EFFECTS OF HYDROALCOHOLIC SEED EXTRACTS OF *ARISTOLOCHIA ELEGANS* ON RENAL AND HEPATIC FUNCTION OF RATS

BY

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DECLARATION

I, MWITWA CHILESHE, this 10th day of August, declare that this dissertation represents my own work. This work has not been done in Zambia before and neither has it been published for any qualification at the University of Zambia or any other University. Various sources to which I am indebted are clearly indicated in the text and in the references.

CANDIDATE: MWITWA CHILESHE

Signed ……………………………………Date……………………………

APPROVAL

The Board of examiners has approved this dissertation to partially fulfil the requirements for the award of the Master of Science degree in Pharmacology at the University of Zambia.

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Supervisor………………………………… Signature………………………….. Date…………………...

ABSTRACT

Aristolochia species grow in different parts of Zambia and are used globally for medicinal purposes. Many Aristolochia species have been associated with adverse effects including renal and hepatic dysfunction. This study investigated the effects of hydro-alcoholic seed extract of *Aristolochia elegans* growing in Zambia on renal and hepatic function of rats.

The study investigated the phytochemical content of the seed extracts of *Aristolochia elegans* and an *in vivo* experimental design with Wistar albino rats was used to investigate effect of extract on biomarkers of renal and hepatic function. Twenty five rats were divided into five equal groups. Group 1 (negative control) received normal saline orally, groups 2, 3, and 4 received 500, 333, and 167mg/kg oral doses of *A. elegans* crude extract respectively, group 5 (positive control) received 1000mg/Kg Paracetamol orally, for ten days. Blood samples were collected from the rats by cardiac puncture and the sera were analyzed for sodium, potassium, chloride, creatinine, urea, Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Total protein and Albumin.

Sub chronic toxicity testing showed that renal function biomarkers; sodium, potassium, urea and chloride concentrations in the test groups did not differ significantly from the concentrations in the negative control group $p > 0.05$. Creatinine levels in the negative controls 43.8µmol/L +/- 7.3, 95% CI [23.7-63.9] was significantly higher than that found in the experimental groups 2, 3 and 4, 22.4+/-1.7 95% CI [17.8-6.9], 22.1 +/- 2.0, 95% CI [16.6-27.5] and 24.3+/- 1.7 8µmol/L 95% CI [19.6-28.9], respectively, P= 0.002, however concentrations of both test groups and negative control were within expected ranges for rats. Albumin, Alkaline phosphatase, Alanine amino transferase and Total protein concentrations in the test groups did not differ significantly from the concentrations in the negative control $p > 0.05$.

The study concludes that *Aristolochia elegans* hydro alcoholic seed extract did not appear to exert any effect on renal and hepatic function at the doses administered. We suggest further studies on the patho-morphological effects of the extract to provide more insight.

Keywords: *Aristolochia elegans* seed extract, Wistar albino rats, renal function, hepatic function

DEDICATION

This work is dedicated to my loving husband Bhagoo Stephen Lumamba and our beautiful daughter Isabella Lumamba.

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ACRONYMS AND ABBREVIATIONS

AA	Aristolochic acid
Ae	*Aristolochia elegans*
ALD	Approximate lethal dose
ALP	Alkaline phosphatase
ALT	Alanine amino transferase
AST	Aspartate aminotransferase
AAN	Aristolochic acid nephropathy
LD50	Median lethal dose
PO	Per Oral
UNZA	University of Zambia
UTH	University Teaching Hospital

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CHAPTER 1: INTRODUCTION

1.1 Background

Research of efficacy and toxicity of herbal medicines is paramount in modern day science as the plant world is considered a rich source of medicines (Shakya, 2016). A number of important drugs have plant origin including Artemether, Digoxin and Pilocarpine. Despite the beneficial medicinal properties of plants they can also possess severe adverse effects which have to be carefully studied to protect the public. (Pelkonen *et al*, 2014)

From ancient times, plants have been used to treat a variety of conditions (Rivera *et al*, 2013). World Health Organization (WHO) approximates that 75-80% of the world's population uses plant medicines either in part or entirely (Ekor, 2013). In developing countries use of medicinal herbs is part of the cultural system in the communities, further the herbs provide a primary source of health care in those communities (Mukherjee, 2002). WHO estimates that In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with fever resulting from malaria is the use of herbal medicines at home (WHO, 2002).

The use of herbal medicines has increased globally (Pan *et al*, 2014), a number of factors have been attributed to this increase including; various claims on the efficacy or effectiveness of certain plants, strong consumer interest in preventative medicine, high cost and side effects of modern drugs, dissatisfaction with allopathic medicine and the perception that botanicals are safe and free from side effects and movement towards self-medication (Bandaranayake, 2006). This increase in the use of herbal medicines has raised serious concerns and questions regarding their quality, safety and efficacy (Hussin, 2001).

The increase in use of herbal remedies has created a need for increased research in this area, to provide sufficient information to the public and health care professionals on the quality, safety and risks associated with herbal medicine use (Ekor, 2013).

Adverse drug reactions are defined by the World Health Organization as “response to a drug that is noxious and unintended, and which occurs at doses used in man for prophylaxis, diagnosis and therapy” (Schatz and Weber, 2015). Adverse drug reactions contribute largely to morbidity and mortality globally; they are reportedly one of the leading causes of death in the western world (Pirmohamen et al., 2004). Adverse effects do not only occur with synthetic drugs but can also be a consequence of herbal medicines. Adverse events such as stroke, heart attacks, liver toxicities, seizures, insomnia, pain and death have been associated with use of herbs (International Agency for research on Cancer, 2010). Some commonly used herbs have been suspected of causing cancer including *Rubia tinctorum, Morinda officinalis* and *Senecio riddellii* (International Agency for research on Cancer, 2010)

Aristolochia is a genus that belongs to the Aristolochiaceae family, which has 500 species (Dey *et al*, 2011); it is a significant genus medicinally. It extensively grows in tropical Asia, Africa and South America. Aristolochia is used for various medicinal purposes especially in Chinese medicine, some of the medicinal uses include; *A. debilis*, in China is used for the treatment of snakebite, tuberculosis and as an antihypertensive agent (Wu *et al*, 2004). *A. albida* which is natively found in West Africa has been used traditionally for various ailments including skin diseases, dysentery, gastrointestinal colic’s and snakebites (Irvine, 1961). The leaves of *Aristolochia elegans* are pounded and diluted in water and used to treat east coast fever of cattle in Bulamogi county Uganda (Tabuti, 2003). *A. elegans* ethanolic extract has also been shown to exhibit antimitotic and antiviral activities (Shi *et al,* 2004).

Aristolochia indica is used to treat cholera, fever, bowel troubles, ulcers, leprosy and, snake bites It is also used as an emmenagogue, arbortifacient, antineoplastic, antiseptic, anti-inflammatory and antibacterial (Dey and Nath De, 2011) . Extracts obtained from European snakeroot (*Aristolochia clematitis*) were formerly licensed for use against abscesses, eczemas and other long-lasting skin diseases and as a non-specific stimulant of the immune system but in 1982; they were withdrawn due to suspected carcinogenic effects (Heinrich *et al*, 2009).

Studies have revealed that plants from Aristolochiaceae family have a variety of medicinal purposes. However these plants have also been associated with toxic effects such nephropathy and hepatocellular damage. The aristolochic acid found in this species is known to cause rapidly progressive interstitial fibrosis and urethelial and hepatic cancer (Wing-Tat *et al*, 2007). The toxic components of *Aristolochia* species are known as aristolochic acids, they are a series of substituted nitrophenanthrene carboxylic acids. The main constituents are 3, 4 methylenedioxy-8-methoxy-10-nitrophenanthrene-1-carboxylic acid and its derivative, aristolochic acid II. The aristolochic acids appear to occur throughout the plants and have been found in the roots, stem, herb and fruit. (European Medicines agency, 2005). The main target of AA in the kidney seems to be the tubular compartment. Aristolochic acid causes tubular cell apoptosis by activating mitochondrial permeability, it also causes progressive interstitial renal fibrosis with loss of tubules predominantly located in the superficial cortex. Reduced megalin expression leads to impaired receptor-mediated endocytosis and is responsible for increased excretion of low molecular weight proteins leading to proteinuria (Gao *et al*, 2000). Aristolochic acid has also been shown to cause hepatocellular degeneration i.e. fatty degeneration and hepatocellular necrosis (Belay, 2011). At a molecular level, the difference between the two aristolochic acids is that aristolochic acid I contains a methoxyl group at the eighth position where aristolochic acid II does not (Figure 1.1). This could account for the differences in toxicity.

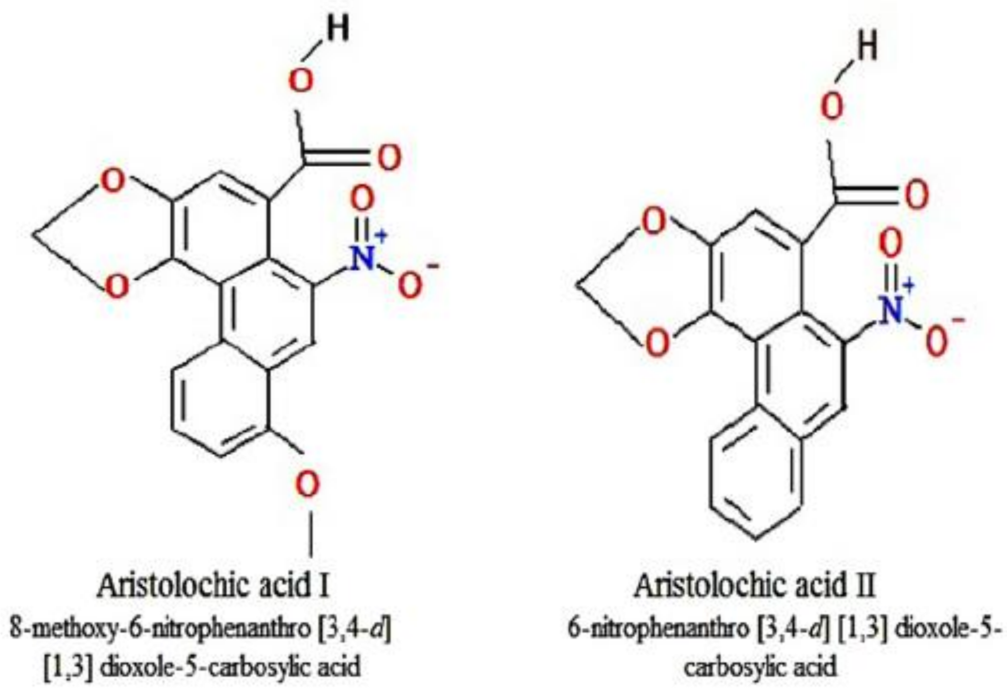

Figure 1.1: Chemical structure of Aristolochic acid I and II (Source: Huan et al, 2006)

A study done revealed that aristolochic acid I caused acute tubular necrosis , extensive cortical interstitial fibrosis and weight loss in treated rats where aristolochic acid II caused little necrosis, no interstitial fibrosis and no weight loss in the rats, Both aristolochic acid I and II were found to produce DNA adducts (Shibutani *et al*, 2007). From the perspective of molecular pharmacology a hallmark of the nephropathy caused by aristolochic acids are covalent 7-(deoxyadenosin-N6-yl) aristolactam I and 7- (deoxyguanosin- N2-yl)-aristolactam I(AL) adducts formed after metabolic activation. These adducts persist for an extended period, which facilitates its detection in target tissues (Grollman *et al*, 2007).

Aristolochia elegans Mast has been employed in folk medicine for various purposes such as; as an expectorant, an antitussive, an antiasthmatic, an analgesic, an antihistamine, a detoxicant agent, an antidote against snake bites and toothache, as a purgative, an insecticide, and as an antispasmodic. In Mexican traditional medicine, this plant is used as antimicrobial, antitumor, antidiarrheal, antipyretic and emmenagogic agent (Shi *et al*, 2004; Jimenez *et al*, 2005). *Aristolochia elegans Mast (*syn. *A. littoralis)* is a plant that can be found worldwide, (Izquierdo *et al*, 2010). It is commonly known as guaco, duck flower, or elephant foot. It is a perennial climbing vine with large pipe-shaped flowers that are shaped like a traditional Dutchman's pipe (Skolen and Csurhes, 2009).) It can grow from 3 m to 4.5 m high on supporting vegetation. Leaves grow 3–10 cm long, 3–12 cm wide on a 5 cm stalk (Kleinschmidt and Johnson, 1977). The plant has broad heart-shaped leaves, each with a distinctive curved leaf base (Figure 1.2).

Figure 1.2: Aristolochia elegans growing in Lusaka, Kaunda square.

In Zambia *Aristolochia, elegans* is locally known as Kalulusha in Chichewa language. The seeds of the plant are used in folk medicine in Zambia for a number of conditions such as abdominal pains, diarrhea, ulcers, dysmenorrhea and Malaria. Patients are required to chew unspecified quantities of the seeds for a period of 3 to 10days. To the author’s knowledge, there is no published data on the traditional use of *Aristolochia elegans* in Zambia. *Aristolochia elegans* belongs to the genus Aristolochia, which has been associated with aristolochic acid induced nephropathy, liver damage and carcinogenicity (Arlt et al, 2002; Belay, 2011). Investigations of the renal and hepatic toxicity of plants in this family have revealed different results based on quantities of crude extracts and species used. Some studies have revealed distortions in biomarkers of liver and kidney function indicating toxicity, as well as indications of carcinogenicity based on histological findings such as dysfunctional DNA adducts, necrosis and hyperplasia of the urothelium. Other studies however have shown no signs of toxicity at certain dosages of Aristolochic acid and crude extracts from the Aristolochiaceae family. It is therefore important that the effects of *Aristolochia elegans* growing in Zambia, both medicinal and toxic be investigated at various dosages to provide adequate and evidence based information on our locally growing Aristolochia species. This study investigated the renal toxic and hepatotoxic effects of the plant in order to provide relevant information to the public. All medicines need to fulfill the basic requirements for safety and efficacy (Kunle *et al*, 2012).

1.2 Problem Statement.

Adverse effects of drugs are a major contribution to morbidity and mortality worldwide (Schatz and Weber, 2015). The increasing use of herbal medicines means that there is increased potential for adverse effects and drug interactions (Hussin, 2001). Traditional healers in Zambia use *Aristolochia elegans* in folk remedies. Aristolochic acid is a major constituent of Aristolochia species, several studies report adverse effects related to aristolochic acid including aristolochic acid nephropathy, urinary tract cancer and hepatocellular necrosis (Wojcikowaki et al, 2004). There is no information or regulation regarding the safety of the use of this folk medicine in Zambia. This study was designed with the purpose of providing information on whether Aristolochia elegans extract impairs renal and hepatic function.

1.3 Research Questions

In order to address the problems highlighted above, the study attempted to answer the following questions:

Does the seed extract of *A.elegans* affect liver and kidney function parameters?

1.4 Objectives

1.4.1 General Objective:

To determine the effect of *Aristolochia elegans* on liver and kidney function parameters in Wistar albino rats

1.4.2 Specific Objectives

1. To determine the effect of hydroalcholic seed extract of *Aristolochia elegans* on sodium, potassium, chloride, urea and creatinine in rats as biomarkers of kidney function.
2. To determine effects of hydroalcoholic seed extract of *Aristolochia elegans* on Alanine amino transferase (ALT), Aspartate amino transferase (AST), Alkaline phosphatase (ALP), Total protein and Albumin in rats as biomarkers of liver function.
3. To determine dose dependent effect of *Aristolochia elegans* extract on Kidney and Liver function parameters.

1.5 Justification of the Study

Herbal medicines use is widely practiced in Zambia, there has been reports of toxicities due to herbal remedies but lack of proper documentation limits information. One of the herbs used by Zambian traditional healers in preparation of folk remedies is *Aristolochia elegans*. To the knowledge of the researcher, no study has been conducted in Zambia to investigate the safety of these preparations, despite literature evidence of the toxicity of certain species in this family of plants. Some countries advocate for controlled use of the Aristolochia species whilst others advocate for a total ban, as there is no consensus on what constitutes a safe dose (Deng, 2003). It is therefore important for continued research to be carried out on the safety of these plants to increase the body of information. Further carrying out investigations on locally grown species is essential as plant active ingredients, most of which are secondary metabolites vary from country to country, due to differences in climate, soil and other ecological factors (Figueiredo *et al*, 2008). For these reasons the investigator believes that this study will be important to public health.

1.6 Scope of study

The scope of this study was to determine the effects of hydroalcholic seed extracts of *Aristolochia elegans* on kidney and liver function. It investigated the effects of sub-acute administration of seed extracts of *Aristolochia elegans* on renal and hepatic functions. It utilized an in vivo experimental design using Wistar albino rats. It did not however study the histological, hematological, behavioral changes or other pathophysiological parameters, which although would have been complementary to the findings of the study were not studied because of resource limitations. This was purely a pre-clinical study and did not involve human participants and there was no attempt to determine the prevalence of these pathologies among the population that use this plant. Further studies to this effect have been recommended.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview

Aristolochia species grow in different parts of the world and are used for various medicinal purposes, however reports have revealed that these plants also have toxic adverse effects. One of the earliest reports of *Aristolochia spp* toxicity involved renal failure and upper tract urothelial carcinoma in cases of young Belgian women who took slimming pills containing Chinese herbs, the women developed rapidly progressive renal interstitial fibrosis which ultimately led to chronic renal failure, and about 5% of the women developed end-stage renal disease, investigations implicated *Aristolochia spp* (Vanherweghem *et al*, 1993). This case and other growing evidence of aristolochic acid toxicity led to the use of products containing aristolochic acid to be banned in several countries (Wu and Wang, 2013).

2.2 Herbal medicine use

In Africa up to 90% and in India 70% of the population depend on traditional medicine to help meet their health care needs (Iris and Wachtel-Galor, 2011). In Ghana, about 70% of the population depends primarily on traditional medicines (Roberts, 2001). About 27 million South Africans use traditional medicines to treat a variety of ailments (Lekotjolo, 2009). The relative ratios of traditional practitio2ners and university trained doctors in Africa give an idea of the widespread use of herbal medicine, examples include; In Kenya Rural (Kilungu) 1 traditional practitioner for 143-345 people compared to 1 medical doctor to 987 people, in Zimbabwe 1 traditional practitioner to 600 people compared to 1 medical doctor 6250 people and in Tanzania 1traditional practitioner for 400 people compared 1 medical doctor 33000 people (Chatora, 2003).

2.3 Adverse drug reactions

Adverse drug reactions can be defined as an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration to the dosage regimen, or withdraw of the product (Edwards and Aronson, 2000). They are a major contributor to health problems globally, studies done show that adverse drug reactions have an incidence of 2.4%-13.8% (Pirmohamed *et al*, 2004). The actual incidence of adverse drug reactions cannot be quantified as they are sometimes not reported or can go unrecognized because some mimic some natural diseases. Adverse drug reactions have a wide spectrum of presentation from minor symptoms such as nausea to those causing death in 0.1%–0.3% of hospitalized patients (Schatz and Weber, 2015). Around 5% of all hospital admissions are the result of an adverse drug reaction, and around 10%–20% of inpatients will have at least one adverse drug reaction (ADR) during their hospital stay (Pirmohamed *et al*, 2004). In a cross sectional study conducted in South Africa, it was observed that the most common adverse drug reactions which led to hospitalization were: renal impairment, hypoglycemia, liver injury, and hemorrhage (Mountan *et al*, 2016) in addition to causing morbidity and mortality adverse drug reactions also increase the cost of health care (Jonsson, 2007).

2.4 Adverse effects of herbal remedies

Due to long standing use of herbal remedies, they are usually considered safe and have less rigorous clinical and non-clinical regulation requirements (Moreira *et al*, 2014). Despite proven efficacy of herbal remedies, they have also been associated with various adverse effects and Interactions (Zhang *et al*, 2015). Studies have revealed toxicities associated with various herbal medicines including, hepato toxic and renal toxic effects of *Ephedra sinica*, antagonistic effects of grape fruit juice on cytochrome P450, *Mentha spicata* causing nephrotoxicity in rats and *Opuntia Megcantha* causing elevated plasma creatinine and urea (Asif, 2012). *Mentha Pulegium* induces abortion and causes renal failure leading to death; *Teucrium Chamaedrys* causes fatal hepatitis, *Gaultheria Procumbens* causes acid-base disturbances, endocrine abnormalities, fluid and electrolyte imbalances (Brown, 2002), *Larrea Tridentate* is known to cause hepatotoxicity, *Pausinystalia yohimbe*, is known to cause systemic lupus erythematosus. Willow bark (*Salix daphnoides*) has been implicated in the causation of renal papillary necrosis (Jha, 2010).

Food and Drug Administration (FDA) received 2621 reports of serious medical problems related to immunological, musculoskeletal, hepatic and renal dysfunction as well as mutagenicity involving herbal products between January 1993 and October 1998 (Saxena *and* Pambora, 2003). One of the most serious complications resulting from use of herbal remedies is acute renal failure (Luycx *et al*, 2002). In Africa, use of herbal products accounts for an estimated 35% of all cases of acute renal failure (Luycx *et al*, 2002). Reported cases most probably do not reflect the actual frequency of adverse reactions caused by traditional herbal preparations, as most cases go unreported (Hussin, 2001).

2.5 Phytochemical constituents of *Aristolochia species*

The Aristolochia species are reported to contain aristolochic acids and esters, aristolactams, aporphines, protoberberines, isoquinolines, benzylisoquinolines, amides, flavonoids, lignans, biphenyl ethers, coumarins, tetralones, terpenoids, benzenoids, steroids, and others with extensive physiological activities (Latha, 2016). Organic extracts or essential oil of leaves, stems and roots of Aristolochia elegans mast contain; Alkaloids, lignans, neolignans, monoterpenoids, diterpenoids, sesquiterpenoids, tetralones, isoquinolines, porphyrins, biphenyl ethers, aristolactolactams, and aristolochic acid dimers (Jimenez *et al*, 2012).

2.6 Biochemistry reference ranges for Rats

Various studies have revealed the average biochemistry reference ranges of renal and hepatic function biomarkers for Wistar albino rats (Table 2.1).

Table 2.1 Reference ranges for renal and hepatic function parameters of Wistar albino rats

Parameter	Range
Sodium mmol/L	139-150
Potassium mmol/L	5.2-7.8
Chloride mmol/L	99-114
Creatinine umol/L	17.7-70.7
Total protein g/L	5.4-8.6
Albumin g/dL	3-4.9
Urea mmol/L	11.42-19.82
Alanine amino transferase U/L	108-375
Alkaline Phosphatase U/L	303.7- 462.3

Values obtained from: Charles River Techn, Kaynur et al, 2006, Johnson- Delanry, C., 1996, Matsuzawa , 1993.

2.7 Aristolochia species toxicity reports

A number of reports due to ingestion of herbal remedies containing Aristolochia species have been made in China. Two series of 58 and 51 Aristolochic acid nephropathy cases respectively, were reported in 2001 most of them after the intake of the liver tonic *Longdan Xieganwan* that contained Caulis *Aristolochia Manshuriensis*, five additional aristolochic acid nephropathy cases were also observed in Hong Kong, following the substitution of the nontoxic Herbal Solani Lyrati by *Aristolochia mollissimae* (Poon *et al*, 2007). Five cases of Aristolochic Acid Nephropathy were diagnosed in Hong Kong, which led to withdraw of AA containing products in Hong Kong (Wing-Tat *et al*, 2007). No health disorders were reported in a study done in Uganda, in individuals who had reportedly used *Aristolochia elegans* for treatment of malaria in a 1-2 year period (Belay, 2011). China/Taiwan reported a dose-dependent relationship with development of urinary tract cancer in the use of *Aristolochia elegans* (Ernst, 2010).Ten cases of acute renal failure, caused by overdose of Caulis Aristolochiae Manshuriensis were reported in China between 1964 and 1988 (Ma and Cheung, 2005). Endemic (Balkan) nephropathy, a devastating renal disease affecting people in rural areas of Bosnia, Bulgaria, Croatia, Romania, and Serbia has been linked to the consumption of Aristolochic acids containing food. (Grollman *et al*, 2007).

2.8 Toxicity studies on Aristolochia species

One of the best-known herb-induced chronic kidney disease is aristolochic acid nephropathy (Jha, 2010). The aristolochic acids found in *Aristolochia* species have been found to have renal toxic effects and carcinogenic properties (Pacheco *et al*, 2009).

A sub chronic toxicity study was conducted in Korea where aqueous extracts of *Aristolochia Fructus* was administered to Sprague- Dawley rats for ninety days, no significant difference in serum biochemistry of renal function parameters and hematology between the test and controls were found at lower doses (21.35 and 213.5 mg/kg), however nephrotoxicity and hyperplasia were found only in Rats receiving the highest dose i.e. 2135 mg/kg (Hwang *et al*, 2006). In a study conducted in China, *Aristolochia Fructus* extracts were administered to Rats for fourteen days, no significant change in levels of serum Creatinine, Blood Urea Nitrogen and Urinary Albumin between test and control groups were found (Zhou *et al*, 2012).). A study was conducted in Japan where three strains of inbred mice, BALB/c, C3H/He and C57BL/6 received 2.5 mg/kg of aristolochic acid I or aristolochic acid sodium salt daily by intraperitoneal or oral administration, 5 days a week for 2 weeks, this study revealed severe tubular injury in BALB/c and C3H/He mice but only mild tubule interstitial changes were seen in C57BL/6 mice, serum creatinine and blood urea nitrogen were increased in BALB/c and C3H/He mice (Sato N, 2004). A study which was conducted in Belgium where salt depleted male wistar rats received daily subcutaneous injections of 1mg/kg (low dose) and 10 mg/kg body weight aristolochic acid for 35 days revealed glucosuria, proteinuria and elevated serum creatinine levels on days 10 and 35 in the high dose group whereas no significant effect was noted in the low dose group (Debelle *et al*, 2002). Another study was conducted in Belgium where female white rabbits were injected intraperitoneally with 0.1mg/kg aristolochic acid, 5 days a week for 17 to 21 months, all test animals developed renal hypo cellular interstitial fibrosis and had increased serum creatinine, glucosuria, tubular proteinuria and anemia (Cosyns *et al*, 2001). A study was conducted in Germany in which the nephrotoxic action of aristolochic acid (AA) was investigated in female Wistar rats given single doses of 10, 50 and 100mg/kg by gastric tube, results showed elevated creatinine and urea levels in the 100mg/kg group only, Renal lesions developed within 3 days, the effect also being dose dependent (Mengs and Stotzem, 1993). A study was done in India where alcoholic extracts of *Aristolochia indica L*, was administered to Wister albino rats at a dosage of 1500mg/kg body weight for 28 days, the results showed that urea nitrogen, creatinine, alanine amino transferase, aspartate amino transferase and uric acid levels were significantly higher in the test than the controls(Mridula *et al*, 2011). A study was conducted in Algeria were rats where given a single dose of aqueous extracts of *Aristolochia longa* 5000mg/kg, the study revealed no significant difference in serum urea and creatinine levels between the control and test groups (Benarba *et al*) . A study was done in Nigeria by Abdulhakeem *et al* (2017) who reported significant elevations in Alkaline phosphatase, Alanine amino transferase and Aspartate amino transferase after 14-day administration of ethanolic extracts of *Aristolochia ringen* to Rats at a dose of 75, 150, 300mg/kg body weight. A study done in Uganda revealed severe damage to kidney and liver of Balb c mice after administration of both ethanol and ether crude extracts of *Aristolochia elegans* seeds, dosages administered were 500, 1000, 2000, 3000, 4000 and 5000mg/kg body weight, four out of ten treated Balb c mice also developed hemorrhages in the stomach, which was a strong indication of carcinogenic property of this plant material (Belay, 2011).

2.9 Summary

Various studies reveal that plants from the Aristolochia species have harmful effects on the kidney and liver function, other studies however revealed no distortion in biomarkers of renal and hepatic function at certain dosages. Toxicity studies involving crude extracts found in literature did not carry out identification and quantification of aristolochic acid in the extracts used, making potency comparisons between different species difficult. There is no consolidated information as to the concentration at which aristolochic acid content begins to be toxic which brings about the current situation where some countries such the United Kingdom, Taiwan and Canada as have completely banned the use of aristolochic acid containing plants (Wu and Wang, 2013) and other countries advocate for controlled use, as the plant has many medicinal purposes. Countries like Uganda are exploring the use of *Aristolochia elegans* in treatment of malaria (Ernst, 2010). Further most studies conducted are on chronic use of the plant species, leaving an information gap regarding acute and sub-acute usage which is practiced here in Zambia. Lastly there are very few toxicity studies on *Aristolochia elegans* specifically. It is therefore cardinal for studies on this plant species which is currently being used in folk medicine in Zambia to be extensively investigated .This study is therefore significant as it investigates the phytochemical composition of locally growing *Aristolochia elegans*, conducts aristolochic acid identification and quantification and studies the effects of the seed extracts of *Aristolochia elegans* when administered sub-acute, as it is used in Zambia.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Design

The study was an in vivo experimental study, using Wistar albino rats. An equal number of male and female rats were used.

3.2 Study site

Experimental procedures were conducted at University of Zambia; Chemistry Department and Department of Physiological Sciences, Pharmacology laboratory in Lusaka, Zambia.

3.3 Plant collection and identification

The dried fruits of *A. elegans* were collected in February from Lusaka West, with the help of a herbalist. A Herbarium specimen of harvested plant was prepared and was botanically identified by a qualified plant taxonomist at the University of Zambia, Department of Biological Sciences.

3.4 Chemicals

Aristolochic acid I European pharmacopeia standard and TLC Silica gel 60 F_{254} Aluminum plate were obtained from Merck (Darmstadt, Germany). Methanol 99% , Chloroform 99%, Ethanol 100% and Normal saline was obtained from Kansama investment limited and manufactured by Xilong Scientific Co.,Ltd. Chloroform 99%, Acetic acid 99.8%, Sulfuric acid (H2SO4) 98%, Hydrochloric acid (HCL) 37%, Ferric chloride 99.9% and metallic Magnesium 98% were obtained from Merck (Darmstadt, Germany).

3.5 Plant processing and extraction

The seeds were manually isolated and size reduced using a blender (1- minute rotations done two times) until consistent. 320.8 grams of size- reduced seeds were soaked in 2 liters of 80% ethanol. Extract obtained would be considered a hydro alcoholic extract because the solvent used was a mixture of alcohol and water. The size reduced seeds were soaked for 48 hours, thereafter the solution was filtered using Macherey-Nagel MN 615.Ø150mm filter paper in a clear chemical free conical flask. Ethanol was removed using BUCHI Rota vapor R11 and water bath at 40°C (Belay, 2011). The stock was placed in air tight amber colored glass bottles and was transported in a cooler box and stored in a freezer at less than 0°C for 24 hrs. It was brought to room temperature 2 hours before reconstitution with normal saline for the experiment.

Calculations for obtaining 80% ethanol:

Concetration1 X Volume1= Concetration2 X Volume2

100% ethanol X Volume1 = 80% X 2000mls

Volume 1 = 1600 mls of 100% ethanol

Therefore 1600mls of 100% ethanol was mixed with 400mls of water to obtain 2000 mls of 80% ethanol.

Extractive value:

The extractive value was calculated as follows:

$$\frac{\text{weight of extract}}{\text{Weight of plant material}} \; X\ 100 = Extractive\ value$$

3.6 Phytochemical analysis

Test for Tanins

Braymer's test: 2mls of the aqueous extract was stirred with 2mls of distilled water and three drops of FeCL3 solution were added. The formation of a green precipitate was an indication for the presence of tanins.

Test for saponins

Froth test: 5mls of aqueous extract was shaken vigorously with 5mls of distilled water in a test tube and warmed. The formation of a stable foam was taken as an indication for presence of saponins.

Test for sterols and triterpenoids

The Liebermann- Bouchard reaction: 10mls of hydro-alcoholic extract were evaporated into a porcelain capsule, the residue was dissolved in 0.5ml chloroform, and 0.5ml acetic anhydride were then added. The resulted solution was passed into a dry test tube, followed by the addition of 2 mls concentrated sulfuric acid (H2SO4), at the test tube bottom. In the case where sterols or triterpenoids are present in solution, a red brown or violet ring appears after 5-10 min at the contact zone between the two liquids as well as a green-bluish or violet upper layer.

Test for flavonoids

Shinoda reagent test: 2 fragments of metallic Mg were mixed with 3ml extract, then 0.5ml concentrated hydrochloric acid (HCL) were added, after 5 minutes a red color appears-for the flavonols, orange-for the flavons red-violaceous- characteristic to flavanones .

Test for alkaloids

3mls of aqueous extract was stirred with 1ml of 1% HCL on a steam bath, Mayer's reagent was then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids

Test for glycosides

Keller- Kiliani test: 2mls of extract was dissolved in 2mls of glacial acetic acid containing one drop of FeCL3 solution. The mixture was then poured into a test tube containing 1ml of concentrated HSO4. A brown ring at the interphase indicates the presence of a deoxy sugar, characteristic of cardenolide glycosides.

3.7 TLC analysis

Thin layer chromatography (TLC) was used as a preliminary step for the detection of Aristolochic acid in *A. elegans* seed (Abdelgadir *et al*, 2011). Protocols used were according to A Concise Quality Control Guide Manual Volume II on Essential Drugs and Other Medicines Accompanying the Global Pharma Health Fund (GPHF). 1.33mg of AAI was dissolved in 10mls of methanol, 0.103 grams of *A. elegans* Crude extract was dissolved in 10mls ethanol and both were sonicated for 5 minutes. 2 µL of the standard and sample each were spotted on TLC Silica gel 60 F_{254} Aluminum plate. The plate was developed in a glass chamber previously saturated for 15 minutes with chloroform: methanol: acetic acid (6:4:1; v/v/v) as a development solvent. The dried chromatoplates were observed in the dark under UV light at 366 nm using the CAMAG UV Chamber. The presence of Aristolochic acid was indicated by oval shaped black spots and the Retention factor (Rf) value of the aristolochic acid was calculated using the formula:

$$\text{Retention Factor (Rf)} = \frac{\text{Distance moved by the spot}}{\text{Distance moved by the solvent front}}$$

3.8 Quantitative determination of Aristolochic acid I using High Performance Thin Layer Chromatography (HPTLC)

HPTLC was used to quantify the amount of Aristolochic acid after preliminary detection (Kamboj and Saluja, 2013).

An automatic sample applicator (Camag Linomat V, Switzerland) equipped with a Hamilton 100-µL syringe was employed for samples application on the HPTLC plate. Chromatographic separations were performed on 20×10 cm glass backed plates pre-coated with 0.2 mm layers of silica gel and fluorescent indicator with a 254 nm excitation wavelength (Kieselguhr 60F-254, E. Merck, Germany). A Camag twin-trough chamber for 20×10 cm plates, with a stainless steel lid, was used for ascending development of the plates. Densitometric scanning was performed on Camag TLC scanner 4, operated with CATS3 software while the source of radiation was a tungsten lamp.

Pre-conditioning: After selection of the chromatographic layer, plates were prewashed with methanol, and were then activated at 70 °C for 60 min.

Sample application: By means of an automatic applicator the samples were spotted with a constant application rate of 5 s/µL in the form of bands of 6 mm in width. The space between bands was 4 mm. The distance from the left edge and the bottom of the plate was kept at 30 and 20 mm respectively.

Suitable mobile phase: The following solvent mixtures were used for development of the chromatoplate: Chloroform: Methanol: water (6:4:1)

Chromatographic development: The tank was saturated for 15 min before insertion of the spotted plate. Plates were developed with 10 mL of mobile phase in strict light-protected conditions. The development distance was approximately 70 mm.

Detection and scanning: After development, the plate was dried in open air for 5 min. Densitometric scanning was then performed in the absorbance mode and at the speed of 4 mm/s, using the tungsten light source at 430 nm (λ_{max} for the compounds). Monochromator bandwidth was kept at 30 nm and the dimension of slit was set at 5 mm in length and 2 mm in width.

3.9 Laboratory Animals

The Rats were obtained from the animal shelter, Biology Department at UNZA main campus. The animals were kept at average room temperature and relative humidity. The mice had free access to water and were fed standard rodent pellets. Habituation conditions were 12 hr. dark/light cycles. An equal number of female and male rats were used and were stored in plastic cages for the duration of the experiment. The rodents were handled in a humane manner with the help of trained animal handler's at Ridgeway campus.

3.10 Inclusion criteria

Healthy, mature male and female Wistar albino rats weighing between 90 – 200g

3.11 Exclusion criteria

Pregnant rats and rats weighing less than 90g were excluded from the study

3.12 Acute toxicity test and determination of Median Lethal Dose (LD50)

The Median lethal dose of extract, which is the dose that produces death in 50% of the test population was investigated. This was done in order to establish dosages of extract to be used in sub-acute toxicity testing. The acute oral toxicity was performed according to the OPPTS (office of prevention, pesticides and toxic substance) guidelines following the Up and down method. Wistar albino rats were fasted overnight, food but not water was withheld. Rats were subjected to 12hr light and dark cycles. The limit test is a test conducted on five animals at a dose of 2000 or 5000mg/kg body weight. The first animal is dosed with the limit dose, if it dies the main test is conducted, if the animal survives the rest of the animals are dosed, if three out of the five animals die, the main test is performed. This test is conducted where the experimenter has information indicating that the test material is likely to be non toxic below a certain dose, it was not conducted in this experiment because there is no information on the toxicity of the test compound in Zambia therefore the main test was performed. The main test involved dosing the first animal at 1.75mg/kg body weight, via the oral route using an oral feeding needle. If the animal survives, the dose for the next animal is increased by a factor of 3.2 (half log units), if it dies, the dose for the next animal is decreased by a similar dose progression, each animal was observed for a period of 48 hours, before making a decision on whether and how much to dose the next animal. The dosing is stopped when once the following stopping criteria are met.

1) 3 consecutive animals survive at the upper end of the limit dose.
2) 5 reversals occur in any 6 consecutive animals tested. A reversal is a situation where nonresponse is observed at some dose, and a response is observed at the next dose tested or vice versa.

3) At least 4 animals have followed the first reversals and the specified likelihood- ratios exceed the critical value of 2.5.

The control rats received normal saline. At the end of the dosing the Median lethal dose is calculated using likelihood ratios.

3.13 Experimental Design

Twenty five Wistar albino rats were used in total, the rats were divided into five groups, 2 control groups and 3 test groups i.e. 5 rats for each group. Based on Median Lethal dose testing. Three doses were chosen i.e. 500mg/kg, 333mg/kg and 167mg/kg to be administered to 3 test groups over a period of 10 days. Doses chosen were similar to doses used in literature (Abdulhakeem *et al* 2017; Belay, 2011; Hwang *et al*, 2006). The positive control used was Paracetamol 1000mg/kg (Pimple et al, 2007).

Group 1: Negative Control to received normal saline Per Oral (PO)

Group 2: received 500mg/kg of *A.elegans* extract PO

Group 3: received 333mg/kg of *A.elegans* extract PO

Group 4: received 167mg/kg of *A.elegans* extract PO

Group 5: Positive control received 1000mg/kg of paracetamol PO

3.13.1 Working Solutions

Two working solutions of the extract were prepared as follows:

Working solution 1: obtained by dissolving 3.5grams of extract in 50mls of normal saline giving a concentration of 70mg/ml

Working solution 2: obtained by dissolving 3.5 grams of extract in 100mls of normal saline giving a concentration of 35mg/ml

Working solution for Paracetamol: obtained by dissolving 1000mg of paracetamol in 10mls of normal saline giving a concentration of 100mg/ml.

3.13.2 Dose Volumes

Dose volumes were calculated using the following formula:

$$\text{Dose volume} = \frac{\text{Dose to be administered to rat}}{\text{Concentration of extract}} \text{ X weight of Rat}$$

All dose volumes administered were 2mls and below depending on the weight of the rat, therefore once daily oral administration was done using an oral feeding needle for a period of 10 days. On the 11th day, blood was collected from the rats using cardiac puncture and taken to the lab for biochemical analysis of outcome measures (Ladokun et al, 2015; Etuk et al, 2009).

3.14 **Blood Collection**

Cardiac puncture was used to collect the blood. Rats were anesthetized using Diethyl ether; a ball of cotton wool suspended on a string was soaked with 0.8ml Diethyl ether and placed in a 1-liter container with the Rat. After ten minutes, sedation was confirmed using toe pinch reflex; the rat was placed in dorsal recumbency, a 23G needle was inserted at a 30-degree angle under the xiphoid process of the sternum. Light pressure was applied on the plunger of the syringe until 2mls of blood was obtained and placed in a chemically clean specimen bottle.

3.15 Biochemical analysis

After collection of blood via cardiac puncture, the blood was allowed to clot and retract, serum was removed using a pipette and placed in chemically clean specimen bottle. Centrifugation was done at 3000 RPM for 3 minutes. Controls Cat. No. ODC0003 and ODC0004 with values determined by Beckman Coulter AU480 system were used. The Beckman Coulter AU480 analyzer was then used to automatically compute the concentration of each sample.

For Renal function: creatinine, urea, sodium, potassium and chloride concentration were measured.

For Liver function: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Albumin and Total Protein concentrations were measured.

3.16 Determination of dose dependent effect

Dose dependent effect was determined by analyzing statistical difference of the means ±SE of biomarker concentrations between the different dose groups. Significance level used was p 0.05.

3.17 Ethical considerations

The laboratory animals were handled according to Institutional Animal Care and Use Committee (ICAUC) i.e.:

Laboratory animals were handled with the help of personnel trained to handle laboratory animals at ridgeway campus. The animals were housed in plastic cages and not more than 3 rats were housed in a single cage to avoid crowding. Plastic feeder bottles were mounted on the cages to provide water for the rats, and standard rodent pellets were used to feed the rats. Habituation conditions for the rats were 12 hr dark/light cycles and were kept at average room temperature and humidity. Least number of rats required to achieve the objectives of the study were used. Female and male rats were housed in separate cages during the study to prevent mating. The rats were handled in a gentle, quiet, humane manner, amount of pain or distress caused was reduced to a minimum by anesthetizing the rats during blood collection and euthanization was done whilst rats were still under anesthesia. Further the duration of the study was limited to that only required to achieve the objectives of the study and no further.

Ethical approval was obtained from ERES CONVERGE IRB and permission to use laboratories was sought from Head of Departments.

3.18 Data analysis

Data were analyzed quantitatively using SPSS statistical software version 21 and Graph Pad Prism Version 7. Descriptive statistics included determination of the mean ±SE for each group with confidence interval set at 95%. The mean Values for each variable were compared using one way ANOVA after verification of normality of data distribution by Shaphiro – wilk normality test and equality of variances by Levens test. In Post-Hoc analysis, Turkey Post-Hoc analysis was used where equal variances were assumed and Dunnett T3 was used were equal variances were not assumed, significance level used was P 0.05

CHAPTER 4: RESULTS

In this chapter, the dissertation presents the results of the study, which includes results of median lethal dose, phytochemical analysis, sub-acute toxicity testing, and results of statistical analysis of means.

4.1 Extractive value

$$\frac{\text{weight of extract}}{\text{Weight of plant material}}\ X\ 100 = Extractive\ value$$

$$Extractive\ value = \frac{24.7g}{320.8g}\ X\ 100 = 8.5\%$$

4.2 Phytochemical analysis

Indicators for presence or absence of phytochemical constituents were observed to determine the phytochemical composition of *Aristolochia elegans* seed extract (Figure 4.1 and Table 4.1)

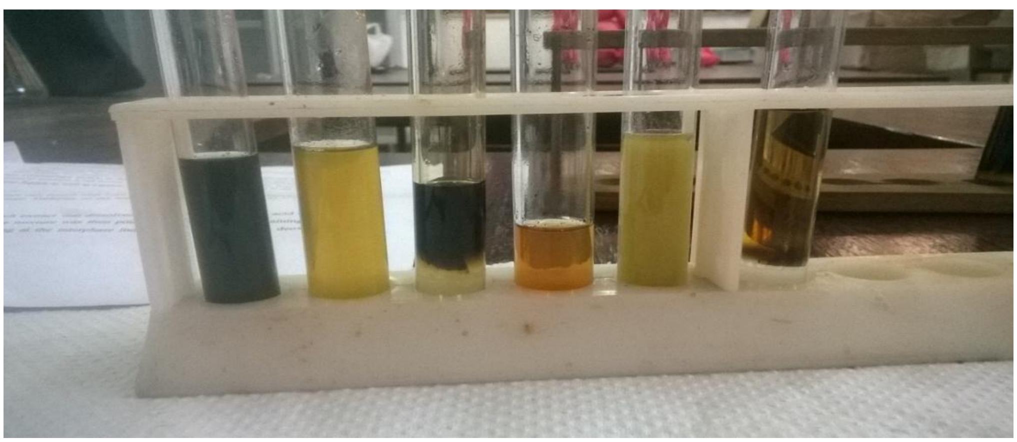

Figure 4.1.Phytochemical analysis of the hydroalcholic seed extract of *Aristolochia elegans* revealed the presence of tanins, sterols, triterpenoides, flavons, alkaloids and glycosides.

Table 4.1. Phytochemical screening of Aristolochia elegans mast seed crude aqueous extract

Phytochemical compound	Test	Positive indicator	Observation	Result
Alkaloids	Mayers's test	Turbidity of resulting precipitate	Turbid precipitate	**Positive**
Flavonoids	Shinoda reagent test	Red color for flavonols, orange for flavons, red-violaceous for flavanones	Orange	**Positive for flavons**
Saponins	Foam test	Persistent foam	No foam	**Negative**
Sterols	Liebermann – Bouchard reaction	Red brown or violet ring appears after 5-10minutes at the contact zone of the two liquids as well as a gree-bluish or violet upper layer	Red ring at the contact zone	**Positive**
Glycosides	Keller- Kiliani test	Brown ring at the interphase	Brown ring at interphase	**Positive**
Tannins	Braymer's test	Formation of green precipitate	Greenish precipitate	**Positive**

Key: (+) positive test, (-) negative test

4.3 Thin layer chromatography

The shape, color, distance moved and Retention Factor of developed spots after application of crude seed extract of *Aristolochia elegnas* and Aristolochic acid standard to chromatography plate revealed the presence of Aristolochic acid in Aristolochia elegans seed extract (Figure 4.2 and 4.3)

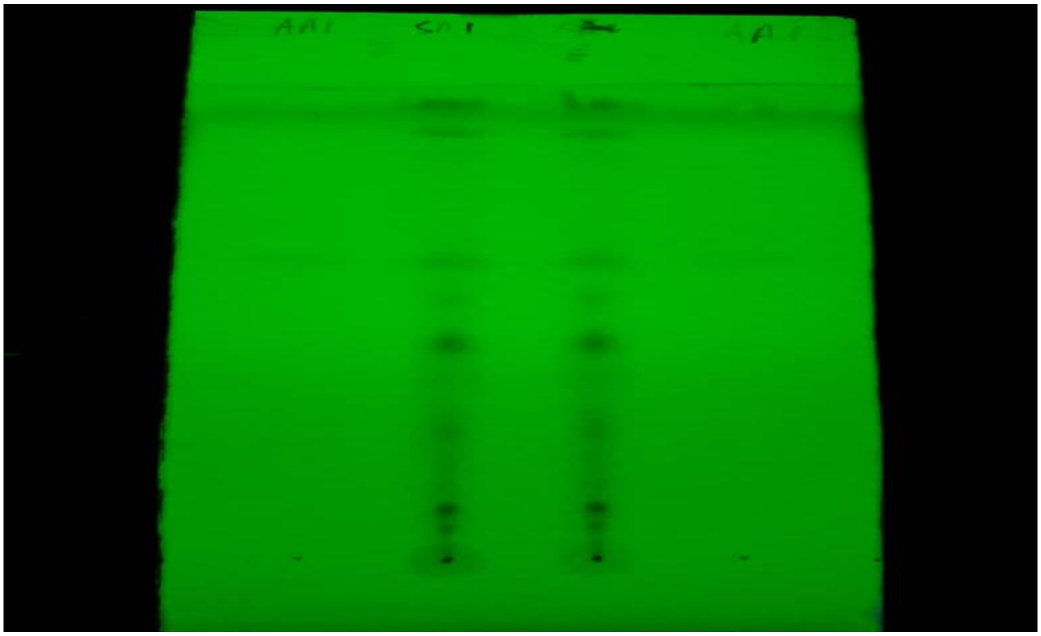

Figure 4.2. TLC analysis revealed presence of aristolochic acid I in the crude extracts of Aristolochia elegans.

Retention Factor (Rf) of standard = $\frac{46}{73} = 0.63$

Retention Factor (Rf) of extract = $\frac{46}{73} = 0.63$

4.4 Quantitative determination of AAI using HPTLC (High Performance Thin Layer Chromatography)

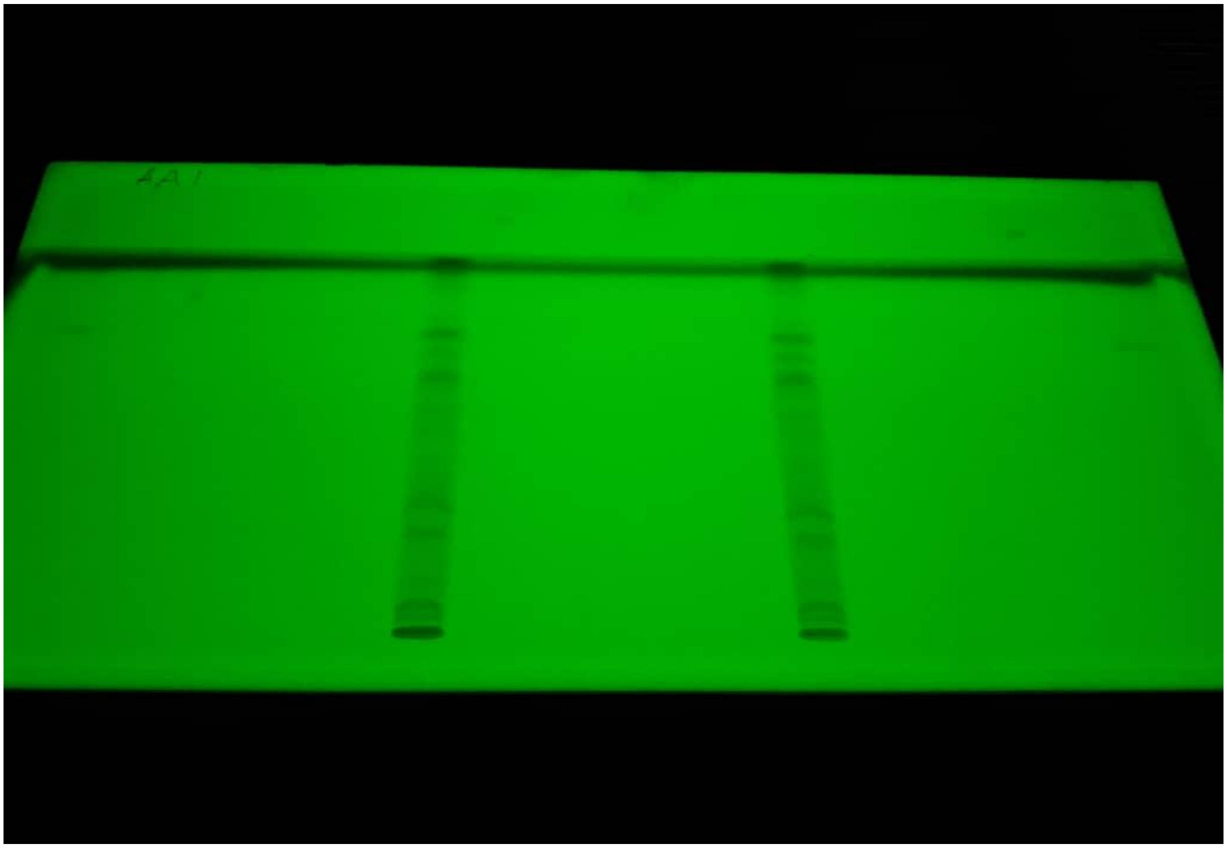

Figure 4.3. HPTLC chromatogram of Aristolochic acid I standard and *Aristolochia elegans* seed extract.

Densitometric scanning was used to determine and plot spectra comparison of the area of Aristolochic acid in the Aristolochic acid standard and *Aristolchia elegans* crude extract (Figure 4.4 and Table 4.2). The determined areas were then used to determine the quantity of Aristolochic acid in *Aristolochia elegans* seed extract (Table 4.3).

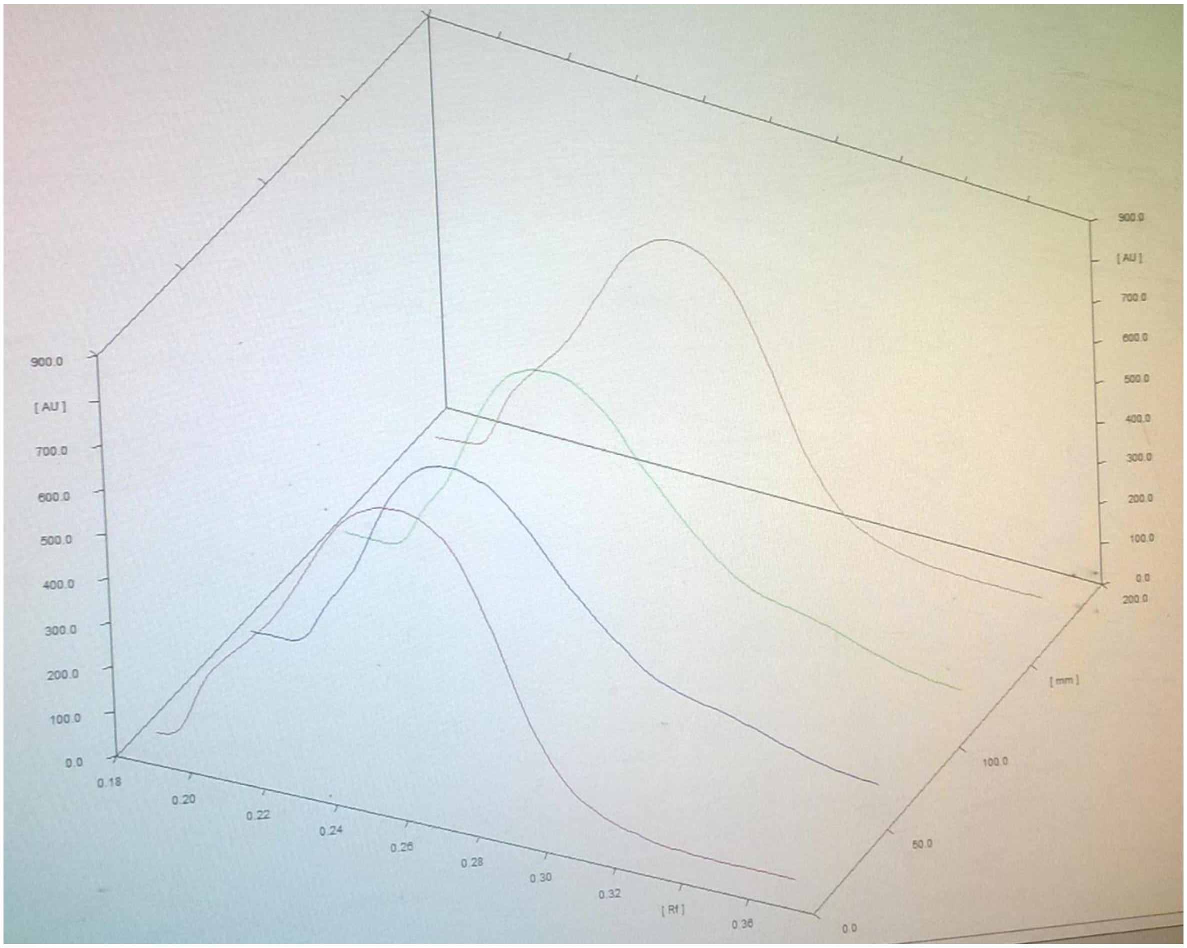

Figure 4.4.Spectra comparison of Aristolochic acid I between standard and crude extract. Plot 1 and 4 represent standard, plot 2 and 3 represent crude extract.

Table 4.2. Aristolochic acid I standard and Aristolochia elegans seed extract profiles

Track	Substance	Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	Standard	1	0.19 Rf	0.9 AU	0.25 Rf	647.5 AU	`100%	0.34 Rf	0.4	28093.2 AU	100%
2	Extract	1	0.19Rf	0.6 AU	0.24 Rf	500.6 AU	100%	0.37 Rf	0.7	22152.1 AU	100%
3	Extract	1	0.19Rf	0.0 AU	0.25 Rf	509.7 AU	100%	0.37 Rf	0.4	22502.6 AU	100%
4	Standard	1	0.19Rf	5.6 AU	0.26 Rf	624.9 AU	100%	0.35 Rf	0.0	26237.2 AU	100%

Table 4.3. Analysis of Densitometric scanning results

	Area	Average Area	Concentration
Standard 1	28093.2		
Standard 2	26237.2	27165.2	
Sample 1	22152.1		
Sample 2	22502.6	22327.35	
Mass of standard weighed/mg			1.33
Mass of sample weighed/mg			102.68
Analyte conc mg/sample			1.09314
Analyte in sample mg/kg			10646.09
AAI Concentration g/kg of Crude extract			**10.65g/kg**

4.5 Acute toxicity testing and determination of LD50

None of the Rats died while conducting the lethal dose determination up to a dose of 5000mg/kg. Therefore, the study concluded that the Median Lethal Dose (LD50) of the extract was greater than 5000mg/kg (Table 4.4)

Table 4.4: Determination of Median Lethal Dose (LD50) of *Aristolochia elegans* seed extract

Group	Dose mg/kg body weight							
	1.75	5.5	17.5	55	175	550	1750	5000
Treatment group	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive
Control (Normal saline)	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive

4.6 Effects of Aristolochia elegans on renal function parameters

Serum concentrations of renal function biomarkers were computed using the Beckman Coulter AU480 analyzer (Table 4.5).

Table 4.5: Kidney function biomarker concentrations in 25 Rat models after 10 day treatment with Aristolochia elegans seed extract

Treatment group		Sodium mmol/L	Potassium mmol/L	Chloride mmol/L	Urea mmol/L	Creatinine μmol/L
	1	137	3.5	104	7.36	70.2
	1	137	3.6	106	9.33	44.2
Group 1 (Negative control)						
1		145	3.9	104.1	6.35	32.4
	1	139	3.9	106.2	10.38	28.8
	1	146	5.6	106.1	9.54	43.4
	2	140	4.7	103.6	9.77	22.2
	2	143	4.2	104.2	8.03	22.2
Group 2 (500mg/kg)						
2		140	4.9	105.7	8.96	21.5
	2	140	4.8	103.8	7.81	17.8
	2	140	5.1	104.6	7.46	28.1
	3	144	4	103	8.81	20.7
	3	138	4.4	102.3	7.92	27.3
Group 3 (333mg/kg)						
3		139	3.7	103.2	6.94	25.9
	3	146	4	112.2	8.2	17.1
	3	139	4.9	104.9	7.86	19.3
	4	140	5.1	107.8	7.6	27.3
	4	136	4.5	102.5	8.51	21.5
Group 4 (167mg/kg)						
4		138	4.2	105.8	7.47	28.8
	4	139	4.5	107.9	6.57	23.7
	4	138	5.3	103.9	7.33	20
	5	138	4.9	107	8.69	28.1
	5	136	3.7	104	6.71	27.3
Positive control						
(paracetamol 1000mg/kg)		142	3.9	105	7.36	20.7
	5	143	3.7	109.3	7.8	27.3
	5	141	4.9	104	11.22	20.7

Comparison of the means using ANOVA revealed that the serum concentrations of sodium, chloride and urea in the test groups were not significantly different from the concentration in the negative control group. Creatinine serum concentrations were significantly lower in the test groups than in the negative control group (Table 4.6).

Table 4.6: Mean renal function biomarker concentrations after 10 day administration of *Aristolochia elegans* extract. Values reported as Means + SE and 95% CI with n= 5 per treatment group

Parameter	Treatment Group	Mean serum values	Negative control	p Value
Serum Sodium mmol/L	500mg/kg of A.e	140.6 +/- 0.6 [138.9-14.3]	140.8 +/- 1.96 [135.4-146.2]	0.72
	333mg/kg of A.e	141.2 +/- 1.6 [136.8-145.6]	140.8 +/- 1.96 [135.4-146.2]	1
	167mg/kg of A.e	138.2 +/- 0.7 [136.4-140]	140.8 +/- 1.96 [135.4-146.2]	0.79
	Positive control	140.0+/1.3 [136.4-143.6]	140.8 +/- 1.96 [135.4-146.2]	0.28
Serum Potassium mmol/L	500mg/kg of A.e	4.7 +/- 0.2 [4.3-5.2]	4.1 +/-0.4 [3.0-5.2]	0.32
	333mg/kg of A.e	4.2 +/- 0.2 [3.6-4.8]	4.1 +/-0.4 [3.0-5.2]	1
	167mg/kg of A.e	4.7 +/- 0.2 [4.1- 5.3]	4.1 +/-0.4 [3.0-5.2]	0.35
	Positive control	4.2 +/- 0.6 [3.4- 5.0]	4.1 +/-0.4 [3.0-5.2]	1
Serum Chloride mmol/L	500mg/kg of A.e	104.4 +/-0.4 [103.3-105.4]	105.3+/0.5 [103.9-106.7]	0.98
	333mg/kg of A.e	105.1 +/-1.8 [100.1-110.2]	105.3+/0.5 [103.9-106.7]	1
	167mg/kg of A.e	105.6+/1.1 [102.6-108.5]	105.3+/0.5 [103.9-106.7]	1
	Positive control	105.9 +/- 1.0 [103- 108.7]	105.3+/0.5 [103.9-106.7]	1
Serum Urea mmol/L	500mg/kg of A.e	8.4 +/- 0.4 [7.2-9.6]	8.6 +/- 0.7 [6.5- 10.7]	1
	333mg/kg of A.e	7.9 +/-0.3 [7.1-8.8]	8.6 +/- 0.7 [6.5- 10.7]	0.92
	167mg/kg of A.e	7.5 +/-0.3 [6.6-8.4]	8.6 +/- 0.7 [6.5- 10.7]	0.64
	Positive control	8.4 +/-0.8 [6.2-10.5]	8.6 +/- 0.7 [6.5- 10.7]	1
Serum Creatinine µmol/L	500mg/kg of A.e	22.4 +/-1.7 [17.8-6.9]	43.8 +/- 7.3 [23.7-63.9]	0.004
	333mg/kg of A.e	22.1 +/- 2.0 [16.6-27.5]	43.8 +/- 7.3 [23.7-63.9]	0.003
	167mg/kg of A.e	24.3 +/- 1.7 8 [19.6-28.9]	43.8 +/- 7.3 [23.7-63.9]	0.008
	Positive control	24.8+/1.78 [20.1-29.5]	43.8 +/- 7.3 [23.7-63.9]	0.1

4.7Effects of Aristolochia elegans on liver function parameters

Serum concentrations of liver function biomarkers were computed using the Beckman Coulter AU480 analyzer (Table 4.7).

Table 4.7: Liver function biomarker concentrations in 25 Rat models after 10 day treatment with Aristolochia elegans seed extract

Treatment group	Alkaline Phosphatase U/L	ALT U/L	Total Protein g/L	Albumin g/L
1	336	272.8	72.4	31.4
1	545.1	186	69.6	32.9
Group 1 (Negative control)	418.1	128.9	73.2	32.7
1	223.4	171.1	65.6	30.7
1	296.1	197.5	86.9	36.6
2	423.1	142.4	67.3	34.2
2	362.2	172.9	69.3	34
Group 2 (500mg/kg)	439.8	102.7	70.1	25.9
2	312.2	196.1	75.1	35.8
2	398.8	356.8	72	33.6
3	485.2	166.2	61.8	31
3	403.5	158	75.4	36
Group 3 (333mg/kg)	302.3	99.7	68.3	34.5
3	392.2	226.1	59.9	24.9
3	443.5	251.5	69.7	33.2
4	217.5	124.6	65.8	33.2
4	310.7	172.4	73.5	33.8
Group 4 (167mg/kg)	306.9	172.4	71.8	35.8
4	493.2	142.7	60.2	30.5
4	491.2	154.7	62.2	32.5
5	262.5	299.6	61.6	30.4
5	278.3	420.3	73	36
Positive control (paracetamol 1000mg/kg)	701.8	361.1	67.8	34.5
5	377.6	293.2	74.7	36.2
5	709.9	316.9	63.2	32.7

The serum concentrations of liver function biomarkers in the test groups were not significantly different from the concentration in the negative control group, except for Alanine amino transferase revealed a significant difference with p= 0.001. Post hoc analysis however revealed that the significant difference was between negative control and positive control (Table 4.8)

Table 4.8: Mean serum liver function biomarker concentrations after 10 day administration of *Aristolochia elegans* extract. Values reported as Means + SE and 95% CI with n= 5 per treatment group

Parameter	Treatment Group	Mean serum values	Negative control	p Value
Alanine amino transferase U/L (± SE), CI	500mg/kg of A.e	194.2 +/- 43.6 [73.2-315.1]	191.3 +/- 23.5 [126.1-256.4]	1
	333mg/kg of A.e	180.3 +/-26.8 [105.9-254.7]	191.3 +/- 23.5 [126.1-256.4]	1
	167mg/kg of A.e	153.4+/9.1 [128.0-178.7]	191.3 +/- 23.5 [126.1-256.4]	0.87
	Positive control	338.2 +/- 23.7 [172.4-304.0]	191.3 +/- 23.5 [126.1-256.4]	0.01
Alkaline Phosphatase U/L (±SE), CI	500mg/kg of A.e	387.2 +/- 22.8 [323.8-450.7]	363.7 +/- 55.2 [210.5-516.9]	1
	333mg/kg of A.e	405.3 +/- 30.5 [320.6-490.1]	363.7 +/- 55.2 [210.5-516.9]	0.99
	167mg/kg of A.e	363.9 +/- 55.0 [211.3-516.5]	363.7 +/- 55.2 [210.5-516.9]	1
	Positive control	588.9 +/- [419.5-758.2]	363.7 +/- 55.2 [210.5-516.9]	0.02
Albumin g/L (±SE), CI	500mg/kg of A.e	32.7 +/- 1.7 [27.9-37.5]	32.9 +/- 1.0 [30.0- 35.7]	1
	333mg/kg of A.e	31.9 +/1.9 [26.5-37.3]	32.9 +/- 1.0 [30.0- 35.7]	0.99
	167mg/kg of A.e	33.1 +/- 0.9 [30.8-35.6]	32.9 +/- 1.0 [30.0- 35.7]	1
	Positive control	34.0 +/- 1.1 [30.9- 37.0]	32.9 +/- 1.0 [30.0- 35.7]	0.98
Total Protein g/L (±SE), CI	500mg/kg of A.e	70.8 +/- 1.3 [67.1- 74.4]	73.5 +/- 3.6 [63.6- 83.5]	0.95
	333mg/kg of A.e	67.0 +/-2.8 [59.2-74.8]	73.5 +/- 3.6 [63.6- 83.5]	0.45
	167mg/kg of A.e	66.7 +/- 2.6 [59.5-73.9]	73.5 +/- 3.6 [63.6- 83.5]	0.4
	Positive control	68.1 +/-2.6 [60.9-75.2]	73.5 +/- 3.6 [63.6- 83.5]	0.61

4.8. Post hoc analysis of means between different dose groups for determination of dose dependent effect.

There was no significant difference between concentrations of serum sodium in the different dose group's p> 0.05 (Table 4.9)

Table 4.9: Dunnett T3 Post Hoc analysis for Sodium (Na)

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Sodium	Negative Control	High dose	0.41	0.72
		Medium dose	0.44	1
		Low dose	0.44	0.79
		Reference Drug	0.4	0.28
	High Dose	Negative Control	0.41	0.72
		Medium Dose	0.26	0.41
		Low Dose	0.25	1
		Reference Drug	0.19	0.4
	Medium Dose	Negative Control	0.44	1
		High Dose	0.26	0.41
		Low Dose	0.29	0.6
		Reference Drug	0.24	0.05
	Low Dose	Negative Control	0.44	0.79
		High Dose	0.25	1
		Medium Dose	0.29	0.58
		Reference Drug	0.23	0.58
	Reference Drug	Negative Control	0.4	0.28
		High Dose	0.18	0.4
		Medium Dose	0.24	0.05
		Low Dose	0.23	0.58

There was no significant difference between concentrations of serum potassium in the different dose group's $p > 0.05$ (Table 4.10)

Table 4.10: Turkey Post Hoc analysis for potassium (K)

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Potassium	Negative Control	High dose	0.37	0.43
		Medium dose	0.37	1
		Low dose	0.37	0.46
		Reference Drug	0.37	1
	High Dose	Negative Control	0.37	0.43
		Medium Dose	0.37	0.59
		Low Dose	0.37	1
		Reference Drug	0.37	0.62
	Medium Dose	Negative Control	0.37	1
		High Dose	0.37	0.59
		Low Dose	0.37	0.62
		Reference Drug	0.37	1
	Low Dose	Negative Control	0.37	0.46
		High Dose	0.37	1
		Medium Dose	0.37	0.62
		Reference Drug	0.37	0.65
	Reference Drug	Negative Control	0.37	1
		High Dose	0.37	0.62
		Medium Dose	0.37	1
		Low Dose	0.37	0.65

There was no significant difference between concentrations of serum Chloride in the different dose group's p> 0.05 (Table 4.11)

Table 4.11: Turkey Post Hoc analysis for Chloride (Cl)

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Chloride	Negative Control	High dose	1.53	0.98
		Medium dose	1.53	1
		Low dose	1.53	1
		Reference Drug	1.53	1
	High Dose	Negative Control	1.53	0.98
		Medium Dose	1.53	0.99
		Low Dose	1.53	0.93
		Reference Drug	1.53	0.87
	Medium Dose	Negative Control	1.53	1
		High Dose	1.53	0.99
		Low Dose	1.53	1
		Reference Drug	1.53	1
	Low Dose	Negative Control	1.53	1
		High Dose	1.53	0.93
		Medium Dose	1.53	1
		Reference Drug	1.53	1
	Reference Drug	Negative Control	1.53	1
		High Dose	1.53	0.87
		Medium Dose	1.53	1
		Low Dose	1.53	1

There was no significant difference between concentrations of serum Urea in the different dose group's $p > 0.05$ (Table 4.12).

Table 4.12: Turkey Post Hoc analysis for Urea

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Urea	Negative Control	High dose	0.79	1
		Medium dose	0.79	0.92
		Low dose	0.79	0.64
		Reference Drug	0.79	1
	High Dose	Negative Control	0.79	1
		Medium Dose	0.79	0.98
		Low Dose	0.79	0.77
		Reference Drug	0.79	1
	Medium Dose	Negative Control	0.79	0.92
		High Dose	0.79	0.98
		Low Dose	0.79	0.98
		Reference Drug	0.79	0.98
	Low Dose	Negative Control	0.79	0.63
		High Dose	0.79	0.77
		Medium Dose	0.79	0.98
		Reference Drug	0.79	0.8
	Reference Drug	Negative Control	0.79	1
		High Dose	0.79	1
		Medium Dose	0.79	1
		Low Dose	0.79	0.8

There was significant difference in serum Creatinine concentrations between negative control and test groups $p < 0.05$, but there was no significant difference between concentrations in the different dose group's $p > 0.05$ (Table 4.13).

Table 4.13: Turkey Post Hoc analysis for Creatinine (Cr)

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Creatinine	Negative Control	High dose	5.09	0.004
		Medium dose	5.09	0.003
		Low dose	5.09	0.008
		Reference Drug	5.09	0.1
	High Dose	Negative Control	5.09	0.004
		Medium Dose	5.09	1
		Low Dose	5.09	1
		Reference Drug	5.09	1
	Medium Dose	Negative Control	5.09	0.003
		High Dose	5.09	1
		Low Dose	5.09	1
		Reference Drug	5.09	1
	Low Dose	Negative Control	5.09	0.008
		High Dose	5.09	1
		Medium Dose	5.09	1
		Reference Drug	5.09	1
	Reference Drug	Negative Control	5.09	0.01
		High Dose	5.09	1
		Medium Dose	5.09	0.982
		Low Dose	5.09	1

There was no significant difference between concentrations of serum Alkaline Phosphatase in the different dose group's p> 0.05 (Table 4.14)

Table 4.14: Dunnett T3 Post Hoc analysis for Alkaline Phosphatase (ALP)

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Alkaline Phosphatase	Negative Control	High dose	59.7	1
		Medium dose	63.1	0.99
		Low dose	77.9	1
		Reference Drug	114.1	0.98
	High Dose	Negative Control	59.7	1
		Medium Dose	38.1	1
		Low Dose	59.5	1
		Reference Drug	102.5	0.99
	Medium Dose	Negative Control	63.1	1
		High Dose	38.1	1
		Low Dose	62.9	1
		Reference Drug	104.4	1
	Low Dose	Negative Control	77.9	1
		High Dose	59.5	1
		Medium Dose	62.9	1
		Reference Drug	114	0.98
	Reference Drug	Negative Control	114.1	0.98
		High Dose	102.5	0.99
		Medium Dose	104.4	1
		Low Dose	114	0.98

There was a significant difference of serum Alanine amino transferase concentrations between Negative control and positive control, $p = 0.01$. But there was no significant difference between concentrations in the negative control and the test groups, $p > 0.05$ (Table 4.15)

Table 4.15: Turkey Post Hoc analysis for Alanine amino transferase (ALT)

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Alanine amino transferase	Negative Control	High dose	39	1
		Medium dose	39	1
		Low dose	39	0.87
		Reference Drug	39	0.01
	High Dose	Negative Control	39	1
		Medium Dose	39	1
		Low Dose	39	0.83
		Reference Drug	39	0.011
	Medium Dose	Negative Control	39	1
		High Dose	39	1
		Low Dose	39	0.96
		Reference Drug	39	0.05
	Low Dose	Negative Control	39	0.87
		High Dose	39	0.83
		Medium Dose	39	0.96
		Reference Drug	39	0.001
	Reference Drug	Negative Control	39	0.01
		High Dose	39	0.01
		Medium Dose	39	0.01
		Low Dose	39	0.001

There was no significant difference between concentrations of serum Total Protein in the different dose group's p> 0.05 (Table 4.16)

Table 4.16: Turkey Post Hoc analysis for Total Protein

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Total Protein	Negative Control	High dose	3.79	0.95
		Medium dose	3.79	0.45
		Low dose	3.79	0.4
		Reference Drug	3.79	0.61
	High Dose	Negative Control	3.79	0.95
		Medium Dose	3.79	0.86
		Low Dose	3.79	0.82
		Reference Drug	3.79	0.95
	Medium Dose	Negative Control	3.79	0.45
		High Dose	3.79	0.86
		Low Dose	3.79	1
		Reference Drug	3.79	1
	Low Dose	Negative Control	3.79	0.4
		High Dose	3.79	0.82
		Medium Dose	3.79	1
		Reference Drug	3.79	1
	Reference Drug	Negative Control	3.79	0.61
		High Dose	3.79	0.95
		Medium Dose	3.79	1
		Low Dose	3.79	1

There was no significant difference between concentrations of serum Albumin in the different dose group's p> 0.05 (Table 4.17)

Table 4.17.: Turkey Post Hoc Analysis for Albumin

Dependent Variable	(i) Study Group	(j) Study Group	Std. Error	Sig
Serum Albumin	Negative Control	High dose	1.98	1
		Medium dose	1.98	0.99
		Low dose	1.98	1
		Reference Drug	1.98	0.98
	High Dose	Negative Control	1.98	1
		Medium Dose	1.98	0.99
		Low Dose	1.98	1
		Reference Drug	1.98	0.97
	Medium Dose	Negative Control	1.98	0.99
		High Dose	1.98	0.99
		Low Dose	1.98	0.97
		Reference Drug	1.98	0.84
	Low Dose	Negative Control	1.98	1
		High Dose	1.98	1
		Medium Dose	1.98	0.97
		Reference Drug	1.98	0.99
	Reference Drugs	Negative Control	1.98	0.98
		High Dose	1.98	0.97
		Medium Dose	1.98	0.84
		Low Dose	1.98	0.99

CHAPTER 5: DISCUSSION

5.1 Discussion

The increase in use of medicinal herbs globally, has increased the need for toxicity studies to be conducted on herbal medicines (Hussin, 2001). The study investigated phytochemical content of *Aristolochia elegans* seed extract, presence and quantity of aristolochic acid in the crude extract. Further acute toxicity and the effects of sub-acute consumption of hydro-alcoholic seed extracts of *Aristolochia elegans* on biomarkers of liver and renal function in rats was investigated.

Phytochemical analysis of the hydroalcholic seed extract of *Aristolochia elegans* revealed the presence of tanins, sterols, triterpenoides, flavons, alkaloids and glycosides. Plants which possess tanins have astringent, hemostatic, antiseptic and toning properties, these plants can be used to treat burns, wounds and contain diarrhea (Aldred, 2009). Human studies have shown that plant sterols significantly reduce plasma total and LDL cholesterol, sterols have also been shown to have beneficial effects in colon cancer and prostate hyperplasia (Moghadasian, 2000). Triterpenoids have been shown to have cytotoxic effects on tumor cells and are anti-inflammatory (Bishayee, 2011). Flavons have been shown to have antioxidant and free radical scavenging activities (Asif, 2013). Alkaloids though poisonous have various medicinal properties including anti-inflammatory and anticancer properties (Cassiano, 2011). The phytochemical constituent of *Aristolochia elegans* could explain the wide medicinal properties of the plant.

Thin Layer Chromatography (TLC) separation of crude extracts and visualization by UV light offers a practical and rapid procedure for the detection of aristolochic acids in botanical products (Abdelgadir *et al*, 2011). Results of TLC analysis revealed presence of Aristolochic acid I in the crude extract. This finding is consistent with phytochemical studies that have shown presence of Aristolochic acid I in plants of the Aristolochia species (Latha, 2016). High Performance Thin

Layer Chromatography is both a qualitative and quantitative technique that can be used to detect and quantify specific constituents in plant extracts (Shuayprom et al, 2015). Good separation of crude extract was achieved using mobile phase Chloroform: Methanol: water (6:4:1), Analysis using denstomeric scanning at 430nm in absorbance mode determined the quantity of Aristolochic acid I in the crude extract to be 10g/kg. This finding was similar to findings in other quantitative determination studies of aristolochic acid in some plants in the Aristolochia species such as; a study conducted in Sudan which found AA-I amount to be 12.98 g/kg in *Aristolochia bracteolata L*. Other studies reveal less quantities of AA-I in some species of Aristolochia such as a quantification of AAI in Aristolochia manshuriensis in Korea was found to be 2.85g/kg (Hwang, 2012) .This shows how active ingredients or secondary metabolites in plants can vary from country to country, due to differences in climate, soil and other ecological factors, making studies of locally grown plants highly important.

Sub chronic toxicity testing showed that renal function biomarkers; sodium, urea and chloride concentrations in the test groups did not differ significantly from the concentrations in the negative control group $p > 0.05$. Serum creatinine levels were significantly lower in the test groups compared to the negative control, $p= 0.013$. A dysfunction in kidney function would have resulted in increased serum creatinine levels in the test groups, low serum creatinine levels are a reflection of reduced muscle mass (Patel *et al*, 2013). Though this finding was significant it was not indicative of kidney dysfunction. Furthermore, despite the serum concentrations of creatinine in the test groups being lower than that of the negative control, the concentrations in both the test groups and negative control were still in the normal expected ranges for rats i.e. 17.7-70.7 µmol/L (Kaynur et al, 2006). Although the relationship between *Aristolochia elgans* extract and muscle mass was not examined in this study, further analysis might benefit from this. Results also showed that liver function biomarkers; Albumin, Alkaline phosphatase and Total protein concentrations in the test groups did not differ significantly from the concentrations in the negative control $p > 0.05$. ANOVA analysis of Alanine amino transferase showed significant difference $p = 0.001$, however post hoc analysis revealed that the significant difference was between negative control and positive control as expected due to hepatic necrosis caused by large doses of paracetamol, there was no significant difference of serum Alanine amino transferase concentrations between test groups and negative control group. Aristolochic acid is known to form dysfunctional DNA adducts with various organs in the body including the liver and the kidneys, the DNA adducts result in genotoxic mutations which leads to renal interstitial fibrosis, loss of corticol tubules and hepatocellular damage (Singh and Prakash, 2011). This results in increased serum concentrations of sodium, potassium, chloride, urea, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin and total protein. Studies however have revealed that the toxic Aristolochic acid I is metabolized by cytochrome P450 enzymes specifically CYP1A to a detoxification metabolite 8-hydroxyaristolochic acid I (AAIa) (Xiao and Ren, 2008). It is therefore possible that at the dosages used in this study the livers of the Rats were able to adequately metabolize and detoxify the aristolochic acid I in the extract. Our findings are consistent with previous reports including; a sub chronic toxicity study in which *Aristolochia Fructus* was administered to rats for ninety days, no significant difference in serum biochemistry of renal function parameters and hematology between the test and controls were found at lower doses (21.35 and 213.5 mg/kg), nephrotoxicity and hyperplasia were found only in Rats receiving the highest dose i.e. 2135 mg/kg (Hwang *et al*, 2006). The findings were also consistent with a study in which *Aristolochia Fructus* extracts were administered to Rats for fourteen days, no significant change in levels of serum creatinine, Blood Urea Nitrogen and urinary Albumin between test and control groups were found (Zhou *et al,* 2012). Another study were rats where given a single dose of *Aristolochia longa* 5000mg/kg, revealed no significant difference in serum urea and creatinine levels between the control and test groups (Benarba *et al,* 2016). These findings are further supported by the fact that there are very few patients found with aristolochic acid nephropathy after consuming aristolochia for short periods and at low doses (Li *et al*, 2001; Arlt *et al*, 2004).

Other studies however show different findings; A study conducted in which 1500mg/kg body weight of *Aristolochia indica L*, was administered to Wister albino rats for 28 days, urea nitrogen, creatinine, alanine amino transferase, aspartate amino transferase and uric acid levels were significantly higher in the test than the controls (Mridula *et al*, 2011). A study done by Abdulhakeem *et al* (2017) reported significant elevations in Alkaline phosphatase, Alanine amino transferase and Aspartate amino transferase after 14-day administration of *Aristolochia ringen* at doses of 75, 150 and 300mg/kg body weight. Differences in findings could be due to differences in aristolochic acid I content in plants used, however comparisons could not be made as quantification of aristolochic acid in extracts used were not conducted in cited studies.

Dose dependent effect of a drug is determined by an increase or reduction in an effect as the dose is altered, the study did not reveal any dose dependent effects of extract on liver and kidney biomarker concentrations. This finding was different from findings in other studies such as the one conducted by Benzakour et al (2011) which demonstrated dose dependent effects of *Aristolochia longa* in a sub-chronic toxicity study, *Aristolochia longa* was examined in two groups of mice, one group received 1.25g/kg/day of aqueous extract and the other group received 2.5g/kg/day of extract. There was no toxicity found in the group receiving the lower dose with respect to renal and liver function parameters as well as histopathogical analysis of kidney and liver, whereas AST, ALP and creatinine were significantly elevated in the group receiving the higher dose, histopathological analysis of the kidney and liver in the high dose group revealed damage. Dose dependent effect was also demonstrated in a study conducted in Germany in which the nephrotoxic action of aristolochic acid (AA) was investigated in female Wistar rats given single doses of 10, 50 and 100mg/kg by gastric tube, results showed elevated creatinine and urea levels in the 100mg/kg group only, Renal lesions developed within 3 days, the effect also being dose dependent (Mengs and Stotzem, 1993). Dose dependent effect could not have been seen in this study due to the fact that none of the doses investigated affected kidney and liver function as revealed by lack of significant changes in concentrations of biomarkers of kidney and liver function in the test groups.

The results of this study provided important information on phytochemical content of *Aristolochia elegans* growing in Zambia and lays important ground work for toxicity testing of *Aristolochia elegans* in Zambia which could be further built on.

Limitations of the study

The study did not investigate the pathomorphological effects of the extract on the kidneys and liver, which could reveal more on the toxicity of the extract. Aspartate amino transferase was not analyzed because blood was collected via cardiac puncture, the heart contains high levels of AST and damage to heart tissue and increased cardiac activity could result in elevated AST levels. Baseline concentrations of parameters studied could not be obtained due to ethical guidelines on blood collection from rats which require 4 weeks recovery time after drawing 1.6 – 2.4 mls blood (Diehl, 2001) .This would not be feasible for a sub-acute toxicity study of this nature, which would require blood being drawn from the rats on day 0 and day 11. Negative and positive controls were therefore used as employed in literature reviewed (Benarba *et al,* 2016 ; Hwang *et al*, 2006 Patel *et al*, 2013; Mridula *et al*, 2011).

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

Hydroalcholic seed extracts of *Aristolchia elegans* did not appear to exert any effect on renal and hepatic function parameters investigated at the doses administered. Hydroalcoholic seed extracts of *Aristolochia elegans* did not exhibit dose dependency at the doses administered.

6.2 Recommendations

The study recommends Histopathological studies on the effects of Aristolochia elegans seed extract to be conducted. Studies to analyze higher dosages for renal and liver toxicity are recommended and lastly studies to optimize the dose for therapeutic effect are recommended.

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APPENDICES

APPENDIX I: PLANT IDENTIFICATION

THE UNIVERSITY OF ZAMBIA

DEPARTMENT OF BIOLOGICAL SCIENCES

Telephone: 291777/8 or Direct: 291531
Telegrams: UNZA LUSAKA
Telex UNZALUZA44370
Fax: +260-211-253952

P.O. Box 32379
Lusaka
Zambia

Your ref:
Our ref:

IDENTIFICATION RESULTS

Date: 6th September, 2017

Name of client: Mwitwa Chileshe

Number of specimens: 1

Specimen name (s):

Aristolochia elegans Mast. (Aristolochiaceae)

Identified by: Mrs Florence Nyirenda (MSc.).

Designation: Scientist / Herbarium Assistant

Signature: [signature]

THE UNIVERSITY OF ZAMBIA
SCHOOL OF NATURAL SCIENCES
08 SEP 2017
DEPT. OF BIOLOGICAL SCIENCES
P.O. BOX 32379, LUSAKA.
TEL: +260 211 291531

APPENDIX II: LIST OF REAGENTS

- Ethanol 80%
- Normal Saline
- Diethyl Ether
- Aristolochic acid I European pharmacopeia standard
- Methanol
- Chloroform
- Acetic acid
- Sulfuric acid (H2SO4)
- Hydrochloric acid (HCL)
- Potassium iodide
- Mercuric Chloride,
- Ferric chloride
- metallic Magnesium

APPENDIX III: INFORMED CONSENT FROM HERBALIST (TRADITIONAL HEALER)

The University of Zambia

School of Medicine

Department of Physiological Sciences

CONSENT FORM FOR: Herbalist

RESEARCH TITLE: Effects of Hydro alcoholic seed extracts of *AristolochiaElegans* on Renal and Hepatic function of Rats

RESEARCHER: Mwitwa Chileshe

I have been given information about the study " Effects of Alcoholic extracts of *AristolochiaElegans* on Renal and Hepatic function of Rats" and discussed the project with Mwitwa Chileshe who is conducting this research as part of a Master's Degree in Pharmacology supervised by Prof. Christian ChinyereEzeala in the department of Physiological Sciences at the University of Zambia.

I understand that if I consent to participate in this research, I will be required to assist the researcher in collecting seeds of *AristolochiaElegans*(Kalulusha) and will be paid a fee of K500. I understand the purpose of the study which is to establish whether *Aristolochiaelegans*has toxic effects on the kidney and liver of Wistar albino rats and that they are no personal risks and burdens. I have had an opportunity to ask Mwitwa Chileshe any questions I may have about the research and my participation.

I understand that my participation in this research is voluntary, I am free to refuse to participate and I am free to withdraw from the research at any time. If I have any enquiries about the research, I can contact Mwitwa Chileshe (0978889203) and/or Prof. Christian ChinyereEzeala (0967781355) or if I have any concerns or complaints regarding the way the research is or has been conducted, I can contact ERES CONVERGE IRB (+260 0955155633, 33 Joseph Mwila Road, Rhodes Park, Lusaka).

APPENDIX III CONTINUED: INFORMED CONSENT FROM HERBALIST (TRADITIONAL HEALER)

By signing below I am indicating my consent to participate in the research. I understand that the information obtained from this research will be primarily used in a Master's Degree thesis and will also be used in summary form for journal publication. I consent for it to be used in that manner.

Signed Date

....................................12 ..../03../17

Name

DAVID KWAMWENDA

245932/51/1

APPENDIX IV: APPROVAL LETTER FROM ERES CONVERGE IRB

ERES CONVERGE

33 Joseph Mwilwa Road
Rhodes Park, Lusaka
Tel: +260 955 155 633
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I.R.B. No. 00005948
F.W.A. No. 00011697

14th February, 2017

Ref. No. 2016-Dec-002

The Principal Investigator
Ms. Mwitwa Chileshe
Plot 1034, Salama Park
LUSAKA.

Dear Ms. Chileshe,

RE: EFFECTS OF ALCOHOLIC EXTRACTS OF *ARISTOLOCHIA ELEGANS* ON RENAL HEPATIC FUNCTION OF RATS.

Reference is made to your corrections. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	Ordinary	Approval No. **2016-Dec-002**
Approval and Expiry Date	Approval Date: 16th February, 2017	Expiry Date: 15th February, 2018
Protocol Version and Date	Version-Nil	15th February, 2018
Information Sheet, Consent Forms and Dates	• English.	15th February, 2018
Consent form ID and Date	Version-Nil	15th February, 2018
Recruitment Materials	Nil	15th February, 2018
Other Study Documents	-	15th February, 2018
Number of participants approved for study	-	15th February, 2018

Where Research Ethics and Science Converge

APPENDIX IV CONTINUED: APPROVAL LETTER FROM ERES CONVERGE IRB

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- ERES Converge IRB does not "stamp" approval letters, consent forms or study documents unless requested for in writing. This is because the approval letter clearly indicates the documents approved by the IRB as well as other elements and conditions of approval.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Prof. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON

APPENDIX V: APPROVAL LETTER FROM UTH

REPUBLIC OF ZAMBIA

MINISTRY OF HEALTH

University Teaching Hospital

P/Bag Rw 1X
Lusaka - Zambia

Fax: +260 211 250305
e-mail: mduth@yahoo.com

Tel: +260 211 253947 (Switch Board)
+260 211 251451

OFFICE OF THE SENIOR MEDICAL SUPERINTENDENT

Our Ref:

23rd February, 2017

Your Ref:

Mwitwa Chileshe
Plot No: 1034 Salama Park
LUSAKA

Dear Madam,

REF: PERMISSION TO CONDUCT RESEARCH AT THE UNIVERSITY TEAHCING HOSPITAL

This letter is written in response to your letter dated 22nd February, 2017 in which you requested to carry out a research entitled "***Effects of Alcohol Extracts of Aristolochia elegans on renal and hepatitis function of rats***".

I notice that your study is primary done in the Laboratory animal (Rats) and analysis will be done by our Laboratory at University Teaching Hospital.

Kindly liase with the Head of Laboratory and Revenue to make sure services of patients, are not disrupted and that test are paid for.

Your co-operation will greatly be appreciated.

Yours faithfully,

Dr. R. Zulu
Ag/Senior Medical Superintendent
Adult Hospital
UNIVERSITY TEACHING HOSPITAL