NARINGENIN ATTENUATES NEUROBEHAVIOURAL DEFICITS AND BIOCHEMICAL PERTURBATIONS INDUCED BY CHRONIC HYPOXIC STRESS IN MICE

BY

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A Thesis in Department of Pharmacology & Therapeutics

Submitted to the Postgraduate College

University of Ibadan, Ibadan.

JULY, 2021

CERTIFICATION

I certify that this work is an original research carried out by Abimbola Sadiat Olugbemide, Department of Pharmacology & Therapeutics, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan.

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DEDICATION

This research work is dedicated to God, Almighty, King of kings, and Lord of lords for seeing me through and for making this research work a success.

ACKNOWLEDGEMENT

My immense gratitude goes to the All-knowing God, revealer of all secrets. The giver and sustainer of life. Hitherto He has helped me!

I am very grateful to everybody that has contributed to the success of my program particularly my supervisor, Professor Solomon Umukoro for his great understanding, support, encouragement and guidance towards the completion of this project.

I sincerely appreciate Dr. B. Ben Azu, Dr. A.G. Bakare, Dr. A.M. Ajayi, Dr Wumi Femi-Akinlosotu for their great contribution towards the completion of this project.

Kudos to all my lecturers: Professor F. A. Fehintola (the H.O.D), Professor A. Sowunmi, Professor Catherine O. Falade, Professor O.G Ademowo, Professor Grace O. Gbotosho, Professor E.O. Iwalewa, Dr. A.O. Aderibigbe, Dr. Aduragbenro A. Adedapo, Dr. O. Abiodun, Dr. O.S. Michael, Dr. J.A Badejo for their efforts in making the department conducive for research work.

I thank all other students of Neuropharmacology Unit, for being suppotive and encouraging throughout the research period, especially Dr B. Ben-Azu, Dr. Oritoke M. Aluko, Mr. Abayomi Adelaja, Dr. T.A. Edeviere, Dr. Mrs Toyin Akinniyi..

I appreciate Mr.W.B. Adegoke, chief technologist and other technologist: Mr.M.O. Olatunde and Mr. O.P. Akintoye, for their assistance, as well as the administrative staff; my colleagues and friends, all of Department of Pharmacology & Therapeutics.

My profound gratitude goes to my parent Mr. and Mrs. Fatai Ajibola Omotosho, my baby sister Morolake thank you for believing in me. To my husband, Revd. Barrister Abiodun Olugbemide, my adorable Omotola and Olamide you have contributed in no small way to this success story. I am grateful to many more people who have in one way or the other contributed to the success of this research work but whose names are not mentioned, I acknowledge your sincere effort and time.

MAY GOD BLESS YOU ALL

ABSTRACT

Hypoxic stress is known to induce depression, cognitive dysfunction and anxiety-related complications through the activation of oxidative and inflammatory signaling pathways. Thus, inhibition of these pathways might mitigate hypoxic stress-induced neurobehavioural deficits. Experimental studies have shown that naringenin improves neurobehavioural disorders induced by ischemic stroke *via* inhibition of oxidative stress, neuroinflammation and neurodegeneration. However, there is paucity of information on its protective effects against neurobehavioural deficits induced by Chronic Hypoxic Stress (CHS). Hence, this study was designed to evaluate the effects and biochemical mechanisms of naringenin on CHS-induced depression, memory deficit and anxiety related behaviours in mice.

Thirty-five male Swiss-mice (20-22 g) were distributed into 5 groups (n=7) and treated intraperitoneally. Mice in groups I (non-stress control) and 2 (stress-control) received 5%-DMSO (vehicle), while groups 3-5 were treated with 10, 25 and 50 mg/kg analytical grade of naringenin, daily for 14 days. Thirty minutes after daily treatment, each mouse in group 2-5 was subjected to 15 minutes hypoxic-stress in an air-tight 250 mL cylindrical vessel for 14 consecutive days. The neurobehavioural phenotypes (locomotor activity, anxiety, depression and memory) were evaluated on day 15 using standard experimental procedures. Thereafter, the animals were euthanized and the harvested brains were processed for determination of malondialdehyde, reduced glutathione (GSH), nitrite, superoxide-dismutase and catalase using standard biochemical techniques. Serum corticosterone and brain Tumor Necrosis Factor-alpha (TNF-α), and interleukin-1β were assayed using ELISA kits. The expressions of Inducible Nitric Oxide Synthase (iNOS), Nuclear Factor Kappa-B (NF-kB) and Brain Derived Neurotrophic Factor (BDNF) were determined using immunohistochemical techniques. The histomorphological changes of the amygdala were also determined using hematoxylin and eosin, and cresyl violet stains. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

Naringenin (25 and 50 mg/kg) relative to stress-control significantly attenuated CHS-induced locomotor deficit (11.71±0.57 and 12.29±0.57 vs 8.29±0.68) and prolonged immobility time in the test for depression (104.40±9.31 and 139.70±8.34 vs 197.40±6.83sec). It also reduced anxiety-like behaviours but did not ameliorate memory deficit induced by CHS. Naringenin (10, 25 and 50 mg/kg) reduced malondialdehyde concentration (36.23±0.96, 40.65±1.60, 67.39±0.32 vs 79.86±4.26 μmol/g tissue) and increased GSH levels (20.85±0.63, 21.99±0.74, 21.65±0.46 vs 17.50±0.50 μmol/g tissue). It also restored the altered brain nitrite content and superoxide dismutase activity but not catalase. Naringenin (25 and 50 mg/kg) reduced CHS-induced increase in the brain contents of TNF-α (37.43±0.63 and 38.84±2.21 vs 50.14±2.26 pg/mL) and interleukin-1β (190.60±11.19, 157.60±6.09 vs 245.70±8.54 pg/mL). The CHS-induced increased brain expressions of iNOSand NF-kBimmunopositive cells were attenuated by naringenin. It also increased BDNF expressions but did not alter serum corticosterone. Histomorphological distortions and loss of neuronal cells in the amygdala induced by CHS was reduced by naringenin.

Naringenin attenuated depression and anxiety like behaviours induced by chronic hypoxic stress. The mechanism was via neuroprotection relating to inhibition of oxidative stress, proinflammatory cytokines, expressions of inducible nitric oxide synthase and nuclear factor kappa-B, and upregulation of brain derived neurotrophic factor expressions.

Keywords: Naringenin, Chronic hypoxic stress, Inflammatory mediators, Brain derived neurotrophic factor

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LIST OF ABBREVIATIONS

AD: Alziemers Disease

BDNF: Brain derived neurotrophic factor

DMSO: Dimethyl sulfoxide

DTNB: 5, 5'-Dithio-bis- (2-nitrobenzoate)

GSH: Glutathione

HOD: Head of Department

HPA: Hypothalamus Pituitary Adrenal

IL-6: Interleukin-6

iNOS: Inducible nitric oxide synthase

IP: Intra peritoneally

MDA: Malondialdehyde

NF-kB: Nuclear factor kappa-B

NG: Naringenin

PG: Panax ginseng

RNS: Reactive Nitrite Species

ROS: Reactive Oxygen Species

SOD: Superoxide-dismutase

TBA: Thiobarbituric acid

TCA: Trichloroacetic acid

TNF-α: Tumor necrosis factor-alpha

TST: Tail suspension test

CHAPTER ONE

INTRODUCTION

1.1 Contextual framework

In perspective of neurobiology, stress has been referred to as conditions capable of distorting body homeostasis occasioned through extrinsic and intrinsic forces, which are counteracted through complex mechanisms of physiological as well as behavioural processes tailored towards restoration of ideal body dynamic equilibrium critical for living organisms' survival as well as optimal health functioning (Chrousos and Gold, 1992; Tsiogos *et al*., 2016). Optimal body equilibrium has been known to be subjected to constant aversive conditions, otherwise described as stressors, either perceived (psychological) or real (physical). Stress, may therefore be regarded as disharmonious condition that activate adaptive stress response that tend to abolish threat to body homeostatic mechanisms. Response to stress has been shown to entails interrelated complex molecular, cellular as well as neuroendocrine apparatus collectively known to be stress systems (Chrososs and Gold, 1992; Chrousos, 2009). It has been reported that autonomic nervous system (ANS) including hypothalamic pituitary adrenal (HPA) axis were the principal components of the stress system that interact with some vital brain regions as well as other body organs in the periphery for efficient mobilisation of defensive mechanisms crucial in mitigating stress pathologies (Tsiogos *et al*., 1994; Tsiogos *et al*., 2016).

Adaptive mechanisms are known to be activated in order to restore stability of internal environment meant for promotion of survival of living organisms (McEwen, 2006; Karatsoreosand McEwen, 2011). Although there are different types of stressors, it has been observed that the key circuitries underpinning stress responses in different situations are essentially identical (McEwen, 2006; Logan and Barksdale, 2008). However, functional deficiency of adaptive mechanisms due to chronic stress leads to disruption of

body homeostasis and constellations of many physiological derangements (Gold, 2015; Tsigos *et al*., 2016). Disequilibrium between allostasis and allostatic load in favour of the later has been shown to be the major driver of multiple pathological derangements associated with chronic stress (Logan and Barksdale, 2008). However, there are emphasis on implementation of strategic measures in promoting resilience against stress, thereby promoting health outcomes. It has been reported that these strategies are expected to attenuate aversive reactions precipitated by persistent stress, as important steps towards mitigation of harmful consequences of prolonged stress on mankind (Logan and Barksdale, 2008).

It has been succinctly established that diverse acute-stressors elicit clusters of time-limited physical as well as behavioral changes that are relatively similar in their patterns of presentations, collectively described as stress syndrome (Chrousos, 2009; Tsigos *et al*., 2016). These time-bound changes known to be adaptive in nature, are initiated for the purposes of protecting as well as promoting the likelihoods of survival of living organisms. However, it has been recognized that as the intensity of the stress increases and prolongs, failure in cellular adaptive mechanisms and manifestations of multiple organs pathologies ensue (Gold, 2015; Tsigos *et al*., 2016). Nevertheless, stressors have been described as having dual effects on body physiological functioning (Chakravarty *et al*., 2013). For example, within certain limit, stress such as acute stress, may produce positive effects on the body such as enhancing alertness, memory performances and stamina to cope with unfavorable life situations (Chakravarty 2013). Thus, under certain situations; stress can be pleasant, rewarding and promoting positive reinforcement essentials in emotions as well as intellectual capability of the organisms (Dorn and Chrousos,1993; Tsigos *et al*., 2016). For example, sexual engagements are pleasurable, even though stressful (Gold, 2015).

Development of diseases may result from changes in organism ability to adequately adapt to intense aversive circumstances. Moreover, diverse harmful effects of stress on array of physiologic functions such as metabolism, growth, reproduction, immuno-competence, behaviour, and personality development have been reported (Tsigos *et al*., 2016). Specifically, involvements of prolonged adversity of high intensity have been severally

documented in genesis of diverse illnesses such as psychiatric disorders (endogenous depression, cognitive decline, psychosis, anxiety and addictive tendency), endocrine dysfunctions (diabetes mellitus as well as infertility), cardiovascular disorders (hypertension, cardiac arrhythmia as well as myocardial infarction), gastrointestinal disorders (ulcerative colitis and peptic ulcer) as well as immune dysfunctions (Zhu *et al*., 2014; Gold, 2015).

Generally, the stress response consists of three major phases namely; the alarm, adaptation and exhaustion. The alarm phase is due to enhanced activation of adrenal glands and sympathetic nervous system. It is closely connected with the increase in the production of adrenaline and steroids, which are meant to help the organisms to effectively cope with the stressor (Panossian, 2017, Panossian *et al*., 2018). The alarm phase is also characterized with elevation of heart rate, blood pressure and increased glucose utilization (Panossian, 2017). The stage of adaptation involves activation of protective mechanisms to effectively combat the stressors and attenuate its damaging effects that typified exhaustion stage (Mattson, 2008; Panossian *et al*., 2018). Nevertheless, adaptation breakdown leads to the phase of exhaustion, which is characterised by inability of living organisms to adapt to tasking aversive conditions (McEwen *et al*., 2015; Oken *et al*., 2015; Sardessai *et al*., 1993). In exhaustion phase, organism reaches its elastic limit; hence can't cope and disease pathologies starts to result from collapse in organism's homeostatic mechanisms (Aluko *et al*., 2015). Many stressors such as infections, exposures to chemical agents, psychosocial as well as physical factors have been alluded to provoking multiple organs pathologies (Shaper *et al*., 2014; Oken *et al*., 2015; Caruso *et al*., 2018). Nevertheless, the brain due to its high venerability factors has been shown to suffer more severely from hypoxia (Chao and Xia, 2010); Shimoda and Polak, 2011).

Hypoxic-stress has been regarded as a medical term ensuing from decreased in oxygen delivery to various body-tissues that imposes greater deleterious effects on brain cells (Chen *et al.,* 2020). Decreased oxygen supply to brain cells for even a short period of time has been shown to produce irreversible impairment to neuronal integrity. Hypoxic stress has been labelled as a potent stressor because its ability to disrupt body

physiological performances further attesting to the fact that constant delivery of oxygen is a precondition for existence of aerobic-organisms. Brain cells, indeed, have been noted as the most first set of body-cells to suffer from hypoxia, and died almost instantly during inadequate oxygen. Brian cells have been alluded to as the sensitive entities to oxygen deficiency due to low antioxidant profile and increased metabolic activity (Tomar *et al.*, 1984; Liu *et al.*, 2014). Brain damage occasioned by oxygen deficiency and reduced perfusion are commonly labelled as hypoxic-injuries. Diverse circumstances such as stroke, myocardial infarction, extreme hypotension, asthma, suffocation, drowning, carbon monoxide-toxicity are known to affect oxygen delivery to brain cells (Lutz, 1992; Malhotra *et al.*, 2001; Busl and Greer, 2010; Chen *et al.*, 2020).

Although inadequate distribution of body-oxygen to brain cells is generally known to impact diverse incapacitating effects (Chen *et al.*, 2020), convulsive-episodes typified immediate occurrences in rodents subjected to anoxic-stress. These forms of convulsive-episodes are denoted as anoxic-tolerance time or anoxic-convulsions (Tomar *et al.*, 1984). It has been said that increased anoxic-tolerance time is a signal of capabilities of adaptogens (Tomar *et al.*, 1984). Increased threshold to anoxic-convulsion, therefore, serves as indications of efficient adaptive mechanisms against diverse stressors as well as health promoting benefits of adaptogens (Panossian *et al.*, 2018).

Abundant reports exist in literature recognizing HPA axis as prime neuroendocrine pathway in mediating cortisol secretions followed by induction of oxidative stress, neuroinflammation as well as alterations of additional biochemical entities orchestrating diverse organs pathologies that epitomized intense stress (Radley *et al*., 2015; Taylor *et al*., 2016; Caruso *et al*., 2018). Indeed, excess cortisol accompanying chronic stress-responses has been shown to participate in losses of multiple neuronal circuitries through upstream mechanisms involving inflammation, coupled with oxidative stress (Tsigos *et al.*, 2016; Panossian, 2017). It is been shown that psychopathologic abnormalities connected with intense are the resultant consequences cortisol-provoked neuronal destructive oxidant substances; ROS/NOS species (Panossian, 2017; Panossian *et al.*, 2018). Meanwhile, additional proinflammatory mediators that further damage neuronal constituents are produced, which in turns provoke more reactive oxygen species, hence,

creating a vicious cycle for pertubation of neurodegeneration that epitomized intense stress-evoked depression as well as memory decline (Tonnies and Trushina, 2017).

Increased cortisol level produced during stress response contributes to losses of diverse neuronal circuitries through induction of free radical formation (Oken *et al.,* 2015; Tsigos *et al.,* 2016). Abundant evidences abound in literature alluding to the involvement of the neuroimmune pathways as mediators of cortisol production that initiates formation of inflammatory as well as additional molecular entities orchestrating intense stress-evoked pathologies of diverse body organs (Dedovic *et al*., 2009; Carty *et al*., 2010; Oken *et al*., 2015). Meanwhile, adrenal glands stimulation for cortisol secretion has long been recognized as one of the early effects of stress on the body. Some of these cortisol-mediated effects include elevated blood glucose and free fatty acids levels through gluconeogenesis and lipogenesis as sources of energy in the battle against intense stress (Kulkarni and Juvekar, 2008; Efferth and Koch, 2011; Tsigos *et al.,* 2016). However, excess cortisol production implicated in neurological dysfunctions during persistent stress occurs through induction of neuronal-damaging oxidants species (Panossian, 2017; Panossian *et al.,* 2018).

Damaged neurons are also known to further produce more proinflammatory mediators, hence, perpetuating degeneration of diverse neuronal circuitries that epitomized neuropsychiatric illnesses caused by intense stress (Pansossian *et al.,* 2018; Tonnies and Trushina, 2017). Thus, neuronal antioxidant deficiency is being alluded to unchecked inflammatory insults as well as enhanced susceptibility to neuronal cells death (Mattson *et al.,* 2008; Panossian, 2017; Peng *et al.,* 2020). However, inflammatory responses to chronic hypoxia involves diverse intracellular signaling pathways (*Busl and Greer; 2010;* Deepti *et al*., 2019). The NF-kβ has been described as the major transcriptional regulatory factor as mediator of secretion of proinflammatory cytokines and immune functions (Deepti *et. al.,* 201). Increased TNF-α during hypoxic stress, for example, was shown to down-regulated expressions of brain-derived neurotrophic factor (BDNF) (Tian *et al.,* 2013; Gold, 2015).

It has been established that intense stress reduced the beneficial effects of BDNF in the brain, hence, genesis of anxiety, depression as well as cognitive decline (Gold, 2015).

Some of the functions ascribed to BDNF include promotion of neurogenesis, neural stem-cells survival, neuronal proliferations that underpin diverse neuropsychiatric illnesses (Autry and Monteggia, 2012; Gold, 2015). Persistent stressor of high intensity or hypercortisolism has been said to underlie depression as well as decreased brain BDNF found in preclinical investigations (Santarelli *et al.*, 2015). Chronic stress damages hippocampus and other brain regions involved in controlling emotion, moods, voluntary movements as well as cognition, further implicating intense stress in addiction, depression, Parkinson's disease, and Alzheimers disease (deKloet *et al*., 2005; McEwen *et al*., 2015). Infact, decreased dendritic spines as a form of dendritic remodeling in hippocampal CA3 neurons of rodents subjected to chronic stress have been documented (Alfarez *et al*., 2003). Significant increase in amygdala dendritic arbors and spine population has been revealed in postmortem brains taken from depressed patient. Hence, reduced neurogenesis in these brain regions have been found to play predominant roles in chronic stress-induced loss of cognition (Gold, 2015).

Strategies toward management of intense stress and promotion of effective resilience might mitigate the impacts of aversive situations (McEwen, 2007). It should be emphasized that any management approach adopted should be directed at the ability of the animals to cope better in the face of intense stress (McEwen, 2007; Compas, 2006). Thus, implementation of strategies that promote resilience might improve health outcomes (Logan and Barksdale, 2008). It has been noted that inefficient resilience is a byproduct of maladaptive response, hence, genesis of diverse pathologies such as anxiety, depression and loss of cognition (McEwen, 2007; Logan and Barksdale, 2008). Compounds with anti-stress property otherwise known as adaptogens, have been reported to promote resistance against stressors (Panossian *et al*., 2018). Adaptogens have been described as compounds from natural sources that act nonspecifically to assist the body to combat stress and return body system to normal homeostasis (Panossian *et al*., 2018).

Naringenin is a well-known naturally occurring dietary flavanone present in various vegetables and fruits. Diverse citrus including oranges, mandarins, grapefruit, lemons and limes are known to be very rich in naringenin (Alam *et al.*, 2014). Thus, consumption of

fruits as well as vegetables abundant in naringenin on regular basis has been recommended as potential strategy for mitigating chronic diseases including metabolic diseases, CVS disorders, as well as neurodegenerative diseases (Alam *et al.,* 2014). These potential usefulness of naringenin have been alluded to its antioxidant and anti-inflammatory properties (Alam *et al*., 2014). It was documented through experimental studies that flavonoid significantly decreases the release of various mediators of inflammatory conditions, including inducible NO synthase, TNF-αand cyclooxygenase-2, in 3-nitropropionic acid–treated rats (Gopinath *et al*., 2012).

Naringenin is popularly known for its potent free radicals scavenging capability as well as suppression of lipid peroxidation (Cavia-Saiz *et al*., 2010). Superoxide and hydroxyl radicals are scavenged by this flavonoid (Cavia-Saiz *et al*., 2010). Moreover, studies have documented diverse CNS activities of naringenin in various *in vivo* as well as *in vitro* experimental models, collectively alluding to the therapeutic potentials of this flavonoid in neuropsychiatric illnesses (Khan *et al*., 2012; Raza *et al*., 2013; Lou *et al*., 2014; Ghofrani *et al*., 2015). Specifically, for example, naringenin was reported to improve cognitive functions and reduced degeneration of neuronal cells in preclinical studies (Khan *et al*., 2012; Yang *et al*., 2014). It was further shown to ameliorate functional disturbances provoked by ischemic stroke through inhibition of neuroinflammation associated with NF-κB signaling (Raza *et al*., 2013).

The potential benefits of naringenin for treatment of inflammatory conditions of CNS domain such as Alzhiemer's disease were also highlighted based on its neuroprotective and cytokine signaling inhibitory activities (Wu *et al*., 2015). The capability of this compound in attenuating neurotoxicity evoked by iron-overload through mechanism related to inhibition of oxidative pathway has been further documented (Chtourou *et al*., 2014). More recently, naringenin was shown to have attenuated cluster of interrelated neurobehavioural derangements evoked by psychosocial stressor in mice by inhibiting cholinesterase, oxidative stress as well as pro-inflammatory cytokines (Umukoro *et al*., 2018). However, extensive literature survey showed paucity of information on naringenin, relating to chronic hypoxia-evoked neurobehavioural derangements.

1.2 Justification of the study

Hypoxia is a stressful condition precipitated through inadequate delivery of oxygen to various body tissues (Kubová and Mares, 2007; *Busl and Greer, 2010*). Studies have established that prolonged hypoxia evoked irreversible injury to diverse brain regions, which in turn triggers manifestations of various neurobehavioural derangements including depression, intense anxiety, aggressiveness, memory decline, and motor dysfunctions (*Malhotra et al. 2001;* Kubová and Mares, 2007; *Busl and Greer, 2010*). The high venerability of brain cells to hypoxia of persistent nature triggers degeneration of multiple neural pathways, which has been ascribed to its low antioxidant defense status as well as high rate of metabolism (Tomar *et al*., 1984; Liu *et al*., 2014). In addition, loss of neuronal antioxidant protective mechanism induces neuroinflammation, which further compromised neuronal integrity and consequently enhancedneuronal cell loss (Panossian *et al*., 2018). Thus, neuroinflammation closely connected with enhanced ROS formation and, particularly insufficient scavenging machineries have been recognized as the major driver to inefficient cellular adaptive mechanisms to persistent stress and development of neuro-pathologies (Tonnies. *et al.* 2017).

Despite many decades of understanding the basis of deleterious effects of prolonged hypoxic conditions, slight advancement in terms of development of effective therapeutics has been recorded that are capable of combating neuropathological consequences thereby improving both physical and mental health in persons recuperating from its debilitating impacts (Chen *et al*., 2020). Nevertheless, there are growing evidences showing that antioxidant and anti-inflammatory agents might be effective in antagonizing multiple pathways involved in neuropathological complications evoked by chronic hypoxic stress. Based on this awareness, effects of naringenin on chronic hypoxia-evoked neurobehavioural sequelae, and likely biochemical mechanisms underpinning its action in mice were investigated in this research.

1.3 Aim of the study

This research was designed to investigate effects ofnaringenin on chronic hypoxic stress-evoked neurobehavioural complications, and likely biochemical mechanisms underpinning its action in mice.

1.4 Objectives of study

- To investigate effects of naringenin on latency to anoxic convulsions, blood glucose contents, serum corticosterone and oxidative stress biomarkers in mice subjected anoxic-stress.
- To investigate naringenin effects on chronic hypoxic stress-evoked neurobehavioural complications in mice.
- To evaluate effects of naringenin on glucose and corticosterone contents provoked through chronic hypoxic stress in mice.
- To investigate naringenin effects on chronic hypoxic-stress evoked increase in oxidative stress parameters as well as pro-inflammatory cytokines.
- To determine naringenin effects on chronic hypoxia-altered brain immuno-positive cells (NF-kB, INOS and BDNF) expressions
- To evaluate probable neuroprotective benefit of naringenin on cyto-architectural distortions of amygdala elicited by chronic hypoxic stress in mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 Stress neurobiology

Optimal body equilibrium is subjected to constant aversive conditions, otherwise described as stressors, either perceived (psychological) or real (physical). Stress, may therefore be regarded as disharmonious conditionthat activate adaptive stress response that tend to abolish threat to body homeostatic mechanisms. Response to stress has been shown to entails interrelated complex molecular, cellular as well as neuroendocrine apparatus collectively termed stress system (Chrousos, 2009). It has been reported that hypo-thalamic pituitary adrenal (HPA) pathway including adrenergic counterpart of autonomic nervous system (ANS) were highlighted as principal components of stress systems that interact with some vital brain regions as well as other body organs in the periphery for efficient mobilisation of defensive mechanisms crucial in mitigating stress pathologies (Tsiogos *et al*., 1994; Tsiogos *et al*., 2016).

Adaptive mechanisms have been said to be activated in order to restore stability of internal environment meant for promotion of survival of living organisms (McEwen, 2006; Karatsoreosand McEwen, 2011). Although there are different types of stressors, it has been observed that the key circuitries underpinning stress responses in different situations are essentially identical (McEwen, 2006; Logan and Barksdale, 2008). However, functional deficiency of adaptive mechanisms due to chronic stress leads to disruption of body homeostasis and constellations of many physiological derangements (Gold, 2015; Tsigos *et al*., 2016). Disequilibrium between allostasis and allostatic load in favour of the later has been shown to be the major driver of multiple pathological derangements associated with chronic stress (Logan and Barksdale, 2008). Allostasis has been described as extension of homeostasis, which represents adaptation processes of

complex interrelated physiological mechanisms to persistent challenging stressful circumstances (Logan and Barksdale, 2008). Meanwhile, allostatic load has been alluded to the consequent of long-term effects precipitated through adaptation failure otherwise allostasis, that often lead to pathological derangements or chronic illnesses (Logan and Barksdale, 2008). However, there are emphasis on implementation of strategic measures to promote resilience against stress, thereby promoting health outcomes. It has been reported that these strategies are expected to attenuate aversive reactions precipitated by persistent stress, as important steps towards mitigation of harmful consequences of prolonged stress on mankind (Logan and Barksdale, 2008).

2.2.Resilience: a patten of stress coping mechanism

Resilience has been regarded as ability to boost resistance against stress (Fig. 2.1) and to adapt as well as function successfully in challenging traumatic circumstances (Russo *et al*., 2012); McEwen *et al*., 2015; Karatsoreos and McEwen, 2011). However, McEwen (2002) defined resilience as a condition of a successful allostasis meant to minimize wear and tear when faced with aversive circumstances. Thus, resilient organisms are known to efficiently adapt to challenging aversive situations, hence, are less prone to pathological damages (Russo *et al*., 2012; McEwen *et al*., 2015; Karatsoreos and McEwen, 2011). Moreover, Dver and McGuiness (1998) defined resilience in term of the tendency of organisms to bounce back from challenging circumstances, which was also linked to improved health benefits despite prevailing pathological conditions. It has also been explained using analogy of elasticity of a rubber band phenomenon, in which a rubber band returns to its initial state after being stretched to certain limits and released. However, when stretched many times, it may lose its elastic nature over time resulting in failure to bounce back to the initial state (Logan and Barksdale, 2008). When excessive force or too much force is applied on it, the rubber band may also lose the elastic behaviour or even break. Finally, there are also some rubber bands with original structural defect, hence, are more susceptible to being broken even with lesser force when compared others (Logan and Barksdale, 2008). The rubber band analogies are typical features of responses relating to concept of allostatic burden. Thus, individuals, who have greater adaptive energy (greater resilience) tend to exhibit higher levels of resistance to

devastating consequences of allostatic burden compared with persons having low adaptive strength as depicted by rubber band analogy (Logan and Barksdale, 2008).

Psychosocial and pharmacological interventions instituted in a timely fashion have been shown to enhance resilience. In fact, the practice of resilience techniques was found to increase the effectiveness of social intervention programs in Hong Kong youth (Lee *et al*., 2007). The connection between allostasis and resilience therefore may provide insight on how resilience might retard the genesis of many chronic diseases, as well as highlighting the need to identify processes of resilience and neural adaptative compensatory mechanisms (Fig. 2.1). However, allostasis concept has provided good theoretical frameworks through which mechanisms involved in resilience could be explained especially how resilience increases hippocampal capacity to withstand challenging situations contribute to chronic illnesses (Russo *et al*., 2012); McEwen *et al*., 2015). Thus, appropriate intervention strategies to boost brain resilience as well as promoting mental health can be initiated. Most importantly, allostasis and resilience concepts will provide insight into multiple biochemical pathways orchestrating decline in resilience and disease vulnerability induced by chronic stress (Fig. 2.1).

2.3 Historical perspective of concept of stress

Although, concrete description of events associated with stress were ascribed to Hans Selye, an American physiologist, Walter Bradfort Cannon after series of studies was the first person to make compilations of effects of different stimuli on visceral structures. These compilations described bodily changes that occurred in response to nociceptive impulses such cold, hunger, exercise as well as strong emotions (Tan and Yip, 2018). It was reported that Cannon documented those functions directed at supporting body energy were immediately stimulated or suppressed temporally for mobilization of great energy towards restoring stability during periods of challenging circumstances (Tan and Yip, 2018). The energy mobilization was described as a way of preparing living organisms for escape, attack or defenses that epitomized fight or flight mechanism (Tan and Yip, 2018). However, homeostasis was later coined by Cannon premised on Claude Bernard concept of milieu interieur (internal medium). Cannon further noted that blood as well as other body fluids enclosing cells constitute internal milieu through which exchanges of

substances between cells occurred, which must always be kept constant or suitable for cellular functions, irrespective of the changes that might have taken place in external environment *(*Logan and Barksdale, 2008; Tan and Yip, 2018).

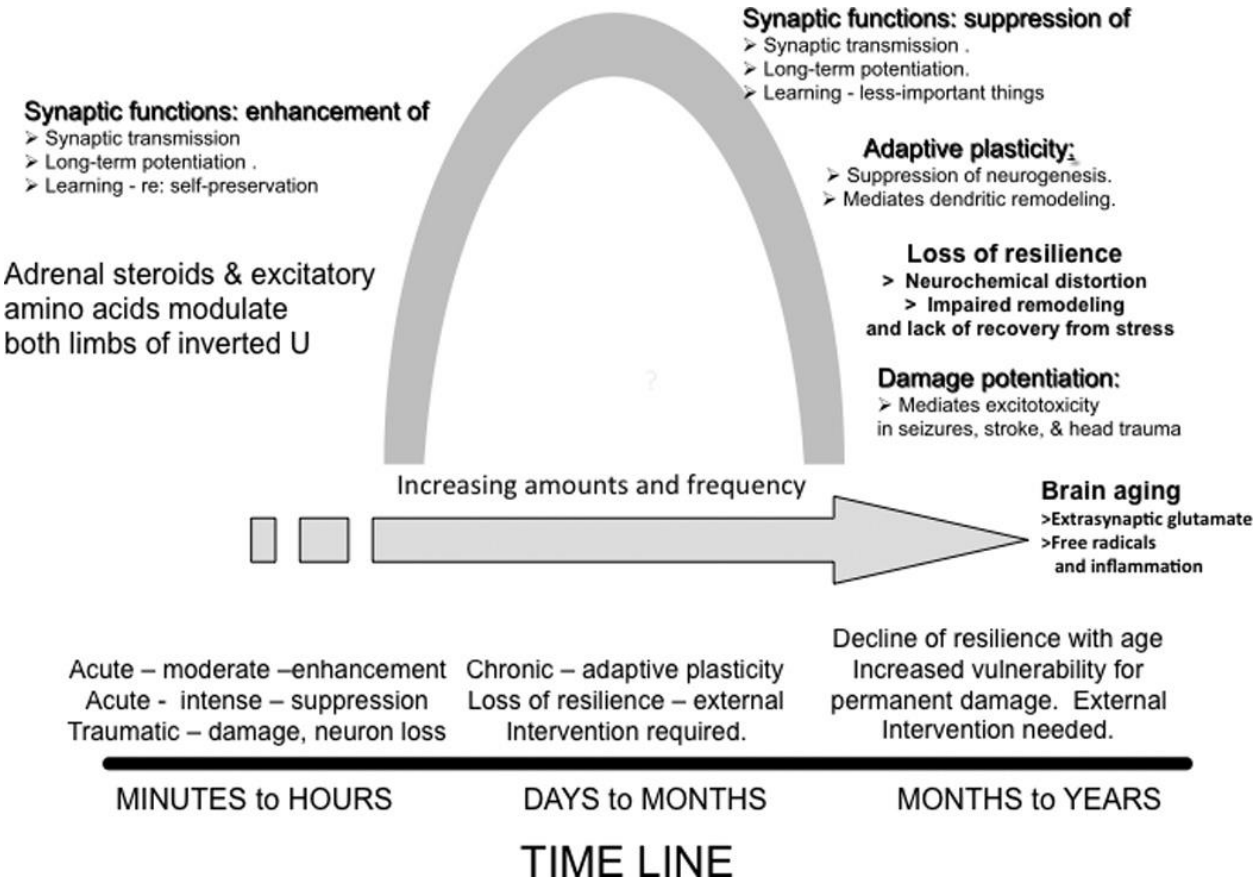

Fig. 2.1. Multiple neurochemical and biochemical pathways involved in resilience and pathological consequences (McEween *et al*. 2015).

Cannon further described homeostasis as the organism's ability of stabilizing body systems in direction of dynamic equilibrium state. This has contributed substantially to the understanding of stress responses and its biological consequences (Tan and Yip, 2018). However, Hans Selye, widely referred to as the father of stress studies was the first that provided concrete experimental data on biological impacts of different stressors, which stemmed from his clinical observations. Selye observed that patients that were suffering from different diseases showed similar patterns of symptoms, constituting what he described as he described as a unitary syndrome. He also observed similar signs in animals injected with hormones from ovarian cow extract (Tan and Yip, 2018). He also discovered that the effects that were produced by hormones of the ovary were likewise reproduced through injection of different extractives from various organs and toxic agents.

Selye noted that irrespective of modes of preparations, these hormonal constituents produced similar effects including increase in sizes of adrenal glands, intestinal ulcers, thyme as well as lymph nodes enlargements, comprising what he described as the general adaptation syndrome (Selye, 1950). According to Selye, this adaptation syndrome connotes set of non-specific changes occurring in three phases: alarm stage, resistance stage as well as phase of exhaustion signaled collapsed state of living organisms (Selye, 1950). Thus, forming the backbone for formulating stress theory and development of stress concept from extensions of the physical laws of elasticity evolved by Hook (Tan and Yip, 2018). In fact, stress terminology has been well described in physics as forms interactions which impose a force and trigger resistance raised against it. It is on record that Selye, was the first person to extend stress into medical circle as non-specific body responses to any burden imposed upon it (Selye, 1976).

Selye also pointed out limitations of homeostasis to include its inability to ensure stability of body systems all alone by itself during stressful circumstances. This drawback made him to introduce the concept of heterostasis through which new steady states could be formed by administration of substances capable of stimulating adaptive mechanisms (Tan and Yip, 2018). It has been noted however, that heterostasis was likely the cornerstone for the development of the new allostatic paradigm that was first formulated in the late 1980s

(Sterling and Eyer, 1988; McEwen, 1998; Schulkin, 2004). Nevertheless, homeostasis, which still remained prominent in medical physiology, is thought to be the major mechanism for physiological regulation in ensuring stability through constancy of the internal environment (Tan and Yip, 2018).

Homeostasis, which was based on principles of negative feedback mechanism through which reactive strategy to perturbations were detected accompanied by elicitation of corrective measures that orchestrate the restoration toward pre-perturbation states (McEwen, 1998; Schulkin, 2004). Thus, basic tenet of homeostatic regulations entails initiation of responses that act cooperatively in a well-organized fashion to protect cellular functions pivotal to wellbeing of the organisms (Tan and Yip, 2018). Meanwhile, allostasis is being viewed as a process for archiving equilibrium through changes occasioned by regulating the set-points responsible for physiological readjustments in order to meet the demand imposed by stressors (McEwen, 1998; Schulkin, 2004). More specifically, Sterling and Eyer (1988) that introduced the word allostasis to indicate the neurobiological mechanisms underpinning the adaptive ability of an organism to imposed stressors through alteration of its defensive mechanisms needed to meet the demand of the new challenging situation.

It has been noted that allostasis has three basic tenets: (1) states that anticipatory response, relying upon past experiences or learning from past events is the most efficient strategy of regulation of stress (2) holds that defensive system changes its function in order to efficiently resist the extra burdens imposed by stressors, and (3) declares that effective control is through a central command unit, the brain, which regulates activations/deactivations of multiple pathways that affect one or more regulated variables, so as to archieve the most cost-beneficial compromises (McEwen, 1998; Schulkin, 2004; Logan and Barksdale, 2008).

The concept of allostasis was preferred over homeostasis, premised on ideas that homeostasis was incapable of addressing some critical issues relating to physiological regulation, particularly on its dependence on reactive responses as well as its insistence on defending an imaginary fixed set- point (Ganzel *et al*., 2010). Interestingly, it is important to mention that increasing number of scientists are embracing allostasis-

concept insisting that it has many merits over the canonical view of homeostasis in physiological context as well as in relationship to health and diseases (Ganzel *et al*., 2010). Moreover, the scope of allostasis has been broadened to embrace factors relating to impacts of psychosocial as well as socioeconomic stressors, and how regulatory readjustments are initiated to reduce the biological consequences of them (Ganzel *et al*., 2010). McEwen and Wingfield (2010), for example, believed that the concept of allostasis serves as a useful tool in clarifying some ambiguities connected with stress and homeostasis. In addition, Peters and McEwen (2012) described allostasis-concept in term of an active process through which living entities adjust to potential threats to their survival as well as changes in their immediate environment (also known as stressors) so as to archieve homeostatic stability and promote survival. However, no indications in literature suggesting that homeostatic principles were initially formulated to address the effects of intricacies of psychosocial stress on physiological functions pivotal to health and diseases. These reasons make allostasis appear to be a source of threat to almost a century doctrine and preeminence of homeostasis (Tan and Yip, 2018).

It is important to mention that Langley (1973) regarded homeostasis as a self-regulating negative feedback-mechanism for ensuring constancy of internal milieu. However, contemporary experts believed that negative feedback mechanism does not get activated except there are perturbations of regulated variables indicating inadequacy of homeostasis to account for stress-related physiological derangements. Although negative feedback mechanism is an integral component of physiological regulatory system, a new model such as allostasis that prevents perturbations through modifications of past experiences as well as learning to make priori changes in anticipation of impending harm, and mitigation of the impacts should be pursued (Tan and Yip, 2018). This argument was further extended by Berridge (2004), who believed that anticipatory behaviours directed at regulated variables from becoming deviated are not homeostatic in nature since the responses are not triggered by perturbations of the regulated variables.

Over the last three decades, the concept of allostasis has gained increasing popularity with a lot of scientists advancing in the directions, which were not included in the original essay. Although Sterling and Eyer (1988) regarded allostasis as a path to achieving

efficient regulatory systems in bodily functions with minimal expenditure of energy, other scientists had redefined allostasis as a process, which occurs when regulation of key variables takes place at considerable underlying cost, hence, allostasis has been linked to pathological consequences. This has eventually led to the introduction of allostatic load by McEwen and Stellar (1993) for justification of the cost of ensuring stability to prolonged activation of compensatory effectors.

2.4 Allostatic load: Impact of persistent stress

Mediation of physiological responses via HPA-axis, ANS, cardiovascular, immune as well as metabolic systems are meant in offering protective and adaptive capability to living organisms during aversive conditions. These processes of adaptation are collectively known as allostasis (McEwen and Stellar, 1993; Ramsay and Woods, 2014) which play vital role in restoration of stability. However, the process of adaptation to chronic stressor comes with a price, and this cost had been labelled as allostatic load (McEwen and Stellar, 1993). The word allostatic load was coined by McEwen and Stellar, (1993) to described wears as well as tears on bodily organs including brain regions involved in adaptive responses to challenging events (Fig. 2.2). This cost may also manifest in forms of pathologies via overproduction of chemical mediators released in response to persistent stress.

Persistent elevated levels of cortisol, for example, were shown to damage hippocampus as well as impairment of neurogenesis that leads to interference with cognition, and upcoming adaptation to stressors (Sapolsk *et al*., 1986; Ramsay and Woods, 2014). Allostatic load also occurs via depletion of stress response systems particularly the immune system, which leads to compromised immunocompetence and consequently higher rates of infection as well as susceptibility to cancer (Karatsoreos and McEwen, 2011). Moreover, increased allostatic burden may also sets in through inability of the body to stimulate specific stress response systems or over activation in some cases (Ramsay and Woods, 2014). However, there are several circumstances, whereby the chronicity of stressors and ensuing physiological responses induce tissue injury thereby aggravating existing illnesses as well as predispose susceptible individuals to diseases, commonly described as maladaptation. These long-term effects of organisms' ability to

accumulate some measures of stress have been described as allostatic load (Ramsay and Woods, 2014), the wear and tear due to chronic hyperactivity or hypoactivity of stress response systems (Karatsoreos and McEwen, 2011). Factors capable of inducing maladaptation were therefore described as pathological-stressors. It has been noted that outcomes of pathological stress on individuals are based not only duration, severity, and stressor-identity, but also genetic factors, memory of past-experiences, as well as psychosocial care.

Allostatic systems are known to participate actively in stress-coping strategy and mediating adaptation, and are most valuable once they are swiftly mobilized and terminated when no longer wanted. However, pathological derangements set in when their effects are prolonged or not promptly terminated. Moreover, inefficiency in engaging allostatic systems also induce overload on the body, with consequential manifestations of multiples pathologies (Ramsay and Woods, 2014; Karatsoreos and McEwen, 20011). This is agreement with the notion that the effects of previous stressors are long-term carry over sequelae of daily stress or intense-adversity. In many cases, allostatic burden can be enormous enough to cause severe or fatal symptoms as described in Selye's exhaustion phase of stress response. Thus, allostasis and allostatic load concepts permit elucidation of the dynamism associated with adaptive capacity of organisms and also delineate individual differences in the capability to mitigate stressors.

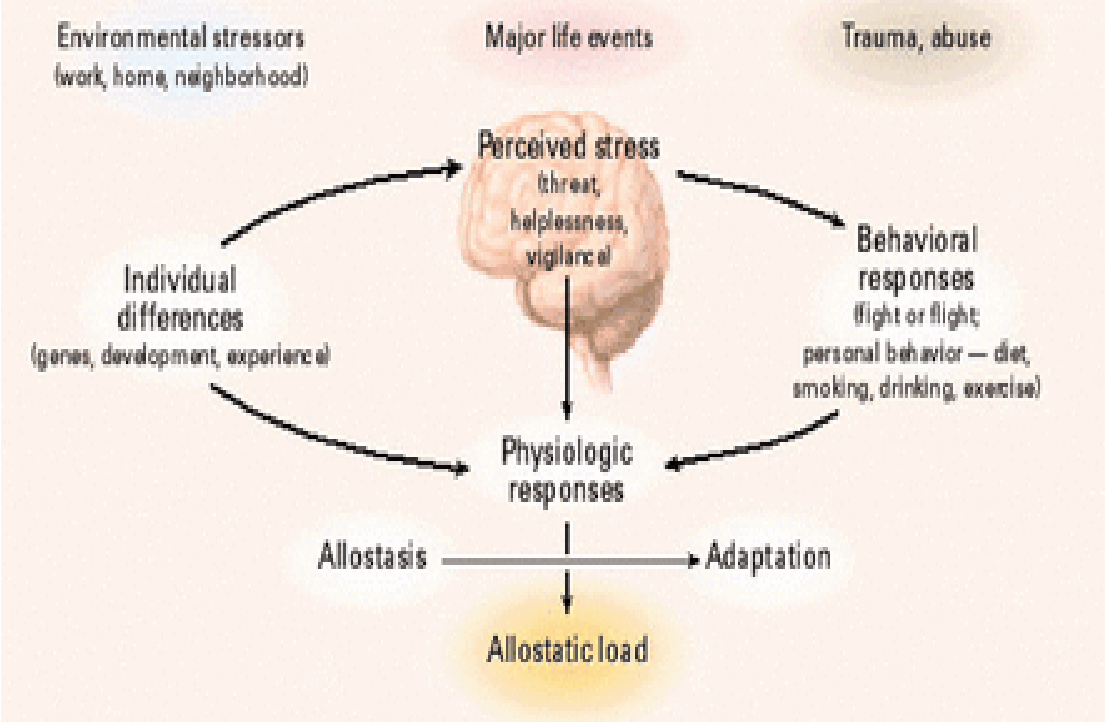

Fig. 2.2: Interplay between allostasis, behavioral and physiological responses to different stressors (McEwen, 1998)

2.5. General Adaptation Syndrome

Selye was supposed to be the foremost scientist to have designated stress as nonspecific signs as well as symptoms of illnesses (Perdrizet, 1997; Tan and Yip, 2018). Stress story begins with the identification of several female sex hormones that were yet unexplored. It was reported that Selye isolated cow ovaries and injected prepared hormonal extract into female rats and measured the responses (Tan and Yip, 2018). The autopsies produced trio of shocking discoveries such as adrenal glands enlargements, atrophy of lymphatic systems and peptic ulcers. Injection of the hormonal extract also produced the same characteristic features. He also extended his investigations through subjecting rats to different stressors such as on cold roof of buildings, rotating treadmill that obligatory for the animals to continue running to remain stable (Tan and Yip, 2018). The findings were identical in presentation of adrenal enlargement, lymphatic atrophy and peptic ulcers in all the experiments. Selye recognized that his findings were expressions of s Claude Bernard's milieu intérieur and linked HPA axis to the manner the body managed stressors. These discoveries led him to propose that stress is an integral component of living organisms in the entire life journey in dealing with unpredictable challenges and Selye described the entire response to chronically applied stressors as general adaptation syndrome, which is also known as Selye's Syndrome in literature (Perdrizet, 1997; Tan and Yip, 2018).

2.6 Phases of stress response

Studies have shown that elaborate neurobiological systems have evolved to orchestrate coordinated responses best suited for any given condition for specific for individuals. In healthy individuals, physiological responses are rapidly turned on and off in synchronization to severity and duration of stressors, and limiting its potentially harmfulness ((Perdrizet, 1997; Tan and Yip, 2018).). However, according to the model of general adaptation syndrome, the adaptive response that organisms go through when exposed to stress entails three series of phases or stages (Perdrizet, 1997; Tan and Yip, 2018).

Phase 1 (alarm phase): During alarm phase, the body first reaction to stressors is to recognize the existence of danger and prepare to deal with the threat, a response commonly identified as “fight or flight response”. Animals are known to decide quickly whether is more viable to flee or to fight with the stimulus that the threat poses, a mechanism engraved in every living organism. This phase is always associated with activation of HPA axis, a portion of the endocrine system regulating stress responses as well as impacts of stressors on various bodily functions. The central nervous system and adrenal glands are also activated (Perdrizet, 1997; Tan and Yip, 2018). During this phase, stress hormones such as cortisol, adrenaline as well as noradrenaline are liberated to offer instant source of energy to boosted coping strategies (Fig. 2.3) and also to activate physical activities that requires fighting or running away. However, an excess of adrenaline results in elevation of blood pressure that could damage blood vessels of the heart and brain thereby predisposing susceptible individuals to heart attacks and stroke (Godoy *et al*., 2018). Also, excessive production of cortisol during this stage may also cause damage to body cells and muscle tissues. This stage takes away so much energy from other body systems (immune system), hence, increasing vulnerability to illnesses.

Phase 2 (Resistance stage): This stage is the phase of adaption, characterized by activation of adaptative machineries of cells aimed at restoring body hemostasis. It is known that most of the physical and biochemical changes that occurred during the alarm phase are reversed or attenuated in phase 2. While this phase has been regarded as period at which coping as well as adaptation arises, the capability to resist stresses is recognized to be increased (Fig. 2.3) but can be limited if the stress persists (Godoy *et al*., 2018**)**. Hence, the need for the use of adaptogens, which are compounds obtained from plants that can non-specifically act to combat stress and boost cellular capacity of the body to cope with stressors.

Phases 3 (Phase of exhaustion): This phase arises when stressor is extreme and protracted, which leads to depletion of adaptive resources of the organisms. The resistance to stressor is gradually depleted (Fig. 2.3) and failures of bodily functions occurred due to compromised immune-status. In Selye’s view, those subjugated to longstanding stress of high intensity might suffer heart-attacks or prone to infections owing to loss of efficient

adaptation. Phase 3, which is due to breakdown in adaptation is characterized by high levels of cortisol (McEwen and Gianaros, 2010; Godoy *et al*., 2018**)**. Cortisol has been implicated in several diseases, behavioural maladaptation (chronic depression and alcoholism.), lowered resistance to infections. Specifically, this phase is frequently connected with illnesses of psychosomatic-domain, ulcers, high blood pressure, asthmatic-attacks, migraines, arthritic-pains and in extreme cases, suicidal tendency and death (McEwen *et al*., 2015).

2.7 Stress-response system

Despite plethora of adverse situations impact on the life, living organisms are endowed with defensive mechanisms against both internal and external stressors as well as restoration of homeostatic balance. It has been known that reaction to harmful insults is activated through multifaceted interconnected pathways collectively denoted to as stress systems (Fig. 2.4) that integrate wide range of brain structures involved in detecting noxious stimuli as well as interpreting them as either real or potential threat or stress (McEwen *et al*., 2015). The sympathetic as well as the cholinergic counterpart, HPA axis, immune system, and molecular processes in various bodily cells function in diverse manners to effect adaptation through allostasis i.e achieving dynamic equilibrium via activation of these systems. However, it is known that some chemical-mediators exhibit biphasic effects, producing beneficial effects as well as promoting pathologies once their action is on longer in tone with each other (i.e increased allostatic burden or over-load). Activeness of the protective adaptive mechanism for body organs against allostatic burden or over-load are two opposing sides of the physiological responses of persons passing through everyday challenges of life (Ramsay and Woods, 2014**;**McEwen *et al*., 2015).

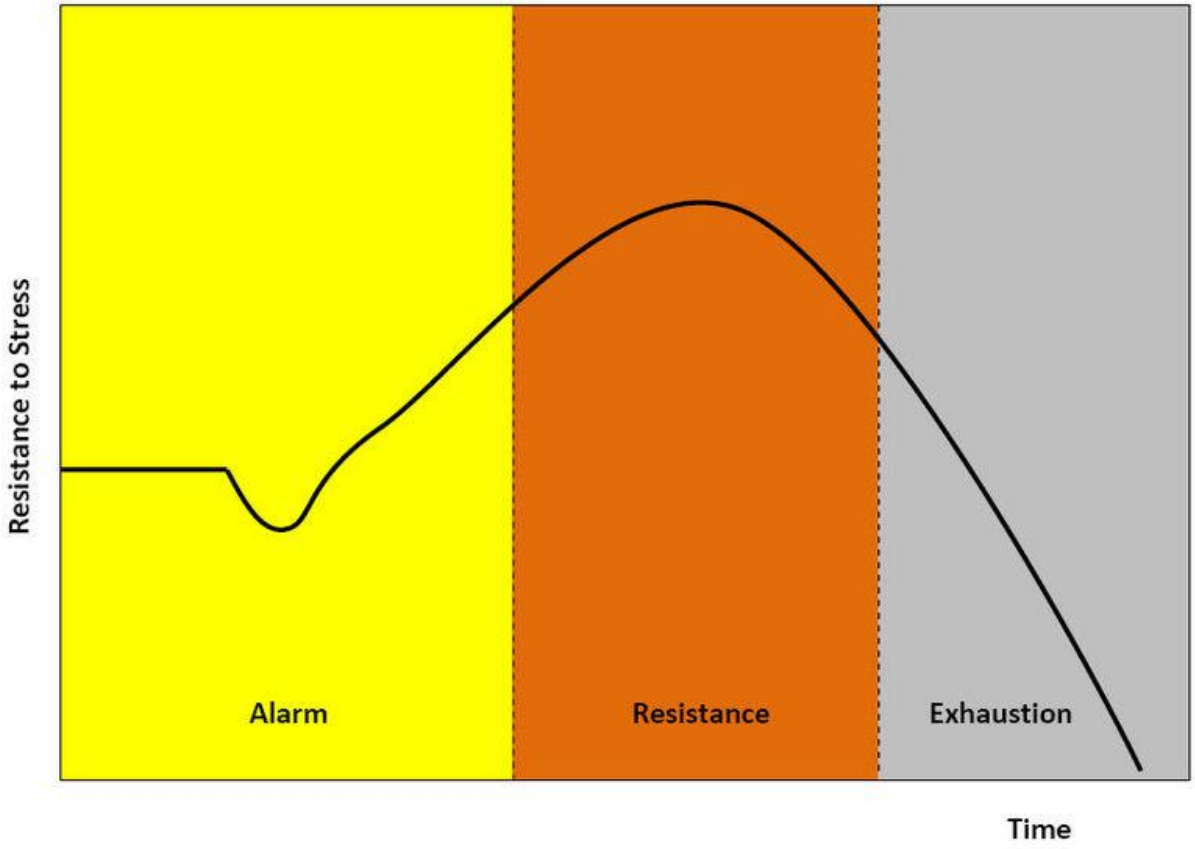

Fig. 2.3: Phases of physiological responses to stress.

The perception of any events as stressors depends on the brain, which promotes stimulation of two prominent components of the stress system; sympathetic-adreno-medullar axis and HPA axis(Fig. 2.4) to release a variety of chemical mediators (Godoy *et al*., 2018**)**. Specifically, stimulation of these two structures resort to specific stressors that lead to synchronized empowering responses aimed at restoring homeostatic stability. In order to achieve this effect, the stress-response system specifically enhances mobilization of energy, immune system activation, altered metabolism, suppression of digestive function as well as reproductive system (Fig. 2.4). The proinflammatory signalling pathways in combination with these CNS-mediated effects, evoke changes in the machinery of cellular functions, synaptic, and neuronal plasticity (McEwen, 2007; Godoy *et al*., 2018). Taken together, these brain-body effects are known to mediate changes in physiological functions and behavioural phenotypes that enable adaptation and survival or pathological consequences in extreme situation (Godoy *et al*., 2018).

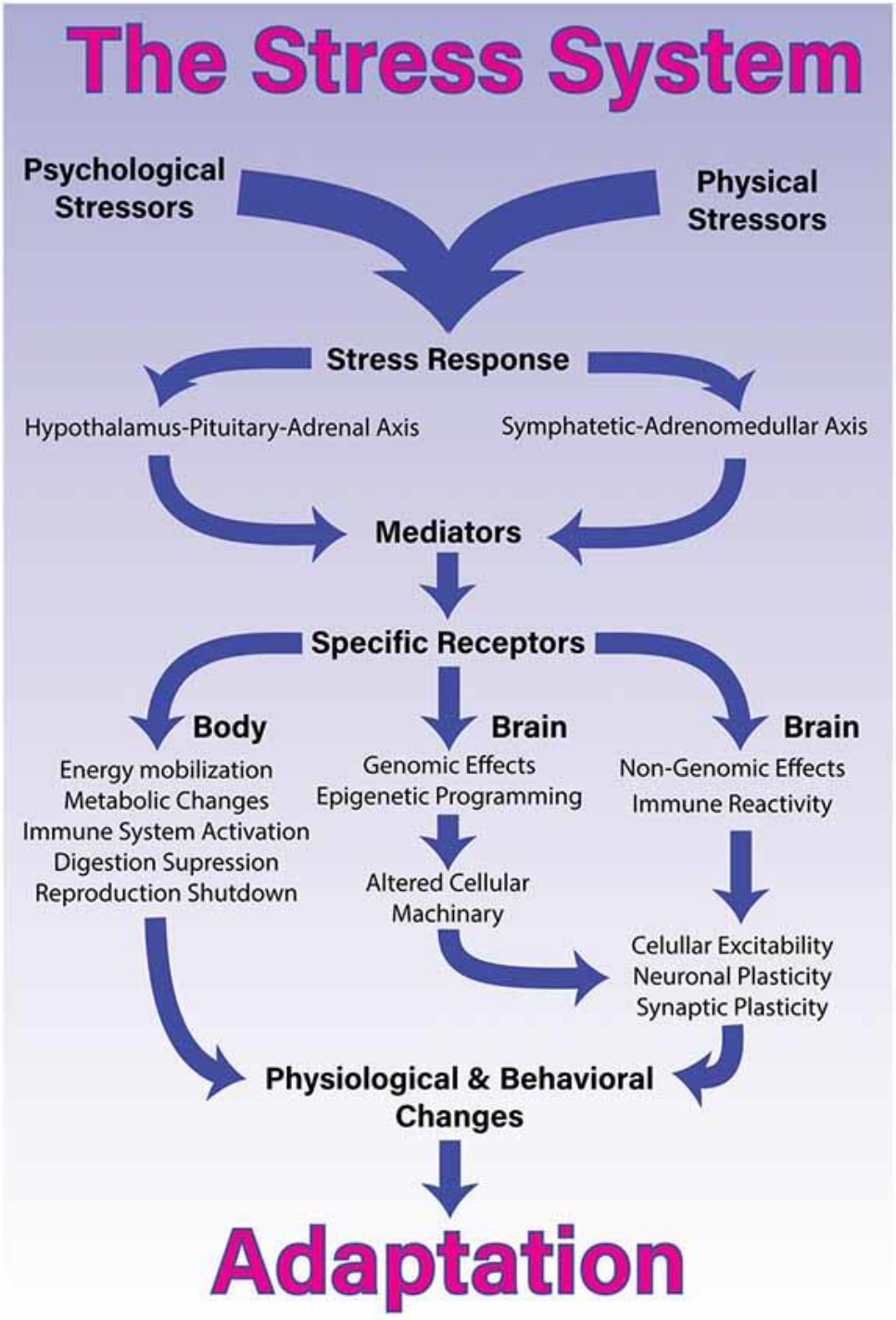

Fig. 2.4: **Stress systems involved in coping mechanisms during stressful situations (Godoy *et al.*, 2018).**

2.8. Central role of brain regions in mediation of stress response

The role of various brain regions in mediating stress-responses has been emphasized through the well-recognized capacity of neurons to promptly respond to aversive circumstances (McEwen and Gianaros, 2010). It is well-known that the brain has the capability to alter its architecture, molecular profiles and its neurochemical substrates during the time of exposure to stressors as well as directing sundry bodily systems including immune, cardiovascular and metabolic functions (McEwen and Gianaros, 2010). The neuronal pathways in healthy brains are known to be refashioned through previous experiences, which enhance modifications of behavioral responses suitable for the prevailing circumstances, for example, the individual becoming apprehensive in anticipation of likely harmful situations (McEwen and Gianaros, 2010).

It has been reported that the healthy brain is more resilient and neuronal pathway adjusts readily to novel settings through changes in gene expression (McEwen and Gianaros, 2010). However, morbid brains are less plastic and displayed maladaptive neuronal-plasticity, hence, unable to adapt appropriately (McEwen and Gianaros, 2010). Extreme stimulation of excitatory amino acids, enhanced through glucocorticoids has been implicated in irreversible damages as key steps underlying irreparable stimulation of cascades resulting AD pathology (McEwen, 2007; Godoy *et al*., 2018). The perception of stress as real or potential threats, therefore, triggers release of harmful chemical substances, which in turns interact with their respective receptors peripherally as well as centrally to cause stress (Godoy *et al*., 2018).

The degree of which neuronal cells adapt to diverse stressors in course of their developmental stage is evidenced through experimental-findings that immature neurons were more resistant to hypoxic-condition (Remero *et al.,* 2003; Shirai *et al.,* 2006) when compared with their mature counterparts. The decrease in neuronal cell's resistance to diverse stressors at various time of development was interpreted as reflection of incessant exposure of living organisms to traumas during course of their life journey. However, inherent amount of stress occurs naturally with aging (Godoy *et al*., 2018). Consequently, irrespective of how fit an individual is adapted to its life challenging events, functional decline occurs eventually. Hence, aging has been viewed as added layers of stresses

experienced throughout life instead an event coming later in biological equation of existence (Hayflick, 2007).

2.9 Principal brain regions involved in mediation of stress responses

The brain plays prominent roles in perceptions of stress as well as coordination of response to stress since it is the principal initiator as well as centre of stress-resiliency, and susceptibility. Reports have also shown that one of the primary functions of the brain is ascertain what challenges are and also regulate both behavioural and physiological responses to stressors (McEwen and Gianaros, 2010). Nevertheless, pinpointing precisely, which brain regions are responsible for specific aspects of stress responses has been fairly difficult. The brain is well recognized to work in network-like fashions to transmit stressful information across its various specific regions suggesting that negative consequences of chronic stress are rooted in neural communication dysfunctions (Ulrich-Lai and Herman, 2009). However, the three most important brain structures implicated in stress responses are the hippocampus, prefrontal cortex and amygdala (McEwen and Morrison, 2013). These brain structures, which are interconnected (Fig. 2.5) have been implicated in regulation of both behavioural as well as physiological stress-responses that might be adaptive during short term or maladaptive during long term bases (McEwen and Gianaros, 2010).

It has been recognized that stress reactivity occurs from two-ways communications; the brain and other vital body systems such autonomic, cardiovascular, and immune systems through neural as well as endocrine pathways underlying memory, and behaviour (McEwen and Gianaros, 2010). It has been reported that the bidirectional stress mechanisms might be protective in some cases of short-term adaptation. However, it might be leads to dysregulation of body functions and ill health in susceptible persons, in chronically stressful circumstances (increased allostatic load) (McEwen and Gianaros, 2010).

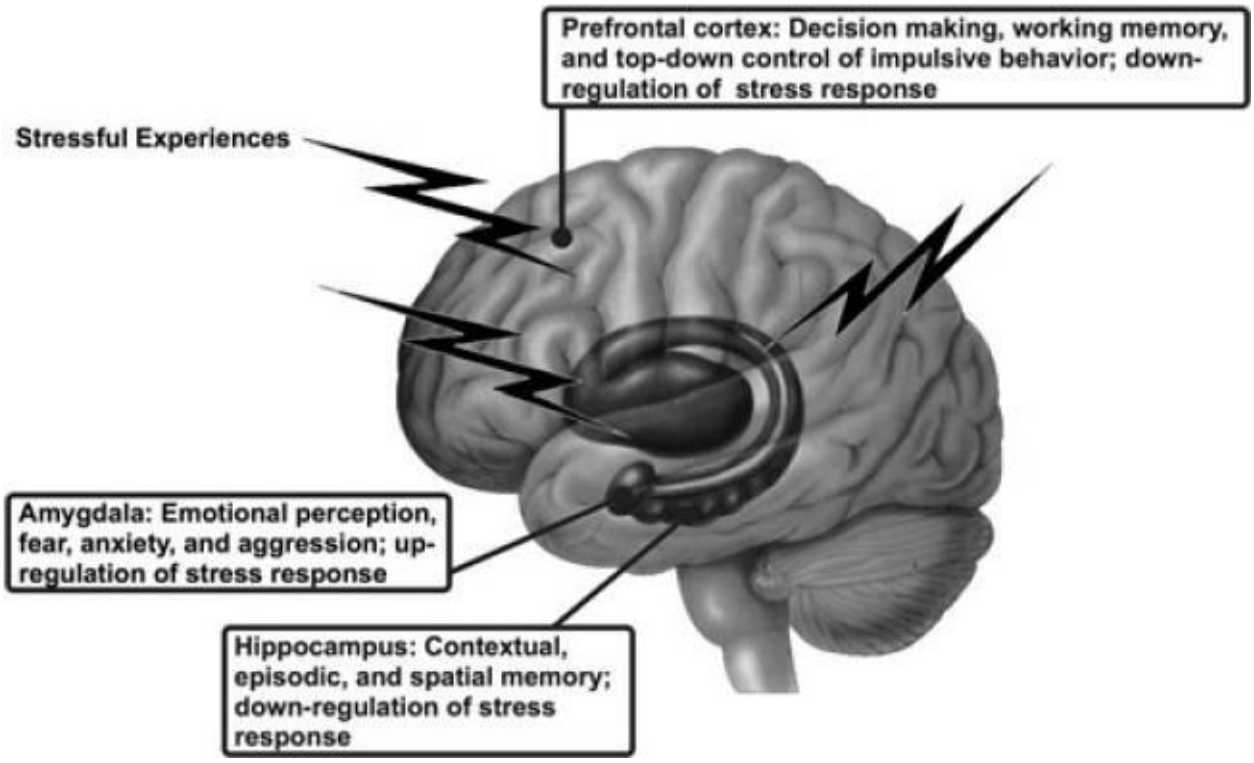

Fig. 2.5.Schematic diagram of the location and key functions of brain regions involved in regulation of cognitive and emotional functions (McEwen and Gianaros, 2010).

2.9.1 Functional role of hippocampus in stress response

Hippocampus, amygdala, and prefrontal cortex are interrelated neural brain structures s(Fig. 2.6) responsible for coordinating neuroendocrine, immune, autonomic functions and behavioural responses in terms of coping with challenging situations (McEwen and Gianaros, 2010).Hippocampus, a prominent component of the limbic system, found in the medial temporal lobe of the brain, was alluded to be first structure asides hypothalamus as major target of stress- hormones (Maras and Baram, 2012). It plays influential roles in memory and processing of contextual aspects of emotions as well as regulating visceral functions, including HPA axis. It has receptors for adrenal steroids as well as principal metabolic hormones that disturb its functions (McEwen *et al*., 2015).

During stressful conditions, the hippocampal neurons are known to shrink and show reduced number of neuronal cells (McEwen *et al*., 2015). Shrinkage of hippocampal neurons has also been revealed in numerous conditions of mental illnesses; schizophrenia as well as depression (McEwen *et al*., 2015). Cortisol content has been linked to reduced size of the hippocampal region and accounts why intense stress impaired hippocampal dependent-learning and neuroplasticity (McEwen, 1999). Neuroplasticity is known to enable the hippocampus in regulation stress reactivity (McEwen and Chattarji, 2004). Since the brain can restructure itself through life experiences revealed that resilience can be enhanced through pharmacological intervention that might yield potential benefits. Nevertheless, hippocampus is a well know brain- region of high vulnerability to chronic stresses (McEwen and Morrison, 2013). Specifically, persistent stresses have been reported to cause hypertrophy of CA3 pyramidal neurons that are connected to glutamatergic system via mossy fibers (Fig. 6). These CA3 neurons are noted for their high vulnerability to chronic-stress, which might not be unconnected with high presence of glucocorticoid receptors. Hippocampal-CA1 as well as dentate gyrus is also very prone to the deleterious effects of intense stress (McEwen and Morrison, 2013). It might be pointed out that the dentate gyrus is commonly known as the spot for adult brain neurogenesis (McEwen, 1999).

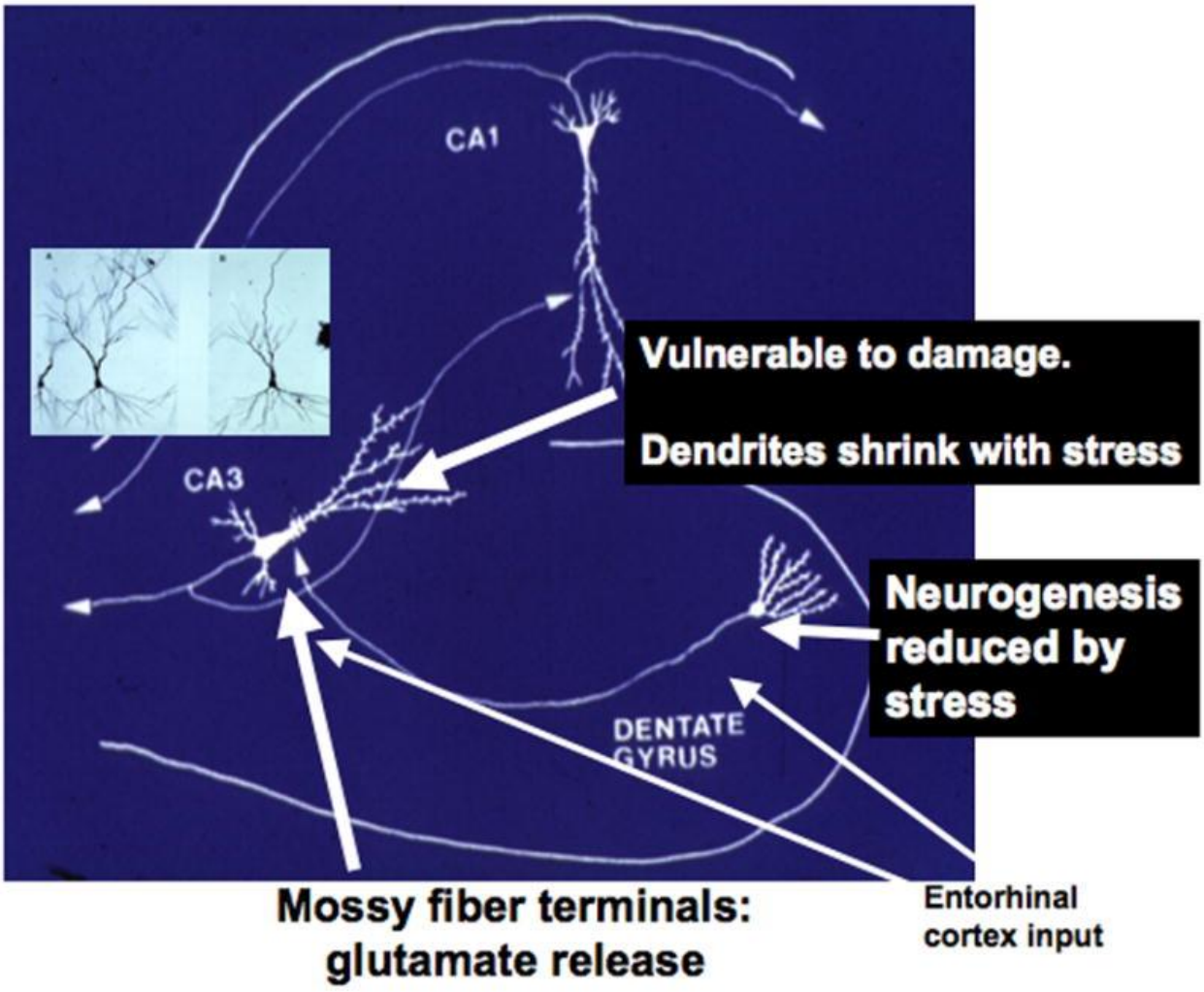

Fig. 2.6: Effects of chronic stress on hippocampal CA3 pyramidal neurons (McEwen, 1999).

2.9.2 Functional role of the amygdala in mediation of stress response

The amygdala brain region consists of discrete cells found in anterior temporal-lobes close to hippocampal-region (Fig. 5). Specifically, it has been described as tiny almond-like structure making part of the limbic-system. This brain structure is known to be active in processing of emotions and modulation of stress-response mechanisms, particularly in context of anxiety or fearful situations (Ponomare *et al*., 2010; McEwen and Gianaros, 2010). Amygdala is widely regarded as critical brain region involved in cortical processes that coordinate stress-provoked alterations in behaviours and peripheral responsiveness in contexts of aversive circumstances. It is instructive to note that neuronal cells in basolateral amygdala have been shown to enlarge after sessions of chronic immobilization stress as well as increased spine density (Vyas *et al*., 2002; McEwen and Gianaros, 2010). Meanwhile, medial amygdala-neurons have been reported to display reduced spine density after chronic stress (McEwen *et al*., 2015; McEwen and Gianaros, 2010; Godoy *et al*., 2018). These changes were also followed by increased anxiety suggesting that chronic stress promotes restructuring as well as dysfunctions of amygdala (McEwen, 1999; Godoy *et al*., 2018; Vyas *et al* 2002).

2.9.3. Role of prefrontal cortex in neurobiology of stress

As depicted in Fig. 5, the prefrontal cortex is situated anteriorly within the frontal lobes of the brain and basically instrumental to higher cognitive functions such as working memory and executive functions. It functions also in controlling stress and other threat-related circumstances through stimulation of coping-mechanisms within subcortical limbic regions including hippocampus, amygdala, and hypothalamus (McEwen and Gianaros, 2010). Neuronal cells within the medial prefrontal cortex have been reported to display dendritic degeneration and loss of spines after chronic stresses (Liston *et al*., 2006; McEwen and Gianaros, 2010). Hypertrophy of the prefrontal cortex caused by chronic stress was reported to produce impairment of executive and cognitive functions (Liston *et al* 2006).

2.10 Autonomic nervous system in stress-response

The ANS, a major component of the nervous system plays central role in mediation of reaction to stresses. Its primary function in mediating response to persistent stress was in the context of the classical description of the fight and flight response many years ago, which centered on activation of the sympathetic nervous system. The sympathetic nervous component mediates the classical phenomenon of the "fight or flight" response that leads to shift of bodily energy towards confronting dangers or escaping from enemies. It has been shown to exhibit diverse neuronal pathways essential for regulation of responses to multiple stressors (Yaribeygi *et al*., 2017).

Noradrenergic sympathetic nerve fibers also supply vasculatures including the gut, which release noradrenaline that has been shown to modify functions of the immune cells including proliferation, differentiation, cell trafficking, and cytokine secretion and autoimmune state in vulnerable organisms (Yaribeygi *et al*., 2017). Stimulation of sympathetic system, for example, has been depicted to induce systemic secretion of IL-6 from immune cells and other inflammatory mediators via activation of HPA axis. The observed effects of this system on immune cells are in consonant with reports showing the negative effects of stressors on immune functions (Yaribeygi *et al*., 2017).

The sympathetic system responds to stress by sending signals to adrenal glands for release catecholamines and cortisol that trigger the heart to beat faster; resulting in elevation of blood pressure. Respiration rate also increased and elevation of blood glucose levels as energy source for dealing with emergency situations. The response of this system to stressors is known to be very fast with the goal to make the body well fitted for the crisis. The body typically goes back to its normal stress-free state immediately the crisis has abated (American Psychological Association, 2014). However, continuous stimulation of the sympathetic system in period of persistent stress triggers physical reactions that lead to damage of bodily organs. Thus, it might be concluded that what persistent stimulation of the nervous system does to the entire body outweighs what stress does to the nervous system. Indeed, abnormal autonomic regulation under conditions of allostatic load has been recognized as key factor in the pathophysiology of cardiovascular disorders (Yaribeygi *et al*., 2017).

2.11 Hypothalamic pituitary adrenal axis in mediation of stress-responses

The hypothalamus; a tiny part of the brain found beneath the thalamus and on top of the brainstem serves as a linkage between the nervous system and endocrine systems. This connection helps in regulating the hypothalamus for the purpose of secretion of hormones into blood stream that cause profound long-lasting effects on bodily functions. It secrets various hormones such as corticotrophin-releasing hormone, which in turn stimulate pituitary gland for further production of hormonal substances during exposure to stressors (O'Connor *et al.,* 2000;Godoy *et al*., 2018).

It is well established that potent stressors activate HPA axis through glucocorticoid-related feedback mechanisms regulated by some prominent neuronal structures such hippocampus, amygdala as well as prefrontal cortex. This feedback machinery may be enhanced in certain chronic ill health conditions in attempt combat persistent stressors. Hypercortisolism that typified HPA overactivity, has been alluded to as a classical sign of generalized stress of high intensity (Yaribeygi *et al*., 2017; Godoy *et al*., 2018). Indeed, HPA hyper-activeness has been reported in some patents with endogenous depression, panic disorder or that suffered sexual abuse (Yaribeygi *et al*., 2017; Godoy *et al*., 2018). Decline in glucocorticoid receptor expressions provoked by persistent stress have been established (Yaribeygi *et al*., 2017; Godoy *et al*., 2018). Decrease in central glucocorticoid receptors might be related to its reversible downregulation or permanent destruction of glucocorticoid-containing neurons. Indeed, diverse patterns of stress-induced HPA-dysregulations have been reported in persons that had suffered post-traumatic stress-disorder as well as chronic fatigue syndrome **(**Godoy *et al*., 2018).

2.12. Excitatory amino acids in mediation of stress response

Excitatory amino acids, particularly glutamate, play prominent roles in structural as well as functional brain changes because it is a key excitatory- transmitter and its excess has been linked to cellular injury and neuroinflammation. Studies have shown that chronic stress produced shrinkage of apical dendrites of hippocampal CA3 neurons and elevated extracellular glutamate levels (Treccani *et al*., 2014). It has been reported that corticosterone acts through membrane linked mineralocorticoid receptors and

glucocorticoid receptors to promote glutamate release. Blockade of N-methyl-D-aspartate (NMDA) receptors and interfering with excitatory activation of ion channels impedes stress-induced dendritic remodeling. Precisely, stress-evoked NMDA-mediated dendritic remodeling has occurred in frontal cortex neurons (Treccani *et al*., 2014). Excess glutamatergic activity has been implicated in the genesis of traumatic stress and ischemia that are characterized by irreversible loss of neuronal cells orchestrated via mechanisms known to be aggravated by glucocorticoids.

In addition, unchecked overflow have shown to contribute to endogenous depression, dendritic shrinkage as well as reduced neurogenesis in experimental animals (Treccani *et al*., 2014). Persistent stress damages not only hippocampal CA3 and dentate gyrus neurons but also medial amygdala as well as prefrontal cortex (Treccani *et al*., 2014).It has also been noted that uncontrolled glutamate overflow plays key role in aging and dementia, medical conditions closely connected with prolonged stress. Studies have demonstrated that treatment of rats with agents that abolish glutamate release delayed aging based on preservation of spatial memory and dendritic spines (Popoli *et al*., 2012).

2.13 Epigenetics of individual differences

Adaptive responses of organisms to persistent stress are known to be governed through intricate multiplicity of genetic, developmental as well as environmental features (Gold, 2015; Tsigos *et al.,* 2016). The gene-environment interactions influence brain development and its adaptability whereas epigenetic event drives how social as well as physical environment act to influence brain functions and bodily organs throughout life courses (Hunter and McEwen; 2013; Reul, 2014). More specifically, it has been defined as measures above the levels the genome, which control expressions of genetic processes without changing the DNA system. Epigenetic data might shed some light upon some trajectories of adaptive or maladaptive machineries that might necessitate pharmacological applications. Individual's features that permit these adaptive or maladaptive consequences rest upon the uniqueness of neural capacity of the individuals, which in most cases depend largely on the experiences gained in the course of life journey, particularly in the early years of life (Gold, 2015; Tsigos *et al.,* 2016). These environmental influences might promote abnormal brain cytoarchitecture as well as

altered epigenetic machinery that could leads to failures in appropriate gene expressions in respond to new challenges.

Early life-events concerning parental care in humans have been described to play key roles in both mental and physical health as established through studies on effects of adversity on childhood experiences (Reul, 2014). Thus, environmental factors in conjunction with past life experiences are critical determinants of our possible health outcomes even though the genetic factors are also important (Reul, 2014; Griffiths and Hunter, 2014). Diverse alleles of frequently occurring genes are known to govern how people will react to stressors or life events. For example, the short variant of serotonin transporter has been linked to some neuropsychiatric illnesses and people having this allele are known to be more vulnerable to adversity and exhibiting depressive illness (McKittrick *et al*., 2000).

2.14. Physical and psychological stressors

In context of perception, stress can be categorized as physical or interoceptive stressor and psychological or exteroceptive stressor. Physical or interoceptive stressors might induce apparent tissue damage such as mucosal inflammation or tissue irritation through excessive production of gastric acid that might lead to initiation of systemic inflammation via releasing pro-inflammatory cytokines (Yaribeygi *et al*., 2017). It is known that these cytokines activate HPA via corticotropin releasing factor. Diverse mechanisms, including cytokine-evoked vagal nerve stimulation been implicated on how they drive systemic inflammatory signal into the brain (Godoy *et al*., 2018). The eventual outcome of peripheral cytokine-induced HPA simulation is for elevation of plasma cortisol levels as well as increased secretion of mediators associated with chronic inflammation.

The severity of psychological stressors has been shown to depend on the chronicity of the stress, age and individuals' perception to aversive-situations. Numerous forms of psychological stressors produce long-lasting effects on the sensitivity of individuals to stress and genesis of chronic diseases in the future. For example, early life adversity such as separation of infants from their mothers during critical periods of development induced everlasting hypersecretion of cortisol and over reactivity of the locus coeruleus, a brain

area concerned with mediation of aggression and violent tendency (Halfon *et al*., 2014). Child abuse or neglect has also been demonstrated to altered HPA axis reactivity stress resulting in several psychopathologies (Karatsoreos and McEwen, 2011). Furthermore, exposure to stressors, perceived as life-threatening such as rape, loss of beloved ones, kidnapping, bullying or combative situations as well as natural disasters have been reported to alter sympathetic as well as HPA sensitivity to aversive stimuli as well as enhanced recalls of such ordeals Karatsoreos and McEwen, 2011). Prolonged stress such as financial difficulty, ill-health of spouses and loss of job, particularly, when perceived as threatening can induce increase in allostatic burden that midwifed several pathological abnormalities (Karatsoreos and McEwen, 2011). These pathological stressors are major causes of neuropsychiatric diseases and violent tendencies that hold strong implications for public health.

2.15 Types of Stress

2.15.1 Acute Stress

Acute stress can be viewed as a type of threat that is often perceived almost immediately and is closely related to fight or flight phenomenon (Neupert *et al.,* 2006). The threat may be regarded any circumstances that is perceived, even subconsciously, as a potential danger and often associated with hyper-arousal response (Larzelere *et al.,* 2008). Acute stress has been alluded to as a reaction to a threat typified by increased sympathetic discharges that primes living organisms for fighting or fleeing. Hans Selye designated it years after as the fight and flight response that he labelled as the first phase of the general adaptation syndrome that typified response to threats amongst living organisms (Larzelere *et al.,* 2008). However, He further categorized the syndrome into three-phases; the alarm, adaptation and exhaustion.

Nearly the observed physiological effects that featured during the alarm phase correspond to rapid release of catecholamines followed by sustained slow rise in glucocorticoids secretion as well as rapid elevation of brain excitatory amino acids (Moghddam, 1993). These reactions were judged to be mandatory for the restoration of dynamic equilibrium required for the survival of the organism in time of acute stress but adverse effects may

ensue when the process is sustained (Sapolsky, 1996). Long lasting stress has indeed been shown to alter biological equilibrium that primes for diverse illnesses as a consequent of maladaptation (McEwen, 1998).

2.15.2 Chronic stress

Chronic stress has been regarded as a threat that is sustained for very a long period of time that keeps the body systems in a state of constant physiological arousal or overload (Pasquali, 2006). It is known to affect all the body organs of the body, disrupting nearly every system in the body. Chronic stressors precipitate diverse pathological manifestations such asthma, back pain, fatigue, headache, irritable bowel syndrome, ulcers, CVS disorders and reduced immune function.

Specifically, it promotes vulnerability to various pathologies including anxiety, endogenous depression, heart attack, stroke, and also contribute to infertility as well as hasten the ageing process. Prolonged stress can cause structural and functional rewiring of the brain, making other bodily organs more susceptible to anxiety as well as depression. Persistent-stressors alter signal transduction pathways, certain neurotransmitters, neurotrophic factors and cell adhesion molecules. Altered glutamatergic pathways have been identified in the mediation of excitotoxic cascade mechanisms that orchestrate neuronal degeneration or neurotoxicity during prolonged stress. N-methyl-D-Aspartate (NMDA)-mediated responses are known to increase after chronic stress further confirming the excitotoxicity of glutamate in chronic stress (Kole *et al.*, 2002). However, the levels of the stress hormone cortisol (Kole *et al.*, 2002) is the most commonly used measure of chronic stress.

2.16 Factors influencing the response to stress

Numerous factors that have to do with the severity and duration of the stressor and individuals' traits affect response to stress (Larzelere *et al.* 2008). Stress reactivity is also modified by events related early life events (parental, postnatal and adolescence), as well as adulthood and other factors such as nutritional status, and mental as well as physical activity). Socioeconomic status of the individuals has been highlighted as a dominant factor in modulating stress reactivity. Pre-existing stress-related disorders like anxiety or

depression also modulate the capacity to respond to a stressful event (Larzelere *et al.* 2008). The precise functioning of the neuroimmune and endocrine networks also preconditions the individual's capacity to respond appropriately to subsequent stressful events. Thus, the functional status of the individuals affects his adaptive mechanism and resilience against intense stress. However, disruption of neuroimmune and endocrine communication might make the persons to respond to stress inappropriately, and more vulnerability to diseases (Larzelere *et al.* 2008).

2.17 Effects of stress on the body

2.17.1 Musculoskeletal system

Chronic stress affects muscle functions tailored against possible injury and pain. There is increased muscle tension in times of persistent stress. Chronic stress causes the muscles in the body to be in constant state of overactive (America Psychological Association, 2014) and stretched, which might even promote stress-related disorders. Good examples of these disorders include tension-type headache and migraine headaches, which are often connected with chronic muscle tension in the shoulders, neck and head. The major determinant injured person to suffer from chronic pain is how he responds to the injury. Muscle tension, and eventual muscle atrophy owing to chronic stress-related musculoskeletal disorders have been cited in literature. This can be controlled through the practice of relaxation techniques that have been reported to relieve muscle tension and reduced the incidence of certain stress-related disorders, such as headache and well-beings (Kloet *et al.*, 2006).

2.17.2. Respiratory system

Chronic stress can trigger breathing difficulty, as often experienced in the fight or flight phenomenon. Acute stress induces hyperventilation and also triggers asthma attacks through induction of bronchoconstriction (American Psychological association, 2014). Studies have demonstrated that long-lasting stress aggravate chronic inflammatory responses of the airway resulting in damage and steady deterioration of lung function via tissue remodeling (Forsythe *et al*., 2004). Several factors such as poverty, racial or ethnic discrimination (Fig. 7) are known to be connected with genesis of asthma (Landeo-

Gutierrez *et al*., 2019). Findings from clinical investigations have also established that patients suffering from asthma responded with increased bronchial tone to distressful situations.

Exposures to environmental factors including allergens as well as air pollutants are usually related with increased risk of asthma and its consequences. Though, more recent investigations have documented that persistent psychosocial stressor are additional environmental triggers of asthmatic attacks (Barnthouse and Jones, 2019). Furthermore, chronic stress has been reported to alter the extent of the airway inflammatory responses in similar way to irritants, allergens as well as infections in persons with asthma (Barnthouse and Jones, 2019). In fact, studies have shown a close association between chronic stressors and risk of asthmatic attacks and antiasthma drugs (Barnthouse and Jones, 2019). Populations studies have also revealed that psychosocial stressors triggered by racial discrimination also modify asthma-related outcomes (Barnthouse and Jones, 2019).

Chronic stress has been shown to reduce response to short-acting β_2-agonists and corticosteroids (Miller and Chen, 2006) and children with high levels of chronic stress were reported to exhibit lower bronchodilator response than those with lower levels of chronic stress (Brehm *et al*., 2015). Prolonged stress has been found to worsen asthma symptoms through the combined effects of neurotransmitters and chemical mediators involved in autonomic regulation of bronchoconstriction and inflammation of the airways (Fig. 8). It has also been found to alter expression of immunologic genes, beta-adrenergic and glucocorticoid receptor genes and cytokine regulation, all of which contribute to asthma pathophysiology and therapeutic failures among patients undergoing intense stress (Barnthouse and Jones, 2019). Thus, intense adversity plays key roles in asthmatic attacks through mechanisms relating to downregulation of the β_2-adrenergic and glucocorticoid receptors in susceptible individuals, perhaps due to persistent secretion of catecholamines (Chen and Miller, 2007;Landeo-Gutierrez, 2019).

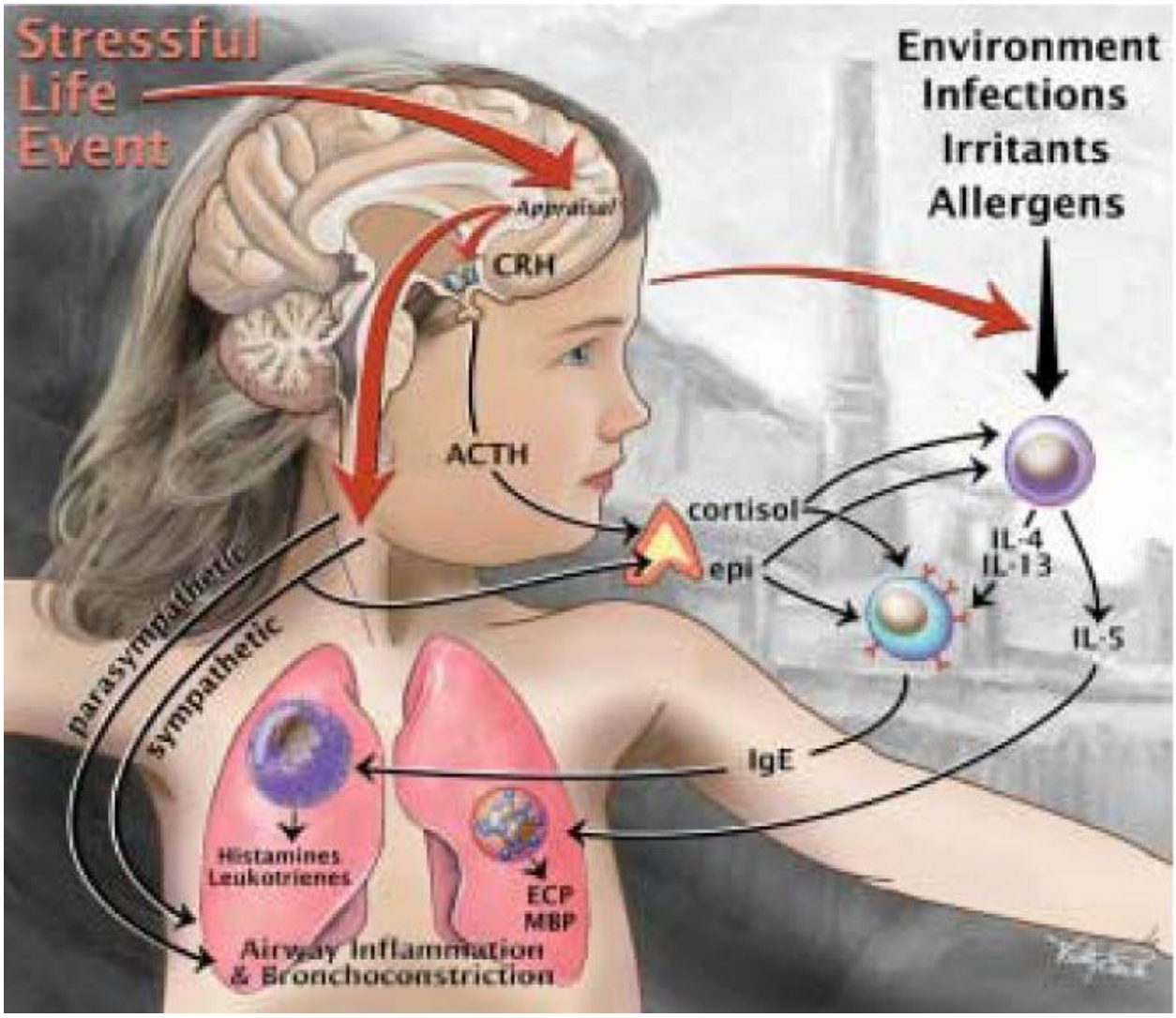

Fig.2.7. Interaction of psychological stressors with environmental factors in asthma pathology (Chen and Miller, 2007).

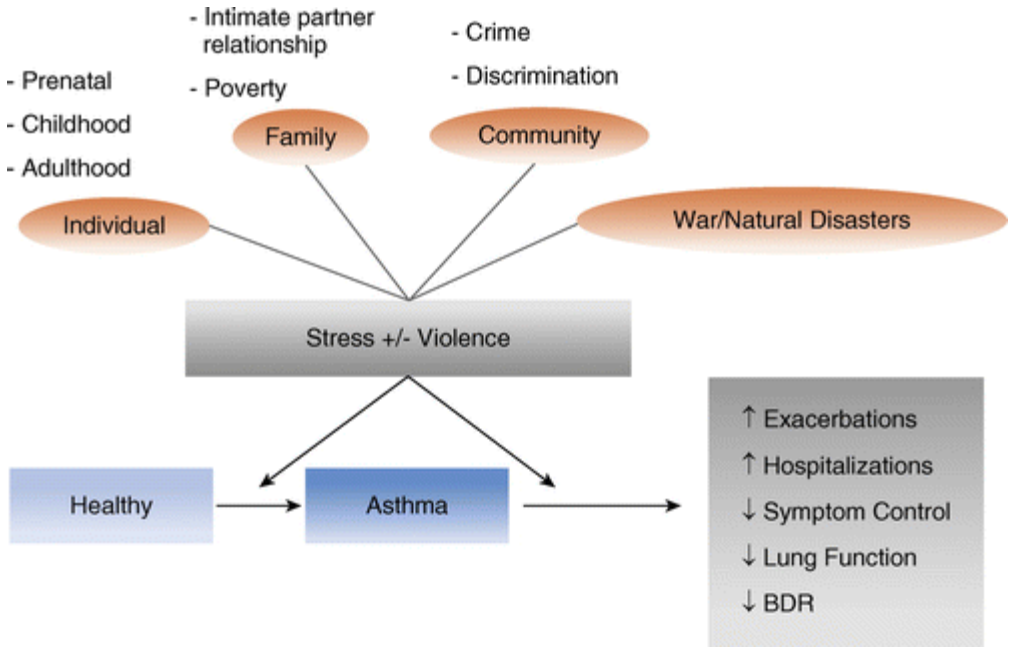

Fig.2.8. Exposure to violence, chronic stress, and asthma. BDR = bronchodilator response (Chen and Miller, 2007).

2.17.3. Cardiovascular function

The cardiovascular system (CVS) consists of the heart as well as blood vessels, which act together for purposes of providing nourishment and oxygen to several bodily organs. It is critically involved in mediation of reactions to both acute and chronic stressors. Stressors of acute dimension induce an increase in heart rate and contractions of cardiac muscle through the actions of catecholamines and cortisol (Yaribeygi *et al*., 2017). Stress, whether acute or chronic has long been established to produce deleterious effects CVS functions (Rozanski *et al*., 1999). It has been documented that intense stress evoked abnormal autonomic activity and indirectly altered CVS activity. Stress-evoked sympathetic nervous system stimulation leads to elevation in heart rate, force of myocardia contraction, vasodilation in arteries of skeletal muscles, narrowing of veins, contraction of the arteries in the kidneys, and also decreased renal sodium excretion

Stress has been noted to affect vascular endothelial cells, increased the risk of thrombosis and ischemia as well as increased platelet aggregation (Rozanski *et al*., 1999; Yaribeygi *et al*., 2017). Meanwhile, key effects of persistent stressors on the CVS have been shown to be related to blood pressure elevation through mechanisms associated with stimulation of autonomic sympathetic nervous system. Stress also alters lipid levels and vascular changes, resulting in increased risk of arrhythmia including other CVS disorders. It has been documented that intense stress evoke alpha-adrenergic activation followed by coronary vasoconstriction, increased heart rate and oxygen demand. Thus, increasing the risk of CVS diseases such as hypertension, heart attack, myocardial infarction and stroke (Rozanski *et al*., 1999; American Psychological Association, 2014; Yaribeygi *et al*., 2017).

Persistent stress also contributes to inflammation of the circulatory system, principally the coronary arteries, which might be additional possible pathway for translating stress to heart attacks (Yaribeygi *et al*., 2017). It also seems that the way persons respond to stressors affect cholesterol levels (American Psychological Association, 2014). Above all, effects of persistent stress do not exist in isolation but are often worsen through unhealthy behaviours such as poor diet, inadequate physical activity, tobacco use, and poor adherence to medications (Yaribeygi *et al*., 2017).

2.17.4. Immune system

Immunity has been viewed as a process of mounting resistance of living organisms against pathogenetic infections and also having suitable resilience to predisposition to diseases, allergy, and autoimmune diseases (Sompayrac, 2016). Lymphocytes have been recognized as key cellular component of immune system. Lymphocytes are known in mediating immune responses through cytokines release, as well as priming B- cells for production of antibodies and killer cells, which in turns kill antigen expressing cells (Sompayrac, 2016). Persistent stress had been found to suppress innate and adaptive immune responses thereby triggering inflammation as well as suppressing immuno-protective cells (Sompayrac, 2016, Dhabhar, 2014).

Although inflammation has long been regarded as adaptive response to tissue injury or infections. Inflammatory conditions of persistent nature, have been linked to multiple pathologies (Dhabhar, 2014). Inflammation of high chronicity provoked through intense stressful conditions is also closely connected with increased risk for numerous diseases such as infectious, cancers, depression and autoimmune diseases (Dhabhar, 2014). Possible mechanisms linking intense stress and inflammation in the genesis of diverse diseases is premised on the fact that chronic stressors cause glucocorticoid receptor resistance thereby interfering modulation inflammatory insults (Cohen *et al*., 2012).

Studies have further provided convincing evidences showing the capability of persistent stress modifying cellular machineries that orchestrate diverse illnesses. Bartolomucci and coworkers (2003), for example, reported that persistent stressor evoked inflammatory responses through release of certain cytokines. Consequently, mice injected with proinflammatory cytokines exhibited abnormal motor function, poor social interaction, anhedonia, memory decline, and increased pain sensitivity (Dantzer, 2009). Taken together, these findings showed how chronic inflammation causes symptoms of endogenous depression as well as sickness behaviors during times of stress of high intensity (Dantzer *et al*., 2008).

Persistent stressors of high intensity also alter humoral/cellular immune responses to pathogenic invasions, hence, promoting risks influenza and common cold and other

infectious diseases. Indeed, causal association among persistent stress and predisposition to infections particularly common cold are succinctly documented in literature (Sompayrac, 2016, Dhabhar, 2014). Loneliness, a type of psychosocial stressor, has been found to worsen viral infections and showed that symptoms of common cold were more severe in persons with loneliness (LeRoy *et al*., 2017). Many studies have shown that stress disrupts immune system through mechanism principally linked to suppression of corticosteroids (Elenkov, 2004; Dantzer *et al*., 2008; Cohen *et al*., 2012).

Stress of persistent nature has been reported to elicit immuno-senescence through suppression of immune functions, which is typified in elderly persons (Mathur *et al*., 2016). Decline in T-cell function that is instrumental to chronic low-grade inflammation has been highlighted as one of the major characteristic features of immuno-senescence (Wu and Meydani, 2008). Characteristically, elderly people in comparing to other age groups have higher proinflammatory cytokines (IL-6, TNF) in circulation (Michaud *et al*., 2013). The functional capability of immune-protective cells defends the body pathogens has been reported be grossly suppressed by high levels of circulating higher proinflammatory cytokines (Dantzer *et al*., 2008; Cohen *et al*., 2012; Michaud *et al*., 2013). Consequently, reduced immunity associated with aging underpins increased vulnerability of older persons to stressors (Michaud *et al*., 2013).

The influences of chronic-stressors on immune cells are not mediated only through glucocorticoids but also via catecholaminergic as well as neuroendocrine pathways. Susceptivity immune cells to persistent stress are a cluster of abnormal regulatory reciprocal influences existing between immune and central nervous systems (Sompayrac, 2016, Dhabhar, 2014). The immune system obtains signals from discrete brain-regions and neuroendocrine pathway through the autonomic nervous system, and relays information back to the brain via cytokines. This interconnection forms a long-loop regulatory feedback system critical in coordinating physiological responses to stressors and inflammation (Michaud *et al*., 2013).

2.17.5. Endocrine system

Glucocorticoid is known to mediate the effects of intense stress on the endocrine functions as in antagonizing actions of growth hormones, sex steroids on muscle and bone anabolism (Charmandari *et al*., 2005). Chronic-stress has been connected with increased visceral adiposity, reduced lean body mass as well as reduced osteoblastic activity that often-typified patients with Cushing's syndrome and melancholic depression. It is also linked with hypertension, hypercoagulation and atherosclerotic-CVS diseases, all of them displaying increased HPA axis activity (Charmandari *et al*., 2005). The relationship between chronic-stress and metabolic syndrome-related manifestations has also been established in cynomolgus monkeys (Charmandari *et al*., 2005). Increased gluconeogenesis has been as one of the key features of persistent stressful conditions as well as insulin resistance due to HPA axis deregulation. Thus, HPA axis is being viewed as contributor to poor diabetes control in patients going through emotional distress (Charmandari *et al*., 2005). In fact, chronic stimulation of HPA axis has been established in diabetic patients who later developed diabetic neuropathy (Charmandari *et al*., 2005). Thus, persistent stimulation of stress systems might lead to vicious cycle of hyperglycemia, hyperlipidemia thereby increasing insulin resistance and diabetic complications.

Growing evidences indicate that chronic-stress plays significant roles in type-2 diabetes mellitus (Hackett and Steptoe 2017). Insulin resistance, for example, has been closely related to potent stress of persistent nature (Tsuneki *et al*., 2013). Obesity, diabetes mellitus, and high visceral adiposity, which are interacted metabolic syndrome, are key sources of chronic inflammation (Donath and Shoelson, 2011). Chronic stress during early life also increased the risk for obesity and diabetes (Hughes *et al*., 2017).

2.17.6. Stress and cancer

Studies in the field of psychoneuroimmunology have suggested the role of potent stressors and absence of social engagement as potential risks for carcinogenesis (Moreno-Smith *et al*., 2010). Glucocorticoids, norepinephrine and epinephrine released during stressful conditions have been reported to exhibit numerous effects on tumor pathology

(Moreno-Smith *et al*., 2010). Glucocorticoid-mediated mechanisms as well as stimulation of sympathetic nervous system have been shown to deplete immune-mechanisms thereby contributing to tumor growth (Lutgendorf and Andersen, 2015). Chronic stressors evoked inflammation resulting in loss of protective immune-armory accounting for predisposition to cancer (Dhabhar, 2014). Growth and progression of tumors have been to be influenced by IL-6, IL-12, IFN-γ as well as TNF (Dolan *et al*., 2017; Mostofa *et al*. 2017).

The propensity of prolonged stress of high intensity has been found to alter anti-tumor specific responses to immunogenic tumor cells. Studies have established that maltreatment during childhood and adulthood adversity caused inefficient immune defense mechanisms (Fagundes *et al*. 2012). Findings from preclinical investigations further revealed that rodents exposed intense ultraviolet-light stressor had squamous cell carcinoma more quickly as well as reduced immunity compared with non-stressed mice (Saul *et al*. 2005; Illi *et al*. 2012).

2.17.7. Gastrointestinal complications and stress

The outcomes of chronic-stress on nutrition and gastrointestinal tract (GIT) have been categorized into two functions, first on appetite (Yaribeygi *et al*., 2017). The appetite-effect has been linked to N-methyl-D-aspartate receptor (Sadeghi *et al*., 2015). Nevertheless, the influence of chronic stressors on feeding behaviours indicates linear relationship between nutrition and intense adversity (Ghanbari *et al*., 2015). Secondly, as shown in Fig. 9, chronic stressors also affect functions of GIT; absorption, intestinal permeability, mucus and acid secretion (Yaribeygi *et al*., 2017). Stress of high chronicity promotes GIT inflammation through secretion of inflammatory mediators resulting in increased permeability and lymphocyte recruitments (Collins, 2001). Lymphocyte aggregation in turns leads to formation of inflammatory agents that further aggravate gastrointestinal inflammatory diseases (Collins, 2001). Truly, several inflammatory diseases including Crohn's disease as well as gastric ulcer are closely connected intense-stressors (Hommes *et al*., 2002). Furthermore, irritable bowel syndrome, a disease of inflammatory origin has been established to be highly interrelated to chronic-stress (Yaribeygi *et al*., 2017).

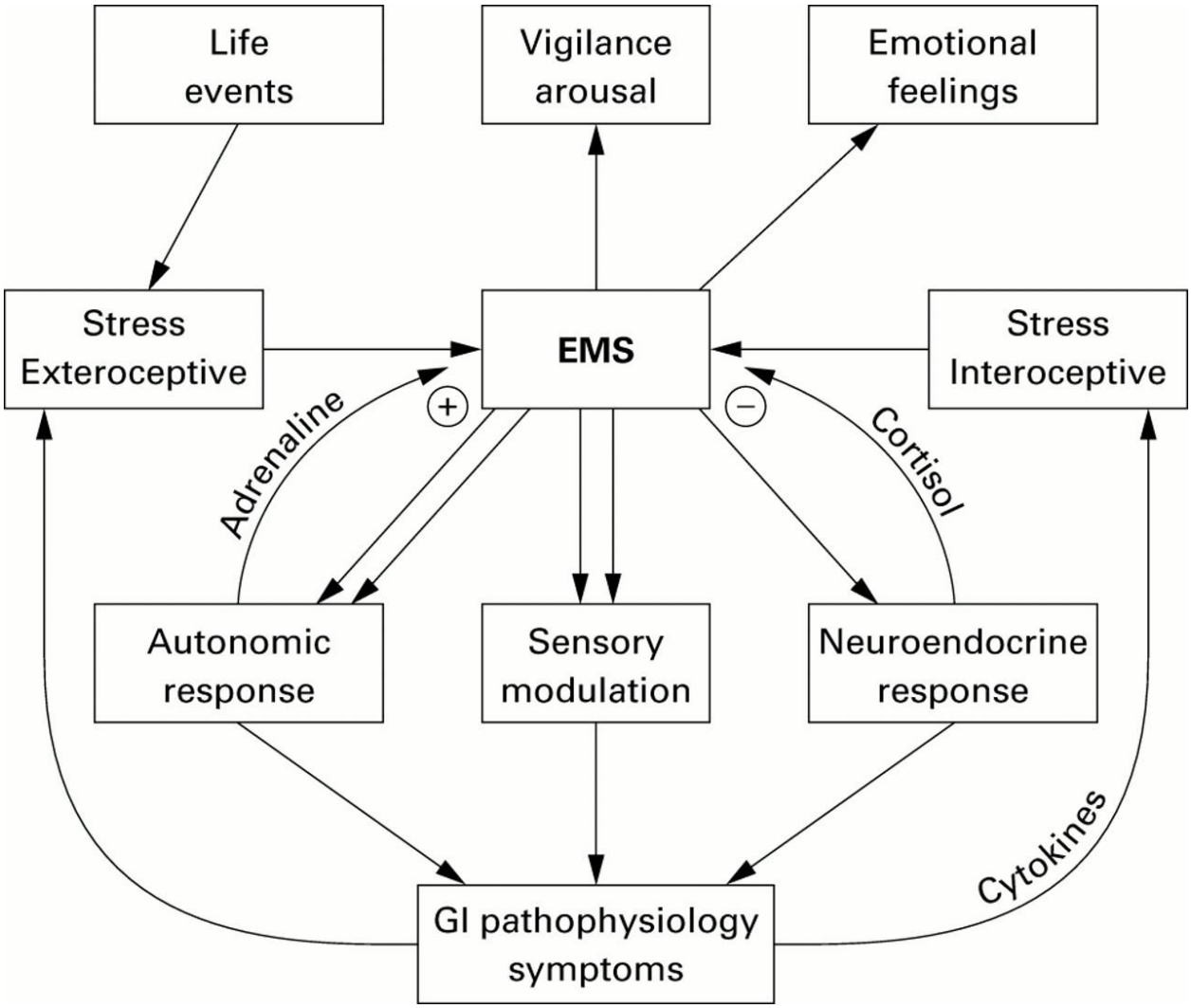

Fig.2.9. Possible mechanisms involved in stress-induced gastrointestinal pathologies (Mayer, 2000)

2.17.8. Reproductive System

Intense stress reduces reproductive capability and even completely abolishes reproduction in lower organisms (Wingfield and Sapolsky, 2003). Hypothalamic pituitary gonadal axis and HPA axis are known to share a number of mediators such as steroid hormones. Thus, stress-induced stimulation of HPA axis will definitely compromise reproduction via altering steroids formation. The fall in reproduction in period of aversive circumstances has been loosely ascribed to the belief that glucocorticoids redirect resources/energy from reproductive performance and individual's sextual engagements (Wingfield and Sapolsky, 2003). It has been observed for example, that in salmons as well, marsupials died subsequently after breeding because they still engage in reproduction during intense stress. The proximate reason for their death has been ascribed to excess glucocorticoids for catabolism of essential proteins (Wingfield and Sapolsky, 2003).

Excess amounts of cortisol disturb testosterone formation, sperm production and maturation as well as induction of impotence, also known as erectile dysfunction. Similarly, intense stress has been reported to alter menstruation amongst women in diverse ways. Chronic stress causes irregular menstruation, painful periods and fluctuations in lengths of the cycle. It may also worsen premenstrual symptoms including mood fluctuations, pains, bloating and irritability. Emotional distress might also worsen diverse symptoms experienced by menopausal women. Chronic-stress is also known to reduce sexual desire or pleasure (American Psychological Association, 2014).

2.17.9. Stress and aging process

Aging is key risk factor enhancing susceptibility to cognitive decline produced by chronic-stressors. Intense stress-evoked activation of glucocorticoid pathway has been established as the major contributor to variability in aging processes. Stress impact on the individual can be seen, from the susceptibility manifested by the individual across the life span, and also on the particular life time on the individual, the effect stress will have will be evident on cognitive function. Stress is usually known to accelerate the aging process and appearance of the individual. Caruso and coworkers (2018) also noted that intense

adversity contribute to exacerbation of cognitive deficits in elderly persons (Caruso *et al*., 2018).

Early-life adversities are known to elicit profound disabling-effects on cognition during adulthood. High cortisol levels and reduced feedback HPA-sensitivity has been linked to aging processes. Involvement of chronic-stress on cognition in aging is also supported by findings of Issa *et al.,* (1990) showing that rats regarded as inferior (as opposed to good) learners as in aged humans had impaired memory as well as elevated corticosterone contents. Additionally, Radley and coworkers (2015) reported that increased glucocorticoid contents followed by abnormal negative feedback regulation of HPA axis are integral component of aging (Radley *et al*., 2015).

In addition, prolonged stress of high intensity elevated oxidative stress and shorten telomere length in healthy premenopausal women (Epel *et al*., 2004; Rizvi *et al*., 2014). Researches have shown shortened telomeres were linked to aging, apoptosis, carcinogenesis as well as increased incidence of numerous diseases (Shammas, 2011). Intense adversity also alters telomere length over life journey (Rizvi *et al*., 2014). Studies have also shown that children exposed to intense aversive conditions had shorter telomeres later in life (Caruso *et al*., 2018).

2.18. Animal models of stress

Diverse animal procedures for elucidating stress-related mechanisms have been established and widely utilized to investigate agents having potential-benefits against stress-invoked pathologies in rodents. Studies relating to stress neurobiology over the years had centered on elucidation of pathological outcomes, therapeutic modalities and unraveling mechanisms underpinning stress responses. The development of suitable animal paradigms that typically reflect stress reactivity and its neurobiological consequences has witnessed several modifications over the years (Marcelo *et al.,* 2007). Moreover, it has been advanced that the ideal animal models should have the capability of replicating the critical aspects of stress responses and mimics the natural course of its multiple devastating outcomes in humans. Nevertheless, no single of the currently available procedures can completely replicate the biological impacts of chronic stressors

in human beings. For instances, some of the existing models are more suitable in reproducing physical stress-responses (Kvetnansky *et al.,* 1970) while others appropriately replicate psychosocial stress-responses and the accompanying behavioural changes (Marcelo *et al.,* 2007). Another confounding issue relates to the fact that chronic stressors cause massive HPA axis stimulation and neuroendocrine dysfunctions accompanied by widespread organ pathologies compared to acute stress, which in some cases might even be beneficials. Thus, appreciate models should have the flexibility in elucidating the key aspects of stress responses of high reproducibility. However, it has been recognized that no stress model is without limitations such as predictability. Meanwhile, animal stress models have been divided broadly into physical and psychological stress models.

2.18.1 Physical stress models

Animal models of stress of physical stressors domain are categorized below:

- Temperature changes-evoked stress; immersion in cold water with no way of escaping.
- Immobilization-evoked stress
- Stress produced through electric foot shock
- Forced swimming endurance
- Anoxic tolerance test

Fluctuation in temperature-evoked stress

Acute changes in environmental temperature might evoke stressful situations by stimulating the thermoregulatory epicenter located in the hypothalamus followed by HPA axis activation. This event initiates immediate release of adrenocortical hormones in blood stream, which are the primers mediators of acute stress responses (Sapolsky *et al.,* 1986). Exposure to extreme cold environment such as immersion in cold water or confinement in cold setting has been used for provoking acute stress responses. In the immersion in cold water model, rodents are independently kept in a vessel containing cold at about 4^0C, and allowed to swim to exhaustion (Retana-Marquez *et al.,* 2003). The time to exhaustion is then measured and the animal taken back to it home cage after

drying. This type of stressor suitable for acute stress studies because it can be done within comparatively short period of time. However, it has major drawback of the animals developing resistance in adapting to the setting on chronic exposure (Pitman, *et al.*, 1988).

In the cold-confined stress model, the rodents are separately kept in fridges at 4^0C. For acute stress, the animals are usually exposed once for some period of time, e.g for 15 minutes. However, the animals are exposed repeatedly for chronic stress responses (Kvetnansky *et al*., 1971). This scenario also triggers activation of HPA axis followed by sharp rise glucocorticoids as response to the stressor (Kvetnansky *et al*., 2002; Staratakis *et al.*, 1995). Nevertheless, it also suffers the drawback of the likelihood of developing resistance during chronic exposure.

Immobilization-evoked stress

This model is widely utilized as a stressor for elucidating physiological, neurobehavioural and biochemical changes that typified stress responses (Kvetnansky, 1970). This paradigm can be executed two possible ways; involving immobilizing the animals (rats) on semicylindrical acrylic tube (4.5cm diameter and 12cm long) with air passages or immobilizing their limbs on boards using adhesive tape. Head movement is usually restricted through the use of metal loop, coiled around their necks. The animals immobilized for about three hours for acute stress-responses while for about for 7 days for chronic stress- responses. The main merit of this model is that it induces inescapable physical and mental stresses, to which adaption rarely occurs (Dronjak, *et al.*, 2006).

Electric foot shock-provoked stress

Rodents are well known to be highly sensitive to even mild electric shock and respond swiftly to this type of stressor. This paradigm entails exposure of rodents to electric foot shock of varying intensity and duration to provoke stressful conditions. The animals are usually exposed to the stressor of electric shock in a chamber with an electrified floor. The unavoidable electric foot shocks with intensity of 3 mA of frequency of 1 per second over 5 min period are delivered to the animals. For acute stress-responses, one exposure is enforced whereas repeated exposures are instigated for chronic stress-responses (Retana-

marquez, 2003). However, electric shock of 60 minutes (0.15 mA shock with a mean inter-shock interval of 60 seconds) has also been adopted (Taysse, 2005). It has the advantage of high sensitivity but has the hazard of electric shock causing death of the animals.

Forced swim-endurance test

This test is premised on the findings that animals exposed to inescapable settings, displayed signs typifying stress-responses. In this stress model, rodents are made to swim in cylinder containing water at normal temperature until the time of exhaustion. One exposure for acute stress while more than one for its adoption for chronic stress (Ferry *et al.*, 1991; (Kitchen *et al.*, 1990). The parameters commonly measured indicative of stress-responses include latency to immobility, time spent in active swimming aa well as latency to exhaustion. Although, it is safe stress-model, adaptation to chronic swimming and inter-strain differences have been reported (Armario, 1995).

2.18.2 Stress models of psychological domains

Animal models of stress of psychological domains include:

- Neonatal isolation-evoked stress
- Predatory stressors
- Day-night light variations
- Noise-evoked stress

Stress model of neonatal isolation

It has been documented that early-life stresses of neonatal isolation have severe health consequences in adulthood (Kuhn *et al.*, 1990; Herman *et al.*, 1997; Kosten *et al.*, 2005). The procedure entails isolation of litters' inbred strains of rodents from their mothers for a specified period of time, usually about 21 days (Kosten *et al.,* 2004). This model has offered a suitable tool for demonstrating the effects of lifetime stressors on susceptibility to psychiatric disorders such as depression, anxiety, cognitive deficits, addiction as well as aggressive tendency (Kosten *et al.,* 2005).

Predatory stress-model

It is well known that encountering predative organisms have been described as one of the greatest terrifying and stressful events living organisms could experience that characteristically trigger the flight or fight response (Lupien *et al.,* 2006). Such stressors cause immediate stimulation of sympathetic nerve resulting in instant increase in blood catecholamines and cortisol. Direct encounter with specific predator has been widely employed for elucidating biochemical as well as physiological derangements triggered by intense stress (Adam *et al*., 1993; Marilia *et al.,* 2007).

In this stress paradigm, mice are exposed to natural predator like cat for a brief period (Blanchard *et al.,* 1998). Changes in behavioural patterns including locomotion, shrieking-like voices as well as neuroendocrine changes are measured after stress sessions (Blanchard *et al.,* 1998). It is relevant to note that this paradigm is useful for induction of acute stress-responses but incidence of habituation to predators can occur, justifying its suitability for acute stress-model.

Changes in day/light-evoked stress

Variations in diurnal patterns induce weighty health consequence upon living organisms (Artcheson *et al.,* 1975). Acute stress-responses have been produced in experimental organisms through subjection to unexpected fluctuations in day-night light patterns (Koseten *et al.,* 2005). The pineal body has been implicated in circadian variations through mediation of melatonin secretion (Nicholson *et al.,* 1985). This chemical agent is known to be secreted from pineal body during responses to dim light while serotonin acting as its functional antagonist in mediating bright light-responses. In fact, sleep-awake events are mediated via the serotonin-melatonin pathway (Bermudez *et al.,* 1983; Hamm *et al.,* 1983).

In this stress-paradigm, the organisms are exposed to light variations via alteration of the light/dark cycles for specific period of time (Mercelo *et al.,* 2007). It is an appropriate method for causing acute stress-responses but can also be adopted for induction of

chronic stress-responses (Rai *et al.,* 2003). It also has the problem of adaptation to the stressor after repeated exposures (Mercelo *et al.,* 2007).

Noise-evoked stress-model

Excessive noise has been identified as potent stressor with great capability of distorting body hemostasis and triggering pathological consequences. Noise has been reported to cause increase in catecholamine and alter intracellular calcium concentrations (Paparelli *et al.,* 1992). Ramsey (1982) noted that noise becomes a potent stressor when it goes beyond 90 dB. It has been shown to induce oxidative stress via depletion of antioxidant enzymes (Samson, 2005). This stressor can be induced in laboratory animals by exposing them to a loudspeaker (15 W) powered by noise generator (0-26 kHz) mounted about 30 cm above each cage. The noise-level may be fixed at 100 dB and monitored using sound level meter (Ravindrari *et al.,* 2005).

2.18.3 Unpredictable chronic mild-stress paradigm

This chronic-stress paradigm involves exposing living organisms to diverse stressors of modest intensity in randomized ways for the purposes of preventing onset of adaptation. Both physical as well as psychosocial stressors are utilized in this model. It is well reported that the multiple stressors paradigm induced arising concentrations of corticosterone suggesting susceptibility of the HPA axis and inability of the animals to adapt (Magarino *et al.,* 1995; (Blanchard *et al.,* 1998). This paradigm has been confirmed as one the most suitable model for elucidation of the neurobiology of chronic stress-mechanisms since it lacks the issue relating to adaptation and also an excellent tool for long-term effects of stressors.

Practically, this model entails subjecting rodents to diverse stressors of both psychosocial and physical domains for some specified period of time. The stressors commonly used include immobilization, overnight sleep deprivation, and rotation of the cage (Ortiz *et al.,* 1996). Wet saw dust bedding, electric foot shock, predators or predator's feaces as well as urine (Anisma *et al*., 2007). Numerous modifications of this model in terms of the applied-stressors as well as time, and sequence of exposure are replete in literature (Ortiz *et al.,* 1996; Renard *et al.,* 2005, Ladd *et al.,* 2004; (Anisman *et al*., 2007).

2.18.4. Hypoxic stress

Hypoxic-stress has been regarded as a medical term ensuing from decrease in oxygen delivery to various body-tissues that imposes greater deleterious effects on brain cells (Taylor *et al*., 2016; Shimoda and Polak, 2011). Decreased oxygen supply to brain cells for even a short period of time has been shown to produce irreversible impairment to neuronal integrity. Hypoxic stress has been labelled as a potent stressor because its ability to disrupt body physiologic performances further attesting to the fact that constant delivery of oxygen is a precondition for existence of aerobic-organisms (*Lutz, 1992;* Taylor *et al*., 2016; Shimoda and Polak, 2011). The brain cells, indeed, have been noted as the most first set of body-cells to suffer from hypoxia, and died almost instantly during inadequate oxygen. Brian cells have been alluded to as the sensitive entities to oxygen deficiency due to low antioxidant profile and increased metabolic activity (Tomar *et al.*, 1984; Liu *et al.*, 2014). Brains damage occasioned by oxygen deficiency and reduced perfusion are commonly labelled as hypoxic-injuries. Diverse circumstances such as stroke, myocardial infarction, extreme hypotension, asthma, suffocation, drowning, carbon monoxide-toxicity are known to affect oxygen delivery to brain cells (Lutz, 1992; Malhotra *et al.*, 2001; Busl and Greer, 2010; Chen *et al.*, 2020).

2.19. Effects of hypoxic stress on brain functions

Brain hypoxic injury produces widespread effects and long-term disabilities affecting the behaivours and mental health status of susceptible individuals (Chen *et al.*, 2020). Some notable deleterious consequences following hypoxia include impaired cognition, anxiety, depression and abnormal motor functions. Seizures are also common manifestations of intense hypoxic state leading to severe brain injury. Hypoxia-evoked loss of consciousness and its duration have been highlighted as major predictors of the extent of brain-injury and possible outcomes of recovery. Meanwhile, some specific brain regions including the hippocampus, prefrontal cortex, amygdala and basal ganglia are known to displayed more vulnerability to chronic hypoxic-stress.

The hippocampal region, a well-known entity for cognition is very vulnerable to oxygen deficiency. Loss of cognition is a notable sign associated with cerebral hypoxic-stress resulting in speech disorders as well as information processing. Intense hypoxic-stress might also deregulate HPA axis thereby disrupting body-homeostatic mechanisms. Damage to structural integrity of the prefrontal cortex triggered impairment in executive functions such as synthesizing and integrating complex information for decisions making, which may lead to inability to act efficiently social settings,diverse personality-changes such as aggressiveness, mood-fluctuations, poor social interaction are prominent signs of prefrontal cortex injury. Injury to the basal ganglia is closely connected with motor dysfunctions such as abnormal movements and postures.

2.20 Mechanisms of hypoxic stress-induced neurologic deficits

Although inadequate distribution of body-oxygen to brain cells is generally known to impact diverse incapacitating effects (Chen *et al.*, 2020), convulsive-episodes typified immediate occurrences in rodents subjected to anoxic-stress. These forms of convulsive-episodes are denoted as anoxic-tolerance time or anoxic-convulsions (Tomar *et al.*, 1984). It has been said that increased anoxic-tolerance time is a signal of capabilities of adaptogens (Tomar *et al.*, 1984). Increased threshold to anoxic-convulsion, therefore, serves as indications of efficient adaptive mechanisms against diverse stressors as well as health promoting benefits of adaptogens.

The HPA axis has been recognized as the prime neuroendocrine pathway in mediating cortisol secretions followed by induction of oxidative stress, neuroinflammation as well as alterations of additional biochemical entities orchestrating diverse organs pathologies that epitomized intense stress ((Radley *et al*., 2015; Taylor *et al*., 2016; Caruso *et al*., 2018). Indeed, excess cortisol accompanying chronic stress-responses has been shown to participate in losses of multiple neuronal circuitries through upstream mechanisms relating to oxidative stress (Dedovic *et al*., 2009; Radley *et al*., 2015; Taylor *et al*., 2016; Caruso *et al*., 2018). It is been shown that psychopathologic abnormalities connected with intense are the resultant consequences cortisol-provoked neuronal destructive oxidant substances; ROS/NOS species (Panossian, 2017; Panossian *et al.*, 2018). Meanwhile, additional proinflammatory mediators that further damage neuronal

constituents are produced, which in turns provoke more reactive oxygen species, hence, creating a vicious cycle for pertubation of neurodegeneration that epitomized intense stress-evoked depression as well as loss of cognition (Tonnies and Trushina, 2017).

2.20.1. Role of oxidative stress/nitrergic stress

It is well known that unguided formation of oxidant molecules including ROS/RNS underpins oxidative and nitrergic stresses in causing tissue damages as well as enhancement of aging-process (Dai *et al*.., 2014). Specifically, the disequilibrium in the fine balances existing between ROS-formations and antioxidant mechanisms is the driver of cellular oxidation (Fig. 2.10). Thus, cellular oxidation ensues whenever the formation of oxidant species exceeds antioxidant capability. The principal origins of ROS are traceable to oxidases, which produce O_2- through donation of an electron to oxygen from reduced counterpart of NADPH. Diverse enzymes including superoxide dismutase and myeloperoxidase play functional roles in formation of oxidant species such as H_2O_2 s (Pourova *et al*., 2010).

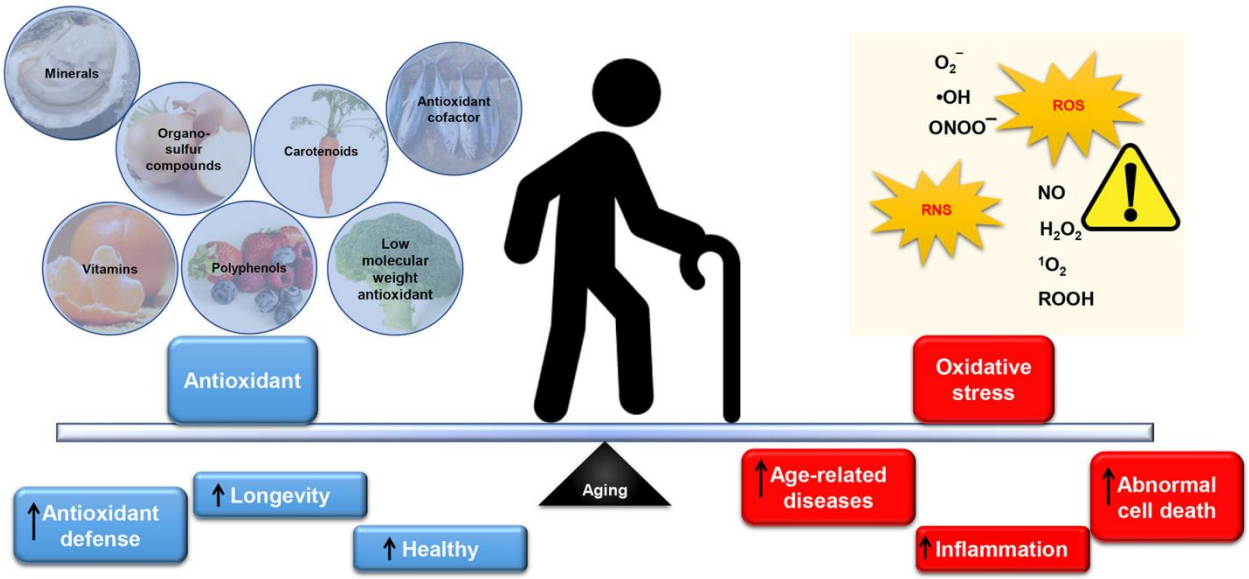

Fig. 2.10: Balance of antioxidants and oxidative stress (Tan *et al.*.,2018)

It has been stated that ROS, including superoxide anion (O_2-), hydroxyl (OH^-), peroxyl (ROO-) and alkoxyl (RO-), are majorly formed during normal body metabolism within mitochondria respiratory chains. They are highly unstable, having one or more unpaired electrons that render them to be very reactive in nature. These oxidant species are notably recognized for their capacity to interact readily with macromolecules including lipids, proteins and DNA. Physiological report have established the physiological functions in control of immune response, inflammation, synaptic plasticity, learning, and memory at low concentrations (Karihtala and Soini, 2007). Nevertheless, their destructiveness in causing diverse tissue oxidations and damage to macromolecular entities, when in excess, are widely documented in literature (Karihtala and Soini, 2007).

Nitric oxide (NO-), peroxynitrite (ONOO-), nitrogen dioxide (NOO-)- the key free radicals of RNS domains (Zhao *et al*., 2013). It is well documented that the principal origin of nitric oxide is through biological biosynthesis utilizing nitric oxide synthases (NOS). Three isotypes of the enzymes have by characterized; namely, the two-constitutively expressed endothelial (eNOS) and neuronal (nNOS) isoforms as well as inducible (iNOS) type. The eNOS are essentially involved in regulation of endothelial functions and immune system (Zhao *et al*., 2013). The iNOS are closely connected with induction of inflammation whereas the nNOS might contribute to neuronal signalling mechanisms (Zhao *et al*., 2013).

Diverse neurobiological-mechanisms underlying neuronal cell death due to chronic-hypoxic stress have been ascribed partly to elevated oxidative and inflammatory pathways. It has been established that the genetic-deletion of NADPH oxidase, cyclooxygenase-2 as well as iNOS mitigate hypoxia-provoked neuronal apoptosis and cognitive decline, suggesting that oxido-neuroinflammation played key in neuropathology caused by hypoxia (Smith *et al*., 2013). It has been reported that stimulation of antioxidant mechanisms (Figure 2.11) represents the early step in mitigating oxidative insults on cell-constituents (Mattson *et al.*, 2008; Panossian, 2017; Panossian *et al.*, 2018). Therefore, losses of neuronal antioxidant-protective mechanisms play key role in inducing neuroinflammation as well as increased susceptibility to neuronal cell degeneration. The inhibitory effect of antioxidants on auto oxidation through

interference with the activity free radicals formation. The efficacy of antioxidants is related to scavenging of oxidant species, which trigger peroxidative tissue injury (Gaschler and Stockwell, 2017). Conversely, intracellular-glutathione deficiency predisposes to neuronal cell losses through promoting mitochondrial membrane degradation in response to increased formation of oxidant species by mitochondrial machinery (Lohan *et al*., 2018).

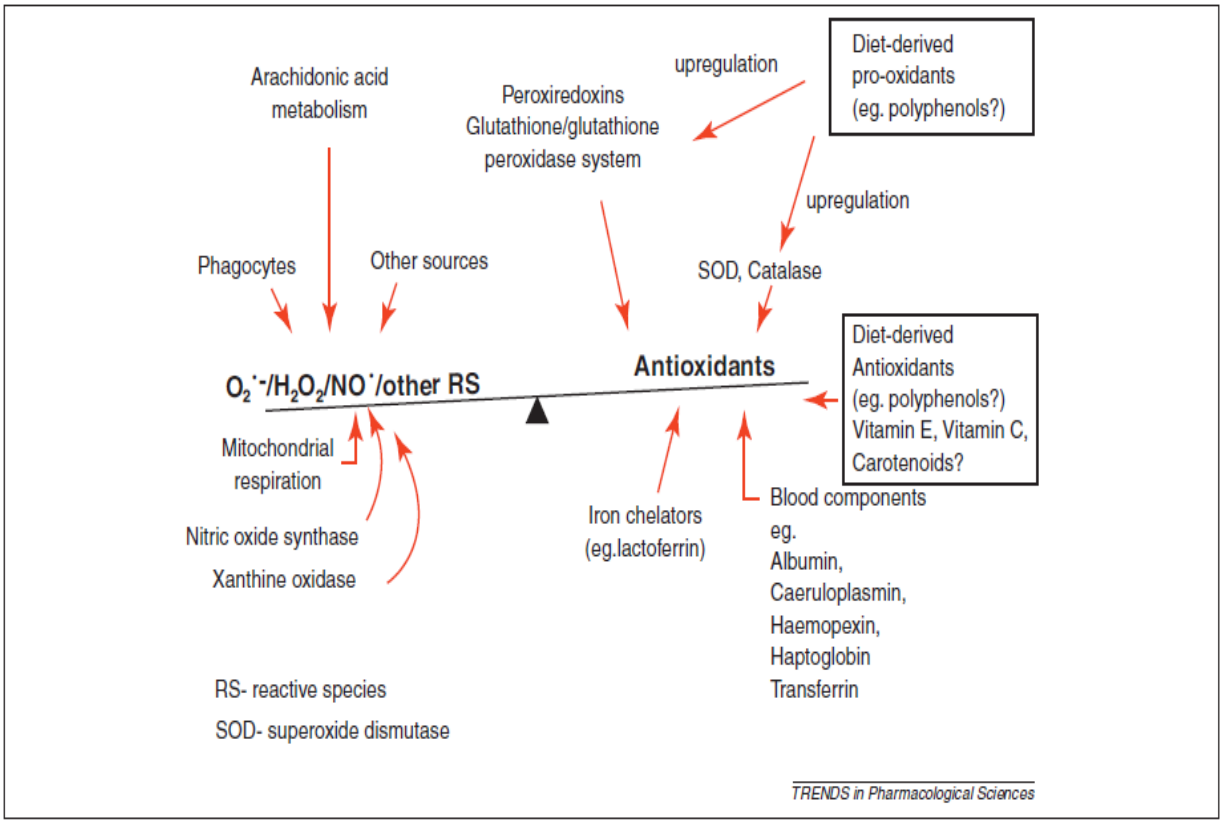

Figure 2.11: Balance between antioxidants and free reactive species (Halliwell *et al* 2005).

2.20.2. Inflammatory pathway in hypoxic stress-induced neurologic deficits

Increased cortisol level produced during stress response contributes to losses of diverse neuronal circuitries through induction of free radical formation (Oken *et al.,* 2015; Tsigos *et al.,* 2016). Abundant evidences abound in literature alluding to the involvement of the neuroimmune pathways as mediators of cortisol production that initiates upstream formation of inflammatory as well as additional molecular entities orchestrating intense stress-evoked pathologies of diverse body organs (Oken *et al.,* 2015; Panossian *et al.,* 2018). Meanwhile, adrenal glands stimulation for cortisol secretion has long been recognized as one of the early effects of stress on the body. Some of these cortisol-mediated effects include elevated blood glucose and free fatty acids levels through gluconeogenesis and lipogenesis as sources of energy in the battle against intense stress (Kulkarni and Juvekar, 2008; Efferth and Koch, 2011; Tsigos *et al.,* 2016). However, excess cortisol production implicated in neurological dysfunctions caused during persistent stress occurs through induction of neuronal-damaging oxidants species (Panossian, 2017; Panossian *et al.,* 2018).

Additionally, it has been established that damaged neurons further produce more proinflammatory mediators, hence, perpetuating degeneration of diverse neuronal circuitries that epitomized neuropsychiatric illnesses caused by intense stress (Pansossian *et al.,* 2018). Thus, deficiency in neuronal antioxidant protection have been alluded to unchecked neuroinflammation as well as enhanced susceptibility to neuronal cells death (Tonnies and Trushina, 2017). Diverse intracellular signallings are known to be involved in inflammatory responses to hypoxic-stress (*Busl and Greer; 2010;* Deepti *et al*., 2019). The NF-kβ has been described as the major transcriptional regulatory factor as mediator of secretion of proinflammatory cytokines and immune functions (Aggarwal *et al*., 2012; Panossian *et al.,* 2018; Chen *et al.,* 2020; Peng *et al.,* 2020). For example, increased TNF-α during hypoxic stress was shown to down-regulate expressions of brain-derived neurotrophic factor (BDNF) (Tian *et al.,* 2013; Gold, 2015).

2.20.3. Brain-derived neurotrophic factor in neuropsychiatric derangements

It has been established that intense stress reduced the beneficial effects of BDNF) in the brain, hence, genesis of anxiety, depression as well as cognitive decline (Gold, 2015). Some of the functions ascribed to BDNF include promotion of neurogenesis, neural stem-cells survival, neuronal proliferations that underpin diverse neuropsychiatric illnesses (Park and Poo, 2013; Gold, 2015). Uncontrollable and prolonged stressors or sustained hypercortisolism in experimental animals have been said to underlie depression as well as decreased brain BDNF (Santarelli *et al.,* 2015).

2.21 Management of stress

Strategies toward management of intense stress and promotion of effective resilience might mitigate the impacts of aversive situations (McEwen, 2007). It should be emphasized that any management approach adopted should be directed at the ability of the animals to cope better in the face of intense stress (McEwen, 2007; Compas, 2006). Thus, implementation of strategies that promote resilience might improve health outcomes (Logan and Barksdale, 2008). It has been noted the inefficient resilience is a byproduct maladaptive response, hence, genesis of diverse pathologies such as anxiety, depression and loss of cognition (McEwen, 2007; Logan and Barksdale, 2008)

Stress control can be described as an effort to decrease the adverse impacts of stress on organisms. It is worth-noting that methods for stress management but only suitable for individuals who display symptoms of a disease or disorder, but also for healthy individuals. The implementation of these techniques to regular routine is an efficient instrument for improving health and protecting lives, which also assists a treasured mediation for the population at large. Health promotion lives, which also assists as a treasured mediation for the population at large. Health promotion could play a fundamental role amongst the various approaches to improving health by developing and implementing measures to decrease or avert distress.

The methods of stress management can be widely classified as biological or psychological. The psychological stress management techniques attempt to regulate the body’s stress reaction by modifying our way of thinking about the stressors. These

techniques/practices on stress boasters and strength. The biological stress management techniques attempt to regulate the body's stress reaction by decreasing physiological activities. These techniques include advanced muscle relaxation, autogenic drills, recreation, biofeedback and directed imaginations (Varvoglil and Darviril, 2011). Nevertheless, drug therapy is by far the most famous biological way of managing stress (Nemeroff and Vale, 2005). Drugs used to handle stress were once called 'minor calming agents', but now substituted gradually with other drugs called 'anxiolytics. Anxiolytics have the impact of reducing the heightened physiological activity without causing fatigue. They contain diazepam and benzodiazepines. They influence brain gamma-aminobutyric acid (GABA) concentrations, which prevents other chemical substances from stimulating brain neurons. This reduces the excitement rate in the brain and make the individual feel calmer. In the management of stress, anti-depressants are also utilized, but beta-blockers such as propranolol are more frequently prescribed. These medications do not influence the chemical signaling of the brain, but instead, they decrease the activity of the sympathetic nervous system, also known as the fight or flight response system.

Beta-blockers can decrease physiological activity by preventing the impacts of adrenaline and noradrenaline, such as increased heart rate and blood pressure, within the organism (Nutt, 2008). The primary strength of the anxiolytics is that they act rapidly and almost instantly reduce stress. Its use id thus justifiable, as long as the stressor is not a long-term but short-term. The drug therapies are as well comparatively cheap and extensively accessible when equated to psychological techniques of stress management. In addition, drugs do not entail any interval or preference in compassion with psychological methods. There are, yet, a variety of medication-released flaws like unpleasant side effects such as somnolence, weariness, dwindled vigilance, and lightheadedness.

The benzodiazepines also induce physical dependency and tolerance-based drug addiction. Beta-blockers as well as anti-inflammatory agents may also be necessary to attenuate pathological derangements with allostatic overload (McEwen, 2007) on the cardiovascular system and the brain. They can also create abstinence syndrome due to anxiety relapses, hence it is challenging for individuals to disassociate from the drug usage. Furthermore, the source of stress impact is not addressed by these standard drugs.

Even if they have a soothing impact, the stressor doesn't end. It is an emotional approach instead of a problem-oriented approach to coping. Nevertheless, 'adaptogens' are collection of plants utilized for managing and treating stress and its associated ailments (Davydov and Krikorian, 2000). Moreover, the need for continuous researches focused on identification of biomarkers of more relevant to allostatic load and development of appropriate interventions against chronic stress-evoked human diseases have been recommended (McEwen, 2003).

It has been established that adaptogens are substances of natural origins that exhibit broad-based spectrum of health-benefits. They are known to demonstrate multiple mechanisms of action relating to nonspecific effects in mitigating diverse pathologies during intense stress (Panossian and Wikman, 2010; Panossian, 2017). More specifically, the potentials of adaptogens in promoting health have been alluded to activation of adaptive mechanisms associated with resilience in time of persistent stressors (Panossian and Wikman, 2010; Panossian *et al.,* 2018). It should be emphasized that Panossian and Wikman (2009) had earlier alluded adaptogens as new-classes of metabolic regulators that promote organism's capability to adapt to stressors and also prevent damages through such stressors. Thus, the distinctiveness of adaptogens entails their non-specificity of actions and reinforcement of cellular defense-machineries compromised in times of intense stress (Panossian and Wikman, 2010; Panossian, 2017).

It has been established that the health benefits offered by adaptogens in mitigating diverse pathologies caused by intense stress centered essentially on inhibiting the release of oxidant species, proinflammatory cytokines in response to stimulation of the HPA axis (Panossian *et al.,* 2007; Wiegant *et al.,* 2008; Panossian and Wikman, 2010; Panossian, 2017). It has been clearly demonstrated that these substances regularized distorted cortisol contents as well as trigger anti-apoptotic proteins, neuroprotective-pathways as well as antioxidant systems during intense stress (Panossian and Wikman, 2009; Panossian, 2017; Panossian *et al.*, 2008). They are further recognized to boost energy reserve and also enhanced immune function in tackling chronic stress (Wagner, 2005; Panossian and Wikman, 2010).

Studies have shown adaptogenic property of a variety of plants such as *Rhodiola rose, Panax ginseng, Ginkgo biloba, Ocimum sanctum, Withaniasomnifera, Schizandra chinensis,* and *Bryonia alba,* with some of them already available for relief of stress (Ellis and Reddy, 2002; Hovhannisyan *et al.,* 2015; Panossian *et al.,* 2018). Precisely, Panax*ginseng constituent, ginseng, for example, has* been shown to exhibit adaptogenic property and widely used for stress-related disorders (Ellis and Reddy, 2002; Hovhannisyan *et al.,* 2015). Additional interventions of non-pharmacological domains advocated for managing intense stress include adjustment of lifestyles to health-promoting activities such as adequate sleep, exercise, social interactive networks etc (McEwen, 2003). However, the need to precisely assess relevant bio-makers indicative intense stress or overload in order to initiate interventions early enough for mitigation of illnesses caused by persistent stress have been advocated in literature (McEwen, 2003).

2.22 Natural sources of Naringenin

Naringenin is a well-known occurring dietary flavanone presence in various vegetables and fruits. Diverse citrus including oranges, mandarins, grapefruit, lemons and limes are known to be very rich in naringenin. Naringenin as depicted in figure 1, is a flavanone glycoside having molecular formula of $C_{27}H_{32}O_{14}$ and molecular mass of 580.4g. Its content is very high in various citrus species, serving as its potential sources. This compound is known to be accountable for distinctive bitter taste of grapefruit (Alam *et al.,* 2014). Naringenin also said be abundant in cereals like millets or sorghum bicolor, the source of Jobelyn, an African food supplement that has gained international recognition as anti-stress and blood-booster. Thus, regular consumption of fruits as well as vegetables abundant in naringenin might be relevant in mitigating chronic diseases including obesity, CVS disorders, metabolic syndromes, and neurodegenerative diseases (Alam *et al.,* 2014).

2.23 Pharmacokinetic properties of naringenin

Pharmacokinetic investigations in rat demonstrated that naringenin undergo rapid metabolic degradation in the liver, with formation of glucuronide metabolites (Ishil *et al.,* 1997; Wang *et al.,* 2006). Gut microflora are said to degrade naringenin in the intestine

as well (Choudhury *et al.,* 1999). Therefore, hepatic metabolism tends to limit its oral bioavailability and its plasma concentrations. It was further reported that a single dose of naringenin given to rats either through intravenous bolus or oral route had rapid conjugation (Choudhury *et al.,* 1999).

Significant serum concentrations of naringenin sulphates and glucuronides were detected almost entirely in bloodstream of rats. It crosses blood brain barrier (BBB) readily and exhibited high BBB permeability (Youdim *et al.,* 2004). The easy penetration of naringenin into the brain might be accounting for its diverse CNS effects. Pharmacokinetic analysis showed that naringenin is rapidly absorbed as its conjugated forms after oral administration (Alam *et al.,* 2014). Nevertheless, it has poor oral bioavailability due to extensive pre-systemic metabolism; partly through liver enzymes and intestinal bacteria (Youdim *et al.,* 2004).

2.24 Pharmacology of naringenin

Naringenin is known to exhibit numerous pharmacological activities ranging from systemic to CNS conditions (Alam *et al.,* 2014). Naringenin demonstrate antihyperglycemic effect in streptozotocin-nicotinamide-induced diabetes in rats (Annadurai *et al.,* 2012). The antihyperglycemic activity was linked to suppression of oxidative stress and inflammation in hepatic as well pancreatic tissues of diabetic rats. It also elevated nitric oxide formation and improved acetylcholine-mediated endothelial function in thoracic aortic ring preparations (Ikemura *et al.,* 2012). It also induces vasodilation in obese rats as well as streptozotocin-treated rats (Fallahi *et al.,* 2012). Calcium-dependent potassium channels are involved in mediating vascular relaxation and naringenin was shown to enhance calcium-activated K+ currents in rat tail artery (Fallahi *et al.,* 2012). Naringenin supplementation also demonstrated plasma-lipid lowering effect in experimental models of hyperlipidemia and obesity.

The cardioprotective and hepatoprotective effects of naringenin has been reported in literature (Mojzisova *et al.,* 2009; Qin *et al.,* 2008). It also prevented isoproterenol-induced myocardial infarction and also reduced oxidative stress as well as decreased inflammatory cells in cardiac muscles of rats given isoproterenol (Mojzisova *et al.,* 2009).

Naringenin was reported to lower the elevated plasma transaminase activity in cadmium-induced liver toxicity in rats (Renugadevi and Prabu, 2010). It further lowered lipid peroxidation and increased antioxidant enzymes (superoxide dismutase, catalase) in liver cells (Renugadevi and Prabu, 2010).

Various investigations have demonstrated anti-inflammatory as well as antioxidant activities of naringenin (Chtourou *et al.,* 2014). It inhibits release of inflammatory mediators including iNOS, TNF-α as well as cyclooxygenase-2 evoked by 3-nitropropionic acid in rats (Gopinath *et al.,* 2012). Naringenin exhibited potent scavenger of free radicals and reduced lipid peroxidation (Cavia-Saiz *et al.,* 2010). Both superoxide and hydroxyl radicals are scavenged by it *in vitro* (Cavia-Saiz *et al.,* 2010). Numerous CNS properties of this compound reported in literature are potential pointers to therapeutic usefulness naringenin in psychopathological ailments (Khan *et al.,* 2012; Raza *et al.,* 2013; Yang *et al.,* 2014; Ghofrani *et al.,* 2015).

Earlier findings on naringenin showed improved cognitive functions and reduced neurodegeneration in experimental studies (Baluchnejadmojarad and Roghani, 2006; Khan *et al.,* 2012; Yang *et al.,* 2014). Its central antioxidant and anticholinesterase effect were said to account for the amelioration of memory dysfunction evoked by excess sugar (Rahigude *et al.,* 2012). The reduction of Aβ level and inflammation might be playing some roles in beneficial effects in memory dysfunctions. Prophylactic treatment with naringenin was shown to mitigate ischemic stroke through inhibiting NF-kB-mediated neuroinflammation. It further exerted protective effects against lipopolysaccharide-evoked microglial activation and 6-hydroxydopamine-induced Parkinson's disease (Wu *et al.,* 2015; Lou *et al.,* 2014). Naringenin also exhibited neuro-protection against stroke through suppressing NF-kappa B pathway (Raza *et al.,* 2013). Preclinical studies further demonstrated that it decreased iron-evoked neurotoxicity through inhibiting oxidative stress (Chtourou *et al.,* 2014).

CHAPTER THREE

MATERIALS AND METHODOLOGIES

3.1 Source and housing of laboratory animals

In this research, male Swiss mice weighing 22 ±2 g utilized in this research were procured from University of Ibadan Central Animal Facility. They were housed at room temperature in standard cages and had unhindered access to rodents' pellet diet as well as distilled water. The mice were subjected to two weeks of acclimatization before beginning the research. University of Ibadan Animal Care and Use Research Ethics Committee approved the research protocols (UI-ACEREC/19/143).

3.2 Drugs and chemicals

The substances used in the research include naringenin-NG, thiobarbituric acid-TBA, (5',5' Dithiosis-(2-nitrobenzoate)-DTNB), trichloroacetic acid-TCA (Burgoyne Burbidges& Co., Mumbai, India), IL-6,TNF-α (biolegend ELISA kits, USA),NF-κB,iNOS and BDNF (bioVision ELISA kits, USA).

3.3 Equipment

Centrifuge (ATKE), spectrophotometer (Spectrumlab), pH meter (EDT instruments), weighing balance (Ohaus), test tubes, Eppendorf tubes, tube racks and dissecting kits were used in this experiment.

3.4 Drug preparation and dosages of naringenin

Previous procedure reported by Umukoro *et al*. (2018) was followed in the preparation of naringenin solution using 5% dimethyl sulfoxide (DMSO), which served as vehicle.

Different doses (10, 25 and 50 mg/kg) of naringenin used for the research were chosen in accordance to results gotten through earlier investigations (Umukoro *et al*., 2018).

3.5 Experimental Procedures

3.5.1 Evaluation of the effect of naringenin on anoxic convulsions in mice

This experiment was carried out utilizing the experimental procedure previously reported by Caillard *et al*. (1975). Mice were grouped on random basis into 5 (7 animals in each group), and those in group 1 were given i.p. injection of 10 mL/kg of vehicle (DMSO) that served as non-stress control. However, mice in groups 2-5 received intraperitoneal injection of naringenin (10, 25, 50 and 100 mg/kg respectively). Mice allocated to groups 2-5 were afterwards subjected to anoxic stress through individual placement in an air-tight cylindrical 250 mL capacity vessel 30 min after treatment. They were afterwards, observed for latency to convulsions (anoxic-tolerance time) and then removed immediately from the vessel for their home cages.

3.5.2 Determination of naringenin effects on anoxia-evoked increased glucose and corticosterone contents

Immediately after the anoxic convulsion test, few drops of blood taken through the mouse tail were utilized for assay for plasma glucose content using glucometer tape (Accu-Chek Roche). For assay of serum corticosterone, 1 mL blood was taken via cardiac puncture followed by centrifugation 3000rpm for 15 min. Corticosterone concentration was then assayed in the serum using ELISA Kit (Oxford Biomedical Research, USA) in accordance with instructions highlighted by the manufacturer.

3.5.3 Brain-tissue preparation of anoxic-stress mice for biochemical assays

The brain tissues of the stressed and non-stress mice were prepared after they have been euthanized using ether anesthesia. The isolated brains were then weighed followed by homogenization using 10% w/v of 0.1M phosphate buffer of pH 7.40. The homogenates in respective groups were divided into different portions for determination of glutathione, malondialdehyde as well as nitrite contents.

3.3.1 Reduced glutathione (GSH) assay

The technique described by Moron and coworkers (1979) was adopted in assaying for brain GSH-content of mice that suffered anoxia. About 0.4 mL of each sample with 0.4 mL of 20% trichloroacetic acid were reacted together, which was accompanied by centrifugation utilizing cold centrifuge at 4^0C at 10,000 rpm for 20 min. Each sample supernatant (0.25 mL) with 2 mL 0.6 mM DTNB were then reacted together. Then, 0.2 M phosphate buffer of pH 8.0 was added for adjustment of the volume 3 mL. Afterwards, absorbances were taken spectrophotometrically (412 nm) against blank reagent and reduced GSH contents, presented as micromoles per gram tissue.

3.3.2 Assay for malondialdehyde contents

This was determined using the procedure earlier described by Okhawa (1979). Simply, 0.5 mL of distilled-water, 1.0 mL trichloroacetic acid (10%) were reacted with the sample (0.5 mL) followed by centrifugation at 2000 rpm for 10 min. Afterwards, each supernatant (0.9 mL) was reacted with 0.1 mL thiobarbituric acid (0.375%). The resultant solution was then heated in water bath at 80^{o}C for 40 min. Absorbance of each supernatant was read spectrophotometrically at 532 nm after the solution had cooled to room temperature. Molar extinction coefficient of $1.56x10^5$ was used in calculating the MDA contents (presented as micromoles per gram tissue).

3.3.3 Assay of nitrite contents

This was carried out using Griess reagent in accordance with a previously reported technique by Green *et al* (1982). Precisely, 100 µL Gresis reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% of N-1 naphthy1 ethylene diamine dihydrochloride) were reacted with each supernatant (100 µL) and absorbance was determined afterwards at 540 nm. Brain nitrite contents were projected from standard curve gotten from 0-100 µM of sodium nitrite.

3.4 Effects ofNaringeninon chronic hypoxic stress

The procedure described by Caillard *et al* (1975), with slight modifications, was used for evaluating effects of naringenin on chronic hypoxic stress using 250 mL air-tight

cylinder-shaped capacity container, whereby mice were independently kept in the vessel daily for 14 days for 15 min each. Specifically, there was random distribution of the mice into 5 treatment-groups (7 mice per group). Precisely, groups I (non-stress control) and 2 (stress-control) mice received i.p injection of 10 mL/kg of 5% DMSO (vehicle) whereas groups 3-5 had intraperitoneal injection of naringenin (10, 25 and 50 mg/kg), daily for 14 days. Based on results obtained from preliminary investigations of the effect of naringenin on anoxic stress, the dose of 100 mg/kg was removed from the chronic hypoxic stress studies. Afterwards, mice in treatment group 2-5 were subjected to multiple hypoxic condition after 30 min of treatment as described above. The neuro-behavioural functions including memory, motor function, anxiety as well as depression were done using the appropriate mazes in this sequence on day 15. The mazes were cleaned using alcohol (70%) in order to eliminate residual odour after each test.

3.5 Assessment of neurobehavioural performance

3.5.1 Assessment of memory functioning

The memory capacity of the hypoxic-stress mice, was assessed on day 15 based on alternation behaviour using Y-maze paradigm in accordance to the method reported by Casadesus and coworkers (2006). This apparatus is made up of three arms; namely A, B and C that were arranged at 120^0. Briefly, mice were placed independently into arm A of Y-maze; alternation behaviours across the arms in correct sequence: ABC, CAB or BCA but not BAB or ABA were then monitored for 5 min period. The accurate alternation (%), which depicts memory function was determined by means of: (Total alternation number/Total number of arms entires-2) X 100 (Casadesus *et al.,* 2006).

3.5.2 Test of locomotor activity

The number of lines crossing-episodes was employed for the assessment of the motor performance of the hypoxic-stressed mice using open field apparatus. Specifically, mice positioned independently into central arena of the open field box were assessed for motor function as soon as the test for memory was done. Thereafter, lines crossing episodes were noted for 5 min duration.

3.5.3 Test for anxiety using elevated-plus maze

The procedure reported by Pellow *et al* (1985), with mice placed independently on the edge of the open-arm and head facing the center of the maze was employed for the anxiety study. Afterwards, patterns of arm entry, duration (s) of stay in open as well as closed arms were noted for 5 min. Arm entry occurs whenever the mice feet fully entered one of the arms.

3.5.4 Light/Dark Box for anxiety

The anxiety behaviour was further investigated based on the patterns of transition in the light/dark box earlier reported by (Bourin and Hascoet, 2003) whereby mice were independently positioned within center of the bright portion of the apparatus. Thereafter, time spent (seconds) in both compartments of the apparatus were captured for 5 min. Thereafter, data were presented as duration of time spent in both compartments.

3.5.5 Tail suspension test for depression

This test is founded upon the notion of onset immobility in rodents subjugated to inescapable aversive stress as reported by Steru *et al* (1985). Precisely, mice independently held to a rod raised to 50 cm height with adhesive-tape applied 1cm from the tail tip. Immobility period (s) was then captured for 4 min after 2 min delay. Immobility was envisaged whenever the mouse assumed motionless state or with little passive activity.

3.6 Blood glucose and corticosterone assays in hypoxic-stressed mice

After the behavioural testing, few drops of blood taken through the mouse tail were utilized for assay of plasma glucose content using glucometer tape (Accu-Chek Roche). For assay of serum corticosterone, 1 mL blood was taken via cardiac puncture followed by centrifugation at 3000rpm for 15 min. Corticosterone concentration was then assayed in the serum using ELISA Kit (Oxford Biomedical Research, USA) in accordance with instructions highlighted by the manufacturer.

3.7 Brain-tissues preparations of hypoxic-stress mice for biochemical assays

The brain tissues of the stress and non-stress mice prepared have been euthanized using ether anesthesia. The isolated brains were weighed followed by homogenization using 10% w/v of 0.1M phosphate buffer of pH 7.40. The homogenates in respective groups, divided into different portions for determination of various biochemical parameters.

3.7.1 Assay for malondialdehyde (MDA) contents

This was determined using the procedure earlier described by Okhawa *et al* (1979). Simply, 0.5 mL of distilled-water, 1.0 mL trichloroacetic acid (10%) were reacted with the sample (0.5 mL) followed by centrifugation at 2000 rpm for 10 min. Afterwards, each supernatant (0.9 mL) was reacted with 0.1mL thiobarbituric acid (0.375%). The resultant solution was then heated in water bath at 80^{o}C for 40 min. Absorbance of each supernatant was read spectrophotometrically at 532 nm after the solution had cooled to room temperature. Molar extinction coefficient of $1.56x10^{5}$ was used in calculating the MDA contents (presented as micromoles per gram tissue).

3.7.2 Reduced glutathione (GSH) assay

The technique described by Moron *et al*(1979) was adopted in assaying for brain GSH content in mice that suffered hypoxia. About 0.4 mL of each sample with 0.4 mL of 20% trichloroacetic acid were reacted together, which was accompanied by centrifugation utilizing cold centrifuge at temperature of 4^{0}C at revolution of 10,000 rpm for a period of 20 min before reacting 0.25 mL supernatant with 2 mL 0.6mM DTNB. Then, 0.2 M phosphate buffer of pH 8.0 was added for adjustment of the volume to 3 mL. Afterwards, absorbances were taken spectrophotometrically (412 nm) against blank reagent and reduced GSH contents, presented as micromoles per gram tissue.

3.7.3 Superoxide dismutase (SOD) assay

The assay for brain SOD was done as outlined in the procedure previously described (Mistra and Fridovich, 1972). The brain tissue supernatant (0.1 mL) was reacted with 0.05 M of 2.6 mL carbonate-buffer of pH 10.2 and reaction initiated by adding 0.5 mL of 0.3 mM adrenaline after equilibration. ELISA microplate-reader was used to measure the

changes in absorbances for 60s intervals for 3min at 480 nm. SOD activity presented based on units of adrenaline consumed (units/mg protein).

3.7.4 Assay for catalase activity

This assay is based on the procedure described by Goth (1990), which involves the use colorimetric assay principled upon the formation of yellow complex with molybdate and H_2O_2 with the addition of catalase. Precisely, 50 µL of each supernatant was reacted with 60 mM phosphate buffer of pH 7.4. This was accompanied by incubation of enzymatic step within 3 min, which was stopped through addition of 100 µL ammonium molybdate (64.8 mM) in sulfuric acid. Microplate reader (MICRO READ 1000, Belgium) was employed for the reading of the absorbance at 405 nm. Catalase activity was then presented in Unit/mg protein.

3.7.5 Assay for nitrite contents

This was done using Griess reagent in accordance with a previously reported technique by Green *et al.*, (1982). Precisely, 100 µL Gresis reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% of N-1 naphthy1 ethylene diamine dihydrochloride) were reacted with each supernatant (100 µL), absorbance was read at 540 nm afterwards. Brain nitrite contents were projected from standard curve gotten from 0-100 µM of sodium nitrite.

3.7.6 Assay for protein contents

The method of Gornall *et al*, (1949) was employed for estimating protein contents for SOD and catalase. Precisely, 50 µL sample supernatant was pipetted into 96 well plates, which was followed by addition of biuret (150 µL). Microplate reader (GDMI, Belgium) was employed for the reading of the absorbance at 540 nm after 30 min of incubation.

3.7.7 Proinflammatory cytokines assays

Brain proinflammatory cytokines (TNF-α and IL-6) contents were assayed using ELISA kit (BioLegend, USA) in accordance with instructions of the manufacturers. The readings were done at 450 nm with the aid of microplate reader (MICRO READ 1000, Belgium) wherein the concentrations were expressed as pg/mL.

3.8 Immunohistochemical assays

ELISA kit (BioVesion, USA) was used for immunohistochemical determination of brain immune-positive cells expressions-NF-kB, iNOS as well as BDNF in accordance with instructions highlighted by manufacturers. Briefly, sections of each brain (20 µm thick), were cut using cryostat and each slice kept after washing in free-floating condition at 40°C for various immunohistochemical studies. The staining processes involved tissue deparaffinization followed by series of processing before each slide was incubated with the NF-kB, iNOS as well as BDNF primary antibody as described by the manufacturers. Photomicrographs of dried stained slides were acquired with Leica ICC50 RE Digital Camera (Germany) with computer interface (Magnafire) and Olympus BX-51 Binocular research microscopeand immuno-positive cells' expressions were done using image J software.

3.9 Histological studies of amygdala

The transverse sections of the amygdala (5-6µm thick) portion of brains fixed in 10% formaldehyde were processed sterotaxically using microtomy (Leica rotary microstome) followed by routine methods of paraffin embedment for histological studies. The procedure reported by Emokpe *et al*., (2019) for staining with H &E for neuronal cell morphology. However, cresyl violet staining was done as earlier reported (Laila *et al*., 2019). Each prepared slides were then observed with research microscope (Olympus CH Japan). Each photomicrograph was afterwards developed using digital camera of Sony type.

Then, viable neurons represented by well rounded-shaped, intact cytoplasmic cells, without obvious alterations were then determined using research microscope (Olympus CH (Japan) at x 960 magnification and graticule. Thereafter, neuronal volumes were estimated by comparing spheres volumes, wherein, the smallest and largest diameters of the neurons were determined. The volume of the neuronal cell was then calculated from the mean of the diameters (Laila *et al.*, 2019).

3.10 Analysis of data

Graph Pad Prism software version 5.0 was used for analysis of the data, which were presented as Mean ± standard error of mean. Data analysis was done using One-way ANOVA and Newman-Keuls *post-hoc* test, and ($p < 0.05$) was adopted as statistically significance.

CHAPTER FOUR

RESULTS

The results of the effects of naringenin (NG) on latency to anoxic convulsions, blood glucose contents, serum corticosterone, oxidative stress biomarkers as well as chronic hypoxicstress-evoked neurobehavioural complications and probable biochemical underlying mechanisms are presented as follows:

4.1 Effect of naringenin on anoxic-convulsions

Naringenin effect on latency to convulsions evoked by anoxia in mice is depicted in Fig. 4.1. Intraperitoneal doses of naringenin (10-100 mg/kg) failed to produce significant delay in latency to anoxic stress-evoked convulsions in mice.

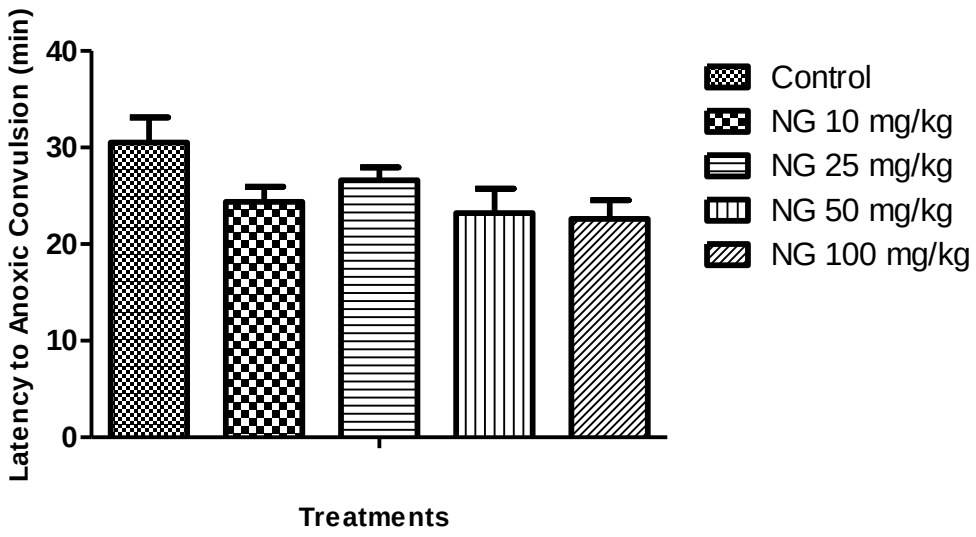

Figure 4.1. **Naringenin (NG) did not prolong latency to anoxic-convulsions in mice.** Each bar is a representative of mean ± standard error of mean in seven mice in different groups.

Control = 5% DMSO

4.2 Effects of naringenin on brain contents of malondialdehyde, glutathione and nitrite of mice subjected to anoxic-tolerance test

Figures 4.2 to 4.4 showed naringenin effects on brain contents of malondialdehyde, glutathione and nitrite in anoxic-stressed mice. Intraperitoneal injection of naringenin (10-50 mg/kg) decreased malondialdehyde accompanied by restoration of glutathione ($p < 0.05$) contents in brains of anoxic-stress mice. However, as presented in figure 4.4, intraperitoneal injection of naringenin did not produce any significant differences in brain concentrations of nitrite when compared with control (Fig.4. 4).

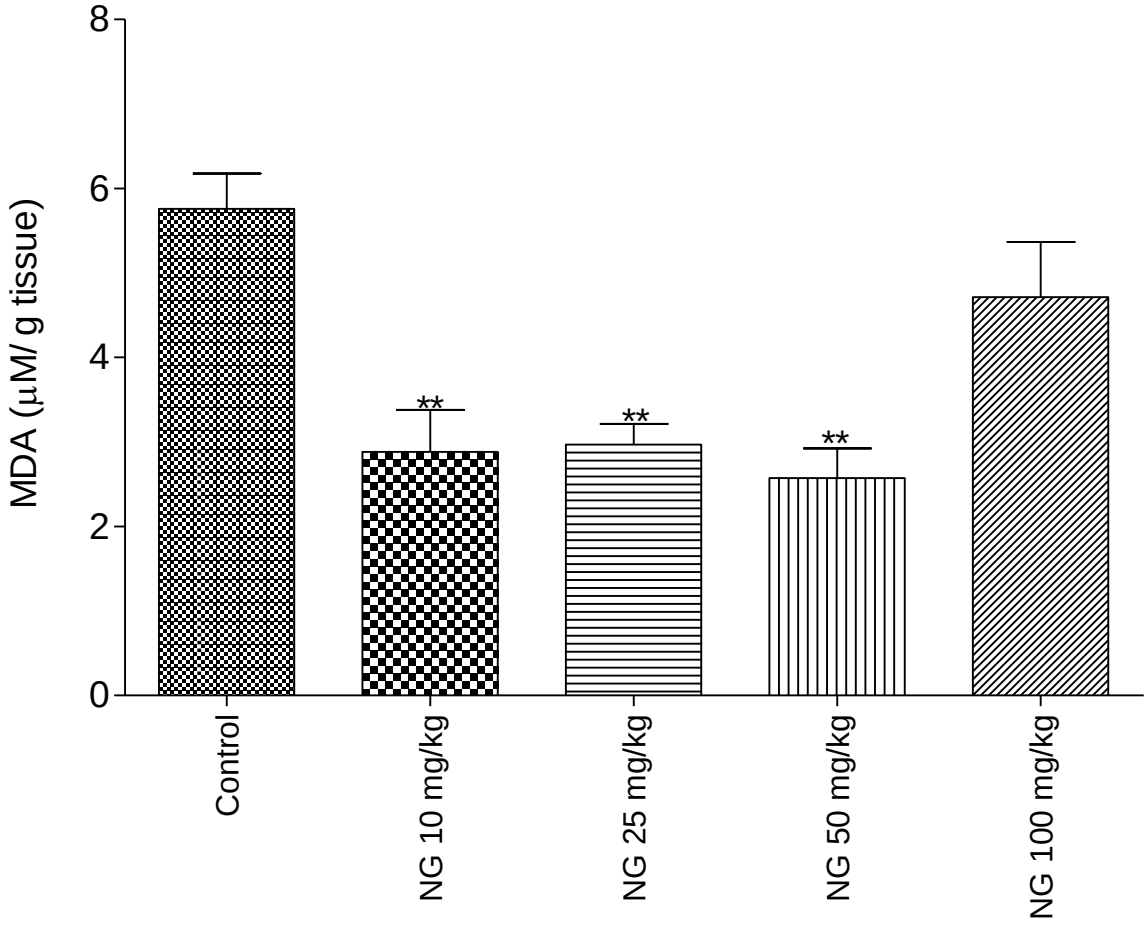

Fig. 4.2. Naringenin (NG) reduces malondialdehyde (MDA) brain content in mice that suffered anoxic-stress.

Data presented are representative of mean ±standard error of mean in seven mice in different groups. *p< 0.05 versus control (ANOVA followed by Newman-Keuls *post-hoc* test).

Control = 5% DMSO

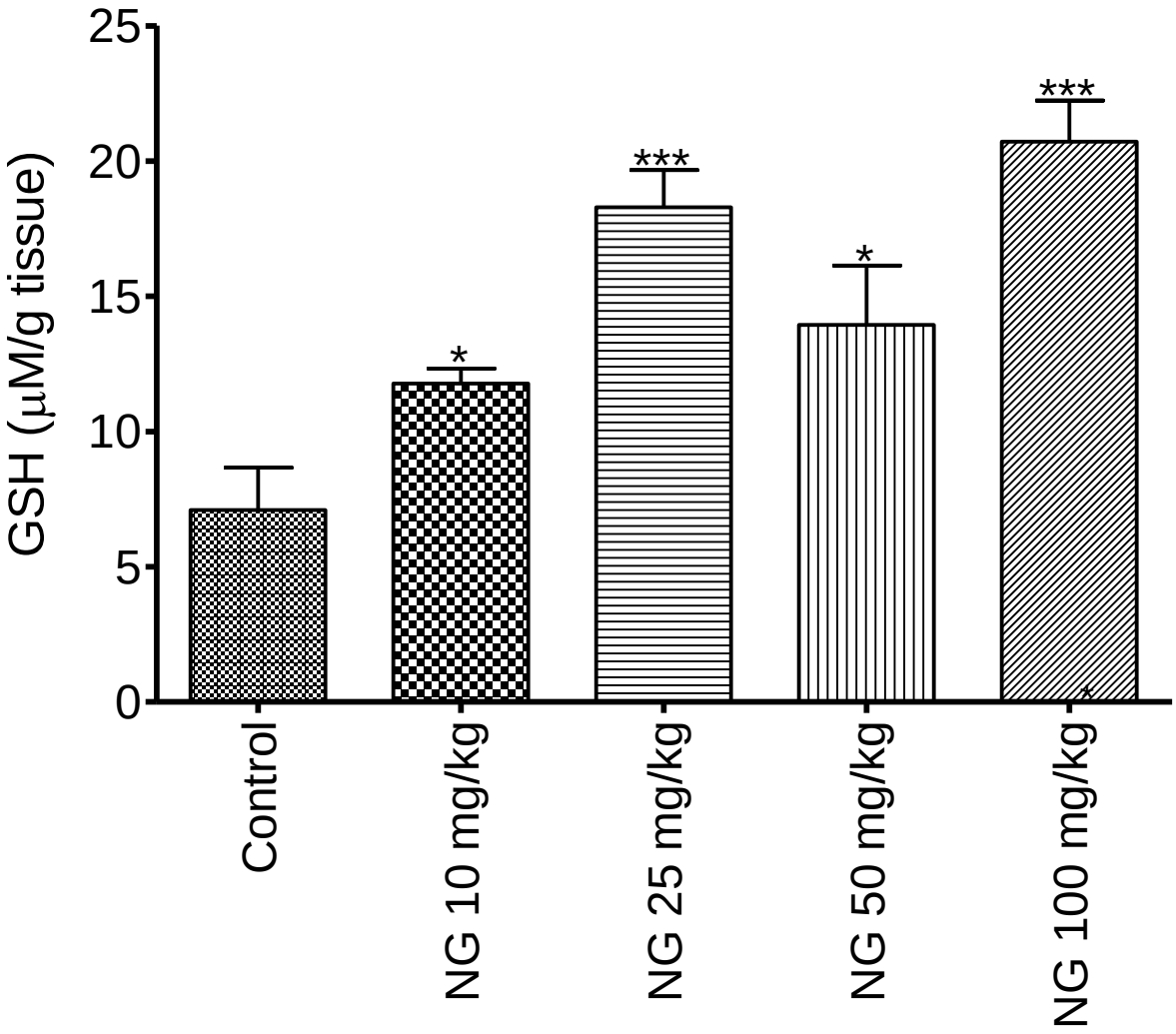

Fig 4.3. Naringenin (NG) elevated brain glutathione (GSH) contents in mice that suffered anoxic stress condition.

Values presented arerepresentative of mean ± standard error of mean in 7 mice across groups. *$p < 0.05$ versus control (ANOVA followed by Newman-Keuls *post-hoc* test). Control = 5% DMSO

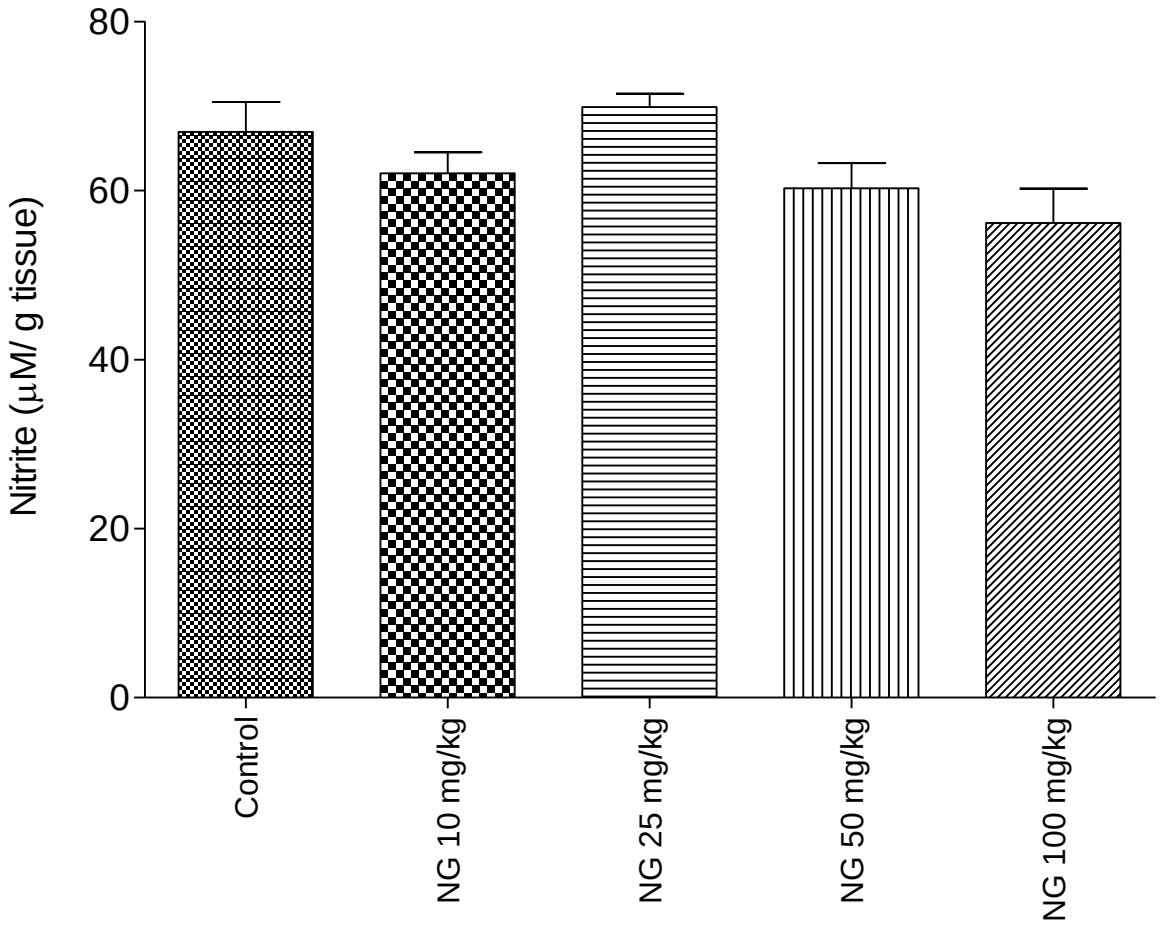

Figure 4.4. Naringenin (NG) did not alter brain nitrite levels of anoxic-stressed mice

Bars are representative of mean ± standard error of mean in seven mice in different groups.

Control = 5% DMSO

4.3 Naringenin enhances motor function in chronic hypoxic-stress mice

Figure 4.5 showed that chronic hypoxic-stress reduced locomotor performance in mice when compared with non-stress control ($p < 0.05$). However, intraperitoneal injection of naringenin at all doses produced significant ($p < 0.05$) improvement in motor function when compared with chronic hypoxic-stress group (Fig. 4.5).

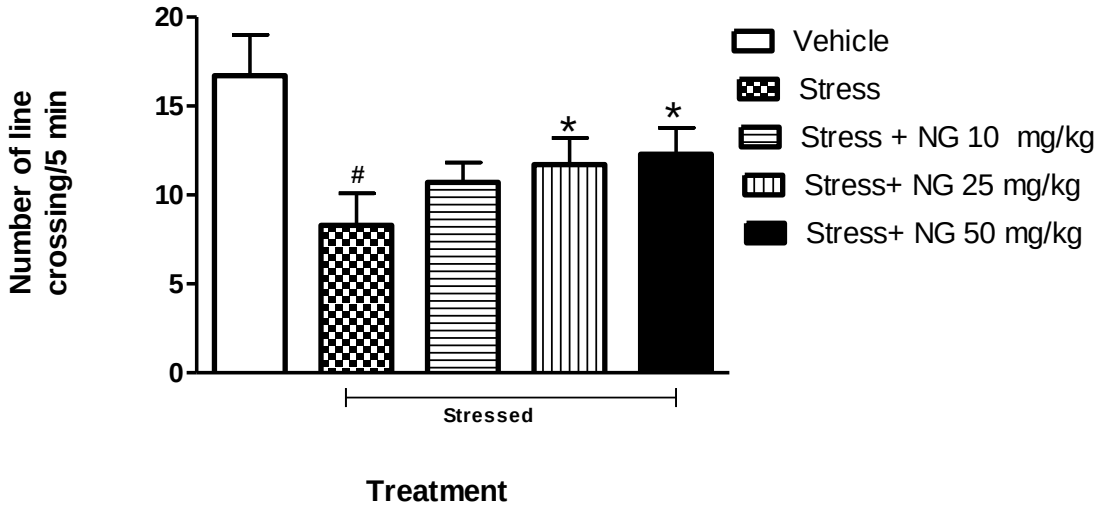

Fig.4.5. Naringenin (NG) improves motor function in chronic hypoxic-stressed mice.

Bar signifies mean ± standard error of mean of seven mice across groups. #P< 0.05 versus vehicle; *p< 0.05 as against stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

4.4 Naringenin improves chronic hypoxic stress-evoked depression-like symptoms in mice

Naringenin effect on chronic hypoxic stress-induced depression-like symptoms is presented through figure 4.6. Mice exposed to chronic hypoxic-stress condition showed increased period of immobility relative to non-stress control. However, naringenin administered intraperitoneally (10-50 mg/kg) decreased period of immobility induced by chronic hypoxic condition in mice (Fig.4. 6).

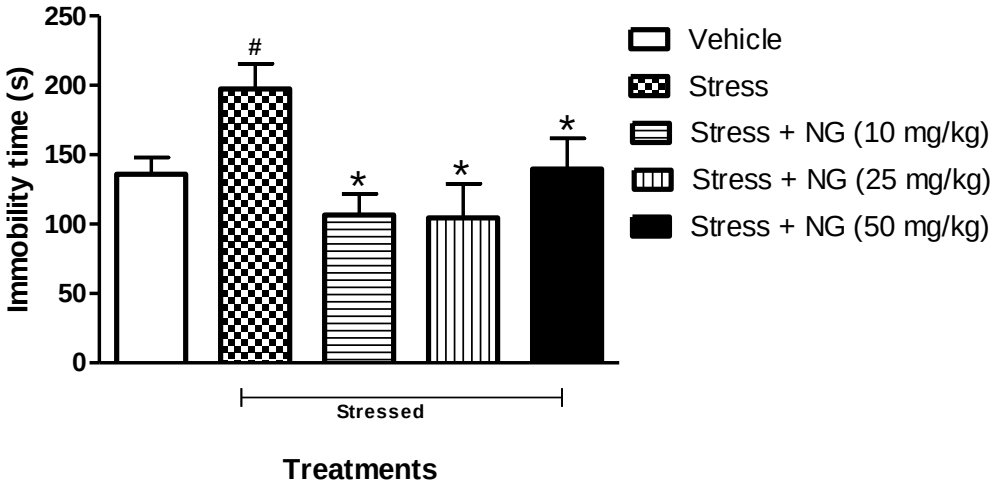

Fig. 4.6: Naringenin (NG) ameliorates symptoms of depression in mice evoked by chronic hypoxic condition.

Data are representative values of mean ± standard error of mean in seven mice in different groups. #p< 0.05 compared with vehicle; *p < 0.05 versus stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

4.5 Naringenin reduces hypoxic-stress-induced anxiety in mice

Naringenin effect on chronic hypoxic stress-induced anxiety-like behaviour is presented in Fig. 4.7-8. The increased duration of stay in the closed arm of the elevated plus maze induced by hypoxic stress was significantly ($p < 0.05$) reduced by intraperitoneal of administration of 10 and 25 mg/kg doses of naringenin (Fig. 4.7). Intraperitoneal of administration of naringenin (10 and 25 mg/kg) also reduced duration of time stayed in the dark compartment when compared with hypoxic stress group (Fig. 4.8).

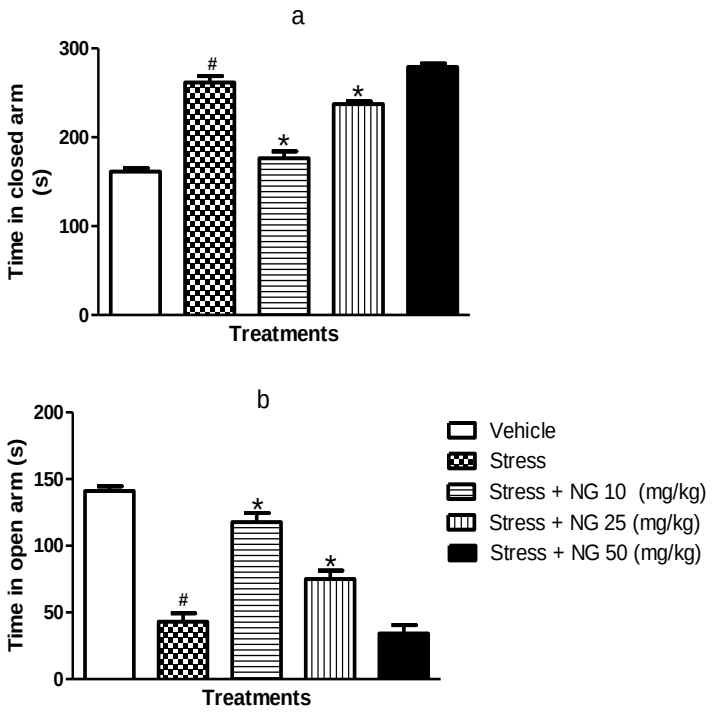

Fig. 4.7: Naringenin (NG) alters the length of time spent (s) in closed arm (A) and open arm (B) of the elevated plus-maze in chronic hypoxic-stressed mice.

Data signified mean ± standard error of mean in seven mice across the groups. #$p< 0.05$ compared with vehicle; *$p< 0.05$ versus stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

A B

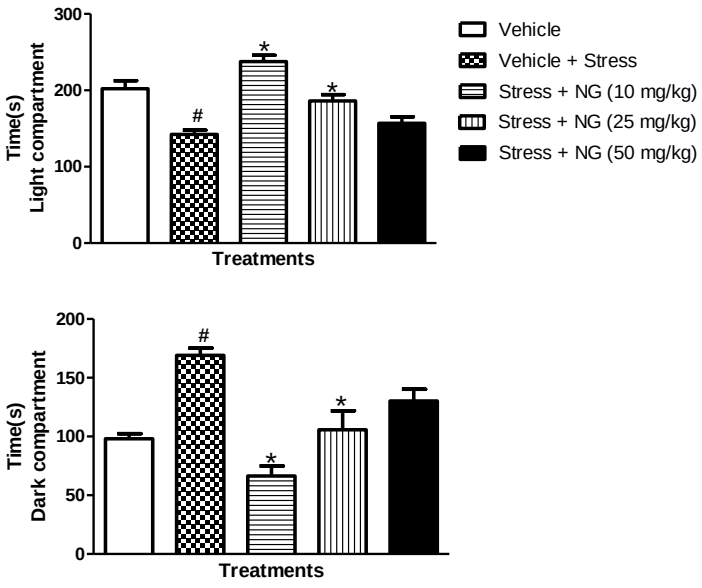

Fig. 4.8: Naringenin (NG) affects performance of mice that suffered chronic hypoxia in the light/dark transition paradigm.

Bar represents mean ± standard error of mean in seven mice in different groups. #$p< 0.05$ versus vehicle; *$p< 0.05$ versus stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

4.6 Effect of naringenin on chronic hypoxia-induced memory deficit

Mice subjected to chronic hypoxia had decreased percentage alternation when compared with vehicle treated group. However, intraperitoneal injection of naringenin (10-50 mg/kg) did not alter percentage alternation when compared with chronic hypoxic stress group.

4.7. Naringenin improves chronic hypoxic-stress-induced increase in glucose and corticosterone contents

Chronic hypoxic stress significantly ($p < 0.05$) increased blood-glucose concentration as well as corticosterone serum content in mice relative to non-stress control (Fig. 4.9). However, intraperitoneal doses of 10-50 mg/kg of naringenin reduced the blood glucose content (Fig.4.9) but not corticosterone concentration when compared with hypoxic stress group.

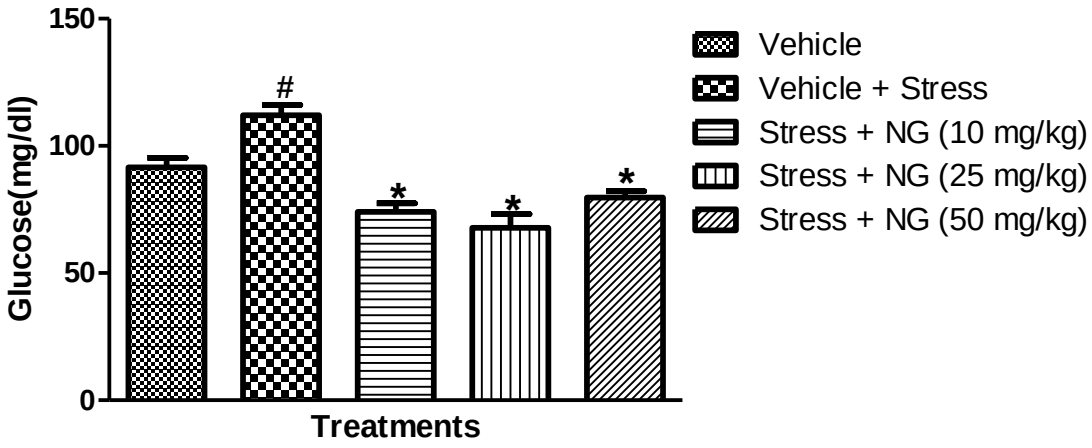

Fig. 4.9. Naringenin(NG) reduces chronic hypoxia-evoked blood glucose elevation in mice

Data represent mean ± standard error of mean in seven mice in different groups. #$p< 0.05$ as against vehicle; *$p< 0.05$ versus stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

4.8. Naringenin effects on chronic hypoxia-evoked malondialdehyde (MDA), glutathione (GSH) and nitrite (NO) in mice brains

The effect of naringenin on MDA, GSH and nitrite in the brains of mice exposed to chronic hypoxia are shown in figures 4.10-11. Chronic hypoxia induced significant ($p < 0.05$) elevation of MDA and NO concentrations accompanied by reduced GSH content in mice brains. However, intraperitoneal doses (10-50 mg/kg) of naringenin significantly ($p < 0.05$) reduced the brain MDA, NO and elevated GSH contents relative to chronic hypoxic stress group (Fig. 4.10-11).

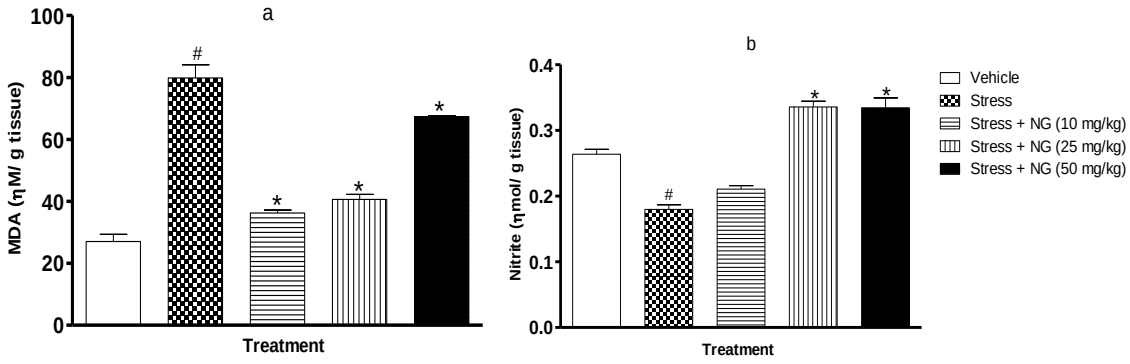

Fig. 4.10. Increased malondialdehyde (MDA) and nitrite (NO) contents evoked by chronic hypoxia were attenuated by naringenin (NG) in mice brains.

Values signified mean ± standard error of mean in seven mice across the groups. #$p< 0.05$ as against vehicle; *$p< 0.05$ versus stress- control (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

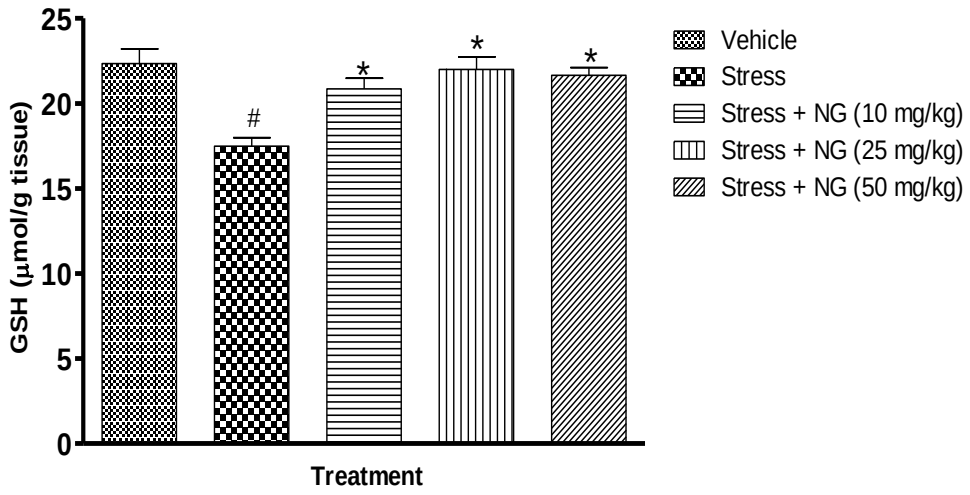

Fig. 4.11. Naringenin (NG) restores the deregulated brain concentrations of glutathione (GSH) induced by chronic hypoxic-stress in mice.

Bars represent mean ± standard error of mean in seven mice across the groups #$p < 0.05$ as against vehicle; *$p < 0.05$ versus stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

4.9. Effects of naringenin on chronic hypoxic stress-induced alterations in activities of antioxidant enzymes in mice brains

As shown in Figure 4.12, exposure of mice to chronic hypoxic stress produced significant ($p < 0.05$) decreases in brain antioxidant enzymes (superoxide- dismutase (SOD) and catalase) relative to non-stress control. However, intraperitoneal doses of naringenin did not significantly ($p > 0.05$) change the brain catalase enzyme activity in mice relative to stress-control (Fig. 4.12). However, naringenin at 50 mg/kg increased SOD activity ($p < 0.05$) in comparison with stress-control (Fig. 4.12).

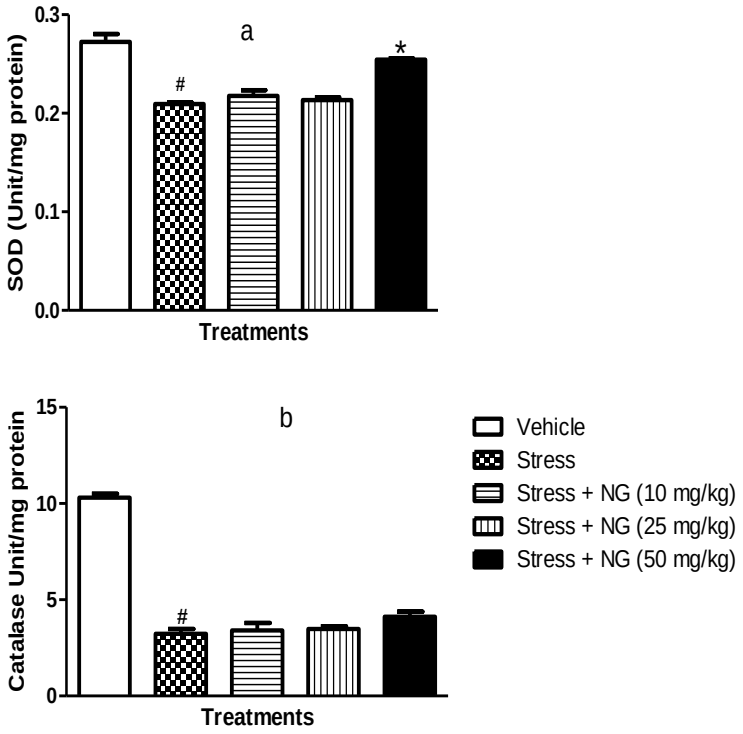

Fig. 4.12. Naringenin (NG) effects on chronic hypoxic stress-evoked dysfunctions in brain activities of antioxidant enzymes in mice

Data are representative mean ± standard error of mean in seven mice in different groups. #$p < 0.05$ versus vehicle; *$p < 0.05$ versus stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO, SOD = Superoxide dismutase.

4.10. Naringenin effects on pro-inflammatory cytokines in chronic hypoxic stressed mice

Figure 4.13 shows the effects of naringenin on brain contents of chronic hypoxic stress induced increase in pro-inflammatory cytokines in mice. Intraperitoneal injection of naringenin (10-50 mg/kg) attenuated the increased TNF-α and IL-1β contents induced by chronic hypoxia in mice ($p < 0.05$) (Fig. 4.13).

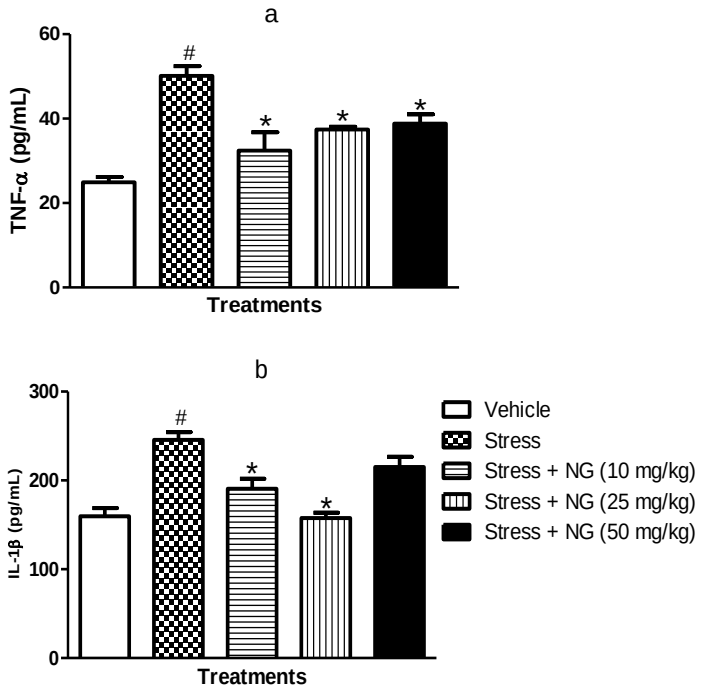

Fig. 4.13. Naringenin (NG) reduces chronic hypoxia-induced elevation in brain proinflammatory cytokines

Data are representative of mean ± standard error of mean in seven mice in different groups. #$p < 0.05$ as against vehicle; *$p < 0.05$ versus stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO, Interleukin-1beta = IL-1β, Tumour Necrosis Factor alpha = TNF-α

4.11 Naringenin reduces hypoxic-stress-induced INOS and NF-KB expressions

Figures 4.14-15 show that mice exposed to chronic hypoxic stress had increased brain NF-kB and INOS immuno-positive cells expressions ($p < 0.05$). However, intraperitoneal injection of naringenin significantly ($p < 0.05$) reduced immuno-positive cells of INOS and NF-kB expressions in mice exposed to chronic hypoxic stress (Fig. 4.16-17).

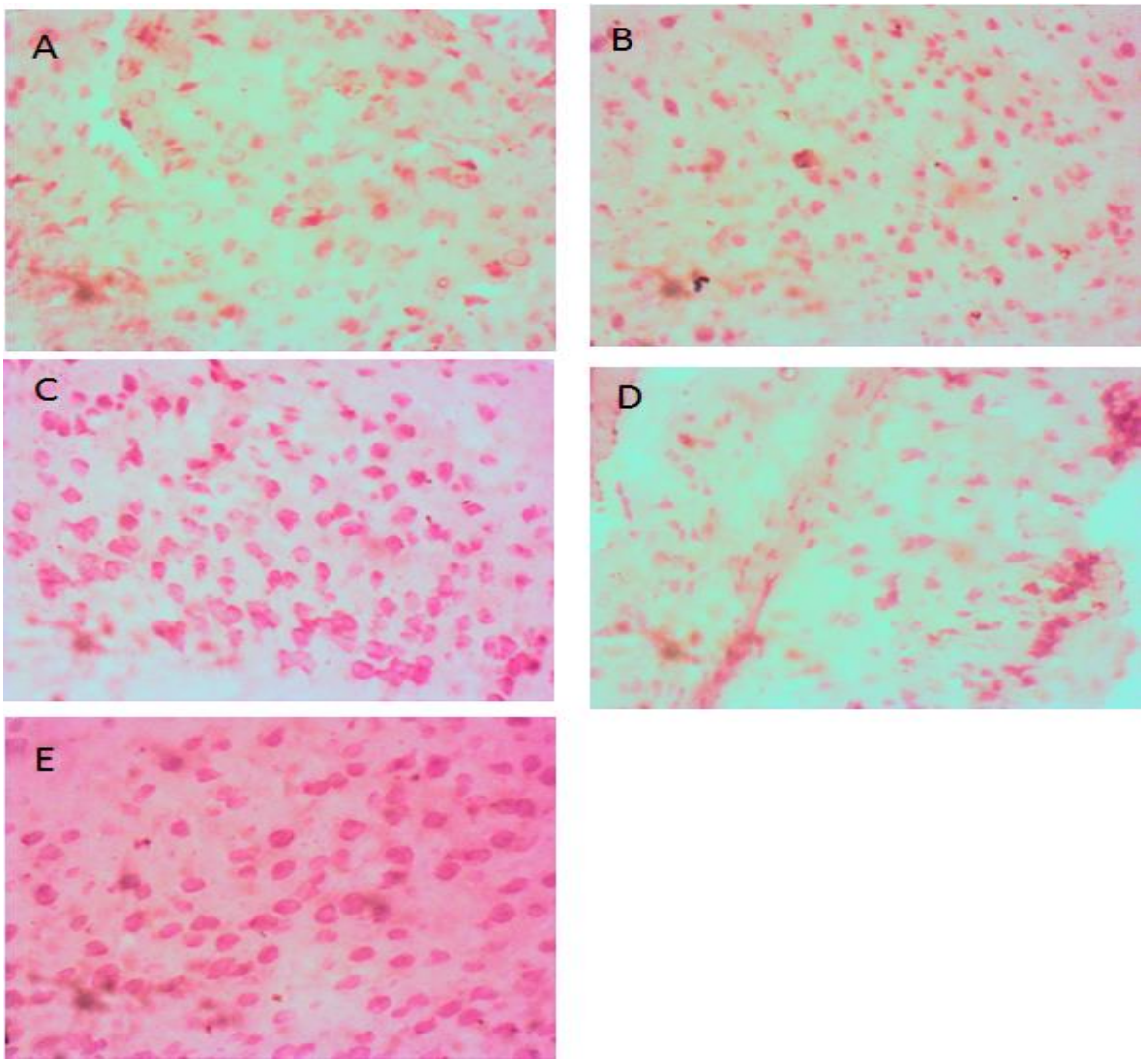

Fig. 4.14. Photomicrographs of brain immuno-positive cells of iNOS of mice that suffered chronic hypoxia. Vehicle 5% DMSO (A); Stress-control (B); Stress + naringenin (10 mg/kg) (C); Stress + naringenin (25mg/kg) (D); Stress + naringenin (50 mg/kg) (E). Magnification: x100.

Slides A, C and D show low immuno-positive cell expressions.

Slides B and E show high immuno- positive expressions.

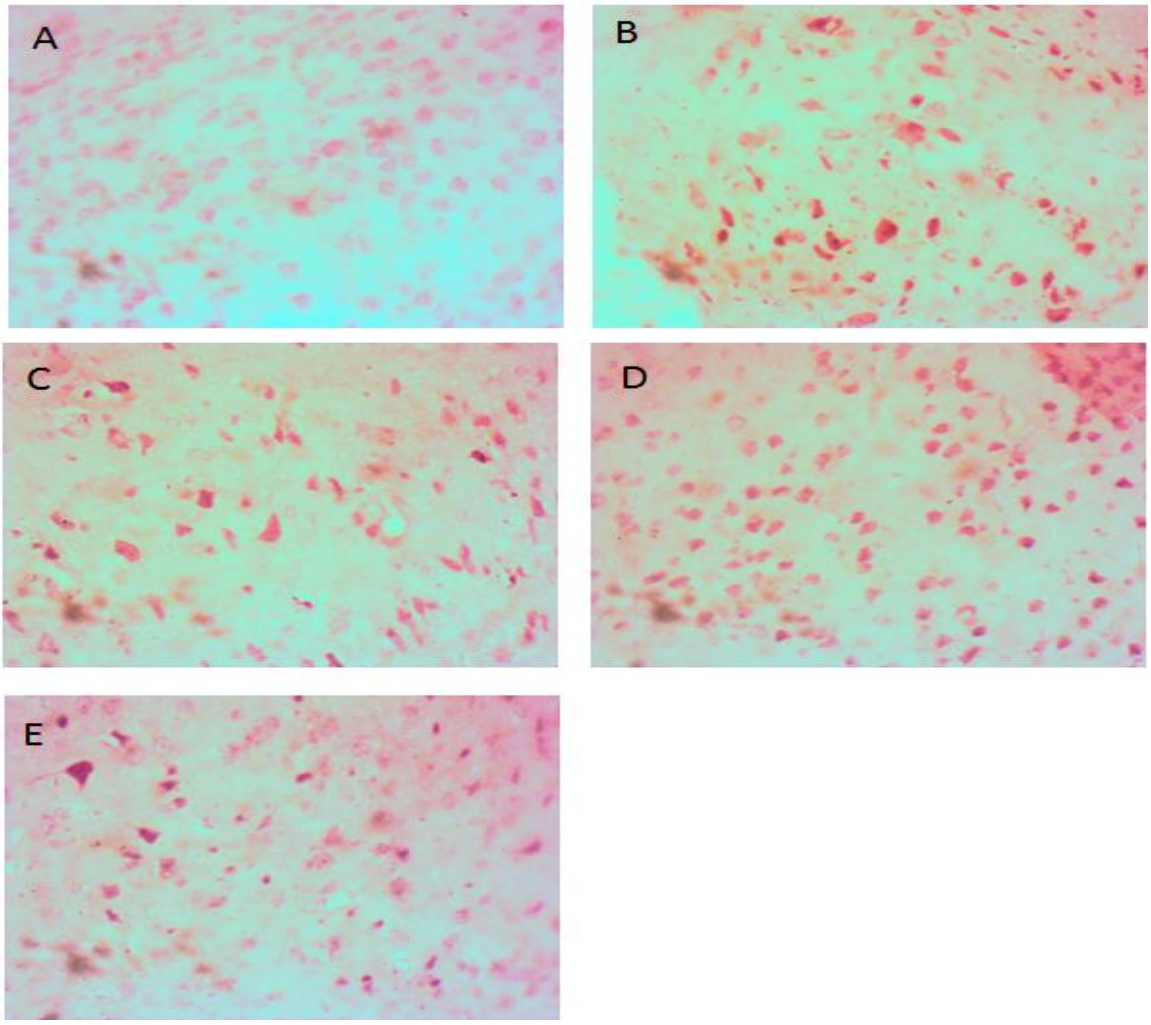

Fig. 4.15. Photomicrographs of brain immuno-positive cells of NF-kB mice that suffered chronic hypoxia. Vehicle 5% DMSO (A); Stress-control (B); Stress + naringenin (10 mg/kg) (C); Stress + naringenin (25mg/kg) (D); Stress + naringenin (50 mg/kg) (E). Magnification x100

Slides A, C and D show low immuno-positive cell expressions.

Slides B and E show high immuno- positive expressions.

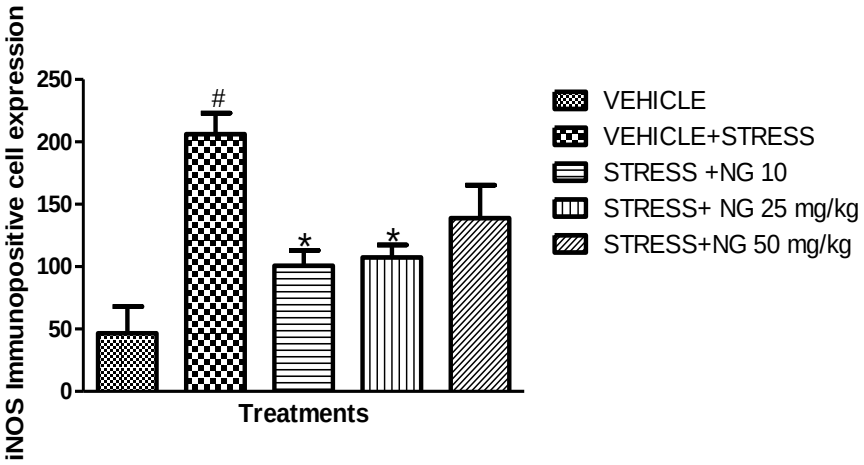

Fig. 4.16. Naringenin (NG) reduces chronic hypoxic stress-evoked increased expressions in brain immuno-positive cells of inducible Nitric Oxide Synthase (iNOS) in mice.

Data are representative of mean ± standard error of mean in seven mice in different groups. #$P< 0.05$ versus vehicle; *$P< 0.05$ as against stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

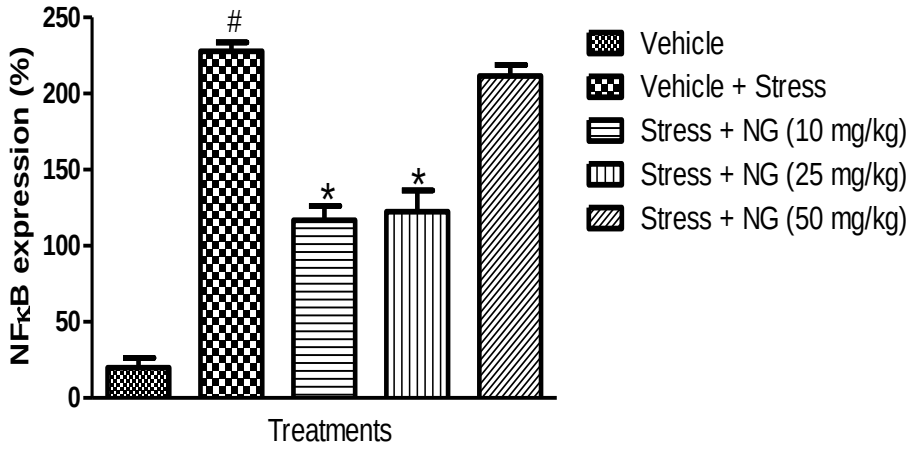

Fig.4.17. Naringenin (NG) reduces chronic hypoxia-evoked increased brain immuno-positive cells of Nuclear Factor Kappa B (NF-kB) expressions of mice.

Data are representative of mean ± standard error of mean in seven mice in different groups. #$P< 0.05$ versus vehicle; *$P< 0.05$ as against stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle 5% DMSO

4.12. Naringenin reduces brain BDNF immune-positive cell expressions induced by chronic hypoxic-stress in mice

As shown in Figure 4.18, chronic hypoxic stress produced significant decrease in expressions of brain BDNF immuno-positive cells relative to non-stress control ($p < 0.05$). However, intraperitoneal injection of naringenin in doses of 10-50 mg/kg significantly ($p < 0.05$) increased BDNF immuno-positive cells expressions in mice exposed to chronic hypoxia (Fig. 4.19).

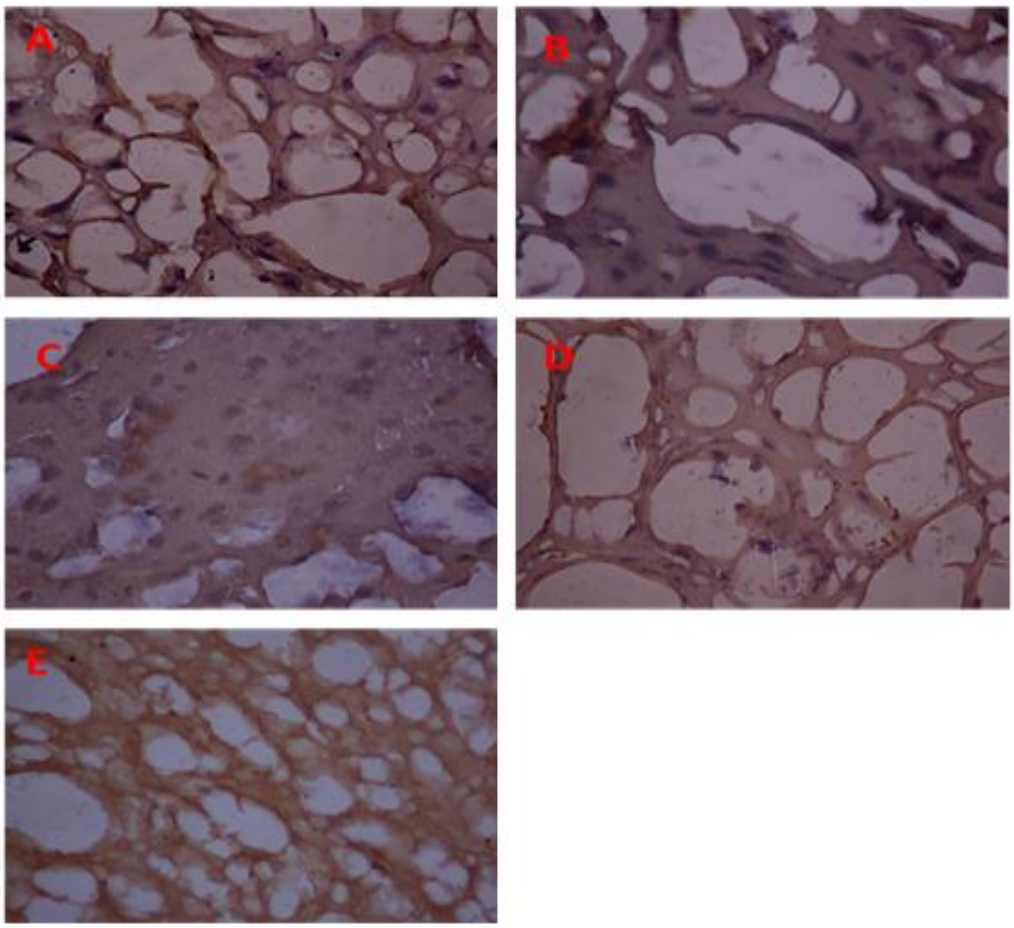

Fig. 4.18. Photomicrographs of brain immuno-positive cells of BDNF of mice that suffered chronic hypoxia.

Vehicle (A); Stress-control (B); Stress + naringenin (10mg/kg) (C); Stress + naringenin (25mg/kg) (D); Stress + naringenin (50 mg/kg) (E). Magnification: x 400

Vehicle = 5% DMSO

Slides B, D and E show low immuno-positive cell expressions.

Slides A and C show high immuno- positive expressions.

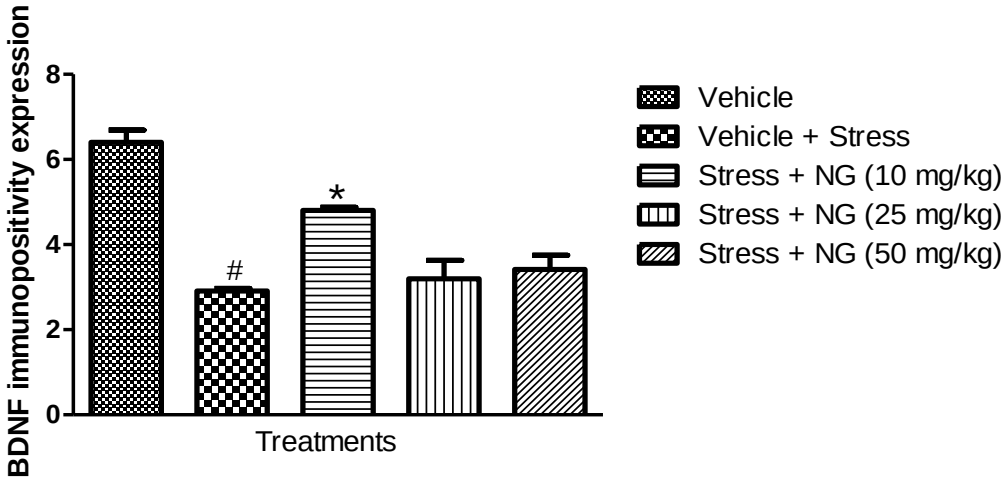

Fig. 4.19. Naringenin (NG) increases brain immuno-positive cells of Brain Derived Neurotrophic Factor (BDNF) expressions of mice.

Data are representative of mean ± standard error of mean in seven mice in different groups. #P< 0.05 versus vehicle; *P< 0.05 as against stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

4.13. Effect of naringenin on chronic hypoxic stress-induced cytoarchitectural distortion of amygdala of mice

Photomicrographs and density of neuronal cells viability counts of amygdala of mice exposed to chronic hypoxic-stress are shown in figure 4.20. Chronic hypoxic stress significantly ($p < 0.05$) decreased the population of amygdala neuronal cells viability. However, intraperitoneal injection of naringenin (10-50 mg/kg) did not prevent the loss of viable amygdala neuronal cells.

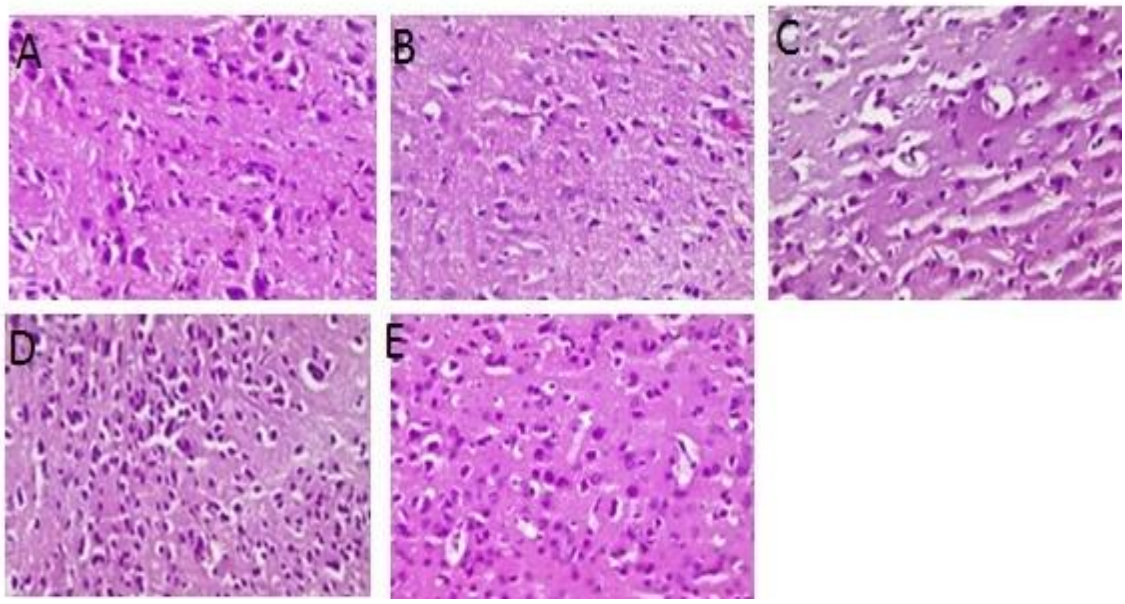

Fig. 4.20. Effect of naringenin (NG) on histological changes of H&E-stained section of amygdala of mice exposed to chronic hypoxic stress.

Vehicle (A); Stress-control (B); Stress + naringenin 10 mg/kg (C); Stress + naringenin 25mg/kg (D); Stress + naringenin 50 mg/kg (E).

Slide A showed normal histomorphology. Slide B showed decreased population of viable neuronal cells. Slides C, D and E indicate reduced viable neurons. M*agnification: ×400.*

Vehicle = 5% DMSO

4.14 Effect of naringenin on chronic hypoxia-induced decreased amygdala neuronal volume

Photomicrographs as well as amygdala neuronal volume of mice exposed to chronic hypoxic- stress are shown in figures 4.21 – 4.22 . Mice exposed to chronic hypoxia had reduced amygdala neuronal volume in comparison to vehicle. However, intraperitoneal dose of 10 mg/kg naringenin demonstrated significant reversal in decreased amygdala neuronal volume of mice in comparison with chronic hypoxia.

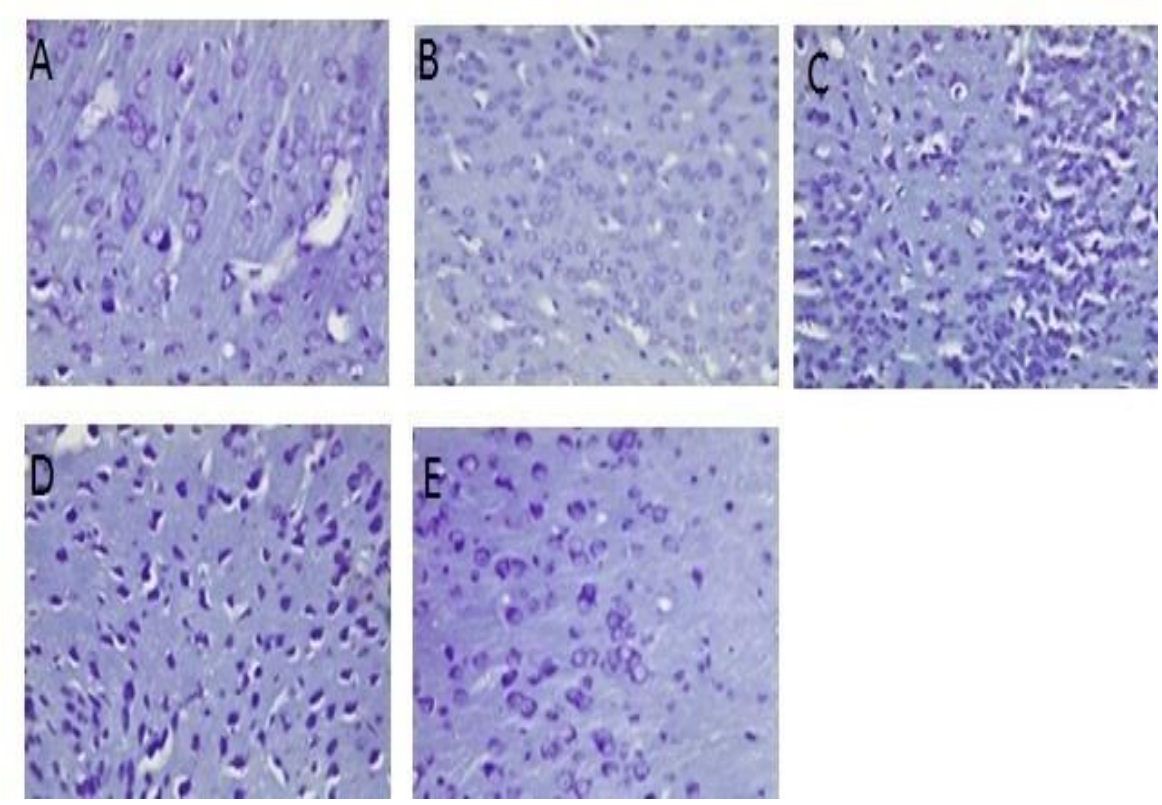

Fig. 4.21. Changes in morphological feature of creosyl-stained amygdala section of mice that suffered chronic hypoxia. Vehicle = 5% DMSO

Vehicle (A); Stress + control (B); Stress + naringenin (10 mg/kg) (C); Stress + naringenin (25mg/kg) (D); Stress + naringenin (50 mg/kg) (E). Magnification ×*400*

Slides A, C, D and E show relatively normal Nissyl bodies.

Slide B show shrinked Nissyl bodies.

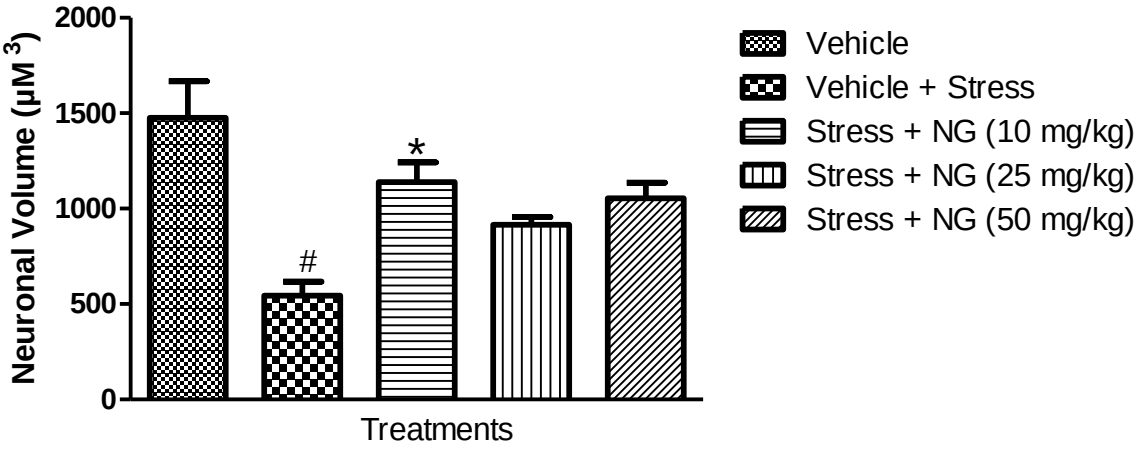

Fig. 4.22. Effect of naringenin (NG) on chronic hypoxia-induced decreased amygdala neuronal volume.

Data are representative of mean ± standard error of mean in seven mice in different groups. #$P< 0.05$ versus vehicle; *$P< 0.05$ as against stress group (ANOVA followed by Newman-Keuls *post-hoc* test).

Vehicle = 5% DMSO

CHAPTER FIVE

DISCUSSION

The results of this study revealed that the first appearance of convulsion otherwise known as latency to convulsion induced by anoxic-stress in mice was not significantly modified by intraperitoneal injection of naringenin. In addition, no major differences were recorded in levels of glucose in the blood of mice subjected to acute anoxic tolerance test when compared with those given various doses of naringenin. Furthermore, intraperitoneal injection of naringenin did not alter nitrite brain levels as compared with the anoxic-stressed mice. However, brain level of malondialdehyde was reduced by naringenin but it increased glutathione concentrations in mice that experienced anoxic-stress.

Anoxic stress has been regarded as aversive condition characterized by a state of oxygen deprivation to various body-tissues including the brain cells (Chen *et al.,* 2020). Anoxic condition has severe multiple effects on the body organs especially the brain as short period of oxygen deprivation been shown to produce irreversible impairment of neuronal integrity. Thus, anoxic-stress has been labelled as one of the most potent stressors because of its capability to disrupt body physiological mechanisms, further attesting to the fact that constant delivery of oxygen is a precondition for existence of aerobic-organisms (Lutz, 1992; Deept *et al*., 2019). Brain cells, indeed, have been noted as the first set of body-cells to suffer severely from inadequate oxygen, and die almost instantly during anoxia. Brian cells have been alluded to as the most sensitive entities to oxygen deficiency due to low antioxidant profile and increased metabolic activity (Tomar *et al.,* 1984; Liu *et al.,* 2014).

Although various disabling effects can be caused by inefficient oxygen delivery to brain cells (Chen *et al*., 2020), seizures are common immediate manifestations seen in animals subjected to anoxic stress (Hansen, 1985; Liu *et al*., 2014; Kubova and Mares, 2007; Caillard *et al.,* 1975). It has been noted that seizure episodes of this domain should be appropriately described as anoxic convulsions (Caillard *et al*., 1975; Tomar *et al*., 1984; Kubova and Mares, 2007). It has further been described as anoxic tolerance time suggesting how long the organism can cope or endure this severe stressor (Caillard *et al*., 1975; Tomar *et al*., 1984). Anoxic condition has been shown to be connected with efficiency of GABAergic neuronal pathway (Losad, 1988; Liu *et al.,* 2014), which perhaps might be underpinning convulsive seizures precipitated by oxygen deprivation in the air tight-cylindrical vessel. Thus, it is expected that organisms with sufficient resilience and efficient adaptive mechanisms should have greater capability to withstand the devastating consequences of anoxic stressor (Hansen, 1985; Liu *et al*., 2014). Actually, agents adjudged to be adaptogens are known to protect against anoxic convulsion and elevate the seizure-threshold to anoxic convulsions to certain degree (Tomar *et al*., 1984). However, intraperitoneal doses of naringenin used in this study did not offer any protection against anoxic convulsions in mice.

One of the early consequences of any stressor is the induction of blood glucose elevation through gluconeogenesis and decreased cellular uptake of glucose (Kulkarno and Juvekar, 2008; Efferth and Koch, 2011; Tsigos *et al*., 2016). Increase in blood glucose is meant to help maintain cellular homeostasis and serve as a source of energy to enable the organism to cope better with the stressor (Kulkarni and Juvekar, 2008; Efferth and Koch, 2011). Anoxic stress can also promote oxidative stress in body organs, especially the brain through activation of metabolic pathway. Furthermore, increased oxidative stress may also ensues following activation of HPA axis suggesting that targeting the oxidative pathway in the early phase of stress-responses as well as chronic hypoxic-stress might modulate upstream neuronal circuitries relevant in mediating certain psychopathological disorders (Mattson *et al*., 2008; Tsigos *et al*., 2016; Panssian *et al*., 2018). Thus, this investigation was anchored on evaluating the effects of naringenin,a neuroprotective biflavonoid on chronic hypoxia-evoked amnesia ,anxiety-related disorders, depression and the mechanisms underpinning its action in mice.

Naringenin was found to improve motor function and also attenuated symptoms associated with endogenous depression and anxiety triggered by chronic hypoxic-stress in mice. It has been recognized that the major triggers involved in neuroinflammation as well as increased vulnerability to neurodegeneration in hypoxic-brains are closely related to activation of oxidative pathway and losses of neuronal cellular antioxidant protective-mechanisms (Peng *et al*., 2020). Hence, appropriate pharmacological interventions relating to modulations of neuroinflammatory signals as well as oxidative pathway might be germane in mitigating chronic stress-evoked neuropsychiatric complications (Panossian *et al*., 2018). Based on these notions, diverse biochemical signalling pathways relevant to intense stress-evoked neuropathological derangements were investigated. Findings from these investigations revealed that naringenin enhanced antioxidant cellular armories as evidenced by elevated GSH content and catalase-activity as well as decreased oxidant molecules as judged by reduced MDA as well as nitrite levels caused by chronic hypoxic-stress in mouse-brain. Naringenin further caused a dose-dependent down-regulation of brain iNOS and NF-k_B immuno-positive cells expressions, and also suppressed pro-inflammatory cytokines (TNF-α and IL-6). Additionally, naringenin at a relevant dose restored loss of brain-BDNF immuno-positive cells precipitated by exposure of mice to chronic hypoxic-stress.

The inability of living organisms to efficiently and timely respond to prolonged adversity of high intensity has been highlighted as the major genesis for developing cluster of psychiatric illnesses such as depression, anxiety, memory decline and addictive tendency (Alo *et al*., 2016; Tsigos *et al*., 2016). Large body of data have established that stress of high chronicity elicited typical features of emotional disturbances consistent with core symptoms of motor dysfunctions, depression, anxiety and memory decline (Mineur *et al.,* 2006; Alo *et al.,* 2016). Hence, predisposition to depression and its severity, with diverse manifestations such as apprehension, worry, aggressiveness as well as suicidal propensity, for example, are core evidences of sustained intense stress. Interestingly, findings from this investigation also demonstrated that chronic hypoxic-stress produced symptoms akin to endogenous depression, memory decline, anxiety and motor dysfunctions.

There are several clinical reports in literature validating the devastating effects of hypoxia on both mental and physical health of the individuals as cases of anxiety, depression as well as physical disabilities have been observed in persons with hypoxic conditions (Young., 2013; Godwin *et al.*, 2012). Incidences of depression and suicide, for example, have been shown to be much higher in individuals residing in high altitudes, patients suffering from chronic hypoxic conditions such as asthma, as well as chronic obstructive pulmonary diseases (Schneider *et al*., 2010). Thus, these clinical reports further validate that chronic hypoxic-conditions are capable of damaging some critical neuronal cells thereby triggering some pathological changes consistent with etiology of endogenous depression as well as related-illnesses. It might be opined that findings of naringenin alleviating the signs of endogenous depression, and anxiety in mice that suffered chronic hypoxia suggest promising beneficial-effect in conditions relating to prolonged stress of high intensity. Moreover, it is important to highlight the findings from Umukoro *et al*., (2018), which showed that this flavonoid alleviated the symptoms of endogenous depression provoked by persistent psychosocial stress via suppression of oxidative stress, proinflammatory cytokines as well as corticosterone.

Several investigations alluding to capability of persistent stress of high intensity causing release of excess cortisol that initiates diverse neuropathological disorders through upstream mechanisms relating to stimulation of inflammatory and oxidative pathways have been documented (Panossian *et al*., 2018). Findings from this study revealed that chronic hypoxic-stress distorted biomarkers of oxidative stress, corticosterone and depleted antioxidant capability in brains of mice. Cortisol-evoked ROS/NOS-formation in times of prolonged stress of high intensity has been shown to underpin neurodegeneration ascribed to endogenous depression as well as memory decline (Mattson *et al*., 2008; Panossian *et al*., 2018). It has been established that one of the first lines of defense of neuronal cells against offensive oxidant species involves activating antioxidant defense machineries that act collectively to confer neuroprotection (Mattson *et al*., 2008, Panossian *et al*., 2018). Studies have also revealed excess cortisol caused depletion of endogenous antioxidants, thereby, exposing neuronal cells to the rising destructiveness of reactive intermediates (Mattson *et al*., 2008; Panossian *et al*., 2018).

Experimental data obtained from this study showed potentials of naringenin in suppressing brain MDA and nitrite concentrations in hypoxic-stressed mice. It also demonstrated GSH-boosting effect followed by enhancement of catalase function in mice-brains after chronic hypoxic-stress, which suggest increased antioxidant cellular-defense capability. Moreover, it is pertinent to state that efficacies of naringenin have been succinctly confirmed in diverse neurological conditions in rodents through mechanisms involving free radicals scavenging as well as lipid peroxidation inhibition (Lou *et al*., 2014; Wu *et al*., 2015; Umukoro *et al*., 2018). Taken together, naringenin's capability in ameliorating symptoms of endogenous depression as well as anxiety in hypoxic-stressed mice, might be related to suppressing oxidative stress as well as augmenting neuronal antioxidant defense integrity.

It has been shown that damaged neuronal-cells within the vicinity of excess oxidant entities in times of prolonged stress of high intensity initiate neuroinflammation, which in turns evoke degeneration of diverse neuronal circuitries (Tonnies *et al*, 2017). In fact, several chemical mediators and intracellular signals have been highlighted as the principal drivers of neuroinflammation in hypoxic conditions (Chen *et al*., 2020). For example, hypoxia activates NF-kB pathway to evoke pro-inflammatory cytokines release as documented in previous studies (Taylor *et al*., 2016; Cheng *et al*., 2020; Gold, 2015). The functions of pro-inflammatory cytokines have been well established in endogenous depression and sickness behaviours through mechanisms relating to loss of neuronal function (Cohen *et al*., 2012; Dantzer, 2009; Dhabhar, 2014).

Hence, pharmacological interventions directed against NF-kB-driven neuroinflammation might yield therapeutic advantage in managing neurological consequences precipitated by hypoxicstress. Interestingly, findings from this study revealed that the promising beneficial effects of naringenin in alleviating symptoms of endogenous depression and related conditions might be mediated through suppression of pro-inflammatory cytokines (TNF and IL-6) in mice that suffered chronic hypoxic condition. In addition, the ability of naringenin to reduce NF-kB as well as iNOS immuno-positive cells expressions further support involvement of inhibition of inflammatory mediators as part of the mechanism of its action against hypoxic-stress. This suggestion is in agreement with several reports

showing that naringenin inhibited proinflammatory cytokines and also elicited neuroprotective capability (Yi *et al*., 2014; Tayyab *et al*., 2019; Khan *et al*., 2012; Ghofrani *et al*., 2015). Taken together, these reports provide additional information suggesting naringenin capability in improving neurologic functions compromised by prolonged stress of high intensity.

It has been established that intense stress reduced the beneficial effects of BDNF) in the brain, hence, genesis of anxiety, depression as well as cognitive decline (Gold, 2015). Some of the functions ascribed to BDNF include promotion of neurogenesis, neural stem-cells survival, neuronal proliferations that underpin diverse neuropsychiatric illnesses (Greenberg *et al*., 2009; Tian *et al*., 2013). Prolonged stress of high severity as well as hypercortisolismhave been further implicated in the genesis of endogenous depression as well as decreased brain BDNF in rodents (Santarelli *et al*., 2015; Tayyab *et al*., 2019).

It has been reported that reduced BDNF expression contribute in several ways to the loss of dendritic arbors and synaptic connections in prefrontal cortex as well as the hippocampal neurons (Autry *et al*, 2012; Gold, 2015). Prolonged hypoxia has been shown to elicit morphological changes of the hippocampal integrity via inhibition of BDNF expression through several mechanisms relating to its receptor as well as endoplasmic reticulum stress biomarkers (Deepti *et al*., 2019). Although, detailed molecular mechanisms underlying the roles of BDNF in intense hypoxia-evoked cell-death areyet to be elucidated, distortions in BDNF signalling appear to be involved in loss of neuronal cells and reduced amygdala as well as hippocampal volumes in depressed persons (Autry *et al*, 2012).

Although naringenin was found to cause increase in BDNF immune-positive cells expression, this effect was only significant at the lowest dose. Nevertheless, earlier investigations have clearly established that naringenin ameliorated the symptoms of endogenous depression and also demonstrated neuroprotective ability evoked by chronic-stress through augmentation of BDNF repression (Tayyab *et al*., 2019). However, the implications of these findings as it relates to naringenin capability in alleviating the symptoms of endogenous depression and anxiety in animals that suffered from hypoxia required more extensive investigations.

Over the years, effects of prolonged stress on integrity of neuronal functioning as it relates to psychiatric illnesses have been extensively studied. Postmortem studies have established that changes in amygdala morphological features and functioning underlie altered moods, including symptoms of depression, anxiety, as well as panic behaviours (Sheline *et al*., 1998; Bellani *et al*., 2011; Haukvik *et al*., 2014). The roles of the amygdala in chronic stress reactivity were further confirmed through abolition of increases in glucocorticoids during stress engagements upon lesions of the amygdala (Carty *et al*., 2010;Kaufman and Charney, 2001). Electrophysiological stimulation of the amygdala was also shown to precipitate anxious behaviors, suggesting its involvement in emotional reactively to intense stressors (Rosen and Schulkin, 1998).

It has been noted that issues involving cortisol-evoked oxidative stress and inflammatory insults underpin amygdala dysfunctions (Carty *et al*., 2010). For example, reduced amygdala size has been reported in neonatal hypoxia (Carty *et al*., 2010). In addition, reduced amygdala volume was also reported in persons who had suffered hypoxia (Kaufman and Charney, 2001). Findings from this present study further showed that intense stress altered amygdala functioning as evidenced by reduced amygdala volume.

It is pertinent to state that agents with neuroprotective capability are known to mitigate pathological consequences precipitated by prolonged aversive conditions on various neuronal cells (Aoyama, 2006; Aoyama and Nakaki, 2013; Tonnies and Trushina, 2017). Specifically, studies have also documented that naringenin showed potential efficacies in diverse CNS disorders via neuroprotection and anti-neuroinflammation (Luo *et al*., 2014; Ghofrani *et al*., 2015; Tayyab *et al*., 2019). Additionally, it also restored histomorphological distortions as well as increased amygdala neuronal cell volume in this study but did not significantly increase the amygdala neuronal cell viability. However, inability of naringenin to produce significant increase in viable cells may not be unconnected with the perception that chronic stressors cause irreparable amygdala injury, even when the stressors have been reversed (Carty *et al*., 2010). However, this supposition is open to further investigations and verifications.

CHAPTER SIX

6.1 Summary and Further Studies

The results of this investigation suggest that naringenin improves symptoms of endogenous depression and anxiety in mice that suffered from chronic hypoxic stress through mechanisms relating to positive modulation of NF-kB/BDNF expressions and oxido-inflammatory responses. Taken together, it is opined that the key findings from this research allude to the necessity for developing this flavonoid as potential agent for management of neuropsychiatric illnesses, having oxido-inflammation as the major neuropathological mechanism**.** However, naringenin did not exhibit any protective effect against convulsive episodes induced by anoxic stress in mice.

6.2 Contribution to Knowledge : This present study

- Established that chronic hypoxic-stress model adopted in this investigation evoked neuropsychiatric complications consistent with endogenous depression, anxiety and memory decline in mice.
- Established the capability of chronic hypoxic-stress damaging brain cells to elicit several biochemical alterations in vulnerable persons that are closely consistent with symptoms of endogenous depression and anxiety.
- Revealed potential benefits of naringenin, a flavonoid found in most fruits and vegetables, in ameliorating symptoms of endogenous depression and anxiety evoked by hypoxic-stress in mice.
- Established neuroprotective mechanisms relating to suppression of oxidative stress, proinflammatory cytokines, increased expressions of NF-kB and iNOS, and up regulation of BDNF expressions in mitigation of hypoxic stress by naringenin.

6.3 Recommendation for Further Studies

The effects of naringenin on biochemical perturbations need to be further investigated in specific brain regions especially the amygdala that is more relevant to the pathogenesis of chronic hypoxic stress-evoked manifestations of the symptoms of endogenous depression and anxiety. In addition, there is a need for the elucidation of the brain neurochemical pathways particularly Gamma-aminobutyric acid (GABA) and glutamate in order to provide more insights into additional molecular targets for therapeutic implications of naringenin in hypoxic stress-evoked psychopathologies. Due to poor oral bioavailability of naringenin, future studies involving its nanoparticles formulation and sustained release blends with binding agents for better pharmacological activities are also recommended.

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