# Evaluation of the Antidiarrheal Activity of 80% Methanol Extract of the Aerial Parts of *Ajuga remota Benth (Lamiaceae)* in Mice

Teshager Yacob

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This is to certify that the thesis prepared by Teshager Yacob, entitled: Evaluation of the Antidiarrheal Activity of 80% Methanol Extract of the Aerial Parts of *Ajuga remota Benth (Lamiaceae)* in Mice and submitted in partial fulfillment of the requirements for the Degree of Master of Sciences 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 Antidiarrheal Activity of 80% Methanol Extract of the Aerial Parts of *Ajuga remota Benth* in Mice

Teshager Yacob

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In the Ethiopian traditional medicine, the aerial parts of *Ajuga remota Benth* ( Local name, Armagusa) is used in the treatment of diarrhea. Since this claim has not been investigated scientifically, this study was undertaken to evaluate the anti-diarrheal activity of 80% methanol extract of *A. remota* (MEAR) using experimental models in mice . The MEAR was administered at doses of (200, 400, 600 and 800 mg/kg ) to four groups of mice (six animals per group) orally in castor oil diarrhea model. Two other groups , one as control and the other as standard ( loperamide 5mg/kg ) were used for comparison with the treatment group. The effect of the extract on the other models, enteropooling and gastrointestinal transit models, was also evaluated using the same grouping and dosing . The extract at doses of 400, 600, and 800 mg/kg produced a dose-dependent and significant inhibition both on the frequency and onset of diarrhea. The percentage purging frequency was 53.4%, 66.7%, 79.6% ($p < 0.001$; for all), and 66.7% ($p < 0.001$) at three doses of MEAR (400, 600, and 800mg/kg ) and with loperamide (5mg/kg ), respectively. The percentage inhibition in intestinal fluid accumulation was 42.5%, 62.1% , and 74.2% ( $p < 0.001$; for all ) at doses of 400, 600 and 800 mg/kg of MEAR, respectively. The MEAR also inhibited significantly ($p<0.001$) and in a dose dependent manner both the normal or castor oil induced intestinal transit. Phytochemical screening revealed the presence of secondary metabolites like alkaloids, flavonoids, terpenoids, tannins, phenolics, glycosides, steroids, and saponins which might have accounted for the antidiarrheal activity. To conclude, this study has shown that the hydroalcoholic extract of *A. remota* contains pharmacologically active substances with significant antidiarrheal activity in all the experimental models used in this study .

Key words : Antidiarrheal, castor oil, enteropooling, gastrointestinal propulsion, *Ajuga remota*

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**Acronyms**

| | |
|---|---|
| AEAR | Aqueous extract of *Ajuga remota* |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| ClC−2 | Chloride channel type-2 channels |
| CLCA/CaCCs | Calcium-activated chloride− channel |
| CLD | Congenital chloride diarrhea |
| CT/CTX | Cholera toxin |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanosine monophosphate |
| CD | Crohn's disease |
| DRA | Downregulated in adenoma |
| ETEC | Enterotoxigenic *Escherichia coli* |
| ENaC | Epithelial sodium (Na+) channels |
| IBS | Irritable Bowel Syndrome |
| IBDs | Inflammatory bowel diseases |
| IL | Interleukin |
| MEAR | 80% Methanol extract of *Ajuga remota* |
| NHE | (Na)Sodium-(H) hydrogen exchanger |
| OECD | Organization for economic cooperation development |
| ORS | Oral rehydration solution |
| ORT | Oral rehydration therapy |
| PAT-1 | Putative anion transporter-1 |
| SCFA | Short-chain fatty acids |
| SLC26 | Solute carrier 26 |
| SGLT1 | Sodium/glucose cotransporter 1 |
| UC | Ulcerative colitis |
| UNICEF | United nations international children's emergency fund |
| TGF-β | Transforming growth factor-β |
| VIP | Vasoactive intestinal peptide |
| WHO | World health organization |

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# 1. Introduction

## 1.1. Definition of diarrhea

Diarrhea can be defined in different ways. The WHO defines diarrhea as the passage of three or more loose or liquid stools per day, or more frequently than is normal for an individual (1). Or it can also mechanistically be defined as; " Diarrhea is the frequent passage of liquid feces and it is characterized by increased gastrointestinal motility and secretions " (2). In general, diarrhoea is a symptom marked by rapid and frequent passage of semisolid or liquid fecal material through the gastrointestinal tract and involves both an increase in the motility of the gastrointestinal tract along with increased secretions and a decrease in the absorption of fluid and thus a loss of electrolytes particularly Na+ and water (3).

## 1.2. Classification of diarrhea

On the basis of duration, diarrhea is classified into 3 types: (i) acute diarrhea, when the duration is less than 2 week, (ii) persistent diarrhea, when the duration varies from 2 to 4 weeks, and (iii) chronic diarrhea, when the duration is more than 4 weeks (4). On the basis of pathophysiology, diarrheal syndromes are classified into five major types; secretory or toxin induced diarrhea, osmotic or malabsorption induced diarrhea, inflammatory diarrhea, iatrogenic/drug-induced diarrhea , and functional diarrhea. Most etiologies will have a complex pathophysiology involving one or more of these mentioned mechanisms (5); each of these types of diarrhea are briefly described in the pathophysiology section .

### 1.3. Epidemiology of diarrhea

Among the various disease conditions that inflict human health, diarrheal infections are one of the world's top communicable disease groups by overall death toll (6). Diarrheal disease is one of the leading causes of preventable death in developing countries, especially among children and infants (7). According to the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), there are about 2.5 billion cases of diarrheal disease worldwide every year, and 1.9 million children younger than 5 years of age die from diarrhea each year, mostly in developing countries (8). This amounts to 18% of all deaths of children under the age of five and means that more than 5000 children are dying every day as a result of diarrheal diseases. Of all child deaths from diarrhea, 78% occur in the African and South-East Asian regions, where bouts of diarrhoea are more likely to result in death or other severe outcomes (8,9). Although the estimates of global mortality from diarrhea declined from approximately 4.6 million annual deaths during the mid-1980s to the current estimate of 1.9 million, the morbidity of this syndrome remains substantial and estimates suggest that overall incidence has remained relatively stable over the past two decades (6,9).

The incidence of diarrhoeal diseases varies greatly with the seasons and a child's age (4). The youngest children are most vulnerable: incidence is highest in the first two years of life and declines as a child grows older (9). Seventy two percent of deaths associated with diarrhoea happen in the first 2 years of life (6,8). Diarrhoea remains the second most common cause of death among children under five globally, following closely behind pneumonia (9). The toll is greater than that caused by AIDS, malaria and measles combined. Nearly, one in five child deaths is due to diarrhoea (6). The highest numbers of childhood deaths were in sub-Saharan Africa, where 50% of deaths of children under the age of 5 from diarrhoea occurred in 2011 (6). Just 15 countries account for almost three quarters of all deaths from diarrhoea among children in 2009 (10). Among these 15 countries , China, Democratic Republic of the Congo , India , Nigeria and Ethiopia rank the top 5 countries in the prevalence of diarrhea. These 15 countries account for 53% of total episodes of diarrhoea and 56% of severe episodes of diarrhoea (10).

Diarrhea is the principal cause of physician visits/hospitalizations, morbidity, and loss from work or school that is associated with substantial health-care costs in industrialized countries like in the USA and other Western countries, but relatively few patients die from diarrhea in these countries (5,8,9). Diarrhea is a major cause of mortality in developing countries (5,8). Other direct consequences of diarrhea in children in resource-limited countries include growth faltering, malnutrition, and impaired cognitive development (6,8).

### 1.4. Etiology of diarrhea

The etiology of diarrhea may differ depending on the type of diarrhea that occurs .There are many possible causes of acute diarrhea, but infection is the most common cause (11). More than 90% of cases of acute diarrhea are caused by infectious agents; the remaining 10% or so are caused by medications, toxic ingestions, ischemia, and other conditions (11). Diarrhea could result from virus-induced release of an unknown vasoactive agent from infected epithelium, causing a local villus ischemia and subsequent functional damage to enterocytes (12). Many etiological agents including viruses, bacteria and intestinal parasites are implicated as causative agents in infectious diarrhea (11,15). Among the various types of viruses, Rotaviruses are considered as the most common diarrheal agent. The most common bacteria related to diarrhea include *Shigella spp, Salmonella spp, Campylobacter spp, Aeromonas, Plesiomonas, Vibrio cholerae*, *Clostridium difficile,* and enterovirulent strains of *Escherichia coli* (13,14). Parasites that can cause acute diarrhea include *Entamoeba histolytica*, *Microsporidium*, *Giardia lamblia*, *Cryptosporidium parvum ,Giardia intestinalis,* and *Cyclospora cayetanensis* (11). Noninfectious causes of acute diarrhea include drugs and toxins, laxative abuse, food intolerance, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), ischemic bowel disease, lactase deficiency,Whipple's disease, pernicious anemia, diabetes mellitus, malabsorption, fecal impaction, diverticulosis, and celiac sprue (11). In a diabetic body there is overgrowth of small intestinal bacteria, these bacteria may

overgrow due to intestinal motility and can cause bloating and diarrhea (22). Lactose intolerance is responsible for many cases of acute diarrhea (11,14 ).

Most cases of chronic diarrhea result from functional or inflammatory bowel disorders, endocrine disorders, malabsorption syndromes and drugs (including laxative abuse) (11,13). In contrast to acute diarrhea, most of the causes of chronic diarrhea are noninfectious. Side effects from drugs, habitual use of stimulant laxatives (e.g., senna, cascara, castor oil), chronic ethanol consumption and ingestion of certain environmental toxins (e.g., arsenic) are the most common secretory causes of chronic diarrhea. Bowel resection, mucosal disease, or enterocolic fistula may result in a secretory-type diarrhea (11,14).

Hormones cause a type of secretory diarrhea. Metastatic gastrointestinal carcinoid tumors like gastrinoma and *medullary carcinoma of the thyroid* may produce watery diarrhea (11). In addition, some hormone deficiencies may be associated with watery diarrhea, such as occurs with adrenocortical insufficiency (Addison's disease) (11). Fat malabsorption may lead to a type of diarrhea known as steatorrhea . Intraluminal maldigestion, mucosal malabsorption, or lymphatic obstruction may produce steatorrhea . Inflammatory diarrheas are generally caused by idiopathic IBD which include *Crohn's disease* and *chronic ulcerative colitis* (11). Primary or secondary forms of immunodeficiency may lead to prolonged infectious diarrhea. IBS also causes chronic diarrhea (11). Factitial diarrhea accounts for up to 15% of unexplained diarrhea referred to tertiary care centers (11).

## 1.5. Physiology of intestinal ion and water transport and the Pathophysiology of diarrhea

### 1.5.1. Physiology of intestinal ion and water transport

There is a constant bidirectional flux of water and ions across the small intestinal mucosa, i.e., absorption and secretion (16).The enterocyte, which is organized in a columnar

epithelial monolayer, is responsible for both electrolyte absorption and secretion in the intestine (17) .

**Enterocyte electrolyte absorption**

Fluid movement is secondary to solute movement; thus, solute absorption (and in particular $Na^+$ absorption) is the driving force behind fluid absorption, while solute secretion (most often active Cl- secretion) is the driving force behind water secretion (18). An understanding of the cellular events associated with diarrhea requires an understanding of ion transport with particular attention to active $Na^+$ absorption and active $Cl^-$ secretion (18). Apical electrolyte absorption by the enterocyte can be divided into two major categories: (i) electroneutral absorption and (ii) electrogenic absorption (16,18). Bulk transport of NaCl in the intestinal epithelium is due to electroneutral absorption by luminal $Na^+/H^+$ and $Cl^-/HCO^{-3}$ exchanger. The remaining absorption is electrogenic and is due to absorption via luminal ENaC and transcellular/paracellular absorption of $Cl^-$ (16). ENaC is the major active ion channel for Na+ reabsorption, and has been shown to compensate for electrolyte loss associated with diarrhoea (17). There are nine isoforms of Na–H transporters called Na-H exchangers (NHE1-9) (19). Among these , NHE2 and NHE3 isoforms have an apical localization pattern and are responsible to bulk Na+ absorption as shown in Figure 1(19,21). Sodium and water absorption by enterocytes is mediated by an ATP-dependent active sodium (Na) pump (Na+, K+-ATPase) located on the basolateral membranes of intestinal crypt and villus cells (5). NHE activity is chiefly regulated by cyclic nucleotides, most notably cAMP. Elevations in cAMP and activation of protein kinase A (PKA) were both shown to inhibit NHE3 (19).

With respect to $Cl^-$ ion transport, electroneutral $Cl^-$ absorption is primarily facilitated by two well-characterized anion exchangers which are members of the solute carrier 26 (SLC26) gene family and are expressed on the apical enterocyte membrane: SLC26A3 (also known as down-regulated in adenoma (DRA) and SLC26A6 (also known as putative anion transporter-1(PAT-1) both of which have been demonstrated as $Cl^-/HCO^{3-}$ exchangers, and are present in both the small intestine and the colon (17) as shown in

Figure1. PAT1 expression is predominant in the small intestine, whereas DRA expression is higher in the colon . This would suggest that PAT-1 is more important for $Cl^{-}/HCO^{3-}$ exchange in the upper GI tract, while DRA is favored in the colon. Moreover, PAT-1-mediated $Cl^{-}$ absorption is dependent on NHE3 Na+ absorption (17). The role of DRA in intestinal absorption is better characterized than PAT1 and 30 mutations of DRA are known to be responsible for autosomal recessive congenital chloride diarrhea (CLD, OMIM 214700). CLD was first described in 1945 in patients exhibiting diarrhea with increased chloride content in the stool and metabolic alkalosis . DRA is the predominant mediator of electroneutral chloride uptake in the small intestine (19).

Absorption of ions also occurs by nutrient-coupled transporters, e.g., sodium glucose symporter 1 (SGLT1), primarily responsible for electrogenic Na+ absorption in the small intestine (17). The electrochemical gradient that confers Na+ absorption through ENaC is utilized by various symporters in the small intestine for secondary active nutrient transport into the enterocyte. Mutations in SGLT1 are responsible for glucose/galactose malabsorption (GGM, OMIM 182380) resulting in osmotic diarrhea. Amino acid transporters in the small intestine take advantage of the same Na+ gradient across the epithelia to conduct absorption (19).

**Enterocyte Electrolyte Secretion**

Although the intestine is considered primarily an absorptive organ, fluid secretion is pivotal to its proper physiological function, for example by facilitating the passage of fecal content (17). An estimated eight liters of fluid are being secreted into the intestine on a daily basis , although this fluid is also composed of gastric, pancreatic, and biliary juices as well as saliva, a significant portion of the secreted volume is considered to be *bona fide* enterocyte secretion (5,19). Unlike fluid absorption, however, secretion is primarily active, driven mainly by changes in intracellular $Cl^{-}$ ion gradients, and is regulated by apical ion channels and the activity of several basolateral transporters (20). Intestinal fluid secretion is linked to the creation of an osmotic gradient that drags water into the intestinal lumen (5,17). Three channels have been identified through which Cl− can be secreted into the intestinal lumen, thus creating the driving osmotic gradient for

fluid secretion, namely: (i) the cystic fibrosis transmembrane conductance regulator (CFTR), (ii) calcium-activated Cl− channels (CaCC) and (iii) chloride channel type-2 (ClC−2) channels (20).

Among these apical chloride channels, CFTR has a major role in the process of intestinal chloride/fluid secretion (19).The CFTR is a cAMP-activated $Cl^-$channel, which is embedded as dimers into the apical membrane, with its 12 transmembrane domains forming the pore for $Cl^-$excretion (20). A mutation in the CFTR gene (ΔF508) is responsible for the phenotype of the hereditary disease of cystic fibrosis (CF), which represents the most prevalent genetic disorder in Caucasians (19,20). Neonates suffering from CF often present with a meconium ileus as the first clinical symptom, which is an obstruction with neonatal hyperviscous stool, thought to be a consequence of impaired intestinal fluid secretion (20).

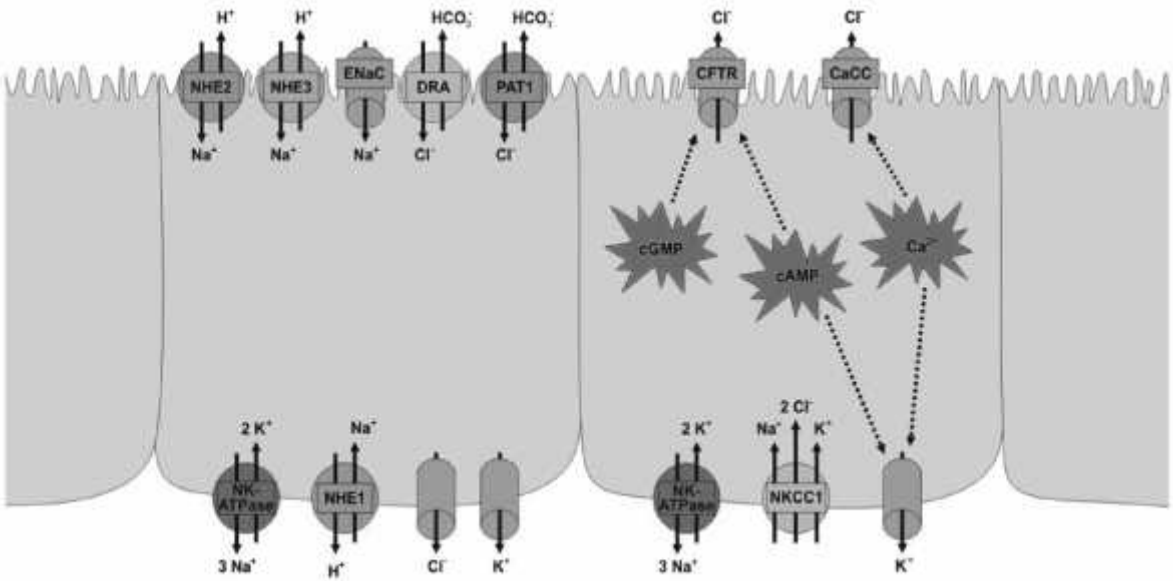


**Figure1:**Summary of intestinal ion transport : absorption and secretion (17) . NHE 2/3 in addition to DRA and PAT-1 anion exchangers import $Na^+$ and $Cl^-$ across the apical membrane into intestinal epithelial cells while exporting a $H^+$ and $HCO^{3-}$ ions. Basolateral NK-ATPase and NHE-1 provides a gradient for the basolateral exit of ions and subsequent transport of water across the intestinal wall (left side, absorption). Chloride is pumped into the cell via the Na2ClK symporter (NKCC1), the gradient for which is provided by the active basolateral NK-ATPase antiporter, which also recycles sodium out of the cell. Thus a drive toward apical exit through cAMP/cGMP activated CFTR or CaCC (right side, secretion).

### 1.5.2. Pathophysiology of diarrhea

The pathophysiology of diarrhea has numerous mechanisms (22). For understanding the pathophysiology of diarrhea, diarrheal syndromes are classified into secretory or toxin induced, osmotic or malabsorption induced, inflammatory, iatrogenic/drug-induced, and functional diarrhea and their pathophysiology is reviewed as follows (5,22).

**Secretory diarrhea**

A number of disease processes produce secretory diarrhea. The basic pathophysiology of secretory diarrhea involves either net secretion of ions (chloride or bicarbonate) or inhibition of net sodium absorption (5). Net intestinal secretion is most often secondary to the stimulation of active chloride secretion and to the inhibition of active absorption of sodium and chloride by messengers such as cyclic AMP .The driving force for intestinal ion secretion can arise from the gut lumen as with infectious diarrhea (enterotoxins), from the subepithelial space (inflammatory mediators), or from the systemic circulation (peptide hormones produced from endocrine tumors or neoplasm's) (22).

The most common cause of secretory diarrhea is infection that is caused by pathogens, which usually affect the small intestine (22,25). Some enterotoxins produced by bacteria trigger signaling molecules such as cAMP, cGMP, or intracellular Ca2+, which, in turn, activate apical Cl− channels leading to an increase in secretion of Cl− and consequently of water. Diarrhea induced by *V. cholerae* and other bacterial enterotoxins like enterotoxigenic *E.Coli* (ETEC ) follow this pathway (22) . Cholera toxin (CT) consists of an A subunit bound to a pentameric ring of B subunits, where the B subunits are responsible for delivery of the A subunit into the cell. The A subunit ADP- ribosylates a GTPase, which regulates adenylate cyclase resulting in elevated cAMP production , which activates protein kinase A (PKA) (19,22,25). Protein kinase A then phosphorylates the regulatory domain of CFTR, leading to $Cl^-$ secretion (25). Cholera toxin triggered elevations of cAMP levels further inhibit $Na^+$uptake by NHE in the villus cell and thereby increase the osmotic driving force for fluid secretion (19).

CaCCs in intestinal epithelial cells provide an important route for $Cl^-$ and fluid secretion in secretory diarrheas and are thought to mediate diarrhea that occurs as a side effect of certain therapies (i.e. chemotherapy or antiretroviral therapy) or as a result of viral infection. Small molecule inhibitors of intestinal CaCCs are predicted to be useful for treating some types of secretory diarrhea (27) .

**Osmotic diarrhea**

Osmotic diarrhea occurs either when nonabsorbable (e.g. sugar alcohols such as mannitol or sorbitol ) or poorly absorbable solutes (e.g. magnesium, sulfates, and phosphates ) are ingested or enterocytes or colonocytes cannot absorb them (5). The osmotic force of the unabsorbed solutes results in driving water and secondarily ions into the gut lumen resulting in diarrhea (22). Malabsorption may also result in osmotic diarrhea, with the malabsorbed nutrients acting as poorly absorbed solutes (26). Absence of disaccharidases , which digest disaccharides to their constituent monosaccharides to permit absorption, as in lactase deficiency results in osmotic diarrhea. Congenital sucrase–isomaltase and trehalose deficiencies are rare causes of disaccharide-induced osmotic diarrhea. The osmotic force of unabsorbed solutes appears to play a major role for the diarrhea induced by celiac disease (22).

**Inflammatory diarrhea**

Inflammation results in diarrhea by many different mechanisms and from a wide variety of etiologies including infections and IBDs. Intestinal inflammation is associated with defects in epithelial barrier function and ion flux, both contributing to impaired fluid homeostasis and diarrhea (23). IBD is one of the most common and important causes of inflammatory diarrhea (24). Inflammation results in mononuclear cells releasing proinflammatory cytokines and eicosanoids, which probably result in decreased absorption but no increase in secretion. The cytokines like TNF-α, interferon-γ, and interleukins (IL-4, -6, -8, -12, -13) and eicosanoids initiated by inflammation down regulate multiple $Na^+$ transporter proteins, including apical transporters NHE1,3, ENaC, and basolateral $Na^+/K^+$-ATPase ion transporters in the colon and small bowel resulting

in Na malabsorption (20, 22-24). Infectious pathogens causing inflammatory diarrheas primarily affect the distal small bowel or the colon. They cause disease by either elaborating cytotoxins or by invading the epithelium with resultant recruitment of inflammatory cells (22). Most of the pathogens causing inflammatory diarrhea do so by producing mucosal damage as well as by stimulating intestinal secretion. In some cases, the organisms elaborate enterotoxins, which stimulate intestinal secretion (5). Exudation or inflammation can contribute to diarrhea when the intestinal epithelium's barrier function is compromised by loss of epithelial cells or disruption of tight junctions as occurs in invasive diarrhea due to *Shigella*/*Salmonella* and inflammatory disease process as in ulcerative colitis (UC) or Crohn's disease (CD) (22,24,26).

**Iatrogenic-/Drug-induced diarrhea**

Diarrhea can also result following certain surgical procedures and usage of certain drugs (22). Diarrhea can follow cholecystectomy in 5–10% of patients which can respond to treatment with bile salt-binding resins in some patients. A number of drugs can cause diarrhea. The pathophysiology of drug-induced diarrhea may involve one or more of the above-mentioned mechanisms. Antibiotic use may alter the bacterial flora in the colon resulting in impaired colonic salvage of malabsorbed carbohydrates resulting in diarrhea (5). Some of the drugs like lactulose may cause osmotic diarrhea, while others may cause secretory diarrhea. Similarly chemotherapeutic drugs may cause diarrhea because of decreased rate of proliferation of the enterocytes (22).

**Functional diarrhea**

Motility disturbances can result in diarrhea as occurring in thyrotoxicosis and opiate withdrawal . *Hyperthyroidism* and certain drugs (e.g., prostaglandins, prokinetic agents) may produce hypermotility with resultant diarrhea (11). Similarly slowing of the motor function of the small intestine as with narcotic use, scleroderma, diabetic autonomic neuropathy, and amyloidosis can result in bacterial overgrowth and hence diarrhea (5).The pathophysiology of functional diarrhea or diarrhea associated with irritable bowel syndrome (IBS) may involve multiple mechanisms. Alteration in colonic transit/motility

and hypersensitivity of the rectum ( in up to 60% of IBS ) may play a role in diarrhea . Disturbances in the neural control (from brain to visceral nerves) and the gut in the form of visceral nociception and abnormal motility mediated by changes in neurotransmitters like serotonin, cholecystokinin, and neurokinins are also proposed to contribute to diarrhea seen in IBS patients (26).

## 1.6. Management of diarrhea

Knowledge of the underlying causative processes in diarrhea facilitates effective treatment and every effort should be made to identify and correct the specific causes of diarrhea (26). Many patients with sudden onset of diarrhea have self-limited illness requiring no treatment or evaluation. However, in severe cases, dehydration and electrolyte imbalances are the principal risk, particularly in infants, children, and frail elderly patients, thus requiring both non-pharmacologic and pharmacological treatment (28) .

### 1.6.1. Prevention of diarrhea

Prevention is the most effective means of combating death due to diarrheal disease. Providing an adequate supply of clean water and appropriate waste disposal technologies including proper hygiene and sanitary practices are vital components of prevention programs (15). Appropriate feeding practices and nutrition including promotion of exclusive breastfeeding for the first 4-6 months of life is aimed at combating diarrheal morbidity of infants. Apart from this, maintaining a steady diet of nutritional, staple foods and supplementation of essential vitamins and minerals such as vitamin A, zinc, and folate in areas of endemic suboptimal nutrition are therefore vital. In addition, broadening immunization coverage for illnesses like Rotavirus and Measles are vital components of diarrhea prevention (15).The WHO now recommends safe and effective Rotavirus vaccines for inclusion in all national immunization programs. Likewise, Measles vaccination is highly effective and reduces the incidence and severity of diarrheal illness in children (15).

### 1.6.2. Non-pharmacological treatment of diarrhea

**Oral rehydration therapy (ORT)**

Oral Rehydration Therapy (ORT) using oral rehydration salts (ORS) is an effective method for preventing dehydration and treating mild to moderate dehydration in children and adults with diarrhea (15). ORT consists of rehydration and maintenance of water and electrolytes , which are the primary treatment goals until the diarrheal episode ends (26). ORT is a cost-effective method of managing acute gastroenteritis and it reduces hospitalization requirements in both developed and developing countries (8). This therapy exploits the fact that nutrient-linked cotransport of water and electrolytes remains intact in the small bowel in most cases of acute diarrhea. Sodium and chloride absorption is linked to glucose uptake by the enterocyte; this is followed by movement of water in the same direction. A balanced mixture of glucose and electrolytes in volumes matched to losses therefore can prevent dehydration (28).

ORS, used in ORT, contain specific amounts of important salts that are lost in diarrhea stool (8). The new lower-osmolarity ORS ( recommended by WHO and UNICEF ) has reduced concentrations of sodium and glucose and is associated with less vomiting, less stool output, lesser chance of hypernatremia, and a reduced need for intravenous infusions in comparison with standard ORS (8). This formulation is recommended irrespective of age and the type of diarrhea. Rice-based oral solution is also a hyposmotically active substrate that elutes glucose without increasing stool or urine outflows (8). Although such solutions can replace diarrheal losses, they do not facilitate reabsorption of secreted fluid and therefore do not lessen diarrhea (8).

Solutions containing extracts from rice or other grains are increasingly being used for oral rehydration and appear not only to replace fluid losses but also to lessen diarrhea (17). Now a days, zinc taken with ORS has been shown to significantly reduce deaths when used as part of the ORT regimen (27). In 2004, WHO and UNICEF recommended the use of a 10- to 14-day zinc treatment together with ORS as a two-pronged approach to treat acute diarrhea in children. Zinc supplements considerably reduce the duration

and severity of diarrhea episodes, decreases stool output, lessens the need for hospitalization, and may also prevent future diarrhea for up to three months (30).

### 1.6.3. Pharmacological treatment of diarrhea

There are a wide variety of antidiarrheal drugs that have been used to treat diarrhea; some of these drugs may help in reducing amount of fluid loss, frequency and consistency of stool (26).The principal utility of nonspecific antidiarrheal agents is to provide symptomatic relief in mild cases of acute diarrhea (26). The various antidiarrheal drugs are grouped into several categories: antimotility agents, antisecretory compounds, adsorbents, antibiotics, bile acid sequestrants, enzymes, and probiotics (26,28,29).

**Antimotility agents** Opioids and their derivatives continue to be widely used in the treatment of diarrhea (29).They act by several different mechanisms, mediated principally through either μ- or δ-opioid receptors on enteric nerves, epithelial cells, and muscle. These mechanisms include effects on intestinal motility (μ receptors), intestinal secretion (δ receptors), or absorption (μ and δ receptors), although most antiperistalic agents act by altering intestinal motility (29). Commonly used antidiarrheals such as diphenoxylate, difenoxin, and loperamide act principally *via* peripheral μ -opioid receptors and are preferred over opioids that penetrate the CNS such as Codeine and morphine (26,29). They may be helpful in secretory diarrhea of mild to moderate severity by reducing the frequency and volume of stools (26,28). Other non-opioid antidiarrheal drugs are calcium channel blockers such as verapamil and nifedipine which can reduce motility and may promote intestinal electrolyte and water absorption (29).

**Antisecretory Agents** There are many drugs that have antisecretory effects on the intestine that work by different mechanisms including inhibition of prostaglandins and effects on cyclic AMP, calmodulin inhibition, and inhibition of gut hormones (28). Bismuth subsalicylate appears to have antisecretory, antiinflammatory, and antimicrobial effects .It is used for the prevention and treatment of traveler's diarrhea, but it also is effective in other forms of episodic diarrhea and in acute gastroenteritis (28). $\alpha_2$ Adrenergic receptor agonists such as *clonidine* can interact with specific receptors on enteric neurons and enterocytes, thereby stimulating absorption and inhibiting secretion

of fluid and electrolytes and increasing intestinal transit time. These agents may have a special role in diabetics with chronic diarrhea, in whom autonomic neuropathy can lead to loss of noradrenergic innervation and also useful for diarrhea caused by opiate withdrawal (28, 29). But severe side effects limit its usefulness (16). Drugs that either mimic or potentiate the effects of antisecretory/proabsorptive hormones or neurotransmitters are proving useful for treatment of diarrhea (16).

Octreotide is a synthetic octapeptide analog of endogenous somatostatin that is effective in inhibiting the severe secretory diarrhea brought about by hormone-secreting tumors of the pancreas and the gastrointestinal tract (16,26). Its mechanism of action appears to involve inhibition of hormone secretion, including serotonin and various other GI peptides (*e.g.,* gastrin, VIP, insulin, secretin, *etc*.). It has direct inhibitory effects on intestinal secretion and stimulatory effects on intestinal absorption (28). Two other somatostatin analogs, lanreotide and vapreotide, have been studied (26,29). Another newer antisecretory agent is Racecadotril. It is a dipeptide inhibitor of enkephalinase that prevents the degradation of endogenous opioids (enkephalins) on the δ -opioid receptor, thereby reducing hypersecretion of water and electrolytes into the intestinal lumen to produce an antidiarrheal effect (28,29). Enkephalins are endogenous opioids that are important enteric neurotransmitters and inhibit intestinal secretion without affecting motility (29). Calmodulin inhibitors, which include chlorpromazine, *and Zaldaride maleate,* a new drug in this class also are antisecretory agents (29).

**Other Agents** Adsorbents are used for symptomatic relief. These products are nontoxic and nonspecific in their action; they adsorb nutrients, bacterial toxins, drugs, and digestive juices like bile salts. Polycarbophil, kaolin and attapulgite are examples of such drugs (26). Adsorbents may work as gels to modify stool texture and viscosity and to produce a perception of decreased stool fluidity (26, 28). Bile acid sequestrants like *Cholestyramine, colestipol,* and *colesevalam* effectively bind bile acids and some bacterial toxins. Cholestyramine is useful in the treatment of bile salt-induced diarrhea. (29). Probiotics are non-pathogenic organisms, for example *Lactobacillus acidophilus* and *Saccharomyces boulardii*, which multiply in the patient's intestine and produce metabolites, which increase acidity of stool and prohibit the growth of enteropathogens.

They prevent the invasion of bacteria in intestine tissue, and produce short chain fatty acids that are beneficial for intestine recovery, and increase the rate of fluid and electrolyte absorption (29).

### 1.6.4. Herbal treatment

Herbal extracts have been used for several millennia to treat diarrheal diseases, and it is estimated that up to 80% of the population in some developing countries is dependent on such options (31). There are an enormous numbers of herbal medicines around the world that are claimed to be effective in treating diarrhea (28). Many plant-derived medicines used in traditional African, American, Asian, European and other indigenous medicinal systems have been recorded in pharmacopeias as agents used to treat diarrhoea (7). Some of the widely used antidiarrheal folk remedies include extracts from blackberry roots and bark, Croton lechleri, Galla chinensis, blueberry leaves and fruit, chamomile leaves, apples, green bananas, wood creosote and Garcinia plant bark and fruit (31). Black and long peppers (32), piperine (33), hot water black tea extracts (BTE) (34), the rhizomes of ginger, *Zingiber officinale* (35), *Egletes viscosa* (36), *Euphorbia hirta* (37) and the essential oil of *Satureja hortensis* (38) only to mention a few are traditionally used antidiarrhoeal herbal formulations and these herbs were also scientifically investigated. Herbs with astringent properties, such as meadowsweet, *Filipendula ulmaria*, agrimony, *Agrimonia eupatoria*, shepherd's purse, *Capsella bursa-pastoris* and cranesbill, *Geranium maculatum*, are traditionally recommended treatments for diarrhoea and are thought to be useful as they bind to the mucosal lining of the small intestine (7).

## 1.7. Rationale for the study

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world (7). Plant extracts are some of the most attractive sources of new drugs and have shown to produce promising results in the treatment of diarrhea (39). Therefore, the search for safe and more effective agents for treatment of diarrheal condition from plant origin has continued to be an important area of active research (40). For this reason, international organizations including WHO have encouraged studies pertaining to the treatment and prevention of diarrheal diseases using traditional medical practices (41). It is therefore important to identify and evaluate the safety and efficacy of available natural medications as alternatives to currently used antidiarrheal drugs, which are not always free from adverse effects (42,43).

According to WHO approximately 80% of world population in developing countries depends on traditional medicines for primary healthcare (44).This is also true for Ethiopia that nearly 80% of human population and 90% of livestock rely on traditional medicine especially on medicinal herbs for the treatment of various disease conditions including diarrhea (45). In particular ,the use of medicinal plants to treat gastrointestinal disorders such as diarrhea and dysentery occupied the major place in the traditional settings of our community, although there are few pharmacological and phytochemical studies which support their use (45,56). The study of medicinal plants with the purpose to provide pharmacological evidence that may explain its traditional therapeutic use is of great importance (1). In this regard medicinal plants and traditional medicine knowledge play an important role in the health care system of the country (44). *Ajuga remota Benth* is one such plant that is used traditionally for treating diarrheal condition (56). Hence, this study may evaluate the putative antidiarrheal activity of the crude extract of this plant with a view to authenticate its acclaimed use by the traditional practitioners. The finding of this study might provide, on a preliminary basis, a clue about the nature of phytochemical constituents' responsible for the antidiarrheal activity of the plant and the possible mechanisms of antidiarrheal action. In addition to this, these findings lend pharmacological credence to the anecdotal, ethnomedicinal use of medicinal plants as remedies for diarrhea and might indicate the need for additional research in this area.

### 1.8. *Ajuga remota Benth*

The plants of genus *Ajuga* are evergreen, clump-forming rhizomatous annual or perennial herbaceous flowering species in the mint family, Lamiaceae, with most plants native to Europe, Asia, and Africa, but also growing in Australia and North America (46). A genus with 40-50 species, mainly in subtropical and temperate regions of the world ; *ajuga* is one of the 266 genera of the family. The *Ajuga* plants grow to 5-50 cm tall, with opposite leaves, which are attractive; the flowers are two lipped and tubular, and mostly blue, purple or yellow in color (46). Plants of the *Ajuga* genus and some compounds isolated from these plants have medicinal value and of ecological and economic importance (46). The species *A. remota* is also known by other names such as *A. integrifolia Buch.-Ham, A. bracteosa Wall.ex.Benth* (47) .

*Ajuga remota* is an erect rhizomatous pubescent herb found growing in the grasslands and other geographic parts of East Africa especially in Kenya and Ethiopia as shown in Figure 2 (48). In Ethiopia, one of the vernacular name of Ajuga remota is Armagusa (oromiffaa ), the name given by the community that uses this plant for the management of diarrhea (56) .The herb is not eaten by animals, birds or insects. This is probably due to the very bitter taste of almost all its parts (48). Phytochemical investigations on *Ajuga remota* have identified numerous compounds that include; *neo*-Clerodane diterpenoid**s** (ajugarin I, II, III, IV &V, and others) (49), phytoecdysteroids; sterol glycoside, 3-*O*-β-glucopyranosylstigmasta-5, 25-diene, stigmasterol and ergosterol-5,8-endoperoxide (50), iridoid glycosides (48,51), flavonol glycosides (48); kaempferol 3-O- α-rhamnoside, quercetin 3-O- β-glucoside, quercetin 3-O- rutinoside. Apart from this, four iridoid glycosides have been isolated from the underground part (48,51). In addition, phytochemical screening of the crude hydroalcoholic extract for secondary metabolites revealed the presence of alkaloids, saponins, terpenoids, glycosides, anthraquionones, steroids, tannins, flavonoids and phenolic compounds (52).

Ethnopharmacological surveys have revealed that some 20 species of *Ajuga* plants are used in traditional medicine mostly in Africa, Asia and China (46). In East Africa, plants

of genus *Ajuga* have been used as a remedy for fever, toothache, severe stomachache, dysentery, high blood pressure, malaria, oedema, pneumonia and liver problems (46,48). In North Africa, *Ajuga* plants are used to treat diabetes and hypertension, as a panacea (cure-all), specifically for gastrointestinal disorder*s*, and as an anthelmintic (46). In traditional Chinese pharmacopoeia, plants of the genus *Ajuga* are known to produce a diuretic effect . Other reported activities of *Ajuga* plants include antibacterial, antifungal, antihypertensive, anti-inflammatory, antimalarial, antimycobacterial, antioxidant, antipyretic, antitumor, larvae and insect antifeedant , and insect growth inhibitor activity (46).

Experimental studies on the plant has investigated that it possess the following biological/pharmacological properties which include diuretic activity (52), antimalarial (48), analgesic (53), anti-Human Immunodeficiency Virus Type 1 (HIV-1) and Type 2 (HIV-2 ) (54), antioxidant /oxygen scavenging activity (55), antihyperglycemic activity, antipyretic, antispasmodic action, cancer prevention, antihypertensive, antimycobacterial, cardiac stimulant, Larvicide (against mosquito ), and Lipoxygenase inhibition activities, as well as anthelimenthic (44,54). Other ethnobotanical claims of the plant include treatment of diarrhea, dysentery, gout, jaundice, amenorrhea, yellow fever, as antiinfective. It is also used externally for burns, boils and syphilis (56-58).

Figure 2.Photograph of *Ajuga remota* plant

## 2. Objectives

### 2.1. General objective

- To evaluate antidiarrheal activity of 80% methanol extract of *Ajuga remota Benth* in mice

### 2.2. Specific objectives

- To evaluate the effect of 80% methanol extract of *A. remota Benth* on castor oil induced diarrhea in mice
- To assess anti-enteropooling effect of 80% methanol extract of *A. remota Benth* on castor oil induced enteropooling in mice .
- To evaluate the effect of the 80% methanol extract of *A. remota Benth* on normal/castor oil induced gastrointestinal motility using charcoal meal test
- To test the acute oral toxicity of the plant
- To screen the phytochemical constituents of the 80% methanol extract of the plant *A. remota Benth* .

## 3. Materials and Methods

### 3.1. Drugs and chemicals

Distilled Water (Ethiopian Pharmaceutical Manufacturing, Ethiopia), Loperamide HCl [Daehwa pharmaceutical, Republic of Korea], Castor oil [Remkaln General Trading P.LC., Amman Pharmaceutical Industries Co., Jordan), Methanol $^{RPE}$ [CARLO ERBA reagents, German], Tween 80 (Atlas Chemical Industries Inc.), Activated Charcoal (India), Chlorform ( Finkem Laboratory Reagent, India), Atropine sulphate Inj.(0.1%) (Jeil Pharm.Co.,Ltd., Korea), ( the chemicals that were obtained from Department of pharmacognosy, Addis Ababa University, Addis Ababa, Ethiopia include Glacial acetic acid, Ammonia, Ferric chloride ($FeCl_3$), Mayer's reagent, Wagner's reagent, Hydrochloric acid (HCl), Sulphuric acid ($H_2SO_4$ ), Benzene and Acetic acid ), were used in the study .

### 3.2. Plant materials

The aerial parts of the plant *Ajuga remota Benth (Lamiaceae)* were collected from a place called Karakore, which is located in Kolfe Qaraniyo sub-city, Woreda 02 near to Medhanealem church in western part of Addis Ababa in December 2013. Identification and authentication of the plant specimens were done by a taxonomist at the National Herbarium, College of Natural Sciences and Computation, Addis Ababa University, where a voucher specimen (Voucher Specimen number TY001 ) has been deposited for future reference.

### 3.3. Experimental animals

Swiss albino mice of either sex (20-30g) were obtained from the animal house unit of School of pharmacy of Addis Ababa University, Addis Ababa, Ethiopia . The animals were housed in polypropylene cages (6–10 animals per cage) under standard environmental conditions (a 12 h/12 h light/dark cycle). In this standard laboratory condition, they were acclimatized for a period of 7 days before beginning the actual experiment. There after, the animals were provided with the standard laboratory pellet

and tap water *ad libitum*. Food was withdrawn 18-20 h before the beginning of the experiments but water was allowed except during antienteropooling test. The care and handling of animals were in accordance with internationally accepted guidelines for use of animals (59) and the procedure was approved by the School of Pharmacy Ethics Committee, Addis Ababa University (IRB/SoP/ 02/03/2010).`

### 3.4. Extraction of plant material

After collection , the aerial parts of the plant were cleaned to remove dust and dirt then sliced to smaller pieces and dried at room temperature under shade for more than 3 weeks. The dried and sliced pieces were then powdered finely using mortar and pestle and subjected to extraction.

#### 3.4.1. Preparation of 80 % methanol extract

Maceration technique was used for the extraction of plant material for 80% methanol**.** One hundred gram of the aerial parts of the powdered plant material was subjected to maceration process with about 500ml of 80% methanol at room temperature for 72h while shaking occasionally. The extract was then filtered through a muslin cloth and Whatman No. 1 filter paper and the marc was remacerated twice using the same volume of solvent to exhaustively extract the plant material. The methanol was then removed from the extract by evaporation under reduced pressure using a rota vapor (BUCHI Rotavapour R-200, Switzerland) at 40°C. The extract was then dried using a lyophilizer to remove the remaining water .The resulting dry hydroalcoholic extract was weighed and calculated for percentage yield, which was (15.5gm; yield 15.50 % w/w) . The dried plant extract was reconstituted with 2% tween 80 for oral administration.

#### 3.4.2. Preparation of aqueous extract

Hundred gram of the finely powdered plant material was placed in a container (Erlenmeyer flask) mixed with 400ml of distilled water, as used traditionally, and allowed to stand for a period of 3 days while shaking occasionally using Orbital shaker that was revolving at 150 rpm. The mixture was then strained, the marc pressed and the

liquids clarified by filtration using Whatman No.1 filter paper and muslin cloth, after standing. The marc was remacerated twice using the same volume of solvent to exhaustively extract the plant material . The extract was then dried using a Lypholizer (freeze dryer). The freeze-dried extract was then collected , well packed in plastic vials and kept in a refrigerator until further used for the experiment. A dried aqueous extract was obtained with percentage yield of 17% w/w .

### 3.5. Acute oral toxicity test

Acute oral toxicity was conducted according to OECD guideline 425 (60). Five female albino mice of 6-8 weeks age (each for aqueous and 80% methanol extract) were randomly selected and food (but not water) was withheld for 3-4 hours before beginning the test. The extract was administered at a single oral dose of 2000mg/kg dissolved in distilled water and 2% tween 80 v/v for the aqueous and 80 % methanol extract respectively. Then the animals were housed separately in cages and observed for any signs of toxicity or death initially for 24 hours and further for a period of 14 days .

### 3.6. Grouping and dosing

Animals were randomly assigned into six groups each consisting of 6 mice of either sex (weighing 20-30 g) and were fasted for 18 hr before the test with free access to water for antidiarrheal test. Negative controls were treated with the vehicle used for reconstitution (10 ml/kg ), 2% tween 80 v/v. Positive controls were treated with standard drug, loperamide Hcl(5mg/kg) or Atropine sulphate ( 3mg/kg) depending of the type of the model used (i.e. in the castor oil induced diarrhea and antienteropooling models loperamide 5mg/kg was used , while in the normal or castor oil induced gastrointestinal transit models atropine sulphate 3mg/kg was used as a standard ) . Four treatment groups were treated with four different doses of MEAR ; 200, 400, 600 and 800mg/kg orally by using oral gavage. Dose selection was made based on the pilot test performed prior to commencement of the actual experiment. Since the aqueous extract showed very little antidiarrheal activity at the larger doses (400mg or 800mg) used compared to the control group during the pilot test, the experiment was conducted completely using the

hydroalcoholic extract except for one model. For the aqueous extract, animals were randomly assigned into four groups each consisting of 6 mice of either sex .

### 3.7. Antidiarrheal activity

#### 3.7.1. Castor oil-induced diarrhoea in mice

The experiment was carried out according to the methods described by Yadav and Tangpu (61). After 1 h of treatment with extract, 2% tween 80 or standard drug, diarrhoea was induced by administration of 0.5 ml of castor oil orally to each mouse. All administrations were carried out orally using oral gavage . The mice were then housed individually in transparent metabolic cages, the bottom of which was lined with white sheet of paper for observation of the number and consistency of fecal droppings . The papers were changed every hour to make the faecal droppings visible for counting and to check stool consistency. During observation period of 4h, the onset of diarrhoea, the number and weight of both dry and wet stools excreted by the animals were recorded and compared with the control for assessing the antidiarrhoeal activity. The onset was measured as the time interval in minutes between the administration of castor oil and the appearance of the first diarrheal stool .The total number of diarrheal feces of the control group was considered 100%. Percent inhibition (PI) was calculated as follows;

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

#### 3.7.2. Castor oil-induced enteropooling

Intraluminal fluid accumulation was determined using the method of Maxwell (2),with slight modifications. After one h of treatment with extract, 2% tween 80 (v/v) or standard drug , 0.5 ml castor oil was administered orally to these animals to induce diarrhea. One h after castor oil treatment, all mice were sacrificed by cervical dislocation, and the small intestine was ligated both at the pyloric sphincter and at the ileocecal junctions and dissected out. The small intestine was weighed. The intestinal contents were collected by

milking into a graduated tube and the volume was measured. The intestines were reweighed and the differences between full and empty intestines were calculated.

### 3.7.3. Normal gastrointestinal transit in mice

Normal gastrointestinal transit was investigated in mice according to the methods described (62,63). One h after the administration of 2% Tween 80 (v/v), extract or standard drug, each animal was given 1 ml standard charcoal meal (10% activated charcoal suspension in 2% tween 80 ). The mice were then sacrificed 1 h after the administration of the charcoal meal, the abdomen were opened and the small intestine was immediately isolated. The length of the intestine from pylorus to the caecum (LSI) and the distance traveled by the charcoal (LM) were measured. The peristaltic index (PI) for each mouse was calculated, expressed as percentage of the distance traveled by the charcoal meal relative to the total length of the small intestine. The percentage inhibition relative to the control was also calculated as:

PI = LM/LSI x 100%

Where: PI = Peristaltic Index

LM = Length of Charcoal Meal; LSI = Length of Small Intestine

$$\% \text{ Inhibition} = \frac{[(\text{Mean of distance travelled by marker of(Control} - \text{Test) group }]}{\text{Mean of distance travelled by marker of Control group}} \times 100$$

### 3.7.4. Castor oil induced gastrointestinal transit in mice

This experiment was carried out using the method described (64). One h after the administration of 2% Tween 80 (v/v), extract or standard drug, castor oil (0.5ml/mouse) was administered orally to these animals to induce diarrhea. After one h of castor oil administration, all animals received 1 ml of charcoal meal marker (10% charcoal suspension in 2 % Tween 80) orally. All the animals were sacrificed after 1 h of marker administration and the small intestine was rapidly dissected out and placed on a clean

white sheet of paper that was stretched on top surface of the table. The intestine was carefully inspected and the distance travelled by charcoal meal plug from the pylorus to caecum was measured and expressed as a percentage of the total distance of the small intestine . The length of the whole intestine was also measured**.**

**In vivo-antidiarrheal index (ADI)**

The *in vivo* antidiarrheal index (ADI *in vivo*) was then expressed according to the formula developed by Aye-Than as described (65).

$$\textbf{ADI } \textit{in vivo} = \sqrt[3]{(\textbf{D freq} \times \textbf{G meq} \times \textbf{P freq})}$$

where, $\mathbf{D}_{\mathbf{freq}}$ is the delay in defecation time or diarrheal onset (as % of control),

$\mathbf{G}_{\mathbf{meq}}$ is the gut meal travel reduction (as % of control), and

$\mathbf{P}_{\mathbf{freq}}$ is the purging frequency, or reduction in the number of stools (as % of control).Each of these parameters were calculated using the formula below;

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

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

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

### 3.8. Phytochemical screening of *Ajuga remota*

The methanol extract was tested for the presence of bioactive compounds by using the following standard methods (66-68).

**Test for saponins**

Crude extract of 0.5g was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins (66).

**Test for glycosides (Keller-kilani test )**

Solvent extract of 0.5 gram was dissolved in 2.0 ml of glacial acetic acid containing one drop of ferric chloride (FeCl3) Solution. This was then under laid with 1.0 ml of concentrated sulphuric acid. A brown ring obtained at the interface indicated the presence of glycosides (67).

**Test for phenols**

The Solvent plant extract of 0.5gram was treated with few drops of neutral ferric chloride solution 5%, intense colour developed indicates the presence of phenols. (67,68).

**Test for flavonoids ( Shinoda test )**

The solvent extract (5 ml, corresponding to 1 gram of plant material) was taken and treated with a few drops of concentrated HCl and magnesium turnings (0.5 gram ). The presence of flavonoids was indicative if pink or magenta – red colour developed within 3 minutes (67).

**Test for steroids**

Crude extract of O.5gram was mixed with 2ml of chloroform and concentrated H2SO4 was added

sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated $H_2SO_4$ and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids (66).

**Test for terpenoids**

Crude extract of 0.5 gram was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated $H_2SO_4$ was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids (66).

**Test for Anthroquinones (Borntreger's test)**

5 gram of plant extract was shaken with 10 ml of Benzene. This was filtered and 5.0 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of violet colour in the ammonical (lower) phase indicated the presence of free hydroxyl anthroquinones (67).

**Test for alkaloids**

Crude extract of 0.5gram was mixed with 2ml of 1% HCl and heated gently. Mayer's And Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids (67).

**Test for tannins**

Crude extract of 0.5gram was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of tannins (66).

### 3.9. Statistical analysis

The results were expressed as mean ± SEM and they were analyzed statistically using One way ANOVA followed by post-hoc tukey test to find out significance difference between control group against each test groups separately. The value of $P<0.05$ was considered statistically significant. Dose-dependent effect was confirmed using Multiple linear regression analysis . Data were tested for a normal distribution using Shapiro-Wilk's normality test and other graphical methods like normal probability plot (e.g. Q-Q plot ) for regression analysis.

## 4. Results

### 4.1. Acute oral toxicity test

Oral administration of MEAR and AEAR produced no visible signs of toxicity in the animals at the limit dose of 2000 mg/kg body weight of the mice. No mortalities were recorded during the 48 hrs. In addition, no toxic symptoms were observed and neither food nor water intake was found to be reduced during the period of 14 days .

### 4.2. Castor oil-induced diarrhea

All mice in the control group produced either wet stools or watery diarrhea starting from one hour after castor oil administration . Pretreatment of mice at the doses of 400, 600, and 800 mg/kg of MEAR caused a concentration -dependent and significant decrease in the frequency of stooling (reduction in number of both wet stools and total number of stools) (Regression coefficient ;$R^2$ =1, for both ), decrease in the weight of wet stools ($R^2$ =.963), and delay in the onset of diarrhea ($R^2$=0.995). The percentage purging frequency relative to controls (number of wet stools) was 53.7%, 66.7%, 79.6 % ($p < 0.001$), and 66.7% ($p < 0.001$) at three doses of MEAR (400mg/kg, 600mg/kg, and 800mg/kg) and with loperamide (5mg/kg ), respectively as shown in Table 1. The percentages relative to controls for diarrhea onset were 93.7%, ($p < 0.01$), 145.7%, and 183.5% ($p < 0.001$) at doses of 400, 600, and 800 mg/kg of MEAR, while with loperamide (5mg/kg) this value was 137.3 ($p < 0.001$) compared to controls as shown in Table 1. The extract at the dose of 200mg/kg had no significant effect on both purging frequency and diarrhea onset . The extract when administered at the dose of 800mg/kg, orally, produced maximal inhibitory effect on all the diarrhea parameters investigated .

The castor oil induced diarrhea model was also conducted using the aqueous extract and the study has shown that this extract lacks significant antidiarrheal activity ( Data not shown).Therefore, further studies using this extract was not performed.

Table1. Effect of the hydroalcoholic extract of *Ajuga remota* on castor oil diarrhea model

| Group (mg/kg) | Onset of diarrhea (min) | Mean wt. of wet stools in 4 h (g) | Mean no. of wet stools in 4 h | Total no. of Stools in 4 h | PI |
|---|---|---|---|---|---|
| **Control** | 67.00±2.51 | .69±.04 | 9.00±1.09 | 11.00±1.26 | |
| **Loperamide5** | 174.33±14.99$^{a_3}$ | .22±.05$^{a_3}$ | 3.00±.73 $^{a_3}$ | 4.00±.07 $^{a_3}$ | 66.7 % |
| **MEAR 200** | 94.67±1.64$^{b2c3d2}$ | .53±.02$^{b3c3d2}$ | 6.83±.60$^{b2c3d2}$ | 10.00±.57$^{b2c3d2}$ | 12.9% |
| **MEAR 400** | 151.33±1.42$^{a3f1}$ | .35±.01$^{a3}$ | 4.17±.30$^{a3c1}$ | 6.00±.73$^{a2f2}$ | 53.7 % |
| **MEAR 600** | 164.67±15.14$^{a3}$ | .28±.05$^{a3}$ | 3.00±.63 $^{a3}$ | 4.00±.85$^{a3}$ | 66.7 % |
| **MEAR 800** | 190.00±19.65 $^{a3}$ | .15±.05$^{a3e1}$ | 1.83±.60$^{a3}$ | 2.00±.94 $^{a3}$ | 79.6% |

Values are expressed as Mean ± SEM . (n = 6 mice) [a] against control, [b] against standard, [c] against 800 MEAR , [d] against 600 MEAR, [e] against 400 MEAR ,[f] against 200 MEAR [1] $p < 0.05$, [2] $p<0.01$, [3]$p<0.001$. MEAR refers to 80% methanol extract of *Ajuga remota*, control: groups treated with 10ml/kg 2% tween 80, Loperamide 5: loperamide Hcl 5mg/kg, numbers refers to dose in mg/kg, PI refers to percent inhibition of defecation

### 4.3. Castor oil induced enteropooling

Percentage inhibition in intestinal fluid accumulation was 42.4 %, 62.1%, and 74.2% ($p < 0.001$; for all) at the doses of 400, 600 and 800 mg/kg of MEAR, respectively, compared to the control group as shown in Figure 3. The standard drug, loperamide Hcl ( 5mg/kg), also inhibited the intestinal fluid accumulation with a percentage inhibition of 66.6% ($p < 0.001$) as shown in Figure 3. Pretreatment with the different doses of the hydroalcoholic extract showed a dose-dependent inhibition ($R^2$ =0.981**)** in castor oil induced intestinal fluid accumulation. Here also the extract when administered at the dose of 200mg/kg had no significant effect on intestinal fluid accumulation, compared to the

control, whereas the 800mg/kg dose produced maximal inhibitory effect on the intestinal fluid accumulation . Similar results were obtained for the weight of intestinal content at the different doses of the extract, compared to the control group as shown in Figure 4.

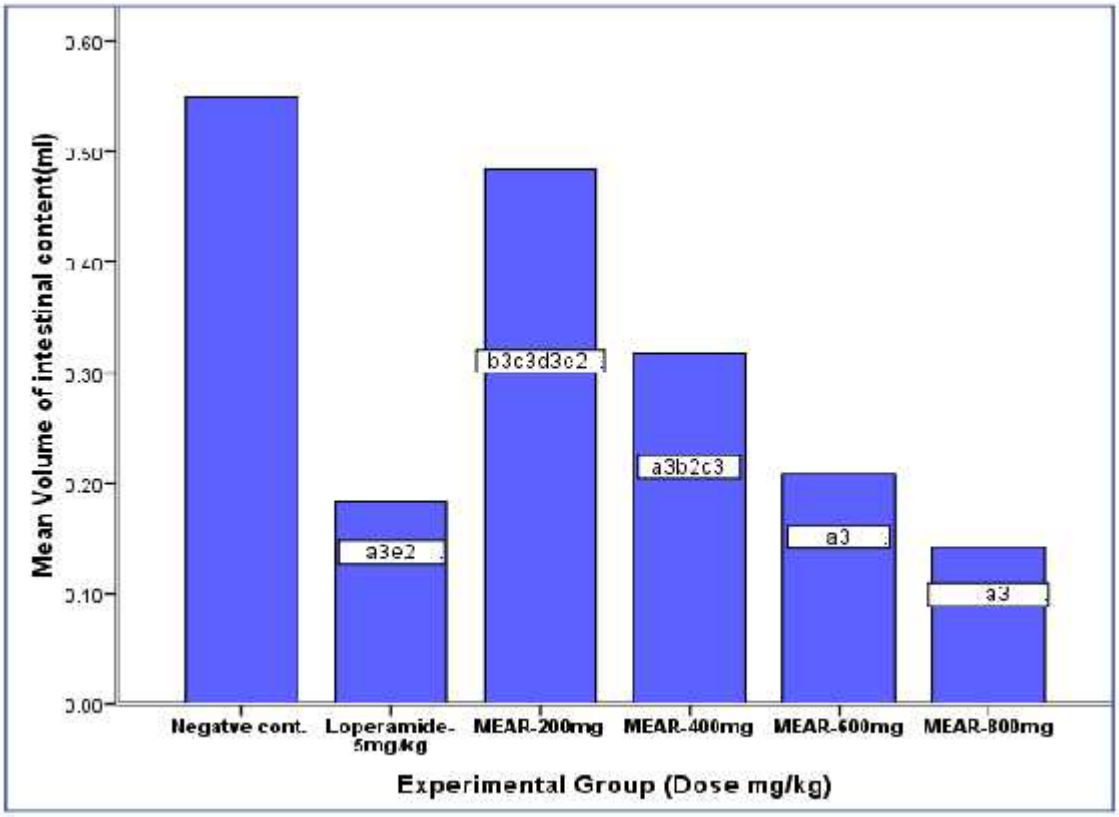


**Figure 3**: Effect of MEAR on castor oil-induced enteropooling (on volume of intestinal content ) in mice. (n = 6). Data are mean ± SEM . [a] against control, [b] against standard, [c] against 800 MEAR, [d] against 600 MEAR, [e] against 400 MEAR, [1] $p < 0.05$, [2] $p<0.01$, [3]$p<0.001$. MEAR refers to 80% methanol extract of *Ajuga remota*, control: groups treated with 10ml/kg 2% tween 80 .

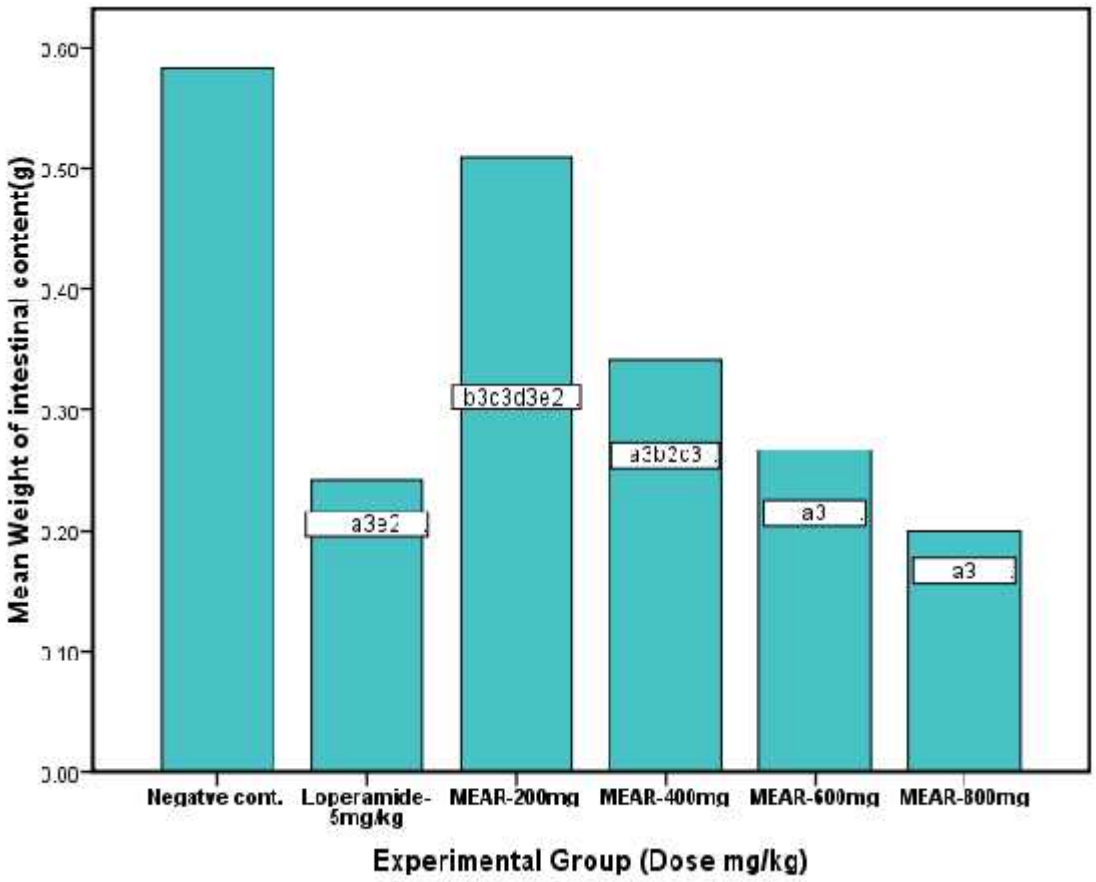


**Figure 4**: Effect of MEAR on castor oil-induced enteropooling ( weight of intestinal content ) in mice. (n = 6 ). Data are mean ± SEM. [a] against control, [b]against standard, [c] against 800 MEAR, [d] against 600 MEAR, [e] against 400 MEAR, [1] $p < 0.05$, [2] $p<0.01$, [3]$p<0.001$. MEAR refers to 80% methanol extract of *Ajuga remota*, control: groups treated with 10ml/kg 2% tween 80

### 4.4. Normal gastrointestinal propulsion in mice

The 80% methanol extract of the plant decreased propulsion and consequently the percentage of intestinal transit of the charcoal meal through the gastrointestinal tract during normal gut transit as compared with control group (2% tween 80). The MEAR inhibited the normal intestinal transit of charcoal meal plug by 35.4 %, 49.1%, and 63.9% *($p<0.001$)* at doses of 400mg/kg , 600mg/kg and 800mg/kg , respectively, while atropine sulphate at the dose of 3mg/kg showed 55.0% (*$p<0.001$)* inhibition as shown in Table 2 . The extract inhibited significantly ($p<0.001$) the normal intestinal propulsion in a dose dependent manner ($R^2=1.000$) . The extract at the dose of 800mg/kg produced maximal

inhibition in intestinal propulsion in the normal intestinal transit. The extract at the dose of 200mg/kg had no significant effect compared to the control on the normal intestinal transit of mice as shown in Table 2 .

Table 2 . Normal gastrointestinal transit in mice treated with the hydroalcoholic extract of the aerial parts of *Ajuga remota*

| Group (mg/kg) | Mean of the total length of intestine (cm) | Mean of distance traveled by charcoal meal (cm) | Peristaltic index (PI ) | (%) Inhibition |
|---|---|---|---|---|
| **Control** | 57.77±1.09 | 40.74±1.25 | 70.51 | 00.0 |
| **Atropine** | 55.96±1.28 | 18.33±.56 $^{a3e1}$ | 32.76 | 55.0 |
| **MEAR 200** | 55.71± 1.11 | 37.56±1.51$^{b3c3d3e3}$ | 67.43 | 7.7 |
| **MEAR 400** | 53.58±0.93 | 26.28±2.15$^{a3b1}$ | 49.05 | 35.5 |
| **MEAR 600** | 54.47±1.17 | 20.70±1.18$^{a3}$ | 38.00 | 49.2 |
| **MEAR800** | 55.30±1.21 | 14.69 ±.55$^{a3\ e3}$ | 26.58 | 63.9 |

Values are expressed as mean ± SEM . ($n$ = 6 mice ) $^{a}$ against control , $^{b}$ against standard, $^{c}$ against 800 MEAR , $^{d}$ against 600 MEAR, $^{e}$ against 400 MEAR ; $^{1}$ P<0.05, $^{2}$ p<0.001, $^{3}p$<0.001 . 200, 400, 600 and 800 mg/kg represent doses of MEAR; Control: control group taking 10ml/kg 2% tween 80, Atropine : Atropine sulphate 3mg/kg

### 4.5. Castor oil induced gastrointestinal transit in mice

The charcoal meal moved farther in the castor oil induced intestinal transit compared to the normal intestinal transit. The MEAR inhibited the intestinal transit of charcoal meal induced by castor oil by 33.3%, 53.8 % and 64.6% *(p<0.001)* at doses of 400mg/kg , 600mg/kg and 800mg/kg , respectively, while atropine sulphate at the dose of 3mg/kg showed 61.4 % (*p<0.001)* inhibition as shown in Figure 5. The extract inhibited significantly (p<0.001) in castor oil induced intestinal transit in a dose dependent manner ($R^2$=1.000) . The inhibitory effect of the extract in castor oil-induced intestinal transit is almost comparable to that of the normal intestinal transit. The extract at the dose of 800mg/kg produced maximal inhibitory effect in the castor oil-induced intestinal transit.

The extract at the dose of 200mg/kg had no significant effect on the intestinal transit compared to the control as shown in Figure 5.

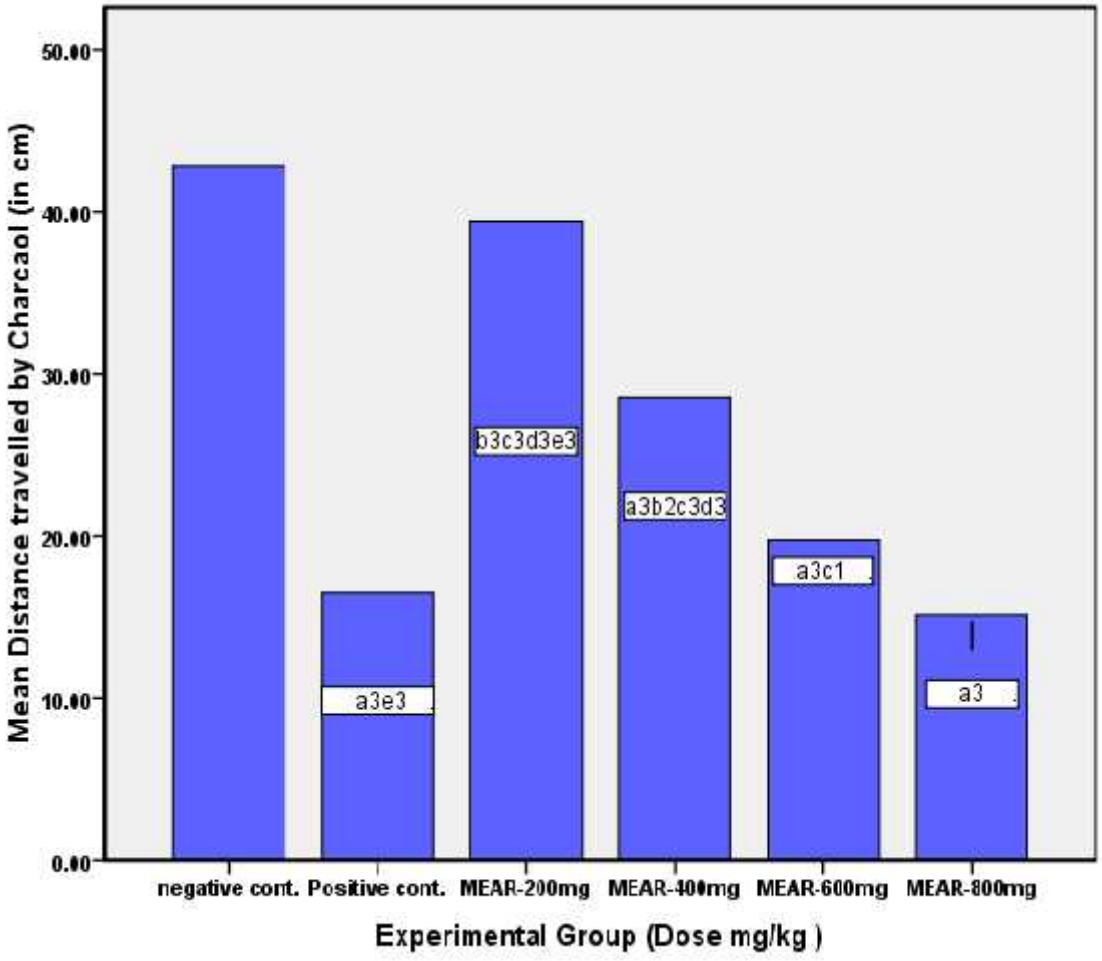


**Figure 5**: Effect of MEAR on castor oil -induced gastrointestinal transit in mice. (n = 6). Data are mean ± SEM. [a] against control ,[b] against standard ,[c] against 800 MEAR ,[d] against 600 MEAR ,[e] against 400 MEAR , [1] $p < 0.05$, [2] $p<0.01$, [3]$p<0.001$. MEAR refers to 80% methanol extract of *Ajuga remota*, control: groups treated with 10ml/kg 2% tween 80

### 4.6. In vivo *antidiarraheal index*

The antidiarrheal index (ADI) is a measure of the combined effects of these different components of diarrhea such as purging frequency, onset of diarrheal stools, and intestinal fluid accumulation. Results for the *in vivo antidiarraheal indices* were 16.3% , 60.8%, 80.5 % and 98.1 % at the doses of 200, 400, 600 and 800 mg/kg, p.o. of MEAR respectively, while the 800mg/kg of the extract gave a maximum index of 98.1% as shown in Figure 6.The extract produced antidiarrheal activity as shown by the antidiarrheal index (ADI) in a dose dependent manner ($R^2$=1.000) .

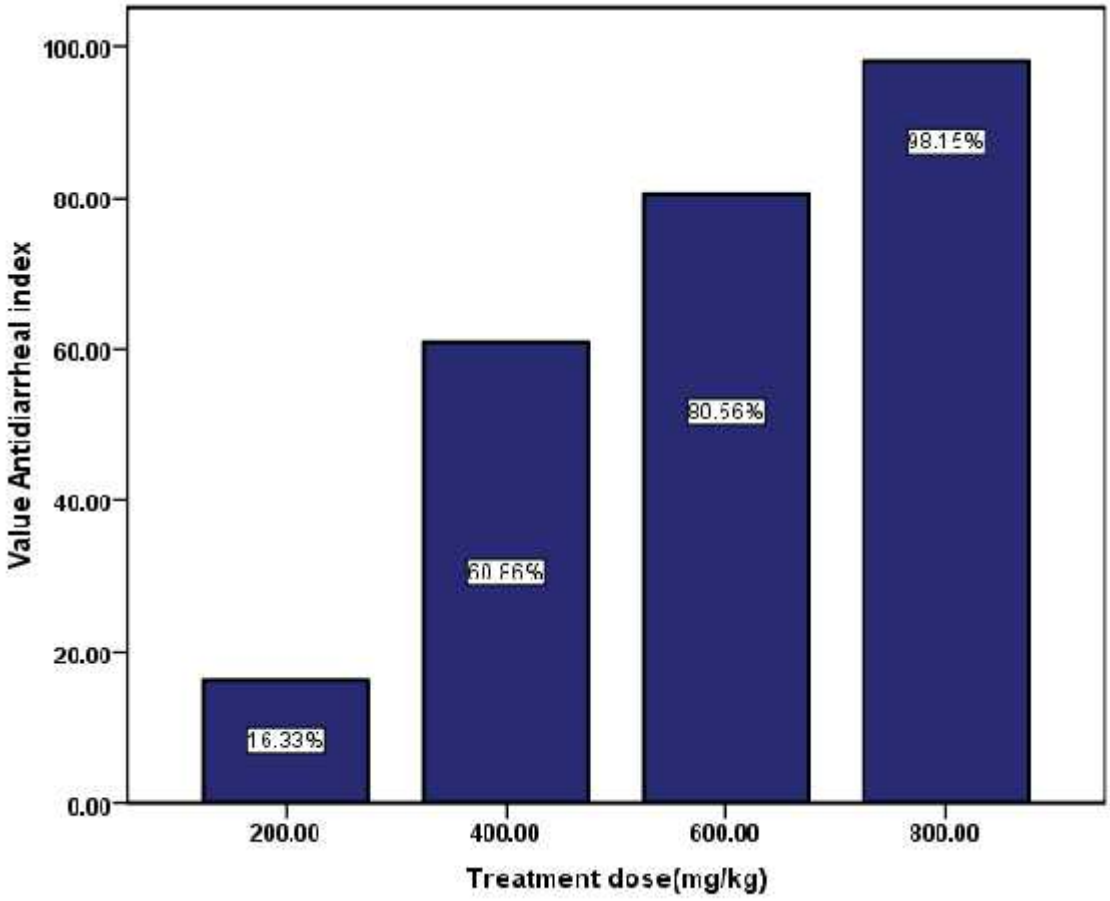


**Figure 6**: Antidiarrheal index of 80% methanol extract of *Ajuga remota*

### 4.7. Phytochemical Analysis

The phytochemical analysis of MEAR tested positive for alkaloids, flavonoids, terpenoids, tannins, phenolics, anthraquinones, glycosides ,steroids ,and saponins as shown in Table 3.

Table 3: Phytochemical analysis of 80% methanol extract and aqueous extract of *Ajuga remota* .

| | **Alkaloids** | **Tannins** | **Saponins** | **Glycosides** | **Phenols** | **Flavonoids** | **Terpenoids** | **Anthraquinones** | **Steroids** |
|---|---|---|---|---|---|---|---|---|---|
| **80% methanol extract of *Ajuga remota*** | + | + | + | + | + | + | + | + | + |

Key: ( + )= presence of phytocompounds ( -) = Absence of phytocompounds

## 5. Discussion

In an attempt to investigate the medicinal use of the aerial parts of *Ajuga remota* in gastrointestinal disorders, such as diarrhea, this study was carried out to evaluate the antidiarrheal activity of the plant extract in experimental animals, and the possible underlying mechanism . The antidiarrhoeal studies carried out reveal that MEAR possesses antidiarrhoeal activity in all the models of diarrhoea used in the present study.

Several studies have validated traditionally used antidiarrheal plants based on animal studies by investigating the bioactivity and effects of these plants on intestinal function that have antispasmodic effects, delay gastrointestinal transit, suppress gut motility, stimulate water adsorption or reduce electrolyte secretion (7). This study has also tried to chemically and biologically evaluate the acclaimed properties of the plant *Ajuga remota Benth* using the different animal models. Relevantly, the hydroalcoholic extract of the aerial parts of *Ajuga remota* exhibited a statistically significant reduction in the incidence and severity of diarrheal stools produced in mice. The extract showed a dose-dependent inhibition in all diarrheal parameters: onset of diarrhea, weight of wet stools , number of wet stools, and total number of stools as compared to the control group . This result is in accordance with previous claims in respect of antidiarrhoeal herbs. Antidiarrhoeal plants are known to reduce number of wet stools , consistency of fecal droppings as well as delay in the onset of diarrhea as reported for *Pterocarpus erinaceus* (2), *Saussurea lappa Clarke* (13), *Lithocarpus dealbata and Urena lobata* (61).

The specific constituent (s) responsible for the anti-diarrheal properties of the aerial parts of this plant is yet to be identified. However, the antidiarrhoeal activity of medicinal plants has been attributed to the presence of bioactive agents such as tannins, alkaloids, saponins, flavonoids, steroids , phenolics and terpenoids (7,39,40,83). The phytochemical analysis of the hydroalcoholic extract of the aerial parts of *Ajuga remota* revealed the presence of many of the secondary metabolites which include tannins, alkaloids, saponins, flavonoids, steroids, terpenoids, glycosides, anthraquinones and phenolic

compounds .This finding is in line with a recent study on the phytoconstituents of the plant (52,55); this might be due to the similarity in geographic location (i.e. almost identical whether condition) where the plant specimens were collected. Of these bioactive agents found in the plant extract, tannins, flavonoids, phenolics, terpenoids and alkaloids may partly be responsible for the observed pharmacological properties of the plant (7,39,40).

Castor oil was used in this study to induce diarrhoea since castor oil model , incorporates both secretory and motility diarrhea ( 39,69,70). Castor oil is composed principally of ricinoleic acid (71,72). It induces diarrhoea by the formation of ricinoleic acid in the upper intestine by one of the several mechanisms proposed below (39,73,74). The use of castor oil induced diarrhea model in this study is logical because the autacoids and PGs are involved in the pathophysiology of diarrhea and these have been implicated in the causation of diarrheas in man (82).The ricinoleic acid being poorly absorbed induces change in the mucosal permeability, electrolyte transport and intestinal peristalsis, leading to a hypersecretory response and diarrhoea (3,75). The ricinoleic acid readily forms ricinoleate salts with $Na^+$ and $K^+$ in the lumen of the intestine. Ricinoleate has several actions that could account for its anti-absorptive effect on the mucosa. It inhibits the enzyme Na+- K+ ATPase and increases the permeability of the intestinal epithelium (3,71). Thus, one possible anti- diarrheal activity of the extract against castor oil induced diarrhea may be attributed to its anti-electrolyte permeability action (71).

Ricinoleic acid also causes local irritation and inflammation of the intestinal mucosa leading to PG release, which causes an increase in the net secretion of water and electrolytes into the small intestine (71,75). Inhibitors of prostaglandin biosynthesis delayed castor oil induced diarrhea (84). Based on these facts, it seems reasonable to suggest that the extract may reduce PG induced secretion of water and electrolytes due to the inhibition of prostaglandin synthesis (75). Investigations of the mode of action indicate that tannins and flavonoids present in the plant may increase colonic water and electrolyte reabsorption (7). Thus, in general the antidiarrheal activity of the extract against experimentally induced diarrhea by castor oil may be attributed to an increase in

fluid and electrolytes absorption and/or to a slowing down of intestinal transit, allowing more time for absorption to occur since castor oil induces acceleration of the intestinal transit because of its motor activities and prevent absorption of water and solutes in the lower intestine (77).

Studies on castor oil - induced intestinal fluid accumulation showed that the extract reduced both the weight and volume of intraluminal contents. These effects, which are direct consequences of reduced water and electrolytes secretion into the small intestine, suggest that the extract may enhance electrolyte absorption from the intestinal lumen consistent with inhibition of hypersecretion (78). The secretory diarrhoea is associated with an activation of $Cl^-$ channels, causing $Cl^-$ efflux from the cell, the efflux of $Cl^-$ results in massive secretion of water into the intestinal lumen and profuse watery diarrhoea (71,79-81).The extract may inhibit the secretion of water into the lumen by reverting this mechanism . Again it is likely that the enhanced electrolyte absorption by the extract may have encouraged the absorption of other intestinal solute contents like nutrients that in turn may have created an osmotic gradient across enterocytes which stimulated water absorption (78). Thus, another possible antidiarrheal activity of the extract could be the reduction in net fluid and solutes secretion, since a great part of the laxative effect of castor oil is related to increase in fluid secretion (77) and this fluid content is the principal determinant of stool volume and consistency (71).

The involvement of muscarinic receptor effect was confirmed by increased production of both gastric secretion and intraluminal fluid accumulation induced by castor oil. Thus, the hydroalcoholic extract may inhibit the secretion of water into the intestinal lumen and this effect is partly mediated by both $\alpha 2$-adrenoceptor and muscarinic receptor systems (70). Tannins present in plant, denature proteins in the intestinal mucosa forming protein tannate complex. The complex formed coat over the intestinal mucosa and makes the intestinal mucosa more resistant to chemical alteration and reduces secretion (40,46), i.e. anti-electrolyte permeability action, further more this complex coat over the mucosa may prevent the PGE2 induced enteropooling (74). In addition, flavonoids present antioxidant properties which are presumed to be responsible for the inhibitory effects

exerted upon several enzymes including those involved in the arachidonic acid metabolism (39), thus, reducing prostaglandin induced fluid secretion. Furthermore, previous studies has also shown that the plant possesses anti-oxidant activity (55). Anti-enteropooling effect of the extract is more relevant because the prevention of enteropooling helps in the inhibition of diarrhea, especially by PGE2 induced diarrhea as it is involved in the onset of diarrhea in intestinal mucosal cells (75). Suppression of the intestinal fluid accumulation by the extract might also suggest the inhibition of gastrointestinal function (69).

The hydroalcoholic extract of *Ajuga remota* inhibited gastrointestinal propulsion in both the normal or castor oil induced intestinal transit. This finding suggests that the extract acts on all parts of the intestine. The hydroalcoholic extract of *Ajuga remota* (400, 600 and 800 mg/kg) and the anti-muscarinic drug, atropine (3mg/kg) decreased the propulsive movement in the charcoal meal study in normal intestinal transit. A decrease in the motility of gut muscles increases the stay of substances in the intestine. This allows better water and electrolyte absorption. It is therefore presumed that the reduction in the intestinal propulsive movement in the charcoal meal model may be due to antispasmodic properties of the extract (61). Antidiarrhoeal treatment in a patient is achieved through the objective of therapy which includes increasing resistance to flow ( segmental contraction and decrease propulsion) and increased mucosal absorption or decreasing secretion (63,74). This is indicative of the ability of the plant to alter normal peristaltic movement and hence decrease the movement of materials in the intestinal tract allowing greater time for absorption (63,74). MEAR changed normal intestinal transit in mice at doses showing antidiarrheal activity . Since the extract shows antipropulsive effect on normal intestine similar to Atropine it seems likely that the antidiarrheal effect could be due to anticholinergic activity .

Investigations on the mode of action indicate that Phytochemicals like phenolics, alkaloids and terperoids act by inhibiting intestinal motility (7). Apart from this, flavonoids has been ascribed to their ability to inhibit intestinal motility and hydro-electrolytic secretion (7,41) as well as contractions induced by spasmogenics (77). Studies on the functional role of tannins also reveal that they could also reduce the

peristaltic movements and intestinal secretions by reducing the intracellular $Ca^{2+}$inward current or by activation of the calcium pumping system (which induces the muscle relaxation) (64) i.e. tannins exhibit spasmolytic and calcium channel blocking (CCB)activities (86) . Sesquiterpenes, diterpenes, terpenes, and other terpenoid derivatives are known for inhibiting release of autocoids and prostaglandins, thereby inhibit the motility and secretion induced by castor oil (85).

Hypermotility characterizes forms of diarrhea where the secretory component is not the causative factor (78). Pre-treatment with the extract suppressed the propulsive movement of charcoal meal plug through the gastrointestinal tract in castor oil induced gut transit which clearly indicates that the aerial parts of the plant extract may be capable of reducing the frequency of stooling in diarrheal conditions. The extract inhibited significantly the gastrointestinal propulsion in a dose dependent manner . Delay in gastric motility causes further absorption of water from feces and may additionally contribute to reducing its watery texture. Castor oil-induced gastrointestinal hypermotility has been suggested to be indirectly mediated by the cholinergic system since it is inhibited by atropine, a known anticholinergic agent . From the results of this study, it is likely that the extract inhibits gastrointestinal hypermotility in diarrhea through anticholinergic effect since the extract reduced gastrointestinal motility as well as gastric contents and watery texture of diarrheal stools thus leading to the much desired reduction in frequency of stooling in diarrheal disease (78). In addition, the alkaloids present in the plant extract are known to exhibit anticholinergic activity (86) .

The antidiarrheal index (ADI) is a measure of the combined effects of these different components of diarrhea such as purging frequency, onset of diarrheal stools, and intestinal fluid accumulation (59). The hydroalcoholic extract of the aerial parts of *Ajuga remota* produced a dose-dependent antidiarrheal index which implies that the plant extract produces antidiarrheal activity in a dose-related manner .

The plant has also been shown to possess antibacterial, antiviral, antifungal, and antihelimenthic properties (39, 52). Such activities of the plant could account additional benefit providing a wider cover for its use in diarrhea of different etiologies including

those of infectious diarrheas and thus can serve as a profitable alternative for treating such diarrheas (39,52). This study has been undertaken by using a crude hydroalcoholic extract, and that the different biological activities assayed herein, may not be due to a single constituent since the crude extracts contain several compounds acting on different mechanisms. In addition, the interplay of the constituents in the crude extract may result in better activity due to synergism or may lead to a decrease in toxicity and it is possible that pure compounds may not necessarily behave in the same manner as the natural extracts (7,67).

## 6. Conclusion

Results of this study has investigated that the hydroalcoholic extract of *Ajuga remota* contains pharmacologically active substances with significant antidiarrhoeal activity in all experimental models used in the study . These attributes may provide the rationale for the use of *Ajuga remota* for treating diarrhea and hence , on a preliminary basis it can be claimed as a potential therapeutic option in the effective management of diarrhea, thus supporting its folkloric use by traditional healers for this purpose .

## 7. Recommendations

Based on the findings of the present study, the following recommendations are forwarded for further studies :

- Further studies should be directed to fractionate and purify (isolate ) the crude extract in order to identify and characterize the bioactive compounds of the extract that are responsible for the anti-diarrhoeal activity .

- Further studies are required to ascertain the precise mechanism of action of the antidiarrheal activity of the plant

- Studies on other models like Ex-vivo model should be carried out to assess the antispasmodic effect of the extract

- Further toxicological studies needs to be done to confirm absence of toxicity though the extracts of the plant appear to be safe under this study .

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