Evaluation of the anti-diarrheal activity of 80% methanol extract and solvent fractions of the fruits of *Mimusops kummel* A. DC. (Sapotaceae) in mice

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A Thesis submitted to the Department of Pharmacology, School of Medicine, College of Health Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology.

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This is to certify that the thesis prepared by Mulugeta Molla, entitled "Evaluation of the anti-diarrheal activity of 80% methanol extract and solvent fractions of the fruits of *Mimusops kummel* A. DC. (Sapotaceae) 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.

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ABSTRACT

Evaluation of the anti-diarrheal activity of 80% methanol extract and solvent fractions of the fruits of *Mimusops kummel* A. DC. (Sapotaceae) in mice

Mulugeta Molla

Addis Ababa University, 2016

Mimusops kummel A. DC. *Eshe, Shiye* is one of the medicinal plants traditionally used for the treatment of diarrhea in Ethiopian folklore medicine. However, the anti-diarrheal activity of the fruits of this plant has not been scientifically validated. The aim of this study was therefore to investigate the anti-diarrheal activities of both 80% MeOH and solvent fractions of the fruits of *Mimusops kummel* A. DC. using mice models of diarrhea. The 80% MeOH was prepared by maceration and the fractions were obtained by successive fractionation of the crude extract with solvents of increasing polarity (chloroform and n-butanol) followed by distilled water. The anti-diarrheal activities of the 80% MeOH and solvent fractions were investigated using castor oil induced diarrhea, intestinal transit and enteropooling models in mice. The anti-diarrheal index (ADI) was determined by combining all diarrhea indicators to evaluate the relative effect of the extract or fractions. For the 80% MeOH and solvent fractions, group I served as a negative control and received vehicle (distilled water or 2% tween-80, 10 ml/kg) orally; groups II-IV were test groups and received various doses (100, 200 and 400 mg/kg) of the extract or fractions, respectively and group V served as a positive control and given a standard drug, 3 mg/kg loperamide hydrochloride orally. In the castor oil induced diarrheal model, the 80% MeOH significantly delayed onset of diarrhea and reduced the number and weight of wet and total feces at all tested doses significantly as compared with the negative control ($p<0.05$). In this model the chloroform, n-butanol and aqueous fractions significantly delayed onset of diarrhea at all tested doses. The n-butanol and aqueous fractions at 200 mg/kg and 400 mg/kg and chloroform fraction at 400 mg/kg significantly reduced the number and weight of wet feces when compared with negative control ($p<0.05$). The n-butanol and aqueous fractions at all tested doses and the chloroform fraction at 400 mg/kg significantly decreased the number and weight of total fecal output. Results from the charcoal meal test revealed that the 80% MeOH as well as all the fractions produced a significant antimotility effect at all tested doses as compared with the

negative control ($p<0.05$). In the enteropooling test, the 80% MeOH as well as the n-butanol and aqueous fractions (at all tested doses) produced a significant decline in the volume and weight of intestinal contents, whereas the chloroform fraction revealed appreciable effect ($p<0.05$) only at 400 mg/kg when compared to negative control. The highest ADI was obtained with the dose of 400 mg/kg of the 80% MeOH, which was comparable to that produced by the standard drug, 3 mg/kg loperamide hydrochloride. Generally, this study demonstrated that the 80% MeOH, n-butanol and aqueous fractions produced anti-diarrheal activities due to dual inhibitory effect on castor oil induced intestinal motility and fluid secretion, with the aqueous fraction being the most active fraction among solvent fractions in all the three models. This research finding provides a scientific evidence for acclaimed traditional use of *Mimusops kummel* fruits for treatment of diarrheal diseases and recommends further research to characterize molecules with anti-diarrheal activity.

Key words: anti-diarrheal activity, castor oil induced diarrhea, gastrointestinal transit, *Mimusops kummel*

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LIST OF ABBREVIATIONS/ACRONYMS

ADI	Anti-Diarrheal Index
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
ASA	Amino Salicylic Acid
ATP	Adenosine Triphosphate
CaCC	Calcium Activated Chloride Channel
cAMP	Cyclic Adenosine Monophosphate
CDC	Center for Disease Control
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	Cyclic Guanosine Monophosphate
CI_{95}	95% Confidence Interval
CREB	cAMP Response Element Binding Protein
CSA	Central Statistical Agency
80% MeOH	80% Methanolic extract
GI	Gastrointestinal
GIT	Gastrointestinal Tract
HIV	Human Immunodeficiency Virus
IBD	Irritable Bowel Disorder
LD_{50}	Median Lethal Dose
NO	Nitric Oxide
OECD	Organization for Economic Cooperation and Development
ORS	Oral Rehydration Solution
ORT	Oral Rehydration Therapy
PATH	Program for Appropriate Technology in Health
PKA	Protein kinase A
UNICEF	United Nations International Children's Emergency Fund
WGO	World Gastroenterology Organization
WHO	World Health Organization

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1. INTRODUCTION

1.1. Definition and classification of diarrhea

The term diarrhea is derived from the Greek words (*dia* = through, *rhein* = to flow or run), denoting increased fluidity and frequency of fecal discharges (EI Mouzan, 1995). The World Health Organization (WHO) defines diarrhea as the passage of three or more loose or liquid stools per day, or as having more stools than is normal for that person (WHO, 2011). Frequency and consistency are variable from person to person (Barbara *et al*., 2009). In addition to these sometimes weight and water content of the stools are used to define diarrhea, characterized by a situation in which an adult daily stools weight greater than 300 g per day and contains 60-95% water generally indicates diarrhea**.** A combination of frequency, stool consistency and stool weight could be taken into account for defining diarrhea (Guerrant *et al*., 2001; Patil *et al*., 2011).

Based on time course, diarrhea can be classified as acute, persistent and chronic diarrhea. Acute diarrhea is defined as three or more loose bowel movements in a 24 hour period (Gregorio *et al*., 2009) and the duration is less than 2 weeks (Halsey, 2009). Furthermore, acute diarrhea could be either acute watery or acute bloody diarrhea (Mohanta *et al*., 2010). Acute watery diarrhea is associated with significant fluid loss and rapid dehydration in infected individual. The pathogens that generally cause acute watery diarrhea include *Vibrio cholera* and *Escherichia coli* bacteria, as well as *Rotavirus*. Acute bloody diarrhea, on the other hand, is marked by visible blood in the stools. It is associated with intestinal damage and nutrient losses in an infected individual. The most common cause of bloody diarrhea is *Shigella* (Guerrant *et al*., 2001; Mohanta *et al*., 2010). Diarrhea is said to be persistent if the duration varies from 2 to 4 weeks, and chronic if it lasts more than 4 weeks in duration (Guerrant *et al*., 2001).

1.2. Epidemiology of diarrhea

Although diarrhea is a preventable and treatable disease, it remains the second leading cause of mortality among children under five years of age worldwide next to respiratory infections and kills more young children than AIDS, malaria, measles, injuries and all other post-neonatal conditions combined (Kaplan *et al*., 2013; WHO, 2013). According to the WHO and the United Nations International Children's Emergency Fund (UNICEF), there are about 2.5 billion cases of

diarrheal disease worldwide every year, and 1.9 million children younger than five years of age die due to diarrhea each year, mostly in developing countries (Farthing and Salam, 2012). This amounts to 18% of all deaths of children under the age of five and means that more than 5000 children are dying every day as a result of diarrheal diseases (Farthing and Salam, 2012; Johansson, 2009).

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 *et al*., 2012). In the African regions, there were 26% severe episodes of diarrhea and the highest numbers of childhood deaths were in sub-Saharan Africa accounted to 50% of deaths due to diarrhea occurred in 2011 (Fischer *et al*., 2013).

According to the Central Statistical Agency demographic and health survey report, the two-week prevalence of diarrhea among children under five years of age was 13% in Ethiopia (CDC, 2013; CSA, 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, the Federal Ministry of Health (2012) and several community-based studies have shown that diarrheal disease is a major public health problem that causes morbidity and mortality in children (Awoke, 2013; Mamo and Hailu, 2014; Tamiso *et al*., 2014).

1.3. Etiology and prevention of diarrhea

Diarrhea is a common symptom of gastrointestinal infections which can be caused by a wide range of pathogens, including bacteria (*Salmonella typhi*, *Escherichia coli, Campylobacter*, *Vibrio cholerae*, *Shigella flexneri* etc), viruses (*Rotavirus*, *Norovirus*, *Adenovirus, Enterovirus, Cytomegalovirus* etc) and protozoa (*Cryptosporidium* and *Entamoeba histolytica*) (Kaplan *et al*., 2013; Toyin *et al*., 2012; WGO, 2008). These pathogens present in the gut causing disruption of normal fluid secretion and motility, and stimulating the gut to expel the contents. Most pathogens are transmitted from the stool of one person to the mouth of another via contaminated food or water (faecal-oral transmission) (Panda *et al*., 2012). *Rotavirus* and *Escherichia coli* are the two most common etiological agents of diarrhea in developing countries (WHO, 2013).

Eating of unsuited foods, putrefaction of food in the intestinal tract, fermentation caused by incomplete carbohydrate digestion, nervous irritability, use of antibiotic drugs and excessive intake of laxatives can also cause diarrhea (Singh and Verma, 2012). Diarrhea also occurs frequently in post-transplantation patients who are receiving immunosuppressive drugs (Sellin, 2001).

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

1.4. Normal intestinal physiology and pathophysiology of diarrhea

1.4.1. Normal intestinal physiology

The human small intestine and colon perform important functions including the secretion and absorption of water and electrolytes, the storage and subsequent transport of intraluminal contents. Alterations in fluid and electrolyte handling contribute significantly to diarrhea (David and Camiller, 2004).

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 and Clarence, 2008; Shah, 2004).

There is a constant bidirectional flux of water and ions across the small intestinal mucosa, that is, absorption and secretion. In the small intestine, solute movement creates the osmotic force for fluid movement. For instance, sodium absorption drives fluid reabsorption, while active chloride secretion contributes to water secretion in secretory diarrhea (Binder, 2005; Binder and Reuben, 2005). Sodium and water absorption by enterocytes is mediated by an active, ATP-dependent active sodium pump (Na^+, K^+-ATPase) located on the basolateral membranes of intestinal crypt and villus cells. Small intestinal Na^+ absorption is mediated primarily by two mechanisms: a

glucose- or amino acid-stimulated cotransport in which Na^+ accompanies the other solute and a coupled Na^+-Cl^- mechanism. The latter is a combination of Na^+-H^+ exchange and Cl^--HCO_3^- exchange. Short-chain fatty acid (SCFA)-mediated Na^+ absorption and aldosterone-sensitive Na^+ absorption occur in the colon (Binder, 2005). Among the various mechanisms described, the coupled Na^+-Cl^- pathways are primarily regulated by cyclic adenosine monophosphate (cAMP) levels and also by cyclic Guanosine Monophosphate (cGMP) and intracellular Ca^{2+} levels (Field, 2003). In addition to the transporters, there are multiple extracellular factors regulating epithelial ion transport-paracrine, immunological, neural and endocrine factors, termed together as a single regulatory system known as PINES (paracrine-immuno-neuroendocrine system) (Mourad *et al.*, 1995).

In addition to the absorptive and secretary function of the intestine, motor functions also play a key role in facilitating digestion and absorption of fluids and nutrients. Synchronized migrating motor complexes normally occur during fasting in the stomach and small bowel with increased contractions following feeding with the total small bowel transit time of approximately 3 h for the food to reach the colon (Kerlin *et al.*, 1982). In the colon, there is further reabsorption with the ascending and transverse colon serving as reservoirs and with the sigmoid and rectum serving as volitional reservoirs (Proano *et al.*, 1990). Any disturbance in the coordinated flux of water and ions, and motility can result in the clinical syndrome of diarrhea.

1.4.2. Pathophysiology of diarrhea

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).

Four general pathophysiologic mechanisms disrupting water and electrolyte balances are the basis of diagnosis and therapy of diarrhea. These are changes in active ion transport by decreased sodium absorption or increased chloride secretion, increase in luminal osmolarity, increase in tissue hydrostatic pressure and change in intestinal motility. These mechanisms have been

related to four broad clinical diarrheal groups: secretory, osmotic, inflammatory and altered motility diarrhea (Spruill and Wade, 2008).

Secretory diarrhea is caused by an increase in water and ions (chloride or bicarbonate) movement to the intestinal lumen, the final effect is an increase in the net secretion of ions (chloride or bicarbonate) and inhibition of the net absorption of sodium and water (Crombie *et al*., 2013). Secretory diarrhea may arise from infectious and non-infectious causes. The most common cause of secretory diarrhea is infectious agents, which produce enterotoxins that interact with receptors and lead to an augmented secretion (Shah, 2004). Most causes of secretory diarrhea alter the second messenger system through alteration in cAMP, cGMP or intracellular Ca^{2+} regulated ion transport pathways and alterations in these mediators cause CFTR or CaCC-mediated Cl^- secretion and inhibition of small intestinal coupled Na^+-Cl^- transport. Paracellularly, sodium follows the chloride to maintain charge balance and water escapes from the cells to maintain osmotic balance. This efflux of water and electrolytes is manifested as watery diarrhea. Secretory diarrhea persists in spite of fasting (Barbara *et al*., 2009; Hoque *et al.*, 2012; Strasinger and Di Lorenzo, 2008).

Osmotic diarrhea occurs when there is a dysfunction in the ability of the intestine to reabsorb fluid as it flows through the lumen. This may be caused by incomplete breakdown or malabsorption of nutrients in the small intestine allowing a larger and more liquid mass to enter the colon. This fecal matter then creates a negative osmotic gradient causing leakage of more fluid into the gut increasing the stool volume. This type of diarrhea can be caused by decreased enzymatic availability (lactose intolerance), a genetic abnormality that decreases or eliminates the ability of the body to absorb certain nutrients (celiac sprue), sugars that are poorly absorbed (sorbitol, mannitol or lactose), laxatives, magnesium containing antacids and antibiotic administration as well as malabsorption of certain fat. Other causes have more to do with changes within the bowel that decrease the ability to reabsorb fluid and nutrients as the stool is propelled through the lumen which includes malnutrition, resection of parts of the bowel and inflammation of the bowel due to infection or disease processes. 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; Strasinger and Di Lorenzo, 2008).

Inflammatory diarrhea is a gastrointestinal (GI) disorder that may encompass all of the pathophysiologic mechanisms. For example, inflammation with resultant injury to the intestine may lead to malabsorption of dietary macronutrients which in turn, creates a luminal osmotic gradient. Additionally, particular infectious agents may induce secretion of fluid into the lumen and blood in the gut may alter intestinal motility (Garrett and Esther, 2012).

Diarrhea due to abnormal intestinal motility can enhance bacterial overgrowth within the intestine. Increased motility shortens the contact period of chyme with mucosa. Therefore in consequence of this change, the volume of chyme in the large intestine increases which causes faster evacuation. The consequent abbreviation of contact of chyme with mucosa results in a decrease in water absorption. Diarrhea due to abnormal intestinal motility is usually present in coincidence with irritable bowel syndrome, after gastrectomy, vagotomy, diabetic neuropathy, sclerodermia and thyrotoxicosis (Barbara *et al*., 2009).

1.5. Management of diarrhea

The therapeutic goals of diarrhea treatment are to prevent excessive water, electrolyte and acid-base disturbances; provide symptomatic relief and manage secondary disorders causing diarrhea (William and William, 2006). Most diarrheal illnesses are self-limited and require no specific intervention other than hydration and dietary modification (Lawler and Wallace, 2003).

Fluid and electrolytes

Fluid replacement is not a treatment to relieve diarrhea but rather an attempt to restore fluid balance. Oral rehydration therapy (ORT) is the administration of appropriate solutions by mouth to prevent or correct diarrheal dehydration. It is a cost-effective method of managing acute gastroenteritis and it reduces hospitalization requirements in both developed and developing countries (WGO, 2012).

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 and Clarence, 2008). The absorption of glucose molecules which is found in the ORT is not

affected by diarrhea and this further increases the absorption of Na^+ through Na^+-glucose co-transport mechanism and water follows Na^+ down its concentration gradient (Goodall, 2014).

Zinc supplement

Diarrhea is a commonly associated problem in children with zinc deficiency and it also leads to excess zinc losses. Clinical studies have shown that a 10 to 14 day treatment course with zinc effectively reduces the duration and severity of both acute and persistent diarrhea (Patel *et al*., 2010a; Penny, 2013). Additional study revealed that zinc supplementation resulted in 13% reduction in the mortality of children due to diarrhea (Yakoob *et al*., 2011). Zinc is found to be safe in HIV infected children and also known to reduce morbidity in these children (Shimelis *et al*., 2008). It is critical for cellular functions, cellular growth and supports proper functioning of the immune system, increase ORS uptake and reduces inappropriate drug use (Bhandari *et al*., 2008).

Antisecretory agents

Antisecretory agents are drugs that decrease the secretion of fluid by blocking chloride channels, inhibiting production of secretogues agents, increasing the availability of endogenous opioids, etc (Eberlin *et al*., 2012; Farthing, 1999). These drugs include the following.

Crofelemer simultaneously blocks two distinct chloride channels, CFTR and CaCC, leading to inhibition of chloride ion secretion. This reduces efflux of sodium and water, which in turn reduces the frequency and consistency of diarrhea (Chordia and MacArthur, 2013; Cottreau *et al*., 2012). This drug was approved by the United States Food and Drug Administration (FDA) for the symptomatic relief of non-infectious diarrhea in adult patients with HIV/AIDS on antiretroviral therapy (FDA, 2012).

Octreotide is a long-acting synthetic analogue of somatostatin which is effective in inhibiting the severe secretory diarrhea brought about by hormone-secreting tumors of the pancreas and the GIT. Its mechanism of action appears to involve inhibition of hormone secretion, including serotonin and various other GI peptides (e.g. gastrin, vasoactive intestinal polypeptide, insulin, secretin, etc). Octreotide as an antisecretory agent is very effective in management of acute infective diarrhea in adults (McQuaid, 2009; Mehta *et al*., 2012).

Racecadotril is a prodrug which inhibits inactivation of endogenous opioid peptides (enkephalins) (Eberlin *et al*., 2012). The enkephalins in turn activate delta receptor that induces a selective increase in chloride absorption via inhibition of adenylate cyclase (Farthing, 1999; 2006; Salazar *et al*., 2000). Racecadotril inhibited secretion induced by cholera toxin (Primi *et al*., 1999) and *Rotavirus* infection (Guarino *et al*., 2009) and it does not produce enteropooling and rebound constipation (Turvill and Farthing, 1997).

Other antisecretory agents include phenothiazine and zaldaride maleate which inhibits hormonal stimulation of cAMP and calmodulin (calcium-binding protein) (Aikawa and Karasawa, 1998; Holmgren *et al*., 1978).

Antimotility agents

These drugs prolong intestinal transit time, thereby reducing the amount of fluid lost in the stool. Loperamide and diphenoxylate with atropine sulfate have been shown to slow transit time within the intestine to permit more re-absorption of fluid. They act by intestinal μ opiate receptors, leading to increased intestinal transit time (Kent and Banks, 2010). In addition, these drugs have antisecretory action in the human jejunum (Al-Abri *et al*., 2005; De Luca and Coupar, 1993).

Antimotility drugs are contraindicated in severe diarrhea and in diarrhea caused by invasive pathogens. This is due to the fact that pooling of large fluid volumes in paralyzed bowel loops and enhanced tissue invasion by the organisms or delay their clearance from the bowel and hence the disease condition may aggravate (Manatsathit *et al*., 2002; Field, 2003).

Antimicrobials

To minimize development of drug-resistant organisms, antibiotics should not be used for relatively mild, self-limited infectious diarrheas. According to the infectious diseases society of America's guidelines for the management of acute infectious diarrhea, empirical antibiotics are commonly recommended without obtaining a fecal specimen in patients with travelers' diarrhea or febrile dysenteric illness, especially those believed to have moderate-to-severe invasive diseases. Fluoroquinolones are recommended as the first drugs of choice for the empirical treatment of adult patients. But nowadays, due to development of resistance they are not widely used (Guerrant *et al*., 2001; Yang *et al*., 2008). Rifaximin is a rifamycin-based, nearly non-absorbable, gut selective antibiotic with an excellent safety profile. It was approved in Italy in

1987 and in the United States in 2004 for the treatment of several GI diseases, particularly acute infectious diarrhea such as travelers' diarrhea secondary to non-invasive *Escherichia coli* (Koo and Dupont, 2010; Ojetti *et al*., 2009).

Anti-inflammatory agents

There are few specific treatments of irritable bowel disorder (IBD) associated diarrhea, in part because of the complexity and limited understanding of mechanisms (Urayama and Chang, 1997). Most of the currently used preparations have actions that inhibit various steps of the inflammatory cascades. The common ant-inflammatory agents for the management of IBD are derivatives of 5-aminosalicylic acid (5-ASA) which act by inhibiting arachidonic acid metabolism, decreasing the synthesis of both leukotrienes and prostaglandins. Though it inhibits both COX and LOX, decreased prostaglandins production appears to play a minor role in the therapeutic effect. Moreover, 5-ASA is a potent scavenger of free radicals (Dhaneshwar, 2014; Vermeire *et al*., 2012).

Miscellaneous products

Probiotics are live microorganisms which can prevent or ameliorate diarrhea through receptor competition, competition for essential nutrients, inhibition of epithelial and mucosal adherence of pathogens and translocation (Scaldaferri *et al*., 2012), introduction of lower colonic pH thereby favoring the growth of non-pathogenic species (Hempel *et al*., 2012), stimulation of immunity or production of antimicrobial substances and production of short chain fatty acids that are beneficial for intestine recovery and increase the rate of fluid and electrolyte absorption, when administered in adequate amounts (Friedman, 2012; Sartor, 2005). Commonly used strains include *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus casei* GG, *Lactobacillus bulgaricus* and *Saccharomyces boulardii* (Bergogne-Berezin, 2000; Sartor, 2005). A systematic review and meta-analysis study suggests that probiotics are associated with a reduction in antibiotic associated diarrhea and *Clostridium difficile* diarrhea (Allen *et al*., 2013; Hempel *et al*., 2012).

Prebiotics are non-digestible food ingredients that may benefit the host by selectively stimulating bacteria in the colon that confer health benefits to the host. The most commonly used prebiotics are inulin-type fructans (inulin, oligofructose and fructooligosaccharides) (Roberfroid *et al*.,

2010). Synbiotics refer to preparations in which probiotic organisms and prebiotics are combined (Hempel *et al*., 2012).

Dietary modifications

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

Investigational anti-diarrheal agents

The intestinal ion transport mechanisms which operate using intracellular signalling mechanisms are pharmacological target in the search for antisecretory agents. Calcium and the calcium-binding protein calmodulin and CFTR protein are among the targets on trial. 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 (PATH, 2013; 2014).

1.6. Traditional medicine

Since the time immemorial, medicinal plants have played an invaluable role in the development of potent therapeutic agents. Despite the development of vast spectrum of approaches for diarrheal management, World Health Organization (WHO) estimates that approximately 80% of the daily healthcare needs of the people in developing countries still rely on traditional medicine for the management of diarrhea (Ojewole *et al*., 2010). In developing countries, a majority of people living in rural areas almost exclusively use traditional medicine in treating all sorts of diseases including diarrhea, which is very common and recurring disease in community. This is also true in some developed countries where the use of modern medicine is predominant (Goyal *et al*., 2007; Patel *et al*., 2010b; Saralaya *et al*., 2010). Medicinal plants are potential sources of anti-diarrheal drugs (Almeida *et al*., 1989; Maikere-Faniyo *et al*., 1989). Considering this fact the WHO has constituted a diarrhea disease control programme (DDC) aimed at a holistic approach to include all aspects for treatment and prevention of diarrheal diseases depending on traditional medical practices, together with the evaluation of health education and prevention approaches. This may reduce mortality rate in developing countries due to diarrhea (Atta and Mouneir, 2004; Damiki and Siva, 2011; Hossain *et al*., 2012; WHO, 2004).

Different studies revealed that the plants showed anti-diarrheal activity by reducing intestinal secretion and motility. The anti-diarrheal activities of medicinal plants have been attributed to the presence of bioactive compounds such as tannins, alkaloids, saponins, flavonoids, steroids, terpenoids and phenolic compounds (Edeoga *et al*., 2005; Komal *et al*., 2013; Umer *et al*., 2013). In recent years, there has been a great interest in herbal remedies for the treatment of number of ailments. Ethnobotanical studies indicate a range of medicinal plants such as the bark extract of *Albizia gummifera*, leaf extract of *Calpurnia aurea* and *Myrtus communis*, root extract of *Ensete ventricosum* and *Caylusea abyssinica*, seed extract of *Coffea arabica* and eating fruits of *Mimusops kummel* have been widely used for the management of diarrhea by traditional healers in Ethiopia (Teklehaymanot and Giday, 2007).

1.7. The experimental plant

Mimusops kummel belongs to Kingdom - *Planate*; Phylum - *Magnoliophyta*; Class - *Magnoliopsida*; Order - *Ericales*; Family - *Sapotaceae* and Genus - *Mimusops.* The genus

Mimusops as described by Linnaeus in 1753 contained 57 species that are widespread throughout the tropical, subtropical and various oceanic islands areas of the world up to 2,100 m altitude (Alain *et al*., 2013). The name of the genus *Mimusops* comes from Greek words *mimo* meaning ape and *ops* meaning resembling (Palmer and Pitman, 1972).

Mimusops kummel is a deciduous tree small-to medium-sized tree up to 25 (-35) m high, containing latex; bole up to 100 cm in diameter; bark deeply grooved, dark grey; crown dense, ovoid; young densely red-brown pubescent branches. Its fruit is an ellipsoid to ovoid berry up to 2.5 cm long, orange-red when ripe, containing a single large seed. The fruits taste pleasant and are used for making juice and alcoholic drinks. *Mimusops kummel* is commonly found in secondary forests, around lakes, in moist or dry evergreen upland forests and woodlands. *Mimusops kummel*, which is depicted below, is native to Guinea, Cote D'Ivoire, Eritrea, Ethiopia, Kenya, Uganda, Tanzania and Malawi (Alain *et al*., 2013).

Mimusops kummel is commonly known as Red milk wood, Bullet wood (English), *Eshe*, *Shiye* (Amharic), *Kummel*, *Lelle* (Tigrigna), *Bururi*, *Qoladi*, *Mito* (Oromifaa) and *Danga* (Wolaytigna) (Alain *et al*., 2013; Teklehaymanot and Giday, 2007).

Photographs of *Mimusops kummel*

Mimusops kummel fruits are principally used in Ethiopia for treatment of diarrhea as well as in the treatment of amoeba (eating fruits) (Teklehaymanot and Giday, 2007). Apart from this, the seeds are used to treat ascariasis and the bark is used as a treatment for anaemia, asthma and malaria. Ethnopharmacological study revealed that hydroalcoholic extract of *Mimusops kummel* root has shown promising activity against *Culex quinquefasciatus*, *Anopheles gambiae* Giles and *Neisseria gonorrhoeae* (Alain *et al*., 2013).

1.8. Rationale for the study

Despite the availability of many drugs for treating diarrhea, majority of them suffer from adverse effects like the induction of bronchospasm and vomiting by racecadotril (Tormo *et al*., 2008); intestinal obstruction and constipation by loperamide (Pankaj, 2006) and undesirable central effects by long term use of morphine and its analogs (Khansari *et al*., 2013). ORT has been the mainstay of treatment of diarrhea; however it does not reduce the frequency of stools or the number of diarrheal days. This treatment often fails in the high stool output state (WGO, 2012). Moreover, there is an increasing threat of drug resistance, side effects, superinfection and the possibility of induction of disease producing bacteriophages by antibiotics. Due to these problems, WHO encourages studies for the treatment and prevention of diarrheal diseases depending on traditional medical practices (WHO, 2004). There are many plants which are traditionally used for the treatment of diarrhea, among which *Mimusops kummel* is one of them. This study attempted to validate the traditional use of this plant and to further ascertain in which fraction (s) the constituents responsible for anti-diarrheal activity are concentrated so as to provide a clue about the nature of the phytochemical constituents responsible for its action and the possible modes of action. The finding of this research could be used as an input in searching of new anti-diarrheal agent that might solve problems associated with the conventional anti-diarrheal drugs. It could also give direction for traditional users on different way of preparation and use of the plant. In addition, the results of this study help the scientific community to further investigate the plant *Mimusops kummel* by initiating advanced studies on molecular mechanisms and formulation of plant source drugs by identifying the specific agent responsible for the anti-diarrheal effect.

2. OBJECTIVE

2.1. General objective

To evaluate the anti-diarrheal activities of 80% MeOH and solvent fractions (chloroform, n-butanol and aqueous) of *Mimusops kummel* fruits in mice

2.2. Specific objectives

- To evaluate the effect of 80% MeOH and solvent fractions of *Mimusops kummel* fruits on castor oil induced diarrhea in mice
- To evaluate anti-motility activity of 80% MeOH and solvent fractions of *Mimusops kummel* fruits on castor oil induced intestinal transit using charcoal meal test in mice
- To evaluate anti-enteropooling effect of 80% MeOH and solvent fractions of *Mimusops kummel* fruits on castor oil induced enteropooling in mice
- To identify the secondary metabolite classes in the 80% MeOH and solvent fractions of *Mimusops kummel* fruits
- To assess acute toxicity of 80% MeOH of *Mimusops kummel* fruits in mice

3. MATERIALS AND METHODS

3.1. Drugs and chemicals

Castor oil (Amman Pharmaceutical Industries, Jordan), activated charcoal (Acuro Organics Ltd, New Delhi), loperamide hydrochloride (Daehwa Pharmaceuticals, Republic of Korea), distilled water (Ethiopian Pharmaceutical Manufacturing Factory, Ethiopia), methanol (Blulux, India), n-butanol (Carlo Erba reagents, France), chloroform (Finkem Laboratory Reagent, India), tween-80 (Atlas Chemical Industries Inc, India), glacial acetic acid, sulfuric acid, ammonia, hydrochloric acid and ferric chloride (BDH Laboratory Supplies Poole, England), benzene (Fisher Scientific, UK), acetic anhydride, ethyl acetate, Mayer's and Dragendorff's reagents (May and Baker LTD Dagenham, England) were used in this study. All the chemicals and solvents were of analytical grade.

3.2. Collection and preparation of plant materials

Fresh ripe fruits of *Mimusops kummel* A. DC. were collected from Sisa, Dera woreda, Amhara region, located about 595 km north of the capital Addis Ababa, Ethiopia in November, 2015. Botanical identification and authentication were done by the kind cooperation of an expert botanist, Dr. Getachew Addis at the Ethiopian Public Health Institute (EPHI), Traditional and Modern Medicine Research Directorate. A voucher specimen (number MM-01) was deposited in the institute's herbarium for future reference. The fruits were initially washed using distilled water to remove dust materials and dried at room temperature under shade for 14 days. The fruits were then chopped into small pieces and ground into coarse powder mechanically using a clean porcelain mortar and pestle. The powder sample was weighed and stored in air sealed polythene bags in locked cupboard at room temperature until extraction commenced.

3.3. Experimental animals

Healthy Swiss albino mice of either sex, weighing 25-30 g and aged 6-8 weeks, which breaded in animal house of EPHI were used for the experiment. The animals were housed in metal cages (5 to 8 animals per cage) under standard conditions, maintained on a 12:12 hr light-dark cycle at ambient room temperature and had free access to food (pellet diet) and water being *ad libitum* up to the time of experimentation. The animals were acclimatized to the laboratory condition for

one week before being subjected to experimental protocol (Umer *et al*., 2013). The animals were handled according to the guideline for the care and use of experimental animals (National Research Council, 2011).

3.4. Ethical approval

Ethical approval was obtained from the Scientific and Ethics Committee of the Department of Pharmacology, School of Medicine, Addis Ababa University.

3.5. Preparation of plant extract and fractions

3.5.1. Preparation of 80% methanolic extract

The crude methanolic extract was prepared by cold maceration technique. For preparation of the crude extract of plant material, about 500 g of coarsely powdered fruits were weighed using sensitive digital weighing balance (Mettler Toledo, Switzerland). The coarse powder was taken in a clean, conical flask and 80% methanol (1:5 (w/v)) was added in flask. The flask with its contents was sealed with cotton plug and aluminum foil and kept for a period of 48 hours at room temperature accompanying intermittent shaking using mini orbital shaker (Bibby Scientific Limited Stone Staffo Reshire, UK) that was revolving at 120 rpm to enhance the efficient extraction. The entire mixture was first filtered through a funnel plunged with muslin cloth two times and then the filtrate was passed through Whatman filter paper (Number 1) (Maidstone, UK). After filtration, the residue was re-macerated two times for a total of 96 h in order to obtain a better yield. The marc was pressed and the combined filtrate was then evaporated using a rotary evaporator (Buchii model R-200, Switzerland) set at 40 ºC to remove the solvent, then the filtrate was placed in an oven at 45 ºC for one week to remove the remaining solvent. The residue was then freeze dried at -20 ºC. The resultant filtrates were pooled together and concentrated in a lyophilizer (Operan, Korea vacuum limited, Korea) to remove water. It rendered a solid residue of yellowish color which was designated as the 80% MeOH. A total of 105 grams (percentage yield of 21%) of dried extract was obtained and stored in an air tight container in deep freezer (-20 ºC) until used for further investigation.

3.5.2. Fractionation of the crude extract

Fractionation of the crude extract was carried out using different organic solvents. Eighty gram of crude extract was dissolved completely in 400 ml of distilled water and this solution was taken in a separating funnel. An equal volume of chloroform was added into it and was shaken vigorously. The mixture was separated in two layers by a separating funnel without shaking. The chloroform layer (lower) was removed. The partition with chloroform was repeated two times more. All of the chloroform layers were combined and subjected to evaporation using a rotary evaporator (Buchii model R-200, Switzerland) set at 40 ºC to get the chloroform fraction, then the filtrate was placed in an oven at 45 ºC for one week to remove the remaining solvent and 10.93 grams (11.50%) was obtained. The aqueous layer was taken into a separating funnel; 400 ml of n-butanol was added into it. The upper layer in this case was n-butanol, which was separated and the procedure was repeated two times. The separated n-butanol layers were pooled and evaporated using a rotary evaporator (Buchii model R-200, Switzerland) set at 40 ºC to obtain the n-butanol fraction, then the filtrate was placed in an oven at 45 ºC for two weeks to remove the remaining solvent and 11.40 grams (12%) was obtained. The remaining aqueous layer (lower in this case) was concentrated in a lyophilizer (Operan, Korea vacuum limited, Korea) to remove water and a total of 16.15 grams (17%) of aqueous fraction was obtained. After drying, the solvent fractions stored in an air tight container in refrigerator until used for evaluation of the anti-diarrheal activity and phytochemical constituents.

3.6. Animal grouping and dosing

In all experimental models, the animals were randomly assigned into five groups (negative control, three test groups and positive control) comprising of six animals per group. All groups were provided with their respective treatments using oral gavage. Group I served as the negative control and received vehicle (10 ml/kg body weight) (distilled water for 80% MeOH and 2% tween-80 for chloroform, n-butanol and aqueous fractions), group II, III and IV received 100, 200 and 400 mg/kg of the 80% MeOH or solvent fractions of *Mimusops kummel* orally, while group V received standard drug (loperamide hydrochloride, 3 mg/kg) per orally. Loperamide hydrochloride served as a standard drug for castor oil induced diarrhea, small intestine transit time and enteropooling models. Dose selection was made based on the acute oral toxicity test as well as pilot study and then 100, 200 and 400 mg/kg doses were designated as low, moderate and

high dose, respectively in the present study. The stock solutions were prepared fresh on the day of experiment.

3.7. Determination of *in vivo* anti-diarrheal activity

3.7.1. Castor oil induced diarrhea model

The method described by Maniyar *et al*. (2010) and modified by Umer *et al*. (2013) was followed for the current study. In this experiment, 30 Swiss albino mice of either sex were deprived of food for 18 h with free access to water and divided randomly into five groups of six mice each, labeled groups I-V. Group I received 10 ml/kg of distilled water or 2% tween-80, group II, III and IV received 100, 200 and 400 mg/kg of 80% MeOH or solvent fractions, respectively, while group V was given loperamide hydrochloride 3 mg/kg orally. After 1 hour of treatment with the distilled water, respective doses or standard drug, diarrhea was induced by administration of 0.5 ml of castor oil orally to each mouse. Following their administration, the animals were placed individually in cages in which the floor is lined with white paper for collection of fecal matters. The transparent paper was changed every hour for a total of 4 hours. During the observation period of 4 hours, the onset of diarrhea (the time interval in minutes between the administration of castor oil and the appearance of the first diarrheal stool), number and weight of wet stools, total number and total weight of fecal output were recorded for individual mouse. Finally, percentage of diarrheal inhibition as well as percentage of weight of wet and total fecal output were calculated by using the formulas described below (Akindele *et al*., 2014; Ara *et al*., 2013).

$$\text{Percentage of inhibition (\%)} = \frac{\text{Mean number of WFC - Mean number of WFT}}{\text{Mean number of WFC}} \times 100$$

Where, WFC = Wet Feces in Control group and

WFT = Wet Feces in Test group.

$$\text{Percentage of wet fecal output (\%)} = \frac{\text{Mean weight of wet feces of each treatment group}}{\text{Mean weight of wet feces of control}} \times 100$$

$$\text{Percentage of total fecal output (\%)} = \frac{\text{Mean fecal weight of each treatment group}}{\text{Mean fecal weight of control}} \times 100$$

3.7.2. Castor oil induced gastrointestinal motility test

Gastrointestinal transit (motility) was investigated in mice using the method of Aye-Than *et al*. (1989) as described by Rahman *et al*. (2012). Before the commencement of the experiment, the mice of either sex were fasted for 18 h, but allowed free access to water and randomly allocated into five groups of six animals per group to determine the effect of the 80% MeOH and each fraction on gastrointestinal transit of a marker meal. The mice in group I (negative control group), received 10 ml/kg of distilled water or 2% tween-80 orally. The mice in groups II-IV were treated with the graded doses of the 80% MeOH or solvent fractions (100, 200 and 400 mg/kg by oral route, respectively). The mice in group V (positive control group) were treated with loperamide hydrochloride at the dose of 3 mg/kg orally. After an hour of dosing, all the mice were challenged with 0.5 ml of castor oil orally to induce diarrhea. One ml charcoal meal (5% activated charcoal suspension in distilled water) was administered orally 1 hour after castor oil administration. Thirty minutes later each mouse was then sacrificed by cervical dislocation, the abdomen was opened and the small intestine was immediately dissected out from pylorus to caecum and placed length wise on a white paper. Thereafter, the distance travelled by the charcoal meal from the pylorus and the total length of the intestine were measured. The peristaltic index (PI) expressed as percentage of the distance traveled by the charcoal meal relative to the total length of the small intestine as well as the percentage inhibition of movement as a function of the control were calculated by using the following formulas.

$$\text{Peristaltic index (PI)} = \frac{\text{Mean distance travelled by charcoal meal}}{\text{Mean length of small intestine}} \times 100$$

$$\text{Percentage of inhibition (\%)} = (Dc - Dt)/Dc \times 100$$

Where, Dc = Mean distance travelled by the charcoal in the control group and

Dt = Mean distance travelled by the charcoal in the test group.

3.7.3. Castor oil induced enteropooling

The effects of 80% MeOH and solvent fractions on intraluminal fluid accumulation were determined using a method described by Ezeja and Anaga (2010) and Mehmood and Gilani (2010). Prior to the experiment, the animals of either sex were fasted for 18 h, randomly divided into five groups consisting of six mice in each group; they were pretreated with distilled water or

2% tween-80 (10 ml/kg), extract or fractions (100, 200 and 400 mg/kg) and standard drug (loperamide hydrochloride 3 mg/kg) orally. After 1 h of treatment, 0.5 ml of castor oil was administered and animals were sacrificed by cervical dislocation 1 h following castor oil administration. The abdomen of each animal was then opened; the small intestine was removed, tied with thread at the pyloric end and the ileocaecal junction. The dissected small intestine was weighed and intestinal contents were then collected by milking into a graduated tube and their volume was measured. Weight of the intestine after milking was taken and the difference between full and empty intestines was calculated. Finally, the percentage inhibitions of intestinal secretion (volume and weight) were calculated relative to the negative control using the following formulas.

$$\text{Percentage of inhibition by using MVIC} = \frac{\text{MVICC-MVICT}}{\text{MVICC}} \times 100$$

Where, MVIC = Mean Volume of Intestinal Content,

MVICC = Mean Volume of Intestinal Content of Control Group and

MVICT = Mean Volume of Intestinal Content of Test Group.

$$\text{Percentage of inhibition by using MWIC} = \frac{\text{MWICC-MWICT}}{\text{MWICC}} \times 100$$

Where, MWIC = Mean Weight of Intestinal Content,

MWICC = Mean Weight of Intestinal Content of Control Group and

MWICT = Mean Weight of Intestinal Content of Test Group.

3.7.4. *In vivo* anti-diarrheal index

The *in vivo* anti-diarrheal index (ADI) for 80% MeOH and solvent fractions was determined by combining three parameters taken from the aforementioned models. It was then expressed according to the following formula developed by Aye-Than *et al* (1989).

$$\textit{In vivo}\ \text{ADI} = \sqrt[3]{Dfreq\ xGmeq\ xPfreq}$$

Where, Dfreq is the delay in defecation time as percentage of negative control,

Gmeq is the gut meal travel reduction as percentage of negative control and

Pfreq is the reduction in purging frequency in the number of wet stools as percentage of negative control.

3.8. Preliminary phytochemical screening

The qualitative phytochemical investigations of 80% MeOH as well as chloroform, n-butanol and aqueous fractions of *Mimusops kummel* fruits were carried out as per standard tests (Bhandary *et al*., 2012; Sasidharan *et al*., 2011; Zohra *et al*., 2012).

Test for terpenoids

To 0.25 g of 80% MeOH and each fraction, 2 ml of chloroform was added in a test tube. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

Test for saponins

To 0.25 g of 80% MeOH and each fraction, 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously for 2-3 minutes and observed for a stable persistent froth. Formation of froth or foam indicated the presence of saponins.

Test for tannins

About 0.25 g of 80% MeOH and each fraction was boiled in 10 ml of water in a test tube and then filtered. Three drops of 0.1% ferric chloride were added to the filtrate. Presence of tannins was confirmed by the formation of brown greenish or blue-black color.

Test for flavonoids

Ten ml of ethyl acetate was added to 0.25 g of 80% MeOH and 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 ammonial layer indicated the presence of flavonoids.

Test for cardiac glycosides

To 0.25 g of 80% MeOH and each fraction diluted to 5 ml in water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. 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

glacial 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 0.25 g of 80% MeOH and each fraction with 2 ml chloroform. Then, 1 ml of concentrated sulfuric acid was added. The color changed from violet to blue or green indicated the presence of steroids.

Test for alkaloids

About 0.5 g of 80% MeOH and 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 5 ml 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.

Test for Anthroquinones

About 0.25 g of 80% MeOH and each fraction was shaken with 10 ml of benzene. This was filtered and 5ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of violet color in the ammonical (lower) phase indicated the presence of free hydroxyl anthroquinones.

Test for Phenols

About 0.25 g of 80% MeOH and each fraction was treated with few drops of neutral ferric chloride solution 5%, the appearance of a greenish precipitate indicated the presence of phenols.

3.9. Acute oral toxicity test

Acute oral toxicity test for 80% MeOH of *Mimusops kummel* fruits was performed according to the Organization for Economic Cooperation and Development (OECD) guideline 425 (2008). Five female albino mice of 6-8 weeks were used for each test. All mice were fasted (food but not

water) for 4 h before and 2 h after the administration of the extract. First, a sighting study was performed to determine the starting dose. For this, a single female mouse was given 2000 mg/kg of the extract as a single dose by oral gavage. Since no death was observed within 24 h, additional four mice were used, and administered the same dose of the extract. The animals were housed separately in cages and observed continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicity, food and water intake and mortality.

3.10. Data analysis

The data were analyzed using the software Statistical Package for Social Sciences (SPSS), version 20. The experimental results obtained from this study were expressed as mean ± CI_{95} (95% confidence interval). The statistical analysis of data was done using one-way analysis of variance (ANOVA) followed by Tukey's Post hoc test for multiple comparisons, which was used to compare results among groups. Differences were considered statistically significant if p values were less than 0.05. Coefficient of determination (R^2), using linear regression analysis, was used where appropriate. The analyzed data were then presented using tables.

4. RESULTS

4.1. Effects on castor oil induced diarrheal model

In the course of observation for 4 hours after castor oil administration, as presented in Table 1, the 80% MeOH of the fruits of *Mimusops kummel* significantly delayed the onset of diarrhea and reduced the number and weight of wet and total stools at doses of 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.05$) and 400 mg/kg ($p<0.05$) as compared with the negative control. Moreover, a significance difference was not obtained when the effects of 100 mg/kg were compared with 200 mg/kg, 400 mg/kg and standard drug in all of the parameters except in delaying the onset of diarrhea. Besides, the data revealed that the percentage of diarrheal inhibitions were 71.40% ($p<0.05$), 76.70% ($p<0.05$) and 85.70% ($p<0.05$) at the doses of 100 mg/kg, 200 mg/kg and 400 mg/kg, respectively. The maximum dose of this extract (400 mg/kg) produced the maximum percentage inhibition of defecation and the lowest percentage of mean fecal output when compared with the tested doses of the extract and positive control (79.40% at the dose of 3 mg/kg).

Amongst the solvent fractions, the aqueous fraction of *Mimusops kummel* fruits significantly prolonged the time of diarrheal induction and decreased 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 inhibitions obtained as compared to control were 60.82% ($p<0.05$), 68.59% ($p<0.05$) and 70.59% ($p<0.05$) at the doses of 100 mg/kg, 200 mg/kg and 400 mg/kg aqueous fractions, respectively. The aqueous fraction also showed a significant reduction in weight of both wet and total fecal output at 100 mg/kg ($p<0.05$), 200 mg/kg ($p<0.05$) and 400 mg/kg ($p<0.05$) when compared to the negative control. Similarly, the n-butanol fraction significantly decreased the frequency and weight of wet and total feces at doses of 200 mg/kg ($p<0.05$) and 400 mg/kg ($p<0.05$), with the highest percentage of diarrheal inhibition (56.82%, $p<0.05$) obtained at the latter dose of this fraction compared to the negative control. On the contrary, the chloroform fraction was devoid of significant anti-diarrheal activity on castor oil induced diarrhea at 100 mg/kg and 200 mg/kg doses as compared with the negative control. In addition, 100 mg/kg and 200 mg/kg chloroform fraction did not have any significant effect on weight of wet and total fecal outputs.

As depicted in Table 1, there was a dose-dependent reduction in the percentage of weight of wet and total fecal outputs in 80% MeOH (R^2=1.000; R^2 =0.997, $p<0.05$), chloroform fraction (R^2=0.939; R^2=0.992, $p<0.05$) and n-butanol fraction (R^2=0.984; R^2=0.974, $p<0.05$), respectively, with 400 mg/kg of the 80% MeOH displaying the highest effect (15.22% and 16.33%). As compared to the standard drug (28.26 % and 34.69 %), the 80% MeOH at its 400 mg/kg revealed the greatest effect to lessen the percentage of fecal output. Besides, both the aqueous and n-butanol fractions revealed moderate reduction in the percentage of both wet and total fecal outputs, with 400 mg/kg aqueous fraction showing the maximum reduction among the solvent fractions. On the other hand, the chloroform fraction showed minimum inhibition of percentage of both wet and total fecal outputs.

Table 1. Anti-diarrheal effects of 80% MeOH and solvent fractions of the fruits of *Mimusops kummel* on castor oil induced diarrheal model in mice

Extracts	Dose administered	Onset of diarrhea (min.)	No of wet feces	Total No of feces	Average weight of wet feces (gm)	Average weight of total feces (gm)	% reduction	% wet fecal outputs	% total fecal outputs
	Control	44.33 ± 1.65	10.50 ± 1.18	11.83 ± 0.94	0.46 ± 0.11	0.49 ± 0.12	------	------	------
80%	100mg/kg	67.00 ± 1.13 *† ‡₸	3.00 ± 0.72 *	4.17 ± 1.55 *	0.16 ± 0.06 *	0.21 ± 0.05 *	71.40	34.78	42.86
MeOH	200 mg/kg	93.33 ± 1.63 *† ₸	2.83 ± 0.94 *	3.50 ± 0.67 *	0.13 ± 0.03 *	0.17 ± 0.03 *	76.70	28.26	34.69
	400 mg/kg	110.67 ± 1.63 *†	1.50 ± 0.84 *	2.33 ± 0.83 *	0.07 ± 0.03 *	0.08 ± 0.04 *	85.70	15.22	16.33
	3 mg/kg loperamide	124.17 ± 1.09 *	2.17 ± 0.60 *	2.67 ± 0.65 *	0.13 ± 0.02 *	0.17 ± 0.02 *	79.40	28.26	34.69
	Control	53.17 ± 2.05	8.50 ± 0.84	9.67 ± 1.10	0.41 ± 0.02	0.45 ± 0.03	------	------	------
	CF100mg/kg	72.00 ± 2.02 *† ‡₸	7.67 ± 1.21 † ₸	9.33 ± 0.97 † ₸	0.40 ± 0.10 † ₸	0.44 ± 0.09 † ₸	9.76	97.56	97.78
	CF200 mg/kg	86.50 ±1.50 *† ₸	5.83 ± 0.94 * †	7.50 ± 0.98 *†	0.30 ± 0.06 †	0.37 ± 0.06 †	31.41	73.17	82.22
	CF400 mg/kg	101.17 ± 2.74 *†	5.17 ± 0.94 * †	7.17 ± 0.60 *†	0.22 ± 0.06 *	0.27 ± 0.05 *	39.18	53.66	60.00
	n-BF100mg/kg	88.33 ± 1.73 *† ‡₸	6.83 ± 1.18 † ₸	7.33 ± 0.97 *†	0.32 ± 0.03 *† ₸	0.33 ± 0.03 *† ₸	19.65	78.05	73.33
Solvent	n-BF200 mg/kg	102.17 ± 2.1 *† ₸	4.67 ± 1.30 * †	6.33 ± 0.82 *†	0.29 ± 0.02 *† ₸	0.31 ± 0.01 *† ₸	45.06	70.73	68.89
fractions	n-BF400 mg/kg	123. 17 ± 1.78 *†	3.67 ± 1.30 *	5.50 ± 0.84 * †	0.18 ± 0.02 *†	0.22 ± 0.02 *†	56.82	43.90	48.89
	AF100mg/kg	92.67 ± 1.40 *† ‡₸	3.33 ± 0.66 *	3.83 ± 0.60 *	0.26 ± 0.02*† ‡₸	0.27 ± 0.02 * †	60.82	63.41	60.00
	AF200 mg/kg	113.83 ± 1.1 *† ₸	2.67 ± 0.82 *	3.83 ± 0.94 *	0.18 ± 0.01 * †	0.22 ± 0.03 * †	68.59	43.90	48.89
	AF400 mg/kg	122.33 ± 0.97 *†	2.50 ± 1.10 *	3.67 ± 0.82 *	0.18 ± 0.02 * †	0.21 ± 0.02 *	70.59	43.90	46.67
	3 mg/kg loperamide	134.83 ± 1.38 *	1.67 ± 0.66 *	2.67 ± 0.66 *	0.13 ± 0.02 *	0.16 ± 0.02 *	80.35	31.71	35.56

Values are expressed as mean ± CI_{95} (n = 6); analysis was performed using one way ANOVA followed by Tukey's Post hoc test; * compared with control values; † compared with loperamide; ‡ compared with 200 mg/kg; ₸ compared with 400 mg/kg, *†‡₸ $p<0.05$, 80% MeOH = 80% methanolic extract, CF = chloroform fraction, n-BF = n-butanol fraction, AF = aqueous fraction, CI_{95} = 95% confidence interval. Controls are 10 ml/kg distilled water (for 80% MeOH) and 2% tween-80 (for chloroform, n-butanol and aqueous fractions).

4.2. Effects on castor oil induced gastrointestinal motility

As presented in the Table 2, the 80% MeOH significantly inhibited the intestinal transit of charcoal meal at all tested doses. The data revealed that the percentage reduction of gastrointestinal transit of charcoal was 57.66% ($p<0.05$), 63.14% ($p<0.05$) and 68.98% ($p<0.05$) at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg, respectively. The activity of the 80% MeOH at 400 mg/kg was comparable to that of the standard anti-diarrheal drug, loperamide hydrochloride (66.06% at the dose of 3 mg/kg).

All the three fractions of fruits of *Mimusops kummel* significantly inhibited gastrointestinal motility of charcoal meal at all tested doses as compared to vehicle treated group and the maximum effect was achieved by the aqueous fraction at 400 mg/kg, the charcoal meal traversed 56.45% of the total length of the small intestine.

Table 2. Effects of 80% MeOH and solvent fractions of the fruits of *Mimusops kummel* on castor oil induced gastrointestinal motility in mice

Extracts	Dose administered	Length of small intestine (cm)	Distance moved by the charcoal meal (cm)	Peristaltic index (%)	% inhibition
	Control	56.83 ± 0.79	45.67 ± 0.97	80.36 ± 1.76	------
80% MeOH	100 mg/kg	52.50 ± 1.10	19.33 ± 0.97 *†‡∓	36.81 ± 1.39 *†‡∓	57.66
	200 mg/kg	53.67 ± 0.97	16.83 ± 0.94 *∓	31.35 ± 1.35 *∓	63.14
	400 mg/kg	57.50 ± 0.84	14.17 ± 1.18 *	24.64 ± 2.05 *	68.98
	3 mg/kg loperamide	58.67 ± 0.97	15.50 ± 0.84 *	26.42 ± 1.29 *	66.06
	Control	55.17 ± 1.38	47.83 ± 0.94	86.74 ± 1.76	------
	CF100 mg/kg	53.67 ± 1.49	42.17 ± 1.71 *†‡∓	78.56 ± 2.02 *†‡∓	11.83
	CF200 mg/kg	54.83 ± 1.55	36.00 ± 1.43 *†∓	65.64 ± 1.50 *†∓	24.73
	CF400 mg/kg	54.67 ± 1.30	32.50 ± 1.50 *†	59.42 ± 1.55 *†	32.05
	n-BF100 mg/kg	55.83 ± 0.94	38.67 ± 0.97 *†‡∓	69.31 ± 2.73 *†‡∓	19.15
Solvent fractions	n-BF200 mg/kg	55.67 ± 1.40	33.00 ± 1.68 *†∓	59.24 ± 1.74 *†∓	31.06
	n-BF400 mg/kg	54.17 ± 1.18	27.67 ± 1.40 *†	51.05 ± 1.72 *†	42.20
	AF100 mg/kg	55.17 ± 0.94	30.17 ± 1.18 *†‡∓	54.74 ± 2.90 *†‡∓	36.92
	AF200 mg/kg	55.83 ± 0.94	24.17 ± 0.94 *†∓	43.30 ± 1.92 *†∓	49.47
	AF400 mg/kg	55.00 ± 1.13	20.83 ± 1.18 *†	37.87 ± 1.90 *†	56.45
	3 mg/kg loperamide	55.50 ± 0.84	17.33 ± 0.97 *	31.23 ± 1.66 *	63.77

Values are expressed as mean ± CI_{95} (n = 6); analysis was performed using one way ANOVA followed by Tukey's Post hoc test; * compared with control values; † compared with loperamide; ‡ compared with 200 mg/kg; ∓ compared with 400 mg/kg, *†‡∓ $p<0.05$, 80% MeOH = 80% methanolic extract, CF = chloroform fraction, n-BF = n-butanol fraction, AF = aqueous fraction, CI_{95} = 95% confidence interval. Controls are 10 ml/kg distilled water (for 80% MeOH) and 2% tween-80 (for chloroform, n-butanol and aqueous fractions).

4.3. Effects on castor oil induced enteropooling

In the gastrointestinal enteropooling test, the 80% MeOH of the fruits of *Mimusops kummel* showed significant reduction in both the average volume (except the low dose, 100 mg/kg) and weight of intestinal contents at all tested doses as compared with the negative control ($p<0.05$). However, there was no statistically significant difference in terms of the volume of intestinal fluid and weight of intestinal contents at the doses of 200 mg/kg and 400 mg/kg when compared with the standard drug, loperamide hydrochloride (at the dose of 3 mg/kg). As the data revealed in Table 3, the percentage inhibition of volume of intestinal contents was found to be 25.49% ($p<0.05$), 39.22% ($p<0.05$) and 50.98% ($p<0.05$) at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg, respectively.

The aqueous and n-butanol fractions reduced the volume and weight of the intestinal contents significantly at all tested doses. Maximum percentage inhibition of the volume of intestinal contents was observed at 400 mg/kg, being 56.32% ($p<0.05$) and 57.47% ($P<0.05$) for aqueous and n-butanol fractions, respectively. Generally, both the aqueous and n-butanol fractions showed comparable percent reduction of both volume and weight of intestinal contents at all tested doses. On the contrary, the chloroform fraction was devoid of any significant inhibitory effect on the volume and weight of intestinal contents up to 200 mg/kg as compared with the negative control. However, significant inhibitory effects were seen at dose of 400 mg/kg being 42.53% ($p<0.05$) and 32.33% ($P<0.05$) for percent reduction of volume and weight of intestinal contents, respectively as shown in Table 3.

Table 3. Effects of 80% MeOH and solvent fractions of the fruits of *Mimusops kummel* on castor oil induced enteropooling in mice

Extracts	Dose administered	Mean volume of small intestinal content (gm)	% inhibition	Mean weight of small intestinal content (ml)	% inhibition
	Control	0.85 ± 0.08	------	1.21 ± 0.10	------
80%	100 mg/kg	0.63 ± 0.11	25.49	1.02 ± 0.17 *†‡₸	15.63
MeOH	200 mg/kg	0.52 ± 0.13 *	39.22	0.80 ± 0.02 *	33.61
	400 mg/kg	0.42 ± 0.09 *	50.98	0.69 ± 0.01 *	43.02
	3 mg/kg loperamide	0.45 ± 0.11 *	47.06	0.71 ± 0.01 *	41.36
	Control	0.87 ± 0.10	------	0.99 ± 0.10	------
	CF100 mg/kg	0.75 ± 0.11 †₸	13.79	0.83 ± 0.07 †	16.16
	CF200 mg/kg	0.57 ± 0.10 †	34.48	0.72 ± 0.10 †	27.30
	CF400 mg/kg	0.50 ± 0.11 *	42.53	0.67 ± 0.10 *†	32.33
	n-BF100 mg/kg	0.63 ± 0.11 *†₸	27.59	0.70 ± 0.10 *†₸	29.31
Solvent	n-BF200 mg/kg	0.58 ± 0.12 *†	33.32	0.60 ± 0.02 *†	39.40
fractions	n-BF400 mg/kg	0.37 ± 0.11 *	57.47	0.53 ± 0.08 *	46.45
	AF100 mg/kg	0.53 ± 0.08 *†	39.08	0.63 ± 0.02 *†₸	36.35
	AF200 mg/kg	0.42 ± 0.10 *	51.72	0.54 ± 0.02 *†	45.50
	AF400 mg/kg	0.38 ± 0.12 *	56.32	0.45 ± 0.03 *	54.53
	3 mg/kg loperamide	0.30 ± 0.07 *	60.91	0.42 ± 0.04 *	57.58

Values are expressed as mean ± CI_{95} (n = 6); analysis was performed using one way ANOVA followed by Tukey's Post hoc test; * compared with control values; † compared with loperamide; ‡ compared with 200 mg/kg; ₸ compared with 400 mg/kg, *†‡₸ $p<0.05$, 80% MeOH = 80% methanolic extract, CF = chloroform fraction, n-BF = n-butanol fraction, AF = aqueous fraction, CI_{95} = 95% confidence interval. Controls are 10 ml/kg distilled water (for 80% MeOH) and 2% tween-80 (for chloroform, n-butanol and aqueous fractions).

4.4. *In vivo* anti-diarrheal index

The *in vivo* anti-diarrheal index (ADI) was measured by considering three parameters as shown in Table 4. These are delay in defecation (time of onset, Dfreq), gut meal travel distance (Gmeq) and purging frequency in number of wet stools (Pfreq). Results from the determination of *in vivo* ADI revealed that the greatest *in vivo* ADI was achieved at the dose of 400 mg/kg of 80% MeOH (96.00%) which is comparable to the standard drug, loperamide hydrochloride at the dose of 3 mg/kg (98.12%). Moreover, the highest anti-diarrheal index was observed at the maximum dose of each fraction. However, amongst all solvent fractions, aqueous fraction showed the highest *in vivo* ADI (80.33%) at the dose 400 mg/kg. Both 80% MeOH and solvent fractions showed dose-dependent increment in ADI value: 80% MeOH (R^2=0.913), chloroform fraction (R^2=0.887), n-butanol fraction (R^2=0.941) and aqueous fraction (R^2=0.832).

Table 4. *In vivo* anti-diarrheal indices of 80% MeOH and solvent fractions of the fruits of *Mimusops kummel*

Extracts	Dose administered	Delay in defecation (time of onset in min., Dfreq) (%)	Gut meal travel distance (Gmeq) (%)	Purging frequency in number of wet stools (Pfreq) (%)	Anti-diarrheal Index (ADI)
80% MeOH	100mg/kg	51.14	57.66	71.40	59.49
	200mg/kg	110.53	63.14	76.70	81.19
	400mg/kg	149.65	68.98	85.70	96.00
	3 mg/kg loperamide	180.10	66.06	79.40	98.12
Solvent fractions	CF100mg/kg	35.41	11.83	9.76	15.99
	CF200mg/kg	62.69	24.73	31.41	36.52
	CF400mg/kg	90.28	32.05	39.18	48.40
	n-BF100mg/kg	66.13	19.15	19.65	29.20
	n-BF200mg/kg	92.16	31.06	45.06	50.53
	n-BF400mg/kg	131.65	42.20	56.82	68.09
	AF100mg/kg	74.29	36.92	60.82	55.05
	AF200mg/kg	114.09	49.47	68.59	72.88
	AF400mg/kg	130.07	56.45	70.59	80.33
	3 mg/kg loperamide	153.58	63.77	80.35	92.32

80% MeOH = 80% methanolic extract, CF = chloroform fraction, n-BF = n-butanol fraction and AF = aqueous fraction

4.5. Preliminary phytochemical screening

Evaluation of the preliminary phytochemical screening of the 80% MeOH of the fruits of *Mimusops kummel* revealed the presence of alkaloids, saponins, tannins, phenols, terpenoids and flavonoids. Steroids, anthraquinones and glycosides were absent in 80% MeOH of the plant. Amongst the solvent fractions, the data revealed that only the aqueous fraction tested positive for flavonoids. On the other hand, saponins and tannins were detected in both aqueous and n-butanol fractions. Phenols were common across all solvent fractions. Terpenoids were also observed in both chloroform and n-butanol fractions. Amongst all fractions, the aqueous fraction appeared to be relatively rich in secondary metabolites as shown from Table 5.

Table 5. Preliminary phytochemical screening of the 80% MeOH and solvent fractions of the fruits of *Mimusops kummel*

Secondary metabolites	**Crude extract**	**Solvent fraction**		
	80% MeOH	**Chloroform fraction**	**n-Butanol fraction**	**Aqueous fraction**
Saponins	+	_	+	+
Tannins	+	_	+	+
Steroids	_	_	_	_
Phenols	+	+	+	+
Flavonoids	+	_	_	+
Alkaloids	+	+	_	+
Terpenoids	+	+	+	_
Anthraquinones	_	_	_	_
Glycosides	_	_	_	_

80% MeOH = 80% methanolic extract, + = present and - = absent

4.6. Acute oral toxicity test

Eighty percent methanolic extract of the fruits of *Mimusops kummel* was studied for oral acute toxicity at dose of 2000 mg/kg by oral route. The extract produced no mortality and any apparent signs of toxicity when observed for first four hours with 30 min interval and followed by daily observations for 14 days following oral administration of a single dose of 2000 mg/kg. In addition, neither food nor water intake was found to be reduced during the period of 14 days.

The absence of mortality and signs of overt toxicity up to five times the maximum effective dose of the extract suggested that 80% MeOH has a wider safety margin and median lethal dose (LD_{50}) value is greater than 2000 mg/kg in mice.

5. DISCUSSION

Traditionally, people customarily use plant(s) or plant-derived preparations considering them to be efficacious against diarrheal disorders without any scientific basis to explain the action of such plants (Atta and Mouneir, 2005). The aim of the present study was to experimentally evaluate the folkloric acclaimed use and safety profile of *Mimusops kummel* fruits, which are regarded to confer protection in diarrhea in Ethiopian traditional medicine. Several studies have validated the use of anti-diarrheal medicinal plants by investigating the biological activity of extracts of such plants, which have antispasmodic effects, delay intestinal transit, suppress gut motility, stimulate water absorption, or reduce the intraluminal fluid accumulation (Atta and Mouneir, 2005; Gutiérrez *et al*., 2013). Therefore, in the present study, the 80% MeOH and solvent fractions of *Mimusops kummel* fruits that have not been studied so far for its anti-diarrheal activity was substantiated with scientific evidence using experimentally induced diarrhea models namely, castor oil induced diarrhea, charcoal meal gastrointestinal motility and castor oil induced enteropooling in mice.

The anti-diarrheal activity of the 80% MeOH and solvent fractions of *Mimusops kummel* fruits were investigated for their *in vivo* anti-diarrheal activities in all the three models using castor oil as diarrhea inducing agent. It is well documented that castor oil causes diarrhea due to its active metabolite, ricinoleic acid (Ammon *et al*., 1974; Jebunnessa *et al*., 2009) by hypersecretory response, which stimulates peristaltic activity in the small intestine, leading to changes in the electrolyte permeability of the intestinal mucosa and thus increases the volume of intestinal content by preventing the reabsorption of water (Mbagwu and Adeyemi, 2008; Rehman *et al*., 2013). Its action also stimulates the release of inflammatory mediators (prostaglandins E and histamine) which cause stomach cramp and diarrhea due to the effect on the smooth muscle and secretion. Thereby prevents the reabsorption of sodium chloride and water (Gatne *et al*., 2008; Rajat *et al*., 2013). The castor oil model therefore, incorporates both secretory and abnormal motility diarrhea (Yegnanarayen and Srotri, 1982). Among the several mechanisms proposed to explain the diarrheal effect of castor oil are inhibition of intestinal Na^+, K^+-ATPase activity, thus reducing normal fluid absorption (Humber, 2002; Imam *et al*., 2012), activation of adenylate cyclase or mucosal cAMP-mediated active secretion (Capasso *et al*., 1994) and nitric oxide (Uchida *et al*., 2000). Therefore, the use of castor oil as diarrhea inducer for all models is

plausible as it mimics the pathophysiologic processes and allows the observation of measurable changes in the number of stools, intestinal transit and enteropooling. Loperamide hydrochloride, the standard drug, apart from regulating the gastrointestinal tract, have also been reported to slow down transit in the small intestine. Loperamide hydrochloride at present is one of the most efficacious and widely employed anti-diarrheal agents and effectively antagonizes the action of castor oil due to its antimotility and antisecretory property (Salgado *et al*., 2005).

The result of the present study showed that the 80% MeOH of *Mimusops kummel* was found to be effective against diarrhea induced by castor oil in different models. The first model being castor oil induced diarrheal model that assesses the potential of a test substance as having an overall anti-diarrheal activity regardless of its effect on gut motility and/or intestinal secretion. The onset of defecation, the number and weight of fecal output, more importantly wet feces, were determined as main parameters. Hence, agents which inhibit the number and weight of feces are considered to have anti-diarrheal activity (Eberlin *et al*., 2012). In the current study, there was a statistically significant ($p<0.05$) reduction in the number and weight of both wet and total fecal output and delayed the initiation of diarrhea with the highest effects observed at 400 mg/kg in the test groups in the experiment. The 80% MeOH tested at 100 mg/kg, 200 mg/kg and 400 mg/kg like the standard drug, loperamide hydrochloride significantly inhibited the frequency of defecation droppings when compared to untreated control mice ($p<0.05$). The significant reduction in frequency of defecation (number of wet stools and total number of stools), weight of wet stools and weight of total stools signifies the efficacy of 80% MeOH of *Mimusops kummel* fruits as anti-diarrheal agent. This result is in support of previous claims in respect of anti-diarrheal herbs. Anti-diarrheal plants are known to reduced number of wet stools as reported for *Eremomastax speciosa* and *Xylocarpus granatum* (Oben *et al*., 2006; Rouf *et al*., 2007). Since castor oil produces diarrhea by preventing fluid and electrolyte absorption and thus resulting in intestinal peristalsis **(**Rehman *et al*., 2013), one of the probable mechanism of anti-diarrheal activity of the test extract *Mimusops kummel* may be its ability to enhance fluid and electrolytic absorption through the gastrointestinal tract.

Moreover, the significantly ($p<0.05$) prolonged time of induction of diarrhea, decreased frequency of fecal parameters (number of wet feces and total number of feces) following the administration of the 80% MeOH suggest its anti-diarrheal activity at all the dose levels. This

assertion was further corroborated with the increased inhibition of defecation. The comparable percentage of inhibition of defecation in the 200 mg/kg and 400 mg/kg doses of the extract and loperamide hydrochloride suggest that the plant extract may have loperamide hydrochloride-like action in exerting its anti-diarrheal activity. The extract might have exerted its anti-diarrheal activity via anti-secretory mechanism as evident from reduction in total number of wet feces. Furthermore, this anti-diarrheal activity could have resulted from the inhibitory activity of 80% MeOH of *Mimusops kummel* on prostaglandins synthesis, nitric oxide and platelet activating factors production, as inhibitors of prostaglandins, nitric oxide syntheses and platelet activating factors are known to delay diarrhea induced by castor oil (Adzu *et al*., 2003; Sunil *et al*., 2001; Tangpu and Yadav, 2004). Similar effects were reported in several studies by Qnais *et al*. (2005), Akindele and Adeyemi (2006) and Appidi *et al*. (2010) following the administration of aqueous leaf extracts of *Juniperus phoenicia*, *Byrsocarpus coccineus* and *Hermania incana*, respectively. In the present study, the percent inhibition by the 100 mg/kg was lower than the 200 and 400 mg/kg, indicating higher dose of the crude extract do have a much better anti-diarrheal effect. This might be because it is a sub-threshold (sub-effective) dose. This implies that a relatively high dose of the extract is needed to produce an anti-diarrheal. This is in line with other reports of other species of plants in which extracts of plants shown to exert anti-diarrheal effect at higher doses (Trease and Evans, 1989).

To further evaluate the nature of the active constituents responsible for the anti-diarrheal activity of the plant, the 80% MeOH was successively fractionated by solvents of differing polarity and the anti-diarrheal activity of the fractions were evaluated using castor oil induced diarrheal model.

The aqueous, n-butanol and chloroform fractions (at 400 mg/kg) produced significant anti-diarrheal effects in all parameters in castor oil induced diarrheal model, with the aqueous fraction being the most active fraction. In addition, both aqueous and n-butanol fractions significantly decreased the number of wet, total feces and weight of both wet and total stooling at 200 mg/kg as compared with the negative control ($p<0.05$). The chloroform fraction significantly decreased the number of wet and total feces but not weight of both wet and total stooling at 200 mg/kg dose. However, its low dose, 100 mg/kg, did not have significant effect in altering any of the aforementioned parameters when compared to the negative control. This may be associated with

lower or insignificant antimotility and/or antisecretory effects that may account for the coverage of some or none of the parameters in castor oil induced diarrheal model. Generally, aqueous and n-butanol fractions had comparable anti-diarrheal effects, but the effects were lower than that of the 80% MeOH.

The chloroform fraction, however, was found to be active either at the moderate and high doses or at the high dose. The insignificant activity of the chloroform fraction at the low dose might be due to the inability of secondary metabolites to reach sufficient concentration. This argument is supported by the fact that activity would be apparent with increasing dose of the fraction. This could possibly suggest that the localization of the active ingredients in the aqueous and n-butanol fractions. Moreover, it is plausible to assume that more polar secondary metabolites could be responsible for inhibition of the diarrheal parameters (onset of diarrhea, number of wet stools, total number of stools, weight of wet stools, weight of total stools) measured. This study was in line with other studies in which the aqueous and n-butanol fractions of different plants reduced the number and weight of stooling (Poli *et al*., 2015; Sangram *et al*., 2015; Sylvie *et al*., 2014).

Generally, the fractions showed differences in potency in castor oil induced diarrheal model in the rank order of; aqueous fraction > n-butanol fraction > chloroform fraction in all parameters. The difference in rank order of potency could emanate from the differential distribution of the secondary metabolites as depicted in Table 5.

The need for phytochemical screening has become imperative, since many plants accumulate biologically active, complex organic chemicals (secondary plant metabolites) in their tissues. In the present study, the preliminary phytochemical screening of the 80% MeOH of *Mimusops kummel* fruits revealed the presence of alkaloids, tannins, saponins, phenols, terpenoids and flavonoids, and these phytoconstituents could be responsible for the significant *in vivo* anti-diarrheal activity of *Mimusops kummel*.

Although the exact mechanism of the anti-diarrheal action of 80% MeOH of *Mimusops kummel* could not be established in this study, a number of investigators have shown that constituents such as alkaloids, tannins, saponins, phenols, terpenoids and flavonoids in general are responsible for anti-diarrheal action of different plant extracts through different mechanisms (Kota *et al*., 2012; Kubacey *et al*., 2012; Shemsu *et al*., 2013). These constituent may mediated

the anti-diarrheal activity of the fruit extract of *Mimusops kummel*. The overall possible mechanism may be due to, inhibition of release of prostaglandins thus inhibiting the motility and secretion induced by castor oil or alteration of the activity of Na^+, K^+-ATPase or activation of chloride channels by the fruit extract of the *Mimusops kummel* which is still to be understood.

The anti-diarrheal activities of the 80% MeOH as well as active fractions might also be due to inhibition of active secretion of ricinoleic acid, resulting in the activation of Na^+, K^+-ATPase activity that in turn promotes absorption of Na^+ and K^+ in the intestinal mucosa which is linked with a decrease in number and weight of wet feces. This effect could probably be linked to the presence of tannins and flavonoids in the 80% MeOH and aqueous fraction and tannins in the n-butanol fraction, which are shown to promote colonic absorption of water and electrolytes (Kubacey *et al*., 2012). By contrast, the chloroform fraction showed modest anti-diarrheal activity at its maximum dose. Tannins and flavonoids are lacking in this fraction and hence the probable synergistic anti-diarrheal activities are no longer available. Furthermore, terpenoids, alkaloids, phenols and saponins also exhibit anti-diarrheal activity. Therefore, these constituents might also be attributable for the overall anti-diarrheal effects of the 80% MeOH and solvent fractions with possible variation in distribution patterns of polarity across the fractions.

Most efficacious and widely employed anti-diarrheal drugs act by decreasing the intestinal motility and/or inhibit secretion of intestinal contents (Gutiérrez *et al*., 2013). Hence, further confirmation of the possible mode of action was tested on intestinal motility and enteropooling models, respectively.

Charcoal meal test in mice is a method used to study the effect of drugs on the motility of intestine. In the present study, it was shown that the 80% MeOH significantly suppresses the propulsion of charcoal marker at all tested doses as compared to negative control. The percentage inhibition of charcoal marker at 400 mg/kg dose (68.98%, $p<0.05$) of this extract was observed to be almost comparable to the standard drug (66.06% at the dose of 3mg/kg). This finding suggests that the extract has the ability to influence the peristaltic movement of intestine thereby indicating the presence of an intestinal antimotility activity. This decrease in GI motility facilitates the absorption of electrolytes and then water (Kent and Banks, 2010), which might be responsible for the decrease in the number and weight of wet feces observed with the extract.

Both the aqueous and n-butanol fractions had statistically significant antispasmodic effects with the highest effect revealed at 400 mg/kg of aqueous fraction (56.45%, $p<0.05$). Even though the chloroform fraction had a significant effect on the charcoal meal test; it failed to show a significant effect on number and weight of wet feces, particularly at the low dose. This could be explained by the fact that antimotility effect might not be a necessary and sufficient factor for counteracting diarrhea and it should be supplemented with a certain degree of antisecretory activity. This study is in line with other studies where doses of various extracts having lower or insignificant effects in some of the parameters of the castor oil induced diarrheal model might have significant antispasmodic effects (John *et al*., 2008; Rajabhau *et al*., 2011; Sumithra *et al*., 2012).

Castor oil induces diarrhea in mice due to the action of ricinoleic acid formed by the hydrolysis of the oil, and it stimulates contraction of the intestine (Rajat *et al*., 2013). The 80% MeOH and all fractions significantly slowed down charcoal meal transit in the GIT, showing that these fractions could have inhibitory effects on the release of autocoids and prostaglandins.

Flavonoids have been demonstrated to inhibit contractions induced by spasmogens (Capasso *et al*., 1988). Again, flavonoids present in the extract of *Loesclia Mexicana* are known to inhibit the release of autocoids and prostaglandins, thereby inhibiting the motility induced by castor oil (Perez *et al*., 2005) and inhibit small intestinal transit (Brijesh *et al*., 2009). Studies on the functional role of tannins, which are present in the 80% MeOH, aqueous and n-butanol fractions, reveal that they could also bring similar functions by reducing the intracellular Ca^{2+} inward current or by activation of the calcium pumping system, which induces the muscle relaxation (Belemtougri *et al*., 2006). In addition, tannins are known to act as anti-diarrheal agents by the denaturation of proteins in the intestinal mucosa forming protein tannates which make the intestinal mucosa more resistant and hence, reduce peristaltic movement by virtue of which so many different plant species has been reported to possess anti-diarrheal potential (Atta and Mouneir, 2005). Terpenoids which are present in the methanolic extract of *Maranta arundinacea* leaves were able to reduce the altered propulsive movement induced by castor oil (Vaghasiya *et al*., 2011). It is well known that terpenoids can act as antispasmodic agents by involving calcium antagonism (Shabana *et al*., 2005).

Diarrhea is usually considered as a result of altered motility and fluid accumulation within the intestinal tract (Umer *et al*., 2013). The third model used in the present study was enteropooling model that aimed to assess the secretary components of diarrhea. In this model, the 80% MeOH, aqueous and n-butanol fractions significantly reduced the average volume and weight of intestinal contents at all tested doses when compared to the negative control group ($p<0.05$). This may promote reabsorption of materials in the intestine due to decrease propulsion of material in the intestinal tract, and the extract and fractions might have exerted their anti-diarrheal action by antisecretory mechanism. On the contrary, the chloroform fraction had a significant inhibition of intestinal fluid accumulation only at the high dose (400 mg/kg). This further supports the significant activity of these fractions on the number and weight of wet feces on the castor oil induced diarrheal model, which is in agreement with other study by which the anti-enteropooling effects of the extract is related with its anti-diarrheal effect (de Sales *et al*., 2015). In fact, the ability of chloroform fraction to reduce the volume and weight of intestinal contents only at 400 mg/kg ($p<0.05$) lends further support to the absence of statistically sound anti-diarrheal effects in the castor oil induced diarrheal model. The significant inhibition of the castor oil-induced enteropooling by aqueous fraction in mice suggests that the extract probably produces relief in diarrhea through its spasmolytic and anti-enteropooling effects. These findings are in consonance with the observations reported by Abdullahi *et al.* (2001) for aqueous root extract of *Terminalia avicennoides* (Combretaceae); Aniagu *et al.* (2005) for aqueous root extract of *Guiera senegalensis* (Combretaceae), Akindele and Adeyemi (2006) for aqueous leaf extract of *Byrsocarpus coccineus* (Connaraceae), and Mbagwu and Adeyemi (2008) for aqueous whole plant extract of *Mezoneuron benthamianum* (Caesalpinaceae).

Ricinoleic acid might activate the nitric oxide pathway and induce nitric oxide (NO) dependent gut secretion (Uchida *et al*., 2000). Other studies also confirmed that NO is involved in the causation of diarrhea and this is counteracted by agents that inhibit NO synthesis (Izzo *et al*., 1996). Similarly, study by Capasso *et al*. (1994) have implicated elevated nitric oxide in the pathogenesis of diarrhea, a disease which was prevented by the intraperitoneal injection of nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester (2.5-50 mg/kg twice) to rats. Therefore, the decrease in the concentration of nitric oxide in the small intestine of extract treated animals may be one of the mechanisms by which the extract exhibits its anti-diarrheal effect. In addition, phytochemical constituents such as flavonoids (Duarte *et al*., 2014;

Messaoudene *et al*., 2011), tannins (Ishii *et al*., 1999) and terpenoids (Jang *et al*., 2004) are implicated in the inhibition of nitric oxide synthesis by suppressing the expression of iNOS enzyme. Therefore, unlike the chloroform fraction, the pronounced inhibition of castor oil induced intestinal fluid accumulation could possibly be related to the presence of flavonoids and tannins that increase the reabsorption of electrolytes and water by hindering castor oil mediated NO synthesis in the 80% MeOH and aqueous fraction. Furthermore, the enteropooling induced by castor oil, probably occurs through stimulation of cyclic AMP/GMP production, and phosphorylation of cystic fibrosis transmembrane conductance regulators (CFTRs). This consequently leads increased secretion of fluids and electrolytes (mainly Cl^- and Na^+) (Bakare *et al*., 2011; Ratnaike *et al*., 2000). Tannins decrease fluid secretion by inhibiting CFTR and CaCC, by generating protein-precipitating reaction to the GI mucosa and due to their high antioxidant capacity (Ashok and Upadhyay, 2012; Ren *et al*., 2012; Wongsamitkul *et al*., 2010). Indeed, it is well established that the liberation of ricinoleic acid from castor oil also causes irritation and inflammation of intestinal mucosa, leading to release of prostaglandins E2, which results in stimulation of secretion (Ezenwali *et al*., 2010; Rajat *et al*., 2013). *In vitro* and *in vivo* experiments have shown that flavonoids, terpenoids and saponins are able to inhibit the intestinal secretory response induced by prostaglandins E2, thereby inhibit secretion induced by castor oil (Dosso *et al*., 2011; Hamalainen *et al*., 2011; Perez *et al*., 2005).

Apart from this, the enteric nervous system also stimulates intestinal secretion through neurotransmitters such as acetylcholine. On the other hand, intestinal absorption can be stimulated with α2-adrenergic agents, enkephalins and somatostatin (Bern *et al*., 1989; Fedorak *et al*., 1985). Secondary metabolites such as flavonoids have been shown to inhibit gastrointestinal release of acetylcholine (Lutterodt, 1989). Again, flavonoids could stimulate α2-adrenergic receptors in the absorptive cells of the gastrointestinal tract (Di Carlo *et al*., 1993). The regulation of transepithelial fluid transport in the gastrointestinal tract is based on not only electrolyte transport but also water transport by aquaporin (AQP) type water channels. Certain tannins were found to inhibit aquaporins (AQPs) 2 and 3 expressions *in vivo* and *in vitro via* down regulating protein kinase A/cAMP response element binding protein (PKA/CREB) signal pathway, which partially accounts for the antisecretory and hence anti-diarrheal effects (Liu *et al*., 2014).

Thus, the pronounced antisecretory activity of the 80% MeOH as well as the aqueous and n-butanol fractions could presumably be related to the existence and hence synergistic effects of flavonoids, tannins, terpenoids and saponins. The highest antisecretory effects of 80% MeOH might be associated with the nature and relative abundance of these secondary metabolites compared to the fractions. The lack of action for the chloroform fraction could be explained by the same corollary, as these constituents except terpenoids were lacking from this fraction. The significant effect of chloroform fraction to reduce the volume and weight of intestinal contents at 400 mg/kg might be due to the accumulation of terpenoids that possess inhibitory effect on NO production (Jang *et al*., 2004). Since the constituents found in the solvent fractions might not relate qualitatively and quantitatively, the n-butanol fraction reduce the volume of intestinal contents more than aqueous fraction at 400 mg/kg dose. These phytochemical constituents may have anti-diarrheal activities *via* a multitude of mechanisms and act either independently or in concert to accomplish the overall anti-diarrheal effect.

The *in vivo* ADI is a measure of the combined effects of three parameters such as delay in onset of diarrheal stools, intestinal motility and purging frequency in number of wet stools (Hussain *et al*., 2009; Okpo *et al*., 2011). Generally, high ADI value indicates a measure of how much effective an extract is in treating diarrhea (Ching *et al*., 2008; Prasad *et al*., 2014). The 80% MeOH and all solvent fractions showed a dose dependent increase on ADI (R^2 = 0.913 for 80% MeOH, 0.887 for chloroform fraction, 0.941 for n- butanol fraction and 0.832 for aqueous fraction), suggesting the dose dependency nature of the 80% MeOH and solvent fractions in this parameter. The highest *in vivo* ADI value was produced by the 80% MeOH at its high dose which is directly related with its anti-diarrheal activity in all of the three models. Thus, reinforcing the notion that this extract is endowed with the best anti-diarrheal activity compared to solvent fractions. Moreover, the aqueous fraction showed the highest ADI value at its maximum dose as compared to the other fractions. Conversely, the chloroform fraction, which had little anti-diarrheal activity in all models, exhibited the lowest ADI, pointing to the fact that ADI is a useful parameter in ranking anti-diarrheal agents.

Thus, the present study indicates that 80% MeOH of *Mimusops kummel* possess significant anti-diarrheal activity due to its inhibitory effect both on GI propulsion and fluid secretion. The inhibitory effect of the fruit extract justified the use of the plant fruit as a non-specific anti-

diarrheal agent in folklore medicine. Interestingly, its dual antimotility and antisecretory effects observed in the current study, reinforcing a notion that *Mimusops kummel* can possibly be a good candidate for diarrheas of diverse etiologies.

In the present investigation, the acute toxicity profile of the fruits of *Mimusops kummel* was determined based on OECD guideline 425 (OECD, 2008). On this test, the median lethal dose (LD_{50}) was found to be >2000 mg/kg for the 80% MeOH. Generally, if the LD_{50} value of the test chemical is more than 3 times the minimum effective dose, the substance can therefore be categorized under experimentally safe substances for use (OECD, 2001). Since the 80% MeOH had LD_{50} value of more than three times of the minimum effective dose (100 mg/kg), it was taken as a good candidate for further studies. Beyond its role for dose determination, LD_{50} can also be used for classification of chemicals. According to WHO hazard classification systems, the 80% MeOH of the fruits of *Mimusops kummel* with LD_{50} >2000 mg/kg is designated as 'unlikely to be hazardous' (WHO, 1992). Hence, based on the safety profile of the 80% MeOH and prior absence of any toxicity data regarding the plant, further toxicity studies were not done on the solvent fractions.

6. CONCLUSION

The results of the present study revealed that the 80% MeOH of *Mimusops kummel* fruits is endowed with a promising anti-diarrheal activity. Moreover, all the three fractions possessed varying degree of anti-diarrheal activity, with the aqueous fraction being the most active fraction followed by the n-butanol fraction and then chloroform fraction in all the three models used in the current study. The overall anti-diarrheal activities of the 80% MeOH and solvent fractions were associated with dual inhibitory effects on castor oil induced gastrointestinal motility and fluid secretion. The anti-diarrheal activities may be ascribed to the presence of bioactive secondary metabolites including flavonoids, tannins, terpenoids, saponins, phenols and alkaloids that act either individually or collectively to bring about the overall anti-diarrheal effect. The highly polar constituents found in the aqueous fraction may have better anti-diarrheal activities than the semi polar and non-polar constituents, in n-butanol and chloroform fractions. These findings provide a scientific support for folkloric repute of *Mimusops kummel* fruits as treatment of diarrheal diseases.

7. RECOMMENDATIONS

Based on the findings of the present study, the following recommendations are suggested to further investigate the experimental plant in depth.

- *In -vitro* studies of the fractions on isolated tissue preparations should be done to support the *in vivo* methods.
- Activity guided fractionation, isolation and purification of the extracts possibly n-butanol and aqueous fractions to identify the active components.
- Further pharmacological studies should be conducted to ascertain the precise mechanism (s) of action of the fractions in producing anti-diarrheal activity.
- Further toxicological studies such as sub-acute, sub-chronic and chronic toxicities should be done in order to assess the long term safety profile of the extract.

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