CHAPTER ONE

INTRODUCTION

1.0. INTRODUCTION

1.1. BACKGROUND OF STUDY

Plants continue to be a major source of medicines as they have been throughout human history. A 2008 report from the Botanic Gardens Conservation International (representing botanic gardens in 120 countries) revealed that 5 billion people still use medicinal plants to partly cater for their health care needs. According to the World Health Organization, medicinal plants would be the best source of a variety of drugs (Toroglu, 2011). The fact that plants synthesize a wide variety of chemical compounds that possess important biological functions accounts for this very important role of medicinal plants in health care. These chemical compounds known as phytochemicals, have been reported to possess beneficial effects on health on long-term basis and can be used to effectively treat diseases that affects humans. About 12,000 of such compounds that have been isolated so far have been estimated to be less than 10% of the total plant active ingredients available (Tapsell *et al*., 2006).

Currently, plant-derived drugs constitute about 25% of conventional medications used today (Rao *et al.*, 2004). Some of these drugs were obtained from plants reported to be potentially toxic. Examples of such drugs are colchicine from *Colchicum autumnale* used in the management of gout; and digoxin from *Digitalis purpurea* used in the management of heart failure. Some medicinal plants in their crude form have also been reported to produce better pharmacological activity than their isolated active components, and in some cases, their isolated active components are more toxic (CHEMEXCIL, 1992) or less efficacious (Kicklighter *et al*., 2003) than the crude extract. Hence the need to advance the development of standardized medicinal plant formulations for clinical use among

populations who will use them anyway; either because they have more confidence in the herbal formulations or they do not have access to conventional medicines.

In spite of wide-spread use of medicinal plants, especially in developing countries, such as Nigeria, many medicinal plants are yet to be comprehensively studied scientifically to validate their folkloric use (Gurib-Fakim, 2006), with the aim of optimizing their medicinal potential. One of such plants is *Aristolochia ringens* Vahl. (Aristolochiaceae). *Aristolochia* species of plants have a long history of use in traditional medicine and have in the past two decades attracted much interest, being the subject of numerous chemical and pharmacological studies (Wu *et al*., 2004). However, there have been reports of the potential toxicity of plants of the Aristolochiaceae family. In spite of this, they are still much in use. *A. ringens* is one of the very commonly used species in Nigeria, though mostly in small doses and for short durations in the management of several disorders including worm infestation, oedema, gastrointestinal and inflammatory disorders (Odugbemi, 2008).

1.2. STATEMENT OF THE PROBLEM

About 80% of Nigerians are rural dwellers who do not have access to adequate healthcare facilities (Abdulraheem *et al*., 2012). While this is the case, even available health care facilities are underutilized partly due to illiteracy among rural people, traditional conservatism, transportation and communication difficulties, among others. In addition, many rural dwellers depend more on herbs for managing their ailments. Side effects and therapeutic failure with orthodox drugs, resulting in reduced patients' compliance, further leading to treatment failures are much reported (Vincent and Furnham, 1996). Some of the reported adverse effects are due to synthetic processes involved in the drugs' development as well as their integrated excipients (Pifferi and Restani, 2003). These

challenges have fostered the search for alternative therapeutic options, such as medicinal plants.

In addition, the practice of only orally handing down information on ethnomedicinal use of plants has limited the available information on plants. This is largely due to the fact that the bearers of such information sometimes pass on, without having shared the knowledge. Some of such information are reportedly on the verge of being lost (Gurib-Fakim, 2006). The proper documentation of evidence-based report of the medicinal uses of plants can forestall the danger of further loss of such vital information.

Effective management of several disorders associated with diarrhoea (Boschi-Pinto *et al.*, 2008) and inflammatory diseases (Rao and Knaus, 2008) are still a challenge; hence, the need to discover more efficacious remedies for these conditions. The need to face up to the effective control of treatment resistant hypertension (blood pressure that remains above treatment goal in spite of concurrent use of 3 antihypertensive agents of different classes (Calhoun *et al*., 2008)) also brings to bear a critical demand for more effective treatment options for hypertension.

The common belief that medicinal plants are safer than orthodox drugs (Okigbo and Mmeka, 2006), have resulted in increased use and sometimes indiscriminate use of medicinal plants including potentially toxic ones. Although, *Aristolochia* species are generally known to be toxic, no documented report on the toxicity profile of *A. ringens* is currently available, despite its continuous use in Nigeria.

1.3. OBJECTIVES OF THE STUDY

1.3.1. General objective

To determine the pharmacological basis for the medicinal use of the aqueous root extract of *A. ringens* (AR) and to evaluate its toxicity.

1.3.2. Specific objectives

The specific objectives of this study are to:

i. evaluate the toxicity of the aqueous root extract of *A. ringens*.
ii. investigate the antidiarrhoeal activity of the aqueous root extract of *A. ringens*.
iii. determine the antiinflammatory, analgesic and central nervous system depressant activities of the aqueous root extract of *A. ringens*.
iv. evaluate the effect of the extract and its fractions on some haemodynamic parameters and
v. determine the phytochemical components of the extract.

1.4. SIGNIFICANCE OF THE STUDY

The significance of this study lies in the determination of the toxicological and pharmacological properties of *A. ringens.* The toxicological aspect of this study will provide relevant information on the toxicological profile of *A. ringens*; given the much reported toxicity of *Aristolochia* species and their continued use in Nigeria.

The pharmacological aspect of this study will validate the folkloric use of the plant as an antidiarrhoeal, antiinflammatory, analgesic and CNS depressant remedy. Other medicinal properties of *A. ringens* may also be discovered in the course of the pharmacological and toxicological investigations. The study would also provide information on the possible mechanisms of actions of *A. ringens*.

Phytochemical investigations in this study will provide information on the content of aristolochic acid (the toxic component of *Aristolochia* plants) and other compounds in the extract and fractions of *Aristolochia ringens*.

1.5. OPERATIONAL DEFINITION OF TERMS

Antidiarrhoeal index$_{\text{in vivo}}$ (ADI *in vivo*): A measure of combined effects of the different components of diarrhoea such as purging frequency, onset of diarrhoeal stools as well as the intestinal transit.

Analgesic: A substance that relieves pain**.**

Antioxidant: A substance that has the potential to protect against oxidative stress by preventing the action of free radicals.

Autonomic nervous system: The part of the peripheral nervous system that controls visceral functions.

BST: Brine shrimp lethality test, carried out to assess the effect of test substance on the survival of *Artemia salina.*

CNS depressant: Central nervous system depressing agent, a substance that inhibits or suppresses excitatory activities in the brain and spinal cord.

Cyclic adenosine monophosphate (cAMP): An important second messenger produced by membrane enzyme adenyl cyclase activation, by the alpha subunit of a G protein.

Diarrhoea: The passing of three or more loose stools per day or more frequently than is normal for the individual.

Enteric nervous system (ENS): One of the main divisions of the autonomic nervous system, also known as intrinsic nervous system; and it consists of a mesh-like system of neurons that governs the function of the gastrointestinal system.

Inflammation: A protective and defense mechanism of the body to disturbed homeostasis due to conditions such as infection and injury that results in systemic and local effects. Signs of inflammation include, pain, redness, swelling and heat in the affected region.

I.P.: Intraperitoneal route of drug administration, administration of drug into peritoneal cavity.

LC_{50}: The median lethal concentration that will produce mortality in 50% of the population tested.

LD_{50}: The median lethal dose in milligram per body weight (usually in kilogram) that will produce mortality in 50% of the population tested.

Medicinal plant: Plant with properties that can be used to cure or manage ailments.

Peristaltic index (PI): The distance travelled by charcoal meal, relative to the total length of small intestine usually expressed in percentage.

Phlogistic: An agent used to induce inflammation e.g. carrageenan and xylene.

Phytochemicals: These are chemical compounds that occur naturally in plants; they are usually reposnsible for the biological action observed with medicinal plants.

p.o.: (*per os)*, A latin expression meaning "by mouth". It refers to the oral route of drug administration i.e. administering drug into the mouth.

Prostaglandins (PGs): One of a number of hormone-like substances derived from arachidonic acid that mediates various actions including inflammation, dilation and constriction of blood vessels.

s.c.: Subcutaneous route of drug administration, drug administration into the layer beneath the skin.

Vehicle: A substance acting as a solvent in which medicinally active ingredient can be administered.

Writhe: Contraction of the abdominal muscle followed by extension of the hind limbs, a painful response in experimental animals in response to noxious stimli.

CHAPTER TWO

LITERATURE REVIEW

2.0. LITERATURE REVIEW

2.1. THE GENUS *ARISTOLOCHIA*

The *Aristolochia* genus is an important genre, widely used in traditional medicine (Wu *et al*., 2004). The name "*Aristolochia*", which means the best delivery or birth, is thought to be of ancient Greek origin and it reflects centuries of use in obstetrics. *Aristolochia* species have long been known for their wide use in traditional medicine. The Aristolochiaceae family of plants consists of about 500 species mostly distributed along tropical, subtropical, and mediterranean regions of the world (Neinhuis *et al*., 2005; Wanke *et al*., 2007). These plants have been cultivated as ornamentals (Wu *et al*., 2002) and used as abortifacients, emmenagogues (Che *et al*., 1984), analgesics, anticancers, anti-inflammatory agents, sedatives, muscle relaxants, antihistaminergics, antiallergics, antihelminthics, antimicrobials and antimalarials (Kubmarawa *et al*., 2007). They have also been used in the management of stomach ache, abdominal pain, rheumatism (Yu *et al*., 2007), wounds, skin diseases, different types of poisonous bites and stings (Wu *et al*., 2004). According to a report by the International Agency for Research on Cancer, *Aristolochia species* have been used as diuretics and in the treatment of oedema (IARC, 2002). Wu *et al*. (2004) have described a number of other biological properties of the *Aristolochia* genus.

Aristolochia species have also been reported as sources of several physiologically active compounds of different classes. Aristolochic acid derivatives with various carbon skeletons; aporphines, benzylisoquinolines, isoquinolines, protoberberines protopines, amides, chlorophylls, mono-, sesqui-, and diterpenoids, lignans, biphenyl ethers, flavonoids, tetralones, benzenoids, and steroids have been identified from different *Aristolochia* species (Wu *et al*., 2005).

Aristolochia is one of the intensely investigated genera and a large number of papers have been published on the production of physiologically important metabolites by *Aristolochia* (Kuo *et al*., 2012). The lignans purified from *A. arcuata;* talaumidin (3), galgravin (5), aristolignin (6), nectandrin A (7), isonectandrin B (8), and nectandrin B (9) all exhibited neuroprotective bioactivity (Zhai *et al*, 2005). In addition, (−)-hinokinin (24), (−)-cubebin (26), (−)-pluviatolide (28), and (−)-haplomyrfolol (29) from *A. constricta* also produced antispasmodic activity. This was also the case with one diterpenoid, (−)-kaur-16-en-19-oic acid (32) (Zhang *et al.,* 2008). Protoberberines 18-21 from *A. constricta* exhibited anti-addictive effects (Capasso *et al.*, 2006). Licarin A (162), licarin B (163), and eupomatenoid-7 (164) reported from *A. taliscana* (León - D í a z *et al.* 2010); and 7,9-dimethoxytariacuripyrone (13), 9-methoxytariacuripyrone (14), and aristololactam I (15) from *A. brevipes* (Navarro-García *et al.*, 2011) showed antimycobacterial action. Licarin A (162) was the most active compound, with minimum inhibitory concentrations (MICs) of 3.12-12.5 μg/mL against H37Rv, four mono-resistant H37Rv variants and 12 clinical multi-drug resistant isolates of *M. tuberculosis*, as well as against five non-tuberculous *Mycobacteria* (NTM) strains. Chagas disease is a chronic illness caused by *Trypanosoma cruzi*, and the major cause of morbidity and mortality in many regions of South America. *A. cymbifera* (used as an abortifacient and an emmenagogue as well as in the treatment of fever, diarrhoea, and eczema) was purified to yield (−)-kusunokinin (54) and (−)-copalic acid (55), which were active against *T. cruzi* (Sartorelli *et al.*, 2010).

The potentially vast usefulness of this plant genus appears to have been limited by the reported progressive nephropathy and urothelial cancer they have been reported to cause in humans (Martinez *et al*., 2002; Meinl *et al*., 2006). As a result, the distribution of herbal medicines containing *Aristolochia* extracts has been prohibited in many countries

(Neinhuis *et al*., 2005). These toxic effects have been reported to be due to aristolochic acids reported to be present in this plant genus (Balachandran *et al*., 2005).

2.1.1. Use of *Aristolochia* plants in Nigeria

In Nigeria, several species of this genus including *A. albida, A. indica* and *A. ringens* are used in the management of various ailments. Users claim that they are highly efficacious when used singly or in combination with other medicinal plants. The antidiarrhoeal activity of one such combination, which includes 2 species of this genus (*A. albida* and *A. ringens*) have been demonstrated in our laboratory (Adeyemi *et al*., 2003). The root of *Aristolochia ringens* is largely used in Nigeria for the management of ailments such as gastrointestinal disturbance, asthma, diabetes and oedema (Odugbemi, 2008). According to a personal communication with Miss Adebola Adebayo, a graduate of the University of Lagos, Nigeria, a pinch of the root of *A. ringens* is usually taken by her family to alleviate gastrointestinal disturbance. Several personal communications with tradomedical practioners in Lagos, Nigeria also revealed the use of these species to manage conditions such as haemorrhoids, fibroids and diabetes. Small portions of the root of *A. ringens* packed into small rolls (Figure 1) are commonly seen among the whares of traditional medicine sellers in various parts of Lagos. Table 1 below highlights some of the uses of *Aristolochia* species in some parts of Nigeria. To the best of our knowledge, there is yet to be any report on the toxic effect of the plant among its users in Nigeria, who claim that it is highly efficacious, hence its continued usage.

Table 1: Use of *Aristolochia* species in Nigeria

Region/ethnic group(s)	***Aristolochia* species**	**Conditions Used to manage/Uses**	**References**
Hausa, Fulani (Northern Nigeria)	*A. albida*	Cancers and inflammation	Abubakar *et al*. (2007)
Kaduna (Northern Nigeria)	*A. bracteata*	Spice	Kayode and Ogunleye (2008)
Ijesha (South western Nigeriaa)	*A. ringens*	Pile	Kayode *et al*. (2008)
South western Nigeria	*A. ringens*	Asthma	Sonibare and Gbile (2008)
South western Nigeria	*A. albida*	Diabetes mellitus	Soladoye *et al*. (2012)
South western Nigeria	*A. repens*	Helminthic infestations	Idu *et al*. (2010)
South western Nigeria	*A. ringens*	Haemorrhoids	Soladoye *et al*. (2010)

2.2. *ARISTOLOCHIA RINGENS*

A. ringens, also known as *Howardia ringens*, is a perennial bushy glabrous climber, native to tropical America, and introduced to most West African countries as a garden ornamental. It has become naturalized in roadside bushes in Sierra Leone, and in many places in Nigeria (Burkill, 1985). It is a fast growing vine that grows up to 20-30 feet high but can also spread to about 3-4 feet wide. It has beautiful dark green heart shaped leaves that are paler underneath (Figure 2). They are about 8 cm long by 5 cm wide and grow closely together to create a dense mass of foliage. The plant produces a number of long fragrant flowers scattered among the drooping leaves. These outrageous looking flowers are solitary in the leaf axils, ebracteolate, geniculate with mottled red, yellow, green and purple shades on a creamy-yellow background (Figure 2). They have subglobose utricle that are about 7 cm long without syrinx and straight tubed. Emerging from the side of the utricle at a sharp angle, are limb-like 4 cm projections with 2 superposed lobes. The upper lobe is obovate-spatulate, not deflected, 4-5 cm wide and up to 8 cm long, while the stiffly erect lower lobe is narrowly lanceolate and up to 16-20 cm long. The flowers' gynostemium is usually 6-lobed; the lobes spreading, coroniform, 1 cm high, 8 mm broad; with anthers 2.5-3.0 cm wide, dehiscence acropetal, septifragal. It has hypanthium, which is usually bent. Its fruits are capsule green maturing to brown and opening to release numerous fertile seeds that are narrowly cordate, flat, about 7 mm wide, 12 mm long and 0.2 mm thick (Burkill, 1985).

Figure 1: The root of *A. ringens* as sold in the local market.

Figure 1: *A. ringens* in its natural habitat. (Adapted from www.flickr.com)

2.2.1. Classification

Kingdom**:**	Plantae (plants)
Subkingdom:	Trancheobionta (vascular plants)
Super division:	Spermatophyta (seed plants)
Division:	Magnoliophyta (flowering plants)
Class:	Magnoliopsida (dicotyledons)
Subclass:	Magnoliidae
Order:	Aristochiales
Family:	Aristolochiaceae (birthwort family)
Genus:	*Aristolochia*
Species:	*Aristolochia ringens*

2.2.2. Distribution

A. ringens is native to northern South America (Venezuela and Brazil) and western South America (Peru). It is now naturalized to west central tropical Africa (Zaire) and West tropical Africa (Nigeria and Cote D'Ivoire). It is also cultivated in other parts of the tropics including Ghana (Burkill, 1985; Neuwinger, 2000).

2.2.3. Common names

English-	Gaping Dutchman's pipe,
Yoruba-	Akogun
Hausa-	Duman Dutse

2.2.4. Phytochemistry

According to Stashenko *et al*. (2009), aristolochic acid II is present in the flower of *A. ringens*; essential oils in the leaves and stem, while sesquiterpenes and monoterpenes have

been mainly isolated from the essential oil in the leaves and stem respectively. Wu *et al*. (2005) also reported the presence of monoterpenoids such as limonene; and sesquiterpenoids such as caryophyllanes. Its composition of magnesium, phosphorus, calcium, iron, zinc, copper and other elements have also been reported (Olabanji *et al*., 2008).

2.2.5. Ethnomedicinal uses

A ringens is used in traditional medicine for the management of several ailments including haemorrhoids, snake bite, rheumatoid arthritis, fibroids, gastrointestinal disturbances, diabetes (Olabanji *et al*., 2008) and asthma (Sonibare and Gbile, 2008). Its root is used in relieving body swelling, joint pains, ulcers and its leaves are used for treating general body rashes (Odugbemi, 2008). A teaspoon of the root extract is taken 3 times daily for 3 days for deworming in Nigeria (Idu *et al*., 2010). Senegalese use its root as an antidote to snake venom (Neuwinger, 2000). It is used in South America for the treatment of ulcer, colic, fever and snake bite (Van Wyk and Wink, 2004). Despite these reported uses, there is yet to be documented reports of scientific studies to determine the pharmacological basis for such uses.

2.2.6. Biological activities

The antifeedant activity of *A. ringens* has been reported against *Sitophilus zeamais,* the maize weevil. In a study by Arannilewa *et al*. (2006), the petroleum ether extract of *A. ringens* (1%) showed insecticidal activity, causing 100% mortality of *S. zeamais* by the third and fourth day after application. The methanolic extract of *A. ringens* has also been shown to possess potentials for antitrypanosomal activity (Osho and Lajide, 2014).

2.3. TOXICOLOGICAL ASSESSMENT OF MEDICINAL PLANTS

Toxicological testing, also known as safety testing, is conducted to determine the degree to which a substance can damage living or non-living organisms. Regulations relating to human health require that every new substance intended for therapeutic use be evaluated preclinically for its safety before administering it to human volunteers and/or patients. Toxicity studies in appropriate animal models are therefore commonly used to assess the potential health risks of such compounds to humans. Such toxicity studies basically assess the toxicity potential of the substance, and the risk is determined by considering the probability of exposure to a particular hazard at certain levels of exposure (Klaassen and Eaton, 1991; Asare *et al*., 2011).

Medicinal plants are usually considered safer than synthetic drugs due to the fact that they are plants grown naturally, like food crops. In addition to this, there is a dearth of reports on the adverse effects produced by herbal drugs. As a result, medicinal plants have become increasingly used for therapeutic purposes, and are often abused in many cases. Several reports are now revealing the fact that medicinal plants may not be completely excluded from the possibility of being toxic.

Phytotoxicity is of immense importance in phytomedicine. The prescription and use of traditional medicine is still largely unregulated; this results often in the danger of misadministration, especially of potentially toxic plants. In addition, plants commonly used in traditional medicine are assumed to be safe based on the fact that they are natural and have been in use over centuries, before any attention was paid to the toxicity of potentially toxic ones. However, recent scientific investigations have shown that many plants used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic (Ferrira *et al*., 1999). The need to assess the toxicity potential of medicinal

plants cannot be over-emphasized. In toxicity assessments, one of the simplest biological responses to monitor is lethality, since there is only one criterion; the target organism is either dead or alive. The lethality response and other adverse effects of medicinal plants are evaluated in phytotoxicity assessments.

Test for lethality in a simple zoological organism, such as the brine shrimp, *Artemia salina,* can be used as a simple tool to guide screening and fractionation of physiologically active plant extracts (Montanher *et al*., 2002). *Artemia* is a genus of aquatic crustaceans characterized by features such as adaptability to wide ranges of salinity (5 to 250 g/L) and temperature (6 to 35 ^{o}C). *Artemia* species have short life cycle, a high adaptability to adverse environmental conditions, high fecundity and a parthenogenetic reproduction method (with nauplii or cysts production). They have small body size, and are able to adapt to varied nutrient resources as they are non-selective filter feeders. Their intrinsic features make them suitable organisms for use in toxicology; guaranteeing reliability, feasibility and cost-effectiveness in routine and/or research toxicological investigations. The brine shrimp toxicity test (BST) is an an efficient, inexpensive and relatively rapid procedure, requiring only low amounts of sample (Meyer *et al*., 1982). The assay is considered a useful tool for preliminary assessment of toxicity, and it has been used for the detection of fungal toxins and for determination of plant extracts' and heavy metals' toxicity (Carballo *et al*., 2002). It involves the exposure of brine shrimps to test substance for the purpose of determining the effect of the test substance on their survival (Meyer *et al*., 1982). This general bioassay is also considered useful for the determination of the potential of a substance to produce a broad range of biological activities. One basic premise in BST is that toxicology is simply pharmacology at a higher dose, thus if we find toxic compounds, lower non-toxic dose of such compounds, might elicit a useful, pharmacological, perturbation on a physiologic system (Montanher *et al*., 2002; Sorgeloos

et al., 1978). It has been shown that BST correlates reasonably well with cytotoxic and other biological properties (McLaughlin, 1991; Yogesh *et al*., 2012).

Acute, sub-acute, sub-chronic and chronic toxicity tests are common assessments used to determine the toxicological profile of test compounds. Acute toxicity tests provide information on the adverse effects of a substance that result either from a single exposure or from multiple exposures within a short period (usually less than 24 hours) (US EPA, 1998). They provide preliminary information on the toxic nature of a material for which no other toxicological information is available. Such information is especially essential in cases of accidental ingestion of a large amount of the material; determination of possible target organs that require close examination; decision on vital tests to be conducted in repeated-dose toxicity tests; and selection of doses for short-term and sub-chronic toxicity tests (Gad and Chengelis, 1988).

To be described as acute toxicity, the adverse effects should occur within 14 days of the administration of the substance. In most acute toxicity test methods, LD_{50} is determined via percentage mortality of animals in the test population within 24 hours of exposure to test substance. Acute toxicity is distinguished from chronic toxicity, which describes the adverse health effects from repeated exposures, often at lower levels, to a substance over a longer period.

The objective of chronic or sub-chronic toxicity studies is to characterize the profile of a substance in a mammalian species (primarily rodents) following prolonged and repeated exposure of at least 3 groups of animals to at least 3 doses of the test substance respectively. The effect of the test substance on treated animals is usually compared to the effect on animals in a control treated group. Male and female animals are used, with at

least 20 animals per sex in each group. The frequency of exposure normally is daily, but this may vary depending on the route chosen (oral, dermal or inhalation). Repeated dose toxicity studies are conducted to evaluate the adverse effects of a test substance on prolonged use, and are carried out to provide information in this regard. Valuable information on target organs, possibility of cumulative effects, and an estimate of the dose at which there is no observable adverse effect as well as the toxic dose are obtained from such studies. Body weight measurements and regular detailed observations should be done in the course of exposure. At the end of the exposure period, haematological, biochemical, and histological examinations may be carried out (OECD, 2009).

To determine the adverse or toxic effects as well as the LD_{50} of the test drugs upon single exposure to a relatively large dose, an acute toxicity test is performed. Sub-chronic exposure to the test drug may also be performed, at the end of which, biochemical and haematological examinations of blood samples, as well as histological examination of tissues excised from the animals, are carried out. This would enable the observation of various markers of the health status or pathological conditions of the animals tested.

2.4. DIARRHOEA

Diarrhoea has been defined by the World health Organization as the passing of three or more loose stools per day or more frequently than is normal for the individual**.** It occurs when the gut's water, electrolyte and nutrient absorption capacity is impaired; an increase in gut motility has also been implicated (Parashar *et al*., 2009). This excessive passage of loose stool could be due to a number of infectious (e.g. viral or bacterial) and non infectious (e.g. medications and malabsorptions) causes. The loss of fluid through diarrhoea can lead to dehydration and electrolyte imbalance that may be life-threatening, particularly in young children, the elderly and people who are malnourished. It has been

reported that diarrhoea is a common cause of death in developing countries. Children under five years old have about two episodes of diarrhoea yearly (Parashar *et al*., 2009) and about 1.9 million die from diarrhoea annually, resulting in about 19% mortality due to diarhoea in children less than five years old (Boschi-Pinto *et al*., 2008).

Castor oil (obtained by pressing the seed of castor oil plant, *Ricinus communis*) is a stimulant and irritant laxative that has been used experimentally to induce diarrhoea due to the action of its metabolite, ricinoleic acid, obtained by the hydrolytic action of intestinal lipase (Iwao and Terada 1962; Watson and Gordon 1962). This laxative action has been reported to be due to impairment of water and electrolyte reabsorption leading to hypersecretion (Ammon *et al*., 1974), as well as sensitization of the intramural neuron of the gut by ricinoleic acid. This results in increased peristalsis and consequently, diarrhoea. Castor oil-induced diarrhoea in rats have also been shown to involve activation of tachykinin receptors, NK_1 and NK_2, by endogenous tachykinins such as substance P and neurokinin A. Inhibitors of these tachykinins have potential to act as antidiarrhoeal agents, whose action can be free from the constipating effect of opioids (Croci *et al*., 1997). More recent reports also indicate increased gastric motility (more prominent in the colon) and secretory actions mediated via tachykinin receptor activation (Kastin, 2013). In addition, Tunarua *et al*. (2012) reported that the laxative effect of ricinoleic acid is mediated via its interaction with the prostanoid receptor, EP_3. Several reports have identified the role of some prostaglandins (PGs) (including $PGF_{2\alpha}$ and PGE_2) in the pathogenesis of diarrhoea. Prostaglandins have been reported to increase secretion of fluid and electrolyte by the activation of adenylate cyclase and subsequent increase in cyclic AMP (Marshall *et al*., 2002).

2.4.1. Management of diarrhoea

This involves various approaches including;

- Supportive therapy-fluid and electrolyte replacement,
- Antidiarrhoeal symptomatic treatment using antimotility drugs to reduce stool, frequency and any other symptoms such as abdominal pain,
- Antisecretory drug therapy aimed at reducing faecal loss, and
- Specific therapy such as antimicrobial chemotherapy to reduce duration and severity of the illness.

2.4.1.1. Supportive therapy

Fluid and electrolyte replacement

Oral fluid and electrolyte replacement (regarded as the corner stone of treatment) is usually sufficient unless the person is vomiting and/or losses are very severe. Dehydration is usually quicker in infants and young children and therefore early administration of an oral rehydration solution (ORS) is advised to prevent severe dehydration and acidosis. For the initial management of severe dehydration in infants and young children, intravenous rehydration therapy is advisable. Recommended oral replacement fluids are glucose-electrolyte solutions known collectively as oral rehydration solutions (ORS). Oral rehydration therapy (ORT) has been a life saving therapy for many patients with severe diarrhoea. The scientific principle and rationale for this therapy is based on active carrier mediated sodium-glucose cotransport (Farthing, 1998). Advancements have been made as regards the administration of ORTs including changes in sodium concentration from 90 mmol/l to 75 mmol/l and inclusion of complex substrates such as cereals and defined polymers to give cereal-based ORSs (Carsburn-Jones and Farthings, 2004).

Cereal-based ORS has the advantages of yielding ORS of low osmolality, delivering an increased amount of substrate (in the form of rice starch polymer for example) along with some protein, which will furhther drive active sodium absorption. However, cereal based ORS has only a significant advantage in cholera but not in other diarrhoeal states (Gore *et al*., 1992).

2.4.1.2 Antidiarrhoeal therapy

Two major classes of antidiarrhoeal agents are used to reduce stool frequency, abdominal cramps, and possibly stool volume. They are antimotility and antisecretory agents.

Antimotility agents

Examples include loperamide and a diphenoxylate-atropine combination. These drugs modestly reduce faecal loss by increasing intestinal transit time and enhancing the potential for reabsorption of fluid and electrolytes. Loperamide is usually the first line treatment in self therapy. It is a synthetic opiate agonist that activates μ opioid receptors in the myenteric plexus of the large intestine. These receptors are situated presynaptically on the endings of the parasympathetic cholinergic innervation of the intestinal smooth muscle and they exert facilitatory effect on smooth muscle contractility (Wood and Galligan, 2004). Activation of μ receptors by loperamide inhibits release of acetylcholine and thus relaxes smooth muscular tone in the gut wall (Regnard *et al*., 2011), resulting in the enhancement of phasic colonic segmentation and inhibition of peristalsis, thus increasing intestinal transit time (Chen *et al*., 2012). In addition, muscarinic acetylcholine receptors on secretory epithelial cells in the gut wall mediate stimulation of fluid and electrolyte secretion into the intestinal lumen by parasympathetic activity. Inhibition of acetylcholine release by loperamide will thus also have an antisecretory activity (Wood and Galligan, 2004). Consequently, loperamide reduces daily faecal volume, decreases fluid and

electrolyte loss, and increases stool viscosity and bulk density. Morphine is also an opioid agonist employed in this study as a reference standard.

Antisecretory agents

The enteric nervous system (ENS) is involved in the promotion of intestinal secretion. A number of neurotransmitters including acetylcholine, vasoactive intestinal peptide and serotonin have been identified in the ENS. They are thought to be involved in intestinal secretion and are therefore potential pharmacological targets for the treatment of watery diarrhoea (Farthing, 2002). Octreotide, an octapeptide analogue of somatostatin, is used to treat secretory diarrhoea associated with diabetes, chemotherapy, and hormone secreting tumours of the pancreas and gastrointestinal tract. Its mechanism of action includes inhibition of the secretion of hormones such as serotonin and vasoactive intestinal peptide.

Racecadotril, another antisecretory agent indicated for symptomatic treatment of acute diarrhoea in both adults and children, is a prodrug rapidly absorbed from the gut and hydrolyzed in the plasma to its active metabolite, thiorphan (Matheson and Noble, 2000). It interacts with the opioid neurotransmitter system in the gut wall, but unlike loperamide, it is an inhibitor of the enzyme, neutral endopeptidase 24.11 (enkephalinase), which degrades endogenous opioid peptides Met- and Leu-enkephalin (Roques *et al*., 1980). These enkephalins are endogenous opioid neurotransmitters synthesized and secreted by interneurons of the ENS. They act on cholinergic neurones, enterochromaffin cells, and secretory epithelial cells to coordinate gastrointestinal function. Enkephalins interact preferentially with the δ opiate receptors densely located on secretory epithelial cells. Activation of these receptors leads to reduced secretion of water and electrolytes mediated by a decrease in cellular cyclic adenosine monophospate (cAMP) (Matheson and Noble,

2000). By inhibiting the breakdown of enkephalins, thiorphan facilitates this antisecretory activity.

Antimicrobial agents

Infectious diarrhoea such as those caused by dysenteric shigellosis, cholera, and pseudomembranous enterocolitis may require antimicrobial treatment. A number of evidences show that antimicrobial agents can reduce the severity and duration of some intestinal infections, especially in infections that produce acute watery diarrhoea. However, in the case of acute infectious diarrhoea, systematic use of antimicrobial therapy is not recommended because the aetiology may not be bacterial, the disease is generally self-limiting and there is a risk of development of antibiotic resistance. In such cases, symptomatic treatment can be prescribed straight away. Only if diarrhoea persists despite appropriate symptomatic treatment should patients be evaluated further to identify pathogen and possibly consider antimicrobial therapy (Faure, 2013). Tetracycline, ciprofloxacin and metronidazole (antibacterials); diloxanide fuorate and metronidazole (antiprotozoals); albendazole and praziquantel (antihelminthics); and ganciclovir and foscarnet (antivirals) are examples of antimicrobials in the management of infectious diarrhoea.

2.4.1.3. Other interventions

Bismuth salicylate has shown efficacy in the treatment of traveller's diarrhoea. It effectively reduces the number of unformed stools by approximately 50%; this is attributed to the antisecretory action of its salicylate moiety but it is also thought to have antibacterial and anti-inflammatory properties. It is not a popular drug of choice, as a large number of tablets must be taken (up to eight tablets) and has a delayed onset of action (up to four hours). It can interfere with the absorption of other medications and has some

unpleasant side effects including tinnitus and black tongue and stool effect (Gorbach, 1990).

Cystic fibrosis transmembrane regulator protein, integral to the chloride channel on the apical membrane of the intestinal epithelial cell, is an essential component of the secretory process (Mathews *et al*., 1999). Drugs that inhibit this protein will be potentially useful for the management of secretory diarrhoea. SP303 (now known as crofelemer), a naturally occurring polyphenolic polymer with chloride channel blocking activity was shown to have antisecretory action. It was approved in December 2012 by the Food and Drug Administration (FDA) for the symptomatic relief of non-infectious diarrhoea in patients with HIV/AIDS on antiretroviral therapy. This approval was given for Silax Pharmaceuticals Limited; and SP303 was given the brand name, Fulyzaq®.

Probiotics are live microorganisms that when administered in adequate amount confer a health benefit on the host. The mechanism of action of these probiotics is thought to be related to competition in the gut with pathogenic microorganisms for nutrients or adhesion sites. It is also possible that probiotics secret certain molecules that inhibit the growth of pathogenic microorganisms. Probiotic microorganisms are generally administered as spores that can resist transit through the highly acidic *milieu* of the stomach and then germinate and proliferate in the less hostile environment of the intestinal lumen. However, these microorganisms fail to colonize the gastrointestinal tract persistently and they disappear from the faeces within days when supplementation ceases. The main therapeutic application of probiotics has been the treatment and prevention of antibiotic-associated diarrhoea. Examples of probiotics used include *Lactobacillus* GG and *Saccharomyces boulardii* (Allen *et al*., 2004).

Medicinal plants have also been used in the management of diarrhoea. Examples of such include *Acacia burkei, Brachylaena transvaalensis, Cissampelos hirta, Sarcostemma viminale, Psidium guajava, Catharanthus roseus* and *Melia azedarach* (Wet *et al*., 2010). Adeyemi *et al*. (2003), demonstrated the antidiarrhoeal effect of an extract consisting of 5 plants including *A. ringens*. In this study, the effect of the aqueous root extract of *A. ringens* alone on gastric motility and diarrhoea induced by castor oil was investigated.

2.5. INFLAMMATION

Although they are separate conditions, pain and inflammation are nearly always associated with each other; and pain was once viewed as always following inflammation. Inflammation is the tissue's response to injurious stimulants including a wide variety of noxious agents such as antibodies, physical injuries or infections. The ability to stage an inflammatory response is essential for survival in the face of environmental pathogens and injury. In some situations and disease conditions however, the inflammatory response may be exaggerated and sustained without apparent benefit and even with severe adverse consequences. The immune system is often involved with inflammatory disorders, demonstrated in allergic reactions, with many immune system disorders resulting in abnormal inflammation. Non-immune diseases with etiological origins in inflammatory processes include cancer, atherosclerosis, and ischaemic heart disease (Cotran *et al*., 1999). Inflammatory responses normally characterized by redness (rubor), heat (calor), swelling (tumor), pain (dolor) and sometimes loss of function (function laesa) which subside as the inflammatory process subsides, occur in four distinct transient phases. Each phase is apparently mediated by different mechanisms namely;

1. an acute phase characterized by transient local vasodilation, increased blood flow (leading to the redness and warmth in the affected area); and increased capillary permeability (resulting in oedema as fluid seeps into interstitial space),

2. a second phase characterized by phagocytosis of offending agents by tissue macrophages and production of neutrophils,
3. a phase characterized by peripheral orientation of leukocytes; pavementing- adhesion of leukocytes to endothelial lining of blood vessel of injured area; diapedesis- movement of leukocytes through capillary wall; chemotaxis - leukocytes drawn to injury site, and
4. a proliferative phase, that features tissue degeneration and fibrosis; that may proceed to chronic inflammation

Many mechanisms are involved in the promotion and resolution of the inflammatory process. One of these mechanisms entails adhesive interactions; involving adhesion molecules such as E-, P-, and L-selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and leukocyte integrins. These molecules are involved in the adhesion of leukocytes and platelets to the endothelium at inflammation sites (Cotran *et al*., 1999).

Activated endothelial cells play a key role in directing circulating cells to inflammatory sites. Cell adhesion occurs by recognition of cell-surface glycoproteins and carbohydrates on circulating cells due to the augmented expression of adhesion molecules on resident cells. Thus, endothelial activation results in leukocyte adhesion as the leukocytes recognize newly expressed L-selectin and P-selectin on the endothelium. Some traditional non-steroidal anti-inflammatory drugs (NSAIDs) may interfere with adhesion by inhibiting expression or activity of some of these cell-adhesion molecules. Recruitment of inflammatory cells to sites of injury involves the concerted interactions of several types of soluble mediators. These include the complement factor C5a, platelet-activating factor, and the eicosanoid LTB_4, all of which can act as chemotactic agonists that can cause the

characteristic movement or orientation of inflammatory cells along a chemical concentration gradient to inflammation sites.

Several cytokines also play essential roles in orchestrating the inflammatory process, especially interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), which have been considered as principal mediators of the biological responses to bacterial lipopolysaccharide (LPS, also called endotoxin). They are secreted by monocytes and macrophages, adipocytes, and other cells. Working in concert with each other and various cytokines and growth factors (including IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF)), they induce gene expression and protein synthesis in a variety of cells to mediate and promote inflammation (Lowry, 1993).

Prostanoids including prostglandins (PGs) and prostacyclins are among important mediators of inflammation. Intradermal, intravenous, or intra-arterial injections of small amounts of prostaglandins mimic many components of inflammation. Administration of PGE_2 or prostacyclin (PGI_2) causes erythema and an increase in local blood flow. Such effects may persist for up to 10 hours with PGE_2 and is partly due to the capacity of prostaglandins to counteract the vasoconstrictor effects of substances such as norepinephrine and angiotensin II; properties not generally shared by other inflammatory mediators (Ueno and Oh-ishi, 2002). Although PGs such as PGE_1 and PGE_2 cause oedema, it is not clear if they can increase vascular permeability in the postcapillary and collecting venules without the participation of other inflammatory mediators (e.g., bradykinin, histamine, and leukotriene C_4 (LTC_4). Furthermore, PGE_1 is not produced in significant quantities except under rare circumstances such as essential fatty acid deficiency. Unlike LTs, prostaglandins are unlikely to be involved in chemotactic

responses, even though they may promote the migration of leukocytes into an inflamed area by increasing blood flow.

2.5.1. Anitinflammatory drugs

These include

- immune selective,
- steroidal and
- non-steroidal anti-inflammatory drugs (NSAIDs).

2.5.1.1. Immune selective antiinflammatory drugs (ImSAIDs)

These are a class of peptides discovered to have diverse biological properties, including anti-inflammatory properties. ImSAIDs alter the activation and migration of inflammatory cells (Bao *et al*., 2006). The ImSAIDs represent a new category of anti-inflammatory agents and are unrelated to steroid hormones or NSAIDs. They were discovered by scientists evaluating biological properties of the submandibular gland and saliva. Early work in this area demonstrated that the submandibular gland released a host of factors that regulate systemic inflammatory responses and modulate systemic immune and inflammatory reactions. It is now well accepted that the immune, nervous, and endocrine systems communicate and interact to control and modulate inflammation and tissue repair.

When a neuroendocrine pathway is activated, it results in the release of immune-regulating peptides from the submandibular gland due to neuronal stimulation from sympathetic nerves. This pathway, which is the cervical sympathetic trunk-submandibular gland (CST-SMG) axis is a regulatory system that plays a role in the systemic control of inflammation (Mathison *et al*., 1994). Early work in identifying factors

that played a role in the CST-SMG axis lead to the discovery of a seven amino acid peptide, submandibular gland peptide-T (SGP-T). SGP-T was reported to have biological activity and thermoregulatory properties related to endotoxin exposure (Mathison *et al*., 1997). SGP-T, an isolate of the submandibular gland, was reported to demonstrate its immunoregulatory properties and potential role in modulating the cervical sympathetic trunk-submandibular gland (CST-SMG) axis, and subsequently shown to play an important role in the control of inflammation. The potent anti-inflammatory activities, both in vivo and in vitro, of the tripeptide Phe-Glu-Gly (FEG), a carboxyl terminal peptide of the prohormone SMR1 (submandibular rat-1) identified in the rat submandibular salivary gland has been reported (Morris *et al*., 2009; Mathison *et al*., 2010).

According to this review, FEG attenuates the cardiovascular and inflammatory effects of endotoxaemia and anaphylaxis, by inhibition of hypotension, leukocyte migration, vascular leak, and disruption of pulmonary function and intestinal motility. The mechanism of action of FEG is reported to be via alteration of activated inflammatory cells, (especially neutrophils), by regulating integrins and inhibiting intracellular production of reactive oxygen species (Mathison *et al*., 2010).

2.5.1.2. Steroidal anti-inflammatory drugs

Glucocorticoids are potent anti-inflammatory agents, capable of relieving inflammatory conditions, regardless of the cause of the inflammation. Examples include hydrocortisone, dexamethasone, beclomethasone and triamcinolone. Their primary anti-inflammatory mechanism is via lipocortin-1 (annexin-1) synthesis. Lipocortin-1 suppresses phospholipase $A_{2,}$ thereby blocking eicosanoid production, and inhibiting various leukocyte mediated inflammatory events including epithelial adhesion, emigration, chemotaxis, phagocytosis, respiratory burst, etc. In other words, glucocorticoids not only

suppress immune response, but also inhibit two main products of inflammation; prostaglandins and leukotrienes. They inhibit prostaglandin synthesis at the level of phospholipase A_2 as well as at the level of cyclooxygenase/PGE isomerase (COX-1 and COX-2) activity (Goppelt-Struebe *et al*., 1989).

Glucocorticoids marketed as anti-inflammatories are often topical formulations, such as nasal sprays for rhinitis or inhalers for asthma. These preparations have the advantage of only affecting the targeted area, thereby reducing side effects or potential interactions with other drugs or food. In this case, the main compounds used are beclomethasone, budesonide, fluticasone, mometasone and ciclesonide. In rhinitis, sprays are used. For asthma, glucocorticoids are administered as inhalants with a metered-dose or dry powder inhaler (Rang *et al.*, 2003).

Mechanism of action of glucocorticoids

Glucocorticoid activity is mediated through processes including transactivation (which involves translocation) and transrepression, both of which refer to the genomic mechanism of glucocorticoid anti-inflammatory action. A nongenomic mechanism of action of glucocorticoids has also been described (Alangari, 2010). Glucocorticoids bind to ligand gated cytosolic glucocorticoid receptor (GR). The newly formed receptor-ligand complex translocates itself into the cell nucleus, where it binds to glucocorticoid response elements (GRE) in the promoter region of the target genes resulting in the regulation of gene expression. This process is commonly referred to as transactivation (Newton, 2000). In this process, some genes coding for certain proteins are up regulated. The proteins have a wide range of effects, including, for example:

- anti-inflammatory action– lipocortin I, p11/calpactin binding protein and secretory leukoprotease inhibitor 1 (SLP1)
- increased gluconeogenesis – glucose-6-phosphatase and tyrosine aminotransferase

In the process of transrepression, the activated hormone receptor interacts with specific transcription factors such as activating protein 1 and nuclear factor-kappaB and prevents the transcription of targeted genes. By this process, glucocorticoids are able to prevent the transcription of proinflammatory genes such as those that code for cytokines including interleukins IL-1B, IL-4, IL-5, and IL-8, chemokines, tumour necrosis factor α and granulocyte macrophage colony stimulating factor.

The ordinary glucocorticoids do not distinguish between transactivation and transrepression and thus influence both the immune genes and the genes regulating the metabolic and cardiovascular functions. Intensive research is aimed at discovering selectively acting glucocorticoids that will be able to repress only the immune system (Newton and Holden, 2007). Genetically modified mice that express a modified GR incapable of DNA binding still showed the anti-inflammatory effects but not the gluconeogenesis produced by glucocorticoids (Reichardt *et al*., 2001). This result strongly suggests that most of the desirable anti-inflammatory effects are due to transrepression, while the metabolic effects mainly arise from transactivation.

Glucocorticoids have been shown to exert a number of rapid actions that are independent of the regulations of gene transcription. Membrane associated glucocorticoid receptors have been shown to mediate lymphocytolysis (Cato *et al*., 2002). In addition, some glucocorticoids have been shown to rapidly inhibit the release of the inflammatory PGE_2. This effect is blocked by the glucocorticoid receptor antagonist, mifepristone (RU-486)

and is not affected by protein synthesis inhibitors. These data together suggest a nongenomic mechanism of action (Revollo and Cidlowski, 2009).

2.5.1.3. Nonsteroidal antiinflammatory Drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate inflammation and pain by inhibiting the cyclooxygenase (COX) enzyme and preventing the synthesis of prostaglandins. They are also used in the management of pain and are discussed under management of pain in this review.

2.6. PAIN

Pain has been defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Bonica, 1979). It is the most common reason for physician consultation and it is a major symptom in many medical conditions that can significantly interfere with a person's quality of life and general functioning (Breivik *et al*., 2008). Pain has multiple causes, and people respond to it in multiple and individual ways. The pain that one individual pushes his/her way through might be incapacitating to another. Even though the experience of pain varies from one person to the next, it is possible to categorize the different types of pain.

2.6.1. Pain classification

Various types of pain have been described. Based on clinical observations and therapeutic responses, pain has been categorized based on:

- Duration and pattern of occurrence: Based on this, pain has been classified into acute and chronic pain. Traditionally, the distinction between acute and chronic

pain relies on an arbitrary interval of time from onset; the two most commonly used markers being 3 months and 6 months since the onset of pain (Turk and Okifuji, 2001). Some theorists and researchers have placed the transition from acute to chronic pain at 12 months (Spanswick and Main, 2000). Others apply acute to pain that lasts less than 30 days, chronic to pain of more than six months duration, and subacute to pain that lasts from one to six months (Thienhaus and Cole, 2000). Pain classification based on duration is necessary for identifying the pathology and for deciding appropriate referrals.

- Severity (e.g. mild, moderate or severe pain); this form of classification is useful for determining how urgently attention is required and what kind of medication is more appropriate.
- Region or location of the body involved (e.g. chest pain, neck pain, pelvic pain): classification in this way is necessary for proper description of pain and to also decipher how serious the pain may be.
- Physiology (e.g. nociceptive, neuropathic and inflammatory pain): Pain classification based on physiology of the pain makes it possible to explain the situations to affected individual and helps to identify the most appropriate treatment.
- Pathology (e.g. migraine, arthrithis, angina, labour and cancer pain): This classification is useful in the sense that protocols exist for managing various conditions of pain. One limitation of this sort of classification is the fact that one pathology can result in different types of pain depending on the stage of the pathological condition. An example is cancer pain, which can be further classified as follows:

  Initial phase

  - Duration –Acute

- Severity – Severe
- Location - Lower back
- Physiology – Nociceptive, inflammatory
- Pathology - Bone metastasis

Late phase

- Duration - Chronic
- Severity - Severe
- Location - Lower back and legs
- Physiology - Neuropathic
- Pathology - Tumour compressing on nerve root

2.6.2. Nociception

Nociception describes the process by which nociceptors (pain receptors) upon activation by noxious stimuli transmit signals through the spinal cord to the brain for the normal processing of pain that results in the interpretation, perception, and response to pain. Noxious stimuli refer to stimuli that are damaging or potentially damaging to normal tissue. Such stimuli can be mechanical, thermal or chemical in nature. Transduction, transmission, perception and modulation of pain are basic processes involved in nociception (McCaffery and Pasero, 1999).

2.6.2.1. Transduction of pain

Transduction begins when the free nerve endings nociceptors of C fibres and A-delta (Aδ) fibres of primary afferent neurones respond to noxious stimuli. Nociceptors are exposed to noxious stimuli when tissue damage and inflammation occur as a result of conditions such as trauma, surgery, infection, and ischaemia. Nociceptors are highly localized in somatic

structures (skin, muscles, connective tissue, bones and joints) and visceral structures (such as liver and gastro-intestinal tract).

In addition to the Aδ and C fibres that carry noxious sensory information, there are primary afferent Aβ fibres that carry non-noxious stimuli (Serpell, 2006). These fibre types possess different characteristics that allow the transmission of particular types of sensory information.

- Aβ fibres are highly myelinated and of large diameter, thus allowing rapid signal conduction (>40 metres per second). They have low activation threshold, and usually respond to light touch and also transmit nonnoxious stimuli.
- Aδ fibres are lightly myelinated and of smaller diameter (2-5 μm), hence conduct more slowly than Aβ fibres (5-15 metres per second). They have high and low activation threshold and respond to mechanical and thermal stimuli. They carry rapid, sharp localized pain and are responsible for the initial reflex response to acute pain.
- C fibres are unmyelinated and are also the smallest (<2 μm) type of primary afferent fibre. Hence they demonstrate the slowest conduction (<2 metres per second). C fibres are polymodal, responding to chemical, mechanical and thermal stimuli. With high activation threshold, C fibre cause sensation of slow, diffuse, dull and burning pain.

Three categories of noxious stimuli have been identified as follows:

- mechanical (e.g. pressure, swelling, abscess, incision, tumour growth);
- thermal (e.g. burn, scald);

- chemical (e.g. excitatory neurotransmitter, toxic substance such as acetic acid, ischaemia, infection).

The noxious stimuli cause release of chemical mediators from the damaged cells. These chemical mediators (including prostaglandin, bradykinin, serotonin, substance P, and histamine) activate and/or sensitize the nociceptors to the noxious stimuli. In order for a pain impulse to be generated, an exchange of sodium and potassium ions (de-polarisation and re-polarisation) occurs at the cell membranes. This results in an action potential and generation of a pain impulse (McCaffery and Pasero, 1999).

2.6.2.2. Transmission of pain

The transmission process occurs in three stages. The pain impulse is transmitted from the site of transduction along the nociceptor fibres to the dorsal horn in the spinal cord; from the spinal cord to the brain stem; and through connections between the thalamus, cortex and higher levels of the brain. The C and Aδ fibres terminate in the dorsal horn of the spinal cord. There is a synaptic cleft between the terminal ends of these afferent fibres and the nociceptive dorsal horn neurones (NDHN). In order for the pain impulses to be transmitted across the synaptic cleft to the NDHN, excitatory neurotransmitters (e.g. adenosine triphosphate, glutamate, calcitonin gene-related peptide, bradykinin, nitrous oxide and substance P) are released, which bind to specific receptors in the NDHN. The impulse generated from this interation is then transmitted from the spinal cord to the brain stem and thalamus via two main nociceptive ascending pathways namely; the spinothalamic pathway and the spinoparabrachial pathway. Since the brain does not have a discrete pain centre, when impulses arrive in the thalamus they are directed to multiple areas in the brain for processing (McCaffery and Pasero, 1999).

2.6.2.3. Perception of pain

This is where pain becomes a conscious multidimensional experience. The multidimensional experience of pain has affective-motivational, sensory-discriminative, emotional and behavioural components. When the painful stimuli are transmitted to the brain stem and thalamus, multiple cortical areas are activated and responses are elicited (Brooks and Tracey, 2005). The areas are briefly discussed below.

- The reticular system: This area is involved with the autonomic and motor response to pain. It is also responsible for warning the individual to do something to alleviate the pain (Lamont *et al*., 2000). For example, automatically removing a hand when it touches a hot saucepan. It also has a role in the affective-motivational response to pain such as looking at and assessing the injury to the hand once it has been removed from the hot saucepan.
- Somatosensory cortex: This is involved with the perception and interpretation of sensations. It identifies the intensity, type and location of the pain sensation and relates the sensation to past experiences, memory and cognitive activities. It identifies the nature of the stimulus before it triggers a response, for example, where the pain is, how strong it is and what it feels like (Farquhar-Smith, 2007).
- Limbic system: This is responsible for the emotional and behavioural responses such as responses relating to attention, mood, and motivation.

2.6.2.4. Modulation of pain

The multiple complex descending modulatory pain pathways (DMPP) regulate pain modulation. They are involved in changing or inhibiting transmission of pain impulses in the spinal cord and can lead to either an increase (excitatory) or decrease (inhibition) in the transmission of pain impulses. Descending inhibition involves the release of inhibitory

neurotransmitters that block or partially block the transmission of pain impulses, and therefore produce analgesia. Inhibitory neurotransmitters involved with the modulation of pain include endogenous opioids (enkephalins and endorphins), serotonin (5-HT), norepinephrine (noradrenaline), gamma-aminobutyric acid (GABA) and neurotensin (Brooks and Tracey, 2005). Endogenous pain modulation helps to explain the wide variations in the perception of pain in different people as individuals produce different amounts of inhibitory neurotransmitters. Endogenous opioids are found throughout the central nervous system (CNS) and prevent the release of some excitatory neurotransmitters, for example, substance P, therefore, inhibiting the transmission of pain impulses (Brooks and Tracey, 2005).

2.6.3. Chronic pain

This form of pain could be nociceptive or neuropathic. Nociceptive pain may also be divided into deep and superficial; and deep pain into deep somatic and visceral. Superficial pain is initiated by the stimulation of nociceptors in the skin or superficial tissues. While deep somatic pain is initiated by activation of nociceptors in ligaments, tendons, bones, blood vessels, fasciae and muscles, and is dull, aching, poorly-localized pain; visceral pain is such that originates in the viscera (internal organs). Visceral pain may be well-localized, but can also be extremely difficult to locate. Referred pain, which refers to pain sensation located in an area distant from the pathology or injury site, has been described for several visceral regions that had been damaged or inflamed (Coda and Bonica, 2001). Neuropathic pain on the other hand, is further divided into peripheral (originating in the peripheral nervous system) and central (originating in the brain or spinal cord) (Bogduk and Merskey, 1994).

2.6.3.1. Pathophysiology of chronic pain

The exact mechanisms involved in the pathophysiology of chronic pain are complex and remain unclear. It is believed that following injury, rapid and long-term changes occur in parts of the CNS that are involved in the transmission and modulation of pain (Ko and Zhuo, 2004). Various changes in the spinal cord have been suggested as leading to chronic pain. These include central sensitization, wind-up, and microglial activation. Central sensitization results from low and high frequency nociceptive stimulations, and can lead to increased excitability of dorsal horn neurones, manifested as increased spontaneous discharge, increased receptive field size (possibly a cellular basis of secondary hyperalgesia) and increased responsiveness to innocuous stimulation of the peripheral receptive field (possibly a cellular basis of allodynia). According to Henry (2008), wind-up is initiated by high threshold, C-fibre strength, stimuli delivered at 3 Hz or more to induce cumulative depolarization.

2.6.4. Neuropathic pain

Neuropathic pain can be defined as pain initiated or caused by a primary lesion or dysfunction in the nervous system which may result from:

- cancer;
- trauma (e.g. complex regional pain syndrome, chronic post-surgical pain)
- infection (e.g. post-herpetic neuralgia; nociceptive neurons damaged by *Vericela zoster* virus)
- ischaemia (e.g. diabetic neuropathy)
- chemically induced pain, for example, as a result of chemotherapy (Farquhar-Smith, 2007).

Some types of neuropathic pain may develop due to damage to peripheral nervous system, causing the pain fibres to transmit pain impulses repetitively and become increasingly sensitive to stimuli. Neuroplasticity characterized by abnormal neuronal sprouting in the peripheral nervous system and within the dorsal horn of the spinal cord may also develop. This sprouting may result in additional generation and increased transmission of pain impulses. Neuropathic pain can be characterized by burning, dull, aching, tingling or electric shock-like sensations.

2.6.5. Management of pain

Algiatry (pain management) employs interdisciplinary measures to ease the suffering and improve the quality of life of people living with pain. It may entail physiotherapy as well as psychology and pharmacotherapy approaches. Pain sometimes is alleviated promptly once the underlying trauma or pathology has resolved, and is treated by one practitioner with analgesics and (occasionally) anxiolytics. Effective management of chronic pain, however, frequently requires the coordinated efforts of the management team, depending on the severity, nature and duration of pain. Pharmacotherapeutic approaches to pain management may include the use of medications such as NSAIDs, opioid analgesics, tricyclic antidepressants and anticonvulsants.

2.6.5.1. Nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs, including the traditional nonselective and the subclass of selective cyclooxygenase-2 (COX-2) inhibitors, have anti-inflammatory and analgesic actions. NSAIDs are a chemically heterogeneous group of organic acids that share certain therapeutic actions and adverse effects. The classes of NSAIDs based on their chemical structures include:

- Salicylic acid derivatives (e.g. aspirin, olsalazine, sulfasalazine and diflunisal)
- Paraaminophenol derivatives (e.g. acetaminophen)
- Indole and indene acetic acids (e.g. indomethacin, sulindac and etodolac)
- Heteroaryl acetic acids (e.g. tolmetin, diclofenac and ketorolac)
- Anthranilic acids (Fenamates) (e.g. mefenamic acid , meclofenamate and flufenamic acid)
- Arylpropionic acid derivatives (e.g. ibuprofen, naproxen, fenoprofen, flurbiprofen, oxaprozin and ketoprofen)
- Enolic acids (e.g. piroxicam and meloxicam)
- Alkanones (e.g. nabumetone)
- COX-2 selective inhibitors (diaryl-substituted pyrazoles e.g. celecoxib and sulfonanilides e.g. nimesulide)

Mechanism of action and therapeutic effects of NSAIDs

NSAIDs are known to inhibit the synthesis of prostaglandins in all cell types (Vane, 1971). They generally do not inhibit the formation of other inflammatory mediators such as other eicosanoids including the leukotrienes. While the clinical effects of these drugs are explicable in terms of prostaglandin synthesis inhibition, substantial inter- and intra-individual differences in clinical response are known.

At higher concentrations, NSAIDs also are known to reduce production of superoxide radicals; induce apoptosis; inhibit the expression of adhesion molecules; decrease nitric oxide synthase; decrease proinflammatory cytokines (e.g. tumour necrosis factor- alpha (TNF-α) and interleukin 1 (IL-1)); modify lymphocyte activity and alter cellular membrane functions (Livshits and Seidman, 2010). However, there are differing opinions

as to whether these actions might contribute to the anti-inflammatory activity of NSAIDs at the concentrations attained during treatment.

There are analgesics that are commonly associated with anti-inflammatory drugs but that have no anti-inflammatory effects. An example is paracetamol, called acetaminophen in the U.S. As opposed to NSAIDs, which reduce pain and inflammation by inhibiting COX enzymes, paracetamol has recently been shown to block the reuptake of endocannabinoids (Mathison and Davidson, 2006), which only reduces pain, likely explaining its minimal effect on inflammation.

Inhibition of prostaglandin biosynthesis by NSAIDs

This is a major known mechanism of action of NSAIDs. Cyclooxygenase (COX) or prostaglandin H_2 synthase (PGHS) is the enzyme that catalyzes the first two steps in the biosynthesis of the prostaglandins (PGs) from the substrate arachidonic acid (AA). AA is a polyunsaturated omega-6 fatty acid obtained from phospholipids components of cell membranes. The steps involved in prostaglandin biosynthesis include the oxidation of AA to the hydroperoxy endoperoxide, PGG_2 and its subsequent reduction to the hydroxyl endoperoxide, PGH_2. The PGH_2 is transformed by a range of enzymatic and nonenzymatic mechanisms into primary prostanoids; PGE_2, PGF_2, PGD_2, PGI_2, and TXA_2 (Figure 3). Therapeutic doses of NSAIDs reduce prostaglandin biosynthesis by blocking COX.

There are two forms of COX; COX-1 and COX-2. COX-1 is a primarily constitutive isoform found in most normal cells and tissues at constant amounts. On the other hand, COX-2 activity is normally absent from cells, but when induced, its levels increase and decrease in a matter of hours after a single stimulus. Most of the stimuli known to induce COX-2 are those associated with inflammation, examples of which include bacterial

lipopolysaccharide (LPS), cytokines such as IL-1, IL-2 and TNF-α. The anti-inflammatory cytokines; IL-4, IL-10, and IL-13, will decrease induction of COX-2, as will the corticosteroids (Otto and Smith, 1995). However, COX-2 is also constitutively expressed in certain areas of kidney and brain. The physiological roles of COX-1 have been deduced from the deleterious side effects of NSAIDs, which while inhibiting PG biosynthesis at inflammatory sites, also inhibit constitutive PG biosynthesis in the gastrointestinal tract. COX-1 provides PGs in the stomach and intestine to maintain the integrity of the mucosal epithelium and its inhibition leads to gastric damage, hemorrhage, and ulceration.

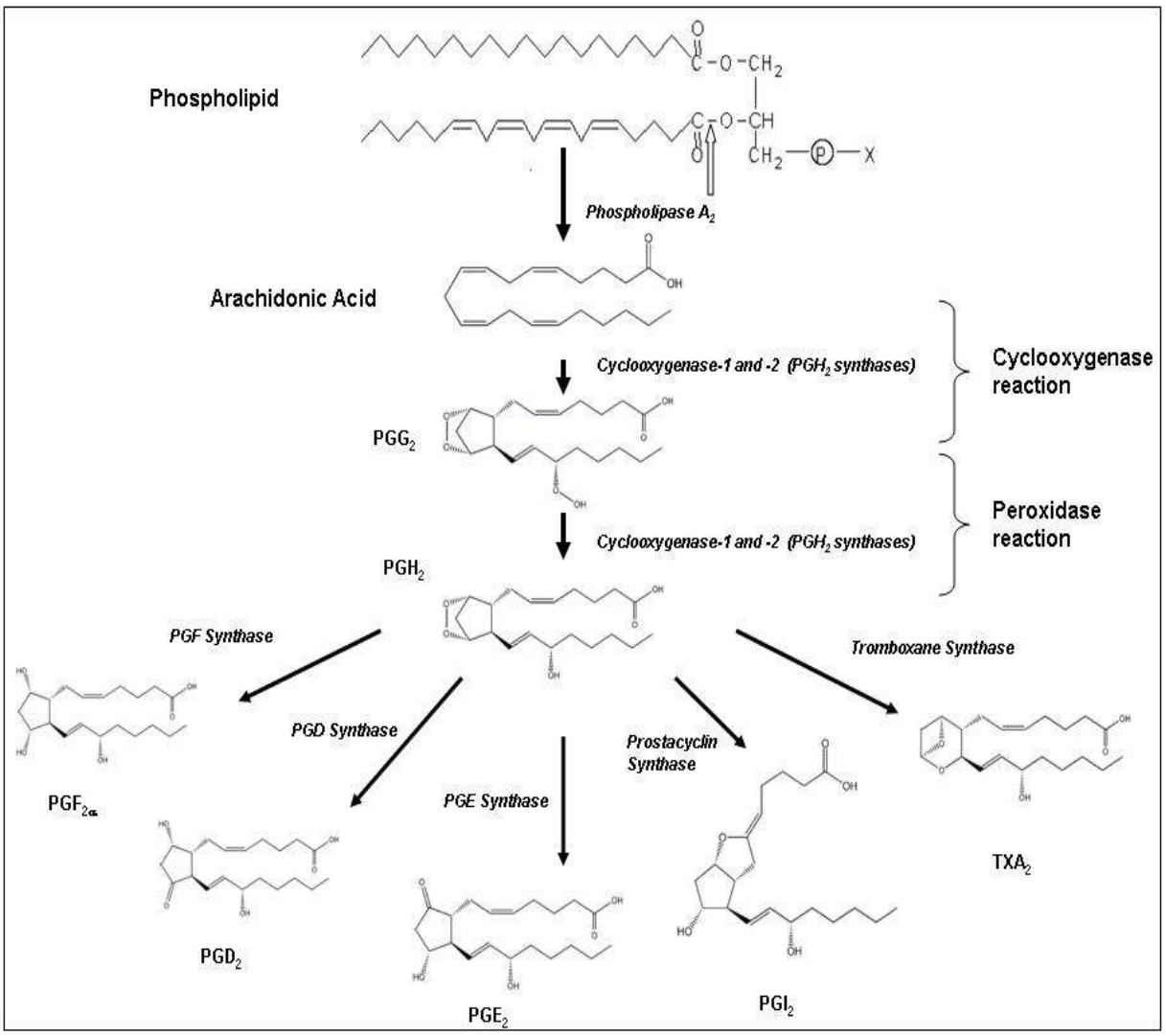


Figure 3: Biosynthesis of eicosanoids (Adapted from Vane *et al*., 1998).

Adverse reactions to NSAIDs

Analgesic and anti-inflammatory medications are widely used in all age groups for the control of pain, inflammation and fever of diverse etiologies. A large proportion of the population is exposed to these drugs, making them a major cause of untoward reactions (Sanchez-Borges *et al*., 2010). The prevalence of adverse reactions to NSAIDs ranges from 10 to 25% of all adverse reactions to drugs. Significant morbidity and mortality have been attributed to their use (Shah *et al*., 2001). After beta lactam antibiotics, hypersensitivity reactions to NSAIDs are the second main cause of hypersensitivity to drugs. Acute manifestations affect the respiratory tract (aspirin exacerbated respiratory disease), the skin (urticaria and angioedema), or are generalized (anaphylaxis) (Sanchez-Borges *et al*., 2010).

Various organs and systems including gastrointestinal, renal, cutaneous, central nervous and vascular systems in the body are adversely affected due to NSAIDs exposure (Table 2). Long-term use of NSAIDs can cause gastric erosions, which can become gastric ulcers and in extreme cases, severe haemorrhage, that could result in death. The risk of death as a result of use of NSAIDs can be as much as 1 in 12,000 for adults aged 16–45 years. The risk increases almost twentyfold for those over 75 years. Other dangers of NSAIDs include exacerbation of asthma, kidney damage, increased risk of myocardial infarction and stroke (Trelle *et al*., 2011).

Table 2: Common and shared adverse reactions to NSAIDs.

System	**Manifestation**
Gastrointestinal	Abdominal pain, nausea, anorexia, gastric erosions/ulcers, anaemia, gastrointestinal haemorrhage and diarrhea.
Renal	Salt and water retention, hyperkalaemia, decreased urate excretion and oedema.
Central nervous system	Headache, vertigo, dizziness, depression, low seizure threshold and hyperventilation.
Platelets	Inhibition of platelet activation, propensity for bruising and increased risk of haemorrhage.
Uterus	Prolongation of gestation.
Vascular	Closure of ductus arteriosus.

(Adapted from Roberts and Morrow, 2008).

2.6.5.1.1. Diclofenac

Diclofenac is one of the most commonly used NSAIDs in some regions, including Europe. The selective inhibitor of COX-2, lumiracoxib is an analogue of diclofenac. Diclofenac has analgesic, antipyretic, and anti-inflammatory activities. It appears to reduce intracellular concentrations of free AA in leukocytes, perhaps by altering its release or uptake. The selectivity of diclofenac for COX-2 resembles that of celecoxib, and as is the case with COX-2 selective inhibitors, observational studies have raised the possibility of a cardiovascular hazard from chronic therapy with diclofenac (Wise, 2013).

Diclofenac is rapidly absorbed, extensively protein bound (99%), with relatively short half life of 1 to 2 hours and peak plasma concentration attained in 2 to 3 hours. It undergoes substantial first-pass effect, such that only approximately 50% of it is systemically available. Diclofenac accumulates in synovial fluid after oral administration, which may explain why its duration of therapeutic effect is considerably longer than its plasma half life. It is metabolized in the liver by CYP2C subfamily of cytochrome P450 enzyme to 4-hydroxydiclofenac (the principal metabolite) and other hydroxylated forms. Following glucuronidation and sulfation, the metabolites are excreted in the urine (65%) and bile (35%) (Kirchheiner *et al.*, 2003).

Diclofenac is approved in the U.S. for the long-term symptomatic treatment of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis (100–200 mg in divided doses). Three formulations are available: an intermediate-release potassium salt, Cataflam®, a delayed-release form, Voltaren®, and an extended-release form, Voltaren-XR®. Diclofenac is also useful for short-term treatment of acute musculoskeletal pain, postoperative pain, and dysmenorrhea (Katzung, 2007). Diclofenac is also available in combination with

misoprostol, a PGE_1 analogue (ARTHROTEC). An ophthalmic solution of diclofenac is available for treatment of postoperative inflammation following cataract extraction.

Diclofenac produces side effects including gastrointestinal effects in about 20% of patients, leading to discontinuation of its use by about 2% of patients. Modest reversible elevation of hepatic transaminases in plasma occurs in 5–15% of patients. Transaminases measurement during the first 8 weeks of therapy with diclofenac is encouraged and the drug should be discontinued if abnormal values persist or if other signs or symptoms develop. Other untoward responses to diclofenac include rashes, allergic reactions, fluid retention, and oedema and rarely, impairment of renal function. Diclofenac is not recommended for children, nursing mothers, or pregnant women. Consistent with its preference for COX-2, diclofenac does not interfere with the antiplatelet effect of aspirin. Given these observations, diclofenac is not a suitable alternative to a selective COX-2 inhibitor in individuals at risk of cardiovascular (Wise, 2013) or cerebrovascular disease.

2.6.5.2. Opioid analgesics

Examples include oxycodone, hydromorphone, oxymorphone, fentanyl, tapentadol and morphine. Opioid analgesics have been used as medicinal agents, especially for the treatment of acute and chronic pain, for thousands of years. Ancient Greeks first identified and used these medicines, which were originally derived from opium — the latex of immature seed capsules of the poppy flower (*Papaver somniferum*) (Ballantyne and Mao, 2003). From these simple beginnings, opioid analgesics have become a mainstay of medical therapy used by millions of patients each year. While numerous drugs have been developed for the treatment of different types of pain, no single class of agent has replaced or reached the same level of usefulness for the treatment of moderate to severe pain as have opioid analgesics (Coluzzi and Pappagallio, 2005).

Opioid analgesics are often the first line of treatment for many painful conditions and may offer advantages over NSAIDs. Opioid analgesics, unlike some nonopioid analgesics, have no true "ceiling dose" for analgesia and do not cause direct organ damage; however, they do have several side effects, including constipation, drowsiness, and respiratory depression (Coluzzi and Pappagallio, 2005). Morphine is the most commonly used opioid analgesic in postoperative pain management, but sometimes other agents, such as hydromorphone, a hydrogenated ketone of morphine are preferred (Eisenberg *et al*., 2009).

Mechanism of action of opioid analgesics

Opioid analgesics bind to a number of different receptors at presynaptic nerve terminal and postsynaptic neuron throughout the body; such receptors include the mu, delta, and kappa opioid receptors (Coluzzi and Pappagallio, 2005). The binding to these different receptors results in both the therapeutic and adverse effects of opioid analgesics. Genetic variations in the structure of these receptors can partially explain interindividual responses, including some adverse reactions to these agents (Rollason *et al*., 2008).

Stimulation of presynaptic opioid receptors results in inhibition of release of neurotransmitters including noradrenaline, acetylcholine and the neuropeptide, substance P (Stein, 1995). Opioid receptors involved in pain transmission and control are present in many regions of the nervous system, including primary afferent neurons, spinal cord, midbrain and thalamus. The opioid drugs produce analgesia by actions at several levels of the nervous system, including inhibition of neurotransmitter release from the primary afferent terminals in the spinal cord (Stoelting and Hillier, 2006) and activation of descending inhibitory controls in the midbrain. Changes in pain sensitivity by opioids in inflammatory pain have been reported to be influenced by interactions at N- methyl, D-aspartate (NMDA) receptor (Rodríguez-Mufioz, 2012).

Neurotransmitter release from neurons is normally preceeded by depolarization of the nerve terminal and Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. Drugs may inhibit neurotransmitter release by a direct effect on Ca^{2+} channels to reduce Ca^{2+} entry, or indirectly by increasing the outward K^{+} current, thus shortening the duration of the action potential. Opioids produce both of these effects because opioid receptors are apparently coupled via G-proteins directly to voltage sensitive K^{+} channels (to cause opening for K^{+} efflux) and N- type voltage-sensitive Ca^{2+} channels (to cause inhibition for reduced Ca^{2+} influx). Increased outward movement of K^{+} is the most likely mechanism for the postsynaptic hyperpolarization and inhibition of neurons activated by opioids. Opioids also interact with other intracellular effector mechanisms, the most important being the adenylate cyclase system (McDonald and Lambert, 2005). The resultant effect of these is reduced neuronal cell excitability, leading to a reduction in transmission of nerve impulses along with inhibition of neurotransmitter release (Figure 4).

Adverse reactions to opioid analgesics

Adverse reactions to opioid analgesics may include constipation, nausea, vomiting, and somnolence, which may be reasons for discontinuation of drug use (Kalso *et al*., 2004). Early cessation or limitation of pain treatment due to adverse reactions can result in the inadequate treatment of pain. There are reports that excessive use of opioid analgesics may lead to a state of hyperalgesia (Ballantyne and Mao, 2003), thus prompting some physicians to be concerned about using opioid analgesics for pain control. When opioids are used for prolonged periods, drug tolerance, chemical dependency and addiction may occur (Carinci and Mao, 2010).

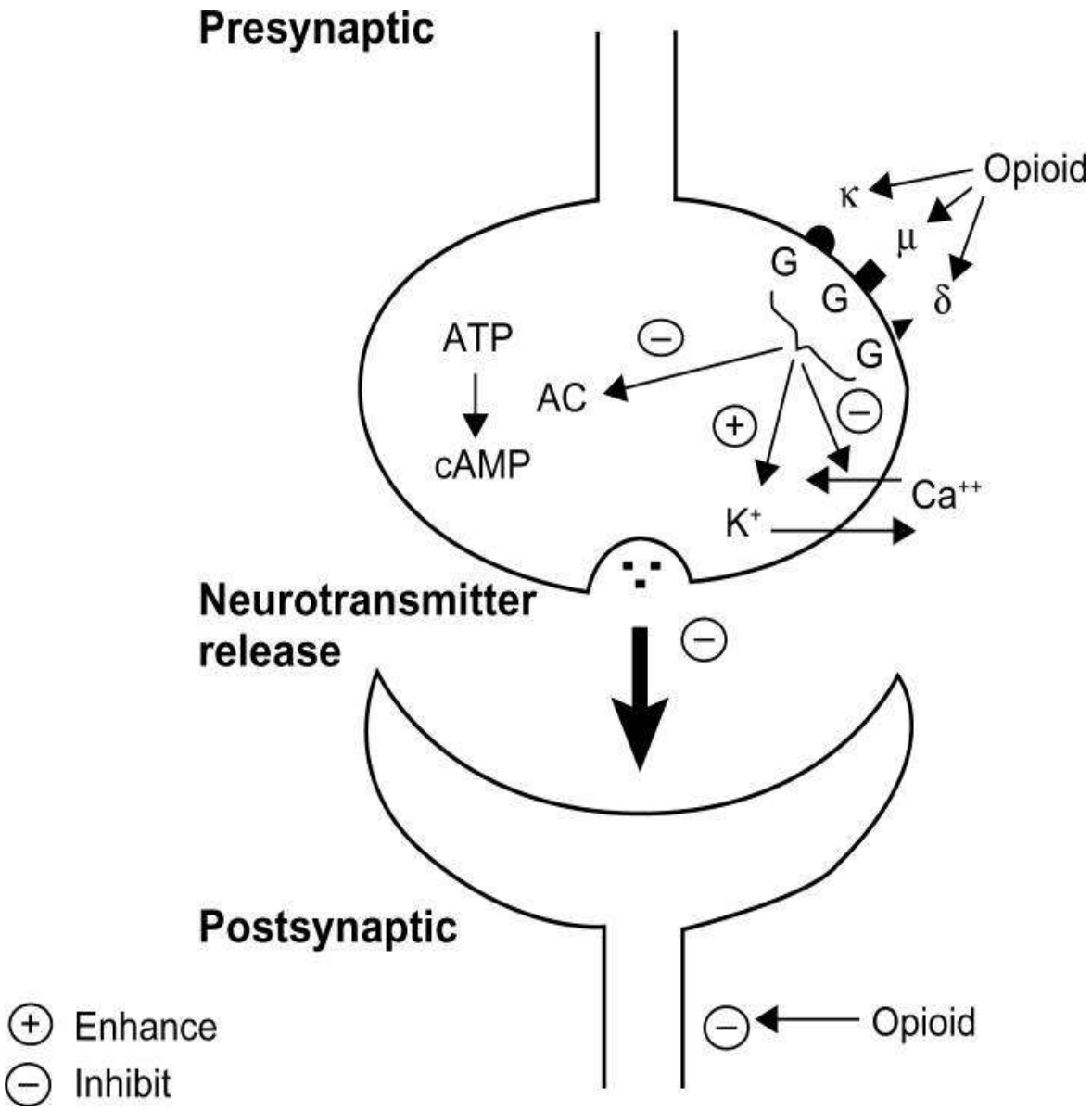


Figure 4: Schematic representation of the mechanism of action of opioids (Adapted from Chahl, 1996). AC-adenylate cyclase, G-G protein, ATP-adenosinetriphosphate, cAMP-cyclic adeninemonophosphate

2.6.5.3. Antidepressants and antiepileptic drugs

Some antidepressant and antiepileptic drugs are used in chronic pain management and act primarily within the pain pathways of the central nervous system, though peripheral mechanisms have been attributed as well. These mechanisms vary and are generally more effective in neuropathic pain disorders as well as complex regional pain syndrome (Jackson, 2006). Drugs such as gabapentin have been widely prescribed for the off-label use of pain control. The list of side effects for these classes of drugs are typically much longer than for opiate or NSAIDs; and many antiepileptics cannot be suddenly stopped without the risk of seizure.

2.6.5.4. Other analgesics

Other drugs are often used as adjuncts to analgesics for the alleviation of various types of pain, and parts of the overall pain experience, hence, they are called adjuvant medications. Gabapentin (an anti-epileptic) not only exerts effects on neuropathic pain, but can potentiate opiates (Caraceni *et al*., 2012). Grapefruit juice may also potentiate opiates (Nieminen *et al*., 2010), by inhibiting CYP450 enzymes in the liver, thereby slowing metabolism of the drug. Orphenadrine, cyclobenzaprine, trazodone and other drugs with anticholinergic properties are useful in conjunction with opioids for neuropathic pain. Orphenadrine and cyclobenzaprine are also muscle relaxants, and therefore particularly useful in painful musculoskeletal conditions. Clonidine has found use as an analgesic for this same purpose (Hall *et al*., 2001).

Chronic pain is one of the most commonly cited reasons for the use of medical marijuana (Lucas, 2012). Evidence of medical marijuana's pain mitigating effects is generally conclusive (Zogopoulos *et al*., 2013). Traditional medicine practice involves the use of some medicinal plants in the alleviation of pain. Plants used include *Alafia barteri, Mesua*

ferrea, Amaranthus spinosus, *Blumea perrothetiana* and *Amaralia bignoniflora* among others (Odugbemi, 2008).

In this study, various preclinical tests using animal models of pain and inflammation were used to determine the anti-inflammatory and analgesic potentials of the aqueous root extract of *A. ringens.* These models involved the use of phlogistic and algogenic agents to induce conditions of inflammation and pain in experimental animals respectively. The effects of the extract in these conditions were compared to those of appropriate reference drugs. Examples of phlogistics employed include carrageenan, egg albumin, and xylene. Acetic acid, acetylcholine and formalin are the algogens used to induce pain.

2.7. HYPERTENSION

Every heart beat results in the pumping of blood to the whole body through arteries. Blood pressure refers to the force of blood pushing up against the walls of blood vessel. Hypertension, also referred to as high blood pressure, is a condition in which the arteries have persistently elevated blood pressure >140/90 mmHg. Isolated diastolic hypertension (systolic blood pressure (SBP) < 140 mmHg, dialstolic blood pressure (DBP) > 90 mmHg) is more common in younger people, and isolated systolic hypertension (SBP > 140 mmHg, DBP < 90 mmHg) is the more common form of hypertension in older people. In 2000, it was estimated that 25% of the world's adult population were hypertensive, and predicted that this would rise to 29% by 2025. By the age of 60, more than one-half of adults in most regions of the world will be hypertensive (Williams, 2010).

Hypertension is a prevalent risk factor for cardiovascular diseases, affecting over 1 billion people worldwide (Rosamond *et al*., 2007). A continuous relationship between blood pressure and cardiovascular risk from blood pressure values as low as 115/75 mmHg has

been reported. The relationship is steeper for stroke than it is for coronary heart disease, and is magnified by age (Williams, 2010). There is a doubling in risk of stroke and ischaemic heart disease mortality, for every 20/10 mmHg increase in blood pressure.

Two forms of hypertension, namely primary and secondary hypertension, have been described. The more common form is primary hypertension (also known as essential or idiopathic hypertension), accounting for 90–95% of all cases of hypertension. Essential hypertension is the form of hypertension that by definition has no identifiable cause (Carretero and Oparil, 2000). It tends to be familial and is likely to be the consequence of a complex interaction involving:

(1) genetic predisposition,

(2) life style and environmental influences, and

(3) disturbances in vascular structure and neurohumoral control mechanisms.

Numerous common genetic variants with small effects on blood pressure have been identified (Kosugi *et al.*, 2009) as well as some rare genetic variants with large effects on blood pressure (Haslam and James, 2005). But the genetic basis of hypertension is still poorly understood (Halperin *et al*., 2008). More than 50 genes have been examined in association studies with hypertension, and the number is constantly growing. One of these genes is the angiotensinogen *AGT* gene (e.g. M235T), studies on which showed that increasing the number of genes increases the blood pressure; a possible cause of hypertension (Dickson and Sigmund, 2006).

Life style and environmental influences have been implicated in the exploding prevalence of hypertension in economically developing regions. Lifestyle changes with the most

important influences on blood pressure include increased sodium intake, excess alcohol consumption, obesity and smoking. A characteristic finding in essential hypertension is an inappropriate increase in peripheral vascular resistance relative to the cardiac output. This is due to remodelling of small arteries (arterioles), characterized by an increase in their media/lumen ratio, but it is not clear whether these changes are a consequence or a cause of raised blood pressure. The functional integrity of large conduit arteries, i.e. the aorta, which becomes stiffer, also influences the development of hypertension, especially systolic hypertension. Endothelial dysfunction and decreased nitric oxide production are found in hypertension, but are more likely a consequence than a cause of elevated blood pressure (Williams, 2010). The specific role of the renin–angiotensin–aldosterone system in the development of essential hypertension remains unclear, but therapeutic agents that inhibit this system have proved to be very effective treatments. The sympathetic nervous system is also known to be involved in the acute and chronic regulation of blood pressure (Williams, 2010).

2.7.1. Management of hypertension

The treatment of hypertension is usually directed towards reducing risk rather than treating symptoms. There is an international consensus that an optimal treatment target should be less than 140/90 mmHg, with a lower target of less than 130/80 mmHg or 120/80 mmHg proposed for higher-risk individuals, i.e. those with established cardiovascular or renal disease or diabetes. Although early studies focused primarily on diastolic blood pressure as the treatment target, systolic blood pressure is invariably more difficult to control and managing it should be an important aspect of hypertensive patients' care. The most effective lifestyle interventions for reducing blood pressure include;

- modification of diet to induce weight loss
- regular aerobic exercise
- restrictions in alcohol and sodium intake

With respect to pharmacotherapeutical approach, many patients will require more than one drug to control blood pressure as monotherapy is rarely sufficient. The blood pressure response to an individual class of blood pressure-lowering medication is heterogeneous, hence there is no 'perfect drug' for every patient. Some trials have indicated that certain comorbidities or target organ damage, provide compelling indications for inclusion of specific classes of drug therapy in the treatment regimen (Williams, 2010).

Drugs used in the management of hypertension include:

- Diuretics: These include thiazide diuretics (such as hydrochlorothiazide); loop diuretics (such as furosemide and bumetanide); potassium-sparing diuretics (such as triamterene and amiloride that inhibibit Na^+ channels, and spironolactone that inhibits aldosterone action). They lower blood pressure initially by the suppression of tubular reabsorption of sodium, thus increasing sodium and water excretion and reducing cardiac output, but during long-term therapy their major haemodynamic effect is due to reduction of peripheral vascular resistance. Adverse reactions to diuretics include reduction of insulin sensitivity causing modest increase in blood glucose; they also increase serum uric acid (may precipitate gout), triglycerides, and LDL cholesterol (Rang *et al.*, 1999).
- β-Adrenergic blocking agents: Examples include propranolol, metoprolol, nadolol, carteolol, atenolol, betaxolol, bisoprolol, pindolol, acebutolol, penbutolol, labetalol and carvedilol. They cause a decrease in heart rate, cardiac output, renin release

and subsequent inhibition of angiotensin II and aldosterone production as well as reduced peripheral resistance. Adverse reactions to β-adrenoceptor antagonists include withdrawal syndrome (characterized by nervousness, tachycardia and angina); reduced myocardial reserve and peripheral vascular insufficiency; exacerbation of asthma, diabetes; increased plasma triglycerides and decreased HDL cholesterol, especially with propranolol. CNS adverse reactions may include lassitude, mental depression, insomnia, nightmares; while gastrointestinal effects may include diarrhoea, nausea, vomiting (Katzung, 2007).

- α_1-Adrenoceptor antagonists: These include prazosin, terazosin, and doxazosin. They block postsynaptic α-adrenergic receptors, relax vascular smooth muscle, and reduce blood pressure due to the lowering of peripheral vascular resistance. First dose syncope and reflex tachycardia are common adverse effects (Carruthers, 1994).
- Ganglion-blocking agents: Trimethaphan and mecamylamine are examples non-depolarizing competitive blockers. They are not currently in use clinically because of toxicity (Oates and Stokes, 1982).
- Central sympatholytics: Agents such as clonidine, guanfacine and guanabenz, stimulate central alpha-2 receptors, thereby reducing sympathetic outflow. These drugs are effective in decreasing heart rate, contractility and vasomotor tone, however, they cause sedation and are usually not first line therapies (Engelman, 1988).
- Angiotensin-converting enzyme (ACE) inhibitors: Examples include enalapril, lisinopril and captopril. Their primary mode of action is inhibition of the renin-angiotensin-aldosterone system, but they also inhibit bradykinin degradation, stimulate the synthesis of vasodilating prostaglandins and, sometimes, reduce sympathetic nervous system activity. Adverse reactions include chronic dry cough

that may require stopping the drug in up to 10% or more of patients; dizziness that may not be related to the degree of blood pressure lowering and skin rashes, which may be observed with any ACE inhibitor. Taste alterations and angioedema also occur in some patients (Rang *et al*., 2003).

- Angiotensin II receptor blockers (ARBs): Examples include losartan, valsartan, irbesartan, candesartan, telmisartan, and eprosartan. They act by competitive inhibition of angiotensin II receptor and their effect is more specific on AT-II action, with little or no effect on bradykinin production or metabolism. In terms of adverse reactions, there is the risk of hypotension, renal failure (in patients with bilateral renal artery stenosis), hyperkalaemia and very rarely, angioedema (Katzung, 2007).
- Calcium channel blocking agents: These include phenylalkylamines (e.g. verapamil), benzothiazepines (e.g. diltiazem) and the dihydropyridines (1^{st} generation e.g., nifedipine; 2^{nd} generation e.g. felodipine; 3^{rd} generation e.g. amlodipine). In addition to their antianginal and antiarrhythmic effects, these calcium channel blockers also dilate peripheral arterioles and thereby reduce blood pressure by inhibiting calcium influx into arterial smooth muscle cells. Among these, verapamil has more cardiac effect (decreasing cardiac output) and nifedipine has more vasodilating effect. The most common side effects of calcium channel blockers are headache, peripheral oedema, bradycardia, and constipation (especially with verapamil in the elderly). The dihydropyridines are more likely to produce symptoms of vasodilatation, such as headache, flushing, palpitations, and peripheral oedema. Calcium channel blockers have negative inotropic effects and may cause or exacerbate heart failure in patients with cardiac dysfunction. Amlodipine is the only calcium channel blocker reported to be safe in patients with severe heart failure (Golan, 2008; Katzung, 2007).

- Direct arterial vasodilators: Agents such as minoxidil and hydralazine are also antihypertensives with relatively limited use. Hydralazine increases cyclic guanosine monophosphate (cGMP) levels, increasing the activity of protein kinase G (PKG). Active PKG adds an inhibitory phosphate to myosin light-chain kinase (MLCK) – a protein involved in the activation of cross-bridge cycling (i.e. contraction) in smooth muscle. This results in blood vessel relaxation (Le *et al*., 2010). It dilates arterioles more than veins. The mechanism of action of hydralazine is not known. Minoxidil, on the other hand, appears to increase potassium conductance in vascular smooth muscles, and the resultant hyperpolarization inhibits action potential necessary for muscle contraction. Both drugs can cause reflex tachycardia (particularly minoxidil) and fluid retention. These side effects can be managed with the addition of a beta-blocker and/or a diuretic. Neither drug is effective for sustained periods. They are usually reserved for the short-term treatment of refractory hypertension, especially in patients with renal failure. Hydralazine can cause a lupus-like syndrome, and minoxidil can produce hair growth (and is sold for the purpose) (Sica, 2007).
- Sodium nitroprusside breaks down non-enzymatically to form nitric oxide. NO activates guanylate cyclase in vascular smooth muscle and increases intracellular production of cGMP. cGMP activates protein kinase G, which activates phosphatases which inactivate myosin light chains. Sodium nitroprusside is an extremely potent arteriolar and venous dilator that is used intravenously for rapid control of hypertensive crises and for blood pressure control during operations (Murad, 1986).

Medicinal plants are also used in the management of hypertension. *Adenia ciscampeloides, Erythrophleum suavenolens, Ocimum gratissimum* and *Lipia multiflora* are examples of such medicinal plants. The antihypertensive action of plants such as *Vitex dordiana,*

Phyllanthus amarus and *Lepidium latifolia* have been investigated (Etuk, 2006). In this study, the effect of *A. ringens* and its fractions on blood pressure were investigated in spontaneously hypertensive rats (SHRs), a strain of genetically hypertensive rats. The animal model of hypertension using SHRs is the most studied model of essential hypertension (Pinto *et al*., 1998).

2.8. PHYTOCHEMICALS

Phytochemicals are naturally occurring chemical components of medicinal plants. Secondary metabolites of medicinal plants are a group of phytochemicals responsible for the pharmacological actions of such plants. Being stationary autotrophs, plants have to cope with a number of challenges, including having to engineer their own pollination, local fluctuations in the supply of the simple nutrients they require to synthesize their food, and the coexistence of herbivores and pathogens in their immediate environment. Plants have therefore evolved secondary biochemical pathways that allow them to synthesize a raft of chemicals, often in response to nutrient deprivation (Reymond *et al*., 2000; Kennedy and Wightman, 2011). These secondary metabolites can be unique to specific species. They do not play any role in the plants' primary metabolic requirements, but increase their overall ability to survive and overcome local challenges by their influence on the plants' interaction with their environment (Kennedy and Wightman, 2011). The energy invested in their synthesis, which is usually far in excess of that required to synthesize primary metabolites can be an indication of how essential these secondary metabolites are to plants. Some of the roles of secondary metabolites include a host of general, protective roles including:

- Antioxidant; free radical-scavenging
- UV light-absorbtion

- Antiproliferative effects
- Defence against infectious microorganisms
- Managing inter-plant relationships; as allelopathic defenders of the plant's growing space against competitor plants.

They are also involved in determining or modifying the plant's relationship with more complex organisms. Their primary role here is often viewed as being one of feeding deterrent, hence the bitter and/or toxic nature of many phytochemicals to potential herbivores (Wink, 2003). However, since plants also have to foster a number of symbiotic relationships in order to survive, secondary metabolites play a role in the attraction of pollinators and other symbiotes through the colours and scents they give the plants. Provision of indirect defenses for the plant is also achieved by attracting the natural enemies of their herbivorous attackers. This may be through provision of attractive chemical milieu for the predator or alternatively, via indirect phytochemicals synthesis and release in response to tissue damage by the herbivore (Wink, 2003; Kennedy and Wightman, 2011).

Phytochemicals can be grouped by the chemical nature of their putative active components. Some of the major phytochemical groups include alkaloids, terpenes, and phenolic compounds (Kennedy and Wightman, 2011).

2.8.1. Alkaloids

Alkaloids are a structurally diverse group of over 12,000 cyclic nitrogen-containing compounds found in over 20% of plant species (Kennedy and Wightman, 2011). They are mostly bitter in taste. The recorded use of alkaloids for medicinal purposes dates back some 5000 years (Goldman, 2001). Examples include local anaesthetic and stimulant,

cocaine; stimulant, caffeine; analgesic, morphine; antibacterial, berberine; anticancer agent, vincristine; antihypertensive, reserpine; cholinomimetic, galantamine; spasmolytic, atropine; vasodilator, vincamine; antiarrhythmic, quinidine; and the antimalarial drug, quinine.

Alkaloids vary in terms of their properties; while most alkaloids contain oxygen in their molecular structure and are usually colorless crystals at ambient conditions, oxygen-free alkaloids, such as nicotine or coniine, are typically volatile, colourless, oily liquids. Coloured alkaloids include berberine (yellow) and sanguinarine (orange). Alkaloids are mostly weak bases, but some, such as theobromine and theophylline, are amphoteric. In terms of solubility, many alkaloids dissolve poorly in water but readily dissolve in organic solvents, such as diethyl ether, chloroform or 1,2-dichloroethane. Many alkaloids and acids form salts of various strengths that are usually soluble in water and alcohol, and poorly soluble in most organic solvents. Exceptions include scopolamine hydrobromide, which is soluble in organic solvents.

Most alkaloids are bitter and/or poisonous when ingested. Alkaloid production in plants appeared to have evolved in response to feeding by herbivorous animals. Plant alkaloids produce various reponses on the central and autonomic nervous systems. They act as narcotics, sedatives, tonics and stimulants; and are capable of bowel and uterine stimulations. They also produce laxative, emetic, expectorant and antiasthmatic actions among others (Oliver, 1960).

2.8.2. Phenolics

Phenolics, sometimes called phenols, are a class of chemical compounds comprising of a hydroxyl group (-OH) attached to an aromatic hydrocarbon. Many phenolics naturally

occur in plants; approximately 10,000 of such compounds have been identified so far (Metcalf, 1987). The simplest compound with this structural motif is phenol, which itself does not occur in plants. Plant phenols are mostly products of the phenylpropanoid pathway. Phenolics range from simple low-molecular weight compounds, such as the simple phenylpropanoids, coumarins, and benzoic acid derivatives, to more complex structures such as flavanoids, stilbenes, and tannins (Ralston *et al*., 2005).

Flavonoids represent the largest and most diverse of the complex phenolics, encompassing some 6000 compounds, all of which share a common underlying structure of two 6-carbon rings, with a 3-carbon bridge, which usually forms a 3rd ring. Flavanoids can be subdivided according to modifications of this basic skeleton into chalcones, flavones, flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanins (Bowsher and Tobin, 2008).

Phenolics play some ecological roles including constitutive and induced roles in toxicity and feeding deterrence in insects (Diaz-Napal *et al*., 2010). They are also induced in the face of bacterial or fungal attack; provide scent, colour, and flavour to attract symbiotic insects. They manage symbiotic relationships with soil bacteria and deter herbivores. They also act as phagostimulant and allelopathics in their relationship with other plants. Alongside these roles, many phenolic compounds also play antioxidant roles and facilitate absorption of UV light (Diaz-Napal *et al*., 2010; Kennedy and Wightman, 2011).

Biologically, plant phenolics are strong antioxidants that can prevent oxidative damage to biomolecules of deoxyribonucleic acid, lipids and proteins. Such damage has been implicated in chronic ilnesses such as cancer, metabolic and cardiovascular disorders. The antioxidant action of phenolics and flavonoids is well documented (Pereira *et al*., 2009).

They exert their antioxidant activity by scavenging free radicals, chelating trace metals and binding proteins with suppression of their enzymatic activity (Seabra *et al*., 2006).

Tannins make up a large group of polyphenols generally capable of tanning leather or precipitating gelatin from solution (Haslam, 1996), a property refered to as astringency. The fact that tannins are usually highly concentrated in lost tissues from plants (e.g. old and dying leaves, outer cork, heartwood and galls) suggests that they might represent metabolic waste products. However, some evidences show that they also play deterrent roles against insect and fungal attacks on plants (War *et al*., 2012). Therapeutically, tannins have been reported to be effective for wound healing (Oliver, 1960), as astringents, poison antidotes, antibacterial and antihelminthics (Reynolds, 1982).

2.8.3. Aristolochic acids

Aristolochic acids are bitter flavoured, slightly water soluble nitrophenanatrene carboxylic acids group of carcinogenic, mutagenic, and nephrotoxic compounds commonly found in plants of the Aristolochiaceae family such as *Aristolochia* and *Asarum* plant species, popularly used in Chinese herbal medicine (Nolin and Himmelfarb, 2010; Heinrich *et al*., 2009). Examples include aristolochic acids I, II and IVa. Aristolochic acid I is the most abundant and active; found in almost all *Aristolochia* species (Wu *et al*., 2005). Although earlier studies by Kupchan and Meriano (1968) and Rosenthai *et al*. (1989) demonstrated the tumour inhibitory and anti-inflammatory effects of aristolochic acid I; today it is very much reported that exposure to aristolochic acids is associated with carcinogenicity (Arlt *et al*., 2002). In spite of the fact that the US Federal Drug Agency (FDA) had advised industries to remove products that contain aristolochic acid from the market, since 2001, products are still present in the market for consumption (Adewunmi and Ojewole, 2004; Schaneberg and Khan, 2004). In some quarters, however, attempts are being made to optimize the medicinal potentials of aristolochic acid. In 2009, Xiao *et al*. reported that β-

napthoflavone, a putative chemopreventive agent inhibits the nephrotoxic effect of aristolochic acid. According to Balachandran *et al*. (2005), structure activity relationship report on aristolochic acid, revealed the importance of functional groups, nitro ($-NO_2$), methoxy ($-OCH_3$) and hydroxyl (-OH) groups in the toxic activity of aristolochic acids. The presence of a nitro group in aristolochic acid structure renders it very toxic, more toxic than methoxy- substituted aristolochic acid. The hydroxyl group on the other hand, renders aristolochic acid, less or non toxic (Balachandran *et al*., 2005).

CHAPTER THREE

METHODOLOGY

3.0. METHODOLOGY

3.1. EQUIPMENT

Haematological auto-analyzer (Hitachi, 902, Tokyo, Japan), biochemical auto-analyzer (Mindray, Shenzhen, China), flame photometer, N-400ME photomicroscope (CEL-TECH Diagnostics, Hamburg, Germany), microscope (Olympus, Tokyo, Japan), CODA non-invasive blood pressure monitor (Kent Scientific Corporation, Torrington, USA), PFP 7 flame photometer (Jenway, Staffordshire, UK), pressure transducer (P23 ID Gould, Statham Instruments, London, UK), data acquisition system (PowerLab®, ADInstruments, Bella Vista, Australia), bridge amplifier, Quad Amp (ADInstruments, Bella Vista, Australia), Windows XP computer (LG, Seoul, South Korea), Power Wave X340 microplate reader (Bio-Tek Instrumenting, Vermont, USA), Agilent HPLC apparatus coupled with UV detector (Agilent Technologies Incoroporated, Santa Clara, USA), HPLC (Shimadzu Corporation, Kyoto, Japan), RF 10AXL fluorescence detector (Shimadzu Corporation, Kyoto, Japan) and hypersil GOLD column 250 x 4.6 (Thermoscientific, Pittsburgh, USA).

3.2. PLANT MATERIAL

The root of *Aristolochia ringens* Vahl. (Aristolochiaceae) were collected from a local market in Mushin, Lagos, Nigeria. It was identified and authenticated by Mr. T.K. Odewo of the Department of Botany and Microbiology, University of Lagos, Nigeria where a herbarium specimen was deposited with voucher number LUH 4061.

3.3. EXPERIMENTAL ANIMALS

Artemisia salina (brine shrimps) were kindly supplied by Dr A.A. Sowemimo of the Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos, Nigeria. The shrimps were allowed a 24 hour period to hatch in sea water to produce nauplii.

Adult albino mice (12-30 g) or rats (100-220 g) of either sex obtained from the Laboratory Animal Centre of the College of Medicine, University of Lagos, Nigeria, were used for the experiment. Some of the rats used in this study were also obtained from Animal Housing Centre of the University of Ibadan, Nigeria. The animals were allowed to acclimatize, kept under standard environmental conditions (temperature 23±1^{o}C, 12 hour light/12 hour dark cycle and 60% humidity) and had access to feed (Livestock Feeds, Lagos, Nigeria) and water *ad libitum*. The experimental procedures were carried out in accordance with the United States National Institute of Health's Guide for the Care and Use of Laboratory Animals (2011).

Spontaneously hypertensive rats (180-320 g) were obtained from the Animal Research Unit and Service Centre (ARASC) of Universiti Sains Malaysia. Ethical approval for the use of these rats was obtained from Universiti Sains Malaysia Animal Ethics Committee.

3.4. PREPARATION OF PLANT EXTRACT

Air-dried root (100 g) was macerated in 1000 ml of distilled water and placed in a refrigerator at 4 °C for 5 days. It was then filtered and the filtrate dried in an oven (Gallenhamp®, England) at 40 °C. The percentage yield of the aqueous root extract of *A. ringens* (AR) obtained was 5.9% (w/w). For some of the experiments, the extract was further subjected to liquid-liquid partitioning to obtain different fractions of the extract.

3.5. LIQUID-LIQUID PARTITIONING OF AR

This was done as described by Otsuka (2005). Five (5) g of dried aqueous root extract of *A. ringens* was dissolved in 10 ml of distilled water as the base solvent. This was then further extracted with different solvents in increasing order of polarity as follows; petroleum ether (100 ml), dichloromethane (350 ml), ethylacetate (300 ml), and n-butanol

(300 ml) to obtain 5 respective fractions including the resulting aqueous fraction, which were then concentrated. These fractions and the crude extract served as the samples for the aristolochic acid I detection and quantification described under section 3.8.5.

In another aspect of the phytochemical study on the aqueous root extract of *A. ringens*, the extract (25 g) with distilled water as a base solvent was again partitioned into petroleum ether (500 ml), chloroform (3,100 ml), n-butanol (1, 400 ml) and aqueous fractions. The fractions obtained were used for determination of tannin, phenolic and flavonoid contents described under section 3.8.3., as well as for antioxidant activity test using 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) assay described under section 3.8.4. These fractions were also tested for effects on some haemodynamic parameters described in section 3.7.4.3.

3.6. TOXICOLOGICAL INVESTIGATIONS

3.6.1. Brine shrimp lethality test

Artemia salina (brine shrimp) lethality test was carried out using a modification of the procedure described by McLaughlin (1991). AR (0.5 ml of 50, 500 and 5000 µg/ml) was put into separate test tubes and made up to 5 ml with filtered sea water to give final concentrations of 10, 100 and 1000 µg/ml respectively. Ten shrimp nauplii were added (using a micropipette) to each of the test tubes; the control consisted of sea water to which also 10 nauplii were added. After 24 hours, the number of surviving shrimps were counted using a magnifying lens and recorded. All experimental assays were done in triplicates. The LC_{50} was calculated using probit analysis (Sowemimo *et al*., 2007)

3.6.2. Acute toxicity tests

Mice of either sex were fasted for 12 hours prior to testing. For the oral acute toxicity test, a group of mice were given AR (10 g/kg). On the other hand, for the intraperitoneal acute

toxicity test, mice were randomly allotted to 4 groups of 5 animals each. Mice in 3 of these groups received AR intraperitoneally at 200, 400, and 600 mg/kg respectively, while mice in the 4th group were given the vehicle, distilled water (10 ml/kg) intraperitoneally. Symptoms of toxicity and mortality in each group for the first 2 hours and for an additional 22 hours were noted. The animals that survived in different groups were further observed for 2 weeks for signs of delayed toxicity. The median lethal dose (LD_{50}) was estimated using Log-dose probit analysis (Miller and Tainter, 1944; Amida *et al*., 2007).

3.6.3. Subchronic toxicity study

Male and female Wistar rats were randomly divided into four groups of 10 animals per sex. For 30 days, group 1 received distlled water (vehicle) (5 ml/kg daily orally) and groups 2–4 were given AR at 10, 50 and 250 mg/kg respectively, daily orally. These doses represent one-fifth of the pharmacologically active dose, the pharmacologically active dose, and five times the pharmacologically active dose respectively (Amida *et al*., 2007). The most active dose in the investigation of the extract's effect in the anti-inflammatory, analgesic and hypotensive activities, was used as the pharmacologically active dose. Body weights were determined weekly and the animals were observed for signs of abnormalities throughout the study. At the expiration of 30 days, some of the rats in each group were fasted overnight and sacrificed; blood sampes and tissues of the kidney and liver, were collected for haematological and biochemical analyses. The other rats in each group were further cared for without giving the extract for another 14 days before sacrificing them and their blood and tissue samples were also collected for analyses.

Histology

The kidney and liver tissues obtained from experimental animals were fixed in 10% formo-saline, dehydrated in alcohol, embedded in paraffin, and cut into 4–5 μm thick

sections. The sections were stained with hematoxylin-eosin for histological examination using a photomicroscope. Slides were examined using ×40, ×100, and ×400 objectives respectively by Dr N.O. Orah of the Pathology Department of the College of Medicine, University of Lagos, Nigeria.

Effect of AR on haematological parameters

Haematological parameters were analyzed using automated haematology analyzer. Parameters evaluated include haematocrit, red blood cell (RBC) count, haemoglobin, platelet count, total white blood cell (WBC) count, mean cell haemoglobin concentration (MCHC), mean red cell volume (MCV), and mean cell haemoglobin (MCH).

Effect of AR on biochemical parameters

Serum samples were analyzed for biochemical parameters including creatinine, urea, albumin, bilirubin, total protein, cholesterol, triglycerides and urea. The concentration of liver enzymes such as alkaline phosphatase (ALP), aspartate transaminase (AST) and alanine transaminase (ALT) were also analyzed using standard protocols using the screen master automated spectrophotometer and corresponding reagent kits (Randox, 1993; Yakubu *et al*., 2006)

Effect of AR on electrolytes

The concentration of some serum electrolytes was also determined using methods described by Yakubu *et al.* (2006) and Oze *et al*. (2007). Sodium and potassium concentration were measured using flame photometry; chloride and bicarbonate concentration using titrimetric method, and calcium concentration by cresol phthalein complexone method.

Effect of AR on endogenous antioxidants

The levels of *in vivo* antioxidants; superoxide dismutase (SOD), using autooxidation of epinephrine method (Sun and Zigma, 1978), catalase (CAT) using Goth's colorimetric method (Goth, 1991) and reduced glutathione (GSH) using a method described by Sedlak and Lindsay, (1968) in liver and kidney tissues of AR exposed male and female rats were determined.

Semen analysis

This was done to assess seminal fluid for motility, count and morphology of sperm. The procedure was carried out as described by Ogli *et al*. (2009). Testes with ipsilateral epididymis of sacrificed male rats were removed after incision on the scrotum. Subsequently, semen was expelled out of the epididymis into a beaker placed in water bath at 36°C.

Sperm motility

Ten (10) to 15 µl of semen was placed on a slide such that the spermatozoa were evenly distributed and covered with a glass cover slip. After appropriate focus of the microscope lens, several fields of the specimen were examined for motility at x40 magnification. The number of motile sperms out of a total of 100 spermatozoa was determined.

Sperm count

A one (1) in 20 dilution of semen in sodium bicarbonate-formalin was thoroughly mixed and poured into a Neubauer ruled chamber using a Pasteur pipette. The number of spermatozoa in an area of 2 sq mm was counted at x10 magnification.

Sperm morphology

A thin smear of the liquefied well-mixed semen made on a slide, was fixed with 95% v/v ethanol while still wet for 5–10 minutes and then allowed to air-dry after which it was washed with sodium bicarbonate-formalin solution. Dilute carbon fuchsin (1 in 20) was then applied to cover and stain smear for 3 minutes and then rinsed off with water. Dilute Loeffler's methylene blue (1 in 20) was also used to cover the smear for 2 minutes (to achieve counterstaining), and then rinsed off with water. The slide was then allowed to dry. Normal and abnormal spermatozoa were examined using the x40 objective of the microscope. Estimation of percentage normal as against abnormal sperm morphology was determined from a hundred spermatozoa.

3.7. PHARMACOLOGICAL ACTIVITIES

3.7.1. Tests for antidiarrhoeal activity

3.7.1.1. Normal intestinal transit

Mice were allotted to groups of 5 animals each. Treatment was carried out in different groups with distilled water (10 ml/kg, p.o.), morphine (10 mg/kg, s.c.) and AR (100, 200, and 400 mg/kg, p.o.). Thirty minutes later, charcoal meal (0.2 ml/mouse, p.o.) made up of 10% charcoal suspension in 5% gum acacia was given to each mouse. Mice were sacrificed 30 minutes after meal administration and the small intestine immediately isolated. The peristaltic index (PI), which is the distance travelled by charcoal meal relative to the total length of small intestine expressed in %, was then determined for each mouse (Aye-Than *et al*., 1989; Adeyemi and Akindele, 2008).

3.7.1.2. Castor oil-induced intestinal transit

Mice were allotted to groups of 5 animals each. The groups of mice recieved distilled water (10 ml/kg, p.o.), morphine (10 mg/kg, s.c.) or AR (100, 200 or 400 mg/kg, p.o.), 30

minutes before administration of castor oil (0.2 ml/mouse, p.o.). Isosorbide dinitrate (150 mg/kg, p.o.) was administered 30 minutes before AR (400 mg/kg p.o.) in one group while yohimbine (1 mg/kg, s.c.), pilocarpine (10 mg/kg, s.c.), phentolamine or propranolol (1 mg/kg, i.p.) were administered 15 minutes before AR (400 mg/kg, p.o.) in some other groups respectvely. Thirty minutes after the administration of castor oil, the animals were given standard charcoal meal. In one group, castor oil was given 15 minutes before administration of AR (400 mg/kg, p.o.). All animals in each treatment group were sacrificed 30 minutes after administration of the charcoal meal, and the small intestine immediately isolated. Peristaltic index (PI) for each mouse was then determined (Adeyemi and Akindele, 2008).

3.7.1.3. Castor oil-induced diarrhoea

Groups of 5 mice each were treated with AR (100-400 mg/kg, p.o.), morphine (10 mg/kg, s.c.) or vehicle (10 ml/kg, p.o.) 30 minutes before the administration of castor oil (0.2 ml/mouse) (Adeyemi *et al*., 2003). Each mouse was kept for observation under a glass funnel, the floor of which was lined with paper and observed for 4 hours. The parameters observed were; the onset of diarrhoea, number of wet faeces, total number of faecal output, weight of wet faeces and total weight of faecal output. A numerical score based on stool consistency was assigned as follows,

- normal stool = 1
- semi-solid stool = 2, and
- watery stool = 3

The *in vivo* antidiarrhoeal index ($ADI_{in\ vivo}$) was then expressed according to the formula:

$$ADI_{in\ vivo} = \sqrt[3]{D_{freq} \times G_{meq} \times P_{freq}}$$

Where D_{freq} is the delay in defaecation time or diarrhoea onset (in % of control),

G_{meq} is the gut meal travel reduction (in % of control), and

P_{freq} is the purging frequency reduction i.e. number of stool reduction (in % of control) (Adeyemi *et al*., 2003).

3.7.1.4. Castor oil-induced enteropooling

Rats (5 per group) were pretreated with AR (200-400 mg/kg, p.o.) or distilled water (10 ml/kg, oral). One hour later, the rats received castor oil (2 ml/rat, p.o.) and were sacrificed 30 minutes later (Adeyemi *et al*., 2003). The entire small intestine was then removed after ligation at the pyloric end and ileocaecal junction, and weighed. The intestinal content was expelled into a measuring cylinder and volume measured. The intestine was reweighed and the difference between full and empty intestines was determined (Yasmeen *et al*., 2010).

3.7.1.5. Gastric emptying

Rats fasted for 24 hours prior to the experiment were randomly allotted to two groups of five animals each. The first group received distilled water (10 ml/kg, p.o.) while the second group was given AR (400 mg/kg, p.o.). One (1) hour later, 3 ml of semi-solid test meal (10% charcoal suspension in 2% methylcellulose) was administered to the rats. In another 1 hour, the animals were sacrificed, laparatomized and the stomachs removed. The full stomachs were weighed, emptied and rinsed. Excess moisture was removed and the empty stomach reweighed. The difference between full and empty stomach was subtracted from the weight of 3 ml test meal to give the quantity emptied from the stomach during the test period (Droppleman *et al*., 1980).

3.7.2. Tests for anti–inflammatory activity

3.7.2.1. Carrageenan-induced rat paw oedema.

Carrageenan (0.1 ml of 1% w/v) dissolved in distilled water was injected into the sub-plantar region of the right hind paw of rats (Winter *et al*., 1962; Okpo *et al*., 2001). The paw size of the rats was measured before injection of carrageenan (C_o) and at intervals of 1 hour for 6 hours after carrageenan injection using the cotton thread method (Bamgbose and Naomesi, 1981). AR (10-100 mg/kg), vehicle (10 ml/kg) or indomethacin (10 mg/kg) were orally administered to respective groups of rats one hour before carrageenan injection. The mean increase in paw swelling was measured and the percentage inhibition was calculated.

3.7.2.2. Egg albumin-induced rat paw oedema

Adult albino rats of either sex were used. Animals were fasted for 12 hours and deprived of water only during the experiment. Each group of rats orally received AR (10-100 mg/kg), indomethacin (10 mg/kg) or distilled water (10 ml/kg) 30 minutes before 0.1 ml of fresh undiluted egg albumin was injected into the sub-planter surface of the right hind paw of the rats. Paw sizes were measured before and 1, 2 and 4 hours after egg albumin injection (Akah and Nwambie, 1994).

3.7.2.3. Formaldehyde-induced inflammation

Rats were injected 0.1 ml of 10% formaldehyde (in normal saline) into the plantar aponeurosis of the left hind paw on the first and third day of the test. The extract (10–100 mg/kg, p.o.), distilled water (10 ml/kg, p.o.) or diclofenac (10 mg/kg, i.p.) were administered daily for 10 days. The rat paw circumference was measured daily for 10 days. The percentage inhibition of the mean increase in the paw oedema of rats in each group was calculated on the tenth day and compared with the control (Akindele and

Adeyemi, 2007; Owoyele *et al*., 2008).

3.7.2.4. Xylene–induced ear oedema test

Albino mice fasted overnight prior to commencement of the experiment were divided to 6 groups of 5 animals each. AR (10-100 mg/kg), dexamethasone (1 mg/kg) or distilled water (10 ml/kg) were administered orally to rats in the respective groups. Thirty minutes later, inflammation was induced in mice by topical application of 30 µl of xylene on the inner surface of the right ear. The animals were sacrificed 15 minutes later under ether anaesthesia and the left and right ears excised and trimmed to equal sizes. The difference between the ear weights was taken as the oedema induced by xylene (Adeyemi *et al*., 2002).

3.7.2.5. Effect on castor oil-induced diarrhoea

Three groups of 5 mice each were treated with AR (50 mg/kg, p.o.), indomethacin (10 mg/kg, p.o.) or distilled water (10 ml/kg, p.o.) respectively 30 minutes before administration of castor oil (0.2 ml/mouse). Each mouse was kept for observation under a glass funnel, the floor of which was lined with a white paper and observed for 4 hours. The parameters observed were; the onset of diarrhoea and diarrhoeal score (Mayureen and Ilavarasan, 2009).

3.7.3. Test for analgesic activity

3.7.3.1. Acetic acid-induced writhing test

Albino mice of either sex were divided into 6 groups of 5 mice each. The vehicle (10 ml/kg), diclofenac (5 mg/kg) or AR (10-100 mg/kg) were administered orally to various groups respectively 1 hour before intraperitoneal injection of 0.6 % (v/v) 10 ml/kg of glacial acetic acid. Naloxone (1 mg/kg, i.p.), glibenclamide (2 mg/kg, p.o.) or haloperidol

(1 mg/kg, i.p.) was administered 30 minutes (for perenteral exposure) or 1 hour (for oral exposure) before the administration of the extract (50 mg/kg, p.o.) in some of the mice. The number of writhes (contraction of the abdominal muscle followed by extension of the hind limbs) was counted for 30 minutes. A reduction in the number of writhes compared to control was taken as an indication of analgesic activity (Adeyemi *et al*., 2011a).

3.7.3.2. Acetylcholine-induced writhing test

Albino mice of either sex were divided into 6 groups of 5 mice each. The vehicle (10 ml/kg), diclofenac (5 mg/kg) or AR (10-100 mg/kg) were administered orally to various groups respectively, 1 hour before intraperitoneal injection of 10 mg/kg acetylcholine. The number of writhes was counted for 15 minutes (Adeyemi *et al*., 2008).

3.7.3.3. Formalin-induced pain

The method used was similar to that described previously (Shibata *et al*., 1989; Adeyemi *et al*., 2011b). Twenty microlitres of 1% formalin was injected into the subplantar surface of the right hind paw of mice. The time (in seconds) spent in licking and biting of the injected paw was taken as duration of pain response. This was measured for 5 minutes after formalin injection (first phase) and 15–30 minutes after formalin injection (second phase). Vehicle (10 ml/kg), AR (10–100 mg/kg, p.o.) or diclofenac (5 mg/kg, p.o.) were administered to separate groups of mice, 1 hour before formalin injection.

3.7.3.4. Tail clip test

A metal artery clip was applied to the root of mice tail to induce pain (Bianchi and Franceschini, 1954; Adeyemi *et al*., 2004). A sensitivity test was carried out in this manner, and animals that did not attempt to dislodge the clip within 10 seconds were discarded. Responsive mice were allotted to groups of five animals each. The tail clip was

applied 1 hour after oral administration of extract (10–100 mg/kg) and the vehicle (5 ml/kg p.o.) or 30 minutes after subcutaneous administration of morphine (10 mg/kg).

3.7.3.5. Hole board test

This study was carried out using a board (40 × 40 cm) with 16 equidistant holes (1 cm diameter × 2 cm depth). Each mouse was placed on the board and allowed to move freely and dip its head into the holes. This behaviour indicates exploratory activity. The number of head dips in 5 minutes was recorded. The test was carried out 60 minutes after administration of the extract (10–100 mg/kg, p.o.), diazepam (3 mg/kg, p.o.) and vehicle (5 ml/kg, p.o.) to different groups of mice respectively (File and Wardill, 1975; Adeyemi *et al*., 2010).

3.7.3.6. Open field test

The open field is a 400 × 400 × 300 mm box with dark stripes that divides it into 16 quadratic blocks. Each mouse was placed on the floor of the box 1 hour after oral administration of AR (10-100 mg/kg), diazepam (3 mg/kg) and vehicle (5 mg/kg). For a 5 minutes period, the number of line crossings made was recorded (Mahendra and Bisht, 2011).

3.7.3.7. Hexobarbitone-induced sleep

Mice in different groups were administered vehicle (5 ml/kg, p.o.), AR (10-100 mg/kg, p.o.) and diazepam (3 mg/kg, i.p.) respectively. One hour later, hexobarbitone (100 mg/kg, i.p.) was in turn administered to each mouse. The onset and duration of sleep were noted. When there was any doubt, the animal was placed gently on its back again and if it righted itself within 1 minute, it was taken that the sleep end point has been reached (Mujumdar *et al*., 2000).

3.7.4. Effect of AR on some haemodynamic parameters

3.7.4.1. Chronic effect of AR on blood pressure and heart rate

Four groups of 5 spontaneously hypertensive rats (SHRs) each were orally administered, AR (25-50 mg/kg), enalapril (3 mg/kg) and vehicle (5 ml/kg) for 21 days. Blood pressure and heart rate were measured using the tail cuff method with CODA non-invasive blood pressure monitor (Kent Scientific Co-operation, USA) on days 0, 7, 14 and 21. On these days, 24-hour urine samples were also obtained by placing each rat singly in metabolic cages for 24 hours (Lessa *et al*., 2008).

3.7.4.2. Effect of AR on urine volume and electrolytes

The volume of urine collected was measured using measuring cylinder; and the urine Na^+ and K^+ concentrations were determined using a Jenway PFP 7 flame photometer.

3.7.4.3. Acute effect of AR on blood pressure and heart rate

Spontaneously hypertensive rats fasted for 12 hours, were anaesthetized with pentobarbitone (60 mg/kg, i.p.), after which cannulae were inserted into the right carotid artery, left jugular vein and the trachea for measurement of haemodynamic parameters, administration of drugs and ease of air passage respectively. Haemodynamic parameters including systolic and diastolic blood pressures as well as heart rate were determined using a cannula connected to pressure transducer, linked to a data acquisition system through a bridge amplifier, using lab chart (version 6.1.1.) software (ADInstruments, Australia). The data acquisition system was connected to a Windows XP computer via a PowerLab USB driver (version 2.0.3). The measurement of haemodynamic parameters was noted before and after the administration of AR (6.25-50 mg/kg) and corresponding equal volumes of the vehicle. In some experiments, the effect of the extract at 50 mg/kg was also noted in the presenc of intravenously administered atropine (1 mg/kg) or hexamethonium (20

mg/kg). The effect of the chloroform, butanol and aqueous fractions of AR were also determined.

3.8. PHYTOCHEMICAL INVESTIGATIONS

3.8.1 Preliminary phytochemical screening

Preliminary phytochemical screening of AR was carried out using methods described by Sofowora (1993).

3.8.1.1. Test for oils

Few drops of extract were spotted on filter paper and allowed to evaporate to dryness. A transluscent spot on the paper was taken as evidence for the presence of oil.

3.8.1.2. Test for alkaloids

Half a gramme of extract was added to 5 ml of 10% $HCl_{(aq)}$ and warmed on a steam bath. This was filtered and a few drops of Meyer's reagent added to 1 ml of filtrate. Light yellow colouration with white precipitate was taken as evidence for the presence of alkaloids.

3.8.1.3. Test for tannins

A portion of AR was added to 5% ferric chloride. A blue black or blue green precipitate was taken as an evidence for the presence of tannins.

3.8.1.4. Test for saponins

The ability of saponnin to produce frothing in aqueous solution was used as basis for this test. A portion of AR was added to water in a test tube and shaken. Frothing which persists on warming was taken as evidence for the presence of saponins.

3.8.1.5. Test for phlobatannins

A portion of the extract was boiled with dilute hydrochloric acid. Deposition of red precipitate was taken as evidence for the presence of phlobatannins.

3.8.1.6. Test for reducing sugars

The extract (2 ml) was mixed with 5 ml of Fehling's solution A and B. A brick red precipitate was taken as evidence for the presence of reducing sugars.

3.8.3. Quantitative determination of tannin, flavonoid and phenolic contents of AR and its fractions

3.8.3.1. Determination of tannins in AR and fractions

Fifteen (15) µl of AR (5 mg/ml) and its fractions were added to 6 µl of Na_2CO_3 (0.25 mg/ml) respectively. Two hundred and seventy-nine (279) µl distilled water was then added to the mixture, which was allowed to incubate for one and half hours. Absorbance was then read at 760 nm using a microplate reader. Tannic acid (0.0005, 0.005, 0.05 and 0.5 mg/ml) was used to obtain standard calibration curve. The tannic acid estimation was done in triplicate and results were expressed as tannic acid equivalents.

3.8.3.2. Determination of total flavonoids in AR and fractions

Sixty (60) µl standard flavonoid, quercetin (0.0005-0.5 mg/kg), AR or its fractions (5 mg/kg) was mixed with 90 µl $NaNO_2$ (5%) and 90 µl of $AlCl_3$ (10%) reagent in a 96-well microplate and allowed to incubate at room temperature for 6 minutes. NaOH (60 µl of 1M) was then added and the mixture allowed to stand for 30 min before the absorbance of the reaction mixture was read at 510 nm using the microplate reader. All samples were analysed in triplicate and the results were expressed as quercetin equivalent (Wu *et al*., 2013).

3.8.3.3. Determination of phenolics in AR and fractions

For the determination of the phenolic contents of AR and its petroleum ether, chloroform, butanol and aqueous fractions, the Folin-Ciocalteu method as described by Barros *et al*. (2007) was applied but modified for use with microplates. Twenty three (23) µl of AR and fractions (5 mg/ml) were added to 23 µl of Folin-Ciocalteu reagent respectively. After 3 minutes, saturated Na_2CO_3 was added to the mixture, which was made up to 130 µl with distilled water. The reaction was kept in the dark for 90 minutes, after which the absorbance was read using a microplate reader at 725 nm. Gallic acid (0.0005-0.5 mg/ml) was used to obtain standard calibration curve. Estimation of the phenolic contents was carried out in triplicate and results were expressed as gallic acid equivalents.

3.8.4. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) assay

To determine the radical scavenging activity (RSA) of AR and its fractions against 2,2-diphenyl-1-picrylhydrazyl (DPPH), 0.01 ml of AR (0.0005-0.5 mg/ml), its fractions (0.0005-0.5 mg/ml) or blank was added to 0.29 ml of DPPH 10^{-4} M respectively and the absorbance (A) determined at 517 nm after 30 minutes incubation in the dark at room temperature. Radical scavenging activity (RSA) was calculated in percent using the following formulae:

$$\% \text{ RSA} = (A_{DPPH} - A_{sample} / A_{DPPH.} - A_{blank}) \times 100.$$

3.8.5. High performance liquid chromatography (HPLC) analyses of AR

3.8.5.1. Identification and quantification of phenolics in AR using HPLC

HPLC analysis for phenolic contents of AR was conducted using Shimadzu HPLC apparatus coupled with a flourimetric detector. The phenolic compounds were detected at excitation and emission wavelengths, λex/λem=226/420 nm. Solvent gradients were formed using the dual pump system by varying the proportion of solvent A [water–acetic

acid (97:3] to solvent B (methanol). The solvent gradient elution programme used was as follows (total run time of 60 minutes); 100% solvent A /0% solvent B at 0 minutes; 90% A/10% B at 10 minutes, 30% A/70% B at 40 minutes, 100% A/0% B at 44 to 50 minutes. Under these conditions, 20 µl of sample (AR 20 mg/ml) or standard phenolics (25-100 µg/ml) were injected. All sample analyses were done in triplicate. The phenolic contents of AR were detected by matching the retention time and their spectral characteristics against those of standards. Quantitation was made according to the calibration curves of respective standard compounds.

3.8.5.2. Aristolochic acid I detection and quantification using HPLC

3.8.5.2.1. Sample preparation

The concentrates of AR and petroleum ether, dichloromethane, ethylacetate, butanol and water fractions were dissolved in 0.1 M NaOH to obtain 200 mg/ml of each sample. Two (2) ml of each sample was then passed under vacuum through a 1 ml capacity solid-phase extraction column previously washed with 2 ml of methanol, followed by 2 ml of water. The column was then washed with 2 ml of 0.1 M NaOH followed by 2 ml of a mixture containing 2 volumes of glacial acetic acid, 28 volumes of water and 70 volumes of methanol. The sample was then eluted with 0.5 ml of a mixture of 98% methanol and 2% concentrated ammonia. The extracts thus obtained were then evapourated to dryness and dissolved in 0.5 ml of 0.1 M NaOH.

3.8.5.2.2. Chromatographic procedure

The chromatographic procedure was carried out using stainless steel column (25 cm × 4.6 mm) packed with base-deactivated octadecylsilyl silica gel for chromatography (4 µm) (Genesis C18), maintaining the column temperature at 30°C. A mixture of 45 volumes of 0.1% v/v of orthophosphoric acid and 55 volumes of acetonitrile was the mobile phase

used at a flow rate of 1 ml per minute and a detection wavelength of 225 nm. The identity of any peaks suspected to be due to arisotolochic acid I was clarified by ultraviolet (UV) detector. Ten microlitres of different concentrations of the reference standard solution was injected respectively and allowed to run first for 5 minutes. A calibration curve of peak area vs concentration was plotted with data obtained from the chromatogram. Ten microlitres of the test samples were also injected and allowed to undergo same process as the refercnce standard. The concentration of aristolochic acid I in the test samples were determined from the calibration curve obtained with the reference standard, aristolochic acid I (Sigma Aldrich, St. Louis, USA).

3.8.6. Gas chromatography-mass spectroscopy (GC-MS) analysis of AR

GC-MS analysis of the aqueous root extract of *A. ringens* was performed using GC coupled to MS-Engine. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and sample injection volume of 2 µl was employed. The injector temperature was maintained at 290°C and the ion-source temperature was 300°C. A solvent delay of 5 minutes and transfer temperature of 300°C were used. Mass spectra were taken at scan interval of 0.5 second and fragments from 28 to 400 Da. The total GC-MS run time was 30 minutes. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Interpretation on mass-spectrum was conducted using the database of the Wiley online library. The spectra of unknown components was compared with the spectra of known components stored in the Wiley online library

3.9. Statistical analysis

Results obtained were expressed as mean ± SEM. Experimental data obtained from the studies were analyzed using the one way analysis of variance (ANOVA) followed by Turkey's multiple comparison test, two way ANOVA followed by Bonferroni's post test or Student's t test as appropriate, using Graph Pad Prism 5 statistical package. Results were considered significant at $p<0.05$.

CHAPTER FOUR

RESULTS

4.0. RESULTS

4.1. TOXICOLOGICAL INVESTIGATIONS

4.1.1. Brine shrimp toxicity test

In the brine shrimp toxicity test, mortality was observed at all the concentrations tested. AR (1000 µg/ml) produced 95% mortality in brine shrimps. The LC_{50} of AR in this test was found to be 175 µg/ml (Figure 5).

4.1.2. Acute toxicity tests

The extract (10 g/kg) produced no visible morbidity or mortality at 24 hours after single oral exposure, and for up to 2 weeks of observation in the treated mice. Administration of the extract intraperitoneally produced a dose-dependent severity in abnormal gait, decreased responsiveness to the environment, writhing and pilo-erection. These effects were seen at 200, 400 and 600 mg/kg respectively. A probit log-dose analysis showed a median lethal dose (LD_{50}) of 407.38 mg/kg (Figure 6).

4.1.3. Subchronic toxicity study

Effects of AR on body weight

AR at 10 mg/kg significantly increased the body weight of male rats by the 3rd and 4th week of exposure (Figure 7). In the female rats on the other hand, it was observed that the body weights were significantly ($p<0.05$) reduced by AR at all tested doses by the 4th week, while only the therapeutic and supratherapeutic doses caused the significant reduction by the 3rd week (Figure 8).

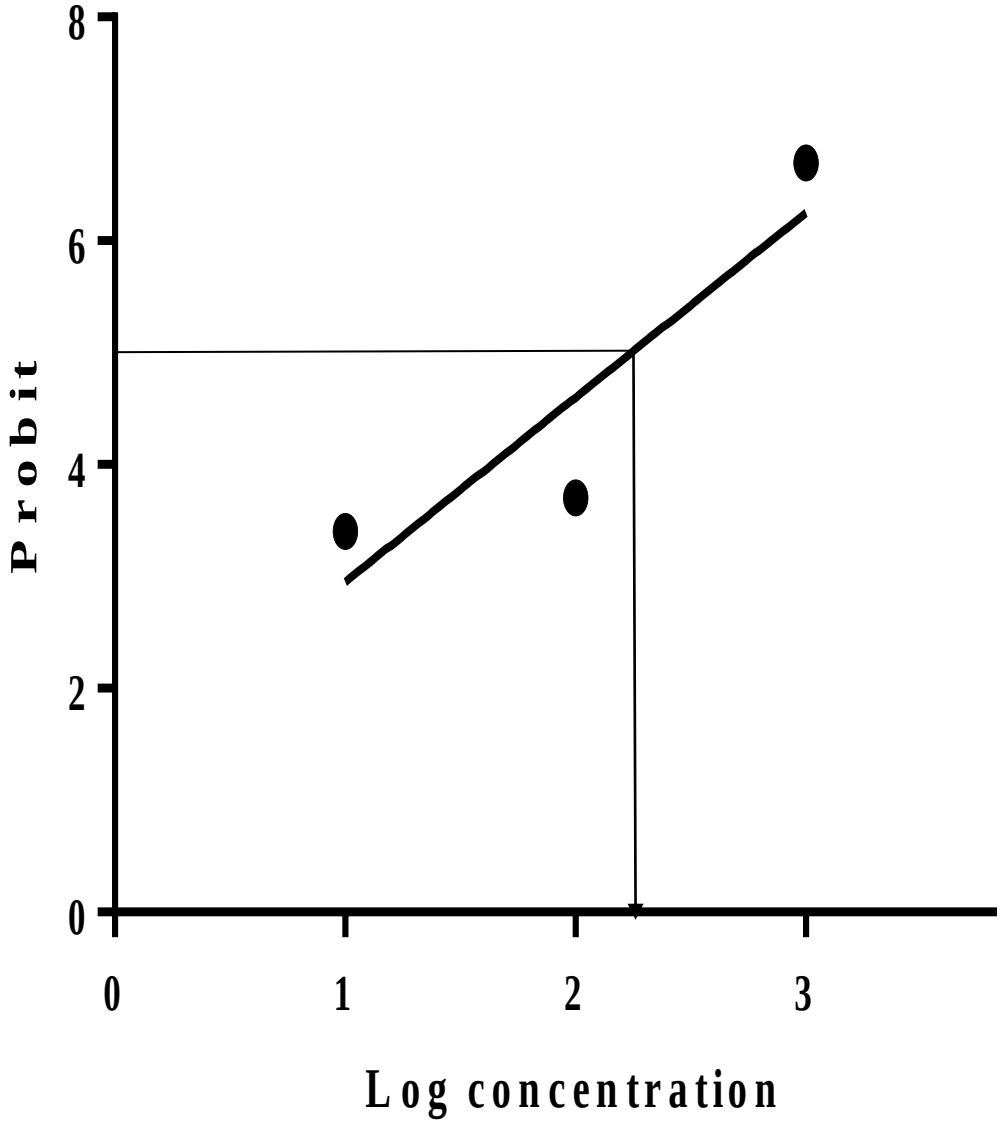


Figure 5: Graph of probit of lethality response of brine shrimps to AR versus log concentration of AR. LC_{50} = 175 µg/ml.

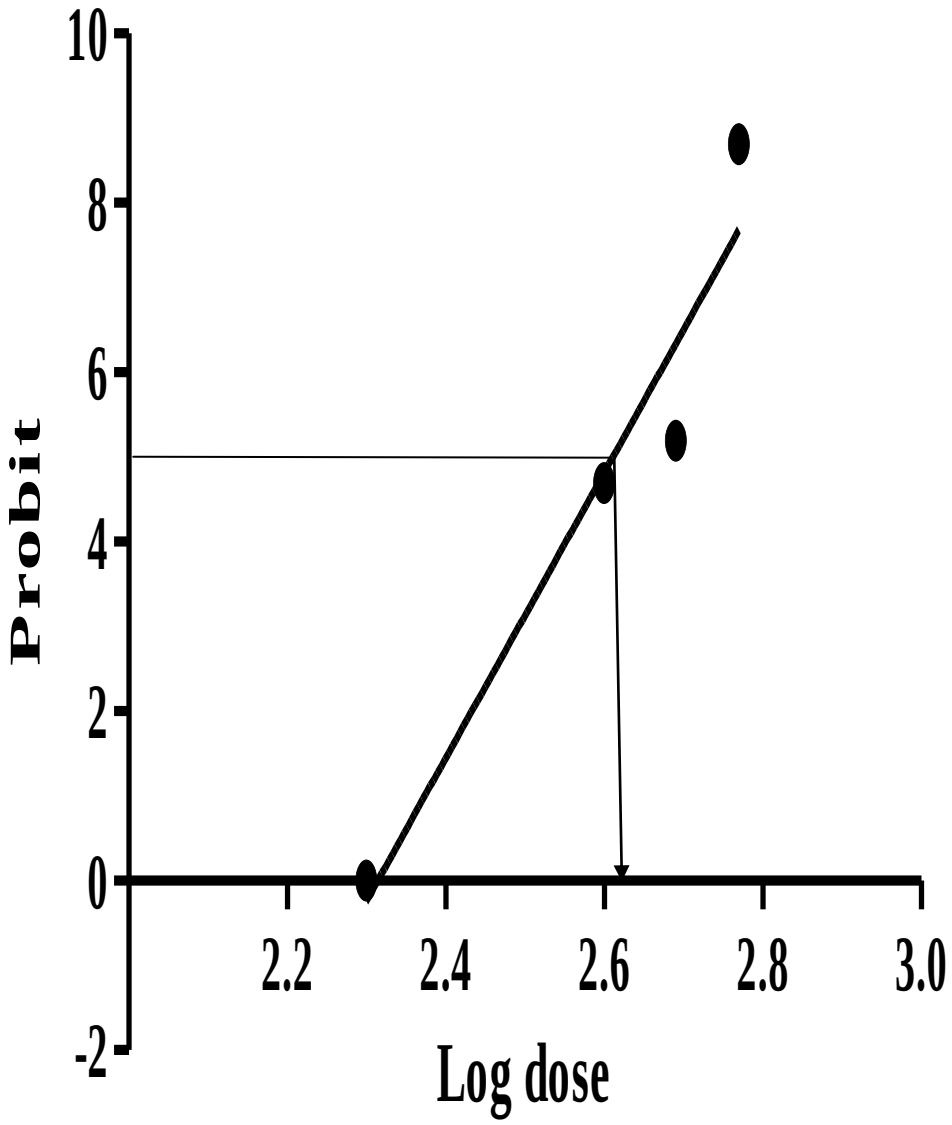


Figure 6: Graph of probit vs. log dose for intraperitoneal acute toxicity test in mice. LD_{50} = 407.38 mg/kg.

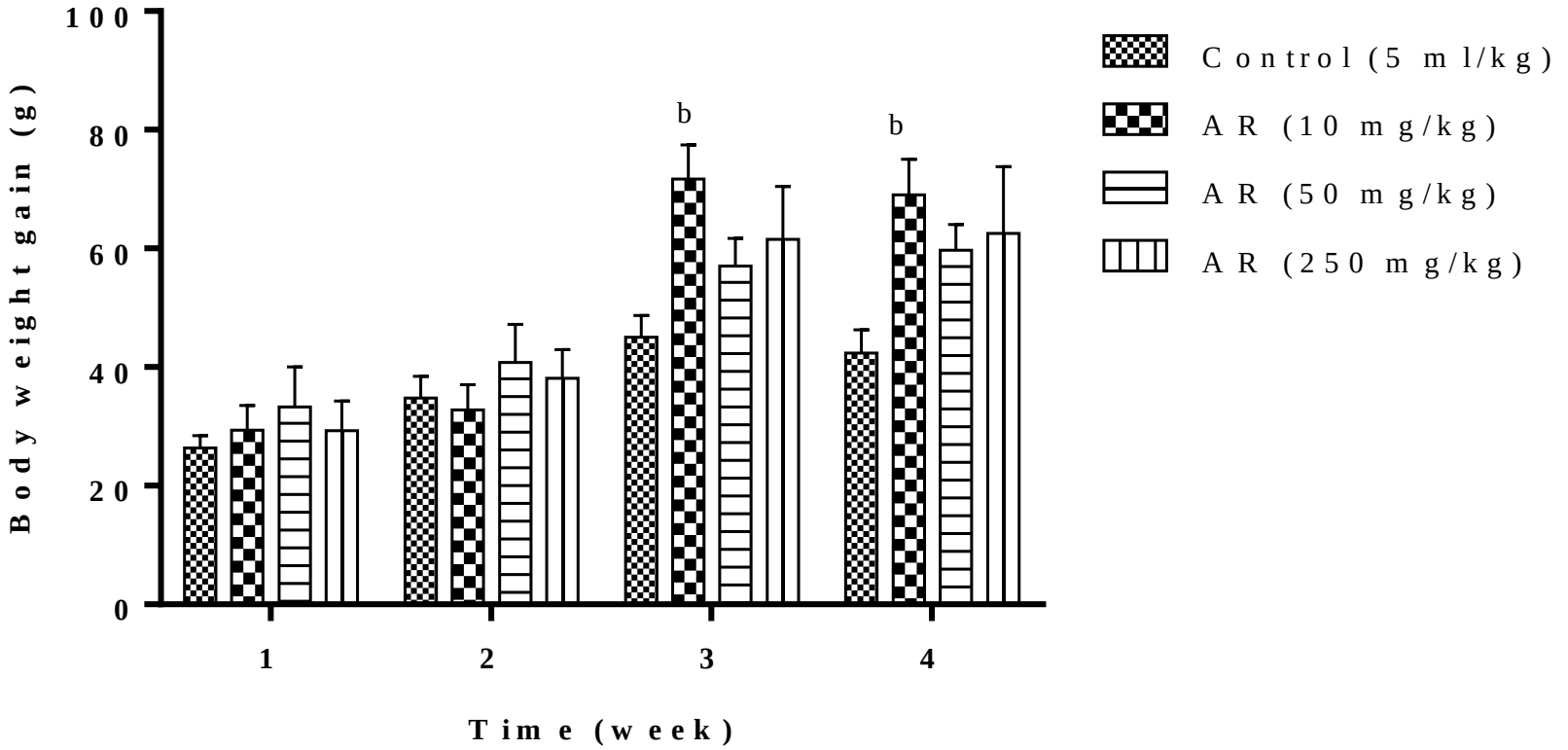


Figure 7: Effect of AR on body weight of male rats during the 30 days exposure to AR. Bars represent mean ± SEM. (n=10) $^{b}p<0.01$ vs. control. (One way ANOVA followed by Tukey's multiple comparison test).

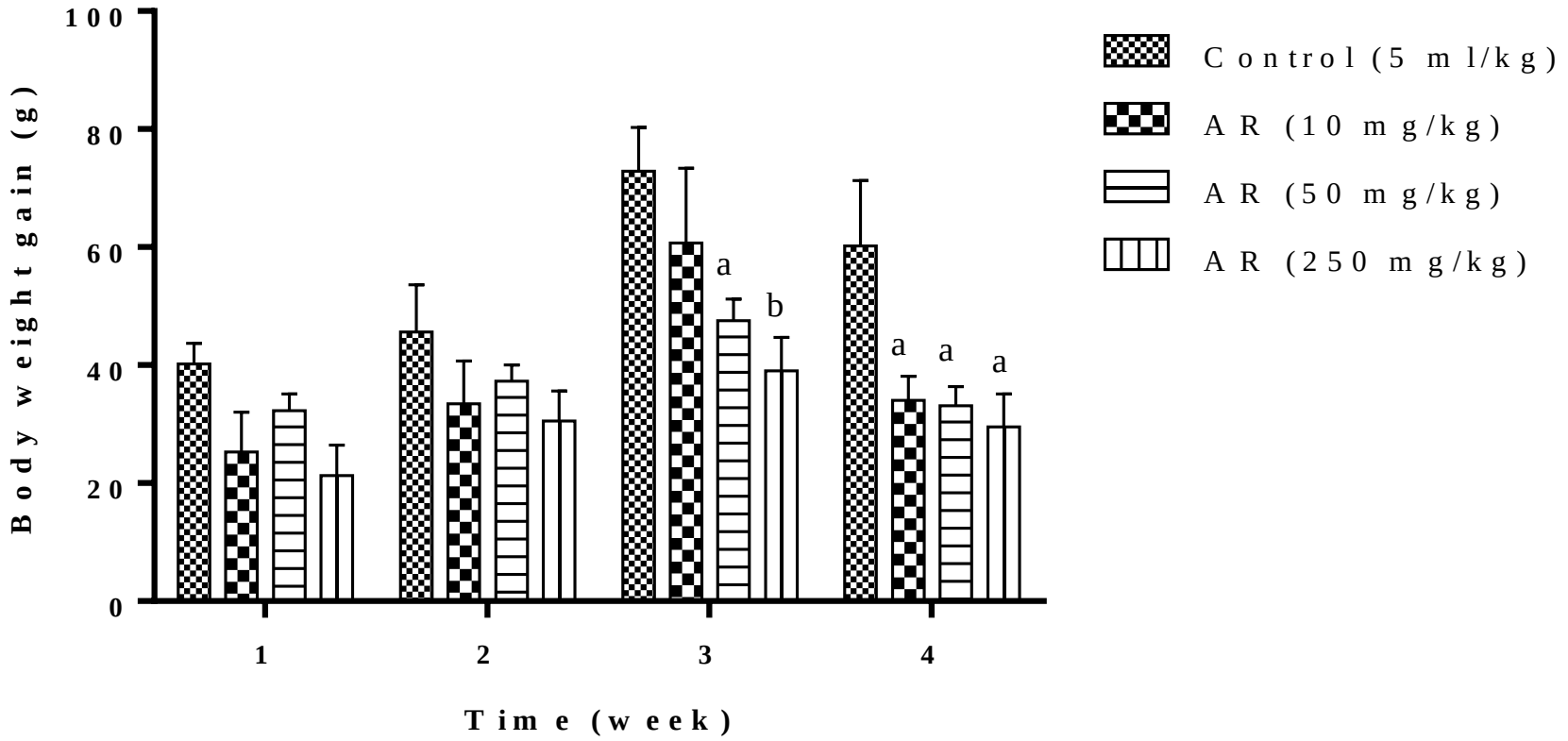


Figure 8: Effect of AR on body weight of female rats during the 30 days exposure to AR. Bars represent mean ± SEM. (n=10) [a]p<0.05, bp<0.01 vs. control. (One way ANOVA followed by Tukey's multiple comparison test).

Effect of AR on organs

AR produced no significant change in the organ weights of male and female rats (Tables 3 and 4 respectively). The histology carried out revealed absence of histopathology of the kidney and liver of male rats treated with AR (10, 50 and 250 mg/kg) (Figures 9 and 10).

Effect of AR on haematologic parameters

In male rats, by the end of the 30 days exposure, AR significantly ($p<0.01$) reduced haematocrit level at 250 mg/kg. At 10 and 50 mg/kg of AR, haematocrit and haemoglobin levels were significantly increased compared to the 250 mg/kg dose. No significant change was observed in the haematologic parameters tested at the expiration of the reversibility pe xriod (Table 5). On the other hand, in the female rats, MCV was significantly reduced by AR at 250 mg/kg by the end of the 30 days exposure period. After the reversibility period, MCV and WBC of female rats treated with AR (250 mg/kg) were significantly decreased. A significant ($p<0.01$) reduction of MCV was also observed at 10 mg/kg of AR. Significant ($p<0.05$) increase in lymphocytes level of AR (10 mg/kg) treated rats was also observed (Table 6).

Effect of AR on biochemical parameters

At the end of the 30 days exposure period, AR significantly reduced triglycerides at 10-250 mg/kg in male rats. All the other parameters tested were not significantly affected by AR. In the reversibility test however, AR (10 and 50 mg/kg) treated rats showed significant increase in urea compared to the control and AR (250 mg/kg) treated rats (Table 7). In the female rats, after the 30 days treatment, AR was found to significantly ($p<0.01$) reduce ALP at 10 mg/kg and significantly ($p<0.05$) reduce creatinine at 250 mg/kg. In the reversibility test, AR at 10 and 50 mg/kg significantly reduced ALT and albumin, and at 10 mg/kg significantly ($p<0.05$) reduced urea (Table 8).

Table 3: Effect of AR on organ weights of male rats

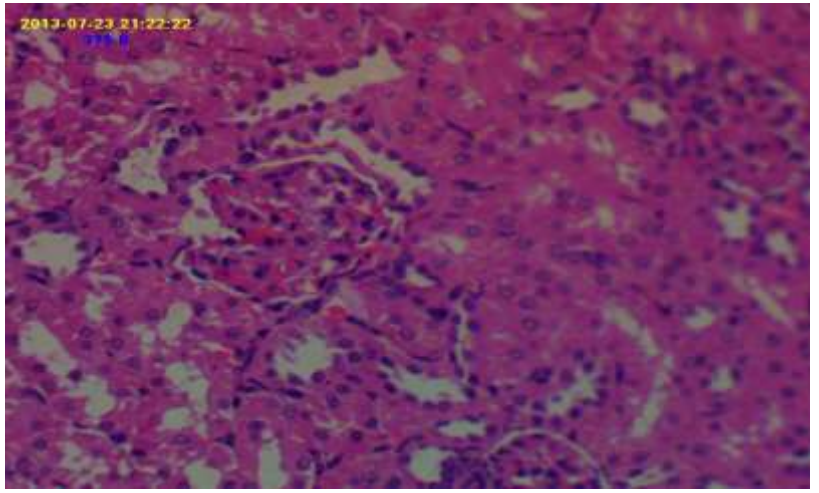
Organ	**After 30 days treatment period** **Organ weight (g per 100 g body weight)**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
Liver	2.75±0.72	2.78±0.20	3.40±0.44	2.74±1.13
Kidneys	0.71±0.08	0.64±0.05	0.71±0.07	0.61±0.20
Brain	0.84±0.07	0.79±0.14	0.97±0.07	0.91±0.10
Heart	0.36±0.02	0.35±0.06	0.40±0.05	0.30±0.12
Lungs	0.99±0.37	0.79±0.29	0.95±0.21	0.72±0.22
Pancreas	0.47±0.19	0.30±0.06	0.41±0.04	0.36±0.19
Spleen	0.50±0.08	0.40±0.11	0.51±0.11	0.33±0.12
Testes	1.23±0.04	1.10±0.14	1.31±0.15	0.99±0.16

Values are mean ± S.E.M. (n=5). $P<0.05$ (One way ANOVA followed by Tukey's multiple comparison test).

Table 4: Effect of AR on organ weights of female rats

Organ	**After 30 days treatment period** **Organ weight (g per 100 g body weight)**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
Liver (g)	3.44±0.06	3.42±0.34	3.51±0.18	3.69±0.32
Kidney (g)	0.73±0.06	0.80±0.08	0.73±0.06	0.78±0.06
Brain (g)	1.08±0.11	1.10±0.01	1.04±0.05	1.15±0.04
Heart (g)	0.42±0.05	0.47±0.03	0.38±0.05	0.43±0.04
Lung (g)	0.80±0.12	0.99±0.04	0.90±0.13	1.10±0.23
Pancreas (g)	0.33±0.05	0.44±0.09	0.39±0.07	0.42±0.02
Spleen (g)	0.42±0.04	0.47±0.06	0.37±0.07	0.48±0.11
Ovaries (g)	0.09±0.01	0.10±0.02	0.11±0.01	0.10±0.01

Values are mean±S.E.M. (n=5). $P<0.05$ (One way ANOVA followed by Tukey's multiple comparison test).



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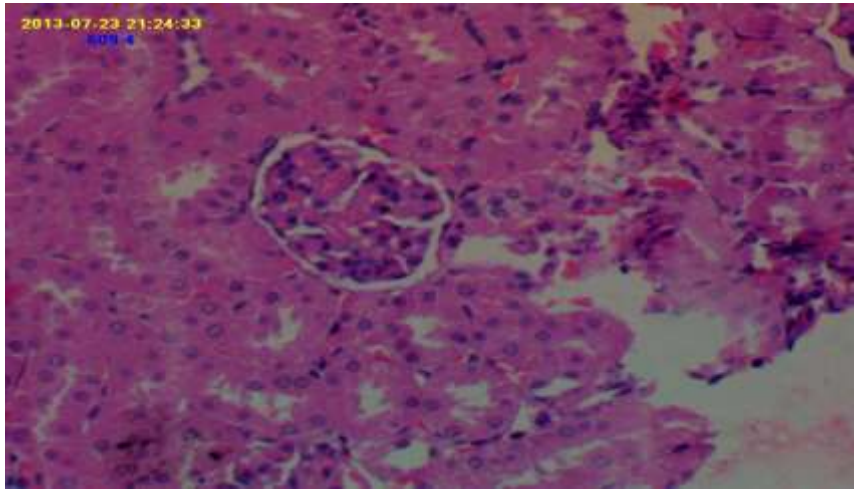

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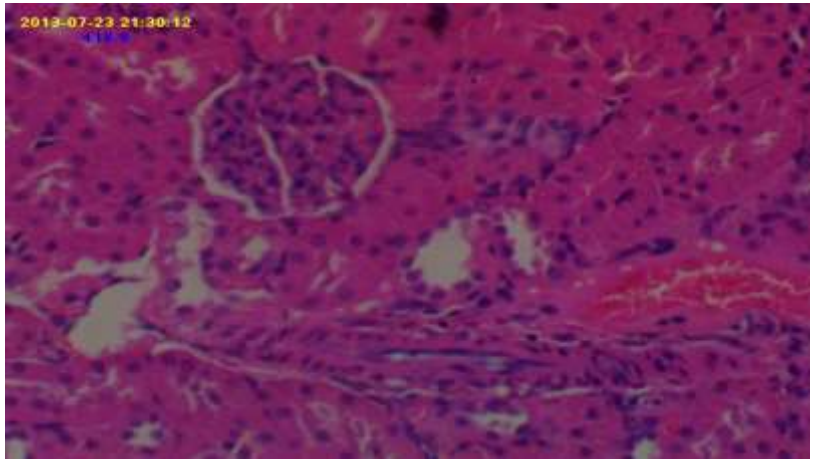

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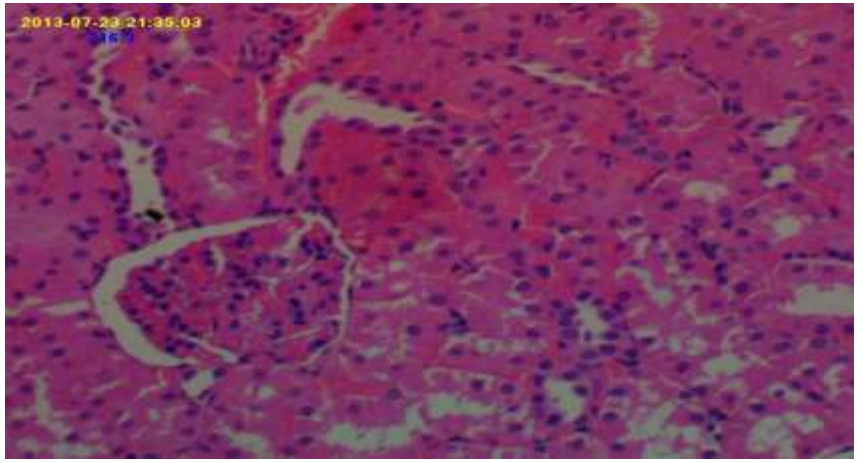
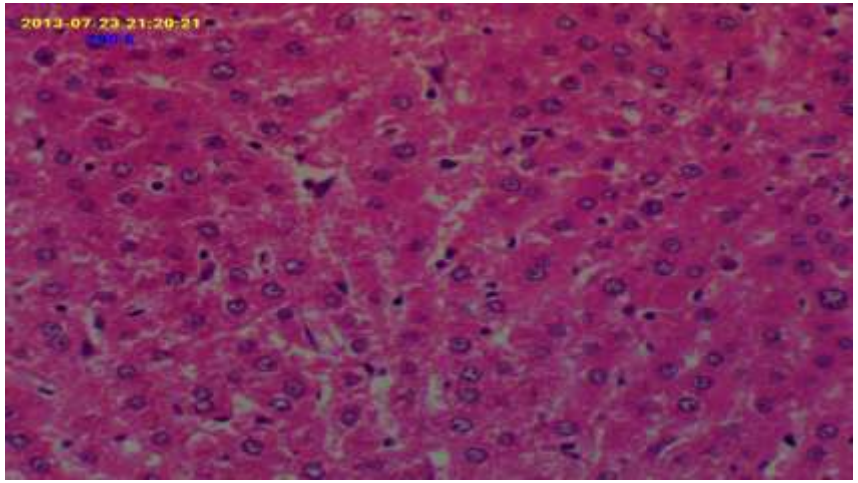


D

Figure 9: Photomicrographs of the kidney of male rats treated with vehicle (A), AR at 10 mg/kg (B), AR at 50 mg/kg (C) and AR at 250 mg/kg (D). Magnification x 40.



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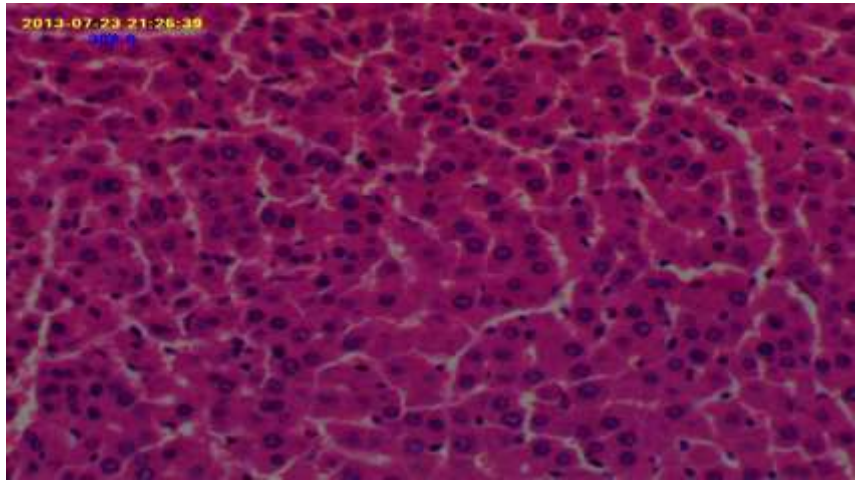

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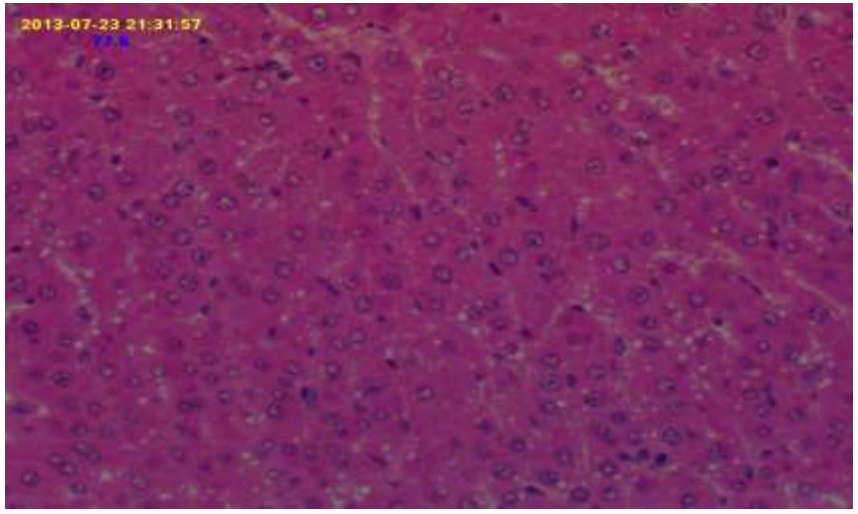

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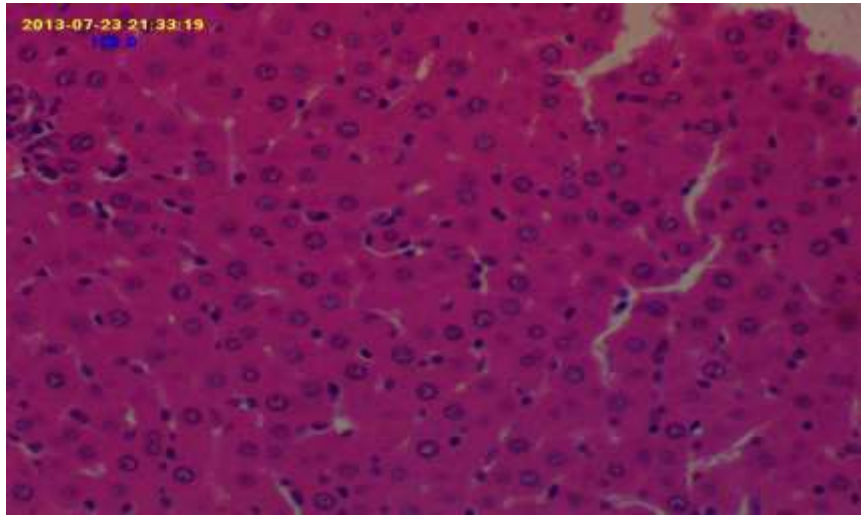


D

Figure 10: Photomicrographs of the liver of male rats treated with vehicle (A), AR at 10 mg/kg (B), AR at 50 mg/kg (C) and AR at 250 mg/kg (D). Magnification x 40

Table 5: The effect of AR on haematology of male rats

Haematologic parameter	**After 30 days treatment period**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
RBC (10^{12}/l)	07.72±0.33	7.87±0.38	7.73±0.23	6.80±0.20	8.03±0.44	8.63±0.31	7.91±0.22	8.02±0.23
Haemoglobin (g/dl)	13.40±0.31	14.35±0.50$^{\beta}$	13.41±0.23$^{\alpha}$	11.97±0.46	15.43±0.49	15.38±0.47	14.48±0.35	15.43±0.34
Haematocrit (%)	46.23±1.49	47.18±2.47$^{\beta}$	42.84±0.79$^{\alpha}$	36.20±1.51^{b}	48.85±2.00	47.00±1.26	43.94±0.95	46.95±0.39
MCH (pg)	18.40±0.33	18.83±0.35	18.46±0.27	18.50±0.83	18.00±0.17	17.90±0.39	18.46±0.41	18.55±0.20
MCHC (g/dl)	30.78±0.25	31.48±0.43	32.00±0.64	31.35±0.48	31.85±0.24	32.30±0.40	32.54±0.47	31.65±0.40
MCV (fl)	60.03±0.85	60.05±1.90	58.04±1.06	58.65±2.14	57.88±0.52	54.15±0.63	57.20±1.08	56.70±0.31
Platelet (10^{9}/l)	736.0±68.8	748.8±63.6	712.0±26.1	689.3±103.5	701.8±51.3	637.5±27.5	708.0±106.3	742.0±52.4
WBC (10^{9}/l)	7.43±0.37	9.15±1.02	9.13±0.57	7.61±0.42	12.10±1.28	11.53±0.30	10.34±1.31	9.45±0.84
Neutrophil (%)	28.85±1.12	30.38±4.45	29.14±2.58	38.00±4.57	25.48±8.23	29.80±2.73	25.35±2.44	27.20±3.52
Lymphocyte (%)	61.25±1.81	60.73±1.27	62.60±1.95	50.50±3.28	66.35±8.45	63.83±1.76	64.06±3.73	62.90±3.03

Values are mean±S.E.M. (n=4-5) b$p<0.01$ vs control; $\alpha p<0.05$, $\beta p<0.01$ vs AR 250 (One way ANOVA followed by Tukey's multiple comparison test). Packed cell volume (PCV), red blood cell (RBC) count, total white blood cell (WBC) count, mean cell haemoglobin concentration (MCHC), mean red cell volume (MCV), and mean cell haemoglobin (MCH).

Table 6: The effect of AR on haematology of female rats

Haematologic parameter	**After 30 days treatment period**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
RBC (10^{12}/l)	7.19±0.19	7.18±0.22	7.02±0.25	7.13±0.38	7.44±0.41	7.75±0.09	8.39±0.35	7.43±0.15
Haemoglobin (g/dl)	13.53±0.14	13.53±0.57	12.44±0.31	12.28±0.95	14.00±0.11	44.28±0.32	14.60±0.57	13.65±0.35
Haematocrit (%)	39.90±8.81	45.95±1.72	36.14±5.94	35.93±8.16	44.33±0.43	40.85±0.73	44.98±1.49	41.35±0.61
MCH (pg)	18.80±0.39	19.48±0.51	17.76±0.23	17.80±0.36	18.43±0.34	17.48±0.71	17.90±0.25	17.80±0.17
MCHC (g/dl)	28.35±0.63	29.35±1.47	28.78±0.40	29.35±0.26	32.13±0.48	32.33±0.26	32.70±0.14	32.18±0.49
MCV (fl)	68.70±2.20	66.45±1.47	62.98±1.52	60.88±1.40[a]	57.83±1.45	52.10±0.44[b]	54.40±0.74	53.65±0.70[a]
Platelet (10^9/l)	632.0±44.1	654.0±88.0	763.2±47.8	681.3±60.4	617.3±20.8	676.0±71.2	553.8±65.7	577.3±46.2
WBC (10^9/l)	7.78±1.24	5.98±0.92	6.02±0.66	9.45±1.33	11.08±1.03	7.93±1.15	9.84±1.01 [β]	4.45±0.22[b]
Neutrophil (%)	27.53±5.85	33.48±4.56	34.86±2.28	29.78±3.35	30.78±1.02	12.25±0.62	26.26±1.03	30.97±14.72
Lymphocyte (%)	64.08±5.55	58.48±4.43	54.92±2.53	59.83 ±5.46	59.08±4.02	81.90±0.91[a]	66.04±2.22	51.55±9.64

Values are mean±S.E.M. (n=4-5) ap<0.05, bp<0.01 vs control; βp<0.01 vs AR 250 (One way ANOVA followed by Tukey's multiple comparison test). Packed cell volume (PCV), red blood cell (RBC) count, total white blood cell (WBC) count, mean cell haemoglobin concentration (MCHC), mean red cell volume (MCV), and mean cell haemoglobin (MCH).

Table **7:** Effect of AR on biochemical parameters of male rats

Biochemical parameter	**After 30 days treatment period**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
AST (IU/L)	212.10±18.40	215.80±21.20	190.30±14.90	177.20±37.10	93.10±32.60	124.40±4.20	143.90±7.70	97.30±11.60
ALT (IU/L)	49.03±03.10	52.25±03.82	56.10±03.31	55.33±05.63	67.43±16.90	72.45±02.80	98.87±08.54	63.80±10.76
ALP (IU/L)	146.40±21.00	108.60±5.00	146.30±13.50	157.40±12.50	146.10±30.90	287.60±34.40	241.40±34.00	190.90±42.90
Urea (mg/dL)	6.33±0.43	8.20±0.73	8.82±2.03	9.30±1.10	4.25±0.56	7.35±0.50 $^{a\beta}$	6.73±0.31$^{a\alpha}$	3.88±0.97
Creatinine (µmol/L)	46.89±2.65	58.24±5.45	56.89±1.28	64.80±7.13	34.96±11.76	54.80±10.86	45.08±3.41	32.40±13.83
Bilirubin (µmol/L)	3.87±0.12	3.85±0.03	4.03±0.06$^{\alpha}$	3.70±0.091	1.10±0.58	1.75±0.15	1.30±0.16	0.68±0.11
Albumin (g/L)	37.30±1.08	41.85±1.39	38.64±1.71	35.63±1.79	31.95±6.01	39.60±0.42	36.73±0.90	32.00±7.99
Total protein (g/L)	72.13±2.67	74.88±3.65	74.52±1.36	71.93±3.27	57.88±9.66	73.60±1.25	79.20±4.21	55.45±14.99
Cholesterol (mmol/L)	1.93±0.14	1.86±0.10	2.09±0.21	2.25±0.12	1.95±0.35	2.32±0.08	1.61±0.24	1.25±0.25
Triglycerides (mmol/L)	2.38±0.29	0.97±0.19^{b}	1.14±0.21^{b}	1.31±0.15^{a}	1.48±0.51	0.99±0.01	0.82±0.11	0.80±0,29

Values are mean ± S.E.M. (n=4-5) $^{a}p<0.05$, $^{b}p<0.01$ vs control; $^{\alpha}p<0.05$, $^{\beta}p<0.01$, $^{\gamma}p<0.001$vs AR 250 (One way ANOVA followed by Tukey's multiple comparison test).

Table 8: Effect of AR on biochemical parameters of female rats

Haematologic parameter	**After 30 days treatment period**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR0 (250 mg/kg)**
AST (IU/L)	194.90±18.06	178.00±22.82	207.20±27.74	135.90±29.99	129.90±9.05	149.50±11.09	113.40±16.86	125.20±3.73
ALT (IU/L)	44.07±05.81	41.45±03.15	53.74±06.59	44.10±13.43	88.70±11.61	43.20±2.35$^{c\alpha}$	59.45±2.13^{b}	71.63±0.38
ALP (IU/L)	166.80±26.12	90.00±5.27^{b}	136.50±10.80	114.90±6.63	230.70±2.70	229.70±27.80	182.50±3.90	203.10±3.29
Urea (mg/dL)	8.87±0.54	8.98±1.18	8.54±0.62	8.45±0.43	5.60±0.11	3.45±0.12^{a}	4.20±0.56	4.73±0.17
Creatinine (µmol/L)	71.04±4.12	66.98±2.10	68.95±3.85$^{\alpha}$	50.24±2.85^{a}	44.13±0.84	30.27±10.01	39.71±5.21	49.17±0.69
Bilirubin (µmol/L)	3.78±0.30	3.93±0.06	3.86±0.11	3.68±0.26	1.35±0.10	2.43±1.24	2.07±0.18	1.88±0.30
Albumin (g/L)	41.30±1.15	38.20±2.99	41.04±1.01	37.23±1.77	45.40±0.82	30.15±3.69^{b}	31.07±1.97^{b}	40.10±3.27
Total protein (g/L)	59.35±14.66	75.15±3.48	77.58±3.24	60.33±12.02	76.40±0.59	66.00±10.24	75.07±7.47	79.93±0.46
Cholesterol (mmol/L)	1.80±0.53	1.81±0.16	1.87±0.11	1.53±0.37	1.44±0.05	1.33±0.39	1.72±0.31	1.51±0.01
Triglycerides (mmol/L)	0.92±0.25	0.70±0.05	0.86±0.11	0.68±0.19	0.70±0.02	0.75±0.24	0.92±0.19	1.09±0.09

Values are mean±S.E.M. (n=4-5) $^{a}p<0.05$, $^{b}p<0.01$ vs control; $^{\alpha}p<0.05$, $^{\beta}p<0.01$, $^{\gamma}p<0.001$vs AR 250 (One way ANOVA followed by Tukey's multiple comparison test).

Effect of AR on Electrolytes

There was no significant alteration in the serum sodium, potassium and bicarbonate concentrations of male rats by the end of the 30 days treatment and after the reversibility period, compared to the control group. A significant reduction of serum chloride by 10 mg/kg of AR compared to 250 mg/kg AR was however observed with the 30 days exposure (Table 9). In the female rats, there was no significant alteration in the serum electrolytes assayed in the 30 days treatment and reversibility test (Table 10).

Effect of AR on endogenous antioxidants

By the end of 30 days exposure, no significant alteration in the level of reduced glutathione (GSH), superoxide dismutase (SOD) or catalase was observed in the liver and kidney homogenates of AR treated male rats compared to control. In the reversibility test, a dose-dependent decrease in SOD significant at 250 mg/kg was observed in the kidney of male rats treated with AR. In the liver of these rats, AR (250 mg/kg) significantly increased GSH and SOD levels compared to control and AR (10 and 50 mg/kg); no significant alterations in catalase was observed (Table 11). After 30 days of exposure to AR (50 and 250 mg/kg), liver and kidney GSH, SOD and catalase of female rats were not significantly altered, relative to control. However, in the reversibility test, kidney SOD was significantly ($p<0.001$) reduced by AR (250 mg/kg) as it was in the male rats; while kidney and liver GSH and catalase were unaltered (Table 12).

Semen analysis

AR produced no significant alteration in morphology or motility in the 30 day exposure period and in the reversibility test period. However, significant reduction in sperm count was observed in the reversibility test in AR (10 and 250 mg/kg) treated rats (Table 13).

Table 9: Effect of AR on serum electrolytes of male rats

Serum electrolyte	**After 30 days treatment period**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
Sodium (mmol/L)	103.50±2.18	109.50±3.50	105.00±2.20	102.80±2.50	131.50±16.50	102.00±12.00	101.00±3.50	120.50±1.50
Potassium (mmol/L)	8.60±1.05	8.45±1.45	12.38±2.28	11.10±1.20	23.10±1.00	30.50±6.50	30.70±1.19	30.50±11.50
Chloride (mmol/L)	110.00±3.58	107.80±5.83$^{\alpha}$	122.40±4.76	138.30±11.01	91.00±5.00	77.50±4.50	79.33±0.33	86.50±1.50
Bicarbonate (mmol/L)	20.00±1.35	19.00±1.35	21.20±3.97	20.75±2.50	12.50±0.50	15.00±2.00	13.67±0.88	12.50±2.50

Values are mean±S.E.M. (n=4-5) $^{\alpha}p<0.05$ vs AR 250 (One way ANOVA followed by Tukey's multiple comparison test).

Table 10: Effect of AR on serum electrolytes of female rats.

Serum electrolyte	**After 30 days treatment period**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
Sodium (mmol/L)	96.50±2.40	105.50±4.03	99.40±1.33	84.25±12.24	117.00±1.00	102.50±17.50	162.30±13.12	123.00±4.00
Potassium (mmol/L)	14.45±1.68	10.68±1.99	7.12±1.99	7.23±2.12	19.10±6.10	28.25±5.05	39.13±14.68	20.65±3.65
Chloride (mmol/L)	127.80±15.12	127.00±12.70	133.20±5.68	120.0±4.88	88.00±2.00	90.50±5.50	96.00±4.72	91.00±2.00
Bicarbonate (mmol/L)	16.00±1.08	18.25±2.18	16.60±0.93	19.25±2.02	10.00±3.00	9.00±1.00	10.67±1.33	10.00±3.00

Values are mean±S.E.M. (n=4-5). $P<0.05$ (One way ANOVA followed by Tukey's multiple comparison test).

Table 11: Effect of AR on liver and kidney antioxidant indices of male rats

Antioxidant enzyme/ oxidative stress marker	**After 30 days treatment period**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
Liver glutathione (U/mg)	2.39±0.19	2.43±0.17	2.41±0.18	2.05±0.12	1.28±0.18	1.00±0.14$^{\gamma}$	1.37±0.01$^{\alpha}$	1.80±0.05^{a}
Liver superoxide dismutase (U/mg)	4.85±0.21	4.44±0.36	4.03±0.26	4.04±0.08	2.63±0.36	1.93±0.24$^{\gamma}$	2.83±0.07$^{\beta}$	4.06±0.07^{b}
Liver catalase (U/mg)	19.46±1.00	19.64±1.97	18.71±1.34	18.29±0.30	10.96±1.71	8.76±1.23	11.18±0.66	15.32±0.53
Kidney glutathione (U/mg)	1.92±0.16	2.02±0.20	2.09±0.42	1.49±0.26	2.09±0.20	2.04±0.04	2.30±0.08	2.00±0.07
Kidney superoxide dismutase (U/mg)	3.83±0.16	4.51±0.34	4.70±0.89	2.66±023	4.65±0.15	4.29±0.44	3.69±0.09	3.01±0.20^{b}
Kidney catalase (U/mg)	17.42±0.86	19.27±1.61	20.10±3.76	12.92±1.67	17.96±0.57	16.43±0.79	17.02±0.73	16.55±0.79

Values are mean±S.E.M. (n=4-5) $^{a}p<0.05$, $^{b}p<0.01$ vs control; $^{\alpha}p<0.05$, $^{\beta}p<0.01$, $^{\gamma}p<0.001$vs AR 250 (One way ANOVA followed by Tukey's multiple comparison test).

Table 12: Effect of AR on liver and kidney antioxidant indices in female rats

Haematologic parameter	**After 30 days treatment period**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**
Liver glutathione (U/mg)	2.17±0.20	2.13±0.05	2.14±0.12	1.91±0.06	0.67±0.34	1.20±0.09	1.19±0.04	3.55±2.35
Liver superoxide dismutase (U/mg)	4.01±0.12	3.73±0.14	3.63±0.27	3.33±0.07	2.03±0.20	2.17±0.15	2.36±0.14	2.44±0.05
Liver catalase (U/mg)	17.16±0.96	16.25±0.58	16.65±0.86	15.78±0.26	7.45±0.75	9.76±0.88	9.47±0.39	9.94±0.45
Kidney glutathione (U/mg)	1.50±0.17	1.49±0.17	2.01±0.13	1.89±0.05	2.13±0.13	2.05±0.19	2.41±0.06	2.08±0.07
Kidney superoxide dismutase (U/mg)	2.88±0.30	2.94±0.44	4.06±0.18	3.94±0.23	3.88±0.25	3.26±0.23	$3.72\pm0.13^{\gamma}$	2.38 ± 0.11^{c}
Kidney catalase (U/mg)	11.04±1.34	$14.94\pm2.30^{\beta}$	9.52±1.47	6.10±1.30	16.25±1.14	14.47±1.15	16.73±0.31	15.87±0.71

Values are mean±S.E.M. (n=4-5) $^{c}p<0.001$ vs control; $^{\beta}p<0.01$, $^{\gamma}p<0.001$vs. AR 250 (One way ANOVA followed by Tukey's multiple comparison test).

Table 13: Effect of AR on semen parameters

Parameter	**After 30 days exposure**				**After reversibility period**			
	Control (5 ml/kg)	**AR (10 mg/kg)**	**AR (50 mg/kg)**	**AR (250 mg/kg)**	**Control (5 ml/kg)**	**AR (10 mg/kg)**	**AR50 (50 mg/kg)**	**AR (250 mg/kg)**
Sperm count (million/ml)	55.31±6.82	36.13±7.47	45.25±1.91	34.69±4.46	61.25±1.25	41.88±3.13[a]	48.75±1.25	46.25±2.50[a]
Sperm morphology (%)	12.50±1.11	12.25±2.42	17.60±1.08	17.25±1.93	11.00±1.00	13.00±3.00	17.00±1.00	19.00±1.00
Sperm motility (%)	40.75±5.30	36.00±6.40	41.60±1.89	31.75±4.03	35.50±10.50	39.00±1.00	44.00±1.00	43.50±1.50

Values are mean±S.E.M. (n=4-5) [a]$p<0.05$ vs control (One way ANOVA followed by Tukey's multiple comparison test).

4.2. PHARMACOLOGICAL ACTIVITIES

4.2.1. Tests for antidiarrhoeal activity

4.2.1.1. Normal intestinal transit

The charcoal meal traversed the farthest distance of the total length of the small intestine, in the control group. AR (100-400 mg/kg) produced a significant dose-dependent decrease in the intestinal transit, the peak effect being observed at 400 mg/kg (Figure 11).

4.2.1.2. Castor oil-induced intestinal transit

AR (100-400 mg/kg) produced a significant dose-dependent inhibition of intestinal transit induced by castor oil. This inhibition of peristalsis peaked at 400 mg/kg with 65.3% inhibition, which is comparable to the 76.1% inhibition by 10 mg/kg morphine. Comparing the preventive and curative effects of AR, AR administration 15 minutes after castor oil (curative) produced an apparently lower effect (45.7% inhibition) on gut propulsion than AR administered 30 minutes before castor oil (65.3% inhibition) (Figure 12). The effect of AR (400 mg/kg) was not significantly influenced by isosorbide dinitrate (150 mg/kg) and yohimbine (1 mg/kg), but was significantly ($p<0.001$) inhibited by pilocarpine (10 mg/kg), phentolamine and propranolol (1 mg/kg) (Figure 13).

4.2.1.3. Castor oil-induced diarrhoea

After castor oil administration, the mice in the control group produced copious diarrhoea. Pretreatment with AR (100-400 mg/kg), however, caused significant dose-dependent delay in the onset of diarrhoea. It also produced significant dose-dependent decrease in the number and weight of wet stools relative to the control group (Table 14). The extract caused a significant dose-dependent reduction in diarrhoea score. The greatest antidiarrhoeal index *in vivo* ($ADI_{in\ vivo}$) of the extract was found to be 81.79% at 400 mg/kg, which is comparable to 86.85% $ADI_{in\text{-}vivo}$ produced by morphine (Table 15).

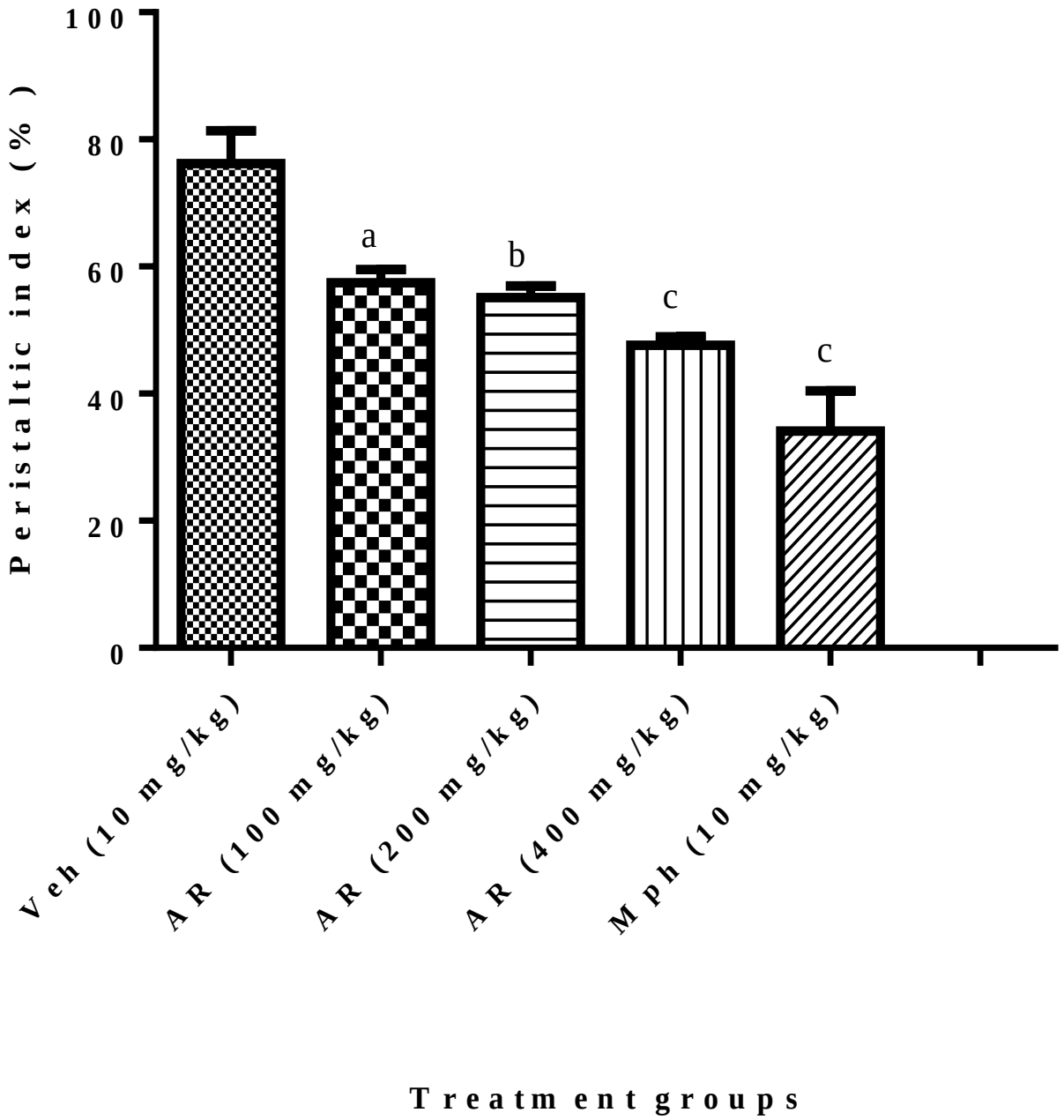


Figure 11: Effect of AR (100-400 mg/kg) on normal intestinal transit. Bars are mean values ± S.E.M. [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs. vehicle (One way ANOVA followed by Tukey's multiple comparison test). Veh (vehicle), AR (aqueous root extract of *A. ringens*), Mph (morphine)

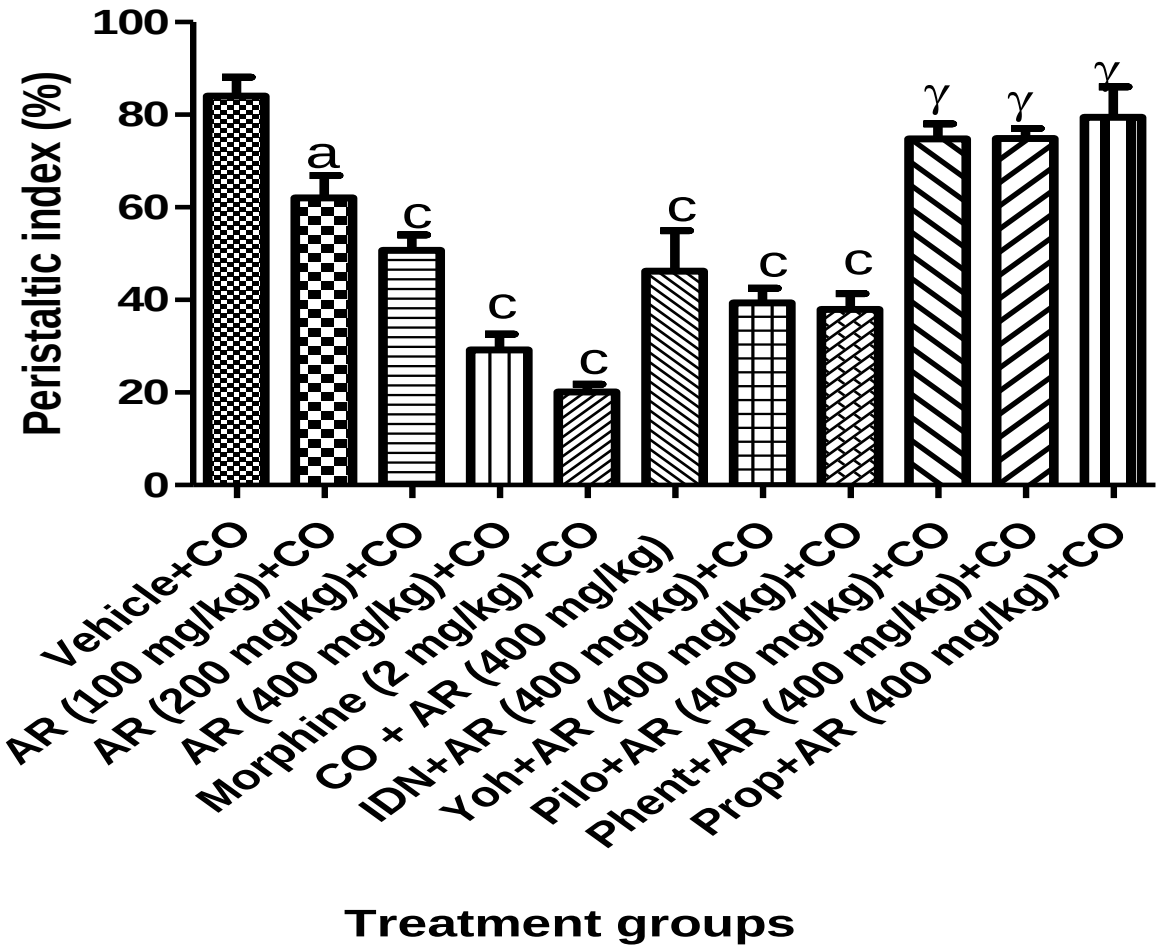


Figure 12: Effect of AR on castor oil (CO 0.2 ml per mouse) induced intestinal transit in the presence and absence of isosorbide dinitrate (IDN 150 mg/kg), yohimbine (Yoh), pilocarpine (Pilo 10 mg/kg), phentolamine (Phent 1 ml/kg) or propanolol (Prop 1 ml/kg). Bars are mean ± S.E.M. $^{a}p<0.05$, $^{c}p<0.001$ vs. vehicle, $^{\gamma}p<0.001$ vs. extract (400 mg/kg) + CO (One way ANOVA followed by Tukey's multiple comparison test).

Table 14: Effect of AR in castor oil-induced intestinal diarrhoea test in mice

Group	Dose (mg/kg)	Diarrhoea onset (min)	Number of wet stools	Total number of stools	Weight of wet stools (g)	Total weight of stools (g)	Diarrhoea score	Protection (%)
Vehicle (ml/kg)	10	49.80±4.25	12.20±0.37	14.00±0.45	0.24±0.03	0.27±0.03	33.00±1.70	-
AR	100	159.60±5.41[c]	3.80±0.58[c]	8.20±1.83[b]	0.08±0.01[c]	0.16±0.03[a]	13.60±2.24[b]	58.78
AR	200	187.20±3.65[c]	3.20±0.34[c]	6.60±1.12[c]	0.04±0.01[c]	0.15±0.02[b]	10.80±1.56[c]	67.27
AR	400	230.00±3.30[c]	1.40±0.24[c]	2.60±0.51[c]	0.02±0.01[c]	0.04±0.00[c]	5.00±0.44[c]	84.84
Morphine	2	234.80±3.81[c]	1.40±0.40[c]	1.80±0.49[c]	0.01±0.00[c]	0.03±0.01[c]	3.40±1.07[c]	89.60

Values are mean ± S.E.M (n=5). [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs. control (One way ANOVA followed by Tukey's multiple comparison test)

Table 15: The *in vivo* antidiarrhoeal index ($ADI_{in\ vivo}$) of AR

Group	**Dose (mg/kg)**	**Diarrhoeal onset (minute)**	**D_{freq}**	**Peristaltic index (%)**	**G_{meq}**	**Number of wet stools**	**P_{freq}**	**$ADI_{in\ vivo}$**
Vehicle	10 (ml/kg)	49.80±4.25	-	84.08	-	12.20±0.37	-	-
AR	100	159.60±5.41[c]	57.73	62.00	26.26	3.80±0.58[c]	68.85	47.08
AR	200	187.20±3.65[c]	72.24	50.68	39.72	3.20±0.34[c]	73.77	59.60
AR	400	230.00±3.30[c]	94.74	29.22	65.25	1.40±0.24[c]	88.52	81.79
Morphine	10	234.80±3.81[c]	97.27	20.10	76.09	1.40±0.40[c]	88.52	86.85

Values are mean ± SEM (n=5). [c]P<0.001 vs. control (One way analysis of variance followed by Tukey's multiple comparison test).

4.2.1.4. Castor oil-induced enteropooling

Oral administration of castor oil produced intestinal fluid volume of 2.28±0.45 ml. Pretreatment of rats with the extract (400 mg/kg, p.o.) 1 hour before castor oil administration decreased the weight and volume of intestinal content, but this effect was not significant (Table 16).

4.2.1.5. Gastric emptying

The extract (400 mg/kg) reduced the quantity of test meal emptied in 1 hour relative to control, but this effect was not significant (Table 17) within the period of observation.

4.2.2. Test for antiinflammatory activity

4.2.2.1. Carrageenan-induced inflammation

AR (10-100 mg/kg) significantly reduced the extent of increase in rat paw size. A peak effect of 57.10% was observed by the 6^{th} hour at 25 mg/kg of the extract. This was comparable to the 57.90% peak reduction by indomethacin (10 mg/kg) at the 3^{rd} hour, by which time AR (50 mg/kg) produced a comparable 52.60% reduction in paw size increase (Table 18).

4.2.2.2. Egg albumin-induced rat paw oedema

The extract (10-50 mg/kg) produced a time and dose-dependent significant (at the 2^{nd} hour) reduction in the paw size increase induced by egg albumin. Its peak effect of 65.60% was observed by the 3^{rd} hour at 50 mg/kg. This was comparable to the 63.90% peak reduction in paw size by diclofenac (10 mg/kg) (Table 19).

Table 16: Effect of AR on Castor oil induced enteropooling.

Group	**Dose (mg/kg)**	**Weight of intestinal content (g)**	**Volume of intestinal content (ml)**
Vehicle	10 (ml/kg)	1.95 ±0.26	2.28±0.45
AR	400	1.54±0.21	1.40±0.12

Values are mean ± S.E.M. (n= 5). P<0.05 (Unpaired Student's t test).

Table 17: Effect of AR on gastric emptying.

Treatment	**Dose (mg/kg)**	**Difference between full and empty stomach weight (g)**	**Quantity emptied (g)**
Vehicle	10 (ml/kg)	1.31±0.13	1.74±0.13
AR	400	1.48±0.17	1.57±0.17

Values are mean ± S.E.M (n= 5). P<0.05 (Unpaired Student's t test).

Table 18: Effect of AR on carrageenan-induced rat paw oedema.

Treatment Groups	**Dose (mg/kg)**	**Increase in paw sizes (cm)**					
		1st hour	**2nd hour**	**3rd hour**	**4th hour**	**5th hour**	**6th hour**
Vehicle	10 (ml/kg)	0.40±0.04	0.51±0.06	0.56±0..06	0.57±0.06	0.46±0.06	0.35±0.06
AR	10	0.30±0.04 (25.00)	0.43±0.06 (15.70)	0.41±0.04 (26.80)	0.38±005 (33.30)	0.24±0.02[b] (47.80)	0.17±0.02[a] (51.40)
AR	25	0.25±0.02 (37.50)	0.39±0.04 (23.50)	0.37±0.02 (33.9)	0.28±0.03[b] (50.9)	0.23±0.02[b] (50.00)	0.15±0.02[a] (57.10)
AR	50	0.25±0.02 (37.50)	0.26±0.03 (49.00)	0.29±0.06 (48.20)	0.27±0.02[b] (52.60)	0.23±0.03[b] (50.00)	0.15±0.03[a] (57.10)
AR	100	0.38±0.05 (05.00)	0.47±0.07 (08.00)	0.37±0.05 (33.90)	0.31±0.04[b] (45.60)	0.33±0.04 (28.30)	0.29±0.03 (17.14)
Indomethacin	10	0.35±0.05 (12.50)	0.33±0.08 (54.50)	0.34±0.13 (39.3)	0.24±0.06[c] (57.9)	0.28±0.05[a] (39.10)	0.22±0.0 (37.10)

Values are mean ± SEM. (n=5) [a]$p<0.05$, [b]$p<0.01$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test). Values in parenthesis are percentage inhibition.

Table 19: Effect of AR on egg albumin-induced rat paw oedema.

Treatment Group	**Dose (mg/kg)**	**Increase in paw size (cm)**		
		1st hour	**2nd hour**	**4th hour**
Vehicle	10 (ml/kg)	0.88±0.10	0.77±0.08	0.61±0.08
AR	10	0.79±0.08 (10.20)	0.57±0.01[a] (26.00)	0.41±0.06 (32.70)
AR	25	0.73±0.05 (17.05)	0.51±0.04[b] (33.80)	0.33±006[a] (45.90)
AR	50	0.54±0.02[b] (38.60)	0.45±0.02[c] (41.50)	0.21±0.02[c] (65.60)
AR	100	0.62±0.05 (29.50)	0.50±0.03[b] (35.10)	0.21±0.02[c] (65.60)
Diclofenac	10	0.63±002 (28.40)	0.53±0.05[a] (31.20)	0.22±0.03[c] (63.90)

Values are mean ± SEM (n=5) [a]$p<0.05$,. [b]$p<0.01$, [c]$p<0.001$ vs. control (One way analysis of variane followed by Tukey's multiple comparison test). Values in parenthesis are percentage inhibition.

4.2.2.3. Formalin-induced rat paw oedema

The extract (10-100 mg/kg), like diclofenac, showed significant ($p<0.001$) reduction in the extent of increase of rat paw size. A dose-dependent reduction was observed at 10-100 mg/kg, with a peak effect of 50.00% at 50 mg/kg. This peak effect was greater than the 40.80% reduction by diclofenac (10 mg/kg) (Table 20).

4.2.2.4. Xylene-induced ear oedema

AR (25-50 mg/kg) significantly reduced the extent of ear size increase in mice. The peak effect of 84.78% reduction produced by the extract (50 mg/kg) was greater than the 65.21% reduction by dexamethasone (1 mg/kg) (Table 21).

4.2.2.5. Prostaglandin inhibition via castor oil-induced diarrhoea test

The extract (50 mg/kg), like diclofenac (10 mg/kg), produced a significant ($p<0.001$) delay in the onset of diarrhoea induced by castor oil. AR and diclofenac also significantly reduced the diarrhoea score (Table 22).

4.2.3. Analgesic activity

4.2.3.1. Acetic acid–induced mouse writhing

AR (10-50 mg/kg) caused significant reduction in the number of writhes by the mice. Its peak reduction in the number of writhes of 37.77%, which was observed at 50 mg/kg, was comparable to the 39.95% reduction by diclofenac (5 mg/kg) (Figure 13). Naloxone (1 mg/kg) and glibenclamide (2 mg/kg) significantly inhibited the effect of AR, while haloperidol exerted no significant effect on the action of AR in the study (Figure 14).

Table 20: Effect of AR on formalin-induced rat paw oedema.

Treatment groups	**Dose (mg/kg)**	**Increase in paw size (cm)**	**% Inhibition**
Vehicle	10 (ml/kg)	1.20±0.03	-
AR	10	0.94±0.03[c]	21.70
AR	25	0.84±0.03[c]	30.00
AR	50	0.60±0.03[c]	50.00
AR	100	0.78±0.03[c]	35.00
Diclofenac	10	0.71±0.03[c]	40.80

Values are mean ± SEM (n=5). [c]$p<0.001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test).

Table 21: The effect of AR on xylene-induced ear oedema

Treatment Group	**Dose (mg/kg)**	**Increase in ear weight (g)**	**% Inhibition**
Vehicle	10 (ml/kg)	0.046±0.012	-
AR	10	0.026±0.004	43.40
AR	25	0.018±0.004[a]	60.86
AR	50	0.007±0.002[c]	84.78
AR	100	0.013±0.006[b]	71.74
Dexamethasone	1	0.016±0.002[a]	65.21

Values are mean ± SEM (n=5) [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test).

Table 22: Effect of AR in the test for delay in castor oil-induced diarrhoea (prostaglandin inhibition assay).

Treatment groups	**Dose (mg/kg)**	**Diarrhoea onset**	**Diarrhoea score**
Vehicle	10 (ml/kg)	50.17±3.49	30.67±3.83
AR	50	94.50±4.97[c]	20.17±2.17[a]
Diclofenac	5	235.30±4.67[c]	1.83±0.98[c]

Values are mean ± SEM (n=6). [a]$p<0.05$, [c]$p<0.001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test).

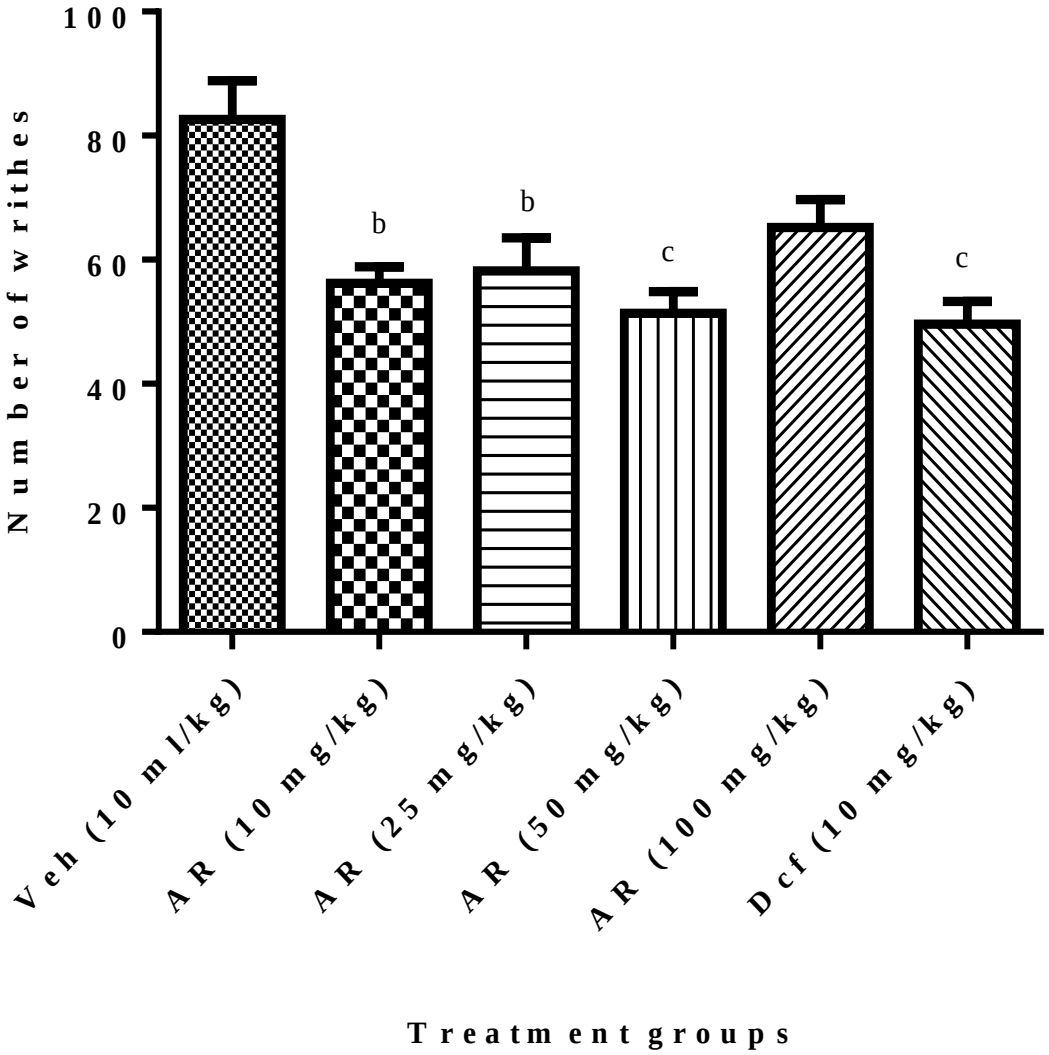


Figure 13: Effect of AR in acetic acid-induced mouse writhing test. Bars are mean ± SEM (n=5). $^{b}p<0.01$, $^{c}p<0001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test). Veh (vehicle), AR (aqueous root extract of *A. ringens*), Dcf (diclofenac)

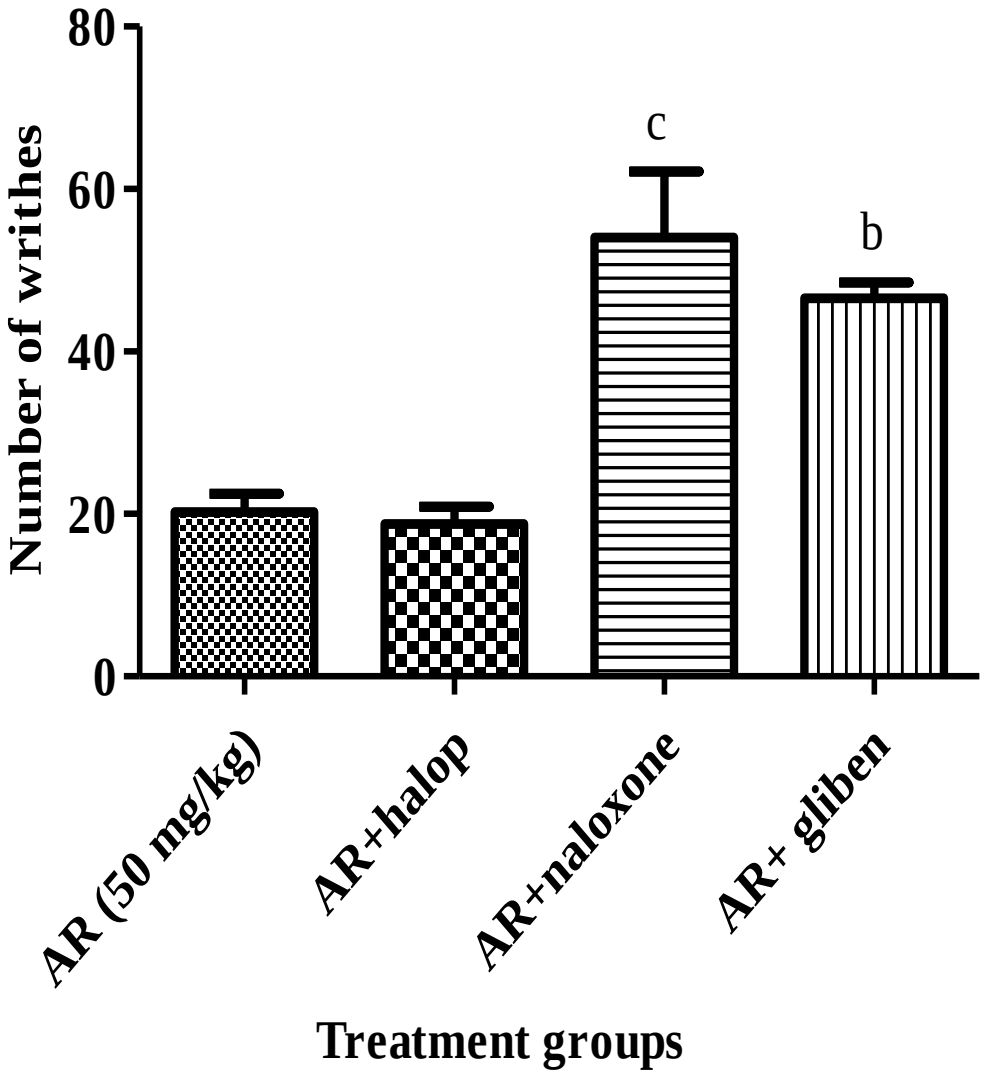


Figure 14: Effect of AR (aqueous root extract of *A. ringens*) (50 mg/kg) in the absence and presence of halop (haloperidol), naloxone, and gliben (glibenclamide) in acetic acid-induced mouse writhing test. Bars are mean ± SEM (n=5). $^{b}p<0.01$, $^{c}p<0001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test).

4.2.3.2. Acetylcholine-induced mouse writhing

A significant reduction in the number of writhes was produced by the extract (10-50 mg/kg) and diclofenac (5 mg/kg). This effect of the extract peaked at 50 mg/kg, with a 70.0% reduction. A reduction of 92.5% by diclofenac was also observed (Figure 15).

4.2.3.3. Formalin-induced pain test

The extract (10-100 mg/kg) produced significant dose-dependent reduction in the cumulative time spent in paw licking and biting reaction by the mice in both phases of the test. This effect peaked at 50 mg/kg with a 63.58% reduction, which was greater than the 40.66% reduction produced by 5 mg/kg diclofenac in the first phase. In the second phase of the test, the effect of the extract also peaked at 50 mg/kg reducing reaction time by 58.69%. This was also greater than the 33.31% reduction by diclofenac (5 mg/kg) (Table 23).

4.2.3.4. Tail clip test

AR (25-50 mg/kg) and morphine (10 mg/kg) significantly increased the time taken for mice to react to the pain due to the pressure induced by the clip. The peak effect of the extract was observed at 25 mg/kg; the pain threshold of the mice was increased by up to 12.7 seconds, unlike the control group that had reacted by 2.8 seconds (Figure 16).

4.2.3.5. Hole board test

AR (25-50 mg/kg) significantly ($p<0.001$) decreased the number of head dips in the hole board test. This effect, which peaked at 50 mg/kg (60% reduction) was found to be greater than that of diazepam (47% reduction) (Figure 17).

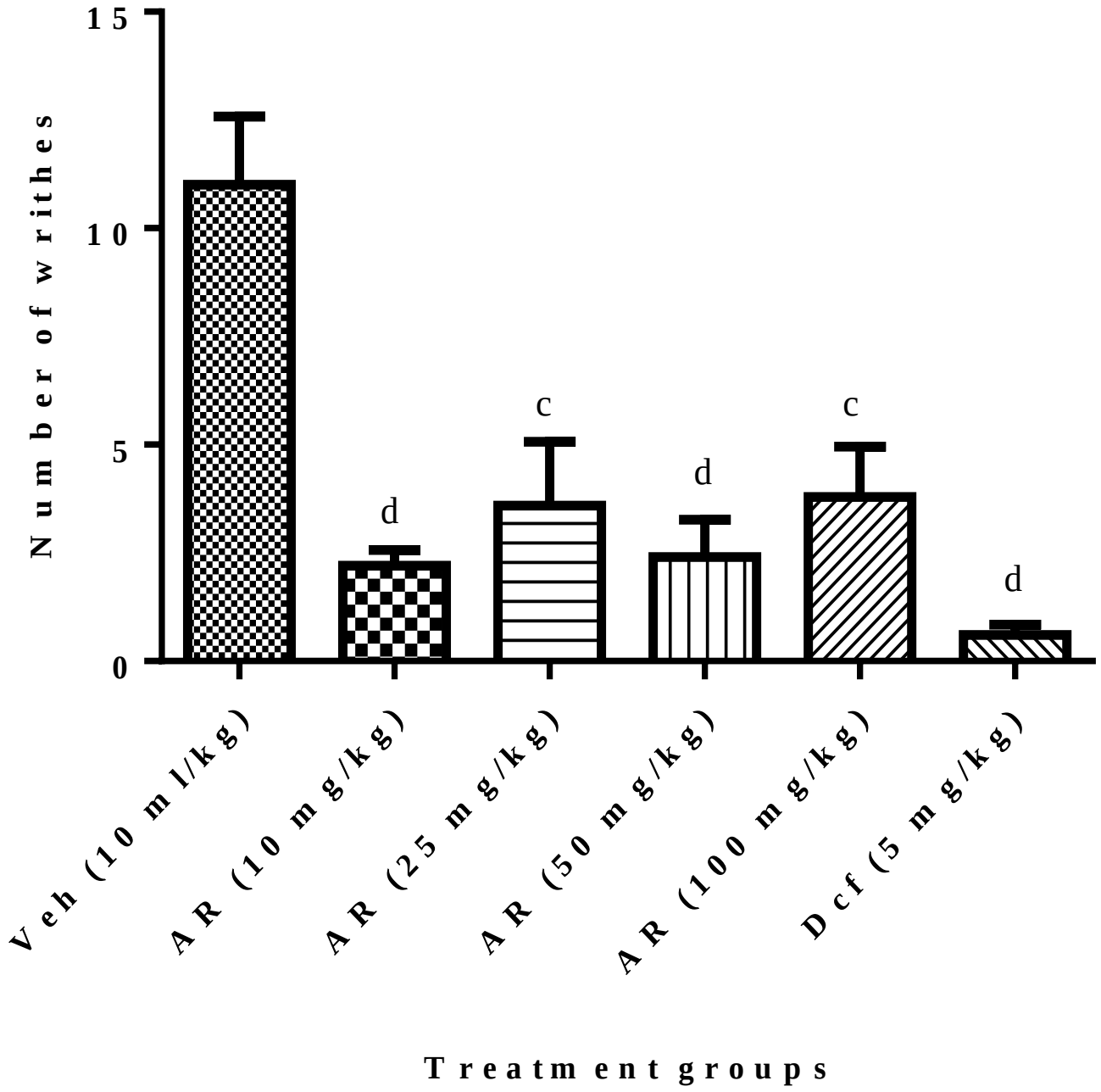


Figure 15: Effect of AR in acetylcholine-induced mouse writhing test. Bars represent mean ± SEM (n=5). $^{c}p<0.001$, $^{d}p<0.0001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test).

Table 23: The effect of AR on formalin-induced pain

Treatment groups	**Dose (mg/kg)**	**Reaction time (s)**			
		0-5 minutes	**% inhibition**	**15-30 minutes**	**% Inhibition**
Vehicle	5 (ml/kg)	103.80±7.12	-	128.70±5.64	-
AR	10	64.40±5.64[b]	37.96	86.83±9.71[b]	32.53
AR	25	49.80±7.58[c]	52.02	84.67±4.53[c]	34.21
AR	50	37.80±5.81[c]	63.58	53.17±3.51[c]	58.69
AR	100	42.00±5.07[c]	59.54	67.17±4.77[c]	47.81
Diclofenac	5	61.60±8.12[b]	40.66	85.83±7.67[c]	33.31

Vaules are mean ± SEM (n=6). [b]$p<0.01$, [c]$p<0.001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test).

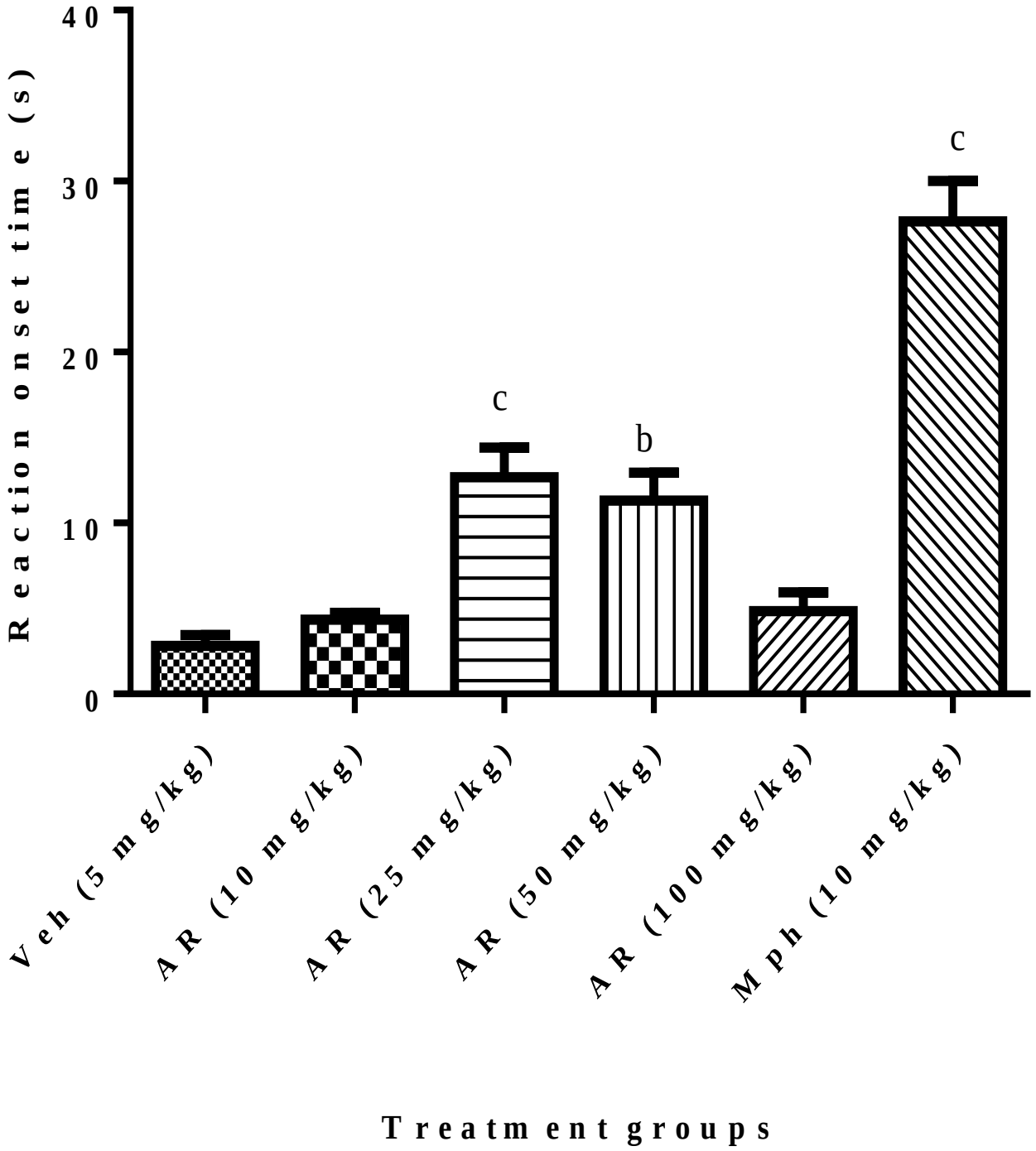


Figure 16: Effect of AR in tail clip test in mice. Bars represent mean ± SEM. [b]$p<0.01$, [c]$p<0.001$ vs control (One way analysis of variance followed by Tukey's multiple comparison test). Veh (vehicle), AR (aqueous root extract of *A. ringens*), Mph (morphine)

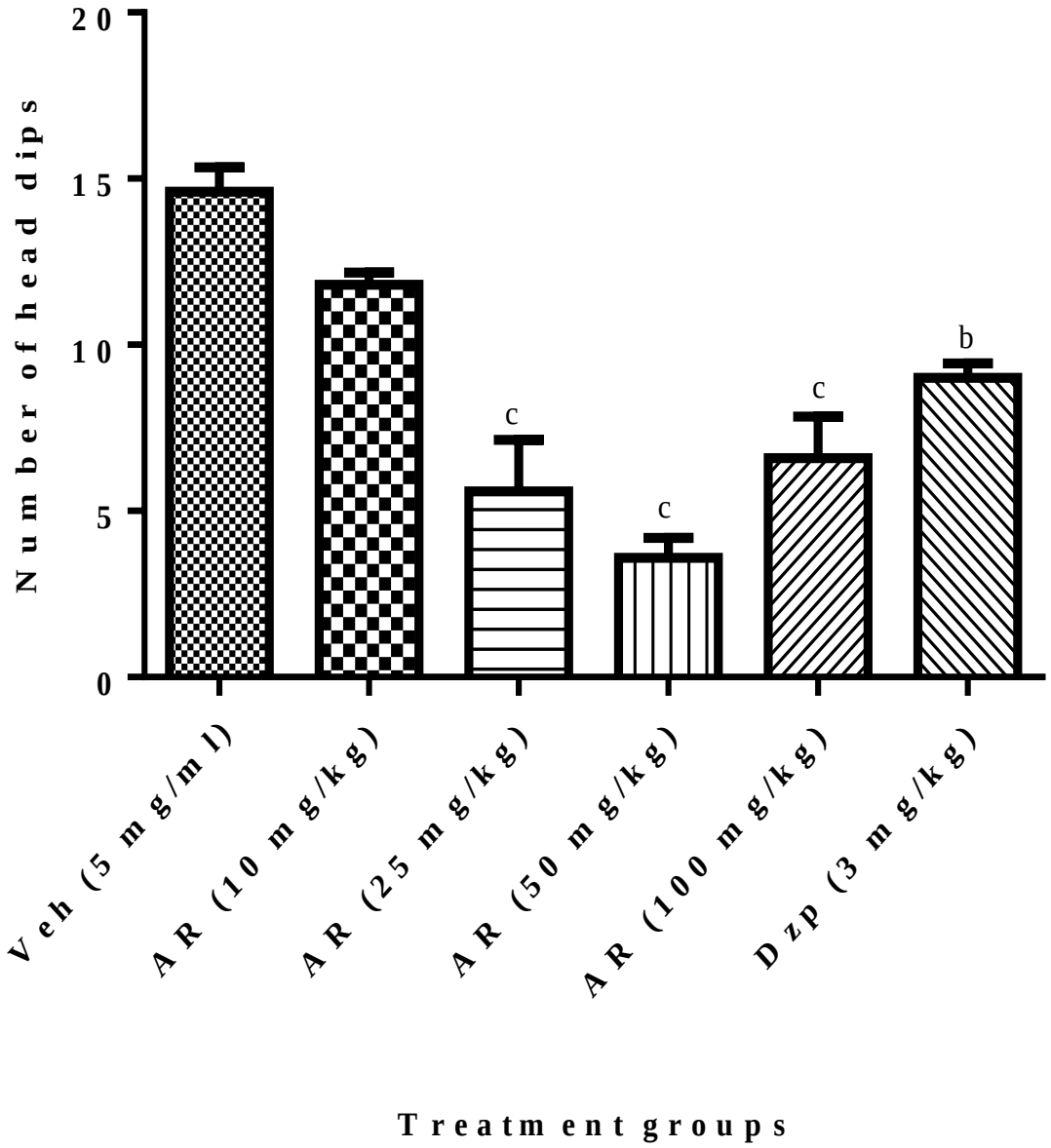


Figure 17: Effect of AR in the hole board test in mice. Values are mean±SEM, $^{b}p<0.01$ $^{c}p<0.001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test). Veh (vehicle), AR (aqueous root extract of *A. ringens*), Dzp (diazepam).

4.2.3.6. Open field test

In the open field test, AR (50 mg/kg) significantly ($p<0.05$) reduced the number of sectional crossings by mice. This effect was greater than that observed with diazepam (3 mg/kg) treated mice (Figure 18).

4.2.3.7. Hexobarbitone-induced sleep test

In the hexobarbitone-induced sleep test, AR (100 mg/kg) significantly ($p<0.001$) increased the duration of sleep. It however produced no significant effect on the onset of sleep (Figure 19).

4.2.4. Effect of AR on some haemodynamic parameters

4.2.4.1. Effect of 21 days treatment ofAR on blood pressure and heart rate of SHRs

Administration of AR (25 and 50 mg/kg) orally for 21 dayssignificantly reduced systolic and diastolic blood pressure in male spontaneously hypertensive rats. The peak reduction of systolic and diastolic blood pressure by AR at 50 mg/kg was observed by the 21st day. This reduction from 165±6.3/124±3.3 mmHg (blood pressure before treatment i.e. at day 0) to 131±1.8/91±3.7 mmHg was comparable to the reduction from 157±7.2/118±5.3 mmHg to 126±8.9/86±6.7 mmHg produced by enalapril (3 mg/kg). The effect of the extract was evident by the 7th day of administration and continued through the 14th and 21st day of exposure. No significant alteration in heart rate by AR was observed except on the 14th day of the study when a reduction from 401±15 to 337±07 beats per minute was observed in SHRs receiving AR (50 mg/kg) (Table 24).

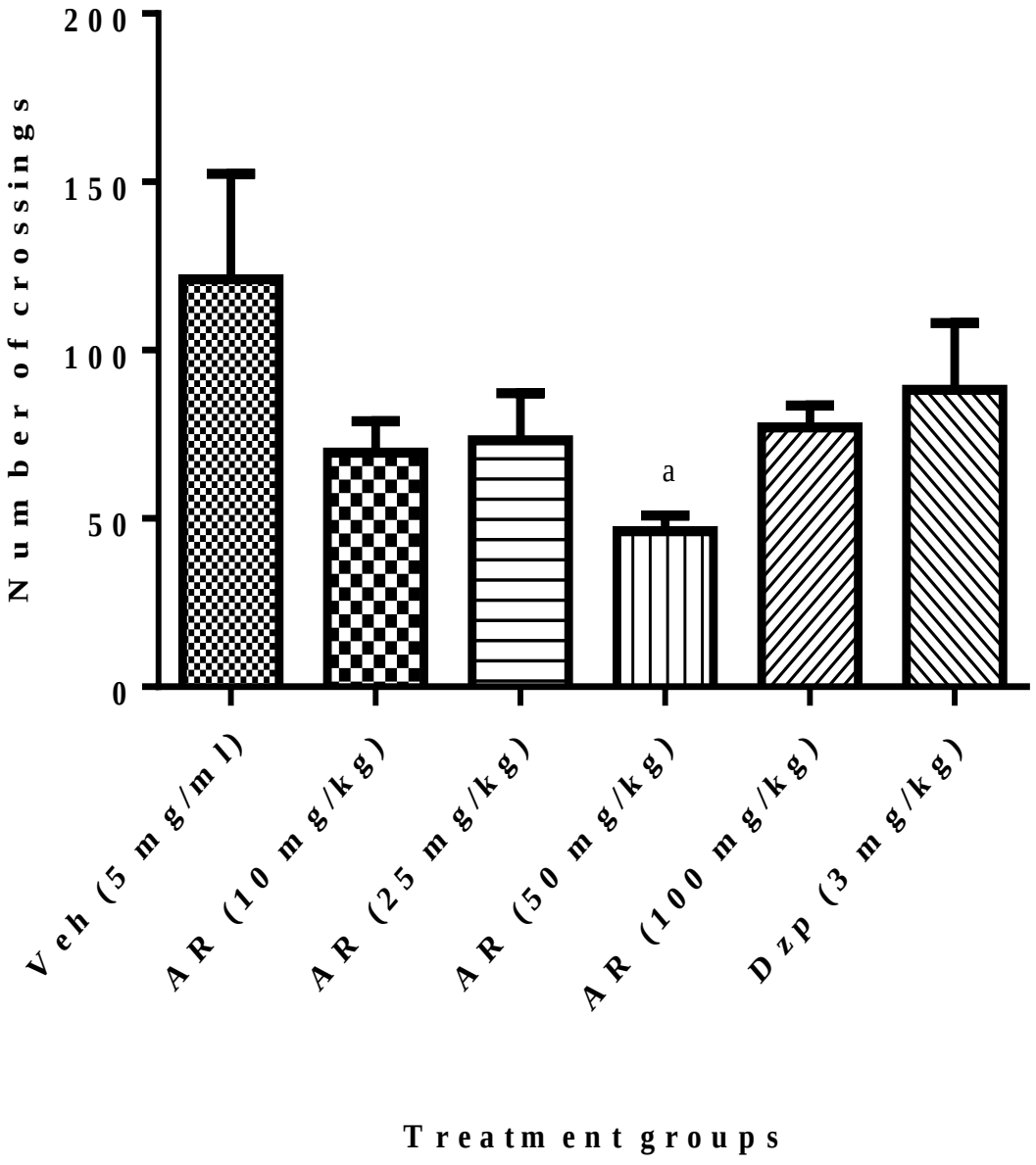


Figure 18: Effect of AR in the open field test in mice. Values are mean±SEM, $^{a}p<0.05$ vs control (One way analysis of variance followed by Tukey's multiple comparison test). Veh (vehicle), AR (aqueous root extract of *A. ringens*), Dzp (diazepam)

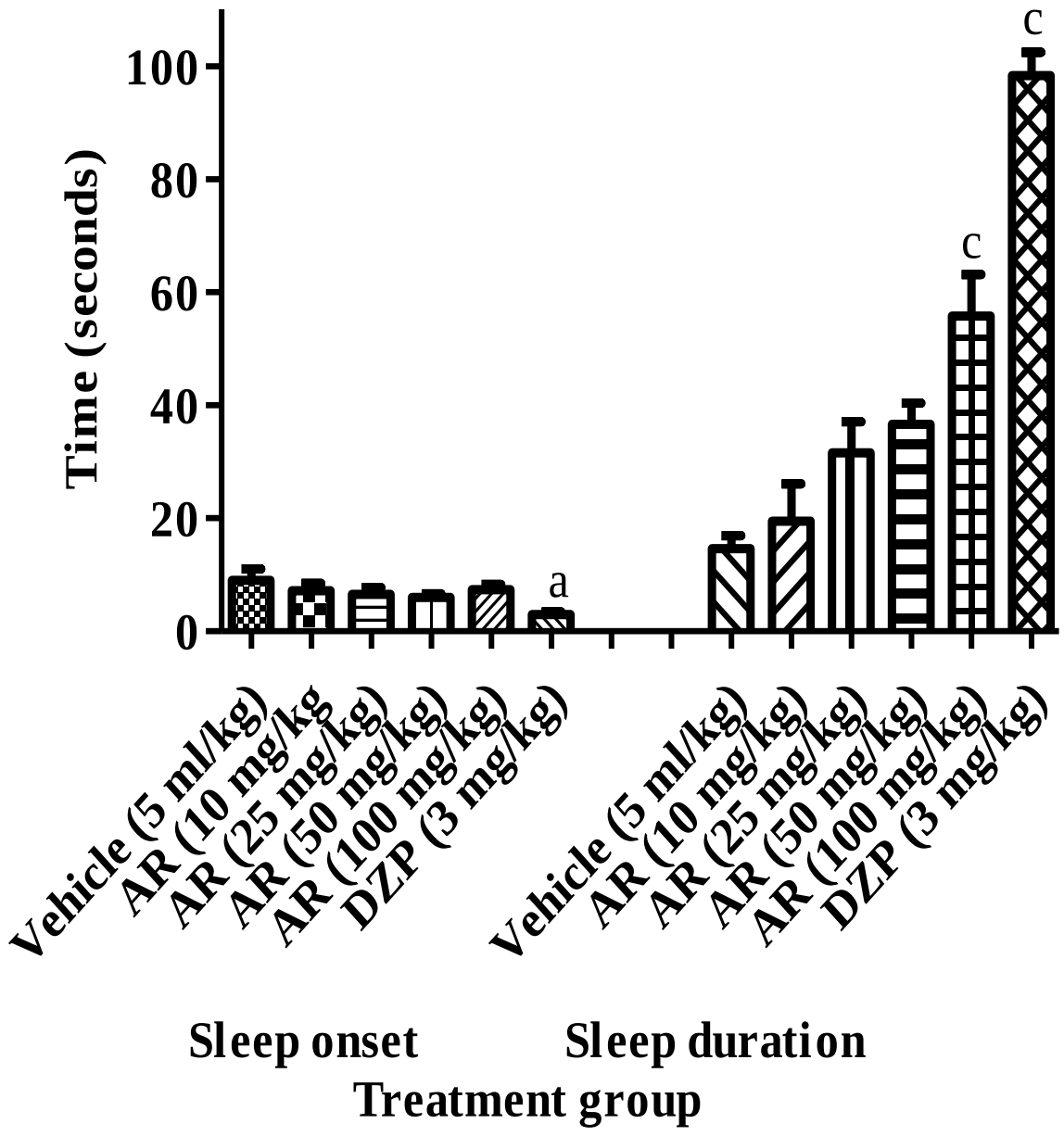


Figure 19: Effect of AR in hexobarbitone-induced sleep test. Values are mean ± SEM. (n=5) $^{a}p<0.05$, $^{c}p<0.001$ compared to control (One way analysis of variance followed by Tukey's multiple comparison test). DZP-diazepam.

Table 24: Effect of 21 days treatment of AR on blood pressure and heart rate of SHRs

Group	**Dose (mg/kg)**	**Day 0**	**Day 7**	**Day 14**	**Day 21**
Systolic blood pressure (mmHg)					
Control	10 (ml/kg)	161.2±2.9	168.4±0.8	168.7±4.1	157.6±4.2
AR	25	165.6±3.0	149.5±3.1	145.9±2.6[a]	137.0±3.9
AR	50	165.1±6.3	148.5±4.6[a]	140.0±7.3[c]	131.6±1.8[c]
Enalapril	3	157.0±7.2	133.6±3.9[d]	135.0±4.4[d]	137.1±8.9[a]
Diastolic blood pressure (mmHg)					
Control	10 (ml/kg)	117.9±1.9	125.8±1.7	128.7±1.0	115.2±3.5
AR	25	124.0±3.5	108.0±1.0[b]	105.3±2.6[c]	98.1±3.2[a]
AR	50	124.2±3.3	104.2±1.9[c]	106.6±4.0[c]	91.0±3.7[d]
Enalapril	3	118.1±5.3	94.3±4.7[d]	99.3±3.5[d]	86.1±6.7[d]
Heart rate (BPM)					
Control	10 (ml/kg)	404±28	367±06	402±24	434±34
AR	25	382±10	358±11	358±07	374±10
AR	50	401±15	391±09	337±07[a]	372±07
Enalapril	3	410±20	382±17	397±17	425±27

Vaules are mean ± SEM. [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$, [d]$p<0.0001$ vs. control (Two way analysis of variance followed by Bonferoni's post hoc test).

4.2.4.2. Effect of AR on urine volume and electrolytes

In the assay to determine the effect of AR on urine volume and electrolytes, it was observed that AR produced no significant change in 24 hours urine output of SHRs on days 7, 14 and 21 of AR exposure. No significant changes in sodium and potassium concentrations of these urine samples were observed (Table 25).

4.2.4.3. Effect of acute administration of AR on blood pressure and heart rate of SHRs

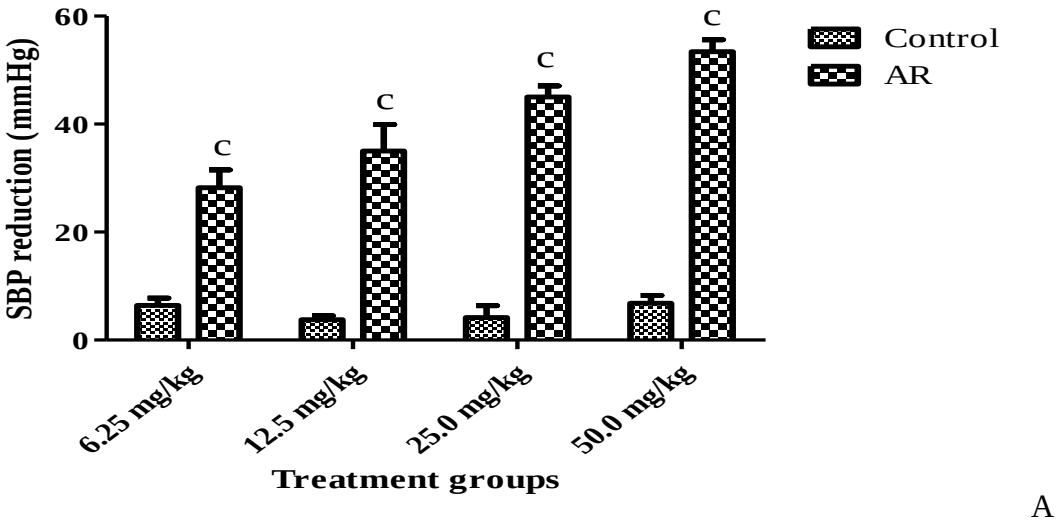
In the experiment to determine the effect of acute intravenous exposure of SHRs to AR (6.25-50 mg/kg), significant dose-dependent reductions in systolic and diastolic blood pressures were observed in SHRs. The greatest effect was observed at 50 mg/kg with reductions of 53.4±2.2 and 49.2±2.8 mmHg in systolic and diastolic blood pressures respectively. A dose-dependent reduction in heart rate, significant at 25 and 50 mg/kg was also observed with intravenous exposure of SHRs to AR. The extract (50 mg/kg) reduced heart rate by 14±2.3 beats per minutes (Figure 20). The reduction of blood pressure and heart rate by AR (50 mg/kg) was significantly inhibited by hexamethonium (20 mg/kg) and atropine (1 mg/kg). Hexamethonium inhibited AR-induced reduction in systolic blood pressure, diastolic blood pressure and heart rate by 61%, 51.6% and 69.6% respectively. Atropine also inhibited AR-induced reduction of these parameters by 66.5%, 61.9% and 73.9% respectively (Figure 21).

In the aspect of the study to evaluate the effect of intravenously administered AR and its fractions, butanol and aqueous fractions significantly reduced blood pressure and heart rate of SHRs. The butanol fraction produced the greatest reduction, with systolic and diastolic blood pressure reductions by 67±3.8 and 68.4 mmHg respectively at 25 mg/kg and heart rate reduction by 40±7 beats per minute at 50 mg/kg. The reduction of these haemodynamic parameters observed with the aqueous fraction was comparable to that of the extract. The chloroform fraction on the other hand, produced no significant effect on these parameters (Figure 22).

Table 25: Effect of AR on urine volume and electrolytes of SHRs

Group	**Dose (mg/kg)**	**Day 0**	**Day 7**	**Day 14**	**Day 21**
Urine volume (ml)					
Control	10 (ml/kg)	5.88±1.25	8.36±1.68	11.10±4.50	7.12±1.17
AR	25	6.10±0.73	7.20±2.03	8.100±3.04	7.90±2.06
AR	50	5.40±0.80	6.80±1.00	6.70±1.64	5.90±0.60
Urine sodium (nM/l)					
Control (ml/kg)	10	165.5±30.3	129.3±0.74	128.5±13.2	130.8±18.4
AR	25	165.0±40.9	137.8±20.4	134.6±14.3	155.00±5.40
AR	50	155.5±12.6	138.8±07.1	131.5±19.8	121.0±15.1
Urine potassium (mM/l)					
Control (ml/kg)	10	489.4±24.0	269.3±25.5	337.3±122.2	395.0±33.5
AR	25	393.3±29.4	250.0±57.4	282.3±22.3	318.8±25.1
AR	50	387.7±59.4	331.0±35.4	2493±62.1	276.8±31.1

Vaules are mean±SEM. P<0.05 (Two way analysis of variance followed by Bonferoni's post hoc test).



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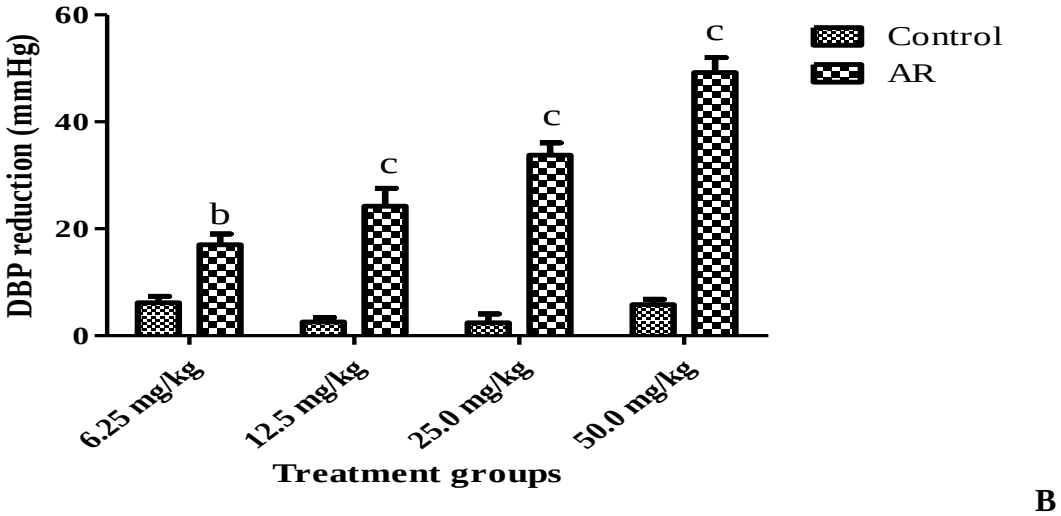

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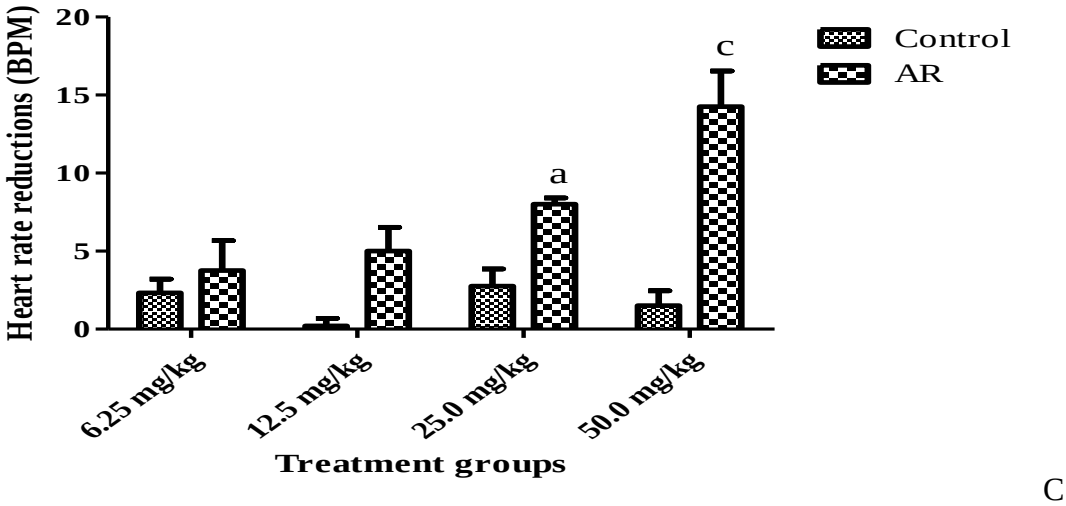

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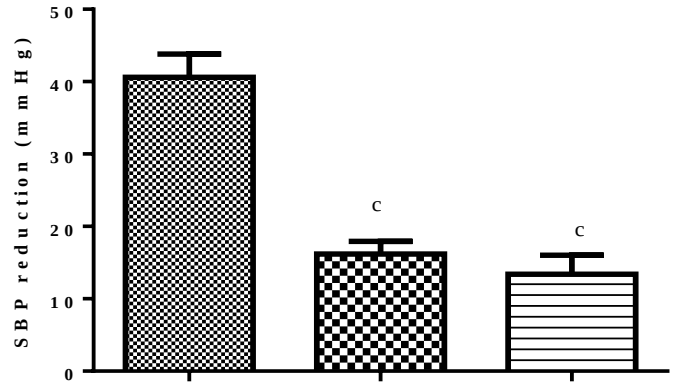
Figure 20: Effect of acute administration of AR on systolic blood pressure (A), diastolic blood pressure (B) and heart rate (C). Bars represent mean ± SEM. [a]p<0.05, [b]p<0.01, [c]p<0.001 vs. control (Two way analysis of variance followed by Bonferroni post test)



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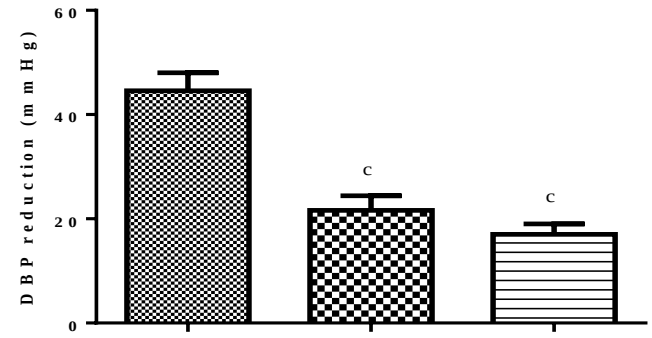

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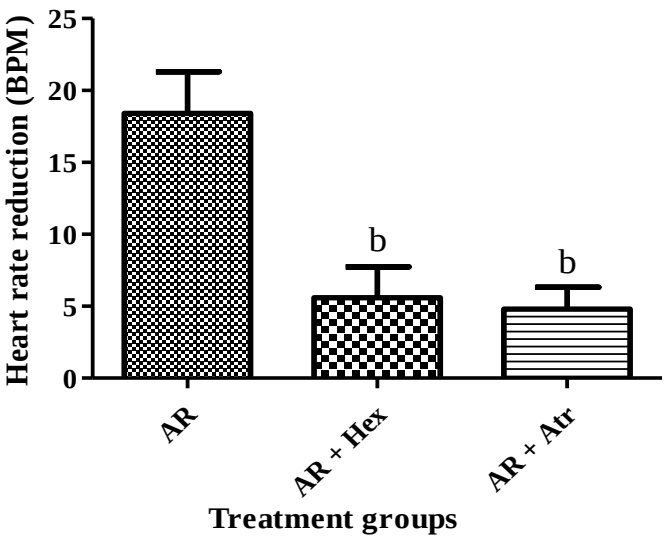

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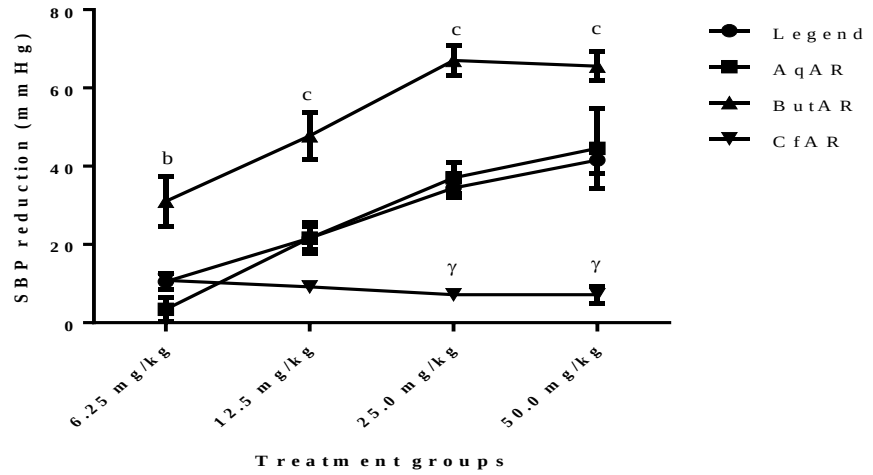
Figure 21: Effect of AR on systolic blood pressure (A), diastolic blood pressure (B) and heart rate (C) in the absence and presence of 20 mg/kg hexamethonium (Hex) and 1 mg/kg atropine (Atr). Bars represent mean ± SEM.$^{b}p<0.01$, $^{c}p<0.001$ vs. control (One way analysis of variance followed by Tukey's multiple comparison test).



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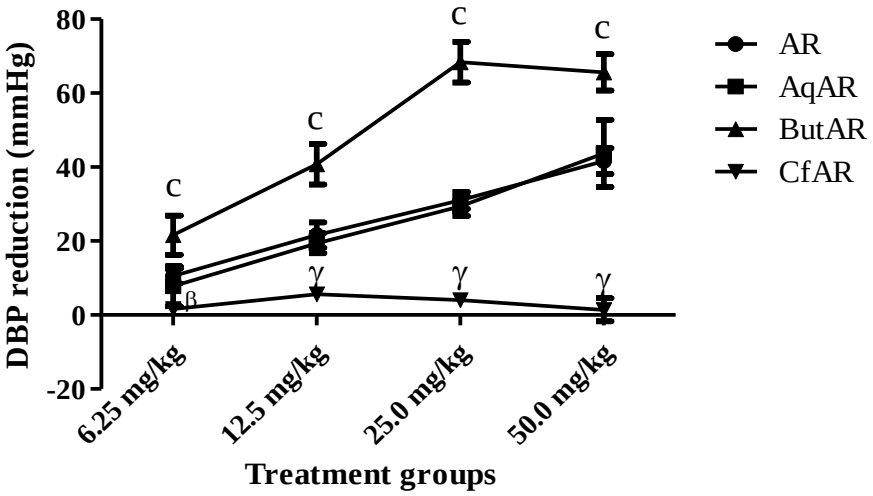

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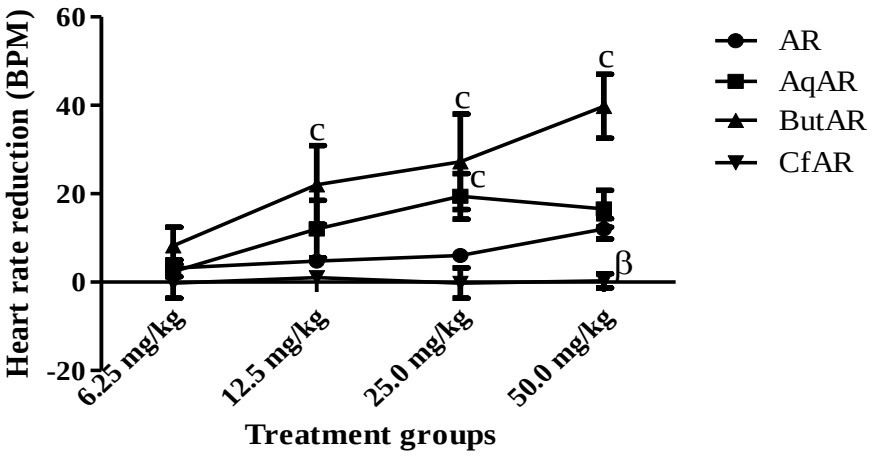

C

Figure 22: The effect of AR and fractions on systolic blood pressure (A), diastolic blood pressure (B) and heart rate (C). Lines represent mean ±S.E.M. $^{b}p<0.01$, $^{c}p<0.001$ vs. control; $^{\beta}p<0.01$, $^{\gamma}p<0.001$ vs. AR (Two way ANOVA followed by Tukey's multiple comparison test). AR-aqueous root extract of *A ringens*, AqAR-aqueous fraction of AR, ButAR-butanol fraction of AR, CfAR-chloroform fraction of AR.

4.3. PHYTOCHEMICAL INVESTIGATIONS

4.3.1. Preliminary phytochemical screening

The preliminary phytochemical screening of AR revealed the presence of oil, alkaloids, saponins, reducing sugars, tannins and phlobatannins (Table 26).

4.3.2. Quantitative determination of total tannins, flavonoids and phenolics in AR and its fractions

In the study to determine the concentration of these components in AR and its fractions, it was observed that the aqueous root extract of AR contains 26.00±0.00 mg/g tannic acid equivalent, 48.00±0.01 mg/g quercetin equivalent and 222.00±0.00 mg/g gallic acid equivalent of tannins, flavonoids and phenolics respectively. The chloroform fraction of AR had the highest content of tannins (130.00±0.03 mg/g tannic acid equivaent), while the butanol fraction contained the most flavonoids (80±0.01 mg/g quercetin equivalent) and phenolics (316.00±0.00 mg/g gallic acid equivalent) (Table 27).

4.3.3. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) assay

In this study, AR and its fractions (0.0005-0.5 mg/ml) scavenged free radicals. AR produced 65% inhibition of free radical generation at 0.5 mg/ml. Of the fractions of AR tested, the butanol fraction showed the greatest inhibition (77% inhibition) at 0.5 mg/ml; this action was greater than that of the reference flavonoid, quercetin (67% inhibition), at the same concentration (Figure 23).

Table 26: The result of preliminary phytochemical screening of AR

Test	**Observation**	**Inference**
Test for oil: Few drops of AR placed on filter paper and allowed to evaporate	Translucence observed	Presence of oil detected
Test for alkaloids: 0.5 g of extract added to 5 ml of 10% $HCl_{(aq)}$ was warmed on steam. 1 ml of filtrate added to few drops of Meyer's reagent	Light yellow colouration with white precipitate	Alkaloids detected
Test for saponins: Dilute extract shaken vigorously and allowed to stand for a few minutes	Frothing observed	Saponins may be present
Test for reducing sugars: 2 ml AR added to 5 ml Fehling's A and B solutions	Green colouration with precipitate	Reducing sugars may be present
Test for tannins: AR added to 2-3 drops of ferric chloride	Dirty green precipitate	Tannins detected
Test for phlobatannins: AR added to 5 ml of 10% HCl and boiled	Red precipitate	Phlobatannins present

Table 27: Quantitative analysis of tannin, flavonoid and phenolic contents of AR and its fractions

AR/fraction	**Tannins (mg tannic acid equivalent per g of dried extract)**	**Flavonoids (mg quercetin equivalent per g of dried extract)**	**Phenolics (mg gallic acid equivalent per g of dried extract)**
AR	26.00±0.00	48.00±0.01	222.00±0.00
PeAR	26.00±0.02	34.00±0.01	100.00±0.03
CfAR	130.0±0.03	50.00±0.02	198.00±0.04
ButAR	20.00±0.01	80.00±0.01	316.00±0.00
AqAR	26.00±0.00	34.00±0.01	174.00±0.05

Values are mean ± S.E.M. AR-aqueous root extract of *A ringens*, PeAR-petroleum ether fraction of AR, CfAR-chloroform fraction of AR, ButAR-butanol fraction of AR, AqAR-aqueous fraction of AR.

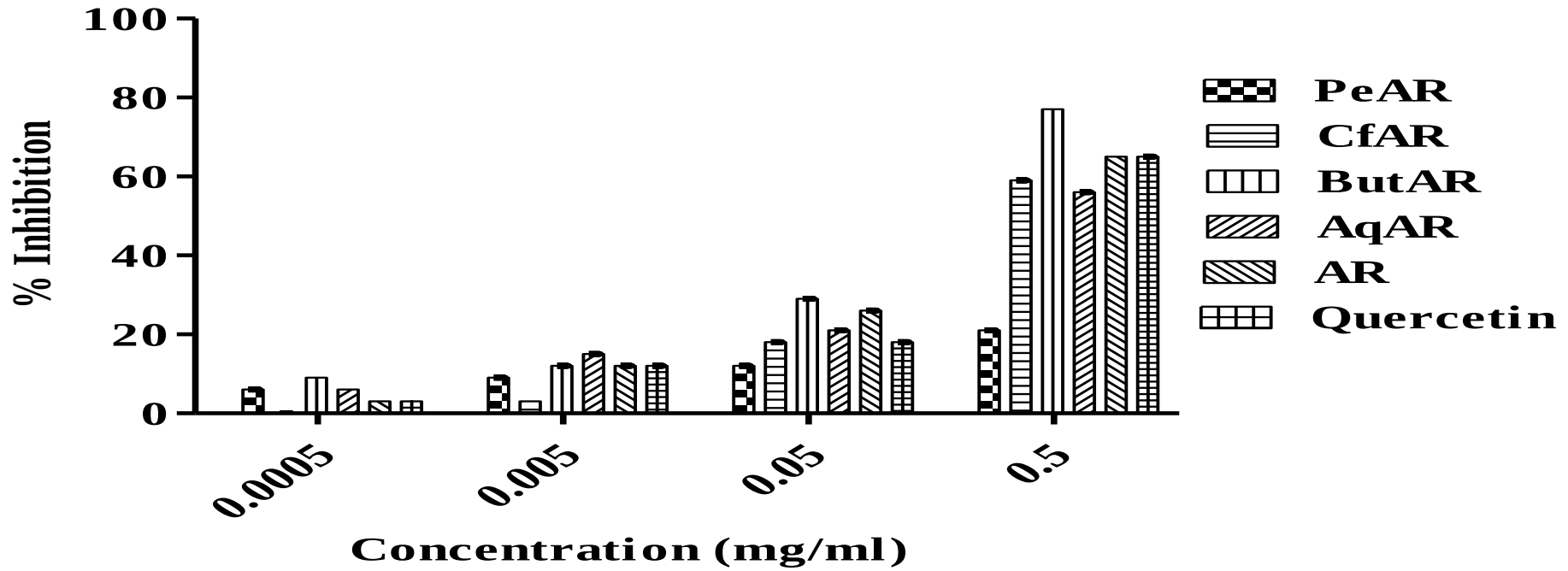

Figure 23: Effect of AR and its fractions in the 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) assay. Bars represent mean ± S.E.M. PeAR-petroleum ether fraction of AR, CfAR-chloroform fraction of AR, ButAR-butanol fraction of AR, AqAR-aqueous fraction of AR.

4.3.4. Identification and quantification of phenolics in AR using HPLC

In the HPLC analysis to identify and quantify specific phenolics in AR, 4-hydroxybenzoic acid, caffeic acid, catechin hydrate, coumaric acid, chlorogenic acid, ferulic acid, quercetin and sinnapic acid were assayed for. Of these, 4-hydroxybenzoic acid and quercetin were detected and found to be 0.469±0.90 and 2.29±33.0 mg 4-hydroxybenzoic acid and quercetin equivalents per g of AR respectively. Figures 24 and 25 show the chromatograms of AR aligned with those of 4-hydroxybenzoic acid and quercetin respectively.

4.3.5. Aristolochic acid I detection and quantification using HPLC

AR was found to contain 0.03±0.02 milligram equivalent of aristolochic acid per gramme of AR. Its butanol and aqueous fractions showed no detectable concentration, while its petroleum ether, dichloromethane and ethylacetate fractions were shown to possess 0.65±2.47, 0.21±2.87 and 0.01±0.11 mg equvalemt aristolochic acid respectively per gramme of the extract (Table 28). Figure 26 shows the chromatograms of aristolochic acid, dichloromethane and aqueous fractions of AR respectively.

4.3.6. Gas chromatography-mass spectroscopy (GC-MS) analysis of AR

GC-MS analysis of AR revealed the presence of 14 compounds including 1,2, benzenediol; 3,7, dioxa-2,8-disilanonane; 2,2,4,4,8-pentamethyl-; ethane-$D_{1;}$ 1,2,-cyclohexanediol, 1-methyl,4-(1-methylethenyl)-; 3-isoproxy-1,1,1,7,7,7-hexamethyl- 3,5,5-tris (trimethylsiloxy) tetrasiloxane; and 1,1,6- trimethyl-1,2-dihydronaphthalene. Figure 27 shows the GC-MS chromatogram of AR, while the detected compounds, their retention times and relative abundance are presented in Table 29.

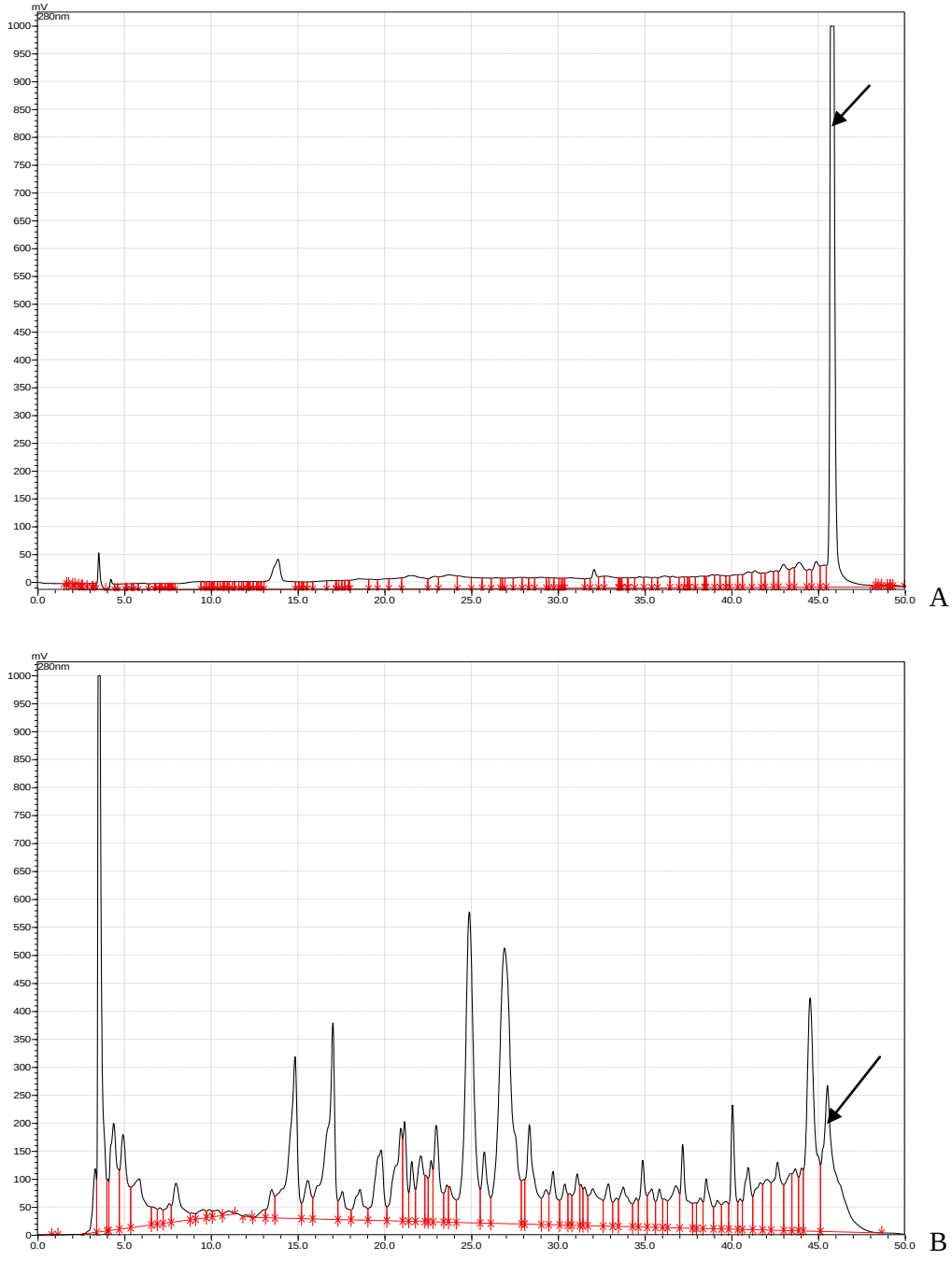

Figure 24: HPLC chromatogram of reference standard, 4- hydroxybenzoic acid (A) and the aqueous root extract of *A.ringens* (B)

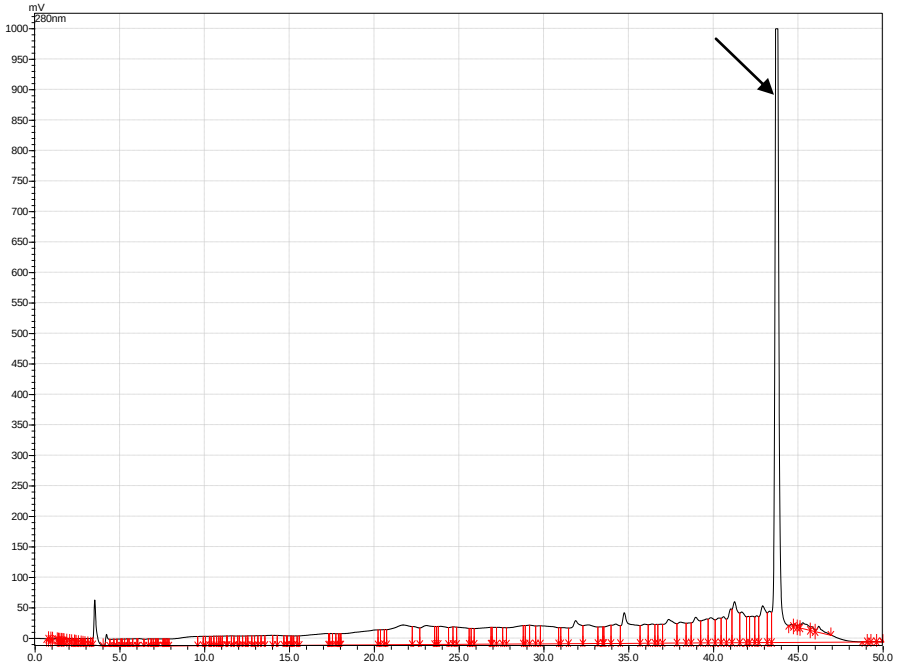

Figure 25: HPLC chromatogram of reference standard, quercetin (A) and the aqueous root extract of *A.ringens* (B).

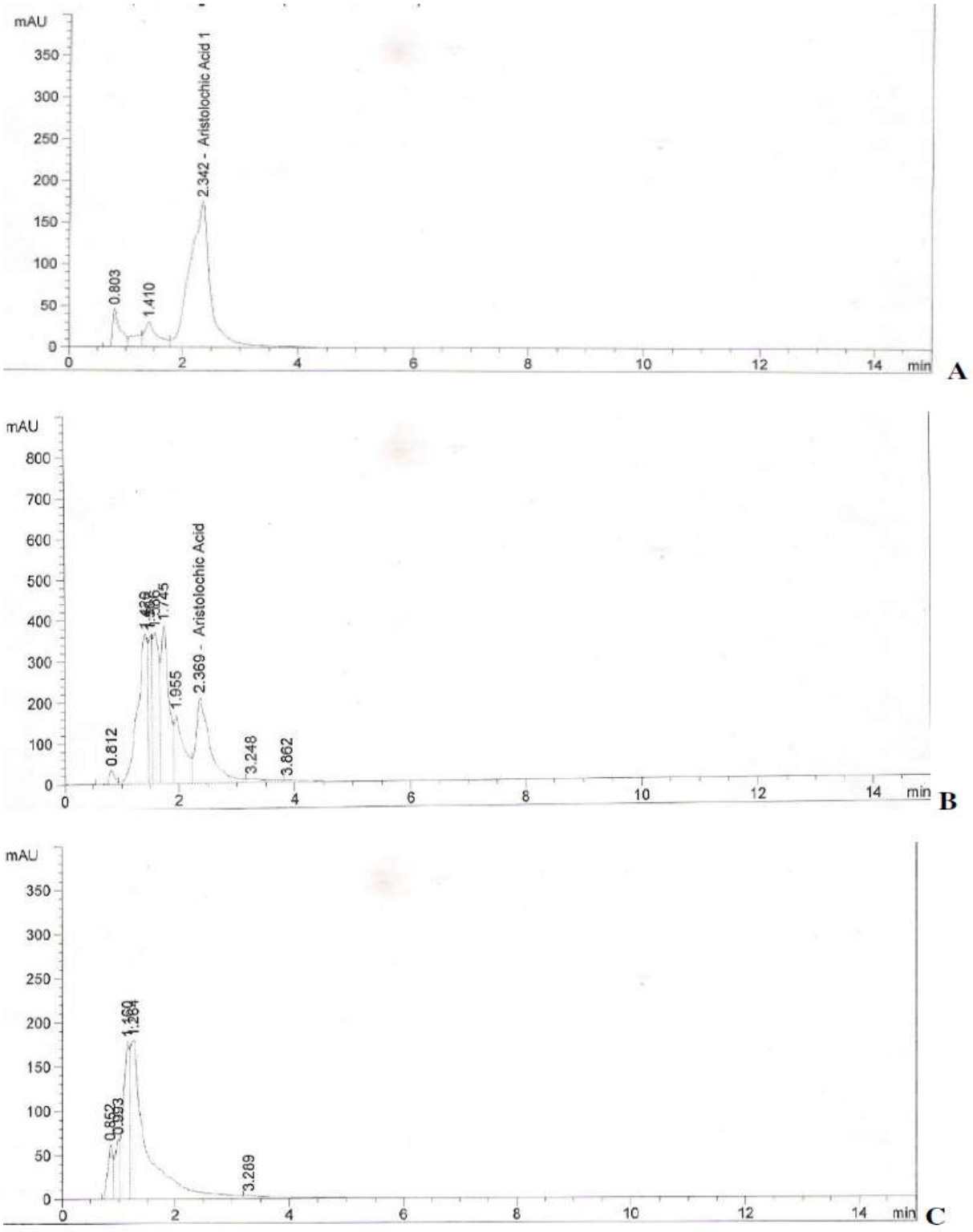

Figure 26: Chromatogram of standard aristolochic acid I (A), dichloromethane fraction of AR (B) and aqueous fraction of AR (C) at 225 nm.

Table 28: Aristolochic acid in AR and fractions obtained via HPLC analysis.

AR and fraction	**Concentration (mg aristolochic acid equivalent per g AR)**
Petroleum ether	0.65±2.47
Dichloromethane	0.21±2.87
Ethyl acetate	0.01±0.11
n-butanol	0.00±0.00
Aqueous	0.00±0.00
Crude extract (AR)	0.03±0.02

Values are mean±S.E.M.

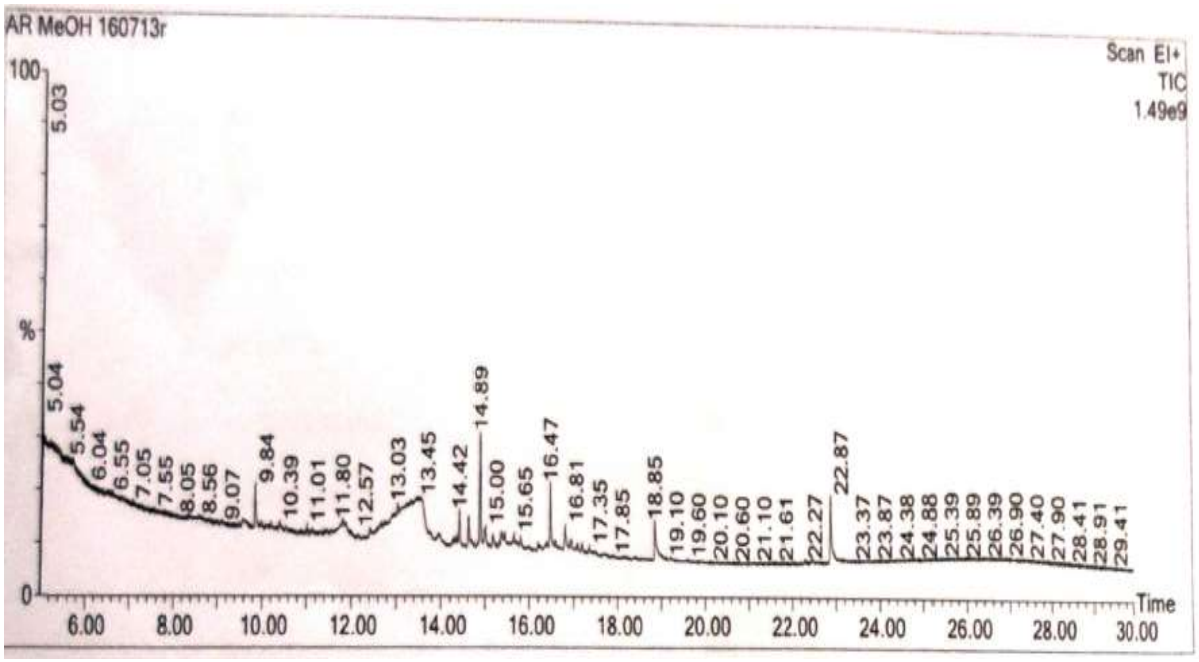

Figure 27: GC-MS chromatogram of the aqueous root extract of *A. ringens*

Table 29: Phytochemicals identified in AR using GC-MS analysis

S/N	RT	Compound name	Relative abundance (%)
1	8.352	1,2, benzenediol	9.045
2	8.572	3,7, dioxa-2,8-disilanonane, 2,2,4,4,8-pentamethyl-	2.571
3	8.607	Hexadeuterodiborane	4.398
4	8.782	Ethane-D_1	3.208
5	9.848	1,2, -cyclohexanediol, 1-methyl,4-(1-methylethenyl)-	2.524
6	11.810	3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy)tetrasiloxane	3.218
7	12.653	1,1,6-trimethyl-1,2-dihydronaphthalene	3.166
8	13.033	(1Aα,6á(S*),6Aα-3.4-dihydro-4-(1,1A,6,6A-tetrahydro-6-hydroxycycloprop[A]inden-6-yl)-1(2H)-naphthalenone	14.144
9	13.549	5-cyclohexene -1,2,3,4-tetrol, (1α2α3α4α)-(+-)	36.555
10	14.887	2H Cyclopropa(a)napthale-2-one, 1, 1A,4,5,6,7, 7A,7B-octahydro 1,1,7,7A tetramethyl-, (1Aα7α,7Aα,7Bα)	4.840
11	16.471	Duvatriendiol	4.039
12	18.848	2-(phenyl)-6-(tert-butyl)pyrimidin-4(3H)-one	4.272
13	22.871	Spiro[acridine-9(1OH)indene],2,3-dihydro3´3´-dimethyl	5.571
14	26.742	Delta (1,1)-biurea	2.460

CHAPTER FIVE

DISCUSSION

5.0. DISCUSSION

5.1. TOXICOLOGICAL EVALUATION OF AR

5.1.1. Brine shrimp lethality test

Brine shrimp lethality test is used to determine the ability of a test compound to produce mortality in laboratory-cultured brine shrimp. It is also a convenient method for monitoring biological activities of natural products (Baravalia *et al*., 2012). In this study, the mortality of brine shrimps increased with increasing concentration of the extract. The LC_{50} of the extract was found to be 175 µg/ml. According to brine shrimp lethality bioassay standard, compounds with LC_{50} less than 1000 µg/ml are considered bioactive in the toxicity evaluation of plant extracts (Meyer *et al.*, 1982). Such compounds are also considered to be potentially cytotoxic.

5.1.2. Acute toxicity tests

In this study, no morbidity or mortality was observed in mice treated with AR up to 10 g/kg orally, indicating that it has an oral LD_{50} greater than 10 g/kg. This shows that the aqueous extract of *A. ringens* may be safe on oral acute exposure in mice. According to Clarke and Clarke (1977) a substance that failed to cause lethality at 10 g/kg can be considered relatively non-toxic. However, mice exposure to AR intraperitoneally showed dose-dependent severity in ataxia, a neurological sign consisting of lack of voluntary coordination of muscle movements, which suggests central nervous system involvements of AR. Writhing, which could have been due to the route of exposure, was also observed. An LD_{50} of 403.78 mg/kg was obtained for AR administered via the intraperitoneal route. The intraperitoneal route

usually allows for a higher bioavailability of drugs due to increased absorption surface area; hence the potential for toxic response and/or lethality at lower doses. According to Looms and Hayes (1996), compounds with LD_{50} of 50 to 500 mg/kg are considered moderately toxic. Intraperitoneally administered AR can therefore be said to be moderately toxic to laboratory mice.

5.1.3. Subchronic toxicity study

In this study, AR showed beneficial and toxic effects on prolonged (30 days) exposure in laboratory rats. Other than a significant increase in weight gain at the subtherapeutic dose of 10 mg/kg, no significant change in the extent of body weight gain was observed in male rats. However, significant reduction in weight of female rats was observed by the 3^{rd} and 4^{th} week of exposure. Such difference in effect between male and female rats may be accounted for by the hormonal difference between them. According to Teo *et al*. (2002), reduction in weight would be indicative of an adverse reaction to administered drug. No significant alteration was observed in the weights of vital organs of male and female rats in the study. The histology of examined kidney and liver of male rats also revealed no histopathology as no lesion or abnormal histology was observed in the treated rats compared to the control rats. Normocellular glomerular tufts were displayed on a background containing tubules and no necrosis was observed. The liver appeared normal with preserved hepatic architecture and hepatocytes arranged as radial plates. No cytoplasmic inclusions or inflammation was observed.

Effect of AR on haematologic parameters

Haematology is essential for the early identification of pathological conditions. Variations in the size, shape and number of blood cells can give early insight into the general state of blood and the bone marrow, as well as clinical factors that may influence them. In this study, AR showed no significant alterations in the red blood cell count of male and female rats, indicating that the extract may have no influence on the number of erythrocytes; possibly having no significant effect on the synthesis or destruction of erythrocytes. To ensure adequate tissue oxygenation, sufficient haemoglobin level must be maintained. AR did not significantly alter the haemoglobin level of the male and female rats in this study.

Although, AR did not affect erythrocyte and haemoglobin levels, it significantly influenced haematocrit, the volume percentage of erythrocytes in blood. Haematocrit is usually considered in conjunction with haemoglobin and red cell count, to determine the presence and type of anaemia. In this study, while the subtherapeutic and therapeutic doses of AR showed haematocrit levelscomparable to control. AR at the supratherapeutic dose significantly reduced haematocrit in male rats, giving a value of 36.20±1.51%. Compared to the vehicle treated rats, which gave haematocrit value of 46.23±1.49%, the extract at the supratherapeutic dose may be said to be capable of causing anaemia in male rats. However, according to Johnson-Delaney (1996), the normal haematocrit range in laboratory rats is 37.6-50.6%, while another report by the University of Minnesota Board of Regents (2009), reveals that the normal range for laboratory rats is 36-64%. Thus, while from this study it appears that AR at the supratherapeutic dose may cause anaemia, the possibility that AR-induced haematocrit reductions may be considered to be within acceptable limits should also be noted.

The effect of AR on red blood cell indices, to further obtain information on the haematology of treated rats, was also determined. Red blood cell indices refer to a range of blood parameters that provide information on haemoglobin content and size of erythrocytes. The indices are valuable in the morphological classification of anaemias (Williams, 1983). They comprise mean cell haemoglobin concentration (MCHC), mean cell volume (MCV) and mean cell haemoglobin (MCH). MCHC measures the weight of haemoglobin in a standard volume of blood, MCH measures the weight of haemoglobin in average erythrocytes and MCV measures the average volume of erythrocytes, which either increases or decreases in accordance to average red cell size (Williams, 1983). Of these indices, only MCV of female rats was significantly affected in the study. By the end of 30 days AR exposure, MCV of female rats was significantly reduced (60.88±1.40 fl) by AR at the supratherapeutic dose. It was also significantly reduced by the extract both at the subtherapeutic (52.10±0.44 fl) and supratherapeutic (53.65±0.70 fl) doses in the reversibility study. These values are within the normal range of 48-70 fl, highlighted by the University of Minnesota, Board of Regents, (2009). However, compared to the control rats in this study, these significant MCV reductions by AR could be an indication of its potential to cause anaemia of the microcytic type. According to Massey (1992), microcytic anaemia is characterized by low MCV.

White blood cell (WBC) count measures the number of leukocytes. After 30 days exposure, AR produced no significant alterations in the WBC of male and female rats. However, at the end of 2 weeks following the ceasation of exposure, female rats, previously exposed to AR (250 mg/kg) showed significant WBC reduction. Low counts may be seen with suppression of the immune system, bone marrow deficiency or failure (Dinauer and Coates, 2008). This

finding therefore indicates a possible delayed onset immune system depressant action of AR at the supratherapeutic dose.

The assay that caters for specific types of white blood cells is referred to as 'differential'. In conjunction with the total white blood cell count, the level of the various leukocyte types gives information about the status of the immune system. Neutrophils and lymphocytes are the two types of white blood cells examined in this study. It was found that while the level of relative neutrophils and lymphocytes of male and the relative neutrophils of female rats were unaltered at the end of the periods of exposure and reversibility, the relative lymphocytes of female rats treated with 10 mg/kg of AR was significantly ($p<0.05$) elevated (81.90±0.91%) 2 weeks after ceasation of AR exposure. Although, this elevation may be considered to be within the normal range for laboratory rats (65-85%), as reported by the Board of Regents of the University of Minnesota, (2009), compared to control group in this study, AR may be said to produce lymphocytosis.

The extract does not appear to have any significant effect on platelets as there was no significant change in the platelet count of male and female rats in the periods of AR exposure and reversibility.

Effect of AR on biochemical parameters

The effect of AR on biochemical parameters was performed to determine the action of AR majorly on liver and kidney functions. Among the assayed biochemical parameters are serum aminotransferases, aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Assay on these enzymes are most frequently used as specific determinants of hepatocellular necrosis. ALT is found predominantly in the liver, with clinically negligible concentrations found in the kidneys, heart, and skeletal muscle; AST on the other hand, is well distributed in the liver, heart, skeletal muscle, kidneys, brain, and erythrocytes (David *et al*., 1990). As a result, ALT is a more specific indicator of liver dysfunction than AST, as AST may be elevated in diseases affecting other organs.

AST is usually found to be more highly expressed than ALT (David *et al*., 1990), as was observed in this study. AR produced no significant change in serum AST of male and female rats as well as the ALT of male rats. This may be indicative of the absence of deleterious effect of AR on the liver of male rats, as was also noted in the normal liver histology of male rats in the study. ALT level of female rats was unaffected in the AR exposure period, but was significantly reduced in female rats treated with AR (10 and 50 mg/kg) and maintained at 250 mg/kg in the reversibility study. These findings reveal that at the tested doses of AR, leakage of these enzymes into blood from damaged hepatocytes does not occur.

The effect of AR on liver function was further determined by assaying for alkaline phosphatase (ALP), a hydrolase enzyme responsible for dephosphorylating several molecules, including nucleotides, proteins, and alkaloids. ALP is distributed in various tissues throughout the body, but is particularly concentrated in tissues such as liver, bone and kidney. ALP of male rats in this study was not significantly affected by AR; while reduction in ALP of female rats was observed at 10 mg/kg of AR. Such reductions are reported to be rare, but can be due to malnutrition, which may be underscored by the reduction in body weight of these animals

seen in this study. The role of malnutrition in reduced ALP levels has been reported (Berk and Korenblat, 2007; Pratt, 2010). However, the fact that ALP level was not significantly elevated, show that AR did not induce its over-production or leakage from damaged hepatocytes, as is usually observed with hepatotoxic substances.

Bilirubin, a reddish yellow pigment in bile, blood and urine is mainly synthesized in the liver as an intermediate product of haem degradation. Its main physiological role is as a cellular antioxidant (Baranano *et al*., 2002). In this study, AR produced no significant alteration in the serum bilirubin concentration in male and female rats; suggesting a possible non-interference with bilirubin concentration in its actions. In terms of liver function, this could be an indication that hepatic excretion of bilirubin is normal, hence liver function in this regard can be said to be normal with AR exposure.

The effect of AR on total proteins and albumin were also determined. Total protein measures several different proteins, with albumin being the most abundant. Serum albumin is a globular protein essential for maintaining the oncotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues, among other functions. In this study, the concentration of serum albumin and total protein in the male rats was found to be unaltered. The female rats serum albumin was also not altered, except in the reversibility study, in which the serum albumin of female rats treated with AR (10 and 50 mg/kg) was found to be significantly reduced. Low albumin levels can be seen in conditions resulting in protein loss, reduction in synthesis (denoting liver dysfunction) and abnormal distribution of albumin. Albumin levels have been reported to be reduced in chronic liver diseases (Sclavo,

1987). However, findings such as reduced levels of ALT and insignificant effect of extract on other biomarkers of liver disease in the study show no additional evidence of liver disease. Hypoalbuminaemia is not specific for liver disease and may also occur in protein malnutrition and nephrotic syndrome (Daniel and Marshal, 1999).

Triglycerides are the body's storage forms of fat and are determined together with cholesterol in lipid profiling to assess an individual's risk of cardiovascular disorder. In the study, while cholesterol level in male and female rats, and triglycerides in female rats were insignificantly altered by AR; triglycerides level in male rats was significantly decreased. Such hypotriglyceridaemic effect indicates that AR may possess cardioprotective potentials, capable of preventing conditions that could arise from artherogenic disorders.

To determine the effect of AR on renal function, serum urea and creatinine (nitrogenous end products of metabolism) were assayed for. While AR produced no significant alteration in the urea and creatinine levels of male rats by the end of the 30 days exposure; significant increase in serum urea with relatively normal creatinine of these rats was observed by the end of the reversibility period. Hence, while the initial observation may indicate that AR may not alter the kidney's filteration of these substances in male rats, the finding in the reversibility study is indicative of a delayed onset effect of AR in this regard. Such high urea accompanied by normal creatinine has been reported to possibly indicate dehydration (Vivanti *et al*., 2008). On the other hand, serum creatinine was reduced significantly in female rats treated with AR (250 mg/kg) by the end of the 30 days exposure period, while serum urea was found to be significantly reduced in female rats treated with AR at 10 mg/kg in the reversibility test.

These findings show that AR enhances the excretion of urea and creatinine in female rats. Malnutrition is also reported to be implicated in low serum urea and creatine concentrations (Waring *et al*., 2008; Beddhu *et al*., 2003).

Effect of AR on electrolytes

Kidney function test may also include electrolytes test, as electrolytes are usually excreted renally. All known higher life forms require a subtle and complex electrolyte balance between intracellular and extracellular environments; the maintenance of precise osmotic gradients of electrolytes is important. Such gradients affect and regulate the hydration of the body as well as blood pH, and are critical for nerve and muscle action, among other functions (Ganong 2005). In this study, sodium, potassium, chloride and bicarbonate concentrations were not significantly altered by AR at all the doses tested; showing that the extract may cause no significant alteration in systems such as the renal system, that regulate the concentration of these electrolytes. Thus indicates normal kidney function in this regard.

Determination of endogenous antioxidant indices

In addition to the kidney and liver function tests carried out in the assay for biochemical parameters, the effect of antioxidants indices in the liver and kidney was determined to provide additional information on the effect of AR on these vital organs. Primary antioxidant enzymes responsible for protection from reactive free radicals, include superoxide dismutase and catalase. Glutathione is a tripeptide antioxidant that prevents damage caused by reactive oxygen species to important cellular components (Pompella *et al*., 2003). By the end of the 30 days exposure and reversibility test periods, liver glutathione level was found to be

significantly unaltered in female rats, while a dose-dependent increase (significant at 250 mg/kg) was observed by the end of the reversibility test, in male rats. This indicates that AR may possess potential hepatoprotective effect, as glutathione would prevent hepatic damage due to oxidative stress, by enhancing elimination of reactive free radicals.

Superoxide dismutase (SOD) is an important antioxidant enzyme that prevents damage to cells. Its substrate (O^{2-}) is responsible for direct damage of biological macromolecules and for generating other reactive oxygen species. Superoxide dismutase keeps the concentration of superoxide radicals at low levels, thus preventing oxidative stress (Fridovich, 1997). While no significant effect on liver and kidney SOD was noted by the end of AR exposure period; two weeks after ceasation of exposure, AR was found to increase liver SOD and glutathione, suggesting hepatoprotective potential of AR in male rats. On the other hand, kidney SOD, which was maintained in comparison to control, with the subtherapeutic and therapeutic doses of AR, was significantly reduced by AR at the supratherapeutic dose in both male and female rats 2 weeks after treatment ceasation. Thus, at the supratherapeutic dose, AR may deplete kidney SOD, thus predisposing the kidney to oxidative stress-induced damage.

Catalase is a tetramer polypeptide enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al*., 2004). Thus, it is a very important enzyme in protecting cells from oxidative damage by reactive oxygen species. It is reported as having one of the highest turnover of all enzymes, in that one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen in a second (Goodsell, 2004). In this

study, AR did not significantly alter the kidney or liver catalase levels in the male or female rats, indicating a possible lack of interference with catalase levels in these rats, by AR.

Semen analysis

The effect of AR on male reproductive system was also investigated. Semen analysis evaluates certain characteristics of semen and the sperm contained therein. It is done to evaluate male fertility. Collection techniques and precise measurement method may influence results. While no significant effect of AR on sperm motility and morphology was observed, sperm count, which was found to be unaltered by the end of the 30 days exposure, was significantly reduced in the reversibility study, showing a possible delayed onset effect. This therefore calls for the need to exercise caution in the use of the plant's extract by males, considering the adverse effect it may pose to their reproductive capacity.

5.2. PHARMACOLOGICAL ACTIVITIES

5.2.1. Antidiarrhoeal activity

Castor oil was used as a diarrhoegenic agent in this study to induce diarrhoea. This is due to the ability of its hydrolytic metabolite, ricinoleic acid (Iwao and Terada, 1962) to impair water and electrolyte reabsorption leading to hypersecretion (Ammon *et al*., 1974). It has also been used to induce increased intestinal transit, which is also an important feature of diarrhoea (O'Donnell *et al*., 1990).

The antidiarrhoeal potential of the aqueous root extract of *A. ringens* is demonstrated by its significant dose-dependent decrease in the intestinal transit of charcoal meal in both normal

and castor oil-induced intestinal transit tests. This effect was observed to peak (with 65.3% inhibition) and to be comparable to that of morphine (10 mg/kg) (76.1 % inhibition) at 400 mg/kg. The fact that there was no significant difference in the inhibition of intestinal transit it produced at 400 mg/kg administered before and after the castor oil showed that it has potential for both preventive and curative effects on increased gut motility in diarrhoea.

In this study, yohimbine, an α_2-adrenergic antagonist, and isosorbide dinitrate, a nitric oxide donor did not significantly influence the action of the extract on intestinal propulsion. This suggests little or no effect of AR on α_2-adrenoceptors in the gastrointestinal tract and also, no significant interaction with the enteric neuron nitric oxide activity by AR. However, phentolamine, a non-selective α adrenoceptor blocker, propranolol, a non-selective β adrenoceptor blocker and pilocarpine, a muscarinic receptor agonist, significantly altered the action of the extract on intestinal propulsion. These suggest that AR probably interacts with α_1 adrenoceptor, β adrenoceptor and muscarinic receptor in its action against diarrhoea.

Clinically, diarrhoea may result from disturbed bowel function, in which case; there is impaired intestinal absorption, hypersecretion of fluid and electrolyte in the intestine, and a rapid bowel transit (Gurgel *et al*., 2001). The induction of diarrhoea by castor oil results from the action of ricinoleic acid formed by hydrolysis of the oil. Ricinoleic acid sensitizes the intramural neurons of the gut and stimulates intestinal transit, allowing for the expulsion of content (O'Donnell *et al*., 1990). This was observed to be inhibited by the extract, as it delayed the onset and the number of diarrhoeal stools.

The $ADI_{in\ vivo}$ is a measure of the combined effects of different components of diarrhoea such as purging frequency, onset of diarrhoeal stools as well as the intestinal frequency. The extract (100-400 mg/kg) produced a dose-dependent increase in the antidiarrhoeal index with its peak effect (81.79) at 400 mg/kg being comparable to that of morphine (86.85) at 10 mg/kg.

5.2.1. Antiinflammatory activity

Carrageenan-induced paw oedema is a useful model to assess the contribution of mediators involved in vascular changes associated with acute inflammation. The development of oedema in the rat hind paw upon injection of carrageenan has been described as a biphasic event. The first phase (0-1 hour post-carrageenan injection) is reported to be due to the release of histamine, serotonin and bradykinin (Di Rosa *et al*., 1971), while the second accelerating phase of swelling (1-6 hour post-carrageenan injection) has been reported to correlate with an increased production of prostaglandins (Di Rosa and Willough, 1971; Di Rosa *et al*., 1971). Siebert *et al*. (1994) reported the involvement of the inducible cyclo-oxygenase (COX-2) in oedema induced by carrageenan in the hind paw (Salvemini *et al*., 1996).

The extract (10-100 mg/kg) decreased the extent of increase in paw size of the rats at all the time intervals in the carrageenan-induced rat paw oedema model. Significant effects were observed within the 4^{th} to 6^{th} hour period. Although indomethacin produced the greatest peak effect observed at the 3^{rd} hour, by the 6^{th} hour, when its effect had become insignificant, the inhibition of oedema by the extract (10-50 mg/kg) was still significant, pointing to a longer duration of action of the extract. These findings therefore indicate the possibility that AR possesses cyclooxygenase inhibitory activity.

In addition, the second phase of carrageenan-induced inflammation has also been reported to involve neutrophil infiltration and production of reactive free radical species derived from them (Salbemini *et al*., 1996; Dordevic *et al*., 2007; Sofidiya *et al*., 2010). This shows that AR may also exert anti-inflammatory activity via inhibition of neutrophil infiltration, prevention of free radical generation, and/or enhancement of free radical scavenging.

Egg albumin, a potent histamine releaser (Cantani, 2008), induces rat paw oedema by causing mast cells to release histamine, which has been associated with increased vasodilatation and increased permeability of blood vessels leading to exudation of plasma proteins and fluids into the tissues (Anosike and Obidua, 2010; Harriot *et al*., 2004). In this model, AR (10-50 mg/kg) also produced a dose-dependent reduction of the rat paw sizes throughout the 4 hours of observation. The peak effect of the extract (50 mg/kg) observed in the 4^{th} hour was higher than that of diclofenac (10 mg/kg). This effect of the extract may be due to mechanisms such as mast cell stabilization and antihistaminergic activity.

The formaldehyde test has been in use for some time in the evaluation of arthritic inflammation (Brownlee, 1950; Owoyele *et al*., 2008; Singh *et al*., 2011). In this model, the animal experiences inflammatory pain both in the limbs and joints. The release of inflammatory mediators such as bradykinin has been implicated in the formaldehyde-induced inflammation (Damas and Liegeolis, 1999). The extract (10-100 mg/kg) produced significant ($p<0.05$) inhibition of rat paw oedema throughout the ten days study period. This shows the potential of the extract in alleviating arthritic and chronic inflammation.

The xylene-induced ear oedema model in mice is more sensitive to steroidal anti-inflammatory drugs e.g. dexamethasone (Zaninir *et al*., 1992). The inhibitory effect of the extract on ear oedema suggests the possible inhibition of phospholipase A_2, the enzyme involved in the release of arachidonic acid leading to synthesis of inflammatory mediators, eicosanoids via lipo-oxygenase and cyclo-oxygenase pathways (Hirata *et al*., 1980).

The test for the delay in castor oil-induced diarrhoea has been reported to be useful for determining the inhibition of prostaglandin by the test compound as one of its possible mechanism of antiinflammatory action (Awouters *et al*., 1978; Mayuren and Ilavarasan, 2009). The extract (50 mg/kg) significantly ($p<0.001$) delayed the onset of diarrhoea and also significantly ($p<0.05$) reduced the diarrhoeal score. These findings indicate a possible inhibition of prostaglandin synthesis by the extract.

5.2.3. Analgesic activity

Acetic acid-induced writhing is a sensitive procedure for the evaluation of peripherally acting analgesics (Gené *et al*., 1998). The reduction in the number of writhes by AR shows that it can inhibit mediators of pain, as also shown in the investigation on the antiiflammatory effect of the extract. Acetic acid is reported to induce pain by liberating endogenous substances such as serotonin, histamine, prostaglandins, bradykinins and substance P, which stimulate local peritoneal receptors to mediate pain, which is manifested by the abdominal constriction response by mice on acetic acid intraperitoneal administration (Bentley *et al*., 1983). An involvement of prostaglandinds E_2 and $F_{2\alpha}$ (Derardt *et al*., 1980) as well as lipoxygenase derived products (Roberts and Morrow, 2008) have also been implicated (Paschapur *et al*., 2009).

Several intracellular pathways lead to the opening of ATP-sensitive potassium channels, (K_{ATP} channels) and consequently, resulting in analgesia. Nitric oxide donors such as sodium nitroprusside for instance, activate the soluble form of guanylyl cyclase, resulting in the synthesis of cyclic GMP (cGMP), which in turn brings about the opening of K_{ATP} channels and subsequent analgesia (Soares *et al*., 2000). NSAIDs such as ketorolac and diclofenac are reported to produce antinociception by activation of L-arginine-nitric oxide-cGMP-K_{ATP} channel pathway. According to White *et al*. (1993), atrial natriuretic peptide activates the particulate form of guanylyl cyclase also resulting in cGMP synthesis, leading to K_{ATP} channel opening and antinociception (Paz–Camos *et al*., 2012). There is also evidence that compounds such as cromakalin and pinacidil that directly open K_{ATP} channels, cause pain relief. The findings in this study reveal the possibility that ATP-sensitive K^+ channel opening is one mechanism by which AR induces antinociception. Administration of K_{ATP} channel antagonist, glibenclamide, before AR significantly reversed the antinociceptive activity of AR. This suggests that AR produced its antinociceptive activity possibly through an influence on the opening of K_{ATP} channel. This channel opening allows for the efflux of K^+ ion, leading to membrane hyperpolarization state, which reduces the membrane excitability associated with nociception (Lawson, 1996).

The possible involvement of opioid receptor interaction in the analgesic effect of AR was demonstrated. Apart from the similarity of actions shared by AR and morphine in some aspects of this study, opioid antagonist, naloxone also inhibited the analgesic action of AR. Haloperidol, a dopaminergic antagonist, on the other hand, did not significantly alter the

effect of AR, suggesting a possible lack of involvement of the dopaminergic system in the analgesia produced by the extract in acetic acid-induced algesia.

Acetylcholine has been regarded as an algogenic agent, which induces pain by irritating serous membrane of the peritoneal cavity and evoking abdominal contraction (writhing) by its activation of nociceptors (Steen and Reeh, 1993) in this region. The extract also reduced the number of writhes induced by acetylcholine.

AR was also found to inhibit both the first and the second phases of the formalin induced pain test. The phases of this test measures pain of both neurogenic (first phase) and inflammatory origin (second phase). The first phase (0–5 minutes) has been reported to be due to direct stimulation of nociceptors and measures centrally mediated effects. It is usually insensitive to anti-inflammatory agents. The second phase (15–30 minutes) on the other hand, is dependent on peripheral inflammation and changes in central processes due to the release of chemical mediators (that stimulate nociception), from damaged cells thus inducing pain (Hunskaar and Hole, 1987). The test measures the response to a long lasting nociceptive stimulus similar to clinical pain (Tjolsen *et al*., 1992) and is recommended as a tool in basic pain research for studying the mechanisms of analgesic agents (Paschapur *et al*., 2009). Agents that act primarily on the CNS inhibit both phases of the test, while peripherally acting drugs inhibit the late phase. The ability of AR to inhibit both phases of the formalin test shows its involvement in both central and peripherally mediated analgesia, probably by prostaglandin synthesis inhibition, as well as central inhibition mechanism(s). The effect of the extract on centrally mediated pain may also be seen in the inhibition of its analgesic effect by naloxone,

in the aceticacid-induced writhing test, indicating an opioid-like effect characteristic of centrally acting opioid analgesics.

In addition, it has been reported that formalin excites sensory neurons by directly activating TRPA1, a cation channel that plays an important role in inflammatory pain. TRPA1 is a member of the Transient Receptor Potential family of cation channels that is highly expressed by a subset of C-fibre nociceptors (Story *et al*., 2003; Nagata *et al*., 2005). In a study by McNamara *et al*. (2007), it was found that formalin induces robust calcium influx in cells expressing TRPA1 channels, and these responses were attenuated by a TRPA1-selective antagonist. Moreover, sensory neurons from TRPA1-deficient mice lacked formalin sensitivity. At the behavioural level, pharmacological blockade or genetic ablation of TRPA1 produced marked attenuation of the characteristic paw flinching, licking, and lifting responses resulting from intraplantar injection of formalin, as was observed with AR in this study. Thus the findings in this study show that AR may be capable of inhibiting TRPA1 mediation of formalin-induced pain.

The tail-clip test is used for elucidation of centrally mediated antinociceptive responses, which focuses mainly on changes above the spinal cord (Vongtau *et al*., 2004). The significant increase in pain threshold by AR (25-50 mg/kg) in this model again suggests its involvement with central pain pathways. Pain is centrally modulated via a number of complex processes including opiate, dopaminergic, descending noradrenergic and serotonergic systems (Pasero *et al*., 1999; Paschapur *et al*., 2009). The analgesic effect produced by the extract may be via central mechanisms involving some of these receptor systems.

To show the possible involvement of central activity of AR in its analgesic action, the activity of AR in hole board, open field and barbiturate-induced sleep tests were carried out. These models are classical models for evaluating central nervous system activity of substances. The reduction of head dip behaviour in the hole board test, sectional crossings in the open field test, as well as prolongation of hexobarbitone-induced sleep by AR, showed its CNS depressant and sedative activities. Supportive evidence to this is the report by File and Wardill (1975), which indicates that reduction in the number of head dips is a measure of sedative activity. Authors such as Adzu *et al.* (2002) and Viswanatha-Swamy *et al.* (2006) also suggested that a decrease in the number of head dips reveals a depressant effect by the administered substance.

Further evidence of the central sedative activity of AR is demonstrated by its ability to enhance the duration of sleep induced by hexobarbitone. It is well known that CNS depressants such as diazepam bind to the barbiturates site associated with a Cl^- ionopore on the $GABA_A$ receptor complex. This leads to an increase in the duration of opening of Cl^- ionopore, resulting in the hyperpolarisation of post-synaptic neurons (Rang *et al*., 2003; Martinez-Vazqueza *et al*., 2012) and a consequent prolongation of the resulting inhibitory effect. The combination of 100 mg/kg of hexobarbitone and 100 mg/kg of AR caused prolongation of sleep duration, as did the same dose of hexobarbitone in combination with 3 mg/kg of diazepam. It may thus seem that the GABAergic system (implicated in diazepam action) may also be involved in AR-induced enhancement of the sedative effects of hexobarbitone.

Given the findings of this study, it can be inferred that a central action is partly involved in the analgesia produced by AR. Some medications such as thiazines (e.g. xylazine) have been reported to cause CNS depression resulting in sedation and analgesia (Short, 1987).

5.2.4. Haemodynamic activity

Hypertension has long been regarded as a major public health problem worldwide; more than 20% of the world's population suffers from high blood pressure (Caceres *et al*., 1987; Kearney *et al*., 2005). It remains the most common risk factor for cardiovascular and cerebrovascular disorders. In spite of all the progress made in its prevention, detection, and management, it remains a major challenge (Kearney *et al*., 2005). Natural products may be alternative solution in the treatment of human hypertension. Various plant extracts have demonstrated potent hypotensive effect in various models of hypertension including models that use spontaneously hypertensive rats, which is a valid animal model of essential hypertension (Eddouks *et al*., 2005).

Daily administration of AR via the oral route for 21 days, caused significant reduction of systolic and diastolic blood pressure in conscious spontaneously hypertensive rats. This effect of the extract, which was observable by the 7^{th} day of exposure was found to be comparable to the hypotensive effect of enalapril (3 mg/kg). Change in heart rate was only observed by the 14^{th} day of exposure to AR at 50 mg/kg. Such nearly insignificant effect on cardiac rate has been reported previously of potentially hypotensive medicinal plants such as *Lepidium sativum* and *Fraxinus excelsior* (Haloui *et al*., 2000). Diuretics are among the class of drugs used in the management of hypertension. In the aspect of the study to investigate the effect of

AR on urine volume and electrolyte, it was observed that AR showed no diuretic effect; as urine volume, sodium and potassium were unaffected through out the study period. This shows that the blood pressure lowering action of AR is not mediated by diuretic mechanism.

The results of the present study also showed that AR, on intravenous administration, induces a dose-dependent acute hypotensive effect on anaesthetized SHRs; reducing significantly, systolic and diastolic blood pressure as well as heart rate, the reduction of which was significant at 25 and 50 mg/kg. Consistent with the results in the 21 days oral exposure, AR produced greater reductions in the systolic blood pressure compared to diastolic blood pressure. Compared to oral administration, intravenous administration resulted in greater reduction of blood pressure, which shows that oral administration of AR may reduce the pharmacological effect of the extract, perhaps due to hepatic metabolism of the orally administered extract, thus reducing its bioavailability.

To examine the possible mechanism involved in this response, the extract's effect on the haemodynamic parameters studied were determined in the presence of atropine (a non-selective muscarinic antagonist) and hexamethonium (an autonomic ganglion blocker). Pre-treatment of rats with hexamethonium and atropine significantly inhibited the hypotensive effect of the plant extract; reducing significantly, the extent of systolic and diastolic blood pressure reductions as well as heart rate reduction. This suggests that the extract's mechanism of hypotensive action may involve interferences with transmissions at the autonomic ganglia and muscarinic receptors.

In the study to investigate the action of the fractions of AR; the effect of its chloroform, butanol and aqueous fractions were compared to that of its crude form. Its butanol fraction was found to be the most active. The effects of the butanol fraction of AR on blood pressure and heart rate were significantly greater than those of AR, while the effects of its aqueous fraction were comparable to that of AR. Compared to AR, its chloroform fraction produced no significant effect on blood pressure and heart rate, suggesting that it does not contain principles with activity in this regard. The effect observed with butanol fraction may be due to its relatively high content of phenolics and flavonoids; as it was shown to possess the highest concentration of these components in the study. Phenolics and flavonoids have been reported to possess many beneficial effects including antihypertensive actions (Ahmed *et al*., 2005).

5.3. PHYTOCHEMICAL ANALYSES

Knowledge of the chemical constituents of plants used for therapeutic applications is desirable as such information will be valuable for drug develpment (Mojab *et al*., 2003). In this study, preliminary phytochemical analysis of AR revealed the presence of certain phytochemicals, which could be said to be responsible for the observed therapeutic actions of *A. ringens*. The phytochemicals include oils, saponins, alkaloids, tannins, reducing sugars and phlobatannins.

Various reports on the pharmacological actions of these phytochemicals isolated from plants are available in literature. Silva *et al*. (2003) reported the analgesic and anti-inflammatory actions of some essential oils extracted from some medicinal plants. In 2000, Hajhashemi and some other researchers reported antispasmodic and antidiarrhoeal activities of the oil extract

identified in a medicinal plant. Alkaloids have been shown to possess analgesic (Sawant *et al*., 2004), antidiarrhoeal (Kavitha *et al*., 2004), sedative (Xu *et al*., 2007), anti-inflammatory (Jiao *et al*., 2011) and antihypertensive (Dangi *et al*., 2002) effects. Saponins have been reported to possess pharmacological actions such as analgesic, anti-inflammatory and antioxidant effects (Zhao *et al*., 2013). The antihypertensive effect of saponins has also been reported (Rhiouani *et al*., 2001).

Total tannins, flavonoids and other phenolics were also determined using quantitative methods of analyses. From this aspect of the study, AR was found to contain as much as 26 mg tannic acid equivalent per g of AR. Its petroleum ether, chloroform, butanol, and aqueous fractions were also found to contain tannic acid ranging from 20 to 130 mg tannic acid equivalent per g of AR. This appreciable concentration of total tannins in AR and its fractions reveal that indeed some of the actions of AR can be accounted for by its tannin content. For instance, tannins have specifically been reported to produce decrease in feed intake, growth rate and feed efficiency (Chung *et al*., 1998); this may partly explain the weight loss observed in treated female rats in the subchronic toxicity study.

Phenolics are reported to be highly ubiquitous in nature. The total flavonoids and phenolics assay showed that AR and its fractions contain very appreciable concentrations of phenolics. Phenolics, which include flavonoids and tannins are known to be highly effective in free radical scavenging and are therefore used to manage ailments in which oxidative stress are implicated (Saeed *et al*., 2012). The presence of these phytochemicals in *A. ringens* and its

fractions is therefore highly significant in this study. Of the fractions tested, the butanol fraction of AR was shown to possess the highest concentration of flavonoids and phenolics.

DPPH assay is an antioxidant test procedure that employs 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), which has an unpaired electron and exhibits a stable violet colour in methanol solution. It is commonly used for evaluation of the free radical scavenging activity of antioxidants (Oyatzu, 1986). The assay is based on the reduction of DPPH in the presence of a hydrogen-donating antioxidant, resulting in the formation of the non-radical form, (DPPH-H) (Blois, 1958; Li *et al*., 2012). In this assay, the butanol fraction of AR, which showed the highest concentration of phenolics and flavonoids, produced the most antioxidant activity.

More specifically, HPLC analysis revealed the presence of phenolic, 4-hydroxybenzoic acid, and flavonoid, quercetin, in AR, the medicinal properties of which can be largely attributable to inhibition of free radical activity. In fact, the antioxidant activity of 4-hydroxybenzoic acid (Merkl *et al*., 2010) and quercetin (Davis *et al*., 2009) has been reported. The analgesic and anti-inflammatory activities of quercetin have also been demonstrated (Davis *et al*., 2009).

Aristolochic acid I has been reported as the most abundant (Wu *et al*., 2005) and most toxic of aristolochic acids (Balachandran *et al*., 2005). In the study to identify and quantify the aristolochic acid I content of AR, the extract was found to contain the acid, which however appeared to be separated into less polar solvents in the process of liquid-liquid partitioning; leaving the more polar solvents, butanol and water, free of detectable concentrations. A study

in our laboratory showed that these fractions without aristolochic acid, possess anti-inflammatory activity. This finding show that *A. ringens,* in spite of belonging to the potentially toxic *Aristolochia* family of medicinal plants, is a potential source of useful phytoactives that can be developed into tolerable and effective medicine.

To determine volatile components present in AR, GC-MS analysis was performed. The analysis revealed the presence of 14 volatile compounds in the extract. The first compound detected was 1,2, benzenediol, with relative abundance of 9.045%. It is also known as catechol; derivatives of which have been reported to possess antioxidant properties (Korkolainen *et al*., 1996), The compound, 5-cyclohexene-1,2,3,4-tetrol, (1α2á3α4á)-(+-) showed the most relative abundance of 36.55%. It is also known as conduritol B (Ryoal Society of Chemistry, 2014) and is reported to be a potent inhibitor of β-glucosidase (Santa Cruz Biotechnology, 2014). Inhibitors of glucosidase enzymes have been reported to possess antifeedant, antiviral, antidiabetic and anticancer activities (Pandey *et al*., 2013). The next most abundant compound was (1Aα,6á(S*),6Aα-3.4-dihydro-4-(1,1A,6,6A-tetrahydro-6-hydroxycy-cloprop[A]inden-6-yl)-1(2H)-naphthalenone, with a relative abundance of 14.144%. The compound, 3-isopropoxy-1,1,1,7,7,7-hexamethyl- 3,5,5-tris (trimethylsiloxy) tetrasiloxane (3.218% relative abundance), which had earlier been identified in *Commelina benghalensis* (Mahadkar *et al*., 2013) was also detected in AR. *Commelina benghalensis* is a medicinal plant of the Commelinaceae family used to manage inflammatory and other conditions. Another compound detected was 1,1,6-trimethyl-1,2-dihydronaphthalene, previously identified in *Moringa oleifera*, whose anti-inflammatory, antihypertensive and antitumour actions have also been reported (Chuang *et al*., 2007).

6.0. CONCLUSION

The findings in this study show that the aqueous root extract of *A. ringens* can be said to be relatively safe on acute oral exposure, but moderately toxic on acute intraperitoneal exposure in mice. It may produce unwanted effects such as weight loss in females and delayed onset low sperm count in males on prolonged periods of exposure (up to 30 days). The findings in the pharmacological investigation on the extract show that it possesses antidiarrhoeal, antiinflammatory, analgesic and antihypertensive activities, which form the basis for its use in traditional medicine. Phytochemical analyses revealed that the extract contains several active components including phenols (such as quercetin and 4-hydroxybenzoic acid) and volatile compounds (such as 1,2, benzenediol and 5-cyclohexene-1,2,3,4-tetrol, (1α2á3α4á)-(+-)-), which could be responsible for the activities observed with the extract.

7.0. SUMMARY OF FINDINGS

In line with the objectives of the study, the following summary has been made from the findings in this study.

Objectives of the study	**Summary of findings**
1. To evaluate the toxicity of AR using appropriate toxicity test procedures	a. The aqueous root extract of *A. ringens* showed potential for biological activity with an LC_{50} of 175 µg/ml in brine shrimps. b. The extract is considered relatively safe on acute oral exposure with an LD_{50} greater than 10 g/kg. c. Its LD_{50} on intraperitoneal administration is 407. 38 mg/kg. d. Upon subchronic exposure for 30 days, the extract appeared to be relatively safe at the doses tested. Some adverse effects such as weight loss in the female rats and haematological alterations (haematocrit reductions in male rats, MCV reduction in female rats) were observed. Delayed onset reduction of sperm count and kidney SOD in male rats; and reduced ALT, kidney SOD, urea and creatinine in female rats, were also observed.
2. To investigate the antidiarrhoeal activity of AR using appropriate antidiarrhoeal test models	a. The aqueous root extract of *A. ringens* possesses significant antidiarrhoeal activity. b. Its action may be mediated by its action on adrenoceptors and muscarinic

	receptors in the GIT, and by its inhibition of secretory action of prostaglandin.
3. To determine its anti-inflammatory, analgesic and CNS activities using standard pharmacological models.	a. The extract possesses significant antiinflammatory and analgesic activities, inhibiting the actions of various phlogistics and algogens. b. Its mechanism of action appears to include inhibition of cyclooxygenase and/or prostaglandin activity; antioxidant action and inhibition of phospholipase A_2. The possible interaction with opioid receptors and ATP-dependent potassium channel may also be implicated in its analgesic action. c. The plausible role of its CNS depressant and/or sedative action in its analgesic activity was demonstrated.
4. To evaluate the effect of the extract and its fractions on haemodynamic parameters using appropriate models.	a. The extract possesses hypotensive activity in spontaneously hypertensive rats. b. This effect may be mediated via its interaction at the autonomic ganglia and muscarinic receptors. It does appear that diuresis is not involved in the extract's hypotensive effect. c. Its butanol fraction is the most active against hypertension in spontaneously hypertensive rat
5. To determine the phytochemical	a. Phytochemical studies showed that AR

components of the extract using phytochemical analytical methods.	contains alkaloids, tannins, saponin, phlobatannins and reducing sugars. b. The relatively high concentrations of phenolics in the extract using quantitative assays and the presence of 4-hydroxybenzoic acid and quercetin as well as aristolochic acid I were shown. c. GC-MS analysis revealed the presence of 14 volatile compounds.

7.0. CONTRIBUTIONS TO KNOWLEDGE

- The efficacy of the aqueous root extract of *A. ringens* against diarrhoea, inflammation, algesia and hypertension was demonstrated for the first time in this study, showing that novel drugs could be developed from *A. ringens*, for the management of these conditions.

- The acute and toxicity profiling of the aqueous root extract of *Aristolochia ringens* suggests relative safety of the extract given orally, however with potential to induce weight loss, haematological alterations and delayed onset sperm count reduction and impact on endogenous antioxidant indices.

- Phytochemical investigations on the aqueous root extract of *A. ringens* revealed phytochemicals responsible for its actions as well as the presence of aristolochic acid I, which was however undetected in its active butanol and water fractions.

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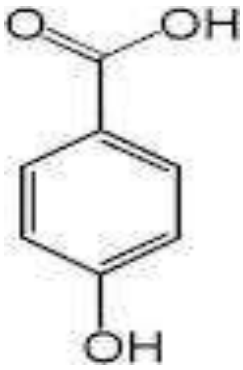
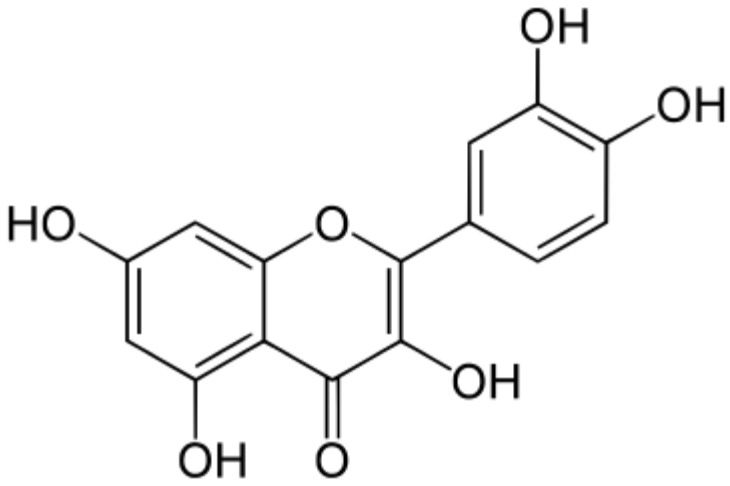
APPENDIX I

Structures of phenolics detected in the aqueous root extract of *A. ringens*



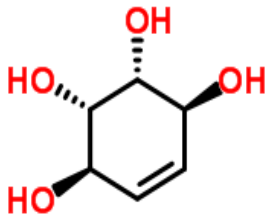
4-hydroxybezoic acid



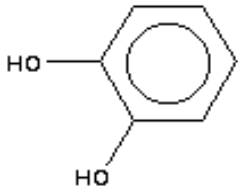
Quercetin

APPENDIX II

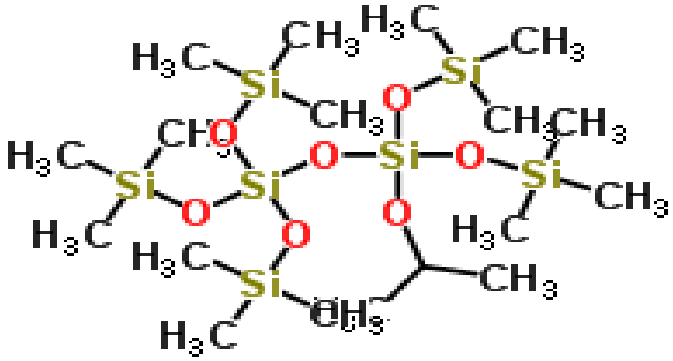
Structure of some of the volatile comounds present in the aqueous root extract of *A. ringens*



5-cyclohexene-1,2,3,4-tetrol, (1α2á3α4á)-(+-)-



1,2, benzenediol



3-isoproxy-1,1,1,7,7,7-hexamethyl- 3,5,5-tris (trimethylsiloxy) tetrasiloxane