THE ROLES OF MOLECULAR MODELING STRATEGIES IN VALIDATING THE EFFECT OF CHRYSIN ON SODIUM ARSENITE-INDUCED CHROMOSOMAL AND DNA DAMAGE

BY

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DECLARATION

I declare that the work in this dissertation entitled "**THE ROLES OF MOLECULAR MODELING STRATEGIES IN VALIDATING THE EFFECT OF CHRYSIN ON SODIUM ARSENITE-INDUCED CHROMOSOMAL AND DNA DAMAGE**" was carried out by me in the Department of Biochemistry. 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 or diploma at this or any other Institution.

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CERTIFICATION

This dissertation entitled "**THE ROLES OF MOLECULAR MODELING STRATEGIES IN VALIDATING THE EFFECT OF CHRYSIN ON SODIUM ARSENITE-INDUCED CHROMOSOMAL AND DNA DAMAGE**" by **BABANGIDA SANUSI**meets the regulations governing the award of the degree of Masters of Science of Ahmadu Bello University, Zaria, and is approved for its scientific contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents, Mal. SanusiSaleh and Haj. AminaSanusi for their love which has been my driving force till now and forever.

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ABSTRACT

Arsenic is a major environmental toxicant as well as a human carcinogen which is present in large amounts in the environment. Biotransformation of arsenic generates reactive methylated species which can bind and facilitate chromosomal and DNA damage. This work investigated the effects of chrysin on sodium arsenic-induced damage on lipid, protein and chromosome of male Wistar rats.Rats were divided into six groupsand treated daily as follows: normal control; 1a and 1b, 10mg/kg sodium arsenite as negative control, 10mg/kg chrysin as positive control, co-administration of sodium arsenite and chrysin, chrysin followed by sodium arsenite and sodium arsenite followed by chrysin. At the end of the experiment, the animals were sacrificed and lipid peroxidation, protein carbonyl and DNA fragmentation in liver, blood, brain and bone marrow cells micronuclei were assayed for. *In silico* molecular docking of S-adenosyl-methionine-dependent methyltransferase in the presence of chrysin was conducted. Chrysin significantly ($p<0.05$) decreased the level of lipid peroxidation, protein carbonyls and DNA fragmentation in blood, liver and brain tissues compare to group treated with sodium arsenite only. Chrysin significantly ($p<0.05$) reduced the level of micronuclei generated in bone marrow cells. Furthermore, chrysin was able to dock into the active site of SAM-dependent methyltransferase with strong hydrogen bond and hydrophobic interactions.The binding energy of the docking was -99.82kJ/moland predicted inhibition constant (Ki) of 0.959μM.Chrysin at 10mg/kg bodyweight was shown to exhibits ameliorative, preventive and curative in effects. This study might have unraveled the beneficial effects of chrysin against sodium arsenite-induced chromosomal and DNA damage, which could be due to inhibition of SAM-dependent methyltransferase.

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LIST OF ABBREVIATIONS

AS3MT	Arsenic methyltransferase
AS^{V}	Arsenate
AS^{III}	Arsenite
$AS^{III}(GS)_3$	Arsenotriglutathione
CA	Chromosomal Aberrations
CAT	Catalase
DSBs	Double Stranded Breaks
DPA	Diphenylamine
DNPH	2,4-dinitrophenylhydrazine
DMA^{V}	Dimethylarsinic Acid
GLUT-4	Glucose Transporters 4
GSH	Reduced Glutathione
GPx	Glutathione Peroxidase
HDAC	Histone Deacetylase
HNE	4-Hydroxy-nonenal

iAs	Inorganic Arsenic
$L^{\cdot}$	Lipid Radical
$LOO^{\cdot}$	Lipid peroxyl Radical
LOOH	Lipid Hydroperoxide
MCF-7	Michigan Cancer Foundation-7
MN	Micronucleus
MDA	Malondialdehyde
MMA^{V}	Monomethylarsenous Acid
mPCEs	Micronucleated Polychromatic Erythrocytes
PI3K	Phosphoinositol-3-kinase
ROS	Reactive Oxygen Species
SAM	S-adenosyl-methionine
SCE	Sister Chromatid Exchanges
TTE solution	Tris base, Triton X-100 and EDTA solution
TMA	Trimethylarsinic Acids
TNF	Tumor Necrosis Factor
TBA	Thiobarbituric Acid

TBARS	Thiobarbituric Acid Reacting Substances

CHAPTER ONE

1.0 INTRODUCTION

Arsenic is a major environmental toxicant as well as a human carcinogen which is present in large amounts in the environment(Martinez *et al.,* 2011). When ingested, it affect different organs of the body through induction of oxidative damage(Sadia*et al.,* 2015). Arsenic compounds are ubiquitous in nature and found in the environment via industrial or agricultural processes as well as some medical applications(Aliyu *et al*., 2013). They are widely used for many industrial purposes, including mining, smelting, herbicidal products, medicines, wine preservatives and electronics(Cheng *et al.,* 2011). Consumption of arsenicals through contaminated water is prevalent in many areas of the world(Aliyu *et al*., 2013).Arsenic in drinking water is a global environmental health problem(Cheng *et al*., 2011). Both acute and chronic arsenic exposures have been associated with a higher than normal risk of skin, lung and bladder cancer(Riedmann *et al*., 2015; Ajani and Olufunke, 2016). It is also known that arsenic interact with other substances, metals inclusive there by potentiating or depressing its effects(Aliyu *et al*., 2012).

The most common forms of arsenic are water-soluble, arsenite (the trivalent form, As^{III}) and arsenate (the pentavalent form, As^{V}). Trivalent arsenic is more toxic than pentavalent arsenic and its inorganic forms are more toxic than organic forms(Moktar *et al.,* 2008). After ingestion, the dissolved arsenicals are readily absorbed through the gastrointestinal tract and distributed in the blood to the liver, kidney, spleen, lung and many other organs of the body(Moktar*et al.,* 2008). Once in the tissues, arsenic exerts its toxic effects through several mechanisms, the most significant of which is, the reversible combination with sulfhydryl's groups and inhibition of

numerous other cellular enzymes(Muhammad and Ibrahim,2015), especially those involved in cellularglucose uptake, gluconeogenesis and fatty acid oxidation(Kumar, 2015). It also alters multiple cellular pathways including expression of growth factors, suppression of cell cycle checkpoint proteins, promotion of apoptosis, inhibition of DNA repair(Amal *et al.,* 2012), decreasing immunosurveillance and increasing oxidative stress(Sadia*et al*., 2015). Arsenic generates reactive oxygen species which also include dimethyl arsenic peroxyl ([$(CH_3)_2$AsOO]) and dimethyl arsenic radical [$(CH_3)_2$As](Monica *et al.,* 2011; Odunola *et al*., 2013;Klibet *et al.,* 2015). The major metabolic pathway of inorganic arsenic in humans is its methylation in liver(Amal *et al.,* 2012). This was proved by the presence of monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in urine and bile(Rezaul *et al.,* 2010). These reactions are catalyzed by a cytosolic enzyme, arsenic (+3 oxidation state) methyltransferase (AS3MT), which uses S-adenosylmethionine (AdoMet) as a methyl group donor(Mohamad and Aliyu, 2013). Because DMA has a shorter circulating half-life than does inorganic arsenic (iAs), arsenic-exposed individuals with higher proportion of MMA and a lower proportion of DMA in their urine, have an increased risk of developing adverse health outcomes(Niedzwiecki *et al*., 2016; Odunola *et al*., 2013). The As^{III} methylation by AS3MT is proposed to have two rounds of reaction(Martinez *et al*., 2011); the first round produces MMA^{V} which is then reduced to MMA^{III}, followed by a second round of methylation to DMA^{V}, a third round of methylation lead to formation of TMA (Trimethylarsinic acid)(Zuzana *et al.*, 2016). However, the methylated arsenic intermediates can be more toxic than inorganic As^{V} or As^{III}, which means under certain circumstances methylation activity can exacerbate arsenic toxicity(Florêncio, 2016). Therefore, arsenic methylation serves as one of decisive factor to determine the overall arsenic induced

toxicities(Zuzana *et al.,* 2016), as a result, the modulation of this pathway could be a viable strategy in combating arsenic induced-toxicity.

In recent years, there is a tremendous interest in the possible role of nutrition in prevention of disease(Kumar, 2015). In this context, antioxidants, especially those derived from natural sources such as medicinal plants, antioxidant rich foods(Aliyu *et al*., 2012) and herbal based drugs are gaining more attention (Klibet *et al*., 2015). Beside, these functional based curative foods can be obtained with ease in our communities. Chrysin (CHR) is a natural flavanoid(Janeiro *et al.,* 2005), biologically active compound extracted from many plants, acacia honey(Muhammad and Ibrahim, 2015; Muhammad *et al.*,2015) andpropolis(Woo *et al.,* 2004; Patel, 2015; Sun *et al*., 2016).Chrysin and its derivatives, in particular, have been reported to possess a broad range of biological activities, including anti-oxidative, anti-viral and anti-inflammatory properties(Woo *et al*., 2004; Khan *et al*., 2010; Girgis*et al.,* 2015) and also have been observed to display anti-cancer potential(Li *et al*., 2011). It is also known in promoting cell death by perturbing cell cycle progression(Samarghandian *et al.,* 2011; Li *et al*., 2011; Odunola *et al*., 2013; Muhammad and Aliyu, 2013).Chrysin inhibit tumor angiogenesis *in vivo*(Li *et al.,* 2010), which is a key step in cancer cell metastasis(Li *et al.,* 2010). Previous work on acacia honey and propolis revealed antioxidant property in sodium arsenite-induced oxidative damage (Muhammad and Ibrahim, 2015) andchrysin has been reported to be one of the active principles. However, the previous work was on crude acacia honey which necessitates the need to investigate the ameliorative, preventive and curative potentials of chrysin against sodium arsenite-induced chromosomal and DNA damage *in vivo*.

1.1 STATEMENT OF RESEARCH PROBLEM

Arsenic contamination of drinking water is a serious environmental problem worldwide because of the large number of contaminated sites that have been identified and the large number of people at risk(Hei and Filipic., 2004).Inorganic arsenic is naturally present at high levels in the groundwater of a number of countries (WHO, 2011). About 160 million people, mainly in developing countries, are being poisoned by arsenic in their drinking water (Karagas *et al*., 2015).The World Health Organization (WHO, 2011) has established a permissible limit of arsenic as its guideline value for drinking water quality ($10\mu gL^{-1}$) that has been estimated to pose 'a minimal risk' to cancer based on human cancer data and cost benefitbalance (NRC, 2001). Moreover, the United States Environmental Protection Agency (USEPA, 2001) has also recommended areference dose (RfD) of arsenic ($0.3l\mu gkg^{-1}dl^{-1}$) as its permissiblelimit of daily exposure in humans with almost no likelihood of anyadverse effect(Khan *et al*., 2013).A research by Garba*et al*. (2012) revealed high concentration of arsenicbeyond the guideline value for drinking water in all the eight local governmentareas of Kaduna North Senatorial District.

1.2 JUSTIFICATION

Chrysin can be found in many naturally occurring plants such as passion flower, propolis, chamomile, mushroom and honey (Sun *et al*., 2016).Several studies in recent years have showed the ability ofacacia honey to mitigate the effects of arsenicals-induced chromosomal and DNA damage (Muhammad and Ibrahim, 2015a).The effect of individual components of acacia honey on arsenicals-induced chromosomal and DNA damage has not been elucidated. These individual bioactive components of acacia honey include chrysin among others. However, to the best of our

knowledge, the potential of chrysin against sodium arsenite-induced chromosomal and DNA damage has not been investigated.

1.3 AIM

The aim of this study is to investigate the effects of chrysinon sodium arsenite-induced chromosomal and DNA damage and evaluate its inhibitory effects on SAM-dependent methyltransferase*in silico.*

1.4 OBJECTIVES

1. To determine the effect of chrysin on sodium arsenite-induced lipid peroxidation in liver, blood and brain tissues.
2. To determine the effect of chrysin on sodium arsenite-induced clastogenicity in bone marrow cells and DNA fragmentation in blood, liver and brain tissues.
3. To determine the effect of chrysin on sodium arsenite-induced protein damage in blood, liver and brain tissues.
4. To determinethe inhibitory effect of chrysin on SAM-dependent methyltransferase*in silico*.

1.5 NULL HYPOTHESIS

Chrysin does not have any effect on sodium arsenite-induced chromosomal and DNA damages.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 SOURCES OF ARSENIC EXPOSURE

Arsenic is one of the most abundant elements in the Earth's crust. Chemically, it is classified as metalloid, exhibiting organic and inorganic forms (Martinez *et al.,* 2011).Arsenic have caused lots of health hazards to human population (Kumar, 2015).Its poisoning has become a major worldwide environmental concern because millions of people have been exposed excessively through contaminated drinking water (Bose *et al.,* 2011). Humans are exposed to arsenic (As) primarily from air, food and water, however, elevated inorganic arsenic in drinking water is the major cause of arsenic toxicity, in addition to occupational, enviromental and industrial pollution (Mazumder, 2000).Human activities have caused an accumulation of arsenic in soils through production of arsenic-based pesticides, fungicides, herbicides, insecticides, paints, wood preservatives, lasers, transistors, semiconductors, smelting of arsenic ores, mining processes and fuel utilization (Sharma and Saumya, 2013).Therefore, exposure to arsenicals in our environment need to be given a serious concern, since it is associated with a number of pathological conditions.

2.1.1 Biotransformation of Arsenics

About 80–90% of ingested $As^{[III]}$ or $As^{[V]}$ is absorbedfrom the gastrointestinal tract (Hossain *et al*., 2013). Inorganic arsenicals are absorbed by intestine, methylated in the liver and excreted through urine(Hirano *et al*., 2004). In living system, arsenic act through a variety of mechanism influencing numerous signal transduction pathways resulting in a vast range of cellular effect

that include apoptosis, growth inhibition or inhibition of differentiation and angiogenesis (Hornhardt *et al.,* 2006). Metabolism of arsenic generates a variety of genotoxic and cytotoxic species, that can damage DNA directly and indirectly, through the generation of reactive oxygen species (ROS) and induction of DNA adducts, strand breaks, cross links and inhibition of the DNA repair process.It is also postulated to affect maintenance of normal DNA methylation patterns, chromatin structure and genomic stability (Martinez *et al.,* 2011).

Although multiple pathways such as inhibition of DNA repair, methylation status and co-carcinogenesis with other environmental toxicants have been proposed, one common theme that has emerged is the role of reactive radical species in the pathogenesis of arsenic-induced diseases (Hei and Filipic.,2004).Arsenic has been implicated in covalent interactions with the thiol groups in proteins causing instability on the structure-function relationship of proteins (Aliyu *et al.,* 2013). Its administration has also been reported to compromises theintegrity of the liver ofmouse,rat,fishand goat (Aliyu *et al.,* 2013). The fate of ingested or inhaled inorganic arsenic (iAs) in the humanbody is largely dependent on its valence state(Marchiset-ferlay and Savanovitch, 2011). The two most common valence states of iAs to which humans might be environmentally exposed are the trivalent (As^{III}) and pentavalent(As^{V}) forms, respectively(Marchiset-ferlay and Savanovitch, 2011). The inorganic arsenicals are known to be taken up by the liver, transformed to monomethylated (MMA) and dimethylated arsenicals (DMA) through consecutive reductivemethylation and then excreted into urine as pentavalent methylated arsenic form with only small amount accumulates in biological structures rich in keratin, such as hair and nail (Marchiset-ferlay and Savanovitch, 2011). Biotransformation of arsenic inside cellis a process through which arsenate is converted into arsenite and then transformed into mono-, di- andtrimethylated products as indicated in the following reactions:

$As^{III}O^{3-}_3 + CH^{+}_3 CH_3 As^{V}O^{3-}_2 + 2e \qquad CH_3 As^{III}O^{2-}_2 + CH^{+}_3 \; (CH3)_2 As^{V}O^{-}_2 + 2e^{-} \longrightarrow$

$$(CH_3)_2 As^{III}O^{-}_2 + CH^{+}_3 \longrightarrow (CH_3)_3 As^{V}O^{-}$$

These reactions point to the important fact that methylation of arsenic is associated with reduction of As^{V} to As^{III} (Ventura-Limas*et al.,* 2011). Some literatures suggested that the antioxidant glutathione (GSH) plays an important role in arsenic biotransformation as a reducing agent and has been shown to be linked to the formation of arsenotriglutathione (As^{III} $(GS)_3$), a complex in which As^{III} is bound to the thiol moieties of the cysteinyl residues of three GSH molecules (Ventura-Lima *et al.,* 2011). Marchiset-ferlay and Savanovitch.(2011) showed that reduction of arsenate to arsenite can also be catalyzed by an arsenate reductase enzyme that requires the presence of inosine and a thiol compound.

$$As\ (GS)_3 + CH_3^{\pm} \longrightarrow CH_2 As\ (GS)_2 \longrightarrow (CH_2)_2 AsGS$$

Hence, monitoring arsenic interactions in biological system may give an insight toward positive measures against oxidative damage to biomolecules.

2.1.2 Role of SAM-dependent methyltransferase in arsenic toxicity

The key point in the metabolism of arsenicals is the repetitive reduction of pentavalent forms of arsenic and oxidative methylation of trivalent forms of arsenic(Hirano *et al*., 2004).The mechanism by which iAs exerts its toxic effects is very complex because its metabolism involves more than five metabolites, all of which can produce toxic effects (Hei and Filipic, 2004). This methylation process uses S-adenosylmethionine(SAM) as a methyl group donor, through a SAM-dependent$As^{[III]}$-methyltransferase, initially isolated from rat liver and a human homologue of cytochrome19 (Dheeman *et al*., 2014). This enzyme catalyzes the transfer of a methyl group from SAM to $As^{[III]}$ producing methylated and dimethylated arsenic compounds(Tokumoto *et al*.,

2014). Trivalent methylated species, such monomethylarsonous acid ($MMA^{[III]}$) and dimethylarsinic acid ($DMA^{[III]}$), have been detected in the urine of patients chronically exposed to iAs in drinking water. Derivate methylated species from iAs metabolisms are considered relevant agents during arsenic toxicities, specially through induction of oxidative stress and impairing DNA repair processes (Martinez *et al.*, 2011). Arsenic biomethylation is associated with arsenic-induced related cancers by conversion of inorganic arsenic (iAs) into carcinogenic trivalent MAs(III) and DMAs(III)(Dheeman *et al*., 2014). Thus, targeting SAM-dependent methyltransferase may reduce arsenic related toxicities.

2.1.3 Chromosomal and DNA damage

Chromosomes are under constant assault by DNA damaging agents, such assaults include DNA double strand breaks (DSBs) and if not repaired in a timely and accurate manner can lead to genomic instability and rearrangement (Morales *et al*., 2016).Genomic instability is characterized by an increased frequency of changes in the genetic material of the cells and it's one of the primary causes of diseases (Lúcia *et al*., 2015; Morales *et al*., 2016). Reactive oxygen species (ROS) generate DNA double stranded breaks with chemically modified ends(Tong *et al*., 2016). It has been reported that arsenic can inhibit the activities of thiol containing enzymes such as DNA ligase, thereby causing defects during DNA replication, repair, recombination and joining of single and double-stranded DNA breaks (Muhammad *et al*.,2015). Since, SAM is the methyl group donor used by DNA methyltransferases to maintain normal epigenetic patterns in all human cells, arsenic is also postulated to affect the maintenance of normal DNA methylation patterns, chromatin structure and genomic stability(Qiu *et al*., 2015).Lihua and Yi. (2013) have also demonstrated that arsenite can induce notonly chromosome aberrations (CA), sister chromatid exchanges (SCE) and micronuclei (MN) in animal cell, plant cells and human cells

but also cell death in cultured human and animal cells. Inorganic arsenic is known to damage chromosomes due to covalent binding between iAs and DNA structures, it has been proposed that most of the DNA damage observed during iAsexposure is indirect, occurring mainly as a result of ROS induction which generates DNA adducts, DNA strand breaks, cross links and chromosomal aberrations (Martinez *et al.,* 2011; Chuaire *et al*., 2016). Micronucleus test is one of the most suitable genotoxicity test, others such as chromosomal aberration test, peripheral blood micronucleus, mouse lymphoma assay have been in use for studying antimutagenic activity of various potential plant dietary sources (Abrevaya *et al.,* 2007; Sumanth and Chowdary, 2010).

DNA damage due to oxidative reactions may arise from injuries at a basic DNA sites, oxidation of purine and pyrimidine bases, to single and double strand breaks in DNA molecules (Fahri *et al.,* 2014). Hydroxyl radical is considered as the most potent agent responsible for oxidative DNA damage which can give rise to many base lesions and attack deoxyribose sugar forming sugar radicals (Duraisingh and Lodish, 2001). Reactive species induce deletion and insertion of single base pair in DNA helix with resulting damages in the native structure of DNA (Kryston *et al*., 2011). Breaks in DNA may also result from damaged DNA replication forks or from oxidative destruction of deoxyribose residues (Jena, 2012). Various strategies has been commonly used for the detection and quantification of DNA damage (Kumari *et al.,* 2008). Spectrophotometric methods involved light absorption by DNA fragment in a test sample (Sellins and Cohen, 1987). Consequently, arsenic-induced damage to biomolecules can accumulate and exacerbate chromosomal and DNA damage leading to genomic instability.

2.2 CHRYSIN

The recent explosion of interest in the bioactivity of the flavonoids of higher plants is due, at least the potential health benefits of these polyphenolic components of major dietary constituents (Manzolli *et al.,* 2015).Flavones originate from flavanones by 2, 3-desaturation and this oxidation may be considered as a branch point reaction in the flavonoid pathway towards final flavone and flavonols products (Fu *et al.,* 2007). Chrysin (5,7-dihydroxy-2-phenyl-4H-chromen-4-one) is a flavone which is used as a dietary supplement found in many plants such as honey, passion fruit (*passiflora sp*.), *Oroxylumindicum,Matricariachamomilla* (Jung, 2016).Compared with other flavonoids, few studies have been carried out in order to determine the beneficial effects of chrysin and some of these investigations yielded promising results (Manzolli *et al.,* 2015). It has many reported biological activities such as anti-oxidant, anti-inflammatory, anti-diabetic, anxiolytic properties as well as vaso-relaxant effects (Mizar *et al.,* 2015). Chrysin has a double bond between C-2 and C-3 and the ringBwill be coplanar with the ringsA and C due to the conjugation(Mizar *et al*., 2015). Themechanism of action as antioxidants seems to involvethe ability of phenols to scavenge radicals by an H-atom, or electron transfer process in which the phenol is convertedinto a phenoxyl radical resulting in the formation of the semiquinone, which can donate a further electron to form the quinone (Janeiro *et al.,* 2005). These properties of chrysincan servea promising potentials in curtailing chromosomal and DNA damage.

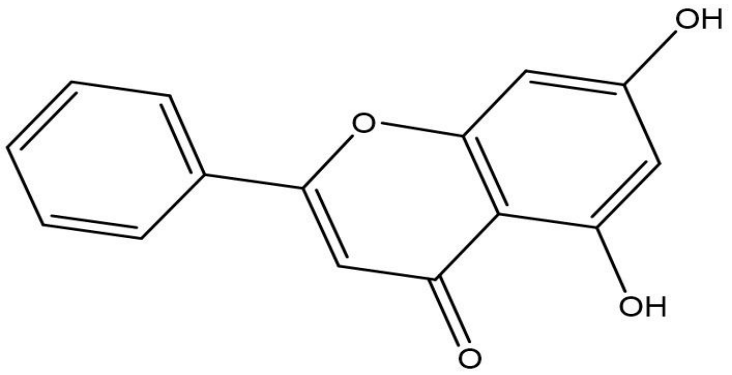


Figure 2.1: Structure of Chrysin Drawn Using ChemDraw Ultra (v 12. 0.12)

2.2.1 Sources of chrysin

i. Honey

Honey is a viscous, supersaturated sugar solution derived from nectar gathered and modified by the honeybee (*Apismellifera*) (Aliyu *et al.,* 2013). Honey composed of at least 300 compounds having fructose and glucose in highest concentrations with phenolic acids [p-hydroxybenzoic, ferulic and t-cinnamic acid], five free flavonoids [pinobanksin, apigenin, pinocembrin, chrysin andacacetin] and abscisic acid (Aliyu *et al.,* 2013). Several health-beneficial effects which includes gastroprotective, hepatoprotective, reproductive, hypoglycemic, antioxidant, antihypertensive, antibacterial, anti-fungal and anti-inflammatory have been demonstrated(Muhammad and Ibrahim,2015). Therefore, dietary use of honey can serve a substitute means as a source of chrysin for human consumption.

ii. Propolis

Propolis is a bee-metabolized resinous substance (bee glue) from plant sap and gums with a number of biological effects ranging from antimicrobial, antioxidant, anti-inflammatory, antidiabetic, dermatoprotective, anti-allergic, laxative and immunomodulatory to anticancer (Patel, 2016).Some of the active components of propolis were identified as caffeic acid phenyl ester, chrysin, artepillin C, nemorosone, galangin, cardanol (Patel, 2016). Thus, the use of propolis in complementary medicine can address oxidative damage linked to arsenic toxicities.

iii. Other sources

Chrysin can also be found in plant sources such as *Passifloracaerulea*, *Passiflora incarnate*, *Oroxylumindicum*, *Matricariachamomilla*, *Pleurotusostreatus*and natural products (Jung, 2016). Hence, it broaden the opportunistic usage and access to chrysin as complementary active food ingredient.

2.2.2 Biological roles of chrysin

Chrysin has been reported to exhibit a number of remarkable beneficial pharmacological effects with the most important and highly reported antioxidant activity (Michel*et al.,* 2014).Chrysin alleviates inflammation through inhibition of cyclooxygenase pathway, histamine, NF-κB, pathway, tumor necrosis factor- alpha, iNOS, cytokines and activation of peroxisome proliferator-activated factor (Jung, 2016). In addition, chrysin inhibits the activities of α-Glucosidase, thus decreasing glucose absorption in blood, induction of GLUT-4 translocation in adipocyte cells, suggesting an increase of glucose uptake into those cells (Jung, 2016). Antioxidant activity of chrysin have also known to play important roles in protection of

pancreatic beta cells damage (Patel*et al.,* 2016). Thirdly, chrysin has also been demonstrated to inhibit the activation of human immunodeficiency virus in models of latent infection through inhibitory activity of reverse transcriptase, integrase and protease activities (Woo *et al.,* 2004) and inhibit casein kinase II involved HIV-1 transcription and replication (Jung, 2016).

Moreover, chrysinshows anticancer activities in various cancer cell lines, which include inhibition of thePI3K/Akt pathway, activation of caspase-3, 8 and depletion of cellular glutathione (Li *et al.,* 2011). It also exhibit anti-proliferative potential in melanoma cells *in vitro*(Odunola *et al.,* 2013).Chrysin, as an HDAC inhibitor, arrests the cell cycle through induction of p21 protein, thus suppresses cellular proliferation (Khoo *et al.,* 2010).Chrysin as an aromatase inhibitor potentially reduces tumor growth inestrogen receptor-positive breast cancer MCF-7 cells(Khan *et al.,* 2010). Furthermore, in atherosclerosis, the major factor is oxidative metabolism of plasma low density lipoproteins by oxygen free radicals which caninjures endothelial cells. Chrysin can scavenge ROS production and increases SOD, GPx and CAT activities (Weng *et al.,* 2005).Chrysin alsocontrols cholesterol efflux and suppresses TNF-alpha-induced adhesion molecule expression (Khoo*et al.,* 2010). In addition, chrysin was reported to enhance the level of testosterone by inhibiting the aromatase enzyme which converts testosterone into estradiol as a dietary supplement in the form of capsule (Khan *et al.,* 2010). Lastly, aged mouse brain shows memory declines, but chrysin prevents age-related cognitive decline by mitigating the decrease of brain-derived neurotropic factor and ROS in the prefrontal cortex and hippocampus of aged mice (Jung, 2016). Altogether, these report cumulatively suggest that chrysincould be beneficial and valuable agent for the management of oxidative stress related disease.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

Chrysin(Sigma Aldrich St. Louis, MO, USA), (254.24g/mol, 284-286^{o}C melting point and 97% purity), Thiobarbituric acid (Sigma Aldrich St. Louis, MO, USA), Giemsa (Sigma Aldrich St. Louis, MO, USA), Phosphate buffered saline (PBS) (Sigma Aldrich St. Louis, MO, USA), 2, 4-dinitrophenylhydrazine (KEM light laboratories). All other chemicals and reagents used were of analytical grade.

3.1.2 Experimental animals

Forty two healthy male Wistar rats of 110-200g weight range were obtained from Animal House, Faculty of Pharmaceutical Sciences, Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria and maintainedin propylene cages under normalenviromental conditions. The animals were fed with standard laboratory animals feed and water *ad libitum*by placing the feeder containing feed of a known weight in the cagesand water in a graduated feeder.Ethical clearance for the use of laboratory animals was obtained from Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC).

3.2 METHODS

3.2.1 Grouping of the experimental animals and treatments

i. *Animals grouping*

The experimental animals were randomly divided into seven (7) groups of six animals each, in separate cages. The grouping comprised of group 1a, 1b, 2, 3, 4, 5 and 6, respectively. The work was carried out in two sets of experiments; the first set comprised of groups 1a, 2, 3 and 4, and were treated for one week while the other set comprises of groups 1b, 5 and 6, treated for two weeks, respectively. These sets of experiment were designed to investigate whether or not the effect of chrysin against sodium arsenite-induced damage is ameliorative (due to interaction between chrysin and sodium arsenite), preventive (its ability to halt the damage caused by sodium arsenite), or curative (to heal the damage caused by sodium arsenite).

ii. *Treatments*

The doses of chrysin (10mg/kg body weight) and sodium arsenite (10mg/kg body weight) were prepared for each group based on their mean body weight and the treatment was carried out orally using cannula on the basis of 5ml/kg of mean body weight for each group (Aliyu*et al*., 2013). Group 1a and 1b animals served as normal control and were treated with distilled water (5ml/kg body weight), group 2 animals were treated with sodiumarsenite (10 mg/kg body weight) (Sadia*et al*., 2015), group 3 animals were treated with chrysin (10mg/kg bodyweight) (Manzolli *et al*., 2015), group 4 animals were simultaneously treated with chrysin and sodium arsenite, group 5 animals were treated with chrysin (10 mg/kg body weight) for the first week followed by sodium arsenite (10 mg/kg body weight) for the second week, group 6 animals were treated with sodium arsenite (10 mg/kg body weight) for the first week followed with chrysin (10 mg/kg body weight) for the second week. The animals in group 1a, 2, 3 and 4 were sacrificed on the seventh (7) day of treatment whereas, group 1b, 5 and 6 were sacrificed on the fourteenth (14) day of treatment. The feed intake, water intake and body weight were monitored on daily basis while organs weights were taken immediately after sacrifice.

3.2.2Sample collection and preparation

After the last administration, the rats were fasted for 24hrs and sacrificed by decapitation following mild chloroform anaesthesia. Blood, brain, liver tissues and femurs were collected. The tissues were homogenized in 1:10 with 0.01M phosphate buffer (pH 7.4). The supernatant (brain and liver) was collected after centrifugation at 3500xg at 4^0C for 10 min. 1:1 blood dilution was also prepared and kept for further analysis. Each time the samples were outside the freezer, they were kept on ice bags. Bone marrows cells from the femurs were immediately used for micronucleus assay.

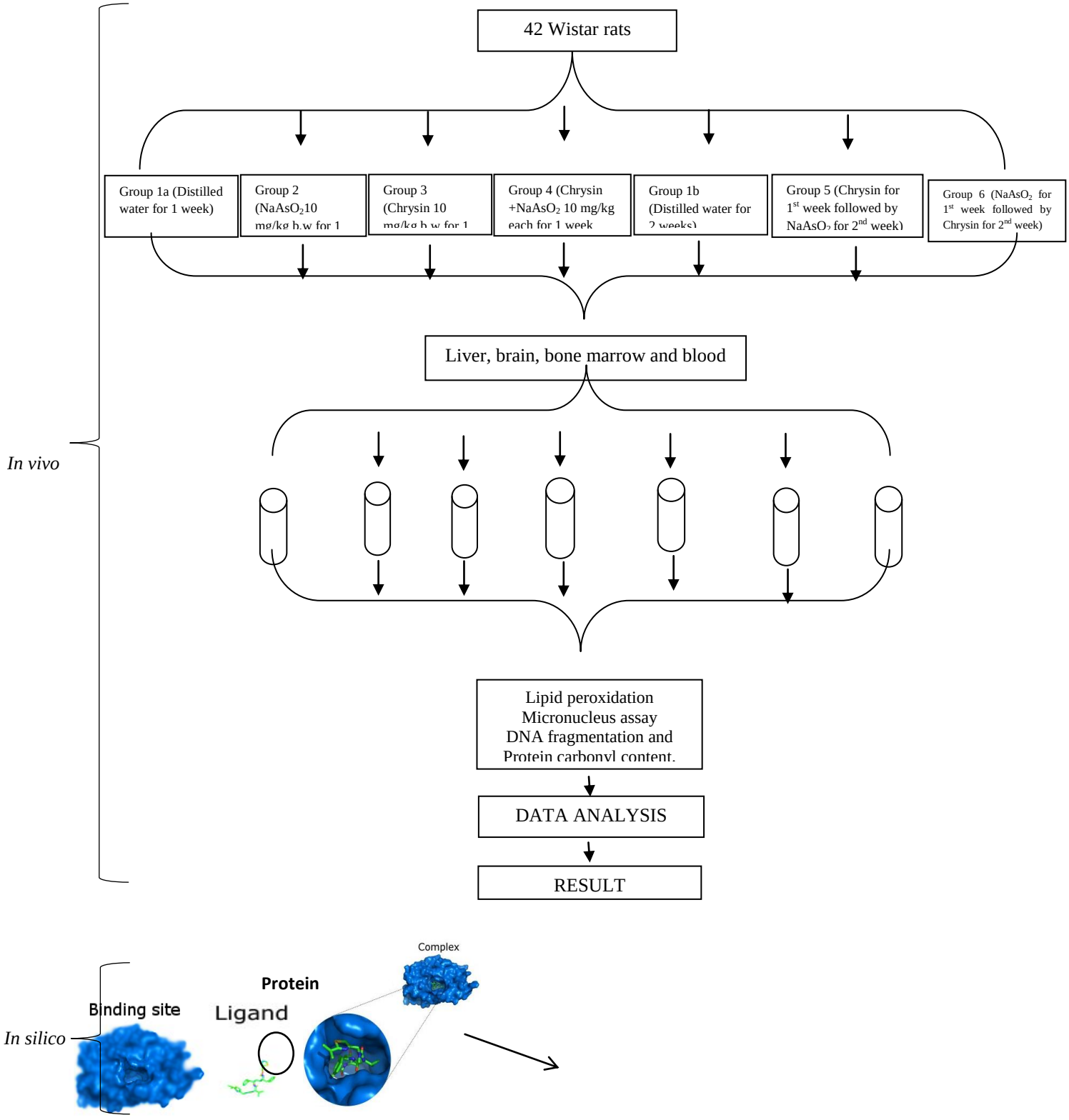


Figure 3.1: Flow Chart of Experimental Design

3.2.3 Determination of lipid peroxidation

Lipid peroxidation was determined using thiobarbituric acid reacting substances according to Ohkawa *et al*. (1979)and with slight modification with Trichloroacetic acid (15%) and Thiobarbituric acid (0.67%). Exactly 2ml of 15% trichloroacetic acid was added into a test tube, 2 ml of Thiobarbituricacid was added and followed by 100µl of the tissue homogenate. The mixture was incubated at 80^{o}C for 30 minutes in a water bath and allowed to cool for some time and centrifuged at 300rpm for 10minutes. A clear supernatant was collected and absorbancemeasured at 535nm wavelength. Malondialdehyde concentration was determined using the molar absorptivity of 1.56×10^{-5}.

3.2.4 Bone marrow cells micronucleus assay

The bone marrow was aspirated from femur and a smears were prepared on a chemically clean glass slides and stained with Giemsa stain. The smears wereanalyzed under oil immersion using light microscope (1000 Magnification) for the presence of Micronuclei (MN) in polychromatic erythrocytes (PCE). Micronuclei was determined by counting a total of about 2000 erythrocytes per animal i.e. about 500 erythrocytes were examined per field for the presence of micronuclei (Gonza *et al.*, 2003).

3.2.5 DNA fragmentation assay using diphenylamine coupled assay

Quantitative DNA fragmentation assay was carried out according to the method of Sellins and Cohen., (1987). The liver and brain were broken using mortar and pestle in phosphate buffer (0.01M, pH 7.4). The homogenate was centrifuged at200×g to obtained the supernatant (S) and

at 20000×g after treating the pellet with Tris-tritonX100-EDTA solutionto obtained supernatant (T) for 10min each and the supernatant was precipitated. Trichloroacetic acid (12.5%) at 4°C was added to the pellet (B) and quantified using a diphenylamine reagent after hydrolysis in 5% TCA at 90°C for 20min. Percentage of DNA fragmentation refers to the ratio of DNA in the supernatant (fragmented) to the total DNA recovered in both supernatant and pellet (fragmented plus intact DNA). The absorbance was taken at 560nm using 5% TCA as the blank. The percentage of the fragmented DNA was calculated using the formula:

$$\%fragmented\ DNA = \frac{(S+T)}{S+T+B} \times 100$$

Where S, T and B are the absorbance at 560nm of fragmented DNA in the S, T and B fractions, respectively.

3.2.6Determination of protein carbonyl content

Protein carbonyl content was determined using DNPH method (Levine *et al.*, 1990) as modified by Cristina *et al.*(2014) as denominated DNPH alkaline method. Exactly, 400µL of 2, 4 Dinitrophenylhydrazine (10mMin 2.5M HCl) was added to 400 µL of sample, after 10 min of incubation, 200µL of NaOH (6M) was added. Absorbance was read at 450 nm after 10 min of incubation at room temperature against a blank containing equal volume of phosphate buffer in place of the sample. The molar absorption coefficient of 22,000M^{-1} cm^{-1} was used to calculate the concentration of protein carbonyls groups and the result was presented as nmol/mg of protein.

3.2.7 Molecular modeling of S-adenosyl-L-methionine-dependent methyltransferase

The structure of chrysin (Figure 1) was sketched usingChemBioDraw program and saved as an SD file. It was uploaded into the Discovery Studio 2.5 (Accelrys Inc., San Diego, CA, USA) and optimized with a Chemistry at Harvard Macromolecular Mechanics (CHARMm) force field, while their partial charges were calculated via the Momany–Rone option(Borisaand Bhatt, 2015;Chen and Chen, 2015). A smart minimizer algorithm was used to minimize the resulting structures, which performs 1000 steps of the steepest descent with a RMS gradient tolerance of three, which was followed by a conjugate gradient minimization.

The X-ray diffraction crystal structure of human S-adenosyl-L-methionine-dependent methyltransferase **(**NSUN5) protein at resolution 1.65Å (PDB ID: 2B9E) was retrieved from Protein Data Bank. All protein preparation and minimization were done using tools and protocols in the Discovery Studio 2.5v. A CHARMm force field was used to type the complex. While using the protein preparation protocol, hydrogen atoms was added to the complex, after which water molecules were removed and the pH of the protein was set to almost neutral value. A sphere binding site with a nine Armstrong (Å) radius was defined around the bonded ligand to identify the binding site of the protein structure. The CDOCKER (CHRMm-based DOCKER) program was used as the scoring function to chrysin into crystal structure of S-adenosyl-L-methionine-dependent methyltransferases (2B9E) binding pocket. CDOCKER is a CHARMm-based docking tool that considers the receptor rigid and generates several prime random

orientations for ligand within the receptor active site followed by the molecular dynamics (MD) based simulated annealing and the final refinement by minimization(Wu and Yang., 2002).

The PDB files of the docked position were generated for best pose of compound using Accelrys discovery studio to generate a three-dimensional ligand binding interaction, before it was imported to chimera to view the hydrophobic structure of the interaction pocket of the ligand in the receptor (2B9E). Furthermore, the 2-dimensional view of the hydrogen bond and hydrophobic interactions of 2B9E-Chrysin complex were drawn using a Ligplot+ v.1.4.5 program.

3.3 STATISTICAL ANALYSIS

The results were presented as mean ± standard deviation (SD). The data were analyzed using One-way Analysis of Variance (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS) software, SPSS Inc., Chicago, Standard version 20.00. $p<0.05$ was considered statistically significant for differences in mean using the least of significance difference (LSD).

CHAPTER FOUR

4.0 RESULTS

4.1 Feed Intake

The results of feed intake are presented in table 4.1 and 4.2, although, fluctuation in feed intake was observed as the day progress. Feed intake to body weight ratio showed administration of sodium arsenite to cause a decline in feed intake.

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	FI/BW
Normal control	182.0	186.0	175.0	183.0	181.3	186.0	157.0	1.5
10 mg/kgb.w$NaAsO_3$	161.2	127.5	210.0	182.8	105.7	125.7	101.6	1.1
10 mg/kgb.wChrysin	168.9	143.0	149.0	193.9	151.2	88.7	143.0	1.2
10 mgkgb.w$NaAsO_3$ + 10 mg/kg b.wChrysin	93.6	152.1	163.2	181.4	157.0	136.4	110.6	1.1

Table 4.1.Effect of Various Treatments on Feed Intake in Male Wistar Rats during One Week Treatments

Values are expressed as feed intake of various treatment groupsin grams (g) (n=6), FI = Mean Feed Intake, BW = Mean Body Weight, FI/BW = Feed Intake by Body Weight ratio, b.w = Body Weight. The treatments were carried-out for one week.

Table 4.2.Effect of Various Treatments on Feed Intake in Male Wistar Rats during Two Weeks Treatments

Treatment	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**FI/W**
Normal control (2 weeks)	182.0	186.0	175.0	183.0	181.3	186.0	157.0	172.0	219.0	199.1	144.6	189.5	184.1	102.0	1.28
10 mg/kg b.wChrysin (1st week) followed by 10 mg/kg b.w$NaAsO_3$ (2nd week)	172.3	148.1	168.3	211.2	186.6	186.0	173.0	179.0	186.0	200.4	170.9	215.8	150.2	86.0	1.31
10 mg/kg b.w$NaAsO_3$ (1st week) Followed by 10 mg/kg b.wChrysin (2nd week)	138.9	160.2	93.8	189.0	136.0	142.6	115.0	148.0	191.6	194.2	147.3	205.0	164.5	107.4	0.78

Values are expressed as feed intake of various treatment groupsin grams (g) (n=6), FI = Mean Feed Intake, BW = Mean Body Weight, b.w = Body Weight, FI/BW = Feed Intake by Body Weight ratio, Chrysin (1st week) followed by Sodium Arsenite (2nd week) and Vice-versa to access the preventive and curative effect of chrysin against arsenite induced toxicity.

4.2 Water Intake

The results of water intake are presented in table 4.3 and 4.4, same pattern of fluctuation was observed as recorded in feed intake. Water intake to body weight ratio also showed administration of sodium arsenite to cause a fluctuations in feed intake.

Table 4.3.Effect of Various Treatments on Water Intake in Male Wistar Rats during One Week Treatments

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	WI/BW
Distilled water	105.0	110.0	150.0	130.0	145.0	100.0	90.0	1.0
10 mg/kg b.w$NaAsO_3$	84.0	55.0	35.0	55.0	80.0	70.0	50.0	0.5
10 mg/kg b.wChrysin	73.0	57.0	50.0	50.0	50.0	55.0	60.0	0.5
10 mg/kg b.w$NaAsO_3$ + 10 mg/kg b.wChrysin	110.0	20.0	30.0	40.0	50.0	50.0	50.0	0.4

Values are expressed as water intake of various treatment groupsin milliliter (ml) (n=6), WI = Mean Water Intake, BW = Mean Body Weight, WI/BW = Water Intake by Body Weight ratio (ml/g), b.w = Body Weight. The treatments were carried-out for one week.

Table 4.4.Effect of Various Treatments on Water Intake in Male Wistar Rats during Two Weeks Treatments

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	WI/BW
Normal control (2 weeks)	105.0	110.0	150.0	130.0	145.0	100.0	90.0	120.0	70.0	85.0	55.0	50.0	30.0	70.0	0.68
10 mg/kg b.wChrysin (1st week) followed By 10 mg/kg b.w $NaAsO_3$ (2nd week)	100.0	114.0	106.0	110.0	60.0	30.0	70.0	38.0	42.0	105.0	78.0	60.0	40.0	40.0	0.53
10 mg/kg b.w $NaAsO_3$ (1st week) followed by 10 mg/kg b.wChrysin (2nd week)	100.0	25.0	25.0	25.0	50.0	70.0	50.0	26.0	50.0	105.0	45.0	50.0	80.0	45.0	0.27

Values are expressed as water intake of various treatment groupsin milliliters (ml) (n=6), WI = Mean Water Intake, BW = Mean Body Weight, b.w = Body Weight, WI/BW = Water Intake by Body Weight ratio (ml/g), Chrysin (1st week) followed by Sodium Arsenite (2nd week) and Vice-versa to access the preventive and curative effect of chrysin against arsenite induced toxicity.

4.3 Bodyweight

As shown in (Table 4.5 and 4.6)there was a significant ($p<0.05$) increase in body weight as the administration progresses in control groups (1a and 1b), chrysin treated, co-administered as well as group five and six. The same pattern of fluctuations was also observed as it indicate in feed and water intake and these could explain the reason for fluctuations in body weight as the days increase. The decrease in body weight was also observed in sodium arsenite treated groups with negative percentage weight change of -8.4 when compare to percentage weight change of 5.9 observed in normal control group (1a). Co-administration of sodium arsenite and chrysin (ameliorative) significantly ($p<0.05$) increases the body weight (-5.1) when compared with sodium arsenite (SA) treated group. The ability of chrysin to play both preventive and curative roles was also seen in group 5 and 6 with percentage weight change of -6.18 and -0.81 when compare with 9.62 in normal control (1b) group. Therefore, chrysin was able to cause increase in body weight in both co-administration, preventive and curative designs.Percentage weight change (%WC) was calculated as final body weight minus initial body weight divided by initial body weight, multiply by one hundred.

Table 4.5.Effect of Various Treatments on the Body Weight in Male Wistar Rats during One Week Treatments

Treatment	**IBW (g)**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**%WC**
Normal control	109.5±6.4	116.0±2.8*	117.0±1.4*	112.5±0.7*	116.5±0.7*	115.0±1.4*	115.5±0.7*	116.0±1.4*	5.9
10 mg/kg b.w$NaAsO_3$	142.5±10.6	142.5±10.6	137.5±17.7	137.5±17.7	137.5±17.7	136.5±14.8	133.5±13.4	130.5±14.8	-8.4
10 mg/kg b.wChrysin	120.0±1.4	125.5±0.7*	123.0±1.4*	121.5±0.7	121.5±3.5	121.0±4.2	125.0±5.7	123.0±2.8	2.5
10 mg/kg b.w$NaAsO_3$ + 10 mg/kg b.wChrysin	136.0±12.7	140.5±14.8	138.0±14.1	129.5±12.0	128.0±8.5	124.5±6.4*	129.0±7.1	129.0±7.1	-5.1

Values are expressed as means ± SD bodyweight of various treatment groups (n=6), IBW = Initial body weight, WC = Weight Change within the week, b.w = Body Weight, * difference is significant when compare with IBW across the row ($p<0.05$ and n=6). The treatments were carried-out for one week.

Table 4.6.Effect of Various Treatments on the Body Weight in Male Wistar Rats during Two Week Treatments

Treatment	**IBW(g)**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day10**	**Day11**	**Day12**	**Day13**	**Day14**	**%WC**
Normal control (2 weeks)	119.5±	116.0±	117.0±	112.5±	116.5±	115.0±	115.5±	116.0±	118.5±	121.0±	126.0±	126.0±	128.5±	131.0±	131.0±	9.62
	6.4	2.8	1.4	0.7*	0.7	1.4	0.7	1.4	21.9	21.2	24.0	21.2	21.9	24.0	22.6	
10 mg/kg b.wChrysin (1st week)followed	134.3±	145.0±	140.3±	144.0±	144.6±	144.0±	145.3±	144.6±	141.5±	135.5±	131.5±	129.5±	127.0±	126.0±	126.0	-6.18
by10 mg/kg b.w**$NaAsO_3$ (2nd week)**	8.5	14.1	15.2	16.1	17.4	17.0	15.5	18.5	1.4*	0.7*	0.7*	4.2	1.4*	7.1	±7.1	
10 mg/kg b.w$NaAsO_3$ (1st week) followed	186.0±	185.5±	186.5±	186.0±	195.0±	192.0±	192.0±	171.5±	188.5±	172.5±	173.5±	175.0±	178.5±	181.5±	184.5	-0.81
byb.w10 mg/kg Chrysin (2nd week)	7.7	9.1	12.0	4.3	8.4	7.0	7.0	2.1*	2.1	2.1*	0.7*	2.8*	0.7*	0.7	±3.5	

Values are expressed as means ± SD bodyweight of various treatment groups, IBW = Initial body weight, * difference is significant when compare with IBW across the row ($p<0.05$ and n=6), WC = Weight Change within 2 weeks, b.w = Body Weight,Chrysin (1st week) followed by Sodium Arsenite (2nd week) and Vice-versa to access the preventive and curative effect of chrysin against arsenite induced toxicity.

4.4 Organ Weight

There was nosignificant ($p>0.05$) change in organ weight and relative organ weight as shown in (Table 4.7 and 4.8) during one and two weeks of treatments when compare with the various treatment groups.

Table 4.7.Effect of Various Treatments on the Organ Weight in Male Wistar Rats after One Week of Treatments

Treatment	**Normal control**	**10 mg/kg b.w$NaAsO_3$**	**10 mg/kg b.wChrysin**	**10 mg/kg b.w$NaAsO_3$ +10 mg/kg b.wChrysin**
Liver Weight (g)	5.03±0.04	5.30±1.10	4.50±0.30	4.80±0.50
%Relative Liver Weight	4.33±0.01	4.00±0.40	3.60±0.20	3.70±0.40
Brain Weight (g)	1.42±0.14	1.30±0.20	1.40±0.09	1.40±0.10
%Relative Brain Weight	1.20±0.11	1.00±0.07	1.10±0.07	1.10±0.10

Values are expressed as means ± SD (n=6),[a] Mean difference is significant ($p<0.05$) when compared with normal control group,[b] Mean difference is significant ($p< 0.05$) when compared with Sodium Arsenite treated group, b.w = Body Weight. The treatments were carried-out for one week.

Table 4.8.Effect of Various Treatments on the Organ Weight in Male WistarRats after Two Weeks Treatments

Treatment	**Normal control (2 weeks)**	**10 mg/kg b.wChrysin (1^{st} week)followed by10 mg/kg b.w$NaAsO_3$ (2^{nd} week)**	**10 mg/kg b.w$NaAsO_3$ (1^{st} week) followed by10 mg/kg b.wChrysin (2^{nd} week)**
Liver Weight (g)	6.02±1.50	4.60±0.50	6.50±0.50
%Relative Liver Weight	3.40±0.10	3.60±0.60	3.60±0.40
Brain Weight (g)	1.45±0.20	1.40±0.10	1.60±0.10
%Relative Brain Weight	0.89±0.33	1.10±0.20	0.90±0.04

Values are expressed as means ± SD (n=6), [a] Mean difference is significant ($p<0.05$) when compared with normal control group,[b] Mean difference is significant ($p< 0.05$) when compared with Sodium Arsenite treated group,b.w = Body Weight, Chrysin (1^{st} week) followed by Sodium Arsenite (2^{nd} week) and Vice-versa to access the preventive and curative effect of chrysin against arsenite induced toxicity.

4.5Lipid Peroxidation in Liver, Blood and Brain Tissues of Male Wistar Rats

The results indicated that sodium arsenite caused significant ($p<0.05$) increase in the level of MDA in the liver, blood and brain when compared with normal control groups(1a and 1b), (Figure 4.1). However, the difference was observed to be lower inchrysin treated group, co-administered group (ameliorative) and group 5 and 6 (preventive and curative) which received both sodium arsenite and chrysin in different patterns.Furthermore, the level of malondialdehyde decrease considerably across various organs, it was highest in brain followed by liver and blood.

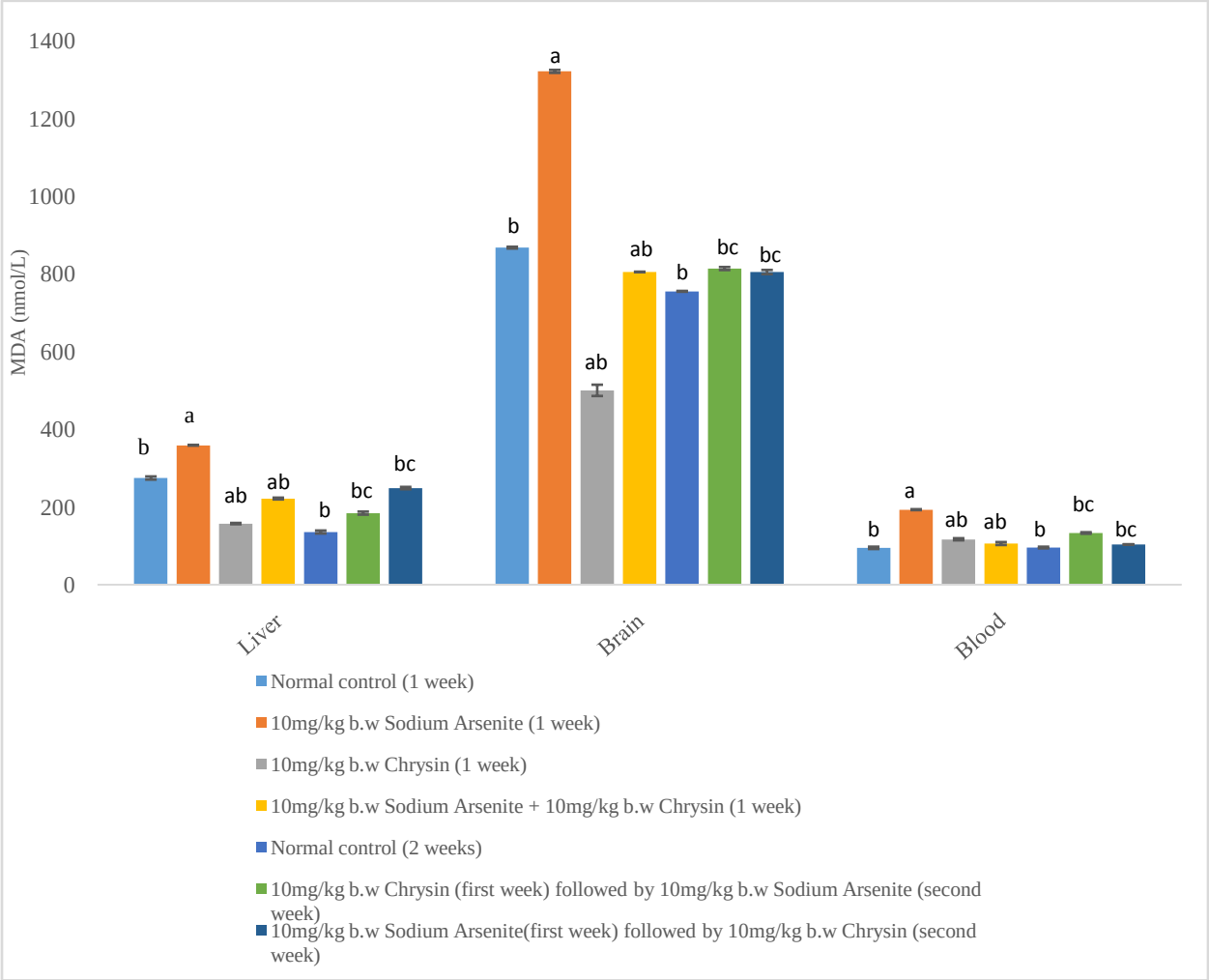


Figure 4.1 Effect of Chrysin on Sodium Arsenite-induced Lipid Peroxidation in Liver, Blood and Brain Tissue of Male Wistar Rats. Values are expressed as means ± SD (n=6), [a, c] Mean difference is significant ($p < 0.05$) when compared with normal control group (1a and 1b, respectively) in each organ, [b] Mean difference is significant ($p < 0.05$) when compared with Sodium Arsenite treated group in each organ respectively, MDA = Malonyl-aldehyde.

4.6Protein Carbonyl in Liver, Blood and Brain Tissues of Male Wistar Rats

The results indicated that sodium arsenite caused significant ($p<0.05$) increase in the level of protein carbonyl in the liver, blood and brain when compared with normal control groups (1a and 1b), (Figure 4.2). The difference was observed to be less in co-administered group (ameliorative) and group 5 and 6 (preventive and curative) which received both sodium arsenite and chrysin in different patterns.However, this decrease in protein carbonyl was higher in blood followed by brain and liver, respectively.

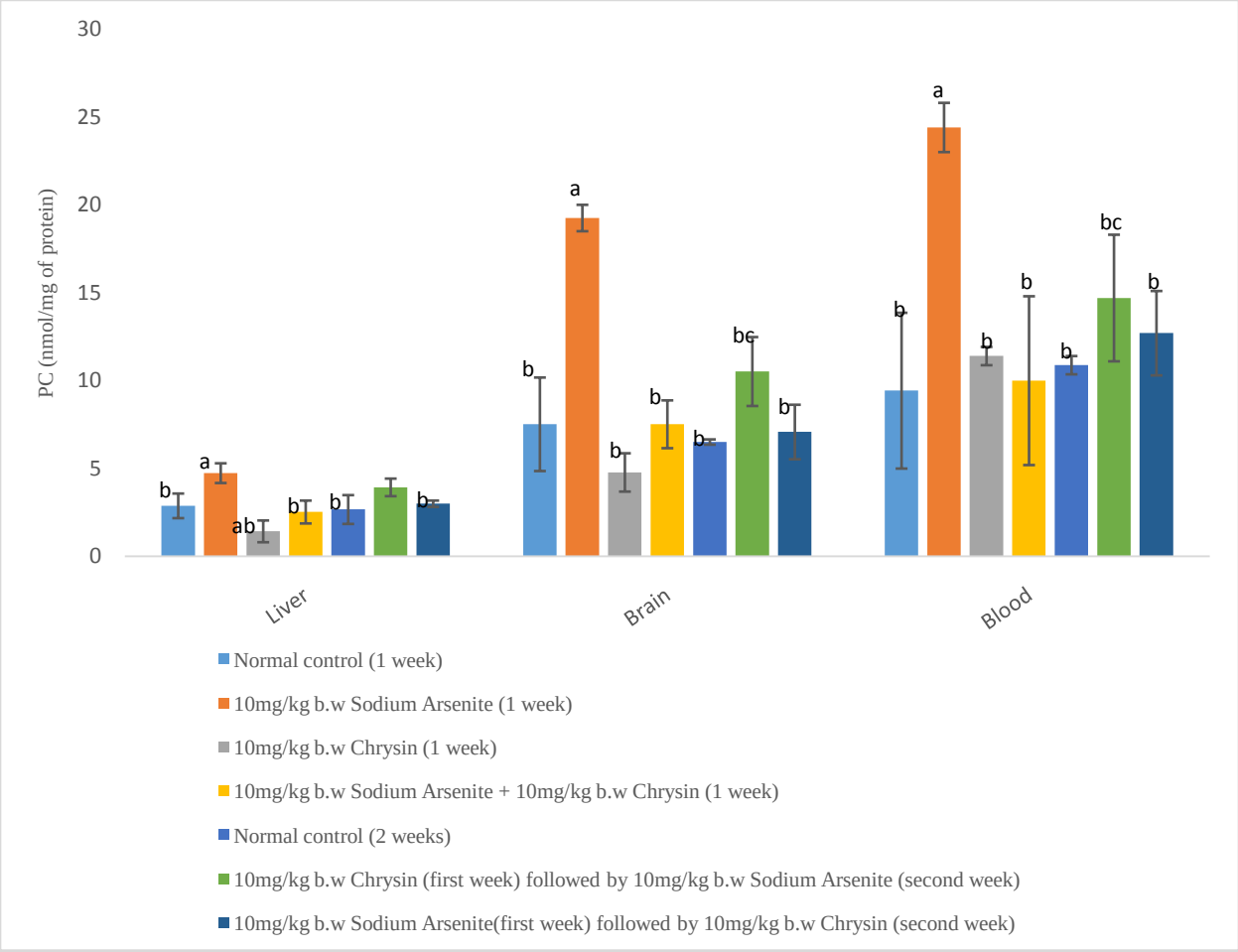


Figure 4.2 Effect of Chrysin on Sodium Arsenite-induced Protein Damage in Liver, Blood and Brain Tissue of Male Wistar Rats. Values are expressed as means ± SD (n=6), [a, c] Mean difference is significant ($p < 0.05$) when compared with normal control group (1a and 1b,

respectively) in each organ, [b] Mean difference is significant ($p < 0.05$) when compared with Sodium Arsenite treated group in each organ respectively, PC= Protein Carbonyl.

4.7DNA Fragmentation in Liver, Blood and Brain Tissues of Male Wistar Rats

The results indicated that sodium arsenite caused significant ($p<0.05$) increase in the level of DNA fragmentation in the liver, blood and brain when compared with normal control groups (1a and 1b), (Figure 4.3). This difference was observed to be less in co-administered group (ameliorative) and group 5 and 6 (preventive and curative) which received both sodium arsenite and chrysin in different patterns.Level of decrease in DNA fragmentation was observed to be highest in the liver followed by brain and blood, respectively.

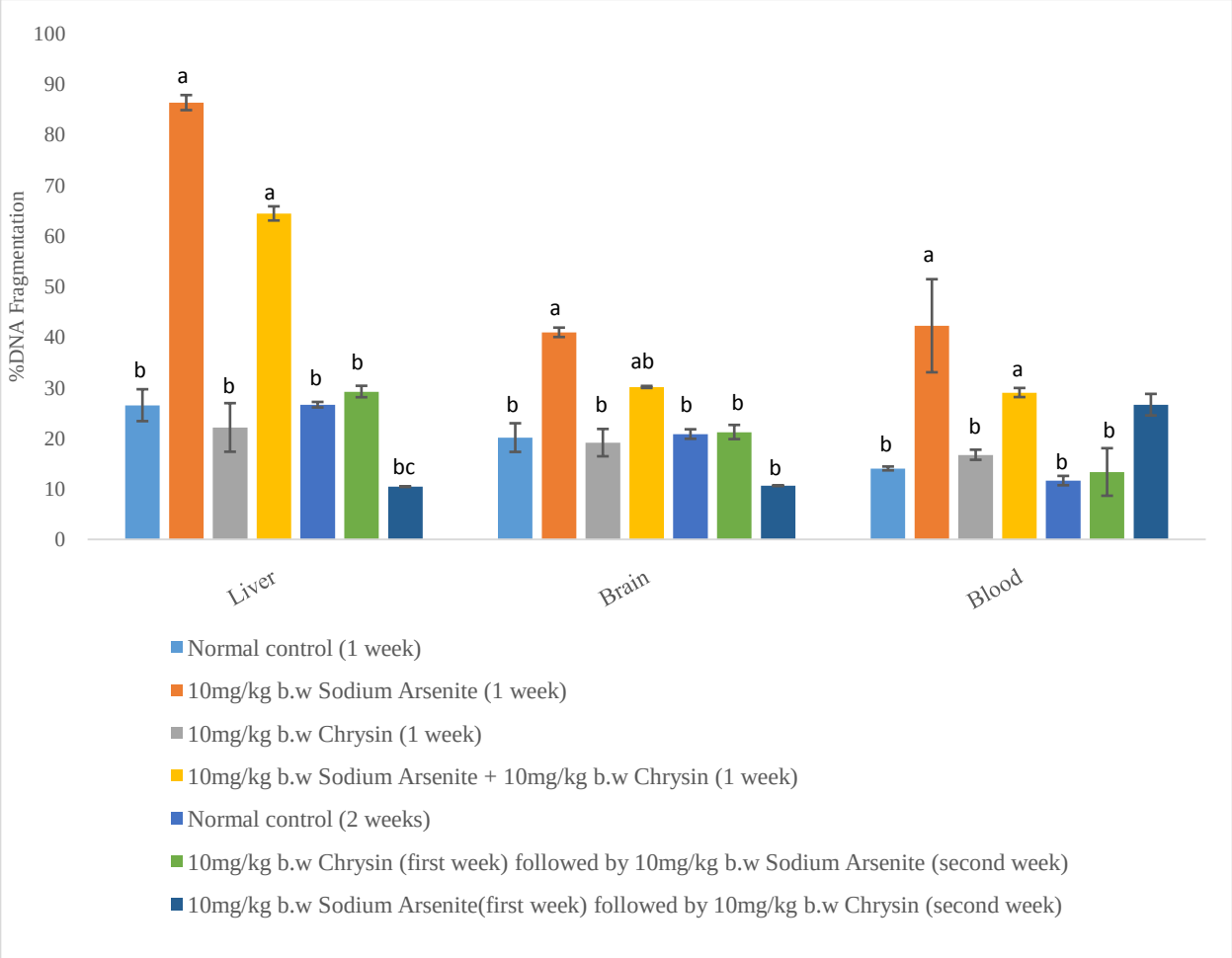

100
90
80
70
60
50
40
30
20
10
0
%DNA Fragmentation
a
b
b
a
b
b
bc
a
b
b
ab
b
b
b
a
b
b
a
b
b
Liver
Brain
Blood
Normal control (1 week)
10mg/kg b.w Sodium Arsenite (1 week)
10mg/kg b.w Chrysin (1 week)
10mg/kg b.w Sodium Arsenite + 10mg/kg b.w Chrysin (1 week)
Normal control (2 weeks)
10mg/kg b.w Chrysin (first week) followed by 10mg/kg b.w Sodium Arsenite (second week)
10mg/kg b.w Sodium Arsenite(first week) followed by 10mg/kg b.w Chrysin (second week)

Figure 4.3 Effect of Chrysin on Sodium Arsenite-induced DNA fragmentation in Liver, Blood and Brain Tissue of Male Wistar Rats. Values are expressed as means ± SD (n=6), [a, c] Mean difference is significant ($p < 0.05$) when compared with normal control group (1a and 1b, respectively) in each organ, [b] Mean difference is significant ($p < 0.05$) when compared with Sodium Arsenite treated group in each organ respectively.

4.8 Frequency of Micronucleated Polychromatic Erythrocytes in bone marrow cells

As expected, the clastogenic effect of sodium arsenitein bone marrow cells of Wistar rats was observed to be significantly ($p<0.05$) higher in sodium arsenite treated group (Figure 4.4, Plate I and II).Co-administration of sodium arsenite with chrysin significantly ($p<0.05$) ameliorate this effect,when compare to sodium arsenite control group and normal control (1a) group. Preventive and curative effect of chrysinwas observed in group 5 and 6 with higher reduction in the level of micronucleus when compare to sodium arsenite treated group and normal control group (1b), respectively.

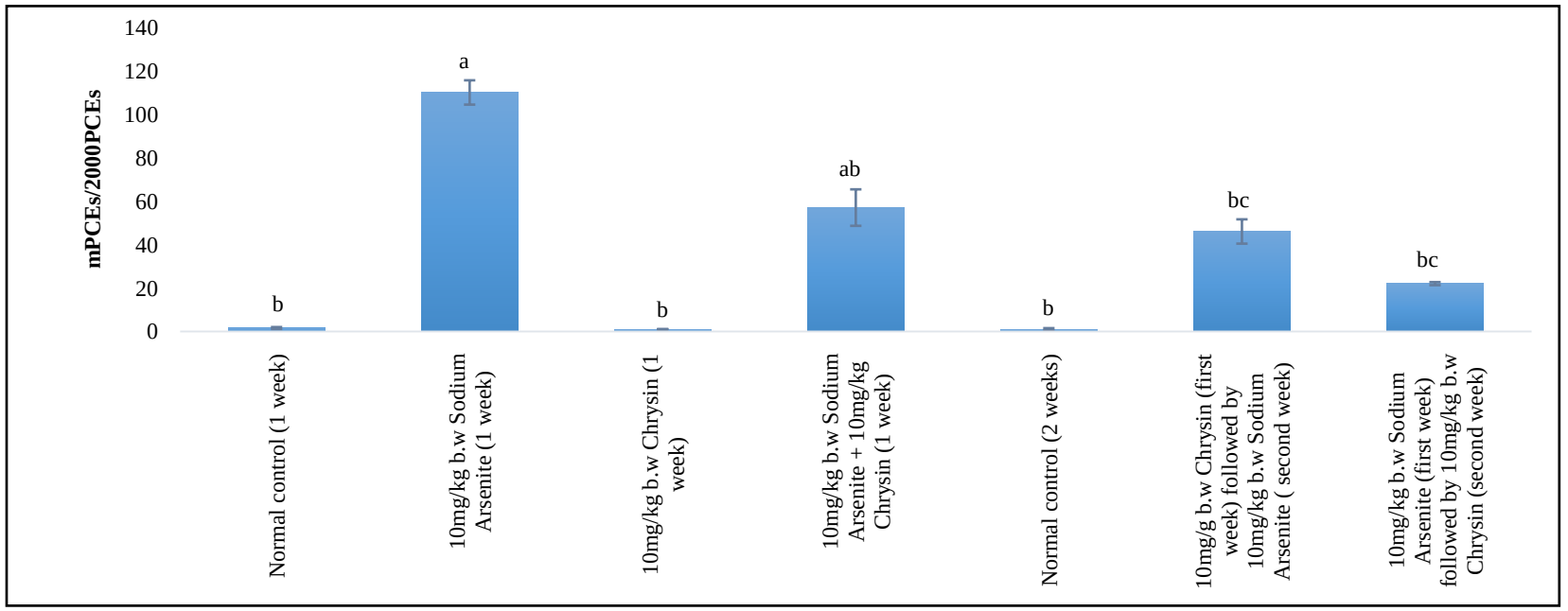


Figure 4.4 Effect of Chrysin Against Sodium Arsenite-induced Chromosomal Damage in Bone Marrow Cells of Male WistarRats.Values are expressed as means ± SD (n=6), [a] Mean difference is significant ($p< 0.05$) when compared with normal control group (1a), [b] Mean difference is significant ($p< 0.05$) when compared with Sodium Arsenite group, [c] Mean difference is significant ($p< 0.05$) when compared with normal control group (1b), mPCEs = micronucleated polychromatic erythrocytes, b.w = Body Weight, 1 week in bracket indicate treatments were carried-out in one week, Chrysin (1^{st} week) followed by Sodium Arsenite (2^{nd} week) and Vice-versa to access the preventive and curative effect of chrysin against arsenite induced toxicity.

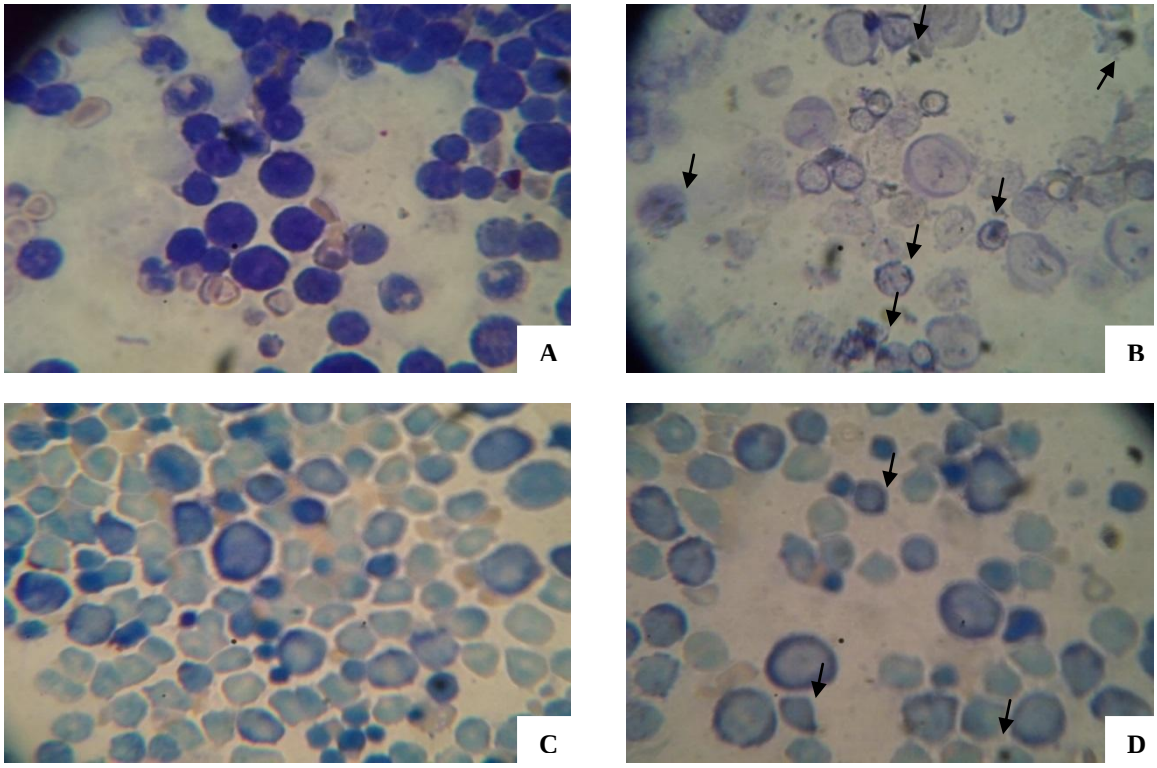


Plate I. Photomicrograph of the Bone Marrow Cells of Male Wistar Rats showing the Presence of Micronucleated Polychromatic Erythrocytes (mPCEs) (Week 1). Magnification X100, 5% Giemsa Stain, Arrow Pointing mPCEs and ABCD Indicating normal control,10 mg/kg b.w Sodium Arsenite, 10 mg/kg b.wChrysin, 10 mg/kg b.wChrysin + 10 mg/kg b.w Sodium Arsenite respectively.The treatment were carried out for one week.

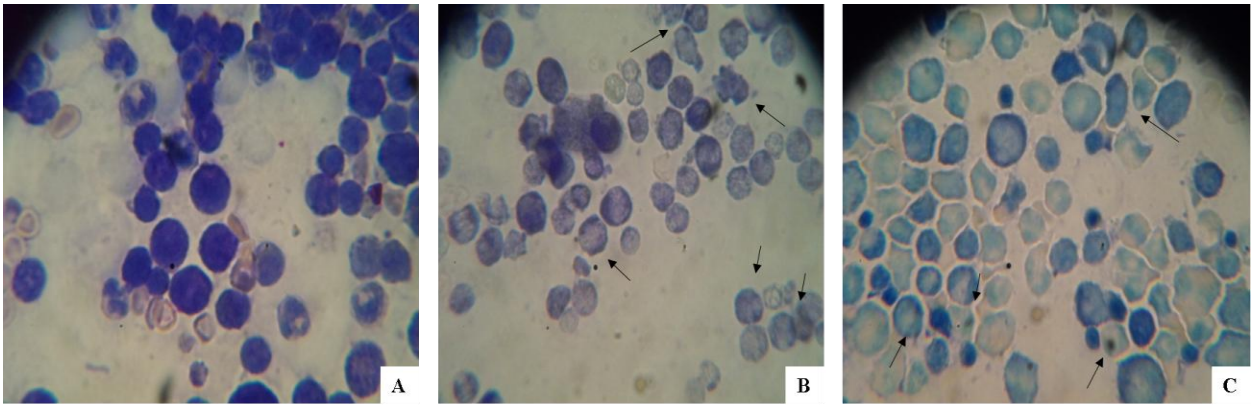


Plate II. Photomicrograph of the Bone Marrow Cells of Male Wistar Rats showing the Presence of Micronucleated Polychromatic Erythrocytes (mPCEs) (Week 2). Magnification X100, 5% Giemsa Stain, Arrow Pointing mPCEs and ABC Indicating normal control, 10 mg/kg b.wChrysin (1^{st} week) followed by 10 mg/kg b.w Sodium Arsenite (2^{nd} week), 10 mg/kg b.wSodium Arsenite (1^{st} week) followed with 10 mg/kg b.wChrysin (2^{nd} week) to access the preventive and curative effect of chrysin against arsenite induced toxicity.

4.9 Molecular Modeling of SAM-dependent Methyltransferase

The structure of chrysin (Figure 2.1) was docked into the binding site of S-adenosyl-L-methionine-dependent methyltransferase and the optimal conformation was identified (Figure 4.2). According to the docking result of the molecule, it is evident that the compound was adequately docked since the orientations are similar as a whole, validating the good quality of the docking model. To further elucidate the interaction between chrysin and the active site of 2B9E, the amino acids interacting with the ligand vis-à-vis the hydrophobicity were also determined, respectively (Figure 3a and 4a). Similarly, the 2-dimensional view of the hydrogen bond and hydrophobic interactions of 2B9E-chrysin complex were shown, respectively(Figure 4.3b and 4.4b). It was revealed that the binding pocket of 2B9E-Chrysin complex was made of Lys240, Ser215, Asp212, Gln211, Leu210, Cys369, Asn239 and S-adenosyl-L-methionine-dependent methyltransferases.Chrysin was found to have a high binding affinity for the receptor 2B9E, which was given as -99.82kJ/mol and an inhibition constant (Ki) of 0.959μM. This is obvious from the structure of chrysin which have a hydrophobic pocket to bind effectively with the receptor. The OH and C=O groups bind to about three amino acids in the binding pocket which influences the high binding constant by introducing some non-covalent interaction (Figure 4.3a). The presence of S-adenosyl-L-methionine (SAM) in NSUN5 protein also influences the binding energy value of chrysinwithin the complex (Figure 2 and 3). This can be seen in the interactions between chrysin and its binding site, where Lys240 served as bridge connecting chrysin and SAM (the main enzyme substrate) which may invariably alter the binding capacity of the latter and eventually affects the enzyme activity. The multiple ligand-binding interactions is also an indication of the potential of chrysin to inhibit S-adenosyl-L-methionine-dependent methyltransferases.

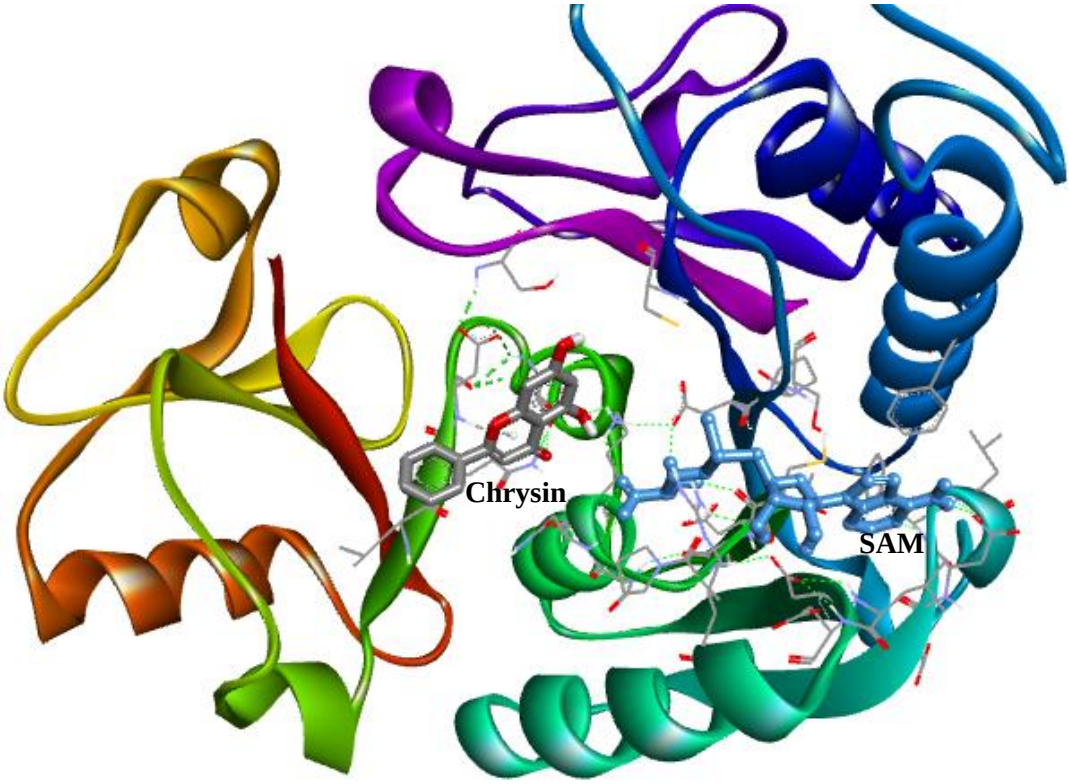


Figure 4.5. Predicted structure of the best docking pose conformation ofchrysinon 2B9E protei

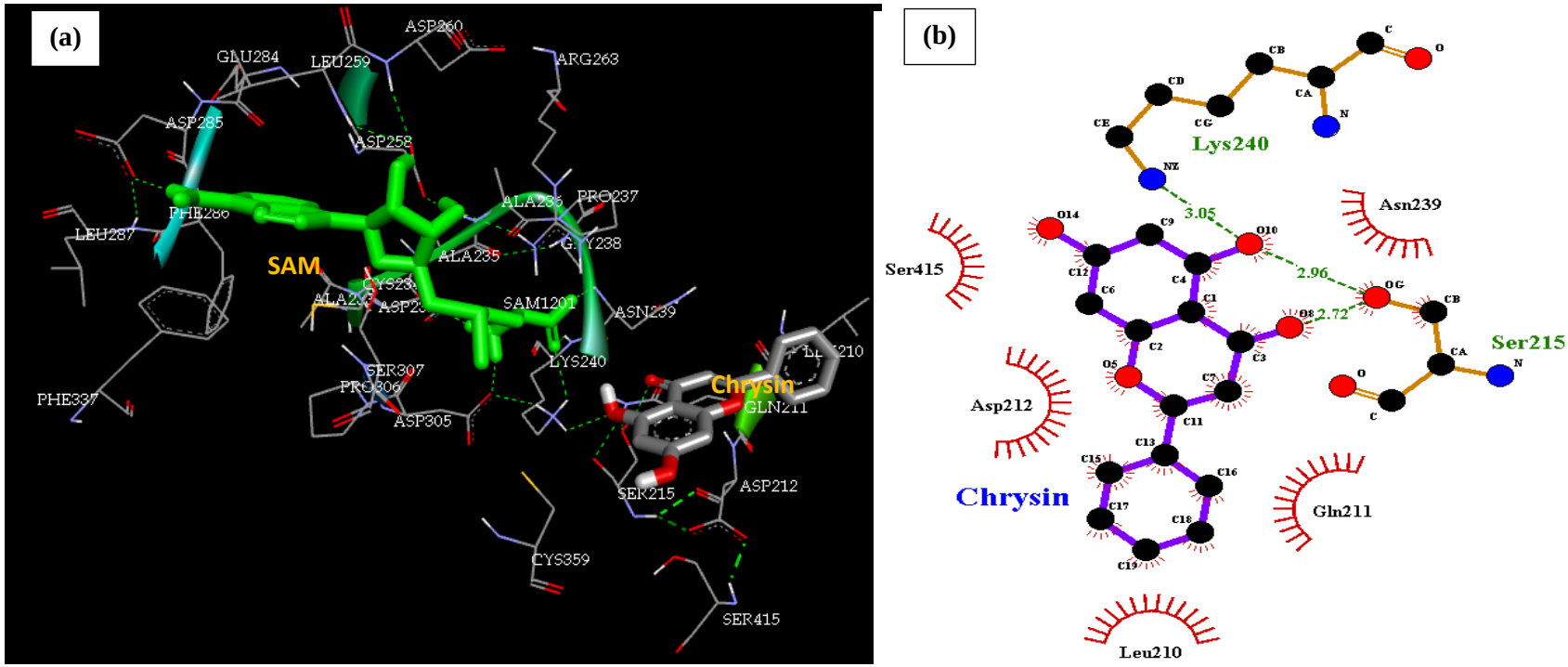


Figure 4.6.Illustration of the interacting amino acids within the active site of SAM-dependent methyltransferase 2B9(a) Chrysin with surrounding amino acids of 2B9E; (b)Hydrogen bond interaction between chrysin andamino acid within 2B9E active site.

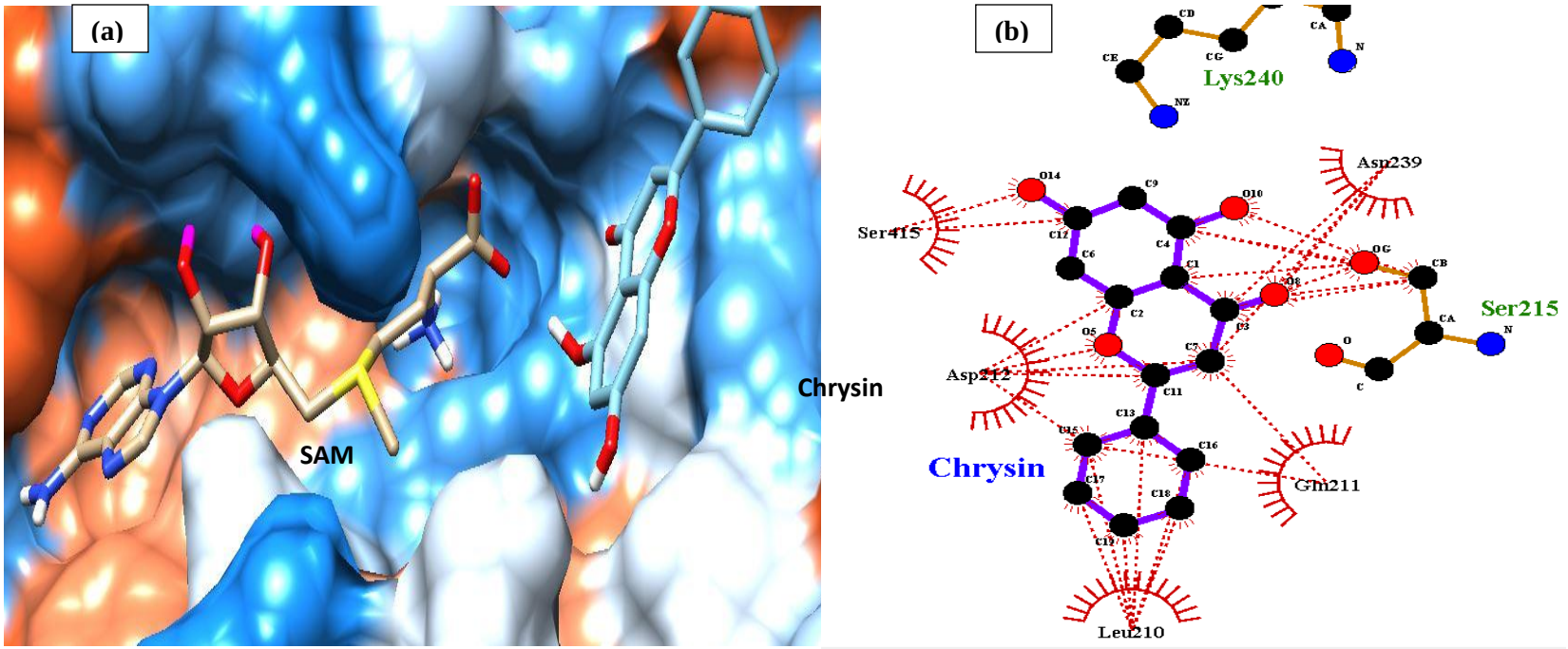


Figure 4.7.Illustration of hydrophobic interaction between chrysin and 2B9E Active Site(a) Chrysin with 4NEV (hydrophobicity surface)at the active binding site, in which the brown and blue regions represent oxygen and nitrogen atoms, respectively, whereas white regions indicate carbon or hydrogen atoms.(b) 2D illustration of the hydrophobic interaction between the binding modesof 2B9E-Chrysin.SAM =S-adenosyl-L-methionine.

CHAPTER FOUR

4.0 DISCUSSION

Arsenic contamination of drinking water from various sources has been reported in many parts of the world including developed and developing countries (Eunice *et al.,* 2016). Arsenic produces oxidative damage by disturbing the pro-oxidant–antioxidant balance, because of the high affinity for the sulfhydryl groups of reduced glutathione (GSH), affecting the maintenance of thiol-disulfide balance (Yousef, 2008). Multiple pathways such as inhibition of DNA repair, methylation status and carcinogenesis have also been proposed as the mechanism of arsenic induced-chromosomal and DNA damage(Hei and Filipic, 2004). It was also proposed that most of the DNA damage observed during iAs exposure is indirect, occurring mainly as a result of reaction of secondary oxidative products with DNA generating adducts, strand breaks, cross links and chromosomal aberrations(Martinez *et al*., 2011). Therefore, oxidative products of lipid peroxidation and protein damage can form DNA crosslink leading to chromosomal aberration and sister chromatids formation(Valko *et al.,* 2006). These effects results in enhanced production of hydrogen peroxide (H_2O_2) and free radicals that promote cellular injury and death (Rana *et al*., 2011), which may lead to various chronic diseases includes cancer, atherosclerosis among others (Atawodi, 2011a; Manoj and Padhy, 2013). Accordingly, the use of agents with powerful antioxidant properties remains the main approaches used to reduce metal-induced toxicity (Kumar, 2015).These are mostly via free radical scavenging ability of flavonoids in protecting human body from oxidative damage(Atawodi *et al.,* 2011b; Solomon and Odunola,2012).The present study, examined the possible ameliorative, preventive and curative roles of chrysin, a flavanoid grouped under flavones on sodium arsenite-induced chromosomal and DNA damage in male Wistar rats.

The findings indicate that chrysin was able to increase body weight of the experimental animal which was affected by sodium arsenite. The increase in body weight improvement was observed to be higher in a group that received sodium arsenitefor one week followed with chrysin for another week. Co-administration of sodium arsenite and chrysin in group four suppress this reduction in body weight which is slightly higher when compare with normal control (1a) group. This is in agreement with the findings of Muhammad and Aliyu,(2013) which shows increase in body weight after daily consumption of acacia honey in which chrysin is one of the active component. The observed reduction in the mean body weight of experimental rats treated with sodium arseniteonly when compared with control group in this study is in agreement with previous studies of Adegunlola*et al.*,(2012), Akin-idowu*et al*.(2015)who reported that exposure to low levels of arsenic altered hormonal function in the glucocorticoid system. This system play an important role in glucose regulation as well as carbohydrate, lipid and protein metabolism (Akin-idowu, 2015) and their dysfunction has been linked to weight loss/gain, which may explain the loss in weight observed.

Heavy metal ions, such as iron, copper, cadmium, mercury, nickel, lead and arsenic, can induce generation of reactive radicals and cause cellular damage via depletion of antioxidant enzymes activities, increase in lipid peroxidation and reaction with nuclear proteins and DNA (Rana *et al*., 2011; Birben *et al*., 2012). Lipid peroxidation leads to oxidative degradation of lipids via the process in which free radicals remove electrons from polyunsaturated fatty acids in cell membrane (Odunola *et al*., 2013). The resulting lipid peroxide radical, disrupts biological membranes having deleterious effect to their structure and function (Ayala *et al*., 2014). Oxidative products of lipid peroxidation such as MDA andacroleincan initiate the formation of DNA adducts thereby exacerbating chromosomal breakage. The present study shows that sodium

arsenite was able to significantly ($p<0.05$) increasethe level of thiobarbituric acids reacting substance in liver, blood and brain tissue. Interestingly, co-administration of sodium arsenite and chrysin was observed to ameliorate this damage when compared with normal control (1a) group. The concentration of MDA was higher in brain followed by liver and then blood.This findings is in conformity with previous data(Atawodi*et al.*,2011a and Adegunlola*et al.*,2012). Preventive and curative roles of chrysin against oxidative lipid damage in this study was observed to be significant ($p<0.05$) in group five and six when compare to sodium arsenite treated group and normal control groups (1b). Curative property of chrysin observed in group six could be attributed to its mainly antioxidant activity due to the presence of hydroxyl group at position at 5 and 7 of the flavan ring that can donate proton thereby quenching the chain of lipid oxidation induced by sodium arsenite(Janeiro *et al*., 2005; Łukaszewicz, 2011; Bilto *et al.*, 2012). This protective role of chrysin on sodium arsenite-induced generation of thiobarbituric acid reacting substances might be due to the ability of chrysin to increase the activities of catalase, superoxide dismutase, glutathione, glutathione peroxidase (Manzolli *et al*., 2015) and inhibit the expression of some prooxidant enzymes such as matrix metalloproteinase (Łukaszewicz, 2011; Jung, 2016) and possibly SAM-dependent methyltransferase in the case of arsenic toxicity(Florêncio, 2016). These of course, may possibly enhance DNA repair system(Wells *et al.*, 2010; Xiang *et al*., 2015).

Reactive oxygen species generated from metabolism of arsenite can cause fragmentation of the peptide chain, alteration of electrical charge of proteins, cross-linking of proteins and oxidation of specific amino acids (Birben *et al*., 2012). Products of protein oxidation were reported to bind to DNA leading to double and single strand breaks(Valko *et al*., 2006; Martinez *et al*., 2011). Oxidative damage to proteins results in a multitude of products(Natarajan *et al.*, 2016) and one

of the most commonly used markers to assess protein oxidation is via the determination of protein-bound carbonyls (Łukaszewicz, 2011; Weber *et al.,* 2015). Data from this study shows a significant increase ($p<0.05$) in the level of protein carbonyl in liver, blood and brain tissue of sodium arsenitetreated experimental rats. However, co-administration of sodium arsenite and chrysin demonstrate a significant ($p<0.05$) decrease in the level of protein carbonyl in comparison to sodium arsenite onlytreated group. Likewise, significant ($p<0.05$) decrease in protein carbonyl also was observed in group five and six when compare to sodium arseniteonly treated and normal control (1b)group. The protective and ameliorative effects of chrysin on protein carbonyl has been attributed to that ability of chrysin to appease the level reactive oxygen species induced by arsenite metabolism through radical scavenging and increase in the expression level of antioxidant enzymes(Patel, 2015; Jung, 2016). This might as well be linked to inhibition of SAM-dependent methyltransferase being the major enzymes involved in arsenic metabolism and generation of reactive oxygen species.

DNA fragmentation has been used as an index of DNA damage, apoptosis and/or necrosis possibly due to chemical-induced genotoxicity mediated by oxidative stress(Muhammad *et al*., 2016). Sodium arseniteled to significant ($p<0.05$) increase in the level of DNA damage in the liver, blood and brain tissue when compare with the normal control groups. But, co-administration of sodium arsenite with chrysin successfully reduced the level of DNA damage when compared to normal control (1a) group. Preventive and curative role of chrysin on sodium arsenite-induced chromosomal and DNA damage was observed in group five and six, respectively. The damage in DNA can be linked to the fact that arsenic can act as co-mutagen by reacting with thiol containing enzymes such as DNA ligase resulting in defective DNA

replication/repair, recombination and joining of single and double-stranded DNA breaks(Odunola *et al*., 2013).

Sodium arsenite is a clastogen causing chromosomal breakage, which interacts with other substances like metals to potentiate its effect (Muhammad and Ibrahim, 2015). Several studies have demonstrated that arsenite can induce chromosomal aberrations (CA), sister chromatid exchanges (SCE) and micronuclei (MN) in different cells types(Lihua and Yi, 2013). Inorganic arsenic induce chromosomal damage through covalent binding between iAs and DNA structures and crosslinking of DNA with oxidative bio-products (Martinez *et al*., 2011). Level of chromosomal damage has been reported in this study based on the frequency of micronuclei in polychromatic erythrocytes (PCEs). We have clearly demonstrated that sodium arsenite treatment significantly ($p< 0.05$) induced the formation of micronuclei in the polychromatic erythrocytes (mPCEs) of the rat bone marrow cells. These observation is consistent with earlier results on clastogenic potential of sodium arsenite in bone marrow (Solomon and Odunola, 2012). Chrysin was able to significantly ($p<0.05$) reduced the clastogenic effect induced by sodium arsenite observed in co-administered group as well as group five and six. This improvement may be due to ability of chrysin to donate a proton thereby quenching the sequential oxidation reactions of macromolecules as well as inhibition of methylation process of sodium arsenite leading to the formation of unstable dimethylarsinicperoxylradicals.

Sodium arsenite when ingested is bio-transformed to mono and dimethyl arsenic by S-adenosyl-L- methionine (SAM)-dependent methyltransferase which are more polar and easily excreted. However, when the level of dimethyl arsenic becomes high, it spontaneously reacts with superoxide radical to generate dimethyl arsenic peroxyl radical that can negatively affect the antioxidant system via a covalent binding to thiol group in proteins. This leads to generation of

ROS that can directly affect protein and DNA or indirectly via lipid peroxidation, peptide crosslinking initiating chromosomal and DNA damage with fatal metabolic consequences(Odunola *et al*., 2013). Therefore, the modulation of S-adenosyl-L- methionine (SAM)-dependent methyltransferase could be a viable strategy in combating arsenicals-induced toxicities. Based on the*in silico* studies, chrysin was able to comfortably docked in to the active site of S-adenosyl-L- methionine (SAM)-dependent methyltransferase via strong hydrogen bond and hydrophobic interactions. The present data was consistent with the previousobservations(Ramesh *et al.,*2015)where strong hydrogen bonds were formed between the hydroxyl groups on chrysin with polar amino acid residues in the binding pocket of some proteins in addition to the strong hydrophobic interactions. These might possiblysubstantiate the observed ameliorative, preventive and curative effects of chrysin against sodium arsenite-induced chromosomal and DNA damage in the present study, asS-adenosyl-L- methionine (SAM)-dependent methyltransferase is considered to be the major facilitator (Odunola *et al*., 2013).Thus,present study provide an insight into the possible role of chrysin in DNA repair system.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

1. Chrysin decreased the level of lipid peroxidation in the liver, blood and brain tissues of Wistar rats induced by administration of sodium arsenite.
2. Chrysin caused a decrease in the frequency of micronucleated polychromatic erythrocytes (mPCEs) in bone marrow cells and the level of DNA fragmentation in the liver, blood and brain tissues of male Wistarrats exposed to sodium arsenite.
3. Administration of chrysin also cause a decrease in the level of protein oxidation in the liver, blood and brain tissues of rats treated with sodium arsenite.
4. Chrysin has the potential to bind SAM-dependent methyltransferase*in silico*with greater binding affinity (-99.82kJ/mol) and inhibition constant (Ki) of 0.959μM.

6.2 Conclusion

In conclusion, findings from this study revealed that chrysin confers both ameliorative, preventive and curative effects against sodium arsenite-induced chromosomal and DNA damage in male Wistar rats,which might be due to inhibition of SAM-dependent methyltransferase. This enzyme is actively involved in arsenic toxicity and its inhibition could serve a signal to initiate DNA repair process.

6.3 Recommendations

Further work to ascertain the effects of chrysin on SAM-dependent methyltransferase and DNA repair enzymes *in vivo* is recommended.

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APPENDIX I

EXPECTED OUTCOMES

1. Since chrysin has the ability to donate proton, it is expected to reduce oxidative damage and lower the level of malondialdehyde induced by sodium arsenite.
2. Chrysin is expected to prevent chromosomal breakage and DNA damage thereby maintaining the integrity of genetic materials.
3. Chrysin is expected to play a positive role in preventing a cluster of protein damage due to sodium arsenite-induced toxicities.
4. Chrysin is expected to inhibit the activity of SAM-dependent methyltransferase involved in arsenic metabolism and *in silico*modelling can be used to show that it can stabilized the enzyme's active site.

APPENDIX II

CONTRIBUTIONS TO KNOWLEDGE

1. Chrysin among other components may be responsible for the ameliorative, preventive and curative effects shown by Acacia honey, propolis, chamomile and medicinal mushroom against lipid peroxidation.
2. Chrysin can provide protection against oxidative protein damage induced by arsenic toxicities.
3. Chrysin shows anticlastogenic/ antigenotoxic effect by controlling chromosomal and DNA fragmentation.
4. Inhibitory potential of chrysin against SAM-dependent methyltransferase may serve a target against arsenic-related toxicities.

APPENDIX III

1. Protective Effects of Chrysin Against Sodium Arsenite-induced Oxidative Stress and DNA Damage in Liver, Blood and Brain of Male Wistar Rats

	Treatment	MDA (nmol/L)	PC (nmol/mg of protein)	% DNA fragmentation
Liver	Normal control	274.20 ± 4.07^{b}	2.88 ± 0.70^{b}	26.50 ± 3.15^{b}
	10 mg/kg b.w $NaAsO_3$	358.50 ± 0.90^{a}	4.74 ± 0.56^{a}	86.30 ± 1.49^{a}
	10 mg/kg b.wChrysin	156.90 ± 1.60^{ab}	1.43 ± 0.62^{ab}	22.10 ± 4.80^{b}
	10 mg/kg b.w $NaAsO_3$ + 10 mg/kg b.wChrysin	221.30 ± 2.30^{ab}	2.53 ± 0.65^{b}	64.40 ± 1.40^{a}
Blood	Normal control	94.42 ± 2.99^{b}	9.43 ± 4.43^{b}	14.00 ± 0.38^{b}
	10 mg/kg b.w $NaAsO_3$	192.70 ± 1.20^{a}	24.40 ± 1.40^{a}	42.20 ± 9.20^{a}
	10 mg/kg b.wChrysin	116.50 ± 2.70^{ab}	11.40 ± 0.52^{b}	16.70 ± 1.00^{b}
	10 mg/kg b.w $NaAsO_3$ + 10 mg/kg b.wChrysin	105.50 ± 3.80^{ab}	10.00 ± 4.80^{b}	29.00 ± 0.90^{a}
Brain	Normal control	867.17 ± 2.25^{b}	7.52 ± 2.66^{b}	20.10 ± 2.83^{b}
	10 mg/kg b.w $NaAsO_3$	1320.90 ± 3.80^{a}	19.25 ± 0.75^{a}	40.90 ± 0.93^{a}
	10 mg/kg b.wChrysin	499.80 ± 14.50^{ab}	4.78 ± 1.09^{b}	19.10 ± 2.70^{b}
	10 mg/kg b.w $NaAsO_3$ + 10 mg/kg b.wChrysin	804.80 ± 0.49^{ab}	7.52 ± 1.36^{b}	30.10 ± 0.20^{ab}

Values are expressed as means ± SD (n=6) [a] Mean difference is significant (P<0.05) when compared with normal control group (1a) along the column in each organ respectively, [b] Mean difference is significant (P < 0.05) when compared with Sodium Arsenite treated group along the column in each organ respectively, MDA = Malonyl-aldehyde, PC = Protein Carbonyl. The treatments were carried-out for one week.

2. Protective Effects of Chrysin Against Sodium Arsenite-induced Oxidative Stress and DNADamage in Liver, Blood and Brain of Male Wistar Rats

	Treatment	**MDA (nmol/L)**	**PC (nmol/mg of protein)**	**% DNA fragmentation**
Liver	Normal control	135.18±3.82	2.67±0.82	26.60±0.54
	10 mg/kg b.wChrysin (1st week) followed by 10 mg/kg b.wSodium Arsenite (2nd week)	183.80±3.90[a]	3.93±0.50	29.20±1.13
	10 mg/kg b.wSodium Arsenite (1st week) followed by 10 mgb.wChrysin (2nd week)	248.30±3.00[a]	3.00±0.18	10.40±0.07[a]
Blood	Normal control	95.28±2.09	10.88±0.52	11.60±0.92
	10 mg/kg b.wChrysin (1st week) followed by 10 mg/kg b.wSodium Arsenite (2nd week)	132.70±1.90[a]	14.70±3.60[a]	13.30±4.70
	10 mg/kg b.wSodium Arsenite (1st week) followed by 10 mg/kg b.wChrysin (2nd week)	103.30±0.40[a]	12.70±2.40	26.60±2.13
Brain	Normal control	754.76±1.07	6.51±0.15	20.80±0.94

10 mg/kg b.wChrysin (1st week) followed by 10 mg/kg b.wSodium Arsenite (2nd week)	813.40±3.90[a]	10.52±1.96[a]	21.20±1.40
10 mg/kg b.wSodium Arsenite (1st week) followed by 10 mg/kg b.wChrysin (2nd week)	804.40±5.40[a]	7.08±1.55	10.60±0.08

Values are expressed as means ± SD (n=6), [a] Mean difference is significant (P<0.05) when compared with normal control group (1b) along the column in each organ respectively, MDA = Malonyl-aldehyde, PC = Protein Carbonyl, Chrysin (1st week) followed by Sodium Arsenite (2nd week) and Vice-versa to access the preventive and curative effect of chrysin against arsenite induced toxicity.

APPENDIX IV

REAGENT PREPARATIONS

Tissue Homogenate

1. The tissues were rinsed with ice-cold saline solution (0.9% NaCl).

3. Tissue homogenate was prepared (1g of tissue in 10ml chilled phosphate buffer, 10 mM, pH 7.4).

4. The homogenate was centrifuge to remove large particles (3000xg at 4°C for 10 minutes).

5. An aliquot of the supernatant was used for protein determination.

6. The remaining samples were kept in ice for other analysis.

LIPID PEROXIDATION

15% Trichloroacetic acid (TCA)

Exactly, 75 g of TCA was dissolved in 200 ml of distilled water and the solution was made to 500ml using distilled water.

0.67% Thiobarbituric acid (TBA)

Exactly, 1.005g of TBA was dissolved in 50ml of distilled water and the volume was made to 150ml by adding more distilled water.

MICRONUCLEUS ASSAY

Sodium citrate solution (2.2%)

Exactly, 2.2g of sodium citrate salt was dissolved in 40ml of distilled water and volume was made up to 100ml.

5% Giemsa stain

Exactly, 5 ml of stock giemsa solution was added in 95 ml of phosphate buffer (10mM, pH 7.4) and mixed clearly.

DNA FRAGMENTATION

5% Trichloroacetic acid (TCA)

Exactly, 5grams of TCA was dissolved in 20 ml of distilled water and the solution was made to 100ml.

25% Trichloroacetic acid (TCA)

Exactly, 50grams of TCA was dissolved in 100ml of distilled water and the solution was made to 200ml.

Diphenylamine solution

Exactly, 10ml glacial acetic acid was added to 150mg diphenylamine in a 50ml polypropylene tube and mixed thoroughly by repeated inversion until complete dissolution. Thereafter, 150µl concentrated sulfuric acid was added and mixed thoroughly followed by 150µl acetaldehyde solution (2% v/v) and mixed thoroughly. This solution was only preparedfresh when needed.

PROTEIN DAMAGE

Hydrochloric acid solution (2.5M)

Specifically, 20ml of distilled water was added into 44.66ml of 12.3M hydrochloric acid contained in 500ml flat bottom flask and swirled to mix and the volume was made to 180ml.

Preparation of 2, 4- dinitrophenylhydrazine (10mM)

Precisely, 0.4356g of the reagent (2,4-dinitrophenylhydrazine) was dissolved in 50ml of 2.5M hydrochloric acid and the volume was made up to 220ml with the same acid solution.

10% TCA

Strictly, 10g of TCA was dissolved in 20 ml of distilled water and the solution was made to 100ml.

20% TCA

Exactly, 20g of TCA was dissolved in 20 ml of distilled water and the solution was made to 100ml.