Evaluation of the anti-diarrheal activity of 80% methanol extract and solvent fractions of the leaves of *Lantana camara Linn* (Verbenaceae) in mice

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A Thesis submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences in partial fulfillment of the requirement for the Degree of Master of Science in Pharmacology.

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This is to certify that the thesis prepared by Getnet Mengistu, entitled "Evaluation of the anti-diarrheal activity of 80% methanol extract and solvent fractions of the leaves of *Lantana camara Linn* (Verbenaceae) in mice" and submitted in partial fulfillment for the requirements of the Degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Evaluation of the anti-diarrheal activity of 80% methanol extract and solvent fractions of the leaves of *Lantana camara* L. (Verbenaceae) in mice

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Addis Ababa University, 2015

Lantana camara L. is one of the medicinal plants traditionally used for the treatment of diarrhea in Ethiopia. Although the antidiarrheal activity of the crude extract of the leaves of this plant was evaluated elsewhere, no work has been performed in the Ethiopian plant. The aim of this study was therefore to further confirm the antidiarrheal activity of 80% methanol extract and fully evaluate the solvent fractions using mice model of diarrhea. The 80% methanol extract was prepared by maceration and the fractions were obtained by successive soxhlet extraction with chloroform and methanol followed by maceration of the marc with water. The antidiarrheal activity of the extract and fractions were investigated using castor oil induced diarrhea (for 80% methanol extract), enteropooling and small intestine transit models (the three models for the fractions). The test groups received various doses (100, 200 and 400 mg/kg) of the extract or fractions, whereas positive controls received Loperamide (3 mg/kg) and negative controls received vehicle (2% tween 80 or distilled water, 10 ml/kg). In the castor oil induced diarrheal model, the 80% methanol extract delayed onset of defecation, at 200 mg/kg and 400 mg/kg, and reduced the number and weight of feces at all tested doses significantly as compared to the negative control. In this model the methanol and aqueous fraction at all tested doses and chloroform fraction at 200 mg/kg and 400 mg/kg significantly reduced the number and weight of wet feces when compared with negative control. In the enteropooling test, the methanol and aqueous fractions significantly reduced the weight and volume of intestinal fluid at all tested

doses, whereas the chloroform fraction significantly reduced the weight of intestinal content only at 400 mg/kg when compared to negative control. Results from the charcoal meal test revealed that all the fractions produced a significant anti-motility effect at all tested doses as compared to negative control. This study confirmed the antidiarrheal activity of the crude extract and further revealed all the three fractions possessed varying degree of antidiarrheal activity, with the methanol fraction being the most active fraction in all the three models.

Key words: antidiarrheal activity, castor oil induced diarrhea, anti-enteropooling, gastrointestinal transit, *Lantana camara*

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This thesis is dedicated to my lovely sister **Kidist Mengistu** who passed away when I was first year postgraduate student.

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LIST OF ACRONYMS

AAD	Antibiotic Associated Diarrhea
ADI	Anti-Diarrheal Index
ANOVA	Analysis of Variance
ASA	Amino Salicylic Acid
CAMP	Cyclic Adenosine Monophosphate
CaCC	Calcium Activated Chloride Channel
CDC	Center for Disease Control
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CGMP	Cyclic Guanosine Monophosphate
CSA	Central Statistical Agency
DDC	Diarrhea Disease Control Programme
GIT	Gastrointestinal Tract
HAART	Highly Active Anti-Retroviral Therapy
HIV	Human Immunodeficiency Virus
IBD	Irritable Bowel Disease
NO	Nitric Oxide
ORT	Oral Rehydration Therapy
PI	Peristaltic Index
SEM	Standard Error of the Mean
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 of diarrhea

The term diarrhea is derived from the Greek language (*dia* = through, *rhein* = to flow), denoting increased fluidity and frequency of fecal discharges (EI Mouzan, 1995). The British Society of Gastroenterology defines diarrhea as the abnormal passage of loose or liquid stools more than three times daily and/or a volume of stool greater than 200 g/day in adults (Thomas *et al.,* 2003). Since it is difficult to quantitate consistency of the stool and the weight is affected by the type of food consumed (e.g. excess fiber have stool weights of 300 g/day or more with normal consistency), a combination of frequency, stool consistency and stool weight should be taken into account for defining diarrhea (Guerrant *et al*., 2001; Talley *et al.,* 1994)

Based on duration of symptoms, diarrhea can be classified into acute, persistent and chronic diarrhea. Diarrhea lasting less than 2 weeks is considered acute. It is typically self-limiting and resolves quickly with no lasting sequelae. Persistent diarrhea starts suddenly as acute diarrhea with duration varying from 2 to 4 weeks, On the other hand, chronic diarrhea lasts longer than four weeks (Bliss *et al.,* 2006; Hall, 2010).

1.2. Epidemiology of diarrheal disease

Eventhough diarrhea is a preventable and treatable disorder, it remains the second leading cause of mortality among children under five years of age next to respiratory infections and kills more young children than AIDS, malaria and measles combined (Liu *et al*., 2012; WHO, 2013). According to the estimates of child mortality issued by the United Nations Children's Fund (UNICEF), pneumonia and diarrhea continue to be the leading killers of children under the age of five worldwide. Pneumonia and diarrhea are responsible for 15% and 9% of global child deaths, respectively, together claiming the lives of more than 1.5 million under-five children in 2013. These

diseases predominantly affect developing countries and of all child deaths from diarrhea, 78% occur in the African and South-East Asian regions (Liu *et al.,* 2012; Pneumonia and Diarrhea Progress Report, 2014; UNICEF, 2013a, b; WGO, 2012).

Ethiopia's pneumonia and diarrhea mortality rate is the fifth highest in the world next to India, Nigeria, Pakistan and the Democratic Republic of Congo (Pneumonia and Diarrhea Progress Report, 2014). Diarrhea is the second leading cause of death across all ages next to lower respiratory infections and the two-week prevalence of diarrhea among children under five years of age was 13% in Ethiopia (CDC, 2013; CSA, 2011). The Federal ministry of health (2012) and several community-based studies have shown that diarrheal disease is a major public health problem that causes morbidity and mortality in children (Mengistie *et al*., 2013; Mohammed and Tamiru, 2014; Tamiso *et al.*, 2014).

1.3. Etiology of diarrhea

Diarrhea is usually a symptom of diseases in the intestinal tract which can be caused by a variety of bacterial (*Escherichia coli*, *Vibrio cholerae*, Shigella species etc.), viral (Rota virus, Norovirus, Cytomegalovirus etc.) and parasitic organisms (protozoa and helminthes) (WGO, 2008). Rotavirus and *E. coli* are the two most common etiological agents of diarrhea in developing countries (WHO, 2013). Cryptosporidium has been the most frequently isolated protozoan pathogen among children seen at health facilities and frequently found among human immunodeficiency virus (HIV) positive patients (WHO and UNICEF, 2009).

Drugs which can affect the normal flora in the gastro intestinal tract (GIT) can cause diarrhea which is called antibiotic associated diarrhea (AAD). The mechanisms by which antibiotics lead to AAD include disturbance of the composition and function of

the normal intestinal flora, overgrowth of pathogenic microorganisms and allergic and toxic effects of antibiotics on intestinal mucosa or pharmacologic effects on motility (Hogenauer *et al*., 1998; Wistrom *et al*., 2001). In addition, diarrhea may be associated with highly active anti-retroviral therapy (HAART), which may be caused by a variety of mechanisms, including increased calcium dependent chloride conductance, cellular apoptosis, necrosis and decreased proliferation of intestinal epithelial cells (MacArthur and DuPont, 2012).

1.4. Normal intestinal physiology and pathophysiology of diarrhea

The normal alimentary tract receives, digests and absorbs large amounts of nutrients, water and electrolytes. In adults, about two liters of liquid are ingested and six to seven liters of salivary, gastric, biliary and intestinal fluids are secreted daily. Since only 100 to 200 ml of water is present in stool each day, more than 95% of fluid is efficiently absorbed by the GIT (Andres, 1988; Debongnie and Phillips, 1978).

There is a constant bidirectional flux of water and ions across the small intestinal mucosa, i.e., absorption and secretion. In the intestine, solute movement creates the osmotic force for fluid movement. For instance, sodium absorption drives fluid reabsorption and active chloride secretion contributes to water secretion (Binder, 2005; Binder and Reuben, 2005). Small intestinal sodium absorption is mediated primarily by two mechanisms: a glucose or amino acid stimulated co-transport in which Na accompanies the other solute and a coupled Na^{+}–Cl^{-} mechanism. The latter is a combination of Na^{+}–H+ exchange and Cl–HCO_3 exchange. Short-chain fatty acid mediated Na absorption and aldosterone sensitive Na absorption occurs in the colon (Binder, 2005; Field, 2003).

Among the various mechanisms described, the coupled Na^{+}–Cl^{-} pathways are primarily regulated by cAMP, cGMP and intracellular Ca^{++} levels which facilitate the phosphorylation and opening of the cystic fibrosis transmembrane conductance regulator (CFTR) and calcium activated chloride channel (CaCC) via cAMP, cGMP and Ca^{++} dependent protein kinases, resulting in secretion of chloride ions into the lumen (Field, 2003; Luo *et al.,* 1998;). Accumulation of negatively charged chloride anions in the crypt cells 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) In addition to the transporters, there are multiple extracellular factors regulating epithelial ion transport; paracrine, immunological, neural, and endocrine factors, termed together as a single regulatory system (Mourad *et al.*, 1995).

In addition to the absorptive and secretary function of the intestine, motor functions also play a key role in facilitating digestion and absorption of fluids and nutrients. Synchronized migrating motor complexes normally occur during fasting in the stomach and small bowel with increased contractions following feeding with the total small bowel transit time of approximately 3 h for the food to reach the colon (Kerlin *et al*., 1982). In the colon, there is further reabsorption with the ascending and transverse colon serving as reservoirs and with the sigmoid and rectum serving as volitional reservoirs (Devroede and Phillips, 1969). Any disturbance in the coordinated flux of water and ions and motility can result in the clinical syndrome of diarrhea.

Four general pathophysiologic mechanisms disrupting water and electrolyte balances are the basis of diagnosis and therapy of diarrhea. These are change in active ion transport by decreased sodium absorption or increased chloride secretion, change in

intestinal motility, increase in luminal osmolarity and increase in tissue hydrostatic pressure. These mechanisms have been related to four broad clinical diarrheal groups: secretory, osmotic, exudative/inflammatory and altered intestinal transit diarrhea (Spruill and Wade, 2008).

Secretory diarrhea is caused by an increase in water and electrolytes (Chloride or bicarbonate) movements to the intestinal lumen, the final effect is the increase of secretion and decrease of absorption of net sodium and water (Navaneethan and Giannella, 2010). Typically, infectious agents are the cause of secretory diarrhea but any substance that causes fluid to be pulled into the bowel can be the cause. Most causes of secretory diarrhea alter the second messenger system through alteration in cAMP, cGMP or intracellular Ca^{++} regulated ion transport pathways and alterations in these mediators cause CFTR or CaCC-mediated Cl secretion and inhibition of small intestinal coupled Na^{+}-Cl- transport. Paracellularly, sodium follows the chloride to maintain charge balance and water escapes from the cells to maintain osmotic balance. This efflux of water and electrolytes is manifested as watery diarrhea (Barrett, 2000; Strasinger and Di Lorenzo, 2008).

Osmotic diarrhea occurs when there is a dysfunction in the ability of the intestine to reabsorb fluid as it flows through the lumen. This may be caused by incomplete breakdown or malabsorption of nutrients in the small intestine allowing a larger and more liquid mass to enter the colon. This fecal matter then creates a negative osmotic gradient causing leakage of more fluid into the gut increasing the stool volume. This type of diarrhea can be caused by decreased enzymatic availability (lactose intolerance), a genetic abnormality that decreases or eliminates the ability of the body to absorb certain nutrients, sugars that are poorly absorbed (sorbitol, mannitol or lactose), laxatives, magnesium containing antacids and antibiotic administration as

well as malabsorption of certain fat. Other causes have more to do with changes within the bowel that decrease the ability to reabsorb fluid and nutrients as the stool is propelled through the lumen which includes malnutrition, resection of parts of the bowel and inflammation of the bowel due to infection or disease processes (Bliss *et al.* 2006; Kent and Banks, 2010; Strasinger and Di Lorenzo, 2008).

When the intestines are not functioning normally, motility can be either increased or decreased and both can lead to diarrhea. Increased motility results in faster transport of stool through the bowel so there is less chance for re-absorption of fluid from the large intestine. Counter-intuitively, decreased motility can also lead to diarrhea. Typically, decreased mobility will lead to constipation, which, in its most severe form, can allow a large bolus of stool to form in the lower intestine and cause an impaction. The stool behind this bolus may become liquid again due to the action of bacteria on the stool. This results in liquid stool leaking around the bolus and causing diarrhea (Bliss *et al*., 2006; Field, 2003)

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

1.5. Management of diarrhea

The aim of diarrhea treatment is to prevent or reverse dehydration, shorten the duration of the illness and to reduce the period that a person is infectious (Grimwood and Forbes; 2009).

1.5.1. Non-pharmacological management

Fluid and electrolytes

Fluid replacement is not a treatment to relieve diarrhea but rather an attempt to restore fluid balance. Oral rehydration therapy (ORT) is the administration of appropriate solutions by mouth to prevent or correct diarrheal dehydration. It is a cost-effective method of managing acute gastroenteritis and it reduces hospitalization requirements in both developed and developing countries (WGO, 2012). Patients with diarrhea who are not dehydrated may replace fluid by drinking flat soft drinks such as ginger, ale, tea, fruit juice, broth or soup. Severe diarrhea may require the use of parenteral solutions such as ringer lactate or normal saline solution to replace large and life threatening fluid losses (Beverly and Clarence, 2008). The absorption of glucose molecules which is found in the ORT is not affected by diarrhea and this further increases the absorption of Na^+ through Na^+- glucose co-transport mechanism and water follows Na^+ down its concentration gradient (Goodall, 2014).

Probiotics and prebiotics

Probiotics are live microorganisms which can prevent or ameliorate diarrhea through receptor competition, competition for nutrients, inhibition of epithelial and mucosal adherence of pathogens, introduction of lower colonic pH favoring the growth of nonpathogenic species, stimulation of immunity or production of antimicrobial substances, when administered in adequate amounts (Allen *et al*., 2010; FAO/WHO; 2002; Rolfe, 2000). Commonly used strains include lactobacilli, bifido bacteria and saccharomyces. A systematic review and meta-analysis study suggests that probiotics are associated with a reduction in AAD and *C. difficile* diarrhea (Allen *et al*., 2013; Hempel *et al*., 2012).

Prebiotics are non-digestible food ingredients that may benefit the host by selectively stimulating bacteria in the colon that confer health benefits to the host. The most commonly used prebiotics are inulin-type fructans (inulin, oligofructose, fructo-oligosaccharides) (Roberfroid *et al.,* 2010). Synbiotics refer to preparations in which probiotic organisms and prebiotics are combined (Hempel *et al.,* 2012).

Zinc supplement

Diarrhea is a commonly associated problem in children with zinc deficiency and it also leads to excess zinc losses. Clinical studies have shown that a 10 to 14 day treatment course with zinc effectively reduces the duration and severity of both persistent and acute diarrhea (Fischer-Walker *et al.,* 2009; Patel *et al*., 2010). Zinc is found to be safe in HIV infected children and also known to reduce morbidity in these children (Shimelis *et al*., 2008). It is critical for overall health, growth, development and supports proper functioning of the immune system, increase ORS uptake and reduces inappropriate drug use (Bhandari, 2008; Bhutta *et al.,* 2000).

1.5.2. Conventional drugs

The main problems that cause diarrhea are over secretion of fluid and electrolyte into the intestinal lumen, decrease absorption of fluid or altered motility. So drugs are used to correct these problems and include the following.

Antisecretary agents

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

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

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

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

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

Antimotility agents

These drugs prolong intestinal transit time; thereby reducing the amount of fluid lost in the stool. Loperamide and diphenoxylate with atropine sulfate have been shown to slow transit time within the intestine to permit more re-absorption of fluid. They act by intestinal μ opiate receptors, leading to increased intestinal transit time (Kent and Banks, 2010). In addition these drugs have antisecretary action in the human Jejunum (De Luca and Coupar, 1993; Hughes *et al.,* 1984).

Anti-infectives

According to the infectious diseases society of America's guidelines for the management of acute infectious diarrhea, empirical antibiotics are commonly recommended without obtaining a fecal specimen in patients with travelers' diarrhea or febrile dysenteric illness, especially those believed to have moderate-to-severe invasive diseases. Fluoroquinolones are recommended as the first drugs of choice for the empirical treatment of adult patients. But nowadays, due to development of resistance they are not widely used (Guerrant *et al*., 2001; Yang *et al*., 2008). Rifaximin is a rifamycin-based, nearly non-absorbable antibiotic with an excellent safety profile. It was approved in Italy in 1987 and in the United States in 2004 for the treatment of several GI diseases, particularly acute infectious diarrhea such as travelers' diarrhea secondary to noninvasive *E. coli* (Koo and Dupont, 2010; Ojetti *et al*., 2009).

Anti-inflammatory agents

There are few specific treatments of irritable bowel disorder (IBD) associated diarrhea, in part because of the complexity and limited understanding of mechanisms (Urayama and Chang, 1997). Most of the currently used preparations have actions that inhibit various steps of the inflammatory cascades. The common antinflammatory

agents for the management of IBD are derivatives of 5-aminosalicylic acid (5-ASA) which act by inhibiting arachidonic acid metabolism, decreasing the synthesis of both leukotrienes and prostaglandins. Moreover, 5-ASA is a potent scavenger of free radicals (Dhaneshwar, 2014; Vermeire *et al*., 2012).

1.5.3. Traditional medicine

Despite the availability of vast spectrum of approaches for diarrheal management, a vast majority of the people in developing countries rely on herbal drugs for the management of diarrhea. WHO has encouraged studies for treatment and prevention of diarrheal diseases using traditional medical practices and constituted a diarrhea disease control programme (DDC), which includes studies of traditional medicine practices together with the evaluation of health education and prevention approaches (Akuodor, 2011; Damiki and Siva, 2011; WHO, 2004).

There are many herbal plants available throughout the world that possesses anti-diarrheal activity with lesser side effect than the conventional drugs. On top of this, different studies suggested that the plants showed anti-diarrheal activity by reducing intestinal motility and secretion. Furthermore, tannins, alkaloids, flavonoids and terpenoids are the main chemical constituents which are responsible for the anti-diarrheal activity of plants (Komal *et al*., 2013). Ethnobotanical studies indicate a range of medicinal plants such as the bark extract of *Ficus vasta*; leaf extract of *Calpurnia aurea, Croton macrostachyus Del, Artemisia afra Jacq*; fresh juice of *Citrus lemon*, seed extract of *Coffea Arabica* and *Lepidium sativum*; root extract of *Caylusea abyssinica* have been widely used for the management of diarrhea by traditional healers in Ethiopia (Birhanu, 2013; Mesfin *et al*., 2013; Teklehaymanot and Giday, 2007).

1.6. The experimental plant

Lantana Camara L. belongs to Kingdom - *Planate*; Subkingdom - *Tracheobionta*; Superdivision - *Spermatophyta*; Division - *Magnoliophyta*; Class - Magnoliopsida; Subclass - *Asteridae*; Order - *Lamiales*; Family - Verbenaceae and Genus - *Lantana* (Sangeetha *et al*., 2015). The family Verbenaceae comprises 100 genus and about 2600 species distributed in tropical and subtropical regions around the world. The genus Lantana as described by Linnaeus in 1753 contained seven species; six from South America and one from Ethiopia (Munir, 1996).

Lantana camara L. is the most widespread species of this genus, growing luxuriantly at elevations up to 2000 m in tropical, sub-tropical and temperate regions (Kalita *et al.*, 2012). It is a medium sized aromatic shrub with tetrangular stem. The plant grows up to 1 to 3 meters and it can spread to 2.5 meter in width. The leaves are generally oval or broadly lance shaped and has a yellow green to green color. Leaves and stem are covered with rough hairs (Sangeetha *et al*., 2015). Flower heads contain 20-40 flowers; the color varies from white, cream, yellow, orange pink, purple and red. Flowering occurs all around the year if adequate moisture and light are available (Lonare *et al*., 2012). The fruits are produced in clusters, green in color and turn black when ripe. Root system is very strong and it gives out new fresh shoots even after repeated cuttings (Kalita *et al.*, 2012).

L.camara (Figure 1) is known by several common names such as black sage, cuasquito, angel lip, flowered sage, shrub verbena, white sage and wild sage all over the world (Saraf *et al*., 2011). In Ethiopia, it has a vernacular name of michi-charo (Sheko ethnic group), Enaro (Maale ethnic communities) and Yewef kollo (Gedeoffa and Amharic language) (Giday *et al*., 2010; Kidane *et al.*, 2014; Mesfin *et al*., 2009).

Figure 1. Photograph of *Lantana camara*

L.camara had been reported to be toxic to grazing animals such as cattle, sheep and goats. The toxic effects have usually been attributed to a series of pentacyclic triterpenes, in which lantadenes A and B are typical members (Sharma *et al*., 1988).

Despite its toxicity, various parts of this plant have been used in traditional medicine for treatment of different diseases in different countries. For instance, in Asian countries, leaves were used to treat cuts, rheumatisms, ulcers and as a vermifuge. Decoctions of leaves were also applied externally for the treatment of leprosy and scabies (Ghisalberti, 2000). In Tamil nadu, India, leaf juice is used to treat dysentery (Johnsy *et al.,* 2013) and leaves are boiled in water and drunk two times a day for a week to treat malaria (Kareru *et al.,* 2007). In Central and South America, the leaves were used to treat sores, chicken pox and measles (Hidayat *et al*., 2011) and diarrhea (Hernández *et al*., 2003). The flower extracts were also used in folk medicine for the

management of several disorders including cancer, asthma, tumors, bilious fevers, chicken pox, eczema, measles, ulcers, swellings, high blood pressure, catarrhal infections, rheumatism, tetanus and malaria (Madiha et al., 2013).

In Ethiopia, the fresh leaves of the plant which is prepared by crushing and rubbing, was used for the treatment of skin infection. In addition, the fresh or dried roots of the plant are also used for the treatment of gonorrhea (Lulekal *et al*., 2008). It's leaves, applied locally on the face, are used to treat febrile illness (Giday *et al*., 2010). It has been also used for the treatment of diarrhea in Wonago Woreda, Southern Nations Nationalities and Peoples Regional State (Mesfin *et al.*, 2009) and Peoples in Libo-Kemkem District, South Gondar (Addisie *et al*., 2011).

L.camara has been also extensively studied for its medicinal properties by scientific methods. Ethnopharmacological studies revealed that the leaves of *L. camara* have been proven to have activities including antibacterial (Agrawal *et al.*, 2012; Saikia and Sahoo, 2011; Salada *et al*., 2015), antifungal (Passos *et al*., 2012), antimycobacterial (Kirimuhuzya *et al*., 2009), antithelmentic (Jitendra *et al*., 2011), antioxidant (Rabia and Asghari, 2013), wound healing (Mahmood *et al*., 2009), antimotility (Sagar *et al*., 2005), antinociceptive and anti-inflammatory (Silva *et al.*, 2015), antimalarial (Gabi *et al*., 2011), antiulcerogenic (Sathish *et al*., 2011), anxiolytic (Kazmi *et al*., 2013) and anti-leishmaniasis (Rachel *et al*., 2012) activities. Apart from this, the stem, stem bark, flower and aerial parts extract were proved to have antifilarial, antidiarrheal, antiviral and antiallergic activities, respectively (Kanagavalli *et al*., 2011; Mahajan *et al*., 2014; Namita *et al*., 2007; Tadesse, 2015). Furthermore chemical investigation of extracts from the leaves of *L.camara* showed the presence of saponins, carbohydrates, tannins, flavonoids, steroids and terpenoids (Kazmi *et al*., 2013; Mahajan *et al*., 2014; Sathish *et al*., 2011). In addition, different

compounds including verbascoside, essential oils, ursolic acid, stearoyl glucoside and a thrombin inhibitor were isolated (Herbert *et al*., 1991; Kazmi *et al*., 2012; Saikia and Sahoo, 2011).

1.7. Rationale for the study

Despite the availability of many drugs for treating diarrhea, majority of them suffer from adverse effects like induction of bronchospasm and vomiting by Racecadotril; and intestinal obstruction and constipation by loperamide (Pankaj, 2006). ORT has been the mainstay of treatment of diarrhea; however it does not reduce the frequency of stools or the number of diarrheal days. This treatment often fails in the high stool output state (Singh and Narayan, 2008). Moreover, there is an increasing threat of drug resistance, side effects, superinfection and the possibility of induction of disease producing bacteriophages by antibiotics. Due to these problems, WHO encourages studies for the treatment and prevention of diarrheal diseases depending on traditional medical practices (WHO, 2004). There are many plants which are traditionally used for the treatment of diarrhea, among which *L.camara* is one of them. The antidiarrheal activity of the crude extract of the leaves of this plant is reported elsewhere (Pabillaran *et al.*, 2014; Sagar *et al.*, 2005). This study attempted to validate the traditional use of this plant and to further ascertain in which fraction (s) the constituents responsible for antidiarrheal activity are concentrated so as to provide a clue about the nature of the phytochemical constituents responsible for its action and the possible mechanisms of action. In addition, the results of this study help the scientific community to further investigate the plant *L. camara* by initiating advanced studies on molecular mechanisms and formulation of plant source drugs by identifying the specific agent responsible for the antidiarrheal effect.

2. OBJECTIVE

2.1. General objective:

To evaluate the antidiarrheal activities of 80% methanol extract (80ME) and solvent fractions (chloroform, methanol and water) of the leaves of *L camara* in mice

2.2. Specific objectives

- To assess acute toxicity of 80ME of *L.camara* leaves in mice
- To assess acute toxicity of the solvent fractions of *L.camara* leaves in mice
- To evaluate the effect of 80ME of *L.camara* leaves on castor oil induced diarrhea in mice
- To evaluate the effect of solvent fractions of *L.camara* leaves on castor oil induced diarrhea in mice
- To evaluate the effect of the solvent fractions of *L.camara* leaves on the weight and volume of intestinal contents in mice
- To evaluate the effect of the solvent fractions of *L.camara* leaves on small intestine transit in mice
- To determine the phytochemical constituents of the 80ME and solvent fractions of *L.camara* leaves

3. MATERIAL AND METHODS

3.1. Drugs and chemicals

Distilled water (Ethiopian Pharmaceutical Manufacturing Factory, Ethiopia), castor oil (Amman Pharmaceutical Industries, Jordan), activated charcoal and loperamide (Acuro Organics Ltd, New Delhi), methanol and chloroform (Research Lab Fine Industries, India), tween 80 (UNI-CHEM Chemical Reagents, India), glacial acetic acid, ammonia, hydrochloric acid and ferric chloride (BDH Laboratory Supplies Poole, England), acetic anhydride and Mayer's reagent (May and Baker LTD Dagenham, England), and Dragendroff's reagent and sulfuric acid (Fisher Scientific, UK) were used in the study.

3.2. Plant collection

The fresh leaves of *L.camara* were collected from Bishoftu, about 48 km South-east of Addis Ababa, Ethiopia in December 2014. Identification and authentication of the plant specimen was done at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University and a voucher specimen was deposited with voucher number GM 002/2014 for future reference. The leaves were washed gently by rinsing on running water to remove dust particles, air dried under shade for five days and then size reduced into coarse powder with mortar and pestle.

3.3. Experimental animals

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

accordance with the guideline for the care and use of laboratory animals (Institute for Laboratory Animal Research, 1996; OECD, 2008) and approved by the department of pharmacology and clinical pharmacy.

3.4. Preparation of plant extract and fractions

The extraction was performed using cold maceration technique for the 80ME and successive soxhlet extraction followed by maceration for the solvent fractions. One hundred fifty grams of the coarse powder of *L.camara* leaves was weighed by sensitive digital weighing balance (Mettler Toledo, Switzerland). The coarse powder was soaked in a flask containing 80% methanol (1:5 (w/v)) and then placed on a shaker (Bibby Scientific Limited Stone Staffo Reshire, UK) tuned to 120 rpm with occasional shaking for 72 h at room temperature. The extract was then filtered first using gauze and then Whatman grade No 1 filter paper (Schleicher and Schuell Microscience Gmbh, Germany) and the marc was re-macerated for a second and third time by adding another fresh solvent. The filtrates from the three batches were combined and concentrated in a rotary evaporator (Buchi Rota Vapor R-200, Switzerland) with temperature of 40^0C. The concentrated filtrate was then frozen in a refrigerator and dried in a lyophilizer (Operan, Korea Vacuum Limited, Korea). The calculated yield was 18%. The extract was then stored in deep freezer (-20 ^{o}C) until used for the experiment.

For the solvent fractions, two hundred fifty grams of dry powder of the plant material was subjected to successive soxhlet extraction with solvents of differing polarity (chloroform and methanol) followed by maceration with water. Each time 50 g of the powder was extracted with 400 ml of chloroform in the extraction chamber. The extracting solvent was heated until clear liquid contents of the chamber siphoned into

the solvent flask (Agrawal *et al.*, 2012; Sathish *et al*., 2011). The chloroform fraction was filtered with Whatman No.1 filter paper and concentrated using rotary evaporator followed by oven at room temperature for 12 h. The marc was then collected and dried at room temperature to remove chloroform and was then re-extracted using absolute methanol following the same procedure as described before, to get the methanol fraction. The marc of absolute methanol fraction was then collected and dried at room temperature to remove methanol.

Finally, the dried marc was cold macerated in an Erlenmeyer flask with distilled water and allowed to stand at room temperature for a period of 72 h with occasional shaking using mini orbital shaker. It was then filtered using gauze followed by Whatman grade No 1 filter paper. The marc was re-macerated for a second and third time by adding another fresh solvent. The filtrates from the three batches were combined together and left over night in a deep freezer and then dried using lyophilizer. After drying, percentage yield of all fractions was determined. The yields of chloroform, absolute methanol and aqueous fractions were 3.2%, 5.6% and 4.5%, respectively. The fractions were then stored in deep freezer (-20 ^{o}C) until used for the experiment.

3.5. Acute oral toxicity test

Acute oral toxicity test for 80ME and solvent fractions of the leaves of *L.camara* was performed according to the OECD guideline 425 (2008). Five female albino mice of 6-8 weeks were used for each test. All mice were fasted for 4 h before and 2 h after the administration of the extract/fractions. First, a sighting study was performed to determine the starting dose. For this, a single female mouse was given 2000 mg/kg of the extract/fractions as a single dose by oral gavage. Since no death was observed within 24 h, additional four mice were used for each of the extract and fractions, and

administered the same dose of extract/fractions. The animals were observed continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicity, food and water intake and mortality.

3.6. Animal grouping and dosing

In all models, animals were randomly divided into five groups (negative control, positive control and three test groups) comprising of six animals per group. Negative controls received vehicle (10 ml/kg) (2% Tween-80 for 80ME and chloroform fraction; and distilled water for methanol and aqueous fractions); and positive controls received loperamide (3 mg/kg) in all models. The test groups (group 3, 4 and 5) received different doses (100, 200 and 400 mg/kg respectively) of the extract/fractions orally which were determined based on the acute oral toxicity test and pilot study.

3.7. Determination of antidiarrheal activity

3.7.1. Castor oil induced diarrhea

The method described by Awouters *et al* (1978) and Igboeli *et al* (2015) was followed for this study. Swiss albino mice of either sex were fasted for 18 h with free access to water and grouped and treated as described under section 3.6. One hour after dosing, each mouse was given 0.5 ml of castor oil orally for induction of diarrhea and placed individually in cages in which the floor is lined with white paper. The transparent paper was changed every hour for a total of four hours. During the observation period, the onset of diarrhea (the time interval in minutes between the administration of castor oil and the appearance of the first diarrheal stool), number and weight of wet stools, total number and total weight of fecal output were recorded. Finally, percentage of fecal output and diarrheal inhibition were calculated by using the formulas described below.

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

Where, WFC = wet feces in control group and WFT = wet feces in test group

$$\%\ \text{of fecal out put} = \frac{\text{mean fecal weight of each treatment group}}{\text{mean fecal weight of control}} * 100$$

3.7.2. Castor oil induced enteropooling

The effects of the fractions on intraluminal fluid accumulation were determined using the method described by Robert *et al* (1976). Animals were fasted for 18 h, grouped and treated as described under section 3.6. After 1 h of treatment, 0.5 ml of castor oil was administered and animals were sacrificed by cervical dislocation 1 h following castor oil administration. The abdomen of each animal was then opened; the small intestine was ligated at both the pyloric sphincter and the ileo-cecal junction, and dissected. The dissected small intestine was weighed and intestinal contents were then collected by milking into a graduated tube and volume of the contents was measured. Weight of the intestine after milking was taken and the difference between the two weights was then recorded. Finally, percentage of reduction of intestinal secretion (volume and weight) was calculated relative to the negative control using the following formula

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

Where, MVIC – Mean Volume of Intestinal Content
MVICC - Mean Volume of Intestinal Content of Control Group
MVICT - Mean Volume of Intestinal Content of Test Group

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

Where, MWIC – Mean Weight of Intestinal Content
MWICC - Mean Weight of Intestinal Content of Control Group
MWICT - Mean Weight of Intestinal Content of Test Group

3.7.3. Gastrointestinal motility test

Animals were fasted for 18 h with free access to water and divided and treated as described under section 3.6 one hour before administration of 0.5 ml castor oil. One ml of the marker (5% activated charcoal suspension in water) was administered orally 1 h after castor oil treatment. The animals were then sacrificed by cervical dislocation 1 h after charcoal meal and the small intestine was dissected out from pylorus to caecum and placed length wise on a white paper. The distance travelled by the charcoal meal and the total length of the intestine was then measured. The peristaltic index and percentage of inhibition were calculated by using the following formula (Aye-than *et al*., 1989; Igboeli *et al*., 2015).

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

Where, Dc: Mean distance travelled by the charcoal in the control group and
Dt: Mean distance travelled by the charcoal in the test group

$$\text{Perstalstic inedx(PI)} = \frac{\text{Distance traveled by charcoal meal}}{\text{total length of small intestine}} * 100$$

3.7.4. In vivo antidiarrheal index

The *in vivo* antidiarrheal index (ADI) for the positive control and solvent fractions was determined based on the data from the above tests using the formula developed by Aye-Than *et al* (1989).

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

Where, Dfreq is the delay in defecation time as % of negative control
Gmeq is the gut meal travel reduction as % of negative control and
Pfreq is the reduction in the number of stools as % of negative control.

3.8. Preliminary phytochemical screening

The qualitative phytochemical investigations of 80ME and chloroform, methanol, and aqueous fractions of leaves of *L.camara* were carried out using standard tests (Kalita *et al.,* 2011; Sasidharan *et al*., 2011).

Test for terpenoids

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

Test for saponins

To 0.25 g of 80ME and each fraction, 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of froth indicates the presence of saponins.

Test for tannins

About 0.25 g of 80ME and each fraction was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added to the filtrate. A brownish green or a blue-black precipitate indicated the presence of tannins.

Test for flavonoids

About 10 ml of ethyl acetate was added to 0.2 g of 80ME and each fraction, and heated on a water bath for 3 min. The mixture was cooled and filtered. Then, about 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammonial layer indicated the presence of flavonoids.

Test for cardiac glycosides

To 0.25 g of 80ME and each fraction diluted to 5 ml in water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides..

Test for steroids

Two ml of acetic anhydride was added to 0.25 g of 80ME and each fraction with 2 ml sulfuric acid. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for alkaloids

0.5 g of 80ME and each fraction was diluted to 10 ml with acid alcohol, boiled, and filtered. To 5 ml of the filtrate, 2 ml of dilute ammonia and 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Dragendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Dragendorff's reagent) was regarded as positive for the presence of alkaloids

3.9. Data analysis

The data was analyzed by using SPSS16.0 for Windows. Results obtained from the study are expressed as mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used to compare results among groups. Linear regression was used where appropriate. Differences were considered statistically significant if p values are less than 0.05.

4. RESULTS

4.1. Acute oral toxicity test

The acute oral toxicity test of 80ME and solvent fractions of leaves of *L.camara* indicated that neither the 80ME nor the solvent fractions caused gross behavioral changes and mortality within 24 h as well as in the next 14 days, indicating that the LD50 of the extract and fractions were greater than 2000 mg/kg in mice.

4.2. Effects of 80% methanol extract on castor oil induced diarrhea

In the castor oil induced diarrheal model, 80ME of leaves of *L.camara* delayed the onset of defecation at 200 mg/kg ($p<0.05$) and 400 mg/kg ($p<0.001$) as well as reduced the frequency of defecation (number of wet feces and total number of feces) at all tested doses significantly as compared to the negative control. Results from the experiment also revealed that all the tested doses of 80ME significantly ($p<0.001$) reduced the weight of feces (weight of wet feces and weight of total feces) when compared with negative control as depicted in Table 1.

Moreover, there was also a significant difference between the minimum (100 mg/kg) and maximum (400 mg/kg) tested doses of the extract in delaying the onset of defecation and reducing the frequency of defecation. The higher tested dose of 80ME (400 mg/kg) showed the maximum percentage inhibition of defecation and the lowest percentage of mean fecal output when compared with the tested doses of the extract and positive control as shown in Table 1.

Table 1. Effects of 80% methanol leaf extract of *Lantana camara* on castor oil induced diarrhea in mice

Group	onset of diarrhea (min)	# of wet feces	# of total feces	Weight of wet feces	Weight of total feces	% inhibition of defecation	% of fecal output
Control	49.5 ± 1.63	7.5 ± 0.34	8.83 ± 0.40	0.36 ± 0.016	0.41 ± 0.024	-	-
80ME100	79.2 ± 3.70 ^{c3}	4.00 ± 0.36 ^{a3b1c1}	5.67 ± 0.49 ^{a1b1c1}	0.16 ± 0.015 ^{a3}	0.20 ± 0.016 ^{a3}	46.7	48.8
80ME200	124.5 ± 11.85 ^{a1}	3.50 ± 0.22 ^{a3}	4.00 ± 0.52 ^{a3}	0.14 ± 0.006 ^{a3}	0.15 ± 0.011 ^{a3}	53.3	36.6
80ME400	186.0 ± 22.07 ^{a3}	1.83 ± 0.60 ^{a3}	2.67 ± 0.88 ^{a3}	0.09 ± 0.029 ^{a3}	0.11 ± 0.034 ^{a3}	75.6	26.8
Loperamide	138.0 ± 21.66 ^{a2}	2.17 ± 0.48 ^{a3}	2.83 ± 0.70 ^{a3}	0.13 ± 0.027 ^{a3}	0.14 ± 0.030 ^{a3}	71.1	34.1

Data are expressed as mean ± SEM (n=6); analysis was performed with One-Way ANOVA followed by Tukey test; ^{a} compared to negative control; ^{b} compared to loperamide 3mg/kg; ^{c} compared to 400mg/kg; $^{1}p<0.05$, $^{2}p<0.01$, $^{3}p<0.001$; 80ME, 80 % methanol extract; Negative controls received 2% Tween 80 in water.

4.3. Effects of solvent fractions on castor oil induced diarrhea

As shown in Table 2, the chloroform fraction showed a significant reduction in number and weight of wet feces at 200 mg/kg ($p<0.01$) and 400 mg/kg ($p<0.001$) compared to the negative control. The higher dose (400 mg/kg) was the only dose that significantly reduced the number of total feces ($p<0.05$) and weight of total feces ($p<0.001$) compared to the negative control. This fraction didn't, however, show significant effect on delaying the onset of defecation at all tested doses when compared with the negative control.

The methanol fraction showed a significant effect in all parameters (onset of diarrhea, number and weight of feces) measured in this model at all tested doses when compared with the negative control. This fraction showed the maximum percentage inhibition of defecation at 400 mg/kg (64.3 %) when compared with other fractions but the reduction was slightly lower than that of the standard drug (67.2%) as shown in Table 2.

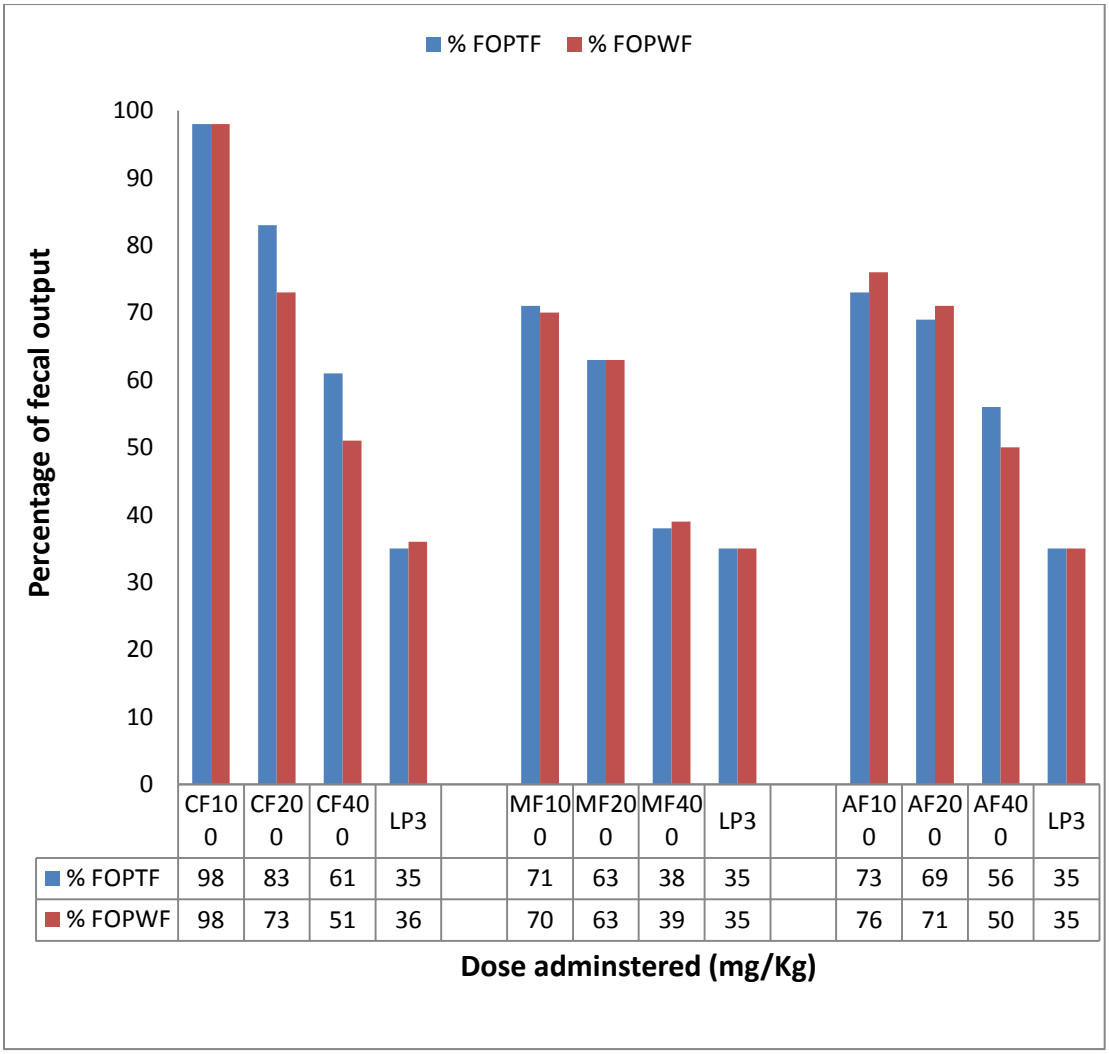
The aqueous fraction significantly reduced the number and weight of feces at all tested doses but significantly prolonged the onset of diarrhea only at 400 mg/kg when compared with the negative control (Table 2).

The effect of solvent fractions on percentage of fecal output of both wet and total feces showed that the methanol fraction had the lowest percentage of fecal output when compared with other fractions of leaves of *L.camara* as shown in Figure 2.

Table 2. Effects of solvent fractions of the leaves of *Lantana camara* on castor oil induced diarrhea in mice

Group	onset of diarrhea (min)	# of wet feces in 4 hr.	# of total feces in 4 hr.	Weight of wet feces	Weight of total feces	% inhibition of defecation
Control	47.8 ± 2.1	9.30 ± 0.62	9.83 ± 0.40	0.45 ± 0.01	0.46 ± 0.01	-
CF100	65.3 ± 4.1 ^{b3}	8.33 ± 0.49 ^{b3d2}	9.50 ± 0.50 ^{b3}	0.44 ± 0.08 ^{b3c2d3}	0.45 ± 0.01 ^{b3d3}	10.4
CF200	77.8 ± 1.9 ^{b2}	6.50 ± 0.22 ^{a2b3}	7.67 ± 0.42^{b3}	0.33 ± 0.07 ^{a2b3d2}	0.38 ± 0.02 ^{b3d1}	30.1
CF400	90.8 ± 2.2 ^{b1}	5.67 ± 0.42 ^{a3b2}	7.17 ± 0.70 ^{a1b3}	0.23 ± 0.10 ^{a3}	0.28 ± 0.01 ^{a3b2}	39.0
Loperamide	144.0 ± 23.0 ^{a3}	2.33 ± 0.67 ^{a3}	2.50 ± 0.76 ^{a3}	0.16 ± 0.38 ^{a3}	0.16 ± 0.04 ^{a3}	74.9
Control	42.17 ± 1.8	11.67 ± 0.67	12.5 ± 0.88	0.46 ± 0.01	0.48 ± 0.01	-
MF100	88.67 ± 2.1 ^{a1b1}	8.00 ± 0.36 ^{a3b3c2d3}	9.17 ± 0.83 ^{a3b3d3}	0.32 ± 0.01 ^{a3b3d3}	0.34 ± 0.01 ^{a3b3d3}	31.4
MF200	101.67 ± 1.4 ^{a2}	6.00 ± 0.36 ^{a3b1}	6.83 ± 0.31 ^{a3}	0.29 ± 0.02 ^{a3b3d3}	0.30 ± 0.01 ^{a3b3d3}	48.6
MF400	122.67 ± 5.6 ^{a3}	4.17 ± 0.31 ^{a3}	4.33 ± 0.33 ^{a3}	0.18 ± 0.01 ^{a3}	0.18 ± 0.01 ^{a3}	64.3
Loperamide	136.83 ± 22.5 ^{a3}	3.83 ± 0.48 ^{a3}	4.50 ± 0.72 ^{a3}	0.16 ± 0.01 ^{a3}	0.17 ± 0.02 ^{a3}	67.2
Control	42.17 ± 1.8	11.67 ± 0.67	12.5 ± 0.88	0.46 ± 0.01	0.48 ± 0.01	-
AF100	70.50 ± 1.7 ^{b2}	8.83 ± 0.17 ^{a2b3c1d3}	9.17 ± 0.40 ^{a1b2}	0.35 ± 0.02 ^{a3b3d3}	0.35 ± 0.02 ^{a3b3d1}	24.3
AF200	81.00 ± 3.0 ^{b2}	6.67 ± 0.42 ^{a3b2}	8.00 ± 0.73 ^{a2b1}	0.33 ± 0.02 ^{a3b3d2}	0.33 ± 0.02 ^{a3b3}	42.8
AF400	96.00 ± 4.6 ^{a1}	5.50 ± 0.43 ^{a3}	7.50 ± 0.62 ^{a3b1}	0.23 ± 0.02 ^{a3b1}	0.27 ± 0.02 ^{a3b2}	52.9
Loperamide	136.83 ± 22.5 ^{a3}	3.83 ± 0.48 ^{a3}	4.50 ± 0.72 ^{a3}	0.16 ± 0.01 ^{a3}	0.17 ± 0.02 ^{a3}	67.2

Data are expressed as mean ± SEM (n=6); analysis was performed with One-Way ANOVA followed by Tukey test; ^{a} compared to negative control; ^{b} compared to loperamide 3mg/kg; ^{c} compared to 200 mg/kg; ^{d} compared to 400 mg/kg; $^{1}p<0.05$, $^{2}p<0.01$, $^{3}p<0.001$; CF, chloroform fraction; MF, methanol fraction; AF, aqueous fraction; Negative controls received 10 ml/kg 2% Tween 80 in water or distilled water.



	CF100	CF200	CF400	LP3		MF100	MF200	MF400	LP3		AF100	AF200	AF400	LP3
% FOPTF	98	83	61	35		71	63	38	35		73	69	56	35
% FOPWF	98	73	51	36		70	63	39	35		76	71	50	35

Figure 2. Percentage of mean fecal output of solvent fractions of the leaves of *Lantana camara*

(CF= chloroform fraction; MF= methanol fraction; AF= aqueous fraction
%FOPTF/%FOPWF = Percentage of fecal output of total/wet feces)

4.4. Effect of solvent fractions on castor oil- induced enteropooling

In the gastrointestinal enteropooling test, the chloroform fraction significantly reduced the weight of intestinal content only at 400 mg/kg ($p<0.05$) but didn't have significant effect on the volume of intestinal fluid at all tested doses. The methanol and aqueous fractions reduced the weight and volume of intestinal fluid at all tested doses significantly when compared to the negative control. The highest effect on both weight and volume of intestinal content was achieved by the methanol fraction at 400 mg/kg when compared with other fractions as shown in Table 3.

Table 3. Effects of solvent fractions of the leaves of *Lantana camara* on castor oil induced enteropooling in mice

Group	Mean weight of small intestinal content (gm)	% of inhibition	Mean volume of small intestinal content(ml)	% of inhibition
Control	0.96 ± 0.060	-	0.77 ± 0.049	-
CF100	0.84 ± 0.043 [b2]	12.5	0.74 ± 0.057 [b1]	3.9
CF200	0.73 ± 0.049	23.9	0.55 ± 0.043	28.6
CF400	0.69 ± 0.044 [a1]	28.1	0.52 ± 0.031	32.5
Loperamide	0.49 ± 0.096 [a3]	49.0	0.32 ± 0.108 [a3]	58.4
Control	1.27 ± 0.086	-	1.03 ± 0.080	-
MF100	0.83 ± .0.039 [a3b1c1]	34.6	0.50 ± 0.037 [a3]	51.4
MF200	0.66 ± 0.051 [a3]	48.0	0.46 ± 0.033 [a3]	55.3
MF400	0.59 ± 0.037 [a3]	57.3	0.33 ± 0.033 [a3]	67.9
Loperamide	0.59 ± 0.038 [a3]	53.5	0.31 ± 0.037 [a3]	69.9
Control	1.27 ± 0.086	-	1.03 ± 0.08	-
AF100	0.81 ± 0.040 [a3]	36.2	0.65 ± 0.043 [a2b2c1]	36.9
AF200	0.76 ± 0.054 [a3]	40.2	0.58 ± 0.070 [a3b1]	43.7
AF400	0.67 ± 0.066 [a3]	47.2	0.34 ± 0.080 [a3]	67.6
Loperamide	0.59 ± 0.038 [a3]	53.5	0.31 ± 0.037 [a3]	69.9

Data are expressed as mean ± SEM (n=6); analysis was performed with One-Way ANOVA followed by Tukey test; [a] compared to negative control; [b] compared to loperamide 3mg/kg; [c] compared to 400 mg/kg; [1]$p<0.05$, [2]$p<0.01$, [3]$p<0.001$; CF, chloroform fraction; MF, methanol fraction; AF, aqueous fraction; Negative controls received 2% Tween 80 in water or distilled water.

4.5. Effect of solvent fractions on castor oil induced gastrointestinal motility

All the three fractions of leaves of *L.camara* significantly inhibited gastrointestinal motility of charcoal meal at all tested doses as compared to negative control and the maximum effect was achieved by the methanol fraction at 400 mg/kg (54.7%) as shown in Table 4.

Table 4. Effects of solvent fractions of the leaves of *Lantana camara* on castor oil induced gastrointestinal motility in mice

Group	Mean length of small intestine (cm)	Mean distance travelled by charcoal meal (cm)	Peristaltic index(PI)	% of inhibition
control	55.8 ± 1.3	46.2 ± 1.2	82.8 ± 2.5	-
CF100	54.3 ± 0.8	40.7 ± 0.8 [a2b3c2d3]	74.9 ± 1.3 [a1b3c3d3]	11.9
CF200	55.5 ± 1.4	34.8 ± 1.0 [a3b3d1]	62.7 ± 0.6 [a3b3d1]	24.7
CF400	55.2 ± 0.9	30.5 ± 1.3 [a3b3]	55.2 ± 2.0 [a3b3]	34.0
Loperamide	54.0 ± 0.8	16.7 ± 0.5 [a3]	30.9 ± 1.0 [a3]	63.8
control	55.8 ± 1.0	49.2 ± 1.4	87.9 ± 1.3	-
MF100	56.3 ± 1.2	35.7 ± 1.0 [a3b3c2d3]	63.3 ± 1.3 [a3b3c3d3]	27.4
MF200	55.3 ±1.5	30.0 ± 1.2 [a3b3d3]	54.1 ± 0.7 [a3b3d3]	39.0
MF400	55.8 ± 1.6	22.3 ± 0.6 [a3b1]	40.2 ± 2.0 [a3b2]	54.7
Loperamide	55.8 ±1.4	17.3 ± 0.6 [a3]	31.1 ± 1.1 [a3]	64.8
control	55.8 ± 1.0	49.2 ± 1.4	87.9 ± 1.3	-
AF100	56.4 ± 1.2	39.3 ± 1.7 [a3b3e3]	69.7 ± 2.8 [a3b3c2d3]	20.1
AF200	56.2 ±1.2	33.9 ± 1.5 [a3b3]	60.2 ± 2.0 [a3b3]	31.1
AF400	54.7 ±1.2	29.7 ± 0.9 [a3b3]	54.2 ± 1.0 [a3b3]	39.6
Loperamide	55.8 ±1.4	17.3 ± 0.6 [a3]	31.1 ± 1.1 [a3]	64.8

Data are expressed as mean ± SEM (n=6); analysis was performed with One-Way ANOVA followed by Tukey test; [a] compared to negative control; [b] compared to loperamide 3mg/kg; [c] compared to 200 mg/kg; [d] compared to 400 mg/kg; [1]$p<0.05$, [2]$p<0.01$, [3]$p<0.001$; CF, chloroform fraction; MF, methanol fraction; AF, aqueous fraction; Negative controls received 2% Tween 80 in water or distilled water.

4.6. *In vivo* anti-diarrheal index

Results from the determination of *in vivo* ADI revealed that the ADI increased with dose for each fraction (R^2 = 0.988 for CF, 0.996 for MF and 0.997 for AF) and the methanol fraction at its higher tested dose had the maximum ADI when compared with other tested doses of all fractions but less than the ADI of loperamide as shown in table 5.

Table 5. *In vivo* anti-diarrheal index of solvent fractions of the leaves of *Lantana camara*

Group	Delay in defecation time (%)	Gut meal travel reduction (%)	Purging frequency in number of wet feces (%)	Anti-diarrheal index (ADI)
CF100	36.6	11.9	10.4	16.5
CF200	62.7	24.7	30.1	36.0
CF400	89.9	34.0	39.0	49.2
Loperamide	201.1	63.8	74.9	98.7
MF100	110.3	27.4	31.4	45.6
MF200	141.1	39.0	48.6	64.4
MF400	190.9	54.7	64.3	87.6
Loperamide	224.5	64.8	67.2	99.2
AF100	67.2	20.1	24.3	32
AF200	92.1	31.1	42.8	49.7
AF400	127.6	39.6	52.9	64.4
Loperamide	224.5	64.8	67.2	99.2

(CF= Chloroform fraction; MF= Methanol Fraction; AF= Aqueous Fraction)

4.7. Preliminary phytochemical screening

The preliminary phytochemical screening of 80ME revealed the presence of all tested constituents except steroids, alkaloids and glycosides. From the solvent fractions, only the chloroform fraction showed the presence of steroids and, alkaloids were exclusively found in the aqueous fraction as shown in Table 6.

Table 6. Preliminary phytochemical screening of 80% methanol extract and solvent fractions of the leaves of *Lantana camara*

		Solvent fractions		
Metabolites	80% Methanol extract	Chloroform fraction	Methanol fraction	Aqueous fraction
Terpenoids	+	+	+	-
Flavonoids	+	-	+	-
Tannins	+	-	+	+
Saponins	+	-	+	+
Steroids	-	+	-	-
Alkaloids	-	-	-	+
Glycosides	-	-	-	-

+ = presence, - = absence

5. DISCUSSION

L.camara is one of the medicinal plants traditionally used for treatment of various aliments including diarrhea. The antidiarrheal activity of the crude extract of the leaves of *L.camara* was reported earlier (Pabillaran *et al.,* 2014; Sagar *et al.,* 2005). This study explored whether geographical variation could have an effect on activity and furthered the work by looking at on which fractions the active constituents are concentrated.

Despite its wide spread medicinal use, *L.camara* is listed as one of the poisonous plants for grazing animals such as cattle and sheep (Sharma *et al*., 1988). But different studies indicated that intoxication only occurs when enough plant material (>1% of body weight) is ingested, particularly when starving animals are conduced to pasture where the plant grows freely (Ghisalberti, 2000). Indeed, acute toxicity studies done on 80ME and fractions (the present study) as well as other studies done on the leaves (Pour *et al.,* 2011) and other parts of the plant (Pour and Sasidharan, 2011; Tadesse, 2015) showed that the plant has LD_{50} > 2000 mg/kg. Based on the method of WHO hazard classification, both 80ME and solvent fractions with LD50 > 2000 mg/kg is designated as 'unlikely to be hazardous'' (WHO, 1975).

To study the antidiarrheal activity of the 80ME and solvent fractions castor oil was used to induce diarrhea in all the three models. It induces diarrhea through a pathophysiological mechanism induced by its active metabolite, ricinoleic acid. Once castor oil is administered orally, it is broken down into recinoleic acid, a hydroxylated fatty acid, by the action of intestinal lipases in the intestinal lumen and considerable amounts of ricinoleic acid is absorbed in the intestine (Kulkami and Pandit, 2005; Watson and Gordon, 1962). Ricinoleic acid then mediates its action by binding with EP_3 prostanoid receptors on smooth muscle cells (Tunaru *et al.,* 2012). The metabolite

is shown to increase secretion of sodium and water through its ultrastructural alterations in the villous tips of the intestinal mucosa (Cline *et al.,* 1976), which is further confirmed by its cytotoxicity to isolated cells (Gaginella *et al.,* 1977). Other studies indicate that hydroxyfatty acids (including ricinoleic acid) induce fluid and electrolyte secretion secondary to their stimulation of an active anion secretory process (Racusen and Binder, 1979). Normal intestinal fluid absorption is also impaired by castor oil through inhibition of intestinal Na^+/K^+ ATPase activity (Gaginella *et al.,* 1978). In addition, castor oil (ricinoleic acid) also alters the motility of GI smooth muscles (Matias *et al.,* 1978). Therefore, the use of castor oil as diarrhea inducer for all models is plausible as it mimics the pathophysiologic processes.

Both the quantity and quality of chemical constituents found in medicinal plants, which are responsible for their medicinal activity, is affected by different factors including geographical location. Since previous studies were done in other countries (India and Philippines) (Pabillaran *et al*., 2014; Sagar *et al*., 2005), the antidiarrheal activity of the 80ME of leaves of *L.camara* was evaluated to check if geographical location and other factors affect the activity of the plant. For this purpose, castor oil induced diarrheal model was used, as this model is the general model to test the antidiarrheal activities of different substances (Awouters *et al.,* 1978; Niemegeers *et al*., 1984). Diarrhea is characterized by an increase in frequency and/or weight of feces (Guerrant *et al*., 2001; Talley *et al.,* 1994) and this model mainly evaluates the effect of an agent on frequency and weight of defecation. Hence, agents which inhibit the frequency and weight of feces are considered to have antidiarrheal activity (Eberlin *et al.,* 2012; Farthing, 1999). The extract significantly reduced the frequency of defecation and the weight of feces at all tested doses. Moreover, it prolonged onset of diarrhea at the middle and higher doses, which could probably related to its

antimotility and antispasmodic effect (Ghodake *et al.*, 2013; Sagar *et al*., 2005). These findings collectively indicate that the difference in geographical location might not affect the antidiarrheal activity of this plant.

To further evaluate the nature of the active constituents responsible for the antidiarrheal activity of the plant, the powder of the leaves was successively fractionated by solvents of differing polarity and the antidiarrheal activity of the fractions were first evaluated by using castor oil induced diarrheal model.

Eventhough there is no single agreed parameter to determine the activity of substances in this model, the antidiarrheal activity of agents is mostly expressed by percentage of inhibition, which is determined by using number of wet feces (Igboeli *et al*., 2015). The fractions inhibited the number of wet feces in a dose dependent manner (R^2 =0.999 for MF; 0.972 for AF and 0.955 for CF) but the chloroform fraction didn't show significant effect at its lower dose. In this parameter, the methanol fraction at its higher dose (400 mg/kg) showed the maximum effect (64.3%), which is comparable with the effect of loperamide (67.2%). Consistent to the above finding the methanol and aqueous fractions exhibited significant inhibition at all tested doses by reducing all parameters related to number and weight of feces, with the methanol fraction being the most active fraction. The chloroform fraction, however, was found to be active either at the middle and higher doses or at the higher dose. The insignificant activity of the chloroform fraction at the lower dose might be due to the inability of secondary metabolites to reach sufficient concentration. This argument is supported by the fact that activity would be apparent with increasing dose of the fraction.

As regards to onset, while the chloroform fraction was devoid of any action, the methanol fraction and the aqueous fraction were active at all doses and the higher dose, respectively. This finding once again reinforces the notion that the methanol fraction is the most potent and active fraction, as a larger dose was needed for the aqueous fraction to delay the urgency of diarrhea. Moreover, it is plausible to assume that semi-polar secondary metabolites could be by and large responsible for increasing onset of diarrhea, as the aqueous fraction contains more polar constituents. Although the chloroform fraction displayed effect on number and weight of feces with increasing doses, it did not have any effect on onset, even with the higher dose used in the current study.

Generally, the fractions showed differences in potency in this model in the rank order of; methanol fraction > aqueous fraction > chloroform fraction in all parameters and this is line with other studies (Sanni *et al*., 2015). The difference in rank order of potency could emanate from the differential distribution of the secondary metabolites as depicted in Table 6.

It is widely known that castor oil is metabolized into ricinoleic acid in the gut, which in turn irritates and causes inflammation in the intestinal mucosa, resulting in release of inflammatory mediators, such as prostaglandins and histamine (Luderer *et al*., 1980). The prostaglandins thus released promote vasodilatation, smooth muscle contraction and mucus secretion in the small intestine. The inhibitors of prostaglandins biosynthesis are therefore considered to delay castor oil-induced diarrhea (Pierce *et al*., 1971; Robert *et al*., 1976). Flavonoids, terpenoids and steroids have been shown to inhibit production of prostaglandins (Awad *et al*., 2004; Fernandez *et al*., 2001; Hamalainen *et al*., 2011). Thus, different extent of significant

antidiarrheal activity observed in the fractions could be due to the presence/absence of these secondary metabolites in the respective fractions.

The anti-diarrheal activity of the fractions might also be due to inhibition of active secretion of ricinoleic acid, resulting in the activation of Na^+, K^+ ATPase activity that promotes absorption of Na^+ and K^+ in the intestinal mucosa (Gaginella *et al.*, 1978) which is linked with a decrease in frequency and weight of feces. This effect could probably be linked to the presence of terpenoids in the chloroform fraction, terpenoids, flavonoids and tannins in the methanol fraction and tannins in the aqueous fraction, which are shown to promote colonic absorption of water and electrolytes (Palombo, 2006).

Mostly, antidiarrheal agents act by decreasing secretion and/or reducing the propulsive movement of GI smooth muscles. So to further get information about the mechanism for the antidiarrheal activity, the fractions were evaluated by enteropooling and GI motility tests.

In the GI enteropooling test, the methanol and aqueous fractions significantly reduced the weight and volume of intestinal fluid at all tested doses when compared to the negative control group. This further supports the significant activity of these fractions on the frequency and weight of wet feces on the castor oil induced diarrheal model, which is in line with other study by which the anti-enteropooling effects of the extract is related with its antidiarrheal effect (de Sales *et al*., 2015). In fact, the ability of chloroform fraction to reduce the weight of intestinal content only at 400 mg/kg ($p<0.05$) lends further support to this notion. .

Ricinoleic acid provoked a marked net secretion of fluid, which is driven by an active ion secretory process and concomitantly inhibited the absorption of solutes from

human jejunum (Ammon *et al*., 1977; Racusen and Binder, 1979). Tannins decrease fluid secretion by inhibiting CFTR and CaCC, by generating protein-precipitating reaction to the GI mucosa and due to their high antioxidant capacity (Ashok and Upadhyay, 2012; Ren *et al.*, 2012; Wongsamitkul *et al.*, 2010). Furthermore, Mascolo *et al* (1993, 1994) reported that ricinoleic acid might activate the nitric oxide pathway and induce nitric oxide (NO) dependent gut secretion. Other studies also confirmed that NO is involved in the causation of diarrhea and this is counteracted by agents that inhibit NO synthesis (Izzo *et al*., 1996). A previous study indicates that different solvent extracts of *L.camara* inhibit NO synthesis through suppression of iNOS protein (Basu and Hazra, 2006). In addition, phytochemical constituents such as flavonoids (Duarte *et al.*, 2014; Raso *et al.*, 2001), tannins (Ishii *et al*., 1999) and alkaloids (Kondo *et al*., 1993) are implicated in the inhibition of nitric oxide synthesis by suppressing the expression of iNOS enzyme.

Thus, the pronounced antisecretory activity of the methanol and aqueous fraction might be due to the combined effect of the different constituents (flavonoid and tannin for methanol fraction; tannin and alkaloids for aqueous fraction) on intestinal secretion. The lack of action for the chloroform fraction could be explained by the same corollary, as these constituents were lacking from this fraction. The significant effect of chloroform fraction to reduce the weight of intestinal content at 400 mg/kg might be due to the accumulation of terpenoids that possess inhibitory effect on NO production (Jang *et al.*, 2004).

In the castor oil induced GI motility test, all the three fractions significantly inhibited GI motility of charcoal meal at all tested doses as compared to negative control. This decrease in GI motility facilitates the absorption of electrolytes and then water (Kent and Banks, 2010), which might be responsible for the decrease in the frequency and

weight of feces observed with the fractions. Eventhough the chloroform fraction had a significant effect on the charcoal meal test; it failed to show a significant effect on frequency and weight of wet feces, particularly at the lower dose. This could be explained by the fact that antimotility effect per se might not be a necessary and sufficient factor for counteracting diarrhea and it should be supplemented with a certain degree of antisecretory activity. .

In addition to its well documented secretory effect, castor oil, through recinoleic acid, alters the motility of GI smooth muscles (Matias *et al.*, 1978). All fractions significantly slowed down charcoal meal transit in the GIT, showing that these fractions could have inhibitory effects on the excitatory neurotransmitters in the GIT thus leading to relaxation of the gut muscles and slowing down motility (Guyton and Hall, 2001). This assumption is further supported by the antispasmodic activity of the methanol extract of the leaves of this plant by antagonizing the actions of acetylcholine (Sagar *et al*., 2005).

Flavonoids, which are abundant in the methanol fraction, have been demonstrated to decrease contraction caused by spasmogenes (Macauder, 1986) and inhibit small intestinal transit (Dicarlo *et al.*, 1993; Ghayur *et al.*, 1997; Meli *et a1.*, 1990; Viswanathan *et al.*, 1984). Studies on the functional role of tannins, which are present in the aqueous and methanol fractions, reveal that they inhibit GI movement by reducing the intracellular Ca^{2+} inward current or by activation of the calcium pumping system (Belemtougri *et al*., 2006) as well as by forming protein tannates, which make the intestinal mucosa more resistant and hence, reduce peristaltic movement (Ashok and Upadhyay, 2012). Terpenoids which are present in the methanol and chloroform fraction demonstrated antispasmodic activity and have an inhibitory effect on GI motility (Jalilzadeh-Amin and Maham, 2015, Palombo, 2006).

Furthermore Sagar *et al* (2005) showed that the methanol leave extract of *L.camara* had more pronounced effect on neostigmine induced motility than on normal GI motility which indicates that the extract might not interfere with the normal function of GIT.

ADI is a measure of the combined effects of different components of diarrhea such as defecation frequency, onset of diarrheal stools and intestinal motility (Aye-Than *et al*., 1989). The fractions showed a dose dependent increase on ADI (R^2 = 0.988 for CF, 0.996 for MF and 0.997 for AF) and the highest ADI was produced by the methanol fraction at its higher dose which is directly related with its antidiarrheal activity in all of the three models.

In addition to its effect on the secretion and motility of GI tract, as shown in this study, *L.camara* had effect on micro-organisms that cause diarrhea (Agrawal *et al.,* 2012; Saikia and Sahoo, 2011; Salada *et al*., 2015; Passos *et al*., 2012). From this it can be postulated that this plant can also be used for infectious diarrhea.

6. CONCLUSION

The present study confirmed the antidiarrheal activity of the crude extract of the leaves of *L.camara* and further revealed that all the three fractions possessed varying degree of antidiarrheal activity, with the methanol fraction being the most active fraction followed by the aqueous fraction and then chloroform fraction in all the three models. The antidiarrheal activities of the fractions could be attributed to the presence of bioactive agents including flavonoids, tannins, terpenoids, saponins, steroids and alkaloids that act individually or collectively. In addition, the results from the present study suggest that compounds ranging from semi-polar to polar are more likely to be responsible for the observed effect.

7. RECCOMENDATION

- Traditional healers should appropriately use the leaves of *L. camara* for treating diarrhea
- Further toxicological studies such as sub-acute and chronic toxicities should be done in order to assess the long term effect of the extract and fractions.
- Further work should be conducted to understand the mechanism (s) of action of the fractions in producing antidiarrheal activity.
- *Ex -vivo* studies of the fractions on isolated tissue preparations should be done to support the *in vivo* methods
- Further studies should be done to isolate, purify and identify pharmacologically active principle (s) responsible for the antidiarrheal activities of the plant.

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