Safety Evaluation of a Polyherbal Antihypertensive Mixture Used in Ghana in Mice

by

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DECLARATION

I hereby declare that this submission is my own work towards the Doctor of Philosophy Degree and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

I dedicate this Thesis to my Dearest Mummy, the late

Mrs. Ellen Kate Asumeng Koffuor

ABSTRACT

Herbal medicine in being used extensively globally with the notion that they are natural and therefore relatively safe compared to other forms of medicine. This study therefore assessed the safety of an FDB approved polyherbal anti-hypertensive used extensively in Ghana. Using ICR mice, the effect of 32 - 550 mg/kg/day of the product on; general health, locomotory activity, muscular coordination and strength, vestibular function, organ functions, hematological, lipid, and histopathological profiles, urine content, pentobarbitone-induced sleeping time, cytochrome P450 activity and reproductive toxicity was assessed. One hour post-treatment observation in acute toxicity studies showed sedation and lethargy and a significant dose-dependent reduction ($P \leq 0.05$-0.001) in locomotion, rearing, centering, grip strength, muscle coordination, and righting response. These observations were however not significant 24 h post-treatment. Acute toxicity studies also showed no significant differences in hematological profile (except in WBC), liver function, kidney function (in plasma urea), and urine content up to 10 days post-treatment. A 45-day sub-chronic toxicity study showed no physical deterioration, and observable clinical and autonomic toxic symptoms. Among other vital organs, only the liver showed significant decrease ($P \leq 0.01$-0.001) in organ weight to body weight ratio with treatment. WBC increased significantly ($P \leq 0.05 - 0.001$) while RBC, MCV, and RBC distribution width were not affected. HGB decreased significantly ($P \leq 0.001$) after 23 days but normalized. HCT, PCT and PDW increased significantly ($P \leq 0.001$) and MCH and MCHC were significantly reduced ($P \leq 0.01$-0.001). Plasma albumin

decreased ($P \leq 0.01 - 0.001$) initially but later increased significantly ($P \leq 0.001$) globulin levels increased significantly ($P \leq 0.001$) hence a significant increase ($P \leq 0.001$) in total protein. ALT, AST, ALP increased significantly ($P \leq 0.01$) after 23 days post-treatment but decreased very significantly ($P \leq 0.001$) below the control after 45 days. Direct, indirect, and total bilirubin increased significantly ($P \leq 0.001$) with duration of treatment. Plasma urea decreased significantly ($P \leq 0.001$) but creatinine increased significantly ($P \leq 0.001$) with higher dosing and longer treatment time. Total cholesterol and HDL reduced very significantly ($P \leq 0.001$) initially but returned to normal while TAG, VLDL, and LDL were significantly very high ($P \leq 0.001$) initially but reduced by day 45. Urine analysis showed no significant changes. The liver, kidney and spleen showed histopathological changes. Pentobarbitone-induced sleeping time was prolonged. Liver cytochrome P450 level decreased very significantly ($P \leq 0.001$). Observations made suggested that the product is not lethal but had CNS depressant, anxiolytic, and probably muscle relaxant activity which affected activity and neurological behavior. It did not have abnormal proliferative effect on blood forming cells but caused microcytic-anisocytic anaemia. Liver synthetic and excretory functions colud be affected with its use. Concomitant administration with other drugs could result in some drug interaction as the product inhibits CYP 450. Within limits of the doses administered in this study, it did not cause reproductive toxicity.

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LIST OF ABBREVIATIONS

ALP	*Alkaline phosphatase*
5-HT	5-hydroxytrypamine
ALT	*Alanine aminotransferase / Alanine transaminase*
AMPA	*α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid*
AST	*Aspartarte aminotransferase / Aspartate transaminase*
CHOL	Cholesterol
CNS	Central Nervous System
CSRPM	Centre for Scientific Research into Plant Medicine
CYP450	Cytochrome P450
FDB	Food and Drugs Board
FI	Fertility Index
GABA	Gamma aminobutyric acid
GGT	*Gamma glutamyl transferase*
GHS	Ghana Health Service
GLP	Good Laboratory Practice
GOT	*Glutamic oxaloacetic transaminase*
GP	Gestation Period
GPT	*Glutamic pyruvate transaminase*
HCT	Hematocrit
HDL	High Density Lipoprotein
HGB	Hemoglobin
ICR	Imprinting Control Region
LBI	Live Birth Index
LDL	Low Density Lipoprotein
LW	Litter weight
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume

MI	Mating Index
NMDA	N-Methyl-D-Aspartate
NOAEL	No-observable-adverse-effect-level
PHA	Polyherbal antihypertensive
PLT	Platelet
RBC	Red Blood Cell
RDW-CV	Red Blood Cell Distribution Width -Coefficient Variation
RWD-SD	Red Blood Cell Distribution Width - Standard Deviation
STP	Society of Toxicologic Pathology
TAG	Triglyceride
VLDL	Very Low Density Lipoprotein
VTA	Ventral Tegmented Area
WBC	White Blood Cell
WHO	World Health Organisation
WI	Weaning Index

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 INTRODUCTION

The use of herbal medicine has been on the increase in many developing and industrialized countries (Fakeye *et al.*, 2009; Ernst, 2003) probably due to patients' dissatisfaction with conventional allopathic medicines in terms of effectiveness and/or safety, satisfaction with therapeutic outcome (Huxtable, 1990; Abbot, 1997), easy accessibility, reduced cost, and the perception that herbal medicines are inherently safe (Fakeye *et al.*, 2009). Herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients (WHO, 2008). In some Asian and African countries, over 80 % of the population depend on traditional healing modalities, including herbal remedies, for health maintenance and therapeutic management of disease (WHO, 2002 a).

In Ghana, it is estimated that more than 60 % of the population uses traditional medicine in many instances to help meet some of the primary health care needs. Many people use both orthodox and traditional medicine to treat diabetes, epilepsy, stroke, hypertension, cardiovascular disorders, breast, cervical and prostate cancers, infertility, erectile dysfunctions, etc**.** In fact it would be accurate to say that more than 90 % of the population in Ghana has used some form of traditional medicine at one point in their life (Youpele, 2009). Most herbal users

perceived that herbs were efficacious, and in some instances, more efficacious than conventional medicines.

Cardiovascular diseases are chronic diseases that cause death in virtually all industrialized countries (Whelton, 1994). Hypertension, one such cardiovascular disease, is a primary risk factor for heart disease and stroke, the leading cause of death worldwide (Chockalingam *et al.*, 2006). The World Health Report 2002 identified hypertension as the third ranked factor for disability-adjusted life years (Chockalingam *et al.*, 2006). Recent analyses have shown that as of the year 2000, there were 972 million people living with hypertension worldwide, and it is estimated that this number will escalate to more than 1.56 billion by the year 2025 (Kearney *et al.*, 2005).

Hypertension is now widely reported in Africa and is the most common cause of cardiovascular disease on the continent. It is estimated that more than 20 million Africans are affected, majority of which live mainly in urban areas (Addo *et al.*, 2007). Prevalence ranges from 25-35 % in adults aged 25-64 years, and its prevention and control could avoid at least 250,000 deaths in Africa per year (WHO report, 2002).

In Ghana, the prevalence of hypertension in urban Accra is 28.3 % (crude) and 27.3 % (age-standardized) (Amoah, 2003). In Kumasi and surrounding communities, the prevalence is 28.7 % (Cappuccio *et al.*, 2004). The Ghana

Health Service indicates that hypertension is the number one killer disease in Ghana today. It is now the second most reported medical condition but was the fifth as at 2006 (GHS News Release, 2007). The upsurge of hypertension has led to an increase in the demand for alternative antihypertensive medicines. The use of herbal products has therefore become very necessary in our healthcare systems because morbidity and mortality from chronic diseases is still on the increase.

Herbal remedies are very effective in hypertension (Napier, 2011). Garlic (*Allium sativum*), Hawthorn berries, Garcinia (*Garcinia cambogia*), Ginger (*Zingiber officinale*), Thankuni (*Centella asiatica*), Shepherd's Purse (*Capsella bursa-pastoris*), and Gingko Biloba, just to mention a few, can help to cleanse, widen and relax blood vessels and reduce cholesterol lining the vessels (Napier, 2011). Counterfeit, poor quality or adulterated herbal products are serious patient safety threats. This has prompted drug regulatory agencies in many countries (including Ghana) to raise safety concerns on the use of these products because little or no toxicological studies have been conducted on most of these products.

An aqueous antihypertensive preparation made from bark and leaf extracts of *Persea americana*, and *Vernonia amygdalina* respectively, claimed to be effective as an antihypertensive by most users, was studied. This product has been registered by the Food and Drugs Board (FDB). Though there are no reports that this polyherbal antihypertensive is causing health hazards, a safety assessment and reproductive toxicity profile of the product will be beneficial as all medicines have

the potential of causing health hazards. This study therefore sought to assess the safety profile of the polyherbal anti-hypertensive product in ICR mice.

1.2 THE CONCEPT OF TOXICITY

Toxicity is defined as the state or quality of being poisonous or capable of causing harm to exposed humans or animals or the degree to which a substance is poisonous or can cause harm to exposed humans or animals (Schimelpfening, 2010). Toxicity can refer to the effect on a whole animal, as well as the effect on a substructure of the animal, such as a cell (cytotoxicity) or an organ (organ toxicity), such as the liver (hepatotoxicity). In the context of pharmacology, toxicity occurs when a person has accumulated too much of a drug in his bloodstream, leading to adverse effects within the body. Drug toxicity may occur when the dose given is too high or the liver or kidneys are unable to eliminate the drug from the bloodstream, allowing it to accumulate in the body (Schimelpfening, 2010). A central concept of toxicology is that effects are dose-dependent; even water can lead to water intoxication when taken in large enough doses, whereas for even a very toxic substance such as snake venom there is a dose below which there is no detectable toxic effect (Hyman, 2012).

1.3 REQUIREMENTS FOR TOXICITY TESTING

For every drug developed to be approved for use in humans and animals, it has to go through years of testing for efficacy and other pharmacokinetic parameters, as well as safety before approval. Toxicity testing for drugs was not very important

and was very relaxed in the 19th century, but that changed in the 20th century after the tragedy in 1937, when a drug labeled "Elixir of Sulfanilamide," killed more than 100 people. The US congress then passed laws that required safety testing of drugs on animals before they could be marketed for human use (Ballentine, 1981).

In the 1960s there was the "Thalidomide tragedy", which caused the laws to be expanded to cover safety testing on pregnant animals before a drug is sold (Gad, 2007). In the post-war era when sleeplessness was prevalent, thalidomide was marketed to a world hooked on tranquilizers and sleeping pills. At the time, one out of seven Americans took them regularly (Gad, 2007). The demand for sedatives was even higher in some European markets, and the presumed safety of thalidomide, the only non-barbiturate sedative known at the time, gave the drug massive appeal (Gad, 2007). Sadly, tragedy followed its release. The drug interfered with a babies' normal development, causing many a baby to be born with phocomelia (shortened, absent, or flipper-like limbs). This catalyzed the beginnings of the rigorous drug approval and monitoring systems in place at the United States Food and Drug Administration (FDA) today (Fintel *et al.*, 2009). Presently toxicological testing is required by all Food and Drug controlling agencies before a product is approved for use (O'Connell, 2008).

Safety studies employ qualitative and quantitative scientific methods to determine the adverse effects of chemicals on living organisms with a view to preventing and minimizing the degree and duration of undesirable effects (Asiedu-Gyekye *et al.*,

2004). It involves performing controlled testing in the laboratory (in *in vitro* assay and in animal models) to determine the toxicity of a compound or drug to a tissue, organ, or system at various concentrations. A safety study is usually performed before administration to humans begins for use as medicines.

Toxicity can be acute, sub-chronic, or chronic. Toxicity studies are designed to supplement the human experience in defining possible toxicity from short-term and prolonged exposure or long after such exposure has been discontinued. This is an important requirement, especially if the preparations are being developed for use as over-the-counter medicines. Under such circumstances, the client is unlikely to have the benefit of the practitioner or manufacturer on matters of medicine therapy-related adverse reactions. In view of these, plant medicine requires safety assessment to facilitate documentation of possible side/adverse effects in order to enable informed choices to be made during patient management for better health care. Safety testing is therefore a legal requirement in Ghana.

1.4 TYPES OF TOXICITY

1.4.1 Acute Toxicity

Acute toxicity is produced by a pharmaceutical when it is administered in one or more doses over a period not exceeding 24 hours (CDER, 1996). Acute toxicity is studied by using a rising dose until signs of toxicity become apparent. Current European legislation demands that "acute toxicity tests must be carried out in two or more mammalian species" covering "at least two different routes of

administration" (EU Directive, 2004). Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use. The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans (Boxenbaum and DiLea, 1995; CDER, 1996).

Acute toxicity studies in animals are conducted using two routes of drug administration: the route intended for human administration, and intravenous administration, if feasible. When intravenous dosing is proposed in humans, use of this route alone in animal testing is sufficient (CDER, 1996). Animals are observed for 14 days after drug administration for possible delayed toxicity. All mortalities, clinical signs, time of onset, duration, and reversibility of toxicity are recorded.

Acute toxicity studies in animals provide the primary safety data supporting single dose safety/kinetic studies in humans (e.g., a study screening multiple analogues to aid in the selection of a lead compound for clinical development). These are designed to assess dose-response relationships and pharmacokinetics. Clinical pathology and histopathology are monitored at an early time and at termination (i.e., ideally, for maximum effect and recovery).

The studies are designed so that the maximum amount of information is obtained from the smallest number of animals. Calculating lethality parameters (e.g., LD_{50}) using large numbers of animals, as was done previously, is not recommended. This test was removed from OECD international guidelines, replaced by methods such as the fixed dose procedure, which use fewer animals and cause less suffering (CFHS, 2000; FRAME, 2006). To avoid causing excessive pain or tissue damage in the animals, pharmaceuticals with irritant or corrosive characteristics are not administered in concentrations that produce severe toxicity solely from local effects (CDER, 1996).

1.4.2 Sub-Acute/Sub-Chronic Toxicity

Sub-acute toxicity studies involve administering a drug in doses recommended doses (below the level at which it causes rapid poisoning) over a period of about four to six weeks, in order to discover if any toxic drug metabolites build up over time (EU Directive, 2004). Subchronic toxicity is the ability of a substance upon repeated or continuous exposure to cause toxic effects for more than one year but less than the lifetime of the exposed organism (Fauci *et al.*, 2008). Sub-chronic or repeat dose studies are conducted to determine the no-observed-adverse-effect level (NOAEL) and cumulative toxicity over a period of 30 - 90 days. This is carried out after initial toxicity information has been obtained by acute testing. Extrapolation of the result of sub-chronic toxicity to humans is valid only to a limited degree. Its real usefulness lies in its ability to provide information on health hazards that are likely to arise from repeated exposure to the plant medicine

of interest by the route of administration used over a limited period of time. It may give indication of organs that are adversely affected by the preparation and the possibility of product accumulation. Sub-chronic studies can also facilitate the selection of dose levels for chronic studies and for establishing safety criteria for human exposure to the substance. The sub-chronic study is not capable of determining toxic effects that have a long latency period for development, such as carcinogenicity (Asiedu-Gyekye *et al.*, 2004) and therefore chronic toxicity studies are required.

In sub-chronic toxicity studies, test animals are observed for apparent sign of toxicity or behavioural alterations during the experimental period. Blood is collected for hematological studies. The sera of test animals are also used in liver and kidney function tests to determine levels of blood urea nitrogen, creatinine, total protein, albumin, total bilirubin, direct bilirubin, *alkaline phosphatase* (ALP), serum *Alanine transaminase* (ALT), *aspartate transaminase* (AST), and serum *gamma glutamyl transferase* (GGT) (Siharat *et al*., 2007). Tests to determine blood levels of glucose are performed. The lipid profile is also determined. Organ to body weight ratio for selected organs is also important in this study. In sub-chronic studies, internal organs of the animals are examined after they have been sacrificed by histopathological methods (Siharat *et al*., 2007). Effects of the products on some enzymes like cytochrome P450 is also of interest.

1.4.3 Chronic Toxicity

Chronic toxicity is the ability of a substance or mixture of substances to cause harmful effects over an extended period, usually upon repeated or continuous exposure, sometimes lasting for the entire life of the exposed organism (Fauci *et al.*, 2008). Testing for chronic toxicity can last up to two years and, in the European Union, is required to involve two species of mammals, one of which must be non-rodent (EU Directive, 2004). Studies that continue for longer than 10 % of a test subjects life span are considered chronic

1.5 PHYSICAL AND CLINICAL OBSERVATION IN SAFETY ASSESSMENT

Clinical observations entail the recording of effects that can be detected by direct observation, such as abnormal gait and body weight. They often provide the first indication of which physiological systems are being affected by the test agent. Mice should be observed regularly throughout the in-life portion of a toxicity study. The type and frequency of these observations should be tailored to meet the scientific objectives of the specific study. The simplest form of clinical observation is an observation for survival and moribundity. This or a higher level of observation must be conducted at least once daily in all toxicity studies. The next level of observation is an observation for clinical signs of toxicity, such as an abnormal level of spontaneous motor activity, abnormal gait, abnormal respiration, and abnormal quantity or quality or faecal output (Shayne, 2007). Physical observation of experimental animals in toxicity studies is very important as it

provides information on the effect of the test drug on the skin, eyes, and mucous membrane. It also provides useful information on the function of the respiratory, digestive, circulatory, as well as the autonomic and central nervous system function, somatomotor function, and general behavior.

1.5.1. Studying Organ Weight to Body Weight Ratio

The evaluation of organ weights in toxicology studies is an integral component in the assessment of pharmaceuticals, chemicals, and medical devices (Sellers *et al*, 2007). The most frequent measure used to interpret drug effects on organ weights in toxicological experiments is the ratio of the organ weight to the animal's body weight. Organ weights are widely accepted in the evaluation of test agent-associated toxicities (Black, 2002; Bucci, 2002; Wooley, 2003). Organ weight changes are often associated with treatment-related effects. The choice of appropriate organs to weigh in toxicology studies involves understanding the test agent's mechanism of action, metabolism and toxicokinetics; the physiology of the test species; and the cumulative data set from previous studies of the same or similar compounds or materials. The Society of Toxicologic Pathology (STP) recommends that organ weights be included routinely in multidose Good Laboratory Practice (GLP) general toxicity studies with durations from 7 days to 1 year (Long *et al.*, 1998).

The STP also recommends that liver, heart, kidneys, brain, adrenal glands, and testes (preferably from sexually mature animals) should be weighed in all species

in multidose GLP general toxicology studies of 7 days to 1 year in duration. Alterations in liver weight may suggest treatment-related changes including hepatocellular hypertrophy (e.g., enzyme induction or peroxisome proliferation) (Amacher *et al*., 2006; Juberg *et al*., 2006). Liver weights may be elevated in studies of less than 7 days duration for potent hepatic enzyme-inducing compounds. Elevated heart weight may be the only evidence of myocardial hypertrophy that is often macroscopically and microscopically difficult to recognize. Changes in kidney weight may reflect renal toxicity, tubular hypertrophy or chronic progressive nephropathy. Variations in adrenal gland weight may indicate hypertrophy, hyperplasia, or atrophy associated with stress, endocrinopathies, or test article effects (Greaves, 2000). Changes in brain weights are rarely associated with neurotoxicity. The utility of brain weight rests in the ability to calculate organ weight to brain weight ratios. Some consider evaluation of organ weight to brain weight ratios helpful when terminal body weights are affected by the test article or to normalize organ weight data when there is large inter-animal variability. The STP recommends collection of brain weights so that organ weight to brain weight ratios may be calculated if needed.

1.5.2 Hematological and Serum Biochemical Analysis in Toxicity Studies

Haematology and clinical biochemical parameters are monitored during the course of toxicity studies to assess the potential target organ toxicity of pharmaceuticals. The investigations permit an assessment to be made of the effects of test materials

on the peripheral blood picture and the function of various organs such as the liver, kidney and bone marrow among others (James, 1993).

1.5.2.1 Hematological Profile

To determine the intravascular effects and bone marrow activity in treated animals, hematological studies are conducted (Siharat *et al*., 2007). Estimation of red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count (total and differential), and platelet count. Also, measurement of blood clotting potential, such as prothrombin time or activated partial thromboplastin time are done in hematological studies (Asiedu-Gyekye *et al*., 2004). Hemoglobin, hematocrit and red blood cell indices are helpful in differential diagnosis of anaemia (Gregg and Voigt, 2000).

1.5.2.2 Serum Biochemical Analysis

Clinical pathology data can make important contributions with respect to dosage selection procedures, definition of 'no-effect' doses, elucidation of toxic mechanisms, screens for specific manifestations of toxicity and investigative studies intended to clarify risk-benefit perspectives. The assay of some key "marker" enzymes in the tissue has been shown to be important indicators of monitoring damage to such tissues. The measurement of activities of various enzymes in tissue and body fluids play a significant role in the investigation on the extent of toxicity of drugs, tissue enzymes can also indicate tissue cellular damage

caused by chemical compounds long before structural damage that can be picked by conventional histological techniques (Yakubu *et al.,* 2006).

1.6 HISTOPATHOLOGICAL EXAMINATION IN TOXICITY STUDIES

Histopathology refers to the examination of a biopsy or surgical specimen, after the specimen has been processed and histological sections have been placed onto glass slides (Mitchell *et al*., 2008).

Evaluation of the pathological alterations induced in laboratory animals by novel drugs represents the cornerstone of their safety assessment before they can be first tried on patients. This preliminary assessment, which is based largely on conventional histopathological techniques, represents a major contribution to the development of new treatments for both human and animal diseases (Greaves, 2000).

The basic paradigm of dosing laboratory animals with various doses of new drug for increasing periods of time accompanied by careful clinical observations, biochemical and hematological monitoring followed by histopathological examination of the tissues remains essentially unaltered and has withstood the test of time. The pathologist is not only required to evaluate alterations to organs and tissues and any relationship that they might have to drug treatment but also to assess the likely relevance any treatment-related findings might have for patients (Greaves, 2000).

In sub-chronic toxicity studies, full gross necropsy on all animals and histopathological examinations of organs and tissues are made at the end of the period of study (Asiedu-Gyekye *et al*., 2004). Histopathological examination of tissues starts with surgery, biopsy, or autopsy. The tissue is removed from the body, and then placed in a fixative which stabilizes the tissues to prevent decay. The most common fixative is 10 % buffered formalin (Mitchell *et al*., 2008).

1.7 CYTOCHROME P450, DRUG INTERACTION AND TOXICITY

The cytochrome P450 family (CYP450) is a large and diverse group of enzymes which catalyze the oxidation of organic substances. These enzymes are most predominant in the liver but can also be found in the intestines, lungs and other organs (Kolars *et al*., 1994; Guengerich, 1994; Wheeler and Guenthner, 1990; Philpot, 1991). The substrates of CYP450 enzymes include metabolic intermediates such as lipids, steroidal hormones as well as xenobiotic substances such as drugs. The most common reaction catalyzed by CYP450 is a mono-oxygenase reaction (Sigel *et al*., 2007).

CYP450 belong to the superfamily of proteins containing a heme cofactor and are therefore called hemoproteins. Cytochromes (CYPs) use a variety of small and large molecules as substrates in enzymatic reactions. Often they form part of multi-component electron transfer chains, called P450-containing systems.

CYP450 have been named on the basis of their cellular (cyto) location and spectrophotometric characteristics (chrome): when the heme iron is reduced, P450 enzymes absorb light at wavelengths near 450 nm, identifiable as a characteristic Soret peak. CYP enzymes have been identified in all kingdoms of life, i.e., in animals, plants, fungi, bacteria (Danielson, 2002).

There are numerous isoforms of CYP450. These include: CYP3A4 (by far the most important), CYP2D6, CYP2C9, CYP2C19, CYP1A2, CYP2E1, and CYP2A6. Members of the CYP3A subfamily are the most abundant cytochrome enzymes in humans. They account for 30 % of the CYP450 enzymes in the liver (Shimada, 1994) and are also substantially expressed in the intestines (70 %). Members of this subfamily are involved in many clinically important drug interactions (Slaughter and Edwards, 1995).

Drug interactions involving the CYP450 isoforms generally result from one of two processes, enzyme inhibition or enzyme induction. Enzyme inhibition usually involves competition with another drug for the enzyme binding site. This process usually begins with the first dose of the inhibitor and onset and offset of inhibition correlate with the half-lives of the drugs involved (Dossing *et al*., 1983). This process may lead to accumulation of drugs which may result in toxicity to tissue.

Enzyme induction occurs when a drug stimulates the synthesis of more enzyme protein enhancing the enzyme's metabolizing capacity (Murray and Reidy, 1990).

It is somewhat difficult to predict the time course of enzyme induction because several factors, including drug half-lives and enzyme turnover, determine the time course of induction. Inhibitors will decrease metabolism of substrates and generally lead to increased drug effect (unless the substrate is a pro-drug) and sometimes toxicity. Inducers will increase metabolism of substrates and generally lead to decreased drug effect (unless the substrate is a pro-drug).

Inhibitors include antidepressants (e.g. nefazodone, fluvoxamine, fluoxetine, sertraline, paroxetine, venlafaxine), ketoconazole, itraconazole, and fluconazole (von Moltke *et al.*, 1996). Others are cimetidine, clarithromycin, diltiazem, and erythromycin. Inducers include carbamazepine, dexamethasone, phenobarbital, phenytoin, and rifampin

1.8 REPRODUCTIVE TOXICITY

Reproductive toxicity is a study of the adverse effects of a drug on sexual function and fertility in adult males and females. It includes developmental toxicity studies which involves studying the offspring for toxic symptoms.

The definitions presented below are adapted from those agreed at the IPCS/OECD Workshop for the Harmonisation of Risk Assessment for Reproductive and Developmental Toxicity, Carshalton, UK, 17-21 October, 1994 (OECD Monograph Series on Testing and Assessment No. 17, 1998). Reproductive toxicity may be expressed as alterations to the female or male reproductive organs,

the related endocrine system, or pregnancy outcomes. The manifestation of such toxicity may include, but not be limited to, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behavior, fertility, gestation, parturition, lactation, developmental toxicity, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems (EPA, 1996).

By the definition, reproductive toxicity can be subdivided into two main headings;

- Adverse effects on reproductive ability or capacity.
- Adverse effects on development of the offspring.

1.8.1 Adverse Effects on Reproductive Ability or Capacity

Any effect of chemicals that would interfere with reproductive ability or capacity is classified as adverse. This may include, but not be limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behavior, fertility, parturition, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems. Adverse effects on or via lactation can also be included in reproductive toxicity. This is because it is desirable to be able to classify chemicals specifically for adverse effect on lactation so that a specific hazard warning about this effect can be provided for lactating mothers.

1.8.2 Adverse Effects on Development of the Offspring

Taken in its widest sense, developmental toxicity includes any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation. However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide hazard warning for pregnant women and men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means adverse effects induced during pregnancy, or as a result of parental exposure. These effects can be manifested at any point in the life span of the organism. The major manifestations of developmental toxicity include; death of the developing organism, structural abnormality, altered growth, and functional deficiency.

1.9 A REVIEW ON THE POLYHERBAL ANTIHYPERTENSIVE

The polyherbal product is a prepackage aqueous preparation made from leaves and bark of *Persea americana* and *Vernonia amygdalina.*

1.9.1 *Persea americana* Mill (Family: Lauraceae)

Persea americana (avocado, alligator pear or butter pear) is a tree native to Central Mexico (Chen *et al.*, 2008). It is cultivated in tropical and sub-tropical climates around the world.

Description: The avocado tree may be erect, usually to 30 ft (9 m) but sometimes to 60 ft (18 m) or more, with a trunk 12 to 24 in (30-60 cm) in diameter, (greater in very old trees) or it may be short and spreading with branches beginning close to the ground. Almost evergreen, being shed briefly in dry seasons at blooming time, the leaves are alternate, dark-green and glossy on the upper surface, whitish on the underside; variable in shape (lanceolate, elliptic, oval, ovate or obovate), 3 to 16 in (7.5-40 cm) long. Those of the Mexican race are strongly anise-scented. Small, pale-green or yellow-green flowers are borne profusely in racemes near the branch tips. They lack petals but have 2 whorls of 3 perianth lobes, more or less pubescent, and 9 stamens with 2 basal orange nectar glands. The fruit, pear-shaped, often more or less necked, oval, or nearly round, may be 3 to 13 in (7.5-33 cm) long and up to 6 in (15 cm) wide. The skin may be yellow-green, deep-green or very dark-green, reddish-purple, or so dark a purple as to appear almost black, and is sometimes speckled with tiny yellow dots, it may be smooth or pebbled, glossy or dull, thin or leathery and up to 1/4 in (6 mm) thick, pliable or granular and brittle. In some fruits, immediately beneath the skin there is a thin layer of soft, bright-green flesh, but generally the flesh is entirely pale to rich-yellow, buttery and bland or nutlike in flavor. The single seed is oblate, round, conical or ovoid, 2 to 2.5 in (5-6.4 cm) long, hard and heavy, ivory in color but enclosed in two brown, thin, papery seed coats often adhering to the flesh cavity, while the seed slips out readily. Some fruits are seedless because of lack of pollination or other factors (Morton, 1987).

Persea americana is known to have hypotensive or antihypertensive effects (Adeboye *et al.,* 1999; Imafidon and Okunrobo, 2009), wound healing properties (Nayak *et al.,* 2008), antibacterial activity (Olaeta, 2007) and glycaemic effects (N'guessen *et al.,* 2009).

1.9.2 Vernonia amygdalina (Family: Asteraceae)

Vernonia amygdalina also known as "bitter leaf" is a widely used medicinal plant in Africa for its antihypertensive effects (Lawal *et al.,* 2010). *Vernonia amygdalina* is a shrub that grows predominantly in tropical Africa. Leaves from this plant serve as vegetable and culinary herb in soup (Argheore et al., 1998). In traditional Nigerian homes, extracts of the plant are used as tonic, in the control of tick and treatment of cough, feverish condition, constipation and hypertension (Regassa 2000; Kambizi and Afolayan 2001; Amira and Okubadejo 2007). *Vernonia amygdalina* extracts may help suppress, delay, or kill cancerous cell in many ways (Jisaka *et al.,* 1993; Colditz *et al.,* 1995; Blanco *et al.,* 2001; Sweeney *et al.,* 2005; Song *et al.,* 2005; Izevbigie *et al.,* 2004; Opata and Izevbigie, 2006).

Vernonia amygdalina may provide anti-oxidant benefits (Erasto, 2007). Many studies have shown that *V. amygdalina* extracts may strengthen the immune system through many cytokines (including NFκB, pro inflammatory molecule) regulation (Sweeney *et al.,* 2005). Studies conducted using streptozotocin-induced diabetic laboratory animals showed that *V. amygdalina* administration decreased blood glucose by 50% compared to untreated diabetic animals (Nwanjo, 2005).

The plant is traditionally used by small farmers in Western Africa, does possess anthelminthic anti-parasitic properties (Ademola and Eloff, 2011).

1.10 ANIMAL EXPERIMENTATION IN SAFETY ASSESSMENT

Animal testing, also known as Animal Experimentation, Animal Research, or *in vivo* testing, is the use of non-human animals in experiments. Worldwide it is estimated that the number of vertebrate animals—from zebrafish to non-human primates—ranges from the tens of millions to more than 100 million used annually (Cohn, 2010). Invertebrates, mice, rats, birds, fish, frogs, and animals not yet weaned are not included in the figures; one estimate of mice and rats used in the United States alone in 2001 was 80 million (Carbone, 2004 a). Most animals are euthanized after being used in an experiment (Carbone, 2004 b).

1.10.1 The Laboratory Mouse

Mice have been domesticated for centuries, even millennia, and have been used in scientific research since the 1600s (Suckow *et al.*, 2001). However, development of the laboratory mouse as a research model really began with genetic experiments in the early 1900s. Today, the laboratory mouse aside from being recognized as the preeminent model for modern genetic research, is used in a variety of other types of research, including cancer, immunology, toxicology, metabolism, developmental biology, diabetes, obesity, aging, and cardiovascular research (Suckow *et al.*, 2001). They are prized for many qualities, including their small size, short generation time, and ease of breeding within the laboratory. The fact

that they are genetically the best characterized of all mammals, increases their value for all fields of study. Mice belong to the order *Rodentia,* and most of the mice used in research belong to the genus *Mus.* Both genetically diverse and genetically defined mice are used in research. NIH Swiss, Swiss Webster, ICR, and CD-1 are among the most commonly used genetically diverse stocks. There are over 3000 genetically defined strains (Suckow *et al.,* 2001).

1.10.1.1. Behavior of Laboratory Mice

In general, the domestic mouse is not terribly aggressive and will attempt to evade rather than confront. The handler is most at risk when attempting to grasp or restrain the animal, as this may cause the animal to attempt biting. Occasionally, a mouse will bite a handler who is reaching toward it. Although a social species, mice will fight with one another and may inflict serious injuries. This is particularly true with males and with some strains. Fighting may be related to the establishment of hierarchy and defense of territory (Suckow *et al.,* 2001). Mice are most active in the evening and are therefore classified as nocturnal. In the laboratory environment, substantial activity is also noted during daytime as well. Mice commonly demonstrate burrowing and nesting behavior, and therefore abundant bedding and other material that encourages such behavior, should be supplied. As an animal with a small body mass to surface area ratio, these behaviors also serve to help the mouse maintain body temperature (Suckow *et al.,* 2001).

1.10.1.2 Normative Values in Laboratory Mice

1.10.1.2.1 Basic Biological Parameters

Typical values for miscellaneous biological parameters (Table 1), clinical chemistry (Table 2), urine (Table 4), and hematology (Table 3) are available. (Note: Significant variation of values may occur between individual mice, strains and stocks, laboratories, and methods of sampling. It is imperative that individual laboratories establish normal values for their specific facility.)

1.10.1.2.2 Clinical Chemistry Parameters

Approximate values for clinical chemistry parameters are shown in Table 2. The values represent ranges in mean values reported for mice between 1 and 12 months of age. Table 2 represents data from mice of various strains, sexes, and laboratory and housing conditions.

Table 1: Basic biological parameters for laboratory mice

Parameters	**Typical/Normal Values of a Mouse**
Life Span	2-3 years
Adult Body Weight	20-40g
Food Intake	12-18g/100g Body Weight/day
Water Intake	15ml/100g Body Weight/day

Table 2: Normal clinical chemistry values for laboratory mice

Parameters	Typical/Normal Values of a Mouse
Glucose	106-278mg/dL
Creatinine	0.5-0.8mg/dL
Calcium	9-12mg/dL
Alanine aminotransferase (ALT)	26-120 IU/L
Aspartarte aminotransferase (AST)	69-191 IU/L
Protein	43-64 g/L
Total Bilirubin	0.3-0.8 mg/dL

NB. The values in the tables above are for adult mice between the ages of 1 and 12 months; the table represents data obtained from male and female mice of various strains and laboratory and housing conditions. Reported ranges and standard deviations for some parameters are very large.* (Suckow *et al.*, 2001)

1.10.1.2.3 Urinalysis of Laboratory Mice

Evaluation of mouse urine is complicated by the small volumes that are usually available. For studies that require multiple or quantitative urinalysis, 24 hour urine collections are usually obtained by using metabolic cages as described in Chapter 2 (note that in Table 4, some values are provided per 24 hour). Increased drinking (polydipsia) and increased urination (polyuria) are typical of diabetes and some types or stages of renal disease. Sick mice, like other sick animals, may drink less than normal, resulting in decreased urine output. In addition, treatment of drinking water with chlorine, acid, or antibiotic can affect palatability of water, and may reduce water intake and urine output. Some mice chew and grind food, resulting in the disappearance of more food than is ingested. Much of this ground food, along with feces, can contaminate urine samples, resulting in abnormal urine sediment and bacterial growth that can alter protein and glucose values. Finally, small

volumes of liquid are susceptible to evaporation because of their relatively large surface areas, so most 24 hour collections of mouse urine are prone to evaporation artifact.

The common laboratory evaluations involved in urinalysis are tests for color, specific gravity, protein, glucose and evaluation of sediment. Typical values are shown in Table 4. Urine sediment is the non-liquid material that remains after urine is centrifuged. This material is evaluated microscopically for the presence of cells, casts, crystals, and bacteria. With 24 hour urine specimens, it is not uncommon to find contaminating plant material (from ground feed), bacteria, and pinworm eggs (in infested colonies, from fecal contamination). *Klossiella muris* is a protozoal parasite that can infect mouse kidneys and may be discerned in urine specimens, but it is very rare in modern, well-maintained mouse colonies. Gastrointestinal protozoa or their cysts and pinworm eggs may be discerned in fecal-contaminated urine specimens (Suckow *et al.*, 2001).

Table 3: Typical hematological values for laboratory mice

Parameters	**Reported Mean Values**	**Units**
Packed cell volume	38.5-45.1	%
Red blood cell number	5.0-9.5	10^6 cells/mm^3
Hemoglobin concentration	10.9-16.3	g/dL
MCV	48.0-56.0	fL
MCH	11.9-19.0	Pg
MCHC	25.9-35.1	g/dL
Platelets	1084-1992	10^3 platelets/µL
White blood cells	3.0-14.2	10^3 cells/µL

Table 4: Normal values of urine analytes for laboratory mice

Parameter	Approximate Normal Value
Color	Clear or slightly yellow
Volume	0.5-2.5 mL/24 h
Specific gravity	1.030
pH	5.0
Glucose	0.5-3.0 mg/24 h
Protein	0.6-2.6 mg/24 h

NB. The values in the tables above are for adult mice between the ages of 1 and 12 months; the table represents data obtained from male and female mice of various strains and laboratory and housing conditions. Reported ranges and standard deviations for some parameters are very large.* (Suckow *et al.*, 2001)

1.10.1.2.4 Hematology

Hematology is the study of blood and usually refers to the study of its cellular components, including erythrocytes or red blood cells, leukocytes or white blood cells, and platelets. Blood can be analyzed with automated equipment (automated complete blood count) and by microscopic examination of stained blood smears. EDTA is the preferred anticoagulant for evaluating the morphology of blood cells, whereas heparin may interfere with staining or assessment of morphology. Hematology values can vary with mouse strain/stock, age, sex, blood sampling method, environmental conditions, pathogen status, and laboratory. The reference values provided below are based on values from various strains, ages, sex, and laboratory conditions. They may provide a useful starting point, but individual laboratories should attempt to establish specific normal values based on these variables.

1.11 CONTROL OF HERBAL REMEDIES

The Food and Drugs Law of 1992 (PNDCL 305B) which established the Food and Drugs Board (FDB), put the control, manufacture, importation, exportation, distribution, use and advertisement of food, drug, cosmetics, medical devices and household chemicals under the preview of the board to ensure their safety, quality and efficacy. The Drug Division contributes to the attainment of the functions of the FDB for safeguarding the public by ensuring that all medicines on the market meet appropriate standards of safety, efficacy, and quality by evaluating all information submitted in the registration dossiers, pre-registration inspection and drug quality analysis report.

The Drug Evaluation and Registration Department has a Herbal Medicine Unit. Its functions include:

- Registration, processing and evaluation of all herbal medicines applications.
- Evaluation of toxicological and clinical information as well as therapeutic data submitted from the Centre for Scientific Research into Plant Medicine (CSRPM), Noguchi Memorial Institute for Medical Research, Faculty of Pharmacy, KNUST-Kumasi and the Department of Pharmacology, Korle-Bu Teaching Hospital.
- Products that are recommended for registration are issued with registration numbers which are valid for one year in case of locally manufactured products and three years for imported herbal drug.

1.13 AIM AND OBJECTIVES

The aim of the study was to establish the safety or otherwise for use of the polyherbal preparation claimed to be effective as an antihypertensive.

1.13.1 Specific Objectives

The following are some specific objectives of the study:

- To determine the acute and delayed toxicities associated with the use of the polyherbal product.
- To determine the effect of the polyherbal on normal behaviors
- To investigate the effect of the product on some vital organs of the body.
- To determine if the product has effect on the hematological profile
- To investigate a possible effects of the product on liver and kidney function
- To assess a possible effect of the product on lipid profile
- To investigate the possible interactions that may occur on concomitant administration with other medication
- To determine the possible effect on reproductive abilities

By this assessment, adverse effects and disorders associated with normal dosing and overdosing, and multiple and single exposure to the product, if any, will be established. Some recommendations then can be made to the FBD on tests that need to be performed on herbal products before approval for registration.

1.14 JUSTIFICATION OF THE STUDY

This study would be of immense significance to the consumer, the manufacturer, the regulatory bodies and the nation as a whole. The findings will instil confidence in both the manufacturers and the users. With the manufacturers it is going to be a source of income and employment and ease the socio-economic burden of the populace as production will increase. Herbal treatments are the most popular form of traditional medicine, and are highly lucrative in the international marketplace. Annual revenues in Western Europe reached US$ 5 billion in 2003-2004. In China, sales of products totaled US$ 14 billion in 2005. Herbal medicine revenue in Brazil was US$ 160 million in 2007 (WHO, 2002).

With the use of the product, the economic burden on individuals and health facilities will reduce, and morbidity and mortality from hypertension and other cardiovascular diseases will reduce. Pharmaceuticals are prohibitively expensive for most of the world's population, half of which lives on less than $2 U.S. per day (DaSilva *et al.*, 2002).

The burden on Government to provide so many antihypertensives will reduce and pressure on health facilities will decrease. Productivity may go up with subsequent socio-economic development. Regulatory bodies could use protocols from this study to assess the safety of other products.

CHAPTER TWO

2.0 ACUTE TOXICITY STUDIES

2.0 INTRODUCTION

Acute toxicity describes the adverse effects of a substance which result either from a single exposure or from multiple exposures in a short space of time; usually less than 24 hours (Moser, 1999). To be described as acute toxicity, the adverse effects should occur within 14 days of the administration of the substance.

The mean lethal dose, or the LD_{50}, which is the dose of a medication that will cause 50 % of the population being tested to die, is a very important measure of acute toxicity. However, if no deaths occur, it does not mean that there was no toxicity. The harmful effects (local and/or systemic) that can occur from a single exposure by any route of administration include rashes associated with the skin and mucous membranes, labored breathing, constipation, emaciation, skin eruptions, abnormal posture, hemorrhage, sedation, diarrhea, polyuria, polydipsia, polyphagia, anorexia, rhinorrhoea/nasal congestion, loss of autonomic reflexes, decreased locomotor activity, neuromuscular inco-ordination and collapse, hyperesthesia, hypothermia, twitching, spasticity, convulsion, writhing, and respiratory depression among others.

2.1 MATERIALS AND METHODS

2.1.1 Animals and Husbandry

Male Imprinting Control Region (ICR) mice at 3-4 weeks of age were obtained from the Department of Pharmacology, KNUST, animal house and acclimatized for 2 weeks prior to initiation of dosing. During this period, mice were observed (physical; in-life) daily and weighed. At initiation of treatment, animals were approximately 5 weeks old. Individual weights of mice placed on test were within ± 30 % of the mean weight for each sex. All mice were examined during the acclimatization period to confirm suitability for study.

Animals were housed in stainless steel, wire mesh cages during the acclimation and the experimental periods. The mice were kept under ambient light/dark cycle, room temperature and relative humidity. The animals had free access to pelleted mice chow (GAFCO, Tema, Ghana) and water daily.

This study was conducted in compliance with all appropriate parts of the Animal Welfare Act Regulations: 9 CFR Parts 1 and 2 Final Rules, Federal Register, Volume 54, No. 168, August 31, 1989, pp. 36112–36163 effective October 30, 1989 and 9 CFR Part 3 Animal Welfare Standards; Final Rule, Federal Register, Volume 56, No. 32, February 15, 1991, pp. 6426–6505 effective March 18, 1991.

2.1.2 The Polyherbal Antihypertensive (PHA)

A prepackage aqueous preparation made from leaves and bark of *Persea americana,* and *Vernonia amygdalina* respectively was the product under study and would be referred to as PHA. The decoction of batch number AH 002 was obtained from retail outlets in Kumasi in the Ashanti Region of Ghana. The suspension was dark brown in colour. PHA was condensed under low temperature and pressure using a Buchi Rotor Evaporator (Rotavapor R-210, Switzerland) and dried in a Gallenkamp hot air oven (Oven 300 plus series, England) maintained at a 40^{o}C for 24 hours. Dosing of PHA was based on the manufacturer's recommendation which was calculated to be 55 mg/kg/day. Dosing was a single event at a volume of 10 ml/kg body weight. Individual dose volumes were calculated based on the animal's most recent recorded body weight. The oral route of administration was used because it is the intended human exposure route.

2.1.3 Acute and Delayed Toxicity Test

The mice were assigned to treatment groups 1-6, with ten (10) in a group. Group 6 was the non-drug treatment group (control). PHA was administered at doses of 55, 110, 165, 275, and 550 mg/kg (representing the stated daily dose, two, three, five and ten times the daily dose respectively) by gavage. Observation for clinical and behavioral symptoms of toxicity and mortality were made by filming for the first 60 minutes after which observations were made hourly for 24 hours and then daily thereafter for 14 days. The time of onset, intensity, and duration of these symptoms, if any, was recorded.

2.1.4 Hematological Profile

Blood samples from untreated mice (control) and 550 mg/kg PHA-treated mice were collected into MediPlus K3 EDTA tubes (Sunphoria Co. Ltd., Taiwan) before treatment, 24 hours after treatment, and then 10 days post-treatment and sent to the KNUST Hospital for hematological assessment using the BC-3000 Plus Auto hematology Analyzer (Mindray, Shenzhen, China). Hematological parameters measured are as shown in Table 5.

2.1.5 Urine Analysis

A semi-quantitative biochemical test was performed on fresh urine samples obtained from mice in metabolic cages (Ugo Basile Biological Research Equipment, Comerio, Va, Italy) prior to treatment, 24 hours, and then 10 days post-treatment using urine reagent test strips (Taytec Enterprises Inc., Missisauga, Canada). Parameters measured were pH, calcium, protein, specific gravity, protein, blood, bilirubin, ketone, glucose, and ascorbic acid. The color, appearance and smell of the urine samples were also noted.

2.1.6 Assessment of Liver and Kidney Function

Prior to PHA treatment (control) and then 10 days post-treatment, blood samples for liver and kidney function tests were collected and centrifuged (temperature: 25°C, speed: 4000 *g*) for 5 minutes using the Mikro 220R [Hettich Zentrifuge, USA] machine to obtain the plasma. In the kidney function tests, blood urea was determined by an enzymatic colorimetric test using the urea cromatest Kit [Linear

Chemicals SL, Barcelona, Spain] and blood creatinine was determined by the creatinine kinetic colorimetric method using the creatinine cromatest Kit [Linear Chemicals SL, Barcelona, Spain]. Absorbances were measured using a microtitre plate reader (ELx808^{tm} UV Biotek Instruments Inc., Winooski, Vermont, USA) and plasma concentrations of urea and creatinine were calculated. In the liver function tests, *aspartarte aminotransferase* (AST), *alanine aminotransferase* (ALT), *alkaline phosphatase* (ALP), *gamma glutamyl transferase* (GGT), total protein, and albumin were analyzed using methods stated in reagent kit [Fortress Diagnostics Ltd, Antrim, UK]. Absorbances were measured.

2.1.7 Data Collection and Analysis

Observations were filmed using a digital camera (FujiFilm FinePix S700, Fujifilm Corporation, USA). The statistical analysis of data obtained were made using GraphPad Prism Version 5.0 [GraphPad Software, Inc. USA]. Statistical estimates were made with One-way Analysis of Variance (ANOVA) followed by Bonferonni's multiple comparisons test [post test] at a confidence level of 95 %. Probability values less than or equal to 5 % ($P \leq 0.05$) were considered significant.

2.2 RESULTS

2.2.1 Acute and Delayed Toxicity Test

After administration of PHA, no deaths were recorded at any of the dose levels over the entire experimental period (14 days). There was no lacrimation, salivation, urination, labored breathing, constipation, emaciation, skin eruptions, abnormal posture, hemorrhage, sedation, diarrhoea, polyuria, polydipsia, polyphagia, anorexia, rhinorrhoea/nasal congestion, loss of autonomic reflexes, neuromuscular inco-ordination and collapse, hyperesthesia, hypothermia, twitching, spasticity, convulsion, writhing, tremors, fasciculations and respiratory depression. Observations of gait in all groups of animal did not show, staggering, wobbly gait, hind limb exaggeration, overcompensating, and/or splayed movements, feet (primarily hind feet) point outward from body, forelimbs dragging and/or showing abnormal positioning, nor walking on toes (the heels of the hind feet are perpendicular to the surface). The mice did not show completely flattened, pelvis flat on surface or pelvis low, dragging somewhat, but did show a hunched, or raised up back. However, there was sedation, lethargy, and reduced activity one hour post-treatment but this effect was not seen after 24 hours.

2.2.2 Hematological Profile

Hematological analysis showed no significant difference ($P > 0.05$) between all parameters measured of the treated and untreated groups, except for a decrease ($P \leq 0.05$) in white blood cell count over time (Table 5). RBC values recorded were all indicative of normal values typical of laboratory mice i.e. within 5-9.5 x 10^6

cells/mm^3 of blood (Suckow *et al.*, 2001). Hemoglobin levels recorded were within normal range (10-16.3 g/dl) and so were the MCV (48-56 fL), MCH (11.9-19.0 pg), and the MCHC (25.9 35.1 g/dl). There were no discrepancies in the RBC distribution widths between the treated and untreated animals over the entire study period. Even though platelet number was much lower compared with normal values (1084-1992 x 10^3 platelets/µl of blood), there were no significant variation among values obtained for treated and untreated animals. A similar trend was seen with MPV, PDW, and PCT.

2.2.3 Urine Analysis

Urinalysis of treated and untreated mice, showed no significant changes except for the increase in pH which became slightly basic (from 5.4 ± 0.55 to 7.8 ± 1.60) and the change of urine color from straw to amber after 24 hours of drug treatment but returns to normal (Table 6). Urine protein was also high compared to normal values of urine analytes for laboratory mice (20–40 mg/24 h) (Beynon and Hurst, 2003). That notwithstanding, variation among treatment groups were not significant. Urine colour, smell, appearance and volumes were similar to the control.

2.2.3 Assessment of Liver and Kidney Function

In the liver function tests, there were no significant differences ($P > 0.05$) in AST, ALT, and GGT levels. ALP and total protein levels increased significantly ($P \leq 0.01$-0.001) 24 h after treatment but by day 10, levels were not significantly

different from that of the control (Table 7). In the kidney function tests, change in plasma creatinine concentration was not significantly, while plasma urea increased significantly ($P \leq 0.05$) (Table 8).

Table 5: Hematological assessment values obtained before, 24 h, and 10 days after treatment of ICR mice with 550 mg/kg of PHA

	Control	24 hours post-treatment	10 days post-treatment
WBC ($x10^9$/L)	8.48 ± 2.45	7.27 ± 4.67	2.87 ± 1.46*
HGB (g/dl)	13.98 ± 1.47	14.92 ± 1.13	13.87 ± 1.05
RBC ($x10^{12}$/L)	7.52 ± 0.63	7.67 ± 0.75	7.28 ± 0.69
HCT (%)	39.48 ± 4.14	41.33 ± 2.69	37.57 ± 3.15
MCV (fL)	52.53 ± 2.38	51.35 ± 0.60	51.82 ± 3.40
MCH (pg)	18.52 ± 0.78	18.47 ± 0.29	19.03 ± 1.07
MCHC (g/dl)	35.35 ± 0.24	35.35 ± 0.24	36.88 ± 0.76
RDW-CV (%)	14.73 ± 0.64	16.17 ± 0.34	16.35 ± 0.93
RDW-SD (fL)	28.25 ± 1.85	29.22 ± 0.62	29.68 ± 2.55
PLT ($x10^9$/L)	700.00 ± 134.51	706.33 ± 120.53	791.83 ± 197.64
MPV (fL)	6.28 ± 0.57	6.48 ± 0.35	6.18 ± 0.67
PDW	16.52 ± 0.71	17.07 ± 0.58	16.47 ± 1.21
PCT (%)	0.43 ± 0.06	0.46 ± 0.07	0.49 ± 0.12

Values recorded are means and standard deviations (n=6). Values obtained for the various parameters before treatment (control, 24 h and 10 days post treatment are not significantly different from each other but for WBC. * implies $P \leq 0.05$. Level of significance was analyzed using One-Way Analysis of Variance (ANOVA) followed by Dunnet's post-hoc test.

Table 6: Semi-quantitative urine analysis values obtained before, 24 h, and 10 days after treatment with 550 mg/kg of PHA

Parameters	Before Drug Treatment	24 h after Treatment	Day 10 after Treatment
pH	5.4 ± 0.55	7.8 ± 1.60	5.6 ± 0.54
Nitrite (mg/dL)	(-)	(-)	(-)
Urobilinogen (mg/dL)	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00
Protein (mg/dL)	72.0 ± 38.3	86.0 ± 31.3	58.0 ± 38.3
Specific gravity	1.000 ± 0.000	1.004 ± 0.002	1.000 ± 0.000
Blood(Ery/uL)	(-)	(-)	(-)
Bilirubin (mg/dL)	(-)	(-)	(-)
Ketones (mg/dL)	(-)	(-)	(-)
Glucose (mg/dL)	(-)	(-)	(-)
Ascorbic (mg/dL)	32.0 ± 11.0	36 ± 8.0	32 ± 11.0
Volume	1.14 ± 0.28	1.24 ± 0.27	1.10 ± 0.20
Color	Straw	Amber	Straw
Appearance	Clear	Clear	Clear
Smell	Pungent	Pungent	Pungent

Values are Means ± Standard Deviations (n=5). (-) implies "negative"

Table 7: Liver function test results obtained before, 24 h, and 10 days after treatment with 550 mg/kg of PHA.

	Control	24 h post-treatment	10 days post-treatment
AST/GOT (U/I)	8.85 ± 6.80	12.08 ± 9.47	12.51 ± 11.87
ALT/GPT (U/I)	8.23 ± 4.83	3.88 ± 3.11	5.42 ± 4.98
GGT (µmol/l)	4.17 ± 2.24	7.33 ± 9.36	3.40 ± 2.32
ALP (U/I)	16.74 ± 9.09	44.53 ± 4.09 ***	13.62 ± 8.21
Total protein (g/l)	58.93 ± 14.87	97.65 ± 28.07 **	45.70 ± 7.92
Albumin (g/l)	26.94 ± 21.79	23.59 ± 5.35	15.15 ± 2.41

Values are Means ± Standard Deviations (n=6). ** implies P ≤ 0.01; * implies P ≤ 0.001. Level of significance was analyzed using One-Way Analysis of Variance (ANOVA) followed by Dunnet's post-hoc test. ALT/GPT = *Alanine Transaminase/Glutamic Pyruvate Transaminase*, AST/GOT = *Aspartate transaminase/Glutamic Oxaloacetic transaminase*, ALP = *Alkaline Phosphatase*, GGT = *Gamma GlutamylTransferase*.**

Table 8: Plasma urea and creatinine concentrations obtained for kidney function tests before 24 h, and 10 days after treatment with 550 mg/kg of PHA

	Control	24 h post-treatment	10 days post-treatment
Urea (mmol/l)	26.68 ± 19.46	41.72 ± 16.88	56.92 ± 12.15 *
Creatinine (µmol/l)	0.49 ± 0.45	0.60 ± 0.57	0.45 ± 0.42

Values are Means ± Standard Deviations (n=6). Values for plasma creatinine obtained for PHA-treated mice were not significantly different from that of the control. * implies P ≤ 0.05. Level of significance was analyzed using One-Way Analysis of Variance (ANOVA) followed by Dunnet's post-hoc test.

2.3 DISCUSSION

Observations made at 1-2 hour intervals for 24 hours and then daily for 14 days makes the study convenient for an acute and delayed toxicity study because most of the observable symptoms of acute toxicity occur within the first 1-2 hours of drug administration. No death recorded implies that the product was not lethal even when up to ten times the daily dose was taken as a single dose. Sedation, lethargy and reduced activity observed with the administration of PHA may be a result of CNS depression. CNS depression often results from the use of depressant drugs such as alcohol, opioids, barbiturates, benzodiazepines, and general anesthetics. When these are used, effects may include anxiolysis, analgesia, sedation, somnolence, dissociation, muscle relaxation, lowered blood pressure/heart rate, respiratory depression, anesthesia, and anticonvulsant effects.

PHA could be exerting its CNS depressant effect through a number of different pharmacological mechanisms, the most prominent of which include facilitation of GABA and/or opioid activity. The principal inhibitory neurotransmitter in the CNS is Gama-aminobutyric acid (GABA), which is widely distributed throughout the brain. Approximately 60-75% of all synapses in the CNS are GABAergic (Schwartz, 1988). GABA binding to GABA-A receptors (one of the three GABA receptors) mediate fast inhibitory synaptic transmissions; they regulate neuronal excitability and rapid changes in mood. Thus, the seizure threshhold, anxiety, panic, and response to stress (i.e., the “fight or flight” response) are regulated by GABA-A receptors (Neilson *et al.*, 1991; Borden *et al.*, 1994).

There are at least five possible mechanisms by which drugs can increase the availability and activity of GABA.

(1) Stimulation of GABA-A receptors. GABA-A receptors are coupled to chloride ion channels; activation of the GABA-A receptor induces increased inward chloride ion flux, resulting in membrane hyperpolarization and neuronal inhibition (Graham *et al.*, 1996)

(2) Increasing the release of GABA from glial cells.

(3) Inhibition of GABA transaminase, (the enzyme that metabolizes GABA)

(4) Increases in GABA synthesis and release

(5) Inhibition of reuptake of GABA by neurons and glial cells

Most reviews conclude that opioids produce minimal impairment of human performance on tests of sensory, motor, or attentional abilities. Recent studies have been able to show some impairments caused by morphine, which is not surprising, given that morphine is a central nervous system depressant. Morphine has resulted in impaired functioning on critical flicker frequency (a measure of overall CNS arousal) and impaired performance on the Maddox Wing test (a measure of deviation of the visual axes of the eyes). Few studies have investigated the effects of morphine on motor abilities; a high dose of morphine can impair finger tapping and the ability to maintain a low constant level of isometric force (i.e. fine motor control is impaired (Kerr *et al.*, 1991) though no studies have shown a correlation between morphine and gross motor abilities.

An inhibition of adrenergic, histamine and/or acetylcholine activity and alteration of the effects of dopamine in the CNS could also result in a decrease in motor activity. PHA therefore could have an effect on neurological behavior.

RBC values being normal indicate that PHA does not cause hemolysis of RBCs and does not have hemorrhagic tendencies. The normal levels of HGB, MCV and RDW confirm this. Hemoglobin concentration is expected to be proportional to the RBC count as hemoglobin is found in red blood cells. Some herbal product can cause massive hemolysis soon after they have been administered which results in low RBC counts and increased bilirubin and urobilinogen levels in blood and therefore in urine (bilirubin was negative in urine analysis while urobilinogen level was low). Abnormal and accelerated destruction of red cells and an increased breakdown of hemoglobin would result in increased bilirubin level (mainly indirect-acting) with jaundice and increased fecal and urinary urobilinogen (Schick, 2011).

It cannot be said that the liver was not injured by drug administration as indicated by the insignificant differences in AST and ALT levels measured between the control and treated groups. Liver transaminases are not indicators of liver dysfunction but are biomarkers of liver injury in a patient with some degree of intact liver function (McClatchey, 2002; Mengel and Schwiebert, 2005). However calculating the AST/ALT ratios 24 h and 10 days post-treatment (3.11 and 2.3 respectively) suggests that PHA possibly causes acute hepatitis at very high doses.

The AST/ALT ratio is sometimes useful in differentiating between causes of liver damage. An AST/ALT ratio (which is normally less than 1.0) greater than 2.0, it is more likely to be associated with viral hepatitis, alcoholic hepatitis or hepatocellular carcinoma. When greater than 1.0 but less than 2.0, it is likely to be associated with cirrhosis (Gopal and Rosen, 2000; Nyblom *et al.*, 2004; Nyblom *et al.*, 2006).

The liver's synthetic abilities necessary for normal vital functions was not impaired as albumin levels were not significantly different between control and treated animals. Although ALP levels were elevated significantly 24 h post treatment, it cannot be said vividly as an indicator of liver injury because ALP is associated with cellular membrane and elevated levels may be caused by injury to the liver, bone, kidney, intestine, placenta, or leucocytes. In the liver, ALP is located in the bile canaliculi. Biliary obstruction induces increased synthesis of ALP and spillage into the circulation (Mengel and Schwiebert, 2005).

The high blood urea nitrogen levels observed in the kidney function tests can indicate kidney dysfunction, but because blood urea nitrogen is also affected by protein intake and liver function, it is therefore not a specific indicator of kidney function. Blood creatinine, a more sensitive indication of kidney dysfunction (Ford-Martin and Best, 2011), was however not elevated. This could imply that PHA has no detrimental effect on the kidney. This was confirmed in the urine analysis results which showed no observable differences in all measured

parameters between PHA - treated and untreated mice. Mice as well as other rodents secrete substantial amounts of protein in their urine, which is not generally observed in humans or other mammals (Kwak *et al.*, 2011). Urinary proteins are detected in both male and female mice, although the total concentration in female urine is 4 times lower than that in male urine (Cheetham *et al.*, 2009). The major urinary proteins (MUPs) are the predominant proteins in mouse urine (Hurst and Beynon, 2004).

2.4 CONCLUSION

PHA is not lethal up to a dose of 550 mg/kg but in treated mice it shows signs of sedation and CNS depression. Plasma ALP levels were elevated. Sub-chronic toxicity studies are recommended to further assess the safety for use of this product.

CHAPTER THREE

3.0 SUB-CHRONIC TOXICITY ASSESSMENT

3.1 INTRODUCTION

Although exposure of experimental animals to single large doses of PHA did not result in lethality, it cannot be concluded that the product is safe to use, as some cell, tissue, organs, and/or systems, could have been negatively affected. This could affect the normal physiological functions of organs and body systems after the exposure. Secondly, for some drugs recommended doses could result in cell and tissue damage after prolonged use; as in the treatment of chronic diseases; which poses a health risk in the near future. Repeat dose toxicity studies (sub-chronic or chronic) need to be carried out therefore after initial information on toxicity has been obtained from an acute toxicity test. This is usually necessary to evaluate toxic characteristics of the drug that could arise over a period of time (duration of exposure normally depends on nature of chemical and circumstances). Observations recorded from clinical sign, changes in; organ weight, body weight, food consumption and water intake, serum chemistry and hematology, functionality of some vital organs, histology of some of the tissues etc., would be useful information on the safety profile of the drug.

3.2 MATERIALS AND METHODS

3.2.1 Dosing of PHA

Dosing of the product was done based on the manufacturer's recommendations. ICR mice were grouped (n=5) and received vehicle or 36, 72, or 180 mg/kg of the product by gavage. Dosing was once daily for 45 days at a volume of 10 ml/kg body weight. Individual dose volumes were calculated based on the animal's most recent recorded body weight. The oral route of administration was used because it is the intended human exposure route.

3.2.2 Antemortem Evaluative Parameters

During the 45-day dosing period, cage-side observations for mortality and emaciation were made morning and evening. Daily observations of the skin for hair loss, open or closed lesions, or abnormal masses, secretions and excretions from the eye, nose, oral cavity, anus, and external genitalia, as well as pupil size, and respiratory pattern and "chattering" were made. Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypy (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilation, walking backward) were assessed. Lethargy, aggression and hunch appearance was also noted once daily for any signs of toxicity. Symptoms of toxicity evaluated for included, constipation, hemorrhage, sedation, diarrhoea, polyuria, polydipsia, polyphagia, anorexia, rhinorrhoea/nasal congestion, loss of autonomic reflexes, decreased locomotory activity, neuromuscular inco-ordination and collapse, hyperesthesia, hypothermia, twitching, spasticity, writhing, and

convulsion. Mice were weighed twice weekly. Food and water intake were determined weekly.

Blood samples were collected for clinical pathology prior to necropsy on day 23 and 45 of treatment, and compared with the untreated. Hematological analysis was performed on EDTA-preserved blood in MediPlus K3 EDTA tubes (Sunphoria Co. Ltd., Taiwan) using KX-21 N Automated Haematology Analyser (Sysmex Corporation, Chuo-ku, Kobe, Japan). Liver and kidney function tests, and lipid profile was performed on plasma obtained from coagulated blood using the Flexor Junior clinical chemical analyzer (Vital Scientific B.V, The Netherlands). Immediately following dosing, mice were placed in metabolic cages (Ugo Basile Biological Research, Comerio, Va, Italy). A 24-hour urine sample was collected while the mice were allowed food and water *ad libitum*. Urine analysis was also performed using urine test strips (Taytec, Mississauga, Canada). Parameters monitored are listed in Table 9.

Table 9: Parameters monitored in the hematological and clinical chemistry assessment of blood male and female ICR mice treated with the test antihypertensive medication for 45 days

	Parameters Monitored
Hematology	White Blood Cell Count (WBC), Red Blood Cell Count (RBC) Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red Blood Cell Distribution Width (RDW-CV & RWD-SD), Platelet Count (PLT)
Liver Function Tests	Albumin, Globulins, Total protein, *Alanine aminotransferase* (ALT), *Aspartarte aminotransferase* (AST), *Alkaline phosphatase* (ALP), Bilirubin Direct, Bilirubin Indirect, Bilirubin Total, *Gamma glutamyl transferase* (GGT)
Kidney Function Tests	Creatinine, Urea, Sodium, Potassium, Chloride
Lipid Profile	Cholesterol, Triglycerides, High Density Lipoproteins (HDL), Very Low Density Lipoproteins (VLDL) , Low Density Lipoprotiens (LDL)
Urine Analysis	pH, Protein (PRO), Specific gravity (SG), Blood (BLO), Bilirubin (BIL), Ketone (KET), Glucose (GLU), Ascorbic acid (ASC), Color, Appearance, Smell

3.2.3 Postmortem Evaluation

Following physical examination and blood sampling, mice were sacrificed by cervical dislocation. The brain, heart, kidney, liver, lung, spleen, testes, uterus, ovaries, were excised, trimmed of fat and connective tissue, blotted dry with filter paper and weighed. The organ weight to body weight ratio was calculated for each

organ. Tissue samples for histological evaluation were obtained on all organs and preserved in 10 % phosphate-buffered formalin. Tissue for histopathological evaluation was embedded in paraffin, sectioned, and hematoxylin/eosin stained specimens prepared. Specimens from all mice in the control and different drug treated dose groups were examined microscopically.

3.2.4 Statistical Analysis

Data obtained from organ weight to body weight ratio, semi-quantitative urine analysis, hematology, and blood chemistry determination between treated and untreated groups of animals which have conventionally been used to evaluate the toxic effect of the test sample was analyzed using GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Data presented are mean ± SD and analyzed by one-way ANOVA followed by Dunnet's multiple comparisons test (post test). $P \leq 0.05$ was considered statistically significant in all analysis.

3.3 RESULTS

3.3.1 Physical (In-life) Evaluation

No mortality occurred during the study. Weekly clinical observations recorded during the study were considered common findings in laboratory mice and unrelated to the test antihypertensive product. There were no secretions from the eye, ear, nose, anus, and external genitalia, no "wasting", audible "chattering", alopecia, and pallor in the eyes. The mice were not lethargic, they fed well and

their stool showed no signs of diarrhea. There were no ocular findings, decreased motor activity and neurological conditions. There was no significant test article effect on body weight in either sex.

3.3.2 Organ Weight to Body Weight Ratio

There were no significant changes in weights for the heart, uterus, ovaries, testis, lung, kidney and spleen between the control and treated mice (Tables 10, 11, and 12). The brain indicated a significant increase in weight ($P \leq 0.5$-0.001) after 23 days of treatment. The weight however, reduced after 45 days of treatment showing no significant changes between the control and treated mice (Table 10, 11, and 12). There were no significant changes ($P > 0.05$) in liver weight between the control and treated mice at a treatment dose of 36 mg/kg/day. Higher doses however recorded decreasing weight ($P \leq 0.001$) with increasing treatment periods (Table 10, 11, and 12).

3.3.3 Hematological Profile

Hematological evaluation (Table 13) did not show any increase in WBC count in the 36 mg/kg treatment groups over the 45-day period. WBC counts however increased significantly ($P \leq 0.01$ - 0.001) in the 72 mg/kg and 180 mg/kg treated groups over the period. The number of erythrocytes did not increase significantly in all treatment groups over the entire period of study except the 36 mg/kg treated group which there was a slight increment ($P \leq 0.05$). The HGB decreased very significantly ($P \leq 0.001$) in all the three dose levels of treatment when measured

after 23 days but the HGB started increasing again and by day 45, the decrease seen in the 32 mg/kg and the 72 mg/kg treated groups compared to the control group have reduced ($P \leq 0.05$), and that of the 180 mg/kg treated group had HGB levels comparable to the control. The HCT increased gradually ($P \leq 0.05$) (with 32 mg/kg of the preparation administered) through a significant increase ($P \leq 0.01$) (23 days of treatment with 72 mg/kg and 180 mg/kg) to a very significant increase ($P \leq 0.001$) in all treatment groups over the entire study period.

MCV was not significantly ($P > 0.05$) affected by the administration of 36 mg/kg/daily over the 45-day period. It however significantly reduced ($P \leq 0.01$) within 23 days of treatment with 72 mg/kg/daily but at the end of the study period, the MCV had normalized. Treatment with 180 mg/kg/daily very significantly ($P \leq 0.001$) reduced MCV but by day 45, the MCV was just slightly lower ($P \leq 0.05$) than the normal. The MCH significantly decreased ($P \leq 0.001$) in all treatment groups over the 23-day period but seemed to be returning to normal by the end of the 45th day with only slight reductions ($P \leq 0.05$) compared to normal values. The MCH for the 180 mg/kg treated group however still had a significantly low ($P \leq 0.01$) value. MCHC values for the entire study period for all treatment groups were very significantly low ($P \leq 0.001$). The RBC distribution width was not affected throughout the study. However looking at the standard deviation values obtained for the RDW i.e. RDW-SD, there were very significant increases ($P \leq 0.001$) in the 36 mg/kg treated groups over the

Table 10: Percentage organ weight to body weight ratio of ICR mice treated with 36 mg/kg of PHA in a toxicity study.

	% ORGAN WEIGHT TO BODY WEIGHT RATIO								
	Testis	**Lungs**	**Kidney**	**Spleen**	**Liver**	**Heart**	**Brain**	**Uterus**	**Ovaries**
Control	0.69 ± 0.19	0.60 ± 0.03	1.35 ± 0.08	0.67 ± 0.23	5.82 ± 0.19	0.50 ± 0.05	0.96 ± 0.08	0.50 ± 0.12	0.15 ± 0.02
Day 23	0.76 ± 0.02 ns	0.64 ± 0.06 ns	1.31 ± 0.04 ns	0.63 ± 0.32 ns	5.70 ± 0.67 ns	0.47 ± 0.04 ns	1.18 ± 0.04 **	0.61 ± 0.03 ns	0.14 ± 0.02 ns
Day 45	0.65 ± 0.02 ns	0.68 ± 0.03 *	1.30 ± 0.08 ns	0.57 ± 0.10 ns	5.62 ± 0.49 ns	0.52 ± 0.04 ns	0.92 ± 0.15 ns	0.59 ± 0.02 ns	0.16 ± 0.02 ns

Values are Means ± Standard Deviations (n=5). * implies $P \leq 0.05$; ** implies $P \leq 0.01$.

Table 11: Percentage organ weight to body weight ratio of ICR mice treated with 72 mg/kg of PHA in a toxicity study.

	% ORGAN WEIGHT TO BODY WEIGHT RATIO								
	Testis	**Lungs**	**Kidney**	**Spleen**	**Liver**	**Heart**	**Brain**	**Uterus**	**Ovaries**
Control	0.69 ± 0.19	0.60 ± 0.03	1.35 ± 0.08	0.67 ± 0.23	5.82 ± 0.19	0.50 ± 0.05	0.96 ± 0.08	0.50 ± 0.12	0.15 ± 0.02
Day 23	0.79 ± 0.05 ns	0.57 ± 0.03 ns	1.28 ± 0.08 ns	0.64 ± 0.11 ns	4.86 ± 0.36 ***	0.42 ± 0.02 *	1.21 ± 0.03 ***	0.55 ± 0.02 ns	0.14 ± 0.02 ns
Day 45	0.64 ± 0.05 ns	0.64 ± 0.07 ns	1.31 ± 0.04 ns	0.51 ± 0.18 ns	4.81 ± 0.24 ***	0.48 ± 0.04 ns	0.98 ± 0.08 ns	0.56 ± 0.03 ns	0.16 ± 0.02 ns

Values are Means ± Standard Deviations (n=5). * implies $P \leq 0.05$; * implies $P \leq 0.001$.**

Table 12: Percentage organ weight to body weight ratio of ICR mice treated with 180 mg/kg of PHA in a toxicity study.

	% ORGAN WEIGHT TO BODY WEIGHT RATIO								
	Testis	**Lungs**	**Kidney**	**Spleen**	**Liver**	**Heart**	**Brain**	**Uterus**	**Ovaries**
Control	0.69 ± 0.19	0.60 ± 0.03	1.35 ± 0.08	0.67 ± 0.23	5.82 ± 0.19	0.50 ± 0.05	0.96 ± 0.08	0.50 ± 0.12	0.15 ± 0.02
Day 23	0.69 ± 0.11 ns	0.58 ± 0.10 ns	1.35 ± 0.08 ns	0.85 ± 0.25 ns	4.32 ± 0.48 **	0.43 ± 0.01 ns	1.06 ± 0.04 *	0.03 ± 0.03 ***	0.02 ± 0.02 ***
Day 45	0.71 ± 0.12 ns	0.57 ± 0.05 ns	1.39 ± 0.09 ns	0.42 ± 0.06 ns	4.03 ± 0.44 ***	0.51 ± 0.02 ns	0.98 ± 0.05 ns	0.53 ± 0.02 ns	0.16 ± 0.02 ns

Values are Means ± Standard Deviations (n=5). * implies $P \leq 0.05$; ** implies $P \leq 0.01$, * implies $P \leq 0.001$.**

period. The 72 mg/kg group had a slightly significant increase ($P \leq 0.01$) by day 23 and by day 45 the variation was very significant. In the 180 mg/kg treated groups increases in RDW-SD were just significant ($P \leq 0.05$) for the period of study.

The platelet number increased very significantly ($P \leq 0.001$) in all treatment groups over the 45-day study period. There were insignificant changes ($P \leq 0.05$) in MPV in all treatment groups over the 45-day period except in the 180 mg/kg treated group after 45-days of treatment. PDW increased very significantly ($P \leq 0.001$) in all groups throughout the treatment period and so was the PCT.

3.3.4 Liver Function Tests

The liver function tests (Table 14) indicates that albumin levels reduced very significantly ($P \leq 0.001$) in all treatment groups by 23 days of drug treatment. Albumin level however increased significantly ($P \leq 0.001$) above the normal by day 45 after treatment in all groups. Globulin levels increased significantly ($P \leq 0.001$) in all treatment groups and therefore total protein over the entire treatment period.

ALT levels increased significantly ($P \leq 0.01$) in all the treatment groups after 23 days of treatment. It however decreased significantly ($P \leq 0.001$) below the control in all treatment groups after 45 days. A similar trend was seen with AST. ALP levels also increased very significantly ($P \leq 0.001$) after 23 days but

decreases very significantly below the control in the 32 mg/kg treated group after 45 days. The 72 mg/kg group had a slight increase ($P \leq 0.05$) over the control after 45 days while level of ALP remained insignificantly high ($P > 0.05$) compared to the control at the end of the study period. GGT values were too low to be detected in all the treatment groups at day 23 but by day 45, GGT levels were high enough to be detected.

Both the direct and indirect bilirubin as well as the total bilirubin was not significantly affected on treating the mice with the different concentrations of the test product for 23 days. However, in all treatment groups there were very significant increases ($P \leq 0.001$) in direct and indirect and therefore total bilirubin levels by day 45.

3.3.5 Kidney Function Tests

The kidney function tests (Table 15), indicated very significant decrease ($P \leq 0.001$) in blood urea in all treatment over the entire study period. Creatinine levels increased very significantly in all the treatment groups after 45 days of treatment but changes in creatinine levels were not significant when measured 23 days after treatment was initiated. Sodium, potassium and chloride levels were not significantly affected when measured on day 23 but by day 45, levels of these ions have reduced very significantly.

Table 13: The effect of 36, 72, and 180 mg/kg of PHA on the hematological profile of ICR mice

Parameters	Control	36 mg/kg		72 mg/kg		180 mg/kg	
		Day 23	Day 45	Day 23	Day 45	Day 23	Day 45
WBC ($x10^9$/L)	4.38 ± 0.61	5.38 ± 0.65 ns	5.05 ± 0.85 ns	5.55 ± 0.48 **	5.3 ± 0.47 *	6.4 ± 0.46 ***	6.05 ± 0.35 ***
HGB (g/dl)	13.84 ± 0.90	11.13 ± 0.75 †††	12.70 ± 0.22 †	11.98 ± 0.35 †††	12.73 ± 0.17 †	11.10 ± 1.07 †††	13.55 ± 0.06 ns
RBC ($x10^{12}$/L)	7.58 ± 0.45	7.57 ± 0.22 ns	8.58 ± 0.20 *	7.99 ± 0.26 ns	8.11 ± 0.30 ns	7.65 ± 0.54 ns	8.20 ± 0. 27 ns
HCT (%)	36.02 ± 3.79	40.1 ± 2.06 *	44.45± 0.87 ***	41.95± 1.95 **	46.08± 0.71 ***	42.83 ± 2.52 **	48.15± 0.52 ***
MCV (fL)	54. 2 ± 1.42	53.40 ± 1.94 ns	52.01 ± 0.33 ns	50.83 ± 1.73 ††	55.60 ± 0.22 ns	50.78 ± 1.90 †††	51.50 ± 1.17 †
MCH (pg)	16.80 ± 1.03	14.8 ± 0.68 ††	14.73 ± 0.17 †	14.7 ± 0.61 †††	15.33 ± 0.05 †	14.30 ± 0.67 †††	14.73 ± 0.48 ††
MCHC (g/dl)	36.74 ± 0.74	27.90 ± 0.48 †††	28.40 ± 0.08 †††	28.73 ± 0.60 †††	27.60 ± 0.08 †††	28.20 ± 0.35 †††	28.18 ± 0.33 †††
RDW-CV (%)	15.30 ± 1.19	14.83 ± 0.71 ns	16.45 ± 1.31 ns	15.03 ± 1.18 ns	15.93 ± 1.63 ns	15.15 ± 5.17 ns	17.55 ± 1.17 ns
RDW-SD (fL)	24.78 ± 2.52	29.70 ± 0.32 ***	30.35 ± 0.33 ***	29.03 ± 1.71 *	31.13 ± 1.88 ***	30.75 ± 4.35 *	30.78 ± 1.00 *
PLT ($x10^9$/L)	794.0 ± 70.26	1303.0 ± 192.26 ***	1400.0 ± 84.49 ***	1087.0 ±136.77 ***	1014.0 ± 16.10 **	1034.0 ± 90.71 **	1214.0 ± 143.15 ***
MPV (fL)	6.22 ± 0.68	5.73 ± 0.13 ns	5.80 ± 0.22 ns	5.78 ± 0.17 ns	6.08 ± 0.13 ns	6.15 ± 0.21 ns	5.55 ± 0.13 †
PDW	16.52 ± 1.33	6.50 ± 0.18 †††	6.58 ± 0.40 †††	6.7 ± 0.22 †††	6.95 ± 0.13 †††	7. 08 ± 0.46 †††	6.38 ± 0.15 †††
PCT (%)	0.50 ± 0.09	2.55 ± 0.06 ***	2.72 ± 0.46 ***	2.75 ± 0.37 ***	3.8 ± 0.54 ***	4.35 ± 1.16 ***	2.40 ± 0.27 **

Values are Means ± Standard Deviations (n=5) . ns implies $P > 0.05$ for insignificant increments or decrements. For significant increments: * implies $P \leq 0.05$; ** implies $P \leq 0.01$, * implies $P \leq 0.001$. For significant decrements: † implies $P \leq 0.05$; †† implies $P \leq 0.01$, ††† implies $P \leq 0.001$.**

Table 14: The effect of 36, 72, and 180 mg/kg of PHA on liver function tests in ICR mice

Parameters	Control	36 mg/kg		72 mg/kg		180 mg/kg	
		Day 23	Day 45	Day 23	Day 45	Day 23	Day 45
Albumin (g/l)	35.5 ± 0.59	31.5 ± 0.46 †††	38.2 ± 0.55 ***	32.1 ± 0.68 †††	37.4 ± 0.51 ***	32.8 ± 1.76 ††	38.9 ± 0.30 ***
Globulins (g/l)	12.1 ± 0.70	28.7 ± 1.26 ***	41.3 ± 1.10 ***	30.9 ± 1.06 ***	43.7 ± 1.41 ***	26.1 ± 1.11 ***	47.2 ± 0.24 ***
Total protein (g/l)	48.2 ± 0.37	60.8 ± 1.46 ***	79.2 ± 0.92 ***	63.0 ± 2.57 ***	81.6 ± 1.15 ***	59.1± 1.35 ***	86.2 ± 0.30 ***
ALT/GPT (U/I)	60.1 ± 2.21	81.4 ± 1.72 ***	32.1 ± 0.50 †††	67.5 ± 1.52 **	42.3 ± 8.98 †††	70.1 ± 7.01 **	38.9 ± 2.33 †††
AST/GOT (U/I)	195.3 ± 1.98	296.8 ± 9.75 ***	92.3 ± 0.81 †††	236.7 ± 2.19 **	118.2± 29.79 †††	219.3 ± 17.5 **9	110.9 ± 4.8 †††
ALP (U/I)	113.0 ± 8.45	200.9 ± 0.3 0 ***	65.2 ±8.09 †††	171.2 ± 1.17 ***	126.3 ± 9.21 *	156.4 ± 24.42 ***	126.3 ± 9.21 ns
Bil. Direct (µmol/l)	1.68 ± 0.08	1.9 ± 0.13 ns	2.5 ± 0.19 ***	1.7± 0.05 ns	2.8 ± 0.22 ***	1.7 ± 0.20 *	2.4 ± 0.18 ***
Bil Indirect(µmol/l)	0.61 ± 0.22	0.7 ± 0.18 ns	1.3 ± 0.12 ***	0.6 ± 0.07 ns	1.6 ± 0.11 ***	0.5 ± 0.20 *	1.4 ± 0.21 ***
TBil (µmol/l)	2.34 ± 0.15	2.6 ± 0.11 *	3.8± 0.18 ***	2.3 ± 0.13 ns	4.4 ± 0.27 ***	2.2 ± 0.10 ns	3.8 ± 0.18 ***
GGT (µmol/l)	R	R	0.3 ± 0.1	R	0.2 ± 0.07	R	0.2 ± 0.07

Values are Means ± Standard Deviations. N=5. . ns implies *P* > 0.05 for insignificant increments or decrements. For significant increments: * implies *P* ≤ 0.05; ** implies *P* ≤ 0.01, * implies *P* ≤ 0.001. For significant decrements: †† implies *P* ≤ 0.01, ††† implies *P* ≤ 0.001.**
ALT/GPT = *Alanine Transaminase/Glutamate Pyruvate Transaminase,* AST/GOT = *Aspartate transaminase/Glutamic Oxaloacetic transaminase,* ALP = *Alkaline Phosphatase,* Bil = Bilrubin; TBil = Total bilirubun, GGT = *Gamma GlutamylTransferase*. R = means plasma concentration is too low to be detected

Table 15: The effect of 36, 72, and 180 mg/kg of PHA on kidney function tests in ICR mice.

Parameters	Control	36 mg/kg		72 mg/kg		180 mg/kg	
		Day 23	Day 45	Day 23	Day 45	Day 23	Day 45
Creatinine (µmol/l)	37.20 ± 0.45	40.2 ± 2.96 ns	43.6 ± 7.25 ns	35.7 ± 0.51 ns	78.5 ± 15.85 ***	36.74 ± 0.40 ns	81.0 ± 0.61 ***
Urea (mmol/l)	15.04 ± 0.56	10.4 ± 1.06 †††	5.6 ± 0.36 †††	10.6 ± 0.39 †††	5.9 ± 0.67 †††	8.5 ± 0.58 †††	6.4 ± 0.05 †††
Sodium (mmol/l)	143.82 ± 1.51	157.2 ± 6.67 *		150.2 ± 1.08 ns		144.42 ± 3.26 ns	
Potassium (mmol/l)	8.19 ± 0.40	9.0 ± 0.3 ns		10.3 ± 0.51 ns		9.3 ±0.17 ns	
Chloride (mmol/l)	111.92 ± 1.25	118.2 ± 6.51 **		111.6 ± 1.84 ns		109.12 ± 3.26 ns	

Values are Means ± Standard Deviations (n=5). ns implies $P > 0.05$ for insignificant increments or decrements. For significant increments: * implies $P \leq 0.05$; ** implies $P \leq 0.01$, * implies $P \leq 0.001$. For significant decrements: ††† implies $P \leq 0.001$.**

3.3.6 Lipid Profile

Lipid profile tests (Table 16) revealed that total cholesterol reduced significantly ($P \leq 0.001$) in the 36 mg/kg and the 72 mg/kg treated groups but did reduced significantly in the 180 mg/kg group after 23 days of treatment but after 45 days, total cholesterol level was not significantly affected ($P > 0.05$) in all treatment groups. Levels of TAG were significantly very high ($P \leq 0.01 - P \leq 0.001$) in all treatment groups after 23 days of treatment. Levels of TAG however reduced after 45 days of treatment and compared to the control was insignificant ($P > 0.05$) in all the treatment groups. Levels of VLDL increased significantly ($P \leq 0.001$) in all the treatment groups 23 days after drug treatment, however, after 45 days of treatment, VLDL levels reduced significantly ($P \leq 0.001$) below the control in all the groups. Levels of LDL were significantly very high ($P \leq 0.001$) in all treatment groups after 23 days of treatment but dropped such that by 45 days of treatment, levels were significantly lower ($P \leq 0.001$) below the control in all the groups. HDL, like cholesterol reduced significantly ($P \leq 0.001$) in the 36 mg/kg and the 72 mg/kg treated groups but the reduction was not significant ($P > 0.05$) in the 180 mg/kg group after 23 days of treatment but after 45 days, total cholesterol level compare to the control group was no significantly affected ($P > 0.05$) in all treatment groups.

3.3.7 Urine Analysis

In the urine analysis (Table 17) of treated and untreated mice, there were no significant changes over time except for increases in pH and change of urine color from straw to amber within 24 hours of treatment.

Table 16: The effect of 36, 72, and 180 mg/kg of PHA on lipid profile of ICR mice .

Parameters	Control	36 mg/kg		72 mg/kg		180 mg/kg	
		Day 23	Day 45	Day 23	Day 45	Day 23	Day 45
CHOL (mmol/l)	2.8 ± 0.06	2.0 ± 0.02.†††	2.8 ± 0.06 ns	2.2 ± 0.07 †††	2.7 ± 0.12 ns	2.7 ± 0.14 ns	2.8 ± 0.12 ns
TAG (mmol/l)	1.0 ± 0.08	1.5 ± 0.01 ***	1.0 ± 0.08 ns	1.2 ± 0.05 **	0.9 ± 0.12 ns	1.7 ± 0.07 ***	0.9 ± 0.01 ns
HDL (mmol/l)	1.6 ± 0.02	1.2 ± 0.03 †††	1.6 ± 0.02 ns	1.2 ± 0.05 †††	1.5 ± 0.07 ns	1.6 ± 0.03 ns	1.6 ± 0.06 ns
VLDL (mmol/l)	0.7 ± 0.04	0.8 ± 0.02 ***	0.5 ± 0.03 †††	0.8 ± 0.02 ***	0.4 ± 0.06 †††	0.8 ± 0.04 ***	0.4 ± 0.01 †††
LDL (mmol/l)	0.5 ± 0.02	0.8 ± 0.06 ***	0.3 ± 0.11 ††	0.7 ± 0.03 ***	0.4 ± 0.02 ††	0.7 ± 0.05 **	0.2 ± 0.01 †††

Values are Means ± Standard Deviations (n=5). ns implies P > 0.05 for insignificant increments or decrements. For significant increments: * implies $P \leq 0.05$; ** implies $P \leq 0.01$, * implies $P \leq 0.001$. For significant decrements: †† implies $P \leq 0.01$, ††† implies $P \leq 0.001$.**
CHOL = Cholesterol; TAG = Triacylglycerides, HDL = High Density Lipoprotein; VLDL = Very Low Density Lipoprotein; LDL = Low Density Lipoprotein

Table 17: The effect of 36, 72, and 180 mg/kg of PHA on urine composition of ICR mice

		36 mg/kg			72 mg/kg			180 mg/kg		
Parameters	**Control**	**Day 1**	**Day 23**	**Day 45**	**Day 1**	**Day 23**	**Day 45**	**Day 1**	**Day 23**	**Day 45**
pH	5.4 ± 0.55	7.8 ± 1.60	5.6 ± 0.54	5.2 ± 0.44	5.6 ± 0.54	5.8 ± 0.44	5.6 ± 0.54	8.2 ± 1.80	5.2 ± 0.44	5.6 ± 0.54
NIT (mg/dL)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
URO (mg/dL)	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00	0.2 ± 0.00
PRO (mg/dL)	72 ± 38.3	86 ± 31.3	58 ± 38.3	86 ± 31.3	86 ± 31.3	86 ± 31.3	100 ± 0.00	86 ± 31.3	100 ± 0.00	86 ± 31.3
SG	1.000 ± 0.000	1.004 ± 0.002	1.000 ± 0.000	1.00 ± 0.000	1.003 ± 0.003	1.000 ± 0.000	1.000 ± 0.000	1.004 ± 0.002	1.000 ± 0.000	1.000 ± 0.000
BLO(Ery/uL)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
BIL (mg/dL)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
KET (mg/dL)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
GLU (mg/dL)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
ASC (mg/dL)	32 ± 11.0	36 ± 8.0	32 ± 11.0	28 ± 11.0	28 ± 11.0	32 ± 11.0	36 ± 8.0	32 ± 11.0	24 ± 8.9	32 ± 11.0
Volume	1.14 ± 0.28	1.24 ± 0.27	1.10 ± 0.20	1.16 ± 0.20	1.22 ± 0.08	1.22 ± 0.22	1.08 ± 0.22	1.18 ± 0.16	1.22 ± 0.08	1.14 ± 0.15
Color	Straw	Amber	Straw	Straw	Amber	Straw	Straw	Amber	Straw	Straw
Appearance	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Smell	Pungent	Pungent	Pungent	Pungent	Pungent	Pungent	Pungent	Pungent	Pungent	Pungent

Values are Means ± Standard Deviations. N=5 Protein (PRO), Specific gravity (SG), Blood (BLO), Bilirubin (BIL), Ketone (KET), Glucose (GLU), Ascorbic acid (ASC), (-) = Negative/Trace quantities undetectable by the test strip.

3.3.8 Histopathological Examination

Photomicrograph from the histological examination showed some changes in the liver, kidney, and spleen between control (Plate 1, 4 & 7) and treated groups of animals. Histological changes observed in the liver at 23 and 45 days of test product treatment was assessed to be fibrosis and cirrhosis at higher doses (180 mg/kg) and prolonged administration (45 days) of the test drug (Plate 2 & 3). Photomicrograph of the kidney showed glomerular inflammation (Plates 5 & 6). The red pulp of the spleen had very mild congestion after 23 days (Plate 8) but normalized after 45 days (Plate 9). The heart, lungs, testes, and uterus did not show any observable histological changes.

PHOTOMICROGRAPHS OF AFFECTED TISSUE IN HISTOPATHOLOGICAL STUDIES

1. LIVER

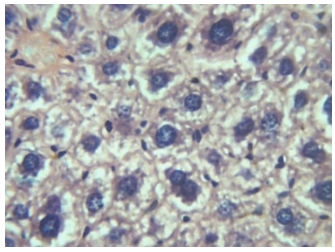

Plate 1: A photomicrograph of a normal liver of an ICR mouse. Magnification X40; Stain: Haematoxylin & Eosin

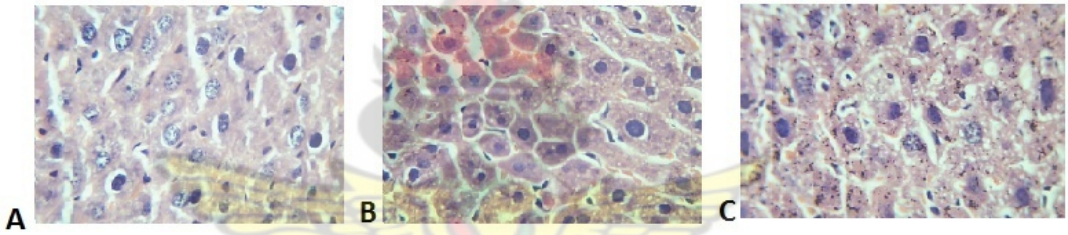

Plate 2: Photomicrographs of the liver of ICR mice showing fibrosis after 23 days of treatment with 36, 72, and 180 mg/kg of PHA (i.e. A, B, C respectively) in histopathological studies in a subchronic toxicity test. Magnification X40; Stain: Haematoxylin & Eosin

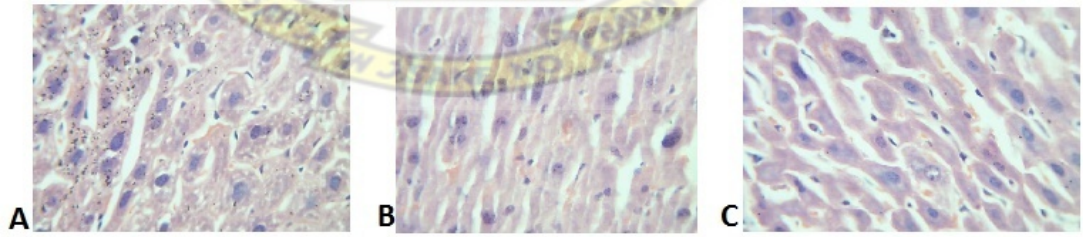

Plate 3: Photomicrographs of the liver of ICR mice showing cirrhosis after 45 days of treatment with 36, 72, and 180 mg/kg of PHA (i.e. A, B, and C respectively) in histopathological studies in a subchronic toxicity test. Magnification X40; Stain: Haematoxylin & Eosin

KIDNEY

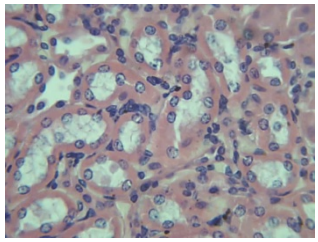

Plate 4: A photomicrograph of a normal kidney of an ICR mouse. Magnification X40; Stain: Haematoxylin & Eosin

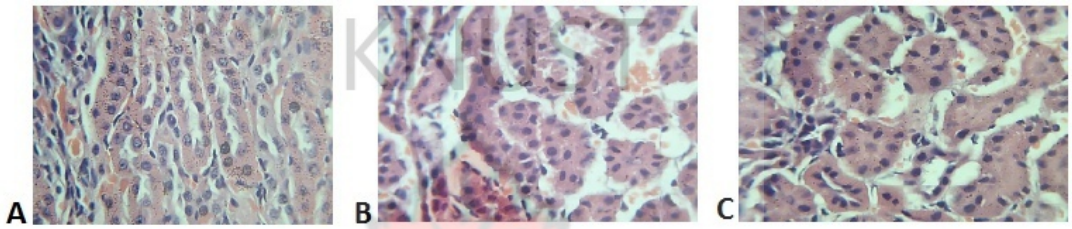

Plate 5: Photomicrographs of the kidney of ICR mice showing glomerular inflammation after 23 days of treatment with 72 and 180 mg/kg of PHA. A, B, and C represents treatment with 36, 72, and 180 mg/kg of PHA respectively in histopathological studies. Magnification X40; Stain: Haematoxylin & Eosin

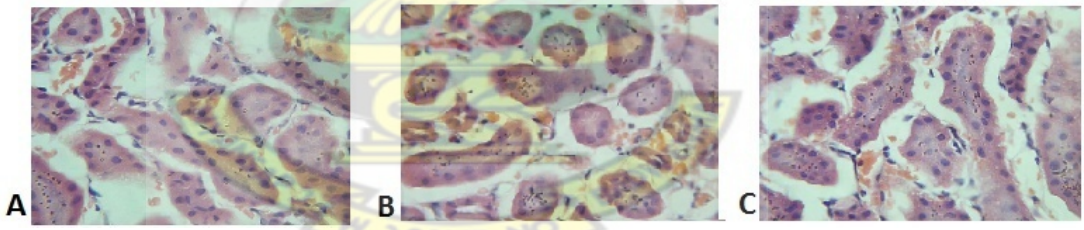

Plate 6: Photomicrographs of the liver of ICR mice showing glomerular inflammation after 45 days of treatment with 36, 72, and 180 mg/kg of PHA (A, B, and C respectively) in histopathological studies. Magnification X40; Stain: Haematoxylin & Eosin

SPLEEN

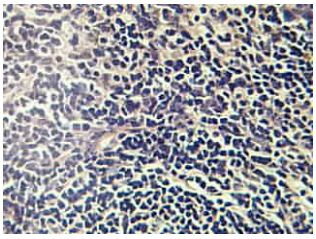

Plate 7: A photomicrograph of a normal spleen of an ICR mouse. Magnification X40; Stain: Haematoxylin & Eosin

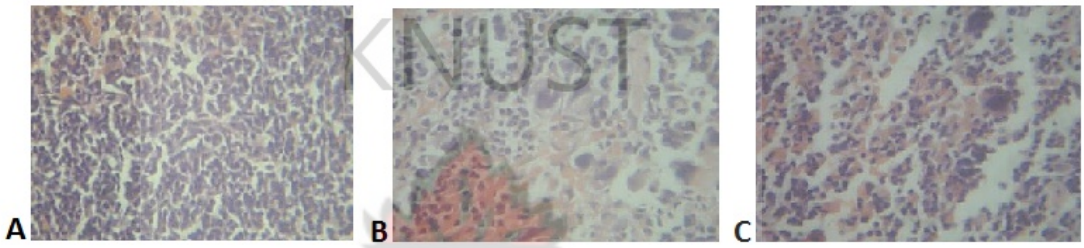

Plate 8: Photomicrographs of the spleen of ICR mice showing mild red pulp congestion after 23 days of treatment with 72, and 180 mg/kg of PHA in histopathological studies. A, B, and C represents treatment with 36, 72, and 180 mg/kg of PHA respectively. Magnification X40; Stain: Haematoxylin & Eosin

Plate 9: Photomicrographs of the liver of ICR mice showing no effect after 45 days of treatment with 36, 72, and 180 mg/kg PHA (i.e. A, B, C respectively) in histopathological studies. Magnification X40; Stain: Haematoxylin & Eosin

3.4 DISCUSSION

The “no effect on body and skin” observed in the physical (In-life) evaluation suggests that the product under study may not have any allergic or carcinogenic effect on skin. It may not cause hypersensitization and neurogenic inflammation. The combination of vasodilation and protein extravasation following activation of nociceptors has been termed "neurogenic inflammation" (Richardson and Vasko, 2002). Allergic reactions and cutaneous neurogenic inflammation can result in hyperesthesia, pruritis, and hyperplasia and carcinomas which cause pain at the site of development and may attract scratching, biting, licking, and increased locomotor activity. When mice do not sleep, they lose their fur, look debilitated, unkempt (an evidence of underlying illness) and unhealthy.

Lacrimation, miosis, rhinorrhoea, salivation, urination, defecation, and labored breathing which are usual signs of muscarinic hyperactivity (autonomic nervous system effect) can be seen by observation of the eye, nose, mouth, the external genitalia, anus, skin and fur as well as the body as a whole. During parasympathetic discharge, acetylcholine binds to muscarinic receptors found in these tissues resulting in these observations. Activation of M_3 receptors in the urinary bladder results in stimulation of detrusor muscle and relaxes the trigone and sphinter muscle of the bladder thus promoting voiding of urine (Katzung, 2006). M_3 activation in the iris circular smooth muscle causes miosis. In the gastro-intestinal tract, M_3 muscarinic effect is increased motility due to increased

muscle tone. It also increases secretions into the tract and relaxes the sphinter muscles, hence frequent defecation and sometimes diarrhea. A block in activity results in constipation. Piloerection is often seen in stressed mice: It starts when a stimulus such as cold or fright or a sympathomimetic agent causes a discharge from the (involuntary) that triggers contraction of the little arrectores pilorum muscles. Contraction of these muscles elevates the hair follicles above the rest of the skin so the hair seems to "stands on end." Piloerection is also a (rare) symptom of some diseases, such as temporal lobe epilepsy, some brain tumors, and autonomic hyperreflexia. Since this was not noticed in the experimental animals, it may suggest that the product may not cause autonomic hyperreflexia.

Bronchiolar smooth muscle contraction (activation of M_3 muscarinic receptors) coupled with reduction in sinoatrial node firing and atrial contraction as a result of M_2 muscarinic receptor activity results in laboured breathing. There was no “chattering”. Chattering is a stertorous breathing noise presumably due to mucopurulent material in the airways, nose or both and is an indication of repiratory tract disorder (Suckow *et al.,* 2001). It may be drug-induced: drugs that have antigenic property. It therefore indicates that the product may not induce allergic reactions.

Some of the observations are CNS-mediated. CNS excitation results in clonic or tonic movements, excessive grooming, repetitive circling (stereotypy) or self-

mutilation, walking backward (bizarre behavior) changes in gait and posture, twitching, spasticity and seizures (convulsion). A depression of the CNS results in decreased locomotory activity, sedation, respiratory depression, neuromuscular inco-ordination and collapse, anorexia and therefore reduced feeding habits that leads to loss of weight and emaciation. Muscle relaxants effects are usually noticed as a decrease in locomotor activity. Pain and inflammation in some parts of the body can be realized as writhing, change in gait and body posture and decreased locomotor activity. Observations showed normal movements with no treatment related symptoms.

Polyuria, polydipsia, polyphagia, are related to carbohydrate (glucose) metabolism. These effects did not show with the use of the product indicating that the product has no effect on insulin production and secretion, and no effect on insulin receptor activity.

The product did not seem to have any "wasting effect" (overall body condition was evaluated to be normal). Mice that are thin or abnormally small compared to littermates in the no treatment group (control) may have underlying illness or disorder (Suckow *et al.*, 2001). There was no pallor in the eyes. Pallor in the eyes could be due to a condition of anaemia.

Medicine cause sudden and gradual deaths by inducing respiratory failure and/or organ failure. Organ failure means that the brain, heart, liver, lungs, kidney, bone

marrow, and skeletal muscles among others fail to function the way they should. An increase in the inhibitory effect of GABA, and paralysis of the muscles that control respiration would result in the slowing and eventual stopping of breathing which leads to respiratory failure. Hyperthermia due to dopamine-over activity, increase in blood pressure, and vasoconstriction can lead to brain damage. Hyperthermia can also lead to multiple organ failure which results in death. The product may not have caused any respiratory arrest, cardiac arrest and organ failure and hence no deaths from this study but further investigations need to be carried out to investigate and verify its safety for use as an antihypertensive agent.

Organ weight change is a sensitive indicator of treatment-induced changes to organs e.g. increase in the weight of an organ might be indicative of a neoplasia, tumour, oedema, inflammation or hypertrophy. On the other hand, decrease in weight of an organ might indicate cell damage, tissue damage, necrosis or atrophy.

It was observed that, the weight of the brain increased by the end of 23 days for each of the concentrations of the herbal preparation. This could have been an indication of hyperplasia of brain cells. Changes in brain weights are rarely associated with neurotoxicity (Sellers *et al.*, 2007). The brain is a vital organ that has effective repair mechanisms and immediately repairs any abnormality imposed on it (Zhao *et al* 2003). Thus prolonged use of the herbal preparation does not have any significance on the weight of the brain.

Increase in concentration of the herbal preparation was observed to decrease the weight of liver significantly in the treated groups of animals. The liver is the main organ for metabolism of xenobiotics in the body. Alterations in liver weight may be suggestive of treatment-related changes including hepatocellular hypertrophy or atrophy (e.g., enzyme induction or peroxisome proliferation) (Amacher *et al*., 2006; Juberg *et al*., 2006). The reduction of the weight of the liver with increasing dose might be due to the metabolic end products or the herbal preparation itself inducing hepatocellular atrophy thus decrease in liver weight. There were no significant changes observed in the weights of the following organs between control and treated animals: kidney, spleen, heart, lung, testes, uterus and ovaries. Changes in kidney weight may reflect renal toxicity, tubular hypertrophy or chronic progressive nephropathy (Greaves, 2000). The weight of the spleen may enlarge due to infections, anemia and cancer but may reduce probably due to atrophy of its red or white pulp. The weight of the heart may change due to myocardial hypertrophy or atrophy (Greaves, 2000). Changes in testes weights may reflect changes in seminiferous tubules or interstitial oedema (Creasy, 2002). Reproductive organ weights (uterus and ovaries) in females which change significantly may be attributed to secondary effects of treatment on the reproductive cycle rather than a direct toxic effect of a test drug.

Even though organ weights provide useful signals indicating effects by test samples, it is important that its interpretation is done together with

histopathological findings. This is because over reliance on statistical significance alone in evaluating organ weight changes are not satisfactory (Gad *et al*., 2002)

There were significant increases in WBC count in ICR mice receiving the higher dose of the preparation. The high counts recorded however were still within the proposed range for mice quoted as 3.0–14.2 x 10^3 cells/µl (Suckow *et al*, 2001). The increase in WBC count could be caused by the product because it becomes a foreign component of the internal environment when adsorbed into the blood from the gut and this attracts an immune system response. Results on RBC count indicated insignificant increases. The significant decrease in hemoglobin concentration recorded after 23 days of product-treatment could possibly be a result of the formation of microcytic red blood cells with reduced hemoglobin within the cell. This will bring the entire blood hemoglobin concentration down. This defect seemed to have corrected later on in the study period as hemoglobin concentration was approaching normal values. The product therefore seems to cause some form of anaemia (possibly microcytic anaemia) in the early days of administration. Aplastic anaemia is ruled out because the RBC count did not reduce. The possibility of causing microcytic anaemia is confirmed by the significant reductions observed with results obtained for the MCV, MCH, and MCHC (RBC indices). If anemia is observed, RDW test results are often used together with mean corpuscular volume (MCV) results to figure out what the cause of the anemia might be. The RDW-CV was insignificant over the period but the RDW-SD increased significantly. Iron deficiency anemia initially presents with a

varied size distribution of red blood cells, and as such shows an increased RDW. An elevated RDW, that is red blood cells of unequal sizes, is known as anisocytosis. Intense reticulocyte replication always gives rise to anisocytosis.

The HCT was elevated; an indication of an increased volume occupied by RBCs. Comparing the experimental HCT with the proposed HCT that has been documented, values were still within the proposed range. From the RBC count, HCT, RBC indices and RBC morphology it could be deduced that the product cause microcytic-anisocytic anaemic when used for a while The results showed an increase in platelet count without any significant change in MPV. The elevated platelet number was confirmed by the elevated PLT as the volume occupied is directly proportional to the number of cell. This suggests that there is product-related activation of thrombocyte production which may increase the risk of thrombosis. A low MPV measurement may be associated with leukemia. An elevated MPV, on the other hand, can indicate an increased risk of stroke and cardiovascular disease (thrombosis). The insignificant changes in the mean platelet volume suggests that the bone marrow is manufacturing platelets normally and therefore reduces the suspected risk of causing thrombosis and other related cardiovascular disorders.

The PDW was significantly high. This suggests that there is high variation in platelet width. This is expected because there is drug-induced platelet production and newly produced platelets tend to be larger than older ones.

Combining the results obtained from the liver function tests, it can be deduced that the test product causes an inflammatory disorder which leads to the development of some form of cirrhosis in the liver in the initial stages of administration. The cellular destruction reduces albumin levels while the inflammation raises globulin levels with a net increase in total protein levels. The product affects the functionality of the liver as was evidently seen by the rise in ALT, AST. This problem however appear to resolve with time. The product also causes a decrease in the liver's excretory function evidently seen by raising ALP and GGT and total bilirubin (direct and indirect bilirubin).

The liver is incapable of adequately removing bilirubin in a timely manner due to blockade of the bile ducts, inflammatory disorders leading to cirrhosis, or due to the overproduction of bilirubin. Because the liver has a large excess of bilirubin excreting capacity, the plasma conjugated bilirubin level does not become elevated until the liver has lost at least one half of its excretory capacity. The marked rise observed at the end of the treatment period, indicates a time-dependent hepatic excretory impairment since no significant changes were present after 23 days. Also, usually, high bilirubin levels are characteristic of idiosyncratic injury, i.e. drug toxicity unrelated to dosage (Kumar *et al*, 2010). The observed seemingly reversal of the drug induced liver injury (DILI) i.e. initial rise followed by a fall in transaminases and ALP level with continued exposure could be due to a process called adaptation (Watkins *et al*, 2006).

Urea levels recorded over the entire study period were significantly low. This could suggest a normal kidney function but an impaired liver function because blood urea nitrogen is affected by liver function. The liver produces urea in the urea cycle as a waste product of the digestion of protein. It is therefore possible that the product had effect on the liver rather than the kidney. Standard biochemical textbooks cite acute liver failure (decreased urea synthesis) for low urea nitrogen concentrations (Lum and Leat-Khourl, 1989). It is also affected by protein intake.

The urea test is usually done in conjunction with a blood creatinine (a more specific indicator of kidney function) (Metheny, 2000). Creatinine levels even though were insignificantly affected when measured 23 days after initiation of treatment, were significantly high after 45 days of treatment at all dose levels. With normal kidney function, the amount of creatinine in the blood remains relatively constant and normal since creatinine produced is affected very little by liver function (Toto, 1998; Woodrow, 2003). This gives an indication that the kidney's function was not affected in the early stages of the treatment.

The reduction in cholesterol observed in the first half of the treatment stage could be due to an interference of adsorption from the intestine by the product, or perhaps interference of the synthesis of cholesterol by the liver and/or intestines (as observed in the LFT). Slightly less than half of the cholesterol in the body derives from biosynthesis *de novo*. Biosynthesis in the liver accounts for

approximately 10 % and in the intestines approximately 15 %, of the amount produced each day. It could also be due to an enhanced peripheral utilization of cholesterol (manufacturer of steroid hormone and bile).These probably could be a contribution to its antihypertensive effect. Levels however returned to normal after 45 days of treatment. There could be several factors coming into play e.g. liver function returning to normal (as observed in LFT) with an increase in cholesterol synthesis (homeostatic mechanism), reduction in peripheral utilization or even an increased intake from diet. Looking at the results for total cholesterol alone it can be deduced that the test product cannot be a risk factor for heart and other cardiovascular diseases.

The rise in plasma TAG, VLDL, and LDL levels and the lowering of HDL in the first half of the experimental period could have been product related. Based on these alone, the product would not support its antihypertensive claims as it may rather increase the risk for cardiovascular disorders. The conditions however reversed and TAG and HDL levels returned to normal as VLDL and LDL levels dropped very significantly below normal values. A decrease in TAG, VLDL, and LDL implies a reduced risk of artherosclerosis, heart disease, and cardiovascular disorders.

Histological changes observed in the liver after 23 days and 45 days of test product treatment was described as periportal fibrosis and cirrhosis. Liver fibrosis is the excessive accumulation of extracellular matrix proteins including collagen that

occurs in most types of chronic liver diseases (Bataller and Brenner, 2005). Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation. Long term administration of the preparation might have resulted in the derangements in the synthesis and degradation of matrix by injuring mesenchymal cells (Miniño *et al.,* 2004).This therefore, eventually develops into cirrhosis.

Histological (biopsy) diagnosis classifies the severity of fibrosis into five stages, S0 to S4. S0 means no fibrosis. S4 is cirrhosis. In between, S1 is a mild fibrosis only seen at the portal area. S2 is a moderate stage of fibrosis, between portal areas, but without the destruction of the lobular structure. S3 is severe fibrosis. At this stage, there is fibrostic bridging between portal areas and between portal areas and center veins. At S4, in addition to S3's changes, there are pseudo-lobules formed and this stage is the final stage, cirrhosis. Liver fibrosis is the net result of the imbalance between the collagen fiber synthesis and decomposition. When fiber synthesis is very active and the decomposition is suppressed, fibrosis will progress.

Cirrhosis is always developed from fibrosis. Although, fibrosis and cirrhosis are different, they are closely related. They are two distinguished pathological conditions. At the fibrosis stages, the amount of collagen increases and the ratio of fibro-connective tissue verses liver cellular tissue increases. But at this stage, the liver lobular structures are intact. There is no pseudo-lobule formation. Cirrhosis

consists of two pathological features: fibro-connective tissue hypertrophy and pseudo-lobule formation. At the cirrhosis stage, the liver's fundamental structure is deformed, and the framework of the liver begins collapse. Thus, reversal is more difficult at this stage (Zhang, 2005).

Long term use of the preparation at high concentration causes inflammation of the glomerulus. This might have resulted from necrosis of glomerular cells. Renal excretion would therefore be impaired. The preparation would therefore increase the toxicity of other drugs administered concurrently with it. The red pulp of the spleen was congested after 23 days which returned to normal after 45 days. The congestion could have resulted due to increased levels of White Blood Cells (WBC) and platelets (PLT) which are present in the pulpasplenica (Junqueira and Carneiro, 2005). These high WBC and PLT coupled with the RBC might have caused the initial blockade of the splenic sinuses which is an integral part of the pulpasplenica. This will eventually impair the filtering ability of the spleen. The absence of significant changes in the heart, testes, uterus, and the lungs signifies that the extract has no effect on them. The herbal preparation therefore might not cause inflammation of the heart; testes and lungs with prolong administration.

3.5 CONCLUSION

The polyherbal antihypertensive product did not cause any observable CNS adverse effect but causes microcytic-anisocytic anaemia in the early stages of use. It may be detrimental to the liver and kidney when used in higher doses. Use of this antihypertensive could however be safe to use when used in lower doses. The no-observable-adverse-effect-level (NOAEL) is 32 mg/kg/day. Monitoring of the blood, liver and kidney may be required with its use.

CHAPTER FOUR

4.0 NEUROBEHAVIORAL ASSESSMENTS

4.1 INTRODUCTION

Neurobehavioral evaluations are an important component of toxicity testing for the neurotoxic potential of drugs (Moser, 1999). Behavioral test for toxicity are designed to demonstrate effects on behavior of animals; the quantitative evaluation of activity and specifically locomotor types of activity of experimental animals. Observations made during standard toxicity studies or specialized neurotoxicity studies can provide information important for identifying and/or characterizing neurotoxic effects.

A protocol that includes a framework for the systematic recording of observations and manipulations, such as a functional observational battery (FOB), is an integral part of neurobehavioral screening. A neurobehavioral test battery can be composed of a variety of endpoints, usually chosen to assess an array of neurological functions, including autonomic, neuromuscular, sensory, and excitability (Moser, 1999).

4.2 MATERIALS AND METHODS

4.2.1 Behavioral Assessment

The animals used for the study and the experimental protocols are as described in the previous experiment. In the behavioral assessment, the spontaneous activity of untreated ICR mice and mice treated with 55 and 550 mg/kg of PHA were

observed 1 hour post-treatment in an open field (previously described by Schiørring, 1979, with modification). The number of times a mouse crossed a line (motion), reared, or walked through the center of the open field (centering), were observed one hour after administration of the herbal product. The procedure was repeated 24 hours post-treatment. The assessment was done with 0.08 mg/kg diazepam; as depressant/anxiolytic and 100 mg/kg caffeine; as a stimulant/psychoactive reference drugs respectively.

4.2.2 Neuromuscular Assessment

Neuromuscular tests for evaluating motor coordination and muscular strength was carried out using the grip strength test and the rotarod test as described by Moser (1999).

4.2.2.1 Grip Strength Test

In the grip strength test, untreated as well as mice treated with 55 and 550 mg/kg of PHA respectively, were hung on a line and observed for how long they could hang and walk from the middle of the line to safety at the edge of the line. Animals that hang but did not move and those that fell off the line were not considered. The threshold period was three minutes.

4.2.2.2 Rotarod Test

The test is used to evaluate the activity of drugs interfering with motor coordination. Naive mice were placed on a 32 mm diameter horizontal wooden rod

of the rotarod equipment (Model 7600; Ugo Basile, Italy) rotating at a speed of 20 rpm. Mice capable of remaining on the top for 3 min or more, in three successive trials were selected for the study. The selected animals were divided into the no treatment (control), 55 mg/kg and 550 mg/kg PHA groups (n = 5) on the test day. One hour after administration of doses each group of animals was then placed on the rod. The time taken (within three minutes) for each animal in a group to fall off the rotating rod was recorded. The total of all the recorded times per group was estimated and compared with the control. The procedure was repeated 24 hour post treatment. The assessment was done with diazepam as the reference drug

4.2.2.3 Righting Reflex Test

In this test, the untreated as well as the mice treated with 55 and 550 mg/kg PHA respectively, were held in a supine position (on its back) and then quickly released to see if they would immediately flip over to resume to a normal standing position. This procedure was done 1 hour and then 24 hours post-treatment. The assessment was done with caffeine and diazepam as the reference drug. The depressant action in the righting reflex tests was scored as no effect (-), slight depression (+), moderate depression (++), strong depression (+++), very strong depression (++++). A trained observer unaware of the experiment assigned the score for the general behavioral studies.

4.2.3 Data Collection and Analysis

Observations were filmed using a FujiFilm FinePix S700 Digital Camera (Fujifilm Corporation, USA). Tracking of spontaneous activity (motion, rearing, centering),

rotarod and grip strength tests was done with the aid of Camtasio Studio version 6.0 (Techsmith corporation software, USA). The statistical analysis of data obtained and plotting of graphs were made using GraphPad Prism Version 5.0 [GraphPad Software, Inc. USA]. Statistical estimates were made with One-way Analysis of Variance (ANOVA) followed by Bonferonni's multiple comparisons test [post-test] at a confidence level of 95 %. Probability values less than or equal to 5 % ($P \leq 0.05$) were considered significant.

4.3 RESULTS

Results from the open field examination show that there were significant dose-dependent decreases ($P \leq 0.001$) in locomotor activity (mostly walking with little or no running) and rearing (Figure 3), and a significant decrease ($P \leq 0.01$) in centering (Figures 3) comparable to the diazepam-treated groups (Figure 1) but not with the caffeine treated groups (Figure 2). The video recording revealed that the mice showed very low reactivity and arousal (somewhat slow, sluggish, slight exploratory movements) comparable to the effects of diazepam. With the manipulative tests, it was observed that PHA-treated mice had significant dose-dependent reductions ($P \leq 0.01 - P \leq 0.001$) in motor coordination and grip strength (Figures 4 and 5), and righting response (++); comparable to diazepam-treated mice. These observations however were not significant after 24 hours post-treatment.

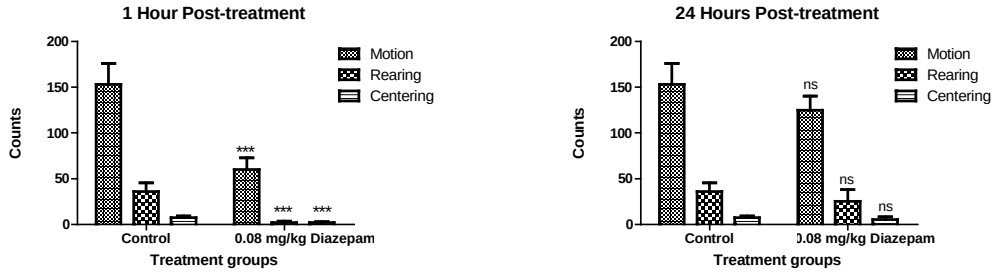

Figure 1: Effect of 0.08 mg/kg Diazepam on motion, rearing and centering in ICR mice in an open field observation 1hour and 24 hours post-treatment. * implies P ≤ 0.001; ns implies P > 0.05.**

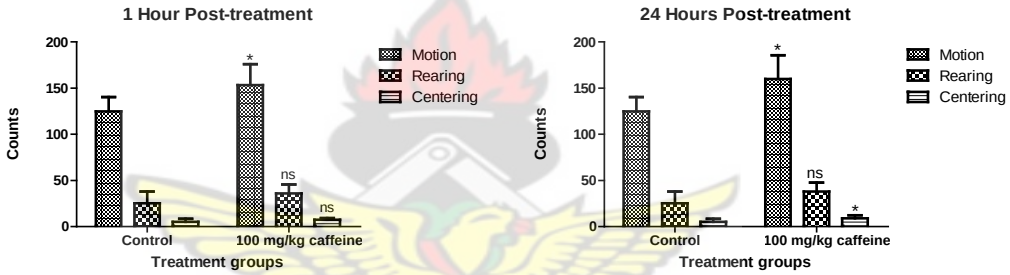

Figure 2: Effect of 100 mg/kg Caffeine on motion, rearing and centering in ICR mice in an open field observation 1hour and 24 hours post-treatment. * implies P ≤ 0.05; ns implies P > 0.05.

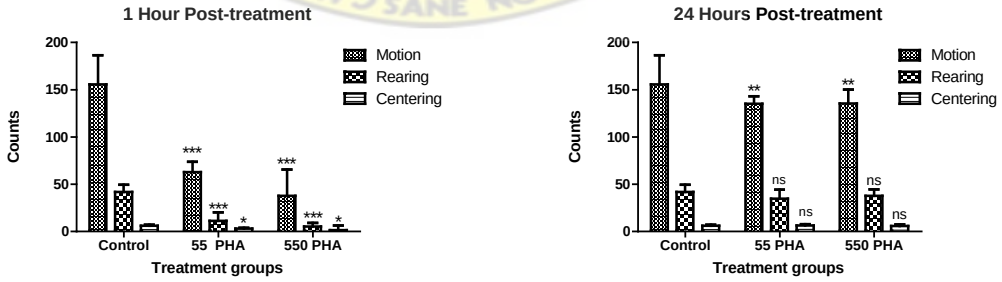

Figure 3: Effect of 55 and 550 mg/kg of PHA on motion, rearing and centering in ICR mice in an open field observation 1 hour and 24 hours post-treatment. * implies P ≤ 0.001; * implies P ≤ 0.001; ns implies P > 0.05.**

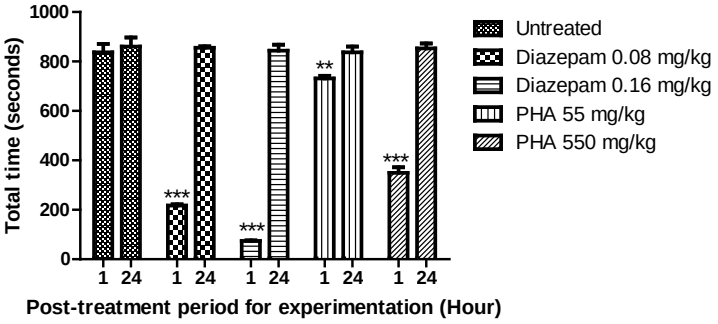

Figure 4: The effects of 0.08 and 0.16 mg/kg Diazepam, and 55 and 550 mg/kg PHA on motor coordination and muscular strength using the rotarod for neurobehavioural assessment during an acute toxicity study. * implies P ≤ 0.001, ** implies P ≤ 0.01**

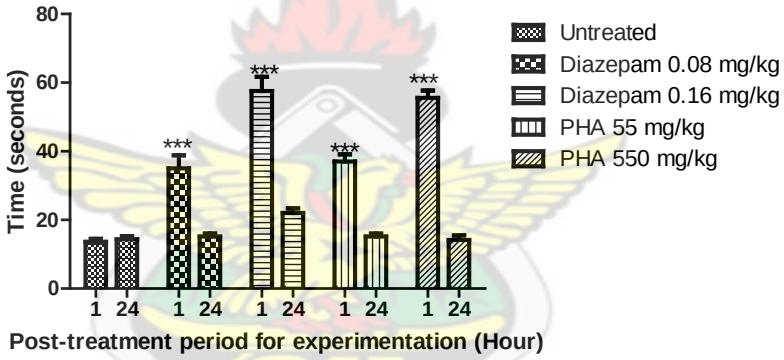

Figure 5: The effects of 0.08 and 0.16 mg/kg Diazepam, and 55 and 550 mg/kg PHA on motor coordination and muscular strength using the grip strength test for neurobehavioural assessment during an acute toxicity study. * implies P ≤ 0.001.**

4.4 DISCUSSION

Neurobehavioural studies revealed significant decreases in spontaneous activity within one hour of drug administration, and returning to normal within 24 hours post-treatment. A similar trend was observed with motor coordination and muscular strength.

Decreasing spontaneous activity, motor coordination, and muscular strength could imply that PHA has CNS depressant, anxiolytic and/or muscle relaxant effect (similar effects were seen in diazepam-treated groups) and thus may be acting by: activating inhibitory $GABA_A$, inhibiting excitatory ∝-*amino-3-hydroxy-5-methyl-4-isoxazole propionic acid* (AMPA) receptors**,** inhibiting the N-Methyl-D-Aspartate (NMDA) receptor (inhibits an excitatory effect), and/or potentiating the action of serotonin (5-HT) at excitatory $5\text{-}HT_3$ receptors (Hobbs *et al.,* 1996). Serotonin (5-HT) potentiates the inhibitory effect of dopamine on dopaminergic ventral tegmental area (VTA) neurons (Brodie and Bunney, 1996). Since these receptors are often localized on inhibitory interneurons, enhanced activation results in CNS inhibitory effects. It could dissolve into lipid membranes affecting the function of membrane proteins, such as receptors and ion channels.

The $GABA_A$ receptor is an inhibitory channel which when activated decreases neuronal activity and enhances the effects of GABA (Atack, 2005). When GABA binds to its site on the $GABA_A$ receptor more chloride ions enter and cause

hyperpolarization of the post-synaptic neuron resulting in an enhanced central nervous system depressant effect (Riss *et al*, 2008; Barondes, 1999).

AMPA receptors are the main mediators of fast excitatory neurotransmission in the CNS (Dingledine *et al.,* 1999). Inhibiting these excitatory AMPA receptors therefore results in CNS depression. The NMDA receptor is an ionotropic receptor that allows for the transfer of electrical signals between neurons in the brain and in the spinal column. For electrical signals to pass, the NMDA receptor must be activated. To remain activated, an NMDA receptor must bind to glutamate and to glycine. Chemicals that deactivate the NMDA receptor would inhibit excitatory effects resulting in CNS depression.

A possible PHA depressant activity on the limbic system, thalamus, and hypothalamus, could account for its anxiolytic effects. Though CNS depressant effect has been observed as an adverse effect with the use of PHA, it could possibly be the mechanism by which the polyherbal exerts its antihypertensive effect.

4.5 CONCLUSION

PHA has CNS depressant, anxiolytic, and probably muscle relaxant activity which affects neurological behaviors. These effects are desirable in antihypertensive therapy.

CHAPTER FIVE

5.0 POTENTIAL DRUG INTERACTIONS

5.1 INTRODUCTION

In Ghana, majority of patients taking herbal medicines in the management of hypertension are also on some orthodox medicines. Although most herbal medical practitioners claim safety with the concomitant use of both herbal and orthodox medicines, the possibility of drug interaction cannot be ruled out. A large proportion of the patients do not inform their health care givers as to the use of herbs with allopathic medicines, and most physicians and pharmacists do not enquire about herb use of their patients, probably believing that there is no such need. Studies have shown that consulting with physicians does not prevent patients from co-administering prescription medicines and herbal medicines (Molassiotis *et al.*, 2005; Howell *et al.*, 2006; Fakeye *et al.*, 2007). More than one-third of the ambulatory hypertensive patients interviewed in a Nigerian hospital were found to be using herbal medicine (Nwako and Fakeye, 2009). Although almost all the plants used by the respondents have proven ethnopharmacological and folkloric uses, this practice could be risky as far as the health of the individual is concerned.

Although some of the biological activities, such as reduction of blood cholesterol, antioxidant and blood-pressure-lowering properties, possessed by some of the herbs may be beneficial, its concomitant administration with allopathic medicines needs to be monitored with adjustment of doses, if necessary, and decisions on suitability of use of herbs can then be made with the patient, if need be. The latter

may be necessary especially if an antihypertensive agent is being concomitantly administered with a herb that possesses blood pressure-lowering effect.

Quite a number of physicians and pharmacists believe that there may be drug–herb interactions, (Adisa and Fakeye, 2006; Fakeye and Onyemadu, 2008) but not much effort is made to investigate patients' herb use. This oversight might have contributed to some of the incidences of side effects experienced as a result of herb use by some patients, which could have been prevented if they had been advised appropriately e.g. gingko interacts with aspirin with the potential of increased risk of bleeding, ginseng may interact (unpredictable) with warfarin hence the concomitant use may lead to a risk of prolonged bleeding, and St. John's wort may decrease theophylline's plasma concentration, thereby reducing its therapeutic effect. It is in this light that the effect of PHA on; cytochrome P450 (liver microsomal enzyme) and pentobarbitone-induced sleeping time is being studied for possible drug interactions.

5.2 METHODOLOGY

5.2.1 Pentobarbitone-Induced Sleeping Time

ICR mice were groups into six with 5 animals per group and received vehicle, 0.08 mg/kg diazepam (i.p), 100 mg/kg caffeine (i.p), or 36, 72 and 180 mg/kg PHA (p.o). Thirty minutes afterwards each animal was injected with sodium 40 mg/kg pentobarbital (i.p). The time which elapsed from the injection to the loss of the rightness reflex (onset of sleep) and the times from the loss of rightness reflex to awakening (duration of sleeping) were registered for each animal.

5.2.2 Cytochrome P450 Assay

ICR mice were groups into four with 10 animals per group and received either vehicle or 80 mg/kg ketoconazole, 100 mg/kg phenobarbitone, or 36, 72 and 180 mg/kg PHA by gavage. Dosing was once daily for 14 days at a volume of 10 ml/kg body weight.

5.2.2.1 Preparation of Tissue Homogenates

After the two-week treatment period, the mice were euthanized and their livers were rapidly excised and immediately placed in ice-cold 0.25 M sucrose to wash off excess blood and to cool the liver. The liver was then blotted dry, weighed and added to four times its weight of 0.25 M sucrose, to obtain a 20 % (w/v) homogenate. The liver was finely chopped with scissors and homogenized with Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged in a refrigerated centrifuge to isolate subcellular fractions

5.2.2.2 Preparation of Post-Mitochrondrial Supernatant

Post-mitochondrial supernatant was prepared by centrifuging the homogenate in a refrigerated centrifuge at 3000 *g* for 15 minutes to pellet intact cells, cell debris, nuclei and mitochondria. The resultant supernatant (the post-mitochondrial supernatant) was carefully decanted. This contains the microsomal plus soluble (cell sap) fractions of the cell.

5.2.2.3 Preparation of Subcellular Tissue Fractions

Aliquot (10 ml) of post-mitochondrial supernatant were mixed with 88 mM $CaCl_2$, such that 0.1 ml of 88 mM $CaCl_2$ was added per ml of supernatant (final $CaCl_2$ concentration is 8 mM) and left to stand on ice for 5 minutes, with occasional gentle swirling. The mixture was then centrifuged at 6000 g for 15 minutes, the supernatant discarded and the pellet resuspended by homogenization in 5 ml of 0.1 M Tris buffer, pH 7.4, yielding the microsomal suspension.

5.2.2.4 Spectral Determination of CYP450

CYP450 is a heamoprotein and use is made of the fact that when the haem iron is reduced and complexed with carbon monoxide, a characteristic absorption spectrum results. The reduced, carbon monoxide difference spectrum of CYP450 absorbs maximally at around 450 nm and the extinction coefficient for the wavelength couple 450-490 nm has been accurately determined to be 91 $mM^{-1}cm^{-1}$, thus allowing quantitative determination of this haemoprotein.

Tissue samples (0.1 ml) were diluted in 10 ml phosphate buffer (pH 7.4) containing 20 % (v/v) glycerol to approximately 2 mg/ml. Two milliliter (2 ml) quantities of the diluted samples were then put into sample cuvettes and baseline recordings made at wavelengths between 400-500 nm. A few grains of solid sodium dithionite were added to the sample cuvettes with gentle stirring and carbon monoxide (produced by mixing 12.5 ml concentration sulfuric acid and 25 ml formic acid in an infusion bottle which was plugged with a perforated rubber stopper containing a giving set) bubbled through the content for approximately 30 s. The spectrum was then re-scanned from 400-500 nm and cytochrome P450 content was calculated using Beer's law and assuming a cuvette path length of 1 cm:

$$\text{Cytochrome P450 (nmol/ml diluted)} = \frac{\text{Absorbance difference (nm) x 1000}}{\text{Extinction coefficient } (mM^{-1}cm^{-1})}$$

5.2.3 Statistical Analysis

The observations are presented as mean ± SEM. Significant differences among means of the group were determined by one-way ANOVA using Graph Pad Prism for windows version 5.00 (Graph Pad Software, San Diego, CA, USA). Significant differences between pairs of groups were calculated using Dunnet's multiple comparison test with level of significance set at $P \leq 0.05$.

5.3 RESULTS

5.3.1 Pentobarbitone-induced Sleeping Time

There was a significant dose-dependent reduction ($P \leq 0.05$-0.001) in the onset and duration of sleep in mice treated with PHA. This effect was similar ($P \leq 0.001$) to animals treated with diazepam. The onset and duration of sleep for the caffeine treated group was not significantly different from that of the vehicle treated group (control).

5.3.2 Cytochrome P450 Assay

Compared to the control, there was a very significant decrease ($P \leq 0.001$) in the CYP450 level in ICR mice treated with ketoconazole and PHA. In the phenobarbitone treated group however, there was a significant increase ($P \leq 0.01$) in cytochrome P450 levels.

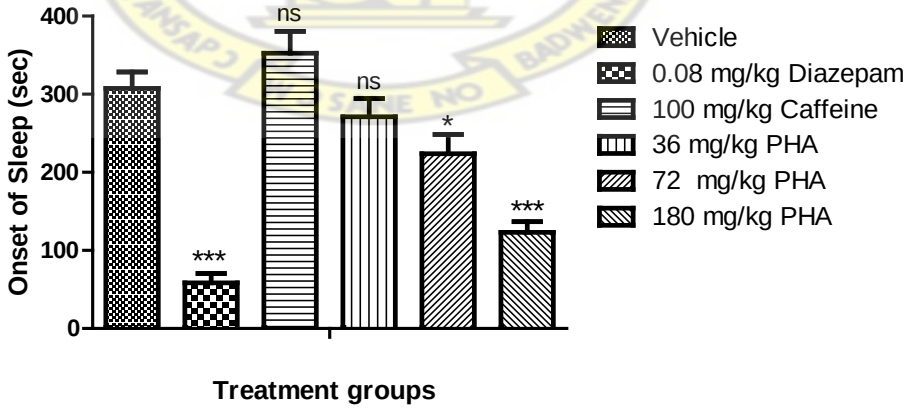

Figure 6: The effect of Diazepam, Caffeine and PHA on the onset of sleep induced by 40 mg/kg Pentobarbitone in ICR mice. For significant differences compared to the control: * implies $P \leq 0.001$, * implies $P \leq 0.05$, ns implies $P > 0.05$. n=5**

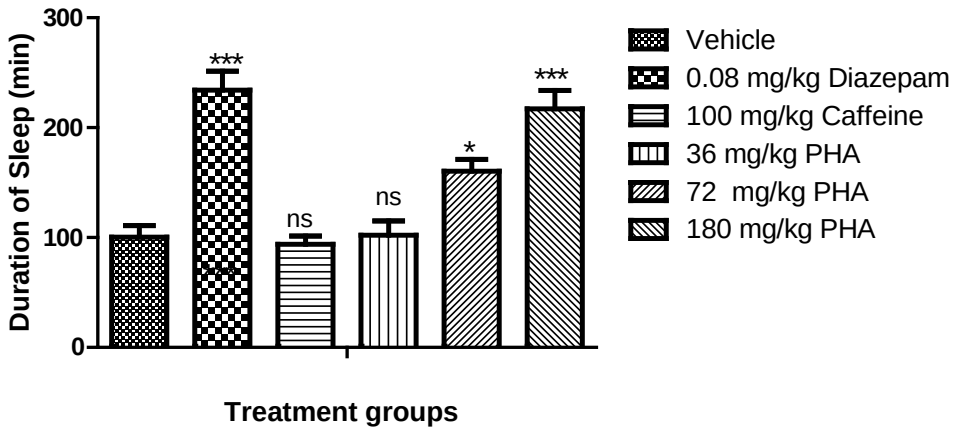

Figure 7: The effect of Diazepam, caffeine and PHA on the duration of sleep induced by 40 mg/kg pentobarbitone in ICR mice. For significant differences compared to the control: * implies $P \leq 0.001$, * implies $P \leq 0.05$, ns implies $P > 0.05$. n=5**

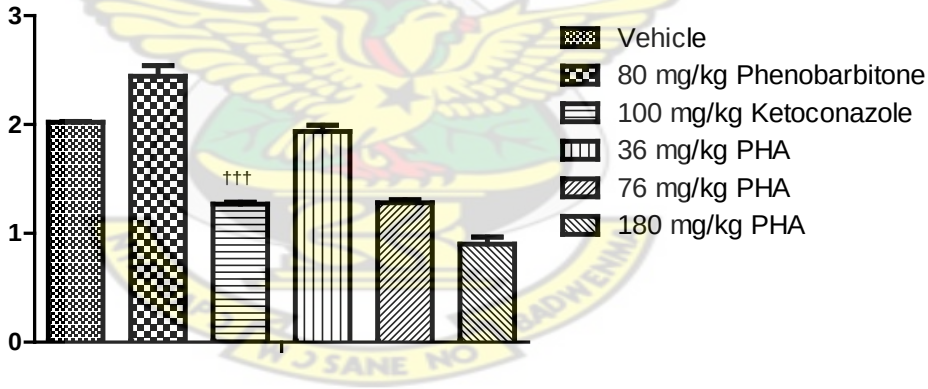

Figure 8: The effect of Phenobarbitone, Ketoconazole, and PHA on the total CYP450 of ICR mice after a two-week treatment period. For significant increments compared to the control: ** implies $P \leq 0.01$. For significant decrements compared to the control: ††† implies $P \leq 0.001$; ns implies $P > 0.05$. n=3

5.4 DISCUSSION

PHA was observed to cause sedation, lethargy and reduced activity suggesting CNS depression during the acute and delayed toxicity study. This was confirmed by the reduced locomotor activity and possible muscle relaxant effect observed in the neurobehavoiural assessment of PHA. The CNS depressant activity of PHA has been confirmed again by the fact that it decreases significantly the onset of pentobarbitone-induced sleep and prolongs the duration of sleep which can be useful in cases of hypertension, in accordance with its ethnopharmacological use. It should be pointed out that activity suggested by observation of the enhanced sleeping time may be due to interference with barbiturate enzymatic metabolism. Through secondary hepatic metabolic inactivation barbiturates will lose their affinity for the GABA receptor complex and thus lose their CNS depressant property (DeRuiter, 2004). Therefore for the CNS depressant effect to persists on concomitant administration with PHA implies that there is a possible drug interaction, hence the determination of PHA's effect on CYP450 enzyme in the liver.

The major pathways by which the action of barbiturates are terminated include the following: oxidation of substituents at C5 by CYP2C19, conjugation of the heterocyclic ring by glucosides, oxidative N-dealkylation of the nitrogen, and oxidative desulfurization of 2-thiobarbiturate to yield more hydrophilic barbiturates.

The liver is responsible for the metabolism of most compounds in the human body. These metabolic processes that do occur in the body are catalysed by liver enzymes. The principal enzyme involved in the oxidative metabolism of drugs and other xenobiotics is CYP450. Knowledge of the effect of drugs on cytochrome P450 activity is essential if we are to avoid drug interactions and improve therapeutic efficacy (Park *et al*., 1995). For this reason, the effect of the plant extract on the liver microsomal enzyme CYP450 was investigated to determine if the metabolic activity of the liver was compromised. From the experimental results it was confirmed that the extract was an enzyme inhibitor.

The very significant reduction in CYP450 levels caused by ketoconazole treatment shows that there is a significant inhibition of the enzyme in the mice. Ketoconazole is known to be a cytochrome P450 enzyme inhibitor (von Moltke, *et al*., 1996). Specifically, it inhibits the CYP3A subfamily; the most abundant cytochrome enzymes in humans which account for 30 % of the cytochrome P450 enzymes in the liver (Shimada, 1994) and are also substantially expressed (about 70 %) in the intestines. Members of this subfamily are involved in many clinically important drug interactions (Slaughter and Edwards, 1995) hence an inhibition of the enzyme has a lot of clinical implications. A similar reduction in CYP450 concentration was seen with PHA. This implies that PHA is could possibly be a CPY450 inhibitor. In the phenobarbitone, enzyme inducer, (Cupp and Tracy, 1998) treated mice there was an increase ($P \leq 0.05$) the levels of the total CYP450 enzymes.

The CYP450 enzyme inhibitory property of PHA (which was comparable to ketoconazole) will affect the liver's ability to effectively metabolize most compounds in the human body. The inhibitory property could possibly have been achieved by binding tightly to CYP450 heme iron (Katzung, 2005) hence reducing the levels of active enzymes to metabolize xenobiotics. There is therefore the possibility of drug interaction when taken with, say, anti-arhythmics (e.g. quinidine) and sulphonylurea anti-diabetics to cause high plasma concentration or prolonged duration of action leading to toxicity (BNF, 2009). Co-administration of the product with anti-HIV protease inhibitors could reduce the clearance of the protease inhibitor and resultant toxicity (Hobbs *et al.*, 1996). This herbal antihypertensive is most often taken concurrently with orthodox antihypertensives by patients. The metabolism and clearance of the orthodox antihypertensives could be reduced resulting in an extended duration of action with a possible hypotensive effect.

5.5 CONCLUSION

This study has shown that PHA shortens the onset of sleep and prolongs the duration of sleep by inhibiting CYP450 enzymes. It is therefore advised that caution should be taken when PHA is used concomitantly with orthodox antihypertensive preparations and sedative-hypnotics since it could potentiate their activity.

CHAPTER SIX

6.0 REPRODUCTIVE TOXICITY

6.1 INTRODUCTION

Reproductive toxicity is the occurrence of biological adverse effects on the reproductive systems of males and/or females that may result from exposure to drugs and chemical agents. The toxicity may be expressed as alterations to the male or female reproductive organs, the related endocrine system, or pregnancy outcomes (EPA, 1996). Medications designed to treat symptoms and cure disease can also cause unanticipated problems with the reproductive system; even in therapeutic doses. Such medication could affect reproduction in the female by decreasing libido (and hence mating), affecting ovulation, fertilization, implantation, and gestation. They could also affect the developing implant and cause still birth or could affect the health of the offspring. In the same way, such medications could affect male reproductive abilities by decreasing libido, causing impotence and hence reducing mating. Drugs made to control high blood pressure such as Clonidine, Thiazides, Spironolactone and β-blockers may cause erectile dysfunction in males. Drugs that affect the central nervous system such as monoamine oxidase inhibitors, selective Serotonin reuptake inhibitors, tricyclics, anxiolytics, alcohol, opioids and cocaine could decrease sperm quantity or quality which does not augur well for fertilization (Srilatha, *et al.*, 1999; Wegner, 2010). Some even enhance male potency but may make the males sterile. Social drugs like alcohol, tobacco and even too much caffeine could cause male infertility. Street drugs like Marijuana, Heroin and Methadone used to treat heroin addiction

can decrease sperm quantity or quality, negatively affecting fertility (Wegner, 2010).

This study has shown that PHA has CNS depressant, anxiolytic, and probably muscle relaxant activity which affects neurological behaviors. Within limits of acute and delayed toxicity, however, the product is safe to use. Sub-chronic toxicity evaluation of this polyherbal mixture suggests that it may be safe to use when used in lower doses but could potentially provoke liver and kidney damage in higher doses. It has also been observed that the product shortens the onset of sleep and prolongs the duration of sleep possibly by inhibiting CYP450 enzymes. It is therefore proposed that caution should be taken on the concomitant use of PHA and allopathic antihypertensives and/or hypno-sedatives as it could potentiate their activity. PHA needs to be studied to determine whether or not this commonly used product on the Ghanaian market has the tendency of causing reproductive toxicity in both males and females.

6.2 METHODOLOGY

6.2.1 Reproductive Toxicity in Female ICR Mice

The reproductive toxicity in female mice was carried out by a method described by Ansah *et al.,* (2010). Four groups of female ICR mice (n=10) were used in the study. Treatment group I was the control and received vehicle only. Treatment groups II, III, and IV received 36, 72, and 180 mg/kg of PHA respectively, daily for two weeks. After the two-week treatment period, the female mice were regrouped by subdividing each treatment group into two (n=5) and labeled as follows: IA, IB, IIC, IID, IIIE, IIIF, IVG, and IVH. Two male mice were introduced into each of the eight female groups. Treatment of female mice in groups IA, IIC, IIIE, and IVG was continued with vehicle, 36, 72, and 180 mg/kg PHA respectively through the gestation period and up to 21 days after parturition. Treatment with vehicle and PHA in groups IB, IID, IIIF, and IVH was discontinued just after the males were cohabited with the females. Formation of vaginal plug was taken as evidence of successful mating. The time taken to mate after cohabitation, number of mice mated, impregnation, fertility, gestation period, litter size, live births and offspring survival were observed. Reproductive indices which include Mating Index (MI), Fertility Index (FI), Live Birth Index (LBI) and Weaning Index (WI) were determined.

6.2.2 Reproductive Toxicity in Male ICR Mice

Four groups of male mice (n=5) were used in the study. Treatment group I was the control and animal received vehicle only. Treatment groups II, III, and IV received 36, 72, and 180 mg/kg of PHA (respectively) daily for 21 days. After the treatment period, the male mice were introduced into groups of female mice as described as follows. Female mice were grouped into eight (n=5) as follows: A, B, C, and D. Two male mice from group I (control) were introduced into female group A; two 36 mg/kg PHA-treated males from group II were introduced into female group B; two 72 mg/kg PHA-treated males from group III were introduced into female group C; and two 180 mg/kg PHA-treated males from group IV were introduced into female group D. Formation of vaginal plug was taken as evidence of successful mating. Time taken to mate, number of mice mated, impregnation, fertility, gestation period, litter size, live births and offspring survival were observed. Mating Index (MI), Fertility Index (FI), Live Birth Index (LBI) and Weaning Index (WI) were determined

6.2.3 Evaluation of Fertility in Male ICR Mice

6.2.3.1 Epididymal Spermatozoa Assay

For epididymal spermatozoa counts, the method described by Meistrich (1989) was used with slight modifications. Four groups of male mice (n=5) were used in the study. Group 1; the control was given received vehicle only. Groups 2, 3, and 4 received 36, 72 and 180 mg/kg respectively of PHA daily for 3 weeks. Animals from each group were then euthanized by cervical dislocation and the wet weight

of the left caudal epididymis and testis was taken and recorded to the nearest 0.1 mg. To prevent the loss of secretory fluid, the base of each seminal vesicle was grasped with forceps before removing. By mincing the caudal epididymis in 20 ml physiological saline at 37°C, a spermatozoa suspension was obtained for evaluation of semen parameters using the Ceti magnum-T/trinocular microscope for fluorescence (Medline Scientific limited, UK) under an objective lens magnification 40X as follows:

6.2.3.1.1 Spermatozoa Concentration

A drop of the spermatozoa suspension was delivered onto the counting chamber of the Improved Neubauer Haemocytometer (Depth 0.1mm, Area: 1/400 mm^2; Yancheng Cordial Lab Glassware Co. Ltd, Jiangsu, China (Mainland) and allowed to stand for 5 minutes for sedimentation, after which spermatozoa were counted from five large squares (volume: 0.5 mm^3) and spermatozoa concentration expressed as number of spermatozoa per ml.

6.2.3.1.2 Spermatozoa Motility

After introducing a drop of spermatozoa suspension onto the counting chamber of the Improved Neubauer Haemocytometer, non-motile spermatozoa numbers were first determined from five large squares, followed by a total spermatozoa count (motile and non-motile). The number of motile spermatozoa was calculated. Spermatozoa motility was estimated as: percentage of motile spermatozoa to the total spermatozoa counted.

6.2.3.1.3 Spermatozoa Viability

This technique is used to differentiate between live and dead spermatozoa. A drop of the eosin stain was added to the spermatozoa suspension and delivered onto the counting chamber of the Improved Neubauer Haemocytometer and allowed to stand for 5 minutes at 37°C. On examination under the microscope (40X), the head of dead spermatozoa were stained red while the live spermatozoa were not stained. Spermatozoa viability was estimated as: percentage of live spermatozoa to the total spermatozoa counted.

Statistical Analysis

The observations are presented as mean ± SD. Significant differences among means of the group were determined by one-way ANOVA using Graph Pad Prism for windows version 5.0 (Graph Pad Software, San Diego, CA, USA). Significant differences between pairs of groups were calculated using the Dunnet's multiple comparison test with level of significance set at $P \leq 0.05$.

6.3 RESULTS

6.3.1 Reproductive Toxicity in Female and Male ICR Mice

Data obtained indicated that PHA did not have any detrimental effect on mating behavior in both female and male mice as time taken to mate after cohabitation between PHA-treatments and that of the control were not significantly different, and mating index was always 100 % (Table 18, 19, and 20). It was also observed that there were no serious effects on fertility as the lowest fertility index (recorded

in only one treatment group) was 75 % (Table 19). Treatment of both female and male mice with PHA did not affect gestation period which was approximately 21 days. PHA did not have any effect on litter number and litter weight and all pregnant mice gave birth to live litter (live birth index was nearly 100 %) with weights similar to untreated mice (Table 19 and 20). Almost all litter in all treatment groups was alive at day 21 after birth; recording 100 % weaning index (Table 19 and 20).

6.3.2 Evaluation of Fertility in Male ICR Mice

Weights of the caudal epididymis recorded and that of the testis were not significantly different from the control (Table 21). Spermatozoa concentrations, spermatozoa motility and viability after semen analysis were also not different from that of the control group (Table 21).

Table 18: Comparison of time to mate, number of mice mated, and number of females pregnant for female pretreatment with PHA for 14 days only prior to mating, and female mice pretreated with PHA for 14 days prior to mating with continuing treatment.

Treatments	PHA Treatment	Time taken to mating (days)	№ of mice mated	№ of Pregnant females
Pretreatment for 14 days followed by mating and continued treatment during gestation	I A (control)	3.4 ± 0.83	5	4
	II C (36 mg/kg)	3.4 ± 0.89 ^{ns}	5	5
	IIIE (72 mg/kg)	3.2 ± 0.83 ^{ns}	5	4
	IVG (180 mg/kg)	3.2 ± 1.09 ^{ns}	4	3
Pre-treatment for 14 days only followed by mating	IB (control)	3.4 ± 1.14	4	4
	IID (36 mg/kg)	3.4 ± 0.89 ^{ns}	5	4
	IIIF (72 mg/kg)	3.4 ± 0.89 ^{ns}	4	4
	IVH (180 mg/kg)	3.2 ± 1.09 ^{ns}	5	4

Values quoted are Means + SD (n=5). There was no significant difference between treated groups and the control as established using one way Analysis of Variance (ANOVA). ns imply P > 0.05.

Table 19: Comparison of reproductive indices for female pretreatment with PHA for 14 days only prior to mating, and female mice pretreated with PHA for 14 days prior to mating with continuing treatment during gestation and up to 21 days after parturition.

	PHA-Treatments	MI (%)	FI (%)	GP (days)	Litter №	LBI (%)	LW (g)	WI (%)
Pretreatment for 14 days followed by mating and continuing treatment during and after gestation	I A (control)	100	80	21.0±0.32	10.4±0.51	100±0.00	1.60±0.083	100±0.00
	II C (36 mg/kg)	100	100	20.8±0.20 ^{ns}	9.2±0.58 ^{ns}	98.2±1.82 ^{ns}	1.68±0.080 ^{ns}	100±0.00 ^{ns}
	IIIE (72 mg/kg)	100	80	21.6±0.24 ^{ns}	10.2±0.58 ^{ns}	100±0.00 ^{ns}	1.63 ±0.082 ^{ns}	94.8±3.37 ^{ns}
	IVG (180 mg/kg)	100	75	21.4±0.24 ^{ns}	9.0±0.45 ^{ns}	100±0.00 ^{ns}	1.62 ±0.65 ^{ns}	100±0.00 ^{ns}
Pre-treatment for 14 days only followed by mating	IB (control)	100	100	21.4±0.40	10.0±0.7	92.6±3.25	1.62 ±0.092	96.4±3.7
	IID (36 mg/kg)	100	80	20.8±0.20 ^{ns}	9.2±0.37 ^{ns}	95.6±2.72 ^{ns}	1.64 ±0.084 ^{ns}	100±0.00 ^{ns}
	IIIF (72 mg/kg)	100	100	21.0±0.24 ^{ns}	10.0±0.78 ^{ns}	100±0.00 ^{ns}	1.67±0.087 ^{ns}	100±0.00 ^{ns}
	IVH (180 mg/kg)	100	80	20.6±0.25 ^{ns}	9.6±0.25 ^{ns}	100±0.00 ^{ns}	1.66 ±0.062 ^{ns}	100±0.00 ^{ns}

Values are Means + SD (N=5). There was no significant difference between treated groups and the control as established using one way Analysis of Variance (ANOVA). Mating Index (MI): The number of mated females/Number of females cohabited; Fertility Index (FI): Percentage mated female/Number of pregnant females; Gestation Period (GP); Live Birth Index (LBI): Number of live offspring/Number of offspring delivered; Litter weight (LW); Weaning Index (WI): Number of offspring at day 21/number of offspring delivered.

Table 20: Reproductive indices for female mice mated with male mice treated with 36, 72 and 180 mg/kg PHA for 21 days.

PHA Treatment (mg/kg)	Time taken to mating (days)	№ of mice mated	№ of Pregnant females	MI (%)	FI (%)	GP (days)	Litter №	LBI (%)	LW (g)	WI (%)
A (control)	3.2 ± 0.83	5	5	100	100	21.0±0.32	9.2±0.62	100±0.00	1.60± 0.083	100±0.00
B (36)	3.2 ± 1.09 ^{ns}	5	4	100	80	21.8±0.20 ^{ns}	10.4±0.26 ^{ns}	98.2±1.82 ^{ns}	1.68± 0.080 ^{ns}	100±0.00 ^{ns}
C (72)	3.4 ± 1.14 ^{ns}	5	5	100	100	21.6±0.24 ^{ns}	10.8±0.42 ^{ns}	100±0.00 ^{ns}	1.63 ± 0.082 ^{ns}	94.8±3.37 ^{ns}
D (180)	3.4 ± 0.89 ^{ns}	5	5	100	100	21.4±0.24 ^{ns}	10.3±0.45 ^{ns}	100±0.00 ^{ns}	1.64 ± 0.065 ^{ns}	100±0.00 ^{ns}

Values are Means + SD (N=5). Differences between treated groups and the control was established using one way Analysis of Variance (ANOVA); ns imply P > 0.05. Mating Index (MI): The number of mated females/Number of females cohabited; Fertility Index (FI): Percentage mated female/Number of pregnant females; Gestation Period (GP); Live Birth Index (LBI): Number of live offspring/Number of offspring delivered; Litter weight (LW); Weaning Index (WI): Number of offspring at day 21/number of offspring delivered.

Table 21: The Effect of 36, 72 and 180 mg/kg PHA treatment on male-specific toxicities in ICR Mice

Treatments	Paired weight of caudal epididymis (mg)	Paired weight of testis (mg)	Spermatozoa concentration (x 10^6cells/ml)	Spermatozoa motility (%)	Spermatozoa viability (%)
Control (untreated)	0.0237 ± 0.004	0.133 ± 0.048	9.22 ± 1.125	93.45 ± 2.734	96.39 ± 2.78
36 mg/kg PHA	0.0242 ± 0.002 ^{ns}	0.133 ± 0.060 ^{ns}	9.37 ± 0.677 ^{ns}	94.67 ± 2.18 ^{ns}	95.55 ± 2.59 ^{ns}
72 mg/kg PHA	0.0228 ± 0.004 ^{ns}	0.132 ± 0.021 ^{ns}	10.04 ± 0.448 ^{ns}	92.88 ± 1.96 ^{ns}	97.01 ± 3.49 ^{ns}
180 mg/kg PHA	0.0245 ± 0.004 ^{ns}	0.134 ± 0.075 ^{ns}	9.86 ± 0.829 ^{ns}	95.82 ± 3.07 ^{ns}	96.02 ± 2.88 ^{ns}

Values quoted are Means + SD (n=5). Differences between treated groups and the control was established using one way Analysis of Variance (ANOVA); ns imply P > 0.05.

6.4 DISCUSSION

Reproductive toxicity risk assessment to evaluate the potential toxicity of drugs to the human male and female reproductive systems and to developing offspring focuses on reproductive system function as it relates to sexual behavior, fertility, pregnancy outcomes, and lactating ability, and the processes that can affect those functions directly (UNECE, 2004). The endpoints that measure characteristics that are necessary for successful sexual performance and procreation are couple-mediated, female-specific, and male-specific (EPA, 1996; Gupta, 2011).

Couple-mediated endpoints are those in which both sexes can have a contributing role if both partners are exposed. These include mating rate, time to mating (time to pregnancy), pregnancy rate, delivery rate, gestation length, litter size (total and live), number of live and dead offspring (fetal death rate), offspring gender, birth weight, offspring survival, and external malformations and variations (Nikolaidis, 2011). Male-specific endpoints of reproductive toxicity include monitoring organ weights (testes, epididymides, seminal vesicles, prostate, pituitary) and sperm evaluation (sperm number count) and quality (morphology and motility) (Linder *et al.*, 1992).

Although PHA did not have any adverse effect on mating behavior in both females and males, a useful indicator of impaired reproductive function may be the length of time required for each pair to mate after the start of cohabitation (EPA, 1996). An increased interval between initiation of cohabitation and evidence of mating

suggests abnormal estrous cyclicity in the female or impaired sexual behavior in one or both partners (Bivens and Olster, 1997; Dominguez and Hull, 2010). A fertility index between 80-100 % as observed confirms the “no-effect” on mating behavior. Sexual behavior reflects complex neural, endocrine, and reproductive organ interactions and is therefore susceptible to disruption by a variety of toxic agents and pathologic conditions. Interference with sexual behavior in either sex by drugs represents a potentially significant reproductive problem which is an evidence of impaired sexual receptivity and copulatory behaviour (Jones and López, 2006; Rodrigues-Alves *et al.*, 2008).

Treatments did not affect the gestation period, however, significant shortening of gestation, can lead to adverse outcomes of pregnancy such as decreased birth weight and offspring survival (Taylor *et al.*, 1989). Significantly longer gestation may be caused by failure of the normal mechanism for parturition and may result in death or impairment of offspring if dystocia (difficulty in parturition) occurs.

PHA did not have any significant effect on litter size (number) and litter weight and live birth index which could imply that other reproductive endpoints such as ovulation rate, and fertilization rate were normal and implantation number was good (Lambert *et al.*, 1991; Vallet, 2000). Furthermore, litter size, litter weight and live birth index were not significantly affect probably indicating that there were no pre-implantation or post-implantation losses, as well as internal malformations and variations which could affect the numbers of live and dead offspring.

Birth weight measured on the day of parturition for the PHA treatments and the control were not significantly different from each other. Birth weights, are influenced by intrauterine growth rates, litter size, and gestation length (which were found to be normal) (US EPA, 1996). Individual pups in large litters tend to be smaller than pups in smaller litters. Thus, reduced birth weights attributed to large litter size was not considered an adverse effect. When prenatal or postnatal growth is impaired by an acute exposure, compensatory growth after cessation of dosing could obscure the earlier effect.

Weaning index recorded was 100 %. This could be due good postnatal structural and functional development devoid of PHA effect. Offspring survival is dependent on birth weight, sex, and normality of the individual, as well as the litter size, lactational ability of the dam, and suckling ability of the offspring (US EPA, 1996). Although all weight and survival endpoints can be affected by toxicity of an agent, either by direct effects on the offspring or indirectly through effects on the ability of the dam to support the offspring, PHA treatment had no effect.

Weights of the caudal epididymis and that of the testis recorded were not significantly different from the control. Weight is an excellent index of either the biochemical or the anatomical state of reproductive organs (WHO 2000; Fisch *et al.,* 2002; Carlsen *et al,*. 2004). The male reproductive organs for which weights may be useful for reproductive risk assessment include the testes, epididymides,

seminal vesicles (with coagulating glands), and prostate. Reproductive organ size (testes and seminal vesicles) occupies a place of special importance among morphological measures because of its direct implication in fertility (Ansah *et al.*, 2010). Reproductive organ sizes are markers of the timing of puberty (Argyropoulos and Shire 1989) and testicular weight is connected total sperm count in mice (Krzanowska 1971; Hunt and Mittwoch 1987; Chubb 1992). This relatively low interanimal variability suggests that absolute testis weight should be a precise indicator of gonadal injury.

Sperm concentrations, sperm motility and viability after semen analysis were also not different from that of the control group. Although effects on sperm production can be reflected in other measures such as testicular spermatid count or cauda epididymal weight, no surrogate measures are adequate to reflect effects on sperm morphology or motility. The ability to detect a decrease in testicular sperm production may be enhanced if spermatid counts are available. Therefore, the conservative approach should be taken that, within the limits indicated in the sections on those parameters, statistically significant changes in measures of sperm count, morphology, or motility as well as number of normal sperm should be considered adverse effects.

6.5 CONCLUSION

The endpoints that measure characteristics that are necessary for successful sexual performance and procreation were not adversely affected with the use of the product under study, therefore, as far as reproductive toxicity is concerned, the polyherbal antihypertensive mixture is safe to use within limits of the doses administered in this study.

CHAPTER SEVEN

7.0 GENERAL DISCUSSION

Many herbs (herbal medicines) have been used for a long time for claimed health benefits. They contain phytochemicals as their pharmacologically active components (Pribitkin, 2005), and are sometimes used as dietary supplements to improve health. They are sold as tablets, capsules, powders, teas, extracts and fresh or dried plants. Plants and derivatives of plants play a key role in world health and have long been known to possess biological activity; it has been documented that 30 % of allopathic drugs are derived from plants (Burns, 2000). According to the World Health Organization about 80% of the world's population living in developing countries rely essentially on plants for primary health care (McKay and Blumberg; 2007).

Herbal medicines have been extensively used in developed countries with the claim that they are natural and relatively safe compared to other forms of medicine (Gurib-Fakim; 2006). Although the overall incidence of adverse effects from herbal products appears to be low compared to those associated with the synthetic forms, harm from herbal products can still occur because of the inherent toxicity of the plant, as well as from contamination, adulteration, plant misidentification, and interactions with other herbal products or pharmaceuticals. Some may interact with other drugs being taking when used over a longer period of time such as when used in treating chronic diseases. Though, not much could be said about Ghana in

relation to toxicity of herbal preparation (probably because of poor documentation), no one has can rule out the fact that there are a number of herbal preparations whose toxicity evaluation have not been well-documented therefore are likely to cause serious adverse effects on the health and reproduction of individuals. This study has not only assessed the toxicity profile of the polyherbal antihypertensive sold and used in Ghana but has also provided a scientific protocol for assessment of the safety for use of other herbal preparations by the Food and Drugs Board in Ghana prior to acceptance and registration.

An antihypertensive herbal product was selected because hypertension has been identified as the third ranked factor for disability-adjusted life years (WHO report, 2002). Hypertension is one of the primary risk factors for heart disease and stroke, the leading causes of death worldwide (Chockalingam *et al.*, 2006). It has a worldwide prevalence of an estimated 600 million people (WHO report, 2002), causes 5 million premature deaths each year worldwide and 13 % of global fatalities (WHO report, 2002). In US alone, 7.5 million people above 20 years are hypertensive. Every one in three adults has hypertension with the cause of 90-95 % of hypertension cases not known (American Heart Association, 2010).

According to a medical news summary released on the 14th of January 2005, scientists are now claiming that 1 in 3 adults in the world will have high blood pressure by 2025 (News Scotsman, 2005). The increase amounts to a 60 % increase which gives a total of 1.56 billion high blood pressure sufferers by 2025.

The biggest increase in prevalence was expected to be in developing (increase of 24 %) and third world countries (increase of 80 %) (WHO report, 2002).

In the African region, it is estimated that more than 20 million people are affected, mainly in the urban areas. Prevalence ranges from 25-35 % in adults aged 25-64 years, and its prevention and control could avoid at least 250,000 deaths in Africa per year (WHO report, 2002).

Observations made after acute and delayed toxicity studies indicated no deaths up to a dose of 550 mg/kg (ten times the daily dose taken as a single dose) of the polyherbal antihypertensive. No death recorded implies that the product was not lethal up to dosage stated hence the LD_{50} is above this dose. This does not mean that the product is safe to use. Besides, calculating lethality parameters using large numbers of animals, as was done previously, is no longer recommended. Replacement with fixed dose procedure which uses fewer animals and cause less suffering is now preferred (CFHS, 2000; FRAME, 2006). Toxicity, by its definition, is the state or quality of the drug/product being poisonous or capable of causing harm to exposed humans or animals or the degree to which a substance is poisonous or can cause harm to exposed humans or animals (Schimelpfening, 2010). Toxicity can be the effect on a whole animal, as well as the effect on a substructure of the animal, such as a cell (cytotoxicity) or an organ (organ toxicity), such as the liver (hepatotoxicity), or a system (e.g. reproductive toxicity) which may be seen as clinical and behavioural changes. This prompted the cage-

side observation for clinical signs and symptoms and behavioural changes, study of the liver and kidney function, as well as hematological and urine analysis after a single exposure of high doses of the product from the time of exposure up to 14 days post exposure.

Clinical symptoms such as lacrimation, salivation, urination labored breathing, constipation, emaciation, skin eruptions, abnormal posture, hemorrhage, sedation, diarrhoea, polyuria, polydipsia, polyphagia, anorexia, rhinorrhoea/nasal congestion, loss of autonomic reflexes, neuromuscular inco-ordination and collapse, hyperesthesia, hypothermia, twitching, spasticity, convulsion, writhing, tremors, fasciculations and respiratory depression are often seen with acute and delayed toxicity. Other observations include staggering, wobbly gait, hind limbs exaggerated, overcompensating, and/or splayed movements, feet (primarily hind feet) point outward from body, forelimbs dragging and/or showing abnormal positioning, nor walking on toes (the heels of the hind feet are perpendicular to the surface) (Vashishtha, 2009).

Some herbal product can cause massive hemolysis soon after they have been administered resulting in a low RBC counts and an increased plasma and urine bilirubin and urobilinogen levels.

Many drugs are implicated in the development of fulminant hepatic and renal failure. Idiosyncratic drug reactions may occur with virtually any medication.

Acetaminophen (also known as paracetamol and *N* -acetyl-p-aminophenol) may lead to liver failure as a result of intentional or accidental overdose. In the US Acute Liver Failure (ALF) study, unintentional acetaminophen use accounted for 48% of cases, whereas 44% of cases were due to intentional use; in 8% of cases, the intention was unknown (Sood and Katz, 2011). Some herbal or alternative medicines, after acute exposure, have also been associated with idiosyncratic reactions resulting in liver damage and renal impairment (Vanderperren *et al.*, 2005; Luyckx and Naicker, 2008).

Urine analysis in toxicology is usually necessary to detect the presence of unusual substances in urine which may be indicators of acute systemic toxicity. Substances from destruction of blood, liver and kidneys, as well as toxicity which can result in metabolic disorders can be detected in urine.

Results from clinical observation, hematological analysis, liver and kidney function tests, and urine analysis did not show any detrimental effect of PHA on experimental animal. However there were behavioural changes seen as sedation, lethargy and reduced activity observed one hour post-treatment. This could be as a result of CNS depression and/or muscle relaxation. This prompted the investigation into the neurobehavioural effect of PHA and chronic toxicity studies in experimental animals.

Behavior test for toxicity are designed to demonstrate effects on behavior of animals; the quantitative evaluation of activity and specifically locomotor types of

activity of experimental animals. Neurobehavioral evaluations are an important component of testing for the neurotoxic potential of chemicals.

The very significant dose-dependent decreases (Figure 3) in locomotor activity, rearing, and centering and the significant reduction in motor coordination and muscular strength in the neurobehavioural assessment confirms CNS depression and/or muscle relaxation effect. Locomotor activity is considered as an index of alertness and a decrease in it is indicative of sedative activity (Lowry *et al.*, 2005).

CNS depression is considered as an affective disorder characterized by change in mood, lack of interest in the surroundings, apathy, loss of energy, and psychomotor retardation (Hoskeri *et al.*, 2011). The CNS depressant activity may be due to the increase in the concentration of GABA in brains (Nagarjun *et al.*, 2003). Since the neurobehavioural effect observed was similar to that observed in the diazepam-treated group it could be speculated that PHA could be acting by; activating inhibitory $GABA_A$, inhibiting excitatory AMPA receptor**s,** inhibiting the NMDA glutamate receptor (inhibits an excitatory effect), and/ or potentiating the action of serotonin (5-HT) at excitatory 5-HT_3 receptors (Hobbs *et al.*, 1996). Serotonin (5-HT) potentiates the inhibitory effect of dopamine on dopaminergic ventral tegmental area (VTA) neurons (Brodie and Bunney, 1996). Since these receptors are often localized on inhibitory interneurons, enhanced activation results in CNS inhibitory effects. It could dissolves into lipid membranes affecting the function of membrane proteins, such as receptors and ion channels.

The $GABA_A$ receptor is an inhibitory channel which when activated decreases neuronal activity and enhances the effects of GABA (Atack, 2005). When GABA binds to its site on the $GABA_A$ receptor more chloride ions enter and cause hyperpolarization of the post-synaptic neuron resulting in an enhanced central nervous system depressant effect (Riss *et al*, 2008; Barondes, 1999).

∝-*Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid* (AMPA) receptors are the main mediators of fast excitatory neurotransmission in the CNS (Dingledine *et al.*, 1999). Inhibiting these excitatory AMPA receptors therefore results in CNS depression. The NMDA receptor is an ionotropic receptor that allows for the transfer of electrical signals between neurons in the brain and in the spinal column. For electrical signals to pass, the NMDA receptor must be activated and to remain activated, an NMDA receptor must bind to glutamate and to glycine. Chemicals that deactivate the NMDA receptor would inhibit excitatory effects resulting in CNS depression.

Subcronic toxicity studies reveal the potential of PHA being hepatotoxic and nephrotoxic. The liver as organ plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. It produces bile, an alkaline compound which aids in digestion via the emulsification of lipids. The liver's highly specialized tissues regulate a wide variety of high-volume biochemical reactions, including the synthesis and breakdown of small and

complex molecules, many of which are necessary for normal vital functions (Maton *et al.*, 1993). Drugs are rendered more hydrophilic by biochemical processes in the hepatocyte, yielding water-soluble products that are excreted in urine or bile (Weinshilboum, 2003) This hepatic biotransformation involves oxidative pathways, primarily by way of the cytochrome P-450 enzyme system (Guengerich, 2001). After further metabolic steps, which usually include conjugation to a glucuronide or a sulfate or glutathione, the hydrophilic product is exported into plasma or bile by transport proteins located on the hepatocyte membrane, and it is subsequently excreted by the kidney or the gastrointestinal tract (Lee, 2003). A drug being hepatotoxic will not only cause new disorders or affect drug metabolism but can potentially cause drug interaction (Jaeschke *et al.*, 2002).

The kidneys excrete a variety of waste products produced by metabolism. It participates in whole-body homeostasis, regulating acid-base balance, electrolyte concentrations, extracellular fluid volume, and regulation of blood pressure. The kidney helps regulate blood pressure by producing an enzyme called renin. When blood pressure falls below normal levels, the kidneys secrete renin into the bloodstream, thereby activating the renin-angiotensin-aldosterone system, which in turn raises blood pressure. A person with kidney failure is less able to regulate blood pressure and tends to have high blood pressure. If renal perfusion is adversely affected however then chronic use (as in long term therapy in hypertension) could produce paradoxical hypertension.

Formation of urine is also the function of the kidney. For organic substances, active transport across the renal proximal tubule followed by elimination via the urine is a major pathway in this detoxification process (Anzai and Endou, 2007). Recently, a large number of drug transport proteins belonging to several different gene families have been identified and have found to be expressed in renal proximal tubules (Anzai and Endou, 2007). These transporters, in combination with relatively high renal blood flow, predispose the kidney to increased toxic susceptibility. Understanding of the molecular mechanism of renal drug transport is essential to achieve desired therapeutic outcomes in response to drug interactions (Anzai and Endou, 2007). A drug being nephrotoxic therefore will not only cause disorders in the body but could also affect drug elimination which could cause drug interaction (Dugo *et al.*, 2010).

Drug interactions occur when the efficacy or toxicity of a medication is changed by administration of another substance. Pharmacokinetic interactions often occur as a result of a change in drug metabolism (Dresser *et al.*, 2000). PHA was found to inhibit CYP450 enzymes. In Ghana, majority of patients taking herbal medicines in the management of hypertension are also on some orthodox medicines. Although most herbal medical practitioners claim safety with the concomitant use of both herbal and orthodox medicines, the possibility of drug interaction cannot be ruled out. For example QT prolongation can occur when an inhibitor is coadministered with terfenadine, astemizole, cisapride or pimozide.

Rhabdomyolysis has been associated with the coadministration of some 'statins' and CYP3A4 inhibitors. Symptomatic hypotension may occur when CYP3A4 inhibitors are given with some dihydropyridine calcium antagonists, as well with the phosphodiesterase inhibitor sildenafil. Excessive sedation can result from concomitant administration of benzodiazepine or nonbenzodiazepine hypnosedatives with CYP3A4 inhibitors. Ataxia can occur with carbamazepine, and ergotism with ergotamine, following the addition of a CYP3A4 inhibitor (Dresser *et al.,* 2000). These examples given therefore suggest that PHA may not be safe on concomitant administration with allopathic medicines and other herbal remedies.

PHA is however safe on the reproductive system. It implies that it did not; cause alterations in sexual behavior, decreases in fertility, or loss of the fetus during pregnancy. Therefore, in the female it may not change sexual behavior, onset of puberty, cyclicity, fertility, gestation time, pregnancy outcome, and lactation: all can disrupt a woman's ability to successfully reproduce. Exposure to lead, for example, can result in menstrual disorders and infertility. In males, use of PHA will not affect sperm count or shape, alter sexual behavior, and/or increase infertility.

7. 1 CONCLUSION

- Acute toxicity studies indicated that PHA is not lethal up to a dose of 550 mg/kg but in treated mice it shows signs of sedation and CNS depression.

- Sub-chronic toxicity however revealed that PHA may be safe to use in lower doses but may cause fibrosis and cirrhosis of the liver and glomerular inflammation in the kidney when used in higher doses. Monitoring of the liver and kidney may therefore be required with the use of this product.
- The No-Observable-Adverse-Effect-Level (NOAEL) is 32 mg/kg/day.
- After neurobehavioral assessment it was established that PHA has CNS depressant, anxiolytic, and probably muscle relaxant activity which affects neurological behaviours. These effects are however desirable in antihypertensive therapy.
- Studying the potential for drug interaction, PHA was found to inhibit CYP450 enzymes. It shortens the onset and prolongs the duration of sleep. It is advised that caution should be taken when the antihypertensive product is used concomitantly with orthodox antihypertensive preparations and sedative-hypnotics since it could potentiate their activity.
- As far as reproductive toxicity is concerned, PHA is safe to use within limits of the doses administered in this study.

7.2 RECOMMENDATIONS

A chronic toxicity study on PHA is recommended for further identification of the hazardous potential like carcinogenicity and mutagenicity.

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APPENDIX

VALUES OBTAINED IN THE NEUROBEHAVIOURAL TESTS

Table 22: Effect of 0.08 mg/kg Diazepam on motion, rearing and centering in ICR mice in an open field observation 1 hour and 24 hours post-treatment

	Control	1 h post-treatment	24 h post treatment
Motion	153.3 ± 22.80	60.1 ± 12.65 ***	124.8 ± 15.5 ns
Rearing	36.1 ± 9.40	2.3 ± 1.34 ***	25.3 ± 12.8 ns
Centering	7.4 ± 1.90	2.2 ± 0.92 ***	5.3 ± 3.1 ns

***** implies $P \leq 0.001$; ns implies $P > 0.05$. n=10**

Table 23: Effect of Caffeine (100 mg/kg) on motion, rearing and centering in ICR mice in an open field observation 1 hour and 24 hours post-treatment

	Control	1 h post-treatment	24 h post treatment
Motion	124.8 ± 15.5	153.3 ± 15.5 *	159.9 ± 25.9 ns
Rearing	25.3 ± 12.8	36.1 ± 9.4 *	37.9 ± 9.9 ns
Centering	5.3 ± 3.1	7.4 ± 1.9 ns	9.0 ± 3.2 ns

*** implies $P \leq 0.05$; ns implies $P > 0.05$.**

Table 24: Effect of 55 mg/kg and 550 mg/kg of PHA on motion, rearing and centering in ICR mice in an open field observation 1 hour and 24 hours post-treatment

		55 mg/kg PHA		550 mg/kg PHA	
	Control	1 h	24 h	1 h	24 h
Motion	155.6 ± 30.9	62.6 ± 11.0 ***	135.0 ± 8.3 ns	37.5 ± 28.0 ***	135.5 ± 14.7 ns
Rearing	41.6 ± 7.6	11.3 ± 8.8 ***	34.8 ± 9.5 ns	5.0 ± 4.0 ***	37.5 ± 7.1 ns
Centering	6.0 ± 1.16	3.0 ± 0.82 *	6.3 ± 1.3 ns	1.3 ± 5.0 ***	5.5 ± 1.9 ns

***** implies $P \leq 0.001$; * implies $P \leq 0.001$; ns implies $P > 0.05$. n=10**

Table 25: The effect of Diazepam and PHA on motor coordination and muscular strength in the rotarod test

	1 h post-treatment	24 h post treatment
Control	837.3 ± 58.1	860.3 ± 63.3
Diazepam 0.08 mg/kg	217.0 ± 8.19 ***	855.0 ± 11.14 ns
Diazepam 0.16 mg/kg	73.7 ± 5.13 ***	844.0 ± 41.9 ns
PHA 55 mg/kg	731.0 ± 18.7 *	836.7 ± 41.5 ns
PHA 550 mg/kg	349.7 ± 38.1 ***	853.3 ± 33.9 ns

Values are means ± SD * implies $P \leq 0.001$, * implies $P \leq 0.05$, ns implies $P > 0.05$; n=3**

Table 26: The effect of Diazepam and PHA on motor coordination and muscular strength in the grip strength test

	1 h post-treatment	24 h post treatment
Control	13.6 ± 2.1	14.4 ± 2.1
Diazepam 0.08 mg/kg	35.0 ± 8.6 **	15.2 ± 1.9 ns
Diazepam 0.16 mg/kg	57.6 ± 9.1 ***	22.0 ± 3.0 ns
PHA 55 mg/kg	37.0 ± 4.7 **	15.2 ± 1.9 ns
PHA 550 mg/kg	55.6 ± 4.7 ***	14.2 ± 2.9 ns

Values are means ± SD. * implies $P \leq 0.001$, ** implies $P \leq 0.01$, ns implies $P > 0.05$; N=5**

VALUES OBTAINED IN THE CYTOCHROME P450 ASSAY

Table 27: The effect of PHA, Phenobarbitone and Ketoconazole on the total CYP450 of ICR mice after a two-week treatment period.

	Control	76 mg/kg PHA	180 mg/kg PHA	80 mg/kg Ketoconazole	100 mg/kg Phenobarbitone
Mean	2.02 ± 0.01	1.28 ± 0.045†††	0.90 ± 0.12†††	1.27 ± 0.034 †††	2.44 ± 0.17**

For significant increments compared to the control: ** implies $P \leq 0.01$. For significant decrements compared to the control: ††† implies $P \leq 0.001$

VALUES OBTAINED FOR PENTOBARBITONE-INDUCED SLEEPING TIME

Table 28: The effects of Caffeine, Diazepam and PHA on Pentobarbitone-induced sleeping time in ICR mice

Treatments	Onset of Sleep (sec)	Duration of Sleep (min)
Vehicle	307.6 ± 46.6	100.4 ± 23.6
0.08 mg/kg Diazepam	58.6 ± 26.6 ***	234.2 ± 38.6 ***
100 mg/kg Caffeine	352.6 ± 62.2	93.8 ± 16.7
36 mg/kg PHA	271.2 ± 52.3	102.2 ± 29.2
72 mg/kg PHA	223.8 ± 55.2 *	160.4 ± 23.8 *
180 mg/kg PHA	123.0 ± 31.2 ***	217.2 ± 37.3 ***

Values are means ± SD (n=5). For significant differences compared to the control: * implies $P \leq 0.001$; * implies $P \leq 0.05$.**