EVALUATION OF ANTIDIARRHEAL ACTIVITY OF AQUEOUS AND 80% METHANOL SEED EXTRACT OF *CALPURNIA AUREA*- Benth (FABACEAE) IN MICE.

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This is to certify that the thesis prepared by Achenef Bogale, entitled: Evaluation of antidiarrheal activity of aqueous and 80% methanol seed extract of *Calpurnia aurea*- Benth (fabaceae) 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 antidiarrheal activity of aqueous and 80% methanol seed extract of *Calpurnia aurea*-Benth (fabaceae) in mice.

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Calpurnia aurea B. is one of the medicinal plants claimed to have antidiarrheal potential, but with limited scientific evidence. This study was aimed at investigating the antidiarrheal and antibacterial activity of aqueous and 80% methanol seed extracts of the plant in mice and selected diarrhea causing bacterial strains. The antidiarrheal activity was evaluated using castor oil induced diarrheal model, prostaglandin induced anti-enteropooling and castor oil induced charcoal meal test in mice of either sex, while antibacterial activity was assessed on *Shigella soni, Salmonella typhimurium, Escherichia Coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* using disk diffusion and micro-dilution techniques. The *in vivo* test groups received a graded dose (60, 120 and 240 mg/kg) of both aqueous and 80% methanol seed extract, whereas positive controls received loperamide (3 mg/kg) and negative controls received distilled water (10 ml/kg). Pretreatment of mice at the stated doses caused a significant reduction in frequency of wet stools and watery content of diarrhea as well as in delaying onset of diarrhea as compared to controls. Both extracts showed a dose-dependent inhibition in all models used. The extracts also showed significant ($p<0.05$) inhibition in intestinal motility in castor oil induced models. In addition, both extracts exhibited variable activity against the selected bacterial strains; a better effect was seen with 80% methanol seed extract. In conclusion, the results obtained in this study suggest that both aqueous and 80% methanol seed extracts of *Calpurnia aurea* have beneficial effect in controlling diarrhea through antisecretory and antimotility activites, with marginal antibacterial activity, lending support to the traditional use of the plant for the same purpose.

Key words: Antidiarrheal activity, Castor oil induced diarrhea, Gastrointestinal transit, Anti-enteropooling, *Calpurnia aurea*

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Contents

Abbreviations/acronyms

ADI	Anti-diarrheal Index
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
CaCCs	Calcium activated Chloride Channel
cAMP	Cyclic Adenosine Monophosphate
CCBs	Calcium Channel Blockers
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFU	Colony forming unit
cGMP	Cyclic Guanosine Monophosphate
EAEC	Enteroaggregative *Escherichia Coli*
EHEC	Enterohemorrhagic *Escherichia Coli*
ETEC	Enterotoxigenic *Escherichia Coli*
FAO	Food and Agriculture Organization of the United Nation
GI	Gastrointestinal
IBDs	Inflammatory Bowel Diseases
IBS	Irritable Bowel Syndrome
IDSA	The guideline of Infectious Diseases Society of America
IL	Interleukin
NO	Nitric Oxide
OECD	Organization for Economic Cooperation Development
ORS	Oral Rehydration Solution
PINES	Paracrine-immuno-neuroendocrine system
PA	Pyrrolizidine Alkaloids
RPM	Rotation per-minute
UNICEF	United Nations International Children's

	Emergency Fund
WGO	World Gastroenterology Organization
WHO	World Health Organization

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1. Introduction

1.1 Definition and classification of diarrhea

The term diarrhea, originates from Greek (dia = through) and Latin (rheen = flow or run), is defined as the rapid transit of gastric contents through the bowel (Earnest *et al*., 2018; Whyte & Jenkins, 2012). The World Health Organization (WHO) defines diarrhea as the passage of three or more loose or liquid stools per day, or as having more stools than is normal for that person (2011). According to Polage *et al*. (2012) diarrhea is the passage of loose or watery stools at least three times in one day duration. However, consistency of the stool matters for the patients rather than its number. The problem with stool consistency is its difficulty to quantify. Due to this, in addition to frequency and consistency of the stool, it is recommended to consider weight of the stool. Mostly, the normal stool output range between 100 and 200 g/day. Therefore, it is possible to consider stool weight >200 g/day as diarrhea. Keeping this in mind, it is important to exclude conditions when some people who consume excess fiber have stool weights of 300 g/day or more with normal consistency which does not necessarily mean diarrhea (Navaneethan & Giannella, 2011).

If duration considered as a criterion, diarrhea can be classified into acute (less than 2 weeks), persistent (from 2 to 4 weeks), and chronic diarrhea (more than 4 weeks). This classification is critical for diagnostic and treatment purpose. Diarrhea can also be categorized based on its pathophysiology into secretory, osmotic, inflammatory, iatrogenic or drug-induced, and functional-/motility-related diarrhea. The previously mentioned scenarios can be due to infectious or non-infectious causes (Navaneethan & Giannella, 2011).

1.2 Epidemiology of diarrhea

All through human history, diarrheal disease has been a major health problem. In the past, it was often deadly and disease outbreaks distribute alarmingly, threatening large populations (Thiagarajah *et al*., 2015). In accordance with the joint report by WHO and the United Nations International Children's Emergency Fund (UNICEF) (2009), around 2.5 billion cases of diarrheal disease worldwide every year, and 1.9 million children younger than five years of age die due to diarrhea each year with the lion share of the burden laid on developing countries. It contributes 18% of all deaths of children under the age of five i.e. more than 5000 children are dying every day as a result of diarrheal diseases (Farthing *et al*., 2013; UNICEF/WHO, 2009).

Even though diarrhea is a preventable and treatable disease, it is the second leading cause of mortality among children under five years of age worldwide, next to respiratory infections. It killed more young children than AIDS, malaria, measles, injuries and all other post-neonatal conditions combined (Kaplan *et al*., 2013; WHO, 2013). In the earlier time, diarrheal diseases were often fatal and disease outbreaks distribute quickly, affecting large populations (Thiagarajah *et al*., 2015). Today, despite the successful interventions such as oral and intravenous rehydration therapy, diarrheal diseases remains one of the leading causes of morbidity and mortality in the world. Globally, diarrhea is the third largest cause of morbidity and the sixth largest cause of mortality among population of all ages. A recent data states that about 3-5 billion diarrheal illnesses and 5-10 million diarrhea-related deaths occur annually among those living in Africa, Asia, and Latin America (Kedir, 2015).

Diarrhea is most common in packed living conditions coupled with poor hygiene and malnutrition (Bakare *et al*., 2011; Tadesse *et al*., 2014). It is a major contributor to malnutrition, and also causes rapid dehydration in infant and elderly people, which could lead to death if treatment is not given (Bakare *et al*., 2011).

Regarding to prevalence of diarrheal disease, it is correlated closely with climate and economic development. The most severely affected regions are developing countries in sub-Saharan Africa and South Asia. This is corroborated by the fact that just 15 countries account for almost three quarters of all deaths from diarrhea among children in 2009. Among these, China, Democratic Republic of Congo, India, Nigeria and Ethiopia are the top 5 countries (Christopher, 2009).

On top of that, as Faure (2013) pointed out, incidence and mortality of diarrheal diseases are greatest among children younger than one year of age. Then it declines incrementally with eight out of ten of these deaths occurring in the first two years of life. Childhood gastroenteritis is the main cause of mortality of children; with 18% of cause specific deaths are among children under five years of age in 2000–2003. The incidence of acute gastroenteritis in children is mainly frequent in areas without access to clean water (Bryce *et al*., 2005). It is estimated that about 88% of diarrheal deaths worldwide are attributable to unsafe water, inadequate sanitation, and poor hygiene (Lopez & Mathers, 2006).

In Africa, including Ethiopia, each child on average suffers from five episodes of diarrhea per year (Kedir, 2015). The number of childhood deaths decreased only by 4% in Africa from 2000 to 2008. It is due to inadequate interventions and high poverty rate (Tambe *et al*., 2015).

Despite the success of interventions such as oral and intravenous rehydration therapy, diarrheal diseases still remain one of the leading causes of morbidity and mortality in the world. According to the latest Global Burden of Disease Study, about 2.39 billion of diarrheal cases reported globally. From this, an estimated 1.31 million deaths occurred annually (Troeger *et al*., 2017). Lower and middle income countries such as Africa and south-east Asia tend to took higher incidence and case-fatality ratios (Mokomane *et al*., 2018). In accordance with the Central Statistical Agency demographic and health survey report, diarrhea contributes to more than 13% child deaths in Ethiopia (CDC, 2016). As UNICEF (2012) reported, diarrhea also accounted for 14% death of under-five children in Ethiopia.

1.3 Etiology and prevention of diarrhea

Diarrheal disease can be either infectious or non-infectious; with infection pathogenesis contributing to the major part worldwide. In infectious diarrhea, the potential causative pathogens include enterotoxin-producing bacteria, such as *Vibrio cholerae* and Enterotoxigenic *Escherichia coli*; viruses, such as *rotavirus*; enteroinvasive bacteria, such as *Shigella* and *Salmonella*; and parasites, such as Giardia, *Entamoeba histolytica* and *Cryptosporidium parvum* (Navaneethan & Giannella, 2011; Thiagarajah *et al*., 2015). These pathogens present in the gut causing disruption of normal fluid secretion and motility, and stimulating the gut to expel the contents. Most pathogens are transmitted from the stool of one person to the mouth of another via contaminated food or water (fecal-oral transmission) (Panda *et al*., 2012). *Rotavirus* and *Escherichia coli* are the two most common etiological agents of diarrhea in developing countries (WHO, 2013).

When diarrheal symptoms worsen or become chronic in the absence of an identifiable infectious organism (virus, bacterium, protozoan) or when occur acutely due to medication and food intolerance or when happened chronically due to primary gastrointestinal (GI) disease, such as inflammatory bowel disease it is categorized under noninfectious Diarrhea (Carlson *et al*., 2016). Rare congenital disorders, for instance microvillus inclusion disease, familial diarrhea syndrome and tufting enteropathy, as well as peptide-secreting neuroendocrine tumors are also known to cause severe secretory diarrhea. Though noninfectious causes of diarrhea are immense in developed countries, infectious causes of diarrhea are still represent a huge proportion of the burden (Thiagarajah *et al*., 2015).

Measures for prevention of diarrhea include: exclusive breastfeeding for the first 6 months of life, safe drinking water, improved sanitation, personal and food hygiene, and *Rotavirus* vaccination. Furthermore, informing parents about the routes of transmission of enteropathogens and preventive measures are important to tackle the problem (Singh & Verma, 2012).

1.4. Normal intestinal physiology and pathophysiology of diarrhea

1.4.1. Normal intestinal physiology

The human small intestine and colon perform important functions such as secretion and absorption of water and electrolytes, storage and transport of intraluminal contents. Under healthy physiological conditions, about 8 liters of fluids (2 liter of ingested fluids and 6 liter from salivary, biliary, gastric, and pancreatic secretions) delivered to the upper small bowel. Most of this fluid is reabsorbed before reaching the distal small bowel so that only about 1 liter of fluid enters the colon. The colon in turn reabsorbs almost all of this fluid and leaving usually less than 200 ml to be excreted in the stool. Since the colon has the capacity to reabsorb up to a maximum of 3–4 liters of fluid it can recap much of the fluid that might be lost due to the small intestinal mal-functioning. Disruption in this fluid and electrolyte handling leads to formation of diarrhea (Navaneethan & Giannella, 2011).

Ions, primarily Na^+, Cl^-, $HCO3^-$, and K^+, and solutes, mainly glucose are the major drivers of the movement of fluid between the intestinal lumen and blood (Thiagarajah *et al*., 2015). Fluid absorption or secretion involves the coordinated activity of membrane transporters located on the apical (lumen-facing) and basolateral (circulation-facing) epithelial membranes (Thiagarajah & Verkman, 2012).

A constant bidirectional flux of water and ions across the small intestinal mucosa is always in function, i.e., absorption and secretion. Sodium absorption drives fluid reabsorption, while active chloride secretion contributes to water secretion in secretory diarrhea (Binder, 2005; Binder & Reuben, 2005). Sodium and water absorption by enterocytes is mediated by an active, ATP-dependent active sodium pump (Na^+, K^+-ATPase) located on the basolateral membranes of intestinal crypt and villus cells. Small intestinal Na^+ absorption is driven primarily by two mechanisms: a glucose- or amino acid-stimulated co-transport in which Na^+ accompanies the other solute and a coupled Na^+-Cl^- mechanism. The latter is a combination of Na^+-H^+ exchange and Cl^--HCO_3^- exchange. Short-chain fatty acid (SCFA)-mediated Na^+ absorption and aldosterone-sensitive Na^+ absorption occurs in the colon (Binder, 2005). Among the various mechanisms described, the coupled Na^+-Cl^- pathways are primarily regulated by cyclic adenosine monophosphate (cAMP) levels and also by cyclic Guanosine Monophosphate (cGMP) and intracellular Ca^{2+} levels (Field, 2003). In addition to the transporters, there are multiple extracellular factors (paracrine, immunological, neural and endocrine) regulating epithelial ion transport, termed together as a single regulatory system known as PINES (paracrine-immuno-neuroendocrine system) (Mourad *et al.*, 1995).

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

1.4.2. Pathophysiology of diarrhea

The pathophysiological mechanisms underlying the loss of intestinal fluid in diarrhea have been a subject of debate for decades. The leading hypothesis up to the 1970s was that most diarrheas ensued because of altered GI motility. Later on, it has become increasingly apparent that a disturbance in the epithelial transport of ions and water is a major cause of intestinal fluid loss even if motility disturbances may contribute (Lundgren, 2002). Four general pathophysiologic mechanisms disrupting water and electrolyte balances are the basis of diagnosis and therapy of diarrhea. These are changes in active ion

transport by decreased sodium absorption or increased chloride secretion, increase in luminal osmolarity, increase in tissue hydrostatic pressure and change in intestinal motility. These mechanisms have been related to four broad clinical diarrheal groups: secretory, osmotic, inflammatory and altered motility diarrhea (Spruill & Wade, 2008).

Secretory diarrhea happens when there is an increase in the amount of fluid being pulled into the lumen of the bowel. Hence, the ability of the intestines to reabsorb is altered. This leads to an increase in the net secretion of ions (chloride or bicarbonate) and inhibition of the net absorption of sodium and water (Sisson, 2011). It may arise from infectious or non-infectious causes. Infectious agents, which produce enterotoxins that interact with receptors and lead to an augmented secretion, are the most common cause of secretory diarrhea (Shah, 2004). They function by altering the second messenger system through modification of cAMP, cGMP or intracellular Ca^{2+} regulated ion transport pathways. Alterations in these mediators cause CFTR or CaCC-mediated Cl^- secretion and inhibition of small intestinal coupled Na^+-Cl^- transport. Paracellularly, sodium follows the chloride to maintain charge balance and water escapes from the cells following sodium ion to maintain osmotic balance. This efflux of water and electrolytes is manifested as watery diarrhea. Therefore, Secretory diarrhea persists in spite of fasting (Hoque *et al*., 2012; Strasinger & Di-Lorenzo, 2008).

Osmotic diarrhea occurs when there is a dysfunction in the ability of the intestine to reabsorb fluid as it flows through the lumen. This may be caused by incomplete breakdown or malabsorption of nutrients in the small intestine allowing a larger and more liquid mass to enter the colon. This fecal matter then creates a negative osmotic gradient causing leakage of more fluid into the gut and increasing the stool volume. This type of diarrhea can be caused by decreased enzymatic availability (lactose intolerance), a genetic abnormality that decreases or eliminates the ability of the body to absorb certain nutrients (celiac sprue), sugars that are poorly absorbed (sorbitol, mannitol or lactose), laxatives, magnesium containing antacids and antibiotic administration as well as malabsorption of certain fat. Other causes have more to do with changes within the bowel that decrease the ability to reabsorb fluid and nutrients as the stool is propelled through the lumen which includes malnutrition, resection of parts of the bowel and inflammation of the bowel due to infection or disease processes. Unlike secretory diarrhea that continues with fasting, osmotic diarrhea disappears with fasting or termination of ingestion of the offending agent (Crombie *et al*., 2013; Strasinger & Di-Lorenzo, 2008).

Inflammatory diarrhea is a GI disorder that may include all of the pathophysiologic mechanisms. For instance, inflammation with resultant injury to the intestine may lead to malabsorption of dietary macronutrients which in turn, creates a luminal osmotic gradient. Additionally, particular infectious agents may induce secretion of fluid into the lumen and blood in the gut may alter intestinal motility (Garrett & Esther, 2012). Diarrhea due to abnormal intestinal motility can enhance bacterial overgrowth within the intestine. Increased motility shortens the contact period of chyme with mucosa. Therefore in consequence of this change, the volume of chyme in the large intestine increases which causes faster evacuation. The consequent abbreviation of contact of chyme with mucosa results in a decrease in water absorption. Diarrhea due to abnormal intestinal motility is usually present in coincidence with irritable bowel syndrome, after gastrectomy, vagotomy, diabetic neuropathy, sclerodermia and thyrotoxicosis (Barbara *et al*., 2009).

1.5 Management of diarrhea

Physiologically, diarrhea is considered favorable to the GI tract as it offers an important mechanism of flushing away detrimental luminal substances (Valeur *et al*., 2009). However, when the loss of fluids and electrolytes surpasses the body's capacity to replace the losses it becomes pathological. Thinking about this, the treatment is aimed at preventing or reversing dehydration, shortening the duration of the illness and reducing the period that a person is remain infectious (Grimwood & Forbes, 2009). Overall, there are three main approaches to the treatment of diarrhea. These are supportive therapy, antidiarrheal therapy and specific therapy.

1.5.1 Supportive therapy (Fluid and electrolyte replacement therapy)

Regardless of the cause the major dread with diarrhea is dehydration (Casburn-Jones & Farthing, 2004; Sisson, 2011). For that reason, keeping adequate hydration or bringing back hydration is the first and vital step in the treatment of diarrheal diseases, mainly of acute-onset diarrhea (Soriano & Vaziri, 2011). Oral rehydration therapy (ORT) is a significant aspect in the prevention of severe dehydration (Sisson, 2011; Soriano & Vaziri, 2011) and acidosis (Casburn-Jones &Farthing, 2004). In severe dehydration in infants and young children, intravenous fluids are desirable (Casburn-Jones & Farthing, 2004). Although ORS effectively treats dehydration when administered appropriately, it does not change fluid losses, diarrheal output or duration of illness.

1.5.2 Antidiarrheal therapy

While every struggle should be made to pinpoint and correct the specific causes of diarrhea, in numerous cases, causes that are specific and potentially treatable are often not detectable and symptomatic therapy alone is commonly indicated (Manatsathit *et al*., 2002). Two classes of antidiarrheal agents useful for reducing stool frequency, abdominal cramps and possibly stool volume are broadly applied (Casburn-Jones & Farthing, 2004). These are antimotility and antisecretory agents.

a) Antimotility Agents

Antimotility agents afford symptomatic relief and serve as useful adjuncts to antibiotic therapy in the treatment of diarrhea. The accepted mechanism of action for antimotility drugs is increasing intestinal transit time and augmenting the potential for reabsorption of fluid and electrolytes (Casburn-Jones & Farthing, 2004; Thiagarajah *et al*., 2015). They embrace loperamide, diphenoxylate, codeine, and other opiates (Manatsathit *et al*., 2002). From these antimotility drugs, Loperamide and diphenoxylate are μ-opioid agonists that are usually used for mild, nonspecific diarrhea (Thiagarajah *et al*., 2015). However, such antimotility agents are contraindicated in diarrhea caused by invasive pathogens because the induced intestinal stasis may enhance tissue invasion by the organisms or delay their clearance from the bowel (Manatsathit *et al*., 2002; Casburn-Jones & Farthing, 2004). Hence, bloody diarrhea with high fever, immunocompromised host and septicemic prone conditions with diarrhea should not be given this group of drugs. Antimotility agents are also not suggested for children and young infants due to the potential for central nervous system side effects and the theoretical possibility of respiratory depression (Casburn-Jones & Farthing, 2004). In addition to opiates, calcium channel blockers (CCBs) such as verapamil and nifedipine were found to have an important role in the treatment of diarrhea resulting from microscopic colitis and diarrhea-predominant IBS due to their prolongation of colonic motility (Soriano & Vaziri, 2011). A centrally acting α_2-adrenergic agonist clonidine is an alternative non opioid agent that works mainly by an alteration of gut motility with an effect on intestinal transport. It stimulates sodium and chloride absorption and inhibits chloride secretion by interacting with its receptor on enterocytes and is primarily prescribed for patients with diabetic diarrhea, although it can also be used for secretory diarrhea of unknown etiology and diarrhea associated with opiate (Soriano & Vaziri, 2011).

b) Antisecretory Drugs

Numerous drugs that were shown to have antisecretory effects *in vitro* are known by a variety of different mechanisms including: inhibition of prostaglandins (PGs) and effects on cAMP, calmodulin inhibition, inhibition of gut hormones and enkephalinase, inhibition of chloride channels (Manatsathit *et al*., 2002). Such drugs are capable of stimulating absorption directly and reduce secretion of water and electrolytes in GI tract, decrease propulsion and increase contact time of intestinal content with mucosal surface, which in turn favors absorption. They include codeine, loperamide, diphenoxylate, lidamidine, bismuth subsalicylate, racecadotril and clonidine (Velázquez *et al*., 2012). Serotonin receptor antagonists particularly antagonists of 5-HT_3 receptor were found to inhibit extrinsic sensory neuron stimulation (which can inhibit nausea, vomiting, stomach pain and bloating) and lessen peristalsis and secretory reflex. Therefore, they were found to have a central role in governing motility and secretion of the gut (Manatsathit *et al*., 2002).

1.5.3 Specific therapy

A different medication groups that are intended to reducing duration and severity of diarrhea are antimicrobials. Due to the self-limiting nature of the disease and the difficulty and delay in identifying the pathogen, the cause of most of acute diarrheal cases usually remains unknown (Kotwani *et al*., 2012). However, epidemiologically, viral pathogens such as *rotavirus* accounts for 70% to 80% of all diarrheal episodes. Antimicrobial therapy for self-limiting and non-infectious diarrhea is generally not encouraged, since it is a means for the development of drug resistance microbes. It can also increase the risk of side effects and higher costs (Kotwani *et al*., 2012). However, in cases of proven infectious diarrhea with known pathogenic agents, definite therapeutic intervention using antimicrobial drugs targeting the causative microbes may be applied.

A large body of evidence indicates that antimicrobial agents can reduce the severity and duration of some intestinal infections, especially in those bacteria and infections that produce acute watery diarrhea (Casburn-Jones & Farthing, 2004). However, the helpfulness of a particular antimicrobial agent depends on the etiologic agent and its antibiotic sensitivity. A study by Sisson (2011) suggested that antibiotics should be used selectively only in case of traveler's diarrhea (in which *E. coli* is the likely pathogen and treatment can shorten the duration of the illness), persistent diarrhea

(suggestive of giardiasis), febrile diarrheal illnesses consistent with invasive disease and *C. difficile* infection.

1.6 Traditional medicine

Since the time immemorial, medicinal plants have played a priceless role in the development of potent therapeutic agents. Despite the development of immense spectrum of approaches for diarrheal management, a study by Agbor and Naidoo (2015) estimates that approximately 80% of the daily healthcare needs of the people in developing countries still rely on traditional medicine for the management of diarrhea. In developing countries, a majority of people living in rural areas almost entirely use traditional medicine in treating all sorts of diseases including diarrhea, which is very common and recurring disease in community. This is also factual in some affluent countries where the use of modern medicine is predominant (Bodeker *et al*., 2003).

Natural products have a unique chemical diversity which results in varying biological activities and drug-like properties. Across the globe there are various herbal plants that possesses antidiarrheal activity, which could be credited to the existence of several bioactive compounds such as tannins, alkaloids, saponins, flavonoids, steroids, and terpenoids within the plants (Otshudi *et al*., 2000; Komal *et al.*, 2013; Umer *et al*., 2013).

In recent years, there has been a great interest in herbal remedies for the treatment of number of ailments. Ethnobotanical studies indicate the use of a range of medicinal plants such as the bark extract of *Albizia gummifera*, leaf extract of *C. aurea* and *Myrtus communis*, root extract of *Ensete ventricosum* and *Caylusea abyssinica*, seed extract of *Coffea arabica* and eating fruits of *Mimusops kummel* for the management of diarrhea by traditional healers in Ethiopia (Teklehaymanot & Giday, 2007).

1.7 The Experimental plant

C. aurea (Figure 1) is a genus of flowering plants within the family of fabaceae. The genus embraces shrubs or small trees in or along the margin of forests in many parts of Ethiopia. It is widely distributed in Africa from Cape Province to Eritrea. The Southern part of India is also additional habitat for the plant (Dula & Zelalem, 2018, Coates, 1983). It is among traditional plants that are claimed to have antidiarrheogenic potential in many areas of Ethiopia. They are accessible, affordable, and sustainable.

However, research output on the scientific information concerning the safety, and efficacy of these plants is not enough.

C. aurea is one of the most commonly used plants in traditional medicine to treat vast medical conditions and parasitic infestation, both in humans and animals (Hutchings, 1996). It is known by several local names, cheka in Afaan Oromo and digita in Amharic. In many regions of Ethiopia, different part of *C. aurea* have been used to treat Amoebiasis, Giardiasis, Malaria, Diarrhoea, Rabies, Diabetes, & Hypertension (Giday *et al*., 2006. Teklehaymanot & Giday, 2007).

Even though the antidiarrheal and antibacterial effect of the 80% methanol leaf extract of *C. aurea* has been studied (Umer *et.al*, 2003), the test is performed by castor oil induced diarrhea model only. The antimotility and antienteropooling effect of the plant was not tested. In addition, the preliminary phytochemical screening studies that compared the leaves and seeds of this plant indicated that the content of alkaloids and tannins are more concentrated in seeds than leaves. Although the seeds of *C. aurea*, like that of the leaf, is claimed to treat diarrhea in Ethiopia, it is not still scientifically investigated. Therefore, the present study aimed to investigate the *in vivo* antidiarrheal and *in vitro* antimicrobial activities of aqueous and 80% methanol seed extract of *C. aurea.*

Figure 1---Photographs of different parts of the *C. aurea* plant taken from the study site

1.8 Rationale for the study

Despite the availability of many drugs for treating diarrhea, majority of them suffer from adverse effects like induction of bronchospasm and vomiting by racecadotril (Tormo *et al*., 2008); intestinal obstruction and constipation by loperamide (Pankaj, 2006) and undesirable central effects by long term use of morphine and its analogs (Khansari *et al*., 2013).

ORS has been the mainstay of treatment for diarrhea. However, it does not reduce the frequency of stools or the number of diarrheal days. This treatment often fails in the high stool output state (WGO, 2012). Moreover, there is an increasing threat of drug resistance, side effects, super-infection and the possibility of induction of disease producing bacteriophages by antibiotics. Due to these problems, WHO (2004) encourages studies for the treatment and prevention of diarrheal diseases depending on traditional medical practices. Furthermore, the ethno-medicinal approach for diarrhea treatment is practical, cost effective, and reasonable (Mishra *et al*., 2016).

There are many plants which are traditionally used for the treatment of diarrhea. *C. aurea* is one of them. This study attempted to validate the traditional use of this plant by evaluating the possible antidiarrheal (*in vivo*) and antimicrobial (*in vitro*) properties of the seed extract of *C. aurea*, in order to establish the claimed biological activities. The finding of this research could be used as an input in searching for new antidiarrheal agent that might solve problems associated with the conventional antidiarrheal drugs. It could also give direction for traditional users on different ways of preparation and use of the plant. In addition, the results of this study help the scientific community to further investigate the plant by initiating advanced studies on molecular mechanisms and formulation of plant source drugs by identifying the specific agent responsible for the antidiarrheal effect.

2. Objective of the study

2.1 General objective

To investigate the antidiarrheal and antimicrobial effect of aqueous and 80% methanol seed extract of the *C. aurea*.

2.2 Specific objectives

- To test the acute oral toxicity of *C. aurea* seeds
- To investigate the effect of aqueous and 80% methanol seed extract of *C. aurea* on castor oil induced diarrhea in mice.
- To assess anti-enteropooling effect of aqueous and 80% methanol seed extract of *C. aurea* on prostaglandin induced enteropooling in mice.
- To investigate the effect of the aqueous and 80% methanol seed extract of *C. aurea* on normal and castor oil induced gastrointestinal motility using charcoal meal test.
- To identify antibacterial effect of aqueous and 80% methanol seed extract of *C. aurea* on selected bacterial strains.

3. Materials and Methods

3.1 Drugs and chemicals

Castor oil (Amman Pharmaceutical Industries, Jordan), activated charcoal (Acuro Organics Ltd,New Delhi), loperamide hydrochloride (Medochemie Ltd, Cyprus(EU)), misoprostol (Mylan Laboratories Ltd., India), distilled water (department of Pharmaceutics and social pharmacy of Addis Ababa University), methanol (Blulux, India), petroleum ether(Carlo Erba Regents S.A.S, Italy), McFarland standard (Remel, Lenexa Kansas 66215, USA), Brain Heart Infusion (BHI) (Difco Laboratories, Detroit Michigan, USA), ciprofloxacin disk 5mcg (Ecton DickinsonPty Ltd, Australia), muler hinton agar(Himedia laboratories Pvt Ltd, India), muller hinton broth (Himedia laboratories Pvt Ltd, India) were used in this study. All chemicals and solvents were of analytical grade.

3.2 Plant materials

The seeds of *C. aurea* (fabaceae) were collected from Addis Zemen which is located in south Gondar zone of Amhara region. After collecting the plant, identification and authentication was done by taxonomists and a voucher specimen (001AB) was deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University for future reference.

3.3 Experimental animals

Adult mice of both sex (20-30g) was obtained from animal house unit of School of pharmacy of Addis Ababa University. The animals were housed in polypropylene cages (6–10 animals per cage) under standard environmental conditions on a 12 h light–dark cycle with free access to pellet food and water *ad libitum*. The animals were acclimatized for a week before beginning the actual experiment. All experiments were conducted during the light period. All the protocols were approved by the School of Pharmacy Ethics Committee and were conducted according to the guideline for the care and use of laboratory animals (Herling, 2016).

3.4 Test strains

Standard strains of *Salmonella typhimurium* (ATCC13331), *Shigella soni* (ATCC12022), *Pseudomonas aeruginosa* (ATCC27853), *Staphylococcus aureus* (ATCC25923) and *Escherichia coli* (ATCC25922), were obtained from Akililu Lema Institute of Pathobiology, Addis Ababa University.

3.5 Extraction and preparation of the Plants

The ripe and dried seeds of *C. aurea* were initially washed using distilled water to remove dust materials. The seeds were then grinded by using grinder and powdered coarsely using mortar and pestle prior to extraction.

3.5.2. Preparation of 80 % methanol extract

The powder of *C. aurea* seeds were extracted using 80% methanol through maceration technique. One hundred gram of coarse powder of the plant material was subjected to maceration process with about 500ml of 80% methanol at room temperature for 72 h. For proper mixing, the plant material was shaken with the solvent continuously on a horizontal orbit shaker. The mixture was then filtered through a muslin cloth and *Whatman No. 1* filter paper and the marcs were re-macerated 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 at 40°C. The extract was then dried using a lyophilizer to remove the remaining water and the resulting dry hydro-alcoholic extract of the plant was weighed and percentage yield was found to be 15.5%. Finally, the dried extracts of the plant was stored at -20°C and was reconstituted with distilled water when oral administration is desired.

3.5.2. Preparation of aqueous plant extract

Cold maceration technique was used for extraction of the plant material. During the process, 100 g of the coarse powder was soaked in an Erlenmeyer flask with 1 L of distilled water and then placed on a shaker tuned at 120 rpm with occasional shaking for 72 h at room temperature.

The extract was filtered first using a muslin cloth and then *Whatman No-1* filter paper and the marc was re-macerated for a second and third time by adding another fresh solvent. The filtrates were left overnight in a deep freezer and then were lyophilized using freeze dryer. After drying, percentage yield

of crude aqueous seed extract of *C. aurea* was found to be 15.5%. The dried plant extract was reconstituted with distilled water for oral administration (Tadesse *et al*., 2017).

3.6 Acute Toxicity Test

Initially, acute toxicity test was done based on the limit test recommendations of Organization for Economic Cooperation and Development (OECD) 425 Guideline (OECD, 2008). First, two female mice were fasted for 4 h and then loaded with 2000 mg/kg of either of the extracts, orally. The mice were then observed for physical or behavioral changes. Since both mice died within 30 min of administration of each extract, based on OECD 425 guide line, the main test procedure was performed. The mice were observed continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicity such as change in food and water intake and also for mortality.

The main test procedure was performed by using a default dose progression factor of 3.2. Based on the recommendation of OECD 425 guideline, since estimate of the substance's lethality is not available, the starting dose for testing procedure was determined to be 175 mg/kg. Then by using the dose progression factor of 3.2, the next doses 550 mg/kg and 1750 mg/kg, tested successively. Each Single animal was dosed in sequence at 48 h intervals. Treatment of an animal at the next dose was delayed until survival of the previously dosed animal was confirmed.

3.7 Grouping and Dosing

Thirty mice (for each solvent extracts) of either sex (weighing 20-30g) were randomly divided into five groups of six mice each and were fasted for 18 h before the test with free access to water. Group I served as negative control and treated with 10 ml/kg normal saline. Group II serving as a positive control was treated with Loperamide (3 mg/kg). Group III, IV and V were given 60mg/kg, 120mg/kg, and 240mg/kg of methanol and aqueous extracts of the *C. aurea* seeds orally. Misoprostol, a PGE_2 analog, was used in entropooling model to induce diarrhea while castor oil is replaced in the remaining models for the same function. All doses were administered orally and maximum volume administered was 10 ml/kg.

3.8 Determination of antidiarrheal activity

3.8.1 Castor Oil-Induced Diarrhea

Antidiarrheal activity of the plant extract was evaluated using the castor oil-induced diarrheal model in mice described by Umer *et al* (2013). Forty eight mice of either sex were randomly divided into eight (the control group remain the same) groups of six mice each and were used after overnight fasting (Earnest *et al*., 2018).

Mice were dosed as described in Grouping and dosing section. One hour after dosing, 0.5 ml of castor oil was administered to each mouse orally. 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 fecal droppings visible for counting and to check stool consistency. Diarrhea was graded as follows: Normal pelleted feces (0), discrete soft-formed feces (1), soft-formed feces (2), soft watery stool (3), and watery stool with little solid matter (4) (Earnest *et al*., 2018).

The animals were observed for a period of 4 h, during which the onset of diarrhea, the number and weight of both dry and wet stools excreted by the animals were recorded and compared with the control for assessing the antidiarrheal 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 % and percentage of diarrheal inhibition for wet and watery content of feces was determined using the following formula:

$$\% \text{ inhibition} = \frac{AWFC - AWFT}{AWFC} \times 100$$

Where AWFC = average weight of feces in the control group and AWFT = average weight of feces in the test group (Earnest *et al*., 2018).

3.8.2 Prostaglandin (PGE_2)-induced enteropooling

In this technique, prostaglandin served as a diarrhea producing agent. Forty eight mice of either sex were randomly divided into eight (the control group remain the same) groups of six mice each and were used after overnight fasting (Earnest *et al*., 2018).

Mice were dosed as described in Grouping and dosing section. Misoprostol administered 1 h after dosing. Then, 1 h after administration of 100 µg/kg of PGE2, all rats were sacrificed by cervical dislocation, and the small intestine accumulated with fluid was ligated both at the pyloric sphincter and at the ileocecal junctions and dissected out. The tied intestine was weighed (m1); its content emptied into a graduated cylinder and its volume was measured. Then, the emptied intestine was weighed (m_0) and the difference between the empty and intact intestine was used to calculate percentage inhibition (reduction in fluid accumulation) of intestinal secretion relative to control group using the following formula:

$$\% \text{ inhibition} = \frac{(A-B)}{A} \times 100$$

Where *A* = average volume or weight of intestine in control group and *B* = average volume or weight of intestine in test groups (Earnest *et al*., 2018).

The volume of the intestinal content was read directly from the graduation while the mass was obtained as (m1-m0) g.

3.8.3 Charcoal Meal test in normal mice

Experimental procedure described by Bahekar & Kale (2015) was used for the evaluation of antidiarrheal activity in mice with slight modification. Thirty mice (for each plant extracts) of either sex were randomly divided into five groups of six mice each and fasted for 18 h prior to the test, but were allowed free access to water. One hour after dosing as described in dosing section, each animal was given 1ml of freshly prepared charcoal meal (10% active charcoal suspension in 2 % tween 80) orally. One hour after charcoal administration, animals were sacrificed. The abdomen was opened and small intestine from the pylorus to caecum was taken out. The distance travelled by the charcoal meal in the intestine, from the pylorus to the caecum was measured and expressed as the percentage of distance covered using the following formula:

$$\% \text{ of transit inhibition} = \frac{To-T1}{To} \times 100$$

T_0 = total length of intestine

T1 = distance travelled by charcoal in intestine

% inhibition = $\frac{mean\ of\ distance\ traveled\ by\ marker\ of\ (control-test)group}{mean\ of\ distance\ traveled\ by\ marker\ of\ control\ group} \times 100$

3.8.4 Charcoal meal test following induction of diarrhea

The effect of the *C. aurea* seed extract on gastrointestinal motility was evaluated as described by Umer *et al.* (2013) with some modification. Forty eight mice of either sex were randomly divided into eight (the control group remain the same) groups of six mice each and were used after overnight fasting (Earnest, *et.al*. 2018). One hour after a dosing, 0.5 ml of castor oil was administered to each mouse orally. After 1 h of castor oil administration, all animals received 1ml of charcoal meal marker (10 % charcoal suspension in 2 % tween 80) orally and all of them were sacrificed after 30 min of marker administration. The small intestine was dissected out and the distance travelled by charcoal meal from the pylorus to caecum was measured and expressed as a percentage of the total distance of the small intestine. The intestine of each mouse was immersed in formalin to arrest peristalsis and then washed in clean tap water before measuring the distance travelled by the charcoal meal. Charcoal movement was expressed as a peristaltic index (PI) as follows:

PI = $\frac{A}{B} \times 100$

Where *A* = distance travelled by charcoal meal and *B* = length of full intestine.

Percentage inhibition is also calculated as follows:

Percentage inhibition = $\frac{APIC-APIT}{APIC} \times 100$

Where APIC = average PI of control and APIT = average PI of test group (Earnest, *et.al*, 2018).

3.8.5 *In vivo* antidiarrheal index (ADI)

In vivo antidiarrheal index (ADI) of treated groups was determined using data from castor oil induced diarrhea, enteropooling, and gastrointestinal motility tests using the formula developed by Aye-Than as described below (Mekonnen *et al*., 2018).

ADI *in vivo* = $\sqrt[3]{\mathrm{DDT} \times \mathrm{GMT} \times \mathrm{IFA}}$

Where;

DDT is the delay in defecation time or diarrheal onset (as % of control)

GMT is the gastrointestinal motility by charcoal travel reduction (as % of control)

IFA is the reduction in the intestinal fluid accumulation (as % of control).

$$\text{DDT} = \frac{\text{Onset of diarrhea in minute of the (test} - \text{negative control) group}y}{\text{Onset of diarrhea in minute of the negative control group}x} \times 100$$

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

$$\text{IFA} = \frac{\text{Mean weight of wet stools of (negative control} - \text{treated)group}}{\text{Mean weight of wet stools of negative control group}} \times 100$$

3.9 Antimicrobial activity

3.9.1 Inoculum preparation and standardization

The bacteria were selected based on availability and considering the likely bacterial strains that can cause diarrhea for which the experimental plant is indicated traditionally. Nutrient agar was prepared following the manufacturer's protocol. After cooling the media to about 45^0c, it was poured to a prelabelled sterile petridishes aseptically and allowed time for congealing of the agar. The standard pathogenic bacteria were then inoculated and spread on the respective prepared agar using inoculating wire loop following aseptic condition and incubated for 24 h at 37^0c.

The bacterial turbidity of each of bacterium was prepared and standardized by following the guideline of Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2012). The bacterial suspension in a broth was prepared by the growth method as follows. After preparing nutrient broth in distilled water, 5 ml of the broth was transferred to test tubes and autoclaved. Isolated colonies of the same morphological type of each bacterium from 3-5 wells were picked up by wire loop from fresh agar plates of bacterial culture and aseptically transferred into prelabelled test tubes containing the sterile nutrient broth and incubated for about 6 h. The turbidity of the inoculum tube was adjusted visually by either adding bacterial colonies or by adding sterile normal saline solution to that of the already prepared 0.5 McFarland standard which is assumed to contain a bacterial concentration of $1x10^8$ CFU/ml. The adjustment and comparison of turbidity of inoculum tube and that of 0.5 McFarland standard was performed by visually

observing them with naked eye against a 0.5 McFarland turbidity equivalence standard card with white background and contrasting black lines in the presence of adequate light.

3.9.2 Agar well diffusion

Agar well diffusion method was used to determine antimicrobial activity. Diluted inoculums (0.1 mL) of test organism (10^8A CFU/mL) were spread on Muller-Hinton agar plates. Wells of 6 mm diameter was punched into the agar medium with sterile cork-borer under aseptic conditions and was filled with a serial dilution of 50 µl of plant extract starting from 1000 mg/ml, solvent blank and standard antibiotic (ciprofloxacin 5µg). The plate was kept at room temperature for 2 h for diffusion and then incubated for 24 h at 37°C.

Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms (Mukherji *et al*., 1995). Ciprofloxacin (5µg disc) was used as a reference standard and distilled water was used as a control. The growth was compared with the reference as well as the control. Each experiment was repeated three times.

3.9.3 Determination of Minimum Inhibitory Concentration (MIC)

The extracts of both solvents that showed antibacterial activity by agar well diffusion method were subjected to serial microbroth dilution technique to determine minimum inhibitory concentration (MIC) as described by previous studies (Gahlaut & Chhillar, 2013; Rouis *et al*., 2013). Serial dilutions were prepared from 1000 mg/ml of the plant extract using distilled water to make 1000, 500, 250, 125, 62.5, 31.25, and 15.625 mg/ml. The wells were inoculated with 0.1 mL aliquot of test organisms (10^8 CFU/mL) having serial dilutions of the extract (50 µl, each). The micro plate was incubated at 37°C ± 1°C for 24 h. Dilution of the extract corresponding to respective test organism showing no visible growth was considered as MIC.

3.9.4 Determination of Minimum Bactericidal Concentration (MBC)

The MBC is defined as the lowest concentration where no bacterial growth is observed. This was determined by aseptically sub-culturing the contents of wells from the MIC results for individual bacterium to antimicrobial free agar as described in different studies (Nikolic *et al*., 2014; Powthong *et al*., 2012; Rouis *et al*., 2013). In this technique, the contents of all wells containing a concentration of

test material above the MIC value from each triplicate, in the MIC determination test, was streaked using a sterile wire loop on MHA aseptically and incubated at 37ºC for 24 h. The lowest concentration of each extract which showed no bacterial growth after incubation was observed for each triplicate and noted as the MBC. The average value was taken for the MBC of test material against each bacterium.

4.0 Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). The experimental results were analyzed using the software Statistical Package for Social Sciences (SPSS), version 20 and statistical significance was determined by one way analysis of variance (ANOVA) followed by Tukey Kramer post Hoc test. P-value of less than 0.05 was considered as statistically significant. The analyzed data is then presented using tables.

4. Results

4.1 Acute Toxicity Test

Aqueous and 80% methanol seed extract of *C. aurea* was studied for acute toxicity at a dose of 2000 mg/kg by oral route. However, mice that received either of the extract died 30 min following administration of the extract. Based on OECD 425 guide line, the main test procedure was performed and dose estimator of each extract was determined to be 1200 mg/kg. Accordingly, the graded dose of 60, 120, and 240 mg/kg of each extract was used for the experiment.

4.2 Effect of the aqueous and 80% methanol seed extracts of *C. aurea* on castor oil-induced diarrhea

During the four hours observation period, all mice in the control group had either wet stool or watery diarrhea. In case of water extract, pretreatment of mice at the dose of 60mg/kg ($p<0.05$), 120mg/kg ($p<0.05$) and 240mg/kg ($p<0.001$) significantly delayed the onset of diarrhea. The 80% methanol extract, however, produced a significant ($p<0.001$) effect on the onset only at the higher dose (240 mg/kg). Both extracts at all dose levels were able to significantly ($p<0.001$) reduce the frequency of diarrhea (Table 1). Moreover, both extracts delayed the onset of diarrhea (R^2=1.00 for methanol extract and 0.946 for aqueous extract) and reduced the frequency of defecation (R^2=0.80 for aqueous extract and 0.893 for methanol extract) in a dose dependent manner as compared to the negative control. For each extract, the percentage of inhibition for both total weight of wet diarrhea and watery content of diarrhea relative to negative controls was calculated. The data revealed that, except for the lower dose of the aqueous extract, all the other doses of both extracts produced a significant ($p<0.05$) decrease in both total weight and watery content of diarrhea compared to controls. Otherwise, there was no detectable difference between standard and extracts as well as among various doses of both extracts (Table 1).

Table 1---effect of the aqueous and 80% methanol seed extracts of *C. aurea* on castor oil induced diarrhea model in mice

Groups	Onset of Diarrhea	Total stool frequency in 4hrs	Total weight of wet diarrhea	% Inhibition of total wet fecal output	Weight of watery content of wet stools	% Inhibition of watery content of wet stool
DW	79.83±2.78	9.17±1.11	1.29±0.08	---	0.69±0.11	---
L3	147.00±2.89^{a1}	2.33±0.21^{a3}	0.56±0.09^{a2}	56.59%	0.14±0.03^{a1}	79.71%
CAW60	151.67±19.23^{a2}	3.17±0.75^{a3}	0.82±0.21	36.43%	0.38±0.18	44.93%
CAW120	156.00±22.27^{a2}	2.17±0.54^{a3}	0.65±0.17^{a1}	49.61%	0.24±0.11	65.23%
CAW240	177.83±7.67^{a3}	1.83±0.31^{a3}	0.48±0.07^{a2}	62.80%	0.11±0.03^{a2}	84.06
CAM60	103.17±10.08	2.5±0.34^{a3}	0.81±0.15^{a1}	37.21%	0.32±0.13^{a1}	53.62%
CAM120	126.33±20.73	1.67±0.21^{a3}	0.54±0.11^{a3}	58.14%	0.18±0.05^{a2}	73.91%
CAM240	203.67±20.77^{a3}	1.17±0.17^{a3}	0.38±0.05^{a3}	70.54%	0.11±0.01^{a3}	84.06%

Values are expressed as Mean ± S.E.M (n = 6), analysis was performed using One way ANOVA followed by tuckey post-hoc test, Comparison was made among different groups: ^{a} compared to control; $^{1}p < 0.05$, $^{2}p < 0.01$, $^{3}p < 0.001$. DW= distilled water, L= loperamide, CAW= *C. aurea* water extract & CAM= *C. aurea* 80% methanol extract.

4.3 Effect of the aqueous and 80% methanol seed extracts of *C. aurea* on prostaglandin induced enteropooling.

Percentage inhibition in intestinal fluid accumulation was 33.96%, 50.94% and 60.92% for water extract and 39.62%, 52.83% and 62.26% for 80% methanol extract, for 60 mg/kg, 120 mg/kg, and 240 mg/kg doses, respectively (Table 2). In both cases, the anti-secretory effect of the plant increased with dose (R^2=0.96 for aqueous extract and, 0.92 for 80% methanol extract). Both aqueous and methanol seed extracts of C. *aurea* showed a significant reduction ($p<0.05$) in average weight as well as volume of

small intestine content at all doses. Furthermore, 240mg/kg dose of water extract of *C. aurea* seed reduced mean volume of intestinal content significantly as compared to that of 60mg/kg. However, there was no statistically significant difference in terms of volume of intestinal fluid and weight of intestinal contents when all doses of both extracts were compared with the standard drug.

Table 2---Effects of the aqueous and 80% methanol seed extracts of *C. aurea* on prostaglandin induced entropooling in mice

Group	**Mean-weight of small intestinal content (gm)**	**% inhibition**	**Mean-volume of small intestinal content(ml)**	**% inhibition**
DW	0.61±0.04	---	0.53±0.02	---
L3	0.27±0.06^{a2}	55.74%	0.27±0.04^{a3}	49.06%
CAW60	0.34±0.09^{a1}	44.26%	0.35±0.05^{a1e1}	33.96%
CAW120	0.35±0.04^{a1}	42.62%	0.26±0.04^{a3}	50.94%
CAW240	0.21±0.04^{a3}	65.57%	0.17±0.03^{a3}	67.92%
CAM60	0.37±0.06^{a1}	39.34%	0.32±0.03^{a2}	39.62%
CAM120	0.34±0.07^{a2}	44.26%	0.25±0.04^{a3}	52.83%
CAM240	0.27±0.02^{a3}	55.74%	0.2±0.05^{a3}	62.26%

Values are expressed as Mean ± S.E.M (n = 6), analysis was performed using One way ANOVA followed by tuckey post-hoc test, Comparison was made among different groups: ^{a} compared to control; ^{e} to 240 mg/kg aqueous $^{1}p < 0.05$, $^{2}p < 0.01$, $^{3}p<0.001$. DW= distilled water, L= loperamide, CAW= *C. aurea* water extract & CAM= *C. aurea* 80% methanol extract.

4.4 Effect of the aqueous and 80% methanol seed extracts of *C. aurea* on castor oil induced gastrointestinal propulsion

Both aqueous and 80% methanol seed extracts of *C. aurea* exhibited a statistically significant ($p<0.001$) anti-motility effect against castor oil induced diarrhea compared to negative controls (Table 3). Both extracts significantly inhibited the intestinal transit of charcoal meal at all doses, with the higher dose exhibiting the maximum effect (63.4% for aqueous 61.1% for 80% methanol extract). The effect was dose dependent, R^2 being 0.733 for aqueous extract and 0.861 for 80% methanol extract. The standard drug produced a significantly higher effect compared to controls ($p<0.001$) as well as lower dose ($p<0.001$) of both extracts.

Table 3-Effects of the aqueous and 80% methanol seed extracts of *C. aurea* on castor oil induced gastrointestinal transit in mice

Group	Total length of small intestine (cm)	Distance moved by the charcoal meal (cm)	Peristalsis index (%)	%Inhibition
DW	56.00±1.13	44.17±1.33	79.00±2.63	---
L3	53.83±1.28	16.67±1.33^{a3c3}	30.81±1.96^{a3c3}	62.26%
CAW60	52.67±0.88	30.67±1.98^{a3b3d3e3}	58.29±3.74^{a3b3d3e3}	30.22%
CAW120	51.50±1.45	18.83±2.34^{a3c3}	36.63±4.52^{a3c3}	57.37%
CAW240	54.00±1.32	16.17±1.05^{a3c3}	29.82±1.31^{a3c3}	63.39%
CAM60	52.00±1.81	22.17±2.73^{a3}	42.23±3.93^{a3e1}	49.81%
CAM120	55.67±1.17	18.83±2.47^{a3}	34.03±4.69^{a3}	57.37%
CAM240	59.83±1.05	17.17±1.45^{a3}	28.55±2.08^{a3c1}	61.13%

Values are expressed as Mean ± S.E.M (n = 6), analysis was performed using One way ANOVA followed by tuckey post-hoc test, Comparison was made among different groups: ^{a} compared to control, ^{b} to standard drug, ^{c} to 60 mg/kg aqueous , ^{d} to 120 mg/kg aqueous , ^{e} to 240 mg/kg aqueous; ^{1}p <0.05, ^{2}p <0.01, ^{3}p<0.001. DW= distilled water, L= loperamide, CAW= *C. aurea* water extract & CAM= *C. aurea* 80% methanol extract.

4.5 Effect of the aqueous and 80% methanol seed extracts of *C. aurea* on normal gastro intestinal transit in mice

Both aqueous and 80% methanol seed extracts of *C. aurea* tended to decrease the intestinal transit of the charcoal meal through the GI compared to the control group (Table 4). The percentage of inhibition of both extracts as well as the standard was under 30%, indicating the transit time for charcoal was shorter in normal than castor oil treated animals. Nevertheless, the effect was dose dependent (R^2=0.60 and R^2=0.81 for water and 80% methanol extract, respectively). The inhibition obtained with the standard drug, however, was significantly greater (29.6%, p<0.05) than the control group.

Table 4---Effects of the aqueous and 80% methanol seed extracts of *C. aurea* on normal gastrointestinal transit in mice

Group	Total length of small intestine (cm)	Distance moved by the charcoal meal (cm)	Percent of transit inhibition	%Inhibition
DW	52.83±1.76	35.50±0.99	32.38±3.31	---
L3	54.00±0.97	25.00±2.88a1	53.70±5.14^{a1c1}	29.58%
CAW60	51.67±1.52	31.83±3.74	32.58±7.93^{b1}	10.34%
CAW120	51.00±2.45	26.67±1.45	47.65±2.01	24.87%
CAW240	53.33±0.95	26.50±1.38	50.17±2.90	25.35%
CAM60	50.83±1.40	31.00±3.48	38.68±7.01	12.68%
CAM120	52.17±0.70	26.83±2.96	48.36±5.94	24.42%
CAM240	53.83±2.51	25.33±2.64	51.70±6.32	28.65%

Values are expressed as Mean ± S.E.M (n = 6), analysis was performed using One way ANOVA followed by tuckey post-hoc test, Comparison was made among different groups: ^{a} compared to control, ^{b} to standard drug, ^{c} to 60 mg/kg aqueous, ^{1}p <0.05, ^{2}p <0.01, ^{3}p<0.001. DW= distilled water, L= loperamide, CAW= *C. aurea* water extract & CAM= *C. aurea* 80% methanol extract.

4.6 The *in vivo* Antidiarrheal Index

The antidiarrheal index for the different doses of the extracts is presented in Table 5. The index was relatively higher for the aqueous than the 80% methanol extract.

Table 5---*In Vivo* antidiarrhea index of aqueous and 80% methanol seed extracts of *C. aurea*

Extracts	Dose administered	Delay in defecation (time of onset in minute, Dfreq %)	Gut meal travel distance, (Gmeq %)	Reduction in Intestinal fluid accumulation (%)	Antidiarrheal index(ADI)
Aqueous extract	60mg/kg	47.37%	30.22%	33.96%	36.50%
	120mg/kg	48.83%	57.37%	50.94%	52.26%
	240mg/kg	55.11%	63.39%	67.92%	61.91%
80%methanol extract	60mg/kg	22.62%	49.81%	39.62%	35.47%
	120mg/kg	36.81%	57.37%	52.83%	48.14%
	240mg/kg	60.80%	61.13%	62.26%	61.39%
Loperamide	3mg/kg	45.69%	62.26%	49.06%	51.87%

Values are expressed as % inhibition of different parameters of different models and the combined effect is calculated as ADI.

4.7 Antimicrobial activity

Both aqueous and 80% methanol seed extracts of *C. aurea* showed marginal antimicrobial activity against the selected strains, except *P. aeuroginosa*, where the extracts were found to be completely inactive. The 80% methanol seed extract appeared to be somewhat better than the aqueous seed extract (Table 6 & 7). Among the selected bacterial strains *S. typhimurium* was the most sensitive one in terms of MIC and MBC. Its growth was highly inhibited at a concentration of 250 mg/ml and completely killed at a concentration of 500 mg/ml. In terms of zone of inhibition, however, *E. coli* showed better effect. Although the test was performed up to a concentration of 7.8mg/ml for both extracts, the smallest dose with antibacterial activity against the selected strains was 125 mg/ml.

Table 6---Antimicrobial effects of both aqueous and 80% methanol seed extracts of *C. aurea* using disk diffusion techniques

		Zone of inhibition			
		Name of bacterial strain			
Category of test	Concentration	*E.coli* (ATCC25922)	*S.aureus* (ATCC25923)	*S.soni* (ATCC12022)	*S.typhimurium* (ATCC13331)
Methanol extract	1000mg/ml	12.7 ± 0.17	11.57 ± 0.09	9.50 ± 0.18	9.20 ± 0.06
	500mg/ml	11.54 ± 0.34	10.00 ± 0.06	---	8.70 ± 0.1
	250mg/ml	9.9 ± 0.06	7.5 ± 0.01	---	8.00 ± 0.15
	125mg/ml	9.51 ± 0.11	7.25 ± 0.13	---	7.34 ±0.09
	Cipro5µg/disc*	28 ± 0.15	26.00 ± 0.15	28.00 ± 0.17	27.00 ± 0.1
Aqueous extract	1000mg/ml	---	8.73 ± 0.09	---	8.70 ± 0.15
	500mg/ml	---	7.7 ± 0.02	---	7.30 ± 0.15
	250mg/ml	---	6.48 ± 0.14	---	---
	125mg/ml	---	---	---	---

Values are expressed as Mean ± S.E.M (n=3). The negative control showed no antibacterial activity *= positive control, Cipro=Ciprofloxacin, --- = no activity. The values are the average of triplicate tests. ATCC---American Type Culture Collection

Table 7---Antimicrobia1 effects of both aqueous and 80% methanol seed extracts of *C. aurea* using micro-dilution techniques

Minimum inhibitory concentration and minimum bactericidal concentration				
Organism	Methanol extract		Aqueous extract	
	MIC	MBC	MIC	MBC
E.coli **(ATCC25922)**	500 mg/ml	1000 mg/ml	---	---
S.aureus **(ATCC25923)**	500 mg/ml	1000 mg/ml	1000 mg/ml	---
S.typhymurium **(ATCC13331)**	250 mg/ml	500 mg/ml	500 mg/ml	1000 mg/ml
S.soni**(ATCC12022)**	1000 mg/ml	---	---	---

MIC---minimum inhibitory concentration, MBC---minimum bactericidal concentration, the values are the average of triplicate tests. ATCC---American Type Culture Collection. --- = no activity.

5. **Discussion**

This study was conducted to evaluate the *in vivo* antidiarrheal and *in vitro* antibacterial activity of aqueous and 80% methanol seed extracts of *C. aurea* in mice and selected bacterial strains, respectively, and the probable underlying mechanism. The results showed that the plant possesses antidiarrheal and minimal antibacterial activity in models used.

It is well known that castor oil causes diarrhea due to its active metabolite, ricinoleic acid (Ammon *et al*., 1974; Jebunnessa *et al*., 2009). Ricinoleic acid causes irritation and inflammation of the intestinal mucosal lining leading to release of PGs, which is responsible for motility and secretion as well as prevention of NaCl and water reabsorption (Ezeja *et al*., 2012; Kumar *et al*., 2014; Sharma *et al*., 2015; Wansi *et al*., 2017).

Its action also stimulates the release of inflammatory mediators (PGs E and histamine) which cause stomach cramp and diarrhea due to its effect on the smooth muscle and secretion, thereby preventing the reabsorption of NaCl and water (Gatne *et al*., 2008; Sarin *et al*., 2013). The castor oil model therefore, embraces both secretory and abnormal motility diarrhea (Yegnanarayen & Srotri, 1982).

Among the several mechanisms proposed to explain the diarrheal effect of castor oil, one is inhibition of intestinal Na^+/K^+-ATPase activity. Thus, reducing normal fluid absorption (Humber, 2002; Imam *et al*., 2012), through activation of adenylate cyclase or mucosal cAMP-mediated active secretion (Capasso *et al*., 1994) and NO release (Uchida *et al*., 2000). Therefore, the use of castor oil as diarrhea inducer is plausible as it mimics the pathophysiologic processes and allows the observation of measurable changes in the number of stools, intestinal transit and enteropooling.

In addition to inducing diarrhea by administering castor oil (that induces secretion of PG through its metabolite ricinoleic acid), administering synthetic PG analogue (misoprostol) directly is also another option. Among the physiological compounds that are known to disturb motility of the GI tract, PGs are the major ones. One or more of these substances might function as intermediates between polypeptide hormones and the adenyl cyclase-cyclic AMP pathway. The presence of digested food in the intestine stimulates the absorption of water and electrolytes. PGE_2 induces diarrhea by inhibiting absorption of glucose, thus resulting in accumulation of fluid in the intestinal lumen. PGE_2 agonists act on PG receptors coupled to G-protein that makes use of inositol triphosphate (IP_3), diacylglycerol (DAG), or

cAMP transducer mechanism. Activation of E-type prostanoid receptor-1 (EP1) causes contraction of smooth muscles via IP3 /DAG or cAMP, which results in secretion of water and electrolytes. In this regard, agents that have the potential to inhibit the activity of PGs could be useful in preventing the enteropooling effect of PGE_2 (Ernest *et al*., 2018). Furthermore, exogenous $PGF_2\alpha$ given to initiate labour or abortion frequently provokes diarrhea as a side effect, and some women experience diarrhea during menses (Rees & Rhodes, 1976). Another study by Riviere and his colleagues testing diarrhea inducing abilities of PGE_2 confirmed its dose and time dependent effect. The study further demonstrated that PGE_2 at a dose of 200 µg /kg also produce accumulation of fluid in the small intestine as well as the colon (enteropooling). However, based on the study, PGE_2 treatment altered neither gastric emptying nor GI propulsion. Due to this evidence PG used only for testing of enteropooling effect (1991). Furthermore, this smooth muscle stimulating action of PGs has been shown to be blocked by loperamide in several laboratory animals (Dodge *et al*., 1977).

Loperamide hydrochloride (the standard drug) apart from regulating the GI tract, have also been reported to slow down transit in the small intestine. Loperamide at present is one of the most efficacious and widely employed antidiarrheal agents and effectively antagonizes the action of castor oil due to its antimotility and antisecretory properties (Salgado *et al*., 2005).

In the present study, both aqueous and 80% methanol seed extracts of *C. aurea* exhibited significant antidiarrheal activity by reducing castor oil and PG induced diarrhea in all models used in mice. Although both extracts showed a significant difference from negative control, there was no apparent difference between the two extracts. The most likely reason could be the presence of similar secondary metabolites in both extracts. In the castor oil induced diarrhea model, both extracts delayed the onset of diarrhea, decreased the frequency of purging, reduced the number of wet stools and inhibited severity of diarrhea. In some of the measured parameters, the effect of 80% methanol seed extract of *C. aurea* was somewhat (but not significant) better than the water extract. This is probably due to the presence of mid-polar to polar bioactive compounds that are found in the methanol (less polar solvent than water) extract of the plant. Both flavonoids and phenolic compounds having antioxidant properties (Mostafa *et al*., 2014) appear to be responsible for the inhibitory effects exerted upon several enzymes including those involved in the arachidonic acid metabolism, thus, reducing PG induced fluid secretion (Yaecob *et al*., 2016). Although phytochemical constituents like tannins and saponins (that are endowed with antidiarrheal activity) exist in both solvent extracts, they are mostly found in large amount in methanol

extract (Muthukumaran *et al*., 2016). In addition to this, phytochemical constituents in the water extract are more prone to hydrolysis and could potentially lose their antidiarrheal effect (Tiwari *et al*., 2011).

There was a statistically significant ($p<0.05$) reduction in the number and weight of both wet and watery content of fecal output as well as a delayed onset of diarrhea in both extracts. The effect was increased in a dose-dependent manner. This infers that a relatively high dose of the extract is needed to produce a better antidiarrheal effect. This is in line with other reports of other species of plants in which extracts of plants shown to exert antidiarrheal effect in a dose dependent manner (Trease & Evans, 1989)

The significant reduction in frequency of defecation (number of wet stools), weight of wet and watery content of stools signifies the efficacy of both aqueous and 80% methanol seeds extract of *C. aurea* as antidiarrheal agent. This result is in support of previous claims about antidiarrheal plants. Antidiarrheal herbs are known to reduce number of wet stools as reported for *Eremomastax speciosa* and *Xylocarpus granatum* (Oben *et al*., 2006; Rouf *et al*., 2007). Since castor oil produces diarrhea by preventing fluid and electrolyte absorption and thus resulting in intestinal peristalsis (Rehman *et al*., 2013), one of the possible mechanism of antidiarrheal activity of the test extract *C. aurea* might be due to its ability to facilitate fluid and electrolyte absorption through the GI tract.

Moreover, the significantly ($p<0.05$) prolonged time of induction of diarrhea, decreased frequency of fecal parameters (number of wet feces) following the administration of both extracts suggest its antidiarrheal activity at all dose levels. This finding was further supported with the increased inhibition of defecation. The comparable percentage of inhibition of defecation at 240 mg/kg doses of the extract with loperamide hydrochloride suggests that the plant has a promising effect and may serve as an alternative agent in the future. The extracts might have exerted their antidiarrheal activity via anti-secretory mechanism as evident from reduction in total number of wet feces and weight of watery content of diarrhea. Furthermore, this antidiarrheal activity could have resulted from the inhibitory activity of both extracts on PGs synthesis, NO and platelet activating factors production, as these modes of action are known to delay diarrhea induced by castor oil (Adzu *et al*., 2003; Bajad *et al*., 2001; Tangpu & Yadav, 2004). Similar effects were reported in several studies by Qnais *et al*. (2005), Akindele and Adeyemi (2006) and Appidi *et al*. (2010) following the administration of aqueous leaf extracts of *Juniperus phoenicia*, *Byrsocarpus coccineus* and *Hermania incana*, respectively.

Percentage inhibition of diarrhea calculated as a function of weight of watery content of diarrhea is higher than that of weight of wet stool diarrhea in both aqueous and 80% methanol seed extracts. This indicates that the most probable mechanisms of the plant extracts are increasing absorption or decreasing secretion or both, of fluid and electrolytes. This is a salient point since this nature of the plant may fill the less proabsorptive and antisecretory property of the standard drug loperamide.

For further evaluation of the mechanism of antidiarrheal activity, the study was extended to determine the anti-enteropooling effect. In PG induced enteropooling, both aqueous and 80% methanol seed extracts of *C. aurea* significantly inhibited the intestinal fluid accumulation and weight of intestinal content at all levels of the tested doses as compared to the negative control. The effect of both extracts against PG induced fluid accumulation is comparable. It may be due to the presence of responsible active metabolites to inhibit fluid accumulation in both extracts comparably. Furthermore, 240mg/kg dose of water extract of *C. aurea* seed reduced mean volume of intestinal content significantly as compared to that of 60mg/kg. This finding further strengthen that the plant extract has a dose dependent anti-enteropooling effect. The anti-enteropooling activity of the plant could be credited to the presence of secondary metabolites including terpenoids, steroids, flavonoids and tannins. Terpenoids (Prakash, 2017), flavonoids (Hamalainen *et al*., 2011) and steroids (Awad *et al*., 2004) have been shown to inhibit production of PGE_2, which are known to play a critical role in the stimulation of intestinal secretions through causing secretion of water and electrolytes (Pierce *et al*., 1971). Tannins decrease fluid secretion by inhibiting CFTR and CaCC, by generating a protein-precipitating reaction to the GI mucosa (Tadesse *et al*., 2017), which make the intestinal mucosa more resistant to chemical alteration (Balaji *et al*., 2012; Yaecob *et al*., 2016).

PG might also activate the NO pathway and induce NO-dependent GI secretion (Degu *et al*., 2016) through the stimulation of cAMP and cGMP concentration (Liang *et al*., 2005). A growing body of evidence indicates that bioactive compounds such as terpenoids (Jang *et al*., 2004; Zhao *et al*., 2016; Prakash, 2017), Alkaloids (Kondo *et al*., 1993; Zhao *et al*., 2016) and flavonoids (Rupesh *et al*., 2014) are implicated in the attenuation of NO synthesis. Therefore, the anti-enteropooling effect of both extracts could probably be through NO pathway interruption.

Moreover, small intestine is extrinsically innervated by both parasympathetic and sympathetic divisions of the autonomic nervous system (Tortora & Derrickson, 2009). Parasympathetic system stimulates intestinal secretion through neurotransmitters such as acetylcholine and vasoactive intestinal peptides

(VIP) while sympathetic one stimulates intestinal absorption through α2 adrenergic agents such as enkephalins and somatostatins. Secondary metabolites such as flavonoids from plant sources could stimulate α2 adrenergic receptors in the absorptive cells of the GI tract (Degu *et al*., 2016). In addition to regulating electrolyte movement, transepithelial fluid transport in the GI tract is also controlled by managing aquaporin (AQP) type water channels. Tannins were found to inhibit aquaporins (AQPs) 2 and 3 expressions *in vivo* and *in vitro* via down regulating protein kinase A/cAMP response element binding protein (PKA/CREB) signal pathway, which partially accounts for the anti-secretory and hence antidiarrheal effects (Liu *et al*., 2014). Therefore, anti-secretory activity of both extracts could probably relate to the existence and synergistic effects of flavonoids, tannins, terpenoids and saponins. Keeping this in mind, in the present study, the plant extracts more likely reduced diarrhea by either stimulating reabsorption of electrolytes and water through sympathetic activation or by inhibiting the fluid secretion into the intestine by altering parasympathetic activity. The anti-entropooling effect of the standard drug loperamide is somewhat lower than its effect on motility (Table 2). However, this is not unexpected and without reason. A study by Schiller *et al*. confirmed that the major antidiarrheal mechanism of action of loperamide is reduction of intestinal motility rather than proabsorptive or anti-secretory effect (1984).

Increasing intestinal motility is one way of increasing formation of diarrhea. To evaluate the antimotility effect of the extracts, the test was performed by using charcoal meal as a marker. GI motility is mainly regulated by the sympathetic and parasympathetic nerves, with the latter acting as the most important factor. Escalating activation of the parasympathetic nerves enhances intestinal transit, while increasing activation of the sympathetic nerves inhibits it (Tortora & Derrickson, 2009). Loperamide, which was used as a standard drug, was known to suppress movement of the charcoal meal due to its anticholinergic, antihistamine and PG blocking effects (Kartm & Adaikan, 1977). Beside cholinergic receptors, activation of the sympathetic system via α2 adrenergic receptors in the GI tract is able to inhibit peristaltic activity, reduce muscle tone, alleviate gastric emptying and promote defense of stomach mucosa (Suleyman, 2012; Beserra *et al*., 2016). The reduction in distance travelled can be used as a tool in order to explain the intestinal smooth muscle relaxation. From previous knowledge, contractions of all smooth muscles absolutely depend on the presence of Ca^{2+} which activates the contractile elements and their relaxation, a mechanism implicated in the antidiarrheal effect of different drugs. Therefore, the plant could have caused the reduction in distance travelled by the charcoal meal through increasing the intracellular Ca^{2+} release.

In this study, the charcoal meal test demonstrates that the graded doses of both aqueous and 80% methanol seed extracts significantly reduced intestinal propulsive movement in castor oil induced intestinal transit as compared to the negative controls. The inhibitory effect of the extracts in castor oil-induced intestinal transit was greater as compared to that of the normal intestinal transit. According to literatures, drugs with antidiarrheal effects are well known for reducing GI contractions and thereby slows the intestinal transit (Kumar *et al*., 2014), allowing more time for better absorption of water and electrolytes (Ihekwereme *et al*., 2016; Yaecob *et al.*, 2016). The observed effect is therefore possible due to the extracts' ability to inhibit the intestinal movement, which in turn accounts for the antidiarrheal effect of the extracts of the plant. In other words, the more the intestinal motility the greater would be the inhibitory effect of the extracts. The importance of this finding should not be underestimated since the related development of constipation, a major problem of most of conventional drugs including loperamide, as a side effect would be lower. The inhibitory effect on the intestinal transit seems comparable for both extracts which could be attributed to the presence of comparable level and type of secondary metabolites that are responsible for antimotility effect of the plant. Phytochemical constituents such as polyphenols, flavones, alkaloids, saponins and terpenoids that are previously detected in the 80% methanol extract of *C. aurea* seeds (Nega *et al*., 2015) are the most likely agents that endow the plant with antidiarrheal properties.

Tannins were shown to inhibit 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, attributed by spasmolytic and calcium channel blocking activities (Yaecob *et al*., 2016; Wansi *et al*., 2017). Flavonoids are also known to inhibit intestinal motility (AL-Maamori, 2011) through relaxing intestinal smooth muscles (Damabi *et al*., 2010; Yaecob *et al*., 2016), while terpenoids, on the other hand, were reported to inhibit intestinal motility and secretion induced by castor oil by inhibiting the release of autacoids and PGs (Yaecob *et al*., 2016). Furthermore, anticholinergic agents are known to inhibit GI hypermotility as indicated earlier.

The *in vivo* antidiarrheal index (ADI) is a measure of the pooled effects of different components of diarrhea such as reduction in GI motility, onset of diarrheal stools, and intestinal fluid accumulation (Yaecob *et al*., 2016). As indicated in the literature, the higher the ADI value, the better the effectiveness of the extract in curing diarrhea (Tadesse *et al*., 2017). The ADI value further corroborated that both extracts of the plant have comparable antidiarrheal activity with the standard drug.

The aqueous and 80% methanol seed extracts of the *C. aurea* found to have a marginal antibacterial activity on most of the tested organisms and virtually no activity on *P. aeruginosa*. Considering MIC and MBC, better effect seemed to be observed against *S. typhymurium*, as its growth was inhibited at a concentration of 250 mg/ml and completely killed at a concentration of 500 mg/ml 80% methanol seed extract of *C. aurea*. In terms of zone of inhibition, however, *E. coli* showed better response. The difference may be due to difference in sensitivity of the organisms to the plant extract, i.e. the 80% methanol seed extract of *C. aurea* may be bactericidal against *S. typhymurium* and bacteriostatic against *E. coli*. The hydro-alcoholic extract of the plant was somewhat better than that of the aqueous extract both in terms of its effectiveness and strain coverage. The difference tends to be due to the difference in polarity and availability of the phytochemical constituents and the vulnerability of aqueous extracts to hydrolysis. More suitable explanation for the decrease in activity of aqueous seed extract can be credited to the enzyme polyphenol oxidase, which degrades polyphenols in water extracts. In methanol extracts, the enzymes are, however, inactive. More importantly, water soluble flavonoids, typically anthocyanins, and phenolics have no antimicrobial significance. In addition to this, methanol was found to extract saponins, which have antimicrobial activity, better than water (Tiwari *et al*., 2011). However, for both extracts tested, since this minimal effect is observed at a higher dose of the plant extract it is impossible to consider antibacterial action of the plant as an appreciable mechanism of diarrhea treatment.

Although the plant is blessed with all of previously mentioned activities, it is not free from toxicity. The degree of intoxication is contingent on the amount of the plant extract administered. The higher the dose the more severe the toxicity. A study assessing the *in vivo* antimalarial activity of hydromethanolic leaf extracts of *C. aurea* supports this finding (Eyasu *et al*., 2013). In contrast to this, another study by Umer *et al*. (2013) that assessed the antidiarrheal effect of 80% methanol leaf extract of *C. aurea* reported that this plant is safe even up to 5000 mg/kg dose. Plant toxins are usually secondary metabolites that are produced and secreted by plants. Secondary metabolites, such as alkaloids, cyanogenic glycosides and furanocoumarins are the most frequently reported source of plant poisoning (Osman *et al*., 2013). A preliminary study that screened the phytochemical constituents of *C. aurea* seeds confirmed that alkaloids and glycosides are parts of the plants secondary metabolites (Nega *et al*., 2015). Among the alkaloids, the pyrrolizidine subtypes are the principal plant metabolites that pose a serious health threat to humans via foodborne plant intoxication. Pyrrolizidine alkaloids (PAs) are particularly abundant in three plant families, namely Asteraceae, Fabaceae and Boraginaceae (Osman *et al*., 2013). Photochemistry of Ethiopian *C. aurea* studied by Asres and his colleagues (1985) further supported that

the plant is endowed with many types of alkaloids, including PAs. In this regard, *C. aurea*, which is belonging to the family of Fabaceae and confirmed to have PAs, could potentially be toxic and judicious use is warranted.

6. Conclusion

The results obtained in the present study suggest that the *C. aurea* seed has significant antidiarrheal activity probably related to its pro-absorptive, antisecretory and antimotility effects. Both extracts had comparable activity, indicating that medium polar to polar constituents contributing to the observed effect. The plant does not seem to have appreciable antimicrobial effect, ruling out this activity as a possible mechanism.

7. Recommendation

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

- Further studies should be conducted to isolate, purify and identify bioactive principle(s) responsible for the antidiarrheal activities of both extracts.
- Further studies are required to ascertain the precise mechanism of action of the antidiarrheal activity of the plant.
- Performing antidiarrheal activity tests should be done with various solvent fractions.
- Both sub-acute and chronic toxicology of the plant should be investigated.
- The community should be informed not to take seeds of the plant in excess and also to use other less toxic alternatives.

8. References

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