THE ADAPTOGENIC EFFECT OF APPLE CIDER VINEGAR (ACV) IN CHRONIC RESTRAINT- STRESSED WISTAR RATS

By

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DECLARATION

I declare that the work in this dissertation entitled: **"The Adaptogenic Effect of Apple Cider Vinegar (ACV) In Chronic Restraint- Stressed Wistar Rats"** has been carried out by me in the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other institution.

_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled **THE ADAPTOGENIC EFFECT OF APPLE CIDER VINEGAR (ACV) IN CHRONIC RESTRAINT- STRESSED WISTAR RATS** by Rukayya Adebisi ABDULRAUF, meets the regulations governing the award of the degree of Master of Science degree (M.Sc) in Human Physiology of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Stress is a prime factor for most of the illnesses in human life. Chronic stress exposure has been reported to cause anatomical and physiological brain damage, immunosuppression, infertility and damage to various internal organs. The function of stress hormones; corticosterone and epinephrine is believed to preserve homeostasis but these hormones may turn out to be detrimental in excess or if they persist in blood when no more required. Thirty (30) Adult wistar rats of both sexes (n=30), weighing 150-200 g were divided into 3 groups each consisting of a male and female subgroup and given the following treatments once a day for 21 days: Normal control group received 1 ml distilled water orally, the restraint stress (RS) group were exposed to chronic restraint stress 6 hours daily while the Apple cider vinegar (ACV)-treated group received 4 ml/kg of apple cider vinegar orally in addition to chronic restraint stress 6 hours daily. The rats were sacrificed after the experimental period and blood was collected via cardiac puncture and the sera obtained and used for determination of serum level of Corticosterone and Epinephrine as well as Malondialdehyde, Superoxide Dismutase and Catalase activity. Preliminary phytochemical screening on apple cider vinegar gave positive tests for flavonoids, anthraquinones, sterols and triterpenoids as well as tannins. ACV (4ml/kg) showed adaptogenic activity by attenuating lipid peroxidation ($p<0.05$) i.e decreased serum malondialdehyde level (655.52 nmol/mg of protein and 492.92 nmol/mg of protein in the male and female rats respectively) while upregulating endogenous superoxide dismutase activity (26.52 U/ml in male rats and 24.8 U/ml in female rats respectively). Polyphenols and flavonoids in ACV may have ameliorated existing lipid peroxidation and enhanced endogenous antioxidant enzyme activity in response to restraint procedure. However, ACV (4ml/kg) did not decrease ($p<0.05$) serum levels of stress hormones corticosterone (315.43 ng/ml and 334 ng/ml) and epinephrine (38.14 pg/ml and 42 pg/ml) in male and female rats respectively. Females showed a higher stress response with

respect to the stress hormones but this was inconsistent with the antioxidant enzymes. In conclusion, ACV is a potential adaptogen and response to anti-stress therapy in chronic restraint stress wistar rats may be similar in both sexes.

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LIST OF ABBREVIATIONS

ACV- Apple Cider Vinegar

ANOVA- Analysis of Variance

CA- Catecholamines

cAMP- Cyclic Adenosine Monophosphate

CAT-Catalase

CRF- Corticotrophin Releasing Factor

CRH- Corticotropin Releasing Hormone

CRS-Chronic Restraint Stress

CuZnSOD- Copper Zinc Soperoxide Dismustase

DHEA- Dehydroepiandrosterone

DNA- Deoxyribonucleic Acid

EDTA-Ethylene Diamine Tetra acetic Acid

ELISA-Enzyme-Linked immunosorbent Assay

ERT- Estrogen Replacement Therapy

GAS- General Adaptation Syndrome

GC- Glucocorticoids

GLR- Glucocorticoid Receptors

GnRH- Gonadotropin Releasing Hormone

GPX-Glutathione peroxidase

GR- Glutathione Reductase

HCl- Hydrochloric Acid

HPA-Hypothalamo-pituitary Adrenal Axis

Ig- Immunoglobulin

LH- Luteinizing Hormone

MDA-Malondialdehyde

MnSOD- Manganese Superoxide Dismutase

NK- Natural Killer

nNOS. Neuronal Nitric Oxide Synthase

NO- Nitric Oxide

NOS- Nitric Oxide Synthase

OD-Optical Density

PHGPX- Phospholipid hydroperoxide Glutathione Peroxidase

ROS- Reactive oxygen species

RS- Restraint Stress

SA- Sympathoadrenal Axis

SEM- Standard Error of Mean

SNSR- State of Non Specific Resistance

SOD-Superoxide Dismutase

SPSS- Statistical Package of Social Sciences

TBA- Thiobabituric Acid

TBARS- Thiobabituric Acid Reacting Substances

TCA- TricholoroAcetic Acid

CHAPTER 1

1.0 INTRODUCTION

1.1 Background to The Study

Stress has been defined in a multitude of ways by experts in various fields. It is a state of disturbed homeostasis due to internal or external sources such as physical and psychological stimuli known as stressors (Moazzam *et al*., 2013). Researches have shown that many clinical disorders can either be induced or aggravated by stress (Adeoye *et al*., 2009).

Stress is followed by activation of the hypothalamopituitary adrenal and sympathoadrenal axes. These axes serve as a neuroendocrine stress response systems and play vital role in the maintenance of homeostasis (Dhabhar, 2009; Zafir and Banu, 2009; Saxena and Saxena, 2012). Hormones such as Corticotrophin releasing factor (CRF), adrenocorticotrophic hormone (ACTH), glucocorticoids (GCs), and catecholamines (CA) are released from the activated axes and are involved in the stress response (McEwen, 2008; Jameel *et al.*, 2014).

The stress response also includes increased heart rate and blood pressure, increase blood glucose level, protein catabolism, mobilization of free fatty acids and immunosuppression. These reactions to stress are life-saving in the short term (acute stress) but become disruptive and damaging in the long term (chronic stress) (Moazzam *et al.*, 2013).

Immobilization and restraint are regarded as stressful conditions. They result in alterations or disturbances in various body systems thus influencing many physiological aspects of an organism. Physical restraint is a well-known stress model, which increases oxidative processes. Severe and persistent stress elevates reactive oxygen species (ROS) production by metabolic and physiological processes, causing cellular damage (Fazzino *et al*., 2010; Tavakoli *et al.*, 2012; Mosavat *et al*., 2014). Females are known to be more vulnerable to stress and exhibit hyperactivity of the hypothalamopituitary adrenal axis function in

comparison to male animals and this has been identified to be at the level of the hypothalamus (Chatterjee *et al*., 2006).

The word vinegar comes from the French word "vin aigre" meaning sour wine (Waleed, 2012). It can be made from almost all carbohydrates that are fermentable such as dates, sorghum, apples, pears, melons, potatoes and host of others. History has it that a Babylonian courtier in 500 BC discovered wine which formed from unattended grape juice leading to the eventual discovery of vinegar and its use as a food preservative (Mahmoodi *et al.,* 2013). Vinegar is made via two fermentation processes; alcoholic fermentation and acetic acid fermentation. Firstly, yeast (*Saccromyces Crevisae*) is used to convert natural food sugars to alcohol and then in the second process, the alcohol is converted to vinegar by acetic acid bacteria (acetobacter) (Johnston and Gaas, 2006).

Apple cider vinegar (ACV) is a liquid product of fermentation of apples. It has a brownish yellow color. It has been in use for hundreds of years. In the year 400 BC, Hippocrates, the father of modern medicine prescribed the mixture; oxymel made of honey and apple cider vinegar for treatments of persistent coughs and other diseases (Johnston and Gaas, 2006). It was also used during the American civil war for dis-infecting the wounds of soldiers. It is an important element in Asian, European, Western and other traditional cuisines of the world (Soltan and Shehata, 2012).

The beneficial effects of apple cider vinegar are attributed to its acetic acid content, apple pectin and to polyphenolic compounds and antioxidant flavonoids. It has also been reported to contain important minerals, vitamins, probiotic enzymes as well as organic acids such as propionic acid, lactic acid, malic acid, chlorogenic acid etc. It contains a higher concentration of organic acids and phenolic compounds compared to other vinegar types (Soltan and Shehata, 2012; Mohamad *et al*., 2015). The "mother of vinegar" which is a cobweb like or jelly like mass which sinks at the bottom of the vinegar after the fermentation processes gives

ACV its prebiotic quality. It is said to comprise of proteins, enzymes and beneficial bacteria (Shariatpanahi *et al*., 2014).

Even though the existence and use of apple cider vinegar dates back to ancient and even prophetic times, only in recent years has its therapeutic properties being evaluated. It has been widely used in various dosage forms in alternative medicine for several conditions such as diabetes (Johnston and Gaas, 2006; Soltan and Shehata, 2012; Mahmoodi *et al*., 2013), obesity (Iman *et al.*, 2015; Atik *et al*., 2016), hypertension (Jabir *et al*., 2011; Balubaid, 2012), hyperlipidemia (Shishehbor *et al*., 2008; Beheshti *et al.*, 2012; Hamed and Matar, 2014), to enhance wound healing (Waleed, 2012) and relieve varicosities (Atik *et al*., 2016). It has also been shown to possess hepatoprotective properties, have significant antifungal action against candida albicans and aspergillus niger (Jabir *et al.*, 2011) as well as anti-*Helicobacter pylori* activity (Shariatpanahi *et al.*, 2014).

An adaptogen is a substance with a non-specific effect which increases the resistance of the recipient to a variety of physical, chemical, or biological stressors and various phytochemicals, antioxidants and vitamins are said to possess significant adaptogenic properties (Head and Kelly, 2009).

1.2 Statement of Research Problem

Stress is a problem that cannot be overlooked as it has deleterious effects on individuals and their productivity. It is a prime factor for most of the illnesses in human life (Parameaswari *et al.*, 2015). Chronic stress exposure to psychological stress in humans and restraint stress in animals has been reported to cause anatomical and physiological brain damage (Murthy *et al*., 2013), immunosuppression (Dharhbor, 2009), infertility (Shirledge and Cidwolski, 2010; Nsonwu-Anyawu *et al.*, 2015), and damage to various internal organs among a host of others. It has also been implicated in the pathogenesis of diabetes, obesity and even hypertension (Amin *et al*., 2016).

The function of stress hormones is believed to preserve homeostasis but these hormones may turn out to be detrimental in excess or if they persist in blood when no more required. As a result, they are likely to take part in the resultant oxidative stress and tissue damage as well as in the pathophysiology of many diseases (Zafir and Banu, 2009; Moazzam *et al*., 2013; Lodhi *et al*., 2014).

1.3 Justification

Restraint as a stress model combines both psychological and physical components of stress without any painful stimulation in addition to producing robust increases in oxidative stress and plasma concentrations of ACTH and glucocorticoids in rats (Moolchandani, *et al*., 2008; Zafir and Banu, 2009; Jameel *et al.,* 2014). The predominant glucocorticoid in rodents, like rats is corticosterone. Therefore, levels of serum corticosterone may serve as an appropriate indicator of stress in rats. The increase in epinephrine secretion as a result of activation of the sympathetic nervous system is also a typical physiological reaction to stress (Lodhi *et al.,* 2014).

Acetic acid, pectin, and various polyphenols and flavonoids in apple cider vinegar have been reported to possess significant therapeutic properties such as antioxidant, antihyperglycemic and antihyperlipidemic properties (Beheshti, 2012; Naziroglu *et al.,* 2012; Mohamad, 2015). This has led to the emergence of apple cider vinegar as one of the natural remedies under investigation for their medicinal properties.

1.4 Aim of the study

The aim of this study was to determine the adaptogenic effect of apple cider vinegar (ACV) in chronic restraint-stressed wistar rats.

1.5 Specific objectives

i. To determine the effect of apple cider vinegar on serum corticosterone level in chronic restraint-stressed male and female wistar rats.

ii. To determine the effect of apple cider vinegar on serum epinephrine level in chronic restraint stressed male and female wistar rats.

iii. To determine the effect of apple cider vinegar on serum malondialdehye (MDA) level and superoxide dismutase (SOD) and catalase (CAT) activity in chronic restraint-stressed male and female wistar rats.

iv. To determine the phytochemical constituents of Apple cider vinegar.

1.4 Null Hypothesis

Apple cider vinegar does not have an adaptogenic effect in chronic restraint-stressed wistar rats.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 STRESS AND OXIDATIVE STRESS

2.1.1 Stress

The physiologic state of homeostasis in the body is susceptible to various perturbations by intrinsic and extrinsic events, whether actual or perceived **(**Guilliams and Edwards, 2010). Stress can be defined as any physical or physiological factor that results in an interruption of the body's homeostasis (Lukaczer and Panico, 2011). It can be induced in experimental animals in various forms e.g. immobilization, forced swim, exposure to cold environment, starvation e.t.c (Fyiad, 2012). Normally stress induced changes are compensatory, self-limiting and adaptive. However when stressful events of any nature exceeds certain threshold limits, the changes become rather irreversible leading to altered homeostasis and exhaustion (Ittiyavirah and Sajid, 2013).

The hypothalamopituitary adrenal (HPA) axis and the sympathoadrenal axes (sympathetic nervous system) are the key components of the stress axis. The actions of these key regulatory centers and their respective hormones are influenced by genetic, environmental and developmental factors (Guilliams and Edwards, 2010).

2.1.2 The Hypothalamo-Pituitary Adrenal (HPA) and the Sympatho-Adrenal (SA) axes

The hypothalamopituitary adrenal (HPA) and the sympathoadrenal (SA) axes are stress reaction neural as well as biochemical pathways which are activated during conditions considered by the brain as stressful. The activation of the HPA axis is an indication that stress response mechanisms have been set into motion. The general adaptation syndrome (GAS) is the process by which animals generally react to stress and the central component of GAS is

the HPA axis. The hypothalamopituitary adrenal axis regulation includes three processes that are interconnected: diurnal rhythm maintenance, stress or threat reaction/response activation, and using negative feedback mechanisms to re-establish basal activity (Smith, 2014). The hypothalamus contains neuroendocrine neurons that synthesize and secrete corticotrophin releasing hormone (CRH) which regulates the pituitary gland and stimulates the production of adrenocorticotropic hormone (ACTH) which in turn stimulates the adrenal cortices to produce glucocorticoids, mainly cortisol. The glucocorticoids act on the hypothalamus and pituitary, to suppress the production of CRH and ACTH, thus completing the negative feedback circuit. Adrenaline and Noradrenaline are secreted by the adrenal medulla via sympathetic nervous stimulation and local effects of cortisol. They are the key components of the sympatho-adrenal axis. These hormones positively feedback to the pituitary and increase the production of ACTH. The physiochemical responses of the axes are easily triggered by non-physical events. Grief, excitement, fear, anxiety, guilt and embarrassment-all can trigger a robust HPA and SA axes response (Guilliams and Edwards, 2010).

2.1.3 The stress response

Stress response comprises the neural and endocrine adaptations that collectively re-establish homeostasis (Lukazer and Panico, 2011). Irrespective of the diverse stressors placed upon animals, a similar physiological response occurs as reported by Hans Selye (Kelly, 2001). The brain is the first line of defense in stressful situations (Ittiyavirah and Sajid, 2013*;* Smith 2014).

There are no real stress receptors in the brain that are only activated in times of extreme or acute stress. Rather, the responses that have been observed as reactions to stress are ever-present. However, there are thresholds that, when exceeded, cause a reaction that is considered to be a stress response. The hypothalamus is a very important participant in the stress response. Its circuitry communicates with many other brain areas including the

pituitary, which is responsible for the release of several important hormones. To successfully combat stress and stressful situations, adaptation is required. Adaptation might be best thought of as the ability to be exposed to a stressor, while responding with either decreased or no characteristic hormonal perturbations. Adaptation also implies being capable of rapidly reassuming homeostasis after the stressor is withdrawn (Kelly, 2001).

Stress, unlike any other pathological condition, triggers a non-specific response and influences multiple physiological systems. It causes certain changes in the structure and chemical composition of the body, which can be accurately appraised. Some of these changes are merely signs of damage, while others are manifestations of the body`s defensive adaptive reactions These changes caused by stress are collectively called the General Adaptation Syndrome (Head and Kelly, 2009).

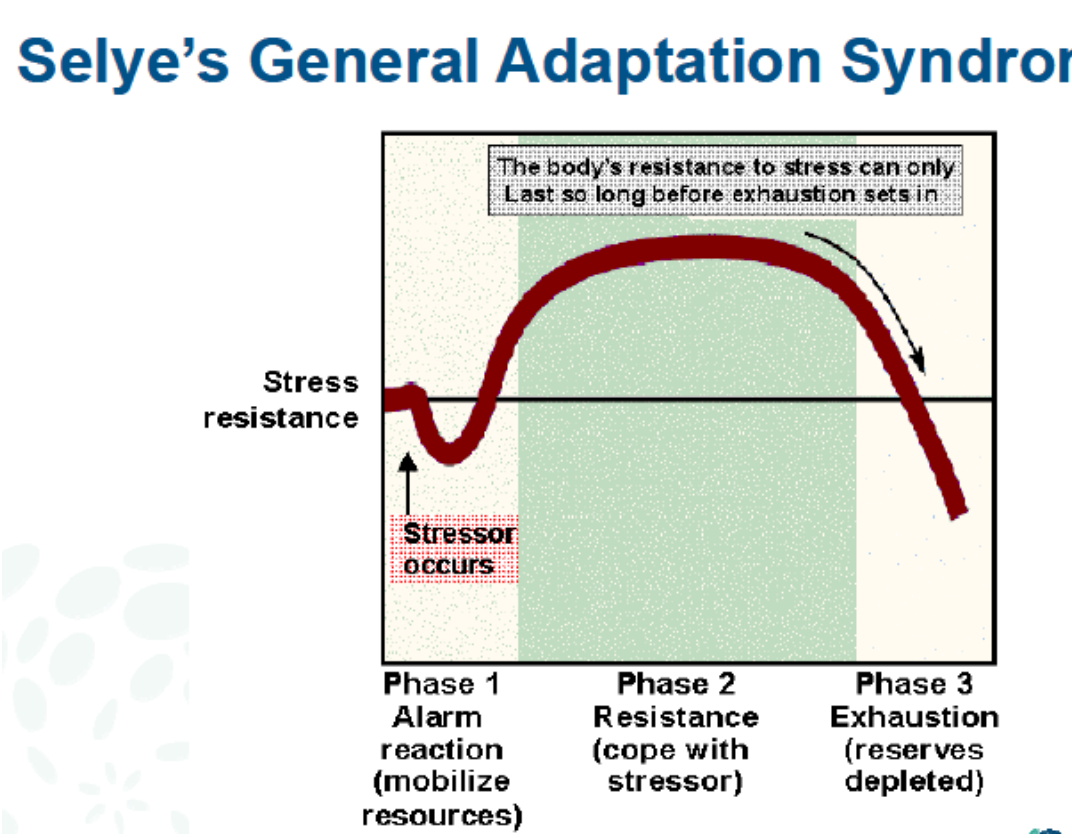


Figure 2.1 General Adaptation syndrome (adapted from google images)

Figure 2.1 illustrates the body's stress response. The body responds to stress in phases: first the alarm phase, then the resistance phase, and sometimes the exhaustion phase.

The alarm-phase stress response is seen through the secretion of catecholamine hormones, which include epinephrine (also known as adrenaline) and norepinephrine. When the hypothalamus detects stress on the body, it sends a signal through the spinal cord to the adrenal medulla, the inner section of the adrenal gland. The cells in the adrenal medulla secrete catecholamine hormones, which induce what is commonly known as the fight or flight response .This response includes an increase in heart rate and blood pressure, bronchodilatation, and other regulations that prepare the body for physical activity (Konduru, 2011).

The resistance phase stress response is the body's attempt to reverse the imbalance that the stressor caused. This response is mediated through the Hypothalamo-pituitary-adrenal (HPA) Axis. The HPA Axis is a signaling pathway in which a stressor is detected by the hypothalamus, which then releases corticotrophin-releasing hormone (CRH). This hormone is then detected by the pituitary gland, which responds by secreting another hormone adrenocorticotropic hormone (ACTH).The ACTH released by the pituitary travels through the blood stream and is eventually detected by the adrenal cortex, which then releases glucocorticoids, including cortisol, as the end of the cycle of positive feedback mechanisms. Finally, in order to maintain a balanced level of hormones in the blood, the cortisol released is then detected by the hypothalamus, which results in a negative feedback mechanism to decrease the release of CRH and subsequently inhibit the rest of the process. The signaling and detecting involved in the HPA axis is distinct to each individual – the amount released and the sensitivity of the receptors varies between organisms. The glucocorticoids that are eventually released from this process are responsible for coping with stress (Panossian *et al.*, 2012).

If the resistance phase fails to restore homeostasis, then the exhaustion-phase kicks in, characterized by the chronic stress response, which results in the breakdown of the body`s defense mechanism . Muscles become fatigued and the body is depleted of resources required to combat stress. Continued exposure to stress during the stage of exhaustion leads to the development of diseases (Goh and Warner, 2012) (Fig 2.1).

2.1.4 Acute Stress

Acute stress has been defined as stress that lasts for a period of minutes to hours (Dhabhar, 2009). Acute stress is experienced in response to an immediate perceived threat. The autonomic nervous system is activated and there is an increase in cortisol and epinephrine levels, higher heart rate, quickened breathing, etc. The fight-or-flight response then kicks in. The term "fight-or-flight" was coined by Walter Cannon in the 1920s when he first described the acute stress response as a theory where an animal reacts to danger "with a general discharge of the sympathetic nervous system". Once the danger is averted, the body returns to homeostasis by activating the relaxation response, which is the opposite of the fight-or-flight response, characterized by reduction of heart rate, decreased production of stress hormones, slower breathing, vasodilation, etc (Sapolsky, 2007; Konduru, 2011) . Acute stress responses promote adaptation and survival via responses of neural, cardiovascular, autonomic, immune and metabolic systems (McEwen, 2008).

2.1.5 Chronic Stress

Chronic stress is stress that persists for several hours per day for weeks or months (Dhabhar, 2009). It is a state of on-going physiological arousal which occurs when the body experiences many stressors or a single stressor continuously. It represents a state of dyshomeostasis resulting from the inability of the adaptive stress response to cope with the intensity or the frequency of various stressors (Saraswathi *et al.,* 2010). Chronic stress can promote and

exacerbate pathophysiology through the same systems that are dysregulated in acute stress (McEwen, 2008) e.g. chronically increased heart rate and blood pressure – produce a chronic wear and tear on the cardiovascular system that can result, over time, in disorders such as stroke and heart attacks (Goh and Warner, 2012; Lodhi *et al*., 2014). Chronic stress can also result in suppression of the immune system, speeding up of the ageing process, infertility, e.t.c (Sapolsky, 2007; Konduru, 2011).

2.1.6 Restraint Stress

Physical restraint of a laboratory animal is the use of manual or mechanical means to limit some or all of an animal's normal movement for the purpose of examination, collection of samples, drug administration, therapy, or experimental manipulation". Some common restraint methods used with laboratory animals include 'squeeze' cages, manual restraint, and restraint boxes or chutes (McMillan *et al*., 2014; Smith, 2014). Rodent restraint stress has been used in modelling human disease for over 85 years and in modelling psychological disease for 35 years (Vorhees *et al.*, 2013).

Acute restraint stress is known to inflict changes upon the brain and inhibition of movement elicits panic and anxiety in rats. Restricted physical activity has been found to elevate stress hormones more than forced physical activity (Jameel *et al*., 2014). Restraint Stress elicits immediate reactions from the nervous and endocrine systems. Membrane lipids in the brain can undergo oxidative damage upon physical restraint stress as this type of stress can trigger brain inflammatory responses including the release of several inflammatory cytokines, free radicals, and the release of protein oxidation marker myeloperoxidase culminating in damaged brain cells (Kim *et al*., 2013). In the induction of restraint stress, the animals are not physically compressed and, therefore, do not experience pain, as such, the stress procedure is believed to be largely psychological in nature due to the feeling of confinement by the animal (Mehta *et al*., 2012).

The effectiveness of restraint as a procedure in inducing stress has been reported by studies. Restraint stress exposure in experimental animals has been shown to cause a significant increase in the levels of catecholamines and corticosterone and this is a well-known finding in response to acute stress; which is the outcome of the stimulation of adrenal cortex and locus coeruleus (Lin *et al*., 2013; Lodhi *et al.,* 2014; Maghsoudi *et al*., 2014). Even two hours of immobilization stress to rats may decrease the testicular steroid concentration along with an increase in the levels of serum corticosterone. It has been suggested that the rise in corticosterone is responsible for the decline in testosterone (Lodhi *et al*., 2014). Twenty one (21) days exposure of rats to restraint stress induced dendritic atrophy in pyramidal cells of the hippocampus (Touyarot and Sandi, 2002). Restraint stress also causes hyperalgesia (Ibironke and Mordi, 2011). It causes a significant elevation in the rate of lipid peroxidation, reduction in nucleic acids and protein in the brain of rats. It has been suggested that restraint results in the generation of reactive oxygen species which may propagate the initial attack on lipid rich membranes of the brain to cause lipid peroxidation (Fyiad, 2012).

2.1.7 Oxidative stress

Oxidative stress, a measure of steady levels of reactive oxygen species (ROS) or oxygen radical in the biological system, is a resultant of overproduction of free radicals i.e. reactive oxygen species (ROS), which exceeds the body's antioxidant defense mechanisms. Reactive oxygen species (ROS) including superoxide anion radical, hydroxyl radical, and hydrogen peroxide, are formed during normal metabolic processes and is exaggerated during stress, can cause oxidative damage of all major groups of biomolecules (DNA, proteins, lipids and small cellular molecules) in the body system (Mehta *et al*., 2012). Normally, antioxidants neutralize ROS and thus help to prevent occurrence of oxidative stress (Mallick *et al.,* 2015). Free radicals play an important role in many cellular processes. At low levels, ROS modulate gene expression, cell proliferation and they can induce transcription and apoptosis. They neutralize

or block the activity of free radicals, thus preventing tissues and cells from damage (Fyiad, 2012). Oxidative stress has been implicated in the development of cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction amongst others (Radomska-Lesniewska *et al*., 2015)

2.1.8 Lipid peroxidation

Lipids are among the primary targets of oxidative stress. During stress, increased production of reactive oxygen species (ROS) leads to increased lipid peroxidation resulting in dyslipidemia which is thought to play an important role in pathogenesis of many degenerative diseases, such as atherosclerosis, damage to DNA, carcinogenesis, and Coronary Artery Disease (Lodhi *et al.*, 2014; Mallick *et al*., 2015)

2.1.9. Antioxidants

Substances that neutralize or keep free radicals or reactive oxygen species at physiological levels are known as antioxidants. They are "substances present at low concentration, compared to oxidizable substrate which significantly delays or prevent the oxidation of the substrate".

Medicinal plants are important sources of biologically active antioxidants. Natural antioxidants such as polyphenols which are abundant in fruits and vegetables, have also received great attention and have been studied extensively, since they are effective free radical scavengers and are assumed to be less toxic than synthetic antioxidants (Doreddula *et al.*, 2014; Radomska-Leśniewska *et al.*, 2015). Mitochondria are the major producers of free radicals or reactive oxygen species (ROS) in the body and some of the adaptive effects of polyphenols that modulate oxidative stress appear to act through the mitochondria. Some antioxidants (endogenous) are made naturally in the body, but others should be obtained from exogenous (external) sources, including the diet (fruits, vegetables and grains), and different natural dietary supplements (Stevenson, 2012). Studies have shown that exogenous

antioxidants can help prevent the free radical damage associated with inflammatory diseases and development of cancer in animal models (Francini and Sebastiani, 2013).

The first line of defense is the preventive antioxidants, which suppress the formation of free radicals e.g Glutathione peroxidase, glutathione-s-transferase, phospholipid hydroperoxide glutathione peroxidase (PHGPX), and peroxidase. The second line of defense is the antioxidants that scavenge the active radicals to suppress chain initiation and/or break the chain propagation reactions, it involves antioxidants such as vitamin E, uric acid, bilirubin, albumin, and thiols. The third line of defense is the repair and *de novo* antioxidants. The proteolytic enzymes, proteinases, proteases, and peptidases, present in the cytosol and in the mitochondria of mammalian cells, recognize, degrade, and remove oxidatively modified proteins and prevent the accumulation of oxidized proteins (Lobo *et al*., 2010; Franchini and Sebastiani, 2013).

2.2 BIOMARKERS OF STRESS AND OXIDATIVE STRESS

Biomarkers of stress include the stress hormones as well as other biomolecules which mediate the stress response. Biomarkers can be used to measure the physiological responses. It has been suggested that males and females respond to stress differently (Konduru, 2011).

2.2.1 Stress Hormones

This includes glucocorticoids and catecholamines. Glucocorticoids regulate catecholamine biosynthesis in the adrenal medulla and catecholamines stimulate adrenocorticotropic hormone release from the anterior pituitary (Moazzam *et al*., 2013).

2.2.1.1 Glucocorticoids; Cortisol

Cortisol is a glucocorticoid steroid hormone synthesized from cholesterol by the adrenal cortex. It has important immunosuppressive and anti-inflammatory effects .It is also involved in the conversion of stored fats and proteins into carbohydrates. It stimulates the release of

catecholamines thus leading to an increase in heart rate and blood pressure. When released in excess it leads to suppression of growth, digestive and reproductive activities (Kim *et al.*, 2008). Cortisol is secreted in response to various stressful situations. Adrenocorticotropic hormone (ACTH), a pituitary hormone, stimulates the secretion of cortisol by a feedback mechanism (Paakkonen and Leppaluoto, 2002). Cortisol modulates cellular activity through intracellular glucocorticoid receptors (GR) found in most tissues. Under normal conditions, plasma cortisol level is about 375nmol/L. Like the stress response in general, cortisol is intended to shunt cellular processes away from long-term metabolic processes and toward those that function primarily for immediate survival and homeostasis. Thus, the negative feedback loop of cortisol on its own secretion is designed to limit long-term exposure of tissues to these short-term catabolic and immunosuppressive actions (Dhabhar, 2009). Chronic and repeated stressors can lead to one or more forms of HPA axis dysregulation, altering appropriate cortisol secretion and affecting end-organ function (Guilliams and Edwards, 2010).

The amount of cortisol in the blood undergoes diurnal variation. The level peaks in the early morning and reaches its lowest level three to five hours after the onset of sleep. Cortisol can be measured from urine, saliva or serum. Cortisol measurements directly capture the status of HPA axis function. Studies have suggested that during acute stress, there is a spike in cortisol levels following exposure to the acute stressor and the levels return to normal once the stress has been resolved. But in chronic stress, levels remain elevated for a period of time before normalizing (Panossian *et al*., 2007).

2.2.1.2 Catecholamines

These are epinephrine and norepinephrine and dopamine synthesized by the adrenal medulla. Norepinephrine is formed by hydroxylation and decarboxylation of tyrosine while epinephrine by methylation of norepinephrine. Phenylethanolamine-N-methyltransferase, the

enzyme that catalyzes the formation of epinephrine from norepinephrine is found in the brain and adrenal medulla and is induced by glucocorticoids. Catecholamines are released in response to emergency situations and their function helps prepare the body to such situations. They increase heart rate, glucose levels, decrease digestion and decrease the activity of the parasympathetic nervous system as part of the flight or fight response (Azad *et al.,* 2015). Studies in rats have suggested that plasma levels of catcholamines are elevated in chronic stress (Paakkonen and Leppaluoto, 2002).

Catecholamines in biological fluids occur in only small quantities, as such the analysis method for their determination should be both selective and sensitive. Immunoassay has emerged as the most powerful tool that fit these requirements with simplicity, specificity and sensitivity in the selective detection of various physiological, biological and environmental substances at trace levels (Kim *et al*., 2008). Enzyme-linked immunosorbent assay (ELISA) is an immunological technique that includes enzyme to detect the presence of analyte in sample (Whiting and Dogue, 2009).

Activation of the hypothalamo-pituitary adrenal axis which is associated with acceleration in oxidative stress via unbalanced redox, including excessive production of mitochondrial reactive oxygen species (Kim *et al.,* 2015), increasing the toxicity of oxygen radical generators (Jorgensen *et al.,* 2013), metabolism of norepinephrine and dopamine which leads to production of free radicals and reactive oxygen species (ROS) (Jafari *et al.,* 2014) and release of catecholamines and glucocorticoids.

2.2.2 Nitric Oxide (NO)

Nitric Oxide (NO), a signaling molecule that is formed from L-arginine and oxygen and whose synthesis is modulated by the enzyme NO synthase (NOS). Nitric Oxide is a gas and free radical and is concerned with modulating neuronal processes and regulating the function of neurons. NOS associated with brain functions is referred to as neuronal NOS, or nNOS.

Stress by restraint increases nNOS in several brain areas. These areas are known to be incorporated with the autonomic nervous system and so it is possible that NO is actually regulating sympathetic output to the periphery either directly or indirectly. Stressful stimuli are known to initiate the transcription of NOS with a resultant increase in nitrergic activity in the HPA axis. This suggests that the regulation of stress hormones, adrenocorticotropic hormone (ACTH) and corticosterone may be under the control of NO (Smith, 2014)**.** Nitric oxide levels can estimated by fluorometric, colorimetric, chemiluminescence as well as chromatographic assays (Bryan and Grisham, 2007).

2.2.3 Oxidative Stress Biomarkers

Reactive oxygen species are kept at physiologically optimal levels by the endogenous antioxidant defense systems as well as natural exogenous antioxidants derived from the diet (Naziroglu *et al.,* 2012). The endogenous antioxidant system, include the antioxidant enzymes which also serve as biomarkers and give a reflection of the oxidative state of the system; superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GLR) (Petrulea *et al*., 2012).

2.2.2.1 Superoxide Dismutase (SOD)

The superoxide dismutases; MnSOD (Manganese superoxide dismutase) and CuZnSOD (Copper-Zinc superoxide dismutase) convert superoxide anions to hydrogen peroxide H_2O_2, which is not in itself a radical, but easily forms one. Hydrogen peroxide is then transformed to water by Catalase or by Glutathione peroxidase. Isoforms of SOD are variously located within the cell. CuZn-SOD is found in both the cytoplasm and the nucleus. Mn-SOD is confined to the mitochondria, but can be released into extracellular space (Djordjevic *et al.,* n. d.).

2.2.2.2 Catalase

Catalase is used by cells to rapidly decompose hydrogen peroxide to water and gaseous oxygen. Hydrogen peroxide is a harmful by product of normal metabolic reactions which must be quickly converted in less dangerous substances to prevent cellular damage. Catalase activity is consistently found to be elevated in liver, heart and aorta, as well as in the brain (Lobo *et al.,* 2010).

2.2.4 Biomarker of Lipid Peroxidation

Malondialdehyde (MDA), also referred to as TBARS (TBA Reacting Substances), is one of the lipid peroxidation products frequently used to determine the oxidant/antioxidant balance in individuals as they are stable and easily measurable. The assay is based on the reactions of thiobarbituric acid with malondialdehyde produced during lipid peroxidation (Mallick *et al*., 2015). The level of TBARS is found to increase in oxidative stress (Mehta *et al*., 2012).

2.3 Effects of Chronic Stress on some Body Systems

2.3.1 Effects of Stress on the Nervous system

The brain is the key organ of the stress response because it determines what is threatening and, therefore, potentially stressful, and also controls the behavioral and physiological responses (McEwen, 2008). The hippocampus is one of the most sensitive and malleable regions of the brain, it expresses a high level of receptors for stress hormones and is also very important in cognitive function (Jorgensen *et al.,* 2013). Cortisol is able to cross the blood brain barrier and bind to receptors in the hippocampus. Long term production of stress hormones is able to induce memory impairments, shrinking and atrophy of the hippocampus (Konduru, 2011). Certain types of acute stress and many chronic stressors suppress neurogenesis (Maghsoudi *et al*., 2014). Stress can induce acute and lethal injury due to free radical attacks in brain tissues since the brain contains large amounts of polyunsaturated fatty acids (Fyiad, 2012).

2.3.2 Effects of Stress on the Immune System

Stress hormones are regarded as being immunosuppressive. Chronic restraint stress compromises the immune status of rats by decreasing the levels of immunoglobulins; serum IgA, IgE, IgG and IgM and lymphocyte count (Moazzam *et al.,* 2013). Stress has also been suggested to play a central role in the incidence and progression of cancers (Lin *et al.*, 2013). Acute psychological stressors and moderate physical exercise transiently enhance immune responses while it suppresses the immune response during chronic stress. In addition, acute stress has also been shown to increase antibody production. It is thus concluded that depending on the duration and severity, psychological tension and physical stress can enhance or suppress the immune system in both humans and animals (Yin *et al*., 2000; Guilliams *et al*., 2010).

2.3.3 Effects of Stress on the Reproductive System

Stress suppresses reproductive functions through released stress hormones. Animal studies have shown that acute and chronic immobilization stress, result in decreased sex hormone levels along with an increase in the levels of serum corticosterone. It has been suggested that rise in corticosterone is responsible for the decline in sexual function (Lodhi *et al*, 2014). Stress causes ineffective pulsation of the "LH Surge". Increased cortisol may decrease the manufacture and activity of progesterone, estrogens, DHEA, and testosterone, this is known as the '*cortisol steal*' or 'pregnenolone steal" (Lukazer and Panico, 2011). Chronic exposure to stressors increases HPA axis activity and concomitantly reduces hypothalamo-pituitary gonadal axis activity. This antagonistic relationship between both these axes has been proposed to underlie the inhibition of reproductive function due to stress (Retana-Ma´rquez *et al*., 2003; Andriyas and Lal, 2015).

2.4 Apple Cider Vinegar (ACV)

Apple cider vinegar (ACV) is a liquid product from fermentation of apples. The word vinegar comes from the French word “vin aigre” meaning sour wine. Vinegar is produced by fermentation of ethanol by acetic acid bacteria, which yields acetic acid. Apple cider vinegar is made from cider or apple must (pressed juice that contains the skin, stems and seeds of the fruit) and it has a brownish-yellow color. It is usually sold unfiltered and unpasteurized with the bacterial culture (or mother of vinegar) still part of the mixture (Waleed, 2012). ACV is very acidic because it contains about 5-6% acetic acid (Budak *et al.*, 2015). Apple and apple products (juice, cider, vinegar) are commonly consumed worldwide. Apple polyphenols contain mainly polyphenolic acid derivatives and other flavonoids. Generally, these polyphenols are distributed in the whole fruit, with higher concentrations present in the peel rather than in the flesh. Phenolic substances were increased by fermentation (Boyer and Liu, 2004).

Figure 2.2: Braggs Apple Cider Vinegar (Braggs, 1912)

2.4.1 Composition

Many medicinal components that are good for health have been reported in natural apple cider vinegar, such as

Organic acids : - oxalic acid, Acetic acid ,succinic acid , citric acid, formic acid, ascorbic (20.5%) , succinic acid, malic acid, lactic acid, propionic acid, syringic acid, vanilic acid, galacturonic acid and fumaric acid, Gallic acid, caffeic acid, Chlorogenic acid, Syringic acid, vanillic acid, Ferulic acid, coumaric acid

Vitamins: A, B_1, B_5, $B_{6,\ B12,}$ C and E

Minerals; silicon, fluorine, iron, sulphur, calcium, magnesium, sodium, chlorine, phosphorus, potassium, zinc, selenium

Flavonoids: Catechin, Pyrogallol Salycillic, quercetin, quercetin glycosides, Rutin (Rt) and Quercetin (Qc) with b-cyclodextrin, kaempferol, Catechin, epicatechin, Cyanidin-3-glucoside, Resveratrol

(Shahidi *et al*., 2008; Soltan and Shehata, 2012; Budak *et al.*, 2015)

2.4.2 Physiological Effects of Apple Cider Vinegar

2.4.2.1 Apple cider vinegar and oxidative stress

Apple cider vinegar has been reported to ameliorate oxidative stress as well as lipid peroxidation. Apple cider vinegar decreased lens oxidative injury in ovaiectomized mice (Naziroglu *et al*., 2012). This is by virtue of its rich phenolic content as well as its flavonoids and carotenoids. Phenolic compounds are secondary metabolites in plants and plant products that contribute to their antioxidant activities Antioxidant strength is measured by both phenolic and non-phenolic compounds, as they reverse free radical activities by decomposing peroxides and unpaired oxygen molecules, and adsorbing and neutralizing the free radicals to render them harmless to the body (Mohamad *et al.*, 2015).

2.4.3.2 Apple cider vinegar and Blood glucose

The antihyperglycemic effect of apple cider vinegar is attributed to its active constituents; acetic acid and other organic acids which may enhance insulin secretion from beta cells. It is proposed that the acetic acid in vinegar may interfere with digestion of starch molecules there by reducing the amount of glucose absorbed into the blood stream after meal, Others suggest that vinegar slows the rate of gastric emptying and thus delays carbohydrate absorption and improves satiety, and the acetic acid enhances uptake of glucose from the blood stream into tissues thereby maintaining blood glucose concentration (Abu-Zaiton, 2011; Iman, 2015; Soltan and Shehata, 2012).

2.4.3.4 Apple Cider Vinegar and Lipid Profile

The hypolipidemic and hypocholesterolemic ability of ACV has been attributed to polyphenols and its acetic acid content which are reported to have lipolytic effect thus suppressing the accumulation of body fat and liver lipids by up regulation of genes for fatty-acid-oxidation- related proteins (Beheshti *et al.,* 2012, Soltan and Shehata, 2012; Hamed and Matar, 2014; Mohamad *et al.,* 2015).

2.4.4 Phytochemicals in Apple Cider Vinegar

2*.4.4.1 Polyphenols:*

Polyphenols are a structural class of organic compounds characterized by the presence of large multiples of phenol structural units. They are the most abundant plant secondary metabolites with highly diversified structures (Kabera *et al.,* 2014). They are divided into simple phenols e.g phenolic acid such as benzoic acid, caffeic acid, chlorogenic acids and polyphenols such as phenylpropanoids and flavonoids (Budak *et al.,* 2015; Francini and Sebastiani, 2013; Vladimir-kenezevic *et al.,* 2012). Some polyphenols are efficient inhibitors of sulfotransferase and can change the activity of thyroid hormones, steroids and catecholamines thus taking part in the stress response. Anti-inflammatory, antibacterial, cardioprotective, anti-oxidant, immunostimulating and anti-anaemic effects have been

correlated with polyphenols (Rzepecka-stojko *et al.,* 2015). Phenolic compounds such as phenypropanoids, phenylethane derivatives and lignans which bear structural resemblance to catecholamines suggest an effect on early stages of stress regulated by the sympathoadrenal nervous system (Panossian and Wikman, 2010).

2.4.4.2 Flavonoids

Flavonoids are the phytochemicals with the skeleton of a chromane ring with aromatic rings attached at the 2- or 3- positions. They are the most abundant class of polyphenols (Vladimir-Knezevic *et al.,* 2012). Flavonoids act by interacting with functional proteins such as enzymes and receptors as their primary target. Some flavonoids are though to act upon the lipid bilayer and modify the membrane physicochemical properties. Flavonoids may ameliorate stress by upregulating cellular stress resistance. Membrane interactive flavonoids inhibit lipid peroxidation and protect cell membranes against oxidative stress (Tsuciya, 2015). The antioxidant properties of flavones allow them to demonstrate potential application as protective and attenuating agents in oxidative stress (Lotankar *et al.,* 2016). There are seven classes of flavonoids, they are: flavons, flavonols, flavonones, flavanes, anthocyanidin, isoflavons and chalcones (Budak *et al.,* 2015; Rzepecka-stojko *et al.,* 2015)

2.4.4.3 Terpenoids

They are aromatic phytochemicals widely distributed in herbs and botanical supplements giving them their characteristic flavour e.g cinnamon, clove, ginger etc. Terpenoids have been used to manage diabetes, inflammatory, microbial and cardiovascular diseases. They also possess some anticonvulsant activity. They include, steroids, carotenoids and triterpenes (Kabera *et al*., 2014). Triterpenes act by activating the adaptive cellular stress pathways (Lee *et al.,* 2014). They also act by targeting anionic phospholipids e. g. phospahtidylglycerol. They are reported to possess antioxidant effect (Vladimir-knezevic *et al*., 2012). Tetracyclic

triterpenes resemble corticosteroids and suggest that they may influence chronic stress response (Panossian and Wikman, 2010).

2.4.4.4 Anthraquinones

They are a class of phytochemicals with a 9, 10- anthraquinone skeleton. They are derivatives of phenolics and have been used traditionally for management of diabetes, burns, fatigue osteoathritis etc (Nandagoapalan *et al.*, 2016).

2.6 Adaptogens

Adaptogens are substances of plant origin which can increase the ability of organism to adapt to a variety of environmental stressors and to avoid stress- induced body damage (Panossian *et al.,* 2012). They possess anti-fatigue and anti-stress activities that can increase mental and physical working performance against a background of fatigue or stress (Domene, 2013; Ittiyavirah and Sajid, 2013).

The term "adaptogen" was coined in 1947 by Russian scientist, Lazarev who described the unexpected effects of Dibazol (2-benzylbenzimidazol)), an arterial dilator developed in France. Dibazol was found to improve the body resistance to stress (Panossian *et al.,* 2012; Domene, 2013). He then defined an "adaptogen" as an agent that allows an organism to counteract adverse physical, chemical, or biological stressors by generating non-specific resistance (Kelly 2001). Adaptogens have a broad range of therapeutic effects without causing any disturbance to the normal functioning of the organism (Mehta *et al*., 2012). The increase in body resistance to external stress conditions is considered an indication of adaptogenic activity (Grace *et al.,* 2009).

Typically these adaptogens prevent or at least decrease certain hormonal changes, such as the increased level of cortisol or corticosterone, that are characteristic of a chronic stress reaction (Panossian *et al*., 2007; Panossian and Wikman, 2012). They are proposed to be amphoteric in nature i.e potentiating the stress response when needed as in acute stress and inhibiting or dampening it when it is becoming deleterious to the body systems as in chronic stress (Pawar and Hugar, 2012).

Chemically, the active components of stress-protective plants and adaptogens can be formally divided into three main groups, namely, tetra (penta)cyclic terpenoids, phenyl- and phenylethyl-propanoids and derivatives, and oxylipins (Grace *et al*., 2009; Panossian and Wikman, 2010).

Since adaptation to stress is associated with the interactions of numerous mediators of the nervous, endocrine and immune systems, and is regulated at all levels of organization (cellular, regulating systems and whole organism). It is very unlikely that different stress protectors have the same mechanism of action (Panossian *et al.,* 2007). Researchers have identified a number of adaptogenic substances in recent times. The most recognized and established adaptogens are extracts of three plants namely: S*chizandra chinensis, Eleutherocsus senticosus* and *Rhodiola rosea* and the trio have been synthesiszed into a single formular known as ADAPT-232 (Panossian and Wangner, 2000).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Animals

Thirty adult wistar rats of both sexes weighing between 150g and 200g were used for this study. They were purchased and housed in the animal house of the department of human physiology, Ahmadu Bello University Zaria under laboratory conditions and had free access to feed and water.

3.1.2 Drugs and chemicals

Raw, Organic and Unfiltered apple cider vinegar (5% acetic acid) was purchased from Braggs (voucher number #G049343406). Enzyme-linked immune-absorbent assay (ELISA) kit for corticosterone (No. GA-E0504RT) and epinephrine (No. GA-E0553RT) were purchased from GenAsia Biotech Co. Ltd.

3.1.3 Equipments

UNIEQUIP ELISA plate reader, Weighing scale, syringes, EDTA bottles, cotton wool, plastic cages, restraint stress cages, cannula, chloroform pool, saw dust, bench centrifuge, normal saline solution, animal feeds.

3.2 Methods

3.2.1 Phytochemical Screening Test

About 5 mls sample of Apple Cider Vinegar was subjected to series of phytochemical screening tests according to standard protocols.

3.2.1.1 Molisch's Test

A few drops of the Molisch reagent were added to the sample. This was followed by the addition of 1.0 ml of concentrated Sulphuric acid down the side of the test tube, the mixture was allowed to stand for 2 minutes and then it was diluted with 5.0 ml of water. The appearance of a red colour at the interphase of the two layers confirms the presence of carbohydrates (Sofowora, 2008).

3.2.1.2 Bontragers test

The sample was combined with 1 mL of 1%v/v sulfuric acid and 1 mL of 5%w/v ferric chloride solution. This mixture was then stirred and boiled in a water bath for 5 minutes. Then, the separated ferric chloride layer was placed in a test tube, and approximately a half volume of 1% w/v potassium hydroxide solution was mixed in and stirred well. A rose-pink to red color was produced in the basic layer indicating the presence of anthraquinones (Harborne, 1998; Sofowora, 2008).

3.2.1.3 Fehlings Test

The sample was combined with equal volumes (1-2 mL) of Fehling A (copper sulfate in distilled water) and Fehling B (potassium tartrate and sodium hydroxide in distilled water).

Then, the sample was boiled in the water bath for 20 minutes. A brick red precipitate was formed, indicating a presence of reducing sugar glycoside (Sofowora, 2008).

3.2.1.4 Kedde-killani's Test

The sample was added to ethanol or methanol in a tray. Then a few drops of Kedde A (2% w/v of 3, 5-dinitrobenzoic acid solution in methanol) was added, followed with the same amount of Kedde B (1% w/v sodium hydroxide in methanol). A purple color was formed, which was a positive result. The mixture of Kedde A and Kedde B solutions giving a light pink color, was a color blank control (Sofowora, 2008).

3.2.1.5 Froth test for Saponins

1-2 mL of distilled water was mixed into the sample and shaken vigorously for approximately 5 minutes. Formation of a stable froth for at least 10 minutes indicated a positive result (Sofowora, 2008).

3.2.1.6 Liberman-Buchard test

The sample was mixed with chloroform and a few drops of concentrate sulfuric acid. The mixture was then shaken well and allowed to stand for a few minutes. A red color formed in the chloroform layer indicated a presence of sterols. Formation of yellow color indicated a presence of triterpenoids (Sofowora, 2008).

3.2.1.7 Sodium hydroxide test

The sample was added to 10% w/v sodium hydroxide solution. Formation of an intense yellow color, which turned colorless after addition of dilute acid solution, indicated a presence of flavonoids (Sofowora, 2008).

3.2.1.8 Mayers/Dragendorff's test

The sample was mixed with 1 mL of 1%v/v sulfuric acid, then a few drops of Dragendorff's reagent (potassium bismuth iodide solution) was added. Alkaloids produce an orange to reddish brown precipitate with these reagents (Harborne, 1998).

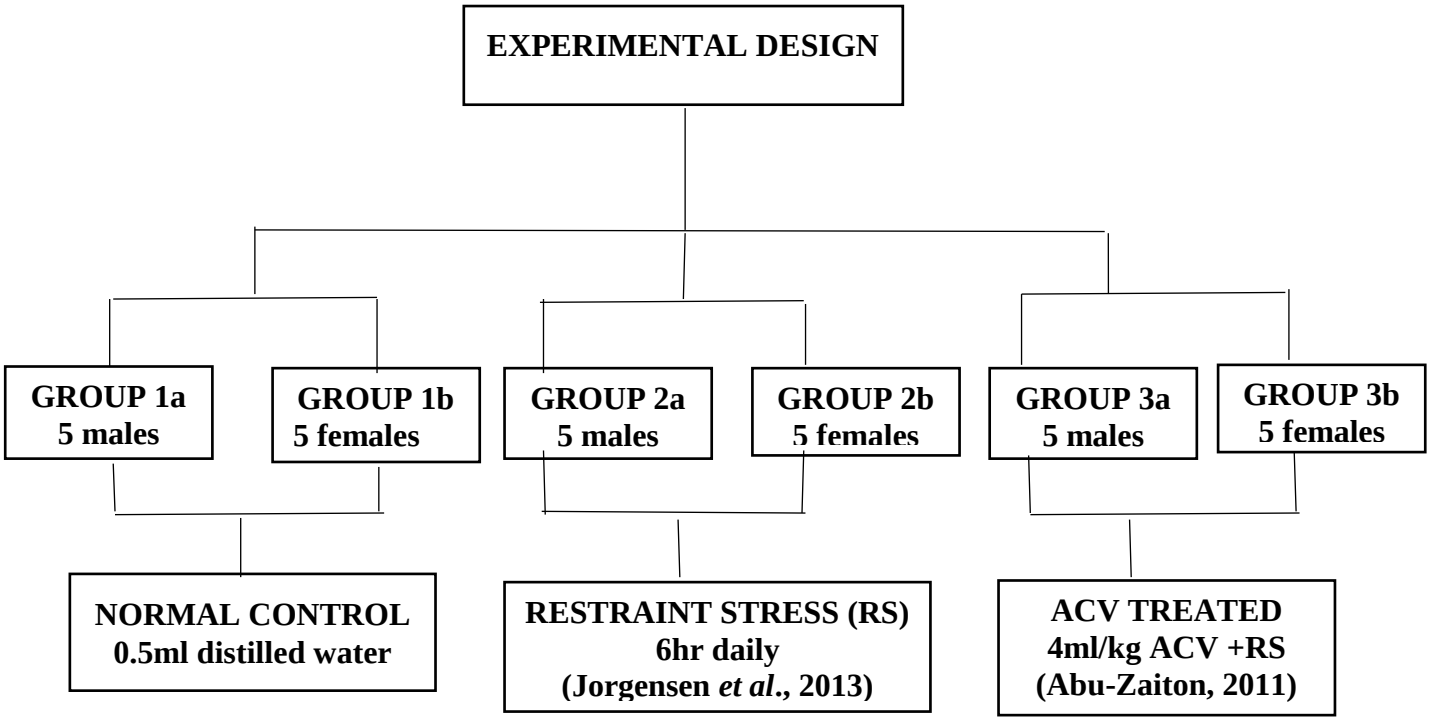
3.2.1.9 Ferric choloride test for tannins

The sample was added to ferric chloride TS, a blue-black or dark blue-green precipitate appeared indicating a positive result (Sofowora, 2008).

3.2.2 Experimental Design

The thirty adult wistar rats comprised 15 males and 15 females, the males were weighed and randomly divided in to three groups of five rats each namely; the normal control group, the restraint stress group and the apple cider vinegar treated group.

This same procedure was also applied to the female rats and then the following treatments were administered once daily for 21 days as summarized by the chart below:



3.2.3 Induction of Restraint Stress

Restraint stress was induced by the method of Smith (2012). Small restraint cages made from Perspex were used. Each rat was housed individually in a multi-compartment cage in which they fit in tightly for the induction of restraint stress but the control group was left in their cages but without access to food or water during the restraint period. The rats were exposed to chronic restraint stress, 6 hours daily for 21 days. This procedure was carried out in the animal house of the department of human physiology, Ahmadu Bello University Zaria.

Plate I: Induction of restraint stress

3.2.4 Collection of Blood Samples

On the 22nd day, after the last dosing of the four groups, the animals were sacrificed using chloroform anesthesia. Blood samples of about 5 ml was drawn from all groups via cardiac

puncture. The samples were allowed to coagulate and then centrifuged and the sera obtained for the various analyses.

3.2.5 Determination of Serum Hormonal Levels

3.2.5.1 Determination of Serum Corticosterone Level

The serum level of corticosterone was determined using enzyme Linked immune-absorbent assay (ELISA) kit according to manufacturer's instructions. The principle of the assay kit was based on the biotin double antibody sandwich technology to assay rat corticosterone.

Briefly, 40 µl of the sample, 10µl of the anti-corticosterone antibodies labeled with biotin and 50µl streptavidin-HRP were added to wells pre-coated with corticosterone monoclonal antibody and then incubated for 60minutes at 37^0C. After incubation, the unbound enzymes were then removed using a washing solution which had earlier been diluted with distilled water 20 times. 50 µl each of chromogen solution A and B were then added and incubated for another 10minutes at 37^0C for color development. Next, 50µl of stop solution was added to each well to stop the reaction, the solution immediately changed color from blue to yellow with the effect of the acid. The shades of solution and the concentration of rat corticosterone are positively correlated. The assay was then performed by taking the blank as zero and the absorbance (OD) of each well measured under 450nm wavelength. The standard curve was then obtained by plotting the standards concentration against their corresponding OD values. Then according to the OD value of samples, the concentration of the corresponding sample was extrapolated (Assay range: 5ng/ml-1000 ng/ml; sensitivity: 2.51 ng/ml).

3.2.5.2 Determination of Serum Epinephrine Levels

The serum level of epinephrine was determined using enzyme Linked immune-absorbent assay (ELISA) kit according to manufacturer's instructions. The principle of the assay kit was based on the biotin double antibody sandwich technology to assay rat Epinephrine.

Briefly, 40µl of the sample, 10 µl of EPI antibodies and 50 µl streptavidin-HRP were added to wells pre-coated with Epinephrine monoclonal antibody and then incubated at 37^0C for 60minutes. After incubation, the unbound enzymes were removed using the washing solution which was provided. 50 µl each of chromogen solution A and B were then added and incubated for another 10minutes at 37^0C away from light for color development. 50 µl of stop solution was added to stop the reaction. The solution immediately changed color from blue to yellow with the effect of the acid. The shades of solution and the concentration of rat Epinephrine are positively correlated.

The assay was then performed by taking the blank as zero and the absorbance (OD) of each well measured under 450nm wavelength. The standard curve was obtained by plotting concentration of the standards against their corresponding OD values. Then according to the OD value of the samples. The concentration of the corresponding sample was extrapolated (Assay range: 1pg/ml-80pg/ml; sensitivity: 0.55 pg/ml).

3.2.6 Determination of Biomarker of lipid peroxidation.

Lipid peroxidation was measured by the modified method of Niehaus and Samuelson (1968) as described by Akanji *et al*. (2009).

Principle:

Estimation of lipid peroxidation is based on the reaction of Malondialdehyde (MDA) produced during lipid peroxidation with thiobabituric acid (TBA) forming a pink coloured MDA-TBA adduct that absorbs strongly at 535nm.

Reagents:

15% tricholorocetic acid (TCA) solution.

0.37% Thiobabituric Acid (TBA) solution.

0.25N Hydrochloric acid (HCL) solution.

Procedure:

The above reagents TBA-TCA-HCL were mixed in a ratio of 1:1:1 to prepare the working reagent. To 0.15ml of serum, 2.0ml of working reagent was added and placed in water bath at 90^{o}C for 60 minutes. The mixture was cooled and centrifuged at 3000× for 5 mins. The absorbance of the pink supernatant was measured at 535nm against reference blank containing 0.15ml distilled water instead of serum.

Calculation:

The MDA was calculated using the molar extinction coefficient of 1.56×10^{5} cm^{-1} M^{-1}

MDA conc= absorbance/ 1.56×10^{5} $cm^{1-}M^{-1} \times 1$

3.2.7 Determination of Biomarkers of Oxidative Stress

3.2.7.1 Determination of Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined by a method described by ***Fridovich*** (1986).

Principle:

The ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenaline at pH 10.2 forms the basis of this assay.

Reagents:

0.05M phosphate buffer solution, pH 7.8.

0.05 Carbonate buffer solution, pH 10.2.

0.3 mM Adrenaline: The solution was prepared fresh.

Procedure:

An aliquot (0.1ml) of serum was diluted in 0.9ml of distilled water to make 1:10 dilution. Then 0.20 ml of the diluted serum was added to 2.5ml of 0.05M Carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3mM Adrenaline. The reference mixture contained 2.5ml of 0.05M Carbonate buffer, 0.3ml of 0.3mM Adrenaline and 0.20 ml of distilled water. Absorbance was measured every 30 seconds up to 150 seconds at 480nm.

Calculations:

Increase in absorbance per minute = $(A_5-A_1)/2.5$

A_5 (absorbance after 5 minutes); A_1 (absorbance after 1minute)

$$\% \text{ Inhibition} = 100 - \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance of blank}} \times 10$$

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute

*3.2.7.2 Determination of Catalase Activity***.**

Catalase activity was determined using the method described by ***Sinha (1972).***

Principle:

The method is based on the reduction of Dichromate in acetic acid to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of perchromic acid as unstable intermediate. Chromic acetate so produced was measured colorimetrically using spectrophotometer at 570nm. This is because dichromate has no absorbance at 570nm and does not interfere with the determination of catalase.

Reagents:

5% potassium heptaoxochromate (VI), ($K_2Cr_2O_7$) solution

0.2 M Hydrogen peroxide (H_20_2)

Dichromate/Acetic acid solution: exactly 5% potassium heptaoxochromate (VI), $K_2Cr_2O_7$, was mixed with glacial acetic acid in the ratio 1:3 (w/v) and stored in brown bottle at room temperature.

Procedure:

To 2.0ml of serum, 1ml of 0.01M phosphate buffer was added followed by 0.4ml of 2M H_2O_2 into two separate test tubes labelled T0 and T1 (blank and test respectively). Exactly 2ml of potassium Dichromate: glacial acetic acid reagent was added to test tube T0 and immediately placed in a water bath at 80^{o}C for 10 minutes. To test tube T1, 2ml of potassium Dichromate: glacial acetic acid reagent was added exactly 10 minutes after addition of H_2O_2 and placed in water bath at 80^{o}C for 10 minutes. The test tubes were cooled to room temperature and absorbance was read at 570 nm against a blank containing 2.4ml distilled water, 1ml 0.01M phosphate buffer and 2ml potassium Dichromate: glacial acetic acid reagent. It was placed in water bath at 80^{o}C for 10minutes.

Calculations:

Catalase activity was determined from the concentration of H_2O_2 in test tube T1 that was neutralized by catalase.

3.2.8 Statistical Analysis

All data was expressed as mean ± standard error of mean (S. E. M.) and analysed using two-way analysis of variance (ANOVA), followed by Tukey' post-hoc test to compare the level

of significance between groups using SPSS version 20.0. Values of $p < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 PHYTOCHEMICAL SCREENING OF APPLE CIDER VINEGAR

CONSTITUENT	TEST	INFERENCE
Carbohydrates	Molisch test	+
Anthraquinones	Bontragers test	+
Glycosides	Fehlings test	+
Cardiac Glycoside	Kelle-killani test	+
Saponins	Froth test	–
Steroids and triterpenes	Liberman Buchard test	+
Tanins	Ferric chloride test	+
Flavonoids	Sodium hydroxide	+
Alkaloids	Mayers test	-
	Dragendorff	-

KEY: + present, - absent

4.2 Effect of Apple Cider Vinegar (ACV) on serum level of Corticosterone in restraint-stressed male and female wistar rats.

In the male rats, the serum corticosterone level was significantly ($p<0.05$) higher in the restraint stressed group (329.86±3.49 ng/ml) when compared to the normal control group (109.14±0.67 ng/ml). The ACV-treated rats (315.43±14.37 ng/ml) showed a decreased serum corticosterone compared to the restraint group although not statistically significant ($p<0.05$).

In the female rats, the restraint group showed a significantly ($p<0.05$) higher serum corticosterone (391±22.77 ng/ml) than the normal control group (223±3.23 ng/ml). The serum corticosterone was decreased although not significant ($p<0.05$) in the ACV-treated group (334±15.28 ng/ml).

The serum corticosterone was statistically significantly ($p<0.05$) higher in the normal control female rats (223±3.23 ng/ml) than in the male rats (109.14±0.67 ng/ml). The same observation was made regarding the restraint stress group female (391±22.77 ng/ml) and male rats (329.86±3.49 ng/ml) ($p<0.05$). However in the ACV treated group, there was no statistically significant ($p<0.05$) difference between the male (315.43±14.37 ng/ml) and female (334±15.28 ng/ml) rats serum corticosterone level (Fig 4.1).

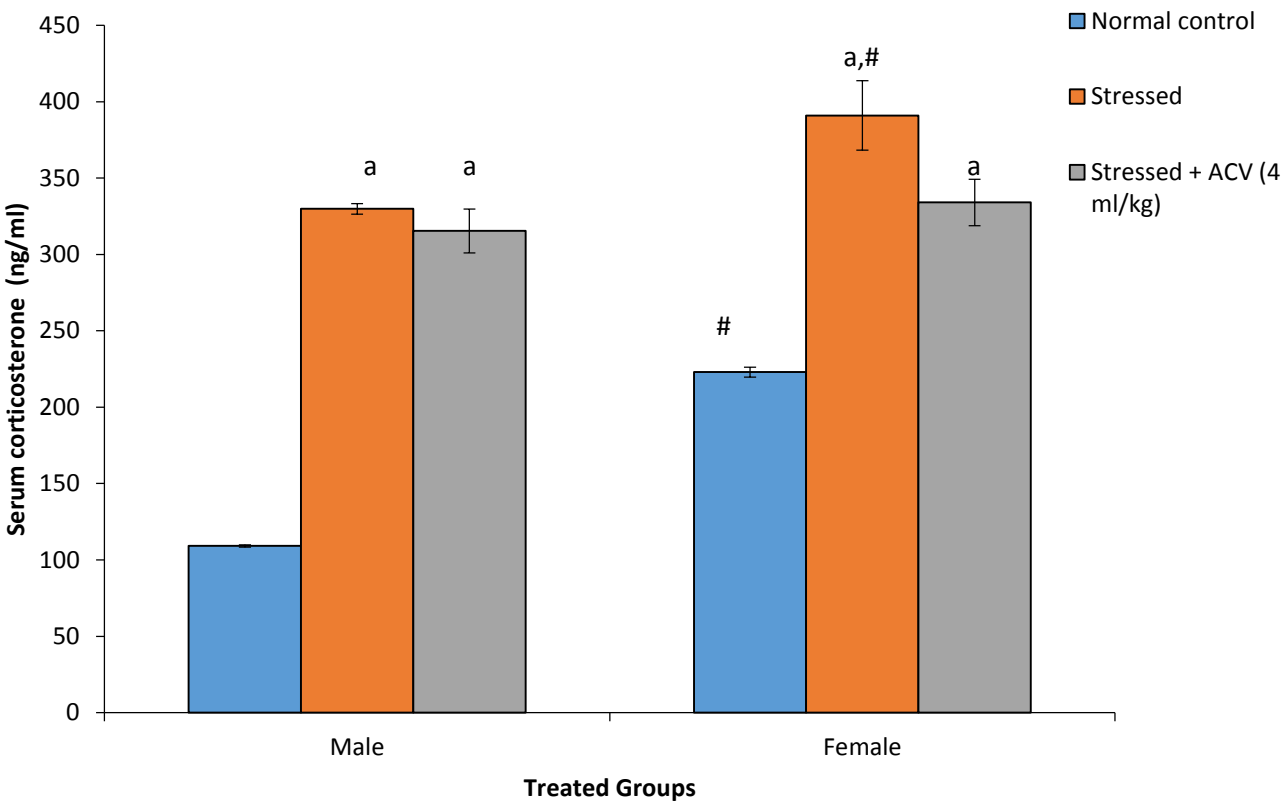


Figure 4.1: Serum levels of corticosterone in restraint-stress male and female wistar rats treated with Apple Cider Vinegar (ACV) a= statistically significant compared to normal control group of the same sex, #= statistically significant between different sexes of the same group

4.3 Effect of Apple Cider Vinegar (ACV) on serum level of epinephrine in restraint-stressed male and female wistar rats.

In the male rats, there was no statistically significant difference ($p<0.05$) in serum epinephrine level between the restraint stress group and the normal control group (41.29±1.09 pg/ml). The serum epinephrine was decreased in the ACV-treated group (38.14±4.2 pg/ml) when compared to the restraint stress group (43±1.36 pg/ml) although not statistically significant ($p<0.05$).

In the female rats, the serum epinephrine level increased but not statistically significant ($p<0.05$) in the restraint stress group (48.86±6.3 pg/ml) when compared to the normal control group (43±1.99 pg/ml). There was also no significant difference ($p<0.05$) between the ACV-treated (42±1.33 pg/ml) and restraint stress group (48.86±6.3 pg/ml) though the serum epinephrine level was lower in the ACV-treated group (42±1.33 pg/ml).

There was no significant ($p<0.05$) difference in serum epinephrine level between the male (41.29±1.09 pg/ml) and female rats (43±1.99 pg/ml) in the normal control group. The restraint stress male (43±1.36 pg/ml) and female rats (48.86 ±6.3 pg/ml) also did not show any statistically significant difference ($p<0.05$) in serum epinephrine level. Serum

epinephrine level was also not significantly different ($p<0.05$) in the male (38.14±4.2 pg/ml) and female (42±1.33 pg/ml) ACV-treated rats (Fig 4.2)

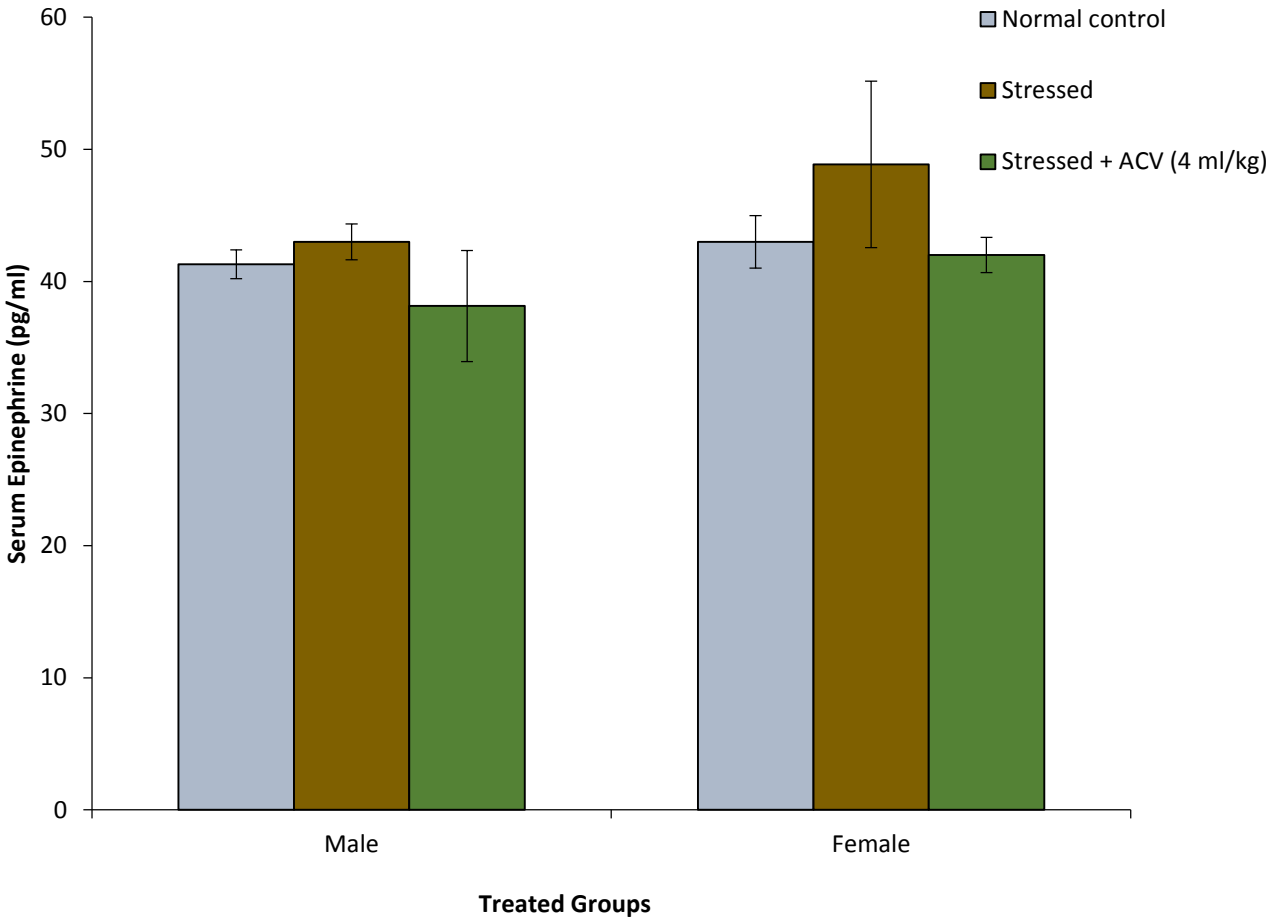


Figure 4.2: Serum levels of epinephrine in restraint-stress male and female wistar rats treated with Apple Cider Vinegar (ACV)

4.4 Effect of Apple Cider Vinegar (ACV) on serum level of Malondialdehyde (MDA) activity in restraint- stressed male and female wistar rats.

In the male rats, serum Malondialdehyde level was increased in the restraint stress group (710.32±26.45 nMol/mg of protein) when compared to the normal control group (547.28±3.33 nMol/mg of protein) although not statistically significant ($p<0.05$).The ACV-treated group (655.52±15.49 nMol/mg of protein) showed a decreased serum malondialdehyde level also not statistically significant ($p<0.05$) when compared to the restraint stress group (710.32±26.45 nMol/mg of protein).

In the female rats, there was non-significant increase ($p<0.05$) in serum Malondialdehyde level in the restraint stress group (740.68±14.8 nMol/mg of protein) when compared to the normal control group (574.52±4.05 nmol/mg of protein). The ACV-treated group (492.92±8.15 nMol/mg of protein) however showed a significant ($p<0.05$) decrease in serum Malondialdehyde level when compared to the restraint stress group (740.68±14.8 nMol/mg of protein).

There was no significant difference ($P<0.05$) in the serum malondialdehyde level between the normal control male (547.28±3.33 nMol/mg of protein) and female rats (574.52±4.05 nmol/mg of protein). The serum malondialdehyde level between the restraint stress male (710.32±26.45 nMol/mg of protein) and female rats (740.68±14.8 nMol/mg of protein) also did not show any significant difference ($p<0.05$). However, in the ACV-treated rats, the female rats (492.92±8.15 nMol/mg of protein) had a significantly ($p<0.05$) lower serum Malondialdehyde level when compared to the male rats (655.52±15.49 nMol/mg of protein) (Fig 4.3)

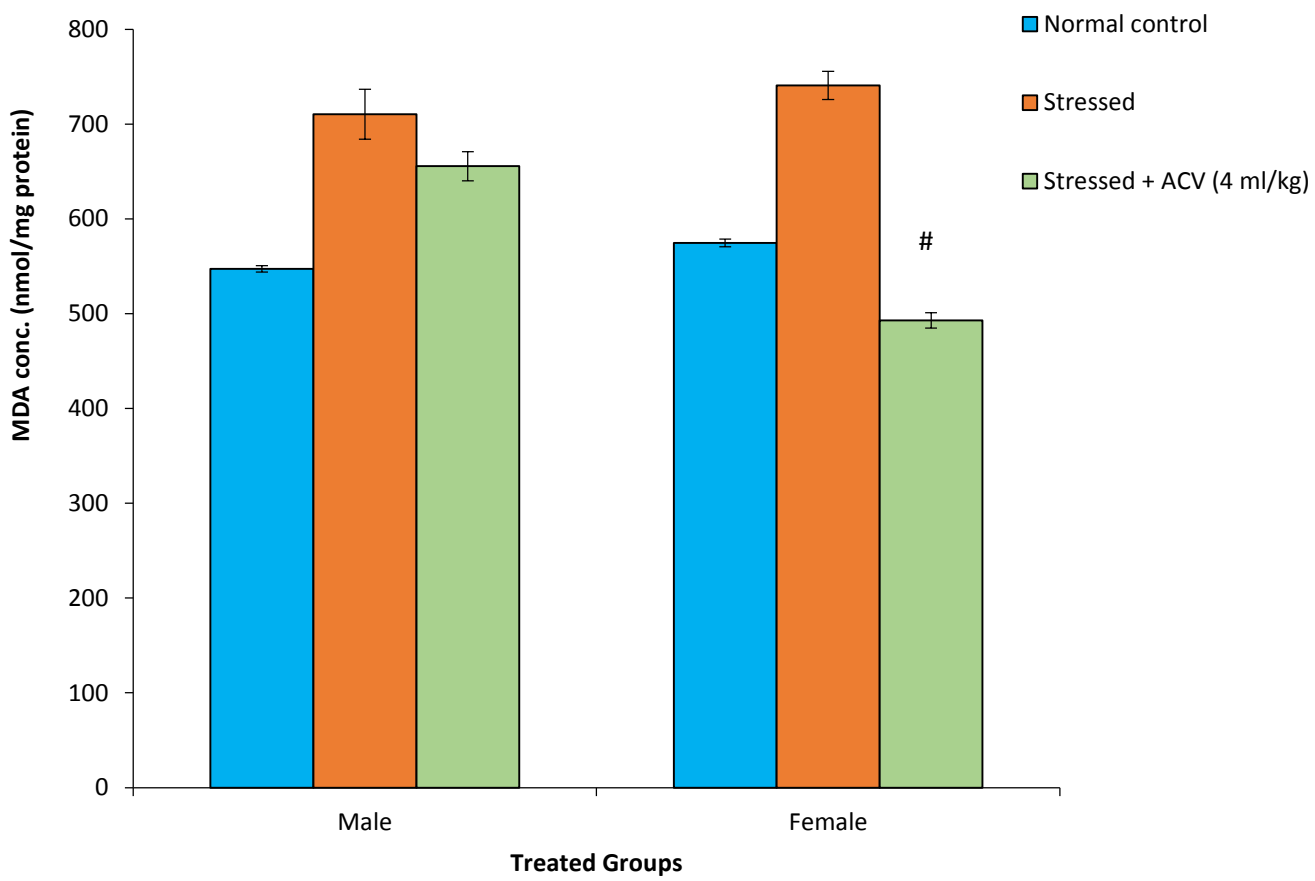


Figure 4.3 : Serum level of Malondialdehyde (MDA) in restraint stress male and female wistar rats treated with Apple Cider Vinegar (ACV) #= statistically significant difference between males and females of the same group.

4.5 Effect of Apple Cider Vinegar (ACV) on serum level of superoxide Dismutase (SOD) activity in restraint- stressed male and female wistar rats.

In the male rats, there was no significant difference ($p<0.05$) in the serum level of Superoxide Dismutase activity between the normal control (16.98 ±0.5 U/ml) and the restraint stress group (18.86±0.21 U/ml). The ACV-treated group (26.52±0.39 U/ml) showed a significantly ($p<0.05$) higher serum Superoxide Dismutase activity when compared to the restraint stress group (18.86±0.21 U/ml).

In the female rats, serum Superoxide Dismutase activity did not show significant difference ($p<0.05$) in the restraint stress group (17.62±0.66 U/ml) when compared to the normal control group (14.74±0.22 U/ml). However, in the ACV-treated group (24.8±1.11 U/ml), serum Superoxide Dismutase activity increased significantly ($p<0.05$) when compared to the restraint stress group (17.62±0.66 U/ml).

There was no significant difference in serum Superoxide Dismutase activity between the male (16.98 ±0.5 U/ml) and female (14.74±0.22 U/ml) normal control group of rats. In the restraint stress group also, serum Superoxide Dismutase activity did not show significant difference between the male (18.86±0.21 U/ml) and female group (17.62±0.66 U/ml) of rats. There was no significant difference between the serum superoxide dismutase activity in the male rats 26.52±0.39 U/ml) of the ACV-treated rats when compared to the female rats (24.8±1.11 U/ml) (Fig 4.4).

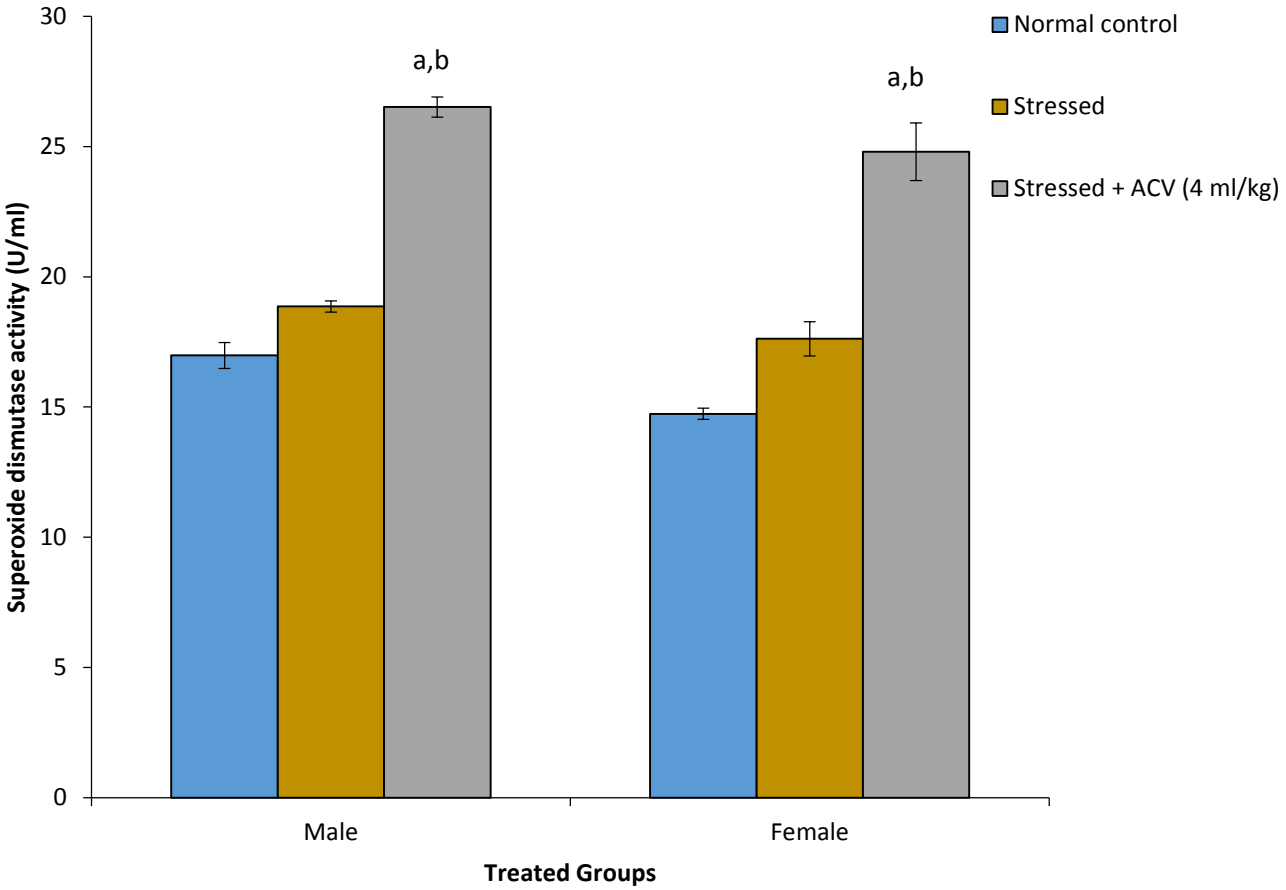


Figure 4.4**:** Serum levels of Superoxide dismutase (SOD) activity in restraint stress male and female wistar rats treated with Apple Cider Vinegar (ACV) , a= statistically significant compared to normal control, b=statistically significant compared to restraint stress group

4.6 Effect of Apple Cider Vinegar (ACV) on serum level of Catalase (CAT) activity in restraint- stressed male and female wistar rats.

In the male rats, there was no significant ($p<0.05$) difference in the serum catalase activity between the restraint stress group (4.22±0.19 U/mg of protein) when compared to the normal control group (3.79± 0.32 U/mg of protein). There was however a significant ($p<0.05$) increase in serum Catalase activity in the ACV-treated group (5.34±0.29 U/mg of protein) when compared to the restraint stress group (4.22±0.19 U/mg of protein).

In the female rats, serum Catalase activity was significantly higher in the restraint stress group (6.3±0.14 U/mg of protein) when compared to the normal control group (3.88 ±0.29 U/mg of protein). Serum catalase activity was decreased significantly ($p<0.05$) in the ACV-treated group 3.67±0.17 U/mg of protein) when compared to the restraint stress group (6.3±0.14 U/mg of protein)

There was no significant difference ($p<0.05$) in serum Catalase activity between the male (3.79± 0.32 U/mg of protein) and female (3.88 ±0.29 U/mg of protein) normal control group of rats. In the restraint stress group, serum Catalase activity was significantly ($p<0.05$) higher in the female rats (6.3±0.14 U/mg of protein) when compared to the male rats (4.22±0.19 U/mg of protein). The serum catalase activity was significantly ($p<0.05$) higher in the female rats 3.67±0.17 U/mg of protein) when compared to the male rats (5.34±0.29 U/mg of protein) of the ACV-treated group (Fig 4.5).

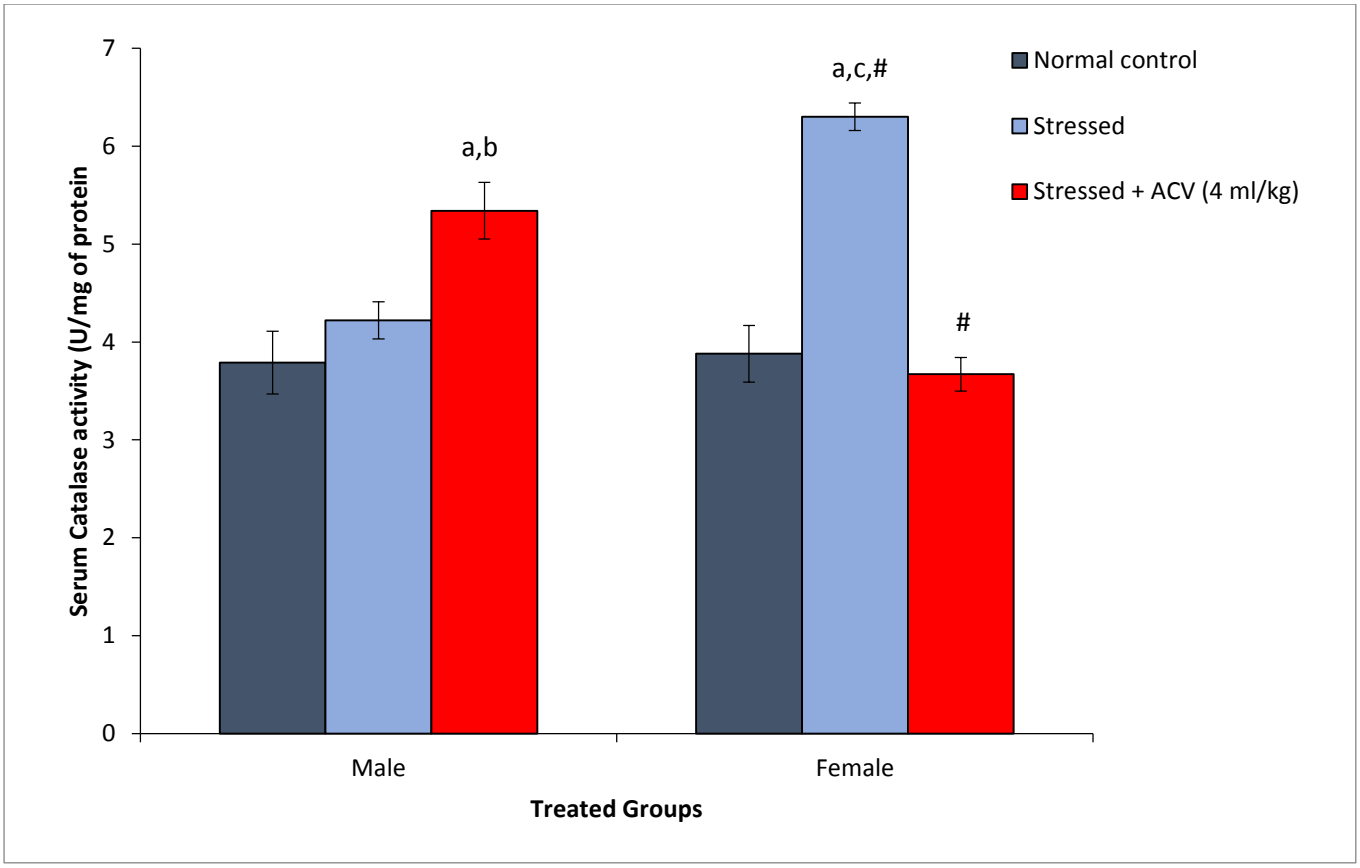


Figure 4.5: Serum levels of Catalase (CAT) activity in restraint stress male and female wistar rats treated with Apple Cider Vinegar (ACV), # = statistically significant difference between different sexes of the same group, a= statistically significant compared to normal control group, b=statistically significant compared to restraint stress group, c= statistically significant compared to ACV-treated group

CHAPTER FIVE

5.0 DISCUSSION

Stress produces an endocrine and metabolic response, a strong feature of which is the increase of the stress hormones mainly corticosterone and catecholamine level. Long-term exposure to elevated stress hormones has been associated with several pathologies (Popovic and Pajovic, 2010). This study aimed to determine the potential adaptogenic or anti-stress effect of apple cider vinegar (ACV) in adult wistar rats subjected to chronic restraint stress.

Adult wistar rats of both sexes were treated with apple cider vinegar (ACV) and exposed to chronic restraint stress 6 hours daily for 21 days. The result of the present study showed that chronic restraint stress caused an increase in the serum levels of corticosterone and epinephrine, indicative of the presence of stress induced by restraint. The increase in serum corticosterone level observed in rats exposed to chronic restraint stress alone could have been due to stimulation of hypothalamopituitary axis (HPA) which leads to an increased corticosterone release in response to the restraint. This observation is similar to that of Popovic and Pajovic, (2010), Malisch *et al.,* (2007) and Vorhees *et al.,* (2013).This observation however disagrees with the work of Bowman *et al*., (2001).

The significantly higher corticosterone level in the female stressed rats compared to the male stressed rats could have been be due to the higher level of corticotrophin releasing hormone found in the female hypothalamus thus making the hypothalamopituitary adrenal (HPA) axis more reactive relative to that of the males. It could also have been due to a suppressive effect of androgens on the hypothalamopituitary adrenal axis by decreasing secretion of adrenocorticotropic hormone and corticosterone and increasing central negative feedback sensitivity (Chatterjee, 2006). This observation agrees with the reports of Bowman *et al*., (2006) and Malisch *et al*., (2007).

Although, stress also results in the stimulation of sympathetic nervous system via the sympathoadrenal axis, which results in increased epinephrine level, the reverse was the case

in this study as no significant increase was seen in restraint stressed rats. This could be because, epinephrine is more essential in the activation of the fight or flight response (acute stress) rather than in chronic stress. As such, it could have been that there were elevated epinephrine levels in the first few days of the stress induction i. e. acute phase of the stress to the animals then the levels may have dropped considerably as the study period progressed. Lodhi *et al.,* (2014) reported that epinephrine levels are significantly elevated in acute restraint stressed rats. It could also be because acute tress tends impact monoamine levels and this may influence catecholamine levels more than chronic stress, while chronic stress places greater demands on the hypothalamopituitary adrenal axis (Kelly, 2001).

The slight increased serum levels of oxidative stress biomarkers; malondialdehyde, superoxide dismutase and catalase activity seen in the restraint stress rats maybe indicative of enhanced oxidative stress from the chronic stress induction. It could be that the biomarkers were significantly elevated in the initial stages of the study in response to the resultant oxidative stress and have been depleted at the chronic stress phase resulting in the observed slight difference in the stressed group. Glucocorticoids are reported to generate free radicals especially as stress progresses i.e chronic stress .This is in agreement with the works of Fyiad (2012) and Jafari *et al*., (2014) who observed a significant increase in malondialdehyde levels in restraint stressed rats when compared to the normal control rats. Ozkol *et al*., (2011) also reported sustained elevation in oxidative stress biomarkers; superoxide dismutase, glutathione peroxidase and catalase levels in repeated restraint rats. The elevated levels of biomarkers of oxidative stress in the restraint stress rats disagrees with the findings of Abd- Aziz *et al*., (2014) who observed an increased malondialdehyde level but attenuated levels of other antioxidant enzymes; superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities in stressed rats.

From this study, Apple cider vinegar (ACV) treatment appears to have some adaptogenic effect. Preliminary phytochemical screening of Apple cider vinegar revealed the presence of triterpenes and phenolics and these represent two out of three compounds into which adaptogens have been grouped with the third being oxylipins (Panossian *et al.,* 2007). The decrease in corticosterone level, although not significant as observed in the Apple Cider Vinegar- treated rats may be due to this adaptogenic effect. Apple cider vinegar may decreased the sensitivity of various components of the hypothalamopituitary-adrenal axis to prevent excessive stress induced hormonal elevation in the treated rats (Head and Kelly, 2009). The decrease in serum level of epinephrine in the ACV treated group can also be attributed to a possible adaptogenic effect of apple cider vinegar, it may have inhibited a stress induced increase in serum catecholamine level by dampening the stress response to chronic restraint (Pawar and Hugar, 2012). There were no observable sex differences in response to ACV treatment. This could mean that both sexes respond to anti-stress therapy equally.

Apple cider vinegar treatment attenuated serum Malondialdehyde activity, this could be due to the protective effect of ACV in ameliorating the excessive lipid peroxidation that may have occurred in the animals due to chronic stress. The polyphenols in Apple Cider Vinegar (ACV) may have acted by interrupting the free-radical chain of oxidation by donating hydrogen from phenol's hydroxyl groups, thereby forming stable free radicals, which do not initiate or propagate further oxidation of lipids (Fyiad, 2012). This is in agreement with work of Naziroglu *et al.,* 2012. Decreased lipid peroxidation as evidenced by lower level of serum malondialdehyde activity in female rats may be due to the effect of female sex hormones which are reported to have antioxidant ability in addition to the antioxidant effect of the ACV thereby causing a decrease in lipid peroxidation in females than in males. The observation that females were significantly less sensitive to this oxidative stress was the first indication of

a possible protection afforded to women that may be due at least in part to circulating hormones such as oestrogen (Wactawski-Wende *et al*., 2009).

The increased serum level of superoxide dismutase activity observed in the apple cider vinegar treated group could be due to the antioxidant effect of ACV by enhancing the antioxidant capability of the rats or upregulating the synthesis or release of endogenous superoxide dismutase in response to increased oxidative stress. Additionally, Phenolic acids as well as vitamins in ACV can scavenge superoxide anion and free radicals in vivo resulting in a potent antioxidant activity (Budak *et al.,* 2014). The decrease in serum catalase activity in the ACV treated group could be due to action of ACV in inhibiting over production of reactive oxygen species by virtue of its high concentrations of flavonoids, anthraquinones and triterpenes as revealed by preliminary phytochemical screening carried out (Boyer and Liu, 2004; Naziroglu *et al*., 2012).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Apple cider vinegar (ACV) (4ml/kg) did not attenuate serum level of stress hormones; corticosterone and epinephrine in male and female rats exposed to chronic restraint stress. ACV ameliorates lipid peroxidation and upregulates endogenous superoxide dismutase activity in chronic restraint stressed male and female wistar rats.

6.2Recommendation

i. Phenolics such as triterpenes and flavonoids contained in apple cider vinegar should be studied for anti-stress activity.
ii. Other forms of experimental stress should be used to further confirm the adaptogenic effect of Apple cider vinegar.
iii. The study should be repeated using a higher dose of Apple Cider Vinegar

Contributions to Knowledge

i. Apple cider vinegar (4ml/kg) did not attenuate the serum level of stress hormones corticosterone (315.43±14.37 ng/ml and 334±15.28 ng/ml) ($p<0.05$) and epinephrine (38.14±4.2 and 42±1.33 pg/ml) ($p<0.05$) in both male and female adult wistar rats respectively exposed to chronic restraint stress.
ii. Apple cider vinegar (4ml/kg) ameliorates lipid peroxidation as shown by decrease in serum level of malondialdehyde activity (655.52±15.49 and 492.92±8.15 nmol/mg protein respectively) ($p<0.05$) in male and female chronic restraint stressed wistar rats.
iii. Apple cider vinegar administration (4ml/kg) boosts endogenous antioxidant activity as indicated by increased serum of superoxide dismutase activity (26.52±0.39 and

24.8±1.11 U/ml) ($p<0.05$) in male and female adult wistar rats exposed to chronic restraint stress respevtively.

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APPENDIX

TABLE 1: Serum corticosterone level following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Corticosterone(ng/ml)
Normal Control (0.5ml distilled water)	166.07±15.87
Restraint Stress (6hour daily)	360.79±14[a]
Apple cider Vinegar (ACV) treated	324.93±10.42

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$). a= significantly different compared to control

TABLE 2: Serum corticosterone level in males and female rats following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Corticosterone (ng/ml)	
	Males	**Females**
Normal Control (0.5ml distilled water)	109.14±0.67	223±3.23*
Restraint Stress (6hour daily)	329.86±3.49	391±22.77*
Apple cider Vinegar (ACV) treated	315.43±14.37	334±15.28

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$).
*= significantly different

TABLE 3: Serum Epinephrine level following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Epinephrine (pg/ml)
Normal Control (0.5ml distilled water)	42.14±1.54
Restraint Stress (6hour daily)	45.93±1.09
Apple cider Vinegar (ACV) treated	40.09±1.04[a]

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$). a= significantly different compared to restraint stress group

TABLE 4: Serum epinephrine level in males and female rats following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Epinephrine (pg/ml)	
	Males	**Females**
Normal Control (0.5ml distilled water)	41.29±1.09	43±1.99
Restraint Stress (6hour daily)	43±1.36	48.86±6.3
Apple cider Vinegar (ACV) treated	38.14±4.2	42±1.33

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$).

TABLE 5: Serum Malondialdehyde (MDA) activity following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Malondialdehyde (nmol/mg of protein)
Normal Control (0.5ml distilled water)	560.9±5.17
Restraint Stress (6hour daily)	725.5±15.6[a]
Apple cider Vinegar (ACV) treated	542.72±15.6[b]

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$). a,b= significantly different

TABLE 6: Serum level of Malondialdehyde (MDA) activity in males and female rats following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Malondialdehyde (nmol/mg of protein)	
	Males	**Females**
Normal Control (0.5ml distilled water)	547.28±3.33	574.52±4.05
Restraint Stress (6hour daily)	710.32±26.45	740.68±14.8
Apple cider Vinegar (ACV) treated	655.52±15.49	492.92±8.15*

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$). *= significantly different

TABLE 7: Serum Superoxide dismutase (SOD) activity following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Superoxide Dismutase (U/ml)
Normal Control (0.5ml distilled water)	15.86±0.45
Restraint Stress (6hour daily)	18.24±0.39[a]
Apple cider Vinegar (ACV) treated	25.66±0.62[ab]

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$). a=significantly different from normal control ab= significantly different from normal control and restraint stress

TABLE 8: Serum level of Superoxide dismutase (SOD) activity between males and female rats following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Superoxide dismutase (U/ml)	
	Males	**Females**
Normal Control (0.5ml distilled water)	16.98±0.5	14.74±0.22
Restraint Stress (6hour daily)	18.86±0.21	17.62±0.66
Apple cider Vinegar (ACV) treated	26.52±0.39	24.8±1.11

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$).

TABLE 9: Serum Catalase (CAT) activity following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Catalase (U/mg of protein)
Normal Control (0.5ml distilled water)	3.84±0.2
Restraint Stress (6hour daily)	5.26±0.36[a]
Apple cider Vinegar (ACV) treated	4.5±0.32

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$).a= significantly different compared to control

TABLE 10: Serum level of Catalase (CAT) activity between males and female rats following 21 days oral administration of apple cider vinegar (ACV) and chronic restraint stress (RS)

Treatment Groups	Catalase (U/mg of protein)	
	Males	**Females**
Normal Control (0.5ml distilled water)	3.79±0.32	3.88±0.29
Restraint Stress (6hour daily)	4.22±0.19	6.3±0.14*
Apple cider Vinegar (ACV) treated	5.34±0.29	3.67±0.17*

± = SEM; Means with different superscripts within columns are statistically significant ($P < 0.05$). *= significantly different