ANTIDIARRHEAL ACTIVITY OF CASHEW TREE BARK AND LEAF EXTRACTS FORTIFIED WITH ZINC IN RATS

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TITLE PAGE

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APPROVAL PAGE

This is to certify that the thesis titled ''Antidiarrheal Activity of Extracts of Cashew Tree Bark and Leaves Fortified with Zinc in Rats'' submitted by David Ebuka .E. for the award of Master of Science Degree in Applied Biochemistry Department is a complete record of the research work carried out by him under my supervision.

....	....
Dr. S.C Udedi	Dr. Igbokwe
Supervisor,	Ag. Head of Department,

DEDICATION

This work is dedicated to God of Light in whom there is no variation.

ACKNOWLEDGEMENT

I wish to express my gratitude to my supervisor, Dr S. C. Udedi who without his close supervision and criticism; this work wouldn't have been completed. Thank you, Dr Udedi for being a mentor.

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Finally, to my parents Mr and Mrs David Ngwu, I appreciate all the love and encouragements you have been showing to me during all my life. I thank God for coming out of your loins.

ABSTRACT

This study investigated the antidiarrheal activity of aqueous extracts of cashew bark and leaf fortified with zinc in rats. Diarrhea was induced in rats by the administration of 2 ml of castor oil with control group receiving normal saline. The aqueous extracts (100, 250, and 500 mg/kg) and zinc (15, 30 and 45 mg/kg) was administered orally to different groups of rats in order to evaluate their antidiarrheal effect. The diarrheal stool and concentration of colonal nitric oxide (NO), mg/100ml was determined. At oral doses of 15 mg/kg zinc, 500 mg/kg bark and 500 mg/kg leaf extracts, diarrheal stool was significantly ($p<0.01$) reduced by 44% (6.67± 1.53), 56% (5.33±2.08) and 42% (7.00±1.00) respectively against castor oil control group 100% (12.0 ±1.73). Also, there was a corresponding reduction in colonal nitric oxide concentration (1.29±0.07, 1.42±0.14 and 1.40±0.05)mg/100ml respectively for 15 mg/kg zinc, 500 mg/kg bark and 500mg/kg of leaf extracts compared to the castor oil control group (1.62±1.73)mg/100ml. Though, 500 mg/kg of both cashew extracts showed a significant ($p<0.01$) decrease in diarrheal stool with values of (5.33±2.08) 56% and (7.00 ±1.00) 42% for cashew bark and leaf extracts respectively, the zinc fortified 500 mg/kg aqueous extracts of bark 70% (3.67±1.53) and leaf extracts 64% (4.33±1.53) showed a better significant ($p<0.01$) reduction in diarrheal stool output. These results suggested that the extracts of bark

and leaf fortified with zinc showed a higher antidiarrheal activity than un-fortified extracts. Furthermore, the effect of zinc-fortified loperamide(a standard antidiarrheal drug) with a value of 75% (3.00±0.70) showed a better antidiarrheal effect than non fortified loperamide with a value of 66%(4.00±2.65). This was possibly as a result of the ability of the extracts to stimulate the re-absorption of water from the intestinal lumen. Also, the reduction in colonal nitric oxide concentration suggests that zinc or the extracts might have an inhibitory effect on the activity of nitric oxide synthase enzyme. The LD_{50} was evaluated. Animals treated with 2900 and 5000mg/kg died within 48 and 24 hours after treatment, respectively. Also, serum ALT and AST levels in extract-treated rats at 1.44 and 2.87g/kg doses were significantly different from those of the control and may indicate hepatotoxicity. But the serum levels of ALP, TB and TP in treated animals were statistically same with those of the control.

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LIST OF ABBREVIATIONS

NO Nitric oxide

NOS Nitric oxide synthase

CNSL Cashew nut shell liquid

ORS Oral rehydration solution

MT Metallothionein

ETEC Enterogenic *E.coli*

EPEC Enteropathogenic *E.coli*

ADP Adenosine disphosphate

ATP Adenosine trisphosphate

cAMP Cyclic adenosine monophosphate

cGMP Cyclic guanosine monophosphate

COX Cyclooxygenase

LPS Lipopolysaccharide

ALT Alanine aminotransferase

ALP Alkaline phosphatase

TB Total Bilirubin

TP Total protein

iNOS Inducible nitric oxide synthase

eNOS Endothelial nitric oxide synthase

nNOS Neuronal nitric oxide synthase

CHAPTER ONE

INTRODUCTION

Diarrhea is the passing of large amount of loose stool, more than 300g in 24 hours. It occurs when there is an irritation of the mucous membrane of the small intestine resulting in an imbalance between secretion and absorption of water. The irritated gut becomes very active, contracting excessively and irregularly. Diarrhea is often classified as osmotic or secretory. It is considered osmotic if luminal substances are responsible for the induction of the fluid secretion, but secretory if endogenous substances, often referred to as secretagogues, induce fluid secretion that persists even when the patient is fasting, (Kazi and Henry, 2006). Central to all diarrheal disorder is the induction of fluid and electrolyte secretion in one or more segments of the small intestine, the large intestine or both. In secretory diarrhea, secretagogue,nitric oxide,affect ion transport in the intestine both by stimulating chloride secretion and by inhibiting sodium and chloride absorption. Secretory diarrhea occurs when there is a net secretion of water into the intestinal lumen regulated upon the effect of the secondary messenger: cAMP, cGMP and nitric oxide (Kazi and Henry, 2006).

Castor oil produces diarrheal effects due to its most active component, ricinoleic acid through a hypersecretory response. They inhibit intestinal Na^+/K^+ ATPase activity, activate adenylate cyclase, and stimulate

prostaglandin and nitric oxide, (Francesco *et a*l, 1994). These bring about changes in electrolyte and water transport and increases peristaltic activity. Ricinoleic acid, a monosaturated 18-carbon fatty acid, is unusual in that it has a hydroxyl functional group on the twelfth carbon. This functional group causes ricinoleic acid (and castor oil) to be unusually polar and allows chemical derivatization that is not obtained with most other seed oil.

Nitric oxide, an endogenous signaling molecule and one of the products of L-arginine catalysed by nitric oxide synthase enzyme has been found to act as a mediator of pathological conditions of diarrhea. Nitric oxide has been considered as a regulator of basal intestinal water transport, as a mediator of pathological conditions where disturbance in water transport plays a role and as an effector substance in both laxatives and antidiarrheal agents, (Mourad *et al*, 1999). Its concentration acts as a secondary messenger which triggers and activated guanylate cyclase. The smooth muscle relaxation induced by nitric oxide results to an: increased cAMP and cGMP which inhibits calcium entry into the cell and decreases intracellular calcium concentration: activation of potassium ion channels which leads to hyperpolarisation and relaxation: stimulation of cGMP-dependent protein kinase that activates myosin light chains which leads to smooth muscle relaxation (Mourad *et al,* 1999). These homeostatic mechanisms can be exploited by pharmacological agents to promote ion absorption and reduce ion secretion.

Zinc is an essential micronutrient and the second most abundant trace element in cells and tissues. Zinc has been considered an important anti-inflammatory factor, protecting cell membrane from oxidative damage and there has been claim of its use in the treatment of diarrhea (Li cui, *et al* 1999). X-ray crystallography for all the isoforms of nitric oxide synthase show a zinc thiolate cluster (ZnS_4) formed by a zinc ion coordinated in a tetrahedral conformation with pairs of symmetrically oriented and phylogenetically conserved cysteine residue at the dimer interface. The stabilization of the dimer interface of zinc thiolate is the key to its catalytic activity (Mourad *et al*, 1999). Although zinc is redox active in aqueous solution, it maintains partial cationic character even in tetracordinated complexes such as zinc thiolate clusters.

RATIONALE OF STUDY:

To find an alternative, readily available and more effective antidiarrheal agent, since loperamide, the standard antidiarrheal drug is both addictive and less effective.

To evaluate the antidiarrheal effect of zinc and zinc enriched extracts since it has been reported by public health practitioners as having a therapeutic effect.

AIMS AND OBJECTIVES

To investigate antidiarrheal activity of aqueous extracts of cashew tree barks and leaves as reported by traditional practitioners

To understand the effect of the extracts on endogenous mediators of diarrhea, nitric oxide and indirectly its enzyme, nitric oxide synthase (NOS)

To compare the antidiarrheal activity of cashew tree bark extracts and cashew tree leaves extracts.

CHAPTER TWO

LITERATURE REVIEW

2.1 CASHEW PLANT (*Anarcadium occidentale*)

The cashew plant, *Anacardium occidentale* is native of Central and South America its main center of variation in Eastern Brazil. The name cashew is derived from ACAJU, a portuege word. It is a member of genius Anacardium of the family Anacardiaceae. It has about 400 species made up of shrubs and tree which are now grown in many parts of the world, where its growth and production is not limited by cold. Over 50% annual cashew productions of 400,000 tones come from South Asia and East Africa especially India and Tanzanians while West Africa produces small quantities of cashew (Opeke; 2000). Cashew is a multipurpose tree of the Amazon that grows up to 15 m high (fig 2.1). It has a thick and tortuous trunk with branches so winding that they frequently reach the ground. Cashew trees are often found growing wild on the drier sandy soils in the central plains of Brazil and are cultivated in many parts of the Amazon rainforest (Bicalho *et al*, 2001).

The cashew tree produces many resources and products. The bark and leaves of the tree are used medicinally, and the cashew nut has international appeal and market value as food. Even the shell oil around the nut is used medicinally and has industrial applications in the plastics and

resin industries for its phenolic content (Mota *et al*, 1985). Then, there is the pseudo-fruit-a swollen peduncle that grows behind the real fruit that yields the cashew nut. The pseudo-fruit, a large pulpy and juicy part, have a fine sweet flavor and are commonly referred to as the "cashew fruit" or the "cashew apple." (Almajali *et al*, 2000) Fresh or frozen cashew fruit concentrate is as common a juice product in South American food stores as orange juice is in the United States. It is very perishable, and therefore, no fresh cashew fruit is exported into the United States or Europe from South America (Laurens *et al*, 1987).

Figure 2.1: Cashew Tree

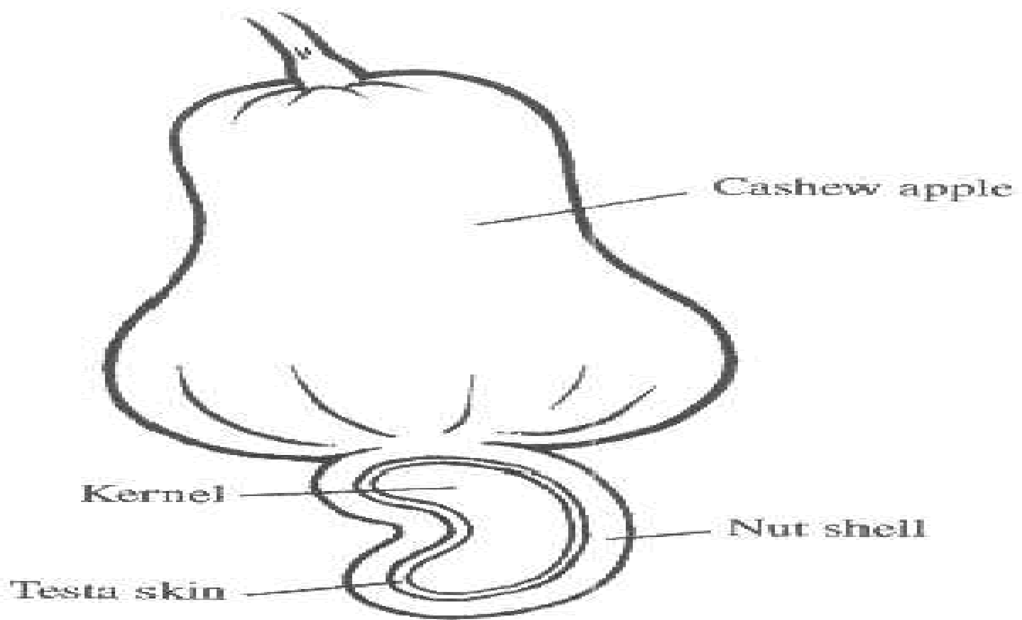


Figure 2.2: Cashew fruit

2.1.1 CASHEW NUT

The cashew nut is defined botanically as the fruit. It grows externally in its own kidney-shaped hard shell at the end of this pseudo-fruit, or peduncle. The nut kernel inside is covered with an inner shell, and between the two shells is a thick, caustic, and toxic oil called cardol. Cashew nuts must be cleaned to remove the cardol and then roasted or boiled to remove the toxins before they can be eaten (Allan G and Andrew H , 1941).

The cashew kernel is a rich source of fat (46 percent) and protein (18 percent) and is a good source of calcium, phosphorus and iron (Bilcalho *et al*, 2001). It has a high percentage of polyunsaturated fatty acids, in particular, the essential fatty acid linoleic acid (Bilcalho *et al*, 2001). The tart apple is a source of vitamin C, calcium and iron (Franca, 1993). The bark, leaves, gum and shell are all used in medicinal applications. The leaves and bark are commonly used to relieve toothache and sore gums, and the boiled water extract of the leaf or bark is used as mouths wash (Shaik *et al*, 2009). A paste of barks ground in water is used in topical applications for the cure of ringworm; in this form it can however act as an irritant and should not be applied to sensitive skin or to children (Bush, 2006). The root has been used as a purgative (Bush, 2006). Fibres from the leaves can be used to strengthen fishing lines and nets, and as folk remedies for calcium deficiency and intestinal colic, as well as a vitamin supplement (Akinpelu *et*

al, 2001). The water-resistant wood is used for boats and ferries, while the resin, in addition to having industrial uses, is used as an expectorant, cough remedy and insect repellent (Alfredo *et al,* 2009).

The cashew nut kernel is constituted of three different portions - the shell, the kernel and the adhering testa (Figure 2). The primary product of cashew nuts is the kernel, which is the edible portion of the nut and is consumed in roasting and in confectionery (Timothy *et al* 1994)

2.1.2 CASHEW NUT SHELL LIQUID (CNSL)

The cashew nut shell contains a viscous and dark liquid, known as cashew nut shell liquid (CNSL), which is extremely caustic. It is contained in the thin honeycomb structure between the soft outer skin of the nut and the harder inner shell. The CSNL content of the raw nut varies between 20 and 25 percent (Hergaty *et al,* 1981).

Cashew nut shell liquid (CNSL) is an important and versatile industrial raw material there are more than 200 patents for its industrial applications. It is particularly used as a raw material for phenolic resins and friction powder for the automotive industry (brake linings and clutch disks). In drum-brake lining compounds, cashew resins are used as fillers, and may also be used as binders (Kudi, 1999). In disc pads, the role of cashew resin is restricted to the use of friction dust as filler. The advantage of the cashew resins

compared with synthetic phenolic resins is that they are more economical and produce a softer material, which gives a quieter braking action. CNSL is also used in moldings, acid-resistant paints, foundry resins, varnishes, enamels and black lacquers for decorating vases, and as insecticides and fungicides (Kudi, 1999). In tropical medicine, CNSL has been used in treating leprosy, elephantiasis, psoriasis, ringworm, warts and corns (Zang *et al*, 1996). After extracting the CNSL, the cashew nut shells can be burned to provide heat for the decorticating operation or can be used in the manufacture of agglomerates. Together with the testa, it may be used either in the manufacture of dyestuff or to provide durability to hammocks and fishing lines (Bob *et al*, 1954).

2.2 USES OF THE CASHEW TREE HERBAL MEDICINE

Native to the Northeast coast of Brazil, cashew was domesticated long before the arrival of Europeans at the end of the fifteenth century. It was "discovered" by European traders and explorers and first recorded in 1578. It was taken from Brazil to India and East Africa, where it soon became naturalized. In sixteenth-century Brazil, cashew fruits and their juice were taken by Europeans to treat fever, sweeten breath, and "conserve the stomach." (Nimral *et al,* 1996)

The cashew tree and its nuts and fruit have been used for centuries by the indigenous tribes of the rainforest, and it is a common cultivated plant in

their gardens. The Tikuna tribe in northwest Amazonia considers the fruit juice medicinal against influenza, and they brew a tea of leaves and bark to treat diarrhea. The Wayãpi tribe in Guyana uses a bark tea as a diarrhea remedy and colic remedy for infants. Tribes in Suriname use the toxic seed oil as an external worm medicine to kill botfly larvae under the skin (Nimral *et al;* 1996). In Brazil, a bark tea is used as a douche for vaginal discharge and as an astringent to stop bleeding after a tooth extraction. A wine made from the fruit is used for dysentery in other parts of the Amazon rainforest. The fruit juice and a bark tea are very common diarrhea remedies throughout the Amazon today, used by *curanderos* and local people alike (Kudi *et al;* 1999).

In Peruvian herbal medicine today, cashew leaf tea (called *casho*) is employed as a common diarrhea remedy; a bark tea is used as an antiseptic vaginal douche; and the seeds are used for skin infections. In Brazilian herbal medicine, the fruit is taken for syphilis and as a diuretic, stimulant and aphrodisiac (Bush; 2006). A leaf tea is prepared as a mouthwash and gargles for mouth ulcers, tonsillitis, and throat problems and is used for washing wounds (Kubo *et al;* 1999). An infusion and/or maceration of the bark are used to treat diabetes, weakness, muscular debility, urinary disorders, and asthma. The leaves and/or the bark is also used in Brazil for eczema, psoriasis, scrofula, dyspepsia, genital problems, and venereal diseases, as well as for impotence, bronchitis, cough, intestinal colic,

leishmaniasis, and syphilis-related skin disorders. North American practitioners use cashew for diabetes, coughs, bronchitis, tonsillitis, intestinal colic, and diarrhea, and as a general tonic (Franca *et al;* 1996).

2.3 PLANT PHYTOCHEMICAL CONTENT

In addition to being delicious, cashew fruit is a rich source of vitamins, minerals, and other essential nutrients. It has up to five times more vitamin C than oranges and contains a high amount of mineral salts. Volatile compounds present in the fruit include esters, terpenes, and carboxylic acids (Swanston *et al;* 1989). The bark and leaves of cashew are a rich source of tannins, a group of plant chemicals with documented biological activity. These tannins, in a 1985 rat study, demonstrated anti-inflammatory and astringent effects, which may be why cashew is effective in treating diarrhea (Swanston *et al;* 1989). Anacardic acids are found in cashew, with their highest concentration is in the nutshells. Several clinical studies have shown that these chemicals curb the darkening effect of aging by inhibiting tyrosinase activity, and that they are toxic to certain cancer cells (Helier *et al;* 1973).

The main chemicals found in cashew nut are alanine, alpha-catechin, alpha-linolenic acid, anacardic acids, anacardol, antimony, arabinose, caprylic acid, cardanol, cardol, europium, folacin, gadoleic acid, gallic acid, gingkol, glucuronic acid, glutamic acid, hafnium, hexanal, histidine, hydroxybenzoic

acid, isoleucine, kaempferols, L-epicatechin, lauric acid, leucine, leucocyanidin, leucopelargonidine, limonene, linoleic acid, methylglucuronic acid, myristic acid, naringenin, oleic acid, oxalic acid, palmitic acid, palmitoleic acid, phenylalanine, phytosterols, proline, quercetin-glycoside, salicylic acid, samarium, scandium, serine, squalene, stearic acid, tannin, and trans-hex-2-enal tryptophan (De souza *et al;* 1994).

2.4 BIOLOGICAL ACTIVITIES AND CLINICAL RESEARCH

Cashew antimicrobial properties were first documented in a 1982 *in vitro* study. In 1999, another study was published indicating it had good in vitro antibacterial activity against *E. coli* and *Pseudomonas (Mourad; 1999)*. Most recently, a 2001 study reported that a bark extract exhibited in vitro antimicrobial activity against 13 of 15 microorganisms tested (Bilcalho *et al;* 2001). In 1999, researchers reported that cashew fruit exhibited antibacterial activity against the Gram-negative bacterium *Helicobacter pylori*, which is now considered to cause acute gastritis and stomach ulcers (Mourad *et al*, 1999). Its effectiveness against leishmanial ulcers also was documented in two clinical studies. Finally, two studies (one in mice and the other in rats) in 1989 and 1998 document the protective quality of a leaf extract against lab-induced diabetes, although the extract did not act as hypoglycemic as some others, it did stabilize blood glucose levels near pretest levels (Jurberg *et al*, 1995).

2.5 CURRENT PRACTICAL USES

The different products produced from this tree offer a wide range of applications. The fruit is used to make highly nutritive snacks and juices, and fruit extracts are now being used in body-care products (Bilcalho; 2001). Because of its high amount of vitamin C and mineral salts, cashew fruit is used as a catalyst in the treatment of premature aging of the skin and to remineralize the skin (Bilcalho; 2001). It is also an effective scalp conditioner and tonic and is often used in shampoos, lotions, and scalp creams for the conditioning activity of its proteins and mucilage. Cashew leaf or bark tea is still widely used throughout the tropics as an effective diarrhea and colic remedy, considered gentle enough for children (Franca;1993). Unfortunately, there are not many cashew products available in the U.S. market, besides of course, cashew nuts (Franca *et al*, 1993).

2.6 PHYTOCHEMICAL SCREENING OF LEAVES AND STEM OF CASHEW TREE

The values of bioactive substances present in Anacardium occidentale such as tannins, carbohydrates, glycosides, resins, sterols, phlobatanins, flavonoids and alkaloids were closely related to those earlier reported by Abulude (2007) on woody plants such as *Mangifera indica*, *Techtona garandis* and *Cola nitida*, (Hassan *et al;* 2004, Ogukwe *et al;* 2004 and

Enomfon *et al;* 2004). Recently, Abulude *et al;* (2010) reported the phytochemical constituents of an aqueous and ethanolic extract of cashew.

Table 2.1: Phytochemical constituents of aqueous and ethanolic extracts of cashew leaf and stem (*Anacardium occidentale).*

	Water		Ethanol	
	Leaf	Stem	Leaf	Stem
Carbohydrate	+++	+++	-	++
Tannins	+++	++	-	++
	++	++		++
Glycosides	++		++	
Resins	++	+++	+++	-
Phlobatannins	–	-	-	-
Flavanoides	++	++	++	++
Alkaloids	++	++	++	++
Sterols	+	-	-	-

+++ - High concentration, ++ - Moderate concentration, + - Low concentration, - _ Absence

Source: Abulude F.O (2007). Electronic Journal of Environment, Agriculture and Chemistry

It has been reported that the presence of bioactive substances in plants play a role in preventing colorectal carcinoma, hyperchloestrolcamia and renal calculi (Rao *et al.*, 1994). It is documented that the presence of saponins can control human cardiovascular disease and reduces cholesterol; also tannins may provide protection against microbiological degradation of dietary proteins in the semen (Rao *et al*, 1994).

Generally, woody plants are versatile plant materials having a wide range of local therapeutic applications, the leaves, roots, barks and seeds are found to be antipyretic, laxative, analgesic, antifungal, antibacterial and non inflammatory (Zhang *et al*, 1996).

2.7 DIARRHEA

Diarrhea can be classified in several ways. It is both a symptom and a sign. As a symptom, it is whatever the patient says it is: a decrease in consistency, an increase in the number or volume of bowel movements, or any combination thereof. As a sign, diarrhea is an increase in stool weight or volume of more than 300 g or ml per 24 hrs in a person on a western diet. The distinction between chronic and acute diarrhea is arbitrary: it is determined by duration. Diarrhea is generally considered acute when it lasts less than two or three weeks. Such diarrhea is frequently caused by an infectious agent, usually self-limiting and often resolves without treatment (Henry, 2006).

2.8 CLASSIFICATION OF DIARRHEA

Diarrhea is often classified as osmotic or secretory. It is considered osmotic if luminal substances are responsible for the induction of the fluid secretion and it is considered secretory if endogenous substances, often refered to as secretagogues, induce fluid secretion that persists even when the patient is

fasting. Osmotic diarrhea can be distinguished from secretory diarrhea by determining the electrolyte concentration in the stool. If there is an osmotic gap-that is, a substantial difference between the stool osmolality and twice the concentration of sodium and potassium in the stool, the diarrhea is osmotic and if not, it is secretory (Rao; 1994). It is more helpful to access the effect of a fast on stool output: when diarrhea ceases with fasting, a dietary nutrient is likely to be the cause. If diarrhea persists unabatedly with fasting, a dietary nutrient is not likely to be the cause. In the former case, malabsorption of carbohydrates, fats or both is probably implicated. The most common cause of carbohydrate malabsorption is lactose intolerance, secondary to primary lactase deficiency or lactase non-persistence (Henry, 2006).

2.9 INDUCTION OF FLUID IN DIARRHEA

Central to all diarrhea disorders is the induction of fluid and electrolyte secretion in one or more segments of the small intestine, the large intestine or both. In secretory diarrhea, secretagogues affect ion transport in the intestine both by stimulating chloride secretion through the activation of the cystic fibrosis transmembrane regulator and by inhibiting sodium and chloride absorption (Read and Low-Beer, 1971).

Intestinal ion transport is a massive phenomenon that includes transepithelial fluxes of >20 Liters of fluid per day. Ion transport is the

direct target of enteric pathogenic agents that cause secretory diarrhea (Timothy *et al*; 1994). The major driving force of ion transport is active NaCl absorption. The colonic epithelium has both absorptive and secretory functions. Ion transport is characterized by a net absorption of NaCl, associated with water, leading to extrusion of small volume of stool. The colonocyte can balance both absorption and secretion and it is able to switch from absorption to secretion when stimulated by secretagogues. The maximal absorptive capacity of the colon can reach 4.5 L/day so that diarrhea (I e, increased water loss) will not occur unless the ileocecal flow rate exceeds the absorptive capacity and/or the colonic mucosa itself is secreting (Read and Low-Beer; 1971)

2.10 ZINC

Zinc is one of the essential trace elements in humans and animals. Its deficiency may occur in various disorders. Zinc deficiency often develops with gastrointestinal manifestations including diarrhea, abdominal pain, vomiting and fever (Shaik *et al*; 2009), indicating that the intestine is one of the tissues most sensitive to zinc deficiency. Zinc is one of several micronutrients that have recently received increasing attention because of their importance in maintaining the health and nutrition of humans. The history of the recognition of the essential role of zinc in biology is relatively brief. In 1986, Raulin demonstrated that zinc was essential in a biological system through studies of the growth of *Aspergillus niger.* Over 50 years

later in 1926, zinc was shown to be essential for higher plants. Shortly later in 1934, zinc was reported by Todd *et al* to be an essential nutrient for rats. By the 1950s, the importance of zinc for animal husbandry was clear: skin lesions (parekeratosis) in swine caused by zinc deficiency were reported in 1955 and the essential role of zinc for the growth of chickens was established in 1958. At this time zinc was also recognized as a necessary micronutrient for humans, but its ubiquity made it seem unlikely that it would be associated with any specific clinical problems. Human zinc deficiency was first described in the early 1960s, and since then it has been recognized as a common deficiency in human population (Kazi and Henry, 2006). Over the past decade there has been an explosion of knowledge and understanding of the biochemical function of zinc at subcellular levels. Zinc is present in the body almost as Zn^{2+} bound to many cellular proteins. Its high affinity for electrons allow interactions with amino acid side chains and the formation of cross-links within and between polypeptides that can modify tertiary protein structure and function (Li cui *et al;* 1999). Zinc is ideally suited to play a central role in intracellular metabolism that even minor impairment of zinc nutrition can potentially lead to multiple biological and clinical effects. Zinc plays a role in cellular growth and differentiation, and thus adverse effects of zinc deficiency are most prominent in tissues with cell turnover, especially the immune system. Recently, there has been a rapid expansion of the public health literature of

the beneficial effects of zinc supplementation in populations where zinc deficiency and infectious diseases are prevalent (Kazi and Henry; 2006).

2.13 ABSORPTION, TRANSPORT AND METABOLISM OF ZINC

The total zinc content of the human body is 23-38 mmol (1.5-2.5 g) and is slightly less than that of total body iron (Alpelian *et al;* 1981). Skeletal muscle accounts for the greatest proportion (60%) of total body zinc because, despite its lower concentration, it comprises the largest part of total body mass (Alpelian *et al;* 1981). Only a small proportion of total body zinc (0.5%) is found in circulating blood, of which 75% is found in erythrocytes and 10-20% in plasma. Thus >95% of body zinc content is intracellular (Kudi *et al;* 1999). Under controlled conditions, apparent absorption of zinc is 33%. Zinc is absorbed from the small intestine, primarily from the duodenum and jejunum and to a lesser extent the ileum, but there is no consensus as to the exact contribution of each anatomical region (Wendy *et al;* 2001). The absorption in various sections of the intestine is influenced by the extent of digestion of food, transit time, and specific factors that bind zinc (e.g, phytic acid). Body zinc homeostasis involves a balance between absorption of dietary zinc and endogenous secretion of zinc through adaptive regulation that is controlled by its dietary supply of. Adaptation of absorption to dietary zinc intake is controlled in the intestine (Brenden *et al;* 1995). There is general consensus that absorption involves two processes: a carrier mediated components

(saturable) and a non-mediated (non-saturable) diffusion component-both of which appear to be a function of luminal zinc concentrations (Tepperman *et al;* 1993). A major portion of zinc is absorbed by a carrier-mediated process that is most active at low luminal zinc concentrations (Vivien *et al;* 1994). In humans, the rate of zinc absorption from perfused jejunum is proportional to the luminal zinc concentration over the range 0.1-1.8 mmol/L and is saturable with luminal zinc concentration >1.8 mmol/L. Luminal zinc concentration after a meal in humans is estimated at 100 mmol/L. This suggests that with normal dietary in-takes, zinc is absorbed by the mediated mechanism (Samans *et al;* 1949). The transcellular movement of zinc is modulated by metallothionein (MT). The level of dietary zinc intake is directly related to the expression of the MT gene in the intestine (Jasinki *et al;* 1994). MT acts as an expandable zinc pool within enterocytes that can impede zinc movement out of these cells by acting as a transient intracellular buffer of Zn^{2+} .(Jasinki *et al;* 1994) As MT synthesis is elevated in response to increased dietary zinc, the absorption of zinc declines. The regulation of zinc absorption does not occur if synthesis of MT is inhibited (Jasinki *et al;* 1994). Vesicular transfer of zinc has also been proposed as an alternative mechanism for the transcellular movement of zinc. Zinc transport also occurs through a non-mediated process in the brush border that does not require energy (Rachmilewitz *et al;* 1995). However, most of the non-mediated zinc absorption reflects paracellular rather than

transcellular movement (Mark *et al;* 1993). Zinc is absorbed by this non-mediated mechanism when luminal zinc concentrations are high, such as following supplementation.

Many factors influence zinc absorption including other minerals and trace elements, proteins, vitamins, phytic acid, physiological factors, and disease processes. Studies have shown that iron interfere with zinc absorption (Richard *et al;* 1994). Both inorganic iron and heme iron inhibit zinc absorption in humans, but iron has no effect on zinc absorption from meals. The amount of iron may determine its effect on zinc absorption, and only pharmacological doses appear to decrease zinc uptake (Angelo *et al;* 1994). Copper has little direct effect on zinc absorption, but long-term ingestion of large amounts of zinc can interfere with copper absorption, leading to anemia (Zhang *et al;* 1996). There is no indication that calcium impairs zinc absorption at the levels found in human diets. Calcium and zinc are absorbed by different transport mechanisms, which may explain why calcium has little effect on zinc absorption (Nimral *et al;* 1996). Calcium supplements, however, may lower zinc absorption through an effect within the intestinal lumen that increases zinc loss.

Zinc is rapidly transported to the liver, mainly by albumin, after transfer from the intestine to the portal circulation. Albumin is the principal zinc-binding protein in plasma, and albumin level may alter zinc absorption. Zinc is transported to extrahepatic tissues in plasma (Bob; 1954). Although

plasma zinc comprises only a small fraction of total body zinc and its level is maintained by homeostatic processes, it does flunctuate markedly in response to dietary intake and a variety of physiological processes (Bob; 1954). Plasma zinc concentrations are related to a variety of external stimuli including the level of dietary zinc intake, meals, fasting and acute infections. Plasma or serum zinc concentrations tend to reflect the level of zinc intake when long-term dietary zinc intakes are low (Wapnir *et al*, 1990).

Evidence from studies of isolated cells suggests that the zinc-binding protein MT is a factor in regulating zinc metabolism. MT is inducible by dietary zinc through the metal response element (MRE) and metal-binding transcriptional factor 1(MTF-1) mechanism of transcriptional regulation (Jasinki *et al*, 1994). Dietary regulation of MT expression appears to constitute an autoregulation system wherein increased MT synthesis is linked to increased zinc binding within cells. MT may act as a Zn^{2+} buffer, controlling the free Zn^{2+} level or helping to coordinate an intracellular pool that is responsive to both hormones and diet. The small intestine plays a key role in the homeostatic control of zinc metabolism through regulation of absorption of exogenous dietary zinc and intestinal conservation of endogenous zinc (Samans; 1949). Both of these processes are responsive to changes in zinc status and both can also be affected by changes in dietary factors that may inhibit absorption of exogenous and endogenous zinc.

Diseases, including diarrhea and malabsorption, can also affect these processes (Samans*;* 1949).

The biological role of zinc is characterized by three major functions: catalytic, structural and regulatory. The catalytic role of zinc is required for the biological function of >300 enzymes covering all six classes of enzymes (Kazi *et al;* 2002). An enzyme is generally considered a zinc metalloenzyme if removal of zinc causes reduction in activity without affecting the enzyme protein irreversibly and reconstitution with zinc restores activity. In these enzymes, the zinc ion is located at the active site and directly participates in the catalytic mechanism and interacts with the substrate molecules (Alfredo *et al;* 2009).

It is difficult to establish an unequivocal direct link between signs of zinc deficiency or toxicity and defects in individual enzymes in complex organisms. A physiological defect would occur only if the zinc-dependent enzyme was a rate-limiting step in a critical biochemical pathway or process (Jurgerg *et al;* 1995). Possible zinc metalloenzyme –disease relationships include alcohol dehydrogenase and zinc deficiency in alcoholic liver disease and decreased thymidine kinase mRNA activity, which partially explains growth retardation in zinc-deficient animals (Brenden *et al;* 1995).

2.12 ZINC AND DIARRHEA TREATMENT

Zinc is an essential micronutrient and is the second most abundant trace element in cells and tissues. It has been considered an important anti-inflamatory factor, protecting cell membranes from oxidative damage (Henry; 2006). Because there are only minimal zinc stores in the body, its bioavailability is determined by a balance among its dietary intake, intestinal absorption and losses through urine, skin and the intestinal tract. Diarrhea with severe zinc deficiency has been observed in children in developing countries, (Desouza *et al* 1994). Studies linking diarrheal diseases with Zn deficiency first were described in reports of low plasma Zn levels in children with acute dehydrating diarrhea. Intestinal zinc loss has been reported in children with acute dehydrating diarrhea (Kubo *e tal* 1994). These findings prompted studies of Zn supplementation in children with diarrhea. Recently, Zn supplementation-ORS has been known to reduce substantially the duration and severity of diarrhea in children with both acute and persistent diarrhea. In addition, Zn has been shown to be effective on a prophylactic basis in reducing pneumonia and diarrhea (Rao *et al* 1994). The initial suggestion of the mechanism by which zinc was effective in improving was that Zn administration corrected an underlying micronutrient deficiency that had contributed in some way to the diarrhea. Nonetheless, the pathophysiological mechanisms that either link zinc deficiency with severe diarrhea or explain the efficacy of Zn in reducing

diarrhea are not understood. The study of Bhatnagan *et al* 2001 is of interest because it was hospital based and involved acute diarrhea with dehydration and showed that total stool output in zinc-treated children was reduced by 28% more than in the control group. The results of a meta-analysis of Zn treatment trials in children further supported the usefulness of zinc as a treatment of acute diarrhea in child death program (Hergarty *et al*, 1981). Thus, there is a compelling body of clinical data that zinc likely is effective both in the treatment of acute diarrhea and its prophylaxis.

All these successful clinical studies concluded that the possible mechanism for the beneficial effect of zinc treatment on the duration of diarrhea included the following: improved absorption of water and electrolytes by the intestine (by yet unidentified mechanism): faster regeneration of gut epithelium (Bush *et al*, 2006): increased levels of enterocyte brush-border enzymes: an enhanced immune response leading to increased clearance of the pathogen responsible for diarrhea from the intestine. Most antidiarrhoeal drugs induce cation absorption and/or inhibit anion secretion. The demonstration that sodium and glucose transport are coupled in the small intestine and that adenosine 3^1 5^1 cyclic monophosphate (cAMP) does not inhibit glucose stimulation of solute and water absorption led to the introduction of ORS for the treatment of diarrhea. A very recent publication established that Zn inhibits cAMP-induced cl-secretion by inhibiting basolateral potassium channels in in-vitro

studies with rat ileum (Helier *et al*, 1973). This study also showed specificity of Zn to cAMP-activated potassium ion channels because Zn did not block calcium-mediated potassium channels. The basolateral potassium channel is currently a target of considerable target for drug development in secretory diarrhea. Because this study was not performed in zinc deficient animals, it provided evidence that Zn likely is effective in the absence of Zn deficiency.

2.13 ZINC AND MUCOSAL CELL FUNCTION

Zinc also plays a key role in maintenance of gut mucosal cells. Zinc blocks basolateral potassium (Kþ) channels and thus inhibits cAMP-induced chloride-dependent fluid secretion, a major control point for fluid secretions in the large intestine (Mendes *et al*, 1990). The mechanism(s) by which zinc may act as an enteroprotective have not yet been determined. Treatment with ORS would have its greatest effect on reducing fluid loss by increasing small intestine absorption. Zinc inhibits cAMP-induced chloride secretion by specifically inhibiting basolateral Kþ channels with no blockage of calcium-mediated channels in in-vitro studies with rat ileum (Hoque *et al*, 2005). Zinc also inhibits cholera toxin-induced, but not E. coli heat-stable enterotoxin-induced, ion secretion in cultured Caco-2 cells. One study (Laurens *et al*, 1987) showed that cAMP acted as the intracellular effector of heat-labile enterotoxin-induced fluid secretion. Guanosine 3^{i},5^{i}-cyclic monophosphate (cGMP) mediates heat-stable-induced fluid secretion. If

substantiated, then the effectiveness of zinc would be limited to heat-labile-induced diarrhea or to diarrhea mediated by cAMP, but not by either cGMP or intracellular calcium. It has also been reported that the zinc transporter ZnT-1 modulates the permeation of cations through the L-type calcium channel (LTCC), thereby regulating cation homeostasis (Swanston *et al*, 1989). ZnT-1 may thus play a role in cellular ion homeostasis by conferring protection against pathophysiological events linked to cellular calcium or zinc permeation. A micromolar concentration of extracellular zinc could set off a massive release of calcium from intracellular pools in colonocytes. A sustained increase in intracellular calcium level may also augment Kþ efflux and a hyperpolarization of cell membrane potential, leading to an advantageous electrical gradient for chloride secretion.

2.14 ZINC NUTRITURE AND INFECTIOUS DISEASES

Zinc has been demonstrated to modulate host resistance to infectious agents. A mild-to-moderate deficiency of zinc may result in profound effects on overall immune function, with increased susceptibility to diarrheal causing pathogens (including parasites, bacteria, and viruses). Zinc deficiency in developed countries is uncommon, but groups at risk for zinc under nutrition were identified in the National Health and Nutrition Examination Survey (NHANES) III study. The groups at greatest risk of inadequate zinc intake were children (1–3 years), adolescent women (12–

19 years), and elderly people aged more than 71 years (Angelo *et al*, 1994). In developing countries, infections often coexist with multiple nutritional deficiencies that may result from general malnutrition, especially in children and the elderly. Results are available from a large number of RCTs of zinc supplementation in prevention of diarrhea in developing countries. The WHO/UNICEF Zinc Task Force reported a pooled analysis of 12 trials on children having acute diarrhea and four trials on children having persistent diarrhea (WHO, 2006). Zinc had a positive therapeutic effect in the treatment of both acute and persistent diarrhea, that is, it is estimated that zinc supplementation reduces duration of the acute diarrhea episodes by up to 25% (compared with 24% for persistent diarrhea); zinc supplementation decreases the proportion of acute episodes lasting more than 7 days by about 25%, therefore significantly reducing the proportion of diarrhea episodes becoming persistent; and it reduces stool volume by about 30%. Subsequent trials in Bangladesh (Franca, *et* al 1993), India (Young *et al* 1994), and Brazil (Alpelian *et al*, 1981) corroborated these findings.

2.15 ZINC AND BACTERIAL DIARRHEA

Enteric pathogens constitute a major pediatric threat in the developing world through their impact on morbidity and mortality and impairment of physical and cognitive development. It appears likely that a cause-and-effect relationship exists with malnutrition. Although many bacterial pathogens can cause diarrheal diseases, a group of fewer than 10 (including Shigella spp., ETEC, Vibrio cholerae, and possibly Campylobacter jejuni) account for a significant percentage of these diseases in developing countries. Vaccines against these agents offer a potentially effective control measure against these diseases, but safe, practical, and effective vaccines for many of these agents have yet to be realized. Zinc supplementation has been shown to reduce the duration and severity of watery diarrhea in studies worldwide, but its mode of action is not known. Initially, it was believed that zinc was acting to correct or ameliorate zinc deficiency, but this appears not to be the only means of protection because children and animals that are not zinc deficient still benefited from zinc in studies of diarrhea. Recently, Crane et al. (Shaik *et al,* 2009) reported that zinc decreased adherence of enteropathogenic E. coli (EPEC) bacteria to rabbit intestinal epithelium. They also demonstrated that zinc inhibited a key enzyme, ecto-50-nucleotidase, involved in the conversion of 50-AMP to adenosine in the lumen of the intestine. Adenosine triggers fluid secretion from host intestinal cells and also has growth-promoting effects on EPEC

bacteria. The zinc inhibition reduced the secretory response that triggers EPEC-activated watery diarrhea. The authors concluded that these effects should be considered pharmacologic effects of zinc, not zinc-replacement therapy, as they also occur in the absence of zinc deficiency. Cholera is a common disease in many countries of the world. About 230 000 cases in more than 50 countries are reported globally, but the WHO estimates that official notifications make up only approximately 5% of the real burden of cholera (Nimral *et al*, 1996). This means that as many as three million cases and more than 100 000 deaths occur each year. About 85% of episodes are mild-to-moderate in severity. In animal studies, the net water and sodium secretion induced by cholera toxin was four times greater in zinc-deficient compared with zinc-adequate animals. Furthermore, zinc repletion of the zinc-deficient animals elicited a 40% reduction in secretion within 48 h of zinc repletion (Angelo *et al*, 1994). Recently, Roy et al. (Brenden *et al.* 1995) investigated the impact of zinc on severe diarrhea in children caused by cholera.

2.16 NITRIC OXIDE SYNTHASE ISOENZYMES

The enzymes responsible for the synthesis of nitric oxide from L-arginine in mammalian tissues are known as nitric oxide synthases. These enzymes are remarkable for three different reasons: the rapidity, with which they have been characterized, purified and cloned (Richard *et al*, 1994). The first

descriptions of NO synthases demonstrated that the synthesis of nitric oxide requires L-arginine and NADPH and results in the formation of citrullin. Nitric oxide synthesis requires not only these substrates but also from other co-enzymes/co-factors, as well as the presence of calmodulin. Subsequent work demonstrated that molecular oxygen is also a substrate for this reaction, being incorporated into NO and citrullin: thus this reaction apparently constitutes an L-arginine N^6 C^6 dioxygenation (Richard *et al*, 1994). As shown in the figure below, it is now clear that nitric oxide synthesis from arginine is a reaction which involves two separate mono-oxygenation steps. N^6-hydroxyarginine is an intermediate species formed by a reaction requiring one oxygen and one NADPH and the presence of tetrahydrobiopterin (BH_4). This reaction appears to be similar to those carried out by the aromatic amino acid hydroxylases which also required BH_4. The second step in the NO synthase reaction results in the oxidation of N^6-hydroxyarginine to form citrullin and NO. The detailed mechanism of this reaction remains to be determined (Richard *et al*, 1994). The mammalian system in which NO synthesis was first demonstrated were the vascular endothelium, the brain and activated macrophages.

2.16.1 Neuronal constitutive NO synthase (nNOS): This isoenzyme synthesizes NO in neurons in response to glutamate. It is the first isoenzyme to be purified and cloned (Wendy *et al*, 2001). This enzyme is calcium ion and calmodulin dependent and is constitutively expressed at a

high activity in the brain. Both antibody staining and *in situ* or RNA hybridization techniques demonstrate that the expression of the gene encoding this isoenzyme is widespread in rat brain with particularly large amounts of the protein and mRNA being found in the cerebellum. Partial purification and characterization of the NO synthase thought to be responsible for production of the NO by non-adrenergic non-cholinergic nerves also suggests that the same isoenzyme is present in both the peripheral and central nervous systems. Because of this wide expression in different tissues and because of the high activity of nNOS in the brain and skeletal muscle, it is likely that this isoenzyme is responsible for the largest proportion of constitutive NO synthase activity in man (Richard *et al*, 1994).

2.16.2 Endothelial constitutive NO synthase (eNOS): The constitutive NO synthase of vascular endothelial cells appear to be functionally similar to nNOS and distinct from iNOS, the induced isoenzyme found in activated macrophages. This isoenzyme synthesizes NO in vascular and endothelial cells in response to acetylcholine (Wendy *et al*, 2001). Both constitutive enzymes are clearly calcium ion and calmodulin-dependent, unlike the macrophage enzyme. Cloning of the bovine and human constitutive eNOS revealed that eNOS and nNOS are distinct gene products with only 57% amino acid identity shared between the two human proteins. This isoform of NO synthase is probably responsible for the NO synthase activity of vascular endothelium in blood vessels of all types: Northern blot analysis

has revealed the presence of the mRNA for eNOS in human bovine, rabbit and rat vascular endothelial cells derived from conduit arteries, microvessels or veins (Wendy *et al*, 2001).

2.16.3 Inducible NO synthase (iNOS): This type of isoenzyme is induced by cytokines in macrophages and hepatocytes. Following exposure of a wide range of cell types and tissues to cytokines or bacterial products, an NO synthase (s) is induced which is clearly different from nNOS and eNOS. Rodent macrophages have been the source of the most extensively studied inducible calcium ion independent NO synthase (Richard *et al*, 1999). It has a subunit molecular mass of approximately 130kDa and is fully active in the absence of either calcium ion or added calmodulin.

2.17 Zinc tetrathiolate center in NOS

An unexpected finding in the crystal structure of NOS was a zinc tetrathiolate center located at the bottom of the dimer interface. The zinc iron is tetrahedrally coordinated to two cystein (cys^{110} and Cys^{115}) from each subunit. Site-directed mutagenesis has shown that iNOS cys^{115} and the corresponding residue in eNOS cys^{99} are essential for dimer stability (Mark *et al,* 1993). A comparison between the zinc-free and zinc-bound human iNOS oxygenase domain structures demonstrated a net gain of eight hydrogen bonds with zinc binding, which would favour dimer stability. The zinc is positioned equidistant from each heme with one of its ligands, cys^{115}

separated by only four residues from ser[119], which hydrogen-bonds directly to BH_4, suggesting that the zinc center acts in a structural capacity by helping to maintain the integrity of the BH_4 binding site (Mark *et al*, 1993). Studies on nNOS also reported zinc playing a role in dimer stabilization but not affecting enzyme activity, (Samans *et al* 1949).

The ZnS_4 center in both iNOS and eNOS oxygenase structures are surrounded by a large solvent filled inter-subunit cavity of approx.750A^0 molecular surface, which may be a binding site for a so far unidentified ligand, (Helier, *et al* 1973).

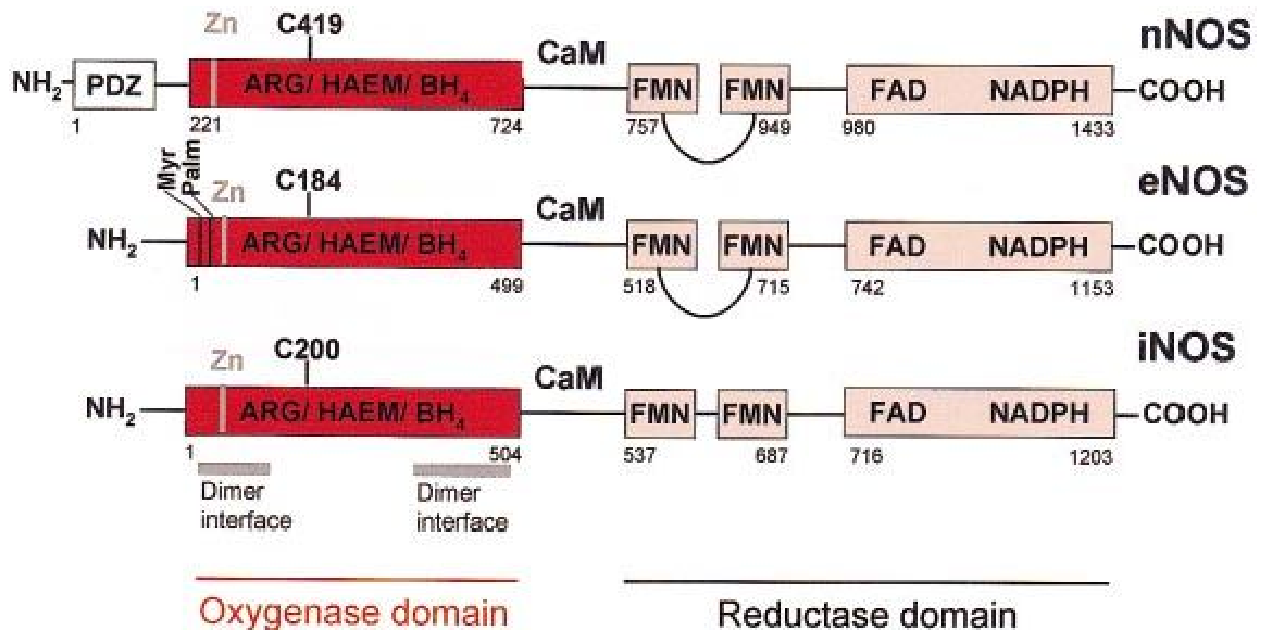


Figure 2.4: Domain structure of human nNOS, iNOS and eNOS

2.18 Feedback Inhibition of Nitric Oxide Synthase by Nitric Oxide

Nitric oxide may itself regulate NOS expression and activity. Specifically, NO has been shown to play an important negative feedback regulatory role on endothelial NOS and therefore vascular endothelial cell function. This process known formally as S-nitrosation (and refered to as S-nitrosylation), has been shown to reversibly inhibit endothelial NOS activity in vascular endothelial cells (Timothy *et al*, 1994). This process may be important because it is regulated by cellular redox conditions and may thereby provide a mechanism for the association between 'oxidative stress' and endothelial dysfunction. The rate-limiting step for the production of nitric oxide may well be the availability of L-arginine in some cell types. Due to the stability of ferrous nitrosyl (Fe^{2+}-NO) complexes, any enzyme that forms a reduced ferrous haem intermediate has the potential to be inhibited by NO. NOS is no exception (Timothy *et al*, 1994). Both iNOS and nNOS can form inhibitory nitrosylspecies during turnover. In the case of iNOS, NO inhibition appears weak and is partly due to build up of the Fe^{3+}-NO complex, but in nNOS, up to 95% of the enzyme in the steady state can be in the tighter Fe^{2+}-NO form. Unlike iNOS, nNOS also seems able to react with NO within the enzyme active site, as the addition of external NOS scavenger has little effect on the inhibition. Stuehr etal recently proposed that in line with recent findings that Fe^{3+}-NO is a final intermediate in the

catalytic cycle, and that the dissociation of NO from this complex competes with its reduction to the Fe^{2+}-NO species (auto-inhibition).

Inhibitors of NO have been described which interact with the NOS enzymes in a variety of ways: different sites, as well as differing time-and substrate-dependence and mechanism of inhibition.

Most inhibitors identified so far are competitive with the substrate L-arginine and have therefore been inferred to be binding at the arginine-binding site, interacting with the conserved glutamate (Glu^{363} of bovine eNOS, Glu^{371} of murine iNOS) which also interacts with the guanidine group of L-arginine, (Bicalho *et al* 2001).

2.19 NITRIC OXIDE

Nitric oxide is an inorganic free radical gas of formular –N=O. The discovery in 1987/88 that vascular endothelial cells are able to synthesize nitric oxide from L-arginine as a transcellular signal was initially received by most biologists with considerable skepticism.

Nitric oxide is an important regulatory factor in physiologic process (Jurgerg, *et al*, 1995). The intestine processes both the calcium-dependent constitutive nitric oxide synthase and the calcium-independent inducible nitric oxide synthase which has been demonstrated under lipopolysaccharide stimulation (Tepperman, *e tal*, 1993). Inducible nitric

oxide synthase when induced, produce a large amount of nitric oxide (Rao, *e tal*, 1994) resulting in a decrease in cellular viability and local intestinal damage. Zinc deficient rats had an inducible nitric oxide synthase gene expressed in the intestine and that interleukin 1 treatment caused many fold enhancements in expression and induced diarrhea (Li cui, *e tal*, 1997). Hence, it could be reasoned that nitric oxide produced by inducible nitric oxide synthase may play a role in the mechanism of zinc deficiency induced damage in the intestine. When nitric oxide is produced, it diffuses across cell membranes freely and equally in all with an average half life of 4seconds (Mota, 1985). The cells in the immediate vicinity can be affected because no efficient scavenger mechanism exists to remove nitric oxide before it can become toxic. On the other hand, it is possible that zinc deficiency resulted in a vulnerability of the intestinal cells to nitric oxide. Malnutrition might be interfering with the proliferating and differentiating processes of intestinal epithelium.

2.19.1 NO: the physiologic messenger

Through the activation of soluble guanylate cyclase and subsequent elevation of guanosine 3^1 5^1 cyclic monophosphate, endothelium-derived relaxing factor which has now been identified as nitric oxide (NO) relaxes vascular smooth muscles (Daniella *et al* 1993). Nitric oxide released from nerves is synthesized from L-arginine by a calcium ion dependent constitutive enzyme, nitric oxide synthase that can be inhibited by certain

analogues of L-arginine (Franscesco *et al* 1991). Recently NO was suggested as neurotransmitter mediating non-adrenergic non-cholinergic relaxations of the intestine including ileal longitudinal muscles from guinea pigs (Mota *et al* 1985) and internal sphincter muscle from humans (Wendy *et al* 2001). Although, a role of NO in the neural regulation of intestinal epithelial function has not been demonstrated, one could be postulated in the light of the finding that activation of guanylate cyclase has been shown to increase chloride secretion in rat intestine (Yong *et al* 1994)

The constitutive isoforms in neuronal or endothelial cells are always present. These nitric oxide synthase isoforms are inactive until intracellular calcium levels increase, the calcium-binding protein calmodulin binds to calcium and the calcium-calmodulin complex binds to and activates nitric oxide synthase. The constitutive nitric oxide synthase isoforms then synthesize small amounts of nitric oxide until calcium level decreases (Angelo *et al*, 1994). This intermittent production of small amount of nitric oxide transmits signals. In contrast, the inducible NOS synthase is normally absent from macrophages and hepatocytes, but when these cells are activated by specific cytokines, an inducible nitric oxide synthase is produced: once produced, it always synthesizes large amounts of nitric oxide. Induced nitric oxide synthase is transcriptionally regulated. The continued production of large amount of nitric oxide kills or inhibits pathogens (Daniella *et al*, 1993).

Nitric oxide diffuses out of the cell that generates it and into the target cells where it interacts with specific molecular targets. The best characterized receptor of nitric oxide is iron, contained in certain proteins as a heme group or as an iron-sulphur complex. Nitric oxide exerts some of its effect by binding to iron-containing enzymes and either activating or inactivating the enzymes. When NO binds to the iron in the heme group of guanylate cyclase, the enzyme is activated. Guanylate cyclase then produces cyclic guanosine monophosphate of which the increase activates other cellular processes. By changing the activity of guanylate cyclase, nitric oxide dilates arteries, signals neurons and kills cells (Alpelian *et al*, 1981).

Another unusual way in which NO affects cell is by facilitating transfer of an ADP-ribose group to an accepting molecule (a process called ADP-ribosylation). Normally, an ADP-ribose group is attached to a protein target by an enzyme, for an example, when cholera toxin ADP-ribosylates a guanosine triphosphate-binding protein. When NO diffuses into a cell, it can cause auto-ADP ribosylation, i.e, ADP ribosylation of a target without enzyme catalysis. For example, NO inactivates the glycolytic enzyme glceraldehyde-3-phosphate dehydrogenation by attaching an ADP-ribose group to it, thereby blocking the production of ATP from glycolysis (Alpelian *et al*, 1981).

2.19.2 NITRIC OXIDE ARTERIAL SMOOTH MUSCLE

A messenger molecule such as acetylcholine receptor on an endothelial cell activates inward calcium currents. Calcium binds to calmodulin and activates endothelial cell nitric oxide synthase, which converts arginine plus oxygen into citrullin and nitric oxide. NO diffuses out of the endothelial cell into an adjacent smooth muscle cell and activates guanylase cyclase by binding to the iron in its heme group. The increase in cyclic guanosine monophosphate causes smooth muscle relaxation, and thus vasodilation (Francesco *et al*, 1994).

2.19.3 Nitric oxide: An immune effector capable of eliminating many pathogens

In contrast to the neuronal and endothelial nitric oxde synthase, the inducible macrophage nitric oxide synthase produces a large amount of nitric oxide. In large quantities, nitric oxide kills almost any nearby cell. It kills or inhibits the growth of many pathogens, including bacteria, fungi, and parasites (Vivien *et al*, 1994).

Because nitric oxide has many molecular targets, it can damage cells in many ways, although precisely how it does so is unclear. Nitric oxide inhibits adenosine triphosphate production at three separate steps: blocking glycolysis by transferring ADP-ribose to glyceraldehyde-3-phosphate dehydrogenase; disrupting the kreb's cycle by binding to the

heme group of cis-aconitase; and inhibiting oxidative phosphorylation by binding to the heme group of ubiquinone reductase. Nitric oxide inhibits DNA synthesis by inactivating ribonucleotide reductase and can damage DNA directly by deamination (Yong *et al*, 1994).

2.20 Transcriptional regulation of macrophage NOS synthase

The production of nitric oxide must be controlled precisely because excessive amounts could damage the host and too little could lead to immunodeficiency (Li cui *et al*, 1999). In contrast to the neuronal and endothelial nitric oxide synthase enzymes that are always present but only active when intracellular calcium levels increase, nitric oxide is normally absent in quiescent macrophages; however once it is synthesized, it is always active. Macrophage nitric oxide synthase is thus transcriptionally regulated. The sequence of signal leading to nitric oxide production begins with an infection that induces immune cell to release cytokines, which in turn signals macrophages to make nitric oxide synthase. For example, a viral infection induces CD^{4+} helper T-lymphocytes or natural killer lymphocytes to produce interferons, or lipopolysaccharides from the cell wall of infecting bacteria cause cell to make tumor necrosis factor (Rachmilewitz *et al*, 1995). Specific combinations of cytokines such as interferons along with either tumor necrosis factor or interleukin, activate intracellular transcription factors inside macrophages such as nuclear factor, to initiate production of nitric oxide synthase. Once synthesized,

macrophage nitric oxide synthase produces nitric oxide continuously. To avoid damaging the host, inhibitory cytokines such as transforming growth factor and mRNA-destabilizing sequence come into play (Richard *et al*, 1994).

2.21 NITRIC OXIDE AND CYCLOOXYGENASE

Nitric oxide enhances cyclooxygenase activity through a mechanism independent of cAMP and suggests that in conditions in which both the NOS and COX systems are present, there is an NO-mediated increase in the production of proinflammatory prostaglandins that may result in an exacerbated inflammatory response (Daniell *et al,* 1993). The data suggests that NO directly interacts with COX to cause an increase in the enzymatic activity.

Production of NO from constitutive NOS is a key regulator of homeostasis, whereas the generation of NO by inducible NOS plays an important role in the host defence response (Mourad *et al*,. 1999). NOS show a number of similarities with cyclooxygenase. COX is the rate-limiting enzyme in the biosynthesis of prostaglandins, thromboxane A2 and prostacyclin (PGI2). In addition to the well characterized constitutive form of COX (COX-1), an inducible isoform of COX (COX-2) is found in endothelial cells, fibroblasts and macrophages after treatment with pro-inflammatory agents including lipopolysaccharide and IL-1B. Anti-inflammatory steroids such as

dexamethasone inhibit the induction of inducible NOS *in-vitro* and *in-vivo* but have no effect on the expression of constitutive NOS. In addition, dexamethasone inhibits IL-1B and LPS-stimulated COX-2 protein synthesis in vitro and in vivo but has no effect on the constitutive form of COX. Many effectors of NO production lead to the simultaneous release of mediators (such as PGE2 and PGI2) from the COX pathway. This is true for the rapidly acting agonists such as bradykinin and for the longer acting agents such as LPS or IL-1B. NO, PGI2 or PGE2 increase the levels of cGMP or cAMP in effector cells (e.g platelets). This synergistic effect may be one of the mechanisms through which the NOS and COX systems operate to amplify a physiological or pathological response (Brenden *et al*, 1995).

Another possible interaction is at the level of the enzyme. In this respect, COX enzymes are potential targets for NO because they contain an iron-heme center at their active site and indeed the vast majority of effects mediated by NO are a consequence of its interaction with iron or iron containing enzymes. For example, the ability of NO to inhibit platelet aggregation and to relax vascular smooth muscle is the result of NO binding to the heme-Fe^{2+} prosthetic group of soluble guanylate cyclase leading to its stimulation and subsequent increase in the levels of cGMP. In the same way, NO interacts with hemoglobin or can exert its cytotoxic effect by interacting with iron-sulphur centers in key enzymes of the respiratory

cycle and DNA synthesis. Thus observations raise the possibility that NO modulates the activity of COX (Helier *et al*, 1973).

2.22 Mechanism of diarrhea induced E.coli heat stable (STa) enterotoxin

Strains of enterotoxigenic E.coli (ETEC) that cause diarrhea in young farm animals have pilli that facilitates adhesion to the intestinal mucosa and electrolytes (Jasinki *e tal*, 1994). ETEC produces two types of enterotoxins: heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT). Heat-labile has been studied extensively and found to be similar to cholera toxin in its structure and function (Richard *e tal*, 1994). On the other hand, ST is unique in its structure and function and consists of two sub-types: STa (methanol-soluble) and STb (methanol-insoluble), (Hergaty *e tal*, 1981). STa has a low molecular weight (2KDa), is poorly immunogenic and induces a secretory response in infant mice (Rao *e tal*, 1983). STa induces secretory diarrhea by stimulating intestinal guanylate cyclase (Almajali *e tal*, 2000). STa binds to a specific receptor, guanylate cyclase C, present in the apical surface of enterocytes and induces intestinal secretion through an increase in the level of cGMP in affected enterocytes (Kubo *e tal* 1994). STa receptors are found throughout the small intestine and the colon of rats and pigs (Mendes *e tal*,.1990). STa-induced diarrhea is associated with enhanced intestinal secretion of Cl- and water (Nimral *e tal*, 1996). Increased secretion of Cl- from target tissues exposed to STa has been directly demonstrated in the cultured human colon carcinoma cell line T84 and in

isolated rat intestinal mucosa. Studies of electrolyte transport in ligated jejuna loops of pigs have also demonstrated an increased net secretion of sodium ion and chloride ion in the presence of crude preparations of STa or cholera toxin (Angelo *e tal*, 1994).

Conclusively, the diarrhea induced by enterotoxigenic E.coli is mediated in large part by the secreted heat-stable enterotoxin (STa), (Brenden *e tal*, 1995). Loss of fluid and electrolytes results from activation of guanylate cyclase enzymes located in the apical surface of the intestinal epithelial cells (Alfredo *e tal*,.2009). Only the particulate form of intestinal guanylate cyclase is stimulated by STa (Bob *e tal*, 1954), in contrast to cholera toxin and E.coli heat-labile enterotoxin which activates the adenylate cyclase of both intestinal and non-intestinal cells (Rachmilewitz *e tal*, 1955). The mechanism of action of STa is not completely understood but it does appear to involve increased intracellular levels of cGMP.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 PLANT MATERIAL

Fresh cashew tree bark and leaves were collected and identified. The samples were washed, chopped into smaller pieces, air dried in the laboratory and then ground to powder.

3.1.2 ANIMALS

Albino wista rats weighing 64-72 grams of both sexes were housed in standard food pellets and water and allowed a one week acclimatization period prior to the study.

3.1.3 DRUGS AND CHEMICALS

Loperamide (reference antidiarrheal drugs), castor oil (laxative agent), zinc gluconate, citrullin solution, diacetylmonoxime, potassium persulphate, saline, trichloroacetic acid, acetate buffer and urease solution were of pharmacological and analytical grade. The chemicals were purchased at sigma chemicals.

3.2 METHODS

3.2.1 PREPARATION OF AQUEOUS EXTRACTS

The powders of both parts were mixed with 250ml distilled and ionized water for 24 hours. The mixture was filtered using a glass funnel, plugged with a sieve cloth. The resulting filtrates were evaporated using gentle heating at 20^0c. The percentage (%) yields of the extracts were calculated.

Weight of extracts after evaporation/weight of extracts before evaporation x 100

3.2.1.1 Percentage yield of cashew bark extract

Weight of cashew tree bark extracts after evaporation = 3.2g

Weight of cashew tree bark extracts before evaporation= (weight of extract + weight of crucible) – weight of crucible

= 63.09g – 56.19g=6.9g

Therefore, % yield= 3.2 x 100/6.9

= 46%

3.2.1.2 Percentage yield of cashew leaves extract

Weight of cashew tree leaves extracts after evaporation =2.9g

Weight of cashew tree leaves extracts before evaporation= (weight of extract + weight of crucible) – weight of crucible

=60.66 – 56.19=4.47g

Therefore, % yield of cashew leaves extracts= 2.9 x 100/4.47

=65%

3.2.2 PREPARATION OF REAGENTS

4 mg Loperamide was dissolved in 5 ml of distilled water.

50 mg zinc gluconate was dissolved in 2 ml of distilled water.

1mg citrullin was dissolved in 100ml of distilled water (0.001%).

3g diacetylmonoxime were dissolved in 100ml distilled water.

1g potassium persulphate was dissolved in 100ml distilled water.

9g of sodium chloride was dissolved in 1000ml distilled water (0.9%w/v saline).

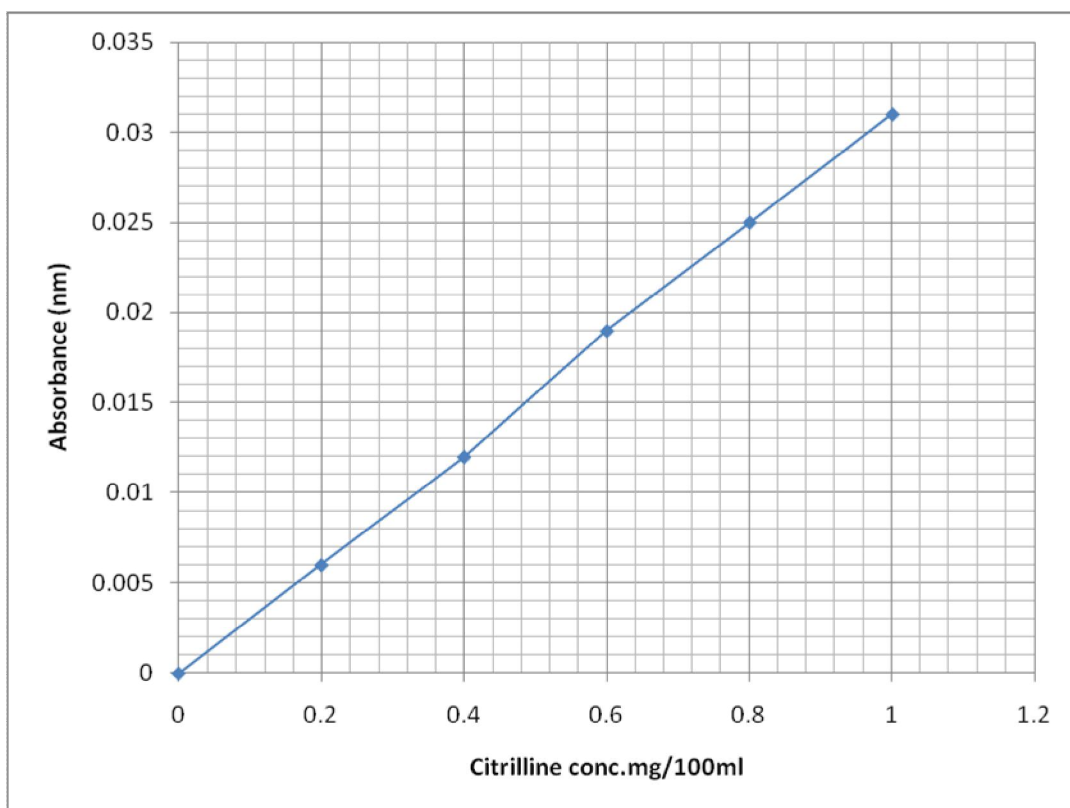
3.2.3 CITRULLINE CALIBRATION TABLE

Citrulline callibration table is prepared using 1mg/100ml citrulline solution in a total volume of 7ml. Six (6) test tubes were used at different concentrations of citrulline solutions with one test tube as a control. Absorbance was read at 440nm.

Tube no	1	2	3	4	5	6
Conc.mg/100ml	0.0	0.2	0.4	0.6	0.8	1.0
Vol.of std (mg/100ml)	0.0	1.4	2.8	4.2	5.6	7.0
Vol.of water (ml)	7.0	5.6	4.2	2.8	1.4	0.0
Conc.Hcl (ml)	4.0	4.0	4.0	4.0	4.0	4.0
Diacetylmonoxime(ml)	0.5	0.5	0.5	0.5	0.5	0.5
Buffer(ml)	0.3	0.3	0.3	0.3	0.3	0.3
Absorbance (nm)	0.000	0.006	0.012	0.019	0.025	0.031

3.2.4 CITRULLINE CALLIBRATION CURVE

A graph of absorbance (nm) was plotted against concentration (mg/100ml).



3.2.5 ANIMAL GROUPS

Adult rats were fasted for 18 hours and divided into different test groups.

3.2.5.1 Zinc Group

Animals were grouped into six (6) groups of three animals each. The first group, the castor oil control group received 2ml castor oil. Group 2, 3 and 4 were the test groups receiving 15mg/kg, 30mg/kg and 45mg/kg respectively. Group five was the standard reference group that received loperamide (5mg/kg). Group 6 received zinc (15mg/kg) + loperamide (5mg/kg).

3.2.5.2 Cashew tree extracts groups

The animals were grouped into seven (7) groups of three animals each. The first group, castor oil control group, received 2ml castor. Groups 2, 3 and 4, the tests groups received 100, 250 and 500mg/kg cashew tree bark extracts respectively. Group 5, 6 and 7, received 100, 250 and 500mg/kg cashew tree leaves extracts respectively.

3.2.5.3 Zinc-fortified cashew tree extracts groups

The animals were grouped into three groups of three animals each. The first group, castor oil control group received 2ml castor oil. Group 2 and 3 received zinc (15mg/kg) + cashew leaves extracts (500mg/kg) and zinc (15mg/kg) + cashew bark extracts (500mg/kg) respectively.

3.2.6 INDUCTION OF DIARRHEA

The method proposed by Meite *et al* (2009) and Shaik *et al* (2009) was found to suit experimental needs. Following the administration of 2 ml castor oil to induce diarrhea, the animals were treated and placed in separate wired cages for observation.

3.2.7 DETERMINATION OF WET /DIARRHEAL STOOL

The cages were covered with filter paper to identify both solid and wet faeces. The total number of faeces and the number of wet faeces passed were recorded over a period of three hours after treatment. The percentage diarrhea inhibition was calculated as a function of the castor oil control: % inhibition= control – test/control x 100

3.2.8 DETERMINATION OF COLONAL CITRULLINE CONCENTRATION

Colonal citrulline level was determined by the method of Allan *et al*, (1947) as an indirect measurement of the level of colonal nitric oxide level. After three hours; the rats were sacrificed, the colon excised,

- Rinsed with saline to remove food and mucus
- Instilled 4ml isotonic saline 0.9% w/v
- Incubated for 15min
- Treated with 0.3ml acetate buffer, PH 5.0
- Added 0.3ml urease solution (to break down urea)
- Added 1ml trichloroacetic acid (for deproteinisation)

- Heated for 2 to 3min in boiling water
- Cooled and filtered
- Took 2ml of filterate
- Added 4ml conc.Hcl to the filterate
- Added 0.5ml diacetylmonoxime solution
- Made it up to 7ml
- Mixed and placed in a boiling water for 9min
- Cooled for 6min to bring the temperature to 65 degrees
- Added 1% potassium persulphate in drops (to develop the colour)
- Read at 440nm
- Recorded the result

3.2.9 INDUCTION OF HEPATOTOXICITY AND LD50 DETERMINATION

Nine rats divided into three animals per group were treated orally with doses (10,100 and 1000mg/kg) of both extracts respectively. They were observed over a 24 h period for gross behavioral change and mortality. No mortality was noted; and three more rats were administered 1600, 2000 and 5000 mg/kg doses of both extracts. Animals treated with 2900 and 5000 mg/kg doses died within 48 and 24 h after treatment respectively. The LD50 was evaluated according to Lorke, 1985.

Another group of four rats each were freely fed with standard feed and water. The extract dissolved in distilled water at 1.44 and 2.87 g/kg were administered. Rats in control group did not receive any treatment for the

same period of two weeks. At the end two weeks, the rats were fasted over night and anaesthetized on the 15th day. Blood was collected and centrifuged at 3000rpm for 10min to obtain the serum. Liver function analysis for serum alanine aminotransferase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP), total bilirubin (TB), and total protein (TP) were performed usin standard methods, Lowry,1951.

3.3 STATISTICAL ANALYSIS

Data were analysed using the students't-test. The results were expressed as mean ± S.E.M. At 99% confidence interval, $p<0.01$ was considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 THE EFFECT OF ZINC ON DIARRHEA

As shown in table 4.1, at 15mg/kg, zinc significantly decreased ($p<0.01$) the total number of wet faeces by 44% (6.67±1.53) upon administration of castor oil (12.0±1.73). Colonal nitric oxide concentration level also decreased significantly from (1.62±1.73) to (1.29±0.07). Zinc-fortified loperamide had a better antidiarrheal activity, 75% inhibition (3.00±0.70) than loperamide 66% inhibition (4.00±2.65). Colonal nitric oxide concentration showed the same pattern.

Table 4.1: The effect of zinc on diarrhea

Treatment	Total no. of feaces	No of wet feaces	% inhibition	Conc of citrullin(mg/100ml)
Castor oil (2ml)	13.7 ±1.53[a]	12.0±1.73	0.00	1.62 ±1.730[a]
Zinc (15mg/kg)	11.66±2.08[ab]	6.67±1.53[b]	44%	1.29±0.070[ab]
Zinc (30mg/kg)	12.7±1.53[ab]	9.30±1.53[b]	22.5%	1.31±0.050[ab]
Zinc (45mg/kg)	12.60±0.58[ab]	9.00±2.65[b]	25%	1.34±0.035[ab]
Loperamide (5mg/kg)	9.67±1.52[ab]	4.00±2.65[b]	66%	0.94±0.040[ab]
Zinc(15mg/kg) + Loperamide	6.60±3.22[bc]	3.00±0.70[b]	75%	0.83±0.066[ab]

Result=mean± S.E.M. [a]significantly higher compared to normal control. [b]Significantly lower than the castor oil control group. [c]Significantly lower than normal group. $P<0.01$

4.2 THE EFFECT OF CASHEW EXTRACTS (BARK AND LEAF) ON DIARRHEA

At 500mg/kg of both extracts, table 4.2, there is a significant reduction, ($p<0.01$) in the number of wet faeces. Cashew bark 56% (5.33±2.08) showed a higher antidiarrheal activity than cashew leaf 42% (7.00 ±1.00). The colonal nitric oxide concentration was lower in cashew leaf (1.40 ±0.05) when compared to cashew bark (1.42±0.14).

Table 4.2: The effect of cashew extracts (bark and leaf) on diarrhea

Treatment	Total no. of feaces	No of wet feaces	% inhibition	Conc of citrullin(mg/100ml)
Castor oil (2ml)	13.7 ±1.53[a]	12.0±1.73	0.00	1.62 ±1.730[a]
Bark extract (100mg/kg)	12.3 ±2.52[ab]	9.70± 1.16[b]	19%	1.55± 0.062[ab]
Bark extract (250mg/kg)	13.33 ±1.53[a]	9.00 ±2.65[b]	25%	1.53 ±0.065[ab]
Bark extract (500mg/kg)	11.0 ±2.00[ab]	5.33± 2.08[bc]	56%	1.42± 0.144[ab]
Leaves extract (100mg/kg)	13.33 ±2.08[a]	11.67 ±1.53	3%	1.56 ±0.065[ab]
Leaves extract (250mg/kg)	10.67 ±1.53[ab]	8.67± 1.15[b]	28%	1.47± 0.095[ab]
Leaves extract (500mg/kg)	11.70 ±1.16[ab]	7.00± 1.00[b]	42%	1.40 ±0.052[ab]

Values are expressed as mean S.E.M. [a]significantly higher compared to normal control. [b]Significantly lower than the castor oil control group. [c]Significantly lower than normal group.P<0.01

4.3 THE EFFECT OF ZINC-FORTIFIED CASHEW EXTRACTS ON DIARRHEA

Zinc-fortified cashew bark 70% (3.67±1.53) showed a higher antidiarrheal activity when compared to zinc-fortified cashew leaves 64% (4.33±1.53). The result showed a similar trend in the level of colonal nitric oxide concentration. Zinc-fortified loperamide showed the most effective antidiarrheal activity 75% (3.00±0.70) and (0.83±0.07) for nitric oxide level.**Table 4 3: The effect of zinc-fortified cashew bark and leaf extracts**

Treatment	Total no. of feaces	No of wet feaces	% inhibition	Conc of citrullin(mg/100ml)
Castor oil (2ml)	13.7 ±1.53[a]	12.0±1.73	0.00	1.62 ±1.730[a]
Zinc(15mg/kg) + tree leaves (500mg/kg)	9.30± 3.06[ab]	4.33±1.53[bc]	64%	1.16 ±0.072[ab]
Zinc(15mg/kg) + tree bark (500mg/kg)	12.00±2.00[ab]	3.67±1.53[bc]	70%	1.02± 0.106[ab]

Results are expressed as mean S.E.M. [a]significantly higher compared to normal control. [b]Significantly lower than the castor oil control group. [c]Significantly lower than normal group. P<0.01

4.4 THE EFFECT OF EXTRACTS ON LIVER FUNCTION PARAMETERS

According to the result shown in table 4.4, the extracts induced significant increase in the levels of ALT and AST. Serum levels of ALP and TP were also dose-related but were not significantly different. TB levels were irregular and either lower than or close to the control. The LD50 per oral in rat was evaluated to be 2154.07 mg/kg (2.154 g/kg)

Table 4.4: Serum levels of Liver Function indices in extracts-treated and normal wistar rats

Serum liver function indicator	Treatment I(2.87g/kg)	Treatment II(1.44g/kg)	Normal rats
ALT(μ/l)	59±10.24	32±2.10	9.58±2.12
ALP(μ/l)	91±8.26	84±9.10	92.20±1.18
AST (μ/l)	128±28.6	89±7.11	9.20±1.28
TB(μmol/l)	0.58±0.03	1.62±0.8	1.63±1.14
TP(g/dl)	7.81±0.61	7.91±1.09	7.81±1.08

CHAPTER FIVE

DISCUSSION AND CONCLUSION

This work established the antidiarrheal effect of cashew extracts as reported by traditional medical practitioners. Both extracts of cashew barks and leaf showed significant reduction in diarrheal stool and concentration of nitric oxide. The reduction in diarrheal faeces could be as a result of its ability to reduce gastrointestinal hyper motility induced by castor oil as reported by Ammon, *et al,* 1974 and Brown and Taylor, 1996. The reduction in the endogenous mediator of diarrhea, nitric oxide by these extracts could suggest an inhibitory effect on nitric oxide synthase, an enzyme that synthesizes nitric oxide in the presence of L-arginine. This suggests that the extracts might contain an inhibitor of NOS which reduced the diarrheal stool output as reported by Mark *et al* 2009 where an inhibitor of NOS also reduced stool output in diarrhea. These findings of this work also agreed with the public health practitioners on the therapeutic use of dietary zinc in the treatment of diarrhea. Though the mechanism by which zinc illicit its mechanism is not well understood, its reduction of colonal nitric oxide concentration suggests an effect on nitric oxide synthase enzyme since the enzyme has a zinc cluster in its structure. The significant reduction in the indicators of diarrhea when the cashew extracts were fortified with zinc indicates a synergistic effect.

This work also revealed that zinc-fortified antidiarrheal drugs should be used as research continues to determine the mechanism behind these actions.

The observed lethal dose (LD50) of 2154.07 mg/kg indicated relative safety. Serum ALT and AST levels in extract-treated rats were significantly different from the control and may indicate hepatotoxicity. But the serum levels of ALP, TB and TP in treated animals were statistically same with those of the control. The findings suggest that the extracts of *A. occidentale* were relatively toxic to rat hepatic systems at oral doses of 1.44 and 2.87 g/kg for the period of administration. However, the hepatotoxicity was minimal since defective hepatic metabolism of bilirubin was not observed. The serum levels of TB in treated animals, which were either lower or close to control supported this. Also TP value did not differ significantly between the control and the treated group. The work done by Okonkwo *et al*, 2010 on hepatotoxicity of inner stem bark extracts agreed with the result of this work.

RECOMMENDATION FOR FURTHER WORK

Further studies on the antidiarrheal effect of these extracts will reveal the active substance(s) responsible for this action. This substance(s) when extracted will show a better antidiarrheal activity than the addictive loperamide drugs. The mechanism by which these extract illicit their actions

need to be well understood. Also, the synergistic effect of zinc and cashew extracts needs further investigation.

REFERENCES

Allan Gornall and Andrew Hunter. A Colorimetric method for determination of citrullin; Pathological Chemistry, University of Toronto (1941); **67**: 650-658

Alfredo, G; Vilforia, B and Carla, A. Colon in acute Intestinal Infection; Journal of Pediatric Gastroenterology and Nutrition (2009); **48**: 558-562

Akinpelu, D. A., Antimicrobial activity of *Anacardium occidentale* bark.Filoterapia (2001); **72**(3): 286–87.

Al-majali, A; Asem, E; Lamar, C and Saeed, A. Studies on the mechanism of Diarrhea induced by E.coli heat stable enterotoxin (STa) in newborn calves. Veterinary Research Communication (2000); **24(5)**: 327-338

Angelo, A; Timothy, S; Nichole, M and Francesco, C. Nitric Oxide as a mediator of the Laxative Action of Magnesium Sulphate. British Journal of Pharmocology (1994); **113**: 228-232

Apelian, D.; Paliwal, M.; Herrschaft, D. C. Casting with Zinc Alloys. Journal of Metals (1981); **33**: 12–19

Bicalho, B., et al.Volatile compounds of cashew apple (*Anacardium occidentale L.*).Z. Naturforsch.(2001); **56**(1–2): 35–9.

Bob, D and Mary, J. The role of accelerated colonic transit in prostaglandin induced diarrhea and its inhibition by prostacyclin. British Journal of Pharmocology (1984); **84**: 157-159

Brenden, J; Ference,L; Steven, M and Salvador,M. Induction of Nitric Oxide Synthase and Microvascular Injury in the Rat Jejunum Provoked by Indomethacin. British Journal of Pharmacology (1995), **216:** 2286-2290

Bush, Douglas Earl; Kassel, Richard. The Organ: An Encyclopedia (2006), p. 679

Charles, C; Dinerman, L and Solomon, H. Nitric Oxide: A physiologic messenger. Basic Science Review

Daniella, S; Thomas, P and Philip, N. Nitric Oxide Activates Cyclooxygenase enzymes. Proc. Natl. Acad. Science (1993);**90**: 7240-7244

de Souza, C. P., et al. The use of the shell of the cashew nut. Anacardium occidentale, as an alternative molluscacide. Rev. Inst. Med. Trop., SÃo Paulo, Brazil (1992); **34**(5): 459–66.

Franca, F., et al. An evaluation of the effect of a bark extract from the cashew (Anacardium occidentale L.) on infection by Leishmania (Viannia) braziliensis. Rev. Soc. Bras. Med. Trop.(1993)**; 26**(3): 151–55.

Francesco, C; Nicola, M; Angelo, A and Timothy, S. Dissociation of Castor-oil Induced Diarrhea and Intestinal Mucosal Injury in Rat. British Journal of Pharmacology (1994); **113**: 1127-1130

Henry, J.B. Causes of Chronic Diarrhea. North England Journal of Medicine (2006); **3553**: 236-239

Hellier, M; Thiramalai, C and Holdsworth, C. The Effect of Amino acids and Dipeptide on Sodium and Water Absorption in Man. Gut (1973); **14**: 41-45

Hergaty, J; Fairclough,P and Clark, M. Jejunal water and electrolyte secretion induced by L-arginine in man. Gut (1981); **22**: 108-113

Jasinski, Stephen M: Zinc. Mineral Year book (1994).

Jurberg, P., et al. Effect of Niclosamide (Bayluscide WP 70), Anacardium occidentale hexane extract and Euphorbia splendens latex on behavior of Biomphalaria glabrata (Say, 1818), under laboratory conditions. Mem. Inst. Oswaldo Cruz (1995); **90**(2): 191–94.

Kazi, M and Henry, J. Zinc in the treatment of Acute Diarrhea: Current Status and Assessment. Gastroenterology (2006); **130**: 2201-2205

Kubo, J., et al. Tyrosinase inhibitors from Anacardium occidentale fruits. J. Nat. Prod. (1994); **57**(4): 545–51.

Kudi, A. C., et al. Screening of some Nigerian medicinal plants for antibacterial activity. J. Ethnopharmacol. (1999); **67**(2): 225–28

Laurens, A., et al. Molluscacidal activity of Anacardium occidentale L. (Anacardiaceae). Ann. Pharm. Fr. (1987); **45**(6): 471–73.

Li Cui, Yogi Tagaki, Masafumi Wasa, and Akira Okada. Nitric Oxide Synthase Inhibitor Attenuates Intestinal Damage Induced by Zinc Defficiency in Rats. Nutrition (1999); **129**: 792-798

Lorke, D. A new approach to practical acute toxicity testing. Arch. Toxicology (1983); **53**; 275-289.

Lowry, O.N; Rosebrough, N.J; Fair,L and Randol R.J. Protein measurement with folin-phenol reagent. Journal of Biological Chemistry(1951); **193**: 265-275.

Mark, J; Halina, S; Somporn, C; Jane, L and David, A. Amelioration of chronic ileitis by nitric oxide synthase inhibition. The Journal of pharmacological and experimental therapeutics (1993);**264:** no 1
Meite, S; Ngnessan, J and Guina, G. Antidiarrheal Activity of Ethyl Acetate Extract of *Morina Morindoides* in Rats. Tropical Journal of Pharmaceutical Research (2009); **8(3)**: 201-207

Mendes, N. M. Molluscacide activity of a mixture of 6-n-alkyl salicylic acids (anacardic acid) and 2 of its complexes with copper (II) and lead (II). Rev. Soc. Bras. Med. Trop. (1990); **23**(4): 217–24.

Mota, M. L. Anti-inflammatory actions of tannins isolated from the bark of *Anacardium occidentale L.* J. Ethnopharmacol. (1985); **13**(3): 289–300.

Mourad, F; Turvill, J and Earthing, M. The Role of Nitric Oxide in Intestinal Water and Electrolyte Transport. Gut (1999); **44**: 143-147

Nimral, K and Tanvir, K. Common Mechanisms of Secretory Diarrhea. Current Science (1996); **9**(9): no 9

Okonkwo, J; Okorie,O; Okonta, J and Okonkwo, C. Sub-chronic hepatotoxicity of *Anacardium occidentale* inner stem bark extract in rats. Indian Journal of Pharmaceutical Sciences (2010); **72(3)**:353-357.

Rao, R; Riviere, P; Pascaud, J and Porreca, F. Tonic Regulation of Mouse Ileal ion transport by Nitric Oxide. The Journal of Pharmacology and Experimental Therapeutic (1994); **269**: no 2

Rachmilewitz, D; Stamler, J; Bachwich, D and Podosky, D. Enhanced Colon Nitric Oxide Generation and Nitric Oxide Synthase Activity in Ulcerative Colitis and Crohn's Disease. Gut (1995); **36**: 718-723

Read, A and Low-Beer, T. Diarrhea: Mechanism and Treatment. Gut (1971): **12**: 1021-1036

Richard, G and Salvador, M. Nitric Oxide Synthase in Mammals. Biochem Journal (1994); **298**: 249-258

Samans, Carl Hubert: Engineering Metals and Their Alloys, Macmillan Co (1949).

Shaikk, B; Nazzim, U and Jebunnessa. Antidiarrheal Activity of Ethanol Bark Extract of Mitragyn Diversifolia. Bangladesh journal of Pharmacology (2009); **14**: 144-146

Swanston-Flatt, S. K., et al. "Glycaemic effects of traditional European plant treatments for diabetes. Studies in normal and streptozotocin diabetic mice." Diabetes Res. 1989; 10(2): 69–73.

Vivien, R and Levin, R. Enterotoxin E.coli STa Activates a Nitric Oxide-Dependent Myenteric Plexus (1994); **2451**: 531-537

Timothy, S; Nicola, M; Angelo, A and Francesco, C. Nitric Oxide as a Mediator of Bisacodyl and Phenolphtalein Laxative Action: Induction of Nitric Oxide Synthase. The American Society of Pharmacology and Experimental Therapeutic (1994); **270**: 1239-1245

Wendy, K; Chris, E and Richard, G. Nitric Oxide Synthase: Function and Inhibition. Biochem Journal (2001), **44:** 143-147

Wapnir, R; Wingertzalan, M and Teichberg, S. L-arginine in low concentration improves rat intestinal water and sodium absorption from oral rehydration solution. Gut (1997); **40**: 602-607

Yong, L; Norman, W; Robert, F and Frank, G. Nitric Oxide is involved in muscle relaxation but not in changes in short-circuit current in rat ileum. The American Physiological Society (1994); **266**:9554-9559

Zhang, Xiaoge Gregory. Corrosion and Electrochemistry of zinc. Springer(1996), p. 93,