Addis Ababa University

College of Health Science

School of Pharmacy

Department of Pharmacology and Clinical Pharmacy

In-vivo and *In-vitro* Mechanistic Study in The Antidiarrheal Activity of Hydro-alcoholic Extract of *Ocimum lamiifolium* HOCHST. EX BENTH Leaves.

By: Dinberu Beyene (B. Pharm)

A Thesis Submitted to

The Department of Pharmacology

and Clinical Pharmacy, School of Pharmacy,

College of Health Sciences

In Partial Fulfillment of The Requirements for The Degree of Master of Science (M.Sc.) in Pharmacology

Addis Ababa, Ethiopia

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Addis Ababa, Ethiopia

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School of Graduate Studies

This is to certify that the thesis prepared by Dinberu Beyene, entitled: "*In-vivo* and *in-vitro* mechanistic study in the anti-diarrheal activities of the hydro-alcoholic extract of *Ocimum lamiifolium* Hochst. Ex Benth leaves'' 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

In-vivo and *in-vitro* mechanistic study in the antidiarrheal activity of the hydro-alcoholic extract of the leaves of *Ocimum lamiifolium* Hochst.ex Benth (Lamiaceae).

Dinberu Beyene

Addis Ababa University, 2023

Ocimum lamiifolium is used in the management of various diseases such as fever, malaria, headache, cough, and gastrointestinal disease (diarrhea). This study was undertaken to evaluate the in-vivo and in-vitro mechanistic studies in the antidiarrheal activity of hydro-alcoholic extracts of the leaves of O. lamiifolium. The anti-diarrheal activity was assessed using a castor-oil-induced diarrhea model, charcoal meal test, and entero-pooling test in mice. The standard drug loperamide 3 mg/kg was given to the positive control. Different doses of the hydro-alcoholic extract of O. lamiifolium (100, 200, and 400 mg/kg were given to the test groups, and distilled water (10 ml/kg) was given to negative controls. The ex-vivo spasmolytic activity was evaluated using organ bath perfusion in isolated guinea pig ileum. The mechanistic study was also explored using a castor-oil-induced diarrheal model in the presence of naloxone (opioid antagonist). In the mechanistic study, the test group received 400 mg/kg extract with naloxone 2 mg/kg, the positive control received loperamide 3 mg/kg with naloxone 2 mg/kg, and the negative control received distilled water 10 ml/kg with naloxone 2mg/kg. In the castor oil-induced diarrhea model, all the tested ingredients significantly prolonged the onset of diarrhea and reduced the number of defecation ($p<0.05$). However, the mean weight of wet and total feces was significantly reduced by only the higher doses (200 and 400 mg/kg) ($p<0.05$). All doses also produced a significant ($p<0.01$) reduction in mean weight and mean volume of intestinal contents in the entero-pooling study. Similarly, in the charcoal meal test, all the study doses of the substance also produced significant ($p<0.001$) antimotility effects. In the mechanistic studies, the percentage inhibition of diarrhea by 400 mg/kg of the extract in the presence of naloxone (2 mg/kg) is 64.69%. In this case, charcoal meal traverse is significantly reduced by the extract compared to the control $p<0.001$. However, in the presence of naloxone (2mg/kg), the percentage inhibition by loperamide 3mg/kg is 6.89%. In the ex-vivo studies, the percentage of response or relaxation produced by the extract was 20%, 65%, and 75% at the doses of 0.1, 0.2, and 0.4 ml respectively. The doses that produced 50% maximal relaxation (EC_{50}) by the extract were 0.18 ml or 1.8 mg of hydro-alcoholic extracts of O. lamiifolium. In conclusion, this study revealed that the hydro-alcoholic leaf extract of O. lamiifolium exhibits considerable anti-diarrheal activity because of its inhibitory effect on gastrointestinal motility and secretion. This is partly mediated through blockage of muscarinic acetylcholine receptors but not opioid receptors.

Key terms: *O. lamiifolium, castor-oil-induced antidiarrheal activity, antimotility, anti-entero-pooling, spasmolytic activities, 80% methanol extraction.*

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

ADI	Anti-Diarrheal Index
AMR	Antimicrobial resistance
cAMP	Cyclic Adenosine Mono Phosphate
CDC	Center for Disease Control and Prevention
cGMP	Cyclic Guanosine Mono Phosphate
GBD	Global Burden of Diseases
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
OECD	Organization for Economic Cooperation and Development
ORS	Oral Rehydration Solution
UNICEF	United Nations Children's Fund
WHO	World health organization

LISTS OF TABLES AND FIGURES

Lists of tables

Lists of figures

1. INTRODUCTION

Diarrhoea is a gastrointestinal disease that causes morbidity and deaths among people, especially children under 5 years old, and most of the time it is preventable and treatable. According to the World Health Organization (WHO) definition diarrhea is the passage of three or more watery feces in 24 hours and an unusual number of diarrhea attacks (Baqui et al., 1991). The intervention for diarrhea is more dependent on the consistency of the feces than the number of appearances.

Diarrhea can be categorized by different means; the duration of the attack and its presentation are the most common. These are acute watery, acute bloody, and persistent diarrhea (WHO, 2005). Acute watery diarrhea (dehydrating diarrhea), which lasts from hours to days, is classified into mild, moderate, and severe based on stool volume. It causes acidosis and dehydration, which leads to death in children (Sarmin et al., 2017). However, this consequence is easily controlled and prevented by using oral rehydration therapy (ORT).

Although many other organisms are responsible for the acute diarrhea; Vibrio cholera, Escherichia coli, and Rotavirus (Kotloff et al., 2013), are common sources of acute watery diarrhea. Lack of appropriate nutrition, insufficient breastfeeding, poor economy, and poor hygiene are some of the factors that predispose children to diarrhea (Smeets & Keita, 2020).

Acute bloody diarrhea contains visible blood in the stool and occurs rarely in children. This illness is subdivided into community-acquired, traveler's, and nosocomial diarrhea on the basis of geography. A traveler's diarrhea is diarrhea acquired through traveling to other geographical areas or crossing international borders, and most of the time it is self-limited.

The most common etiology for acute bloody diarrhea is *Shigella* (Guerrant et al., 2001). However, the etiology of traveler's diarrhea differs geographically. Nosocomial diarrhea is an infection that develops after two or three days of health care visits in other cases. During admission to a healthcare setting for other illnesses, children often acquire diarrhea (ponce et al.,1995). Community-acquired diarrhea is caused by Shigella species, Salmonella and Campylobacter (Guerrant et al., 2001).

Persistent diarrhea starts acutely and persists for fourteen or more days (Bandsma et al., 2019). It is caused most commonly by Giardia lamblia and Cryptosporidium parvum but bacteria are also more common in immune-insufficient persons (Cheng et al., 2005; Dupont, 2016).

1.1. Epidemiology of diarrhea

Globally, 2016 data showed diarrhea became the fifth and eighth leading cause of mortality among children aged below five years (446 000 deaths) and among all ages (1655944 deaths), respectively (Troeger et al., 2018). In India however, the mortality rate due to diarrhea among children under five years old has declined from 309 to 47 in the year from 1990 to 2019 (Behera & Mishra, 2022). Globally, trends of diarrhea from 1980–2015 among children aged under five years also increasingly declined from 21.3% to 5.7% per thousand born. This is due to the increasing coverage of zinc supplementation and ORT (Black et al., 2019).

The African continent accounts for the greatest proportion of deaths from diarrhea (49.63%), and 26% of severe episodes. From this in sub-Saharan Africa, it was one in two childhood deaths in 2011 (Walker et al., 2013). The estimated prevalence of diarrhea among children under 5 years of age in East Africa was 15.86% (Tareke et al., 2022).

The overall prevalence of diarrhea among children under five years old by 2018 in Ethiopia obtained in meta-analysis and systematic review indicates that more than one in five means (22%), with the Afar region accounting for 27% (Alebel, 2018). These indicate that the prevalence is significantly higher. More specifically, two weak prevalence studies in Laelay-Maychew District showed 17.7% (Gebrezgiabher et al., 2019), and Dale District (Sidama zone), 13.6% (Melese et al., 2019). In Addis Ababa, the prevalence is 11.9%, especially watery diarrhea, among those who have low income (less than 50 dollars per month) (M. Adane et al., 2018).

1.2. Pathophysiology of diarrhea

Diarrhea resulted from the disturbance in the absorptive and secretory processes within the bowel (Field, 2003). The basic pathophysiological processes in diarrhea include active secretion, osmosis, exudation/inflammation, and altered motility.

Secretory diarrhea

Watery diarrhea is grouped as either osmotic or secretory. In the patients with secretory one, stool osmolality is accounted for by electrolytes such as Na+ and K+ as well as associated anions. The net production of Cl- /HCO3- or the retardation of net Na+ absorption are the basic mechanisms involved in this type of diarrhea (Schiller, 1999).

The intestinal secretion is because of the activation of active chloride secretion and to the hindering of active absorption of Na and Cl- by the intracellular mediator cyclic adenosine mono-phosphate (Barrett, 2000). As shown in figure 1, the driving force for intestinal ion secretion appeared from infection to the gut lumen (Navaneethan & Giannella, 2008). The secretion also arises from subepithelial space (inflammatory mediators) (Field, 2003), and or from the systemic circulation (peptide hormones produced from endocrine tumors) (Jensen, 1999). Infectious pathogens are common sources of secretory diarrhea; they attach to the mucosa and disturb the absorptive or secretory process of the enterocyte (Schiller, 1999).

The mechanisms in secretory diarrhea include the activation of intracellular mediators, i.e., cAMP, cGMP, and intracellular calcium, which stimulate active chloride secretion from crypt cells and inhibit neutral coupled sodium chloride absorption (Barrett, 2000). Moreover, stimulation of Cl- channels in the apical membrane of enterocytes, such as cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated chloride channels (CaCC), enhances fluid secretion. The change in these mediators causes CFTR-mediated chloride secretion and inhibition of small intestinal-coupled Na-Cl transport.

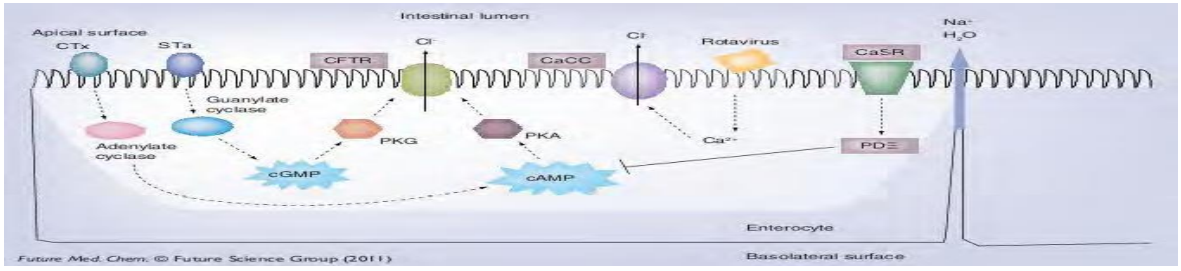


Figure 1: Secretory pathways in the gut epithelium disrupted by diarrhea-causing pathogens

cAMP; CaSR: Calcium-sensing receptor; cGMP: Cyclic guanosine monophosphate; CTx: Cholera toxin; PDE: Phosphodiesterase; PKA: Protein kinase A; PKG: Protein kinase G; STa: Enterotoxigenic Escherichia coli heat-stable toxin (De Hostos et al., 2011).

Osmotic diarrhea

It happens when non-absorbable (mannitol or sorbitol) or erratically absorbable substances such as magnesium, sulfate and phosphates are taken or enterocytes are unable to absorb them. When the osmotic force of such kinds of substances drives fluid and ions into the gut lumen diarrhea happens (Field, 2003).These types of diarrhea may be caused by a lack of enzymes to digest. These are congenital lactase deficiency (lactose intolerance), genetic abnormalities, poorly absorbed sugar, laxatives, and magnesium-containing agents (certain antacids) (Bajor, 2008).

Inflammatory diarrhea

It results from different etiologies like infection and inflammatory bowel disease. The colon is the primary area affected by pathogens. They lead to disease by inducing cytotoxins or by taking over the epithelium with the enlisting of inflammatory cells. Enteroaggregative E. coli (EAEC), entero-hemorrhagic Escherichia coli (EHEC), and C. difficile are cytotoxin-producing non-invasive pathogens. Invasive pathogens causing inflammatory diarrhea include Shigella, Campylobacter, Salmonella, and E. histolytica. They cause inflammatory diarrhea by causing mucosal destruction and by activating intestinal secretion (Binder, 2009; Eisenhut, 2006).

Functional diarrhea

Although the pathophysiology of this functional diarrhea or irritable bowel syndrome (IBS) is well known the cause is unclear. Colonic transit, hypermotility and rectal hypersensitivity are the well-known pathophysiology of functional diarrhea (Grundy, 2002). A study showed that in the Western community, 3%–5% of people were affected by functional diarrhea (Talley et al., 1991). Disturbances in neural control (from the brain to visceral nerves) and the gut in the form of visceral nociception and abnormal motility mediated by alteration in neurotransmitters like serotonin, cholecystokinin, and neurokinins are also proposed to contribute to diarrhea seen in patients with functional diarrhea (Drossman et al., 2002).

1.3. Management of diarrhea

1.3.1. Non-pharmacotherapy

Oral rehydration salt (ORS)

Acute watery diarrhea causes dehydration, which might lead to death due to the losing of important fluid and electrolytes unless treated. Oral rehydration salt is one of the non-pharmacological therapies commonly used for diarrhea, independent of etiology (Guarino et al., 2018). This helps for the replacement of lost fluids and prevents complications due to dehydration. However, despite its proven effectiveness in treating diarrhea, ORS does not reduce the frequency of fluid loss or bowel movements and also does not reduce the duration of illness. However, its combination with *Lactobacillus* GG in the treatment of acute diarrhea results in shorter duration of diarrhea and faster discharge from the hospital (Szajewska & Mrukowicz, 2001). A report from a systematic review conducted in 2016 reveals that the percentage of diarrhea among people who were given ORS for two weeks is 85% in Sierra Leone, followed by Tanzania at 40%, and Ethiopia at 26% (Carvajal-Vélez et al., 2016).

Zinc and minerals

Evidence showed that zinc has an appreciable useful effect on the length of acute diarrhea attack and also has a beneficial effect in reducing associated complications and mortality (Patel et al., 2005). It prevents the incidence and severity of diarrhea through immune and cellular function. The WHO recommends zinc for the management of diarrhea, and it decreases diarrhea-related healthcare visits by 23% (Walker & Black, 2010). The dose of zinc recommended by the WHO for aged older than six months is 20 mg per a day. The recommended dose of zinc for children younger than six months is 10 mg per a day. However, the regularity of the supply of this medicine is a problem, especially in Africa. In sub-Saharan Africa, the proportion of zinc addressed was less than 5% (Carvajal-Vélez et al., 2016). Improving the coverage of zinc is the goal of Ethiopia by 2025 in the global action plan to prevent preventable child death by diarrhea (Qazi et al., 2015).

Probiotics and prebiotics

Probiotics

Although in most countries they are used as food additives, probiotics are increasingly gaining credence as a medicine (Canani et al., 2007). Probiotics are nonpathogenic living microorganisms that encourage health by ameliorating gut microflora, boosting immunity, or repairing damage.

Treatments with probiotics ameliorate the treatment of acute infectious diarrhea in children either by reducing the period of diarrhea or hindering its complication. Probiotic microorganisms hinder the metabolic activities of pathogenic microorganisms and their adhesion to intestinal cells, modulate the gut microflora, and have immune stimulatory or regulatory properties (De Vrese & Marteau, 2007).

The two most studied strains are *Lactobacillus rhamnosus GG* and *Saccharomyces boulardii;* they shorten diarrhea by one day, and both are safe for children (Allen et al., 2010; Van Niel et al., 2002). Although probiotics are believed to have beneficial effects, there is a lack of strong evidence to make any recommendation about their routine use (Guarino et al., 2015).

Prebiotics

The concept of prebiotics was introduced for the first time in 1995 by Glenn Gibson and Marcel Roberfroid. Prebiotics are complex non-digestible carbohydrates used to preferentially stimulate the growth of health-promoting intestinal flora (Gibson et al., 2017). Inulin is an example of indigestible oligosaccharides that fulfill the criteria for prebiotic classification. The major characteristics of prebiotics are resistance to digestive enzymes in the human gut, fermentability by the colonic microflora, and pH-lowering effects, and despite the promising results of animal experiments, there is no report of a successful preventive or therapeutic use of prebiotics in patients with diarrhea (De Vrese & Marteau, 2007).

1.3.2. Conventional medicine

Various anti-motility and anti-secretory agents are available for the symptomatic relief of diarrhea. Diphenoxylate hydrochloride with atropine sulfate, morphine, codeine and loperamide are potent antidiarrheal drugs active in clinical use.

Anti-peristalsis and anti-motility agents

These drugs enhance intestinal transit time and also, they help the reabsorption of essential electrolytes and fluids. The most commonly used drugs are loperamide and the diphenoxylate-atropine combination. Although loperamide in combination with antibiotics used in the management of dysentery, these drugs pose a risk of colonic dilatation associated with infective colitis (Casburn-Jones & Farthing, 2004). Moreover, loperamide is no longer used in children under two years old due to unwanted effects like drowsiness and ileus, as well as death due to paralytic ileus (Bhuta & Tahir, 1990).

Antisecretory agents

Morphine, codeine, and more importantly, loperamide, are good antidiarrheal drugs used for the management of diarrhea of different etiologies. These agents primarily work by reducing intraluminal fluid accumulation and temporarily correcting fluid and electrolyte imbalances in the intestine (Hughes et al., 1984). They produce their effect by stimulating opioid μ receptors in the intestine (Holzer, 2009).

The antisecretory agent racecadotril is an inhibitors of the enzyme enkephalinase that degrades endogenous enkephalin. It reduces hypersecretion and encourages absorption but does not slow intestinal motility (Casburn-Jones & Farthing, 2004). The pro-absorptive activity of racecadotril is due to the augmentation of endogenous enkephalins. *In-vitro* study showed that it increases sodium and chloride absorption (Farthing, 2002).

Although constipation is a common problem with anti-motility agents, racecadotril is devoid of this effect and can be safely used in children (Design, 2000). Furthermore, it is also devoid of central nervous system effects despite its weak anti-secretory effect (Primi et al., 1999). Also, randomized controlled trials showed inspiring evidence for the use of this drug, which provides clinically important symptomatic relief via the reduction of length and severity diarrhea (Mehta et al., 2012). The clinical relevance lies much more in antisecretory than antimotility.

Octreotide, an analog of somatostatin, has relevant effects on the gut secretion caused by neuroendocrine tumors (De Herder et al., 1996). Studies shown in isolated mammalian intestines have an inhibitory effect on water and electrolyte transport in the intestine

(Dharmsathaphorn et al., 1980). It is most often suggested for patients with human immune viruses and post-chemotherapy (Peeters_et_al, 2010).

Intracellular communications are one means of pharmacological target for the regulation of secretory processes in the gut. The drug phenothiazine and chlorpromazine hinder hormonal stimulation of cAMP and likely inhibit the effects of calmodulin (a calcium-binding protein) (Holmgren et al., 1978). However, their hypotensive effect due to their α-adrenergic receptor blocking activity is not used clinically. Another calmodulin inhibitor called zaldaride maleate has shown anti-secretory activity in *in-vivo*; however, it is inefficient compared to the standard (Okhuysen et al., 1995).

Antimicrobial Therapy

Antimicrobials work by either inhibiting the growth or killing the microorganisms in the host (Guilhelmelli et al., 2013; Majumder et al., 2020). They are reliably helpful for the management of diarrhea, particularly bloody diarrhea. They reduce the length of occurrence and halt transmission from one person to another. Antibiotics are recommended in cases of infection (Koletzko & Osterrieder, 2009). The drugs ciprofloxacin, norfloxacin, and rifaximin are the recommended antibiotics for the prophylaxis of traveler's diarrhea (Steffen et al., 2015).

Despite this, for fear of resistance and adverse consequences, the use of prophylaxis is rare and only reserved for highly susceptible individuals (Dupont et al., 2009).

1.3.3. Herbal or traditional medicine

The history of traditional practice is as long as the history of human beings on this earth. In developing countries, including Africa and some Asian countries, the majority of the population depend on traditional practice for their primary healthcare needs (Balemba et al., 2010). In middle-income countries like India, 11.7% of the population uses traditional practice for their different illnesses (Oyebode et al., 2016).

In recent years, there is growing interest and demand for the discovery of modern medicine from its traditional origins (Olajuyigbe and Afolayan, 2012; Yuan et al., 2016). More than one hundred thirty-two (132) different plants are used as a remedy for diarrhea in Ethiopia

(Woldeab et al., 2018). Among these: leaf extract of *Ocimu lamiifolium* (Gedif & Hahn, 2003); *Calpurnia aurea*, *Plagiolepis abyssinica*, *Rhus natalensis*, *Oxalis radicosa*, *Kosteletzkya adoensis* (Woldeab et al., 2018)*; Lantana camara* steam extract (Mesfin et al., 2009), leaf extract of *Croton macrostachyus* (Mesfin et al., 2014); leaf extract of *Hagenia abyssinica*, *Senna didymobotrya*, root extract of *Carissa spinarum* L, *Balanites aegyptiaca*, *Carissa spinarum* (Banchiamlak & Young-dongKim, 2019), *Mangifera indica*, Podocarpus falcatus (Temam & Dillo, 2016) and etc.

Although various plants are used for diarrhea treatment, most of them have not been scientifically evaluated. Some of the plants that were tested for *in-vivo* antidiarrheal activities are *Clutia abyssinica* root extract (Zayede et al., 2020), *Dodonaea viscosa* (Abdela, 2019), *Calpurnia aurea* leaf extract (Umer et al., 2013), *Croton macrostachyus* leaf extracts (Degu et al., 2016), the stem extract of *Lantana camara* (Tadesse et al., 2017), *Mangifera indica* leaves extract (Yakubu & Salimon, 2015), and *O. lamiifolium* leaves extract (Alemu et al., 2022). Therefore, the objective of this study is scientifically evaluating the acclaimed traditional use of the antidiarrheal activities of *O. lamiifolium* in mice.

1.4. The experimental plants

Botanical source and characteristics

The genus *Ocimum* consists of over one hundred fifty (150) species. It is assumed as one of the largest genera of the Lamiaceae family (Ololade, 2014). Most of these species are extremely aromatic and economically helpful since they are the main source of essential oils, medicinal plants, and flavorants (Oualili, 2019).

Ocimum lamiifolium is indigenous medicinal herbs in Ethiopia and is locally named "dama'' in (Guragea), "dama-kasse'' (in Amharic) and "anchabi'' (in Oromifa) language. It is a common herb distributed over a wide part of the country (Getasetegn & Tefera, 2016). It is also distributed in different parts of Africa (Giday et al., 2010). *O. lamiifolium* is an erect, branching subshrub or shrub growing about 0.7 to 3 meters tall as shown in figure 1. Leaves are ovate and opposite, and flowers are pinkish in racemes. The plant grows along roads, in bushland, on forest edges, and on grassland between 1000 and 3000 m (Belay, 2017).

Ethnobotanical Uses of *O. lamiifolium*

The leaves of *O. lamiifolium* are used for many ailments. In Ethiopia the juice of the leaf is administered to the nose to treat pain and fever (Giday et al., 2010). The fresh leaves of *O. lamiifolium* pounded in water are drunk to treat gastrointestinal problems such as diarrhea, amoeba and cough or cold (Gedif & Hahn, 2003; Woldeab et al., 2018). The water juices of the root of *O. lamiifolium* is also used in combination with fresh leaves of *Vernonia amygdalina* and *Clutia abyssinica* for the treatment of diarrheal (Stark et al., 2013). The leaves are also used for undefined gastrointestinal disorders in north Shewa (Selale) (Atnafu et al, 2018). It is also used orally for stomach disorders in Harer, Ethiopia (Bussa & Belayneh, 2020). The leaves also used in combination with the leaves of *Lippia adoensis* and *Fuerstia africana* for general malaise (Kefalew et al., 2015).The leaves also combined with *E. abyssinica* stem bark decocted extract are traditionally used for hepatic disease (Mukazayire et al., 2011). Its leaf powder is also used for the treatment of malaria and measles in pediatrics in Uganda (Nalumansi et al., 2014).

Ethnopharmacological activities of *O. lamiifolium*

The plant *O. lamiifolium* is extensively studied in *in-vivo* as well as *in-vitro* for different activities. The leaf extracts have good *in-vitro* antibacterial activities on both gram positive and gram negative bacteria (Addis et al., 2019; Kifle et al., 2007; Runyoro et al., 2010). Apart from this, *in-vivo* studies showed leaf extract has good anti-malarial activities (Kefe et al., 2016), antipyretic activities (Makonnen et al., 2003), anti-inflammatory (Mequanint et al., 2011) and *in-vitro* anti-oxidant activities (Nair et al., 2016). This plant was also evaluated previously for its *in-vivo* anti-diarrheal activity (Alemu et al., 2022).

Phytochemistry of *O. lamiifolium*

A preliminary phytochemistry of methanol extracts of *O. lamiifolium* leaves showed the presence of alkaloids, saponins, anthraquinones, steroids, glycosides, flavonoids, tannins, total phenol, and terpenoids (Alemu et al., 2022). Alkaloids, flavonoids, and triterpenoids are found in the leaves of *O. lamiifolium is* in appreciable amounts (positive within 5 min). Terpenoids, flavonoids, saponins, tannins, and steroids are also found in ethanol extracts of its leaves (Sahalie et al., 2018). Similarly another report also showed the presence of phenolic acids, flavonoids, glycosides, and sugars in its ethanol and aqueous extracts (Makonnen et al., 2003). The seeds of *O. lamiifolium* contain phenolic compounds identified

at 330 nm. The constituent rosmarinic acid, lithospermic acid, hydroxybenzoic acid, ferulic acid, cinnamic acid, and were detected in these seeds (Hakkim et al., 2008).

Figure 2*:* Photographs of leaves of *Ocimum lamiifolium Hochst. ex benth* (Belay, 2017)

1.5. Rationales for the study

Despite extensive progress that has been made in the reduction of diarrheal burden especially in children, it continues to threaten global public health and is one of the major reasons for the death and morbidity of children (Ugboko et al., 2020). Furthermore, the rate of death is high among low and medium-income countries (Walker et al., 2013). More specifically, the prevalence is more than one in five in Ethiopia (Alebel, 2018).

Various drugs are available for the treatment of diarrhoea disease, such as antisecretory, antimotility, and antimicrobial agents. Antimicrobials are one of the inevitable medicines available for infectious diarrhea. However, the gradual emergence of microbial resistance as well as severe side effects has become a global issue (Majumder et al., 2020). Furthermore, common etiologies for diarrhea developed resistance to currently used medicines (such as fluoroquinolones) (WHO, 2014).

Among the various drugs available for the management of diarrhea, antimotility and antisecretory are also extensively used for the treatment of diarrhea. However, none of them has

been used in the regular management of diarrhea because of the adverse consequence of the majority of anti-diarrhea drugs after prolonged use (Kamm, 1994; Osadebe et al., 2012).

Their use is a concern especially in younger children (Li et al., 2007). More specifically, the anti-cholinergic effects of atropine, which cause dry mouth and urinary retention, and the tolerance effects of diphenoxylate with long-period use are an issue in children (Mehra et al., 2013). Another drug like loperamide causes bronchospasm and constipation (Prakash et al., 1980), it also causes a series of adverse effects like ileus, lethargy, and abdominal distension in children under three years old (Li et al., 2007). There are also unwanted central nervous system effects caused by opioid drugs (Khansari et al., 2013; Wood et al., 1998). This drug also causes constipation, over sedation, and respiratory depression (Choi & Billings, 2002). Nausea, vomiting, bronchospasm and face edema caused by racecadotril (Tormo et al., 2008). Although oral rehydration therapy is essential for the reduction of diarrhea-related deaths in children (Sastry & Burgard, 2005), the attack rate of diarrhea has remained unchanged. Generally, evidence-based medicine has not yet been successful in the announcement of safe and effective drugs for the treatment of gastrointestinal disease (diarrhea) (Riaz et al., 2020).

Therefore, due to this shortcoming of conventional drugs, there is a necessity for reinforcing research into medicinal herbs to search for a new remedy from natural sources (Chaddha V et al., 2013; Rawat et al., 2017). Moreover, the WHO also encourages the finding of new remedies for the treatment of diarrhea using traditional practices (Atta & Mouneir, 2004). There are many natural sources for the search of new agents to treat this disease worldwide. In Ethiopia, more than 132 medicinal plants are used for diarrhea treatment traditionally (Woldeab et al., 2018). This study aimed to investigate the mechanism behind the previous reports on the *in-vivo* antidiarrheal activity of hydro-alcoholic extracts of the leaves of *O. lamiifolium* (Alemu et al., 2022). Moreover, the finding will establish a baseline for further scientific study.

2. OBJECTIVES

2.1. General objective

To investigate *in-vivo* and *in-vitro* mechanistic study on the antidiarrheal activity of hydro-alcoholic extract of the leaves of *O. lamiifolium* Hochst. ex Benth.

2.2. Specific objectives

- To evaluate the acute toxicity profile of hydro-alcoholic extract of the leaves of *O. lamiifolium* Hochst. Ex Benth in mice.
- To re-evaluate the antidiarrheal effect of hydro-alcoholic extract of the leaves of *O. lamiifolium* Hochst. Ex Benth against castor oil-caused diarrhea in mice.
- To re-assess the anti-motility activity of hydro-alcoholic extract of the leaves of *O. lamiifolium* Hochst. Ex Benth on castor oil-caused intestinal transit in mice.
- To re-evaluate the anti-secretory effect of hydro-alcoholic extract of the leaves of *O. lamiifolium* Hochst. Ex Benth on castor oil-caused entero-pooling in mice.
- To assess the *in-vitro* spasmolytic activities of the leaves of *O. lamiifolium* Hochst. Ex Benth in isolated guinea pig ileum.
- To evaluate the opioid pathway in the antidiarrheal activity of hydro-alcoholic extract of the leaves of *O. lamiifolium* Hochst. exBenth in Castor oil-caused diarrhea in mice pretreated with naloxone.
- To determine the total phenol, flavonoid, and alkaloid quantity present in the leaves of *O. lamiifolium* Hochst. Ex Benth.

3. MATERIALS AND METHODS

3.1. Drugs and chemicals

Drugs and chemicals that were used during the study: Loperamide (Daehwa Pharmaceuticals, Republic of Korea), Castor oil (Amman Pharmaceutical Industries, Jordan), distilled water (Ethiopian Pharmaceutical Manufacturing Factory, Epharm, Ethiopia), methanol (Carlo Erba reagents, S.A.S, France), activated charcoal (Acuro Organics Ltd, New Delhi, India), Sodium nitrate, sodium hydroxide, aluminum chloride, citric acid-1-hydrate (pulver, chem, rein, ph. Eur. B.P., Franc), sodium phosphate dibasic (Germany), methanol, bromocresol green, and hydrochloric acid (Loba Chemie Pvt. Ltd.) are obtained from AAU CHS. Acetylcholine chloride, atropine sulfate, and Tyrode's solution (Sodium chloride (NaCl); potassium chloride (KCl); calcium chloride (CaCl2); magnesium chloride (MgCl2); sodium hydrogen carbonate (NaHCO3); sodium dihydrogen phosphate (NaH2 PO4); glucose) are funded by Ethiopian public health institutions (EPHI), and Naloxone hydrochloride (Neon Laboratory Ltd, India) is obtained from Amanuel specialized mental hospital.

3.2. Materials and equipment

Materials and equipment used during study include: UV Spectrophotometer (Jenway Model 6500, England), rotary evaporator (Heidolph, Germany), electronic balance (KERN-ALJ 220-4, Germany), tissue drying oven (Medite Medizintechnik, Germany), Mini orbital shaker (SSM1-STUART), Grass model 7E polygraph, Whatman filter paper (Number 1), separatory funnel, flasks, syringes with needles, gavage, scissors, surgical blade Petri dish, beaker, metered ruler and water bath.

3.3. Experimental animals

Healthy young adult swiss albino mice age ranging from 6-8 weeks weighing 24–35 g was used in the *in-vivo* study. In the *in-vitro* study guinea pigs weighing 250–300 g was used. The mice were obtained from the animal house of the School of Pharmacy, AAU, and the guinea pigs were from the Ethiopian Public Health Institution. In the animal house the mice were kept in plastic cages at room temperature on a 12-hour light and dark cycle with access to food and water. The guinea pig is also kept in a galvanized cage with free access to food like cabbages and other foods at room temperature. For seven days before conducting the experiment, the mice were adapted to the laboratory condition. The mice and pigs were denied food for 18 and 24 hours

respectively prior to the starting of the experiment with free access to water. However, in the case of the entero-pooling model, the animals were withdrawn from both food and water.

3.4. Plant collection

The leaves of *O. lamiifolium* were collected from Butajira area (135 km from AA), Guragea zone, South Nation Nationality People Region, in May 2021. The plant was verified by taxonomist Mr. Melaku Wonafrash, and a voucher specimen (number DB001) was deposited at the National Herbarium, College of Natural and Computational Sciences, AAU for future reference. The leaves of *O. lamiifolium* were washed using distilled water and dried at room temperature under shade for 1 week. Finally, the dried leaves were reduced to a smaller size.

3.5. Extraction of the plant material

3.5.1. Preparation of 80% methanol extracts of the leaves of *O. lamiifolium*.

To 300 grams of dried powder from the leaves were used. Equally divided weights of the plant material were added to two separate Erlenmeyer flasks (2 Liters). To each flask sufficient amounts of methanol (80%) up to 1500 ml were poured in the first round. It was macerated for 72 hours with infrequent shaking. After the extract was filtered using a double-layer muslin cloth and Whatman (Number 1) filter paper, residue was re-macerated for the second time and, finally, the third time by adding new solvent. The result obtained from the three-maceration process was combined and concentrated using a rotary evaporator under decreased pressure at 40°C. Finally, the product was dried in a dry oven at 40°C to remove water and stored in deep freezer. The yield of the product was determined using the formula described below.

$$\text{Percentage of yield} = \frac{\text{Extraction obtained}}{\text{Total amount of material used}} \times 100\%$$

3.6. Acute oral toxicity test

The acute toxicity study was performed on the basis of the Organization for Economic Co-operation and Development (OECD) 425 (OECD, 2022) guideline for the hydro-alcoholic extract of *O. lamifoliium* leaves (80%MEOL). A total of 5 mice were used for the test. Initially, randomly selecting a single female, non-pregnant mouse was done and fasted for 3 hours. The

mice were administered an oral bolus dose of 2gm /kg of the test substance. It was then observed carefully and continuously for the first 30 minutes for any sign of toxicity followed by every four hours for the first 24 hours. Based on the results obtained from the first mouse; additional four mice were enlisted and fasted for three hours. They were administered the same bolus dose of the extract (2 gm/kg) and watched for any sign of toxicity and death in the next fourteen days.

3.7. Grouping and Dosing

A total of thirty mice were used in each *in-vivo* model to conduct the experiment. However, in the opioid transit study only eighteen mice were used. After eighteen (18) hours they were deprived of food, mice were allocated into five groups. In every group there were six mice. All the groups were administered their treatments using oral gavage. One of the groups was assigned as negative control and administered distilled water (10 ml/kg), which was used for the reconstitution of all the drugs. The second group was allocated as positive control and treated with loperamide 3 mg/kg. For the test groups, three different doses were determined based on the acute toxicity test data such as a low dose (half of the middle dose); a middle dose (one-tenth of the dose used during the acute toxicity test) and a higher dose (twice of the middle dose) according to OECD guideline (OECD, 2022). Based on acute toxicity data the three doses were 100, 200 and 400 mg/kg of hydro-alcoholic extracts of *O. lamiifolium* leaves for the test groups. For the opioid transit study animals were grouped into three groups. The negative control treated distilled water 10 ml/kg with naloxone 2 mg/kg, positive control treated loperamide 3 mg/kg with naloxone 2 mg/kg, and for the test group 400 mg/kg with naloxone 2 mg/kg.

3.8. Determination of antidiarrheal activity

3.8.1. Castor oil-induced diarrhea

The mice were randomly grouped into 5 groups of 6 animals in each. The weight of each mouse was recorded. Thereafter, based on their weight they were treated with their dose as explained in section 3.7. One hour after they received their doses, mice were administered 0.5 ml per mouse of castor oil (CO) orally. Then after they were placed individually in the beaker and followed for any change for four hours. The following parameters i.e. Onset of time for the first diarrhea in minutes, number of defecation (i.e., frequency of wet and total stool), and weight of fecal output (wet and total) were documented for each mouse. Finally, the following parameters were calculated (Abdela, 2019; Tadesse et al., 2017).

$$\text{Percentage inhibition (PI)} = \frac{\text{Mean number of wet stools of (control group-test group)}}{\text{Mean number of wet stools of control group}} \times 100$$

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

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

3.8.2. Castor oil-induced charcoal meal test /gut motility test

After the mice were deprived of food for eighteen (18) hours, they were grouped and administered their doses depending on their weight as explained in Section 3.7. After 1 hour, they were administered 0.5 ml of CO for each mouse. Then, 1 hour after the CO treatment, 1 ml of a 5% activated charcoal suspension in distilled water was administered to each mouse as a marker. After one hour, the mice were euthanized by cervical dislocation, and the small intestine (SI) was dissected out from the pylorus to the caecum (Hussaini et al., 2021). The distance from the pyloric sphincter to the mark was measured and expressed as a percentage of the total length of the SI (Tadesse et al., 2017). The following parameters are calculated.

$$\text{Percentage inhibition} = \frac{\text{MPIC} - \text{MPIT}}{\text{MPIC}} \times 100$$

Where MPIC =Mean PI of control and MPIT = Mean PI of test group

$$\text{Peristaltic index (PI)} = \frac{\text{Distance moved by charcoal meal}}{\text{Total length of small intestine (pylorus to caecum)}} \times 100$$

3.8.3. Castor oil-induced entero-pooling activity

The entero-pooling test was determined using the method followed by Degu et al (2016). For 18 hours, all mice were withdrawn from food and water. They were allocated into five groups 6 mice in each as explained in section 3.7. The mice were then administered their doses as explained in Section 3.7. Thereafter one hour later they were administered 0.5 ml of CO per mouse. One hour later the mice were euthanized by cervical dislocation (Hussaini et al., 2021) and the abdomen was opened. The whole length of the SI was carefully removed and weighed. Thereafter the intestinal contents were collected into a graduated tube and measured. The empty intestine was reweighed and reduced from the previous weights of the intestine. Finally, the following parameters were calculated.

$$\text{Percentage of inhibition} = \frac{(\text{MVICC} - \text{MVICT})}{\text{MVICC}} \times 100$$

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

$$\text{Percentage inhibition} = \frac{(\text{MWICC} - \text{MWICT})}{\text{MWICC}} \times 100$$

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

3.8.4. The in vivo anti-diarrheal index (ADI $_{in\text{-}vivo}$)

The *in-vivo* antidiarrheal index (ADI $_{in\text{-}vivo}$) was determined based on the formula described below (Hussain et al., 2009).

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

$$D\ freq = \frac{\text{Onset of diarrhea in minute of the (test -control) group} \times 100}{\text{Onset of diarrhea in minute of the control group}}$$

$$G\ meq = \frac{\text{Distance travelled by the charcoal marker in the (control -test) group} \times 100}{\text{Distance travelled by the charcoal marker in the control group}}$$

$$Pfreq = PI = \frac{\text{Mean number of wet stools of (control-treated) group} \times 100}{\text{Mean number of wet stools of control group}}$$

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

3.8.5. Spasmolytic activity study in isolated guinea-pig ileum

The experiments were conducted as explained previously (Babaei et al., 2008; Ventura-Martínez et al., 2018). For 24 hours, the guinea pigs were withdrawn from food but not water and they were sacrificed using a high dose of ketamine anesthesia. Immediately after the abdomen was opened, about a 20 cm-long piece of ileum was taken into a Petri dish containing Thyroid solution. About 2 to 3 cm long pieces of ileum were fixed in a 30 ml tissue bath containing Tyrode's solution. The tissue bath was aerated with a solution of 95% O_2 and 5% CO_2, and maintained at a temperature of 37oC.

The composition of Tyrode's solution per litter was potassium chloride (KCl=0.2g), Sodium chloride (NaCl=8g), Sodium dihydrogen phosphate (NaH2PO4=0.05g), Magnesium dichloride (MgCl2=0.1g), sodium hydrogen carbonate (NaHCO3 1g), calcium dichloride (CaCl2=0.2g), and glucose 1g (pH 7.4).

The lower part of the segment of the ileum was connected to the metal tissue hook. The opposite part was attached by a cotton thread to an isotonic transducer and connected to an oscillograph. For each tissue, a 1-gram preload was applied. The tissue was washed several times in a 5-minute interval using Tyrode mixture and allowed to stabilize for half hour prior to isotonic contractions of agonist were recorded.

Different doses of the agonist with a concentration of 1 µg/ml were tested, and responses to a sub-maximal dose of agonist were recorded. Thirty seconds was used for agonist contact time along with a five-minute interval between each dose. Once the tissue was equilibrated with

reproducible effects from the doses of the agonist, the extract was tested. This was done by adding, every 5 minutes, graded doses (10 mg/ml) of the hydro-alcoholic extract of *O. lamiifolium* to the tissue bath, and followed by a constant concentration of Ach (0.27×10^{-2} μM). The response of the tissue was recorded on a computerized data processing system via an isotonic force transducer (Ventura-Martinez et al., 2020).

Finally, the height of each response was measured and determined as a percent of response or relaxation. From the height of response produced by agonist at the dose of 0.4ml each response of the extract was reduced and calculated as percentage of response.

3.8.6. Castor oil-induced diarrhea in mice pretreated with naloxone

The antidiarrheal mechanism of action of the hydro-alcoholic extract of *O. lamiifolium* was evaluated to investigate the presence of an opioid pathway (Oliveira et al., 2021). The mice were grouped and treated as explained in section 3.7. First all the mice were treated with naloxone 2 mg/kg subcutaneously after they were deprived of food for 18 hours. 30 minutes later the mice were administered their respective dose as explained in section 3.7. After 30 minutes mice were administered CO (0.5 ml/mouse). Thereafter, the mice were administered 1 ml of a 5% charcoal suspension in distilled water 1 hour after CO treatment. One hour later, the abdomens of the mice were opened after they were euthanized by cervical dislocation. The distance moved by marker from the pyloric sphincter and the total length of the SI were measured using a metered ruler. Finally, the formula described under section 3.8.2. were calculated (Tadesse et al., 2017).

3.9. Quantitative determination of phenols, flavonoids, and alkaloids

Determination of total phenol

The Folin-Ciocalteu method was used to estimate the quantity of total phenol present in the hydro-alcoholic extracts of *O. lammifolium* (Mahalle and Gupta, 2021). First serial dilution of gallic acid (100, 50, 25, 12.5, and 6.25 μg/ml) in distilled water was prepared to set up a calibration curve. Then one ml of each solution of gallic acids was added into test tubes. Five ml of distilled water and 0.5 ml of Folin-Ciocalteu were poured into the test tubes. Five minutes later, 1.5 ml of a 20% sodium carbonate solution was poured. Thereafter, the test tube was made up to 10 ml using distilled water and incubated for 90 minutes. Lastly, the absorbance of the solution was measured using UV spectrophotometry at 760 nm. The procedure was repeated for the extract at 100 μg/mL and blank (distilled water) instead of gallic acid. The total phenolic

quantity was calculated using a standard curve of gallic acid (y = 0.0098x-0.1294, R^2 = 0.966). At the end the results obtained were expressed as mg of gallic acid equivalent per gram of dry extracts (Nigatu et al., 2021). The experiment was repeated three times.

Determination of total flavonoid content

The determination of the total flavonoid content of hydro-alcoholic leaf extracts of *O. lamiifolium* was based on the aluminum chloride method (Patel et al., 2010). First serial dilutions of the standard (quercetin) at concentrations of 1, 0.50, 0.25, 0.125, and 0.065 mg/ml were prepared. A sufficient volume of 10% aluminum chloride ($AlCl_3$), 5% sodium nitrate ($NaNO_{2)}$, 1M sodium hydroxide (NaOH), and a 1 mg/ml extract solution were prepared separately.

One ml of quercetin solution was transferred into test tubes. Thereafter 0.3 ml of 5% sodium nitrate was transferred to the test tube. After 5 minutes, 0.3 ml of 10% aluminum chloride was transferred to the test tube and mixed with the solutions and allowed to rest for 5 minutes. Then, after adding 2 ml of 1M sodium hydroxide to the solutions, the volume was adjusted to 10 ml using distilled water. Finally, after 30 minutes of incubation of the mixed solution, the absorbance was measured in a UV spectrophotometer at 510nm. In the same way the procedure was repeated for 1 ml of the extract and blanks. The total flavonoid quantity was calculated using a standard curve of quercetin (y = 0.145x + 0.0241, R^2 =0.9999). Lastly, the result obtained is expressed as milligram of quercetin equivalent per 100 gram of dry extracts (Nigatu et al., 2021). All procedures were conducted three times.

Determination of total alkaloid content

Quantitative estimation of alkaloids in hydro-alcoholic extracts of *O. lamiifolium* was done based on the method followed by (Ajanal et al., 2012).

Preparation of bromocresol green and phosphate buffer

A mixture of bromocresol green (BCG) (34mg), 2N sodium hydroxide (1.5 ml) and distilled water (2.5 ml) were heated to make a completely dissolved solution. After the mixture was dissolved well the volume was adjusted to 500 ml by adding additional water to prepare BCG. A phosphate buffer solution (PH, 4.7) was prepared with 35.8g of Na_2HPO_4 and 21.02 g of citric acid. They were poured into flasks separately and dissolved with distilled water. Then after the volume for each solution was made up to 500 ml. Finally, the two mixtures were mixed and labeled to use later.

Preparation of atropine curve

First serial concentrations of standard solution (Atropine), (120, 60, 30, 15, and 7.5 µg/ml) were prepared in methanol. Then after 1 ml of standard was added into separatory funnels. 5 ml of phosphate buffer and 5 ml of BCG mixture were transferred to the filtrates. The mixture was shaken two times using 4 ml of chloroform. Thereafter the chloroform extracts were collected in the Eppendorf tube, and its final volume was made up to 10 ml with chloroform. The absorbance was measured at a wavelength of 470 using. This was done in triplicate.

Separation of alkaloids

Two ml of extract solution at a concentration of one mg/ ml prepared in methanol were mixed with two ml of 2N hydrochloric acid solution and filtered. One ml of the filtrate was transferred to a separatory funnel and washed twice using 5 ml of chloroform. Then after the chloroform extract was avoided and the PH of the rest solution was adjusted to neutral using a 0.1 M sodium hydroxide solution. To the neutralized solution, 5 ml of BCG and 5 ml of buffer mixture were added and shaken. The solution was extracted with 4 and 4 ml of chloroform by vigorous shaking. The extract was then collected in the Eppendorf tube, and its final volume was made up to 10 ml. In the same way this procedure was repeated in the blank (methanol). All procedures were conducted three times. The total alkaloid quantity was calculated using a standard curve of atropine ($y = 0.0022x-0.0133$, $R^2 = 0.9948$). Finally, the estimated total alkaloid quantity was expressed as mg of atropine equivalent per gram of dry extracts.

3.10. Statistical analysis

The final data were expressed as the mean ± standard error of the mean (SEM). The results obtained from study were analyzed using the software Statistical Package for Social Sciences (SPSS), version 25. The statistical significance was resolved by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. A P-value which is less than 0.05 was considered statistically significant.

4. RESULT

4.1. Extraction yields

19.5 g of extract were obtained from 300g of dried powder of *O. lamiifolium* leaves. The calculated yield of the crude methanol extracts is 6.5% (w/w).

4.2. Acute oral toxicity test

The hydro-alcoholic extract of *O. lamiifolium* has a wider safety margin. It produced neither observable toxicity nor death during the fourteen-day observation time after oral administration of oral bolus dose of five times the higher dose of the test substance according to the OECD guideline. There was neither physical nor behavioral change seen during the observations of the experimental mice. This revealed that the LD_{50} value of hydro-alcoholic extracts of *O. lamiifolium* leaves is greater than 2 g/kg in mice.

4.3. Determination of antidiarrheal activity

4.3.1. The effects of hydro-alcoholic extracts of *O. lamiifolium* leaves on a castor oil-induced diarrheal model

As the data presented in Table 1, All doses of hydro-alcoholic extracts of *O. lamiifolium* leaves profoundly delayed the onset time of the first diarrhea appearance and also decreased the number of wet and total stool defecation as compared to the control ($p<0.05$). However, the lower doses (100 mg/kg) could not show meaningful difference compared to the control in some parameters of diarrhea, such as the mean weights of wet and total feces. In all aforementioned parameters of diarrhea, a significant difference was obtained when 100 mg/kg compared with 400 mg/kg of the test ingredient. The 200 mg/kg extracts could not show a statistically significant difference compared with 400 mg/kg in delaying the first diarrhea. Only the higher dose (400 mg/kg) produced comparable results with the loperamide (84.91%), in percent of diarrhea reduction. Moreover, the percentage of mean fecal output in the case of 400mg/kg is less than loperamide 3 mg/kg (figure 3). Furthermore, the percentage of diarrhea inhibition was 36.36% ($p<0.05$), 48.54% ($p<0.05$), and 81.81% ($p<0.001$) at the doses of 100, 200, and 400 mg/kg respectively (Table 1).

Table 1: Effects of hydro-alcoholic extracts of the leaves of *O. lamiifolium* on castor-oil-induced diarrhea

Dose administered	Onset of Diarrhea (Minutes)	Number of wet feces	Total Number of feces	Mean weight of wet feces (in gm)	Mean weight of total feces (in gm)	% of reduction Using No of wet feces.
DW 10ml/kg	61.33±7.14	5.50±1.04	6.16±0.40	0.27±.05	0.37±.05	
MEOL100 mg/kg	104.83±7.70[a 1b3c1]	3.50±1.04[a1 b3c3]	4.33±0.81[a1 b3c3]	0.20±.02[b3c3]	0.33±.04[b3c3]	36.36
MEOL200 mg/kg	158.50±5.24[a 1b1e1]	2.83±0.75[a1 b1c12]	3.33±0.516 [a1b2c1]	0.15±.03[a1b3c 2]	0.25±.05[a2b2c1]	48.54
MEOL400 mg/kg	169.33±7.76[a 3]	1.00±0.63[a3]	2.00±0.89[a3]	0.06±.03[a3]	0.11±.04[a3]	81.81
Loperamide 3mg/kg	174.83±8.20[a 3]	0.83±0.75[a3]	1.83±0.75[a3]	0.04±.03[a3]	0.12±.06[a3]	84.91

Values are expressed as Mean ± SEM (n = 6), One-Way ANOVA was used for the analysis followed by Tukey test; [a] compared to control, [b] to standard drug, [c] to 400 mg/kg, [d] to 200 mg/kg, [e] to 100 mg/kg; [1]P <0.05, [2]P <0.01, [3]P <0.001. DW: Distilled water; MEOL= 80% methanol extracts of *Ocimum lamiifollium*

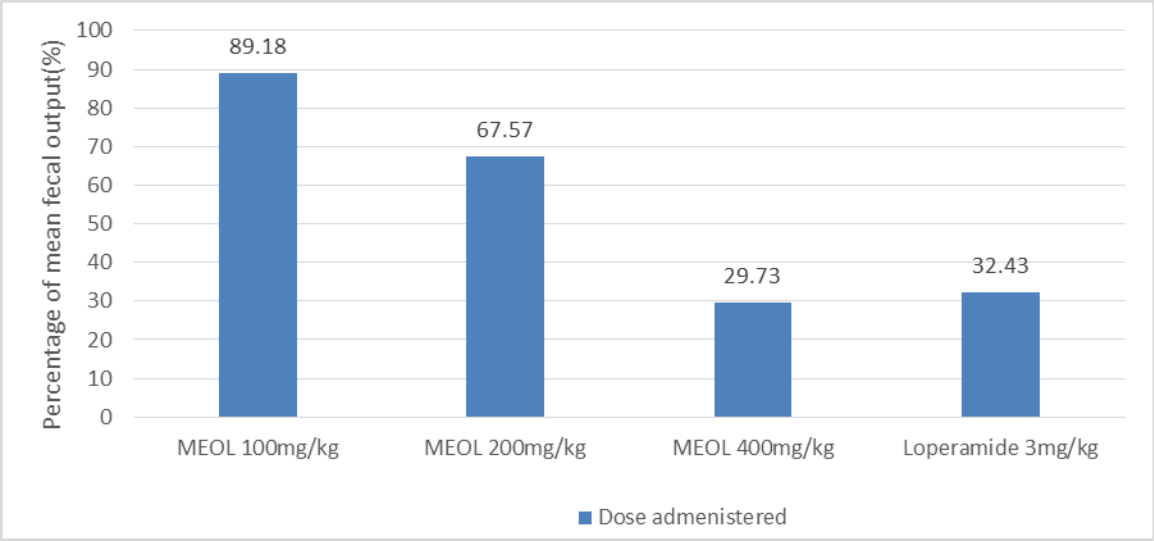


Figure 3: Percentage of mean fecal output of hydro-alcoholic extract of the leaves of *O. lamiifolium* in castor oil-induced diarrheal model.

4.3.2. The effects of the hydro-alcoholic extracts of *O. lamiifolium* leaves on castor oil-induced intestinal transit in mice

The data shown in Table 2, hydro-alcoholic extracts of *O. lamiifolium* leaves profoundly reduced intestinal transit of charcoal meal at all doses of the test substance compared to the control ($p<0.001$). Distance moved by the charcoal marker in case of control was 32.67 ± 1.71 and its peristalsis index =59.22. The distance moved by the test ingredient at the dose of 100, 200 and 400mg/k were 22.33 ± 1, $p<0.001$; 18.83 ± 0.94, $p<0.001$ and 10.58 ± 0.55, $p<0.001$ respectively. Their peristalsis index was 41.35, 34.98 and 19.53 at the dose of from the lower to the higher respectively. The percentage inhibition of gut meal movement was 30.18% ($p<0.001$), 40.93% ($p<0.001$), and 67.02% ($p<0.001$) at a dose of 100, 200, and 400 mg/kg respectively. The higher dose (400 mg/kg) of the test ingredient produced comparable results in the inhibition of gut motility compared to the standard (68.88%, $p<0.001$).

Table 2: Effects of hydro-alcoholic extracts of the leaves of *O. lamiifolium* on castor-oil caused intestinal transit in mice.

Dose administered	Length of small intestine (SI)	Distance moved by charcoal marker(cm)	Peristalsis index (%)	% of inhibition
DW 10 ml/kg	55.17±1.14	32.67±1.71	59.22	
MEOL100 mg/kg	54.00±0.58	22.33±1.00^{a3b3c3}	41.35 ^{a3b3c3}	30.18
MEOL200 mg/kg	53.83±0.98	18.83±0.94^{a3b3c3}	34.98 ^{a3b3c3}	40.93
MEOL400 mg/kg	54.16±0.87	10.58±0.55^{a3}	19.53 ^{a3}	67.02
Loperamide 3 mg/kg	53.33±1.20	9.83±0.60^{a3}	18.43 ^{a3}	68.88

Values are expressed as Mean ± SEM (n = 6), One-Way ANOVA was used for the analysis followed by Tukey test; ^{a} compared to control, ^{b} to standard drug, ^{c} to 400 mg/kg, ^{d} to 200 mg/kg, ^{e} to 100 mg/kg; ^{1}P <0.05, ^{2}P <0.01, ^{3}P <0.001. DW: Distilled water; MEOL= 80% methanol extracts of *Ocimum lamiifollium,* SI=small intestine

4.3.3. The effects of the hydro-alcoholic extracts of *O. lamiifolium* leaves on castor oil-induced entero-pooling.

As presented in Table 3, hydro-alcoholic extract of *O. lamiifolium* profoundly reduced both mean weight and mean volume of intestinal contents. The mean weight and mean volume of intestinal contents of the control were 1.24 ± .01 and 0.89 ± 0.02 respectively. The mean weight of intestinal contents for the test ingredient were 1.12 ± 0.02, 0.81 ± 0.01 and 0.72 ± 0.01 at the doses of 100, 200 and 400 mg/kg respectively. The percentage of inhibition in mean weight of intestinal were found to be 9.68% ($p<0.001$), 34.68% ($p<0.01$), and 41.94% ($p<0.001$) at dose of the lower to the higher respectively. The mean volume of the test ingredients was 0.78 ± 0.01, 0.60 ±0.01 and 0.49 ± 0.02 at the doses of the lower to the higher respectively. Their percentage inhibition of volume of intestinal content was 12.36% ($p<0.01$), 32.58% ($p<0.001$), and 44.94% ($p<0.001$) at doses of 100, 200, and 400 mg/kg respectively (Table 3).

Table 3: Effects of 80% methanol extracts of the leaves of *O. lamiifolium* on intestinal fluid accumulation in mice.

Dose administered	Volume of intestinal contents(ml)	Percent of inhibition	Weight of intestinal contents(gm)	% of inhibition
DW 10 ml/kg	0.89±.02		1.24±.01	
MEOL100 mg/kg	0.78±.01^{a2b3c3d3}	12.36	1.12±.02^{a3b2c2d2}	9.68
MEOL200 mg/kg	0.60±.01^{a3b3}	32.58	0.81±.01^{a2b2c1}	34.68
MEOL400 mg/kg	0.49±.02^{a3}	44.94	0.72±.01^{a3}	41.94
Loperamide 3mg/kg	0.48±.01^{a3}	46.07	0.70±01^{a3}	43.55

Values are expressed as Mean ± SEM (n = 6), One-Way ANOVA was used for the analysis followed by Tukey test; ^{a} compared to control, ^{b} to standard drug, ^{c} to 400 mg/kg, ^{d} to 200 mg/kg, ^{e} to 100 mg/kg; ^{1}P <0.05, ^{2}P <0.01, ^{3}P <0.001. DW: Distilled water; MEOL= 80% methanol extracts of *Ocimum lamiifollium*

4.3.4. *In-vivo* anti-diarrheal index (ADI)

As data presented in Table 4, the result revealed that there is a dose-dependent increment in the ADI value of hydro-alcoholic extracts of *O. lammifolium* leaves. Based on the data shown in the table, the higher *in vivo* ADI (98.8%) was obtained from the higher dose (400 mg/kg) next to the loperamide (102.7).

Table 4: **In-vivo *anti-diarrheal indices of hydro-alcoholic extracts of the leaves of* Ocimum lamiifolium**

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)
DW 10ml/kg	------	------	------	------
MEOL 100mg/kg	70.93	30.18	36.36	42.7
MEOL 200mg/kg	158.44	40.9	48.55	60
MEOL 400mg/kg	176.1	67	81.82	98.8
Loperamide 3mg/kg	185.1	68.88	84.91	102.7

DW=Distilled water, MEOL= 80% methanol extracts of *Ocimum lamiifollium*

4.3.5. The effect of hydro-alcoholic extracts of *O. lamiifolium* leaves on Acetylcholine-induced contraction of isolated Guinea-pig ileum

At micromolar concentrations, acetylcholine induced concentration-dependent contraction of tissue, producing its maximum effect within 30 seconds of contact as shown in Figure 4. When tested on a guinea pig ileum, hydro-alcoholic extracts of *O. lamifoliium* leaves showed a spasmolytic effect at doses ranging from 1 to 4 mg, and an enhanced decline of the height of the contraction was observed with increasing doses (Table 5, Figure 5). The maximal percentages of response or relaxation produced by graded doses of the test ingredient were 20%, 65% and 75% at the dose of 1 mg, 2 mg and 4 mg respectively (Table 5).

Table 5: response of 80% methanol extracts of the leaves of *O. lamiifolium* (10 mg/ml) to acetylcholine (0.68 x 10^{-2} µM) induced contractions of isolated guinea pig ileum.

Dose of Ach and OL	Height of response (mm)	%inhibition (relaxation)	Response
Ach (0.68 x 10^{-3} **µM**)	9	-	Contraction
Ach (0.14 x 10^{-2} **µM**)	9	-	Contraction
Ach (0.27 x 10^{-2} **µM**)	20	-	Contraction
Ach (0.55 x 10^{-2} **µM**)	11	-	Contraction
Ach (0.27 x 10^{-2} **µM**) + OL (1 mg)	16	20	Relaxation
Ach (0.27 x 10^{-2} **µM**) + OL (2 mg)	7	65	Relaxation
Ach (0.27 x 10^{-2} **µM**) + OL (4 mg)	5	75	Relaxation

Ach=Acetylcholine, OL=*Ocimum lamiifolium*

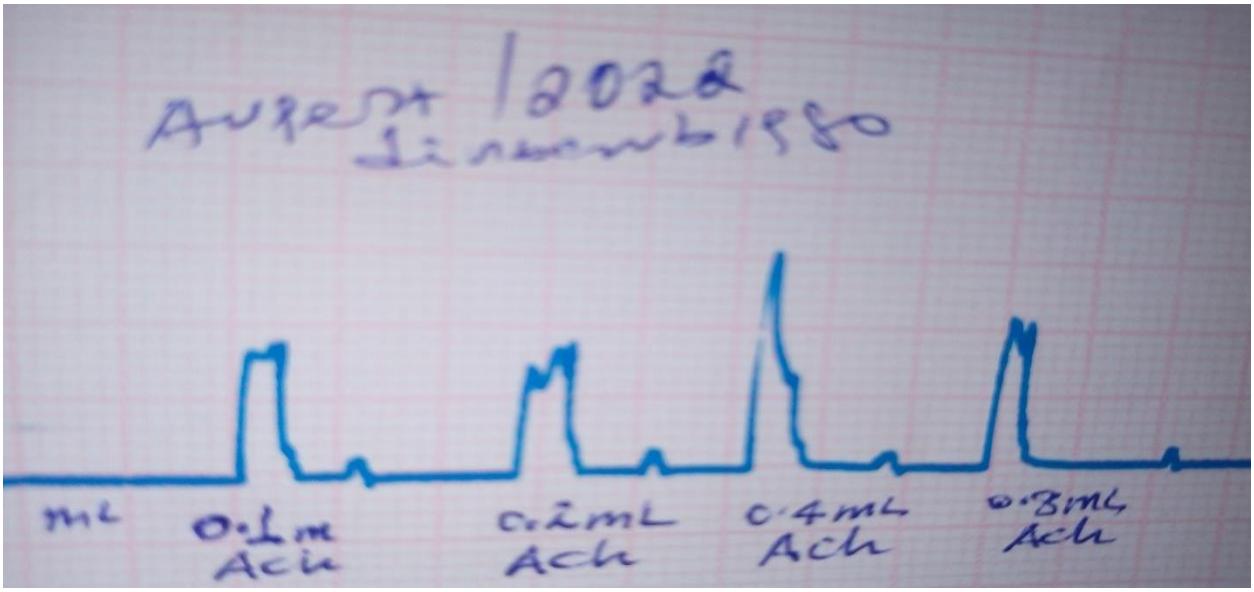


Figure 4: Spasmogenic effects of Acetylcholine on isolated guinea pig ileum preparation.

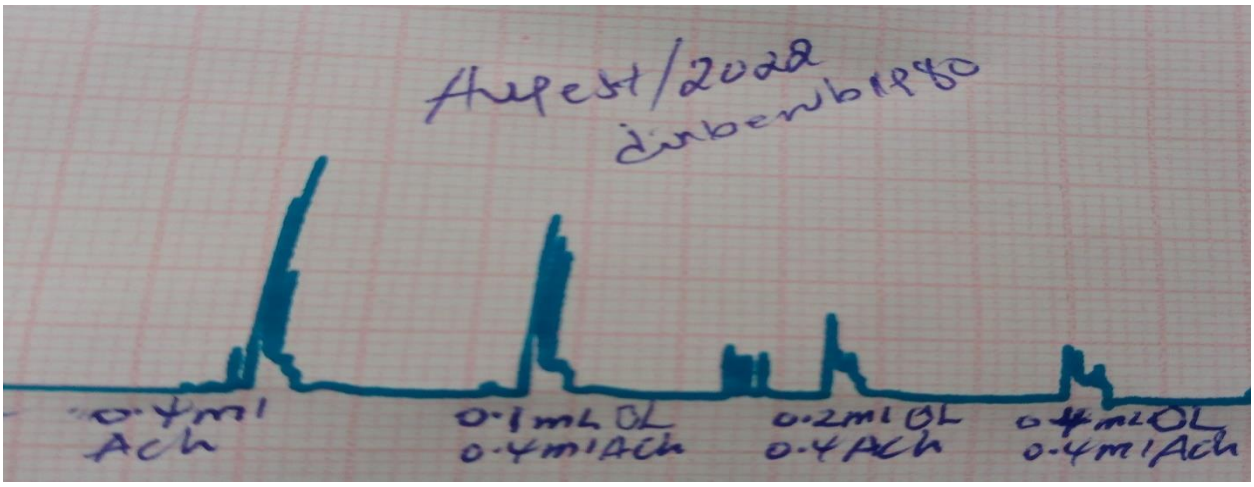


Figure 5: Spasmolytic effects of hydro-alcoholic extracts of *O. lamiifolium* leaves on Acetylcholine induced isolated guinea pig ileum contraction.

Figure 6 shows that the effective dose to produce 50% response (EC_{50}) of the hydro-alcoholic extracts of the leaves *O. lamiifolium* is 1.8 mg. 1.8 mg of the extract produces 50% relaxation. The result is obtained from the graph y=91.353 + 117.19.

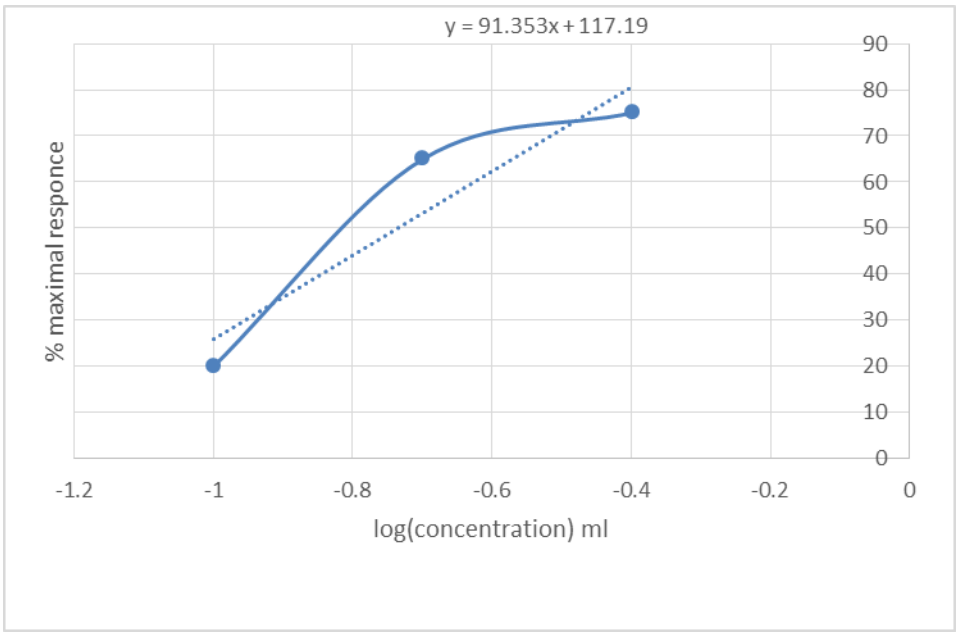
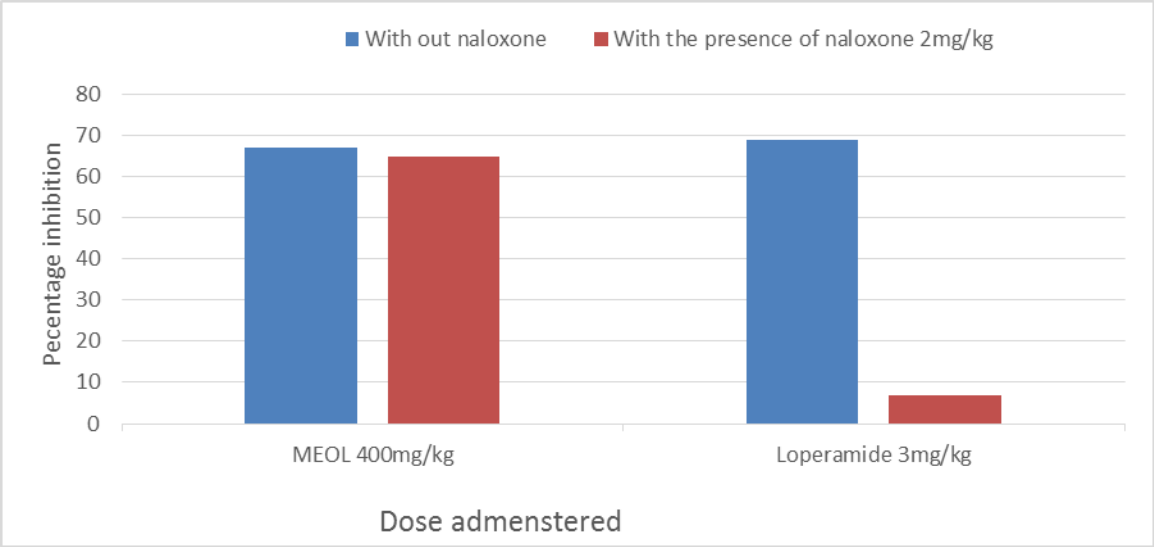


Figure 6: Curve shows log(dose(ml)) of extract versus percent of maximal response.

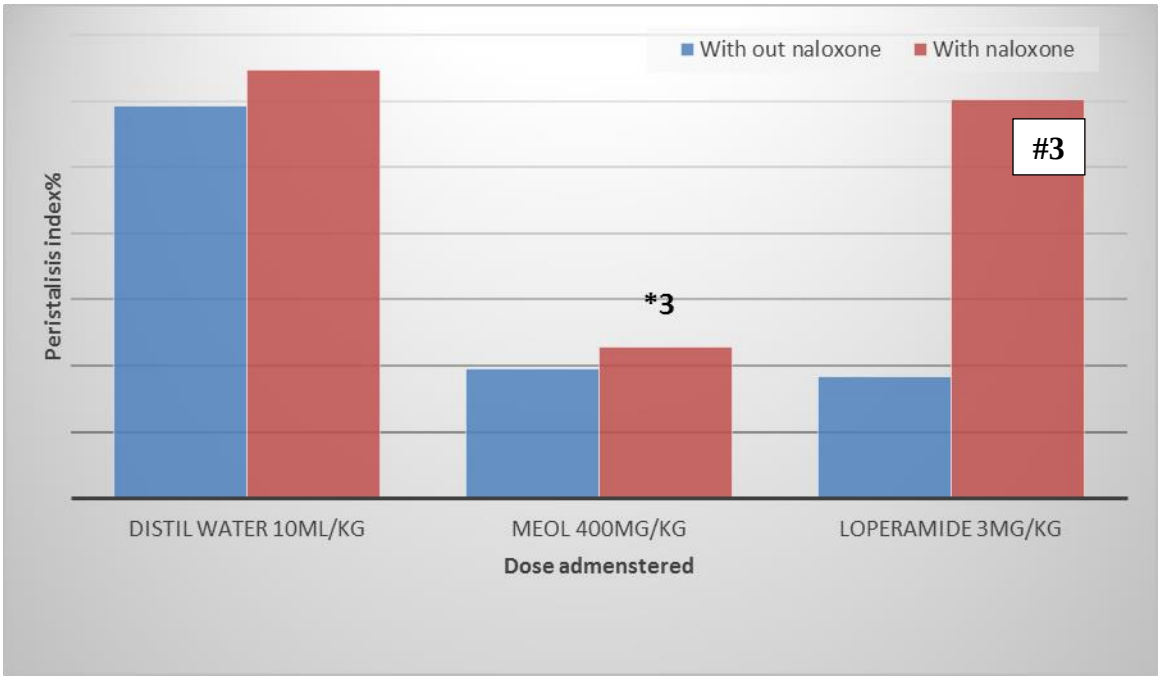
4.3.6. The effects of hydro-alcoholic extracts of *O. lamiifolium* leaves in castor oil-induced diarrhea in mice pretreated with naloxone(antagonist)

In the presence and absence of naloxone, the percentage inhibition by 400 mg/kg of the test substance was 64.68% (figure 7) and 67.02% (Table 3), respectively. In the presence and absence of naloxone, charcoal meal traverse was profoundly reduced by the test substance ($p<0.001$) as shown in figure 8. In the presence and absence of naloxone, the percentage inhibition by loperamide (3 mg/kg) is 6.89% (figure 7) and 68.88% (Table 3), respectively. The charcoal meal traverse is insignificantly reduced by loperamide compared to the control in the presence of naloxone.



MEOL=80%methanol extracts of *O. lamiifolium*

Figure 7: The percentage inhibition of peristalsis in the presence and absence of specific opioid receptor antagonist (Naloxone).



Values are expressed as Mean ± SEM (n = 6), One-Way ANOVA was used; * compared to distil water + naloxone, #compared to loperamide without naloxone; [1]P <0.05, [2]P <0.01, [3]P <0.001**;** MEOL= 80% methanol extracts of *O. lamiifollium*

Figure 8: Peristalsis index in the presence and absence of specific opioid antagonist (Naloxone).

4.4. Total Phenolic, Flavonoid and Alkaloid content of hydro-alcholic extracts of the leaves of *O. lamiifolium*.

The total phenol, flavonoid, and alkaloid content of 80% methanol extracts of the leaves of *O. lamiifolium* is found to be 201.4 mg/gm, 178.6 mg/gm, and 45.6 mg/gm, respectively, as determined from the curves fitted for standards (Figure 9).

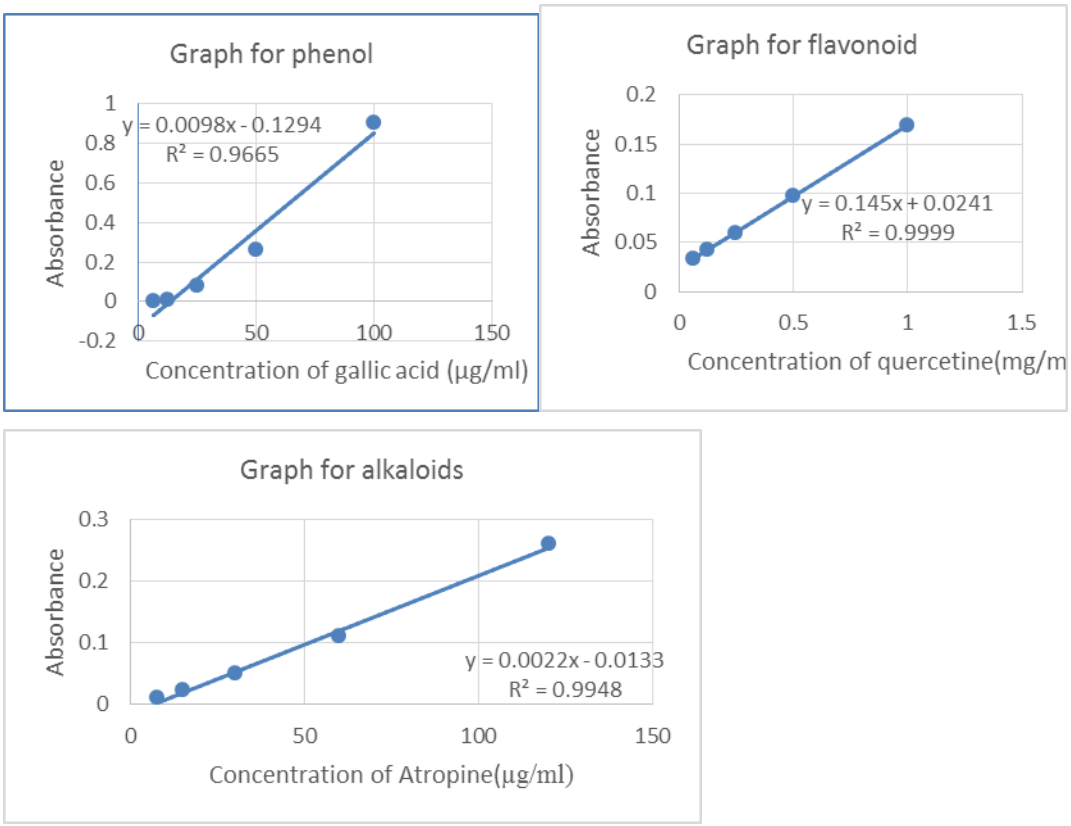


Figure 9: Calibration curves for determination of total phenols, flavonoids and alkaloids.

5. DISCUSSION

Hydro-alcoholic extractions of *O. lamiifolium* leaves resulted in 6.5% (w/w) yields. When compared to its aqueous and chloroform extraction in the earlier studies, this yield is higher (Kefe et al., 2016; Makonnen et al. 2003). This may be because the substance in *O. lamiifolium* leaves dissolves more easily in methanol. Its expanded polarity index (from non-polar to polar) may also be a contributing element.

This plant has undergone phytochemical screening in different laboratories in previous research. The quantitative analysis of the leaves of this plant showed the presence of a significant quantity of phenol, flavonoids, and alkaloids. The quantity of phenol determined in this study is higher than previously determined contents present in the seeds of this plant in India (Hakkim et al., 2008). The difference may be the difference in the plant part used, the geographical difference of the plant grows and or the time of collection.

The acute toxicity studies of the hydro-alcoholic extracts of *O. lamiifolium* leaves revealed that five times the higher dose of the extract did not produce any sign of observable toxicity or death in the experimental mice with in the first 24 hours and post-treatment. This indicates that the oral LD_{50} of hydro-alcoholic extracts of *O. lamiifolium* leaves is greater than 2 g/kg and that it is safe at the acute level. The test was done according to the oral toxicity tests recommended by the OECD guideline (OECD, 2022). It is also reported that sub-chronic toxicity studies in rats at the dose of 400 mg/kg of the aqueous extracts of this plant did not cause any toxicity. However, at the higher dose (600mg/kg) of the aqueous extract showed toxicity (Adane et al., 2023).

The castor oil-caused diarrhea model was used to assess the overall anti-diarrheal potentials of the test ingredient regarding its impact on secretion and motility. Diarrhea is resulted from increased bowel movement, an excessive amount of gut secretion and reduced intestinal reabsorption (Rao et al., 1987). It can be characterized by either hyper-secretory or hyper-motility.

It is well documented that castor oil is used in the study of antidiarrheal activity to induce diarrhea (Suleiman et al., 2017; Umer et al., 2013; Zayede et al., 2020). The metabolism of castor oil by lipase liberates ricinoleic acid in the gut (upper parts of small intestine) (Watson &

Gordon, 1962). The effect of ricinoleic acid in the gut is because of its capability to stimulate the G protein-coupled prostanoid receptor (E3) in the smooth muscle cells of the intestine (Tunaru et al., 2012). This receptor is commonly expressed in uterine and intestinal smooth muscle (Bos et al., 2004), and are activated by this metabolite. When ricinoleic acid binds to the prostanoid receptor it activates the G protein. The activation of this protein activates adenylyl cyclase (AC) and resulted in an increase in the concentration of cAMP (Bos et al., 2004; Mapesa et al., 2021). Moreover, this metabolite also stimulates epithelial cells to produce nitric oxide (NO) and cause the production of prostaglandins (E series) (Mascolo et al., 1993, 1997). Prostaglandins are known to be good diarrhoeagenic agents in experimental animals. Therefore, inhibitors in prostaglandin biosynthesis are believed to inhibit castor oil (ricinoleic acid)-induced diarrhea (Mascolo et al., 1993; Praveen, 2010; Tunaru et al., 2012).

In the castor oil-induced diarrhea model, the hydro-alcoholic extracts of *O. lamiifolium* leaves significantly delayed the time of diarrhea onset and decreased the purging frequency in a dose-dependent fashion at all doses ($p<0.05$). Inconsistent to this study, only 400 mg/kg of the same plant extract produced delay in onset of diarrhea and frequency of defecation (Alemu et al., 2022).

In this study the highest effect was produced by 400 mg/kg in all of the described parameters (Table 1). This result is comparable with the effect obtained from the same plant studied in a different setup (Alemu et al., 2022). A single report studied in Nigeria on aqueous extracts of the leaves of *Ocimum gratissimum* also revealed a dose-dependent decrease in the number of defecation (Ezekwesili et al., 2005). However, in this study only the middle and the higher doses of the test ingredient meaningfully reduced other parameters (i.e., mean weight of wet and total stools). Inconsistent to this, 100 mg/kg of the extract of the same plant reduces the mean weight of wet stools significantly ($p<0.001$) (Alemu et al., 2022). Furthermore, the percentage reduction of diarrhea by the highest dose of the ingredient is 81.81% (Table 1). It is a comparable result with the standard, loperamide 3mg/kg (84.91%). Similarly, the result obtained from the higher dose of the extract is comparable with the standard in the percentage reduction of diarrhea (Alemu et al., 2022). The higher dose (400 mg/kg) of the test ingredient produced a lesser percentage of mean fecal output than the standard and all the other doses (Figure 3). In all

aforementioned parameters, loperamide produced a significant difference compared with the control ($p<0.001$).

The medicinal effects of plants are because of their metabolites. Secondary metabolites such as tannins, alkaloids, saponins, flavonoids, steroids or terpenoids are attributed to anti-diarrheal activities (Kumar, 2010; Odo et al., 2013; Rawat et al., 2017; Yakubu & Salimon, 2015).

The astringent effects of tannin and its capability of forming a protein tannate complex on the mucosa make the intestine more resistant and reduce secretion (Ashok & Upadhyaya, 2012). Steroids such as phytosterols inhibit prostaglandin E2 production (Awad et al., 2004). Both alkaloids (Lema et al., 1986) and flavonoids such as quercetin and kaempferol (Ferrfindiz & Alcaraz, 1991; Osei et al., 2020) are also implicated in the hindering of prostaglandin production through the inhibition of the key enzymes (cyclo-oxygenase one and two and lipoxygenase). Rosmarinic acid (ester of caffeic acid) is phenolic compounds present in *O. lamiifolium* (Amabye & Mussa, 2015; Hakkim et al., 2008). This compound is also attributed to anti-inflammatory activity by inhibiting the expression of cyclo-oxygenase enzymes (Gamaro et al., 2011).

A growing evidence also showed non-steroidal anti-inflammatory drug (NSAID) inhibits castor-oil caused diarrhea (E. Niemegeers, 1978). In the previous study, aqueous and ethanol extracts of *O. lamiifolium* leaves showed anti-inflammatory (Mequanint et al., 2011) and antipyretic activity (Makonnen et al., 2003). This may be due to the inhibition of prostaglandin production.

It is therefore apparently reasonable to say that the antidiarrheal activity of the hydro-alcoholic extracts of the *O. lamiifolium* leaves is related to the inhibition of ricinoleic acid-induced prostaglandin production. Previously, it was reported that the plant *O. lamiifolium* leaves have different secondary metabolites detected through phytochemical screening. These are alkaloids, phenols, flavonoids, tannins, steroids, saponins, glycosides, sugars, and triterpenoids (Makonnen et al., 2003; Nair et al., 2016; Sahalie et al., 2018). Thus, it is reasonable to say they are responsible for the anti-diarrheal activities of the hydro-alcoholic leaf extract of *O. lamiifolium.*

Pre-treatment of this test ingredient also significantly suppressed distance moved by marker in the small intestine in all doses ($p<0.001$) (table 2). This clearly denotes that the leaf extracts have the capacity to reduce the frequency of defecation (diarrhea severity). In contrast to this study, only the higher dose of the same plant extract significantly reduced the distance traveled by

markers studied in other setups (Alemu et al., 2022). Generally, all tested doses of the test ingredient reduced gut motility in a dose-dependent fashion. Furthermore 400mg/kg of the test ingredient produced comparable results with loperamide. This result is in line with other studies on the aqueous stem extract of *L. camara* (Tadesse et al., 2017). This showed that hydro-alcoholic extracts of *O. lamiifolium* have anti-motility or anti-peristalsis activity. One of mechanisms many anti-diarrheal agents work through is the suppression of gut motility (Suleiman et al., 2017). Delaying gut motility contributes to further absorption of fluid from feces and electrolytes in the gastrointestinal tract (Ezenwali et al., 2009; Odo et al., 2013). This denotes the presence of metabolites in the extract.

Certain amines such as serotonin and acetylcholine are ascribed to the stimulation of gut motility. Some of the receptors that are associated with the serotonergic effects are S-HT1, S-HT2, S-HT3 and S-HT4-receptors) and the activation causes gut motility (Camilleri, 2009; Rozé, 1980). Therefore, the anti-serotonin activity may be one possible mechanism by which the test ingredient causes dysmotility.

It is well known that the reference drug loperamide used in this study mediates its effect through the stimulation of opioid receptor (μ-receptor), thereby blocking Ach release. The anti-diarrheal effects of flavonoids and tannins are due to their capacity to inhibit gut motility (Ashok & Upadhyaya, 2012). Hence, the considerable anti-motility effects of the test substance are possibly associated with the combining or synergistic hindering effects of metabolites on castor oil-caused motility.

Diarrhea resulted from a deviation in the balance between absorptive and secretory processes within the bowel and the disturbance of basic pathophysiological processes, including active secretion (Field, 2003). The other model used in this study is the entero-pooling model, which aimed to determine the anti-secretory effects of the test ingredient.

At all tested doses of the test ingredient, both the average volume and weight of intestinal contents were significantly reduced ($p<0.01$) (Table 3). This result is consistent with the study on the middle and the higher doses of the same extract done in different settings (Alemu et al., 2022). This denotes that the crude extract enhances electrolyte and nutrient absorption consistent with the inhibition of hyper secretion. Furthermore, the percentage of inhibition in the case of

both average volume and average weight of intestinal contents by 400 mg/kg has a closer effect with loperamide 3 mg/kg. Therefore, it can be generalized that the hydro-alcoholic extracts of *O. lamiiflium* have anti-secretory activity. It also explains one of the mechanisms of the test substance may be through anti-entero-pooling action.

As stated in the literature, the reason for the antidiarrheal activity of extract is the presence of bioactive (Di Carlo et al., 1993; Rawat et al., 2017). More specifically, the antidiarrheal activity of certain bioactive such as flavonoids (apigenin, flavone, kaempferol, morin) (Di Carlo et al., 1993), and tannins (Kumar, 2010), are also linked to their capacity to interfere with hydro-electrolytic production in the lumen. In addition both metabolites are involved in the enhancement of colonic fluid and electrolyte reabsorption (Palombo, 2006). The fact is that ricinoleic acid is implicated in the activation of the NO pathway and thereby induces NO-dependent intestinal secretion (Mascolo et al., 1993, 1997). A previous study showed that treatment with NO synthase inhibitors in rats causes suppression of ricinoleic acid causing intestinal fluid production and Na+ secretion (Mascolo et al., 1993). Similarly, flavonoids such as apigenin, wogonin, luteolin, tectorigenin, and quercetin (Kim et al., 1999) are believed to interfere with NO synthesis.

A growing evidence also showed a chloride channel, cystic fibrosis transmembrane conductance regulator protein induced secretion can be blocked by the metabolite tannins (Wongsamitkul et al., 2010). This channel is the major route for chloride secretion of certain diarrhea (Verkman et al., 2006). Therefore, either the single or combined effects of metabolites found in this extract are possibly ascribed for the anti-secretory effects.

In general, from this study among three *in-vivo* models evaluated, the effect of the extract is slightly higher than the finding from other setups with the same plants. This may be due to the difference in plant-growing areas. The difference is also possibly due to the time of plant collection, which affects the quantities of bioactive present in the plants (Kabubii et al., 2023).

The effect of extracts in all diarrhea indicators such as onset time of diarrhea appearance, gut motility, and frequency of defecation are all together combined in the determination of the *in-vivo* anti-diarrheal index (Hussain et al., 2009; Than et al., 1989). Generally, a higher anti-diarrheal index value denotes the greater effectiveness of the test substance in diarrhea treatment (Akindele et al., 2014; Prasad et al., 2014). Leaf extracts of *O. lamiifolium* produce a dose-

dependent antidiarrheal index (table 4). Besides this, the higher dose of the test ingredient produces the highest ADI, it is closer to the effect produced by loperamide. Therefore, the higher dose, which produces the highest antidiarrheal index, is the best antidiarrheal dose of the test ingredient.

The anti-diarrheal effect of the hydro-alcoholic extracts of *O. lamiifolium* against castor oil-caused diarrhea in mice indicates either an inhibitory effect on contraction or on electrolyte-out flux. To find out its possible inhibitory effect on intestinal motility, the test substance was further evaluated in *in-vitro* experiments on isolated Guinea pig ileum for its spasmolytic activity using Ach as a contractile agent.

It is proven that the crude extracts of *O. lamiifolium* produced a concentration-dependent drop in spontaneous tissue contraction (Figure 5, Table 5). Therefore, hydro-alcoholic extracts of *O. lamiifolium* leaves blocked the spasmogenic effects of Ach. This is possibly because of its inhibitory activity at any step in the contraction cascade narrated elsewhere. The effective dose for the 50% response (EC50) of this extract is 1.8 mg of the extract concentration (Figure 6). That means 1.8 mg of hydro-alcoholic extracts of *O. lamiifolium* produced 50% maximal relaxation.

The enteric nervous system becomes more active as a result of parasympathetic stimulation, which results in increased secretion and general gut activity as well as an increase in overall blood flow to the intestine. The stimulation of muscarinic receptors (M2, M3) by the parasympathetic nerve mediates its effect in the gut (Shamkuwar, 2021). Stimulating the muscarinic receptors stimulates intracellular communication. This communication increases intracellular calcium levels, which interact with smooth muscle (SM) and cause full contraction, and which also induces diarrhea.

In the previous studies, spasmolytic constituents found in various plants mainly produce their effect via calcium channel blockage. Plants such as *Ocimum basilicum* (Janbaz et al., 2014), *Ocimum selloi* (Souza et al., 2015) and *Ocimum majorana* (Makrane et al., 2018), were produced spasmolytic effects on isolated Guinea pig ileum through calcium channel blockage. The spasmolytic effects of *O. lamiifolium* might also be linked to blocking of this channel. However, further research is needed to validate this notion.

Various drugs also work through the stimulation of the opioid receptor for their anti-diarrheal activity (Holzer, 2009; Pannemans & Corsetti, 2018). The higher dose, which is the best dose of the extract according to ADI value, is evaluated for the opioid pathway in the presence of naloxone (opioid antagonist). In the case of the extract the distance traveled by charcoal meal was not meaningfully affected by the presence of the antagonist as shown in figure 7. This is so suggesting that the anti-diarrheal effect of the study plant is not involved in the opioid pathway. This indicates that the side effect linked to the stimulation of opioid receptors is excluded in the case of the test ingredient, and it is beneficial that this extract will be an alternative to the drug that fails to treat diarrhea through the stimulation of opioid receptors. However, for loperamide, the distance traveled by charcoal meal was significantly affected by the presence of the antagonist compared to the effect of loperamide in the absence of the antagonist ($p<0.001$) (Figure 7). Hence, it can be generalized that the effect of the extract is devoid of opioid receptors.

Interestingly, in addition to the anti-motility and anti-secretory activity proved in these studies, it was also reported that the leaf extract of *O. lamiifolium* has shown promising anti-bacterial activities, including against diarrhea-causing pathogens (Addis et al., 2019; Kifle et al., 2007; Runyoro et al., 2010). Therefore, the substance found in the leaves of *O. lamiifolium* is a good candidate for the anti-diarrheal drug in the management of diarrhea with multiple etiologies.

6. CONCLUSION

The present study showed that the antidiarrheal activity of the hydro-alcoholic extracts of *O. lamiifolium* is partly mediated through blocking of muscarinic acetylcholine receptors. The study also demonstrated that the anti-diarrheal activity of the plant is not involved in the opioid pathway. The present study also showed the presence of a significant amount of phenols, flavonoids and alkaloids in this plant. These anti-diarrheal activities are possibly related to the presence of bioactive agents that act individually or collectively. The study result validates the reliability of the previous studies and gives clues on the mechanism actions of the test ingredient for its antidiarrheal activities. This will give clues for more advanced investigation of the active principles of these plants for the discovery of efficacies and safe anti-diarrheal drugs.

7. FUTURE DIRECTIONS

The subsequent directions are recommended to investigate the experimental plant in-depth:

- Further studies should be done to isolate, identify and purify the pharmacologically active principle(s) responsible for the antidiarrheal activities of the plant.
- Further toxicological evaluation such as sub-acute and chronic toxicity study should be done to assess the long-term safety profile of the extract.
- Further exploring of *ex-vivo* studies on the fraction using isolated tissue preparation.
- Further studies should also be done to explore the possible mechanisms of action of the responsible constituents in the test ingredient.

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