Evaluation of the anti-diarrheal activity of the solvent fractions of *Croton macrostachyus* Hocsht. ex Del. (Euphorbiaceae) leaves in mice

Amsalu Degu

A Thesis submitted to

The Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences

Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacology

Addis Ababa University

Addis Ababa, Ethiopia

October 2014

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Amsalu Degu, entitled "Evaluation of the anti-diarrheal activity of the solvent fractions of *Croton macrostachyus* Hocsht. ex Del. (Euphorbiaceae) leaves in mice" 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:

External Examiner Getnet Yimer (MD,PhD) Signature _____Date_____

Internal Examiner Teshome Nedi (PhD) Signature_____ Date_____

Advisor Ephrem Engidawork (PhD) Signature_____ Date_____

Advisor Workineh Shibeshi (PhD) Signature_____Date_____

_____

Chair of Department

Evaluation of the anti-diarrheal activity of the solvent fractions of *Croton macrostachyus* Hocsht. ex Del. (Euphorbiaceae) leaves in mice

Amsalu Degu

Addis Ababa University, 2014

Abstract

Traditional healers in Ethiopia use a wide range of medicinal plants with antidiarrheal properties. Among these, *Croton macrostachyus* is one such plant claimed to have an antidiarrheal activity in Ethiopian folklore medicine. Previous studies showed that the crude extract is endowed with the claimed property and this study was undertaken to further the claim by screening different fractions for the said activity so that it could serve as a basis for subsequent studies. The fractions were obtained by successive soxhlet extraction with solvents of differing polarity (chloroform & methanol) followed by cold maceration of the marc of the methanol fraction with distilled water. The antidiarrheal activity was evaluated using castor oil induced diarrheal model, charcoal meal test and anti-enteropooling test in mice. The test groups received various doses (300, 400, 500 mg/kg and an additional dose of 1000 mg/kg for the aqueous fraction) of the fractions, whereas positive controls received either Loperamide (3 mg/kg) or Atropine (5 mg/kg) and negative controls received vehicle (10 ml/kg). In the castor oil induced model, the chloroform (at all test doses) and methanol (at 400 & 500 mg/kg) fractions significantly prolonged diarrheal onset, decreased the frequency of stooling and weight of feces. The aqueous fraction was, however, devoid of significant effect at all doses tested. Similarly, in the enteropooling test, whilst the chloroform and methanol fractions produced a significant dose dependent decline in the weight and volume of intestinal contents, the aqueous fraction was without appreciable effect. Results from the charcoal meal test revealed that all the fractions produced a significant anti-motility effect either at all

doses (chloroform fraction) or at middle and higher doses (methanol and aqueous fractions). Taken together, the present study demonstrated that the chloroform and methanol fractions possessed significant anti-diarrheal activity due to its inhibitory effect on castor oil induced gastrointestinal propulsion and fluid secretion. Nevertheless, the aqueous fraction showed only significant anti-motility effect at the higher dose (1000 mg/kg) employed in the study.

Key words: Antidiarrheal activity, Castor oil induced diarrhea, gastrointestinal transit, anti-enteropooling, *Croton macrostachyus*

Acknowledgements

First and foremost, I would like to thank the Almighty God and his mother Saint Mary for the wisdom, capability and strength that has been bestowed upon me during this research project, and indeed, throughout my life. I would like to gratefully and sincerely thank my Advisors: Dr. Ephrem Engidawork and Dr. Workineh Shibeshi for their guidance, understanding, patience, and most importantly, for their provision of unique opportunity to gain a wider breadth of experience in the sphere of education. Besides, I would like to thank my Sister Genet Degu for her support, encouragement, patience, and unwavering love that was undeniably the bedrock upon which the past ten years of my life had been built. My sincere gratitude also goes to Ms. Fantu Assefa for her consistent help in the laboratory activities and Mr. Molla Wale for constant care of the laboratory animals. Finally, I would like to thank Addis Ababa University for funding this study and Ambo University for sponsoring my Postgraduate education.

Table of Contents

List of abbreviations

ADI	Anti-Diarrheal Index
cAMP	Cyclic Adenosine Monophosphate
CDC	Centers for Diseases and Control Prevention
cGMP	Cyclic Guanosine Monophosphate
NO	Nitric Oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
ORS	Oral Rehydration Salt
UNICEF	United Nations International Children's Emergency Fund
WHO	World Health Organization

List of figures

List of tables

1. Introduction

1.1 Definition and classification of diarrhea

According to World Health Organization (WHO), diarrhea can be defined as the passage of unusually loose or watery stools, usually at least three times in a 24h period. However, it is the consistency of the stools rather than the number that is most important (WHO, 2005). A reduction in consistency of stools and an upsurge in frequency of bowel movements greater than three stools per day have often been used as a definition for epidemiological investigations (Guerrant *et al*., 2001).

Based on the duration of diarrheal episodes, diarrhea could be classified as acute, persistent, and chronic diarrhea. Acute diarrhea is defined as an abnormally frequent discharge of semisolid or fluid fecal matter from the bowel lasting less than 14 days, while diarrhea is termed as chronic when it lasts more than four weeks (Fine & Schiller,1999; Mohanta *et al*., 2010). However, persistent diarrhea is an episode of diarrhea, with or without blood that lasts at least for 14-30 days. Acute diarrhea could be acute watery or acute bloody diarrhea. Acute watery diarrhea is associated with significant fluid loss and rapid dehydration in an infected individual lasting for several hours or days. On the other hand, acute bloody diarrhea is marked by visible blood in the stools because of intestinal damage and nutrient losses in an infected individual (Mohanta *et al*., 2010; Pawlowski *et al*., 2009).

1.2 Epidemiology of diarrhea

Pneumonia and diarrhea may not come to cognizance when thinking about the leading killers of children in the world. Malaria and AIDS epidemics have occupied much of the media attention concerning the fatal childhood diseases. Astonishingly, however, equivalent numbers of children die from pneumonia and diarrhea as from malaria,

AIDS, measles, injuries, and all other post-neonatal conditions combined (Mary, 2013). Despite this heavy loss of young life, the uncomplicatedness, and cost-effectiveness of necessary interventions, comparatively few global resources are devoted to confront diarrhea. Apart from this, children living in poor or distant communities are most at risk and evidence shows that children are dying from these preventable diseases since effective interventions are not provided evenhandedly across all communities (WHO & UNICEF, 2013).

Diarrheal diseases account for 1 in 9 child deaths worldwide, making diarrhea the second leading cause of death among children under the age of 5 and responsible for killing around 760, 000 children every year. On top of this, there are nearly 1.7 billion cases of diarrheal disease every year in the world. For children with HIV, diarrhea is even more deadly, the death rate for these children is 11 times higher than the rate for children without HIV. As a result, diarrhea can be considered as a global menace that can culminate in mortality and morbidity due to loss of fluids and electrolytes from the body (CDC, 2012; WHO, 2013).

The global burden of diarrheal incidence and severity of the disease is highest in Southeast Asian and African regions. In addition to this, a study conducted in low and middle-income countries revealed that there were nearly 1.7 billion episodes of childhood diarrhea during 2010 (Fischer Walker *et al*., 2012). Furthermore, in the African regions, there were 26% severe episodes of diarrhea and the highest numbers of childhood deaths were in sub-Saharan Africa, where 50% of deaths from diarrhea occurred in 2011(Fischer Walker *et al*., 2013).

According to the Central Statistical Agency demographic and health survey report, the two-week prevalence of diarrhea among children under 5 years of age was 13% in Ethiopia (Central Statistical Agency, 2011). In addition to this, diarrhea accounted for

14% death of under five children in Ethiopia (UNICEF, 2012). Despite the dearth of nationwide prevalence study in Ethiopia, several community-based studies have shown that diarrheal disease is a major public health problem that causes morbidity and mortality in children (Awoke, 2013; Dessalegn *et al*., 2011; Mamo & Hailu, 2014; Mohammed *et al*., 2013).

1.3 Etiology of diarrheal disease

Diarrhea is a common symptom of gastrointestinal infections caused by a wide range of pathogens, including bacteria *(Escherichia coli*, *Shigella*, *Campylobacter*, *Vibrio cholerae*), viruses (rotavirus), and protozoa (*Cryptosporidium*). However, a handful of organisms are responsible for most acute cases of childhood diarrhea. Among these, rotavirus is the leading cause of acute diarrhea, and responsible for about 40% of all hospital admissions due to diarrhea among under five children worldwide (CDC, 2008; Kotloff *et al*., 2013; WHO, 1999, 2008). Globally, rotavirus is the most common cause of severe diarrhea in children (CDC, 2008). The prevalence of diarrhea due to rotavirus infections is reported to be 38 % in Western India (Chavan *et al*., 2013), 27.94 % in China (Ouyang *et al*., 2012), 32.2 % in Turkey (Ozdemir *et al*., 2010), 45.4 % in Uganda (Nakawesi *et al*., 2010), 83.8 % in Khartoum (Elhag *et al*., 2013) and 26.6 % in Jimma University Specialized Hospital, Ethiopia (Bizuneh *et al*., 2004).

1.4 Physiology of secretion and pathological mechanisms of diarrhea

During normal processes, approximately nine liters of fluid traverse the gastrointestinal tract daily. Of this amount, gastric juice (2L), saliva (1L), bile (1L), pancreatic juice (2L) and intestinal secretions (1L) accounts about seven liters of the transverse fluid across the gastrointestinal tract. However, the remaining two liters are

ingested fluids. Generally, the small intestine and colon absorb 99% of the overall fluid load of about 9 L/day presented to it. Of these 9 L of fluid presented to the intestine, only small amount vestiges in the stool after absorptive processes have occurred (Beverly & Clarence, 2008; Shah, 2004).

Enormous quantities of water are secreted into the lumen of the small intestine during the digestive process. Almost all of this water is also reabsorbed in the small intestine. Regardless of whether it is being secreted or absorbed, water flows across the mucosa in response to osmotic gradients. As the digestion process continues, there is generation of osmotically active molecules that causes lumenal osmolarity to increase dramatically and water is pulled into the lumen. Then, as the osmotically active molecules (maltose, glucose, amino acids) are absorbed, osmolarity of the intestinal contents decreases and water can be absorbed (Richard, 2006a).

The apical or lumenal membrane of crypt epithelial cells contain an ion channel of immense medical significance - a cAMP-dependent chloride channel also known as the cystic fibrosis trans membrane conductance regulator (CFTR).Chloride ions enter the crypt epithelial cell by co-transport with sodium and potassium. Elevated intracellular concentrations of cAMP in crypt cells activate the CFTR, resulting in secretion of chloride ions into the lumen. Accumulation of negatively charged chloride anions in the crypt creates an electric potential that attracts sodium into the lumen, which ultimately results in secretion of NaCl. This in turn creates an osmotic gradient across the tight junction and draws water into the lumen (Richard, 2006a).

The pathophysiological mechanisms underlying the loss of intestinal fluid in diarrhea have been a subject of debate for decades. The leading assumption up to the 1970s was that most diarrheas ensued because of altered gastrointestinal motility. Later on, it has become increasingly apparent that a disturbance in the epithelial transport of ions

and water is a major cause of intestinal fluid loss even if motility disturbances may contribute (Lundgren, 2002).

Diarrhea is a consequence of innumerable pathological conditions instigated by altered gastrointestinal motility and fluid secretion in the intestine lumen which can be elicited by an increased secretion of electrolytes (secretory diarrhea), an enhanced ingestion of osmotic substances (osmotic diarrhea) or the presence of infectious diarrhea as a result of virulent microorganisms (infective diarrhea) (Brijesh *et al*., 2006; Field, 2003; Hughes *et al*., 1982).

Osmotic diarrhea

Absorption of water in the intestines is dependent on adequate absorption of solutes. If excessive amounts of solutes are retained in the intestinal lumen, water will not be absorbed and diarrhea might occur. Osmotic diarrhea may occur due to ingestion of poorly absorbed substrates (mannitol, sorbitol, Magnesium sulphate) and malabsorption of certain carbohydrates because of enzyme deficiency. Since the intestine cannot maintain an osmotic gradient, these unabsorbed ions in the intestinal lumen cause retention of water to maintain an intraluminal osmolarity equal to that of body fluids. Disaccharides (sucrose and lactose) require disaccharidase for their breakdown before absorption. Lactose intolerance results due to deficiency of disaccharidase (lactase), thus the osmotically active lactose causes retention of water in the intestinal lumen and results in osmotic diarrhea. The distinguishing feature of osmotic diarrhea is its disappearance with fasting or termination of ingestion of the offending agent unlike secretory diarrhea that continues with fasting (Crombie *et al*., 2013; Field, 2003; Shah, 2004).

Secretory diarrhea

Large volumes of water are normally secreted into the small intestinal lumen, but a large majority of this water is efficiently absorbed before reaching the large intestine. As a result, diarrhea might occur when there is an imbalance between intestinal secretion and absorption of fluids and electrolytes (Crombie *et al*., 2013; Richard, 2006b).

In the case of secretory diarrhea, either an increase in the net secretion of ions (chloride or bicarbonate) or inhibition of the net absorption of sodium is the mechanism of diarrhea. It is commonly caused by infectious agents, which produce enterotoxins that interact with receptors and lead to an augmented anion secretion (Shah, 2004).

In addition to this, cholera toxin is responsible for the death of millions of people due to the capability of strongly activating adenylyl cyclase and cause a prolonged increase in intracellular concentration of cAMP within the crypt enterocytes. This alteration results in prolonged opening of the chloride channels that are instrumental in secretion of water from the crypts, allowing uncontrolled secretion of water (Field, 2003; Richard, 2006a).

Diarrhea associated with deranged motility

In order for nutrients and water to be efficiently absorbed, the intestinal contents must be adequately exposed to the mucosal epithelium and retained long enough to allow absorption. Disorders in motility that accelerate transit time could decrease absorption, resulting in diarrhea even if the absorptive process was proceeding properly (Richard, 2006b).

In disorders such as thyrotoxicosis and irritable bowel syndrome, intestinal hurry has been linked to abnormal enteric nervous system function. Many endocrine diarrheas, such as those due to peptide-secreting tumors or hyperthyroidism, may lead to diarrhea not only by effects on intestinal electrolyte transport but also by accelerating intestinal motility (Field, 2003; Shah, 2004).On the contrary, decreases in effective motility in the small intestine due to smooth muscle damage and autonomic neuropathy (diabetic) might cause diarrhea because of bacterial overgrowth (Field, 2003).

Inflammatory and infectious diarrhea

The epithelium of the digestive tube is sheltered from insult by a number of mechanisms constituting the gastrointestinal barrier, but like many barriers, it can be breached. Destruction of the epithelium by microbial or viral pathogens results not only in exudation of serum and blood into the lumen but also often associated with widespread destruction of absorptive epithelium. In such cases, absorption of water occurs very inefficiently and results in exudative diarrhea (Field, 2003; Richard, 2006b).

1.5 Management of diarrhea

The therapeutic goals of diarrhea treatment are to prevent excessive water, electrolyte, and acid-base disturbances; provide symptomatic relief; treat curable causes of diarrhea; and manage secondary disorders causing diarrhea (William and William, 2006).

1.5.1 Non pharmacological

A. Fluid and electrolytes

Fluid replacement is the cornerstone therapy for diarrhea. In many parts of the world where diarrheal states are frequent and severe, fluid replacement is consummated using oral rehydration salt(ORS), a measured mixture of water, salts, and glucose (Beverly & Clarence, 2008).

The WHO recognized oral rehydration salt (ORS) comprises 75 mEq/L sodium, 75 mmol/L glucose, 65 mEq/L chlorides, 20 mEq/L potassium, and 10 mEq/L citrate, having a total osmolarity of 245 mOsm/L (WHO, 2002).

When prepared and given correctly, ORS solution offers adequate water and electrolytes to correct the shortfalls associated with acute diarrhea. Potassium is provided to replace the large potassium losses associated with acute diarrhea, especially in infants, thus preventing serious hypokalemia. Citrate is provided to avert or correct base deficit acidosis. Glucose is indispensable because, when it is absorbed, it promotes the absorption of sodium and water in the small intestine regardless of the cause of the diarrhea (WHO, 2005).

Fluid and electrolyte replacement will maintain homeostasis when diarrhea is severe enough to produce substantial fluid loss and electrolyte disturbances (Rita, 2013). Patients with diarrhea who are not dehydrated may replace fluid by drinking flat soft drinks such as ginger, ale, tea, fruit juice, broth, or soup. Severe diarrhea may require the use of parenteral solutions such as ringer lactate or normal saline solution to replace large and life-threatening fluid losses (Beverly & Clarence, 2008).

B. Zinc supplement

Zinc deficiency is widespread among children in developing countries. Zinc has been shown to play critical roles for cellular functions, cellular growth, and function of the immune system. Although the theoretical basis for potential role of zinc has been postulated for some time, convincing evidence of its importance in child health was provided by randomized controlled trials of zinc supplementation (WHO, 2005).

Numerous studies indicated that zinc supplementation significantly reduce the severity and duration of diarrhea in children less than 5 years of age (Haider & Bhutta, 2009; Lukacik *et al*., 2008; Penny, 2013; Roy *et al*., 2008). Additional study revealed that zinc supplementation resulted in 13% reduction in the mortality of children due to diarrhea (Yakoob *et al*., 2011).

WHO and UNICEF are recommending zinc supplementation (20 mg/day for children older than six months & 10 mg/day for infants under six months old for 10-14 days) for the treatment of all diarrhea episodes among children (USAID *et al*., 2005).

1.5.2 Pharmacological

A. Antisecretory agents

These agents temporarily correct the imbalance of electrolytes and fluid in the colon. Bismuth subsalicylate is thought to have antisecretory and antimicrobial effects used to treat acute diarrhea (Beverly & Clarence, 2008). Somatostatin analogues (e.g., octreotide) might block hormone production by the tumor in the case of hormone-secreting neoplasms. Since they also appear to have a direct antisecretory effect on the gut epithelium, they have been employed for treating cancer chemotherapy induced diarrhea (Field, 2003).

Crofelemer is an antidiarrheal medication with unique inhibitory mechanisms at both the CFTR and the calcium-activated chloride channels, which are responsible for chloride secretion and subsequent luminal hydration. It was approved to treat diarrhea in HIV/AIDS patients on antiretroviral therapy. Moreover, Crofelemer may be an important addition to the currently available drugs for the management of secretory diarrhea (Yeo *et al*., 2013).

Racecadotril (acetorphan) is an oral enkephalinase inhibitor used for the treatment of acute diarrhea. By preventing the degradation of endogenous enkephalins, racecadotril reduces hypersecretion of water and electrolytes into the intestinal lumen (Szajewska *et al*., 2007).

Stimulation of alpha two adrenergic receptors on enterocytes promotes fluid and electrolyte absorption and inhibits anion secretion. Loss of adrenergic innervation may play a role in impaired intestinal fluid and electrolyte absorption in diabetic patients with autonomic neuropathy. Anecdotal experience with an α2 agonist, clonidine, suggests its utility in diabetic diarrhea, but severe side effects limit its usefulness (Fedorak *et al*., 1985; Field, 2003).

B. Antiperistaltic (Antimotility) Agents

Antiperistaltic drugs prolong intestinal transit time, thereby reducing the amount of fluid lost in the stool. The two drugs in this category are Loperamide HCl and Diphenoxylate HCl with Atropine sulfate. The atropine is included only as an abuse deterrent. When taken in large doses, the unpleasant anticholinergic effects of atropine negate the euphoric effect of Diphenoxylate. Both Loperamide and Diphenoxylate are effective in relieving symptoms of acute non-infectious diarrhea

and are safe for most patients experiencing chronic diarrhea (Beverly & Clarence, 2008).

C. Anti-infective agents

To minimize development of drug-resistant organisms, antibiotics should not be used for relatively mild, self-limited infectious diarrheas. Use of antibiotic is appropriate when the diarrhea is hemorrhagic, seriously dehydrating, associated with serious systemic signs and symptoms, or of longer than 5 days duration without improvement. Empiric antibiotic therapy is an appropriate approach to traveler's diarrhea. Primary empiric antibiotic choices include fluoroquinolones such as ciprofloxacin or levofloxacin. Azithromycin may be a feasible option when fluoroquinolones resistance is encountered. *Clostridium difficile* induced diarrhea can be treated by withdrawal of the offending antibiotic and oral administration of Metronidazole, Vancomycin (Beverly & Clarence, 2008; Field, 2003), Fidaxomicin (Johnson &Wilcox, 2012), or Rifaximin (Rubin *et al*., 2011).

D. Investigational anti-diarrheal agents

A class of high potency inhibitors of CFTR anion channel (thiazolidinones) has been identified through screening a large number of compounds in transfected cells. One of these (CFTR inhibitor 172), on a single intraperitoneal injection into mice (250 μg/kg), reduced cholera toxin induced small intestine secretion by more than 90% over 6 hours. Hence, Thiazolidinone CFTR inhibitors may be useful in reducing intestinal fluid loss in cholera and other secretory diarrheas (Ma *et al*., 2002). In addition to this, Calcium alumina silicate is under investigation to determine its effectiveness on cancer chemotherapy (Irinotecan) induced diarrhea (Anderson Cancer Center, 2014) and medullary thyroid cancer diarrhea (Salient Pharmaceuticals Incorporated 2012). Another investigational drug, iOWH032(on Phase II clinical trial)

was found to be safe, effective and well tolerated based on the previous findings on animal studies, preclinical toxicology studies, and first-in-human trials in healthy volunteers. Unlike most currently available medications, iOWH032 works to treat diarrheal processes directly by reducing fluid secretion and shorten both the duration and severity of symptoms (PATH, 2013, 2014).

1.5.3 Traditional medicine

Herbal medicines cater about 80% of the health needs of world's population, especially for millions of people in the vast rural areas of developing countries (Kim, 2005; WHO, 2000a). In spite of the fact that a large number of drugs have been developed by the pharmaceutical industry, indigenous phytotherapy is still practiced in several rural areas, using treatments handed down from generation to generation. Moreover, there is a revitalization of interest in herbal products at a global level and the conventional medicine is now beginning to accept the use of scientifically validated botanical source of drugs (Gilani & Rahman, 2005; WHO, 2000b).

In Ethiopia, about 80% of populations relied on traditional medicine due to the cultural acceptability, relatively low cost of traditional medicine and limited access to modern health facilities (Kassaye *et al*., 2006). In addition to this, the government sturdily supports and encourages traditional medicine through its policies despite sustainable use of traditional medicine and their integration with modern medical practice has been limited (Kassaye *et al*., 2006).

There are various herbal plants available throughout the world that possesses anti-diarrheal activity with lesser side effect than the conventional drugs. On top of this, different studies suggested that the plants showed anti-diarrheal activity by reducing intestinal motility and secretion. Furthermore, the antidiarrheal activities of medicinal

plants have been attributed to the presence of bioactive agents such as tannins, alkaloids, saponins, flavonoids, steroids, and terpenoids (Longanga Otshudi *et al*., 2000; Komal *et al*., 2013; Umer *et al*., 2013).

In Ethiopia, wide range of medicinal plants such as the bark extract of *Albizia gummifera*, leaf extract of *Calpurnia aurea* and *Myrtus communis*, seed extract of *Coffea arabica*, root extract of *Ensete ventricosum* and *Caylusea abyssinica* have been widely used for the management of diarrhea without scientific investigation of its safety and therapeutic potentials (Etana, 2010; Teklehaymanot & Giday, 2007).

1.6 The experimental plant

Croton macrostachyus Hochst. ex Del. (Euphorbiaceae) is commonly known as broad-leaved *Croton* (English), Bisana (Amharic), Islami, Tambuk and Tambush (Tigrigna), and Abnga in Berta ethnic group (Flatie *et al*., 2009; Orwa *et al*., 2009). The genus *Croton* consists of approximately 1,300 species that are widespread throughout the tropical and subtropical areas of the world (Salatino *et al*., 2007).The name of the genus *Croton* comes from a Greek word *Kroton*, which means ticks, because of the seeds' resemblance to ticks. The specific epithet is also derived from the Greek macro – (large) and – stachyus (relating to spike) hence with a large spike (Orwa *et al*., 2009). It is a deciduous tree 3-25 m high, crown rounded and open with large spreading branches commonly found in secondary forests, around lakes, in moist or dry evergreen upland forests and woodlands. *C.macrostachyus*, which is depicted in Figure 1, is native to Eritrea, Ethiopia, Kenya, Tanzania, Uganda, and Nigeria (Orwa *et al*., 2009).

Figure 1: Photograph of *Croton macrostachyus*

In areas where it is native, the plant parts are used for treatment of cough (boiled leaf decoction), venereal diseases and tapeworm (root decoction, crushed seeds and leaves in water), to hasten clotting of wounds (fresh leaves juice), remedy for skin rash (Bark from the stems and roots is boiled in water), and as abortifacient and uterotonic (Bark maceration and seed) (Mairura, 2007; Orwa *et al*., 2009; Wilson & Gebre Mariam, 1979).

C. marcostachyus, which is widely distributed throughout tropical Africa, is endowed with a number of ethnomedicinal uses in Ethiopia. Traditionally, the leaves are used to treat Diabetes mellitus (Salatino *et al*., 2007), scabies, hepatitis, jaundice, *Tinea versicolor*, and as an anti-dote for snake and scorpion venom (Flatie *et al*., 2009; Teklehaymanot & Giday, 2007). In addition to this, it is used to treat fever, edema (leaves or young shoots), hemorrhoids (mashed leaves), ear problems (seed

preparations) (Mairura, 2007) and diarrhea (leaves powder mixed with water) (Mesfin *et al*., 2009; Teklehaymanot & Giday, 2007).

Ethnopharmacological studies revealed that hydroalcoholic extracts of *C.macrostachyus* leaves has shown promising activity against *Neisseria gonorrhoeae* (Tefera *et al*., 2010), *Plasmodium berghei* (Bantie *et al*., 2014) and *Mycobacterium tuberculosis* (Gemechu *et al*., 2013). Apart from this, it has analgesic and anti-inflammatory (aqueous and methylene chloride/methanol stem bark extracts) (Kamanyi *et al*., 2009), anti-convulsant and sedative (bark decoction) (Ngo Bum *et al*., 2012) and anti-leishmanial activities (crotepoxide, isolated compound from berries) (Gelaw *et al*., 2012). Phytochemical studies on the genus *Croton* showed the presence of secondary metabolites such as alkaloids, terpenoids, and flavonoids. Amongst them, diterpenoids are the predominant phytochemical constituents in the genus (Salatino *et al*., 2007). Fourteen secondary metabolites have been isolated from *C. macrostachyus* and five of them were isolated from the genus for the first time. The compounds include phenolic and triterpenes of the lupane and hopane groups (Tala *et al*., 2013).

1.7 Rationale for the study

Despite advances of science in the understanding of the causes, treatment, and prevention of diarrheal diseases, significant numbers of children death occur every year (Kosek *et al*., 2003; Thapar & Sanderson, 2004). Moreover, antibiotics are indispensable remedy of infectious disease including diarrhea. However, a significant upsurge of new antibiotic resistance mechanisms has been spreading globally and threatening our ability to combat common infectious diseases (WHO, 2014). According to WHO (2014) antimicrobial resistance global report, there was decreased susceptibility of diarrhea causing pathogens such as *Escherichia coli* (to third

generation cephalosporins, Fluoroquinolones), *Neisseria gonorrhoeae* (to third generation cephalosporins) and *Shigella* species (to fluoroquinolones).

Even if sufficient drugs are available for treating diarrhea, majority of the existing drugs suffer from adverse effects like the induction of bronchospasm, vomiting (racecadotril), intestinal obstruction, constipation (Loperamide) (Pankaj, 2006) and dependency (Diphenoxylate) (Mehra *et al*., 2013). Correspondingly, oral rehydration therapy (ORT) has been widely identified as a crucial factor in the decline of child mortality due to diarrhea (Sastry & Burgard, 2005). However, the attack rate of the disease has persisted and this treatment often fails in the high stool output state (Brijesh *et al*., 2006; Farthing, 2004). In view of this, there is a necessity of strengthening research into medicinal plants to investigate alternative drugs from natural products (Mohammed *et al*., 2009).

Furthermore, the use of medicinal plants for the treatment of diarrhea in folk medicine is a routine practice in many countries of the world. However, in most of the cases these practices handed down from generation to generation empirically without knowing the plausible mechanisms, safety, and efficacy of herbal treatments (Mujumdar *et al*., 2001). Among these plants, the leaves extract of *C. macrostachyus* has a claimed folklore use as an antidiarrheal agent. Preliminary study conducted on the crude hydroalcoholic (80% methanol) extract showed a remarkable antidiarrheal activity (Busa, 2014). Nevertheless, there is a need to do further study such as fractionation so that in the end active principles responsible for the activity could be identified.

2. Objectives of the study

2.1 General objective

- ✓ To evaluate the anti-diarrheal activity of the solvent fractions of *Croton macrostachyus* leaves in mice

2.2 Specific objectives

- ✓ To evaluate the effect of chloroform, methanol and aqueous fractions of *Croton macrostachyus* leaves on castor oil induced diarrhea in mice
- ✓ To evaluate the effect of the solvent fractions of *Croton macrostachyus* leaves on the weight and volume of intestinal contents in mice
- ✓ To evaluate the effect of the solvent fractions of *Croton macrostachyus* leaves on small intestine transit in mice
- ✓ To determine the phytochemical constituents of the solvent fractions of *Croton macrostachyus* leaves

3. Material and methods

3.1 Drugs and chemicals

Castor oil (Amman Pharmaceutical Industries, Jordan), activated charcoal (Acuro Organics Ltd, New Delhi), Loperamide (Daehwa Pharmaceuticals, Republic of Korea), Atropine sulphate (Lab Renaudin, France), distilled water (Ethiopian Pharmaceutical Manufacturing Factory, Ethiopia), Tween 80 (Atlas Chemical Industries Inc, India), chloroform (Finkem Laboratory Reagent, India), and methanol (Fisher Scientific, UK) were used in the study.

3.2 Plant material

Leaves of *C.macrostachyus* were collected from Kolfe keranio (Tateke village) subcity of Addis Ababa City Administration in October 2013. The plant was authenticated by a taxonomist and a voucher specimen (number AD001) was deposited at the National Herbarium of College of Natural and Computational Sciences, Addis Ababa University for future reference. The leaves were washed using distilled water to remove dust materials and dried at room temperature under shade for 14 days. The leaves were then pulverized to coarse powder using mortar and pestle.

3.3 Experimental animals

Healthy Swiss albino mice of either sex, weighing 20–30 g and aged 6–8 weeks were used for the experiment. The animals were obtained from the animal house of Ethiopian Public Health Institute and School of Pharmacy, Addis Ababa University. The animals were kept in plastic cages at room temperature and on a 12h light–dark cycle with free access to pellet food and water *ad libitum*. The animals were acclimatized to laboratory condition for one week prior to the experiments (Umer *et*

al., 2013). All studies were conducted in accordance with the guideline for the care and use of laboratory animals (National Research Council, 2011).

3.4 Preparation of the solvent fractions

Soxhlet and maceration extraction techniques were used for the extraction of plant material. Two hundred gram dry powder of the plant material was subjected to successive soxhlet extraction with solvents of differing polarity (chloroform and methanol) followed by maceration with water.

First, 50 mg of the powdered plant material was placed in the extraction chamber of the Soxhlet apparatus. The extracting solvent (chloroform) in the flask was heated until clear liquid contents of the chamber siphoned into the solvent flask. Each time 50mg of the powdered plant material was extracted with 200ml of solvent in the soxhlet extraction process (Rahman *et al*., 2011). The chloroform fraction was then filtered with Whatman No. 1 filter paper and concentrated using rotary evaporator (Buchi labortechnik AG, Switzerland) under reduced pressure set at 40^{o}C followed by oven at room temperature for 12h (Zavala-Mendoza *et al*., 2013). The marc was collected and dried at room temperature to remove chloroform.

The residue left was next extracted using methanol following the same procedure as described before to get the methanol fraction. Finally, the marc of methanol fraction was collected and dried at room temperature. Then, the whole dried marc was cold macerated in an Erlenmeyer flask with distilled water and allowed to stand at room temperature for a period of 72h with occasional shaking using mini orbital shaker (Bibby scientific limited stone Staffordshire, SI150SA, UK). It was then filtered two times with gauze then through whatman filter paper (NO.1). The residue was re-macerated two times for a total of 6 days in order to obtain a better yield. The marc

was pressed, and the combined liquid was clarified by filtration and then the filtrate was freeze dried in a lyophilizer (Operan, Korea vacuum limited, Korea) to remove water. After drying, percentage yield of all fractions were determined and the yield was of 4.7%, 5.6%, and 3.5% for the chloroform, methanol and aqueous fractions, respectively. The chloroform and methanol fractions were reconstituted in 2% Tween-80, while the aqueous fraction was reconstituted in distilled water.

3.5 Grouping and dosing

The animals were randomly assigned into five groups (chloroform & methanol fractions) and six groups (aqueous fraction) of six animals per group to perform antidiarrheal activity test using three models. The first group was assigned as negative control and administered with vehicle (2% Tween 80 for chloroform & methanol fractions, distilled water for aqueous fraction) at a volume of 10 ml/kg. The second group was assigned as positive control and administered with standard drugs (3 mg/kg Loperamide orally for anti-enteropooling test & castor oil induced diarrhea, 5mg/kg Atropine sulphate intraperitoneally for charcoal meal test). The rest of the groups were treated with various doses of the fractions (300, 400, 500 mg/kg for the chloroform and methanol fractions, and an additional dose of 1000 mg/kg for the aqueous fraction). Dose selection was based on previous study of the crude extract, (Busa, 2014) as well as pilot experiments. The fractions were reconstituted with the respective vehicles during the day of experiment and administered orally.

3.6 Determination of antidiarrheal activity

3.6.1 Castor oil induced Diarrhea

The method described by Umer *et al.* (2013) was followed for this study. Swiss albino mice of either sex were fasted for 18h with free access to water and randomly

allocated and treated as described under section 3.5. One hour after administration of the vehicle or fractions, all animals were given 0.5 ml of castor oil orally using oral gavage and individually placed in cages in which the floor was lined with transparent paper and changed every hour. During an observation period of 4h, the time of onset of diarrhea (the time interval in minutes between the administration of castor oil and the appearance of the first diarrheal stool), frequency of defecation (number of wet feces and total number of fecal output) and weight of feces excreted by the animals were recorded. The percentage of fecal output and diarrheal inhibition was determined according to the following formula (Akah *et al*., 1999; Dosso *et al*., 2012; Ezekwesili *et al*., 2010; Mamza *et al*., 2014; Oben *et al*., 2006; Pillai,1992).

Besides, Calculations were made for the delay in diarrhoeal onset and purging index by comparing with the control group. The *in vivo* anti-diarhhoeal index (ADI) was then expressed according to the formula (Aye-than *et al*., 1989) desribed below.

$$\text{I. \% of inhibition} = \frac{\text{Average number of WFC} - \text{Average number of WFT}}{\text{Average number of WFC}} * 100$$

Where, WFC = average number of wet feces in control group and WFT = average number of wet feces in test group.

$$II. In\ vivo\ anti\ diarrheal\ index\ (ADI) = \sqrt[3]{Dfreq\ xGmeq\ xPfreq}$$

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

$$III. Percentage\ of\ fecal\ output = \frac{Mean\ fecal\ weight\ of\ each\ group}{Mean\ fecal\ weight\ of\ control} * 100$$

3.6.2 Charcoal meal (gastrointestinal motility) test

Mice of either sex were fasted for 18h with free access to water and divided and treated with the vehicle or fractions according to their respective groupings 1h before oral administration of 0.5 ml castor oil by oral gavage. One ml of a marker (5% charcoal suspension in 2% Tween 80) was administered orally 1h after castor oil treatment. The animals were then sacrificed by cervical dislocation after administration of the marker and the small intestine was dissected out from pylorus to caecum. The distance travelled by charcoal meal from the pylorus was measured and expressed as percentage of the total length of small intestine (Chitme *et al*., 2004). Then, the percentage of inhibition and Peristalsis index was expressed using the following formula (Aye-than *et al*., 1989; Hussain *et al*., 2009; Mamza *et al*., 2014).

Percentage of inhibition $= \frac{\mathrm{A}-\mathrm{B}}{\mathrm{A}} * 100$ where A is the distance moved by the charcoal (cm) in the control group, and B is the distance moved by the charcoal (cm) in the treated group.

$$\text{Peristalsis index} = \frac{\text{mean distance traveled by charcoal meal}}{\text{mean length of small intestine}} * 100$$

3.6.3 Anti-enteropooling test

Intraluminal fluid accumulation was determined using the method described by Islam *et al* (2013). Animals of either sex were deprived both food and water for 18h, and grouped and treated as described under section 3.5. After 1h, 0.5ml of castor oil was administered orally. One hour later, the mice were sacrificed by cervical dislocation and the small intestine was ligated at both the pyloric sphincter and the ileocaecal junction. The small intestine was then dissected out and weighed. Later on, intestinal contents were collected by milking into a graduated tube and the volume was

measured. The intestine was reweighed and the difference between the full and the empty intestine was calculated. Finally, percentage of reduction of intestinal secretion and weight of intestinal contents was determined using the following formula (Mamza *et al*., 2014).

$$\text{Mean Percentage inhibition} = \frac{\text{MVICC} - \text{MVICT}}{\text{MVICC}} * 100$$

Where, MVICC is the mean volume of the intestinal content of the control group while MVICT is the mean volume of the intestinal content of the test group.

$$\text{Mean percentage inhibition of intestinal content weight} = \frac{A - B}{A} * 100$$

Where A is the mean weight of intestinal content of the control and B is the mean weight of intestine content of the test group

3.7 Preliminary phytochemical screening

The qualitative phytochemical investigations of the chloroform, methanol, and aqueous fractions of *C.macrostachyus* leaves were carried out using standard tests (Ayoola *et al*., 2008) as described below.

Test for terpenoids (Salkowski test)

To 0.5 g of each solvent fraction of *C.macrostachyus* leaves, 2 ml of chloroform was added. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for saponins

To 0.5 g of each fraction, 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of froth indicates the presence of saponins.

Test for tannins

About 0.5 g of each fraction was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added. A brownish green or a blue-black precipitate indicated the presence of tannins.

Test for flavonoids

About 10 ml of ethyl acetate was added to 0.2 g of each fraction and heated on a water bath for 3 min. The mixture was cooled and filtered. Then, About 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammoniacal layer indicated the presence of flavonoids.

Test for cardiac glycosides (Keller-Killiani test)

To 0.5 g of each fraction diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for steroids

Two ml of acetic anhydride was added to 0.5 g fraction of each sample with 2 ml sulfuric acid. The color changed from violet to blue or green in some samples indicating the presence of steroids**.**

Test for alkaloids

0.5 g of each fraction was diluted to 10 ml with acid alcohol, boiled, and filtered. To 5 ml of the filtrate, 2 ml of dilute ammonia and 5ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Dragendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Dragendorff's reagent) was regarded as positive for the presence of alkaloids.

3.8 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) and analyzed using the Statistical Package for the Social Sciences (SPSS), version 16.0 software. Difference between group means was analyzed with one way analysis of variance (ANOVA) followed by Tukey post Hoc test. $P < 0.05$ was considered as statistically significant.

4. Results

4.1 Effects on castor oil- induced diarrhea in mice

In the castor oil-induced diarrheal model (Table 1), the chloroform fraction of *C. macrostachyus* leaves significantly prolonged the time of diarrheal induction and the frequency of stooling (number of wet feces and total number of feces) in a dose-dependent manner. Data from the experiment revealed that the percentage of diarrheal inhibition compared to controls was 70.4% ($p<0.001$), 92.6% ($p<0.001$), and 96.3 % ($p<0.001$) at the doses of 300, 400, and 500 mg/kg, respectively. In addition, at all the tested doses, the chloroform fraction produced comparable effect with the standard drug, Loperamide (81.5%). The methanol fraction of *C. macrostachyus* leaves significantly prolonged the onset of diarrheal feces and decreased frequency of wet feces defection only at the doses of 400 mg/kg and 500 mg/kg. This fraction produced the highest percentage of diarrheal inhibition (82.2%) at 500 mg/kg, which was comparable with that of the standard drug (82.2 %). On the contrary, the aqueous fraction was devoid of significant anti-diarrheal activity on castor oil induced diarrhea at all tested doses as compared with the negative control (Table 1).

As depicted in Figure 2, there was a dose-dependent reduction in the percentage of mean fecal output among all solvent fractions, with 500 mg/kg of the chloroform fraction displaying the highest effect (12.2%). As compared to the standard drug, both chloroform and methanol fractions at the dose of 500 mg/kg showed the utmost effect to lessen the percentage of mean fecal output. On the other hand, the aqueous fraction, even at 1000 mg/kg showed minimum inhibition of percentage of mean fecal output.

Table 1: Effect of the fractions of *Croton macrostachyus* leaves on castor oil induced diarrheal model in mice

Group	onset time of diarrhea (min)	Total # of wet feces in 4h	Total # of feces	Total weight of feces(gm)	% inhibition of defecation
Control	76±16.71	4.5±0.72	4.67±0.80	0.82±0.19	----
Loperamide 3mg/kg	167.83±25.62^{a1}	0.833±0.31^{a3}	1.83±0.65^{a1}	0.17±0.044^{a1}	81.49
CF300mg/kg	169.83±22.78^{a1}	1.33±0.42^{a3}	1.83±0.65^{a1}	0.29±0.14^{a1}	70.37
CF400mg/kg	217.50±22.50^{a3}	0.33±0.33^{a3}	0.83±0.31^{a2}	0.14±0.13^{a1}	92.59
CF500mg/kg	238.33±1.67^{a3}	0.17±0.17^{a3}	0.167±0.166^{a3}	0.10±0.10^{a1}	96.296
Control	82.5±19.96	4.67±0.49	5.33±0.42	0.83±0.22	----
Loperamide 3mg/kg	167.83±25.62^{a1}	0.833±0.31^{a3}	1.83±0.65^{a1}	0.17±0.044^{a1}	82.16
MF300mg/kg	143.5±25.54	2.5±0.56	3.83±0.48	0.37±0.15	46.47
MF400mg/kg	186.33±22.19^{a1}	1.17±0.48^{a2}	2.50±0.85^{a1}	0.114±0.05^{a1}	74.95
MF500mg/kg	191.33±30.62^{a1}	0.83±0.48^{a3}	1.17±0.60^{a1}	0.11±0.06^{a1}	82.23
Control	42.50±4.16	7.00±0.89	6.67±0.80	0.72±0.13	--------
Loperamide 3mg/kg	120.50±28.07^{a1}	1.50±0.22^{a2c2d2e1f1}	2.00±0.37^{a1c1d1e1}	0.15±0.07^{a1c2d2e2}	78.57
AF300mg/kg	54.17±8.60^{h2I2}	6.83±0.83^{b2g3h3I3}	8.17±1.66^{b2g3h3I3}	0.71±0.096$^{b2\ g3h3I3}$	2.43
AF400mg/kg	55.00±8.79 ^{h2I2}	6.67±1.05^{b2g3h3I3}	7.33±0.99^{b1g3h3I3}	0.69±0.21^{b2g3h3I3}	4.71
AF500mg/kg	60.67±2.58 ^{h2I2}	6.50±1.09^{b1g3h3I3}	7.17±1.11^{b1g3h3I3}	0.68±0.06$^{b2\ g3h3I3}$	7.14
AF1000mg/kg	82.17±28.13 ^{h2I2}	6.17±1.30^{b1g3h3I3}	6.33±1.17^{b1g3h3I3}	0.56±0.11	11.86

Values are expressed as Mean ± S.E.M (n=6), analysis was performed with One-Way ANOVA followed by Tukey test, ^{a} compared to control, ^{b} to standard drug, ^{c} to 300mg/kg, ^{d} to 400mg/kg, ^{e} to 500mg/kg,^{f} to 1000mg/kg, ^{g} to CF300mg/kg, ^{h} to CF400mg/kg and ^{I} to CF500mg/kg; ^{1}P<0.05, ^{2}P<0.01, ^{3}P<0.001; CF=chloroform fraction, MF= methanol fraction and AF=aqueous fraction. Controls received 10 ml/kg of Tween 80 2% in water or distilled water.

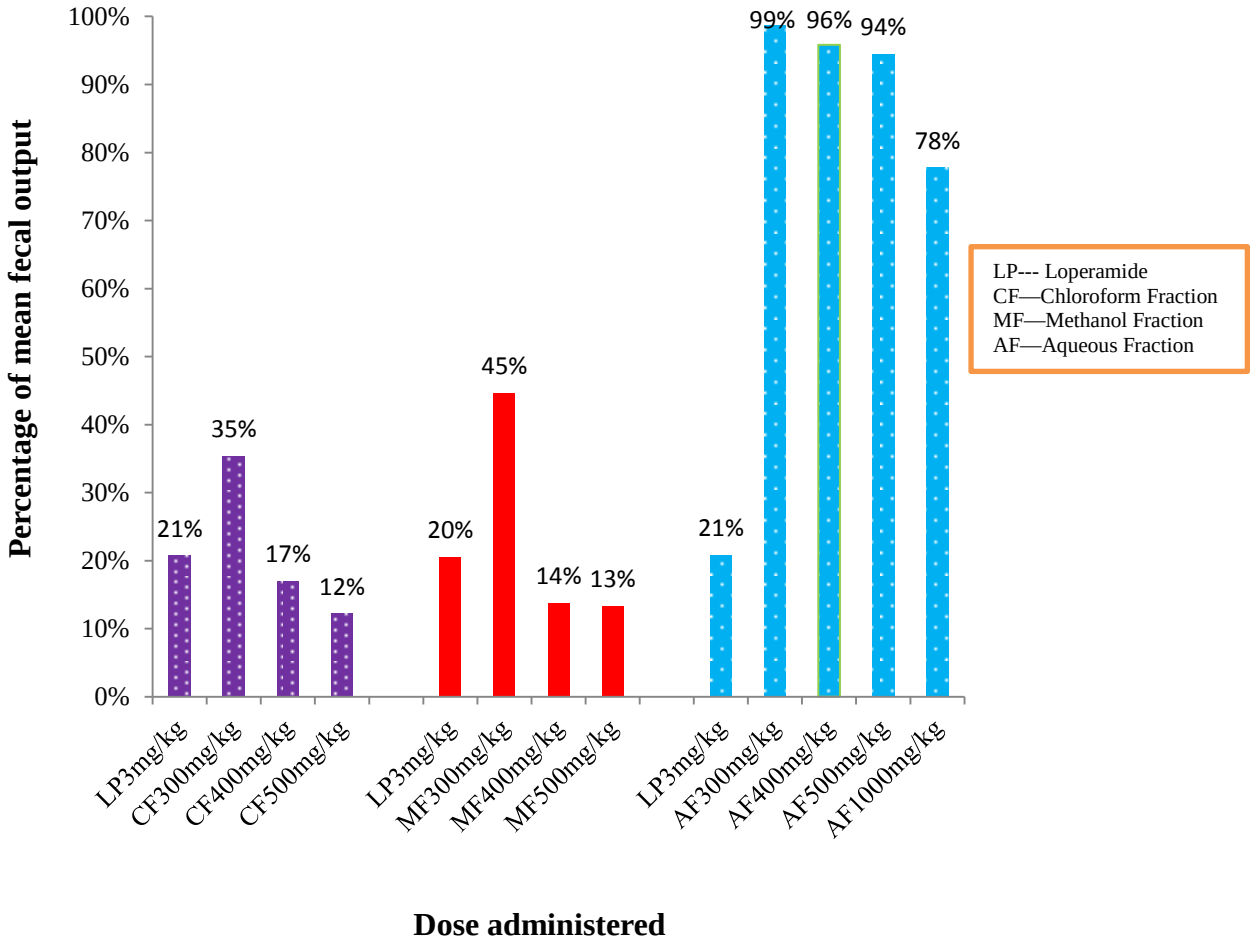

Figure 2: Percentage mean fecal output of the solvent fractions of *Croton macrostachyus* leaves on castor oil induced diarrheal model in mice

4.2 Effects on castor oil- induced enteropooling in mice

In gastrointestinal enteropooling test, the chloroform and methanol fractions of *C. macrostachyus* leaves reduced the volume of intestinal fluid and weight of the intestinal contents significantly in a dose-dependent manner. Maximum percentage inhibition of the volume of intestinal contents was observed at 500 mg/kg, being 76.1% ($p<0.01$) and 75.3% ($P<0.01$) for chloroform and methanol fractions, respectively. Similarly, the uppermost reduction for the weight of intestinal contents was observed at 500 mg/kg for both chloroform and methanol fractions. However, there was no statistically significant difference in terms of the volume of intestinal fluid and weight of intestinal contents when all doses of the chloroform and methanol fractions were compared with Loperamide. On the contrary, the aqueous fraction did not show significant inhibitory effect on the volume and weight of intestinal fluid as compared with the negative control (Table 2).

Table 2: Effect of the fractions of *Croton macrostachyus* leaves on castor oil induced enteropooling in mice

Dose administered	Volume of intestinal content (ml)	% of inhibition	Mean weight of intestinal content(gm)	% of inhibition
Control	0.67±0.21	-------	0.78±0.16	-------
Loperamide 3mg/kg	0.12±0.08^{a2}	82.1	0.21±0.08^{a2}	73.08
CF300mg/kg	0.18±0.054^{a1}	73.13	0.27±0.09^{a2}	65.38
CF400mg/kg	0.17±0.03^{a1}	74.6	0.269±0.06^{a2}	65.51
CF500mg/kg	0.16±0.04^{a2}	76.12	0.248±0.04^{a2}	68.21
Control	0.77±0.17	-------	0.83±0.2	-------
Loperamide 3mg/kg	0.12±0.08^{a3}	84.41	0.21±0.078^{a2}	74.70
MF300mg/kg	0.30±0.05^{a1}	61.04	0.36±0.11^{a1}	56.63
MF400mg/kg	0.20±0.04^{a2}	74.03	0.31±0.04^{a1}	62.65
MF500mg/kg	0.19±0.02^{a2}	75.32	0.29±0.03^{a1}	65.06
Control	0.47±0.06	-------	0.45±0.07	-------
Loperamide 3mg/kg	0.083 ±0.01^{a2c1d1e1f1}	82.34	0.08±0.01 ^{a1c1d1e1f1}	82
AF300mg/kg	0.43±0.098^{b1}	8.5	0.44±0.08 ^{b1}	2.22
AF400mg/kg	0.38±0.07 ^{b1}	19.12	0.43±0.12 ^{b1}	4.44
AF500mg/kg	0.362±0.09 ^{b1}	22.98	0.422±0.09 ^{b1}	6.22
AF1000mg/kg	0.35±0.06 ^{b1}	25.53	0.41±0.05 ^{b1}	8.89

Values are expressed as Mean ± S.E.M (n=6), analysis was performed with One-Way ANOVA followed by Tukey test; ^{a} compared to control, ^{b} to standard drug, ^{c} to 300 mg/kg, ^{d} to 400 mg/kg, ^{e} to 500 mg/kg, ^{f} to 1000 mg/kg, ^{g} to CF300 mg/kg, ^{h} to CF 400mg/kg and ^{I} to CF500 mg/kg; ^{1}P<0.05, ^{2}P<0.01, ^{3}P<0.001; CF=chloroform fraction, MF= methanol fraction and AF=aqueous fraction. Controls received 10 ml/kg of Tween 80 2% in water or distilled water.

4.3 Effects on castor oil- induced intestinal transit in mice

The chloroform fraction significantly inhibited gastrointestinal transit time of charcoal meal at 300 (25.5%, $p<0.05$), 400 (43.4%, $p<0.01$), and 500 (52.4%, $p<0.01$) mg/kg as compared to the control. On the other hand, the methanol fraction of *C. macrostachyus* leaves had statistically significant inhibitory effect (56.1%, $p<0.001$) on gastrointestinal transit time of charcoal meal only at the dose of 500 mg/kg. Interestingly, as compared to the control, the aqueous fraction showed significant inhibition of gastrointestinal transit (44.3%, $p<0.05$) of charcoal meal at the dose of 1000 mg/kg (Table 3).

4.4 *In vivo* anti-diarrheal index

Determination of the *in vivo* anti-diarrheal index revealed that there was an increase in this parameter with dose in chloroform and methanol fractions of *C. macrostachyus* leaves. Moreover, the highest anti-diarrheal index was observed at the maximum dose of each fraction. However, amongst all solvent fractions of *C. macrostachyus* leaves, the chloroform fraction showed the highest anti-diarrheal index at 500 mg/kg (Table 4).

Table 3: Effect of the fractions of *Croton macrostachyus* leaves on castor oil induced intestinal transit in mice

Dose administered	Mean length of small intestine (cm)	Mean Distance traveled by the charcoal meal (cm)	Peristalsis index (%)	% of inhibition
Control	58.75±2.48	55.32±2.78	94.04±1.74	------
Atropine sulphate 5mg/kg (i.p)	60.27±0.46	18.92±2.59^{a3c2}	31.33±4.22^{a3c2}	65.78
CF300mg/kg	57.57±2.38	41.22±0.83^{a1b2}	72.25±3.42^{a1b2}	25.49
CF400mg/kg	58.12±2.18	31.33±4.89^{a2}	54.56±8.71^{a2}	43.36
CF500mg/kg	61.03±0.945	29.00±7.09^{a2}	47.37±11.15^{a2}	52.42
Control	60.05±2.06	56.88±2.73	94.55±1.78	-------
Atropine sulphate 5mg/kg (i.p.)	63.1±1.32	24.13±4.24^{a3c1}	38.71±7.45^{a3c1}	57.58
MF300mg/kg	61.25±1.52	43.25±5.45^{b1}	70.12±8.38^{b1e1}	23.96
MF400mg/kg	66.28±2.49	42.87±5.89	64.59±8.36	24.63
MF500mg/kg	63.07±1.88	25.00±3.39^{a3}	39.78±5.66^{a3c1}	56.05
Control	62.68±3.54	50.65±3.88	80.81±4.02	-------
Atropine sulphate 5mg/kg(i.p.)	61.05±2.86	18.72±3.99^{a1c1d1}	30.66±6.26^{a1c1d1}	63.04
AF300mg/kg	63.55±1.47	50.08±2.65^{b1}	78.8±6.04^{b1f2}	1.12
AF400mg/kg	63.45±1.54	49.25±5.76^{b1f1}	77.62±8.54^{b1f2}	2.76
AF500mg/kg	61.98±3.69	38.23±7.74^{f1}	61.68±11.67	24.5
AF1000mg/kg	68.00±1.53	27.45±2.21^{a1c1d1}	40.53±3.55^{a1c2d2}	44.26

Values are expressed as Mean ± S.E.M (n=6), analysis was performed with One-Way ANOVA followed by Tukey test; [a] compared to control, [b] to standard drug, [c] to 300 mg/kg, [d] to 400 mg/kg, [e] to 500 mg/kg, [f] to 1000 mg/kg, [g] to CF300 mg/kg, [h] toCF 400mg/kg and [I] to CF500 mg/kg; [1]P<0.05, [2]P<0.01, [3]P<0.001; CF=chloroform fraction, MF= methanol fraction and AF=aqueous fraction. Controls received 10 ml/kg of Tween 80 2% in water or distilled water.

Table 4: *In vivo* anti-diarrheal index of the fractions of *Croton macrostachyus* leaves

Dose administered	Delay in defecation time or onset (Dfreq)	Gut meal travel reduction (*G*meq)	Purging frequency in number of wet stools(Pfreq)	*In vivo* anti-diarrheal index(ADI)
Control	----	----	----	----
CF300mg/kg	123.46	23.17	70.44	58.62
CF400mg/kg	186.18	41.98	92.67	89.81
CF500mg/kg	213.59	49.62	96.2	100.65
Control	----	----	----	----
MF300mg/kg	73.94	25.84	46.47	44.61
MF400mg/kg	125.85	31.69	74.95	66.86
MF500mg/kg	131.92	57.93	82.23	85.65
Control	----	----	----	----
AF300mg/kg	27.46	2.49	2.43	5.50
AF400mg/kg	29.41	3.95	4.71	8.18
AF500mg/kg	42.75	27.24	7.14	20.26
AF1000mg/kg	93.34	49.84	11.86	38.10

CF= Chloroform Fraction, MF= Methanol Fraction, AF= Aqueous Fraction

4.5 Preliminary phytochemical screening

Phytochemical screening test revealed that alkaloids were detected in all fractions. However, (flavonoids & cardiac glycosides and terpenoids & steroids) were exclusively found in methanol and chloroform fractions, respectively. Amongst all fractions, the methanol fraction appeared to be rich in secondary metabolites (Table 5).

Table 5: Preliminary phytochemical screening of solvent fractions of *Croton macrostachyus* leaves

Secondary metabolites	Chloroform fraction	Methanol fractions	Aqueous fraction
Alkaloids	+	+	+
Tannins	-	+	+
Saponins	-	+	+
Terpenoids	+	-	-
Steroids	+	-	-
Flavonoids	-	+	-
Anthraquinones	-	-	-
Cardiac Glycosides	-	+	-

+ = present, - = absent

5. Discussion

The use of castor oil as diarrhea inducer is well documented (Akter, 2013; Dahiru, 2006; Rahman *et al*., 2011). Its active metabolite (ricinoleic acid), which is liberated by the action of lipases in the upper part of the small intestine, is responsible for the diarrhea inducing properties (Komal *et al*., 2013; Tripathi, 2008).

Ricinoleic acid produces local irritation and inflammation of the intestinal mucosa, causing the release of prostaglandins that eventually increases gastrointestinal motility, net secretion of water and electrolytes (Horton *et al*., 1968; Robert *et al*., 1976). This effect could occur due to the capability of ricinoleic acid to activate the G protein-coupled prostanoid receptor (EP3) on the smooth muscle cell of the intestine (Tunaru *et al*., 2012). In addition, it forms ricinoleate salts with sodium and potassium in the lumen of the intestine and these salts inhibit sodium-potassium ATPase and increase permeability of the intestinal epithelium, which in turn produces a cytotoxic effect on intestinal absorptive cells (Komal *et al*., 2013).

In the castor oil induced diarrheal model, the chloroform (at all tested doses) and methanol fractions (at 400 and 500 mg/kg) significantly prolonged the time of diarrheal onset, and decreased the frequency of defecation and weight of feces. Besides, the chloroform fraction showed comparable effect with that of the crude extract in this model (Busa, 2014). This study was in line with other studies in which the chloroform extract of different plants reduced the frequency of stooling in a dose-dependent manner (Billah *et al*., 2013; Karthik *et al*., 2011; Mazumder *et al*., 2006). By contrast, the aqueous fraction was devoid of any activity at all tested doses. This could possibly suggest the probable localization of the active ingredients in these two fractions.

Non-steroidal anti-inflammatory drugs (NSAIDs) could inhibit castor oil induced diarrhea apart from its inhibition on prostaglandin synthesis (Awouters *et al*., 1978). Similarly, the aqueous and methylene chloride/methanol extracts from the stem bark of *C. macrostachyus* has also shown analgesic and anti-inflammatory activities like that of NSAIDs (Kamanyi *et al*., 2009). Thus, it is plausible to assume that the antidiarrheal effect of the active fractions could be attributed to inhibition of castor oil-induced prostaglandin synthesis.

Terpenoids such as abietic acid and steroids like phytosterols have been shown to inhibit production of prostaglandin E2 (Awad *et al*., 2004; Fernandez *et al*., 2001), which are known to play a crucial role in the stimulation of intestinal secretions (Bern *et al*., 1989). Thus, the significant antidiarrheal activity observed in the chloroform fraction could probably be attributed to the presence of these secondary metabolites in the fraction.

The anti-diarrheal activity of the active fractions might also be due to inhibition of active secretion of ricinoleic acid, resulting in the activation of Na^+, K^+ATPase activity that promotes absorption of Na+ and K+ in the intestinal mucosa. This effect could probably be linked to the presence of terpenoids in the chloroform fraction and tannins & flavonoids in the methanol fraction, which are shown to promote colonic absorption of water and electrolytes (Palombo, 2006).

Although the chloroform fraction exhibited maximum effect, statistically significant difference was not observed when the antidiarrheal effects of the chloroform and methanol fractions was compared, indicating that both fractions are active in this model. The present result is concordant with other studies, where the chloroform and methanol fractions displayed comparable inhibition of castor oil induced diarrhea (Islam *et al*., 2013; Zavala-Mendoza *et al*., 2013). Despite there appear to be a

differential distribution of active constituents in the two fractions, the comparable activity of the two fractions suggests that different secondary metabolites are endowed with antidiarrheal properties.

In the castor oil induced enteropooling test, treatment of mice with different doses of the chloroform and methanol fractions produced a significant decline in the intestinal fluid accumulation. On the contrary, the aqueous fraction was devoid of significant inhibition of castor oil induced intestinal fluid accumulation at all tested doses. As compared to the crude extract (Busa, 2014) both chloroform and methanol fraction demonstrated higher effect in this model.

Mascolo *et al*. (1993, 1994) reported that the active metabolite of castor oil (ricinoleic acid) might activate the nitric oxide pathway and induce nitric oxide (NO) dependent gut secretion. A growing body of evidence indicates that phytochemical constituents such as terpenoids (Jang *et al*., 2004) and flavonoids (Kim *et al*., 1999, 2004; Messaoudene *et al*., 2011) are implicated in attenuation of NO synthesis. Thus, in contrast to the aqueous fraction, the pronounced inhibition of castor oil induced intestinal fluid accumulation (enteropooling) and the weight of the intestinal content could possibly be linked to the presence of terpenoids (chloroform fraction) and flavonoids (methanol fraction) that increase the reabsorption of electrolytes and water by hindering castor oil mediated NO synthesis. The fact that intestinal fluid accumulation and Na+ secretion induced by castor oil is attenuated by pretreatment of rats with NO synthesis inhibitors (Mascolo *et al*., 1993) reinforces the notion that the anti-enteropooling effect of both fractions could probably be by interfering with the NO pathway.

Alkaloids, which are detected in all fractions, also demonstrated inhibitory effect on NO synthesis (Kondo *et al*., 1993). Nevertheless, due to the successive extraction

method used in this study, most of the alkaloids could be extracted by the chloroform and methanol, and trace amounts might have remained in the aqueous fraction. Consequently, the phytochemical constituents that could inhibit castor oil induced fluid secretion were either absent or present in undetectable amount in the aqueous fraction, explaining why this fraction had lower antidiarrheal effects.

The antidiarrheal effect of flavonoids has been ascribed to their ability to inhibit intestinal motility and hydro-electrolytic secretion (Di Carlo *et al*., 1993; Galvez *et al*., 1993; Rao *et al*., 1997). Flavonoids are also able to inhibit the intestinal secretory response induced by prostaglandins E2 (Hamalainen *et al*., 2011; Sanchez de Medina *et al*., 1997). Moreover, the enteric nervous system stimulates intestinal secretion through neurotransmitters such as acetylcholine and vasoactive intestinal peptide. On the other hand, intestinal absorption can be stimulated with alpha two adrenergic agents, enkephalins, and somatostatin (Bern *et al*., 1989; Fedorak *et al*., 1985). Secondary metabolites such as flavonoids from plant sources could stimulate alpha two adrenergic receptors in the absorptive cells of the gastrointestinal tract (Di Carlo *et al*., 1993). Hence, in contrast to the aqueous fraction, the significant anti-secretory activity of the methanol fraction could probably be related to the existence of flavonoids that in turn stimulate alpha two adrenergic receptors in the enterocytes and promote fluid and electrolyte absorption. However, the chloroform and methanol fractions showed comparable effect in this model despite the better antidiarrheal effect observed in the chloroform fraction. This could perhaps be due to the collective interference of terpenoids and steroids on prostaglandin E2 induced gut secretion (Awad *et al*., 2004; Fernandez *et al*., 2001).

In the evaluation of intestinal transit, results demonstrated that the chloroform fraction significantly reduced the intestinal propulsive movement of charcoal meal at all the

test doses (300,400 and 500 mg/kg body weight) as compared to the negative control. Besides, this fraction showed greater effect in this model as compared to the crude extract in the previous study (Busa, 2014). This is in line with other studies in which the chloroform extract significantly inhibited the distance travelled by charcoal meal (Zavala-Mendoza *et al*., 2013). Interestingly, both the methanol fraction (56.1%, $p<0.001$) as well as the aqueous fraction (44.3%, $p<0.05$) produced substantial inhibition of the peristaltic movement of charcoal meal at the higher dose employed in the present study (500 mg/kg and 1000 mg/kg, respectively). This finding suggests that there is difference in the potency (chloroform>>methanol>>aqueous fraction) of phytochemical constituents that mediate castor oil induced gastrointestinal motility. The potent action of the chloroform fraction could be ascribed to the synergistic effects of terpenoids and alkaloids to prolong the time for absorption of water and electrolytes through hampering peristaltic movement of the intestine. Indeed, alkaloids (Palombo, 2006) and terpenoids (Maciel *et al*., 2000; Palombo, 2006) have been demonstrated to have inhibitory effect on gastrointestinal motility. Although the phytochemical constituents responsible for the antidiarrheal effect are yet to be identified, the amount of phytochemical constituents that are responsible for impeding gastrointestinal motility such as tannins (Almeida *et al*., 1995; Tripathi, 2008; Yadav & Tangpu, 2007) and alkaloids (Palombo, 2006) appear to increase with dose. This could possibly the reason why significant anti-motility effect was observed at the higher dose of the aqueous fraction. However, in this fraction, significant antidiarrheal effect was not observed in other models with increasing in dose. This might be due to the lack of secondary metabolites such as terpenoids (Fernandez *et al*., 2001), steroids (Awad *et al*., 2004) and flavonoids (Hamalainen *et al*., 2011;Sanchez de Medina *et al*., 1997) that could inhibit prostaglandin E2 induced fluid secretion in the intestine.

Unlike the castor oil induced and enteropooling diarrheal model, maximum effect was observed with the methanol fraction (56.1%) rather than the chloroform fraction (52.4%) in charcoal meal test. This could perhaps be linked to the presence of synergistic inhibitory effect of tannins (Almeida *et al*., 1995; Tripathi, 2008; Yadav & Tangpu, 2007) and flavonoids (Di Carlo *et al*., 1993; Galvez *et al*., 1993; Rao *et al*., 1997) on castor oil induced gastrointestinal motility.

Plants that have tannins in their composition can precipitate proteins of the enterocytes, reducing the peristaltic movements and intestinal secretions (Almeida *et al*., 1995; Tripathi, 2008; Yadav & Tangpu, 2007).The layers formed by the precipitate of proteins on the mucosal surface of the enterocytes also inhibit the development of microorganisms, thus explaining the antiseptic action of tannins (Almeida *et al*., 1995). Therefore, based on this fact, the presence of tannins in the methanol fraction might provide a clue to further investigating its role for infectious diarrhea, as the present study did not address this issue.

The anti-diarrheal index (ADI) is a measure of the combined effects of the different parameters of diarrhea such as purging frequency, onset of diarrheal stools and intestinal motility (Hussain *et al*., 2009). Besides, higher ADI value is a measure of the effectiveness of an extract in curing diarrhea (Ching *et al*., 2008; Prasad *et al*., 2014). ADI increased with dose, suggesting the dose dependency of this parameter. The chloroform fraction showed the highest ADI value as compared to the other fractions, reinforcing the notion that this fraction is endowed with the best anti-diarrheal activity among all the solvent fractions. Conversely, the aqueous fraction, which had little or no antidiarrheal activity in most of the models, exhibited the lowest ADI, pointing to the fact that ADI is a useful parameter in ranking antidiarrheal agents

6. Conclusion

The present study revealed that the chloroform and methanol fractions of *C. macrostachyus* leaves possessed significant anti-diarrheal activity due to its inhibitory effect on castor oil induced gastrointestinal propulsion and fluid secretion. Nevertheless, the aqueous fraction showed only significant anti-motility effect at the higher dose (1000 mg/kg) employed in the study. The antidiarrheal activities of these fractions could be attributed to the presence of bioactive agents, which are either non-polar or semi polar, including, among others, tannins, alkaloids, saponins, flavonoids, steroids, and terpenoids that act individually or collectively.

7. Suggestions for future works

- ✓ Further studies should be done to isolate, purify and identify pharmacologically active principle (s) responsible for the antidiarrheal activities of the plant
- ✓ *Ex vivo* studies of the fractions should be done to suggest possible mechanism of actions
- ✓ Further toxicological studies should be done
- ✓ *In vitro* antibacterial activities of the fractions should be done to determine the role of each fraction in infectious diarrhea
- ✓ Further studies that aim to isolate the active principle (s) or elucidating the possible mechanism of action should use either fractions.

8. References

Akah PA, Aguwa CN, Agu RU (1999). Studies on the antidiarrhoeal properties of *Pentaclethra macrophylla* leaf extracts. *Phytother Res* **13**(4):292-295.

Akter S, Sarker A, Hossain S (2013). Antidiarrhoeal activity of rind of *Punica granatum*. *Int Curr Pharm J* **2**(5): 101-104.

Almeida CE, Karnikowski MG, Foleto R, Baldisserotto B (1995). Analysis of antidiarrhoeic effect of plants used in popular medicine. *Rev Saude Publica* **29**(6):428-33.

Anderson Cancer Center (2014).*Calcium Aluminosilicate Anti-Diarrheal in Treating and Preventing Diarrhea in Patients With Metastatic Colorectal Cancer Receiving Irinotecan.*[Online] Available at: https://clinicaltrials.gov/ct2/show/NCT00748215 [Accessed 13 August 2014].

Awad AB, Toczek J, Fink CS (2004). Phytosterols decrease prostaglandin release in cultured P388D1/MAB macrophages. *Prostaglandins Leukot Essent Fatty* Acids **70**(6):511-520.

Awoke W (2013). Prevalence of childhood illness and mothers'/caregivers' care seekingbehavior in Bahir Dar, Ethiopia: A descriptive community based cross sectional study. *Open J Prev Med* **3**(2):155-159.

Awouters F, Niemegeers CJ, Lenaerts FM, Janssen PA (1978). Delay of castor oil diarrhoea in rats: a new way to evaluate inhibitors of prostaglandin biosynthesis. *J Pharm Pharmacol* **30**(1): 41-45.

Aye-than, Kulkarni HJ, Wut-hmone, Tha SJ (1989). Anti-diarrhoeal efficacy of some Burmese indigenous drug formulations in experimental diarrhoeal test models. *Int J Crude Drug Res* **27**(4):195-200.

Ayoola GA, Coker HA, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, *et al* (2008). Phytochemical screening and antioxidant activities of some selected medicinal Plants used for malaria therapy in Southwestern Nigeria. *Trop J Pharm Res* **7**(3): 1019-1024.

Bantie L, Assefa S, Teklehaimanot T, Engidawork E (2014). *In vivo* antimalarial activity of the crude leaf extract and solvent fractions of *Croton macrostachyus* Hocsht. (Euphorbiaceae) against Plasmodium berghei in mice. *BMC Complement Altern Med* **14**:79.

Bern MJ, Sturbaum CW, Karayalcin SS, Berschneider HM, Wachsman JT, Powell D (1989). Immune system control of rat and rabbit colonic electrolyte transport. Role of prostaglandins and enteric nervous system. *J Clin Invest* **83**(6):1810-1820.

Beverly CM, Clarence EC (2008). Constipation, Diarrhea, and Irritable Bowel Syndrome. In: MA.Chisholm-Burns, BG.Wells, TL.Schwinghammer, PM.Malone, JM.Kolesar, JC.Rotschafer, JT. Dipiro, eds. *Pharmacotherapy Principles & Practice.* New York: McGraw-Hill Companies, 307-322.

Billah MM, Khatun H, Parvin S, Islam E, Islam SM, Mia AA, *et al* (2013). Antibacterial, antidiarrhoeal, and cytotoxic activities of methanol extract and its fractions of *Caesalpinia bonducella* (L.) Roxb leaves. *BMC Complement Altern Med* **13**(1):101.

Bizuneh T, S/Mariam Z, Abebe A, Lema E (2004). Rotavirus infection in under-five children in Jimma Hospital, Southwest Ethiopia. *Ethiop J Health Dev* **18**(1):19-24.

Brijesh S, Daswani PG, Tetali P, Rojatkar SR, Antia NH, Birdi TJ (2006). Studies on *Pongamia pinnata* (L.) Pierre leaves: understanding the mechanism(s) of action in infectious diarrhea. *J Zhejiang Univ Sci B* **7**(8):665-674.

Busa Z (2014). *Evaluation of in vivo anti-diarrheal activity of 80% methanolic extract of the Leaves of Croton macrostachyus Hochst. (Euphorbiaceae) in Rats*. Unpublished Master Thesis, Addis Ababa University, Addis Ababa.

CDC (2008). Rotavirus Surveillance- Worldwide, 2001-2008. Morbidity and Mortality Weekly report **57**(46):1255-1257. [Online]Available at:http: //www. cdc. gov/ mm wr/ preview/mmwrhtml/mm5746a3.htm[Accessed 6August 2014].

CDC (2012). *Global Diarrhea Burden.* [Online] Available at: http ://www .cdc .gov / healthy water/global/diarrhea-burden.html [Accessed 5 september 2013].

Central Statistical Agency (2011). *Ethiopia Demographic and Health Survey 2011 Preliminary Report* , Addis Ababa, Ethiopia.

Chavan SC, Agarkhedkar S, Chavan DS, Nagdawane RP, Singhania S (2013). Prevalence of rotavirus diarrhea among children hospitalized in a tertiary care hospital in Western India. *Int J Pharm Biomed Sci* **4**(1): 4-7.

Ching FP, Omogbai Ek, Ozolua RI, Okpo SO (2008). Antidiarrhoeal activities of aqueous extract of *Stereospermum kunthianum* (Cham, Sandrine Petit) stem bark in rodents. *Afr J Biotechnol* **7**(9):1220-1225.

Chitme HR, Chandra R, Kaushik S (2004). Study of antidiarrhoeal activity of *Calotropis gigantea* R.Br. in experimental animals. *J Pharm Pharmceut Sci* **7**(1):70-75.

Crombie H, Gallagher R, Hall V (2013). Assessment and management of diarrhoea. *Nurs Times* **109**(30):22-24.

Dahiru D, Sini JM, John-Africa L (2006). Antidiarrhoeal activity of *Ziziphus mauritiana* root extract in rodents. *Afr J Biotechnol* **5**(10): 941-945.

Dessalegn M, Kumie A, Tefera W(2011). Predictors of under-five childhood diarrhea: Mecha District, West Gojam, Ethiopia. *Ethiop J Health Dev* **25**(3):192-200.

Di Carlo G, Autore G, Izzo AA, Maiolino P, Mascolo N, Viola P, *et al* (1993). Inhibition of intestinal motility and secretion by flavonoids in mice and rats: Structure activity relationships. *J Pharm Pharmacol* **45**(12):1054-1059.

Dosso K, Nguessan BB, Bidie AP, Gnangoran BN, Meite N, Nguessan D, *et al* (2012). Antidiarrhoeal activity of an ethanol extract of the stem bark of *Piliostigma reticulatum* (Caesalpiniaceae) in rats. *Afr J Tradit Complement Altern Med* **9**(2):242-249.

Elhag WI, Saeed HA, Omer el FE, Ali AS (2013). Prevalence of rotavirus and adenovirus associated with diarrhea among displaced communities in Khartoum, Sudan. *BMC Infect Dis* **13**:209.

Etana B (2010). *Ethnobotanical Study of Traditional Medicinal Plants of Goma Wereda, Jima Zone of Oromia Region, Ethiopia.* Unpublished Masters Thesis,Addis Ababa University, Addis Ababa.

Ezekwesili JO, Nkemdilim UU, Okeke CU (2010). Mechanism of antidiarrhoeal effect of ethanolic extract of *Psidium guajava* leaves. *Biokemistri* **22**(2):85-90.

Farthing MJ (2004). Novel agents for the control of secretory diarrhoea. *Expert Opin Investig Drugs* **13**(7):777-785.

Fedorak RN, Field M, Chang EB (1985). Treatment of diabetic diarrhea with clonidine. *Ann Intern Med* **102**(2):197-199.

Fernandez MA, Tornos MP, Garcia MD, De las Heras B, Villar AM, Saenz MT (2001). Anti-inflammatory activity of abietic acid, a diterpene isolated from *Pimenta racemosa var. grissea*. *J Pharm Pharmacol* **53**(6):867-872.

Field M (2003). Intestinal ion transport and the pathophysiology of diarrhea. *J Clin Invest* **111**(7):931-943.

Fine KD, Schiller LR (1999). AGA technical review on the evaluation and management of chronic diarrhea. *Gastroenterology* **116**(6):1464–1486.

Fischer Walker CL, Perin J, Aryee MJ, Boschi-Pinto C, Black RE (2012). Diarrhea incidence in low- and middle-income countries in 1990 and 2010: a systematic review.*BMC Public Health* **12**:220.

Fischer Walker CL, Rudan I, Liu L, Nahir H, Theodoratou E, Bhutta ZA, *et al* (2013). Global burden of childhood pneumonia and diarrhoea. *Lancet* **381**(9875):1405-1416.

Flatie T, Gedif T, Gebre-Mariam T, Asres K (2009). Ethnomedical survey of Berta ethnic group Assosa Zone, Benishangul-Gumuz Regional State, mid-west Ethiopia. *J Ethnobiol Ethnomed* **5**:14.

Galvez J, Crespo ME, Jimenez J, Suarez A, Zarzuelo A (1993). Anti-diarrhoeic activity of quercitrin in mice and rats. *J Pharm Pharmacol* **45**(2):157-159.

Gelaw H, Adane L, Tariku Y, Hailu A (2012). Isolation Of crotepoxide from berries of *Croton macrostachyus* and evaluation of its anti-leishmanial activity. *J Pharmacogn Phytochem* **1**(4):15-25.

Gemechu A, Giday M, Worku A, Amen G (2013). *In vitro* anti-mycobacterial activity of selected medicinal plants against *Mycobacterium tuberculosis* and *Mycobacterium bovis* Strains. *BMC Complement Altern Med* **13**: 291.

Gilani AH, Rahman AU (2005). Trends in ethnopharmacology. *J Ethnopharmacol* **100**(1-2):43-49.

Guerrant RL,Van Gilder T, Steiner TS, Thielman NM, Slutsker L,Tauxe RV, *et al* (2001). Practice guidelines for the management of infectious diarrhea. *Clin Infect Dis* **32**(3):331-351.

Haider BA, Bhutta ZA (2009). The effect of therapeutic zinc supplementation among young children with selected infections: a review of the evidence. *Food Nutr Bull* **30**(1 Suppl):S41-59.

Hamalainen M, Nieminen R, Asmawi MZ, Vuorela P, Vapaatalo H, Moilanen E (2011). Effects of flavonoids on prostaglandin E2 production and on COX-2 and mPGES-1 expressions in activated macrophages. *Planta Med* **77**(13):1504-1511.

Horton EW, Main IH, Thompson CJ, Wright PM (1968). Effect of orally administered prostaglandin E1 on gastric secretion and gastrointestinal motility in man. *Gut* **9**(6):655-658.

Hughes S, Higgs NB, Turnberg LA (1982). Antidiarrhoeal activity of loperamide: studies of its influence on ion transport across rabbit ileal mucosa *in vitro*. *Gut* **23**:974-979.

Hussain Z, Amresh G, Singh S, Rao CV (2009). Antidiarrheal and antiulcer activity of *Amaranthus spinosus* in experimental animals. *Pharm Biol* **47**(10):932–939.

Islam AM, Uddin ME, Chowdhury AU, Rahman M, Habib R, Rahman A (2013). *In vivo* antidiarrheal and cytotoxic potential of different fractions of *Pandanus foetidus* Leaves. *Am J Biomed Sci* **5**(3): 208-216.

Jang DS, Min HY, Jeong YH, Lee SK, Seo EK (2004). Di- and sesqui-terpenoids isolated from the pods of *Sindora sumatrana* and their potential to inhibit lipopolysaccharide-induced nitric oxide production. *Arch Pharm Res* **27**(3):291-294.

Johnson AP, Wilcox MH (2012). Fidaxomicin: a new option for the treatment of *Clostridium difficile* infection. *J Antimicrob Chemother* **67**(12):2788-2792.

Kamanyi A, Mbiantcha M, Nguelefack TB, Ateufack G,Watcho P, Ndontsa BL, *et al* (2009). Anti-nociceptive and anti-Inflammatory activities of extracts from the stem bark of *Croton macrostachyus* (Euphorbiaceae) in mice and rats. *J Complement Integr Med* **6**(1):1-17.

Karthik P, Kumar RN, Amudha P (2011).Anti diarrheal activity of the chloroform extract of *Cayratia pedata* Lam in albino wistar rats. *Pharmacologyonline* **2**: 69-75.

Kassaye KD, Amberbir A, Getachew B, Mussema Y(2006). A historical overview of traditional medicine practices and policy in Ethiopia. *Ethiop J Health Dev* **20**(2):127-134.

Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochem Pharmacol* **58**(5):759-65.

Kim HS (2005). Do not put too much value on conventional medicines. *J Ethnopharmacol* **100**(1-2):37-39.

Kim SJ, Park H, Kim HP (2004). Inhibition of nitric oxide production from lipopolysaccharide-treated RAW 264.7 cells by synthetic flavones: structure-activity relationship and action mechanism. *Arch Pharm Res* **27**(9):937-943.

Komal, Kumar S, Rana AC (2013). Herbal apporaches for diarrhea:A review. *Int Res J Pharm* **4**(1):31-38.

Kondo Y, Takano F, Hojo H (1993). Inhibitory effect of bisbenzylisoquinoline alkaloids on nitric oxide production in activated macrophages. *Biochem Pharmacol* **46**(11):1887-1892.

Kosek M, Bern C, Guerrant RL (2003). The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. *Bull World Health Organ* **81**(3):197-204.

Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, *et al* (2013). Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet* **382**: 209–222.

Longanga Otshudi A, Vercruysse A, Foriers A (2000). Contribution to the ethnobotanical, phytochemical and pharmacological studies of traditionally used medicinal plants in the treatment of dysentery and diarrhoea in Lomela area, Democratic Republic of Congo (DRC). *J Ethnopharmacol* **71**(3): 411-423.

Lukacik M, Thomas RL, Aranda JV (2008). A meta-analysis of the effects of oral zinc in the treatment of acute and persistent diarrhea. *Pediatrics* **121**(2):326-336.

Lundgren O (2002). Enteric nerves and diarrhoea. *Pharmacol Toxicol* **90**(3):109-120.

Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C,Galietta LJ, *et al* (2002). Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin–induced intestinal fluid secretion. *J Clin Invest* **110**(11):1651-1658.

Maciel MA, Pinto AC, Arruda AC, Pamplona SG, Vanderlinde FA, Lapa AJ, *et al* (2000). Ethnopharmacology, phytochemistry and pharmacology: a successful combination in the study of *Croton cajucara*. *J Ethnopharmacol* **70**(1):41-55.

Mairura FS (2007). *Croton macrostachyus* Hochst. ex Delile. In: Schmelzer GH & Gurib-Fakim A (Editors). Prota 11(1): *Medicinal plants/Plantes médicinales 1*. [CD-Rom]. PROTA, Wageningen, Netherlands.

Mamo A, Hailu A (2014). Assessment of prevalence and related factors of diarrheal diseases among under-five year's children in Debrebirehan Referral Hospital, Debrebirehan Town, North Shoa Zone, Amhara Region, Ethiopia. *Open Access Libr J* **1**:1-14.

Mamza UT, Sodipo OA, Khan IZ, Wampana B (2014). Evaluation of antidiarrhoeal Activity of ethanolic extract from the leaves of *Phyllanthus amarus* (Euphorbiacea). *Int J Green Herb Chem* **3**(1):171-178.

Mary KA (2013). *Forgotten Killers: Pneumonia and Diarrhea Prevalence in Third World Countries.*[Online]Availableat:https://www.google.com.et/#q=Forgotten+KillersPneumonia+and+Diarrhea+Prevalence+in+ThirdWorld+Countries[Accessed 5 September 2013].

Mascolo N, Izzo AA, Barbato F, Capasso F (1993). Inhibitors of nitric oxide synthetase prevent castor-oil-induced diarrhoea in the rat. *Br J Pharmacol* **108**(4):861-864.

Mascolo N, Izzo AA, Autore G, Barbato F, Capasso F (1994). Nitric oxide and castor oil-induced diarrhea. *J Pharmacol Exp Ther* **268**(1):291-295.

Mazumder R, Bhattacharya S, Mazumder A, Pattnaik AK,Tiwary PM, Chaudhary S (2006). Antidiarrhoeal evaluation of *Aegle marmelos* (Correa) Linn. root extract. *Phytother Res* **20**(1): 82–84.

Mehra A, Sarkar S, Basu D (2013). Lomotil (diphenoxylate) dependence in India. *Indian J Psychol Med* **35**(3):248-250

Mesfin F, Demissew S, Teklehaymanot T (2009). An ethnobotanical study of medicinal plants in Wonago Woreda, SNNPR, Ethiopia. *J Ethnobiol Ethnomed* **5**:28.

Messaoudene D, Belguendouz H, Ahmedi ML, Benabdekader T, Otmani F, Terahi M, *et al* (2011). *Ex vivo* effects of flavonoids extracted from *Artemisia herba alba* on

cytokines and nitric oxide production in Algerian patients with Adamantiades-Behcet's disease. *J Inflamm* **8**:35.

Mohammed A, Ahmed H, Goji AD, Okpanachi AO, Ezekiel I,Tanko Y (2009). Preliminary anti-diarrhoeal activity of hydromethanolic extract of aerial part of *Indigofera pulchra*in Rodents. *Asian J Med Sci* **1**(2):22-25.

Mohammed S, Tilahun M, Tamiru D (2013). Morbidity and associated factors of diarrheal diseases among under five children in Arba-Minch District, Southern Ethiopia. *Sci J Public Health* **1**(2) :102-106.

Mohanta G, Praveen Kumar NV, Manna PK, Parimalakrishnan S (2010). Recent advances in management of acute diarrhoea in children. *Indian J pharm pract* **3**(3):1-3.

Mujumdar AM, Misar AV, Salaskar MV, Upadhye AS (2001). Antidiarrhoeal effect of an isolated fraction (JC) of *Jatropha curcas* roots in mice. *J Nat Remedies* **1**(2):89-93.

Nakawesi JS, Wobudeya E, Ndeezi G, Mworozi EA, Tumwine JK (2010). Prevalence and factors associated with rotavirus infection among children admitted with acute diarrhea in Uganda. *BMC Pedatr* **10**:69.

National Research Council (2011). *Guide for the Care and Use of Laboratory Animals.* 8th ed. Washington, DC: The National Academies Press.

Ngo Bum E, Ngah E, Ngo Mune RM, Ze Minkoulou DM, Talla E, Moto FC, *et al* (2012). Decoctions of *Bridelia micrantha* and *Croton macrostachyus* may have anticonvulsant and sedative effects. *Epilepsy Behav* **24**(3):319-323.

Oben JE, Assi SE, Agbor GA, Musoro DF (2006). Effect of *Eremomastax speciosa* on experimantal diarrhoea. *Afr J Trad CAM* **3**(1):95-100.

Orwa C, Mutua A, Kindt R, Jamnadass R, Simons A (2009). *Agroforestree Database:a tree reference and selection guide version 4.0* [Online] Availableat: http:// www World agroforestry.org/treedb2/speciesprofile.php?Spid=620# [Accessed 21 June 2014].

Ouyang Y, Ma H, Jin M, Wang X, Wang J, Xu L, *et al* (2012). Etiology and epidemiology of viral diarrhea in children under the age of five hospitalized in Tianjin, China. *Arch Virol* **157**(5):881-887.

Ozdemir S, Delialioglu N, Emekdas G (2010). Investigation of rotavirus, adenovirus and astrovirus frequencies in children with acute gastroenteritis and evaluation of epidemiological features. *Mikrobiyol Bul* **44**(4):571-578.

Palombo EA (2006). Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function. *Phytother Res* **20**(9):717-724.

Pankaj JP (2006). Treatment of disorders of bowel motility and water flux; antiemetics; agents used in biliary and pancreatic diseases. In: L. Laurence, S. John & L. Keith, eds. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 11th ed. New York: McGraw Hill, p. 1038.

PATH (2013). *Antidiarrheal Drug Candidate Moves to Next Phase of Clinical Study*.[Online] Available at:http://www.path.org/news/press-room/435/ [Acessed 14 August 2014].

PATH (2014). *A Phase 2, Randomized, Double-Blind, Placebo-Controlled, Two-Part Trial to Evaluate the Efficacy, Safety, and Tolerability of iOWH032 in the Treatment of Acute Dehydrating Diarrhea in Adult and Pediatric Patients With Cholera*. [Online] Available at:http://clinicaltrials.gov/show/NCT02111304 [Acessed 14 August 2014].

Pawlowski SW, Warren CA, Guerrant R (2009). Diagnosis and treatment of acute or persistent diarrhea. *Gastroenterology* **136**(6): 1874–1886.

Penny ME (2013). Zinc Supplementation in Public Health. *Ann Nutr Metab***62**(suppl 1):31-42.

Pillai NR (1992). Antidiarrhoeal activity of *Punica granatum* in experimental animals. *Int J Pharmacogn* **30**(3): 201-204.

Prasad SK, Laloo D, Kumar R, Sahu AN, Hemalatha S (2014). Antidiarrhoeal evaluation of rhizomes of *Crypotcroyine spiralis* Fish .Ex Wydler:anti motility and antisecretory effects. *Indian J Exp Biol* **52**(2):139-146.

Rahman A, Hasan SN, Sampad KS, Das AK (2011). Antinociceptive, anti diarrheoal and cytotoxic activities of *Rhizophora mucronata* Lamk. *Pharmacologyonline* **1**: 921-929.

Rao VS, Santos FA, Sobreira TT, Souza MF, Melo CL, Silveira ER (1997). Investigations on the gastroprotective and antidiarrhoeal properties of ternatin, a tetramethoxyflavone from *Egletes viscosa*. *Planta Med* **63**(2):146-149.

Richard B (2006a). *Secretion in the Small Intestine.* [Online] Available at: http :// www .vivo .colostate.edu/hbooks/pathphys/digestion/smallgut/secretion.html

[Accessed 14 September 2013].

Richard B (2006b). *Pathophysiology of Diarrhea.* [Online] Available at: http :// www .vivo .colostate.edu/hbooks/pathphys/digestion/smallgut/diarrhea.html[Accessed 9 September 2013].

Rita W (2013). *Oncology-Supportive Care:Nausea,Vomiting and Diarrhea.* [Online] Availableat:http://www.clinicaloptions.com/inPractice/Oncology/ Supportive Care / ch43SuppCare-Nausea.aspx [Accessed 12 September 2013].

Robert A, Nezamis JE, Lancaster C, Hanchar AJ, Klepper MS (1976). Enteropooling assay: a test for diarrhea produced by prostaglandins. *Prostaglandins* **11**(5):809-828.

Roy SK, Hossain MJ, Khatun W, Chakraborty B, Chowdhury S, Begum A, *et al* (2008). Zinc supplementation in children with cholera in Bangladesh: randomised controlled trial. *BMJ* **336**(7638):266-268.

Rubin DT, Sohi S, Glathar M, Thomas T, Yadron N, Surma BL (2011). Rifaximin is effective for the treatment of *Clostridium difficile* associated diarrhea: results of an open-label pilot study. *Gastroenterol Res Pract* **2011**:1-5.

Salatino A, Salatino MF, Negri G (2007). Traditional uses, Chemistry and Pharmacology of *Croton* species (Euphorbiaceae). *J Braz Chem Soc* **18**(1):11-33.

Salient Pharmaceuticals Incorporated (2012). *The Efficacy of CASAD in Patients With Diarrhea Related to Medullary Thyroid Cancer*.[Online]Available at: http :// clinical trials.gov/show/NCT01739634 [Accessed 13 August 2014].

Sanchez de Medina F, Galvez J, Gonzalez M, Zarzuelo A, Barrett KE (1997). Effects of quercetin on epithelial chloride secretion. *Life Sci* **61**(20):2049-2055.

Sastry N, Burgard S (2005). The prevalence of diarrheal disease among Brazilian children: trends and differentials from 1986 to 1996. *Soc Sci Med* **60**(5):923-935.

Shah S (2004). Evaluation of diarrhea: The challenge continues! Part-I. *Indian J Med Sci* **58**(2):75-78.

Szajewska H, Ruszczynski M, Chmielewska A, Wieczorek J (2007). Systematic review: racecadotril in the treatment of acute diarrhoea in children. *Aliment Pharmacol Ther* **26**(6):807-813.

Tala MF, Tan NH, Ndonsta BL, Tane P (2013). Triterpinoids and phenolic commpounds from *Croton macrostchyus*. *Biochem Syst Ecolo* **51**:138–141.

Tefera M, Geyid A, Debella A (2010). *In vitro* anti-*Neisseria gonorrhoeae* activity of *Albizia gummifera* and *Croton macrostachyus. Revista CENIC Ciencias Biologicas* **41**:1-11.

Teklehaymanot T, Giday M (2007). Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. *J Ethnobiol Ethnomed* **3**:12.

Thapar N, Sanderson IR (2004). Diarrhoea in children: an interface between developing and developed countries. *Lancet* **363**(9409):641-653.

Tripathi K (2008). *Essentials of Medical Pharmacology*, 8 ed. New Delhi: Jaypeeb Brothers Medicals Publishers (P) Ltd, New Delhi.

Tunaru S, Althoff TF, Nusing RM, Diener M, Offermanns S (2012). Castor oil induces laxation and uterus contraction via ricinoleic acid activating prostaglandin EP3 receptors. *Proc Natl Acad Sci USA* **109**(23):9179-9184.

USAID, UNICEF,WHO (2005). *Diarrhoea Treatment Guidelines Including New Recommendations for the Use of ORS and Zinc Supplementation for Clinic-Based Healthcare Workers*. [Online] Available at: http://www.who.int/maternalchild adolescent/documents/a85500/en/ [Accessed 19August 2014].

Umer S, Tekewe A, Kebede N (2013). Antidiarrhoeal and antimicrobial activity of *Calpurnia aurea* leaf extract. *BMC Complement Altern Med* **13**:21.

UNICEF (2012). *Pneumonia and Diarrhoea:Tackling the Deadliest Diseases for the World's Poorest Children*[Online] Available at: http://data.unicef.org/child-health/diarrhoeal-disease [Accessed 7 August 2014].

WHO and UNICEF (2013). *End preventable Child Deaths from Penumonia and Diarrhea by 2025: Global Action Plan for Prevention and Control of Pneumonia and Diarrhoea,* Geneva: WHO Library Cataloguing.[Online] Available at: www .who. int/ iris/ bit stream/10665/79200/1/9789241505239_eng.pdf[Accessed 17 July 2014].

WHO (1999). *The Evolution of Diarrhoeal and Acute Respiratory Disease Control at WHO,* Geneva: World Health Organization.[Online] Available at: whqlibdoc.Who.int/hq/ 1999/WHOCHSCAH99.12.pdf[Accessed 17 July 2014].

WHO (2000a). *General Guidelines for Methodologies on Research and Evaluation of Tradational medicine,* Geneva, Switzerland: WHO/EDM/TRM/2000.1.[Online] Available at: whqlibdoc.who.int/hq/2000/WHOEDMTRM2000.1.pdf[Accessed 17 July 2014].

WHO (2000b). *Promoting the Role of Tradtional Medicine in the Health Systems: A strategy for the African Region,* Geneva: World Health Organization.[Online] Available at: http://www.who.int/iris/handle/10665/95467[Accessed 17 July 2014].

WHO (2002).*WHO Drug Information*,16(2). [Online] Available at: http://www.who. int/druginformation [Accessed 30 October 2014].

WHO (2005). *The Treatment of Diarrhoea : A manual for Physicians and Other Senior health Workers*,4th ed. Geneva: WHO Library Cataloguing.[Online] Available at: whqlibdoc.who.int/publications/2005/9241593180.pdf[Accessed 17 July 2014].

WHO (2008). *Weekly Epidemiological Record,* Geneva : World Health Organization.[Online] Available at: www.who.int/entity/wer/2013/wer8802.pdf[Accessed 17 July 2014].

WHO (2013). *Diarrhoeal Disease.* [Online] Available at: http://www.who.int/media centre /factsheets/fs330/en/#content [Accessed 7 September 2013].

WHO (2014). *Antimicrobial Resistance Global Report on Surveillance 2014 [*Online] Available at:http://www.who.int/ drugresistance/documents/surveillancereport/en/ [Accessed 17 August 2014].

William JS,William EW (2006). Diarrhea. In: GW.Barbara, TD.Joseph, S.Terry, H.Cynthia, eds. *Pharmacotherapy Handbook*, 6th ed. New York: McGraw-Hill Companies, 223-224.

Wilson RT, Gebre Mariam W (1979). Medicine and magic in central Tigre: A contribution to the ethnobotany of the Ethiopian plateau. *Econ Bot* **33**(1):29-34.

Yadav AK, Tangpu V (2007). Antidiarrheal Activity of *Lithocarpus dealbata* and *Urena lobata* Extracts: Therapeutic Implications.*Pharm Biol* **45**(3): 223–229.

Yakoob MY, Theodoratou E, Jabeen A, Imdad A, Eisele TP, Ferguson J, *et al* (2011). Preventive zinc supplementation in developing countries: impact on mortality and morbidity due to diarrhea, pneumonia and malaria. *BMC Public Health* **11** (Suppl 3):S23.

Yeo Q, Crutchley R, Cottreau J, Tucker A, Garey KW (2013). Crofelemer, a novel antisecretory agent approved for the treatment of HIV-associated diarrhea. *Drugs Today* **49**(4):239-252.

Zavala-Mendoza D, Alarcon-Aguilar FJ, Perez-Gutierrez S, Escobar-Villanueva MC, Zavala-Sanchez MA (2013). Composition and Antidiarrheal Activity of *Bidens odorata* Cav. *Evid Based Complement Alternat Med* **1**:1-7.