TOXICOLOGICAL EVALUATION OF METHANOLIC EXTRACT AND ALKALOIDAL FRACTION OF *CROTALARIA RETUSA* LINN

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CERTIFICATION

This study was supervised by me and approved in accordance with the partial fulfillment of the requirements for the award of Master of Science (M.Sc.) Degree in Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria.

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Prof. O.O. Oyedapo Date

DEDICATION

To Him, who created life from the molecular level to the most complex of being, who created me in His image out of profound love, my all in all, whose power has always being my strength, the Almighty God I dedicate this work.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABSTRACT

The study was designed to investigate the toxic potentials of methanolic extract and alkaloidal fraction of leaf and stem of *Crotalaria retusa*. This was with a view to understanding its mechanism of toxicity to animals and plants.

Fresh leaves and stems of *C. retusa* were collected, cut into bits, washed, drained and air dried in the laboratory over three (3) weeks and pulverized into powder. Methanolic extract (ME) of powdered material was prepared by soaking 350 g in 800 ml of 70% (v/v) methanol for 48 h, filtered and concentrated to dryness at 35^{o}C on a rotary evaporator. Crude alkaloidal fraction was prepared from ME by a procedure that consisted of acid dissolution with 10% (v/v) hydrochloric acid, basification to pH 9 with 1M sodium hydroxide solution and extraction with chloroform. Rat diets containing 0-5% (w/w) of ME were prepared and administered to fifteen (15) albino rats randomly divided into three (3) groups of five (5) rats each over a period of eighteen (18) days. Brown bean (*Phaseolus vulgaris)* seeds were grown with varying concentrations (0-100 µg/ml) of crude alkaloid fraction with strychnine (10 µg/ml) as reference alkaloid. Leaves and stems were collected from beans seedlings for biochemical analyses. On day 19, rats were sacrificed, dissected and blood was collected by cardiac puncture into an anticoagulant (3.8% w/v trisodium citrate) for plasma preparation and livers were collected asceptically for preparation of tissue homogenates. Plasma and liver homogenates were used for biochemical analyses such as assay of enzyme activities and evaluation of concentrations of metabolites. Analyses of bean seedlings involved determination of percentage germination and evaluation of biochemical parameters in the leaves and stems.

Phytochemical screening of *Crotalaria retusa* methanolic extract revealed the presence of alkaloids, flavonoids, tannins, cardiac glycosides, triterpenoids, steroids and saponins. Administration of

C. retusa methanolic extract supplimented-diets for 18 days caused increase in the activities of plasma L-aspartate and L-alanine aminotransferases, acid and alkaline phosphatases activities while there were decrease in the activities of the enzymes in the liver homogenates of the treated animals. Moreover, activities of plasma α-mannosidase activities decreased ($p<0.05$) significantly, concentrations of creatinine, bilirubin, urea, albumin and sugar also increased significantly in the treated albino rats. Also percentage germination of bean seeds reduced with increasing concentrations of alkaloid fraction. The activities of antioxidant enzymes (superoxide dismutase and catalase) increased with alkaloid concentration in the leaves and stems of bean seedlings. The levels of metabolites (proline, reduced glutathione and ascorbic acid) increased significantly ($p<0.05$) in the stems and leaves of treated bean seedlings. However, there was reduction in the total protein and sugar contents of the leaves and stems of bean seedlings which implied stress. The results of this study revealed that the mechanisms of action of both the extract and its alkaloid fraction involved the induction of oxidative stress that resulted in the generation of reactive oxygen species (ROS).

The study concluded that both the methanolic and chloroform extracts of *C. retusa* were toxic to both plants and animals and that induction of oxidative stress was the underlying mechanism of toxicity.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

: PYRROLIZIDINE ALKALOIDS

Pyrrolizidine alkaloids (PAs) are plant secondary metabolites exclusively biosynthesised by plants against herbivors. It has been estimated that about 6000 plant species world wide, representing 3 % of all flowering plants, may contain PAs (Smith and Culvenor, 1981). PAs are mainly found in the distantly related angiosperm families of the Boraginaceae (all genera), Asteraceae (Senecioneae and Eupatorieae) and Fabaceae (genus *Crotalaria*) (Mattocks and Jukes, 1986). The toxicological effects of acute exposure to PAs to livestock and humans are well known and documented (Wiedenfeld and Edgar, 2011). In particular, human poisoning with 1,2-unsaturated PAs had been demonstrated to cause liver toxicity resulting in development of hepatic veno-occlusive disease (HVOD) (Chen and Huo, 2010). The latest documented outbreak was recorded in Afghanistan in 2008 (Kakar *et al.,* 2010) where flour contaminated with seeds of *Heliotropium lasiocarpum* (Boraginaceae) and milk from goats appeared to be the main sources of poisoning. In laboratory animals, 1 , 2-unsaturated PAs exhibited genotoxicity in different model systems and carcinogenicity was observed following chronic oral exposure to several 1 , 2-unsaturated PAs (Fu *et al.,* 2004; Chen *et al.,* 2010).

Seeds and dusts from pyrrolizidine alkaloids producing plants contaminate cereal grains in many countries of the world (IPCS 1988; ANZFA, 2001). The grain cleaning methods, routinely used in industrialized countries, reduce pyrrolizidine alkaloid contamination to below the level

that could cause acute poisoning by removing the foreign seeds. The methods do not remove the dust components and there are concerns that diseases such as cirrhosis, cancer and pulmonary arterial hypertension could result from chronic exposure to low levels of pyrrolizidine alkaloids in products such as cereals grains even in the industrialized world (Edgar, 2003).

Medicinal plants containing pyrrolizidine alkaloids have also been found to cause significant liver damage, especially in children (IPCS, 1989; Roeder, 1995, 2000). The use of herbal teas made from pyrrolizidine-producing plants to treat minor illnesses was also recognized as a cause of liver diseases in parts of Africa and other tropical and subtropical countries (Roeder, 1995, 2000). While pyrrolizidine alkaloid poisoning was more common in developing countries, where traditional herbal medicines are widely used, industrialized countries such as the USA and UK have also reported pyrrolizidine alkaloids intoxications from consumption of herbal medicines (WHO-IPCS, 1988). Germany, Switzerland and Austria, (where it was claimed in the 1980s by some herbal medicine practitioners that traditional medicinal plants had therapeutic benefits without the undesirable side effects) (WHO-IPCS, 1988) have also reported fatal cases of pyrrolizidine alkaloids intoxication from consumption of traditional herbal products that contained pyrrolizidine alkaloids (Brooks *et al.,*1970).

Many animal products such as milk have also been shown to be sources of pyrrolizidine alkaloids (Molyneux and James, 1990). Human milk from women exposed to pyrrolizidine alkaloids has caused veno-occlusive disease in neonates and infants (Panter *et al.,*1990), eggs (Edgar and Smith, 1999), and there are also reports of pyrrolizidine alkaloids in offal from animals fed plants containing pyrrolizidine alkaloids (IPCS, 1988; ANZFA, 2001).

1.2: Meat as Possible Source of Pyrrolizidine Alkaloids

Many millions of meat–producing livestock are exposed to plants containing pyrrolizidine alkaloids. Pyrrolizidine alkaloids toxicosis is considered to be the most common poisoning of livestock worldwide (Prakash *et al.,* 1999). It is however not yet clear whether hazardous residues of pyrrolizidine alkaloids remain in meat entering the human food chain.

Experiments in which radiolabelled pyrrolizidine alkaloids were given to animals, showed that most of the radioactivity (80%) was excreted within 24 hours (Eastman *et al.,* 1982; Candrian *et al.,* 1985). The residues that persisted was probably dihydropyrrolizidine adducts of sulphydryl, hydroxyl and amino bearing molecules. Following administration of 1, 2-dehydropyrrolizidine ester alkaloids dihydropyrrolizidine adducts have been detected in liver and in blood of experimental animals (Yan *et al.,* 2002).

1.3: Milk as possible source of Pyrrolizidine Alkaloids

Milk has been shown to be another source of pyrrolizidine alkaloid exposure in animals. Both pyrrolizidine free bases and N-oxides were detected in milk (Molyneux and James 1990) but the more water soluble N-oxides, the dominant forms occurring in plants, are thought to be most readily transferred into milk (Molyneux and James,1990). No human cases of pyrrolizidine alkaloid poisoning via milk have been unequivocally established but Huxtable (1989) refered to several instances where veno-occlusive disease occurred in suckling babies in Jamaica where there was no history of direct herbal administration to the infants.