Evaluation of *in-vivo* antidiarrheal activities of 80% methanol extract and solvent fractions of the leaves of *Myrtus communis* Linn (Myrtaceae) in mice

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A thesis paper submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, in partial fulfillment of the requirements of Master of Science Degree in Pharmacology.

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This is to certify that the thesis prepared by Mekonnen Sisay, entitled "Evaluation of *in vivo* antidiarrheal activities of 80% methanol extract and solvent fractions of the leaves of *Myrtus communis Linn* (Myrtaceae) 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 *in-vivo* antidiarrheal activities of 80% methanol extract and solvent fractions of the leaves of *Myrtus communis* Linn (Myrtaceae) in mice

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Myrtus communis L. has a folkloric repute for the management of diarrhea and dysentery in different parts of the world including Ethiopia. However, it has not been scientifically validated yet regarding its safety and efficacy. The aim of this study was to investigate the antidiarrheal effects of both 80% methanol extract (80ME) and solvent fractions of the leaves of *Myrtus communis* L in mice models of diarrhea. The 80ME was obtained using maceration technique. Besides, the fractions were prepared from the leaf powder by successive soxhlet extraction with solvents of increasing polarity (chloroform followed by methanol) and then maceration of the marc of the methanol fraction with distilled water. The antidiarrheal activities of the 80ME as well as solvent fractions were evaluated using castor oil induced diarrhea, intestinal transit and enteropooling models in mice. For the 80ME, Group I served as negative control and received 10 ml/kg of distilled water orally; Group II served as a positive control and given a standard drug (3 mg/kg loperamide orally); Group III-V were test groups and received 100, 200 and 400 mg/kg of the extract respectively. A similar grouping was used for the fractions, except for the aqueous group , where they received 200, 300, 400 mg/kg with an additional dose of 800 mg/kg. In the castor oil induced diarrheal model, the 80ME significantly delayed the diarrheal onset at 200 mg/kg ($p<0.05$) & 400 mg/kg ($p<0.01$) and inhibited the number and weight of fecal output at all tested doses. The chloroform and methanol fractions significantly delayed onset of diarrhea at 400 mg/kg ($p<0.05$) and decreased the frequency and

weight of fecal output (at both 300 & 400 mg/kg). The aqueous fraction demonstrated modest antidiarrheal effect at 800 mg/kg ($p<0.05$). Results from the charcoal meal test revealed that the 80ME at all doses ($p<0.001$) as well as the chloroform and methanol fractions at 300 mg/kg ($p<0.05$) & 400 mg/kg ($p<0.01$; $p<0.001$) produced a significant anti-motility effect. By contrast, the aqueous fraction revealed significant antimotility effect ($p<0.01$) at its maximum dose. Similarly, in entero-pooling test, the 80ME (at all tested doses, $p<0.01$) as well as the chloroform and methanol fractions (at 300 & 400 mg/kg, $p<0.05$) produced a significant decline in the weight and volume of intestinal contents, whereas the aqueous fraction revealed appreciable effect ($p<0.05$) at 800 mg/kg only. Generally, the present study demonstrated that the 80ME, chloroform and methanol fractions produced promising antidiarrheal activities due to dual inhibitory effect on castor oil induced intestinal motility and fluid secretion. Therefore**,** this finding provides a scientific support for acclaimed traditional use of *Myrtus communis* L for treatment of diarrheal diseases.

Key terms: *Myrtus communis*, castor oil, antidiarrheal activity, antimotility, antientero-pooling, 80% methanol extract, solvent fractions

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ACRONYMS

ADI	Anti-Darrheal Index
CaCC	Calcium Activated Chloride Channel
cAMP	Cyclic Adenosine Mono Phosphate
CDC	Center for Disease Control and Prevention
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
cGMP	Cyclic Guanosine Mono Phosphate
CREB	cAMP Response Element Binding Protein
CSA	Central Statistics Agency
EPHI	Ethiopian Public Health Institute
FDA	Food and Drug Administration
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
OECD	Organization for Economic Cooperation and Development
ORS	Oral Rehydration Solution
PATH	Program for Appropriate Technology in Health
UNICEF	United Nations Children's Fund
WGO	World Gastroenterology Organization
WHO	World Health Organization

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1. INTRODUCTION

1.1. Definition and classification

The term diarrhea was derived from the Greek word (dia = through, rhein = to flow), denoting increased fluidity and frequency of fecal discharges (Mouzan, 1995). World Health Organization (WHO) (2013) defines diarrhea as the passage of unusually loose or watery stools, usually at least three times in a 24 h period. It is also an alteration in a normal bowel movement characterized by an increase in the volume, frequency, and weight of stools (Guerrant *et al*., 2001; Talley *et al*., 1994; Thomas *et al*., 2003).

Diarrhea can be classified by several methods with duration of the symptoms being foremost. Diarrhea lasting less than two weeks is considered to be acute (Guerrant *et al*., 2001). Acute diarrhea is typically self-limiting and resolves quickly with no lasting sequelae (World Gastroenterology Organization (WGO), 2008). 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* or *Escherichia coli (E.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 (dysentery) is *Shigella* (Guerrant *et al*., 2001; Mohanta *et al*., 2010).

Persistent diarrhea is an episode of diarrhea, with or without blood that lasts for two to four weeks (Mohanta *et al*., 2010). Chronic diarrhea, on the other hand, lasts longer than four weeks (Abdullah & Firmansyah, 2013; Mouzan, 1995). It can in turn be subdivided in to watery, fatty (malabsorption) and inflammatory diarrhea (Juckett & Trivedi, 2011).

1.2. Epidemiology of diarrhea

According to the WHO and United Nations Children's Fund (UNICEF) (2013), there were about 1.7 billion cases of diarrheal disease worldwide every year, and 760, 000 children younger than 5 years of age died from diarrhea each year, mostly in developing countries. This is approximated to 18% of all deaths of children under the age of five. Besides, based on the Center for Disease Control and prevention (CDC) (2013) report, of all child deaths from diarrhea, 78% occurred in the African and South-East Asian regions. Globally in this age group, diarrhea remains the second leading cause of death (after pneumonia), and both the incidence and the risk of mortality from diarrheal diseases are greatest (CDC, 2013; Mary, 2013; UNICEF, 2014; WGO, 2012).

A systematic review done on diarrheal incidence in low and middle income countries from 1990 to 2010 reported that incidence has declined from 3.4 episodes/child year in 1990 to 2.9 in 2010 (FischerWalker *et al*., 2012). Furthermore, in the African regions, there were 26% severe episodes of diarrhea and the highest numbers of childhood deaths were seen in sub-Saharan Africa regions where 50% of deaths from diarrhea occurred in 2011(FischerWalker *et al*., 2013).

In Ethiopia, according to the Central Statistical Agency (CSA) demographic and health survey report (2011), the two-week prevalence of diarrhea among children under 5 years of age was 13%. Besides, diarrhea accounted for 14% death of under five children (UNICEF, 2012). Moreover, diarrhea remained as one of the top 10 causes of death in Ethiopia (CDC, 2013). More specifically, the prevalence of childhood diarrheal diseases among under five children was reported to be 19.6% in Shebedino district, Southern nations, nationalities and peoples region (Tamiso *et al.*, 2014), 23.8% in Dejen district, Eastern Gojam (Mossie *et al*., 2014), 30.5% in Arba-

Minch district (Mohammed *et al*., 2013), 18.0% in Mecha district, West Gojam (Dessalegn *et al*., 2011), 28.9% in Nekemte town (Regassa *et al*., 2008).

1.3. Etiology of diarrhea

Diarrhea is a common symptom of gastrointestinal infections caused by a wide range of pathogens, including bacteria, viruses and protozoa. Most of which are spread by fecal contamination of water or during unhygienic conditions (Guerrant *et al*., 2001). Rotavirus and *E. coli* are the two most common etiological agents of diarrhea in developing countries (Akinnibosun & Nwafor, 2015; CDC, 2008; WHO, 2013). Rotavirus caused severe and fatal diarrhea in young children worldwide (CDC, 2008). According to the Program for Appropriate Technology in Health (PATH) (2013), Ethiopia is one of the five countries with the greatest rotavirus burden and accounted for 6% of all rotavirus deaths globally. Approximately, 28% of all under five diarrheal disease hospitalizations in Ethiopia was caused by rotavirus. Besides, rotavirus has been observed as the major cause of acute diarrhea among under five children in Jimma University Specialized Hospital (Bizuneh *et al*., 2004). *Cryptosporidium,* on the other hand*,* has been frequently isolated protozoan pathogen among HIV positive patients (WGO, 2012).

Apart from this, chemotherapy induced diarrhea is also a common problem, especially in patients with advanced cancer (Stein *et al*., 2010). Furthermore, children who die from diarrhea often suffer from underlying malnutrition, which makes them more vulnerable to diarrhea (Das *et al*., 2013; WGO, 2012; WHO, 2013).

In Ethiopia, higher risk of diarrhea was seen in households with high number of children and without improved toilet facilities, and children of illiterate mothers (Mengistie *et al*., 2013; Mihrete *et al*., 2014). Besides, latrine availability, home based

water treatment, source of water, disposal of feces and poor hand washing practices were significantly associated with acute diarrheal diseases (Gebrehiwot *et al*., 2015; Godana & Mengiste, 2013; Mossie *et al*., 2014).

1.4. Normal gut physiology and pathophysiology of diarrhea

1.4.1. Normal gut physiology

Secretion and absorption of electrolytes and fluid are two essential functions of the small intestinal epithelial cells. During normal processes, approximately 9 liters of fluid traverse the gastrointestinal tract daily. Generally, the small intestine and colon absorb 99% of the overall fluid load and only small amount vestiges in the stool after absorptive processes have occurred (Beverly & Clarence, 2008; Ghishan & Kiela, 2012; Shah, 2004). Regardless of whether water is being secreted or absorbed, it flows across the mucosa in response to osmotic gradients. As the digestion process continues, there is generation of osmotically active molecules that cause lumenal osmolarity to increase dramatically and water is pulled into the lumen. Then, as the osmotically active molecules are absorbed, osmolarity of the intestinal contents decreases and water can be absorbed (Ghishan & Kiela, 2012; Richard, 2006).

The apical membrane of crypt epithelial cells contain an ion channel of great medical importance, a cAMP-dependent chloride channel known as the cystic fibrosis trans-membrane conductance regulator (CFTR). CFTR chloride channel controls salt and water transport across epithelial tissues (Kirk, 2000; Schultz *et al.,* 1999). Chloride ions enter the crypt epithelial cell by co-transport with sodium and potassium. Elevated intracellular concentrations of cAMP in crypt cells activate the CFTR, resulting in secretion of chloride ions into the lumen. Accumulation of negatively charged chloride anions in the crypt creates an electric potential that attracts sodium into the lumen, which ultimately results in secretion of NaCl. This in turn creates an

osmotic gradient across the tight junction and draws water into the lumen (Richard, 2006). Besides, an increase in intracellular levels of cGMP or Ca^{2+} can also activates CFTR Cl^- channel function (Li *et al*, 2010; Arora *et al*., 2013).

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 diarrhea by and large is ensued because of altered gastrointestinal motility. Later on, it has become increasingly apparent that a disturbance in the epithelial electrolyte and water transport is a major cause of intestinal fluid loss even if motility disturbances may contribute (Lundgren, 2002).

1.4.2. Pathophysiology of diarrhea

Diarrheal syndromes result from disturbances in any of the basic pathophysiological processes including active secretion, osmosis, exudation/inflammation, and altered motility (Field, 2003; Kent & Banks, 2010).

Secretory diarrhea

A number of disease processes produce secretory diarrhea. The basic pathophysiology involves either net secretion of ions (chloride or bicarbonate) or inhibition of net sodium absorption (Schiller, 1999). Net intestinal secretion is most often secondary to the stimulation of active chloride secretion and to the inhibition of active absorption of sodium and chloride by messengers such as cAMP (Barrett, 2000). In many secretory diarrheas, activation of chloride channels in the apical membrane of enterocytes, including the CFTR and calcium activated chloride channels (CaCC), increases fluid secretion (Thiagarajah *et al*., 2015). The driving force for intestinal ion secretion can arise from the gut lumen as with infectious diarrhea (enterotoxins, such as cholera toxin (CTx), *E. coli* or *Yesinia enterocolitica* as shown in figure 1), from the subepithelial space (inflammatory mediators), or from the systemic circulation

(peptide hormones produced from endocrine tumors) (Field, 2003; Gabriel *et al*., 1994; Hostos *et al*., 2011; Li *et al*., 2010). Most causes of secretory diarrhea alter the second messenger systems through alteration in cAMP, cGMP, or intracellular Ca^{2+}-regulated ion transport pathways (Barrett, 2000; Binder, 2005).

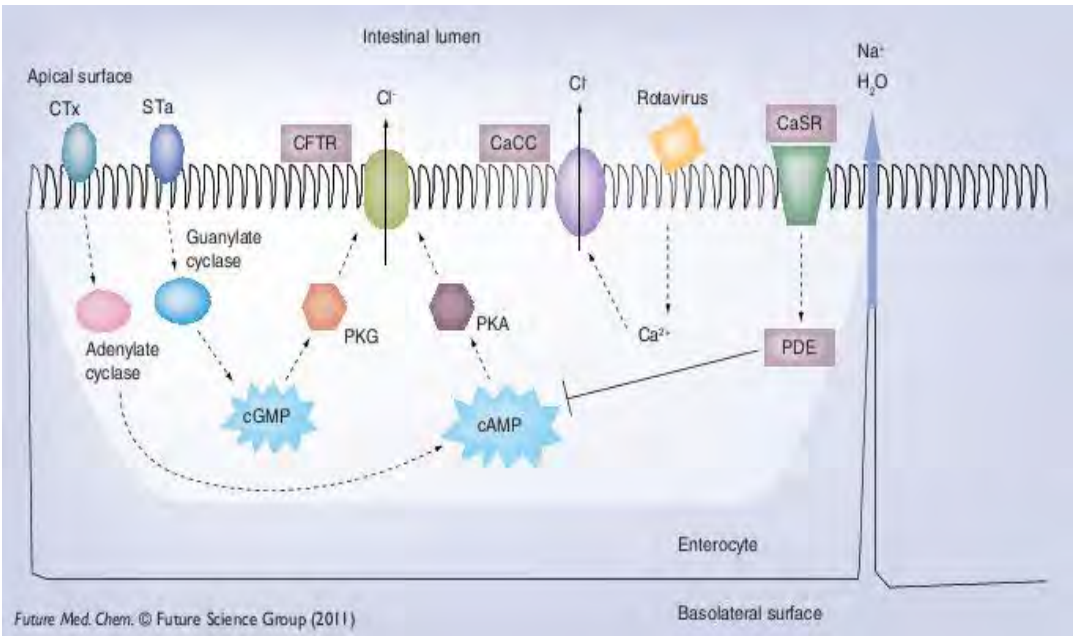

Figure 1: Secretory pathways in the gut epithelium affected by diarrhea-causing pathogens:

CaCC: Calcium-activated chloride channels; cAMP: Cyclic adenosine monophosphate; CaSR: Calcium-sensing receptor; CFTR: Cystic fibrosis transmembrane conductance regulator; cGMP: Cyclic guanosine monophosphate; CTx: Cholera toxin; PDE: Phosphodiesterase; PKA: Protein kinase A; PKG: Protein kinase G; STa: Enterotoxigenic *Escherichia coli* heat-stable toxin (Hostos *et al*., 2011).

Osmotic diarrhea

Osmotic diarrhea occurs either when non absorbable or poorly absorbable solutes are ingested or enterocytes cannot absorb them. This creates a negative osmotic gradient causing leakage of more fluid into the gut increasing the stool volume. The causes of this type of osmotic diarrhea are varied but can be broken down into decreased enzymatic availability (lactose intolerance), a genetic abnormality that decreases or eliminates the ability of the body to absorb certain nutrients (celiac sprue), sugars that are poorly absorbed (polyols), laxatives, magnesium-containing antacids, and

malabsorption of certain fats and bile acids (Field, 2003; Goteborg, 2008; Kent & Banks, 2010).

Inflammatory diarrhea

Certain diarrheal syndromes are caused by inflammation and exudation of the intestinal mucosa and the interaction between cytokines from immunologically reactive cells. Inflammatory diarrhea may result from a wide variety of etiologies including infections and inflammatory bowl diseases (IBDs). Infectious pathogens causing inflammatory diarrhea primarily affect the distal small bowel or the colon. They cause disease by either elaborating cytotoxins or by invading the epithelium with resultant recruitment of inflammatory cells. Most of the pathogens causing inflammatory diarrhea do so by producing mucosal damage as well as by stimulating intestinal secretion (Arora, 2013; Binder, 2009; Eisenhut, 2006).

Functional diarrhea

During normal functioning of the intestines, solids and fluid are moved through the gut with peristaltic waves of the smooth muscles (Choi *et al*., 1997).

The pathophysiology of functional diarrhea may involve multiple mechanisms. Alteration in colonic transit and hypersensitivity of the rectum seen in irritable bowel syndrome (IBS) patients play a role in diarrhea. There is also rapid and increased frequency of high-amplitude propagated contractions after food consumption in IBS. Disturbances in the neural control, visceral nociception and abnormal motility mediated by changes in neurotransmitters are also proposed to contribute to diarrhea in these patients (Drossman *et al*., 2002; Quigley, 2006). Moreover, motility disorders may cause diarrhea through both secretory and osmotic mechanisms. Increased motility may decrease the time for the luminal contents to be in contact with the

epithelium for absorption resulting in secretory diarrhea. On the other hand, slow transit may be associated with bacterial over growth and the ensuing bile acid deconjugation, and steatorrhea (Camilleri, 2004; Choi *et al*., 1997).

1.5. Management of diarrhea

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

1.5.1. Non pharmacological therapy

Oral rehydration solutions

Oral rehydration solutions (ORS) are the first line treatment for diarrhea. Fluid and electrolyte losses due to acute diarrhea can be adequately replaced orally by using glucose-electrolyte solution of optimal concentration (WGO, 2012).

Supplemental zinc therapy, multivitamins, and minerals

Zinc has been shown to play critical roles for cellular functions, cellular growth and function of the immune system. However, its deficiency is widespread among children in developing countries (WHO, 2005). Routine zinc therapy, as an adjunct to ORS is useful in modest reduction of the severity and frequency of diarrheal episodes in children in developing countries. Supplementation with zinc sulfate reduces the incidence, duration and severity of acute and persistent diarrhea (Galvao *et al*., 2013; Lukacik *et al*., 2008). The recommendation for all children with diarrhea is 20 mg of zinc per day for 10-14 days (Infants aged 2 months or younger should receive 10 mg/day for 10-14 days). All children with persistent diarrhea should receive supplementary multivitamins and minerals, including magnesium, each day for 2 weeks (WGO, 2012).

Probiotics, prebiotics and synbiotics

Probiotics including *lactobacilli* and *Saccharomyces cerevisiae* are useful in reducing the severity and duration of acute infectious diarrhea in children. They are undergoing investigation and are emerging as a viable option for the prevention and management of antibiotic-associated, infectious (*Clostridium difficile*), and radiation-induced diarrhea (Eddies & Gray, 2008; Hempel *et al*., 2012; Vrese & Marteau, 2007).

Prebiotics were originally defined by Gibson and Roberfroid (1995) as ‘‘non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health.’’ This criterion is fulfilled only by some indigestible but fermentable carbohydrates such as inulin and lactulose (Vrese & Marteau, 2007). Synbiotics refer to preparations in which probiotics and prebiotics are combined (Hempel *et al*., 2012).

1.5.2. Conventional medicines

Antisecretory agents

These agents temporarily correct the imbalance of electrolytes and fluid in the small intestine and colon (Beverly & Clarence, 2008).

Morphine and, more importantly, loperamide are potent anti-diarrheal agents. This class of drugs is known to be active at μ opiate receptors that mediate their inhibitory effects on intestinal smooth muscle, but there was considerable interest in whether these agents might also have direct antisecretory effects. *In vitro* studies indicated that both morphine and loperamide can inhibit chloride secretion induced by bacterial enterotoxins and prostaglandin E_2 (Hughes *et al*., 1982; McKay *et al*., 1982).

Octreotide is a somatostatin analogue with documented antisecretory activity in neuro-endocrine tumors like gastrinoma and carcinoid syndrome. Besides, octreotide as an antisecretory agent is also very effective in management of acute infective diarrhea induced by enterotoxines in adults (Abbas *et al*., 1996; Mehta *et al*., 2012).

Enkephalins are endogenous opioids in the gut that have pro-absorptive and antisecretory activity in the small intestine. It is thought that enkephalins directly inhibit the activity of adenylate cyclase linked delta (δ) receptors on the enterocyte basolateral membrane. They are degraded by membrane bound metalloproteinase, enkephalinase (Turvill & Farthing, 1997). Racecadotril is an enkephalinase inhibitor, and hence potentiates the action of enkephalins. It does not produce enteropooling and rebound constipation. Besides, it lacks central nervous system side effects despite its weak antisecretory activity (Primim *et al*., 1999). However, there is encouraging evidence that treatment with it can provide clinically relevant symptomatic relief by reducing the severity and duration of diarrheal episodes (Tormo *et al*., 2008).

Intracellular signaling mechanisms are obvious pharmacological targets for the control of intestinal secretory processes. The phenothiazine, chlorpromazine inhibits hormonal stimulation of cAMP and probably inhibits the effects of the calcium-binding protein, calmodulin (Holmgren *et al*., 1978). Zaldaride maleate, another calmodulin inhibitor, has been shown to have antisecretory activity in animal models (Aikawa *et al*., 2000).

Crofelemer is a novel compound (purified proanthocyanidin oligomer) extracted from the stem bark latex of the *Croton lechleri* (Tradtrantip *et al*., 2010). It is a first antidiarrheal agent that simultaneously targets two distinct chloride channels, CFTR and CaCC (Cottreau *et al*., 2012; Tradtrantip *et al*., 2010). Its brand, Fulyzaq, is the

second botanical prescription drug approved by Food and Drug Administration in 2012 (FDA, 2012). Furthermore, it is primarily reserved for treatment of non-infectious diarrhea in HIV/AIDS patients starting antiretroviral therapy (ART) (Chordia & MacArthur, 2013).

Anecdotal experience with an α_2 adrenergic agonist, clonidine, suggests its utility in diabetic diarrhea, but severe side effects limit its usefulness (Fedorak *et al*., 1985).

Antiperistaltic Agents

Antiperistaltic drugs prolong intestinal transit time, thereby reducing the amount of fluid lost in the stool. The drugs in this category include loperamide hydrochloride and diphenoxylate hydrochloride with atropine sulfate. Both agents are effective in relieving symptoms of acute non-infectious diarrhea and are safe for most patients experiencing chronic diarrhea (Beverly & Clarence, 2008). Studies in humans showed that the predominant effects *in vivo* were due to a decrease in the irregular motor activity (phase II) of the migrating motor complex (Schiller *et al*., 1984). Although loperamide have some antisecretory activity, the balance of opinion would attribute its antidiarrheal action to its effects on gut motility (Hughes *et al*., 1982; Kachel *et al*., 1986).

Antimicrobial therapy

Antimicrobials are reliably helpful for children with bloody diarrhea (most likely *shigellosi*s), suspected cholera with severe dehydration etc (Guerrant *et al.,* 2001). Antiprotozoal drugs can be effective for diarrhea, especially for *Giardia, Entamoeba histolytica*, and particularly for *Cryptosporidium*, with nitazoxanide (WGO, 2008). Antimicrobials are considered to be the drugs of choice for empirical treatment of traveler's diarrhea and of community acquired secretory diarrhea when the pathogen

is known. Primary empiric antibiotics include fluoroquinolones such as ciprofloxacin and levofoxacin. Azithromycin may be a feasible option when fluoroquinolone resistance is encountered (Beverly & Clarence, 2008; Navaneethan & Giannella, 2010). Rifaximin is a rifamycin based, nearly non absorbable, gut selective antibiotic with an excellent safety profile (Koo & Dupont, 2010). It was approved in the United States in 2004 for the treatment and chemoprophylaxis of uncomplicated travelers' diarrhea secondary to non-invasive *E. coli* (Hong & Kim, 2011).

1.5.3. Traditional medicines

Since the time immemorial, medicinal plants have played an invaluable role in the development of potent therapeutic agents. Today, it is estimated that about 80% of people in developing countries still rely on traditional medicine for their primary health care. They are currently in demand and their popularity is increasing over time as a potential source of modern medicines (Olajuyigbe & Afolayan, 2012; Pathak & Das, 2013; Tiwari, 2008). Approximately 25% of modern medicines are directly descended from plants first used traditionally. Many others are synthetic analogues built from prototype compounds isolated from plants. Generally, 70% modern medicines are derived from natural products (Pathak & Das, 2013).

There are many herbal plants that possess anti-diarrheal activity with lesser side effect than the conventional drugs. Furthermore, tannins, alkaloids, flavonoids and terpenoids are the main chemical constituents that are responsible for the anti-diarrheal activity of the plants and may be due to antispasmodic and/or antisecretory effects (Komal *et al*., 2013). Antispasmodic activity has been demonstrated for some flavonoids such as quercetin, genistein, and rutin; terpenoids such as himachalol, 1,8-cineol, and tymol; alkaloids such as himbacine, protopine, retuline, and metuenine (Amin & Maham, 2015; Dicarlo *et al*., 1993; Saxena *et al*., 2013). Penta-*m*-digalloyl-

glucose (PDG), hydrolysable tannin extracted from Chinese gallnut, was examined as antisecretory agent both *in vivo* and *in vitro* through inhibiting CFTR mediated chloride channels (Wongsamitkl *et al*., 2010).

A range of medicinal plants such as bark extract of *Albizia gummifera*; leaf extract of *Calpurnia aurea*, *Myrtus communis*, *Artemisia afra* and *Croton marcostachyus*; seed extract of *Coffea arabica*; root extract of *Echinops kebercho*, *Ensete ventricosum*, *Cucumis ficifolius*, *Leonotis ocymifolia* and *Caylusea abyssinica* have been widely used for the management of diarrhea and related gastrointestinal disorders by traditional healers in Ethiopia (d'Avigdor *et al*., 2014; Enyew *et al*., 2013; Etana, 2010; Teklehaymanot & Giday, 2007). Amongst them, the leave extracts of *Calpurnia aurea* (Umer *et al.,* 2013), *Croton marcostachyus* (Degu, 2014); root extracts of *Echinops kebercho* (Shiferie & Shibeshi, 2013) have already evaluated scientifically. However, the therapeutic potentials of some of these medicinal plants have not been validated yet. Therefore, this study was undertaken to evaluate the acclaimed traditional use of *Myrtus communis* L. in diarrheal diseases.

1.6. The Experimental plant

Botanical source and characteristics

Myrtus is a small genus belonging to the family Myrtaceae which includes approximately 100 genera and 3000 species growing in temperate, subtropical and tropical regions of the world (Ozkan & Guray, 2009).

Myrtus communis Linn is the only species of the genus found in the Northern Hemisphere. It is an aromatic evergreen perennial shrub native to Southern Europe, North Africa and West Asia. It is also distributed in South America, North Western Himalaya and Australia and widespread in the Mediterranean region. Myrtus, the

Greek name for Myrtle and communis means common plant growing in groups (Aslam *et al*., 2010; Ozkan & Guray, 2009; Sumbul *et al*., 2011).

In Ethiopia, *Myrtus communis* L has several vernacular names such as Ades (Amharic, Guragegna, Tigregna); Haddus (hararegna), Addisaa, coddoo (Afan Oromo); wobattaa (Welaitigna) (Hedberg *et al*., 2006; Tadesse & Mesfin, 2010).

The common myrtle as depicted in figure 2 has upright stem, 2.4-3 m high. The stem of the plant is branched and dark green leaves are glossy, glabrous, coriaceous, opposite, paired, ovate to lanceolate with stiff structure, aromatic, entire margined, acuminate and 2.5-3.8 cm long. It has axillary white flowers on slender peduncles; medium sized about 2 cm in diameter. They give off a sweet fragrant smell. The berries are pea-sized, orbicular and blue-black. It is highly drought tolerant and needs only little to moderate water. It can grow in damp places, shades as well as full sun up to 800 m altitudes (Aslam *et al*., 2010; Ozkan & Guray, 2009; Sumbul *et al*., 2011).

Figure 2: - Photographs of *Myrtus communis* Linn

Traditional applications

Myrtus communis L is one of the most important drugs being used in Unani system of medicine since ancient Greece period. It is a well-known shrub for its therapeutic, cosmetic and food uses (Sumbul *et al*., 2011). Since time of immemorial, the name and use of *it* have been associated with myth and various rituals (Tadesse & Mesfin, 2010). It has been known more as a decorative hedge plant in Europe. Besides, in Sardinia (Italy) and in the Mediterranean region, it is used to make a liqueur called 'Mirto' and as a culinary herb (Ozkan & Guray, 2009).

Myrtus communis has been frequently used for various ailments like gastric ulcer, diarrhea, dysentery, rheumatism; cosmetic purposes as well as flavoring of food and wines (Sumbul *et al*., 2011). Various parts of it have also been used as folkloric repute for the management of several disorders including hemorrhoid, inflammation, pulmonary diseases (asthma) (Alipour *et al*., 2014), depression, polymenorrhea and wound (Farzae *et al*., 2014). Besides, it has been used as vulnerary, cough suppressant, and digestant effects (Tiwari, 2008), for relieving stress (Akaydin *et al*., 2013), as hypotensive agent and for treatment of eczema and other skin diseases (the decoction of leaf powder) (Sarri *et al*., 2014). Moreover, the leaves have been traditionally used for the treatment of diarrhea in Pakistanian and Indian traditional medicines (Haq *et al*., 2011)**,** in Turkish traditional medicine (leaves and/or fruits are boiled and the stock is drunk) (Dogan & Ugulu, 2013), and in Iranian traditional medicine (Farzae *et al*., 2014).

In Ethiopia, rural women mix the leaf extract of myrtle with raw butter and apply it to their hair for improved bodily scent (Tadesse & Demissew, 1992; Tadesse & Mesfin, 2010). It is also used as antipyretic and sedative agent (Jansen, 1981). In addition, it has been used for the treatment of dandruff (bathing with crushed fresh leaves),

diarrhea and stomach disorders (Juice of the leaf is taken orally in the morning in Zegie peninsula, around Bahirdar) (Teklehaymanot & Giday, 2007), scabies (dried leaf powder mixed with butter is applied topically) (Gebeyehu *et al*., 2014), headache (the leaves are crushed, boiled with water and then drunk) (Getaneh & Girma, 2014).

Ethnopharmacological and phytochemical studies

Extensive ethnopharmacological studies revealed that the leaves have been shown to possess promising antimicrobial activities (Alem *et al*., 2008; Ali *et al*., 2009; Appendino *et al*., 2006; Mansouri *et al*., 2001; Sulaiman *et al*., 2013). Besides, its essential oil showed potent antimicrobial activities against *Helicobacter pylori* (Antonella *et al*., 2007) and clinical strains of *Mycobacterium tuberculosis* (Zanetti *et al*., 2010). It was also effective against all isolates of Aspergillus species (essential oil) (Mohammadi *et al*., 2008), *Candida Albicans* (all crude extracts) (Erdogan *et al*., 2014), *Leishmania tropica* (both essential oil and methanol extracts) (Mahmoudvand *et al*., 2015), and *Plasmodium falciparum in vitro* (essential oil) (Milhau *et al*., 1997).

Apart from this, the leaves have been shown to possess anti-inflammatory activities (Al-Hindawi *et al*., 1989; Feisst *et al*., 2005), antioxidant activities (Bouaziz *et al*., 2015), spasmolytic, bronchodilator and vasodilator activities (Janbaz *et al*., 2013). Various leaf extracts also revealed anti-hyperglycemic (Elfellah *et al*., 1984), antiulcer (Sumbul *et al*., 2010), narcotic analgesic (Twaij *et al*., 2009), antilipidemic and antithrombotic (Khan *et al*., 2014) and anticancer activities (Ogur, 2014). In addition, its essential oil also revealed sedative-hypnotics (Walle *et al*., 2014), anxiolytic (Hailu *et al*., 2011; Moreira *et al*., 2014) and antimutagenic activities (Mimica-Dukic *et al*., 2010).

The leaves of *Myrtus communis* L were investigated to contain small amounts of phenolic acids including caffeic, ellagic and gallic acids, and a flavonoid, quercetin derivatives. On the other hand, flavonoids such as galloyl derivatives of catechin and gallocatechin as well as myricetin derivatives are present in large amounts (Al-Hajjar *et al*., 2012). Similarly, four hydrolyzable tannins, two polyphenolic compounds, and four myricetin glycosides were isolated from the leaves of the plant (Yoshimura *et al*., 2008). The major terpenoids found in the essential oils of the leaves include α-pinene, α-terpineol, linalool, and 1, 8-cineole (Khani & Basavand, 2012).

1.7. Rationale for the study

Remedies for diarrhea have been available for centuries including astringents, opiates and antimicrobial agents. With the passage of time, many problems associated with frequent use of synthetic drugs become prominent like emergence of resistance and severe side effects (Farthing, 2006). Antibiotics are the major remedy of infectious diseases including diarrhea; however, significant increase in antibiotic resistance has been observed in common pathogens worldwide (Hellinger, 2000; Nguyen *et al*., 2006). Bacteria of the genus *Shigella* expressed multiple resistances to various drugs including ampicillin. The genus *Campylobacter* also exhibits significant resistance to quinolones (Selimović *et al*., 2012).

Despite the wide availability of drugs for treating diarrhea, majority of existing drugs suffer from untoward effects like the induction of bronchospasm, and vomiting by racecadotril (Tormo *et al*., 2008); intestinal obstruction and rebound constipation by loperamide (Pankaj, 2006); undesirable central effects by long term use of morphine and its analogs (Khansari *et al*., 2013; Parrish, 2008); upper respiratory tract infections, bronchitis, cough etc by clofelemer (Fulyzaq) (FDA, 2012); acute pancreatitis induced by nifuroxazide (Shindano *et al*., 2007); α-blocker associated

hypotension by phenothiazines (Holmgren *et al*., 1978). Moreover, the attack rate of the disease has remained unchanged and the treatment often fails in the high stool output state with ORS usage (Farthing, 2004; WGO, 2012). Therefore, in recent years, safe alternatives have been sought. There is a need for intensification of research into medicinal plant claim to be effective for the management of diarrheal diseases (Pankaj *et al*., 2006; Pokale & Kushwaha, 2011). Among these plants, the leaves extract of *Myrtus communis* L has acclaimed folklore use as an antidiarrheal agent.

2. OBJECTIVES

2.1. General objective

- To evaluate *in-vivo* antidiarrheal activities of 80ME and solvent fractions of the leaves of *Myrtus communis* L. in mice

2.2. Specific objectives

- To evaluate the acute toxicity profile of 80ME of the leaves of *Myrtus communis* L. in mice
- To evaluate the effect of 80ME and solvent fractions (chloroform, methanol and aqueous) of the leaves of *Myrtus communis* L. on castor oil induced diarrheal model in mice
- To assess anti-motility activity of 80ME and solvent fractions of the leaves of *Myrtus communis* L. on castor oil induced intestinal transit in mice
- To evaluate anti-enteropooling effect of 80ME and solvent fractions of the leaves of *Myrtus communis* L. on castor oil induced entero-pooling in mice
- To determine the phytochemical constituents present in 80ME and solvent fractions of the leaves of *Myrtus communis* L.

3. MATERIALS AND METHODS

3.1. Drugs and chemicals

All solvents used for the extraction process are of laboratory grade. Drugs and chemicals used in the study include: castor oil (Amman Pharmaceutical Industries, Jordan), activated charcoal (Acuro Organics Ltd, New Delhi, India), Loperamide (Daehwa Pharmaceuticals, Republic of Korea), distilled water (Ethiopian Pharmaceutical Manufacturing Factory, Epharm, Ethiopia), Tweens 80 (Atlas Chemical Industries Inc, India), chloroform (Hi-Media Laboratory Reagents, India), methanol (Carlo Erba reagents, S.A.S, France), glacial acetic acid (BDH Laboratory Supplies Poole, England), sulfuric acid (BDH Laboratory Supplies Poole, England), ammonia(BDH Limited poole, England), hydrochloric acid(BDH Laboratory Supplies Poole, England), acetic anhydride (May and Baker LTD Dagenham, England), ferric chloride (BDH Laboratory Supplies Poole, England), Mayer's and Dragendorff's reagents(May and Baker LTD Dagenham, England).

3.2. The plant material

The leaves of *Myrtus communis L.* were collected from Merssa town, Habru woreda, North Wollo zone, Amhara region (490 km North East of Addis Ababa) in October, 2014. The plant was authenticated by a taxonomist and a voucher specimen (number MS002) was deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University (AAU) for future reference. The leaves of *Myrtus communis L* were washed gently, and dried at room temperature under shade for 2 weeks. The dried leaves were then reduced to appropriate size using mortar and pestle.

3.3. Experimental animals

Healthy Swiss albino mice of either sex, weighing 20–30 g and aged 6–8 weeks were used for the experiment. The mice were obtained from animal house of School of Pharmacy, AAU and Ethiopian Public Health Institute (EPHI). The animals were kept in plastic cages at room temperature and on a 12 h light/dark cycle with access to pellet food and water *ad libitum*. Mice were acclimatized to laboratory condition for one week prior to the experiments. Food was withdrawn 18 h prior to the beginning of all the experiments. However, water was accessed except in entero-pooling model, where both food and water were withdrawn. The care and handling was according to international guidelines for the use and maintenance of experimental animals (Institute for Laboratory Animal Research, 1996; National Research Council, 2011; Organization for Economic Cooperation and Development (OECD), 2008).

3.4. Extraction of the plant material

3.4.1. Preparation of 80ME

The extraction was carried out by maceration technique using 80% methanol as a solvent. Hundred fifty gram of the dried powder was weighed using electronic digital balance (Mettler Toledo, Switzerland) and added to an Erlenmeyer flask (2 L) to which 500 ml of 80% methanol solvent was poured in the first round. The plant material was macerated for 72 h with occasional shaking using mini orbital shaker (Bibby scientific limited stone Stafford shire, SI150SA, UK) tuned to 120 rpm. The extract was filtered through double layered muslin cloth followed by Whatman (No.1) filter paper (Schleicher and Schuell Microscience Gmbh, Germany). The marc was then re-macerated for a second and third time by adding another fresh solvent. The resultant filtrates were combined and concentrated using a rotary evaporator (Buchi labortechnik AG, Switzerland) under reduced pressure at 40°C. A dark green paste was

obtained and kept into deep freezer (AFTRON AFF 545, Denmark) to solidify. The residual aqueous solvent was then removed using a lyophilizer (Operon, Korea vacuum limited, Korea). The percentage yield of 80ME was then found to be 16.33% (w/w). Finally, the extract was kept in deep freezer with air tight container until use.

3.4.2. Preparation of solvent fractions

Both Soxhlet and maceration techniques were used for extraction of the plant material. The initial procedure resembles to that of the 80ME except that the dried leaves were pulverized to coarse powder using mortar and pestle and then sieved to maintain uniformity of particle size. From this, 150 g dry powder was subjected to successive soxhlet extraction with solvents of increasing polarity (chloroform and methanol) followed by maceration of the marc of methanol with distilled water (Bainiwal *et al*., 2013; Degu, 2014).

In every batch, 50 mg of the powdered plant material was added in the extraction thimble which in turn was placed into the chamber of Soxhlet apparatus. First, 350 ml chloroform was added into the bottom flask fixed with Soxhlet apparatus and was heated until clear liquid contents of the chamber siphoned into the solvent flask (until exhaustive extraction with the solvent of interest) (Rahman *et al*., 2011). The chloroform fraction was then filtered with suction filter and then concentrated using rotary evaporator under reduced pressure set at 40^{o}C followed by oven at room temperature for 48 h (Zavala-Mendoza *et al*., 2013). The marc in the thimble was collected and then dried overnight at room temperature to remove chloroform.

The residue (marc) left was then extracted using methanol using the same procedure as described for the chloroform fraction to get the methanol fraction except that it was

kept for a week in oven at room temperature for drying. Besides, the marc of methanol fraction was then collected and dried at room temperature.

Finally, the whole dried marc was combined from the three batches and macerated in an Erlenmeyer flask with distilled water and allowed to stand at room temperature for a period of 3 days in each round (total of 9 days) with occasional shaking using mini orbital shaker. The procedure utilized for extraction of 80ME was repeated except that lyophilization rather than vaporization was used to concentrate the extract. After drying, the percentage yields of all fractions were determined and found to be 5.2%, 13.8% and 7.2% for the chloroform, methanol and aqueous fractions, respectively. The fractions were kept in deep freezer with air tight containers till use.

3.5. Acute oral toxicity test

Acute toxicity test was performed according to the OECD 425 (2008) guideline for the 80ME. Initially, a single female mouse was fasted for 3 h and was loaded with 2000 mg/kg of the 80ME as a single dose by oral gavage. It was then observed for any sign of toxicity within the first 24 h. Based on the results of the first mouse, another 4 female mice were recruited and fasted for 3 h. Thereafter, they were given the same dose and were observed for any sign of toxicity or death in the next 14 days.

3.6. Grouping and dosing

Mice were randomly assigned into five groups of six animals each to perform antidiarrheal activities using three models for both 80ME and solvent fractions. All groups were provided with their respective treatments using oral gavage. The first group was assigned as negative control and received a vehicle (distilled water for 80ME, methanol and aqueous fractions; and 2% tweens-80 for the chloroform fraction) at a volume of 10 ml/kg. The second group was assigned as positive control and the standard drug, Loperamide (3 mg/kg) was administered orally for all tests. For

the test groups, three dose levels were determined based on the acute toxicity test (A middle dose, which is one-tenth of the dose utilized during acute toxicity study; a low dose, which is half of the middle dose, and a high dose which is twice of the middle dose) (OECD, 2008). Hence, the test groups were given 100 mg/kg, 200 mg/kg and 400 mg/kg of 80ME of the leaves of *Myrtus communis* L.

Coming to solvent fractions, however, the test groups were treated with various doses of the fractions (200 mg/kg, 300 mg/kg and 400 mg/kg respectively, with additional dose of 800 mg/kg for the aqueous fraction). Appropriate doses for the fractions were selected based on the study carried out using the 80ME as well as a series of pilot studies of each fraction. The 80ME as well as solvent fractions were reconstituted with the respective vehicles at appropriate concentrations. The solutions were prepared fresh on the day of the experiments.

3.7. Determination of antidiarrheal activity

3.7.1. Castor oil induced diarrhea

The method followed by Umer *et al* (2013) was used for this study. Swiss albino mice of either sex were fasted for 18 h and randomly allocated to five groups of six animals each and treated as described under section 3.6. One hour after administration of the respective doses, all animals were given 0.5 ml of castor oil. Thereafter, they were individually placed in cages where the floor was lined with white paper. During an observation period of 4 h, onset of diarrhea (the time interval between the administration of castor oil and the arrival of the first diarrheal stool in minutes), frequency of defecation (the number of wet and total feces) as well as the weight of fecal output (wet and total feces in gm) were recorded for individual mouse.

The percentages of diarrheal inhibition as well as weight of wet and total fecal output were determined according to the formulae I-III (Ara *et al*., 2013; Degu, 2014; Tadesse *et al*., 2014).

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

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

WFT = average number of wet feces in test group.

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

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

3.7.2. Castor oil induced charcoal meal test /gastrointestinal motility

All mice were fasted for 18 h and divided into five groups of six each for 80ME and each solvent fraction and treated as described under section 3.6. 1 h later, 0.5 ml castor oil was administered. Then, 1 ml of marker (5% activated charcoal suspension in distilled water) was administered orally 1 h after castor oil treatment. The animals were then sacrificed after an hour and the small intestine was dissected out from pylorus to caecum. The distance travelled by the charcoal meal from the pylorus was measured and expressed as percentage of the total length of the small intestine from the pylorus to caecum (peristaltic index) as shown in formula I. The percentage of inhibition was then expressed using the formula II (Yasmeen *et al*., 2010; Degu, 2014).

I. $$Peristaltic\ index(PI)\ = \frac{distance\ travelled\ by\ charcoal\ meal}{whole\ \ length\ of\ small\ intestine} * 100$$

II. $$\%\ inhibition\ \ = \ \ \frac{PI\ of\ control\ group - PI\ of\ test\ group}{PI\ of\ control\ group} * 100$$

3.7.3. Castor oil induced enteropooling activity

Intraluminal fluid accumulation was determined using the method described by Islam *et al* (2013). Mice of either sex were deprived of both food and water for 18 h and divided into five groups of six animals each and treated as described under section 3.6 one hour prior to oral administration of castor oil (0.5ml/mouse). One hour after castor oil administration, the mice were sacrificed by cervical dislocation. The abdomen of each mouse was opened; the whole length of small intestine was then taken from the pyloric sphincter to ileo-caecal junction; ligated at both ends and dissected out carefully. Their full small intestines were weighed and intestinal contents were then collected by gentle milking into a graduated tube and hence the volume of intestinal contents was measured. The intestines were reweighed and the difference between the full and the empty intestines was calculated. Eventually, the percentage inhibitions of the volume and weight of intestinal contents were determined according to the formulae I and II respectively (Mamza *et al*., 2014; Robert *et al*., 1976).

I. $$\text{Percentage of inhibition} = \frac{MVICC - MVICT}{MVICC} * 100$$

Where, MVICC = Mean volume of the intestinal content of the control group, MVICT = Mean volume of the intestinal content of the test group.

II. $$\text{Percentage of inhibition} = \frac{MWICC - MWICT}{MWICC} * 100$$

Where, MWICC = Mean weight of the intestinal content of the control group, MWICT = Mean weight of the intestinal content of the test group.

3.7.4. In vivo anti-diarrheal index

The *in vivo* antidiarrheal index (ADI) for the 80ME, solvent fractions and standard drug were determined by combining three parameters taken from the afforementioned

models. It was then expressed according to the following formula (Aye-than *et al*., 1989; Okpo *et al*., 2011).

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

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

3.8. Preliminary phytochemical screening

The qualitative phytochemical investigations of the 80% methanol extract and the solvent fractions of the leaves of *Myrtus communis* L were carried out using standard tests (Bhandary *et al*., 2012; Farhan *et al*., 2012; Zohra *et al*., 2012)

Test for terpenoids (Salkowski test)

To 0.30 gm of each of 80% methanol and solvent fractions of the leaves of *Myrtus communis*, 2 ml of chloroform was added. Then, 3 ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for saponins (Foam test)

To 0.30 gm of each of 80% methanol and solvent fractions, 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of froth indicates the presence of saponins.

Test for tannins (ferric chloride test)

About 0.30 gm of each of 80% methanol and solvent fractions was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added. A brownish green or a blue-black precipitate indicated the presence of tannins.

Test for flavonoids

About 10 ml of ethyl acetate was added to 0.30 gm of each extracts and heated on a water bath for 3 min. The mixture was cooled and filtered. Then, About 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammoniacal layer indicated the presence of flavonoids.

Test for cardiac glycosides (Keller-Killiani test)

To 0.30 gm of each extracts diluted to 5 ml in water was added to 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for steroids (Liebermann-Burckhardt test)

Two ml of acetic anhydride was added to 0.30 g of each extracts with 2 ml chloroform. Then, 1 ml of concentrated sulfuric acid was added. The formation of dark green color in some samples indicated the presence of steroids**.**

Test for alkaloids (Mayer's and Dragendorff's test)

0.50 g of each extract was diluted to 10 ml with acid alcohol, boiled, and filtered. To 5 ml of the filtrate, 2 ml of dilute ammonia and 5ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Dragendorff's reagent to the other. The formation of a cream (with

Mayer's reagent) or reddish brown precipitate (with Dragendorff's reagent) was regarded as positive for the presence of alkaloids.

3.9. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The experimental results were analyzed using the software Statistical Package for Social Sciences (SPSS), version 16. Statistical significance was determined by one way analysis of variance (ANOVA) followed by Tukey Kramer post Hoc test. P-value of less than 0.05 was considered statistically significant. Coefficient of determination (R^2), using linear regression analysis, was determined where appropriate. The analyzed data were then presented using tables and figure.

4. RESULTS

4.1. Acute oral toxicity test

The 80ME of the leaves of *Myrtus communis* L. produced neither overt toxicity nor death during the 14 days observation period 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. The absence of mortality and signs of overt toxicity up to 5 times the maximum effective dose of the extract suggested that 80ME has a wider safety margin and LD_{50} value greater than 2000 mg/kg in mice.

4.2. Effects on castor oil induced diarrheal model

In the castor oil-induced diarrheal model, as presented in table 1, the 80ME of the leaves of *Myrtus communis* L. significantly prolonged the onset of diarrhea and reduced the frequency and weight of wet and total stools at doses of 200 mg/kg and 400 mg/kg as compared to the control. The 100 mg/kg of the extract, however, showed statistically significant effect only on some parameters of diarrhea:- frequency of wet feces ($p<0.001$) and weight of wet ($p<0.001$) and total ($p < 0.05$) fecal outputs. Moreover, a significance difference was obtained when the effects of 100mg/kg were compared with 400 mg/kg and standard drug in all of the parameters except in delaying onset of diarrhea. Besides, the data revealed that the percentage of diarrheal inhibitions were 42.58% ($p<0.01$), 62.52% ($p<0.001$), and 74.96 % ($p<0.001$) at the doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg respectively. The maximum dose of this extract produced the utmost antidiarrheal effect comparable to the standard drug (72.56%, $p<0.001$).

Amongst the solvent fractions, only 400 mg/kg of the chloroform and methanol fractions produced a significant delay in initiation of diarrhea ($p<0.05$). The percentage of diarrheal inhibitions obtained as compared to control were 51.23%

($p<0.05$) and 58.92 % ($p<0.05$) at the doses of 300 mg/kg and 400 mg/kg chloroform fractions respectively. The chloroform fraction also showed a significant reduction in weight of both wet and total fecal output at 300 mg/kg ($p < 0.05$) and 400 mg/kg ($p< 0.05$). Similarly, the methanol fraction significantly decreased the frequency and weight of wet feces at doses of 300 mg/kg ($p< 0.05$; $p< 0.05$) and 400 mg/kg ($p< 0.05$; $p< 0.01$) respectively, with the highest percentage of diarrheal inhibition (62.67%, $p <0.05$) obtained at the latter dose of this fraction compared to the control. However, 300 mg/kg of both fractions did not have any significant effect on number of total fecal outputs. On the contrary, the aqueous fraction was devoid of any significant delay in onset of diarrhea at all tested doses as compared with the respective negative control even if significant diarrheal reduction was seen at doses of 800 mg/kg (53.33%, $p< 0.05$).

As depicted in Figure 3, there was a dose-dependent reduction in the percentage of weight of wet and total fecal outputs in 80ME (R^2=0.970; R^2 =0.949, $p<0.05$), chloroform (R^2=0.995; R^2=0.955, $p<0.05$), and methanol fractions (R^2= 0.999; R^2=1.000, $p<0.01$) respectively, with 400 mg/kg of the 80ME displaying the highest effect (22.22% and 27.03%). As compared to the standard drug (25.00%, 29.33%), the 80ME at its 400 mg/kg revealed the greatest effect to lessen the percentage of fecal output. Besides, both the chloroform and methanol fraction revealed moderate reduction in the percentage of both mean wet and total fecal outputs with 400 mg/kg methanol fraction showing the maximum reduction among the solvent fractions. On the other hand, the aqueous fraction showed minimal inhibition of percentage of both fecal outputs except at 800 mg/kg.

Table 1:- Antidiarrheal effects of 80ME and solvent fractions of the leaves of *Myrtus communis* Linn 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
80ME	Control	76.67 ±7.99	6.67 ±0.49	7.00 ± 0.52	0.36 ± 0.02	0.37 ± 0.02	-------
	100mg/kg	109.67 ± 12.15	3.83 ± 0.70^{a2b1f1}	4.50 ± 0.72^{b1f1}	0.20 ± 0.02^{a2b2f2}	0.21 ± 0.02^{a1b1f1}	42.58
	200 mg/kg	145.00 ± 21.77^{a1}	2.50 ± 0.50^{a3}	3.00 ± 0.68^{a2}	0.14 ± 0.03^{a3}	0.15 ± 0.03^{a2}	62.52
	400 mg/kg	173.83 ±18.03^{a2}	1.67 ± 0.49^{a3}	2.67 ±0.72^{a2}	0.08 ± 0.02^{a3}	0.10 ± 0.03^{a3}	74.96
	3 mg/kg loperamide	161.50 ± 16.93^{a2}	1.83 ±0.40^{a3}	2.83 ± 0.60^{a2}	0.09 ± 0.02^{a3}	0.11 ± 0.02^{a3}	72.56
Solvent fractions	Control	80.17 ± 4.34	6.50 ± 0.43	6.83 ± 0.54	0.35 ± 0.03	0.36 ± 0.04	-----
	CF200mg/kg	123.33 ± 23.81	4.00 ± 1.00	4.33 ± 1.08	0.20 ± 0.05	0.21 ± 0.06	38.46
	CF300 mg/kg	140.50 ± 19.99	3.17 ± 0.65^{a1}	3.67 ± 0.76	0.16 ± 0.03^{a1}	0.17 ± 0.04^{a1}	51.23
	CF400 mg/kg	152.00 ± 21.01^{a1}	2.67 ± 0.76^{a1}	3.17 ± 0.83^{a1}	0.13 ± 0.04^{a1}	0.15 ± 0.04^{a1}	58.92
	3 mg/kg loperamide	165.83 ± 33.17^{a1}	1.67 ± 0.76^{a2}	2.33 ± 1.05^{a1}	0.08 ± 0.04^{a2}	0.09 ± 0.04^{a2}	74.31
	Control	69.33 ± 8.98	7.50 ± 1.34	8.17 ± 1.28	0.42 ± 0.05	0.45 ± 0.05	------
	MF200mg/kg	104.33 ± 6.14	5.17 ± 0.40^{b1}	5.67 ± 0.49^{b1}	0.29 ± 0.03^{b1}	0.30 ± 0.04^{b1}	31.07
	MF300 mg/kg	136.00 ± 29.02	3.83 ± 0.87^{a1}	4.33 ± 1.05	0.22 ± 0.06^{a1}	0.24 ± 0.06^{a1}	48.93
	MF400 mg/kg	155.50 ± 26.89^{a1}	2.83 ± 0.95^{a1}	3.50 ± 1.23^{a1}	0.14 ± 0.06^{a2}	0.17 ± 0.06^{a2}	62.67
	3 mg/kg loperamide	166.83 ± 25.23^{a1}	1.83 ± 0.70^{a2}	2.33 ± 0.92^{a2}	0.09 ± 0.03^{a3}	0.11 ± 0.04^{a3}	75.56
	Control	69.33 ± 8.98	7.50 ± 1.34	8.17 ± 1.28	0.42 ± 0.05	0.45 ± 0.05	------
	AF200mg/kg	70.83 ± 6.53^{b2j1n1}	6.83 ± 0.70^{b2i1j1m1n1}	7.67 ±0.67^{b2g1i1j1n1}	0.39 ± 0.02^{b3g2i2j2n2}	0.42 ± 0.02^{b3g2i1j2n2}	8.93
	AF300 mg/kg	77.67 ± 4.86^{b1j1n1}	6.33 ± 0.72^{b2j1n1}	6.67 ± 0.76^{b1j1n1}	0.35 ± 0.03^{b2g1i1j1n1}	0.36 ± 0.04^{b2i1j1n1}	15.60
	AF400 mg/kg	81.00 ± 3.53^{b1}	5.67 ± 0.62^{b1j1n1}	6.50 ± 0.62^{b1j1}	0.32 ± 0.02^{b2j1n1}	0.33 ± 0.02^{b1j1n1}	24.40
	AF800 mg/kg	134.67 ± 32.67	3.50 ± 0.99^{a1}	4.33 ± 1.22^{a1}	0.18 ± 0.05^{a2}	0.21 ± 0.05^{a2}	53.33
	3 mg/kg loperamide	166.83 ± 25.23^{a1}	1.83 ± 0.70^{a2}	2.33 ± 0.92^{a2}	0.09 ± 0.03^{a3}	0.11 ± 0.04^{a3}	75.56

Values are mean ±SEM (n= 6); analysis was performed using one way ANOVA followed by Tuckey post hoc test; , [a] compared with control values; [b] compared with loperamide; [c] compared with 100 mg/kg; [d] compared with 200 mg/kg; [e]compared with 300 mg/kg; [f] compared with 400 mg/kg; [g] compared with 800 mg/kg, [h] compared with CF200, [i] compared with CF300, [j] compared with CF400, [k]compared with MF200, [m] compared with MF 300, [n] compared with MF 400; [1]$p<0.05$, [2]$p<0.01$, [3]$p<0.001$; CF= chloroform fraction, MF= methanol fraction, AF=aqueous fraction. Controls are 10 ml/kg- distilled water (for 80ME, methanol and aqueous fractions) and 2% tweens-80 (for chloroform extract).

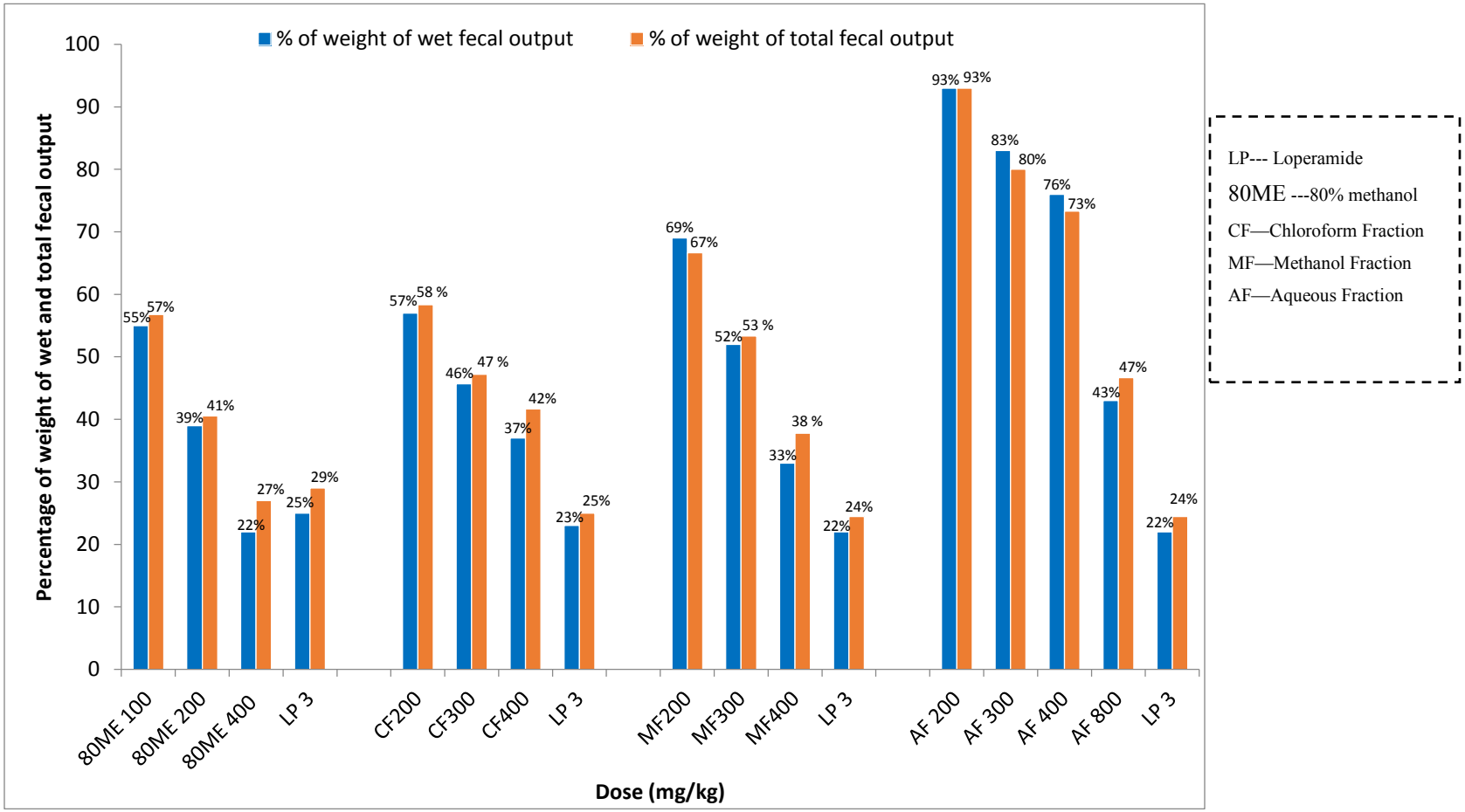

Figure 3: Percentage of weight of fecal output of the 80ME and solvent fractions of the leaves of *Myrtus communis L* on castor oil induced diarrheal model in mice

4.3. Effects on castor oil induced intestinal transit in mice

As presented in the Table 2, the 80ME 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 33.54% ($p< 0.001$), 46.12% ($p< 0.001$), and 62.31% ($p <0.001$) at doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg respectively. The maximum dose of this extract showed comparable anti-motility effects to that of the standard (59.61%, $p< 0.001$).

The chloroform fraction also significantly inhibited the gastrointestinal transit of charcoal meal at doses of 300 mg/kg (35.11%, $p<0.05$) and 400 mg/kg (44.86 %, $p<0.01$) as compared to the control. In addition, the methanol fraction had statistically significant inhibitory effect on gastrointestinal transit of charcoal meal at the doses of 300 mg/kg and 400 mg/kg with the utmost inhibitory effect observed by the latter dose amongst all solvent fractions (47.54%, $p<0.001$). On the contrary, the aqueous fraction produced a significant decrease in the propulsive movement of the charcoal meal through the small intestine only at the dose of 800 mg/kg (38.61%, $p<0.01$) as compared to the respective control.

Table 2:- Effects of 80ME and solvent fractions of the leaves of *Myrtus communis* L on gastrointestinal transit in mice

Types of Extracts	Dose administered	Length of small intestine (cm)	Distance moved by the charcoal meal (cm)	Peristaltic index (%)	% inhibition
80ME	Control	56.17 ± 1.42	36.67 ± 1.94	65.09 ± 2.25	-------
	100 mg/kg	58.33 ± 0.80	25.17 ± 2.43^{a3b2f2}	43.26 ± 4.37^{a3b2f2}	33.54
	200 mg/kg	58.67 ± 1.12	20.05 ±1.09^{a3}	35.07 ± 2.27^{a3}	46.12
	400 mg/kg	58.17 ± 2.01	14.33 ± 1.65^{a3}	24.53 ± 2.53^{a3}	62.31
	3 mg/kg loperamide	56.33 ± 1.28	14.83 ± 0.91^{a3}	26.29 ± 1.34^{a3}	59.61
Solvent fractions	Control	57.67 ± 1.76	36.17 ± 4.18	62.33 ± 6.06	------
	CF200 mg/kg	56.33 ± 1.08	25.17 ± 3.46^{b1}	44.88 ± 6.39^{b1}	27.99
	CF300 mg/kg	59.50 ± 1.09	24.00 ± 1.39^{a1}	40.44 ± 2.60^{a1}	35.11
	CF400 mg/kg	60.17 ± 1.89	20.67 ± 2.43^{a2}	34.37 ± 3.91^{a2}	44.86
	3 mg/kg Loperamide	56.83 ± 1.11	14.00 ± 1.41^{a3}	24.58 ± 2.32^{a3}	60.56
	Control	58.50 ± 0.67	36.83 ± 3.21	62.81 ± 5.11	-------
	MF200 mg/kg	59.17 ± 1.38	29.17 ± 1.96^{b2}	49.47 ± 3.47^{b2}	21.24
	MF300 mg/kg	59.50 ± 1.41	24.00 ± 3.33^{a1}	40.29 ± 5.48^{a1}	35.85
	MF400 mg/kg	59.00 ± 1.59	19.17 ± 3.27^{a3}	32.95 ± 5.99^{a3}	47.54
	3 mg/kg Loperamide	56.33 ± 1.28	13.67 ± 1.52^{a3}	24.20 ± 2.55^{a3}	61.47
	Control	58.50 ± 0.67	36.83 ± 3.21	62.81± 5.11	------
	AF200 mg/kg	56.83 ± 0.95	34.00 ± 1.83^{b3g1j1n1}	59.79 ± 2.98^{b3g1j1n2}	4.81
	AF300 mg/kg	56.83 ± 1.92	31.00 ± 2.05^{b2g1}	54.91 ± 4.33^{b2g1n1}	12.58
	AF400 mg/kg	59.17 ± 0.98	29.50 ± 3.09^{b2}	49.79 ± 4.95^{b2}	20.73
	AF800 mg/kg	56.83 ± 1.17	21.83 ± 3.55^{a2}	38.56 ± 6.29^{a2}	38.61
	3 mg/kg Loperamide	56.33 ± 1.28	13.67 ± 1.52^{a3}	24.20 ± 2.55^{a3}	61.47

Values are mean ±SEM (n= 6); analysis was performed using one way ANOVA followed by Tuckey post hoc test; , [a] compared with control values; [b] compared with loperamide; [c] compared with 100 mg/kg; [d] compared with 200 mg/kg; [e]compared with 300 mg/kg; [f] compared with 400 mg/kg; [g] compared with 800 mg/kg, [h] compared with CF200, [i] compared with CF300, [j] compared with CF400, [k]compared with MF200, [m] compared with MF 300, [n] compared with MF 400; [1]$p<0.05$, [2]$p<0.01$, [3]$p<0.001$; CF= chloroform fraction, MF= methanol fraction, AF=aqueous fraction. Controls are 10 ml/kg- distilled water (for 80ME, methanol and aqueous fractions) and 2% tweens-80 (for chloroform extract).

4.4. Effects on castor oil induced enteropooling

In intestinal fluid accumulation test, the 80ME of the leaves of *Myrtus communis* L showed significant reduction in both the average volume and weight of intestinal contents at all tested doses as compared to control. As the data revealed in table 3, the percentage inhibition of volume of intestinal contents was found to be 25.58% ($p<0.01$), 38.37% ($p<0.001$), and 46.51% ($p<0.001$) at doses of 100 mg/kg, 200 mg/kg, 400 mg/kg respectively.

The chloroform and methanol fractions reduced the volume and weight of the intestinal contents significantly at doses of 300 mg/kg and 400 mg/kg only. Maximum percentage inhibition of the volume of intestinal contents was observed at 400 mg/kg, being 38.46 % ($p<0.01$) and 40.96% ($P<0.01$) for chloroform and methanol fractions, respectively. Generally, both the chloroform and methanol fraction showed comparable percent reduction of both volume and weight of intestinal contents at all tested doses. On the contrary, the aqueous fraction was devoid of any significant inhibitory effect on the volume and weight of intestinal contents up to 400 mg/kg as compared with the respective control. However, significant inhibitory effects were seen at dose of 800 mg/kg being 33.74% ($p<0.01$) and 30.90% ($P<0.01$) for percent reduction of volume and weight of intestinal contents respectively (Table 3).

Table 3:- Effects of 80ME and solvent fractions of the leaves of *Myrtus communis* L on gastrointestinal fluid accumulation in mice

Extracts	Dose administered	Volume of intestinal contents (ml)	% inhibition	Weight of intestinal contents (gm)	% inhibition
80ME	Control	0.86 ± 0.07	-------	1.12 ± 0.03	-------
	100mg/kg	0.64 ± 0.04^{a2b1f1}	25.58	0.91 ± 0.05^{a2b1f1}	18.75
	200 mg/kg	0.53 ±0.03^{a3}	38.37	0.77 ± 0.06^{a3}	31.25
	400 mg/kg	0.46 ± 0.02^{a3}	46.51	0.70 ± 0.02^{a3}	37.50
	3 mg/kg loperamide	0.47 ± 0.04^{a3}	45.35	0.71 ± 0.03^{a3}	36.61
Solvent fractions	Control	0.78 ± 0.08	--------	1.02 ± 0.07	--------
	CF200mg/kg	0.61 ± 0.07^{b1}	21.79	0.85 ± 0.07^{b1}	16.67
	CF300 mg/kg	0.53 ± 0.04^{a1}	32.05	0.75 ± 0.04^{a1}	26.47
	CF400 mg/kg	0.48 ± 0.04^{a2}	38.46	0.69 ± 0.03^{a2}	32.35
	3 mg/kg loperamide	0.43 ± 0.06^{a2}	44.87	0.66 ± 0.05^{a3}	35.29
	Control	0.83 ± 0.06	-----	1.10 ± 0.03	-------
	MF200mg/kg	0.67 ± 0.04^{b1}	19.28	0.93 ± 0.07^{b1}	15.45
	MF300 mg/kg	0.58± 0.03^{a1}	30.12	0.81 ± 0.04^{a1}	26.36
	MF400 mg/kg	0.49 ± 0.08^{a2}	40.96	0.73 ± 0.08^{a2}	33.64
	3 mg/kg loperamide	0.47 ± 0.04^{a3}	43.47	0.68 ± 0.02^{a3}	38.18
	Control	0.83 ± 0.06	------	1.10 ± 0.03	-------
	AF200mg/kg	0.76 ± 0.04^{b2g1j1n1}	8.43	1.02 ± 0.05^{b3g2j2n1}	8.18
	AF300 mg/kg	0.73 ± 0.07^{b1j1n1}	12.05	0.98 ± 0.07^{b2g1j1}	10.91
	AF400 mg/kg	0.68 ± 0.07	18.07	0.93 ± 0.05^{b1}	15.45
	AF800 mg/kg	0.55 ± 0.02^{a2}	33.74	0.76 ± 0.03^{a2}	30.90
	3 mg/kg loperamide	0.47 ± 0.04^{a3}	43.47	0.68 ± 0.02^{a3}	38.18

Values are mean ±SEM (n= 6); analysis was performed using one way ANOVA followed by Tuckey post hoc test; , [a] compared with control values; [b] compared with loperamide; [c] compared with 100 mg/kg; [d] compared with 200 mg/kg; [e]compared with 300 mg/kg; [f] compared with 400 mg/kg; [g] compared with 800 mg/kg, [h] compared with CF200, [i] compared with CF300, [j] compared with CF400, [k]compared with MF200, [m] compared with MF 300, [n] compared with MF 400; [1]$p<0.05$, [2]$p<0.01$, [3]$p<0.001$; CF= chloroform fraction, MF= methanol fraction, AF=aqueous fraction. Controls are 10 ml/kg- distilled water (for 80ME, methanol and aqueous fractions) and 2% tweens-80 (for chloroform extract).

4.5. *In vivo* antidiarrheal index

The *in vivo* antidiarrheal 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. The greatest *in vivo* ADI was achieved at the dose of 400 mg/kg of 80ME (83.96%) which is comparable to the standard drug, loperamide (78.22%). Among the solvent fractions, methanol fraction showed the highest *in vivo* ADI (71.81%) at doses of 400 mg/kg. Both 80ME and solvent fractions showed dose dependent increment in ADI value (80% methanol extract (R^2=0.944), chloroform fraction (R^2=0.997), methanol fraction (R^2=0.991), aqueous fraction (R^2=0.999)).

Table 4:- *In vivo* antidiarrheal indices of 80ME and solvent fractions of the leaves of *Myrtus Communis*

Extracts	Dose administered	Delay in defecation (time of onset in Min, Dfreq) (%)	Gut meal travel distance (Gmeq) (%)	Purging frequency in number of wet stools (%)	*In vivo* antidiarrheal index (ADI)
80ME	Control	-------	-------	-------	-------
	100mg/kg	43.04	33.54	42.58	39.47
	200 mg/kg	89.12	46.12	62.52	63.58
	400 mg/kg	126.72	62.31	74.96	83.96
	3 mg/kg loperamide	110.64	59.61	72.56	78.22
Solvent fractions	Control	-------	------	------	-----
	CF200mg/kg	53.84	27.99	38.46	38.69
	CF300 mg/kg	75.25	35.11	51.23	51.34
	CF400 mg/kg	89.59	44.86	58.92	61.87
	3 mg/kg loperamide	106.85	60.56	74.31	78.34
	Control	------	------	------	------
	MF200mg/kg	50.48	21.24	31.07	32.18
	MF300 mg/kg	96.16	35.85	48.93	55.25
	MF400 mg/kg	124.29	47.54	62.67	71.81
	3 mg/kg loperamide	140.63	61.47	75.56	86.76
	Control	-------	-------	--------	-------
	AF200mg/kg	2.16	4.81	8.93	4.53
	AF300 mg/kg	11.54	12.58	15.60	13.13
	AF400 mg/kg	16.83	20.73	24.40	20.42
	AF800 mg/kg	94.24	38.61	53.33	57.89
	3 mg/kg loperamide	140.63	61.47	75.56	86.76

CF =chloroform fraction, MF=methanol fraction, AF= aqueous fraction

4.6. Preliminary phytochemical screening

Evaluation of the preliminary phytochemical screening of the 80ME of the leaves of *Myrtus communis* L. revealed the presence of terpenoids, flavonoids, tannins, glycosides and saponins but alkaloids and steroids were absent. Amongst the solvent fractions, the data revealed that alkaloids were not detected in all solvent fractions and trace amounts of steroids were detected in the chloroform fraction only. On the other hand, terpenoids and flavonoids were detected in both chloroform and methanol fractions. Tannins were common across all fractions. Glycosides and saponins were also observed in both methanol and aqueous fractions. Amongst all, the 80ME and the methanol fraction appeared to be relatively rich in secondary metabolites (Table 5).

Table 5:- Preliminary phytochemical screening of the 80 % methanol extract and solvent fractions of the leaves of *Myrtus communis* L.

Constituents	Crude extract	Solvent fraction		
	80ME	Chloroform	Methanol	Aqueous
Cardiac glycosides	+	_	+	+
Flavonoids	+	+	+	-
Alkaloids	_	_	_	_
Saponins	+	_	+	+
Steroids	_	+	_	_
Tannins	+	+	+	+
Terpenoids	+	+	+	_

+ = present, - = absent

5. DISCUSSION

Medicinal plants, although assumed to be safe, are potentially toxic which necessitates investigation of their safety status (Getahun, 1976; Ifeoma & Oluwakanyinsola, 2013). It is therefore important to properly evaluate their safety and efficacy profile of plants that are under use in traditional medicines. The need for newer, more effective, cheaper and most importantly safer antidiarrheal drugs has become a paramount issue to tackle this present situation (Komal *et al*., 2013; Kumar *et al*., 2010). This study was aimed to validate the safety and effectiveness of *Myrtus communis* L as antidiarrheal agent.

The acute toxicity profile of the leaves of *Myrtus communis* L was determined based on OECD guideline 425 (OECD, 2008). On this test, the LD_{50} was found to be > 2000 mg/kg for the 80ME. Generally, if the LD_{50} value of the test chemical is more than 3 times the minimum effective dose, the substance is considered as a good candidate for further studies (Carol, 1995). Since the 80ME had an 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 80ME of the leaves of *Myrtus communis* with LD_{50} > 2000 mg/kg is designated as 'unlikely to be hazardous'(WHO, 1975). Hence, based on the safety profile of the 80ME and prior absence of any toxicity data regarding the plant, further toxicity studies were not done on the solvent fractions.

The plant material was investigated for its *in vivo* antidiarrheal activities in all the three models using castor oil as diarrhea inducing agent. Diarrhea occurs when there is an imbalance between the secretary and absorptive processes of gastrointestinal tract and/or when there is an alteration of motility of intestinal smooth muscles

(Gidudu *et al*., 2011; Talley *et al*., 1994). The use of castor oil as diarrhea inducer is well documented (Okpo *et al*., 2011; Rahman *et al*., 2011; Shiferie & Shibeshi, 2013; Tadesse *et al*., 2014). When administered orally, it produces irritant laxative effect mediated by its active metabolite, ricinoleic acid, a hydroxylated fatty acid released by intestinal lipases. Ricinoleic acid produces local irritation and inflammation of the intestinal mucosa, causing the release of prostaglandins that eventually increase gastrointestinal motility, net secretion of water and electrolytes (Horton *et al*., 1968; Robert *et al*., 1976). Ricinoleic acid mediates the aforementioned pharmacological effects of castor oil *via* specifically activating EP_3 prostanoid receptors. In mice lacking EP_3 receptors, the laxative effect induced *via* ricinoleic acid is absent (Tunaru *et al*., 2012). Besides, it forms ricinoleate salts with Na^+ and K^+ in the lumen of the intestine and these salts inhibit Na^+/K^+ ATPase; increase permeability of the intestinal epithelium, which in turn produces a cytotoxic effect on intestinal absorptive cells (Cline *et al*., 1976; Gaginella *et al*, 1977, 1978). It also induces fluid and electrolyte secretion secondary to their stimulation of an active anion secretory process which is most likely to be mediated by cAMP (Racusen & Binder, 1979). Therefore, the use of castor oil as diarrhea inducer for all models is plausible as it resembles the pathophysiologic processes and ensures the face validity of actual diarrheal diseases in human and animals.

The first model being castor oil induced diarrheal model assesses the potential of a test substance as having an overall antidiarrheal activity regardless of its effect on gut motility and/or intestinal secretion. The onset of defecation, the frequency and weight of fecal output, more importantly wet feces, were determined as main parameters. The 80ME (at 200 mg/kg and 400 mg/kg) significantly delayed the initiation of diarrhea and reduced the number and weight of both wet and total fecal output with the highest

effects observed at 400 mg/kg in all of the aforementioned parameters. The lower dose (100 mg/kg) of this extract, however, showed significant effect on some of the parameters in this model: - frequency of wet feces ($p<0.01$), weight of wet ($p<0.01$) and total ($p<0.05$) fecal output. It did not have statistically significant effect on delaying onset of diarrhea and reducing frequency of total feces. This might be linked to the interference of dry feces which are less reliable to indicate diarrhea in cases of total fecal output. In addition, doses having lower antimotility and/or antisecretory effects are less likely to address all the parameters measured in this model. This was in agreement with other studies where plants having comparable antispasmodic and/or antisecretory effects failed to extend initiation of diarrhea (Degu, 2014; Tadesse *et al*., 2014).

Diarrhea is characterized by fecal urgency and incontinence (WGO, 2012; WHO, 2013). Substances exhibiting antidiarrheal activity may have a potential to retard the onset of diarrhea significantly as seen in 200 and 400 mg/kg 80ME. Based on the WHO (2013) criteria, however, a decrease in consistency and an increase in frequency of bowel movements to greater than 3 stools per day generally describes diarrhea. Even though diarrhea has been defined over time by various scientific groups and health organizations in different ways, greater emphasis is given on the consistency of stools rather than the number. Normally, stool is 60-90% water; diarrhea usually occurs when the percentage exceeds 90% (CDC, 2013; Gidudu *et al*., 2011; WHO, 2013). Therefore, determination of percentage inhibition has mainly focused on the reduction of frequency of wet, but not total, fecal outputs as a good marker of antidiarrheal activity.

Diarrhea is also presented with an increase in weight of defecation (Mouzan, 1995; Thomas *et al*., 2003; WHO, 2013). Accordingly, the 80ME displayed a dose-

dependent reduction in percentage of weight of wet fecal output (R^2=0.970, p< 0.05) and total fecal output (R^2=0.949, p<0.05), indicating the antidiarrheal potential of the 80ME in this model. This study is concordant with other studies in which the crude extract of different plants reduced the frequency and weight of stools in a dose-dependent manner (Okpo *et al*., 2011; Rajamanickam *et al*., 2010; Shiferie & Shibeshi, 2013; Tadesse *et al*., 2014).

Coming to solvent fractions, both the chloroform and methanol fractions (at 400 mg/kg) produced significant effects in all parameters in this model. In addition, both of these fractions significantly decreased the frequency of wet, but not total, feces and weight of both wet and total stooling at 300 mg/kg. Similar to 100 mg of crude extract, 300 mg/kg of both fractions failed to significantly extend onset of diarrhea as compared to their respective controls. The lowest dose, 200 mg/kg, of both fractions, however, did not have significant effect in altering any of the aforementioned parameters compared to controls. 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 general model. Generally, these fractions had comparable antidiarrheal effects, but the effects were lower than that of the 80ME.

Looking at the dose dependency nature of the fractions, the methanol fraction (R^2 =0.994) appeared to have a steeper slope than that of the chloroform fraction (R^2=0.980). Similarly, methanol fraction had also revealed sharper reduction in weight of both wet and total fecal outputs respectively (R^2= 0.999; R^2=1.000, p<0.01) as compared to chloroform fraction (R^2= 0.995; R^2=0.955, p<0.05). The methanol fraction is more likely to lose potency at lower dose unlike chloroform fraction which retains antidiarrheal activity within narrow limits along all dose ranges. This might be attributed to qualitative and quantitative differences in bioactive constituents of these

fractions. On the contrary, the aqueous fraction was devoid of significant delay in onset of diarrhea at all tested doses but significant reduction in the number and weight of fecal output were observed at 800 mg/kg. Most of the doses of the aqueous fraction (up to 400 mg/kg) also failed to demonstrate any significant effect on the subsequent models. This could possibly suggest that the localization of the active ingredients in the chloroform and methanol fractions. This study was in line with other studies in which the chloroform and methanol fractions of different plants reduced the frequency and weight of stooling (Billah *et al*., 2013; Degu, 2014; Karthik *et al*., 2011; Mazumder *et al*., 2006).

Non-steroidal anti-inflammatory drugs (NSAIDs) could inhibit castor oil induced diarrhea (Awouters *et al*., 1978). Similarly, 80% ethanolic extracts of *Myrtus communis* L showed potent anti-inflammatory activity in a previous study (Al-Hindawi *et al*., 1989). This was further supported by the fact that isolated constituents from the leaves of the plant (myrtucommulone, semi-myrtucommulone and non-prenylated acylphloroglucinols (phlorotannins)) are known to suppress the biosynthesis of eicosanoids both *in vivo* and *in vitro* (Feisst *et al*., 2005). Thus, it is reasonable to assume that the antidiarrheal effect of the 80ME and solvent fractions, with possible variation in distribution patterns, could be partly ascribed to inhibition of castor oil-induced prostaglandin synthesis.

Flavonoids such as quercetin showed antidiarrheal activity against castor oil and PGE_2 -induced diarrhoea in mice *via* increasing the colonic fluid absorption in the presence of secretagogue compounds (Gálvez *et al*., 2011). Tannins have also exhibited broad-spectrum antidiarrheal properties possibly due to increasing trans-epithelial resistance and inhibiting the CFTR and CaCC chloride channels (Ren *et al*., 2012). Certain terpenoids such as 1, 8-cineole and abietic acid have demonstrated

antidiarrheal properties *via* dual antispasmodic and antisecretory activities (Amin & Maham, 2015; Fernandez *et al*., 2001). Besides, steroids like phytosterols have been shown to inhibit production of prostaglandin E_2 (Awad *et al*., 2004), which are known to play a crucial role in the stimulation of intestinal secretions (Bern *et al*., 1989).

The anti-diarrheal activities of the 80ME 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. This effect could probably be linked to the presence of terpenoids, tannins and flavonoids which are shown to promote colonic absorption of water and electrolytes (Palombo, 2006) in the 80ME, chloroform and methanol fractions. By contrast, the aqueous fraction showed modest antidiarrheal activity at its maximum dose. Flavonoids, steroids and terpenoids are lacking in this fraction and hence the probable synergistic antidiarrheal activities are no longer available. Apart from this, predominately tannins might be responsible for the antidiarrheal activity. Most of the aforementioned secondary metabolites such as flavonoids (quercetin and cathechin derivatives), terpenoids (1,8-cineole), and tannins (gallotannins, ellagitannines and phlorotannins) were screened from the leaves of this plant so far (Al-Hajjar *et al*., 2012; Khani & Basavand, 2012; Yoshimura *et al*., 2008). Therefore, these constituents might be attributable for the overall antidiarrheal effects of the 80ME and solvent fractions with possible variation in distribution patterns of polarity across the fractions.

Most antidiarrheal drugs act by decreasing the intestinal motility and/or inhibit secretion of intestinal contents (Hughes *et al*., 1982; Kachel *et al*., 1986; McKay *et al*., 1982). Hence, further confirmation of the possible mechanism of action was tested on intestinal motility and entero-pooling models, respectively.

The reduction of gastrointestinal motility is one of the mechanisms by which many antidiarrheal agents can act (Beverly & Clarenc, 2008; Schiller *et al*., 1984). It was observed that the 80ME significantly suppresses the propulsion of charcoal marker in all tested doses. In the present study, the percentage inhibition of charcoal marker at 400 mg/kg dose (62.31%, $p< 0.001$) of this extract was observed to be almost comparable to the standard. 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.

Besides, both the chloroform and methanol fractions had comparable antispasmodic effects with the highest effect revealed at 400 mg/kg of methanol fraction (47.54%, $p<0.001$). On the other hand, only the maximum dose of aqueous fraction (800 mg/kg) showed substantial antimotility effect. The middle dose (300 mg/kg) of both the chloroform and methanol fractions also showed statistically significant effects in this model. The lower dose (200 mg/kg) of chloroform and methanol fractions and most of the doses of aqueous fraction, however, failed to demonstrate significant antimotility effects indicating lack of statistically sound antidiarrheal activities seen in the first model. This study is in line with other studies where doses of various extracts having lower or insignificant antispasmodic effects might have significant effects in some or noon of the parameters of the castor oil induced diarrheal model (Degu, 2014; Okpo *et al*, 2011; Taddesse *et al*, 2014).

Previous study on isolated tissue preparations *in vitro* demonstrated that 70% methanol extract of the leaves of *Myrtus communis* L possess bronchodilator, spasmolytic and vasodilator activities (*via* inhibiting spontaneous, K^+ and carbachol induced smooth muscle contractions) possibly due to dual blockade of cholinergic receptors and voltage dependent calcium channels (Janbaz *et al*., 2013). Therefore, it

is plausible to assume that the *in vivo* antimotility effect of the 80ME and solvent fractions could be partly ascribed to anticholinergic and/or calcium channel blocker effects. It is in line with several related studies where *in vitro* mechanistic studies were correlated with *in vivo* antimotility activities (Khan and Gilani, 2009; Mehmood *et al*., 2011; Shah *et al*., 2010).

Furthermore, naturally occurring flavonoids such as Catechin, Isoliquiritigenin, showed antispasmodic, bronchodilator and vasodilator activities probably due to calcium channel antagonist effects (Amira *et al*, 2008; Chen *et al*., 2009; Ghayur, 2007). The higher antispasmodic effects observed in 80ME and the first two active fractions might be due to the presence of flavonoids that are missing in the aqueous fraction. Although the phytochemical constituents responsible for the antidiarrheal effect are yet to be identified, the amount of phytochemical constituents that is responsible for impeding gastrointestinal motility such as tannins appear to increase with dose (Almeida *et al*., 1995; Yadav & Tangpu, 2007). Similarly, studies on the functional role of tannins also 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 (Yadav & Tangpu, 2007).This could possibly be the reason why significant anti-motility effect was observed at the higher dose of the aqueous fraction.

Diarrheal syndromes result from varieties of pathophysiological processes (Field, 2003; Kent & Banks, 2010). The third being enteropooling model was aimed to assess the secretary components of diarrhea. In this model, the 80ME extract showed significant reduction in both the average volume and weight of intestinal contents at all tested doses as compared to control. Besides, both the chloroform and methanol fraction showed comparable percent reduction of both volume and weight of intestinal

contents at all tested doses. On the contrary, the aqueous fraction was devoid of significant inhibition of intestinal fluid accumulation except at the additional dose (800 mg/kg). As compared to the 80ME, both the chloroform and methanol fractions demonstrated lower effects. This model further supports that lower doses of the fractions (200 mg/kg of chloroform and methanol as well as most of the doses of aqueous) did not have any significant anti-enteropooling effects, along with insignificant antimotility effects, indicating the absence of statistically sound antidiarrheal effects in the first model.

Mascolo *et al*. (1993, 1994) reported that the active metabolite, ricinoleic acid might activate the nitric oxide pathway and induce nitric oxide (NO) dependent gut secretion. A growing body of evidence indicates that phytochemical constituents such as terpenoids (Jang *et al*., 2004) and flavonoids (Kim *et al*., 1999, 2004; Messaoudene *et al*., 2011) are implicated in attenuation of NO synthesis. Therefore, unlike the aqueous fraction, the pronounced inhibition of castor oil induced intestinal fluid accumulation could possibly be related to the presence of terpenoids and flavonoids that increase the reabsorption of electrolytes and water by hindering castor oil mediated NO synthesis in the 80ME, chloroform and methanol fractions.

Apart from this, the antidiarrheal effects of flavonoids and tannins have also been ascribed to their ability to inhibit hydro-electrolytic secretion in the intestine through various mechanisms (Di Carlo *et al*., 1993; Galvez *et al*., 1993; Kumar *et al*., 2010). The enteric nervous system also stimulates intestinal secretion through neurotransmitters such as acetylcholine. On the other hand, intestinal absorption can be stimulated with α_2-adrenergic agents, enkephalins, and somatostatin (Bern *et al*., 1989; Fedorak *et al*., 1985). Secondary metabolites such as flavonoids could stimulate α_2-adrenergic receptors in the absorptive cells of the gastrointestinal tract (Di Carlo *et*

al., 1993). Furthermore, tannins are astringents that either bind and precipitate or shrink proteins of luminal surface of intestine (Ashok & Upadhyaya, 2012). Particularly, hydrolysable tannins extracted from Chinese gallnut were also examined as antisecretory agent both *in vivo* and *in vitro via* inhibiting CFTR chloride channels (Wongsamitkl *et al*., 2010). 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 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 antidiarrheal effects (Liu *et al*., 2014).

In contrast to the aqueous fraction, the significant antisecretory activities of the 80ME as well as the chloroform and methanol fractions could probably be related to the existence and hence synergistic effects of flavonoids, tannins and terpenoids. The highest antisecretory effects of 80% methanol extract might be associated with the nature and relative abundance of these secondary metabolites compared to the two fractions. In aqueous fractions, however, tannins may play an important role as antisecretory agent which increase with dose escalation. Since the independent nature of the extraction processes utilized, the constituents found in the 80ME and solvent fractions might not relate qualitatively and quantitatively. These phytochemical constituents may have antidiarrheal activities *via* a multitude of mechanisms and act either independently or in concert to accomplish the overall antidiarrheal effect.

The *in vivo* ADI is a measure of the cube root of combined effects of three parameters such as purging frequency in number of wet stools, delay in onset of diarrheal stools and intestinal motility (Aye-than *et al*., 1989; Okpo *et al*., 2011). Generally, higher ADI value indicates a measure of how much effective an extract is in treating diarrhea

(Ching *et al*., 2008; Prasad *et al*., 2014). ADI was increased with dose, suggesting the dose dependency nature of this parameter. The 80ME showed highest *in vivo* ADI value among all extracts with corresponding doses, reinforcing the notion that this extract is endowed with better antidiarrheal activity compared to solvent fractions. Moreover, the methanol fraction showed the highest ADI value at its maximum dose as compared to the other fractions. Conversely, the aqueous fraction, which had little antidiarrheal activity in most of the models, exhibited the lowest ADI, pointing to the fact that ADI is a useful parameter in ranking antidiarrheal agents.

Interestingly, extensive studies revealed that the leaves of *Myrtus communis* L have been shown to possess promising antimicrobial activities against several microorganisms including diarrhea causing pathogens (Alem *et al*., 2008; Ali *et al*., 2009; Antonella *et al*., 2007; Appendino *et al*., 2006; Mansouri *et al*., 2001; Sulaiman *et al*., 2013; Zanetti *et al*., 2010). Therefore, in addition to its dual antimotility and antisecretory effects observed in this study, its overwhelming antimicrobial properties reinforcing a notion that *Myrtus communis* L. can possibly be a good candidate for diarrheas of diverse etiologies including those with infectious component.

6. CONCLUSION

This study revealed that the 80ME of the leaves of *Myrtus communis* L. is endowed with a promising anti-diarrheal activity. Moreover, the chloroform and methanol fractions were also found to possess substantial anti-diarrheal activities. However, the aqueous fraction showed modest antidiarrheal effect at its maximum dose (800 mg/kg) used in the study. The overall antidiarrheal activities of the 80ME and solvent fractions were associated with dual inhibitory effects on castor oil induced gastrointestinal motility and fluid secretion. Their antidiarrheal activities may be ascribed to the presence of bioactive secondary metabolites, ranging from non-polar to polar, such as flavonoids, tannins, terpenoids and steroids that act either individually or in concert to bring about the overall antidiarrheal effect. What is more, the non-polar and semi polar constituents, in chloroform and methanol fractions, may have better antidiarrheal activities than highly polar constituents found in the aqueous fraction. These findings provide a scientific support for folkloric repute of *Myrtus communis* L as treatment of diarrheal diseases.

7. FUTURE DIRECTIONS

The following recommendations are suggested to further investigate the experimental plant in depth.

- Further studies should be done to isolate, purify and identify pharmacologically active principle (s) responsible for the antidiarrheal activities of the plant
- Further toxicological studies such as sub-acute, sub-chronic and chronic toxicities should be done to assess the long term safely profile of the extracts
- *Ex -vivo* studies of the fractions on isolated tissue preparations should be done to support the *in vivo* methods
- Further studies that aim to elucidate the possible mechanism of actions of responsible constituents in each extracts.

8. REFERENCES

Abbas Z, Moid I, Khan AH *et al* (1996). Efficacy of octreotide in diarrhea due to *vibrio cholera*: a randomized control trial. *Annals of Tropical Medical Parasitology* **90**:507-513.

Abdullah M and Firmansyah MA (2013). Clinical approach and management of chronic diarrhea. *The Indonesian Journal of Internal Medicine* **45**: 2.

Aikawa N, Karasawa A, Ohmori K (2000). Effect of zaldaride maleate, an antidiarrheal compound, on intracellular cyclic nucleotide-mediated intestinal ion secretion in rats. *European Journal of Pharmacology* **394**: 233–236.

Akaydin G, Şimşek I, Arituluk ZC *et al* (2013). An ethno-botanical survey in selected towns of the Mediterranean sub region (Turkey). *Turkish Journal of Biology* **37**: 230-247.

Akinnibosun FI and Nwafor FC (2015). Prevalence of diarrhea and antibiotic susceptibility test in children under 5 years at University of Benin Teaching Hospital, Nigeria. *International Research Journal of Public and Environmental Health* **2**: 49-55.

Alem G, Mekonnen Y, Tiruneh M *et al* (2008). *In vitro* antibacterial activities of crude extract of Myrtus *communis* on common human pathogens. *Ethiopian Medical Journal* **46**: 63-69.

Al-Hajjar MA, Al-Shahiry KF, Al-Abache SM (2012). Isolation and Identification of some active constituents of *Myrtus Communis* in Iraq by using HPLC, FTIR techniques. *Iraqi National Journal of Chemistr*y **45**:11-22.

Al-Hindawi MK, Al-Deen IH, Nabi MH *et al* (1989). Anti-inflammatory activity of some Iraqi plants using intact rats. *Journal of Ethno pharmacology* **26**:163-168.

Ali MR, Hussin MS, Kadum MM *et al* (2009). *In-vitro* Antimicrobial Activities of *Myrtus communis* and Cyperus rotundus L. Extracts. *Al- Mustansiriya Journal of Science* **20**:1.

Alipour G, Dashti S and Hosseinzadeh H (2014). Review of pharmacological effects of *Myrtus communis* L. and its active constituents. *Phytotherapy Research* **28**: 1125–1136.

Almeida CE, Karnikowski MG, Foleto R, *et al* (1995). Analysis of antidiarrheal effect of plants used in popular medicine. *Rev Saude Publication* **29**:428-33.

Amin GJ and Maham M (2015). The application of 1,8-cineole, a terpenoid present in medicinal plants, inhibits castor oil-induced diarrhea in rats. *Pharmaceutical Biology* **53**:594-599.

Amira S, Rotondo A, Mulè F (2008). Relaxant effects of flavonoids on the mouse isolated stomach: Structure-activity relationships (SAR). *European Journal of Pharmacology* **599**:126–130.

Antonella D, Giovanna B, Paola M *et al* (2007). *In vitro* activity of essential oil of *Myrtus communis* L. against *Helicobacter pylori*. *International Journal of Antimicrobial Agents* **30**:562-563.

Appendino G, Maxia L, Bettoni P *et al* (2006). Antibacterial galloylated alkylphloroglucinol glucosides from myrtle (*Myrtus communis*). *Journal Natural Products* **69**: 251-254.

Ara A, Salehein M, Ahmed NU *et al* (2013). Anti-diarrheal activity and acute toxicity of methanolic bark extract of *Adenanthera pavonina* linn (Fabaceae) and its elemental composition. *Turkish Journal of Pharmaceutical Scienc*es **10**: 263-272.

Arora K (2013). Macromolecular Complexes of Cystic Fibrosis Transmembrane Conductance Regulator Alter Fluid Transport in Inflammatory Bowel Disorders. *Health Science Center*, the University of Tennesse.

Ashok PK and Upadhyaya K (2012). Tannins are astringent. *Journal of Pharmacognosy and Phytochemistry* **1**: 2278- 4136.

Aslam S, Ganaie KA, John AQ *et al* (2010). Family Myrtaceae in Kashmir *Myrtus communis* L: A new record for the shrub world of Kashmir Himalayas. *Academia Arena* **2**:42-43.

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

Awouters F, Niemegeers CJ, Lenaerts FM *et al* (1978). Delay of castor oil diarrhea in rats: A new way to evaluate inhibitors of prostaglandin biosynthesis. *Journal of Pharmacy and Pharmacology* **30**: 41-45.

Aye-than, Kulkarni HJ, Wut-hmone, *et al* (1989). Antidiarrheal efficacy of some Burmese indigenous drug formulations in experimental diarrheal test models. *International Journal of Crude Drug Research* **27**:195-200.

Bainiwal LK, Vijayvergia P, Vijayvergia R (2013).Determination of preliminary phytoconstituents, total phenolic and flavonoids contents in the roots, leaves and stems of *Cleome viscosa* Linn. *International Journal of Biological & Pharmaceutical Research* **4**: 891-895.

Barbara G (2006). Diarrhea In: Pharmacotherapy Handbook, 6th edition, *New York, McGraw-Hill*, pp. 223-227.

Barrett (2000). New insights into the pathogenesis of intestinal dysfunction: secretory diarrhea and cystic fibrosis. *World Journal of Gastroenterology* **6**: 470-474.

Bern MJ, Sturbaum CW, Karayalcin SS *et al* (1989). Immune system control of rat and rabbit colonic electrolyte transport, Role of prostaglandins and enteric nervous system. *Journal of Clinical Investigation* **83**:1810-1820.

Beverly CM and Clarence E (2008). Constipation, diarrhea and irritable bowel syndrome in: pharmacotherapy principles & practice, *New York: McGraw-Hill Companies*, pp307-322.

Bhandary SK, Kumari S, Bhat VS *et al* (2012). Preliminary phytochemical screening of various extracts of *Punica granatum* peel, whole fruit and seeds. *Nitte University Journal of Health Science* **2**: 4

Billah MM, Khatun H, Parvin *et al* (2013). Antibacterial, antidiarrhoeal, and cytotoxic activities of methanol extract and its fractions of Caesalpinia bonducella (L.) Roxb leaves. *BMC Complementary and Alternative Medicine* **13**:101.

Binder HJ (2005). Intestinal fluid and electrolyte movement, In: Medical physiology, 11th edition, *Philadelphia, PA: Elsevier*, pp. 931–946.

Binder HJ (2009). Mechanisms of diarrhea in inflammatory bowel diseases. *Annals of Newyork Academy of Science* **11**:285–293.

Bizuneh T, S/Mariam Z, Abebe A *et al* (2004). Rotavirus infection in under five children in Jimma University Hospital, Southwest Ethiopia. *Ethiopian Journal of Health Development* **18:**19-24.

Bouaziz A, Khennouf S, Zarga MA *et al* (2015). Phytochemical analysis, hypotensive effect and antioxidant properties of *Myrtus communis* L. growing in Algeria. *Asian Pacific Journal of Tropical Biomedicin*e **5**:19-28.

Camilleri M (2004). Chronic diarrhea: a review on pathophysiology and management for the clinical gastroenterologist, *Clinical Gastroenterology & Hepatology* **2**: 198–206.

Carol A (1995). Acute, Sub chronic and chronic Toxicology. In: CRC editors. Handbook of Toxicology. U.S.: *CRC Press*: 51-104.

CDC (2008). Rotavirus Surveillance-Worldwide, 2001-2008. *Morbidity and Mortality Weekly report* **57**:1255-1257. [Online] Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5746a.htm[Accessed 1 August 2015].

CDC (2013). Global Diarrheal Burden. [Online] Available at: http://www.cdc.gov/healthy water/global/diarrhea-burden.html [Accessed 5 August, 2015].

Central Statistical Agency (2011). Ethiopia demographic and health survey 2011-preliminary report, Addis Ababa, Ethiopia.

Chen G, Zhu L, Liu Y, *et al* (2009). Isoliquiritigenin, a flavonoid from licorice, plays a dual role in regulating gastrointestinal motility *in vitro* and *in vivo. Phytotherapy Research* **23**:498–506,

Ching FP, Omogbai Ek, Ozolua RI, *et al* (2008). Antidiarrheal activities of aqueous extract of *Stereospermum kunthianum* (Cham, Sandrine Petit) stem bark in rodents. *African Journal of Biotechnology* **7**:1220-1225.

Choi MG, Camilleri M, O'Brien MD *et al* (1997). A pilot study of motility and tone of the left colon in diarrhea due to functional disorders and dysautonomia. *American Journal of Gastroenterology* **92**:297–302.

Chordia P and MacArthur RD (2013). Crofelemer, a novel agent for treatment of non-infectious diarrhea in HIV-infected persons. *Expert Review of Gastroenterology and Hepatology* **7**:591-600.

Cline WS, Lorenzsonn V, Benz L, *et al* (1976). The effects of sodium ricinoleate on small intestinal function and structure. *The Journal of Clinical Investigation* **58**: 380-390.

Cottreau J, Tucker A, Crutchley R *et al* (2012). Crofelemer for the treatment of secretory diarrhea. *Expert Review of Gastroenterology and Hepatology* 6: 17-23.

d'Avigdor E, Wohlmuth H, Asfaw Z *et al* (2014). The current status of knowledge of herbal medicine and medicinal plants in Fiche, Ethiopia. *Journal of Ethnobiology and Ethnomedicine* **10**:38.

Das SK, Chisti MJ, Huq S *et al* (2013). Etiology of diarrhea among severely malnourished infants and young children: Observation of urban-rural differences over one decade in Bangladesh. *Food and Nutrition Science*s **4**, 233-239.

Degu A (2014) Evaluation of *in vivo* anti-diarrheal activity of solvent fractions of the Leaves of *Croton macrostachyus* Hochst (Euphorbiaceae) in mice. Unpublished Master Thesis, Addis Ababa University, Addis Ababa.

Dessalegn M, Kumie A & Tefera W (2011). Predictors of under-five childhood diarrhea: Mecha District, West Gojam, Ethiopia. *Ethiopian Journal of Health Development* **25**:3.

Di Carlo G, Autore G, Izzo AA *et al* (1993). Inhibition of intestinal motility and secretion by flavonoids in mice and rats: Structure activity relationships. *Journal of Pharmacy and Pharmacology* **45**:1054-1059.

Dogan Y and Ugulu I (2013). Medicinal Plants Used for Gastrointestinal Disorders in Some Districts of Izmir Province, Turkey. *Ethno Medicine* **7**: 149-161.

Drossman DA, Camilleri M, Mayer EA *et al* (2002). AGA technical review on irritable bowel syndrome. *Gastroenterology* **123**:2108–2131.

Eddins C and Gray M (2008). Are probiotic or symbiotic preparations effective for the management of *clostridium difficile*-associated or radiation-induced diarrhea? *Journal of Wound Ostomy Continence Nursing* **35**: 50-58.

Eisenhut M, (2006). Changes in ion transport in inflammatory bowel diseases. *Journal of Inflammation* **3**:5.

Elfellah MS, Akhter MH, Khan MT (1984). Anti-hyperglycaemic effect of an extract of *Myrtus communis* in streptozotocin-induced diabetes in mice. *Journal Ethno pharmacology* 11:275-281.

Enyew A, Asfaw Z, Kelbessa E *et al* (2013). Status of medico-cultural commercial plants at Fiche town market, Ethiopia. *International Journal of Pharmaceuticals and Health care Research* **01**:227-236.

Erdogan EA, Goksen G, Everest A (2014). Anti-candidal activities of some *Myrtus Communis* L. extracts obtained using accelerated solvent extraction (ASE). *Journal of Applied Biology and Biotechnology* **2**: 012-014.

Etana B (2010). Ethno-botanical Study of traditional medicinal Plants of Goma Wereda, Jima Zone of Oromia Region, Ethiopia. Unpublished Masters Thesis, Addis Ababa University, Addis Ababa.

Farhan H, Rammal H, Hijazi A *et al* (2012). Phytochemical screening and extraction of polyphenol from stems and leaves of a Lebanese *Euphorbia macrolada schyzoceras* Boiss. *Annals of Biological Researc*h **3**:149-156.

Farthing MJ (2004). Novel agents for the control of secretory diarrhea. *Expert Opinion of Investigational Drugs* **13**:777-785.

Farthing MJ (2006). Antisecretory drugs for diarrheal Disease. *Digestive Diseases* **2**:47–58.

Farzae MH, Abbasabadi Z, Shams-Ardekani MR (2014). A Comprehensive review of plants and their active constituents with wound healing activity in traditional Iranian Medicine, *Wounds* **26**:197-206.

FDA (2012). FDA approves first anti-diarrheal drug for HIV/AIDS patients, US Food and Drug Administration, USA. [Online] available at: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm333701.htm [Accessed 10 September 2015]

Fedorak RN, Field M, Chang EB (1985. Treatment of diabetic diarrhea with clonidine. *Annals Internal Medicine* **102**:197-199.

Feisst C, Franke L, Appendino G (2005). Identification of molecular targets of the oligomeric non-prenylated acylphloroglucinols from *Myrtus communis* and their implication as anti-inflammatory compounds. *Journal of Pharmacological and Experimental Therapeutics* **31**:389-396.

Fernandez MA, Tornos MP, Garcia MD *et al* (2001). Anti-inflammatory activity of abietic acid, a diterpene isolated from *Pimenta racemosa* var. grissea. *Journal of Pharmacy and Pharmacology* **53**:867-872.

Field M (2003). Intestinal ion transport and the pathophysiology of diarrhea. *Journal of Clinical Investigation* **111**:931–943.

FischerWalker CL, Perin J, Aryee MJ *et al* (2012). Diarrhea incidence in low and middle income countries in 1990 and 2010: a systematic review. *BMC public health* **12**:220.

FischerWalker CL, Rudan I, Liu L, *et al* (2013). Global burden of childhood pneumonia and diarrhea. *Lancet* **381**:1405-1416.

Gabriel SE, Brigman KN, Koller BH *et al* (1994). Cystic fibrosis heterozygote resistance to cholera toxin in the cystic fibrosis, mouse model. *Science* **266**:107-109.

Gaginella TS, Haddad AC, Go VL *et al* (1977). Cytotoxicity of ricinoleic acid and other intestinal secretagogues on isolated intestinal epithelial cells. *Journal of Pharmacology and Experimental Therapeutics* **201**: 259-266.

Gaginella TS, Philips SF, Dozois RR *et al* (1978). Stimulation of adenylate cyclase in homogenates of isolated intestinal epithelial cells from hamsters. Effects of gastrointestinal hormones, prostaglandins and deoxycholine and recinoleic acid. *Gastroenterology* **74**: 11-15.

Galvao TF, Thees MF, Pontes RF *et al* (2013). Zinc supplementation for treating diarrhea in children: a systematic review and meta-analysis. *Rev Panam Salud Publica* **33**:370–7.

Gálvez J, Crespo ME, Jiménez J *et al* (2011). Antidiarrheal activity of quercitrin in mice and rats. *Journal of Pharmacy and Pharmacology* **45**: 157–159.

Gebeyehu G, Asfaw Z, Enyew A *et al* (2014).ethno botanical study of traditional medicinal plants and their conservation status in Mecha wereda, West Gojam Zone of Ethiopia. *International Journal of Pharmaceutical and Health care Research* **02**:137-154.

Gebrehiwot EM, Berheto TM, Worku A *et al* (2015). Childhood Diarrhea in Central Ethiopia: Determining Factors for Mothers in Seeking Modern Health Treatments. *Science Journal of Clinical Medicine* **4**: 4-9.

Getahun A (1976). Some common medicinal and poisonous plants used in Ethiopian folk medicine. Addis Ababa University, Addis Ababa, Ethiopia.

Getaneh S and Girma Z (2014). Ethnobotanical study of medicinal plants in Debrelibanose wereda, Central Ethiopia. *African Journal of Plant Science* **8**:366-379.

Ghayur MN, Khan H, Gilani AH (2007).Antispasmodic, bronchodilator and vasodilator activities of (+) Catechin, a naturally occurring flavonoids. *Archives of Pharmaceutical Research* **30**: 970-975.

Ghishan FK and Kiela PR (2012). Small intestinal ion transport. *Current Opinion of Gastroenterology* **28**: 130 -134.

Gibson GR and Roberfroid MB (1995). Dietary modulation of the colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition* **125**: 1401–1412.

Gidudu J, Sack DA, Pina M *et al* (2011). Diarrhea: Case definition and guidelines for collection, analysis, and presentation of immunization safety data. *Vaccine* **29**: 1053–1071.

Godana W and Mengiste B (2013). Environmental factors associated with acute diarrhea among children under five years of age in derashe district, Southern Ethiopia. *Science Journal of Public Health* **1**: 119-124.

Goteborg AB (2008). Bile acid induced diarrhea pathophysiological and clinical aspects, department of internal medicine, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg. *Intellecta Docusys, Västra Frölunda*, Sweden.

Guerrant RL, Gilder TV, Steiner TS *et al* (2001). Practice guidelines for the management of infectious diarrhea. *Clinical Infectious Diseases* **32**: 331-351.

Hailu E, Engidawork E, Asres K (2011). The Essential oil of *Myrtus communis* L produces a non-sedating anxiolytic effect in Mice Models of Anxiety. *Ethiopian Pharmaceutical Journal* **29**: 1-12.

Haq F, Ahmad H, Alam M (2011). Traditional uses of medicinal plants of Nandiar Khuwarr catchment (District Battagram), Pakistan. *Journal of Medicinal Plants Research* **5**: 39-48.

Hedberg I, Kelbessa E, Edwards S *et al* (2006). Flora of Ethiopia and Eritrea, Addis Ababa University, Ethiopia, volume 5:539-540.

Hellinger W (2000). Confronting the problem of increasing antibiotic resistance. *South Medical Journal* **93**: 842-848.

Hempel S, Newberry SJ, Maher AR *et al* (2012). Probiotics for the Prevention and Treatment of Antibiotic-Associated Diarrhea, A Systematic Review and Meta analysis, *Journal of American Medical Association* **307**:1959-1969.

Holmgren J, Lange S, Lonnroth I (1978). Reversal of cyclic AMP-mediated intestinal secretion in mice by chlorpromazine. *Gastroenterology* **75**:1103–1108.

Hong KS and Kim JS (2011). Rifaximin for the treatment of acute infectious diarrhea. *Therapeutic Advances in Gastroenterology* **4**: 227 -235.

Horton EW, Main IH, Thompson CJ *et al* (1968). Effect of orally administered prostaglandin E1 on gastric secretion and gastrointestinal motility in man. *Gu*t **9**: 655-658.

Hostos EL , Choy RK, Nguyen T (2011).Developing novel antisecretory drugs to treat infectious diarrhea. *Future Medicinal Chemistry* **3**, 1317–1325.

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

Ifeoma O and Oluwakanyinsola S (2013). Screening of herbal medicines for potential toxicities Abuja, Nigeria: *National Institute for Pharmaceutical Research and Development* [Online] Available at: http://dx.doi.org/10.5772/54493[Accessed 15 July 2015].

Institute for Laboratory Animal Research (ILAR) (1996).Guide for the care and use of laboratory animals. Washington DC, USA: *National Academy Press*.

Islam AM, Uddin ME, Chowdhury AU *et al*. (2013). *In vivo* antidiarrheal and cytotoxic potential of different fractions of *Pandanus foetidus* Leaves. *American Journal of Biomedical Sciences* 5(3): 208-216.

Janbaz KH, Nisa M, Saqib F *et al* (2013). Bronchodilator, vasodilator and spasmolytic activities of methanolic extract of *Myrtus communis* L. *Journal of Physiology and Pharmacology* **64**:479-84.

Jang DS, Min HY, Jeong YH *et al* (2004). Di- and sesqui-terpenoids isolated from the pods of Sindora sumatrana and their potential to inhibit lipopolysaccharide-induced nitric oxide production. *Archives of Pharmaceutical Research* **27**:291-294.

Jansen, PCM (1981). Spices, condiments and medicinal plants in Ethiopia, their taxonomy and agricultural significance, *Centre for Agricultural Publishing and Documentation*, Wageningen, the Netherlands, 274.

Juckett G and Trivedi R (2011). Evaluation of Chronic Diarrhea. *American Family Physician* **84**:1119-1126.

Kachel G, Ruppin H, Hagel J *et al* (1986). Human intestinal motor activity and transport: effects of a synthetic opiate. *Gastroenterology* **90**: 85–93.

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

Kent AJ and Banks MR (2010). Pharmacological management of diarrhea. *Gastroenterology Clinics of North America* **3**: 495-507.

Khan A and Gilani AH (2009). Antispasmodic and bronchodilator activities of *Artemisia vulgaris* are mediated through dual blockade of muscarinic receptors and calcium influx. *Journal of Ethno pharmacology* **126**:480–486.

Khan RA, Feroz R, Jemil M *et al* (2014). Hypolipidemic and antithrombotic evaluation of aqueous extract of *Myrtus communis* L. *African Journal of Pharmacy and Pharmacology* **8**: 235-239.

Khani A and Basavand F (2012). Chemical composition and insecticidal activity of myrtle (*Myrtus communis* L.) essential oil against two stored-product pests. *Journal of Medicinal Plants and By-products* **2**: 83-89.

Khansari MR, Sohrabi MR , Zamani F (2013). The Usage of Opioids and their Adverse Effects in Gastrointestinal Practice: A Review. *Middle East Journal of Digestive Disclosur*e **5**: 5–16

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

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

Kirk KL (2000). New paradigms of CFTR chloride channel regulation. *Cellular and Molecular Life Sciences* **57**: 623 – 634.

Komal, Kumar S, Rana A (2013). Herbal approaches for diarrhea: A review, *International Research Journal of Pharmacy* **4**:31-38.

Koo HL and Dupont HL (2010). Rifaximin: A unique gastrointestinal-selective antibiotic for enteric diseases. *Current Opinion in Gastroenterology* **26**: 17 -25.

Kumar R, Sharma RJ, Bairwa K *et al* (2010). Pharmacological review on natural antidiarrheal agents. *Der Pharmaceuticals and Chemicals* **2**: 66-93.

Li C, and Naren AP (2010). CFTR chloride channel in the apical compartments: spatiotemporal coupling to its interacting partners. *Integrated Biology* **2**:161-177.

Liu C, ZhengY, Xu W *et al* (2014). Rhubarb tannins extract inhibits the expression of Aquaporins 2 and 3 in Magnesium Sulphate-induced diarrhea model. *Bio-Medical Research International* **14:3-5.**

Lukacik M, Thomas RL, Aranda JV (2008). A Meta-analysis of the Effects of Oral Zinc in the Treatment of Acute and Persistent Diarrhea. *Pediatrics* **121**: 2.

Lundgren O (2002). Enteric nerves and diarrhea. *Pharmacology and Toxicology* **90**:109-120.

Mahmoudvand H, Ezzatkhah F, Sharififar F (2015). Antileishmanial and cytotoxic effects of essential oil and methanolic extract of *Myrtus communis* L. *Korean Journal of Parasitology* **53**: 21-27.

Mamza UT, Sodipo OA, Khan IZ *et al* (2014). Evaluation of antidiarrhoeal activity of ethanolic extract from the leaves of *Phyllanthus amarus* (Euphorbiacea). *Internatioal Jouranl of Green Herb Chemicals* **3**:171-178.

Mansouri S, Alireza FA, Teimour GT *et al* (2001). Antibacterial activity of the crude extracts and fractionated constituents of *Myrtus communis*. *Pharmaceutical Biology* **39**: 399-401.

Mary KA (2013). *Forgotten Killers:Pneumonia and Diarrhea Prevalence in Third WorldCountries.*[Online]Available at: https://www.google.com.et/#q=Forgotten+KillersPneumonia+and+Diarrhea+Prevalence+in+ThirdWorld+Countries[Accessed 5 September 2014].

Mascolo N, Izzo AA, Autore G, *et al* (1994). Nitric oxide and castor oil-induced diarrhea. *Journal of Pharmacology and Experimental Therapeutics* **268**:291-295.

Mascolo N, Izzo AA, Barbato F *et al* (1993). Inhibitors of nitric oxide synthetase prevent castor-oil-induced diarrhea in the rat. *British Journal of Pharmacology* **108**:861-864.

Mazumder R, Bhattacharya S, Mazumder A *et al* (2006). Antidiarrheal evaluation of *Aegle marmelos* (Correa) L. root extract. *Phytotherapy Res*earch **20**: 82–84.

McKay JS, Linaker BD, Higgs NB *et al* (1982). Studies of the antisecretory activity of morphine in rabbit ileum *in vitro*. *Gastroenterology* **82**: 243–247.

Mehmood MH, Siddiqi HS, Gilani AH (2011). The antidiarrheal and spasmolytic activities of Phyllanthus emblicaare mediated through dual blockade of muscarinic receptors and Ca^{2+} channels. *Journal of Ethno pharmacology* **133**:856–865.

Mehta S, Khandelwal PD, Jain VK *et al* (2012). A Comparative study of racecadotril and single dose octreotide as an anti-secretory agent in acute infective diarrhea. *Journal of the Association of Physicians of India* **60**:12-15.

Mengistie B, Berhane Y, Worku A (2013). Prevalence of diarrhea and associated risk factors among children under-five years of age in Eastern Ethiopia: A cross-sectional study. *Open Journal of Preventive Medicine* **3**: 446-453.

Messaoudene D, Belguendouz H, Ahmedi ML *et al* (2011). *Ex vivo* effects of flavonoids extracted from *Artemisia herba* alba on cytokines and nitric oxide production in Algerian patients with Adamantiades-Behcet's disease. *Journal of Inflammation* **8**:35.

Mihrete TS, Alemie GA, Teferra AS (2014). Determinants of childhood diarrhea among under five children in Benishangul Gumuz Regional State, North West Ethiopia. *BMC Pediatrics* **14**:102.

Milhau G, Valentin A, Benoit F *et al* (1997). *In vitro* antimalarial activity of 8 essential oils. *Journal of Essential Oil Research* **9**: 329-333.

Mimica-Dukic N, Bugarin D, Grbovic S *et al* (2010). Essential oil of *Myrtus communis* L: As a potential antioxidant and antimutagenic agents. *Molecules* **15**: 2759-2770.

Mohammadi R, Esfahani SH, Shadzi S *et al* (2008). Antifungal activity of *Myrtus communis* L essential oil against clinical isolates of Aspergillus. *Journal of Isfahan Medical School 26*: 105-111.

Mohammed S, Tilahun M, Tamiru D (2013). Morbidity and associated factors of diarrheal diseases among under five children in Arba-Minch district, Southern Ethiopia, 2012. *Science Journal of Public Health* **1**:102-106.

Mohanta G, Praveen K, Manna P *et al* (2010). Recent advances in management of acute diarrhoea in children. *Indian Journal of Pharmacy Practice* **3**:1-3.

Moreira MR, Salvadori MG, Almeida AA *et al* (2014). Anxiolytic-like effects and mechanism of (−)-myrtenol: A monoterpene alcohol. *Neuroscience Letters* **579**: 119–124.

Mossie TB, Tadesse DG, Kassa AW (2014). Childhood diarrheal disease among under five children at Dejen district, Northwest Ethiopia. *American Journal of Health Research* **2**: 177-181.

Mouzan M (1995). chronic diarrhea in children: Part I. Physiology, pathophysiology, etiology. *Saudi Journal of Gastroenterology* **1**: 37-42.

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

Navaneethan U and Giannella R (2010). Diarrhea Diagnostic and therapeutic advances. Chapter1 in: Guandalini S. and Vaziri H.editors, e-ISBN 978-1-60761-183-7.*Springer science+Busines media* p 1-16

Nguyen TV, Van PL, Huy CL *et al* (2006). Etiology and epidemiology of diarrhea in children in Hanoi, Vietnam. *International Journal of Infectious Diseases* **10**: 298—308.

OECD (2008). Guidelines for Testing of Chemicals: Guideline 425: Acute Oral Toxicity. Paris, France. The Organization of Economic Co-operation and Development.

Retrieved from http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-ofchemicals-section-4-health-effe.

Ogur R (2014). Studies with *Myrtus communis* L: Anticancer properties. *Journal of Intercultural Ethnopharmacology* **3**:4.

Okpo SO, Ching FP, Ekeleme IC (2011). Evaluation of the anti-diarrheal activity of the aqueous extract from leaves of *Pterocarpus santalinoides*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* **2**:590.

Olajuyigbe OO and Afolayan AJ (2012). Ethnobotanical survey of medicinal plants used in the treatment of gastrointestinal disorders in the Eastern Cape Province, South Africa. *Journal of Medicinal Plants Research* **6**: 3415-3424.

Özkan AMG and Güray CG (2009). A Mediterranean *Myrtus communis* L. (Myrtle), Plants and Culture: seeds of the cultural heritage of Europe, Turkey. Retrieved from http://www.uni-graz.at/~katzer/ engl/Myrtcom.html.

Palombo EA (2006). Phytochemicals from traditional medicinal plants used in the treatment of diarrhea: modes of action and effects on intestinal function. *Phytotherapy Research* **20**: 717–724.

Pankaj JP (2006). Treatment of disorders of bowel motility and water flux; antiemetics agents used in biliary and pancreatic diseases. In: L. Laurence, S. John & L. Keith, Goodman and Gilman's the pharmacological basis of therapeutics - 11th edition, *New York: McGraw Hill*, p. 1038.

Parrish CR (2008). Opioid analgesics and the gastrointestinal tract. *Practical Gastroenterology* 2:64.

PATH (2013). Rotavirus disease and vaccines in Ethiopia, vaccine access and delivery global program, a *catalyst for global health*, USA. Retrieved from www.path.org/annual-report/2013/

Pathak K and Das RJ (2013). Herbal Medicine: A Rational Approach in Health Care System, *International Journal of Herbal Medicine* **1**: 86-89.

Pokale S and Kushwaha R (2011). A review on antidiarrheal activity of herbals. *International Journal of Research in Pharmaceutical and Biomedical Science* ***2****:2229-3701.*

Prasad SK, Laloo D, Kumar R, *et al* (2014). Antidiarrheal evaluation of rhizomes of *Crypotcroyine spiralis* Fish.Ex Wydler: antimotility and antisecretory effects. *Indian Journal of Experimental Biology* **52**:139-146.

Primim P, beuno L, baumer P *et al* (1999). racecadotril demonstrates intestinal antisecretory activity *in vivo*. *Ailment pharmacology therapy* **13**:3-7.

Quigley EM (2006). Changing face of irritable bowel syndrome. *World Journal of Gastroenterology* **12**:1–5.

Racusen LC and Binder HJ (1979). Ricinoleic Acid Stimulation of Active Anion Secretion in Colonic Mucosa of the Rat. *Journal of Clinical Investigation* **63**:743-749.

Rahman A, Hasan SN, Sampad KS, *et al* (2011). Antinociceptive, antidiarrheal and cytotoxic activities of *Rhizophora mucronata* Lamk. *Pharmacologyonline* **1**: 921-929.

Rajamanickam V, Rajasekaran A, Anandarajagopal K *et al* (2010). Antidiarrheal activity of *Dodonaea viscosa* root extracts. *International Journal of Pharmaceutical and Biological Sciences* **1**:4

Regassa V, Birke W, Deboch B (2008). environmental determinants of diarrhea in under-five children in Nekemte town, Western Ethiopia. *Ethiopian Journal of Health Sciences* **18**: 2.

Ren A, Zhang W, Thomas HG *et al* (2012). A Tannic acid-based medical food, Cesinex exhibits broad-spectrum antidiarrheal properties: A mechanistic and clinical study. *Digestive Diseases Science* **57**:99–108.

Richard B (2006). Secretion in the small intestine. Retrieved from:- http://www.vivo.colostate.edu/hbooks/pathphys/digestion/smallgut/secretion.html.

Robert A, Nezamis JE, Lancaster C *et al* (1976). Enteropooling assay: a test for diarrhea produced by prostaglandins. *Prostaglandins* **11**:809-828.

Sarri M, Mouyet FZ, Benziane M *et al* (2014). Traditional use of medicinal plants in a city at steppic character (M'sila, Algeria). *Journal of Pharmacy and Pharmacognosy Research* **2**:31- 35.

Saxena M, Saxena J, Nema R *et al* (2013). Phytochemistry of medicinal plants. *Journal of Pharmacognosy and Phytochemistry* **1**:6.

Schiller LR (1999). Secretory diarrhea. *Current Gastroenterology Report* **1**:389.

Schiller LR, Santana CA, Morawski SG *et al* (1984) Mechanism of the antidiarrheal effect of loperamide, *Gastroenterology*, 86: 1475–1480.

Schultz BD, Singh AK, Devor DC *et al* (1999). Pharmacology of CFTR chloride channel activity. *Physiological Reviews* **79**:109–144.

Selimović BM, Babić T, Stojanović P (2012). Bacterial Etiology of Diarrheal Syndrome. *Scientific Journal of the Faculty of Medicine* **27**:55-62.

Shah AJ, Bhulani NN, Khan SH *et al* (2010). Calcium channel blocking activity of *Mentha longifolia* L. explains its medicinal use in diarrhea and gut spasm. *Phytotherapy Research* **24**:1392–1397.

Shah S (2004). Evaluation of diarrhea: The challenge continues! Part-I. *Indian Journal of Medical Sciences* **58**:75-78.

Shiferie F and Shibeshi W (2013). *In vivo* antidiarrheal and *ex-vivo* spasmolytic activities of the aqueous extract of the roots of *Echinops kebericho* (Asteraceae) in rodents and isolated guinea-pig ileum. *International Journal of Pharmacy and Pharmacology* **2**: 110-116.

Shindano A, Marot L & Geubel AP (2007). Nifuroxazide-induced acute pancreatitis: a new side-effect for an old drug. *Acta Gastroenterology Belg* **70:**32-3.

Stein A, Voigt W, Jordan K (2010).Chemotherapy-induced diarrhea: pathophysiology, frequency and guideline-based management. *Therapeutic Advances in Medical Oncology* **2**: 51-63.

Sulaiman GM, Abd-ul-Wahed HE, Abd-ul-Wahab AI *et al* (2013). Antimicrobial Activity of Silver nanoparticles synthesized by *Myrtus communis* extract. *Journal of Engineering and Technology* **31**:3.

Sumbul S, Ahmad M A, Asif M, *et al* (2010). Evaluation of *Myrtus commun*is L berries (common myrtle) in experimental ulcer models in rats. *Human Experimental Toxicology* **29**:935-944.

Sumbul S, Ahmad MA, Asif M (2011). *Myrtus communis* Linn: A review. *Indian Journal of Natural Products and Resources* **2**:395-402.

Tadesse M and Demissew S (1992) Medicinal Ethiopian Plants: Inventory, identification and classification. In: Sue Edwards and Zemede Asfaw, (Eds.). Plants used in African Traditional Medicine as Practiced in Ethiopia and Uganda, Botany 2000: *East and Central Africa. NAPRECA Monograph* **5**: 1-24.

Tadesse M and Mesfin B (2010).A review of selected plants used in the maintenance of health and wellness in Ethiopia, *Ethiopian Journal for Research and Innovation Foresight* **2**: 85-102.

Tadesse WT, Hailu AE, Gurmu AE *et al* (2014). Experimental assessment of antidiarrheal and antisecretory activity of 80% methanolic leaf extract of *Zehneria scabra* in mice. *BMC Complementary and Alternative Medicine* **14**:460.

Talley NJ, Weaver AL, Zinsmeister AR (1994). Self-reported diarrhea:what does it mean? *American Journal of Gastroenterology* **89**: 1160–1164.

Tamiso A, Yitayal M & Awoke A (2014). Prevalence and determinants of childhood diarrhea among graduated households, in rural area of Shebedino district, Southern Ethiopia, 2013. *Science Journal of Public Health* **2**: 243-251.

Teklehaymanot T and Giday M (2007). Ethno-botanical study of medicinal plants used in Zegie Peninsula, Northwestern Ethiopia. *Journal of Ethnobiology and Ethnomedicine* **3**: 12.

Thiagarajah JR, Donowitz M, Verkman AS(2015).Secretory diarrhea: mechanisms and emerging therapies. *National Reviews of Gastroenterology and Hepatology* **12**: 446–450.

Thomas PD, Forbes A, Green J *et al* (2003). Guidelines for the investigation of chronic diarrhea. *Gut* **52**: 1–15.

Tiwari S (2008).Plants:A Rich Source of Herbal Medicine. *Journal of Natural Products* **1**:27-35.

Tormo R, Polanco I, Lindo ES *et al* (2008). Acute infectious diarrhea in children: new insights in antisecretory treatment with Racecadotril. *Acta Paediatrica* **97**: 1008–1015.

Tradtrantip L, Namkung W, and Verkman AS (2010).Crofelemer, an antisecretory antidiarrheal proanthocyanidin oligomer extracted from Croton lechleri, targets two distinct intestinal chloride channels. *Molecular Pharmacology* **77**:69–78, 2010.

Tunaru S, Althoff TF, Nüsing RM *et al* (2012). Castor oil induces laxation and uterus contraction *via* ricinoleic acid activating prostaglandin EP_3 receptors. *Proceedings the National Academy of Sciences* **109**: 9179–9184.

Turvill JL and Farthing MJ (1997). Enkephalins and enkephalinase inhibitors in intestinal fluid and electrolyte transport. *European Journal of Gastrohepatology* **9**:877-80.

Twaij H and El-Jalil HA (2009). Evaluation of Narcotic (Opioid like) analgesic activities of medicinal plants. *European Journal of Scientific Research* **33**:179-182.

Umer S, Tekewe A, Kebede N (2013). Antidiarrheal and antimicrobial activity of *Calpurnia aurea* leaf extract. *BMC Complementary and Alternative Medicine* **13**:21.

UNICEF (2012) Pneumonia and Diarrhoea: Tackling the Deadliest Diseases for the World's Poorest Children [Online] Available At:

http://www.unicef.org/eapro/Pneumonia_and_Diarrhoea_Report_2012.pdf[Accessed 8 October 2014].

UNICEF (2014). Committing to Child Survival: A Promise Renewed. Progress Report 2014. Retrievedfrom http://files.unicef.org/publications/files/APR_2015_web_15Sept14.pdf

Vrese M and Marteau PR (2007). Probiotics and Prebiotics: Effects on diarrhea. *The Journal of Nutrition* **137**:803-811.

Walle M, Walle B, Zerihun L *et al* (2014). Sedative-hypnotic like effect of the essential oil from the leaves of *Myrtus communis* on mice. *American Journal of Biomedical and Life Sciences* **2**:70-77.

WGO (2008). acute diarrhea, World Gastroenterology Organization practice guideline. Retrieved from http://www.worldgastroenterology.org/guidelines/global-guidelines/acute-diarrhea.

WGO (2012). Acute diarrhea in adults and children: a global perspective, *World Gastroenterology Organization Global Guidelines*. [Online] Available at: http://www.worldgastroenterology.org/UserFiles/file/guidelines/acute-diarrhea-english-2012.pdf[Accseed 5 June 2015].

WHO (1975). Hazard classification-acute LD50 values of formulated products. In: The guidebook to the registration of public health pesticides and repellents against Vectors. Retrivied from http://app.nea.gov.sg/cms/htdocs/article.asp?pid=1211.

WHO (2005). The Treatment of diarrhea: a manual for physicians and other senior health. 4th ed. Geneva: *WHO Library Cataloguing*. [Online] Available at: http://whqlibdoc.who.int/publications/2005/9241593180.pdf [Accessed 10 Sept 2014]

WHO and UNICEF (2013). End preventable Child Deaths from Penumonia and Diarrhea by 2025: Global Action Plan for Prevention and Control of Pneumonia and Diarrhoea, Geneva: WHO Library Cataloguing. [Online]Available at: www.who.int/iris/bitstream/10665/79200/1/9789241505239eng.pdf [Accessed 7 July 2015].

Wongsamitkul N, Sirianant L Muanprasat C (2010). Plant-derived hydrolysable tannin inhibits CFTR chloride channel: A potential treatment of diarrhea. *Pharmaceutical Research* **27**: 490-497

WHO (2013). Diarrheal disease. [Online] Available at: http://www.who.int/mediacentre/factsheets/fs330/en. [Accessed October 8, 2014].

Yadav AK and Tangpu V (2007). Antidiarrheal Activity of *Lithocarpus dealbata* and *Urena lobata* extracts: Therapeutic implications. *Pharmaceutical Biology* **45**: 223–229.

Yasmeen M, Prabhu B, Agashikar NV (2010). Evaluation of the antidiarrheal activity of the leaves of *Ixora coccinea* Linn in rats. *Journal of Clinical Diagnosis Research* **4**: 3298-3303.

Yoshimura M, Amakura Y, Tokuhara M *et al* (2008). Polyphenolic compounds isolated from the leaves of *Myrtus communis*. *Journal of Natural Medicine* **62**:366–368.

Zanetti S, Cannas S, Molicotti P, *et al* (2010). Evaluation of the antimicrobial properties of the essential oil of *Myrtus communis* L. against clinical strains of *Mycobacterium* spp. *Inter disciplinary Perspectives of Infectious Diseases* **1**: 1-3.

Zavala-Mendoza D, Alarcon-Aguilar FJ, Perez-Gutierrez S, *et al* (2013). Composition and antidiarrheal activity of *Bidens odorata* Cav. *Evidence Based Complementary and Alternative* Medicine **1**:1-7.

Zohra SF, Meriem B, Samira S *et al* (2012). Phytochemical Screening and identification of some compounds from Mallow. *Journal of Natural Products and Plant Resources* **2**:512-516.