



ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Evaluation of the diuretic activity of the aqueous and 80% methanolic extracts of the leaves of *Ajuga remota* B. (Lamiaceae) in mice

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment for the requirement of the Master of Science degree in Experimental Pharmacology.

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Table of Contents

LIST OF ACCRONYMS	i
LIST OF TABLES	iii
LIST OF FIGURES	iv
ACKNOWLEDGEMENTS	v
ABSTRACT	vi
1. INTRODUCTION	1
1.1. Definition and History of Diuretics	1
1.2. Renal Anatomy and Physiology	2
1.3. Mechanisms and sites of action of diuretics	5
1.4. Therapeutic Importance of Diuretics	6
1.5. Adverse effects	7
1.6. Diuretic Resistance	8
1.7. Novel Diuretics	8
1.7.1. Adenosine A1 receptor antagonists	8
1.7.2. The Vaptans	9
1.8. Botanical Diuretics	10
1.9. <i>Ajuga remota</i> Benth. (Lamiaceae)	10
1.9.1. The Genus <i>Ajuga</i>	10
1.9.2. The use of <i>Ajuga</i> plants in traditional medicine	11
1.9.3. Compounds isolated from plants of the genus <i>Ajuga</i>	11
2. OBJECTIVE.....	13
2.1. General Objective	13
2.2. Specific Objectives	13
3. MATERIALS AND METHODS	14
3.1. Drug and Chemicals	14
3.2. Experimental Animals.....	14
3.3. Collection of the Plant	14
3.4. Extraction of the Plant	14
3.4.1. Aqueous Extraction	15
3.4.2. Hydroalcoholic Extraction.....	15

3.5. Acute Toxicity Test.....	15
3.6. Phytochemical Screening.....	15
3.7. Grouping and Dosing of animals	17
3.8. Diuretic Activity	18
3.9. Analytical Procedures	19
3.10. Statistical Analysis	19
4. RESULTS.....	20
4.1. Acute Toxicity Study	20
4.2. Phytochemical Screening.....	20
4.3. Diuretic Activity: Effect on Urine Volume.....	20
4.3.1. Aqueous Extract.....	20
4.3.2. Hydroalcoholic Extract.....	24
4.4. Saluretic Activity: Effect on electrolyte content of the urine	26
4.4.1. Aqueous Extract.....	26
4.4.2. Hydroalcoholic Extract.....	28
4.5. Urinary pH.....	30
4.5.1. Aqueous Extract.....	30
4.5.2. Hydroalcoholic Extract.....	31
4.6. Electrolyte Content of the Extracts	31
5. DISCUSSION.....	32
6. CONCLUSIONS	38
7. RECOMMENDATIONS	39
8. REFERENCES	40

LIST OF ACCRONYMS

ACE	Angiotensin Converting Enzyme
ADH	Anti Diuretic Hormone
ADHF	Acute Decompensated Heart Failure
AQP1	Aquaporin 1
BMD	Bone Mineral Density
CA	Carbonic Anhydrase
CAI	Carbonic Anhydrase Inhibitors
CFEX	Chloride Formate Exchanger
CHD	Coronary Heart Disease
CHF	Congestive Heart Failure
DCT	Distal Convoluted Tubule
DHF	Decompensated Heart Failure
DW	Distilled Water
EHNRI	Ethiopian Health Nutrition Research Institute
EPFSA	Ethiopian Pharmaceutical and Fund Supply Agency
FR10	10 mg/kg of Furosemide
GFR	Glomerular Filtration Rate
NHE3	Sodium Hydrogen Exchanger 3
HF	Heart Failure
IV	Intravenous
PCWP	Pulmonary Capillary Wedge Pressure

PCT	Proximal Convoluted Tubule
PGs	Prostaglandins
RBF	Renal Blood Flow
TAL	Thick Ascending Limb
V1R	Vasopressin Receptor 1
V2R	Vasopressin Receptor 2

LIST OF TABLES

Table 1. Effect of aqueous extract of the leaves of <i>A. remota</i> on urine volume in mice.....	28
Table 2. Effect of hydroalcoholic extract of the leaves of <i>A. remota</i> on Urine volume in mice.....	30
Table 3. Effect of aqueous extract of the leaves of <i>A. remota</i> on 5 h urinary electrolyte excretion in mice.....	32
Table 4. Effect of hydroalcoholic extract of the leaves of <i>A. remota</i> on 5 h urinary electrolyte excretion in mice.....	34

LIST OF FIGURES

Fig 1. Diagram showing the anatomy of kidney and nephron.....	4
Fig 2. Tubule transport systems and main sites of action of diuretics.....	7
Fig 3. Photograph of the plant <i>A. remota</i>	17
Fig 4. Time course of diuresis in mice treated with different doses of aqueous <i>A. remota</i> extract and furosemide	27
Fig 5. Effect of the aqueous extract of the leaves of <i>A. remota</i> on urinary pH.....	35
Fig 6. Effect of hydroalcoholic extract of the leaves <i>A. remota</i> on urinary pH	36

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ABSTRACT

In the Ethiopian traditional medicine the leaves of *Ajuga remota* (Local name 'Armagusa') is used as diuretic agent. Since the diuretic activity of this plant had not been investigated in scientifically controlled studies, the aim of the present study was to evaluate the diuretic potential of the aqueous and hydroalcoholic extracts of the leaves of *A. remota* in mice after acute oral administration.

Adult mice were administered orally either aqueous (250 mg/kg, AA250; 500 mg/kg, AA500 and 1000 mg/kg, AA1000) or 80% methanolic (250 mg/kg, AM250; 500 mg/kg, AM500 and 750 mg/kg, AM750) extract and urine output and electrolyte contents were quantitated up to 5 h and compared with those administered with furosemide 10 mg/kg (F10) and distilled water (CON).

The hydroalcoholic extract increased diuresis significantly ($p < 0.01$) only at the maximum dose, while the aqueous extract increased diuresis at moderate (AA500, $p < 0.01$) and higher (AA1000, $p < 0.01$) doses by the end of the fifth hour compared to CON. Results on electrolyte excretion showed both the aqueous and hydroalcoholic extracts had an increased natriuresis at the maximum doses ($p < 0.001$ for AA1000 and $p < 0.01$ for AM1000), while the kaliuresis effects were smaller at those doses when compared with standard, thus the plant had a beneficial potassium saving effect at these doses. Phytochemical screening of both extracts revealed the presence of secondary metabolites like phenolic compounds, tannins, saponins, flavonoids, terpenoids, steroids, and cardiac glycosides which might account for the diuretic activity. The plant was also found to be safer at a maximum dose of 5000 mg/kg for both extracts.

In conclusion the results indicated significant diuretic activity at different doses and thus provide evidence for its folkloric use. And the different components like flavonoids are thought to be responsible for the evidenced diuretic activity.

Key Words: Furosemide, Diuretics, *A. remota*, Natriuresis, Kaliuresis.

1. INTRODUCTION

1.1. Definition and History of Diuretics

The term 'diuresis' signifies an increase in urine volume while 'natriuresis' refers to an increase in renal sodium excretion. And the important natriuretic drugs usually also result in water excretion, they are usually called diuretics, and the increase in sodium excretion is assumed (Ives and Warnock, 2004). Drug-induced diuresis is beneficial in many life-threatening disease conditions such as congestive heart failure (CHF), nephritic syndrome, cirrhosis, renal failure, hypertension, and pregnancy toxemia (Camarago *et al.*, 2004). Naturally occurring diuretics include caffeine in coffee, tea, and cola, which inhibit Na⁺ reabsorption and alcohol in beer, wine and mixed drinks, which inhibit secretion of anti diuretic hormone (ADH) (Koti and Purnima, 2008).

The term diuretic derived from the Greek diouretikos, meaning "to promote urine." Although infusion of saline or ingestion of water would therefore qualify as being diuretic, the term diuretic usually connotes a drug that can reduce the extracellular fluid volume by increasing urinary solute or water excretion. The term aquaretic has been applied to drugs that increase excretion of solute free water, distinguishing these drugs from traditional diuretics, which enhanced solute with water excretion (Okusa and Ellison, 2008).

In 1919, the ability of organic mercurial antisyphilitics to affect diuresis was discovered by Vogl, the then medical student. This observation led to the development of effective organic mercurial diuretics, drugs that were used commonly until the 1960s (Eknoyan *et al.*, 1975). In 1937, the antimicrobial, sulfanilamide, was found to cause metabolic acidosis in patients. Carbonic anhydrase (CA) had been discovered in 1932; it was known that sulfanilamide inhibited this enzyme. Soon, more potent sulfonamide based carbonic anhydrase inhibitors (CAIs) were developed, but these drugs suffered from side effects and limited potency (Eknoyan *et al.*, 1975).

Further developments to explore the possibility that modification of sulfonamide-based drugs could lead to drugs that enhanced sodium chloride rather than sodium bicarbonate excretion resulted in studies of metadisulphonamides, and gave rise eventually to the first modern, orally active diuretic chlorothiazide in 1957, and by early 1960s its

congeners (thiazide diuretics). This group of drugs ushered in the modern era of diuretic therapy and revolutionized the clinical treatment of edema. The search for more potent classes of diuretics continued, based on the structure of chlorothiazide and sulfonamyl derivatives. This led to the development of ethacrynic acid and furosemide (Jackson and Chabbers, 2001).

Although all these compounds proved to be very effective in promoting sodium excretion, they all caused potassium loss, and this prompted the search for potassium sparing diuretics. Aldosterone antagonists such as spironolactone, introduced in 1962, partially satisfied this requirement, but they had several drawbacks. Numerous compounds were screened and eventually amiloride and triameterene emerged (Rang *et al.*, 1999).

1.2. Renal Anatomy and Physiology

Mammalian kidneys are paired. They are located posteriorly near the lower ribs. When viewed anteriorly, the right kidney is situated behind the liver and the left kidney is below the spleen. Each kidney normally receives its blood supply from a single renal artery and renal vein. The urine formed in each kidney is drained via a single ureter into the bladder. The kidney is subdivided into cortex, outer medulla, and inner medulla or papilla (Fig1). The papilla is the pyramid shaped distal portion of the inner medulla, which extends into the renal pelvis. In all mammals, urine exits from the tip of the papilla (e) via the ducts of Bellini into the renal pelvis, which is an expanded upward extension of the ureter (Sands and Verlander, 2010).

The basic urine-forming unit of the kidney is the nephron, which consists of a filtering apparatus, the glomerulus, connected to a long tubular portion that reabsorbs and conditions the glomerular ultrafiltrate. Each human kidney is composed of approximately one million nephrons (Jackson, 2006).

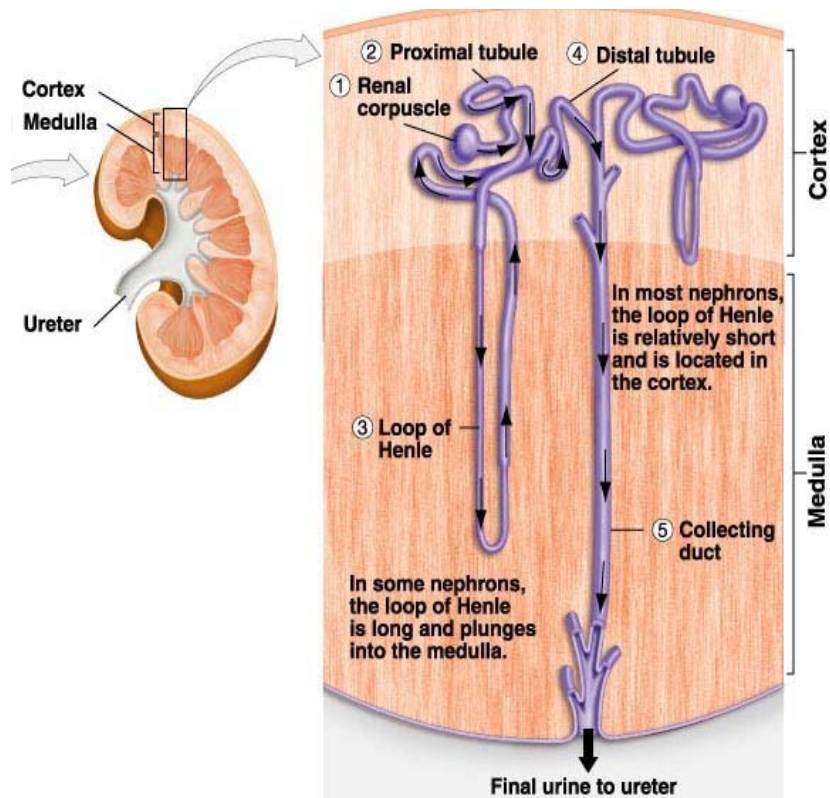


Fig 1. Diagram showing the anatomy of kidney and nephron

The glomerulus is the filtering unit of the kidney, and each glomerulus consists of several capillary loops. Blood enters the glomerulus through the afferent arterioles and exits via the efferent arterioles; the glomerular capillaries are located between these two arteriolar systems (Tisher and Brenner 1989). The portion of plasma that crosses the glomerular capillary wall and enters bowman's space is an ultrafiltrate composed of water, solute, and other small molecules. The glomerular capillary wall forms both a size and charge barrier, and is generally less permeable to negatively charged molecules than neutral or positively charged molecules of the same size. Thus, the normal glomerular ultrafiltration barrier blocks the passage of cells and large anionic proteins. In disease states, both the size barrier and charge barrier are compromised and result in the appearance of proteins and cells in the ultrafiltrate and urine (Valtin 1973; Maddox and Brenner 1991).

The proximal convoluted tubule (PCT) is responsible for reabsorbing 50–60% of the glomerular ultrafiltrate. Thus, it is a site for high volume reabsorption, but not for regulation of the final composition of the urine (Sands and Verlander, 2010). The PCT reabsorbs solute isosmotically. Water is reabsorbed through the aquaporin-1 (AQP1)

water channel (Preston *et al.*, 1992; Nielsen *et al.*, 1993) located in both the apical and basolateral plasma membranes. Water reabsorption is driven by reabsorption of sodium chloride and sodium bicarbonate via a variety of transcellular and paracellular mechanisms, including sodium–proton antiporters (NHE3), sodium–bicarbonate cotransporters (NBC), and a chloride–formate antiporter (CFEX). Under normal conditions, most of the filtered bicarbonate is reabsorbed (Sands and Verlander. 2010).

As the tubule dives into the medulla, middle zone of the kidney, the tubule becomes narrower and forms a loop (Loop of Henle) that reenters the cortex as the thick ascending limb (TAL) that travels back to near the glomerulus. Because the interstitium of the medulla is very hyperosmotic and the Loop of Henle is permeable to water, water is reabsorbed from the Loop of Henle and into the medullary interstitium (Jackson, 2006). This loss of water concentrates the urine within the Loop of Henle. The TAL, which is impermeable to water, has a cotransport system ($\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransporter) that reabsorbs sodium, potassium and chloride at a ratio of 1:1:2. Approximately 25% of the sodium load of the original filtrate is reabsorbed at the TAL. From the TAL, the urine flows into the distal convoluting tubule (DCT), which is another site of sodium transport (~5% via Na^+/Cl^- cotransporter) into the cortical interstitium (the DCT is also impermeable to water) (Mekonnen *et al.*, 2006).

At last, the tubule goes back into the medulla as the collecting duct and then into the renal pelvis where it joins with other collecting ducts to exit the kidney as the ureter. The distal segment of the DCT and the upper collecting duct has a transporter that reabsorbs sodium (about 1-2% of filtered load) in exchange for potassium and hydrogen ion, which are excreted into the urine. It is important to note two things about this transporter. First, its activity is dependent on the tubular concentration of sodium, so that when sodium is high, more sodium is reabsorbed and more potassium and hydrogen ion are excreted. Second, this transporter is regulated by aldosterone, which is a mineralocorticoid hormone secreted by the adrenal cortex. Increased aldosterone stimulates the reabsorption of sodium, which also increases the loss of potassium and hydrogen ion into the urine (Harlan, 2007). Water is reabsorbed in the collecting duct through special pores that are regulated by ADH, which is released by the posterior pituitary. ADH increases the permeability of the collecting duct to water, which leads to increased water reabsorption, a more concentrated urine and reduced urine outflow.

Nearly all of the sodium originally filtered is reabsorbed by the kidney, so that less than 1% of originally filtered sodium remains in the final urine (Mekonnen *et al.*, 2010).

1.3. Mechanisms and sites of action of diuretics

Diuretics act on multiple sites of action, thus their mechanism of action is dependent on these sites. Thus diuretics are classified based on either their site of action or mechanism of action as shown in the diagram below (Fig 2).

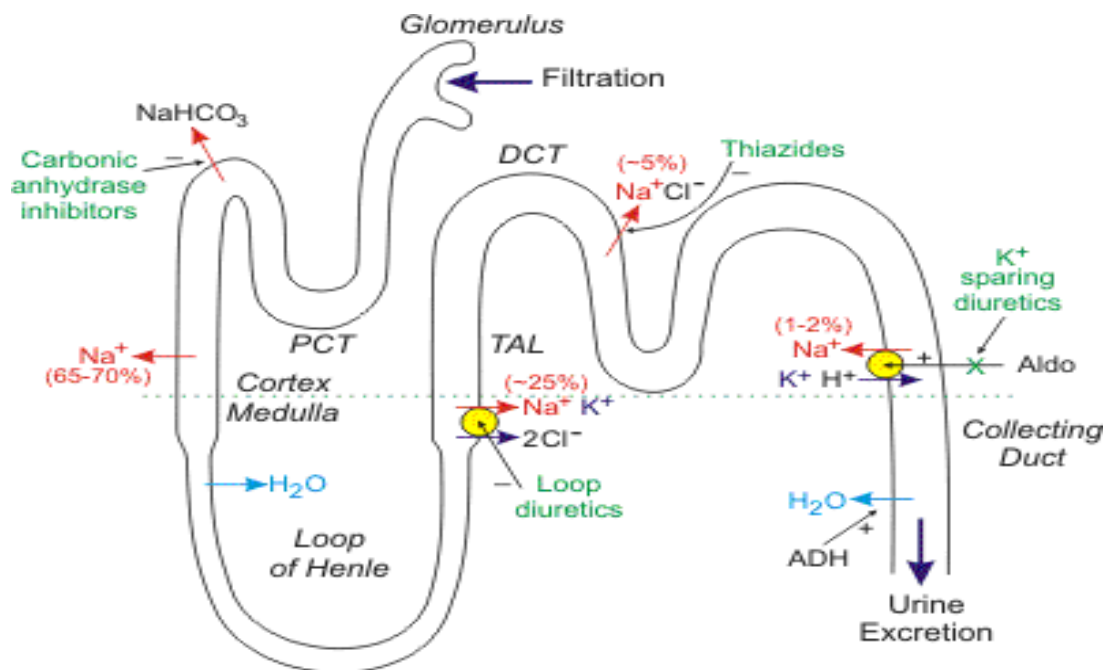


Fig 2. Tubule transport systems and main sites of action of diuretics (Richard, 2007)

Although osmotic agents do not act directly on transport pathways, the rate of transport of ions is affected. The most pronounced effect observed with these diuretics is a brisk diuresis and natriuresis. The mechanisms by which they produces diuresis are thought to be secondary to (i) an increase in osmotic pressure in the proximal tubule fluid and Loop of Henle thereby retarding the passive reabsorption of water and (ii) an increase in renal blood flow and washout of the medullary tonicity (Mark and Ellison, 2008).

CAIs (e.g. acetazolamide, dichlorphenamide, methazolamide) inhibit the transport of bicarbonate out of the PCT into the interstitium by inhibiting the CA enzyme, which leads to less sodium reabsorption at this site and therefore greater sodium, bicarbonate

and water loss in the urine. CAIs are the weakest of the diuretics and seldom used in cardiovascular disease. Their main use is in the treatment of glaucoma (Mekonnen *et al.*, 2010).

Loop or high ceiling diuretics such as furosemide, ethacrynic acid, and bumetanide, act mainly on the medullary and TAL to block $\text{Na}^+/\text{K}^+/\text{Cl}^-$ and cause a pick diuresis far greater than which occurs with other diuretics (Tripathi, 2003). The site of action of thiazide diuretics is in the distal convoluted tubule. These drugs inhibit an electroneutral Na^+/Cl^- cotransporter located on the luminal surface. There is no direct effect of the thiazides on K^+ transport in this segment. Rather, these agents are associated with increased renal K^+ excretion through their effects to increase distal Na^+ delivery in the setting of increased mineralocorticoid activity (Palmer and Naderi, 2007).

The site of action of potassium-sparing diuretics is the distal tubule. This segment is a heterogeneous structure composed of at least four cell types: DCT cells, connecting tubule cells, intercalated cells, and principal cells. The collecting duct is the final site of sodium chloride reabsorption where almost 3% of the filtered load is reabsorbed. In addition to actions on the cortical collecting duct, these diuretics inhibit Na^+ and K^+ transport by the connecting tubule, which is an important site of aldosterone stimulated Na^+ absorption and K^+ secretion (Bachmann *et al.*, 1999).

1.4. Therapeutic Importance of Diuretics

According to recent estimates by the World Health Organization (WHO), approximately one-third of all deaths (16.7 million people) around the globe results from cardiovascular diseases (WHO, 2003), which constitute the major cause of death worldwide. Conditions such as hypertension lead to other types of diseases, such as stroke and kidney and heart diseases, and hence need to be treated (Gasparotto *et al.*, 2009).

Amongst these cardiovascular diseases, CHF is a major cause of morbidity and mortality worldwide (Faris *et al* 2002). Diuretic therapy is an essential part of heart failure (HF) management in patients with fluid retention. The primary indication for diuretic use in HF is to alleviate the signs and symptoms of pulmonary or systemic venous congestion rather than altering disease progression. The use of diuretics in HF

has been associated with activation of the renin-angiotensin-aldosterone and sympathetic nervous systems (Mielniczuk *et al.*, 2008).

Decompensated heart failure (DHF) is also caused by excessive fluid retention or fluid in the wrong place, so it is one of the targets of diuretics therapy. Many patients with DHF present with dyspnea secondary to pulmonary edema. This edema can be managed by reducing volume load using diuretics; intravenous (IV) diuretics are the most commonly employed therapy (John *et al.*, 2006). Practice guidelines recommend that acute decompensated heart failure (ADHF) patients with evidence of volume overload be treated with escalating doses of diuretics, although it is recognized that such therapy can be associated with worsening renal function (Givertz *et al.*, 2007).

Hypertension (systolic/diastolic blood pressure greater than 140/90 mm Hg) is associated with increased morbidity with respect to coronary heart disease (CHD), CHF, left ventricular hypertrophy, nephropathy, retinopathy, central nervous system changes, vascular disorders, and stroke (Kannel, 1996). Diuretics have been used in the management of hypertension for approximately four decades. They have demonstrated blood pressure-lowering efficacy and a proven ability to prevent strokes, myocardial infarction, and CHF (Matthew and Moser, 2000).

1.5. Adverse effects

Diuretics make up one of the most common causes of hyponatremia, with an estimated incidence of 11% in 1 series of 114 geriatric patients (Liamis *et al.*, 2008). Diuretic-induced hyponatremia is caused almost exclusively by thiazide or thiazide-like agents. Loop diuretics, by inhibiting sodium chloride reabsorption in the TAL of the Loop of Henle, reduce the osmolarity of the medullary interstitium. Consequently, loop diuretics rarely are associated with hyponatremia because they impair both the renal concentrating and diluting mechanisms (Liamis *et al.*, 2008).

All aldosterone-receptor blockers act as facultative natriuretics and potassium and hydrogen retaining diuretics. Consequently, they may cause hyperkalemia, hyponatremia, or metabolic acidosis at the doses that are therapeutically effective in hypertension, HF, hepatic edema, and other conditions, depending upon the patient's biological characteristics, renal function, disease, sodium intake, and therapeutic

regimen. Hyperkalemia and hyponatremia have ominous prognoses if allowed to progress (Ariel *et al.*, 2005).

1.6. Diuretic Resistance

In some initially ill patients, conventional doses of diuretics do not always result in optimal diuresis. In such cases, patients are considered “diuretic resistant.” Three mechanisms of diuretic resistance have been suggested. The most common and the first mechanism is the concept of rebound sodium retention. For example, 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 (Asare, 2009). The second mechanism is post-diuretic effect, a compensatory sodium-retention process that begins as the diuretic action wanes. The body has compensated by absorbing more sodium, partially nullifying the effect of the drug (Asare, 2009).

The third 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. For example, the diuretic response of furosemide reportedly falls by as much as 40% by the third day of treatment, depending on the degree of volume depletion (Wilcox *et al.*, 1983).

1.7. Novel Diuretics

1.7.1. Adenosine A1 receptor antagonists

Adenosine is an important modulator of renal physiology that acts via adenosine A1 receptors to mediate glomerular afferent arteriole constriction, reabsorption of sodium in the proximal tubule, and tubuloglomerular feedback (Vallon *et al.*, 2006). Hence in the kidney, adenosine functions are mediated through adenosine A1 and A2 receptors, which are coupled to Gi and Gs, respectively. Adenosine A1 receptors are also localized in the proximal tubules and A1 receptor mRNA has been identified in other nephron segments. Studies in which selective adenosine A1 receptor antagonists have been infused systemically or directly into the kidney have shown immediate diuresis (water excretion) and natriuresis (sodium excretion). This is consistent with A1 receptors mediating sodium transport in the proximal tubule. Therefore, adenosine A1

receptors are located in two key tissues in the kidney relevant to fluid balance control: the afferent arteriole and the proximal tubule (Welch, 2002).

The primary pharmacologic rationale for use of adenosine A1 receptor antagonists in acute HF is based on the hypothesis that inhibition of these receptors will increase renal blood flow (via inhibition of adenosine A1 receptor-mediated renal vasoconstriction of the afferent glomerular arteriole) and enhance diuresis without triggering tubuloglomerular feedback. Proof of concept for this mechanism has been demonstrated in preclinical pharmacology studies and clinical studies of at least 2 adenosine A1 receptor antagonists, BG9719 (Gottlieb *et al.*, 2002) and rolofylline (KW- 3902, MK-7418) (Cotter *et al.*, 2008). The synthetic adenosine A1 receptor antagonist rolofylline is a xanthine derivative with higher selectivity for A1 than for A2 adenosine receptors (approximately 160:1). In Phase II studies, rolofylline enhanced diuresis in patients with acute HF and significantly increased glomerular filtration rate (GFR) and renal plasma flow in patients with HF (Givertz *et al.*, 2007).

1.7.2. The Vaptans

Similar to other neurohormones that are activated in CHF, circulating ADH is elevated in patients with CHF. The precise role of ADH in the pathophysiology of cardiovascular disease is controversial. ADH acts via three receptor types: V1a, V1b (V3), and V2. ADH regulates various physiological processes including vascular tone regulation, cardiovascular contractility and body fluid regulation through activation of V1a and V2 receptors, respectively (Lee *et al.*, 2003).

The recent development of nonpeptide orally active ADH-receptor antagonists has allowed reevaluation of the precise role of ADH in experimental animal models of hypertension and HF (Punniyakoti *et al.*, 2008). Tolvaptan is a modified benzazepine derivative that was selected as a potent human V2-receptor (V2R) antagonist through a series of structural conversions of mozavaptan. Tolvaptan exerts an aquaretic effect by blocking the V2 receptors at the renal collecting ducts and thereby inhibiting water reabsorption. ADH binding studies of this agent reported a 29:1 (V2:V1a) receptor selectivity in cloned human ADH receptors (Yamamura *et al.*, 1998).

1.8. Botanical Diuretics

There is growing interest in the health benefits of herbs and botanicals (Foote and Cohen, 1998). In line with this, there are an increasing number of published articles claiming that plants or plant-derived active principles may function as mild diuretic agents. A large majority of this research has determined the degree of clinical support for the traditional use of common or folklore medicines. Such evidence is needed in order to determine whether there is any scientific basis for their use (Wright *et al.*, 1998). Many investigators demonstrated that studies of herbal plant used in traditional medicine as diuretics were in progressive elevation in the last decades, and might be a precious tool used in human pathology treatment (Jouad *et al.*, 2001).

There are several plant species and genera reported to possess diuretic effects. Some of the promising plants includes *Spilanthes acmella* (Ratnasooriya *et al.*, 2004), *Rungia repens* (Basu and Arivukkarasu, 2006), *Petroselinum sativum* and *Spergularia purpurea* (Jouad *et al.*, 2001), *Withania alata* (Marti-Herrera *et al.*, 2007), *Smilax canariensis* (Abdala *et al.*, 2008), *Hibiscus sabdariffa* (Odigie *et al.*, 2003), *P. sellowianus* (Hnatyszyn *et al.*, 1999), *Sambucus mexicana* (Caceres *et al.*, 1987) and *S. nigra* (Beaux *et al.*, 1999).

1.9. *Ajuga remota* Benth. (Lamiaceae)

A. remota is an erect rhizomatous pubescent herb found growing in the grasslands of Kenya and other parts of East Africa. The herb is not eaten by animals, birds or insects. This is probably due to the very bitter taste of almost all its parts (Fig 3).

1.9.1. The Genus *Ajuga*

The plants of genus *Ajuga* are evergreen, clump-forming rhizomatous annual or perennial herbaceous flowering species in the mint family, Lamiaceae, with most plants native to Europe, Asia, and Africa, but also growing in Australia and North America. There are at least 301 species of the genus *Ajuga* with many variations; *ajuga* is one of the 266 genera of the family Lamiaceae. The *Ajuga* plants grow to 5-50 cm tall, with opposite leaves, which are attractive. The flowers are two lipped and tubular, and mostly blue, purple or yellow in color. Many *Ajuga* plants are used in horticulture as groundcover or border, and in rock gardens, but some are regarded as weeds. Some *ajuga* species have a large number of varieties, which are used in gardens because of

their varied blooms of different colors. Many plants of the *Ajuga* genus and some compounds isolated from these plants have medicinal value and of ecological and economic importance (Israili and Lyoussi, 2009).

1.9.2. The use of *Ajuga* plants in traditional medicine

Ethnopharmacological surveys have revealed that some 20 species of *Ajuga* plants are used in traditional medicine mostly in Africa, Asia and China. The pharmacology and therapeutic value of plants including *Ajuga reptans* has been described as early as 1948 (Israili and Lyoussi, 2009).

In East Africa, plants of the genus *Ajuga* have been used as a remedy for fever, toothache, dysentery, and high blood pressure. In North Africa, *Ajuga* plants are used to treat diabetes and hypertension, as a panacea (cure-all), specifically for gastrointestinal disorders, and as an anthelmintic (Israili and Lyoussi, 2009). Other reported activities of *Ajuga* plants include antibacterial, antifungal, anti-inflammatory, antimalarial/antiplasmodial antitumor, larvae and insect antifeedant (Israili and Lyoussi, 2009), antihypertensive (Odek-Ogunde, *et al.*, 1993 and Ragunathan and Abay, 2009), diuretic (Aliotta and Pollio, 1994; Ragunathan and Abay, 2009), antimycobacterial (Cantrell *et al.*, 1999), antioxidant (Chenni *et al.*, 2007), antipyretic (Baytop, 1984), insect growth inhibitor (Camps and Coll, 1993) activity.

1.9.3. Compounds isolated from plants of the genus *Ajuga*

Chemical investigations have led to the isolation of a large number of compounds, including phytoecdysteroids, neo-clerodane- diterpines and diterpinoids, triterpines specific sterols, anthocyanidin-glucosides and iridoid glycosides, quinols, withanolid, flavonoids, triglycerides and essential oils. Among the many plants containing phytoecdysteroids, the plants belonging to *Ajuga* genus are unique for the great variety of such compounds produced, which exert a broad spectrum of biological and pharmacological actions (Israili and Lyoussi, 2009).



Fig 3. Photograph of the plant *Ajuga remota* Benth.

Although most of the diuretics proved to be very effective in promoting sodium excretion, all cause potassium loss and prompted the search for potassium sparing diuretic. Hence, search for a new diuretic agent that retains therapeutic efficacy and yet devoid of potassium loss is justified (Koti and Purnima, 2008).

Many indigenous drugs had been claimed to have diuretic effect in traditional system of medicine but they were not properly investigated. It has been documented that a number of plants are used as diuretic agents traditionally and some of them are confirmed for their claimed activities by various researches. *A. remota* is one of the plants found in Ethiopia which are widely used traditionally in Bahirdar Zuria to treat high blood pressure and stomach pain (Ragunathan and Abay, 2009). In order to advocate the ethnobotanical uses of *A. remota*, the biological effects of the plant should be supported by scientific data.

2. OBJECTIVE

2.1. General Objective

To assess the diuretic effect of the aqueous and hydroalcoholic extracts of the plant *A. remota* B. in mice.

2.2. Specific Objectives

- To evaluate the effects of the aqueous and hydroalcoholic extracts of *A. remota* on urine volume.
- To determine the electrolytes excretion (saluretic) effect of the plant *A. remota*
- To determine the pH and electrolyte excretion pattern thereby to predict the possible mechanism of action
- To assess acute toxicity profile of the plant

3. MATERIALS AND METHODS

3.1. Drug and Chemicals

Absolute methanol (EPFSA, Addis Ababa, Ethiopia); Normal Saline, Distilled water and Furosemide (EPHARM, Addis Ababa, Ethiopia). Dragendrof's reagent, 10% Ethanolic ferric chloride, Hexane, Dilute Ammonia, Acetic Anhydride, Concentrated Sulfuric Acid, Glacial Acetic Acid, 5% Ethanolic Ferric Chloride, 1% Aqueous Hydrochloric acid, and Chloroform were purchased from BDH, Poole England.

3.2. Experimental Animals

Adult albino mice bred in the animal house of Ethiopian Health and Nutrition Research Institute (EHNRI) and having weights ranging from 20 to 30 g and 8 weeks of age were used for the experiment. The animals were housed under standard environmental conditions ($25\pm 1^\circ\text{C}$, $55\pm 5\%$ humidity and 12 h/12 h light/dark cycle). The animals were allowed free access to tap water and standard laboratory pellet. The care and handling of mice were in accordance with the internationally accepted standard guidelines for use of animals (Vogel, 2007).

3.3. Collection of the Plant

The leaves of *A. remota* were collected from a place called Sebeta, a few kilometers West from Addis Ababa, Ethiopia in December 2010. The plant was identified as *A. remota* at the National Herbarium, Addis Ababa University. A voucher specimen (Voucher No. E001) was deposited in the Herbarium.

3.4. Extraction of the Plant

The leaves of *A. remota* were sliced to smaller pieces and dried at room temperature in the shade for more than two weeks. The dried and sliced pieces of the leaves were then powdered finely and extracted as follows.

3.4.1. Aqueous Extraction

200 g of the dried powder of leaves of *A. remota* was boiled at 100°C in 1000 ml of distilled water for 30 min, in the same manner as prepared traditionally, cooled to room temperature for 15 min. The decoction obtained was centrifuged, filtered, and placed in an oven until dried. The dried extract was collected and weighed. The approximate yield of the dry extract was 11.5% (w/w). The dried plant extract was reconstituted with distilled water (DW) for oral administration.

3.4.2. Hydroalcoholic Extraction

400 g of dried powder of leaves of *A. remota* was macerated with about 600 ml of 80% methanol for 24 h. The extract was then filtered and the marc was remacerated twice using the same volume of 80% methanol to exhaustively extract the plant material. The methanol was then removed from the extract by evaporation under reduced pressure using a rota vapor (BUCHI Rotavapour R-200, Switzerland) at 40 °C. The resulting dry extract was weighed and calculated for percentage yield which was 9.95% (w/w). The dried plant extract was reconstituted with DW and given orally.

3.5. Acute Toxicity Test

Six groups of female mice of 25±5gm of body weight were formed, a control (vehicle) and five groups treated with different doses (350-5000 mg/kg) of aqueous and hydroalcoholic extract. The animals had access to food and water *ad libitum* and were observed for clinical signs during the 24 h period following per os administration. Lethality was assessed using death within 7 days as an index of toxicity (Camargo *et al*, 2004).

3.6. Phytochemical Screening

Phytochemical screening tests were carried out on the aqueous and hydroalcoholic extracts of the plant *A. remota* using standard procedures to identify the constituents as described elsewhere (Trease and Evans, 1989).

3.6.1. Test for Phenolic Compounds (Ferric Chloride Test)

The extract was diluted to 5 ml with an appropriate solvent. To this a few drops of neutral 5% ferric chloride solution was added. A dark green color indicates the presence of phenolic compounds.

3.6.2. Test for Tannins

About 0.5 g of the dried powdered sample was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

3.6.3. Test for Saponins

The extract was diluted with an appropriate solvent and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. 2 cm layer of foam indicates the presence of saponins.

3.6.4. Test for Flavonoids

5 ml dilute ammonia solution was added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4 . A yellow coloration indicates the presence of flavonoids. The yellow coloration disappears on standing.

3.6.5. Test for Terpenoids

5 ml of each extract was mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface shows positive results for the presence of terpenoids.

3.6.6. Test for Steroids

The extract was mixed with 2 ml of acetic anhydride. To this 1 or 2 drop of concentrated H_2SO_4 was added slowly along the sides of the test tubes. An array of color change shows the presence of steroids.

3.6.7. Test for Alkaloids

i. Mayer's test: To a few ml of the filtrates, a drop of Mayer's Reagent (MR) was added by the side of the test tube. A creamy or white precipitate indicates that the test is positive.

ii. Hager's Test: To a few ml of the filtrates, a drop of Hager's Reagent (HR) was added by the side of the test tube. Precipitate formation indicates the test that is positive.

3.6.8. Test for Anthraquinones

Few ml of the extract was mixed with benzene. To this, 10% ammonia solution was added. The formation of pink color at the interface indicates the presence of anthraquinones.

3.6.9. Test for Cardiac Glycosides (Keller-Killiani Test)

5 ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayered with 1 ml of concentrated H₂SO₄. A brown ring of the interface indicates a deoxy sugar characteristic of cardenolides.

3.7. Grouping and Dosing of animals

Animals were randomly assigned into five groups each consisting of 8 mice for diuretic test. Negative controls were treated with the vehicle used for reconstitution (2 ml/100gm of body weight, CON). Positive controls were treated with standard drug, furosemide 10 mg/kg (Fr10). Three treatment groups in each test were treated with different doses of the extract as follows: the aqueous extract at doses of 250 mg/kg (AA250), 500 mg/kg (AA500), and 1000 mg/kg (AA1000); and hydroalcoholic extract at doses of 250 mg/kg (AM250), 500 mg/kg (AM500), and 750 mg/kg (AM750).

Dose selection was made based on the acute toxicity test performed prior to the commencement of the experiment. Thus, doses had been selected based on the outcome of the toxicity study.

3.8. Diuretic Activity

Diuretic activity was determined following the methods used by Lahlou *et al.* (2007) with slight modification. Each male mouse was placed in an individual metabolic cage 24 h prior to commencement of the experiment for adaptation and then fasted overnight with free access to water. The animals were pretreated with physiological saline (0.9% NaCl) at an oral dose of 0.15 mL/10 g body weight (BW), to impose a uniform water and salt load (Benjumea *et al.*, 2005). Each group was then treated as described in section 3.7 orally by gavage. Immediately after administration, the mice were individually placed in a metabolic cage. Urine was then collected and measured for a total of 5 h at 1, 2, 3, 4, and 5 h after dosing. The urine was then filtered and finally stored at -20 °C for further electrolyte analyses (Benjumea *et al.*, 2005).

The following parameters were calculated in order to compare the effects of the extracts and furosemide 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 a drug (Formula -2). A parameter known as diuretic activity was also calculated. To obtain diuretic activity, the diuretic action of the extract was compared to that of the standard drug in the test group (Formula – 3) (Mukherjee, 2000).

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

$$\text{Diuretic Action} = \frac{\text{Urinary excretion of treatment groups}}{\text{Urinary excretion of control group}} \quad (\text{Formula-2})$$

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

3.9. Analytical Procedures

Sodium, potassium and chloride levels of urine and the plant extract were analyzed. Sodium and potassium concentrations were determined by making use of flame photometry, and chloride concentration was quantified using, Ion Selective Electrode (ISE) analyzer (AVL 9181 Electrolyte Analyzer, Roche, USA). The flame photometer worked 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 equipment prior to analysis with different levels of standards.

3.10. Statistical Analysis

Data are expressed as mean \pm S.E.M (standard error of mean) of eight mice for the test. Statistical analysis of the data were performed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Significant differences were set at *P* values lower than 0.05.

4. RESULTS

4.1. Acute Toxicity Study

The mice were observed for 15 days to see if aqueous and hydroalcoholic plant extracts had acute toxicity in mice. And, even at 5000 mg/kg, the *A. remota* extracts did not provoke any visible signs of toxicity. This had been evidenced by absence of tremor, loss of weight, lethargy, paralysis, stress or adverse behaviors. In addition, there was also no sign of diarrhea and none of the treated mice were dead.

4.2. Phytochemical Screening

The extracts (both aqueous and hydroalcoholic) of the leaves of the plant *A. remota* had been explored for the composition of medicinally active compounds and both were found to be positive for phenolic compounds, saponins, steroids, cardiac glycosides, flavonoids, tannins and terpenoids. On the other hand alkaloids and anthraquinones were only found in the hydroalcoholic extract.

4.3. Diuretic Activity: Effect on Urine Volume

4.3.1. Aqueous Extract

The aqueous extract of the plant produced diuresis which appeared to be dose-dependent (Table 1). And the time course of action of diuresis is also depicted in (Fig 4). AA250 did not produce better diuresis than the vehicle in the first and second hour, but starting from the third hour a very slight increase in urine volume was produced and then an increased diuresis by about 10.5% had been recorded at the fifth hour, which was not found to be significant. Mice treated with AA500 had an increased diuresis starting from the second hour of urine collection but a significant diuresis was produced starting from the fourth hour (68%, $p < 0.05$) and a maximum increase of (93.3%) at the fifth hour ($p < 0.01$) when compared with CON animals. However, the highest dose of AA1000 produced diuresis which was significant (82.2%, $p < 0.01$) starting from the very first hour and a maximum increase of diuresis (96%, $p < 0.01$) was recorded at the fifth hour.

Fr10 treated mice produced diuresis which was significant as compared to CON group, starting from the first hour (90%, $p < 0.01$) and continued until the end of the fifth hour (95%, $p < 0.01$), thus the onset of diuresis is almost one hour (Table 1).

The standard drug Fr10 had a significant diuretic effect than that of AA250 ($p < 0.001$) but had comparable effect with AA500 at the end of the fifth hour, while the maximum dose (i.e. AA1000) produced an effect which was better than that of the standard drug. This could be revealed from the diuretic activity of AA500, AA1000 and Fr10 which were 1.93, 1.96 and 1.95 respectively (Table 1).

When the different doses of the aqueous extracts compared each other, the highest dose, AA1000, produced diuresis which was significant starting from the first hour ($p < 0.001$) and continued till the end of the fifth hour ($p < 0.01$) as compared with AA250. With regard to the middle dose (AA500), AA1000 produced a significant diuresis only at the first hour of urine collection ($p < 0.01$) but volume was not significant thereafter. Indeed, as time went by both doses appeared to exhibit similar effectiveness, as they had comparable diuretic action (1.93 and 1.96 for AA500 and AA1000 respectively) (Table 1).

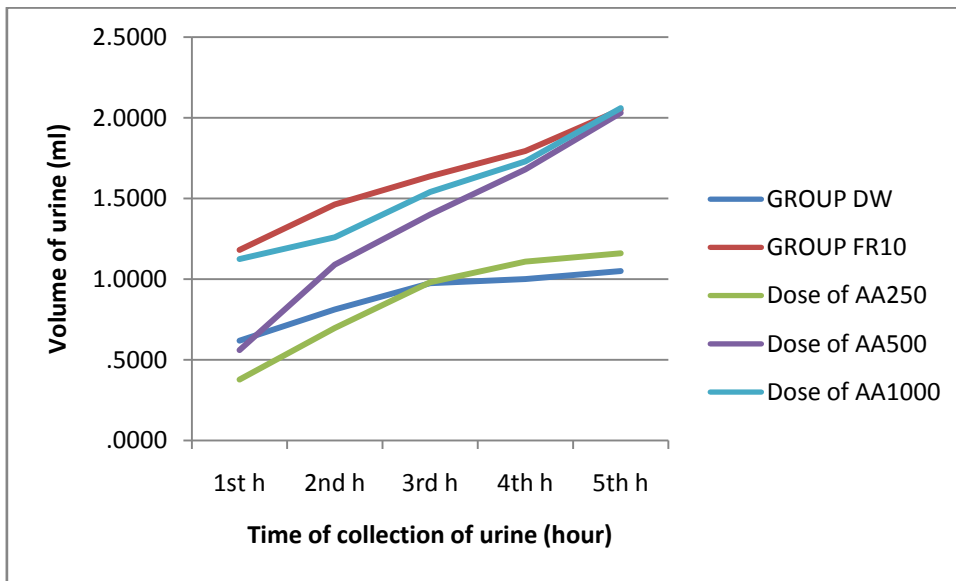


Fig 4. Time course of diuresis in mice treated with different doses of aqueous *A. remota* extract: N = 8, AA250- 250 mg/kg, AA500- 500 mg/kg, AA1000- 1000 mg/kg and Fr10- Furosemide 10 mg/kg

As shown in Fig 4 diuresis increased from the first hour and continued to increase throughout the hours of collection. And the volume showed an increasing response at the different doses of the treatment groups. AA250 had shown better diuresis than the vehicle starting from the third hour though it was not significant but the medium and maximum doses had produced significant diuresis by the end of the fifth hour.

Table 1: Effect of aqueous extract of the leaves of *A. remota* on diuresis in mice (n=8)

Group	Volume of Urine (ml)					Diuretic action	Diuretic activity
	1h	2h	3h	4h	5h		
CON	0.62 ± 0.13	0.81 ± 0.11	0.97 ± 0.11	1.0 ± 0.11	1.05 ± 0.10	1.0	
Fr10	1.18 ± 0.07 ^{a1}	1.46 ± 0.09 ^{a3}	1.64 ± 0.08 ^{a1}	1.79 ± 0.09 ^{a2}	2.05 ± 0.11 ^{a2}	1.95	1.0
AA250	0.38 ± 0.04 ^{b3,c3}	0.69 ± 0.05 ^{b3,c2}	0.98 ± 0.17 ^{b2}	1.11 ± 0.18 ^{b2,c1}	1.16 ± 0.17 ^{b3,c2,d1}	1.10	0.56
AA500	0.56 ± 0.08 ^{b3,c2}	1.09 ± 0.07	1.41 ± 0.12	1.68 ± 0.13 ^{a1}	2.03 ± 0.13 ^{a2}	1.93	0.99
AA1000	1.13 ± 0.13 ^{a2}	1.26 ± 0.14 ^{a1}	1.54 ± 0.19 ^{a1}	1.73 ± 0.17 ^{a2}	2.06 ± 0.20 ^{a2}	1.96	1.01

^a:against control, ^b:against standard, ^c:against AA1000 mg/kg, ^d:against AA500 mg/kg, ¹: P<0.05, ²:P<0.01, ³:P<0.001

AA250: extract 250 mg/kg, AA500: extract 500 mg/kg, AA1000: extract 1000 mg/kg, Fr10: Furosemide 10 mg/kg CON: control group

4.3.2. Hydroalcoholic Extract

For the hydroalcoholic extract, diuresis was not as effective as that of aqueous extract. The effect was not significant for the first two doses (AM250 and AM500) compared to CON group. Although AM750 resulted in an increased diuresis starting from the first hour (38.7%), it failed to reach statistical significance, however, increase in diuresis started to become significant at the fourth hour (57%, $p<0.05$), and produced maximum effect at the fifth hour (91.4%, $p<0.01$) when compared with the CON (Table 2). Furosemide on the other hand produced a significant diuresis starting from the first hour.

Both AM250 ($p<0.001$) and AM500 ($p<0.01$) produced a lower diuretic effect as compared to that of Fr10, while the maximum dose (AM750) had an effect which was comparable to that of furosemide i.e. with diuretic action of (1.92 vs 1.95) (Table 2).

Among the different doses of treatment groups, the maximum dose of AM750 produced an increased diuresis which was significant at the first hour ($p<0.01$), third hour ($p<0.01$) and a maximum increase had occurred by the end of the fifth hour ($p<0.001$) when compared with AM250. On the other hand, AM750 had resulted in diuresis which was significant at the first hour ($p<0.05$), fourth hour ($p<0.05$) and at the end of the fifth hour ($p<0.01$) compared to AM500.

Table 2: Effect of hydroalcoholic extract of the leaves of *A. remota* on diuresis in mice (n=8)

Group	Volume of Urine (ml)					Diuretic action	Diuretic activity
	1h	2h	3h	4h	5h		
CON	0.62 ± 0.13	0.81 ± 0.11	0.97 ± 0.11	1.0 ± 0.11	1.05 ± 0.10	1.0	
Fr10	1.18 ± 0.07 ^{a1}	1.46 ± 0.09 ^{a3}	1.64 ± 0.08 ^{a1}	1.79 ± 0.098 ^{a2}	2.05 ± 0.11 ^{a2}	1.95	1.0
AM250	0.45 ± 0.46 ^{b3,c2}	0.62 ± 0.07 ^{b3}	0.68 ± 0.72 ^{b3,c2}	0.84 ± 0.07 ^{b3,c2}	1.1 ± 0.85 ^{b3,c3}	1.06	0.54
AM500	0.48 ± 0.09 ^{b3,c1}	0.71 ± 0.09 ^{b3}	0.91 ± 0.12 ^{b3}	1.08 ± 0.13 ^{b2,c1}	1.31 ± 0.11 ^{b2,c2}	1.25	0.64
AM750	0.86 ± 0.03	0.98 ± 0.08	1.32 ± 0.13	1.57 ± 0.16 ^{a1}	2.01 ± 0.19 ^{a2}	1.92	0.98

^a:against control, ^b:against standard, ^c:against AM750mg/kg, ¹: P<0.05, ²:P<0.01, ³:P<0.001

AM250: extract 250 mg/kg, AM500: extract 500 mg/kg, AM750: extract 1000 mg/kg, Fr10: Furosemide 10mg/kg CON: control group

4.4. Saluretic Activity: Effect on electrolyte content of the urine

4.4.1. Aqueous Extract

The urine samples collected over the five hours were analyzed for the electrolyte content (Na^+ , K^+ , and Cl^-) and presented in Table 3. Whilst AA250 tended to decrease sodium loss by 17.1%, AA500 increased by 34.4% compared to CON group. By contrast, AA1000 significantly increased sodium loss by 69.2% ($p < 0.001$). Fr10 increased Na^+ excretion by 68.9% ($p < 0.001$) which was significant over the five hour periods.

Urinary K^+ excretion was measured for all treatment groups, and Fr10 had only shown significant K^+ loss with 106.8% of ($p < 0.001$) compared to the CON group. AA250 showed a slight kaliuresis (31.6%) and that of AA500 even showed more increased K^+ excretion (66.7%) albeit not found to be significant at both doses when compared to CON group. But the maximum dose of the aqueous extract showed a relatively lower amount of potassium excretion even when compared with that of the CON group. In the case of Cl^- the three extract groups produced an increased excretion in the urine which was 15.1%, 32.9% and 88.6% ($p < 0.001$) for AA250, AA500 and AA1000, respectively (Table 3).

The first two doses of the extract showed a lowered excretion of urinary Na^+ than Fr10, while the highest dose (AA1000) had a comparable effect with the standard. However, K^+ excretion of Fr10 significantly exceeded both AA250 ($p < 0.05$) and AA1000 ($p < 0.001$) but not that of AA500. For Cl^- , however, there was not any significant difference in between the extracts and the standard drug (Table 3). Table 3 also shows that the saluretic indices of Na^+ and Cl^- of the extract at the highest dose and Fr10 were comparable (1.69, 1.87 vs 1.68, 1.67), while the saluretic index of K^+ for the highest dose was very smaller than Fr10. In addition, the Na^+/K^+ ratio of AA1000 was higher than Fr10. $\text{Cl}^-/\text{Na}^+ + \text{K}^+$ was also calculated and AA500 had the lowest value (0.38).

Comparing the different doses of the extract, AA1000 and AA500 had produced a significant natriuresis as compared with AA250 with $p < 0.01$ and $p < 0.001$ respectively, but with regard to K^+ excretion the medium dose (AA500) had the highest kaliuresis as compared to AA1000 ($p < 0.05$). The excretion of Cl^- , however, did not show a significant difference in between the doses.

Table 3: Effect of aqueous extract of the leaves of *A. remota* on 5h urinary electrolyte excretion in mice (n=8)

Group	Urinary electrolyte concentration (mmol/L)			Saluretic Index			Na ⁺ /K ⁺	Cl ⁻ / Na ⁺ +K ⁺
	Na ⁺	K ⁺	Cl ⁻	Na ⁺	K ⁺	Cl ⁻		
DW	59.97 ± 7.83	43.7 ± 5.80	44.2 ± 2.58				1.37	0.43
Fr10	101.3 ± 6.01 ^{a3}	92.07 ± 13.85 ^{a3}	73.85 ± 11.27	1.68	2.1	1.67	1.10	0.38
AA250	49.70 ± 2.74 ^{b3,c3}	57.5 ± 5.94 ^{b1}	50.87 ± 10.58	0.83	1.32	1.13	0.86	0.47
AA500	80.62 ± 4.89 ^{e2}	72.85 ± 2.66 ^{c1}	58.75 ± 8.34	1.34	1.67	1.33	1.11	0.38
AA1000	101.5 ± 6.98 ^{a3}	38.08 ± 1.81 ^{b3}	83.37 ± 2.71 ^{a3}	1.69	0.87	1.87	2.66	0.59

^a:against control, ^b:against standard, ^c:against AA1000mg/kg, ¹: P<0.05, ²:P<0.01, ³:P<0.001

Saluretic Index=mmol of electrolyte of test group/mmol of electrolyte of control group

AA250:extract *A. remota* 250 mg/kg, AA500: *A. remota* extract 500 mg/kg, AA1000: *A. remota* extract 1000 mg/kg, Fr10: Furosemide 10 mg/kg CON: control group

4.4.2. Hydroalcoholic Extract

Similarly the five hours urine obtained from the hydroalcoholic extracts were analyzed for the same electrolytes. The urinary Na⁺ excretion showed an increasing pattern as it was 6.3%, 50.7% and 69.6% ($p < 0.01$), for the respective doses of AM250, AM500 and AM750 when compared with CON group. On the other hand, K⁺ excretion had increased from AM250 to AM500 but in case of AM750 the excretion of K⁺ was the lowest as compared to CON. The excretion of Cl⁻ had also showed an increasing manner of 26.1%, 28.6%, and 76% ($p < 0.05$) for AM250, AM500 and AM750 respectively (Table 4).

Na⁺ excretion of AM250 and AM500 was lesser as compared to Fr10, but AM750 have had comparable excretory effect. On the contrary, K⁺ excretions for the first two doses were higher than that of Fr10 but there was not a significant relationship in between the extracts and the standard drug. In the case of Cl⁻, excretion there was not any significant difference between the standard and the three doses of the extract. The saluretic indices had also been calculated similarly and closer results were obtained for Na⁺ and Cl⁻ between the highest dose of the extract and Fr10 (1.69, 1.76 vs 1.68, 1.67). Cl⁻/Na⁺+K⁺ value also been calculated and the AM500 provided the least value (0.29) (Table 4).

For the different doses of hydroalcoholic extract, both AM750 and AM500 had resulted in a significant Na⁺ excretion when compared with AM250 i.e. $p < 0.05$ and $p < 0.01$ respectively. In the case of K⁺ excretion, AM500 had the highest kaliuretic effect when compared with AM750 ($p < 0.05$). But Cl⁻ excretion did not show a significant excretion difference in between the different doses of the extract.

Table 4: Effect of hydroalcoholic extract of the leaves of *A. remota* on 5h urinary electrolyte excretion in mice (n=8)

Group	Urinary electrolyte concentration (mmol/L)			Saluretic Index			Na ⁺ /K ⁺	Cl ⁻ /Na ⁺ +K ⁺
	Na ⁺	K ⁺	Cl ⁻	Na ⁺	K ⁺	Cl ⁻		
CON	59.97 ± 7.83	43.7 ± 5.80	44.2 ± 2.58				1.37	0.43
Fr10	101.3 ± 6.01 ^{a3}	92.07 ± 13.85 ^{a3}	73.85 ± 11.27	1.68	2.1	1.67	1.10	0.38
AM250	63.75 ± 7.3 ^{b1,d1}	97.5 ± 13.2 ^{c1}	55.74 ± 6.23	1.06	2.23	1.26	0.65	0.34
AM500	90.37 ± 5.8 ^{a1,e1}	100.6 ± 11.7 ^{c2}	56.82 ± 8.06	1.51	2.30	1.29	0.89	0.29
AM750	101.7 ± 4.72 ^{a2,e2}	53.5 ± 2.95	77.78 ± 9.71 ^{a1}	1.69	1.22	1.76	1.90	0.50

^a:against control, ^b:against standard, ^c:against AM750mg/kg, ^d:against AM500mg/kg, ^e:against AM250mg/kg. ¹: P<0.05, ²:P<0.01, ³:P<0.001

Saluretic Index=mmol of electrolyte of test group/mmol of electrolyte of control group

AM250: *A. remota* meth. 250mg/kg, AM500: *A. remota* meth. 500mg/kg, AM750: *A. remota* meth. 1000mg/kg, Fr10: Furosemide 10mg/kg CON: control group

4.5. Urinary pH

The urinary pH was measured. And the different treatment groups of both aqueous and hydroalcoholic extracts had resulted in the production of relatively alkaline urine.

4.5.1. Aqueous Extract

The pH had shown an increasing order from AA250 (7.57) to AA1000 (7.72). The CON group had produced the lowest pH and the standard group gave rise to slightly alkaline urine of 7.45, but the difference in between groups found to be insignificant.

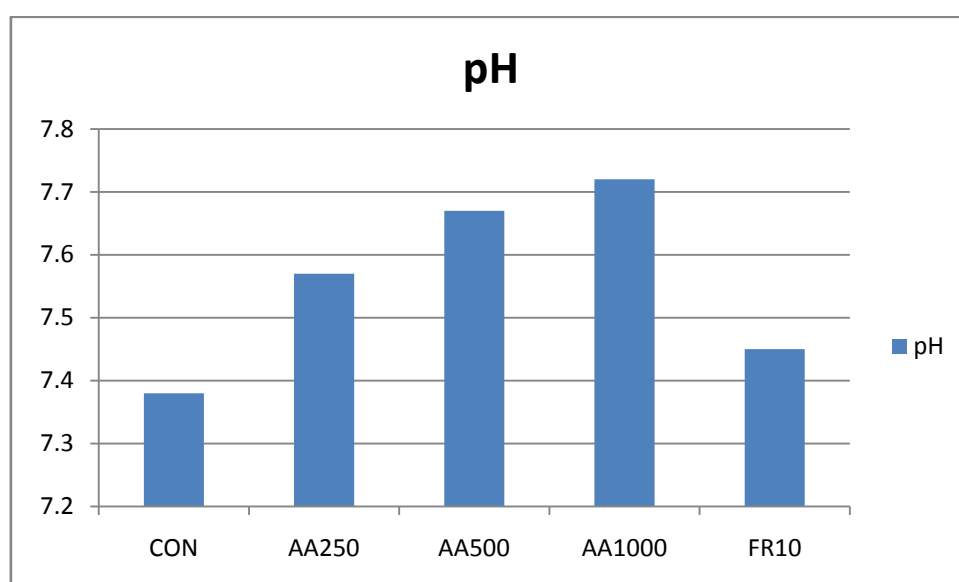


Fig 5. Urinary pH of the aqueous extract of the leaves of *A. remota* and the controls

4.5.2. Hydroalcoholic Extract

Similarly the pH increased in the case of hydroalcoholic extract of the plant. AM250 had the lowest pH of 7.3 and AM750 with the most alkaline pH of 7.88. With the negative controls produced the least pH, the standard drug had produced a slightly alkaline urine. And there was not any significant difference between the extracts and the controls.

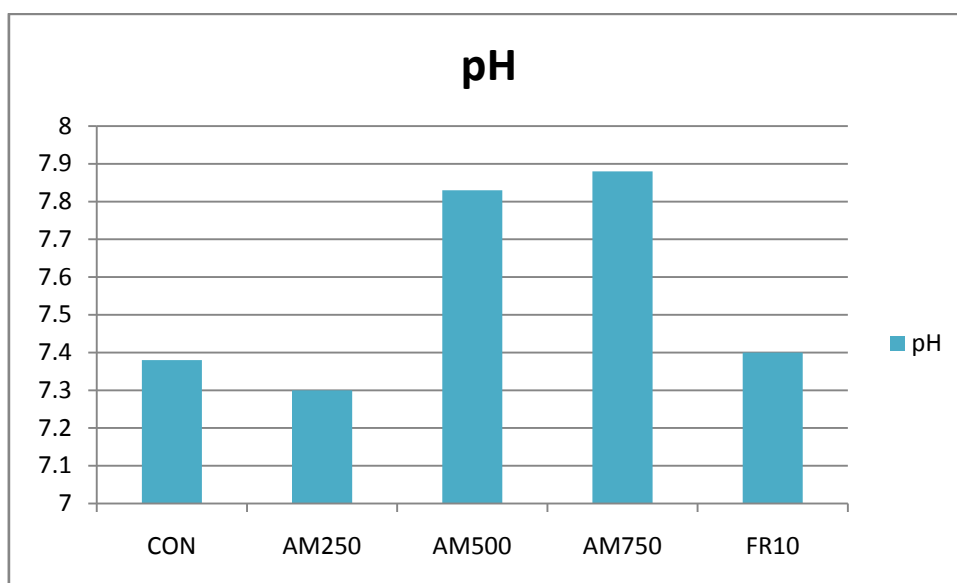


Fig 6. Urinary pH of the 80% methanolic extract of the leaves *A. remota* and controls

4.6. Electrolyte Content of the Extracts

The electrolyte content of both aqueous and hydroalcoholic extract had been explored so as to rule out the interference on the result. The result showed that Na^+ and Cl^- amounts were below detection level. And K^+ content was found to be 35.3, 40.7 and 47.1 mmol/lit for AA250, AA500 and AA1000, respectively, in the case of aqueous extract. And a lower values of 12, 20 and 23.5 mmol/lit for AM250, AM500 and AM750, respectively, in case of hydroalcoholic extract.

5. DISCUSSION

Diuresis has two components: increase in urine volume (water excretion) and a net loss of solutes (i.e. electrolytes) in the urine (Jackson, 2006). These processes result from suppression of renal tubular reabsorption of water and electrolytes into the blood stream. In the present study, therefore, both volume and electrolyte parameters were measured to evaluate the diuretic effect of the plant extracts. Loop diuretics such as furosemide can increase the urinary flow rate, also they are strongly saluretic in as much as they increase urinary sodium and chloride excretion that is why in this study furosemide was used as positive control.

The plant was prepared as a decoction in order to simulate the way it is traditionally used, by boiling the leaves for about thirty minutes in water. And with the assumption the active ingredient (s) responsible for the claimed diuretic activity might not be soluble in water adequately; hydroalcoholic extract of the plant was also included in the study.

Previous studies on diuretic agents have found it to be advantageous to ‘pre-treat’ or ‘prime’ the test animals with various fluids. Since diuretics are employed clinically in the treatment of edema, it would be highly important to demonstrate effectiveness in the presence of electrolyte and water (Nedia *et al.*, 2004). Thus, the saline was administered to simulate edema.

In view of urine output, both aqueous and 80% methanolic extracts of the leaves of the plant *A. remota* showed an increased diuresis as compared to the control group. Compared to hydroalcoholic extract, the aqueous extract produced a better diuretic effect. This difference in their effect could be seen in the different doses used in this study. The minimum doses of both extracts did not produce an effect and this could be accounted by the lack of enough concentration of active components which were responsible for the diuretic activity at these lower doses. But in case of the medium doses, while the aqueous extract AA500, was able to produce significant diuresis in 5 h (Table 1), the same dose of the hydroalcoholic extract was devoid of any appreciable effect. Increasing the dose did affect the diuretic effect produced especially by the aqueous extract. For e.g., the diuretic effect produced by AA1000 was higher than that achieved by AA500 (2.06 ± 0.20 Vs 2.03 ± 0.13) (Table 1). Moreover, the diuretic

activity (0.99) of AA500, closer to Fr10 (1.0), was lower than that of AA1000 (1.01). On the other hand, AM750 produced a diuretic effect of (2.01 ± 0.19) , which was yet lower than both the medium and maximum doses of aqueous extract. It is therefore possible to suggest that the ingredient (s) of the plant material responsible for the diuretic effect could probably be more polar and hence better extracted in water than 80% methanol.

Comparison of the two extracts of *A. remota* indicates that the aqueous extract showed an increase in diuresis which appeared to be dose-dependent. And, the diuretic activity of the extracts of *A. remota* at their highest respective doses was a moderate type, since their values were 1.01 and 0.98 for AA1000 and AM750 respectively. Diuretic activity is considered to be good if it is more than 1.50, moderate if it is within 1.00 and 1.50, little if it is within 0.72-1.00 and nil if it less than 0.72 (Gujral *et al.*, 1955).

The diuretic action of the plant extracts, particularly, at highest doses was generally high and quantitatively similar to that of Fr10. Although the lower doses of the extracts produced diuretic effect significantly lower than Fr10, both AA1000 and AM750 of the aqueous and hydroalcoholic extract, respectively, were able to produce effects comparable to that of Fr10 at the fifth hour (Table 1 and 2) which clearly shows that the extracts have a potential to induce diuresis markedly as those of known synthetic diuretics.

Furthermore, the onset of the diuretic action of the most effective dose of the extracts such as AA1000 was sufficiently rapid and had a fairly long duration of action as it produced its significant effect from the first hour ($P < 0.01$) to the fifth hour ($P < 0.01$) (Table 1). The difference in the time of onset of the diuretic action of the different doses and between extracts may be related to the gastrointestinal absorption characteristics of the active principle (s) which are responsible for the evidenced diuretic activity. This is an appealing diuretic profile as it would curtail the frequency of administration, in addition to a decreased risk of hypokalemia. Though, the onset and duration of action remained to be addressed in detail in other researches on the most active doses.

The effect of the extracts on water excretion was accompanied by urinary electrolyte excretion effect, since there appeared to be an increased salt excretion as compared to the control group, which supports the idea that the diuretic effect of *A. remota* was of

the saluretic type in contrast to aquaretic type which is typical feature of most phytodiuretic agents (Martín-Herrera *et al.*, 2007). In case of Na⁺ and Cl⁻, the minimum and medium doses of both extracts did not show any significant effect when compared to the CON group. But the maximum doses of AA1000 and AM750 did have an interesting natriuretic effect; it is thus highly beneficial in different edematous conditions. The ratio Na⁺/K⁺ was calculated as indicator of natriuretic activity. Values greater than 2.0 indicate a favorable natriuretic effect and if the ratio exceeds 10.0, it would have potassium-sparing effect (Vogel, 2007). The Na⁺/K⁺ values were calculated and showed the natriuretic effect to be, 2.66 and 1.90 for AA1000 and AM750, respectively, which further strengthens the higher natriuretic effect of the extracts at these doses. So the aqueous extract at the dose of AA1000 had the best natriuretic effect as its value was greater than 2.0, and the hydroalcoholic extract (AM750) also had a value of 1.90 which was around the acceptable value to have natriuretic effect.

Regarding K⁺ excretion, it is easy to observe that *A. remota* at doses of 1000 mg/kg (aqueous) and 750 mg/kg (hydroalcoholic) showed an interesting K⁺- saving effect, whose values were nearer to those of the CON group and as a loop diuretic, furosemide by acting in the TAL resulted in hypokalemia. But in the case of *A. remota* extracts of AA1000 and AM750, there happened to be a K⁺- saving effect, when compared to Fr10 group and the difference found to be significant in the case of AA1000. This fact may point out that the extract at their peak doses exhibited advantageous effect with respect to hypokalemia, one of the potential adverse effects of furosemide. But still even higher doses are needed to be investigated to definitely assure if this K⁺- saving effect would show a dose dependent manner henceforth.

In contrast with the previous assays carried out comparing aqueous and methanol extracts of some plants which showed an interesting K⁺-saving effect at low and intermediate doses (Martín-Herrera *et al.*, 2007), in the present study of *A. remota* for both extracts the above K⁺-saving effects were observed only at their maximum doses (i.e. AA1000 and AM750). It is probable that at low dosages of the aqueous and methanol extracts of *A. remota*, the substances responsible for the K⁺- saving effect were not found in sufficient concentrations as occurred with the maximum dose. And this could be highly associated with the amount of the active principle that should be

reached to the site of action, so at higher doses there would be a sufficient amount of active component to distribute and reach the receptors, so as to produce the effect. In addition the interaction between active principles probably have been highly pronounced at these doses, as there might exist components which probably are not found at lower doses at sufficient amount. And this seems to account for the existence, at least of two different mechanisms, one of which produces notable diuresis with a sparing of potassium and another with very strong diuresis in which there is a clear tendency to lose the K^+ - conservative effect (Martín-Herrera *et al.*, 2008).

In view of the mechanistic study of the plant extracts, it is possible that *A. remota* extracts exerted diuretic effect by inhibiting tubular reabsorption of water and electrolytes as such action has been suggested for some other plants. The possibility of direct action of potassium content of *A. remota* extract on diuretic effect is not considered particularly in case of hydroalcoholic extract, since the K^+ content of the extract was very low in comparison with the salt concentration obtained from other plants (Sripanidkulchai *et al.*, 2001). Regarding to the hydroalcoholic extract, it should be pointed out that, in contrast to the aqueous extract in whose water preparation it occurs a removal of salts, with the hydroalcohol this salts removal does not generate. Thus the notable diuretic effect produced by the hydroalcoholic extract at the maximum dose reaffirmed the concept that the diuretic activity of *A. remota* was not solely due to its content of potassium salts. So it is possible to rule out the osmotic mechanism of action that could occur due to the higher salt content of the plant at least for the hydroalcoholic extract. Even if, this difference in potassium content might contribute for a discrepancy in diuretic effect between the aqueous and hydroalcoholic extracts, since the potassium content was relatively higher in the case of aqueous extract.

Loop diuretics like furosemide increases urinary flow rate and urinary excretion of sodium, potassium and chloride, by inhibiting $Na^+K^+2Cl^-$ symporter in the TAL and inhibiting CA enzyme (Mekonnen *et al.*, 2010). Aqueous and hydroalcoholic extracts of *A. remota* produced diuresis and saluresis, but at the highest doses Na^+ and Cl^- excretion were similar with that of furosemide, however with regard to K^+ the extracts had a lower K^+ excretion at these doses nothing like furosemide which significantly produced kaluresis, so the mechanism was unlikely to be the loop diuretics type. It is also possible to exclude the thiazide like type mechanism either, as these diuretics

relatively increase the urinary K^+ level more and alters the urinary Na^+/K^+ ratio, but the extracts from the plant *A. remota* showed a K^+ -saving effect at their maximal doses.

The Cl^-/Na^++K^+ ratio was calculated and showed the extent of CA inhibitory effect; CA inhibition can be excluded at ratios between 1.0 and 0.8. With decreasing ratios slight to strong CAI can be assumed (Vogel, 2007). The Cl^-/Na^++K^+ amount was calculated for both extracts and the intermediate doses, AA500 and AM500 showed the strongest CA inhibitory effect with values of 0.38 and 0.29 respectively, thus the strongest CA inhibition effect at these middle doses might have contributed to the highest K^+ loss compared to the other doses. So it is plausible to postulate that one of the possible mechanisms of action of these extracts could be CA inhibition type. And still the maximum doses produced the highest diuresis, even though the medium doses had the lowest Cl^-/Na^++K^+ ratio, thus there ought to be another mode of action which manifested at the peak doses.

In determination of the urinary pH the extracts showed a relative increase in the pH values as compared to the controls, so this strengthens that CA inhibition as one of the mechanisms of action of the plant. Thus, these reductions of potassium excretion at the maximum doses of the extracts along with the resulted alkalization of the urine might give clue on the probability of the plant acting as modest potassium-saving diuretics.

The active principle/s responsible for the diuretic effects of the hydroalcoholic and aqueous extracts of this species is/are, so far, not known, so it is not identified which compounds are exactly responsible for the diuretic, natriuretic and kaliuretic activities of *A. remota* but preliminary phytochemical analysis carried out with the hydroalcoholic and aqueous extracts revealed the presence of polar compounds such as flavonoids and steroids. One can suppose that these substances might be responsible, at least in part, for the observed diuretic activity and that they may act individually or synergistically. Previous studies have demonstrated also that there are several compounds which could be responsible for the plants diuretic effects such as flavonoids, saponins or organic acids (Maghrani *et al.*, 2005). The effect may be produced by stimulating regional blood flow or initial vasodilatation, or by producing inhibition of tubular reabsorption of water and anions, with the result in both cases being diuresis (Martín-Herrera *et al.*, 2008).

In the toxicological evaluation of *A. remota*, it was revealed that the plant did not produce any sign of acute toxicity in mice even at larger dose (5000 mg/kg) of both aqueous and hydroalcoholic extracts. The absence of acute toxicity at this dose confirmed the safe nature of this plant since doses which seem clearly higher than the usual dosage in traditional medicine failed to elicit any toxic symptoms when given to Swiss albino mice. This result suggests that the LD₅₀ of the plant is higher than 5000 mg/kg.

To sum up, the present study supports the ethnomedical use of *A. remota* for its diuretic effect. Although, the active component (s) remained unknown, based on the pattern of excretion of water, sodium and potassium, it appears that the plant could possibly have more than one mechanism of action which contributes to the potassium-saving and natriuretic effect especially at their maximal doses. Multiple mode of action had been reported with some herbal medications (Jaykody *et al.*, 2011). Thus, adding up to the predicted CA inhibitory effect there must be another active component (s) which reaches effective concentration at maximum doses of the extracts that contributes to the potassium saving and highest diuretic effect of the plant *A. remota*.

6. CONCLUSIONS

Looking at the data shown in the results from both aqueous and hydroalcoholic extracts of *A. remota* there had been a very interesting saluretic diuresis noted especially in case of aqueous extract. The diuretic action of the plant extracts especially at the peak doses had been comparable to the standard drug. And the maximum doses of both extracts had more or less a moderate diuretic activity, since their values were near 1.0.

From the electrolytes analyzed and urinary pH it was plausible to assume that the plant could have multiple mode of action, CAI mechanism being one of them.

Finally, the data seem to indicate that this diuretic effect could be associated with the presence in the plant of active principles of highly polar nature, where the flavonoids and steroids might be the main chemical protagonists for this activity.

The safe nature of the plant in addition to the evidenced diuretic effect from both extracts in the present study provides further support to explain the traditional folk-medicine use of *A. remota* to treat high blood pressure especially by the peoples around Bahirdar Zuria.

7. RECOMMENDATIONS

- Investigation of specific component (s) responsible for the diuresis should be analyzed from the different fractions of the crude extracts
- Investigation of higher doses to further confirm if the resulted diuretic and saluretic effect shows dose dependency
- Further investigations are necessary to determine the precise mechanism by which the extracts of *A. remota* affect diuresis and urinary electrolytes excretion especially to confirm the evidenced *in-vivo* CAI effect on mice through further *in-vitro* tests.
- The chronic toxicity profile of the plant also should be performed so as to prove the safety in the long term use

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Declaration

I, the undersigned, declare that this thesis work is my original work and has not been presented for a degree in any other university.

Name: _____

Signature: _____

This thesis has been submitted for examination with my approval as a university advisor.

Name: _____

Signature: _____

Place and Date of submission: Addis Ababa, Ethiopia, September, 2011