Evaluation of *in vivo* Anti-diarrheal Activity of 80% Methanolic Extract of the Leaves of *Croton macrostachys* Hochst. (Euphorbiaceae) in Rats

Zebenay Bussa

A Thesis Submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy in Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacology.

Addis Ababa University

Addis Ababa, Ethiopia

March, 2014

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Zebenay Bussa, entitled: Evaluation of *in vivo* anti-diarrheal activity of 80% methanolic extract of the leaves of *Croton macrostachys* Hochst. (Euphorbiaceae) in rats and submitted in partial fulfillment of the requirements for the degree of master of science in pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

Internal Examiner_____ Signiture_____ Date_____

External Examiner_____ Signiture_____ Date_____

Advisor _____ Signiture_____ Date_____

Advisor _____ Signiture_____ Date_____

Evaluation of *in vivo* anti-diarrheal activity of 80% methanolic extract of the leaves of *Croton macrostachys* Hochst. (Euphorbiaceae) in rats

Zebenay Bussa,

Addis Ababa University, 2014

ABSTRACT

Diarrhea is a common cause of death in developing countries. It is also one of the primary causes of morbidity and mortality on a global scale, leading to 1 billion disease episodes and 1.8 million deaths each year, among children under five years of age. Due to limitations associated with various treatments available, the need for developing newer drugs is imperative. This study aimed at investigating the *in vivo* anti-diarrheal activity of 80% methanolic extract of leaves from the traditionally used medicinal plant, named *Croton macrostachys*. The effect of the 80% methanolic extract in rodents on castor oil-induced diarrhea, intestinal transit, and enteropooling was evaluated at doses of 200, 400, and 600 mg/kg body weight. The anti-diarrheal index (ADI) was calculated combining all diarrhea indicators to see the relative effect of the extract. In castor oil induced diarrhea, the extract produced a significant ($p < 0.001$) reduction in the severity and frequency of diarrhea, and significantly delayed onset of diarrhea at all doses tested. It was equally effective to that of loperamide in this model at 600 mg/kg by providing 100 % protection against diarrhea. In the castor oil induced intestinal transit test, the extract produced a significant ($p < 0.01$) decrease in propulsion with peristaltic index (PI) values of 40.03 ± 3.00 and 39.48 ± 2.27, respectively at doses of 400 and 600 mg/kg versus 56.38 ± 1.76 % for control. However, the effect was not significant at 200 mg/kg. The extract also showed a significant reduction in weight and volume of intestinal fluid accumulation, at doses

of 200, 400, and 600 mg/kg. The highest ADI was obtained with the dose of 600 mg/kg of the extract, which was comparable to that produced by the standard. In conclusion, the results obtained showed that 80% methanolic extract of the leaves of *Croton macrostachys* contain some biologically active principles that are active against diarrhea, and this may be the basis for its traditional use for treating diarrhea. Therefore, it is a good candidate for further fractionation of the extract and study.

Key Words: *Croton macrostachys*, Castor Oil, Anti-diarrheal Activity, Loperamide, Atropine, Anti-diarrheal index

ACKNOWLEDGEMENTS

I praise the name of Almighty God who gave me power and patience in every endeavor of my life.

I would like to express my genuine appreciation to my advisors Dr. Ephrem Engidawork and Dr. Teshome Nedi for their advice and encouragement while I was working on my thesis research.

I want to thank the laboratory technician of Pharmacology Core Lab, W/ro Fantu, for her unlimited cooperation. I would also like to thank the laboratory technician of Pharmacognosy Core Lab, Ato Haile, for his cooperation.

My indebtedness is also extended to the animal attendants of Pharmacology Department, Ato Molla Wale, for his constant care of the experimental animals and support.

I wish to thank the School of Graduate Studies of AAU for extending its support in the course of the study. I further wish to give my gratitude to the Department of Pharmacology and Clinical Pharmacy of AAU for providing me with laboratory equipment, chemicals, and other supports.

I would also like to thank all my academic colleagues for their supportive opinions and suggestions.

Finally, I would deeply thank Wollega University for sponsoring me.

Contents

LIST OF FIGURES viii

LIST OF TABLES ix

LIST OF ABBREVATIONS x

1. INTRODUCTION 1

1.1 Definition and Classification of Diarrhea 1

1.2 Epidemiology of Diarrhea 2

1.3 Etiology and Prevention of Diarrhea 3

1.4 Normal Intestinal Physiology and Pathophysiology of Diarrhea 3

1.4.1 Normal Intestinal Physiology 3

1.4.2 Patophysiology of Diarrhea 5

1.5 Management of Diarrhea 8

1.5.1 Non-Pharmacological 8

1.5.2 Pharmacological Method 9

1.5.3 Herbal Treatment 13

1.7 *Croton macrostachys* 14

1.6 Rationale for the Study 15

2. OBJECTIVES 17

2.1 General Objective 17

2.2 Specific Objectives 17

3. MATERIALS AND METHODS 18

3.1. Drugs and Chemicals 18

3.2 Plant Material 18

3.3 Experimental Animals 18
3.4 Plant Extraction 19
3.5 Grouping and Dosing 19
3.6 Determination of Anti-diarrheal Activity 20
3.6.1 Castor Oil Induced Diarrhea 20
3.6.2 Charcoal Meal Test 20
3.6.3 Castor Oil-Induced Enteropooling 21
3.7 Statistical Analysis 22
4. RESULTS 23
4.1 Castor Oil-Induced Diarrhea 23
4.2 Charcoal Meal Test 25
4.3 Castor Oil-Induced Enteropooling 28
5. DISCUSSION 30
6. CONCLUSION 36
7. RECOMMENDATION 37
REFERENCES 38

LIST OF FIGURES

Figure 1: Photograph of *Croton macrostachys*.....15

Figure 2: Effect of 80% the methanolic extract of the leaves of *Croton macrostachys* on number of wet stool and total number of stools.....24

Figure 3: Effect of 80% methanolic extract of the leaves of *Croton macrostachys* extract on onset of diarrhea.....25

LIST OF TABLES

Table 1: Anti-diarrheal activity of 80% methanolic extract of the leaves of *Croton macrostachys* H. against castor oil induced diarrhea in rats. 24

Table 2: Effect of 80% methanolic extract of the leaves of *Croton macrostachys* H. on Castor oil induced small intestinal transit in rats. 26

Table 3: In vivo anti-diarrheal index of 80% methanolic extract of the leaves of *Croton macrostachys* H. 27

Table 4: Effect of 80% methanolic extract of the leaves of *Croton macrostachys* H. extract on castor oil induced enteropooling in rats. 29

LIST OF ABBREVATIONS

5-HT	Serotonin
AAD	Antibiotic Associated Diarrhea
ACh	Acetyl Choline
ADI	anti-diarrheal index
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	One-Way Analysis of Variance
C	*Croton*
cAMP	Cyclic Adenosine Monophosphate
CD	Crohn's Disease
cGMP	Cyclic Guanosine Monophosphate
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
IBDs	Inflammatory Bowel Diseases
IBS	Irritable Bowel Syndrome
LTC4 & D4	Leukotriene C4 and D4
Na, K-ATPase	Adenosine Triphosphate (ATP)-Dependent Active Sodium (Na) Pump
NO	Nitric oxide
OECD	Organization of Economic Cooperation and Development

ORS	Oral Rehydration Solution
ORT	Oral Rehydration Therapy
PINES	Paracrine–Immuno-Neuroendocrine System
SCFA	Short-Chain Fatty Acid
SEM	Standard Error of the Mean
SNNPR	Southern Nations, Nationalities and People's Region
SPSS	Statistical Package for Social Science
TGF-β	Transforming Growth Factor-β
TMP-SMX =	Trimethoprim-Sulfamethoxazole
WHO	World Health Organization

1. INTRODUCTION

1.1 Definition and Classification of Diarrhea

Diarrhea is a symptom marked by rapid and frequent passage of semisolid or liquid fecal material through the gastrointestinal tract and involves both an increase in the motility of the gastrointestinal tract along with increased secretions and a decrease in the absorption of fluid and thus loss of electrolytes particularly Na^+ and water (Rang *et al*., 2003). It is one of the most common clinical signs of gastrointestinal disease, but also can reflect primary disorders outside the digestive system. Diarrhea is considered to be present if one of the following conditions are met: (a) stool weight of greater than 200 g per day, (b) more than 2 stools per day for more than 30 days, (c) more than 3 stools per day for more than 7 days, (d) more than 3 stools per day, looser than usual, for more than 3 days; or (e) more than 3 stools per day, with a change in frequency or consistency (Sarin, 2012).

Based on time course, diarrhea may be classified as acute, persistent, and chronic. Acute diarrhea is defined as three or more loose bowel movements in a 24-hour period (Gregorio *et al*., 2009) and the duration is less than 2 weeks (Halsey, 2009). Diarrhea is said to be persistent if the duration varies from 2 to 4 weeks, and chronic if it lasts more than 4 weeks in duration (Guerrant *et al*., 2001). It can also be classified based on the etiology as infectious or non-infectious. Non-infectious diarrhea, e.g. due to irritable bowel syndrome, results from a complex interaction of immune and neuronal factors. The mechanisms of diarrhea caused by various pathogens can be classified as inflammatory or non-inflammatory (Hostos *et al*., 2011). Diarrhea can also be classified based on its pathophysiology into secretory,

osmotic, inflammatory, iatrogenic/drug related, and functional/motility-related diarrhea (Guandalini and Vaziri, 2011).

1.2 Epidemiology of Diarrhea

Although diarrhea is a preventable disease, it remains the second leading cause of death (after pneumonia) among children aged under five years worldwide (Kaplan *et al*., 2013). Diarrhea is one of the primary causes of morbidity and mortality on a global scale, leading to 1 billion disease episodes and 1.8 million deaths each year (Chou *et al*., 2010). It is also one of the leading causes of morbidity and mortality in developing countries among under the same age group (Regassa *et al*., 2008). It kills more young children than AIDS, malaria and measles combined (Hostos *et al.*, 2011).

Around 50% of deaths among children under five occur in sub-Saharan Africa and 40% in South Asia (Kaplan *et al.*, 2013). Such areas are also with higher case-fatality rates compared to children living in high-income countries due to lack of access to quality health care and timely and effective treatment with oral rehydration solution (ORS) and zinc (Sutariya *et al*., 2011).

Ethiopia is among the five mostly poor and populous countries which account about half of the world's deaths due to pneumonia and diarrhea, along with four other countries such as India, Nigeria, Democratic Republic of the Congo, and Pakistan (UNICEF, 2012). Poverty, crowding, contaminated water supplies (Bakare *et al.*, 2011), childhood underweight, suboptimal breastfeeding, unsafe drinking water and sanitation, vitamin A deficiency, and zinc deficiency all contribute to its incidence(Kaplan *et al.*, 2013).

1.3 Etiology and Prevention of Diarrhea

Diarrheal diseases are caused by a variety of pathogens including viruses (for example, rotavirus), bacteria (*Cholera*, *Shigella* and enterotoxigenic *Escherichia coli* (ETEC)), protozoa (*Cryptosporidium* and *Entamoeba histolytica*) and helminthes. These pathogens present in the gut causing disruption of normal fluid secretion and motility and stimulating the gut to expel the contents. Most pathogens are transmitted from the stool of one person to the mouth of another via contaminated food or water (faecal-oral transmission) (Kaplan *et al.*, 2013; Panda *et al.*, 2012). Overeating or eating of wrong foods, putrefaction of food in the intestinal tract, fermentation caused by incomplete carbohydrate digestion, nervous irritability, use of antibiotic drugs, and excessive intake of laxatives can also cause diarrhea (Singh and Verma, 2012). Diarrhea also occurs frequently in post-transplantation patients who are receiving immunosuppressive drugs (Sellin, 2001).

Measures for prevention of diarrhea include: exclusive breastfeeding for the first 6 months of life, safe drinking water, improved sanitation, personal and food hygiene and rotavirus vaccination. The parents should also be informed about the routes of transmission of enteropathogens and preventive measures (Singh and Verma, 2012).

1.4 Normal Intestinal Physiology and Pathophysiology of Diarrhea

1.4.1 Normal Intestinal Physiology

As diarrhea is the end result of a derangement in the normal physiology of the intestinal handling of water and electrolyte, an understanding of these processes is essential to appreciate the pathophysiological changes that lead to diarrhea.

All segments of intestine from duodenum to distal colon have mechanisms for both absorbing and secreting water and electrolytes (Field, 2003).

There is a constant bidirectional flux of water and ions across the small intestinal mucosa, i.e., absorption and secretion. Absorption occurs in villus cells and secretion largely by crypt cells (Binder and Reuben, 2005). Sodium and water absorption by enterocytes is mediated by an active, ATP-dependent active sodium pump (Na^+, K^+-ATPase) located on the basolateral membranes of intestinal crypt and villus cells. In the intestine, solute movement creates the osmotic force for fluid movement. Na^+ absorption drives fluid reabsorption, while active Cl^- secretion contributes to water secretion in secretory diarrhea. Small intestinal Na^+ absorption is mediated primarily by two mechanisms: a glucose- or amino acid-stimulated cotransport in which Na^+ accompanies the other solute and a coupled Na^+–Cl^- mechanism. The latter is a combination of Na^+–H^+ exchange and Cl^-–HCO_3^- exchange. Short-chain fatty acid (SCFA)-mediated Na^+ absorption and aldosterone-sensitive Na^+ absorption occur in the colon (Binder, 2005). Among the various mechanisms described, the coupled Na^+–Cl^- pathways are primarily regulated by cyclic adenosine monophosphate (cAMP) levels and also by cyclic Guanosine Monophosphate (cGMP) and intracellular Ca^{2+} levels (Field, 2003). In addition to the transporters, there are multiple extracellular factors regulating epithelial ion transport – paracrine, immunological, neural, and endocrine factors, termed together as a single regulatory system known as PINES (paracrine–immuno-neuroendocrine system) (Mourad *et al.*, 1995).

In addition to the absorptive and secretary function of the intestine, motor functions also play a key role in facilitating digestion and absorption of fluids and nutrients. Synchronized migrating motor complexes normally occur during fasting in the

stomach and small bowel with increased contractions following feeding with the total small bowel transit time of approximately 3 h for the food reaches the colon (Kerlin *et al.*, 1982). In the colon, there is further reabsorption with the ascending and transverse colon serving as reservoirs and with the sigmoid and rectum serving as volitional reservoirs (Proano *et al*., 1990). Any disturbance in the coordinated flux of water and ions and motility can result in the clinical syndrome of diarrhea.

1.4.2 Patophysiology of Diarrhea

For better understanding of the pathophysiology of diarrhea, it is classified as secretory or toxin induced, osmotic or malabsorption induced, inflammatory, iatrogenic/drug-induced, and functional diarrhea. Most etiologies will have a complex pathophysiology involving one or more of these mentioned mechanisms.

i) Secretory Diarrhea

The basic pathophysiology involves either net secretion of ions (chloride or bicarbonate) or inhibition of net sodium absorption (Hoque *et al.*, 2012; Poley and Hofmann, 1976).

Most causes of secretory diarrhea alter the second messenger systems through alteration in cAMP, cGMP, or intracellular calcium-regulated ion transport pathways (Binder, 2005; Field, 2003; Hoque *et al.*, 2012) Alterations in these mediators cause the cystic fibrosis transmembrane conductance regulator (CFTR) mediated Cl^- secretion and inhibition of small intestinal-coupled Na^+–Cl^- transport (Hoque *et al.*, 2012; Salazar-Lindo, 2011). CFTR is a chloride channel and is the primary driver of secretion in cases of diarrhea caused by enterotoxigenic bacteria (Hostos *et al*., 2011).

Secretory diarrhea may arise from infectious and non-infectious causes. The most common cause of secretory diarrhea is infection, which usually affect the small intestine (Schiller, 1999). Infectious secretagogues include the viruses: rotavirus and norovirus, enterotoxigenic *E. coli* (ETEC), *Vibrio cholerae*, *Giardia*, and *Cryptosporidium* infections (Hoque *et al*., 2012). Non-infectious secretagogues include chemicals produced by certain types of cancer, prostaglandins produced in patients with bowel inflammation and substances not well absorbed such as fatty acids and bile acid (Bliss *et al*., 2006). Secretory diarrhea persists in spite of fasting (Mercadante, 1995).

ii) Osmotic Diarrhea

Osmotic diarrhea occurs when osmotically active but poorly absorbable solutes in the intestine draw water to them because the gastrointestinal mucosa is not able to maintain an osmotic gradient (Sellin, 2001). The causes of osmotic diarrhea are varied but can be broken down into decreased enzymatic availability (lactose intolerance), a genetic abnormality that decreases or eliminates the ability of the body to absorb certain nutrients (celiac sprue), poorly absorbable sugars (sorbitol, mannitol or lactose) (Strasinger and Di Lorenzo, 2008), and poorly absorbable solutes (magnesium, sulfates, and phosphates) (Hammer *et al.*, 1989). This fecal matter then creates a negative osmotic gradient causing leakage of more fluid into the gut increasing the stool volume and resulting in diarrhea (Field, 2003). Osmotic diarrhea typically lessens after fasting or stopping the causative agents (Mercadante, 1995).

iii) Inflammatory Diarrhea

Inflammatory diarrhea may result from a wide variety of etiologies including infections and Inflammatory Bowel Diseases (IBDs). Infectious pathogens causing

inflammatory diarrhea primarily affect the distal small bowel or the colon (Pawlowski *et al.,* 2009). They cause disease either by elaborating cytotoxins or by invading the epithelium with resultant recruitment of inflammatory cells (Navaneethan and Giannella, 2008). Most of the pathogens causing inflammatory diarrhea do so by producing mucosal damage as well as by stimulating intestinal secretion. Agents responsible for inflammatory diarrhea include agents such as enterotoxins, cytokines, prostaglandins, and nitric oxide (NO) that could be produced in the course of the disease (Urayama and Chang, 1997). IBDs are also one of the most common and important causes of inflammatory diarrhea (Binder, 2009). The cytokines and eicosanoids initiated by inflammation down regulate the ion transporters in the colon and small bowel resulting in Na^+ malabsorption (Amasheh *et al.,* 2004; Thevarajah *et al*., 2005).

iv) Drug-Induced Diarrhea

There are a number of drugs that are known to cause diarrhea either as a side effect or as the desired effect of the drug. The mechanism of causing diarrhea can vary from drug to drug. Antibiotic use may alter the bacterial flora in the colon resulting in impaired colonic salvage of malabsorbed carbohydrates (McFarland, 2006) and emergence of pathogenic organisms such as *Clostridium difficile* (Bergogne-Berezin, 2000) to cause antibiotic associated diarrhea (AAD). Some of the drugs like lactulose may cause osmotic diarrhea, while others may cause secretory diarrhea. Both coffee and theophylline increase intracellular cAMP, opening of chloride channels and increasing secretion, while erythromycin interacts with the motilin receptors increasing the motility to cause diarrhea. Prostaglandin analogs (e.g. misoprostol) can affect the intestine at many level including permeability, motility, transport of electrolytes as well as affecting peptides that stimulate secretion Similarly

chemotherapeutic drugs may cause diarrhea because of decreased rate of proliferation of the enterocytes (Sellin, 2001).

v) Functional Diarrhea

When the intestines are not functioning normally, motility can be either increased or decreased and both can lead to diarrhea. Increased motility may decrease the time for the luminal contents to be in contact with the epithelium for absorption resulting in secretory diarrhea, like in irritable bowel syndrome (IBS) (Drossman *et al., 2002;* Prior *et al.*, 1990; Vassallo *et al.*, 1992*)* and disturbances in the neural control (Grundy, 2002; Mamikunian *et al*., 2009). On the other hand, slow transit as occurring in diabetes mellitus and scleroderma may be associated with bacterial overgrowth and the ensuing bile acid deconjugation, poor micelle formation, and steatorrhea (Camilleri, 2004).

1.5 Management of Diarrhea

The mainstay of managing diarrheal diseases is determination and correction of fluid depletion (water and electrolyte depletion), shock and acidosis, maintenance of nutrition, and drug therapy (e.g., anti-diarrheal agents and antimicrobial therapy). The relative importance of each is governed by the severity and nature of diarrhea (Singh and Verma, 2012).

1.5.1 Non-Pharmacological

Most diarrheal illnesses are self-limited and require no specific intervention other than hydration and dietary modification (Lawler and Wallace, 2003).

i) Oral Rehydration Therapy (ORT)

Oral rehydration therapy (ORT) is the administration of fluid by mouth to prevent or correct dehydration that is a consequence of diarrhea. According to Langsten and Hill (1995), it is the cornerstone of treatment to prevent dehydration especially for acute watery diarrhea. Oral rehydration salt (ORS) solution is the fluid specifically developed for ORT. It simply consists of electrolytes (sodium and potassium chloride) and glucose, which promotes water absorption (Hostos *et al.*, 2011). A more effective, lower-osmolarity ORS (with reduced concentrations of sodium and glucose, associated with less vomiting, less stool output, and a reduced need for intravenous infusions in comparison with standard ORS) has been developed for global use (Farthing *et al*., 2008).

ii) Dietary Modification

Dietary modifications can also significantly alter the course of certain gastrointestinal conditions. For instance, it is evident that avoidance of lactose or gluten-containing foods can greatly benefit patients with lactose intolerance or celiac disease, respectively (Guandalini and Vaziri, 2011). For patients with prominent dumping, dietary modification comprising frequent small, dry meals that are high in protein and low in carbohydrate and substances that prolong the absorption of carbohydrate, such as pectin, may be useful (Mercadante, 1995). Dietary substances that may aggravate IBS symptoms such as fatty foods (which delay stomach emptying but also stimulate the lower bowels leading to bloating and discomfort and diarrhea), beans and gas producing foods (which can produce bloating and diarrhea), as well as alcohol and caffeine should be avoided (Drossman *et al.*, 2002).

1.5.2 Pharmacological Method

i) Antimicrobials

Routine empirical use of antimicrobials in the treatment of acute watery diarrhea is neither necessary nor appropriate. Antimicrobials should be used only for specific enteric pathogens and a given clinical severity; this approach involves a thoughtful clinical evaluation of each case. *Cholera* and *shigellosis* are among the few gastrointestinal infections in which a specific antimicrobial could meaningfully shorten the disease severity, decrease the risk of complications and reduce its transmission (Salazar-Lindo, 2011).

Antiprotozoal, e.g. nitazoxanide, drugs can be very effective for diarrhea in children, especially for *Giardia*, *Entamoeba histolytica*, and *Cryptosporidium* (Halsey, 2009). Antimicrobials such as ciprofloxacin and norfloxacin are to be considered the drugs of choice for empirical treatment of traveler's diarrhea in which ETEC or other bacterial pathogens are likely causes and of community-acquired secretory diarrhea when the pathogen is known (Akalin, 1993; Guerrant *et al*., 2001). Azithromycin is widely available and has the convenience of single dosing. Treatment for *amoebiasis* should, ideally, include diloxanide furoate following metronidazole, to get rid of cysts that may remain after metronidazole treatment. Trimethoprim-sulfamethoxazole (TMP-SMX) is a reasonable alternative to quinolones for empiric treatment of children and patients with sensitivity to quinolones or in areas where quinolone-resistant organisms are prevalent (Guerrant *et al*., 2001). Vancomycin, metronidazole, bacitracin or fusidic acid can also be indicated in severe cases of AAD particularly those related to *Clostridium difficile* (Bergogne-Berezin, 2000; Hogenauer *et al*., 1998).

However, the clinical benefit of antimicrobial therapy must be carefully weighed against the cost, the risk of adverse reactions, harmful eradication of normal intestinal flora, the induction of Shiga toxin production, and the increase of antimicrobial resistance (Guerrant *et al.*, 2001; Lori, 2008). Woman taking contraceptives should be

advised that antibiotics may decrease the efficacy of oral contraceptives because of the change in intestinal bacteria and possible altered absorption of contraceptive hormones in the intestine (Bauer and Wolf, 2005).

ii) Probiotics (biotherapeutic agents)

Biotherapeutic agents or probiotics are live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance. Potentially, probiotics maintain or restore gut microecology during or after antibiotic treatment through the following mechanisms: receptor competition, competition for nutrients, inhibition of epithelial and mucosal adherence of pathogens and translocation (Scaldaferri *et al*., 2012), lower colonic pH thereby favoring the growth of nonpathogenic species (Hempel *et al*., 2012), stimulation of immunity, or production of antimicrobial substances (Friedman, 2012). These agents have been used in the treatment and prevention of AAD. Most common probiotics include *Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei* GG, *Lactobacillus bulgaricus, Saccharomyces boulardii* (Bergogne-Berezin, 2000). As probiotics are living organisms given to ill patients, the potential for adverse reactions exists (McFarland, 2006).

iii) Antimotility and Antisecretory Drugs

In mild diarrhea, antimotility agents (e.g., loperamide) will lessen stool frequency and, by increasing the time of contact with the gut epithelium, will also lessen stool volume (Field, 2003). They might be recommended in routine noninvasive and non-inflammatory diarrhea, and should be avoided in bloody febrile patients. Loperamide inhibits intestinal peristalsis and has mild antisecretory properties, and is preferred for symptomatic treatment in diarrhea (Al-Abri *et al.*, 2005*).*

Other drugs such racecedotril (an enkephalinase inhibitor) (Field, 2003), octreotide (somatostatin analogue) (Jensen, 1999), and Crofelemer (CaCCs, Calcium sensitive Chloride Channels inhibitor) (Hostos *et al.*, 2011) are antisecretory antidiarrheal drugs found to inhibit secretion in different diarrheal conditions. Selective 5-HT3 receptor antagonists such as cilansetron and alosetron are also effective drugs in relieving pain and normalizing bowel frequency as well as reducing urgency (Drossman *et al.*, 2002).

iv) Anti-inflammatory Agents

Most of the preparations of this group have actions that inhibit various steps of the arachidonic acid cascade. Some are to decrease the production of many immune and inflammatory mediators that actively inhibit mucosal absorption (Urayama and Chang, 1997). Example of drugs in this group include sulfasalazin (potently inhibits cyclooxygenase (Sharon *et al.*, 1978) and also affect 5-lipoxygenase activity both in intestinal mucosa and neutrophils (Dreyling *et al*., 1987)), zileuton (a selective inhibitor of 5-lipoxygenase), and glucocorticoids (inhibition of phospholipase A2 activity) (Laursen *et al*., 1994). These drugs are mainly indicated in diarrhea due to IBDs.

Other groups of drugs like bile acid resins (e.g. cholestyramine), α2 agonists (e.g. clonidine), and proton pump inhibitors (e.g. omeprazol) can also be indicated for treatment of diarrhea caused by ileal surgery, diabetes mellitus, and gastrinoma syndrome, respectively (Mercadante, 1995). Food supplements such as zinc (Singh and Verma, 2012), folate, vitamin A, magnesium and copper reduce the duration and severity of diarrheal episodes in children in developing countries (Kulkarni *et al*., 2012; Sarin, 2012). Supplements like zinc can also have adverse effects such as

epigastric pain, lethargy, and fatigue if given in high doses (Lazzerini and Ronfani, 2008). Several adsorbents like cholestyramine, kaolin, pectin, activated charcoal can also be used (Sarin, 2012).

1.5.3 Herbal Treatment

Despite immense technological advancement in modern medicine, many people in the developing countries still rely on traditional healing practices and medicinal plants for their daily health care needs (Singh and Verma, 2012). According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Hossain *et al.*, 2012). It is often noted that 25% of all drugs prescribed today come from plants. This estimate suggests that plant-derived drugs make up a significant segment of natural product– based pharmaceuticals (Rout *et al*., 2009). Herbal products from medicinal plants are preferred because of less testing time, higher safety, efficiency, cultural acceptability and lesser side effects (Prasad *et al*., 2012).

There have been numerous reports of the use of traditional plants for the treatment of diarrheal diseases. The main chemical constituents in plants found to be responsible for anti-diarrheal activity are tannins and tannic acid, flavonoids, alkaloids, sesquiterpenes, diterpenes, terpenes and terpenoids (Sarin, 2012). The chemical compounds present in herbal products are a part of the physiological functions of living organisms, and hence they are believed to have better compatibility with the human body (Prasad *et al*., 2012). Plant extracts can have antispasmodic effects, delay gastrointestinal transit, suppress gut motility, stimulate water adsorption or reduce electrolyte secretion (Palombo, 2006). These activities may explain the benefits of using particular plants in the treatment of diarrheal disease. *Acacia catechu* (Sarin,

2012), *Chiranthodendron pentadactylon Larreat* (Velázquez *et al*., 2012), *Bombax buonopozense* (Singh and Verma, 2012), *Croton blanchetianus* Baill, *Croton rhamnifolius* Willd, and *Croton argyroglossum* Baill (Siqueira *et al*., 2012) are few examples, selected randomly, of herbal medicines proven to have anti-diarrheal activity.

1.7 *Croton macrostachys*

Croton macrostachys Hochst. ex Del. belongs to Euphorbiaceae family and to the genus croton (Mairura, 2007). *C. macrostachys* has a vernacular name of Bisana (Amharic), Asisi (Agew), Abnga (Berta ethinic group), and Bissano (Wonago). It is a shrub or tree 2-25m long. Leaves are large, green and turn orange before falling. Flowers are creamy to yellow-white colored with sweet scent, dioecious or at least on separate shoots. Fruits are green when young, turn gray at maturity and mature while still on the tree (Bantie *et al*., 2014).

C. macrostachys is found on forest edges along rivers, around lakes, in moist or dry evergreen upland forests, woodlands, wooded grasslands or clump bush land and along roadsides. The altitudinal range so far recorded for the plant is 500-2350m. In Ethiopia this plant is found in Tigray, Gonder, Gojam, Wollo, Shewa, Arsi, Wellega, Illuababora, Kefa, Sidamo, Bale and Harerge. In Africa, it is distributed West to Guinea, South to Angola, Zambia, Malawi and Mozambique (Amenu, 2007).

In West Africa, different plant parts are taken to treat constipation, stomach-ache and female infertility (Mairura, 2007). *C. macrostachys* is very common plant in Cameroonian traditional medicine. Its roots and fruits are used for constipation, diabetes and as purgative. Leaves are used to treat cough. Roots are used for malaria, venereal illnesses and like antidiabetic (Mbiantcha *et al*., 2013). Croton oil, extracted

from its seeds, has also been used in traditional Chinese medicine to treat severe constipation since the seed of the plant can cause diarrhoea (Mairura, 2007).

In Ethiopia, *C. macrostachys* (Fig. 1) has many uses, and the best known among is as herbal preparation to treat human health problems (Lulekal *et al.*, 2008). It is used as an antidote for snake and scorpion venom (Flatie *et al*., 2009), for treatment of abdominal cramp, wound (Bekalo *et al*., 2009), skin disorders, malaria (Mesfin *et al*., 2009), and headache (Amenu, 2007). Leaf powder of *C. macrostachys* mixed with water is taken orally for treatment of diarrhea in Zegie Peninsula, Northwestern part of Ethiopia (Teklehaymanot and Giday, 2007). According to Mesfin *et al* (2009), *C. macrostachys* was also the preferred plant among the medicinal plants that were reported by more informants as a remedy to diarrhea.

Figure 1: Photograph of *Croton macrostachys*

1.6 Rationale for the Study

According to an estimate, over 80% of the developing world's population still rely on traditional medicines (mainly herbs) to cater to their health care needs despite

advances in modern medicine, which is mainly attributed to strong cultural belief, accessibility, and affordability (Bashir *et al*., 2011). WHO also encourages studies for the treatment and prevention of diseases like diarrhea on the basis of traditional medical practices (Singh and Verma, 2012). Despite such a high dependency, many of them have not been investigated for their described effects (Manigaunha *et al*., 2010). This wide use of traditional medicines in developing countries like Ethiopia plus the already existing medicinal use of the various species can be taken as an opportunity to investigate the scientific relevance of the plants.

C. macrostachys is one of the famous medicinal plants in Ethiopia, and has been used in the treatment of diarrhea in Northwestern part of Ethiopia, Zegie Peninsula, (Teklehaymanot and Giday, 2007) without any scientific proof for safety & efficacy. Thus investigating safety & efficacy of this plant in animal model could give valuable information in this regard. The finding of this research could be used as an input in searching of new anti-diarrheal agent that might solve problems associated with the conventional anti-diarrheal drugs. It could also give direction for traditional users on different way of preparation and use of the plant. In addition, it could help the scientific community to further investigate the plant *C. macrostachys* by initiating advanced studies on molecular mechanisms and formulation by identifying the specific agent responsible for its anti-diarrheal effect.

2. OBJECTIVES

2.1 General Objective:

To assess the *in vivo* anti-diarrheal activity of 80% methanolic extract of the leaves of *Croton macrostachys* in rats.

2.2 Specific Objectives

- ✓ To evaluate the effect of 80% methanolic extract of the leaves of *C. macrostachys* on castor oil induced diarrhea in rats.
- ✓ To evaluate the effect of 80% methanolic extract of the leaves of *C. macrostachys* on castor oil induced small intestinal transit using charcoal meal test in rats.
- ✓ To assess the anti-enteropooling effect of 80% methanolic extract of the leaves of *C. macrostachys* on castor oil induced enteropooling in rats.

3. MATERIALS AND METHODS

3.1. Drugs and Chemicals

Distilled Water (Ethiopian Pharmaceutical Manufacturing, Ethiopia), Methanol (Blulux, India), Castor oil (Amman Pharmaceutical Industries Co, Jordan), Activated Charcoal (The British Drug House, Ltd., London), Atropine sulphate (Sigma-Aldrich Chemie GMbH, Germany), Loperamide (Daehwa Pharmaceutical, Republic of Korea), and Tween 80 (BDH limited, England) obtained from local vendors were used.

3.2 Plant Material

The leaves of *C. macrostachys* were collected from Sebeta, 25 km west of Adiss Ababa, Oromia Region, Ethiopia in March 2013. Identification and authentication of the plant specimens were done by Ato Melaku Wendafrash (Seniour Botanist) at the National Herbarium, Department of Biology of Addis Ababa University, where a specimen was deposited with voucher number LK00023 for future reference.

3.3 Experimental Animals

Wistar Albino rats (150-250 g) of either sex bred and maintained at the animal house of School of Pharmacy, Addis Ababa University, were used. They were housed in a standard environment. Animals were acclimatized for one week to the experimental environment and provided with a commercial food and water *ad libitum*. The rats were maintained and handled according to international guidelines for use and maintenance of experimental animals (OECD, 2001).

3.4 Plant Extraction

The plant material was thoroughly washed with tap water to remove dirt and soil. The leaves were air dried at room temperature under shade and reduced to appropriate size by grinding. Crude extract was prepared by cold maceration technique. A total of 400 g dried leaves were extracted by maceration (100 g of dried leave in 600 ml of 80% methanol) for 72 h. The mixture was first filtered using gauze and then the filtrate was passed through Whatman filter paper (No 3, 15 cm size with retention 6 µm). After filtration, the residue was re-macerated for another 72 h two times. The combined filtrate was then evaporated using rota vapor (Buchi labortechnik AG, Switzerland) set at 40 ºC to remove the solvent. The residue was then freeze dried using a lyophylizer (Gperon, Korea) set at -56^{o}C of temperature and 96 torr of pressure. After drying, a total of 71.2 g of dry extract was harvested (17.8 % yield). The dried extract was placed in a screw cup vials and stored in a refrigerator until use.

3.5 Grouping and Dosing

The animals were randomly divided into five groups of 6 animals each for the anti-diarrheal tests. In each anti-diarrheal model, there were a negative control, a positive control, and three test groups. The negative control groups were treated with Tween 80 (2 % in water), positive control groups were given the standard drug loperamide (10 mg/kg) in castor oil induced diarrhea and anti-enteropooling models, and atropine sulphate (10 mg/kg) in charcoal meal test. The test groups were treated with 200, 400, and 600 mg/kg doses of the leaf extract of *C. macrostachys*. Dose determination was done by a pilot test. All administrations were done via the oral route and maximum volume administered was 2 ml.

3.6 Determination of Anti-diarrheal Activity

3.6.1 Castor Oil Induced Diarrhea

Following grouping, each animal fasted for 24 h was placed in individual cage, the floor of which was lined with white paper and replaced every hour. Diarrhea was induced by administering 2 ml of castor oil orally to each rat. Each animal received either vehicle or treatment one hour before castor oil according to the respective grouping as described under section 3.5. Onset of diarrhea, number of diarrheal episodes, frequency of defecation, weight of wet stools, and total weight of stools were recorded for each animal, for a total of 4 h. The results were recorded by taking the vehicle groups as 100% and calculated as percentage inhibition for the extracts and positive control groups (Jain *et al*., 2011) using the formula below (Shiramane *et al.*, 2011).

$$Percentage\ of\ protection(\%) = \frac{Mean\ weight\ of\ stool\ of\ control - Mean\ weight\ of\ stool\ of\ drug/extract\ treated}{Mean\ weight\ of\ stool\ of\ control} \times 100$$

3.6.2 Charcoal Meal Test

Animals were fasted for 24 h with free access to water and treated according to their respective grouping 30 min before the administration of castor oil. One ml of marker (5% charcoal suspension in 2% Tween 80) was administered orally 30 min after castor oil treatment. The animals were sacrificed after 30 min of charcoal administration, and the small intestine, from the pylorus to the cecum, was rapidly removed and laid out on white paper. The tissue was then inspected and the distance traversed by the charcoal meal was measured. This distance was calculated as a

percentage of the whole intestine length using the following relation (Akuodor *et al*., 2011).

$$Persistalisis\ Index(PI) = \frac{Distance\ travelled\ by\ the\ charcoal\ meal}{Total\ length\ of\ small\ intestine} \times 100$$

$$\%\ of\ inhibition = \frac{PI\ of\ negative\ control - PI\ of\ drug/extract\ treated}{PI\ of\ negative\ control} \times 100$$

The *in vivo* anti-diarrheal index (ADI) was then calculated according to the formula shown below (Ching *et al*., 2008; Franca *et al*., 2008; Shoba and Thomas, 2001; Vogel, 2002)

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

Where: Dfreq = Delay in defecation time or diarrheal onset (in % of control), Gmeq = Gut travel reduction (in % of control) and Pfreq = purging frequency as number of stool reduction (in % of control).

3.6.3 Castor Oil-Induced Enteropooling

Intraluminal fluid accumulation was determined by using the method described by Adeyemi and Akindele (2008). Rats were grouped as described earlier and deprived of food and water for 18 h prior to the experiment. Animals were then dosed according to their grouping 30 min prior to castor oil administration. Thirty minutes after castor oil administration all the rats were sacrificed, and then the small intestine was removed, tied with thread at the pyloric end and the ileocaecal junction. After taking weight of the intestine, the content was milked into a graduated tube and volume was measured. The weight of the intestine was taken again and the difference

between the full and empty intestine was calculated. Percentage reduction of intestinal secretion (volume) and weight of intestinal content was calculated relative to the negative control (Adeyemi and Akindele, 2008).

$$Percentage\ of\ reduction(\%) = \frac{Mean\ weight/volume\ of\ intestinal\ content\ of\ the\ control - Mean\ weight/volume\ of\ intestinal\ content\ of drug/extract\ treated}{Mean\ weight/volume\ of\ intestinal\ content\ of\ the\ control} \times 100$$

3.7 Statistical Analysis

Results are expressed as mean ± standard error of mean (SEM) of responses. All the results were analyzed statistically using SPSS Software Ver. 16.0 and the statistical significance was determined using One-way Analysis of Variance (ANOVA) followed by Tukey Post-hoc test. Linear regression analysis was also used to correlate dose with response. *P* value less than 0.05 was considered to be significant.

4. RESULTS

4.1 Castor Oil-Induced Diarrhea

Castor oil increased the mean number of defecation and hastened the onset of diarrhea in the negative control group. It also increased the mean number of diarrheic feces and the mean weight of stool in this group. This increase was regarded as maximum (100%). As presented in Table 1 and Figs 2 & 3; all doses of the extract produced a significant delay ($p<0.001$) in onset of diarrhea and also significantly decreased ($p<0.001$) the mean number of defecation, diarrheic feces, and weight of stool compared to controls. Percent protection conferred by the different doses of the extract was 93.20%, 97.99%, and 100% for 200mg/kg, 400mg/kg, and 600mg/kg, respectively. Moreover, diarrhea was seen in a few numbers of rats at 200 and 400 mg/kg and no diarrhea at all was observed at 600 mg/kg. Thus, the plant extract at 600mg/kg was as effective as the standard drug, loperamide, since no wet feces were recorded in both cases. Correlation between the responses (percentage protection against defecation and diarrheic faces) and dose increment showed no statistically significant relation, indicating; percentage protection by *C. macrostachys* was not dose-dependent. There was also no significant difference in producing effect between different doses of the extract as well as the extract and the standard drug compared to the control.

Table 1: Anti-diarrheal activity of 80% methanolic extract of the leaves of *Croton macrostachys* H. against castor oil induced diarrhea in rats.

Group	Dose(in mg/Kg)	Weight of Wet Stools(g)	Total Weight of Stools(g)	% inhibition of defecation	% inhibition diarrheic feces
Control	-	2.45 ± .38	2.98 ± .59	-	-
CM	200	.07± .07*	.17 ± .11*	94.41*	93.20*
CM	400	.06 ± .06*	.06 ± .06*	99.59*	97.99*
CM	600	.00 ± .00*	.00 ± .00*	100*	100*
Loperamide	10	.00 ± .00*	.00 ± .00*	100*	100*

Data are expressed as mean ± SEM (n = 6). *p < 0.001 against control group. CM, *Croton macrostachys* leaves.

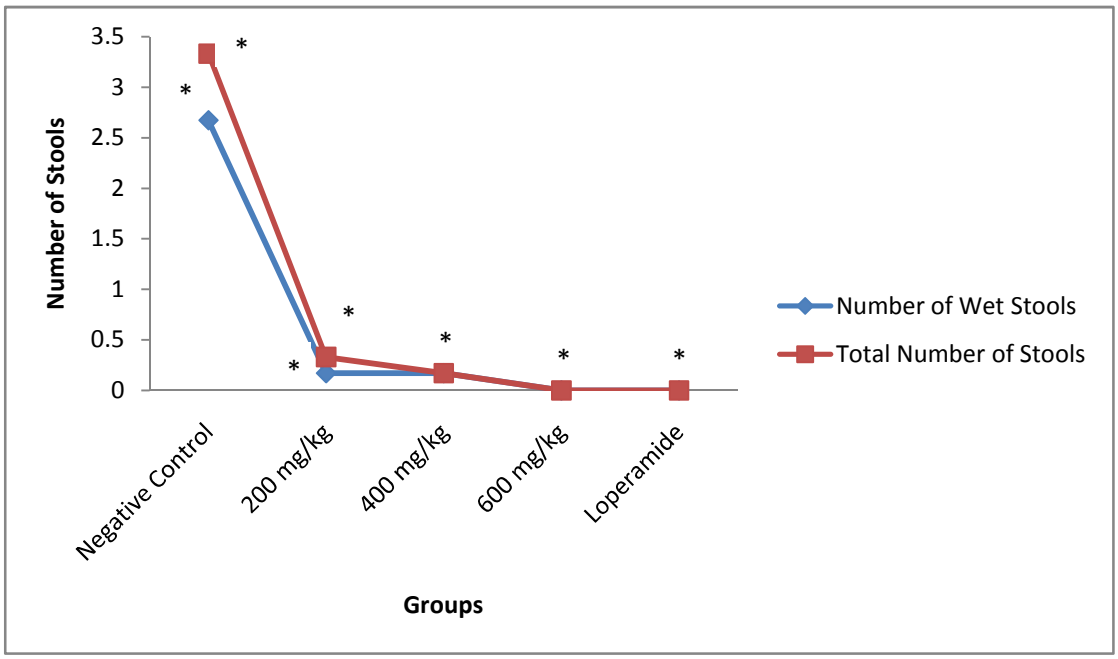

Figure 2: Effect of 80% the methanolic extract of the leaves of *Croton macrostachys* on number of wet stool and total number of stools (dry + wet) observed in the period of 4 hr. Data are expressed as mean ± SEM (n = 6). *p < 0.001 against control group.

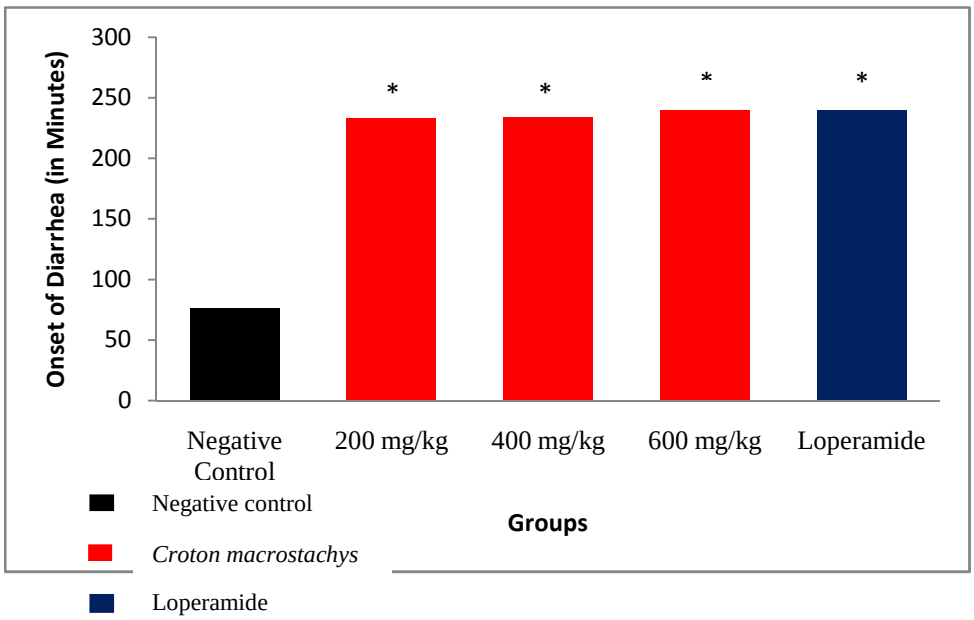

Figure 3: Effect of 80% methanolic extract of the leaves of *Croton macrostachys* extract on onset of diarrhea observed in the period of 4 hr. Data are expressed as mean ± SEM (n = 6). *p < 0.001 against control group.

4.2 Charcoal Meal Test

C. macrostachys significantly decreased ($p<0.01$) perstalitic index at 400 mg/kg (29%) and 600 mg/kg (30%), but lacked effect at 200 mg/kg since the result showed no significant difference when compared to negative control group. The standard drug, atropine sulphate, reduced the small intestinal transit significantly ($p<0.001$) with percentage value of 36.1%. There was no significant difference seen in activity between different doses of the extract as well as the extract and the standard drug except at dose of 200 mg/kg at which the difference was statistically significant ($P<0.01$) when compared to the standard (Table 2).

Table 2: Effect of 80% methanolic extract of the leaves of *Croton macrostachys* H. on Castor oil induced small intestinal transit in rats.

Group	Dose	Peristaltic Index (PI) (%)	Inhibition of Diarrhea (%)
Control	-	56.38 ± 1.76	-
CM	200 mg/kg	50.54 ± 2.54	10.4b*
CM	400 mg/kg	40.03 ± 3.00	29.0 a*
CM	600 mg/kg	39.48 ± 2.27	30.0 a*
Atropine sulphate	(10 mg/kg)	36.06 ± 3.64	35.3 a**

Data are expressed as mean ± SEM (n = 6). a, compared to control; b, compared to the standard; *p < 0.01 and **p < 0.001. CM, *Croton macrostachys* leaves.

ADI was calcualted to see the relative effect of the extract (Table 3). The highest ADI was obtained at a dose of 600 mg/kg of the extract, which was comparable to that produced by the standard.

Table 3: *In vivo* anti-diarrheal index of 80% methanolic extract of the leaves of *Croton macrostachys* H.

Group	Dose (in mg/kg)	Delay in defecation (time of onset)	*D*freq	Gut meal travel distance	*G*meq	Purging frequency in number of wet stools	*P*freq	*In vivo* anti-diarrheal index
Control	-	76.33 ± 7.28	-	56.38 ± 1.76	-	2.67 ± .21	-	-
CM	200	232.67 ± 7.14	204.8	50.54 ± 2.54	10.4	.17 ± .17	93.6	58.4
CM	400	233.67 ± 6.33	206	40.03 ± 3.00	29.0	.17 ± .17	93.6	82.4
CM	600	240.00 ± .00	214.4	39.48 ± 2.27	30.0	.00 ± .00	100	86.3

Values are expressed as mean ± SEM (n = 6).

*D*freq is the delay in defecation time or diarrhea onset (in % of control).

*G*meq is the gut meal travel reduction (in % of control).

*P*freq is the purging frequency, as number of stool reduction (in % of control).

CM, *Croton macrostachys* leaves

4.3 Castor Oil-Induced Enteropooling

C. macrostachys was also found to possess anti-enteropooling activity as it significantly decreased both weight and volume of intestinal fluid content in rats at all doses (Table 3). At 200 mg/kg it significantly decreased both volume ($p<0.05$) and weight ($p<0.01$) of intestinal fluid content with percentage reduction of 23.8 and 29.3 %, respectively, against the negative control group. The plant extract also conferred significant protection against intestinal secretion at both 400 and 600 mg/kg, and the effect was in a dose dependent manner ($p<0.05$) as it was checked by making use of linear regression analysis. The extract at 600 mg/kg significantly decreased ($p<0.05$) intestinal secretion compared to its effect at 200 mg/kg. As depicted in Table 3, the standard drug, loperamide, significantly decreased both intestinal secretion and weight of intestinal content in rats.

Table 4: Effect of 80% methanolic extract of the leaves of *Croton macrostachys* H. extract on castor oil induced enteropooling in rats.

Group	Dose	Weight of intestinal content (g)	Percent inhibition (weight) (%)	Volume of intestinal content (ml)	Percent inhibition (volume) (%)
Control	-	4.71 ± .34	-	4.29 ± .29	-
CM	200 mg/kg	3.33 ± .24	29.3a**b***	3.27 ± .21	23.8a*b***c*
CM	400 mg/kg	3.21 ± .34	31,8a*b***	3.12 ± .21	27.3a*b***
CM	600 mg/kg	2.24 ± .18	52.4a***b*	2.23 ± .14	48.0a***
Loperamide	5 mg/kg	1.11 ± .15	76.4a***	1.14 ± .19	73.4a***

Data are expressed as mean ± SEM (n = 6). a, compared to control; b, compared to the standard; c, compared to 600 mg/kg. *p<0.05, **p<0.01, ***p<0.001. CM, *Croton macrostachys* leaves.

5. DISCUSSION

The present study aimed at providing the pharmacological basis for the medicinal use of the leaf extract of *C. macrostachys* in diarrhea using *in vivo* assays in rats. Pilot study was done on both 80% methanolic extract and aqueous extract of the leaves of the plant to determine the solvent which could effectively extract the bioactive components with anti-diarrheal effect and the dose to be administered. The study showed only 80% methanolic extract was found to have anti-diarrheal activity. This might be because methanol efficiently penetrates cell membranes, permitting the extraction of high amounts of endocellular components in contrast to solvents of lower and higher polarity. Hence methanol chiefly dissolves polar constituents together with medium and low polar compounds extracted by co-solublization (Panda *et al*., 2012). Therefore, the observed anti-diarrheal activity on 80% methanolic extract might be due to medium or low polar constituents or synergistic effect of all polar, medium and low polar constituents of the extract. Dose range finding studies also showed that doses below 200 mg/kg were ineffective. Thus, anti-diarrheal activity of leaf extract of *C. macrostachys* was evaluated at doses of 200, 400, and 600 mg/kg body weight.

Castor oil was used in this study to induced diarrhea. Several mechanisms have been proposed to explain the diarrheal effect of castor oil including inhibition of intestinal Na+, K+-ATPase activity to reduce normal fluid absorption, activation of adenylate cyclase or mucosal cAMP mediated active secretion, stimulation of prostaglandin, platelet activating factor (Hossain *et al.*, 2012)$_a$ and nitric oxide formation (Bakare *et al.*, 2011). However, it is well documented that castor oil produces diarrhea due to its most active metabolite, ricinoleic acid by hypersecretory response (Bakare *et al*.,

2011), which causes irritation and inflammation of the intestinal mucosa, leading to release of prostaglandins, which results in stimulation of secretion and motility (Hossain *et al*., 2012)$_b$. The gut wall contains prostaglandins E and F with prostaglandin synthetase activity mainly found in the mucosa (Latha and Reddy, 2009). Prostaglandins are implicated in the pathophysiology of diarrhea. Prostaglandins stimulate secretion by at least two mechanisms. The first is direct activation of the intestinal mucosal cell, where they stimulate active anion secretion and inhibit neutral absorption of sodium and chloride through the activation of adenylate cyclase and generation of cAMP. At concentrations that do not stimulate adenylate cyclase, prostaglandins seem to stimulate calcium influx that is dependent on extracellular calcium, although the mechanism for this action remains unknown. In addition to their direct effects, certain eicosanoids, notably prostaglandin I2 and possibly LTC4, stimulate enteric neurons and release neurotransmitters such as acetylcholine. The peptidoleukotrienes LTC4 and D4 have also been found to be potent stimulators of smooth muscle contraction thereby causing reduced transit time that would contribute to the diarrhea (Urayama and Chang, 1997).

Since the extract of *C. macrostachys* successfully inhibited the castor oil-induced diarrhea, the extract might have exerted its anti-diarrheal action via antisecretory and antimotility mechanisms, as it was evident from the protection observed against diarrheic feces and reduction in frequency of defecation in the test groups at all doses of the extract. The plant also showed delay in onset of diarrhea to almost equivalent extent to the standard drug at all doses. The observed effects of the plant extract might be attributed to the presence of active chemical compounds (Palombo, 2006) present within its leaves. Phytochemical analysis done on 80% methanolic extract of the leaves of *C. macrostachys* collected from similar geographical location showed that it

contain different secondary active metabolites such as alkaloids, saponins, phenolic compounds, cardiac glycosides, tannins, terpenoids and flavonoids (Bantie *et al*., 2014). Investigations of the mode of action of phytochemicals in extracts of different plant species indicated that tannins and flavonoids increase colonic water and electrolyte reabsorption and other phytochemicals such as phenolic compounds and alkaloids act by inhibiting intestinal motility (Palombo, 2006). Again, flavonoids present in the plant extract were reported to inhibit release of autacoids and prostaglandins, thereby inhibit motility and secretion (Hossain *et al.*, 2012)$_a$ induced by castor oil. Flavonoids were also found to display a wide range of biological activities including inhibition of enzymes such as prostaglandin synthase, cycloxygenase, and lypoxygenase (Mukinda, 2005) that might mainly contribute to its anti-diarrheal activity since these enzymes are responsible for the formation prostaglandin and leukotrines, agents involved in pathophysiology of diarrhea. Flavonoids are a large group of polyphenolic compounds and have also been reported to exhibit a wide variety of biological effects such as anti-oxidation, anti-inflammation, anti-platelet, anti-thrombotic action, anti-allergic effects, anti microbial, anti-mutagenic effects, and induce detoxifying enzyme systems such as glutathione S-transferase (Mukinda, 2005). Most of the aforementioned mode of actions might have contributed to its anti-diarrheal effect. The secondary metabolites detected in the leaf extract of *C. macrostachys* might possess similar therapeutic property to the aforementioned plant constituents indicating the observed efficacy against castor oil induced diarrhea might be ascribed to these constituents. Researches done on similar species of plants such as *C. blanchetianus* Baill, *C. rhamnifolius* Willd, and *C. argyroglossum* Baill showed that the percentage content of flavonoids and/or tannins was responsible for their anti-diarrheal activity (Siqueira *et al*., 2012).

Since NO was also reported to involve in pathophysiology of diarrhea (Hossain *et al*., 2012)$_b$, NO scavenging capacity of the extract may help to arrest the chain of reactions initiated by excess generation of NO. NO works as an atypical neural modulator that is involved in neurotransmitter release such as acetylcholine and neuronal excitability, which could have a role in the pathophysiology of diarrhea. NO is also implicated for inflammation, cancer and other pathological conditions (Hossain *et al*., 2012)$_b$. It participates in pathogenic pathways underlying IBD which is mainly involved in pathophysiology of diarrhea (Urayama and Chang, 1997). Various phytochemical components, especially polyphenols (such as flavonoids and tannins etc) were known to be responsible for the free radical scavenging and antioxidant activities of plants (Hossain *et al.,* 2012)$_b$. Hence NO (produced by the action of castor oil in rats gastrointestinal system) scavenging capability of these phytochemicals might be attributed to the anti-diarrheal effect of the leaf extract of *C. macrostachys*.

Pretreatment with the 80% methanolic extract of *C. macrostachys* suppressed the propulsive movement or transit of charcoal meal through the intestinal tract, and this clearly indicates that the leaf extract is capable of reducing the frequency of stools in diarrheal conditions by prolonging intestinal transit time. However, at 200 mg/kg the extract did not show statistically significant effect on intestinal transit, and this may indicate that higher doses are required to produce such effect. However, the same dose of the plant extract showed anti-diarrheal activity in castor oil induced diarrhea model, and this observation may suggest that the plant at low dose exclusively displays antisecretory activity but with increasing dose both antisecretory and antimotility activities are apparent. Reduction in intestinal motility action of the plant might be ascribed to the presence of flavonoids and alkaloids in the extract as they

were mentioned to have such an action (Mukinda, 2005; Palombo, 2006). As revealed in the result section, the plant extract showed almost comparable efficacy to the standard drug at doses of 400 and 600 mg/kg indicating the plant might provide promising antimotility agent at higher doses if further little effort is applied. Delay in small intestinal and colonic motility causes further absorption of salt and water (Mercadante, 1995) from feces by increasing contact time between mucosal absorptive surface and luminal fluid (Urayama and Chang, 1997), and this may additionally contribute to reducing the watery texture of the stool.

In testing antienteropooling effect, the plant extract significantly reduced the volume and weight of intestinal content at all doses. At 200 mg/kg, the plant extract showed no effect in charcoal meal test model; however, significantly exhibited anti-diarrheal effect in the other two models. This indicates that the anti-diarrheal effect of *C. macrostachys* might largely be due to its antienteropooling effect or the dose of the plant extract required to produce antienteropooling effect is lower than the dose needed to produce antimotility effect. The standard drug loperamide showed significally better effect than all doses of the extract. The antienteropooling effect of the plant extract could be due to either a decrease in mucosal secretion or increase in mucosal absorption of water and electrolytes or both. Since electrolyte absorption determines the efficiency of nutrient absorption, it is likely that the enhanced electrolyte absorption by the extract might have encouraged the absorption of other intestinal contents. The solute absorption in any region of the intestine is a function of the rate of water uptake in that region. Thus, the solute absorption enhanced by the action of the plant extract may have created an osmotic gradient across enterocytes which might stimulated water absorption. These observations reasonably suggest that the extract inhibits gastrointestinal hyper-secretion and enteropooling by enhancing

electrolytes, solutes and water absorption from the intestinal lumen (Ezenwali et al., 2010). The antienteropooling activity of the plant extract might be attributed to flavonoids and tannins present. Tannins and flavonoids were found to increase colonic water and electrolyte reabsorption (Palombo, 2006). Inhibition of prostaglandin formation by flavonoids (Mukinda, 2005) might also contribute to its antisecretory effect. Many species of the genus Croton were also reported to have promising antienteropooling activity. Studies on *C. urucurana* indicated that flavonoids present in the plant probably exhibited antisecretory activity (Velázquez *et al*., 2012). Another study on *C. lechleri* indicated that an oligomeric proanthocyanidin (SP-303) extracted from the bark latex of the tree produced high inhibitory effect on cholera toxin-induced fluid accumulation and chloride secretion by blocking the CFTR Cl^- channel (Fischer *et al*., 2004). Tannins were also known to have the effect through denaturing or precipitating the proteins by the formation of protein tannate, thereby causing the intestinal mucosa more resistant and reduces secretion (Hossain *et al*., $2012_{a;}$ Latha and Reddy, 2009; Njoku and Obi, 2009). It has also been confirmed the level of tannins is more concentrated in plants having anti-diarrheal activities (Siqueira *et al.*, 2012).

Finally, the effects of C. macrostachys on all indicators of diarrhea investigated in this study were summed up in the calculation of the ADI as shown in Table 3. The higher the ADI value the greater the effectiveness in the treatment of diarrhea. Maximum ADI of 86.3% was produced by the extract at the dose of 600 mg/kg indicating treatment of diarrhea by the plant extract is highly effective at this dose, a value lower than that elicited by standard (91.8%).

6. CONCLUSION

The result of the present study suggests that the 80% methanolic leaf extract of *C. macrostachys* possessed significant anti-diarrheal activity due to its effect on reduction in onset of diarrheal stool, delay in gastrointestinal propulsion, and inhibition of fluid accumulation in the intestinal tract of rats. Hence, the study supports the claims made by traditional medical practitioners using the plant in the treatment of diarrhea. Based on these findings, it can be assumed that *C. macrostachys* could be a potential source for discovery of new and effective anti-diarrheal drug.

7. RECOMMENDATION

The 80% methanolic extract of the leaves of *C. macrostachys* has been proven to have a promising anti-diarrheal activity; therefore, the following recommendations are forwarded to further study and fully benefit from the plant.

- ✓ It is a good candidate for further fractionation of the extract and study.
- ✓ The activity of the extract should also be evaluated using in vitro method to check its antibiotic effect since it might also be source of drug to treat infectious diarrhea resistant to the conventional medicines.
- ✓ Activity guided isolation should be undertaken to isolate the active principle(s) and develop it into a good lead compound(s).
- ✓ Detailed pharmacological and toxicological studies are needed to evaluate its clear mechanism of action and safety profile.

REFERENCES

Adeyemi, O.O., Akindele, A.J., 2008. Antidiarrhoeal activity of the ethyl acetate extract of *Baphia nitida* (Papilionaceae). J. Ethnopharmacol. 116, 407–412.

Akalin, H., 1993. Quinolones in the treatment of acute bacterial diarrhoeal diseases. Drugs 45, 114–118.

Akuodor, C., Muazzam, I., Usman-Idris, M., Megwas, U., Akpan, J., Chilaka, K., Okoroafor, D., Osunkwo, U., 2011. Evaluation of the antidiarrheal activity of methanol leaf extract of *Bombax buonopozense* in Rats. Ibnosina J. Med. Biomed. Sci. 3, 15–20.

Al-Abri, S., Beeching, N., Nye, F., 2005. Traveller's diarrhoea. Lancet Infect Dis 5, 349–60.

Amasheh, S., Barmeyer, C., Koch, C., 2004. Cytokine-dependent transcriptional down-regulation of epithelial sodium channel in ulcerative colitis. Gastroenterology 126, 1711.

Amenu, E., 2007. Use and Management of medicinal plants by indigenous people of Ejaji area (Chelya Woreda) West Shoa, Ethiopia: An ethnobotanical approach. Addis Abeba University, Addis Abeba, Ethiopia.

Bakare, R.., Magbagbeola, O.A., Akinwande, A.I., Okunowo, O.W., Green, M., 2011. Antidiarrhoeal activity of aqueous leaf extract of *Momordica charantia* in rats. J. Pharmacogn. Phytother. 3, 1–7.

Bantie, L., Assefa, S., Teklehaimanot, T., Engidawork, E., 2014. In vivo antimalarial activity of the crude leaf extract and solvent fractions of *Croton macrostachys* Hocsht.

(Euphorbiaceae) against *Plasmodium berghei* in mice. BMC Complement. Altern. Med. 14, 10 pages.

Bashir, S., Memon, R., Gilani, A., 2011. Antispasmodic and antidiarrheal activities of *Valeriana hardwickii* Wall. rhizome are putatively mediated through calcium channel blockade. Evid. Based Complement. Alternat. Med. 6 pages.

Bauer, K., Wolf, D., 2005. Do antibiotics interfere with the efficacy of oral contraceptives? J Fam Pr. 54, 1079–1080.

Bekalo, T., Woodmatas, S., Woldemariam, Z., 2009. An ethnobotanical study of medicinal plants used by local people in the lowlands of Konta Special Woreda, southern nations, nationalities and peoples regional state, Ethiopia. J. Ethnobiol. Ethnomedicine 5, 15 pages.

Bergogne-Berezin, E., 2000. Treatment and prevention of antibiotic associated diarrhea. Int. J. Antimicrob. Agents 521–526.

Binder, H., 2005. Intestinal fluid and electrolyte movement. Med. Physiol. 931–946.

Binder, H., 2009. Mechanisms of diarrhea in inflammatory bowel diseases. Ann N Acad Sci 1165, 285–293.

Binder, H., Reuben, A., 2005. Nutrient digestion and absorption. Med. Physiol. 947–974.

Bliss, D.Z., Doughty, D.B., Heitkemper, M.M., 2006. Pathology and management of bowel dysfunction, in: Urinary and fecal incontinence current management concepts. St Louis, Mosby Elesevier, pp. 425–456.

Camilleri, M., 2004. Chronic diarrhea: a review on pathophysiology and management for the clinical gastroenterologist. Clin Gastroenterol Hepatol 2, 198–206.

Chou, W., Wu, J., Wang, Y., Huang, H., Sung, F., Chuang, C., 2010. Modeling the impact of climate variability on diarrhea-associated diseases in Taiwan. Sci. Total Environ. 409, 43–51.

Dreyling, K., Hope, W., Peskar, B., 1987. Leukotrienes in Crohn's disease: effect of sulfasalazine and 5-aminosalicylic acid. Adv Prostaglandin Thromboxane Leukot Res 17, 339–343.

Drossman, D., Camilleri, M., Mayer, E., Whitehead, W., 2002. AGA technical review on irritable bowel syndrome. Gastroenterology 123, 2108–2131.

Ezenwali, M., Njoku, O., Okoli, C., 2010. Studies on the anti-diarrheal properties of seed extract of *Monodora tenuifolia*. Int J Appl Res Nat Prod 2, 20–26.

Farthing, M.J., Lindberg, G., Dite, P., Khalif, I., Salazar-Lindo, E., Ramakrishna, B.S., Goh, K., Thomson, A., Khan, A.G., 2008. Acute diarrhea. World Gastroenterol. Organ. Pract. Guidel.

Field, M., 2003. Intestinal ion transport and the pathophysiology of diarrhea. J. Clin. Invest. 111, 931–943.

Fischer, H., Machen, T.E., Widdicombe, J.H., Carlson, T.J., King, S.R., Chow, J.W., Illek, B., 2004. A novel extract SB-300 from the stem bark latex of *Croton lechleri* inhibits CFTR-mediated chloride secretion in human colonic epithelial cells. J. Ethnopharmacol. 93, 351–357.

Flatie, T., Gedif, T., Asres, K., Gebre-Mariam, T., 2009. Ethnomedical survey of Berta ethnic group Assosa Zone, Benishangul-Gumuz regional state, mid-west Ethiopia. J. Ethnobiol. Ethnomedicine 5, 11 pages.

Friedman, G., 2012. The role of probiotics in the prevention and treatment of antibiotic-associated diarrhea and *Clostridium difficile* colitis. Gastroenterol Clin N Am 41, 763–779.

Gregorio, G., Gonzales, M., Dans, L., Martinez, E., 2009. Polymer-based oral rehydration solution for treating acute watery diarrhoea (Review). Cochrane Collab.

Grundy, D., 2002. Neuroanatomy of visceral nociception: vagal and splanchnic afferent. Gut 51, I2–I5.

Guandalini, S., Vaziri, H. (Eds.), 2011. Diarrhea: Diagnostic and Therapeutic Advances. Clin. Gastroenterol.

Guerrant, R., Van Gilder, T., Steiner, T., 2001. Practice guidelines for the management of infectious diarrhea. Clin Infect Dis 331–351.

Halsey, J., 2009. Current Approaches to the Treatment of Gastrointestinal Infections: Focus on Nitazoxanide. Clin. Med. Ther. 1, 263–275.

Hammer, H., Santa Ana, C., Schiller, L., Fordtran, J., 1989. Studies of osmotic diarrhea induced in normal subjects by ingestion of polyethylene glycol and lactulose. J Clin Invest 84, 1056.

Hempel, S., Newberry, S., Maher, A., Wang, Z., V. Miles, J., Shanman, R., Johnsen, B., Shekelle, P., 2012. Probiotics for the prevention and treatment of antibiotic-associated diarrhea. J. Am. Med. Assoc. 307, 1959–1969.

Hogenauer, C., Hammer, H., Krejs, G., Reisinger, E., 1998. Mechanisms and management of antibiotic-associated diarrhea. Clin Infect Dis 27, 702–770.

Hoque, K., Chakraborty, S., Ali Sheikh, I., Woodward, O., 2012. New advances in the pathophysiology of intestinal ion transport and barrier function in diarrhea and the impact on therapy. Anti Infect Ther 10, 687–699.

Hossain, H., Dey, S., Hira, A., Howlader, S., Ahmed, A., Sultana, S., 2012. Evaluation of antidiarrhoeal potential of the ethanolic extract of three bangladeshi medicinal plants. Int. J. Pharm. Phytopharm. Res. 1, 371–374.

Hossain, H., Hassan, M., Jahan, I.A., Nimmi, I., Islam, A., 2012. Antidiarrheal activity, nitric oxide scavenging and total tannin content from the bark of *Ceriops Decandra* (Griff) Ding Hou. Int. J. Pharm. Sci. Res. 3, 1306–1311.

Hostos, E.L., Choy, R.K., Nguyen, T., 2011. Developing novel antisecretory drugs to treat infectious diarrhea. Future Med Chem 3, 1317–1325.

Jain, D., Patel, N., Somani, R., 2011. Antidiarrheal activity of ethanolic heartwood extract of *Pterocarpus Marusupium*. Pharmacologyonline 1, 552–559.

Jensen, R., 1999. Overview of chronic diarrhea caused by functional neuroendocrine neoplasms. Semin Gastrointest Dis 10, 156–172.

Kaplan, W., Wirtz, V., Teeuwisse, A., Stolk, P., Duthey, B., Laing, R., 2013. Priority Medicines for Europe and the World 2013 Update.

Kerlin, P., Zinsmeister, A., Phillips, S., 1982. Relationship of motility to flow of contents in the human small intestine. Gastroenterology 82, 701–706.

Kulkarni, H., Mamtani, M., Patel, A., 2012. Roles of zinc in the pathophysiology of acute diarrhea. Curr Infect Rep 14, 24–32.

Latha, L.., Reddy, P.., 2009. Antimicrobial, antidiarrhoeal and analysis of phytochemical constituents of *Sphaeranthus amaranthoides*. Indian J. Sci. Technol. 2, 45–48.

Laursen, L., Lauritsen, K., Bukhave, K., Rask-Madsen, J., Jacobsen, O., Naesdal, J., 1994. Selective 5-lipoxygenase inhibition by zileuton in the treatment of relapsing ulcerative colitis: a randomized double-blind placebo-controlled multicenter trial. Eur J Gastroenterol Hepacol 6, 209–215.

Lawler, J., Wallace, M., 2003. Diagnosis and treatment of bacterial diarrhea. Curr. Sci. Inc 5, 287–294.

Lazzerini, M., Ronfani, L., 2008. Oral zinc for treating diarrhoea in children. Cochrane Database Syst. Rev. 2.

Lori, A., 2008. Traveler's Diarrhea: An update on prevention and treatment. J. Midwifery Women's Health 53, 251–254.

Lulekal, E., Kelbessa, E., Bekele, T., Yineger, H., 2008. An ethnobotanical study of medicinal plants in Mana Angetu District, southeastern Ethiopia. J. Ethnobiol. Ethnomedicine 4, 10 pages.

Mairura, F.S., 2007. *Croton macrostachys* Hochst. ex Delile. In: Schmelzer. Med. PlantsPlantes Médicinales 1 11.

Mamikunian, G., Vinik, A., O'Dorisio, T., Woltering, E., Go, V., 2009. Neuroendocrine tumors : A comprehensive guide to diagnosis and management.

Manigaunha, A., Ganesh, N., Kharya, M.D., 2010. Morning glory: A new thirst in-search of *de-novo* therapeutic approach. Int. J. Phytomedicine 2, 18–21.

Mbiantcha, M., Nguelefack, T., Ndontsa, B., Tane, P., Kamanyi, A., 2013. Preliminary assessment of toxicity of *Croton macrostachys* stem bark (Euphorbiaceae) extracts. Int. J. Pharm. Chem. Biol. Sci. 3, 113–132.

McFarland, L., 2006. Meta-Analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* Disease. Am. J. Gastroenterol. 101, 812–822.

Mercadante, S., 1995. Diarrhea in terminally ill patients: Pathophysiology and treatment. J. Pain Syptom Manag. 10, 298–309.

Mesfin, F., Demissew, S., Teklehaymanot, T., 2009. An ethnobotanical study of medicinal plants in Wonago Woreda, SNNPR, Ethiopia. J. Ethnobiol. Ethnomedicine 5.

Mourad, F., O'Donnell, L., Dias, J., 1995. Role of 5-hydroxytryptamine type 3 receptors in rat intestinal fluid and electrolyte secretion induced by *cholera* and *Escherichia coli* enterotoxins. Gut 37, 340–345.

Mukinda, J.T., 2005. Acute and chronic toxicity of the flavonoid- containing plant, *Artemisia afra* in rodents (MSc Thesis). University of the Western Cape, Bellville, South Africa.

Navaneethan, U., Giannella, R., 2008. Mechanisms of infectious diarrhea. Nat Clin Pr. Gastroenterol Hepatol 5, 637–647.

Njoku, O., Obi, C., 2009. Phytochemical constituents of some selected medicinal plants. Afr. J. Pure Appl. Chem. 3, 228–233.

Palombo, E.A., 2006. Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: Modes of action and effects on intestinal function. Phytother. Res. 20, 717–724.

Panda, S., Patra, N., Sahoo, G., Bastia, A., Dutta, S., 2012. Antidiarrheal Activities of Medicinal Plants of Similipal Biospher Reserve, Odisha, India. Int J Med Arom Plants 2, 123–134.

Pawlowski, S., Warren, C., Guerrant, R., 2009. Diagnosis and treatment of acute or persistent diarrhea. Gastroenterology 136, 1874–1886.

Poley, J., Hofmann, A., 1976. Role of fat maldigestion in pathogenesis of steatorrhea in ileal resection: fat digestion after two sequential test meals with and without cholestyramine. Gastroenterology 71, 38–44.

Prasad, R., Izam, A., Khan, M., 2012. Jatropha curcas: Plant of medical benefits: Review. J. Med. Plants Res. 6, 2691–2699.

Prior, A., Colgan, S., Whorwell, P., 1990. Changes in rectal sensitivity after hypnotherapy in patients with irritable bowel syndrome. Gut 31, 896–898.

Proano, M., Camilleri, M., Phillips, S., Brown, M., Thomforde, G., 1990. Transit of solids through the human colon: regional quantification in the unprepared bowel. Am J Physiol 258, G856–G862.

Rang, H.P., Dale, M.M., Ritter, J.M., Moore, P.K., 2003. Pharmacology, 5th ed. Elsevier Science Ltd, New Delhi, India.

Regassa, G., Birke, W., Deboch, B., Belachew, T., 2008. Environmental determinants of diarrhea among under-five children. Ethiop. J. Health Sci. 18, 39–45.

Rout, S., Choudary, K.A., Kar, D.M., Das, L., Jain, A., 2009. Plants in traditional medicinal system - future source of new drugs. Int. J. Pharm. Pharm. Sci. 1, 23 pages.

Salazar-Lindo, E., 2011. Acute infectious diarrhoea in children – The Role of drug treatment. Eur. Gastroenterol. Hepatol. Rev. 7, 31–36.

Sarin, av, 2012. Herbal antidiarrhoeals: A Review. Int. J. Res. Pharm. Biomed. Sci. 3, 637–640.

Scaldaferri, F., Pizzoferrato, M., Pecere, S., Forte, F., Gasbarrini, A., 2012. Bacterial flora as a cause or treatment of chronic diarrhea. Gastroenterol Clin N Am 41, 581–602.

Schiller, L., 1999. Secretory diarrhea. Curr Gastroenterol Rep 1, 389.

Sellin, J., 2001. The pathophysiology of diarrhea. Clin. Transplant. 15.

Sharon, P., Ligumsky, M., Rachmilewitz, D., Zor, U., 1978. Role of prostaglandins in ulcerative colitis, enhanced production during active disease and inhibition by sulfasalazine. Gastroenterology 75, 638–640.

Shiramane, R., Biradar, K., Chivde, B., Shambhulingayya, H., goud, V., 2011. *In-vivo* antidiarrheal activitiy of ethanolic extract of *Delonix regia* flowers in experimental induced diarrhea in wistar albino rats. Int. J. Res. Pharm. Chem. 1, 442–447.

Singh, K., Verma, B., 2012. An integrated approach towards diarrhoea. Res. Rev. J. AYUSH 1, 11–33.

Siqueira, C., Cabral, D., Sobrinho, T., Amorim, E., Melo, J., Araujo, T., Albuquerque, U., 2012. Levels of tannins and flavonoids in medicinal plants: Evaluating bioprospecting strategies. Evid. Based Complement. Alternat. Med. 7 pages.

Strasinger, S., Di Lorenzo, M., 2008. Chapter 15: Fecal analysis, in: Urinalysis & body fluids. Philadelphia, Pennsylvania, pp. 245–257.

Sutariya, S., Talsania, N., Shah, C., 2011. Study of prevalence of diarrheal diseases amongst under five population. Natl. J. Community Med. 2, 96–99.

Teklehaymanot, T., Giday, M., 2007. Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. J. Ethnobiol. Ethnomedicine. 3, 11 pages.

Thevarajah, S., Polaneczky, M., Scherl, E., Frissora, C., 2005. Hormonal influences on the gastrointestinal tract and irritable bowel syndrome. Pract. Gastroenterol. 62–74.

UNICEF, 2012. Tackling the deadliest diseases for the world's poorest children.

Urayama, S., Chang, E., 1997. Mechanisms and treatment of diarrhea in inflammatory bowel diseases. Inflamm. Bowel Dis. 3, 114–131.

Vassallo, M., Camilleri, M., Phillips, S., Brown, M., Chapman, N., Thomforde, G., 1992. Transit through the proximal colon influences stool weight in the irritable bowel syndrome. Gastroenterology 102, 102–108.

Velázquez, C., Calzada, F., Bautista, M., Gayosso, J., 2012. Management of secretory diarrhea, in: Current Concepts in Colonic Disorders. InTech, México.