Antidiarrhoeal Principle Rich-Fraction in *Mangifera indica* Leaves and its Mechanisms of Action in Castor Oil-induced Diarrhoeal Rats

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A Thesis Submitted to the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, in fulfilment of the requirement for the Award of Doctor of Philosophy (Ph.D.) Degree in Biochemistry

JULY, 2018

CERTIFICATION

I certify that this work was carried out by Mr. Salimon, Saoban Sunkanmi in the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, under my supervision.

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DEDICATION

This thesis is dedicated to my mother

DECLARATION

I, Saoban Sunkanmi SALIMON (Matric Number - 07/55EH227) declare that this thesis entitled "Antidiarrhoeal Principle Rich-Fraction in *Mangifera indica* Leaves and its Mechanisms of Action in Castor Oil-Induced Diarrhoeal Rats" is the result of a study carried out by me under the supervision of Prof. M.T. Yakubu 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 not been submitted to any University nor is it before any other University for consideration for the Award of a Doctor of Philosophy (Ph.D.) degree in Biochemistry.

Salimon, S.S. (July, 2018).

ACKNOWLEDGEMENTS

All praises, glory and adorations is due to Almighty Allah, the lord of the universe, the master of destiny, the giver and taker of life, the creator of the Heavens and Earth, indeed without His permission and grace, my very existence and the actualization of this achievement will seize to happen. Blessings and salutations to the noble Prophet Muhammed (Peace Be Upon Him), the messenger of Allah, the bringer of faith, the masterpiece of Allah's creation, the bearer of good news, the seal of prophethood, the most virtuous of mankind (S.A.W.). May Allah shower His mercy and blessings on him, his household and the generality of Muslim Ummah until the day of reckoning.

My profound and sincere gratitude goes to my supervisor, Prof. Musa Toyin Yakubu for his guidance, knowledge, constructive criticism, understanding and professionalism. I will forever be in awe of your excellence both academically and as a human. Words are not enough to describe my appreciation of everything you have done with and for me since I walked through your door as an undergraduate student meeting his supervisor for the very first time. From then till date, I can only say Alhamdulillah as this was a huge turning point in my life in many ways I was too shallow minded to understand then and still do not fully understand now. May Allah reward you abundantly in ways you do not understand and continue to ease your task now and forever (Amin).

I truly appreciate the effort, support and contributions of all the Professors in the department staring with the Head, Prof. (Mrs.) E.A. Balogun, M.A. Akanji, H.O.B. Oloyede, C.O. Bewaji; (Mrs.) S.O. Malomo and A.T. Oladiji. My appreciation will be incomplete without mentioning the names of Dr. J.O. Adebayo, R.O. Arise, (Mrs.) F.A. Suleiman, A. Igunnu, M.O. Nafiu, M.O. Salawu, A.-L. Quadri, (Mrs.) R.A. Oyegoke, Mrs. H.F. Muritala, F.A. Abubakar, S. Omoniwa, Mr. O.K. Bello and Mrs. S. Omar for their support and words of encouragement towards the completion of this research.

To my father, who assumed the duty of a mother without forgetting his responsibility as a father for over a decade, indeed everything I am today and would be tomorrow is due to your relentless effort to exercise your duty as a parent and actualize the dream of the woman whom no one can replace in our lives. May Allah forgive her sins, have mercy on her and grant her a place under His shade on the day of resurrection. I pray to Almighty Allah to grant him good health and spare his life to reap the fruit of his labour until it is best for him to leave than to live. I cannot but acknowledge the support from my brothers Adam, Abdul-Baki and Fatiu, your love, prayers and support are what keeps me going during the tough times. I will forever be grateful to you all.

Also, I will like to appreciate Dr. T.O. Ajiboye (CEO, Research Fulcrum) for the knowledge I gained from him during the course of this study as well as his contributions and the conducive research environment towards getting a bulk of my results. Similarly, all the families that have contributed to my life positively starting with the Ayinla family, Olaonipekun, Akindele and Hamzat, may you find support at every turn of your life too.

Friends are like shoes from different boxes, some are loose, some are tight while some are just the right size, they help you walk through life. I am grateful to Allah for the group of people I have as friends, Ayinla Abdul'Aziz, Olaoti Khaleed, Opakunle Khadijat, Dr. Q.O. Nurudeen, Jimoh Rukayat, Osineye Suaib, Ahmed Habeeb and Laura Grouselle without whom my life will be completely different and boring, thanks for being my right, I love you all.

My colleagues, Ogunro, Tonia, Ak, Catalyst, Okeme, Lanre, Officer, Fehin, Abu *et al*. all of whom I share the bond of the struggles of Ph.D., to those that have finished I say congratulations, to those that are still in the struggle, I say never give up the fight and see you on the other side.

Finally, to my first supporter, friend and (hopefully) other half, Abdulsalam Rofiat Adeola, your understanding and patience with me during the struggle is a source of joy. I hope to repay the kind gesture by being the best man I can and strive to keep you happy at all time. To every other person that deserves a mention, please forgive my oblivious mind, may Allah ease your struggles too in this wonderful journey of life.

SALIMON, S.S.

(July, 2018)

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LIST OF ABBREVIATIONS

AChE	–	Acetylcholinesterase
ADI	–	Antidiarrhoeal index
AIDS	–	Acquired immune deficiency syndrome
ALP	–	Alkaline phosphatase
ALT	–	Alanine aminotransferase
AMP	–	Adenosine monophosphate
ASHSP	–	American society of health system pharmacists
AST	–	Aspartate aminotransferase
ATP	–	Adenosine triphosphate
BCG	–	Bromocresol green
cAMP	–	cyclic AMP
CD	–	Celiac disease
CDC	–	Centre for disease control and prevention
CF	–	Cystic fibrosis
CFTR	–	Cystic fibrosis transmembrane conductance regulator
cfu	–	Colony forming unit
cGMP	–	cyclic GMP
CMD	–	Congenital microvillous disease
CMV	–	Cytomegalovirus
COX	–	Cyclooxygenase
DCM	–	Distance travelled by charcoal meal
DNA	–	Deoxyribonucleic acid
DNPH	–	Dinitrophenylhydrazine
DTNB	–	Dithionitrobenzoic acid
EAEC	–	Enteroaggresive *Eschericia coli*
EAF	–	Ethylacetate fraction
EDTA	–	Ethylene diamine tetra-acetic acid
EGTA	–	Ethylene glycol tetra-acetic acid
EHEC	–	Enterohemorrhagic *Eschericia coli*

EIEC	–	Enteroinvasive *Eschericia coli*
eNOS	–	Epithelial cell nitric oxide synthase
ENS	–	Enteric nervous system
EPEC	–	Enteropathogenic *Eschericia coli*
ETEC	–	Enterotoxigenic *Eschericia coli*
GIT	–	Gastrointestinal tract
GMP	–	Guanosine monophosphate
GRA	–	Government residential area
GSH	–	Reduced glutathione
GSSG	–	Oxidized glutathione
HIV	–	Human immunodeficiency virus
HMG	–	Hydroxymethyl glutaryl
HPLC	–	High performance liquid chromatography
IBS	–	Irritable bowel syndrome
Ig A	–	Immunoglobulin A
IL	–	Interleukin
iNOS	–	Inducible nitric oxide synthase
IUCN	–	International union for conservation of nature
IUPAC	–	International union of pure and applied chemistry
IVAC	–	International vaccine access centre
LSI	–	Length of small intestine
MDA	–	Malondialdehyde
Na-K ATPase	–	Sodium-potassium ATPase
NBF	–	n-butanol fraction
nNOS	–	Neuronal tissue nitric oxide synthase
NO	–	Nitric oxide
NSAID	–	Non-steroidal anti-inflammatory drug
NSP	–	Non-structural protein
PDE	–	Phosphodiesterase
PI	–	Peristaltic index

RF	–	Residual fraction
R_f	–	Retention factor
RNS	–	Reactive nitrogen species
ROS	–	Reactive oxygen species
RP	–	Rehydration project
SDS	–	Sodium dodecyl sulphate
SFB	–	Sub-fraction B
SGLT	–	Sodium dependent glucose transporter
SIBO	–	Small intestine bacterial overgrowth
SOD	–	Superoxide dismutase
TBA	–	Thiobabituric acid
TCA	–	Trichloroacetic acid
TLC	–	Thin layer chromatography
TNFR	–	Tumor necrosis factor receptor
UERC	–	University ethical review committee
UIH	–	University of Ilorin herbarium
UNICEF	–	United nations children's fund
USA	–	United States of America
VT	–	Verocytotoxin
WASH	–	Water sanitation and hygiene
WHO	–	World health organization
α-TNF	–	Alpha-tumor necrosis factor

ABSTRACT

Diarrhoea is an alteration in bowel movement characterized by increased water content and frequency of stooling. The aqueous extract of *Mangifera indica* leaves have been validated for their efficacy as an antidiarrhoeal agent. However, the probable mechanism(s) of action remain unknown. The objectives of the study were to: (i) partition the aqueous extract using solvent-solvent extraction; (ii) determine the antidiarrhoeal activity of the solvent-solvent and column chromatographic fractions in female Wistar rats; (iii) establish the probable mechanism of action of the sub-fractions; (iv) evaluate the toxicity of sub-fraction B (SFB) on selected organs; and (v) identify the probable bioactive constituents of SFB.

The aqueous extract of *M. indica* leaves was successively fractionated into ethyl-acetate fraction (EAF), n-butanol fraction (NBF) and residual fraction (RF). The EAF, NBF and RF were evaporated to dryness at 40℃. They were reconstituted and administered to screen for antidiarrhoeal activity using castor oil-induced diarrhoea; enteropooling; and gastrointestinal motility models. Each of the sub-fractions of the RF were also screened for their antidiarrhoeal activity and evaluated for probable mechanism(s) of action. The SFB was also evaluated for its effects on the liver, kidney and small intestine. Data were analysed using One-way Analysis of Variance and Tukey's *Post-hoc* test at $p < 0.05$.

The findings revealed that the:

i. RF at 14.09 and 56.35 mg/kg body weight (bw) significantly prolonged the onset time of diarrhoea, decreased the total number of faeces, wet faeces, water content and increased the inhibition of defecation by 94.29 and 96.34% respectively;
ii. mass and volume of intestinal content significantly decreased at 14.09 mg/kg bw of the RF with an inhibition of 45.20% on intraluminal fluid accumulation;
iii. distance travelled by charcoal meal was shortest in diarrhoeal rats treated with 14.09 mg/kg bw of the RF with 39.79% inhibition of peristalsis;
iv. the antidiarrhoeal index (ADI) was highest at 14.09 mg/kg bw of the RF (55.19%). At 56.35 mg/kg bw, the ADI (49.87%) compared favourably with that of the reference drug (48.50%);
v. highest inhibition of defecation was exhibited by sub-fraction B at 6.47 mg/kg bw while the onset of diarrhoea was most prolonged by sub-fraction A;
vi. antienteropooling and antimotility activities were highest in the sub-fraction A and B treated rats, respectively;
vii. activities of Na-K ATPase, alkaline phosphatase and the concentrations of glucose, Na^+ and K^+ were increased by SFB while the levels of Cl^-, protein carbonyl and nitrite were decreased;
viii. levels of acetylcholinesterase, glutathione, superoxide dismutase and intestinal protein were all increased by SFB;
ix. sub-fraction B at 12.94 mg/kg bw exhibited structural and functional toxicity on the liver kidney and small intestine; and
x. mangiferin and catechin were identified as the probable bioactive constituents of SBF.

The study concluded that the antidiarrhoeal principles were present in the RF while the established mechanisms included antisecretory, anti-inflammatory, antioxidant and modulation of intestinal permeability. The antidiarrhoeal principle rich-SBF may therefore be explored as a lead for drug discovery in the treatment of diarrhoea.

CHAPTER ONE

1.0 Introduction

The gastrointestinal tract (GIT) in rats is a tubular system network that connects the mouth to the anus via the pharynx, oesophagus, stomach, small and large intestine (Figure 1). It is also associated with some other digestive organs such as the pancreas, liver and the gall bladder.

The GIT is dedicated to processing and absorption of fluids and nutrients crucial to the maintenance of good health (Leiper, 2015). For optimal functionality of the GIT, a balance must be maintained between intestinal fluid volume and motility. The intestinal fluid volume is keenly regulated by the intestinal villous epithelial cells through the control of fluid absorption; and secretion across the intestine via intestinal crypt cells (Leiper, 2015). A net fluid absorption driven by osmotic gradients that modulates the movement of solutes (sugars and amino acids) and electrolytes (sodium ions and chloride ions) across the epithelial lining of the lumen, predominate in these opposing processes (Hladky and Barrand, 2014). In contrast, motility is regulated by the activation of enteric nervous system (ENS) responsible for the protection of intestinal epithelial cells (Yu and Li, 2014). The ENS autonomously regulates the endocrine and exocrine functions of the GIT, blood flow, motility, immune and inflammatory processes (Yu and Li, 2014; Sharkey, 2015). Any disturbance in the balance of the intestinal fluid either by decreased absorption or increased secretion, and/or changes in GIT motility usually provoke intestinal disorders, clinically evident as diarrhoea (Kiela and Ghishan, 2016).

1.1 Statement of the Problem

On a daily basis, diarrhoea claims more children lives (2,195) than measles, famine, malaria, human immunodeficiency virus (HIV) and war put together (Liu *et al*., 2012). However, diarrhoea is often neglected by the policy makers at the strategy levels with priority

placed on other diseases such as HIV, malaria, diabetes etc. (Gill *et al.*, 2012). The funds allocated for the control of diarrhoea falls short relative to the global burden of the disease.

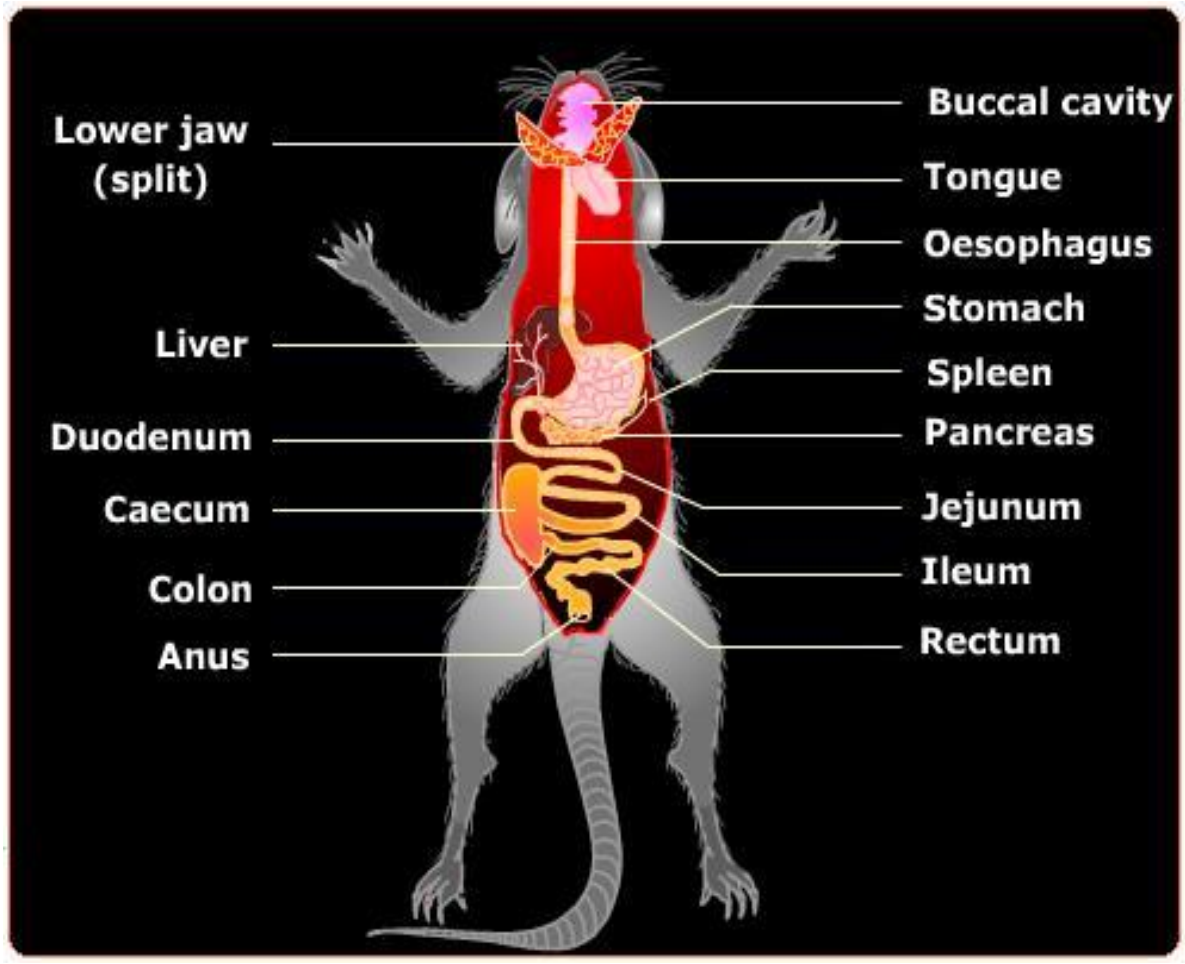


Figure 1: The gastrointestinal tract of a rat

Source: Helander and Fandriks (2014)

This is in-part due to the misplaced priorities of the policy-makers to meet the staggering needs of the general populace. HIV/AIDS (32%), malaria (18%), tuberculosis (14%), measles (8%), pneumonia (8%), malnutrition (7%) and neonatal causes (7%) are all considered to be of more importance than diarrhoea (6%) by the policy makers (Figure 2) (Gill *et al.*, 2012). This in turn has contributed to the alarming rate with which the disease occurs especially in Nigeria where there is shortage or lack of safe water and hygienic practices.

1.2 Justification for the Study

1. The incidence of poverty, natural disasters and complex emergencies (all of which contributes to the prevalence of diarrhoea) is at its peak which makes it a matter of National urgency to develop more effective, cheaper and safer antidiarrhoeal drugs.
2. The alarming increase in the mortality and morbidity of both old and young to this epidemic makes it a major health challenge.
3. Insufficient financing of diarrhoeal control initiatives due to low political will by the policy makers.
4. Although, Yakubu and Salimon (2015) had reported the efficacy of the crude extract of *M. indica* leaves as an antidiarrhoeal agent, the need to isolate the bioactive agent(s) which will eventually serve as lead to drug development is desirable.
5. Consequently, the drug when developed can offer alternative and complementary therapy for the management of diarrhoea.
6. Also, the health care delivery system and economy of the country could be boosted tremendously by the product if eventually developed and patented.

1.3 Overall Objective

The overall objective of this study was to isolate and characterize the antidiarrhoeal principle rich-fraction in the aqueous extract of *M. indica* leaves and establish is probable mechanism(s) of action in female rats.

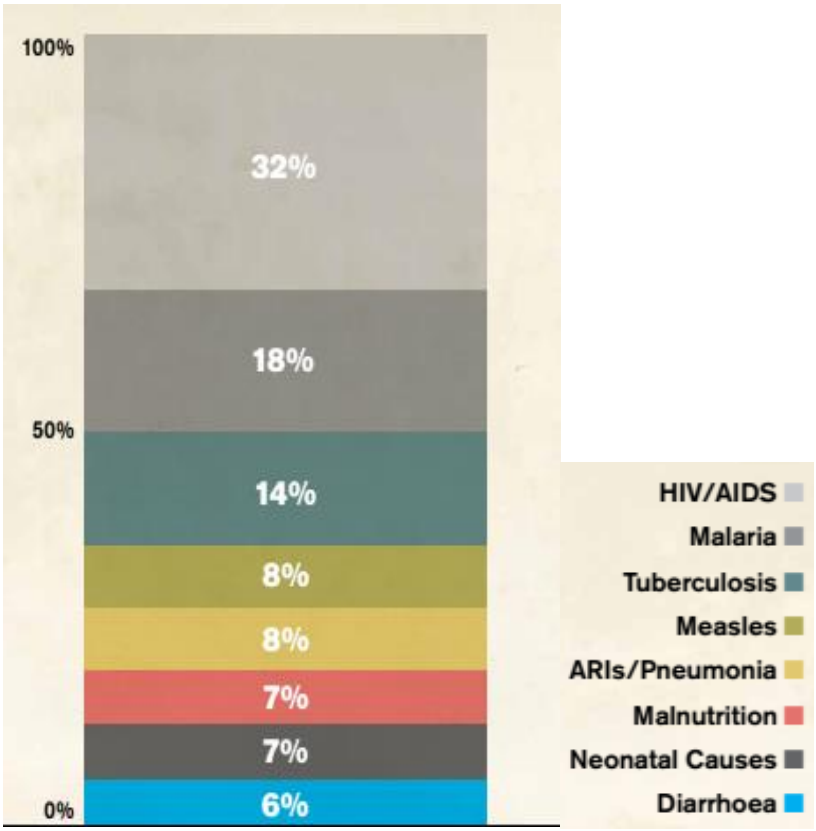


Figure 2: Child health issues as perceived by policy-makers in the order of priority

Source: Gill *et al.* (2012)

1.3.1 Specific Objectives

The specific objectives were to:

1. prepare the aqueous extract followed by successive solvent-solvent partitioning with n-hexane, ethylacetate and n-butanol (in increasing order of polarity);
2. determine the antidiarrhoeal activity of the solvent-solvent and column chromatographic fractions using; castor oil induced-diarrhoea; castor oil induced-enteropooling; and gastrointestinal transit test models;
3. monitor probable mechanisms – antisecretory, anti-inflammatory, antimotility, antioxidant and intestinal permeability;
4. evaluate the toxicological implication(s) of the antidiarrhoeal principle rich-fraction on the liver, kidney and small intestine;

i. Liver function indices; Total protein, albumin, globulin, total and conjugated bilirubin, AST: ALT ratio, Albumin:Globulin ratio, alanine aminotransferases, aspartate aminotransferases, alkaline phosphatase.

ii. Kidney function indices; Serum urea, uric acid, creatinine, Na^{+}–K^{+} ATPase, electrolytes (sodium, potassium and chlorine), serum and urine creatinine, creatinine clearance;

iii. Intestinal function indices; Intestinal protein, protein carbonyl, protein oxidation (%), glucose and alkaline phosphatase

iv. Histological examination of the liver, kidney and small intestine

CHAPTER TWO

2.1 Literature Review

Diarrhoea is an alteration/irregularity in bowel movement characterized by increased water content, volume and frequency of stooling usually three times or more within 24 hours (Baldi *et al*., 2009). However, the frequent passing of formed stools, loose or pasty stools by breast-fed babies are not cases of diarrhoea (Seema *et al*., 2008). The pathophysiology of diarrhoea includes microbial and parasitic infections (Hodges and Gill, 2010), stress (oxidative or physical) (Soderholm, 2012), immune system dysfunction (Schulzke *et al*., 2009), disruption in the GIT integrity and neuro-humoral mechanisms (Spiller, 2004; Vitali *et al*., 2006). Also, diarrhoea can be sequelae to other diseases such as cholera, irritable bowel syndrome (IBS), gastroenteritis (intestinal inflammation and ulcerative colitis), malaria and diabetes mellitus (Gale *et al*., 2007; Baldi *et al*., 2009; Forgacs and Patel, 2011). Diarrhoea can result from the impairment in intestinal fluid secretion (resulting from osmotic load within the intestine), hyper-motility (resulting from rapid intestinal transitions) or hypo-motility (resulting in decreased intestinal fluid reabsorption) or combination of these mechanisms (Vitali *et al*., 2006). Physiologically, diarrhoea may be considered beneficial to the GIT as it provides an important mechanism for the elimination of harmful luminal substances (Valeur *et al*., 2009). However, diarrhoea becomes fatal and pathological when the body is incapacitated to adequately replace the lost fluids and electrolytes (Valeur *et al*., 2009).

Diarrhoea is considered one of the most dangerous GIT disorders as severe cases usually result in death due to dehydration and loss of electrolytes (UNICEF, 2010). According to the World Health Organization (WHO, 2017) report, a global incidence of about 1.7 billion diarrhoeal episodes occur in humans annually resulting in the death of about 525,000 children under the age of five (Routray, 2017). In addition to causing acute disease and mortality,

diarrhoea associated malnutrition could result in stunted growth, non-optimal immune functionality and increased susceptibility to infections. Diarrhoea therefore poses a major health challenge to human, as it could lead to premature mortality, disability and/or increase health-care costs (Townsend *et al*., 2017).

Diarrhoea is also a common illness and major cause of death during complex emergencies and natural disasters as there is usually overcrowding, shortage of food, drugs and water supply (Sharp and Beadling, 2012; Bain *et al.,* 2013). At the onset of an emergency, individuals, especially children differ in their baseline health and nutritional status and as such, face increased risk of exposure to communicable diseases especially diarrhoea and malnutrition while malaria and acute respiratory tract infections are also common (Bain *et al*., 2013).

In animal production, diarrhoea is presumed to impose heavy productivity losses on affected farms, although, the true effect in monetary terms may not be easily appreciated. The apparent on-farm losses include reduction in productivity (wool, milk, egg, meat and their quality), weight loss and abortion as well as increased mortality and morbidity (Lamy *et al.*, 2012). Episodes of diarrhoeal diseases can also affect the export market and hurt consumer's confidence in the farm products (Lamy *et al.*, 2012).

2.2 Prevalence and Mortality Rate of Diarrhoea

Despite the availability of simple management and effective treatment measures, diarrhoea remains not just a common illness but also a global killer accounting for 9 percent of child death within 60 months of age (UNICEF, 2017). Diarrhoea remains second in the leading cause of death among children, following closely behind pneumonia (the forgotten killer) both of which accounts for an estimated 40% child death across the globe annually (UNICEF, 2010). The global burden of incidence and severity of diarrhoea is highest in South Asia and Africa with a death ratio of 72% occurring in the first 2 years of life thus, necessitating

increased emphasis in the preventive practices and treatment option for neonates (Walker *et al*., 2013).

Amouzou *et al.* (2016) reported 1 out of 10 childhood deaths were due to diarrhoea which translates to 526,000 deaths per year, equivalent to a childhood death per minute. Nigeria accounts for an estimate of 150,000 diarrhoeal deaths among children under the age of five (Charyeva *et al.*, 2015). More lives are lost to diarrhoea daily (2,195 children) than measles, famine, human immunodeficiency virus (HIV), malaria and war put together (Liu *et al*., 2012). For HIV-infected children, diarrhoea is even deadlier with a death rate of 11 times greater than those of uninfected children (Elfstrand and Floren, 2010).

About 75% of diarrhoea deaths are concentrated in 15 high burden countries with Nigeria coming second to India (Table 1). Of these 15 countries, six (India, Nigeria, Democratic Republic of Congo, Afghanistan, Pakistan and Angola) have missed the MDG4 target "to reduce by two-thirds the mortality rate among children under-five between 1990–2015" while others like Ethiopia, Indonesia, Bangladesh, China, Niger and Tanzania have met their goals but still remain in the top 15 because of the high burden of diarrhoea in their most vulnerable population (Boschi-Pinto *et al.*, 2008; IVAC, 2015). Despite these statistics, strides made over the last two decades have shown that diarrhoea prevention is not only possible but also cost effective (Townsend *et al*., 2017). Thus, necessitating the continuous and relentless effort to mitigate or completely eradicate the disease from Nigeria and the world at large.

2.3 Classes of Diarrhoea

Diarrhoea is often classified by the duration of symptoms which is very important for diagnosis and treatment of the disease. It can either be acute, persistent or chronic diarrhoea each having various underlying complications. Highlighted below are the three classes of diarrhoea;

Table 1: Child deaths from diarrhoeal disease in high burden countries

Rank	Country	Total number of annual childhood deaths due to diarrhoea
1	India	297,114
2	Nigeria	210,557
3	Pakistan	103,760
4	Democratic Republic of Congo	78,422
5	Angola	54,548
6	Ethiopia	46,888
7	Indonesia	33,737
8	Chad	30,641
9	Afghanistan	30,419
10	Niger	28,164
11	China	27,114
12	Sudan	24,103
13	Bangladesh	24,571,
14	Somalia	23,426
15	United Republic of Tanzania	22,394

Source: IVAC (2015)

1. Acute diarrhoea: It usually last from hours to days but not more than 2 weeks. It can occur in various epidemiological setting which can be hospital acquired, community acquired (such as a refugee camp), or during travels (Guandalini and Vaziri, 2010). *Shigella spp*, enterotoxigenic *Eschericia coli* (ETEC) and enterohemorraghic *E. coli* (EHEC) are the major causes of acute diarrhoea. In some cases, acute diarrhoea may be as a result of physiological response to harmful substance(s) within the bowels (White and Jenkins, 2012). This could be beneficial as it aids the expulsion of harmful microorganisms and toxins out of the system. In immunocompetent victims, acute diarrhoea could be settled without serious medical intervention (Guandalini and Vaziri, 2010).
2. Persistent diarrhoea: Diarrhoea is described as persistent when the symptoms last between 2 – 4 weeks. It is usually predominated by etiologies of infectious origin. More often than always, persistent diarrhoea is associated with high morbidity rate due to the malabsorption of nutrients that often accompany diarrhoea (Guandalini and Vaziri, 2010). Viruses such as norovirus and rotavirus contribute to persistent diarrhoea, *Camphylobacter* and *Salmonella spp*. are rare while enteropathogenic, enteroinvaasive and enteroagressive *E. coli* (EPEC, EIEC and EAEC) are the major bacteria pathogens responsible for persistent diarrhoea (Pawlowski *et al*., 2009). Protozoan infections are the most common cause of persistent diarrhoea in immunocompromised victims (the aged and HIV patients) while *Cryptosporidium* and *Giardia* spp. occasionally cause persistent diarrhoea (Pawlowski *et al*., 2009; Guandalini and Vaziri, 2010).
3. Chronic diarrhoea: It is usually a symptom of a major GIT disturbance (such as IBS, ulcerative colitis, Addison's disease etc) or occasionally from chronic infections which can last for a month or more (Schiller *et al*., 2014). It has been estimated that 1 – 5% of

adults suffer from chronic diarrhoea probably due to their weak immune system or as side effect of a maintenance drug known as drug-induced diarrhoea (Schiller, 2004).

2.4 Aetiologies of Diarrhoea

The most common causes of diarrhoea are due to infections in the small intestine by virus, bacteria, protozoa and parasites. However, diarrhoea can also be of non-infective causes which could be from food allergy, genetic disorders, damage to intestinal mucosal amongst others. Most deaths from diarrhoeal disease are of infectious origin (Liu *et al.*, 2015).

2.4.1 Causes of Infectious Diarrhoea

1. Bacterial agents

Several bacteria such as *Eschericia coli, Campylobacter jejuni, Salmonella spp., Vibrio cholerae, Clostridium difficule, Shigella spp., Yersinia spp.* are frequently cultured from diarrhoeal faeces. Some of these microorganisms and how they caused diarrhoea are described below.

a) *Eschericia coli*: All strains of *E. coli* (EPEC, ETEC, EAEC, EHEC and EIEC) have be isolated and identified as causes of diarrhoea albeit, at varying effects. EPEC predominates in children but rarely found in adults. They attach firmly to the surface of the intestinal mucosal leading to dissolution of the brush border by inducing vesiculation of the microvilli (Lapointe *et al*., 2009). This process in the jejunum and ileum results in a loss of brush border enzymes, dysregulation of intestinal architecture, host inflammatory response and the loss of large absorptive surface (Lapointe *et al*., 2009). ETEC is common in traveller's diarrhoea, they produce diarrhoea through the attachment to intestinal mucosa and elaborate one or both of heat labile and heat stable toxins (Field, 2003). This leads to the stimulation of inflammatory cytokines that causes inflammatory diarrhoea. EIEC corresponds with bloody diarrhoea, they attach to colonic enterocytes, penetrate via endocytotic

mechanism prior to replication. This results in necrosis and loss of large areas of colonic mucosa which leads to dysentery (Viswanathan *et al*., 2009). EHEC, predominant in cattle produce attaching-effacement to the terminal ileal and colonic mucosa and release verocytotoxins (VT 1 or 2) (Karmali *et al.*, 2010; Kang, 2013). These kill colonic enterocytes and produce haemorrhagic colitis and haemolytic uremic syndrome (Karmali *et al.*, 2010). EAEC causes watery and persistent diarrhoea in children and HIV patients respectively (WHO, 2010).

b) *Salmonella spp.*: It is a group of bacteria known to cause enteric fever, gastroenteritis, food poisoning and various other illness. Entry of salmonella into the GIT provokes a cascade of events that culminates into the release of inflammatory cytokines which destroys the intestinal microflora and epithelial cells thereby loosing its protective function. This in turn leads to impaired reabsorption process hence diarrhoea.

c) *Vibrio cholerae*: The causative agent of cholera, an infectious disease characterized by severe watery/secretory diarrhoea and emesis that leads to dehydration and/or death within hours of onset if not treated aggressively. It induces intracellular cAMP generation which stimulates active secretion of intestinal fluid through the stimulation of cystic fibrosis transmembrane conductance regulator (CFTR)-mediated chloride ion secretion and/or inhibition of electrolyte reabsorption (Field, 2003; Satitsri *et al.*, 2016).

d) *Shigella spp.*: It is a virulent, non-capsulated gram negative bacteria commonly known to cause shigellosis. They invade the intestinal epithelium, spread within the cells and induce a potent inflammatory response resulting in the destruction of the mucosal layer thereby causing bloody diarrhoea (Phalipon and Sansonetti, 2007).

Shigella infection in malnourished children often cause growth retardation, impaired nutrition and recurrent infection.

e) *Campylobacter spp.:* Campylobacteriosis an infectious disease majorly caused by *Campylobacter jejuni* is the leading cause of diarrhoea in USA (CDC, 2017). *C. jejuni* penetrates the intestinal mucus layer, secretes proteins that disrupts the integrity of the intestinal epithelial cells. It stimulates the proinflammatory pathway and thus, a large production chemokines, cytokines and innate effector molecules (van Putten *et al.,* 2009). This leads to impairment of the intestinal epithelial cell function resulting in secretory diarrhoea. Campylobacter infection is common in HIV patients as well as adults and children with immunoglobulin deficiencies (Javid and Ahmed, 2017).

2. Viral agents

Viral agents such as rotavirus, calcivirus, norovirus, cytomegalovirus (CMV) and enterovirus have been reported to cause diarrhoea of varied severity. They are the major cause of acute diarrhoea in children. Listed below are some viral agents known to cause diarrhoea;

a) Rotavirus: It is most common cause of gastroenteritis in infants and young children showing symptoms of emesis, fever, severe watery diarrhoea and abdominal pain that can last between 3–8 days. Adults can also be infected but with milder symptom while protection from illness could be obtained by vaccination (CDC, 2016). Infection of rotavirus leads to diarrhoea in different phases from the direct effect of the virus to the indirect effects and host response to the infection. Some of the known mechanisms include malabsorption, secondary to destruction of host enterocytes, activation of the ENS as well as the

active secretion stimulated by the action of the rotavirus non-structural protein (NSP 4) (Mestrovic, 2015).

b) Norovirus: Also known as Norwalk virus is the most common cause of nonbacterial gastrointestinal outbreaks. Symptoms include abdominal cramps, watery diarrhoea, nausea and emesis. In adults, diarrhoea is more common while vomiting is prominent in children (Karst, 2010). Norovirus exploits M-cells for infection, they stimulate alterations in the expression levels of brush border enzymes such as alkaline phosphatase, sucrase and trehalase (Karst, 2010; Karst and Tibbetts, 2016). Also, norovirus infection causes specific histological changes like broadening and blunting of the villi, shortening of the microvilli and apoptosis of enterocytes (Karst, 2010).

c) Cytomegalovirus (CMV): CMV is a very common viral infection but non-symptomatic in healthy individuals. However, infection in immunosuppressed, chemotherapy, AIDS, organ transplant patients can be severe with symptoms such as abdominal pain, bloody diarrhoea, gastrointestinal bleeding, fever, perforation amongst others (Wilcox, 2010; Pant *et al.,* 2013). Diarrhoea usually results from malabsorption and inflammatory disorders (Sue *et al.*, 2016).

3. Protozoan agents

Protozoans capable of causing diarrhoea include but not limited to *Giardia lamblia, Entamoeba histolica, Cyclospora cayetanensis, Isospora belli, Cryptosporidium pervum* and *Giardia intestinalis* (Linscott, 2011). They are responsible for a fraction of the cases of infectious diarrhoea among children in developing countries. *G. lamblia* causes giardiasis and are often accompanied by abdominal distension, weight loss, acute, intermittent and chronic diarrhoea depending on severity. *C. pervum* causes cryptosporidiosis characterized by chronic diarrhoea while *E. histolica* is known to cause

amebiasis with symptoms including amebic colitis, bloating, bloody mucoid diarrhoea, cramps and fever (Hashmey *et al.*, 1997). Although, asymptomatic infection is common with immunocompetent individuals, immunocompromised individuals are more likely to experience various gastrointestinal complaints (Linscott, 2011).

Other microorganisms such as *Staphylococcus aureus, Candida albicans, Trichuris trichiura* and adenoviruses of bacterial, fungal, parasitic and viral origin respectively have also been implicated as potential causes of diarrhoea in both humans and animals.

2.4.2 Non-infectious Causes of Diarrhoea

1. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a disorder of the bowel functions, characterized by hypersensitivity (about 60%) that could manifest in varying degrees of severity. The muscle lining of the intestine normally relaxes and contract intermittently to move chyme along the digestive tract. However, this pattern is compromised in IBS patients resulting in various discomforting symptoms that mainly include diarrhoea and/or constipation, bloating, mucus in stool, incomplete gastric emptying, abdominal pains etc. (Barbara *et al.*, 2014). In more severe cases, IBS may be associated with some non-intestinal symptoms such as sexual dysfunction (lack of libido or pain during intercourse), fibromyalgia syndrome, headache, muscle pains, spasms and so on (Barbara *et al.*, 2014).

The exact cause of IBS is unknown. However, some predisposing factors include infection, altered intestinal microflora, hormonal changes which is sometimes aggravated by stress. Medications are targeted at symptoms while dietary adjustment and psychological intervention could also be employed (Bohn *et al*., 2015).

2. Small Intestine Bacteria Overgrowth

Small intestine bacteria overgrowth (SIBO) is the overgrowth of bacteria in the intestine ranging from 10^6 cfu/ml or more (Saad and Chey, 2014). It has been suggested as a possible cause of IBS but associated with symptoms such as flatulence, bloating, steatorrhea, abdominal distention, bloating as well as chronic diarrhoea. This could be as a result of increased gas production and osmotic load, deconjugated bile acids or toxic by-products following bacterial metabolism in the intestine (Ford *et al*., 2009; Quigley, 2014).

Intestinal microflora may also be altered by antibiotic therapy that suppresses or eradicates a targeted population of microorganism while leaving the resistant microbes to flourish (Quigley, 2014).

3. Congenital microvillous disease

Congenital microvillous disease (CMD) is an autosomal recessive disorder of the intestinal epithelial cells characterized by morphological enterocyte disorder which manifests as a life threatening, severe watery diarrhoea. It is a very rare disorder of unknown origin that causes completely irreversible intestinal failure secondary to diarrhoea. CMD has a very early onset usually within the first few days to about 2 months, patients develop metabolic acidosis, cardiac abnormalities, severe hypernatremia and dehydration (Croft *et al*., 2000).

4. Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder that causes a persistent lung infection, but also impairs the functional capacity of the liver, kidneys, pancreas and intestine. CF, a major cause of pancreatic exocrine failure in children usually arises from the mutation to the CFTR gene located on chromosome 7 which results into a defective cAMP-dependent chloride channel function (Abraham and Sellin, 2012). An increase in intracellular cAMP results in the active secretion of water and electrolytes

into the intestinal lumen causing secretory diarrhoea. Although, pulmonary disease happens to be the major cause of mortality and morbity, 80 – 90% of the patients encounter pancreatic insufficiency and suffer from gastrointestinal symptoms (Walkowiak *et al*., 2008). CF affects exocrine glands, causing them to make thick and sticky secretions thereby blocking the absorption surface of the intestine and pancreatic secretory ducts (Cunningham and Taussig, 2013). This leads to digestive and gastrointestinal problems.

5. Celiac Disease

Celiac disease (CD) is a systemic autoimmune disorder triggered by the ingestion of gluten in genetically susceptible individuals (Lauret and Rodrigo, 2013). It is characterized by inflammation and/or irritation of the small intestine which causes flattening of the epithelial cells and villous atrophy that leads to malabsorption of nutrients (Schuppan *et al*., 2009; Lauret and Rodrigo, 2013). The malabsorption of nutrients leads to the absorption of water into the intestinal lumen through osmosis thereby, causing osmotic diarrhoea (Cunningham and Taussig, 2013). Gluten is a complex protein found in barley, wheat, rye, sorghum and other related grains. Symptoms include chronic diarrhoea, weight loss, abdominal distention, bloating while anaemia, lethargy, stunted growth, osteoporosis and infertility are long term consequences (Schuppan *et al*., 2009). Withdrawal of offensive agent as well as a maintenance of gluten-free diet will end symptoms while the damaged intestine completely heals within 6 – 24 months (Jabri and Sollid, 2009).

6. Castor Oil

Castor oil otherwise known as ricinus oil is a triglyceric fatty acid that occurs in castor plant (*Ricinus communis*). For centuries, castor oil has been used therapeutically either as a stimulant, laxative or its oxytocic effect amongst others

(Tunaru *et al.*, 2012). It is obtained by pressing the seeds of castor plant and can be extremely toxic to humans and animals though, the leaves contain lesser toxins. Symptoms include gastrointestinal and neuromuscular disorders that could persist for several hours. However, application in trace amounts could function as a vaccine for poisoning (Queensland, 2016).

Highly unique for its remarkably near constant composition of fatty acids which include 89.5% ricinoleic acid (12-hydroxyoleic acid), 4.2% linoleic acid, 3.0% oleic acid, 1% stearic and palmitic acid, 0.7% dihydroxystearic acid and 0.3% of linolenic and eicosanoic acid (Burdock *et al.*, 2006). Castor oil has an excellent emollient and lubricating properties. It also has a relatively high viscosity which is uniform and reliable hence, its long-term use as an absolute standard for viscosity (Burdock *et al.*, 2006).

On ingestion, castor oil is digested by the intestinal lipases to release ricinoleic acid, which is easily absorbed by the small intestine. Castor oil induces diarrhoea due to its metabolite ricinoleic acid through the following mechanism;

a) irritation and inflammation of the intestinal mucosal which results in the release of prostaglandins (Ammon *et al.*, 1974);
b) decrease in activity of Na-K ATPase (Ammon *et al.*, 1974);
c) alteration of intestinal water and ion transport flux through the modulation of mucosal cAMP (Ammon *et al.*, 1974);
d) cytotoxic effect on the intestinal epithelial cells (Stewart *et al.*, 1975);
e) alteration in the structural integrity of the intestinal mucosa villi;
f) induction of pro-contractile effects on the intestinal smooth muscles;
g) activation of secretory processes as well as the perturbation of intestinal motility (Stewart *et al.*, 1975).

2.5 Pathophysiology of Diarrhoea

Several cases of diarrhoea have been reported however, the main underlying mechanisms for diarrhoea disease are described below.

2.5.1 Secretory Diarrhoea

This occurs when there is a shift in the balance between secretion and absorption process in the small intestine. It leads to excessive secretion of fluid by the intestinal mucosa which might be due to the release of enterotoxins or cytotoxins (e.g cholera toxin, shigatoxin) causing the activation of specific secretory pathways or inherent anomaly of the enterocyte such as CMV atrophy (Palowski *et al*., 2009). In most cases, the absorption mechanism is still intact but usually overwhelmed thereby causing diarrhoea. Secretory diarrhoea is characterized by high stool volume output, insensitivity to fasting and intact nutrient absorption process (Field, 2003). Mechanisms include the activation of intracellular mediators (such as cAMP, cGMP, calcium) that stimulates the cystic fibrosis transmembrane conductance regulator (CFTR) mediated chloride ion secretion from the crypt cells and inhibit the absorption of coupled sodium chloride with a net intestinal secretion (Chang and Leung, 2014). In most cases, secretory diarrhoea is infectious however, non-infectious causes of secretory diarrhoea also exist (Navaneethan and Giannella, 2010).

Microbial causes include the norovirus, rotavirus, ETEC, *V. cholerae* and *Giardia* which acts by adhering to the mucosal layer to disrupt the secretory/absorptive process of enterocytes. This favour the active secretion of the intestinal mucosa without causing destruction or acute inflammation hence the term noninflammatory diarrhoea (Navaneethan and Giannella, 2010). Some non-infectious causes include malabsorbed bile salts and fatty acids, peptide hormones and neurotransmitters (such as bradykinin, acetylcholine, substance P, serotonins, histamines, prostaglandins) that acts by stimulating intestinal secretion (Navaneethan and Giannella, 2010).

2.5.2 Osmotic Diarrhoea

This occurs where there is the excessive presence of osmotically active substance such as sugar (lactulose, sorbitol) or salt (magnesium sulphate) in the lumen that passively absorbs fluid down the osmotic gradient till it exceeds the absorptive capacity of the gut (Whyte and Jenkins, 2012). In this case, diarrhoea ceases when the offending agent has been withdrawn. Increase in osmotically active substances in the lumen could be due to the;

1. ingestion of non-absorbable or poorly absorbed solutes such as sugar alcohols (mannitol and sorbitol) or osmotic laxatives such as lactulose, magnesium salts, sulphates and phosphates (Whyte and Jenkins, 2012).
2. malabsorption or maldigestion of specific solutes such as in the case of lactose intolerance, glucose-galactose malabsorption and disaccharide deficiency.
3. damaged enterocytes and mucosa thereby dampening the absorption process as is the case with Crohn's disease, Celiac disease and acute gastroenteritis (Whyte and Jenkins, 2012).
4. motility disorders as found in hyperthyroidism, IBS, gastroschisis that causes reduced contact with the lumen and consequently, higher concentrations of luminal solutes (Whyte and Jenkins, 2012).

2.5.3 Inflammatory Diarrhoea

This may occur as a result of destruction of epithelial cells, compromised tight junctions, loss of resorptive area of the mucosa membrane and the release of inflammatory cytokines that stimulates fluid secretion (Navaneethan and Giannella, 2010). Inflammatory diarrhoea could arise from a range of etiologies which include but not limited to ulcerative colitis, irritable bowel disease, infections amongst others. Infectious pathogens cause inflammatory diarrhoea via increased production of cytotoxins or invasion of intestinal epithelial cells which triggers the release of inflammatory cells. Examples of cytotoxin producing non-

invasive organisms include EHEC, EPEC, *C. difficule* while the invasive microbes include *S. flexineri, Yersinia* spp., *E. histolytica, C. jejuni* and *S. typhi* (Navaneethan and Giannella, 2010).

Most of these pathogens inflict intestinal mucosal damage, stimulate intestinal secretion, elaborate enterotoxins (which stimulates secretion) or facilitate the synthesis of inflammatory mediators (which further enhances intestinal secretion as well as mucosal damage). These series of event lead to the production of immature cells (to replace the epithelial cells destroyed) that are deficient in brush-border enzyme and transporters necessary for the optimal absorption of nutrients and water (Palowski *et al*., 2009).

2.5.4 Motility-Related Diarrhoea

This occurs when there is an alteration in intestinal motility which can be a rapid movement (hypermotility) or decreased contractile forces, hence, slower transit (hypomotility). The normal GIT function is a very swift system capable of adjusting to shift in volumes due to food intake, further breakdown of larger particles ingested, movement of chyme to nutrient absorptive sites and expulsion of waste products. These alterations in intestinal motility may cause deleterious changes in absorption process, transport, nutrient intake and feacal output (Whyte and Jenkins, 2012).

Increased intestinal motility implies reduced transit time which translates into poor absorption of water and electrolytes. However, hypomotility could lead to bacterial overgrowth, deconjugation of bile salts or dysentery all of which are predisposing factors to diarrhoea (Sachdev and Pimentel, 2013). Hypermotility is common amongst people that have undergone colectomy or small bowel resection due to the reduction in length of the small intestine (Brown *et al.*, 2015).

2.5.5 Oxidative Stress-Related Diarrhoea

Oxidative stress is the systemic imbalance between the generation of free radicals [reactive oxygen species (ROS) and reactive nitrogen species (RNS)] and the antioxidant defense system (Nita and Grzybowski, 2016). Intestinal production of free radicals may result from the systemic response to a variety of factors such as irritants, infections, inflammation and/or depleted antioxidant system all of which have been implicated as potential causes for diarrhoea (Suzuki *et al.*, 2012).

Intestinal oxidative stress could manifest from lipid peroxidation which facilitates the aberration of intestinal cellular and membrane functions. Also, some of these reactive species could autonomously act as secretagogues or provoke the release of neurotransmitters (acetylcholine and NO) that functions to stimulate increased contraction and motility of the intestine (Nardi *et al*., 2007). Similarly, these sequences of event might trigger the release of inflammatory mediators such as prostaglandins known to provoke diarrhoea (Chang and Leung, 2014).

2.5.6 Other Variants of Diarrhoea

2.5.6.1 Iatrogenic Diarrhoea

Iatrogenic diarrhoea is suspected following the use of new medications or surgical procedure (Yueh *et al.,* 2014). It is also known as drug-induced diarrhoea. In 5 – 10% of cholecystectomy patients, diarrhoea usually occurs as a consequence to the procedure (Chassany *et al*., 2000). Several drugs have been implicated as potential causes of diarrhoea including some homeopathic medications. The influence from these drugs may be as part of their pharmacologic effects, allergic reactions or the expected physiological response to the drug. Some of the group of drugs known to stimulate diarrhoea include alpha-glucosidase inhibitors, osmotic laxatives, NSAIDs, anticholinergics, acetylcholinesterase inhibitors, HMG-CoA reductase inhibitors, antibiotics, theophylline etc. (Jong *et al*., 2012).

a) Acetylcholinesterase inhibitors prevent the breakdown of acetylcholine which stimulates intestinal motility.
b) Osmotic laxatives may stimulate a net secretion of fluids over reabsorption
c) Antibiotics may alter the intestinal microflora which might promote SIBO or dampen colonic salvage of poorly absorbed carbohydrates that consequently leads to diarrhoea.
d) Theophylline, a phosphodiesterase (PDE) inhibitor causes diarrhoea by preventing the regulation of cAMP by PDE. This results in the opening of chloride channels and increased secretion (Abraham and Sellin, 2012).

On suspicion of drug-induced diarrhoea, the easiest solution is to discontinue the use of the drug for alternative medications or treat with antidiarrhoeal agents (Abraham and Sellin, 2012). Although, diarrhoea may resolve with continued use in some cases (Riddle *et al.,* 2016).

2.5.6.2 Fatty Diarrhoea

It is caused by improper digestion or malabsorption of nutrients and clinically associated with steatorrhea and weight loss (Hackert *et al.,* 2014). Some of the major causes of steatorrhea includes cystic fibrosis, chronic pancreatits, Whipple's disease, lactose intolerance and drugs like cholestyramine, orlistat, highly active antiretroviral therapy with about 60 – 80% certainty (Hackert *et al.,* 2014).

2.5.6.3 Idiopathic Diarrhoea

Diarrhoea is said to be idiopathic when the underlying cause remains a mystery (Abraham and Sellin, 2012). It is usually chronic in nature and would require various tests and treatments to rule out potential underlying causes or disease (such as IBS, Addison's disease, Celiac disease, food poisoning) (Abraham and Sellin, 2012).

2.5.6.4 Factitious Diarrhoea

This is an intentional or unintentional self-induction of diarrhoea usually arising from the abuse of laxatives (Abraham and Sellin, 2012). Although, not all cases of factitious diarrhoea involve the abuse of laxatives, some occur as a result of unawareness in the relationship between laxatives and diarrhoea. Also, factitious diarrhoea could be due to the ignorance of a drug user to the capability of a drug to induce diarrhoea (usually as a side effect) or sometimes as a result of individual idiosyncrasy. About 4% of chronic diarrhoea patients may be categorized as factitious diarrhoea (Abraham and Sellin, 2012).

2.5.6.5 Functional diarrhoea

Functional diarrhoea is a gastrointestinal disorder characterized by chronic or recurrent diarrhoea without structural or biochemical alterations (Schiller, 2004). It is characterized by the presence of loose or watery stool in about 75% stool or more without pains (Longstreth *et al*., 2006). It is often lumped up with IBS-diarrhoea but the distinguishing factor between IBS-diarrhoea and functional diarrhoea is the presence of pains (Camilleri *et al.*, 2015).

2.5.6.6 Pseudo-diarrhoea

Pseudo-diarrhoea, also known as hyper-defecation is the increased frequency of stooling but still within the daily weight range of < 300 g (Mitsuhashi *et al.,* 2018). The frequent passing of loose or watery stool by breast fed babies can also be categorized as pseudo-diarrhoea. This can be attributed to their dietary intake that lacks roughages, thereby, nullifying the possibility of formulating hardened stool in their GI system. Feacal impaction or vaginal pessary (a removable device placed in the vagina to support pelvic organ prolapse) could cause pseudo-diarrhoea due to renal obstruction that allows the passage of only liquid stool (Tatar and Trivedi, 2005).

2.6 Clinical Manifestations of Diarrhoea

Diarrhoea is usually characterized by increase in loss of water and electrolytes both of which can be critical to the health and/or survival of affected individuals. The former leads to dehydration while the latter causes electrolyte imbalance (Muhsin and Mount, 2016).

2.6.1 Dehydration

Dehydration can be described as the excessive loss of water by the body to compensate for the normal and regular physiological activities of the body. Dehydration resulting from acute watery diarrhoea is one of the major causes of diarrhoeal death. It leads to the impairment in the functioning of vital organs most especially the kidney (Padlipsky, 2014). Dehydration also causes hypovolemic shock, metabolic acidosis, cardiovascular failure and ultimately death (Padlipsky, 2014). Dehydration can be categorized into three based on the concentration of sodium in the blood; isotonic, hypertonic and hypotonic (RP, 2017).

1. Isotonic dehydration: The osmolar balance in extracellular fluid during dehydration, characterized by a balanced deficit of water and sodium level is known as isotonic dehydration (Kenefick *et al*., 2012).
2. Hypertonic dehydration: It occurs when there is a loss of water in excess of sodium thereby causing hypernatremia. It usually results from the improper replacement of lost fluid and electrolytes, inefficient absorption or intake of hypertonic fluids thereby increasing the serum concentration of sodium. It is the most common among infants with symptoms such as severe thirst and irritation while seizures may occur (Kenefik *et al.,* 2012).
3. Hypotonic dehydration: This is the loss of electrolytes in excess of water. It is similar to hypertonic in that it occurs mostly in children but differs by the deficit of salt in excess of water. It may occur as a consequence of isotonic dehydration from the

intake of replacement fluids that does not properly compensate for the lost salt. Lethargy and seizures are some of the known symptoms.

Dehydration can also be categorized as mild, moderate or severe based on the amount of lost fluid (Kenefick *et al*., 2012; Padlipsky, 2014).

Mild dehydration: Dehydration is described as mild when fluid deficit is less than 5% of the body weight and symptoms include thirst, decreased skin turgor, sunken eyes, lack of tears and dry mucous membrane (Kenefick *et al*., 2012).

Moderate dehydration: When fluid deficit is greater than 5 but less than 10% of body weight, dehydration is moderate. Signs at this stage include some symptoms of hypovolemic shock such as anuria, extreme sensitivity to temperature changes, feeble and rapid pulse, diminished consciousness. The symptoms worsen as water deficit increases and becomes severe.

Severe dehydration: When water deficit is in excess of 10% body weight, it leads to severe dehydration. Severe dehydration is characterized by sunken eyes, absence of tears, intense thirst, dry mucus membrane, anuria, tachycardia, delirium, shrivelled skin and delayed skin turgor (Kenefik *et al.*, 2012). It is a life threatening medical emergency that rapidly leads to death as a result of collapse in circulatory function (Kenefick *et al*., 2012).

2.6.2 Electrolyte Imbalance

Electrolytes are electrically charged minerals found in bodily fluids, tissues and sometimes in urine. Major examples include sodium ion (Na^+), potassium ion (K^+), chloride ion (Cl^-), bicarbonate (HCO_3^-) while others such as calcium ion (Ca^{2+}), magnesium ion (Mg^{2+}) and phosphate ion (PO_4^{2-}) also exist. They are essential for survival as they are vital to the proper functioning of various human organs and tissue such as nerve and muscle function, blood pressure and pH, acid-base balance as well as the regeneration of damaged tissues (Nordqvist, 2016). They are required at a specific range which cannot be exceeded or limited in order to maintain balance. Electrolyte imbalance is a shift in concentration gradient due to

influx or extrusion of electrolytes between the intracellular and extracellular compartment (Reddy and Stutts, 2013). Examples include hypernatremia, hyponatremia, hyperchloremia, hypokalemia, hypocalcemia etc. It can manifest from conditions such as excessive sweating, exercise, vomiting, diarrhoea, dehydration, kidney disease, congestive heart failure (Reddy and Stutts, 2013). Without proper replacement, different symptoms (such as seizure, muscle spasm, bone disorders, nervous system disorder, convulsion, lethargy, twitching, fatigue) can occur depending on the electrolyte out of balance and the level (high or low) (Nordqvist, 2016).

2.7 Mode of Transmission

Diarrhoea is usually spread by the faecal-oral route, which includes the ingestion of contaminated water or food, person-to-person transmission, and direct contact with infected faeces (CDC, 2016). Examples of behaviours that aid the spread of the diarrhoea (Figure 3) include but not limited to;

1. preparing food with hands that have been soiled during defecation and not washed (most especially among food vendors);
2. allowing an infant to crawl, or a child to play in an area where human or animal faeces are present;
3. planting, irrigation and washing of farm products with contaminated water;
4. drinking and cooking from a contaminated water source;
5. diversion of attention by caregivers to other business without proper cleaning up thereby contaminating everything they come in contact with (Rissman, 2015).

Other predisposing factors of diarrhoea include diseases (IBS, hyperthyroidism, Addison's disease), early introduction of milk formula or solid foods to toddlers with immature digestive tract to metabolize the food materials (a common practice among career women), lack of maternal education and care on the predisposing factors of diarrhoea and how to

manage it. This exist mainly amongst the rural communities where the same water source for cooking is used for every other domestic activity such as bath, washing etc. Malnutrition, usually perceived as consequential to diarrhoea could also be a cause of diarrhoea (Nel, 2010). It leads to lack of nutrients, weight loss, stunted growth and ultimately weakened immune system that promotes high susceptibility to causative agents (Nel, 2010). Diarrhoea is usually more frequent, severe and prolonged in malnourished children (CDC, 2016).

2.8 Prevention and Treatment Options

Diarrhoea is a completely preventable and also curable. There is no specific treatment option for diarrhoea but rather, treatment is targeted at the aetiology of the disease as well as symptoms (Riddle *et al.*, 2016).

2.8.1 Implementation of Water, Sanitation and Hygiene Initiative

The lack of access to safe water, poor water supply, sanitation (to facilitate the use of toilets and prevent open defecation), personal and domestic hygiene are some of the leading factors contributing to the onset of diarrhoea as well as various other diseases sequalae to diarrhoea (UNICEF, 2016). The easiest preventive measure for diarrhoea is the implementation of the WASH initiative aimed at the availability of quality water, building of toilets and nurturing of hygienic practices especially handwashing with soap (UNICEF, 2016).



Figure 3: Actions predisposing to diarrhoea

Source: Rissman (2015)

This promotes the prevention and control of transmission of communicable diseases such as diarrhoea. The impact of effective deployment of WASH intervention cannot be overestimated, 68% of the world population use improved sanitation. Furthermore, 91% of the world population now have access to safe water (WHO, 2015).

2.8.2 Oral Rehydration Therapy

This is the replacement of lost fluids and electrolytes to prevent and/or correct dehydration resulting from diarrhoea (Bhan *et al.*, 2012). It is a relatively safe, effective, cheap and easy treatment method that involves the use of oral rehydration salts (ORS) for the prevention of dehydration, replacement of lost fluids, maintenance of nutrition and personal hygiene. ORSs are basically made up of glucose and electrolytes to aid intestinal absorption of water and electrolytes. The constituents of a standard ORS include 75mEq/l sodium, 20 mEq/l potassium, 65 mEq/l chloride, 75 mmol/l glucose an 10 mmol/l citrate to give a total osmolarity level of 245mOsm/l (Routray, 2017). Despite the substantial decrease in diarrhoeal deaths due to dehydration, ORSs still has a limited use in most developed and developing countries (Bhan *et al.*, 2012). Since the adoption of ORS in 1978 as the primary tool to combat diarrhoea, mortality rate from acute diarrhoea had decreased from 5 million to 1.3 million deaths annually (Routray, 2017). However, an increased incidence of transient hyponatremia may occur. The dose of ORS administered is a function of the degree of dehydration. An easy homemade ORS consists of 8 teaspoons of sugar, 1 teaspoon of table salt dissolved in 1 litre of water to which a half cup of orange juice or mashed bananas can be added to improve taste and available electrolytes (WHO, 2015).

2.8.3 Zinc Supplementation

Zinc, a potent prooxidant and anti-inflammatory agent is a vital micronutrient required for cell growth, differentiation and protein synthesis in humans. It also stimulates improved resistance to infections and enhances pathogen clearance (Patel *et al*., 2010; WHO, 2017).

Zinc plays a vital role in intestinal epithelial barrier function (Skrovanek *et al.,* 2014). Deficiency in zinc levels has been attributed to diarrhoea. Some of the established mechanisms of zinc in relation to diarrhoea include;

a) inhibition of cAMP-induced chloride dependent fluid secretion;
b) improved regeneration of intestinal epithelium;
c) improvement in absorption of water and electrolytes;
d) improved immune response and;
e) increase in the activity of brush border enzymes (Walker *et al*., 2009).

Zinc supplementation has been reported to reduce the duration, severity and prevent subsequent episodes of diarrhoea though with increased risk of vomiting. If zinc supplements (mostly available as zinc gluconate, sulphate or acetate) are administered within 10 – 14 days, it significantly decreases the incidence of diarrhoea for about three months (UNICEF, 2010).

2.8.4 Vaccination

Vaccination against known pathogens that causes diarrhoea (especially, rotavirus, *V. cholerae, Salmonella spp.*) has been clarified as an effective way of combating diarrhoea (Venkatesan and Van de Verg, 2015). It provokes the formation of protective antibodies (IgA) which does not only prevent disease but also mitigate the spread of antibiotic resistance. A study by Moor *et al*. (2017) on salmonella-based diarrhoea showed that vaccine-induced high-avidity IgA effectively protected the intestine against pathogens by enchaining growing bacteria and clumped bacteria daughter cells to each other during meiosis. Although, replication process remains effective, the new generations are trapped due to agglutination of the daughter cells. This, prevents the imminent attack on intestinal tissues and promotes effective clearance of pathogen. The IgA driven clump formation also prevents the exchange of genetic materials among bacteria population thereby mitigating or completely eradicating the incidence of resistance crisis (Moor *et al*., 2017).

Evidence of successful vaccinations against other known pathogens such as rotavirus, ETEC, *V. cholerae* also exist in open scientific literature (Soares-Weiser *et al*., 2012; O'Ryan *et al*., 2015; Venkatesan and Van de Berg, 2015) which is important for the successful prevention and spread of diarrhoeal disease.

2.8.5 Probiotics

They are live microbes of beneficial effects, capable of promoting healthy digestive and immune system while also preventing or treating some illness of the GIT (de Vrese and Offick, 2010). Probiotics can be supplied via beverages, foods or as dietary supplements. Various strains of probiotics (such as *Lactobaccilus acidophilus, Bifidobacteria bifidum, Saccharomyces boulardi*) have been reported to inhibit the growth, metabolic activity and adhesion of enteropathogenic microorganisms (*V. cholerae,* ETEC, *Shigella* or *Salmonella spp.*) to the intestinal cells. Proposed mechanisms include:

i. production of bactericidal substances such as acetic, butyric and lactic acids, hydrogen peroxide which effectively kills the pathogens in the gut (Hemaiswarya *et al*., 2013);
ii. decreasing intestinal pH which makes the gut more acidic thereby making it difficult for the survival of harmful bacteria (de Vrese and Marteau, 2007);
iii. adhering to the mucosal layer surface also efficiently prevents the adhesion of harmful bacteria to the intestinal mucosa which effectively prevents them from causing illness (Ribet and Cossart, 2015);
iv. releasing of gut-protective metabolites such as SCFA, conjugate-linoleic acids, arginine and glutamine which alleviates or prevent any harmful effect the intestinal microbes might possess (Coakley *et al*., 2003); and

v. competing for digestible substances and adhesion to the mucosal layer also serves as beneficial effect of probiotics over the harmful organisms (de Vrese and Marteau, 2007).

Other known effects include the engulfment of harmful microbes, regulation of intestinal motility and mucus production, immunomodulatory responses, strengthening of the barrier function on intestinal mucosa as well as the neutralization of toxic metabolites from harmful microorganisms (Coakley *et al*., 2003; de Vrese and Marteau, 2007; Ribet and Cossart, 2015).

2.8.6 Medications

Several over-the-counter drugs have been promoted as antidiarrhoeal agents. Loperamide hydrochloride, atropine sulphate and bismuth subsalicylate stand-out with a reliable and sufficient evidence of efficacy, however, not without some known side effects (Farthing *et al.*, 2013).

2.8.6.1 Loperamide Hydrochloride

Loperamide hydrochloride, an opiate analogue is one of the most widely recommended over-the-counter antidiarrhoeal drug used. It has a molecular weight of 477.045 g/mol, and can also be identified by its molecular formula $C_{29}H_{33}ClN_2O_2$ (Figure 4). It is scientifically known by the International Union of Pure and Applied Chemistry (IUPAC) as 4-(p-chlorophenyl)-4-hydroxy-N,N-dimethyl-a,a-diphenyl-1-piperidinebutyramide monohydrochloride (Elqudaby *et al.*, 2014).

Loperamide differs from other opiates such as diphenoxylate due to its inability to cross the blood brain barrier which makes it incapable of the opiate-like stimulatory effect on the central nervous system (Laveeri *et al*., 2016). It is a symptomatic treatment method targeted at the alleviation of the condition with little or no effect on the primary cause of diarrhoea. Some of the known mechanisms of action of loperamide hydrochloride include;

i. the inhibition of intestinal motility;

ii. inhibition of intestinal secretions in excess of absorption;

iii. modulation of water and electrolyte movement across the bowels;

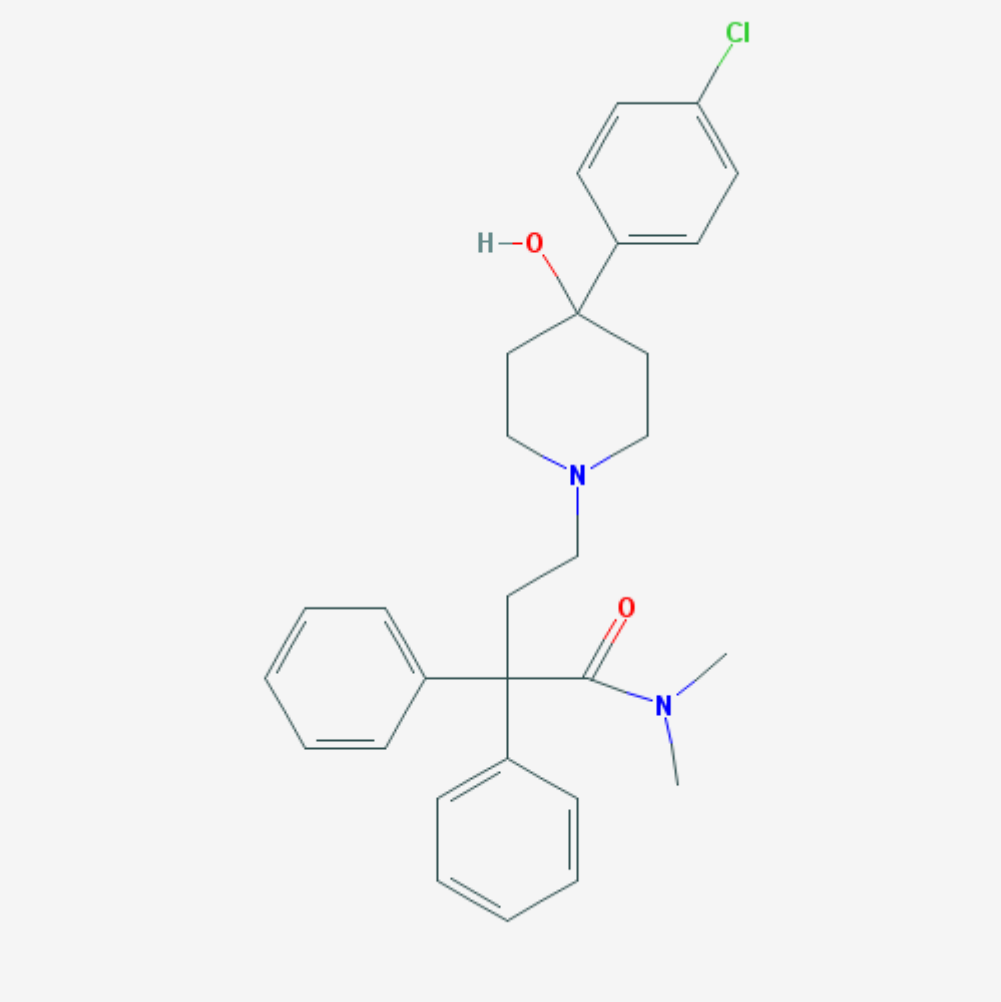


Figure 4: Loperamide hydrochloride

Source: Elqudaby *et al.* (2014)

iv. binding of opiate receptors on the intestinal mucosal which inhibits the release of acetylcholine and prostaglandins, this leads to reduced peristalsis and prolongs transit time to facilitate effective reabsorption process (Laveeri *et al*., 2016).

Though, very effective and potent for both infantile and adult diarrhoea of various pathophysiology, loperamide hydrochloride also causes some unpleasant side-effects such as constipation, abdominal pain and cramps, nausea, emesis, fatigue, dizziness amongst others (ASHSP, 2016). Some of the contraindications of the drug include hypersensitive patients, acute dysentery, ulcerative colitis, bacterial enterocolitis and pseudomembranous colitis (ASHSP, 2016).

2.8.6.2 Bismuth Subsalicylate

Bismuth subsalicylate is another over-the-counter drug used for the treatment of diarrhoea, heartburn, nausea, indigestion, stomach upset and other gastrointestinal disorders (Boyd *et al.,* 2014). It acts as an antacid, with a molecular weight of 363.101 g/mol. Bismuth subsalicylate (molecular formula: $C_7H_6BiO_4$) is also identified by its IUPAC name, {2}-benzodioxabismin-4-one monohydrate. It has been reported to possess some antibiotic and anti-inflammatory activity (Boyd *et al.,* 2014).

It is hydrolysed in the stomach to yield salicylate which is readily absorbed and bismuth salt which is weakly absorbed. The bismuth salt coats the walls of the mucosal layer thereby preventing the adhesion of microorganisms. It has a protection rate of about 65% that diminishes after three weeks (Graham and Lee, 2015). Similar with loperamide, bismuth subsalicylate is also symptomatic and has no effect on the causative agent. Some of its known side-effects include black tongue and stool, constipation, anxiety, dizziness, impaired hearing etc. (Graham and Lee, 2015).

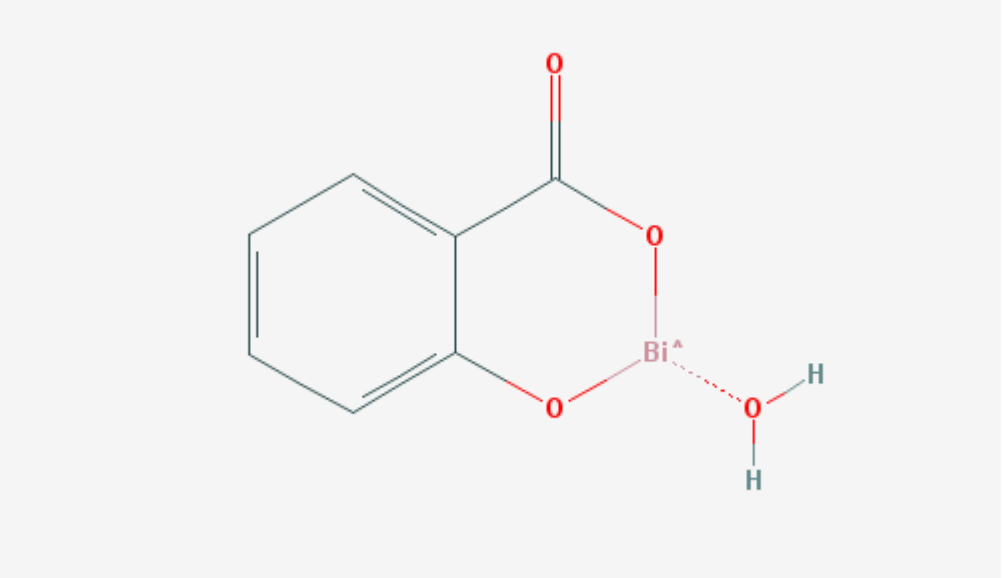


Figure 5: Bismuth subsalicylate

Source: Boyd *et al.* (2014)

2.8.6.3 Atropine Sulphate

Atropine sulphate is a naturally occurring alkaloid originally isolated from *Atropa belladonna* but found in other plants of the family Solanaceae. It has a molecular weight of 387.447 g/mol, chemical formula $C_{17}H_{25}NO_7S$ and IUPAC name; (8-methyl-8-azabicyclo-[3.2.1]-octan-3-yl)-3-hydroxy-2-phenylpropanoate;sulfuric acid (Arvadiya and Dahivelkar, 2013).

It functions mainly as a competitive antagonist of muscarinic cholinergic receptors thereby inhibiting completely, the effect of acetylcholine and other choline esters on the smooth muscles (Kruse *et al.*, 2012). This leads to reduction in the cholinergic effect of acetylcholine therefore decreasing peristaltic activity in the small intestine. Some of the known side effects include, ataxia, blurred vision, tachycardia, restlessness, palpitations and sometimes anhidrosis in adults (inability to sweat properly which might lead to overheating or sometimes hyperthermia; a potentially fatal condition) (Kruse *et al.*, 2012). Toxic doses could lead to circulatory function collapse, delirium, hallucinations, paralysis, coma or even death (Fosnes *et al.*, 2011).

2.8.7 Phytotherapy

Plants have been a mainstay in the treatment of diseases and healing practices since time immemorial (Sofowora *et al.*, 2013). In many developing and developed countries alike, traditional medicines have assumed an important role in major health-care services with most medications being of natural origin (WHO, 2013). Plants have continued to provide directly usable drugs and a great variety of chemical compounds which may serve as precursors for pharmaceutical chemo-synthesis with improved pharmacological properties (Ballabh, 2008; IUCN, 2011).

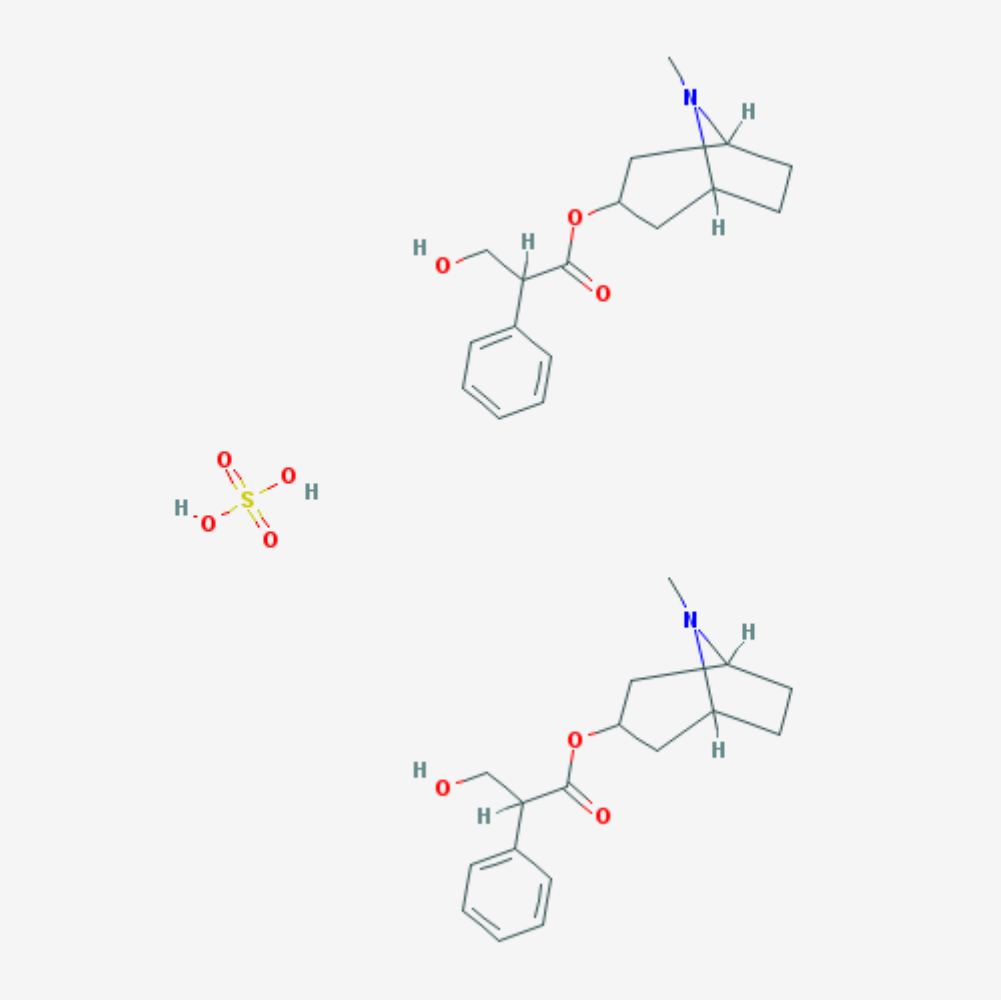


Figure 6: Atropine sulphate

Source: Arvadiya and Dahivelkar (2013)

An estimated 80% of the world population rely on herbal medicine for their primary health care. In order to overcome the menace of diarrhoea in developing countries, the World Health Organization has introduced a programme for diarrhoea control, which involves the use of traditional herbal medicines (WHO, 2009). This is partly due to their economic viability, easy accessibility, ancestral experience and perceived efficacy (WHO, 2009; WHO, 2013). Plant extracts, formulations, or pure natural compounds have been deployed in the control of diverse diseases ranging from cancer, diabetes, malaria, coughs, inflammation and diarrhoea to infections in human and veterinary medicine. Several plants have been explored for their antidiarrhoea activity by herbal medicine practitioners with some already validated with scientific evidence (Gutierrez *et al*., 2007). However, the active components which form the basis for the biosynthesis of pharmaceutical products are largely unknown. The table below shows a number of plants with antidiarrhoea activity and their mode of action (Table 2).

2.8.7.1 *Mangifera indica*

Mangifera indica is a member of the family Anacardiaceae. It is commonly known as mango (English), *aam* (Hindi), *mangue* (French), *Mi wang* (Chinese), *mano* (Ghana), *manga* (Portuguese), *mangoro* (Yoruba – Western Nigeria), *mangwaro* (Hausa – Northern Nigeria) and *mangolo* (Igbo – Eastern Nigeria). Although, native to tropical Asia where it has been cultivated for over 4000 years (Ross, 1999), *M. indica* is now widely grown in different parts of Africa, especially the Southern and Western part of Nigeria where it is mostly valued for its quality fruits, nutritional and medicinal uses (Nwinuka *et al.,* 2008). *M. indica* is a large evergreen tree that grows between 15 to 30 m high, anchored by a long unbranched taproot that can descend to a depth of 6 – 8 m in the ground. It bears green leaves that could be red or yellow (prior to maturity), the leaves (Plate 1) are about 29 – 30 cm long with tiny red or yellowish – green flowers. The fruits vary in colour (green, yellow or red), shape (egg or

kidney shaped) and size with over a thousand varieties available globally (Nwinuka *et al.*, 2008).

Table 2: Medicinal plants with antidiarrhoeal activity and their mode of action(s)

S/N	*Medicinal plant*	Family name	Plant part used	Active component	Mode of action	Reference
1	*Trilepisium madagascariense*	Moraceae	Stem bark	Isoliquiritigenin	Antisecretory	Teke *et al.* (2010)
2	*Pycnocycla spinose*	Umbelliferae	Leaves	6-(4-hydroxy-3-methoxyphenyl)-hexanonic acid	Antispasmodic	Sadraei *et al*. (2014a)
3	*Chiranthodendron pentadactylon*	Malvaceae	Leaves	Epicatechin	Antisecretory	Velazquez *et al*. (2012)
4	*Eriosema chinense*	Leguminosae	Roots	Lupinifolin	Antisecretory, antimotility and antioxidant	Prasad *et al*. (2013)
5	*Salvia ballotiflora*	Acanthaceae	Not specified	19-deoxyicetexone	Increased reabsorption	Perez-Gutierrez *et al*. (2013)
6	*Pycnocycla spinose*	Umbelliferae	Leaves	Isovanillin and iso-acetovanillon	Antimotility	Sadraei *et al*. (2014b)
7	*Trichilia emetica*	Meliaceae	Not specified	Polyphenols	Antioxidant and antibacterial	Konate *et al*. (2015)
8	*Citrus tangerine*	Rutaceae	Leaves	Nobiletin	Antisecretory	Hao *et al.* (2015)

The ethnomedicinal uses of the plant across different cultures are numerous with various parts of the plant being used as astringent, antioxidant, antiseptic, diaphoretic, stomachic, vermifuge and immunomodulator (Nwinuka *et al.*, 2008). *M. indica* can also be used for the treatment of dysentery, rheumatism, typhoid fever, diabetes, insomnia, anaemia, abscess, asthma, mouth infections, diarrhoea, sore throat, irritations, haemorrhage, scurvy, leucorrhoea, hypertension, scorpion and bee stings (Doughari and Manzara, 2008; Shah *et al*., 2010).

Some of the claims previously listed have been supported with scientific evidence. Hannan *et al.* (2013) reported the possibility of sourcing antibiotic substances from acetone leaf extract of mango since it demonstrated antibacterial activity against multidrug resistant *S. typhi*. Also, the ethanolic extract of *M. indica* leaves at 250 mg/kg body weight exhibited profound antidiabetic activity in streptozotocin-induced diabetic mice via stimulation of the release of insulin by the β-cells of the pancreas (Aderibigbe *et al*., 2001). Furthermore, mango fruit peels have been reported to be a novel route for the synthesis of silver nanoparticles (Yang and Li, 2013). The anti-inflammatory activity of the aqueous extract of *M. indica* stem bark was evaluated in experimental murine rats model. The extract found to contain mixtures of polyphenols and flavonoids attenuated colon shortening, body weight loss and diarrhoea all of which are colitis symptoms (Marquez *et al*., 2010). These effects were attributed to the capability of the extract to reduce the levels of inducible nitric oxide synthase (iNOS), α-tumor necrosis factor (α-TNF), cyclooxygenase-2 (COX-2) as well as the expression of tumor necrosis factor receptor-2 (TNFR-2) in colitic tissues while there was reduction in the serum levels of interleukin-6 (IL-6) and α-TNF (Marquez *et al.*, 2010).

Aqueous extract of *M. indica* leaves also exhibited profound antidiarrhoeal activity in castor oil-induced diarrhoea in female Wistar rats by significantly prolonging the onset time

of diarrhoea, decreasing volume of intestinal content and reducing the motility rate (Yakubu and Salimon, 2015).



Plate 1: ***Mangifera indica*** **tree showing the leaves**

2.9 Organs Studied

2.9.1 The Small Intestine

The small intestine is the part of the gastrointestinal tract located between the stomach and the large intestine. It serves as the primary digestive organ where majority of digestion and absorption takes place (Sherwood *et al.*, 2006).

2.9.1.1 Anatomy of the Small Intestine

The small intestine of a fully developed rat is about 100 cm in length, 5 g in weight and 4% of the body weight. The length could be doubled in a cadaver due to the loss of muscle tone (Thiel, 2012). The small intestine (Figure 7) is divided into three structural parts from proximal to distal end; duodenum, jejunum, and ileum (Townsend *et al.*, 2004). The duodenum, the first part of the small intestine with a length of approximately 95 – 100 mm connects the stomach to the small intestine (Vdoviakova *et al.*, 2016). It covers 10% of the total length of the small intestine. Also present in the duodenal wall is the hepatopancreatic ampulla commonly known as the Ampulla of Vater (Tortora and Derrickson, 2008; OpenStax, 2016). This is the point where the bile duct and pancreatic duct meets and open into the duodenum in a volcanic-shaped structure called the duodenal papilla. The jejunum measured at 890 – 1300 mm is the longest part of the small intestine in rats. It forms loops, fill the right part of the abdominal cavity ventrally and links the duodenum to the ileum (Vdoviakova *et al.*, 2016). There is no distinct demarcation between the jejunum and the ileum. At a length of about 20 – 30 mm, the ileum is the shortest part of the small intestine in rats (Vdoviakova *et al.*, 2016). Compared to the jejunum, the ileum is slightly enlarged and thicker. It has more vascular and developed mucosal folds that empties out to the cecum at the ileocecal sphincter (Vdoviakova *et al.*, 2016). The whole of the small intestine is surrounded by the large intestine which empties the bowel through the rectum and out of the anus (Figure 7).

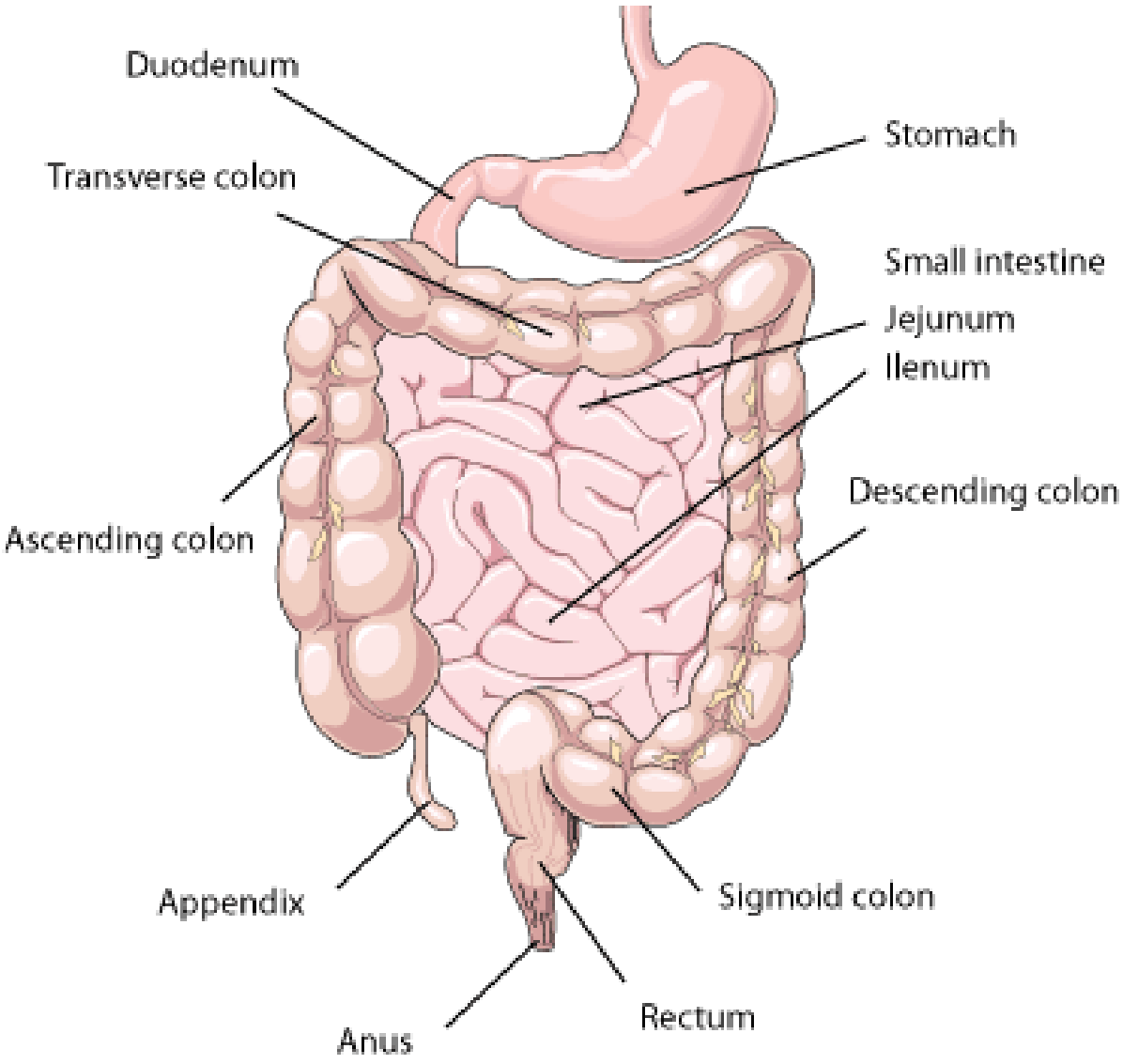


Figure 7: Anatomy of the small intestine

Source: Sherwood *et al*. (2006)

Similar to the alimentary canal, the small intestine is composed of four layers; the mucosa, sub-mucosa, smooth muscle and serosa (Tortora and Derrickson, 2008). However, the intestinal mucosa and submucosa have adaptive features that include; plicae circulares, villi and microvilli to increase the absorptive surface area of the small intestine by 600 folds or more (Helander and Fandriks, 2014).

2.9.1.2 Functions of the Small Intestine

The primary function of the small intestine is to aid the digestion and absorption of nutrients in chyme. However, it is also responsible for some other important functions some of which are as listed (OpenStax, 2016).

i. Neutralization: The small intestine is responsible for the neutralization of chyme from the stomach usually with a pH of 2 or less. This is achieved by the stimulation of the pancreas to release sodium bicarbonate and as a consequence, neutralizes the pH of chyme. Also, the bile juice and mucus have neutralizing effects and thus, protects the small intestine from acid burns.

ii. Microbial Protection: The epithelial cells of the bowels have antimicrobial cells (Paneth cells) which are responsible for the secretion of bactericidal lysozymes thereby killing pathogenic microorganisms that could provoke diarrhoea in the small intestine.

iii. Lipid Emulsification: The digestion of lipids starts majorly in the small intestine. It stimulates the release of bile which emulsifies and break down the lipids into smaller particles for proper digestion. Malabsorption may ensue from the improper digestion of lipids by the small intestine, often manifesting as steatorrhea. The unabsorbed fatty acids are converted to hydroxy fatty acids by intestinal flora which causes impairment in absorption and stimulate the secretion of water and electrolytes (Newnham, 2017).

iv. Digestion: The contraction of intestinal smooth muscles facilitates the mechanical breakdown of food. This is complimented by the release of digestive enzymes (lactase, peptidase, sucrase, maltase, lipase and enterokinase) for the chemical breakdown of carbohydrate, lipids and proteins to their monomeric units. Disorders in digestion could stimulate the absorption of water from the intracellular compartment to the extracellular compartment thereby, causing osmotic diarrhoea.

v. Absorption: About 95% of absorption process takes place in the small intestine. The absorption of water, electrolytes, vitamins, minerals, glucose, amino acids, free fatty acids (and other products of digestion) takes place in the small intestine via various transport mechanisms (diffusion, osmosis, active transport etc). Any derangement in absorption process could lead to an imbalance between the secretory and absorption process causing a net fluid secretion. This in turn leads to secretory diarrhoea.

vi. Immunity: The small intestine also plays an active role in the body immune system. The ileum contains Payer's patches which plays host to lymphocytes that are integral part of the lymphatic system. Furthermore, the intestinal microflora plays supportive role in the body's defense system. The loss of immunoprotective ability by the small intestine could make the organ vulnerable to pathogens capable of causing diarrhoea.

vii. Hormonal Secretion: Different parts and cells of the small intestine are specialized for the secretion of various hormones necessary to facilitate digestion. Some important hormones secreted by the small intestine include;

i) Intestinal gastrin - Stimulate the release of gastric acid

ii) Secretin - Regulation of water homeostasis and stimulate the release of bicarbonate by the pancreas.

iii) Motilin - Stimulation of intestinal peristalsis and production of somatostatin

iv) Cholecystokinin – Suppression of hunger and the release of pancreatic juice and bile into the intestine.

v) Glucose-dependent insulinotropic peptide - Stimulates the release of insulin.

2.9.2 The Liver

This is the large lobed glandular organ of vertebrates, involved in various metabolic processes. It is one of the most important organs in the body.

2.9.2.1 Anatomy of the Liver

The liver is a wedge shaped, soft, flabby, reddish brown organ suspended under the diaphragm. It is composed of four lobes (the left, right, middle and caudate lobes) (Figure 8) all of which can be further divided into lobules (Tortora and Derrickson, 2008). The left and middle lobes form a single lobe but the middle lobe has a deep notch to which the round ligament attached. The right lobe is split into two sub-lobes and the caudate lobe is divided into the paracaval portion and the Spiegel lobe (Contran *et al.*, 2005). The left, right, and caudate lobes has one primary portal branch, whereas the middle lobe has two portal branches. The left and the right sub- and caudate lobes has one large hepatic vein each, whereas three large hepatic veins are located in the middle lobe. The left and right lobes function independently and are separated anteriorly by the falciform ligament and ligament teres hepatis (Abdel-Misih and Bloomston, 2010). The lobules are demarcated by connective tissues containing bile duct, the lymphatic nerve and blood vessels. The gall bladder where bile is stored in humans is absent in rats (Vdoviakova *et al.*, 2016). The sinusoids are formed by fenestrated capillaries from the oxygen rich hepatic artery and the nutrient rich portal veins. The sinusoids contain Kupffer cells that functions in the removal of dead blood cells as well as the phagocytosis of pathogens and other xenobiotics that gets to the liver (Abdel-Misih and Bloomston, 2010).

2.9.2.2 Functions of the Liver

The liver performs various functions in the body all of which are vital to the existence of life. Some of the major functions of the liver are as follows;

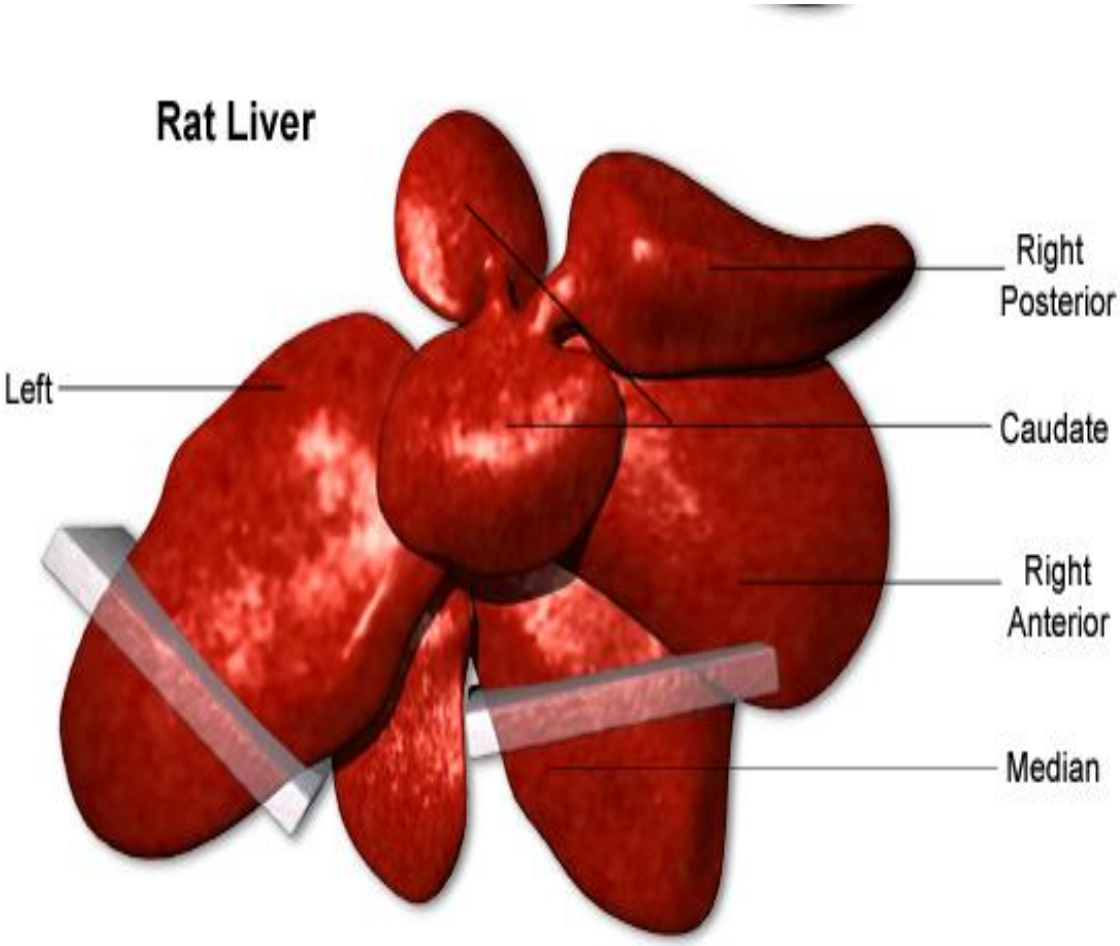


Figure 8: Anatomy of the liver

Source: Atangana and Noutchie (2014)

i. Metabolism: The liver is the central of general metabolism in the body. Protein, lipid and carbohydrate metabolism all take place in the liver. Excess blood glucose is converted and stored as glycogen while the shortage of glucose is regulated by the breakdown of glycogen back to glucose. Plasma proteins such as albumins, globulin, fibrinogens are synthesized in the liver whereas excess amino acids are broken down by deamination process to produce urea while their carbon skeleton are recycled for gluconeogenesis. Similarly, homeostasis of cholesterol, ketone bodies and important lipoproteins (synthesis of HDL for the clearance of LDL, VLDL and chylomicrons) take place in the liver (Contran *et al.*, 2005).

ii. Bile production: The liver in rats also functions in the synthesis of hydrophilic bile acids in low quantities. This is because rats rarely feed on fat-rich foods which facilitates the survival of the animals without gall bladder (Turumin *et al.*, 2013).

iii. Storage: The liver act as a storage organ for certain minerals such as iron and copper, vitamins (A, B, D and K) as well as glycogen.

iv. Detoxification: The detoxification of poison and pathogenic by products takes place in the liver where they are transformed to the more soluble product (via the phase I or phase II reactions) that can be excreted. Ammonia is also converted to urea by the liver.

v. Formation and breakdown of red blood cells: The liver is responsible for the destruction of old red blood cells to produce bilirubin and production of new cells along with the spleen in adults or singly in foetus.

vi. Heat production: The various metabolic products going on in the liver leads to the production of heat as by product which is distributed across the body via blood circulation. However, the immense ability of the liver to regenerate helps and supports the survival of the organ from the wear and tear of a lifetime.

vii. Filtration: The liver also acts as a filter between the blood coming from the gut and the blood in circulation. It rids the body of the antigens, worn out cells, microorganisms, xenobiotics, hormones, proteins and metabolic waste products in circulation. This in turn keeps the blood and the body at optimal functional capacity.

2.9.3 The Kidney

The kidney is an excretory organ important for the filtration of blood for metabolic waste products which are expelled out of the system (Walter, 2004). It occupies the retroperitoneal space between the parietal peritoneum and posterior abdominal wall. The kidney of an adult male rat weighs between 0.9 – 1.2 g (Al-Samawy, 2012). The kidney is a well vascularized organ and as such receives not less than 25% of the cardiac output at rest.

2.9.3.1 Anatomy of the Kidney

The kidney is a pair of bean-shaped organ, located on either side of the spine and surrounded by the ribs, muscles and fats (Al-Samawy, 2012). Directly above each kidney is the adrenal gland which stimulates the release of aldosterone for sodium reabsorption. The parenchyma of the kidney is divided into two major structures, the renal cortex on the outer region and the renal medulla at the inner region. These structures take the shape of 8 to 18 cone-shaped renal lobes, each containing renal cortex surrounding a portion of medulla called a renal pyramid (Figure 9). Urine made by the nephrons are transported to the calyces for excretion via bundles of collecting ducts known as the papillae (Figure 9). The pyramids and renal columns taken together constitute the kidney lobes. Accessory structures such as the blood vessels, ureters, nerves and lymphatics enter and exit the kidney via the renal hilum. Also emerging from the hilum is the renal pelvis (formed by the major and minor calyxes) through which urine is channelled to the ureter via peristalsis (Figure 9). The nephrons function to cleanse the blood of metabolic waste products and maintain balance of the constituents in circulation.

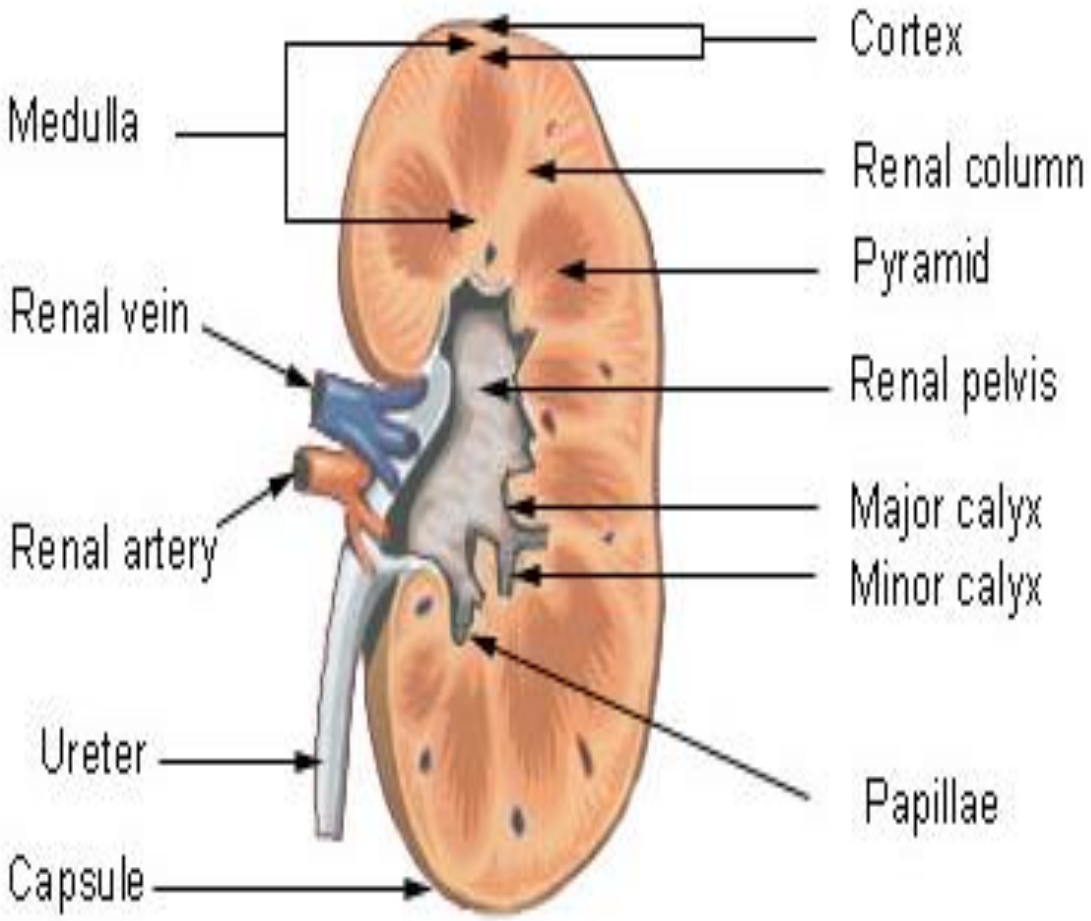


Figure 9: Anatomy of the kidney

Source: Patel (2009)

It is composed of glomerulus in contact with a network of capillary vessels, the proximal tubule, loop of Henle, distal tubule and collecting duct (Al-Samawy, 2012). The glomerulus and the Bowman's capsule together make up the renal corpuscle. All the renal corpuscle, proximal and distal convoluted tubules make up the cortex of the kidney (Cotran *et al.*, 2005).

2.9.3.2 Functions of the Kidney

The kidney is involved in various biological functions which includes excretion, detoxification, regulation of homeostasis and as an endocrine organ. Some of the major functions of the kidney include;

i. Excretion: The kidney is responsible for the removal of metabolic waste products such as creatinine, urea and uric acid all of which can be toxic to the brain or other organs of the body. Similarly, pesticides, drugs, food additives and other non-nutritive compounds in the body are eliminated from the body.

ii. Homeostasis: The kidneys plays an active role in the regulation of blood glucose by reabsorbing blood glucose when in excess or synthesizing during shortage of blood glucose most especially in a fasting state. The water volume is also maintained by the kidneys for balance between the internal and external compartment of the body. The kidney generates 180 litres of filtrate per day, with a large percentage reabsorbed, and allowing for the generation of approximately 2 litres of urine only. This in-turn helps in the regulation of the osmolarity of the body system.

iii. Electrolyte concentration: The serum concentration of electrolytes is monitored and maintained by the kidney thereby preventing osmotic fluxes. The balance between the intracellular and extracellular fluid components also helps to stabilize arterial blood pressure and prevent metabolic acidosis or alkalosis.

iv. Synthetic function: The kidney is responsible for the production of the enzyme renin, angiotensin and vitamin D required for the reabsorption of calcium and phosphorus

thereby promoting mineralization. As an endocrine gland, the kidney is responsible for the secretion of hormones, erythropoietin, aldosterone and vasopressin all of which play important roles in the general metabolism of the body.

v. Toxicity: The kidney is the primary excretory organ which contributes to the susceptibility of the kidney as a target organ for several toxicant. The nephron is the functional unit of the kidney where various biochemical, functional and morphological activities occur. The exposure of the kidney to certain toxicants leads to cellular injury and sometimes derangement or loss of functional capacity.

2.10 Serum

Serum is the supernatant obtained following the centrifugation of a blood sample that has been allowed to clot. Serum differs from plasma, the liquid portion of normal unclotted blood containing the platelets, red and white blood cells. In the serum, fibrinogen and other clotting factors have been removed whereas albumin, globulin, dissolved hormones and electrolytes are retained (Guyton and Hall, 2006).

2.11 Biochemical Parameters Studied

2.11.1 Intestinal Function Parameters

2.11.1.1 Na-K ATPase

Na-K ATPase also known as the sodium pump is an enzyme found in all mammalian cell membranes and responsible for the regulation of ionic gradients across cell membrane which is necessary for normal cellular functioning (Karlish *et al*., 2008). Na-K ATPase is a member of the P-ATPases family that uses the energy released from the hydrolysis of ATP for the active transportation of cations against their concentration gradient. It has three subunits denoted by α, β and γ however, only the former two are required for the pumping of ions (Karlish *et al*., 2008). The α-subunit contains the catalytic binding sites for Na^+, K^+, ATP and inhibitors such as ouabain while the β-subunit is essential for the pumping function of Na-K

ATPase, it stabilizes the α-subunit conformation, chaperone the delivery of the α/β complex to the plasma membrane and necessary for cell-cell adhesion (Geering, 2008; Laursen *et al*., 2013). The activity of the sodium pump is regulated by the γ-subunit, also known as the FXYD protein (Laursen *et al.*, 2013). The α-subunit of the sodium pump has 4 isoforms α (1 – 4), α1 isoform is ubiquitous and found in all cells, α2 and α3 isoforms are keenly expressed by the neuronal tissues, skeletal muscles and cardiac myocytes while α4 is found in the testis and regulates sperm motility (Yan and Shapiro, 2016).

Na-K ATPase is an integral transmembrane protein responsible for the extrusion of $3Na^+$ from the intracellular membrane for every $2K^+$ absorbed in the cell to maintain intestinal epithelial cell ion homeostasis (Gal-Garber *et al*., 2003). The Na-coupled nutrient transport is the primary means of absorption of many nutrients such as glucose, amino acids, vitamins and certain nucleic acids (Saha *et al.,* 2015). The nutrient coupled uptake of Na^+ into the brush border membrane which in turn stimulates extrusion of Na^+ via the basolateral membrane is efficiently maintained by intestinal Na-K ATPase (Saha *et al.*, 2015). Thus, Na-K ATPase plays a significant role in the management and treatment of diarrhoea as the movement of Na^+ and glucose (from oral rehydration) across the brush border membrane promotes the reabsorption of water through the intestinal epithelial cells thereby, promoting rehydration (Hamilton and Crane, 2013).

2.11.1.2 Nitric Oxide

Nitric oxide (NO) is a ubiquitous signalling molecule. It is a short-lived free-radical gas endogenously produced (Arthur *et al.*, 2014). It has been implicated in various physiological and biochemical processes such as secretion, peristalsis, homeostasis and vascular tone, immune function, relaxation of muscles, cardiac contractility and neurotransmission (Coleman, 2001; Moncada *et al*., 1991).

NO being a major cell signalling molecule can exert its activity primarily by activating soluble guanylate cyclase and thus activating cyclic GMP dependent kinases. This initiates a cascade of physiological changes such as the regulation of cGMP-gated ion channels, alterations in phosphodiesterase function and activation of cAMP and cGMP-dependent protein kinases, leading to a further amplification of secretory process in the small intestine (Arthur *et al.*, 2014).

Nitric oxide is synthesized by a complex group of enzymes known as the nitric oxide synthase (NOS) with three distinct isoforms in mammals. They include the neuronal tissue nitric oxide synthase (nNOS), epithelial cell nitric oxide synthase (eNOS) which was the first isoform discovered (Moncada *et al*., 1991). The nNOS and eNOS are also known as constitutive since they are continuously expressed by the neuronal and epithelial cells respectively (Griffith and Steuhr, 1995). However, the third isoform; inducible nitric oxide synthase (iNOS) is synthesized by immunological stimuli which results in a very high and continuous production of NO for several hours or days (Moncada *et al*., 1991; Nathan and Xie, 1994). The upregulation of iNOS causes effects such as nitrosation, nitration and oxidation in tissues where the production of reactive nitrogen species such as peroxynitrites (ONOO) and dinitrogen trioxide (N_2O_3) prevail (Grisham *et al*., 1999). When produced in small quantities (constitutive NO), it confers protective effect and helps to maintain the integrity of intestinal epithelial cells (Vallance *et al.*, 2004). However, when it is produced in large quantities (inducible NO), a broad spectrum of pathophysiological conditions such as septic shock, hypertension, atherosclerosis, carcinogenesis, ischemia and irritable bowel disease has been implicated (Kolios *et al.*, 2004; Arthur *et al.*, 2014).

2.11.1.3 Glucose

Glucose is the product of carbohydrate catabolism absorbed into the blood stream by the small intestine. It is constantly required by the brain, muscles and other tissues as an

important source of energy the lack of which could cause lethargy, seizures, loss of consciousness and/or death. In contrast, prolonged elevation of blood glucose if not converted into glycogen, fatty acids, amino acids or oxidized in other catabolic pathways of the cell, could result in diabetes, renal failure, neuropathy and cardiovascular diseases.

Diarrhoea often causes impairment in the reabsorption of water and electrolytes whereas, the reabsorption of glucose remains intact. The absorption of glucose across the intestinal brush-border membrane is predominantly mediated by the sodium-dependent glucose transporter 1 (SGLT 1), a membrane protein that couples each molecule of glucose absorbed with two molecules of Na+ (Chen *et al.,* 2016). This causes a shift in concentration gradient which stimulates the activity of intestinal Na-K ATPase and results in a net absorption of water over secretion (Wild *et al*., 1999; Alexander and Carey, 2001).

2.11.1.4 Acetylcholinesterase

Acetylcholine is the most abundant neurotransmitter of the enteric nervous system, where it functions in the intestinal transport of water (Nezami and Srinivasan, 2010). Following activation, the cholinergic neurons stimulate the release of acetylcholine into the neuroepithelial junction which in-turn promotes secretory process by the crypts over reabsorption of water across the cell surface (Furness *et al.,* 2014).

Acetylcholine also regulate mucus secretion, proliferation, cytokine production and secretion at the epithelial level whereas at the brush border, modulate splanchnic blood flow, gut motility and immune cell activation (Duboc *et al.* 2016). However, acetylcholinesterase (AChE) functions to terminate the transmission of cholinergic synapse via the hydrolysis of acetylcholine to form acetic acid and thiocholine as shown in Figure 10 (Beri *et al.*, 2013).



Figure 10: Hydrolysis of acetylcholine by acetylcholinesterase

Source: Lee *et al.* (2011)

2.11.1.5 Protein Carbonyl

Protein carbonyl is one of the major oxidative stress markers for protein oxidation (Levine *et al.*, 1990). The carbonyl groups of oxidized proteins are found on the side of the amino acid moieties especially for proline, threonine, arginine and lysine. The small intestine is exposed to various metabolites and metabolic products from the digestion of foods, nutrients, drugs and microbial waste products which essentially leads to ROS accumulation and cause oxidative damage (Cao and Kaufman, 2014). As a consequence, there is increased alteration in functional capacity and integrity of intestinal epithelial cells. Protein carbonyls formed during oxidative damage are chemically stable which makes them a suitable target for measurement (Stadtman and Levine, 2003).

2.11.1.6 Reduced Glutathione

This is the main non-enzymatic antioxidant in the small intestine characterized by the presence of the thiol (-SH) functional group (Sen, 1997). It is a highly ubiquitous hydrophilic moiety found in several cells and tissues in millimolar concentrations. It is a tripeptide composed of the amino acids, glycine, glutamate and cysteine which can exist in the reduced (GSH) or oxidized (GSSG) form. About 95% of the glutathione pool exist in the reduced form and the amount of each form can be a determinant of the cellular redox status which explains its role as an antioxidant in cells (Fang *et al*., 2002; Jones, 2006). GSH is a very potent ROS and free radical scavenger, functions mainly in detoxification by forming conjugates with the various xenobiotics and indigestible metabolites in the body (de Barboza *et al*., 2017). Some of the functions of GSH includes; serving as a thiol buffer for important cellular proteins (such as thioredoxins, metallothionines), acting as a cofactor for various metabolic and signal pathway enzymes, stimulates the regeneration of endogenous ascorbates and tocopherols (Smeyne and Smeyne, 2013). GSH is important for the regulation of intestinal mucosal fluidity, absorption and detoxification processes (de Barboza *et al*., 2017).

The reduction in GSH levels in the intestine has been attributed to the inhibition of electron transport chain which promotes cell apoptosis, ROS generation and decreased production of ATP (Circu *et al.*, 2009; de Barboza *et al.*, 2017).

2.11.1.7 Superoxide Dismutase

Superoxide dismutase (SOD) are a class of closely related enzymes that catalyze the conversion of superoxide anion into oxygen and hydrogen peroxide (Dasuri *et al*., 2013). SODs are present in almost all aerobic cells and extracellular fluids. They contain a metal ion cofactor depending on the isoenzyme, which can be copper, manganese, zinc or iron. In mammals, copper/zinc SOD is present in the cytosol while manganese SOD, the most effective antioxidant system, is present in the mitochondrion (Poyton *et al*., 2009). SOD has been attributed with the prevention of gastrointestinal mucosal cells injury by preventing the formation of peroxynitrite in cells exposed to excess nitric oxide (Klinowski *et al.*, 2006).

2.11.1.8 Catalase

Catalase is cellular enzyme responsible for the hydrolysis of hydrogen peroxide (H_2O_2) to water and oxygen (Imlay, 2003). It is a tetramer of four polypeptide chain each of which is over 500 amino acids long (Nicholls *et al.*, 2001). Catalase contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide (Nicholls *et al.*, 2001). Catalase is present in all prokaryotes and eukaryotes. With the exception of erythrocytes, it is predominantly located in peroxisomes and also found in the cytoplasm and mitochondria of all types of mammalian cells where H_2O_2 is generated (Gandhi and Abramov, 2012). Catalase plays a significant role in cellular antioxidant defense mechanisms by limiting the accumulation of H_2O_2 (Chelikani *et al.*, 2004).

2.11.1.9 Malondialdehyde

Reactive oxygen species degrade polyunsaturated lipids to form malondialdehyde (MDA), an established marker of oxidative stress. It is a reactive aldehyde that causes toxic stress in

cells and forms covalent protein adducts referred to as advanced lipoxidation end products. The varying concentrations of malondialdehyde in different cells can therefore be used to measure the level of oxidative damage in the body (Jaeschke *et al*., 2002). MDA reacts specifically with deoxyadenosine and deoxyguanosine of DNA forming DNA adducts which are mutagenic (Kon *et al*., 2004). The guanidine group of arginine residue condenses with MDA to give 2-aminopyrimidines. Increased level of MDA in the small intestine has been correlated with intestinal mitochondrial injury which causes the perturbation of intestinal permeability function (Zhou *et al.*, 2016).

2.11.1.10 Alkaline Phosphatase

Alkaline phosphatase has been found in many organisms including eukaryotes and prokaryotes. ALP acts on a large variety of naturally occurring and synthetic substrates some of which include 3-glycerophosphate, phenyl phosphate and paranitro phenyl phosphate (Tietz, 1995). ALP is a marker enzyme for endoplasmic reticulum and plasma membrane (Wright and Plummer, 1974). It catalyzes the hydrolysis of various phosphomonoester to give phosphate group and corresponding alcohol, phenol and sugar (Figure 11) but can also function as a transferase when an appropriate acceptor is present.

2.11.1.10.1 Distributions

Alkaline phosphatase has been reported to be present in bone, intestine, crop, liver, kidney, heart, placenta and mammary gland (Lalles, 2014). ALP is present in most tissue but particularly of high concentration in the bone osteoblasts, hepatobiliary tract, intestinal wall, renal tubules and placenta (Kaplan, 2005).

2.11.1.10.2 Isoenzymes

Studies like electrophoretic analysis, substrate dependent reactions and kinetics have demonstrated the existence of different molecular forms of alkaline phosphatase in blood of healthy and diseased person (Sharma *et al.*, 2014). There are 2 isoenzymes (products of

different genes) and several isoforms (produced from post-translational modification of isoenzymes) of ALP. The isoenzymes are produced from intestinal and tissue non-specific ALP genes and differ in amino acid sequence. Isoforms differ in catalytic sites and activity, immunogenicity, and electrophoretic mobility. The major isoforms that can be measured in animals are liver-ALP (L-ALP), corticosteroid-ALP (C-ALP, only in dogs), bone - ALP (B - ALP) and intestinal - ALP (I - ALP) (Sharma *et al.*, 2014).

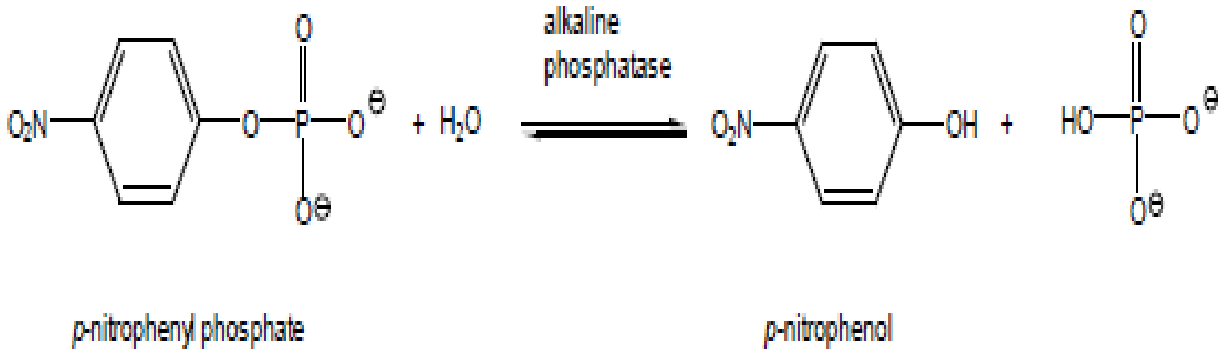


Figure 11: Hydrolysis of paranitrophenyl phosphate by alkaline phosphatase

Source: Wright *et al*. (1972)

a) Intestinal-ALP gene/isoenzyme: This produces the C-ALP and I-ALP isoforms. C-ALP is unique to dogs and is induced by corticosteroids (endogenous or exogenous). Even though it is a product of the intestinal gene, it is actually produced in hepatocytes (Sharma *et al.*, 2014). b) Tissue non-specific-ALP gene/isoenzyme: This produces the remaining isoforms of ALP, including L – ALP, B – ALP, placental – ALP, leukocyte – ALP and germ cells – ALP (Sharma *et al.*, 2014).

2.11.1.10.3 Activators and Inhibitors

Alkaline phosphatase is activated by some divalent ions such as Mg^{2+}, Mn^{2+} and Co^{2+}. Optimal activity was obtained with Mg^{2+} at about 1.0 mol/L. However, glycine and alanine at low concentrations increase serum ALP activity (Bobkova *et al.*, 2013). It is inhibited by Zn^{2+}, Be^{2+}, Cu^{+}, Hg^{2+}, sulphide, cyanide, adrenaline, oxalate, borate, adrenochrome and oxidizing agents (Bobkova *et al.*, 2013).

2.11.1.10.4 Clinical Applications

ALP is released into the blood in various disorders, including obstructive jaundice and some bone diseases. Measurement of the serum level of ALP forms a common clinical test in distinguishing hepatobiliary disease from bone disease (Mittal *et al*., 2011). ALP has been reported to be a prognostic for canine osteosarcoma (Mittal *et al*., 2011). Similarly, increase in alkaline phosphatase activities is also noticed in liver cancer and heart infection. High ALP levels in the liver can show that the bile ducts are blocked. Elevation of ALP in the bones indicates that there could be active bone formation occurring as ALP is a bye product of osteoblast activity (such as the case in Paget's disease of bone). Placental alkaline phosphatase is elevated in seminomas (Berk and Korenblat, 2007). Although, lowered levels of serum ALP are less common than elevation, reduced levels of ALP has been reported in the disease hypophosphatasia, an autosomal recessive disease (Mittal *et al*., 2011).

Intestinal ALP functions majorly in intestinal homeostasis viz; regulating the secretion of bicarbonate ions and duodenal surface pH, absorption of long chain fatty acids, regulation of intestinal microflora and mitigation of inflammatory processes in the small intestine through the detoxification of pathogenic substances (Lalles, 2010; 2014). Administration of exogenous I-ALP reportedly decreased intestinal and systemic production of inflammatory cytokines and improved gut barrier function (Rentea *et al*., 2012; Riggle *et al*., 2013). It also provides protection against SIBO via the maintenance of intestinal microbiome (Alam *et al*., 2014).

2.11.2 Liver Function Indices

Liver function indices are a series of special blood tests to determine whether or not the synthetic, secretory and metabolic function of the liver are intact or compromised. Some of these tests include; total protein, albumin, globulin, bilirubin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. These tests can also help to distinguish between acute and chronic liver disorders.

2.11.2.1 Albumin

Albumin is an un-glycosylated globular protein consisting of 585 amino acids with a molecular weight of about 67 kDa (Caraceni *et al.,* 2013). Serum albumin is the most abundant plasma protein in mammals, produced in the liver and forms a large proportion of all plasma protein constituting about 60% of the total protein (Suzuki, 2006). Albumin is essential for the maintenance of osmotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues (Caraceni *et al.*, 2013). It also acts as a plasma carrier by non-specifically binding several hydrophobic steroid hormones and as a transport protein for hemin and fatty acids. It also plays a role in stabilizing extracellular fluid volume (Caraceni *et al.,* 2013). The liver is the primary cite of albumin

biosynthesis hence, a decrease in serum albumin is attributed to hepatic failure corresponding to about 75% loss of hepatic function (Mazzaferro *et al.,* 2002).

2.11.2.2 Globulin

Globulins are the 'antibody' proteins important for fighting diseases. The globulins are a family of globular proteins with relatively high molecular weights (< 93 kDa). Some globulins are produced in the liver, while others are made by the immune system (Julius, 2010). Serum globulin concentrations comprises of the alpha, beta and gamma globulin fractions based on protein electrophoretic fractionation. A decrease in serum globulin can be associated with impairment in the synthetic function of the liver whereas higher levels might be suggestive of infection, inflammatory disorder or autoimmune diseases (Chaterjee, 2017).

2.11.2.3 Bilirubin

Bilirubin (formerly referred to as hematoidin) is the yellow breakdown product of normal heme (ferroprotoporphyrin IX) catabolism. It is a potentially toxic substance that is safely disposed-off by the body and elevated levels may indicate certain diseases. It is responsible for the yellow colour of stool, bruises, urine, and the yellow discoloration in jaundice (Liu *et al*., 2008). Bilirubin circulates in the bloodstream in two forms: Indirect (or unconjugated) bilirubin and direct (conjugated). Indirect bilirubin which is insoluble travels through the bloodstream to the liver, where it is conjugated to become soluble (Chernecky and Berger, 2008). Heme is converted to bilirubin, which is then transported by albumin from the blood to the liver when it is conjugated. Conjugated bilirubin is released into the bile for storage in the gallbladder or transferred directly to the small intestines. Serum bilirubin is considered a liver function test, as it reflects the liver's ability to take up, process, and secrete bilirubin into the bile (Berk and Korenblat, 2007). Although, for decades bilirubin was considered as a metabolite dangerous for human health, the study by Libor (2005) indicated that bilirubin

exhibits potent antioxidant properties with substantially positive clinical consequences. The demonstration of the potent antioxidant activity by bilirubin, has led to the hypothesis that bilirubin's main physiologic role is as a cellular antioxidant (Liu *et al*., 2008).

2.11.2.3.1 Total Bilirubin

Bilirubin, the orange-yellow bile pigment, is produced from protoporphyrin IX. For each mole of heme catabolized, one mole each of carbon dioxide, bilirubin and ferric ions are produced. In mammals, daily bilirubin production from all sources averages 250-300 mg, 85% of which is derived from heme moiety of the haemoglobin released from senescent erythrocytes that are destroyed in the reticuloendothelial system (Burtis *et al.,* 2012). The remaining 15% of bilirubin is produced from erythrocyte precursors destroyed in the bone marrow and from the catabolism of other heme-containing proteins (such as myoglobin, cytochromes, and peroxidases). The sum total of bilirubin obtained from all these processes that has not been conjugated is termed total bilirubin (Burtis *et al.,* 2012).

2.11.2.3.2 Conjugated Bilirubin

In the liver, conjugation of bilirubin is mandatory to render it soluble, thereby preventing reabsorption by the intestine and facilitate excretion (Kalakonda and John, 2017). This is achieved via the conjugation of the propionic acid side chain of bilirubin with glucuronic acid in a process known as glucuronidation (Kalakonda and John, 2017). The accumulation of conjugated bilirubin in the serum might occur when the excretory function of the hepatic cells have been impaired. When biliary drainage system is compromised or blocked, some of the conjugated bilirubin leaks out of the hepatocytes and appears in the urine, turning it dark amber. The presence of conjugated bilirubin in the urine signifies hepatobiliary dysfunction (Kalakonda and John, 2017).

2.11.2.4 Alanine Aminotransferase

Alanine aminotransferase (ALT) generally referred to as glutamate pyruvate transaminase has its systematic name as L-alanine-2-oxoglutarate aminotransferase. It catalyses the transamination reaction between L-alanine and α-ketoglutarate to form pyruvate and glutamate (Yang *et al.*, 2009) as shown in Figure 12.

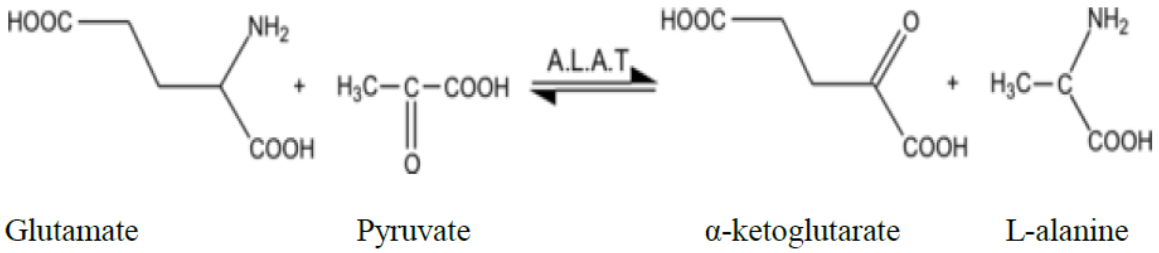


Figure 12: Reaction catalysed by alanine aminotransferase

Source: Yang *et al.* (2009)

2.11.2.4.1 Distribution

ALT is less abundant in human tissues (Burtis *et al.*, 2012), it is native to the cytosol, though small amounts have been found in the mitochondria of liver cells and in the heart muscles (Burtis *et al.*, 2012).

2.11.2.4.2 Isoenzymes

The isoenzymes of ALT (ALT1 and ALT2) show variation in their rate of reaction with substrates as well as their sensitivity to heat. The cytoplasmic and mitochondrial fraction of the ALT shows the same pH sensitivities and immunological properties, though they can be distinguished by their differences in electrophoretic mobilities. The serum levels of ALT1 and ALT 2 is due to liver damage (Rafter *et al.*, 2012).

2.11.2.4.3 Activators and Inhibitors

The activity of alanine aminotransferase is stimulated by a high concentration of aldehydes, oxoacids and ketones. It is inhibited by the presence of accumulated pyruvate which is one of the reaction products (Yang *et al.*, 2009).

2.11.2.4.4 Clinical Applications

The activities of ALT are measured clinically as one of the biomarkers traceable to changes in the pathological condition of the liver (Ozer *et al.*, 2008). The level of serum ALT can be used in the differential diagnosis of liver and heart diseases. The concentration of ALT is much higher in the liver than in other organs. Hepatocellular injury leads to the leakage of ALT from the hepatocyte into the blood, which takes approximately 42 hours to clear off (Yang *et al*, 2014). Although an increase in the activity of ALT can be highly suggestive of liver injury, ALT is not liver-specific because there are extrahepatic sources of circulating ALT such as skeletal or cardiac muscle damage and drugs that promotes the expression of ALT gene (Yang *et al.*, 2014). High levels of ALT are observed in patients with acute hepatitis and acute cirrhosis, while it might not be detected in sera of healthy subjects.

2.11.2.5 Aspartate Aminotransferase

Aspartate aminotransferase (AST) generally referred to as glutamate oxaloacetate transaminse (GOT) has its systemic name as L-aspartate-2-oxoglutarate aminotransferase. It catalyzes the transamination reaction between L-aspartate and α-ketoglutarate to form glutamate and oxaloacetate (Figure 13) at optimum pH of 7.4.

2.11.2.5.1 Distribution

AST is widely distributed in animal tissues with a relatively high concentration in the liver, heart, skeletal muscle and kidney (Ozer *et al.*, 2008). The pancreas, lung and spleen also contain considerable quantities of the enzyme (Ozer *et al.*, 2008). It is found both in the cytosol and in the mitochondria of the cells.

2.11.2.5.2 Isoenzymes

There are two isoenzymes of AST, isoenzymes I and II, located in the cell cytoplasm and mitochondria respectively. They differ in their electrophoretic mobilities, pH variation, immunochemical properties and degree of substrate affinities (Nelson and Cox, 2008). Isoenzyme I (cationic isozymes) has a much greater affinity for aspartate than isoenzyme II (anionic isoenzymes) (Nelson and Cox, 2008).

2.11.2.5.3 Activators and Inhibitors

The activities of AST are inhibited by its product, oxaloacetate. Therefore, it is an allosteric enzyme. The activator of AST is pyridoxal phosphate which acts as a cofactor in the presence of Mg^{2+} (Nelson and Cox, 2008).

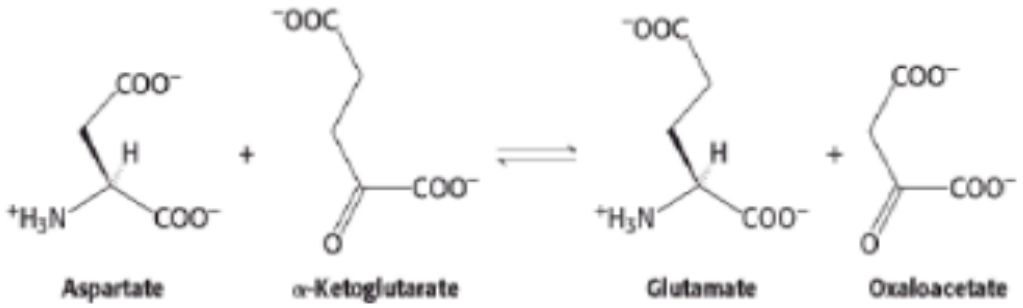


Figure 13: Reaction catalysed by aspartate aminotransferase

Source: Jeremy *et al*. (2002)

2.11.2.5.4 Clinical Applications

AST isoenzyme can be used as marker enzymes in the study of diagnosis of liver diseases caused by drug toxicity or by infection (Nelson and Cox, 2008). Although, ALT is considered as a more specific marker for liver injury than AST, this is due to the expression of AST from other tissues, such as brain, myocardial and skeletal muscle cells (Yang *et al.*, 2014). Clinically, the ratio of AST to ALT which helps in distinguishing between hepatic and extrahepatic injury is more precise. An AST/ALT ratio of 2:1 is indicative of hepatic injury (Ozer *et al*., 2008) whereas an AST/ALT ratio of ≤ 0.4 during recovery from severe acute hepatotoxicity indicates a good prognosis for recovery (McGovern *et al*., 2015).

2.11.3 Kidney Function Indices

Kidney function indices are parameters determined to evaluate the metabolic state and physiology of the kidney (Guyton and Hall, 2006). Alterations in the serum levels of metabolites such as urea, uric acid, creatinine and electrolytes such as Na^+, K^+, Cl^- is an indication of kidney dysfunction (Guyton and Hall, 2006). The result of kidney function tests is important as it can be used for assessing excretory function of the kidney (Guyton and Hall, 2006).

2.11.3.1 Urea

Urea with a molecular weight of 60 kD, is the major nitrogenous waste product resulting from protein catabolism (Burtis *et al.*, 2012). It is a highly soluble and non-toxic compound. In the liver, amino acids are deaminated (either through oxidative or non-oxidative process) to produce ammonia. Ammonia is toxic to the body and is converted to urea via the urea cycle. This is the primary method of nitrogen excretion from the body (Burtis *et al.*, 2012).

The rate of production of urea is accelerated by high protein diet, starvation, tissue damage and sepsis (Burtis *et al.*, 2012). It is present in virtually identical concentrations per unit of water in extracellular and intracellular fluids since it diffuses freely through capillary

walls and cell membranes. Over 90% of the total urea is excreted by the kidney whereas intestinal reabsorption and excretion by the skin account for the minor fraction. Urea is a marker for glomerular function where it is freely filtered from the blood rather than being reabsorbed or secreted by the tubules (Burtis *et al.*, 2012).

2.11.3.2 Uric Acid

Uric acid is a waste product formed from the breakdown of purines (Burtis *et al.*, 2012). Most of the uric acid in the body dissolves in the blood and passes through the kidneys into the urine. Blood urates are completely filtrable and both proximal tubular reabsorption and distal secretion occur (Burtis *et al.*, 2012). A rise in the blood concentrations of uric acid may occur due to a decrease in renal excretion whereas a decrease might be indicative of defective renal tubular reabsorption of uric acid (Burtis *et al.*, 2012). Thus, serum uric acid level can be used to assess kidney function (Cotran *et al.*, 2005).

2.11.3.3 Creatinine

Creatinine is a break-down product of creatine phosphate in muscle (Burtis *et al.*, 2012). It is usually produced at a fairly constant rate by the body (depending on muscle mass). The measurement of blood creatinine as well its renal clearance is used as diagnostic indicator for kidney function (Burtis *et al.*, 2012). Creatinine, present in all body fluids and secretions is freely filtered by the glomerulus. Although, it is not reabsorbed by the renal tubules, there is some trace of tubular secretion (Burtis *et al.*, 2012). High serum creatinine is usually symptomatic of uraemia and chronic renal failure whereas lower values might imply acute tubular necrosis, glomerulonephritis, dehydration and polycystic kidney disease (Edmund and David, 2006; Gowda *et al.*, 2010).

2.11.3.4 Serum Electrolytes

Serum electrolytes are naturally occurring elements and compounds in the body capable of dissociation in water to form anions or cations (Burtis *et al.*, 2012). Electrolytes exist in the

blood as ions of potassium, chlorine, magnesium, sodium and bicarbonate and can be measured by laboratory analysis of the serum. The clinically important electrolytes are Na^+, Ca^{2+}, K^+, HCO_3^{2-}, Cl^-, PO_4^{3-}. They contribute more than 98% of blood serum electrolyte. The most important electrolytes (Na^+, K^+, HCO_3^{2-} and Cl^-) occur primarily as free ions and play key role in water homeostasis, maintenance of pH, osmotic pressure and as cofactors in various enzyme reactions (Burtis *et al.*, 2012). In contrast, significant amounts (> 40%) of Ca^{2+}, Mg^{2+} and other trace elements are bound to proteins such as albumin. Electrolyte concentration in the serum though unstable (due to dietary intake, renal excretion and pathological condition) could serve as indices of kidney functional state (Guyton and Hall, 2006).

2.11.3.4.1 Serum Sodium

Sodium ion, the major cation of extracellular fluid is an electrolyte that plays important roles in maintaining the salt/water balance in the body (Burtis *et al.*, 2012). The kidneys regulate serum sodium levels by excreting the excess after obtaining the average daily requirement. Sodium is freely filtered by the glomeruli. However, about 70 – 80% of the filtered Na^+ is actively reabsorbed at the proximal tubule while a further 20 – 25% is reabsorbed at the loop of Henle along with Cl^- and water (Burtis *et al.,* 2012). Pathological conditions of the kidney (such as renal tubular disease) which result in malabsorption of sodium ion may lead to decreased serum levels of sodium ion. However, hypernatremia can be attributed to a decrease in glomerular filtration or excess loss of water which increases cellular sodium concentration (Burtis *et al.,* 2012).

2.11.3.4.2 Serum Potassium

Potassium ions are the major cation in animal cells (Guyton and Hall, 2006). They are important in neurone (brain and nerve) function, muscle contraction and in influencing osmotic balance between cells and the interstitial fluid (Palmer, 2014). By the nature of their

electrostatic and chemical properties, K^+ are larger than Na^+. Potassium is freely filtered by the glomerulus. Most of the K^+ filtered are reabsorbed in the proximal tubule and loop of Henle, such that < 10% of the filtered load reaches the distal nephron (Palmer, 2014). In the proximal tubule, K^+ absorption is primarily passive and proportional to Na^+ and water. Ion channels and pumps in cell membranes distinguish between the two ions and maintain a low cellular concentration of Na^+. This in turn leads to the active reabsorption of K^+ against the concentration gradient (Palmer, 2014). A reduction in K^+ concentration in body fluids is an indicator of kidney tubular dysfunction and may cause a potentially fatal condition known as hypokalemia (Palmer, 2014).

2.11.3.4.3 Serum Chloride

Chloride ions are the major anion in the extracellular water space. Physiological significance of Cl^- include the maintenance of proper body water distribution, osmotic pressure, and normal acid-base balance in the extracellular fluid compartment (Smith *et al.*, 1999). In the central nervous system, the inhibitory action of glycine and some of the action of γ-aminobutyric acid relies on the entry of Cl^- into specific neurons (Burtis *et al.*, 2012). The amount of serum chloride is carefully controlled by the kidneys. Increased serum concentration of Cl^- occurs in dehydration, acute renal failure, metabolic acidosis and renal tubular diseases (Burtis *et al.*, 2012).

2.12 Histopathology

Histopathology is the study of tissue architecture in a diseased state. It also refers to the microscopic examination of tissue in order to study the manifestations of disease. Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has been processed and histological sections have been placed onto glass slides (Mitchell *et al.*, 2009). Histopathology

observations are crucial if data are to be correctly evaluated as it establishes a correlation with clinical pathology findings.

CHAPTER THREE

3.0 Materials and Methods

3.1 Collection and Authentication of Plant Samples

The plant samples were obtained from the Staff Quarters of University of Ilorin, GRA, Ilorin, Kwara State, Nigeria. The plant samples were authenticated at the Herbarium Unit of the Department of Plant Biology, University of Ilorin, where it was registered under the voucher number (UIH/001/454).

3.2 Experimental Animals

Two hundred healthy, female Wistar rats *(Rattus norvegicus)* with an average weight of 124.10 ± 3.08 g were obtained from the Animal Holding Unit of the Department of Pharmacology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The animals were kept in clean aluminium cages, maintained under well ventilated house conditions (temperature: 28 ± 3^{o}C; photoperiod: 12 hours light and dark cycle; relative humidity: 45-55%) and allowed unrestricted access to rat pellets (Cap Feeds and Flours Mills Ltd, Ibadan, Oyo State, Nigeria) and clean tap water.

3.3 Ethical Clearance

The research was carried out after ethical approval from the University Ethical Review Committee (UERC/ASN/2017/916).

3.4 Assay Kits, Drugs and Chemicals

The assay kits for alanine aminotransferase (ALT) aspartate aminotransferase (AST), albumin, bilirubin, glucose, urea, uric acid and creatinine were obtained from Randox Laboratory Ltd., Co. Antrim, United Kingdom whereas the assay kits for the electrolytes; sodium, potassium and chloride ions were obtained from Teco Diagnostic, Anaheim, CA, USA. The loperamide hydrochloride, atropine sulphate and castor oil were products of Lincoln Pharmaceuticals Ltd., Gujarat, India, Hubei Tianyo Pharma, China, and Bells Sons

and Co. (Druggist) Ltd., Southport, England, respectively. Adenosine 5'-triphosphate disodium salt (ATP), sodium nitroprusside, sodium dodecyl sulphate (SDS), acetyl-thiocholine, ethylene diamine tetra-acetic acid (EDTA), dithionitrobenzoic acid (DTNB) and 2, 4-dinitrophenylhydrazine (DNPH) were products of Sigma Chemical Co, St. Louis, MO, USA. Ethylacetate, n-butanol, diethyl ether and methanol were products of Emsure Merck, Darmstadt, Germany, Lobal Chemie Laboratory Reagents and Fine Chemicals, Lobal Chemie Pvt. Ltd, Mumbai, India, and BDH Chemicals Ltd., Poole, England respectively. The silica gel and TLC plates were factory made by Sorbent Technologies, Norcross GA, USA. All other reagents used were of analytical grade, obtained from Sigma Aldrich, St. Louis, USA.

3.5 Preparation of Plant Extract

Fresh leaves of *M. indica* were oven-dried at 40^{o}C until a constant weight was obtained after which 300 g of the pulverized plant sample was extracted in 3 litres of distilled water. The resulting filtrate was lyophilized to give a yield of 21.12 g corresponding to a percentage yield of 7.04%. This was then subjected to exhaustive extraction in increasing order of polarity to obtain the solvent-solvent fractions; and column chromatography for the sub-fractions used in the present study as illustrated in Figure 14.

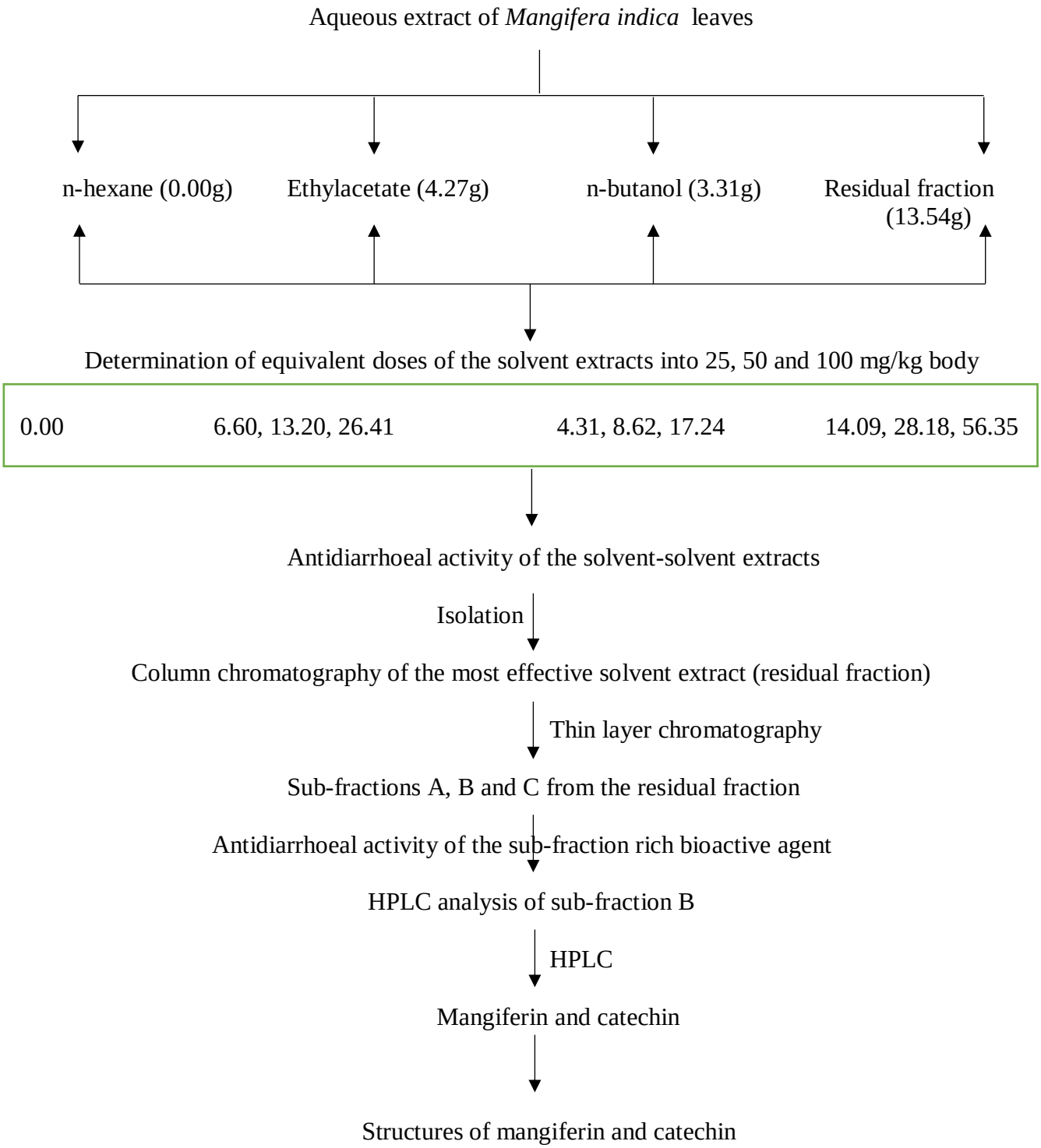


Figure 14: Schematic representation of the isolation procedure for the bioactive compounds

Adapted from Hassan *et al.* (2013)

3.6 Bioactivity Guided Fractionation

The methods described by Kupchan *et al.* (1973) was adopted for solvent-solvent partitioning of the aqueous extract of *M. indica* leaves. The solution of the extract was fractionated successively using ethyl-acetate and n-butanol while also retaining the residue. The fractions obtained (ethyl acetate-, n-butanol- and residual-fractions) were then evaporated to dryness using a rotary evaporator at 40℃. Each of the dried extracts were then tested for their antidiarrhoeal activity using castor oil-induced diarrhoea, castor oil-induced enteropooling and gastrointestinal motility test models.

3.7 Antidiarrhoeal Test Models

3.7.1 Castor Oil-induced Diarrhoea

The model described by Sunil *et al*. (2001) was adopted with slight modification. The test animals were fasted (without food, but water) for 8 hours prior to the commencement of the experiment. Each animal was placed in a cage, the floor of which was lined with blotting paper. Animals in the first (positive control) and second (negative control) groups were orally administered 1 ml each corresponding to 2.0 mg/kg body weight of loperamide hydrochloride and distilled water while the other test groups were administered equal volume of the ethylacetate fraction, n-butanol and residual fractions corresponding to 25, 50 and 100 mg/kg body weight equivalent of the crude extract. At 30 minutes post-treatment, each animal was again treated with 1 ml of castor oil and the time between the administration of the oil and the appearance of the first diarrhoeal drop was noted. The severity of diarrhoea was determined at 1-hour interval for a period of 4 hours by monitoring the diarrhoeal drops on the pre-weighed blotting paper placed beneath the rat cages. The total number of faeces, diarrhoeal faeces and total weight of faeces excreted were expressed as average of six determinations and compared with those of the controls. The percentage inhibition of diarrhoeal defecation in each group was computed and at the end of 4th hour, the animals were sacrificed. The

serum and intestinal supernatants were prepared according to the procedure described by Akanji and Yakubu (2000) for the determination of various biochemical parameters.

3.7.2 Castor Oil-induced Enteropooling

The procedure described by Havagiray *et al*. (2004) was adopted for intraluminal fluid accumulation test with slight modifications. The test animals were fasted for 8 hours before the experiment. Animals in the positive control group were intramuscularly administered 1 ml of atropine sulphate corresponding to 1.0 mg/kg body weight while the negative control group received orally, 1 ml of distilled water respectively. Rats in the test groups received equal volume of the various fractions of the extract. Immediately after these administrations, 1 ml of castor oil was administered orally to each rat in all the groups. After 30 minutes, the rats were sacrificed as described by Akanji and Yakubu (2000). The small intestine was excised and the intestinal contents was squeezed into a measuring cylinder to obtain the volume and mass of the intestinal content. The inhibition of intestinal content was then computed using the expression:

Inhibition of intestinal content (%) = Mass of intestinal fluid (g) $\frac{\text{Control} - \text{Treatment}}{Control} \times 100$

3.7.3 Gastrointestinal Motility Test

This was determined as described by Al-Taher (2008) with slight modification. Thirty minutes after the administration of 2 % Tween 80 (v/v), atropine sulphate and the various fractions of the extract solution, 1 ml of castor oil was administered orally to the animals to induce diarrhoea. After 30 minutes of castor oil administration, all animals received 1 ml of charcoal meal marker (10 % charcoal suspension in 2 % tween 80) orally. All the animals were sacrificed after 30 minutes of charcoal meal administration and the small intestine was rapidly dissected for careful examination. The distance travelled by charcoal meal (DCM) plug from the pylorus to caecum was measured and expressed as a percentage of the total

length of the small intestine (LSI). The peristaltic index (PI) for each rat was calculated, expressed as percentage of the DCM relative to the LSI as;

PI = $\frac{DCM}{LSI} \times 100$.

The percentage inhibition relative to the control was also calculated using the expression:

Percentage Inhibition = Mean of distance travelled by marker $(\frac{\text{Control – Treatment}}{Control}) \times 100$

3.8 Antidiarrhoeal Index

The combined effect of all the diarrhoeal models monitored on the test groups were compared with their respective controls for the determination of antidiarrhoeal index. The ADI was calculated using the mathematical expression described by Than *et al*. (1989) as;

ADI = $\sqrt[3]{Dfreq \times Gmeq \times Pfreq}$

Where: $D_{freq.}$ = Delay in onset time of test subjects as a percentage function of the control;

$G_{meq.}$ = Reduction in peristaltic movement as a percentage function of the control; and

$P_{freq.}$ = Reduction of intraluminal fluid content as a percentage function of the control.

3.9 Column Chromatographic Analysis of the Residual Fraction

The residual fraction of the aqueous extract of *M. indica* leaves was further fractionated using column chromatography as described by Reid and Sarker (2012). A homogenous slurry was prepared by dissolving the residual fraction in the solvent system composed of methanol, ethyl acetate and distilled water in the ratio 5:3:2. This was then adsorbed on silica gel and dried to form a powdery sample. The column was packed with silica gel (100 – 200 mesh size) and the powdered sample at the top of the column for isocratic elution. The column was then eluted with the same solvent system with the eluents collected at 7 minutes interval. A total of 16 eluents were collected and subjected to thin layer chromatography in order to obtain their retention factor (R_f) values.

3.10 Thin Layer Chromatography of the Column Chromatographic Eluents

The procedure described by Singh and Sahu (2005) was adopted for the preparation of thin layer chromatogram. A drop of each eluent was spotted using a capillary tube on a pre-coated thin layer chromatographic (TLC) paper (K_5 – Silica gel, 150A^o layer thickness: Whatmann). The plates were developed in a chromatography tank containing the solvent system: methanol, ethyl acetate and distilled water (5: 3: 2). The plate was withdrawn from the TLC chamber, dried at room temperature and then visualized in an iodine vapour chamber and ultraviolet light (UV 254 nm). Their respective R_f values were determined using the expression:

$$R_f \text{ value} = \frac{\text{Distance travelled by each compound from the base line}}{\text{Distance travelled by the solvent}}$$

The eluents were grouped into three based on their R_f values as sub-fraction A (fractions 1 – 4; $R_f = 0.94$), B (fractions 5 – 12; $R_f = 0.86$) and C (fractions 13 – 16; $R_f = 0.32$).

3.11 Antidiarrhoeal Activity of Sub-Fractions A, B and C

Sub-fractions A, B and C were further evaluated for their antidiarrhoeal activities using castor oil-induced diarrhoea, castor oil-induced enteropooling and gastrointestinal motility test models as previously described in sections 2.8.1, 2.8.2 and 2.8.3. The small intestine was excised for biochemical analysis to determine probable mechanism(s) of action as an antidiarrhoeal agent.

3.12 Preparation of Serum and Tissue Supernatant

The animals were sacrificed following the procedure described by Akanji and Yakubu (2000). The rats were anaesthetized in a glass jar saturated with diethyl ether fumes to make them unconscious. The jugular veins were immediately cut and blood was collected in appropriately labelled centrifuge tubes. This was left to clot at room temperature for 20 minutes and thereafter, centrifuged at 685 × g for 10 minutes. After centrifugation, the supernatant was aspirated using a Pasteur's pipette. The serum was stored in a freezer

maintained at -5℃ and used within 12 hours of preparation for the various biochemical analyses.

After sacrifice, the organs of interest (liver, kidney and/or small intestine) were isolated from the dissected rats. The small intestine was emptied of faecal contents, the kidney was decapsulated and the liver was defatted. Afterwards, all the organs were blotted to remove blood stains. The isolated tissues were weighed, and then stored immediately in ice cold 0.25M sucrose solution. A known weight of the liver, pair of kidney, and small intestine were then homogenized separately in ice-cold 0.25M sucrose solution (1:5 w/v). The homogenates were centrifuged at 1398 × *g* for 15 minutes after which the supernatants were aspirated and stored in the freezer (-5℃) for further analysis.

3.13 Tissue Dilution

The supernatants obtained after centrifugation were then appropriately diluted using 0.25M sucrose solution (Table 3).

3.14 Parameters Adopted for Studying Mechanisms of *M. indica* as an Antidiarrhoeal agent

3.14.1 Antisecretory Mechanism

3.14.1.1 Determination of Na^+-K^+ ATPase Activity

The procedure described by Ronner *et al*. (1977) as modified by Bewaji *et al*. (1985) was adopted.

Principle

ATP is hydrolysed in the presence of appropriate cations to release inorganic phosphate. The amount of inorganic phosphate is measured using the ammonium molybdate-ascorbic acid system. Concentrated sulphuric acid oxidizes ammonium molybdate to molybdic acid, which gives a yellow colour with inorganic phosphate. Ascorbic acid reduces the molybdic

acid to give a blue colouration. The intensity of the colour is proportional to the concentration of inorganic phosphate liberated into the reaction medium.

Table 3: Dilution factor for various assays

Parameters / Organ	Liver	Kidney	Small Intestine	Serum
Acetylcholinesterase	--	--	300	--
Albumin	--	--	--	5
ALP	30	--	30	--
ALT	--	--	--	30
AST	--	--	--	30
Bilirubin	--	--	--	5
Catalase	--	--	60	--
Creatinine	--	30	--	--
Globulin	--	--	--	5
Glucose	--	--	30	--
Reduced glutathione	--	--	60	--
Malondialdehyde	--	--	60	--
Mg^{2+} ATPase	--	300	300	--
Na^{+}, K^{+} and Cl^{-}	--	--	--	5
Na^{+}-K^{+} ATPase	--	300	300	--
Nitrite	--	--	25	--
Protein carbonyl	--	--	30	--
Superoxide dismutase	--	--	60	--
Total Protein	30	--	30	--
Urea	--	30	--	--
Uric Acid	--	30	--	--

Procedure for Determination of Na^+-K^+ ATPase Activity in the Small Intestine

For this test, 400 µl of 200 mM NaCl / 40 mM KCl / 60 mM Tris (pH 7.4) was pipetted into test tube. Thereafter, 20 µl of $MgCl_2.6H_2O$ (80 mM), 20 µl of EGTA (20 mM), 240 µl of distilled water and 20 µl of appropriately diluted supernatant was added. This was mixed and incubated at 37℃ for 5 minutes after which 100 µl of ATP (8 mM) was added. The resulting solution was also mixed and incubated at 37℃ for 30 minutes. Thereafter, 200 µl of SDS (5%) and 2,000 µl of reagent C (mixture of ammonium molybdate/sulphuric acid solution [reagent A] and 9% ascorbic acid [reagent B] in ratio 4:1 v/v) were added. The mixture was left undisturbed at room temperature for 30 minutes for colour development. The blank was similarly prepared except that 20 µl of distilled water was used instead of the 20 µl of the supernatant. The absorbance of the test was read against the blank at 820 nm. The absorbance obtained was then extrapolated from the calibration curve for phosphate (Figure 19) to obtain the concentration of inorganic phosphate.

Calculation:

$$\text{Specific activity (µmol Pi/mg protein/hr)} = \frac{[Pi] \times 2 \times D.F}{1000 \times \text{Protein Conc (mg/ml)}}$$

Where:

[Pi] = Concentration of inorganic phosphate in nmoles (obtained from the calibration curve).

2 = Factor introduced to obtain the amount of Pi released per hour

D.F = Dilution Factor

1000 = factor introduced to convert the Pi released to µmol.

NOTE:

The specific activity of Na^+-K^+ ATPase was obtained by subtracting the specific activity of Mg^{2+}-ATPase from that of Na^+-K^+ ATPase.

3.14.1.2 Assay of Mg^{2+} ATPase Activity

The determination of Mg^{2+}-ATPase activity was carried out following the method described by Fleschner and Kraus-Friedmann (1986).

Principle:

Procedure for Determination of Mg^{2+} ATPase Activity in the Small Intestine

For the test, 400 µl of 240 mM KCl / 60 mM Tris (pH 7.4) was pipetted into test tube. Thereafter, 20 µl of $MgCl_2.6H_2O$ (80 mM), 20µl of EGTA (20 mM), 220 µl of distilled water, 20 µl of appropriately diluted supernatant and 20 µl of Vanadate (0.04 M) was added. This was mixed and incubated at 37℃ for 5 minutes to which 100 µl of ATP (8 mM) was added. The resulting solution was also mixed and incubated at 37℃ for 30 minutes. Thereafter, 200 µl of SDS (5%) and 2,000 µl of reagent C was added. The mixture was left undisturbed at room temperature for 30 minutes for colour development. The blank was similarly prepared except that 20 µl of distilled water was used instead of the 20 µl of the supernatant. The absorbance of the test was read against the blank at 820 nm. The absorbance obtained was then extrapolated from the calibration curve for phosphate (Figure 19) to obtain concentration of inorganic phosphate.

Calculation:

$$\text{Specific activity (µmol Pi/mg protein/hr)} = \frac{[Pi] \times 2 \times D.F}{1000 \times \text{Protein Conc (mg/ml)}}$$

Where;

[Pi] = Concentration of inorganic phosphate in nmoles (obtained from the calibration curve).

2 = Factor introduced to obtain the amount of Pi released per hour

D.F = Dilution Factor

1000 = factor introduced to convert the Pi release to µmoles.

Preparation of Calibration Curve for Inorganic Phosphate

Varying volumes (20-120 µl) of 1 mM $NaH_2PO_4.2H_2O$ were pipetted into six test tubes and distilled water was added to each of the test tube to make up the volume to 1 ml. Thereafter, 2 ml of reagent C was added and the mixtures were left undisturbed at room temperature for 30 minutes. The absorbance of the resulting mixture which was read at 820 nm against reagent blank was then used to plot the calibration curve for inorganic phosphate (Figure 19).

3.14.1.3 Determination of Intestinal Glucose Concentration

The method described by Trinder (1969) was adopted for the determination of intestinal glucose concentration.

Principle

The reaction between hydrogen peroxide, phenol and aminoantipyrine is employed to form a quinone in the presence of peroxidase. Glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-δ-lactone with the concurrent release of hydrogen peroxides. Peroxidase catalyses the hydrogen peroxide formed in a second reaction into H_2O and gluconic acid in the presence of p-hydroxybenzoic acid and 4-aminoantipyrene with the quantitative formation of quinoneimine dye complex. A red-violet colouration was formed and the intensity which is proportional to glucose concentration was read at 510 nm.

Procedure

A known volume (100 µl) of samples and standard were pipetted into test tubes to which 100 µl of reagent R_2 (made up of phosphate buffer at pH 7.0, phenol, glucose oxidase, peroxidase and 4-aminoantipyrene) was pipetted. The mixture was incubated for 10 minutes at 37^0C and the absorbance of the samples and standard were read against the blank at 510 nm.

Calculation:

$$\text{Glucose concentration (mmol/l)} = \frac{A_{sample} \times \text{Concentration of standard}}{A_{standard}}$$

Where; A_{sample} is the absorbance of the sample and;

$A_{standard}$ is the absorbance of the standard

3.14.1.4 Determination of Serum Electrolytes

3.14.1.4.1 Serum Sodium Ion

The serum concentration of sodium ion was determined as described by Tietz (1995).

Principle

This principle is based on energy level of ions in the solution. The ions absorb light energy coming from a gas source which results in a transfer to a higher energy level. The ions later fall back to their ground state when the energy source is exhausted. The greater the number of ions in the sample, the higher the concentration (Tietz, 1995).

Procedure

A known volume (500 µl) of the working standard was added to 10 µl of the appropriately diluted sample while the blank was constituted by replacing the sample with 10 µl of distilled water. The sample was thoroughly mixed and absorbance was read against the reagent blank at 590 nm. The concentration of sodium ion was calculated using the expression:

Calculation:

$$\text{Sodium ion concentration (mEq/l)} = \frac{\Delta A_{sample} \text{ x Standard concentration}}{\Delta A_{standard}}$$

Where ΔA_{sample} is the change in absorbance of the sample; and

$\Delta A_{standard}$ is the change in absorbance of the standard

3.14.1.4.2 Serum Potassium Ion

Flame photometry method described by Tietz (1995) was adopted for the determination of the serum concentration of potassium ion.

Principle

Potassium solution under carefully controlled conditions and when fully sprayed (aspirated) into a burner, dissociates to give neutral atoms in their ground state. Some of these atoms become excited in the flame and move into higher energy states. This in-turn causes a light reflection that passes through a suitable filter into a photo-sensitive element. The amount of current produced is proportional to the concentration of potassium in the original solution.

Procedure

A known volume (250 µl) of the working standard was added to 10 µl of the sample. The blank was constituted by replacing the sample with 10 µl of distilled water while the standard was consisted of 260 µl of the working reagent alone. The sample was mixed together and absorbance read at 770 nm against the reagent blank. The absorbance of the test samples and the standard were used for the calculation of potassium ion using the expression:

Calculation:

$$\text{Potassium ion concentration (mEq/l)} = \frac{\Delta A_{sample} \times \text{Concentration of standard}}{\Delta A_{standard}}$$

Where ΔA_{sample} is the change in absorbance of the sample; and

$\Delta A_{standard}$ is the change in absorbance of the standard

3.14.1.4.3 Serum Chloride Ion

This will be done using mercuric nitrate as described by Tietz (1995).

Principle

Chloride ions in the sample react with mercuric thiocyanate displacing the thiocyanate ion. The free thiocyanate ions formed react with ferric ions to produce a coloured complex. This

ferric coloured complex absorbs light at 436 nm; a rate which is proportional to the concentration of chloride ions in the sample.

Procedure

A known volume (100 µl) of the appropriately diluted sample was dispensed into clean test tubes. The blank was constituted by adding 10 µl of distilled water to the sample while 10 µl of the standard solution was added for the standard. The mixture was incubated for 5 minutes at 37ºC after which the absorbance of the sample and standard was read at 436 nm against the reagent blank. The absorbance of the test samples and the standard were used for the calculation of potassium ion by adopting the following expression:

Calculation:

$$\text{Chloride ion concentration (mEq/l)} = \frac{\Delta A_{sample} \times \text{Concentration of standard}}{\Delta A_{standard}}$$

Where ΔA_{sample} is the change in absorbance of the sample; and

$\Delta A_{standard}$ is the change in absorbance of the standard

3.14.2 Anti-inflammatory Mechanism

3.14.2.1 Determination of Intestinal Alkaline Phosphatase Activity

The method described by Wright *et al.* (1972) was adopted for this assay.

Principle

The amount of phosphate ester that was split within a given period of time is a measure of the phosphatase enzyme. Para-nitrophenyl phosphate is hydrolysed to para-nitrophenol and phosphoric acid at a pH of 10.1. The para-nitrophenol confers a yellowish colour on the reaction mixture and its intensity is read spectrophotometrically at 400 nm.

Procedure

A known volume (1100 µl) of carbonate buffer (0.1 M) and 50 µl of $MgSO_4.7H_2O$ (0.1 M) were added to the test tubes. Then 100 µl of the supernatant (appropriately diluted) was added and incubated at 37ºC for 10 minutes. Thereafter, 250 µl of 10 mM p-nitrophenyl phosphate was added and the solution was incubated for 30 minutes at 37ºC. The reaction was immediately terminated by adding 1000 µl of 1N sodium hydroxide. The blank was constituted by replacing the supernatant with 100 µl of distilled water after which the absorbance was read at 400 nm. Enzyme activity was calculated using the following expression:

Calculation:

$$\text{Enzyme activity (nm/min/ml)} = \frac{\Delta A/\text{min} \times 1000 \times TV \times F}{9.9 \times SV \times L}$$

Where: ΔA/min = Change in absorbance of the reaction mixture per minute;

TV = Total volume of the reaction mixture;

F = Total dilution factor;

SV = Volume of the supernatant

L = Light path length (1cm);

9.9 = Extinction co-efficient of 1 µm of p-nitrophenol in an alkaline solution of 1 ml and 1 cm path length; and

1000 = the factor introduced to enable the enzyme activity to be expressed in nM/min/mg protein.

The specific activity for alkaline phosphatase was calculated from the expression:

Calculation:

Specific enzyme activity (nM/min/mg protein) = Enzyme activity ÷ Protein concentration

3.14.2.2 Determination of Nitric Oxide Concentration

The concentration of nitric oxide was determined as described by Wo *et al.* (2013).

Principle

The principle is based on the reduction of nitrate by copper-coated cadmium and the nitrite produced is determined by diazotization of fuchsin acid under acidic condition, coupling to resorcinol in a slightly alkaline medium.

Procedure

The procedure involved deproteinization of supernatant, activation of cadmium granules and the reduction of nitrate to nitrite.

i. Deproteinization of Supernatant

A known volume (50 µl) of the supernatant was added to 200 µl of 75mmol/l $ZnSO_4$ solution, followed by 250 µl of 55mmol/l NaOH reagent and mixed properly. After 10 minutes of incubation, the solution was centrifuged at 504 × *g* for another 10 minutes. The supernatants were then aspirated and used for the determination of nitrite concentration.

ii. Activation of Cadmium Granules

The acid from the cadmium granules was rinsed 3 times with distilled water. The cadmium granules were then shaken for 2 minutes with 5 mmol/l $CuSO_4$ in glycine-NaOH buffer. The activated copper coated granules were then used within 10 minutes of preparation as the prolonged exposure of activated granules to air diminishes their reductive capacity.

iii. Nitrite Assay

A known volume (100 µl) of glycine-NaOH buffer was pipetted into empty tubes labelled blank and samples. A known volume (200 µl) of the supernatant was added to each of the sample tubes. The volume of the solution was made up to 400 µl in all the sample tubes using distilled water while the supernatant was replaced with distilled water for the blank. The reaction was initiated by the addition of 2.5 mg of freshly activated cadmium granules to each of the test tubes, stirred and left undisturbed for 60 minutes. Afterwards, 200 µl of each

sample were dispensed into an appropriately labelled tube to which 250 µl of ethylene diamine tetra-acetic acid (EDTA) solution, 300 µl of 1.0 mol/l HCl and 30 µl of 1.0 g/l fuchsin acid solution were added to give a final volume of 780 µl; this was thoroughly mixed and incubated at room temperature for 2 minutes. Thereafter, 20 µl of 0.05 mol/l resorcinol solution and 300 µl of 1.0ml/l NH_4OH were added and mixed thoroughly. The absorbance of the samples were determined against the blank at 436nm. The concentration of nitrite in the supernatant was then extrapolated from the calibration curve (Figure 20) and multiplied by 25 (the dilution factor).

Preparation of Calibration Curve for Nitrite

The calibration curve (Figure 20) was prepared by using working standard solution of different concentration of sodium nitrite ($NaNO_2$). A stock standard of NO_2^- was prepared with $NaNO_2$ to yield a concentration of 2.18 mmol/l. A working standard solution was also prepared by diluting the stock standard at the time of assay. Other reagents including fuchsin acid solution, 1.0 g/l; resorcinol solution, 0.05 mol/l; EDTA solution, 0.1 mol/l; ammonium hydroxide, 1.0 mol/l; hydrochloric acid, 1.0 mol/l; buffer solution, glycine-NaOH buffer (pH 9.7) were added (as described earlier) to the concentration series of each standard made at 0, 22, 44, 66, 88, 110, 132, 154, 176 and 198 (µmol/l) and made up to 1,100 µl using distilled water. The absorbance was read at 436 nm.

3.14.3 Antioxidant Mechanism

3.14.3.1 Superoxide Dismutase Activity

The method described by Misra and Fridovich (1972) was adopted for this assay.

Principle

The method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2, 4 (iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) and causes a red dye formation. The superoxide dismutase activity is then measured by the degree of

inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of INT under assay conditions.

Procedure

A known amount (50 µl) of the sample was diluted with 50 µl of 0.01 mol/L phosphate buffer (pH 7) and added to 170 µl of mixed substrate (0.05 mmol/L xanthine, 0.025 mmol/L I.N.T, 40 mmol/L (pH 10.2), 0.94 mmol/L EDTA). This was thoroughly mixed, after which 250 µl of xanthine oxidase (80 U/L) was added to the solution. An increase in absorbance was recorded every 30 s for 150 s at 480 nm. The % inhibition was calculated using the expression:

Calculation:

$$\% \text{ Inhibition} = 100 - \frac{\Delta\text{Absorbance of sample/ min} \times 100}{\Delta\text{Absorbance of blank/min}}$$

The enzyme activity was calculated using the expression:

$$\text{SOD activity (nmol/min/ml)} = \frac{\% \text{ Inhibition} \times 1000 \times \text{dilution factor}}{50 \times 0.05}$$

Where: 0.05 is the volume of sample used;

1000 = factor introduced to enable the expression of enzyme activity in nmol/min/ml;

50 = Inhibition of the rate of reduction of INT per unit SOD

The specific activity of SOD was determined from the expression:

$$\text{Specific enzyme activity (nmol/min/mg protein)} = \frac{\text{SOD activity (nmol/min/ml)}}{\text{Protein concentration (mg/ml)}}$$

3.14.3.2 Catalase Activity

The activity of catalase was determined as described by Aebi (1974).

Principle

The method is based on the ability of catalase to reduce hydrogen peroxide (H_2O_2) to water and oxygen. One unit of catalase will decompose 1.0 µmole of H_2O_2 per minute at pH 7.0 and 25℃.

Procedure

Tissue supernatant (10 µl) was added to test tube containing 190 µl of 50 mM phosphate buffer (pH 7.0). Reaction was initiated by the addition of 100 µl of freshly prepared 30 mM H_2O_2 after which the absorbance was read at 240 nm for 60 seconds. The molar extinction coefficient of H_2O_2 (43.6M cm^{-1}) was used to compute the catalase activity. One unit of activity is equal to 1 mmol of H_2O_2 degraded per minute and was expressed as units per milligram of protein.

3.14.3.3 Reduced Glutathione

The level of reduced glutathione was determined according to the method described by Ellman (1959).

Principle

The acid soluble sulfhydryl groups (non-protein thiols of which more than 93% is reduced glutathione) form a yellow coloured complex with dithionitrobenzoic acid (DTNB). The absorbance of the coloured complex is read at 412 nm.

Procedure

A known amount (500 µl) of the tissue supernatant was mixed with 100 µl of 25 % TCA and kept on ice for few minutes. The solution was then centrifuged at 1754 × g for 10 minutes. Afterwards, 300 µl of the resulting supernatant was mixed with 700 µl of 0.2 M sodium phosphate buffer (pH 8) and 2000 µl of 0.6 mM DTNB (prepared in 0.2 M buffer, pH 8). The yellow colour obtained was read after 10 minutes at 412 nm against a blank which contained 100 µl of 5 % TCA instead of the supernatant. A calibration curve (Figure 21) was

prepared using different concentrations (10-50 nmoles) of GSH in 300 µl of 5 % TCA. The GSH content was extrapolated from the calibration curve and expressed as nmols/mg protein.

3.14.4 Antimotility Mechanism

3.14.4.1 Acetylcholinesterase

Acetylcholinesterase (AchE) activity was determined by following the procedures described by Ellman *et al.* (1961).

Principle

The principle is based on the cleavage of acetylthiocholine by AChE to release thiocholine which reacts with the sulphydryl end of DTNB to form thionitrobenzoic acid. This anion leads to a yellow colouration that absorbs light maximally at 412 nm.

Procedure

A known volume of the sample (50 µl) was mixed with 1 ml of phosphate buffer (0.1 M, pH 8.0), 50 µl of DTNB, 10 mM and 20 µl acetylthiocholine iodide (78 mM). The change in absorbance was monitored at 412 nm for 3 minutes. The enzyme activity (n-moles of substrate hydrolyzed/min/mg protein) was determined from the expression:

Calculation:

$$\text{Acetylcholinesterase activity} = \frac{\Delta A \times Vol_t \times 1000}{1.36 \times 10^4 \times LP \times Vol_s \times \text{protein concentration}}$$

Where ΔA = Change in absorbance

Vol_t = Total volume of the assay

1.36×10^4 = extinction coefficient of DTNB

LP = Light path = 1

Vol_s = Volume of sample used (ml)

3.14.5 Intestinal Barrier Function

3.14.5.1 Malondialdehyde

The procedures described by Ohkawa *et al.* (1979) was adopted for the determination of malondialdehyde (MDA).

Principle

The principle involves the formation of an adduct with TBA. The adduct formed (MDA-TBA) during the reaction in acidic medium is extracted to the organic layer of the reaction mixture and the absorbance was measured at 532 nm.

Procedure

A solution containing 200 µl of the tissue homogenate, 750 µl of 0.8 % TBA, 750 µl of acetic acid (20 %, pH 3.5) and 300 µl of distilled water was kept in a boiling water bath for 1 hour at 95℃. Thereafter, the solution was removed from the water bath and cooled. Then, 500 µl of distilled water and 2500 µl of butanol:pyridine mixture (15:1) was added to the reaction tube, thoroughly mixed and centrifuged at 1000 × *g* for 10 minutes. The absorbance of the clear supernatant obtained was then read at 532 nm against butanol:pyridine mixture. MDA content was determined using the molar extinction coefficient of 1.56×10^6.

Calculation:

Concentration of MDA = $\frac{Absorbance\ of\ sample}{Extinction\ coefficient}$

3.14.5.2 Determination of Intestinal Total Protein

The concentration of intestinal total protein was determined using Biuret reagent method described by Gornal *et al.* (1949).

Principle

The principle is based on the formation of purple coloured complex of cupric ion in alkaline medium which interacts with peptide bond. The intensity of the purple colouration is due to the coordination between the cupric ions and the unshared electron pair of nitrogen

and oxygen in the peptide bond to form the coloured coordination complex. The intensity of the purple colour is proportional to the total protein concentration.

Procedure

A known volume (400 µl) of Biuret reagent was added to 100 µl of the appropriately diluted supernatant. This was mixed thoroughly and left undisturbed for 30 minutes at room temperature for colour development. The blank was constituted by replacing the sample with equal volume of distilled water. The absorbance was read against blank at 540 nm. The concentration of protein in the sample was calculated by comparing with those on the calibration curve (Figure 22) for egg albumin. Concentration of the protein in the sample was then extrapolated from the calibration curve for egg albumin using the expression below:

Protein concentration (mg/ml) = $C_s \times F$

Where: C_s = Corresponding protein concentration from the calibration curve

F = dilution factor

Preparation of Calibration Curve for Protein

A protein standard, egg albumin stock solution (10 mg/ml) was prepared. Varying volumes of the stock solution corresponding to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µl were dispensed into cleaned test tubes. The volumes were made up to 100 µl with distilled water after which 400 µl of Biuret reagent was added, making the total volume of the prepared solutions to 500 µl. The solutions were left undisturbed for 30 minutes at room temperature after which the absorbance was read at 540nm.

3.14.5.3 Determination of Protein Carbonyl

The concentration of intestinal protein carbonyl was determined as described by Levine *et al.* (1990).

Principle

This method is based on the reaction between DNPH and protein carbonyls to form a Schiff base which produces the corresponding hydrazine; the absorbance of which was read at 370 nm.

Procedure

An aliquot (100 µL) of test samples was dispensed in test tubes to which equal volume of 10mM DNPH and 2.5 M HCl were added. This was mixed thoroughly and incubated at room temperature for 1 hour with intermittent stirring. Afterwards, equal volume of 10% (w/v) trichloroacetic acid (TCA) was added and centrifuged at 3500 × g for 20 minutes to obtain the protein pellets. The supernatant was aspirated and discarded while the precipitates were re-washed using 10% TCA. Furthermore, the precipitate was washed thrice using ethanol and ethyl acetate (1:1 v/v) in order to rid the solution of any trace of unreacted DNPH and lipid remnants. The resulting solution was dissolved in 2 ml of guanidine hydrochloride and incubated at 37℃ for 10 minutes. The insoluble materials were removed by centrifugation at 1000 × g for 10 minutes while the carbonyl content was determined by reading the absorbance at 370 nm. The test samples were read against the control and carbonyl content was calculated using the following expression:

Calculation:

Protein carbonyl (µM/mg of protein) = $\frac{\Delta A \times df \times 1.2 \times 10^4}{22000 \times Protein\ concentration}$

Where ΔA is the change in absorbance (Sample – Control);

df is the dilution factor;

22000 is the molar absorption coefficient and

1.2×10^4 is the extinction coefficient of protein carbonyl.

3.14.5.4 Protein Carbonylation

The percentage of protein oxidized to protein carbonyl was determined by adopting the expression:

Protein carbonylation (%) = $\frac{Protein\ carbonyl}{Total\ protein} \times 100$

The sub-fraction that exhibited the most profound antidiarrhoeal activity was then subjected to high performance liquid chromatography (HPLC) for identification of potential bioactive agents.

3.15 Identification of Bioactive Agents

The most active sub-fraction was subjected to high performance liquid chromatography (HPLC) following the procedures established by Sasidharan *et al.* (2011). The chromatographic analysis was carried out on a reversed phase C_{18} column with acetonitrile/water (75:25) mixture under gradient elution. A known quantity of the dried sample (0.5 g) was dissolved in 5 ml of ethanol (40 %) for 24 hours. The mixture was then filtered. Afterwards, the supernatant was evaporated to dryness in a boiling water bath. The resulting dry powder was then dissolved in acetonitrile and water in the ratio 3:1. An aliquot (5 µl) of the sample was then injected into the sample chamber of the HPLC machine for detection with UV at 280 nm.

3.16 Toxicity Study

The most active sub-fraction B of the residual fraction of the aqueous extract of *M. indica* leaves was evaluated for toxicity on the liver, kidney and small intestine of female Wistar rats. Twenty-four matured female rats were randomized into four groups (A, B, C and D) of six animals each. Rats in group A were the control and received 1 ml of distilled water only for 72 hours whereas rats in groups B, C and D received equal volume of the most active sub-fraction of the residual fraction corresponding to 3.24, 6.47 and 12.94 mg/kg body weight respectively. The rats were sacrificed 24 hours post administration and the serum and tissue homogenates were obtained as described earlier in section 3.2.

3.16.1 Determination of Liver Function Indices

3.16.1.1 Total Protein

The total protein concentration was determined as described in section 3.14.5.2.

3.16.1.2 Serum Albumin

The procedure described by Doumas *et al.* (1971) was adopted for the determination of albumin in the serum of the animals.

Principle

The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3', 5, 5'-tetrabromocresol sulphonapthalein (bromocresol green, BCG). The albumin BCG complex absorbs maximally at 578 nm; the absorbance being directly proportional to the albumin concentration in the sample.

Procedure

A known volume (300 µl) of reagent 2 was added to test tubes containing 10 µl of the appropriately diluted serum. The blank and standard were constituted by replacing the serum with equal volume of distilled water and reagent 1 respectively. The solution was then incubated at 37℃ for 10 minutes and the absorbance was read at 630 nm.

Calculation

$$\text{Albumin concentration (g/L)} = \frac{A_{sample} \times \text{Concentration of standard}}{A_{standard}}$$

Concentration of standard = 50.3 g/L

Where ΔA_{sample} is the change in absorbance of the sample; and

$\Delta A_{standard}$ is the change in absorbance of the standard

3.16.1.3 Serum Globulin

The concentration of serum globulin was determined by adopting the expression described by Tietz (1995).

Serum globulin = Total protein – Serum albumin

3.16.1.4 Albumin/Globulin Ratio

The concentration of serum albumin:globulin was determined by adopting the expression:

Albumin-Globulin ratio = $\frac{Concentration\ of\ serum\ albumin}{Concentration\ of\ serum\ globulin}$

3.16.1.5 Serum Bilirubin

Bilirubin was determined using the method described by Jendrassik and Grof (1938).

Principle

Direct (conjugated) bilirubin in the serum reacts with diazotized sulphanilic acid in alkaline medium to form blue coloured complex while total bilirubin was determined in the presence of caffeine, which releases albumin bound bilirubin.

Direct Bilirubin

A known volume (100 µl) of reagent 1, 25 µl of reagent 2 and 500 µl of 0.9% NaCl were added to 50 µl of the sample. The blank was constituted the same way as the test except for the absence of 25 µl of reagent 2. The solution was mixed and incubated for 10 minutes at 25℃, after which the absorbance was read at 546 nm against the sample blank.

Total Bilirubin

A known volume (100 µl) of reagent 1, 25 µl of reagent 2 and 500 µl of reagent 3 were added to 50 µl of the sample. The blank was constituted the same way as the test except for the absence of 25 µl of reagent 2. The solution was mixed and incubated for 10 minutes at 25℃. Then, 100 µl of reagent 4 was added to all the solutions (including the blank), this was then mixed and incubated for 15 minutes at 25℃. The absorbance of the samples were read at 578 nm against the sample blank.

Calculation

Total bilirubin (µmol/L) = $185 \times A_{TB}$

Conjugated bilirubin (µmol/L) = 246 × A_{DB}

Where A_{TB} = Absorbance of total bilirubin and A_{DB} = Absorbance of direct bilirubin

3.16.1.6 Determination of Alanine Aminotransferase Activity

The method described by Reitman and Frankel (1957) was adopted for the determination of activity of alanine aminotransferase in the female rats.

Principle

Alanine aminotransferase activity was determined by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenyl hydrazine.

Procedure

A known volume (500 µl) of reagent 1 was added to each test tube containing 100 µl of the enzyme source and incubated for 30 minutes at 37℃. Then, 500 µl of reagent 2 was added after which the solution was thoroughly mixed and left undisturbed for 20 minutes at 25℃. The reaction was then terminated by adding 500 µl of 0.4N sodium hydroxide. The blank was constituted by replacing the enzyme source with 100 µl of distilled water. The solution was mixed and absorbance read against blank after 5 minutes at 468 nm. The enzyme activity was extrapolated from the calibration curve (Figure 23).

3.16.1.7 Determination of Aspartate Aminotransferase Activity

The method described by Reitman and Frankel (1957) was adopted for the determination of the activity of aspartate aminotransferase.

Principle

The enzyme catalyses the reversible reaction involving α–ketoglutarate and L-aspartate to form L-glutamate and oxaloacetate. Aspartate aminotransferase can then be determined by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenyl hydrazine.

Procedure

A known volume (500 µl) of reagent 1 was added to each test tube containing 100 µl of the enzyme source (appropriately diluted) and incubated for 30 minutes at 37℃. Then, 500 µl of reagent 2 was added and the solution was thoroughly mixed and left undisturbed for 20 minutes at 25℃ after which the reaction was immediately terminated by adding 0.5 ml of 0.4N sodium hydroxide. The blank was constituted by replacing the enzyme source with 100 µl of distilled water. The solution was again thoroughly mixed after which the absorbance was read against blank after 5 minutes at 468 nm. The enzyme activity was obtained from the calibration curve (Figure 24).

3.16.1.8 AST/ALT Ratio

The AST:ALT was determined by adopting the expression:

Calculation

$$\text{AST/ALT ratio} = \frac{Concentration\ of\ serum\ AST}{Concentration\ of\ serum\ ALT}$$

3.16.1.9 Determination of Liver Alkaline Phosphatase

This was determined following the procedure of Wright *et al.* (1972) as earlier described for intestinal ALP in section 3.14.2.1.

3.16.2 Determination of Kidney Function Indices

3.16.2.1 Creatinine

This was determined as described by Bartels and Bohmer (1972).

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 read spectrophotometrically at 490 nm.

Procedure

A known volume (500 µl) of the working reagent was added to 50 µl of the sample (appropriately diluted) while the standard was constituted by replacing the sample with 50 µl

of the standard solution. The solution was mixed, after which the absorbance (A_1) of both the standard and samples were recorded after 30 seconds at 492 nm. Exactly 2 minutes later, the absorbance (A_2) of the standard and samples were recorded again.

Calculation

$$\text{Concentration of creatinine (µmol/L)} = \frac{\Delta A_{sample} \times \text{Concentration of standard}}{\Delta A_{standard}}$$

Concentration of standard = 182 µmol/L

$A_2 - A_1$ = Difference in absorbance of sample or standard between 30 – 120 seconds

Where; ΔA_{sample} is the change in absorbance of the sample; and

$\Delta A_{standard}$ is the change in absorbance of the standard

3.16.2.2 Creatinine Clearance Test

The rate of clearance of creatinine by the glomerulus was computed using the expression:

Calculation:

$$\text{Creatinine clearance} = \frac{Uc \times V}{Sc}$$

Where U_c = urine creatinine;

V = volume of urine; and

S_c = serum creatinine

3.16.2.3 Serum Urea

The concentration of serum urea was determined by adopting the method described by Veniamin and Vakirtzi (1970).

Principle

When urea is heated in strongly acidic conditions with substances such as diacetyl ($CH_3COCOCH_3COC{=}NaOH$), yellow condensation compounds are formed; the reaction is intensified by the presence of polyvalent ions such as ferric ion, which leads to the formation of red coloured complex. The intensity is proportional to the concentration of urea in the sample.

Procedure

A known volume (100 µl) of reagent 1 was added to test tubes containing 10 µl of the appropriately diluted sample, blank and standard; both of which were constituted by replacing the diluted samples with 10 µl of distilled water and standard working reagent respectively. The reaction constituent was thoroughly mixed and incubated at 37℃ for 10 minutes. Thereafter, 2500 µl of reagent 2 and 3 were sequentially added to the test tubes (sample, blank and standard). The reaction constituent was thoroughly mixed and incubated again at 37℃ for 15 minutes. Absorbance of the sample and standard was read against the blank at 546 nm.

Calculation

$$\text{Concentration of urea (mmol/L)} = \frac{\Delta A_{sample} \times \text{Concentration of standard}}{\Delta A_{standard}}$$

Concentration of standard = 13.37 mmol/L

Where ΔA_{sample} is the change in absorbance of the sample; and

$\Delta A_{standard}$ is the change in absorbance of the standard

3.16.2.4 Serum Uric Acid

Serum uric acid was determined following the procedure described by Tietz (1995).

Principle

The principle behind the determination of uric acid in the serum is based on the reduction of phosphotungistic acid by uric acid in the presence of sodium carbonate or cyanide to give

blue complex, which can be read spectrophotometrically at a wavelength of 620 nm.

Procedure

A known volume (500 µl) of the reagent was added to test tubes containing 20 µl of the serum (appropriately diluted). The blank and standard were constituted by replacing the serum with equal volume (20 µl) of distilled water and standard working reagent respectively. The mixture was incubated for 5 minutes at 37℃ after which the absorbance of sample and standard were read spectrophotometrically against reagent blank at 520 nm.

Calculation

$$\text{Concentration of uric acid (mg/dl)} = \frac{\Delta A_{sample} \times \text{Concentration of standard}}{\Delta A_{standard}}$$

Concentration of standard = 10.48 mg/dl

Where ΔA_{sample} is the change in absorbance of the sample; and

$\Delta A_{standard}$ is the change in absorbance of the standard

3.16.2.5 Determination of Kidney Na-K ATPase

The activity of the sodium pump in the kidney was determined as previously described for intestinal ATPase in section 3.14.1.1.

3.16.2.6 Determination of Serum Electrolytes

The serum concentrations of sodium, potassium and chloride ions in the animals were determined as previously described in section 3.14.1.4.

3.16.3 Determination of Intestinal Function Indices

The concentrations of intestinal glucose, protein, protein carbonyl as well as the activity of intestinal ALP and the percentage carbonylation of intestinal protein were determined as previously described in sections 3.14.1.3, 3.14.5.2, 3.14.5.3, 3.14.2.1 and 3.14.5.4 respectively.

3.17 Histological Examination

Histological examination of the liver, kidney and small intestine was carried out following the procedures described by Krause (2001).

Procedure

The selected organs were excised from the experimental animals and fixed in 10% (v/v) formal saline, dehydrated through ascending grades of ethanol (70%, 90% and 95% v/v), cleaned in xylene and embedded in paraffin wax (melting point 56℃) (Krause, 2001). Tissue sections were prepared as described by Drury and Wallington (1973) and stained with haematoxylin/eosin (H&E). The processed histology slides were read under a light microscope (Nikon 90*i*, Tokyo, Japan). Photomicrographs of the liver, kidney and small intestine were captured at a magnification mg. × 400; using Presto! Image Folio package software.

3.18 Data Analysis

Data were expressed as means ± SEM of six determinations. Statistical analysis was performed using One-way Analysis of Variance and Tukey's *post-hoc* test. The data were considered statistically significant at $P < 0.05$ using GraphPad Prism Version 6.01 (GraphPad Software, Inc., California, USA).

CHAPTER FOUR

4.0 Results

4.1 Effect of the Solvent-solvent Fractions on Castor Oil-induced Diarrhoea

4.1.1 Antidiarrhoeal Activity of the Ethyl Acetate Fraction of the Aqueous Extract of *M. indica* Leaves

The ethyl acetate fraction (EAF) of the aqueous extract of *M. indica* leaves significantly ($p < 0.05$) prolonged the onset time of diarrhoea by 2, 3 and 4 folds in a dose dependent manner. Also, the number of wet faeces, fresh weight and water content of the faeces significantly ($p < 0.05$) decreased at all the doses investigated. However, the percentage inhibition of defecation which was highest at 26.41 mg/kg body weight of the EAF was 31.42%, whereas a completely absent inhibition and 25.71% inhibition were recorded at 6.60 and 13.30 mg/kg body weight respectively (Table 4).

4.1.2 Antidiarrhoeal Activity of the n-butanol Fraction of the Aqueous Extract of *M. indica* Leaves

The n-butanol fraction (NBF) of the aqueous extract of *M. indica* leaves significantly ($p < 0.05$) prolonged the onset time of diarrhoea by 3, 5 and 2 folds at 4.31, 8.62 and 17.24 mg/kg body weight respectively when compared with the distilled water treated control diarrhoeal rats (Table 5). The total number of faeces, fresh weight and water content of faeces in diarrhoeal rats treated with 4.31 mg/kg body weight of the NBF compared favourably with the distilled water treated diarrhoeal rats whereas, there was a significant ($p < 0.05$) decrease at 8.62 and 17.24 mg/kg body weight (Table 5). The percentage inhibition of defecation at 4.31 mg/kg body weight was essentially similar to the distilled water treated rats. However, the inhibitions recorded at 8.62 and 17.24 mg/kg body weight of the NBF were 80 and 85% lower than the reference drug treated rats respectively (Table 5).

4.1.3 Antidiarrhoeal Activity of the Residual Fraction of the Aqueous Extract of *M. indica* Leaves

The residual fraction (RF) of the aqueous extract of *M. indica* at 14.09, 28.18 and 56.35 mg/kg body weight significantly ($p < 0.05$) prolonged the onset time of diarrhoea by 28, 11 and 17 folds respectively (Table 6). The total number of faeces as well as the number of wet faeces, fresh weight and water content of faeces significantly ($p < 0.05$) decreased at all the doses investigated. The percentage inhibition of defecation (52.57%) as well as the fecal parameters of rats treated with 28.18 mg/kg body weight of the RF compared favourably with those of the rats that were administered 2.00 mg/kg body weight of loperamide hydrochloride (Table 6). However, there was 62 and 69% increase in the inhibition of defecation at 14.09 and 56.35 mg/kg body weight of the RF of the aqueous extract of *M. indica* respectively (Table 6).

4.2 Effect of the Solvent-solvent Fractions on Castor Oil-induced Enteropooling

4.2.1 Anti-enteropooling Activity of the Ethyl Acetate Fraction of the Aqueous Extract of *M. indica*

The volume and mass of intraluminal fluid content significantly ($p < 0.05$) decreased in rats treated with the EAF of the aqueous extract of *M. indica* leaves. However, the decrease in mass and volume of intraluminal content at all doses investigated were relatively higher when compared with the reference drug (atropine sulphate) treated rats (Table 7). The percentage inhibition of intestinal fluid accumulation of the diarrhoeal rats treated with 6.60, 13.20 and 26.40 mg/kg body weight of the EAF of the aqueous extract of *M. indica* (32.29, 31.76 and 35.08% respectively) were significantly ($p < 0.05$) lower compared with the reference drug treated diarrhoeal rats which produced inhibition of 59.69% (Table 7).

Table 4: Effects of the ethylacetate fractions of *Mangifera indica* leaves on castor oil-induced diarrhoea

Fecal Parameters	Loperamide	Distilled water	Ethyl acetate fractions		
Dose (mg/kg body weight)	2.0	0	6.60	13.20	26.41
Onset time (minutes)	46.03 ± 2.51^{b}	5.13 ± 0.81^{a}	9.86 ± 0.88^{c}	15.87 ± 2.14^{d}	20.55 ± 2.07^{e}
Total number of faeces	9.00 ± 0.71^{b}	12.25 ± 0.48^{a}	11.75 ± 0.85^{a}	8.75 ± 0.25^{b}	8.00 ± 0.41^{b}
Number of wet faeces	3.75 ± 0.63^{b}	8.75 ± 0.38^{a}	8.75 ± 0.48^{a}	6.50 ± 0.51^{c}	6.00 ± 0.41^{c}
Fresh weight of faeces (g)	3.19 ± 0.53^{b}	6.61 ± 0.44^{a}	5.88 ± 0.37^{a}	5.25 ± 0.29^{c}	4.60 ± 0.27^{d}
Water content of faeces (ml)	1.51 ± 0.29^{b}	4.78 ± 0.23^{a}	4.47 ± 0.25^{a}	2.91 ± 0.12^{c}	3.07 ± 0.12^{c}
Inhibition of defecation (%)	57.14	0.00	0.00	25.71	31.42

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 5: Effects of n-butanol fractions of *Mangifera indica* leaves on castor oil-induced diarrhoea

Fecal Parameters	Loperamide	Distilled water	n-butanol fractions		
Dose (mg/kg body weight)	2.0	0	4.31	8.62	17.24
Onset time (minutes)	46.03 ± 2.51^{b}	5.13 ± 0.81^{a}	15.65 ± 1.33^{c}	24.17 ± 1.95^{d}	9.23 ± 1.01^{e}
Total number of faeces	9.00 ± 0.71^{b}	12.25 ± 0.48^{a}	12.50 ± 1.04^{a}	10.25 ± 0.25^{c}	10.67 ± 1.20^{bc}
Number of wet faeces	3.75 ± 0.63^{b}	8.75 ± 0.38^{a}	10.00 ± 0.41^{a}	7.75 ± 0.25^{a}	8.00 ± 1.00^{a}
Fresh weight of faeces (g)	3.19 ± 0.53^{b}	6.61 ± 0.44^{a}	6.74 ± 0.15^{a}	5.93 ± 0.17^{c}	6.11 ± 0.51^{c}
Water content of faeces (ml)	1.51 ± 0.29^{b}	4.78 ± 0.23^{a}	4.61 ± 0.18^{a}	4.13 ± 0.13^{c}	3.58 ± 0.19^{c}
Inhibition of defecation (%)	57.14	0.00	0.00	11.43	8.57

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 6: Effect of the residual fractions of *Mangifera. indica* leaves on castor oil-induced diarrhoea

Fecal Parameters	Loperamide	Distilled water	Residual fractions		
Dose (mg/kg body weight)	2.0	0	14.09	28.18	56.35
Onset time (minutes)	46.03 ± 2.51^{b}	5.13 ± 0.81^{a}	139.44 ± 15.19^{c}	57.68 ± 5.13^{d}	83.51 ± 7.04^{e}
Total number of faeces	9.00 ± 0.71^{b}	12.25 ± 0.48^{a}	1.75 ± 0.63^{c}	7.00 ± 0.71^{d}	2.33 ± 0.33^{e}
Number of wet faeces	3.75 ± 0.63^{b}	8.75 ± 0.38^{a}	0.50 ± 0.19^{c}	4.15 ± 0.23^{b}	0.32 ± 0.03^{c}
Fresh weight of faeces (g)	3.19 ± 0.53^{b}	6.61 ± 0.44^{a}	0.14 ± 0.01^{c}	3.72 ± 0.27^{b}	0.19 ± 0.06^{c}
Water content of faeces (ml)	1.51 ± 0.29^{b}	4.78 ± 0.23^{a}	0.02 ± 0.00^{c}	2.50 ± 0.13^{d}	0.04 ± 0.00^{c}
Inhibition of defecation (%)	57.14	0.00	94.29	52.57	96.34

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 7: Effect of ethylacetate fractions from *Mangifera indica* leaves on castor oil-induced enteropooling

Fecal Parameters	Atropine sulphate	Distilled water	Ethyl acetate fractions		
Dose (mg/kg body weight)	2.0	0	6.60	13.20	26.41
Mass of intestinal contents	2.31 ± 0.15^{b}	5.73 ± 0.81^{a}	3.88 ± 0.06^{c}	3.91 ± 0.03^{c}	3.72 ± 0.16^{c}
(g)	1.81 ± 0.29^{b}	4.60 ± 0.23^{a}	3.00 ± 0.07^{c}	3.15 ± 0.11^{d}	2.89 ± 0.10^{d}
Intraluminal fluid accumulation (ml)	59.69	0.00	32.29	31.76	35.08
Inhibition of intestinal fluid accumulation (%)					

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

4.2.2 Anti-enteropooling Activity of the n-butanol Fraction of the Aqueous Extract of *M. indica*

The mass and volume of intestinal content following the treatment of diarrhoeal rats with 4.31 and 8.62 mg/kg body weight of the NBF of the aqueous extract of *M. indica* leaves compared favourably with the distilled water treated control diarrhoeal rats (Table 8). However, treatment with 17.24 mg/kg body weight of the NBF significantly ($p < 0.05$) decreased the mass and volume of intraluminal fluid content with a percentage inhibition of 25.31% (Table 8). In contrast, the percentage inhibitions produced by the 4.31 and 8.62 mg/kg body weight of the NBF fractions were lower (46 and 100% respectively).

4.2.3 Anti-enteropooling Activity of the Residual Fraction of the Aqueous Extract of *M. indica*

The mass and volume of intestinal content significantly ($p < 0.05$) decreased at all doses investigated whereas, the inhibition of intestinal fluid accumulation significantly increased when compared with the distilled water treated control rats (Table 9). However, the percentage inhibitions obtained at 14.09, 28.18 and 56.35 mg/kg body weight were significantly lower by 24.30, 42.10 and 29.84% respectively when compared with the reference drug treated rats with a percentage inhibition of 59.69% (Table 9).

Table 8: Effect of n-butanol fractions from *Mangifera indica* leaves on castor oil-induced enteropooling

Fecal Parameters	Atropine sulphate	Distilled water	n-butanol fractions		
Dose (mg/kg body weight)	2.0	0	4.31	8.62	13.24
Mass of intestinal contents (g)	2.31 ± 0.15^{b}	5.73 ± 0.81^{a}	4.95 ± 0.19^{a}	6.03 ± 0.34^{a}	4.28 ± 0.36^{c}
Intraluminal fluid accumulation (ml)	1.81 ± 0.29^{b}	4.60 ± 0.23^{a}	4.00 ± 0.10^{c}	4.50 ± 0.22^{d}	3.77 ± 0.18^{d}
Inhibition of intestinal accumulation (%)	59.69	0.00	13.61	0.00	25.31

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 9: Effect of residual fractions from *Mangifera indica* leaves on castor oil-induced enteropooling

Fecal Parameters	Atropine sulphate	Distilled water	Residual fractions		
Dose (mg/kg body weight)	2.0	0	14.09	28.18	56.36
Mass of intestinal contents (g)	2.31 ± 0.15^{b}	5.73 ± 0.81^{a}	3.14 ± 0.01^{c}	3.75 ± 0.22^{d}	3.33 ± 0.06^{c}
Intraluminal fluid accumulation (ml)	1.81 ± 0.29^{b}	4.60 ± 0.23^{a}	2.05 ± 0.07^{c}	2.50 ± 0.11^{d}	2.44 ± 0.10^{d}
Inhibition of intestinal accumulation (%)	59.69	0.00	45.20	34.56	41.88

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

4.3 Effect of the Solvent-solvent Fractions on Gastrointestinal Motility

4.3.1 Antimotility Effects of Ethyl Acetate Fraction from the Aqueous Extract of *M. indica* Leaves

The EAF of the aqueous extract of *M. indica* leaves significantly ($p < 0.05$) decreased the peristaltic index of the rats compared with the distilled water control rats (Table 10). However, the highest decrease in peristaltic index was exhibited by the reference drug treated rats. The inhibition of peristalsis however, was highest at 26.41 mg/kg body weight (30.73%) which compared favourably with the reference drug treated rats (33.45%). At 6.60 and 13.20 mg/kg body weight on EAF, the percentage inhibition of peristalsis obtained was relatively lower (20.77 and 12.76% respectively) (Table 10).

4.3.2 Antimotility Effects of n-butanol Fraction from the Aqueous Extract of *M. indica* Leaves

There was a dose dependent increase in the distance travelled by the charcoal meal plug as well as the peristaltic index (Table 11). However, the percentage inhibition of peristalsis obtained at 4.31, 8.62 and 17.24 mg/kg body weight of the NBF were respectively lowered by 60.75, 67.56 and 85.44% compared to the atropine sulphate treated rats (Table 11).

4.3.3 Antimotility Effect of the Residual Fraction from the Aqueous Extract of *M. indica* Leaves

At 28.18 mg/kg body weight of the RF, the distance covered by the charcoal meal plug was highest and lowest at 14.09 mg/kg body weight. The distance travelled by charcoal meal plug and peristaltic index obtained at 14.09 mg/kg body weight of the RF compared favourably with the reference drug treated rats (Table 12). The percentage inhibition of peristalsis was highest at 14.09 mg/kg body weight of the RF treated rats with an increase of 18.95% compared with the reference drug treated animals. However, the percentage inhibition obtained at 28.18 and 56.35 mg/kg body weight of the RF were 54.05 and 8.13% lower than the reference drug treated rats respectively (Table 12).

Table 10: Effect of the ethylacetate fraction from *Mangifera indica* leaves on gastrointestinal motility of the female Wistar rats

Fecal Parameters	Atropine sulphate	Distilled water	Ethyl acetate fractions		
Dose (mg/kg body weight)	2.0	0	6.60	13.20	26.40
Length of small intestine (cm^3)	94.03 ± 2.50^b	87.15 ± 0.90^a	88.52 ± 2.11^a	89.95 ± 1.90^a	88.40 ± 2.00^a
Distance travelled by charcoal meal (cm^3)	56.00 ± 2.28^b	84.15 ± 3.11^a	66.67 ± 1.15^b	73.41 ± 3.06^c	57.13 ± 4.19^b
Peristaltic index	59.56	96.56	75.32	81.61	64.63
Inhibition of peristalsis (%)	33.45	0.00	20.77	12.76	30.73

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 11: Effect of n-butanol fraction from *Mangifera indica* leaves on the gastrointestinal motility of the female Wistar rats

Fecal Parameters	Atropine	Distilled water	n-butanol fraction		
Dose (mg/kg body weight)	sulphate				
	2.0	0	4.31	8.62	17.24
Length of small intestine (cm^3)	94.03 ± 2.50^b	87.15 ± 0.90^a	92.12 ± 1.33^b	95.07 ± 4.95^b	88.70 ± 3.25^a
Distance travelled by charcoal meal (cm^3)	56.00 ± 2.28^b	84.15 ± 3.11^a	73.15 ± 1.20^b	75.02 ± 3.91^c	80.05 ± 1.77^b
Peristaltic index	59.56	96.56	79.41	78.91	90.25
Inhibition of peristalsis (%)	33.45	0.00	13.07	10.85	4.87

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 12: Effect of residual fraction from *Mangifera indica* leaves on the gastrointestinal motility of the female Wistar rats

Fecal Parameters	Atropine sulphate	Distilled water	Residual fraction		
Dose (mg/kg body weight)	1.0	0	14.09	28.18	56.35
Length of small intestine (cm^3)	94.03 ± 2.50^{b}	87.15 ± 0.90^{a}	89.74 ± 3.05^{ab}	92.15 ± 6.10^{b}	93.12 ± 4.09^{b}
Distance travelled by charcoal meal (cm^3)	56.00 ± 2.28^{b}	84.15 ± 3.11^{a}	50.67 ± 2.17^{b}	71.22 ± 5.81^{c}	58.29 ± 2.08^{b}
Peristaltic index	59.56	96.56	56.46	77.29	62.60
Inhibition of peristalsis (%)	33.45	0.00	39.79	15.37	30.73

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

4.4 Antidiarrhoeal Index of the Solvent-solvent Fractions of the Aqueous Extract of *M. indica* Leaves

The computed antidiarrhoeal index was 0 at 6.60 mg/kg body weight of the EAF, 4.31 and 8.62 mg/kg body weight of the NBF (Table 13). The highest ADI index computed was 55.19 obtained at 14.09 mg/kg body weight of the RF (Table 13). The ADI for all the solvent-solvent fractions were in the order; 14.09 mg/kg body weight of the RF > 56.35 mg/kg body weight of the RF/Reference drug > 26.41 mg/kg body weight of the EAF/28.18 mg/kg body weight of the RF > 13.20 mg/kg body weight of the EAF > 17.24 mg/kg body weight of the NBF > 6.60 mg/kg body weight of EAF = 4.31 and 8.62 mg/kg body weight of the NBF (Table 13).

4.5 Chromatographic Separation of the Residual Fraction from the Aqueous Extract of *M. indica* Leaves

Column chromatographic separation of the residual fraction gave a total of 16 eluents which were further grouped into three (sub-fractions A, B and C) based on their R_f-values (Table 14).

4.6 Effect of the Sub-fractions A, B and C on Castor Oil-induced Diarrhoea

Sub-fraction A from the residual fraction significantly ($p < 0.05$) prolonged the onset time of diarrhoea in castor oil-induced diarrhoeal rats and significantly ($p < 0.05$) decreased the total number of faeces, number of wet faeces, fresh weight and water content of faeces when compared with the distilled water treated control diarrhoeal female rats (Table 15). Although, the sub-fraction B significantly ($p < 0.05$) reduced the onset time of diarrhoea, the episodes of diarrhoea as well as the weight and volume of water content were also significantly ($p < 0.05$) decreased. Similar to sub-fraction B, the onset time of diarrhoea significantly ($p < 0.05$) decreased in the diarrhoeal rats treated with sub-fraction C of the residual fraction while the

number of wet faeces, weight of faeces, water content and diarrhoeal episodes significantly ($p < 0.05$) decreased (Table 15).

Table 13: Antidiarrhoeal index of the solvent-solvent fractions from *M. indica* leaves

Solvent-solvent fractions Equivalent dose (mg/kg body weight)	Loperamide/Atropine sulphate	Distilled water	Antidiarrhoeal index		
	2 / 1	0	25	50	100
Ethyl acetate	48.50	0.00	0.00	22.72	32.36
n-butanol	48.50	0.00	0.00	0.00	10.18
Residual	48.50	0.00	55.19	30.34	49.87

KEY:

Fraction/Equivalent dose	Ethyl acetate	n-butanol	Residual
25	6.60	13.20	26.41
50	4.31	8.62	17.25
100	14.09	28.18	56.35

Table 14: Retention factor of column chromatographic fractions from the residual fraction of *Mangifera indica* leaves

Pooled sub-fractions	Chromatographic fractions	Spot R_f values	
		1	2
A	1	0.96	
	2	0.95	
	3	0.95	
	4	0.92	0.89
B	5	0.88	
	6	0.88	
	7	0.86	
	8	0.85	
	9	0.85	
	10	0.85	
	11	0.85	
	12	0.84	0.50
C	13	0.33	
	14	0.32	0.12
	15	0.32	0.13
	16	0.31	0.08

The highest delay in the onset of diarrhoea was obtained in the reference drug (loperamide) treated rats, whereas the most profound decrease in diarrhoeal episodes as well as the weight and volume of water content was obtained in the sub-fraction B treated diarrhoeal animals (Table 15). Sub-fractions A and B increased the inhibition of defecation by 10.10 and 22.83% respectively whereas there was 9.83% decrease in sub-fraction C treated rats when compared with the reference drug treated animals (Table 15).

4.7 Effect of the Sub-fractions A, B and C on Castor Oil-induced Enteropooling

The intestinal fluid accumulation was significantly ($p < 0.05$) reduced both in volume and mass by all the three sub-fractions. However, the reductions in mass and volume of intestinal fluid content by sub-fractions A, B and C were 31.28, 18.33 and 36.92% lower compared with the reference drug treated female rats (Table 16).

4.8 Effect of Sub-fractions A, B and C on Gastrointestinal Motility

The peristaltic index was significantly ($p < 0.05$) decreased by 42.35, 28.45 and 18.95 % in sub-fractions A, B and C treated rats respectively when compared with the distilled water treated control animals (Table 17). The decrease in peristaltic index in sub-fraction A treated rats compared favourably with the reference drug treated animals (Table 17).

The most profound percentage inhibition of peristalsis was exhibited by the rats treated with 4.19 mg/kg body weight of sub-fraction A with an increased inhibition of 14.50% when compared with the atropine sulphate treated rats (Table 17). However, the percentage inhibition of peristalsis computed in the sub-fractions B and C treated rats were 22.01 and 55.15% lower compared with the reference drug treated rats (Table 17).

4.9 Antidiarrhoeal Index of Sub-fractions A, B and C of the Residual Fraction

The computed ADI value for the sub-fractions A and B compared favourably but were significantly ($p < 0.05$) lower than the reference drug treated rats (Figure 15). The computed ADI was in the order; reference drug > sub-fractions A and B > sub-fraction C (Figure 15).

Table 15: Effect of sub-fractions A, B and C of the residual fraction from *Mangifera indica* leaves on castor oil-induced diarrhoea

Fecal Parameters	Distilled water	Loperamide	Sub-fractions of the residual fraction		
			A	B	C
Dose (mg/kg body weight)	0	2.0	4.19	6.47	3.43
Onset time (minutes)	47.33 ± 0.11^{a}	145.67 ± 5.82^{b}	120.00 ± 20.14^{c}	20.47 ± 0.13^{d}	38.31 ± 2.52^{e}
Total number of faeces	14.02 ± 0.62^{a}	6.00 ± 1.71^{bc}	5.00 ± 0.03^{c}	2.84 ± 0.21^{d}	6.33 ± 0.54^{b}
Number of wet faeces	10.33 ± 1.08^{a}	3.67 ± 0.13^{b}	3.00 ± 0.19^{c}	2.15 ± 0.22^{d}	4.32 ± 0.03^{e}
Fresh weight of faeces (g)	6.46 ± 0.14^{a}	2.55 ± 0.20^{b}	2.17 ± 0.05^{b}	1.12 ± 0.27^{c}	2.97 ± 0.18^{b}
Water content of faeces	3.31 ± 0.19^{a}	1.51 ± 0.29^{b}	1.48 ± 0.05^{b}	0.50 ± 0.13^{c}	2.35 ± 0.12^{d}
(ml)	0.00	64.47	70.96	79.19	58.18
Inhibition of defecation (%)					

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 16: Effect of sub-fractions A, B and C of the residual fractions of *M. indica* leaves on castor oil-induced enteropooling

Fecal Parameters	Distilled water	Atropine sulphate	Sub-Fractions of the Residual fraction		
			A	B	C
Dose (mg/kg body weight)	0	1.0	4.19	6.47	3.43
Mass of intestinal contents (g)	7.80 ± 0.16^{a}	1.80 ± 0.16^{b}	4.24 ± 0.22^{d}	3.23 ± 0.16^{c}	5.46 ± 0.57^{e}
Intraluminal fluid accumulation (ml)	4.96 ± 0.11^{a}	0.99 ± 0.09^{b}	4.40 ± 0.12^{a}	3.18 ± 0.12^{c}	4.71 ± 0.18^{a}
Inhibition of intestinal fluid accumulation (%)	0.00	76.92	45.64	58.59	30.00

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 17: Effect of sub-fractions A, B and C of the residual fraction from *Mangifera indica* leaves on intestinal transit of female Wistar rats

Fecal Parameters	Distilled water	Atropine sulphate	Sub-Fractions of the Residual fraction		
			A	B	C
Dose (mg/kg body weight)	0	1.0	4.19	6.47	3.43
Length of small intestine (cm^3)	88.42 ± 3.66^a	88.15 ± 0.17^a	82.40 ± 3.88^a	85.45 ± 2.13^a	89.72 ± 5.13^a
Distance travelled by charcoal meal (cm^3)	80.55 ± 1.11^a	45.28 ± 1.22^b	40.17 ± 0.75^d	53.04 ± 3.26^c	64.73 ± 1.84^e
Peristaltic index	91.10	51.37	48.75	62.55	72.15
Inhibition of peristalsis (%)	0.00	43.79	50.13	34.15	19.64

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

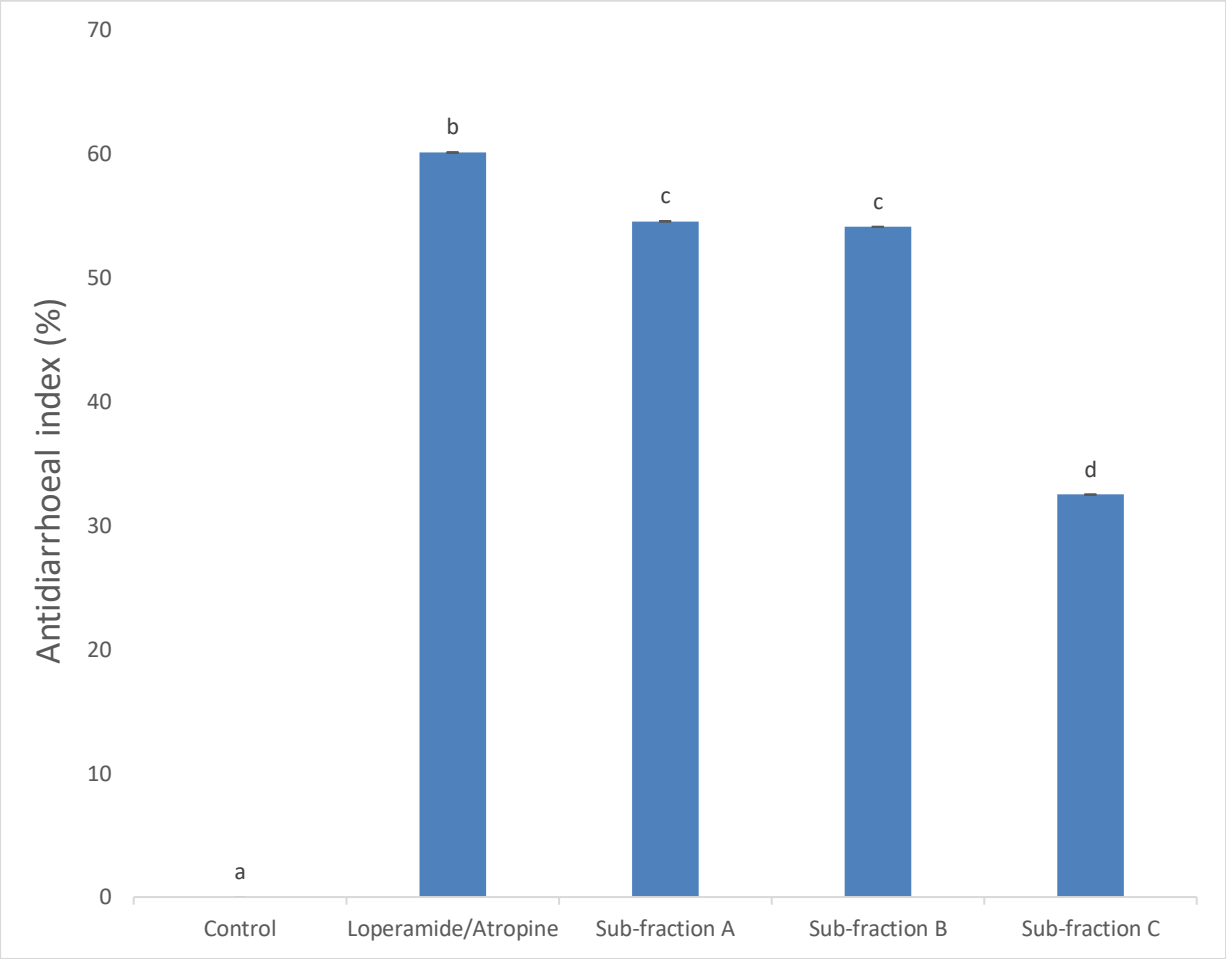


Figure 14: Antidiarrhoeal index of the sub-fractions A, B and C of the residual fraction from the aqueous extract of *Mangifera indica* leaves

4.10 Mechanism of Action

4.10.1 Antisecretory Mechanism

The serum concentrations of Na^+ and Cl^- significantly decreased in diarrhoeal rats treated with loperamide hydrochloride, sub-fractions A and B while the concentration of K^+ significantly increased when compared with the distilled water treated control animals (Table 18). Similarly, the concentration of intestinal glucose significantly increased in diarrhoeal animals treated with reference drug as well as the sub-fractions A and B of the residual fraction from the aqueous extract of *M. indica* leaves (Table 18).

Furthermore, sub-fraction B significantly ($p < 0.05$) increased the activity of intestinal Na-K ATPase in the diarrhoeal rats in a manner similar to the reference drug treated rats. However, the activity of intestinal Na-K ATPase was no affected by the sub-fraction A (Table 18).

4.10.2 Anti-inflammatory Mechanism

The treatment of diarrhoeal animals with sub-fraction A had no effect on the activity of intestinal ALP. In contrast, treatment with sub-fraction B of the residual fraction significantly ($p < 0.05$) increased the activity of intestinal ALP and compared favourably with the reference drug treated rats (Table 19). Nitrite concentrations were significantly ($p < 0.05$) decreased in diarrhoeal rats administered 4.19 and 6.47 mg/kg body weight of sub-fractions A and B respectively when compared with the distilled water treated control animals (Table 19). The decrease in nitrite concentration by sub-fraction B was greater when compared with the reference drug treated group (Table 19).

4.10.3 Antioxidant Mechanism

Treatment of the diarrhoeal rats with the reference drug, sub-fractions A and B significantly ($p < 0.05$) increased the concentration of reduced glutathione as well as the

activities of catalase and superoxide dismutase when compared with the distilled water treated rats (Table 20).

4.10.4 Antimotility Mechanism

The administration of 4.19 and 6.47 mg/kg body weight of sub-fractions A and B respectively significantly ($p < 0.05$) increased the activity of intestinal acetylcholinesterase (Figure 16). The highest increase in activity of acetylcholinesterase was produced by the sub-fraction A (123.01%) whereas, the activity was increased by 31.46 and 55.87% in the sub-fraction B and reference drug treated group respectively when compared with the control (Figure 16).

4.10.5 Intestinal Barrier Function

The treatment of diarrhoeal animals with sub-fraction A of the residual fraction had no effect on the concentration of intestinal MDA, protein, protein carbonyl and percentage carbonylation (Table 21). In contrast, sub-fraction B significantly ($p < 0.05$) decreased the concentration of intestinal MDA in a manner that compared favourably with the reference drug treated rats (Table 21). Furthermore, the concentration of intestinal protein significantly ($p < 0.05$) increased in diarrhoeal rats treated with 6.47 mg/kg body weight of sub-fraction B and 2.00 mg/kg body weight of loperamide hydrochloride whereas, there was a significant ($p < 0.05$) decrease in intestinal protein carbonyl content (Table 21). The decrease in intestinal protein carbonyl content was higher in the sub-fraction B treated diarrhoeal rats (57.22%) than the reference drug treated animals (25.41%) (Table 21).

The percentage decrease in protein carbonylation was 39.06, 4.73 and 65.24% in diarrhoeal rats treated with 2.00 mg/kg body weight of loperamide hydrochloride, 4.17 mg/kg of sub-fraction A and 6.47 mg/kg body weight of sub-fraction B respectively (Table 21).

Table 18: Antisecretory effect of sub-fraction B of the residual fraction in castor oil-induced diarrhoeal rats

Parameters	Distilled water	Loperamide	Sub-fraction A	Sub-fraction B
Dose (mg/kg body weight)	0	2.0	4.19	6.47
Na^+ (mEq/l)	252.23 ± 21.51^a	66.47 ± 7.24^b	296.67 ± 30.14^c	214.16 ± 8.87^c
K^+ (mEq/l)	1.89 ± 0.14^a	2.67 ± 0.10^b	3.37 ± 0.09^c	3.31 ± 0.19^c
Cl^- (mEq/l)	42.34 ± 1.50^a	37.45 ± 0.45^b	31.94 ± 0.34^c	29.95 ± 1.56^c
Glucose (mmol/l)	15.11 ± 0.33^a	18.27 ± 0.10^b	17.83 ± 0.22^b	20.01 ± 0.45^c
Na-K ATPase (µmolPi/mg prot/hr)	812.12 ± 5.33^a	1156.64 ± 14.37^b	863.33 ± 7.15^c	1190.03 ± 11.20^b

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 19: Anti-inflammatory effect of sub-fractions A and B of the residual fraction in castor oil-induced diarrhoeal rats

Parameters	Distilled water	Loperamide	Sub-fraction A	Sub-fraction B
Dose (mg/kg body weight)	0	2.0	4.19	6.47
Alkaline phosphatase (IU/L)	17.81 ± 0.21^{a}	20.10 ± 0.66^{b}	17.30 ± 0.73^{a}	19.49 ± 0.17^{b}
Nitrite (µmol/L)	171.06 ± 9.15^{a}	75.52 ± 3.10^{c}	93.17 ± 4.80^{b}	23.85 ± 2.06^{d}

Values are mean of 6 replicates ± SEM, values with different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

Table 20: Antioxidant effect of sub-fraction B of the residual fraction in castor oil-induced diarrhoeal female Wistar rats

Parameters	Distilled water	Loperamide	Sub-Fraction A	Sub-Fraction B
Dose (mg/kg body weight)	0	2.0	4.19	6.47
SOD (nmol/min/ml)	25.13 ± 0.26^{a}	87.25 ± 2.08^{b}	90.00 ± 1.93^{b}	89.40 ± 1.58^{b}
Catalase (kU)	10.85 ± 0.21^{a}	13.50 ± 1.47^{b}	16.42 ± 0.33^{c}	17.94 ± 0.54^{c}
GSH $\times 10^{-4}$ (µmol/ml/min)	6.94 ± 0.35^{a}	7.51 ± 0.82^{ab}	8.04 ± 0.05^{b}	8.01 ± 0.14^{b}

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

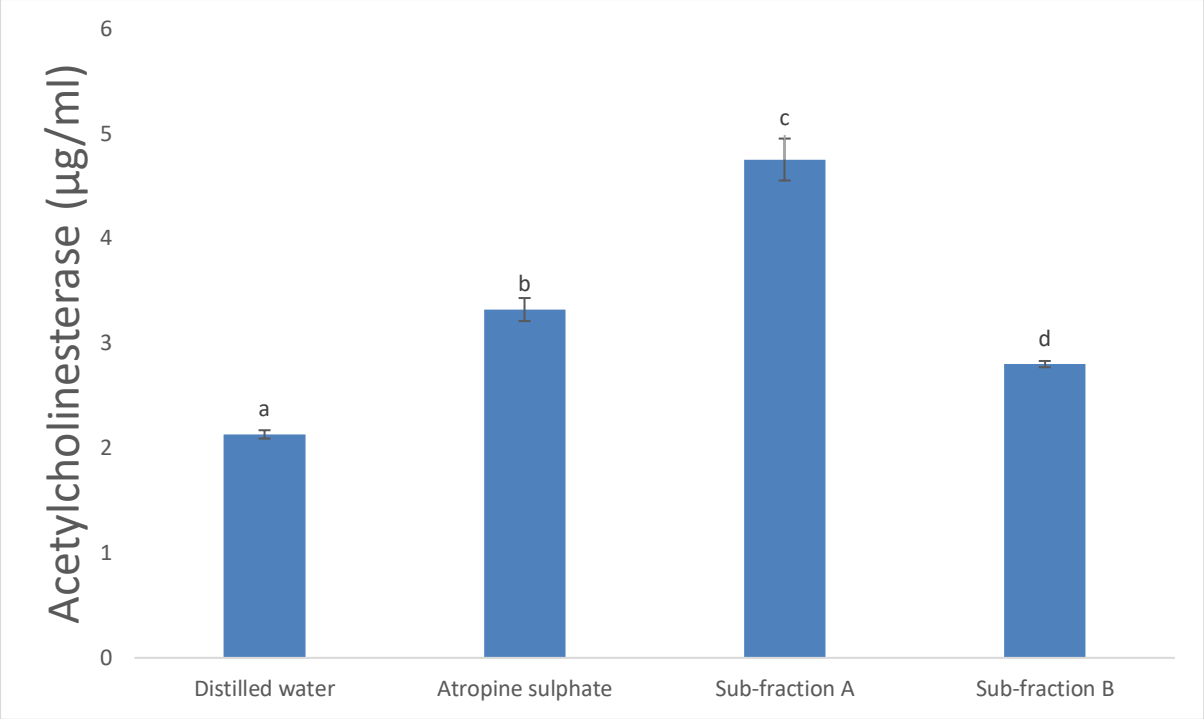


Figure 15: Antimotility effect of the sub-fractions A and B on the activity of acetylcholinesterase in gastrointestinal transit test

Table 21: Effect of the sub-fractions A and B of the residual fraction on intestinal permeability in castor oil-induced diarrhoeal rats

Parameters	Distilled water	Loperamide	Sub-fraction A	Sub-fraction B
Dose (mg/kg body weight)	0	2.0	4.19	6.47
MDA × 10^{-6} ($nm^{-1}cm^{-1}$)	5.10 ± 0.21^{a}	1.98 ± 0.03^{b}	4.58 ± 0.13^{d}	1.16 ± 0.02^{c}
Intestinal protein (mg/ml)	21.43 ± 0.30^{a}	26.23 ± 0.65^{b}	20.60 ± 0.45^{a}	26.37 ± 1.02^{b}
Protein carbonyl (µm/mg)	13.42 ± 0.11^{a}	10.01 ± 0.53^{b}	12.29 ± 0.88^{a}	5.74 ± 0.13^{c}
Protein carbonylation (%)	62.62	38.16	59.66	21.77

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

4.11 Histoarchitectural Changes of the Small Intestine in Sub-fractions A and B Treated Rats

The histological examination of the small intestine of distilled water treated diarrhoeal animals revealed lymphocytic follicular hyperplasia with mucosal inclusion bodies (Plate 2a). Compared with the control, the treatment of diarrhoeal animals with the reference drug shows mucosal hyperplasia, mild degeneration of mucosal villi as well as a dilated blood vessel (Plate 2b). The histoarchitecture of the small intestine in sub-fraction A treated rats revealed mucosal inclusion bodies with polymorph infiltration, whereas there was mucosal hyperplasia with an essentially normal microvilli in sub-fraction B treated rats (Plates 2c and 2d).

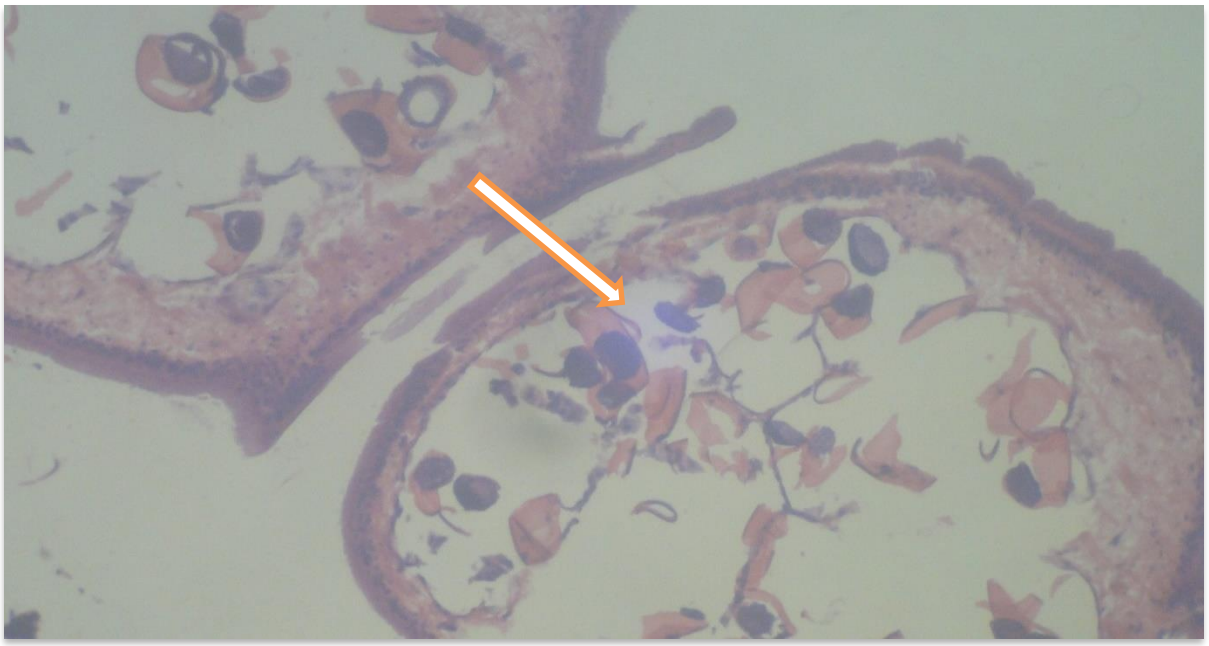


Plate 2a: Cross section of the small intestine of distilled water treated diarrhoeal rats (mg. × 400; H & E)

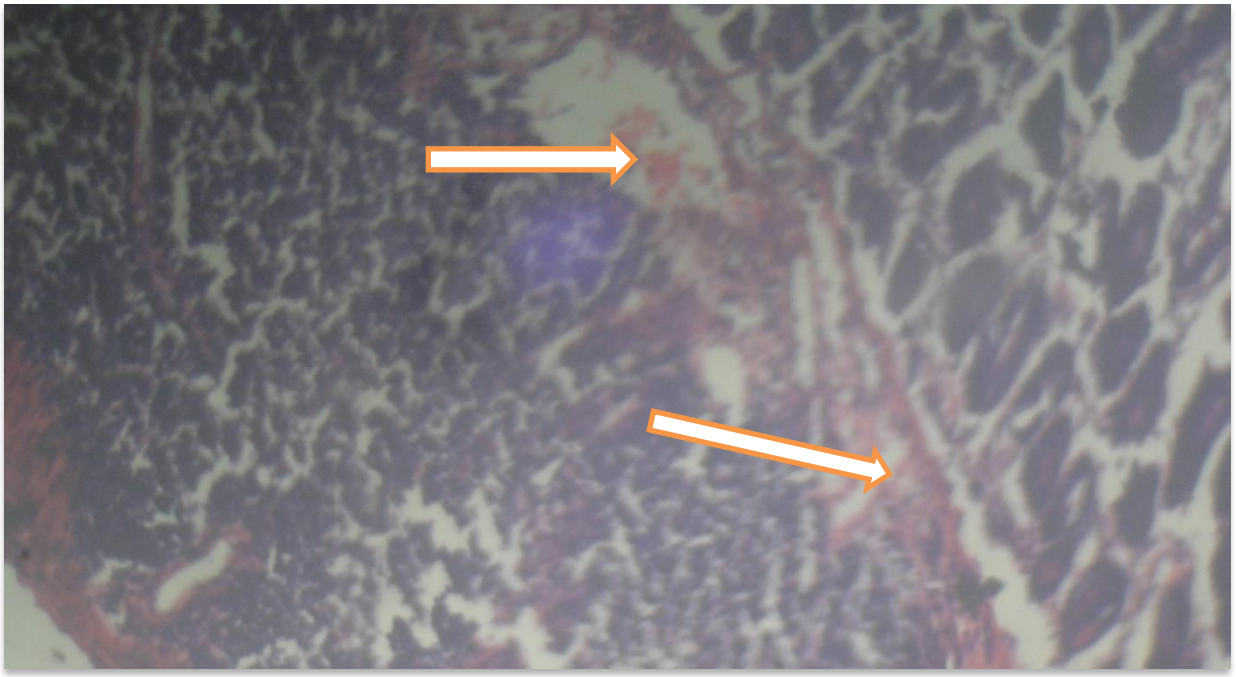


Plate 2b: Cross section of the small intestine of rats treated with 2 mg/kg body weight of loperamide hydrochloride (mg. × 400; H & E)

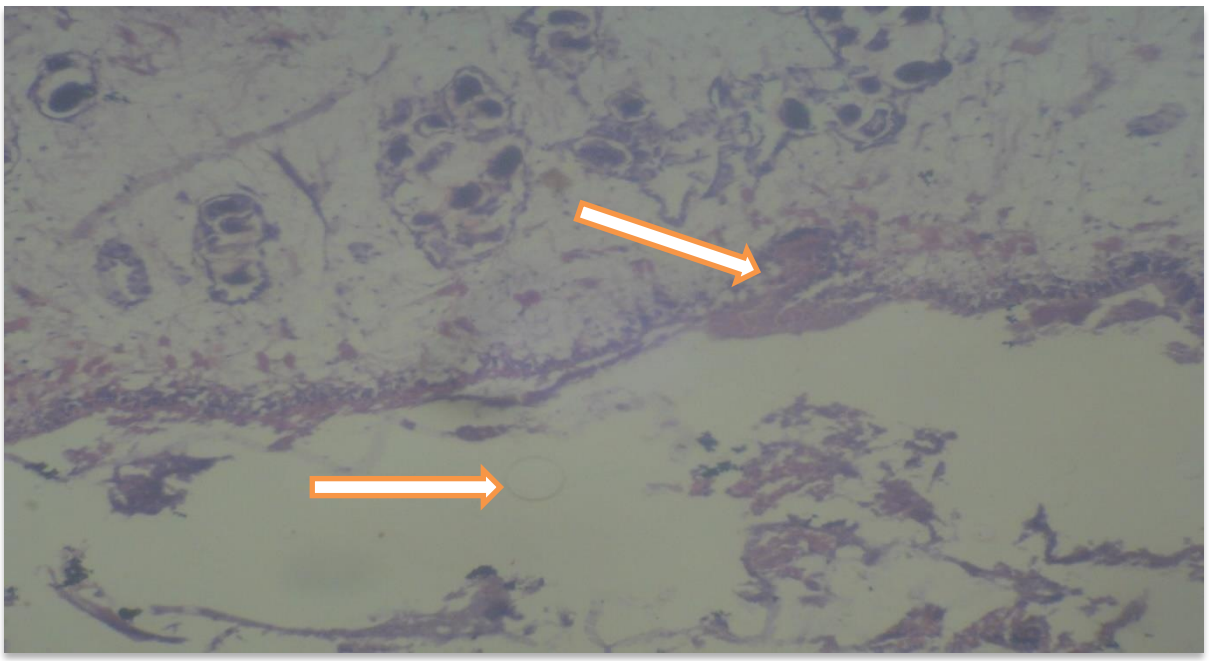


Plate 2c: Cross section of the small intestine of rats treated with sub-fraction A of the residual fraction from the aqueous extract of *M. indica* leaves (mg. × 400; H & E)

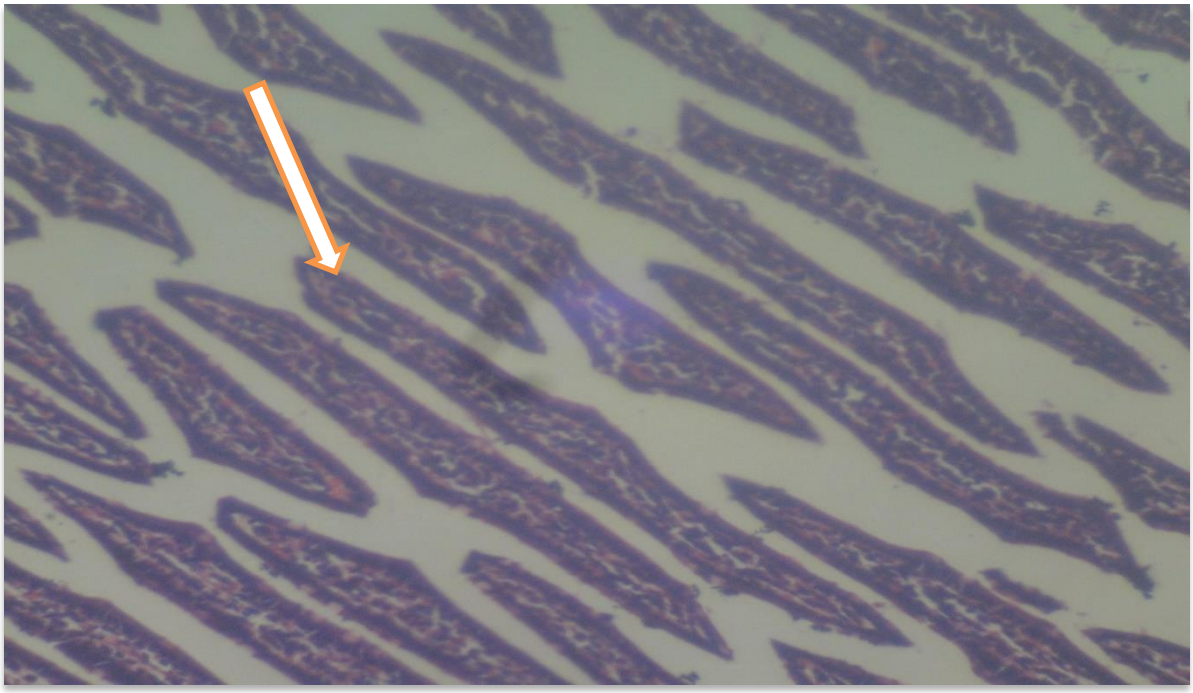


Plate 2d: Cross section of the small intestine of rats treated with sub-fraction B of the residual fraction from the aqueous extract of *M. indica* leaves (mg. × 400; H & E)

4.12 Constituents of Sub-fraction B

The HPLC analysis of sub-fraction B revealed the presence of two chemical compounds, mangiferin and catechin (Plate 3). Mangiferin (molecular formula: $C_{19}H_{18}O_{11}$), a four-ringed polyphenolic compound (Figure 17) had a peak value of 14.033 while catechin (molecular formula: $C_{15}H_{14}O_6$) also a polyphenolic compound but three-ringed (Figure 18) had a lower peak at 3.116 (Plate 3).

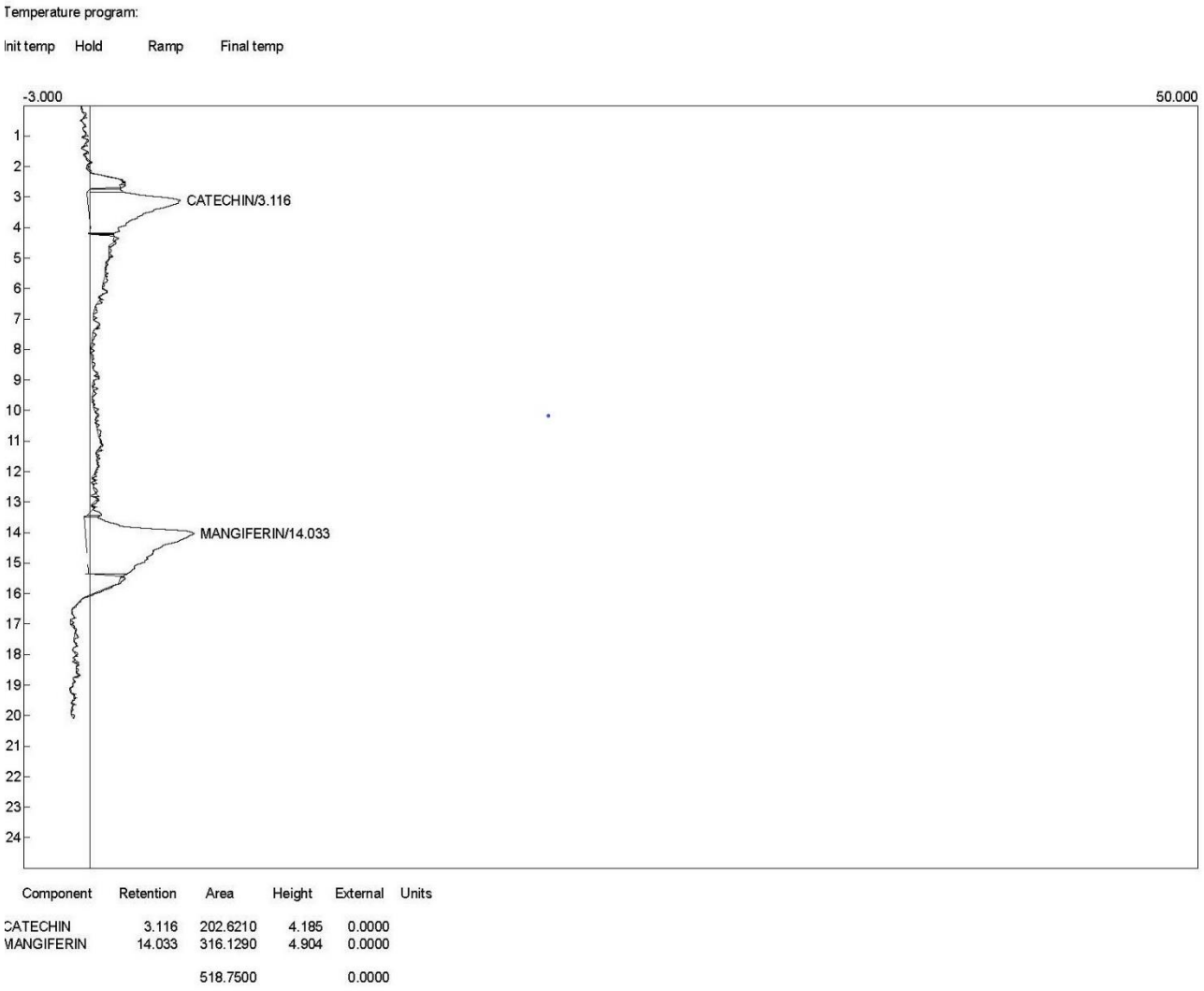


Plate 3: HPLC fingerprint of sub-fraction B from the residual fraction of the aqueous extract of *Mangifera indica* leaves

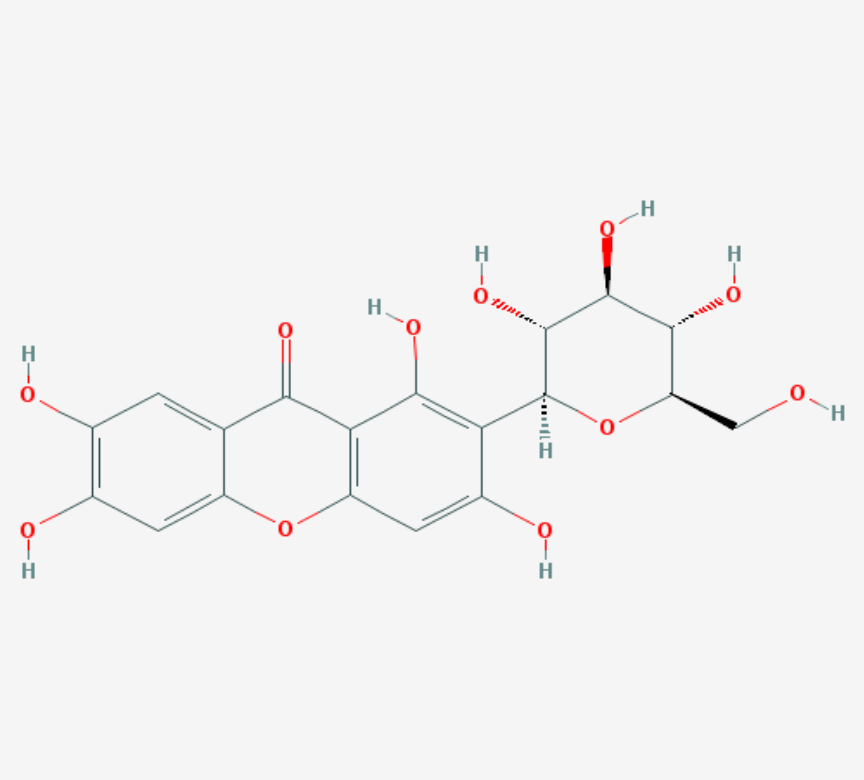


Figure 16: Structure of mangiferin

Source: Lou *et al.* (2012)

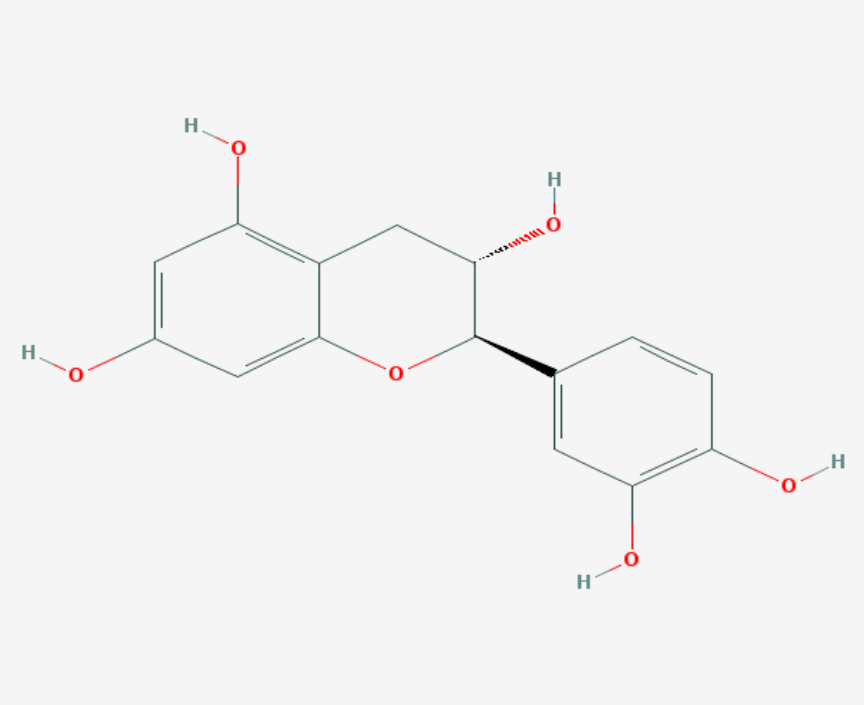


Figure 17: Structure of catechin

Source: Rasouli *et al.* (2017)

4.13 Liver Function Indices

The administration of 3.24, 6.47 and 12. 94 mg/kg body of sub-fraction B significantly ($p < 0.05$) increased the concentration of serum protein and globulin in a dose dependent manner whereas, the concentration of serum albumin increased only in rats treated with 6.47 and 12.94 mg/kg body weight of sub-fraction B (Table 22). The computed albumin/globulin ratio significantly ($p < 0.05$) increased in the rats treated with 3.24 and 6.47 mg/kg body weight of sub-fraction B. In contrast, the computed albumin/globulin ratio significantly ($p < 0.05$) decreased in rats treated with 12.94 mg/kg body weight of sub-fraction B (Table 22). Furthermore, the concentration of serum total bilirubin significantly ($p < 0.05$) decreased in rats treated with 3.24 mg/kg body weight of sub-fraction B whereas, there was no significant alteration in total bilirubin levels in rats treated with 6.47 and 12.94 mg/kg body weight of sub-fraction B (Table 22). At 3.24 and 6.47 mg/kb body weight of sub-fraction B, the concentration of direct bilirubin was not significantly different from the control however, there was a significant ($p < 0.05$) decrease at 12.94 mg/kg body weight of sub-fraction B (Table 22).

At all the doses investigated, the activities of liver AST and ALT significantly ($p < 0.05$) increased in a dose dependent manner whereas there was no significant effect on serum AST and ALT activities. Compared to the control, the liver AST/ALT ratio computed decreased by 9.47 and 43.68 % in rats treated with 3.24 and 6.47 mg/kg body weight of sub-fraction B respectively while an increase of 42.11% was observed at 12.94 mg/kg body weight (Table 22). The activity of liver ALP significantly ($p < 0.05$) increased in rats administered 6.47 and 12.94 mg/kg body weight of sub-fraction B while, the activity of the enzyme was significantly ($p < 0.05$) increased in a dose dependent manner in the serum (Table 22).

Table 22: Liver function indices of female Wistar rats after administration of sub-fraction B from *Mangifera indica* leaves

Parameters	Distilled water	Sub-fraction B of the residual fraction		
Dose (mg/kg body weight)	0	3.24	6.47	12.94
Total protein (mg/ml)	8.55 ± 0.30^{a}	10.01 ± 0.09^{b}	18.31 ± 1.68^{c}	22.59 ± 1.45^{d}
Albumin (mg/ml)	5.19 ± 0.63^{a}	6.39 ± 0.88^{a}	12.16 ± 0.46^{d}	9.63 ± 0.21^{c}
Globulin (mg/ml)	3.36 ± 0.01^{a}	3.62 ± 0.00^{b}	6.15 ± 0.02^{b}	12.96 ± 0.01^{c}
Albumin/Globulin ratio	1.55 ± 0.00^{a}	1.77 ± 0.00^{b}	2.05 ± 0.00^{c}	0.74 ± 0.00^{d}
Total bilirubin (µmol/L)	15.87 ± 1.15^{a}	9.72 ± 0.40^{c}	13.57 ± 0.95^{b}	14.47 ± 0.82^{b}
Direct bilirubin (µmol/L)	10.46 ± 0.83^{a}	9.23 ± 0.10^{b}	10.33 ± 0.29^{a}	8.64 ± 0.55^{b}
Liver AST (U/L)	22.20 ± 0.71^{a}	21.74 ± 0.83^{a}	32.54 ± 0.51^{b}	81.50 ± 1.07^{b}
Serum AST (U/L)	7.12 ± 0.09^{a}	6.95 ± 0.11^{a}	7.39 ± 0.45^{a}	6.88 ± 0.70^{a}
Liver ALT (U/L)	11.66 ± 0.14^{a}	12.66 ± 0.26^{b}	30.28 ± 0.34^{c}	30.19 ± 1.18^{c}
Serum ALT (U/L)	5.33 ± 0.22^{a}	5.25 ± 0.30^{a}	6.02 ± 0.08^{b}	6.53 ± 0.10^{c}
Liver AST/ALT ratio	1.90 ± 0.02^{a}	1.72 ± 0.00^{c}	1.07 ± 0.00^{b}	2.70 ± 0.05^{d}
Liver ALP (IU/L)	19.24 ± 0.79^{a}	20.73 ± 0.59^{a}	30.59 ± 0.60^{b}	28.76 ± 0.91^{b}
Serum ALP (IU/L)	8.00 ± 0.35^{a}	10.72 ± 0.05^{b}	11.26 ± 0.33^{c}	13.50 ± 0.12^{d}

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

4.14 Kidney Function Indices

The administration of 6.47 and 12.94 mg/kg body weight of sub-fraction B dose dependently increased the serum concentration of urea. In contrast, the level of uric acid was not altered at all doses investigated (Table 23). Although, there was no regular pattern on the effect of sub-fraction B in serum creatinine level, however, there was a dose dependent increase in urine creatinine. All the doses of sub-fraction B investigated significantly ($p < 0.05$) increased the creatinine clearance rate, notably at 6.47 mg/kg body weight that produced a clearance rate 7 folds more than the control (Table 23). The activity of kidney Na-K ATPase as well as the concentration of serum sodium ion significantly ($p < 0.05$) increased in rats treated with 3.24 and 6.47 mg/kg body weight of sub-fraction B. In contrast, there were significant ($p < 0.05$) decreases in serum sodium ion and the activity of kidney Na-K ATPase the rats treated with 12.94 mg/kg body weight of sub-fraction B (Table 23).

Although, the serum concentrations of potassium and chloride ion were not significantly different from their respective controls in rats treated with 3.24 and 6.47 mg/kg body weight of sub-fraction B. However, there were significant ($p < 0.05$) decreases in serum potassium and sodium ions following the administration of 12.94 mg/kg body weight of sub-fraction B (Table 23).

Table 23: Kidney function indices of female Wistar rats after administration of sub-fraction B from *Mangifera indica* leaves

Parameters	Distilled water	Sub-fraction B of the residual fraction		
Dose (mg/kg body weight)	0	3.24	6.47	12.94
Serum urea (mmol/L)	3.04 ± 0.12^{a}	2.99 ± 0.05^{a}	3.70 ± 0.11^{b}	4.72 ± 0.15^{c}
Serum uric acid (mg/dl)	2.11 ± 0.08^{a}	2.14 ± 0.39^{a}	2.20 ± 0.04^{a}	2.22 ± 0.02^{a}
Serum creatinine (µmol/ml)	3.78 ± 0.12^{a}	3.70 ± 0.06^{a}	1.51 ± 0.02^{b}	7.11 ± 0.13^{c}
Urine creatinine (µmol/ml)	0.43 ± 0.02^{a}	0.72 ± 0.05^{b}	1.45 ± 0.01^{c}	2.46 ± 0.20^{d}
Creatinine clearance	1.14	1.95	9.60	3.46
Na-K ATPase (µmolPi/mg protein/hr)	750.14 ± 11.19^{a}	770.20 ± 30.40^{a}	920.33 ± 10.59^{b}	690.73 ± 21.84^{c}
Serum Na^{+} (mEq/L)	221.13 ± 12.74^{a}	289.45 ± 25.10^{b}	316.46 ± 15.77^{c}	155.32 ± 18.95^{d}
Serum K^{+} (mEq/L)	2.65 ± 0.11^{a}	2.72 ± 0.07^{a}	2.57 ± 0.21^{a}	3.32 ± 0.03^{b}
Serum Cl^{-} (mEq/L)	88.74 ± 2.54^{a}	93.96 ± 5.06^{a}	93.22 ± 2.10^{a}	101.16 ± 10.18^{b}

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

4.15 Intestinal Function Indices

There was a significant decrease in intestinal protein at 3.24 mg/kg body weight whereas, the doses of 6.47 and 12.94 mg/kg body weight of sub-fraction B significantly ($p < 0.05$) increased the concentration of intestinal protein when compared with the control (Table 24). There was no significant alteration in the concentration of intestinal glucose following the administration of 3.24 mg/kg body weight of sub-fraction. However, the doses of 6.47 and 12.94 mg/kg body weight significantly ($p < 0.05$) increased the concentration of intestinal glucose (Table 24). Sub-fraction B at all doses investigated significantly ($p < 0.05$) increased the concentration of protein carbonyl with a corresponding increase in percentage protein carbonylation (Table 24). The activity of intestinal ALP in rats treated with 3.24 and 6.47 mg/kg body weight of sub-fraction B compared favourably with the control whereas, there was a significant increase in activity of intestinal ALP at 12.94 mg/kg body weight (Table 24).

Table 24: Intestinal function indices of female Wistar rats after administration of sub-fraction B from *Mangifera indica* leaves

Parameters	Distilled water	Sub-fraction B of the residual fraction		
Dose (mg/kg body weight)	0	3.24	6.47	12.94
Total protein (mg/ml)	14.32 ± 0.10^{a}	11.10 ± 0.29^{b}	15.42 ± 0.37^{c}	15.35 ± 1.04^{c}
Glucose (mmol/l)	7.30 ± 0.11^{a}	7.12 ± 0.09^{a}	8.25 ± 0.22^{b}	8.51 ± 0.30^{b}
Protein carbonyl (µm/mg)	1.12 ± 0.03^{a}	3.45 ± 0.06^{c}	1.59 ± 0.20^{b}	8.73 ± 0.16^{d}
Protein carbonylation (%)	7.82 ± 0.00^{a}	31.08 ± 0.00^{c}	10.31 ± 0.00^{b}	56.87 ± 0.00^{d}
Alkaline phosphatase (IU/L)	11.94 ± 0.65^{a}	11.85 ± 0.33^{a}	12.77 ± 0.81^{a}	14.10 ± 0.70^{b}

Values are mean of 6 replicates ± SEM, different superscripts across the rows for each parameter are significantly different at $P < 0.05$.

4.16 Histological Examination of the Liver, Kidney and Small Intestine of Rats Treated with 3.24, 6.47 and 12.94 mg/kg body weight of Sub-fraction B

The histoarchitecture of the liver of the distilled water treated rats was essentially normal with dilated blood vessels, portal tract and intact hepatocytes (Plate 4a). There was no histoarchitectural changes in the liver of rats treated with 3.24, 6.47 and 12.94 mg/kg body weight of sub-fraction B (Plates 4b, 4c and 4d).

The kidney of the distilled water treated rats were within the normal histology with the presence of tough fibrous capsule, glomerular cortex and medulla (Plate 5a). There were no treatment related changes in the kidney of rats administered 3.24 mg/kg body weight of sub-fraction B (Plate 5b). However, the kidney of rats administered 6.47 mg/kg body weight of sub-fraction B revealed a normal Bowman's capsule, with congested capillaries (Plate 5c) while the kidney of rats administered 12.94 mg/kg body weight of sub-fraction B exhibited dilated blood vessels with tubular infiltration (Plate 5d).

The histoarchitecture of the distilled water treated control rats revealed an essentially normal feature with mucosal hyperplasia (Plate 6a) while the 3.24 mg/kg body weight treated rats revealed fat necrosis with glandular hyperplasia (Plate 6b). The small intestine of the 6.47 mg/kg body weight treated rats revealed mucosal hyperplasia with thickness of muscle layer (Plate 6c) whereas there was extensive degeneration of mucosal villi as well as the submucosal layer in the 12.94 mg/kg body weight treated rats (Plate 6d).

Plate 4a: Cross section of the liver of control rat treated with distilled water only (mg. × 400; H & E)

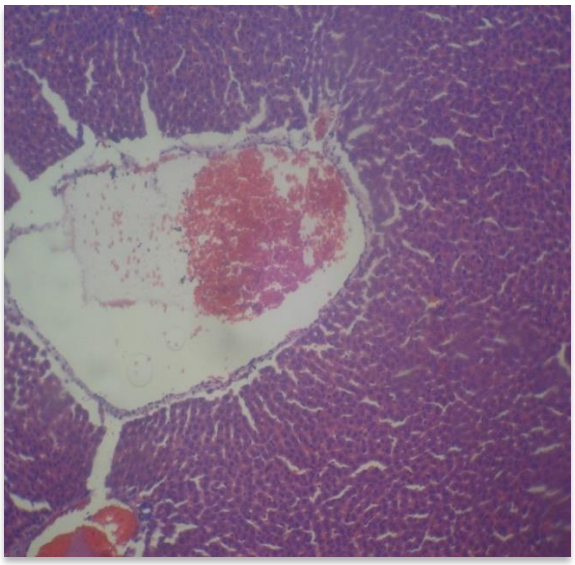


Plate 4b: Cross section of the liver of rat treated with 3.24 mg/kg body weight of sub-fraction B (mg. × 400; H & E)

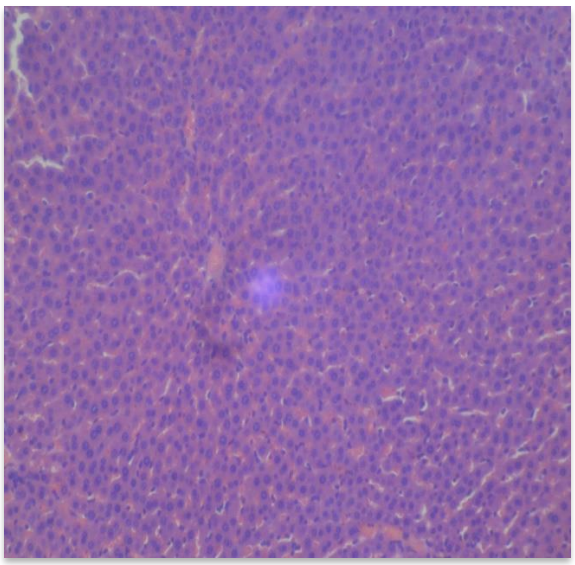


Plate 4c: Cross section of the liver of rat treated with 6.47 mg/kg body weight of sub-fraction B (mg. × 400; H & E)

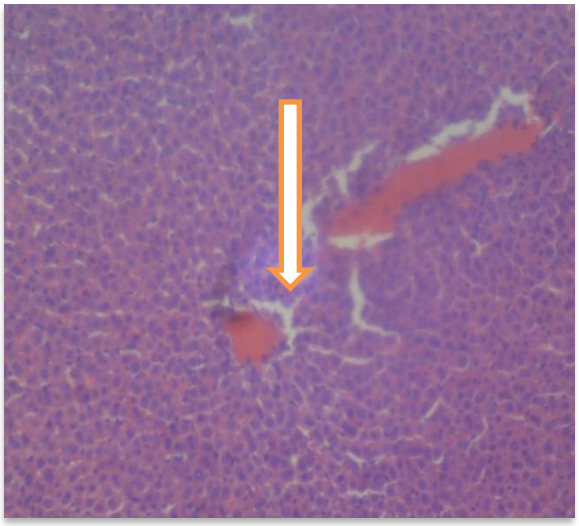
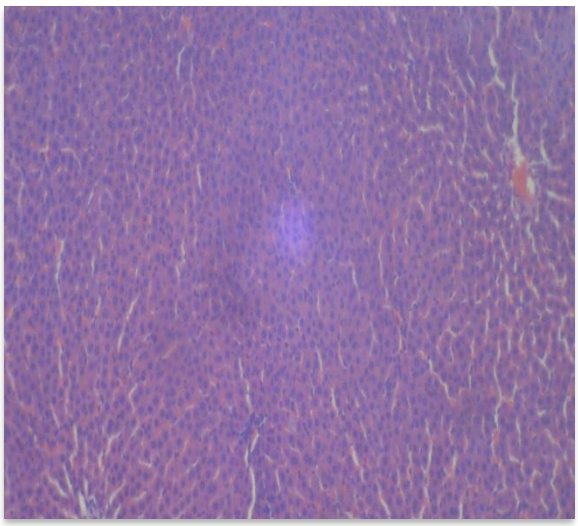


Plate 4d: Cross section of the liver of rat treated with 12.94 mg/kg body weight of sub-fraction B (mg. × 400; H & E)



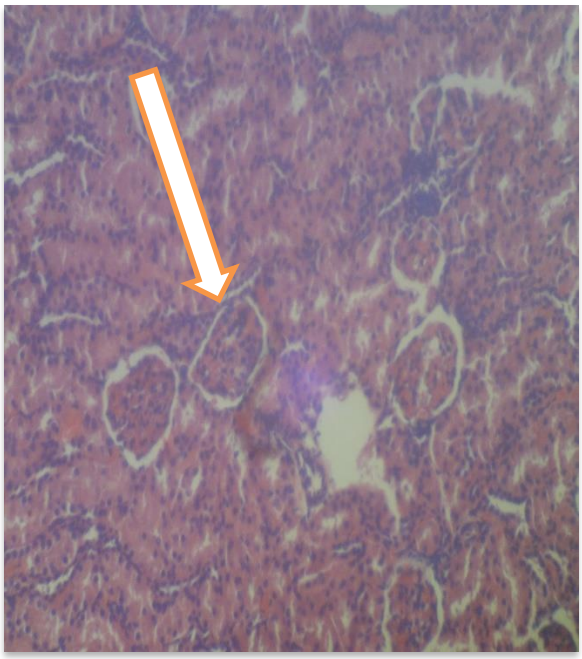


Plate 5a: Cross section of the kidney of control rat treated with distilled water (mg. × 400; H & E)

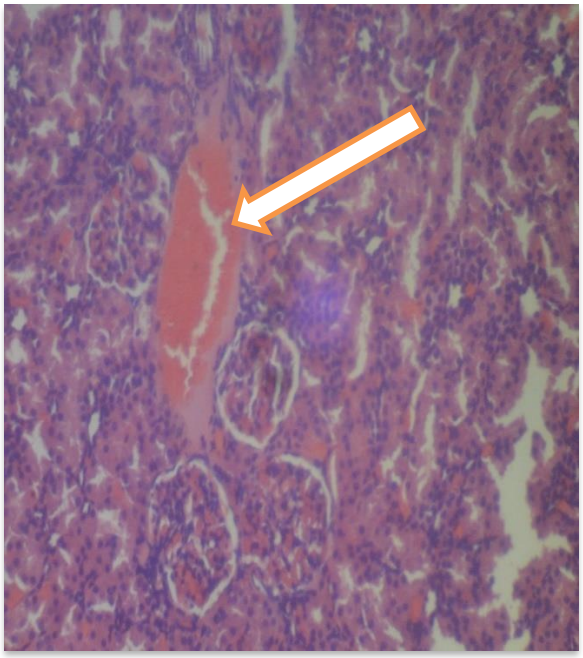


Plate 5d: Cross section of the kidney of rats treated with 12.94 mg/kg body weight of sub-fraction B (mg. × 400; H & E)

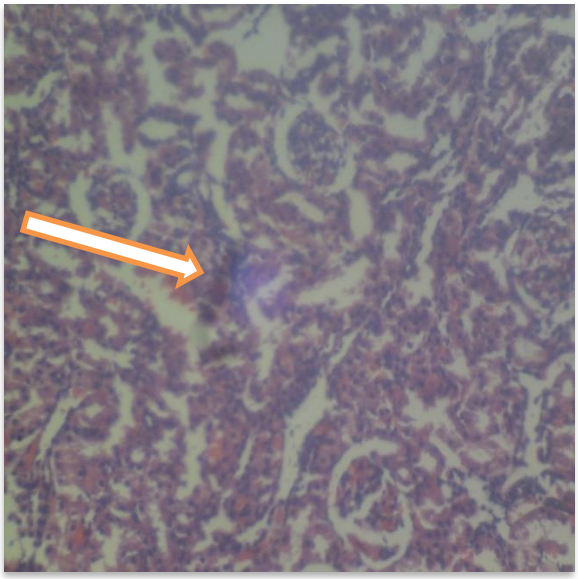


Plate 5b: Cross section of the kidney of rats treated with 3.24 mg/kg body weight of sub-fraction B (mg. × 400; H & E)

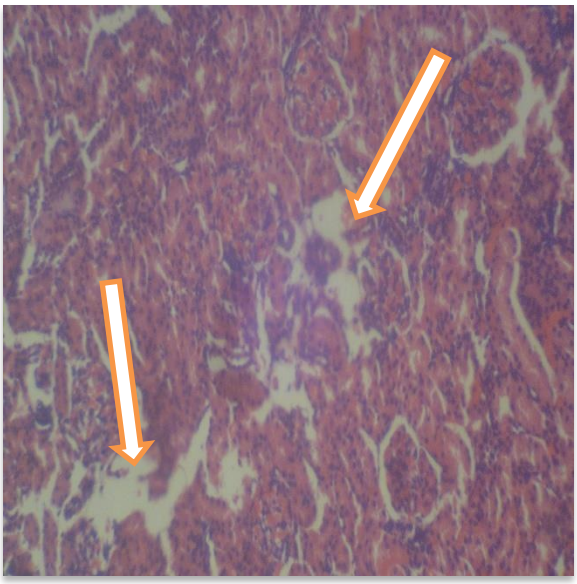


Plate 5c: Cross section of the kidney of rats treated with 6.47 mg/kg body weight of sub-fraction B (mg. × 400; H & E)

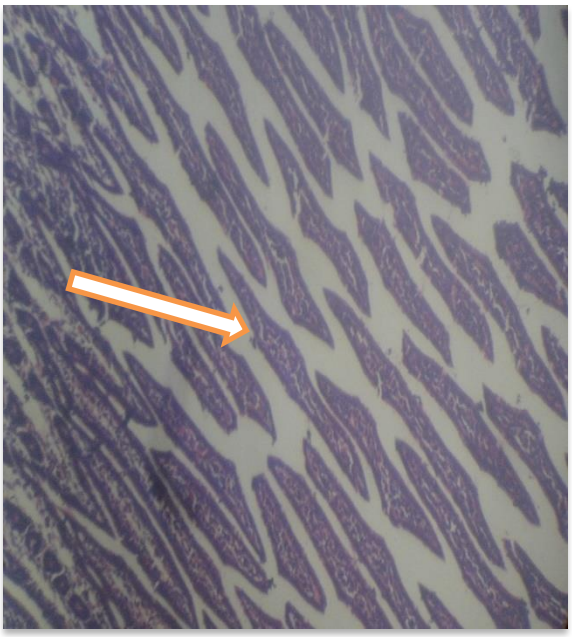
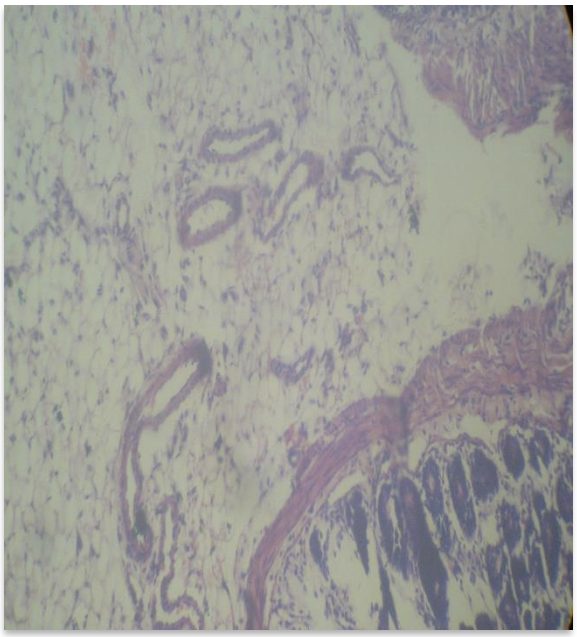


Plate 6a: Cross section of the small intestine of control rat treated with distilled water (mg. × 400; H & E)

Plate 6b: Cross section of the small intestine of rat treated with 3.24 mg/kg body weight of sub-fraction B (mg. × 400;



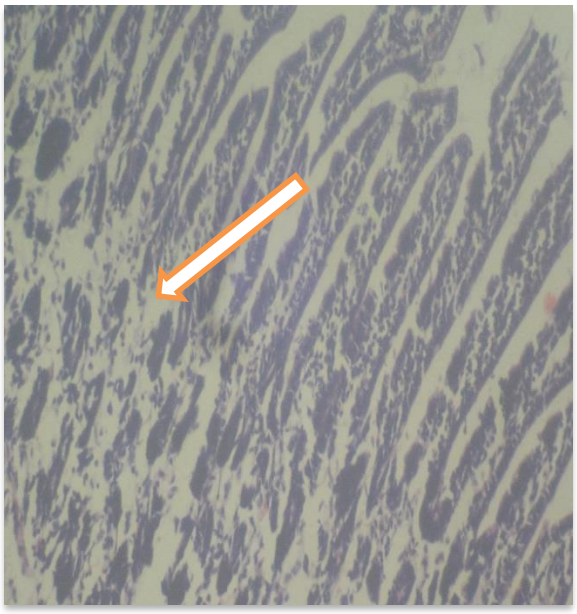
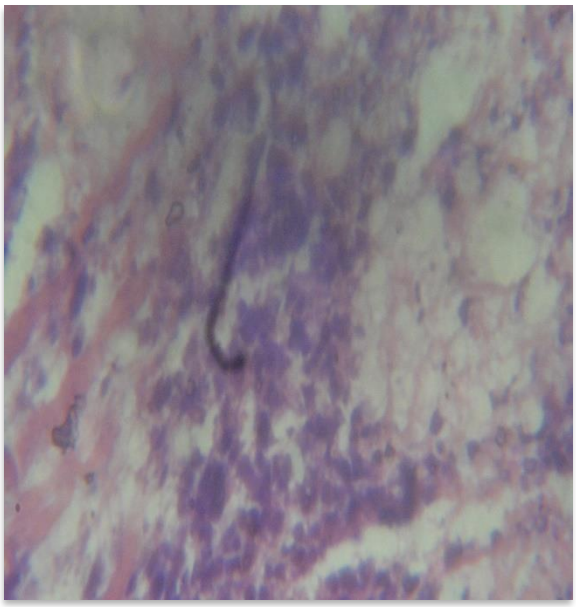


Plate 6c: Cross section of the small intestine of rat treated with 6.47 mg/kg body weight of sub-fraction B (mg. × 400;

Plate 6d: Cross section of the small intestine of rat treated with 12.94 mg/kg body weight of sub-fraction B (mg. × 400; H & E)



CHAPTER FIVE

5.0 Discussion

The use of plants or plant derived supplements are usually perceived to be efficacious in the treatment of various ailments, some of which are without an established or known scientific breakthroughs for their treatments (such as sickle cell anaemia, cancer etc.). However, the successful isolation of biogenic compounds from medicinal plants will not only aid the validation of folkloric knowledge but, also add value to indigenous plants, support plant conservation and promote the preservation of ancient knowledge. This in-turn, will also contribute to research and development in the pharmaceutical industries for the production of new drugs to treat various diseases.

The pharmacological study by Yakubu and Salimon (2015) already established the aqueous extract of *M. indica* leaves as an antidiarrhoeal agent. The aqueous extract at 100 mg/kg body weight did not produce any episode of diarrhoea and exhibited a maximum (100%) inhibition of defecation. The distance travelled by charcoal meal, volume and mass of intestinal fluid were reduced whereas the activity of Na-K ATPase was elevated. The study concluded that the aqueous extract of *M. indica* leaves possess antidiarrhoeal activity against castor oil-induced diarrhoea, enteropooling and gastrointestinal transit. However, this study was undertaken to fill the lacuna of the probable mechanisms and the biogenic molecule(s) of *M. indica* leaves that was responsible for the antidiarrhoeal activity earlier reported by Yakubu and Salimon (2015).

5.1 Antidiarrhoeal Activity of Solvent-solvent Fractions of the Aqueous Extract of *M. indica*

5.1.1 Castor Oil-induced Diarrhoea

Castor oil has a long history and use for the induction of diarrhoea due to its metabolite ricinoleic acid which functions to perturb electrolyte transport (Gaginella, 1994), intestinal

smooth muscles contractility (Izzo *et al.*, 1993), decrease or completely inhibit Na-K ATPase activity (Gaginella and Bass, 1978), interfere with oxidative metabolism (Gaginella *et al.*, 1975), stimulate mucosal cyclic AMP (Gaginella *et al.*, 1978) as well as a cytotoxic effect on intestinal epithelial cells (Bretagne *et al.*, 1981).

The distilled water treated diarrhoeal rats exhibited increased frequency of stooling, high number and weight of faeces, water content as well as a short onset time. This implies that castor oil treatment had induced diarrhoea by stimulating the active secretion of intestinal mucosal cells, smooth muscle contraction and mucus secretion all of which caused an imbalance in the secretory and reabsorption mechanisms (Brijesh *et al.*, 2009). These resulted in increased volume of intestinal content and prevention of reabsorption process which lead to a net fluid secretion (Yakubu and Salimon, 2015).

The EAF at 6.60 mg/kg body weight had no effect on the diarrhoeal rats whereas, at 13.20 and 26.41 mg/kg body weight, the episodes of diarrhoea were mitigated. The decrease in the episodes of diarrhoea by the 13.20 and 26.41 mg/kg of the EAF treated rats suggests the fraction contains some antidiarrhoeal compounds. However, the reductions were not comparable with the reference drug as well as the crude extract that produced a complete inhibition of defecation. This implies that the EAF might not be the richest fraction with the antidiarrhoeal principles.

The NBF at 4.31 mg/kg body weight had no effect on the diarrhoeal rats while there were reductions in the diarrhoeal episodes and fecal parameters monitored at 8.62 and 17.24 mg/kg body weight. The absence of antidiarrhoeal activity at 4.31 mg/kg body weight of the NBF implies the fraction cannot be explored for the antidiarrhoeal principle from the aqueous extract of *M. indica* leaves. The reductions in the number of faeces, weight and water content of faeces at 8.62 and 17.24 mg/kg body weight of the NBF were similar to those of the EAF

treated with 13.20 and 26.41 mg/kg body weight. Thus, were not the best option for the exploration of the antidiarrhoeal principle from the aqueous extract of *M. indica* leaves.

Furthermore, the RF at all doses investigated exhibited the most profound antidiarrhoeal activity as evidenced by the prolongation of the onset time, decrease in the episodes of diarrhoea as well as a high rate of inhibition of defecation. The antidiarrhoeal activity of the RF was more pronounced at 14.09 mg/kg body weight where the onset time was delayed 3 folds more than the reference drug, rate of inhibition of defecation was relatively higher and frequency of stooling was restored back to normalcy. This implies that the RF from the aqueous extract of *M. indica* leaves is rich in antidiarrhoeal principles from the crude extract.

5.1.2 Castor Oil-induced Enteropooling

During diarrhoea, there is increased production of secretagogues which promotes secretion over reabsorption process and thus, leads to intestinal fluid accumulation (Sebai *et al.*, 2014). The EAF at all doses investigated attenuated the rate of intraluminal fluid accumulation as evidenced by the decrease in mass and volume of intestinal content. This implies the enhancement of reabsorption of water and electrolytes by the EAF and consequently the attenuation of intestinal fluid accumulation (Tangpu and Yadav, 2004). The NBF at 8.62 mg/kg body weight had no effect on the accumulation of intraluminal fluid which is evident of the absence of anti-enteropooling activity. However, there were reductions in the rate of intraluminal fluid accumulation at 4.31 and 13.24 mg/kg body weight of the NBF. The RF from the aqueous extract of *M. indica* leaves also produced inhibitory effect on intestinal fluid accumulation. This might not be unconnected to the presence of some anti-enteropooling compounds capable of inhibiting the production of secretagogues (such as prostaglandins, histamines, acetylcholine etc). The reduction in the masses and volume of intestinal content in castor oil-induced enteropooling by the EAF, NBF and RF suggests the fractions could stimulate reabsorption of water and electrolytes thus, the attenuation of

intestinal fluid accumulation. It is worthy of note that the anti-enteropooling activity exhibited by the RF at 14.09 mg/kg body weight was similar to the reference drug, atropine sulphate.

5.1.3 Gastrointestinal Motility

The cytotoxic effect of ricinoleic acid on the intestinal epithelial cells as well as the induction of pro-contractile effect on smooth muscles constitute the short transit time in diarrhoeal episodes (Stewart *et al.*, 1975; Palombo, 2006). Cytotoxicity on the epithelial cells causes the loss of adult cells on the brush border of the intestinal mucosal membrane thereby leaving young immature cells with lesser absorptive capacity (Lapointe *et al.*, 2009). As a consequence, there is increased volume and motility of intestinal content.

An increase in intestinal propulsive movement leads to short reabsorption time which translates into diarrhoea. The decrease in peristaltic index by the EAF, NBF and RF at all doses investigated suggests a suppression in gastrointestinal motility. Although, all the fractions from the aqueous extract of *M. indica* leaves exhibited inhibition of peristalsis, the RF at 56.35 mg/kg body weight and the EAF at 26.40 mg/kg body weight were statistically comparable with the reference drug. The percentage inhibition of peristalsis produced by the RF at 14.09 mg/kg body weight was relatively higher than the reference drug (atropine sulphate). Therefore, the increased inhibition of peristalsis indicates prolongation in the gastrointestinal transit time for the reabsorption of water and electrolytes which also translates into a decrease in intestinal motility (Tangpu and Yadav, 2004).

The results from this study contrasts those reported by Nwidu *et al.* (2014) and Degu *et al.* (2016) where the ethyl acetate fraction of *Carpolobia lutea* (Polygalaceae) leaves and the chloroform fraction of *Croton macrostachyus* (Euphorbiaceae) leaves exhibited the most profound antidiarrhoeal activities respectively. These disparity in findings could be attributed

to the differences in plant families or the chemistry of the bioactive components present in the various plants.

5.1.4 Antidiarrhoeal Index

ADI is a measure of the combined effect of three parameters of diarrhoea (delay in onset time, reduction in peristaltic index and reduction in intraluminal fluid content). The higher the ADI, the more the effectiveness of a drug in the treatment of diarrhoea (Prasad *et al.*, 2014). The findings from this study revealed that the 14.09 mg/kg body weight of the RF had the highest ADI value which was relatively higher than the reference drug. This implies that the RF at 14.09 mg/kg body weight is the richest fraction in antidiarrhoeal bioactive principles of all the solvent-solvent fractions from the aqueous extract of *M. indica* leaves.

Furthermore, the antidiarrhoeal activity exhibited by the RF at 14.09 mg/kg body weight were closely related to the findings from the study by Yakubu and Salimon (2015). Although, the study by Yakubu and Salimon (2015) reported that the onset of diarrhoeal faeces was completely averted by the aqueous extract of *M. indica* leaves at 100 mg/kg body weight. The findings from the present study followed similar pattern with a prolonged onset time of diarrhoea as well as a relatively high (> 95%) inhibition of defecation. The reduction in antidiarrhoeal activity of the RF from the aqueous extract could be attributed to the loss of some important metabolites that might have acted in synergy with the bioactive component to produce the observations reported from the previous study.

5.2 Antidiarrhoeal Activity of Sub-fractions A, B and C of the Residual Fraction from the aqueous extract of *M. indica*

5.2.1 Effects on Castor Oil-induced Diarrhoea

The delayed onset of diarrhoeal faeces by sub-fraction A as well as the decrease in fecal parameters (total number of faeces, fresh weight and water content of faeces) suggests the fraction inhibited diarrhoeal episodes. Although, there was a relatively low onset time of

diarrhoea in the sub-fraction B treated rats, the reductions in fecal parameters were more pronounced than the sub-fractions A, C and reference drug treated rats. This implies that the fraction has little (or no) effect on motility, rather, it stimulated a reduction in secretion and enhanced the reabsorption of water and electrolytes in the small intestine which completely averted the episodes of diarrhoea. Also, the early onset time in sub-fraction B treated rats suggests the fraction facilitated the excretion of the offensive agent from the intestinal lumen thereby restoring normal bowel activity. Similarly, the sub-fraction C of the residual fraction from the aqueous extract of *M. indica* leaves stimulated an early onset of diarrhoea. However, the episodes of diarrhoea were not completely averted like in sub-fraction B treated rats. The difference in antidiarrhoeal activities between the sub-fractions A and B implies the separation of the variety of bioactive components present in the RF into the various sub-fractions thereby exhibiting different modes of action. Also, it can be postulated that some of the bioactive components from sub-fraction B were present in the sub-fraction C but in limited quantities which was just enough to decrease the incidence of diarrhoea in the sub-fraction C treated rats.

5.2.2 Effect on Castor Oil-induced Enteropooling

An increase in net fluid absorption can be achieved by a drug via the stimulation of intestinal epithelial cells to enhance their rate of absorption or decrease the rate of peristalsis in the gut. This causes prolonged reabsorption time which promotes absorption and decrease the volume of stool thus, the inhibition of intraluminal fluid accumulation (Yacob *et al.*, 2016). The decrease in volume of intraluminal fluid by the sub-fraction B treated rats implies the fraction exerted some stimulatory effect on the intestinal epithelial cells to facilitate the reabsorption of water and electrolyte. The decrease in mass of intestinal content can be attributed to the absorption of intestinal solutes (Rode *et al.*, 2013) that otherwise might have caused a shift in concentration gradient and promote water secretion into the intestinal lumen.

Although, the mass of intestinal content was decreased by the sub-fractions A and C whereas, the volume of intraluminal fluid was not. This implies the presence of poorly absorbable solutes (ricinoleic acid) in abundance which develops into osmotic diarrhoea (Whyte and Jenkins, 2012) through the absorption of water and electrolytes into the intestinal lumen. None of the sub-fractions from the residual fraction could stimulate reabsorption as the reference drug atropine sulphate. This can be attributed to its anticholinergic effect (Izzo *et al.*, 1999) whereby the stimulation of reabsorption process is coupled with a decrease in peristaltic movement hence, there is increased reabsorption time.

5.2.3 Effect on Gastrointestinal Motility

All the sub-fractions (A, B and C) from the residual fraction caused a reduction in gastrointestinal motility as evidenced by the reduction in peristaltic index. The reduction in intestinal motility as complemented by the suppression of intestinal propulsive movement implies a decreased cholinergic effect. Thus, translating to a lower movement of intestinal smooth muscle cells and increased inhibition of peristalsis. It is worthy to note that the decrease in peristaltic movement by sub-fraction A was more profound than the reference drug treated rats. This suggests that the prolonged delay in onset time by sub-fraction A in the castor oil-induced diarrhoeal model might not be unconnected to the reduction in intestinal transit with a similar trend observed in the sub-fraction B treated rats where there was a relatively lower inhibition of peristalsis. The difference in antimotility activity notably in the sub-fractions A and B further supports the suggestion of the presence of different bioactive agents.

5.2.4 Antidiarrhoeal Index of Sub-fractions A, B and C

The ADI is a useful parameter for rating antidiarrhoeal agents with relevance to their activity (Degu *et al.*, 2016). Both of the sub-fractions A and B exhibited profound antidiarrhoeal activity, hence, the ADI was necessary to establish the most potent fraction.

The computed ADI suggests both fractions were rich in the suspect antidiarrhoeal bioactive principle from the aqueous extract of *M. indica* leaves with values greater than 50%. Hence, the two fractions were subjected to further analysis to establish the most profound fraction with respect to their mechanism of action.

5.3 Studies on the Probable Mechanism(s)

5.3.1 Antisecretory Effect

Diarrhoea has been reported to cause impairment in the reabsorption of water and electrolytes while the absorption of glucose remains intact (Saha *et al.*, 2015). There are several mechanisms for the absorption of Na^+ in the intestinal lumen, the most prominent of which is the co-transport with glucose mediated by SGLT1 (Chen *et al.*, 2016). Therefore, the increased availability of glucose in the sub-fraction B treated diarrhoeal rats implies the improved availability of substrate to facilitate the transport of Na^+ across the apical membrane which in turn stimulates the reabsorption of water. Therefore, the reduction of fecal weight, water content and episodes of diarrhoea in the diarrhoeal rats administered 6.47 mg/kg body weight of sub-fraction B can be attributed to the stimulation of water and electrolyte reabsorption exerted by the increased absorption of glucose probably via the enhancement of SGLT1 expression (Kiela and Ghishan, 2016). In contrast, there was a relatively high serum concentration of Na^+ in sub-fraction A treated rats which implies that the absorption of water and electrolytes have been compromised. In the absence of serum glucose that proffers alternative pathway for the absorption of water and electrolytes, there was continuous accumulation of fluid and electrolytes as evidenced by the relatively high-water content in the diarrhoeal rats treated with the sub-fraction A of the residual fraction.

Na-K ATPase is responsible for the maintenance of ion homeostasis in intestinal epithelial cells (Gal-Garber *et al.*, 2003). Moreso, an increase in glucose absorption has been reported to stimulate the activity of intestinal Na-K ATPase (Wild *et al.*, 1999; Alexander and Carey,

2001). Hence, the upregulation in the expression of intestinal Na-K ATPase might not be unconnected to the increase availability of glucose in the sub-fraction B treated diarrhoeal rats. The increased turn-over of Na-K ATPase in sub-fraction B treated rats also facilitates the cellular uptake of K^+ coupled with the downhill movement of Na^+ across the intestinal brush border membrane (Field, 2003). This triggers a shift in concentration gradient that favours the absorption of water osmotically and inhibition of active secretion by intestinal epithelial cells (Field, 2003). In contrast, the activity of intestinal Na-K ATPase was not modulated by the sub-fraction A treatment thereby suggesting a different mechanism other than antisecretory effect. Cyclic AMP functions to stimulate the cystic fibrosis transmembrane regulator (CFTR) Cl^- channel of the intestinal epithelial cells which leads to the active secretion of Cl^- with limited or complete inhibition of the absorption of Na^+ (Schuier *et al.*, 2005). The reduction in serum Cl^- in the sub-fraction B treated rats implies the fraction might contain a stimulant for intestinal phosphodiesterase important for the regulation of cAMP. The suppression of cAMP activates the closure of the Cl^- channel by blocking CFTR thus, sustaining the electrical driving force required for the extrusion of Cl^- (Field, 2003; Casburn-Jones and Farthing, 2004; Schuier *et al.*, 2005). However, the concentration of Cl^- remained high in the sub-fraction A treated diarrhoeal rats thereby suggesting the stimulation of the CFTR Cl^- channel by castor oil-treatment was not ameliorated by this fraction (Casburn-Jones and Farthing, 2004).

The K^+ channels of the intestinal epithelial cells are also involved in secretory and absorption process by hyperpolarizing the mucosal membrane which provides the driving force for electrogenic transports such as the co-transport of Na^+ and glucose (Palmer, 2014). Therefore, the increase in K^+ in the sub-fraction B treated rats further emphasizes the restoration of absorption process. As evident from the results obtained in this study, the sub-fraction B of the residual fraction from the aqueous extract of *M. indica* leaves exhibited

profound antisecretory effects whereas the sub-fraction A had no effect on intestinal hypersecretion.

The modulation of electrolyte disorder as well as the increase in the concentrations of serum glucose by sub-fraction B was consistent with those reported by Prasad *et al.* (2013) where the crude extract and bioactive fractions from *Eriosema chinense* roots increased total carbohydrate and corrected electrolyte imbalance in diarrhoeal animals. Also, the increased activity of Na-K ATPase was consistent with our previous study (Yakubu and Salimon, 2015) and a similar effect was reported by Sahoo *et al.* (2016) where the flavonoidal fraction of *Apium leptophyllum* fruits enhanced the activity of Na-K ATPase.

5.3.2 Anti-inflammatory Effect

The expression of intestinal alkaline phosphatase (ALP) spans through the length of the small intestine. The highest expression occurs at the duodenum and phosphorylation activity is at its peak at the ileum (Lalles, 2014). It is secreted into the intestinal lumen and blood stream by the microvilli of the brush border and functions to regulate the secretion of bicarbonate, stimulate the absorption of intestinal long chain fatty acids (LCFA) and neutralize lipopolysaccharides (LPS) (Fawley and Gourlay, 2016). This series of event culminates into the maintenance of intestinal microbiota and anti-inflammatory effect by the intestinal ALP (Fawley and Gourlay, 2016). The increased activity of intestinal ALP by the sub-fraction B and the reference drug treated rats suggest that the fraction could stimulate the release of intestinal ALP which in turn might promote the absorption of the LCFA ricinoleic acid present in the lumen following treatment with castor oil.

Nitric oxide (NO) is an enteric nervous system neurotransmitter that confers protection on the intestinal mucosal and modulates intestinal motility (Vallance *et al.*, 2004). An increase in the synthesis of this neurotransmitter can stimulate the release of inflammatory cytokines such as prostaglandins, interleukins, leukotrienes and alters intestinal motility which

increases the severity of diarrhoea disease (Stanzel *et al.*, 2008). Elevated levels of NO in the distilled water treated diarrhoeal rats can be attributed to the upregulation of inducible nitric oxide synthase (iNOS) which provokes the release of inflammatory cytokines that are known to induce or aggravate the severity of diarrhoea (Nathan and Xie, 1994; Kim, 2011). As a consequence, the surface of intestinal epithelial cells might have been coated thereby preventing effective reabsorption due to reduction in the surface area of the microvilli for absorption (Cotton *et al.*, 2011). NO is produced in small amounts under physiological condition hence, the reduction in concentration of intestinal NO by the sub-fraction B can be attributed to the down-regulation of iNOS. The inhibition of iNOS alleviates the release of prostaglandins and other inflammatory cytokines (Kim, 2011). This may contribute to the reduction of intestinal fluid accumulation reported in this study. Furthermore, the decrease in excessive secretion of NO by the sub-fraction B might be in part due to the increased activity of intestinal ALP via the modulation of the NO/prostaglandin pathway.

Contrary to the stimulatory effect of the sub-fraction B on the expression of intestinal ALP and inhibition of NO, the sub-fraction A could not stimulate the activity of intestinal ALP while the concentration of intestinal NO was reduced. This implies that the sub-fraction A had little to no effect on the inflammatory mediators of the intestinal mucosa. The inhibition of nitric oxide biosynthesis by the sub-fraction B conforms with the study reported by Prasad *et al.* (2013) and Sahoo *et al.* (2016).

5.3.3 Antioxidant Effect

Several studies have reported the disruption of intestinal redox balance during diarrhoea (Rao *et al.*, 2008; Sebai *et al.*, 2014; Jabri *et al.*, 2016). The production of ROS and RNS in the intestinal lumen could provoke the incidence of diarrhoea by acting as secretagogues; stimulating the release of inflammatory cytokines (such as prostaglandins) and; facilitating the contraction of intestinal smooth muscles (Circu and Aw, 2012). The proper maintenance

of intestinal GSH is imperative to normal health and functioning of the tissue (Aw, 2005). Some of the known sources of intestinal GSH include dietary intake, intracellular *de novo* synthesis or from hepatic biliary secretions (Zhang *et al.*, 2005; Circu and Aw, 2012). GSH functions to scavenge intestinal mucosal ROS and RNS, facilitate electron transport chain and increase ATP production (Circu *et al.*, 2009; de Barboza *et al*., 2017). Therefore, the increase in intestinal mucosa GSH by the reference drug, sub-fractions A and B treated rats suggest the presence of a potent antioxidant capable of stimulating the availability of intestinal GSH and the prevention of cellular oxidative damage (Fang *et al.*, 2002). Furthermore, the increase in intestinal GSH by the sub-fractions A and B might suggest enhancement of ATP production required for various metabolic process in the small intestine (such as Na-K ATPase, glucose metabolism and electron transport).

SOD and catalase are responsible for the termination of free radical processes (Nair *et al.*, 2006). SOD has been implicated with the prevention of intestinal mucosal cell injury (Klinowski *et al.*, 2006) while catalase prevents the protrusion of intestinal microvilli (Ruh *et al.*, 2000). The reduction in activities of intestinal antioxidant enzymes in the diarrhoeal rats implies the accumulation of ROS/RNS resulting in increased lipid peroxidation. The repletion of superoxide dismutase and catalase by the sub-fractions A and B suggests the presence of exogenous molecule(s) capable of bolstering the cellular antioxidant system (Jabri *et al.*, 2016). The alleviation of oxidative stress by the sub-fractions A and B in a manner similar to the reference drug thereby identifies the restoration of redox balance as another mechanism with which the sub-fractions A and B of the residual fraction from *M. indica* leaves exerts its antidiarrhoeal activity. The increase in activities of SOD and catalase by sub-fractions A and B were consistent with previous studies reported by Rao *et al.* (2008), Sebai *et al.* (2014) and Jabri *et al.* (2016).

5.3.4 Antimotility Effect

Acetylcholine exerts excitatory effect and stimulates the contraction of intestinal smooth muscle cells which results in increased intestinal propulsion and active secretions (Furness *et al.*, 2014; Duboc *et al.*, 2016). Acetylcholine also activates the CFTR Cl^- channel and stimulates the release of inflammatory cytokines which leads to intestinal secretion (Nezami and Srinivasan, 2010; Duboc *et al.*, 2016). The hydrolytic function of acetylcholinesterase (AChE) thereby makes it an important marker for the regulation of intestinal motility. The elevation of intestinal AChE activity in the sub-fraction A treated rats might be attributed to the activation of α-adrenergic receptors at the tight junction thereby inhibiting the sympathetic stimulation of gastrointestinal motility by acetylcholine (Browning and Travagli, 2014). Furthermore, the increase in intestinal AChE by sub-fraction A of the residual fraction from *M. indica* leaves implies inhibition in the transmission of cholinergic synapse and therefore, decreased intestinal motility (Browning and Travagli, 2014). The decrease in gastrointestinal motility by the sub-fraction A is corroborated by the high inhibition of peristaltic movement reported in this study. Although, the sub-fraction B also stimulated the activity of intestinal AChE, the effect was not comparable with the reference drug or sub-fraction A. This implies increased biosynthesis of intestinal acetylcholine and its cholinergic effect which manifested as the short reabsorption time and enhancement of intestinal motility reported.

5.3.5 Intestinal Permeability

The intestinal mucosal barrier is a complex structure that confers both physical and chemical protection on the intestinal mucosa (Konig *et al.*, 2016). It is composed of the epithelial cell lining, vascular endothelium and mucus layer that separates the intracellular milieu from the external luminal compartment (Cummings *et al.*, 2004). Disruption of the barrier causes increased intestinal permeability which in turn facilitates the translocation of

harmful substances, pathogens as well as the loss of tight junction complex function important for the regulation of the flow of water and electrolytes across cell membranes (Konig *et al.,* 2016).

Oxidative stress plays a key role in the initiation and maintenance of diarrhoeal symptoms (Jabri *et al.,* 2016). The ROS and RNS react with the membrane phospholipids of intestinal epithelial cells to initiate and promote peroxidation thereby causing intestinal injury and cellular malfunction (Kakkar *et al.,* 1992). The relatively high levels of intestinal MDA and protein carbonyl (both of which are products of peroxidation) in the sub-fraction A treated rats suggests perturbation of intestinal barrier function. Although, MDA has cytotoxic effect on the intestinal microvilli, there is a continuous replenishment of enterocytes with new young cells (with decreased functional capacity) by the crypt stem cells to replace the damaged cells (McCann and Simpson, 2006). The absence of complete restoration in fecal parameters by the sub-fraction A can therefore be attributed to the derangement in mucosal barrier function due to the presence of immature enterocytes. The sub-fraction B completely averted the incidence of diarrhoea which could be indicative of the restoration of barrier function and/or inhibition of peroxidation by the bioactive constituent of sub-fraction B in the diarrhoeal rats. This is evidenced by the decreased intestinal MDA and protein carbonylation reported in this study. Intestinal proteins (such as the aquaporins, tight junctions, occludin, claudins etc.) play vital roles in the maintenance of the integrity of intestinal membrane (Haschek, 2010). Oxidative damage of intestinal proteins can lead to lysis or cross linking of proteins which results in loss of activity, viability of the cell membrane function and ultimately impairment of permeability process (Haschek, 2010). The increased secretion of proteins and decreased level of protein carbonylation in the sub-fraction B treated rats could imply the stimulation of protein biosynthesis as a pathophysiological response to improve and/or restore barrier function following the cytotoxic effect from castor oil treatment. The

decrease in intestinal protein in the sub-fraction A treated diarrhoeal rats as complemented by increased carbonylation suggest the disruption of intestinal epithelial barrier function. The increase in intestinal total protein by the sub-fraction B was also similar to that reported by Prasad *et al.* (2013) while the reduction in MDA levels was similar to the recent findings by Jabri *et al.* (2016).

5.4 Histoarchitectural Changes

Maintenance of the structural integrity of the small intestine is intrinsic to the proper functioning of the organ. Any alteration to which could lead to derangement or complete disruption of intestinal barrier function. The lymphocytic follicular hyperplasia in the distilled water treated rats might be an immunological response to the inflammation as a result of castor oil treatment. However, the presence of inclusion bodies with polymorph infiltration in sub-fraction A treated rats further corroborates the derangement of barrier function thereby allowing the influx of foreign substances in the cell. Also, the perturbation of cellular integrity in the sub-fraction A treated rats can be attributed to the cytotoxic effect of MDA which was relatively high in the diarrhoeal rats (McCann and Simpson, 2006). The presence of dilated blood vessels in the reference drug treated animals can be indicative of gastrointestinal bleeding. This might imply ischemic injury of the intestinal mucosal due to the various chemical assaults which could lead to organ failure and other possible side effects of loperamide hydrochloride (Oliveira *et al.*, 2014; ASHSP, 2016). The presence of follicular hyperplasia with essentially normal microvilli as observed in the sub-fraction B treated rats suggests protective effect of the fraction on structural integrity of the intestine (Haschek, 2010).

Overall, studies on the mechanism of action of the sub-fractions A and B has been able to establish the sub-fraction B as the most potent fraction with antidiarrhoeal bioactive principles. Although, the sub-fraction A exhibited the most profound antimotility effect

whereas the sub-fraction B exhibited anti-secretory, antioxidant and restoration of intestinal permeability function. The sub-fraction B of the residual fraction was subjected to further analysis.

5.5 Biogenic Constituents

Mangiferin (2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), a natural polyphenol has been proven to exert strong antioxidant activity attributed to the presence of multiple hydroxyl groups and its xanthonoid structure with C-glucosyl linkage (Pal *et al.*, 2015; Saha *et al.*, 2016). The restoration of intestinal redox balance reported in the sub-fraction B treated rats might therefore be attributed to the presence of mangiferin. Also, mangiferin forms complex with phospholipids thereby enhancing intestinal permeability function (Saha *et al.*, 2016). The concentrations of intestinal permeability function parameters were upregulated in this study. Therefore, the amelioration of intestinal permeability in diarrhoeal rats treated with the sub-fraction B might also be implicated to the effect of mangiferin on the brush border membrane. Similarly, the increase in intestinal glucose concentration reported in the sub-fraction B treated rats might be attributed to the cleavage of the glucose moiety of mangiferin thereby facilitating the antisecretory effect reported on the sub-fraction. Furthermore, mangiferin has been reported to inhibit the transcriptional activity of NF-κB and other proinflammatory mediators such as iNOS, cyclooxygenase and α-tumor necrosis factor (Dou *et al.*, 2014; Saha *et al.*, 2016). Therefore, the anti-inflammatory activity exhibited by the sub-fraction B might as well be attributed to the natural xanthoid, mangiferin.

Catechins have also been validated for their anti-inflammatory, antimicrobial, antioxidant and anticarcinogenic effects (Hirao *et al.*, 2010; Gupta *et al.*, 2014; Zanwar *et al.*, 2014). Therefore, the antioxidant, antisecretory and anti-inflammatory effects exhibited by the sub-fraction B of the residual fraction from the aqueous extract of *M. indica* leaves on castor-oil

induced diarrhoea, enteropooling and gastrointestinal motility might not be unconnected to the presence of mangiferin and catechin.

5.6 Toxicological Implications

In herbal medicine, there is the common idea or belief that herbal drugs are very safe and free of side effects which is false. Several studies have established the toxicological implications of medicinal plants with hundreds of constituents of highly toxic effect (such as digitalis, solanine, pyrrolizidine, amygdalin, myristicin etc) (Pittenger, 2002; Lee *et al*., 2005; Barceloux, 2009; Krenzlok and Mrvos, 2011). Therefore, it is necessary to evaluate the potential toxicity of the sub-fraction B from the aqueous extract of *M. indica* leaves on the liver, kidney and intestine of the female Wistar rats.

5.6.1 Liver Function Indices

Serum total protein consists of albumin and globulin which are related to the synthetic function of the liver. The increase in serum total protein, albumin and globulin in the sub-fraction B treated rats implies increased *de-novo* synthesis of protein. The albumin/globulin ratio which can vary from between 1.2 – 2.5 may depict diseased state of the body where low levels could imply an autoimmune disorder, inflammation or liver cirrhosis whereas high levels suggest synthetic disorder or malabsorption problems (Carol, 2017). The albumin/globulin ratios of rats treated with 3.24 and 6.47 mg/kg body weight of sub-fraction B of the residual fraction were essentially normal thereby suggesting the synthetic function of the liver was intact. The reduction of albumin/globulin ratio in rats administered 12.94 mg/kg body weight of sub-fraction B might imply an inflammatory response by the liver due to chemical assault on the organ (Carol, 2017).

Bilirubin, a hepatobiliary biomarker is a product of hemolysis usually transported to the liver by albumin where it is conjugated via glucuronidation (Dzoyem *et al*., 2014). The irregular pattern in serum total and conjugated bilirubin at 3.24 and 6.47 mg/kg body weight

might imply an adaptation of the liver to the effect of the sub-fraction B of the residual fraction. The reduction in conjugated bilirubin in rats treated with 12.94 mg/kg body weight of the extract might not be unconnected to the depreciation in serum albumin required to transport bilirubin from the serum to the liver where it is conjugated. This implies that the excretory function of the liver was compromised following the administration of 12.94 mg/kg body weight of the sub-fraction B.

ALT is a cytoplasmic enzyme with relatively high serum levels in hepatocyte damage while the serum concentration of AST is an indicator of the extent of the tissues' damage. ALT is specific for hepatocytic inflammation of the liver, characterized by increased activity of about 10 – 20 folds (Feldman *et al.*, 2010; Singh *et al*., 2011). Although, the enzymes are synthesized by the liver, the activity of the enzymes in the serum could indicate the loss of cellular integrity by the organ thereby causing leakage to the serum (Yakubu *et al.*, 2015). The elevation in activities of liver AST and ALT without a corresponding increase in the serum might suggest increased *de novo* synthesis of the enzymes. An increase in AST/ALT suggests progressive depletion of the functional capacity of the liver which could be in form of liver fibrosis, cirrhosis or specify the presence of a congenital disease such as hyperthyroidism or Wilson's disease (Sayal *et al*., 1997; Giannini *et al*., 2003; Eisenbach *et al*., 2007). The liver AST/ALT ratio was elevated in the rats treated with 12.94 mg/kg body weight of the sub-fraction B might imply liver dysfunction. ALP is an important biomarker for cholestasis in the liver, anchored to the cell membrane by the glucophosphatidyl inositol (GPI) proteins which are cleaved by bile acids therefore, resulting to increased serum activity of ALP as they are released from the cell membrane (Dzoyem *et al*., 2014). Hence, the increased activity of serum ALP in rats administered 6.47 and 12.94 mg/kg body weight of sub-fraction B might be attributed to drug-induced cholestasis while the increased production of liver ALP might be attributed to the homeostatic effect of the liver to compensate for the

ALP lost to the serum (Ramaiha, 2007; Singh *et al*., 2011). The increased activity of serum ALP might imply hepatocellular leakage from the liver to the serum (Yakubu *et al.* 2015).

5.6.2 Kidney Function Indices

Urea, a major end product of protein catabolism is synthesized by the hepatocytes and mainly excreted by the kidney. The concentration of blood urea is equivalent to that present in the glomerular filtrate hence, decrease glomerular filtration leads to increased blood urea (Tietz, 1995). The elevation in the level of serum urea at 6.47 and 12.94 mg/kg body weight can be attributed to the increased *de novo* synthesis of total protein by the liver thereby causing the increased production of urea. Also, the increase in serum urea might be attributed to impairment of glomerular filtration function which implies renal dysfunction (Burtis *et al.*, 2012). Uric acid, an end product of purine catabolism was not affected by the administration of the sub-fraction B at all doses investigated and therefore, implies the renal glomerular and tubular function was not compromised (Burtis *et al*., 2012). Creatinine is a metabolic waste product of muscle metabolism generated by the spontaneous degradation of creatine to creatinine with the generation of energy (Dzoyem *et al*., 2014). Creatinine clearance by the glomerulus from the blood to urine occurs at a fairly constant rate and therefore proffers an insight to glomerular filtration rate. An elevation in serum creatinine level signifies a kidney disease or loss of kidney function (Edmund and David, 2006). The decrease in serum creatinine in 6.47 mg/kg body weight treated rats might imply decreased production of creatine by the liver or increased glomerular filtration rate as evidenced by the creatinine clearance rate (Park *et al*., 2013). The elevated level of serum creatinine in rats treated with 12.94 mg/kg body weight might suggest uremia or impaired kidney function (Gowda *et al.*, 2010). However, the high creatinine clearance in the 12.94 mg/kg body weight treated rats suggests the increase in serum creatinine might not be entirely due to a loss of kidney function but from increased production (Park *et al*., 2013). Arginine and glycine are

precursors for creatine synthesis hence, the increase in serum creatinine might not be unconnected to the increased synthesis of protein in the liver (Thomas *et al*., 2015).

Renal Na-K ATPase plays an integral role in the active translocation of Na^+ and K^+ across the membrane thereby maintaining acid-base balance (Wingo and Cain, 1993; Ferguson *et al*., 2008). Impairment in the activity of renal Na-K ATPase has been attributed to distortion of renal function whereas an increased activity had been reported to enhance renal function (Hussein and Lokhandwala, 2003). The sub-fraction B at 3.24 mg/kg body weight did not cause any alteration in the activity of renal Na-K ATPase which implies a healthy state renal function. An increase in activity of renal Na-K ATPase has been attributed to the enhancement and glomerular and tubular functions hence, the elevation of renal Na-K ATPase at 6.47 mg/kg body weight could imply enhanced renal function (Hussein and Lokhandwala, 2003) whereas the reduction in the activity of renal Na-K ATPase at 12.94 mg/kg body weight could be an indication of kidney dysfunction.

Electrolytes (Na^+, K^+ and Cl^-) are freely filtered by glomeruli and readily reabsorbed at the proximal tubule, distal tubule and the loop of Henle (Burtis *et al.*, 2012). High serum levels of Na^+ could imply dehydration while low levels could be attributed to loss of reabsorption process by the tubules that could lead to hyponatremia (Thurman and Berl, 2013). The increase in concentration of serum Na^+ at 3.24 and 6.47 mg/kg body weight might imply loss of water and extracellular fluid volume while the reduction at 12.94 mg/kg body weight is suggestive of poor reabsorption consequent of derangement in tubular function (Dzoyem *et al.,* 2014). The concentrations of serum K^+ and Cl^- were not affected following treatment with 3.24 and 6.47 mg/kg body weight of the sub-fraction B which implies the tubular function was not adversely affected. The elevated levels of serum K^+ and Cl^- at 12.94 mg/kg body weight indicates a tubular dysfunction or the manifestation of acid-base imbalance (Koch and Taylor, 1992).

5.6.3 Intestinal Function Indices

Intestinal protein functions in the intestinal permeability, an increase or decrease in which will cause drastic changes in permeation rate. The increase in intestinal protein at 6.47 and 12.94 mg/kg body weight might imply the regenerative effect of the gastrointestinal epithelium to replace damaged cells (Circu and Aw, 2012). This in turn causes enhancement of intestinal barrier function. However, the increase in intestinal protein carbonyl coupled with the high rate of protein carbonylation at 12.94 mg/kg body weight might be suggestive of increased peroxidation which might dampen the functional capacity of intestinal epithelial cells (Haschek, 2010). Glucose acts as an energy source for the small intestine while also facilitating the rate of absorption for water and electrolytes across the brush border membrane (Wright *et al.*, 2012; Kiela and Ghishan, 2016). The increase in intestinal glucose at 6.47 and 12.94 mg/kg body weight which can be attributed to improved availability from external source which might be implicated with enhancement of the reabsorption in intestinal epithelial cells (Wright *et al.*, 2012). Elevated levels of intestinal ALP in rats treated with 12.94 mg/kg body weight of the sub-fraction B might suggest a pathophysiological response of the organ to alleviate inflammation (Fawley and Gourlay, 2016).

5.7 Histopathology of the Liver, Kidney and Small Intestine

The absence of any histoarchitectural changes in the liver of the experimental animals treated with sub-fraction B at all doses investigated despite the alterations in functional parameters implies the fraction exerted functional toxicity on the liver without causing alterations on the structural integrity of the organ. The absence of histoarchitectural changes in the kidney of rats treated with 3.24 mg/kg body weight of sub-fraction B implies there was no treatment related changes. However, the presence of congested capillaries and dilated blood vessels in rats treated with 6.47 and 12.94 mg/kg body weight might be an indication of deterioration in kidney function. Furthermore, the absence of structural changes on the

intestinal epithelial cells at 3.24 mg/kg body weight indicates absence of structural toxicity at while the further thickening of muscle layer at 6.47 mg/kg body weight suggests damage of intestinal enterocytes without any deleterious effect on the underlying lamina propyria (Haschek, 2010). The derangement in structural integrity at 6.47 mg/kg body can be completely restored within few days due to the regenerative ability of the crypt stem cells (McCann and Simpson, 2006; Haschek, 2010). However, the extensive degeneration of mucosal villi and submucosal layer at 12.94 mg/kg body weight could be suggestive of mucosal collapse and loss of barrier function (Haschek, 2010). As a consequence, there might be ulceration and haemorrhage at the intestinal mucosa. Although, no study has reported the toxicity of mangiferin (Saha *et al.*, 2016), the functional and structural toxicity exhibited by the sub-fraction B might therefore be attributed to the presence of some other metabolites capable of exerting the toxic effects reported in this study.

CHAPTER SIX

6.0 Summary of Findings

1. The residual fraction exhibited the most profound antidiarrhoeal activity in castor oil-induced diarrhoea, enteropooling and gastrointestinal motility.
2. The computed ADI was highest in the diarrhoeal rats treated with the 14.09 mg/kg body weight of the residual fraction.
3. Both sub-fractions A and B exhibited substantial antidiarrhoeal activities while the sub-fraction C did not exhibit any substantive antidiarrhoeal activity.
4. The sub-fractions A exerted the most profound antimotility effect whereas the sub-fraction B exhibited antisecretory, anti-inflammatory and modulation of intestinal barrier function.
5. Mangiferin and catechin were detected in the sub-fraction B; the sub-fraction with the most antidiarrhoeal activity.
6. Sub-fraction B exhibited functional and structural toxicities on the kidney and small intestine at 12.94 mg/kg body weight.
7. The pharmacological dose of 6.47 mg/kg for antidiarrhoeal activity was found to be relatively safe and can therefore serve as a relatively cheaper and safer lead for drug development.

6.1 Conclusion

The study concluded that the sub-fraction B of the residual fraction exhibited the antidiarrhoea activity reported in the aqueous extract of *M. indica* leaves. The mechanisms of action of the sub-fraction B on chemical induced diarrhoea include restoration of intestinal secretory and absorptive function, inhibition of inflammation and stimulation of intestinal barrier function. The antidiarrhoeal activity of the sub-fraction B can be attributed to the presence of mangiferin and catechin thus, could be explored as a potential therapeutic agent in the treatment of diarrhoea.

6.2 Recommendations

Due to the limitations and findings from this study, a further study is required to determine the antidiarrhoeal activity of mangiferin and catechin singly and in combination.

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APPENDIX

Preparation of Reagents

0.25M Sucrose solution

85.575g sucrose was dissolved in small quantity water and made up to 1 litre with distilled water.

Biuret reagent

1.5 g of copper sulphate ($CuS0_4.5H_20$) and 6.0 g of sodium potassium tartarate ($NaK.C_4H_4O_6.4H_2O$) were dissolved in distilled water to make 500 ml solution (solution A). 3 g of sodium hydroxide (NaOH) was dissolved in distilled water to make 300 ml (Solution B). Solution B was then added slowly to solution A with continuous stirring and the resulting solution transferred to a one-litre standard flask and the volume made up to the marked level with distilled water. The solution was stored in a polythene bottle.

2,4-Dintrophenylhydrazine (1mM)

200 mg of 2,4-dinitrophenylhydrazine was dissolved in hot 1N HCl and allowed to cool. The solution was then made up to 1000 ml, using more 1N HCl, in a standard flask.

Alkaline phosphatase Assay Reagent Preparation

Carbonate Buffer (0.1M, pH 10.1)

0.84g of sodium bicarbonate ($NaHCO_3$) was dissolved in 100 ml of distilled water (0.1M NaHCO3) to give solution A. 1.06g of sodium bicarbonate (Na2CO3) was dissolved in distilled water to made up to 100 ml (0.1M Na2CO3) to give solution B. Solution A was added to Solution B with continuous stirring until pH of the mixture comes to 10.1

Para-nitro Phenyl phosphate (pNPP, 10mM)

0.37114g of para-nitro phenyl orthophosphate was dissolved in distilled water and made up to 100 ml in a 100 ml standard volumetric flask.

Sodium hydroxide (1N)

40 g of sodium hydroxide pellet was dissolved in small quantity of distilled water and made up to 1 litre in a one-litre standard volumetric flask.

Magnesium sulphate (0.1M)

2.464 g of magnesium sulphate (MgSO4.7H2O) was dissolved in distilled water to make 100 ml solution.

Reagent Preparation for assay of ATPase activities

200ml NaCl/400mM KCl/60mM Tris buffer (pH 7.4)

2.922g of NaCl, 0.745g of KCl and 1.8165 of Tris were dissolved in 200ml of distilled water in a beaker. The pH was adjusted to 7.4 using HCl and the solution was made up to 250 ml with distilled water in a standard volumetric flask.

240mM KCl/60mM Tris buffer (pH 7.4)

4.473g of KCl and 1.8165g of Tris were dissolved in 200 ml of distilled water in a beaker. The pH was adjusted to 7.4 using HCl and the solution was made up to 250 ml with distilled water in a standard volumetric flask.

0.04M Vanadate

0.07356g of orthovanadate salt was dissolved in 10 ml of distilled water.

8mM ATP solution

0.22g of ATP was dissolved in distilled water and the solution was made up to 50 ml.

20mM EGTA solution

0.3804g of EGTA was dissolved in distilled water and made up to 50 ml.

80mM MgCl2.6H2O solution

0.8132g of MgCl2.6H2O was dissolved in distilled water and made up to 50 ml.

5% Sodium dodecyl sulphate (SDS) solution

5g of SDS was dissolved in distilled water and made up to 100 ml.

Ammonium molybdate / sulphuric acid solution (Solution A)

4g of ammonium molybdate was dissolved in 18 ml of conc. H2SO4, and thoroughly stirred. The solution was then poured into 400 ml of distilled water in a measuring cylinder and then made up to 620 ml by adding more distilled water.

9% Ascorbic acid solution (Reagent B)

9g of ascorbic acid was dissolved in distilled water and made up to 100 ml.

Reagent C

This was prepared for immediate usage by adding reagent A and B in ratio 4:1 (v/v).

Reagent for Catalase

0.2M 30% hydrogen peroxide

0.3mL of 30% hydrogen peroxide was added to a little quantity of distilled water and made up to 100mL in 100mL of standard volumetric flask.

5% Dichromate

5g of K2CrO7 was dissolved in 50mL of distilled water and made up to 100mL with water in a 100mL volumetric flask.

Dichromate/glacial acetic acid (1:3)

100mL of $K_2Cr_2O_7$ was mixed with 300mL of glacial acetic acid.

Phosphate buffer (0.01M, pH 7.8)

Solution 1: 1.7907g of disodium hydrogen phosphate (Na_2HPO_4) was dissolved in a little quantity of distilled water and made up to 500mL with distilled water in a 500mL volumetric flask. Solution 2: 0.78005g of sodium dihydrogen phosphate (Na_2HPO_4) was dissolved in a little quantity of distilled water and made up to 500mL with distilled water in a 500mL volumetric flask. Solutions 1 and 2 were added together with continuous stirring until a pH of 7.8 was obtained.

Reagent for Superoxide dismutase

0.3mM Epinephrine

0.01374g was dissolved in a little quantity of distilled water and made up to 250mL in a 250mL volumetric flask

Carbonate buffer (0.05M, pH 10.2)

Solution 1: 1.05g of sodium hydrogen carbonate ($NaHCO_3$) was dissolved in a little quantity of distilled water and made up to 250mL with distilled water in a 250mL volumetric flask.

Solution 2: 3.2875g of disodium carbonate ($Na_2CO_3.10H_2O$) was dissolved in a little quantity of distilled water and made up to 250mL with distilled water in a 250mL volumetric flask.

Solution 1 and 2 were added together with continuous stirring until a pH of 10.2 was obtained.

Reagent for reduced glutathione

25% trichloroacetic acid (TCA)

25g of TCA was dissolved in distilled water and made up to 100mL in a 100mL volumetric flask

5, 5‟-Dithiobis- (-2-Nitro-benzoic Acid (DTNB)

0.11892 g of DTNA was dissolved in distilled water and made up to 500mL in a volumetric flask.

0.2M phosphate buffer (pH 8.0)

Solution 1: 35.814g of disodium hydrogen phosphate (Na_2HPO_4) was dissolved in a little quantity of distilled water and made up to 500mL with distilled water in a volumetric flask.

Solution 2: 15.601g of sodium dihydrogen phosphate (Na_2HPO_4) was dissolved in a little quantity of distilled water and made up to 500mL with distilled water in a volumetric flask.

Solution 1 and 2 were added together with continuous stirring until a pH of 8 was obtained.

Reagent for Malondialdehyde

0.7% Thiobarbituric acid (TBA)

0.7g of TBA was dissolved in a little quantity of distilled water and made up to 100mL with distilled water in a 100mL volumetric flask.

20% Trichloroacetic acid (TCA)

20g of TCA was dissolved in a little quantity of distilled water and made up to 100mL with distilled water in a 100mL volumetric flask.

Reagent for Histopathology

10% buffered formalin

8.5g of sodium chloride was dissolved in distilled water to make 500 ml solution. 100 ml of formalin was slowly added with continuous stirring and made up to 1 litre with distilled water.

Composition of the reagents prepared in assay kits

Reagents for aspartate transaminase (AST)

0.4 N Sodium Hydroxide

16 g of sodium hydroxide was dissolved with small quantity of distilled water in a beaker and made up to 1 litre in a one litre volumetric flask.

Reagent R1 composition

Tris Buffer (pH 7.8) 110 mmol/l

L-Aspartate 340 mmol/l

Lactate dehydrogenase ≥ 4000 U/l

Malate dehydrogenase ≥ 750 U/l

Reagent R2 composition

CAPSO 20 mmol/l

2-Oxoglutarate 85 mmol/l

NADH 1.05 mmol/l

Reagents for alanine transaminase (ALT)

0.4 N Sodium Hydroxide

16 g of sodium hydroxide was dissolved with small quantity of distilled water in a beaker and made up to 1 litre in a one litre volumetric flask.

Albumin

R1 – Bromocresol green concentrate and succinate buffer

Total and Conjugated Bilirubin

R1- 29 mmol/l of sulphanilic acid and 0.17 N hydrochloric acid

R2- 38.5 mmol/l of sodium nitrite

R3- 0.26 mol/l of caffeine and 0.52 mol of sodium benzoate

R4-0.93 mol/l of tartrate and 1.9 N sodium hydroxide

Creatinine

R1a – Picric acid

R1b – Sodium hydroxide

Urea

R1 – Sodium nitropusside (37 mL) and Urease (1 mL)

R2 – Phenol

R3 – Sodium hypochlorite

CALIBRATION CURVES

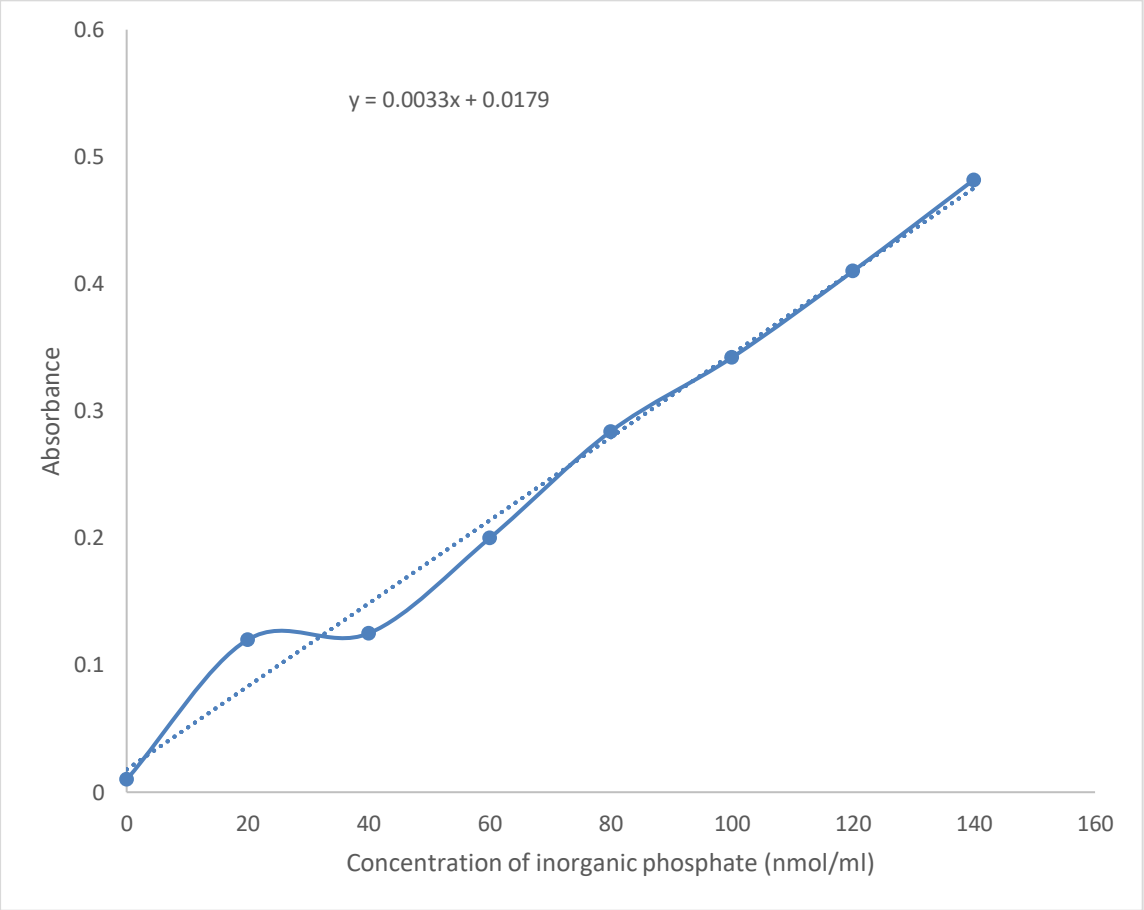


Figure 18: Calibration curve for inorganic phosphate

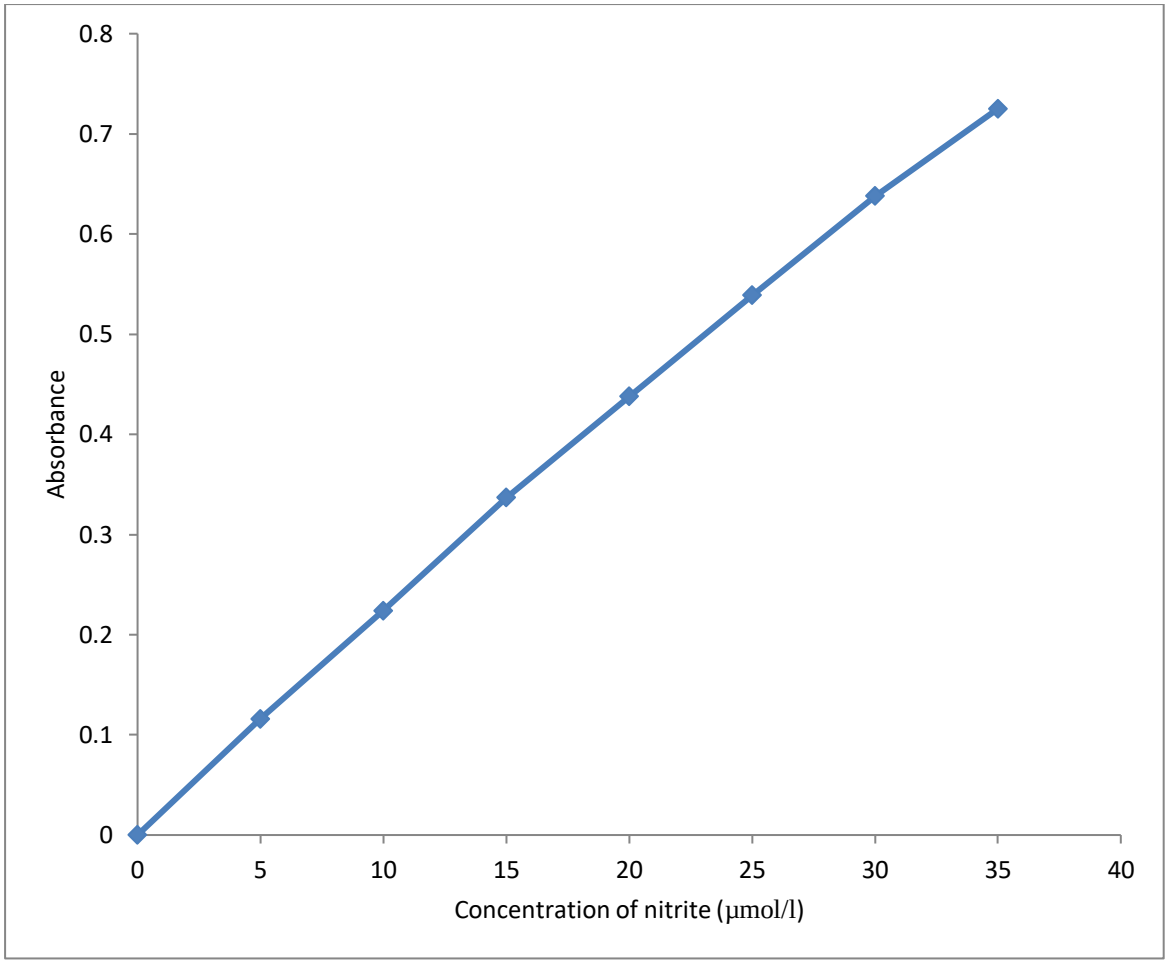


Figure 19: Calibration curve for nitrite

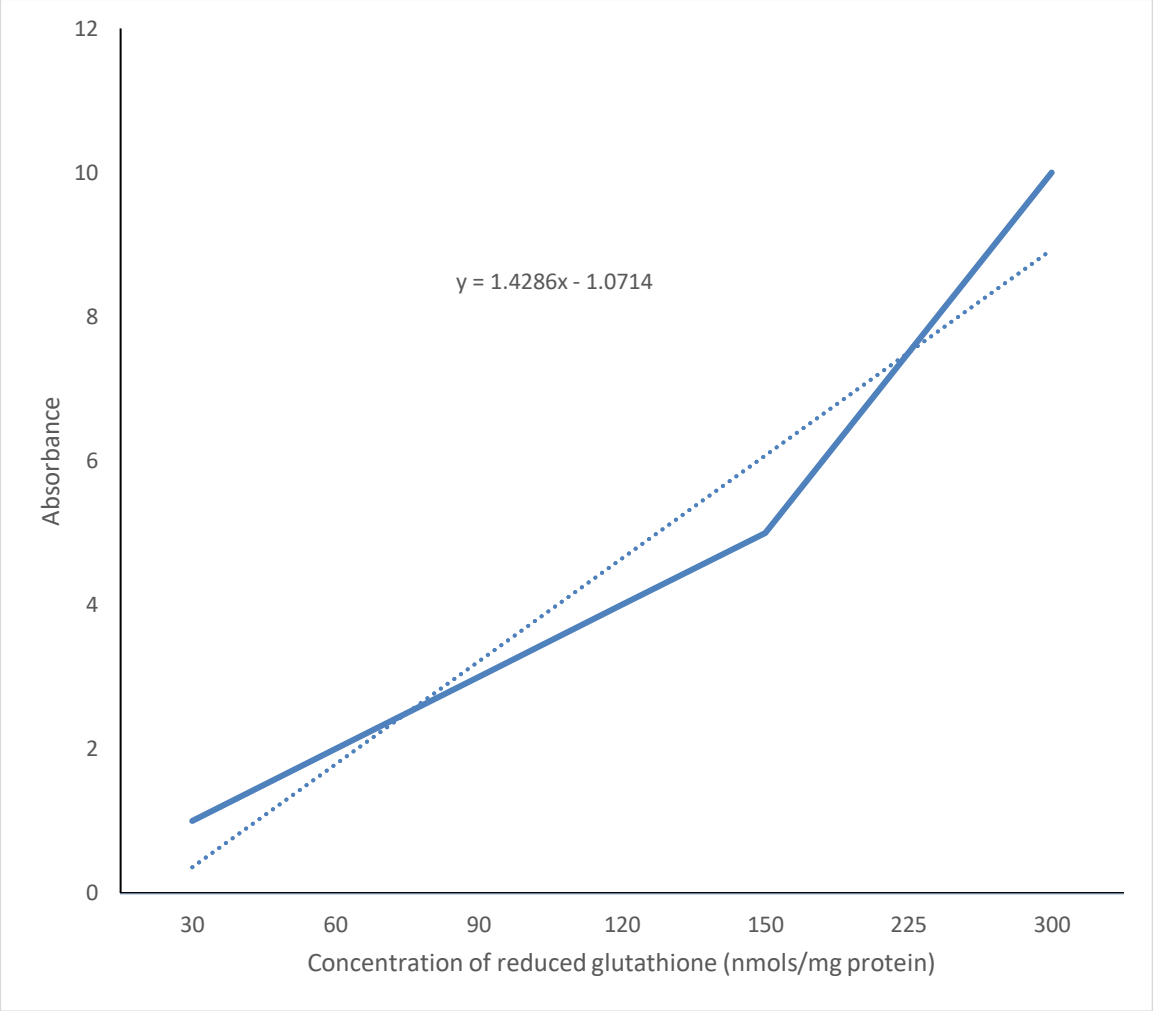


Figure 20: Calibration curve for reduced glutathione

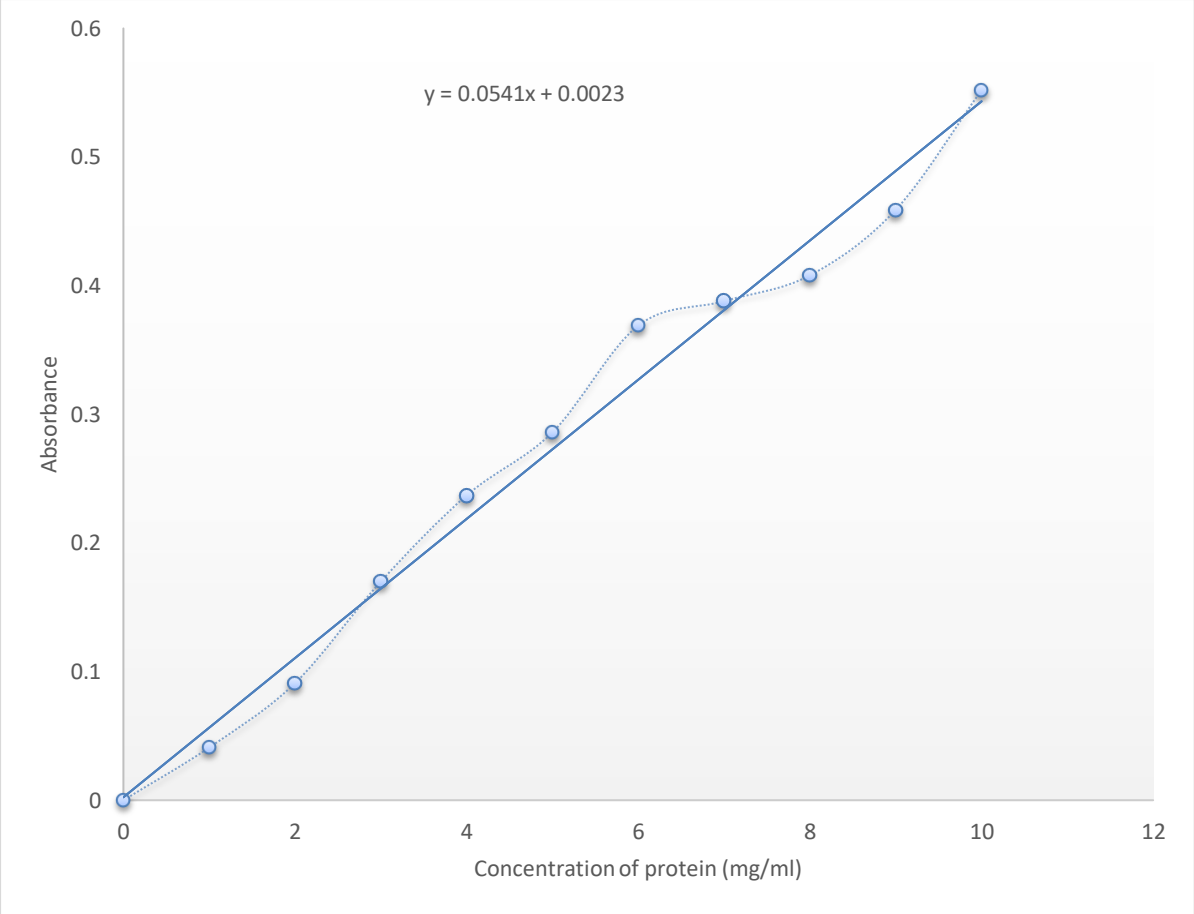


Figure 21: Calibration curve for total protein

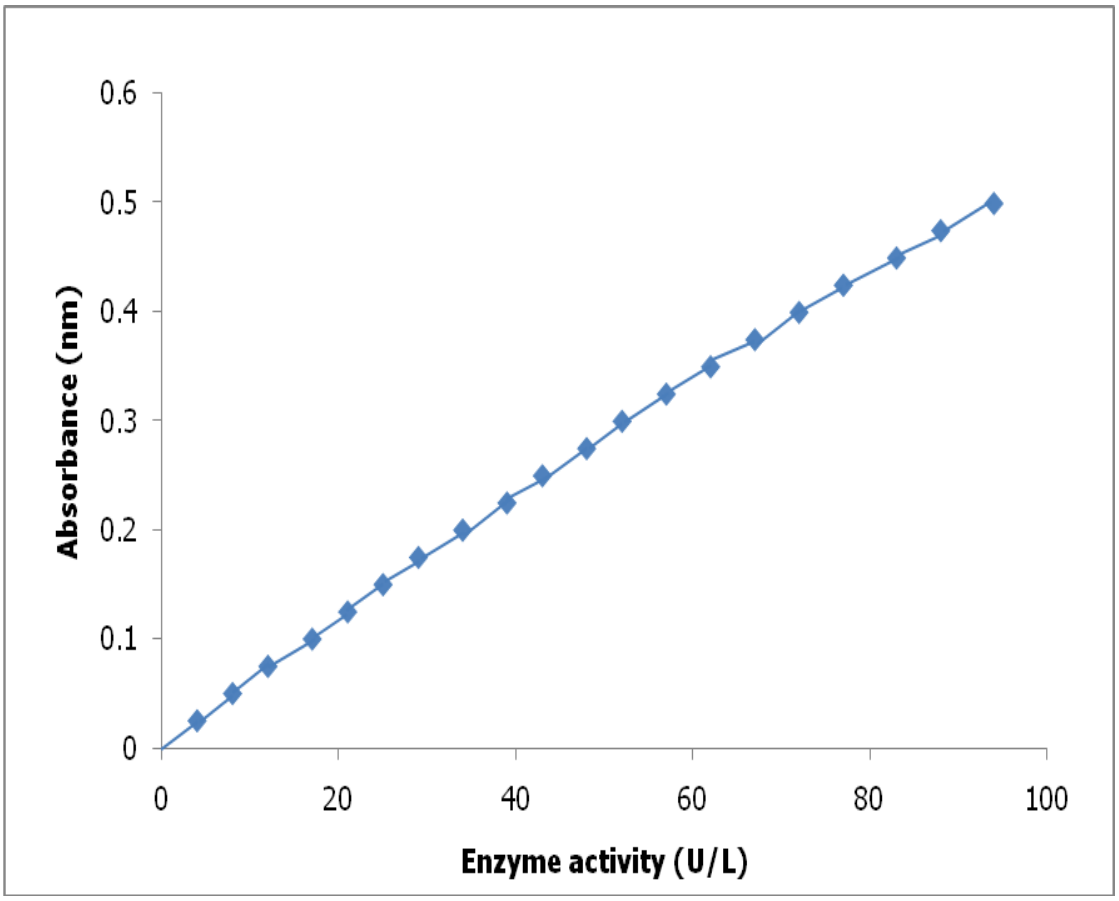


Figure 22: Calibration curve for alanine aminotransferase

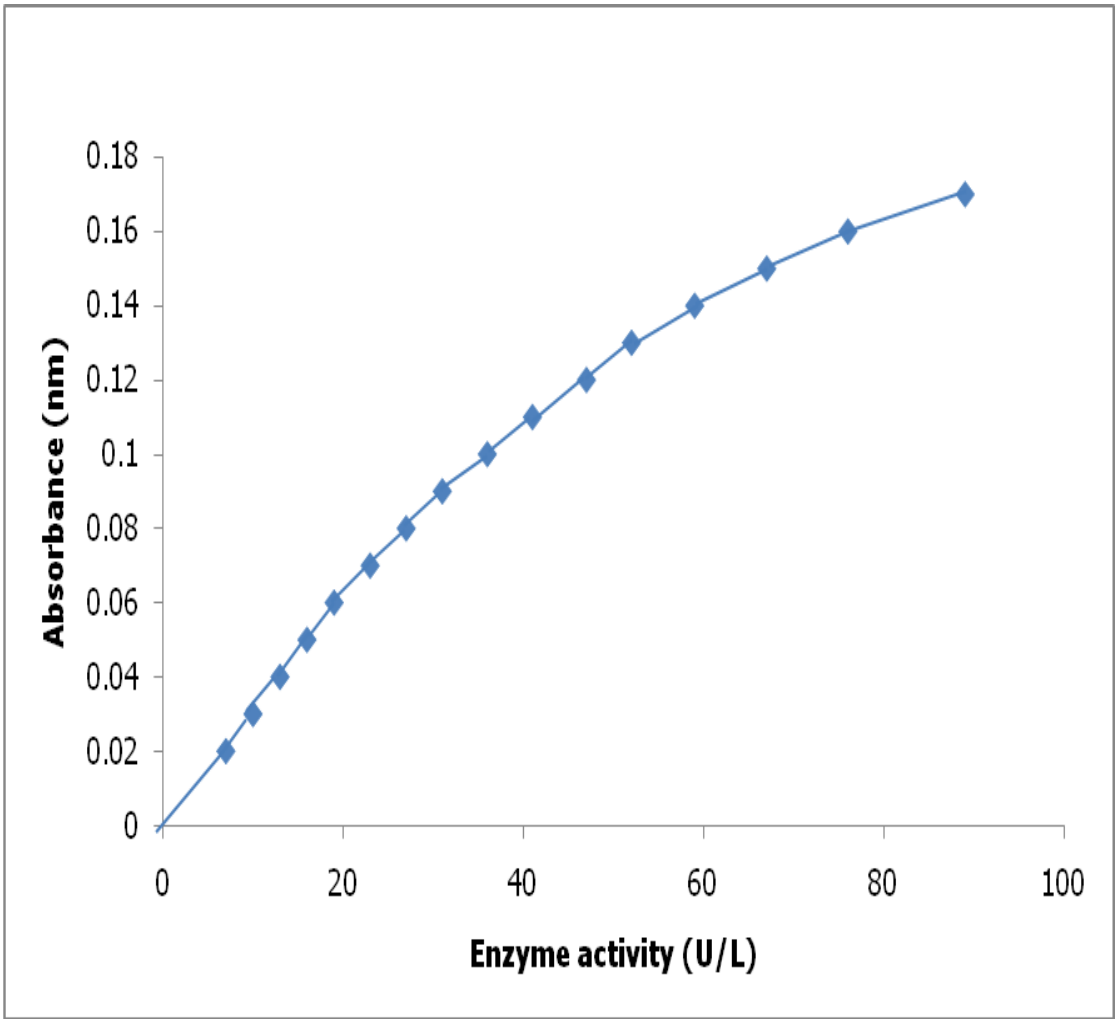


Figure 23: Calibration curve for aspartate aminotransferase