NEUROBEHAVIOURAL, HAEMATOLOGICAL AND BRAIN BIOCHEMICAL CHANGES IN WISTAR RATS SUBJECTED TO PSYCHOLOGICAL STRESS AND ADMINISTERED WITH LYCOPENE

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Declaration

I hereby declare that the work in this thesis titled "NEUROBEHAVIOURAL, HAEMATOLOGICAL AND BRAIN BIOCHEMICAL CHANGES IN WISTAR RATS SUBJECTED TO PSYCHOLOGICAL STRESS AND ADMINISTERED WITH LYCOPENE" was performed by me in the department of Department of Veterinary Physiology, Ahmadu Bello University, Zaria, under the supervision Prof. J. O. Ayo, Dr. T. Aluwong and Dr. A. Mohammed. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any institution.

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| Name of student | Signature | Date |

Certification

This thesis titled "NEUROBEHAVIOURAL, HAEMATOLOGICAL AND BRAIN BIOCHEMICAL CHANGES IN WISTAR RATS SUBJECTED TO PSYCHOLOGICAL STRESS AND ADMINISTERED WITH LYCOPENE" by Ogundeji, Tunde meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dedication

This thesis is dedicated to God, the Almighty.

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My appreciation goes to my supervisors: Prof. J. O. Ayo, Dr. T. Aluwong and Dr. A. Mohammed for their contributions and guidance in making this work a success.

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Abstract

The aim of the study was to evaluate neurobehavioural, haematological and brain biochemical changes in Wistar rats subjected to psychological stress (that is, fear/anxiety-induced stress) and administered with lycopene. Twenty-eight adult male Wistar rats were randomly assigned to four groups each comprising seven animals: unstressed (U-Control), unstressed + lycopene (U+Lyco), stressed (S-Control) and stressed + lycopene (S+Lyco). During the 14-day period of the experiment, olive oil (1 ml/kg/day) was administered singly or mixed with lycopene (10 mg/kg/day) by gastric gavage to each rat in the groups. U-Control rats were administered olive oil only while U+Lyco rats were administered with lycopene mixed with olive oil. S-Control rats were subjected to psychological stress and administered olive oil only while S+Lyco rats were subjected to psychological stress and administered lycopene mixed with olive oil. S-Control and S+Lyco were subjected to psychological stress by exposure to the following tests/tasks at specified periods: open-field test on days 3 and 10; escapable foot-shock on day 13 followed by a situational reminder on day 14; excitability score test on day 14; inclined plane performance task and forepaw grip time task on days 1, 7 and 14. U-Control and U+Lyco did not receive such exposure. At the end of the study period, brain and blood samples collected after sacrificing the animals were analyzed for brain MDA and electrolytes concentrations; activities of superoxide dismutase, catalase and acetylcholinesterase; PCV, Hb, RBC, WBC, DLC, TP as well as EOF and erythrocyte MDA concentrations. The results showed that psychological stress causes behavioural, sensorimotor and cognitive deficits; decreased brain superoxide dismutase, catalase and acetylcholinesterase activity; brain electrolytes imbalance and haematological alteration. Lycopene ameliorated the effect of psychological

stress on behavioural, cognitive, sensorimotor performance, brain biochemical and haematological parameters. In conclusion, ameliorative effect of lycopene on psychologically-induced changes may be due to its antioxidant property.

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List of Abbreviations

Ache: Acetylcholinestarase

ACTH: Adrenocorticotropic hormone

ASD: Acute stress disorder

AT: Ambient temperature

Band: Band neutrophils

BNST: Bed nucleus of stria terminalis

CAT: Catalase

CRH: Corticotropin-releasing hormone

CSD: Central square duration

CSF: Central square frequency

Cu: Cupper

DBT: Dry-bulb temperature

DLC: Differential leucocyte counts

DNA: Deoxyribonucleic acid

DTNB: 5,5-dithiobis-2-nitrobenzoeic acid

EOF: Erythrocyte osmotic fragility

Eos: Eosinophils counts

FFS: Fight-flight syndrome

GABA: Gamma amino butyric acid

GAS: General adaptation syndrome

GMT: Greenwich mean time

GSH: Gluthathione

GSH-Px: Glutathione peroxidases

GSSG: Oxidized gluthathione

H_2O_2: Hydrogen peroxide

Hb: Haemoglobin

HDL: High-density lipoprotein

HI: Heat index

HPA: Hypothalamic-pituitary-adrenal axis

LDL: Low-density lipoprotein

LHPA: Limbic–hypothalamo–pituitary–adrenal axis

Lym: Lymphocytes counts

MCHC: Mean corpuscular haemoglobin Concentration

MCV: Mean Corpuscular Volume

MDA: Malodialdehyde concentration

MHC: Mean haemoglobin Concentration

Mn: Manganese

Mon: Monocytes counts

N/L Ratio: Neutrophils lymphocytes ratio

NaCl: Sodium chloride

NADPH: Nicotinamide adenine dinucleotide phosphate

Neut: Neutrophils counts

NO_2: Nitrogen dioxide

PCV: Packed cell volume

PSA: Prostate specific antigen

RBC: Erythrocytes counts

RH: Relative humidity

RNA: Ribonucleic acid

ROS: Reactive oxygen species

S+Lyco: Stressed and administered lycopene mixed with olive oil

S-Control: Stressed control and administered olive oil

SOD: Superoxide dismutase

TBARS: Thiobarbituric acid reactive substance

TP: Total protein

U+Lyco: Unstressed and administered lycopene mixed with olive oil

U-Control: Unstressed control and administered olive oil

VLDL: Very-low-density lipoprotein

WBC: Leucocytes counts

WBT: Wet-bulb temperature

Zn: Zinc

CHAPTER 1
INTRODUCTION

Maintenance of sound performance is vital in productivity and work output in animals. Domestic animals, especially dogs and cats used for security purpose and some captive wild animals that are used in circus show worldwide, need to maintain adequate psychological performance in terms of behavioural and cognitive responses. One source of threat to the maintenance of sound performance is fear/anxiety-induced stress. Although some recent studies have suggested that there may be separate fear and anxiety systems (Davis, 2006), body stress response mechanisms to fear and anxiety are apparently similar (Shin and Liberzon, 2010). Fear/anxiety-induced stress refers to psychological abnormality that occurs following an exposure to an events which causes intense fear and/or anxiety, consequently, resulting in abnormal stimulation of the body stress response mechanisms. Thus, fear/anxiety response may activate the stress response system via the limbic-hypothalamo-pituitary-adrenal (LHPA) axis, leading to physiological or pathological changes. The concept of a stress response system as it relates to activation of LHPA axis and secretion of stress hormones like corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol may be highly relevant to the neurocircuitry of fear and anxiety (Shin and Liberzon, 2010). An important and often overlooked aspect of the fear/anxiety neurocircuitry is its overlap and interaction with the neurocircuitry that coordinates the stress response system. Research findings suggest a significant overlap in structures involved in fear/anxiety and stress responses (Mayberg *et al.*, 1999; Heim and Nemeroff, 2001; Liberzon and Martis, 2006). Several reports have shown psychological conditions associated with abnormal stimulation of the stress

response mechanism in domestic animals (von Borell, 2001; Lay and Wilson, 2004). Abnormal stimulation of the stress response mechanism may results in depletion of bioprotective molecules in the antioxidant defense system, leading to an increased generation of reactive oxygen species which in turn results in oxidative stress (Maier and Watkins,1998; Chihuailaf *et al.,* 2002; Lykkesfeldt and Svendson, 2007).

Management of fear/anxiety-induced stress

and the related disorders usually includes a combination of pharmacological substances, short-term psychotherapy or both. In humans, a variety of medications have been shown to be beneficial in the management of abnormal psychological conditions before subsequent development into more severe situations (Maxmen and Ward, 1995). There is paucity of information on the potential effects of lycopene in the management of fear/anxiety-induced stress in animals.

Lycopene is one of the most potent antioxidants among dietary carotenoids. Although chemically a carotenoid, lycopene has no vitamin A activity. It widely occurs in many fruits and vegetables, particularly in tomatoes. It is the red pigment of ripe tomatoes with strong antioxidant property, which can neutralize oxygen derived free radicals (Rafi *et al.,* 2007). Due to its strong colour, lycopene is used as a colouring agent in food additives and it is non-toxic. Like all carotenoids, lycopene is a polyunsaturated hydrocarbon (Figure 1.1). It is a hydrophobic, acyclic isomer of beta-carotene and is longer than any other carotenoid. The eleven conjugated double bonds in lycopene give the compound its deep red colour and are responsible for its antioxidant activity.

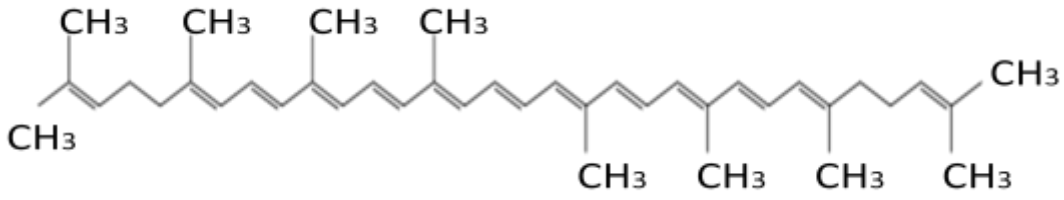

Figure 1.1: Chemical formula of all-*trans* lycopene ($C_{40}H_{56}$)

Early research findings suggested some amelioration of cardiovascular disease, cancer, diabetes, osteoporosis and even male infertility following lycopene administration. Indeed, lycopene has been suggested to be the most powerful carotenoid quencher of singlet oxygen (Di Mascio *et al.*, 1989), produced during exposure to ultraviolet light, a primary cause of skin ageing (Berneburg *et al.*, 1999). As an antioxidant, lycopene has a singlet-oxygen-quenching ability twice as high as that of beta-carotene and 100 times higher than that of alpha-tocopherol, which in turn has 125 times more quenching action than the water soluble gluthathione (Shi and Le Mague, 2000; Atessahin et al., 2005).

1.1 STATEMENT OF RESEARCH PROBLEMS

Relieve of animal suffering and improvement of animal production are cardinal objectives in care and use of domestic animals. Studies aim at achieving these goals become significant considering the importance of maintenance of animal well-being in productivity and work output. The relevance of investigating the effects of psychological conditions on productivity and work output in domestic animals has long been identified (Fox, 1968). In humans, fear/anxiety-induced stress is a psychological condition that occurs in response to exceptional physical and/or psychological stressor (Bryant, 2006). It may be caused by exposure to events that induce intense fear and/or anxiety. In domestic animals, such events are potent stressors with relevance to the well-being of the animals, leading to severe

damage to specific target organs and tissues or even death in some species (von Holst, 1997). Such potent psychological stressors include situation of uncertainty, social pressure, novel environment, weaning, isolation and fear (von Borell, 2001; Lay and Wilson, 2004), transportation and introduction into new environment (Minka and Ayo, 2011). Although the clinical symptoms of fear/anxiety-induced stress in humans are characterized by anxiety-related symptoms (Bryant, 2006), the symptoms are, apparently, similar in animals. Fear/anxiety-induced stress usually appears within minutes of exposure to the stressor and may either resolve itself with time or progress into a more severe condition. This may have adverse effects on productivity and work output of animals through induced behavioural changes, reminiscent of enhanced anxiety and depression-like behaviour (Koolhass *et al.,* 1990; Armario *et al.,* 2008). In addition to adverse effects on productivity and work output of animals, reports have shown that mere exposure to a single episode of a psychological stressor may alter the response of animal to drugs such as diazepam (Antelman *et al.,* 1988; Armario *et al.,* 2008). Research findings from humans and rodents indicates that psychological stress is a more potent stressor than physical stress (Lay and Wilson, 2004).

1.2 JUSTIFICATION OF STUDY

Various anxiety-like effects of psychological stress are likely to use different brain pathways and neurochemical mechanisms as observed in studies using rats (Armario *et al.,* 2008). This clearly suggest that not all aspects of psychological stress involve similar neurobiological process. Several pharmacologic agent have been use to investigate the effects of psychological stress on neurochemistry of body response. For instance, the effect of anisomycin, a protein synthesis inhibitor, on cat exposure (Adamec *et al.,* 2006; Cohen *et al.,* 2006). Findings from such studies usually serve as guide in the use of existing

treatments and possible development of new treatments approach. However, there is paucity of information on the use of lycopene, an antioxidant, in providing insight on the proximate mechanism of psychological stress in animals. Thus, expanding the scope of the study of antioxidants with the aim of understanding their physiological role, particularly in abnormal psychological condition, may be of great therapeutic and prophylactic value.

Nutritional supplements, including lycopene, are consumed not only for the maintenance of general well-being, but also for the management of certain disease conditions. Lycopene exerts ameliorative effects on cardiovascular disease (Kohlmeir *et al.,* 1997; Englehard *et al.,* 2006), cancer (Rao *et al.,* 1999; Nahum *et al.,* 2001; Kucuk *et al.,* 2002; Giovannucci *et al.,* 2002; Etminan *et al.,* 2004), diabetes (Giovannucci *et al.,* 1995), osteoporosis (Rao *et al.,* 2003; Rao, 2005) and even male infertility (Mohanty *et al.,* 2001; Mangiagalli *et al.,* 2010). However, there is dearth of information on the benefit of lycopene in the management of fear/anxiety-induced stress. Besides its use in providing insight on the pathophysiologic mechanism of psychological stress in animals, exploring its potential benefits in psychologically-induced abnormality may be of value.

Currently, efforts are geared towards investigating the antioxidant properties of lycopene and possible correlation between lycopene consumption and general health. Although best known as an antioxidant, both oxidative and non-oxidative mechanisms are involved in lycopene's bioprotective activity. Other mechanisms of action of lycopene that are also being investigated include modulation of intercellular gap junction communication, hormonal and immune system, and metabolic pathways (Khan *et al.,* 2008). The potential benefit of lycopene in the management of neurodegenerative conditions with clinical

symptoms of cognitive impairment, emotional disturbances and movement abnormality indicates that it may exerts some positive effects on the nervous system (Kumar and Kumar, 2009). This may be through its antioxidant properties, the mechanisms of which are yet to be elucidated. It is conceivable that the findings from this study may be of value in further understanding the physiological responses to fear/anxiety-induced stress, and the proximate biochemical mechanism of lycopene actions in abnormal stress response to fear and anxiety.

1.3 GENERAL AIM OF THE STUDY

The general aim of this study was to evaluate neurobehavioural, haematological and brain biochemical changes in Wistar rats subjected to psychological stress (that is, fear/anxiety-induced stress) and administered with lycopene.

1.4 SPECIFIC OBJECTIVES OF THE STUDY

1. To evaluate the effects of lycopene administration on behavioural responses of Wistar rats.
2. To determine the effects of lycopene administration on learning and short-term memory of Wistar rats.
3. To evaluate the effects of lycopene administration on excitability, neuromuscular coordination and motor strength of Wistar rats.
4. To determine the effects of lycopene administration on malondialdehyde concentration, activities of superoxide dismutase, catalase and acetylcholinesterase in the brain of Wistar rats.

5. To determine the effects of lycopene administration on erythrocytes MDA concentration, erythrocytes osmotic fragility, leucocytes as well as other erythrocytes parameters of Wistar rats.

1.5 RESEARCH HYPOTHESES

1. Psychological stress does not alter behavioural responses, cognitive ability, sensorimotor performance, haematological and brain biochemical parameters of Wistar rats.
2. Lycopene does not affect psychologically-induced changes in behavioural responses, cognitive ability, sensorimotor performance, haematological and brain biochemical parameters of Wistar rats.

CHAPTER 2
LITERATURE REVIEW

2.1 FEAR, ANXIETY AND STRESS RESPONSE

Fear is an emotional response to a known or definite threat, while anxiety is often a response to an imprecise or unknown threat. The studies on anxiety disorder have its roots in the study of fear response in animal models and brain responses to psychological stressors in healthy animals (Shin and Liberzon, 2010). Fear/anxiety-induced stress refers to psychological abnormality that occurs following an exposure to an event which causes intense fear and/or anxiety, consequently, resulting in abnormal stimulation of the body stress response mechanisms. This may leads to oxidative stress which in turn result in behavioural, physiological and biochemical alterations. It is becoming increasingly clear that specific characteristics of experience (for example, novelty, social pressure and fear) are very relevant for body stress response activation (Abelson *et al*, 2007). Thus, research on fear/anxiety neurocircuitry offers information on the brain circuits that may underlie understanding abnormal response, to inform possible development of new treatments and the prediction of treatment response (Shin and Liberzon, 2010).

Excessive fear is a key component of anxiety-related psychological disorder, therefore, most studies on anxiety-related psychological abnormality is closely intertwined with that of fear circuits in animal models. Key components of fear circuitry include the amygdala (and its subnuclei), nucleus accumbens [including bed nucleus of stria terminalis (BNST)], hippocampus, ventromedial hypothalamus, periaqueductal gray, a number of brain stem nuclei, thalamic nuclei, insular cortex, and some prefrontal regions (mainly infralimbic cortex) (Davis, 2006; Maren, 2008; Quirk and Mueller, 2008). These regions have their

respective roles in the various components of fear processing such as the perception of threat or unconditioned stimuli, the pairing of an unconditioned stimulus and conditioned response (learning/conditioning), the execution of efferent components of fear response, and the modulation of fear responses through potentiation, contextual modulation, or extinction. A large volume of experimental work has examined the neurocircuitry associated with fear responses, mainly in rodents, using primarily fear conditioning, inhibitory avoidance, and fear-potentiated startle models (Shin and Liberzon, 2010).

Some parts of the brain region are implicated in both fear/anxiety and stress responses, suggesting that the responses are interrelated and may influence one another. Major subcortical components of the LHPA axis (for example, hypothalamus, hippocampus, amygdala, and BNST) have also been identified as key components of fear/anxiety neurocircuitry, even though sometimes involving different subnuclei, for example paraventricular nuclei versus ventromedial hypothalamus for LHPA and fear neurocircuitry, respectively. More recently with the introduction of *in-vivo* imaging methodologies in LHPA/stress research, the role of cortical structures like the insula and dorsal medial prefrontal cortex in the activation and inhibition of stress response respectively has been reported (Liberzon and Martis, 2006). Subgenual anterior cingulate cortex is also involve in self-induced sadness and depression (Mayberg *et al*, 1999). Together, these findings suggest a significant overlap in structures involved in the stress response and those involved in fear/anxiety responses; for example, medial prefrontal cortex, insula, amygdala, hippocampus, and BNST (Shin and Liberzon, 2010). Finally, with respect to neurotransmitters involved, CRH is likely involved in the orchestration of both LHPA axis activity and many anxiety/fear responses (Heim and Nemeroff, 2001).

2.2 STRESS RESPONSE SYSTEM

Selye (1963) defined stress as any biological response elicited when an animal perceives threat to its homeostasis. Such biological response to threat are referred to as stress response (Moberg, 2000; Virdin and Kidd, 2009). Factors that can provoke a state of threatened homeostasis and initiate stress response are psychological, physiological or environmental in nature (Chrousos and Gold, 1992), and are collectively called stressors (stress factors). Stressors are stimuli which can originate internally and/or externally. They initiate stress response via the hypothalamic-adrenal medullary (the sympathetic nervous system) and the HPA neural pathways, consequently resulting in changes (Maier and Watkins, 1998; Garcia-Bueno *et al.*, 2008). The changes may be physiological, but may result in oxidative stress if there is excess stimulation of the stress response mechanism. Oxidative stress is an imbalance in the oxidant/antioxidant system in favour of excessive production of oxidants (Altan *et al.*, 2003; Durackova, 2010). It is characterized by pre-pathological manifestations that may eventually lead to death (Selye,1946). Thus, the study of stress response system is pivotal in understanding the concept of stress in animals.

2.2.1 Specific and Non-Specific Stress Response

Stress response can be categorized as specific (short-term) and non-specific (long-term) (Siegel, 1980; Virdin and Kidd, 2009). Specific stressors are typically short-term (such as a sudden increase in environmental temperature) and the animals typically respond to specific stressors by trying to combat the stressor. Specific stress response was referred to as fight-flight syndrome (FFS). It involves the hypothalamic-adrenal medullary stress response system which comprise the hypothalamus and sympathetic neural pathway to the

adrenal medulla and release of adrenaline by the adrenal medulla (Garcia-Bueno *et al.*, 2008).

The non-specific stress response involve hypothalamic-pituitary-adrenocortical (HPA) stress response system, which was conceptualized by Hans Selye as general adaptation syndrome (GAS) (Selye,1946). In this case, animal subjected to a stressor takes measures to adapt to the stressor rather than dealing with it directly. Selye distinguished three phases of such stress response as: (i) alarm (Cannon's emergency concept), followed by (ii) resistance (that is by the release of corticosteroids), which if unsuccessful result in (iii) exhaustion of the response system (pre-pathological state) and eventually death.

2.2.2 Factors that can Provoke a Stress Response

These factors are refer to as stressors and they can be categorized into exogenous and endogenous stressors. Exogenous stressors that can provoke a stress response are further categorized into psychological (for example, fear, anxiety, depression and social pressure), patho-physiological (e.g. age, pain, pregnancy, genetics, and physical abnormalities) and environmental/physical factors. Examples of environmental/physical factors include nutritional status, handling, radiation, cigarette smoke, certain organic solvents and pesticides, sun radiation, mycotoxins, trauma, hyperoxia and over-exercising, drug consumption and medication.

Endogenous stressors that can provoke a stress response include ischaemia or reperfusion injury following surgery or artery blockage (Reed, 1995). This may result in inhibition/reduction in the level of antioxidants which affect the ability of the body to scavenge increased level of free radical.

2.2.3 Mechanism of Stress Response

When an animal first encounters a stressor, sensory detectors transformed that information into neural signals that are transmitted to either or both cognitive and non-cognitive center of the nervous system to generate a coordinated response to the challenge (von Borell, 2001). Cognitive brain centres such as (cerebral cortex) perceive threats and act to initiate this response mechanism via nervous signals that activate CRH in the hypothalamus (Johnson *et al.,* 1992). CRH has neuro-transmitter function in the brain (von Borell, 2001). For example, it activates the sympathetic adrenomedullary system, resulting in elevation of plasma adrenaline concentration, arterial blood pressure and heart rate (Garcia-Bueno *et al.,* 2008). Failed attempts to combat or flee from the stressor immediately result in the activation of the hypothalamic-pituitary-adrenocortical (HPA) stress response system (Siegel, 1980; Virdin and Kidd, 2009). When this system is activated, the hypothalamus produces corticotrophin-releasing factor, which then activates the anterior pituitary to increase synthesis and secretion of ACTH and other metabolites (Axelrod and Reisine, 1984; Garcia-Bueno *et al.,* 2008). Secretion of ACTH causes the cells of the adrenal cortical tissue to proliferate and secrete glucocorticoid. Increasing glucocorticoid concentration in the blood may inhibit ACTH secretion from the pituitary. However, the release of ACTH is dependent on the intensity of the stressors and modulated by steady-state sensitive feedback mechanism. Mild stressors may, therefore, be gradually inhibited by the glucocorticoid feedback, whereas severe stressors are not inhibited (Axelrod and Reisine, 1984; von Borell, 2001).

The activities of catecholamines and glucocorticoid in animals is associated with increased physiological activities, involving utilisation of oxygen with accompanied production of

free radicals at the cellular level. During normal metabolic reaction, four electrons are added to around 95-98% from the 85-90% of the inhaled oxygen in other to form water. The remaining 2-5% is reduced into metabolites called free radicals in the cells. Thus, free radicals are formed as a normal end product of cellular metabolism arising from either the mitochondrial electron transport chain or from stimulation of nicotinamide adenine dinucleotide phosphate (NADPH) (Valko, *et al.,* 2007; Sordillo and Aitken,2008).

2.2.4 Roles of Free-Radicals in Stress Response

2.2.4.1 Beneficial Effects of Free Radicals

A certain physiological level of ROS is crucial for the regulation of cell function such as the activation of nuclear transcription factors, gene expression and a defense mechanism to target tumour cells and microbial infections (Simon *et al.,* 2000; Durackova, 2010). Superoxide anion may serve as a cell growth regulator (Lee, *et al.,* 2003). Singlet oxygen can attack various pathogens and induce physiological inflammatory response (Stief 2003). Nitric oxide is one of the most widespread signaling molecules and it participates in every cellular and organ function in the body. Nitric oxide acts as a neurotransmitter and an important mediator of the immune response (Fang *et al.,* 2002).

2.2.4.2 Adverse Effects of Oxidative Stress

Despite the fact that oxygen is necessary for biological oxidation of substrates for energy for survival and activity, the action of oxygen at cellular level may really be two-sided (Durackova, 2010). Adverse effects of oxidative stress comprise direct and harmful effects on cellular function and structure. Among these detrimental effects on cells, lipid

peroxidation plays a crucial role (Benzie, 1996). Lipid peroxidation, as a consequence of oxidative stress, can cause damage to cell membranes directly by altering membrane fluidity, permeability and integrity (Halliwell, 2006; Friedman, 2010). Oxidative attack can also lead to damage to DNA, carbohydrate, protein and other molecules through self-perpetuating chain reaction and toxic by-products. Lipid peroxide can form cyclic peroxides, which can decompose to highly cytotoxic products such as aldehydes and alkoxyl radical. These cytotoxic compound react aggressively with living tissues, thereby disrupting the cell membrane and diffuse from the lipid membrane to damage other cells. Such disruption leads to changes in permeability, viscosity, secreting activity, and enzymatic inactivation. The processes constitute patho-biochemical mechanisms involve in initiation or progression phase of various diseases (Wiseman and Halliwell, 1996; Sordillo and Aitken, 2008).

It becomes apparent that an imbalance in protection against free radical and their generation can be associated with pathogenesis of wide variety of chronic diseases. Coronary heart disease, carcinogenesis, cataract and age related macular degeneration, neuronal diseases, neuromuscular diseases, and a large number of other important diseases such as Parkinson's disease and Alzheimer's disease, have been related directly or indirectly with oxidative stress (Diplock *et al.*, 1998; Durackova, 2010). In addition, during the past decade, several lines of evidence have convinced scientists that oxidative stress play an important role in ageing (Beckman and Ames, 1998). The ROS theory of ageing suggest that ageing is caused by the deleterious effects of accumulated free radicals throughout the lifespan (Cheeseman and Slater, 1993; Lee, *et al.*, 2003).

2.3 ANTIOXIDANT DEFENSE SYSTEM

Oxidative stress is rigorously controlled by multiple defense line, which includes enzymatic and non-enzymatic scavengers and quenchers (Vani *et al.,* 2010). These defenses either stop the free-radical chain reaction or divert the free radicals to a less deleterious target. The endogenous enzymes involved are three groups of metal-binding proteins.

i. The first comprises superoxide dismutases (SOD), which dismutases the highly reactive superoxide free radicals (O_2.) to the less reactive species H_2O_2 (Teixeira, 1998) at the mitochondria (Mn-dependent SOD), nucleus and cytoplasm (Cu/Zn-dependent SOD).

ii. The second group are catalase (CAT) which scavenge hydrogen peroxide (H_2O_2) and convert it to water and molecular oxygen (Mates and Sanchez-Jimenez, 1999). Catalase are found in peroxisomes in most tissues and they probably serve to remove peroxides generated by peroxisomal oxidase enzymes.

iii. The third group are glutathione peroxidases (GSH-Px), which are the major enzymes that remove H_2O_2 generated by SOD in the cytosol and mitochondrial by oxidizing the tripeptide gluthathione (GSH) into its oxidized form (GSSG) (Halliwell and Gutteridge, 1986).

The antioxidant enzymes are complimented by small-molecule antioxidants, some of which are derived exclusively from the diet (Cantuti-Castelvetri *et al.,* 2000). Major non-enzymatic defenses include GSH by itself, the tocopherols, carotenoids and ascorbic acid. The mechanisms by which these antioxidants act at the molecular and cellular level include roles in gene expression and regulation, apoptosis and signal transduction. Thus, antioxidant molecules are involve in fundamental metabolic and homeostatic processes

(Elsayed, 2001). Indirect antioxidants function are mediated by enzymes that restore endogenous antioxidant levels; for example, GSH level are replenished on reduction of GSSG by the enzyme gluthathione reductase. Antioxidant defense system have evolved to defend the body against free radicals but may become overwhelmed during period of chronic oxidative stress (Vani *et al.,* 2010). Because these defense systems are not 100% effective, the body have also develop enzyme system to repair damage that free radical inflicts DNA, RNA and proteins (Winrow *et al.,* 1993; Durackova, 2010). The balance between free radical generation and antioxidant defenses may be a crucial force in chronic disease prevention (Machlin and Bendich, 1987).

2.4 LYCOPENE

Lycopene is one of the most potent antioxidants among dietary carotenoids. It is a bright red carotene and a member of the carotenoid family of phytochemicals. Although used as food colourant for many years, the investigation into the health benefits of lycopene did not begin until the last part of the 20th century. However, in a relatively short period of time, an increasing body of evidence based on laboratory, animal, and population-based research supports the role of lycopene in health and disease, and as a potential natural food antioxidant.

2.4.1 Physicochemical and Biochemical Properties

Several studies have investigated the properties of lycopene (Botsoglu *et al.,* 2004; Bhom, 2007; Linsheld *et al.,* 2007; Mangiagalli *et al.,* 2010). Although lycopene is chemically a carotene, it has no vitamin A activity because it lacks the beta-ionone ring of alpha- and beta-carotene. Lycopene (Fig. 1.1) is an hydrophobic, acyclic isomer of beta-carotene with highly unsaturated hydrocarbon containing 11 conjugated bonds. The presence of

eleven conjugated double bonds gives the compound its deep red colour and is responsible for its powerful antioxidant activity.

It has strong antioxidant property which can neutralize oxygen-derived free radicals (Rafi *et al.,* 2007). It is able to react with singlet oxygen and various radical cation, and has the highest Trolox-equivalent antioxidant capacity value of all carotenoids (Rice-Evans *et al.,* 1997). Its all-*trans* isomeric form may efficiently quench singlet oxygen and trap peroxyl radicals. As an antioxidant, lycopene has a singlet-oxygen-quenching ability twice as high as that of beta-carotene (vitamin A relative) and 100 times higher than that of alpha-tocopherol (vitamin E relative), which in turn has 125 times more quenching action gluthathione (water soluble) (Di Mascio *et al.,* 1989; Shi and Le Mague, 2000; Atessahin *et al.,* 2005). Although, best known as an antioxidant, both oxidative and non-oxidative mechanisms are involved in lycopene's bioprotective activity. One non-oxidative activity is regulation of gap-junction communication between cells. Lycopene participates in a host of chemical reactions hypothesized to prevent carcinogenesis and atherogenesis by protecting critical cellular biomolecules, including lipids, proteins, and DNA (Khan *et al.,* 2008).

2.4.2 Sources of Lycopene

Lycopene widely occurs in various kinds of fruits and vegetables, particularly in tomatoes. It is the red pigment of ripe tomatoes and watermelon (Tadmor *et al.,* 2005; Frusciante *et al.,* 2007; Perkins-Veazie *et al.,* 2003, 2007) and some fruit and vegetable (Nguyen and Schwarts, 1999). Dietary sources and nutritional supplements constitute the major source of lycopene intake. Fruits and vegetables that are high in lycopene include gac (*Momordica cochinchinensis*), tomatoes, watermelon, pink grapefruit, pink guava, papaya, red bell pepper, sea buckthorn, wolfberry (goji, a berry relative of tomato), and rosehip. Not all red

fruits contain lycopene e.g. strawberries, cherries. Although gac has the highest content of lycopene than any known fruit or vegetable, tomatoes and tomato-based sauces, juices and ketchup account for more than 85% of the dietary intake of lycopene for most people due to gac's rarity outside its native region of southeast Asia (Rao and Rao, 2007). The lycopene content of tomatoes depends on species and increases as the fruit ripens (Khan *et al.*, 2008). Within tomato products, lycopene is most prominent making up 60 - 64% of the carotenoids present. Other phytonutrients that are also present in tomatoes and tomato products such as phytoene (10 - 11%), gamma-carotene (10 - 11%), neurosporine (7% - 9%), phytofluene (4% - 5%), betacarotene (1% - 2%), delta-carotene (1% - 2%) and lutein (trace to 1%) (Clinton, 1998). Recent studies have shown lycopene to act synergistically with these phytonutrients in providing its beneficial properties.

Although, lycopene is available in a supplement form, there is, apparently, a synergistic effect when it is obtained from the whole fruit instead, where other components of the fruit enhance lycopene's effectiveness. Most lycopene supplements are oil-based in form of soft gel capsule. The enteric coating is a neutral polymer coating placed around the capsule to protect it from stomach acids. This ensures that it gets to the upper intestine where the supplement nutrients can be properly absorbed. When absorbed from the small intestine, lycopene is transported in the blood by various lipoproteins and accumulates in the liver, adrenal glands, and testes (Bramley, 2000).

2.4.3 Bioavailability and Isomerization

Normally, lycopene obtained from plants tends to exist in an all-*trans* configuration, the most thermodynamically stable form. However, the double bonds are subjected to isomerisation (Halloway *et al*., 2000). As a polyene, it undergoes *cis*-*trans* isomerization

induced by light, thermal energy and chemical reactions. Bioavailabilty implies the fraction of carotenoid that is absorbed by the body and available for use in physiological function (Tanumihardjo, 2002). For lycopene, studies have indicated that the bioavailability of the *cis*-isomer of lycopene is greater than the all-*trans* isomer (Boileau *et al*., 1999). This may be because *cis*-isomer are more soluble in bile acid micelles and so preferencially incorporated into chylomicrons (Bramley, 2000). Remaining to be determined, however, is whether or not cis-isomers are biologically more effective than trans-isomers once in the body (Rao and Agarwal, 2000).

Not all sources of lycopene are equally bioavailable. Ingested in its natural *trans*-form, such as is prominent in tomatoes, lycopene is poorly absorbed (Rao and Agarwal, 2000). Heat processing of tomatoes and tomato products induces isomerisation of lycopene from all-*trans* to *cis* configuration, which in turn increases its bioavailability (Stahl and Sies, 1992; Clinton, 1998; Agarwal *et al.,* 2001). More significantly, the chemical form of lycopene is altered by the temperature changes involved in processing. Heat processing improves bioavailability by increasing *cis*- to *trans*-ratio. Unlike other fruits and vegetables, where nutritional content such as vitamin C is diminished upon cooking, heat processing of tomatoes increases the lycopene bioavailability. Lycopene is absorbed better from heat processed foods than unprocessed sources (Bohm and Bitsch, 1999). Thus, lycopene in tomato paste is four times more bioavailable than in fresh tomatoes. For this reason, tomato sauce is a preferable source as opposed to raw tomatoes (Stahl and Sies, 1996; Shi and Le Mague, 2000).

In addition to the importance of its conformation at the time of ingestion, bioavailability of lycopene is also affected by factors such as amount of lycopene consumed, food matrix, dietary fibre and fat, interaction with other carotenoids, and interference with proper micelle formation (Blakely *et al.*, 1994; Castenmiller and West, 1998; Bohm and Bitsch, 1999; van Het Hof *et al.*, 2000; Shi and Le Mague, 2000; Tanumihardjo, 2002). For instance, the presence of other carotenoids, such as beta-carotene that share a common LDL-mediated absorption pathway, can also influence lycopene absorption (Johnson *et al.*, 1997). Lycopene is absorbed better from lipid–rich diets (Bohm and Bitsch, 1999). Since lycopene is fat-soluble (as are vitamins, A, D, E and beta-carotene), absorption into tissues is improved when oil is added to the diet. Carotenoid bioavailability is increased by presence of fat (De Pee and West, 1996; van Den Berg, 1999; Tanumihardjo, 2002).

Carotenoid bioavailability is decreased by dietary fibre (Erdman *et al.*, 1986). While most green leafy vegetables and other sources of lycopene are low in fats and oils, lycopene is tightly bound to vegetable fibre. Generally, lycopene is enclosed in cells of the tissue and only a portion of the lycopene that is present is absorbed. Therefore, processing makes the lycopene more bioavailable by increasing the surface area available for digestion. For example, processed tomato products such as pasteurised tomato juice, soup, sauce, and ketchup contain the highest concentrations of bioavailable lycopene from tomato-based sources. Cooking and crushing tomatoes (as in the canning process) and serving in oil-rich dishes (such as spaghetti sauce or pizza) greatly increases assimilation from the digestive tract into the bloodstream. Hence, mechanical processing of tomato products help to breakdown cell wall and release lycopene from food matrix, thereby increasing its bioavailability (Hill and Rogers, 1969; Helzsouer *et al.*, 1989).

2.4.4 Pharmacokinetics and Tissue Distribution

After ingestion, lycopene is incorporated into lipid micelles in the small intestine. These micelles are formed from dietary fats and bile acids, and help to dissolve the hydrophobic lycopene and allow it to permeate the intestinal mucosal cells by a passive transport mechanism. When absorbed from the small intestine, lycopene is transported in the blood by various lipoproteins and accumulates in the liver, adrenal glands, and testes. Little is known about the liver metabolism of lycopene, but like other carotenoids, lycopene is incorporated into chylomicrons and released into the lymphatic system. It is then distributed in blood plasma, initially in very-low-density lipoprotein (VLDL) and chylomicrons fractions but later in low-density lipoprotein fractions (LDL) and high-density lipoprotein (HDL) fractions of the serum. The highest levels are found in the LDL (Stahl and Sies, 1996; Bramley, 2000).

Lycopene is mainly distributed to fatty tissues and organs such as the adrenal glands, liver, and testes. In human dietary intervention research, serum lycopene levels significantly increase after consuming tomato foods or lycopene supplementation (Rao and Agarwal, 1998; Rao, 2004). However, unlike other carotenoids, lycopene levels in serum or tissues do not correlate well with overall intake of fruits and vegetables. In human plasma, lycopene is the most predominant carotenoid, present naturally in greater amounts than beta-carotene and other dietary carotenoids. This perhaps indicates its greater biological significance in the human defense system. Its level is affected by several biological and lifestyle factors. Lycopene is found in most human tissues, but is not deposited uniformly. These differences in tissue distribution suggest that there are specific mechanisms for the preferential deposition of lycopene, particularly in the adrenals and testes (Bramley, 2000).

2.4.5 Toxicity

This is an important consideration, but so far there are no known adverse effects from taking lycopene other than from excessive intake. Lycopene is non-toxic and is commonly found in the diet. A high intake of foods containing lycopene is not known to cause any harmful side-effects. However, in a middle-aged woman who had prolonged and excessive consumption of tomato juice, the skin and liver were coloured orange-yellow and elevated levels of lycopene in her blood occurred. After three weeks on a lycopene-free diet, the skin colour returned to normal (Stahl and Sies, 1996). This discolouration of the skin is known as lycopenodermia (INFMB, 2000) and is non-toxic.

Very little is known about the potential for adverse effects from high doses of supplemental lycopene. The Institute of Medicine at the National Academy of Sciences did not establish a tolerable upper intake level for carotenoids (including lycopene) when it reviewed these compounds in year 2000. Recommended average daily intake of lycopene range from 0.70 to 25.20 mg/day. However, based on human research, recent recommendations for the daily intake of lycopene suggest 7 mg/day (Rao and Shen, 2002). At this level of intake, circulatory lycopene concentration is maintained at a level consistent with that shown to reduce lipid peroxidation and result in other beneficial effects of lycopene. Some research findings indicate that under certain circumstances, lycopene (and other carotenoids) can become oxidized in the body, and may subsequently behave like free radicals and cause cellular damage. Cigarette smoke, for example, may cause lycopene to become oxidized. This may explain, at least in part, the research findings that cigarette smokers who take carotenoid supplements may have an increased risk of cancer or heart disease.

2.4.6 Potential Health Benefits

The evidence in support of the role of lycopene in the prevention of chronic diseases is primarily epidemiological in nature. However, tissue culture, animal experiments and more recently human intervention studies are providing convincing evidence in support of the epidemiological observations. Lycopene is not modified to vitamin A in the body so can be accessible for other benefits such as antioxidation. Dietary supplementation with lycopene results in health benefits due to improvement in antioxidative status and in the prevention of severe chronic disease (Tapiero *et al.,* 2004; Wu, *et al.,* 2004; Bhuvaneswari and Nagini, 2005; Rao *et al.,* 2006; Avci and Durak, 2007).

Similarly, several trials in healthy men and women have demonstrated the ability for a variety of doses of dietary lycopene (5 to 150 mg/day) from foods and tomato oleoresin extract to exert a positive significant influence on serum biomarkers considered to relate to atherogenesis and cancer risk (Rao and Agarwal, 1998; Rao *et al.,* 1999). These effects include decreased serum lipid peroxidation,(Rao and Agarwal, 1998; Rao and Shen, 2002; Rao, 2004) decreased serum LDL oxidation (Rao and Agarwal, 1998; Rao and Shen, 2002; Rao, 2004) and decreased protein oxidation (Rao, 2004, Rao and Shen, 2002). Human research has also shown lycopene supplementation to result in significantly increased total antioxidant potential (Rao, 2004) and a trend towards decreased serum DNA oxidation (Rao and Agarwal, 1998).

2.4.6.1 Lycopene and Cancer

There have been several studies that analyze the anti-cancer properties of lycopene, although research has been primarily inconclusive. With respect to lycopene's role in the

prevention of cancer, Giovannucci (1999) reviewed 72 epidemiological studies and found the consumption of tomatoes and tomato products or circulating levels of lycopene to be inversely related to risk of cancers, most prominently, cancers of the prostate gland, lung, and stomach. The results from this study (which examined case-control dietary studies, prospective dietary studies and blood specimen-based investigations) also suggested a reduced risk of other cancers, including pancreatic, colorectal, esophageal, oral, breast, and cervical cancers. Other cancers investigated did not show clear relationships to the intake of tomatoes, tomato products or lycopene.

Lycopene may reduce risk of cancer by activating special cancer preventive enzymes such as phase II detoxification enzymes, which remove harmful carcinogens from cells and the body (Giovannucci *et al.,* 2002). It is an inhibitor of human cancer cell proliferation and it was found that, unlike cancer cells, human fibroblasts were less sensitive to lycopene, and the cells gradually escaped growth inhibition over time. Lycopene is also found in lung tissue and is valuable in protecting lymphocytes from NO_2 damage found in lung cancer. In addition to its inhibitory effect on basal endometrial cancer cell proliferation, lycopene also was found to suppress insulin-like growth factor-I stimulated growth. Insulin-like growth factors are major autocrine/paracrine regulators of mammary and endometrial cancer cell growth. Therefore, lycopene interference in this major autocrine/paracrine system may open new avenues for research on the role of lycopene in the regulation of endometrial cancer and other tumours (Levy *et al.*,1995). In different studies however, lycopene was even found to have an inhibitory effect on cataract development (Pollack *et al.,* 1997) and several different kinds of cancer cells including breast and endometrial cancer cells

(Nahum *et al.,* 2001), prostate carcinoma cells (Giovannucci *et al.*, 2002), and colon cancer cells (Narisawa *et al.,* 1996).

Another meta-analysis of observational studies examined 11 case-control and 10 cohort studies to determine the effect produced by a daily intake of tomato products (Etminan *et al.*, 2004). It was concluded that tomato products can play a role in the prevention of prostate cancer. However, the beneficial effect of consuming tomato products was found to be modest and restricted to high amounts of intake. In a case-control study that examined prostate cancer, it was found that serum and prostate tissue lycopene levels were significantly lower in prostate cancer patients when compared with their age-matched controls (Rao *et al.,* 1999). The findings suggest that prostate cancer patients either lack the ability to absorb lycopene efficiently or utilise lycopene rapidly due to increased oxidative stress. This case-control study also revealed significantly higher serum prostate specific antigen (PSA) and protein oxidation in prostate cancer patients compared with controls. No significant relationship was observed between serum beta-carotene and PSA in prostate cancer cases compared with controls. Finally, a recent study with prostate cancer patients has also shown a possible prophylactic effect of lycopene in the shrinkage and regression of prostate tumors (Kucuk, *et al.,* 2001; Kucuk, *et al.,* 2002).

2.4.6.1 Lycopene and Cardiovascular Disease

Another area of research is that of lycopene and cardiovascular disease. In general, epidemiological studies exploring lycopene and cardiovascular disease show significant associations between the intake of tomatoes and serum, plasma, and adipose tissue levels of lycopene. However, some studies have failed to show such associations. In a large

multicenter case-control study, the relationship between adipose tissue antioxidant status (alpha- and beta-carotene and lycopene) and acute myocardial infarction were evaluated in 662 cases and 717 controls (Kohlmeir *et al.,* 1997). Adipose antioxidant levels were measured because they are considered to be a better marker of long-term exposure than serum lycopene. After adjusting for a range of dietary variables, higher lycopene but not alpha- or beta-carotene adipose tissue levels were found to be protective against myocardial infarction risk in non-smokers. Also related to cardiovascular disease, mildly hypercholesterolaemic individuals with grade-1 hypertension taking 15 mg/day of lycopene from tomato oleoresin extract had significantly decreased systolic and diastolic blood pressure compared to placebo (Engelhard *et al.,* 2006).

2.4.6.3 Lycopene and Bone Health

Recently, research has begun to explore the potential effects lycopene to work against the onset of bone disease. Although not fully understood, there is evidence that oxidative stress caused by reactive oxygen species is associated with the pathogenesis of osteoporosis. In a recent *in-vitro* study of bone marrow prepared from rat femurs, it was demonstrated that lycopene, in the absence or presence of parathyroid hormone, inhibited osteoclastic mineral resorption and formation of tartrate-resistant acid phosphatase, positive multinucleated osteoclasts, as well as the reactive oxygen species produced by osteoclasts (Rao *et al.,* 2003). The authors suggested that this finding may be important in the pathogenesis, treatment and prevention of osteoporosis (Rao *et al.,* 2003). Clinical studies are now being conducted to study the role of lycopene in osteoporosis (Rao, 2005). Researchers have studied the relationship between lycopene and bone resorption in postmenopausal women.

They found higher lycopene intake and higher serum lycopene to be associated with lower bone resorption (Rao, 2005). Based on the results from this study, the researchers are now conducting a lycopene intervention study with postmenopausal females to evaluate the relationship between lycopene and the risk of osteoporosis.

2.4.6.4 Lycopene and Male Infertility

Several studies have shown the effect of lycopene on male infertility (Martino *et al.,* 2006; Goyal *et al.,* 2007; Turk *et al.,* 2007; Mangiagalli *et al.,* 2010). In one study of 50 volunteers with low active sperm counts, 35 volunteers (70%) experienced an improvement in sperm count, 30 (60%) had improved functional sperm concentrations, 27 (54%) had improved sperm motility, 23 (46%) had improved sperm motility index, and 19 (38%) had improved sperm morphology following consumption of 8 mg/day of lycopene supplementation from tomato oleoresin extract (Mohanty *et al.,* 2001). Further studies are now being undertaken to confirm these preliminary observations and to gain further understanding into the role of lycopene in male infertility.

CHAPTER 3
MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS AND MANAGEMENT

Adult male Wistar rat was used as a model animal in this study. Twenty-eight, 10 - 14 week old Wistar rats weighing 190 - 220 grams were procured from the animal house of the National Institute of Trypanosomiasis Research, Kaduna. They were kept in the animal house of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria at ambient temperature of 25 ± 0.8 ^{o}C and a relative humidity of 86.14 ± 3.44 %. The animals were housed seven animals per cage and on a 12 hour light-dark cycle (light during 7:00 A.M. to 19:00 P.M.). They had access to standard pellet chow and drinking water *ad libitum*. They were pre-conditioned to the experimental environment for two weeks prior to the commencement of the experiment.

3.2 MEASUREMENT OF THERMAL MICRO-ENVIRONMENT PARAMETERS

Daily dry- and wet- bulb temperature of the experimental room were recorded using a wet- and dry-bulb thermometer (Brannan®, England) at 07:00 h, 13:00 h and 18:00 h (GMT+1). The parameters were taken daily throughout the experimental period, lasting 14 days. Relative humidity (RH) values were obtained from dry- and wet- bulb temperature using conversion table from the bulletin of the US Weather Bureau No. 1071. Temperature-humidity index (HI) values were calculated from ambient temperature and relative humidity using heat index calculator (Jackson, 2009).

3.3 CHEMICALS AND LYCOPENE

Lycopene (10 mg capsule, General Nutrition Corporation, Pittsburgh, U.S.A.) was obtained from a reputable pharmaceutical sales outlets. It was reconstituted in olive oil (*Goya en espana,* S.A.U., Savilla, Spain) to appropriate working concentration. All chemicals and solvents used were of analytical grade and were also obtained from a reputable pharmaceutical sales outlets.

3.4 EXPERIMENTAL GROUPS AND TREATMENTS

The twenty-eight, adult, male Wistar rats were randomly assigned to four groups, each comprising seven animals: unstressed control (U-Control), unstressed + lycopene (U+Lyco), stressed control (S-Control) and stressed + lycopene (S+Lyco). The dose of lycopene used is 10 mg/kg/day and was based on previous study by Kumar and Kumar (2009). During the 14-day period of the experiment, olive oil (1 ml/kg/day) was administered by gastric gavage singly or mixed with lycopene to each rat in the groups. U-Control rats were administered olive oil only while U+Lyco rats were administered with lycopene mixed with olive oil. S-Control rats were subjected to fear and administered olive oil only while S+Lyco rats were subjected to fear and administered lycopene mixed with olive oil.

3.5 EXPERIMENTAL PROTOCOL

U-Control and U+Lyco did not receive such exposure, but S-Control and S+Lyco were subjected to psychological stress (that is, fear/anxiety-induced stress) by exposure to the following tests/tasks at the specified periods:

(i) Open-field test as described by Zhu *et al.* (2001) with little modification by Brown *et al.* (2004) on days 3 and 10.

(ii) Escapable foot-shock as described by Zhu *et al.* (2001) and Ambali *et al.* (2010) on days 13 and 14.

(iii) Excitability score test as described by Maria *et al.* (2002) with little modification by Ayo *et al.* (2006) and Adeiza and Minka (2010) on day 14.

(iv) Incline plane performance task as described by Petrich (2006) and Ambali *et al.* (2010) and forepaw grip time as described by Abou Donia *et al.* (2001) and Ambali *et al.* (2010) on days 1, 7 and 14.

3.6 EVALUATION OF NEUROBEHAVIOURAL RESPONSES

All assessments were conducted in fully wake condition between the hour of 9:00 - 16:00 hour.

3.6.1 Assessment of Behavioural Response in Open-Field Apparatus

The effects of lycopene on locomotion, exploratory activity and anxiety behaviour of each rat were studied using open-field test as described by Zhu *et al.* (2001) with little modification, that is a central square in the middle of the open-field apparatus as described by Brown *et al.* (2004). Briefly, the open-field apparatus was constructed using a cardboard box (50 cm × 50 cm × 46 cm high) with clear Plexiglas on the inner surface. The floor was divided into 24 equal squares with a central square (18 cm × 18 cm), drawn in the middle of the open-field. Each rat was placed in the central square and observed for 5 minutes and the following behaviours were recorded during the next 3 minutes after initial 2 minutes of habituation: Ambulation: the number of grid lines it crossed with all the four paws (ii) Central square frequency: frequency of entering into the central square with all four paws

(iii) Central square duration: time spent in the central square. (iv) Rearing: the number of times the animal stood on its hind limbs (v) Grooming: number of times the animal made the following responses: grooming of the face, licking/cleaning and scratching the various parts of the body (vi) Stretch-attend postures: the number of times there is forward elongation of the head and shoulders (head is usually low to the platform) followed by retraction to the original position (vii) Defaecation and urination: the number of faecal boli excreted and urine streak produced respectively, and (viii) Immobilisation (freezing): It was recorded to have occurred, when a rat had its eyes open, holding its head against the gravity, but without any head, body or limb movement. Between tests, the apparatus was cleaned with soapy water followed by 70 % ethanol to avoid interfering odours of the previous rat.

3.6.2 Assessment of Cognitive Ability

The effect of lycopene on cognitive ability of each rat was assessed via learning and short-term memory test as described by Zhu *et al.* (2001) and Ambali *et al.* (2010). Briefly, learning test was conducted 48 hours (on day 13) to the termination of the study using step-down inhibitory avoidance learning test. The apparatus used for the learning test was an acrylic chamber (40 × 25 × 25 cm), consisting of a floor made of parallel 2-mm-calibre stainless steel bar, spaced 1 cm apart. An electric shock was delivered through the floor bars. Each animal was gently placed on the platform. Upon stepping down, the rat immediately received a single 80-volt foot shock. For rat that did not return to the platform, the foot shock was repeated every five seconds. A rat was considered to have learned the avoidance task, if it remained on the platform for more than two minutes. The number of foot-shocks was recorded as an index of learning acquisition. Short-term memory was

assessed in individual rats from each group using step-down inhibitory avoidance learning test, 24 hours (on day 14) after the assessment of learning. The apparatus used for the memory test was the same as that used for the assessment of learning. In this test, each rat was again placed gently on the platform. The time that the rat remained on the platform was recorded as the index of memory retention. Staying on the platform for two minutes was counted as maximum memory retention (ceiling response).

3.6.3 Assessment of Excitability Score

The excitability score of each rat was assessed using excitability score test as described by Maria *et al.* (2002) with little modification by Ayo *et al.* (2006) and Adeiza and Minka (2010). Briefly, each rat was held by the tail upside down and kept in such position for 30 seconds. The level of reaction of the animal was graded and scored as follows: (i) Score 1 (calm): Rat did not show any sign of wriggling and paw movement. (ii) Score 2 (occasional shakes): Rat responded through gentle wriggling and movement of forepaw. (ii) Score 3 (repeated shakes): Rat responded through stronger wriggling and movement of forepaw. (iv) Score 4 (violent shakes): Rat responded through vigorous wriggling, strong movement of fore- and hind limb as well as successfully climbed the tip of its tail.

3.6.4 Assessment Of Neuro-Muscular Coordination

This was done by assessing each rat in all the group using incline plane performance task as described by Petrich (2006) and Ambali *et al.* (2010). The apparatus consisted of: rough wooden plank with thick foam pad at its bottom end, a vertical wooden bar with several notches (angles were measured and marked on them) and an iron rod. The apparatus set-up was obtained by propping the wooden plank on the iron rod attached to the vertical wooden

bar. The plank was initially raised to an inclination of 35^0**,** and thereafter gradually increased stepwise by 5^0 until the subject could no longer stay (without sliding down) on the plank for 3 seconds. The task was performed with the head of the rat facing left- and then right-hand side of the experimenter. The highest angle at which each rat stayed and stood horizontally facing each direction was recorded. Two trials were performed for each testing period.

3.6.5 Assessment of Motor Strength

This was evaluated from the forepaw grip time (Abou-Donia *et al.*, 2001) and Ambali *et al.* (2010) by having each rat hung down from a 5-mm diameter wooden dowel gripped with both forepaws. The time spent by each rat before releasing the grip was recorded in seconds.

3.7 BLOOD AND BRAIN COLLECTION

On 15^{th} day (that is, 24 hours after the last test was conducted), each of the overnight fasted rat was sacrificed using light ether anaesthesia and blood was collected via cardiac puncture into heparinized sample bottles for haematological analysis. The brains were removed, forebrain dissected out and cerebellum discarded. They were weighed and then homogenized immediately in a known volume of ice-cold phosphate buffer. The supernatants were separated by centrifugation at 2,000 × *g* for 5 min and stored in ice-pack until assay was done.

3.8 DETERMINATION OF BRAIN BIOCHEMICAL PARAMETERS

Malondialdehyde concentration, activities of superoxide dismutase and catalase in the supernatant obtained from the brain samples were analyzed using commercial kits

(Northwest Life Science Specialties, LLC, Vancouver, U.S.A.). Electrolytes concentration were determined using an auto-analyser (Bayer Express Plus, Bayer, Germany. Serial number: 15950).

Cholinesterase activity was also determined in the supernatant obtained from the brain samples as described by Ellman *et al.* (1961) using acetylthiocholine iodide (30 µl final concentration = 0.5 mmol) as substrate and 5,5-dithiobis-2-nitrobenzoeic acid (DTNB; 200 µl; final concentration = 0.33 mmol). Assay tubes were completed to 1 ml with sodium phosphate buffer (pH 8) as described by Canadas *et al.* (2005). The emzyme activity was calculated relative to protein concentration.

3.9 DETERMINATION OF HAEMATOLOGICAL PARAMETERS

Blood samples obtained from each rat in an heparinized sample bottles was used for analysis of the following haematological parameters:

3.9.1 Erythrocytes malondialdehyde concentration

2.5 ml of heparinized blood sample obtained from each animal was centrifuged at 4000 × g for 5 minutes and plasma discarded. Washing of erythrocytes three times in cold isotonic saline (0.9% w/v) was done to obtain erythrocyte packets which was used to analyze for MDA concentrations using commercial kits (Northwest Life Science Specialties, LLC, Vancouver, U.S.A.).

3.9.2 Other haematological parameters

Determination of the following haematological parameters was conducted as described by Schalm *et al.* (1975): packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell count (RBC), white blood cell count (WBC), differential leucocyte count (DLC).

Total protein concentration was determined from blood plasma as described by Knowles *et. al.,* (1993).

3.9.3 Erythrocytes osmotic fragility

Determination of erythrocyte osmotic fragility was based on method described by Faulkner and King (1970) and modified by Oyewale (1992). It is an indirect method of determination of oxidative stress status**.** Briefly, 1 ml of freshly obtained blood collected into heparinized sample bottles from each rat was pipette into a set of test tubes containing 0.0, 0.1, 0.3, 0.5, 0.7, 0.9 g/dl of sodium chloride stock solution (pH 7.4). This was followed by careful mixing and incubation for 30 min at room temperature (26-28°C). Thereafter, the test tubes was centrifuged at 800 × g for 10 min using a centrifuge IEC HN-SH (Damon/IEC Division, UK). The supernatant was transferred into a glass cuvette. The concentration of haemoglobin in the supernatant was measured colorimetrically by reading the absorbance at a wavelength of 540 nm using a spectrophotometer (Jenwey, 6405, Japan). The percentage haemolysis was calculated using the following formula:

$$\text{Percentage haemolysis} = \frac{\text{Optical density of test solution}}{\text{Optical density of standard water}} \times 100$$

3.10 STATISTICAL ANALYSES

Values obtained were expressed as mean ± SEM. Data were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison *post-hoc* test. The program used for the analysis was GraphPad Prism 4.0 Version from GraphPad Software, San Diego, U.S.A. Values of $P < 0.05$ were considered significant.

CHAPTER 4
RESULTS

4.1 EFFECTS OF LYCOPENE ADMINISTRATION ON NEUROBEHAVIOURAL RESPONSES OF WISTAR RAT

4.1.1 Behavioural Response in Open-Field Apparatus

Behavioural responses of Wistar rats as observed in open-field apparatus on the 3rd and 10th day of lycopene administration showed that the antioxidant did not exert any significant effect on most of the parameters. Although, there was increased immobilisation ($P < 0.05$) and stretched-attend posture ($P < 0.01$) in the S+Lyco group, compared to the S-Control group on the 3rd day. The increase disappeared by 10th day of lycopene administration. In the open-field exploration, lycopene administration for 10 days elicited significant increase in central activity in the S+Lyco group compared to the S-Control group, as evidenced by increase in centre square frequency ($P < 0.001$) and duration ($P < 0.001$). Although no significant effect were observed between the two groups at 10th day of lycopene administration, there was 10 % and 20 % increases in ambulation and rearing frequency, respectively, in the S+Lyco group compared to the S-Control group (Table 4.1).

4.1.2 Cognitive Ability Analysis

Learning acquisition (13th day), as measured by the number of times a rat received foot shock, was significantly ($P < 0.01$) higher in the S+Lyco group compared to the S-Control group (Figure 4.1). The duration a rat stayed on the platform, a measure of memory retention (on 14th day), were not significantly different between S+Lyco group and S-Control group. However, the duration of the period rats stayed on the platform was 12.5 % higher in the S+Lyco group compared to the S-Control group (Figure 4.2).

Table 4.1: Effects of lycopene administration on behavioural responses of Wistar rats in open-field apparatus (n = 7). Unit for freezing and CSD are in seconds, while others are in number of times. *P < 0.05, **P < 0.01,***P < 0.001.

Parameters	DAY 3		DAY 10	
	S-Control	S+Lyco	S-Control	S+Lyco
Line Crossing	26.43 ± 4.95	25.43 ± 7.31	19.57 ± 4.52	24.86 ± 6.43
Central Square Frequency (CSF)	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.14	1.86 ± 1.79***
Central Square Duration (CSD)	0.00 ± 0.00	0.00 ± 0.00	0.43 ± 0.43	2.86 ± 2.86***
Rearing Frequency	6.43 ± 1.67	6.57± 1.66	4.86 ± 1.53	9.43 ± 2.85
Grooming	2.86 ± 0.88	2.00 ± 0.62	3.14 ± 0.71	2.43 ± 0.78
Stretched-Attend Posture	21.43 ± 1.11	25.14 ± 3.94**	14.29 ± 2.27	19.14 ± 2.83
Urination	0.57 ± 0.20	0.57 ± 0.30	0.14 ± 0.14	0.29 ± 0.18
Defeacation	3.14 ± 0.91	4.14 ± 0.80	4.43 ± 0.84	3.43 ± 1.11
Immobilization (freezing)	4.86 ± 2.65	13.14 ± 6.18*	12.29 ± 5.10	7.71 ± 3.55

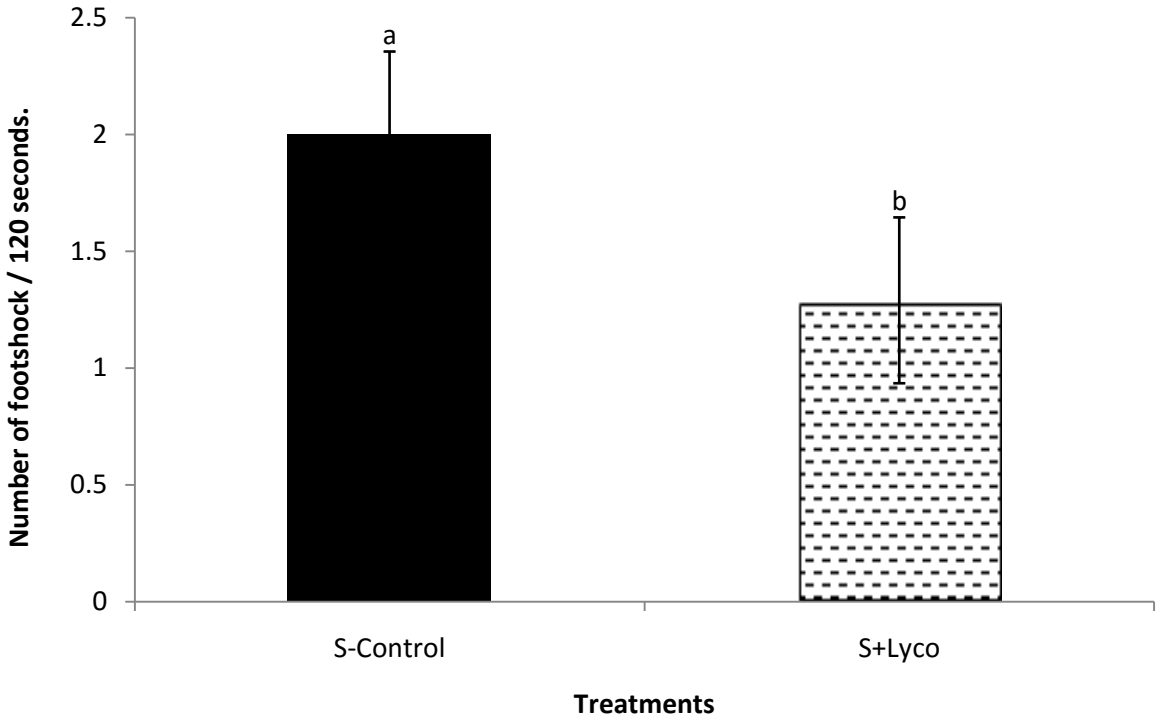

Figure 4.1: Effects of lycopene administration on learning in Wistar rats (n = 7). [a,b] P-Values with different superscript letters are significantly ($P < 0.01$) different.
S-Control = Stressed control; S+Lyco = Stressed + Lycopene

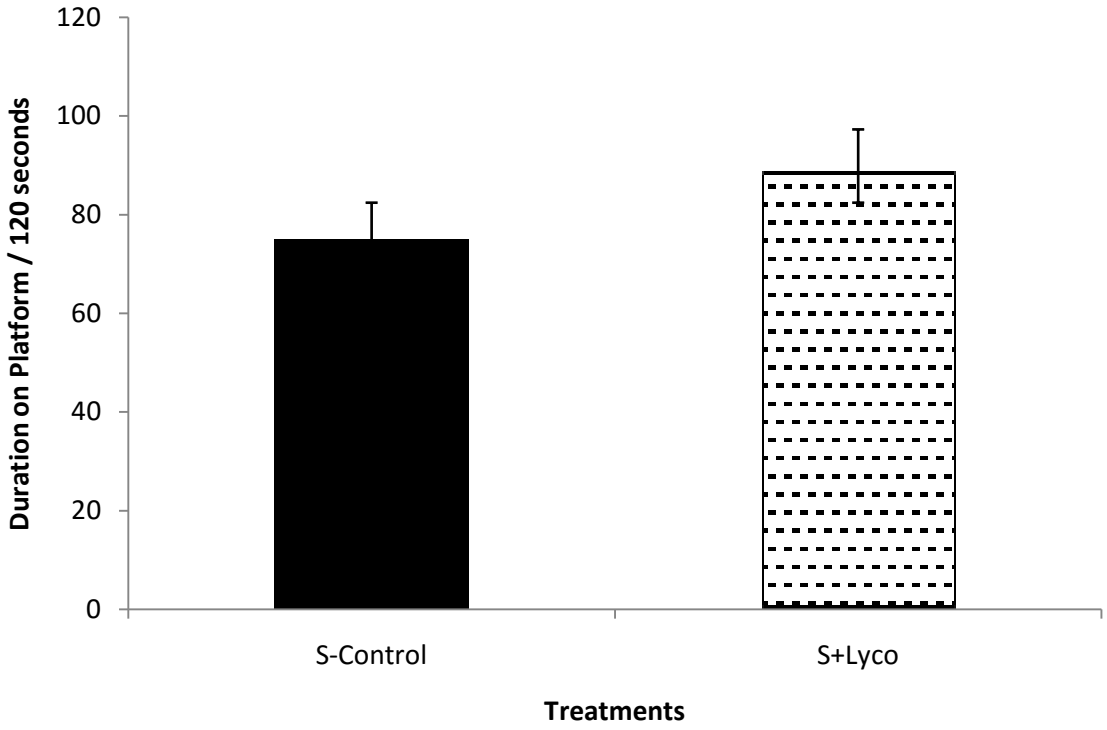

Figure 4.2: Effects of lycopene administration on memory in Wistar rats (n = 7).
S-Control = Stressed control; S+Lyco = Stressed + Lycopene

4.1.3. Excitability Score Analysis

Effects of lycopene on excitability score of each rat on 14th day of the administration are shown Figure 4.3. None of the rats in the S+Lyco group had excitability score of one (1), while 14.3 ± 0.7 % of those in the S-Control group had excitability score of one. Excitability score of two (2) was recorded in 57.1 ± 2.9 % of the rats in S-Control group, and the value was significantly ($P < 0.001$) higher than that of 42.9 ± 2.2 % recorded for rats in the S+Lyco group. 42.9 ± 2.2 %, of rats in the S+Lyco group had excitability score of three (3), and the value was significantly ($P < 0.001$) higher than 14.3 ± 0.7 % recorded in the S-Control group. 14.3 ± 0.7 % of rats in each of the groups had excitability score of four (4).

4.1.4 Neuro-Muscular Coordination

Neuro-muscular coordination was measured by the angle at which the animal slipped off the inclined plane apparatus. The dynamics indicates that rats in the S+Lyco group showed a decrease in angle of slip from day 0 up to day 7, and then followed by an increase up to day 14. However, the S-Control group showed an increase in angle of slip from day 0 up to day 7, and then followed by an decrease up to day 14. For the 14 days of lycopene administration, a significant ($P < 0.05$) increase in angle of slip was obtained in rats administered with lycopene compared to that of the control group (Figure 4.4a and 4.4b).

4.1.5. Motor Strength

Motor strength as measured by the time spent by each rat before releasing their grip from the wooden dowel are shown in Figure 4.5a and 4.5b. The dynamics indicates that rats in the S+Lyco and S-Control groups showed a decrease in grip time from day 0 up to day 7,

and then followed by an increase up to day 14. For the 14 days of lycopene administration, the grip time of rats showed no significant difference between S+Lyco group and S-control group.

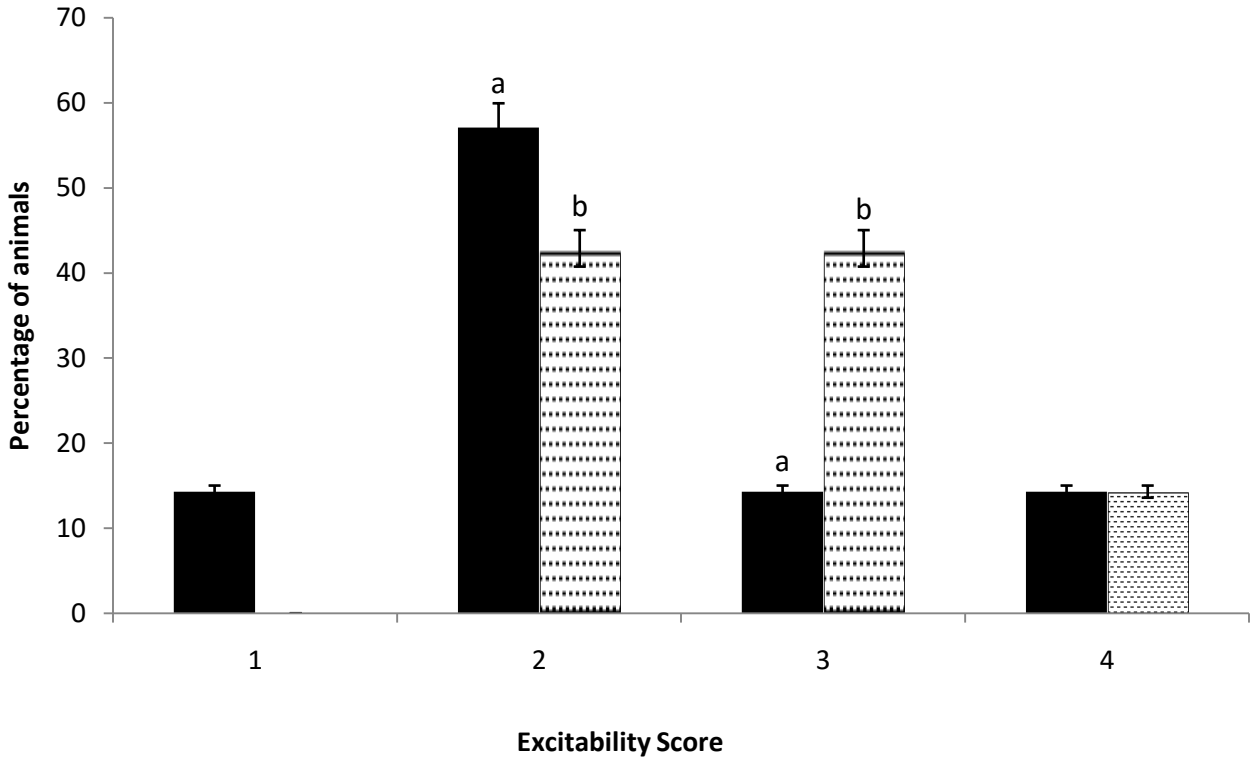

Figure 4.3: Effects of lycopene administration excitability score in Wistar rats (n = 7). [a,b] P-Values with different superscript letters are significantly (P < 0.001) different. S-Control = Stressed control; S+Lyco = Stressed + Lycopene

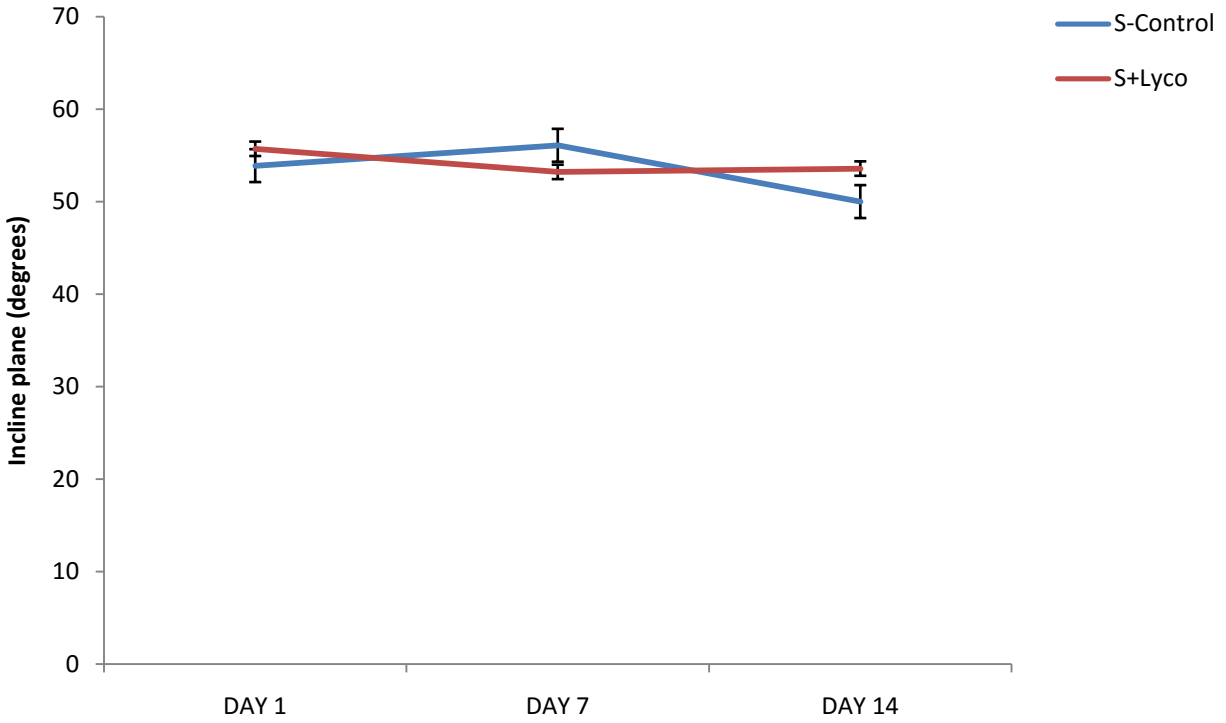

Figure 4.4a: Effects of lycopene administration on dynamics of neuro-muscular coordination in Wistar rats

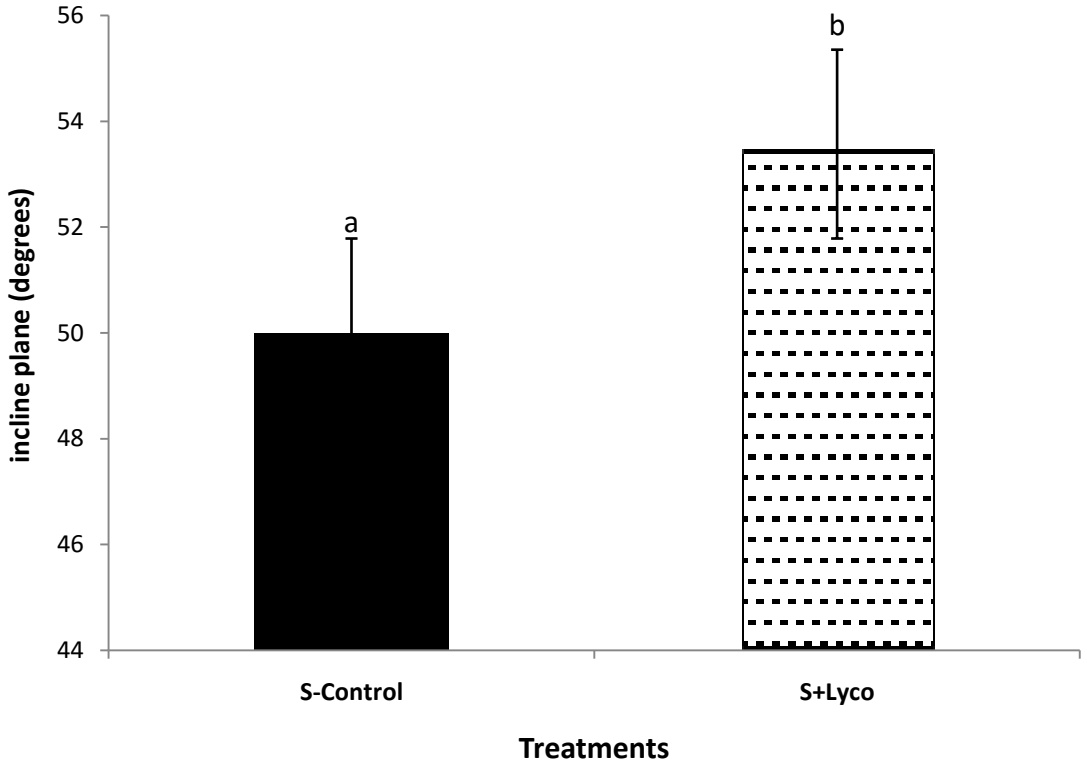

Figure 4.4b: Cumulative effects of lycopene administration on incline-plane performance in Wistar rats at day 14 (n = 7).
[a,b] P-Values with different superscript letters are significantly ($P < 0.05$) different. . S-Control = Stressed control; S+Lyco = Stressed + Lycopen

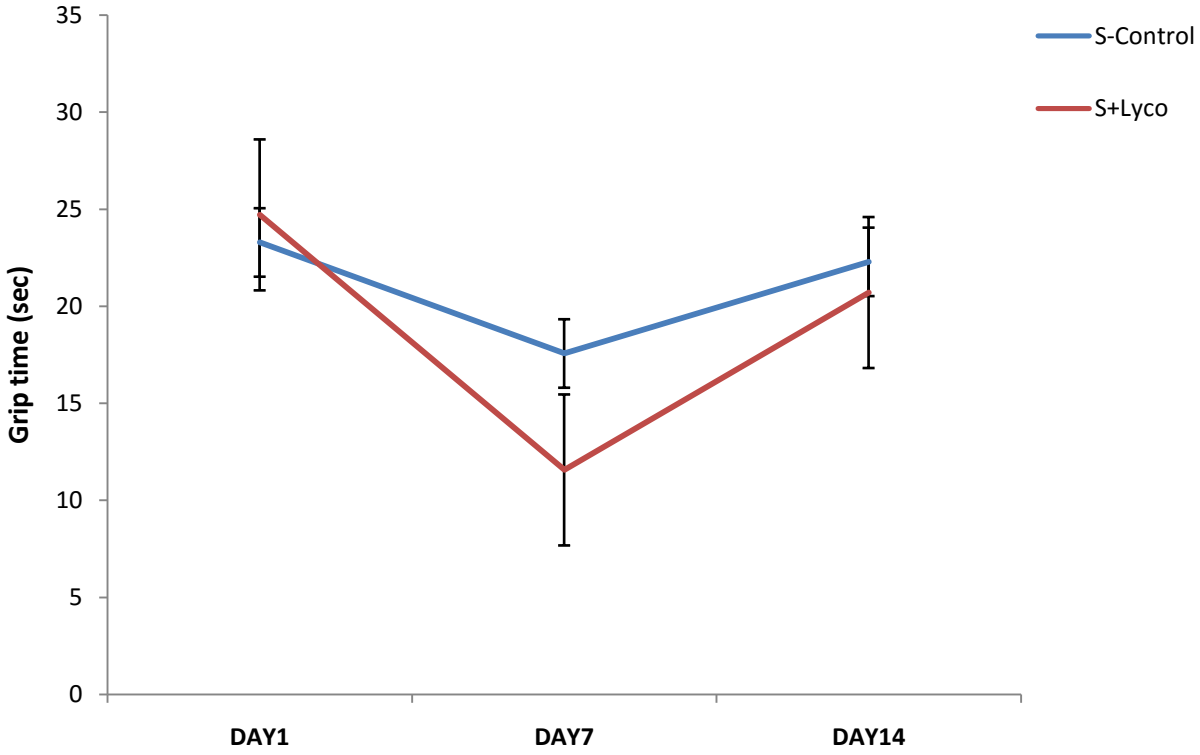

Figure 4.5a: Effects of lycopene administration on dynamics of motor strength in Wistar rats

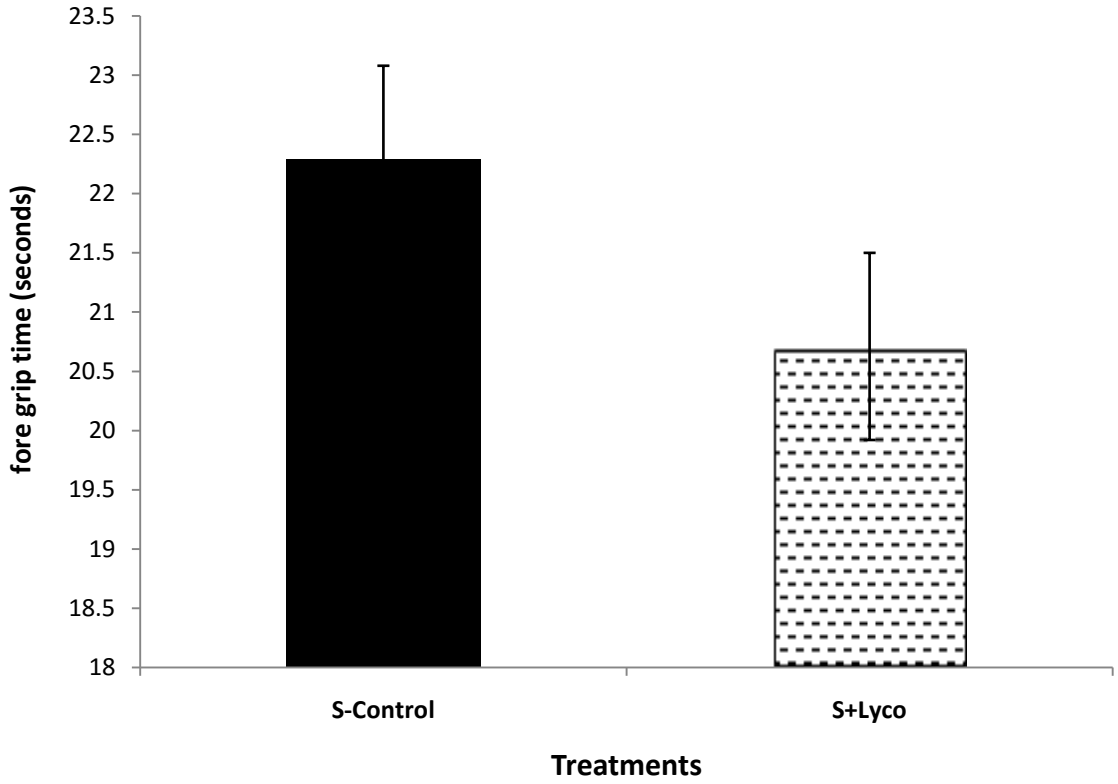

Figure 4.5b: Cumulative effects of lycopene administration on fore-grip time in Wistar rats at day 14 (n = 7).
S-Control = Stressed control; S+Lyco = Stressed + Lycopene

4.2. EFFECTS OF LYCOPENE ADMINISTRATION ON BRAIN BIOCHEMICAL PARAMETERS OF UNSTRESSED AND PSYCHOLOGICALLY STRESSED WISTAR RATS.

4.2.1. Brain Lipoperoxidation

Comparison between the unstressed (that is, U-Control and U+Lyco) and stressed (S-Control and S+Lyco) groups, indicate a significant decreases in MDA concentration in the brain of S+Lyco rats compared to those obtained in U-Control ($P < 0.01$) and U+Lyco ($P < 0.001$) groups. Within the stressed group, a significant decrease in MDA concentration in the brain was recorded in S+Lyco rats ($P < 0.05$) when compared to that of the S-Control. However, there was no significant difference in concentrations obtained within the unstressed groups (Table 4.2).

4.2.2. Superoxide Dismutase

There was a significant decrease in SOD activity in the brain of S-Control ($P < 0.01$) and S+Lyco ($P < 0.01$) rats in comparison to that of the U-Control group. However, within the unstressed and stressed groups, there was no significant difference in SOD activities (Table 4.2).

4.2.3. Catalase

Comparison between the unstressed and stressed groups showed a significant decrease in CAT activity in the brain of S-Control rats in comparison to those of U-Control ($P < 0.05$) and U+Lyco ($P < 0.001$) groups. Similarly, significant decrease in CAT activity in the brain of S+Lyco rats was recorded when compared to U-Control ($P < 0.05$) and U+Lyco ($P < 0.001$) groups. However, within the unstressed groups and the stressed groups, there was no significant difference in CAT activity (Table 4.2).

4.2.4 Brain Electrolytes Activity

No significant difference was recorded in the brain Sodium, Potassium and Calcium concentrations of all the group examined (Figure 4.6a, b, d). However, a significant ($P < 0.05$) increase in the brain Chloride concentration was recorded in S+Lyco compared to S-Control (Figure 4.6c).

4.2.4 Acetylcholinesterase Activity

No significant difference was recorded in the brain acetylcholinesterase activity of all the groups examined, both between and within the unstressed and stressed groups (Figure 4.7).

Table 4.2: Effects of lycopene administration on oxidant and some endogenous antioxidants activities in the brain of unstressed and psychologically stressed Wistar rats (n=5). [a,b] Values with different superscript are significantly different.

Parameters	U-Control	U+Lyco	S-Control	S+Lyco
MDA (nmol/mg of protein)	1.91 ± 0.11^{a}	2.16 ± 0.11^{a}	1.83 ± 0.07^{a}	1.41 ± 0.08^{b}
SOD (IU/mL of protein)	2.21 ± 0.11^{a}	1.91 ± 0.15	1.50 ± 0.12^{b}	1.50 ± 0.11^{b}
CAT (IU/mL of protein)	60.29 ± 2.63^{a}	66.00 ± 1.90^{a}	49.29 ± 2.54^{b}	48.43 ± 2.88^{b}

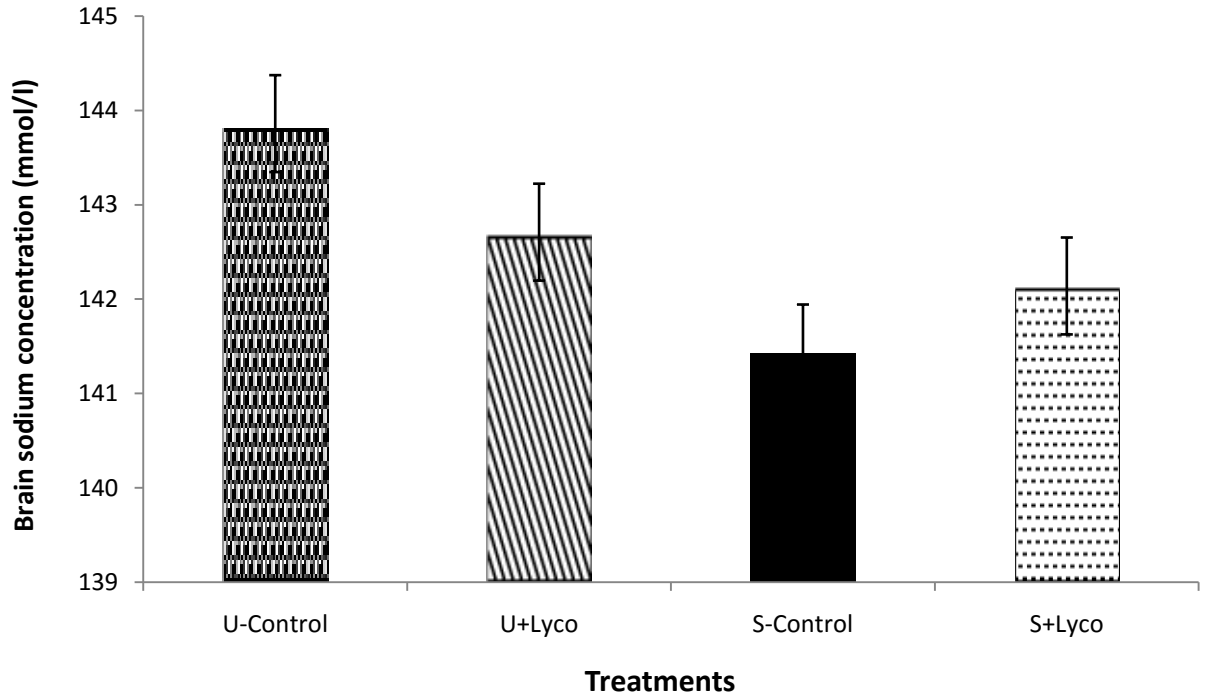

Figure 4.6a: Effects of lycopene administration on brain sodium concentration in unstressed and psychologically stressed Wistar rats (n = 7). U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

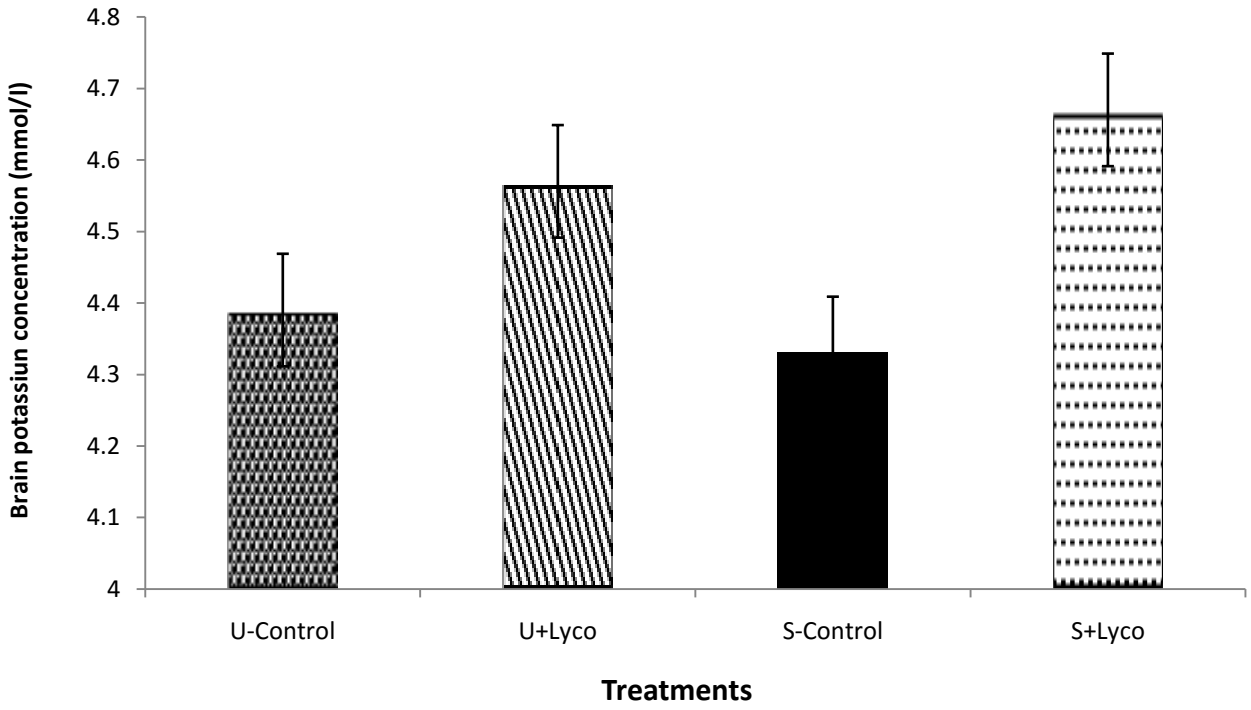

Figure 4.6b: Effects of lycopene administration on brain potassium concentration in unstressed and psychologically stressed Wistar rats (n = 7). U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

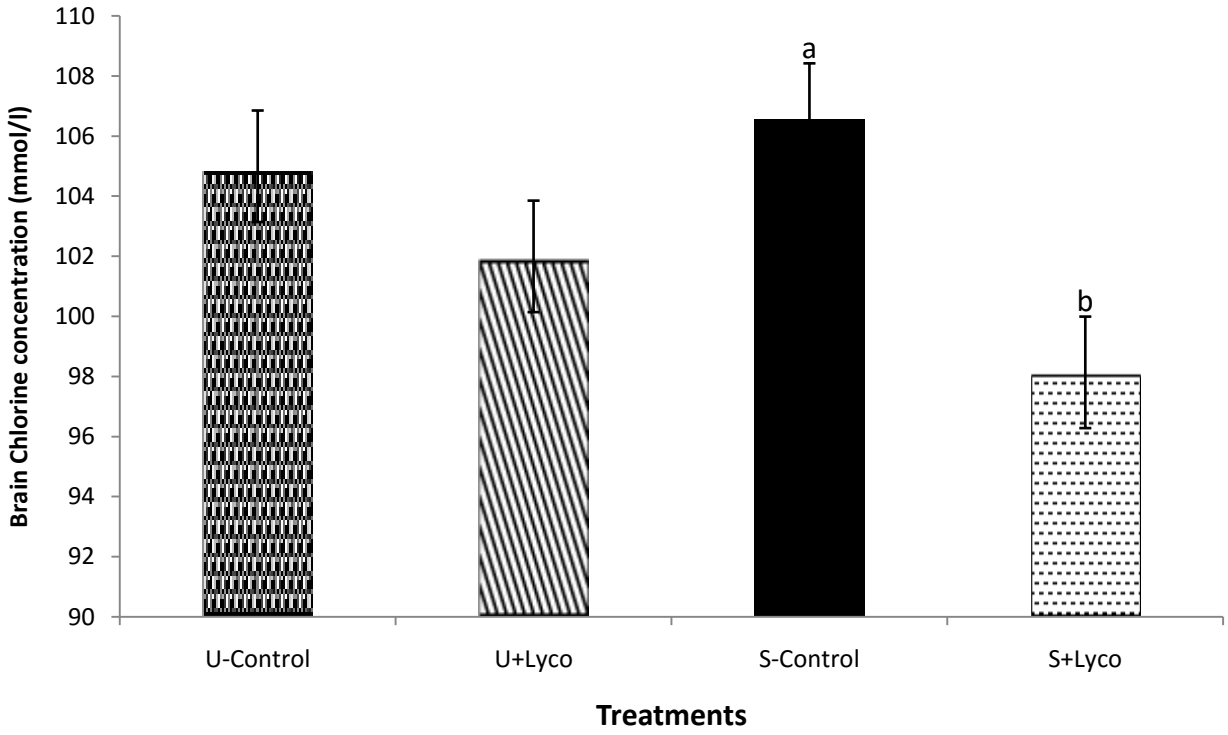

Figure 4.6c: Effects of lycopene administration on brain chloride concentration in unstressed and psychologically stressed Wistar rats (n =7). [a,b] P-Values with different superscript letters are significantly ($P < 0.05$) different. U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

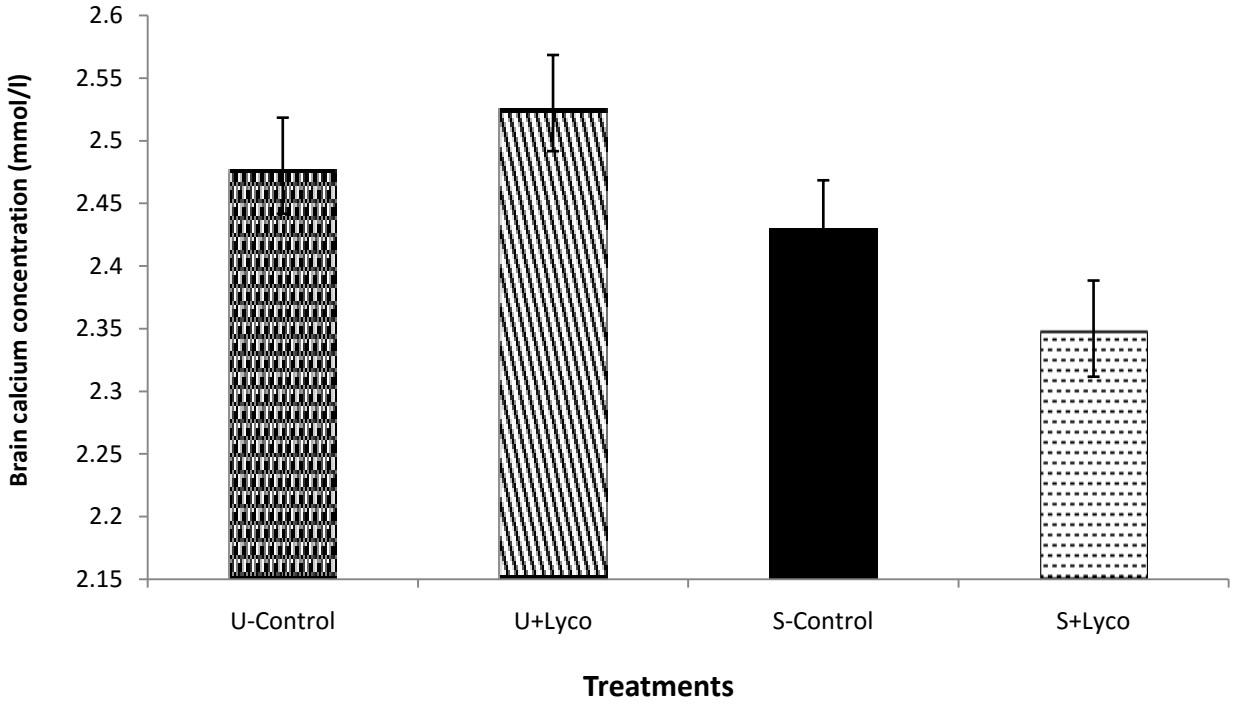

Figure 4.6d: Effects of lycopene administration on brain calcium concentration in unstressed and psychologically stressed Wistar rats (n = 7). U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

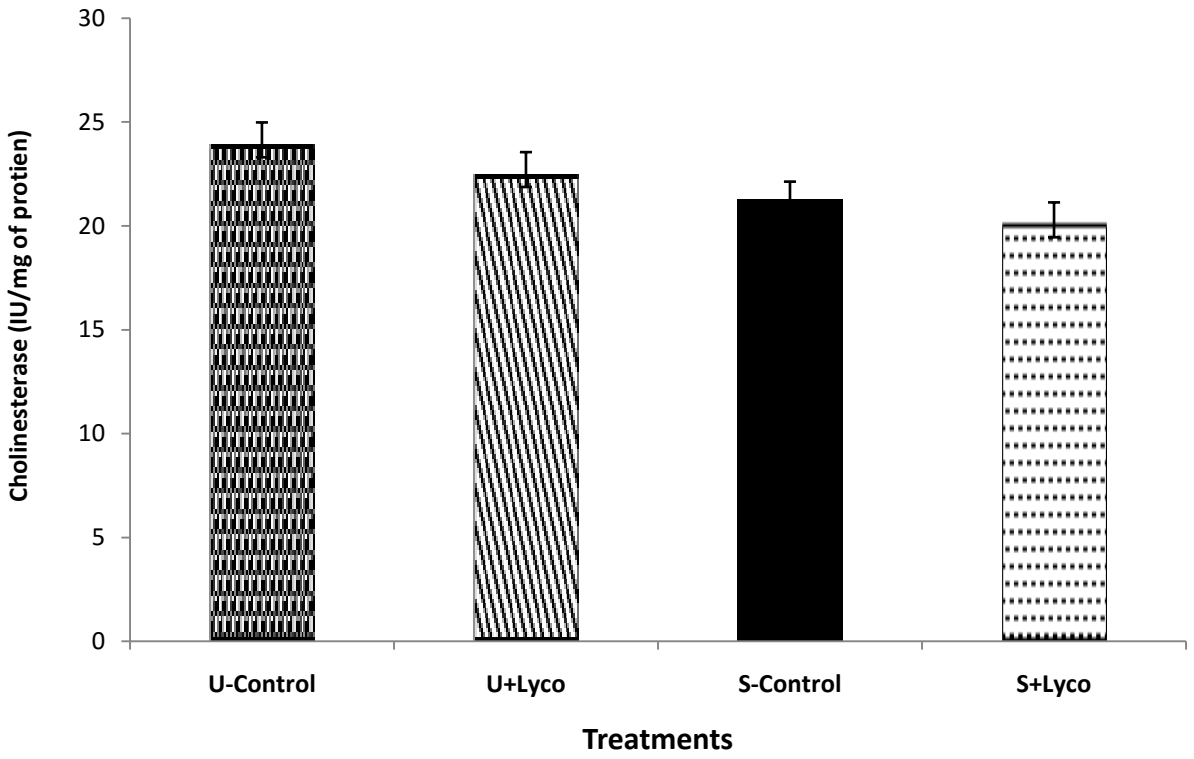

Figure 4.7: Effects of lycopene administration on acetylcholinesterase activity in the brain of unstressed and psychologically stressed Wistar rats (n = 5). U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

4.3. HAEMATOLOGICAL PARAMETERS OF UNSTRESSED AND PSYCHOLOGICALLY STRESSED WISTAR RATS ADMINISTERED WITH LYCOPENE.

4.3.1 Erythrocytes malondialdehyde concentration

There was no significant difference in the values obtained when U-Control and U+Lyco groups were compared. However, the erythrocytes MDA concentration of U-Control group was significantly ($P < 0.01$) lower than that of S-Control group (Figure 4.8).

4.3.2 Other haematological parameters

There was no significant difference in PCV, Hb, RBC, WBC, Neut, Lym, Mon, Eos, Band as well as Lym : Neut ratio values both between and within groups (Table 4.3 and 4.4). However, TP value was significantly ($P < 0.05$) increased in the S+Lyco group compared to S-Control (Table 4.3).

4.3.3 Erythrocyte osmotic fragility

Significant ($P < 0.05$) changes in percentage erythrocyte fragility were recorded at 0.7, 0.5 and 0.3 % of NaCl. There was 100 % haemolysis at 0.0 % of NaCl (distilled water). No significant changes in erythrocyte fragility were observed at 0.1 % of NaCl. Comparison between U-Control and U+Lyco groups showed that U+Lyco group was lower than that of U-Control group at 0.3 % of NaCl. No significant changes in erythrocyte fragility was observed when S-Control and S+Lyco groups were compared. A significant increase was recorded in S+Lyco group when compared with U+Lyco group at 0.5 and 0.7 % of NaCl. Similarly, significant increase was recorded in S-Control group, when compared with U-Control group at 0.7 % of NaCl (Figure 4.9).

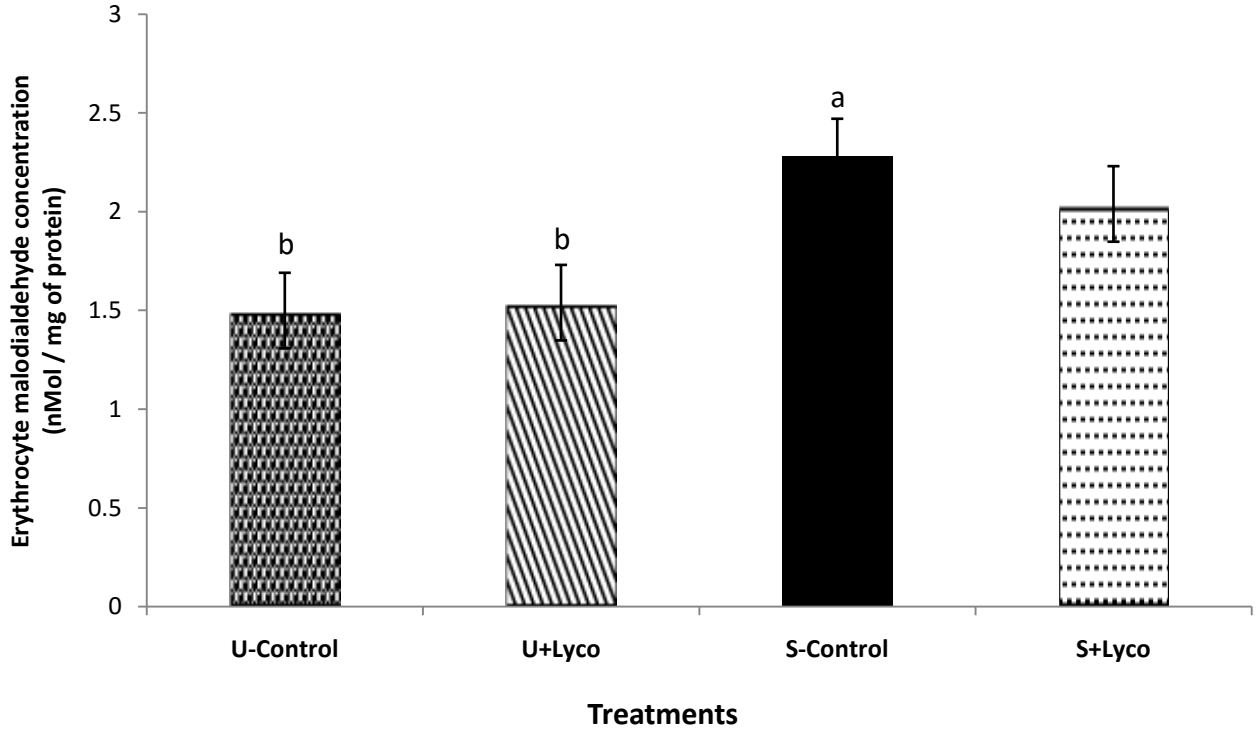

Figure 4.8: Effects of lycopene administration on erythrocyte malondialdehyde concentration of unstressed and psychologically stressedWistar rats (n = 5). [a,b] P-Values with different superscript letters are significantly ($P < 0.05$) different. U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

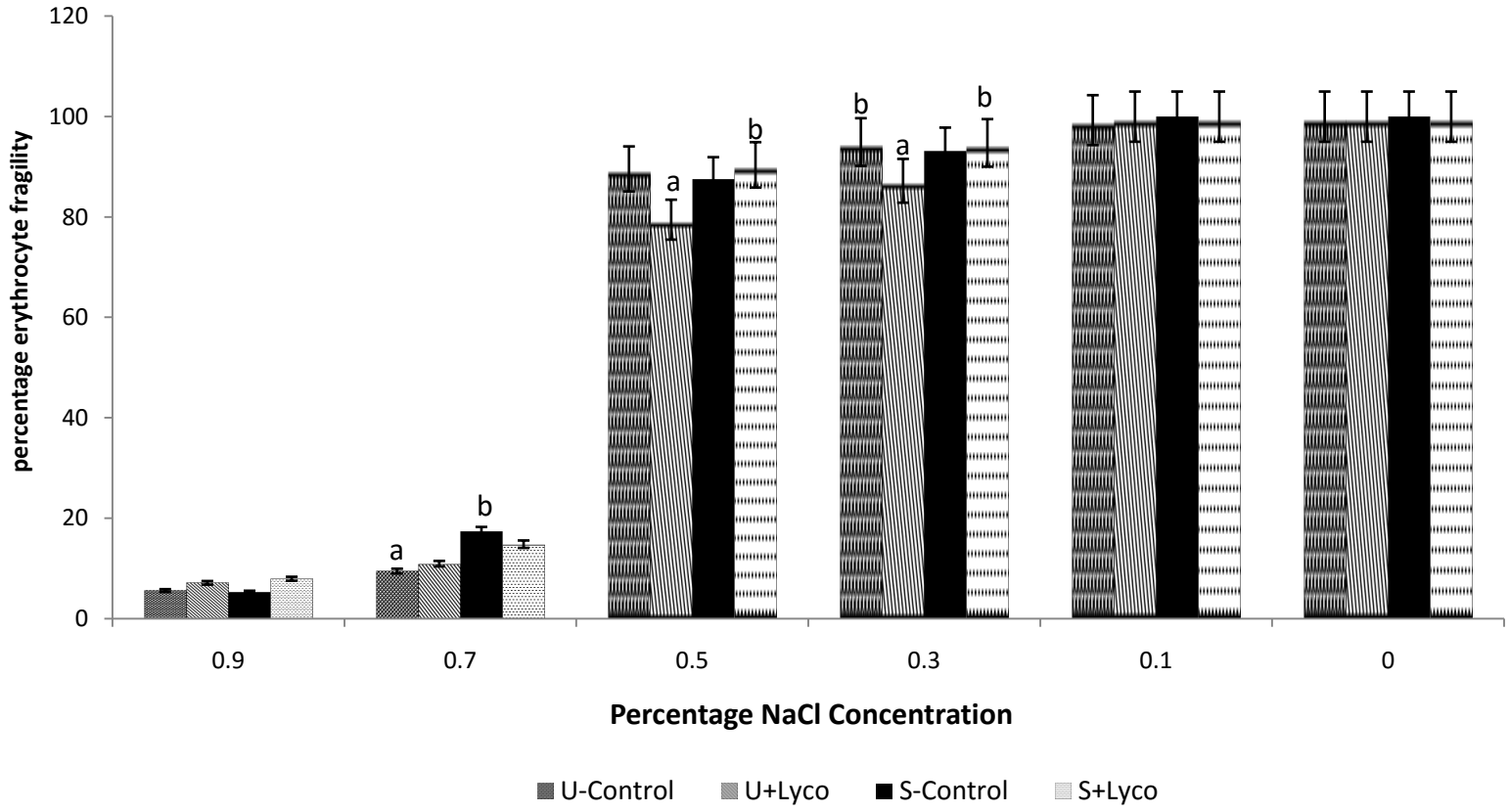

Figure 4.9: Effects of lycopene administration on erythrocyte osmotic fragility in of unstressed and psychologically stressed Wistar rats (n = 5). [a,b] P-Values with different superscript letters are significantly ($P < 0.05$) different. U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

Table 4.3: Effects of lycopene administration on some erythrocytes parameters and total protein of unstressed and psychologically stressed Wistar rats (n=5). [a,b] Values with different superscript are significantly ($P < 0.05$) different. U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

Parameters	U-Control	U+Lyco	S-Control	S+Lyco
PCV (%)	50.40 ± 1.94	49.40 ± 1.44	52.60 ± 0.51	53.20 ± 0.92
Hb (g/dl)	16.78 ± 0.65	16.44 ± 0.49	17.50 ± 0.17	17.72 ± 0.30
RBC ($\times 10^{12}/\mu L$)	8.20 ± 0.27	8.10 ± 0.25	8.72 ± 0.15	8.84 ± 0.16
TP (g/dl)	6.48 ± 0.19	6.44 ± 0.15	6.36 ± 0.19[a]	7.00 ± 0.06[b]

Table 4.4: Effects of lycopene administration on leucocytes counts of unstressed and psychologically stressed Wistar rats (n=5). U-Control = Unstressed control; U+Lyco = Unstressed + Lycopene; S-Control = Stressed control; S+Lyco = Stressed + Lycopene

Parameters	U-Control	U+Lyco	S-Control	S+Lyco
WBC ($\times 10^3/\mu L$)	18.68 ± 2.00	18.08 ± 1.30	17.14 ± 2.34	18.08 ± 0.81
Neut ($\times 10^3/\mu L$)	19.60 ± 4.10	20.60 ± 2.50	20.00 ± 0.84	23.80 ± 3.25
Lym ($\times 10^3/\mu L$)	81.80 ± 5.52	76.20 ± 3.28	73.80 ± 1.32	78.20 ± 3.26
Mon ($\times 10^3/\mu L$)	0.00 ± 0.00	1.20 ± 0.49	2.00 ± 0.55	1.80 ± 0.49
Eos ($\times 10^3/\mu L$)	0.60 ± 0.60	1.80 ± 0.49	2.80 ± 0.37	1.40 ± 0.60
Band ($\times 10^3/\mu L$)	0.00 ± 0.00	0.80 ± 0.37	1.40 ± 0.40	1.00 ± 0.45
N/ L Ratio	0.26 ± 0.08	0.28 ± 0.04	0.27 ± 0.01	0.30 ± 0.04

CHAPTER 5
DISCUSSION

The open-field test provides simultaneous measures of locomotion, exploration and anxiety. In this study, exposure to the open-field apparatus following three days of lycopene administration showed increased anxiety as evidenced by significant increase in stretch-attend postures and immobilisation. This agrees with the report of Blanchard *et al.* (2001) and Brown *et al.* (2004) that a high frequency of stretch-attend postures, a "risk assessment" behavioural parameter signifying that an animal is hesitant to move from its present location to a new position, indicates high level of anxiety. It also agrees with the findings that increased immobilisation (freezing behaviour) indicates high level of anxiety (Menon and Dandiya, 1969; Sinha and Ray, 2004). In this study, three days of lycopene administration did not confer any positive effect following exposure to the novel-environment of the open-field apparatus. This may requires further investigation. However, lycopene reduces anxiety and increases exploratory behaviour in Wistar rats following 10 days of administration prior to exposure to the open-field apparatus. This is evidence by increase in central square entries and the duration of time spent in the central square of the open-field apparatus. This result agrees with the report by Brown *et al.* (2004) that a high frequency/duration of the behavioural parameters indicates high exploratory behaviour and low anxiety level. From the observation in the open-field apparatus, lycopene showed anxiolytic effect following 10 days of its administration. This may be attributed to one of its anti-stress properties which enhances post-inhibitory synaptic functions, and perhaps GABAergic activity in the critical neurons, responsible for the regulation of fear/anxiety response. The GABAergic system is responsible for the motivation of fear/anxiety-related response (Kelvens, 1997). Gamma amino butyric acid (GABA), the major neurotransmitter

for fast inhibitory synaptic transmission (Olsen, 2002), functions by triggering the opening of GABA-gated chloride channels ($GABA_A$ receptors) that in turn leads to the inhibition of body muscles (Schofield et al., 1987; Jorgensen, 2005). The result of the present study further agrees with similar findings on benzodiazepine derivatives, such as diazepam, whose anxiolytic effect is due to its anti-stress property (Nirmal, 2008), and probably potentiation of GABA actions (Haefely, 1994; Olsen, 2002).

The present study demonstrated the role of lycopene in fear learning and short-term memory in Wistar rats. Rats administered with lycopene learnt significantly faster and had relatively higher short-term memory retention compared to that of the control. This finding may be attributed to strong antioxidant property of lycopene (Atessahin *et al.,* 2005; Kumar and Kumar, 2009). Fear is a potent psychological stressor, capable of provoking the sympatho-adrenal system and leading to physiological changes which in turn result in increased oxidative stress (Maier and Watkin, 1998; Laur, 2002). Oxidative damage caused by free radicals have been linked to neurodegenerative diseases (Cantuti-Castelvetri *et al.,* 2000; Atessahin *et al.,* 2007), including learning and memory impairment (Kuhad *et al.,* 2008; Kumar and Kumar, 2009).

The excitability score test exploits the reaction of the animal to handling, and on this basis the level of reaction can be graded or scored (Maria *et al.,* 2002; Ayo *et al.,* 2006; Adeiza and Minka, 2010). The present study showed that none of the rat administered lycopene had score of 1, instead majority obtained the score of 2 and 3, with greater percentage having score of 3. This indicates that lycopene increased performance of Wistar rats, probably via enhancement of GABAergic system. Potentiating of GABA action have been shown to decrease cortisol action (Liakakos *et al.,* 1974; Brikas, 1994), which otherwise

favour body activities leading to excess free radical production. The ability of excitable membrane (such as nerves and muscles) to generate action potential may reflects a greater level of performance. Free radical have been shown to decrease viability and integrity of nerve cell membrane that in turn hampers action potential generation (Halliwell, 2006; Friedman, 2010).

Poor neuromuscular coordination observed in the S-Control group in this study may be due to induction of oxidative stress by the animal's natural fear of falling response. Indeed, psychological stressors such as fear has immense ability to provoke acute stress reaction (Maier and Watkin, 1998; Laur, 2002), leading to symptoms that include poor coordination. In this study, the effects of fear on motor coordination was attenuated in rats administered with lycopene as they exhibited better performance in the inclined plane task. In addition to its potent free-radical scavenging property, this findings may be attributed to the ability of lycopene to prevent excess stimulation by excitotoxic glutamate, by favouring the opening of chemically-regulated, gated channels for chloride ions. This in turn may enhance inhibitory postsynaptic potential, which is very vital for neural control of body movement and other brain functions (Mailly, *et al.*, 1999; Phillis and Oregan, 2003; Das *et al.*, 2008).

A decrease in forepaw grip time, recorded in this study in rats administered lycopene may not necessarily reflect a true comparison of motor strength between the two groups examined. The reason being that improved performance reflects enhanced coordination and excitability score as demonstrated in the present study. Therefore, the duration of time spent by each rat, administered lycopene, to hold the wooden dowel decreased due to hyper activity.

Stress causes metabolic response that consequently results in the generation of superoxide anion, hydrogen peroxide and hydroxyl radical ions as the major ROS in the bio-systems, which provoked cell lipid membrane peroxidation (Nirmal, 2008). Antioxidant study in the brain of rats revealed a decreased in the levels of SOD and CAT activities in the stressed groups compared to unstressed groups. This indicates that administration of psychological stressors triggered free-radical generation in the brain of the stressed groups. Endogenous antioxidants such as SOD and CAT maintained the TBARS level in the stressed group (S-Control) within range comparable to the unstressed (U-Control and U+Lyco). Administration of lycopene significantly reduce TBARS level in S+Lyco group compared to S-Control as well as that of U-Control and U+Lyco groups. This may probably be due to its various antioxidant defense strategies, most especially the scavenging of two of the ROS: singlet molecular oxygen and peroxyl radicals (Atessahin *et al.,* 2005). Peroxyl radicals are good oxidising agents (Decker,1998). They can abstract hydrogen from other molecules with lower standard reduction potential, and this reaction is frequently observed in the propagation stage of lipid peroxidation (Lee *et al.,* 2003). Very often the alkyl radical formed from this reaction can react with oxygen to form another peroxyl radical, resulting in chain reaction (Lee *et al.,* 2003). Some peroxyl radicals break down to liberate superoxide anion or can react with each other to generate singlet oxygen (Halliwell and Gutteridge, 1985). Results of the present study further indicate the anti-stress properties of lycopene and that it may be exerting ameliorative effects on psychologically-induced deficits. However, it did not have any significant effect on psychologically-induced SOD and CAT alterations.

The significant increase in erythrocyte MDA concentration in stressed groups compared to unstressed groups indicates that fear increased free-radical induced lipid peroxidation in the erythrocytes and their membranes. This agrees with the report by Shin and Liberzon (2010) that there is a significant overlap in structures involved in the fear/anxiety response and stress response, and CRH is likely involved in the coordination of both LHPA axis activity and many fear/anxiety responses. Another finding in the present study suggesting links between fear and stress response is the slight increase in PCV, Hb and RBC, obtained in the stressed groups compared to the unstressed groups. This may be attributed to the effect of the fear response causing the activation of the sympathetic nervous division of the autonomic nervous system to elicit the stress response via the LHPA axis. This leads to a myriad of neuroendocrine changes, including release of catecholamines. The release of catecholamines and their subsequent effect lead to splenic contraction (Szygula, 1990; Tauler *et al.,* 2006) and thus stimulation of erythropoietin production (Duca *et al.,* 2006), causing increase in erythrocytes in the blood circulation. The subsequent increase in circulating mass of erythrocytes favour mechanical disruption of erythrocyte cell membrane as result of physical compression against the wall of blood vessels and capillaries (Senturk *et al.,* 2001; Tauler *et al.,* 2006). In addition to this, constant exposure to high oxygen tension, high level of iron, richness in polyunsaturated fatty acid (Kollanjiappan *et al.,* 2002) lack of nucleus and other organelles in the erythrocyte (Dordevic *et al.,* 2008) render the erythrocyte membrane to be highly vulnerable to lipid peroxidative alterations. Erythrocyte osmotic fragility test gives an in-vitro measure of the tensile strength of erythrocyte membrane. The greater the percentage of erythrocyte osmotic fragility, the weaker the tensile strength of erythrocyte membrane. Therefore,

sequel to the in-vivo weakening of the erythrocyte membrane due to fear-induced free-radical activities, a significant increase was observed in percentage erythrocyte osmotic fragility of S-Control group compared to U-Control group as well as in S+Lyco group compared to U+Lyco group. This further demonstrates that fear response causes disturbances in the integrity of the erythrocyte cell membrane integrity, and thus prone the cell to lysis.

The finding of this study reveals that the insignificant decrease in percentage erythrocyte osmotic fragility in S+Lyco group compared to S-Control group at 0.7 % NaCl concentration, which positively correlated with same in erythrocyte MDA concentration result, suggest that lycopene may be beneficial in ameliorating fear-induced oxidative damage to erythrocytes. This effect of lycopene may be attributed to protection of serum lipoproteins from oxidative stress, although conclusive studies have not been shown (Rice-Evans *et al.,* 1997; Bramley, 2000). Serum lipoproteins such as high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL) transport cholesterol from the liver and vice-versa. Cholesterol, an important component of cell membrane, act as free-radical scavenger. Relationships between serum lipid and erythrocyte membrane lipid show that the change in serum lipids may increase susceptibility of the erythrocytes to lipid peroxidation (Ulana *et al.,* 1985; Clemens and Bursa-Zanetti, 1989). Alteration in the level of serum lipid may occur following oxidative damage to molecules such as serum lipoproteins. The result of the present study suggest that lycopene protect the serum lipoprotein from oxidative damage by increase in total plasma protein concentration. This finding agrees with the reports that proteins, particularly albumin because of its large amount in plasma, exert antioxidant property in the body

(Halliwell, 1988; Bourdon and Blache, 2001; Roche *et al.*, 2008). Although the mechanism underlying its role in the increase in total protein concentration remain to be elucidated, preferential deposition of lycopene in organs such as liver may be implicated (Bramley, 2000).

Comparison between percentage erythrocyte osmotic fragility of U-Control and U+Lyco groups reveals increasing protective ability of lycopene as the osmotic stress of the erythrocytes increased; that is, increasing hypotonicity. However, the amelioration of fear-induced oxidative damage to the erythrocytes became limited as *in-vitro* osmotic stress increased. This is evidenced by insignificant difference in percentage erythrocyte osmotic fragility of S-Control and S+Lyco groups. This requires further investigation.

CHAPTER 6
SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Fear/anxiety-induced stress causes:

1. Impairment in learning and short-term memory.
2. Deficit in neuromuscular coordination and excitability.
3. Decreased brain superoxide dismutase, catalase and acetylcholinesterase activities.
4. Alteration in brain electrolytes balance.
5. Increase EOF and erythrocyte lipid peroxidation.
6. Increase in PCV, Hb, RBC and total protein.

Lycopene reduces anxiety, and improves explorative activity and cognition via its potent antioxidant action. The deficit in neuromuscular coordination and excitability was ameliorated by lycopene by prevention of lipid peroxidation. Lycopene reduces the effect of fear/anxiety-induced stress response on erythrocyte cells probably through its antioxidant activities which increases total plasma protein concentration in the blood.

6.2 Conclusion

In conclusion, fear/anxiety-induced stress in Wistar rats caused behavioural, cognitive and sensorimotor deficits as well as haematological changes, and the ameliorative effect of lycopene on psychologically-induced changes may be due to its antioxidant property.

6.3 Recommendations

6.3.1 Specific Recommendations

Further studies on lycopene should be conducted to elucidate the proximate mechanism of its inhibitory activity. Investigation on the beneficial effect of lycopene on the erythrocytes of animals subjected to an increase in *in-vitro* osmotic stress is also required.

6.3.2 General Recommendations

It is recommended that lycopene may be of adjunctive benefit in ameliorating the adverse effects of psychological stress in animals.

REFERENCES

Abelson J. L., Khan S., Liberzon I., Young E. A. (2007). HPA axis activity in patients with panic disorder: review and synthesis of four studies. *Depression and Anxiety*, 24: 66-76.

Abou-Donia, M. B., Goldstein, L. B., Jones, K. H., Abdel-Rahaman, A. A., Damodaran, T., Dechkovskaia, A. M., Bullman, S. L., Amir, B. E. and Khan, W. A. (2001). Locomotor and sensorimotor performance deficits in rats following exposure to pyridostigmine bromide, DEET and permethrin alone and in combination. *Toxicological Science*, 60: 305-314.

Adamec, R., Burton, P., Blundell, J., Murphy, D. I. and Holmes, A. (2006). Vulnerability to mild predator stress in serotonin transporter knockout mice. *Behavioural Brain Research,* 170: 126-140.

Adeiza, A. A. and Minka, N. S. (2010). Neuromuscular behaviour of Wistar rats administered methanol extract of *Ximenia americana. Archives of Applied Science Research,* 2(5): 217-225.

Agarwal, A., Shen, H., Agarwal, S. and Rao, A. V. (2001). Lycopene content of tomato products: its stability, bioavailability and *in vivo* antioxidant properties. *Journal of Medicinal Food,* 4: 9-15.

Altan, O., Pabuccuoglu, A., Konyalioglu, S. and Bayraktar, H. (2003). Effect of heat stress on oxidative stress, lipid peroxidation, and some stress parameters in broilers. *British Poultry Science,* 44: 545-550.

Ambali, S. F., Idris, S. B., Onukak, C., Shittu, M. and Ayo, J. O. (2010). Ameliorative effects of vitamin C on short-term sensorimotor and cognitive changes induced by acute chlorpyrifos exposure in Wistar rats. *Toxicology and Industrial Health,* 26: 547-558.

Antelman, S. M., Knopf, S., Kocan, D., Edward, D. J. and Nemeroff, C. B. (1988). One stressful event blocks multiple actions of diazepam for up to at least a month. *Brain Research,* 445: 380-385.

Armario, A., Escorihuela, R. M. and Nadal, R. (2008). Long-term neuroendocrine and behavioural effects of a single exposure to stress in adult animal. *Neuroscience and Behavioural Reviews,* 32: 1121-1135.

Atessahin, A., Yilmaz, S., Karahan, I., Ceribas, A. O. and Karauglu, A. (2005). Effects of lycopene against cisplant-induced nephrotoxicity and oxidative stress in rats. *Toxicology,* 212: 116-123.

Atessahin, A., Ceribas, A. O. and Yilmaz, S. (2007). Lycopene, a carotenoid, attenuates cyclosporine-induced renal dysfunction and oxidative stress in rats. *Basic Clinical Pharmacology and Toxicology,* 100: 372-376.

Avci, A. and Durak, I. (2007). Tomato and cancer. *In:* Durak, I. (Ed.), *Nutracuetical and Natural Products in Cardiovascular Diseases, Cancer and Haemorrhoids,* Transworld Research Network, Trivandrum, India, Pp. 41-46.

Axelrod, J. and Reisine T. D. (1984). Stress hormones: Their interaction and regulation. *Science,* 224: 452–459.

Ayo, J. O., Minka, N. S. and Mamman, M. (2006). Excitability scores of goats administered ascorbic acid and transported during hot-dry conditions. *Journal of Veterinary Science,* 7(2): 127-131.

Beckman, K. B. and Ames, B. (1998). The free radical theory of ageing mature. *Physiological Review,* 78: 547-581.

Benzie, I. F. (1996). Lipid peroxidation: a review of causes, consequence, measurement and dietary influences. *International Journal of Food Science and Nutrition,* 46: 233-261.

Berneburg, M., Grether-Beck, S., Kurten, V., Ruzicka, T., Briviba, K. and Sies, H. (1999). Singlet oxygen mediates the UVA-induced generation of the photo ageing-associated mitochondrial common deletion. *Journal of Biological Chemistry,* 274(22): 15345–15349.

Bohm, V. and Bitsch, R. (1999). Intestinal absorption of lycopene from different matrices and interaction with other carotenoids , lipid status and antioxidant capacity of human plasma. *European Journal of Nutrition,* 38: 118-1181

Bhom, V. (2007). Are tomatoes good for the heart? European Union project: LYCOCARD present its first result. *Ernahrung-Umschau,* 54: 446-449.

Bhuvaneswari, O. and Nagini, S. (2005). Lycopene: a review of its potential as an anticancer agent. *Current Medicinal Chemistry - Anti-cancer Agents,* 5(6): 627-635.

Blakely, S. R., Brown, E. D. Babu, U., Grundel, E. and Mitchell, G. V. (1994). Bioavailability of carotenoids in tomatoes paste and dried spinach and their interaction with canthaxanthin. *Federation of American Society of Experimental Biology Journal,* 8: 192.

Blanchard, D. C., Griebel, G. and Blanchard, R. J. (2001). Mouse defensive behaviours: Pharmacological and behavioural assays for anxiety and panic. *Neuroscience and Behavioural Reviews,* 25: 205-218.

Boileau, A. C., Merchen, N. R., Wasson, K., Atkinson, C. A. and Erdman Jr., J. W. (1999). *Cis*-lycopene is more bioavailable than *trans*-lycopene in *in-vitro* and *in-vivo* in lymph-cannulated ferret. *Journal of Nutrition,* 129: 1176-1181.

Botsoglu, N., Papageorgiou, G., Nikolakakin, I., Florou-paneri, P., Giannenan, I., Dotas, V. and Sinapi, E. (2004). Effect of dietary tomato pulp on oxidative stability of Japanese quail meat. *Journal of the Agricultural and Food Chemistry*, 52: 2982-2988.

Bourdon, E. and Blache, D. (2001). The importance of proteins in defence against oxidation. *Antioxidant and Redox Signalling*, 3: 293-311.

Bramley, P. M. (2000). Is lycopene beneficial to human health? *Phytochemistry,* 54: 233-236.

Brikas, P. (1994). The role of GABA receptors in control of omasal myoelectrical activity of sheep. *Research in Veterinary Science,* 56: 69-74.

Brown, R. E., Gunn, R. K., Schellinck, H. M., Wong, A. A. and O'Leary, T. P. (2004). Anxiety, exploratory behavior, and motor activity in 14 inbred strains of mice. MPD - Brown1 Project Protocol. Retrieved October 20, 2011, from www.phenome.jax.org/db/q?rtn=project/docstatic&doc=wehlsten1/welsten1_protocol.

Bryant, R. A. (2006). Non-PTSD post-traumatic disorders: acute stress disorder. *Psychiatry*, 5(7): 238-239

Canadas, F., Cardona, D., Davila, E. and Sanchez-santed, F. (2005). Long-term neurotoxicity of chlopyrifos spatial learning impairment on repeated acquisitionin a water maze. *Toxicological Science,* 85: 944-951.

Cantuti-Castelvetri, Shukitt-Hale, B. and Joseph, J. A. (2000). Neurobehavioural aspects of antioxidants in ageing. *International Journal of Developmental Neuroscience,* 18: 367-381.

Castenmiller, J. J. M. and West, C. E. (1998). Bioavailability and bioconversion of caroteniods. *Annual Review of Nutrition,* 18: 19-38.

Cheeseman, K. H. and Slater, T. F. (1993). An introduction to free radical biochemistry. *British Medical Bulletin*, 49: 481-493.

Chihuailaf, R. H., Contreras, P. A. Wittwer, F. G. (2002). Pathogenesis of oxidative stress: consequence and evaluation in animal health. *Veterinaria Mèxico,* 33(3): 265-283.

Chrousos, G. P. and Gold, P. W. (1992). The concepts of stress and stress system disorders. Overview of physical and behavioural homeostasis. *Journal of America Medical Association,* 267(9): 1244-1252.

Clemens, M. R. and Bursa-Zanetti, Z. (1989). Lipid abnormalities and peroxidation of erythrocytes in nephrotic syndrome. *Nephron,* 53: 325-329.

Clinton, S. K. (1998). Lycopene: chemistry, biology, and implications for human health and disease. *Nutrition Review,* 56: 35-51.

Cohen, H., Kaplan, Z., Matar, M. A., Loewenthal, U., Koslovsky, N. and Zohar, J. (2006). Anisomycin, a protein synthesis inhibitor, distrupts traumatic memory consolidation and attenuates posttraumatic stress response in rats. *Biological Psychiatry,* 60: 767-776.

Das, A., Belagodu, A., Reiter, R. J., Ray, S. K. and Banik, N. L. (2008). Cytoprotective effects of melatonin on C6 astroglial cells exposed to glutamate excitotoxicity and oxidative stress. *Journal of Pineal Research,* 45(2):117-24.

Davis, M. (2006). Neural systems involved in fear and anxiety measured with fear-potentiated startle. *American Psychologist,* 61: 741-756.

De Pee, S. and West, C. E. (1996). Dietary caroteniods and their role in combating vitamin A deficiency: a review of the literature. *European Journal Clinical Nutrition,* 50: S38-S53.

Decker, E.A. (1998). Antioxidant mechanism. *In*: Akoh, C. C. and Min, D. B. (eds.), *Food, Lipids, Chemistry, Nutrition and Biotechnology*, New York: Marcel Dekker, Pp. 397-401.

Di Mascio, P., Kaiser, S. and Sies, H. (1989). Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Archives of Biochemistry and Biophysics,* 274(2): 532-538.

Diplock, A. T., Charleux, J. L., Crozier-Willi, G., Kok, F. J., Rice-Evans C., Roberfroid, M., Stahl, W. and Vina-Ribes J. (1998). Functional food science and defence against reactive oxidative species. *British Journal of Nutrition,* 80(Suppl.): S77-S112.

Dordevic, N. Z., Barbic, G. M., Markovic, S. D., Ognjanovic, B. K., Stajn, A. S., Zikic, R. V., and Saicic, Z. S. (2008). Oxidative stress and change in antioxidant defense system in erythrocyte of preeclampsia in women. *Reproductive Toxicology,* 25: 213-218.

Duca, L., Da Ponte, A., Cozzi, M., Carbone, A., Pomati, M., Nava, I., Cappellini, M. D. and Fiorelli, G. (2006). Change in erythropoiesis, iron metabolism and oxidative stress after half-marathon. *Internal and Emergency Medicine,* 1(1): 30-34.

Durackova, Z. (2010). Some current insights into oxidative stress. *Physiological Research,* 59: 459-469.

Ellam, G. L., Courtney, K. D., Andres, Jr. V. and Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacoclogy,* 7: 88-90.

Elsayed, N. M. (2001). Antioxidants mobilisation in response to oxidative stress: a dynamic environmental-nutritional interaction. *Nutrition,* 17: 828-834.

Engelhard, Y. N., Gazer, B. and Paran, E. (2006). Natural antioxidants from tomato extract reduce blood pressure in patients with grade-1 hypertension: a double-blind, placebo-controlled pilot study. *American Heart Journal,* 151: 100.e6-100.e1.

Erdman Jr., J. W., Fahey, G. C. and White, C. B. (1986). Effects of purified dietary fiber source on β-carotene utilization by the chick. *Journal of Nutrition,* 116: 2415-2423.

Etminan, M., Takkouche, B. and Caamano-Isorna, F. (2004). The role of tomato products and lycopene in the prevention of prostate cancer: a meta-analysis of observational studies. *Cancer Epidemiology Biomarkers and Prevention,* 13: 340-345.

Fang, Y.Z., Yang, S. and Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutrition,* 18: 872-879.

Faulkner, W.R. and King, J.W. (1970). *Manual of Clinical Laboratory Procedures,* Chemical Leather Company, Ohio, U.S.A., 345 Pp.

Fox, M. W. (1968).Veterinary ethology. *In:* Fox, M. W. (Ed.), *Abnormal Behaviour in Animals*. W. B. Saunders, U. S. A. 12-20 Pp.

Friedman, J. (2010). Why is the nervous system vulnerable to oxidative stress? *In*: Gadoth, N. and Gobel, H. H. (Eds.), *Oxidative Stress and Free Radical Damage in Neurobiology*, Springer Science+Bisiness Media, Pp. 20-21.

Frusciante, L., Carli, P., Ercolano, M. R., Pernice, R., Di Matteo, A., Fogliano, V. and Pellegrini, N. (2007). Antioxidant nutritional quality of tomato. *Molecular Nutrition and Food Research,* 51(5): 609-617.

Garcia-Bueno, B., Caso, J. R. and Leza J. C. (2008). Stress as an inflammatory condition in brain: Damaging and protective mechanisms. *Neuroscience and Behavioural Reviews,* 32: 1136-1151.

Giovannucci, E. (1999). Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. *Journal of the National Cancer Institute,* 91(4): 317-331.

Giovannucci, E., Ascherio, A., Rimm, E. B., Stampfer, M. J., Colditz, G. A. and Willett, W. C. (1995). Intake of carotenoids and retinol in relation to risk of prostate cancer. *Journal of the National Cancer Institute,* 87(23): 1767-1776.

Giovannucci, E., Willett, W. C., Stampfer, M. J., Liu, Y. and Rimm, E. B. (2002). A prospective study of tomato products, lycopene, and prostate cancer risk. *Journal of the National Cancer Institute,* 94(5): 391-396.

Goyal, A., Chopra, M., Lwaleed, B. A., Birch, B. and Cooper, A. J. (2007). The effects of dietary lycopene supplementation on human seminal plasma. *BJU International,* 99: 1456-1460.

Haefely, W. (1994). Allosteric modulation of $GABA_A$ receptor channels: a mechanism for interaction with a multitude of CNS functions. *In*: Mohler, H. and Daprada M. (Eds.), *The Challenge of Neuropharmacology*, Basel: Editiones Roche, Pp. 15-39.

Halliwell, B. and Gutteridge, J. M. C. (1985). The importance of free radicals and catalytic metal ions in human diseases. *Molecular Aspects Medicine,* 8(2): 89-193.

Halliwell, B. and Gutteridge, J. M. C. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Archives of Biochemistry and Biophysics,* 246: 501-514.

Halliwell, B. (1988). Albumin: an important extracellular antioxidant? *Biochemistry and Pharmacology,* 37: 569-571.

Halliwell, B. (2006). Oxidative stress and neurodegeneration: where are we now? *Journal of Neurochemistry,* 97: 1634-1658.

Halloway, D. E., Yang, M., Paganga, G., Rice-Evans, C. A. and Bremley, P. M. (2000). Isomerization of dietary lycopene during assimilation and transport in plasma. *Free Radical Research,* 32: 93-102.

Heim, C. and Nemeroff, C. B. (2001). The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biological Psychiatry,* 49: 1023-1039.

Helzsouer, K. J., Comstock, G. W. and Morris J. S. (1989). Selenium, lycopene, α-tocopherol, β-carotene, retinol, and subsequent bladder cancer. *Cancer Research,* 49: 6144-6148.

Hill, H. M. and Rogers, L. J. (1969). Conversion of lycopene into β-caroteneby choloroplast of higher plants. *Biochemistry Journal,* 113: P31-P32.

Institute of Medicine, Food and Nutrition Board (INFMB) (2000). *Beta-Carotene and Other Carotenoids. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*. National Academy Press, Washington D.C., Pp. 325-400.

Jackson, K. Y. (2009). Heat index calculator. National Weather Service Forecast Office. Retrieved October 20, 2011, from http:/www.crh.noaa.gov/jkl/?n=heat_index.calculator

Johnson, E.O., Kamilaris, T.C., Chrousos, G.P. and Gold, P.W. (1992). Mechanism of stress: A dynamic overview of behavioural homeostasis. *Neuroscience and Biobehavioural Reviews,* 16: 115-130.

Johnson, E. J., Qin, J., Krinsky, N. I. and Russell R. M. (1997). Ingestion by men of a combined dose of beta-carotene and lycopene does not affect the absorption of beta-carotene but improves that of lycopene. *Journal of Nutrition,* 127: 1833-1837.

Jorgensen, E.M. (2005). GABA. *In*: Joshua, M. K. (Ed.), *WormBook.* The *C. Elegans* Research Community. Retrieved November, 20, 2011, from http://www.wormbook.org.

Kelvens, C. (1997). *Fear and Anxiety.* California State University, Northridge, U.S.A. Retrieved October, 20, 2011, from http://www.csun.edu/~vcpsy00h/students/fear.htm

Khan, N., Afaq, F. and Mukhtar, H. (2008). Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxidant and Redox Signalling,* 10(3): 475-510.

Knowles, T. G., Warris, P. G., Brown, S. N., Kestin, S. C., Rhind, S. M., Edwards, J. E., Anil, M. H., and Dolan, S. K. (1993). Long distance transport of lambs and the time needed for subsequent recovery. *The Veterinary Record,* 133: 286-293.

Kohlmeir, L., Kark, J. D. and Gomez-Gracia, E. (1997). Lycopene and myocardial infarction risk in the EURAMIC Study. *American Journal of Epidemiology,* 46: 618-626.

Kollanjiappan, K., Manoharan, S. and Kayalvizhi, M. (2002). Measurement of erythrocyte lipids, lipid peroxidation, antioxidant and osmotic fragility in cervical cancer patients. *Clinica Chimica Acta,* 326: 143-149.

Koolhaas, J. M., Hermann, P. M., Kemperman, C., Bohus, B., van den Hoofdakker, R. H. and Beersma, D. G. M. (1990). Single social defeat in male rat induces a gradual but long lasting behavioural change: a model of depression? *Neuroscience Research and Communications*, 7: 35-41.

Kucuk, O., Sarkar, F. H. and Sakr, W. (2001). Phase II randomized clinical trial of lycopene supplementation before radical prostatectomy. *Cancer Epidemiology Biomarkers and Prevention*, 10: 861-868.

Kucuk, O., Sarkar, F. and Djuric, Z., Sakr, W., Pollak, M. N., Khachik, F., Banerjee, M., Bertram, J. S. and Wood Jr., D. P. (2002). Effects of lycopene supplementation in patients with localized prostate cancer. *Experimental Biology and Medicine*, 227: 881-885.

Kuhad, A., Sethi, R. and Chopra, K. (2008). Lycopene attenuates diabetes-associated cognitive decline in rats. *Life Sciences*, 83: 128-134.

Kumar, P. and Kumar, A. (2009). Effects of lycopene and epigallocatechin-3-gallate against 3-nitropropionic acid induced cognitive dysfunction and gluthathione depletion in rats: a novel nitric oxide mechanism. *Food and Chemical Toxicology*, 47: 2522-2530.

Laur, D. (2002). *The Anatomy of Fear and How It Relates to Survival Skills Training.* Integrated Street Combatives. . Retrieved October, 20, 2011, from http://www.iwcbooks.com/articles/anatomy.htm

Lay Jr., D. C. and Wilson, M. E. (2004). Considerations when using physiological data in assessing animal well-being . *Journal of Animal and Veterinary Advances*, 3: 613-625.

Lee, J., Koo, N. and Min, D. B. (2003). Reactive oxygen species, aging, and antioxidative nutraceuticals. *Comprehensive Reviews in Food Science and Food Safety*, 3: 21-33.

Levy, J., Sharoni, Y., Danilenko, M., Miinster, A., Bosin, E., Giat, Y. and Feldman, B. (1995). Lycopene is a more potent inhibitor of human cancer cell proliferation than either alpha-carotene or beta-carotene. *Nutrition and Cancer*, 24(3): 257-266.

Liakakos, D., Douglas, A. and Jouramani, G. (1975). Inhibitory effect of ascorbic acid on cortisol secretion following adrenal stimulation in children. *Clinica Chemica Acta*, 65: 251-255.

Liberzon, I. and Martis, B. (2006). Neuroimaging studies of emotional responses in PTSD. *Annals of the New York Academy of Science*, 1071: 87–109.

Linsheld, B. L., Canene-Adams, K. and Erdman Jr., J. W. (2007). Lycopenoids: Are lycopene metabolites bioactive ? *Archives of Biochemistry and Biophysics,* 458: 136-140.

Lykkesfeldt, J. and Svendson, O. (2007). Oxidants and antioxidants in disease: oxidative stress in farm animals. *The Veterinary Journal,* 173: 502-511.

Machlin, L. J. and Bendich, A. (1987). Free radical-tissue damage: protective role of antioxidant nutrients. *Federation of American Society of Experimental Biology Journal,* 1: 441-445.

Maier, S. F. and Watkins, L. R. (1998). Cytokines for psychologists: Implications for bidirectional immune-to-brain communication for understanding behaviour, mood, and cognition. *Psychoneuroimmunology Reviews,* 105: 83-107

Mailly, F., Marin, P., Isreal, M., Glowinski, J. and Premont J. (1999). Increase in external glutamate and NMDA receptor activation contribute to H_2O_2-induced neuronal apoptosis. *Journal of Neurochemistry,* 73: 1181-1188.

Mangiagalli, M. G., Martino, P. A., Smajlovic, T., Guidobono Cavalchini, L. and Marelli, S. P. (2010). Effect of lycopene on semen quality, fertility and native immunity of broiler breeders. *British Poultry Science,* 51(1): 152-157.

Maren, S. (2008). Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats. *European Journal of Neuroscience,* 28: 1661–1666.

Maria, G. A. M., Villaroel, G., Chacon, G. and Gebresenbet, G. (2002). Scoring system for evaluating the stress to cattle of commercial loading and unloading. *The Veterinary Record,* 154: 818-821.

Martino, P. A., Mangiagalli, M. G., Marelli, S. P., Smajlovic, T. and Guidobono Cavalchini, L. (2006). Effect of lycopene on semen bacteriological status and native immunity in roosters. *World Poultry Science Journal, XII European Poultry Science Conference, Verona,* 62: 415.

Mates, J. M. and Sanchez-Jimenez, F. (1999). Antioxidant enzymes and their implications in pathophysiologic processes. *Frontiers in Bioscience,* 4: 339-345.

Maxmen, J. S. and Ward, N. G. (1995). *Essential Psychopathology and its Treatment* - revised for *DSM-IV* (2nd Ed.). W. W. Norton, New York, 280 Pp.

Mayberg, H. S., Liotti, M., Brannan, S. K., McGinnis, S., Mahurin, R. K. and Jerabek, P. A. (1999). Reciprocal limbic-cortical function and negative mood: converging PET findings in depression and normal sadness. *American Journal of Psychiatry,* 156: 675–682.

Menon, M. K. and Dandiya, P. C. (1969). Behavioural and brain neurohormonal changes produced by acute heat stress in rats: influence of psychopharmacological agents. *European Journal of Pharmacology,* 8: 284-291.

Minka, N. S. and Ayo, J. O. (2011). Behavioural responses, traumatic injuries and live weight losses in ostrich (*Struthio camelus*) chicks transported by road during hot-dry conditions. *Archiv Tierzucht,* 54: 635-647.

Moberg, G. P. (2000). Biological response to stress: Implications for animal welfare. *In*: Moberg, G. P. and Mench, J. A. (Eds.), *The Biology of Animal Stress.* CABI Publishing, UK., Pp. 1-21.

Mohanty, N. K., Sujit, K., Jha, A. K. and Arora, R. P. (2001). Management of idiopathic oligoasthenospermia with lycopene. *Indian Journal of Urology,* 18(1): 57-61.

Nahum, A., Sharoni, Y., Prall, O. W., Levy, J., Hirsch, K., Watts, C. K., Danilenko, M. (2001). Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27(Kip1) in the cyclin E-cdk2 complexes. *Oncogene,* 20(26): 3428-3436.

Narisawa, T., Fukaura, Y., Hasebe, M., Ito, M., Nishino, H., Khachik, F., Murakoshi, M., Uemura, S. and Aizawa, R. (1996). Inhibitory effects of natural carotenoids, alpha-carotene, beta-carotene, lycopene and lutein, on colonic aberrant crypt foci. *Cancer Letters,* 107(1): 137-142.

Nguyen, M. L. and Schwarts, S. J. (1999). Lycopene: Chemical and biological properties. *Food Technology,* 53: 38-45.

Nirmal, J., Babu, C. S., Harisudhan, T. and Ramanathan, M. (2008). Evaluation of behavioural and antioxidant activity of *Cytisus scoparius* Link in rats exposed to chronic unpredictable mild stress. *BMC Complementary and Alternative Medicine,* 8: 15doi.10:1186/1472-6882-8-15

Olsen, R. W. (2002). GABA. In: Davis, K. L., Charney, D., Coyle, J. T. and Nemeroff, C., *Neuropsychopharmacology: The Fifth Generation of Progress*. American College of Neuropsychopharmacology, Pp. 159-168.

Oyewale, J.O. (1992). Effects of temperature and pH on the osmotic fragility of erythrocytes of domestic fowl (*Gallus domesticus*) and guinea fowl (*Numida maleagris)*. *Research in Veterinary Science,* 52: 1-4.

Perkins-Veazie, P., Collins, J. K., Edward, A., Wiley, E. and Clevidence, B. (2003).Watermelon: Rich in the antioxidant lycopene. *Acta Horticulturae,* 628: 663-668.

Perkins-Veazie, P., Collins, J. K., Clevidence, B., Wu, G. Y. (2007). Watermelon and health. *Acta Horticulturae,* 731: 121-127.

Petrich, C.E. (2006). Effect of NMDA receptor antagonist MK-801 on recovery from spinal cord injury in rats given uncontrollable stimulation. A senior honours thesis submitted to the office of honours programme and academic scholarships, University undergraduate research fellow, Texas A & M University. Retrieved April 17, 2007, from http://txspace.tamil.edu/bistream/960.1/3701/1/petrichThesis.pdf.

Phillis, J. W. and Oregan, M. H. (2003). Characterization of modes of release of amino acids in the ischemic/reperfused rat cerebral cortex. *Neurochemistry International,* 43: 461-467.

Pollack, A., Madar, Z., Eisner, Z., Nyska, A. and Oren, P. (1997). Inhibitory effect of lycopene on cataract development in galactosemic rats. *Metabolic Pediatric Systemic Ophthalmology,* 19(20): 31-36.

Quirk, G. J. and Mueller, D. (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology*, 33: 56–72.

Rafi, M. M., Yadav, P. N. and Reyes, M. (2007). Lycopene inhibits LPS-induced proinflammatory mediator inducible nitric oxide synthase in mouse macrophage cells. *Journal of Food Science,* 72: S069-S074

Rao, A. V. and Agarwal, S (1998). Bioavailability and in vivo antioxidant properties of lycopene from tomato products and their possible role in the prevention of cancer. *Nutrition and Cancer,* 31: 199-203.

Rao, A. V., Fleshner, N. and Agarwal, S. (1999). Serum and tissue lycopene and biomarkers of oxidation in prostate cancer patients: a case-control study. *Nutrition and Cancer,* 33: 159-164.

Rao, A. V. and Agarwal, S. (2000). Role of antioxidant lycopene in cancer and heart disease. *Journal of the American College Nutrition.* 19: 563-569.

Rao, A. V. and Shen, H. (2002). Effect of low dose lycopene intake on lycopene bioavailability and oxidative stress. *Nutrition Research,* 22:1125-1131.

Rao, A. V. (2004). Processed tomato products as a source of dietary lycopene: bioavailability and antioxidant properties. *Canadian Journal of Dietetic Practice and Research,* 65: 161-165.

Rao, A. V., Ray, M. R. and Rao, L. G. (2006). Lycopene. *Advances in Food and Nutrition Research,* 51: 99-164.

Rao, A. V. and Rao, L. G. (2007). Carotenoids and human health. *Pharmacological Research,* 55 (3): 207-216.

Rao, L. G., Krishnadev, N., Banasikowska, K. and Rao, A. V. (2003). Lycopene I - effect on osteoclasts: lycopene inhibits basal and parathyroid hormone-stimulated osteoclast formation and mineral reabsorption mediated by reactive oxygen species in rat bone marrow cultures. *Journal of Medicinal Food*, 6: 69-78.

Rao, L. G. (2005). Will tomatoes prevent osteoporosis? *Endocrinology Rounds*, 5(2): 118-135.

Reed, D.J. (1995). Toxicity of oxygen. *In*: De Matteis and Smith, L.L. (Eds.), *Molecular and Cellular Mechanism of Toxicity*, CRC Press, New York., Pp. 35-68.

Rice-Evans, C. A., Sampson, J., Bramley, P. M. and Holloway, D. E. (1997). Why do we expect carotenoids to be antioxidant *in-vivo*? *Free Radical Research*, 26: 382-398.

Roche, M., Rondeau, P., Singh, R.N., Tarnus, E., Bourdon, E. (2008). The antioxidant properties of serum albumin. *FEBS Letters*, 582: 1783-1787.

Schalms, O.W., Jain, N.C. and Carroll, E.J. (1975). *Veterinary Heamatology*, 3rd ed. Lea and Fibiger, Philadelphia.

Schofield, P.R., Darlison, M.G., Fujita, N., Burt, D.R., Stephenson, F.A., Rodriguez, H., Rhee, L.M., Ramachandran, J., Reale, V., Glencorse, T.A. (1987). Sequence and functional expression of the GABA A receptor shows a ligand-gated receptor super-family. *Nature*, 328: 221–227.

Selye, H. (1946). The general adaptation syndrome and the disease of adaptation. *Journal of Clinical Endocrinology*, 6: 117-230.

Selye, H. (1963). Stress and the adaptation syndrome. *In: Encyclopaedia of Medicine, Surgery and Specialties.* F. A. Davis Company, New York, Vol. XIII, Pp. 365-366.

Senturk, U. K., Gunduz, F., Kuru, O., Aktekin, M. R., Kipmen, D., Yalcin, O., Bor-Kucukatay, M., Yesilkaya, A. and Baskurt, O. K. (2001). Exercise-induced oxidative stress affects erythrocytes in sedentary rats but not exercise-trained rats. *Journal of Applied Physiology*, 91(5): 1999-2004.

Shi, J. and and Le Mague, M. (2000). Lycopene in tomatoes: chemical and physical properties affected by food processing. *Critical Review in Food Science and Nutrition*, 40: 1-42.

Shin, L. M. and Liberzon, I. (2010). The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology*, 35: 169-191.

Siegel, H. S. (1980). Physiological stress in birds. *Bioscience*, 30: 529-533.

Simon, H.U., Haj-Yehia, A. and Levi-Schaffer, F. (2000). Role of reactive oxygen species in the apoptosis induction. *Apoptosis,* 5: 415-418.

Sinha, R. K. and Ray, A. K. (2004). An assessment of changes in open-field and elevated plus-maze behaviour following heat stress in rats. *Iranian Biomedical Journal,* 8(3): 127-133.

Sordillo, L. M. and Aitken, S. L. (2008). Impact of oxidative stress on the health and immune function of dairy cattle. *Veterinary Immunology and Immunopathology,* doi: 10.1016/j.vetimm.2008.10.305

Stahl, W. and Sies, H. (1992). Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans. *Journal of Nutrition,* 122: 2161-2166.

Stahl, W. and Sies, H. (1996). Lycopene: a biologically important carotenoid for humans? *Archives of Biochemistry and Biophysics,* 336(1): 1-9.

Stief T. W. (2003). The physiology and pharmacology of singlet oxygen. *Medical Hypotheses,* 60: 567–72.

Szygula, Z. (1990). Erythrocytic system under the influence of physical exercise and training. *Sports Medicine,* 10: 181–197.

Tadmor, Y., King, S., Levi, A., Davis, A., Mier, A., Wasserman, B., Hirschberg, J. and Lewinsohn, E., (2005). Comparative fruit colouration in watermelon and tomato. *Food Research International,* 38: 837-841.

Tanumihardjo, S. A. (2002). Factors influencing the conversion of carotenoids to retinol: bioavailability to conversion to bioefficacy. *International Journal for Vitamin and Nutrition Research,* 72: 40-45.

Tapiero, H., Townsend, D. M. and Tew, K. D. (2004). The role of carotenoids in prevention of human pathologies. *Biomedicine and Pharmacotherapy,* 58: 100-110.

Tauler, P., Aguilo, A., Gimeno, I., Fuentespina, E., Tur, J.A. and Pons, A. (2006). Response of blood cell antioxidant enzyme defences to antioxidant diet supplementation and to intense exercise. *European Journal of Nutrition,* 446(4): 187-95.

Teixeira, H. D., Schumacher, R. I. and Meneghini, R. (1998). Lower intracellular hydrogen peroxide levels in cells over expressing CuZn-superoxide dismutase. *Proceedings of the National Academy of Sciences of the United States of America,* 95: 7872-7875.

Turk, G., Atessahin, A., Sonmez, M., Yuce, A. and Ceribasi, A. O. (2007). Lycopene protects against cyclosporine A-induced testicular toxicity in rats. *Theriogenology,* 67: 778-785.

Ulana, L. B., Brewer, G. T. and Hill, G. M. (1985). Effects of dietary cholesterol on erythrocyte peroxidantstress *in vitro* and *in vivo*. *Archives of Biochemistry and Biophysics,* 835: 434-440.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. Mazur, M. and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and disease. *International Journal of Biochemistry and Cell Biology,* 39:44-84.

van Den Berg, H. (1999). Carotenoids interactions. *Nutrition Reviews,* 57: 1-10.

van Het Hof, H. F., West, C. E., Weststrate, J. A., and Hautvast, J. G. (2000). Dietary factor that affect the bioavailabilty of carotenoids. *Journal of Nutrition,* 130: 503-506.

Vani, R., Reddy, S. S. and Asha, D. S. (2010). Oxidative stress in erythrocytes: a study of the effect of antioxidant mixtures during intermittent exposure to high altitude. *International Journal of Biometerology.* 54: 553-562.

Virden, W. S. and Kidd, M. T. (2009). Physiological stress in broilers: Ramifications on nutrient digestibility and responses. *Journal of Applied Poultry Research,* 18 :338–347.

von Borell, E.H. (2001). The biology of stress and its application to livestock housing and transportation assessment. *American Society of Animal Science,* 79(E. Suppl): E260-E267

von Holst, D. (1997). The concept of stress and its relevance for animal behaviour. *Advances in the Study of Behaviour,* 27: 1-131.

Winrow, V. R., Winyard, P. G., Morris, C. J. and Blake, D. R. (1993). Free radicals in inflammation: second messengers and mediators of tissue destruction. *British Medical Bulletin,* 49*:* 506-522.

Wiseman, H. and Halliwell, B. (1996). Damage from DNA by reactive oxygen and nitrogen species: role of inflammatory disease and progression to cancer. *Biochemistry Journal,* 313: 17-29.

Wu, K., Erdman Jr., J. W., Schwartz, S. J., Platz, E. A., Leitzmann, M., Clinton, S. K., De Groff, V., Willett, W.C. and Giovannucci, E. (2004). Plasma and dietary carotenoids, and the risk of prostate cancer: A nested case-control study. *Cancer Epidemiology, Biomarkers and Prevention,* 13: 260-269.

Zhu, H., Robin, W., Rockhold, R.W., Baker, R.C., Kramer, R.E. and Ho, I.K. (2001). Effect of single or repeated dermal exposure to methyl parathion on behaviour and blood cholinesterase activity in rats. *Journal of Biomedical Sciences*, 8: 467-474.

LIST OF APPENDICES

Appendix 1: Behavioural data of Wistar rats obtained from the open-field apparatus (line crossing)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	40	42	18	21
2	34	42	12	22
3	14	5	2	35
4	38	17	34	22
5	35	23	36	56
6	14	49	19	17
7	10	0	16	1
Mean ± S.E.M.	26.43 ± 4.95	25.43 ± 7.31	19.57 ± 4.52	24.86 ± 6.43

Appendix 2: Behavioural data of Wistar rats obtained from the open-field apparatus (central square frequency)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	1	1
5	0	0	0	12
6	0	0	0	0
7	0	0	0	0
Mean ± S.E.M.	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.14	1.86 ± 1.79

Appendix 3: Behavioural data of Wistar rats obtained from the open-field apparatus (central square duration)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	3	0
5	0	0	0	20
6	0	0	0	0
7	0	0	0	0
Mean ± S.E.M.	0.00 ± 0.00	0.00 ± 0.00	0.43 ± 0.43	2.86 ± 2.86

Appendix 4: Behavioural data of Wistar rats obtained from the open-field apparatus (rearing frequency)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	14	9	1	6
2	4	10	2	5
3	4	2	1	9
4	9	5	12	7
5	9	8	7	25
6	4	12	7	12
7	1	0	4	2
Mean ± S.E.M.	6.43 ± 1.67	6.57± 1.66	4.86 ± 1.53	9.43 ± 2.85

Appendix 5: Behavioural data of Wistar rats obtained from the open-field apparatus (grooming frequency)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	1	3	3	4
2	2	0	0	2
3	3	4	3	1
4	5	3	4	6
5	7	1	6	0
6	1	3	4	3
7	1	0	2	1
Mean ± S.E.M.	2.86 ± 0.88	2.00 ± 0.62	3.14 ± 0.71	2.43 ± 0.78

Appendix 6: Behavioural data of Wistar rats obtained from the open-field apparatus (stretched-attend posture)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	25	36	12	21
2	25	27	13	23
3	22	27	8	32
4	21	17	25	15
5	19	29	11	21
6	17	34	11	11
7	21	6	20	11
Mean ± S.E.M.	21.43 ± 1.11	25.14 ± 3.94	14.29 ± 2.27	19.14 ± 2.83

Appendix 7: Behavioural data of Wistar rats obtained from the open-field apparatus (urination frequency)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	0	1	0	0
2	1	0	0	0
3	0	1	0	1
4	1	0	0	1
5	1	2	0	0
6	1	0	0	0
7	0	0	1	0
Mean ± S.E.M.	0.57 ± 0.20	0.57 ± 0.30	0.14 ± 0.14	0.29 ± 0.18

Appendix 8: Behavioural data of Wistar rats obtained from the open-field apparatus (defaecation frequency)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	3	4	4	3
2	5	0	5	0
3	6	5	4	6
4	0	4	7	4
5	3	4	6	3
6	5	7	5	0
7	0	5	0	8
Mean ± S.E.M.	3.14 ± 0.91	4.14 ± 0.80	4.43 ± 0.84	3.43 ± 1.11

Appendix 9: Behavioural data of Wistar rats obtained from the open-field apparatus (freezing/immobilization)

	DAY 3		DAY 10	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	6	0	30	14
2	10	13	28	25
3	18	11	20	0
4	0	13	0	0
5	0	7	8	0
6	0	0	0	5
7	0	48	0	10
Mean ± S.E.M.	4.86 ± 2.65	13.14 ± 6.18	12.29 ± 5.10	7.71 ± 3.55

Appendix 10: Cognitive ability data of Wistar rats obtained from learning test (Day 13) and short-term memory test (Day 14).

	Learning acquisition (No. of foot-shock)		Memory retention (in seconds)	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco
1	1	1	120	120
2	1	1	120	120
3	1	1	12	85
4	1	3	12	30
5	7	1	120	34
6	2	1	120	120
7	1	1	21	120
Mean ± S.E.M.	2.00 ± 0.85	1.29 ± 0.29	75.00 ± 21.24	89.86 ± 15.71

Appendix 11: Excitability score data of Wistar rats obtained from excitability score test (day 14).

	Excitability	
Animal No.	S-Control	S+Lyco
1	3	3
2	3	4
3	3	3
4	4	3
5	5	5
6	3	4
7	2	4
Mean ± S.E.M.	3.29 ± 0.36	3.71 ± 0.29

Appendix 12: Neuro-muscular coordination in of Wistar rats obtained from the incline-plane performance test (in seconds)

	DAY 1		DAY 7		DAY 14	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco	S-Control	S+Lyco
1	57.2	55	60	52.5	55	57.5
2	52.5	57.5	57.5	52.5	52.5	55
3	50	55	55	50	50	52.5
4	55	60	57.5	55	40	55
5	55	55	52.6	50	47.5	52.5
6	55	52.5	55	52.5	55	50
7	52.5	55	55	60	50	52.5
Mean ± S.E.M.	53.89 ± 0.90	55.71 ± 0.90	56.09 ± 0.91	53.21 ± 1.30	50.00 ± 1.97	53.57 ± 0.92

Appendix 13: Muscle strength of Wistar rats obtained from the fore-grip time test (in seconds)

	DAY 1		DAY 7		DAY 14	
Animal No.	S-Control	S+Lyco	S-Control	S+Lyco	S-Control	S+Lyco
1	37	18	35	10	19	14
2	27	15	13	16	50	44
3	39	10	14	19	17	24
4	7	35	9	7	7	12
5	6	17	11	9	6	7
6	20	70	20	5	38	15
7	27	8	21	15	19	29
Mean ± S.E.M.	23.29 ± 4.97	24.71 ± 8.24	17.57 ± 3.35	11.57 ± 1.95	22.29 ± 6.10	20.71 ± 4.79

Appendix 14: Biochemical data obtained from brain samples of Wistar rats (malonaldehyde concentration)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	1.8	2.2	1.8	1.4
2	2.3	2.6	1.6	1.1
3	1.7	2.4	2	1.7
4	1.8	1.8	2.1	1.5
5	1.6	1.9	1.6	1.6
6	1.9	2.2	1.8	1.3
7	2.3	2	1.9	1.3
Mean ± S.E.M.	1.91 ± 0.11	2.16 ± 0.11	1.83 ± 0.07	1.41 ± 0.08

Appendix 15: Biochemical data obtained from brain samples of Wistar rats (superoxide dismutase)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	2.6	1.3	1.6	1.6
2	2.5	1.8	1.3	1.8
3	2.3	2.2	1.9	1.1
4	1.8	1.6	1.2	1.6
5	1.9	1.9	1.8	1.5
6	2.3	2.4	1.1	1.1
7	2.1	2.2	1.6	1.8
Mean ± S.E.M.	2.21 ± 0.11	1.91 ± 0.15	1.50 ± 0.12	1.50 ± 0.11

Appendix 16: Biochemical data obtained from brain samples of Wistar rats (catalase)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	62	67	56	53
2	71	63	47	37
3	63	71	42	45
4	55	58	46	61
5	49	73	43	51
6	59	64	51	44
7	63	66	60	48
Mean ± S.E.M.	60.29 ± 2.63	66.00 ± 1.90	49.29 ± 2.54	48.43 ± 2.88

Appendix 17: Biochemical data obtained from brain samples of Wistar rats (acetylcholinesterase)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	28	26	22	24
2	24	24	18	22
3	22	28	16	15
4	26	24	19	16
5	23	22	24	18
6	22	19	22	24
7	24	16	28	23
Mean ± S.E.M.	24.14 ± 0.83	22.71 ± 1.55	21.29 ± 1.52	20.29 ± 1.46

Appendix 18: Biochemical data obtained from brain samples of Wistar rats (calcium)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	2.66	2.47	2.55	2.33
2	2.41	2.41	2.31	2.41
3	2.47	2.48	2.47	2.37
4	2.45	2.66	2.43	2.25
5	2.48	2.43	2.46	2.27
6	2.46	2.35	2.33	2.35
7	2.41	2.9	2.45	2.5
Mean ± S.E.M.	2.48 ± 0.03	2.53 ± 0.07	2.43 ± 0.03	2.35 ± 0.032

Appendix 19: Biochemical data obtained from brain samples of Wistar rats (sodium)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	143	147	143	140
2	141	138	140	138
3	142	144	140	147
4	146	142	142	142
5	148	142	144	140
6	147	140	146	141
7	140	146	135	147
Mean ± S.E.M.	143.86 ±1.18	142.71 ±1.21	141.43 ± 1.34	142.14 ± 1.34

Appendix 20: Biochemical data obtained from brain samples of Wistar rats (chlorine)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	106	104	110	96
2	100	108	106	102
3	104	106	112	106
4	103	102	107	94
5	114	98	113	96
6	106	96	102	98
7	102	100	96	95
Mean ± S.E.M.	105.00 ± 1.70	102.00 ± 1.63	106.57 ± 2.27	98.14 ± 1.64

Appendix 21: Biochemical data obtained from brain samples of Wistar rats (potassium)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	4.1	4.8	4.3	4.9
2	4	3.9	4.1	3.7
3	4.9	4.4	4.3	4.6
4	4.2	4.2	4.1	5.1
5	4.7	4.8	4.5	5.5
6	4.8	5.3	4.7	4.3
7	4	4.6	4.3	4.6
Mean ± S.E.M.	4.39 ± 0.15	4.57 ± 0.17	4.33 ± 0.08	4.67 ± 0.22

Appendix 22: Haematological data obtained from blood samples of Wistar rats (PCV)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	53	49	53	54
2	51	47	53	55
3	54	46	54	55
4	51	54	51	51
5	43	51	52	51
Mean ± S.E.M.	50.40 ± 1.94	49.40 ± 1.44	52.60 ± 0.51	53.20 ± 0.92

Appendix 23: Haematological data obtained from blood samples of Wistar rats (Hb)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	17.6	16.3	17.6	18
2	17	15.6	17.6	18.3
3	18	15.3	18	18.3
4	17	18	17	17
5	14.3	17	17.3	17
Mean ± S.E.M.	16.78 ± 0.65	16.44 ± 0.49	17.50 ± 0.17	17.72 ± 0.30

Appendix 24: Haematological data obtained from blood samples of Wistar rats (RBC count)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	8.5	8.1	8.8	9
2	8.3	7.8	8.6	9.1
3	8.8	7.4	9.2	9.2
4	8.2	8.9	8.3	8.4
5	7.2	8.3	8.7	8.5
Mean ± S.E.M.	8.20 ± 0.27	8.10 ± 0.25	8.72 ± 0.15	8.84 ± 0.16

Appendix 25: Haematological data obtained from blood samples of Wistar rats (MCV)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	62.4	60.5	60.2	60
2	61.5	60.3	61.6	60.4
3	61.4	62.2	58.7	59.8
4	62.2	60.7	61.5	60.7
5	59.7	61.5	59.8	60
Mean ± S.E.M.	61.44 ± 0.48	61.04 ± 0.35	60.36 ± 0.55	60.18 ± 0.16

Appendix 26: Haematological data obtained from blood samples of Wistar rats (MCHC)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	33.2	33.3	33.3	33.3
2	33.3	33.2	33.2	33.3
3	33.3	33.3	33.2	33.3
4	33.3	33.3	33.3	33.3
5	33.3	33.3	33.3	33.3
Mean ± S.E.M.	33.28 ± 0.02	33.28 ± 0.02	33.26 ± 0.03	33.30 ± 0.00

Appendix 27: Haematological data obtained from blood samples of Wistar rats (MHC)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	20.7	20.1	20	21.1
2	20.5	20	20.5	20
3	20.5	20.7	19.6	20.1
4	20.7	20.3	20.5	19.9
5	19.9	20.5	19.9	20.2
Mean ± S.E.M.	20.46 ± 0.15	20.32 ± 0.13	20.10 ± 0.18	20.26 ± 0.22

Appendix 28: Haematological data obtained from blood samples of Wistar rats (eMDA)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	1.3	1.3	2	1.9
2	1.4	1.2	2.6	1.8
3	1.6	1.5	2.1	2.8
4	1.8	1.8	2.6	1.8
5	1.4	1.9	2.1	1.9
Mean ± S.E.M.	1.50 ± 0.09	1.54 ± 0.14	2.28 ± 0.13	2.04 ± 0.19

Appendix 29: Haematological data obtained from blood samples of Wistar rats (TP)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	6.2	6.2	6.2	6.8
2	6	6.8	7	7
3	7	6.6	6.6	7
4	6.8	6.6	6	7.2
5	6.4	6	6	7
Mean ± S.E.M.	6.48 ± 0.19	6.44 ± 0.15	6.36 ± 0.19	7.00 ± 0.06

Appendix 30: Haematological data obtained from blood samples of Wistar rats (N/L ratio)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	0.18	0.35	0.30	0.22
2	0.23	0.28	0.27	0.39
3	0.57	0.16	0.26	0.36
4	0.12	0.20	0.23	0.32
5	0.22	0.40	0.30	0.22
Mean ± S.E.M.	0.26 ± 0.08	0.28 ± 0.04	0.27 ± 0.01	0.30 ± 0.04

Appendix 31: Haematological data obtained from blood samples of Wistar rats (WBC)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	18	16	12.7	19.5
2	20	16	25.9	15.4
3	14	23	15.2	18
4	25.4	17.2	14.2	17.5
5	16	18.2	17.7	20
Mean ± S.E.M.	18.68 ± 2.00	18.08 ± 1.30	17.14 ± 2.34	18.08 ± 0.81

Appendix 32: Haematological data obtained from blood samples of Wistar rats (Neut)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	15	25	21	17
2	19	21	20	35
3	35	14	20	26
4	11	16	17	23
5	18	27	22	18
Mean ± S.E.M.	19.60 ± 4.10	20.60 ± 2.50	20.00 ± 0.84	23.80 ± 3.25

Appendix 33: Haematological data obtained from blood samples of Wistar rats (Lym)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	85	71	70	76
2	84	75	73	89
3	62	86	78	72
4	96	81	75	72
5	82	68	73	82
Mean ± S.E.M.	81.80 ± 5.52	76.20 ± 3.28	73.80 ± 1.32	78.20 ± 3.26

Appendix 34: Haematological data obtained from blood samples of Wistar rats (Mon)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	0	2	3	2
2	0	2	2	3
3	0	0	0	2
4	0	0	3	2
5	0	2	2	0
Mean ± S.E.M.	0.00 ± 0.00	1.20 ± 0.49	2.00 ± 0.55	1.80 ± 0.49

Appendix 35: Haematological data obtained from blood samples of Wistar rats (Eos)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	0	3	4	3
2	3	2	3	2
3	0	0	2	0
4	0	2	3	2
5	0	2	2	0
Mean ± S.E.M.	0.60 ± 0.60	1.80 ± 0.49	2.80 ± 0.37	1.40 ± 0.60

Appendix 36: Haematological data obtained from blood samples of Wistar rats (Band Neut)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	0	0	2	2
2	0	2	2	2
3	0	0	0	0
4	0	1	2	1
5	0	1	1	0
Mean ± S.E.M.	0.00 ± 0.00	0.80 ± 0.37	1.40 ± 0.40	1.00 ± 0.45

Appendix 37: Erythrocyte osmotic fragility data obtained from blood samples of Wistar rats (0.0 % NaCl concentration)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	100	100	100	100
2	100	100	100	100
3	100	100	100	100
4	100	100	100	100
5	100	100	100	100
Mean ± S.E.M.	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Appendix 38: Erythrocyte osmotic fragility data obtained from blood samples of Wistar rats (0.1 % NaCl concentration)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	100	100	100	100
2	100	100	100	100
3	100	100	100	100
4	96.55	100	100	100
5	100	100	100	100
Mean ± S.E.M.	99.31 ± 0.69	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Appendix 39: Erythrocyte osmotic fragility data obtained from blood samples of Wistar rats (0.3 % NaCl concentration)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	90.32	86.67	88	94.28
2	96.43	81.25	94.87	96.77
3	91.18	85	96.69	97.14
4	100	86.21	92.11	93.1
5	96.77	96.97	94.12	92.54
Mean ± S.E.M.	94.94 ± 1.83	87.22 ± 2.62	93.16 ± 1.48	94.77 ± 0.94

Appendix 40: Erythrocyte osmotic fragility data obtained from blood samples of Wistar rats (0.5 % NaCl concentration)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	87.1	80	80	97.14
2	92.86	68.75	92.31	87.1
3	88.24	75	93.75	94.29
4	86.21	82.76	86.42	86.62
5	93.55	90.91	85.29	86.85
Mean ± S.E.M.	89.59 ± 1.51	79.48 ± 3.72	87.55 ± 2.50	90.40 ± 2.23

Appendix 41: Erythrocyte osmotic fragility data obtained from blood samples of Wistar rats (0.7 % NaCl concentration)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	6.45	11.11	12	14.29
2	8.93	12.5	15	12.9
3	11.77	15	26.56	14.86
4	13.79	8.62	15.78	17.24
5	6.45	7.58	17.65	14.93
Mean ± S.E.M.	9.48 ± 1.46	10.96 ± 1.33	17.40 ± 2.47	14.84 ± 0.70

Appendix 42: Erythrocyte osmotic fragility data obtained from blood samples of Wistar rats (0.9 % NaCl concentration)

Animal No.	U-Control	U+Lyco	S-Control	S+Lyco
1	4.84	4.4	4	7.14
2	7.14	8.33	5.13	6.45
3	5.88	10	6.25	8.57
4	6.9	6.9	5.26	8.62
5	3.23	6.06	5.88	8.96
Mean ± S.E.M.	5.60 ± 0.72	7.14 ± 0.96	5.30 ± 0.39	7.95 ± 0.49

Appendix 43: Daily thermal micro-environmental data of the experimental room for the period of study at 7:00 hour.

DAYS	DBT	WBT	DBT –WBT	RH	HI
1	26	23	3	78	28
2	24	24	0	100	25
3	22	22	0	100	23
4	23	22	1	100	24
5	24	24	0	100	25
6	25	24	1	100	25
7	24	24	0	100	25
8	25	22	3	77	26
9	26	23	3	78	28
10	23	22	1	100	24
11	24	23	1	100	25
12	26	24	2	85	28
13	25	23	2	84	26
14	23	22	1	100	24
Mean ± S.E.M.	24.29 ± 0.34	-	-	93.00 ± 2.66	25.43 ± 0.43

Appendix 44: Daily thermal micro-environmental data of the experimental room for the period of study at 13:00 hour.

DAYS	DBT	WBT	DBT –WBT	HI	RH
1	27	24	3	30	78
2	27	24	3	30	78
3	26	23	3	28	78
4	25	24	1	25	100
5	25	24	1	25	100
6	27	24	3	30	78
7	27	25	2	30	85
8	27	24	3	30	78
9	28	24	4	31	72
10	25	23	2	26	84
11	26	23	3	28	78
12	26	23	3	28	78
13	27	24	3	30	78
14	24	25	-1	25	100
Mean ± S.E.M.	26.21 ± 0.30	-	-	28.29 ± 0.59	83.21 ± 2.56

Appendix 45: Daily thermal micro-environmental data of the experimental room for the period of study at 18:00 hour.

DAYS	DBT	WBT	DBT -WBT	HI	RH
1	28	25	3	32	78
2	26	25	1	28	100
3	28	25	3	32	78
4	27	25	2	30	85
5	27	25	2	30	85
6	28	26	2	33	85
7	29	25	4	33	72
8	28	24	4	31	72
9	23	23	0	24	100
10	26	23	3	28	78
11	26	23	3	28	78
12	27	24	3	30	78
13	28	25	3	32	78
14	25	23	2	26	84
Mean ± S.E.M.	26.86 ± 0.42	-	-	29.79 ± 0.72	82.21 ± 2.31

Appendix 46: Mean daily thermal micro-environmental parameters of the experimental room during the study period

Days	DBT	RH	HI
1	27.00	78.00	30.00
2	25.67	92.67	27.67
3	25.33	85.33	27.67
4	25.00	95.00	26.33
5	25.33	95.00	26.67
6	26.67	87.67	29.33
7	26.67	85.67	29.33
8	26.67	75.67	29.00
9	25.67	83.33	27.67
10	24.67	87.33	26.00
11	25.33	85.33	27.00
12	26.33	80.33	28.67
13	26.67	80.00	29.33
14	24.00	94.67	25.00

Appendix 47: Mean thermal micro-environment parameters of the experimental room during the study period.

Hour of the day (h)	Ambient Temperature (^{O}C)	Relative Humidity (%)	Heat index (^{O}C)
7:00	24.29 ± 0.34^{a}	93.00 ± 2.66	25.43 ± 0.43^{b}
13:00	26.21 ± 0.30^{a}	83.21 ± 2.56	28.29 ± 0.59^{c}
18:00	26.86 ± 0.42^{a}	82.21 ± 2.31	29.79 ± 0.72^{c}
Mean ± S.E.M.	25.79 ± 0.77^{a}	86.14 ± 3.44	27.84 ± 1.28^{a}

Data obtained are represented as Mean ± S.E.M. and compared using Students' *t*-test. P-values with different superscript letters along the same row are significantly different (ab = $P < 0.05$; ac = $P < 0.01$)

Appendix 48: Minimum and maximum thermal micro-environment parameters of the experimental room during the period of study.

Hour of the day (h)	Ambient temperature (^{O}C)			Relative Humidity (%)			Heat Index (^{0}C)		
	Minimum	Maximum	Range	Minimum	Maximum	Range	Minimum	Maximum	Range
7:00	22	26	4	77	100	23	23	28	5
13:00	24	28	4	72	100	28	25	31	6
18:00	23	29	6	72	100	28	24	33	9
Mean ± S.E.M	23	27.67	4.67	73.67	100	26.33	24	30.67	6.67