BIOCHEMICAL STUDY OF ANTIDIARRHOEAL FRACTIONS OF *Annona senegalensis* ROOT AND STEM BARKS IN CASTOR OIL-INDUCED DIARRHOEAL ALBINO RATS

AHMED, MARYAM ABIOLA
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A Ph.D. THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF ILORIN, ILORIN, NIGERIA, IN FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph. D.) DEGREE IN BIOCHEMISTRY

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DECLARATION

I, AHMED, Maryam Abiola with matriculation number 96/047891, declare that this Thesis entitled "Biochemical Study of Antidiarrhoeal Fractions of *Annona senegalensis* Root and Stem Barks in Castor oil-induced Diarrhoeal Albino Rats" is the result of a study carried out by me under the supervision of Dr. R. O. Arise in the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. I confirm that this thesis presents the results of my findings and that its contents are entirely my ideas unless otherwise stated with appropriate referencing. This Thesis has neither been submitted to any University nor under consideration for the award of Doctor of Philosophy (Ph.D.) degree in Biochemistry at any other University.

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AHMED, Maryam Abiola **Date**

CERTIFICATION

I certify that this work was carried out by Ahmed, Maryam Abiola in the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, under my supervision.

…… …….

Dr. R. O. Arise **Date**

Supervisor

APPROVAL PAGE

This thesis has been read and approved as having met the requirement of the Department of Biochemistry and Postgraduate School, University of Ilorin, Ilorin, Nigeria, for the award of Doctor of Philosophy (Ph.D.) degree in Biochemistry.

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Dr. R. O. Arise Date
Supervisor

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Prof. Elizabeth A. Balogun Date
Head of Department

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Prof. S. J. Josiah
External Examiner Date

DEDICATION

This thesis is dedicated to Almighty Allah and to the memories of my late parents; Alhaji Suleiman Usman and Alhaja Munirat Usman.

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All praises, thanks and adorations belong to Almighty Allah for making this work a success. Alhamdulilah Robil Alamin.

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Ahmed, Maryam Abiola

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LIST OF ABBREVIATIONS

EFAR	Ethylacetate fraction of aqueous root
EFAS	Ethylacetate fraction of aqueous stem
DS	Dichloromethane stem
DFAR	Dichloromethane fraction of aqueous root
DR	Dichloromethane root
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2-2, Azinobis-3-ethylbnzo-thiazoline-6-sulfonate
COX	Cycloxygenase
ALP	Alkaline phosphatase
AST	aspartate transaminase
ALT	Alanine transaminase
SOD	Superoxide dismutase
GSH	Reduced Glutathione
GPx	Glutathione Peroxidase
GST	Glutathione transferase
AR	Aqueous root
AS	Aqueous stem
ALB	Albumin
BIL	Bilirubin
CFTR	Cystic fibrosis transmembrane conductance regulator
NHE	Sodium hydrogen exchanger
ENaC	electrogenic sodium channel

GIT	Gastrointestinal tract
TBA	Thiobarbituric acid
SGLT	Sodium glucose transporter
SCFA	Short chain fatty acid
PG	Prostaglandin
ROS	Reactive oxygen species
RNS	Reactive nitrogen specie
NO	Nitric oxide
ENS	Enteric nervous system
TAC	Total antioxidant capacity
PCV	Packed cell volume
CaCC	Calcium chloride channel
IBD	Inflammatory bowel diarrhoea
CNS	Central nervous system
ORT	Oral rehydration theraphy

ABSTRACT

Diarrhoea is a gastrointestinal tract disorder characterized by frequent stooling. The use of the aqueous extract of *Annona senegalensis* root and stem bark for the treatment of diarrhoea has been documented. However, limited information exists on the components, mechanism of action and safety. The aim of the study was to carry out biochemical assessment of the antidiarrhoeal fractions from *A. senegalensis* root and stem barks in castor oil-induced diarrhoeal rats. The objectives of the study were to: (i) evaluate the antidiarrhoeal activity of solvents extract of *A. senegalensis* stem and root barks; (ii) fractionate the bioactive extracts; (iii) explore possible mechanism(s) of action of the bioactive fraction(s); (iv) evaluate the toxicity of bioactive sub-fractions; and (v) identify chemical compounds in the bioactive sub-fractions.

Aqueous, n-hexane and dichloromethane stem and root bark extracts of *A. senegalensis* were screened for antidiarrhoeal activity using castor oil-induced diarrhoeal, gastrointestinal tract (GIT) motility inhibition and antienteropooling models. Bioactive aqueous extract was partitioned into hexane, dichloromethane and ethylacetate fractions. Bioactive fractions were fractionated into sub-fractions using column chromatography. The mechanism(s) of action of the bioactive sub-fractions was determined by evaluating their antioxidant properties, intestinal fluid electrolyte concentration, small intestinal Na^+- K^+ ATPase and cyclooxygenase II activities. Kidney and liver function indices were evaluated after administration of bioactive sub-fractions. Active principles were identified using gas chromatography-mass spectrometry. Data were subjected to analysis of variance and Duncan's multiple range test at $p < 0.05$.

The findings of this study were that:

i. aqueous stem (AS) and dichloromethane root (DR) extracts at 100 mg/kg body weight (bw) significantly decreased the number of wet feaces while GIT motility inhibition and antienteropooling activity were significantly increased by aqueous root (AR) and dichloromethane stem (DS) extracts;
ii. inhibition of defecation was highest (85.25%) in the ethylacetate fraction of AS (EFAS), while antienteropooling and GIT motility inhibition was highest in the dichloromethane fraction (DFAR) and ethylacetate fraction (EFAR) of AR respectively;
iii. sub-fraction 1 of EFAS (EFAS1) and fraction 2 of DR (DR2) at 25 mg/kg bw exhibited the highest inhibition of defecation;

iv. anti-motility activity was highest in sub-fraction 2 of EFAR (EFAR2) at 25 mg/kg bw while antienteropooling activity was highest in fraction 3 of DS (DS3) and sub-fraction 1 of DFAR (DFAR1);

v. EFAS1 had the least IC_{50} for diphenyl-1-picrylhydrazyl, highest hydrogen peroxide scavenging activity, significantly increased Na^+-K^+ ATPase activity and decreased cyclooxygenase II activity;

vi. chloride and Na^+ concentration were significantly decreased by EFAR2 and DS3 respectively.

vii. alanine aminotransferase and Na^+ significantly increased while urea decreased at 200 and 400mg/kg bw of DFAR1, EFAR2, EFAS1, DS3 and DR2;

viii. chemical compounds identified were; catechol and ethyl eicosapentanoate in EFAS1; piperidnyloxy, thiazoline and quinazoline in EFAR2; and aldosterone derivatives in DFAR1 and DS3.

The study concluded that antidiarrhoeal principles present in DS3, EFAS1, DR2, EFAR2 and DFAR1 exhibited different mechanism of action. Fourteen days administration of these principles was not safe. Identified bioactive principles may be explored for the development of antidiarrhoeal drugs.

CHAPTER ONE

INTRODUCTION

1.0 Background of the study

Diarrhoea is defined as an alteration in the normal bowel movement, characterized by an increase in the volume or fluidity, frequency and water content of stool (Baldi *et al*., 2004; Thaper and Sanderson, 2004). Ghai *et al*. (2009) defined diarrhoea as the passage of unusually loose or watery stools for at least three times in 24 hours. The World Health Organization (WHO) in 1995 as well as Singh and Verma (2012) emphasized the importance of the change in the consistency (i.e fluidity) of the stool rather than the frequency or number of stool. It is a major cause of child mortality and infant deaths in low- and middle-income countries (Peter and Umar, 2018). In children, it can cause malnutrition, stunted growth and affect intellectual development (Granthan-McGregor *et al*., 2000; Rodriguez *et al*., 2011). Diarrhoea is a common clinical sign due to decreased intestinal absorption of fluids and increased intestinal electrolyte secretion resulting in loose and watery stool (Baldi *et al.*, 2009; Spiller *et al*., 2009). Diarrhoea is thus, a result of a disruption in the balance between the absorptive and secretory processes of the gastrointestinal tract (Whyte and Jenkins, 2012).

Diarrhoea can be classified based on duration, pathophysiology and etiology (Fabel and Shealy, 2014). Based on etiology, it can be classified as infectious or non-infectious. Diarrhoea can be classified as acute, persistent or chronic based on the duration of the symptoms and the pathophysiologic mechanism may fall into one or more of the following clinical groups- secretory, osmotic, exudative and altered intestinal motility (Guerrent *et al*., 2001; Fabel and Shealy, 2014). Infectious diarrhoea is due to infectious etiology. The causative agents for this infection include

viral, bacterial and protozoan sources which may be contacted through contaminated food and drinks (either as a result of feacal-oral contamination or poor water sanitation among others) (Martin and Jung, 2014). Of all the causative agents, *Escherichia coli* and *Rotavirus* are the most common. Other important ones are *Camphylobacter spp*., *Salmonella spp*., *Shigella spp.* and *Vibrio cholera* (Thapar and Sanderson, 2004). Non infectious diarrhoea can occur due to medication, food intolerances (such as certain types of food poisoning, food allergies, lactose intolerance etc) or due to chronic diseases such as cystic fibrosis, inflammatory bowel diseases (IBD) or some drugs such as antibiotics (Carlson *et al*., 2016).

Diarrhoea is usually managed with oral rehydration therapy (ORT) to reduce severe dehydration. Oral rehydration therapy does not actually stop diarrhoea but will prevent dehydration until the causative agent is eradicated (Whyte and Jenkins, 2012). Thus, ORT does not change fluid losses, diarrhoeal output or duration of the diarrhoea (Thiagarajah *et al*., 2015). In high volume watery diarrhoea such as cholera, replacing fluid losses orally still presents a major challenge due to severe fluid loss (Farthing, 2002). In cases of severe fluid losses, drugs with anti-spasmolitic, antimotility, antioxidative, antibiotics, antisecretory, pro-absorptive and/or anti-inflammatory effect (depending on the causative agents) may be used to treat diarrhoea (Wynn and Fougere, 2007). The problems associated with some of the standard therapies include antimicrobial resistance, drug toxicity, constipation and nausea (Rajeev *et al*., 2010).

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years in developing countries. Several plants such as *Acacia nilotica, Indigo spicata* Forski, *Gmelina arborea, Azadiracta indica, Ficus bengalensis, Moringa oleifera* have been reported to have antidiarrhoeal properties (Agunu *et al*., 2005; Sarin and Bafna, 2012). The major setbacks that affect the use of these antidiarrhoeal plants are; lack of standardization (in terms of

methods and precise dosage), extinction of plants due to overexploitaion and the ability to pose other undesirable side effects such as hepatotoxicity and teratogenic effects by *Indigofera spicata* (Njume and Goduka, 2012; Fletcher *et al*., 2015; Woldeab *et al*., 2018). *Annona senegalensis* root and stem bark mixture is used by the people of Adamawa state for the treatment of diarrhoea (Adzu *et al*., 2005).

1.1 *Annona senegalensis* tree

Annona senegalensis popularly known as African custard apple (English) is known as "gwandar daaji" in Hausa, "uburu-ocha" in Igbo and "abo" in Yoruba languages. It is referred to as "arere" in Ilorin. It is found widely distributed in Central and West Africa. It is a shrub, 2- 6 m tall but may reach 11m under favourable conditions. The unripe fruit is green, turning yellow to orange on ripening. The plant possesses several medicinal uses as shown in Tables 1 and 2. The boiled root-bark is used by Hausas of Northern Nigeria for the management of intestinal disorders and the stem bark is chewed by Senegalese for the relief of stomach ache (Awa *et al*., 2012). The plant is being used as an antihelmintic by local livestock farmers in Nigeria (Alawa *et al*., 2003). The stem bark and leaves are used for the treatment of skin cancer and leukaemia (Abubakar *et al*., 2007). A mixture of its stem and root is used by the people of Adamawa State in Nigeria to treat diarrhoea. The plant has been reported to contain several phytochemicals which include tannins (Jada *et al*., 2014); flavonoids (Jada *et al*., 2015); saponins (Afolabi and Afolabi, 2013); alkaloids (You *et al*., 1995); glycosides and steroids (Ijaiya *et al*., 2014); volatile oils (Ngamo *et al*., 2007; anthocyanins (Mpiana, 2012); ascorbic acid and amino acids (Yisa *et al.,* 2010). Nineteen monoterpenes and sesquiterpenes have been identified in the essential oil of its leaves and fruits in Nigeria, with car-3-ene been the major constituent (Ameen *et al*., 2011).

Figure 1.1: ***Annona senegalensis*** **tree**

1.2 Statement of the problem

Diarrhoea disease is the second leading cause of death in children under five years old (WHO, 2017). It accounts for 8.9% of all death in children aged below five years, 42% of these deaths occur in Nigeria and India (Lancet, 2017). Most of the drugs used for the treatment of diarrhoea have adverse effects such as constipation, abdominal discomfort, nausea (Gralla *et al*., 2005) and antimicrobial resistance from the use of antibiotics (Pariwat *et al*., 2008). To overcome this, World Health Organisation (WHO) included the use of herbal medicine in treating diarrhoea (WHO, 2004). Since then several medicinal plants have gained importance in the treatment of diarrhoea.

The ethnomedicinal use of the root and stem barks of *Annona senegalensis* as a remedy for diarrhoea is well established. However, the principle responsible for the antidiarrhoeal property is yet to be identified. This is very essential as some of these active compounds in medicinal plants are potentially toxic, thus the need to identify the active principle responsible for the activity.

1.3 Justification for the study

The unavailability, unaffordability and side effects of orthodox antidiarrhoeal drugs in the rural areas has made the reliance on herbal medicinal plants more popular. *Annona senegalensis* plant is commonly used for the treatment of diarrhoea (Igoli *et al*., 2005). This study will confirm traditional folklore of the use of *A. senegalensis* root and stem bark as an antidiarrhoeal plant. It will also identify the antidiarrhoeal compounds, and their mechanism of action for further pharmacological development. If the compound is new, it will add to the number of natural products from medicinal plants used in the treatment of diarrhoea. The study will also confirm the safety of prolonged usage of antidiarrhoeal fractions obtained from *Annona senegalensis* root and stem barks.

1.4 Objectives of the study

The aim of the study was to isolate and characterize the antidiarrhoeal rich fractions from the extracts of *A. senegalensis* root and stem barks in castor oil-induced diarrhoeal rats and establish the probable mechanism(s) of action and toxicity of the antidiarrhoeal fractions.

1.4.1 Specific objectives

The specific objectives of the study were to:

I. evaluate the antidiarrhoeal activity of hexane, dichloromethane and aqueous root and stem bark extracts of *A. senegalensis* in castor oil-induced diarrhoeal rats;

II. fractionate and partially purify the bioactive extracts of *Annona senegalensis* root and stem barks;

III. explore possible mechanism(s) of action of the bioactive fraction(s) obtained from *A. senegalensis* root and stem barks by investigating;

   a) *in vitro and in vivo* antioxidant activities of the bioactive fraction(s) obtained from *A. senegalensis* root and stem barks;

   b) effect of the bioactive fraction(s) obtained from *A. senegalensis* root and stem barks on lipid peroxidation by determining the concentration of small intestine malondialdehyde (MDA) of castor oil-induced diarrhoeal rats;

   c) antisecretory and proabsorptive activity of the bioactive fraction(s) obtained from *Annona senegalensis* root and stem barks by evaluating electrolyte concentration in intestinal fluid of castor oil-induced rats;

i) effect of the bioactive sub-fraction(s) obtained from *A. senegalensis* root and stem barks on small intestine Na^+- K^+ ATPase activity of castor oil-induced diarrhoeal rats;

ii) effect of the bioactive fraction(s) obtained from *A. senegalensis* root and stem barks on small intestine cyclooxygenase (COX) II enzyme activity in castor oil-induced diarrhoeal rats;

IV. identify active principles in the bioactive sub-fractions obtained from *A. senegalensis* root and stem barks;

V. evaluate the toxicity of bioactive sub-fractions obtained from *A. senegalensis* root and stem barks on antioxidant enzymes, heamatological, liver and kidney function parameters of castor oil-induced diarrhoeal rats.

CHAPTER TWO

LITERATURE REVIEW

2.1 Gastrointestinal tract

The gastrointestinal tract (GIT) is a continuous tube that stretches from the mouth to the anus (Barrette *et al*., 2010). The gastrointestinal tract's accessory organs include the liver, pancreas and gall bladder as shown in Figure 2.1 (Scanlon, 2011). Functionally, the GIT supplies the body including the gut with nutrients, electrolytes and water by performing five distinct functions: motility, secretion, digestion, absorption and storage (Barrette *et al*., 2010). The proper functioning (i.e absorption and secretion) of the GIT critically depends on the concerted activities of ion pumps, channels, symporters etc located on the apical and basolateral membranes of gastric and intestinal epithelial cells, which operate in coupled systems to mediate trans- epithelial transport (Shull *et al*., 2000).

The gut consists of four main layers: the mucosa which comprises epithelial cells (enterocytes, endocrine cells and others), the lamina propria and the muscularis mucosae; the submucosa: two muscle layers- an inner thick circular and an outer thin longitudinal layer, and a serosal layer (Sayegh and Washington, 2012). The parts of the gastrointestinal tract encountered by the meal or its residues are: ileum, ceacum, colon, rectum and anus as shown in Figure 2.1 (Barrette *et al*., 2010). The tract is divided into segments that restrict the flow of intestinal contents to optimize digestion and absorption. These include: the upper and lower oesaphageal sphincters, the pylorus that retards emptying of the stomach, the illleocecal valve that retains colonic contents (including large number of bacteria) in the large intestine, and the inner and outer anal sphincters.

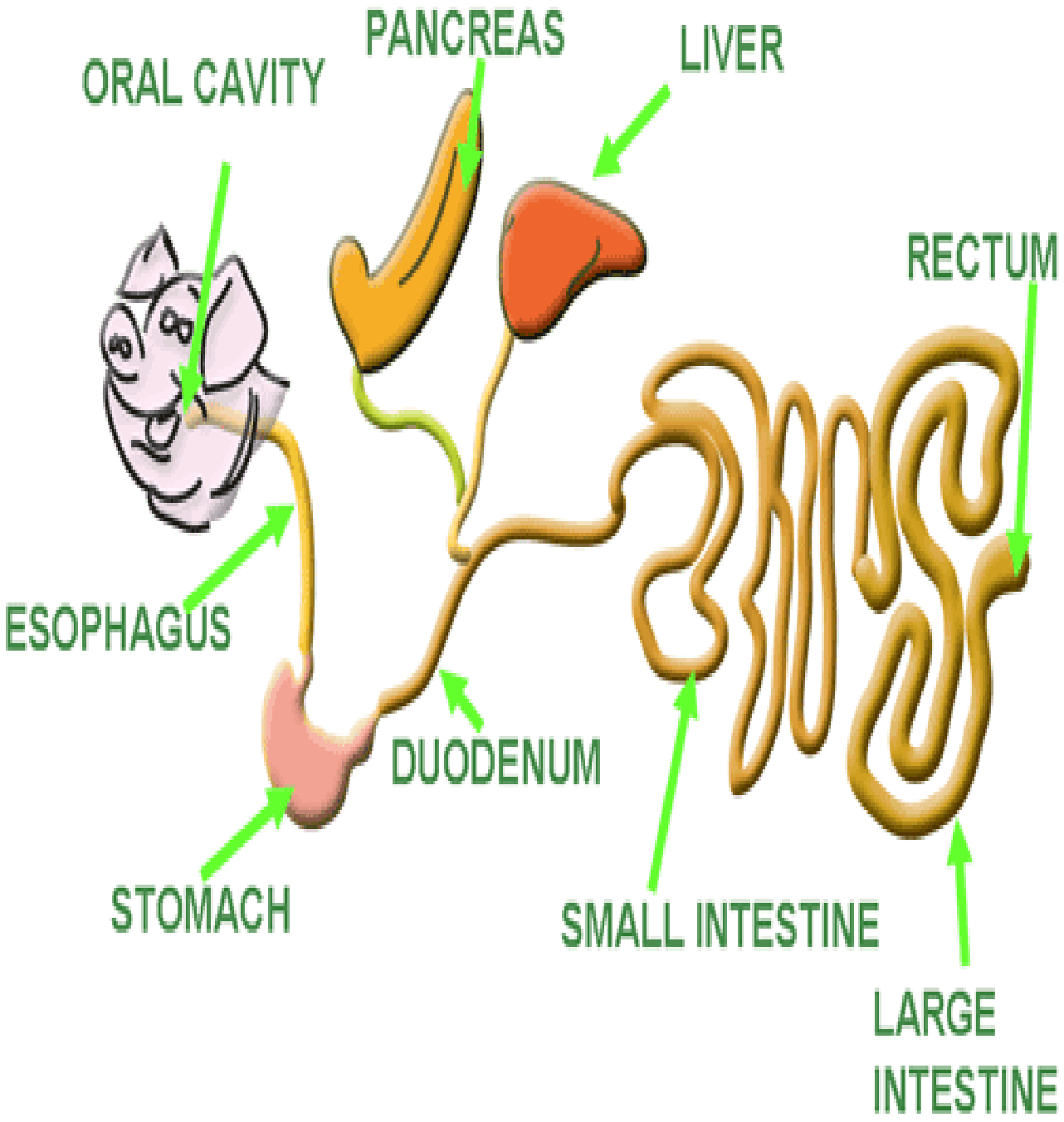

Figure 2.1: Functional parts of the human gastrointestinal tract

Source: Scanlon (2011).

2.1.1 Anatomy of small intestine

The small intestine is a specialized abdominal tubular structure with an adult length of about 6m (Freeman and Thomson, 2004). It can be divided into two segments: duodenum and jejunoileum. The duodenum or proximal portion located near the head, consists of four parts: bulbar, descending, transverse and ascending portions. From the ligament of Treitz, is the more distal small intestine called the jejunoileum that is suspended on a mesentery crossing from left upper to right lower quadrants. The small intestine then enters the large intestine at the ileocecal valve which is a physiolocal spincter that acts to reduce luminal reflux into the small intestine. The proximal and distal parts of the jejunoileum are arbitrarily labeled jejunum and ileum respectively. Jejunum has numerous and thicker folds (plicae circulares) than the ileum (Freeman and Thomson, 2004).

The intestine is composed of functional layers. Adjacent to nutrients in the lumen is a single layer of columnar epithelial cells. This represents the barrier that nutrients must traverse to enter the body (Barrette *et al*., 2010). In general, the intestinal wall is composed of four layers; serosa, muscularis, propria, submucosa and mucosa (Freeman and Thomson, 2004). The epithelial layer maybe divided into villus and crypt regions (Freeman and Thomson, 2004). Villi are fingerlike projections extending into the small intestinal lumen. The mucosal cells in the small intestine have a brush border made up of numerous microvilli lining the apical surfaces (Barrette *et al*., 2010). The microvilli are covered by a layer of epithelial cells with only a few other cell types interspersed (Lynch, 2004). The extensive folding increases surface area providing an optimal situation for absorption. In between the villi, at the base, are extensive pits called crypts of lieberkuhn which contain multipotential cells that can differentiate into absorptive epithelial cells

as they migrate towards the villus tip. A large variety of mucus secreting goblet cells and endocrine cells are found in the crypts (Lynch, 2004). It is lined on its luminal side by a layer that is rich in neutral amino acids and sugars; the glycocalyx. The membranes of the mucosal cells contain glycoprotein enzymes that hydrolyze carbohydrates and peptides (Barrette *et al*., 2010). The mucosa in the intestines contains hormones which includes; enterogastrone, gastric inhibitory polypeptide (GIP), secretin, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), somatostatin and serotonin (Barron, 2010).

The main function of the small intestine is digestion and absorption of nutrients. The main role of small intestine motility is to mix food products with the digestive enzymes to promote contact of chime with the absorptive cells over a sufficient length of bowel and finally to propel reminants into the colon. Hence, the two main types of motility pattern common in the small intestine are migrating motor complex (MMC) and fed pattern (Barron, 2010).

2.1.2 Large intestine

The large intestine extends from the terminal ileum at the ileocecal valve to the rectum. At the terminal ileum, the large intestine becomes the ascending colon, the tranverse colon and the descending colon. The descending colon is followed by the sigmoid colon and the rectum (Scanlon, 2011). The superior and inferior mesenteric arteries and the hypogastric arteries supply blood to the large intestine (Scanlon, 2011). The crypts of lieberkuhn are also present but less extensive in the colon. The crypts contain goblet cells and stem cells as in the small intestine but no endocrine cells (Lynch, 2004). The main function of the large intestine is water absorption. It also absorbs potassium, sodium and chloride, and provides mucus which lubricates the intestinal

wall and holds feaces for further elimination. Motility is slowed down in the colon to allow the absorption of water, sodium and other minerals (Barrette *et al*., 2010).

2.2 Gastrointestinal motility

Digestive and absorptive properties of the gastrointestinal tract depend on a variety of mechanism that soften the food, propel it through the length of the GIT, and mix it with hepatic bile stored in the gall bladder and digestive enzymes secreted by the salivary glands and pancreas. Some of these mechanisms depend on intrinsic properties of the intestinal smooth muscle, others involve the operation of reflexes involving the neurons intrinsic to the gut, refluxes involving the CNS, gastrointestinal hormones, etc (Barrette *et al*., 2010). The general patterns of motility can be divided into four: peristalsis, segmentation and mixing, basic electrical activity (BER), and migrating motor complex (MMC) (Barrette *et al*., 2010). Peristalsis is a reflex response that is initiated when the gut wall is stretched by the contents of the lumen; occurring in all parts of the GIT from the oesaphagus to the rectum. Segmentation and mixing also known as fed pattern occur during meal. This pattern is related to peristalsis, and is promoted by the ENS, but designed to retard the movement of the intestinal contents along the length of the intestinal tract to give ample time for digestion and absorption (Barrette *et al*., 2010). This pattern mainly occurring in the small intestine, mixes intestinal contents with digestive juices. spreading them again and again over the absorptive surfaces of the brush border (Freeman and Thomson, 2004). Migrating motor complex (MMC) occurs during fasting, between periods of digestion. It is characterized by a front of intense spiking activity that migrates down the entire small intestine of luminal content in preparation for next meal (Barrette *et al*., 2010) and to prevent stagnation and bacterial overgrowth (Freeman and Thomson, 2004). It is initiated by motilin (Barrette *et al*., 2010). Basic electrical activity (BER) occurs in the oesophagus and the proximal portion of the stomach. The smooth

muscle of the GIT undergoes spontaneous rhythmic fluctuations in membrane potential between about -65 and -45Mv. The function of the BER is to coordinate peristaltic and other motor activity (Barrette *et al*., 2010).

2.3 Physiology of intestinal secretion and absorption

Absorption in this term refers to the transport of substances from the intestinal lumen through the barrier of the mucosal epithelial cells into the lymphatic system i.e the blood (Caspary, 1992). The small intestine is the most important site of absorption and is constructed such that there is maximum absorptive surface of the epithelium (Caspary, 1992). Absorption is a specific function of the plasma membrane of the intestinal epithelial cells. It involves two processes: movement from the intestinal lumen into the apical (mucosal end of the absorbing cell and movement from the basilar end of the absorbing cell into the subcellular space and subsequently into the circulatory or lymphatic system. The process responsible for the movement across basolateral membranes to the extracellular fluid is often quite different from those responsible for movement across the luminal cell membrane (Barrette *et al*., 2010). The rate of absorption is dependent on factors such as mode of ingestion, gastric emptying, osmolality of the food and the rate of passage (motility) through the small intestine (Caspary, 1987). Motility of the gastrointestinal tract can influence both the rate and extent of absorption.

Secretion of fluid and electrolyte by the gastrointestinal tract helps to lubricate the epithelial surface and protect mucosa from damage as food passes along it (Sidhu and Cooke, 1995). A number of transports and ion channels such as K^+ channels, Na^+- K^+ ATPase, Na^+- K^+-$2Cl^-$ co transporters, cystic fibrosis transmembrance conductance regulator (CFTR) Cl^- channel and other Cl^- channels are involved in intestinal fluid secretion (Barrette and Keely, 2000). Secretion and

absorption can occur in the same epithelial cells but predominantly, absorption occurs in the villi while secretion occurs in the crypts (Thiagarajah *et al*., 2015)

2. 3.1 Absorption of nutrients

2.3.1.1 Carbohydrate

Hexoses and pentoses are rapidly absorbed across the wall of the small intestine (Barret *et al*., 2010). Oligo- and poly- sacharides are not absorbed to any appreciable amount in the small intestine, they have to be broken down to monosacharides before they can be absorbed (Caspary, 1992). The end product of enzymatic hydrolysis of carbohydrates in the small intestine is glucose and fructose (Caspary, 1992). The duodenum and upper jejunum have the highest capacity to absorb these sugars (Lynch, 2004). Glucose and galactose are absorbed via sodium dependent transport across luminal membranes and Na^+-independent facilitative transport across the basolateral membranes; fructose is transported via an apical Na^+-independent transport mechanism (Lynch, 2004). The glucose transporter has three binding sites: one for glucose and the other two binds a molecule of Na^+ each (Loo *et al*., 2013). The inwardly directed downhill gradient of Na^+ provides the driving force for glucose transport. This downhill gradient is maintained by an energy requiring sodium pump (Na^+-K^+ ATPase) located at the basolateral membrane which pumps out Na^+ from the interior of the epithelial cells, thus allowing glucose to accumulate against an electrochemical gradient (Caspary, 1987). Inhibition of the sodium pump leads to inhibition of active glucose transport (Caspary, 1983). This transporter accepts D- glucose and D-galactose and not fructose (Caspary, 1992). Fructose absorption occurs passively via a facilitative glucose transporter (GLUT) family namely; GLUT 5 in the apical membrane and GLUT 2 in the basolateral membrane (Ferraris *et al*., 2018). GLUT 5 is specific for fructose alone while GLUT 2

is also capable of binding glucose and galactose (Douard and Ferraris, 2018). GLUT 2 hs low affinity for fructose (Douard and Ferraris, 2018).

Carbohydrates that cannot be completely absorbed in the small intestine are subjected to bacterial degradation in the colon. This process is an important phenomenon especially with respect to the salvage of energy and the prevention of osmotic diarrhoea (Caspary, 1987). Bacterial fermentation of this carbohydrates results largely in the production of short chain fatty acids (SCFA) which can be reabsorbed efficiently by non-ionic diffusion reabsorbtion in the proximal colon (Luciano *et al*., 1984). Reabsorption of short chain fatty acid (SCFA) reduces the osmotic load of the colon (Caspary, 1992). The reabsorption of short chain fatty acid also serves as an additional energy supply for colonic fluid and electrolyte balance, aids in the absorption of NaCl and helps maintain luminal as well as cytosolic pH (Kuzelmann and Mall, 2002).

2.3.1.2 Protein

Protein digestion products are absorbed as amino acids or as dipeptides by specifc transporters which occur primarily in the duodenum and upper jejenum (Lynch, 2004). L-amino acids are absorbed more rapidly than the corresponding D-isomers. D-amino acids are absorbed solely by passive diffusion whereas most L-amino acids are actively transported out of the intestinal lumen (Barrette *et al*., 2010)

2.4 Active electrolyte absorption

Active electrolyte and fluid absorption can be classified as either nutrient dependent or nutrient independent absorption (Freeman and Thomson, 2004).

2.4.1 Nutrient coupled electrolyte absorption

The single most important process that takes place in the small intestine which makes nutrient absorption possible is the establishment of an electrochemical and concentration gradient of sodium across the epithelial cell boundary of the lumen (Freeman and Thomson, 2004). It was discovered in the 1960's that sugars (glucose and galactose) and amino acids are absorbed across the small intestinal brush border membrane via carriers that couple their movements to that of Na^+ (Schultz *et al*., 1966). The coupling of these nutrients to Na^+ permits the organic solute to be transported uphill i.e from low luminal to higher cell concentration, a gradient opposite to that of Na^+. These organic solutes then move downhill from enterocytes to blood via basolateral membrane carriers that operate independently of ion movements. Fructose is not absorbed by the same channel. The transepithelial Na^+ movement generates a lumen negative transepithelial voltage facilitating paracellular Cl^- and fluid absorption (Kato and Romero, 2011). This is achieved by the Na^+/glucose-linked transporter (SGLT, also known as Slc 5 –proteins) and several Na^+/amino acid and cotransporters (Slc 6, Slc 38 etc) and sodium coupled solute carriers (Kato and Romero, 2011).

The sodium pump (Na^+- K^+ ATPase) then removes Na^+ that entered the enterocytes from the lumen, thereby maintaining a low intracellular Na^+, a high intracellular K^+ and a negative intracellular electric potential (Freeman and Thomson, 2004). The sodium pump provides the potential energy for uphill sugar and amino acid absorption. Absorption of short chain fatty acid (SCFA); propionate, butyrate and acetate serve as an additional energy supply for colonic epithelial cells and has a significant impact on NaCl absorption (Binder and Mehta, 1989). SCFA stimulates electroneutral uptake of Na^+ by acidification of colonocytes and activation of apical Na^+/H^+ and Cl^-/butyrate exchangers (Sellin and De-Soignie, 1998; Rajendran and Binder, 1994).

Chlorine absorption is stimulated by increased HCO_3^- production during SCFA metabolism and stimulation of the apical Cl^-/HCO_3^- exchanger. Absorption of SCFA has a large impact on the regulation of luminal intestinal pH (Kunzelman and Mall, 2002).

2.4.2 Nutrient independent electrolyte absorbtion

In the period between meals when nutrients are absent, sodium and chloride are absorbed together from the lumen by the coupled activity of a sodium hydrogen exchanger (Freeman and Thomson, 2004). The sodium /hydrogen exchanger (NHE) gene family plays an integral role in neutral sodium absorption (Zachos *et al*., 2015). It carries out electroneutral exchange of Na^+ and H^+ (He and Yun, 2010). It is often functionally coupled to the Cl^-/HCO_3^- exchanger (as shown in Figure 2.2). It is reffered to as electroneutral NaCl absorption, and it is a critical mechanism for transepithelial movement of Na^+, Cl^- and HCO_3^- (He and Yun, 2010). The transepithelial Na^+ movement generates a lumen negative (mucosal negative) transepithelial voltage facilitating paracellular Cl^- and fluid absorption (Kato and Romero, 2011). Bulk transport of NaCl in the colonic epithelium is due to this electro-neutral absorption (Kunzelmann and Mall, 2002). The secreted H^+ neutralizes an equal amount of luminal HCO_3^-.

Cell pH adjusts the relative rates of these two exchangers; H^+ extrusion by Na/H exchanger can cause cell alkalization, which stimulates Cl^- entry and HCO_3^- extrusion by Cl^-/HCO_3^- exchanger while the Cl^-/HCO_3^- exchanger increases cell H^+, thereby sustaining Na^+/H^+ exchanger (Freeman and Thomson, 2004). Increase in cell concentrations of cAMP and free Ca^{2+} inhibit the Na^+/H^+ exchanger. Therefore, substances that increase cell concentrations of cAMP and Ca^{2+} such as neurotransmitters and certain luminal substances like bacterial enterotoxins, bile salts and hydrogenated fatty acids can down regulate electrolyte absorption in the small and large intestine

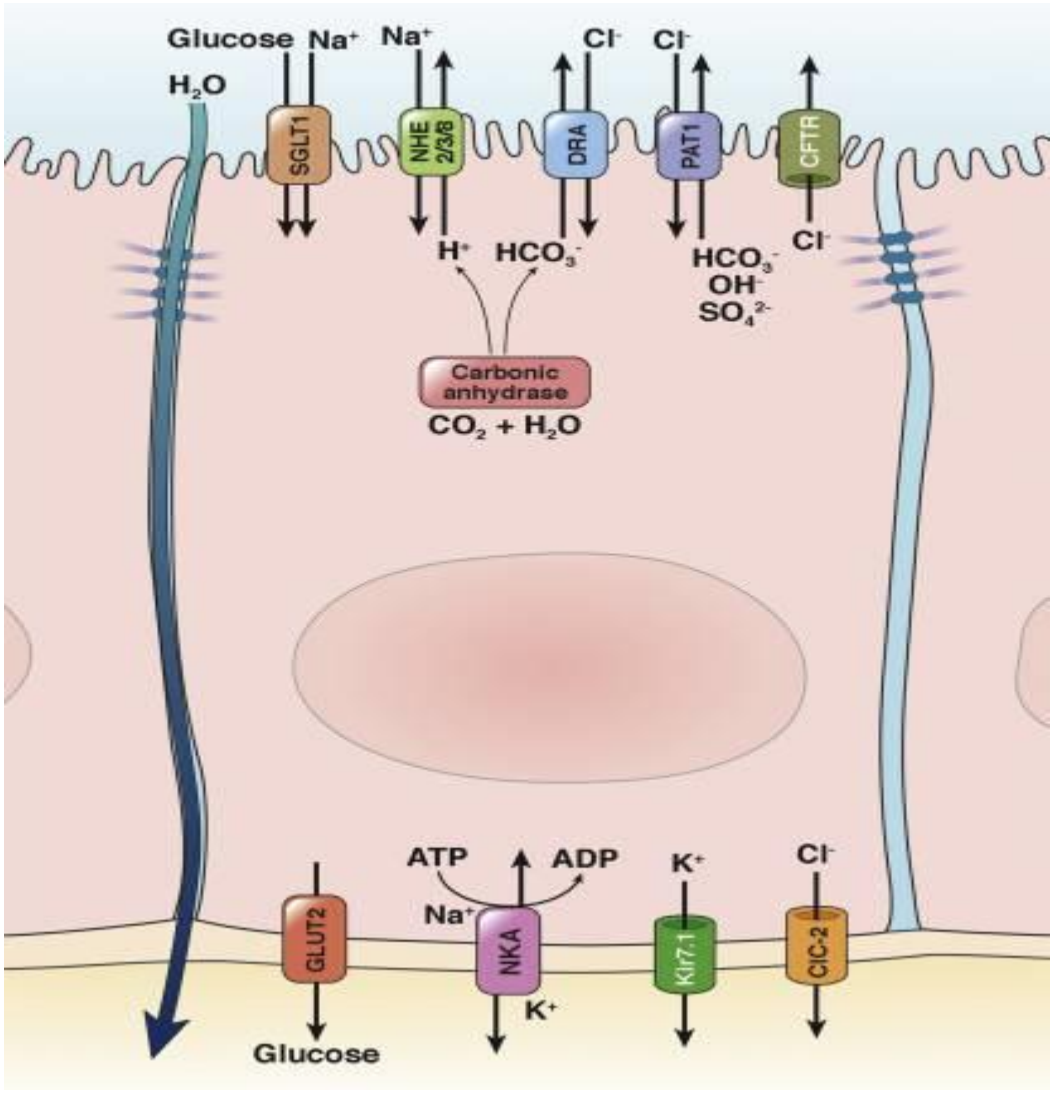

Figure 2.2: The apical Na^+/H^+ exchange in transepithelial sodium, glucose and water absorption

Source: Micheal *et al.* (2017).

via the Na^+/H^+ exchanger (Freeman and Thomson, 2004). Electroneutral absorption is also up and down regulated in response to some G-protein linked receptors, tyrosine kinase coupled receptors and protein kinases (Donowitz *et al*., 2000). Activation of protein kinase C (PKC), Ca^{2+}/Calmodulin – dependent kinase inhibits NHE_3, whereas stimulation of α_1 or β_2- receptors activates NHE_3 (Hall *et al*., 1998; Liu and Gesek, 2001). In the distal colon, an additional electrogenic mechanism for sodium absorption is expressed. In this mechanism, sodium enters across the apical membrane via an epithelial sodium channel, also referred to as electrogenic sodium channel (ENaC) (Kunzelmann and Mall, 2002). ENaC is regulated predominantly by aldosterone (Canessa *et al*., 1994). It is potentially inhibited by amiloride and other related diuretic compounds (Barbry and Hofmann, 1997). In the colon, luminal Na^+ is much lower than in other segments and decreases in a proximal to distal manner generating a lumen negative transepithelial voltage which provides a large driving force for luminal Na^+ uptake via the ENaC (Haas and Forbush, 2000; Kato and Romero, 2011). Sodium that enters the cell are pumped out by the epithelium sodium pump on the basolateral side, while Chlorine that have entered the cytosol via apical Cl^- channels leave the cell via basolateral Cl channels or $Cl^{-/}HCO_3^-$ exchangers (Reddy and Quinton, 1994). Basolateral outward transport of Na^+ by the sodium pump must keep up with the apical Na^+ entry via Na^+ channels (Greger *et al*., 1991). Cystic fibrosis transmembrane regulator (CFTR) regulates both electroneutral and electrogenic absorption of electrolytes in the epithelium (Clarke and Hardine, 1996; Grubb and Boucher, 1997).

2.5 Passive permeability to ions and water

Water is linked to Na^+ absorption, hence flows down the osmotic gradient (Lynch, 2004). There are no large differences in ion concentration because the osmotic equilibration between plasma and lumen is fairly rapid (Freeman and Thomson, 2004). These junctions are more permeable to

cations than anions, therefore the differences in the blood to lumen concentration for Na^+ and K^+ are generally smaller than those for Cl^- and HCO_3^- (Freeman and Thomson, 2004). The colonic epithelium displays lower passive permeability to salt and water (Freeman and Thomson, 2004).

2.6 Active electrolyte secretion

Secretion takes place continuously throughout the small intestine and colon to adjust the local fluidity of the intestinal contents as needed for mixing, diffusion and movement of the meal and its residues along the length of the gastrointestinal tract (Barrette *et al*., 2010). The Na^+-$2Cl^-$-K^+ co–transporter and the Na^+- K^+ ATPase are essential for Cl^- secretion (Kunzelmann and Mall, 2002). Secretory epithelial cells contain Cl^- and K^+ channels in their luminal membranes allowing for secretion of KCl (Kunzelmann and Mall, 2002). Cl^- enters the enterocytes from the interstitial fluid via Na^+-$2Cl^-$-K^+ cotransporters in their basolateral membranes (Barrette *et al*., 2010). Na^+ entering the enterocytes is recycled to the contraluminal solution by the Na/K exchange pump while K^+ entering via the pump and the triple cotransporter diffuses back to the contraluminal side through the K^+ channels (Freeman and Thomson, 2004). The basolateral K^+ channels hyperpolarize epithelial cells and maintain the electrical driving force for Cl^- secretion (Kunzelmann and Mall, 2002). Also, the Na^+ gradient causes Cl^- to accumulate above electrochemical equilibrium. This Cl^- can either; recycle back to the contraluminal solution through the $Na^+/K^+/2Cl^-$ cotransporter or through the basolateral membrane Cl channels or be secreted into the lumen through the luminal membrane Cl channels (CFTR and the Ca^{2+} dependent Cl^- channels) (Freeman and Thomson, 2004; Barrette *et al*., 2010). When chloride is secreted into the lumen, a serosa positive electric potential is generated which provides the driving force for sodium secretion through the paracellular pathway (Freeman and Thomson, 2004). In the resting secretory cell, the luminal Cl^- channels are closed, secretion is activated by different secretagogues that act via different

intracellular messengers (Grotjohann *et al*., 1998; Mall *et al*., 2001) to eventually initiate the opening of Cl^- gate in the luminal membrane of the secretory cell (Freeman and Thomson, 2004).

Some potassium is secreted into the intestinal lumen as a component of mucus via luminal K^+ channels of the enterocytes of the colon (Barrette *et al*., 2010). Bicarbonate is also secreted to the luminal side of the epithelium to produce an intestinal juice of slightly alkaline pH (Kunzelmann and Mall, 2002). Bicarbonate is secreted to the luminal side by either an electrogenic HCO_3^- efflux, a luminal Cl^-/HCO_3^- exchanger or via the $SCFA/HCO_3^-$ exchanger (Hasselblatt *et al*., 2001).

Secretion of electrolytes is paralled by that of macromolecules. The largest macromolecule is mucus, which protects epithelial cells from embrasion and bacterial invasion (Halm and Halm, 2000). Mucus of different composition is released from goblet and crypt columnar epithelial cells upon stimulation with agonists that increase either intracellular cAMP or Ca^{2+}(Epple *et al*., 1997). Jarry *et al*. (2004) reported a link between both prostaglandin E (PGE_2) an adenosine induced Cl^- transport and mucus secretion in differentiated epithelial cells. Figure 2.3 shows ion transporter and channels (both in the secretory and absorptive cell) in the various segments of the gastrointestinal tract.

2.7 Gastrointestinal regulation

Secretion and absorption are controlled by endocrine, paracrine, autocrine, immunologic and neuronal stimuli (Kunzelmann *et al.*, 2000). The gut performs its function by two control system; intrinsic and extrinsic. The intrinsic control system is located between the different layers

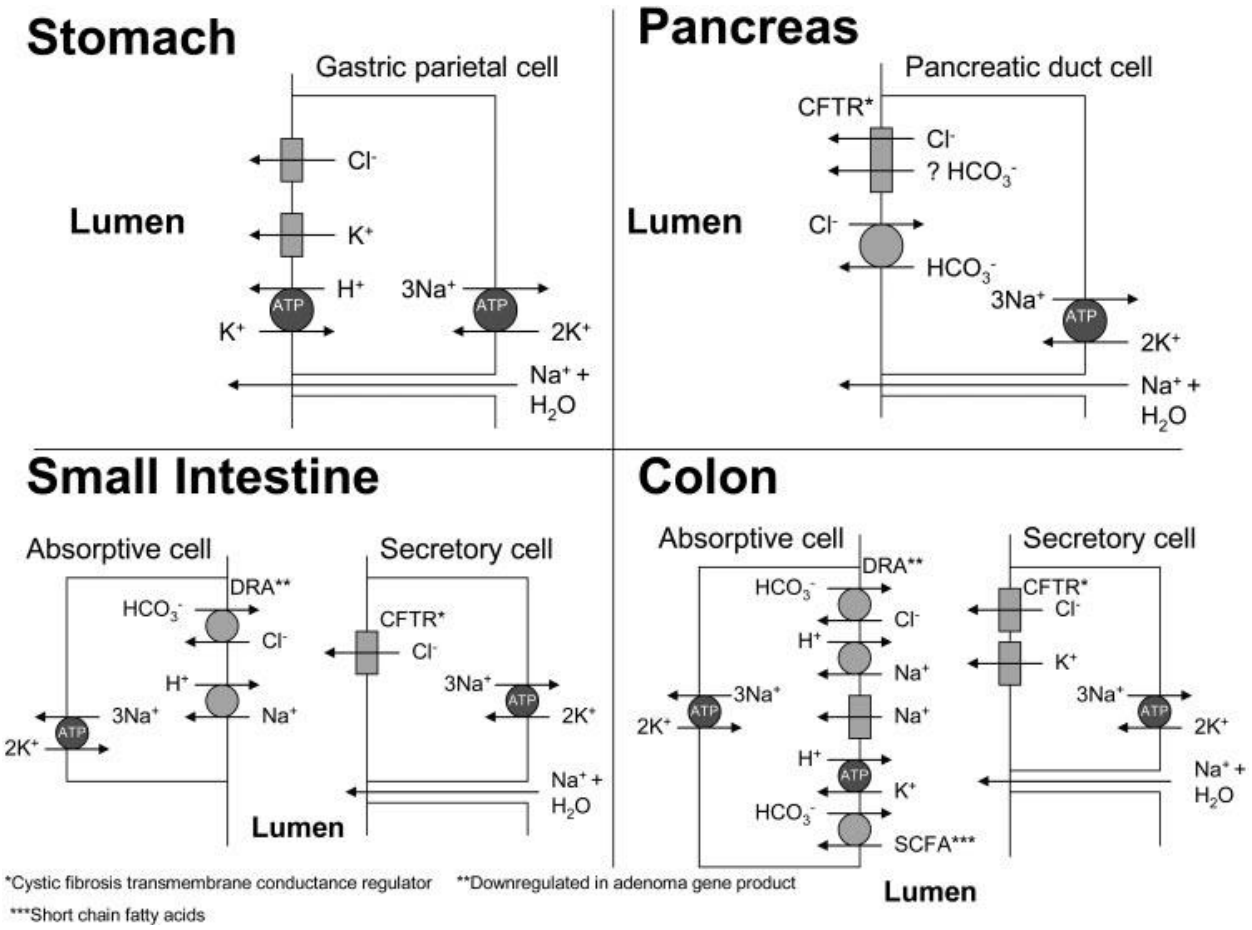

Figure 2.3: Key apical membrane ion transporters and channels in various segments of the gastrointestinal tract

Source: Gennari and Weis (2008).

of the gut, whereas the extrinsic control system resides outside the wall of the gut (Kunzelmann *et al*., 2000). Each of these systems consists of two components namely: nerves and endocrine secretions (Kunzelmann *et al*., 2000). The intrinsic system has two components: the enteric nervous system (ENS) and gut hormones while the extrinsic control system consists of the vagus and splanchnic nerves and the hormone; aldosterone (Kunzelmann *et al*., 2000). Immune mediators or neurotransmitters may act directly on epithelial cells or may induce the release of mediators by other cell types (Kunzelmann *et al*., 2000). The gastrointestinal tract contains the highest number of immune mediators in the body that interact with the intrinsic control system of the gut to regulate some functions of the gastrointestinal tract, including motility and secretion (Sayegh and Washington, 2012).

Secretion and absorption is regulated via the second messengers; Ca^{2+}, cGMP or cAMP and also other mediators such as diacylglycerol and PKC (Kato and Romero, 2011). Enterotoxins, neurotransmitters or drugs increase intracellular cAMP, Ca^{2+} or cGMP (Donowitz and Welsh, 1986; Donowitz *et al*., 1989). The primary targets of intracellular cAMP are PKA and eventually CFTR (Webber *et al*., 1999). CFTR Cl^- channels are activated by PKA dependent phosphorylation and binding of ATP (Kunzelmann, 1999). cGMP induces Cl secretion by stimulation of CFTR through *c*GMP regulated protein kinase G type II. It may also exert additional inhibitory effects on the phosphodiesterase which leads to increase in *c*AMP (Screiber *et al*., 2000). Studies have shown that nitric oxide (NO) is able to increase intracellular *c*GMP and trigger the release of PGE_2 (Kamosinka *et al*., 1997; Vaandrager *et al*., 1997). Increase in intracellular Ca^{2+} activates the basolateral K^+ channels and enhance the exit of luminal Cl^- (Warth *et al*., 1999). cAMP, cGMP and/or Ca^{2+} inhibit NHE_3 (at the apical brush border membrane) and thus activates electrogenic Cl^-

and fluid secretion by activating CFTR on the apical membrane (Kato and Romero, 2011). These signals have no effect on glucose coupled Na^+ absorption (Donowitz and Asarkof, 1982).

2.7.1 Regulation of sodium /hydrogen exchanger (NHE_3) by cAMP, $Ca^{2+}$and cGMP

Sodium /hydrogen exchanger (NHE_3) is one of the main targets of the second messengers; cAMP, Ca^{2+} and cGMP (Kato and Romero, 2011). This inhibition requires both second messenger activated protein kinases and the NHE-regulatory factor (NHERF) scafford proteins; NHERF 1, NHERF 2 and NHERF 3 (Cinar *et al*., 2007; Broere *et al*., 2009). Ca^{2+} mediated inhibition of NHE_3 requires NHERF 2 and NHERF 3 while cGMP rquires NHERF 2 only (Cha *et al*., 2005; Cinar *et al*., 2007). cAMP activates protein kinase A (PKA II) which anchors to NHERF 1 and 2 through the cytoskeletal protein known as ezrin, cytovillin or villin 2, to directly phosphorylate multiple serine residues in the cytoplasmic domain of NHE_3 (Yun *et al*., 1997; Lamprecht *et al*., 1998). Ca^{2+} induces membrane localization of protein kinase C (PKC), which interacts with NHERF2 and α-actinin (Lee-kwon *et al*., 2003) and phosphorylates NHE_3 (Wiederkehr *et al*., 1999). NHE_3 is also activated by decreased intracellular pH or increased cellular metabolism (Turner and Black, 2001).

2.7.2 Regulation of NHE_3 and Na^+/K^+ ATPase by glucocorticoid, serum glucocorticoid regulated kinase (SGK-1) and P13 kinase (P13K)

Electroneutral sodium absorption, Na^+/K^+ ATPase and sodium glucose transporter (SGLT1) are activated in the small intestine by glucocorticoid (Grahammer *et al.*, 2006). NHE_3 is regulated by glucocorticoid via serum-glucocorticoid regulated kinase (SGK-1) (Yun *et al*., 2002), by inducing SGK-1 gene expression through stimulation of P-13 Kinase (Loffing *et al*., 2006; Musch *et al*., 2008). P13K synthesizes phosphatidylinositol 3,4,5- triphosphate, which activates 3-

phosphoinositol-dependent protein kinase-1 (PDK-1) to directly phosphorylate SGK-1 and activate it. Mineralocorticoids also activate NHE_3 in the proximal colon (Lang *et al*., 2006).

2.7.3 Neuroendocrine regulation

Parasympthetic (cholinergic) neurons, cholinergic secretomotor neurons and VIP secretomotor neurons mediate the secretory neural effect (Kato and Romero, 2011). Acetylcholine, a predominant neurotransmitter of the enteric nervous system, is central to the regulation of intestinal water transport (Keely, 2011). Acetylcholine and VIP inhibit electroneutral NaCl absorption and induce electrogenic Cl^- secretion in the small intestine epithelium (Kato and Romero, 2011). Acetylcholine interacts with muscarinic receptors (M_3) which increase cellular Ca^{2+} (Keely, 2011) and VIP receptors which increase cAMP (Cooke, 2000) to mediate secretory response. Acetylcholine induced secretion in response to SCFA occurs in the colonic epithelium and results into a non neuronal acetycholine-mediated secretory response (Sha *et al*., 2009; Keely, 2011).

Substance P, (an 11-amino acid peptide) whose receptor is neurokinin 1 receptor (NK1), has a secretory effect (Cooke *et al*., 1998). The cholinergic and non- cholinergic secretomotor neurons are involved in the secretory effect (Cooke *et al*., 1998). Serotonin also known as 5-OH-tryptamine (5-HT) has prosecretory effect which is mediated predominantly by cholinergic and VIP secretomotor neurons (Kato and Romero, 2011). It also modifies the brush border architecture to reduce NHE_3 function (Gill *et al*., 2008). It is secreted from the enteric nerves in the myentric plexus and enterochromaffin (EC) cells (Hansen and Witte, 2008; Spiller, 2008).

Somatostatin exists in tissues in 2 forms; somatostain 14 and somatostatin 28 (Barrette *et al*., 2010). It is secreted by extrinsic and intrinsic neurons and by D-cells in the pancreatic islet and

gastrointestinal mucosa (Patel, 1999; Barrette *et al*., 2010). Somatostatin inhibits the secretion of gastrin, VIP, glucose, amino acids and tryglycerides. Its analogue activates electroneutral NaCl absorption in the intestine (Barrette *et al*., 2010). Norepinephrine (secreted by the adrenal gland or sympathetic nerve terminal) and other catecholamines increase electroneutral NaCl absorption and decrease electrogenic Cl^- secretion by intestinal mucosa (Kato and Romero, 2011). Catecholamine act at the α- adrenergic receptor coupled with the G proteins G_{12} and G_{13} to antagonize cAMP production (Remaury *et al*., 1993). Opiods cause small intestine absorption (Sternini *et al.*, 2004). Neuropeptide Y inhibits VIP induced cAMP synthesis and Cl^- secretion as well as prostaglandin elicited Cl^- secretion (Kato and Romero, 2011). The antisecretory effect of neuropeptide is mediated by norepinephrine (α-adrenergic receptors) in the ileum (Anthone *et al*., 1991). This absorption effect is mediated by γ_1 receptors in the colonic epithelium (Cox, 2007).

2.7.4 Regulation by the paracrine/endocrine system

Guanylin is a gastrointestinal polypeptide that binds to guanylyl cyclase. Stimulation of guanylyl cyclase increase the intracellular concentration of cGMP which in turn cause increased Cl^- secretion into the lumen (Barrette *et al*., 2010). It acts predominantly in a paracrine fashion to regulate fluid movement (Barrette *et al*., 2010). Peptide YY (PYY) is released from endocrine L-cells of the ileal mucosa following a meal. PYY inhibits secretion in the small intestine (Friel *et al*., 1986).

2.7.5 Regulation by the immune system

Histamine, the most important immune mediator, is secreted by activated mast cells. In the colon, it binds to histamine H_1 receptor to activate electrolyte secretion via an increase in intracellular Ca^{2+} (Cooke and Wang, 1994). Another important secretagogue in the mammalian colon is the

prostaglandin E_2 (PGE_2) synthesized in the arachidonic acid pathway. It is an inflammatory mediator (Kuzellman and Mall, 2000). Stimulation of PGE_2 receptors induces generation of intracellualar cAMP which activates Cl^- secretion and inhibits electroneutral NaCl absorption (Penn *et al*., 1994). T-cell activation via tumor necrosis factor α (TNF-α) and interferon –γ (IFN-γ) action inhibits intestinal Na^+ absorption, increases Cl^- secretion, increase intestinal permeability and causes diarrhoea (Kato and Romero, 2011).

2.8 Diarrhoea

Diarrhoea occurs when there is an imbalance between absorption and secretion of water and electrolytes. This can be be due to decreased absorption or increased secretion (Whyte and Jerkins, 2012). Osmosis, acive secretion, exudation and altered motility are the main causses of diarrhoea (Field, 203).

2.8.1 Types of diarrhoea

Diarrhoea can be basically classified into three major classes; secretory, osmotic and motility disorder diarrhoea as shown in Figure 2.4.

2.8.1.1 Secretory diarrhoea

Secretory diarrhoea is a type of diarrhoea caused by net increase of chloride or bicarbonate and fluid into the lumen (Navaneethan and Giannella, 2010). The result of increase in electrolyte secretion is the decrease in the absorption of sodium and water (Navaneethan and Giannella, 2010). It occurs when there is a disturbance in the balance between absorption and secretion (Velazquez *et al*., 2012). The most common cause of secretory diarrhoea is infection, which usually affect the small intestine (Schiller, 1999). Non-infectious secretagogues include chemicals produced by certain types of cancer, prostaglandins produced in inflammation and substances not

well absorbed such as fatty acids and bile acid (Bliss *et al*., 2006). Secretory diarrhoea persists in spite of fasting (Mercadante, 1995)

2.8.1.2 Osmotic diarrhoea

Osmotic diarrhoea occurs when osmotically active but poorly absorbable solutes in the intestine draw water into the lumen because the gastrointestinal mucosa is not able to maintain an osmotic gradient (Sellin, 2001). The cause of osmotic diarrhoea include; decreased enzymatic availability (lactose intolerance), a genetic abnormality that decreases or eliminates the ability of the body to absorb certain nutrients; poorly absorbable sugars (eg sorbitol, mannitol and lactose) (Strasinger and Di-Lorenzo, 2008); and poorly absorbable solutes (magnesium, sulfates and phosphates) (Hammer *et al*., 1989). This feacal matter creates a negative osmotic gradient causing leakage of more fluid into the gut, increasing stool volume and resulting in diarrhoea (Field, 2003). This type of diarrhoea stops after fasting or termination of ingestion of offending agent (Mercadante, 1995).

2.8.1.3 Diarrhoea due to deranged motility (functional diarrhoea)

Disorders in motility that accelerate transit time could decrease absorption by decreasing the time for the luminal contents to be in contact with the epithelium for absorption. This results in diarrhoea even if the absorption process is proceeding normally (Richard, 2006b). Many endocrine diarrhoea are not only due to effects on intestinal electrolyte transport but also due to accelerated intestinal motility (Field, 2003: Shah, 2004). On the other hand, slow transit or decreased motility in the small intestine due to smooth muscle damage and autonomic neuropathy such as in diabetes mellitus may cause diarrhoea because of bacterial growth (Field, 2003).

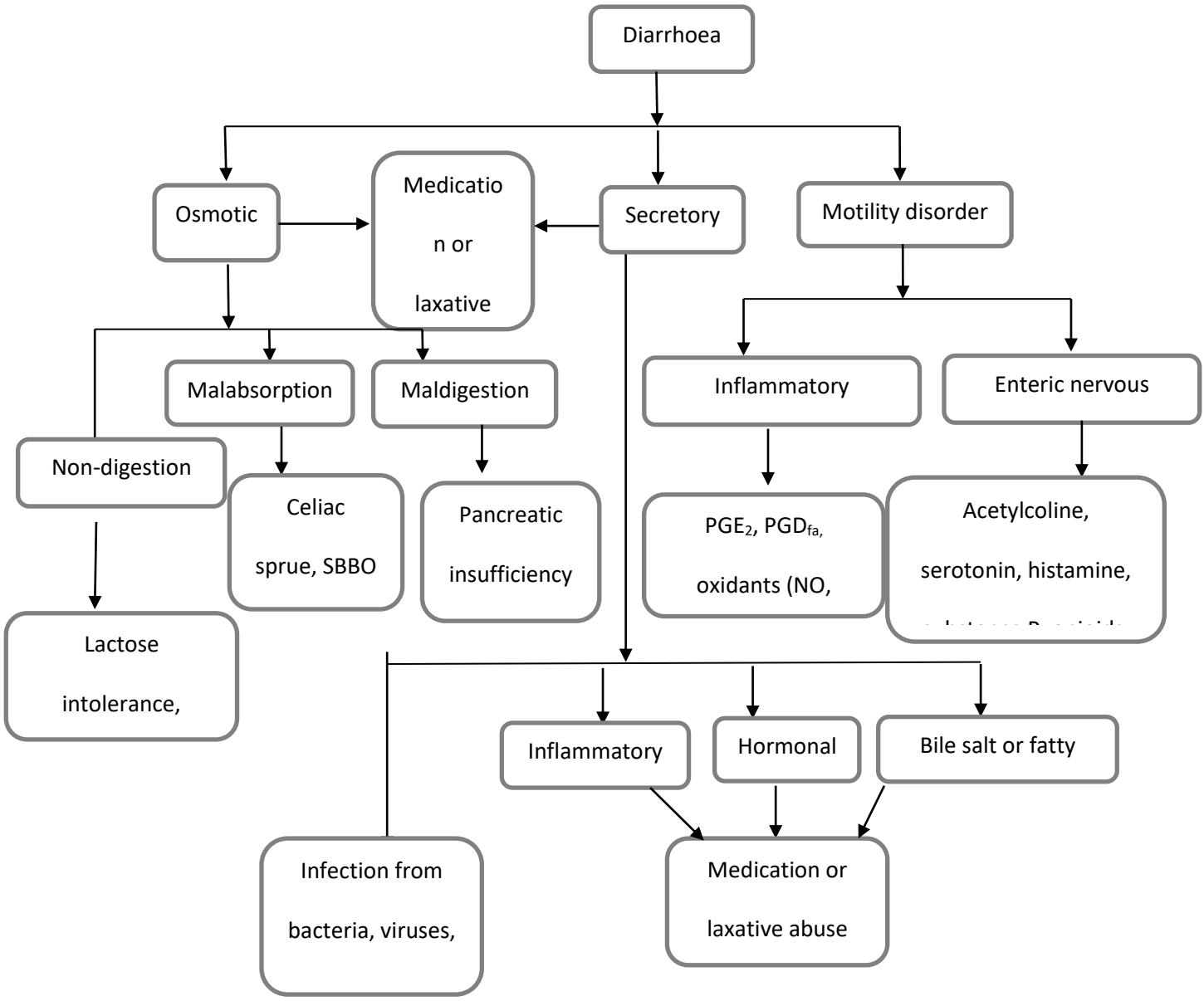

Figure 2.4: Classification of diarrhoea

Source: Sellin (2001)

2.8.1.4 Inflammatory or exudative diarrhoea

Inflammatory bowel disease (IBD) is one of the most common causes of inflammatory diarrhoea (Binder, 2009). Infections also cause inflammatory diarrhoea. Most of the pathogens causing inflammatory diarrhoea act by producing mucosal damage as well as by stimulating intestinal secretion. This primarily occurs in the distal small bowel or the colon (Pawlowski *et al*., 2009). Cytokines and eicosanoids initiated by inflammation down regulate the ion transporters in the colon and small bowel resulting in Na^+ malabsorbtion (Amasheh *et al*., 2004; Thevarajah *et al*., 2005).

2.8.1.5 Drug - induced diarrhoea

Some drugs cause diarrhoea either as side effect or as the desired effect of the drug. Antibiotics cause diarrhoea by altering the bacterial flora in the colon resulting in impaired colonic salvage of malabsorbed carbohydrates (McFarland, 2006) and emergence of pathogenic organisms such as *Clostridium difficili* (Bergogne-Berezin, 2000). Some broad spectrum antibiotics such as ampicillin, metronidazole and neomycin slows down colonic tansit (motility) due to the depletion of the gut microbiome and the associated downregulation of metabolic signaling (Ge *et al.*, 2017). In addition, some antibiotics such as erthyromycin has prokinetic action on the GIT, mediated through motilin receptor stimulating potential (Annese *et al*., 1992). The removal of some commensal organisms by antibiotics could result in decreased carbohydrate digestion (Saunders and Wiggins, 1981) which leads to accumulation of osmotically active substances in the intestinal lumen, thus causing osmotic diarrhea (Young and Schimidt, 2004).

Prostaglandin analogs (e.g misoprostol) can affect the intestine at many levels including permeability, motility, transport of electrolytes as well as affecting peptides that stimulate secretion. Similarly, chemotherapeutic drugs cause diarrhoea by decreasing the rate of proliferation of the enterocytes (Sellin, 2001). The oral hypoglycaemia medication used for the management of diabetes mellitus vis metformin, acarbose and orlistat may induce diarrhoea as a side effect (Gould and Selin, 2009) while the recommended dietary material such as the non-digestive sweetness (sorbitol, mannitol and D-xylose) induce osmotic diarrhoea (Forgacs and Pastel, 2011).

2.9 Pathophysiology of diarrhoea

There are several pathophysiological mechanism of diarrhoea which include microbial and parasitic infections (Hodges and Gill, 2010), stress (oxidative and physical) (Soderholm and Perdue, 2001), altered GIT motility as a result of damage to enteric nervous system (ENS) and intestinal inflammation; dysfunctional immunity (Schulzke *et al*., 2009), disrupt GIT integrity and neurohumoral mechanisms (Vitali *et al*., 2006). It can also occur as a symptom of other diseases such as inflammatory bowel syndrome (IBS), ulcer, cholera, diabetes mellitus and HIV ((Forgacs and Pastel, 2011).

2.9.1 Mechanism of infectious diarrhoea

Some enteric pathogens attach and alter the surface of the invaded cell. Attachment of these pathogens to the apical surface of the enterocyte destroys the adjacent epithelial microvilli and thus forms a pedestal-like structure from the accumulation of cytoskeletal proteins, such as actin, beneath the site of attachment (Thapar and Sanderson, 2004). These pathogens produces enterotoxin which may damage the intestinal epithelial cells resulting in loss of host epithelial cell

layer i.e decrease in intestinal surface area thus decrease fluid absorbtion rate (Laohachai *et al*., 2004). This may in turn cause change in osmotic permeability, resulting from mucosal destruction and changes in fluid homeostatis through the toxins action on ion channels (Ramaroa and Lereclus, 2006). Many pathogens induce diarrhoea by distorting the tight junction complex (Searls, 2000).

A well understood pathogenic mechanism of infectious diarrhoea is that caused by *Rotavirus.* *Rotavirus* attaches to mature enterocytes on the upper third of the villus via Ca^{2+}- dependent endocytosis. *Rotavirus* impairs glucose absorption via two mechanisms: it impairs Na^{+}-glucose co-transport by inhibiting SGLT-1; intestinal diasaccharidases (sucrose, maltase and lactase) responsible for cleaving monosacharides for Na^{+}- glucose co-transport located on the brush border membrane of enterocytes are markedly attenuated in rotaviral enteritis thus causing malabsorbtion and osmotic diarrhoea (Collins *et al*., 1988). *Rotavirus* secretes an enterotoxin called NSP_4, which increases intracellular Ca^{2+} and a resultant increase in chloride secretion (Moeser and Blikslager, 2007). NSP_4 directly damages or interferes with the function of the apical Na^{+}-glucose transporter SGLT as shown in Figure 2.5, further exacerbating diarrhoea (Halaihel *et al*., 2000). It also inhibits Na^{+}/K^{+} ATPase function, thus impairing both NaCl and Na^{+} -linked nutrient transport (Moeser and Blikslager, 2007).

2.9.2 Mechanism of secretory diarrhoea

The basic pathophysiology involves secretion of chloride ions which is accompanied by movement of sodium and water into the intestinal lumen (Hoque *et al*., 2012; Patel *et al*., 2013). Intestinal colonization by pathogenic micro-organisms is a major cause for acquired secretory diarrhoea

(Guerrant *et al*., 1999). Diarrhoeal pathogens and their toxins induce secretory diarrhoea by simultaneously stimulating active Cl^- secretion and inhibiting Na^+ absorbtion across the apical

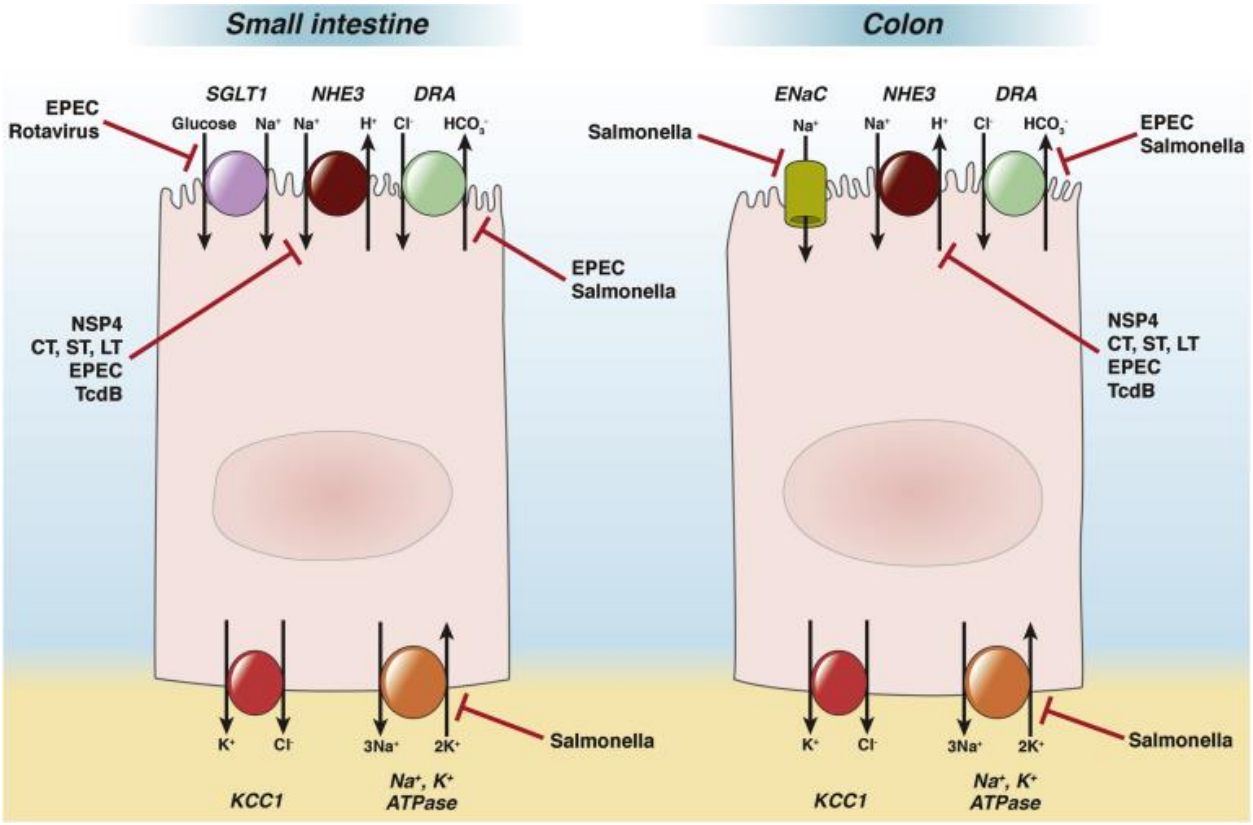

Figure 2.5: *Rotavirus* inhibition of sodium glucose transporter (SGLT)

Source: Das *et al.* (2018)

membrane of electrolyte with resulting massive fluid and electrolyte loss into the GIT (Schuier *et al*., 2005). Bacterial and viral toxin activates luminal cystic fibrosis transmembrane conductance (CFTR) (Kunzelmann and Mall, 2002). CFTR is a cyclic adenylate monophosphate (cAMP)-activated Cl- channel expressed in epithelial cells in the intestine and other fluid-transporting tissues (Thiagarajah and Verkman, 2003). It plays a central role in secretory diarrhoea. Most causes of secretory diarrhoea alter the second messenger system through alteration in cAMP, cGMP or intracellular calcium regulated ion transport pathways as shown in Figure 2.6. Alterations in these mediators cause CFTR – mediated Cl^- secretion and inhibition of small intestinal coupled Na^+-Cl^- transport (Navaneethan and Giannella, 2010). CFTR inhibits ENaC and NHE_3 (Kunzelman, 1999). Therefore, activation of CFTR leads to inhibition of both electrogenic absorbtion via ENaC and electroneutral absorbtion via NHE_3, further contributing to excessive secretion (Kunzelmann and Mall, 2002).

An example of infectious secretory diarrhoea is that caused by *Vibrio cholerea*. Cholera toxin (CT) is the primary virulence factor of *V. cholerea* (Thiagarajah and Verkman, 2005). It is composed of an A subunit and 5 binding (B) subunits arranged in a pentameric ring (Sixma *et al*., 1991). B subunit binds to GM_1 ganglioside receptors that is located at the apical membrane of intestinal epithelial cells (IEC) and becomes internalized (Muanprasat and Chatsudthipong, 2013). Inside the cell, the A subunit causes activation of adenylyl cyclase by activation of the stimulatory G protein Gsα, resulting in elevated concentration of cAMP (Lencer, 2001). Elevation of cAMP

results in activation of the cystic fibrosis transmembrane (CFTR) Cl^- channel in the apical plasma membrane (Kunzelmann and Mall, 2002) as shown in Figure 2.8. This subsequently causes CFTR

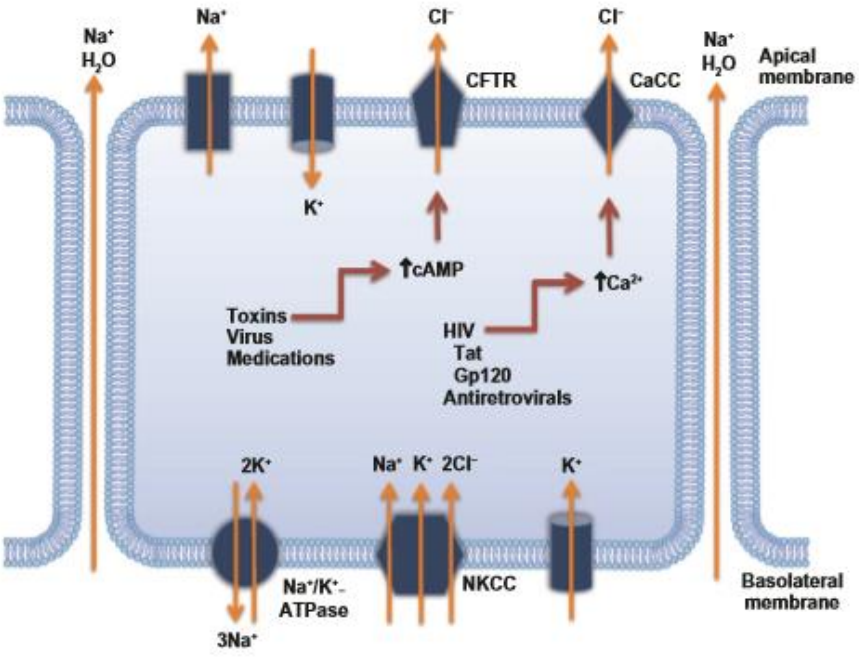

Figure 2.6: Mechanism of secretory diarrhoea

Source: Patel *et al.* (2013)

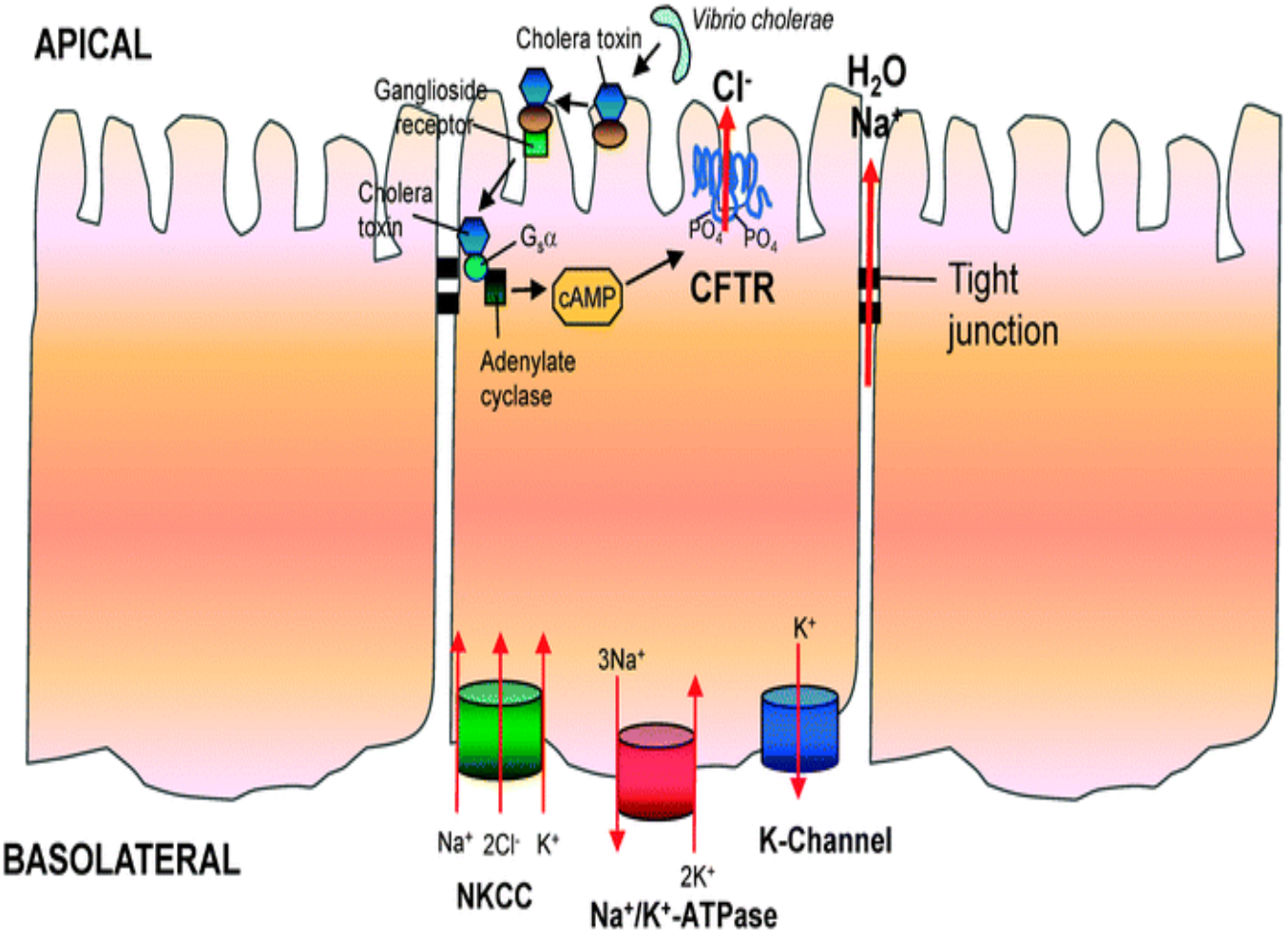

Figure 2.7: Cholera-toxin induced diarrhoea

Source: Das *et al*. (2018).

– mediated Cl^- secretion and the inhibition of electroneutral Na^+ absorption, which in turn causes net fluid secretion and secretory diarrhoea (Field, 2003).

2.9.3 Inflammation and diarrhoea

The physiological defense reponse of the body to any kind of injurious stimulus is called inflammation (Zamora *et al*., 2000). Inflammation is controlled by the presence of chemical mediators each with a specific role in the inflammatory process. These mediators may be exogenous, arising from bacteria or chemical irritants, or endogenous in origin (Zamora *et al*., 2000). The most important endogenous mediators identified include; the vasoactive amines; histamine and serotonin, the kinin system, the arachidonic acid metabolites like prostaglandins and leukotrienes, platelet activating factor, neuropeptides, reactive oxygen species, and inflammatory cytokines (Trowbridge and Emiling, 1997). Some infectious enteric pathogens elicit inflammatory cascade and mediator so as to manifest diarrhoea (Guttman and Finlay, 2009). The immune system assist with the killing of pathogenic micro-organisms and/or the removal of harmful and cell debris (Stables *et al.,* 2010) through the release of numerous pro-inflammatory cytokines, chemokines, eicosanoids such as PGE_2 etc (Conforti *et al*., 2008). The activation of these inflammatory mediators can induce changes in gut motility, neuronal functionality and hydro-electrolyte movement with resultant diarrhoea (Gelberg, 2007).

2.9.3.1 Mechanism of action of inflammatory modulated diarrhoea

There are several mechanisms involved in inflammatory modulated diarrhoea which includes;

2.9.3.1.1 Epithelial barrier disruption

Gastrointestinal epithelium barrier provides a physical defence against hostile environment within the intestinal lumen (Blikslager, 2010). This defence is achieved by interactions between serosal barrier components including the adhesive mucuos gel layer, the mucosal immune system and the tight junction (TJs) (Schenk and Mueller, 2008). The tight junction (multiple protein complexes located around the apical end of the lateral membrane of the epithelial cells) acts as a selective/semi permeable paracellular barriers allowing movement of ion, solutes and water through the intestinal epithelium while also preventing the translocation of luminal antigens, micro-organisms and their toxins into the mucosa (Groschwitz and Hogan, 2009; Guttman and Finlay, 2009). Inflammatory cytokines, reactive oxygen species and pathogen disrupts the intestinal tight junction (TJ) barrier (Guttman *et al*., 2006). Impaired intestinal TJ cause an increase in intestinal permeability resulting in diarrhoea (Schenk and Mueller, 2008).

2.9.3.1.2 Reduced absorption capacity

There is decrease in the total absorptive surface area in an inflamed intestinal tract due to brush border shortening which results into water and electrolyte malabsorbtion (Cotton *et al*., 2011). This creates an osmotic gradient that draws water into the small intestinal lumen and consequently osmotic diarrhoea (Gelberg, 2007; Schulke *et al*., 2009).

2.9.3.1.3 Stimulatory effects on electrolyte secretion

Inflammatory mediators are known for their stimulatory effects on electrolyte secretion and their inhibitory effects on NaCl absorption (Traynor *et al*., 1993). Some of the inflammatory mediators

(e.g PGE_2, LTB_4 and histamine) serves as secretagogue causing excessive secretion of chloride resulting in secretory diarrhoea (Field, 2003).

2.9.3.1.4 Structural changes

Inflammation also cause structural changes to the enteric nervous system (ENS) that ranges from axonal damage to neuronal death (Stanzel *et al*., 2008). Neurotransmitter synthesis, storage and release are also altered by these changes and thus contribute to the altered intestinal motility during the onset and progression of many GIT disorder (Stanzel *et al*., 2008).

2.9.3.2 Prostaglandin and its pathophysiology of diarrhoea

Prostaglandins (PG) are important inflammatory mediators (Botting, 2006). Cyclooxygenase (COX) or prostaglandin endoperoxide synthase (PGHS) is the key enzyme responsible for the synthesis of prostaglandins. COX possess two active site; a cyclooxygenase active site which converts arachidonic acid to the endoperoxide and a peroxidase active site that converts the peroxide- prostaglandin G (PGG_2) to another endoperoxide, PGH_2 (Bothing, 2006). This PGH_2 is then converted to prostaglandin (PG), prostacyclin and thromboxane A_2 by specific synthases (Botting, 2006). There are two isoforms of the cyclooxygenase; cycloxygenase (COX) I and cycloygenase (COX) II. COX I is constitutive and has clear physiological functions which includes its cycloprotective role in the gastrointestinal mucosa (Blobaum and Marnett, 2007). COX II is inducible and is upregulated by a wide variety of stimuli such as cytokines, mutagens, oncogens, growth factor and tumor promoters. COX II is the target of anti-inflammatory drugs because it is responsible for the biosynthesis of prostaglandins in acute inflammatory (Blobaum and Marnett, 2007).

PGE_2 elicit net secretion of fluid, chloride and bicarbonate ions and inhibit sodium absorption (Hirokawa, 2004; Fujii *et al*., 2016). Fujii *et al*. (2016) reported that PGE_2 binds to Gs coupled receptor and mediates cAMP intracellular signalling indicating that the PG-induced active electrolyte secretion is cAMP induced. Prostaglandins affect the motility of the gastrointestinal tract (Karaki and Kuwahara, 2004). It also inhibits intestinal Na^+-K^+ ATPase (Oliveira *et al*., 2009).

2.9.4 Oxidative stress and diarrhoea

During normal metabolic functions, highly reactive compounds called free radicals are generated in the body (Krishnamurthy and Wadhwani, 2012). Free radicals are electrically charged molecules i.e they have an unpaired electron which causes them to seek out and capture electrons from other substances in order to neutralize themselves (Krishnamurthy and Wadhani, 2012). The presence of unpaired/unstable electrons in their outermost shell makes them highly reactive (Noori, 2012). Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called free radical or reactive oxygen specie (ROS). ROS and Reactive Nitrogen specie (RNS) may also be of exogenous origin (Vaskova *et al*., 2012).

Under normal aerobic conditions, cells are always threatened with the effect of ROS (Krishnamurthy and Wadhani, 2012). They could damage the cell and cause death. Human cells possess a robust antioxidant defense systems made up of several antioxidant enzymes in conjuction with a number of low molecular weight antioxidant molecules such as ascorbic acid and vitamns which is used to neutralize these free radicals (Arise *et al.,* 2019). Oxidative stress results whenever there is imbalance between ROS production and antioxidant defence, and may through a series of events deregulate the cellular functions leading to various pathological

conditions (Chitra and Pillai, 2002). Reactive oxygen specie and reactive nitrogen specie are generated from a number of enzymatic and non-enzymatic sources (Orient *et al*., 2007). Generally, most of the ROS and RNS produced by cells occur as a consequence of normal aerobic metabolism, oxidative burst from phagocytes which are part of the mechanisms by which bacteria and viruses are killed, foreign proteins (antigens) denatured and xenobiotic metabolized (Krishnamurthy and Wadhwani, 2012). Consequently, chronic inflammation, infections, exposure to allergens and exposure to drugs or toxins, pesticides and insecticides may all contribute to an increase in the body's oxidant load (Krishmaurhy and Wadhwani, 2012). There is a strong correlation between oxidative stress and some diseases (Arise *et al*., 2019).

Reactive oxygen species (ROS) play a key role in electrolyte loss and enhanced mucosal permeability occurring in chronic diarrhoea (Darmon *et al*., 1993). Some other reactive species such as HOCl and NH_2Cl can act as secretagogues to evoke the release of acetylcholine or other neurotransmitters, thus stimulating the ENS to increase contractility or motility of intestinal tract (Gaginella *et al*., 1992). Oxidative stress increases intestinal water secretion which contributes to the persistence of diarrhoea (Lindley *et al*., 1994). Studies by Nieto *et al*. (2000) shows that chronic diarrhoea impairs or markedly decreases intestinal antioxidant defense system. Oxidative stress induces lipid peroxidation. Also, the production of inflammatory mediators such as prostaglandins, leukotrines, pro-inflammatory cytokines, interleukins, interferons etc leads to changes in the physiological integrity of the cell membrane (Conforti *et al*., 2008*).*

2.9.4.1 Lipid peroxidation and diarrhoea

Lipid peroxidation is one of the consequences of uncontrolled oxidative stress (i.e imbalance between the pro-oxidant and antioxidant levels in favour of pro-oxidants) in cells, tissues and

organs. It is an accumulated effect of reactive oxygen species which leads to deterioration of biological systems (Badmus *et al*., 2011). It is considered the main molecular mechanisms involved in the oxidative damage to cell structures and in the toxicity process that leads to cell death (Repetto *et al*., 2012). It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers (Dianzani and Barrera, 2008). Lipid peroxidation occurs in both plants and animals (Repetto *et al*., 2012).

Lipid peroxidation *in vivo* has been implicated as the underlying mechanisms in numerous disorders and diseases such as cardiovascular diseases, cancer, neurological disorders and aging (El-beltagi and Mohammed, 2013), various gastro-intestinal pathological conditions (Bhattacharya *et al*., 2014), inflammatory (Farooqui and Farooqui, 2011), infectious (Repetto *et al*., 1996) and nutritional diseases (Repetto *et al*., 2010b). Lipid peroxidation is a primary mechanism for intestinal cellular malfunction and can destroy the capacity of membranes to maintain ionic gradient resulting in an aberration in ion transport, particularly affecting potassium efflux and sodium/calcium influx (Dudeja and Ramaswamy, 2006). Lipid peroxidation causes intestinal mucosal damage and cellular malfunction which can destroy the capacity of membranes to maintain ionic gradients. This can result into an aberration in ion transport, particularly affecting potassium efflux and sodium/calcium influx (Dudeja and Ramaswamy, 2006). The production of arachidonic acid metabolite in lipid peroxidation process can also contribute to intestinal dysfunction including diarrhoea.

Lipid peroxidation may be enzymatic and non-enzymatic (Repetto *et al*., 2010b); enzymatic lipid peroxidation is catalyzed by the lipoxygenase family, a family of lipid peroxidation enzymes that

oxygenates free and esterified polyunsaturated fatty acid (PUFA), generating as a consequence, peroxy radicals. Non enzymatic lipid peroxidation and formation of lipid peroxides are initiated by the presence of molecular oxygen and is facilitated by Fe^{2+} (Repetto *et al*., 2010a).

Lipid peroxidation involves three major stages: initiation stage, propagation stage and termination stage (Catala, 2006). The initiation stage of lipid peroxidation occurs when the oxidant abstracts hydrogen from polyunsaturated fatty acids of the cell membrane forming a radical lipid; the propagation stage involves the rearrangement of the lipid radical to form conjugated dienes and can interact with oxygen to form lipid peroxide radicals. The peroxide radicals can in turn abstract hydrogen from lipids to produce lipid hydroperoxides which can be oxidized via reaction with reduced (Fe^{2+}) to lipid alkoxyl radicals and lipid peroxide, thus continuing the chain reaction of lipid peroxidation. In the termination stage, the lipid peroxide radicals in the presence of reduced metals can be degraded to form highly reactive and potent toxic aldehyde such as malondialdehyde (MDA) as shown in Figure 2.8. This chain reaction is usually terminated by endogenous antioxidant molecules by forming non-reactive substances (Catala, 2006).

2.9.4.1.1 Malondialdehyde

Malondialdehyde (MDA) is a secondary lipid peroxidation product. It is an end product generated by decomposition of arachidonic acid and larger polyunsaturated fatty acid (PUFA) through enzymatic and non enzymatic processes (Esterbauer *et al*., 1991). MDA can be generated by

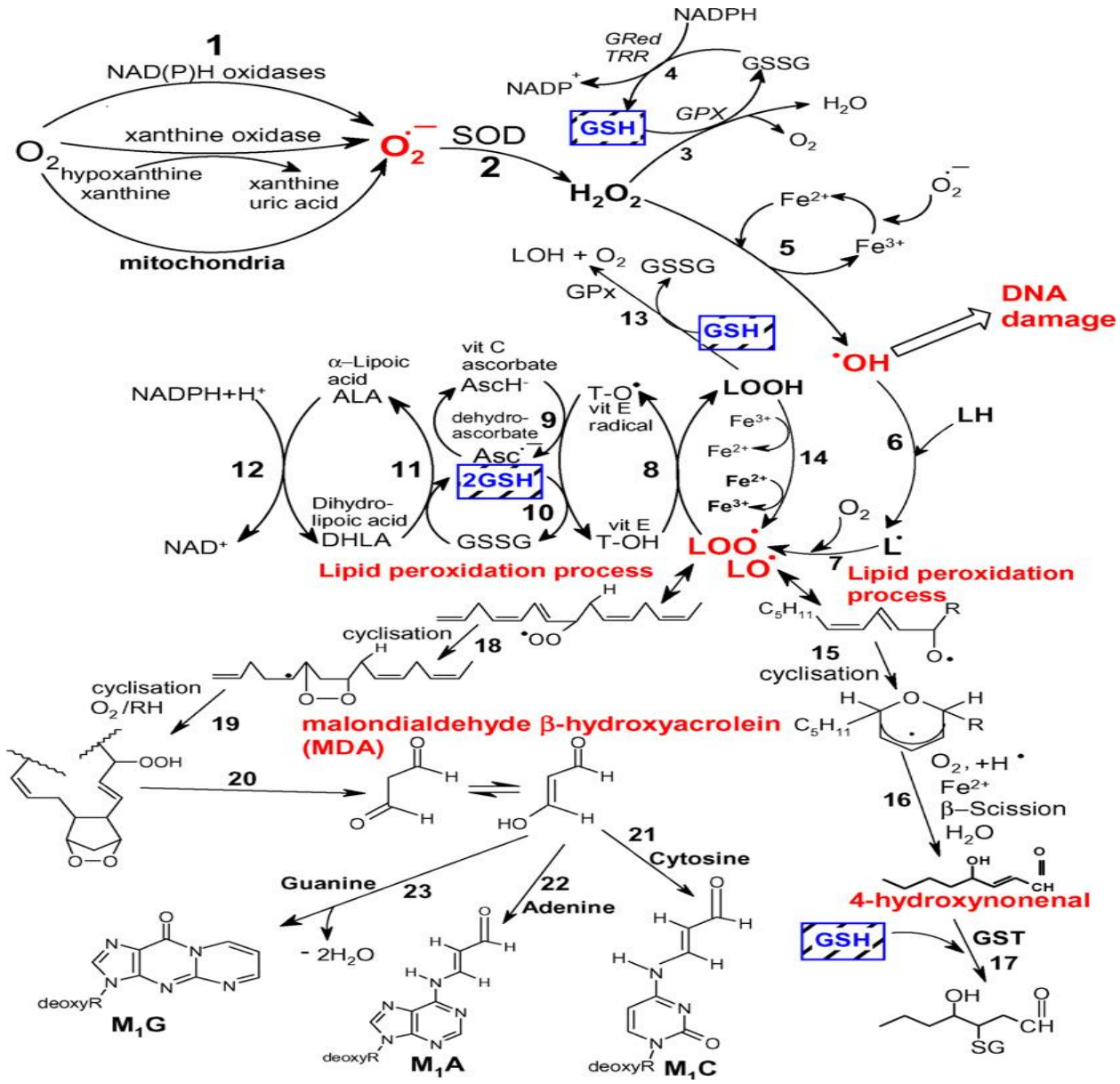

Figure 2.8: Pathways of reactive oxygen specie formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, ,lipoic acid) in the management of oxidative stress

Source: Valko *et al.* (2007).

enzymatic processes during the biosynthesis of thromboxane A_2 (TXA_2). In the non enzymatic process, lipid peroxidation can undergo cyclization by intramolecular radical addition to the cis-double bond homoallylic to the peroxyl group to form a new radical. This free radical formed after cyclization can cyclize again to form bicycle endoperoxide; structurally related to prostaglandins and undergo cleavage to produce MDA. Once formed, MDA can be enzymatically metabolized or can react with cellular and tissue proteins or DNA to form addults, resulting, in biomolecular damages (Ayala *et al*., 2014).

Malonaldialdehyde (MDA) is widely used as a convenient biomarker for lipid peroxidation of omega 3 and omega-6 fatty acids because of its facile reaction with thiobarbituric acid (TBA) (Esterbauer *et al*., 1990). The TBA test is based on the reactivity of TBA towards MDA to yield an intensely coloured chromagen fluorescent red adduct (Sinnhuber *et al*., 1958).

2.9.4 Nitric oxide and diarrhoea

Nitric oxide (NO) is generated from L-arginine by a family of nitric oxide synthases (NOS) (Nathan and Xie, 1994). There are three isoforms of NOS namely; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Nathan and Xie, 1994). The iNOS isoform in contrast to ENOS and nNOS is not expressed constitutively but is induced in a wide variety of cells by various stimulators such as bacterial lipopolyscaharides (LPS), gamma interferon and interleukin (Nathan and Xie, 1994). The potential sources of NO in the gut are the endothelial cells, the intrinsic intestinal tissue (mast cells, epithelium, smooth muscle, neurons) residing and infiltrating leucocytes (neutrophils and monocytes), reduction of luminal gastric nitrates and to a lesser extent denitrification of commensal bacteria (Salzman, 1995). This small signaling molecule is involved in a variety of physiological and pathophysiological actions (Moncada and Higgs,

1995). Physiologically NO mediates several signalling pathways and modulates crucial physiologic processes that are necessary for maintaining normal tissue homeostasis, for example, apoptosis, DNA repair and cell cycle (Hussain *et al*., 2005). In the gut, nitric oxide exhibits a variety of biological actions which includes; platelet aggregation and adhesion (Moncada and Higgs, 1995), relaxation of adjacent non vascular smooth muscle in response to peptidergic nerve stimulation (Synder *et al*., 1991) and modulation of transepithelial ion secretion (Gaginella *et al*., 1995).

The association of iNOS induction with diarrhoea has been suggested for many infectious diarrhoea such as those caused by *Shigella*, *V. cholerea,* and *Rotavirus* (Mourad *et al*., 1999; Fassano, 2001; Borghan *et al*., 2007). iNOS expression is elevated in response to reproductive rotavirus infection and the elevation correlates with occurrence of diarrhoea. Therefore, NO plays a role in rotavirus – induced diarrhoea (Borghan *et al*., 2007).

Nitric oxide is involved in water transport either by acting directly on the epithelium or indirectly by stimulating neuronal refluxes, or by stimulating the release of other agents from the epithelium or the enteric nervous system that can modify water transport or by affecting mucosal blood flow (Mourad *et al*., 1999). Nitric oxide activates soluble guanylate cyclase resulting in an increase in cGMP, a potent activator of intestinal secretion (Brasitus *et al*., 1976; Murad *et al*., 1978). It also stimulates cyclooxygenase activity directly, independent of cGMP (Wilson *et al*., 1996). NO donors induce secretion of fluid and electrolytes into the human colon (Stack *et al*., 1996). NO has proabsorptive effect in the small intestine (Mourad *et al*., 1999). It activates basolateral K^+ channels to mediate the proabsorptive effect (Shirgi- Degen and Beubler,, 1996).

Nitric oxide can combine with free radicals such as peroxide to form the highly toxic peroxynitrite (Radi *et al*., 1991). This could alter normal physiological regulation of electrolytes transport in the small intestine and colon (Mourad *et al*., 1999). NO and other nitrogen oxides could stimulate intestinal secretion by the nature of their free radical structure (Daniel *et al*., 1994, Gaginella *et al*., 1995). NO exerts its antisecretory effect by opening chloride channels (Tamai and Gaginella, 1993). Physiologically, NO promotes fluid absorption but in pathophysiological states, in high concentrations, it is capable of evoking net secretion (Mourad *et al*., 1992). The inhibition of the pathological rather than protective effects may yield therapeutic benefits. Therefore, the development of selective NOS inhibitors is important to dissect out the different aspects of NO function (Mourad *et al*., 1999).

2.9.6 Enteric nervous system and diarrhoea

The enteric nervous system (ENS) is a collection of neurons in the gastrointestinal tract (Furness and Costa, 1980). The ENS closely resembles the central nervous system (CNS) because it coordinates all the activities or function of the gastrointestinal system. It communicates with the CNS through the autonomic network, to provide additional central control for local enteric function (Jones and Blickslager, 2002). The ENS controls the motility, exocrine and endocrine secretions, microcirculation of the gastrointestinal tract and is also involved in regulating immune and inflammatory processes (Lundgren *et al*., 2000). The ENS is involved in the regulation of basal electrolyte and fluid secretion and plays a prominent role in hypersecretory activity associated with many infectious and non-infectious diarrhoea (Jones and Blikslager, 2002). The cross talk among pathogens, epithelium, lamina propia mesenchymal cells and inflammatory cells is integrated by enteric nervous and is central to the pathophysiology of diarrhoea. Also, the ENS

plays a role in regulating local and distant motility alterations which contributes to diarrhoea (Jones and Blikslager, 2002).

The five primary targets of the enteric nerves of the gut are smooth muscle cells responsible for GIT motility, mucosal secretory cells and inflammatory cells, gastrointestinal endocrine cells, the gastrointestinal micro-vasculature and the immunomodulatory and inflammatory cells of the gut (Raj and Ikuo, 1996). Disorders of the ENS may result in secretory, inflammatory or immunological dysfunction of the gut (Raj and Ikuo, 1996). Studies have shown that the mechanism by which cholera toxin stimulates secretion is more complex than stimulating epithelial Cl^- secretion via activation of cAMP -dependent pathway (Raj and Ikuo, 1996; Jones and Bilkslager, 2002). The enteric nervous system (ENS) has a major role in the secretory action of cholera toxin (Raj and Ikuo, 1996). Enteroendocrine cells play a role in cholera toxin secretory responses (Nilson *et al*., 1983). Enteroendocrine cells have receptors for cholera toxin and respond to the toxin by producing 5-hydroxytrptamine (5-HT). The 5-HT sensory neurons trigger a reflux mural pathway that stimulates epithelial Cl^- secretion through the VIPergic secretomotor nerves (Jones and Bilkslayer, 2002). This neural reflex –dependent mechanism also occurs in other enteric bacterial toxins (Jones and Bilkslayer, 2002). The interneurone or secretomotor neurons in the myentric and not the submucosal plexus appeasr to participate. In most intestinal secretory states, the nerve reflexes in the ENS are stimulated to cause intestinal fluid losses (Lundgren *et al*., 2000). Gastrointestinal motility disorders result from developmental abnormality of elements of the ENS such as the interstitial cells (Jones and Bilkslayer, 2002)

Inflammation associated with infectious or non-infectious enterocolitis triggers hypersecretion by a mechanism that involves the ENS (Jones and Blikslager, 2002). Inflammatory response directly

triggers enterocyte secretion through a PG-dependent intestinal hypersecretion (Granella, 1979). Activated neutrophils are important effector cells in the pathophysiology of secretory diarrhoea in infectious entercolitis. These neutrophils (or other granulocytes and mast cells) are recruited to sites of infection to release cytokines and other inflammatory mediators that induce Cycloxygenase activity and subsequent prostaglandin production in mesenchymal fibroblasts. Prostanoids produced by mesenchymal fibroblasts are then capable of stimulatory enterocytes directly (PGE_2) or via enteric nerves (PGI_2). Inflammatory cytokines and other mediators such as 5-HT and histamines may also directly trigger the enteric nervous system to induce epithelial secretion through a PG independent mechanism (Jones and Blikslager, 2002). The inflammatory mediator – 5-HT, activates sensory submucosal neurons to stimulate reflexive epithelial secretion (Jones and Blikslager, 2002). Thus, mucosal secretion is induced by mediators released by neutrophils, mast cells, macrophages, or other cells in inflamed tissues by removing sympathetic brake from secretomotor neurons to the intestinal crypts (Jone and Blikslager, 2002).

2.9.6.1 Regulation of fluid transport by enteric nervous system

Regulation of enterocyte secretion by enteric nervous system (ENS) is complex and includes both sympathetic and parasympathetic pathways and mural reflex network (Jones and Blikslager, 2002). Of all the neurotransmitters and neuromodulators, acetylcholine and vaso-active intestinal peptide (VIP) are the major neurotransmitters stimulating enterocyte secretory activity in both the large and small intestines (Cook and Reddix, 1994). VIP stimulates Cl^- rich fluid secretion by triggering increase in cAMP via its receptors on the epithelium in a similar manner as cholera toxin while acetylcholine activates Cl^- secretion by a calcium dependent signal generated by activation of muscarinic receptors on epithelial cells.

Basically 2 types of PGs; PGI_2 and PGE_2 regulate enterocyte secretion. PGI_2 is approximately 50% inhibited by atropine (Argenzio and Armstrong-Rghoaads, 1996). Also, the secretory response to PGI_2 is partially reduced by vasoactive intestinal peptide (VIP) antagonist (Argenzio and Armstrong-Rghoads, 1996). The neural network underlying the effect of PGI_2 secretion involves ganglionic interneurons and both cholinergic and VIPergic motor neurons that synapse with epithelial cells (Jones and Blikslager, 2002). The stimulation of enteric secretion by PGE_2 is independent of the ENS. The stimulation is a receptor mediated stimulation of the enterocytes (Argenzio and Armstrong-Rghoads, 1996). The enteric nervous system is a target and a potential site of action for antidiarrhoeal drugs (Jones and Bilkslager, 2002).

2.10 Treatment and management of diarrhoea

The main aim of managing diarrhoeal diseases is prevention of excessive water loss, electrolyte and acid-base disturbances; provide symptomatic relief; treat curable causes of diarrhoea and manage secondary disorders causing diarrhoea (Amerine, 2006). There are various ways of managing and /or treating diarrhoea. They can be grouped into pharmacological and non-pharmacological treatment.

2.10.1 Non-pharmacological treatment

2.10.1.1 Oral rehydration therapy (ORT)

Oral rehydration therapy is the first line of treatment for diarrhoea (Singh and Verma, 2012). It is the cornerstone of treatment to prevent dehydration especially for acute watery diarrhoea (Langsten and Hill, 1995). The oral rehydration solution simply consists of electrolytes (Sodium and Pottasium chloride) and glucose, which promotes water absorption (De-Hostos *et al*., 2011).

WHO recommended oral rehydration salt consists of 75 mEq/L sodium, 75 mmol/L glucose, 65 mEq/L chlorides, 20 mEq/L potassium and 10 mEq/L citrate, having a total osmolarity of 245 osm/L (WHO, 2002). Potassium is given to replace the large potassium loses associated wih acute diarrhoea, preventing serious hypokalemia; citrate corrects base deficit acidosis, while the absorption of glucose enhances/promotes the absorption of sodium and water in the small intestine.

2.10.1.2 Zinc supplement

Numerous studies have shown zinc supplementation significantly reduce the severity and duration of diarrhoea (Roy *et al*., 2007; Haider and Bhutta, 2009). Additional study revealed that zinc supplementation resulted in 13% reduction in the mortality of children due to diarrhooea (Yakoob *et al*., 2011). WHO and UNICEF recommended zinc supplementation (20 mg/day for children older than six months and 10 mg/day for infants > 6 months old for 10-14 days) for the treatment of diarrhoea among the children (USAID *et al*., 2005).

2.10.2 Pharmacological treatment

2.10.2.1 Antibiotics

These antimicrobials can either be bactericidal/fungicidal (i.e they can kill microbes directly) or bacteriostatic (i.e they prevent micobes from growing). Some of these antimicrobials interfere with protein synthesis process of the bacteria e.g chloramphenicol, erythromycin, streptomycin and tetracycline. Chloramphenicol and erythromycin binds to the 50s portion of the 70s prokaryotic ribosome to inhibit formation of peptide bond in the growing polypeptide chain: tetracycline interferes with the attachment of tRNA carrying the amino acids to the growing polypeptide chain,

while aminoglycosides like streptomycin and gentamicin interferes with the initial steps of protein synthesis by changing the shape of the 30s portion of the 70s prokaryotic ribosome (Tortoral *et al*., 2004). This intereference causes the genetic code on the mRNA to be read incorrectly (Tortoral *et al*., 2004). Some antibiotics damage the cell membrane by distorting the cell surface, which usually cause interference in the permeability of the plasma membrane and ultimately results in the loss of important metabolites from the microbial cell (Talaro, 2005).

Antimicrobials used in the management of antidiarrhoeal are either unsafe or ineffective because of their indiscriminate use which leads to emergence of resistant strains (Mittal and Mathew, 2001). Antimicrobials are supposed to be used only for specific enteric pathogens like *Vibrio cholerea, Shigella dysenteriae, Giardia intestinalis, Entamoeba histolytica etc* (Singh and Verma, 2012). The use of antibiotic in the treatment of acute infectious diarrhoea should be carefully weighed against the cost, the risk of adverse reactions, harmful eradication of normal intestinal flora and the increase of antimicrobial resistance (Guerrant *et al*., 2001; Lori, 2008).

2.10.2.2 Probiotics (Bio-therapeutic agent)

These are live microbial food supplement which beneficially affects the host by improving the intestinal microbial balance. Potentially, probiotics maintain or restore gut micro-encology during or after antibiotic treatment through the following mechanisms; receptor competition, competition for nutrients, inhibition of epithelial and mucosal adherence of pathogens and translocation (Scalolaferri *et al*., 2012), lowering colonic pH thereby favouring the growth of non-pathogenic species (Hampel *et al*., 2012), stimulation of immunity or production of antimicrobial substances (Friedman, 2012). The most common probiotics used in the treatment of acute diarrhoea includes;

Saccharomyces boulardi, *Bifidobacterium longum*; *lactobacillus acidophilus* and *lactobacillus bulgaricus* (Bergogne-Berezin, 2000).

2.10.2.3 Antisecretory drugs

These drugs inhibit secretion. These drugs include racedotril (an enkaphalinase inhibitor) (Field, 2003); octreotide (somatostatin analogue) (Jensen, 1999) and crofelmer (calcium sensitive chloride channels [CaCCs] inhibitor) (De-Hostos *et al*., 2011). Somatostatin analogues block hormone production by the tumor in the case of hormone secreting neoplasms (Jensen, 1999). Racedrotil prevents the degradation of endogenous enkephalins, thus reducing hypersecretion of water and electrolytes into the intestinal lumen (Szajewka *et al*., 2007). Crofelemer is an addition to the currently available drugs for the management of secretory diarrhoea. It was first approved to treat HIV/AIDS patients on antiretroviral therapy (Yeo *et al*., 2013). It has inhibitory effect on both the cystic fibrosis transmembrane conductance regulator (CFTR) and the calcium activated chloride channels which are responsible for chloride secretion and subluminal hydration (Yeo *et al*., 2013).

2.10.2.4 Antimotility drugs (antiperistaltic)

This group of drug prolongs intestinal transit time, which increases the time of contact with the gut epithelium reducing the frequency and volume of stool (Field, 2003). The two drugs in this category are loperamide and diphenoxylate HCl. Both loperamide and diphenoxylate are effective in relieving symptoms of acute non-infectious diarrhoea (Hanauer, 2008). Loperamide is an opiod receptor agonist and acts on the μ-opioid receptors in the myentric plexus of the large intestine, to decrease its activity, by decreasing the tone of the longitudinal smooth muscle and increasing the tone of circular smooth muscles of the intestinal wall. Thus, increasing the amount of time substances stay in the intestine, allowing for more water to be absorbed out of the feacal matter

(Kasper *et al*., 2005). Loperamide has mild anti-secretory properties (AL-Abric *et al*., 2005). The major side effects of loperamide include abdominal pain, bloating, nausea, dry mouth, dizziness, fatigue, hypersensitive reactions like skin rashes, paralytic ileus in infants and young children which may lead to death (Martindale, 2005)

Diphenoxylate hydrochloride is a synthetic derivative of pethidine with little or no analgesic activity. It reduces intestinal motility. It is metabolized in the liver principally to diphenoxylic acid which has antidiarrhoeal activity (Martindale, 2005). Its adverse side effects include anorexia, nausea, abdominal distension, paralytic ileus, toxic megacolon, pancreatitis, euphoria, restlessness, depression, numbness of extremitis, swelling of the gums etc. young children are more prone to these effects (Martindale, 2005; Kasper *et al*., 2005).

2.10.2.5 Anti-inflammatory agents

These drugs are mainly used in diarrhoea due to inflammatory bowel disease. They inhibit arachidonic acid cascade. Some of these drugs decrease the production of many immune and inflammatory mediators that actively inhibit mucosal absorbtion (Urayama and Chang, 1997). Examples of such drugs include; zieuton (a selective inhibitor of 5-lipoxygenase), glucocorticoids (inhibition of phospholipase A_2 activity) (Laursen *et al*., 1994) and sulfasalazine which inhibits cyclooxygenase and also 5-lipoxygenase activity both in intestinal mucosa and neutrophils (Dreyling *et al*., 1987)

2.11 Potential mechanism and drug target sites in the pharmacological development of antisecretory drugs

The mechanism of action of cholera toxin reveal several potential target areas to design therapeutic agents which includes; the inhibition of adenylate cyclase; the blockage of the active site of the

enzyme located in the A-subunit: the disruption of the assembly of the holotoxin by interrupting the A_2-B interaction (Velazquez *et al*., 2012); inhibitors of enkephalinase and of the cystic fibrosis transmembrane conductance regulator (Thiagarajah and Verkman, 2005; Norimatsu *et al.,* 2012) and inhibition of transport proteins involved in cAMP activated chloride secretion (Velazques *et al*., 2012). There has been a continuous research for drugs that inhibit the secretory process in the enterocytes to help in the control of diarrhoea, but only a few candidates have emerged. Table 2.1 gives a summary of such drugs, their target and their developmental stage.

2.11.1 Antioxidants in diarrhoeal management

Human and animals have endogenous control system for normal oxidative metabolism to occur in the body without damaging cells (Valko *et al*., 2007). During oxidative stress, this defense mechanism is insufficient and thus leads to cell damage. Returning the animal to a more neutral oxidative balance may promote repair of damaged membranes and prevent further damage (Nose, 2000). Antioxidants or free radicals scavengers such as vitamin C, α-tocopherol, superoxide dismutase and catalase have been reported to be beneficial in the treatment of diarrhea (Valko *et al*., 2007). It is found in all eukaryotic cells and it is one of the key non-enzyme antioxidant in the body and generally present in its reduced form, GSH (Bhattarcharygya *et al*., 2014). High intake of fruits and vegetables stimulate GSH- dependent enzymes (Hoensch *et al*., 2002).

Table 2.1: Potential antidiarrhoeal therapies

Name	Target and mechanism	Developmental stage
Antisecretory factor	Inhibition of ENS	Phase III
CaCCinh-AO1	Absorbable CaCCinhibitor	Preclinal
CFTRinh-172	Absorbable CFTR inhibitor	Preclinical
Clotrimazole	K^+ channel inhibitors	FDA approved for other indication
Gallotannins	CaCCinhibitors	Preclinical nutritional supplement
MalH-lectin	Inhibitor of the external pore of CFTR	Preclinical
PPQ/BBO	Absorbable CFTR inhibitor	Preclinical

BBO - benzopyrimido-pyrolo-oxazinediole; CaCC-calcium activated chloride channel; PPQ – pyrimido-pyrrolo-quinoxalinediole.

Source: Thiagarajah *et al*. (2015).

2.11.1.1 Antioxidant compounds (non-enzymatic antioxidants)

2.11.1.1.1 Glutathione

Glutathione is a tri-peptide (Glu-Cys-Gly) whose antioxidant function is facilitated by the sulphydyl group of cysteine. On oidation, the Sulphur forms a thiyl radicak that reacts with a second oxidized glutathione forming a disulfide bond (GSSG) (El-Beltagi and Mohammed, 2013). It is found in all eukaryotic cells and it is one of the key non-enzymatic antioxidant in the body and generally present in its reduced frm, GSH (Bhattarchargya *et al*., 2014). High intake of fruits and vegetables stimulate GSH-dependent enzymes (Hoensh *et al*., 2012).

2.11.1.1.2 Tocopherol (Vitamin E)

Vitamin E is the generic term used for tocopherol and tocotrienols consisting of two rings with a hydrocarbon chain. Both tocopherol and tocotrienols are similar in structure, but the tocotrienols structure has double bonds on the isoprenoids units (El-Beltagi & Mohammed, 2013). Natural vitamin E are known as α, β, γ and δ according to the methyl or proton group that are bound to their benzene rings and the most common and biologically active form is alpha tocopherol (Brigelius –flohe and Traber, 1999). α-tocopherol terminates the activity of lipid peroxidation by scavenging for lipid peroxyl radicals (LOO^{-}) but itself is converted into a reactive radical during this reaction (Vanacker *et al*., 1993). They can interupt free radical chain reactions by capturing the free radicals (El-Beltagi and Mohammed, 2013). The free hydroxyl group on the aromatic ring is responsible for the antioxidant property. It acts by donating the hydrogen atom from the

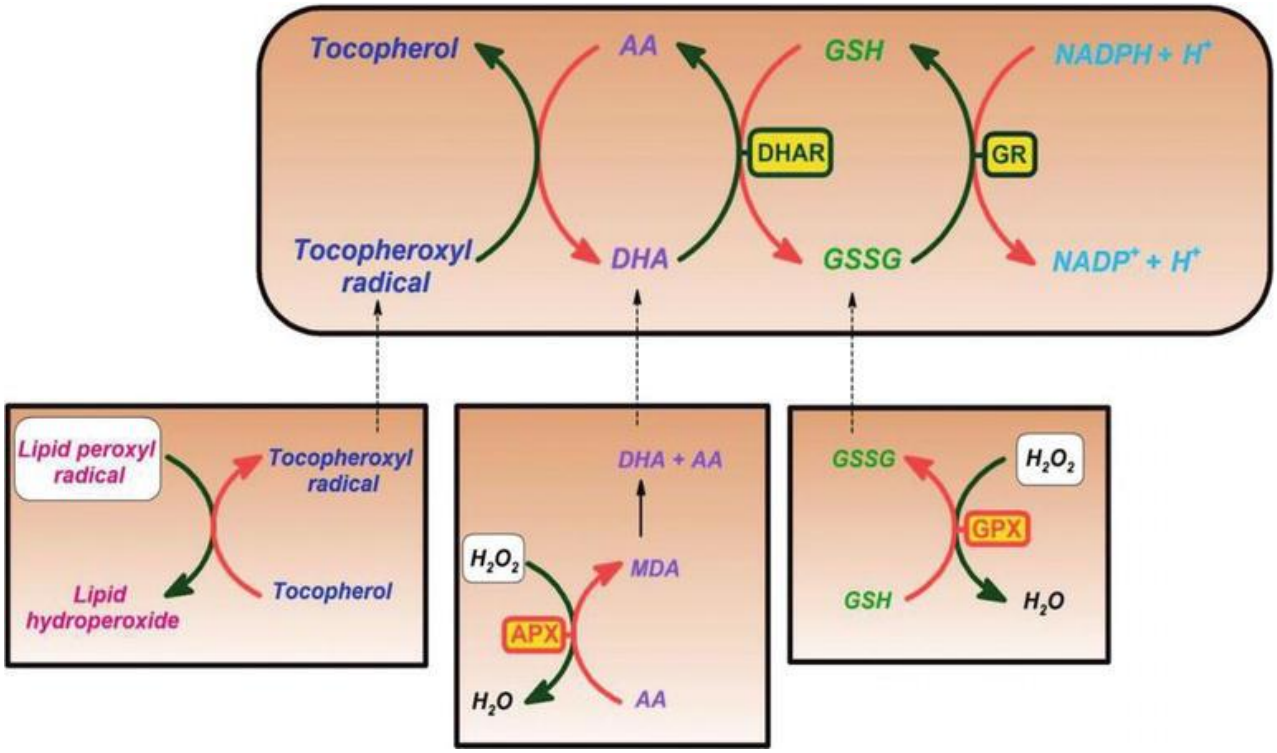

Figure 2.9: Ascorbate and redox cycling antioxidants

AA - ascorbate; DHA - dehydroascorbate; DHAR – semidehydroascorbate reductase; GSH-glutathione; GSSG - semi-glutathione reductase; GR - glutathione reductase; APX - ascorbate peroxidase; and GPX,- glutathione peroxidase
Source: Pehlivan (2017)

2.11.1.1.3 Ascorbic acid (Vitamin C)

Vitamin C, or ascorbic acid is the primary antioxidant in plasma and cells (May *et al*., 1999). It is synthesized in higher plants from hexoses primarily by the direct conversion of D-glucose to ascorbate (Loewus, 1988; El-Beltagi and Mohammed, 2013). Vitamin C is abundant in fresh fruits and vegetables. Ascorbic acid functions as a reductant for many free radicals by donating electrons and thus preventing oxidative stress (El-Beltagi and Mohammed, 2013). It also reduces heavy metal ions (Fe, Cu) that can generate free radicals via the Fenton reaction and thus have pro-oxidant activity (Stoh and Bagchi, 1995). Ascorbic acid can directly scavenge oxygen free radicals with or without enzyme catalysts and can indirectly scavenge them by recycling tocopherol to the reduced form (El-Beltagi and Mohammed, 2013). Ascorbate reacts with superoxide, hydrogen peroxide or the tocopheroxyl radical to form monodehydroascorbic acid and/or dehydroascorbic acid. The reduced forms are recycled back to ascorbic acid by monodehydroascorbate reductase and dehydroascorbate reductase using reducing equivalents from NAD(P)H or glutathione, respectively (El-Beltagi and Mohammed, 2013). Vitamin c has an aromatic ring which is able to delocalize an unpaired electron (Lu *et al*., 2010). Vitamin C ($AscH^-$) in the aqueous phase directly reacts with lipid peroxyl ($ROO^{\cdot}$) and hydroxyl radicals to form water and liqud hydroperoxides respectively as shown in Figure 2.10. It can also neutralize the radical form of other antioxidants such as glutathione radical and vitamin E radical to regenerate theses antioxidants as shown in Figure 2.11.

2.11.1.1.4 Carotenoids

Carotenoids are C40 isoprenoids and tetraterpenes that are located in the plastids of both photosynthetic and non-photosynthetic plant tissues (El-Beltagi and Mohammed, 2013). α and β-

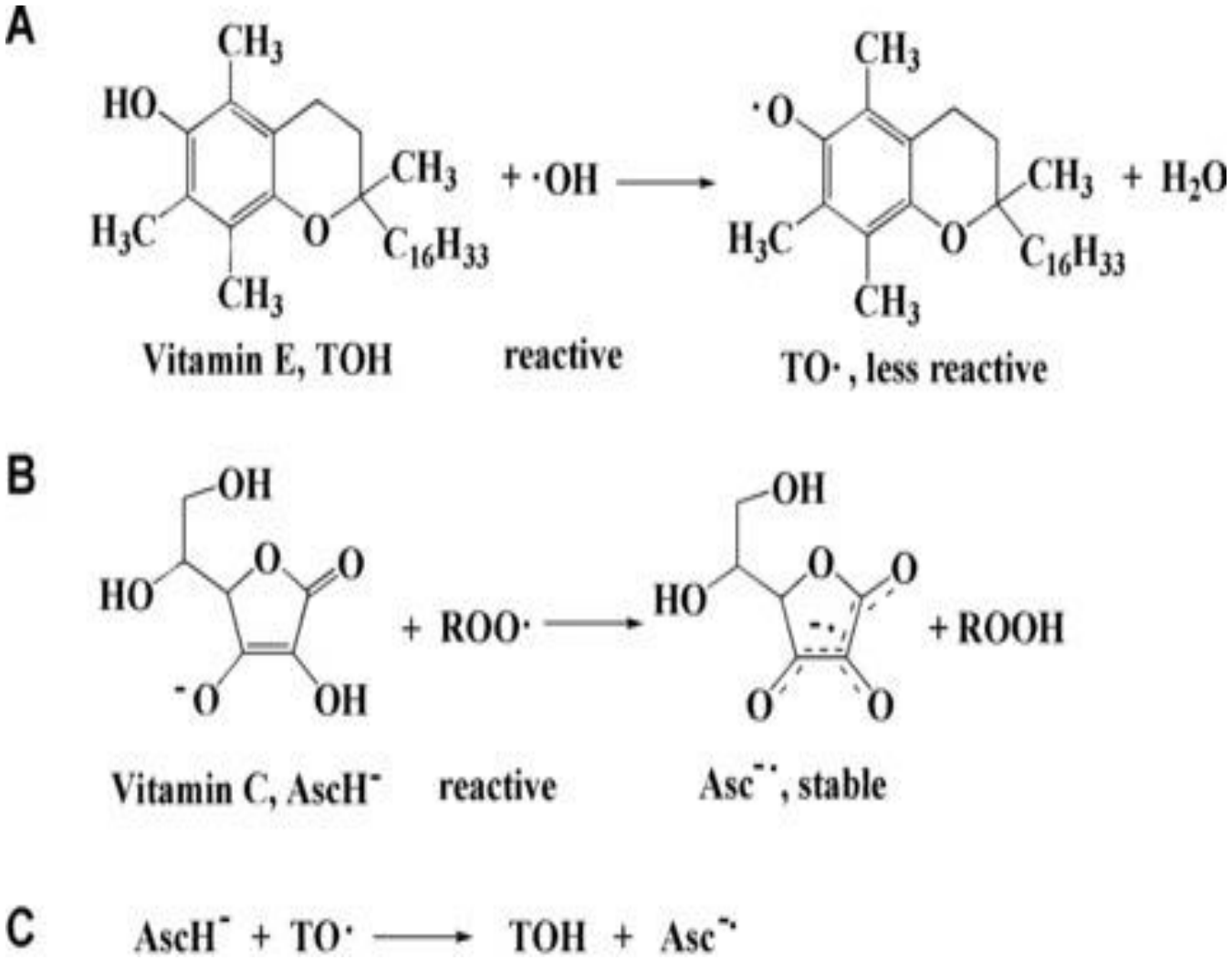

Figure 2.10: Direct reactions of Vitamin E (TOH) with ▪OH (A) and vitamin C ($AscH^-$) with ROO▪ (B) and regeneration of vitamin E from vitamin C

Source: Lu *et al.* (2010)

carotene, lycopene and cryptocanthin are the main carotenoids in food as well as in the body (Gerster *et al.*, 2002). The antioxidant properties of biological carotenoids depend on retinol - binding proteins and other endogenous antioxidants *in vivo*. Carotenoids can prevent lipid peroxidation by either reacting with lipid peroxidation products to terminate chain reactions (Burton and Ingold, 1984); by scavenging singlet oxygen and dissipating the energy as heat (Mathis and Kleo, 1973), by reacting with triplet or excited chlorophyll molecules to present formation of singlet oxygen or by the dissipation of excess excitation energy through the xanthophyll cycle. The conjugated double bond present in most carotenoids is responsible for that antioxidant activity.

2.11.1.1.5 Phenolic compound

Phenols are characterized by at least one aromatic ring bearing one or more hydroxyl groups. (El-Beltagi and Mohammed, 2013). Polyphenols comprise flavonoids, phenols, phenolic acids, lignins and tannins (Bhattachargy *et al*., 2010). Plant phenols are important antioxidants (Pietta, 2000).

Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol - derived radical to stabilize and delocalize the unpaired electron (chain breaking function) and from their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans *et al*., 1997). Flavonoids alter peroxidation kinetics by modifying the lipid packing order and also decreasing fluidity of the membranes (Anora *et al.*, 2000). They also inhibit cyclooxygenase (COX), lipoxygenase (LOX), glutathione transferase (GST), microsomal monooxygenases and NADH oxidase (Brown *et al.*, 1998).

2.12 Antioxidant enzymes

2.12.1 Superoxide dismutase

Superoxide dismutase is a metal ion cofactor requiring enzymes that catalyze dismutation of superoxide ($O_2^{\cdot-}$) into oxygen O_2 and hydrogen peroxide (El-Beltagi and Mohammed, 2013; Bhattachargya *et al*., 2014).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H \xrightarrow{SOD} H_2O_2 + O_2$$

SOD has a central roe in the defense against oxidative stress because it is present in all aerobic organisms and most subcellular compartments that generate activated oxygen (Scandalias, 1993). There are three distinct types of SOD in humans classified on the bases of their metal cofactor; cytosolic copper and zinc containing SOD (Cu-Zn-SOD), manganese requiring mitochondrial SOD (Mn-SOD) and an extracellular Cu-Zn containing SOD (EC-SOD) (Nozik-Grayck *et al*., 2005). Iron containing SOD (Fe-SOD) is present in bacteria and plants but not in vertebrates and yeast, while nickel-containing SOD (Ni-SOD) is present in only prokaryotes (Van *et al*., 1999).

Superoxide formed in the mitochondria is dismuted to hydrogen peroxide by Cu-Zn-SOD present in the mitochondrial intermembranous space and Mn-SOD present in the mitochondrial matrix (Okado-Matsumoto and Fridovich, 2001). Uncharged hydrogen peroxide crosses the mitochondrial membranes and in the cytosol can be scavenged by either cytosolic Cu-Zn-SOD or catalase (Poyton *et al*., 2009). Gastrointestinal mucosal injury can be prevented by superoxide dismutase in the gastrointestinal mucosa (Kohut and Mogis, 1993; Kilnowski *et al*., 1996). The levels of all the three isoforms of SOD increases in intestinal tissues of inflammatory bowel disease (IBD) (Krudenier *et al*., 2003).

2.12.2 Catalase

Catalase (CAT 1.11.1.6) is a heme- containing enzyme that catalyzes the dismutation of hydrogen peroxide into water and oxygen. (El-Beltagi and Mohammed, 2013).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2.$$

The enzyme is found in the peroxisome of all aerobic eukaryotes (Bhattachatyya *et al*., 2014). It is important in the removal of hydrogen peroxide generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, the glyoxylate cycle and purine catabolism (El-Betagi and Mohammed, 2013). A manganese catalase is found in prokaryotes (Zamocky and Koller, 1999). In humans, catalase is found largely in liver, kidney and erythrocyte (Bhattachargya *et al*., 2014).

2.12.3 Glutathione Peroxidase

Glutathione peroxidase is a selenium dependent enzyme, which decompose H_2O_2 and various hydro-and lipid peroxides (Kinnula *et al*., 1993). The classical form of GPx is cellular and dispersed throughout the cytoplasm but GPx activity is also found in mitochondria (Ribas *et al*., 2014). Glutathione peroxidase (GPx) converts glutathione (GSH), a tripeptide consisting of glutamate, cysteine and glycine into oxidized glutathione (also called glutathione disulfide (GSSSG) and during this process reduces H_2O_2 to H_2O and lipid hydroperoxides (ROOH) to corresponding stable alcohols. (Bhattachargya *et al*., 2014). The GPx reaction is coupled to glutathione reductase (GSSG-R) which maintains reduced glutathione (GSH) levels as shown in Figure 2.11. Glutathione peroxidase (GPx) serves an important role in protecting cells from the harmful effect of peroxide decomposition as shown in Figure 2.11 (Bhattachargya *et al*., 2014).

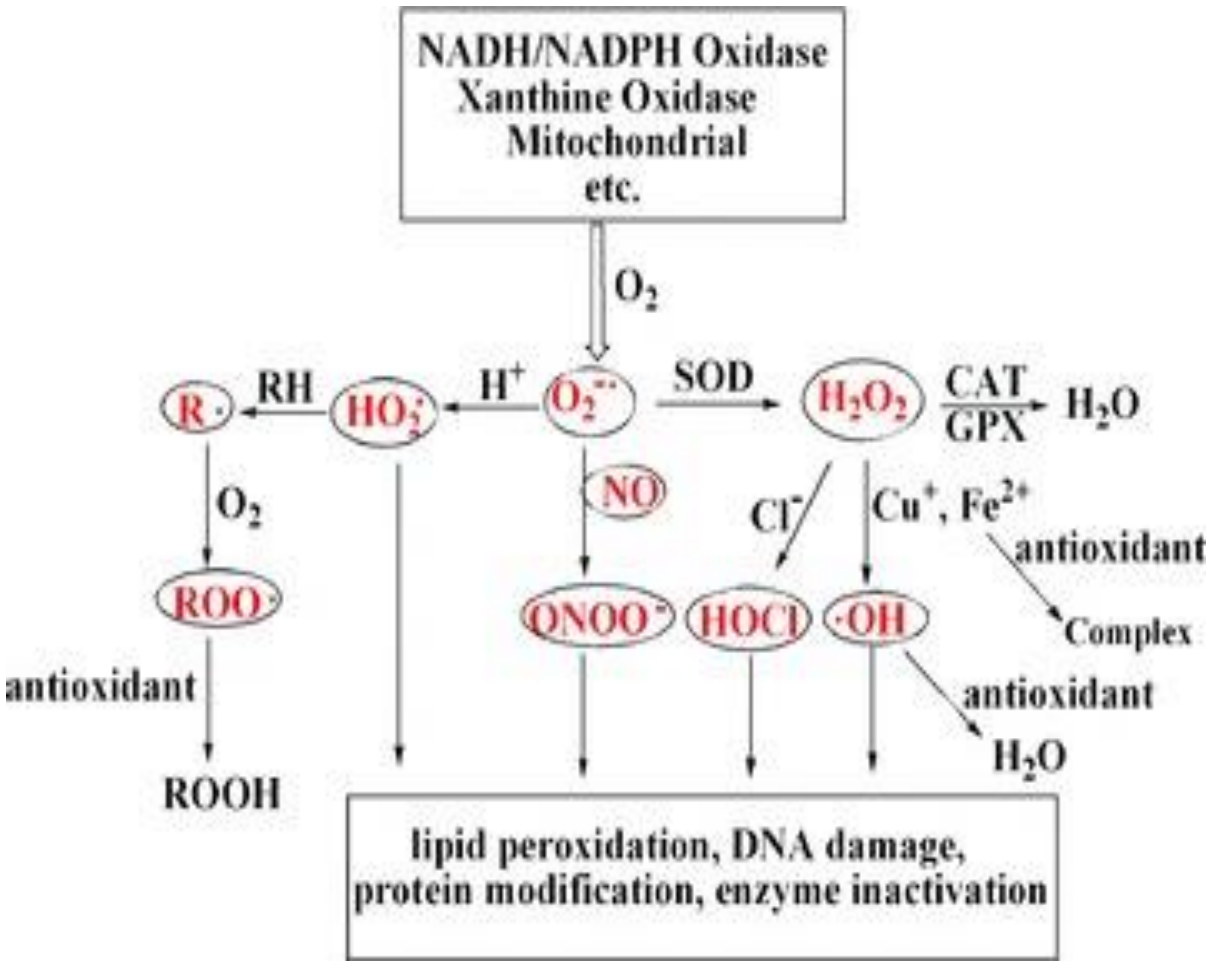

Figure 2.11: Summary of ROS types and sources, and action point of antioxidants.

$O_2^{-\bullet}$, superoxide anion; $HO_2^{\bullet}$, perhydroxyl radical; ▪OH, hydroxyl radical; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; R▪, lipid alkyl radical; RH, lipid; ROO▪, lipid peroxyl radical; ROOH, lipid hydroperoxide; SOD, superoxide dismutase; CAT, catalase and GPX, glutathione peroxidase

Source: Lu *et al*. (2010)

2.12.4 Glutathione reductase

Glutathione reductase (GR or GSR) reduces oxidized glutathione disulfide (GSSG to GSH) (Bhattachatyya *et al*., 2014). This homodimeric enzyme is a flavoprotein disulfide oxidoreductase. Each subunit contains four domains: FAD- binding and NADPH- binding domains (Bhattachargya *et al*., 2014). GR protects red blood cells, hemoglobin and cell membranes from oxidative stress by generating GSH (Change *et al*., 1978).

2.13 Medicinal plant as source of antidiarrhoeal compounds

Medicinal plants have been widely used in alleviating diarrhoeal symptoms in humans and animals (Brijesh *et al*., 2006; Gutierrez *et al*., 2007). Numerous species of these plants have been scientifically validated for their antidiarrhoeal property (Gutierez *et al*., 2007). Plant extracts can have antispasmodic effects, delay gastrointestinal transit, suppress gut motility, stimulate water adsorption or reduce electrolyte secretion (Palombo, 2006). These plants exhibit these activities through various secondary metabolites described below;

2.13.1 Terpenes and triterpenoids

Terpenes are among the most widespread and chemically diverse groups of natural product (Doughari, 2012). They are flammable unsaturated hydrocarbons existing mainly in liquid form and commonly found in essential oils, resins and oleoresins (Doughari, 2012). They have a general formula $(C_5H_8)n$ (Doughari, 2012). They are classified according to the number of isoprene units or numbers of carbon in their skeletal structure (Zwinger and Basu, 2008). Monoterpenes (C10) contain 2 units of isoprene units containing 15 carbon atoms and are major components of many essential oil (Martinez *et al*., 2008). This group of compounds acts as phytoalexins, antimicrobial

and antifeedant in plants. Diterpenes are classically considered to be resins. The triterpenes include steroids, sterols and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity (Doughari, 2012). They inhibit release of autocoids and prostaglandins (Tiwari *et al*., 2011). Several terpenoids have been identified to have good antidiarrhoeal properties via different mechanisms. Niloticane isolated from *acacia nilotica* (L) have antimicrobial effect against *Bacillus subtillis*, *Staphylococcus aureus* and *E. coli* (Elden *et al*., 2010); Oleanolic acid, ursolic acid and betullinic acid from *Chaenomeles speciosa* have the potential of blocking the binding of virulence heat labile unit (LTuB) of *E. coli* enterotoxin to gangloside receptor; Glycyrrhizin from *Glycyrrhiza uralensis* also have LTuB –binding inhibitory potential, therefore they can suppress LT-induced intestinal fluid accumulation (Chen *et al*., 2005); oleanolic acid and echinocystic acid isolated from *Luffa cylindrical* can increase phagocyte index, stimulate macrophage, increase humoral and cell mediated immune responses (Khajuria *et al*., 2007); betulonic acid have PGE inhibition activity (Reyes *et al*., 2006); masilinic acid and oleanolic acid isolated from olive poamace oil have concentration dependent IL-6, TNF-α-modulatory effects in a human mononuclear cell culture assay (Marquez-martin *et al*., 2006). Steroids enhance intestinal absorption of Na^+ and water (Tiwari *et al*., 2011).

2.13.2 Alkaloids

These are the largest group of secondary chemical constituents. Alkaloids refer to a group of heterocyclic nitrogen compounds with remarkable physiological and pharmacological activities (Samy and Gopalakrishnakone, 2008). They are derived from amino acid with various radicals replacing one or more of the hydrogen atoms in the peptide ring. Most alkaloids contain oxygen (Doughari, 2012). The compounds have basic properties and are alkaline (Doughari, 2012). Majority of alkalines exists as solid e.g atropine. Solutions of alkaloids are intensely bitter

(Doughari, 2012). Though, alkaloids have many antidiarrhoeal pharmacological mechanisms such as microbiocidal effect on diarrhoeagenic pathogens, the main antidiarrhoeal effect is that of delayed intestinal transition of bowel materials (Cowan, 1999). They also inhibit the release of autocoids and prostaglandins (Qnais, 2005). Some of the pharmacological important antidiarrhoeal alkaloids include; kurryam, koenimbine, boldine, 8-acetonyldihydronitidine and 8-acetonyldihydroavicine and koenine (Mandal *et al*., 2006). Boldine has good antioxidant property while 8-acetonyldihydronitidine and 8-acetonyldihydroavicine have strong antistaphyloccocial activity (Nissanka *et al*., 2001)

2.13.3 Phenolic compounds

They are the largest category of phytochemical. The three most important groups of dietary phenolics are; flavonoids, phenolic acids and polyphenols (Saxena *et al.*, 2013). Phenolic are hydroxyl group (-OH) containing class of chemical compounds where the OH is bonded directly to an aromatic hydrocarbon group (Saxena *et al*., 2013).

2.13.3.1.1 Phenolic acid

Phenolic acids posses one carboxylic acid functional group (Saxena *et al.*, 2013). Naturally occurring phenolic acids contains two distinctive carbon frame works; the hydroxycinnamic and hydroxybenzoic structures. These compounds have been studied for their properties against oxidative damage which may lead to various degenerative diseases such as cardio vascular diseases, inflammation and cancer (Saxena *et al.*, 2013). Phenolic compounds may be polymerized into larger molecules such as the proanthocyanidins (condensed tannins). It has antimicrobial activity against some strains of bacteria such as *staphylococcus aureus* (Gryglewski *et al.*, 1987). It also has anti-inflammatory and antioxidant activities (Ghasemzadeh *et al.*, 2010).

2.13.3.2 Flavonoids

Flavonoids are polyphenolic compounds with more than one benzene ring in its structure (array of C_{15} aromatic compouds) (Doughari, 2012). They are derived from parent compound known as flavans (Doughari, 2012). More than 4,000 (four thousand) flavonoids have been identified (Harbone and Baxter, 1999). Flavonoids can be classified into different groups such as flavones, flavonols, flavonones, isoflavones and anthocyanidins based on the level of oxidation and pattern of substitution on ring C (Feng, 2017). Flavonoids most commonly occur in plant materials as flavonoid O-glycosides, in which one or more hydroxyl group of the aglycones are bound to a sugar, forming an acid labile glycosides O - C bond (Tsao, 2010). Flavonoids have been reported to exert multiple biological properties including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor (Tapas *et al.*, 2008) but the best described property of almost every group of flavonoids is their ability to scavenge free radicals and chelate metals (Saxena *et al.*, 2013; Vinayagam and Xu, 2015).

The antioxidant effects of flavonoids are enhanced by the number and position of hydroxyl group in the molecule, the catechol structure (O-dihydroxy group in the B ring), presence of unsaturation (C_2 - C_3 double bond) and 4-oxo function (keto double bond at position 4) in the C ring (Heim *et al.*, 2002). Catechol structure possess electron. It donates electron to stabilize radical species. The 4 - oxo in association with the C2-C3 double bond increases the radical scavenging activity by delocalizing electrons from B ring, the 3-OH moiety of the C ring generates an extremely active scavenger. The 5-OH and 7-OH groups may also add scavenging potential in certain cases (Amic *et al.*, 2007). Flavonoids may be capable of binding the transition metal ions, such as copper and

iron, which play a role in glycoxidation, thus preventing metal catalyzed formation of hydroxyl radicals or related species from H_2O_2 (Treml and Smejkal, 2016). Flavonoids also exert antimicrobial activity (Tapas *et al*., 2008).

Flavonoids act as antidiarrhoeal by inhibiting the contraction caused by spasmogens (Ojewole, 2008); inhibiting the release of autocoids and prostaglandins (Alam *et al*., 2008; Sulaiman, 2008) and inhibition of release of acetylcholine by the gastrointestinal tract (Ojewole *et al*., 2008). Flavonoids also stimulate the normalization of deranged water transport across the mucosal cells (Oben, 2006), inhibit intestinal motility and have antisecretory effects (Laure *et al*., 2006; Teke *et al*., 2007; Brijesh *et al*., 2009).

2.13.3.3 Tannin

Tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysacharides, (cellulose, hemicellulose, pectin, etc), alkaloids, nucleic acids and minerals, (Mueller-harvey and Mcallan, 1992; Schofield *et al*., 2001). Tannins can be divided into four major groups based on their structural characteristics; gallotannins, ellagitannins, complex tannins and condensed tannins (Mole and Waterman, 1987; Mangan, 1998). Gallotannins are tannins in which galloyl units are bound to diverse polyol-, catechin-, or triterpenoid units. Ellagitannins are tannins in which at least two galloyl units are C-C coupled to each other and do not contain a glycosidically linked catechin unit. Complex tannins are tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit. Condensed tannins are all oligomeric and polymeric proanthocyanidins formed by linkage of C- 4 of one catechin with C-8 or C- 6 of the next monomeric catechin (Doughari, 2012).

Tannin rich plants are used for the treatment of diarrhoea (Doughari, 2012). They are used as anti-inflammatory and antioxidant pharmaceuticals (Dolara *et al*., 2005). They have the ability to block the binding of β-subunit of heat labile enterotoxin to ganglioside (GM_1), resulting in the suppression of heat labile enterotoxin-induced diarrhoea (Chen *et al.*, 2007). Tannins reduce secretion, inhibit intestinal motility and stimulate normalization of deranged water transport (Laure *et al*.,2006; Suleiman *et al*., 2008; Brijesh, 2009).

2.13.4 Saponin

Saponins are a group of secondary metabolic found widely distributed in the plant kingdom (Saxon *et al*., 2013). They are regarded as high molecular weight compound in which a sugar molecular is combined with triterpene or steroid aglycone (Doughari, 2012). There are two major groups of saponin; steroid saponins and triterpene saponins (Doughari, 2012). They are soluble in alcohol and water and insoluble in non-polar solvents. Saponins play a part in plants defense system and have been included in a large group of protective molecules found in plant, named phytoanticipins or phytoprotectants (Laceille-Dubois and Wagner, 1996.). Saponins impair the digestion of protein and the uptake of vitamin and minerals in the gut and act as antifungal and antiviral agents (Morrissey and Osbourn, 1999; Traore *et al*., 2002).

2.13.5 Anthraquinones

These are derivatives of phenolic and glycosidic compounds. They are solely derived from anthracene (Maurya *et al.*, 2008; Firn, 2010).

2.13.6 Essential oil

Essential oils are the odorous and volatile products of various plant and animal species. Essential oils have a tendancy to evaporate on exposure to air even at ambient conditions and are thus

referred to as volatile oils or ethereal oils (Doughari, 2012). They mostly contribute to the odoriferous constituents or "essences" of the aromatic plants that are used abundantly in enhancing the aroma of some spices (Martinez *et al.*, 2008). Essential oils inhibit the release of autocoids and prostaglandins (Tiwari *et al*., 2011).

2.14 Minerals

Minerals are inorganic substance present in all body tissues and fluids and their presence is necessary for the maintenance of certain physicochemical processes which are essential to life (Hays and Swenson, 1985). They play so many important roles in the activities of the body (Malhotra, 1998, Eruvbetine, 2003). Minerals are broadly classified as macro (major) or micro (trace) elements (Soetan *et al*., 2010). A third category is the ultra trace elements (Soetan *et al*., 2010). The macro minerals are required in amounts greater than 100 mg/dL while the trace elements are required in amounts lesser than 100 mg/dL (Murray *et al*., 2000). The macro minerals include phosphorus, calcium, sodium and chloride, while the micro elements include iron, copper, cobalt, potassium, magnesium, iodine, zinc, manganese, molybdenum, fluoride, chromium, selenium, and sulfur (Erubvetene, 2003). The trace elements Cu, Fe, Se, Mn and Zn are integral part of enzymatic anti-oxidants. Cu and Zn represent an integral part of Cu-Zn superoxide dismutase; Mn an integral part of Mn- superoxide dismutase while Fe is an integral part of Se-GPX (glutathione peroxidase) (Arinola *et al*., 2008c).

2.14.1 Interrelationships and interferences among mineral elements

There are many metabolic and absorptive inter-relationships among minerals. The functions of minerals in humans and animals are inter-related (Soetan *et al*., 2010). For example, a high level of potassium appears to increase the requirement for sodium and vice versa (Merck, 1986). This

occurs as a means of maintaining cation-anion balance (Soetan *et al*., 2010). Plasma phosphorus level is inversely related to the blood calcium level (Hays and Swenson, 1985). Sodium, potassium, calcium, phosphorus and chlorine have both collective and individual role in the function of body fluids (Soetan *et al*., 2010). There is a close relationship between chloride and sodium ions. In animals, excess chloride and a constant level of sodium can result in acidosis whereas, excess sodium and a constant level of chloride results in alkalosis (Soetan *et al*., 2010). Cardiac muscle, skeletal muscle and nervous tissue depend on a proper balance between calcium and magnesium ions. A calcium intake will increase the need for zinc (Hays and Swenson, 1985).

2.14.2 Biochemical functions of mineral/elements in humans and animals

2.14.2.1 Calcium

Calcium functions as a constituent of bones and teeth. It is important in the regulation of nerve and muscle function (Soetan *et al*., 2010). It is required in blood coagulation process; it activates the conversion of prothrombin to thrombin. A number of enzymes such as adenosine triphosphatase, succinic dehydrogenase, lipase etc are activated by calcium (Soetan *et al*., 2010). Dietary calcium and phosphorus are absorbed mainly in the upper small intestine, particularly the duodenum and the amount absorbed is dependent on source, calcium-phosphorus ratio, intestinal pH, lactose intake and dietary levels of calcium, phosphorus, vitamin D, iron, manganese, aluminium and fat (Soetan *et al*., 2010). Absorption of calcium and phosphorus is facilitated by a low intestinal pH. Low pH of the duodenum favours absorption. Lactose also enhances the absorption of calcium (Hays and Swenson, 1985).

2.14.1.2 Phosphorus

Phosphorus is found in every cell of the body and is involved in many metabolic processes (Hays and Swenson, 1985). Phosphorus is a constitutuent of bones, teeth and phosphate buffer. It is important in the formation of high energy compounds such as adenosine triphosphate (ATP) (Soetan *et al*., 2010). Sixty to eighty percent of the total phosphorus of cereal grains and oil seeds exists organically bound as phytic acid. Phytic acid is the hexaphosphoric acid ester of inositol and is found mainly as the Ca-Mg salt called phytin phosphorus. This is largely unavailable to monogastric animals but ruminants can utilize it because of the presence of the enzyme phytase in microorganism's rumen, which hydrolyse the organically bound phosphorus and renders it available for absorbtion.

2.14.1.3 Sodium

Sodium is the major cation in extracellular fluid (Chatterjea and Shinde, 2008). It is involved in the maintainance of osmotic pressure of the body fluids, activates nerves and muscle function, regulates plasma volume and acid-base balance, maintainance of membrane potentials, transmission of nerve impulses and in the process of absorption of monosacharides, amino acids, pyrimidines and bile salts (Murray *et al*., 2000). Sodium metabolism is regulated by aldosterone (Soetan *et al*., 2010). Sodium is absorbed by sodium pump situated in basalateral plasma membrane of intestinal and renal cells. Sodium pump actively transports sodium into extracellular fluids (Chatterjea and Shinde, 2008). Less than 1% of filtered sodium (2500 mmol per day) appears in the urine. Seventy percent (70%) of the filtered sodium is reabsorbed in proximal tubule while 20% of the filtered sodium is further reabsorbed by ascending loope of Henle (Chantterjee and Shinde, 2008).

2.14.1.4 Iron

Iron is one of the most essential trace elements in the body (Chatterjea and Shinde, 2008). Iron exists in the blood mainly as heamoglobin in the erythrocytes and as transferrin in the plasma. It is transported as transferrin, and stored as ferrin (Murray *et al*., 2000). Iron present in the body can be categorized into two; essential or functional iron and storage iron. Essential iron is one which is involved in the normal metabolism of cells while the storage iron is present in two major compounds; ferritin and haemosiderin (Chatterjea and Shinde, 2008). Fe^{2+} is required for conversion of superoxide radical to free hydroxyl (OH^-) radical in the Haber's reaction (Chantterjea and Shinde, 2008).

Iron is essential for the function of enzymes such as catalase, peroxidase and other iron containing enzymes such as xanthine oxidase, cytochrome reductase etc (Chatterjea and Shinde, 2008). It is involved in the synthesis and packaging of neurotransmitters, their uptake and degradation into other iron containing proteins which may directly or indirectly alter brain function (Beard, 2001). Enterocytes in the proximal duodenum are responsible for absorption of iron (Chatterjea and Shinde, 2008). The source of iron has marked effect on its absorbtion. Heme iron which comes from animal products is efficiently absorbed, while non-heme iron which is present in plants is inefficiently absorbed (Hooda *et al.,* 2014). Its absorption depends on the composition of the diet, pH of the intestinal milieu and the state of health of the individual (Chartterjea and Shinde, 2008). Phytates and oxalates inhibit non-heme iron absorption, dietary fibres may also bind the iron or decrease gastrointestinal transit time, rate of non-heme iron absorption decreases as pH becomes more alkaline (Chartterjea and Shinde, 2008). Low phosphate diet increases iron absorption (Soetan *et al*., 2010). Adrenoocorticol hormones (glucocorticoids) play a role in regulating the level of plasma iron. During stress, the hypothalamus, adenohypophysis and adrenal cortex are

activated and this leads to decrease in plasma iron (Hays and Swenson, 1985). The absorbtion of iron is inhibited by profuse diarrhoea (Malhotra, 1998).

2.14.1.5 Potassium

Potassium is the principal cation in intracellular fluid and functions in acid-base balance, regulation of osmotic pressure, muscle contraction of nerve impulse, cell membrane and is required during glycogenesis (Soetan *et al*., 2010). Its metabolism is regulated by aldosterone (Soetan *et al*., 2010). It is continuously filtered by the glomeruli of the kidney and reabsorbed by the cells of proximal convoluted tubules. Damage to body cell memebrane leads to release of k^+ ion into extracellular fluid (Chatterjea and Shinde, 2008).

2.14.1.6 Chloride

Chloride is the major anion in extracellular fluid. It makes up over 60% of the anions in the extracellular fluid. It is involved in fluid and electrolyte balance as well as acid-base balance. Chloride in gastric secretions is derived from blood chloride and is reabsorbed in the latter stages of digestion in the lower intestine (Murray *et al*., 2000).

2.14.1.7 Copper

Copper is a constituent of many enzymes which include cytochrome C oxidase, amine oxidase, catalase, peroxidase, cytosolic superoxide dismutase, lactase etc. It is an essential micro-nutrient necessary for heamatologic and neurologic function (Tan *et al*., 2006). Copper helps in the incorporation of iron in heamoglobin and also assists in iron absorbtion from the gastrointestinal tract (GIT) (Murray *et al*., 2000). Copper is transported by albumin bound to ceruloplasmin. The copper containing protein in liver is called hepatocuperin while that of red blood cells is called erythrocuperin and brain is called cerebrucuperin (Soetan *et al*., 2010). Copper is primarily

absorbed in duedonum. Phytates, zinc, molybedenum, cadmium, silver, mercury and high amount of vitamin C inhibit copper absorbtion (Chatterjea and Shinde, 2008). It helps in the utilization of Fe for heamoglobin synthesis, helps in bone formation and maintainance of myelin sheaths of nerve fibres.

2.14.1.8 Magnesium

Magnesium is the fourth most abundant and important cation in humans (Chatterjea and Shinde, 2008). It is extremely essential for life and is present as intracellular ion in all living cells and tissues (Chatterjea and Shinde, 2008). Magnesium can function as co-factor and as activator to wide spectrum of enzyme actions (Chatterjea and Shinde, 2008). It is a constituent of bones and teeth and exerts an effect on neuromuscular irritability similar to that of Ca^{2+}. High levels depress nerve conduction and low levels may produce hypomagnesemia tetany (Chatterjea and Shinde, 2008). Absorption of copper takes place primarily in small intestine (Chatterjea and Shinde, 2008). Increased GIT motility decreases absorbtion. A damaged mucosa also decreases absorption (Chatterjea and Shinde, 2008). Gastrointestinal disorders that impair absorbtion such as crohn's disease can limit the body's ability to absorb magnesium. Chronic or excessive vomiting and diarrhoea may also result in magnesium depletion. Deficiency disease is secondary to malabsorption, diarrhoea and alcoholism (Soetan *et al*., 2010).

2.14.1.9 Zinc

Zinc is widely distributed in plant and animal tissues and occurs in all living cells (Soetan *et al*., 2010). Zinc forms an integral part of metalloenzyme in the body. Examples of zinc containing enzymes are superoxide dismutase, carbonic anhydrase, leucine aminopeptidase and carboxy peptiadase A. It also plays a role in insulin secretion, growth and reproduction, wound healing and

biosynthesis of mono-nucleotides (Chatterjea and Shinde, 2008). Vitamin A and E metabolism and bioavailability are dependent on zinc status (Szabo *et al*., 1999). Zinc also plays a role in binding of regulatory proteins in DNA and in zinc finger motif (Chatterjea and Shinde, 2008). Only a small percentage of dietary zinc is absorbed and the absorbtion takes place mainly in the duodenum and ileum (Chatterjea and Shinde, 2008). An amount of zinc equivalent to the total absorbed zinc is reabsorbed into the gut in intestinal fluids (Soetan *et al*., 2010).

2.15 *Annona senegalensis*

Annona senegalensis is from the *annonaceae* family and is used as medicinal plants (Ilboudo *et al*., 2010). It is mostly found in the tropical rain forest or savannah (Okoye *et al*., 2011). It has aromatic flowers which are used to flavor food (Okoye *et al*., 2012). The fruit is also consumed as food (Okhale *et al*, 2016). It is used ethnomedicinally for the treatment of a wide array of diseases (Okoye *et al*., 2012). Ethnopharmacological studies have supported the folklore use of all the parts as shown in Table 2.2 and 2.3.

2.15.1 Phytochemical constituents in *Annona senegalensis*

Phytochemical screening of several plant parts of *Annona senegalensis* revealed the presence of numerous secondary metabolite which includes tannin, flavonoids, alkaloids, carbohydrates and saponins in the root bark (Igwe and Nwobodo, 20i4; Ijaiya *et al*., 2014). Sterols and/ or triterpenes, anthocyanes, glucids, coumarins and alkaloids in the roots have also been reported (Konate *et al*., 2012). Gas chromatography–mass spectrometry (GC–MS) study of thee stem bark of *A. senegalensis* revealed the presence of 1.2- benzediol (catechol)., butylated hydroxytoluene (BHT)., n-headecanoic acid, 13-ocadecanoic acid, oleic acid and squalene (Awa *et* al., 2012). Catechol has been reported to be toxic to micro-organsims while kaur-16-en-19-oic acid, a diterpenoid, has been

Table 2.2: Some ethnomedicinal uses of *Annona senegalensis*

Plant part	Form	Uses	References
Leaf	-	Yellow fever	Aiyeloja and Bello, 2006
Leaf	Decoction	Tuberculosis	Ofukwu *et al*., 2008
Stem	-	Snake bites, Hernia	Dambatta and Aliyu, 2011
Root	Infusion	Necrotizing Venom	Emmanuel and Mmoudou, 2015
Root	Infusion	Erectile dysfunction	Faleyimu and Akinyemi, 2010.
Root	Decoction	Male sexual impotence, gastritis	Jiofack *et al*., 2009
Root bark	Decoction	Infectious disease	Magassouba *et al*., 2007

Table 2.3: Researches reporing activities of *Annona senegalensis*

Test	Plant part extract	Result	References
Antihelmintic activity	Ethanolic leaf extract	0.8% w/v of extract caused a 98.33% mortality of the larva but had no effects on egg hatching	Nukenine *et al*., 2006
Antivenomous activity	Root bark	Extract caused reduction in the induced hyperthermia and directly detoxified snake venom by 16 - 33%	Adzu *et al*., 2005
Anticonvulsant activity	Aqueous and methanolic extract of root bark	Curative and protective effect. Prolonged onset of tonic and clonic phases of seizure	Konate *et al*., 2012; Okoye *et al.,* 2009
Cytotoxic activity	Methanolic extract of leaves	Low cytotoxicity with an IC_{50} OF 28. 8 µg/Ml	Ajaiyeoba *et al*., 2006
Astringent/haemostatic activity	Hydro-alcoholic leave extract	Acts on primary haemostasis through vasoconstriction	Dandjesso *et al*., 2012
Antiplasmodial/antimalarial activity	Methanolic root extract	Extract showed activity against the chloroquine resistant strain of *P. falciparum.*	Ajaiyeoba *et al*., 2006
Antioxidant activity	Aqueous leaf extract	Scavenged free radicals in vitro and reversed CCl_4 induced hepatocellular damage in rat.	Ajiboye *et al*., 2010
Anti-inflammatory activity	Ethanolic leaf extract; methanolic stem bark	Induced significant decrease in number of inflammatory cells; increased nociceptive reaction latency in hot plate test	Suleiman *et al*., 2014; Yeo *et al.*, 2011.
Spermatogenic activity	Aqueous leaf extract	Increased sperm concentration and sperm motility but not sperm morphology	Oladele *et al*., 2014.
Antibacterial activity	methanolic stem bark	MIC of 62.5 mg/ml against *S. aureus*; no activity against *E.coli*.	Apak and Olila, 2006.
	methanolic root bark	MIC 0.39 mg/ml on *E. coli*, 3.17 mg/ml on *S. enteriditis* and 25.0 mcg/ml oni	Awa *et al.*, 2012
Antidiarrhoeal activity	Methanolic stem bark	Low dose of the extract decreased intestinal time and attenuates contractile effects of acetylcholine and histamine on rabbit ileum	Suleiman *et al.*, 2008.

reported to be responsible for the antibacterial effects of root bak (Awa *et al*., 2012; Okoye *et al.*, 2012). Triterpenes and flavonoids containing essential oils with antimicrobial activity have been isolated from the stem bark (Chalchat *et al*., 1997; Khallouki *et al*., 2002). All of the parts of *Annona senegalensis* contains essential oils (Nkounkou *et al*., 2010). The plant contains vital minerals such as Ca, Mg, K, Zn, Cu, Pb, Cr. Amino acids and ascorbic acids have been reported to be present in the plant (Yisa *et al*., 2010).

2.15.2 Lethal dose (LD_{50}) of *Annona senegalensis*

The lethal dose (LD_{50}) of the root bark has been estimated to be greater than 5000 mg/kg (Iboudo *et al*., 2010a; Nanti *et al*., 2018) while the lethal dose for the stem bark has been reported to be greater than 2,000 mg/kg (Adisa *et al*., 2019).

2.16 Organs studied

2.16.1 Kidney

The kidney is a compound tubular gland covered by a connective tissue capsule. The components of kidney are arranged in three layers; outer cortex, inner medulla, and the renl sinus (Sembulingam and Sembulingam, 2012). The renal system has the maximum excretory capacity out of all the organs responsible for the removal of waste from the body (Sembulingam and Sembulingam, 2012). The nephron is the functional unit of the kidney (Fry, 2010). The nephron consisits of two main parts: the renal corpuscle and the renal tubule (Fry, 2010). The renal corpuscle consists of a glomerulus and Bowmans' capsule while the renal tubule is composed of proximal tubule, loop of henle (descending an ascending limbs) and distal convoluted tubule (Fry, 2010).

The kidneys have several important functions which can be broadly divided into two: extracellular homeostasis and secretion of hormones (Fry, 2010). The primary function of the kidney is

homeostasis. It is able to accomplish this role by the formation of urine. During the formation of urine, kidney regulates various activities such as excretion of waste products, e.g urea, uric acid, maintenance of acid-base balance, maintenance of electrolyte balance especially sodium. Elimination of sodium is in relation to water balance: it eliminates sodium when osmolarity of body water increases but retains it when the osmolarity decreases (Barrette *et al*., 2010). The hormones secreted includes erythropoietin which regulates red blood cell production in the bone marrow, renin which is the key part of the rennin-angiotensin-aldosterone system aand the active forms of vitamin D (Calcitrol) and prostaglandins (Fry, 2010).

Blood is filtered through the glomerular capillaries into the renal tubules (glomerular filtration) producing an ultrafiltrate which contains not only water products but many other substances which are required by the body and thus needs to bre reabsorbed (Woodrow, 1987; Barrette *et al*., 2010). The volume of the filtrate is reduced and its composition altered in a process called tubular reabsorption (removal of water and solutes from the tubular fluid) and tubular secretion (secretion of solutes into the tubular fluid) (Barrett *et al*., 2010). Many substances including sodium, potassium, chlorine, sugars and amino acids are reabsorbed from the proximal tubule together with water, so that as the filtrate reaches the descending loope its osmolarlity increases (Woodrow, 1987). At the distal tubule, 80% of the original water content of the filtrate has been reabsorbed. The hormones: antidiuretic hormne (ADH) and aldosterone makes the final adjustments to the fluid hormone. Antidiuretic hormone (ADH) alters the permeability of the distal renal tubules to reabsorb sodium (Woodrow, 1987). Other roles of kidney include regulation of blood calcium, long term regulation of arterial blood pressure, stimulation of erythrocytes by secreting

erythropoietin and stimulation of endocrine hormones such as erythropoietin, thrombopoietin, renin 1, 25-dihydrxylcholecalciferol (calcitriol). Damage to the nephrons increases the permeability of the glomerular membrane, allowing molecules including protein to pass into the tubules (Woodrow, 1987)

2.16.2 Liver

The liver is the largest organ in the body (Sembulingam and Sembulingam, 2012). It is rough wedge shaped and lies in the upper abdomen and right side of the abdominal cavity just below the diaphragm (Barrett *et al*., 2010). The liver serves as a filter between the blood coming from the gastrointestinal tract and the blood in the rest of the body (Barrett *et al*., 2010). The liver is made up of many lobes called hepatic lobes. Each lobe consists of many lobules called hepatic lobules which are the structural and functional unit of the liver. The lobule is made up of liver cells called hepatocytes (Sembulingham and Sembulingam, 2012). The liver receives about 1,500 ml/min of blood from the hepatic artery and portal vein; hepatic artery supplies oxygenated blood to the liver. Portal vein brings deoxygenated blood rich in monosacharides and amino acids from stomach, intestine, spleen and pancreas. The blood also contains bile salts, bilirubin, urobilinogen and gastro-intestine hormones (Sembulingam and Sembulingam, 2012). The substances synthesized by hepatic cells, waste products and carbondioxide are discharged into sinusoids. The sinusoids drain into central vein of the lobule and ultimately form hepatic veins (right and left) (Sembulingam and Sembulingam, 2012).

The liver detoxifies the blood (Barrette *et al*., 2010). Detoxification role can be divided into two; physical and biochemical. The physical involves the trapping and breaking down of bacteria and other foreign particles by the kupffer cells. Xenobiotics and other toxins are then converted to

inactive, less lipophilic metabolites by cytochrome P_{450} enzymes in a series of biochemical reactions (Barrette *et al*., 2010). The liver play key roles in fat metabolism, carbohydrate metabolism, including glycogen storage, conversion of galactose and fructose to glucose and gluconeogenesis, maintaianance of blood glucose levels, metabolism of essentially all steroid hormones, synthesis of lipoproteins and convertion of amino acids and two carbon fragments derived from carbohydrate into fats for storage (Barrette *et al*., 2010).

The liver is the only site of albumin synthesis and most of the other plasma proteins (Barrette *et al*., 2010). The main functions of albumin are the regulation of osmotic pressure and as carrier of many substances through out the body. Deamination and transamination of amino acids occur in the liver resulting in the formation of ammonia and urea (Barrett *et al*., 2010). Other liver functions include the synthesis and breakdown of red blood cells and storage of vitamin A, D, K, B_{12} and folic acid (Barrette *et al*., 2010).

CHAPTER THREE

METHODOLOGY

3.1 Plant material

3.1.1 Collection of plant

Fresh stem and root barks of *Annona senegalensis* were collected in Takum, Taraba state between May, 2016 to August, 2016 and authenticated at the Herbarium Unit of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria and was assigned a voucher number UILH/001/449.

3.1.2 Preparation of plant

Freshly collected stem and root barks of *Annona senegalensis* were washed clean and air-dried under shade (room temperature) to constant weight and then pulverized separately using mortar and pestle into powder. The powdered samples were stored in airtight containers and kept at room temperature until required for use.

3.1.3 Extraction procedure

The air-dried powdered plant samples were soaked separately in three solvents; hexane, dichloromethane and water in the ratio 1: 10 for 24 hours at ambient temperature (35°C) with vigorous shaking at 3 h intervals. The crude extracts were filtered using Whatman No. 1 filter paper. Each of the filtrates was evaporated to dryness at 40^{o}C under reduced pressure and the dried substance was stored in airtight bottle until required (Odebiyi and Sofowora, 1991). The crude extract was kept in a desiccator. When required, a known quantity of the extract was dissolved in a known volume of appropriate solvent to obtain the desired concentration.

3.2 Experimental Animals

A total of 455 albino rats of both sexes (135 for antidiarrhoeal activity of the solvent extracts; 185 for the bioactivity guided fractionation of the bioactive extracts/fractions; 35 for the mechanism of action of the antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem barks and 100 for toxicity studies of the antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem barks) weighing between 130 -150 g were obtained from the Animal Breeding Unit of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria. They were housed in well ventilated aluminium cages, and given standard laboratory diet and water *adlibitum.* The rats were handled according to the guidelines for the protection and handling of laboratory animals by the International Council for Laboratory Animal Science (ICLAS) and approved by the ethical committee of the Department of Biochemistry, University of Ilorin, Ilorin, Nigeria.

3.3 Ethical approval

This research was approved by the University of Ilorin Ethical Review Committee and was given an approval number: UERC/ASN/2018/1216.

3.4 Induction of diarrhoea

The procedure described by Awouters *et al.* (1978) was followed to induce diarrhoea. The rats were fasted for 18 hr. with free acess to water. The rats were administered 1 mL castor oil orally using orogastric cannula to induce diarrhoea. The rats were kept in separate metabolic cages lined with filter paper to collect wet stool. Diarrhoea was confirmed by the first appearance of watery stool.

Table 3.1: Animal grouping for evaluation of antidiarrhoeal activity of extracts from *A. senegalensis* root and stem barks

Group	Treatment
Tween control	1.0 mL/kg body weight (b.wt) of 20% Tween
DMSO control	1.0 mL/kg body weight (b.wt) of 20% DMSO
Standard drug control	Diarrhoeal rats treated with 3.0 mg/kg body weight (b.wt) loperamide
Dichloromethane stem extract (DS)	Diarrhoeal rats administered 100 mg/kg body weight (b.wt) of DS extract reconstituted in 20% DMSO
Dichloromethane root extract (DR)	Diarrhoeal rats administered 100 mg/kg body weight (b.wt.) of DR extract reconstituted in 20% DMSO
Hexane stem extract (HS)	Diarrhoeal rats administered 100 mg/kg body weight (b. wt) of HS extract reconstituted in 20% DMSO
Hexane root bark (HR)	Diarrhoeal rats administered 100 mg/kg body weight (b.wt) of HR extract reconstituted in 20% DMSO
Aqueous stem extract (AS)	Diarrhoeal rats administered 100 mg/kg body weight (b.wt.) of AS extract reconstituted in 20% Tween
Aqueous root extract (AR)	Diarrhoeal rats administered 100 mg/kg body weight (b. wt.) of AR extract reconstituted in 20% Tween

Each group contained five adult albino rats
DMSO – Dimethyl sulfoxide

3.5. Evaluation of antidiarrhoeal activity of *A. senegalensis* root and stem barks

Rats used for antidiarrhoeal activity of hexane, dichloromethane and aqueous extract were randomly selected and grouped as shown in Table 3.1. Forty five (45) rats were used for the castor oil - induced stool inhibition experiment while another set of forty five rats were used for the castor oil-induced enteropooling and measurement of gastrointestinal transit time experiments respectively.

3.5.2 Castor oil-induced diarrhoea inhibition

The method described by Gunakkunru *et al*. (2005) was adopted with modifications in the number of rats used. After an hour of induction of diarrhoeal, the extracts/fractions/sub-fractions were administered. The animals were placed separately in metabolic cages over white clean whatman filter paper, which was changed every hour. The severity of diarrhoea was assessed each hour for 4 hours. The total number of diarrhoea faeces of the control group was considered 100%.

% inhibition = (Control - Test) × 100/Control

3.5.3 Measurement of gastrointestinal transit time

The method described by Robert *et al.* (1976) was adopted. One hour after induction of diarrhoea, the extracts/fractions/sub-fractions were administered to the rats. After thirty (30) min of the administration, 1ml of charcoal meal (10% suspension in 5% gum acacia) as a marker diet was given orally to rats in each group. The rats were sacrificed by ether (20% v/v) anesthesia and small intestine carefully separated from mesentery avoiding being stretched. For each animal, gastrointestinal transit was calculated as percentage distance travelled by charcoal meal to the total length of intestine. The inhibitory effect of the extracts/fractions/sub-fractions on gastrointestinal transit was calculated relative to respective group as thus:

$$\% \text{ gastrointestinal transit time} = \frac{\text{Distance travelled by charcoal meal}}{\text{Total length of small intestine}} \times 100$$

3.5.4 Castor oil-induced enteropooling

Castor oil-induced enteropooling was determined by the method of Mascolo *et al*. (1994). One hour after induction of diarrhoea, the extracts/fractions/subfrations were administered orally to the rats. After 1 hour, the rats were sacrificed by ether anesthesia. The edges of the intestine from pyloric to ceacum were tied with thread and the intestine removed and weighed. Intestinal content was collected by milking into a graduated tube and the volume measured. The intestine was reweighed and differences between full and empty intestine calculated.

3.6 Bio-activity guided fractionation of root and stem bark extracts of *Annona senegalensis*

The bioactivity guided fractionation of the crude aqueous root and stem barks as well as that of crude extracts of dichloromethane root and stem barks is schematically shown in Figure 3.1. In this phase of the study, a total of one hundred and eight five rats were used for the bio-activity guided fractionation of root and stem bark extracts of *Annona senegalensis*. The rats were randomly divided into groups. The number of groups for each experiment was determined by the number of fractions/ sub-fractions obtained after fractionation. Each group contained five rats. The rats were administered 25 mg/kg b. wt. of the fractions/sub-fractions obtained after inducing diarrhoea.

3.6.1 Solvent – solvent partitioning of aqueous extract

Solvent- solvent partitioning was done using the method designed by Kupchan *et al.* (1973) and modified by Van-Wagener *et al.* (1993). The partitioned process is schematically shown in Figure 3.2. Crude aqueous extract (5 g) was transferred into a 500 mL beaker. In another beaker 90 mL of methanol was added to 10 mL of water. This mixture was then added to the extract to make a 10% aqueous solution. The 10% aqueous methanol solution was poured into a seperating funnel and

was successively partitoned with hexane, dichloromethane and ethyl acetate in order of increasing polarity. The separating funnel was shaken vigorously, cork opened and allowed to stand until there was clear separation. The fractions were collected and evaporated to dryness using a rotary evaporator at 40^{o}C. All fractions were collected and tested for antidiarrhoeal activity. The most active solvent fraction was subjected to partial purification.

3.6.2 Solvent system selection for purification of antidiarrhoeal fraction

A small amount of the fraction was transferred to a thin layer chromatography (TLC) plate using a pasteur's pipette or a capillary tube. Silica gel was used as stationary phase. Several eluting solvents were tested to see the solvent system that will give a better separation of the components present in the sample. The developed chromatogram in the TLC plate was sprayed with 1:1 mixture of 2% vanillin in 25% ethanol (2 grams in 100 mL); iodine crystals and 95% ethanol and 10% sulfuric acid in 9:1 ratio and placed in a hot plate to reveal the spots / bands. The solvent system that showed the best resolutions of spots (well separated spots and less tailing) was used for column chromatography.

3.6.3 Partial purification of antidiarrhoeal fractions obtained from *A. senegalensis* root and stem barks using column chromatography

The dried fractions obtained were weighed. The ratio of 1.0 g antidiarrhoeal fraction per 20 silica gel powder was used to determine the weight of the silica gel that was placed in a column. The column for the fractionation was 390 mm long with radius of 15 mm. The fraction (1.0 g fraction per 20 g silica gel) was dissolved in the eluting solvent and transferred into the column. Elution was done using the solvent determined by thin layer chromatography (2%, 4%, 6%, 8% and 10%) methanol in dichloromethane for the dichloromethane extracts and ethylacetate: butanol: water:

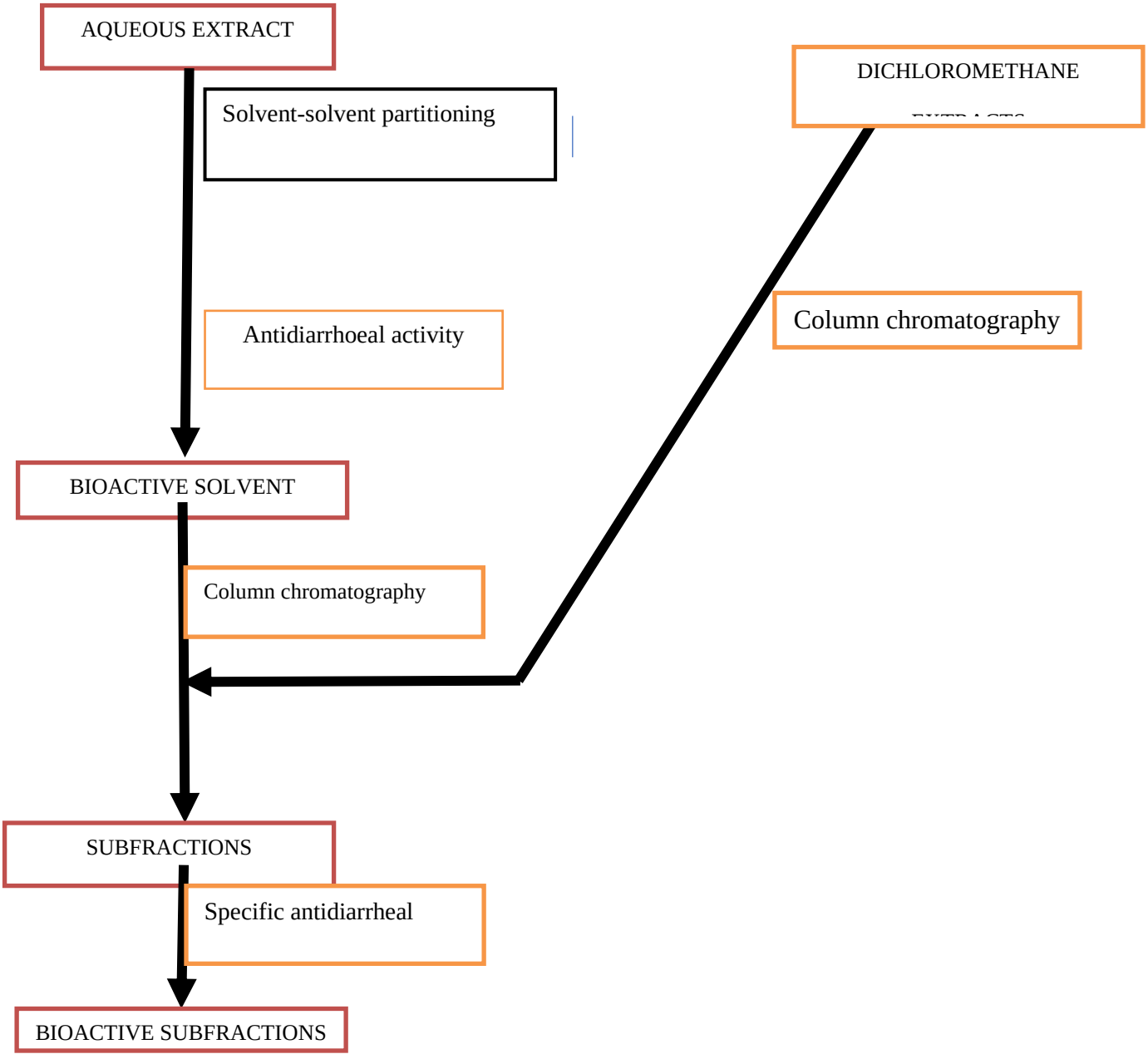

Figure 3.1: A schematic illustration of the bioactivity guided fractionation of the aqueous and dichloromethane root and stem bark extracts of *Annona senegalensis*

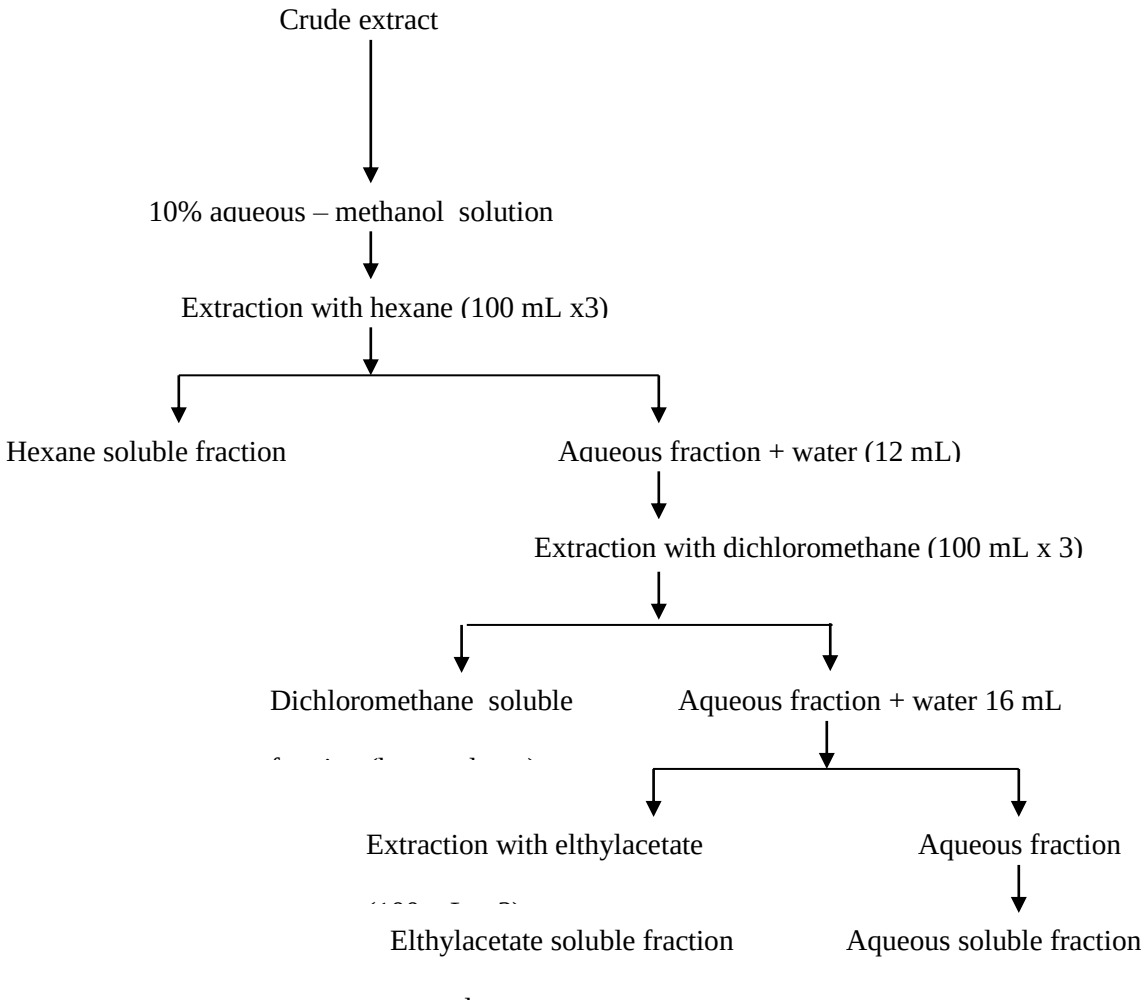

Figure 3.2: Schematic representation of the modified Kupchan method of partitioning crude extracts of *A. senegalensis* root and stem barks

3.7. Evaluation of the mechanism(s) of antidiarrhoeal activity of antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

Table 3.2: Animal grouping for investigation of mechanism(s) of antidiarrhoeal action of the antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem barks

Group	Treatment
DMSO control	Diarrhoeal rats administered 1mL of 20% DMSO
Water control	Diarrhoeal rats administered 1mL of distilled water
DFAR1	Diarrhoeal rats administered 25 mg/kg b. wt DFAR1 reconstituted in water
EFAR2	Diarrhoeal rats administered 25 mg/kg b.wt. EFAR2 reconstituted in water
EFAS1	Diarrhoeal rats administered 25 mg/kg b.wt. EFAS1 reconstituted in water
DR2	Diarrhoeal rats adminitered 25 mg/kg b. wt. DR2 reconstituted in 20% DMSO
DS3	Diarrhoeal rats administered 25 mg/kg b.wt. DS3 reconstituted in 20% DMSO

All groups contained five Albino rats.

DMSO- Dimethyl sulfoxide, DFAR1- sub-fraction 1 of dichloromethane fraction from aqueous root bark, EFAR2- sub-fraction 2 of ethylacetate fraction from aqueous root bark, EFAS1- sub-fraction 1 of ethylacetate fraction from aqueous stem bark, DR2- sub-fraction 2 of dichloromethane root bark extract. DS3 – sub-fraction 3 of dichloromethane stem bark extract.

acetic acid (50:40:5:5)] was used for the aqueous fractions. Eluants were collected in 100mls. The fractions were spotted on thin layer chromatography (TLC) plates. Sub-fractions with the same retention factor (Rf) in the TLC plate were pooled together. All sub-fractions were evaluated for antidiarrhoeal activity. Sub-fractions with the same level of activity were pooled together as one main sub-fractions.

3.7. 1 Preparation of intestinal homogenates and intestinal fluid

The rats were sacrificed by ether anesthesia after an hour of administration of the antidiarrhoeal sub-fractions. The small intestine of each rat was carefully removed. The intestinal content was collected by milking into a test tube. The intestinal fluid was used for the antisecretory activity determination by measuring its Na^+, K^+ and Cl^-concentrations. The small intestine was homogenized in 0.25 M sucrose (1: 4) and its malondialdehyde (MDA) concentration, antioxidant enzyme, Na^+-K^+ ATPase and cyclooxygenase II (COX II) activities were determined according to standard methods.

3.7.2 Evaluation of antioxidant mechanism of the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

3.7.2.1 *In vitro* antioxidant evaluation of the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

The antidiarroeal sub-fractions were screened for *in vitro* antioxidant activity by using the following antioxidant models:

3.7.2.1.1 Determination of 2, 2- diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity of the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

Principle

This method is based on the reduction of 2, 2-diphenyl-1-picrylhydrazl (DPPH) in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the nonradical

form DPPH-H. This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517 nm. The free radical scavenging activity can be measured by using 2, 2-diphenyl-1-picryl-hydrazyl or 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) by the method of McCune and Johns (2002).

Procedure

An aliquot amount (1mL) of various concentrations of the sub-fractions in methanol was added to 4 mL of 0.1 mmol/L methanolic solution of DPPH. A blank probe was obtained by mixing 4 mL of 0.1 mmol/ L methanolic solution of DPPH and 200 µL of deionized distilled water. After 30 min of incubation in the dark at room temperature, the absorbance was read at 517 nm against the prepared blank. Inhibition of free radicals by DPPH in percentage was calculated using the formula-

% inhibition = $100 - [(ABS_{sample} - ABS_{blank})/ ABS_{control}] \times 100$.

3.7.2.1.2 Ferric reducing antioxidant power (FRAP) assay of the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

Principle

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-triazine (TPTZ), forming an intense blue Fe^{2+} – TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The decrease in absorbance is proportional to the antioxidant content (Benzie and Strain, 1996).

Procedure

An aliquot amount (0.2 mL) of the sub-fraction was added to 3.8 mL of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM $FeCl_3.\ 6H_2O$ solution) and the reaction mixture was incubated at 37°C for 30 min and the increase

in absorbance at 593 nm was measured. $FeSO_4$ was used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as nmol $FeSO_4$ equivalents per gram of sample. Ascorbic acid was used as the positive control (Benzie and Strain, 1996)

3.7. 2.1.3 Assay for hydroxyl radical scavenging activity of the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

Principle

Ascorbic acid, iron and EDTA in an aqueous system conspire with each other to generate hydroxyl radicals. The scavenging ability for hydroxyl radicals is measured by the method of Kunchandy and Rao (1990).

Procedure

The reaction mixture (1.0 mL) consisted of 100 µL of 2-deoxy - D - ribose (28 mM in 20 mM KH_2PO_4 -KOH buffer, pH 7.4), 500 µL of the sub-fractions, 200 µL EDTA (1.04 mM) and 200 µM $FeCl_3$ (1:1 v/v), 100 µL of H_2O_2 (1.0 mM) and 100 µL ascorbic acid (1.0 mM). This was incubated at 37ºC for 1 hour. One (1.0) mL of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8%) were added and incubated at 100ºC for 20 min. After cooling, absorbance was measured at 532 nm, against a blank sample. Ascorbic acid was used as a positive control**.**

3.7.2.1.4 Assay for 2, 2 - Azinobis 3-ethylbenzo-thiazoline -6-sulfonate (ABTS) radical scavenging activity of antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

Principle

The reaction is based on the peroxidase substrate, 2, 2-azinobis 3-ethylbenzo-thiazoline -6-sulfonic acid (ABTS**)** forming a relatively stable radical upon an electron oxidation (Bartosz and Bartosz, 1999).

Procedure

ABTS radical was generated by reacting an ABTS aqueous solution (7 mmol/L) with $K_2S_2O_8$ (2.45 mmol/L) in the dark for 16 hours. Aliquot amount (2 mL) of appropriatly diluted sub-fraction was added to 2.0 mL ABTS solution and the absorbance was read at 734 nm after 15 min.

3.7.2.1.5. Determination of total antioxidant capacity of the antidiarhhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

Principle

The total antioxidant capacity of the different antidiarrhoeal sub-fractions were determined spectrophotometrically by phosphomolybdenum method described by Prieto *et al*. (1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the sample and subsequent formation of a green phosphate Mo (V) complex at acidic pH.

Procedure

One (1) mL of each sub-fraction (0.5 $mgml^{-1}$) was mixed with 3 mL reagent solution. The reagent solution contained 0.6 M H_2SO_4. 28 mM sodium phosphate and 4 mM ammonium molybdate. The blank contained 4 mL reagent solution only. The mixtures were incubated at 95^oC for 150 min. The mixture was allowed to cool to room temperature and the absorbance was read at 695 nm. The total antioxidant capacity was expressed as tannic acid equivalent (TAE).

3.7.2.2 Assay of antioxidant enzymes activities in small intestine of castor oil-induced diarrhoeal rats administered antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

3.7.2.2.1 Determination of catalase (CAT) activity

Principle

Catalase activity was determined by the decrease in absorbance due to H_2O_2 consumption (Aebi, 1974).

Procedure

Fifty (50) µL of small intestinal homogenates was diluted with 5 mL, 50 mM phosphate buffer solution (pH 7). Two (2.0) mL of the diluted intestinal homogenate was added to 1 mL of H_2O_2 in sample tube, the blank tube contained 1ml of phosphate buffer and 2 mL of diluted homogenate. It was mixed immediately and the absorbance was taken at 15 seconds and after 30 seconds at 240 nm.

3.7.2.2.2 Determination of reduced glutathione (GSH)

Principle

The sulfhydryl goup of GSH reacts with 5, 5- dithio-bis-2-nitrobenzene acid (DTNB), Ellman's reagent to produce a yellow coloured 5-thio-2- nitrobenzoic acd (TNB). The mixed disulfide; (between GSH – TNB) GS-TNB that is concomitantly produced is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the recycling concentration which is in turn directly proportional to GSH concentration (Burtis and Ashwood, 1996).

Procedure

To 100 µL small intestine homogenates was added, 800 µL double distilled water (DW), 100 µL 50% trichloroacetic acid. It was mixed well by vortex for 10-15 min, and centrifuged for 15 min at 3000 × g. The supernatant was decanted and 400 µL of the supernatant was taken. Tris-EDTA buffer (800 µL) and 20 µL DTNB reagent were added. It was mixed well by vortex. In the same way, blank (deionized distilled water alone) and standards (0.03 g of GSH in a final volume of 100 mL of 0.4 M EDTA) solution were prepared. The absorbance of standard and homogenates were read within 5 min of the addition of DTNB at 412 nm.

3.7.2.2.3 Determination of superoxide dismutase (SOD)

Principle

Superoxide dismutase reacts with O_2^- formed during the oxidation of epinephrine and therefore slows down the rate of formation of the adrenochrome as well as the amount formed. The percentage inhibition with respect to superoxide dismutase is estimated by the increase in absorbance (Sun and Zigman 1978).

Procedure

The reacton mixture (3 µL) contained 2.95 mL of 0.05 M sodium carbonate buffer (pH 10.2), 0.02 mL of intestine homogenate and 0.03 mL of epinephrine. HCl (0.05 mL) was used to initiate the reaction. The reference cuvette contained 2.95 mL buffer, 0.03 mL of substrate epinephrine and 0.02 mL of water. SOD activity was calculated by measuring the change in absorbance at 480 nm for 5 min. Enzyme activity was calculated as described here under.

$$\text{Increase in absorbance per minute} = \frac{A_5 - A_0}{2.5}$$

Where A_0 = absorbance after 0.5 min

A_5 = absorbance after 2.5 min

$$\% \text{ inhibition} = \frac{\text{Increase in absorbance for substrate} \times 100}{\text{Increase in absorbance of blank}}$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute.

3.7.2.3 Evaluation of intestinal barrier function of the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks in castor oil-induced diarrhoeal rats

3.7.2.3.1 Determination of malondialdehyde concentration

Principle

Malondialdehyde (MDA) normally reacts with thiobarbituric acid (TBA) to generate a MDA-TBA adduct. The MDA-TBA adduct can be easily quantified colorimetrically. The optical density of the pink colour formed is directly proportional to the concentration of MDA (Kunchandy and Rao, 1990).

Procedure

Aliquot amount (0.8 mL) of Tris KCI was added to 0.2 mL of the intestinal homogenate and then quenched by addition of 0.25 mL, of TCA; 0.25 mL of TBA was added and the reaction mixture incubated for 45 mm at 80°C and then cooled on ice. The resulting pink-coloured reaction mixture was centrifuged at 4000 × g for 15 min. The absorbance of the clear pink supernatant was then read at 535 nm using distilled water as blank. The concentration of MDA was then calculated thus:

$$\text{MDA (mgldL)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532} \times \text{volume of sample} \times \text{mg protein}}$$

Where E_{532} is molar absorbtivity at 532 nm = 1.56×10^{-5}

3.7.2.4 Evaluation of the antisecretory mechanism of antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem barks in castor oil-induced diarrhoeal rats

3.7.2.4.1 Determination of intestinal fluid potasium concentration

Principle

The amount of potassium present in a sample is determined by using sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension. The turbidity is proportional to potassium concentration (Wooten and Freeman, 1982).

Procedure

Four test tubes were labelled standard, control, blank and sample. One (1.0) mL of potassium reagent (sodium tetraphenylboron 2.1 mM) and 0.01 mL of intestinal fluid was added to the respective test-tubes. It was mixed and allowed to stand at room temperature for 3 min. The absorbance of sample (AT) and standard (AS) were read against reagent blank at 500nm.

Calculation

Potassium (mmol/L) = AT/AS × conc. of standard

3.7.2.4.2 Determination of intestinal fluid chloride concentration

Principle

Chloride ions form a coloured complex according to the following reaction

$2Cl^- + Hg(SCN)_2 \longrightarrow HgCl_2 + 2SCN^-$

$SCN^- + Fe^{3+} \longrightarrow FeSCN^{2+}$.

The intensity of the colour is proportional to the chloride concentration (Wooten and Freeman, 1982).

Procedure

Three test tubes were labelled blank, standard and sample. One (1 mL) of chloride reagent was added to the three samples. Ten (10) µL of intestinal fluid was added to the test tube labelled sample only. Ten (10) µL of standard was added to the test tube labelled standard. It was mixed and incubated for 5 min at 37^{o}C. The absorbance of sample and standard were measured against blank.

Calculation

Chloride (mmol/L) = ($ABS_{sample}/ABS._{standard}$) × 125(standard curve conc.)

3.7.2.4.3 Determination of intestinal fluid sodium concentration

Principle

Sodium and proteins are precipitated together by magnesium uranyl acetate as uranyl magnesium sodium acetate salt. Excess of uranyl salt reacts with potassium ferrocyanide to produce a brownish colour. The intensity of the colour is inversely proportional to the sodium concentration in the specimen and is measured photometrically at 530 nm (Wooten and Freeman, 1982).

Procedure

Two test tubes were labelled standard and test. Ten (10) µL of sodium standard was added to the test tube labelled standard while 10 µL of intestinal fluid was added to the test tube labelled sample. To the three test tubes, 1 mL of precipitating reagents was added. The mixture was shaken vigorously and incubated at room temperature for 5 min. It was centrifuged at 2000 – 3000 rpm for 2 min to obtain a clear supernatant. The supernatant was transferred immediately into a test tube for sodium estimation. Another set of three test tubes were labelled blank, standard and test. One (1 mL) of colour reagent was added to the test tubes labelled standard and test. Twenty (20) µL of precipitating reagent was added to the blank test tube. The mixture was shaken vigorously and

allowed to stand at room temperature for 5 min. The absorbance of sample and standard against blank were measured. Sodium concentration was calculated thus:

$$\text{Sodium concentration (mmol/L)} = \frac{\text{Abs. of blank} - \text{Abs. of Test}}{\text{Abs. Blank} - \text{Abs Standard}} \times \text{standard concentration}$$

3.7.2.5 Assay of small intestine Na^+– K^+ ATPase activity of the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks in castor oil-induced diarrhoeal rats

Principle

Adenosine triphosphatase (ATPase) assay indirectly measures the activity of efflux transporters. Adenosine triphosphate (ATP) cleavage is tightly linked to substrate translocation as the energy for the substrate translocation is derived from ATP hydrolysis. Its hydrolysis yields inorganic phosphate which can be measured colorimetrically. The amount of inorganic phosphate liberated is directly proportional to the activity of ATPase (Glavinas *et al.,* 2008).

Procedure

Intestine homogenates were prepared as described by Straub and Carver (1975). The enzyme source was diluted to a final volume of 400 mL and kept frozen. The Na^+- K^+ ATPase was assayed as described by Suhail and Rizvi (1987). The assay medium contained 100.0 mL NaCl (140 mM), 100 mL of 120 mM KCl, 50 mL of 3 mM $MgCl_2$, and 2.5 mL of 30 mM imidazole (pH 7.25), 50 mL of 5×10^{-4}M ouabain and 25 mL of 6 mM ATP. Incubation was carried out for 30 min at 37^{o}C, the reaction was stopped by adding 3.5 mL of a solution containing 0.5 M H_2SO_4, 0.5% ammonium molybdate and 2% SDS.

Protein concentration was determined by the method of Lowry *et al*. (1951) and inorganic phosphate concentration was determined by the method of Munoz *et al*. (1983). The Na^+ - K^+ ATPase activity was expressed in terms of micro mole of inorganic phosphate /hr/mg protein.

3.7.2.6 Evaluation of anti-inflamatory mechanism of action of antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks in castor oil-induced diarrhoeal rats

3.7.2.6.1 Determination of cyclooxygenase (COX) II activity

Principle

Cyclooxygenase II activity assay utilizes the peroxidase component of cyclooxygenases. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N,N-tetramethyl-p-phenylenediamine (TMPD) at 590nm (Guenzle *et al*., 2019).

Procedure

The standard, blank and intestine homogenate well were prepared. Fifty (50) µL of standard was added to the standard well. Ten (10) µL of intestine homogenate was added to 40 µL of sample diluent in the testing well. One (100) µL of Horseradish peroxidase (HRP)-conjugate reagent was added to each well and was covered with an adhesive strip. They were incubated for 60 min at 37^{o}C. The wells were aspirated and washed repeatedly four times. Fifty (50) µL chromogen solution was added to each well and was gently mixed. They were incubated for 15 min at 37^{o}C and protected from light. Fifty (50) µL of quenching solution was added to each well. Absorbance was read at 450 nm.

3.8 Identification of compounds in the antidiarrhoeal sub-fractions using gas chromatography-mass spectrometry (GC-MS) analysis

Principle

The gas chromatography-mass spectrometry (GC-MS) works on the principle that a giving volatile mixture will separate into its individual compounds when heated. The heated gases are carried in an inert gas such as helium into a column. The separated gases emerge from the column and flows into the mass spectrometry that identifies the compounds by their mass.

Procedure

The identification of compounds in the antidiarrhoeal sub-fraction(s) was performed on a GC-MS equipment (Thermo Scientific Co.) Thermo GC-TRACE ultra version 5.0 Thermo MS DSQ II model. The experimental conditions of the GC-MS system were as follows: DB 5 - MS capillary standard non - polar column with a dimension of 30 Mts, ID: 0.25 mm, film thickness of 0.25 μm. The flow rate of mobile phase carrier gas (Helium) was set at 1.0 ml/min. In the gas chromatography part, temperature programme (oven temperature) was 70°C which was raised to 260°C at 6°C/min and injection volume was 1 μL. Samples dissolved in ethanol were run fully at a range of 50-650 m/z and the results were compared by using Wiley Spectral library search programme.

3.9 Toxicity studies of antidiarrhoeal sub-fractions from *A. seneganlesis* root and stem barks

A total of 100 adult albino rats were divided into 5 main groups for the toxicity study of the five antidiarrhoeal sub-fractions obtained from *A. seneganlesis* root and stem barks. Each group was further subdivided into four sub-groups of five rats each. For each sub-group, group I served as control, groups II, III and IV received 100, 200 and 400 mg/kg b.wt of antidiarrhoeal sub-fractions from *A. Senegalensis* root and stem bark for 14 days respectively. The feed intake of each rat was recorded on a daily basis. The body weight of the rats were taken on the first day of the experiment and (initial weight) and prior to sacrifice (final weight). Change in body weight was calculated as the difference in the final weight and initial weight. The rats were observed for signs of toxicity and mortality throughout the experiment. The rats were sacrificed under anaesthesia on the 15^{th} day. Blood samples were collected into non anti-coagulated and ethylenediaminetetraacetic acid (EDTA) tubes. The non anti-coagulated blood was then centrifuged at 3,000 rpm for 10 min and serum was collected. The rats were quickly dissected. The heart, kidneys, liver and stomach were

removed, washed clean and weighed. The relative organ body weight ratio (ROW) of each rat was calculated as:

ROW = weight of organ (g)/body weight of animal (g).

3.9.1 Liver function test

3.9.1.1 Determination of alanine aminotransferase (ALT) activity

Principle

Alanine aminotransferase (ALT) catalyzes the transfer of an amino group from L- alanine to 2-oxoglutarate to form pyruvate and L- alanine. Alanine aminotransferase is usually determined by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine (Reitman and Frankel, 1957)

Procedure

A standard calibration curve for ALT was prepared by dispensing various volumes (0.1 - 0.7 mL) of 2 mM sodium pyruvate solution (as standard) into different test tubes. The mixtures were shaken and incubated for 30 min at 37°C, 0.001 M 2, 4-dinitrophenylhydrazine (DNPH) (1 mL) was added to each of the test tubes. The mixture was allowed to stand for 20 min at 25°C after which 5 mL of 0.4 N NaOH was added. The mixture was shaken and after 5 min a violet coloured solution developed. The absorbance was read at 546 nm against a reagent blank. The values obtained was plotted against the corresponding concentration of pyruvate.

ALT buffered substrate solution (1 mL) was dispensed into test-tube followed by the addition of 0.2 mL of the enzyme source (serum). This was mixed and incubated for 30 min at 37°C after which 1mL of 0.001 M 2.4-dinitrophenylhydrazinc solution was added and allowed to stand for 20 min at 25°C. 0.4 N NaOH (5 mL) was thereafter added and allowed to stand for 5 min for a purple

colour to develop. The absorbance was read at 546 nm from the spectrophotometer and the value obtained was used to obtain the equivalent activity of ALT by extrapolatng from the standard curve and expressed in U/L (Figure 7- appendix). The blank was constituted as described above except that distilled water was used as substitute for the enzyme source (serum).

3.9.1.2 Determination of aspartate aminotransferase (AST) activity

Principle

Aspartate aminotransferase catalyzes the reversible transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. Aspartate aminotransferase (AST) is usually determined by monitoring the concentration of oxaloacetate hydrazine formed with 2, 4-dinitrophenylhydrazine (Reitman and Frankel, 1957).

Procedure

The procedure for the calibration of AST standard curve and the determination of AST activity in serum is as described for ALT procedure in section 3.9.1.1 except that AST buffered substrate contained aspartate in lieu of alanine.

Calculation: The absorbance value obtained from the spectrophotometer was used to obtain the equivalent activity of AST by extrapolation from the standard curve and expressed in U/L (Figure 8- appendix).

3.9.1.3 Determination of alkaline phosphatase (ALP) activity (colorimetric method)

Principle

Alkaline phosphatase catalyses the hydrolysis of phosphate esters in alkaline buffer and produces an organic radical and inorganic phosphate. p-Nitrophenyl phosphate (p-NPP) is hydrolyzed to p-nitrophenol and phosphoric acid at pH 10.1. The p - nitrophenol confers a yellowish color on the

reaction mixture and its intensity is directly proportional to the enzyme activity (Wright *et al*., 1972).

Procedure

An aliquot amount (2.2 mL) of 0.1 M carbonate buffer (pH 9.0) was dispensed into clean test-tube, 0.2 mL of the serum and 0.1 mL of 0.1 M MgSO4 were added. The mixture was equilibrated in water bath for 10 minutes. An aliquot (0.5 mL) of 19 nM p-nitrophenyl phosphate was added and the mixture incubated at 37^{o} C for 10 min to form a yellowish solution. The reaction was stopped with 2.0 mL of 1.0 N NaOH and the absorbance read at 400 nm. Blank was prepared by adding serum to the test tube labelled blank immediately after stopping the reaction with NaOH.

Activity of ALP was calculated using the expression:

ALP activity (U/L) = $(\Delta OD/\min \times 1000 \times TV \times F)/(9.9 \times SV \times L)$

$\Delta OD/\min$ = Change in optical density of reaction mixture per minute; TV Total volume of the reaction mixture; F Total dilution factor; SV = Volume of enzyme source; L = Light path length (I cm) 9.9 = Extinction co-efficient of 1gm of p-nitrophenol in an alkaline solution of 1mL and 1 cm path length

3.9.1.4 Determination of albumin concentration

Principle

The measurement of serum albumin is based on its quantitative binding to the indicator 3,3',5, 5'-tetrabromo-m cresol sulponephthalein commonly known as bromocresol green (BCG) to produce a blue green complex. The albumin-BCG-complex absorbs maximally at 578nm. The absorbance is directly proportional to the concentration of albumin in the sample (Doumas *et al*., 1971).

Procedure

Bromocresol green (BCG) concentrate was prepared by mixing 25 mL of 75 mmol/L succinate buffer (pH 4.2) with 0.25 mL 0.15 mmol/L bromocresol green and 10 mL 7 mol/L brig 35. One (1) ml of BCG concentrate was pipetted into three test tubes labelled blank, standard and sample. Ten (10) µL of distilled water was added to blank. Ten (10) µL of albumin standard was added to the test tube labeled standard while 10 µL of serum was added to the test tube labeled sample. The optical density (A) was read against blank at 578 nm after 3 min incubation at 35ºC.

Calculation

$$\frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Cstandard} = \text{Csample}$$

3.9.1.5 Determination of total and direct bilirubin concentration

Principle

Sulfanic acid reacts with sodium nitrate to form diazotized sulfanilic acid in the presence of dimethylsufoxide. Total bilirubin reacts with diazotized sulfanillic acid to from azobilirubin (blue color) which can be detected at 540 nm. In the absence of dimethylsulfoxide, only the direct bilirubin reacts to give azobilirubin (Jendrassik and Golf, 1938).

Procedure

Reagent RT1 for the total bilirubin was prepared by adding 100 mL of 3.2 mmol/L sulfanilic acid, 50 mL of 165 mmol/L hydrochloric acid and 50 mL dimethyl sulfoxide. Reagent RD1 contained the other reagents apart from dimethyl sulfoxide. Sodium nitrite (8.6 mmol/L) was used as reagent 2(R2) for both direct and total bilirubin. For total bilirubin determination, the two reagents (RT1 and R2) were mixed as the working reagent in the ratio 125 mL: 25 mL. while for direct bilirubin RD1 and R2 were mixed in the ratio 125 mL: 25 mL. One (1.0) mL of working reagent was pipeted into two test tubes labeled sample and standard. Seventy five (75) µL of serum was added

to the sample testtube while 75 µL of standard as added to te standard testtube. It was mixed well and the absorbance was read at 555 nm after 3 min of incubation at 35ºC. the same procedure was used for the direct bilirubin except that its working reagent did not contain dimethylsulfoxide

Calculation

$$\frac{A_{serum}}{A_{standard}} \times C_{standard} = C_{serum}$$

Where A= absorbance; C = concentration

3.9.2 Kindney function test

3.9.2.1 Determination of urea concentration

Principle

Salicylate and hypochlorite in the reagent react with the ammonium ions liberated from urea in the presence of urease enzyme to form a green complex (2,2-dicarboxylindophenol). The absorbance of the green complex is directly proportional to concentration of urea (Fawelt and Scott, 1960).

Procedure

The sample (0.01) mL of either serum or standard Randox control) was added to 1mL of urease prepared in phosphate buffer containing 63.4 mM sodium salicylate, 5 mM sodium nitroprusside, 1.5 mM EDTA, 18.0 mM sodium hypochlorite and 750 mM sodium hydroxide The mixture was mixed and incubated for 10 min at room temperature ($\leq$ 27°C) with the development of a bluish green coloured solution. The absorbance of the test sample and standard were measured against the blank at 600 nm. Blank was prepared by substituting the sample for distilled water. The urea concentration in the sample was calculated using the following expression:

$$\text{Urea concentration (mg/dl)} = \frac{\text{Absorbance of sample x Concentration of standard}}{\text{A standard}}$$

3.9.2.2 Determination of creatinine concentration in rat serum using colorimetric method

Principle

Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration (Bartels and Brohmer, 1999).

Procedure

The working reagent was prepared by mixing equal volume of 35 mmol/L picric acid and 0.32 mol/L sodium hydroxide. A standard solution of 177 µmol/L of creatinine was prepared. Two (2.0) mL of the working reagent was placed in two macrocuvette and labeled standard and sample. Standard solution (0.2) mL was added to the cuvette labeled standard while 0.2 mL of serum was added to the sample cuvette. The mixture was allowed to stand for 30 secs. The absorbance of the standard and sample were read at 492 nm and recorded as $A1_{standard}$ and $A1_{sample}$ respectively. Two min later, the absorbance A2 of standard and sample was read

Calculation

$$A2 - A1 = \Delta A$$

$$\frac{\Delta A_{sample}}{\Delta A_{standard}} \times 2 = \text{creatinine conc. (mg/dL)}$$

3.9.2.3 Determination of uric acid concentration

Principle

Uric acid concentration in serum was determined as reported by Fossati *et al.* (1980). Uric Acid is converted by uricase to allantoin and hydrogen peroxide, which under the catalytic influence of peroxidase oxidizes 3,5-Dichloro 2-hydroxybenzenesulfonic acid and 4 aminophenazone to form a red-violet quinoneimine compound that is easily detected using UV- spectrophotometry.

Test tubes were labelled standard, reagent blank and samples. 1.0 mL of uric acid reagent was pipetted into each tube after which 0.02 mL of serum was added, the blank and standard were constituted by replacing the serum with 0.02 mL of distilled water and standard respectively. The mixture was mixed and incubated at 37°C for 5 min to give a purple coloured solution.The absorbance was read against the reagent blank at 520 nm. The uric acid concentration was calculated using the formula

$$\text{Uric acid concentration (mg/dL)} = \frac{\text{Absorbance of serum x Concentration of standard}}{\text{Absorbance of standard}}$$

3.9.3 Determination of heamatological parameter

3.9.3.1 Determination of packed cell volume (PCV)

Micro haematocrit tubes were filled up to three quarters (3/4) of its lenghth and then sealed using plasticine. The tubes were placed in a micro haematocrit machine and spun at about 12000 revolutions per minute for exactly 5 min. Micro haematocrit reader was used to evaluate the PCV in percentage

3.9.3.2 Differential white blood cells count

A thin blood film weas made on a clean grease free slide and allowed to air dry at room temperature. The films were stained using GIEMSA stain. The stained films were dried at room temperature and mounted under oil immersion lens. Lymphocyte, monocyte, neutrophil, eosinophil and basophil were counted under light microscope. The white blood cells were identified as; neutrophils, having multilobulated nucleus; eosinophil were having biloted nucleus; monocytes were having horse shoe shape or bean shape nucleus, while the cytoplasms of basophils were highly granulated and their nucleus were obstructed by granules (Ochei and Kolhatkar, 2007).

3.9.3.3 Platelet count

A thoma pipette was used to achieve a 1:200 dilution with formal citratePlatelets ware counted using a high power (40 x) objective in the four large corner squares. The platelets were identified as highly retractile particles. (Ochei and Kolhatkar, 2007).

3.9.3.4 Erythrocyte count

Blood sample was drawn into the red blood cell pipette to the o.5 mark. The excess blood outside the pipette was carefully wiped using cotton or a guaze. Heyen's reagent was drawn into the pipete up to 101 mark. The pipette was rotated rapidly. After five minutes, small volume of the fluid was introduced under a cover slip placed on the counting chamber. The cell was allowed to settle for 3 mins. The counting chamber was placed on the micrsoscope (Ochei and Kolhatkar, 2007). The erythrocyte (red blood cell) was counted in the four corner squaresand in the enter square. Total erythrocyte count was calculated as:

$$\text{Total erythrocyte } (\mu L) = \frac{\text{number of erythrocyte counted} \times \text{dilution fcator}}{\text{Area counted} \times \text{depth}}$$

3.10 Statistical analysis

The computation of the mean and statistical analysis was done using SPSS software version 24..0. Data was expressed as the mean ± SEM of group of five animals which was statistically analyzed with one-way analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT). For all the tests, results with p values < 0.05 was taken to imply statistical significance

CHAPTER FOUR

RESULTS

4.1 Antidiarrhoeal activity of hexane, dichloromethane and aqueous extracts of *Annona senegalensis* root and stem barks in castor oil-induced diarrhoeal rats

Table 4.1 shows the stool inhibition of solvent extracts of *A. senegalensis* root and stem barks in castor oil-induced diarrhoeal rats. Dichloromethane root (DR) and aqueous stem (AS) extracts significantly decreased ($p < 0.05$) the number of wet feaces when compared to their respective negative control (DMSO and Tween). The extracts (DR and AS) had 100% inhibition of wet feaces and was comparable with the group treated with the standard drug (loperamide).

The result of the gastrointestinal tract inhibition and the antienteropooling activity of solvent extracts of *A. senegalensis* root and stem barks is presented in Table 4.2. Aqueous root (AR) and dichloromethane stem (DS) significantly decreased ($p < 0.05$) the volume and weight of intestinal fluid when compared to their respective negative control (Tween and DMSO) and the loperamide group. Dichloromethane stem (DS) and aqueous root (AR) extracts significantly increased ($p < 0.05$) the percentage gastrointestinal tract inhibition when compared with their respective negative control (DMSO and Tween). This significant increase was not comparable with the group treated with the standard drug loperamide.

Table 4.1: Inhibitory effects of solvent extracts of *A. senegalensis* root and stem barks on wett stool in castor oil-induced diarrhoeal rats

Group	No of wet faeces	% inhibition of wet faeces
DMSO	8 ± 0.88^{e}	-
Tween	5 ± 0.33^{d}	-
Loperamide	0 ± 0.00^{a}	100. 00
100 mg/kg b.wt. DS	2 ± 0.05^{c}	61.50
100 mg/kg b.wt DR	0 ± 0.00^{a}	100.0
100 mg/kg b.wt. HS	1 ± 0.07^{b}	57.70
100 mg/kg b.wt. HR	1 ± 0.06^{b}	57.70
100 mg/kg b.wt. AS	0 ± 0.00^{a}	100.00
100 mg/kg b.wt. AR	4 ± 0.33^{d}	10.00

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

DS- Dichloromethane stem bark extract, DR – Dichloromethane root bark extract, HS –Hexane stem bark extract, HR – Hexane root bark extract, AR – aqueous root bark extract, AS – aqueous stem bark extract, DMSO- dimethylsufoxide

Table 4.2: Gastrointestinal tract inhibition and antienteropooling activity of solvent extracts of *A. senegalensis* root and stem barks in castor oil-induced diarrhoeal rats

Group	% GIT inhibition	Volume of intestinal fluid (mL)	Weight of intestinal fluid (mg)
DMSO	5.56 ± 0.24^{a}	3.13 ± 0.24^{d}	2.53 ± 0.14^{b}
Tween	15.21 ± 0.77^{b}	2.03 ± 0.41^{b}	2.50 ± 0.23^{b}
3.0 mg/kg b.wt. Loperamide	45.39 ± 0.53^{e}	4.00 ± 0.23^{g}	4.73 ± 0.19^{e}
100 mg/kg b.wt.DS	28.31 ± 0.90^{d}	1.40 ± 0.12^{a}	1.73 ± 0.26^{a}
100 mg/kg b.wt.DR	12.83 ± 1.00^{b}	3.23 ± 0.03^{e}	3.10 ± 0.26^{c}
100 mg/kg b. wt. HS	4.28 ± 0.34^{a}	3.53 ± 0.23^{f}	4.60 ± 0.10^{e}
100 mg/kg b. wt. HR	5.85 ± 0.27^{a}	2.53 ± 0.15^{c}	3.37 ± 0.27^{d}
100 mg/kg b.wt.AS	25.08 ± 1.26^{c}	2.47 ± 0.22^{c}	3.13 ± 0.07^{c}
100 mg/kg b.wt.AR	36.60 ± 3.58^{d}	1.16 ± 0.10^{a}	1.90 ± 0.16^{a}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

DS- Dichloromethane stem bark extract, DR – Dichloromethane root bark extract, HS –Hexane stem bark extract, HR – Hexane root bark extract, AR – aqueous root bark extract, AS – aqueous stem bark extract.

4.2 Bioactivity guided fractionation of antidiarrhoeal bioactive extracts

Table 4.3 depicts the stool inhibition of solvent fractions obtained from aqueous stem bark extract of *A. senegalensis* in castor oil-induced diarrhoeal rats. The ethylacetate partitioned fraction (EFAS) significantly reduced ($p < 0.05$) the number of wet stools (1 ± 0.03) when compared with the negative control (4 ± 0.27).

The percentage gastrointestinal tract inhibition and enteropooling activity of solvent fractions from aqueous root bark extract of *A. senegalensis* in castor oil-induced diarrhoeal rats is presented in Table 4.4. The dichloromethane partitioned fraction of the aqueous root bark extract of *A. senegalensis* (DFAR) significantly decreased ($p < 0.05$) the weight and volume of the intestinal fluid when compared to the negative control and loperamide while the ethylacetate partitioned fraction (EFAR) had the highest %GIT inhibitory activity ($96.64 \pm 0.80\%$) when compared to the loperamide treated rats ($35.17 \pm 0.81\%$) and the negative control ($19.68 \pm 0.47\%$).

Plate 4.1 shows the chromatogram of solvent fractions from dichromethane stem bark extract (DS) of *A. senegalensis* while Table 4.5 shows the retention factor (R_f) of the fractions from dichloromethane stem bark extract (DS) of *A. senegalensis*. Fraction DS2, DS3 and DS4 had two distinct spots as shown in Plate 4.1. The R_f for the two spots were the same for these fractions (DS2, DS3 and DS4). Sub-fractions DS5 to DS9 had the same Rf value while fractions DS11-DS13, DS14 – DS16 had the same R_f value.

Table 4.3: Inhibiory effect of solvent fractions obtained from aqueous stem bark extract of *A. senegalensis* on wet stool in castor oil induced- diarrhoeal rats

Group	No of wet Feaces	% inhibition of wet faeces
Control	4 ± 0.27^{c}	-
3mg/kg b.wt.Loperamide	0 ± 0.00^{a}	100.00
50 mg/kg b.wt.HFAS	4 ± 0.12^{c}	0.00
50 mg/kg b. wt.EFAS	1 ± 0.03^{a}	83.25
50mg/kg b.wt. DFAS	3 ± 0.23^{b}	33.25
50 mg/kg b.wt. AFAS	2 ± 0.18^{b}	50.00

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

HFAS- Hexane fraction of aqueous stem bark extract, EFAS – ethylacetate fraction of aqueous stem bark extract, DFAS- diethylacetate fraction of aqueous stem bark extract, AFAS- aqueos fraction of aqueous stem bark extract.

Table 4.4: Gastrointestinal tract inhibition and antienteropooling activity of solvent fractions from aqueous root bark extract (AR) of *A. senegalensis* in castor oil - induced diarrhoeal rats

Group	% GIT inhibition	Weight of int. fluid (mg)	Volume of intestinal fluid (mL)
Control	19.68 ± 0.47^{a}	1.25 ± 0.34^{b}	1.95 ± 0.06^{b}
3 mg/kg b.wt Loperamide	35.17 ± 0.81^{b}	1.77 ± 0.07^{b}	1.93 ± 0.18^{b}
50 mg/ kg b.wt HFAR	19.00 ± 0.70^{a}	1.00 ± 0.09^{b}	1.84 ± 0.15^{b}
50 mg/kg b.wt EFAR	96.64 ± 0.80^{d}	2.07 ± 0.08^{c}	3.05 ± 0.23^{c}
50 mg/kg b.wt DFAR	79.03 ± 0.52^{c}	0.84 ± 0.07^{a}	1.03 ± 0.03^{a}
50 mg/kg b.wt AFAR	79.33 ± 0.13^{c}	1.32 ± 0.11^{b}	1.38 ± 0.12^{b}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

HFAR- Hexane fraction of aqueous root bark extract, EFAR – ethylacetate fraction of aqueous root bark extract, DFAR- diethylacetate fraction of aqueous root bark extract, AFAR- aqueos fraction of aqueous root bark extract.

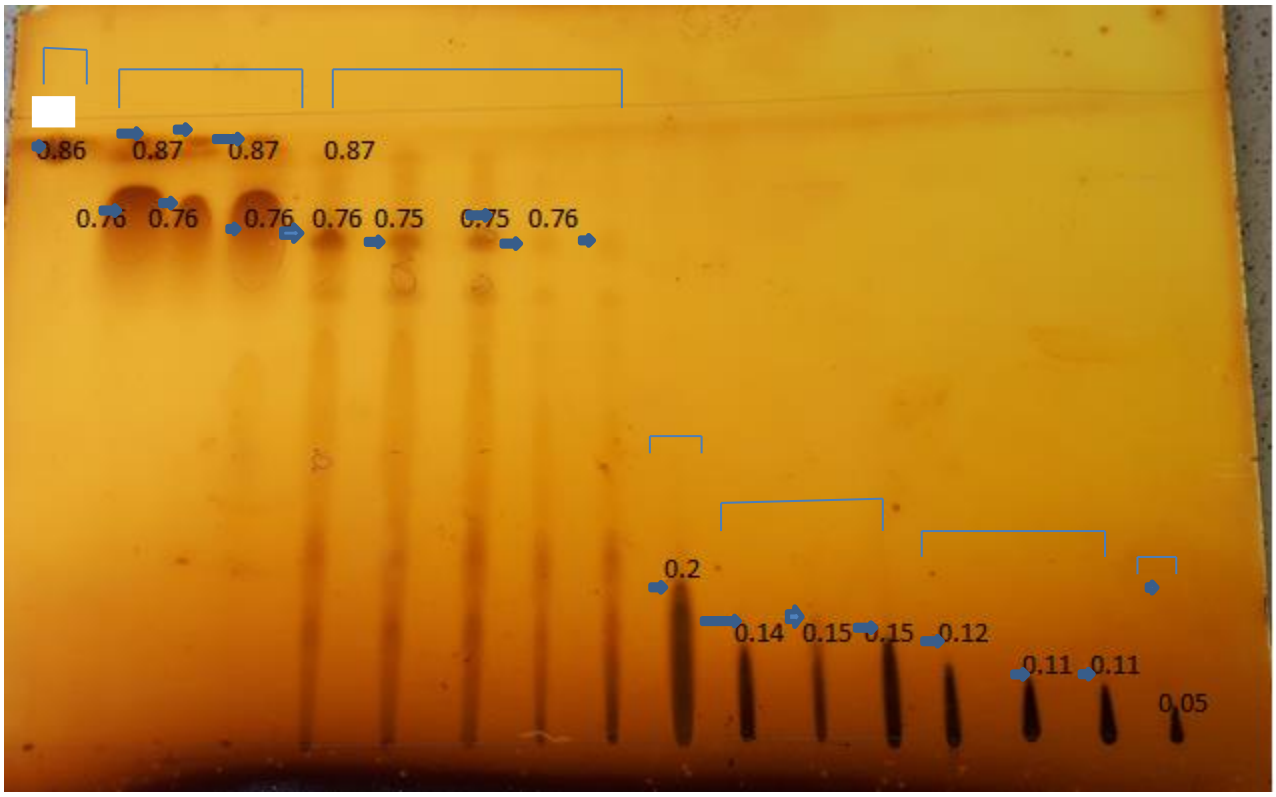

Plate 4.1: Thin layer chromatogram (TLC) of sub-fractions from dichloromethane stem bark (DS) extract of *Annona senegalensis*

Numbers on plate indicates R_f values

Table 4.5: Retention factor (R_f) of sub-fractions from dichloromethane stem bark extract (DS) of *A.senegalensis*

FRACTION	**R_f**	**R_f**
DS1	0.86	
DS2	0.76	0.87
DS3	0.76	0.87
DS4	0.76	0.87
DS5	0.75	
DS6	0.75	
DS7	0.75	
DS8	0.76	
DS9	0.75	
DS10	0.25	
DS11	0.14	
DS12	0.15	
DS13	0.15	
DS14	0.12	
DS15	0.11	
DS16	0.11	
DS17	0.05	

DS- dichloromethane stem bark extract of *A.senegalensis.*

Table 4.6 shows the percentage gastrointestinal tract inhibition and antienteropooling activity of fractions from dichloromethane stem bark extract (DS) of *A. senegalensis*. Fraction 3 (DS3) reduced the volume of the intestinal fluid the most. The weight of the intestinal fluid was not significantly different ($p < 0.05$) from the negative control. DS3 also significantly increased the % GIT inhibition ($p < 0.05$) when compared to the negative control.

The thin layer chromatogram of sub-fractions from dichloromethane fraction of aqueous root bark extract (DFAR) of *A. senegalensis* is shown in Plate 4.2. The plate showed the distance travelled on the thin layer chromatoplate by each of the 10 sub-fractions obtained from column chromatography of DFAR. Table 4.7 shows the R_f values of sub-fractions obtained from DFAR. DFAR1 to DFAR4 had R_f values ranging between 0.61 - 0.62, DFAR5 to DFAR9 had R_f values ranging between 0.70 to 0.72 while DFAR10 had R_f value of 0.40. Plate 4.2 illustrates the pooling together of these sub-fractions into three main sub-fractions based on the similarity of the R_f.

Table 4.8 shows the antienteropooling activity of sub-fractions from dichloromethane fraction of aqueous root bark (DFAR) of *A. senegalensis* in castor oil–induced diarrhoeal rats. Sub-fractions 1, 2 and 3 (DFAR1, DFAR2 and DFAR3) significantly decreased ($p < 0.005$) both the weight and volume of intestinal fluid. The highest anti-enteropooling activity was exhibited by sub-fractions 1 and 2, but the intestinal fluid of sub-fraction 1 (DFAR1) was solid while sub-fraction 2 was liquid.

Table 4.6 Gastrointestianl tract (GIT) inhibition and antienteropooling activity of sub-fractions from dichloromethane stem bark (DS) extract of *A. senegalennsis* in castor oil - induced diarrhoeal rats

Fractions	Weight of intestinal fluid (mg)	Volume of intestinal fluid (mL)	%GIT inhibition
Control	2.33 ± 0.13^{a}	2.50 ± 0.21^{c}	3.14 ± 1.31^{a}
25 mg/kg b.wt DS1	2.33 ± 0.13^{a}	3.73 ± 0.28^{d}	1.96 ± 0.98^{a}
25 mg/kg b.wt DS2	3.60 ± 0.15^{c}	2.97 ± 0.15^{c}	1.20 ± 0.58^{a}
25 mg/kg b.wt DS3	2.67 ± 0.22^{a}	0.63 ± 0.23^{a}	65.80 ± 2.54^{b}
25 mg/kg b.wt DS4	4.67 ± 0.33^{d}	4.57 ± 0.07^{e}	4.00 ± 2.01^{a}
25 mg/kg b.wt DS5	3.00 ± 0.18^{b}	2.77 ± 0.18^{c}	4.88 ± 2.61^{a}
25 mg/kg b.wt DS6	3.67 ± 0.33^{c}	1.77 ± 0.14^{b}	0.00 ± 0.00^{a}
25 mg/kg b.wt DS7	5.37 ± 0.43^{e}	3.13 ± 0.26^{d}	2.43 ± 1.37^{a}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

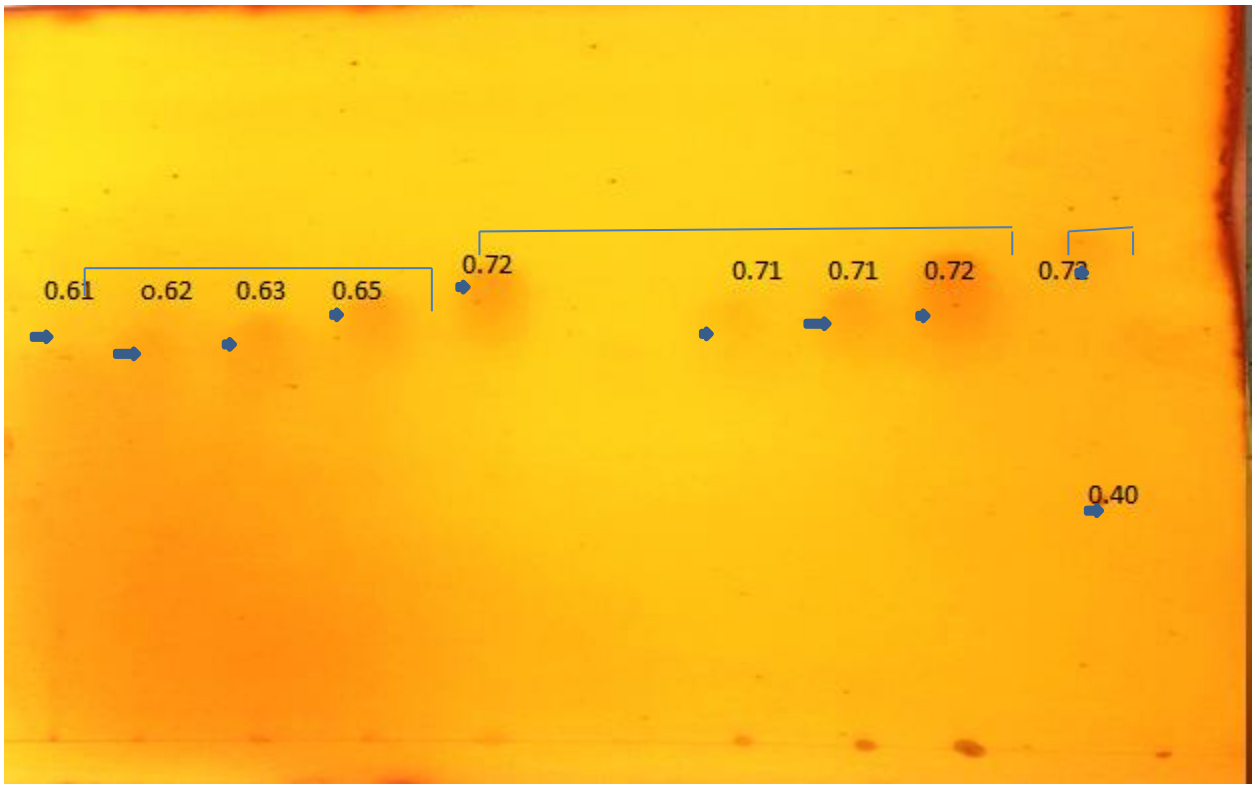

Plate 4.2: **Thin layer chromatogram of sub-fractions from dichloromethane fraction of aqueous root bark extract (DFAR) of *A. senegalensis***

Numbers on plate indicates R_f values

Table 4.7: Retention factor (R_f) of sub-fractions from dichloromethane fraction of aqueous root bark extract (DFAR) of *A. senegalensis*

Fractions	R_f	R_f
DFAR1	0.61	
DFAR2	0.62	
DFAR3	0.63	
DFAR4	0.65	
DFAR5	0.75	
DFAR6	0.70	
DFAR7	0.71	
DFAR8	0.71	
DFAR9	0.72	
DFAR10	0.40	0.75

DFAR - dichloromethane fraction of aqueous root bark extract (DFAR) of *A. senegalensis*

Table 4.8: Antienteropooling activity of sub-fractions from dichloromethane fraction of aqueous root bark extract (DFAR) of *A. senegalensis* in castor oil – induced diarrhoeal rats

Fractions	Weight of intestinal fluid (mg)	Volume of intestinal Fluid (mL)	Descriptive feature
Control	7.74 ± 0.38^{c}	5.00 ± 0.12^{c}	Liquid
25 mg/kg b.wt DFAR1	2.47 ± 0.12^{a}	2.60 ± 0.13^{a}	Solid
25 mg/kg b.wt DFAR2	2.47 ± 0.17^{a}	2.37 ± 0.18^{a}	Liquid
25 mg/kg b.wt DFAR3	3.30 ± 0.14^{b}	3.07 ± 0.26^{b}	Liquid

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

DFAR - dichloromethane fraction of aqueous root bark of *A. senegalensis*

Table 4.10 shows the stool inhibition of sub-fractions from ethylacetate fraction of aqueous extract of *Annona senegalensis* stem bark (EFAS) in castor oil – induced diarrhoeal rats. Sub-fractions 1 and 2 (EFAS1 and EFAS2) significantly reduced ($p < 0.05$) the number of wet stool to 0.00 ± 0.00 when compared to the negative control (3.33 ± 0.23).

The thin layer chromatogram of sub-fractions from dichloromethane root bark extract (DR) of *Annona senegalensis* is shown in Plate 4.3. Table 4.11 shows the retention factor (R_f) of sub-fractions from dichloromethane root bark extract (DR) of *Annona senegalensis.* DR1 to DR6 had R_f values ranging from 0.64 to 0.64. DR7 and DR8 had R_f values of 0.002 and 0.003 respectively.

Table 4.12 shows the stool inhibition of sub-fractions from dichloromethane root bark extract (DR) of *A. senegalensis* in castor oil-induced diarrhoeal rats. Sub-fractions 2 and 3 (DR2 and DR3) significantly ($p < 0.05$) decreased the number of wet stool (0.00 ± 0.00) when compared to the negative control (3.00 ± 0.28).

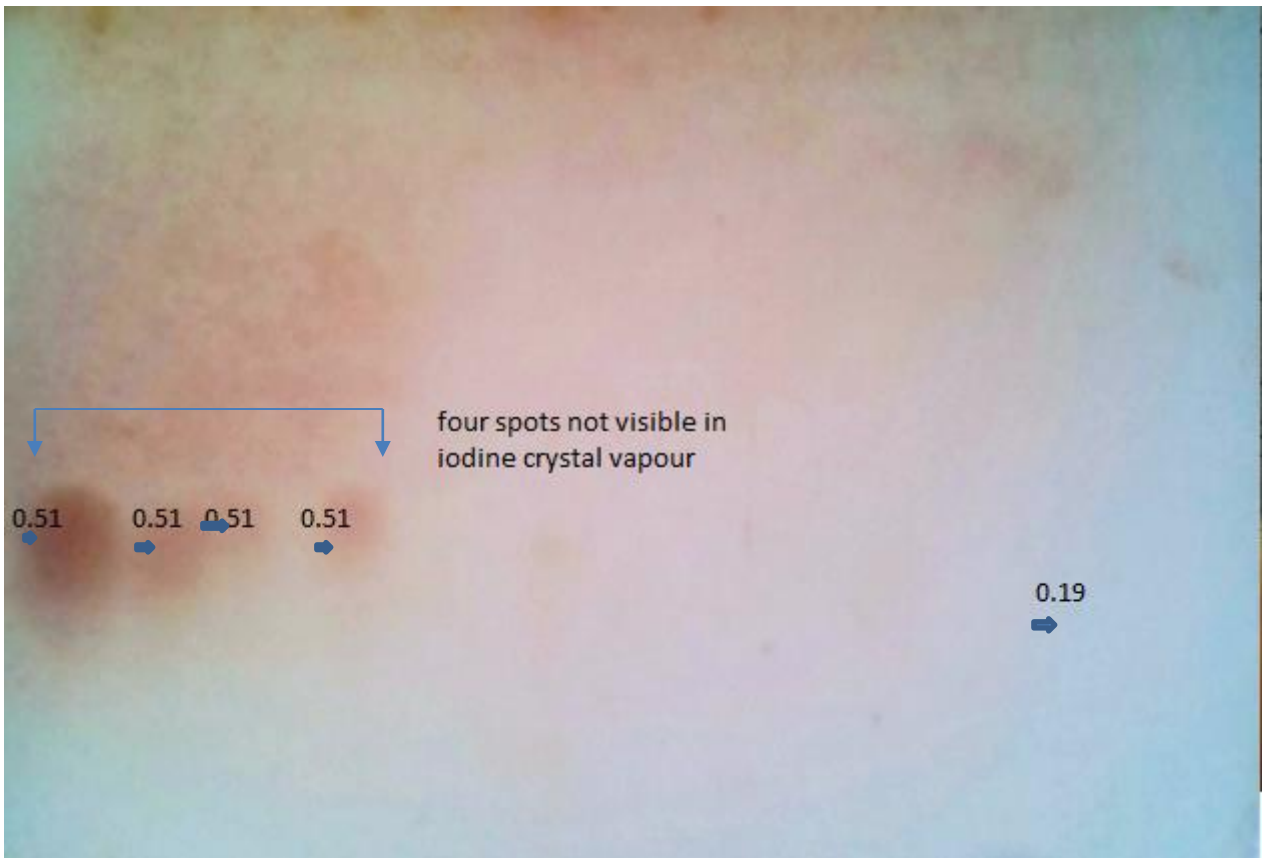

Plate 4.3: Thin layer chromatogram plate of sub-fractions from ethylacetate fraction of aqueous extract of *Annona senegalensis* stem bark (EFAS)

Numbers on plate indicates R_f values

Table 4.9: Retention factor (R_f) of sub-fractions from ethylacetate fraction of aqueous extract of *Annona senegalensis* stem bark (EFAS)

Fraction	R_f
EFAS1	0.51
EFAS2	0.51
EFAS3	0.52
EFAS4	0.51
EFAS5	-
EFAS6	-
EFAS7	-
EFAS8	-
EFAS9	0.19

EFAS - ethylacetate fraction of aqueous extract of *Annona senegalensis* stem bark

Table 4.10: Inhibiory effect of sub-fractions from ethylacetate fraction of aqueous extract of *Annona senegalensis* stem bark (EFAS) on wet stool in castor oil - induced diarrhoeal rats

Fractions	Number of wet stool
Control	3.33 ± 0.23^{c}
25 mg/kg b. wt. EFAS1	0.00 ± 0.00^{a}
25 mg/kg b.wt. EFAS2	0.00 ± 0.00^{a}
25 mg/kg b.wt EFAS3	2.67 ± 0.21^{b}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

EFAS - ethylacetate fraction of aqueous extract of *Annona senegalensis* stem bark

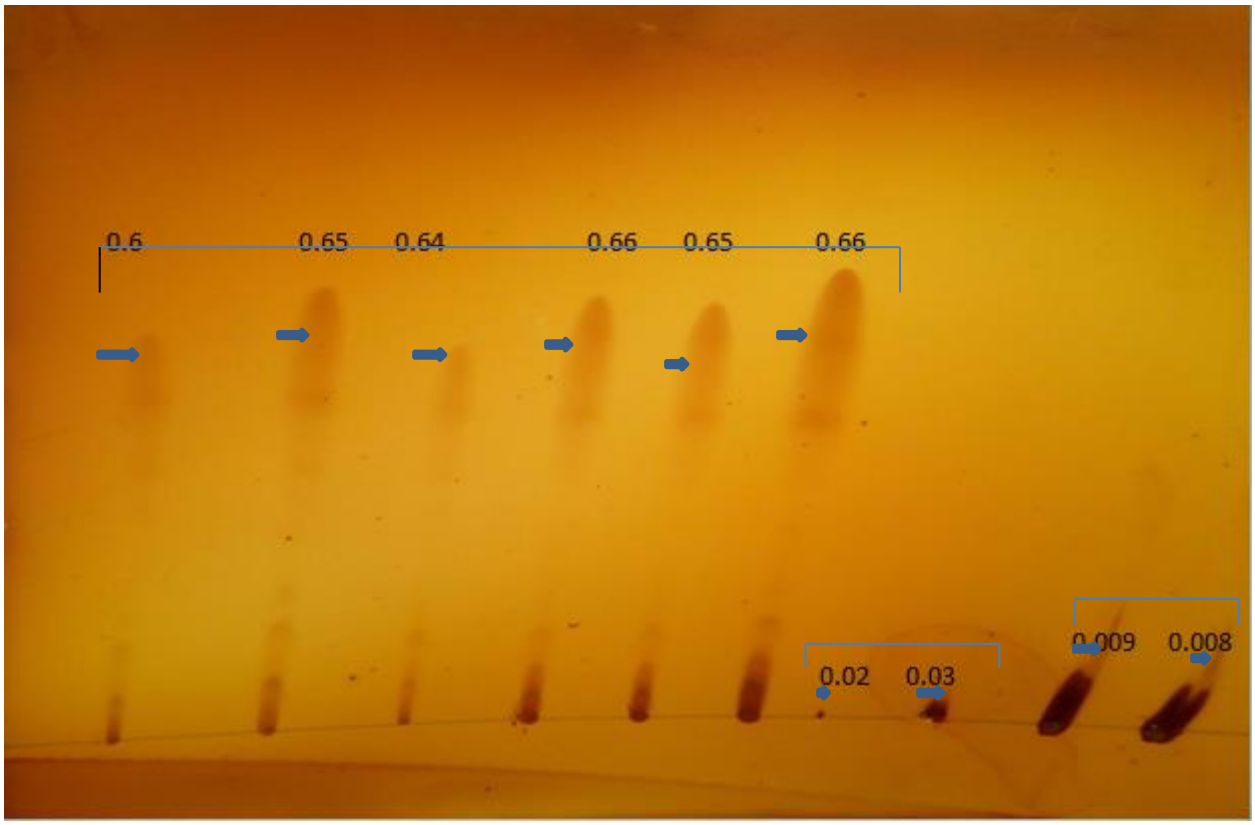

Plate 4.4: Thin layer chromatogram of sub-fractions from dichloromethane root bark extract (DR) of *Annona senegalensis*

Numbers on plate indicates R_f values

Table 4.11: Retention factor (R_f) of sub-fractions from dichloromethane root bark extract (DR) of *Annona senegalensis*

Fractions	R_f
DR1	0.6
DR2	0.65
DR3	0.64
DR4	0.66
DR5	0.65
DR6	0.66
DR7	0.002
DR8	0.003
DR9	0.009
DR10	0.008

DR – dichloromethane root bark extract of *A. senegalnsis*

Table 4.12: Inhibitory effect of sub-fractions from dichloromethane root bark extract (DR) of *Annona senegalensis* on wet stool in castor oil – induced diarrhoeal rats

Fractions	Number of wet stool	% inhibition of diarrhoea stool
DMSO	3.00 ± 0.28^{b}	-
25 mg/kg b.wt. DR1	3.00 ± 0.18^{b}	0
25 mg/kg b.wt. DR2	0.00 ± 0.00^{a}	100
25 mg/kg b.wt. DR3	0.00 ± 0.00^{a}	100

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

DR- dichloromethane root bark extract of *A. senegalensisis*

Plate 4.5 shows the thin layer chromatogram of sub-fractions obtained from ethylacetate fraction of aqueous *Annona senegalensis* root bark extract (EFAR). The plate shows the distance travelled by eleven sub-fractions spotted on the thin layer chromatography plate. The distance travelled by seven Sub-fractions (EFAR1, EFAR2, EFAR3, EFAR4, EFAR5, EFAR9 and EFAR11) were not visible in iodine crystals.

Table 4.13 shows the retention factor of sub-fractions obtained from ethylacetate fraction of aqueous *Annona senegalensis* root bark extract (EFAR). Sub-fractions EFAR5, EFAR6, EFAR7, EFAR8 had R_f between 0.39 – 0.40.

Table 4.14 shows the gastrointestinal transit inhibition of sub-fractions from ethylacetate fractions from aqueous *Annona senegalensis* root bark extracts (EFAR). Results from Table 4.14 shows that EFAR 2 significantly increased ($p < 0.05$) % GIT inhibition (78.75 ± 2.85) when compared to the negative control (52.47 ± 3.23%).

4.3. Mechanism of action of antidiarrhoeal fractions of *Annona senegalensis* root and stem bark

4.3.1 Antioxidant activity of the antidiarrhoeal fractions of *Annona senegalensis* root and stem bark

Table 4.15 shows the result of antioxidant activity of the antidiarrhoeal sub-fractions obtained from *A.* senegalensis root and stem barks. The DPPH IC_{50} for all the sub-fractions were significantly greater ($p < 0.05$) than that of the standard control ascorbic acid (12.09 ± 0.18). Amongst the sub-fractions, EFAS 1 had the least DPPH IC_{50} (24.42 ± 2.95) while DFAR1 had thhighest (72.88 ± 6.58). DS 3 had the highest total antioxidant capacity (TAC). DFAR1 showed the least FRAP activity, H_2O_2 scavenging activity and ABTS IC_{50}. EFAS1 and DR 2 showed the highest H_2O_2 scavenging activity. DR 2 had highest scavenging activity for OH^-.

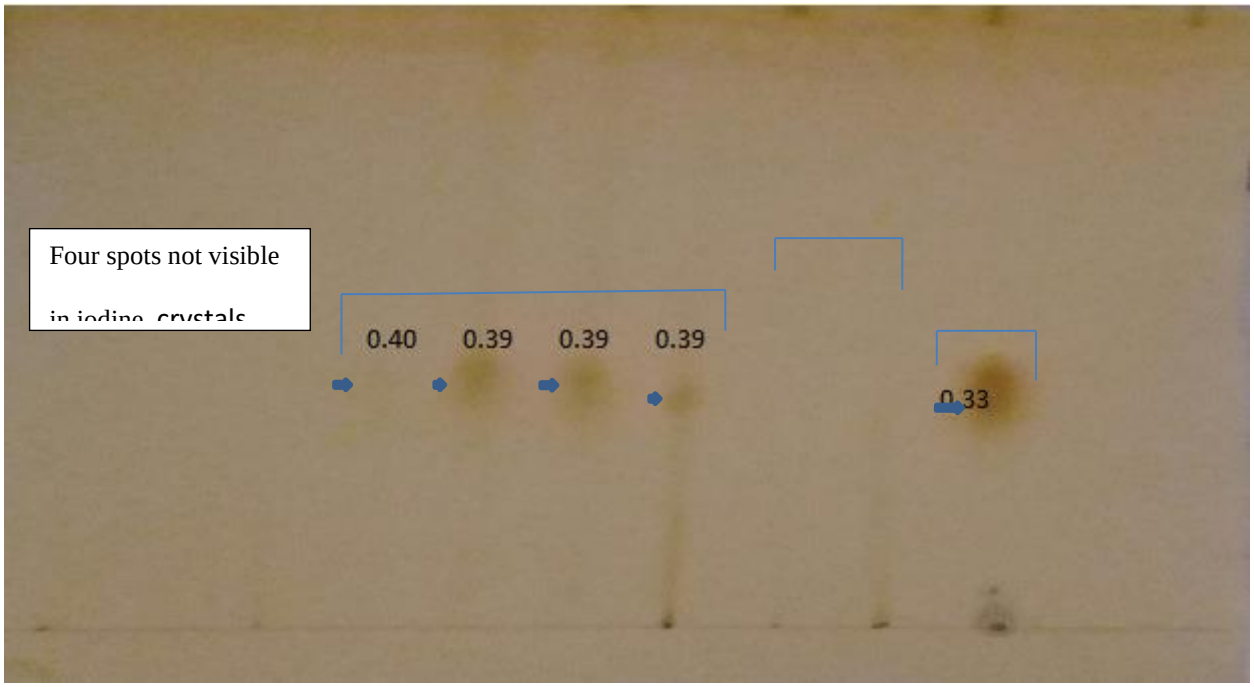

Plate 4.5: Thin layer chromatogram of sub-fractions obtained from ethylacetate fraction of aqueous *Annona senegalensis* root bark extract (EFAR)

Numbers on plate indicates R_f values

Table 4.13: Retention factor of sub-fractions obtained from ethylacetate fraction of aqueous *Annona senegalensis* root bark extract (EFAR)

Fractions	R_f
EFAR1	-
EFAR2	-
EFAR3	-
EFAR4	-
EFAR5	0.40
EFAR6	0.39
EFAR7	0.39
EFAR8	0.40
EFAR9	-
EFAR10	-
EFAR11	0.43

EFAR - ethylacetate fraction of aqueous *Annona senegalensis* root bark extract (EFAR)

Table 4.14: Gastrointestinal transit inhibition of sub-fractions obtained from ethylacetate fraction of aqueous *Annona senegalensis* root bark extract (EFAR) in castor oil-induced diarrhoeal rats

Fraction	% inhibition of gastrointestinal transit
Negative control	52.47 ± 3.23^{b}
25 mg/kg b.wt. EFAR1	24.70 ± 1.81^{a}
25 mg/kg b.wt. EFAR2	78.75 ± 2.85^{c}
25 mg/kg b.wt.EFAR3	50.05 ± 4.20^{b}
25 mg/kg b.wt.EFAR4	53.11 ± 2.87^{b}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

EFAR - ethylacetate fraction of aqueous *Annona senegalensis* root bark extract

Table 4.15: Antioxidant activity of the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

	DPPH (IC_{50}) (mmol/L)	FRAP (nmol/L)	ABTS (IC_{50}) (mmol/L)	H_2O_2 (mol/L)	TAC (tannic acid equiv.)	OH^- (mol/L)
Ascorbic	12.09 ± 0.18^{a}	127.67 ± 0.98^{d}	33.92 ± 0.80^{b}	6.32 ± 0.31^{c}	150.67 ± 1.23^{e}	70.2 ± 0.09^{e}
DFAR1	58.88 ± 4.58^{d}	121.29 ± 1.46^{a}	17.59 ± 0.58^{a}	0.92 ± 0.20^{a}	117.22 ± 1.25^{b}	69.66 ± 0.07^{d}
EFAR2	40.03 ± 0.17^{c}	126.36 ± 0.58^{b}	59.14 ± 0.04^{c}	3.32 ± 0.12^{b}	122.36 ± 0.53^{c}	63.15 ± 0.58^{a}
EFAS1	24.42 ± 1.95^{b}	127.83 ± 1.06^{c}	63.89 ± 1.68^{d}	7.17 ± 0.06^{d}	121.99 ± 3.93^{c}	64.89 ± 0.37^{b}
DR2	43.82 ± 1.04^{c}	128.55 ± 0.70^{c}	64.13 ± 2.68^{d}	7.38 ± 0.04^{d}	108.40 ± 0.16^{a}	70.66 ± 0.29^{e}
DS3	48.43 ± 3.02^{c}	131.31 ± 0.66^{d}	83.02 ± 0.52^{e}	6.20 ± 0.02^{c}	127.50 ± 0.78^{d}	68.69 ± 0.03^{c}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

EFAS1 – sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark, DFAR2 - sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks, EFAR2 - sub-fraction 2 of ethylacetate fraction of *A. senegalensis* root barks, DR2 - sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis*, DS3 - sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis*

Table 4.16 shows the small intestinal antioxidant enzymes activity of antidiarrhoeal sub-fractions obtained from *A. Senegalensis* root and stem barks in castor oil- induced diarrhoeal rats. All the fractions except DS3 significantly increased ($p < 0.05$) the activity of catalase when compared with their respective control (water and DMSO repectively). EFAR2 significantly increased ($p < 0.05$) the activity of GPx, when compared with the group that received only water. DR2 and DS3 also significantly increased ($p < 0.05$) the activity of GPx when compared with the groups that received DMSO. There was significant increase ($p < 0.05$) in the concentration of GSH in rats administered DS3 and DR2 fractions when compared to the group that receved only DMSO. All the Sub-fractions significantly decreased ($p < 0.05$) the activity of SOD when compared with their respective control. EFAR2 had the highest SOD activity when compared with all the treatment groups.

4.3.2 Evaluation of intestinal barrier function of antidiarrhoeal sub-fractions of *Annona senegalensis* root and stem barks

4.3.2.1 Concentration of malondialdehyde (MDA) in small intestine of castor oil-induced diarrhoeal rats treated with antidiarrhoeal sub-fractions of *A. senegalensis* root and stem barks

Figure 4.1 shows the concentration of malondialdehyde (MDA) in small intestine of castor oil-induced diarrhoeal rats treated with antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem barks. There was significant decrease ($p < 0.05$) in the small intestinal concentraton of MDA of rats administered EFAS1 and EFAR2 sub-fractions when compared with the group that received only water. EFAS1 had the least concentration. MDA concentration for DR2 and DS3 fractions were not significantly different ($p > 0.05$) from their control (DMSO).

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Table 4.16: Small intestinal antioxidant enzyme activities and glutathione (GSH) concentration of antidiarrhoeal sub-fractions of *Annona senegalensis* stem and root barks in castor oil- induced diarrhoeal rats

Groups	Catalase (U/L)	Glutathione peroxidase (GPX) (U/L)	GSH (mg/dl)	Superoxide dismutase (SOD) (U/L)
Water	2.28 ± 0.19^{b}	5.59 ± 0.68^{d}	8.12 ± 0.33^{f}	8.01 ± 0.31^{e}
DMSO	0.91 ± 0.22^{a}	2.48 ± 0.09^{a}	4.40 ± 0.35^{a}	9.02 ± 0.63^{f}
25 mg/kg.b.wt. EFAS1	7.60 ± 0.87^{d}	5.61 ± 0.21^{d}	6.97 ± 0.26^{d}	6.38 ± 0.17^{b}
25 mg/kg b. wt.DFAR1	3.00 ± 0.26^{c}	4.01 ± 0.34^{b}	5.17 ± 0.35^{b}	6.90 ± 0.17^{c}
25 mg/kg b.wt .EFAR2	2.83 ± 0.14^{c}	6.85 ± 0.13^{e}	8.53 ± 0.62^{f}	7.80 ± 0.10^{d}
25mg/kg b.wt DR2	8.70 ± 0.23^{e}	4.82 ± 0.46^{c}	6.00 ± 0.70^{c}	6.20 ± 0.08^{b}
25 mg/kg b. wt. DS3	0.99 ± 0.20^{a}	4.98 ± 0.31^{c}	6.21 ± 0.38^{c}	3.84 ± 0.86^{a}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

DMSO – dimethylsulfoxide. EFAS1 – sub-fraction 1 of ethylacetate fraction of *A .senegalensis* stem bark, DFAR2 - sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks, EFAR2 - sub-fraction 2 of ethylacetate fraction of *A .senegalensis* root barks, DR2 - sub-fraction 2 of dichloromethane root bark extract of *A .senegalensis*, DS3 - sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis.*

4.3.3 Evaluation of the antisecretory/proabsorptive mechanism of antidiarrhoeal sub-fractions of *A. senegalensis* root and stem barks

Figure 4.2 shows chloride concentration of intestinal fluids of castor oil-induced diarrhoeal rats after administration of antidiarrhoeal sub-fractions obtained from *Anonna sengalensis* root and stem barks. Only EFAR2 significantly decreased ($p < 0.05$) Cl^- concentration in the intestinal fluid of castor oil-induced diarrhoeal rats. The Cl^- concentration for the DR 2 fraction was not significantly different ($p > 0.05$) from the DMSO control.

Figure 4.3 shows the sodium ion concentration of intestinal fluids of castor oil induced diarrhoeal rats after administration of the antidiarrhoeal sub-fractions obtained from *Anonna sengalensis* root and stem barks. DS3 significantly decreased ($p < 0.05$) the concentration of Na^+ when compared to the DMSO control. DS3 was not significantly different ($p > 0.05$) from the water control.

Figure 4.4 shows the potassium ion concentration of intestinal fluid of castor oil- incduced diarrhoeal rats after administration of the antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem barks. None of the antidiarrhoeal sub-fractions significantly decrease the concentration of K^+ of intestinal fluids when compared to their respective control groups (water and DMSO).

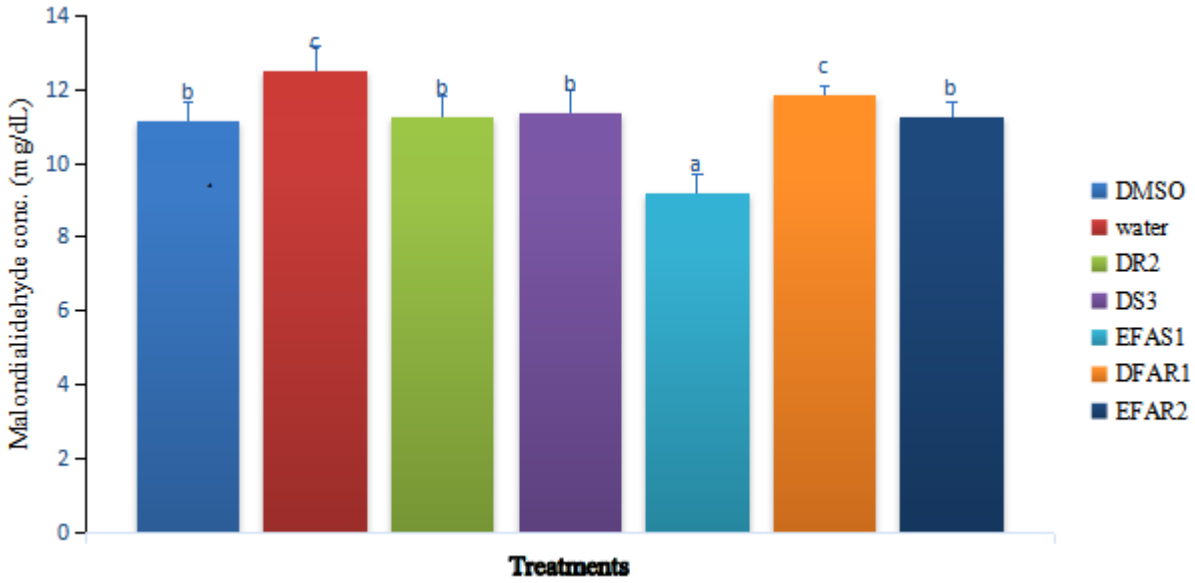

Figure 4.1: Concentration of malondialdehyde (MDA) in small intestine of castor oil-induced diarrhoeal rats treated with antidiarrhoeal sub-fractions of *Annona senegalensis* stem and root bark extracts

Bars are mean of five replicates ± S.E.M. Bars with different superscript down the column are significantly different ($p < 0.05$)

Water: diarrhoeal rats administered distilled water.
DMSO: diarrhoeal rats administered dimethylsulfoxide.
EFAS1: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark,
DFAR: diarrhoeal rats administered with 25 mg/kg b. wt. sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks:
EFAR2: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 2 of ethylacetate fraction of *A. senegalensis* root barks,
DR2: diarrhoeal rats administered 25mg/kg b.wt. sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis*,
DS3: diarrhoeal rats administered 25 mg/kg b.wt. sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis.*

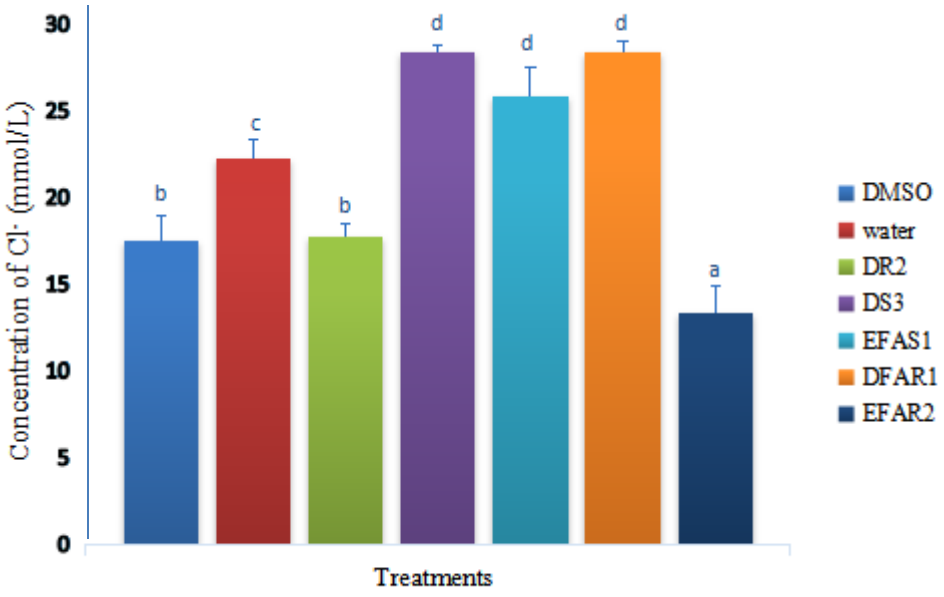

Figure 4.2: Chloride concentration in intestinal fluids of castor oil induced diarrhoeal rats after administration of antidiarrhoeal sub-fractions obtained from *Anonna senegalensis* root and stem bark extracts

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different ($p < 0.05$)

Water: diarrhoeal rats administered distilled water.
DMSO: diarrhoeal rats administered dimethylsulfoxide.
EFAS1: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark,
DFAR: diarrhoeal rats administered with 25 mg/kg b. wt. sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks:
EFAR2: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 2 of ethylacetate fraction of *A. senegalensis* root barks,
DR2: diarrhoeal rats administered 25mg/kg b.wt. sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis*,
DS3: diarrhoeal rats administered 25 mg/kg b.wt. sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis.*

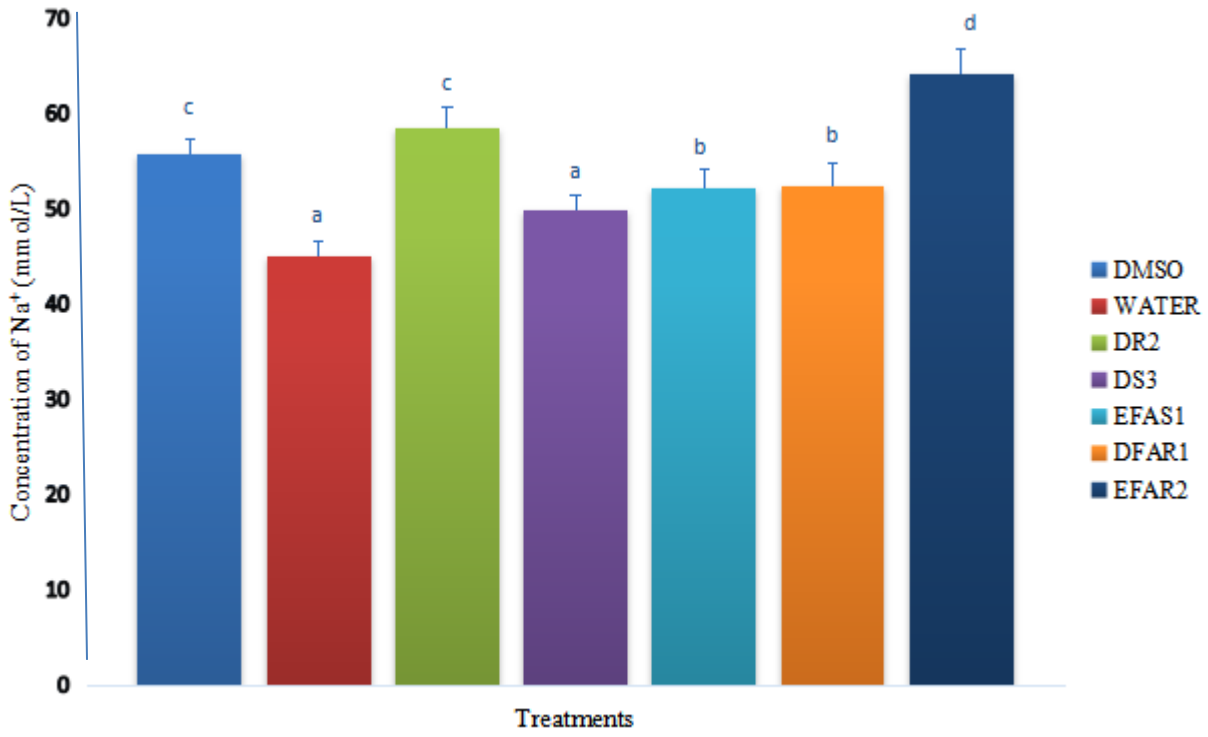

Figure 4.3: Sodium ion concentration of intestinal fluids of castor -oil induced diarrhoeal rats after administration of the antidiarrhoeal sub-fractions obtained from *Anonna sengalensis* root and stem bark extracts

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different from others ($p < 0.05$)

Water: diarrhoeal rats administered distilled water.
DMSO: diarrhoeal rats administered dimethylsulfoxide.
EFAS1: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark,
DFAR: diarrhoeal rats administered with 25 mg/kg b. wt. sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks:
EFAR2: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 2 of ethylacetate fraction of *A. senegalensis* root barks,
DR2: diarrhoeal rats administered 25mg/kg b.wt. sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis*,
DS3: diarrhoeal rats administered 25 mg/kg b.wt. sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis.*

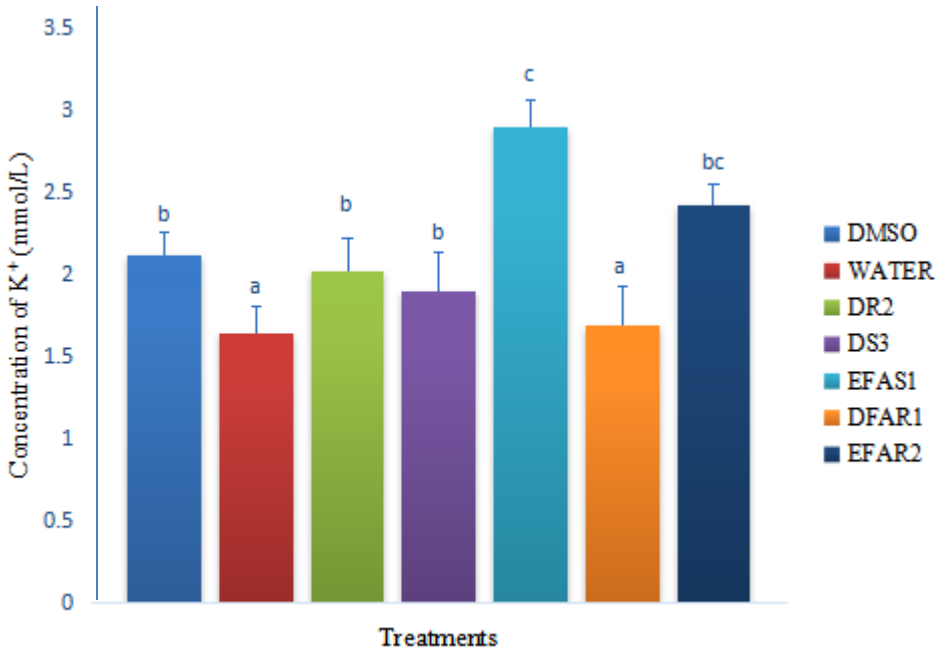

Figure 4.4: Potasium ion concentration of intestinal fluids of castor oil - induced diarrhoeal rats after administration of the antidiarrhoeal sub-fractions obtained from *Anonna sengalensis* root and stem bark extract

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different from others ($p < 0.05$)
Water: diarrhoeal rats administered distilled water.
DMSO: diarrhoeal rats administered dimethylsulfoxide.
EFAS1: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark,
DFAR: diarrhoeal rats administered with 25 mg/kg b. wt. sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks:
EFAR2: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 2 of ethylacetate fraction of *A. senegalensis* root barks,
DR2: diarrhoeal rats administered 25mg/kg b.wt. sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis*,
DS3: diarrhoeal rats administered 25 mg/kg b.wt. sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis.*

4.3.4 Small intestinal Na^+- K^+ ATPase activity of castor oil induced - diarrhoeal rats after treatment with antidiarrhoeal sub-fractions of *Annona senegalensis* stem and root bark extracts

Figure 4.5 shows the small intestinal Na^+- K^+ ATPase activity of castor oil induced - diarrhoeal rats after treatment with antidiarrhoeal sub-fractions obtained from *Annona senegalensis* stem and root bark extracts. EFAS1 significantly increased ($p < 0.05$) the activity of Na^+- K^+ ATPase when compared to the control (water). Small intestinal Na^+- K^+ ATPase acitivity of rats treated with DR2 and DS3 were not significantly different ($p > 0.05$) from that of those treated with DMSO

4.3.5 Evaluation of the anti-inflammatory activity of antidiarrhoeal sub-fractions of *Annona senegalensis* root and stem barks

4.3.5. 1 Cyclooxygenase II (COX II) activity of castor oil - induced diarrhoeal treated rats

Figure 4.6 shows cyclooxygenase II (COX II) activity of castor oil - induced diarrhoeal rats following treatment with the antidiarrhoeal sub-fractions obtained from *Annona senegalensis* stem and root bark extracts. EFAS1, DFAR2 and EFAR2 significantly decreased ($p < 0.05$) the actity of COX II. There was no significant decrease ($p > 0.05$) between the COX II activities of DR2, DS3 and DMSO.

4.4 GC-MS chromatograms of compounds present in antidiarrhoeal sub-fractions of *Annona senegalensis* root and stem bark extracts

The GC- MS chromatogram of sub-fraction 3 of dichlromethane stem extract (DS3) of *A. senegalensis* is shown in Figure 4.7 while Table 4.17 shows the GC- MS analysis of the compounds present in the sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis* (DS3). GC-MS analyses of DS 3 sub-fraction revealed the presence of 24 compounds as shown in Table 4.17. Androst-16-ene 3-one; a steroid was found to be the major compound in this sub-fraction (50.33%). Many minor constiuents were also identified such as allaromandredene oxide

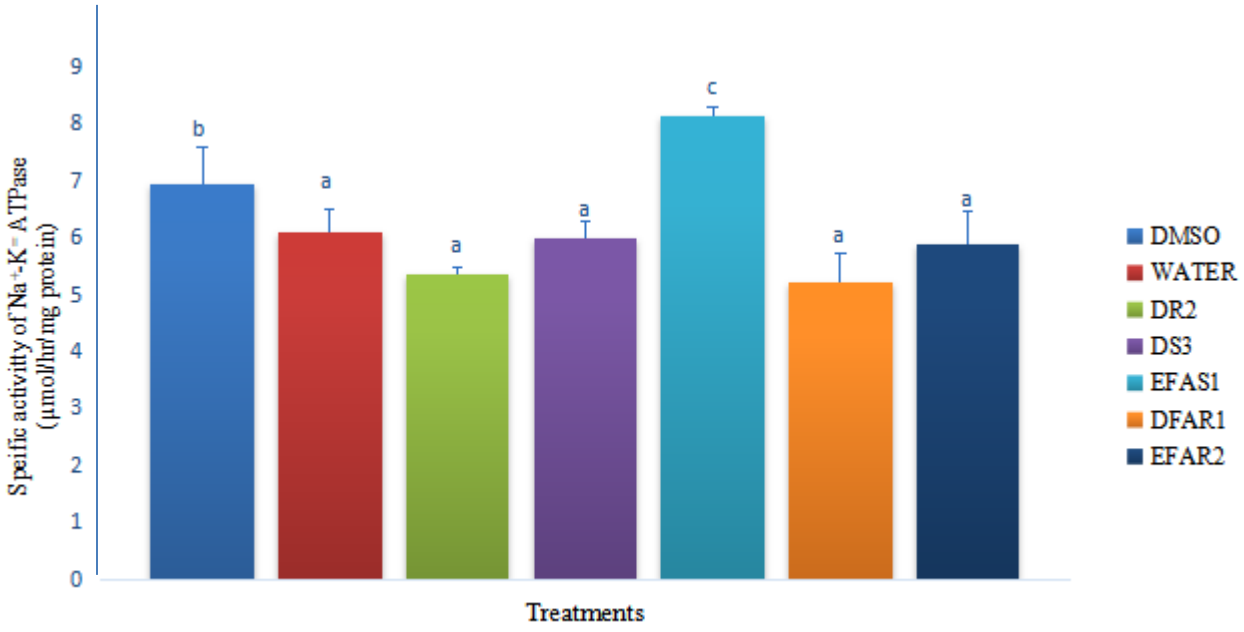

Figure 4.5: Small intestinal Na^+- K^+ ATPase activity of castor oil-induced diarrhoeal rats after treatment with antidiarrhoeal sub-fractions obtained from *Annona senegalensis* stem and root bark extracts

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different from others ($p < 0.05$)
Water: diarrhoeal rats administered distilled water.
DMSO: diarrhoeal rats administered dimethylsulfoxide.
EFAS1: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark,
DFAR: diarrhoeal rats administered with 25 mg/kg b. wt. sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks:
EFAR2: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 2 of ethylacetate fraction of *A. senegalensis* root barks,
DR2: diarrhoeal rats administered 25mg/kg b.wt. sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis*,
DS3: diarrhoeal rats administered 25 mg/kg b.wt. sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis.*

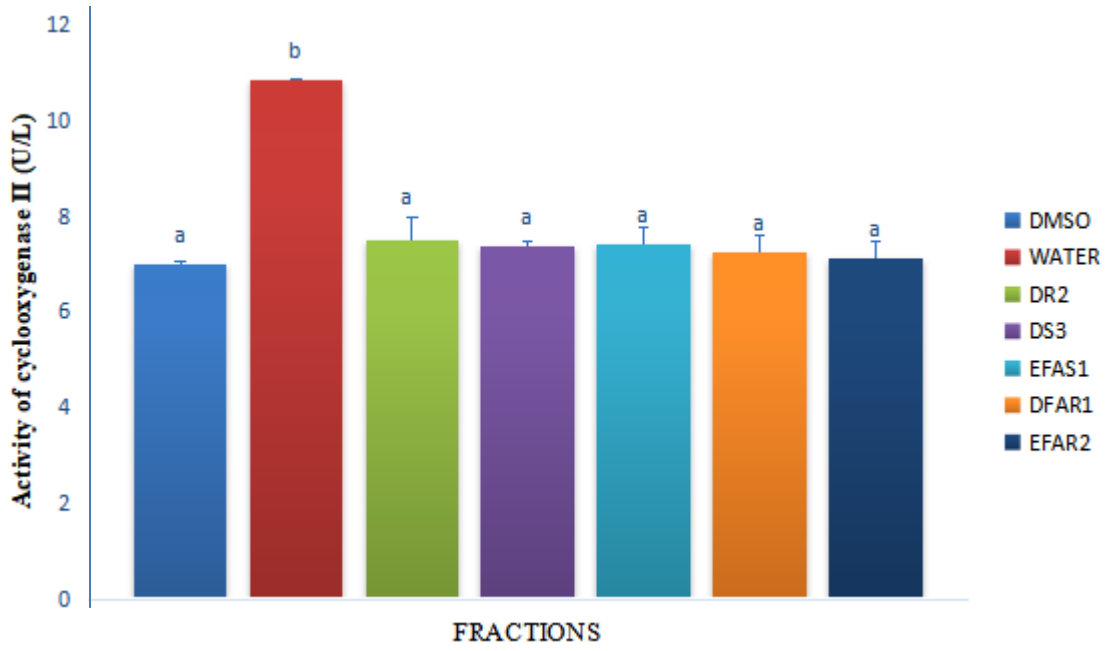

Fig 4.6: Cyclooxygenase II (COX II) activity in castor oil-induced diarrhoeal treated rats

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different ($p < 0.05$)

Water: diarrhoeal rats administered distilled water.
DMSO: diarrhoeal rats administered dimethylsulfoxide.
EFAS1: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark,
DFAR: diarrhoeal rats administered with 25 mg/kg b. wt. sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks:
EFAR2: diarrhoeal rats administered 25 mg/kg b.wt.sub-fraction 2 of ethylacetate fraction of *A. senegalensis* root barks,
DR2: diarrhoeal rats administered 25mg/kg b.wt. sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis*,
DS3: diarrhoeal rats administered 25 mg/kg b.wt. sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis.*

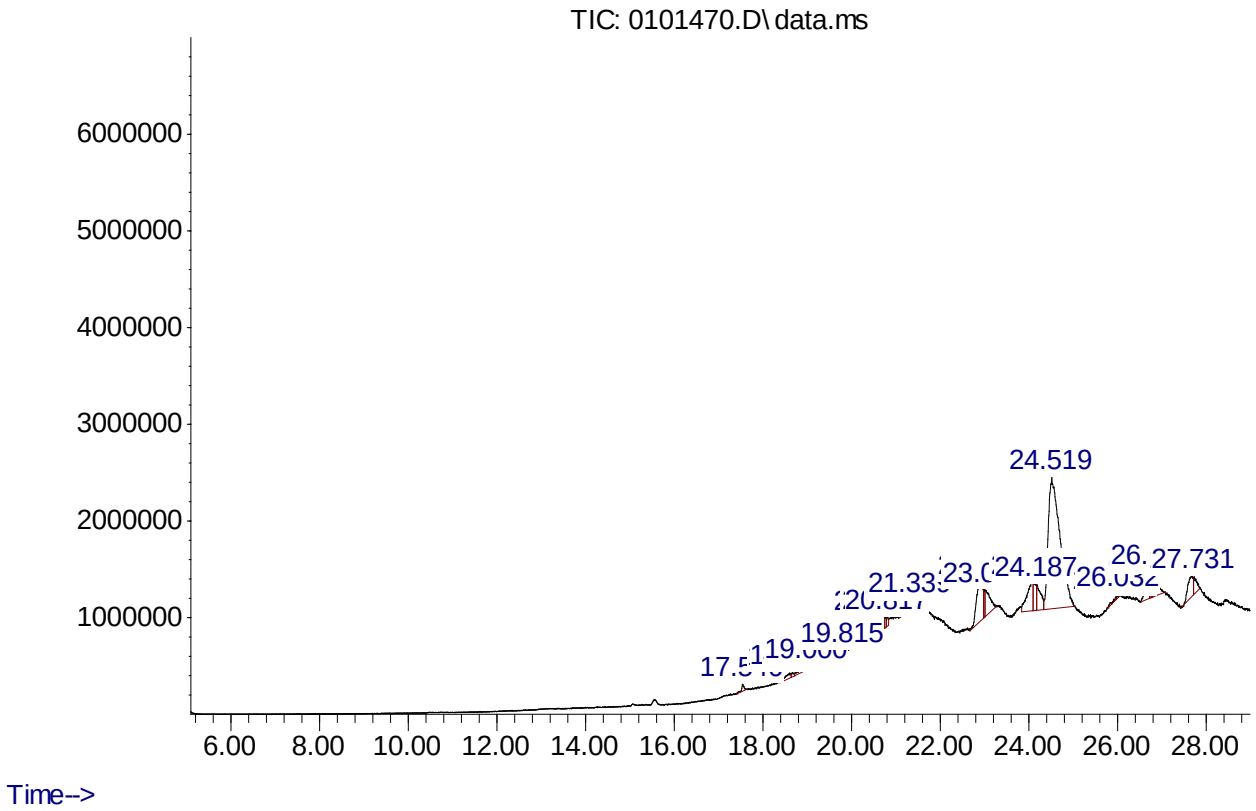

Figure 4.7: GC-MS chromatogram of sub-fraction 3 of dichloromethane stem bark extract (DS3) of *Annona senegalensis*

Table 4.17: Chemical compounds present in sub-fraction 3 of the dichloromethane stem bark extract (DS3) of *Annona senegalensis*

S/N	Compound name	RT(min)	% peak area	Class of compound
1	Benzoic acid, 2,4, bis (trimethyl)oxyl-trimethysilyl ester	17.545	0.55	Ester
2	1-formyl-2,2,6-trimethyl-3-(3 methyl but-2-enyl 6-cyclohexane	18.605	0.84	Aromatic compound
3	Alloaromandendrene oxide	18.671	0.30	Essential oil
4	Isolongifolol, methyl ether	19.812	0.10	Essential oil
5	Alloaromandendrene	20.701	2.55	Essential oil
6	Alloaromandendrene	20.730	0.45	Essential oil
7	Isolongifolol, methyl ether	20.760	0.47	Essential oil
	Naphalene	20.819	0.46	
8	Hexadecanoic acid, methyl ester	21.264	0.69	Fatty acid methyl ester
9	Pentadecanoic acid, 14 methyl ester	21.308	0.14	Fatty acid methyl ester
10	1-formyl-2-2-6-rimethyl-3-cyclohexane	21.338	0.05	
11	Cis 13-octadecanoic acid, methy ester	22.908	7.94	Unsaturated fatty acid methyl ester
12	Cis 13-octadecanoic aid, methyl ester	22.989	1.07	Unsaturated fatty acid methyl ester
13	9-octadecanoicacid, methyl ester	23.026	4.12	Unsaturated fatty acid methyl ester
14	Atis-16-ene	24.078	5.53	Diterpenoid
15	Kaur-16-ene	4.108	2.72	Diterpene alkaloid
16	Androst-16-ene 3-one	24.523	50.33	Steroid Pheromone
17	Cedran-diol	26.033	0.04	
18	Prasterone	26.715	2.74	Anabolic steroid
19	3-α, 17 β, dihydroyestr-4-ene	25.959	0.36	
	Hydroxydehydrosteric acid	26.744	1.01	
20				
21	Kauran-19-oic acid, methyl ester	26.774	0.93	
22	13,17, seco, 5α-pregn-13(18)en-20-one	26.811	3.48	Steroid
23	N-Acridine-9yl,N-(4 fluorophenyl)hydrazine	27.685	3.54	
24	8a(2H)-phenathrenol, 7 ethenyldodecahydro-1,1,4a-en-20-one	27.730	1.70	

(0.30%), cis-13 –octadecanoic acid methyl ester (9.01%), 9-octadecanoic acid, methyl ester (4.12%), atis-16-ene (5.23%), prasterone (2.74%), 13, 17, seco, 5α-pregn 13(18) en-20-one, N-acridine-9-yl,N-(4,fluorophenyl)hydrazine, 8A(2H)-phenathrenol, 7 ethenyldodecahydro-1,1,4a-en-20-one

Figure. 4.8 shows the GC-MS chromatogram of sub-fraction 1 of dichloromethane fraction of aqueous root bark extracts (DFAR1) of *A. senegalensis.* Table 4.18 shows the identified compouds in sub-fraction 1 of dichloromethane fraction of aqueous root bark extracts (DFAR1) of *A. senegalensis.* Figure 4.8 shows 4 peaks obtained from GC-MS analysis of DFAR1. Four compounds were identified from the retention time (RT) obtained as shown in Table 4.18. androstan-3-one, 17 hydroxy-2-methyl (2β, 5β, 17β), a steroid is the major compound in the sub-fraction with 51.98 peak area.

Figure 4.9 shows the GC-MS chromatogram of sub-fraction 1 of ethylactate fraction of aqueous stem bark extracts (EFAS1) of *A. senegalensis.* Table 4.19 shows the identified compouds in sub-fraction 1 of ethylacetate fraction of aqueous stem bark extracts (EFAS1) of *A. senegalensis.* GC-MS analysis of EFAS1 showed 30 peaks with different retention time (RT) values. Twenty one (21) compounds were identified. Catechol was identified with RT values between 6.712 - 6.941 as shown in Table 4.19. Other compounds identified are hexadecanoic acid, kaur-16-ene, ethyl 5,8,11,14,17, icosapentanoate, 2,4,5, pyrimidinetriamine and androstan-3-17-dione 9,11, epoxy with peak area as 1.34%, 1.10%, 19.15%, 43.89%, 2.16% and 0.41% respectively.

Figure 4.10 shows the GC-MS chromatogram of sub-fraction 2 of dichloromethane root bark while Table 4.20 shows the identified compounds in sub-fraction 2 of dichloromethane root bark extracts

of *A. senegalensis*. The GC-MS analysis of DR2 fshowed 35 peaks out of which 28 compounds were identified as shown in Figure 4.10. and Table 4.20 Cyclopentanol (1-methylenecyclopropyl)

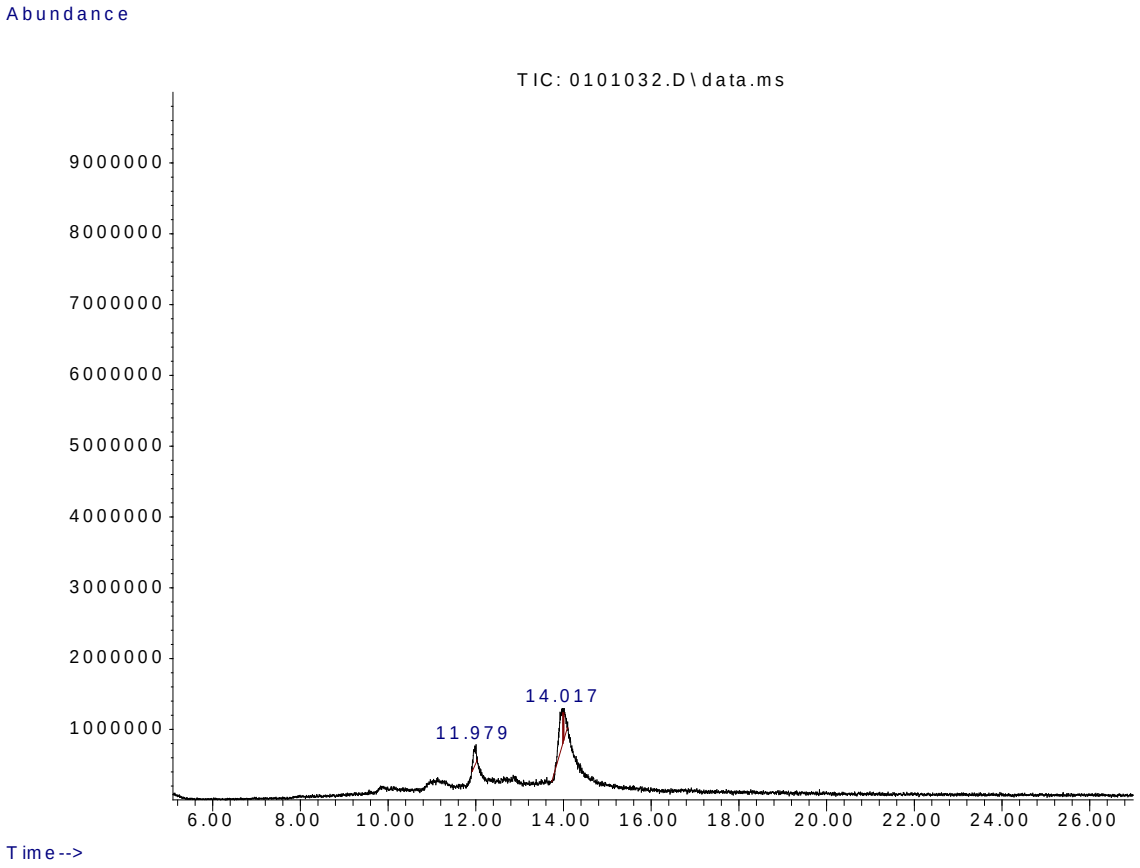

Figure 4.8: GC-MS chromatogram of sub-fraction 1 of dichloromethane fraction from aqueous root bark extract (DFAR1) of *Annona senegalensis*

Table 4.18: Chemical compounds present in sub-fraction 1 of dichloromethane fraction from aqueous root bark extract (DFAR1) of *Annona senegalensis*

S/N	Compound name	RT(min)	% peak area	Class of compound
1	3-Tetradecen-5-yne	11.98	19.36	Essential oil
2	Androstan-3-one, 17 hydroxy-2-methy, (2β,5β, 17β)	13.973	51.98	Steroid
3	Butanimide, N(3 methylphenyl) 2,2,3,3,4,4,4,heptafluoro-	13.996	9.52	
4	Pyrazolo(3,4,b)pyridine-3-one	14.019	19.14	Nitrgen containing heterocyclic

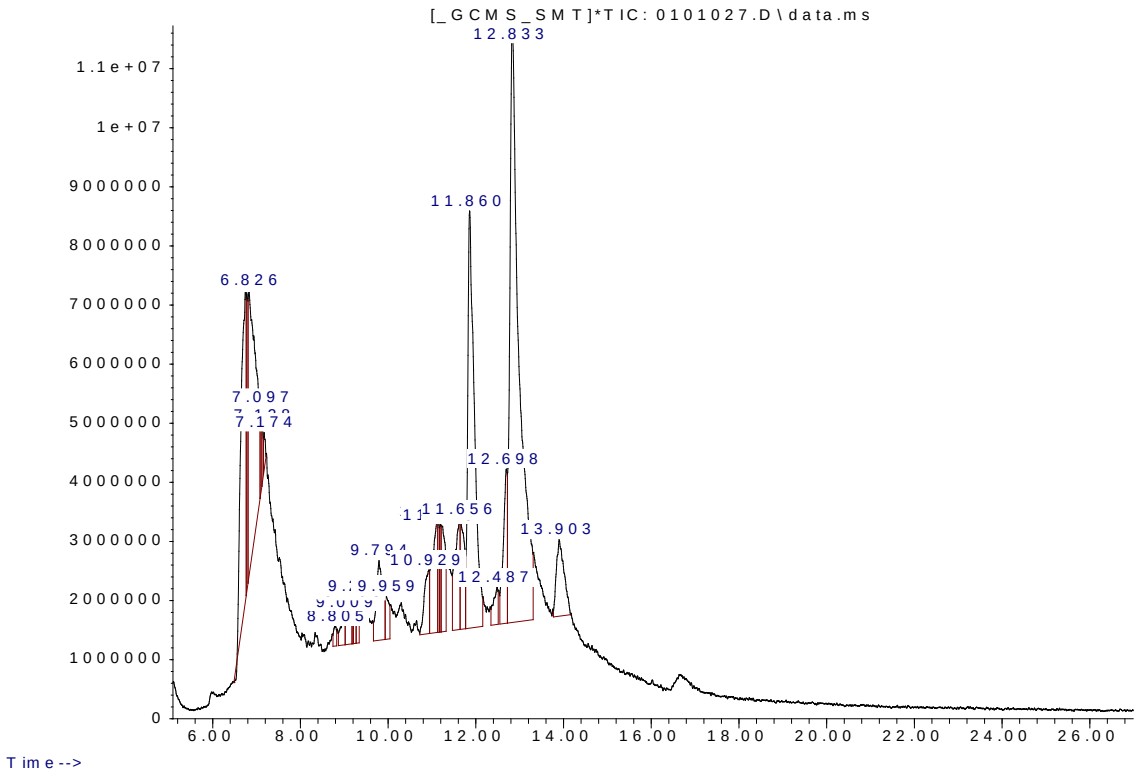

Figure 4.9: GC-MS chromatogram of sub-fraction 1 of ethylacetate fraction from aqueous ethylacetate stem bark extract (EFAS1) of *Annona senegalensis*

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Table 4.19: Chemical compounds present in sub-fraction 1 of ethylacetate fraction from aqueous stem bark extract (EFAS1) of *Annona senegalensis*

S/N	Compound name	RT(min)	% peak area	Class of compound
1	Catechol	6.712	7.78	Phenol
2	Catechol	6.746	2.83	Phenol
3	Catechol	6.775	2.78	Phenol
4.	Catechol	6.821	1.35	Phenol
5	Catechol	6.838	1.94	Phenol
6	Catechol	6.889	1.53	Phenol
7	Catechol	6.941	0.49	Phenol
8	Phenol, 3,4,5, trimethyl	8.064	0.05	Flavonoid
9	3-(1-hydroxy-1-methyl-ethyl) 5 - phenyl isoxazolin -3-ol	8.796	1.07	Heterocyclic
10	6,7, dimethyl triazolo (4,3, -b, 1,2,4) triazine	9.830	0.94	Heterocyclic nitrogen compound
11	n-hexadecanoic acid	10.929	1.34	Fatty acid
12	Diazoprogesterone	11.026	0.28	Steroid
13	Kaur-16-ene	11.187	0.33	Diterpene alkaloid
14	Trachylobane	11.547	0.35	Tetracyclic diterpenes
15	Hexane, 1-chloro-5-methyl	11.656	1.10	Tetracyclic diterpenes
16	Kaur-16-ene	11.856	6.33	Diterpene alkaloids
17	Kaur-16-ene	11.873	12.82	Diterpene alkaloids
18	3,6, Nonadien-1-ol	12.480	0.47	
19	1,8, Nonadiene, 2 methyl 5,7, dimethylene	12.686	4.13	Alkene
20	Ethyl 5,8,11,14,17, icosapentaenoate	12.835	43.89	Fatty acid
21	2,4,5 pyrimidinetriamine	13.899	2.16	
22	Androstan-3,17 dione 9,11, epoxy-	13.950	0.41	Steroid epoxy

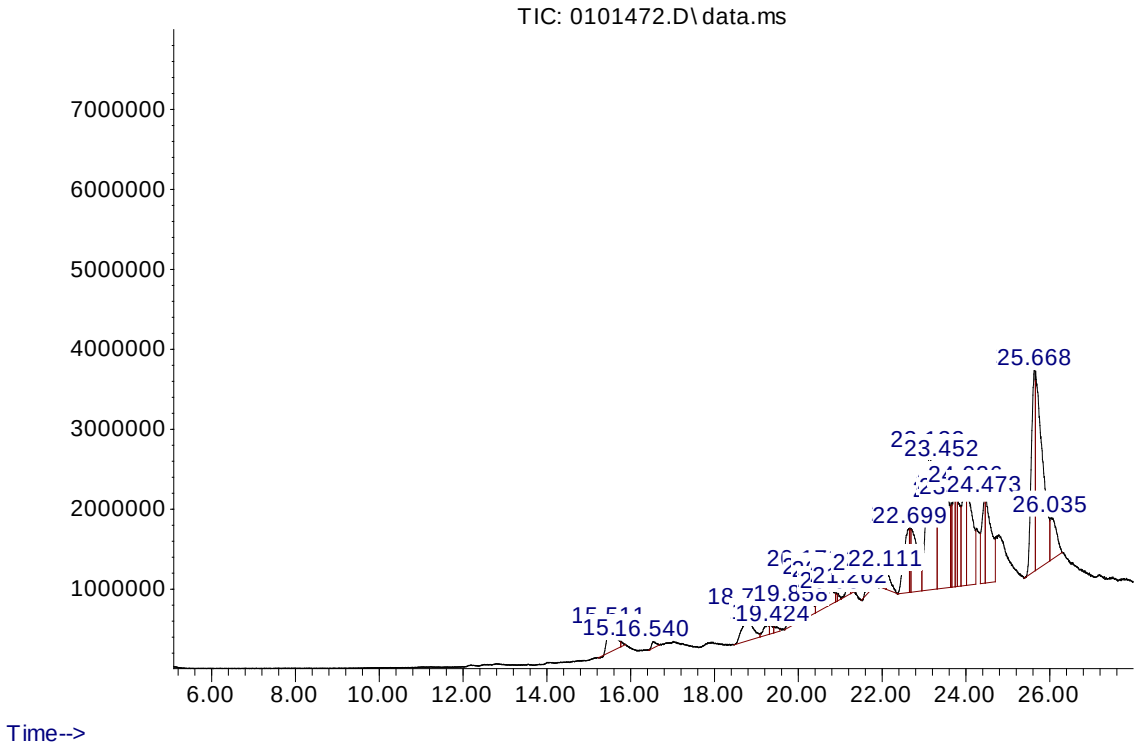

Figure 4.10: GC-MS chromatogram for sub-fraction 2 of dichloromethane root bark extract (DR2)

Table 4.20: Chemical compounds present in sub-fraction 2 of dichloromethane root bark extract (DR2) of *A. senegalensis*

S/N	Compound name	RT(min)	% peak area	Class of compound
1	Caparratriene	15.509	1.94	Sesquiterpene
2	Carophyllene oxide	15.775	0.10	oxygenated sesquiterpene
3.	Phthalic acid, isohexylnon-5-yn-3-yl ester	18.752	2.67	Aromatic hydrocarbon
4	Hexadecanoic acid, methyl ester	19.286	0.57	Fatty acid methyl ester.
5	Hexadecanoic acid, methyl ester	19.331	0.29	Fatty acid methyl ester.
6	Hexadecanoic acid, methyl ester	19.427	0.24	Fatty acid methyl ester
7.	Ethane sulfonamide, 2-(3-aminophenyl)ethylamino	19.856	1.10	
8	6-methyl-8-(2,6,6,trimethyl-1-cyclohexanyl)-3-5,7,octatriene-2-one.	20.167	4.22	Aromatic compound
9	Kaur-16-ene	20.545	2.05	Diterpene alkaloids
10	Kaur-16-ene	20.767	0.98	Diterpene alka;oid
11	7-heptadecene-1-chloro-	20.901	0.13	
12	Azulene 1,2,3,5,6,7,8,,8a octahydro-1,4, dimethyl -7-(1-methylethenyl)	21.723	0.64	Isomer of naphthalene
13	Thumbergol	21.745	0.37	
14	Kauran-16—ene	22.641	3.31	
15	D-Homoandrostane	22.701	4.41	D-Homosteroids
16	Benzanamine, 4,Chloro-N-(2 pyridinylmethylene	23.123	12.18	Aromatic amines
17	Cyclopentanol, (1-methylenecyclopropyl)	23.449	11.19	Cyclic alcohol
18	Androstan-17-ol, 2,3, epoxy 2α, 3α, 5α, 17β)	23.730	2.45	Steroid
19	Kauran-18-al, 17 (acetyloxy)	24.026	5.89	Diterpene alkaloids
20	Acetaphenol	24.471	5.22	Phenol
21	Benzene methanol, 4 [bis 4 methylphenyl) amino]	25.641	7.44	
22	N-Acridine-9-yl-N- (4-fluoro-phenyl)hydrazine	25.671	13.93	Heterocyclic nitrogen containing

was the abundant with 11.19% peak area. Other compounds identified were fatty acid methy ester, sesquiterpene, diterpene alkaloids and some unclassified compounds.

The GC-MS chromatogram of the sub-fraction 2 of ethylacetate fraction of aqueous root bark extract (EFAR2) of *A. senegalensis* is shown in Figure 4.11. The identified chemical compounds in the sub-fraction 2 of ethylacetate fraction of aqueous root bark (EFAR2) of *A. senegalensis* are presented in Tables 4.21. Figure 4.11 shows 56 peaks obtained from GC-MS analysis of EFAR2. Some of the compounds identified include 2(3H)-furanonone, hexanoic acid tetrahydrofurylmethylester, isoamyl layrate, isopropyl palmitate, hexadecanoic acid pentyl ester, 1-2-benzenedicarboxylic acid. Isopropyl palmitate is the most abundant compound found in the sub-fraction with a peak area of 40.69%.

4.5 Toxicity studies

4.5.1 Influence of administration of antidiarrhoeal sub-fractions from aqueous root and stem barks extract of *Annona senegalensis* on weight, total feed intake and mortality of rats

Table 4.22 shows the change in weight, total feed intake and mortality of rats administered antidiarrhoeal sub-fractions from aqueous root and stem barks extract of *A. senegaleesis*. There was no significant difference ($p > 0.05$) in the change in body weight of the rats administered 100, 200 and 400 mg/kg. b.wt. of DFAR1 when compared to the normal control, while there was a significant increase ($p < 0.05$) in the total feed intake of rats administered all test doses of DFAR1 when compared with normal control. The change in body weights of rats administered 400 mg/kg b.wt. EFAR2 was not significantly different ($p > 0.05$) from the control whereas it significantly increased ($p < 0.05$) the total feed intake when compared with the normal control. In contrast, 100

mg/kg b.wt. EFAR2 significantly inccreased ($p < 0.05$) the change in body weight when compared with its control while there was a significant deccrease ($p < 0.05$) total feed intake of rats

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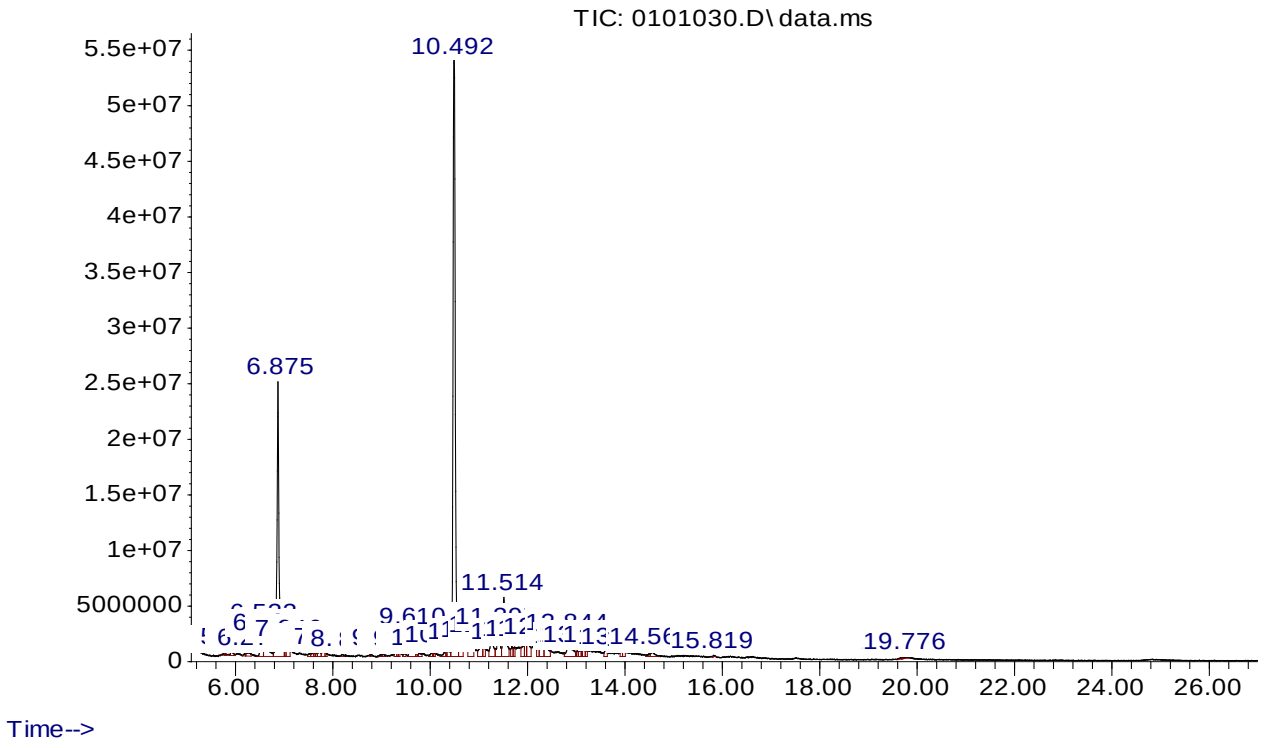


Figure 4.11: GC-MS chromatogram of sub-fraction 2 of ethylacetate fraction of aqueous root stem bark extract (EFAR2) of *A. senegalensis*

Table 4. 21: Chemical compounds present in sub-fraction 2 of ethylacetate fraction of aqueous root stem bark extract (EFAR2) of *A. senegakensis*

S/N	Compound name	RT(min)	% peak area	Class of compound
1	Sulfurous acid	5.745	0.08	Acid
2.	3-methoxy but-1-ene	5.928	0.33	Alkene
3	2-hydroxy -2-methyl-4-heptanone	6.191	0.14	
4	2(3H)-Furanone, dihydro-3-hydroxy-4,4, dimethyl-	6.598	2.17	Gamma butyrlactone
5	Hexanoic acid, tetrahydro furylmethyl ester	6.872	17.02	
6.	Oxirane, propyl	7.010	0.58	
7	Diethyl 2-hydroxy-3-tetrahydrofuran 2-yl) succinate	7.044	0.96	
8	3 Buten -2-ol, 2 methyl	7.513	0.19	Alcohol, component of pheromone
9	Butanoic acid, ethyl ester	7.599	0.24	Ester
10	Propanal, 2 propenylhydrazone	7.731	0.38	
11	3-buten-2-ol, 2 methyl	7.811	0.47	Alcohol
12	Hydroxylamine, o-(3-methylbutyl)	8.188	0.15	
13	1-hexene, 3,4,5, trimethyl	8.989	0.12	Alkene
14	Isobutyl laurate	9.304	0.27	Fatty acid ester
15	1-Piperidinyloxy, 4-hydroxy-2,2,6, 6-tetramethyl-	9.356	0.18	
16	Isoamyl laurate	9.602	2.41	Fatty acid ester
17	17α, hydroxyprogesterone, trimethylsilyl ester, bis (O-methyloxine)	9.790	0.28	Steroid
18	2(Heptyloxycarbonyl) benzoic acid	10.334	0.41	

Table 4. 21: Chemical compounds present in sub-fraction 2 of ethylacetate fraction of aqueous root stem bark extract (EFAR2) of *A.senegakensis* (continued)

S/N	Compound name	RT(min)	% peak area	Class of compound
19	Benzene propanoic acid, β-bromo	10.351	0.54	
20	Isopropyl palmitate	10.494	40.69	Fatty acid ester
21	1-heptene, 4-methyl-	10.597	1.99	Alkene
22	Isopropyl palmitate	10.826	1.20	Fatty acid ester
23	Hexadecanoic acid butyl ester	11.055	1.10	Fatty acid ester
24	Cyclononasiloxane, octadecamethyl	11.095	0.65	
25	Hexadecanoic acid butyl ester	11.261	1.79	Fatty acid butyl ester
26	1-propyl 16-methylheptadecanoate	11.398	2.30	Fatty acid ester
27	Hexadecanoic acid pentyl ester	11.513	4.92	Fatty acid ester
28	Heptasiloxane	11.679	0.56	
29	Diglycolic acid, nonyl 2,4,4, trimethyl pentyl ester	11.719	0.42	
30	5,5 dimethyl thiazoline-2-resorcino	11.925	0.94	
31	N-Benzyl-N-ethyl-p-isopropyl benzamide	12.228	0.62	
32	n-propylheptyl ether	12.268	1.30	
33	Octadecanoic acid,pentyl ester	12.382	1.58	Fatty acid pentyl ester
34	1,2, Benzenedicarboxylic acid diundecyl ester	12.846	2.57	Aromatic nitrogen containing compound
35	Quinazoline-4-(1H)one	13.115	0.20	Keto quinazoline
36	Thumbergol	13.979	0.35	

administered 100 mg/kg b. wt. EFAR2 when compared with its control. There was a significant decrease in the change in weight ($p > 0.05$) of rats treated with 100, 200 and 400 mg/kg b. wt. EFAS1 when compared to the control while there was no significant difference ($p > 0.05$) in the total feed intake when compared with the control. Sixty percent (60%) mortality was recorded on the 13^{th} day at 400 mg/kg b.wt. Administration of DS3 significantly decreased ($p < 0.05$) the change in weight but significantly increased ($p < 0.05$) the total feed intake at all test doses when compared with the normal control. Forty percent (40%) mortality was recorded on the 13^{th} day of administration of 400 mg/kg b.wt DR2. There was significant increase ($p < 0.05$) in the change in weight of rats administered 200 and 400 mg/kg b.wt DR2 and a corresponding significant increase ($p < 0.05$) in feed intake.

Table 4.23 shows the organ to body weight ratio of rats administered antidiarrhoeal Sub-fractions from *Annona senegalensis* root and stem barks. There was a significant increase ($p < 0.05$) in organ to body weight ratio of kidney, heart and stomach of rats administered DFAR1. Out of all of the organs assessed in albino rats administered EFAR2, only the stomach increased significantly when compared to the normal control. There was significant increase in the organ to body weight ratio of the liver and stomach of rats administered EFAS1, DR2 and DS3

Table 4.22: Influence of administration of antidiarrhoeal sub-fractions of aqueous root and stem barks extract of *Annona senegalensis* on change in weight, total feed intake and mortality of rats

Grps	Weight gained (g)	Total feed intake (g)	Mortality (%)
Control	26.00 ± 0.36^{a}	423.33 ± 1.86^{a}	0
100 mg/kg b.wt DFAR1	24.67 ± 0.90^{a}	438.00 ± 1.00^{b}	0
200 mg/kg b.wt DFAR1	16.00 ± 1.22^{a}	458.00 ± 2.90^{c}	0
400 mg/kg b.wt DFAR1	23.67 ± 0.97^{a}	479.00 ± 2.52^{d}	0
Control	26.00 ± 2.06^{b}	423.33 ± 1.86^{a}	0
100 mg/kg b.wt EFAS1	2.00 ± 0.03^{a}	417.33 ± 2.68^{a}	0
200 mg/kg b.wt EFAS1	2.33 ± 0.01^{a}	416.00 ± 7.57^{a}	0
400 mg/kg b.wt EFAS1	3.33 ± 0.21^{a}	393.67 ± 4.91^{a}	40 (13th day)
Control	26.00 ± 0.13^{a}	423.33 ± 1.86^{c}	0
100 mg/kg b.wt EFAR2	56.00 ± 1.51^{b}	398.33 ± 2.19^{a}	0
200 mg/kg b.wt EFAR2	14.67 ± 0.67^{a}	419.33 ± 1.20^{b}	0
400 mg/kg b.wt EFAR2	22.33 ± 1.60^{a}	429.00 ± 1.23^{d}	0
Control	26.00 ± 2.08^{b}	408.00 ± 1.53^{b}	0
100 mg/kg b.wt DR2	21.33 ± 1.80^{a}	422.33 ± 1.45^{d}	0
200 mg/kg b.wt DR2	30.33 ± 2.96^{c}	412.00 ± 3.06^{c}	0
400 mg/kg b.wt DR2	29.67 ± 0.88^{c}	413.00 ± 3.62^{c}	20 (13th day)
Control	26.00 ± 2.08^{c}	408.00 ± 1.53^{a}	0
100 mg/kg b.wt DS3	14.66 ± 0.43^{a}	419.00 ± 0.58^{b}	0
200 mg/kg b.wt DS3	15.33 ± 0.01^{a}	424.33 ± 2.60^{c}	0
400 mg/kg b.wt DS3	17.67 ± 1.67^{b}	428.67 ± 2.60^{d}	0

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

EFAS1 – sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark, DFAR2 - sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks, EFAR2 - sub-fraction 2 of ethylacetate fraction of *A. senegalensis* root barks, DR2 - sub-fraction 2 of dichloromethane root

bark extract of *A. senegalensis*, DS3 - sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis.*

Table 4.23: Organ to body weight ratio of rats administered antidiarrhoeal Sub-fractions from *Annona senegalensis* root and stem barks ($\times 10^{-3}$)

	Kidney	Liver	Heart	Stomach
Control	3.0 ± 0.0^{a}	33.0 ± 1.0^{a}	2.0 ± 0.0^{a}	44.0 ± 1.0^{a}
100 mg/kg.b.wt DFAR1	6.0 ± 0.5^{c}	38.0 ± 2.0^{a}	4.0 ± 0.0^{b}	77.0 ± 1.3^{b}
200 mg/kg b.wt DFAR1	6.0 ± 0.4^{c}	34.0 ± 3.0^{a}	4.0 ± 0.1^{b}	81.0 ± 5.0^{a}
400 mg/kg b.wt. DFAR1	4.0 ± 0.2^{b}	35.0 ± 3.2^{a}	3.0 ± 0.3b	86.0 ± 4.0^{a}
Control	3.0 ± 0.00^{a}	33.0 ± 1.0^{a}	2.0 ± 0.0^{a}	44.0 ± 1.0^{a}
100 mg/kg b.wt. EFAR2	2.0 ± 0.20^{a}	30.0 ± 2.3^{a}	2.0 ± 0.2^{b}	75.0 ± 6.0^{b}
200 mg/kg b.wt. EFAR2	5.0 ± 0.40^{a}	43.0 ± 3.0^{a}	5.0 ± 0.4^{b}	63.0 ± 2.7^{b}
400 mg/kg b.wt EFAR2	5.0 ± 0.45^{a}	42.0 ± 2.0^{a}	5.0 ± 0.3^{b}	99.0 ± 1.1^{c}
Control	3.0 ± 0.0^{a}	33.0 ± 0.1^{a}	2.0 ± 0.0^{a}	44.0 ± 1.0^{a}
100 mg/kg b.wt. EFAS1	3.0 ± 0.3^{a}	42.0 ± 0.2^{b}	3.0 ± 0.1^{a}	111.0 ± 9.1^{b}
200 mg/kg b.wt. EFAS1	3.0 ± 0.1^{a}	40.0 ± 0.4^{b}	2.0 ± 0.0^{a}	121.0 ± 3.0^{c}
400mg/kg b.wt. EFAS1	3.0 ± 0.3^{a}	43.0 ± 0.1^{b}	2.0 ± 0.3^{a}	132.0 ± 3.0^{d}
Control	3.0 ± 0.3^{a}	29.0 ± 0.1^{a}	2.0 ± 0.0^{a}	57.0 ± 0.1^{a}
100 mg/kg b.wt. DR2	3.0 ± 0.3^{a}	40.0 ± 0.3^{b}	2.0 ± 0.1^{a}	97.0 ± 2.7^{b}
200 mg/kg b.wt DR2	3.0 ± 0.3^{a}	$44..0 \pm 0.4^{b}$	3.0 ± 0.3^{a}	82.0 ± 0.6^{b}
400 mg/kg b.wt. DR2	3.0 ± 0.3	40.0 ± 0.1^{b}	3.0 ± 0.2^{a}	95.0 ± 0.6^{b}
Control	$3.0 \pm 0.03^{a.}$	29.01 ± 0.1^{a}	2.0 ± 0.0^{a}	57.0 ± 0.1
100 mg/kg b.wt. DS3	3.0 ± 0.02^{a}	40.00 ± 0.3^{b}	4.0 ± 0.1^{b}	71.0 ± 5.7^{b}
200mg/kg b.wt. DS3	4.0 ± 0.01^{a}	37.00 ± 0.2^{b}	4.0 ± 0.0^{b}	95.0 ± 2.0^{b}
400 mg/kg b.wt. DS3	3.5 ± 0.02^{a}	35.00 ± 0.2^{b}	4.0 ± 0.0^{b}	114.0 3.5

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

EFAS1 – sub-fraction 1 of ethylacetate fraction of *A. senegalensis* stem bark, DFAR2 - sub-fraction 2 of dichloromethane fraction of *A. senegalensis* stem barks, EFAR2 - sub-fraction 2 of ethylacetate fraction of *A .senegalensis* root barks, DR2 - sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis*, DS3 - sub-fraction 3 of dichloromethane stem bark extract of *A .senegalensis.*

4.5.2 Effect of administration of antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem barks on rat liver function parameters

Table 4.24 shows the effect of administration of sub-fraction 1 of dichloromethane fraction from aqueous root bark extract of *A .senegalensis* (DFAR1) on rat liver function parameters. There was significant increase ($p < 0.05$) in alanine aminotransferase (ALT) activity of rats administered 100, 200 and 400mg/kg b.wt. DFAR1 when compared to the normal control. There was no significant difference ($p > 0.05$) in aspartate aminotransferase (AST) activity and albumin concentration at all doses when compared with the normal control. There was significant decrease ($p < 0.05$) in the concentration of unconjugated and conjugated bilirubin in the serum of rats administered all test doses of DFAR1. The sub-fraction also caused a significant decrease in the activity of serum alkaline phosphatase (ALP).

Table 4.25 shows the effect of administration of sub-fraction 1 of ethylacetate fraction from aqueous stem bark extract of *A. senegalensis* (EFAS1) on rat liver function parameters. There was a significant increase ($p < 0.05$) in serum ALT activity of rats administered all test doses of EFAS1 as shown in Table 4.25. Only 400mg/kg b.wt of EFAS1 fraction caused a significant increase in the activity of serum ALP and AST whereas 100 and 200 mg/kg b.wt EFAS1 caused a significant decrease ($p < 0.05$). There was a significant decrease in conjugated and unconjugated biliribun concentration in the serum of rats administered EFAS1.

Table 4.24: Effect of administration of sub-fraction 1 from dichloromethane fraction of aqueous root bark extract of *Annona senegalensis* (DFAR1) on rat liver function parameters

Dose	AST (U/L)	ALP (U/L)	ALT(U/L)	Uncon bil. (mg/dL)	Con. BiI (mg/dL)	Alb (g/L)
Control	34.37 ± 0.43^{a}	163.67 ± 1.16^{c}	36.33 ± 0.14^{a}	2.92 ± 0.23^{c}	2.47 ± 0.00^{c}	0.86 ± 0.05^{a}
100 mg/kg b.wt. DFAR1	23.13 ± 0.76^{a}	101.36 ± 0.65^{a}	75.44 ± 0.18^{b}	2.71 ± 0.13^{b}	2.27 ± 0.12^{b}	1.17 ± 0.15^{a}
200 mg/kg b. wt.. DFAR1	24.67 ± 0.58^{a}	119.60 ± 0.85^{a}	106.67 ± 1.04^{c}	2.53 ± 0.11^{a}	2.12 ± 0.09^{a}	1.27 ± 0.17^{a}
400 mg/kg b.wt. DFAR1	22.07 ± 1.14^{a}	132.69 ± 0.11^{b}	143.00 ± 0.20^{d}	2.42 ± 0.03^{a}	2.03 ± 0.03^{a}	0.98 ± 0.06^{a}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

AST- aspartate aminotransferase, ALP – alkaline phosphatase, ALT – alanine aminotransferase. Unconj. Bil.- unconjugated bilirubin, Conj. Bil.- conjugated bilirubin, Alb. - albumin

Table 4.25: Effects of administration of sub-fraction 1 of ethylacetate fraction of aqueous stem bark extract of *Annona senegalensis* (EFAS1) on rat liver function parameters

Dose	AST (U/L)	ALP (U/L)	ALT (U/L)	Uncon. bil. (mg/dL)	Con. Bil. (mg/dL))	Alb. (g/L)
Control	34.37 ± 0.43^{b}	163.67 ± 1.16^{c}	36.33 ± 0.14^{a}	2.92 ± 0.02^{c}	2.47 ± 0.00^{b}	0.86 ± 0.05^{a}
100 mg/kg b.wt. EFAS1	24.13 ± 0.35^{a}	95.56 ± 0.27^{a}	80.00 ± 0.95^{b}	2.50 ± 0.15^{b}	$2.10 \pm 0.1b^{a}$	0.99 ± 0.04^{a}
200 mg/kg.b.wt. EFAS1	19.00 ± 0.00^{a}	130.40 ± 0.02^{b}	75.67 ± 0.72^{b}	2.64 ± 0.11^{b}	2.22 ± 0.10^{a}	1.25 ± 0.05^{b}
400 mg/kg b.wt. EFAS1	55.73 ± 0.23^{c}	194.11 ± 0.94^{d}	89.32 ± 0.03^{b}	2.36 ± 0.02^{a}	1.97 ± 0.01^{a}	1.27 ± 0.03^{b}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$). AST- aspartate aminotransferase, ALP – alkaline phosphatase, ALT – alanine aminotransferase. Unconj. Bil.- unconjugated bilirubin,

Conj. Bil.- conjugated bilirubin, Alb. - albumin

Table 4.26 depicts the effects of administration of sub-fraction 2 of ethylacetate fraction of aqueous root bark extract of *A. senegalensis* (EFAR2) on rat liver function parameters in rats. There was a significant increase ($p < 0.05$) in the activity of serum alanine aminotransferase (ALT) of rats administerd at all test doses of EFAR 2 when compared to the normal control. There was no significant difference ($p > 0.05$) in serum aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities of rats administered 100, 200 and 400 mg/kg b.wt of EFAR2 when compared with the normal control. The concentration of conjugated and unconjugated bilirubin significantly decreased ($p < 0.05$) when compared with the normal control. There was significant increase ($p < 0.05$) in the concentration of serum albumin at 200 and 400 mg/kg b.wt EFAR2. This increase was not dose dependent.

Table 4.27 shows the effects of administration of sub-fraction 3 of dichloromethane stem bark (DS3) extract of *A. senegalensis* on rat liver function parameters in rats. There was a significant decrease ($p < 0.05$) in serum ALP activity, unconjugated and conjugated bilirubin concentration and a significant increase ($p < 0.05$) in serum ALT activity in rats administered all test doses of DS3 when compared with the normal control.

Table 4.28 shows the effects of administration of sub-fraction 2 of dichloromethane root bark extract of *Annona senegalensis* (DR2) on rat liver function parameters in rats. There was no significant difference ($p > 0.05$) in values of serum activities of aspartate aminotransferase, alkaline phosphatase, alanine aminotransferase, concentration of unconjugated and conjugated bilirubin, and albumin in rats administered DR2 at all doses when compared with the normal control.

Table 4.26: Effect of administration of sub-fraction 2 on ethylacetate fraction of aqueous root bark of *A. senegalensis* (EFAR2) on rat liver function parameters

Dose	AST (U/L)	ALP (U/L)	ALT (U/L)	Uncon.BiI (mg/dL)	Con. BiI (mg/dL)	ALB (g/L)
Control	34.37 ± 1.03^{a}	163.67 ± 1.16^{a}	36.33 ± 0.14^{a}	2.92 ± 0.02^{b}	2.47 ± 0.00^{b}	0.84 ± 0.05^{a}
100 mg/kg b.w. EFAR2	22.33 ± 2.16^{a}	153.43 ± 1.52^{a}	99.33 ± 0.88^{d}	$2.46 \pm 0 .15^{a}$	2.12 ± 0.09^{a}	0.91 ± 0.07^{a}
200 mg/kg b.w. EFAR2	16.20 ± 3.10^{a}	161.43 ± 1.18^{a}	77.33 ± 0.26^{c}	2.48 ± 0.13^{a}	2.09 ± 0.11^{a}	1.18 ± 0.09^{c}
400 mg/kg b.w. EFAR2	33.73 ± 1.11^{a}	168.93 ± 2.11^{a}	54.67 ± 0.14^{b}	2.40 ± 0.14^{a}	2.02 ± 0.12^{a}	0.98 ± 0.04^{b}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)
AST- aspartate aminotransferase, ALP – alkaline phosphatase, ALT – alanine aminotransferase. Unconj. Bil.- unconjugated bilirubin,

Conj. Bil.- conjugated bilirubin, Alb. - albumin

Table 4.27: Effect of administration of sub-fraction 3 of dichloromethane stem bark extract (DS3) on rat liver function parameters

Group	AST (U/L)	ALP (U/L)	ALT (U/L)	Uncon. Bil (mg/dL)	CON. BiI (mg/dL)	ALB (g/L)
Control	24.80 ± 0.10^{b}	103.20± 0.57^{b}	18.66 ± 0.33^{a}	2.35 ± 0.01^{b}	1.98 ± 0.01^{b}	0.98 ± 0.05^{a}
100 mg/kg b.wt DS3	18.13 ± 0.14^{a}	66.63 ± 0.44^{a}	77.00 ± 0.51^{b}	2.16 ± 0.06^{a}	1.81 ± 0.05^{a}	0.88 ± 0.03^{a}
200 mg/kg b.wt. DS3	26.93 ± 0.32^{b}	61.21 ± 0.43^{a}	75.67 ± 0.53^{b}	2.01 ± 0.07^{a}	1.68 ± 0.05^{a}	0.91 ± 0.07^{a}
400 mg/kg b.wt. DS3	23.60 ± 0.30^{b}	57.16 ± 0.04^{a}	85.33 ± 1.03^{b}	1.99 ± 0.06^{a}	1.67 ± 0.05^{a}	0.97 ± 0.10^{a}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

AST- aspartate aminotransferase, ALP – alkaline phosphatase, ALT – alanine aminotransferase. Unconj. Bil.- unconjugated bilirubin, Conj.Bil.-conjugatedbilirubin,Alb.-albumin

Table 4.28: Effect of administration of sub-fraction 2 of dichloromethane root bark extract (DR2) on rat liver function parameters

Group	AST (U/L)	ALP (U/L)	ALT (U/L)	uncon.biI (mg/dL)	con. BiI (mg/dL)	Alb. (g/L)
Control	24.80 ± 0.14[a]	103.20 ± 0.57[a]	18.67 ± 1.33[a]	2.35 ± 0.01[a]	1.98 ± 0.01[a]	0.98 ± 0.05[a]
100 mg/kg b.wt DR2	31.06 ± 0.33[a]	105.33 ± 0.18[a]	19.66 ± 0.21[a]	2.50 ± 0.41[a]	2.10 ± 0.34[a]	0.98 ± 0.12[a]
200 mg/kg b.wt. DR2	26.93 ± 0.32[a]	106.67 ± 1.04[a]	18.67 ± 0.45[a]	2.31 ± 0.11[a]	1.94 ± 0.09[a]	0.87 ± 0.01[a]
400 mg/kg b.wt DR2	23.80 ± 0.30[a]	113.00 ± 0.21[a]	19.00 ± 0.57[a]	2.16 ± 0.06[a]	1.78 ± 0.04[a]	0.89 ± 0.01[a]

Values are expressed as mean ± S.E.M. Different superscript down the column are significantly different ($p < 0.05$).
AST- aspartate aminotransferase, ALP – alkaline phosphatase, ALT – alanine aminotransferase. Unconj. Bil.- unconjugated bilirubin, Conj. Bil.- conjugated bilirubin, Alb. - albumin

4.6.3 Effect of administration of antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem bark extracts on rat kidney function parameters

The effect of administration of sub-fraction 1 of dichloromethane fraction of aqueous root bark of *A. senegalensis* (DFAR) on rat kidney function parameters is presented in Table 4.29. There was significant decrease ($p < 0.05$) in serum urea concentration of rats administered test doses of DFAR1 when compared with the normal control. The decrease was dose dependent. Serum Na^+ and K^+ concentration of rats administered 100, 200, 400 mg/kg b.wt DFAR1 significantly increased ($p < 0.05$) when compared to the normal control.

Table 4.30 shows the effect of sub-fraction 1 of ethylacetate fraction of aqueous stem bark extract of *A. senegalensis* (EFAS1) on rat kidney function parameters. There was significant decrease ($p < 0.05$) in serum urea concentration of albino rats administered 400 mg/kg b.wt EFAS1. Serum Na^+ concentration of rats administered 100, 200 and 400 mg/kg b.wt. EFAS1 significantly increased ($p < 0.05$) when compared to normal control. There was no significant difference ($p > 0.05$) in serum K^+ concentration when compared to the normal control. Serum Ca^{2+} and uric acid concentration of rats administered 100, 200 and 400 mg/kg b.wt. EFAS1 significantly decreased ($p < 0.05$) when compared to the normal control. The decrease was dose dependent.

Table 4.31 shows the effect of sub-fraction 2 of ethylacetate fraction of aqueous root bark extract of *A. senegalensis* (EFAR2) on rat kidney function parameters. There was a significant reduction ($p < 0.05$) in the concentration of serum urea and creatinine of rats treated with 100, 200, 400 mg/kg b.wt. of EFAR2 when compared with the normal control. Serum Na^+ and K^+ concentration of rats administered 100, 200 and 400 mg/kg b.wt. EFAR2 significantly increased ($p < 0.05$) when compared with the normal control.

Table 4.29: Effects of administration of sub-fraction 1 of dichloromethane fraction of aqueous root bark extract of *A. senegalensis* (DFAR1) on serum kidney function parameters in rats

Group	Urea (mmol/L)	Creatinine (mg/mL)	Na^+ (mmol/L)	K^+ (mg/dL)	Ca^{2+} (mg/dL)	Uric acid (mmol/L)
Control	155.26 ± 1.46^c	0.93 ± 0.09^a	226.00 ± 0.01^a	9.62 ± 0.17^a	3.20 ± 0.07^a	4.78 ± 0.06^a
100 mg/kg b wt .DFAR1	78.48 ± 2.70^b	0.96 ± 0.01^a	1950.00 ± 1.99^b	14.56 ± 1.32^b	3.39 ± 0.16^a	5.05 ± 0.24^a
200 mg/kg b.wt. DFAR1	74.28 ± 1.29^a	0.64 ± 0.06^a	2586.36 ± 3.76^b	13.80 ± 0.89^b	2.96 ± 0.18^a	4.41 ± 0.25^a
400 mg/kg b.wt. DFAR1	53.35 ± 7.27^a	0.41 ± 0.03^a	2047.72 ± 1.63^b	14.96 ± 0.60^b	3.23 ± 0.32^a	4.81 ± 0.49^a

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

Table 4.30: Effect of administration of sub-fraction 1 on ethylacetate fraction of aqueous stem bark extract of *A. senegalensis* (EFAS1) on rat kidney function parameters

Group	Urea (mmol/L)	Creatinine (mg/mL)	Na^+ (mmol/L)	K^+ (mg/dL)	Ca^{2+} (mg/dL)	Uric acid (mmol/L)
Control	155.26 ± 1.46^b	0.83 ± 0.005^a	$226.00 \pm .005^a$	9.62 ± 0.17^a	3.20 ± 0.04^c	4.78 ± 0.06^c
100 mg/kg b.wt. EFAS1	138.47 ± 3.23^b	0.09 ± 0.001^a	2134.09 ± 7.22^c	13.60 ± 0.17^a	$2.88 \pm 0 .16^b$	4.30 ± 0.24^b
200 mg/kg b.wt. EFAS1	147.71 ± 2.03^b	0.27 ± 0.009^a	1856.82 ± 4.92^b	9.73 ± 0.28^a	$2.91 \pm 0 .11^b$	4.34 ± 0.16^b
400 mg/kg b.wt. EFAS1	80.41 ± 0.33^a	0.96 ± 0.003^a	1721.55 ± 0.03^b	12.59 ± 0.03^a	2.80 ± 0.03^a	4.11 ± 0.03^a

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

Table 4.31: Effect of sub-fraction 2 of ethylacetate fraction of aqueous root bark extract of *A.senegalensis* (EFAR 2) on rat kidney function parameters

Grp	Urea (mmol/L)	Creatinine (mg/mL)	Na^+ (mmol/L)	K^+ (mg/dL)	Ca^{2+} (mg/dL)	Uric acid (mmol/L)
Control	155.26 ± 1.46^c	$0.83 \pm .005^c$	226.00 ± 0.05^a	9.62 ± 1.18^a	3.20 ± 0.04^a	4.78 ± 0.06^a
100 mg/kg b.wt EFAR2	115.15 ± 5.25^b	$0.50 \pm .002^b$	1963.0 ± 1.16^b	13.11 ± 0.26^b	3.02 ± 0.08^a	4.50 ± 0.11^a
200 mg/kg b.wt EFAR2	125.11 ± 5.73^b	$0.23 \pm .002^a$	1925.0 ± 3.34^b	14.12 ± 0.84^b	3.20 ± 0.09^a	4.60 ± 0.03^a
400 mg/kg b.wt EFAR2	64.09 ± 2.71^a	$0.55 \pm .003^b$	1763.6 ± 5.12^b	14.02 ± 0.34^b	3.13 ± 0.14^a	4.67 ± 0.20^a

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

Table 4.32 depicts the effects of administration of sub-fraction 3 of dichloromethane stem bark extract of *A. senegalensis* in rat kidney parameters. The serum urea concentration of rats administered 200 and 400 mg/kg b.wt. of DS3 significantly increased ($p < 0.05$) when compared with the normal control. Serum Na^+ and K^+ concentration of rats administered 100, 200 and 400 mg/kg b.wt of DS3 significantly increased when compared to the control.

Table 4.33 shows the effect of administration of sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis* on serum kidney parameters. There was significant decrease ($p < 0.05$) in serum urea concentration of rats administered 200 and 400 mg/kg b.wt .DR2 when compared to the normal control. Serum urea concentration of rats administered 100mg/kg b.wt. DR2 was not significantly different ($p > 0.05$) from the normal control. Serum Na^+ concentration of rats administered 100 mg/kg b. wt significantly decreased ($p < 0.05$) when compared to the normal control but the K^+ concentration of the rats significantly increased ($p < 0.05$). In contrast, administration of 400mg/kg b.wt DR2 significantly increased ($p < 0.05$) serum Na^+ concentration while it significantly decreased ($p < 0.05$) serum K^+ concentration.

4.5.4 Effect of administration of antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem bark extracts on some rat serum antioxidant enzyme activities

Table 4.34 shows the effect of administration of sub-fraction 1 of dichloromethane fractions of aqueous root bark extract of *Anonna senegalensis* on rat serum antioxidant enzyme activities. Serum glutathione peroxidase (GPx) activity significantly increased ($p < 0.05$) at all test doses of DFAR1 in a dose dependent manner while there was a corresponding significant decrease ($p < 0.05$) in the activity of superoxide dismutase (SOD) when compared to the control. There was a significant decrease ($p < 0.05$) in catalase activity at all test doses when compared with the control.

Table 4.32: Effect of administration of sub-fraction 3 of dichloromethane stem bark extract of *Annona senealensis* (DS3) on rat kidney function parameters

Group	Urea (mmol/L)	Creatinine (mg/mL)	Na^+ (mmol/L)	K^+ (mg/dL)	Ca^{2+} (mg/dL)	Uric acid (mmol/L)
Control	82.27 ± 3.87^a	0.53 ± 0.005^b	25.32 ± 3.84^a	10.57 ± 0.06^a	3.07 ± 0.07^a	4.59 ± 0.09^a
100 mg/kg b.wt.DS3	80.33 ± 0.72^a	0.18 ± 0.002^a	159.24 ± 2.58^b	14.22 ± 0.58^b	3.37 ± 0.29^a	5.01 ± 0.43^a
200 mg/kg b,wt. DS3	140.16 ± 1.30^b	0.64 ± 0.009^b	161.36 ± 7.48^b	13.92 ± 1.02^b	3.11 ± 0.08^a	5.20 ± 0.12^a
400 mg/kg b.wt. DS3	140.53 ± 1.72^b	0.13 ± 0.001^a	140.91 ± 7.63^b	14.68 ± 0.17^b	3.24 ± 0.04^a	5.06 ± 0.30^a

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

Table 4.33: Effects of administration of sub-fraction 2 of dichloromethane root bark of *Annona senegalensis* (DR2) on rat kidney function parameters

Group	Urea (mmol/L)	Creatinine (mg/mL)	Na^+ (mmol/L)	K^+ (mg/dL)	Ca^{2+} (mg/dL)	Uric acid (mmol/l)
Control	82.27 ± 3.87^b	0.53 ± 0.05^a	25.32 ± 0.38^b	10.57 ± 0.05^b	3.07 ± 0.07^a	4.59 ± 0.09^a
100 mg/kg b.wt. DR2	80.71 ± 2.90^b	0.64 ± 0.01^a	6.13 ± 0.06^a	14.75 ± 1.46^c	3.95 ± 0.66^a	5.88 ± 0.09^a
200 mg/kg b.wt. DR2	30.77 ± 2.75^a	0.50 ± 0.06^a	59.85 ± 1.45^b	11.69 ± 1.39^b	3.01 ± 0.30^a	4.98 ± 0.07^a
400 mg/kg b.wt. DR2	39.51 ± 3.28^a	0.93 ± 0.03^a	2282.54 ± 6.70^c	9.38 ± 1.09^a	3.24 ± 0.04^a	4.77 ± 0.09^a

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

Table 4.34: Effect of administration of sub-fraction 1 of dichloromethane fraction of aqueous root bark of *Annona senegalensis* (DFAR1) on some rat serum antioxidant enzyme activities

Group	GPX	SOD	CAT $\times 10^{-3}$
		(U/L)	
Control	0.28 ± 0.02^{a}	211.57 ± 1.25^{c}	17.67 ± 0.01^{b}
100 mg/kg b.wt. DFAR1	3.28 ± 0.09^{b}	91.21 ± 4.87^{b}	2.67 ± 0.01^{a}
200 mg/kg b.wt. DFAR1	6.50 ± 0.19^{c}	41.46 ± 1.29^{a}	4.35 ± 0.01^{a}
400 mg/kg b.wt. DFAR1	6.53 ± 0.28^{c}	33.17 ± 0.29^{a}	5.50 ± 0.06^{a}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

GPx – glutathione peroxidase,

SOD – superoxide dismutase

CAT – catalase

Table 4.35 depicts the effect of administration of sub-fraction 1 of ethylacetate fraction of aqueous stem bark extract (EFAS1) of *A. senegalensis* on rat serum antioxidant enzyme activities. Serum glutathione peroxidase (GPx) activity in rats administered 100, 200 and 400 mg/kg b. wt. EFAS1 significantly increased ($p < 0.05$) when compared with the normal control. There was a corresponding decrease ($p < 0.05$) in serum superoxide dismutase (SOD) activity. The serum catalase activity in rats administered 400 mg/kg b.wt EFAS1 significantly increased ($p < 0.05$) when compared with the control.

Table 4.36 shows the effect of administration of sub-fraction 2 of ethylacetate fraction of aqueous root bark extract (EFAR2) of *Annona senegalensis* on selected rat serum antioxidant enzyme activities. Serum glutathione peroxidase (GPx) activity significantly increased ($p < 0.05$) at all test doses of EFAR2 when compared to the control. The increase was dose dependent. In contrast, there was significant decrease ($p < 0.05$) in serum superoxide dismutase (SOD) activity in rats administered 100, 200 and 400 mg/kg b.wt. when compared to the normal control. The decrease in superoxide dismutase activity was dose dependent. Administration of EFAR2 to rats significantly increased ($p < 0.05$) serum catalase activity.

Table 4.37 shows the effects of administration of sub-fractions 3 from dichloromethane stem bark extracts of *A. senegalensis* (DS3) on some selected rat serum antioxidant enzyme activities. Administration of 100, 200 and 400 mg/kg b.wt DS3 significantly increased the activity of catalase when compared with the control while 200 and 400 mg/kg b.wt. DS 3 significantly reduced ($p < 0.05$) the activity of SOD. Administration of 400 mg/kg b.wt DS3 significantly increased ($p < 0.05$) GPx activity.

Table 4.35: Effect of administration of sub-fraction1 of ethylacetate fractions of aqueous stem bark (EFAS1) of *A. senegalensis* on some rat serum antioxidant enzymes activities

Group	GPX	SOD	CAT $\times 10^{-3}$
		(U/L)	
Control	0.28 ± 0.02^{a}	211.57 ± 1.25^{c}	17.77 ± 0.14^{a}
100 mg/kg b.wt. EFAS1	4.72 ± 0.40^{c}	24.88 ± 0.01^{a}	17.58 ± 0.40^{a}
200 mg/kg b.wt. EFAS1	2.75 ± 0.26^{b}	40.29 ± 1.09^{b}	15.58 ± 0.12^{a}
400 mg/kg b. wt. EFAS1	2.57 ± 0.25^{b}	49.75 ± 0.07^{b}	40.00 ± 0.25^{b}

Values are mean of three replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

GPx – glutathione peroxidase,

SOD – superoxide dismutase

CAT - catalase

Table 4.36: Effect of administration of sub-fraction 2 of ethylacetate fraction of aqueos root bark extracts of *Annona sengalensis* (EFAR2) on some rat serum antioxidant enzyme activities

Group	GPX	SOD	CAT $\times 10^3$
		(U/L)	
Control	0.28 ± 0.02^{a}	211.57 ± 1.25^{c}	1.77 ± 0.14^{a}
100 mg/kg b.wt. EFAR2	1.05 ± 0.07^{b}	41.46 ± 0.58^{b}	41.67 ± 1.27^{b}
200 mg/kg b. wt. EFAR2	6.25 ± 0.56^{c}	33.17 ± 0.98^{a}	52.33 ± 3.94^{b}
400 mg/kg b. wt. EFAR2	5.42 ± 0.17^{c}	33.16 ± 0.89^{a}	80.37 ± 0.15^{c}

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$).

GPx – glutathione peroxidase,

SOD – superoxide dismutase

CAT - catalase

Table 4.37: Effect of administration of dichloromethane stem bark fraction 3 (DS3) on some rat serum antioxidant enzyme activities

Group	GPX	SOD	CAT×10^{-3}
		(U/L)	
Control	0.81 ± 0.02^{a}	24.88 ± 0.00^{c}	9.32 ± 0.30^{a}
100 mg/kg b. wt. DS3	0.41 ± 0.01^{a}	24.88 ± 1.60^{c}	16.91 ± 0.50^{b}
200 mg/kg b. wt. DS3	0.41 ± 0.08^{a}	16.58 ± 0.87^{b}	21.08 ± 0.26^{b}
400 mg/kg b. wt. DS3	2.50 ± 0.06^{b}	8.29 ± 0.58^{a}	31.25 ± 0.53^{c}

Values are mean of five replicates ± S.E.M. Values with different superscript are significantly different from others ($p < 0.05$)

GPx – glutathione peroxidase,

SOD – superoxide dismutase

CAT - catalase

Table 4.38 shows the effects of administration of sub-fraction 2 from dichloromethane root bark extracts of *A. senegalensis* (DR2) on some rat serum antioxidant enzyme activties. There was no significant difference ($p > 0.05$) in serum glutathione peroxidase (GPx) activity of rats admistered 100, 200 and 400 mg/kg b.wt DR2 when compared with the control. Serum superoxide dismutase (SOD) activity of rats administered 400 mg/kg b. wt DR2 significantly increased ($p < 0.05$) when compared with the control while serum catalase activity of DR2 treated rats ignificantly increased ($p < 0.05$) when compared with the control.

4.5.5 Effect of administration of antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem bark extracts on rat serum malondialdehyde (MDA) concentration in rats

Figure 4.12 shows the effects of administration of sub-fraction 1 of dichloromethane fraction of aqueous root bark extract of *A. senegalensis* (DFAR1) on serum malondialdehyde concentration in rats. Malondialdehyde concentration in serum of rats administered 100, 200 and 400 mg/kg b.wt of DFAR1 significantly increased ($p < 0.05$) when compared with the control.

The effects of administration of sub-fraction 1 of ethylacetate fractions of aqueous stem bark extracts of *A. senegalensis* (EFAS1) on serum malondialdehyde concentration in rats is shown in Figure 4.13. There was a significant increase ($p < 0.05$) in serum malondialdehyde (MDA) concentration at all test doses of EFAS1 when compared with the control.

Figure 4.14 shows the effects of administration of sub-fraction 2 of ethylacetate fractions of aqueous root bark extracts of *A. senegalensis* (EFAR2) on serum malondialdehyde concentration in rats. The concentration of serum malondialdehyde in rats administered 100, 200 and 400 mg/kg b.wt EFAR 2 significantly increased ($p < 0.05$) when compared to the control.

Table 4.38: Effect of administration of sub-fraction 3 of dichloromethane stem bark extract of aqueous root bark of *Annona senegalensis* (DR2) on some rat serum antioxidant enzyme activities

Group	GPX	SOD	CAT × 10^{-3}
		(U/L)	
Control	0.81 ± 0.02[a]	24.88 ± 1.00[a]	9.35 ± 0.01[a]
100 mg/kg b. wt. DR2	2.22 ± 0.09[a]	16.59 ± 0.21[a]	31.65 ± 0.14[d]
200 mg/kg b. wt. DR2	2.12 ± 0.06[a]	24.88 ± 0.47[a]	29.83 ± 0.82[c]
400 mg/kg b. wt. DR2	1.25 ± 0.00[a]	49.75 ± 0.47[b]	21.33 ± 0.18[b]

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

GPx – glutathione peroxidase,

SOD – superoxide dismutase

CAT – catalase

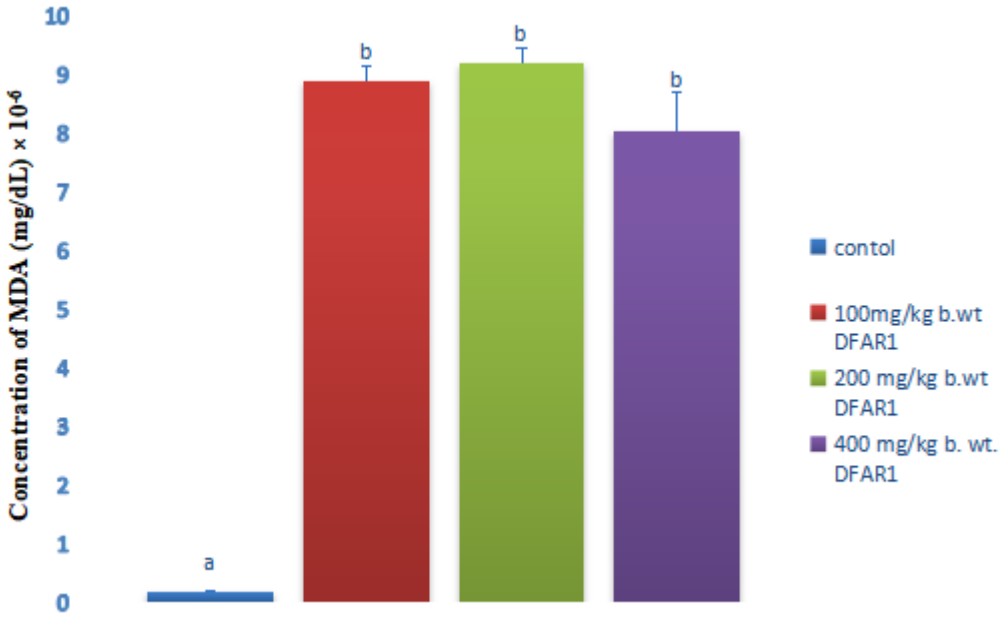


Figure 4.12: Effect of administration of sub-fraction 1 of dichloromethane fraction of aqueous root bark extract of *A. senegalensis* (DFAR1) on rat serum malondialdehyde (MDA) concentration

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different ($p < 0.05$)

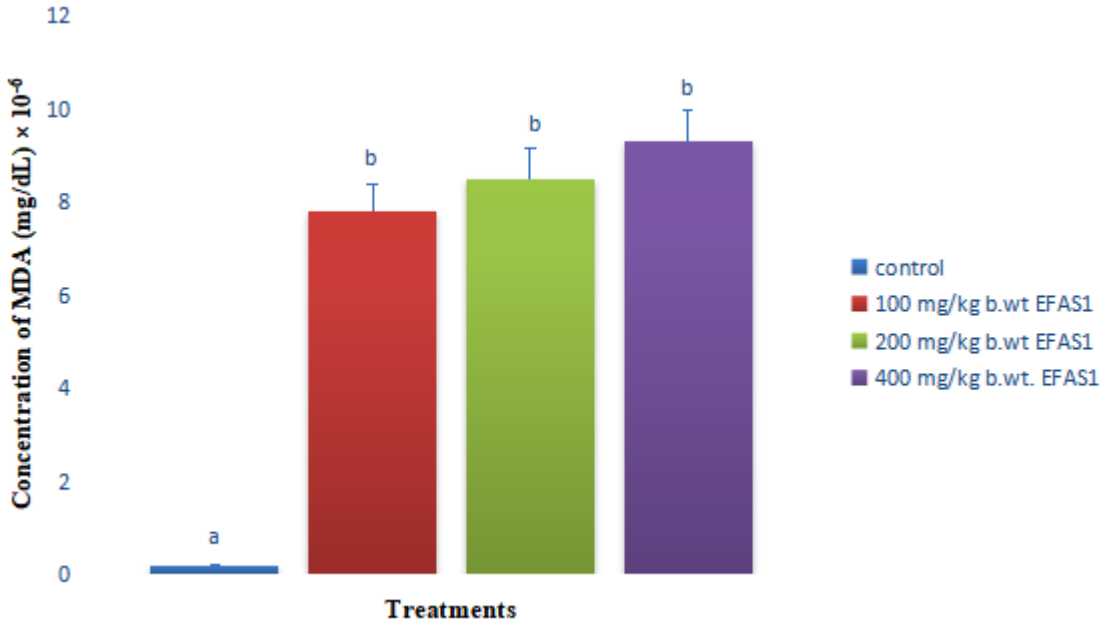
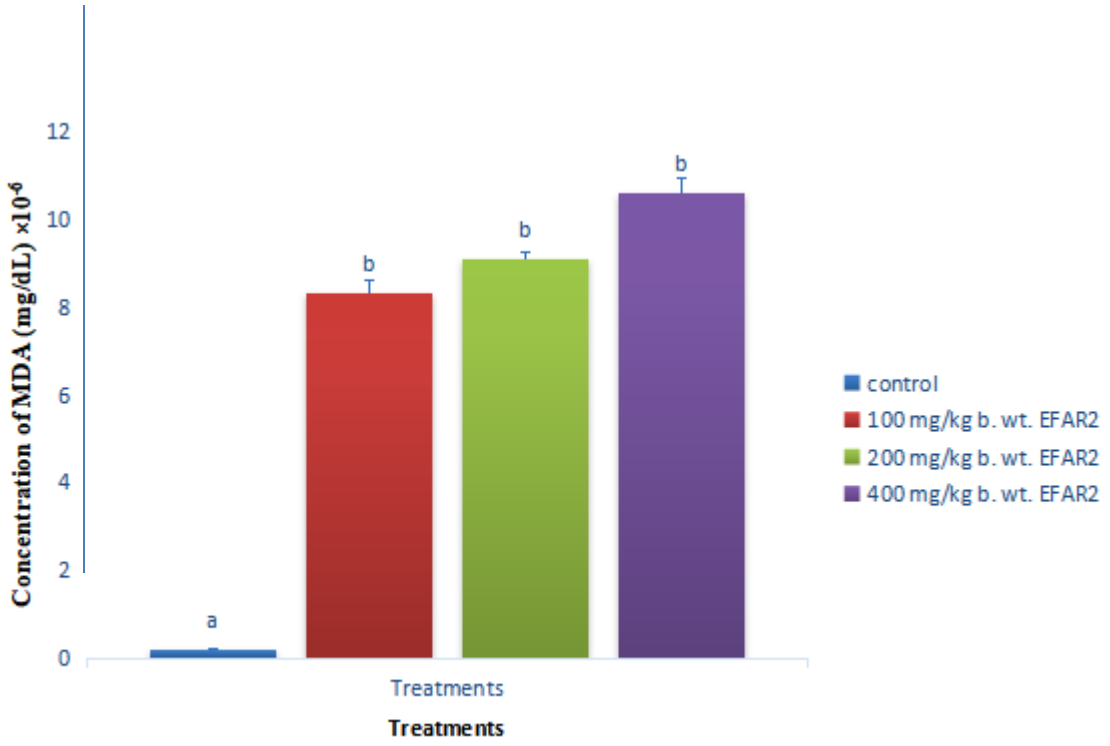


Figure 4.13: Effect of administration of sub-fraction 1 of ethylacetate fraction of aqueous stem bark (EFAS1) of *A. senegalensis* on rat serum malondialdehyde (MDA) concentration

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different ($p < 0.05$)



s

Figure 4.14: Effect of administration of sub-fraction 2 from ethylacetate fraction of aqueous root bark extract (EFAR2) on rat serum malondialdehyde (MDA) concentration

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different ($p < 0.05$)

Figure 4.15 shows the effects of administration of sub-fraction 3 of dichloromethane stem bark extract of *A. senegalennsis* (DS3) on serum malondialdehyde concentration in rats. Serum malondialdehyde (MDA) concentration in rats administered 100, 200 and 400 mg/kg b.wt. of DS3 significantly increased ($p < 0.05$) when compared with the control. Figure 4.16 shows the effects of administration of sub-fraction 2 of dichloromethane root bark extracts of *A. senegalensis* (DR2) on serum malondialdehyde concentration in rats. Serum concentration of malondialdehyde (MDA) in rats administered 100, 200 and 400 mg/kg b.wt. of DR2 significantly increased ($p < 0.05$) when compared to the control.

4.6.6 Selected heamatological indices of rats administered antidiarrhoeal sub-fractions from *Annona senegalensis* root and stem bark extracts

Table 4.39 shows some heamatological indices of rats administered sub-fraction 1 of dichloromethane fraction of aqueous root bark extract of *A. senegalensis* (DFAR1). There was a significant increase ($p < 0.05$) in percentage packed cell volume (PCV) at all test doses of DFAR1 when compared to the control. There was no significant difference ($p > 0.05$) in erythrocytes, basophil, neutrophil, platelets and monocytes when compared with the control.

Table 4.40 shows some heamatological indices of rats administered sub-fraction 1 of ethylacetate fraction of aqueous stem bark extract *A. senegalensis* (EFAS1). There was no significant difference ($P > 0.05$) in PCV, erythrocytes, basophil, neutrophil, platelets and monocytes when compared with the control. There was a significant increase ($p < 0.05$) in lymphocytes concentration in rats administered 400 mg/kg b.wt of EFAS1 when compared with the control.

Table 4.41 shows heamatological parameters in rats administered sub-fraction 2 of ethylacetate fraction of aqueous root bark extract *A. senegalensis* (EFAR2). There was no significant difference ($p > 0.05$) in the studied heamatological parameters of rats administered 100, 200 and 400 mg/kg b.wt EFAR2 when compared to the control.

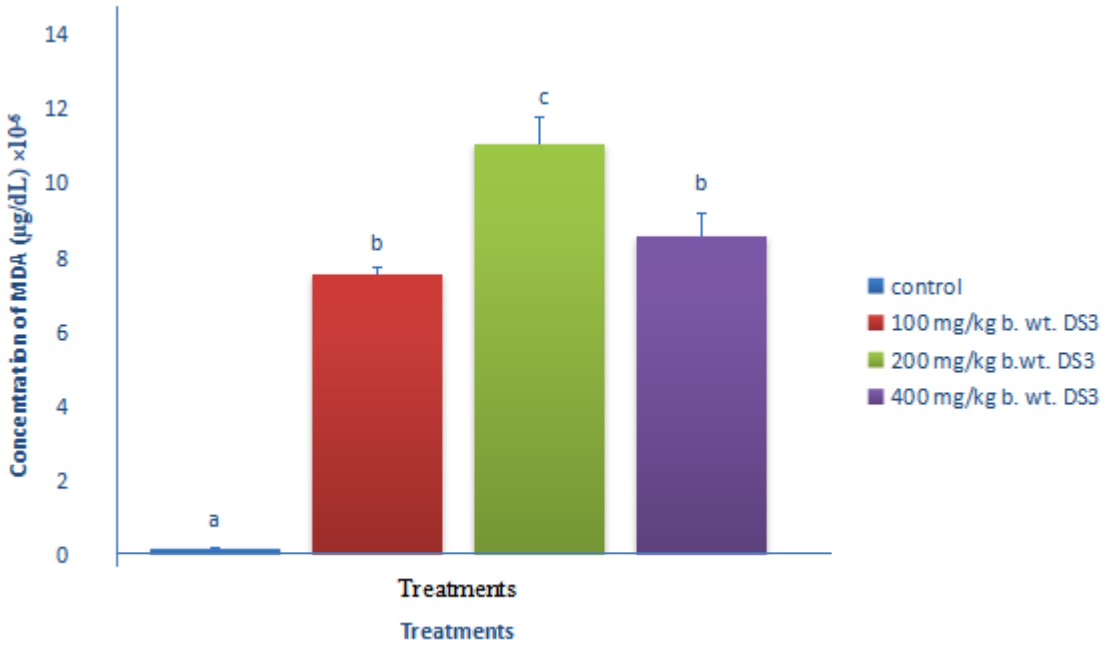


Figure 4.15: Effect of administration of sub-fraction 3 of dichloromethane stem bark extract (DS3) of *A. senegalensis* on rat serum malondialdehyde (MDA) concentration

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different ($p < 0.05$)

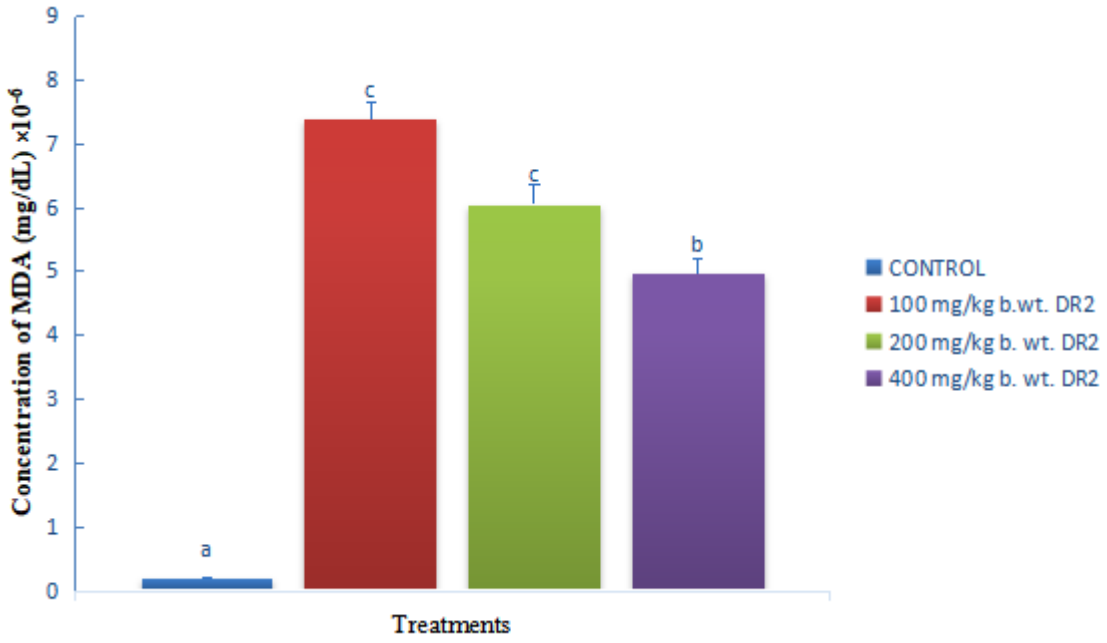


Figure 4.16: Effect of administration of sub-fraction 2 of dichloromethane root bark extract (DR2) of *A. senegalensis* on rat serum malondialdehyde (MDA) concentration

Values are mean of five replicates ± S.E.M. Bars with different superscript are significantly different ($p < 0.05$)

Table 4.39: **Effect of administration of sub-fraction 1 of dichloromethane fraction of aqueous root bark extract (DFAR1) of *Annona senegalensis* on selected heamatological parameters in rats**

GROUP	PCV(%)	Eryt×10^6 µL	Baso×10^6 µL	Neu×10^6 µL	Lym×10^6 µL	Eos×10^6 µL	Mon×10^6 µL	plate×10^6 µL
Control	41.0 ± 0.58^a	0.33 ± 0.03^a	0.33 ± 0.03^a	2.33 ± 0.20^a	30.0 ± 0.58^a	0.3 ± 0.03^a	0.33 ± 0.0^a	2.33±0.17^a
100 mg/kg DFAR1	45.7 ± 0.88^c	1.00 ± 0.09^a	1.33 ± 0.12^a	1.67 ± 0.16^a	29.3 ± 0.95^a	0.0 ± 0.00^a	0.67 ± 0.03^a	1.67± 0.16^a
200 mg/Kg DFAR1	42.3 ± 2.03^b	1.00 ± 0.10^a	1.00 ± 0.09^a	0.00 ± 0.00^a	26.7 ± 1.47^a	0.0 ± 0.00^a	1.00 ± 0.06^a	2.33 ± 0.19^a
400 mg/kg DFAR1	44.7 ± 1.20^b	2.67 ± 0.17^a	2.67 ± 0.19^a	0.67 ± 0.06^a	16.0 ± 0.66^b	0.0 ± 0.00^a	1.00 ±0.06^a	1.67 ±0.13^a

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

PCV – packed cell volume, Eryt. – erythrocytes, Baso – basophils, Neu. – neutrophils, lymp. – lymphocytes, Eos.- eosophils, Mon. – monocytes, Plate- platelet

Table 4.40: Effect of administration of sub-fraction 1 of ethylacetate fraction of aqueous stem bark extract of *Annona sngalensis* (EFAS1) on selected heamatological parameters in rats

GROUP	PCV(%)	Eryt×10^6 µL	Baso×10^6 µL	Neu×10^6 µL	Lym×10^6 µL	Eos×10^6 µL	Mon×10^6 µL	plate×10^6 µL
Control	41.0 ± 0.58^a	0.33 ± 0.03^a	0.33 ± 0.03^a	2.33 ± 0.13^a	30.0 ± 0.58^b	0.3 ± 0.03^a	0.33 ± 0.03^a	2.33 ± 0.22^a
100 mg/kg b. wt. EFAS1	44.7 ± 1.48^a	1.33 ± 0.13^a	0.67 ± 0.06^a	0.67 ± 0.06^a	35.3 ±1.87^b	1.3 ± 0.09^a	0.00 ±0.00^a	2.33 ± 0.12^a
200 mg/kg b. wt. EFAS1	39.0 ± 0.84^a	0.00 ± 0.00^a	1.00 ± 0.10^a	1.67 ± 0.16^a	10.7 ± 0.87^a	0.0 ± 0.00^a	0.33 ± 0.03^a	0.67± 0.06^a
400 mg/kg b. wt. EFAS1	41.7 ± 0.33^a	0.00± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	40.7 ± 2.04^c	0.0 ±0.03^a	0.33 ± 0.03^a	1.67 ± 0.09^a

Values are mean of three replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

PCV – packed cell volume, Eryt. – erythrocytes, Baso – basophils, Neu. – neutrophils, lymp. – lymphocytes, Eos.- eosophils, Mon. – monocytes,Plate.platelet

Table 4.41: Effect of administration of sub-fraction 2 of ethylacetate fraction from aqueous root bark extract of *A. senegalensis* (EFAR2) on selected heamatological parameters in rats

Group	PCV(%)	Eryt×10^6 µL	Baso×10^6 µL	Neu×10^6 µL	Lym×10^6 µL	Eos×10^6 µL	Mon×10^6 µL	plate×10^6 µL
Control	41.0 ± 0.58[a]	0.33 ± 0.03[a]	0.33 ± 0.03a	2.33 ± 0.22[a]	30.0 ± 0.58[a]	0.33 ± 0.03[a]	0.33 ± 0.03[a]	2.33 ± 0.23[a]
100 mg/kg b. wt. EFAR2	42.3 ± 2.03[a]	0.33 ± 0.02[a]	1.33 ± 0.13[a]	0.67 ±0 .06[a]	10.7 ± 0.43[a]	0.33 ± 0.03[a]	0.67 ± 0.06[a]	0.67 ± 0.05[a]
200 mg/kg b. wt. EFAR2	48.0 ± 2.51[a]	1.33 ± 0.11[a]	1.33 ± 0.12[a]	2.00 ± 0.19[a]	23.7 ±0.12[a]	0.67 ± 0.03[a]	0.67 ± 0.05[a]	1.67 ± 0.07[a]
400 mg/kg b. wt. EFAR2	43.3 ± 2.84[a]	0.00 ± 0.00[a]	0.67 ± 0.05[a]	0.00 ± 0.00[a]	19.3 ± 1.08[a]	0.00 ± 0.00[a]	0.67 ± 0.03[a]	2.00 ± 0.06[a]

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

PCV – packed cell volume, Eryt. – erythrocytes, Baso – basophils, Neu. – neutrophils, lymp. – lymphocytes, Eos.- eosophils, Mon. – monocytes,Plate.platelets

Table 4.42 shows heamatological parameters in rats administered sub-fraction 3 of dichloromethane stem bark of *A. senegalensis* (DS3). There was a significant increase ($p < 0.05$) in percentage packed cell volume (PCV) of rats administered 100, 200 and 400 mg/kg b.wt DS3 when compared with the control. There was no significant difference ($p > 0.05$) in monocytes, basophils and platelets at all test doses of DS3 when compared with the control.

Table 4.43 shows some heamatological parameters of rats administered sub-fraction 2 of dichloromethane root bark extract of *A. senegalensis* (DR2). There was a significant increase ($p < 0.05$) in percentage packed cell volume (PCV) of rats administered 100, 200 and 400mg/kg b.wt. DR2 when compared to the control. There was a significant decrease ($p < 0.05$) in erythrocytes of rats administered 100, 200 and 400 mg/kg b.wt. DR2 when compared with the control

Table 4.42: Effect of administration of sub-fraction 3 of dichloromethane stem bark extract of *Annona senegalensis* (DS3) on selected heamatological parametrs in rats

Group	PCV(%)	Eryt×10^6 µL	Baso×10^6 µL	Neu×10^6 µL	Lym×10^6 µL	Eos×10^6 µL	Mon×10^6 µL	plate×10^6 µL
Control	31.0 ± 0.58^a	3.67 ± 0.18^c	0.67 ± 0.03^a	0.33 ± 0.03^a	16.3 ± 0.64^a	1.67 ± 0.15^a	0.67 ± 0.03^a	1.33 ± 0.08^a
100 mg/kg b. wt. DS3	36.0 ± 2.31^b	1.33 ± 0.06^b	0.33 ± 0.03^a	0.33 ± 0.03^a	21.7 ± 2.02^a	0.33 ± 0.03^a	0.00 ± 0.00^a	1.33 ± 0.12^a
200 mg/kg b. wt. DS3	42.7 ± 2.91^c	0.66 ± 0.03^b	1.67 ± 0.03^b	1.00 ± 0.16^a	27.0 ± 1.12^a	1.00 ± 0.09^a	0.00 ± 0.00^a	2.33 ± 0.13^a
400 mg/kg b. wt. DS3	41.7 ± 1.45^c	0.00 ± 0.00^a	0.00 ± 0.00^a	0.33 ± 0.03^a	29.7 ± 1.08	0.67 ± 0.07^a	0.00 ± 0.00^a	1.33 ± 0.13^a

Values are mean of five replicates ± S.E.M. Values with different superscript down the column are significantly different ($p < 0.05$)

PCV – packed cell volume, Eryt. – erythrocytes, Baso – basophils, Neu. – neutrophils, lymp. – lymphocytes, Eos.- eosophils, Mon. – monocytes, Plate. - platelets

Table 4.43: Effect of administration of sub-fraction 2 of dichloromethane root bark extract (DR2) on selected heamatological parameters in rats

Grp (mg/kg)	PCV(%)	Eryt×10^6 µL	Baso×10^6 µL	Neu×10^6 µL	Lym×10^6 µL	Eos×10^6 µL	Mon×10^6 µL	plate×10^6 µL
Control	31.0 ± 0.58^a	3.67 ± 0.18^c	0.67 ± 0.32^a	0.33 ± 0.03^a	16.3 ± 0.64^a	1.67 ± 0.67^c	0.67 ± 0.03^a	1.33 ± 0.08^a
100 mg/kg b. wt. DR2	39.3 ± 1.45^c	0.67 ± 0.07^b	1.33 ± 0.89^a	0.67 ± 0.07^a	19.7 ± 0.55^a	0.67 ± 0.06^b	0.00 ± 0.00^a	1.33 ± 0.07^a
200 mg/kg b. wt. DR2	35.0 ± 1.08^b	0.33 ± 0.03^b	0.33 ± 0.31^a	0.00 ± 0.00^a	26.0 ± 0.98^b	1.00 ± 0.00^b	1.33 ± 0.13^a	1.00 ± 0.10^a
400 mg/kg b. wt. DR2	38.7 ± 1.20^c	0.00 ± 0.00^a	0.33 ± 0.30^a	0.67 ± 0.00^a	11.3 ± 0.23^a	0.00 ± 0.00^a	0.00 ± 0.00^a	1.67 ± 0.07^a

Values are mean of four replicates ± S.E.M. Values with different superscript down the column are significantly different ($p<0.05$)

CHAPTER FIVE

DISCUSSIONS

5.1 Antidiarrhoeal activity of hexane, dichloromethane and aqueous *Annona senegalensis* root and stem bark extracts

The significant reduction in the number of wet faeces by the dichloromethane root (DR) and aqueous stem (AS) extract indicates the anti-secretory potential of these extracts. Generally, reduction in wet stool suggests the ability of the extract to inhibit hyper-secretion of electrolytes and fluid (Usman *et al.*, 2000). The antisecretory activity may be as a result of inhibition of prostaglandin synthesis (Paredes *et al.*, 2016). Prostaglandins (PGE_2) stimulates secretion of chloride ion (Pierce *et al*., 1991). It also suppresses the absorption of sodium by interfering with Na^+ - K^+ ATPase activity, changes electrolyte transport and induces an increase in the permeability of the mucosal cells (i.e stimulating peristaltic activity) to result in hyper secretory response and diarrhoea (Zavala *et al*., 1998).

The significant increase in the percentage inhibition of gastrointestinal tract (GIT) transit as well as the decrease in the weight and volume of intestinal fluid by dichloromethane stem bark (DS) and aqueous root bark (AR) extracts indicates the antimotility and antienteropooling activities of this extract. A reduction in gastrointestinal motility increases the time of stay of gastrointestinal contents in the intestine, thus promoting intestinal water and electrolyte absorption (Mekonnen *et al.*, 2018). The antidiarrhoeal potential of these extracts may therefore be attributed to its antimotility activity. These suggest that the extract contains substances that are able to inhibit GIT motility probably through anticholinergic activity on intestinal mucosa. Anticholinergics prevents diarrhoea by blocking cholinergic stimulation of gastrointestinal tracts (Teferi *et al.*, 2019). Cholinergic stimulation of gastrointestinal mucosa results in increased gastrointestinal motility.

This is in agreement with the report of Suleiman *et al.* (2008) and Teferi *et al.* (2019) that the methanol stem bark extract of *A. Senegalensis* exhibited antimotility activity as observed in this study.

Antidiarrhoeal agents act by either decreasing secretion and/or reducing the propulsive movement of the gastrointestinal smooth muscles (Tadesse *et al.,* 2017). In this study, DS and AR showed better antimotility and antienteropooling activity than AS and DR suggesting that AS and DR exerted its antidiarrhoeal action through another mechanism other than by antimotility and antienterpooling activity. The significant reduction in intraluminal fluid by DS and AR observed in this study may also be as a result of inhibition of prostaglandin biosynthesis. Inhibitors of prostaglandin synthesis impairs diarrhoea by decreasing secretion of fluid into the lumen (Parades *et al.,* 2016). Dichloromethane root (DR) and aqueous root barks (AR) extracts may therefore contain substances that inhibit prostaglandin synthesis.

5.2 Bioactivity guided fraction of bioactive extracts of *Annona senegalensis* root and stem barks

The significant reduction in intraluminal fluid accumulation by dichloromethane fraction of (DFAR) and the significant increase in inhibition of gastrointestinal tract by the ethylacetate fraction of aqueous root bark extracts (EFAR) indicates aqueous root bark extract (AR) contains antimotility agent present in the ethylacetate fraction of aqueous root bark (EFAR) and an antienteropooling agent present in the dichloromethane fraction of aqueous root bark (DFAR). This therefore infers that intraluminal fluid accumulation of AR is not due to its antimotility activity, but due to the presence of both antimotility compound and antienteropooling compounds.

The significant reduction in weight and volume of intestinal fluid of rats administered sub-fraction 3 of dichloromethane stem extract (DS3) and sub-fraction 1 of dichloromethane fraction of

aqueous root bark extract (DFAR) indicates that antienteropooling agents are present in these two sub-fractions (DS3 and DFAR1). The significant reduction in the number of wet stools by ethylacetate fraction of aqueous root bark (EFAS1) and sub-fraction 2 of dichlromethane root bark extract (DR2) indicates that these sub-fractions; EFAS1 and DR2 contains substances that may have antisecretory activity.

5.3 Mechanism of action of antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

5.3.1 Antioxidant mechanism

The low inhibitory concentration for 2,2-diphenyl-1- picrylhydrazyl (DPPH IC_{50}) of EFAS 1, highest H_2O_2 scavenging activity of EFAS 1 and DR2 and the highest ferric reducing antioxidant power (FRAP) activity by DS3 observed in this study show that EFAS1, DR2 and DS3 possess high antioxidant activity and can scavenge for free radicals through various mechanisms. Scavenging of H_2O_2 may be attributed to the presence of phenolic groups in EFAS1 and DR2 that could donate electrons to hydrogen peroxide to neutralize it to water. The FRAP mechanism is through electron donation which terminates the oxidation chain reaction by reducing the oxidized intermediates into the stable form while the DPPH radical scavenging activity indicates the ability of the substance to transfer electrons or donate electrons (Brand-Williams e*t al*., 1995; Pulido *et al.,* 2000; Tachakiffirungrod *et al.,* 2005).

Reactive oxygen species has been implicated in diarrhoea due to its ability to induce oxidative damage of lipids. Oxidative damage of lipids contributes to diarrhoea by disruption of ion transporters due to loss of membrane integrity (Tandon *et al.,* 2004). The ability of these sub-fractions to scavenge for reactive oxygen species imply that they could be used in counteracting the deleterious efforts of reactive oxygen species produced *in vivo*. Thus, the antioxidant activity of EFAS1, DS3 and DR2 may contribute to the antidiarrhoeal potential of these sub-fractions.

Umuokoro and Ashorobi (2005) ascribed the antidiarrhoeal activity of ascorbic acid and ∝-tocopherol in castor oil - induced diarrhoeal rats to their antioxidant property. Onoja *et al.* (2018) also attributed the antidiarrhoeal effects of *B. pinnatum* to antioxidant acitivty.

Castor oil-induced diarrhoea causes a depletion of catalase and glutathione peroxidase (GPx) activities which results in oxidative stress (Micheal and Navdeep, 2014: Rtibi *et al.,* 2017). The significant increase in catalase and glutathione perioxidase activities in castor oil-induced diarrhoeal rats treated with EFAR2, DR2 and DS3 indicates the ability of these sub-fractions to attenuate intestinal hypersecretion caused by oxidative stress. Oxidative stress has been reported to be involved in intestinal hypersecretion (Rao *et al.* 2008). Increased catalase and glutathione peroxidase (GPx) activities observed in this study therefore, provides a first line defense system against intestinal hypersecretion caused by oxidative stress. Jabri *et al*. (2016) also reported the increase in catalase and glutathione peroxidase (GPx) activities of antidiarrhoeal plants.

Superoxide and hydroxyl radicals are the major reactive oxygen species generated in the intestine (Rao *et al.,* 2008). The significant reduction in superoxide dismutase activity in rats administered DS3, DR2, EFAS1, EFAR2 and DFAR1 may be attributed to decrease generation of superoxide radicals. Elevated level of superoxide radical increases the cellular concentration of superoxide dismutase. Increased superoxide dismutase (SOD) activity has been reported to correlate with an increase in castor oil induced fluid accumulation (Shoba and Thomas, 2001). Therefore, the reduction in superoxide dismutase (SOD) activity by the individual administration of DR2, DS3, DFAR1, EFAR2 to rats may result to a decrease in castor oil-induced intraluminal fluid accumulation; suggesting an improvement over diarrhoeal condition by these sub-fractions. The reduction in superoxide dismutase by these sub-fractions may therefore, contribute to the

antidiarrhoeal potential of these sub-fractions. The ability to decrease intraluminal fluid by DR2 and EFAS1 may contribute to the reduction in the number of wet faeces dropping exhibited by DR2 and EFAS1 (Table 4.10 and 4.12). Rao *et al.* (2008) also reported a significant reduction in superoxide dismutase activity by *Gunammon tamala* extract in castor-oil-induced diarrhoeal rats.

Thus, the sub-fractions (EFAS1 and DS3) acts as antioxidant to scavenge for free radicals which will prevent the deleterious effects of free radicals such as acting as secretagogues to induce secretory diarrhea and/or inducing oxidative damage of lipids.

5.3.2 Intestinal barrier function mechanism

Castor oil increases the formation of malondialdehyde in the gastrointestinal mucosa due to oxidative stress indicating an increase in lipid peroxidation (Sebai *et al.* 2014; Rtibi *et al.,* 2018). The reduction in the concentration of malondialdehyde in the small intestine of castor oil - induced diarrhoeal rats by the administration of EFAS1; and EFAR2 may be ascribed to its ability to inhibit lipid peroxidation. This effect may be attributed to the antioxidant activity of EFAS1 and EFAR2 possibly by terminating free radical chain reaction. Ajiboye *et al*. (2010) also ascribed the scavenging activity of the aqueous leaf extract of *A. senegalensis* on DPPH, H_2O_2, superoxide ion and ABTS, as well as ferric reducing power to interference with lipid peroxidation. An imbalance between the production of reactive oxygen species and antioxidant defense produces oxidative stress, which amplifies tissue damage by releasing prooxidative forms of reactive iron that are able to drive fenton chemistry and lipid peroxidation (Gutteridge, 1995).

The ability of EFAS1 to inhibit lipid peroxidation may be ascribed to its ability to scavenge effectively for H_2O_2 (Table 4.15). Hydrogen peroxide (H_2O_2) generation accompanies intestinal hyersecretion in the mucosal intestine (Rtibi *et al.,* 2018). Effective H_2O_2 scavenging activity will

protect against oxidative damage of lipids. The significant increase in GPx activity by EFAR2 may be responsible for its ability to inhibit lipid peroxidation. Glutathione peroxidase (GPx) prevents lipid peroxidation by converting lipid hydroperoxides to a more stable compound. Oxidative damage of lipids in the intestinal mucosa results in loss of membrane integrity and intestinal barrier dysfunction and eventually, disruption in ion transport (particularly affecting potassium efflux and sodium influx), production of arachidonic acid and eventually contributes to diarrhoea (Tandon *et al.,* 2004). Inhibition of lipid peroxidation by EFAS1and EFAR2 prevents intestinal barrier dysfunction and may contribute to the antidiarrhoeal potential of these Sub-fractions. Other antidiarrhoeal plants such as myrithe berries seed extract have been reported to protect against castor oil induced lipid peroxidation and have been reported to contribute to its antidiarrhoeal potential (Adzu *et al*., 2005)

Inhibition of lipid peroxidation by EFAS1 and EFAR2 may protect the intestinal barrier and maintains the integrity of the intestinal cell which will prevent the disruption of its function.

5.3.3 Antisecretory/proabsorptive mechanism of action

The significant reduction in intestinal chloride ion concentration suggests that sub-fraction 1 of ethylacetate fraction of aqueous root (EFAR1) inhibits one or more of the chloride channels. Activation of chloride channels as it occurs in most secretory diarrhoea causes the massive secretion of chloride ions into the intestinal lumen and consequently create an electrochemical gradient and an osmotic force to drive the secretion of sodium and water (Barrett and Keely, 2000). Therefore, suppression of intestinal chloride secretion would reduce the intestinal fluid and electrolyte accumulation. Suppression of intestinal chloride may occur via the inhibition of any of its transport proteins (Muanprasat and Chatsudthipong, 2013; Thiagarajah *et al.,* 2014). EFAR2

therefore contains antisecretory substances that are capable of inhibiting any of the chloride channels

Iinhibition of Na^+ absorption may occur in response to excessive secretion of Cl^- and in other types of diarrhoea (Singh *et al.,* 2014). The significant reduction in Na^+ concentration by sub-fraction 3 of dichloromethane stem bark extract (DS3) suggests a pro-absorptive activity of this sub-fraction (DS3). This pro-absorptive activity is probably by stimulation of any of the sodium channels responsible for the absorption of sodium from the lumen. Sub-fraction 3 of dichloromethane stem bark extract (DS3) may contain substances that are able to stimulate any of the sodium channels [i.e. either sodium hydrogen exchanger (NHE) or electrogenic sodium channels (ENaC)]. The significant reduction in Na^+ concentration in this study can also be attributed to the antimotility activity of DS3 (Table 4.6). Decrease in intestinal motility (i.e. inhibition of gastrointestinal motility) increases the stay of substances in the intestine and allows for better water, electrolyte and nutrient reabsorption (Yadav and Tangpu, 2007). This may be the reason for the reduction in Na^+ concentration.

The sub-fraction (EFAR2) by inhibiting any of the chloride channels (CFTR or CaCC channels) reduces the concentration of intestianal lumen (Barrette and Keely, 2000).. This will restore the electrochemical gradient required for normal reabsorption of other electrolytes.

5.3.4. Stimulation of $Na^+ – K^+$ ATPase activity

The significant increase in Na^+ - K^+ ATPase activity by sub-fraction 1 of ethylacetate fraction of aqueous stem bark extract (EFAS1) indicates that this sub-fraction may contain substances that are capable of stimulating the Na^+ - K^+ ATPase activity. The sodium pump plays an important role in the regulation of electrolyte and water fluxes in the intestinal mucosa (Herfath *et al*., 2003). Na^+ -

K^+ ATPase activity has been reported to decrease in all types of diarrhoea (Tripp *et al.,* 1980; Rachmilewitz *et al*., 1984; Ejderham *et al.,* 1989). Inhibition of this enzyme contributes to intense fluid and electrolyte accumulation which results to diarrhoea. Thus, stimulation of the Na^+ - K^+ ATPase in diarrhoea may alter the accumulation of fluids in the intestine and will consequently inhibit the number of wet stool. Therefore, the antidiarrhoeal activity of this sub-fraction may be attributed to its stimulation of Na^+ - K^+ ATPase.

Stimulation of Na^+- K^+ ATPase by EFAS1 restores the electrochemical gradient which is the driving force for all active electrolyte transport.

5.3.5. Anti-inflammatory mechanism

The significant decrease in the activity of cyclooxygenase (COX) II in rats administered EFAS1, DFAR1 and EFAR2 indicates that they are inhibitors of prostaglandin synthesis. Inhibition of COX II leads to the suppression of prostaglandin synthesis. Prostaglandin, a product of arachidonic acid formed through cyclooxygenase pathways, is known to cause inflammation (Singh, 1999). Inflammation disrupts the epithetical barrier, cause structural changes to the enteric nervous system (ENS) and decrease the total absorptive surface area, resulting into diarrhoea (Schenk and Mueller, 2008; Stanzel` *et al.,* 2008 and Cotton *et al.,* 2011). Prostaglandin, itself may contribute to diarrhea by malabsorption of glucose and sodium, and stimulating secretion of chloride (Robinson *et al*., 2001). Inhibition of prostaglandin synthesis, will therefore suppress diarrhoea. The anti-inflammatory compounds present in EFAR2, EFAS1 and DFAR1 may therefore become candidate compounds for antidiarrhoeal management.

Flavonoids and sesquiterpenoids are known inhibitors of cyclooxygenase (COX) II (Fernandes *et al.,* 2007). Kaur-16-ene isolated from *Annona squamosa* has been shown to have anti-inflammatory property (Chavan *et al.,* 2011). The anti-inflammatory activity of these fraction may

be due to the presence of these phytochemicals in these sub-fractions. The total ethereal leaf extracts of *A. senegalensis* have also been reported to have anti-inflammatory property (Sene *et al.,* 2017).

The sub-fractions DFAR1, EFAS1 and EFAR2 act as anti-inflammatory agent to inhibit prostaglandin synthesis by inhibiting cyclooxygenase II activity. This reduces excessive secretion of electrolytes and water caused by prostaglandins

5.4 Identified antidiarrhoeal compounds

5.4.1 Identifiied antidiarrhoeal compounds in sub-fraction 3 of dichloromethane stem bark extract (DS3) of *Annona senegalensis*

The main compound in fraction DS3, androstan-16-ene-3-one (Table 4.17) is an aldosterone derivative. Aldosterone regulates electrogenic Na^+ absorbtion in the distal colon via the electrogenic sodium channel (ENaC) (Kunzelmann and Mall, 2002). ENaC is the rate limiting factor for electrogenic Na^+ absorption in descending colon (Singh *et al*., 2014). It is downregulated in inflammatory bowel disease (IBD) diarrhoea (Magalhaes *et al*., 2016). Also, activation of cystic fibrosis transmembrane conductance regulator (CFTR) that occurs in secretory diarrhoea inhibits Na^+ absorbtion via ENaC (Kunzelmann and Mall, 2002). The reabsorbtion of Na^+ via the ENaC channel plays an important role in re-absorbtion of water (Halevey *et al*., 1986; Fromm *et al*., 1993). The efficient re-absorbtion of water by DS3 treated castor oil-induced diarrhoeal rats may be due to the presence of androstan-16-ene-3-one. Thus, the aldosterone derivatives present in DS 3 is a good anti-diarrhoeal drug candidate via its antienteropooling activity. Also, Singh *et al.* (2014) reported that drug stimulation of ENaC channel will be a good target for diarrhoea treatment because of its distal location in the GI tract in the intestine in which highly efficient Na^+ absorbtion occurs (Singh *et al*., 2014). Also, the antispasmodic activity of kaurane diterpenes has been reported (Zamilpa *et al*., 2002). Zamilpa *et al*. (2002) reported the spasmolytic activity of

Kaur-16-en-19-oic acid. Kauran-19-oic acid present in DS3 may be responsible for its spasmolytic activity. This antimotility agent, kauran-19-oic acid, slows down the gastrointestinal tract motility. This provides sufficient time for reabsorbtion of water, electolytes and nutrients (Yadav and Tangpu, 2007) and may have contributed to the antienteropooling activity of this fraction.

The 4.12% 9 - octadecanoic acid, methyl ester also present in DS3 sub-fraction may be responsible for its antioxidant property. This fatty acid is an efficient antioxidant (Syeda *et al*., 2011).

5.4.2 Identified antidiarrhoeal compounds in sub-fraction 1 of dichloromethane fraction from aqueous root bark extract of *Annona senegalensis* (DFAR1)

The main compound in DFAR 1, androstan -3-one is also an aldosterone derivative. Aldosterone regulates electrogenic Na^+ absorbtion in the distal colon via the electrogenic sodium channel (ENaC) (Kunzelmann and Mall, 2002). The reabsorbtion of Na^+ via the ENaC channel plays an important role in re-absorbtion of water (Halevey *et al*., 1986; Fromm *et al*., 1993). The efficient re-absorbtion of water by DFAR1 and its ability to reduce the volume of the intestinal fuid (Table 4.8) may be due to the presence of androstan-3-one. Thus, the aldosterone derivatives present in DFAR1 maybe a good anti-diarrhoeal drug candidate with antienteropooling mechanistic property.

Though DFAR1 does not contain hexadecanoic acid, it still inhibited COX II. It therefore follows that the sub-fraction may contain other substances for its COX II inhibitory activity. Triterpenes have been reported to have COX II inhibitory activity (Lee *et al*., 2006). Also, several studies have reported COX II inhibitory activity of polyunsaturated fatty acid (Kaur *et al.*, 2014).

5.4.3 Identified antidiarrhoeal compounds in sub-fraction 1 of ethylacetate fraction of aqueous *Annona senegalensis* stem bark extract (EFAS1)

Catechol, one of the major constituents of EFAS1 fraction is a component of catecholamine. Catechol is a flavonoid. Flavonoids inhibit intestinal motility and hydroelectrolytic secretions (Di-Carlo *et al*., 1993). Catecholamine, an organic compound that has a catechol ring and amine

(Fitzgerald, 2011) stimulates electrogenic NaCl absorption and decrease electrogenic Cl^- secretion by interaction with α-adrenoreceptors on enterocyte (Chang *et al*., 1982; Dharmsathaphorn *et al*., 1984). It acts at the α- adrenergic receptor coupled with the G-proteins to antagonize cAMP production. Donowitz *et al*. (1982) reported that the catechol moiety is important for maximal agonist activity at the α- adrenoreceptors. Therefore, the presence of catechol in EFAS1 may be responsible for the stool inhibition observed in EFAS1 treated castor oil-induced diarrhoeal rats as a result of cAMP production. α-adrenergic agonists also alter the motility of the intestine in a manner similar to that of opiates (Schiller 1994). It also exerts antispasmodic effects via the activation of μ- opiod receptors in enteric neurons (Mehmood and Gilani, 2010). The presence of catechol in this sub-fraction may be responsible for the stool inhibitory activity of this sub-fraction. The antimolity and antisecretory activity of catechol contributes to the overall reduction of wet stool.

Catechol are effective metal chelators. This is because metal ions that prefer octahedral geometry such as Fe^{2+} and Fe^{3+} can co-ordinate up to 3 catecholate (Perron and Brumaghim, 2009). The ability to co-ordinate iron gives it, its antioxidant property because iron is a primary cause of ROS generation. Thus, the ability to prevent iron generated ROS prevents oxidative stress (Perron and Brumaghim, 2009). Also, a catechol type flavonoid – catechin, has been reported to have antioxidant activity. It is able to upregulate antioxidant enzymes and scavenge ROS like superoxide (O^-), hydroxyl (OH^-) and peroxyl radicals (Yoshida *et al*., 2008, Boots *et al*., 2016). Thus, Catechol is able to inhibit lipid peroxide by scavenging for reactive oxygen species (ROS) (Yoshida *et al*., 2008). This antioxidant activity of EFAS1 sub-fraction (Table 4.15), and its abiity to prevent lipid peroxidation (Figure 4.1), may also contribute to its antidiarrhoeal property.

EFAS1 also contains other phytochemicals which might act synergistically to exert their stool inhibitory property. It contains 1.34% n-hexadecanoic acid and 19% kaurane terpenoids (Kaur-16-ene). n-hexadecanoic acid is a competitive inhibitor of phospholipase A_2 (Vasudwa *et al*., 2012). Phospholipase A_2 is responsible for ester bond hydrolysis of membrane phospholipids to release fatty acid and are the initiating steps of inflammation (Vasudwa *et al*., 2012). Inhibition of phospholipase is one of the ways to control inflammation (Vasudewa *et al*., 2012). Also, inhibition of phospholipase A_2 (PLA_2) could deplete the downstream pro-inflammatory metabolites of arachidonic acid and thus, inhibit cycloygenase (COX) II (Dharmappa *et al*., 2009). Also, Hema *et al*. (2011) reported a direct inhibition of COX II enzyme by hexadecanoic acid which acts as a selective anti-inflammatory agent. Therefore, the presence of n-hexadecanoic acid suggests that EFAS1 may have anti-inflammatory property. Anti-inflammatory activity of EFAS1 sub-fraction may also be contributory to the antidiarrhoeal property of this sub-fraction.

Kaurane terpenoids have antispasmolytic activity (Zamilpa *et al*., 2002). Reduction of intestinal motility provides better re-absorbtion of nutrients, electrolyte and water. These actions in combination will provide an overall reduction in diarrhoea which may be manifested in the reduction of wet stool.

The major constituent of EFAS 1, ethyl 5,8,11,14,17, icosapentanoate (Table 4.19**)** is an unsaturated fatty acid ester and may be responsible for the activation of Na^+- K^+ ATPase activity. Silva *et al*. (2016) reported that unsaturated fatty acid is responsible for the activation of Na^+- K^+ ATPase activity. The increased activity might be as a result of these fatty acids inserting themselves in the plasmatic membrane which in turn leads to increase membrane fluidity and subsequent increase in the activity of the Na^+- K^+ ATPase (Zavodnik *et al*., 1996; Ibarguren *et al*., 2014). Membrane fluidity is an important factor in cellular function because the surrounding lipids

can directly modulate the proper function of several proteins (Ibarguen *et al*., 2014). Zavodnik *et al*. (1996) reported that fatty acid methyl ester can cause non-specific interaction with the lipid content of plasma membrane, leading to a disturbance of optimum complementarity between proteins and surrounding lipids inducing changes in the morphology of the membrane and its fluidity, finally leading to changes in the activity of the sodium pump.

Several studies have also shown that the surface tension of the membrane can also be modulated by insertions of fatty acid which alters lipid composition of the bilayer (Cordomi *et al*., 2010; Lopez *et al*., 2012). Silva *et al*. (2016) suggested that specific point modifications on membrane environment might be enough to cause modification of the Na^+- K^+ ATPase. Herfarth *et al*. (2003) reported that compounds that stimulate Na^+- K^+ ATPase activity results in improvement of diarrhoea. This improvement in diarrhoea was seen in the reduction of wet stool by EFAS1 (Table 4.10).

5.4.5 Identified antidiarrhoeal compounds in sub-fractions 2 of dichloromethane root bark extract (DR2) of *Annona senegalensis*

The several phytochemicals present in sub-fraction 2 of dichloromethane root bark extract (DR2) of *Annona senegalensis* may ac together to prevent wet faeces which is characteristic of diarrhoea. The observed inhibition of intestinal motility and secretion by DR2 may be as a result of ssesquiterpene (caparratriene and carophyllene oxide) which has been reported to decrease prostaglandin synthesis (Milanova *et al*., 1995; Nikiema *et al*., 2001). It also contains 1.1% hexadecanoic acid methyl ester which has been reported to be an anti- inflammatory agent (Aparma *et al*., 2012); Kaurane terpenoids - an antispasmotic agent (Zamilpa *et al*., 2002) and androstane derivatives (D-Homoandrostane) (Table 4.20). The significant reduction in wet stool exhibited by DR2 (Table 4.12) may be as a result of the synergistic interaction of these compounds.

5.4.5 Identified antidiarrhoeal compounds in sub-fraction 2 of ethylacetate fraction from aqueous *Annona senegalensis* root bark extract (EFAR2)

Yadav and Tangpu (2007) reported that decrease in the motility of gut muscle (i.e inhibition of gastrointestinal motility) increases the stay of substances in the intestine and allows better water, electrolyte and nutrient reabsorbtion., thus improving diarrhoea. According to Thiagarajah *et al*. (2015), the putative mechanism of action of antimotility drug is increased Na^+ and fluid absorption. The reduced gastrointestinal motility observed in EFAR2 treated castor oil-induced diarrhoea may be due to the presence of unidentified antimotility compounds.

The significant reduction in Cl^- concentration in EFAR 2 treated castor oil –induced diarrhoeal rats might be due to the presence of three compounds; 1-piperidynyloxy; 5,5 dimethyl thiazoline 2-resorcino and qiunazoline-4-1(H)one (Table 4.20). Pongkorpsakol *et al*. (2015) reported that piperidine inhibited cAMP- induced Cl^- secretion via inhibition of cystic fibrosis transmembrane regulator (CFTR), cAMP activated basolateral K^+ channels and Ca-chloride channels (CaCC). They further explained that piperidine is a novel class of drug for the treatment of diarrhoeal disease caused by the intestinal hypersecretion of chloride ion.

Das *et al*. (2018) also reported that diarrhoea might be effectively targeted by small molecules that act by specifically acting on the transporters implicated in the disease. Studies have shown that small molecule modulators of CFTR function could be useful in secretory diarrhoea (Verkman *et al*., 2006). Thiazolidone, pyrimidoquinoxalindione (PPQ)/Benzopyrimido-pyralooxazinedione and glycine hydrides have been identified as CFTR inhibitors (Thiagarajah *et al*., 2004). Thus, the reduction of Cl^- concentration in EFAR2 treated castor oil-induced diarrhoeal rats might be due to inhibition of CFTR channels by any of these compounds present in the sub-fraction or by the synergistic interaction of the three compounds. Inhibition of chloride channels (CFTR and CaCC)

is known to be an attractive strategy for antisecretory drug therapy (Thiagarajah *et al*., 2014). This sub-fraction may therefore be an important antisecretory drug candidate for the treatment of diarrhoea.

5.5 Toxicity study

5.5.1 Influence of administration of antidiarrhoeal sub-fractions of *Annona senegalensis* root and stem barks on feed intake and change in weight of rats

Body weight gain is an important indicator of gross toxicity. The significant increase in feed intake with no significant difference in weight gain as observed in DFAR1 treated rats indicates that the sub-fraction contains antinutrient that interfered with nutrient reabsorption. The significant reduction in body weight with an increase in feed intake exhibited by DR2 treated rats may be suggestive that the sub-fractions contain substances that may have the potential to interfere with nutrient absorption or produce drastic tissue destruction. This may be due to the presence of antinutrients. Drastic toxicity or interference with absorption of nutrients usually reflects in body weight reduction (Nariya *et* al., 2011). Antinutrients such as tannins have been reported to be responsible for decrease in feed intake, feed efficiency, protein indigestibility and growth rate (Chung *et al.,* 1998). They combine with either nutrients in foods such as proteins and iron or proteins of the organism such as digestive enzymes (Kumar and Singh, 1984; Butler, 1992). Inhibition of the absorption of nutrients and the decrease in the activity of digestive enzymes results in reduction of nutritional efficiency (Iboudo *et al.,* 2019).

The significant increase in organ to body weight ratio of kidney, heart and stomach of rats administered DFAR1 amd the liver and stomach of rats administered EFAS1, DR2 and DS3 suggest toxic exposure to they organs. Alteration in body weight and or organ weight has been linked to toxic events arising from exposure to a toxicant (Orisakwe *et al*., 2004; Adeyemi and

Faniyan, 2014). Adverse interaction of plant extract with major organs can cause inflammation and cellular constriction which usually reflects in the organ to body ratio (Devaki *et* al., 2012).

5.5.2 Liver function of rats administered antidiarrheoal sub-fractions obtained from *Annona senegalensis* root and stem barks

Alanine aminotransferase (ALT) is an enzyme predominantly present in the liver. Elevated levels in serum is an indicator of liver damage. Aspartate aminotransferase (AST) is an enzyme present in the cell of the liver, skeletal muscles, kidney, heart and pancrease. Alanine aminotransferase is the primary pathological marker of hepatic dysfunction while aspartate transaminase is used as supplementary marker to substantiate the degree of liver damage (Ozer *et al*., 2008). The significant increase in the level of serum alanine aminotransferase (ALT) of rats administered EFAS1, EFAR2, DFAR1 and DS3 indicates liver damage. Alanine aminotransferase (ALT) is a cytosolic enzyme predominantly present in the liver. Damage to the plasma membrane will lead to leakage of cytosolic contents including the cytoslic enzymes (ALT) into the serum (Ajiboye *et al.,* 2010). Therefore, elevated levels of ALT in the serum is an indicator of hepatocellular injury. The increase in serum ALTof rast administered EFAS1, EFAR2, DFAR1 and DS3 in this study might be as a result of assault of the toxic constituents of these sub-fractions on the plasma membrane of the liver leading to the leakage of these enzymes from the cytosol into the blood.

Albumin is synthesized in the liver. Albumin levels are used to evaluate the synthetic capacity of the liver. With progressive liver disease, serum albumin levels fall reflecting decrease synthesis (Limde and Hyde, 2003). The non significant difference in serum albumin level in rats administered DFAR1, DS3 and DR2 observed in this study suggest that the sub-fractions did not affect the synthetic function of the liver. Bilirubin is a useful index of the excretory function of the liver (Arise *et al.,* 2018). The significant decrease in the serum levels of conjugated and

unconjugated bilirubin of rats administered DFAR1, EFAS1, EFAR2, DS3 and DR2 suggest that administration of these sub-fractions did not affect the excretory function of the liver. The non significant difference in ALT and AST between the normal rats and DR2 treated rats observed in this study indicates that DR2 is not toxic to the liver. In contrast, Okoye *et al.* (2012) reported that *A. senegalensis* root was not hepatotoxic at low concentrations (50 and 100 mg/kg b.wt.) but was hepatotoxic at 400 mg/kg b.wt. Ilboudo *et al*. (2019) also reported that aqueous *A. senegalensis* root bark at doses less than 300 mg/kg b.wt. was not hepatotoxic. All of the sub-fractions except DR2 from the root of *A. senegalensis* was hepatotoxic. This suggests that the non hepatotoxic constituent of the root has been fractionated into DR2.

5.5.3 Kidney function of rats administered antidiarrhoeal fractions obtained from *Annona senegalensis* root and stem barks

The kidney is responsible for removal of metabolic wastes such as urea, ion, and creatinine. The concentration of these metabolites are used to assess the normal functioning of the nephrons. The significant decrease in serum urea concentration of rats administered EFAR1, EFAS1, DFAR2 and DS3 observed in this study, suggest kidney damage. A reduction in urea may be due to high rate of urea excretion (i.e. inhibition of urea reabsorption in the renal tubule of the nephron (Mehrdrad, 2007). Urea is freely filtered into the glomerulus but reabsorbed by urea pumps in the collecting ducts of the nephron (Schrier, 2008). The decreased serum urea concentration in rats administered EFAR1, EFAS1 and DFAR2 observed in this study, therefore suggests that these sub-fractions contain substance capable of inhibiting urea pumps with a resultant decrease in reabsorption of urea and a decrease in urea concentration (Schrier, 2008).

A major role of the kidney is the maintenance of electrolytes balance (Dhondup and Qian, 2017). Chronic renal failure result in sodium retention in the blood (Ray *et al.*, 2015). Increased serum

sodium concentration in rats administered DFAR1, EFAS2, EFAR1, DR2, DS3 treated rats observed in this study indicates chronic renal failure. These sub-fractions may contain substances capable of stimulating any of the sodium pumps responsible for active sodium transportation out of the nephron. Aldosterone present in DFAR1and DS3 sub-fractions has been reported to cause an increase in the tubular reabsorption of Na^+ by stimulating the electrogenic sodium channel (ENaC) leading to decrease in the excretion of sodium in urine and a concomitant increase in the blood (Panda, 1989). Many antidiarrhoeal plants have been reported to have nephrotoxic activity (; Afagnigni *et al.,* 2017; Mba *et al.,* 2017) but none of these studies have been attributed to the toxicity of the antidiarrhoeal constituent of the plant.

5.5.4 Antioxidant enzyme in rats administered antidiarrhoeal sub-fractions obtained from *A. senegalensis* root and stem barks

The increase in catalase and glutathione peroxidase (GPx) activities in rats administered DFAR1, EFAS1, EFAR2 and DS3 observed in this study might have been induced in response to increased generation of reactive oxygen species particularly hydrogen peroxide (H_2O_2). Catalase and glutathione peroxidase catalyses the conversion of H_2O_2 to O_2 and H_2O (Day, 2009). Reactive oxygen species are usually produced during biotransformation of xenobiotic in the liver (Londis and Yu, 2000s). Usually, the antioxidant defense systems including the antioxidant enzymes are induced in response to increased reactive oxygen species production (Livingstone, 2001). Superoxide dismutase (SOD) catalyzes the formation of hydrogen peroxide from superoxide radicals. Its levels are directly related to catalase activity (Gad, 2011). Thus, decreased superoxide dismutase activity should lead to a decrease in catalase activity. This trend was not observed in this study. Decreased activity of superoxide dismutase did not lead to decrease in catalase activity of rats administered DFAR1, EFAR2, EFAS1 and DS3. The decreased activity of serum superoxide

dismutase may be attributed to inactivation by hydrogen peroxide. Hydrogen peroxide can inactivate superoxide dismutase in a feedback mechanism (Hink *et al.,* 2002). It is therefore possible that overproduction of hydrogen peroxide inactivated superoxide dismutase. The activities of the antioxidant enzymes suggest that the fractions induced generation of reactive oxygen species.

5.5.5 Lipid peroxidation in rats administered antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

Malondialdehyde, an oxidative damage product of lipi peroxidation, is the main marker in lipid peroxidation (Zeb and Ulah, 2016). The significant increase in serum malondialdehyde concentration of rats administered DFAR1, EFAS1, EFAR2, DS3 and DR2 suggests that the sub-fractions initiated lipid peroxidation as a result of oxidative stress. Oxidative stress due to toxic effects is usually indicated by an increase in malondialdehyde (Salui and Bawa-Allah, 2012). Oxidative stress causes reactive oxygen species to react with unsaturated lipids in the membranes of cells and organelles thus initiating lipid peroxidation (Arise and Malomo, 2012).

5.5.6 Heamatological parameters of rats administered antidiarrhoeal sub-fractions obtained from *Annona senegalensis* root and stem barks

Heamatological parameters are usually assessed to determine the well being of an animal (Ajayi and Raji, 2012). Heamatological parameters are good indicators of the physiological status of animals (Khan and Zafr, 2005). The significant increase in percentage packed cell volume (PCV) in rats administered DFAR1, DS3, and DR2 indicated that the sub-fractions contain substances that stimulated red blood cell production. Differential white blood cell counts are indicators of an organism's ability to defend the body against invasion from foreign bodies (Jorum *et al*., 2016). The significant increase in lymphocyte count by DFAR1 observed in this study reflects the leukopoietic and immunomodulatory effects of DFAR1. Lymphocytes produce, transport or

distribute antibodies in immune response (Muhammad and Sani, 2018). Elevated lymphocyte count in organisms indicate high degree of resistance to invasion from foreign body (Soetan *et al*., 2010). The increase in lymphocyte count suggests that DFAR1 confer high resistance to assault caused by toxic constituents of the sub-fraction. The leukopoietic and immunomodulatory effects of DFAR1 may be due to the presence of some phytochemicals. Terpenoids, steroids, phenolic compounds and alkaloids have been reported to have leukopoietic and immunostimulatory properties (Wagner, 1999). Androst-16-ene-3-one (steroids), atis-16-ene and or prasterone (anabolic steroids) present in DS3; androstan-3-one-17 hydroxy -2- methyl (steroids) in DFAR1; and kauran-18-al (diterpene alkaloid), kaur-16-ene (diterpene alkaloids) and andrsostan-17-ol,2,3,-epoxy 2α (steroid) present in DR2 may be responsible for the immunostimulatory and leukopoietic property of the sub-fractions.

SUMMARY OF RESEARCH FINDINGS

The research findings are that:

i. aqueous stem (AS) and dichloromethane root (DR) extracts at 100 mg/kg body weight (bw) significantly decreased the number of wet feaces while GIT motility inhibition and antienteropooling activity were significantly increased by aqueous root (AR) and dichloromethane stem (DS) extracts;

ii. inhibition of defecation was highest (85.25%) in the ethylacetate fraction of AS (EFAS), while GIT motility inhibition and antienteropooling activity was highest in the ethylacetate fraction (EFAR) and dichloromethane fraction (DFAR) of AR respectively;

iii. sub-fraction 1 of EFAS (EFAS1) and sub-fractions 2 of DR (DR2) at 25 mg/kg b.wt. exhibited the highest inhibition of defecation;

iv. anti-motility activity was highest in sub-fraction 2 of EFAR (EFAR2) at 25 mg/kg b. wt. while antienteropooling activity was highest in sub-fraction 3 of DS (DS3) and sub-fraction 1 of DFAR (DFAR1);

v. EFAS1 had the least IC_{50} for diphenyl-1-picrylhydrazyl, highest hydrogen peroxide scavenging activity, significantly increased Na^+-K^+ ATPase activity and decreased cycloxygenase II activity;

vi. chloride and Na^+ concentration were significantly decreased by EFAR2 and DS3 respectively.

vii. liver and kidney functional toxicity were observed at 100, 200 and 400 mg/kg b. wt. of DFAR1, EFAR2, EFAS1, DS3 and DR2;

viii. catechol and ethyl eicosapentanoate in EFAS1; piperidnyloxy, thiazoline and quinazoline in EFAR2; and aldosterone derivatives in DFAR1 and DS3 were identified as the responsible antidiarrhoeal compounds.

CONCLUSION

The antidiarrhoeal principles present in DS3, EFAS1, DR2, EFAR2 and DFAR1 exhibited different mechanism of action. Fourteen days administration of these principles was not safe. Identified bioactive principles may be explored for the development of antidiarrhoeal drugs.

RECOMMENDATION

The various antidiarrhoeal sub-fractions obtained in this study should be further purified to obtain the pure bioactive compounds, the specific mechanisms of action of each of the identified compounds and also their toxicological evaluation should be carried out.

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APPENDIX

1. Preparation of ferric reducing assay power (FRAP) reagent

Reagent A (Sodium acetate buffer; 300mM pH 3.6)

Glacial acetic acid (16ml) was added to 3.1g of sodium acetate trihydrate, the solution was then made up to 1 litre with distilled water.

Reagent B (10mM TPTZ solution)

10ml of 40mM HCl was used to dissolve 0.031g of TPTZ at 50^{o}C.

Reagent C (20.0mM ferric chloride solution)

Ferric chloride (0.25g) was dissolved in 10ml of distilled water.

Preparing the FRAP reagent:

25ml of reagent B and C each were added to 25ml of reagent A. The FRAP reagent was maintained at 37^{o}C for a minimum of 10 min.

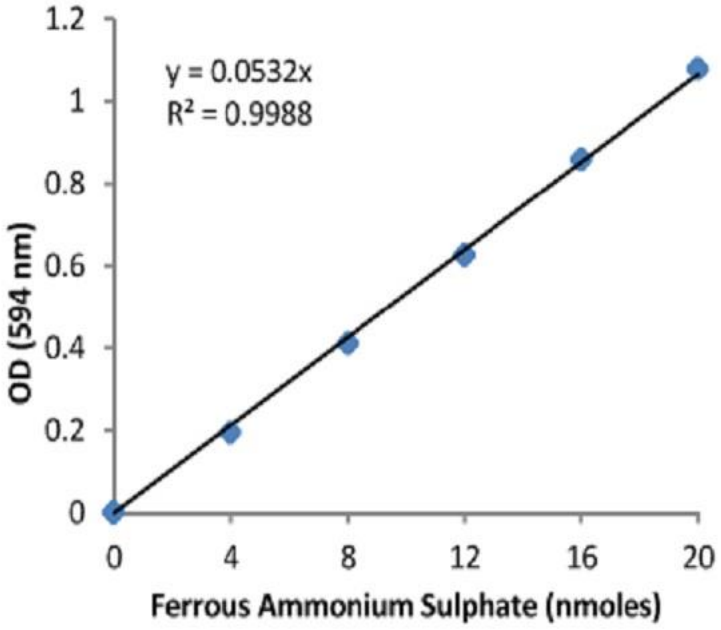

Figure 1: Standard curve for the determination of concentration of ferrous ammonium sulphate released

2. Preparation of molybdate reagent solution for total antioxidant capacity

Reagent A (0.6M H_2SO_4)

33.33ml of concentrated H_2SO_4 (18N) was added to distilled water and was made up to a final volume of 1L with distilled water.

Reagent B (4mM ammonium molybdate)

4.94g of ammonium molybdate was dissolved in 1L of distilled water

Reagent C (28mM sodium phosphate)

3.35g of sodium phosphate was dissolved in 1L of distilled water.

Preparation of molybdate reagent solution

1ml each of reagent A, reagent B and reagent C were added together in 20ml of distilled water and made up to 50ml with distilled water

3. Preparation of TCA-TBA-HCl reagent

15g of trichloroacetic acid (TCA) was dissolved in 100ml of distilled water. 0.37g of thiobarbituric acid (TBA) was also dissolved in 100ml of distilled water and then heated mildly in a water bath. 0.25N HCl was also prepared. TCA-TBA-HCl reagent was prepared by mixing TCA, TBA and HCl in ratio 1:1:1.

4. **Preparation of Phosphate Buffer (100mmol/L pH 7.4) for determination of alkaline aminotransferase activity.**

20.209g of sodium phosphate dibasic heptahydrate was dissolved in 800ml of distilled water. 3.394g of sodium phosphate monobasic monohydrate was added. The reaction was adjusted to PH 7.4 by adding HCl or NaOH. The volume was made up to 1litre.

5. Composition of reagents used

DTNB reagent

29.7mg of DTNB was dissolved in 25ml methanol and distilled water was added to distilled water was added to make up to 1000ml.

Chloride reagent

Chloride reagent contained 2mmol/L mercuric thiocyanate, 40mmol/L ferric nitrate, 45mmol/L nitric oxide and 0.15mmol/L mercuric nitrate. The standard contained 125mmol/L aqueous chloride.

Sodium concentration reagent

Precipitating reagent (R1): this reagent contained 19mmol/L uranyl acetate and 140mmol/L magnesium acetate.

Colour reagent (R2): The colour reagent contained 550mmol/L ammonium thiocyanate and 550mmol/L ammonia.

The sodium standard 150mmol/L sodium standard concentration.

Potassium reagent

2.1mM sodium tetraphenylboron.

Reagents for the determination of aspartate transaminase (AST) activity

Buffer: The buffer contained 10mmol/L phosphate buffer (pH 7.4), 100mmol/L L-aspartate and 2mmol/L α-oxoglutarate.

Reagents for the determination alkaline phosphatase (ALP) activity

Buffer: this was prepared by adding 1mmol/L diethanolamine buffer pH 9.8, with 0.5 mmol/L $MgCl_2$. P-nitrophenylphosphate (10 mmol/L) served as the substrate.

Reagents for the determination of alkaline transaminase (ALT) activity

Buffer: The buffer was made up of 100mmol/L phosphate buffer (pH 7.4), 2.0 mmol/L L-alanine and 2.0 mmol/L α-oxoglutarate.

Reagents for the determination of albumin concentration

BCG concentrate: BCG concentrate was made by adding 75mmol/L succinate buffer PH 4.2 with 0.15mmol/l bromocresol green and 7ml/L Brig 35.

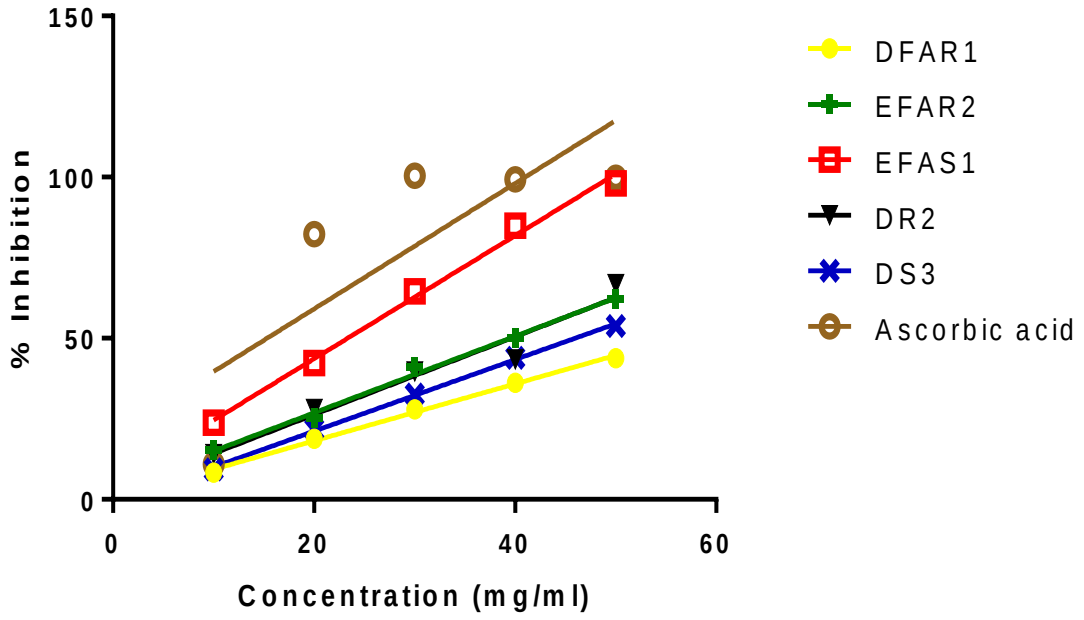

Fig 2: A graph of % DPPH inhibition against concentration of antidiarrhoeal fractions obtained from *Annona senegalensis*.

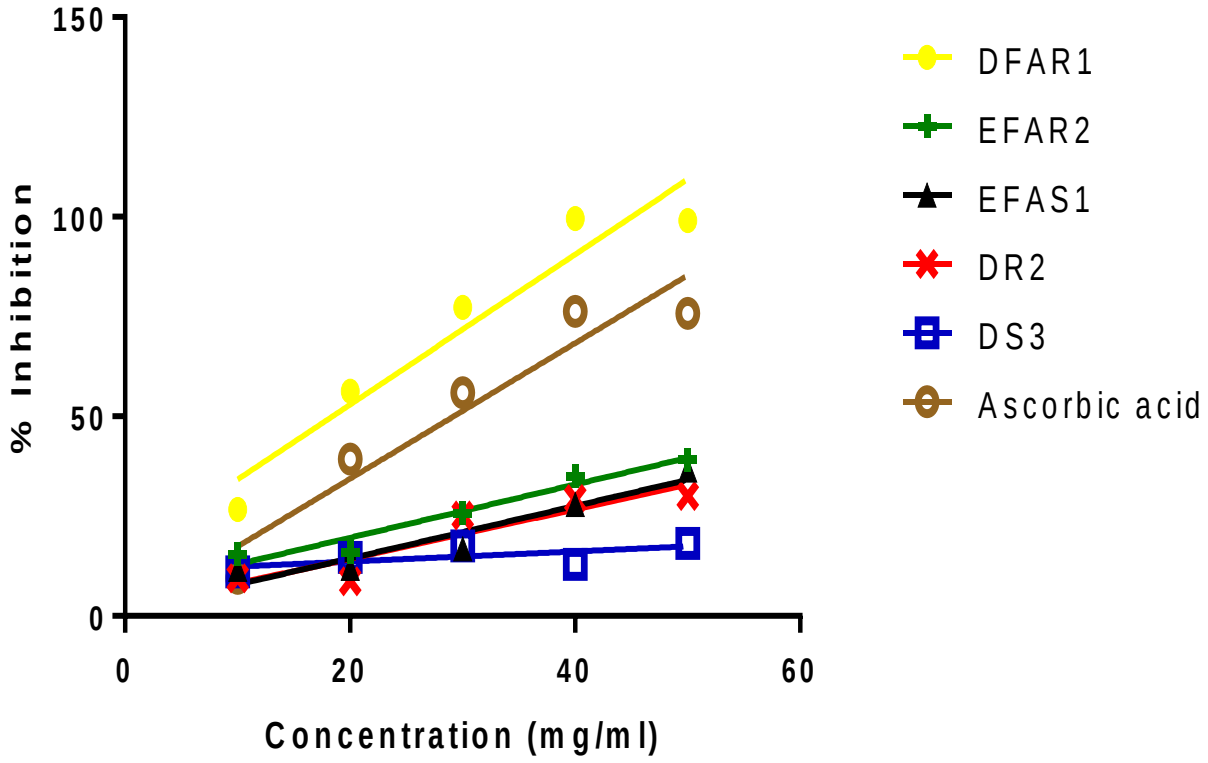

Fig 3: A graph of % ABTS inhibition against concentration of antidiarrhoeal fractions obtained from *Annona senegalensis*

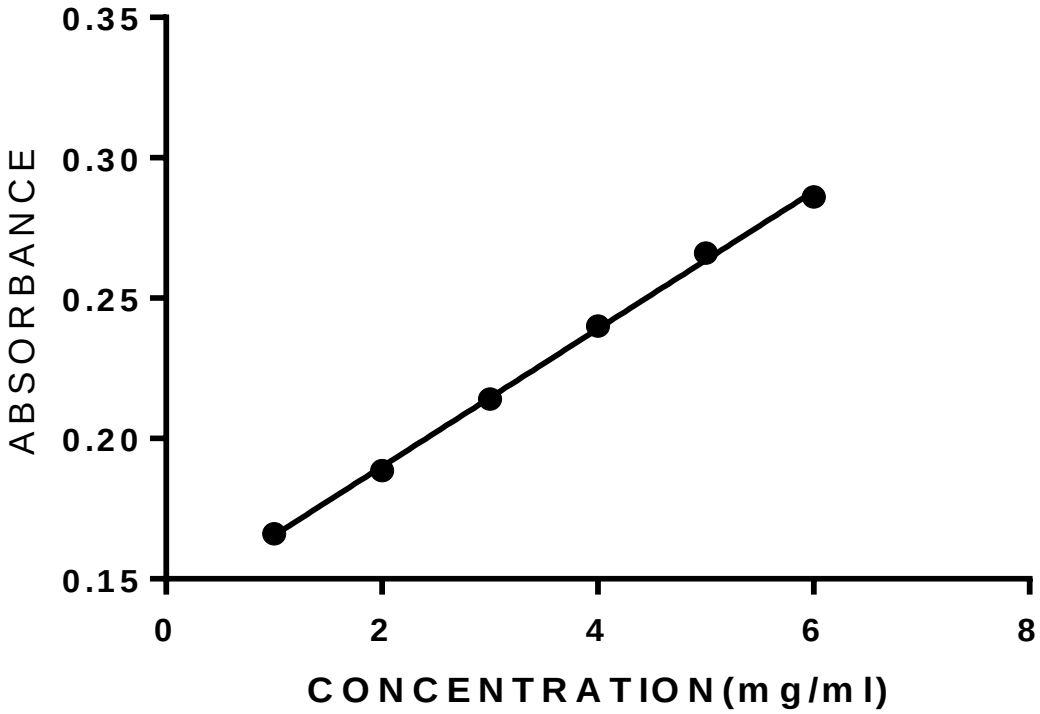

Fig 4: Protein standard curve

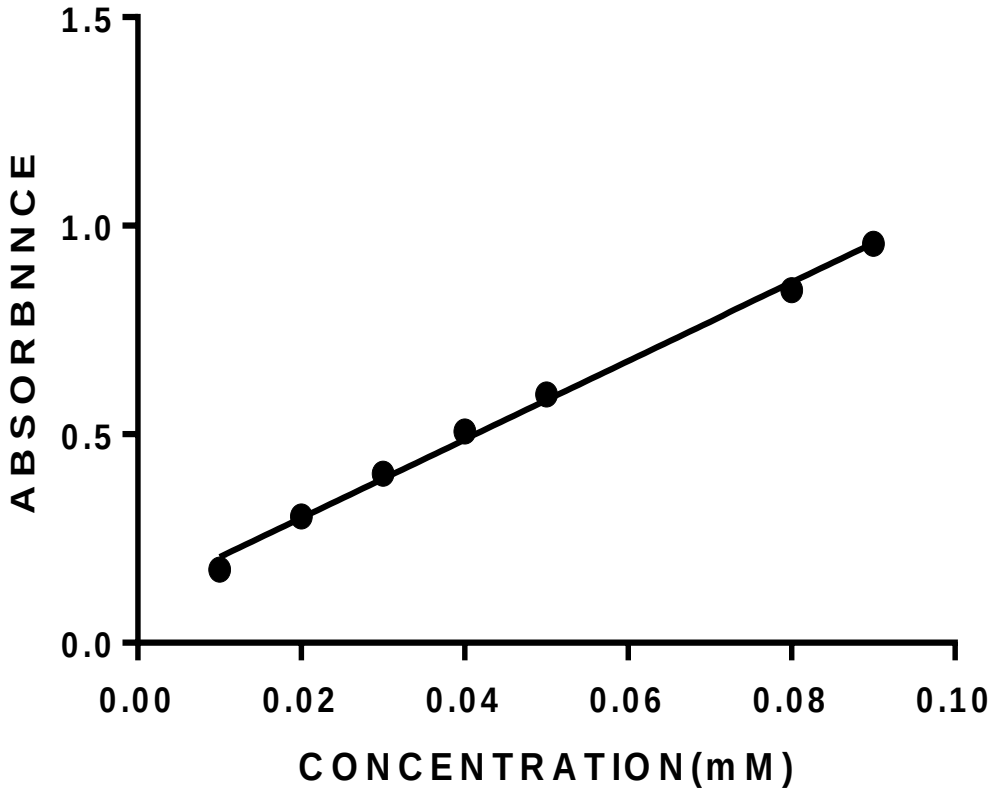

Fig 5: Phosphate standard curve

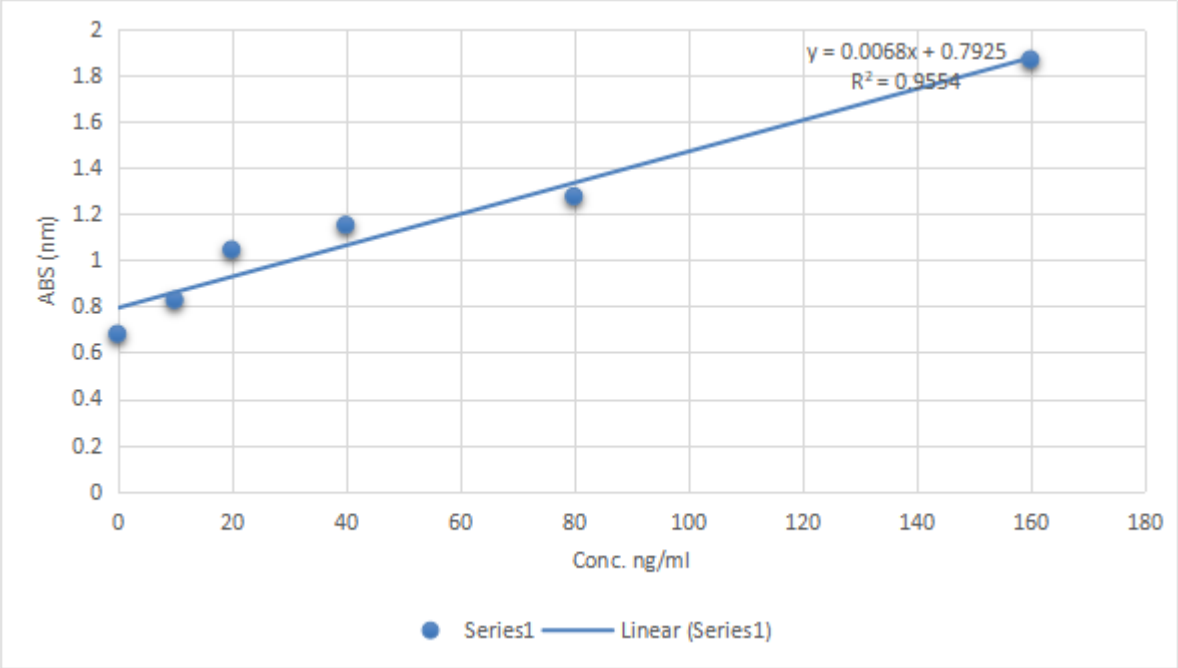

Figure 6: Cyclooxygenase II activity standard curve.

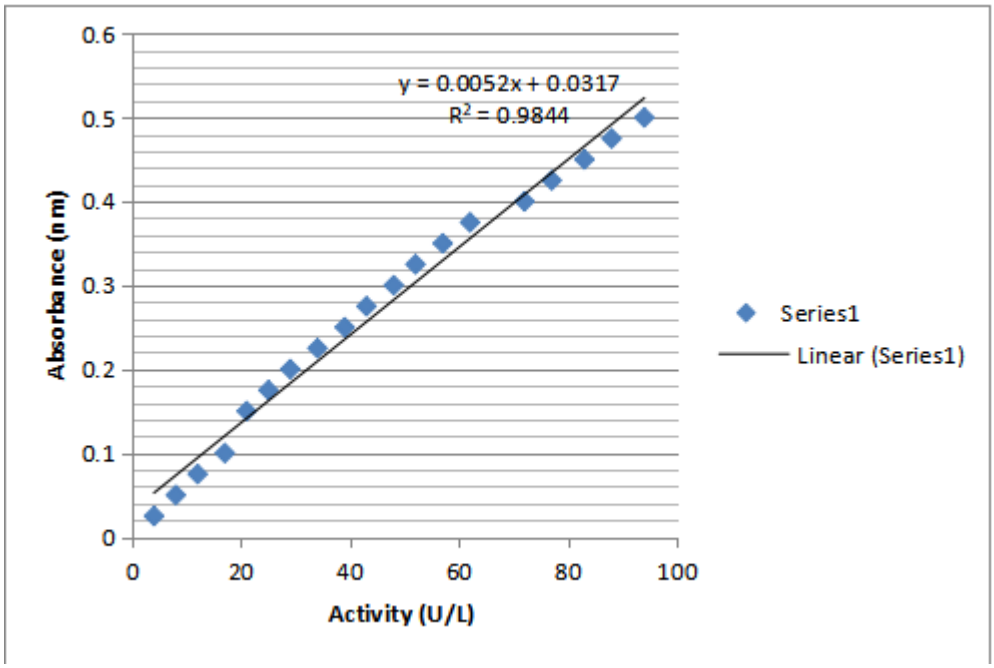

Figure 7: Standard curve for the activity of alanine aminotransferase

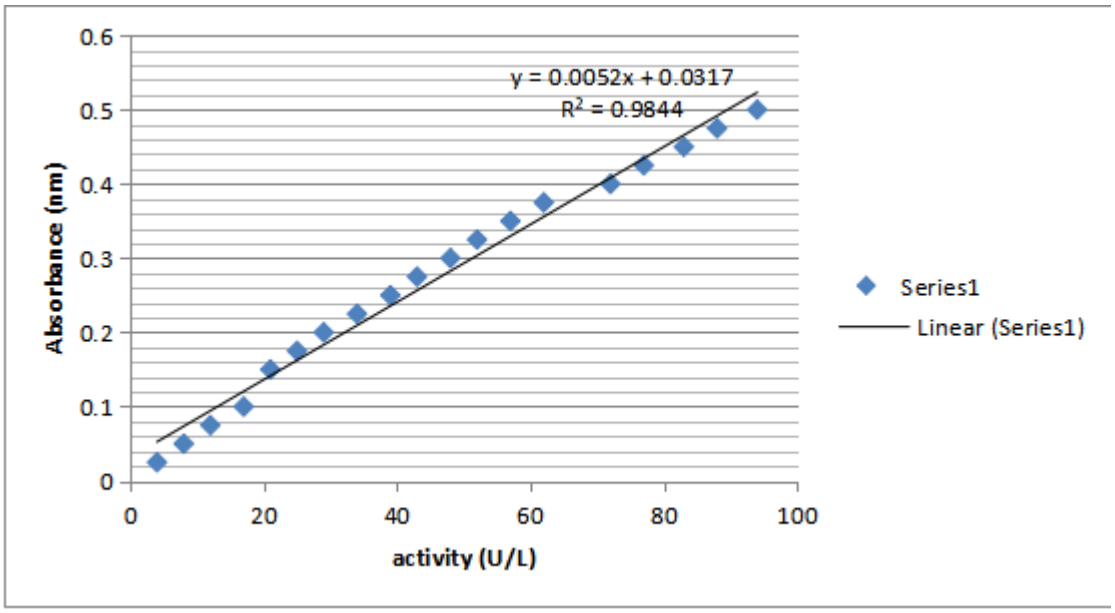

Fig 8: Standard curve for the activity of aspartate aminotransferase