Evaluation of diuretic activity of solvent fractions of 80% methanol root extract of *Clutia abyssinica* Jaub and Spach (Euphorbiaceae) in rats

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This is to certify that the thesis prepared by Birhanu Geta, entitled "Evaluation of diuretic activity of solvent fractions of 80% methanol root extract of *Clutia abyssinica* Jaub and Spach (Euphorbiaceae) in rats" and submitted in partial fulfilment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Abstract

Evaluation of diuretic activity of solvent fractions of 80% methanol root extract of *Clutia abyssinica* Jaub and Spach (Euphorbiaceae) in rats

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Diuretics are drugs that increase the rate of urine flow and sodium excretion, and are used to adjust the volume and composition of body fluids in a variety of clinical conditions. Several diuretic agents are available in the market, but they are associated with a multitude of problems which are not yet solved. These limit their clinical usefulness and calls for the search for new and better compounds. This study aimed to investigate the diuretic activity of different solvent fractions of 80% methanol root extract of *Clutia abyssinica,* an extensively used plant in Ethiopian folk medicine. The roots of *Clutia abyssinica* were subjected to successive maceration followed by liquid-liquid extraction. Male rats were treated with vehicle (distilled water or 2% Tween 80), standard (Furosemide 10 mg/kg) and three doses (100, 200 and 400 mg/kg) of 80% methanol extract and solvent fractions. Parameters, including urine volume, electrolyte concentration and pH were measured. The 80% methanol extract produced significant diuresis ($p<0.001$) at 200 and 400 mg/kg by the end of the fifth hour compared to negative control. Among the solvent fractions, the n-butanol fraction had significant diuresis ($p<0.001$) at the doses of 200 and 400 mg/kg. The aqueous fraction, however, did not induce significant diuresis. On the other hand, the chloroform fraction had no significant diuresis at 100 and 200 mg/kg but had at 400 mg/kg ($p<0.01$). Regarding electrolyte excretion, the 80% methanol extract produced significant natriuresis and kaliuresis at 200 mg/kg and 400 mg/kg ($p<0.001$) compared to negative control. Similarly, the n-butanol fraction had also produced significant natriuresis and kaliuresis ($p<0.001$) at the doses of 200 and 400 mg/kg. Phytochemical screening of the 80% methanol extract and solvent fractions revealed the presence of secondary metabolites, including alkaloids, flavonoids, terpenoids, polyphenols and saponins, which might account for the diuretic activity. These findings collectively indicate that the n-butanol fraction exhibited significant diuretic activity, which could be used as a starting point for further studies.

Keywords: *Clutia abyssinica*, diuresis, natriuresis and kaliuresis.

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Abbreviations

ADH	Antidiuretic hormone
AVP	Arginine vasopressin
CD	Collecting duct
DCT	Distal convoluted tubule
GFR	Glomerular filtration rate
ISE	Ion selective electrode
OECD	Organization for economic cooperation and development
PCT	Proximal convoluted tubule
TAL	Thick ascending limb
UT	Urea transporter

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1. Introduction

1.1 Definition and History of Diuretics

The word *diuretic* is derived from the Greek '*diouretikos*' meaning "to promote urine'' (Okusa and Ellison, 2008). By definition, a diuretic is any substance causing the kidney to secrete excess fluid by blocking the reabsorption of either water itself or electrolytes like sodium, potassium, chloride and bicarbonate. Diuretics also alter the excretion of hydrogen, calcium, magnesium and some other substances like; phosphoric acid and uric acid (Reilly and Jackson, 2011). Diuretic agents are used to adjust the volume and composition of body fluids in a variety of disorders such as congestive heart failure, liver cirrhosis, certain kidney diseases, hypertension, water poisoning and pregnancy toxaemia (Snigdha et al., 2013). Some diuretics, such as acetazolamide, help to make the urine more alkaline and are helpful in increasing excretion of acidic substances in cases of overdose or poisoning (Wile, 2012).

The discovery and use of diuretics for the mobilization of excess body fluid has a long history, which dates back to the beginning of medicine. Paleolithic humans may have discovered the original diuretic when they found caffeine-containing plants whose seeds and bark were used to prepare beverages. The natural substances coffee, tea and cola have diuretic effect and they were used due to their therapeutic importance before the development of conventional diuretics (Okusa and Ellison, 2008).

In 1553, Paracelsus identified Calomel (mercurous chloride) as a diuretic to be used in the management of edema (dropsy). In 1919, Alfred Vogl observed that the injection of organomercurial compounds such as merbaphen for syphilis also caused a substantial diuresis and this led to the development of effective organic mercurial diuretics that were used until 1960s (Lant, 1987; Okusa and Ellison, 2008). In 1937, following the discovery of the enzyme carbonic anhydrase, sulphanilamide antimicrobials were found to cause metabolic acidosis,

mild diuresis and hyperventilation in patients by blocking this enzyme. Schwartz showed that sulfanilamide could induce diuresis in patients with congestive heart failure who were resistant to organic mercurial diuretics (Lant, 1987). Soon, more potent sulphonamide based carbonic anhydrase inhibitors were developed, but these drugs suffered from side effects and limited potency. This led to the development of chlorothiazide by modification of sulphonamides and resulted in enhanced sodium chloride rather than sodium bicarbonate excretion. In the mid-1950s, the first modern diuretic, acetazolamide, an inhibitor of carbonic anhydrase with low diuretic potential, was developed (Safarian et al., 2007). Although various agents that increase urine flow have been described since antiquity, it was not until 1957 that a practical and powerful diuretic agent (chlorothiazide) became available for widespread use (Ernst and Moser, 2009). A few years later, in 1960s, potassium sparing diuretics, were developed (Ellison and Loffing, 2009; Pascaline et al., 2011; Wile, 2012). Potassium sparing agents such as spironolactone is associated with several drawbacks and current development of diuretics shift in focus to diuretics that alter water excretion such as nonspecific agents (Lithium, demeclocycline) to antidiuretic hormone receptor antagonists (Beg and Rizvi, 2015; Amin and Danny, 2016), endogenous diuretics (atrial natriuretic peptide and urodilatin) and novel agents under development (Denton et al., 2013).

1.2 Renal Anatomy and Physiology

The kidneys are a pair of bean-shaped organs lying retroperitoneal (behind the parietal peritoneum) in the superior lumbar region of the posterior abdominal wall. They extend from the level of the 11^{th} or 12^{th} thoracic vertebra superiorly to the 3^{rd} lumbar vertebra inferiorly, and thus they receive some protection from the lower two ribs. The right kidney is crowded by the liver and lies slightly inferior to the left kidney (Marieb, 2012).

An average adult kidney weighs 150 g and is about 12 cm high, 6 cm wide, and 3 cm thick; the size of a clenched fist. The lateral surface of each kidney is convex; the medial surface is concave and has a vertical cleft called the renal hilum, where blood vessels, lymphatic vessels, ureters, and nerves enter and leave the kidney (Guyton and Hall, 2016).

The kidneys are the main organs of homeostasis that maintain the acid-base and water-salt balance of the blood. The primary function of the kidneys is to maintain a stable internal environment for optimal cell and tissue metabolism by getting rid of waste materials such as; urea, mineral salts, toxins, and other waste products of metabolism. The kidneys also conserve water, salts, and electrolytes. At least one kidney must function properly for life to be maintained (Marieb, 2012).

The kidneys are highly vascularized organs. Blood enters each kidney via a renal artery through the hilum, filtered to form urine and leaves through small veins into the renal vein, which drains into the inferior vena cava (Figure 1). Each human kidney contains about 800,000 to 1,000,000 nephrons. As shown in Figure 1, each nephron contains two principal parts: a renal corpuscle, which filters the blood plasma, and a long renal tubule, which converts the filtrate to urine. The renal tubule is divided into four major regions: the proximal convoluted tubule (PCT), nephron loop, distal convoluted tubule (DCT) and collecting duct (CD) (Ives, 2012; Guyton and Hall, 2016). Urine formation begins when a large amount of fluid that is virtually free of protein is filtered from the glomerular capillaries into the Bowman's capsule. After it passes into the

renal tubule, its composition is quickly modified by tubular reabsorption and tubular secretion (Costantini and Kopan, 2010; Guyton and Hall, 2016).

PCT, which arises from the glomerular capsule, is responsible for reabsorbing approximately 66% of filtered sodium ions (Na^+), 85% of sodium bicarbonate ($NaHCO_3$), 65% of K^+, 60% of water, and virtually all of the filtered glucose and amino acids. $NaHCO_3$, NaCl, glucose, amino acids, and other organic solutes are reabsorbed via specific transport systems in the PCT. Potassium ions are reabsorbed via the para-cellular pathway. Water is reabsorbed passively, maintaining osmolality of the proximal tubular fluid at a nearly constant level (Reilly and Jackson, 2011; Guyton and Hall, 2016).

$NaHCO_3$ reabsorption by the PCT is initiated by the action of a Na^+/H^+ exchanger (NHE3) located in the luminal membrane of the proximal tubule epithelial cell. This transport system allows Na^+ to enter the cell from the tubular lumen in exchange for a proton (H^+) from inside the cell. As in all portions of the nephron, Na^+/K^+-ATPase in the basolateral membrane pumps the reabsorbed Na^+ into the interstitium so as to maintain a low intracellular Na^+ concentration (Reilly and Jackson, 2011; Ives, 2012). From PCT, the fluid dives deep into the loop of Henle, which is a hair pin or long U-shaped portion of the renal tubule found mostly in the medulla (Marieb, 2012). The loop of Henle consists of three functionally distinct segments: the thin descending segment, the thin ascending segment, and the thick ascending segment (Figure 1). The thick ascending limb (TAL) of loop of Henle is impermeable to water and transports electrolytes into the interstitium of the kidney, producing a high osmotic pressure of the interstitium. Twenty-five percent of the filtered sodium is reabsorbed using a luminal $Na^+/K^+/2Cl^-$ cotransport mechanism in this region of nephrons. The energy for Na^+ reabsorption derives from the basolateral Na^+/K^+ pump. The effective prevention of a passive water flow with water tight junctions leads to a high osmotic pressure in the renal medulla (Broodbank and Christian 2014).

Although the $Na^+/K^+/2Cl^-$ co-transporter is electrically neutral, its action contributes to excess K^+ accumulation within the cell. Back diffusion of this K^+ into the tubular lumen causes a lumen-positive electrical potential that provides the driving force for reabsorption of cations, including Mg^{2+} and Ca^{2+} via the para-cellular pathway (Reilly and Jackson, 2011; Ives, 2012; Broodbank and Christian 2014). The TAL empties into the DCT, where 5-10% of the filtered sodium and chloride ions are reabsorbed (Reilly and Jackson, 2011). Finally, the urine reaches at the collecting tubule system, which is responsible for only 2-5% of NaCl reabsorption by the kidney (Ives, 2012). The connecting tubule, which coalesces to form the collecting ducts, performs the final adjustment of renal excretion (Guyton and Hall, 2016). It is the site at which mineralocorticoids exert a significant influence (Ives, 2012). Collecting tubules include principal cells, which reabsorb Na^+ and secrete K^+, and two populations of intercalated cells, α and β, which secrete acid (H^+) and base (HCO_3^-), respectively (Ives, 2012; Range et al., 2012; Broodbank and Christian 2014). Unlike other segments of the nephron, principal cell membranes exhibit separate ion channels for Na^+ and K^+. Since these channels exclude anions, transport of Na^+ or K^+ leads to a net movement of charge across the membrane. Because Na^+ entry into the principal cell predominates over K^+ secretion into the lumen, a 10-50 mV lumen negative electrical potential develops. This lumen-negative electrical potential drives the transport of Cl^- back to the blood via the para-cellular pathway and draws K^+ out of cells through the apical membrane K^+ channel. Thus, there is an important relationship between Na^+ delivery to the collecting tubule system and the resulting secretion of K^+. That is, Na^+ delivery to this site enhances K^+ secretion (Ives, 2012; Wile, 2012). The final concentration of the urine depends on water permeability of the collecting ducts carrying the urine through the cortex and medulla (Broodbank and Christian 2014). The permeability of the collecting ducts is regulated with Antidiuretic hormone (ADH). ADH causes the incorporation of additional water channels (aquaporins) into the apical membrane of the principal cells. In the absence of ADH, the

collecting tubule is impermeable to water, and dilute urine is produced. ADH markedly increases water permeability, and this leads to the formation of more concentrated urine (Reilly and Jackson, 2011; Ives, 2012; Wile, 2012).

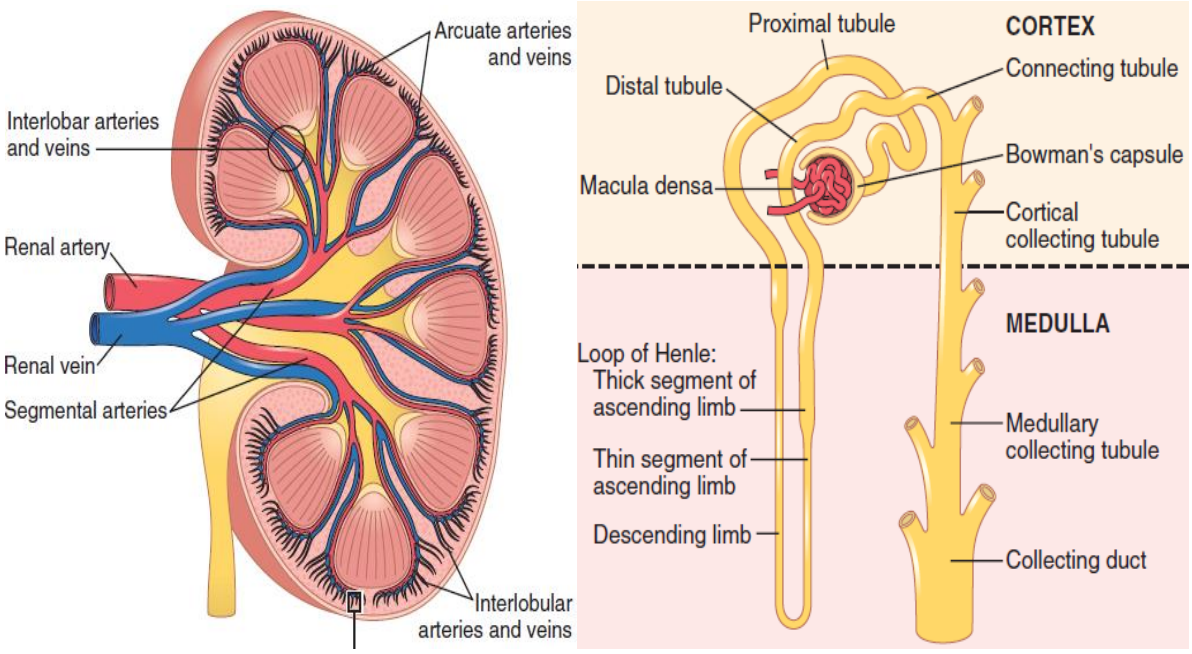

Figure 1. Major anatomical features of the kidney and nephron (Guyton and Hall, 2016).

1.3 Conventional Diuretics

There are around five classes of conventional diuretics that are classified based on the mechanism and site of action with in nephron segment (Cadwallader et al., 2010; Reilly and Jackson, 2011; Ives, 2012). The first classes are loop diuretics (high ceiling diuretics); which are widely used for the symptomatic treatment of heart failure and fluid retention in chronic kidney disease. They act primarily by inhibiting the symporter protein known as $Na^+/K^+/2Cl^-$ co-transporter, which is responsible for sodium and chloride reabsorption over the entire length of the TAL of the loop of Henle. The $Na^+/K^+/2Cl^-$ co-transporter in the TAL normally reabsorbs approximately 25% of the sodium load (Wile, 2012). Inhibition of this pump leads to a significant increase in the distal tubular concentration of sodium, reduced hypertonicity of the surrounding interstitium, and less water reabsorption in the CD. This altered handling of sodium and water leads to both diuresis and increased loss in sodium. Despite their maximum

efficacy, loop diuretics are associated with a wide range of side effects such as hypokalaemia, hyponatraemia, hypotension, hypomagnesaemia, hypocalcaemia, hyperuricemia, metabolic alkalosis and dose related ototoxicity (Snigdha et al., 2013; Smith, 2014).

The second classes are thiazides and thiazide-like or low-ceiling diuretics; which act via inhibition of sodium and chloride reabsorption at the DCT by blocking Na^+-Cl^- transporter (Reilly and Jackson, 2011; Wile, 2012). Since Na^+-Cl^- transporter normally reabsorbs approximately 5% of filtered sodium, these diuretics are less efficacious than loop diuretics in producing diuresis and natriuresis. The most common side effects associated with use of these diuretics are hypokalaemia, metabolic alkalosis, hyperuricemia, hypotension, hyperglycaemia, hyponatraemia and hyperlipidaemia (Ives, 2012; Wile, 2012; Smith, 2014).

The third agents are potassium-sparing diuretics; these agents are further classified into two based on their mechanism of action. Aldosterone receptor antagonists: unlike loop and thiazide diuretics, these agents do not act directly on sodium transport rather, they antagonize the action of aldosterone by inhibiting mineralocorticoid receptors at the distal segment of the distal tubule and sodium channel blockers: which inhibit sodium reabsorption in the distal tubule (Smith, 2014). This causes more sodium and water to pass into the CD and to be excreted in the urine. They are called potassium-sparing diuretics because they do not produce hypokalaemia, but they are associated with severe hyperkalaemia, arrhythmia, metabolic acidosis, gynaecomastia and peptic ulcer disease (Wile, 2012; Snigdha et al., 2013).

The fourth classes are carbonic anhydrase inhibitors; they inhibit the transportation of bicarbonate out of the PCT into the interstitium, which leads to less sodium reabsorption at this site and therefore greater sodium, bicarbonate and water loss in the urine. These are the weakest of all diuretics and are seldom used in the management of cardiovascular disease. Their main use is in the treatment of glaucoma and prophylaxis of mountain sickness (Cadwallader et al., 2010; Reilly and Jackson, 2011). Hypokalaemia and metabolic acidosis are major side effects

for this class of diuretics. The last agents are osmotic diuretics, these are non-reabsorbable substances act by inhibiting sodium and water reabsorption in the proximal tubule, and more importantly, in the loop of Henle. They are not used for the treatment of oedematous conditions because they precipitate the condition due to fluid shift and they are associated with hypotension (Wile, 2012; Snigdha et al., 2013; Smith, 2014).

1.4 Diuretic Resistance

The body responds to diuretic drug therapy in several different ways that can lead to diuretic resistance. Especially, in some critically ill patients, conventional doses of loop diuretics do not always result in optimal diuresis. In such cases, patients are considered diuretic resistant. Diuretic resistance is defined as a failure to achieve the therapeutically desired reduction in edema despite a full dose of diuretic. The causes of diuretic resistance include poor adherence to drug therapy or dietary sodium restriction, pharmacokinetic issues, and compensatory increases in sodium reabsorption in nephron sites that are not blocked by the diuretic (Hoorn and Ellison, 2016).

Two mechanisms of the phenomenon of diuretic resistance have been suggested. The most common is the concept of rebound sodium retention. After administration of loop diuretics, sodium absorption is blocked at the loop of Henle, leading to a pronounced reabsorption of sodium at the distal sites of the nephron. This reabsorption may be sufficient to nullify the prior blockade. Studies in rats have shown that six to eight days of continuous furosemide infusion caused hypertrophy of the DCT, the connecting tubule, and the collecting ducts of the nephron (Asare, 2009). This results in an enhanced capacity for sodium and fluid reabsorption by the hypertrophied distal tubule. This also explains the synergistic response to combination therapy (using a loop diuretic and a thiazide to block sodium re-absorption at the loop of Henle and the distal tubule, respectively) (De Bruyne, 2003; Nag et al., 2011).

The second mechanism is diuretic braking, the decrease in a patient's response to a diuretic after receiving the first dose. In other words, the magnitude of response to each administered dose of diuretic declines with time. Diuretic braking is not a separate mechanism but occurs as a result of rebound sodium retention and post-diuretic effect, a compensatory sodium-retention process that begins as the diuretic action wanes. The complex process, called the braking phenomenon is the adaptation to the drug that is due to change in the structure and function of the kidney itself, activation of the sympathetic nervous system and changes in the several hormone pathways (Asare, 2009; Nag et al., 2011).

When treating diuretic resistance, it is important for the patient to restrict daily consumption of fluid intake, follow a low-sodium diet and avoid taking non-steroidal anti-inflammatory drugs (NSAIDS). The other approach that is remarkably effective for managing diuretic resistance is sequential blockade of the nephron. This is done by combining diuretics that act in different segments of the nephron, usually a loop and a thiazide diuretic, resulting in inhibition of reabsorption at multiple sites (Nag et al., 2011).

1.5 Novel Diuretics

The advancement in molecular techniques and analytical procedures led to an explosion in the understanding of molecular pathophysiology of disease pathways and potential therapeutic targets, which have direct link with the disease and targeting will result in maximum efficacy with minimal adverse effects. Since long-term use of conventional diuretics can have several adverse effects, including hypokalaemia, hyperkalaemia, hyponatraemia, hyperuricemia, hyperlipidaemia and hyperglycemia (Wile, 2012; Snigdha et al., 2013; Smith, 2014); new diuretic targets are being identified and agents are being developed. Novel diuretic agents include adenosine receptor (A1) antagonists, Arginine Vasopressin (AVP) V2 receptor blockers, specific ion channel inhibitors (inhibitors of Kir1.1, Kir4.1/5.1 potassium channels and ClC-Ka/b chloride channels) (Denton et al., 2013), urea transporter blockers (Yang, 2015;

Klein, and Sands, 2016), chloride-bicarbonate exchanger (pendrin) inhibitors and regulatory protein (e.g. SPAK) inhibitors (Denton et al., 2013; Jeong and Hong, 2015).

1.5.1 Adenosine Receptor (A1) Antagonists

Adenosine is an important intermediary metabolite, acting as a building block for nucleic acids and a component of the biological energy currency adenosine triphosphate (ATP). In addition, adenosine functions as a signalling molecule through the activation of four distinct adenosine receptors: A1, A2A, A2B and A3 (Fredholm et al., 2001). These receptors are widely expressed and have been implicated in several biological functions, both physiological and pathological. These include cardiac rhythm and circulation, lipolysis, renal blood flow, immune function, sleep regulation and angiogenesis, as well as inflammatory diseases, ischemia-reperfusion and neurodegenerative disorders (Chen et al., 2013; Sachdeva and Gupta, 2013).

Even though the ubiquitous nature of adenosine and wide spread distribution of its signalling becomes a challenge in developing adenosine receptor ligands for specific clinical applications, there are substances under development. As different genetic based studies confirmed, stimulation of A1 receptor on the glomerular afferent arteriole reduces renal blood flow and glomerular filtration rate (GFR) and on the proximal tubules increases sodium and water reabsorption (Gottlieb et al., 2002). Inhibition of sodium reabsorption in the PCT would be beneficial in diseases associated with volume retention. Induction of diuresis and natriuresis without causing the compensatory decrease in renal function would be beneficial in diuretic resistant patients with volume overload. Therefore, effective inhibition of sodium reabsorption without reducing renal function is desirable and blockade of adenosine A1 receptors may provide such novel therapy. In addition, A1 receptor antagonists may have a role in the treatment of ischemic injury to the kidney by maintaining afferent arteriole vasodilation and preserving the GFR (Chen et al., 2013; Welch, 2015).

1.5.2 Arginine Vasopressin Receptor V-2 Inhibitors (aquaretics)

Conventional diuretics are not ideal therapies for hyponatremia because they can actually exacerbate hyponatremia by inducing excretion of Na^+ in excess of free water (Smith, 2014). Thus, a new class of pharmacological agents called aquaretics that selectively enhance renal water excretion has been developed. AVP by acting on its three (V1a, V1b (V3) and V2) G-protein coupled receptors, plays a central role in regulating body fluid homeostasis, serum osmolality, and vascular tone (Bouley et al., 2008; Amin and Danny, 2016). The main function of AVP is mediated via V2 receptors that are found in the CD segment of nephrons and affect the rate of water excretion through kidneys. When release of AVP is stimulated, the circulating vasopressin binds to the V2 receptors on the principal cells of CD and stimulates insertion of aquaporins on the apical membrane, which in turn result in increased water retention (Reilly and Jackson, 2011; Ives, 2012). In patients with heart failure (HF), there is an increased level of AVP, contributing to such symptoms as edema, dyspnea, and congestion. V2 receptor antagonists result in an increase in net fluid loss, a decrease in body weight and a reduction in the rate of HF exacerbation. Vasopressin receptor antagonists are classified into two classes based on receptor selectivity as selective V2 receptor antagonists (mozavaptan, lixivaptan, satavaptan, tolvaptan) and nonselective receptor antagonists (conivaptan). V2 antagonists are the only class of aquaretics that have been approved by Food and Drug Administration (FDA) for treatment of hyponatremia (Greenberg and Verbalis, 2006). Unlike the conventional therapy, tolvaptan as a selective V2 receptor antagonist could be a new hope for treatment of HF, but still not considered as initial therapy due to lack of studies (Beg and Rizvi, 2015; Amin and Danny, 2016).

1.5.3 Urea Transporter (UT-A1/A3) Inhibitors

Urea transporters (UTs) are a family of membrane channel proteins that are specifically permeable to urea and play an important role in intra renal urea recycling and in urine concentration. There are two types of UT proteins in humans, UT-A and UT-B. The UT-A has six sub families produced by alternative splicing (UT-A1~UT-A6), most of which are expressed in kidney and play an important role in urine concentrating mechanism, which is a passive transport of urea across the plasma membrane in the descending limb of loop of Henle and inner medullary collecting duct (Chen, 2013; Klein and Sands, 2016). UT-B subfamily has only one member, which is extensively expressed in various tissues, such as kidney, testis, brain, bone marrow, spleen and erythrocyte. It is also expressed in endothelia of kidney descending vasa recta (DVR) and mediates the passive transport of urea down its concentration gradient, thus it is indispensable in renal urea recycling and urine concentration (Esteva-font et al., 2015; Yang, 2015; Sun et al., 2016). Although the role of UT-B in urinary concentrating ability suggests the clinical applications of UT-B inhibitors as potential novel diuretics, untoward effects may outweigh due to the extra renal distribution of UT-B (Sands, 2013; Sun et al., 2016). UT-A isoform is of the greatest importance for urinary concentration as it is the rate-limiting step in apical membrane urea transport in the inner medullary collecting duct and hence required to establish the hyper-molar renal medullary interstitium (Yang, 2015). UT-A1, which is regulated by ADH, transports urea across the apical membrane into the intracellular space of luminal cells in the inner medullary collecting duct of the kidneys. It is a passive transporter and reabsorbs up to 70% of the original filtered load of urea. UT-A2 transports urea across the apical membrane into the luminal space of cells in the thin descending loop of Henle of the kidneys. The other variant, UT-A3, transports urea into the interstitium of the inner medullary collecting duct (Sands et al., 2010). The inner medullary collecting duct UTs, UT-A1 and UT-A3, are attractive diuretic drug targets since they are located in the last portion

of the nephron. Inhibition of UTs did not affect GFR or the clearance rate of Na^+, K^+, Cl^- in urine, except for urea. Their inhibition results in diuresis due to urea induced osmosis in the collecting ducts of the kidney. Thus, they should have maximal effects on renal urea reabsorption and minimal downstream effects on electrolyte excretion, in contrast to conventional diuretics that act in more proximal portions of the nephron. Therefore, UTs might be useful as novel diuretic targets to excrete water without disturbing electrolyte balance and based on this, UT inhibitors have been developed (Verkman et al., 2014). They can be widely used to increase renal water excretion in conditions associated with total body fluid overload, including congestive heart failure, cirrhosis and nephrotic syndrome. By disrupting counter current mechanisms and intra-renal urea recycling, UT inhibitors, alone or in combination with conventional diuretics may induce a diuresis in states of refractory edema where conventional diuretics are ineffective (Yang, 2015; Sun et al., 2016). A selective urea transporter UT-A1 inhibitor would be a novel type of diuretic, likely with less undesirable side effects than conventional diuretics, because it acts on the last portion of the nephron (Sands, 2013).

1.6 Botanical Diuretics

In the classical world, herbs were used to treat a variety of diseases and as a diuretic to remove excess body fluid. The diuretic properties of various plants were codified by Dioscorides in 40-90 A.D. Dioscorides wrote the methods by which plants could be transformed into medicines and for what they could be used; such as diuretic agents were important in removing fluids from the body (U.S.NIH, 2012). In recent decades, interest in the health benefits of herbals has grown in Western culture (Al Disi et al., 2016). In some developing nations, up to 80% of the population depends on traditional medicines as part of their primary care (Ekor, 2014). Modern scientific methods have proven the efficacy of several herbal extracts as diuretics. Herbals appear to exert a milder diuretic effect and have fewer side effects compared to their contemporary pharmaceutical counterparts, and may prove clinically useful as first line

therapies or supplements to conventional therapy (Dutta et al., 2014). Most of the herbal diuretics mentioned in Middle Eastern texts written between the 8th and 11th centuries have not been tested by modern methods (Shoja et al., 2015).

Worldwide trend towards the utilization of natural plant remedies has created an enormous need for information about the properties and uses of the medicinal plant (Dutta et al., 2014). There are a growing number of studies purporting diuretic effects with traditional medicines and based on the claims in the folkloric use, several medicinal plants were evaluated scientifically with promising diuretic activity. Some of these plants are; *Olea europaea* (Somova et al., 2003), *Carissa edulis* (Nedi et al., 2004), *Rumex abyssinicus* (Mekonnen et al., 2010), *Ruta graveolens* (Jayakody et al., 2011), *Rumex vesicarius* (Rao et al., 2011), *Achyranthes aspera* (Srivastav et al., 2011), *Trianthema portulacastrum* (Asif et al., 2013), *Ajuga remota* (Hailu and Engidawork, 2014), *Buchanania angustifolia* and *Buchanania lanzan* (Hullatti et al., 2014), C*issampelos pareira* (Sayana et al., 2014), *Nigella sativa* (Asif et al., 2015), *Flueggea leucopyrus* (Ellepola et al., 2015), *Moringa stenopetala* (Geleta et al., 2015), *Vernonia amygdalina* (Olufunsho and Abimbola, 2015), *Thymus schimperi* (Haji and Makonnen, 2016), and *Clutia abyssinica* (Tegegne et al., 2017).

1.7 The Experimental Plant

Clutia abyssinica Jaub and Spach, commonly known as "large or smooth-fruited lightning-bush" in English (Bijekar and Gayatri, 2014), "Fiyele-Feji" in Amharic (Ragunathan and Abay, 2009; Mohammed and Abraha, 2013; Enyew et al., 2014; Birhanu et al., 2015; Limenih et al., 2015), "F'eo" in Oromiffa (Bussmann et al., 2011), is a shrub that belongs to the family Euphorbiaceae (Bijekar and Gayatri, 2014). Geographically, it is distributed from Congo east to Eritrea and Somalia and through eastern Africa; south to Zambia, Angola, Mozambique and South Africa (Kuma and Shibru, 2015).

Clutia abyssinica is a shrub or bush that can range in habit from an erect, large woody herb or shrub growing 1-3 metres tall commonly, or can become a tree up to 6 metres tall rarely. It is found in dry forest, forest remnants, secondary forest and wooded grassland on rocky hillsides and riverine, evergreen thickets at 700-3700 m altitude (Mohammed and Abraha, 2013; Kipkore et al., 2014; Kuma and Shibru, 2015; Zone et al., 2015; Meragiaw et al., 2016).

The leaves of *Clutia abyssinica* turned from green to orange when they become old and die (Figure 2). Flowers are pale green in colour and fruits are green and turned into red when they mature. The stems are brittle and it is propagated only by seed (Matu, 2008).

Traditionally, all parts of *Clutia abyssinica* have many medicinal uses against a variety of diseases. In the eastern Africa, the boiled roots are made into soup, which is taken as a remedy for enlarged spleen and kidney problems, difficulty of urination, headache, stomachache and malaria (Matu, 2008). In Kenya, a concoction made from the boiled roots is used to treat colic pain in infants and erectile dysfunction in adults (Kigen et al., 2014; Kipkore et al., 2014). It also has anti-viral (against coxsackie and polio viruses) and antimalarial use (Njoroge and Bussmann, 2006; Muthaura et al., 2007). Root and leaf decoction is used in the treatment of venereal and skin diseases, chest problems, cancer and fertility in humans (Pascaline et al., 2011; Bijekar and Gayatri, 2014). In Tanzania, the root infusion is used to cure roundworm

infections, to clean kidneys and to treat stomachache (De Boer et al., 2005). In Eritrea, the leaf extract is used to treat gastritis and hypertension (Andemariam, 2010). In Tigray, crushed fresh leaf extracts are used for the treatment of herpes zoster, ring worm, internal parasite infection, black spider bite and leshimaniasis (Teklay et al., 2013; Teklay, 2015). In Welega, fresh leaves are held under the teeth for 20-30 minutes to treat toothache (Megersa et al., 2013). *Clutia abyssinica* leaf extracts are used to treat dysentery, toothache and to kill ecto-parasites (Enyew et al., 2014; Limenih et al., 2015; Meragiaw et al., 2016). It also has antimalarial, insecticidal and repellent properties (Meragiaw and Asfaw, 2014).

In the Kembatta and Oromo ethnic groups, the leaf and root extracts are used in the treatment of eczema and rheumatism (Maryo et al., 2015). In Mana Angetu District, south-eastern Ethiopia, they are used in the treatment of skin infection and bloody diarrhea (Lulekal et al., 2008). In Gondar, the dried root powder with zagol, medab and gracha eshoh for bone fracture by tying at the affected area till cure (Birhanu et al., 2015). In Gojjam, the dried root powder mixed with local beer (*tella*) is given orally in the morning as diuretic (Ragunathan and Abay, 2009).

Some scientific studies revealed its antimalarial (Muthaura et al., 2007), antitrypanosomial (Mergia et al., 2014), analgesic (Koech et al., 2017), anti-inflammatory (Koech et al., 2017), antipyretic (Koech et al., 2017) and diuretic (Tegegne et al., 2017) activities. The medicinal value of *Clutia abyssinica* may be due to its constituents that produce a definite physiological action. Phytochemical analysis of root extracts showed that this plant is found to contain alkaloids, saponins, anthraquinones, phenolics, tannins, terpenoids, flavonoids (Pascaline et al., 2011) and a complex mixture of 5-methyl coumarins (Waigh et al., 1991). But it was not found to contain glycosides (Pascaline et al., 2011).

Figure 2. Photograph of *Clutia abyssinica* (Hyde et al., 2017)

1.8 Rational for the study

Alleviation of diseases and maintenance of good health using herbal medicines is as old as mankind and is the most popular form of healthcare practice known to humanity that has been practiced by all cultures in all ages throughout the history of civilization (Chikezie and Ojiako 2015). About 80% of the world's population living in the developing world rely on herbal medicinal products as a primary source of healthcare (Ekor, 2014). Herbs are effective in the treatment or prevention of various diseases, such as diabetes mellitus, ascites, cardiovascular disorders, renal problems and even cancer (Bijekar and Gayatri, 2014).

Diuretics are one class of medicines that are used in the management of renal and cardiovascular diseases. The most commonly used diuretics, thiazides and loop diuretics have been associated with a number of adverse effects and diuretic resistance (Wile, 2012), which necessitates the search for new and better diuretics. The roots of *Clutia abyssinica* has been shown to produce diuretic activity (Tegegne et al., 2017*).* To further substantiate the diuretic activity of the plant, to determine in which fraction (s) the constituents responsible for diuretic activity are concentrated and in order to provide clue about the nature of the phytochemical constituents responsible for its action, this study was undertaken using the crude extract as well as various fractions of the root of *Clutia abyssinica*. The results of this study could help the scientific community to further investigate *Clutia abyssinica* by initiating advanced studies on molecular mechanisms and formulation of plant source drugs by identifying the specific agent responsible for the diuretic effect.

2. Objectives

2.1 General Objective

- To evaluate the diuretic activity of 80% methanol root extract and solvent fractions of *Clutia abyssinica* in rats

2.2 Specific Objectives

- To assess acute oral toxicity profile of the crude 80% methanol root extract and solvent fractions of *Clutia abyssinica*
- To determine the effect of 80% methanol root extract and solvent fractions of *Clutia abyssinica* on urine volume of rats
- To determine the effect of 80% methanol root extract and solvent fractions of *Clutia abyssinica* on urine electrolyte concentration
- To perform electrolyte analysis of 80% methanol root extract and solvent fractions
- To determine the effect of 80% methanol root extract and solvent fractions of *Clutia abyssinica* on urine pH of rats
- To perform preliminary phytochemical analysis for 80% methanol root extract and solvent fractions

3. Materials and Methods

3.1 Chemicals and Reagents

The following chemicals and reagents were used in the experimental study: methanol (Carlo Erba, France), n-butanol (Fresenius Kabi, India), chloroform (Fisher Scientific, UK), Tween 80 (UNI-CHEM Chemical Reagents, India), the standard drug Furosemide (EPharm, Ethiopia), distilled water (EPharm, Ethiopia), normal saline (Aculife Healthcare Pvt.Ltd. India) and other chemicals and reagents for phytochemical tests. All reagents used were of analytical grade.

3.2 Plant Material

The roots of *Clutia abyssinica* were collected in December 2016 around Debre Markos town, about 300 km northwest of Addis Ababa. Taxonomic identification of the plant specimen was made by Ato Wegie Abebe and a voucher specimen (BG01) was deposited at the National Herbarium, College of Natural Sciences, Addis Ababa University for future reference. The collected roots were thoroughly washed with tap water to remove dirt, soil and any other foreign materials. The roots were then cut into smaller pieces manually and dried under shade at room temperature for a period of 2 weeks. The dried roots were milled in to fine powder by using electrical mill.

3.3 Experimental Animals

Healthy Spargue Daweley rats of either sex with age of 6-10 weeks and having a weight range of 225-270 g inbred in the animal house of School of Pharmacy, Addis Ababa University were used for the experiment. The animals were housed in polypropylene cages (6 rats per cage) under standard environmental conditions (25±5^{o}C, 55±5% humidity and 12 h/12 h light/dark cycle). The animals were allowed free access to tap water and laboratory pellet, and acclimatized to laboratory condition for one week prior to the experiment. Each rat was placed in an individual metabolic cage (metabolic cage for rats, TECHNIPLAST, Italy) 24 h prior to

commencement of the experiment for adaptation. The care and handling of the animals were in accordance with the internationally accepted standard guidelines for use of animals (OECD-425, 2008; National Research Council, 2011). The protocol was approved by the department of Pharmacology and Clinical Pharmacy.

3.4 Extraction of the Plant

Nine hundred gram of the root powder was extracted by cold maceration using 80% methanol as a solvent. Three flasks were used, in which 300 g of the root was soaked in each flask containing a litre of 80% methanol and then placed on a shaker at 120 rpm for 72 h at room temperature. The extract was filtered by using muslin cloth and Whatman grade No 1 filter paper and the marc was re-extracted for the second and third time by adding another fresh 80% methanol. The fluid extracts were combined and concentrated in a rotary evaporator (Buchi, Rotavapor R-210/215, Switzerland) under reduced pressure at 40^{o}C. The concentrated filtrate was then frozen in a refrigerator and dried in a lyophilizer (Lyophilizer, OPR-FDU-5012, Korea). The dried root extract was collected and the yield was found to be 8.2% (w/w).

Two-third of the crude 80% methanol extract (about 49 g) was then successively fractionated using chloroform, n-butanol, and distilled water. First, the crude extract was suspended in 200 ml of warm water and the suspension was shaken in a separatory funnel by adding 50 ml chloroform three times in a separated process. Chloroform fraction was obtained in three separately performed fractionations. Second, the aqueous residue was shaken with 50 ml n-butanol three times separately and n-butanol fraction was obtained. Thereafter, the fractions were concentrated in a rotary evaporator and water bath, and the yield obtained was 7.2 g (14.6%) and 10.3 g (20.9%) for chloroform and n-butanol fractions respectively. The aqueous residue was also lyophilized and a total of 12.6 g (25.6%) of aqueous fraction was obtained. Finally, the crude 80 % methanol extract, chloroform and n-butanol fractions were stored in a

deep freezer (-20 °C), whereas the aqueous fraction was stored in a desiccator until used for the experiment.

3.5 Acute Oral Toxicity Test

Acute oral toxicity test for the crude 80% methanol root extract and solvent fractions of *Clutia abyssinica* was performed according to the organization for economic co-operation and development guideline (OECD-425, 2008). All rats were fasted overnight before and 4 h after administration of the 80% methanol extract and solvent fractions. First, a sighting study was performed to determine the starting dose. For this female rats were used and each rat was given 2000 mg/kg of the 80% methanol extract and solvent fractions as a single dose by oral gavage. Since no death was observed within 24 h, additional four rats were used for each of the 80% methanol extract and solvent fractions, and administered as the same dose mentioned above. The animals were observed continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h for the general signs and symptoms of toxicity (diarrhea, weight loss, tremor, lethargy and paralysis), food and water intake and mortality (OECD, 2008).

3.6 Grouping and Dosing of Animals

Animals were randomly assigned into fifteen groups (two negative controls, positive control and twelve test groups) comprising of six animals per group for diuretic test. Negative controls were treated with the vehicles used for reconstitution (2% Tween 80 in water (2%TW80) for 80% methanol extract, chloroform and n-butanol fraction, and distilled water (DW) for aqueous fraction) and positive controls were treated with standard drug, Furosemide 10 mg/kg (F10). Among the test groups (group 4, 5 and 6) were treated with 100 mg/kg (M100), 200 mg/kg (M200) and 400 mg/kg (M400) doses of 80% methanol root extract. Test groups (group 7, 8 and 9) were treated with 100 mg/kg (CF100), 200 mg/kg (CF200) and 400 mg/kg (CF400) doses of chloroform fraction. Groups 10, 11 and 12 were treated with 100 mg/kg (BF100), 200

mg/kg (BF200) and 400 mg/kg (BF400) doses of n-butanol fraction. Treatment groups (group 13, 14 and 15) received 100 mg/kg (AF100), 200 mg/kg (AF200) and 400 mg/kg (AF400) doses of aqueous fraction. Doses were determined using data from acute toxicity test. Route of administration for all groups was orally using oral gavage and the volume administered was 1mL/100 g.

3.7 Diuretic Activity

Diuretic activity was determined following the method used by Hailu and Engidawork (2014). All animals were subjected to fasting overnight with free access to water. The rats were pre-treated with normal saline at an oral dose of 15 mL/kg to impose a uniform water and salt load. They were divided into groups comprising of six animals per group and dosed as described under section 3.6. Immediately after administration, the animals were placed in metabolic cages (1 rat per cage) specially designed to separate urine and faeces and allow collection of urine into volumetric flask through funnels at the lower portion of the cages. Urine was then collected and measured at 1, 2, 3, 4 and 5 h after dosing and stored at -20°C for electrolyte analysis. At the time of urine collection, no food or water was made available to the animals.

The parameters recorded for each rat were urine volume, urine concentration of Na^+, K^+ and Cl^- and urine pH. The urinary concentration of electrolytes was expressed in terms of mMol/L. The following parameters were also determined in order to compare the effects of extracts with the vehicle and standard on urine excretion. The urinary excretion independent of the animal weight was calculated as total urinary output divided by total liquid administered (Formula-1). The ratio of urinary excretion in test group to urinary excretion in the control group was used as a measure of diuretic action of a given dose of an agent (Formula-2). As the diuretic action is prone to variability, a parameter known as diuretic activity was calculated to compare the diuretic action of the extract in the test group to that of the standard drug (Formula-3) (Hailu and Engidawork, 2014; Geleta et al., 2015).

$$\text{Urinary Excretion} = \frac{\text{Total urinary output}}{\text{Total liquid administered}} \times 100\% \quad (1)$$

$$\text{Diuretic Action} = \frac{\text{Mean urinary excretion of treatment groups}}{\text{Mean urinary excretion of control group}} \quad (2)$$

$$\text{Diuretic Activity} = \frac{\text{Diuretic action of test drug}}{\text{Diuretic action of standard drug}} \quad (3)$$

3.8 Analytical Procedures

Sodium, potassium and chloride levels of urine and the plant extract were analyzed. Sodium and potassium concentrations were determined by using flame photometer, and chloride concentration was quantified by using Ion Selective Electrode (ISE) analyzer (AVL 9181 Electrolyte Analyzer, Roche, Germany). The flame photometer works by flame production when the atom changed from its excited state to the ground state, while the ISE analyzer contains software which permits electrolyte parameter configuration. A calibration was performed automatically in both cases prior to analysis with different levels of standards. The ratios of electrolytes (test/control), Na^+/K^+ and $Cl^-/[K^+ + Na^+]$ were calculated to evaluate the saluretic, natriuretic and carbonic anhydrase activity of the extract and fractions. In addition, pH was directly determined on fresh urine samples by using pH meter. Furthermore, the salt content of the crude 80% methanol root extract and solvent fractions was also determined to rule out its contribution on urinary electrolyte concentration.

3.9 Phytochemical Screening

The qualitative phytochemical investigation of 80% methanol root extract and solvent fractions (chloroform, n-butanol and aqueous) were carried out using standard tests to identify the presence of secondary metabolites like; polyphenols, tannins, saponins, flavonoids, terpenoids, steroids, alkaloids and cardiac glycosides as follows.

Test for tannins

About 0.25 g of 80% methanol extract and each fraction was boiled in 10 mL of water separately in a test tube and then filtered. In each sample, a few drops of 10% ferric chloride

were added and observed for formation of precipitate or colour change. A bluish-black or brownish-green precipitate indicates the presence of tannins (Esatu et al., 2015).

Test for saponins

To 0.25 g of 80% methanol extract and each fraction, 5 mL of distilled water was added. Then, the mixture was shaken vigorously for 2 minutes and observed for formation of a stable persistent froth, which indicates the presence of saponins (Esatu et al., 2015; Gul et al., 2017).

Test for terpenoids

About 0.25 g of 80% methanol extract and each fraction was added in different test tubes. Two mL of chloroform was added in each test tube. Then, 3 mL concentrated sulfuric acid was carefully added in each of them to form a layer. A reddish-brown coloration at the interface indicates the presence of terpenoids (Esatu et al., 2015; Rohini and Padmini, 2016; Gul et al., 2017).

Test for flavonoids

About 0.5 g of 80% methanol extract and each fraction was added in different test tubes. Then, 10 mL of distilled water was added in each test tube and boiled for 5 min. The mixture was filtered while hot and allowed to cool. Few drops of 20% sodium hydroxide solution were added to 1 mL of the cooled filtrate. A change to yellow colour, which on addition of hydrochloric acid changed to colourless solution indicates the presence of flavonoids (Tiwari et al., 2011; Hossain et al., 2013).

Test for cardiac glycosides

About 0.5 g of 80% methanol extract and each fraction was dissolved in distilled water in different test tubes. In 5 mL of each solution, 2 mL of glacial acetic acid containing one drop of ferric chloride solution was added. This was underplayed with 1 mL of concentrated sulfuric acid. Formation of brown ring at the interface indicates presence of cardiac glycosides (Rohini and Padmini, 2016).

Test for alkaloids

About 5 mL of 5% hydrochloric acid was added to 0.5 g of 80% methanol extract and each fraction, and heated on a water bath. When cooled, few drops of Dragendroff's reagent (potassium bismuth iodide) was added. Appearance of the reddish-brown precipitate indicates the presence of alkaloids (Tiwari et al., 2011; Esatu et al., 2015).

Test for polyphenols

To 2 mL of filtered solutions of 80% methanol extract and each fraction, three to four drops of 1% $FeCl_3$ solution were added. Formation of bluish black color indicates the presence of phenols (Esatu et al., 2015; Rohini and Padmini, 2016).

Test for steroids

One mL of 80% methanol extract and each fraction was dissolved in 10 mL of chloroform and equal volume of concentrated sulfuric acid was added by sides of the test tube. The upper layer turns red and sulfuric acid layer showed yellow with green fluorescence, which indicates the presence of steroids (Gul et al., 2017).

3.10 Statistical Analysis

Results of the study are expressed as mean ± standard error of mean (S.E.M). Statistical analysis of the data were performed with one-way analysis of variance (ANOVA) followed by Tukey post hoc multiple comparison test. Dose dependent effects were evaluated by using linear regression. Significant differences were set at p values lower than 0.05.

4. Results

4.1 Acute Oral Toxicity Test

The acute oral toxicity test of 80% methanol root extract and solvent fractions of *Clutia abyssinica* indicated that neither the 80% methanol extract nor the solvent fractions caused gross behavioral changes and mortality within 24 h as well as in the next 14 days, indicating that the median lethal oral dose of the methanol extract and fractions were greater than 2000 mg/kg in rats.

4.2 Diuretic Activity: Effect on urine volume

4.2.1 80% Methanol Extract

The effect of oral administration of 80% methanol root extract of *Clutia abyssinica* on urinary output is shown in Table 1. The 80% methanol root extract produced diuresis which appeared to be a function of dose and time ($r^2 = 0.819$; $p<0.001$). M100 did not produce any detectable difference in urine volume as compared to negative control. M200 and M400, however, produced a significant increase in urine volume with maximum diuresis of 99% ($p<0.001$) and 122% ($p<0.001$), respectively. F10 had better diuresis than M100 ($p<0.001$), a bit higher effect than M200, and a comparable effect with M400 in the period of urine collection. When different doses of the 80% methanol extract were compared with each other, M400 and M200 produced significant diuresis compared to M100 at all time-points. M200 and M400 exhibited a diuretic action of 1.99 and 2.09, respectively. The percent urinary excretion of M200 (106%) and M400 (110%) was higher as compared to negative control (52%).

Table 1: Effect of 80% methanol root extract of *Clutia abyssinica* on urine volume in rats

Group	Volume of urine (mL)					% Urinary Excretion	Diuretic action	Diuretic activity
	1 h	2 h	3 h	4 h	5 h			
2%TW80	1.32 ± 0.15	1.82 ± 0.13	2.26 ± 0.12	2.82 ± 0.18	3.18 ± 0.22	52	1.00	
F10	2.48 ± 0.17^{a3}	3.80 ± 0.13^{a3}	5.00 ± 0.37^{a3}	6.50 ± 0.29^{a3}	6.92 ± 0.27^{a3}	111	2.17	1.00
M100	1.30 ± 0.32^{b2,c1,d2}	1.58 ± 0.37^{b3,c3,d3}	2.67 ± 0.33^{b3,c3,d3}	3.33 ± 0.36^{b3,c3,d3}	4.17 ± 0.48^{b3,c3,d3}	69	1.31	0.60
M200	2.25 ± 0.16^{a1}	3.37 ± 0.33^{a2}	4.41 ± 0.19^{a3}	5.58 ± 0.20^{a3}	6.33 ± 0.21^{a3}	106	1.99	0.92
M400	2.37 ± 0.16^{a2}	3.67 ± 0.21^{a3}	4.95 ± 0.24^{a3}	6.25 ± 0.25^{a3}	6.67 ± 0.21^{a3}	110	2.09	0.97

Each value represents mean ± S.E.M; n=6; [a], against 2%TW80; [b], against standard (F10); [c], against M200; [d], against M400; [1]: $p < 0.05$, [2]: $p < 0.01$, [3]: $p < 0.001$; M100: 80% methanol extract 100 mg/kg, M200: 80% methanol extract 200 mg/kg, M400: 80% methanol extract 400 mg/kg, F10: Furosemide 10 mg/kg, 2%TW80: 2% Tween 80 in water.

4.2.2 Solvent fractions

The effect of solvent fractions of 80% methanol root extract of *Clutia abyssinica* on urinary output is presented in Table 2. The n-butanol fraction produced diuresis, which showed to be a function of dose and time (r^2 = 0.830; $p<0.001$). BF100 did not produce any detectable difference in urine volume compared to negative control. By contrast, BF200 produced significant diuresis starting from the 1st h of urine collection (65%, $p<0.05$) to the end of the 5th h (81%, $p<0.001$) and maximum diuresis was achieved at the end of the 3rd h (88%, $p<0.001$). BF400 also produced diuresis which was significant starting from the 1st h (72%, $p<0.01$) to the end of the 5th h and maximum increase in urine output was recorded at the end of the 5th h (96%, $p<0.001$). In addition, Table 2 also demonstrated that the percent urinary excretion of BF100 (64%), BF200 (94%) and BF400 (98%) was higher as compared to negative control (52%).

F10 had better diuresis than BF100 ($p<0.001$) from the 2nd h onwards. The effect was also significant compared to BF200, particularly at the last two hours ($p<0.01$). Although F10 was able to produce a slightly higher effect than BF400, the effect did not reach statistical significance. This could be seen from the diuretic activity of BF100, BF200, BF400 and F10 which were 0.56, 0.83, 0.90 and 1.00, respectively (Table 2). The comparative diuretic action of BF100 was 1.20. The middle and higher doses exhibited a diuretic action of 1.81 and 1.96, respectively. When different doses of the n-butanol fraction were compared with each other, BF400 and BF200 produced diuresis which was significant starting from the 1st h ($p<0.05$) to the end of the 5th h ($p<0.001$) as compared to BF100.

The chloroform fraction did not produce any detectable difference in urine volume in its lower and middle doses as compared to negative control. Although CF400 produced significantly low diuresis ($p<0.01$) as compared to the standard drug, it produced a maximal increase in diuresis at the end of the 3rd h (77%, $p<0.01$) relative to negative control. This could be revealed from

the diuretic activity of CF400 and F10, which were 0.72 and 1.00, respectively (Table 2). As shown in Table 2, the percent urinary excretion of chloroform fraction was 63%, 72% and 85% for CF100, CF200 and CF400, respectively. There was no statistically significant difference in diuresis across the different doses of the chloroform fraction except at the end of the first ($p<0.01$), second ($p<0.001$) and third hour ($p<0.05$) in which CF400 produced significant diuresis as compared to CF100.

The aqueous fraction did not produce any detectable difference in urine volume at all doses as compared to negative control. From the comparison of fractions, n-butanol fraction had better diuretic activity in its middle (BF200) and higher (BF400) doses followed by CF400. BF400 produced diuresis that reached significant level starting from the 1^{st} h till the end of the 5^{th} h when compared with CF100 and all doses of the aqueous fraction ($p<0.001$). BF200 produced diuresis that was a bit higher than CF400. CF400 produced significant diuresis starting from the 1^{st} h till the end of the 5^{th} h when compared with BF100 and all doses of the aqueous fraction ($p<0.001$).

Table 2: Effect of solvent fractions of *Clutia abyssinica* root extract on urine volume in rats

Group	Volume of urine (mL)					%Urinary Excretion	Diuretic action	Diuretic activity
	1 h	2 h	3 h	4 h	5 h			
2%TW80	1.32 ± 0.15	1.82 ± 0.13	2.26 ± 0.12	2.82 ± 0.18	3.18 ± 0.22	52	1.00	
F10	2.48 ± 0.17^{a3}	3.80 ± 0.13^{a3}	5.00 ± 0.37^{a3}	6.50 ± 0.29^{a3}	6.92 ± 0.27^{a3}	111	2.17	1.00
BF100	1.21 ± 0.31^{b2,c1,d1}	1.33 ± 0.33^{b3,c3,d3}	2.50 ± 0.22^{b3,c3,d3}	3.17 ± 0.17^{b3,c3,d3}	3.83 ± 0.17^{b3,c3,d3}	64	1.20	0.56
BF200	2.18 ± 0.31^{a1}	3.02 ± 0.36^{a1}	4.26 ± 0.24^{a3}	5.28 ± 0.16^{a3,b2}	5.75 ± 0.17^{a3,b2}	94	1.81	0.83
BF400	2.27 ± 0.15^{a2}	3.42 ± 0.20^{a2}	4.38 ± 0.12^{a3}	5.50 ± 0.22^{a3,b1}	6.23 ± 0.21^{a3}	98	1.96	0.90
CF100	1.24 ± 0.10^{b3,d2}	1.42 ± 0.14^{b3,c1,d3}	2.46 ± 0.43^{b3,d1}	3.20 ± 0.44^{b3}	3.86 ± 0.44^{b3}	63	1.21	0.56
CF200	1.58 ± 0.11^{b2}	2.32 ± 0.19^{b3}	3.24 ± 0.22^{b2}	3.85 ± 0.10^{b3}	4.31 ± 0.17^{b3}	72	1.36	0.63
CF400	2.00 ± 0.17^{a1}	2.75 ± 0.31^{a1,b2}	4.00 ± 0.26^{a2}	4.50 ± 0.43^{a2,b2}	5.00 ± 0.37^{a2,b2}	85	1.57	0.72
DW	1.36 ± 0.18	1.72 ± 0.10	2.50 ± 0.13	3.00 ± 0.17	3.24 ± 0.16	53	1.00	
F10	2.48 ± 0.17^{a3}	3.80 ± 0.13^{a3}	5.00 ± 0.37^{a3}	6.50 ± 0.29^{a3}	6.92 ± 0.27^{a3}	111	2.14	1.00
AF100	1.34 ± 0.13^{b3}	1.68 ± 0.15^{b3,d1}	2.80 ± 0.22^{b3}	3.20 ± 0.19^{b3}	3.40± 0.22^{b3}	55	1.05	0.49
AF200	1.38 ± 0.11^{b3}	2.16 ± 0.18^{b3}	2.96 ± 0.30^{b3}	3.18 ± 0.26^{b3}	3.47 ± 0.25^{b3}	56	1.07	0.50
AF400	1.50 ± 0.15^{b3}	2.46 ± 0.27^{a1,b3}	3.47 ± 0.40^{b1}	3.62 ± 0.36^{b3}	3.89± 0.37^{b3}	64	1.20	0.56

Each value represents mean ± S.E.M; n=6; BF, n-butanol fraction; CF, chloroform fraction; AF, aqueous fraction; [a], against negative control; [b], against standard (F10); [c], against 200 mg/kg; [d], against 400 mg/kg; [1]: $p < 0.05$, [2]: $p < 0.01$, [3]: $p < 0.001$; F10: Furosemide 10 mg/kg, 2%TW80: 2% Tween 80 in water; DW: distilled water.

4.3 Saluretic Activity: Effect on urinary electrolyte excretion

4.3.1 80% Methanol Extract

The urine samples collected over the five hours were analyzed for the electrolyte content (Na^+, K^+, and Cl^-) and presented in Table 3. The 80% methanol extract caused significantly increased sodium loss at all doses. M200 and M400 increased sodium excretion by 82% ($p<0.001$) and 99% ($p<0.001$), respectively, compared to negative control. F10 produced the maximum sodium excretion (118%, $p<0.001$) and this effect was significantly greater than M100 ($p<0.001$) and M200 ($p<0.001$). Enhanced potassium excretion was found by M200 (51%, $p<0.001$) and M400 (70%, $p<0.001$) compared to negative control. These values were, however, significantly lower than produced by F10. A similar pattern emerged with chloride excretion to that of potassium, with a little bit difference. M200 (34%, $p<0.01$) and M400 (43%, $p<0.001$) produced significant elimination of chloride than negative control. This time, however, no apparent differences were observed between the standard and the two doses of the extract. As shown in Table 3, the saluretic indices of the standard drug: for Na^+ (2.18), K^+ (2.13) and Cl^- (1.55), were slightly higher than the indices of M400: 1.99, 1.69 and 1.43 for the three ions, respectively. In addition, the Na^+/K^+ ratios of M100 (1.67), M200 (1.79), and M400 (1.75), were higher than the ratio for the standard drug (1.53). The carbonic anhydrase inhibitory activity of M400 and M200, was 0.62 and 0.64, respectively, (Table 3).

Table 3: Effect of 80% methanol root extract of *Clutia abyssinica* on urinary electrolyte excretion in rats

Group	Urinary Electrolyte Concentration (mMol/L)			Saluretic Index			Na^+/K^+	$Cl^-/[Na^+ + K^+]$
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-		
2%TW80	63.21 ± 4.02	42.42 ± 3.85	85.60 ± 5.02				1.49	0.81
F10	138.00 ± 3.42[a3]	90.42 ± 3.49[a3]	132.50 ± 8.82[a3]	2.18	2.13	1.55	1.53	0.58
M100	88.50 ± 2.79[a3,b3,c3,d3]	53.00 ± 2.07[b3,d2]	107.54 ± 5.30	1.40	1.22	1.26	1.67	0.76
M200	115.00 ± 4.59[a3,b3]	64.00 ± 2.61[a3,b3]	114.56 ± 5.28[a1]	1.82	1.51	1.34	1.79	0.64
M400	126.00 ± 4.19[a3]	72.00 ± 3.76[a3,b2]	122.76 ± 7.20[a3]	1.99	1.69	1.43	1.75	0.62

Each value represents mean ± S.E.M; n=6; [a], against 2%TW80; [b], against standard (F10); [c], against M200; [d], against M400; [1]: $p < 0.05$, [2]: $p < 0.01$, [3]: $p < 0.001$; M100: 80% methanol extract 100 mg/kg, M200: 80% methanol extract 200 mg/kg, M400: 80% methanol extract 400 mg/kg, F10: Furosemide 10 mg/kg, 2%TW80: 2% Tween 80 in water.

4.3.2 Solvent fractions

The measured urinary concentration of Na^+, K^+ and Cl^- of rats treated with different solvent fractions of 80% methanol root extract of *Clutia abyssinica* is presented in Table 4.

All doses of the n-butanol fraction produced a significant increase in Na^+ excretion compared to negative control, being 33% for BF100 ($p<0.01$), 72% for BF200 ($p<0.001$) and 87% for BF400 ($p<0.001$). K^+ excretion was significantly enhanced by BF200 (44%, $p<0.01$) and BF400 (56%, $p<0.001$). However, it was only the higher dose of this fraction that produced a significant increase in Cl^- elimination (36%, $p<0.05$). The n-butanol fraction had a consistently less performance than the standard drug in Na^+ and K^+ excretion at all doses. This pattern was, however, changed with Cl^- excretion, where F10 was able to produce a significant increased elimination only compared to BF100.

Saluretic index of the n-butanol fraction appeared to increase with dose for the ions measured, although the index for each ion was invariably lower than the standard drug (Table 4). Nevertheless, the natriuretic activity of this fraction, as determined from the Na^+/K^+ ratio, was better than F10. The urinary electrolyte excretion of rats treated with the chloroform fraction showed significant increase in Na^+ (61%, $p<0.001$) and K^+ (37%, $p<0.05$) excretion only at the higher dose. This dose, however, did not display any detectable change in Cl^- excretion compared to negative control. The standard drug produced a better elimination of Na^+ and K^+ ions than all doses of the chloroform fraction. There were no detectable changes in the urinary excretion of ions with treatment of the aqueous fraction compared to negative control (Table 4). Carbonic anhydrase inhibitory activity measurement was done for different fractions. As it is determined from the ratio of $Cl^-/[Na^+ + K^+]$, the highest carbonic anhydrase inhibitory activity among the fractions, was observed for the n-butanol fraction and the lowest for the aqueous fraction (Table 4). Once again, the standard had a maximal carbonic anhydrase inhibitory activity (0.58).

Table 4: Effect of solvent fractions of *Clutia abyssinica* root extract on urinary electrolyte excretion in rats

Group	Urinary Electrolyte Concentration (mMol/L)			Saluretic Index			Na^+/K^+	$Cl^-/[Na^+ + K^+]$
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-		
2%TW80	63.21 ± 4.02	42.42 ± 3.85	85.60 ± 5.02				1.49	0.81
F10	138.00 ± 3.42^{a3}	90.42 ± 3.49^{a3}	132.50 ± 8.82^{a3}	2.18	2.13	1.55	1.53	0.58
BF100	84.00 ± 2.54^{a2,b3,c3,d3}	50.00 ± 3.24^{b3,d1}	101.84 ± 6.24^{b1}	1.33	1.18	1.19	1.68	0.76
BF200	109.00 ± 4.11^{a3,b3}	61.00 ± 3.23^{a2,b3}	112.20 ± 6.43	1.72	1.44	1.31	1.79	0.66
BF400	118.00 ± 4.31^{a3,b2}	66.30 ± 3.17^{a3,b3}	116.10 ± 6.06^{a1}	1.87	1.56	1.36	1.78	0.63
CF100	68.89 ± 1.86^{b3,d3}	44.16 ± 1.04^{b3}	90.44 ± 2.56^{b3}	1.09	1.04	1.06	1.56	0.80
CF200	73.32 ± 3.32^{b3,d3}	47.61 ± 2.65^{b3}	94.33 ± 6.57^{b3}	1.16	1.12	1.10	1.54	0.78
CF400	102.00 ± 2.43^{a3,b3,c3}	58.00 ± 2.59^{a1,b3}	108.85 ± 4.98	1.61	1.37	1.27	1.76	0.68
DW	62.18 ± 3.75	41.45 ± 3.01	88.00 ± 5.77				1.50	0.85
F10	138.00 ± 3.42^{a3}	90.42 ± 3.49^{a3}	132.50 ± 8.82^{a3}	2.18	2.13	1.55	1.53	0.58
AF100	64.67 ± 2.05^{b3}	39.67 ± 2.59^{b3}	86.60 ± 2.82^{b3}	1.04	0.94	1.01	1.63	0.83
AF200	68.24 ± 0.77^{b3}	44.60 ± 2.69^{b3}	92.53 ± 2.86^{b3}	1.09	1.05	1.08	1.53	0.82
AF400	67.33 ± 3.15^{b3}	44.29 ± 1.38^{b3}	89.40 ± 2.24^{b3}	1.08	1.04	1.04	1.52	0.80

Each value represents mean ± S.E.M; n=6; BF: n-butanol fraction; CF: chloroform fraction; AF: aqueous fraction; [a], against negative control; [b], against standard (F10); [c], against 200 mg/kg; [d], against 400 mg/kg; [1]: $p < 0.05$, [2]: $p < 0.01$, [3]: $p < 0.001$; F10: Furosemide 10 mg/kg, 2%TW80: 2% Tween 80 in water; DW: distilled water.

4.4 Electrolyte content of the extract

Water soluble salts could be present in the extract and solvent fractions, and consequently interfere with the urinary excretion of electrolytes. The content of Na^+, K^+ and Cl^- in 80% methanol extract and solvent fractions was therefore determined to exclude the possibility of interference. The result revealed that there were no detectable levels of the three electrolytes (Na^+, K^+, Cl^-) at all doses in the extracts as tested by the instruments used in this study.

4.5 Urinary pH

The urinary pH was measured and different treatment groups of the crude 80% methanol extract and solvent fractions had resulted in different urine pH.

4.5.1 80% Methanol Extract

Urinary pH measurement revealed that the 80% methanol root extract produced a relatively alkaline urine, ranging from 7.8 (for M100) to 8.78 (for M400), as shown in Figure 3. M200 and M400 produced a significantly increased pH as compared to negative control ($p<0.001$). The standard drug gave rise to alkaline urine like the 80% methanol extract.

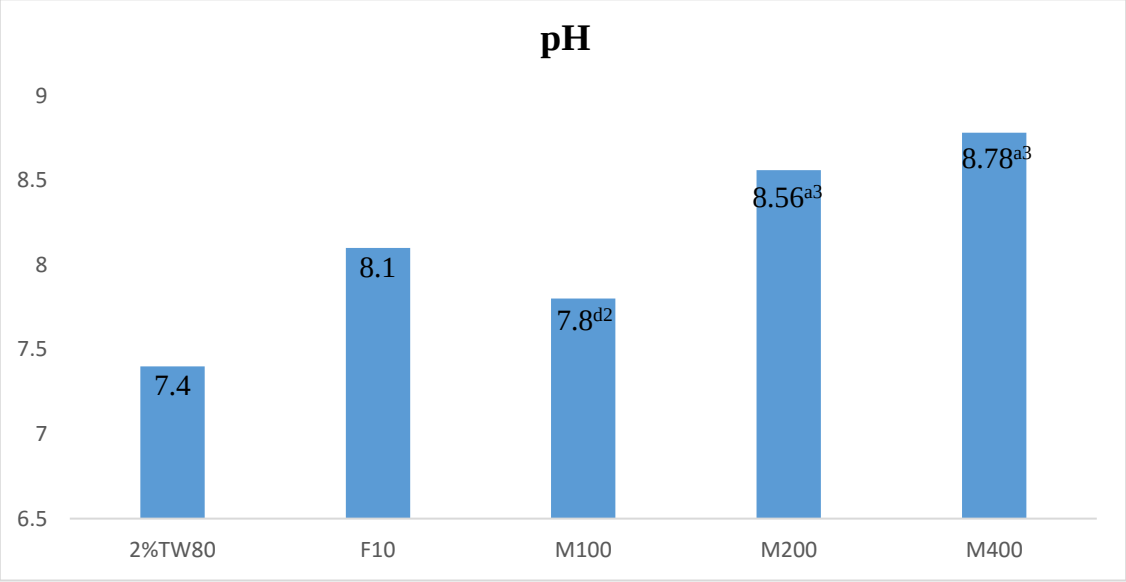

Figure 3. Effect of 80% methanol root extract on urine pH of rats.

Each value represents mean; n=6; [a], against 2%TW80; [b], against standard (F10); [c], against M200; [d], against M400; [1]: $p < 0.05$, [2]: $p < 0.01$, [3]: $p < 0.001$; M100: 80% methanol extract 100 mg/kg, M200: 80% methanol extract 200 mg/kg, M400: 80% methanol extract 400 mg/kg, F10: Furosemide 10 mg/kg, 2%TW80: 2% Tween 80 in water.

4.5.2 Solvent fractions

The effect of solvent fractions on urinary pH is presented in Figure 4. Rats treated with n-butanol fraction produced alkaline urine compared to negative control, which was in the range of 7.8 (BF100) to 8.62 (BF400). BF200 ($p<0.01$) and BF400 ($p<0.001$) produced a significantly higher pH as compared to negative control. CF100 produced the lowest pH and CF400 produced alkaline urine, but the difference between groups found to be insignificant. The aqueous fraction produced significantly low pH at all doses ($p<0.001$) as compared to the middle and higher doses of n-butanol fraction.

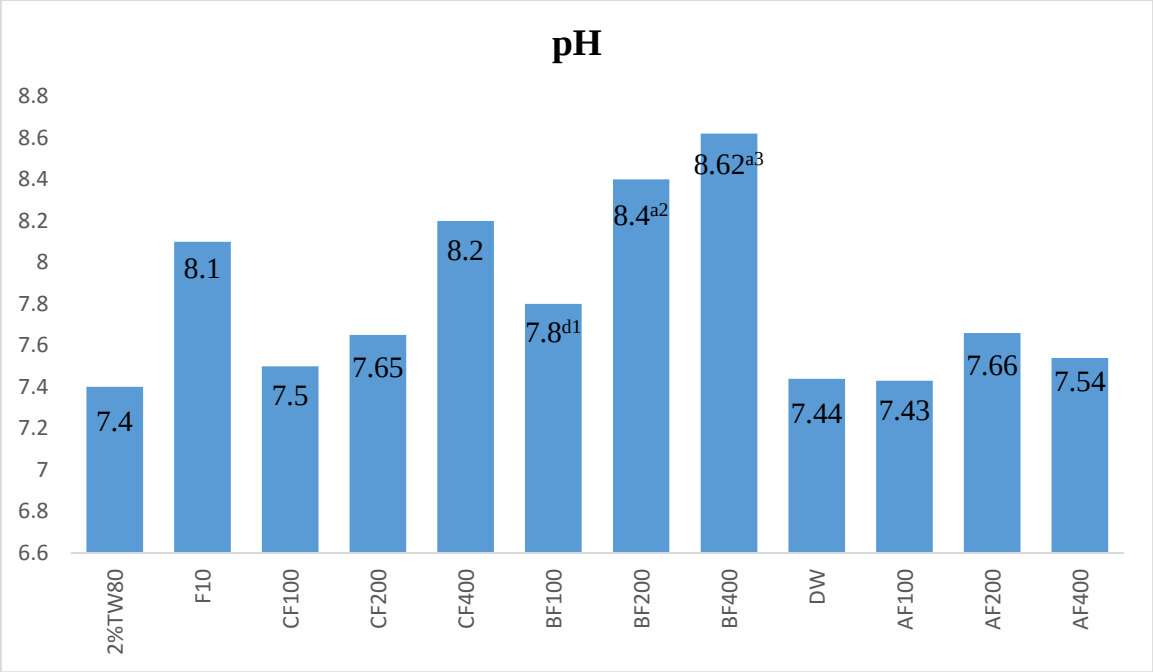

Figure 4. Effect of solvent fractions of 80% methanol root extract on urine pH of rats.

Each value represents mean; n=6; CF, chloroform fraction; BF, n-butanol fraction; AF, aqueous fraction; [a], against negative control; [b], against standard (F10); [c], against 200 mg/kg; [d], against 400 mg/kg; [1]: $p < 0.05$, [2]: $p < 0.01$, [3]: $p < 0.001$; F10: Furosemide 10 mg/kg, 2%TW80: 2% Tween 80 in water; DW: distilled water.

4.6 Phytochemical Screening

The 80% methanol root extract and solvent fractions of *Clutia abyssinica* were tested for the composition of medicinally active compounds. The 80% methanol crude extract and n-butanol fraction were found to be positive for alkaloids, flavonoids, polyphenols, saponins, tannins and terpenoids. Alkaloids, flavonoids, polyphenols, saponins and tannins were detected in chloroform fraction, whereas polyphenols, saponins, tannins and terpenoids were only found in the aqueous fraction (Table-5).

Table 5: Phytochemical screening of 80% methanol root extract and solvent fractions of *Clutia abyssinica*

		Solvent fractions		
Metabolites	80% Methanol extract	Chloroform fraction	n-Butanol fraction	Aqueous fraction
Alkaloids	+	+	+	-
Cardiac glycosides	-	-	-	-
Flavonoids	+	+	+	-
Polyphenols	+	+	+	+
Saponins	+	+	+	+
Steroids	-	-	-	-
Tannins	+	+	+	+
Terpenoids	+	-	+	+

+ = presence, - = absence

5. Discussion

Diuretics are mainly used to adjust water and electrolyte balance in order to decrease fluid volume of the body. Since diuretics increase renal excretion of water and electrolytes, they are often used in the management of pathological conditions such as edema (e.g. in congestive heart failure and certain renal diseases), hypertension, cirrhosis, toxemia and poisoning (Reilly and Jackson, 2011; Snigdha et al., 2013). Several herbal agents are being used as diuretic in order to alleviate edema with minimal adverse effects (Kumar et al., 2010; Dutta et al., 2014). So it would be highly imperative to demonstrate effectiveness of the plant extracts in the presence of electrolytes and water. Thus, saline was administered to simulate edema. This study reports the diuretic and natriuretic effect of different solvent fractions of 80% methanol root extract of *Clutia abyssinica*. The ethno-medicinal application indicates that the dried root of the plant should be drunk daily in the morning by dissolving with local beer (*tella)* (Ragunathan and Abay, 2009). Extraction was performed with 80% methanol to simulate the traditional application.

In view of urine output, 80% methanol root extract showed an increase in diuresis that appeared to vary with dose and time. The 80% methanol extract did not produce visible effect throughout the experiment in its lower dose, but medium and higher doses were able to produce a significant effect throughout the observation time (Table 1). This could probably suggest that the lower dose might be below the minimum effective dose, which cannot elicit diuresis and the other two doses might be large enough to cause significant diuresis. In addition, the diuretic action of 80% methanol extract revealed that M200 and M400 had a value of 1.99 and 2.09, respectively, which are nearly similar to the action of F10 (2.17). The diuretic activity of 80% methanol extract in the two effective doses was a mild type, since their values were 0.92 and 0.97 for M200 and M400, respectively (Table 1). Diuretic activity is considered to be good if

it is more than 1.50, moderate if it is between 1.00-1.50, mild if it is between 0.72-0.99 and nil if it less than 0.72 (Hailu and Engidawork, 2014; Tegegne et al., 2017).

In order to concentrate or separate the active principles, fractionation of the crude 80% methanol root extract was done by using solvents of different polarity. This study showed that the aqueous fraction did not increase urine output significantly at the dose levels employed. This suggests that most of the polar components of the root of the plant might be devoid of any diuretic activity. In contrast, the chloroform fraction produced significant diuresis at 400 mg/kg. This is possibly due to increased concentration of active components in the larger dose, and could indicate that less polar components of the plant might have diuretic activity with increasing concentration. Although BF100 was unable to produce a detectable change in urine volume, BF200 and BF400 produced significant diuresis that increased with dose. This could be surmised from the diuretic activity for the two doses, where BF400 displayed a better activity than B200 (0.9 vs. 0.83). It is also of note that CF400 produced a diuretic effect, which was lower than that of B200 and B400. This collectively suggests that ingredients of the plant responsible for diuretic effect probably are semi-polar and better fractionated by n-butanol than the other solvents used. Moreover, in saline primed rats, BF200 and BF400 caused a significant increase in urine output at the end of the 3rd h of administration as compared to the negative control. In comparison, F10 induced a brisk and significant diuresis within an hour of administration. The delay in the onset of diuresis with the n-butanol fraction may indicate that its diuretic activity is probably mediated via secondary organic metabolites. Interestingly, the diuretic activity of n-butanol fraction was dose-dependent indicating that this effect is intrinsic, genuine and possibly receptor mediated (Al Disi et al., 2016).

Excretion of electrolytes is as important as the excretion of water for treatment of hypertension, peripheral edema and ascites in congestive heart failure (Hock, 2016). The increase in diuresis induced by n-butanol fraction was reflected in a similar manner in urinary ionic excretion. It

significantly increased excretion of urinary electrolytes (Na^+ and Cl^-) in a dose-dependent manner. Although the n-butanol fraction significantly increased urinary excretion of K^+ as compared to negative control, the level was significantly lower than that induced by the standard drug. The ratio of Na^+/K^+ was calculated for aldosterone secretory index (natriuretic activity) and values greater than 2.0 indicate a favourable natriuretic effect, whereas ratios greater than 10.0 indicate a potassium-sparing effect (Hock, 2016). However, the n-butanol fraction did not increase the Na^+/K^+ ratio. This observation suggests that n-butanol fraction is not acting as a potassium-sparing diuretic. Because, potassium-sparing diuretics are usually very weak, have slow onset of action (Ives, 2012) and increase the urinary Na^+/K^+ ratio (Hock, 2016).

Furthermore, n-butanol fraction increased the saluretic index and had a dose-dependent diuresis. Collectively, these observations suggest that n-butanol fraction might act via the mechanism of loop diuretics. Loop diuretics like furosemide increase urinary flow rate and urinary excretion of Na^+, K^+ and Cl^- by inhibiting Na^+-K^+-$2Cl^-$ symporter in the TAL, stimulating production of renal prostaglandins and by inhibiting carbonic anhydrase enzyme in the PCT (Cadwallader et al., 2010; Reilly and Jackson, 2011, Ives, 2012). The larger doses of 80% methanol extract and n-butanol fraction of *Clutia abyssinica* used in the present study produced a similar Na^+ and Cl^- excretion profile to that of the standard. However, there is a difference when K^+ excretion is considered. This could possibly suggest that the mechanism by which the 80% methanol extract and n-butanol fraction produce diuresis is not exactly the same to that of loop diuretics.

In addition, the crude 80% methanol extract and n-butanol fraction exhibited a dose-dependent diuresis, a comparable increase in excretion of Na^+ and Cl^-, in which no increased Na^+/Cl^- ratio (thiazide secretory index) (Jayakody et al., 2011), and altered urinary Na^+/K^+ ratio were observed. These collectively indicate that the crude extract and n-butanol fraction might not

act via thiazide-like mechanism. Thiazide and thiazide-like diuretics the so called low ceiling diuretics have a rapidly flattening dose response curve, and increase urinary flow rate and excretion of electrolytes particularly, Na^+, K^+ and Cl^- by interfering with Na^+-Cl^- co-transporter in the distal tubule and also to some extent by inhibiting carbonic anhydrase enzyme in the PCT (Hullatti et al., 2014).

One of the major adverse effect of loop and thiazide diuretics is hypokalemia, which may require the oral administration of potassium supplements or potassium sparing diuretics that reduce urinary K^+ excretion (Ives, 2012; Wile, 2012; Snigdha et al., 2013; Smith, 2014)). According to the findings of this study, the 80% methanol extract and its n-butanol fraction have K^+ saving effect in comparison to the excretion of other electrolytes. So, this is one advantage of the plant extract over the conventional agents. Since the 80% methanol extract and its n-butanol fraction induced both water and electrolyte excretion, it is possible that they exerted diuretic effect by inhibiting tubular reabsorption of water and electrolytes as such action has been suggested for some other plants (Prabhu et al., 2014). The potassium content of the crude 80% methanol extract and its solvent fractions was undetectable for all doses. Therefore, the possibility of direct action of potassium content of the plant extract on diuretic effect could be excluded.

The ratio of $Cl^-/[Na^+ + K^+]$ is calculated to estimate carbonic anhydrase inhibition. Carbonic anhydrase inhibition can be excluded in the ratios between 1.0 and 0.8. With decreasing ratios, slight to strong carbonic anhydrase inhibition can be assumed (Hock, 2016). The 80% methanol crude extract had carbonic anhydrase indices of 0.64 and 0.62, in the doses of M200 and M400, respectively. Similarly, n-butanol fraction had carbonic anhydrase indices of 0.66 and 0.63, in the doses of BF200 and BF400, respectively (Table 4). Thus, this study indicates that the 80% methanol extract and its n-butanol fraction might have inhibitory action on carbonic anhydrase

enzyme in renal tubules. This may be evidenced from the notion that a significantly increased urinary pH was also observed in the 80% methanol extract and n-butanol fraction treated rats. The active principle(s) responsible for the diuretic effect of the 80% methanol extract and solvent fractions of *Clutia abyssinica* is/are, so far, not known, so it is not identified which compounds are exactly responsible for the diuretic and natriuretic activities. Previous studies showed that alkaloids and flavonoids were found to have diuretic activity (Amirkia and Heinrich, 2014). These plant constituents might elicit their effect by stimulating regional blood flow or initial vasodilation, by producing inhibition of tubular reabsorption of water and electrolytes, or by increasing renal circulation and thus the rate of glomerular filtration eventually culminating in diuresis (Melendez-Camargo et al., 2014).

Preliminary phytochemical analysis done on the 80% methanol crude extract and solvent fractions revealed a variety of secondary metabolites that appeared to be differentially distributed across the extract and fractions (Table 5). It is reasonable to suggest that the phytochemicals shown in Table 5 may act individually or synergistically to produce the diuretic activity of *Clutia abyssinica.* It is possible that flavonoids and alkaloids presents in the 80% methanol root extract and in the two solvent fractions exerted diuretic effect by inhibiting tubular reabsorption of water and electrolytes as such action has been suggested for some other plants (Hullatti et al., 2011; Melendez-Camargo et al., 2014). Flavonoids exhibit diuretic effect associated with potassium-saving activity. They increase diuresis mainly due to angiotensin converting enzyme (ACE) inhibition (Ghosh and Scheepens, 2009; Perez-Vizcaino et al., 2009), increased bioavailability of bradykinin, prostacyclin, and nitric oxide, or exerting inhibitory effect on Na^+/K^+-ATPase (Perez-Vizcaino et al., 2009; Junior et al., 2012). Adenosine A1 receptor antagonists can induce diuresis and Na^+ excretion by direct inhibition of Na^+ re-absorption in proximal tubules, or indirectly by promoting afferent arteriole dilation (Chen et al., 2013; Welch, 2015). Flavonoids were reported to be one of the natural antagonist

ligands for adenosine A1 receptor while antagonistic activity to the receptor is known to associate with diuretic activity (Yuliana et al., 2013). The n-butanol fraction induced a significant increase in diuresis and natriuresis. It increased the urinary output and electrolyte excretion (Na^+, K^+ and Cl^-) as compared to negative control. The results of this study raise the possibility of existence of better diuretic activity in n-butanol fraction by inhibiting tubular reabsorption of water and electrolytes compared to all doses of aqueous and lower and middle doses of chloroform fractions. This might be due to increased concentration of phytochemicals responsible for diuresis in the n-butanol and larger dose of chloroform fractions.

To sum up, this study provided further evidence that the 80% methanol extract as well as the n-butanol fraction possessed a comparable diuretic activity with that of the standard drug. Results obtained from the solvent fractions revealed that there was an increase in volume of urine output in the n-butanol fraction. Therefore, this data seems to indicate that the diuretic effect of the plant is distributed to semi-polar bioactive principles contained in the n-butanol fraction. The wide range of diuretic mechanism is suggested with some herbal medications (Hullatti et al., 20011; Amirkia and Heinrich, 2014). Hence, adding up to the anticipated carbonic anhydrase inhibitory effect, there must be another mode of action that contribute to the highest diuretic effect of the plant.

6. Conclusion

This study provides evidence for the use of *Clutia abyssinica* as a diuretic agent through enhancement of salt and water excretion. Although the active components which are responsible for the observed effect remains to be seen, moderately polar components individually or in synergy act by multiple mechanisms to produce the observed effect. The larger doses of both the crude 80% methanol root extract and n-butanol fraction produced a remarkable diuresis, which was comparable to Furosemide. From the data of electrolyte analysis and urinary pH, it is plausible to assume that the plant could have multiple modes of action.

7. Recommendation

- ✓ Investigation on the specific compound responsible for diuresis should be performed.
- ✓ *In vitro* studies of the fractions on isolated specimen should be done to support the *in vivo* methods
- ✓ The precise site(s) of action, the molecular and cellular mechanism(s) of action of compounds present in the plant *Clutia abyssinica* remain to be elucidated in further studies.
- ✓ Further toxicological studies such as sub-acute, sub-chronic and chronic toxicities should be done to assess the long-term safety profile of the extracts and fractions.

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