

BIOCHEMICAL TOXICOLOGY OF *DESMODIUM ADSCENDENS*

A THESIS SUBMITTED TO THE

**DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY
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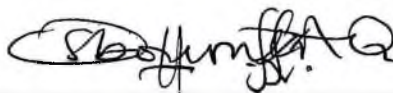
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DECLARATION

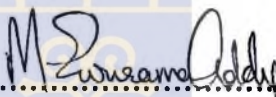
The experimental work described in this thesis was performed by me at the Department of Biochemistry and the Noguchi Memorial Institute for Medical Research (NMIMR) both of the University of Ghana, Legon, and the University of Ghana Medical School Animal House, Korle-Bu under the supervision of Prof. Marian Ewurama Addy, Dr. Laud K.N. Okine and Prof. Alexander K. Nyarko.

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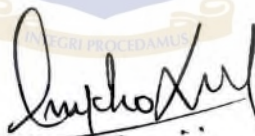
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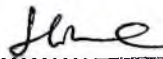
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DEDICATION

To:

God, for all His unspeakable gifts

My parents: Mr. Alfred A. Quaye and Madam
Harriet L. Baddoo.

My sisters and brothers: Winifred, Genevieve, Grace,
Scherazade, Alfred, Frederick and
Horace.

All my friends and loved ones.



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ABSTRACT

Aqueous crude extract of *Desmodium adscendens*, as dispensed at the Centre for Scientific Research into Plant Medicine (CSRPM), was freeze-dried into a powder and used in this study to evaluate its toxicological effects in rats.

For acute toxicity studies, high doses of the freeze-dried extract, were administered orally as single doses to rats, and the behaviours of the animals and organ toxicity were noted. Low, medium and high doses of the plant extract, representing 3, 10, and 30% of the dose that caused 50% lethality over the period of observation were administered to another group of rats in subchronic toxicity studies, and certain biochemical parameters measured as indices of liver and kidney toxicity.

In order to investigate possible drug-drug interaction with other drugs, the effects of the plant material on zoxazolamine paralysis time, thiopentone sleeping time and the induction/inhibition of isozymes of the cytochrome P450 (CYP) enzyme systems were studied.

From the results of the acute toxicity studies, the dose of the plant extract that caused 50% lethality was estimated to be 1125mg/kg body weight and this is about 456 times the prescribed dose in humans. When the extract was chronically administered, no significant differences were observed in levels of γ -glutamyltransferase activity, total protein, total bilirubin, creatinine and blood urea nitrogen concentrations in sera of test and control animals, irrespective of the doses of the extract administered. There were however increases in alanine and aspartate aminotransferase activities, and direct bilirubin concentration with increasing dose levels of the plant extract. Suggesting a

possible hepatocellular damage, and hepatic excretory dysfunction or increased RBC haemolysis respectively.

The plant extract caused a decrease in zoxazolamine paralysis time and prevented thiopentone from causing sleep in test animals as compared to controls. Results of the studies on the hepatic microsomal CYP isozymes suggest that CYP2B1/2B2 are involved in the metabolism of zoxazolamine and thiopentone.

This study has indicated that the doses that caused lethality are much higher than the effective therapeutic dose of the extract of *D. adscendens*. Chronic administration also showed minimal signs of hepatotoxicity at doses 15-150 times higher than the effective therapeutic dose, suggesting that the plant extract may be safe at the therapeutic dose.

Thus although *D. adscendens* may be safe, its induction of CYP2B1/2B2 and inhibition of CYP2E suggest that when the plant extract is co-administered with other drugs, may lead to possible drug interaction which may cause increase in /loss of therapeutic effectiveness or toxicity of the co-administered drug.

CHAPTER ONE

INTRODUCTION

All over the world, medicinal plants or herbal preparations have been used for the treatment of various diseases before the advent of Western medicine. It is therefore not surprising that a number of drugs in use were developed from plant products (Ayensu, 1978).

Eighty percent of the world's population relies entirely on local medicinal preparations made almost exclusively from plants (Lewington, 1990). In Ghana, majority of the population use herbal medicine because they have little or no access to Western or orthodox medicine whereas the medicinal plants are easily accessible. In areas where the people have access to Western medicine, herbal medicine is also used for other reasons such as shortage of imported allopathic drugs and relatively higher cost of treatment with these drugs compared to the medicinal plants, which are found in abundance at virtually no cost. Due to these reasons, plants will continue to be the source of medical care for the majority of the world's poor particularly those in the tropical areas where two-thirds of all the world's plant species exist (Principe, 1989).

Claims of many traditional healers such as herbalist, fetish priests and other "specialists", until recently, have been dismissed by Western medical practitioners due to the fact that traditional medicinal techniques and remedies have frequently not lent themselves to scientific verification. However, such healers more often than not have good knowledge of the efficacies of many plants in addition to being able to identify them. In spite of the rather negative or indifferent attitudes of some medical practitioners towards the use of traditional herbal preparations for medicinal purposes, many countries like Ghana have held on to their folk medicine, especially when a

number of them have been proven to have curative properties and used in the management of several diseases including those declared incurable by Western medicine (Abbiw, 1990; Lewington, 1990).

Quite a large number of plant species and their derivatives are used in different parts of the world for the treatment of diseases. It is estimated that between 35,000 and 70,000 different species of plants have been used as medicines by various peoples of the world and at least some 7,000 different plant-derived medicinal compounds have been introduced into Western pharmacopoeia. Out of these, only about 120 plant-based drugs coming from 95 plant species are prescribed for use worldwide (Lewington, 1990; Principe, 1989). In Africa, use of medicinal plants is the first health care system, and it is available for more than 70% of the population, majority of whom are in poor rural areas.

Approximately 5,000 species only out of about 250,000 flowering plants have had their therapeutic potentials tested and very few out of these have been acknowledged by Western Practitioners to really have therapeutic value. Research institutions like the Center for Scientific Research into Plant Medicine in Ghana are now making use of the knowledge of the traditional healers to develop medicinal plant preparations. The research institutions are identifying local substitutes for imported drugs, testing the therapeutic potentials of plants as pharmaceuticals and seeking to find cures for diseases including those against which Western medicine has so far proved ineffective.

Some reports received by the World Health Organization (WHO) have stated that traditional healers form the basic core of primary health care workers in rural populations in developing countries and that Western and traditional medicine should complement each other. WHO has therefore taken steps to encourage the utilization

of the resources and stressed the role that traditional medicine can play in strengthening the health care system through the Primary Health Care (PHC) in developing countries, especially those found in the tropics. The WHO has been attempting to incorporate traditional medicine into the health care delivery systems of developing countries encouraging traditional healers to receive more official training on one hand, and nurses and doctors in these countries to study the work of the traditional healers and to analyze the properties of the herbs they use on another hand. It has generally been accepted that a fully conversant knowledge of herbal medicine could substantially contribute to providing an acceptable level of health care to the world by the turn of the 21st century (Ayensu, 1978). Prior to the WHO's initial efforts to promote the investigation and development of traditional medicine, several developing countries were taking the necessary steps to begin the integration of herbal medicine as a useful and major part of their medical programmes.

In Africa, the application of herbs for internal and external uses has always been a major factor in the practice of medicine. Some of the applications include the treatment of wounds with concoctions prepared from leaves, bark and roots due to the disinfectant property of certain plant parts, use of alkaloid containing plant families for their healing qualities, and the use of other plant species as anti-parasitic, antibiotic, anti-cancer, diuretic and anti-inflammatory compounds among other uses. Some of the diseases treated with medicinal plants or herbal preparations include colds and fevers, coughs, bronchitis, pains and anxieties, hypo- and hypertension, malaria, psychiatric diseases, menstrual tension and menopausal problems (Lewington, 1990).

The mode of action and the metabolic pathway for eliminating most of these medicinal plant preparations are not known and this has in recent times given rise to

many research activities into plant medicine. Many of these plant preparations are administered in their crude forms as plant extracts but that does not preclude possible toxic effects. There is therefore the need to evaluate potential toxic effects in the usage of medicinal plant preparations as “drugs”, especially when a lot of them are dispensed without monitoring and standardization.

A drug is essentially any molecule that alters a living system’s function through interaction at the molecular level (Craig and Stitzel, 1994). Introduction of drugs into a living system entails absorption and distribution within the system and then subsequent excretion (clearance). The efficacy of an administered drug is largely dependent on its availability, which is determined by the absorption, distribution, and excretion characteristics and the route of administration. Bioavailability is the measure of the rate and completeness of absorption from the site of drug administration and it is affected by individual differences such as age, sex, state of health and environmental factors.

Drugs may elicit their pharmacological effects as administered (unchanged) or may require biotransformation to a more (or less) active metabolite. Metabolic activation of pro-drugs (drugs that have to be activated before their effect is elicited) may give rise to beneficial effects or result in reactive intermediates that can cause tissue damage. The effect of a drug or its metabolite may be due to inhibition or activation of certain enzymes or their isozymes. This is usually found in interaction of drugs where the effect of a drug is increased or decreased due to the effect of another drug present.

Drug metabolism is the major mechanism by which drug action is terminated (Craig and Stitzel, 1994). Termination of drug action is the results of biotransformation of the drug to a product of reduced pharmacological activity with

subsequent excretion by the kidneys. Almost all drugs that undergo metabolic biotransformation are converted to products (metabolites) that are more polar than the original compounds. The increase in polarity generally results in a more rapid rate of excretion in addition to the reduction in pharmacological activity of the drugs. Biological systems are equipped within limits for drug homeostasis and therefore administered drugs must be eliminated at some point from the body to forestall their accumulation and subsequent toxicity.

The efficacy of medicinal plant preparations depends on their active chemical constituents and pharmacological effects. Many of these plants may contain only traces of the medicinal elements; thus large doses of the preparations have to be administered. Systematic screening of plant preparations is a means of finding their efficacy. An index for assessing the efficacy of a drug or a medicinal plant preparation is the median effective dose (ED_{50}), which is the dose at which 50% of individual organisms exhibit the specified effect. The extreme of toxicity resulting in death is assessed by the median lethal dose (LD_{50}). These indices provide a convenient way of comparing the potencies and toxicities of drugs and medicinal plant preparations in both experimental and clinical settings.

The biotransformation of drugs, which are foreign compounds or xenobiotics, involves enzymes. Metabolites have been proposed as specific biochemical indicators of xenobiotic exposure (Payne *et al.*, 1987; Payne, 1976). The enzymes that are induced are distinctly different from those that participate in carbohydrate, protein and lipid metabolism. Identification of the induced enzymes can be used to establish the pathway through which a drug is metabolized. Several biochemical assay systems have been promising in the identification of the metabolic pathway of xenobiotics

including those dependent on elevated levels of enzymes found in Phases I and II elimination reactions (Olsson, 1987; Stegeman, 1981).

Biotransformation of drugs occurs via Phase I and Phase II (conjugation) reactions. The Phase I reactions are catalyzed by the cytochrome P-450 monooxygenase enzyme system which is found in the endoplasmic reticulum. The same monooxygenase enzyme system in tissues other than the liver is used to metabolize endogenous substrates such as arachidonic acid, fatty acids and steroids. The Phase II reactions are catalyzed by transferases that are found in both ER and in the cytosol. Administration of various substances lead to the induction of different isozymes or forms of the Phases I and II metabolizing enzymes; having different molecular weights and exhibiting varying substrate specificities as well as immunochemical and spectral characteristics (Katzung, 1989). Generally, pollutants induce CYP1A1 monooxygenase enzyme system and drugs induce CYP3A4 enzyme system (Gentest Corporation catalog, 2000).

The xenobiotic-metabolizing enzymes are found in large quantities in the liver, making it the dominant organ in drug metabolism (Craig and Stitzel, 1994). Induction results in increased concentrations of the metabolizing enzymes. Therefore elevated concentrations or activities of the hepatic enzymes involved in biotransformation of drugs, and other toxicity studies, such as acute, chronic and subchronic toxicity studies and liver and kidney function tests, can be used to determine toxicological effects of medicinal herbal preparation that are used therapeutically.

Desmodium adscendens is claimed to have several uses as a medicinal plant (Dokosi, 1998; Ayensu, 1978). An extract of *D. adscendens* has been shown to have anti-anaphylactic property both *in vivo* and *in vitro* (Addy and Dzandu, 1986; Addy and Awumey, 1984) and to modulate arachdonic acid metabolism (Addy and

Schwartzman, 1995; 1992; Addy and Burka, 1989; 1988). Brookman-Amissah (1994) also showed that the extract non-competitively inhibits hepatic cytochrome P450 monooxygenase enzyme system *in vitro*. Some work done on *D. adscendens* shows that the plant extract increases cytochrome P-450 concentration (Nanabenyin, 1997) and NADPH-dependent cytochrome P450 reductase activity (Afflu, 1997) in mice hepatic microsomes when administered *in vivo*. The extract could also presumably cause an increase in the concentration and activity of mammalian hepatic transferases, which catalyze the Phase II reaction of the metabolic biotransformation of drugs.

In view of the medicinal uses of *D. adscendens* coupled with the fact that its metabolism is not fully determined, this study was aimed at finding the isozymes of the drug-metabolizing enzymes that may be induced/inhibited by the plant extract and to show whether or not the extract could have toxicological effects, which may be reflected as altered liver and kidney functions.

The specific objectives of the study were to determine:

- (i) the median lethal dose (LD₅₀) in acute toxicity studies.
- (ii) the effects of the extract on liver and kidney function after subchronic administration
- (iii) concentrations and activities of the drug-metabolizing enzymes induced/inhibited after the subchronic treatment with the plant extract.
- (iv) whether the observations in (iii) may affect the metabolism and hence the metabolic effects of other ingested compounds (drug interactions).

CHAPTER TWO

LITERATURE REVIEW

2.1 Toxicology

2.1.1 Introduction to toxicology

It is the right dose of a substance that differentiates a poison from a remedy. Since all chemicals can produce injury or death when under exposed conditions, it is evident that there is no such thing as a completely "safe" chemical. Toxicology is the study of the adverse effects of chemical agents on biological systems or living organisms. There is a wide spectrum of doses needed to produce deleterious effects, serious injury, or death. Some chemicals will produce death in microgram doses and are commonly thought of, as being extremely poisonous or toxic, while others may be relatively harmless following doses in excess of several grams.

Categories of toxicity have been devised on the basis of the wide variation in the dosage of chemicals needed to produce death. Table 2a illustrates an example of such classification, which provides a toxicity rating or class based on the probable lethal oral dose for humans (Klaassen *et al.*, 1986).

Table 2a: Toxicity Rating chart

<u>Toxicity rating</u>	<u>Dosage</u>
Practically nontoxic	>15g/kg
Slightly toxic	5-15g/kg
Moderately toxic	0.5-5g/kg
Very Toxic	50-500mg/kg
Extremely toxic	5-50mg/kg
Super toxic	<5mg/kg

Toxic agents may be classified in terms of their target organs (liver, kidney, lungs, etc.), their use (pesticide, solvent, food additive etc.), their source (animal or plant toxins), their effects (cancer, mutation, liver damage etc.), their physical state (gas, dust, liquid), their labeling requirements (explosive, flammable, oxidizer), their chemistry (aromatic amine, halogenated hydrocarbons etc.), their biochemical mechanism of action (sulfhydryl inhibitor, methemoglobin producer), or by general terms such as irritants, pollutants and corrosives. Combination of classification systems may be needed to provide the best rating system for a specific purpose (Klaassen *et al.*, 1986).

Toxicological effects of a drug may be immediate, occurring or developing rapidly after a single administration or delayed after a lapse of some time. Most drugs produce immediate toxic effects but delayed effects are also observed with carcinogenic and neurotoxic drugs. For pathological injury of effect on a tissue, the effect may be said to be reversible where the tissue regenerates (e.g. liver) or irreversible where the tissue cannot divide and be replaced (e.g. the nervous system).

A drug does not produce adverse or toxic effect in biological systems unless that agent or its biotransformation products reach appropriate sites in the body at concentrations and for lengths of time sufficient to produce the toxic manifestations. The occurrence of a toxic response is dependent therefore on the chemical and physical properties of the drug, the exposure situation and the susceptibility of the biological system. Thus, to characterize fully the potential toxicity of a specific drug, we need to know the type of effect it produces, the dose required to produce the effect, and also information about the exposure and the biologic system or subject.

The major factors that influence toxicity as it relates to exposure situations are the route of administration, and the duration and frequency of exposure.

Two main principles underlying all descriptive animal toxicity testing are that:

- (a) the effects produced by a chemical or drug in laboratory animals, when qualified, are applicable to humans and
- (b) the exposure of experimental animals to toxic agents in high doses is a necessary and valid method of discovering possible hazards in humans.

2.1.2 Toxicity studies

Exposure of test animals to drugs in toxicity studies is divided into acute, subacute, subchronic and chronic. It is very important to know the dosage of a drug that will elicit effective or toxic responses. The median effective dose of a drug is the dose that would protect 50% of the experimental animal population, to express the average sensitivity, and to elicit the desired response. This is expressed as ED_{50} . The ultimate toxic effect of a drug is death and this extreme toxicity response is assessed by the median lethal dose (LD_{50}), which is the dose of a drug that would cause death in 50% of the experimental animal population. Therapeutic index is the ratio of the dose required to produce a toxic effect to the dose needed to elicit the desired therapeutic response; expressed as the ratio of the median lethal dose (LD_{50}) to the effective or therapeutic dose. The effective dose of a chemical or drug is about one-tenth the median lethal dose (Ballantyne *et al.*, 1995).

The first toxicity studies or test performed on a new drug or chemical is acute toxicity (single dose administration). The LD_{50} and other acute toxic effects are determined after one or more routes of administration, in two or more species. The

species most often used are mice and rats (rodents), rabbits (non-rodent) and sometimes dogs. Food is often withheld the night prior to dosing and the studies performed in both adult male and female animals. The number of animals that die in a period of 24-48hrs after a single dose is noted and tabulated. Surviving animals are observed for 14 days for signs of toxicity such as weight changes, lethargy, behavioural modifications, etc. The acute toxicity studies give a quantitative measure of an acute toxicity (LD_{50}), identify the clinical manifestations of acute toxicity, and give dose-ranging guidance for other toxicity studies. Repeated exposures to a test material may be given within a 24-hour period for some slightly toxic or practically nontoxic chemicals (Klaassen *et al.*, 1986). In this study the LD_{50} could not be determined because more animals died after the 24-48hrs period. However, an estimation of the dose that caused 50% lethality of the experimental animals was made and used as the basis for the determination of the doses used in the subchronic studies.

Apart from the rapid assessment of lethality, other signs and symptoms including possible target organs affected, information concerning the rate of detoxification and/or elimination, as well as the duration and intensity of the toxic effects are also assessed in the acute toxicity studies. The results also help or assist investigators in designing subsequent subchronic and chronic studies and estimating the potential hazard to health and well being, as well as planning therapeutic trials of drugs in humans. The major use of LD_{50} is to assess the relative toxicity of a drug or substance in terms of the toxicity associated with other agents tested in the same species, and knowing its level of toxicity.

Repeated exposure of drugs or chemicals is divided into subacute, subchronic and chronic. Subacute toxicity studies are performed to obtain information on the

toxicity after repeated administration and as an aid to establish doses for subchronic toxicity studies. Subacute exposure refers to repeated exposure of a chemical for one month or less, subchronic, for one to three months, and chronic, for more than three months. Subchronic toxicity studies are usually conducted by the route of intended exposure (usually oral), and at least three doses are employed: a high dose that produces toxicity but does not cause more than 10 percent fatalities, a low dose that produces apparently no toxic effects, and an intermediate dose.

Observations on the test animals include mortality, body-weight changes, diet consumption, pharmacological and toxicological signs, hematology, and blood chemistry profiles. Hematology and blood chemistry measurements are usually done prior to, in the middle of, and at the termination of exposure. At the end of the experiments, the gross and microscopic condition of the organs and tissues, and the weight of various organs are recorded and evaluated. The subchronic toxicity studies characterize the dose-response relationship of a test substance and provide data for chronic toxicity studies.

Chronic or long-term toxicity studies are performed similarly to the subchronic studies, except for the period of exposure, which is longer and somewhat dependent on the intended period of exposure in humans. Chronic toxicity studies are often used to determine the carcinogenic potential of drugs and other chemicals, and both gross and microscopic pathologic examinations are made on those animals that survive the chronic exposure as well as those that died early. Other toxicity studies that may also be done are eye and skin irritations, sensitization, teratology and reproduction, mutagenicity, inhalation toxicology, immunotoxicology and toxicokinetics (Ballantyne *et al.*, 1995; Klaassen *et al.*, 1986).

Dose-response relationship is the characteristics of exposure and the spectrum of effects that come together in a correlative relationship, and it is found to be the most fundamental and pervasive concept in toxicology. The dose-response concept is based on the assumptions that the response is due to the chemical administered, its dose or concentration, and that the method used in measuring the relationship is quantifiable and a precise means of expressing toxicity. The relation between the degree of response of the biological system, whatever response is selected for measurement, and the amount of chemical agent administered assumes a consistent typical sigmoidal curve form, with the ordinate labeled response or percentage response and the abscissa, log of dosage. The typical distributed sigmoid curve approaches a response of 0 percent as the dosage decreases and 100 percent as the dosage increases (Goldstein *et al.*, 1974)

The fundamental aim of any toxicological study is to obtain biochemical, physiological and morphological information indicative of toxicity that is reproducible, reliable and dose-related, and which can be interpreted and/or extended to the assessment of health risk in human (Echobichon, 1992).

2.1.3 Biochemical indicators of organ toxicity

Effects of drugs on the liver have been estimated traditionally by measuring the activities of aminotransferases (most often aspartate and alanine aminotransferases) in serum, where they have been released when liver cells are damaged. Many other enzymes including 5-nucleotidase, alcohol, lactate and isocitrate dehydrogenases, leucine aminopeptidase, glutathione-S- and ornithine carbomoyl transferases have also been analyzed for the same liver cell damage. However, since tissues other than the liver also contain these enzymes, their activities

may be elevated in serum also when non-hepatic tissues are damaged. Analysis of specific enzymes such as alkaline phosphatase and γ -glutamyl-transferase has been used to overcome this lack of specificity as biomarkers of hepatic damage, involving biliary excretion (Craig and Stitzel, 1994).

Several other liver function tests including concentrations of serum proteins synthesized in the liver (e.g. albumin, clotting factors), serum concentrations of bile acids, as well as test for hepatic excretory function have also been used, but these also lack specificity since hepatic viral infections and alcohol have been found to affect them. A number of these enzyme activities and liver function tests are carried out in chronic and subchronic toxicity studies. Some of the activities measured, and tests used in the subchronic toxicity studies carried out in this experiment include aspartate and alanine aminotransferases, γ -glutamyltransferase and total serum protein concentrations.

Several different types of indicators have been used as biomarkers of renal damage, and classified as kidney function markers. These include urinary proteins of low and high molecular weights (e.g. albumin, transferrin, retinol-binding globulin, rheumatoid factor, immunoglobulin G), cytotoxicity markers (tubular antigens e.g. BB50, BBA, HF5), enzymes (e.g. N-acetylglucosaminidase, β -galactosidase) in urine, biochemical markers (eicosanoids, fibronectin, kallikrein activity, sialic acid, glucosaminoglycans) in urine, red blood cells negative charges, serum creatinine and blood urea nitrogen (BUN).

Drug-induced kidney damage is typically seen as acute tubular necrosis (ATN), affecting the cells in the proximal tubule. This causes impairment of reabsorption of water, electrolyte, glucose and amino acids thus decreasing glomerular filtration. Increased concentrations of serum creatinine and blood urea

nitrogen are markers of impaired glomerular filtration. These markers were determined in this experiment in assessing nephrotoxicity.

Hepatic and renal anatomy and function contribute to the susceptibility of the liver and the kidneys to toxic effects of drugs. They have high blood flow thus exposing them to relatively high concentrations of drugs and toxic agents. Since the bulk of xenobiotic biotransformation occurs in these organs (especially the liver) effects of agents that undergo bioactivation to toxic species can be significant.

Other measurements used to assess toxicity include biomarkers of pulmonary toxicity, neurotoxicity, reproductive and developmental toxicity, haematological biomarkers of toxicity, immunotoxicity and genotoxicity. These were not studied in this project due to lack of reagents and time.

2.2 Drug usage

Early in human history, a relationship was formed between religion and the use of drugs. In this relationship, religion dominated because it was believed that the sick were demon possessed and that those who could identify the disease and restore health by their proficiency in the use of drugs had powers other than the application of chemical substances in diagnosis, prevention and treatment of diseases, or the use of drugs for intentional modification of normal physiological and biochemical functions (therapeutics). However the attitude on the use of drugs to effect cures changed drastically as more knowledge about the effects of drugs became known. It was later recognized that regularity prevailed in the natural world that was independent of supernatural whim or will.

Early medical practitioners gathered herbs, plants, animals, and minerals; and often blended them into a variety of foul-smelling and ill-flavoured concoctions. The fact that many of these preparations were so distasteful led to an attempt to improve

certain properties of these mixtures to ensure patient use. Individuals who began to search for improved products formulation were largely responsible for the founding of the disciplines of pharmacy (the science of preparing, compounding and dispensing medicines) and pharmacognosy (the identification and preparation of crude drugs from natural sources) (Craig and Stitzel, 1994).

Drugs are molecules that alter functions of living systems by interacting at the molecular level. There are two broad types of drugs depending on the locus of synthesis. These are the endogenous and the exogenous drugs. Endogenous drugs are synthesized in the living system whereas exogenous drugs are introduced from outside as active substances or metabolized into the active substances. Exogenous drugs are foreign to the living systems, and when ingested, attempts are immediately initiated by the body to eliminate them. The means of eliminating exogenous drugs is basically through metabolism and may involve the induction of enzymes that are required for the biotransformation reactions. One of such enzymes is the cytochrome P450 monooxygenase complex or system (Nebert *et al.*, 1991).

2.3 Drug metabolism

2.3.1 General introduction to drug metabolism

Biotransformation of drugs converts them to products that are more polar than the parent compound, generally diminishing the toxicological or pharmacological activity, by forming inactive or less active products. This results in a more rapid rate of renal clearance since renal tubular reabsorption is diminished. However, there are compounds that in themselves have little or no activity (toxicity or therapeutic), but on introduction into the body are metabolized into active species. Examples of such compounds are acetaminophen and benzo(*a*)pyrene.

When elimination of drugs occur primarily by metabolism, the routes of metabolism can significantly affect the drug's safety and efficacy. Individual differences in metabolic rates of elimination that occur via a single metabolic pathway, can lead to large differences in drug and metabolite concentrations in blood and tissues. In some instances, differences exhibit a bimodal distribution indicative of a genetic polymorphism (Guidance for Industry, 1997). When a genetic polymorphism affects an important metabolic route of elimination of a drug, large dosing adjustments may be necessary to achieve the safe and effective use of the drug. Another property that can affect drug safety and efficacy is drug-drug interference due to a common pathway of metabolism, via the cytochrome P450 monooxygenase enzyme system. Although drugs are metabolized by the cytochrome P450 enzyme system, different drugs are metabolized by different sub-families of the enzyme system.

Nifedipine, cyclosporine A and quinidine are metabolized by CYP3A4 and therefore the presence of these drugs causes the induction of the isozyme (Mennier *et al.*, 2000; Thummel and Wilkinson, 1998; Kronbach *et al.*, 1988). CYP3A4 is the most abundant drug-metabolizing enzyme in the human liver (Shimada *et al.*, 1994) and it is involved in the metabolism of over 50% of all drugs used in human (Rendic and DiCarlo, 1997).

Isoniazid, ethanol and acetone cause the induction of the CYP2E subfamily. CYP2E1 phenotyping has used a probe drug, chlorzoxazone, which is metabolized by the isozyme. However, recent studies suggest that CYP1A1, CYP1A2 and possibly CYP3A subfamilies are involved in chlorzoxazone metabolism, limiting the specificity of chlorzoxazone for CYP2E1 phenotyping (Streetman *et al.*, 2000). CYP2E1 also has applications for monitoring alcoholics (Rancy *et al.*, 1999).

CYP2C19 metabolizes several therapeutic agents such as S-mephenytoin, omeprazole, propranolol, and imipramine (Hersberger *et al.*, 2001); and coumarine is metabolized by CYP2A6 (Mennier *et al.*, 2000).

Among the xenobiotic-metabolizing cytochrome P450 isozymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 appear to be the most commonly responsible for the metabolism of drugs (Spatzenegger and Jaeger, 1995). Inhibition of cytochrome P450-mediated metabolism enzymes is often a mechanism for drug-drug interactions (Murray, 1992). In addition, induction of the same isozyme by two different drugs when co-administered can lead to toxicity.

2.3.2 Cytochrome P-450 monooxygenase enzyme system

The cytochrome P-450 monooxygenase enzyme system comprises of structurally and functionally related heme proteins that biotransform both endogenous and exogenous compounds. The endogenous compounds include fatty acids, steroids, prostaglandins and bile salts; and the exogenous compounds include polycyclic aromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's), dioxins, pesticides, petroleum products, drugs and food additives (Ortizde Montellano and Correia, 1995; Nelson *et al.*, 1996).

The reactions catalyzed by these enzymes are of the type generally referred to as monooxygenase (oxidative Phase I) reactions, formerly mixed function oxidase, and are considered important in detoxification pathways. The monooxygenase enzyme system has been found to be substrate specific, the specificities being imposed by the existence of multiple forms of cytochrome P450 (Klotz *et al.*, 1986; Goksoyr, 1985). The essential components of the monooxygenase enzyme system are

cytochrome P450, NADPH-dependent cytochrome P450 reductase and a phospholipid (Yasukochi and Masters, 1976).

Cytochrome P450 is a multigenetic family of haemoproteins, which determine the duration of action of many drugs and play a key role in chemical carcinogenesis and toxicity (Nebert and Gonzalez, 1987; Conney, 1986). Many isozymes of cytochrome P450 exist. The isozymes differ from each other in substrate specificity and catalytic activity (Guengerich *et al.*, 1982). Potentially useful models such as primary hepatocyte cultures have been used to find a correlation between the effects of induction and different isozyme patterns of cytochrome P450 with toxicity of various compounds (Moldeus, 1987).

Selective dealkylation of phenoxazone alkyl derivatives, 7-ethoxyresorufin by CYP1A1 and CYP1A2 in a 7-ethoxyresorufin-O-deethylase (EROD) (Mennier *et al.*, 2000; Burke *et al.*, 1985) and 7-pentoxyresorufin by CYP2B1 and CYP2B2 in 7-pentoxyresorufin-O-depentylation (PROD) (Burke *et al.*, 1985) reactions have been investigated. Hydroxylation of p-nitrophenol by CYP2E1 (Robert *et al.*, 1995), aniline by CYP1A2 and CYP2E1 (Ryan *et al.*, 1985), testosterone by CYP2A1 (Levin *et al.*, 1987), CYP2B1, CYP2C11, and the formation of hydroxytestosterone by CYP3A subfamilies (Waxman, 1988; Sonderfan *et al.*, 1987; Waxman *et al.*, 1983), depending on the position of the hydroxylation have also be established.

The term “induction” was coined by Conney (1967), for the monooxygenase enzyme system to mean an increased production of the cytochrome P450 monooxygenase enzymes via the stimulation of protein synthesis, especially cytochrome P450s.

Induction can be evaluated by measuring increased catalytic activity of monooxygenases (e.g. ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-

O-depentylyase (PROD) which were used in this experiment, and aryl hydrocarbon hydroxylase (AHH) activity, immunochemical detection of induced proteins by Western blotting and enzyme-linked immunosorbent assay (ELISA), and mRNA detection with cDNA probes. The immunochemical detection of cytochrome P450 protein is important as a supplement to the catalytic methods since the enzyme activity in certain exposure situations may be degraded or influenced by inhibiting processes and also due to overlaps of enzyme activities (Goksoyr, 1991).

Substances that induce cytochrome P450 consist of compounds that have a broad spectrum of structural types, uses and pharmacological activities (Davis *et al.*, 1978). The ability to induce the cytochrome P450 enzyme system has been found in most organisms, which is partly linked to the survival of most organisms in contaminated environments.

2.4 Drug-drug interaction

Drug-drug interaction is concerned with the modification of the effect of one drug by the presence of another drug, by direct or indirect means, and is known to occur by a number of mechanisms such as alterations in absorption, distribution, receptor action, metabolism (biotransformation) or excretion of one or both of the interacting drugs. Drug interaction may or may not be of clinical significance, and may also be beneficial or hazardous (Goldstein *et al.*, 1974).

The effects of two drugs given simultaneously will produce a response that may simply be the sum of their individual responses, or may be greater or less than that of the sum of the individual responses. The effects of pharmacological and toxicological interactions may be additive synergistic potentiative or antagonistic. In an additive effect, the combined effect of the two drugs is equal to the sum of the

effect of each drug given alone, and in synergistic effect, the combined effect of the two drugs is much greater than the sum of the effect of each drug given alone. For potentiation effect one drug may not have a toxic effect on a system, but when added to another drug, it tends to boost the action of the latter, and in antagonistic effect, two drugs administered together interfere with each other's action or one interfering with the action of the other.

It is known that a drug may inhibit or speed up the metabolism of another drug or endogenous compounds. Induction of drug-metabolizing enzymes has been found to be affected by drug-drug interaction. The induction of drug metabolizing enzymes by a drug A can increase the concentration of an enzyme which metabolizes another drug B, so that simultaneous administration of the two drugs will cause a rapid breakdown of drug B and therefore its effect not realized. On the other hand, drug A can cause an inhibition of the induction of the enzyme that metabolizes drug B, so that there would be high concentrations of drug B when the two drugs are administered simultaneously, which may lead to toxic effects.

The purpose of induction is to rapidly convert water insoluble xenobiotics to more soluble/conjugatable/excretable metabolites. However, hepatotoxicity can be caused by a number of chemicals that are metabolized by the cytochrome P-450-dependent mixed function oxygenase system to reactive intermediates. Induction of cytochrome P450 isozymes caused by a drug, which can affect the levels of other drugs, metabolized by the same induced cytochrome P450 isozyme causes drug-drug interactions, leading to possible toxicity.

In this study, possible drug-drug interaction was investigated by assaying for the type of drug metabolizing enzymes present in hepatic microsomes prepared from

experimental animals that have been on an extract of *Desmodium adscendens*. The effect of such administration on drugs that induce the cytochrome P-450 monooxygenase enzyme system, and have overt physiological effects, was also studied.

2.5 Medicinal plants

2.5.1 General introduction to medicinal plants

In traditional societies, where human beings enjoy an intimate relationship with the ambient vegetation, man has experimented with many of the plant species. Most were innocuous, serving no purpose, a few were found nourishing, some were distasteful or even toxic, some seemed to relieve symptoms of sickness, and a small number found with narcotic or hallucinogenic effects. An astonishingly good knowledge of the properties of plants has been built from this experimentation, much of which has been passed on orally from generation to generation. Recent discoveries of unbelievably potent and effective properties in plants have convinced the world that it is undoubtedly still neglecting life-saving or health-promoting constituents lurking in many kinds of plants tissue (Ayensu, 1978).

A more realistic environment is now developing and steps are increasingly being taken to examine the merits of traditional medicine in the light of modern science, with the view of adopting effective medical practices and discouraging the harmful ones. There is therefore the recognition that all available resources need to be used if better health care is to be achieved on a worldwide basis. It is now generally accepted that a fully conversant knowledge of herbal medicine could substantially contribute to providing acceptable level of worldwide health care.

Most pharmaceutical houses in both advanced and developing countries, are well established in the practice of extracting active components from numerous plant species in the formation of new drugs; thereby making many of modern medicine derived from traditional plant medicine. For example, nearly half of all prescription drugs produced in Europe are initially derived from raw plants materials, and in the United States of America, over one quarter of the 1,500 million prescriptions dispensed annually are derived from plants. In economically strained developing countries, especially Africa, dependence on medicinal plants is of prime interest as there is so much cost involved in the importation and usage of modern pharmaceutical drugs. Coupled with the fact that majority of the plant species occur in the tropical areas, a number of plants from Africa are being actively worked on as likely candidates for new drugs. In Ghana, herbal medical practice dates back to prehistoric times in both the rural and urban areas. Herbs and other plant species with medicinal value are used in their raw states, processed to obtain extracts, or treated to form derivatives.

The advancement in biotechnology has prompted an increase in the interest in researching into plants as a source of raw materials for developing new pharmaceutical drugs. Pharmaceutical agencies are now reported to be patrolling the tropical forests of developing countries in pursuit of exotic medicinal plants, as their interest now is in screening for natural products with curative properties. Countries like the United States of America have doubled their interest in plants as sources of raw materials for discovering and developing new pharmaceutical products (Komen, 1991).

The importance of plant-derived pharmaceuticals and the size of the world market for such drugs require that research in this field should be strongly encouraged

and financially supported by governments and private pharmaceutical industries. In Ghana, institutions that carry out research into medicinal plants include the Centre for Scientific Research into Plant Medicine (CSRPM), Akwapim Mampong, Council for Scientific and Industrial Research (CSIR), educational institutions such as the Universities, and other (both large and small scale) private research stations. The overall effect of any scientific study on medicinal plants will help to establish and confirm the credibility of the use of herbal medicine and to detect any toxicological effects associated with the plant.

2.5.2 *Desmodium adscendens*

D. adscendens belongs to the family Leguminosae-Papilionoideae, and found in South Africa, South America, India and almost all the West African countries including Ghana. A number of species of this widely distributed plant have been identified, with about sixteen (16) species in Tropical Africa (Hutchison and Daizel, 1954), nine (9) of which occur in Ghana (Irvine, 1961), and four dozens in India (Ghosal and Bhattacharya, 1972). There are two closely related varieties in Ghana: *D. adscendens var adscendens* and *D. adscendens var robustum*, with the former receiving more attention.

D. adscendens is about 30-60cm high with slender branches which have thin soft hairs that are also present on its terminal, broad and elliptical leaflets. It produces white, pink or yellow flowers, which develop into greenish-yellow, one-side indented fruits, covered with thick hairs. It occurs in humid and shady forest areas, mostly found as under-growths. Among the Akans in Ghana, it is called "ananse nkate" meaning spider's groundnuts, "nkatenkate" meaning groundnuts-like (because the leaves resemble those of groundnuts), or "akwan famu" meaning path passes through

(because it grows on either side of forest paths). It has other indigenous names in other Ghanaian native languages.

D. adscendens is found to have many uses depending on where it is found. It is useful as a cover crop and serves as fodder for farm animals besides having insecticide and papermaking values. Other agriculturally useful species include *D. uncinatum*, a pasture plant in Australia; *D. intortum*, a good source of vitamin A, used as forage in the Democratic Republic of Congo (formerly Zaire); and *D. diffusion*, utilized as a source of nitrogen in fertilizers.

In West Africa, the plant is known to have a wide range of medicinal uses. In Ghana and some other West African countries, aqueous extract from ground, boiled and cooled dried leaves of *D. adscendens* is drunk for the treatment of bronchial asthma and the extract from boiled and cooled fresh leaves is drunk as a cure for constipation. The fresh leaves, when pounded or ground and soaked in limejuice, is used for dressing wounds, and when salted and mixed with roasted cornmeal, are eaten for the treatment of dysentery. In Liberia, infusion bath of the leaves is used for managing convulsion and venereal sores, and in Cameroon the ground leaves mixed with guinea-grains is applied on scratches on the chest as a treatment for coughs (Ayensu, 1978). Decoction of the leaves is also used to enhance lactation, to aid parturition, and for alleviating dysmenorrheal pain, diarrhoea and abdominal colic (Ampofo, 1977). The plant extract is actually dispensed at the CSRPM as an anti-asthmatic agent.

Other species of *Desmodium* are also known for their medicinal properties. Preparations from different parts of *D. gangeticum* are used to treat diarrhoea, chronic fever, asthma, abdominal tumors, urinary problems, nasal polyps, and catarrh, and preparations from the roots are also used as astringents, tonics and diuretics (Ayensu,

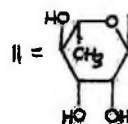
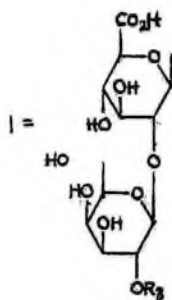
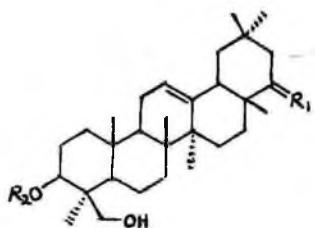
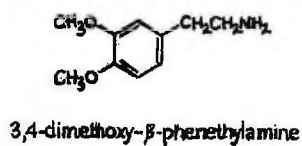
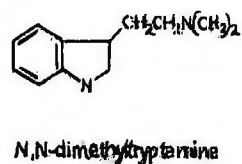
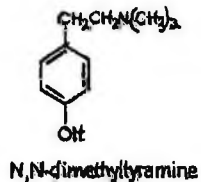
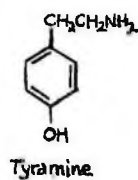
1978). The leaves of *D. triflorum* are used to induce lactation, as a remedy for diarrhoea, dysentery and convulsion, and the roots are used in treating coughs, asthma, and also applied to wounds and abscesses. The alkaloid content of the various parts of *Desmodium* spp., is thought to be the component that confers the medicinal properties (Ghosal and Bhattacharya, 1972; Ghosal *et al.*, 1971;).

A number of chemical components have been isolated from the aqueous extracts of the stem/leaves of *D. adscendens*. These include tyramine, N,N-dimethyltyramine, hordenine, 3,4-dimethoxy- β -phenethylamine, salsoline (a 6-hydroxytetrahydro-isoquinoline derivative), N,N-dimethyltryptamine, and several unidentified indole and other minor basic components such as phenolic compounds (Asante-Poku *et al.*, 1988). Other components are triterpenoid glycosides, dehydrosoyasaponin I (DHS-I), soyasaponins I and III, and soyasapogenol B and E (McManus *et al.*, 1993). DHS-I and soyasaponins I and III have been found to be potent activators of calcium-dependent potassium ion channels involved in airway smooth muscle tone regulation. Figure 2a shows the structures of some of the isolated chemical compounds of *D. adscendens*.

It is the anti-asthmatic and anti-inflammatory properties of *D. adscendens* that have excited and shaped the interest and approach of the various studies on the plant. Asthma, a hypersensitivity phenomenon is characterized by recurrent episodic bouts of coughing, apnoea, tightness of the chest, and wheezing as symptoms. The pathological features are one of airway smooth muscle contraction, mucosal thickening resulting from edema of the lung parenchyma, and cellular infiltration (Katzung, 1989).

Inflammation, a local response to cell injury involving small blood vessels, cells circulating within the vessels, and nearby connecting tissues, has swelling, pain,

heat and redness as symptoms. Many anti-inflammatory drugs function by preventing the formation of mediators, which carry out the inflammatory process, or by blocking the actions of the mediators on target cells whose behaviour is modified (Lagunoff, 1994). Asthmatic patients take drugs to manage the disease. If they also take *D. adscendens*, possible toxicity due to interference or drug-drug interaction may occur if the same cytochrome P450 subfamily induced by the drug is also induced or inhibited by *D. adscendens*.



Compound	R ₁	R ₂	R ₃
Soyasaponin I	H, OH	I	II
Dehydrosoyasaponin I	O	I	II
Soyasaponin III	H, OH	I	H
Soyasapogenol B	H, OH	H	-

Figure 2a: Some isolated chemical constituents of *Desmodium adscendens*

CHAPTER THREE

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1 Rats

White wistar rats (males and females) were obtained from the University of Ghana Medical School Animal House. The rats were kept in groups of forty (40) animals either at the Department of Nutrition and Food Science, University of Ghana, Legon or the University of Ghana Medical School Animal House. The animals were used for the acute and subchronic toxicity studies.

The rats were housed in groups of five or six in clean cages (previously washed with detergent and disinfectant, and dried). They were fed daily with Grower mash, a formulated animal feed purchased from Kosher Feedmills Limited. The nutritional content of the feed was 15-16% protein, 3-5% crude fibre, 1.00% calcium, 0.40% phosphorus, 0.65% lysine and 0.40% salt; with energy content of 2800 Kcal/kg of the feed. The animals were given tap water as their source of drinking water, which was changed daily. Sawdust spread in large rectangular metal plants was placed under the cages housing the animals to collect faeces and urine; and this was also changed regularly, once every week.

3.1.2 Medicinal plant extract

Crude aqueous plant extract of *Desmodium adscendens* as dispensed to patients at the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong-Akwapim was obtained from the Centre.

3.1.3 Chemicals and reagents

The following assay kits: alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total bilirubin, direct or conjugated bilirubin, total serum protein, serum creatinine and blood urea nitrogen (BUN) were obtained from Randox Chemicals Co (Antrim, UK). Nicotinamide adenine dinucleotide phosphate (reduced) (NADPH), resorufin, 7-ethoxyresorufin, 7-pentoxyresorufin, zoxazolamine (2-amino-5-chlorobenzoxazole), bovine serum albumin and perchloric acid were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Glycerol, potassium chloride, copper sulphate pentahydrate, sodium hydroxide, sodium chloride, dipotassium hydrogen phosphate dodecahydrate, magnesium chloride, sodium dithionite, ethanol, and dithiothreitol were obtained from Fluka Garantie.

Dimethylsulphoxide (DMSO) and trichloroacetic acid were obtained from Wako Pure chemical Industries, Japan, Tris (hydroxymethyl) aminomethane and sodium dihydrogen phosphate were obtained from Hopkins and Williams, England, and Ethylenediaminetetraacetic acid (EDTA) and p-nitrophenol were obtained from BDH laboratory, England.

3.2 METHODS

3.2.1 Preparation and administration of plant extract

A volume of 10litres of crude aqueous plant extract of *D. adscendens* was concentrated by evaporating to a small volume using a vacuum rotary evaporator and then freeze dried into a powder. The powdered extract was weighed, dissolved in 0.15M NaCl solution (0.88% standard saline solution), and the resulting solution

administered orally to the test animals using a plastic syringe with a metallic gavaging needle at its end.

3.2.2 Acute toxicity studies

Rats used in the acute toxicity studies were weighed to determine their individual weights before the administrations of the extract. A dose of 10g/kg body weight was administered in a volume of 5ml of the standard saline solution. Two rats (a male and a female) were used at this dose level and the animals observed after the administration. Their physical behaviour, number of deaths and times of deaths from the time or day of administration of the plant extract were noted and recorded.

The dosage was reduced to 8, 5, 3 and 1.0g/kg body weights; using two rats (male and female) at each dose level, based on the observations of each previous dose level. The administration of the extract and observation of the animals at a higher dose level was completed before that for a subsequent lower dose was done. The maximum period of time over which the animals were observed after the administration with the plant extract was two weeks.

Based on the results obtained from the above studies, six (6) different dose levels: 0.5, 0.75, 1.00, 1.25, 1.50 and 1.75g/kg body weights were chosen, prepared and administered to six different groups of animals of six rats each. Each dose was administered in a volume of 1ml of the standard saline solution per animal. The animals were observed over a period of two weeks from the day of administration. The number of deaths that occurred at the various dose levels were noted and recorded. Average of the doses that caused 50% lethality in the acute toxicity studies over the period of observation was calculated and used as the basis for determining the doses of the plant extract used in the subchronic toxicity studies.

All the animals that died during the study were dissected and their organs examined macroscopically. All control animals were given equal volume of the standard saline solution, orally in place of the plant extract administered to the test animals.

3.2.3 Subchronic toxicity studies

Pretreatment for subchronic toxicity studies

Rats used in the subchronic toxicity studies were weighed on days 0, 1 and 2 of administration and then weekly for six (6) weeks. The initial weights were used to calculate the dosage of the plant extract to be administered, and to determine the % weight gained over the period of treatment. Oral administration of the extract was done daily over a period of forty-five (45) days.

Based on the MR_{50} obtained from the acute toxicity studies, three (3) dose levels - high, medium and low - were chosen and used in the subchronic toxicity studies. The medium dose was one-tenth the MR_{50} ($0.1x$), the high dose was $0.3x$ and the low dose was $0.03x$, where x was the median mortality rate dose, MR_{50} . In addition to these groups of animals, there was a control group in which the animals were given equal volumes of the standard saline solution, in place of the plant extract administered at the various dose levels. The administrations were done in a total volume of 0.5ml solution per animal. These groups of animals (the high, medium and low dose groups, and the control group) were used in the preparation of serum and hepatic microsomes for liver and kidney function tests and cytochrome P450-dependent monooxygenase activities respectively.

Two other groups of animals (with 5 animals in each group) and their controls were also given the medium dose over a period of thirty-five (35) days and used in a

zoxazolamine (2-amino-5-chlorobenzoxazole) paralysis test and thiopentone sleeping test. These were done to determine the effect of the plant extract on the metabolism and metabolic effects of zoxazolamine and thiopentone, drugs whose actions are known to be terminated by the cytochrome P-450 monooxygenase enzyme system.

Zoxazolamine paralysis test

Zoxazolamine was weighed and dissolved in dimethylsulfoxide (DMSO) to prepare a dose of 60mg/kg body weight in a total volume of 0.5ml zoxazolamine solution. This was injected intraperitoneally (I.P.) into the animals to become paralyzed, which were then observed and the time that they got out of paralysis noted. The times they could be turned to lie on their backs were taken as the initial times of paralysis and the times they turned to their normal postures as the final times of paralysis. The paralysis times were obtained as the differences between the final and initial times of paralysis (Nyarko *et al.*, 1999).

Thiopentone sleeping test

A volume of twenty millilitres distilled water was added to 1g of thiopentone and the resulting solution shaken gently until a clear solution was obtained, giving a concentration of 50mg/ml. A dose of 100mg/kg body weight in 0.5ml solution was administered intraperitoneally into the animals, and then observed for their sleeping times. The times they could be turned to lie on their backs were taken as the initial sleeping times and the times they turned to their normal postures as the final sleeping times. The differences between the final and initial times were taken as the sleeping times (Nyarko *et al.*, 1999).

Based on the observations made after the first administrations, the dose of the thiopentone was increased to 200mg/kg body weight (doubled) and injected into new group of animals (extract administered and control animals). These were also observed for their sleeping times.

Preparation of Serum

Serum was prepared using blood from animals pretreated for the sub-chronic toxicity studies. At the end of the period of extract administration, the animals were anaesthetized with diethylether and pinned on a dissecting board, with their ventral sides facing upwards. A syringe with needle was inserted into the cardiac chamber of each animal, targeting the heart (cardiac puncture), and then blood drawn into the syringe.

Each blood sample collected was transferred into centrifuge tubes, allowed to clot, and centrifuged at 2,500g for 15 minutes at room temperature using a bench centrifuge. At the end of the centrifugation, the tubes were removed and the supernatant fractions (sera) pipetted into labelled eppendorf tubes and stored at -40°C. The sera were later used to determine the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl aminotransferase (GGT), total and conjugated bilirubin, blood urea nitrogen (BUN) and serum creatinine, for the determination of liver and kidney function.

Preparation of microsomes

The animals used for the sub-chronic toxicity tests, from which blood was drawn for the preparation of serum, were also used in the preparation of the microsomes. The animals were dissected, after blood had been drawn, and their livers

and kidneys rapidly excised. The freshly excised organs were rinsed of blood in a pre-chilled 0.154M KCl solution (rinsing buffer), blotted dry with tissue papers and weighed. A sample of liver weighing 7g from each animal was minced and homogenized in 1:3 volume of ice-cold homogenizing buffer (appendix I), with a Glas-col homogenizer previously placed in ice.

The homogenates were spun in a Hitachi 20PR-52D centrifuge at 16,000g for 10 minutes at 4°C. The supernatant fractions were decanted (discarding the pellets) and centrifuged again at 100,000g for 60 minutes also at 4°C using a Hitachi 80P-7 ultracentrifuge. The supernatant fractions (S9 fractions) were discarded and the microsomal pellets washed with storage buffer, pH 7.4 to remove any haemoglobin that may be present, and to clean the microsomes of cytochrome b₅ contamination. The washed microsomal pellets were resuspended in 2.0ml storage buffer, distributed into labelled eppendorf tubes and stored at -80°C.

The microsomal preparations were then later used in biochemical assays to evaluate the isozymes of CYP present by measuring ethoxyresorufin-O-deethylase, pentoxyresorufin-O-depentylase activity and p-nitrophenol hydroxylation activities.

3.2.4 Clinical Chemistry Tests

Five serum samples each from the high, medium and low dose extract treated rats and the control group were used for the clinical chemistry tests

Total serum protein

The biuret method of total protein determination was used, in which cupric ions in an alkaline medium interact with protein peptide bonds resulting in the

formation of a coloured complex (Henry *et al.*, 1974). The contents of the reagents used are provided in Appendix III.

The procedure used was as follows: The content of the biuret reagent (reagent 1) was diluted with 400ml of double distilled water, rinsing the bottle thoroughly. The content of the blank reagent (reagent 2) was also diluted with 400ml of double distilled water, rinsing the bottle thoroughly. A volume of 1ml of the diluted biuret reagent was pipetted into sample, standard and blank labelled tubes, and 20 μ l of the standard protein solution, serum samples and distilled water added to the appropriate tubes. The contents of the tubes were mixed and incubated at 25 $^{\circ}$ C for 30minutes. The absorbances of the serum samples and the standard were then read against the reagent blank at 546nm.

The total protein concentration of the serum samples were calculated as follows:

$$\text{Total protein concentration} = \frac{A_{\text{samples}}}{A_{\text{standard}}} \times \text{Standard concentration.}$$

Where A_{sample} is the change in absorbance of serum sample and A_{standard} is the change in absorbance of the standard reagent.

Alanine aminotransferase (ALT)

The method used is an optimized standard UV method according to the recommendations by the International Federation of Clinical Chemistry (IFCC) (IFCC, 1980). The principle behind the method is the oxidation of NADH coupled to the reduction of pyruvate to lactate and catalyzed by lactate dehydrogenase. In the presence of alanine aminotransferase, alanine is converted to pyruvate, which is then reduced to lactate using NADH. The contents of the reagents used are provided in Appendix III.

The instructions in the enzyme assay kit was as follows:

A volume of 2ml of the buffer/substrate reagent (reagent 1) was added to one vial of reagent 2 consisting of enzyme, coenzyme and α -oxoglutarate. This was prepared fresh and used. A volume of 0.1ml serum sample initially stored at -40°C was pipetted into 1cm cuvette and 1.0ml of the reconstituted reagent added. The content of the cuvette was mixed and an initial absorbance at 340nm taken after 1miunte. A timer was started simultaneously and the absorbance read again after 1, 2 and 3 minutes. The reaction was carried out at 25°C .

The ALT activity of each serum sample was calculated using the formula:

$$\text{Activity (U/L)} = \frac{\Delta A/\text{min} \times \text{Total assay vol. (ml)}}{E \times \text{lightpath (m)} \times \text{sample vol. (ml)} \times 1000}$$

Where $\Delta A/\text{min}$ = change in absorbance per minute

1000 = factor for converting ml to L

E = Molar absorptivity of p-nitroaniline

OR
$$\text{ALT Activity (U/L)} = 1746 \times \Delta 340\text{nm}/\text{min}$$

Aspartate aminotransferase (AST)

The method used for this determination is also an optimized standard UV method recommended by the IFCC (IFCC, 1980). The principle behind the method is the oxidation of NADH coupled to the reduction of oxaloacetate to malate, a reaction catalyzed by malate dehydrogenase. In the presence of aspartate aminotransferase, aspartate is converted to oxaloacetate, which is used in a redox reaction in which NADH is used and malate formed. The contents of the reagents used are provided in Appendix III.

For this assay, a volume of 2ml of the buffer/substrate reagent (reagent 1) was added to one vial of reagent 2 consisting of the enzyme, the coenzyme and the α -oxoglutarate. This reconstituted enzyme solution was prepared fresh and used. A volume of 0.1ml serum sample initially stored at -40°C was taken through the same procedure of addition of reconstituted reagent, reading and recording of absorbance, and calculation of the AST activity as in the ALT determination.

Gamma-glutamyltransferase (GGT)

In the presence of γ -glutamyltransferase and glycylglycine, γ -glutamyl-p-nitroanilide is converted to γ -glutamylglycylglycine, and p-nitroanilide that is formed measured spectrophotometrically (Ladenson, 1980; Teitz, 1987). The contents of the reagents used are provided in Appendix III.

A volume of 3ml of distilled water was added to one vial of the reagent to obtain the working reconstituted solution. A volume of 1ml of the working reagent was pipetted into test tubes and 0.1ml of the serum samples added and mixed. Initial absorbances were immediately read at 405nm against air and a timer started simultaneously. The absorbances were read again after 1,2 and 3 minutes.

The GGT activities of the serum samples were calculated using the following formula:

$$\text{Activity (U/L)} = \frac{\Delta A_{\text{min}} \times \text{Total assay vol. (ml)}}{E \times \text{lightpath (m)} \times \text{sample vol. (ml)} \times 1000}$$

Where ΔA_{min} = change in absorbance per minute

1000 = factor for converting ml to L

E = Molar absorptivity of p-nitroaniline ($9.9\text{cm}^2 / \text{umol}$ at 405nm)

OR $\text{GGT Activity (U/L)} = \Delta A_{\text{min}} \times 1111$

Total bilirubin

A colorimetric method to determine total bilirubin in the presence of dimethylsulphoxide (DMSO) by the reaction with diazotized sulphanilic acid was used. The contents of the reagents used are provided in Appendix III.

Working diazo reagent was prepared by mixing 30 volumes of reagent 1 and 1 volume of reagent 2. The working reagent was protected from light by wrapping with carbon paper and covered at the top with an opaque paper card. A volume of 1ml of the diazo reagent was pipetted into blank, standard and sample labelled tubes. To the test reactions, a volume of 40ul of serum samples or standard reagent was added to the 1ml diazo reagent whereas in the blank tubes the 1ml diazo reagent was used as the reagent blank. The contents of the tubes were mixed and allowed to stand for 5minutes at 25⁰C. The absorbances of the samples and standard were read at 560nm against the reagent blank.

The concentrations of total bilirubin in the serum samples were calculated using the following formula:

$$\text{Total bilirubin conc. (umol/L)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Standard concentration.}$$

Where A_{sample} is the absorbance of serum samples and A_{standard} is the absorbance of the standard reagent.

Direct (conjugated) bilirubin

A colorimetric method to determine direct bilirubin in the absence of dimethylsulfoxide (DMSO) was used. The contents of the reagents used are provided in Appendix III.

A volume of 2.5ml of reagent 1 was pipetted into blank, standard and sample labelled tubes and a drop of reagent 2 added. In the test reactions, 0.1ml of serum

sample or standard reagent was added, mixed and allowed to stand for exactly 5 minutes at 25⁰C. The absorbances of the samples and standard were read at 560nm against a reagent blank, which contained only 2.5ml of reagent 1 and a drop of reagent 2. The concentration of the direct bilirubin in the serum samples was calculated using the same formula as that used for calculating total bilirubin concentration.

Creatinine

Creatinine concentration was determined by a colorimetric method in which creatinine in an alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex found is directly proportional to the creatinine concentration (Bartels and Bohmer, 1972). The contents of the reagents used are provided in Appendix III.

Equal volumes of 25ml each of picric acid and sodium hydroxide were mixed, and 1.0ml of the working solution obtained pipetted into standard and sample labelled tubes. A volume of 0.1ml of serum sample and standard reagent were added to the appropriate tubes, mixed and incubated for 30 seconds at 25⁰C. The absorbance A₁ of the sample and standard were then read at 492nm against air. Exactly two minutes later, the absorbances A₂ were read again and the change in absorbances (ΔA) computed.

The concentration of the creatinine in the serum samples were calculated using the formula:

$$\text{Creatinine (umol/L)} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 177$$

Where ΔA_{sample} is the change in absorbance of serum samples, ΔA_{standrad} is the change in absorbance of the standard reagent, and the value 177 incorporates the

factor for converting millilitres to litres and the molar absorptivity of the creatinine-picric acid complex.

Blood Urea Nitrogen (BUN)

Blood urea nitrogen concentration was determined by a kinetic method (Young *et al.*, 1975). The principle behind the method is the formation of NAD⁺ from NADH (oxidation of NADH) coupled to the amination of α -oxoglutarate to form glutamate, a reaction catalyzed by glutamate dehydrogenase. The ammonia used in the reaction is obtained from the hydrolysis of urea in the presence of water and urease. The contents of the reagents used are provided in Appendix III.

One vial of the enzyme reagent (reagent 2) was reconstituted with 15ml of buffer (reagent 1) and used. A volume of 1ml of the reconstituted reagent was then pipetted into 1cm light path cuvette. A reagent blank consisting of only 1ml of the reconstituted reagent was also prepared. A volume of 10ul of the samples and the standard reagent was added to the appropriate tubes, mixed and incubated at 37^oC for 30 seconds. Initial absorbances were read at 340nm and a timer started simultaneously. The absorbances were read again after 1, 2 and 3 minutes.

The blood urea nitrogen concentration was calculated using the following formula:

$$\text{BUN concentration (mmol/L)} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{Standard concentration.}$$

Where ΔA_{sample} is the change in absorbance of sample and $\Delta A_{\text{standard}}$ is the change in absorbance of the standard reagent.

3.2.5 Biochemical assays

Protein concentration of microsomal preparation

The biorad reagent method as described by Bradford (1976) was used to determine the protein concentration of the microsomal preparations obtained. The principle is based on protein-dye binding. The microsomal preparations were diluted as follows: 1 in 25, 1 in 50 and 1 in 100 with distilled water and used in the protein concentration determination.

A protein calibration curve was prepared using bovine serum albumin (BSA) as the standard protein. A stock (BSA) solution of concentration 1mg/ml was prepared, and from the stock 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0mg/ml BSA solutions were prepared by pipetting 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0ml into test tubes and making them up to 1ml with distilled water.

The biorad reagent was diluted to 1 in 5 concentration with distilled water and 1ml of the diluted solution was pipetted into previously labelled test tubes. To the test tubes containing the 1 in 5 diluted biorad reagent were added 20ul of the different concentrations of the BSA solutions and the diluted microsomal preparations (each BSA solution and diluted microsomal preparation were added to different test tubes). The content of each test tube was mixed by shaking gently and the mixture left to stand at room temperature for at least 5 minutes. The absorbances of the mixtures were then read at wavelength 595nm. The 0.0mg/ml test sample was used as blank to auto-zero the spectrophotometer before the absorbance reading was taken.

The protein concentrations of the microsomal preparations were determined by directly interpolating the absorbances of the microsomal preparation test samples from the calibrated standard curve.

p-Nitrophenol (PNP) hydroxylation determination

p-Nitrophenol hydroxylation was measured as described by Reinke and Moyer (1985), with modifications as indicated by Nyarko (1994). Based on the results obtained from the protein concentration determination of the microsomal preparations, each PNP hydroxylation reaction was performed in a total volume of 1.5ml reaction mixture.

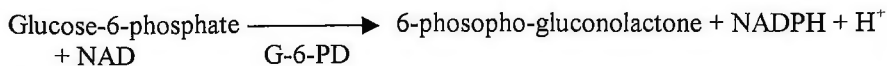
The reaction was carried out by pipetting 1ml of freshly prepared 0.05M Tris buffer, pH 7.4 containing 0.4mg NADPH and 5mM $MgCl_2$ into previously labelled test tubes, and a volume of microsomal preparation containing 1mg protein added to it. The reactions were initiated by the addition of 200nmol of PNP in Tris buffer to each tube. The reaction mixtures were incubated at 37°C in a shaking water bath for 10 minutes. At the end of the incubation period, the reactions were terminated by the addition of 0.5ml $HClO_4$ (0.6N) to each tube.

The mixtures were centrifuged at 2,500g to remove the protein precipitates that were present. Aliquots (1ml) of the supernatants obtained were pipetted into test tubes and each mixed with 100ul of 10M NaOH. The absorbances of the resulting solutions were read at wavelength 546nm against a blank, which contained all the reagents except microsomal preparation, which was replaced with Tris buffer. The amount of 4-nitrocatechol formed was then calculated using a molar absorptivity of $10.28mM^{-1}Cm^{-3}$.

Ethoxyresorufin-O-deethylase (EROD)

Phosphate buffer (1ml) was pipetted into labelled test tubes containing test, blank and standard samples, and 20ul of 50mM NADPH was added to each tube. When there was shortage of NADPH, a NADPH-generating system was prepared as

shown in Appendix I and used for this assay (Gibson and Skett, 1994). This generating system takes advantage of the following reaction:



The components of the NADPH-generating system were dissolved in 0.1M Tris buffer and mixed thoroughly, with the NADP⁺ added just prior to use. A volume of 1ml of the NADPH-generating solution was immediately pipetted into labelled test tubes. Each microsomal preparation has a test reaction and a test blank.

A volume of 100ul of the microsomal preparations, diluted 1 in 25 was added to the mixtures in the test and blank tubes. Cold 10% trichloroacetic acid (0.5ml) was added to the blank tubes, or the tubes were placed in boiling water bath to denature the microsomal protein. All the tubes (test, blank and standard) were pre-incubated in a water bath at 37°C for 5 minutes and then 7-ethoxyresorufin of concentration 1.25nmol in a total volume of 15.1ul added to each tube. The resulting solutions were mixed by shaking gently and incubated also at 37°C for 5 minutes.

After the incubation, 0.5ml cold 10% TCA was added to the test reaction tubes, or placed in boiling water to denature the microsomal protein, and then all the tubes centrifuged at 2,500g for 10minutes. A known concentration of 10nmol/ml standard resorufin in a volume of 100ul resorufin solution was added to the standard tube. The content of the standard tube was used to calibrate the fluorimeter, which had been previously set at an extinction wavelength of 510nm and an emission wavelength of 586nm. A blank-blank tube containing 1,115ul phosphate buffer, 20ul of 50mM NADPH, with or without 0.5ml 10% cold TCA was used to autoblack the fluorimeter.

All the supernatants obtained from the centrifugation of the test and blank tubes were read from the fluorometer at the indicated extinction and emission

wavelengths. Differences between the fluorescence values of the test and blank reaction supernatants of the same microsomal preparation sample were computed and analyzed.

Pentoxoresorufin-O-depentylase (PROD)

The overall procedure, buffer and NADPH-generating cofactor solutions used in the pentoxoresorufin-O-depentylase assay were the same as that used in the ethoxoresorufin-O-deethylase assay. However, 7-pentoxoresorufin of concentration 1.25nmol in a total volume of 35.4ul was added to each tube after the pre-incubation, in place of the 7-ethoxoresorufin used in the EROD assay.

3.2.6 Statistical Analyses

The statistical analyses were performed using the Jandel SigmaStat statistical software, Version 2.0 (1992-1995). Statistical significance between values obtained for samples from the four (4) different groups of animals were evaluated by the student-Newman-Keul's ANOVA. The level of significance was set at $P < 0.05$ in all cases.

CHAPTER FOUR

RESULTS

4.1 Yield of freeze-dried plant extract

A volume of 10litres of crude aqueous plant extract of *D. adscendens* gave 38.25g of freeze-dried powdered plant extract. The concentration of the crude aqueous extract is therefore 3.825mg/ml or 0.3825%.

4.2 Acute toxicity studies

4.2.1 Observation of behaviour of animals

The animals administered with the 10, 8, 5, 3, and 1.0g/kg body weight of the plant extract showed the following physical behaviour: rounded up; piloerection (both fur and whiskers); shiny eyes; became agitated in the first 3-5 minutes and then less active; having diarrhoea; right fore-limb became immobilized and could not be used.

4.2.2 Lethality

The doses of the plant extract administered and the number of days elapsed before deaths of the two animals for each dose group are shown in Table 4a.

As indicated in the table (Table 4a), for the animals administered with an extract dose of 1.0g/kg weight dose, one of the animals died on day six but the other animal survived the 14-day period of observation and beyond.

Table 4a: Effect of dose of plant extract on time of death (no. of days)

of the two animals in each of the five doses administered.

No. of Days	Dose (g/kg body weight)				
	10	8	5	3	1
<1	1				
1	1	2			
2			1		
3			1	1	
4					
5				1	
6					1

The lethalties of different doses of the plant extract (0.5-1.75mg/kg body weight) are shown in Table 4b. It was observed that none of the animals died in the 0.5g/kg, one (1) animal died in the 0.75g/kg, three (3) animals died in both the 1.0 and 1.25g/kg, four (4) animals in the 1.50g/kg, and all the animals in the 1.75g/kg body weight dose groups died. Based on this result the 50% lethality over the period of observation was estimated to be 1.125g/kg (1125mg/kg) body weight.

4.2.3 Observation of some vital organs.

The livers of the animals that died after the administration of the plant extract in the acute toxicity studies had numerous dark brown spots over light brown surfaces compared to uniformly coloured dark brown livers in the control animals, which were not given the plant extract. The kidneys and digestive tracts of the treated animals were darker in colour than those of the control animals. The whitish-brown colours of the lungs in the treated animals were not as shiny as those in the control animals. In addition, these lungs also appeared to be wrinkled.

4.3 Subchronic toxicity studies

4.3.1 Body and organ weight changes

The average initial and final body weights, and the percentage average body weight gained by the two sets of animals used in this study are shown in Table 4c. The percentage average body weights gained were calculated as a percentage over the average initial weights of the animals. There were no differences in body weight gained between the male and female animals

Table 4b: Effect of dose of plant extract on lethality of animals in the acute toxicity test.

Dose (g/kg Body Weight)	No. of Death	% Lethality
1.75	6	100.0
1.50	4	66.7
1.25	3	50.0
1.00	3	50.0
0.75	1	16.7
0.50	0	0.0

N=6 for each dose group.

Table 4c: Effect of dose of plant extract on animal body weight expressed as percentage of the initial weights

(Female).

Weight	Zoxazolamine paralysis test groups		Thiopentone sleeping tests groups	
	Control	Test (Medium Dose)	Control	Test (Medium Dose)
Final	169 ± 18	159 ± 16	231 ± 14	205 ± 16
Initial	116 ± 4	112 ± 5	163 ± 11	143 ± 10
Final-Initial	53	47	68	62
% Gain	45.7	42.0	41.7	43.4

(Male)

Weight	Control	Low Dose	Medium Dose	High Dose
Initial	178 ± 10	165 ± 8	155 ± 6	152 ± 5
Final	270 ± 15	245 ± 17	233 ± 18	225 ± 16
Final-Initial	92	80	78	73
% Gain	51.7	48.5	50.3	48.0

The average weights of the excised livers and kidneys from the treated (low, medium and high dose) and control animals are shown in Table 4d. The weights of the organs are expressed as a percentage of the final body weight.

There were no significant differences in the organs of the different groups of animals when examined macroscopically, and this was confirmed when the results were analyzed statistically.

4.3.2 Zoxazolamine paralysis test

Table 4e shows the times of paralysis of the individual animals, and the average paralysis time of the test and control animals. A dose of 60mg/kg body weight of zoxazolamine dissolved in DMSO caused an average paralysis time of 3hours 27minutes in the control animals and an average of 2 hours 47minutes in the test animals, before they could turn to their normal posture after the administration.

4.3.3 Thiopentone Sleeping Test

For a dose of 100mg/kg body weight of the thiopentone administered, the test animals even though they looked very dizzy and wobbled when they tried to move, they could not be turned to lie on their back; they resisted being placed in that posture. On the other hand, the control animals slept and could be turned to lie on their back. The average sleeping time taken was 9 ± 2 min.

When the dosage was increased to 200mg/kg body weight (doubled) in both the test and the control groups, the animals had very long hours of sleep with about 60% of each group dying.

Table 4d: Effect of dose of plant extract on liver and kidney weights.

Dose groups of animal	Av. Weight of excised livers(g)	Weights of excised livers as % of body weight	Av. Weights of excised kidneys (g)	Weight of excised kidneys as % of body weight
Low dose	8.61 ± 1.27	3.51	1.31 ± 0.16	0.53
Medium dose	8.01 ± 1.69	3.44	1.28 ± 0.19	0.55
High dose	8.42 ± 1.42	3.74	1.31 ± 0.05	0.58
Control	8.80 ± 0.82	3.26	1.39 ± 0.14	0.51

Table 4e: Effect of plant extract on zoxazolamine-induced paralysis time.

Animals	Paralysis Time (mins)	
	Test Group	Control Group
1	157minutes	175 minutes
2	-	209 minutes
3	172 minutes	223 minutes
4	175minutes	230 minutes
5	164 minutes	200 minutes
Average paralysis time	167 ±10 minutes	207 ± 22 minutes

4.4 Clinical Chemistry Tests

4.4.1 Total Serum Protein

There were no statistically significant differences between the average total serum protein concentrations determined in the four (4) animal treatment groups (Figure 4a).

4.4.2 Alanine aminotransferase (ALT)

Alanine aminotransferase activities in the sera of the four (4) animal treatment groups increased with increasing dose levels of the plant extract (Figure 4b). Statistical analysis showed no significant differences between the control, low dose and medium dose groups. Differences between the high dose groups and the three (3) other groups were statistically significant ($P=0.002$).

The average ALT activity of the high dose groups was 69.6%, 52.2% and 47.8% more than those of the control, low dose and medium dose groups respectively.

4.4.3 Aspartate aminotransferase (AST)

γ -Aspartate aminotransferase activities in the sera of the four (4) groups of animals also showed increases with increasing dose levels of the plant extract (Figure 4c). There was no statistically significant difference between the enzyme activities of the low and medium dose groups. However, there was a statistically significant difference between the values for those two groups and the control group. There were also significant differences between the high dose groups and the three (3) other treatment groups ($p<0.001$).

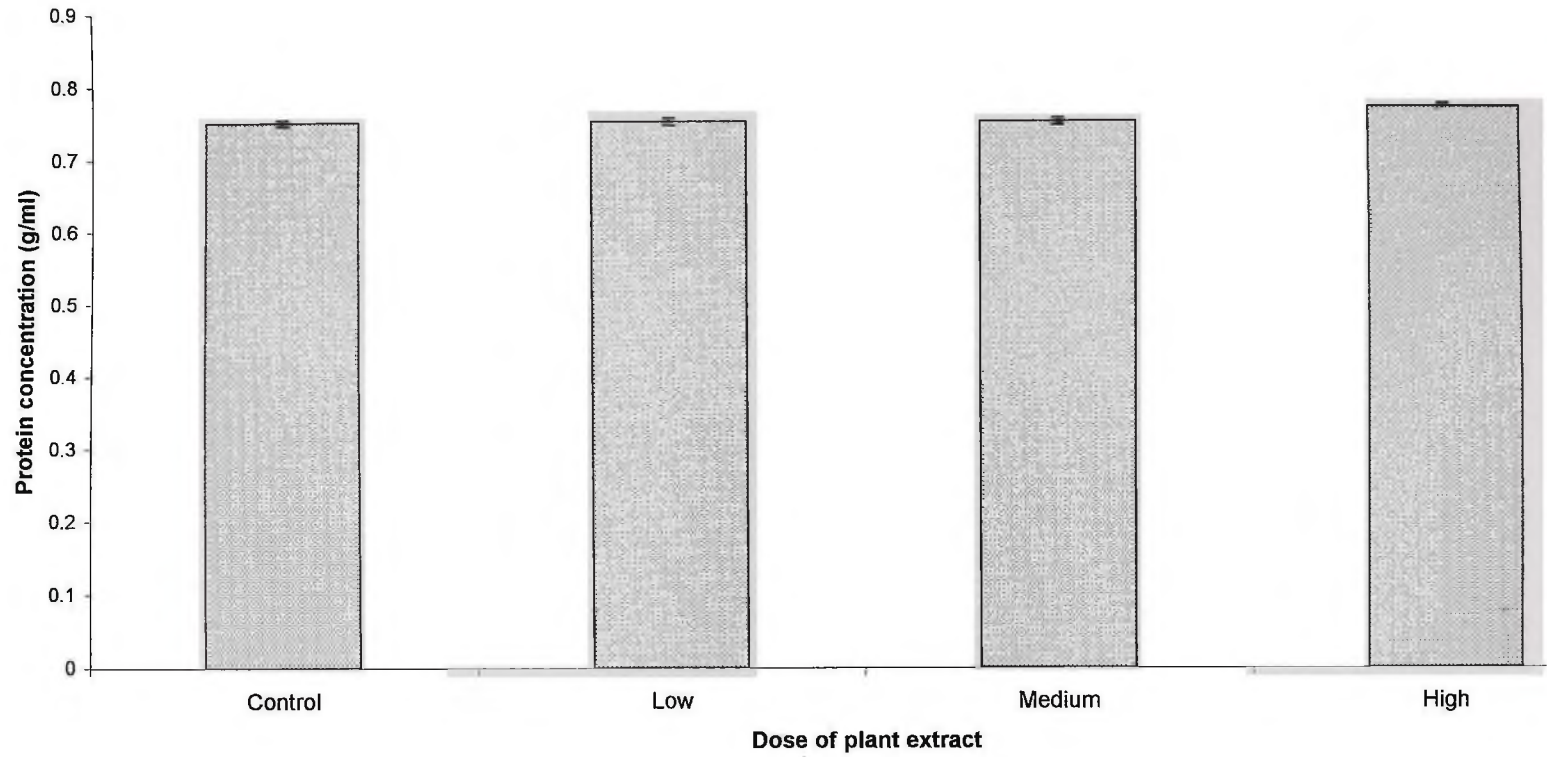
Figure 4a: Effect of plant extract on total serum protein concentrations

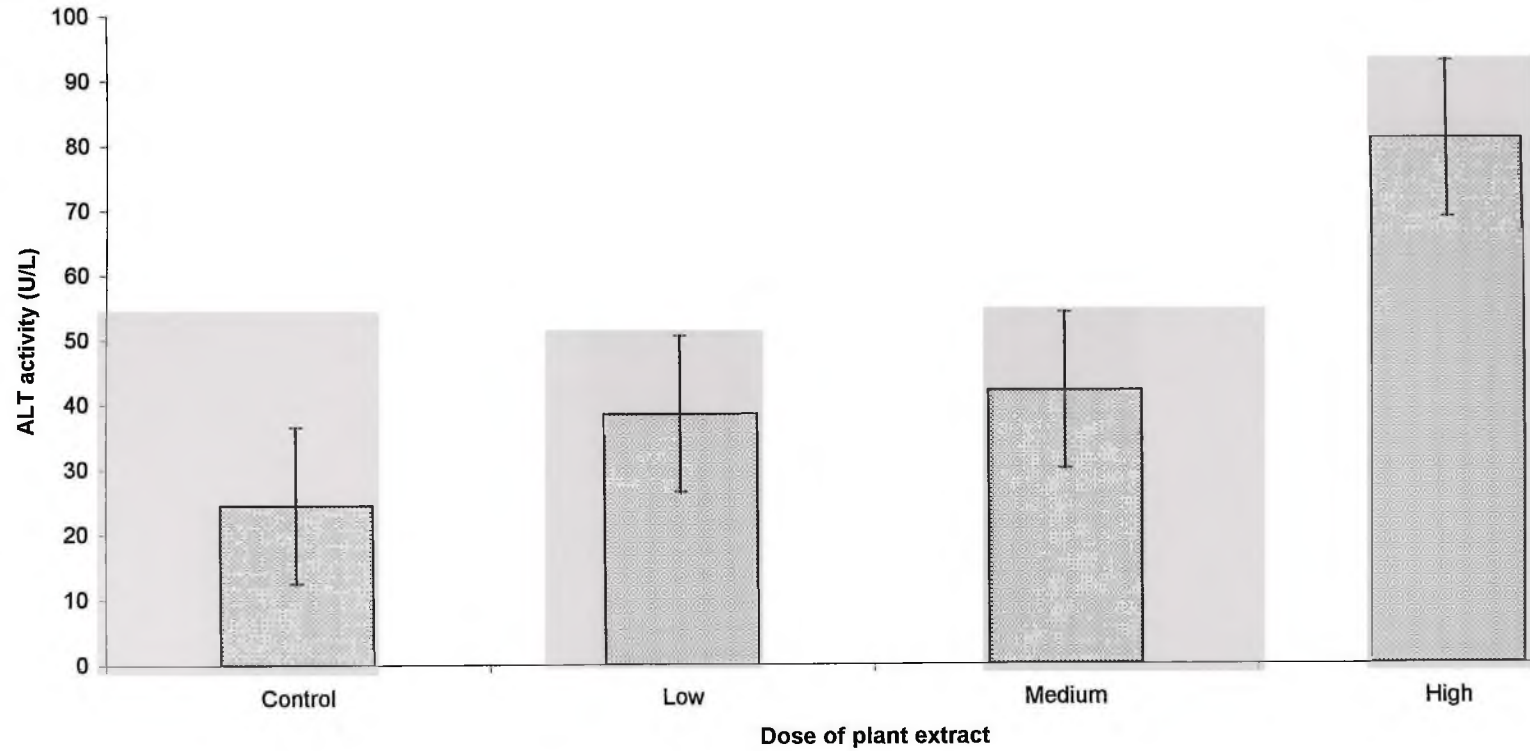
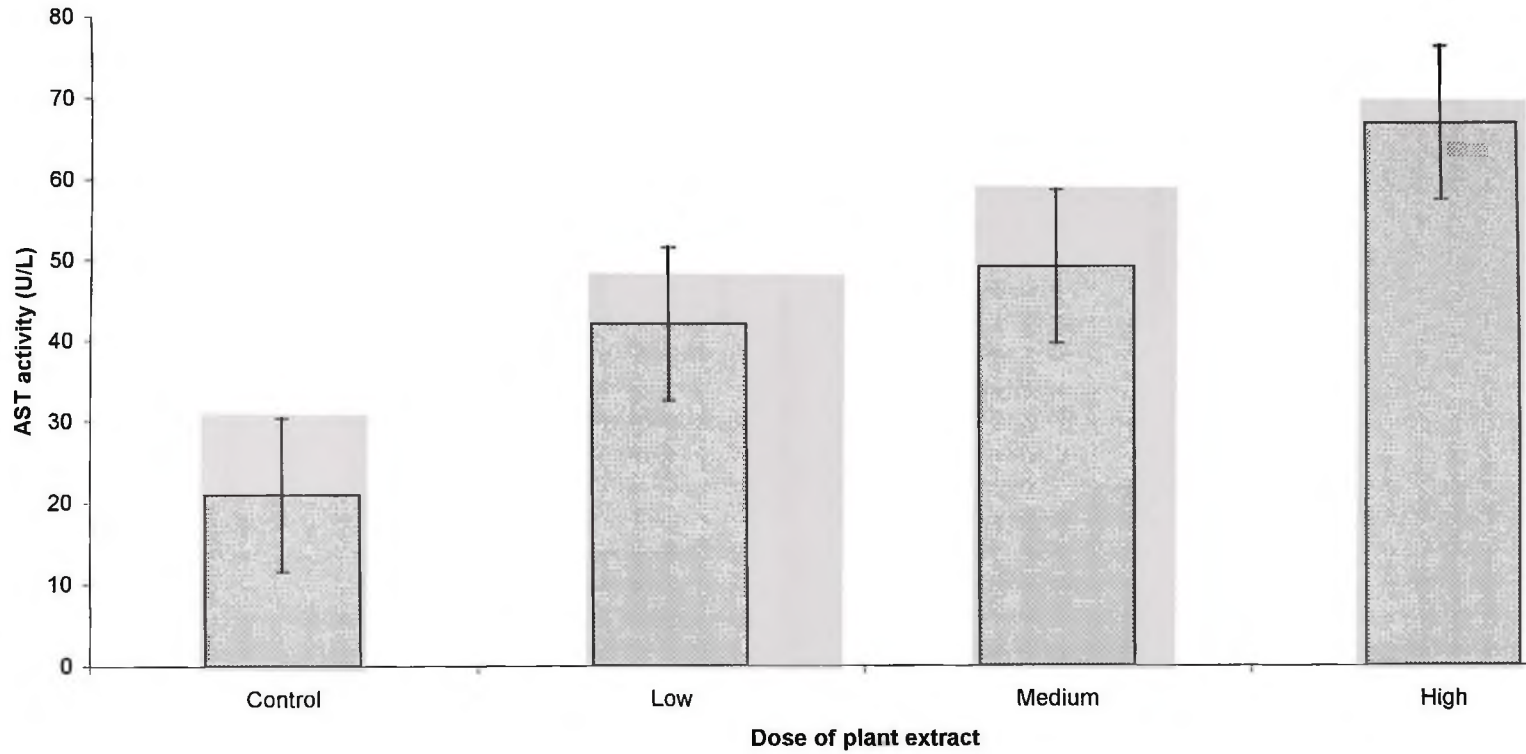
Figure 4b: Effect of plant extract on serum alanine aminotransferase activity

Figure 4c: Effect of plant extract on aspartate aminotransferase activity

4.4.4 Gamma-glutamyltransferase (GGT)

The results of the γ -glutamyltransferase activities determined in the sera of the four (4) animal treatment groups are shown in Figure 4d. There were statistically no significant differences between the average γ -glutamyltransferase activities of the various groups of animals, even though there were slight increases in the activities with increasing doses of the plant extract.

4.4.5. Total bilirubin

The results of the total bilirubin concentration determination in the sera of the four (4) animal treatment groups are shown in Figure 4e. Even though there were minimal increases in the total bilirubin concentrations with increasing dose levels of the plants extract, statistical analysis showed no significant differences between the average total bilirubin concentrations of the various treatment groups.

4.4.6. Direct (Conjugated) bilirubin

The direct bilirubin concentrations determined in the sera of the four (4) animal treatment groups are shown in Figure 4f. Statistically significant differences existed between the high dose group and the three (3) other groups of animals ($p=0.011$), but no significant differences between the control, low dose and medium dose groups.

The average direct bilirubin concentration of the high dose group was 36.3%, 33.4% and 30.3% more than those of the control low dose and medium dose groups respectively.

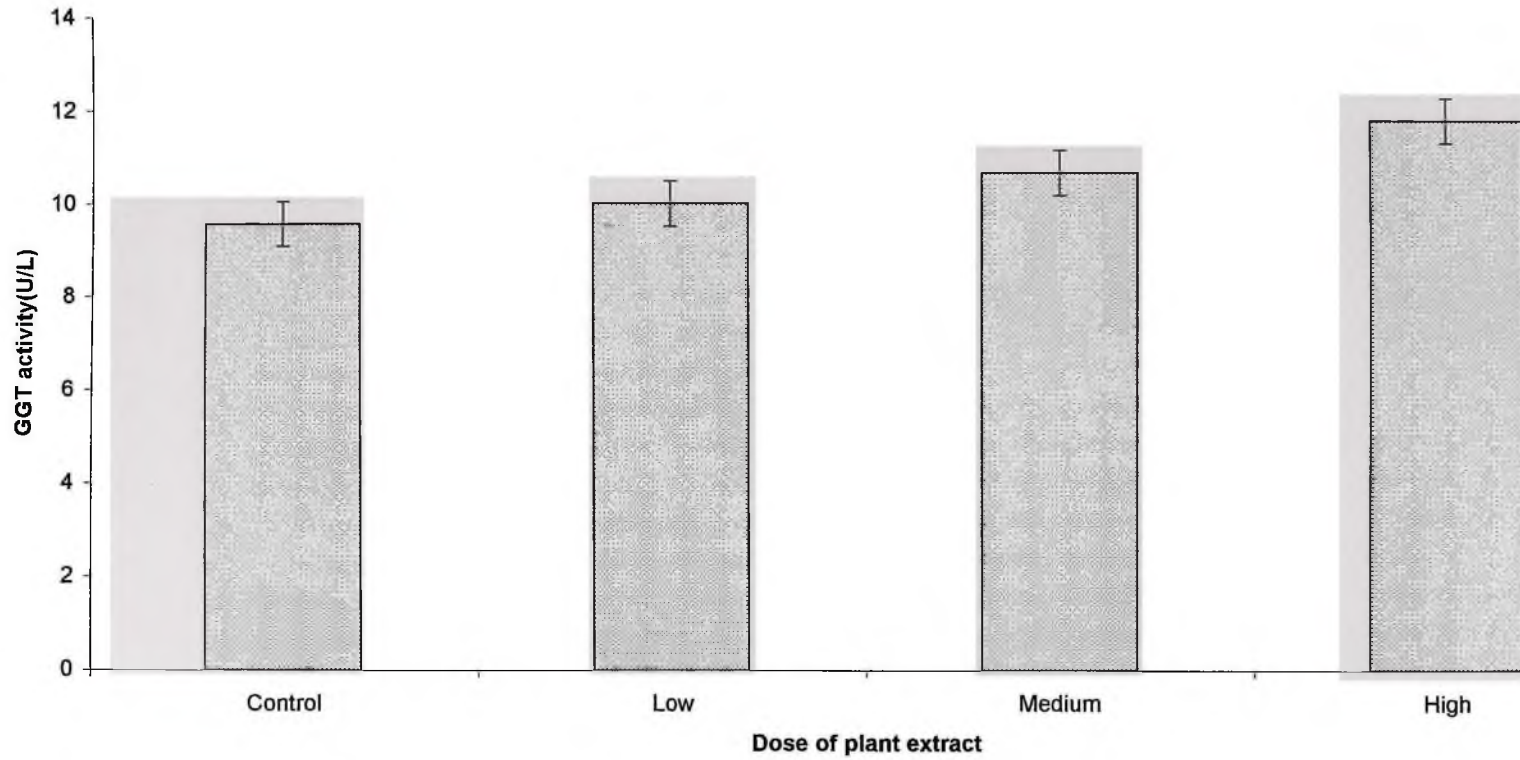
Figure 4d: Effect of plant extract on serum gamma-glutamyltransferase activity

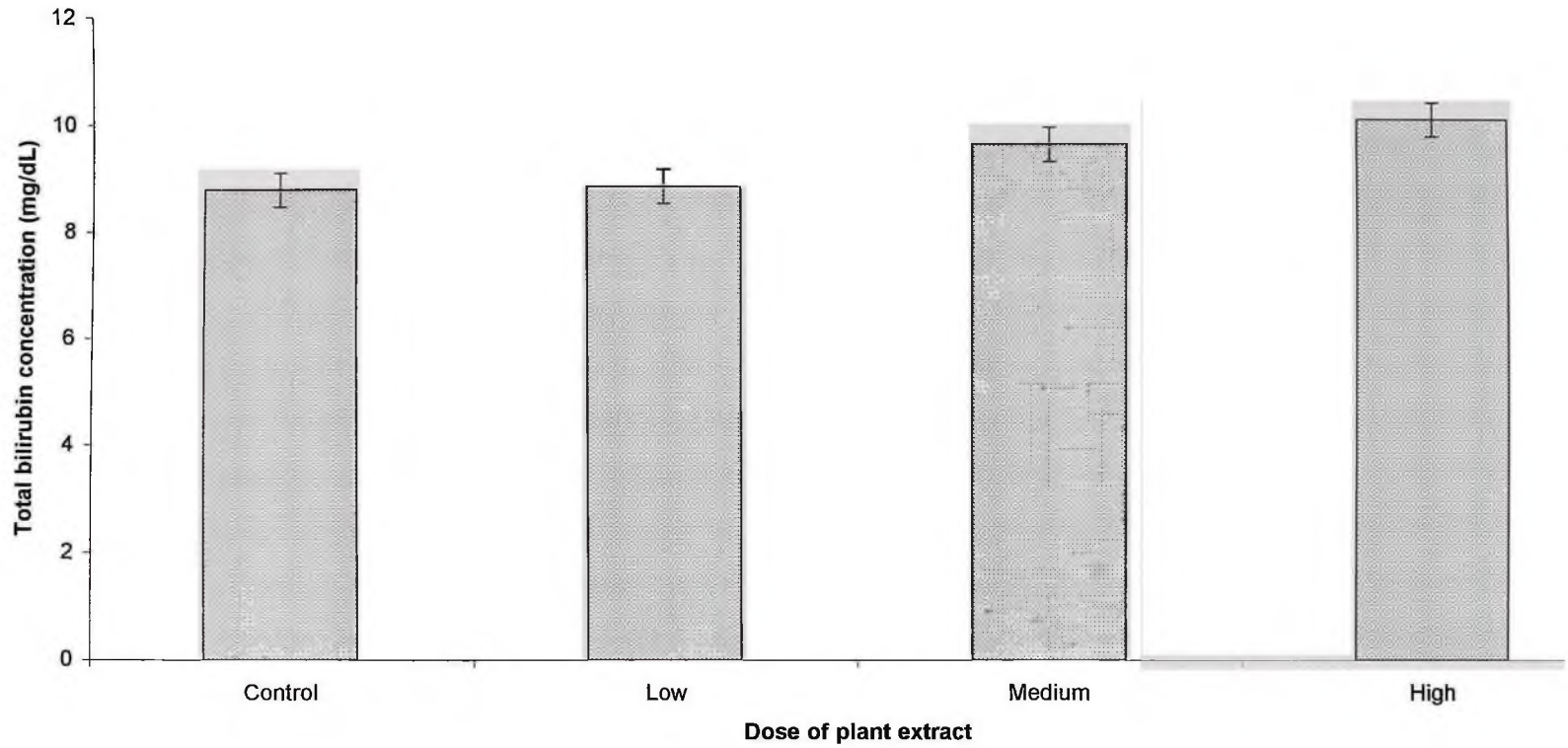
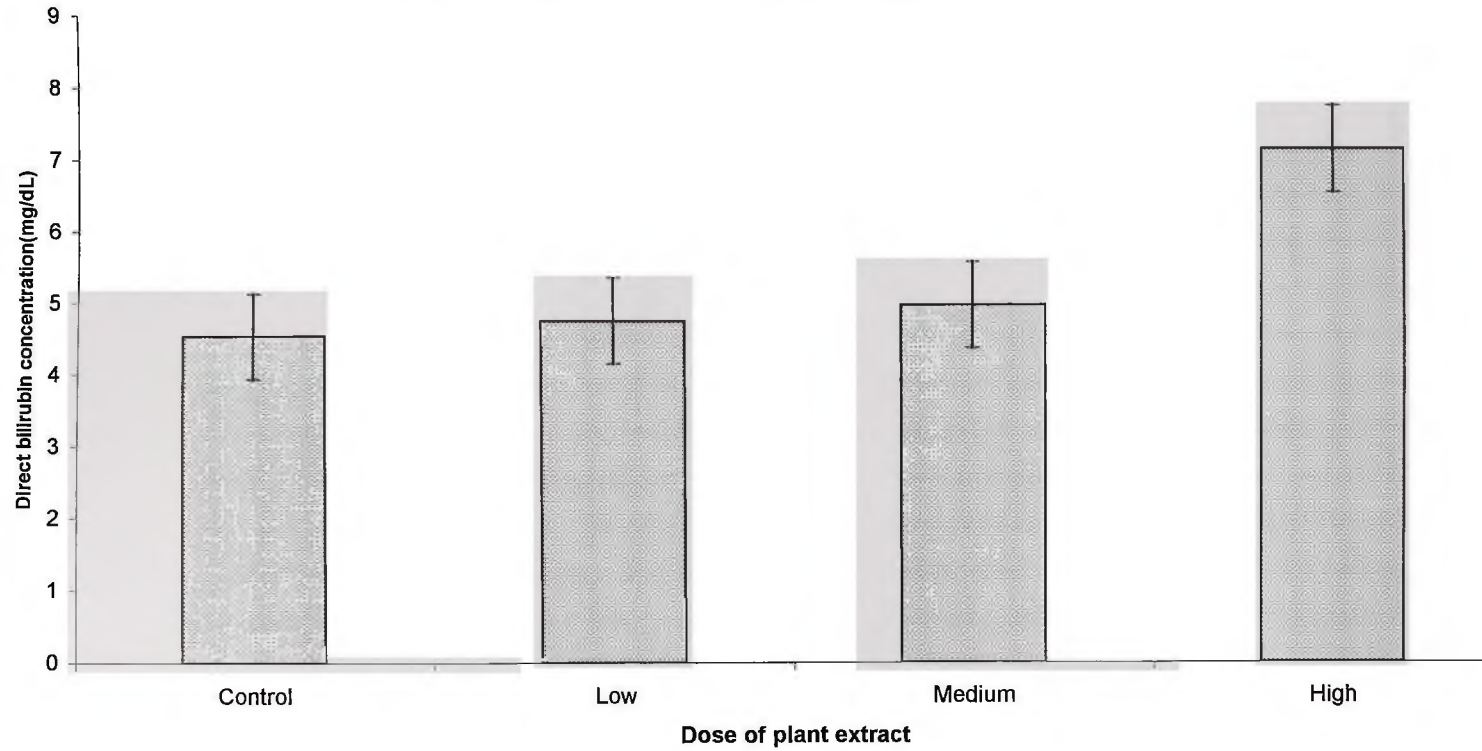
Figure 4e: Effect of plant extract on total serum bilirubin concentrations

Figure 4f: Effect of plant extract on direct serum bilirubin concentrations

4.4.7 Creatinine

There were no statistically significant differences between the average creatinine concentrations determined in the sera of the four (4) animal treatment groups (Figure 4g).

4.4.8 Blood urea nitrogen (BUN)

There were also no statistically significant differences between the average blood urea nitrogen concentrations determined in the sera of the four (4) animal treatment groups (Figure 4h).

4.5 Biochemical Assays

4.5.1 Protein concentrations of microsomal preparations

Figure 4i shows the protein calibration curve from which the protein concentrations of the microsomal preparations were estimated.

The average protein concentrations of the microsomal preparations as estimated from the protein calibration curve and corrected for the dilution factors are shown in Figure 4j. Statistical analysis indicated no significant differences between the control, low, and medium dose groups, but there were significant differences between the high dose group and the three (3) other groups. The high dose group had an average concentration, which is 49.5% more than that of the medium and low dose groups, and 53.5% more than that of the control group.

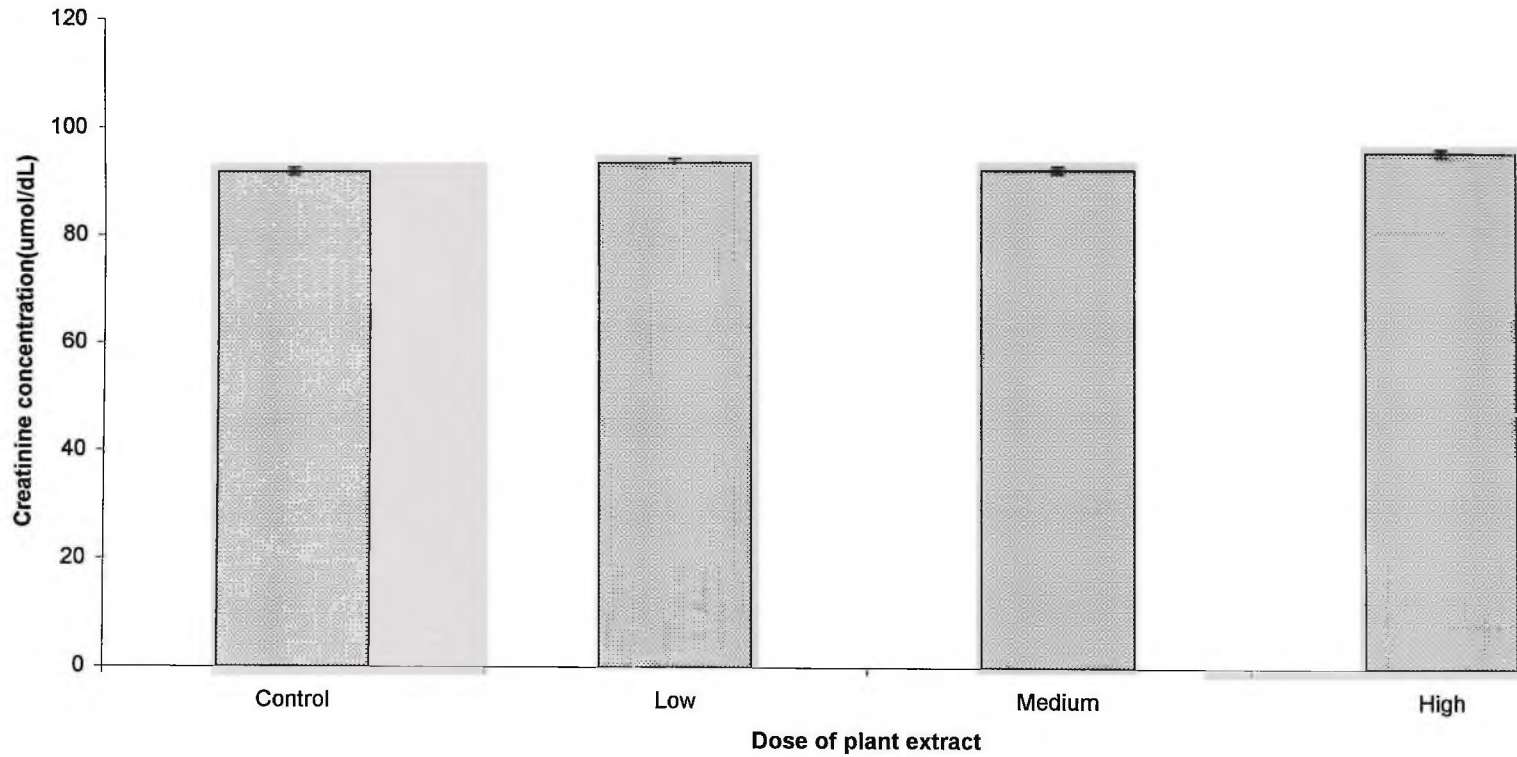
Figure 4g: Effect of plant extract on serum creatinine concentrations

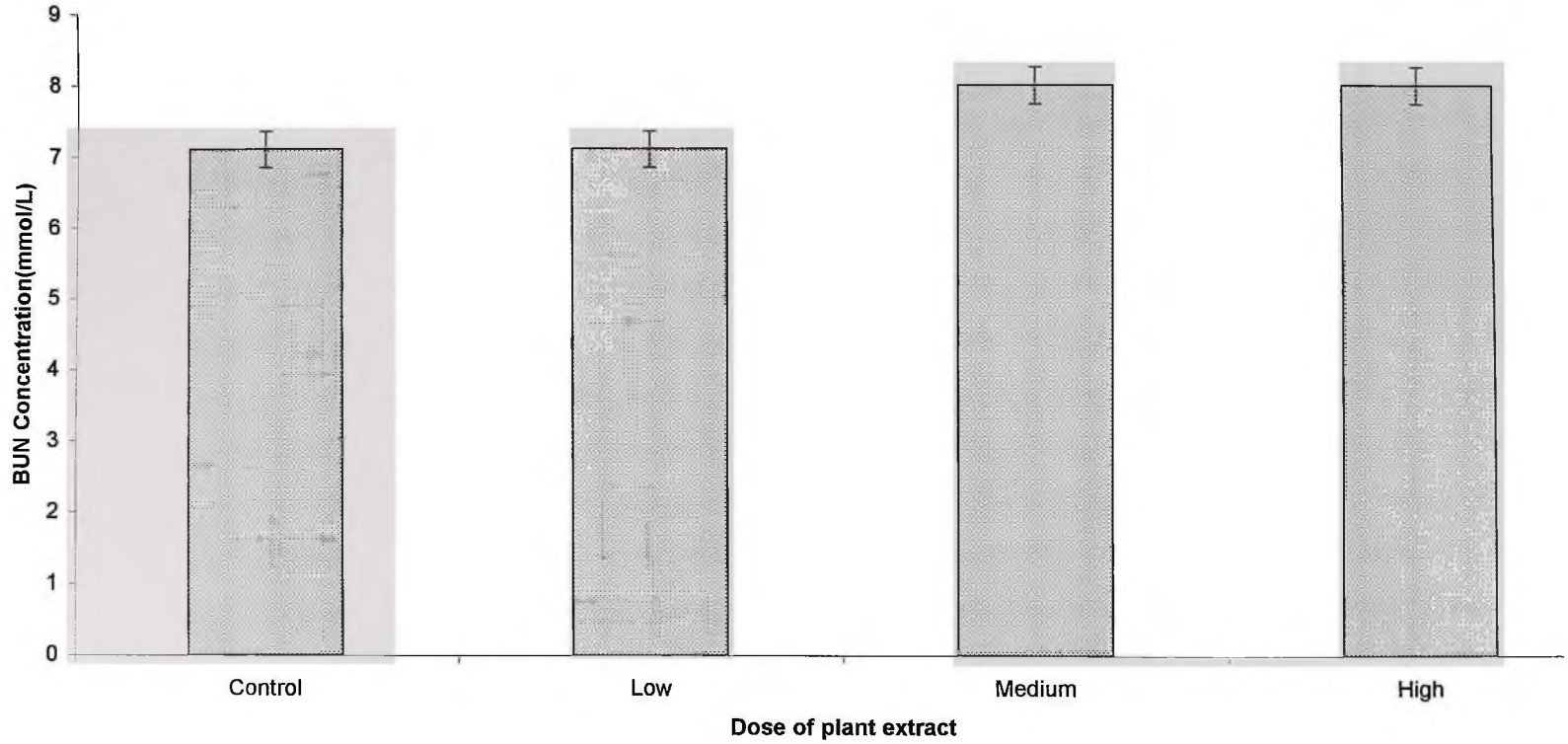
Figure 4h: Effect of plant extract on serum blood urea nitrogen concentrations

Figure 4i: Standard protein calibration curve

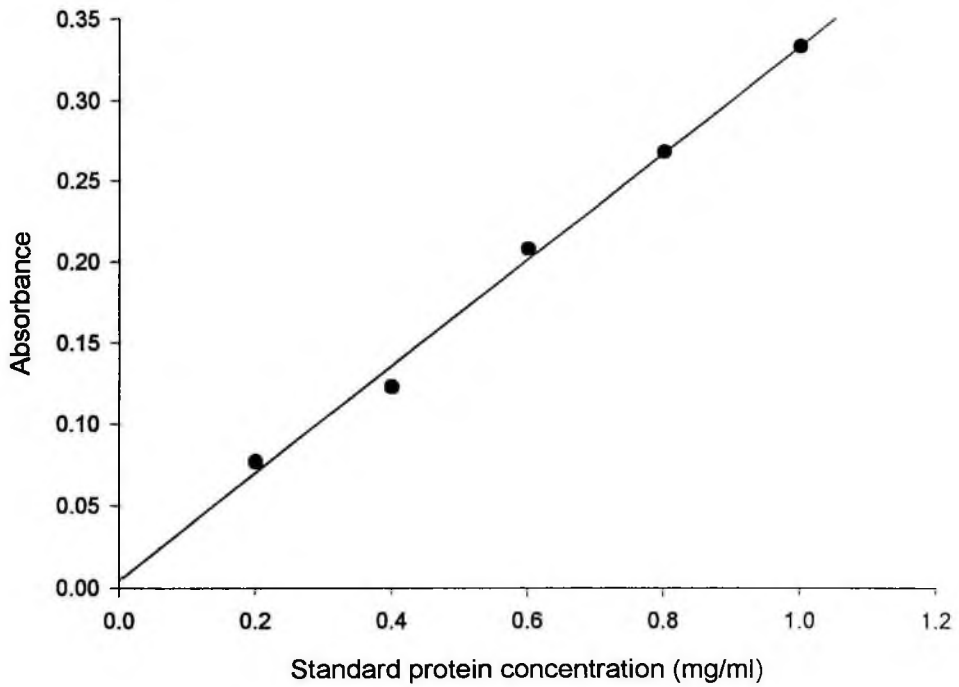
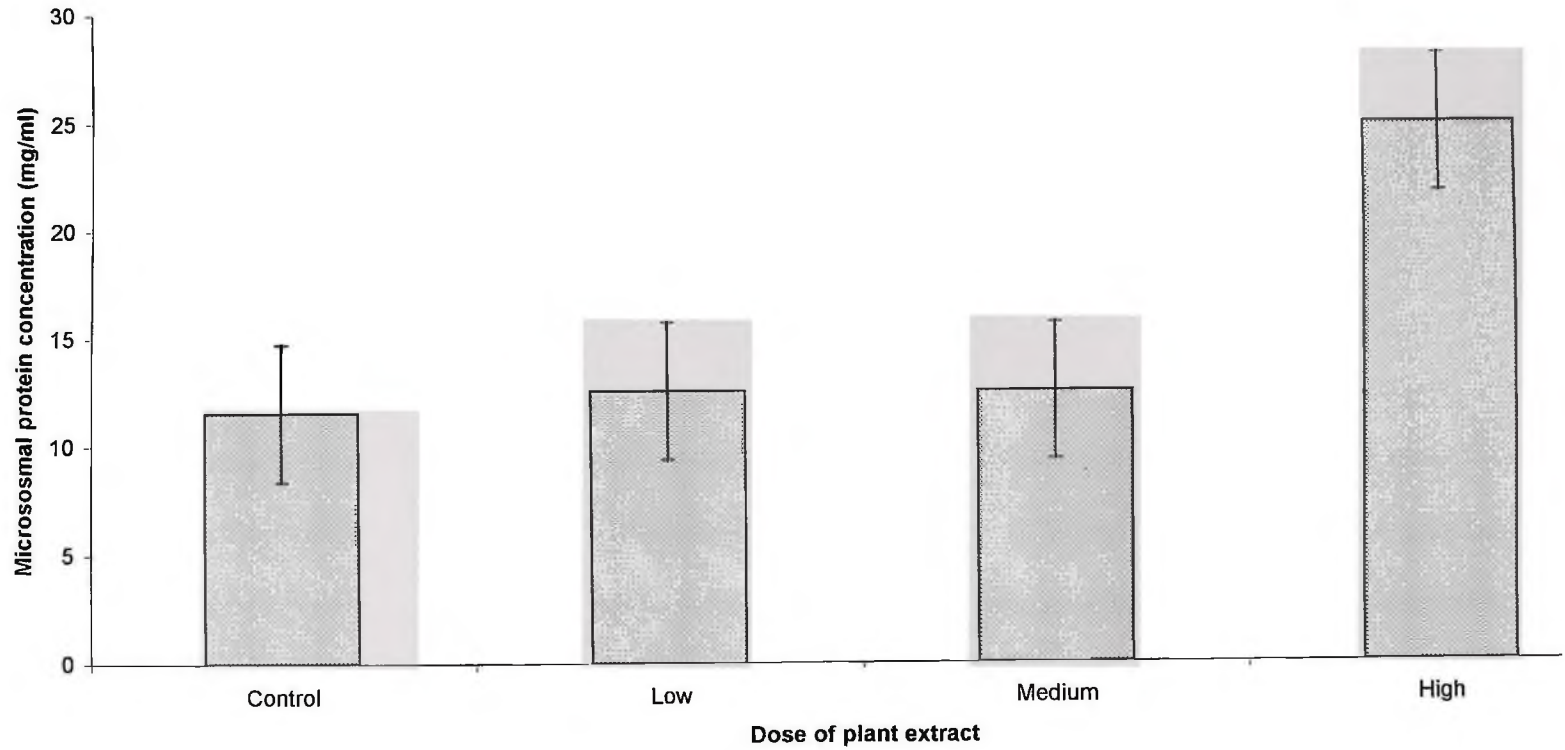


Figure 4j: Effect of plant extract on hepatic microsomal protein concentration

4.5.2 p-Nitrophenol hydroxylation

The microsomal cytochrome P450 monooxygenase enzyme activity measured as p-nitrophenol hydroxylation in the four (4) animal treatment groups decreased with increasing dose levels as shown in Table 4f. Statistical analysis of the results showed significant differences between control and other treatment groups except the low dose group. The p-nitrophenol hydroxylation activities of the low, medium and high dose groups as percentage inhibition of the control group are shown in Figure 4k.

4.5.3 Ethoxyresorufin-O-deethylase (EROD)

The results of the hepatic microsomal CYP450 monooxygenase enzyme activity measured as ethoxyresorufin-O-deethylase in the four (4) animal treatment groups, showed very minimal increases with increasing dose levels (Figure 4l). There were statistically no significant differences between the average ethoxyresorufin-O-deethylase activities of the different groups of animals.

4.5.4 Pentoxyresorufin-O-depentylase (PROD)

The results of the hepatic microsomal enzyme activities, measured as pentoxyresorufin-O-depentylase in the four (4) animal treatment groups, showed increases in activity with increasing dose levels (Figure 4m). There was no statistically significant difference between the control and low dose groups, nor between the medium and high dose groups. However, there were significant differences between the control and low dose groups on one hand and the medium and high dose groups on the other hand ($P=0.001$).

Table 4f: Effect of plant extract on p-nitrophenol hydroxylase activity.

Animal	p-nitrophenol hydroxylation activity (pmol/min/mg protein)			
	Control	Low Dose	Medium Dose	High Dose
1	546.69	551.56	470.28	258.75
2	552.53	470.82	309.34	162.45
3	457.20	638.13	503.89	216.93
4	585.60	414.40	330.74	142.83
5	428.01	449.42	319.07	208.17
Av. Activity	514.01±86.00	497.66±140.47	386.77±117.12	199.05±59.70

Average activity = Mean ± SD/SEM of n = 5

Figure 4k: Effect of plant extract on p-nitrophenol hydroxylase activity of the three (3) different dose groups of animals expressed as percentage inhibition of the control

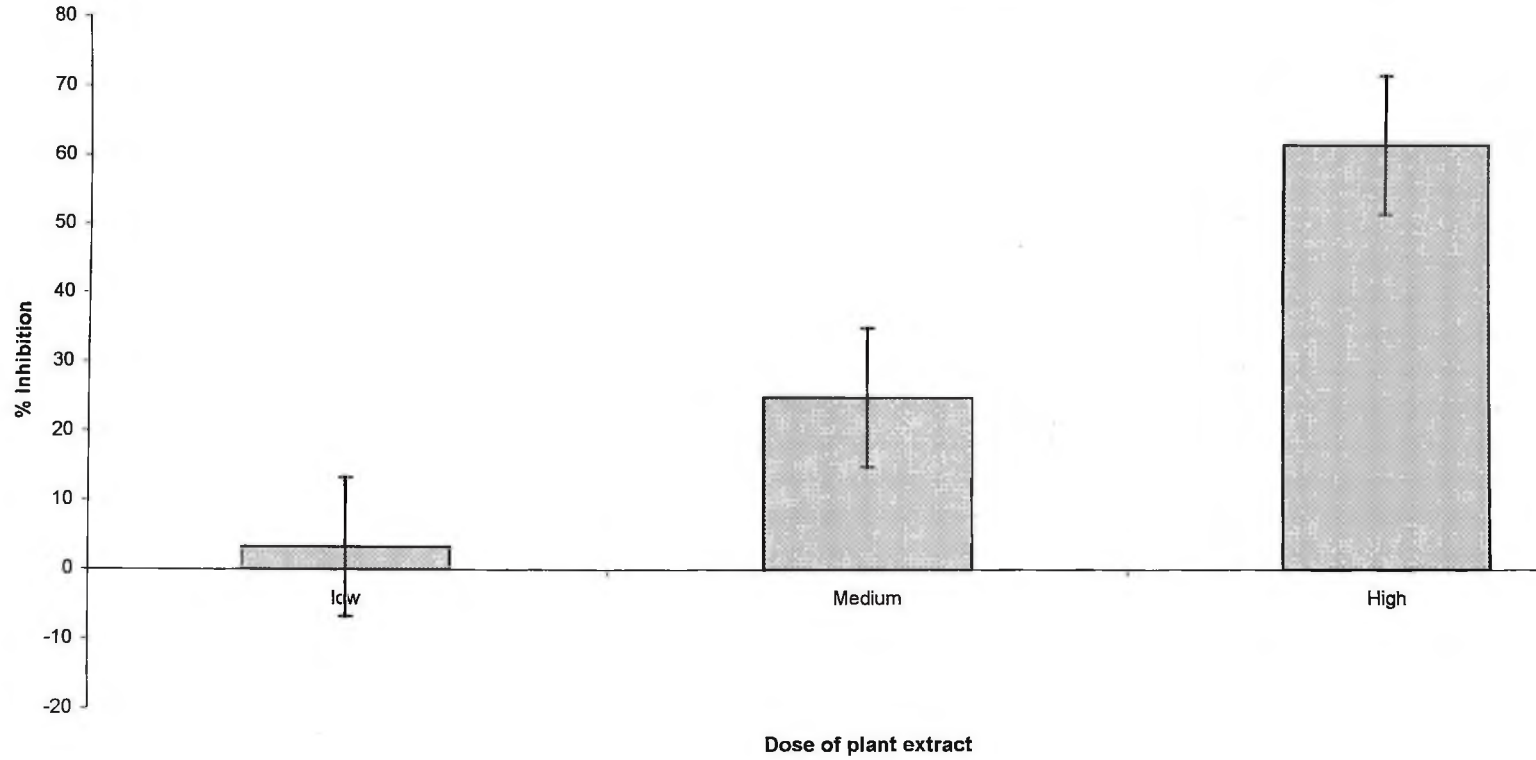


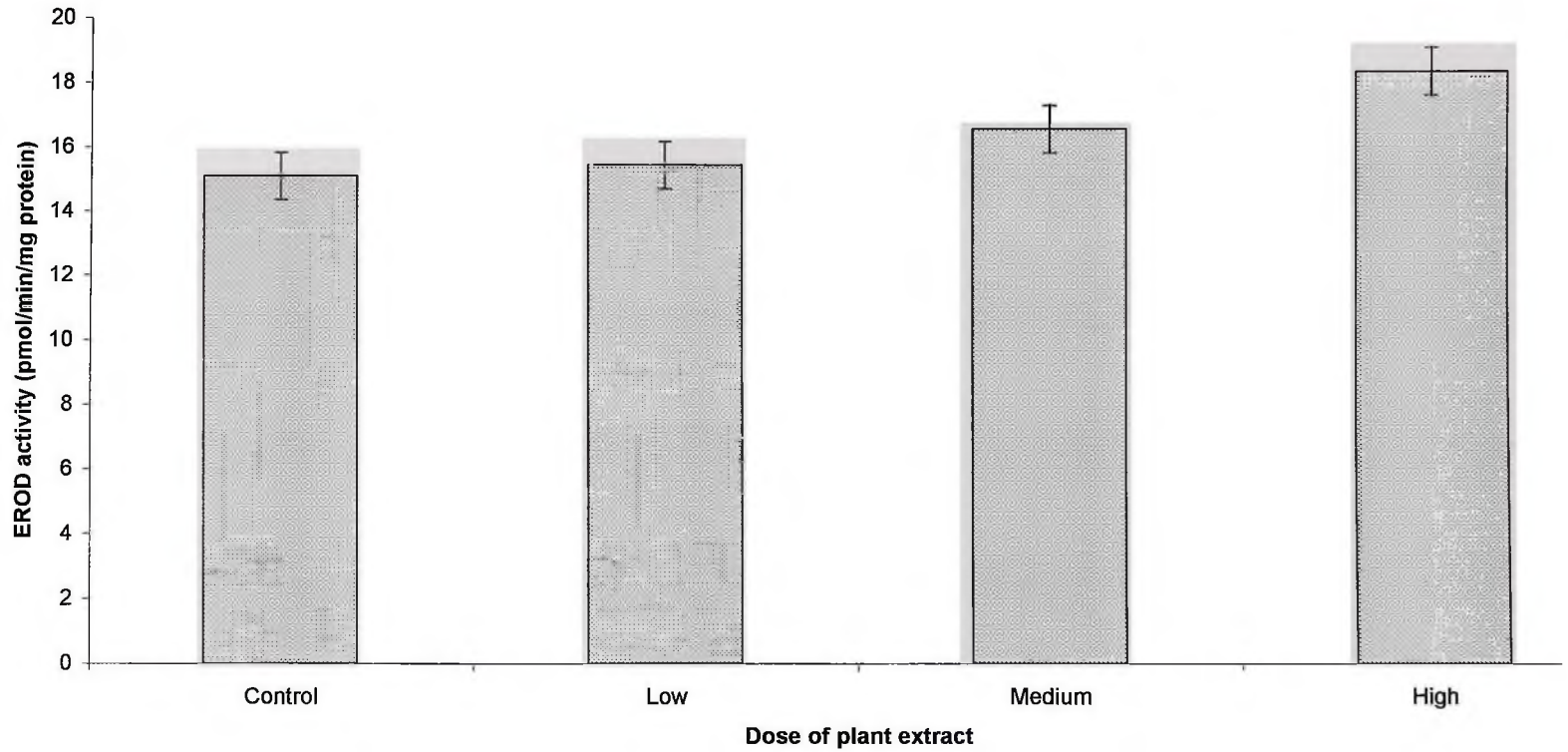
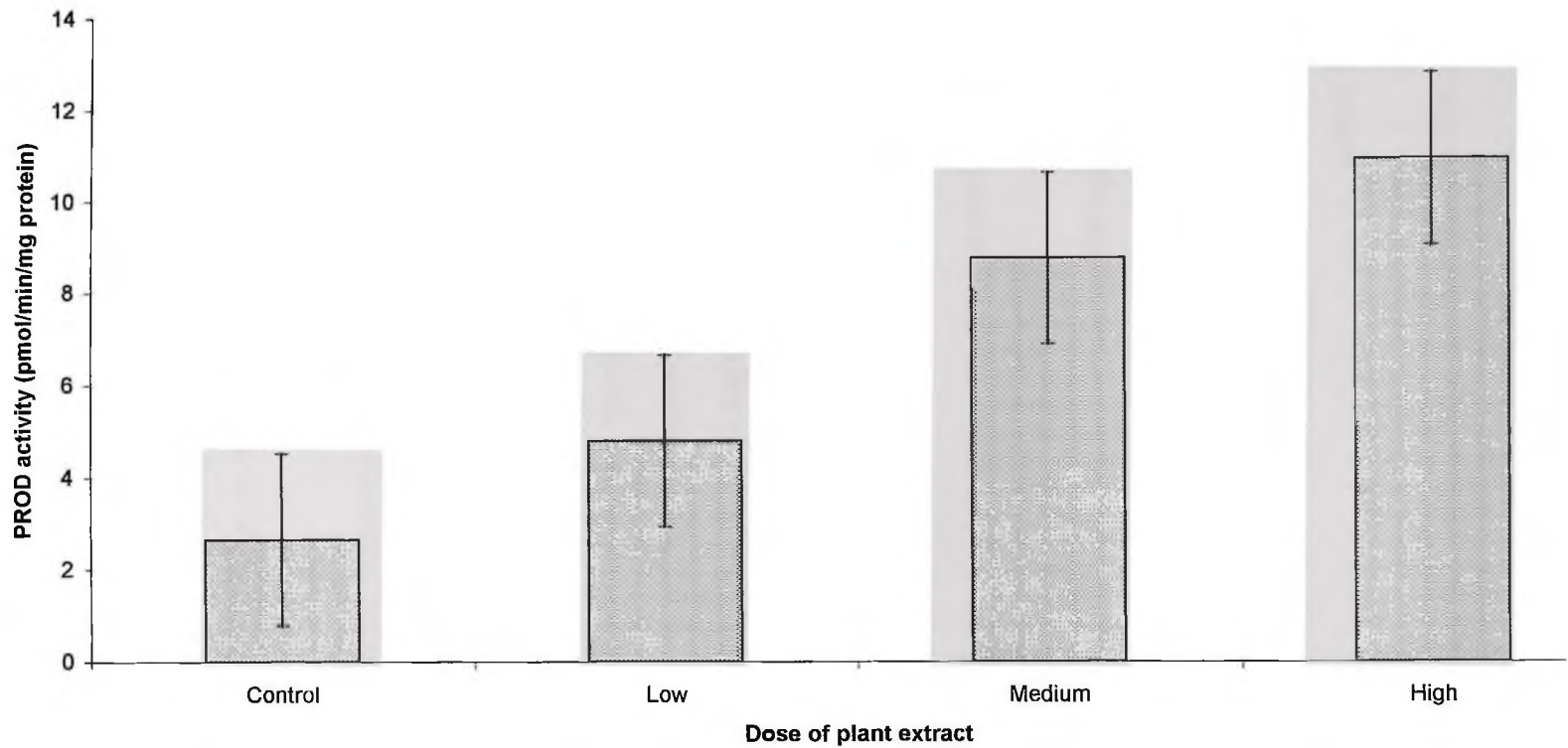
Figure 4l: Effect of plant extract on hepatic microsomal EROD activity

Figure 4m: Effect of plant extract on hepatic microsomal PROD activity

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

The inception of the use of drugs dates back to prehistoric times but drug usage or ingestion of substances for therapeutic purposes today can still cause impaired physiological functions or even lead to death (Echobichon, 1992). It is based on the dangers of drug usage that investigations are carried out to determine how safe or toxic a particular substance is.

Research into medicinal plant preparations has become necessary to validate their potency and efficacy for the diseases or conditions that the herbalists claim to be treated or managed by these preparations. However, the research involves very little or nothing about the possible toxicity of these herbal preparations, almost all of which are used in their crude forms. Crude herbal preparations used for therapeutic purposes can also be toxic, thus the need to investigate their toxicological effects.

The present study on *D. adscendens* was therefore carried out to determine whether apart from its several medicinal uses, the plant preparation has any toxicological effects. Studies on the chemical composition (McManus *et al.*, 1993; Asante-Poku *et al.*, 1988), efficacy (Addy and Burka, 1989; 1988) and mode of action (Addy and Schwartman, 1995; 1992) of the plant extract have been done, but no study has been done on its toxicological effects.

In the therapeutic use to manage asthma, *D. adscendens* is administered orally, thus the oral administration of the plant chosen in this study. The choice of experimental animals (rats) was based on the fact that, in terms of related organisms, rats are among

the species of animals that are related to human with similar physiological and biochemical conditions. Other experimental animals that could have been used include mice, guinea pigs, and rabbits.

In this study, two levels of toxicity tests were used, acute and subchronic toxicity tests. These were done to determine the effect of the plant extract when single high doses are administered, and when relatively lower doses are administered over a period of time. Liver and kidney function tests were used to determine any toxicological effects in the subchronically treated animals because the organs are the most probable ones to be involved in the metabolism and clearance of the plant extract. Therefore damages of tissues that may be caused by the plant extract are likely to occur in these organs. Specific analytes and enzyme activities in serum were measured to aid in the determination of such damages due to the administration of *D. adscendens*.

The assays for cytochrome P450 dependent enzymes were used because the enzyme system has been shown to be involved in the metabolism of many substances foreign to the body, and these include herbal preparations or phyto-medicines (Gillette, 1966). Induction of these isozymes provides useful information about the routes of metabolism of a drug or herbal medicinal preparation. In this study, the cytochrome P450 dependent enzyme assays were used to determine the effect that the plant extract could have on other drugs when co-administered with such drugs.

One of the aims of the study as indicated in this thesis was to find isozyme of drug-metabolizing enzymes that may be affected by the plant extract in its use for the management of asthma. More of the different cytochrome P450 dependent enzyme assays could have been done but due to lack of reagents and equipments only p-

nitrophenol hydroxylation, ethoxyresorufin-O-deethylase and pentoxyresorufin-O-depentyldase assays were done. Again, the isozymes induced due to the plant extract could have been identified using immunological techniques but the reagents necessary for this were not available during these investigations.

The crude aqueous extract of *D. ascendens* was concentrated and freeze-dried into a powdered extract so that the dosages administered into the experimental animals could be quantified. As indicated in the results, the concentration of the crude aqueous extract was 3.826mg/ml. The recommended dosage of the aqueous extract for adult patients as prescribed by the Centre for Scientific Research into Plant Medicine (CSRPM), where it is dispensed is three (3) tablespoonfuls per day, a volume of about 45ml. This volume contains approximately 172mg of the extracted plant material. The average body weight of an adult human being is 70kg, making the prescribed dosage of the crude aqueous extract to be approximately 2.5mg/kg body weight per day.

The 50% lethality estimated was 1.125g/kg (1125mg/kg) body weight. This means that for an average human being of 70kg body weight, a dose of 78.54g of the dry extract should be taken to cause 50% death; assuming that both rats and humans have the same rate of metabolism. This dose is about 456 times the dose prescribed at CSRPM, making this plant preparation therapeutically very safe. Interspecies comparisons of drug metabolism have been questioned by Echobichon (1992). However, a comparable acute toxicity data for *D. adscendens* in humans cannot be established for ethical reasons.

On obtaining the estimated 50% lethality dose, one-tenth the value was used as the medium dose in the subchronic treatment, and the low and high doses were 0.03 and 0.3 of the value respectively. This was to ensure that, the dose range within which the

extract was going to be administered was fairly safe. A medium dose of 112.2mg/kg, a low dose of 33.75mg/kg, and a high dose of 337.5mg/kg body weights per day were therefore used in the subchronic treatment. In some studies done on *D. adscendens*, a dose range of 30 to 100mg/kg body weights per day were used (Dadzie-Mensah, 2000; Afflu, 1997; Nanabenyin, 1997) and found to be effective. These doses fall within the safe dose range obtained and used in this experiment.

In the acute toxicity test in which the animals were administered with very high doses of *D. adscendens*, the animals were observed for behaviours that indicated signs of toxicity of the plant extract. The observed rounding up and piloerection are symptoms of toxicity of the autonomic nervous system. The aggressive behaviour or agitation and severe depression are symptoms of toxicity of the central nervous system. Immobilization (lameness) of the right fore-limb (righting reflex), and increased sensitivity are symptoms of damage to the sensory system. The presence of diarrhoea is a symptom of toxicity to the gastrointestinal system (McNamara, 1976). Therefore the high doses of the plant extract affected certain key organs in the body leading to the behaviours and conditions observed, and the eventual death of the animals. All of the doses except the 1.0g/kg body weight were much higher than the 50% lethality dose, which was very important in determining the dose range of the extract that would be safe. It also provided good information about the potential dangers of the extract when administered at high doses.

The observations following autopsy of the animals that died after the high dose administration, suggest that probably the liver and the lungs were affected by the plant extract. The relatively darker colouration of the kidneys and the digestive tracts of the

treated animals could be due to the dark coloured nature of the extract. However, from the observations made on the behavioural patterns of the animals, it is obvious that other organs or tissues were affected but these could not be identified, as only visible differences or changes between the treated and the control groups were used in identifying the affected organs.

Total serum protein concentration is one of the clinical chemistry tests used in determining liver function (Henry *et al.*, 1974). This is because destruction of liver cells will lead to the release of enzymes and other proteins synthesized in the liver into the blood. These go to increase the protein content of the blood. It is therefore expected that for liver cell damage due to *D. adscendens*, the protein content of the blood will increase. However, the differences between the average total serum protein concentrations measured in the four (4) groups of animals were not statistically significant. This implies that there is no release of enzymes from the liver as a result of hepatic damage due to the plant extract.

The average ALT activity of the medium dose group was close to 50% of the high dose group. In the aspartate aminotransferase determination, the results also showed increases in the average AST activity with increasing dose levels of the plant extract. The results of the average ALT and AST activities suggest some level of tissue damage at the high dose of the plant extract. However, since tissues other than the liver contain these enzymes, the elevation in their activity may or may not be due to liver tissue damage. It has also been reported that hundreds of drugs and other substances affect ALT and AST activities, which may not necessarily be due to tissue damage (Tryding *et al.*, 1996). However, possible hepatocellular damage may have occurred.

The results of the γ -glutamyltransferase measured showed statistically no significant differences between the average enzyme activities in the four (4) groups of animals. Gamma-glutamyltransferase is one of the enzymes whose activity is specific and indicative of liver cell damage (Craig and Stitzel, 1992). It can therefore be inferred from the results that there was no liver tissue damage caused by the plant extract.

There were also no statistically significant differences between the results of the total bilirubin, which showed minimal increases in concentration with increasing dose levels of the plant extract in the different groups of animals. The statistically non-significant differences in the average total bilirubin concentrations suggest no hepatotoxicity due to the plant extract.

The results showed statistically significant differences between the average direct bilirubin concentrations of the high dose group and the other three (3) groups. The average concentration of the high dose group was at least 30% more than those of the other dose groups. The high concentration of the direct bilirubin in the high dose group may be due to the induction of Phase II xenobiotic-metabolizing enzymes (which are conjugating enzymes) in response to high bilirubin levels probably as a result of RBC haemolysis or due to hepatic excretory dysfunction.

The creatinine and blood urea nitrogen results showed no significant differences in their average concentrations when analyzed statistically. The creatinine and blood urea nitrogen concentrations were used as biochemical indices of renal damage. The results therefore suggest that the plant extract administered chronically had no adverse effect on the kidney, even at high dose levels.

From the zoxazolamine paralysis test, a shorter paralysis time was recorded for the test group compared to the control group as shown in Table 4e. It has been documented that chronic use of some medications may modulate cytochrome P450 isozymes, which terminate the effects of drugs such as pentobarbital (PB) and zoxazolamine, or deplete glutathione (GSH), which scavenges reactive metabolites (Pratt and Taylor, 1990). It is therefore inferred from this study that certain isozymes of cytochrome P450 that can also metabolize zoxazolamine were induced by the chronic administration of the plant extract. This caused a higher rate of metabolism of the zoxazolamine when administered, leading to the shorter time recorded for the treated animals to turn to their normal posture as compared to the relatively longer time recorded for the control animals. Therefore one would expect that when zoxazolamine or drugs with the same route of metabolism are administered to patients taking *D. adscendens* regularly, there would be drug-drug interaction, which could lead to the drug being metabolized faster, and therefore being therapeutically ineffective and possibly toxic.

In the thiopentone sleeping test, just like in the case of the zoxazolamine paralysis test, the results tend to indicate that certain isozymes of cytochrome P450 that can also metabolize thiopentone are induced by the chronic administration of the plant extract. The cytochrome P450 isozymes induced metabolize thiopentone as soon as it is administered and therefore effective blood levels that will cause the animals in the test group to go to sleep completely are not achieved. It is therefore likely that in the same way as it was with the zoxazolamine, simultaneous administration of thiopentone or drugs with similar route of metabolism, together with *D. adscendens*, will result in drug-drug interaction, which will interfere with the therapeutic effect of the drug. On doubling

the dose of the thiopentone administered, about 60% of the animals in each group (both test and control) died after very long hours of sleep. This indicates that with the double dose, there was enough drug unmetabolized. This was too high and therefore toxic, causing that high percentage of death. Thiopentone is on the country's essential drug list as a general anaesthetic. Such an interaction is important as higher doses may be required to put patients on this preparation to sleep.

If a subject who is taking the plant extract and these types of drugs (zoxazolamine and thiopentone) is a poor metabolizer (PM) of the drug, he/she is less likely to suffer from taking these drugs than one who is a poor metabolizer but is not on the extract. The latter could have toxic effects from overdose.

The results of the p-nitrophenol hydroxylation showed a decrease in activity with increasing dose levels of the plant. This indicates that increases in dose levels caused an inhibition of the activity of the enzyme involved in the p-nitrophenol hydroxylation or its induction. The inhibition was dose-dependent. Such inhibition would mean that if a drug, which is metabolized by this same enzyme is administered to a subject who is also taking the plant extract to control an asthmatic condition, the drug will be metabolized at a slower rate. The drug will therefore remain in the blood stream for a longer period of time than it should have been, leading to higher concentrations, which could be toxic if the potent drug is toxic. On the other hand, if this subject is an extensive metabolizer (EM) of a drug such that the drug's expected therapeutic effect is not achieved, simultaneous administration with the plant extract will cause the drug to stay in the body for a longer period of time. This would allow enough time for the drug to act and the right therapeutic effect achieved.

The p-nitrophenol hydroxylation assay is used to determine the activity of CYP2E subfamilies (Robert *et al.*, 1995), and can therefore be inferred that the plant extract inhibits the activity or induction of CYP2E isozymes. An example of a drug that is metabolized by the CYP2E enzyme system is isoniazid, an anti-tuberculous drug, which is also on the country's essential drug list.

From the results of the ethoxyresorufin-o-deethylase activity, it was shown that there were no significant differences in the average EROD activity of the four (4) groups of animals (Figure 4m). This may be due to the fact that the plant extract has no significant effect on ethoxyresorufin-O-deethylase as far as induction or inhibition is concerned. The EROD assay is used to determine the induction of CY1A1/1A2 isozymes (Mennier *et al.*, 2000; Burke *et al.*, 1985; Burke and Mayer, 1983). It can therefore be inferred from the results that the plant extract has no effect on CYP1A1/1A2 induction. These isozymes are known to be induced mainly by pollutants (Hoffman *et al.*, 1991). However, propranolol, a cardiovascular drug and tricyclic anti-depressants are known to induce these isozymes.

The results of the pentoxyresorufin-O-depentyase showed increases in activity with increasing dose levels of the plant extract (Figure 4n). Although the increases appeared to be dose-dependent, there were no statistically significant differences. The increases in the average PROD activity due to increasing dose levels of the plant extract indicate induction of a specific cytochrome P450 isozyme. Induction of the enzyme would mean that if a drug, which is metabolized by this CYP isozyme is taken by a subject who is on the plant extract, the drug would be metabolized at a faster rate because of the presence of the drug-metabolizing enzyme induced by the extract. The drug would

5.2 CONCLUSION

Acute (high single dose) exposures or administrations of *D. adscendens* crude extract resulted in toxicity and lethality in rats. A dose of 1.125g/kg body weight of the extract was estimated as the dose that caused 50% lethality in the experimental animals, as compared to only 2.5mg/kg body weight per day as the dose used for the treatment of asthmatic patients.

Chronic administrations of 33.75, 112.5, and 337.5mg/kg body weight resulted in no toxic effect, measured through liver and kidney function tests. It can therefore be concluded that the recommended dose of the crude extract of *D. adscendens* (2.5mg/kg body weight per day) by the Centre for Scientific Research into Plant Medicine (CSRPM), where it is prescribed and dispensed for the treatment of asthma may be safe.

Crude extract of *D. adscendens* inhibited CYP2E subfamily isozymes, had no effect on CYP1A1/1A2 isozymes, induced CYP2B1/2B2 isozymes, the latter isozymes known to be involved in the metabolism of zoxazolamine and thiopentone. There is therefore the possibility of drug-drug interaction, when drugs metabolized by these enzymes are taken by asthmatic patients who are on this plant extract and that may have toxicological consequences.

5.3 RECOMMENDATION

The study could be repeated using other species of animals such as mice and guinea pigs for a more complete toxicology of the plant extract.

The effect of the plant extract on other cytochrome P450 isozymes, especially the CYP3A4 isozyme, which metabolizes greater than 50% of drugs, and the CYP2C subfamily isozymes should be carried out to determine how the plant extract affects them

Confirmation of the identity of the CYP isozymes that were induced or inhibited in this study, through the use of immunochemical techniques would advance the investigations initiated in this study.

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APPENDIX I

PREPARATION OF SOLUTIONS AND REAGENTS

1. Homogenizing buffer, pH 7.4

This was prepared by dissolving 6.980g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 5.605g of KCl and 0.192g EDTA in 200ml distilled water and 58ml of 87% glycerol added to the resulting solution. The pH of the final solution was adjusted to 7.4 with few drops of concentrated NaOH solution. Distilled water was added to a total volume of 500ml.

2. Storage buffer, pH 7.4

Preparation and component of the storage buffer are the same as those for the homogenizing buffer, except that the percentage of glycerol was increased to 30% in the final volume prepared. This was obtained by the addition of 174ml of the 87% glycerol.

3. 0.1M Tris buffer, pH 7.4

This was prepared by dissolving 6.038g of Tris in 200ml of distilled water and 100ml of 100% glycerol added to the resulting solution. The pH was adjusted to 7.4 with a few drops of concentrated NaOH solution and distilled water added to a total volume of 500ml.

4. Phosphate buffer, pH 7.6

This was prepared by dissolving 7.8005g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 300ml of 0.1M NaOH solution and the pH of the resulting solution adjusted with more 0.1M NaOH solution to pH 7.6. The total volume of the phosphate buffer of pH 7.6 obtained was 856ml.

5. 0.88% Standard saline solution (0.15M NaCl)

This was prepared by dissolving 4.39g of NaCl in 400ml of distilled water and the resulting solution made up to 500ml with more distilled water.

6. NADPH-generating solution

The following components were mixed in a total solution volume of 53ml.

0.1M Tris buffer, pH 7.4	42.5ml
0.15M MgCl ₂	5.0ml
0.5M Nicotinamide	5.0ml
Glucose-6-phosphate (4μmol/ml)	3.0ml
Glucose-6-phosphate dehydrogenase (20units/ml)	8.0mg
NADP ⁺	40.0mg

APPENDIX II**CALCULATIONS****1. Dosage of plant extract in sub-chronic toxicity studies**

50% Lethality (LT ₅₀)	= 1,125mg/Kg body weight
Medium dose	= LT ₅₀ x 0.1 = 112.5 mg/Kg body weight per day
Low dose	= LT ₅₀ x 0.03 = 33.7mg/Kg body weight per day
High dose	= LD ₅₀ x 0.3 = 337.5mg/Kg body weight per day

2. Activity of p-nitrophenol (PNP) hydroxylation

PNP Activity	= $\frac{\text{Absorbance}}{\text{Mg protein}} \times 9.7276 \times 10^9 \text{ mol/min/protein}$
PNP Activity	= $\frac{\text{Absorbance}}{\text{Mg protein}} \times 9.7276 \text{ nmol/min/mg protein}$

where 9.7276 is the factor incorporating molar absorptivity of 4-nitrocatechol.

3. Activity of ethoxvresorvfin-o-deethylase (EROD)

EROD fluorescent units of 0.333nmol/ml Standard resorufin = 119.1 units
Therefore, Activity of measured fluorescent units,

$$= \frac{X \times 0.333}{119.1} \times 2.80 \times 10^{-3} \times X \text{ nmol/ml/mg protein}$$

where X is the fluorescent unit measured.

4. Activity of pentoxvresorufin-o-depentylase (PROD)

PROD fluorescent units of 0.333nmol/ml Std resorufin = 105.9 units
Therefore, Activity of measured fluorescent unit

$$= \frac{X \times 0.333}{105.9} = 3.15 \times 10^{-3} \times X \text{ nmol/ml/mg protein}$$

where X is the fluorescent unit measured.

APPENDIX III

CONTENTS AND CONCENTRATIONS OF REAGENTS IN KITS FOR ENZYMES AND ANALYTES ASSAYS

1. Total serum protein

Biuret reagent:	
Sodium hydroxide	100mmol/L
Na-K-tartrate	16mmol/L
Potassium iodide	15mmol/L
Cupric sulphate	6mmol/L
Blank reagent:	
Sodium hydroxide	100mmol/L
Na-K-tartrate	16mmol/L
Standard:	
Protein	60g/L

2. Alanine aminotransferase (ALT)

Reagent 1	
Tris buffer	100mmol/L
L-alanine	0.6mmol/L
Reagent 2	
α -oxoglutarate	15mmol/L
Lactate dehydrogenase	$\geq 1.2 \mu\text{ml}$
NADH	0.18mmol

3. Aspartate aminotransferase (AST)

Reagent 1:	
Tris buffer	84mmol/L
L-Aspartate	260mmol/L
Reagent 2:	
α -oxoglutarate	12mmol/L
Malate dehydrogenase	$\geq 420 \mu\text{L}$
Lactate dehydrogenase	$\geq 600 \mu\text{L}$
NADH	0.18mmol/L

4. Gamma-glutamyltransferase

Tris buffer	71.5 mmol/L, pH 8.25
Glycylglycine	126 mmol/L
L- γ -glutamyl-p-nitroamide	4 mmol/L

5. Total bilirubin

Reagent 1:

Sulphanilic acid	5.8mmol/L
Hydrochloric acid	0.2mol/L
Dimethylsulphoxide	7.0 mol/L
Sodium nitrate (Reagent 2)	0.07mol/L
Standard bilirubin	15 μ mol/L

6. Direct (Conjugated) bilirubin

Reagent 1:

Sulphanilic acid	5.0mmol/L
Hydrochloric acid	0.5mmol/L
Sodium nitrite (Reagent 2)	0.1mol/L
Standard bilirubin	5.0 μ mol/L

7. Creatinine

Standard Creatinine	177 μ mol/L
Picric acid	35mmol/L
Sodium hydroxide	0.35mol/L

8. Blood urea nitrogen

Reagent 1:

Tris buffer	150mmol/L, pH 7.6
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Reagent 2:

Urease	≥ 10 U/L
Glutamate dehydrogenase	≥ 2 U/ml
NADH	0.26mmol/L
Adenosine-5-bisphosphate	3.0mmol/L
α -oxoglutarate	14mmol/L
Standard urea	13.3mmol/L