress-induced reduction in natural killer activity in mouse models. Eleutheroside E was also discovered to perform significant functions in the utilization of stored lipids, reduction of serum triglycerides, lowering of the accumulation of blood urea nitrogen and cause a reduction in muscle lactic acid by enhancing the levels of lactate dehydrogenase, thus protecting muscle tissues (Huang *et al.*, 2011a).

#### 2.7.3.2. *Withania somnifera*

Popularly called Ashwagandha, *Withania somnifera* (L. Dunal, Solanaceae) is a perennial shrub cultivated in India, the Mediterranean, and Africa where it's been used for centuries by Ayurvedic practitioners as a rejuvenating tonic as well as to promote health and longevity. It works by augmenting the body's defense against diseases, delaying the ageing process, revitalizing and augmenting the body's resistance against harmful stimuli and producing a feeling of mental well-being (Bano *et al.*, 2015). The plant attains its 'unique' name from "Ashwa", meaning horse because its root smells like a horse and as a result is believed to provide power like horse when consumed (Tiwari *et al.*, 2014). The pharmacological activities of ashwagandha are based on the occurence of withanolides, a class of steroidal lactones gotten from its roots (Kaur *et al.*, 2013). Occasionally referenced as 'Indian ginseng' due to its ginseng like health promoting effects, it has been reported that ashwagnadha energizes, improves memory, shows anxiolytic effects, has hepatoprotective property, enriches the blood, has potent antioxidant activity, improve the cell-mediated immunity, improves sexual life and reproductive equilibrium and act as powerful adaptogen (Bhattacharya and Muruganandam, 2003; Sandhu *et al.*, 2010).

A research conducted to assess the anti-stress activity of ashwagandha in rats observed stress-induced immunosuppression, memory impairment, stomach ulcer, hyperglycemia, male sexual dysfunction, neuro-depression, and enhanced plasma corticosterone concentration in response to 21 days chronic mild electric footshock stress. The researchers observed that administration of ashwagandha 1 hr prior to footshock reversed immunosuppression, boosted the activity of peritoneal macrophages, mitigated the occurrence and intensity of stress-induced gastric ulcers and enhanced acquired tasks memory (Bhattacharya and Muruganandam, 2003).

### 2.7.3.3. *Panax ginseng*

Ginseng is the root of numerous plant species belonging to the genus Panax (C. Meyer, Araliaceae). This plant genus is composed of several species of which *Panax ginseng* with a medicinal record of over 5,000 years is the most extensively used. It was first grown around 11 BC (Radad *et al.*, 2004) and has been used for centuries in Korea, Japan and China. Today, ginseng is well-known and used globally as a natural medicine. *Panax ginseng* is well known since ancient times as a stimulant, tonic, diuretic, immune booster and digestive aid. This plant includes the active compounds, such as flavonoids, polysaccharides, saponins and volatile oils. The saponins in ginseng typically known as ginsenosides are the principle active ingredients responsible for its many pharmaceutical actions and over 30 diverse ginsenosides have been discovered (Back *et al.*, 1996). Various clinical and pharmacological effects associated with its use have been reported, such as cardiprotective, neuroprotective, immunomodulatory, antihypertensive and antistress activities (Rai *et al.*, 2001).

### 2.7.3.4. *Ginkgo Biloba*

Rightly called the "living fossil", *Ginkgo biloba* L. (Mantissa Plantarum Altera, 1771, Ginkgoceae) is a mythical tree more than 250 million years old which has survived the most severe forms of pollution in the twentieth century, particularly the radioactive waste of the Hiroshima atomic bomb. The tree is now cultivated extensively around the world. Since the time of the ancient Chinese medicine, *Gingko biloba* seeds and leaves have been documented as a source of medicine. Its leaves infusions are drunk in form of teas, leaves extracts formulated into film-coated tablets, syrups or injections are now available around the world for purchase and are one of the most commonly taken herbal medicines (Chan *et al.*, 2007). Today, nearly 500

scientific papers documenting the effects of *Ginkgo biloba* have been published, making it the well-researched botanical medicine available (Mullaicharam, 2013). Like most medicinal plants, *Gingko biloba* contains a cocktail of active constituents such as the flavonoids including quercetin, kaempferol, and isorhamnetins, but the principal constituents that account for the biological and/or pharmacological actions of *Ginkgo biloba* are the trilactonic terpenes ginkgolides and bilobalide (Chan *et al.*, 2007; Mullaicharam, 2013). Various clinical researches validated the effectiveness of its leaves in Alzheimer's disease, diabetic neuropathies, cerebral atherosclerosis and insufficiencies, depression, tinnitus, and vertigo (Shi *et al.*, 2010; Da Silva *et al.*, 2011). In the indigenous Chinese medicine, *Ginkgo* leaves extracts are being used to treat circulatory disorders, cognitive problems, asthma, tinnitus and vertigo (Puttalingamma 2015). Furthermore, extracts of *Gingko* was discovered to have potent adaptogenic activity (Rai *et al.*, 2001).

### 2.7.3.5. *Ocimum sanctum*

Known in English as Holy Basil and Tulsi in Ayurvedic tradition, *Ocimum sanctum* (Family: Lamiaceae) is a native plant of Southeast Asia and India. The plant owes its numerous pharmacological effects to several phytochemical components such as rosmarinic acid, ursolic acid, luteolin, apigenin, β-sitosterol, eugenol and myrcenal (Pattanayak *et al.*, 2010). The plant is recognized by several traditional medical systems for its medicinal values. The leaves are claimed to possess healing power, used in the treatment of fever, cough, eye disorder, ring worm and other skin diseases, headaches, and protection against stress (Kumar *et al.*, 2012). Experimental and clinical investigations showed that Holy basil possess immunomodulatory (Mukherjee *et al.*, 2005), anti-inflammatory (Singh and Majumdar, 1996), hepatoprotective (Lahon and Das 2011), antipyretic (Singh *et al.*, 2007), aruidiabetic, anticoagulant and anti-stress activities (Bathala *et al.*, 2012; Jothie *et al.*, 2016).

### 2.7.3.6. *Rhodiola rosea*

*Rhodiola rosea* (Golden or Arctic root) belong to the genus Rhodiola which comprises of over 200 species, of which about 20 are used in some parts of Asia as traditional medicines (Bawa and Khanum, 2009). This genus of medicinal plants is predominantly found in China, Tibet, Mongolia, and the Himalayan belt, and also in North America and Europe. The most popular

species *Rhodiolla rosea* is commonly used as a nervous system stimulant, antidepressant, anti-fatique, energy booster and in preventing mountain sickness in the indigenous medical systems of Asia and Eastern Europe (Kelly, 2001). *Rhodiola rosea* is classified as an adaptogen as it has the capability to enhance performance and to boost the body's resistance to various stressors (Olsson *et al*., 2009; Chan, 2012). Moreover, Olsson *et al*., (2009) documented that the administration of SHR-5; an extract obtained from *R. rosea* reduced fatique, enhanced attention performance, and reduced stress-induced cortisol response in individuals with burnout and chronic fatigue syndromes (Kelly, 2001). Salidroside, one of the chief components of *R rosea* has been reported to increase endurance and liver glycogen levels in rats exposed to exhaustive swimming (Xu and Li, 2012). Furthermore, salidroside reduced oxidative stress and enhanced antioxidant activity in the liver cells of rats (Xu and Li, 2012).

In addition to its adaptogenic activity, *Rhodiola rosea* have been found to possess neuroprotective (Lee *et al*., 2013), antinociceptive (Doncheva *et al*., 2013), anti-inflammatory (Doncheva *et al*., 2013), hepatoprotective (Wu *et al*., 2009), antidepressant (Yang *et al*., 2014), anticancer (Wang *et al*., 2014) and cardioprotective (Sun *et al*., 2012).

### 2.7.3.7. *Astragalus membranceus*

Astragalus L. is one of the largest genuses of flowering plants in the Leguminosae family. The plants are widely distributed throughout the temperate and arid regions ( Podlech, 2008). One of the Chinese specie; *Astragalus membranceus* (membranous milk-vetch root) is an important herb in Traditional Chinese Medicine which has been used in a wide variety of herbal blends and natural medicines. Its principle active constituents include polysaccharides, saponins, flavonoids, amino acids, and trace elements (Ma *et al*., 2002). It has been prescribed for centuries to boost energy and strengthen immunity (Horne, 2014). Research has shown that *Astragalus membranceus* possess anti-inflammatory (Kim *et al*., 2013), immunoregulatory (Qin *et al*., 2012), anti-tumor (Tian *et al*., 2012), cardioprotective (Ma *et al*., 2013), antidiabetic (Yu *et al*., 2006), and antiaging (Gao *et al*., 2010) activities. Other researches have also revealed its antiviral, antioxidative, hepatoprotective, and neuron protective activities ( Li *et al*., 2014). *Astragalus membranceus* has been suggested to be taken as one of the auxiliary drugs for the treatment of congestive heart failure (Zhou *et al*., 2001).

### 2.7.3.8. *Schisandra chinensis*

*Schisandra chinensis* belongs to the plant family *Schisandraceae*. It is a climbing plant widely distributed in the region of the Russian Far East, Korea, Japan and northeastern China, and is used in Traditional Chinese Medicine as an anti-aging agent, sedative and tonic (Huang *et al*., 2005). *Schisandra chinensis* is renowned as a beauty tonic and is considered to be a youth preserving herb in China. It is said to be a powerful stimulant in sexual weakness/impotence, used in the treatment of gonorrhoea, protracted diarrhoea, dysentery, enuresis, cough, jaundice, wheezing, asthma, urinary tract disorders, exhaustion, diabetes as well as body weakness (Panossian and Wikman, 2008). In healthy subjects, *Schisandra chinensis* increases endurance and accuracy of movement, mental performance and working capacity, and generates alterations in the basal levels of nitric oxide and cortisol in blood and saliva with subsequent effect s on the blood cells, vessels and CNS (Panossian and Wikman, 2008). Numerous clinical trials have demonstrated the beneficial effects of *Schisandra chinensis* on various body systems. In the nervous system, it helps prevent neurologic and psychiatric disord ers (Song *et al*., 2011), in the cardiovascular system, it counteracts hypertension and cardiotonic disorders on the skin, it reduces allergic dermatitis and in the gastrointestinal system, it alleviates acute gastrointestinal diseases, gastric hyper- and hypo-secretion, chronic gastritis, stomach and duodenal ulcers, wound healing and trophic ulcers (Panossian and Wikman, 2008). It has also been reported to possess anticancer activity (Gnabre *et al*., 2010, Smejkal *et al*., 2010).

## 2.8. MORIN HYDRATE

Morin hydrate (3,5,7,2',4'-pentahydroxyflavonol) is a yellow crystalline bioflavonoid. It was initially gotten from the branches of mulberry fig (*Morus alba* L.) and is also found in different herbs and fruits including almond hulls, old fustic, sweet chestnut, onion, seed weeds, mill, osage orange and in red wine. It is also found abundantly in other plants belonging to Moraceae family (Wijeratne *et al*., 2006; Venu Gopal, 2013).


Figure 4: Chemical structure of Morin hydrate (Venu Gopal, 2013)

### 2.8.1. Pharmacological activities of Morin hydrate

#### 2.8.1.1. Neuroprotective activity

Morin hydrate was shown to possess neuroprotective effect in a model of NMDA over activation-induced excitotoxic neuronal death via reduction of ROS generation and activation of the antioxidant system (Campos-Esparza *et al.*, 2009). Morin hydrate was also observed to regulate the NF-κB nuclear translocation (Campos-Esparza *et al.*, 2009) Additionally, Morin hydrate was shown to possess potent neuroprotectant effect in different models of ischemia, rescued neurons from acute injury-induced cell death and lessened neurological defects triggered by ischemic brain injury by attenuating receptor-mediated calcium influx, oxidative stress and apoptosis (Gottlieb *et al.*, 2006). It was also found to diminish free radicals formation and loss of hippocampal CA1 pyramidal cells in rats subjected to experimental ischemia (Gottlieb *et al.*, 2006).

### 2.8.1.2. Antitumor and antiinflammatory activity

In a model of trinitrobenzenesulphonic acid-induced colitis, morin hydrate was observed to show anti-inflammatory activity in rats via down-regulation of certain intestinal inflammatory mediators (Galvez *et al.*, 2001). Also, morin hydrate was found to inhibit elevation of proinflammatory biomarkers in diabetic rats (Alsharari *et al.*, 2014). Studies indicated that morin hydrate inhibits tumor promotion and suppresses tumor growth in several models and these outcomes correlate with down-regulation of certain NF-κB-modulated gene expression (Manna *et al.*, 2007). Moreover, morin hydrate was reported to inhibit chemically-induced carcinogenesis in rats tongue and suppress phorbol ester-induced transformation of hepatocytes (Hsiang *et al.*, 2005).

### 2.8.1.3. Antidiabetic activity

In a rat model of diabetes, morin hydrate was observed to exhibit beneficial role on membrane bound glycoproteins and enzymes by decreasing oxidative stress in streptozotocin-induced diabetic rats (Vishnukumar *et al.*, 2012). Morin hydrate was shown to protect the liver and kidneys of the rats against pathological alterations induced by streptozotocin (Vishnukumar *et al.*, 2012), ameliorate hyperalgesia induced by hyperglycemia and by virtue of its anti-inflammatory and antioxidant actions in nerve cells and reduced neuropathic pain (Alsharari *et al.*, 2014). Furthermore, by means of its potent anti-inflammatory and antioxidant activities, morin hydrate was observed to reduce diabetic osteopenia via its ability to reduce bone loss; a characteristic of diabetic conditions (Abuohashish *et al.*, 2013).

### 2.8.1.4. Hepatoprotective activity

In a rat model of hepatic ischemia-reperfusion, pretreatment with Morin hydrate was shown to reduce liver necrosis through its antioxidant action in inhibiting xanthine oxidase and its ability to protect human red cell membrane from peroxidative attack (Wu *et al.*, 1993). Morin has been shown to modulate liver marker levels and redox status during experimental ammonium chloride-induced hyperammonaemia in rats by virtue of its antioxidant properties and through the removal of excess ammonia and inhibition of NMDA receptor-mediated neurotoxicity (Subash and Subramanian, 2008), exert protective effect against ethanol-induced hepatotoxicity

due to its antioxidant, anti-inflammatory and antifibrotic effect (Bhakuni *et al.*, 2017) and has hepatoprotective potential against acrylamide-induced toxicity by scavenging free radicals and blocking the epoxidation mechanism (Singh *et al.*, 2015).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1. EXPERIMENTAL ANIMALS

Male Swiss mice (22 – 25 g) obtained from the University of Ibadan, Ibadan central animal house were were used in the study. The animals were maintained under a 12,12 h light/dark cycle in the Department of Pharmacology and Therapeutics animal house facility, had unrestricted access to food and water and were made to acclimatize for at least 7 days preceeding the inception of the experiments. The mice were used in line with the NIH Guide for the Care and Use of Laboratory Animals and the experiments were performed following approval by the Ethics Committee of the University of Ibadan (UI-ACUREC/App/2015/067).

## 3.2. DRUGS AND CHEMICALS

Korean ginseng (Korea Pharma co. ltd), Morin hydrate (Sigma-Aldrich, USA), Diazepam (Hoffman-La Roche, Switzerland), Trichloroacetic acid (TCA) (BDH Chemicals Ltd, England), Thiobarbituric acid (TBA) (Guanghua Chemical Factory Co. Ltd., China), Acetic acid (Sigma-Aldrich, Inc., St Louis, USA), Anhydrous disodium phosphate ($Na_2HPO_4$) (BDH Chemicals Ltd, Poole, England), Sodium bicarbonate (Fisons Loughborough Leics, England), Potassium sodium tartarate (Scholarlab, Spain), Potassium iodide (May and Baker, England). Sodium hydrogen bicarbonate (BDH Chemicals Ltd, Poole, England), Sodium hydroxide (J.T Baker Chemicals Co., Phillipsburg, N.J., USA), Phosphoric acid (Sigma Aldrich, Germany), Tris base (BDH Chemicals Ltd, Poole, England), Sulfanilamide (Sigma Aldrich, Germany), Acid sodium phosphate monohydrate ($NaH_2PO_4.H_2O$) (BDH Chemicals Ltd, Poole, England), Copper sulphate (BDH Chemicals Ltd, Poole, England), Formaldehyde (BDH Chemicals Ltd, Poole, England), DTNB (Sigma Aldrich, Co. Germany), Mouse IL-1 beta ELISA (Affymetrix eBioscience, San Diego, California, USA), Corticosterone ELISA Kit (Assay Pro, Germany) Mouse TNF alpha ELISA (Affymetrix eBioscience, San Diego, California, USA) were used in the study.

Morin hydrate was dissolved in normal saline to give a 5 mg/ml stock solution which was then diluted as needed during each of the experiments. Doses used were chosen based on pilot studies and available literature.

### 3.3. EXPERIMENTAL DESIGN

A. Neurobehavioral studies: Animals in these studies were allotted into 6 groups of 5 mice each. Group 1 animals served as control animals and administered the vehicle (VEH: 10 mL/kg normal saline), groups 2 - 5 were given Morin hydrate (MH: 10, 20, 40, and 80 mg/kg, i.p.), whereas group 6 mice were administered diazepam (DIZ: 2 mg/kg, i.p.). The drugs were administered 30 min preceeding the commencement of each experiment.

B. Swimming endurance and anoxic tolerance stress studies: Animals were allotted into 5 groups of 5 mice each. Animals in group 1(control) were given normal saline (VEH: 10 mL/kg) and not subjected to stress regimen. Groups 2 - 4 animals were given Morin hydrate (MH: 5, 10, and 20 mg/kg, i.p.) and subjected to stress, while group 5 mice were administered ginseng (GIN: 25 mg/kg, i.p.) and also subjected to stress.

C. Acute restraint and chronic stress studies: Animals were allotted into 6 groups of 6 mice per group. Group 1 mice (control) received normal saline (VEH: 10 mL/kg) without exposure to stress. Group 2 animals also received the normal saline (VEH: 10 mL/kg) and were exposed to the stress, groups 3 - 5 animals were administered Morin hydrate (MH: 5, 10, and 20 mg/kg, i.p.) and subjected to stress, while group 6 mice were given ginseng (GIN: 25 mg/kg, i.p.) and also subjected to stress.

### 3.4. NEUROBEHAVIORAL STUDIES

#### 3.4.1. Novelty-Induced behaviour

Novelty-induced behaviour (NID) of mice was assessed individually in sequence in the open field. The open field is a rectangular arena of dimension 45 cm x 25 cm x 25 cm, composed of a

wooden hardboard floor which is evenly partitioned into 16 (7 x 7 cm) squares with a surrounding wooden wall having a transparent side made of glass for unobstructed viewing. Animals were allotted into 6 groups of 5 mice each. Group 1 animals received 10 ml/kg VEH, groups 2 - 5 were given 10, 20, 40, and 80 mg/kg MH respectively, and group 6 mice were administered 2 mg/kg DIZ. Each mouse was individually placed in the plexiglas cage immediately after treatment for 30 min during which rearing and grooming were observed and scored. Vertical locomotion (the total number of squares intersected with the four paws) was scored for an additional 5 min. Rearing is connoted as how frequently a mouse raises its fore limbs either in free air or against the cage wall while standing on its hind limbs, while grooming is described as the frequency of body cleaning, face washes and body and pubis picking with mouth and paws. After each session, the cage was wiped clean with 70%v/v of ethanol so as to eliminate odor cues. The experimental time was strictly adhered to (between 9 am and 1 pm daily) so as to avoid alterations in biological rhythm (Ajayi and Ukponmwan, 1994).

### 3.4.2. The Hole board test

The test evaluates exploratory activities that demonstrate the sedative properties of test drugs. The apparatus consists of a board with about sixteen (16) holes through which a mouse can poke its head. Animals were allotted into 6 groups of 5 mice each. Group 1 animals received 10 ml/kg VEH, groups 2 - 5 were administered 10, 20, 40, and 80 mg/kg MH respectively, and group 6 mice were administered 2 mg/kg DIZ. Afterwards, each animal was placed on the hole board 30 minutes following drug administration and the number of head dips into the holes was observed and scored over a 5 min period (Dorr *et al*., 1971).

## 3.5. ACUTE STRESS STUDIES

### 3.5.1. Swimming endurance test

The swimming endurance test was carried out in accordance with the paradigm of Kannur (Kannur *et al*., 2006) with slight variations. Animals were divided into 5 groups of 5 mice each. Group 1 animals received 10 ml/kg VEH, groups 2 - 4 animals received 5, 10, and 20 mg/kg MH respectively while group 5 animals received 25 mg/kg GIN. Animals in group 1 were left

wooden hardboard floor which is evenly partitioned into 16 (7 x 7 cm) squares with a surrounding wooden wall having a transparent side made of glass for unobstructed viewing. Animals were allotted into 6 groups of 5 mice each. Group 1 animals received 10 mL/kg VEH, groups 2 - 5 were given 10, 20, 40, and 80 mg/kg MH respectively, and group 6 mice were administered 2 mg/kg DIZ. Each mouse was individually placed in the plexiglas cage immediately after treatment for 30 min during which rearing and grooming were observed and scored. Vertical locomotion (the total number of squares intersected with the four paws) was scored for an additional 5 min. Rearing is connoted as how frequently a mouse raises its fore limbs either in free air or against the cage wall while standing on its hind limbs, while grooming is described as the frequency of body cleaning, face washes and body and pubis picking with mouth and paws. After each session, the cage was wiped clean with 70%v/v of ethanol so as to eliminate odor cues. The experimental time was strictly adhered to (between 9 am and 1 pm daily) so as to avoid alterations in biological rhythm (Ajayi and Ukponmwan, 1994).

### 3.4.2. The Hole board test

The test evaluates exploratory activities that demonstrate the sedative properties of test drugs. The apparatus consists of a board with about sixteen (16) holes through which a mouse can poke its head. Animals were allotted into 6 groups of 5 mice each. Group 1 animals received 10 mL/kg VEH, groups 2 - 5 were administered 10, 20, 40, and 80 mg/kg MH respectively, and group 6 mice were administered 2 mg/kg DIZ. Afterwards, each animal was placed on the hole board 30 minutes following drug administration and the number of head dips into the holes was observed and scored over a 5 min period (Dour *et al*., 1971).

## 3.5. ACUTE STRESS STUDIES

### 3.5.1. Swimming endurance test

The swimming endurance test was carried out in accordance with the paradigm of Kannur (Kannur *et al*., 2006) with slight variations. Animals were divided into 5 groups of 5 mice each. Group 1 animals received 10 mL/kg VEH, groups 2 - 4 animals received 5, 10, and 20 mg/kg MH respectively while group 5 animals received 25 mg/kg GIN. Animals in group 1 were left

undisturbed in their home cage. A translucent glass container (25 cm height × 18 cm in width) was filled with water at room temperature and animals in groups 2-5 were individually compelled to swim separately 30 min after treatment. Total immobility time was noted for 15 minutes. A mouse is considered immobile whenever it ceased to struggle, slightly moving its limbs just to stay afloat in water.

### 3.5.2. Anoxic tolerance test

The test was performed according to the procedure stated by Konnur (Konnur *et al*., 2006). Here, hermetic vessels of about 250 ml air capacity were used for the experiment. Prior to the experiment, the vessels were made air tight by means of a rubber cork. Animals were divided into 5 groups of 5 mice each. Group 1 animals received 10 ml/kg VEH, groups 2 - 4 animals received 5, 10, and 20 mg/kg MII respectively while group 5 animals received 25 mg/kg GIN. Mice in groups 2 - 5 were individually placed in an airtight hermetic vessel 30 min after treatment, and the onset of convulsion was noted.

### 3.5.3. Acute restraint test

The acute restraint model was carried out according to the procedure of Masood (Masood *et al*., 2003), with minor modifications. In this test, animals were divided into 6 groups of 6 mice each. Groups 1 and 2 animals received 10 ml/kg VEH, groups 3 - 5 animals received 5, 10, and 20 mg/kg MII while group 6 animals received 25 mg/kg GIN for 7 consecutive days. The last treatment was done on day 7 and 30 min afterwards, animals were exposed to stressful stimuli by restraining them in a plastic restrainer for 2 hours, after which the animals were tested for anxiety- and depressive-like behaviours using the EPM and FST paradigms respectively.

## 3.6. CHRONIC STRESS STUDIES

### 3.6.1. Chronic restraint test

In this test, animals were divided into 6 groups of 6 mice each. Groups 1 and 2 animals received 10 mL/kg VEH, groups 3 - 5 animals received 5, 10, and 20 mg/kg MII while group 6 animals received 25 mg/kg GIN for 14 consecutive days, 30 min prior to restraint for 2 h in a mouse

restrainer. On the 14th day, 30 min following the last stress exposure, mice were tested for anxiety- and depressive-like behaviours using the EPM and FST paradigms respectively (Rai *et al.*, 2003).

### 3.6.2. Paradoxical Sleep Deprivation

The 'grid suspended over water' technique was employed. Mice were placed on grids set 2 cm apart from each other balanced inside large plastic cages filled with water to a level of about 1 cm below the surface of the grid in order to deprive them of rapid eye movement (REM) sleep (Shinomiya *et al*, 2003). Mice were divided into 6 groups of 6 mice each. Groups 1 and 2 animals received 10 mL/kg VEH, groups 3 - 5 animals received 5, 10, and 20 mg/kg MH while group 6 animals received 25 mg/kg GIN for 5 days. However, the animals were sleep deprived on the 4th and 5th days (for 48 h). This method is founded on the basis that the animals could only get non-rapid eye movement sleep (NREM) and at the start of REM sleep due to muscle relaxation they fall into the water and are thereafter quickly awakened, and return onto the grid. Nevertheless, it is important to point out that although this methodology focuses basically on REM sleep, a substantial amount of NREM is lost followed by the induction of significant amount of stress. Animal weights were noted at start and end of the experiment with the aim of checking for effect of sleep deprivation on body weight along with possible modulatory role of morin hydrate. On completion of the 48 hour duration of sleep deprivation, the effect of 48 h REM sleep deprivation on motor function of each mouse was determined for 5 min. Afterwards, anxiety levels (using the EPM) and extent of memory impairment (using the Y maze) were assessed.

### 3.6.3. Chronic unpredictable stress

Chronic unpredictable stress regimen was adopted from previous methods (Munhoz *et al.*, 2006, Pandey *et al.*, 2010) with minor adjustments. Mice were exposed to varying stressful conditions for 14 days. Mice were grouped as described in 3.3C; the stressed groups were individually housed. Each specific stressor and duration of exposure for each day are as follows: Day 1: 10 min forced swim; day 2: 24 h cage tilt (90°); day 3: 24 h empty cage; day 4: overnight food and water deprivation; day 5: 3 h lights off during the day; day 6: 10 min of cold isolation at 4°C; day 7: 24 h cage tilt (90°); day 8: 30 min restraint; day 9: 10 min forced swim; day 10: 24 h empty

cage; day 11: 3 h lights off; day 12: food and water deprivation overnight; day 13: 10 min cold isolation at 4°C; day 14: 30 min restraint. The time of stress exposure for each day varied in order to reduce predictability. Unstressed (control) mice were undisturbed except for basic routines like feeding and cage cleaning. Twenty-four hours following the last stress exposure, mice were analysed for CUS –induced anxiety utilizing the EPM, cognitive impairment utilizing the Y maze and spontantaneous locomotor activity.

## 3.7. BEHAVIORAL TESTS

### 3.7.1. Elevated plus maze (EPM) Test

The EPM is a valid behavioural paradigm for estimating the anxiolytic or anxiogenic properties of pharmacological agents in rodents (Walf and Frye, 2007). The apparatus is made of wood comprising of a pair of open arms (30 x 5 x 0.25 cm) which are essentially unprotected boards and a pair of closed arms (30 x 5 x 15 cm) with borders originating from a joint median platform (5 x 5 cm) and raised to 50 cm above ground level. Each mouse was singly positioned at the middle of the maze opposite an open arm. Duration and frequency of exploration in each arm was noted by a blind observer for 5 minutes. After each session, the cage was wiped clean with 70%v/v of ethanol so as to eliminate odor cues. An entry was determined when all paws of the animal have crossed the line between the arm and the middle of the maze. The index of open arm avoidance (IOAA) was interpreted as the level of anxiety and was calculated as:

$$IOAA = 100 - \frac{[\%\ \text{time in open arms} + \%\ \text{entries into open arms}]}{2}$$

### 3.7.2. Forced Swim Test (FST)

The FST was done as earlier reported by Porsolt et al (1977). Mice were individually placed in transparent plexiglass cylinders (25 cm x 18 cm) filled with water to a depth of 15 cm at room temperature and forced to swim for the duration of 6 min. The total time an animal remains immobile (immobility time) in seconds was noted in the course of the concluding 4 min test duration. An animal is judged immobile whenever it ceased to struggle, slightly moving its limbs just to stay afloat in water.

### 3.7.3. Y-maze Test

The test to evaluate memory performance was performed in accordance with the protocol earlier described (Casadesus *et al.*, 2007). The test device comprises of 3 equivalent arms (120°, 41 x 5 x 15 cm) labelled 'A', 'B', and 'C'. The test can be employed in assessing spontaneous alternation performance; a pointer to short term working memory in rodents. A mouse must be able to recall the previous arm it explored and then move on to another until it has completely alternated among the three arms. In this test, each animal was positioned in one arm (mostly arm 'A' for uniformity) and left to explore for 5 min with no restrictions. When an animal enters into an arm of the maze with all of its paws, it was counted as an arm entry and when it enters into all three arms consecutively, it was scored as an alternation. Spatial working memory is represented as the percentage alternation and is evaluated using the formula:

$$\left[\frac{\text{Total complete alternations}}{\text{Total entries} - 2}\right] X\ 100$$

The order of arm entries was noted and each arm of the maze was wiped with 70% v/v ethanol at intervals.

### 3.7.4. Spontaneous motor activity

Spontaneous motor activity was assessed by making use of an automated activity cage. This apparatus captures the horizontal locomotion of animals. The mice were left to habituate for an hour in the test room ahead of the experiment and thereafter, individually positioned at the central point of the cage and left to move without restrictions. The total lines each mouse crossed with the four paws were automatically counted for 5 min. A solution of 70% v/v ethanol was used to wipe the cage in between each session to avoid odour cues.

## 3.8. COLLECTION OF BLOOD FOR GLUCOSE, LIPIDS AND CORTICOSTERONE DETERMINATION

Upon termination of each experiment, blood was drawn from the retro-orbital plexus of animals in the acute restraint, chronic restraint and CUS models into plain tubes following overnight fast. Whole blood was used for glucose test, after which the remaining blood was centrifuged to obtain a serum for the estimation of cholesterol, triglycerides and corticosterone.

### 3.8.1. Measurement of blood glucose

The concentration of glucose in the blood determined utilizing a blood glucose monitoring meter (AccuCheck performer meter) and blood glucose strip (AccuCheck). The strip was placed into the meter and blood was dropped at the tip of it and drawn up into the meter. Immediately the blood glucose reading was displayed on the meter and recorded.

### 3.8.2. Total cholesterol

Total serum cholesterol levels was determined by enzymatic colorimetric method using the assay kit (Spinreact, Spain) as specified by the manufacturer following the method earlier described (Naito, 1984). Briefly, the reagents (containing the enzymes Cholesterol esterase, Cholesterol oxidase, Peroxidase, and 4-Aminophenazone) were dissolved in the buffer and gently mixed in order to completely dissolve the contents to give the working solution. Thereafter, 1.0 mL of working solution was added to 10 µL of serum or cholesterol standard (200 mg/dL) and incubated for 10 min at 25°C. Absorbance (A) of the samples and standard were afterwards taken against the blank at 500 nm. Cholesterol concentration in the sample was obtained from the calculation below:

$$\frac{A_{Sample} - A_{Blank}}{A_{standard} - A_{Blank}} \times \text{Standard conc.} = \text{mg/dL sample cholesterol}$$

### 3.8.3. Serum HDL cholesterol

Serum HDL (good cholesterol) levels was determined by enzymatic colorimetric method using the assay kit (Spinreact. Spain) as stated by the manufacturer corresponding to the method earlier described (Naito, 1984). Briefly, 300 µL of Reagent 1 was mixed with 3 µL of sample or calibrator (lyophilized serum) and allowed to incubate at 25°C for 5 min. Absorbance (A1) of samples and calibrator was taken at 600 nm. Reagent 2 was afterwards added, mixed and incubated at 25°C for 5 min. Absorbance (A2) of samples and calibrator was taken and the increase in absorbance (ΔA= A2 – A1) was calculated. Cholesterol concentration was determined from the equation:

$$\frac{\Delta A_{Sample}}{\Delta A_{Calibrator}} \times \text{Calibrator conc.} = \text{mg/dL sample HDL}$$

### 3.8.4. Serum triglyceride estimation

The concentration of serum triglyceride was determined using enzymatic colorimetric method by means of the assay kit (Spinreact, Spain) as specified by the manufacturer corresponding to the method earlier described (Buccolo and David, 1973). 1 mL of the reagent is mixed with 10 µ L of the sample or standard and the mixture incubated for 5 minutes at 37°C. Absorbance (A) is read at 500 nm. Concentration of triglyceride in the sample is obtained from the equation:

$$\frac{A_{Sample}}{A_{Standard}} \times \text{Standard conc.} = \text{mg/dL sample triglyceride}$$

### 3.8.5. Determination of serum corticosterone

Serum corticosterone was detected utilizing enzyme-linked immunoabsorbent assay (ELISA, Assaypro USA) kit in accordance with the manufacturers' protocol. The assay was performed at room temperature and all chemicals were freshly prepared and brought to room temperature prior to use. Briefly, 25 µL/well of corticosterone standard or sample and 25 µL/well of biotinylated

corticosterone were dispensed into each well, gently mixed and left for 2 h at 25°C to incubate. Thereafter, 200 µL of wash buffer was used to wash the wells each time for 5 washes. 50 µL each of streptavidin-peroxidase conjugate and chromogen substrate were dispensed into each wells one after the other following series of incubations and washes. The reaction was finally terminated with 50 µL/well of stop solution. A blue to yellow colour transformation was observed. Absorbance was immediately read with the aid of a multifunctional plate reader at 450 nm. Unknown sample concentration in ng/mL was extrapolated from the standard curve which was a plot of absorbance against concentration of the standard.

## 3.9. TISSUE PREPARATION

Following behavioural tests in the acute restraint, chronic restraint, PSD and CUS models, mice were sacrificed by cervical dislocation and the brains instantly separated. Each brain tissue was thereafter weighed, homogenized (1:10% w/v) with phosphate buffer (0.1M, pH 7.4), and the homogenates centrifuged at 10,000 rpm for 15 min at 4°C. The resultant supernatants were used to estimate malondialdehyde (MDA), reduced glutathione (GSH) and nitrite concentrations. Supernatants were also reserved for enzyme-linked immunosorbent assays of IL-1β and TNF-α.

### 3.9.1. Protein estimation

Protein concentration in samples was determined as stated by Gornall *et al* (1949). 0.9 mL of distilled water, 0.1 mL sample and 3 mL biuret reagent were mixed together in a test tube and left to incubate at 25°C for 30 min. Absorbance was taken at 540 nm by making use of a spectrophotometer. The standard (1 mg/mL bovine serum albumin) was estimated within a range of 0.01-0.1 mg/mL.

### 3.9.2. Reduced glutathione estimation

Reduced glutathione (GSH) level was determined as stated by Moron *et al.* (1979). Briefly, 0.9 mL of sodium phosphate buffer (0.2 M, pH 8), 0.1 mL sample, 1 mL of TCA (20%) and 1mM EDTA were mixed in test tubes and left for 5 min and afterwards centrifuged at 10,000 rpm for 10 min. Thereafter, 0.25 mL of supernatant obtained along with 2 mL of 0.6 mM DTNB were mixed with

0.75 mL of phosphate buffer in fresh plain tubes and left on the bench for 5 min. Absorbance was thereafter taken at 412 nm. GSH level in brain tissue was expressed as µmol/g tissue.

### 3.9.3. Lipid peroxidation assay

Brain malondialdehyde (MDA) concentration was determined in accordance with the procedure of Wilbur *et al* (1949). 0.1 mL of sample, 1.9 mL of Tris-KCl buffer and 0.5 mL each of TCA (30%) and TBA (0.75%) were mixed in plain test tube and then left to incubate at 96°C in a water bath for 1 h. The mixture was cooled under a running tap and 2 mL of butanol added and thereafter centrifuged at 3,000 rpm for 10 min. Absorbance was read at 532 nm with a spectrophotometer. Molar extinction coefficient of $1.56\times10^{5}$ $M^{-1}$ $cm^{-1}$ was used to evaluate the concentration of MDA in brain samples, expressed as µmoles of MDA / g tissue.

### 3.9.4. Brain nitrite estimation

The concentration of nitrite in brain samples was evaluated by means of the Greiss assay. The Greiss reaction is an indicator of nitrite formation and is based on the principle that when nitrite is detected in a $NO_2^-$ containing sample, a redish pink colouration is formed upon treatment with the Greiss reagent. Briefly, 100 µL of Greiss reagent, 300 µL of sample and 2.6 mL of distilled water were mixed in test tubes and thereafter incubated for 30 min at 25°C. Absorbance was afterwards read at 548 nm. Nitrite level in samples was extrapolated from the standard curve of sodium nitrite (0 – 100 µM) (Green *et al.*, 1982).

### 3.9.5. Interleukin-1 Beta (IL-1β) estimation

The concentration of IL-1β in brain tissues was determined with the aid of ELISA kit (Affymetrix eBioscience, USA) as specified by the manufacturer, following the method earlier described (Cho *et al.*, 2008). All chemicals including the samples and standard solutions were brought to room temperature prior to use. Briefly, ELISA plate was coated with anti-mouse IL-1β capture antibody (100 µL/well), made airtight and left overnight to incubate at 4°C. The wells were aspirated, washed thrice with wash buffer, dried of any residual buffer with the aid of a blotting paper and afterwards blocked with 200 µL/well of diluent. The wells were thereafter incubated for an hour, aspirated and washed after which 100 µL/well of reconstituted and diluted mouse IL-1β lyophilized standard (1000 pg/mL) was added to a well and a 2-fold serial dilution

0.75 mL of phosphate buffer in fresh plain tubes and left on the bench for 5 min. Absorbance was thereafter taken at 412 nm. GSH level in brain tissue was expressed as µmol/g tissue.

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The concentration of IL-1β in brain tissues was determined with the aid of ELISA kit (Affymetrix eBioscience, USA) as specified by the manufacturer, following the method earlier described (Cho *et al.*, 2008). All chemicals including the samples and standard solutions were brought to room temperature prior to use. Briefly, ELISA plate was coated with anti-mouse IL-1β capture antibody (100 µL/well), made airtight and left overnight to incubate at 4°C. The wells were aspirated, washed thrice with wash buffer, dried of any residual buffer with the aid of a blotting paper and afterwards blocked with 200 µL/well of diluent. The wells were thereafter incubated for an hour, aspirated and washed after which 100 µL/well of reconstituted and diluted mouse IL-1β lyophilized standard (1000 pg/mL) was added to a well and a 2-fold serial dilution

of it was perfonned to make an 8 point standard curve. 100 µL/well each of brain supernatant, anti-mouse IL-1β biotin detection antibody, enzyme (Avidin-HRP) and tetramethylbenzidine (TMB) substrate solution were dispensed into each well one after the other following series of incubation and washes (3 times). Finally, the reaction was terminated with 50 µL of stop solution. Reading was taken with the aid of a microplate reader at 450 nm. The unknown sample concentration in pg/mL was extrapolated from the standard curve which was a plot of absorbance against concentration of the standard.

### 3.9.5. Tumour Necrosis Factor-Alpha (TNF-α) estimation

The concentration of TNF-α in brain tissues was estimated using ELISA kit (Affymetrix eBioscience, USA) as specified by the manufacturer, following the method earlier described (Cho *et al.*, 2008). All chemicals including the samples and standard solutions were brought to room temperature prior to use. Briefly, ELISA plate was pre-coated with anti-mouse TNF-α purified capture antibody (100 µL/well), made airtight and left to incubate at 4°C overnight. The wells were aspirated, washed thrice with wash buffer, dried of any residual buffer with the aid of a blotting paper and afterwards blocked with 200 µL/well of diluent. The wells were thereafter incubated for 1 h, aspirated and washed after which 100 µL/well of reconstituted and diluted mouse TNF-α lyophilized standard (1000 pg/mL) was added to a well and a 2-fold serial dilution of it was performed to make an 8 point standard curve. Afterwards, 100 µL/well each of brain supernatant, anti-mouse TNF-α biotin detection antibody, enzyme (Avidin-HRP) and substrate solution were added one after the other following series of incubation and washes. Finally, stop solution (50 µL) was used to terminate the reaction. Absorbance was taken with a plate reader at 450 nm. The unknown sample concentration in pg/ml was extrapolated from the standard curve which was a plot of absorbance against concentration of the standard.

## 3.10. TISSUE PREPARATION FOR HISTOCHEMICAL STUDIES

Following behavioral tests, mice subjected to chronic restraint, PSD and CUS were perfused and fixed with 10 % v/v buffered formalin. Brain tissues were then subjected to the routine method for paraffin wax embedment to obtain paraffin wax embedded tissue blocks. Transverse sections (5-6

μm thick) of the prefrontal cortex and hippocampus were obtained using a microtome and fixed on glass slides.

### 3.10.1. Histology and estimation of neuronal density

Transverse sections of the prefrontal cortex and the hippocampus were stained with hematoxylin and eosin (H & E) in order to display the general histology of those regions (Eltony and Elgayar, 2014). A digital Camera (Optronics) fixed to a light microscope (Olympus BX-51) and a computer interface (MagnaFire) was used in capturing the images. The general morphology of the pyramidal cells was characterized using inter-reader variability. Finally, the quantity of viable neurons in the prefrontal cortex, CA1 and CA3 pyramidal layers were counted and quantitative analysis was performed by ImageJ software (Going, 1991). Viable neurons were identified as round-shaped cells with intact cytoplasmic membranes, having no nuclear condensation or distortion. Neuronal density was calculated as a ratio of viable neurons to square area of the circular view in a section.

### 3.10.2. Immunohistochemistry

Immunohistochemistry staining for iNOS and NF-κB were carried out as stated by the manufacturer following the modified procedure of Song *et al* (2013) by using the avidin-biotin peroxidase complex (HRP/DAB, Merck, Germany. LOT: 2775482). Thin sections (4 μ) of brain tissues were floated and mounted on APES charged glass slides. The tissue sections were deparaffinized. Antigen was retrieved in microwave in citrate buffer solution (10 mM citric acid, pH 6.0) for 10 min and thereafter incubated in 70% methanol with 3% $H_2O_2$ for 10 min, drained and rinsed with distilled water. Blocking solution was then added for 10 min. The antigen was incubated with the primary antibodies (monoclonal iNOS and NF-κB) diluted to 1:100 in PBS at 4°C overnight and thereafter washed thrice with wash buffer. The slides were allowed to incubate for 10 min in biotinylated anti-mice secondary antibody followed by peroxidase-conjugated streptavidin after series of washes with buffer. 3,3-diaminobenzidine (DAB) was afterwards added, rinsed and counterstained with Mayer's hematoxylin. DPX and cover slips were then mounted for examination.

## 3.11. STATISTICAL ANALYSIS

All results are expressed as Mean ± SEM. The results were analyzed using one way ANOVA and Student's Newman Keuls post hoc test. Statistical results were analysed using GraphPad Prism Software (version 4.0) (GraphPad Software Inc., San Diego, CA, USA). A value of $P < 0.05$ was considered to be statistically significant.

# CHAPTER FOUR

# RESULTS

## 4.1. EFFECT OF MORIN HYDRATE ON NOVELTY-INDUCED BEHAVIOUR

The effect of morin hydrate on novelty-induced behaviours in mice is represented in figures 5, 6 and 7. One-way ANOVA revealed a significant difference in rearing and locomotion amongst treatment groups. A significant dose-dependent reduction in rearing and locomotion was observed upon treatment with MH (10, 20, 40 and 80 mg/kg) relative to the VEH group, whereas a significant difference in grooming behaviour from VEH was only observed at 80 mg/kg ($p < 0.05$)

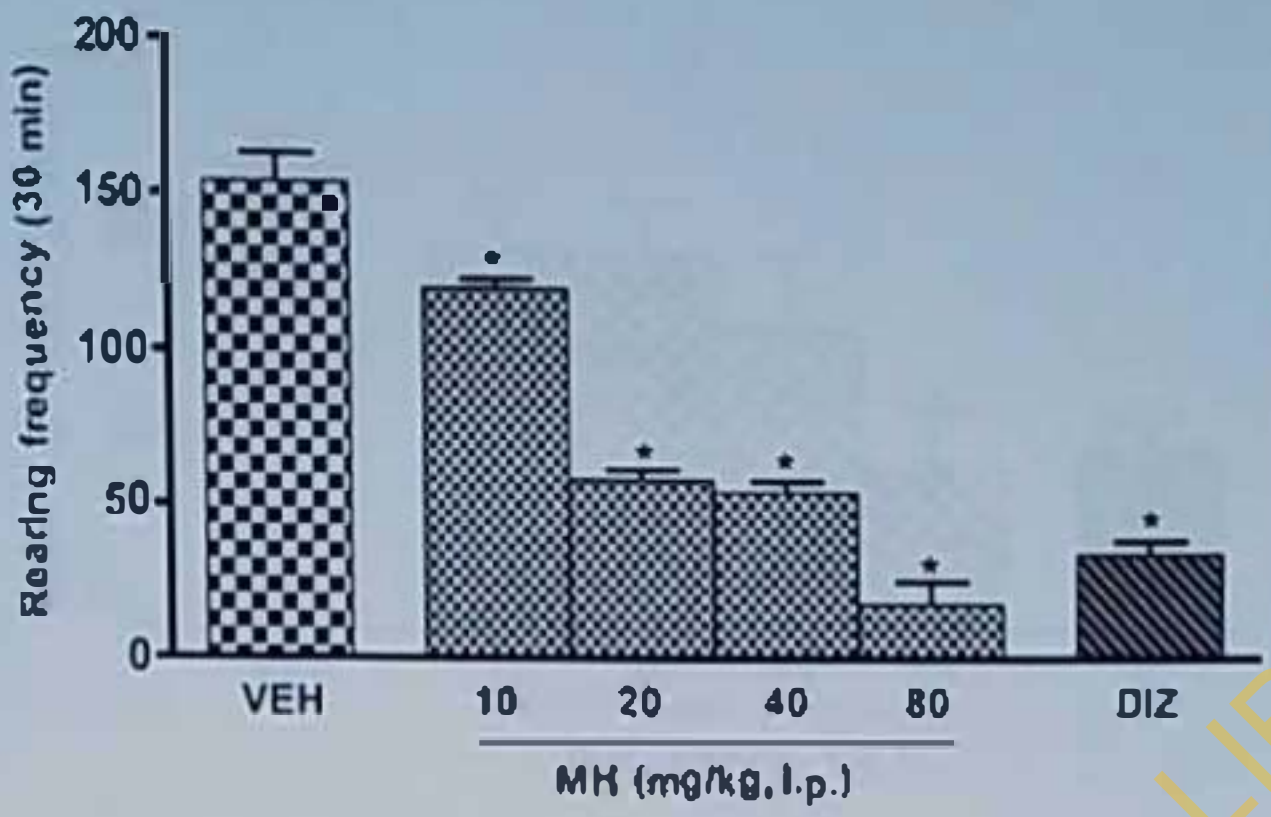



Figure 5: Effect of morin hydrate on novelty induced rearing in the open field.

Each column indicates mean ± SEM, 5 animals / group

* $p < 0.05$ relative to VEH (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml./kg)

MH: Morin hydrate

DIZ: Diazepam (2 mg/kg, i.p.)

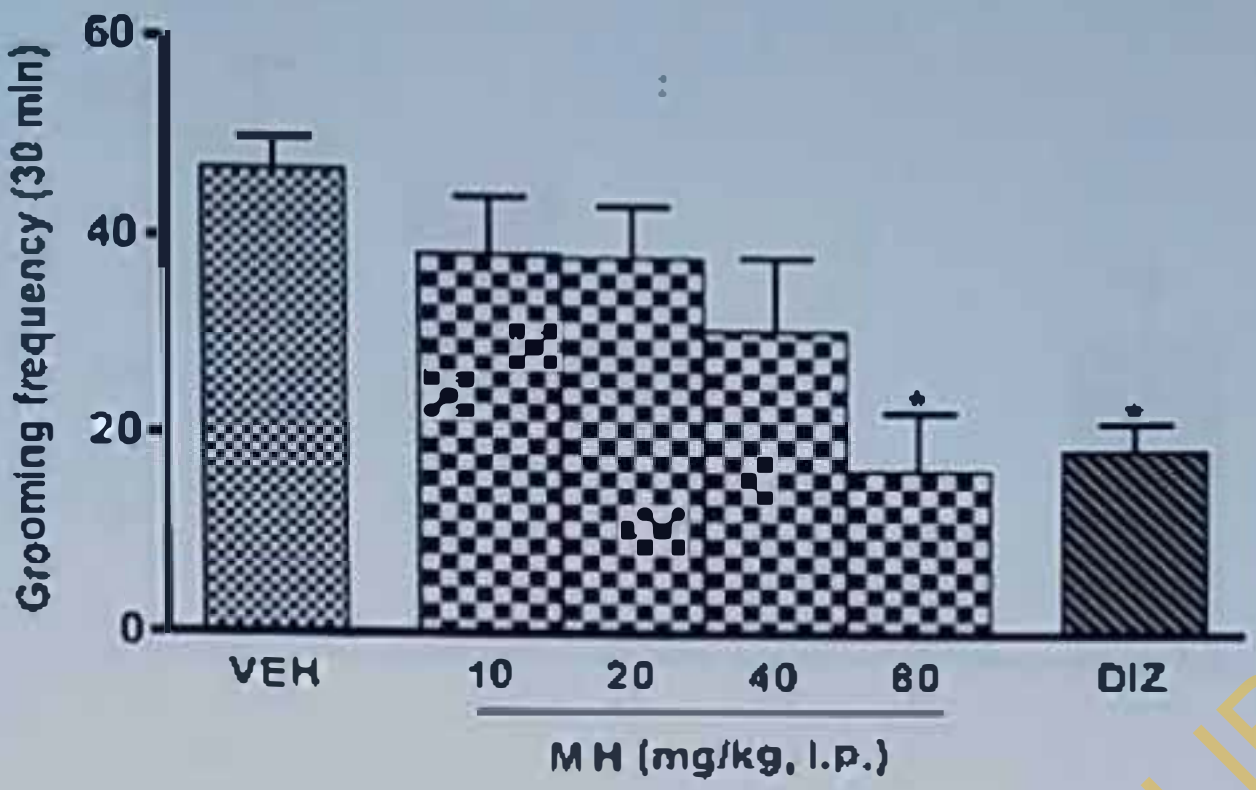



Figure 6: Effect of morin hydrate on novelty induced grooming in the open field.

Each column indicates mean ± SEM, 5 animals / group

• $p < 0.05$ relative to VEH (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

DIZ: Diazepam (2 mg/kg, i.p.)

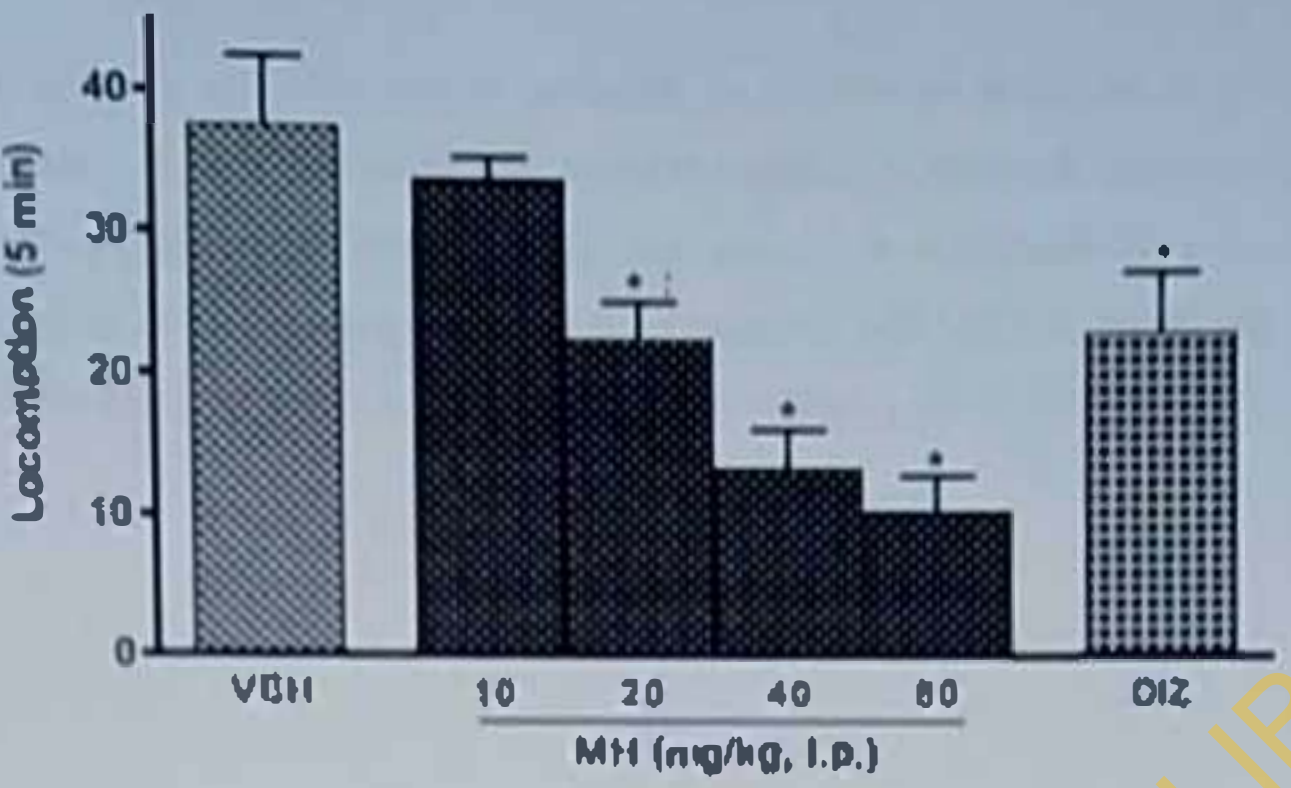



**Figure 7: Effect of morin hydrate on locomotor activity in the open field.**

Each bar represents mean ± SEM, 5 animals / group

* $p < 0.05$ relative to VEH (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

DIZ: Diazepam (2 mg/kg, i.p.)

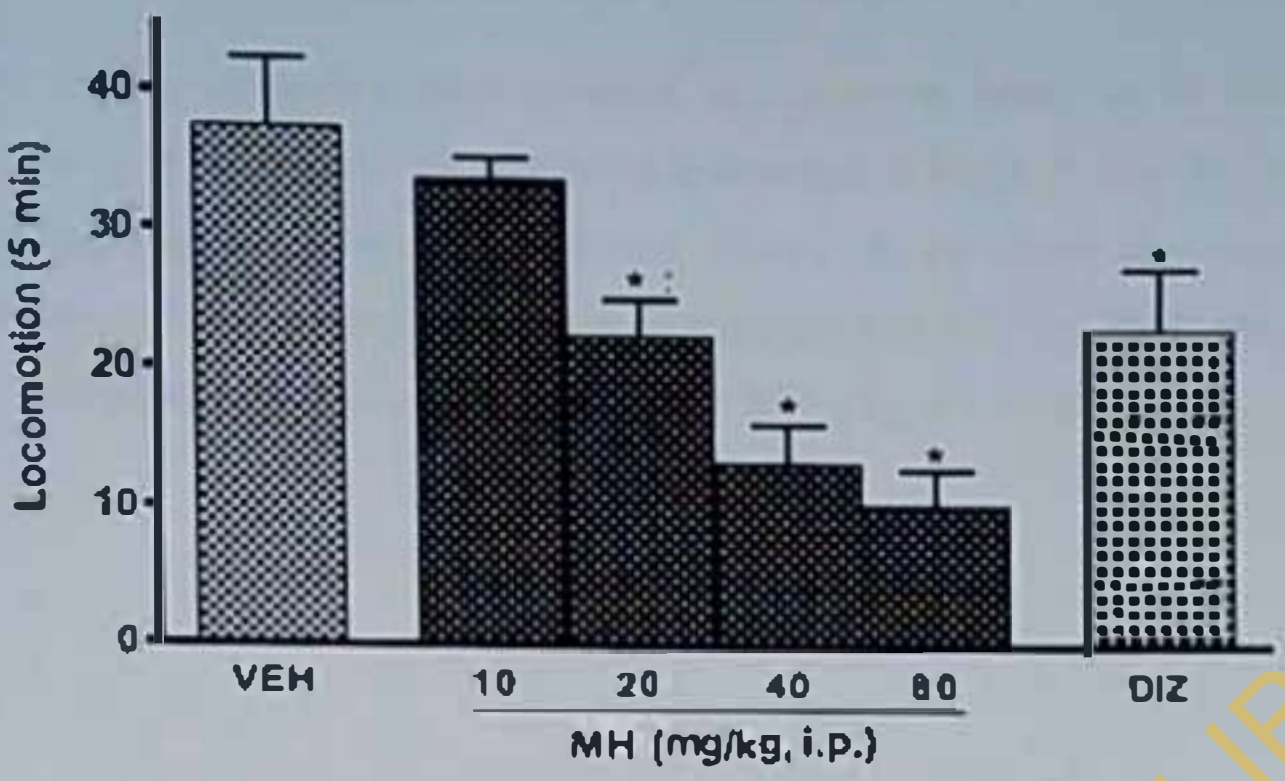



Figure 7: Effect of morin hydrate on locomotor activity in the open field.

Each bar represents mean ± SEM, 5 animals / group

* $p < 0.05$ relative to VEH (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

DIZ: Diazepam (2 mg/kg, i.p.)

## 4.2. EFFECT OF MORIN HYDRATE ON HEAD DIPS

The outcome of morin hydrate treatment on exploratory behaviour of mice as indicated by frequency of head dips in the hole board is indicated in Figure 8. One-way ANOVA showed a significant difference amongst treatment groups. A significant dose-dependent decline in frequency of head dipping was noted upon treatment with MH (10, 20, 40 and 80 mg/kg) relative to VEH group with maximal reduction seen at 80 mg/kg ($p < 0.05$).

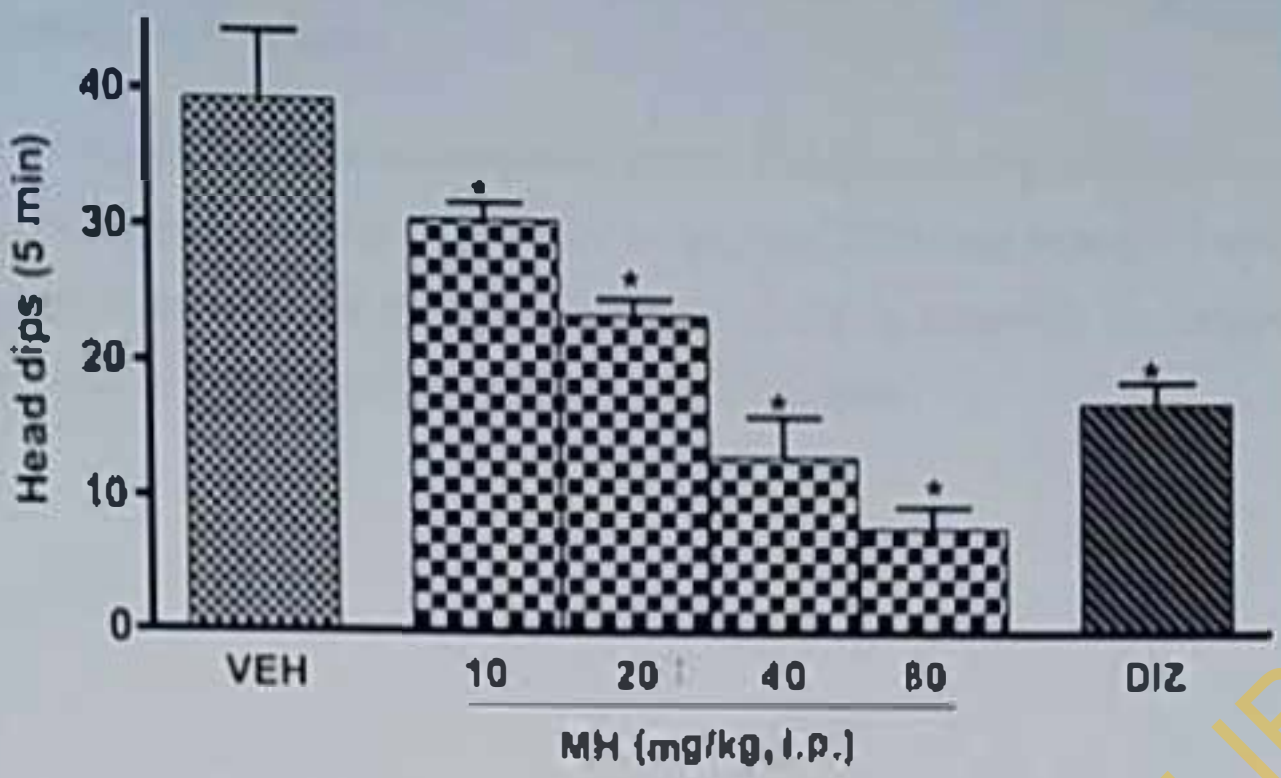



Figure 8: Effects of morin hydrate on head dips

Each column indicates mean ± SEM, 5 animals / group.

* $p < 0.05$ relative to VEH (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

DIZ: Diazepam (2 mg/kg, i.p.)

## 4.3. MORIN HYDRATE REDUCES IMMOBILITY TIME IN THE SWIMMING ENDURANCE MODEL

Effect of morin hydrate on immobility period in the swimming endurance model is indicated in Figure 9. One-way ANOVA disclosed a significant difference amongst treatment groups. Morin hydrate at doses 10 and 20 mg/kg increases swimming endurance by reducing immobility time compared to VEH (Newman Keuls post hoc comparison)

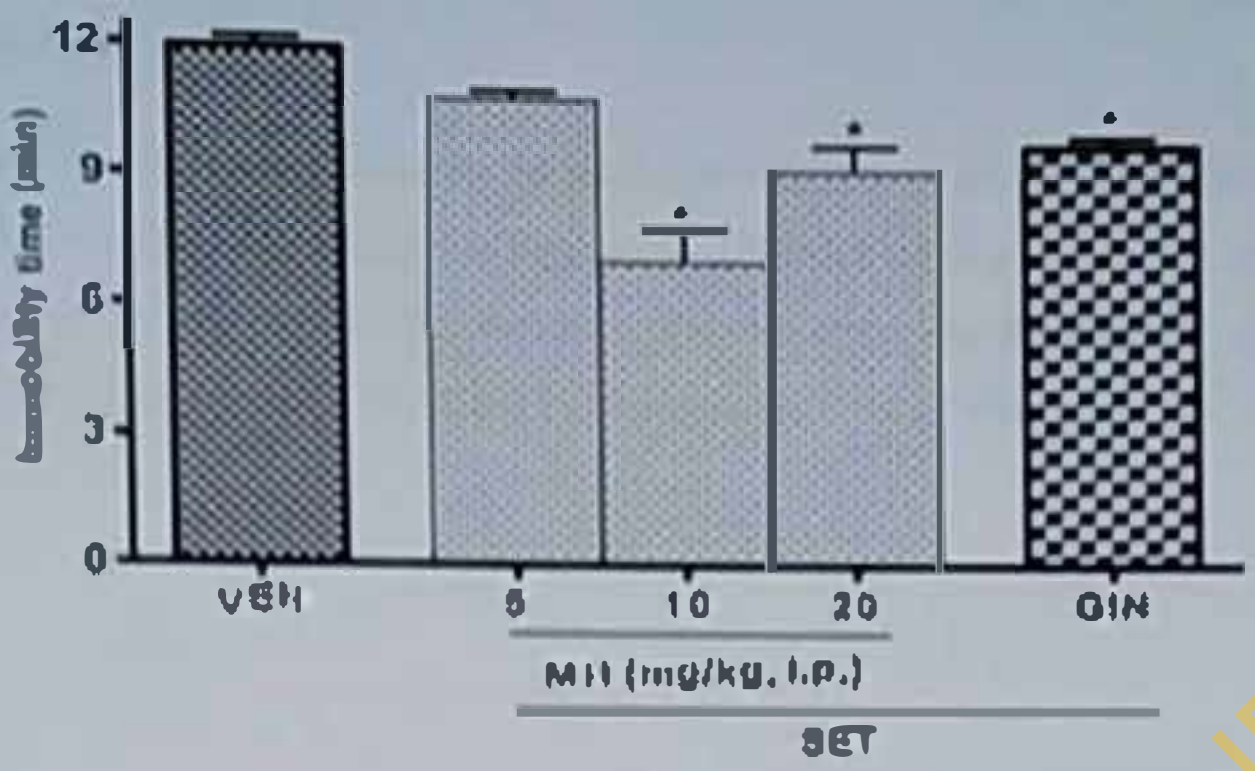



Figure 9: Effect of Morin hydrate on immobility time in the swimming endurance test

Each column indicates mean ± SEM, 5 animals / group.

* $p < 0.05$ compared to control group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MII: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

SET: Swimming Endurance Test

## 4.4. MORIN HYDRATE INCREASES THE ONSET OF CONVULSION IN ANOXIC STRESS

The effect of Morin hydrate on the onset of convulsion in anoxic mice is indicated in Figure 10. One-way ANOVA indicates a significant difference amongst treatment groups. The onset of convulsion which symbolized tolerance to anoxic stress was significantly and dose-dependently increased upon MH (5, 10, 20 mg/kg) treatment relative to VEH group ($p < 0.001$).

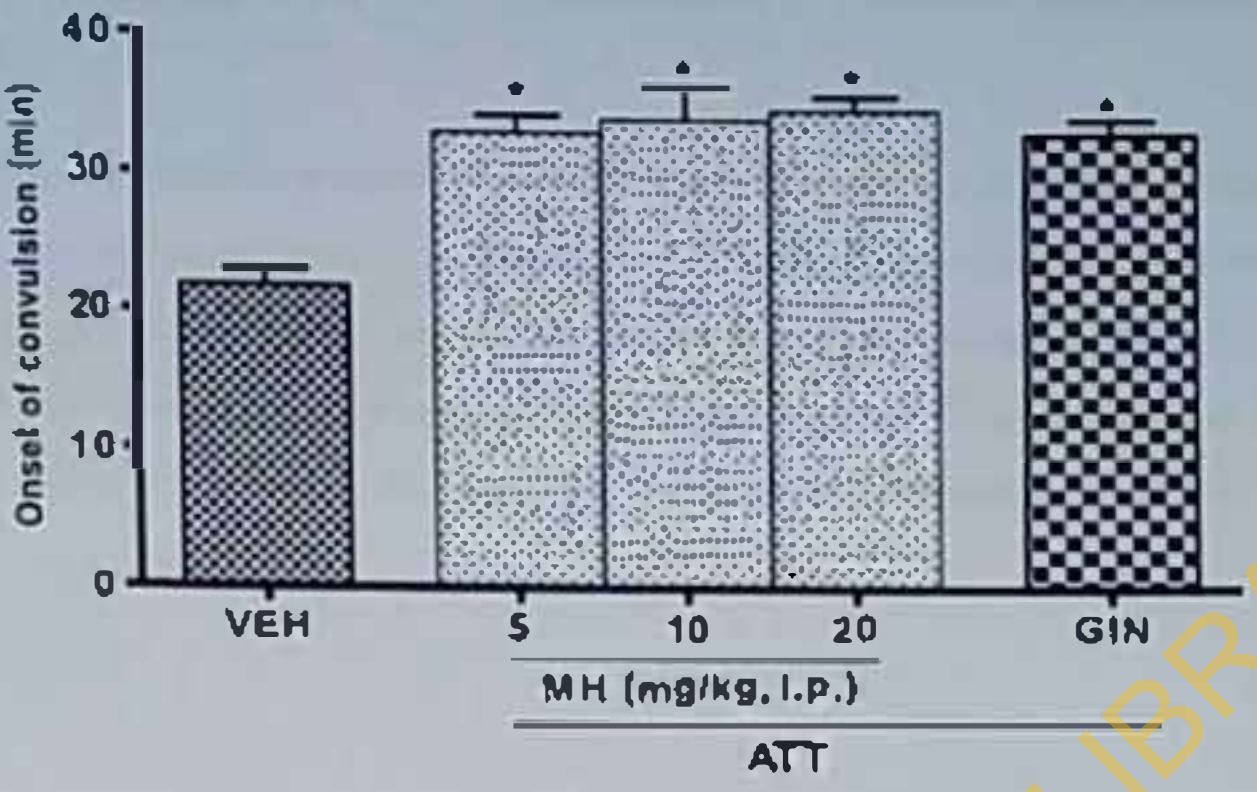



**Figure 10: Effect of Morin hydrate on the onset of convulsion**

Each bar represents mean ± SEM, 5 animals / group

• $p < 0.05$ relative to control (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ATT: Anoxic Tolerance Test

## 4.5. MORIN HYDRATE REDUCES IMMOBILITY TIME IN THE FORCED SWIM PARADIGM

Impact of morin hydrate on immobility period in the FST which is an indicator of depressive-like behaviour is as shown in Figure 11. One-way ANOVA revealed a significant difference amongst treatment groups. Acute restraint stress increased the immobility period significantly ($p < 0.001$) in the VEH acute stress group relative to VEH unstressed group. This increase was significantly ($p < 0.001$) upturned by MH (5, 10, 20 mg/kg), indicating antidepressant-like effect.

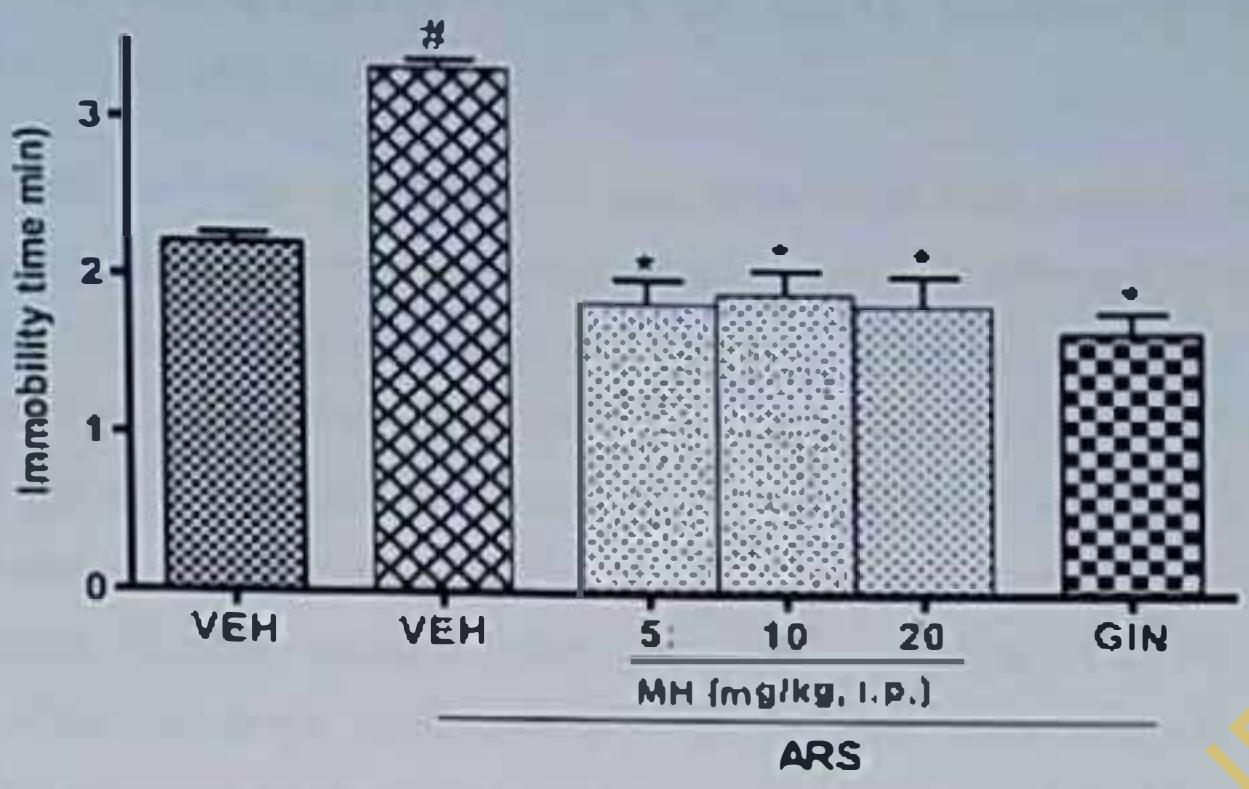



**Figure 11: Immobility in FST after acute restraint stress**

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH ARS group (One-way ANOVA followed by Student-Newman-Keuls posthoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

## 4.6. MORIN HYDRATE MITIGATES ACUTE RESTRAINT STRESS-INDUCED ANXIETY-LIKE BEHAVIOR

Anxiety-like behaviour exhibited by mice subjected to acute restraint stress as indicated by frequency and duration of exploration of both arms of the EPM and the modulatory effect of morin hydrate is shown in Figures 12, 13, 14 and 15. One-way ANOVA indicated a significant difference amongst treatment groups. A significant ($p < 0.001$) decline in frequency and duration of open arms exploration was noted in the VEH acute restraint stress group relative to the VEH unstressed group. Morin hydrate (5, 10, 20 mg/kg, i.p.) reversed this effect in a significant manner, indicating anxiolytic effect. This was further supported by the index of open arm avoidance which was significantly ($p < 0.001$) elevated in the VEH ARS group compared to VEH unstressed group, and was significantly ($p < 0.001$) reversed by morin hydrate (Figure 16).

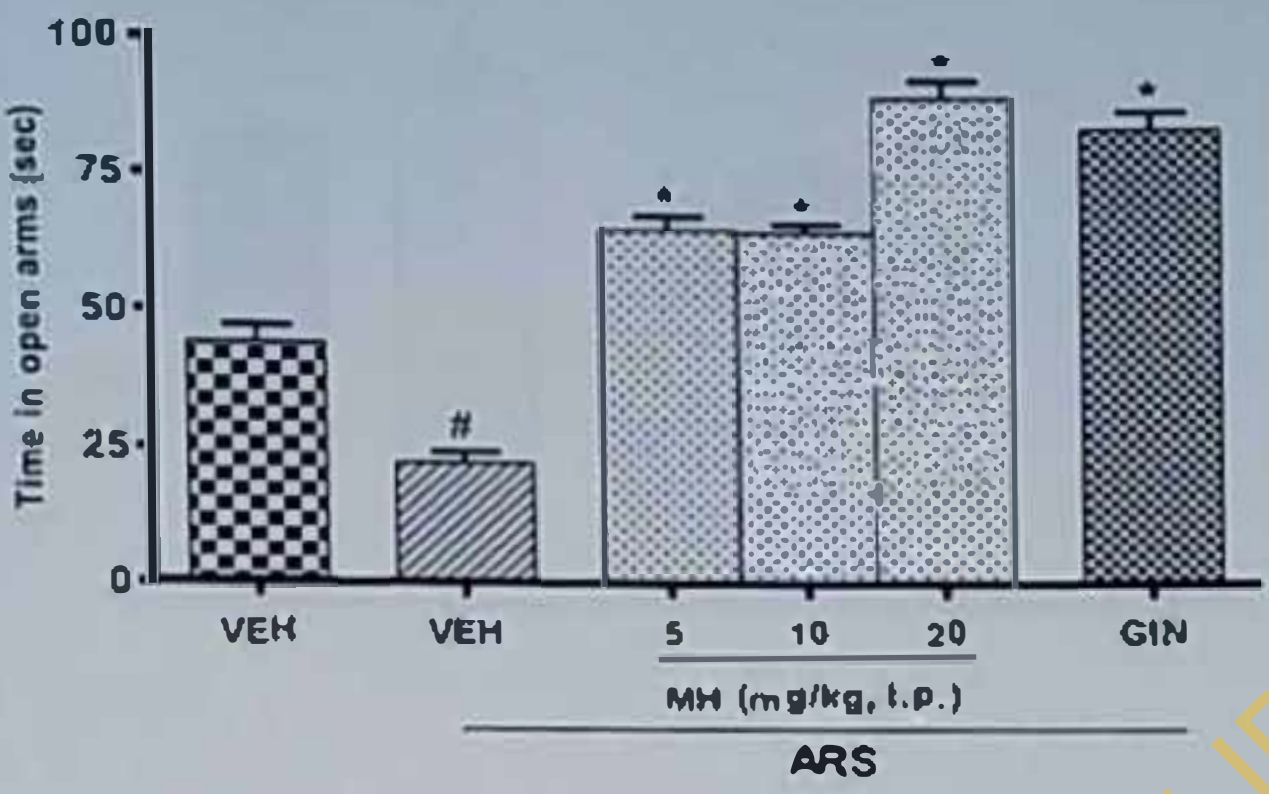





**Figure 12: Effect of Morin hydrate on time spent in the open arms of the EPM following acute restraint stress**

Each column indicates mean ± SEM, n = 6

# $p < 0.01$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH ARS (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

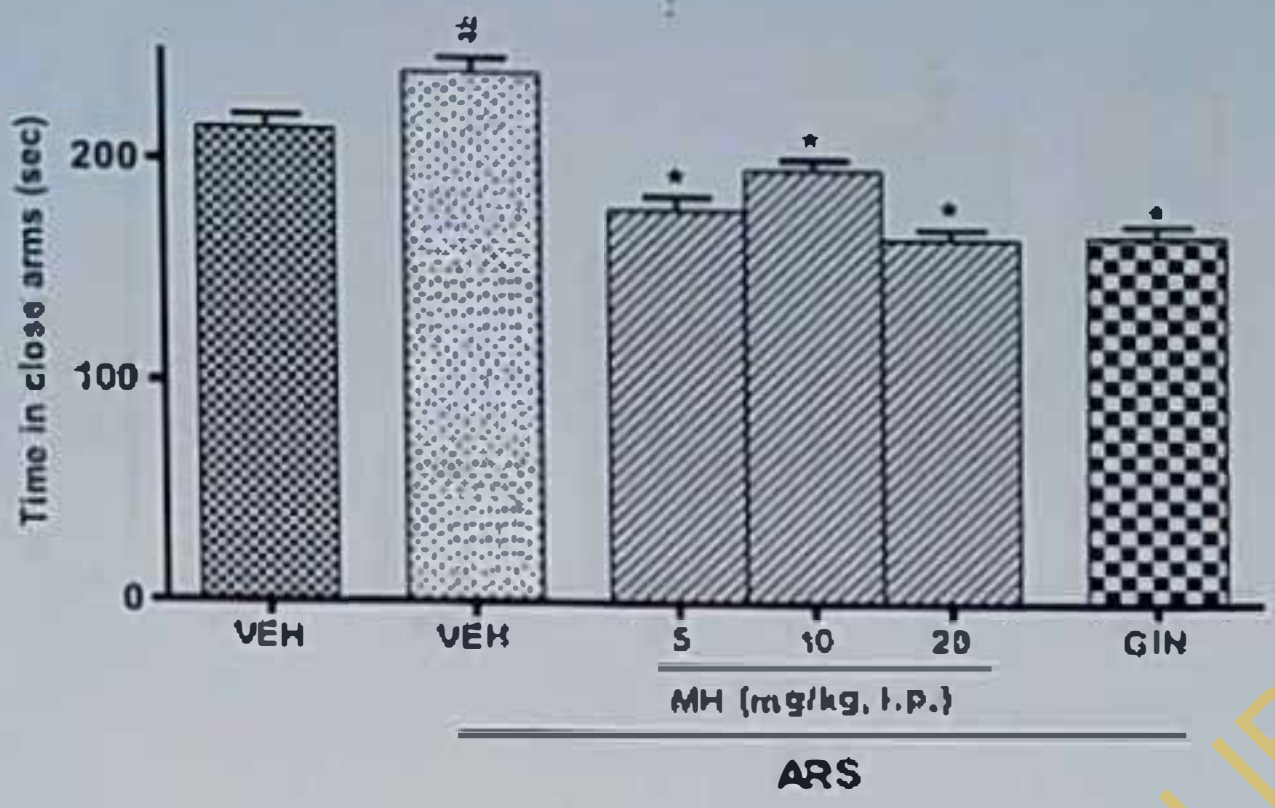



**Figure 13: Effect of Morin hydrate on time spent in the close arms of the EPM following acute restraint stress**

Each column indicates mean ± SEM, n = 6

# p < 0.01 relative to VEH unstressed group

* p < 0.001 relative to VEH ARS (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

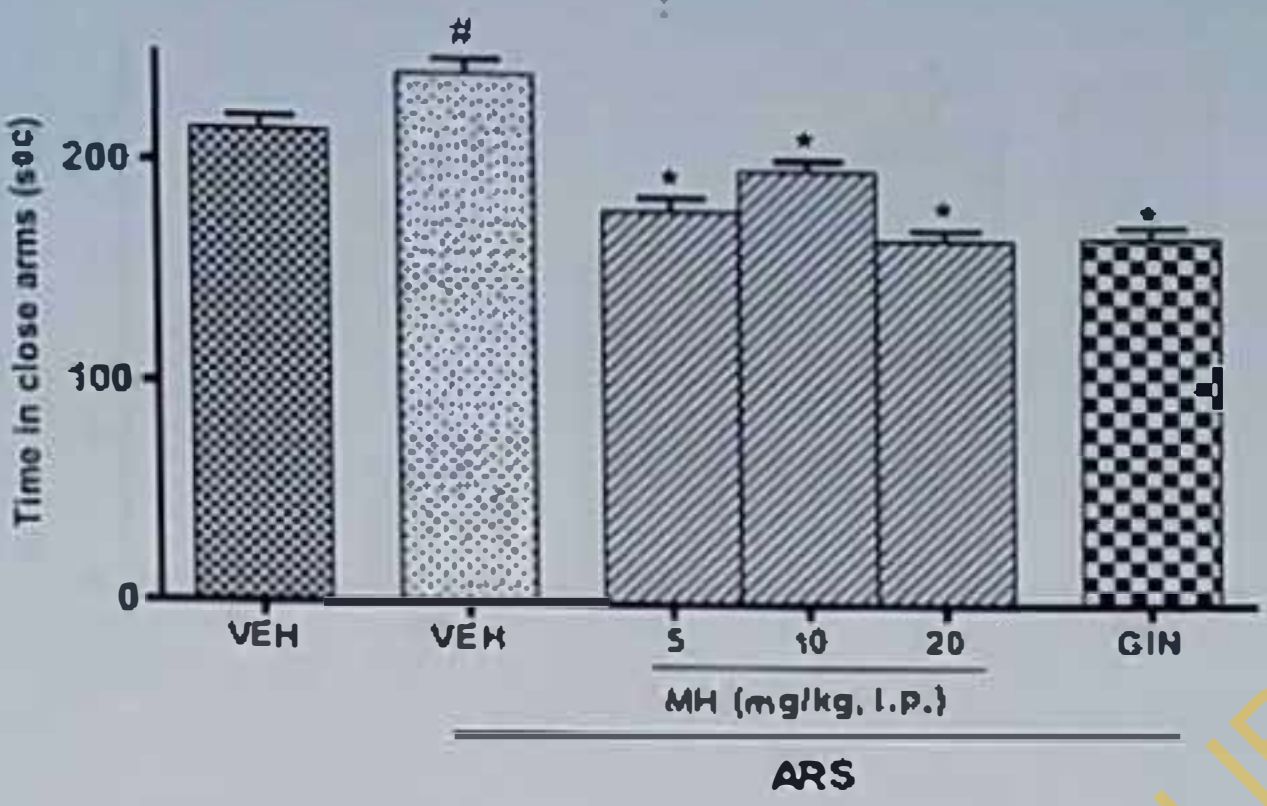



**Figure 13: Effect of Morin hydrate on time spent in the close arms of the EPM following acute restraint stress**

Each column indicates mean ± SEM, n = 6

# $p < 0.01$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH ARS (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

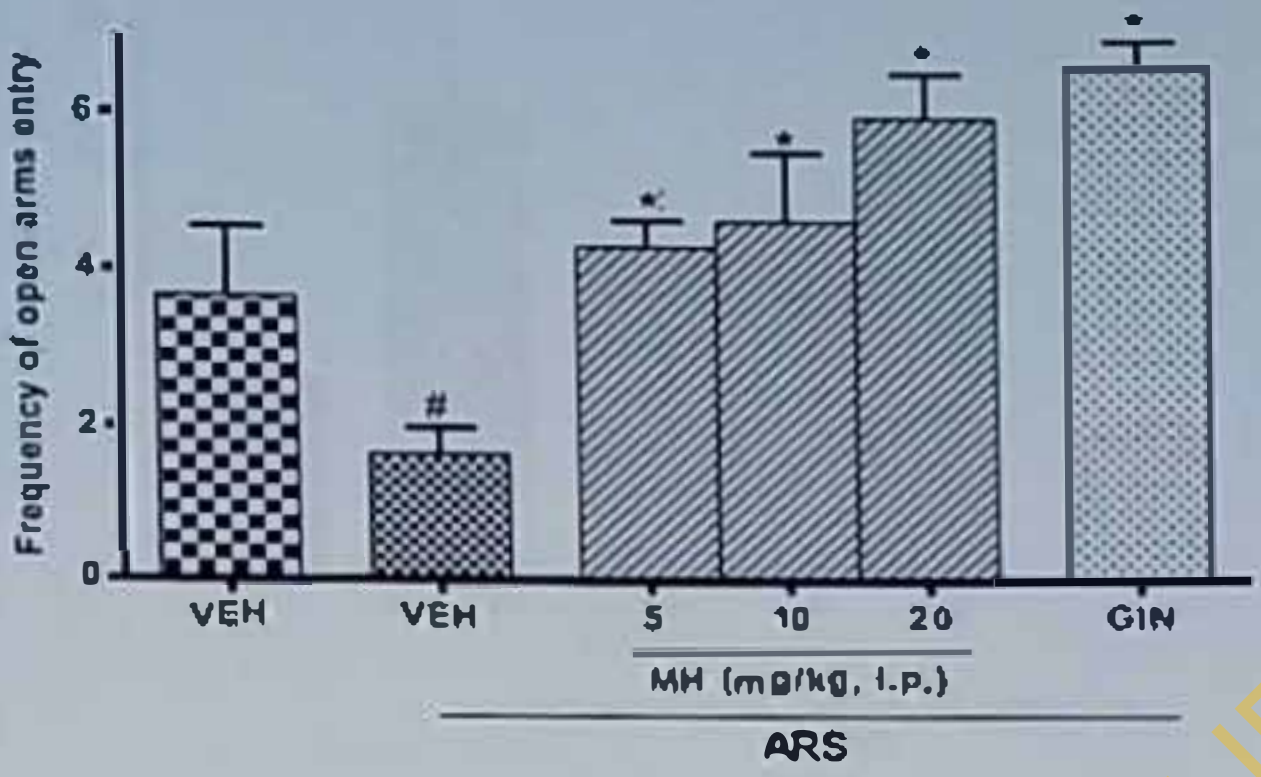



Figure 14: Effect of Morin hydrate on open arms entry in the EPM after acute restraint stress

Each column indicates mean± SEM, 6 animals/group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH ARS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

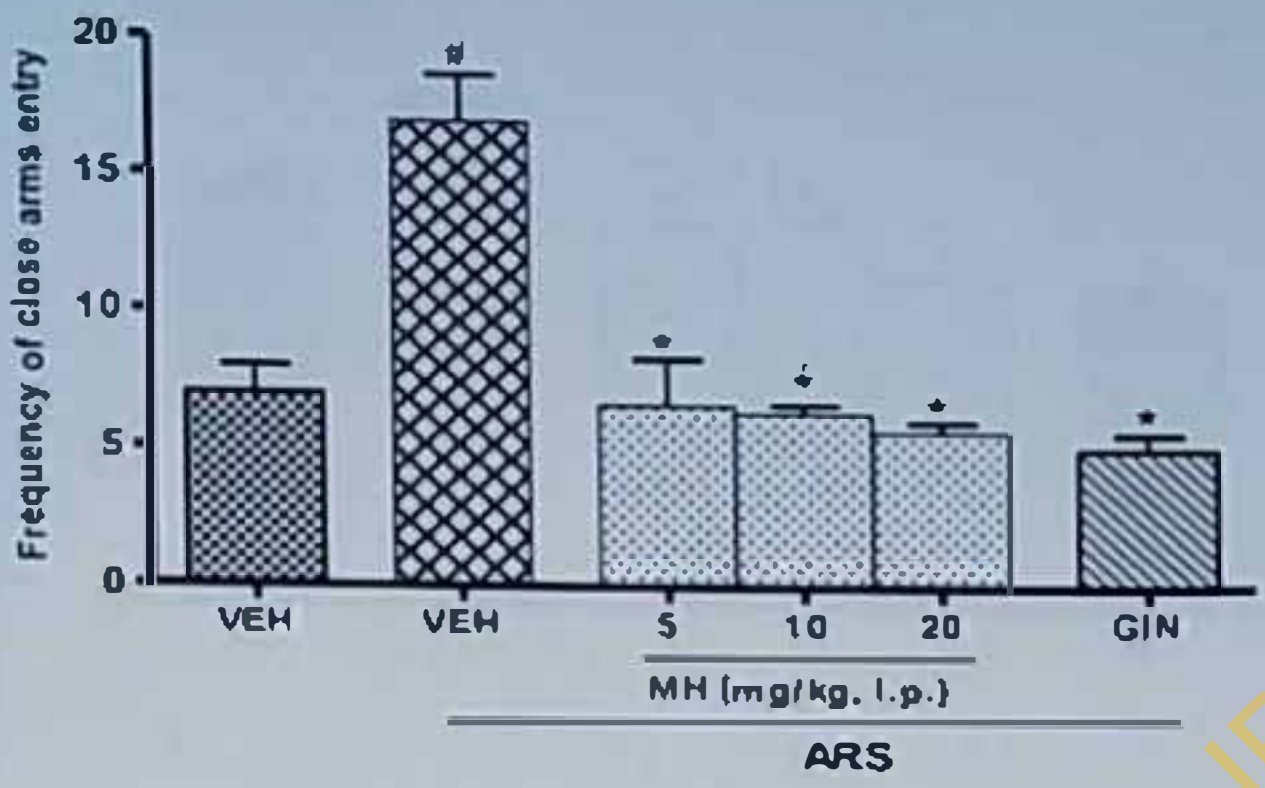



Figure 15: Effect of Morin hydrate on close arms entry in the EPM after acute restraint stress

Each column indicates mean ± SEM, 6 animals/group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH ARS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

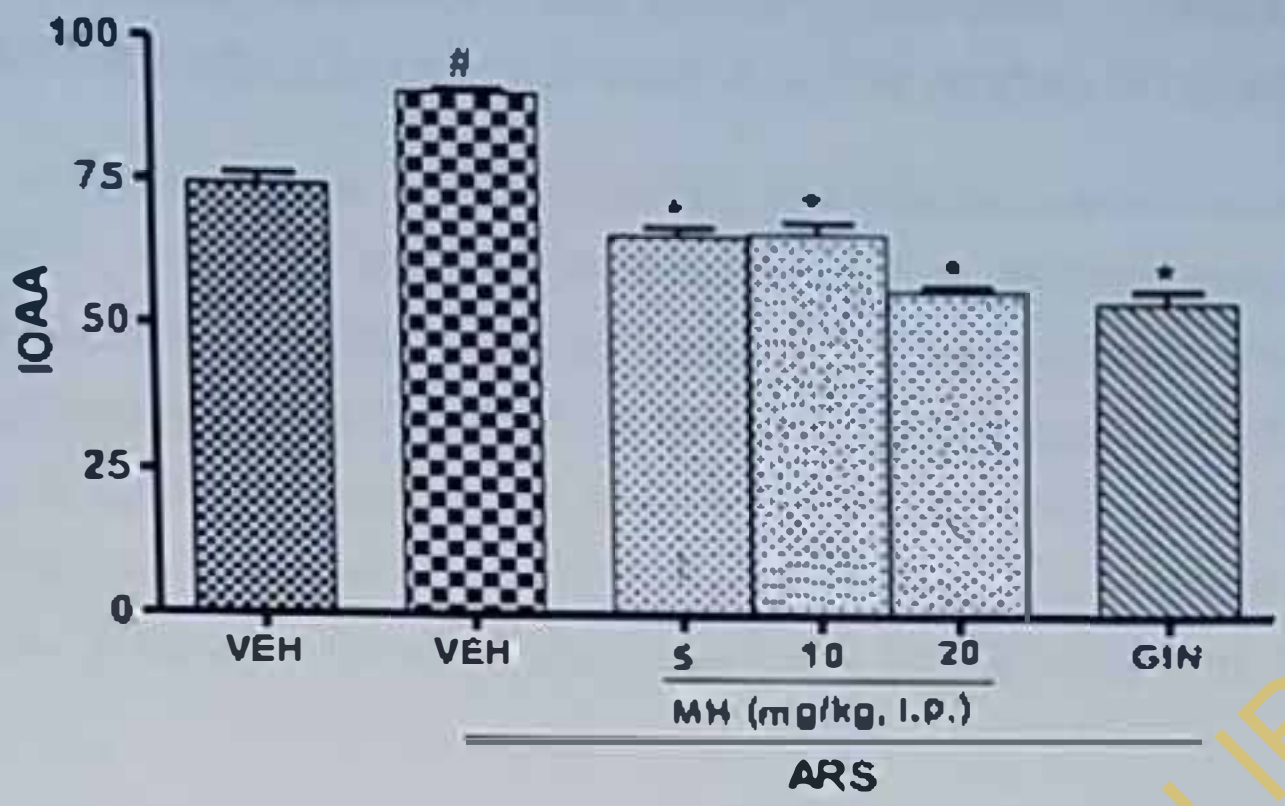



**Figure 16: Effect of Morin hydrate on the Index of open arm avoidance in the EPM after acute restraint stress**

Each column indicates mean ± SEM, 6 animals/group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH ARS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

## 4.7. MORIN HYDRATE INFLUENCES GLUCOSE, CHOLESTEROL AND TRIGLYCERIDE CONCENTRATIONS IN ACUTE RESTRAINT STRESSED MICE

A rise in serum glucose level was observed in mice subjected to acute restraint stress. One way ANOVA indicated a significant difference amongst all treatment groups. Serum glucose concentration was significantly ($p < 0.05$) elevated in VEH acute restraint stress group relative to VEH unstressed group. The MH (5, 10, 20 mg/kg) treatment significantly ($p < 0.001$) upturned this effect. Furthermore, one way ANOVA indicated that acute restraint stress significantly decreased cholesterol level relative to VEH unstressed group. This decrease was reversed by MH (5 and 10 mg/kg). However, MH did not significantly alter triglyceride level (Table 1).

Table 1: Morin hydrate alters glucose, cholesterol and triglyceride concentrations in acute restraint stressed mice

| Treatment (mg/kg) | Blood glucose(mg/dL) | Total cholesterol(mg/dL) | Triglycerides (mg/dL) |
|---|---|---|---|
| $VEH_{(UNS)}$ | 76.1 ± 8.0 | 115.0 ± 2.1 | 130.0 ± 10.1 |
| $VEH_{(ARS)}$ | 168.0 ± 7.6# | 92.7 ± 1.4# | 82.6 ± 1.7# |
| MH 5mg/kg | 134.0 ± 7.4* | 110.0 ± 2.7* | 88.9 ± 3.4 |
| MH 10mg/kg | 112.0 ± 11.0* | 112.0 ± 5.0* | 68.6 ± 2.9 |
| MH 20 mg/kg | 106.0 ± 2.36* | 94.6 ± 5.5 | 70.1 ± 0.97 |
| GIN | 128.0 ± 11.9* | 127.0 ± 0.3* | 68.8 ± 0.42 |

Each result represents mean ± SEM, 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to $VEH_{(ARS)}$ group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

$VEH_{(UNS)}$: Vehicle unstressed group

$VEH_{(ARS)}$: Vehicle acute restraint stress group

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

## 4.8. EFFECT OF MORIN HYDRATE ON ACUTE RESTRAINT STRESS-INDUCED OXIDATIVE STRESS BIOMARKERS IN MICE

Effects of Morin hydrate on changes in oxidative biomarkers in mice exposed to acute restraint stress are indicated in Figures 17, 18 and 19. Figure 17 revealed that in the VEH acute stress group, a significant ($p < 0.01$) reduction was noted in brain GSH level relative to VEH unstressed group. One-way ANOVA revealed that this observed reduction was significantly ($p < 0.05$) reversed by MH (5, 10, 20 mg/kg). Furthermore, acute restraint stress significantly elevated brain MDA ($p < 0.001$) and nitrite ($p < 0.01$) levels in VEH acute stress group relative to VEH unstressed group as shown in Figures 18 and 19 respectively. One way ANOVA revealed that Morin hydrate (5, 10, 20 mg/kg) significantly reversed this effect.

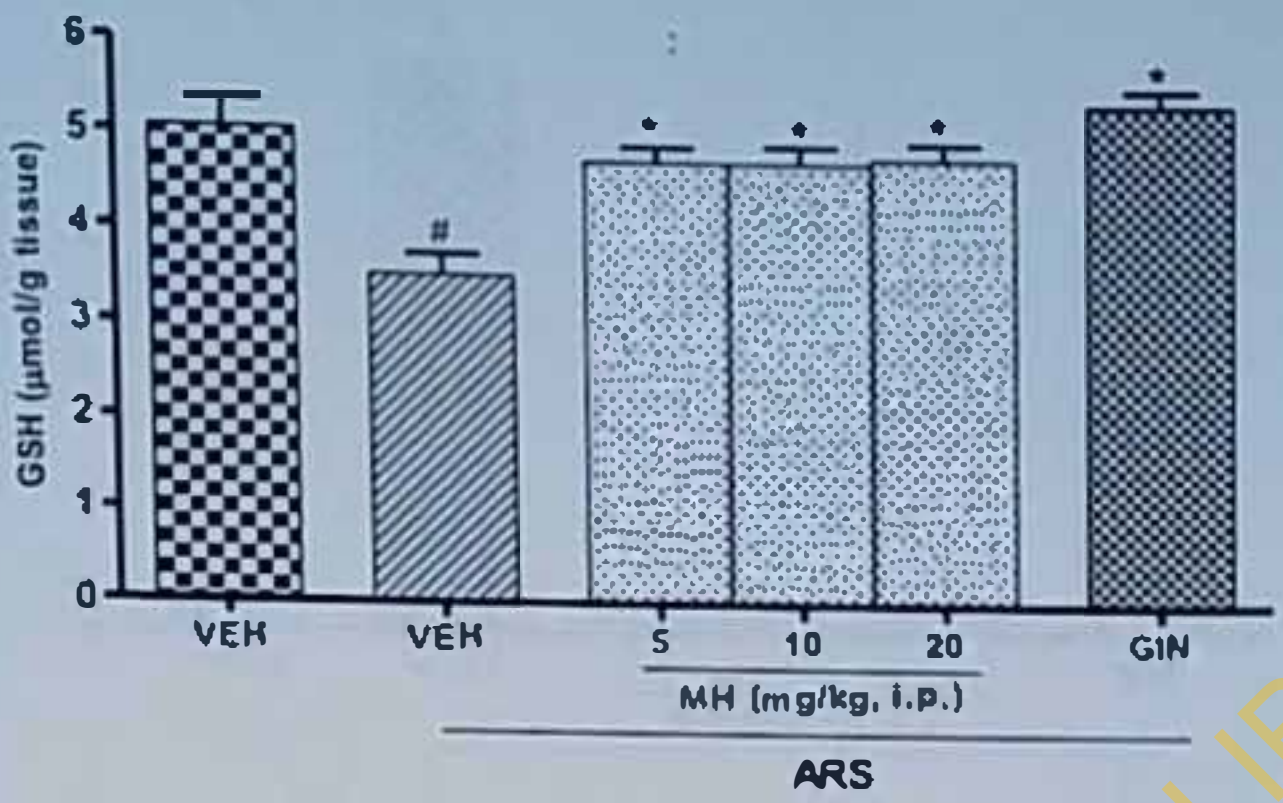



**Figure 17: Effect of Morin hydrate on brain GSH level following acute restraint stress.**

Each column indicates mean ± SEM. 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH ARS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

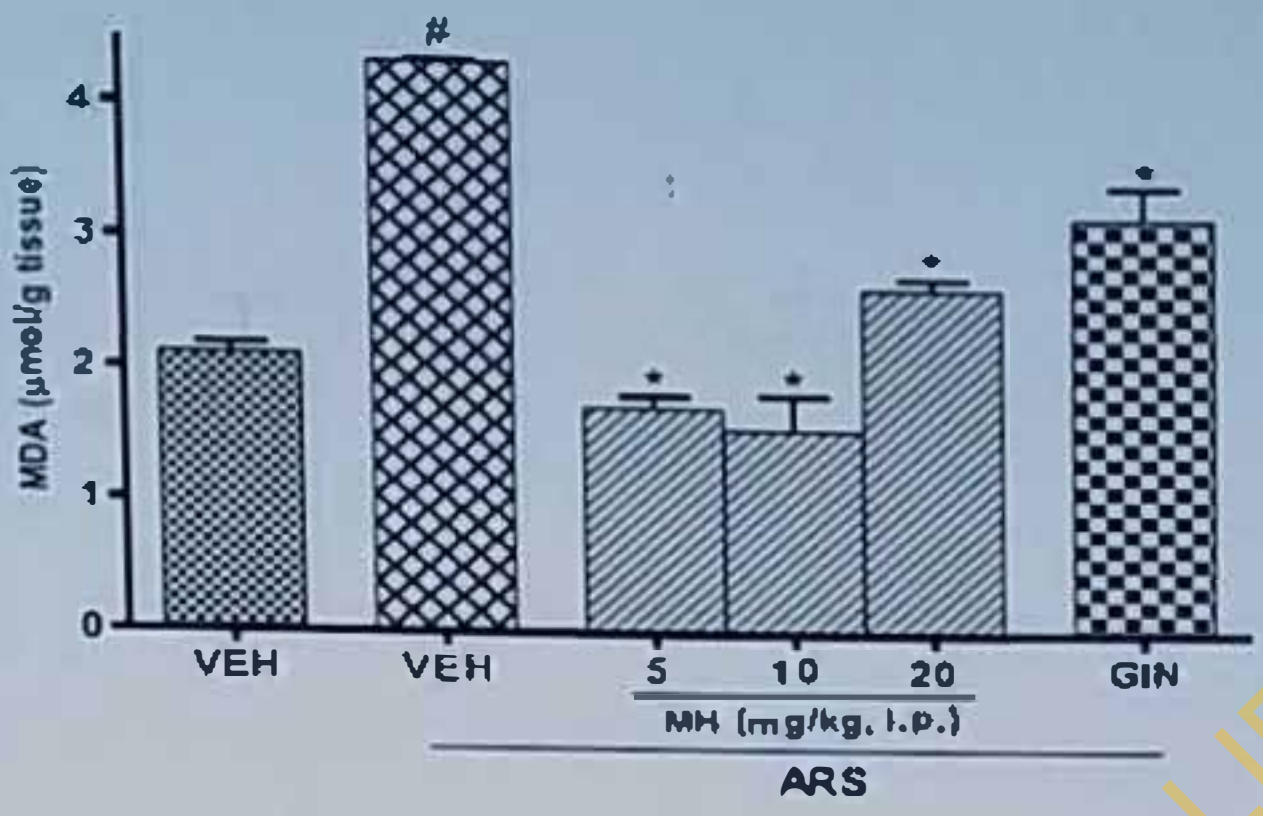



Figure 18: Effect of Morin hydrate on brain MDA level following acute restraint stress.

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH ARS group (One-way ANOVA followed by Student-NewmanKeuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg. i.p.)

ARS: Acute Restraint Stress

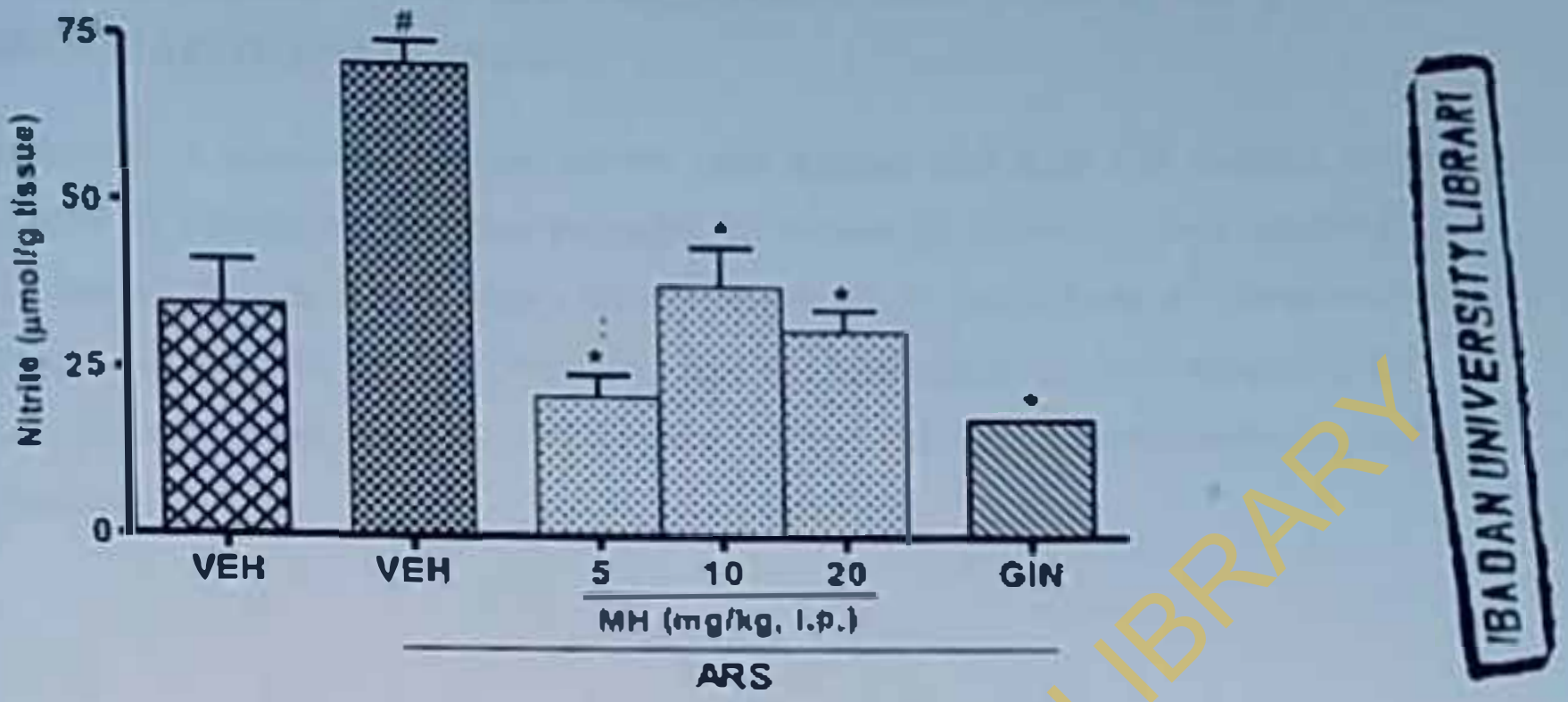





Figure 19: Effect of Morin hydrate on nitrite levels following acute restraint stress.

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.01$ relative to VEH unstressed group

* $p < 0.01$ relative to VEH ARS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

ARS: Acute Restraint Stress

## 4.9. MORIN HYDRATE MITIGATES DEPRESSIVE BEHAVIOUR IN MICE IN THE CHRONIC RESTRAINT PARADIGM

The duration of immobility of chronic restraint stress-exposed mice in the FST model is shown in Figure 20. Chronic restraint stress prolonged the duration of immobility when compared to VEH unstressed group. This mimics a state of despair that is symptomatic of depressive-like behavior. However, this prolonged immobility time was significantly ($p < 0.05$) reduced by MH (5, 10, 20 mg/kg) when compared to VEH chronic restraint stress group, thereby indicating anti-depressant-like effect.

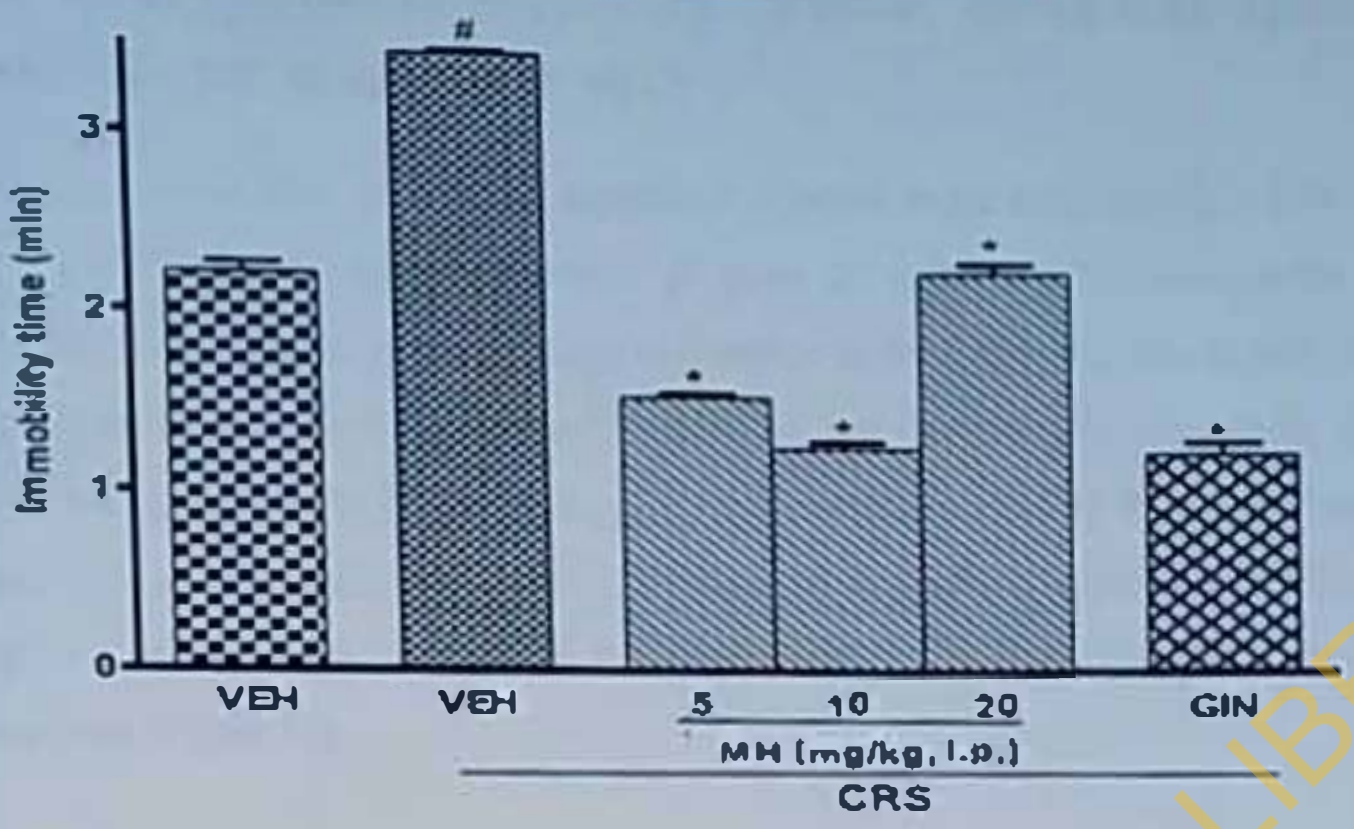



Figure 20: Effect of Morin hydrate on immobility period in the FST after chronic restraint stress.

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.05$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

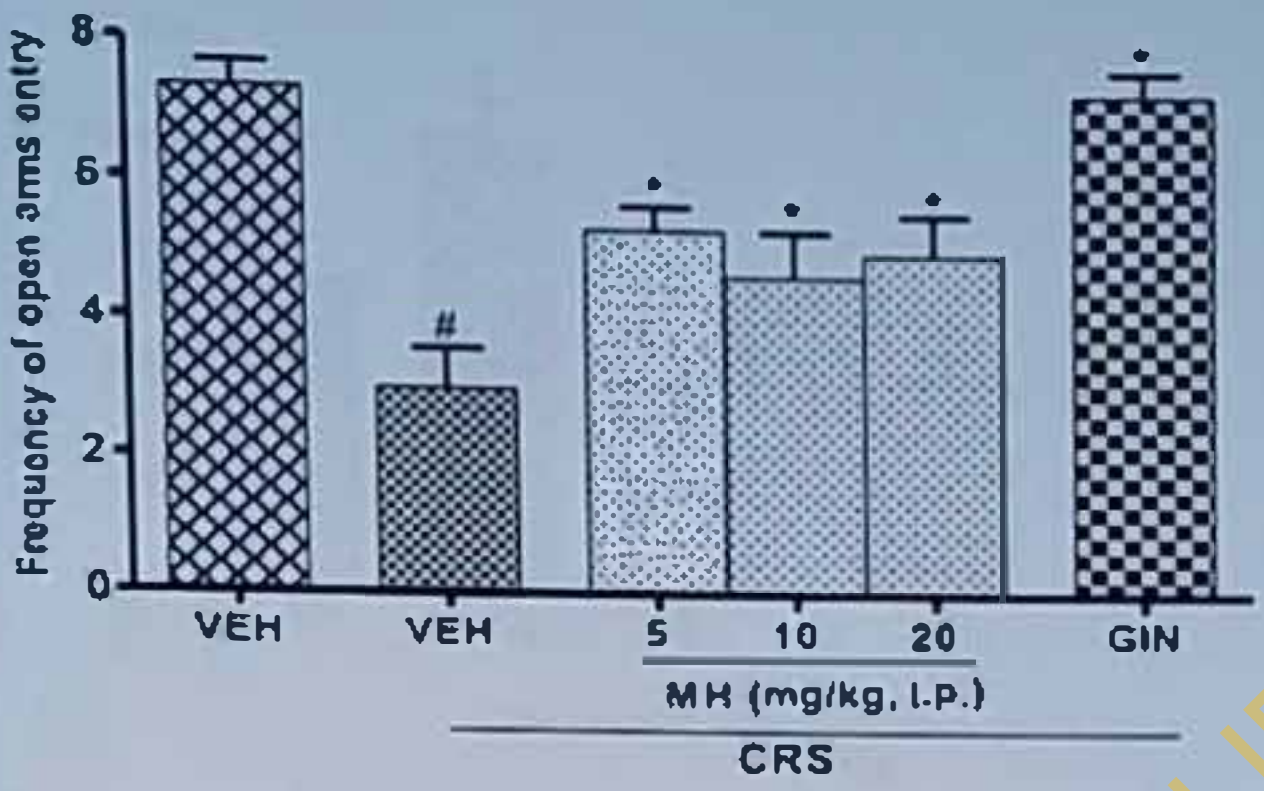
MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

## 4.10. MORIN HYDRATE MITIGATES CHRONIC RESTRAINT STRESS-INDUCED ANXIETY-LIKE BEHAVIOR IN MICE

Chronic restraint stress provoked a significant decrease in the frequency ($p < 0.001$) and duration ($p < 0.001$) of open arms exploration (Figures 21 and 23) and consequently increased the frequency and duration of closed arms exploration in the EPM (Figures 22 and 24). However, a significant ($p < 0.05$) elongation in the duration and frequency of open arms exploration was observed upon MH (5, 10, 20 mg/kg, i.p.) treatment compared to VEH CRS group, indicating anxiolytic activity which was further buttressed by the index of open arm avoidance which was elevated in the VEH CRS group but significantly ($p < 0.001$) reversed by morin hydrate treatment (Figure 25).




Figures 21: Effect of Morin hydrate on the frequency of open arms entry in the EPM following chronic restraint stress exposure.

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.01$ relative to VEH unstressed group

• $p < 0.05$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

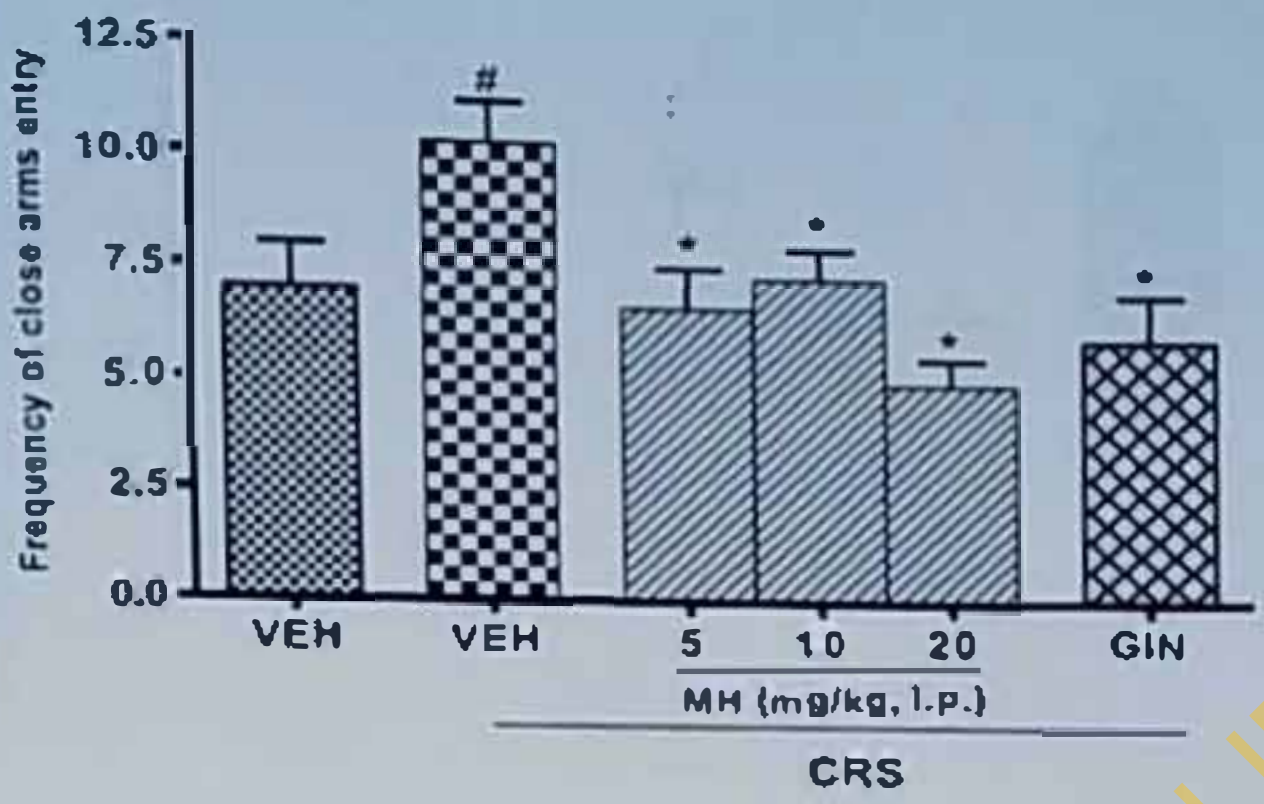



**Figure 22: Effect of Morin hydrate on frequency of close arms entry in the EPM following chronic restraint stress exposure.**

Each column indicates mean ± SEM. 6 animals / group

# $p < 0.01$ relative to VEH unstressed group

* $p < 0.01$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

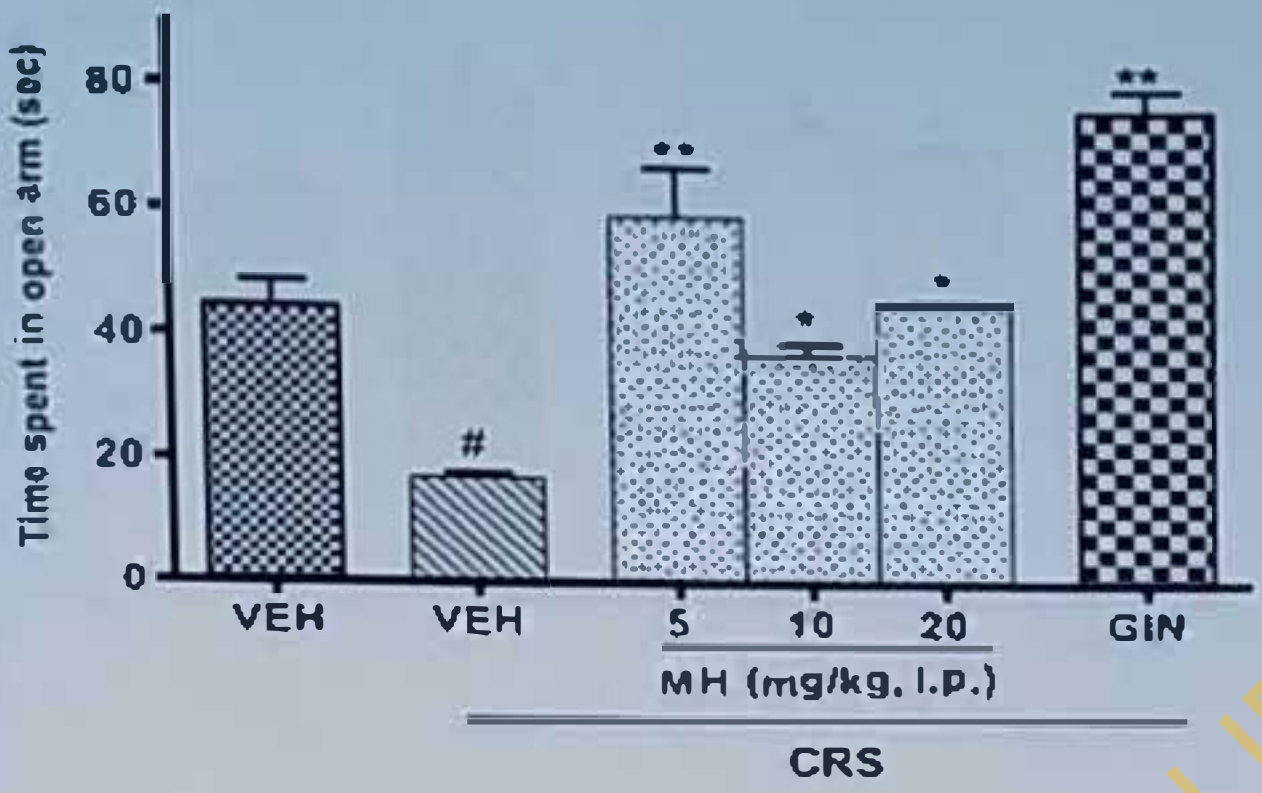





**Figure 23: Effect of Morin hydrate on time spent in the open arms of the EPM following chronic restraint stress exposure.**

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.01$ relative to VEH unstressed group

*, ** $p < 0.01$ and $p < 0.001$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

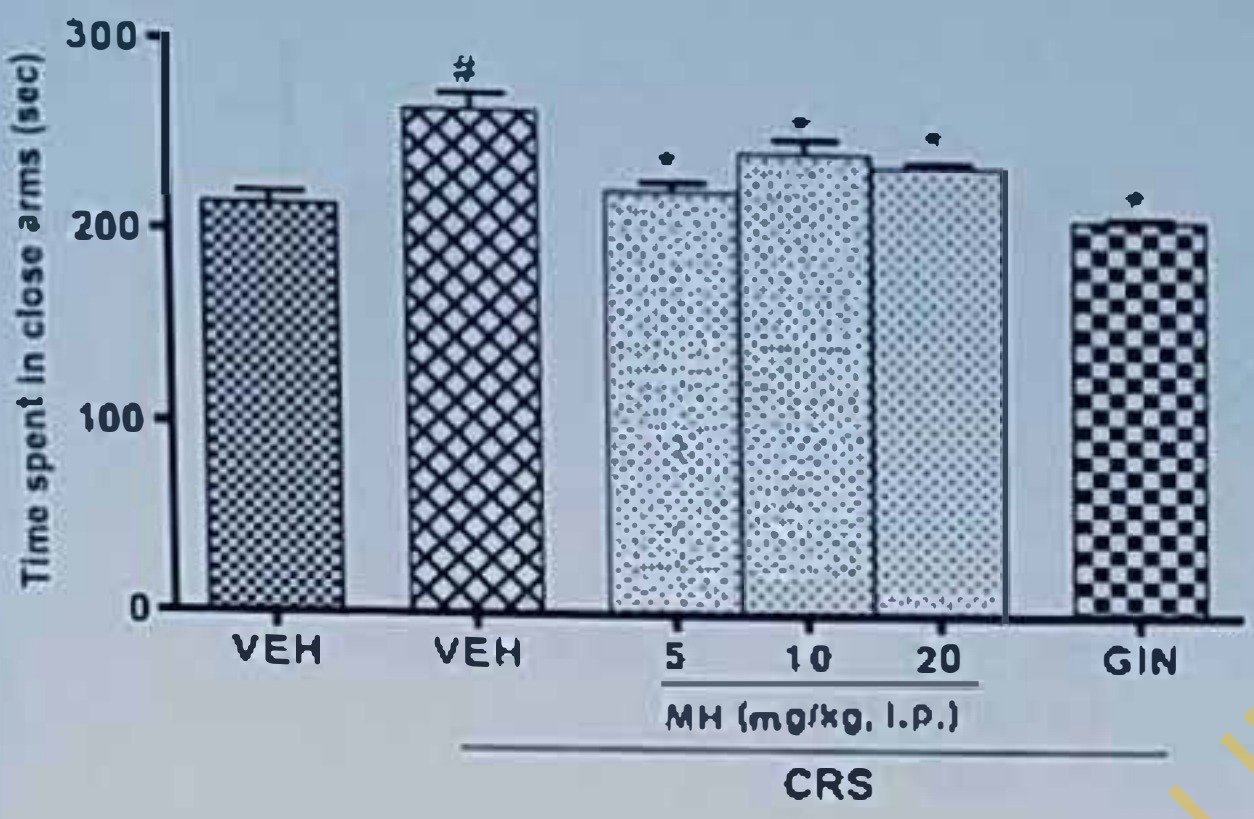



Figure 24: Effect of Morin hydrate on time spent in the EPM following chronic restraint stress exposure.

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

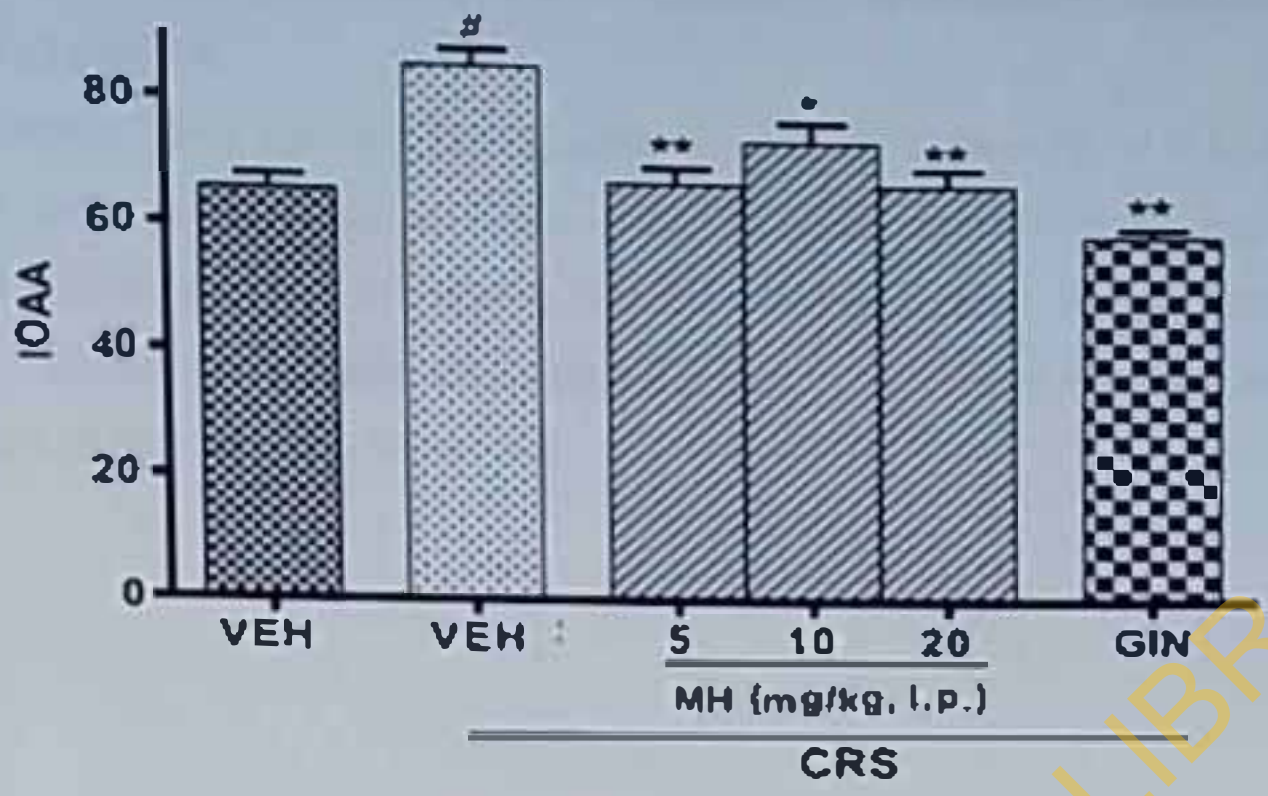



**Figure 25: Effect of Morin hydrate on the index of open arm avoidance in the EPM after chronic restraint stress**

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

*, ** $p < 0.01$ and $p < 0.001$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

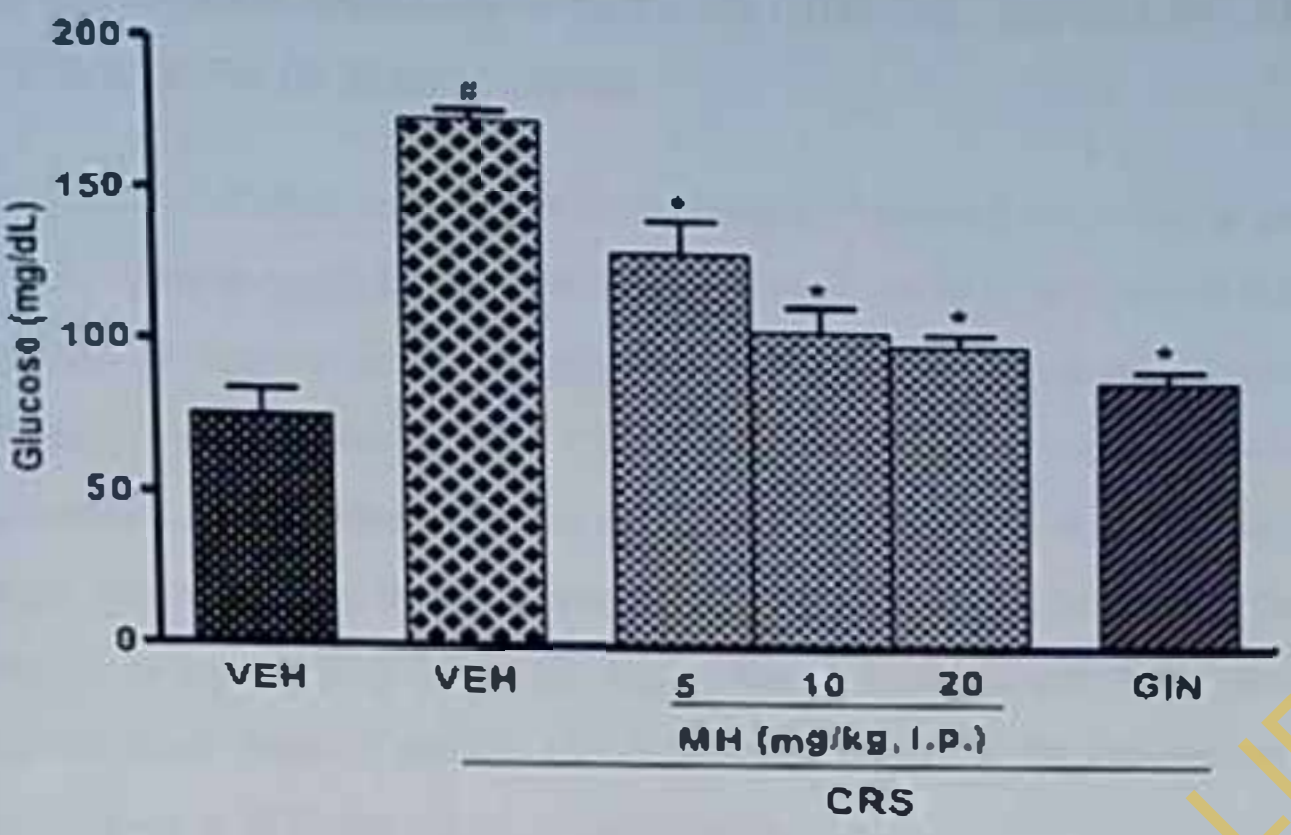
CRS: Chronic Restraint Stress

## 4.11. EFFECT OF MORIN HYDRATE ON CHRONIC RESTRAINT STRESS-INDUCED HYPERGLYCEMIA

Chronic restraint stress caused a rise in serum glucose level. One-way ANOVA indicated a significant difference amongst treatment groups. Chronic restraint stress induced glucose upsurge significantly ($p < 0.05$) relative to VEH unstressed group. A significant ($p < 0.05$) reversal of this chronic restraint stress-induced hyperglycaemia was observed upon treatment with MH (5, 10 and 20mg/kg) (Figure 26).

## 4.11. EFFECT OF MORIN HYDRATE ON CHRONIC RESTRAINT STRESS-INDUCED HYPERGLYCEMIA

Chronic restraint stress caused a rise in serum glucose level. One-way ANOVA indicated a significant difference amongst treatment groups. Chronic restraint stress induced glucose upsurge significantly ($p < 0.05$) relative to VEH unstressed group. A significant ($p < 0.05$) reversal of this chronic restraint stress-induced hyperglycaemia was observed upon treatment with MH (5, 10 and 20mg/kg) (Figure 26).




**Figures 26: The effect of morin hydrate on glucose concentration in chronic restraint stressed mice**

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.05$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test)

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

## 4.12. EFFECT OF MORIN HYDRATE ON CHRONIC RESTRAINT STRESS-INDUCED ALTERATIONS IN SERUM LIPIDS

The impact of chronic restraint stress on changes in serum lipids levels is presented in Table 2. Chronic restraint stress triggers the mobilization of lipids as seen by the significant ($p < 0.05$) elevation of triglycerides and cholesterol relative to VEH unstressed group. This observed elevation was upturned by MH (5, 10, and 20 mg/kg) treatment significantly ($p < 0.05$). Furthermore, chronic restraint stress produced a significant ($p < 0.05$) decline in HDL cholesterol which was potentiated by pre-treatment with Morin hydrate. Serum atheriosclerotic index was noted to be significantly ($p < 0.05$) diminished on treatment with MH relative to VEH chronic restraint stress group (Table 2). This is attributed mainly to the suppression of total cholesterol and increase in HDL cholesterol concentrations.

Table 2: Effect of Morin hydrate on chronic restraint stress-induced changes in serum lipids

| Treatment (mg/kg) | Triglycerides (mg/dl) | Total cholesterol (mg/dl) | HDL cholesterol (mg/dl) | Atherosclerotic index |
|---|---|---|---|---|
| $VEH_{(UNS)}$ | 72.3 ± 2.4 | 55.3 ± 1.8 | 36.0 ± 2.3 | 0.55 ± 0.07 |
| $VEH_{(CRS)}$ | 107.0 ± 2.9# | 76.3 ± 3.5# | 25.0 ± 0.6# | 2.06 ± 0.20# |
| MH 5 mg/kg | 93.7 ± 2.9* | 66.3 ± 1.8* | 34.0 ± 1.2* | 0.96 ± 0.10* |
| MH 10 mg/kg | 89.7 ± 3.2* | 64.7 ± 1.5* | 34.3 ± 0.9* | 0.89 ± 0.10* |
| MH 20 mg/kg | 82.3 ± 3.8* | 58.0 ± 1.5* | 35.7 ± 1.2* | 0.63 ± 0.05* |
| GIN | 72.7 ± 2.3* | 56.0 ± 2.1* | 36.0 ± 1.0* | 0.56 ± 0.09* |

Each result represents the mean ± SEM, 6 animals / group.

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.05$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

$VEH_{(UNS)}$: Vehicle unstressed group

$VEH_{(CRS)}$: Vehicle Chronic Restraint Stress group

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

## 4.13. EFFECT OF MORIN HYDRATE ON CHRONIC RESTRAINT STRESS-INDUCED CHANGES IN OXIDATIVE STRESS BIOMARKERS IN MICE

Chronic restraint stress reduced brain GSH concentration significantly ($p < 0.001$) in comparison to VEH unstressed group. Upon MH (5, 10, 20 mg/kg) treatment, a significant ($p < 0.001$) rise in GSH concentration was noted (Figure 27). Concentration of MDA in brain tissues was found to be significantly ($p < 0.001$) elevated by chronic restraint stress compared with VEH unstressed group, and upon MH (5, 10, 20 mg/kg) treatment, lipid peroxidation was significantly ($p < 0.001$) attenuated (Figure 28). Furthermore, chronic restraint stress significantly ($p < 0.001$) raises brain nitrite levels in the VEH chronic restraint stress group relative to VEH unstressed group as shown in Figure 29. Treatment with MH (5, 10, 20 mg/kg) was also found to reverse this effect significantly ($p < 0.001$).

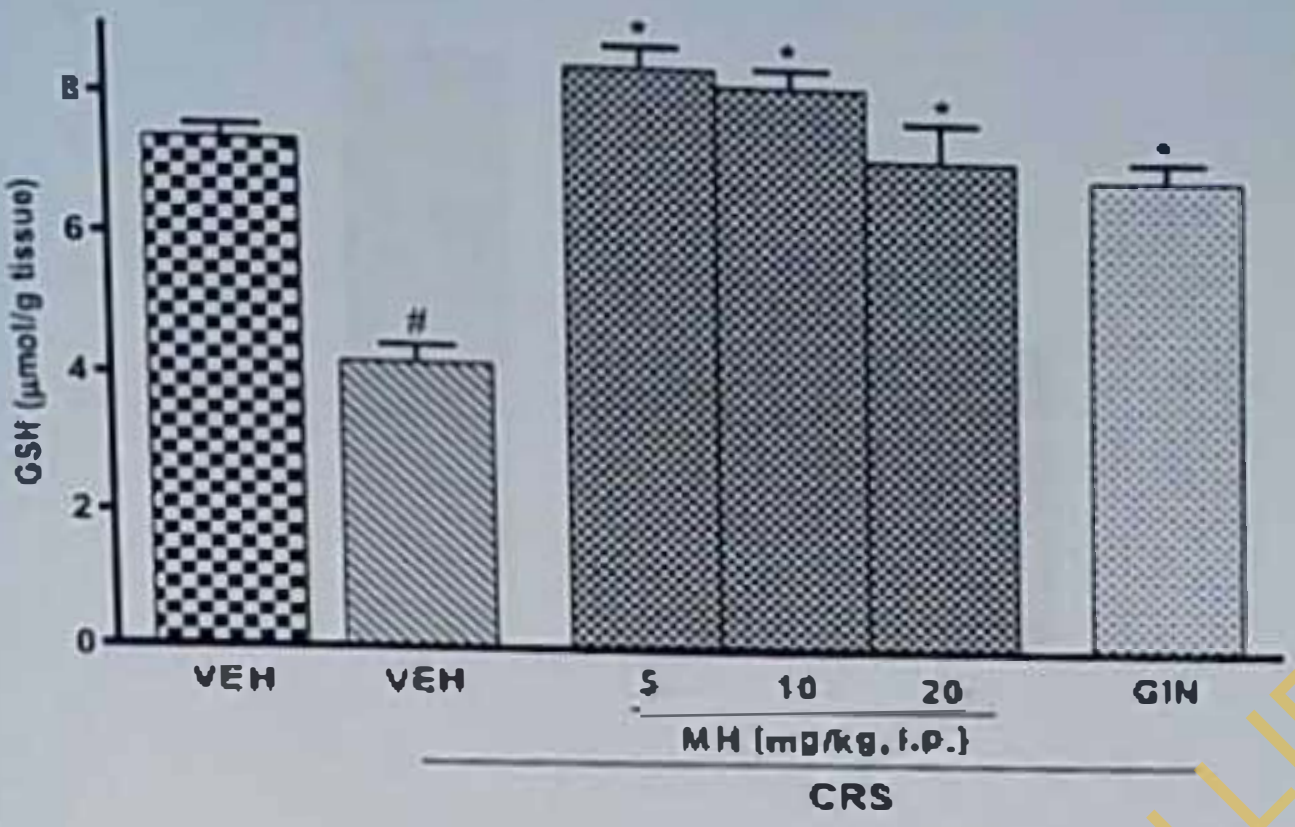



Figure 27: Effect of Morin hydrate on brain GSH levels in mice following chronic restraint stress exposure.

Each column indicates mean ± SEM. 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test)

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

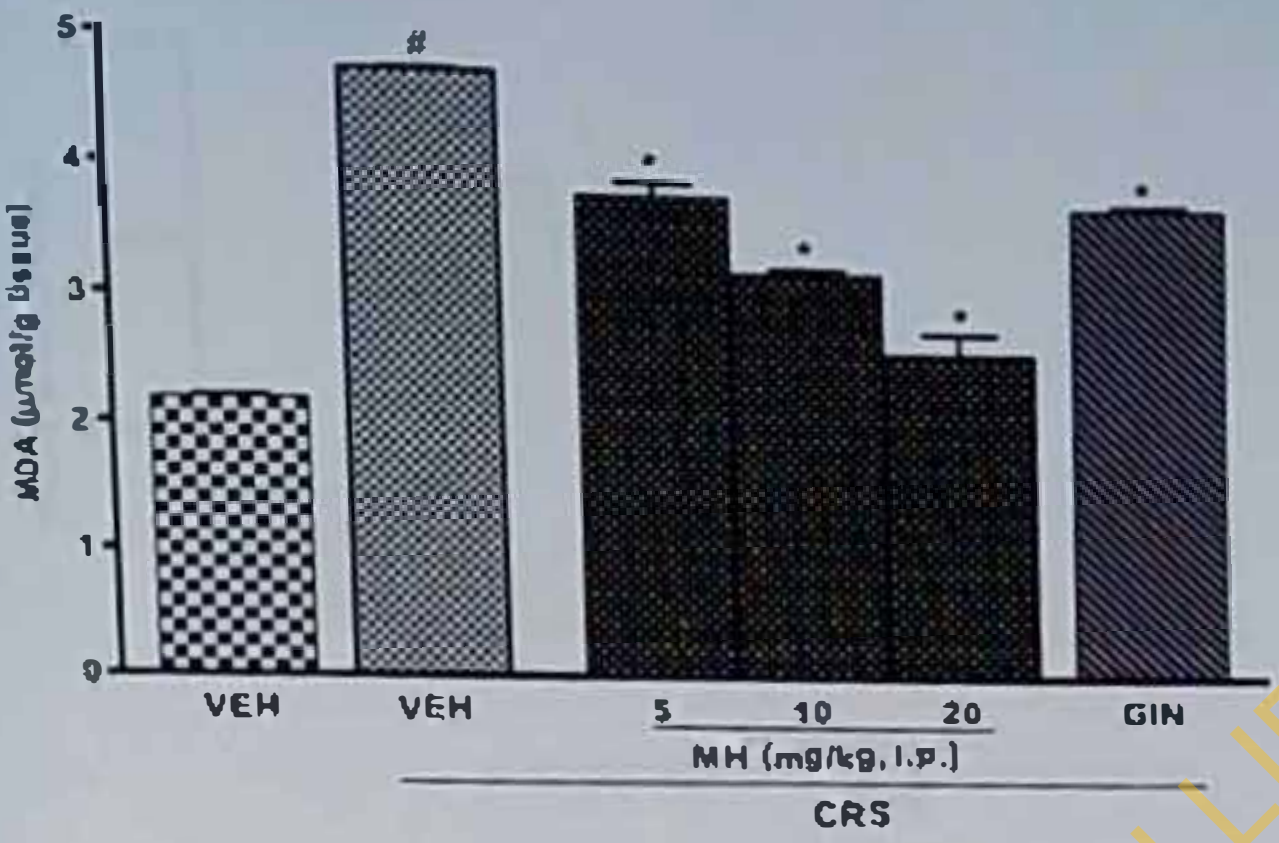





**Figure 28: Effect of Morin hydrate on brain MDA levels in mice following chronic restraint stress exposure.**

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

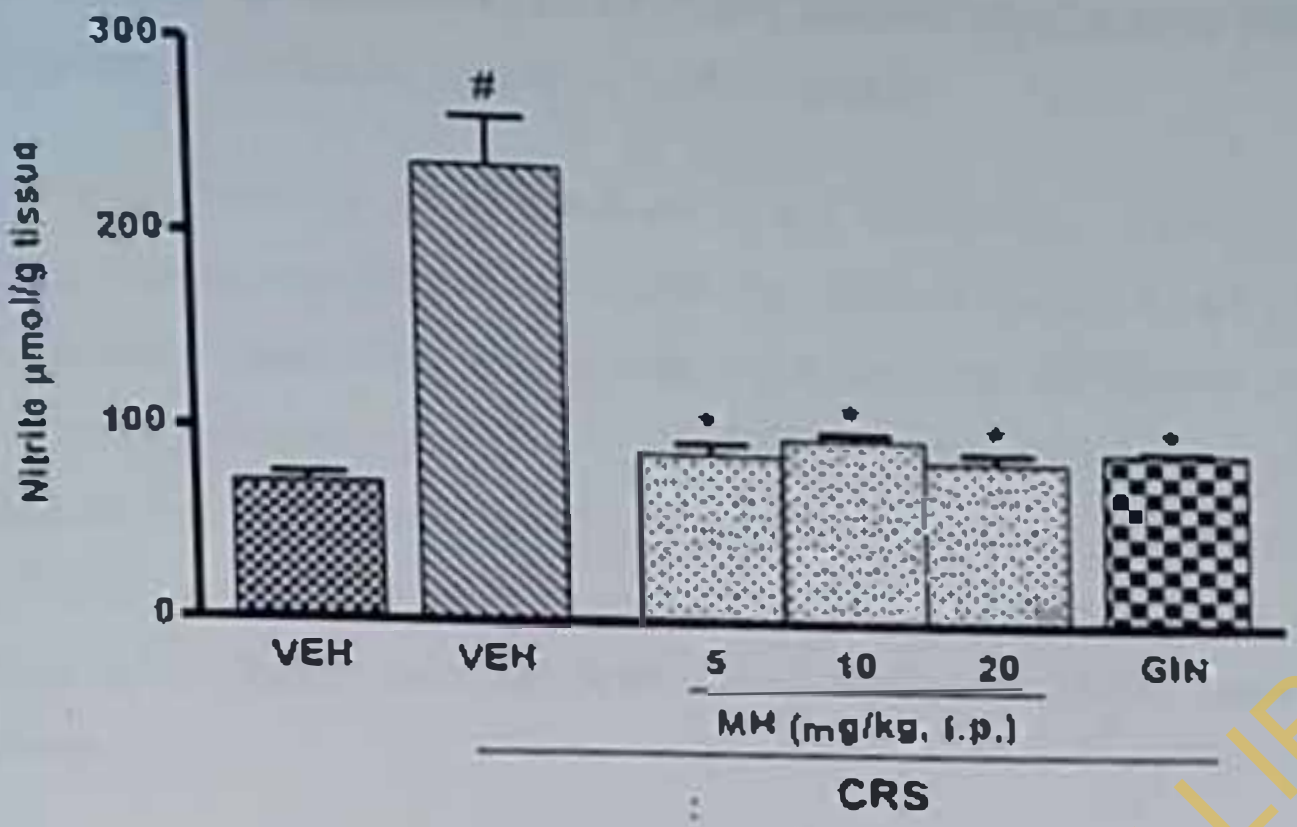



**Figure 29: Effect of Morin hydrate on brain nitrite levels in mice following chronic restraint stress exposure.**

Each column indicates mean ± SEM, 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

## 4.14. EFFECT OF MORIN HYDRATE ON CHRONIC RESTRAINT STRESS-INDUCED NEURONAL DEGENERATION IN MOUSE BRAIN

Histological studies of the prefrontal cortex and hippocampal neurons of mice exposed to chronic restraint stress revealed remarkable morphological changes in each group (Plates 1 and 2). Chronic restraint stress induced some abnormalities and significantly ($p < 0.001$) reduced total viable prefrontal cortex neurons and the hippocampal CA3 neurons relative to the VEH unstressed group (Figures 30 and 31). However, the number of viable neurons was significantly ($p < 0.001$) increased by MH (5, 10, and 20 mg/kg, i.p.) treatment. These results suggest that morin hydrate demonstrated significant protective effects on chronic stress-induced neuronal damage.

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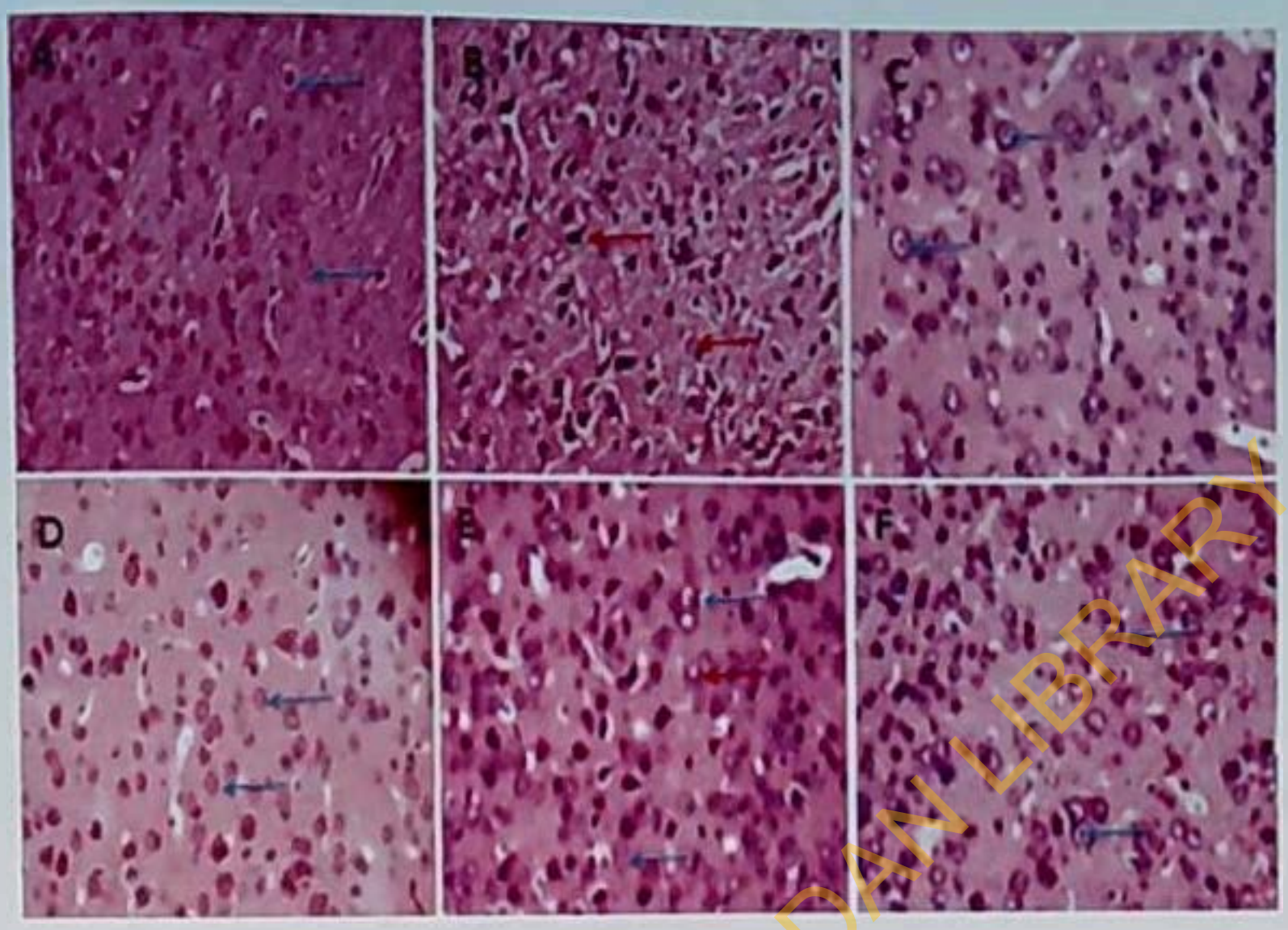


Plate 1: Effect of Morin hydrate on morphology of prefrontal cortex neurons of mice exposed to chronic restraint stress

(A) Vehicle only (normal prefrontal cortex with normal neurons)

(B) Vehicle + CRS (significant loss of neurons and mild necrosis)

(C) MH 5 mg/kg + CRS (normal prefrontal cortex, clean stroma, significant increase in viable neurons)

(D) MH 10 mg/kg + CRS (normal cortex, significant increase in viable neurons)

(E) MH 20 mg/kg + CRS (normal cortex, with viable but few damaged neurons)

(F) GIN + CRS (normal cortex, significant increase in viable neurons)

Blue arrows indicate normal neurons while red arrows indicate indicates damaged neurons. (Original magnification X400).

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

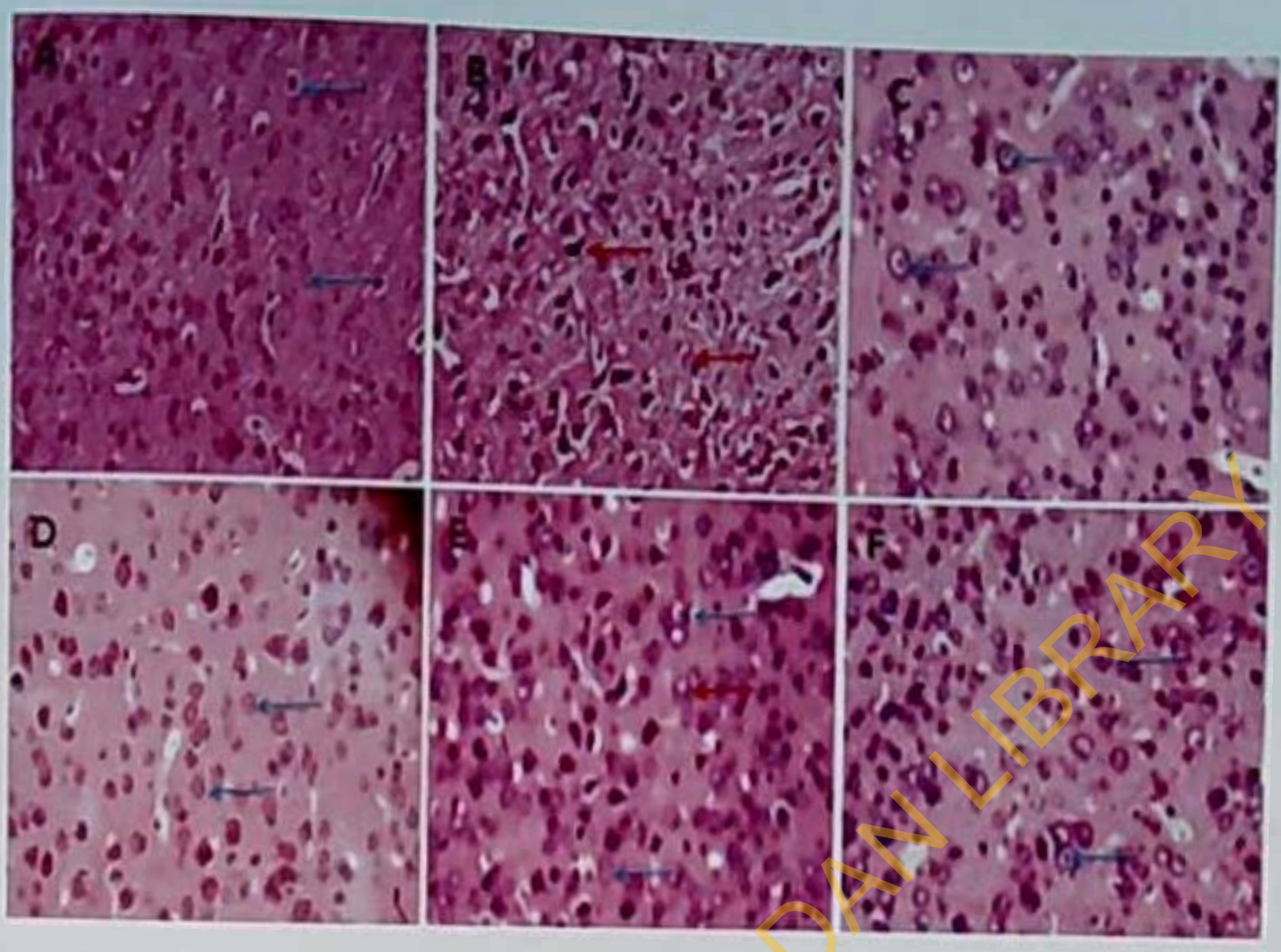


**Plate 1: Effect of Morin hydrate on morphology of prefrontal cortex neurons of mice exposed to chronic restraint stress**

(A) Vehicle only (normal prefrontal cortex with normal neurons)

(B) Vehicle + CRS (significant loss of neurons and mild necrosis)

(C) MH 5 mg/kg + CRS (normal prefrontal cortex, clean stroma, significant increase in viable neurons)

(D) MH 10 mg/kg + CRS (normal cortex, significant increase in viable neurons)

(E) MH 20 mg/kg + CRS (normal cortex, with viable but few damaged neurons)

(F) GIN + CRS (normal cortex, significant increase in viable neurons)

Blue arrows indicate normal neurons while red arrows indicate indicates damaged neurons. (Original magnification X400).

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

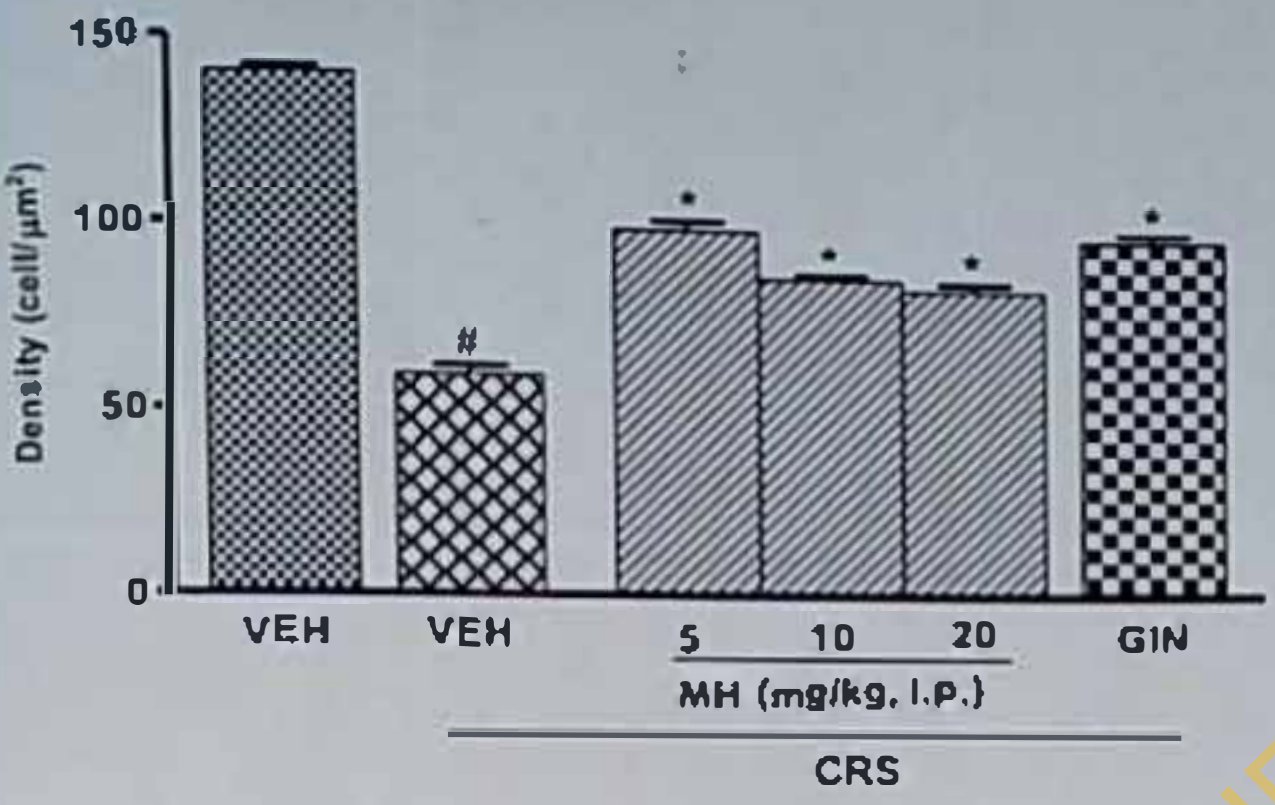


**Figure 30: Effect of Morin hydrate on viable prefrontal cortex neurons following chronic restraint stress exposure.**

Each column indicates mean ± SEM, 3 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CRS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

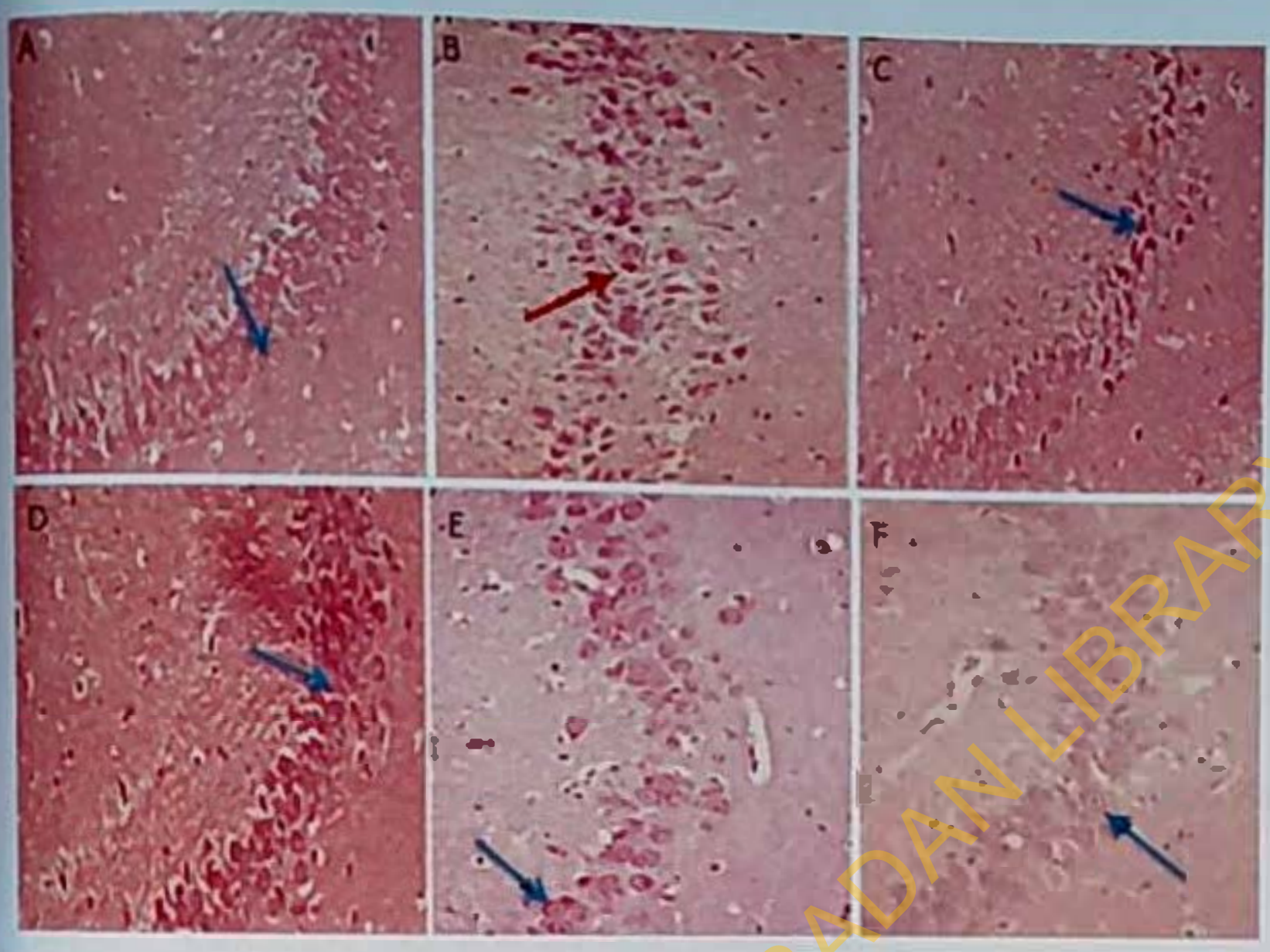




**Plate 2: Impact of Morin hydrate on morphology CA3 hippocampal cells of mice exposed to chronic restraint stress**

(A) Vehicle only (normal layer with intact neurons)

(B) Vehicle + CRS (irregular layer and lots of damaged neurons)

(C) MH 5 mg/kg + CRS (normal layer and significant increase in viable neurons)

(D) MH 10 mg/kg + CRS (regular layer with significant increase in viable neurons)

(E) MH 20 mg/kg + CRS (normal layer with intact neurons)

(F) GIN + CRS (regular layer with significant increase in viable neurons)

Blue arrows indicate normal neurons while red arrows indicate indicates damaged neurons. (Original magnification X400).

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

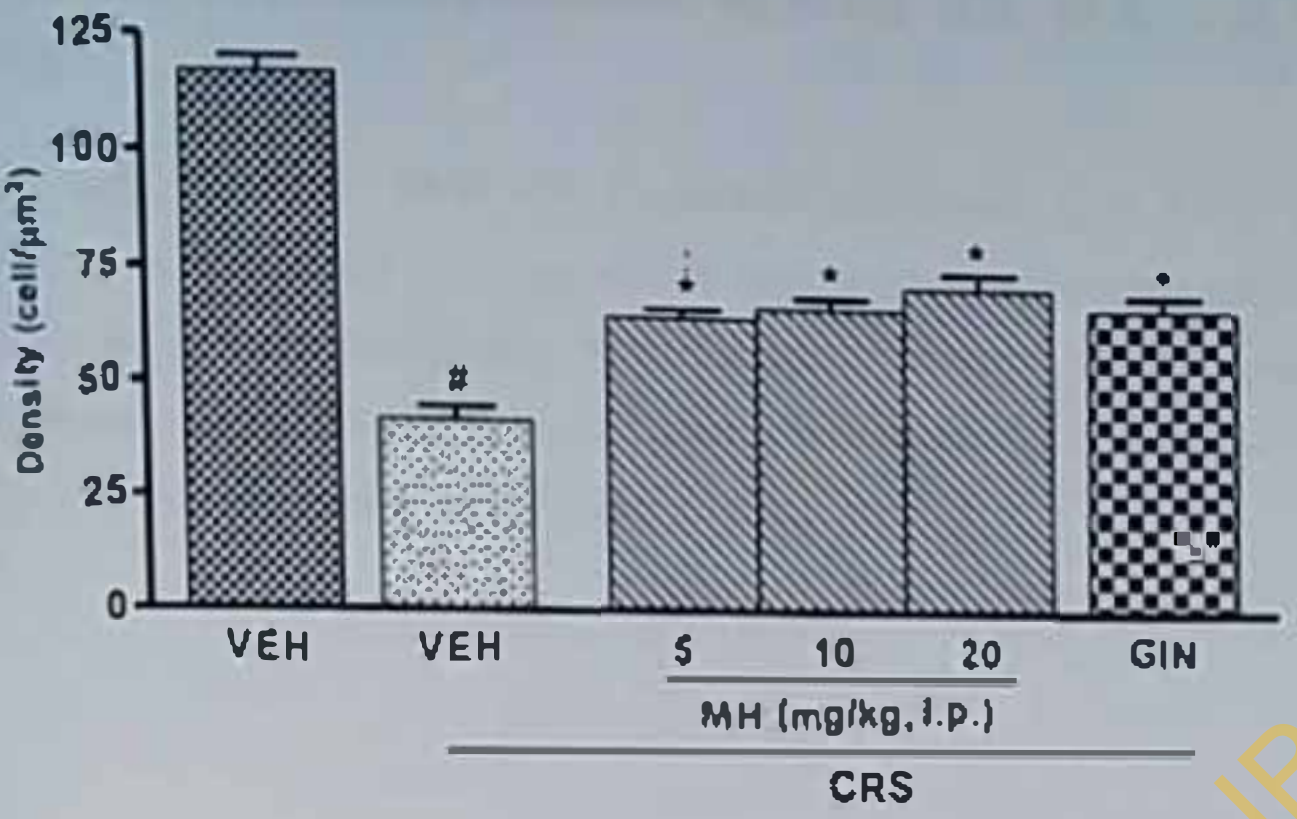


Figure 31: Effect of Morin hydrate on CA3 hippocampal neurons after exposure to chronic restraint stress.

Each column indicates mean ± SEM, 3 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to chronic restraint stress group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

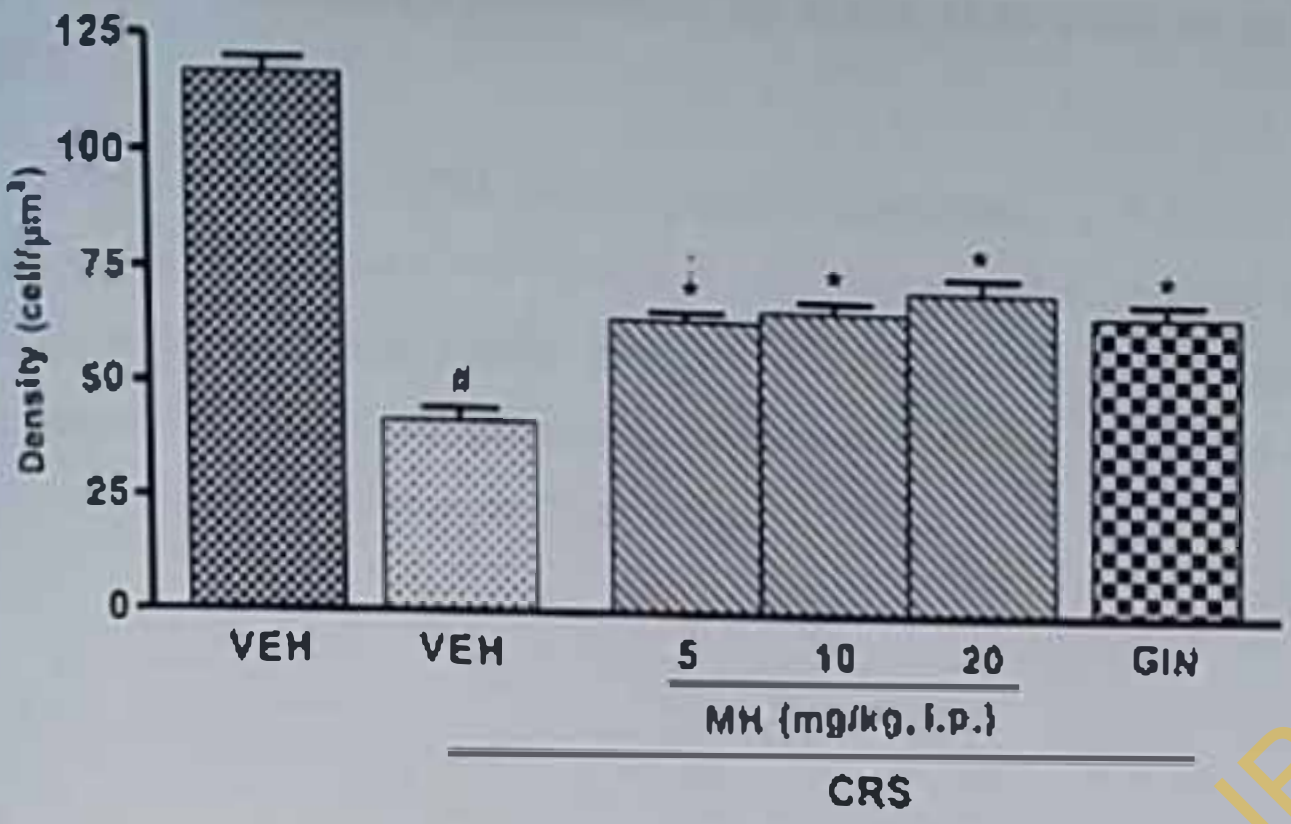


**Figure 31: Effect of Morin hydrate on CA3 hippocampal neurons after exposure to chronic restraint stress.**

Each column indicates mean ± SEM, 3 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to chronic restraint stress group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CRS: Chronic Restraint Stress

## 4.15. MORIN HYDRATE ENHANCES MEMORY FUNCTION IN SLEEP DEPRIVED MICE

Forty eight (48) hours REM sleep deprivation significantly ($p < 0.001$) impaired memory performance as indicated by the decrease in percentage alternation observed in VEH PSD group relative to VEH unstressed group. However, MH treatment (5 and 10 mg/kg, i.p.) induced a significant ($p < 0.05$) improvement in percentage alternation relative to VEH PSD group, indicating memory enhancement (Figure 32).

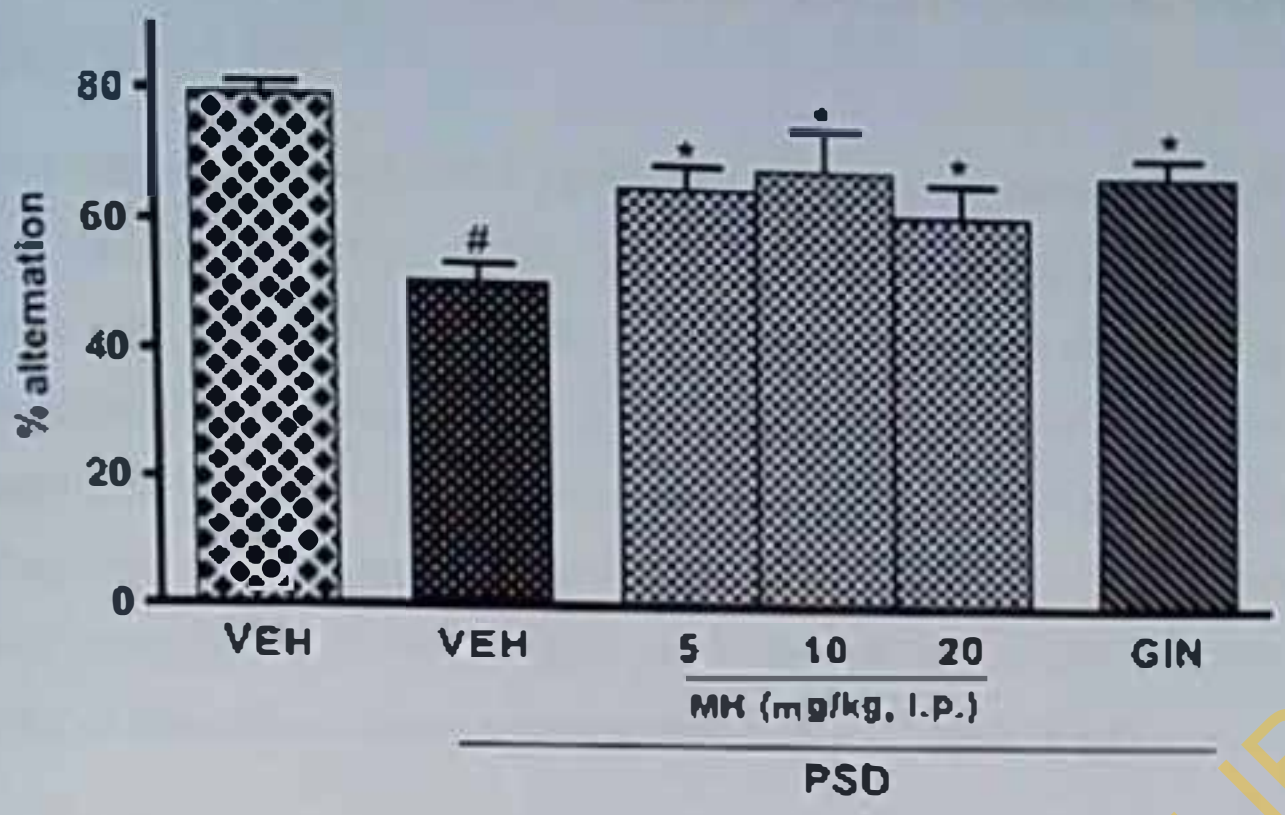


Figure 32: Effects of Morin hydrate on memory performance following PSD

Each column indicates mean ± SEM, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.05$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

## 4.16. MORIN HYDRATE MITIGATES ANXIETY-LIKE BEHAVIOUR IN MICE FOLLOWING PSD

REM sleep deprivation for 48 h significantly reduced the duration ($p < 0.001$) and frequency of open arms exploration ($p < 0.05$), and consequently enhanced the duration and frequency of closed arms exploration in the EPM in a significant manner as shown in Figures 33, 34, 35 and 36. However, a significant ($p < 0.01$) elongation of duration and frequency of open arms exploration was noted upon treatment with MH (5, 10, 20 mg/kg, i.p.) compared to VEH PSD group. This demonstrates the protective effect of MH against anxiety-like behaviours after sleep deprivation. MH (5, 10, 20 mg/kg, i.p.) also significantly ($p < 0.001$) reversed the increase in the index of open arm avoidance compared with VEH PSD group (Figure 37).

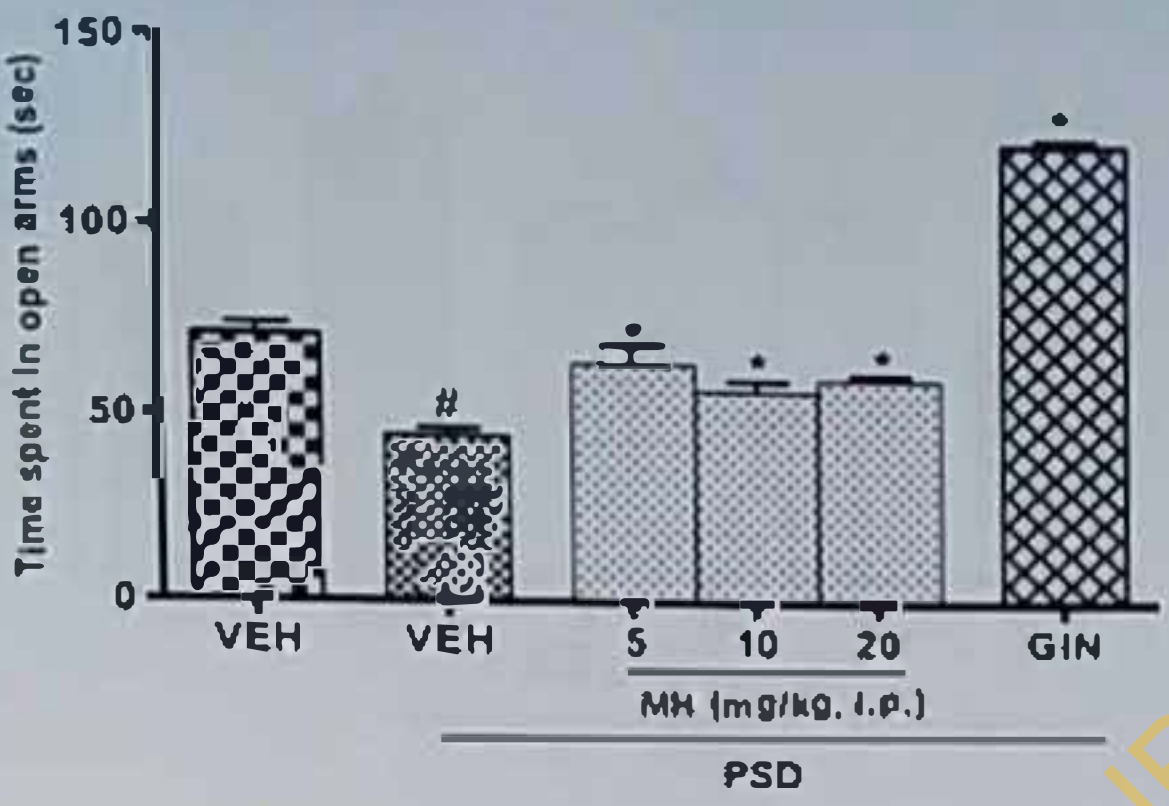


**Figure 33: Effect of Morin hydrate on time spent in the open arms of the EPM following PSD.**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.01$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

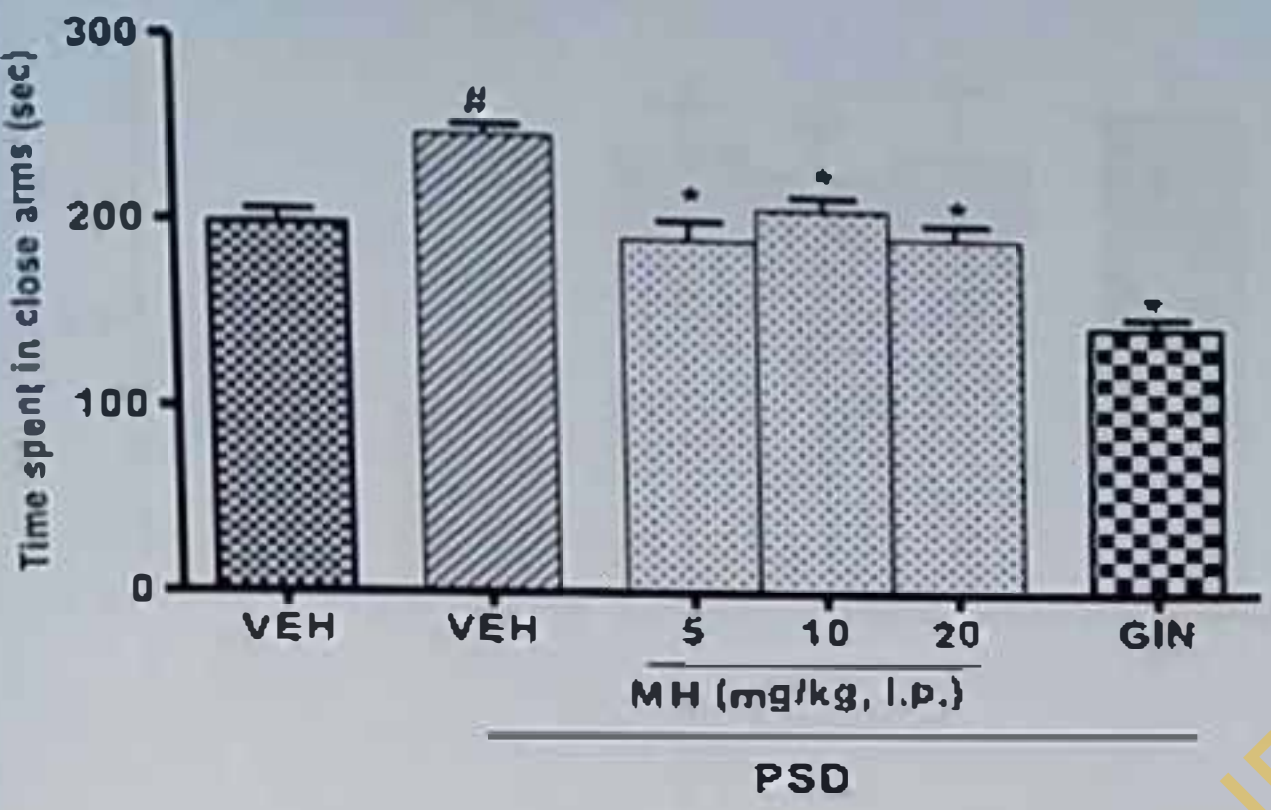


Figure 34: Effect of Morin hydrate on time spent in the close arms of the EPM following PSD.

Each column indicates mean ± S.E.M. 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

• $p < 0.01$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

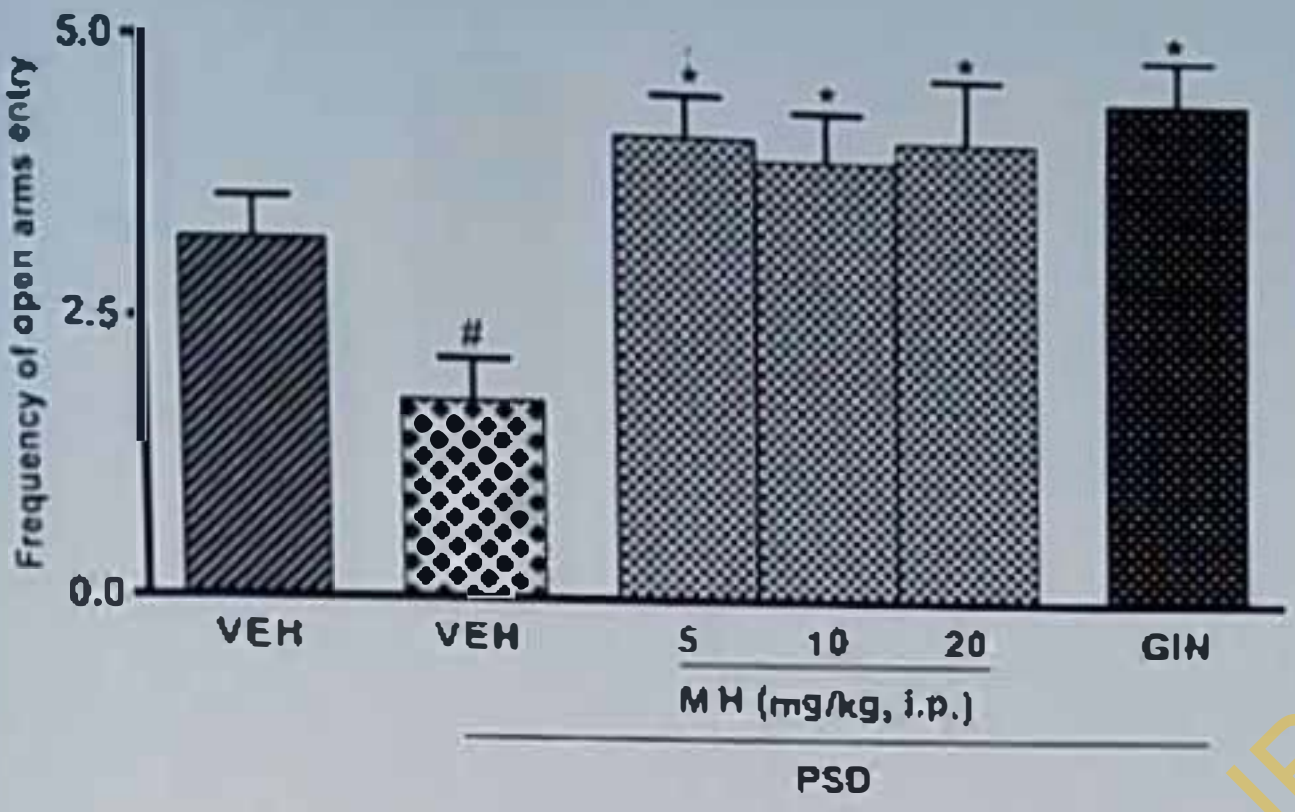


**Figure 35: Effect of Morin hydrate on the frequency of open arms exploration in the EPM following PSD.**

Values represent the means ± SEM, 6 animals / group.

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.01$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

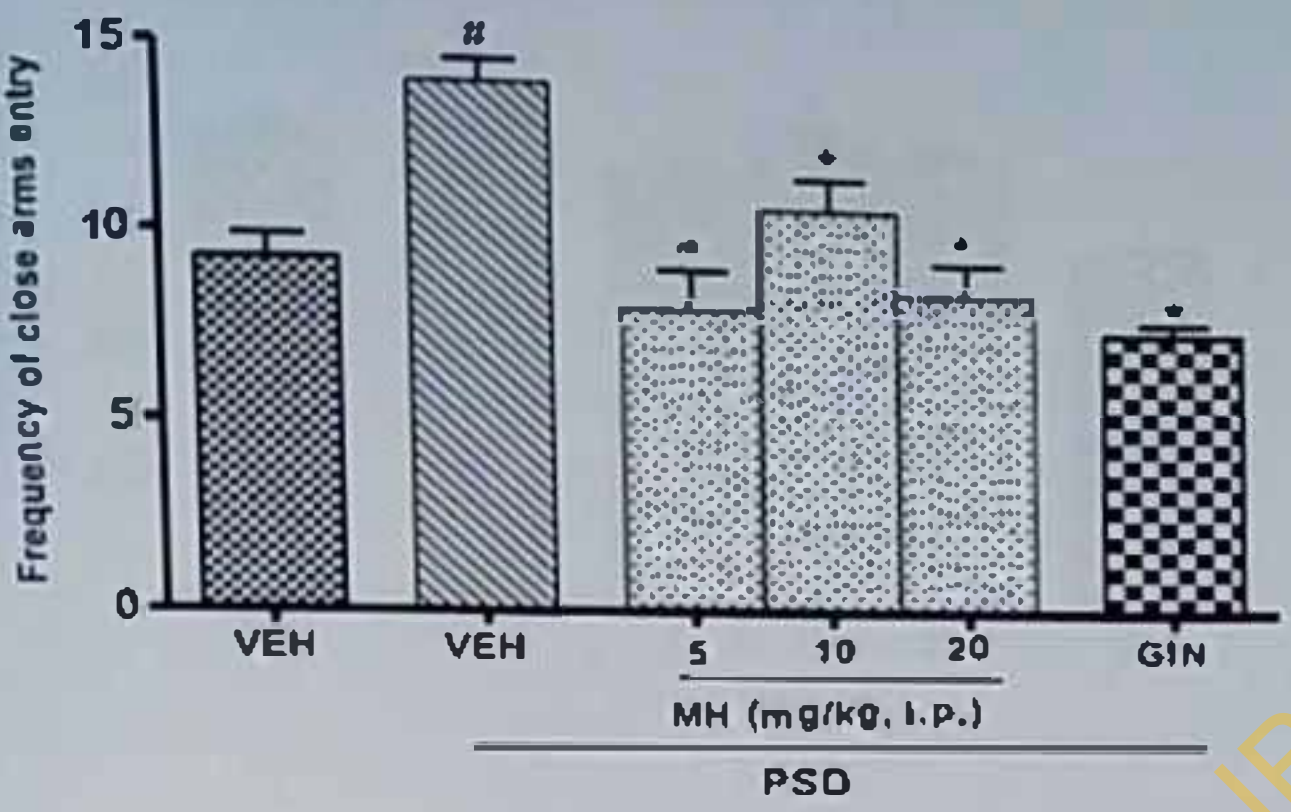


Figure 36: Effect of Morin hydrate on the frequency of close arms exploration in the EPM following PSD.

Values represent the means ± SEM. 6 animals / group.

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.01$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

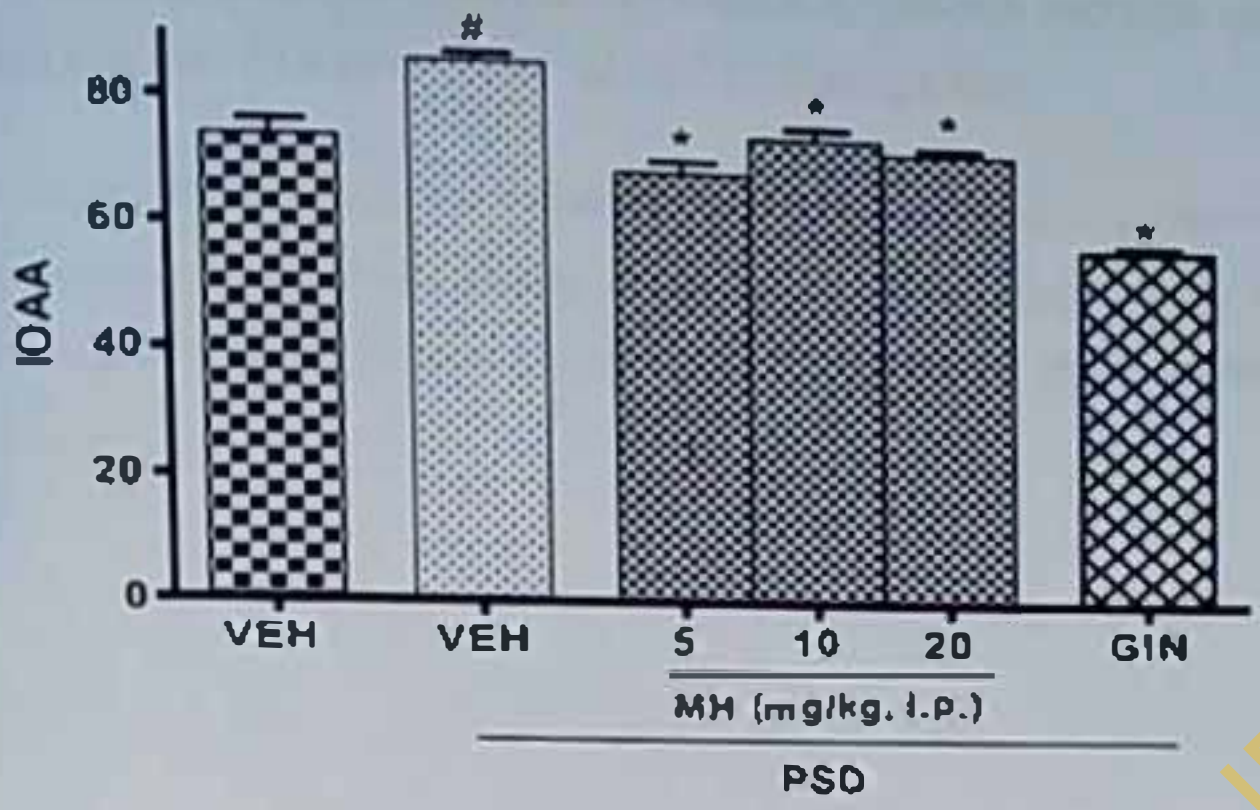


Figure 37: Effect of Morin hydrate on the index of open arms avoidance in the EPM following PSD.

Values represent the means ± SEM, 6 animals / group.

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg i.p.)

PSD: Paradoxical Sleep Deprivation

## 4.17. MORIN HYDRATE MITIGATES SPONTANEOUS MOTOR ACTIVITY IN MICE FOLLOWING PARADOXICAL SLEEP DEPRIVATION

The impact of 48 h sleep deprivation on motor activity of mice is as shown in Figure 38. Sleep deprivation enhanced motor activity significantly ($p < 0.01$) relative to VEH unstressed group. However, MH (5, 10, 20 mg/kg, i.p.) treatment upturned the observed effect significantly ($p < 0.01$).

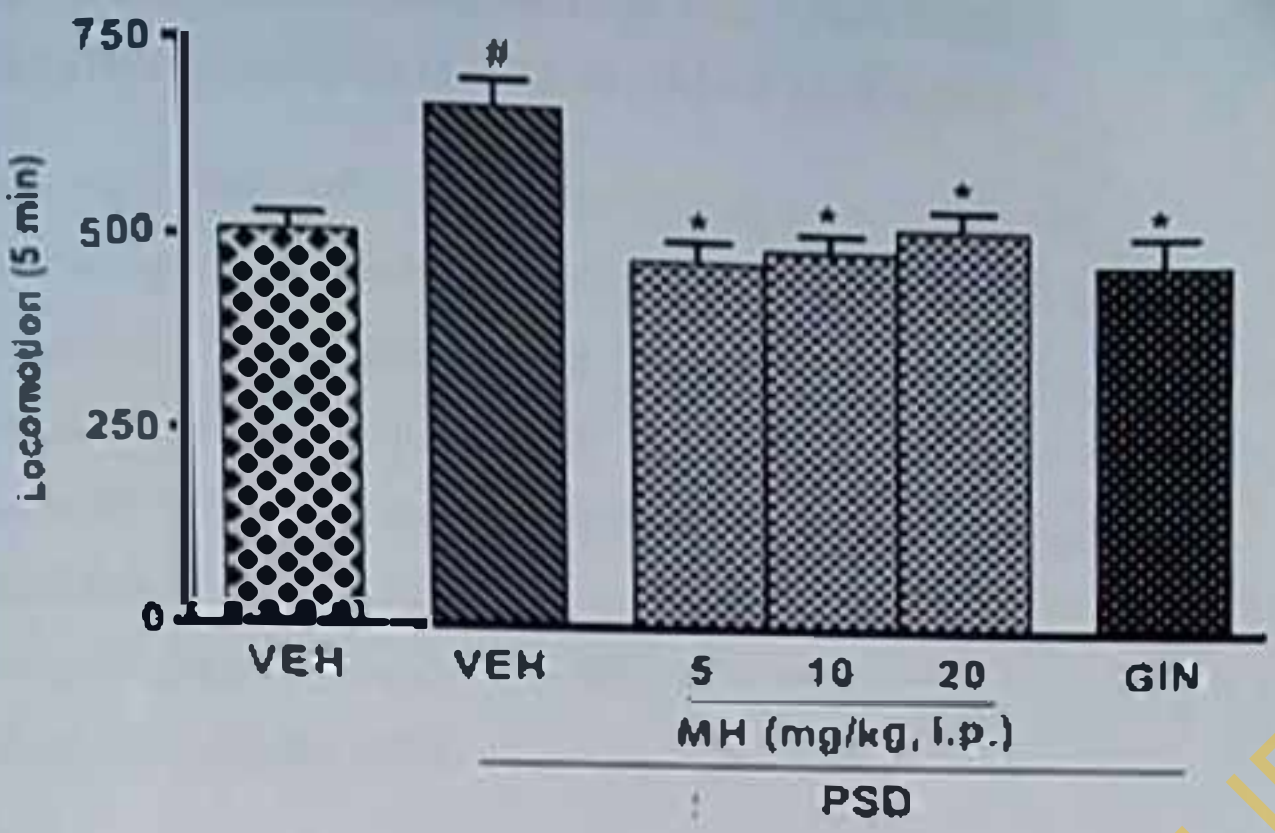


**Figure 38: Effect of Morin hydrate on spontaneous motor activity following PSD.**

Each column indicates mean ± S.E.M. 6 animals / group

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.01$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test.

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

## 4.18. MORIN HYDRATE MITIGATES OXIDATIVE STRESS IN MICE BRAINS FOLLOWING PARADOXICAL SLEEP DEPRIVATION

Forty eight hours sleep deprivation reduced brain GSH levels significantly relative to VEH unstressed group. Treatment with MH (5, 10, 20 mg/kg, i.p.) however, overturned this reduction in brain GSH levels significantly ($p < 0.001$) (Figure 39). Brain MDA levels, an index of lipid peroxidation increased significantly ($p < 0.001$) in the VEH PSD group relative to VEH unstressed group and was significantly reversed by MH (Figure 40). Furthermore, PSD significantly ($p < 0.001$) raised nitrite concentration in the vehicle PSD group relative to VEH unstressed group (Figure 41). The MH (5, 10, and 20 mg/kg, i.p.) treatment also significantly ($p < 0.001$) reversed this effect..

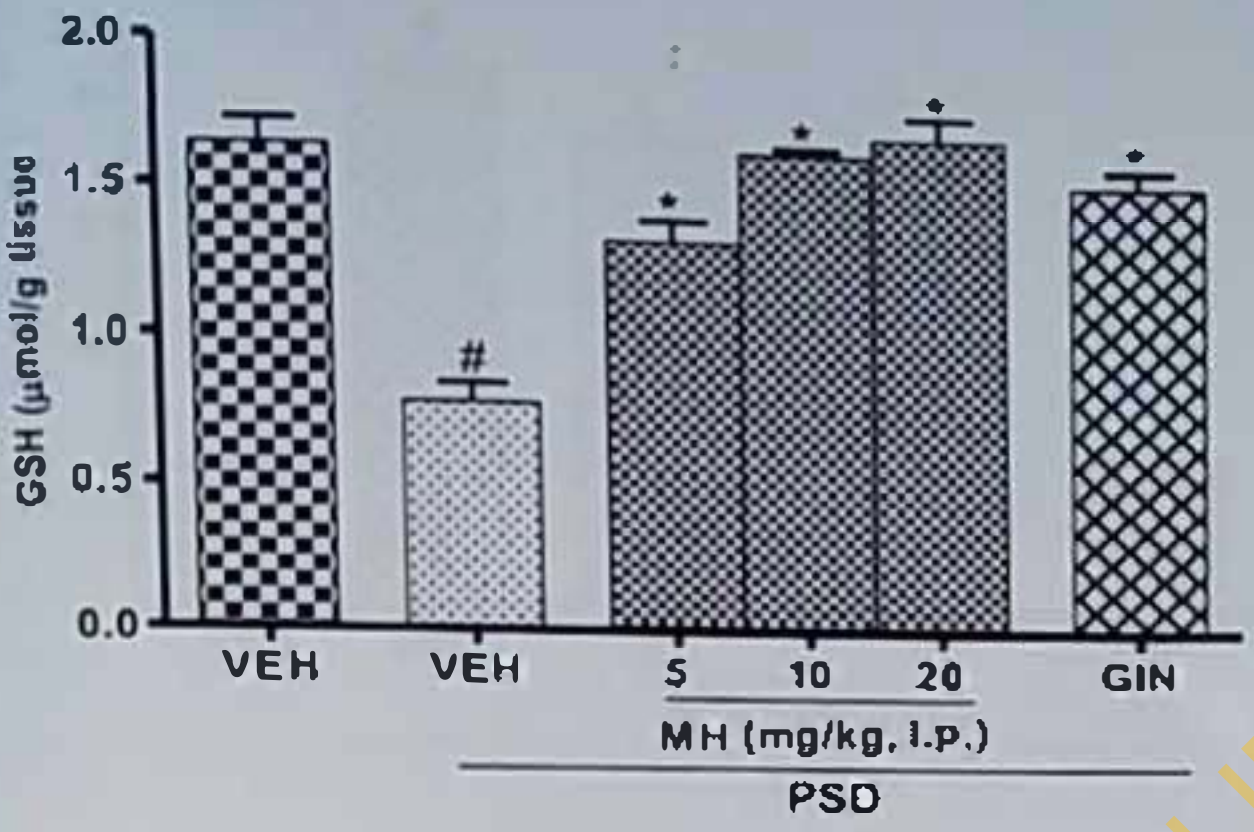


**Figure 39: Effect of Morin hydrate on brain GSH levels following PSD.**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

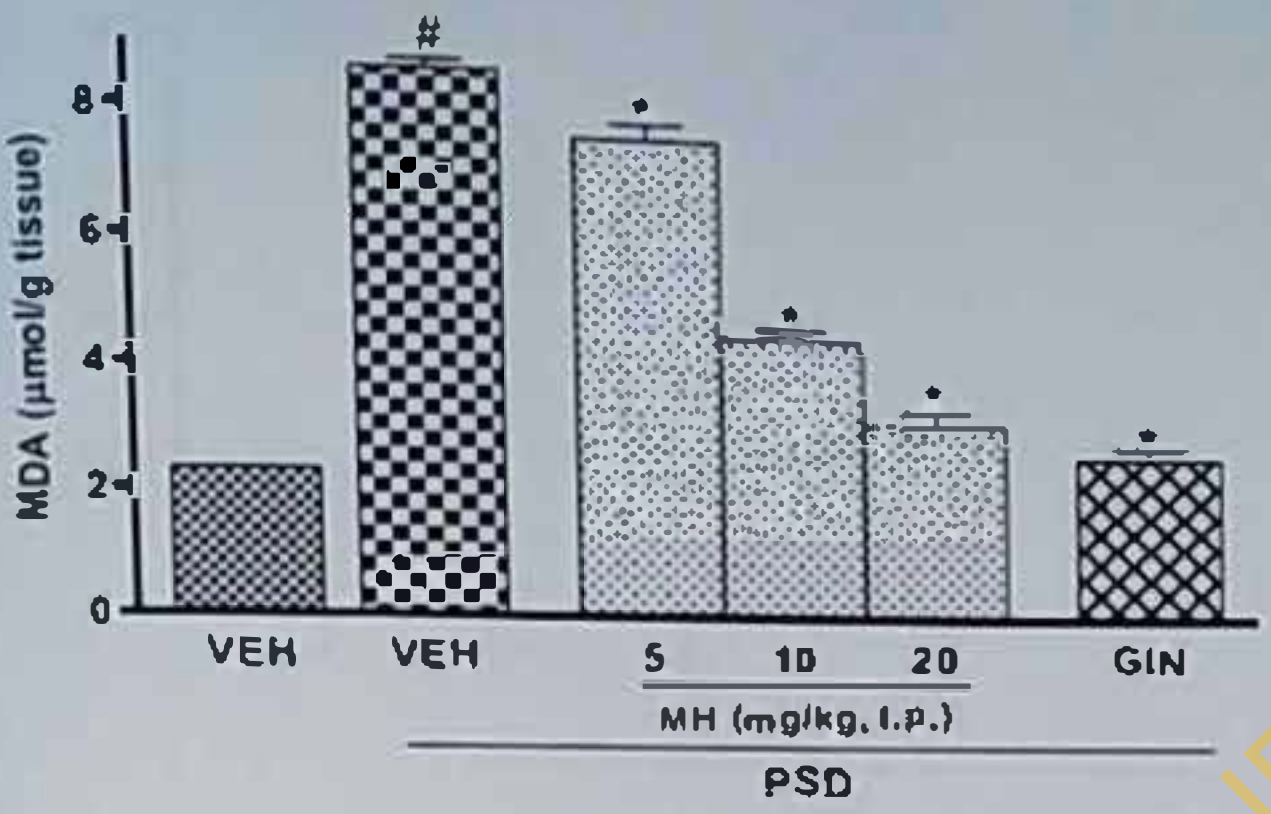


**Figure 40: Effect of Morin hydrate on brain MDA levels following PSD**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

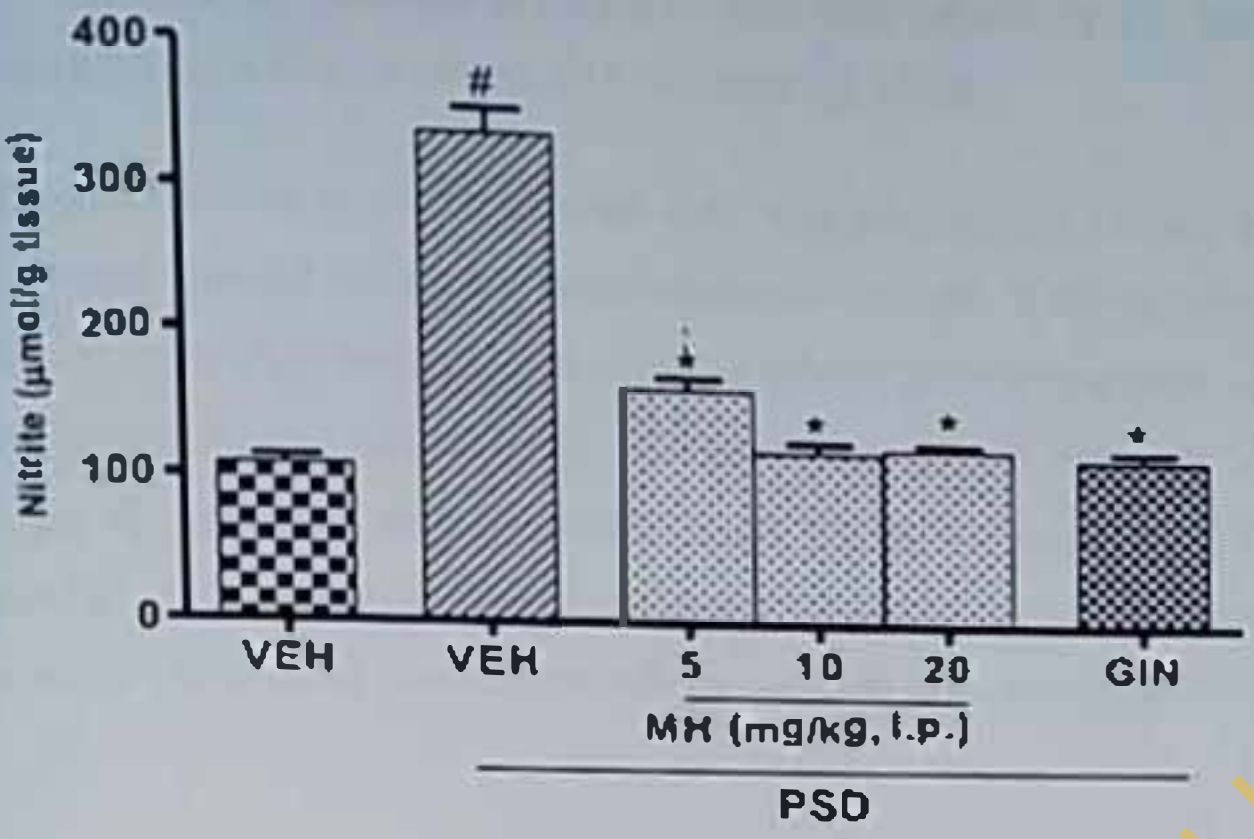


**Figure 41: Effect of Morin hydrate on brain nitrite concentration following PSD**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.01$ relative to VEH PSD group (One-way ANOVA followed by Student-Newmnn-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

## 4.19. EFFECT OF MORIN HYDRATE ON THE DENSITY OF HIPPOCAMPAL CA1 PYRAMIDAL NEURON IN SLEEP DEPRIVED MICE

Histological studies of the hippocampal CA1 pyramidal neurons of mice exposed to 48 h sleep deprivation revealed remarkable neuropathological changes in the representative in each group (Plate 3). Forty eight hours sleep deprivation induced some irregularities in the pyramidal layer and significantly ($p < 0.05$) reduced viable neurons population relative to VEH unstressed group (Figure 42). However, the population of viable neurons was significantly ($p < 0.001$) increased upon MH (5, 10, and 20 mg/kg, i.p.) treatment. These results further buttress the suggestion that the anti-stress action of Morin hydrate might be linked to its neuroprotective property.

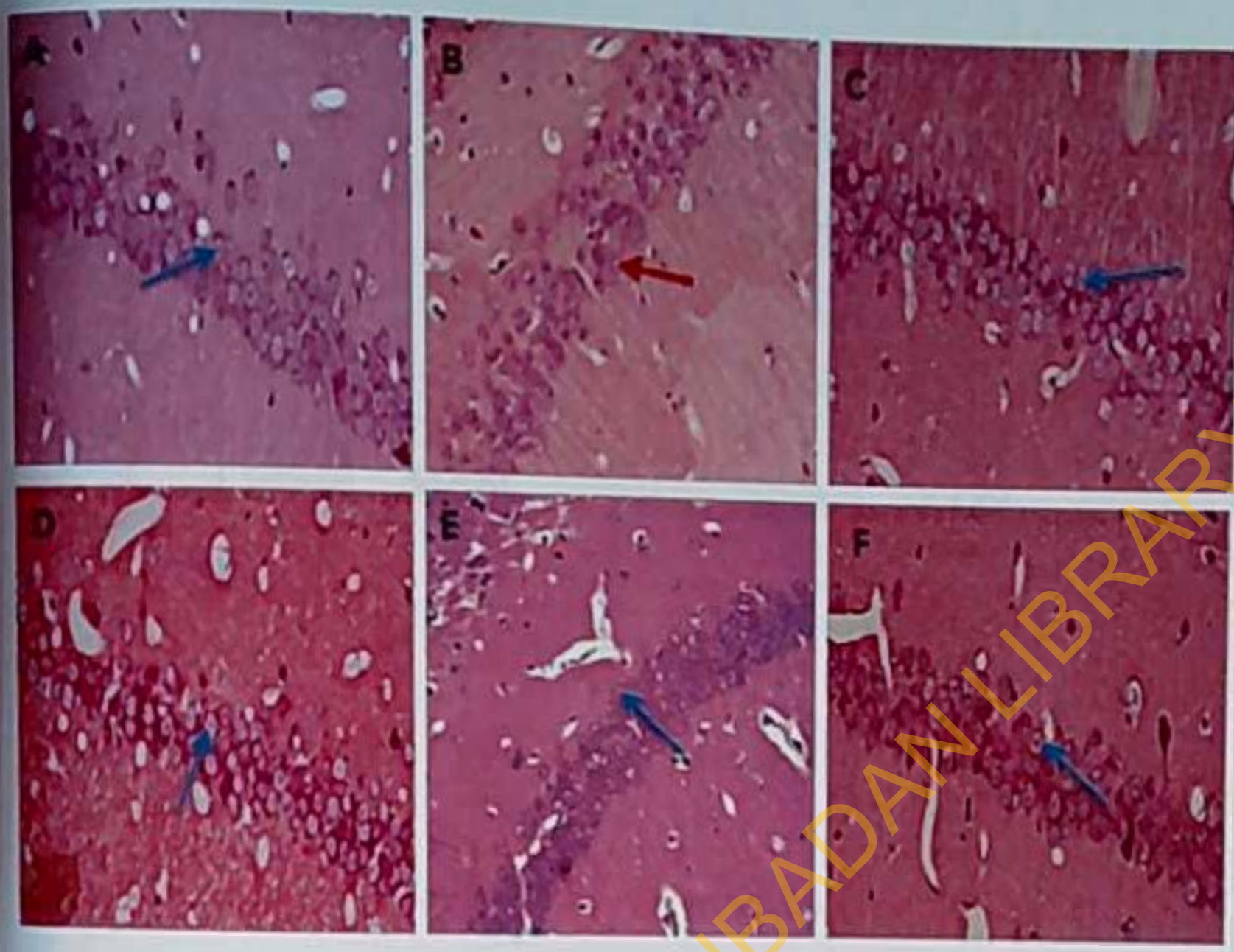


**Plate 3: Effect of Morin hydrate on morphology of neurons in the CA1 pyramidal layer of mice hippocampus following PSD**

(A) Vehicle only (normal layer with intact neurons devoid of any lesion)

(B) Vehicle + PSD (irregular layer and severe necrosis of neurons)

(C) MH 5 mg/kg + PSD (normal layer with very mild diffuse gliosis)

(D) 10 mg/kg + PSD (normal layer with significant increase in viable neurons)

(E) 20 mg/kg + PSD (normal layer with significant increase in viable neurons)

(F) GIN + PSD (normal layer with significant increase in viable neurons)

Blue arrows indicate normal neurons while red arrows indicate indicates damaged neurons. (Original magnification X400).

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

PSD: Paradoxical Sleep Deprivation

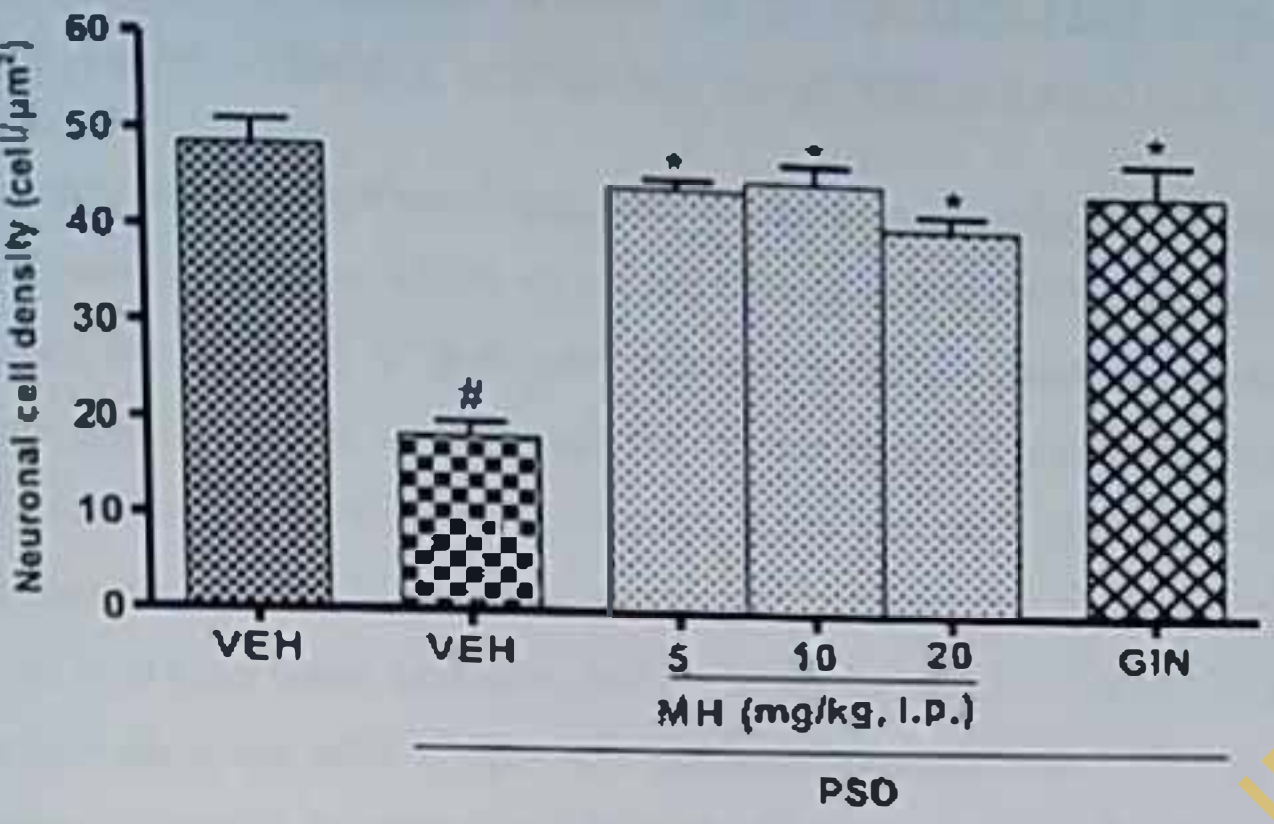


**Figure 42: Effect of Morin hydrate on density of viable CA1 pyramidal neurons of mice following PSD.**

Each column indicates mean ± S.E.M, 3 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH PSD group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i-p.)

PSD: Paradoxical Sleep Deprivation

## 4.20. EFFECT OF MORIN HYDRATE ON ANXIETY-LIKE BEHAVIOR IN MICE FOLLOWING CHRONIC UNPREDICTABLE STRESS EXPOSURE

The effect of Morin hydrate on frequency and duration of exploration in both arms of the EPM are as shown in Figures 43, 44, 45 and 46. Chronic unpredictable stress significantly ($p < 0.05$) reduced the frequency of open arms exploration and as a result increased exploration in the closed arms. The CUS also enhanced the duration of closed arms exploration significantly ($p < 0.05$) relative to the VEH unstressed group. Morin hydrate (5, 10, and 20 mg/kg, i.p.) increased the frequency ($p < 0.05$) and duration ($p < 0.001$) of open arms exploration significantly relative to the VEH CUS group. Moreover, MH (5, 10, 20 mg/kg, i.p.) significantly ($p < 0.001$) reversed the increase in the index of open arm avoidance compared with VEH PSD group (Figure 47), further buttressing the protective effect of MH against anxiety-like behaviours after stressful exposure.

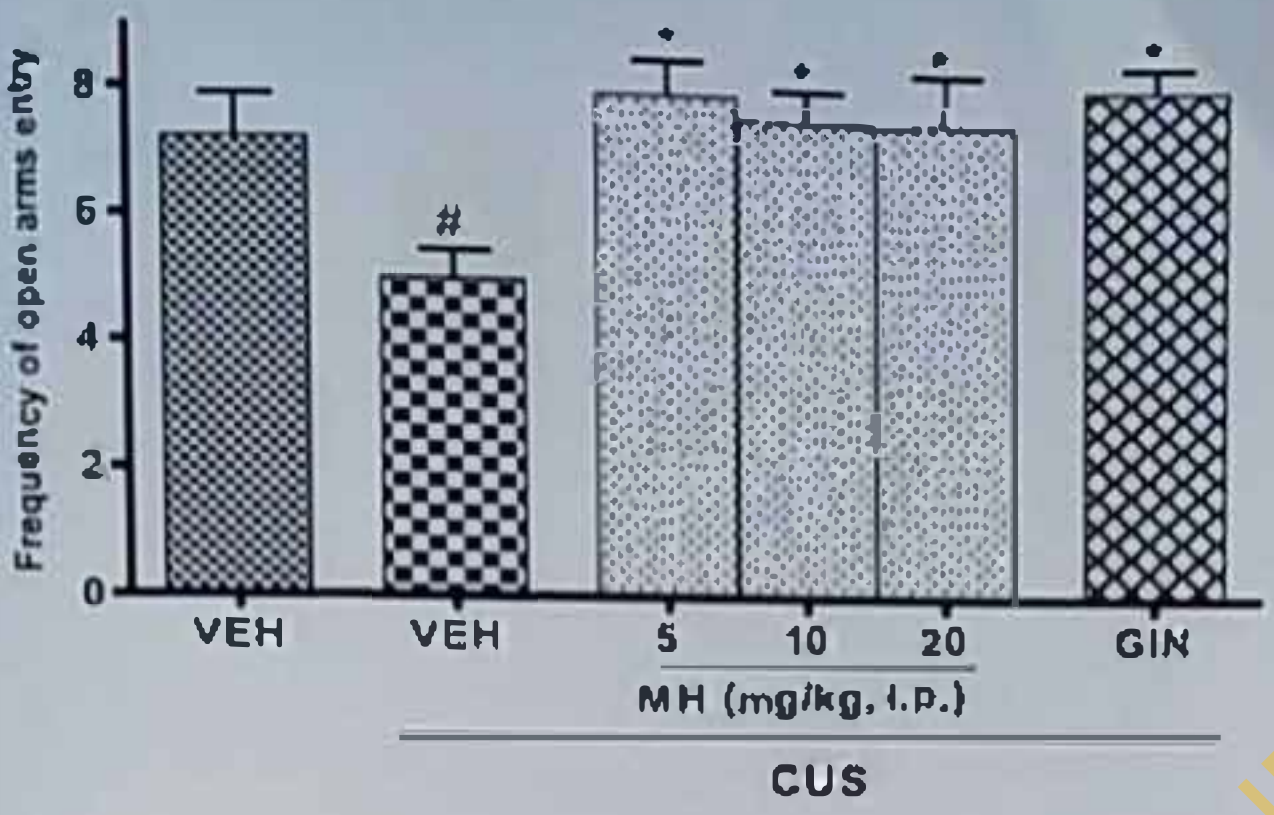


**Figure 43: Effect of Morin hydrate on the frequency of open arms exploration in the EPM following CUS exposure.**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.05$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

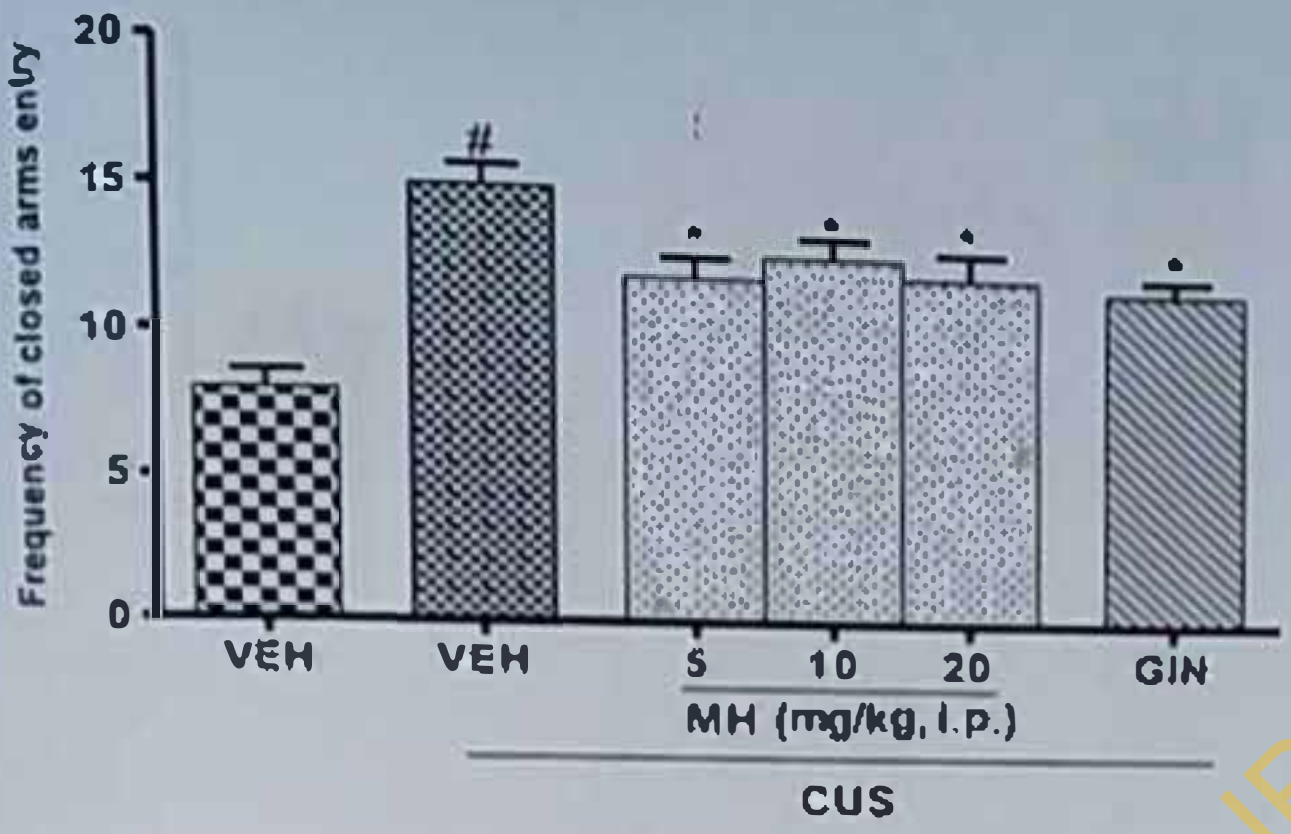


**Figure 4.4: Effect of Morin hydrate on the frequency of close arms exploration in the EPM following CUS exposure.**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.05$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

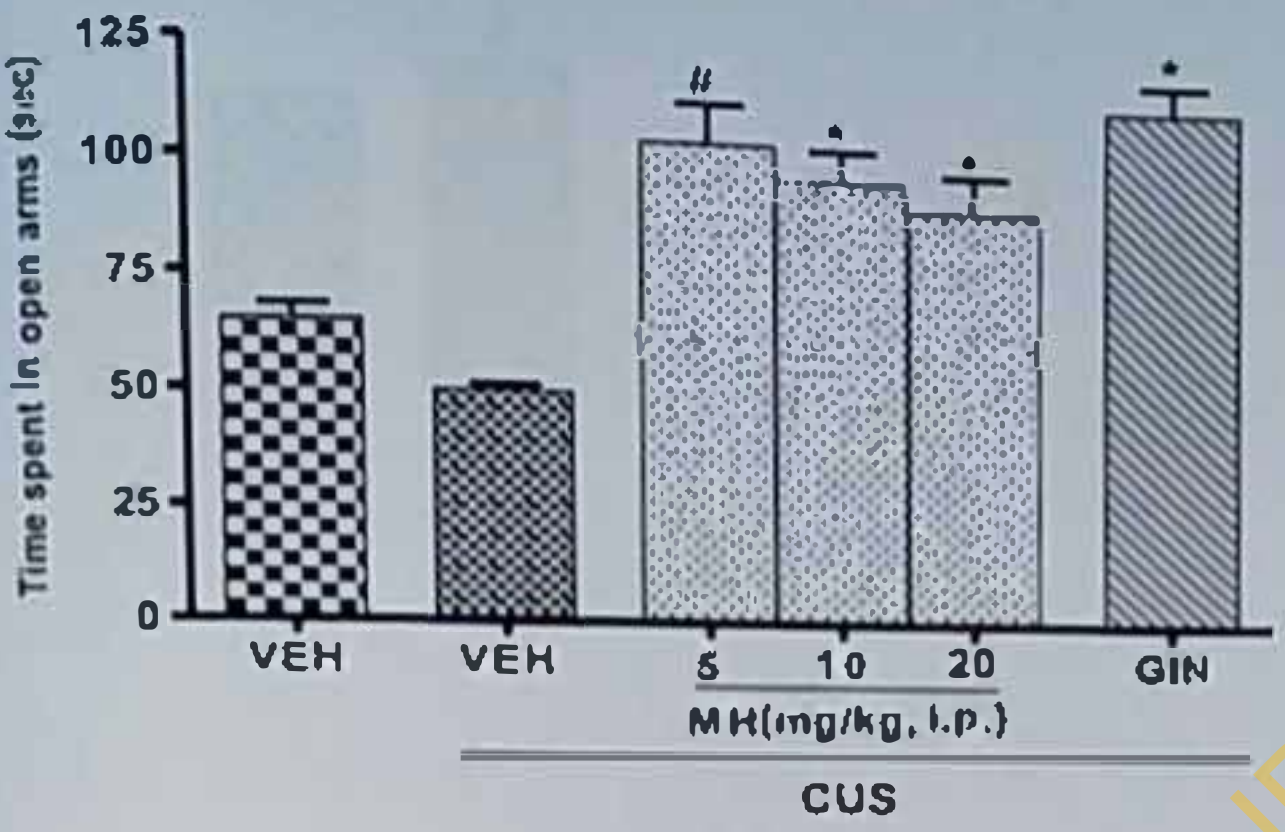


**Figure 45: Effect of Morin hydrate on time spent in open arms of the EPM following CUS exposure.**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls posthoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

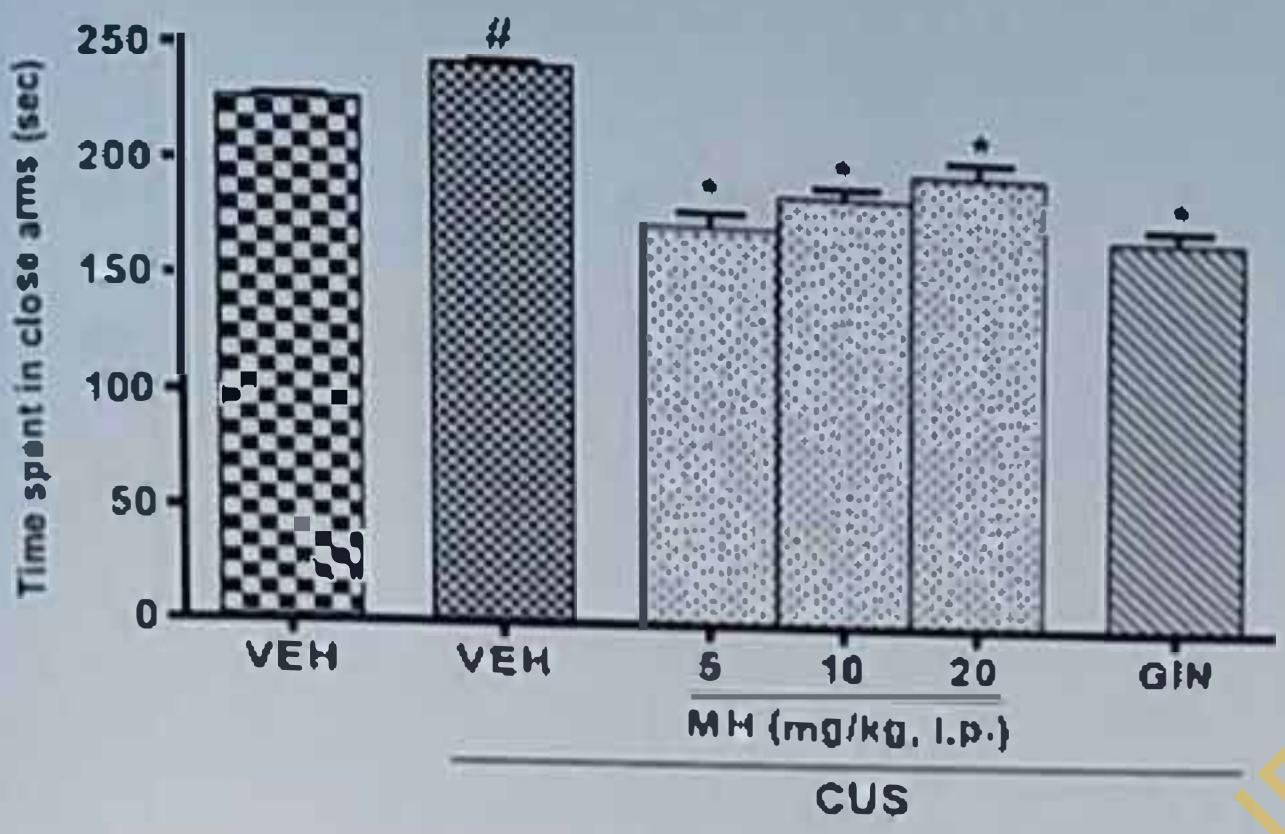


**Figure 46: Effect of Morin hydrate on time spent in the close arms of the EPM following CUS exposure.**

Each column indicates mean ± S.E.M. 6 animals / group

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

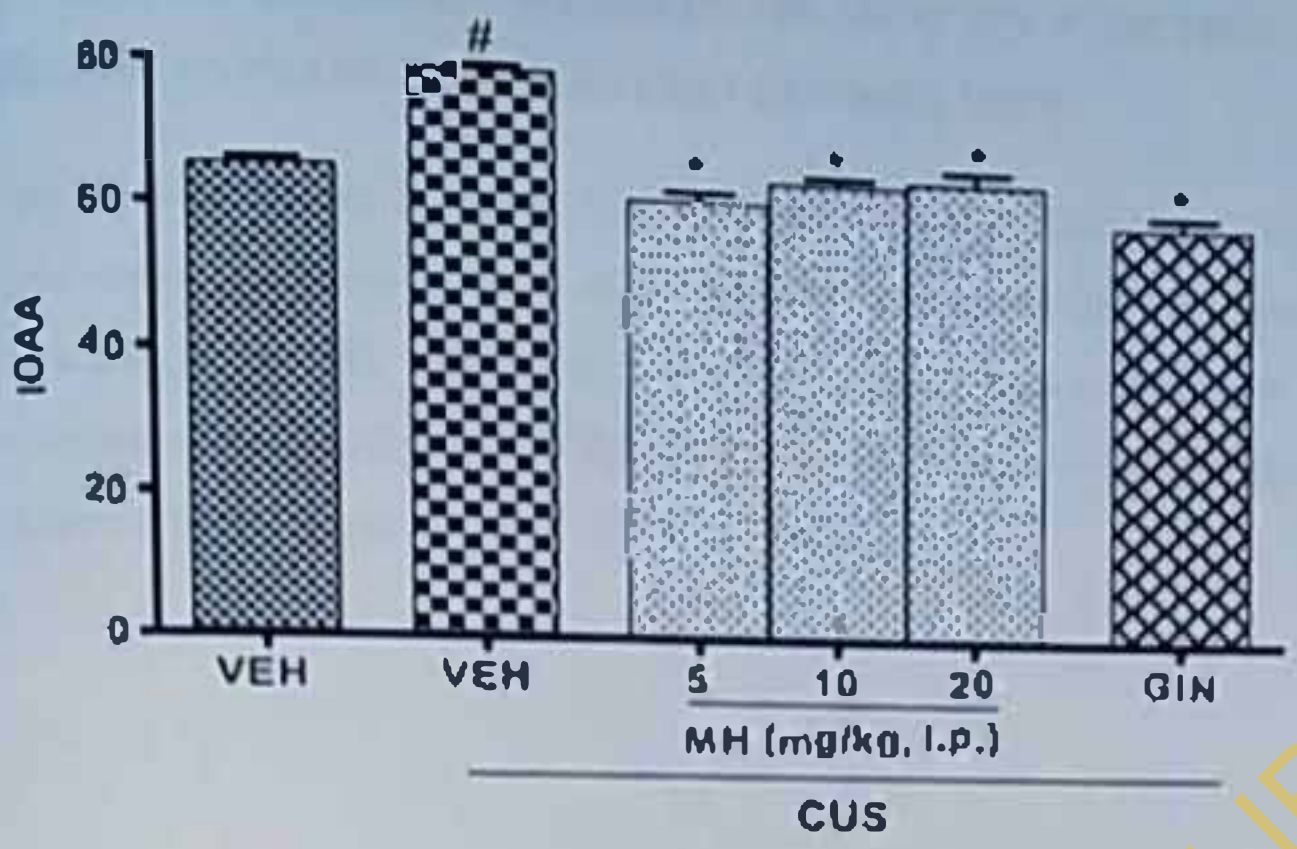


**Figure 17: Effect of Morin hydrate on the Index of open arms avoidance in the EPM following CUS.**

Each column indicates mean ± S.E.M. 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.21. EFFECT OF MORIN HYDRATE ON MEMORY FUNCTION SUBJECTED TO CHRONIC UNPREDICTABLE STRESS-EXPOSED MICE

Stress has been known to impair memory functions and in this study, CUS was observed to impair working memory in the Y maze paradigm as depicted by the reduction in percentage alternation (Figure 48). However, MH (5, 10 and 20 mg/kg) treatment enhanced memory performance significantly ($p < 0.05$) as seen by the increase in percentage alternation in the treatment groups compared to the VEH CUS group.

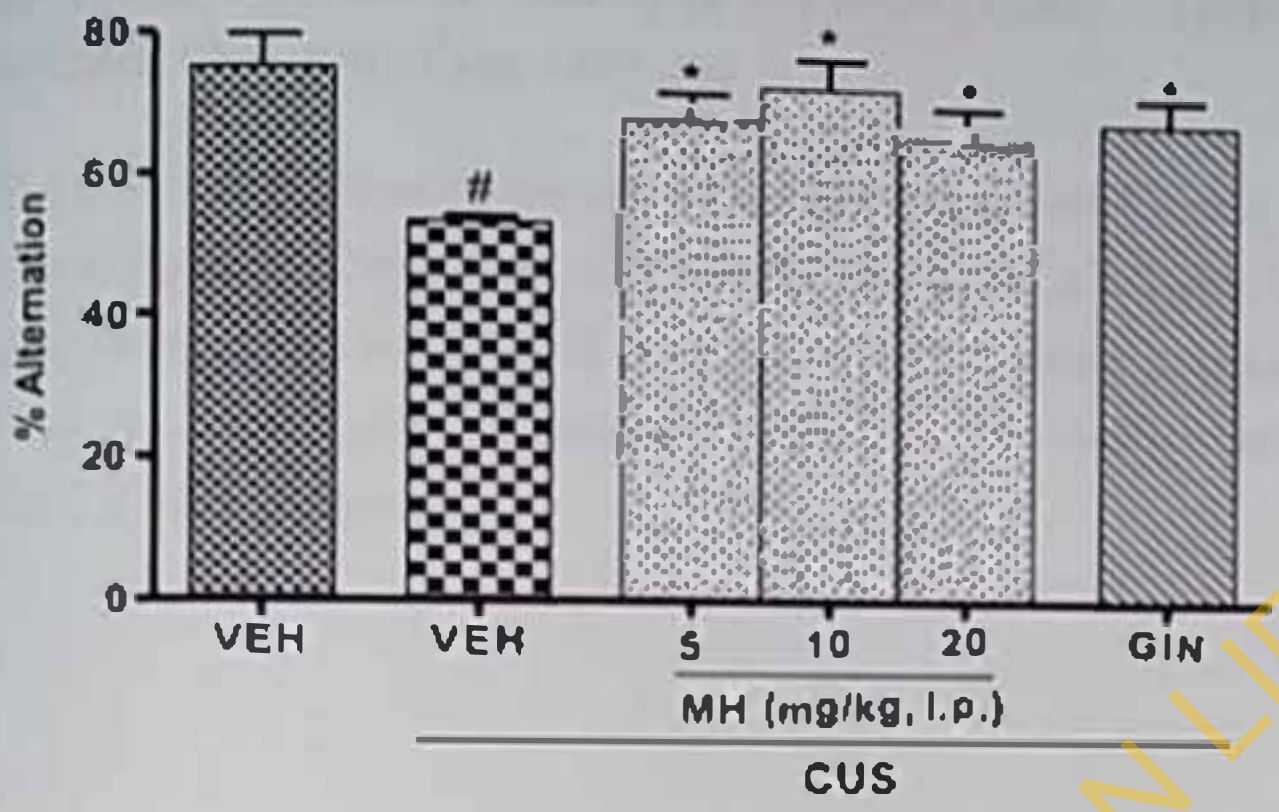


**Figure 48: Effect of Morin hydrate on memory function in CUS-exposed mice.**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.22. EFFECT OF MORIN HYDRATE ON SPONTANEOUS MOTOR ACTIVITY IN CHRONIC UNPREDICTABLE STRESS

Effect of Morin hydrate on the spontaneous motor activity of mice following CUS exposure is as shown in Figure 49. Chronic unpredictable stress significantly ($p < 0.001$) reduced spontaneous motor activity as seen in VEH CUS group relative to VEH unstressed group. However, chronic MH (5, 10, and 20 mg/kg) treatment reversed this observed reduction significantly ($p < 0.001$) relative to VEH CUS group.

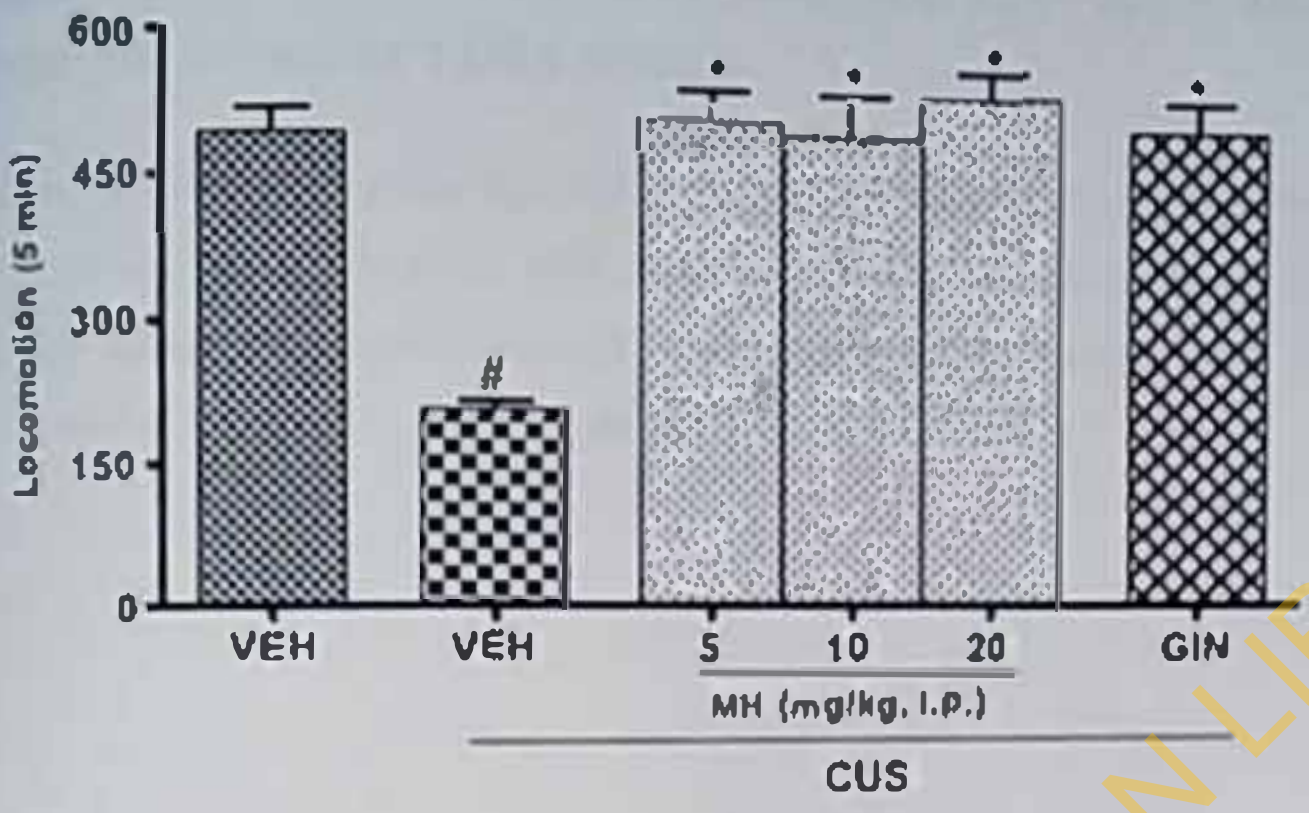


**Figure 49: Effect of Morin hydrate on spontaneous motor activity following CUS.**

Each column indicates mean ± S.E.M. 6 animals / group

# $p < 0.05$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.23. EFFECT OF MORIN HYDRATE ON BLOOD GLUCOSE IN MICE EXPOSED TO CHRONIC UNPREDICTABLE STRESS

Chronic unpredictable stress significantly ($p < 0.001$) heightened blood glucose level as noted in VEH CUS group compared to VEH unstressed group. A significant ($p < 0.001$) overturn of this chronic unpredictable stress-induced hyperglycaemia was observed upon long-term treatment with MH (5, 10 and 20mg/kg) (Figure 50).

## 4.23. EFFECT OF MORIN HYDRATE ON BLOOD GLUCOSE IN MICE EXPOSED TO CHRONIC UNPREDICTABLE STRESS

Chronic unpredictable stress significantly ($p < 0.001$) heightened blood glucose level as noted in VEH CUS group compared to VEH unstressed group. A significant ($p < 0.001$) overturn of this chronic unpredictable stress-induced hyperglycaemia was observed upon long-term treatment with MH (5, 10 and 20mg/kg) (Figure 50).

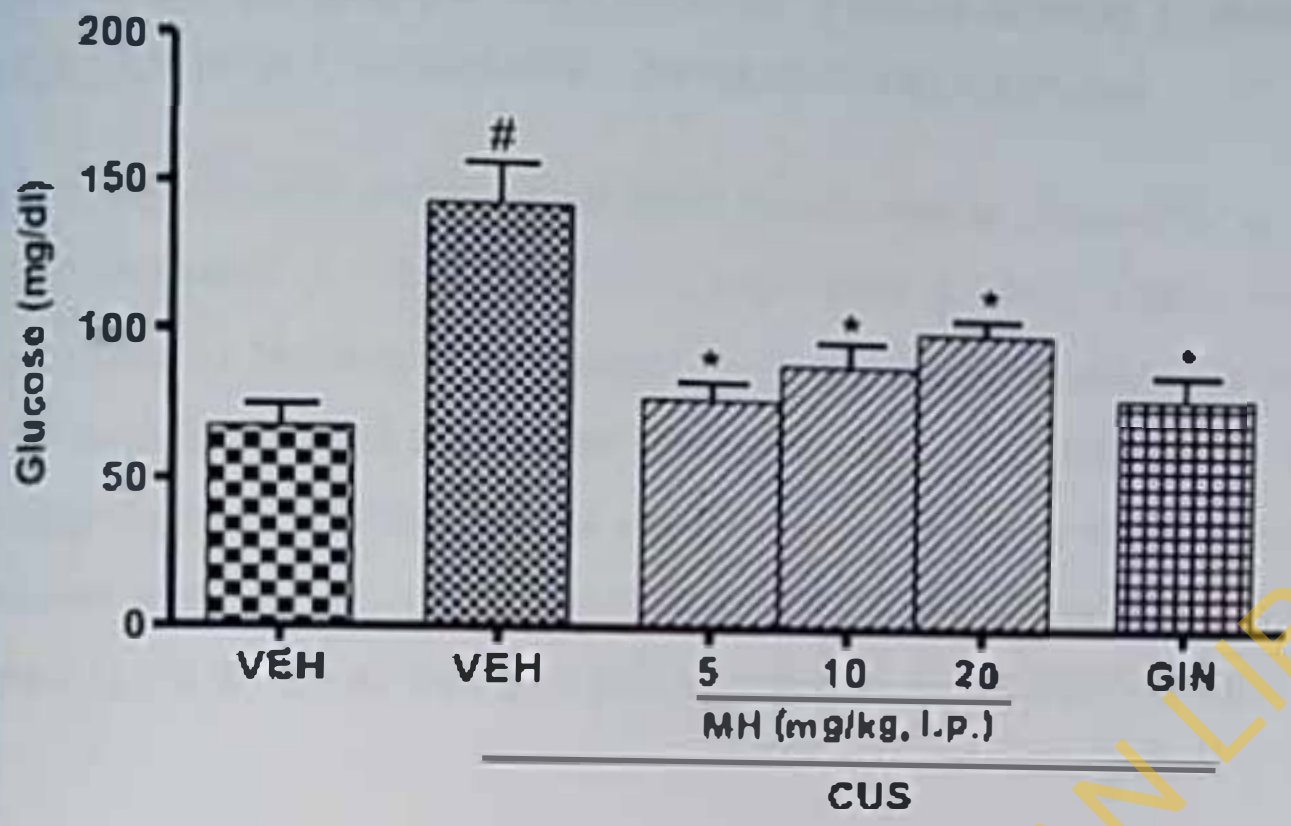


**Figure 50: Effect of Morin hydrate on blood glucose level following CUS.**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.24. MORIN HYDRATE INFLUENCES SERUM LIPIDS CONCENTRATIONS IN MICE EXPOSED TO CHRONIC UNPREDICTABLE STRESS

Chronic unpredictable stress triggers lipids mobilization as observed by the significant increase in total cholesterol ($p < 0.001$) and serum triglyceride ($p < 0.01$) relative to the VEH unstressed group (Table 3). Morin hydrate treatment (5, 10, and 20 mg/kg) attenuated this observed increase in serum cholesterol and triglyceride significantly ($p < 0.05$). Furthermore, CUS reduced HDL cholesterol (good cholesterol) levels significantly ($p < 0.05$), an effect which was overturned by treatment with Morin hydrate. Serum atherosclerotic index was also reduced in a significant manner ($p < 0.001$) in the treatment groups relative to the VEH CUS group.

Table 3: Effect of Morin hydrate on serum lipids in chronic unpredictable stress –exposed mice

| Treatment (mg/kg) | Triglycerides (mg/dl) | Total cholesterol (mg/dl) | HDL cholesterol (mg/dl) | Atherosclerotic index |
|---|---|---|---|---|
| $VEH_{(UNS)}$ | 36.67 ± 1.86 | 46.00 ± 4.16 | 36.67 ± 1.76 | 0.25 ± 0.07 |
| $VEH_{(CUS)}$ | 55.33 ± 1.76$^{\#\#}$ | 77.33 ± 1.86$^{\#\#\#}$ | 25.33 ± 1.76$^{\#}$ | 1.59 ± 0.20$^{\#\#\#}$ |
| MH 5mg/kg | 44.33 ± 2.40$^{*}$ | 65.00 ± 0.58 | 32.00 ± 0.58$^{*}$ | 1.03 ± 0.05$^{**}$ |
| MH 10mg/kg | 44.00 ± 3.05$^{*}$ | 60.67 ± 3.48$^{*}$ | 34.67 ± 3.67$^{*}$ | 0.77 ± 0.09$^{***}$ |
| MH 20 mg/kg | 45.67 ± 2.33$^{*}$ | 59.00 ± 2.65$^{*}$ | 34.00 ± 0.58$^{*}$ | 0.73 ± 0.05$^{***}$ |
| GIN | 45.67 ± 2.73$^{*}$ | 57.67 ± 2.19$^{*}$ | 34.33 ± 0.88$^{*}$ | 0.68 ± 0.03$^{***}$ |

Each result indicates mean ± S.E.M, 6 animals / group

# $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ relative to VEH unstressed group

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman Keuls post-hoc test).

$VEH_{(UNS)}$: Vehicle unstressed group

$VEH_{(CUS)}$: Vehicle chronic unpredictable stress group

MH: Morin hydrate

GIN: Ginseng (25 mg/kg. i.p.)

## 4.25. EFFECT OF PRETREATMENT WITH MORIN HYDRATE ON CHRONIC UNPREDICTABLE STRESS -INDUCED ADRENAL HYPERTROPHY

Exposure of mice to CUS increased adrenal glands weights in a significant manner ($p < 0.01$) as seen in the VEH CUS group relative to VEH unstressed group. Morin hydrate (5, 10, 20 mg/kg i.p.) treatment significantly ($p < 0.01$) inhibited CUS-induced adrenal hypertrophy as compared to the VEH CUS group (Figure 51).

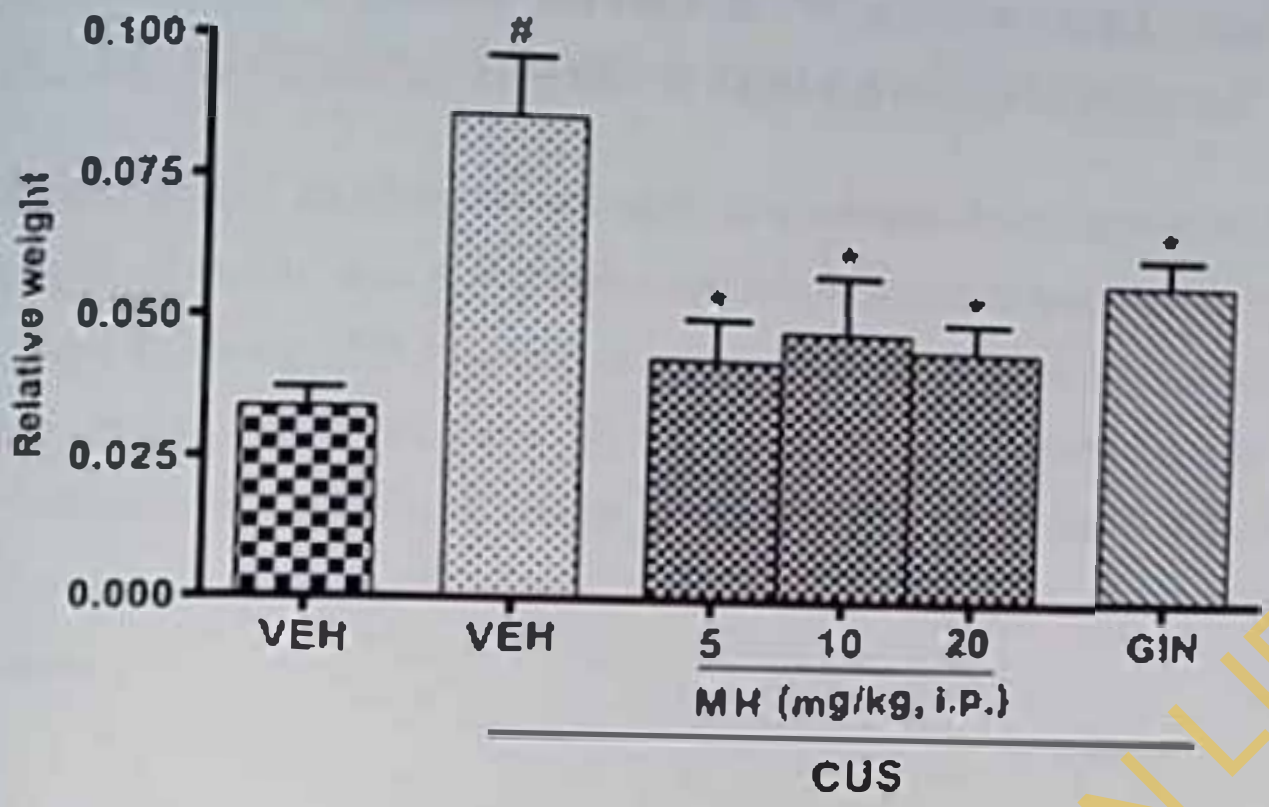


**Figure 51: Effect of Morin hydrate on adrenal gland weight of mice following CUS.**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.01$ relative to VEH unstressed group

* $p < 0.01$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.26. EFFECT OF MORIN HYDRATE ON BLOOD CORTICOSTERONE LEVELS FOLLOWING CHRONIC UNPREDICTABLE STRESS EXPOSURE

The effect of CUS on HPA axis activation as represented by corticosterone levels is as shown in Figure 52. One-way ANOVA revealed that serum corticosterone was significantly ($p < 0.001$) increased following CUS exposure as seen in VEH CUS group relative to the VEH unstressed group. Morin hydrate treatment (5, 10 20 mg/kg, i.p.) attenuated this increase in serum corticosterone in a significant manner ($p < 0.05$). This shows that CUS activated the HPA axis and treatment with Morin hydrate suppresses the activation, demonstrating its anti-stress potential.

## 4.16. EFFECT OF MORIN HYDRATE ON BLOOD CORTICOSTERONE LEVELS FOLLOWING CHRONIC UNPREDICTABLE STRESS EXPOSURE

The effect of CUS on HPA axis activation as represented by corticosterone levels is as shown in Figure 52. One-way ANOVA revealed that serum corticosterone was significantly ($p < 0.001$) increased following CUS exposure as seen in VEH CUS group relative to the VEH unstressed group. Morin hydrate treatment (5, 10 20 mg/kg, i.p.) anenuated this increase in serum corticosterone in a significant manner ($p < 0.05$). This shows that CUS activated the HPA axis and treatment with Morin hydrate suppresses the activation, demonstrating its anti-stress potential.

## 4.26. EFFECT OF MORIN HYDRATE ON BLOOD CORTICOSTERONE LEVELS FOLLOWING CHRONIC UNPREDICTABLE STRESS EXPOSURE

The effect of CUS on HPA axis activation as represented by corticosterone levels is as shown in Figure 52. One-way ANOVA revealed that serum corticosterone was significantly ($p < 0.001$) increased following CUS exposure as seen in VEH CUS group relative to the VEH unstressed group. Morin hydrate treatment (5, 10 20 mg/kg, i.p.) anenuated this increase in serum corticosterone in a significant manner ($p < 0.05$). This shows that CUS activated the HPA axis and treatment with Morin hydrate suppresses the activation, demonstrating its anti-stress potential.

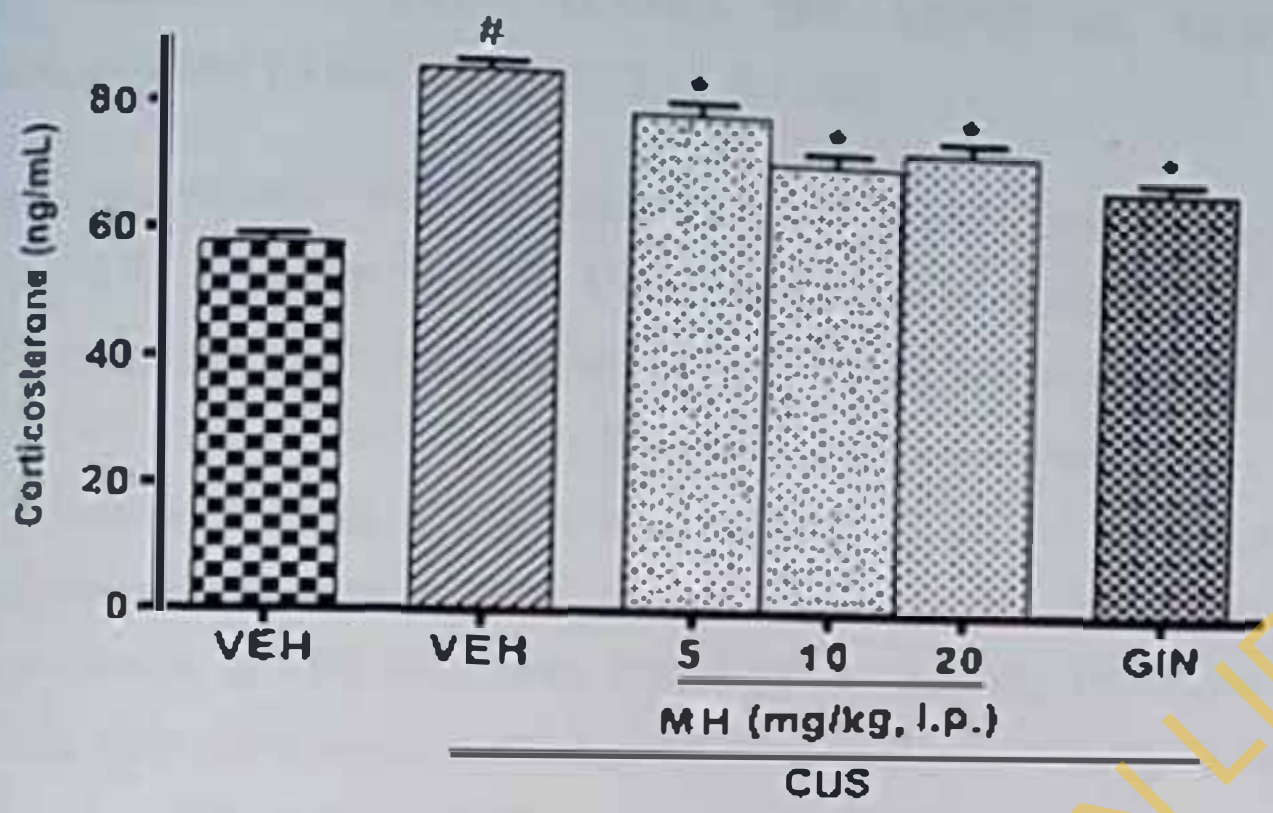


**Figure 52: Effect of morin hydrate on serum corticosterone level in CUS mice**

Each column indicates mean ± S.E.M. 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

• $p < 0.05$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.27. MORIN HYDRATE ALTERS THE LEVEL OF OXIDATIVE STRESS BIOMARKERS IN MICE SUBJECTED TO CUS

Chronic unpredictable stress significantly ($p < 0.001$) reduced brain GSH concentration as seen in the VEH CUS group relative to VEH unstressed group. Morin hydrate treatment (5, 10, 20 mg/kg i.p.) upturned the decrease in GSH concentration significantly ($p < 0.001$) compared to VEH CUS group (Figure 53). Also, brain MDA level was elevated by CUS compared with the VEH unstressed group. Morin hydrate treatment (5, 10, 20 mg/kg i.p.) attenuated lipid peroxidation by reducing brain MDA levels significantly ($p < 0.001$) as seen in the treatment groups relative to VEH CUS group (Figure 54). Furthermore, CUS significantly ($p < 0.001$) elevated brain nitrite level but pretreatment with MH (5, 10, 20 mg/kg) reversed this increase in nitrite concentration in a significant manner ($p < 0.001$) as seen in the treatment groups compared to VEH CUS group (Figure 55).

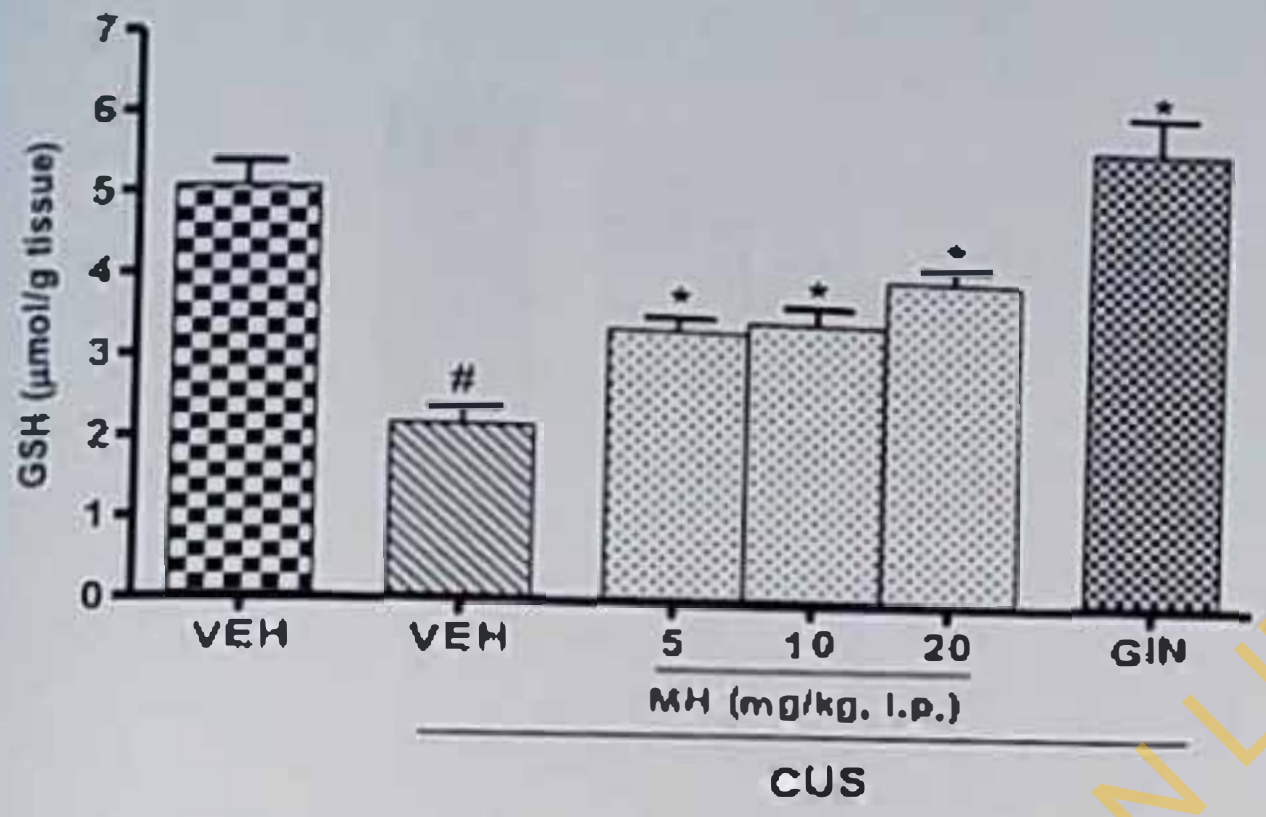


Figure 53: Effect of Morin hydrate on brain GSH level following CUS exposure

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

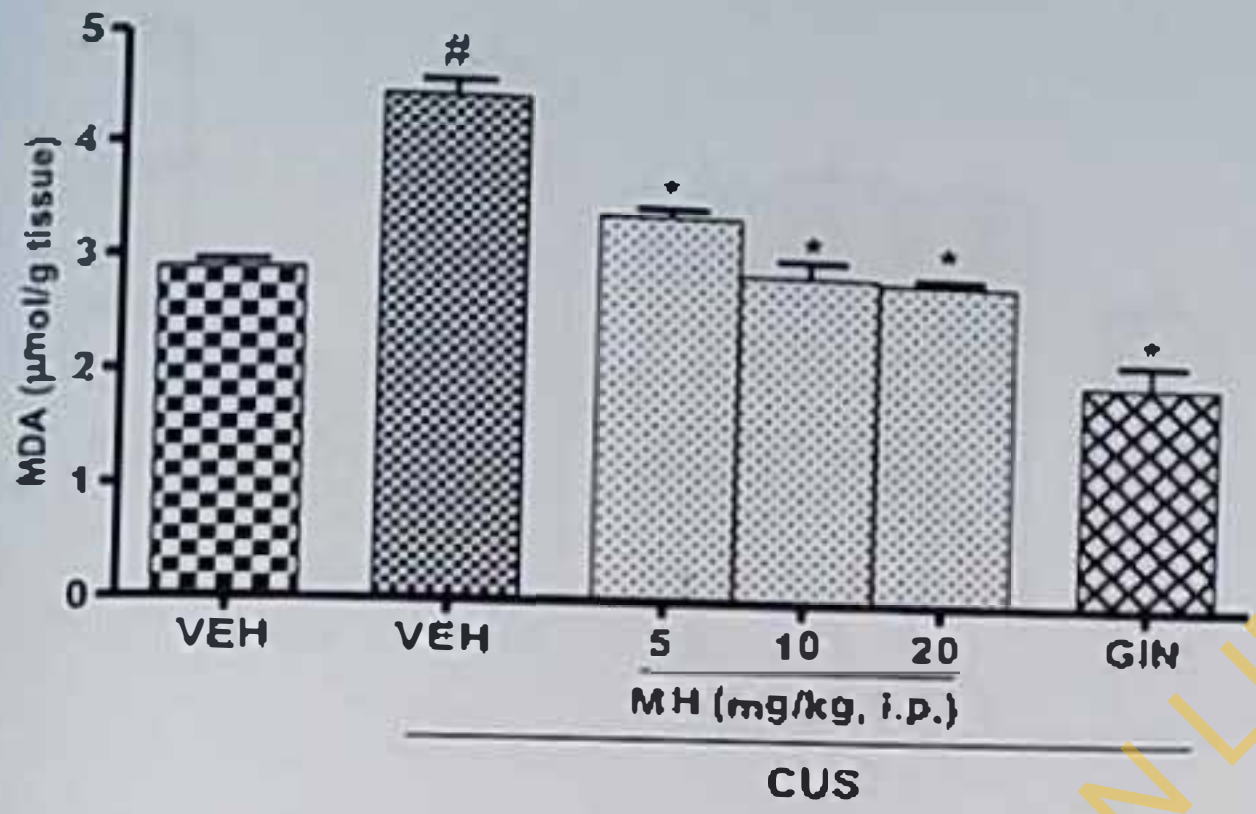


Figure 54: Effect of Morin hydrate on brain MDA level following CUS exposure

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

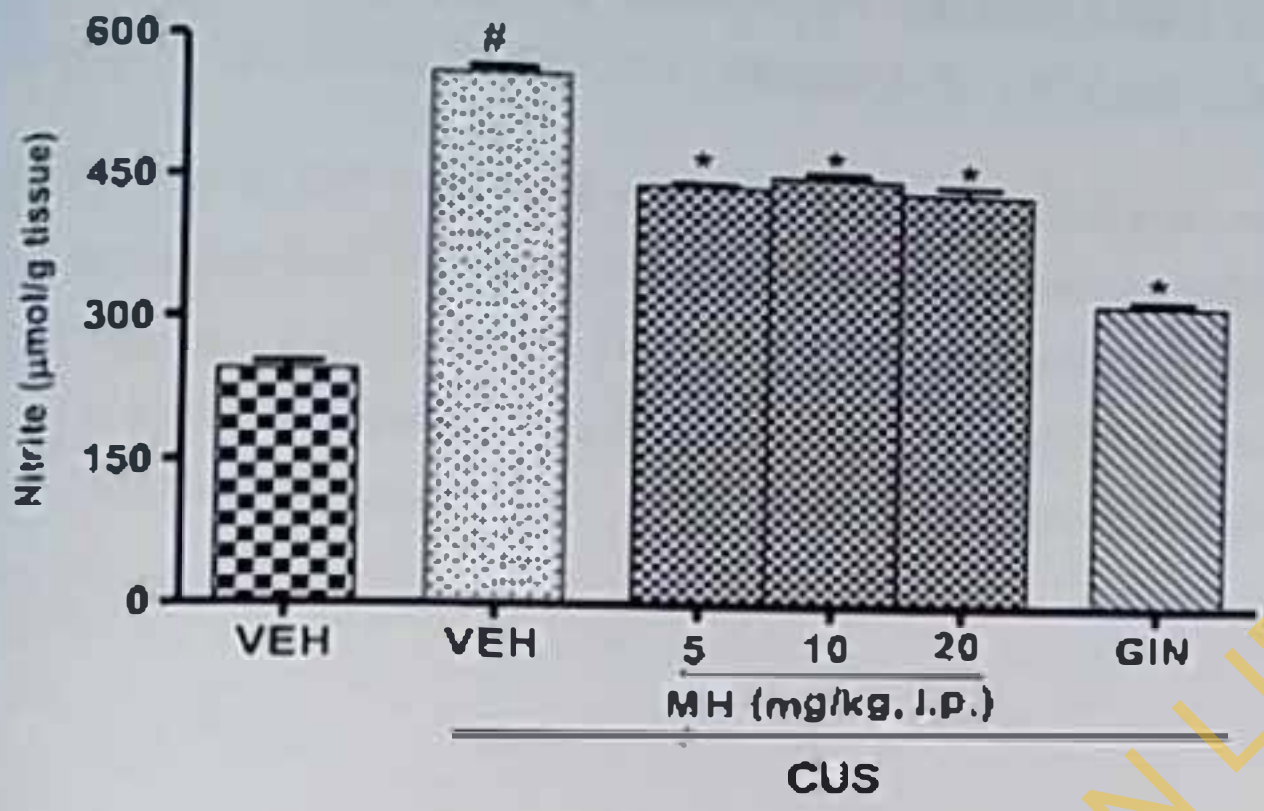


**Figure 55: Effect of Morin hydrate on brain nitrite level in CUS-exposed mice**

Each column indicates mean ± S.E.M. 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.28. EFFECT OF MORIN HYDRATE ON PROINFLAMMATORY CYTOKINES LEVELS FOLLOWING CHRONIC UNPREDICTABLE STRESS EXPOSURE

The expression of TNF-α and IL-1β in mice brains following CUS exposure and the modulatory effect of Morin hydrate is as shown in Figures 56 and 57. Chronic unpredictable stress exposure significantly ($p < 0.001$) enhanced TNF-α and IL-1β concentrations in mice brains as noted in the VEH CUS group relative to the VEH unstressed group. Morin hydrate treatment (5, 10, 20 mg/kg) however, significantly ($p < 0.001$) upturned the observed elevations suggesting anti-neuroinflammatory activity.

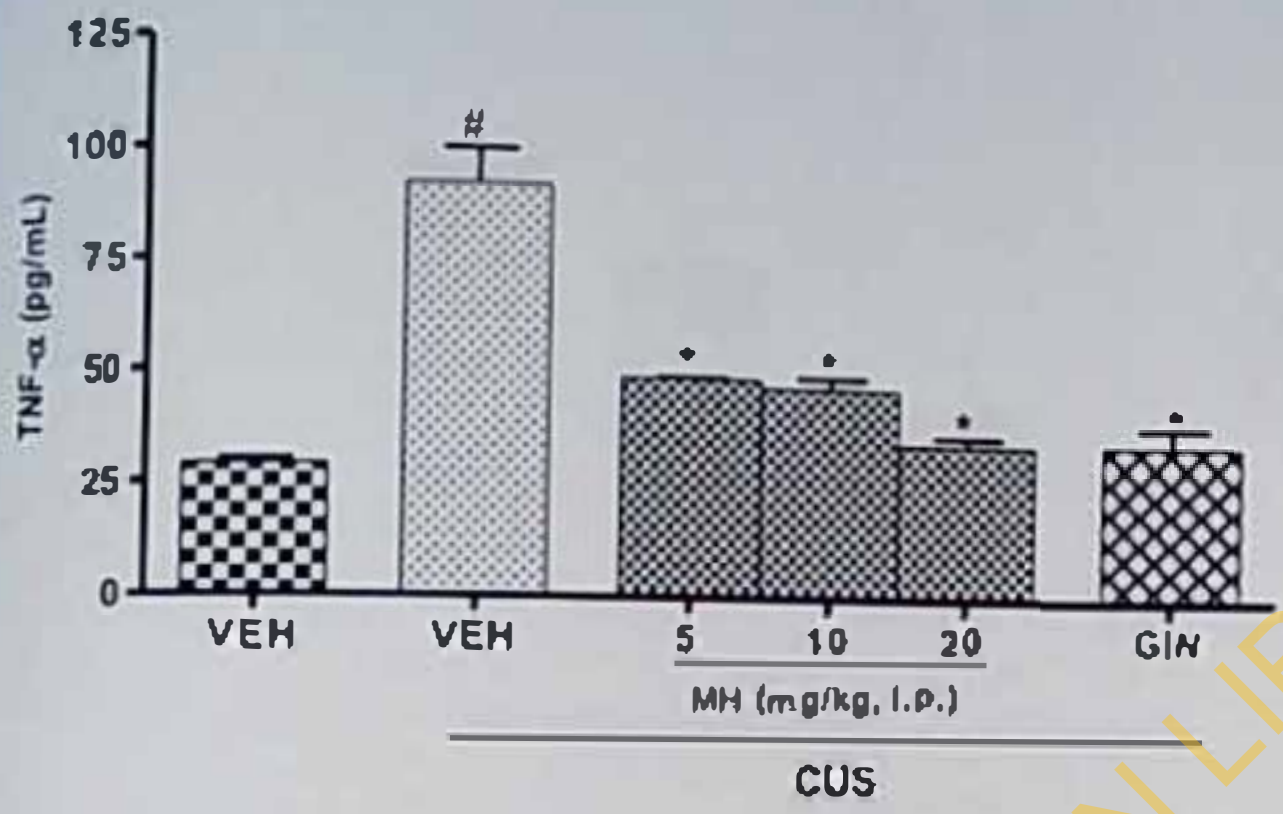


Figure 56: Effect of Morin hydrate on TNF-α level in mice exposed to CUS

Each column indicates mean ± S.E.M. 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml./kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

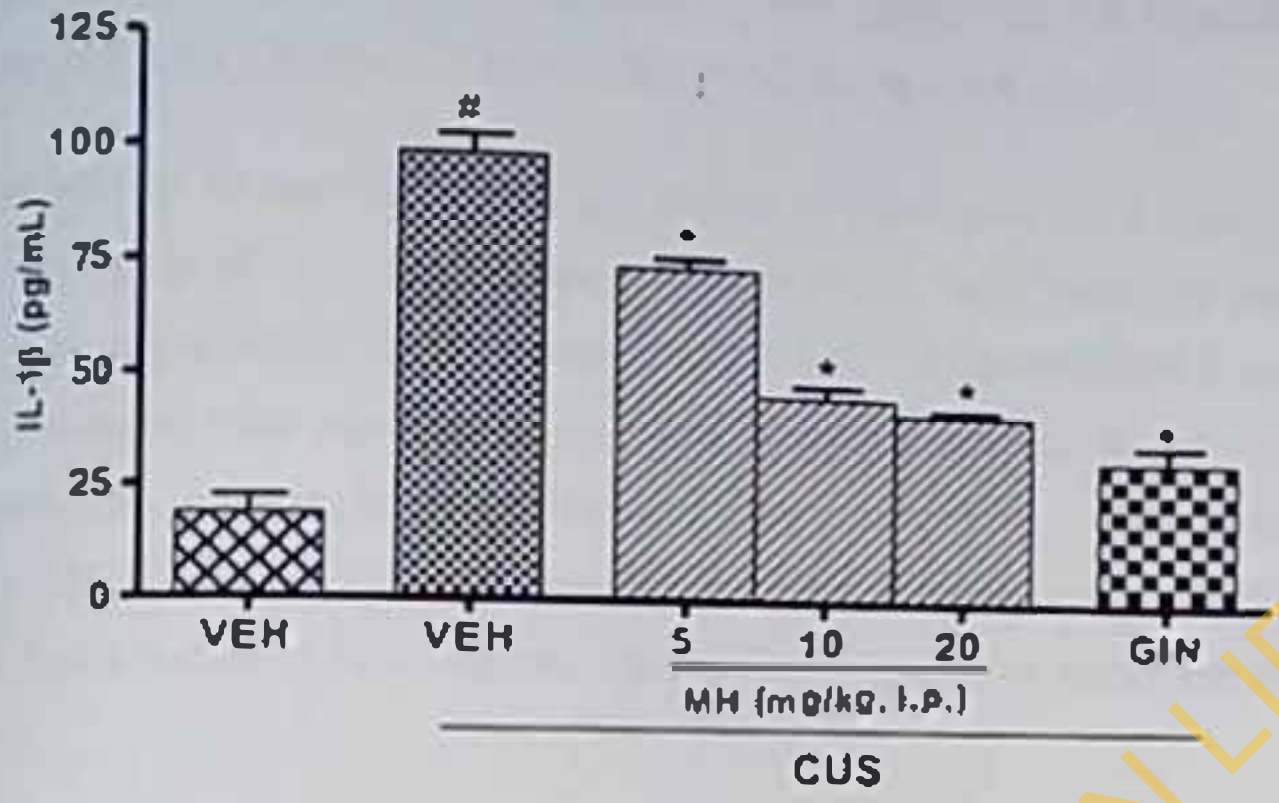


**Figure 57: Effect of Morin hydrate on IL-1β level in mice exposed to CUS**

Each column indicates mean ± S.E.M, 6 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.29. EFFECT OF MORIN HYDRATE ON CHRONIC UNPREDICTABLE STRESS-INDUCED NEURONAL DEGENERATION IN MOUSE BRAIN

The effect of Morin hydrate on CUS-induced structural changes in hippocampal cornu ammonis 3 (CA3) pyramidal layer and dentate gyrus neurons in mice brains are shown in Plates 4 and 5. Chronic unpredictable stress exposure induced some neuropathological changes in the hippocampus. Furthermore, CUS significantly ($p < 0.001$) cause a reduction in the population of viable CA3 pyramidal and dentate gyrus neurons when compared to VEH stressed group, suggesting neurodegeneration (Figures 58 and 59). Administration of Morin hydrate (5, 10, 20 mg/kg i.p.) mitigated the loss of viable neurons which further suggests neuroprotective effect.

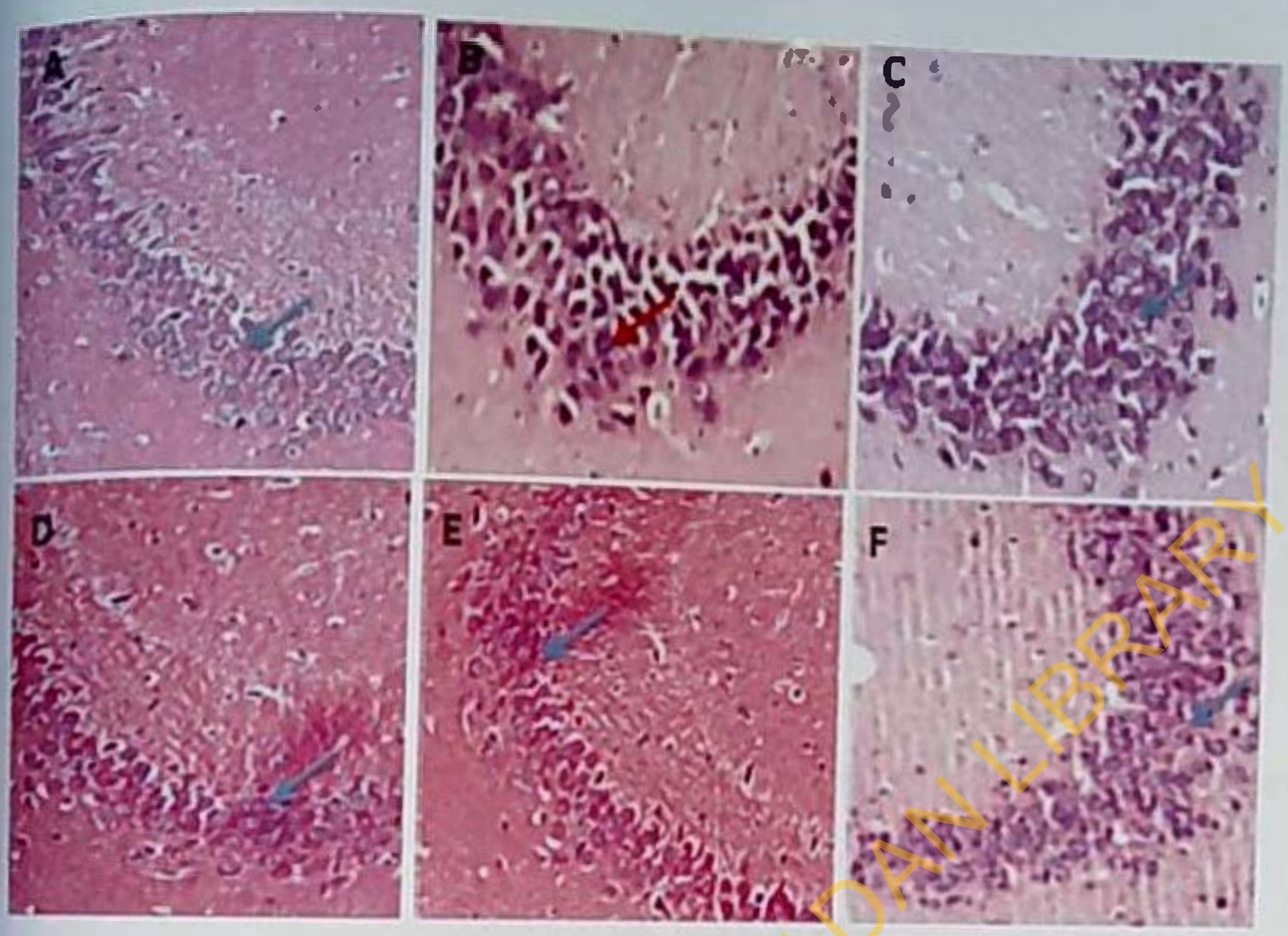


Plate 4: Effect of Morin hydrate on morphology of hippocampal CA3 pyramidal neurons in mice following CUS exposure

(A) Vehicle only (normal pattern of neurons and cellular layers)

(B) Vehicle + CUS (irregular layer and necrosis of neurons)

(C) MH 5 mg/kg + CUS (normal neuronal cells with no visible lesion)

(D) MH 10 mg/kg + CUS (relatively normal neurons)

(E) MH 20 mg/kg + CUS (relatively normal neurons)

(F) GIN + CUS (normal neuronal cells, moderate increase in glial cells: astrocytosis)

Blue arrows indicate normal neurons while red arrows indicate indicates damaged neurons. (Original magnification X400).

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p)

CUS: Chronic Unpredictable Stress

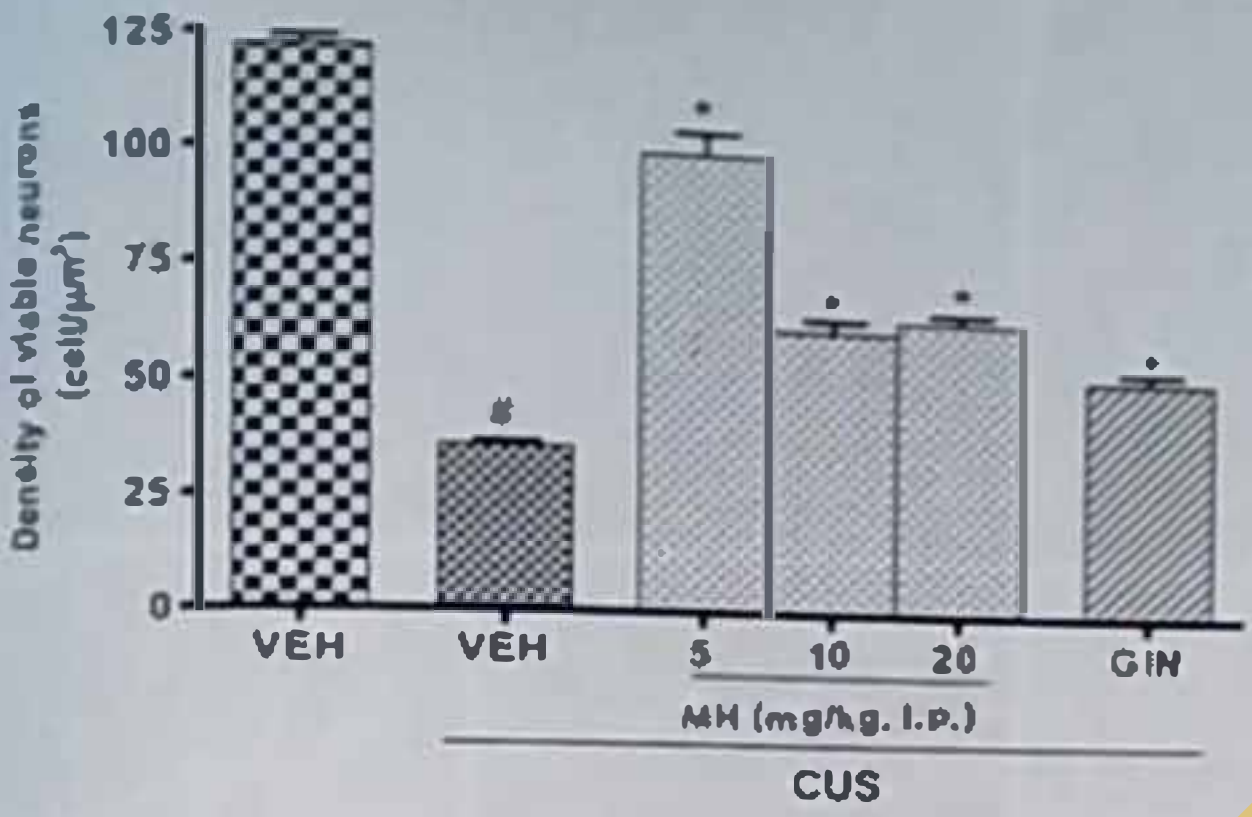


Figure 58: Effect of Morin hydrate on the density of viable CA3 hippocampal neurons in mice following CUS exposure.

Each column indicates mean ± S.E.M. 3 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.01$ and ** $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg. i.p.)

CUS: Chronic Unpredictable Stress

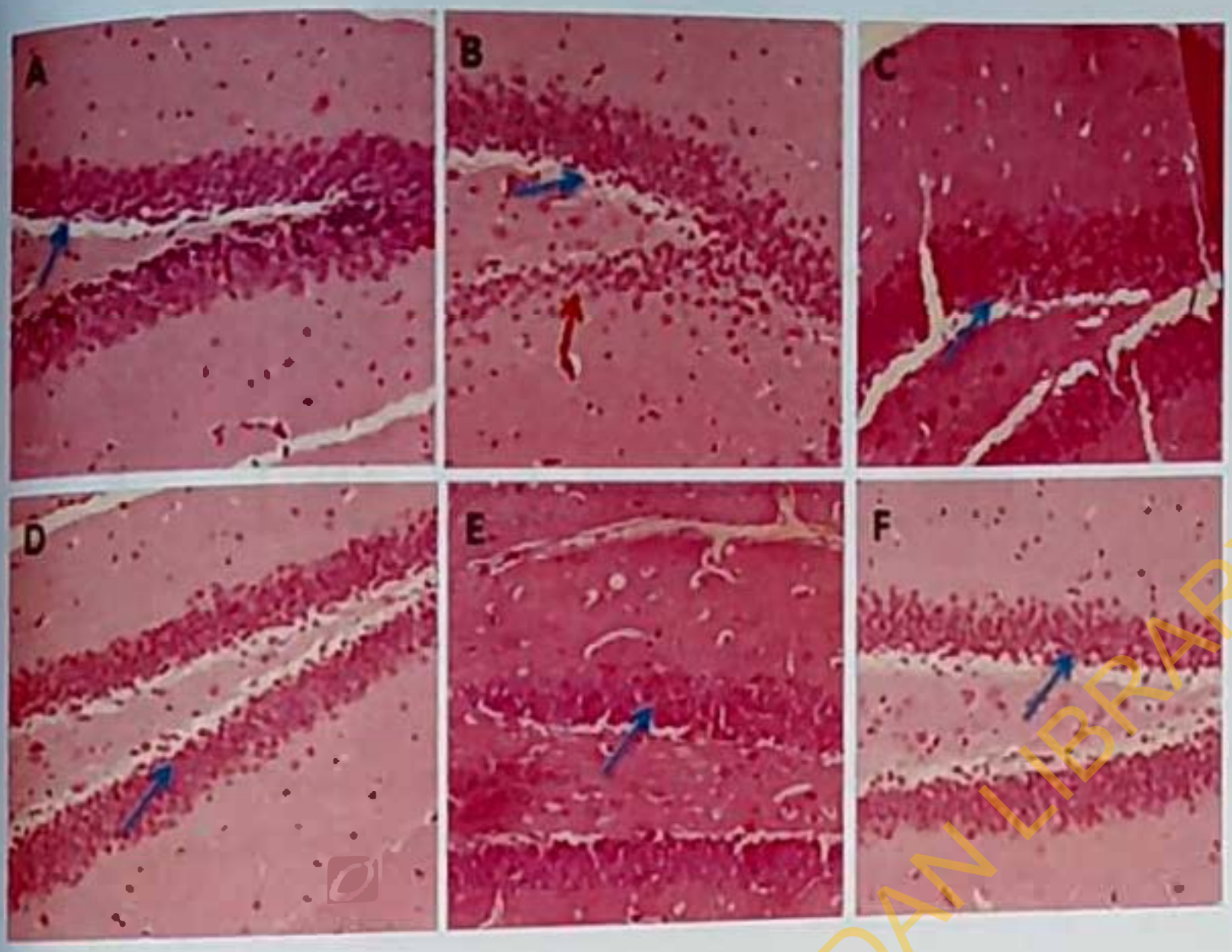


**Plate 5: Effect of Morin hydrate on morphology of the hippocampal dentate gyrus neurons in mice following CUS exposure**

(A) Vehicle only (normal cellular layers and intact neurons)

(B) Vehicle + CUS (irregular layer and severe necrosis of neurons)

(C) MH 5 mg/kg + CUS (normal neurons, few foci of vacuolation with no visible lesion)

(D) MH 10 mg/kg + CUS (regular layer with relatively normal neurons)

(E) MH 20 mg/kg + CUS (relatively ordered layer and normal neurons)

(F) GIN + CUS (normal neuronal cells, moderate increase in glial cells; astrocytosis)

Blue arrows indicate normal neurons while red arrows indicate indicates damaged neurons. (Original magnification X400).

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

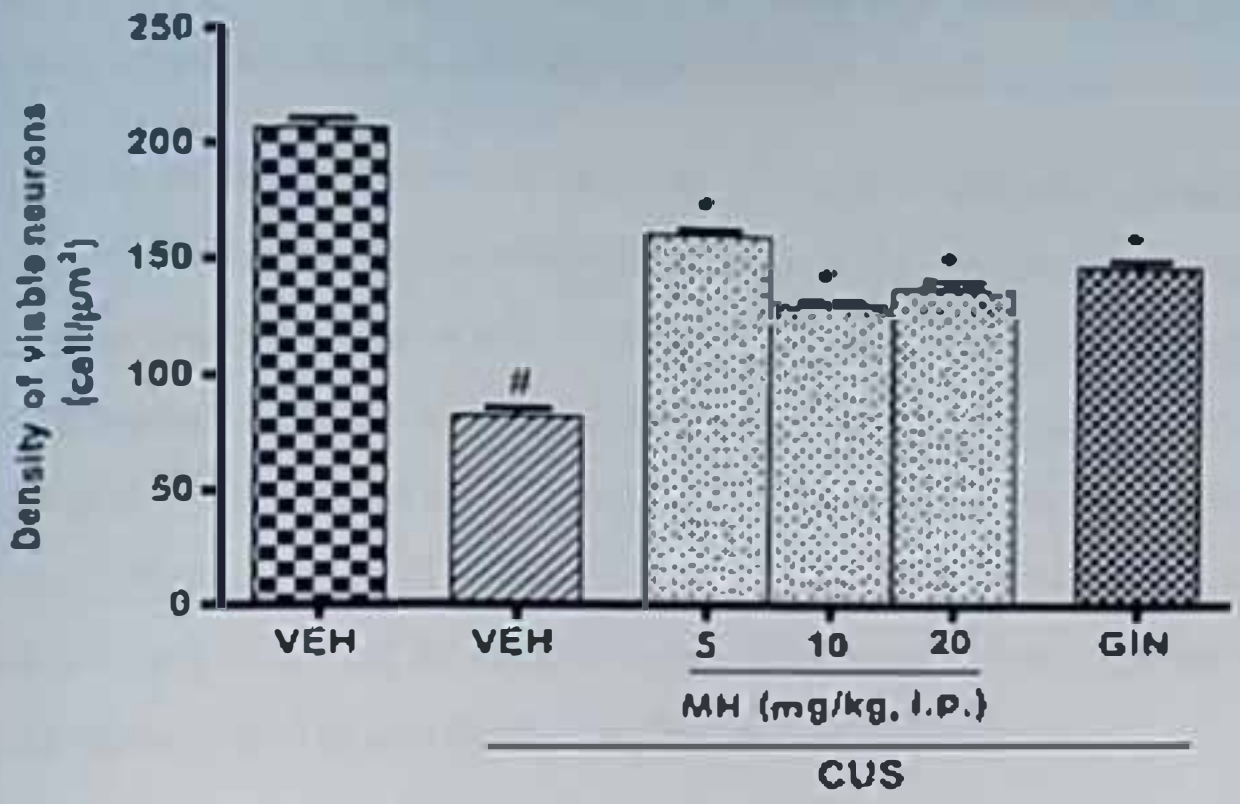


Figure 59: Effect of Morin hydrate on hippocampal dentate gyrus neurons density in mice exposed to CUS.

Each column indicates mean ± S.E.M. 3 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed b) Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

## 4.30. EFFECT OF MORIN HYDRATE ON THE EXPRESSION OF NF-κB AND iNOS IN THE HIPPOCAMPUS FOLLOWING CUS EXPOSURE

The effect of morin hydrate on immunohistochemical alterations caused by CUS and the expressions of iNOS and NF-κB positive cells in the hippocampi of mice is shown in the photomicrographs in Plates 6 and 7. Relative to vehicle only group, CUS augmented iNOS and NF-κB expressions in the hippocampus as indicated by the intensity of brown coloration in the slides as shown in Plates 6 and 7, which was further buttressed by the number of positive cells as shown in figures 60 and 61. Morin hydrate administration (5, 10, and 20 mg/kg i.p.) however, decreased both iNOS and NF-κB expressions. This further suggests that the anti-stress actions of Morin hydrate could be as a result of its neuroprotective effects.

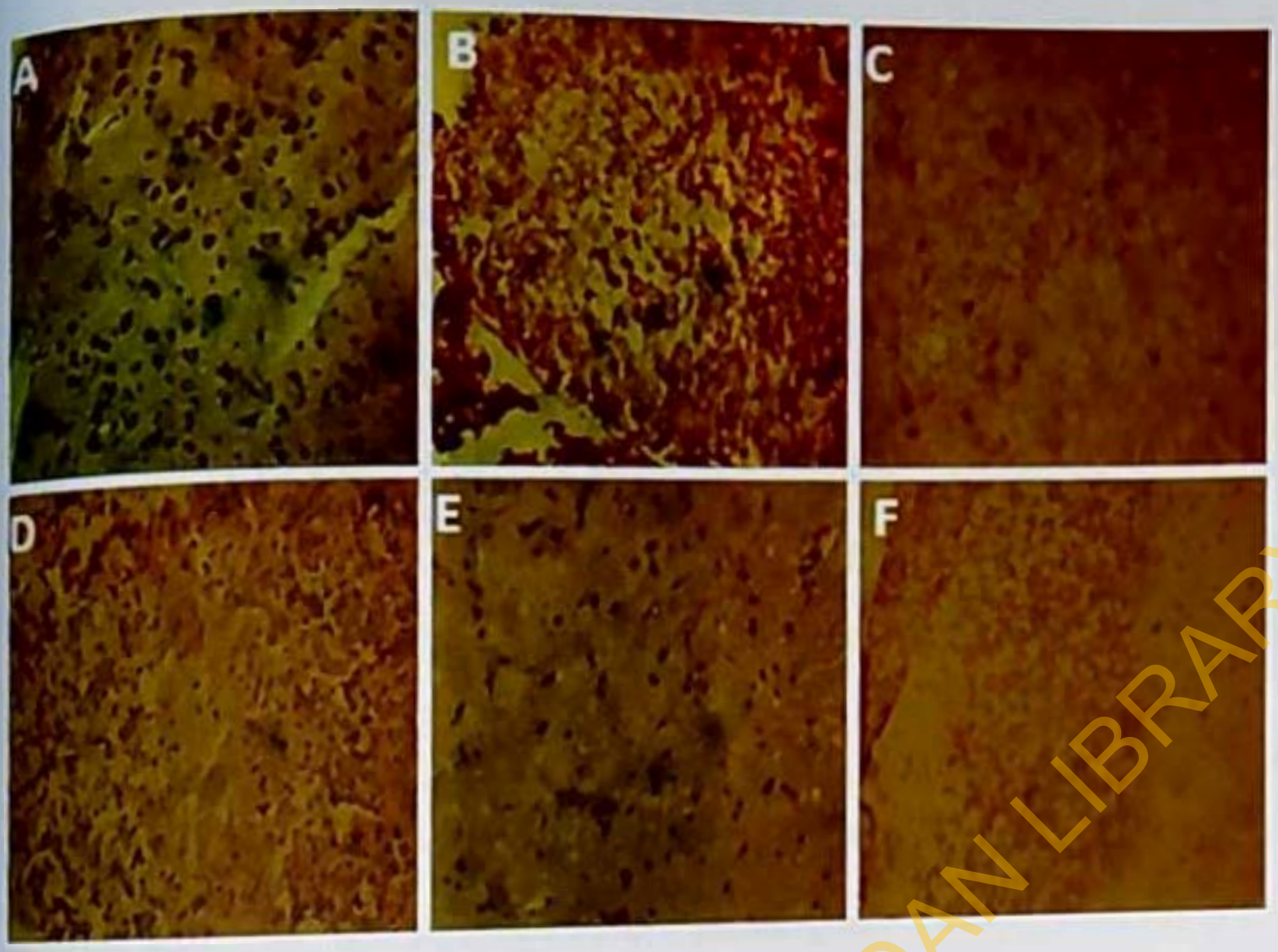


Plate 6: Photomicrograph representing the effect of morin hydrate on Immunohistochemical alterations and expression of iNOS positive cells in the hippocampus following CUS exposure

A: Vehicle only

B: Vehicle + CUS

C: MH 5 mg/kg + CUS

D: MH 10 mg/kg + CUS

E: MH 20 mg/kg + CUS

F: GIN + CUS

(Original magnification X400)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

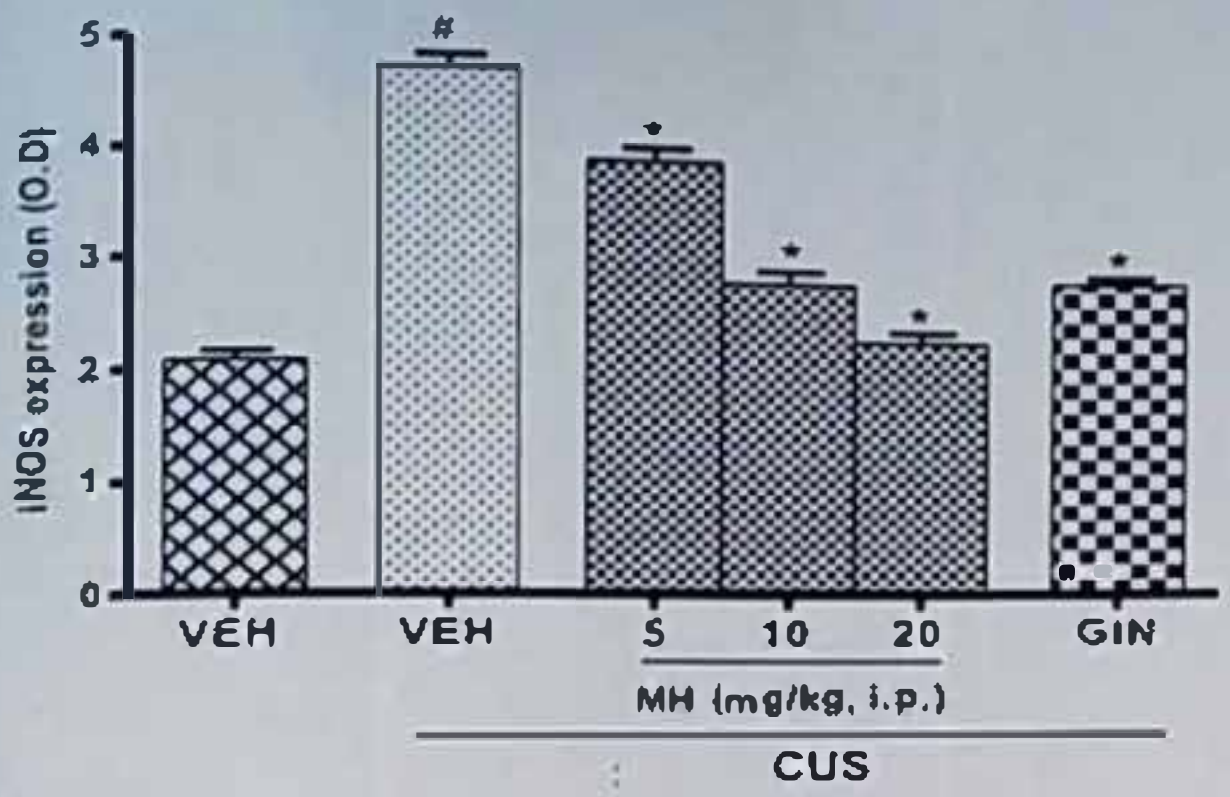


**Figure 60: Effect of Morin hydrate on expression of iNOS positive cells in the hippocampus following CUS exposure.**

Each column indicates mean ± S.E.M, 2 animals / group

# $p < 0.001$ relative to VEH unstressed group

• $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 mL/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

iNOS: Inducible nitric oxide synthase

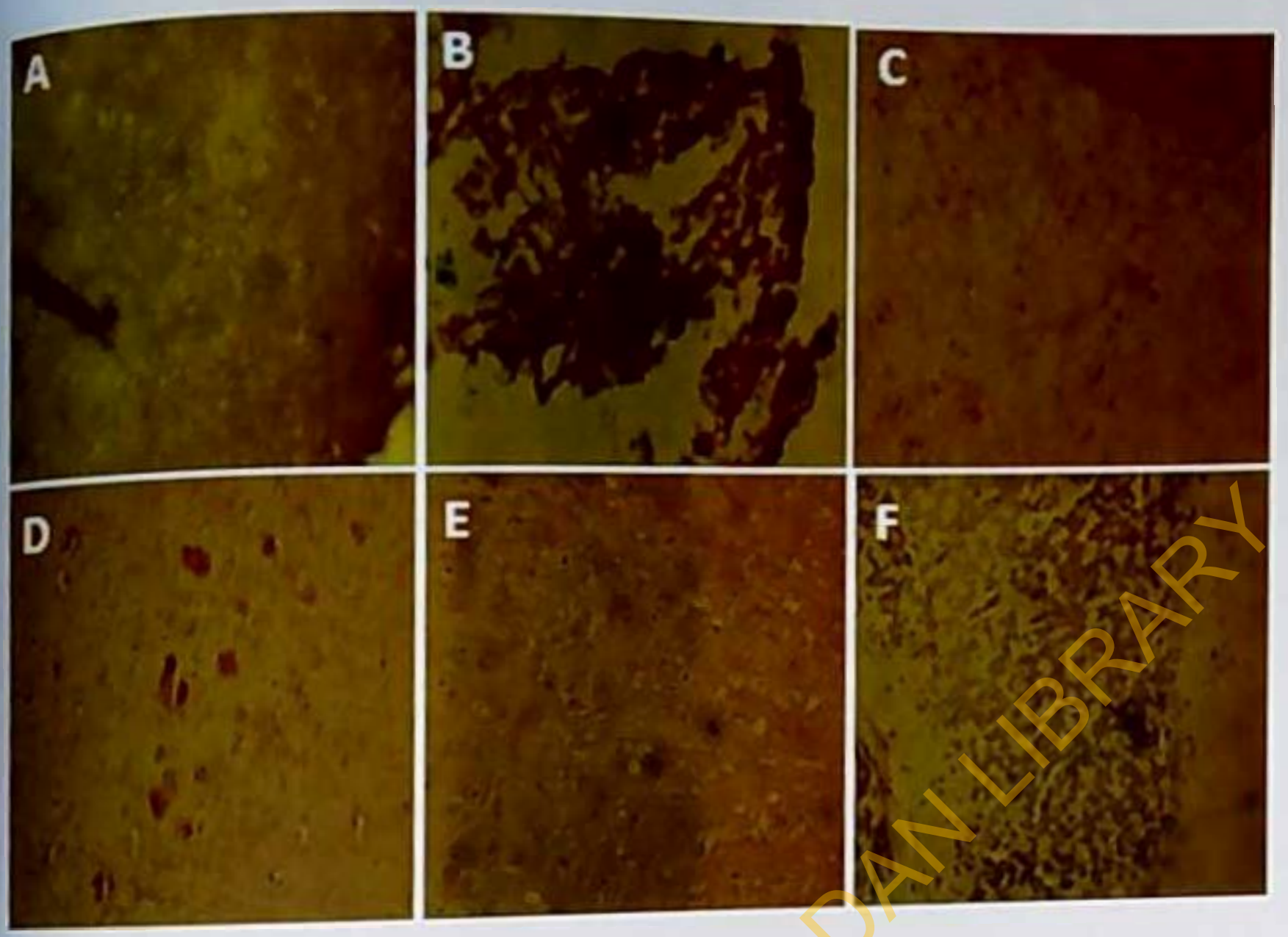


Plate 7: Photomicrograph representing the effect of morin hydrate on immunohistochemical changes and expression of NF-κB positive cells in the hippocampus following CUS exposure

A: Vehicle only

B: Vehicle + CUS

C: MH 5 mg/kg + CUS

D: MH 10 mg/kg + CUS

E: MH 20 mg/kg + CUS

F: GIN + CUS

(Original magnification X400)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

NF-Kb: Nuclear factor kappa B

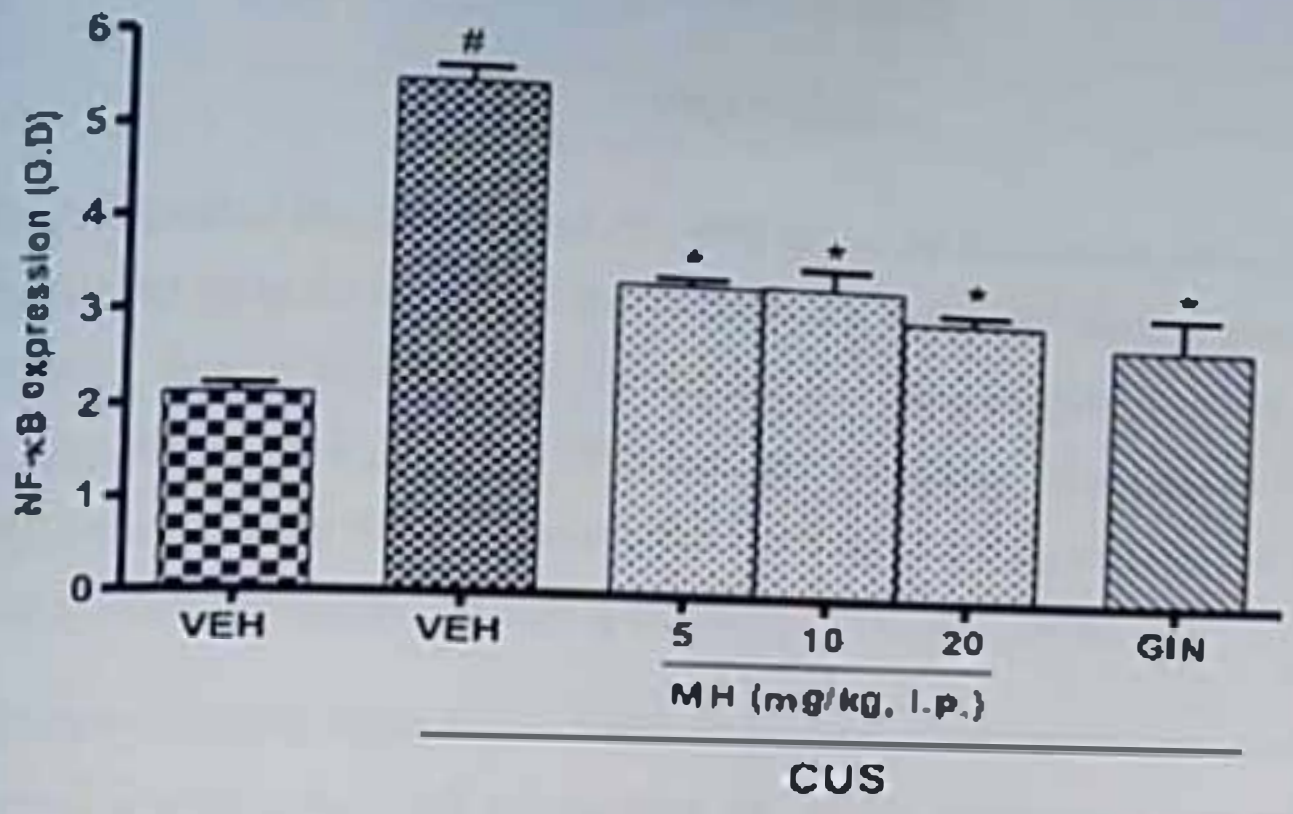


**Figure 61: Effect of Morin hydrate on NF-κB positive cells expression in the hippocampus following CUS exposure.**

Each column indicates mean ± S.E.M. 2 animals / group

# $p < 0.001$ relative to VEH unstressed group

* $p < 0.001$ relative to VEH CUS group (One-way ANOVA followed by Student-Newman-Keuls post-hoc test).

VEH: Vehicle (normal saline 10 ml/kg)

MH: Morin hydrate

GIN: Ginseng (25 mg/kg, i.p.)

CUS: Chronic Unpredictable Stress

# CHAPTER FIVE

# DISCUSSION

The physiological changes induced by stress are under normal circumstances self-limiting and adaptive, but when the stressful event overrides it's 'threshold' limits or maintained for extended periods of time, it becomes irreversible, and most physiological systems become negatively affected (Huang *et al.*, 2015). Therefore, the knowledge of adaptation has emerged which centres on explicating the mechanisms that aid in counteracting extreme and excessive responses to stress while augmenting the coping mechanism (Rai *et al.*, 2003).

Adaptogens are substances that are meant to produce a 'non-specific' resistance status to various stressors in organisms while normalizing the overall physiological function of the body by promoting homeostasis, strengthening and protecting the body against diverse stressors which compromise body systems and functions (Lakshmi and Sudhakar, 2009). Thus, the anti-stress activity of an adaptogen is attributed to its capability to boost adaptation during stressful exposure (Roshan *et al.*, 2010).

The principle on which the swimming endurance model is based is that rodents, when compelled to swim in a confined space initially begin by struggling vigorously in order to escape and eventually become immobile, indicating stress. In the swimming endurance model conducted, it was observed that morin hydrate prolonged struggling time. This increase in endurance is probably due to normalization of blood catecholamine and monoamine oxidase levels or by increase in utilization of the adenosine triphosphate (ATP) pathway. Increase in endurance could also be due diminished muscle glycogenolysis or reduced concentrations of muscle lactic acid and ammonia; which are poisonous by-products of muscular work (Debnath *et al.*, 2011). Like morin hydrate, the flavonoids rutin and quercetin have been shown to increase physical endurance and overall performance of mice in swimming endurance model. The potentials of these two flavonoids is ascribed to their ability to normalize plasma catecholamine and monoamine oxidase level (Lotankar *et al.*, 2016)

Oxygen is a vital element, on which all body functions including cellular respiration are dependent. Anoxic stress tolerance is characterized by shortage of oxygen supply which depicts an environmental stressor and any drug which increases adaptation under anoxic condition by increasing tolerance can act as an anti-stress agent (Singh and Yadav, 2014). The flavonoids rutin and quercetin have been shown to increase stress tolerance probably due to their ability to reduce cerebral oxygen consumption or confer resistance to anoxia, effects that are quite beneficial in the protection of neuronal cells against oxidative stress. It has also been stated that the ability of the two flavonoids to increase stress tolerance could be due to increase in succinate dehydrogenase level in the brain. Succinate dehydrogenase is concerned with the use and preservation of cellular energy which supports the adaptive processes in the course of stressful exposure (Lotankar *et al.*, 2016). In this study, morin hydrate also increased stress tolerance, indicating anti-stress activity.

Restraint model of stress in mice depicts a combination of physical and emotional stress leading to both restricted movement and aggressiveness with evident anxiety and depressive disorders (Kulkarni and Juvekar, 2009). Considerable evidences showing an association between markers of stress and these psychological disorders have accumulated (Goutam *et al.*, 2012). The EPM paradigm has been widely used for assessing anxiety responses and the typical manifestations of anxiety behavior are reduced frequency and duration of open arms exploration. When rodents are exposed to the new environment, they tend to avoid entering the open arm and prefer to stay in the closed arm, which indicates anxiety or fear. Anxiolytic drugs have the characteristics of reducing anxiety reactions of rodents in an elevated plus maze. Animals treated with an anxiolytic tend to explore and stay longer in the open arm relative to their untreated counterparts. Several plants and flavonoids have demonstrated anxiolytic effects as demonstrated by their ability to increase exploration in the open arms and so are largely used as anxiolytics in traditional medicine. Typical examples are *Ginkgo biloba* and the flavonoids linalool, and α-tocopherol (Almeida *et al.*, 2009). This present study revealed a reduction in the duration as well as frequency of open arm exploration which were reversed by treatment with morin hydrate indicating that the test drug could reduce fear and anxiety experienced after exposure to stressful conditions.

The FST model, like the swimming endurance test is based on behavioural despair in response to an inescapable circumstance and/or confinement. The alletation observed in acute stress-induced immobility in the FST suggests that Morin hydrate attenuates stress-induced depressive-like symptoms in mice. The principle on which the swimming endurance model is based is that rodents, when compelled to swim in a confined space initially begin by struggling vigorously in order to escape and then eventually become immobile, indicating stress

Acute restraint stress was earlier reported to induce hyperglycemia in rodents (Sugimoto *et al.*, 1998). Stressful stimuli activate the sympathetic nervous system with concomitant adrenaline secretion from the adrenal medulla. Elevated adrenaline level afterwards inhibits the β cells of the pancreas from secreting insulin, resulting in elevated blood glucose concentration. The hyperglycemic consequence of epinephrine and corticosterone is attributable to enhanced liver glycogenolysis all through acute stress (Kioukia-Fougia *et al.*, 2002). Adrenaline could also stimulate adenyl cyclase in the muscle and adipose tissue, causing an enhancement in intracellular cyclic AMP (cAMP) levels, with the responsibility of facilitating the mobilization of glucose and fany acid reserves in tissues. In this present study, morin hydrate reversed the hyperglycemic effect observed after acute restraint stress. The reference drug ginseng also reversed the acute restraint stress-induced hyperglycemia. Ginseng has previously been indicated to lower hyperglycemia caused by acute stress (Rai *et al.*, 2003).

Stress-induced alterations in specific lipid levels have been documented although the models and stress stimuli producing these alterations have been inconsistent. The depletion of glycogen store in the course of stressful stimuli and corticosterone release instigates gluconcogenesis and mobilization of fat reserves as alternative energy resource. This decreases triglycerides and cholesterol levels (Kioukia *et al.*, 2002). Reduction in serum triglyceride, which act as a rapid source of energy during stressful conditions could also be as a result of catecholamines actions on triglyceride lipase activity in fat tissues (Singh *et al.*, 2001). Acute restraint stress lowers serum cholesterol and triglyceride levels in this study. This could be as a result of mobilization of energy substrates to the particular sites of demand. Moreover, treatment with morin hydrate normalized the cholesterol level but with no effect on triglycerides.

The nervous system has lots of oxidizable substrates, has high oxygen pressure and has low antioxidant capacity making it extremely sensitive to oxidative damage (Metodiewa and Koska,

2000). Stress has been well-documented to enhance free radicals generation which has also been linked to HPA axis hyperactivation with resultant rise in corticosterone discharge (Liu *et al.*, 1996). Also, sympathetic activation in the course of stress elevates respiration rate with concomitant generation of more oxygen for tissues, resulting in additional free radicals production, leading to reactive oxygen specie/antioxidant system imbalance (Halliwell, 1994). These free radicals trigger oxidative stress that cause damages to hippocampal neurons which uphold the homeostatic condition of the HPA axis via negative feed back mechanisms, leading to increase in corticosterone secretion (Halliwell, 1994). Studies have shown that restraint stress induced oxidative damage to the lipid, protein, and DNA in the brains of rodents and natural antioxidant supplements mitigate this effect and increase body performance during exposure to stressful stimuli (Samarghandian *et al.*, 2013). In this study, acute restraint stress provoked lipid peroxidation (as depicted by elevated MDA levels), elevated nitric oxide levels and caused reduction in the glutathione content of the brain. These effects were significantly reversed by morin hydrate basically due to its radical scavenging effects as an account of the free radical scavenging activities of morin hydrate have been previously suited (Venu Gopal *et al.*, 2013).

The outcome of chronic stress which represents an allostatic phase in humans or animals is seen in endocrine alterations (Van Cauter *et al.*, 2007), disruption of sleep patterns (Papale *et al.*, 2005) and behavioural and memory deficits (Stickgold and Walker, 2007). It's an aftermath of the inability of the adaptive stress response to survive a variety of stressors of different frequency or intensity (Saraswathi *et al.*, 2010). Several neurodegenerative diseases have been linked with chronic stress (Pittenger and Duman, 2008).

Various contradicting reports on how chronic stress affects blood sugar levels have been documented. Several studies revealed that chronic stress might induce type I diabetes mellitus in humans, and in different experimental animal model (Mirshekar *et al.*, 2015). Corticosterone released during chronic stress induces glycogenolysis in the liver, resulting in glucose mobilization which contributes to the hyperglycemia observed (Kioukia-Fougia *et al.*, 2002). This elevated glucose level is vital for the maintenance of the availability of ATP to the brain, muscles and other organs of demand (Kioukia-Fougia *et al.*, 2002). An elevation in glucose level was noted in this study which was significantly attenuated by Morin hydrate. Moreover, *Morus*

2000). Stress has been well-documented to enhance free radicals generation which has also been linked to HPA axis hyperactivation with resultant rise in corticosterone discharge (Liu *et al.*, 1996). Also, sympathetic activation in the course of stress elevates respiration rate with concomitant generation of more oxygen for tissues, resulting in additional free radicals production, leading to reactive oxygen specie/antioxidant system imbalance (Halliwell, 1994). These free radicals trigger oxidative stress that cause damages to hippocampal neurons which uphold the homeostatic condition of the HPA axis via negative feed back mechanisms, leading to increase in corticosterone secretion (Halliwell, 1994). Studies have shown that restraint stress induced oxidative damage to the lipid, protein, and DNA in the brains of rodents and natural antioxidant supplements mitigate this effect and increase body performance during exposure to stressful stimuli (Samarghandian *et al.*, 2013). In this study, acute restraint stress provoked lipid peroxidation (as depicted by elevated MDA levels), elevated nitric oxide levels and caused reduction in the glutathione content of the brain. These effects were significantly reversed by morin hydrate basically due to its radical scavenging effects as an account of the free radical scavenging activities of morin hydrate have been previously stated (Venu Gopal *et al.*, 2013).

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*alba*, from which Morin hydrate was originally isolated has been found to possess antidiabetic activity by increasing insulin production (Bagachi *et al.*, 2013).

Stress-induced elevations of circulatory lipids and lipoproteins are biologically and clinically significant as considerable evidences showing an association between circulatory lipid elevations, cardiovascular diseases and markers of stress have been demonstrated (Artham *et al.*, 2008). Chronic stress raises triglyceride levels probably due to "stress eating" (Parker, 2013) and cholesterol levels via mechanisms associated with enhanced HPA axis activity with subsequent increase in catecholamine and cholesterol concentrations (Lakshmi and Sudhakar, 2009; Nayanatara *et al.*, 2012). Catecholamines are known to activate lipolysis via stimulation of β3 adrenoceptors in adipose tissues, and suppress insulin secretion (Shabir *et al.*, 2013). Similarly, glucocorticoids and free fatty acids increase HMG-CoA reductase activity in hepatocytes, promoting cholesterol synthesis (Niaura *et al.*, 1992). Additionally, catecholamines suppress hepatic lipase activity, raising blood LDL, IDL and VLDL concentrations (Niaura *et al.*, 1992). Research have revealed that chronic restraint stress elevates triglyceride and cholesterol levels in rodents (Kulkarni and Juvekar, 2008; Shabir *et al.*, 2013). In this study, Morin hydrate significantly reversed chronic restraint stress-induced increase in triglyceride and cholesterol levels. Furthermore, chronic restraint stress lowered HDL level which mediates cholesterol homeostasis and is highly inversely correlated with heart diseases (Tan *et al.*, 2006). HDL possesses anti-atherogenic property by promoting the efflux of cellular cholesterol and reversing cholesterol transport. Furthermore, HDL cholesterol possesses anti-inflammatory, antioxidant and anticoagulant effects (Wang and Briggs, 2004). In this study, Morin hydrate administration reversed the reduction of HDL induced by chronic restraint stress, and consequently lowered the atherosclerotic index indicating that Morin hydrate may protect against stress-induced elevation of cardiovascular risk indicators.

Chronic restraint stress also triggers excessive free radicals production, instigating lipid peroxidation, particularly in cell membranes. Several researches indicated that chronic restraint stress causes lipid peroxidation and nitrite production, and depleted GSH level (Ahmad *et al.*, 2012). Similarly, chronic restraint stress induced oxidative stress in this study via increasing brain MDA and nitrite levels and decreasing GSH level. However, administration of Morin hydrate ameliorated these effects.

Neurons are the basic functional and structural component of the nervous system. Various factors such as stress alter the structural makeup of neurons, in particular dendritic arbanisation, synaptic junctions, neurochemical components and functions (Hemamalini, 2013). Chronic stress exposure dynamically controls the complexity of dendrites. These stress-induced alterations in dendritic structures are observed in neurons of the prefrontal cortex, amygdala, and hippocampal CA3 pyramidal neurons (Krugers *et al.*, 2010). Dendritic atrophy in these regions is observed during prolong stress exposure which may affect their various functions. This could be due to reduced brain derived neurotropic factor (BDNF) expression, apoptosis of the neurons, glucocorticoid toxicity, reduced functionality of the GABA-ergic network, glutamate-induced excitotoxicity or increased intracellular levels of $Ca^{2+}$ (Hemamalini, 2013). Enhanced $Ca^{2+}$ levels are known to collapse microtubules and activate calcium-activated neural proteinase, an enzyme predominantly found in neurons which controls cytoskeletal proteins disintegration (Hemamalini, 2013). This results in retraction and collapse of dendrite branches, as structural integrity of neuronal processes requires stable microtubules (Hemamalini, 2013). Several anti-stress agents have been shown to rescue neurons from death and reduce dendritic atrophy in stress conditions through several mechanisms and recently, the neuroprotective activities of flavonoids have become the point of focus (Hemamalini, 2013). The mechanisms of flavonoids action could be through induction of neurogenesis, formation of new dendrites (neurostimulating effect), or through corticotrophin releasing factor stimulation, which may in turn cause an increase in hippocampal synaptic efficacy (Hemamalini, 2013). Flavonoids could also protect neuronal cells against stress induced neuronal injury through free radicals scavenging and neuron protection against oxidative damage evoked by excess glutamate release (Lee *et al.*, 2000). A typical example is the flavonoid Myricetin which enhanced survival of hippocampal CA3 neurons and inhibited cognitive impairments in rats with Alzheimer's disease (Ramezani *et al.*, 2016). The outcome of this study further confirmed that chronic restraint stress produced severe neuronal cell necrosis and structural damage in the hippocampus and prefronal cortex. Accordingly, since morin hydrate is shown to possess antioxidant activity, it is hereby hypothesized that the neuroprotection observed on administration of morin hydrate might be through its protection against oxidative stress. Moreover, it was reported that *Morus alba*; the plant from which morin hydrate was originally isolated possess neuroprotective property via its free radicals scavenging capability (Bagachi *et al.*, 2013).

Paradoxical sleep deprivation; a condition not usually explored in most research on stress-induced transformations as a stressor is included as a model to further strengthen the observed anti-stress effect of Morin hydrate. Sleep deprivation leads to various neurobehavioral effects and produce gradual changes in specific neuroendocrine systems in ways akin to what is seen in some stress-related conditions explained in chapter 2 (Pires *et al.*, 2012). This supports the view that insufficient sleep may sensitize individuals to stress-related disorders, by acting on stress systems (Meerlo *et al.*, 2008). Also, these marked behavioral changes may be due to structural remodeling that could aggravate anxiety as well as memory dysfunction and aggression in the hippocampus, prefrontal cortex and amygdala. Furthermore, reports have shown that sleep deprivation influences motor behaviour (Vollert *et al.*, 2011), causes oxidative stress (Gopalakrishnan *et al.*, 2004), impair cognitive functions (McEwen 2007) and eventually results in brain cells damage. Results from previous studies show that sleep deprivation impairs memory function via initiation of oxidative stress as well as HPA axis modulation, which can cause some detrimental physiological effects (Meerlo *et al.*, 2008). The Y-maze as a model for evaluating working memory is centred on the rodent's ability to enter into as many different arms as possible and at the same time attempting to recall the precise order of arms alternation (Rothbart, 2007). This is usually correlated to spatial working memory and it is expected that a mouse prefers exploring a new arm while avoiding a previous one; a measure of memory usually called 'spontaneous alternation' (Rothbart, 2007; Lee *et al.*, 2010). In this study, sleep deprivation reduced percentage spontaneous alternations relative to the unstressed control, suggesting memory impairments. This observation further supports previous studies showing that sleep deprivation impaired memory performance in rodents (McEwen, 2007). However, the capability of morin hydrate to significantly counteract the decline in memory performance caused by sleep deprivation, as indicated by the increase percentage spontaneous alternations suggests its anti-amnesic property that might make it applicable as a remedy for amnesic conditions linked with sleep deprivation.

Anxiety-like behaviour which is a disorder that triggers unnecessary panic and nervousness was assessed using EPM. The usefulness of this model as a behavioural utility test is founded on the fact that rodents are naturally inclined to explore new and differing environments, which include the two closed arms and the potentially dangerous open arms (Crayan and Holmes, 2005). This test is very useful in testing for anxiolytic properties of new compounds as anxiolytics increases

the rodent's inclination towards exploring the open arms than the closed arms. Sleep deprivation caused a significant decline in open arm exploration, which suggests increase in anxiety-like behaviour in this study. This further buttressed the outcome of an earlier study which indicated that anxiety-like behaviour increases with sleep deprivation (Pires *et al.*, 2012). Morin hydrate however exhibited anxiolytic property as it significantly increased open arms exploration, which agrees with previous studies (Mangaiarkkarasi *et al.*, 2012).

Research demonstrated that the neurobiological consequences of sleep deprivation are akin to those of psychostimulants like cocaine and amphetamine which are capable of elevating dopamine function and increasing dopaminergic neuron firing, resulting in hyperlocomotion (Koob and LeMoal, 2008). Indeed, studies in animals have linked sleep deprivation with dopaminergic system hyperactivity as a consequence of increase in dopamine release and elevated dopaminergic neurons firing (Zant *et al.*, 2011). Furthermore, it was reported that acute intake of cocaine-induced hyperlocomotion in mice was aggravated when the animals were sleep deprived for 6 hours (Berro *et al.*, 2014). Accordingly in this study, sleep deprivation for 48 hours provoked behaviours related to hyperlocomotion. Thus, the capability of morin hydrate to reverse hyperlocomotion caused by sleep deprivation, as demonstrated by the reduction in line crossing in the automated activity cage indicates its calming and relaxing effects.

It's been theorized that the effectiveness of the brain's antioxidant mechanism is enhanced by having adequate sleep as studies in sleep deprived rats have showed an elevation in thalamic and hypothalamic oxidative stress (D'Almeida *et al.*, 2000). Accordingly, assaying for oxidative stress markers will provide a good understanding of the damages induced by sleep deprivation (Garg and Kumar, 2008). A study conducted in mice revealed that sleep deprivation by the platform method for 72 hours increased hippocampal oxidative stress (Vollert *et al.*, 2011). Likewise, sleep deprivation for 48 hours triggers lipid peroxidation, raised nitrite and decreased catalase and GSH levels (Kalonia and Kumar, 2007). The result of this study further supports the report as sleep deprivation raises MDA and nitrite levels, whilst decreasing GSH activity. This could participate in the anxiety and memory impairment observed as there are evidences that oxidative stress markers resulting from sleep deprivation promotes impairment of cognitive function. Morin hydrate however attenuated nitrite and MDA levels, and additionally restored

GSH levels in mice brains. This suggests that an underlying mechanism of the stress-protective effects of morin hydrate is via its antioxidant property.

Sleep plays a very vital function in promoting synaptic plasticity, neuronal recovery and maintenance of proper brain function (Meerlo *et al.*, 2009). Sleep deprivation is believed to cause damages to brain functions and in addition accelerate the advancement of neuropathy as several studies have demonstrated that various adult neurogenesis markers are mitigated by sleep deprivation which also hasten neuroinflammation and consequently the neurodegenerative process through either altered levels of neuroprotective markers, heightened sensitivity to neuronal excitatory pathways, increased hippocampal glutamate receptors expression or $Ca^{2+}$-induced excitotoxicity (Torabi-Nami *et al.*, 2013). Reports have also indicated that sleep deprivation can hinder neurogenesis in rats through corticosterone surge (Mirescu *et al.*, 2006; Mirescu and Gould, 2006). Analysing the outcome of sleep deprivation on cognition revealed that the brain region especially susceptible to sleep deprivation is the hippocampus. This brain region participates crucially in emotion together with cognition regulation and an interrelation between hippocampal volume reduction and the origin and symptom of depression and other emotional disorders have been established (Lucassen *et al.*, 2010). Studies carried out in rats revealed that sleep deprivation reduces neurogenesis in the dentate gyrus and initiates neuronal cell death (Hairston *et al.*, 2005). This could be due to diminished antioxidant protection (Czeh and Lucassen, 2007). Moreover in this study, sleep deprivation raised the magnitude of CA1 neurons damage as revealed by H&E staining and as a consequence decreased the population of viable neuronal cells. However, pretreatment with morin hydrate significantly reduced neuronal damage and rescued viable neuronal cells in the hippocampus, suggesting new protective effect.

Studies revealed that when rodents are exposed to one particular type of stressor for prolong period, they tend to adapt to the stressor (Zheng *et al.*, 2009). Thus, in order to rule out the process of adaptation, and to increase the degree of stress-related consequences, this study subjected animals to different stressors at varying durations in the chronic unpredictable stress (CUS) protocol, mimicking the variability of stressors encountered in everyday life. The protocol was also employed to explore the probable molecular mechanisms underlying the anti-stress actions of Morin hydrate. The CUS paradigm is a widely used and applicable stress model in rodents which mimic several behavioural features noted in individuals with depression, anxiety

and mood disorders and causes immunological changes symbolic of chronic stress response (Pittenger and Duman, 2008).

Exposure to CUS can intensify anxiety symptoms in humans and animals (McEwen, 2000). This anxiety symptom either appears after the stressful stimuli has been terminated in humans or could be delayed as seen in some rodents (Pandey *et al*., 2010). The reduction observed in frequency as well as duration of open arms exploration in this study is indicative of anxiogenic effect of CUS. This result further agrees with those obtained in the chronic restraint and PSD models. Also, treatment with Morin hydrate reversed the effect of CUS, as observed in other models. This further confirms that Morin hydrate possess protective effect against stress-induced anxiety.

The mammalian hippocampus is highly populated with glucocorticoid receptors which trigger the alteration of synaptic terminal structures in response to high corticosterone levels (as seen in chronic stress) resulting in neuronal atrophy, hippocampal cell death and ultimately memory impairments (Eichenbaum, 2000). Exposure of rodents to chronic unpredictable stress was reported to alter specific brain structures like the hippocampus, thereby causing deficits in post-stress acquisition and retrieval memory in mice (Bhatia *et al*., 2011). Research indicated that rodents subjected to a variety of physical stressors on a daily basis for 28 days experienced deficiencies in performance and learning in the Morris Water Maze. This was due to excessive free radicals production which inflicts detrimental effects on neurons (Liu and Mori, 1999). Furthermore, various accounts suggestive of the role of elevated cholesterol levels in memory impairment have been documented. Under physiological conditions, cholesterol participates enormously in learning and memory process but is linked with memory impairments when in excess (Biondi, 2006). In this study, CUS impaired memory function as seen in the Y maze test where percentage alternation of stressed mice was reduced. This chronic unpredictable stress-induced memory impairment could be as a result of elevated levels of circulatory cholesterol which was ameliorated by administration of Morin hydrate. This further buttress the finding that Morin hydrate ameliorates stress-induced memory impairment in mice.

Studies have shown that rodents exposed to CUS exhibited reduced locomotor activity which could simulate some characteristics of human motormental retardation, a symptom which

accompanies major depression in humans (Pandey *et al.*, 2010). Also, when placed in a novel environment, rodents generally show increased ambulation, whereas, rodents exposed to CUS exhibit reduced ambulation in a new environment, a discrepancy which could be effectively upturned by the anti-depressant drug escitalopram in accordance with past research on the effectiveness of anti-depressants in reversing inescapable stress-induced hypolocomotion in mice in the open field (Pal and Dandiya, 1994). This study employed an automated activity cage to check the outcome of CUS on locomotion and the role of Morin hydrate. In confirmation of previous studies, it was observed that CUS reduced locomotor activity, an event that was upturned by morin hydrate treatment. This further confirmed the suggestion that Morin hydrate attenuates stress-induced depressive-like symptoms in mice.

Chronic unpredictable stress has been stated to induce hyperglycemia, and increase cholesterol and triglycerides levels in rodents via HPA axis hyperactivation (Bhatia *et al.*, 2010). This hyperglycemia is mediated through circulating corticosterone. Likewise in this study, CUS significantly increased the level of blood glucose, cholesterol and triglyceride, which was attenuated by chronic administration of Morin hydrate. CUS reduced the level of HDL cholesterol, increasing the risk of a cardiovascular disorder as indicated by the atherosclerotic index. Also, this was attenuated by Morin hydrate administration.

Chronic unpredictable stress is also characterized by free radicals generation and HPA axis hyperactivation with consequent corticosterone hypersecretion (Liu *et al.*, 1996). Free radicals on the other hand may be involved in HPA axis hyperactivation and corticosterone hypersecretion by triggering hippocampal neuronal damage (Bhatia *et al.*, 2011). Several accounts on the anti-stress effects of free radicals scavengers like quercetin (Tiwari *et al.*, 2015), curcumin (Bhatia *et al.*, 2011), triphala (Sonkar and Mishra, 2011), vitamin C (Kelly, 1999) and vitamin E (Al-Ayadhi *et al.*, 2006) have been stated. Morin hydrate was also found to mitigate oxidative stress as observed via decline in MDA, nitrite and elevated GSH concentrations. These findings additionally indicate that the antioxidant action of Morin hydrate plays a significant part in its anti-stress effects.

Among the prominent changes which occur during chronic stress are glucocorticoid hypersecretion, adrenal glands hypertrophy as well as increased corticosterone levels (Makara

and Haller, 2001). Prolonged HPA axis activation gives rise to hypertrophy of the adrenal glands. Adrenal gland hypertrophy further revealed that the stress-responsive HPA axis is practically and actively involved (Kenjale *et al.*, 2007). Stressful stimuli stimulates the adrenomedullary response to release adrenaline which then stimulates the pituitary β2 adrenergic receptors to release large amounts of adrenocorticotropin hormone (ACTH) which upon release, stimulates the adrenal cortex as well as the adrenal medulla with concomitant secretion of corticosterone from the adrenal cortex and further epinephrine release from adrenal medulla and an increase in adrenal gland weight to a larger extent (Zafir and Banu, 2009). Several adaptogenic substances such as Curcumin (Bhatia *et al.*, 2011), *Panax ginseng* and *Gingko biloba* (Rai *et al.*, 2003) are indicated to possess anti-stress effects, owing to their ability to decrease stress-induced adrenal hypertrophy and circulating corticosterone. In this study, CUS was found to significantly increase adrenal gland weight with concomitant elevation of serum corticosterone level, indicating hyperactivation of the HPA axis. This was significantly attenuated by long term administration of Morin hydrate.

High levels of glucocorticoids inhibit virtually all components of the inflammatory response. At the cellular level, glucocorticoids possess anti-inflammatory and immunosuppressive actions through alteration of the function and circulation of leucocytes, decreasing inflammatory mediators and cytokines production (Chamondari, 2005). Likewise at the early stage of chronic stress, glucocorticoids downregulate proinflammatory cytokines while upregulating anti-inflammatory cytokines (Tian *et al.*, 2014). The second stage of chronic stress causes HPA axis fatigue and glucocorticoid-resistance, in which the ability of glucocorticoids to curtail cytokines production becomes diminished (Cohen *et al.*, 2012; Tian *et al.*, 2014). As a result, the inflammation related pathways are activated and the genes responsible for proinflammatory cytokine production are in turn activated, resulting in upregulation of the proinflammatory cytokines (Miller *et al.*, 2008).

Chronic unpredictable stress increases proinflammatory cytokines production which then triggers the stimulation of nuclear factor NF-kB, the proinflammatory transcription factor needed for the proinflammatory gene iNOS expression (Li and Kann, 1999). Nitric oxide synthase is concerned with catalyzing the production of nitric oxide; a strong oxidant molecule involved with neurodegeneration. Studies have revealed that stress-induced TNF-α overproduction is

implicated in iNOS expression through a NF-κB - dependent mechanism (Madrigal *et al.*, 2002). Activated NF-κB in turn causes further increase of proinflammatory cytokines to a certain level and unless the sustained stress exposure is removed, the proinflammatory cytokines further increase, inducing an inflammatory response. Flavonoids are renowned for their anti-inflammatory activities via inhibiting the production of the transcription factor NF-κB, along with other pro-inflammatory mediators (Serafini *et al.*, 2010). Among these are the flavonoids epicatechin and isoquercitrin isolated from *Theobroma cacao*, which were shown to inhibit TNFα, and IL-6 mRNA expression as well as nitric oxide secretion (Ramiro *et al.*, 2005). The CUS elevated TNF-α and IL-1β level in mice brains in this study. Also NF-κB and iNOS were seen to be highly expressed, indicating neuroinflammation. Morin hydrate treatment however reduced IL-1β and TNF-α, levels. Moreover, it also reduced NF-κB and iNOS expressions, demonstrating the anti-neuroinflammatory effect of Morin hydrate.

Elevations in the circulating corticosterone levels as observed in CUS can also trigger hippocampal impairment in rodents by increasing basal glutamate levels or cytosolic calcium load in the hippocampus (McEwen, 1999). The result of this study also confirmed that CUS causes structural damages and loss of hippocampal neurons. Moreover, morin hydrate treatment attenuated these effects.

# CHAPTER SIX

# SUMMARY AND CONCLUSION

## 6.1 SUMMARY

The study sought to evaluate the mechanisms of antistress activity of Morin hydrate in mice. Different acute and chronic models of stress were employed. Morin hydrate prolonged swimming time and onset of convulsion in the swimming endurance and anoxic tolerance tests respectively. Morin hydrate significantly attenuated both acute and chronic stress-induced memory impairment, anxiety and depressive like symptoms, and normalized blood glucose cholesterol and triglycerides which were increased by stress. Additional results obtained from the study revealed that Morin hydrate possess antioxidant activity as it significantly reduced MDA and nitrite levels, and increased the endogenous antioxidant, GSH. Furthermore, Morin hydrate inhibits stress-induced adrenal hypertrophy and reduced corticosterone levels, indicating a reduction in the HPA axis hyperactivation, reduced IL-1β and TNF-α levels in mice brains and also significantly reduced the expressions of iNOS and NFkB, and attenuated stress-induced neuronal atrophy.

## 6.2 CONCLUSION

The study hereby concludes that Morin hydrate protects against some detrimental changes induced by acute and chronic stress such as oxidative stress, HPA axis hyperactivation, hyperglycemia, hyperlipidemia and neuroinflammation. The mechanisms of action of Morin hydrate include inhibition of HPA axis hyperactivation, oxidative stress, hyperglycemia, hyperlipidemia and neuroinflammation.

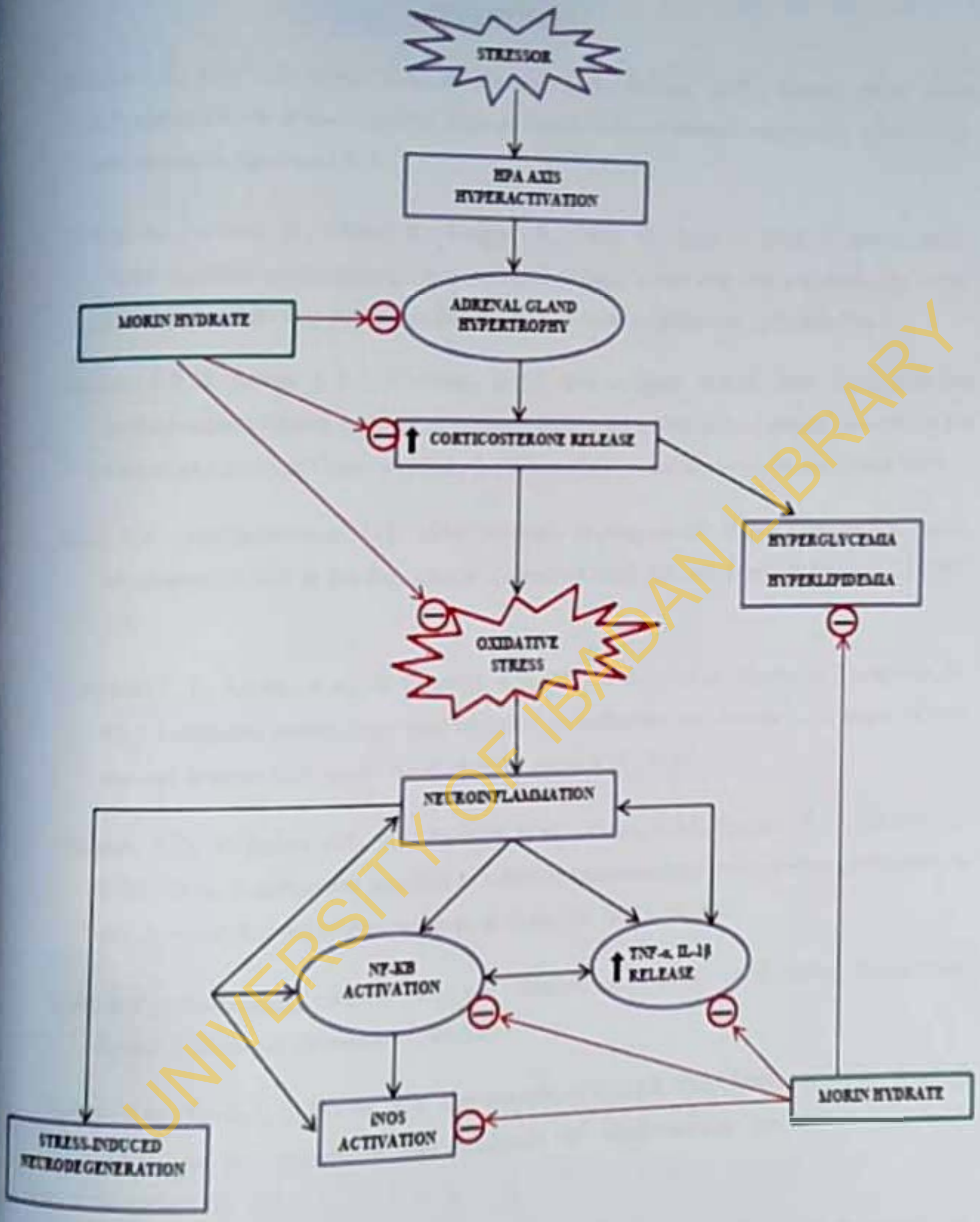


Figure 62: PROPOSED MECHANISMS OF ACTION OF MORIN HYDRATE

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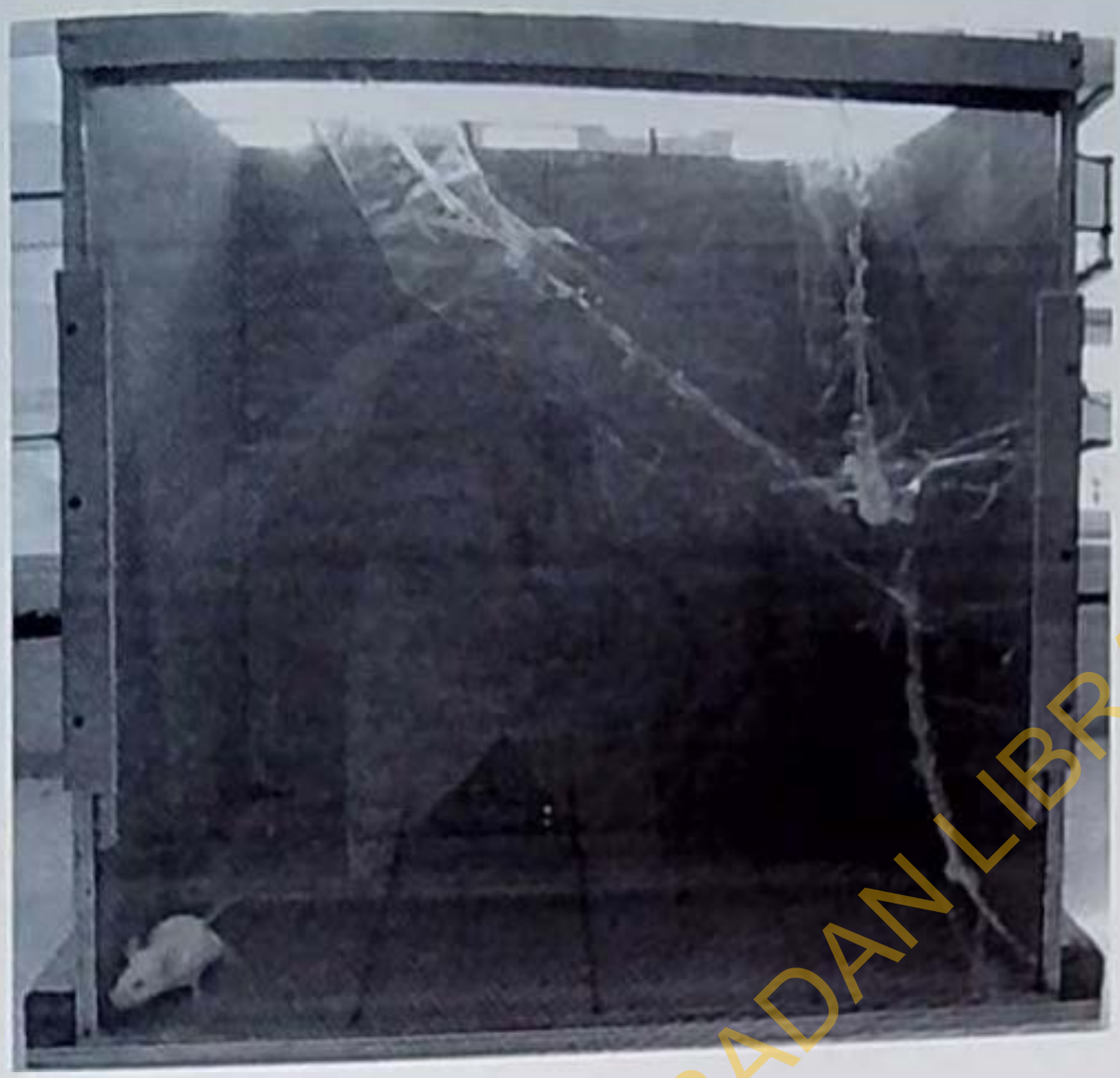
**Appendix**

The hole board apparatus

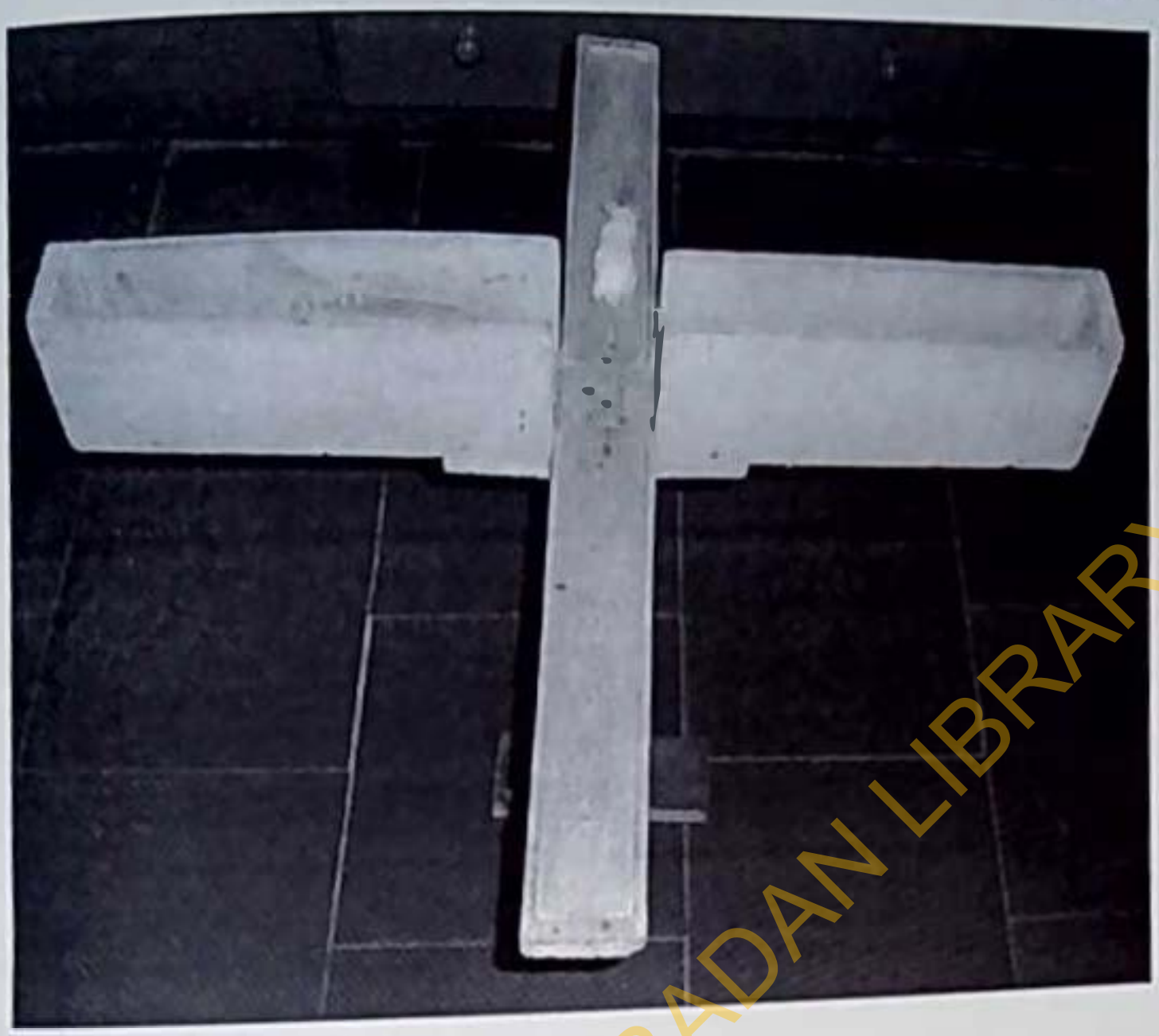


The open field apparatus

Plastic restrainer

The Y maze apparatus

The forced swim apparatus



The elevated plus maze

The elevated plus maze