



**Evaluation of Diuretic activities of hydro-alcoholic extract and solvent fractions of the roots of *Withania somnifera* L. (Solanaceae) in rats**

**By**

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**A thesis submitted to the Department of Pharmacology, School of Medicine, College of Health Sciences, Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology.**

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This is to certify that the thesis prepared by Khaalid Dayib, entitled "Evaluation of the diuretic activity of 80% methanol extract and solvent fractions of *Withania somnifera* roots in rats" and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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## ABSTRACT

### **Evaluation of Diuretic activities of hydro-alcoholic extract and solvent fractions of the roots of *Withania somnifera* L. (Solanaceae) in rats**

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**Addis Ababa University, 2016**

**Background:** *Withania somnifera* (Local name ‘Gisawa’) is used in folkloric medicine for the management of hypertension in different parts of the world including Ethiopia, this may be due to its diuretic activity. However, it has not been yet scientifically validated for its efficacy and safety.

**Objective:** The aim of this study was to investigate the diuretic potential of both hydro-alcoholic (80% methanol) extract and solvent fractions of the hydro-alcoholic root extract of *W. somnifera* in rats.

**Methods and Materials:** The roots of *W. somnifera* used in this study were collected from Addis Ababa, which was then identified and authenticated by a taxonomist. The plant materials were garbled, dried under shade and grounded which were then macerated in 80% methanol to give the crude extract. A portion of the crude 80% methanol extract was further partitioned sequentially using solvents of increasing polarity to give chloroform, n-butanol fractions and the aqueous residue. Rats were randomly divided into five groups; two control groups (positive and negative controls) and three test groups each comprising six rats. Group I served as negative control and received distilled water 10 ml/kg, Group II served as a positive control and was given a standard drug 10mg/kg hydrochlorothiazide, Group III, IV and V were test groups and received 200, 400 and 600

mg/kg of the hydro-alcoholic extract, respectively. And a similar grouping was used for the solvent fractions. Urine output was collected up to 24 h and analyzed for electrolytes.

**Results:** The hydro-alcoholic extract increased diuresis significantly at the doses of 400 and 600 mg/kg ( $p < 0.001$ ), while the solvent fractions of the hydro-alcoholic extract the aqueous and n-butanol fractions significantly increased urine volume ( $p < 0.01$ ) at 400 mg/kg, with maximum urine output at the highest dose. Regarding electrolyte excretion, the larger doses of both hydro-alcoholic extract and aqueous fraction had increased natriuresis ( $p < 0.001$ ), while the effect on kaliuresis were smaller when compared with the standard. Phytochemical analysis revealed the presence of secondary metabolites; include tannins, terpenoids, flavonoids and saponins, which could be the responsible component (s) for the diuretic activity.

**Conclusion:** The results of the present study indicated that the **plant** is endowed with significant diuretic activity providing evidence for its traditional claim. The increased diuresis effects of the crude extracts and fractions may be attributable for presence of increased polarity of phytoconstituent(s).

**Key words:** *Withania somnifera*, hydrochlorothiazide, diuresis, natriuresis and kaliuresis.

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## LIST OF ABBREVIATIONS/ ACRONYMS

A <sub>1</sub> R	A <sub>1</sub> Adenosine Receptor
ARF	Acute Renal Failure
AVP	Argenin Vasopressin Receptor
ADH	Antidiuretic Hormone
ADHF	Acute Decompensated Heart Failure
ANOVA	One-Way Analysis of Variance
AQP2	Aquaporin 2
CA	Carbonic Anhydrase
CD	Collecting Duct
CFEX	Chloride Formate Exchanger
CHF	Congestive Heart Failure
DCT	Distal Convoluted Tubule
EPHI	Ethiopian Public Health Institute
FDA	Food and Drug Authority
GFR	Glomerular Filtration Rate
HF	Heart Failure
ISE	Ion Selective Electrode
NBC	Sodium-bicarbonate Cotransporter
NHE3	Sodium-proton Antiporter
OECD	Organization of Economic Co-operation and Development
PCT	Proximal Convoluted Tubule
S.E.M	Standard Error of Mean

SIADH	Syndrome of Inappropriate Antidiuretic Hormone
SPSS	Statistical Package for Social Sciences
TAL	Thick Ascending Loop
UT-A	Urea Transporter-A
UT-B	Urea Transporter-B
V <sub>2</sub> R	Vasopressin Receptor 2
WHO	World Health Organization

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# 1. INTRODUCTION

## 1.1. History of Diuretics

The term diuretic is derived from Greek word ‘diouretikos’ meaning to promote urine (Ellison, 2013). Although infusion of saline or ingestion of water would qualify as being diuretic, the term diuretic usually represents drug that can reduce the extracellular fluid volume by increasing urinary solute or water excretion (Okusa and Ellison, 2008). The term aquareticis applied to drugs that increases excretion of solute free water differentiating these drugs from traditional diuretics, which enhanced solute and water excretion. By increasing urine flow rate, diuretic usage leads to increased excretion of sodium and water in edematous conditions (Suresh et al., 2010).

The use of diuretics for the treatment of edema has been available since the 16<sup>th</sup> century. In 1553, mercurous chloride (calomel), inorganic mercury, was recorded by Paracelsus to be the first effective diuretic therapy for edema (Marvin *et al.*, 2009; Ellison, 2013). In 1919, the ability of organic mercurial antisphyliotics to affect diuresis was discovered by Vogl, medical student, which led to the development of effective organic mercurial diuretics. However, their toxic side effect, parenteral administration and limited efficacy precluded wide spread use. In the 1930s sulfonamide-based carbonic anhydrase (CA) inhibitors were developed, but these drugs had serious side effects and limited potency (Okusa and Ellison, 2008). However, these findings stimulated researchers to explore the possibility that modification of sulfonamide-based drugs could lead to drugs that enhance sodium chloride rather than sodium bicarbonate excretion.

The result of this program was the synthesis of chlorthiazide and its marketing in 1957, heralding a new era of diuretic drugs termed thiazides (Sterns, 2011). The search for better classes of diuretics based on the structure of chlorthiazide and sulfonamide derivatives resulted in the development of furosemide and ethacrynic. Although, these compounds were proved to be very effective in promoting sodium excretion, they caused potassium loss as unwanted effects. The search for potassium sparing diuretics resulted in the introduction of spironolactone, amiloride and triameterene (George, 2011).

Although most of the diuretics proved to be very effective in promoting sodium excretion, all cause potassium loss and this prompted the search for potassium sparing diuretics. Hence, a search for new diuretic agents that retain therapeutic efficacy and yet devoid of potassium loss is justified (Shankar *et al.*, 2013).

## 1.2. Renal Anatomy and Physiology

The paired kidneys are reddish, bean-shaped organs located just above the waist between the peritoneum and the posterior wall of the abdomen. Because their position is posterior to the peritoneum of the abdominal cavity, they are said to be retroperitoneal organs. The kidneys are located between the levels of the last thoracic and third lumbar vertebrae, a position where they are partially protected by the eleventh and twelfth pairs of ribs. The right kidney is slightly lower than the left because the liver occupies considerable space on the right side superior to the kidney (Gerard and Bryan, 2009).

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.

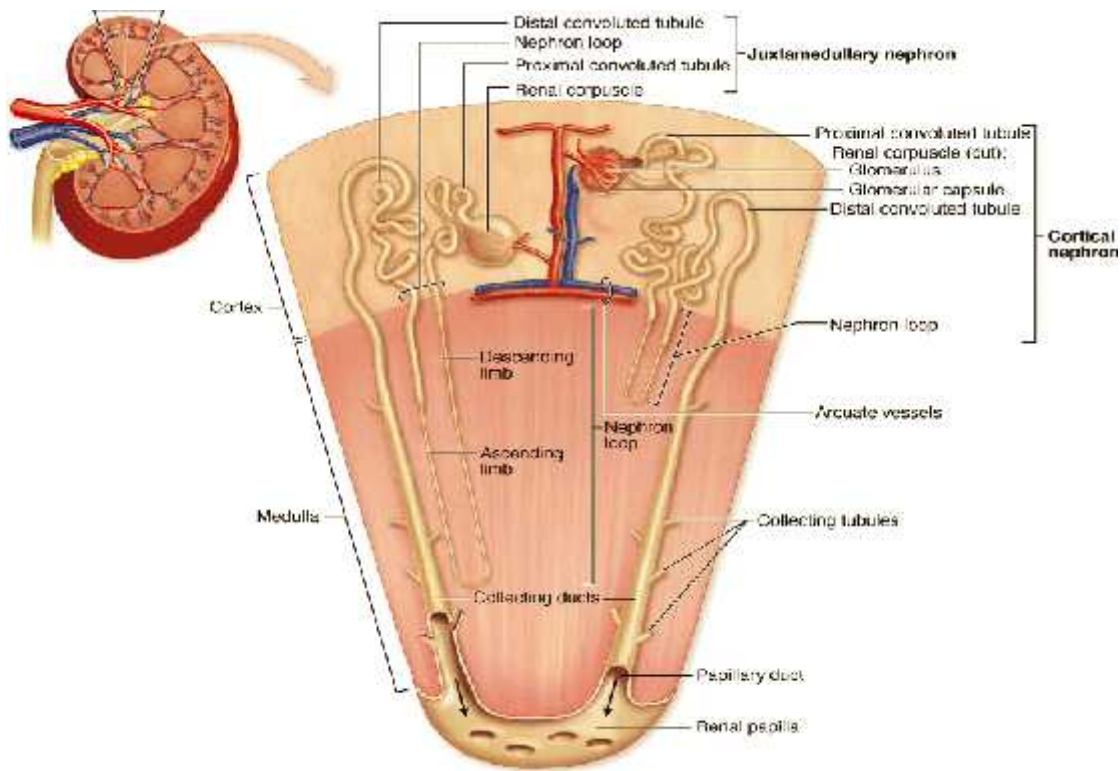


Fig 1. The anatomy of kidney and nephron (Anthony, 2010).

The kidneys receive about a quarter of the cardiac output. From the several hundred litres of plasma that flow through them each day, they filter (in a 70-kg human) approximately 120 litres per day, 11 times the total extracellular fluid volume. This filtrate is similar to plasma apart from the absence of protein. As it passes through the renal tubule, about 99% of the filtered water, and much of the filtered sodium ions, are reabsorbed, and some substances are secreted into it from the blood. Eventually, approximately 1.5 litres is voided as urine per 24 hours under usual conditions (Rang *et al.*, 2007).

The kidneys are highly vascularized organs that play a fundamental role in maintaining body salt and fluid balance and blood pressure homeostasis through the actions of their nephrons (Zhuo and Li, 2013). Nephrons are the structural and functional units of the kidneys. Each kidney contains over one million of these tiny blood processing units, which carry out the processes that form urine. A nephron consists of two principal parts: a renal corpuscle where the blood plasma is filtered and a long renal tubule that processes this filtrate into urine. The renal corpuscle consists of a ball of capillaries called a glomerulus, enclosed in a two layered glomerular (Bowman's) capsule (Rang *et al.*, 2007). 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 fluid that filters from the glomerular capillaries collects in the capsular space between the parietal and visceral layers and then flows into the renal tubule on one side of the capsule. The renal tubules a duct that leads away from the glomerular capsule and ends at the tip of a medullary pyramid. It is divided into four major regions: the proximal

convoluted tubule, loop of Henle, distal convoluted tubule and collecting duct. Each region of the renal tubule has unique physiological properties and roles in the production of urine (*Rang et al.*, 2007).

Urine formation begins when a large amount of fluid that is virtually free of protein is filtered from the glomerular capillaries into 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). PCT which arises from the glomerular capsule is responsible for reabsorbing approximately 65% of the filtrate (Ives, 2012). 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). A metalloenzyme, CA (type IV). Which is found in the luminal and basolateral membranes catalyzes the dehydration and rehydration of carbonic acid to provide  $H^+$  for the exchange (Ives, 2012).

From the proximal tubule, fluid flows in the loop of Henle, which dips into the renal medulla. Each loop consists of a descending and an ascending limb. When the ascending limb of the loop has returned pathway back to the cortex, its wall becomes much thicker, and its referred to as the thick ascending limb (TAL). TAL is relatively impermeable to water but has a high reabsorptive capacity which is about 20-25% of the filtered sodium load. The primary component of reabsorption is the  $Na^+/K^+$  ATPase in the basolateral membrane. By keeping intracellular sodium concentration low, it maintains a favorable electrochemical gradient for sodium entry into the cell across the apical membrane via a

protein carrier capable of transporting sodium, potassium and two chloride ions which is known as the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter. This is a secondary carrier that takes advantage of the gradient of  $\text{Na}^+$  transport to translocate  $\text{K}^+$  and  $\text{Cl}^-$  from the lumen into the TAL cells against their respective electrochemical gradient (Shirley and Unwin, 2010).

From the TAL, the urine courses toward the DCT, where approximately 5-7% of the filtered sodium load is reabsorbed via  $\text{Na}^+/\text{Cl}^-$  cotransporter into the cortical interstitium. As with other nephron segments, transport is powered by a  $\text{Na}^+$  pump in the basolateral membrane. The free energy in the electrochemical gradient for  $\text{Na}^+$  is harnessed by a  $\text{Na}^+/\text{Cl}^-$  symporter in the luminal membrane that moves  $\text{Cl}^-$  into the epithelial cell against its electrochemical gradient. Chloride then exits the basolateral membrane passively via a  $\text{Cl}^-$  channel (Atherton, 2006; Jackson, 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).

The final concentration of the urine depends on the water permeability of the collecting ducts carrying the urine through the cortex and medulla (Nielsen *et al.*, 1999). ADH controls the permeability of these cells to water by regulating the insertion of pre-formed water channels (aquaporin-2, AQP<sub>2</sub>) 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 final urine (Ives, 2012).

### **1.3. Conventional Diuretics**

#### **1.3.1. Mechanism of diuretics and sites of action**

Diuretic drugs act at specific sites along the kidney tubule to inhibit specific sodium reabsorption pathway, thereby increasing renal salt and water excretion. They are categorized into different types based on their distinct Mechanisms and sites of action (Friedman and Hebert, 1997). Many Diuretics suppress sodium and water reabsorption by inhibiting the function of specific proteins that are responsible for the transportation of electrolytes across the epithelial membrane. Osmotic diuretics inhibit water and sodium reabsorption by increasing intratubular osmotic pressure (Michael, 2009). Figure 2 shows major site of diuretic action in renal tubule.

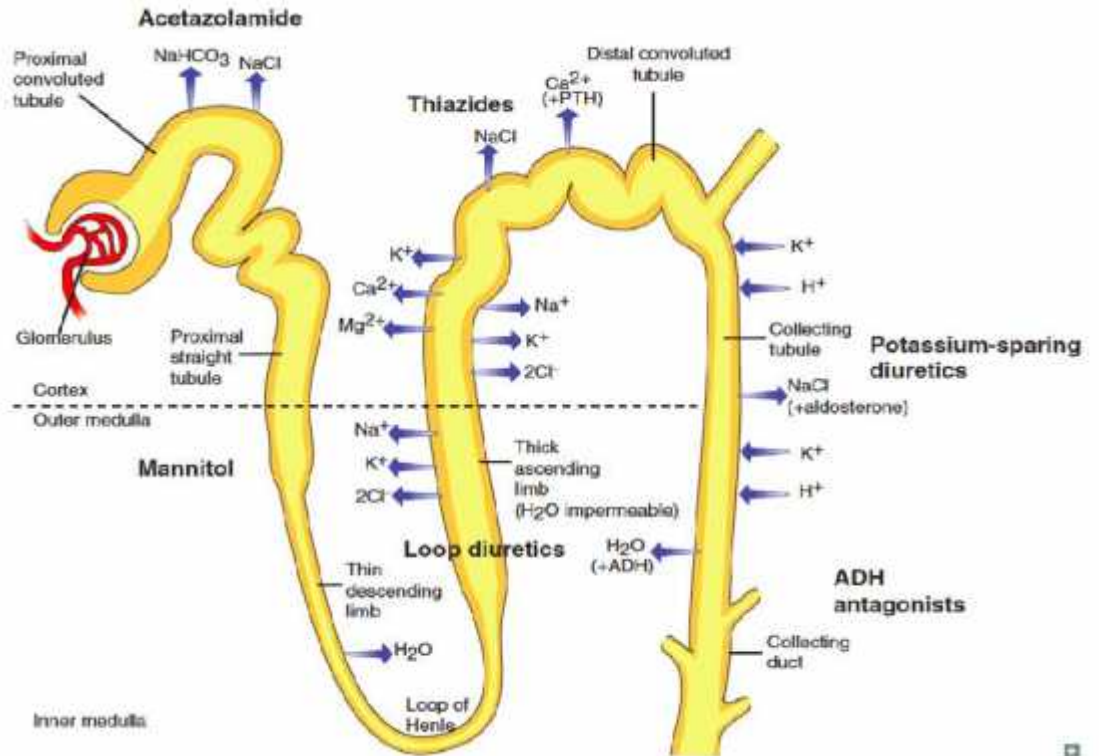


Figure 2: Tubule transport systems and sites of action of diuretics (Bhushan, 2013).

The pharmacological activity of drugs in this group depends entirely on the osmotic pressure exerted by the drug molecules in solution, and not on interaction with specific transport proteins or enzymes. They increase the osmotic pressure in the proximal tubule fluid and loop of Henle, thereby retarding the passive reabsorption of water. Mannitol is the prototypical osmotic diuretic. Other agents considered in this class include urea, glycerin and isosorbide (Garwood, 2009; Ellison, 2013).

CA inhibitors act primarily on proximal tubule cell to inhibit bicarbonate absorption by inhibiting the CA enzyme. They are the first useful orally active diuretics and include acetazolamide, metazolamide and dichlorphenamide (Friedman and Hebert, 1997). Owing inhibitory effect on CA in the proximal tubule, these agents increase bicarbonate

excretion by 25 – 30%. The increase in sodium and chloride excretion is minimal, as most of these ions are reabsorbed by more distal segments (Okusa and Ellison, 2008).

CA inhibitors have a limited therapeutic role as diuretic agents because of weak natriuretic properties. They are used primarily to reduce intraocular pressure in glaucoma and to enhance bicarbonate excretion in metabolic alkalosis (Knepper *et al.*, 2005).

The thick ascending limb (TAL) of loop of Henle actively reabsorbs sodium and potassium chloride via the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter that is responsible for 25% of salt reabsorption. It is not permeable to water and thus is a urine diluting segment. Active sodium reabsorption at TAL contributes to the hypertonicity in medullary interstitium. Loop diuretics act on the TAL of the loop of Henle, inhibiting the transport of sodium chloride out of the tubule into the interstitial tissue by inhibiting the co-transporter on the apical membrane (Smith, 2014).

The site of action of thiazide diuretics is in the distal convoluted tubule. These drugs inhibit an electro-neutral  $\text{Na}^+/\text{Cl}^-$  cotransporter located on the luminal surface. There is no direct effect of the thiazides on  $\text{K}^+$  transport in this segment. Rather, these agents are associated with increased renal  $\text{K}^+$  excretion through their effects to increase distal  $\text{Na}^+$  delivery in the setting of increased mineralocorticoid activity (Palmer and Naderi, 2007).

Potassium sparing diuretics act primarily at the cortical part of the collecting duct and to a lesser extent in the late distal and connecting tubules either by direct blockage of mineralocorticoid receptors (e.g., spironolactone and eplerenone) or blocking epithelial sodium channels in the luminal membrane (e.g., amiloride and triamterene). Since only a

small amount of sodium is reabsorbed here, these agents are capable of limited natriuresis (Ernst and Gordon, 2010).

### **1.3.2. Therapeutic application of Diuretics**

The use of diuretics for therapeutic purposes is not new. They were used for the treatment of dropsy as early as 16th century. Diuretic use in clinical practice spans conditions like edema, hypertension, metabolic acidosis and hyperkalemia. 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. Common clinical strategies to achieve a lowering of blood pressure include the use of diuretics (Gasparotto *et al.*, 2009).

Moreover, the use of diuretics is approved by Food and Drug Administration (FDA) for the treatment and management of life-threatening edema associated diseases such as hypertension, congestive heart failure (CHF), glaucoma, cirrhosis, nephritis syndrome, diabetes insipidus, pregnancy toxemia and liver ailments, the preference of diuretics for the treatment for edema is due to that only diuresis or saluresis breaks the cycle of salt and water retention by the kidney and reduces or eliminates it (Nigwekar and Waiker, 2011). 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).

Furthermore, recent insight into the use of diuretic agent described their application for acute decompensated heart failure (ADHF) as most patients admitted for this type of HF have clinical manifestations of extracellular fluid volume overload, and pulmonary edema, currently, ADHF treatment guidelines recommend using diuretics as first-line therapy and they remain the standard of care for treatment of congestion in patients with the disease (Givertz *et al.*, 2007; Freda *et al.*, 2011). 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).

The term acute renal failure (ARF) has been used to encompass a wide variety of clinical disorders ranging from glomerulonephritis to prerenal azotemia. It is generally defined as a rapid decline usually within hours to weeks in glomerular filtration rate (GFR) and retention of nitrogenous waste products (Kellum, 1997). Diuretic agents are frequently given to augment renal salt and water excretion in the setting of extracellular volume overload. They are also frequently given during ARF in an effort to convert oliguric to nonoliguric ARF, since oliguria has been recognized as a factor for the severity of ARF (Mehta *et al.*, 2002).

In cirrhosis, there is activation of the neurohumoral axis which leads to vasoconstriction. This condition initiates the kidney to begin retaining sodium and water, which is, at least in part, mediated by angiotensin and adrenergic receptors that result in edema. The events

lead to diminished delivery of sodium chloride to the mineralocorticoid receptor sites in the CD, which in turn results in failure of the normal aldosterone escape mechanism and the Na<sup>+</sup> retaining effect of the secondary hyperaldosteronism persists. Thus, inhibition of the secondary hyperaldosteronism with adequate doses of mineralocorticoid-receptor antagonists such as spironolactone has emerged as the primary diuretic therapy in cirrhotic patients (Bansal *et al.*, 2009;Schrier,2011).

### **1.3.3. Adverse Effects of Diuretics**

Depending on the site and mode of action, some diuretics increase excretion of potassium, chloride, calcium, bicarbonate, or magnesium. Some can reduce renal excretion of electrolyte-free water, or protons. Consequently, electrolyte and acid-base disorders commonly accompany diuretic use. Except for the mildly natriuretic collecting duct agents, which are used mainly to limit potassium excretion, all diuretics can cause volume depletion with prerenal azotemia (Greenberg, 2000). Loop and thiazide diuretics may lead to deficiency of the main electrolytes, particularly potassium and sodium. Hypokalemia and hyponatremia to a lesser degree may secondarily cause other metabolic effects. The degree of potassium wastage is directly related to the dose of diuretic in loop and thiazide diuretics (Ernst and Mann, 2011).

Hyponatremia is an important and potentially life-threatening event that can result from diuretic use. Thiazide diuretics seem to carry greater risk than loop diuretics due to their action at the distal segment where dilution occurs, but all diuretics classes are implicated (Sica and Carter, 2011). Additionally, potential toxic effects of loop diuretics are a usually reversible ototoxicity, interstitial nephritis, a few rare dermatological symptoms and arrhythmias due to drug-induced hypokalaemia (Schetz, 2004).

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, heart failure, 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).

#### **1.3.4. Diuretic Resistance**

Diuretic drugs are usually effective treatment for edema when used prudently. However, some patients become resistant to diuretic therapy when treatment with a potent diuretic drug fails to reduce fluid volume to the desired level (Ellison, 1997). The body responds to diuretic drug therapy in several different ways that can lead to diuretic resistance. Some of these responses cause the body to retain sodium and water in the short term (Asare, 2009).

Short term adaptations usually occur with short acting diuretics like furosemide in which urinary drug concentrations decline below the diuretic threshold abruptly. Thereafter, compensatory sodium retention occurs by the kidney during the rest of the day until the next dose of diuretic is administered. This is called post-diuretic salt retention. If sodium intake is high, post-diuretic salt retention can completely abolish the effect of the diuretic and a negative sodium balance is not achieved. Hence, restricting dietary NaCl is beneficial in unmasking the potential effect of the drug (Ellison, 1997; Bruyne, 2003).

For the long term increase of sodium and water retention “the braking phenomenon” is the adaptation to the drug that is due to changes in the structure and function of the kidney itself, activation of the sympathetic nervous system and changes in several hormone pathways (Asare, 2009).

Studies have shown that chronic administration of a loop diuretic induces hypertrophy and hyperplasia in epithelial cells of the DCT, leading to an increased reabsorption of sodium in this segment, thereby blunting the natriuretic effect. Moreover, the action of a diuretic drug to increase the elimination of sodium in one segment of the kidney may result in the delivery of an increased load of sodium to downstream portions of the kidney, which then increases sodium reabsorption (Bruyne, 2003). Addition of a distal diuretic agent such as thiazides to a loop diuretic often achieves a successful diuresis by blocking the nephron sites at which the hypertrophy occurs and also protect sodium reabsorption at distal sites (Opie and Kaplan, 2010).

## 1.4. Novel diuretics

### 1.4.1. Adenosine Receptor Blockers

Adenosine mediates its effects through activation of a family of four G-protein coupled adenosine receptors (ARs), named  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ . These receptors differ in their affinity for adenosine, in the type of G proteins, that they recruit, and finally in the downstream signaling pathways that are activated in the target cells. Among these receptors,  $A_1$  and  $A_{2A}$  ARs are potential therapeutic target for diuretics (Baraldi *et al.*, 2008; Muller and Jacobson, 2011). The  $A_1$  and  $A_{2A}$  receptors possess high affinity while the  $A_{2B}$  and  $A_3$  receptors show relatively lower affinity for adenosine.  $A_1$  and  $A_3$  are coupled to  $G_i$  proteins and inhibit adenylyl cyclase, while  $A_{2A}$  and  $A_{2B}$  receptors activate  $G_s$  proteins to stimulate adenylyl cyclase (Sheth *et al.*, 2014).

In the heart and other vascular beds, adenosine functions as a vasodilator through activation of  $A_{2A}$  receptors. In the kidney, however, activation of  $A_1$  receptors in the afferent arteriole results in vasoconstriction that reduces renal blood flow and glomerular filtration rate (GFR). In addition, adenosine has direct luminal effects, enhancing sodium reabsorption in the PCT (Vallon *et al.*, 2008).

Meanwhile, it was not surprising that blockade of  $A_1$  receptor decreases  $Na^+$  reabsorption and increases  $Na^+$  excretion (i.e.,  $A_1$  antagonists are diuretics). Indeed, selective blockade of renal  $A_1$  receptor rapidly (within minutes) and markedly (3 to 10 fold) increases urinary  $Na^+$  excretion in animals and humans with little or no effect on  $K^+$  excretion. These facts provide the rationale to develop  $A_1$  receptor antagonists as new class of diuretics that may prove useful for the treatment of a variety of cardiovascular disorders.

In fact, A<sub>1</sub> receptor antagonists are in development as diuretics for the management of chronic and acute heart failure and may be useful for other indications such as treatment of liver cirrhosis and hepato-renal syndrome (Jackson *et al.*, 2012). However, A<sub>1</sub> receptor antagonist, rolofylline, which once demonstrated as a promising diuretic (phase II clinical trials) in acute decompensated heart failure with renal impairment of diuretic resistance and in chronic congestive heart failure has withdrawn from study because of central nervous system toxicity and unexpected negative effects on GFR (Ives, 2012; Jackson *et al.*, 2012). Newer A<sub>1</sub> receptor inhibitors that are much more potent and more specific have been synthesized. Several of these (e.g., Avenri [BG9926] and BG9719) are under study and if found to be less toxic than rolofylline, may become available as diuretics (Ives, 2012).

#### **1.4.2. Vasopressin Antagonists**

Arginine vasopressin (AVP), also known as ADH, is a neuropeptide hormone synthesized by hypothalamic nuclei and secreted by the posterior pituitary to play a central role in setting serum and interstitial fluid osmolarity (Bugaj *et al.*, 2009; Aditya and Rattan, 2012). The biological effects of vasopressin are mediated by three receptor subtypes, all of which are members of the G-protein linked receptor family; V1a, V1b (V3) and V2 (Lehrich *et al.*, 2013). When release is stimulated, the circulating vasopressin binds to the vasopressin receptors 2 (V2R) on the principal cells of the collecting duct stimulates insertion of water channels on the apical membrane increasing reabsorption of water, thereby producing an antidiuresis. As a consequence of receptor inactivation, the synthesis and transport of aquaporin-2 water channel proteins into the apical membrane of the collecting duct cells is inhibited (Narayan *et al.*, 2012).

Tolvaptan is an oral, selective vasopressin V<sub>2</sub>-receptor antagonist that acts upon the distal nephron to promote aquaresis (Goldsmith and Gheorghide, 2005). Data from phase 2 trials have shown that this agent can relieve signs and symptoms of congestion and correct hyponatremia, without significantly altering blood pressure (BP) or renal function (Gheorghide *et al.*, 2005). The vaptans are classified into two as selective V<sub>2</sub>R antagonists (mozavaptan, lixivaptan, satavaptan, and tolvaptan) and nonselective vasopressin receptor antagonists (conivaptan) based on receptor selectivity (Jagadeesh *et al.*, 2014).

### **1.4.3. Urea Transporter Inhibitors**

Urea transporter inhibitors like phenylsulfoxyoxazole, benzenesulfonanilide, phthalazinamine, and aminobenzimidazole used as diuretics have potential clinical significance. Urea transporter inhibitors have a different mechanism of action from conventional diuretics, which block salt transport across kidney tubule epithelial cells. Urea transporter inhibitors 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 (Sands, 2013). By disrupting countercurrent mechanisms and intrarenal urea recycling, urea transport inhibitors, alone or in combination with conventional diuretics, may induce a diuresis in states of refractory edema where conventional diuretics are ineffective (Yu *et al.*, 2009). PU-48 evaluated in rat models shows that urea transporter inhibitors may be developed as a novel class of diuretics performing urea-selective diuresis without disturbing electrolyte excretion and metabolism (Yang, 2015).

## 1.5. Botanical Diuretics

Medicinal plants can be important sources of unknown chemical substances with potential therapeutic effects. Besides the world health organization estimated that over 75% of the world's populations still rely on plant-derived medicines, usually obtained on traditional healers for basic health-care needs (Roopesh *et al.*,2011).There is growing interest in the health benefits of herbs and botanicals. In line with this there are an increasing number of published articles claiming that plants or plant-derived actives may function as mild diuretic agents (Adil and Mishra, 2013).

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.*, 2007). Many investigators demonstrated that studies of herbals in folklore use as diuretics were in progressive evaluation and might be valuable tools used in human disease (Kumar *et al.*, 2010).

There are several plant species and genera reported to possess diuretic effects. Some of the promising plants includes *Olea europaea* (Somova *et al.*, 2003), *Rungia repens*(Basu and Arivukkarasu, 2006), *Petroselinum sativum* and *Spergulaa purpurea* (Jouadet *al.*, 2001), *Withania aristata* (Marti-Herrera *et al.*, 2007), *Smilax caninesis* (Abdalaet *al.*,2008), *Hibiscus sabdiffera* (Odigieet *al.*, 2003), *Costus speciosus*in (Prabhu *et al.*, 2014), *Cissampelo spareira* (Sayana *et al.*, 2014) and *Nigella sativa* (Asif *et al.*, 2015).

### 1.6. The experimental plant *Withania somnifera* (solanaceae)

The family Solanaceae comprises about 80 genera and 3000 species, from which 1500 belong to the genus *Solanum*. This genus is widespread over the world although it is concentrated mainly in the tropics and subtropics (Pérez *et al.*, 2007). Solanaceae, or commonly known as potato family is an economically important family of angiosperms with a global distribution. The family ranges from herbs to trees, and includes a number of important spices, weeds, ornamentals, crops, and medicinal plants out of which *Withania somnifera* commonly known as *Asgandh* or *Ashvaganadha* is one of the most potent aphrodisiacs used in traditional systems of medicine like Unani medicine and Ayurveda (Imtiyaz *et al.*, 2013).

*Withania somnifera* (Figure 3) belonging to the family Solanaceae is an evergreen, erect, branching, tomentosa shrub, 30-150 cm in height. The leaves are simple opposite, alternate and the tip of the leaf is acute, and glabrous up to 8 to 12 cm in length. Flowers are greenish of lurid yellow with diameter of 4-6 mm and 1 cm long. Roots are cylindrical, straight and unbranched with 1-2 cm thick (Saidulu *et al.*, 2014)



Figure 3. Aerial part of *Withania somnifera*

*Withania somnifera* or (Gisawa in Amharic), It is used for coughs and asthma, as a narcotic with anti-epileptic activity in Ethiopia and other traditional uses for headache (as a dressing), paludism (malaria), ague, fever, stomachache and as a diuretic (Hassan *et al.*, 2012).

*Withania somnifera* or Gisawa is also found to have antifertility properties and to be traditionally used as a vaginal douche (aqueous extract) for its uterotonic and anti-implantation activity on butanol fraction extract (Desta, 1994).

The root contains several alkaloids including withanine, withananine, withananinine, pseudo withanine, somnine, somniferine, somniferinine (Imtiyazet *al.*, 2013). Leaves are reported to contain withanone, somnitol, glucose, and inorganic salts containing chloride, sulfate, nitrate, sodium and potassium. Leaves are also reported to contain several chlorinated withanolides (Debet *al.*, 2006).

## 1.7. Rationale for the Study

Maintaining an appropriate extracellular volume is too important for overall health and wellbeing of all animals. However, this volume can be altered due to clinical conditions such as congestive heart failure, renal failure, hypertension, syndrome of inappropriate antidiuretic hormone (SIADH) and hypervolemic hyponatremia (Dipiro *et al.*, 2008).

The two commonly used diuretics, that is, thiazides and furosemide, have been associated with many side effects, like disturbances of electrolytes, acid-base and water balance, changes in uric-acid, carbohydrate and lipid metabolism and drug interactions (Khan *et al.*, 2012). Therefore, there is a need to look for safe diuretics.

Herbal medicines are considered to be more economical sources of drugs and also contain synergistic and/or side effects neutralizing potential (Gilani and Atta-ur-Rahman, 2005). Therefore, herbal diuretics can be considered as better therapeutic option, because of their relatively safer and milder actions as compared to diuretics used nowadays which produce several adverse effects due to their strong saluretic effects. As most of the plants contain potassium, along with other nutritional elements like  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ , etc (Jan, 2011). Hence, this study is intended to evaluate the diuretic activity of 80% methanol extract and solvent fractions of roots of *W. somnifera* in rats.

## **2. OBJECTIVES OF THE STUDY**

### **2.1. General Objective**

To evaluate the diuretic activities of 80% methanol extract and solvent fractions of the roots of *W. somnifera* in rats.

### **2.2. Specific Objectives**

- ❖ To evaluate the effects of the 80% methanol extracts and solvent fractions of the roots of *W. somnifera* on urine volume.
- ❖ To assess the electrolyte excretion (saluretic) effect of crude extract and fractional extract of *W. somnifera*
- ❖ To determine the pH of the urine
- ❖ To assess acute toxicity of the crude and solvent fractions of the *W. somnifera*
- ❖ To perform preliminary phytochemical analysis of the crude and fractions

### **3. MATERIALS AND METHODS**

#### **3.1. Drugs and Chemicals**

All solvents used for the extraction process are of laboratory grade. Drugs and chemicals used in the study include: hydrochlorothiazide (Remedica Ltd., Cyprus), Distilled water (Pharmacology lab in School of Medicine), Tween 80 (BDH Chemicals Ltd, England), Normal saline (EPHARM, Addis Ababa, Ethiopia), Absolute Methanol (Carlo Erba reagents, S.A.S, France), chloroform (BDH, Poole, England), butanol (BDH, Poole, England), (glacial acetic acid, H<sub>2</sub>SO<sub>4</sub>, Ammonia, HCl, Acetic anhydride, FeCl<sub>3</sub>, ethyl acetate, Mayer's and Dragendorff's reagents) were obtained from the Department of Pharmaceutical chemistry and Pharmacognosy.

#### **3.2. Experimental Animals**

Healthy Wistar albino rats of either sex, weighing 180 – 250 g were used for the experiment. The rats were obtained from animal house of Pharmacology Department (School of Medicine), AAU and of Ethiopian Public Health Institute (EPHI). The animals were housed in polypropylene cages (6–8 animals per cage) under standard environmental conditions (25±1 °C, 55±5% humidity and 12 h/12 h light/dark cycle). The animals were allowed free access to tap water and laboratory pellet *ad libitum*. 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 animals were in accordance with internationally accepted OECD-420 (2008) guidelines for use of animals.

### **3.3. Collection of the plant**

The roots of *Withania somnifera* were collected from Addis Ababa, Ethiopia (Bole bulbula), in July 2015. The plant was authenticated by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University (AAU) and a voucher specimen number KD001 was deposited for future reference.

### **3.4. Extraction of the plant material**

The roots of *Withania somnifera* was thoroughly washed with tap water to remove dirt and soil and then were sliced to smaller pieces and dried at room temperature in the shade for more than two weeks. The dried and crushed roots were then powdered finely and subjected to extraction.

#### **3.4.1. Hydro-alcoholic (80% methanol) extract**

The extraction was carried out by maceration technique using 80% methanol as a solvent. Accordingly, 600g of the dried powder was weighed using electronic digital balance and then divided into three portions in three different Erlenmeyer flask (200g each for ease of extraction) and was soaked in a closed flask with 200ml of the 80% methanol solvent for each extract in a period of 3 days with occasional agitation using shaker (Bibby scientific limited stone Staffo Reshire, UK), at room temperature. The resultant solution was combined and filtered through double layered muslin cloth followed by whatman (No.1) filter paper. The residue was re-macerated for the second and third times with fresh solvent for a total of 6 days in order to exhaustively extract the marc and to get a better yield. The extract was then concentrated using a rotary evaporator (BÜCHI Rota-vapor R-200, Switzerland) under reduced pressure at 40°C. The concentrated filtrate was frozen

in a refrigerator overnight and then freeze dried in the lyophilizer (Operon, Korea vacuum limited, Korea), to obtain freeze dried crude extract. Finally, the percentage yield of crude extract was found to be 13.65% (w/w), and the dried extract was used for further fractionation.

### **3.4.2. Solvent fractionation**

The 80% methanol extract of *W. somnifera* had better diuretic activity and was subjected further fractionation using chloroform, butanol and aqueous solvent based on their relative solubility. Accordingly, a total of 60g of 80% methanol crude extract of *W. somnifera* was dissolved in 250 ml of distilled water using separatory funnel. The dissolved hydro-alcoholic extract was partitioned with 250 ml chloroform and repeated until the chloroform layer becomes clear. The filtrate was concentrated in a rotary evaporator (Buchi Rota vapor, Switzerland at 80 rpm and 40<sup>0</sup>C) to obtain chloroform fraction and the yield obtained was 12.5 grams (20.8%). The aqueous residue was further partitioned with 250 ml n-butanol. The butanol filtrate was concentrated similarly as chloroform fraction to have butanol fraction and a yield of 14 gm (23.3%) was obtained. The remaining aqueous residue was frozed in deep freezer overnight and then freeze dried with a lyophilizer (Operon, Korea vacuum limited, Korea) and a total of 16.5 gm (27.5%) of aqueous fraction was obtained. All fractions were kept in tightly closed containers in refrigerator at -20<sup>0</sup>C until used for the experiment.

### **3.5. Acute toxicity test**

Acute toxicity test was performed according to the Organization for Economic Cooperation and Development (OECD) 425 (2008) guideline.

Female rats were used for the toxicity study. Initially, a single female rat was fasted food but not water for overnight and was loaded with 2000 mg/kg of the 80 % methanol extract as a single dose by oral gavage. It was then observed for any signs of toxicity within the first 24 h. Based on the results of the first rat; another 4 female rats were recruited and fasted for overnight with food but not water. Thereafter, they were given the same dose and were observed for any sign of toxicity or death in the next 14 days. After acute toxicity test, of the crude extract further acute toxicity test of the fractionation was done to check whether if the fractionations have change on the toxicity profile of the plant. After the conduct of acute toxicity test three dose levels were chosen to evaluate diuretic efficacy of the crude. A middle dose, which is one-tenth of the dose utilized during acute toxicity study; a low dose, which is half of the middle dose, and a high dose which is twice of the middle dose

### **3.6. Grouping and dosing the experimental animals**

The rats were randomly assigned into five groups of each with six animals to perform diuretic activities for both 80% methanol extract and solvent fractions. The first group was assigned as negative control and received with the vehicle used for reconstitution (10ml/kg of body weight). Positive controls were treated with standard drug, hydrochlorothiazide 10 mg/kg (HCT-10). The test groups were given three different

doses of extract as follows: the hydro-alcoholic extract at doses of 200 mg/kg (ME200), 400 mg/kg (ME400), 600 mg/kg (ME600).

For the solvent fractions, the test groups were treated with various doses of the fractions (200 mg/kg, 400 mg/kg and 600 mg/kg respectively. Dose selection was made based on the acute toxicity test performed prior to the beginning of the actual experiment.

The hydro-alcoholic extract as well as solvent fractions were reconstituted with appropriate vehicles and the solutions were prepared fresh on the day of the experiments.

### **3.7. Diuretic activity**

The method of Lahlou *et al.* (2007) was employed in the determination of diuretic activity. The rats were fasted for 18 h with free access to water. Before treatment, all animals received normal saline at an oral dose of 15 mL/kg body weight, to impose a uniform water and salt load (Benjumea et al., 2005). Each group was then administered the extract and vehicle orally by gavages. Immediately after administration, the rats were individually placed in a metabolic cage. During this period no food was made available to the animals. The urine was collected and measured at 1, 2, 3, 4, 5 and 24 h after dosing and stored at  $-20^{\circ}\text{C}$  for electrolyte analysis.

The following parameters were determined in order to compare the effects of the extracts with 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). 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, 2002).

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

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

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

### 3.8. Analytical Procedure

Sodium, potassium and chloride levels of urine were analyzed. The electrolyte concentrations were determined by using Ion Selective Electrode (ISE) analyzer (AVL 9181 Electrolyte Analyzer, Roche, Germany). A calibration was performed automatically prior to analysis with different levels of standards. Ratios of electrolytes;  $\text{Na}^+/\text{K}^+$  and  $\text{Cl}^-/\text{K}^+ + \text{Na}^+$  were calculated to evaluate the saluretic activity of the different extracts. In addition, pH was directly determined on fresh urine samples using a pH meter. Furthermore, the salt content of the extract was determined to rule out its contribution on urinary electrolyte concentration.

### **3.9. Preliminary phytochemical screening**

The qualitative phytochemical investigations of the crude extract, and chloroform, butanol and aqueous fractions of the roots of *Withania somnifera* were carried out using standard tests (Debella, 2002; Ayoola *et al.*, 2008). As described below.

#### **Test for terpenoids (Salkowski test)**

To 0.25g of each of the crude and solvent fractions was added by 2ml of chloroform. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

#### **Test for Saponins**

To 0.25 g of the crude extract and each fraction, 5 ml of distilled water was added in a test tube. Then, the solution was shaken vigorously and observed for a stable persistent froth. Formation of froth indicates the presence of saponins.

#### **Test for tannins**

About 0.25g of each fraction and crude extract was boiled in 10 ml of water in a test tube and then filtered. The addition of a few drops of 0.1% ferric chloride to the filtrate resulting in blue, blue-black, green or blue-green coloration or precipitation was taken as evidence for the presence of tannins.

#### **Test for flavonoids**

About 10ml of ethyl acetate was added to 0.25g of the crude extract and each fraction and heated on a water bath for 3min. The mixture was cooled and filtered. Then, about 4ml of the filtrate was taken and shaken with 1ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammoniacal layer indicated the presence of flavonoids.

### **Test for cardiac glycosides (Keller-Killiani test)**

To 0.25g of the crude extract and each fraction diluted to 5 ml in water was added 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was under lied with 1ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

### **Test for alkaloids**

About 0.25g of the crude extract and each solvent fraction was stirred with 5 ml of 1% HCl on a steam bath. 1 ml of the filtrate was treated with a few drops of Mayer's reagent and was similarly treated with Dragendorff's reagent. Turbidity or precipitation with both reagents was taken as preliminary evidence for the presence of alkaloids.

### **Test for Anthraquinones (Borntrager's Test)**

About 0.5g of sample of each plant extract was shaken with 5ml of chloroform and filtered. A 10% ammonium hydroxide solution (5ml) was added to the filtrate, and the mixture was shaken. The presence of a pink, red or violet color in the ammonical phase was taken as an indication of the presence of anthraquinones(Aiyelaagbe and Osamudiamen, 2009).

### **Test for steroids**

Two ml of acetic anhydride was added to 0.5g of each sample with 2 ml sulfuric acid. The color changed from violet to blue or green in some samples indicating the presence of steroids.

### **3.10. Statistical analysis**

Data are expressed as mean  $\pm$  standard error of mean (SEM). The experimental results were analyzed using the software Statistical Package for Social Sciences (SPSS), version 20. Comparison of urine volume, electrolyte concentration and statistical significance was determined by one way ANOVA followed by Turkey's Post Hoc multiple comparison test. Linear regression was done for dose dependency test. Significant differences was set at p values less than 0.05

## **4. RESULTS**

### **4.1. Acute toxicity test**

The 80% methanol extract as well as solvent fractions of the hydro-alcoholic root extract of *Withania somnifera* produced neither overt toxicity nor death during the 14 days observation period following oral administration of a single dose of 2000 mg/kg. This was confirmed by absence of tremor, weight loss, paralysis, or aversive behaviors. There was no sign of diarrhea and none of the treated rats were dead, indicating that 80% methanol extract and solvent fractions of the hydro-alcoholic root extract had a wider safety margin and LD<sub>50</sub> value greater than 2000 mg/kg in rats.

### **4.2. Diuretic activity: Effect on urine volume**

#### **4.2.1. 80% methanol extract**

Eighty percent of methanol extract of the roots of *Withania somnifera* produced diuresis (Table 1) which appeared to be dose-dependent ( $r^2 = 0.950$ ;  $p < 0.001$ ). ME200 did not produce better diuresis compared to control animals throughout the 24h period. Rat treated with ME400 had an increased diuresis starting from the 1<sup>st</sup> h of urine collection (62%,  $p < 0.01$ ) and maximum increase was observed at the 2<sup>th</sup> h of urine collection (77%,  $p < 0.001$ ) when compared to the control. However, the highest dose of ME600 produced diuresis which was significant at the 1<sup>st</sup> h (81%,  $p < 0.001$ ) and maximum diuresis was recorded at the 2<sup>nd</sup> h (102%,  $p < 0.001$ ) compared to the control.

HCT10 treated rat produced diuresis which was significant as compared to control group, starting from the 1<sup>st</sup> h (85%,  $p < 0.001$ ), and maximum was recorded at the 3<sup>rd</sup> h (122%,

$p < 0.001$ ). The standard drug HCT10 had a significant diuretic effect than that of ME200 ( $P < 0.001$ ), as well as when compared to the ME400 ( $p < 0.01$ ) and comparable effect with ME600 at the end of the 24 h observation. This could be seen from the diuretic activity of ME200, ME400, ME600 and HCT10 which was 0.6, 0.9, 1.02 and 1.00 respectively. When the different doses of the 80% methanol extract compared each other, the highest dose, ME600, produced diuresis which was significant starting from the first hour ( $p < 0.001$ ) and continued till the end of the 24<sup>th</sup> h as compared with ME200. With regard to the ME400 the ME600 produced a significant diuresis on the first hour and continued till the end of the 24<sup>th</sup> h ( $p < 0.01$ ). ME400 increased urine volume that produced significant level starting in the first five hour ( $p < 0.01$ ) and continued up to 24<sup>th</sup>h ( $p < 0.001$ ) when compared to ME200.

Table 1: Effect of 80% methanol extracts of *Withania somnifera* roots on 24 h urine volume in rats

Group	Volume of Urine (mL)						Diuretic Action	Diuretic Activity
	1 h	2 h	3 h	4 h	5 h	24 h		
Control	1.00±0.07	1.33±0.06	1.56±0.05	2.03±0.04	2.48±0.03	5.13±0.04	1.00	
HCT10	2.03±0.04 <sup>a3</sup>	2.55±0.04 <sup>a3</sup>	2.93±0.03 <sup>a3</sup>	3.39±0.06 <sup>a3</sup>	4.76±0.12 <sup>a3</sup>	9.25±0.06 <sup>a3</sup>	1.80	1.00
ME200	1.08±0.07 <sup>b3c3d3</sup>	1.43±0.04 <sup>b3c3d3</sup>	1.65±0.03 <sup>b3c3d3</sup>	2.21±0.06 <sup>b3c3d3</sup>	2.71±0.07 <sup>b3c3d3</sup>	5.56±0.04 <sup>b3c3d3</sup>	1.08	0.60
ME400	1.61±0.04 <sup>a3b3d3</sup>	2.00±0.05 <sup>a3b3d3</sup>	2.53±0.04 <sup>a3b3d3</sup>	3.10±0.03 <sup>a3b1d2</sup>	4.00±0.05 <sup>a3b2d3</sup>	8.24±0.21 <sup>a3b3d3</sup>	1.61	0.89
ME600	2.10±0.03 <sup>a3c3</sup>	2.66±0.06 <sup>a3c3</sup>	2.98±0.05 <sup>a3c2</sup>	3.45±0.08 <sup>a3c2</sup>	5.01±0.05 <sup>a3c3</sup>	9.40±0.07 <sup>a3c3</sup>	1.83	1.02

Each value represents mean ± S.E.M (n=6) and was analyzed by ANOVA followed by Tukey post hoc multiple comparison test.

<sup>a</sup>against control, <sup>b</sup>against standard, <sup>c</sup>against ME400 mg/kg, <sup>d</sup> against ME600 mg/kg; <sup>1</sup>:p < 0.05, <sup>2</sup>:p < 0.01, <sup>3</sup>:p < 0.001; ME200: Methanol extract 200 mg/kg, ME400: Methanol extract 400 mg/kg, ME600: Methanol extract 600 mg/kg, HCT10: hydrochlorothiazide 10 mg/kg, Control: animals treated with distilled water

#### 4.2.2. Aqueous and butanol fractions

Among the solvent fractions, only 600mg/kg and 400mg/kg of the aqueous fractions as well as the 400mg/kg and 600mg/kg of the butanol fraction and 600mg/kg chloroform fractions produced a significant diuresis ( $p < 0.01$ ). The aqueous fraction produced diuresis which appeared to be a dose dependent ( $r^2 = 0.946$ ;  $p < 0.001$ ). As shown in table 2. AF200 did not produce better diuresis compared to control animals throughout the 24h period. AF400 and AF600 produced an increased diuresis starting from the first hour ( $p < 0.01$ ) and ( $p < 0.001$ ) respectively when compared with control group. AF400 ( $p < 0.01$ ) produced a lower diuretic effect as compared to HCT10 with diuretic action of 1.57 vs 1.80, while AF600 had an effect comparable to HCT10 with diuretic action of 1.78 vs 1.80. when different doses of aqueous fraction treated rats compared; AF600 produced an increased diuresis which was significant starting from the first hour, and the maximum increase had occurred at the end of 24 h ( $p < 0.001$ ) when compared with AF200. AF600 produced an increased diuresis starting from the first hour ( $p < 0.05$ ) and continued till the 24 h ( $p < 0.001$ ) compared to AF400. AF400 produced diuresis that reached significant level starting from the first hour ( $p < 0.05$ ) and continued up to 24 h ( $p < 0.001$ ) compared with AF200.

The rats treated with butanol fraction also produced diuresis which appeared to be in a dose dependent manner ( $r^2 = 0.942$ ;  $p < 0.001$ ). As shown in Table 2. BF400 and BF600 produced an increased diuresis starting from the third and first hour ( $p < 0.01$ ), ( $p < 0.001$ ) respectively. BF400 with diuretic action of 1.7 ( $p < 0.01$ ) produced a lower diuretic effect as compared to HCT10 with diuretic action of 1.8, while BF600 had a better effect than BF400.

When different doses of butanol fraction treated rats compared; BF600 produced an increased diuresis which was significant starting from the first hour, and the maximum increase had occurred at the end of 24 h ( $p < 0.001$ ) when compared with BF200. BF600 produced an increased diuresis starting from the second hour ( $p < 0.01$ ) and continued up to 24 h ( $p < 0.001$ ) compared to BF400. BF400 produced diuresis that reached significant level starting from the third hour ( $p < 0.01$ ) and continued till 24 h ( $p < 0.001$ ) compared with BF200.

Table 2: Effects of aqueous and butanol fractions of *Withania somnifera* roots on 24 h urine volume in rats

Group	Volume of Urine (mL)						Diuretic Action	Diuretic Activity
	1 h	2 h	3 h	4 h	5 h	24 h		
Control	1.00± 0.07	1.33±0.06	1.56±0.05	2.03±0.04	2.48± 0.03	5.13± 0.04	1.00	
HCT10	2.03± 0.04 <sup>a3</sup>	2.55±0.04 <sup>a3</sup>	2.93±0.03 <sup>a3</sup>	3.39±0.06 <sup>a3</sup>	4.76± 0.12 <sup>a3</sup>	9.25± 0.06 <sup>a3</sup>	1.80	1.00
AF200	1.05±0.06 <sup>b3c1d3</sup>	1.40±0.05 <sup>b3c1d3</sup>	1.62±0.01 <sup>b3c2d3</sup>	2.15± 0.02 <sup>b3c2d3</sup>	2.58± 0.03 <sup>b3c3d3</sup>	5.48± 0.04 <sup>b3c3d3</sup>	1.07	0.59
AF400	1.36±0.05 <sup>a2b3d2</sup>	1.70±0.05 <sup>a2b3d3</sup>	2.13±0.08 <sup>a3b3d3</sup>	2.53± 0.08 <sup>a3b3d3</sup>	3.35± 0.08 <sup>a3b3d3</sup>	8.05± 0.06 <sup>a3b3d3</sup>	1.57	0.87
AF600	1.71±0.07 <sup>a3c1</sup>	2.15±0.04 <sup>a3c2</sup>	2.70±0.08 <sup>a3c3</sup>	3.05± 0.06 <sup>a3c3</sup>	4.66± 0.12 <sup>a3c3</sup>	9.12± 0.07 <sup>a3c3</sup>	1.78	0.98
BF200	1.02±0.05 <sup>b3f3</sup>	1.36±0.02 <sup>b3f3</sup>	1.60±0.02 <sup>b3e2f3</sup>	2.10± 0.02 <sup>b3e1f3</sup>	2.50± 0.03 <sup>b3e2f3</sup>	5.27± 0.04 <sup>b3e3f3</sup>	1.03	0.57
BF400	1.15±0.06 <sup>b3</sup>	1.51±0.04 <sup>b3f1</sup>	1.95±0.05 <sup>a2b3f1</sup>	2.36± 0.04 <sup>a2b3f1</sup>	3.03± 0.06 <sup>a2b3f3</sup>	7.69± 0.12 <sup>a3b3fe</sup>	1.50	0.83
BF600	1.41±0.05 <sup>a3</sup>	1.88±0.07 <sup>a3e1</sup>	2.26±0.07 <sup>a3e1</sup>	2.68± 0.08 <sup>a3e1</sup>	4.20± 0.11 <sup>a3e3</sup>	8.74± 0.09 <sup>a3e3</sup>	1.70	0.94

Each value represents mean ± S.E.M (n=6) and was analyzed by ANOVA followed by Tukey post hoc multiple comparison test.

<sup>a</sup>against control, <sup>b</sup>against standard, <sup>c</sup>against AF400 mg/kg, <sup>d</sup>against AF600 mg/kg, <sup>e</sup> against BF400 mg/kg, <sup>f</sup> against BF600 mg/kg <sup>1</sup>:p < 0.05, <sup>2</sup>:p < 0.01, <sup>3</sup>:p < 0.001; AF200: aqueous fraction 200 mg/kg, AF400: aqueous fraction 400 mg/kg, AF600: aqueous fraction 600 mg/kg, BF200: butanol fraction 200mg/kg, BF400: butanol fraction 400mg/kg, BF600: butanol fraction 600mg/kg, HCT10: hydrochlorothiazide 10 mg/kg, Control: animals treated with distilled water

### **4.2.3. Chloroform fraction**

In contrary the chloroform fraction was devoid of significant diuresis at the dose of CF200mg/kg and 400. However, significant diuresis was obtained at the dose of 600mg/kg ( $p<0.01$ ). CF600 produced an increased diuresis starting from the first hour ( $p<0.01$ ) and continued up to 24 h ( $p<0.001$ ). CF600 produced much lower diuretic effect as compared to HCT10 with a diuretic action of 1.56 vs 1.80.

Comparing the 80% Methanol extract and the solvent fractions (aqueous, butanol and chloroform), the 80% methanol extract had better diuretic activity than the solvent fractions. ME600 had a better effect than AF600 with a diuretic action of 1.83 vs 1.78 respectively.

Table 3: effect of chloroform fraction roots of *Withania somnifera* on Urine volume

Group	Volume of Urine (mL)						Diuretic Action	Diuretic Activity
	1 h	2 h	3 h	4 h	5 h	24 h		
Control	1.01±0.07	1.32±0.06	1.55±0.05	2.04±0.04	2.47± 0.03	5.13±0.04	1.00	
HCT10	2.03±0.04 <sup>a3</sup>	2.55±0.04 <sup>a3</sup>	2.93±0.03 <sup>a3</sup>	3.39±0.06 <sup>a3</sup>	4.76± 0.12 <sup>a3</sup>	9.25±0.06 <sup>a3</sup>	1.80	1.00
CF200	1.01±0.05 <sup>b3d2</sup>	1.35±0.02 <sup>b3d2</sup>	1.57±0.02 <sup>b3d2</sup>	2.05± 0.03 <sup>b3d2</sup>	2.49± 0.03 <sup>b3d3</sup>	5.19±0.04 <sup>b3d3</sup>	1.01	0.56
CF400	1.10±0.04 <sup>b3</sup>	1.40±0.05 <sup>b3d2</sup>	1.62±0.04 <sup>b3d1</sup>	2.10± 0.06 <sup>b3d1</sup>	2.60± 0.07 <sup>b3d2</sup>	6.10±0.06 <sup>b3d3</sup>	1.19	0.66
CF600	1.33±0.04 <sup>a2</sup>	1.73±0.08 <sup>a3c1</sup>	1.93±0.04 <sup>a2c1</sup>	2.33± 0.07 <sup>a2c1</sup>	3.15± 0.16 <sup>a3c2</sup>	7.98±0.15 <sup>a3c3</sup>	1.56	0.86

Each value represents mean ± S.E.M (n=6) and was analyzed by ANOVA followed by Tukey post hoc multiple comparison test.

<sup>a</sup>against control, <sup>b</sup>against standard, <sup>c</sup>against CF400 mg/kg, <sup>d</sup> against CF600 mg/kg; <sup>1</sup>:p < 0.05, <sup>2</sup>:p < 0.01, <sup>3</sup>:p < 0.001; CF200: chloroform fraction 200 mg/kg, CF400: chloroform fraction 400 mg/kg, CF600: chloroform fraction 600 mg/kg, HCT10: hydrochlorothiazide 10 mg/kg, Control: animals treated with distilled water (2%, tween80 for chloroform fraction)

### 4.3. Saluretic activity: Effect on electrolyte content of the urine

#### 4.3.1. 80% Methanol Extract

The cumulative urine samples collected in the 24 h were analyzed for the electrolyte content ( $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ ) and presented in (Table 4). ME400 (methanol extract 400 mg/kg) increased urinary sodium excretion by 54% which was significant compared to control group. Similarly, ME600 significantly increased sodium loss by 67% ( $p < 0.001$ ) compared to control. HCT10 increased significantly sodium excretion by 75% ( $p < 0.001$ ) compared to control group. Urinary potassium excretion was analyzed for all groups. ME400 resulted an increased potassium excretion by 32% ( $p < 0.001$ ) which was significant when compared control, while ME600 increased potassium by 37% ( $p < 0.001$ ) compared to control, and the maximum potassium excretion was resulted by HCT10 which is 46% ( $p < 0.001$ ) compared to control group.

In the case of chloride ion the three doses of 80% methanol extract resulted an increased excretion in the urine which was 30%, 58%, 77% for ME200, ME400 and ME600, respectively ( $p < 0.001$ ) when compared to control group as shown in Table 3.

Table 3, also shows that the saluretic indices of  $\text{Na}^+$  and  $\text{Cl}^-$  of the extract at the highest dose (ME600) and HCT10 were comparable (1.68, 1.77 vs 1.75, 1.80), while the saluretic index of  $\text{K}^+$  for the highest dose was smaller than HCT10 (1.37 vs 1.46), and the  $\text{Na}^+/\text{K}^+$  ratio of ME600 was higher than HCT10 (1.66 VS 1.63). In addition HCT10.  $\text{Cl}^-/\text{Na}^+ + \text{K}^+$  was also calculated and ME600 had the highest value (0.62).

Comparing the different doses of the extract, ME600 and ME400 had produced a significant natriuresis as compared with ME200 with  $p < 0.001$  and  $p < 0.01$  respectively,

and with regard to  $K^+$  excretion the ME600 and ME400 had also produced significant kaliuresis as compared to ME200  $p<0.01$  and  $p<0.05$  respectively. The excretion of chloride also resulted in a significant difference with  $p<0.001$  of the ME600 and ME400 when compared with ME200.

Table 4: Effect of 80% methanol extracts of *Withania somnifera* roots on urinary electrolyte

Group	Urinary Electrolyte Concentration (mmol/L)			Saluretic Index			Na <sup>+</sup> /K <sup>+</sup>	Cl <sup>-</sup> /Na <sup>+</sup> +K <sup>+</sup>
	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>		
Control	71.9±1.57	53.0±2.41	67.3±2.09				1.36	0.54
HCT10	126.0±1.71 <sup>a3</sup>	77.3±1.21 <sup>a3</sup>	121.0±1.90 <sup>a3</sup>	1.75	1.46	1.80	1.63	0.60
ME200	90.7±1.35 <sup>b3c3d3</sup>	59.3±1.88 <sup>b3c1d2</sup>	87.5±1.78 <sup>b3c3d3</sup>	1.26	1.12	1.30	1.53	0.58
ME400	110.4±1.50 <sup>a3b2d1</sup>	69.7±1.57 <sup>a3b2</sup>	106.0±1.74 <sup>a3b2d2</sup>	1.53	1.29	1.57	1.60	0.59
ME600	121.2±2.25 <sup>a3c1</sup>	72.6±2.14 <sup>a3b1</sup>	119.3±2.00 <sup>a3c2</sup>	1.68	1.37	1.77	1.66	0.62

Each value represents mean ± S.E.M (n=6) and was analyzed by ANOVA followed by Tukey post hoc multiple comparison test.

<sup>a</sup>against control, <sup>b</sup>against standard, <sup>c</sup>against ME400 mg/kg, <sup>d</sup>against ME600 mg/kg; <sup>1</sup>:p < 0.05, <sup>2</sup>:p < 0.01, <sup>3</sup>:p < 0.001; ME200: methanol extract 200 mg/kg, ME400: methanol extract 400 mg/kg, ME600: methanol extract 600 mg/kg, HCT10: hydrochlorothiazide 10 mg/kg, Control: animals treated with distilled water

#### **4.3.2. Aqueous and butanol fraction**

The aqueous fractions showed an increased pattern of urinary sodium excretion significantly at doses of 400mg/kg and 600mg/kg compared to control as presented in (Table 5). AF400 and AF600 increased sodium excretion by 44% ( $p<0.001$ ) and 57% ( $p<0.001$ ), respectively. Similarly, both AF400 and AF600 was also increased potassium and chloride excretion compared to control. AF400 enhance potassium and chloride excretion by 21% ( $p<0.05$ ) and 47% ( $p<0.001$ ) and AF600 increased by 33% ( $p<0.01$ ) and 63% ( $p<0.001$ ), respectively.

Sodium excretion of AF200 and AF400 was lesser as compared to HCT10, but AF600 had comparable effects and this is supported by the comparable saluretic indices of Na of the AF600 (1.56) and the standard drug (1.75). Potassium excretions for all doses were lower than HCT10. In the case of chloride excretion, there was a significant difference between the first two doses of AF200 and AF400 with ( $p<0.001$ ), when compared with HCT10. However, AF600 and HCT10 had comparable effect on chloride excretion.

The saluretic indices had also been calculated and among the aqueous fractions, the closer results were obtained for Na and Cl between AF600 and HCT10 (1.57, 1.63 vs 1.75, 1.80) respectively. From the calculated Cl/Na + K ratio, the AF200 provided the lowest value of 0.48.

When the different doses of Aqueous fraction were compared among each other, both AF400 and AF600 had resulted in significant Na excretion compared to AF200 with ( $p<0.01$ ) and ( $p<0.001$ ) respectively for both doses. AF600 had the highest kaliuresis compared to AF200 and AF400 with ( $p<0.001$ ) and ( $p<0.05$ ) respectively. AF600 also

caused chloride excretion that is significant level when compared with AF200 and AF400 with ( $p<0.001$ ) and ( $p<0.01$ ), respectively.

The effect of the butanol fraction on urinary electrolyte excretion was also depicted in Table 5. Sodium excretion was not significant by BF200 against the control, however it was significantly increased by BF400 (25%;  $p<0.001$ ) and BF600 (47%;  $P<0.001$ ) compared to controls. Sodium excretion resulted by HCT10 was found to be better to BF200 (62%;  $P<0.001$ ), BF400 (40%;  $P<0.001$ ) as well as BF600 (19%;  $p<0.01$ ). an increase of K excretion was noted was not significant for all the doses of butanol fraction. However, BF400 and BF600 enhance Cl excretion by (35%;  $p<0.001$ ) and (48%;  $p<0.001$ ).

When the different doses of the fraction were compared among each other BF400 was significantly higher Na excretion compared to BF200 (16%;  $p<0.05$ ), while BF600 exhibited significantly better Na excretion compared to BF200 (36%;  $P<0.001$ ).

The saluretic indices had also been calculated and among the butanol fractions, the closer results were obtained for  $\text{Na}^+$  and  $\text{Cl}^-$  between BF600 and HCT10 (1.47, 1.48 VS 1.75, 1.80) respectively. From the calculated  $\text{Cl}/\text{Na} + \text{K}$  ratio, the BF200 resulted the lowest value of 0.57.

Table 5: Effect of aqueous and butanol fractions of *Withaniasomnifera* roots on urinary electrolyte excretion in rats

Group	Urinary Electrolyte Concentration (mmol/L)			Saluretic Index			Na <sup>+</sup> /K <sup>+</sup>	Cl <sup>-</sup> /Na <sup>+</sup> +K <sup>+</sup>
	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>		
Control	71.9±1.57	53.0±2.41	67.3±2.09				1.36	0.54
HCT10	126.0±1.71 <sup>a3</sup>	77.3±1.21 <sup>a3</sup>	121.0±1.90 <sup>a3</sup>	1.75	1.46	1.80	1.63	0.60
AF200	83.2±2.45 <sup>b3c3d3</sup>	56.2±3.34 <sup>b3d1</sup>	80.9±2.23 <sup>b3c3d3</sup>	1.16	1.06	1.20	1.48	0.58
AF400	103.8±2.19 <sup>a3b3</sup>	63.9±2.42 <sup>a1b2</sup>	99.1±2.12 <sup>a3b3d2</sup>	1.44	1.21	1.47	1.62	0.59
AF600	112.8±2.71 <sup>a3b3</sup>	70.6±2.21 <sup>a3</sup>	109.8±2.32 <sup>a3b1c2</sup>	1.57	1.33	1.63	1.60	0.60
BF200	77.9±1.71 <sup>b3e3f3</sup>	54.7±3.11 <sup>b3</sup>	75.9±1.79 <sup>b3e3f3</sup>	1.08	1.03	1.13	1.42	0.57
BF400	90.1±2.00 <sup>a3b3f1</sup>	59.9±2.94 <sup>b3</sup>	91.0±1.39 <sup>a3b3</sup>	1.25	1.13	1.35	1.50	0.61
BF600	105.6±1.98 <sup>a3b3e1</sup>	64.8±1.93 <sup>a1b3</sup>	99.9±2.14 <sup>a3b3</sup>	1.47	1.22	1.48	1.63	0.59

Each value represents mean ± S.E.M (n=6) and was analyzed by ANOVA followed by Tukey post hoc multiple comparison test.

<sup>a</sup>against control, <sup>b</sup>against standard, <sup>c</sup>against AF400 mg/kg, <sup>d</sup>against AF600 mg/kg, <sup>e</sup> against BF400mg/kg, <sup>f</sup> against BF600mg/kg; <sup>1</sup>:p < 0.05, <sup>2</sup>:p < 0.01, <sup>3</sup>:p < 0.000; AF200: aqueous fraction 200 mg/kg, AF400: aqueous fraction 400 mg/kg, AF600: aqueous fraction 600 mg/kg, BF200: butanol fraction 200mg/kg, BF400: butanol fraction 400mg/kg, BF600: butanol fraction 600mg/kg, HCT10: hydrochlorothiazide 10 mg/kg, Control: animals treated with distilled water

### 4.3.3. Chloroform fraction

Among the chloroform fractions only the highest dose produced statistically significant increase (37%;  $p < 0.001$ ) in sodium excretion compared to control (Table 6). when the different doses of the chloroform fraction were compared among each other, CF600 exhibited better  $\text{Na}^+$  excretion than CF200 (30%;  $P < 0.01$ ) and CF400 (28%;  $p < 0.01$ ). Furthermore, it showed significantly better  $\text{Cl}^-$  excretion compared to CF200 (30%;  $P < 0.01$ ) and CF400 (25%;  $p < 0.01$ ). However, HCT10 showed significantly better  $\text{Na}^+$  and  $\text{K}^+$  excretion activity ( $p < 0.001$ ) than all doses of the chloroform fraction. With regards to  $\text{Cl}^-$  excretion, HCT10 still had a better excretion profile with all the dose of the fraction ( $p < 0.001$ ).

Table 6: Effect of chloroform fraction of *Withania somnifera* roots on urinary electrolyte excretion in rats

Group	Urinary Electrolyte Concentration (mmol/L)			Saluretic Index			Na <sup>+</sup> /K <sup>+</sup>	Cl <sup>-</sup> /Na <sup>+</sup> +K <sup>+</sup>
	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>		
Control	72.3±1.57	52.8±2.41	68.1±2.09				1.37	0.54
HCT10	126.0±1.71 <sup>a3</sup>	77.3±1.21 <sup>a3</sup>	121.0±1.90 <sup>a3</sup>	1.74	1.46	1.78	1.63	0.60
CF200	73.6±2.25 <sup>b3d1</sup>	53.4±2.59 <sup>b3</sup>	70.8±2.17 <sup>b3d1</sup>	1.02	1.01	1.04	1.38	0.56
CF400	75.1±1.91 <sup>b3</sup>	54.0±3.40 <sup>b3</sup>	73.9±2.18 <sup>b3</sup>	1.04	1.02	1.09	1.39	0.57
CF600	95.9±2.28 <sup>a3b3</sup>	62.9±2.10 <sup>b3</sup>	92.3±2.79 <sup>a1b3</sup>	1.33	1.19	1.36	1.52	0.58

Each value represents mean ± S.E.M (n=6) and was analyzed by ANOVA followed by Tukey post hoc multiple comparison test.

<sup>a</sup>against control, <sup>b</sup>against standard, <sup>c</sup>against CF400 mg/kg, <sup>d</sup>against CF600 mg/kg; <sup>1</sup>:p < 0.05, <sup>2</sup>:p < 0.01, <sup>3</sup>:p < 0.000; CF200: chloroform fraction 200 mg/kg, CF400: chloroform fraction 400 mg/kg, CF600: chloroform fraction 600 mg/kg, HCT10: hydrochlorothiazide 10 mg/kg, Control: animals treated with distilled water (2% tween80 for chloroform fraction).

#### **4.4. Electrolyte content of the extracts**

The electrolyte content of the extracts was examined ( $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$ ) using ISE analyzer in order to rule out possibility of interference, these water soluble salts could be present in the extracts and consequently interfere with the intrinsic diuretic effect of the plant. The result revealed that  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$  content in the 80% methanol extract and solvent fractions were below the detection level.

## 4.5. Urinary pH

The urinary pH was measured. The different treatment groups of hydro-alcoholic and solvent fractions had resulted in different urine pH.

### 4.5.1. Effect of hydro-alcoholic extract on Urine Ph

The pH profile of the groups treated with hydro-alcoholic extract had shown relatively alkaline as shown in Figure 4. For an increasing order from ME200 (7.84) to ME600 (8.35), while the control had produced the lowest pH (7.15) and the standard had produced relatively alkaline (7.52). But there was no any significant difference between the extracts and the control.

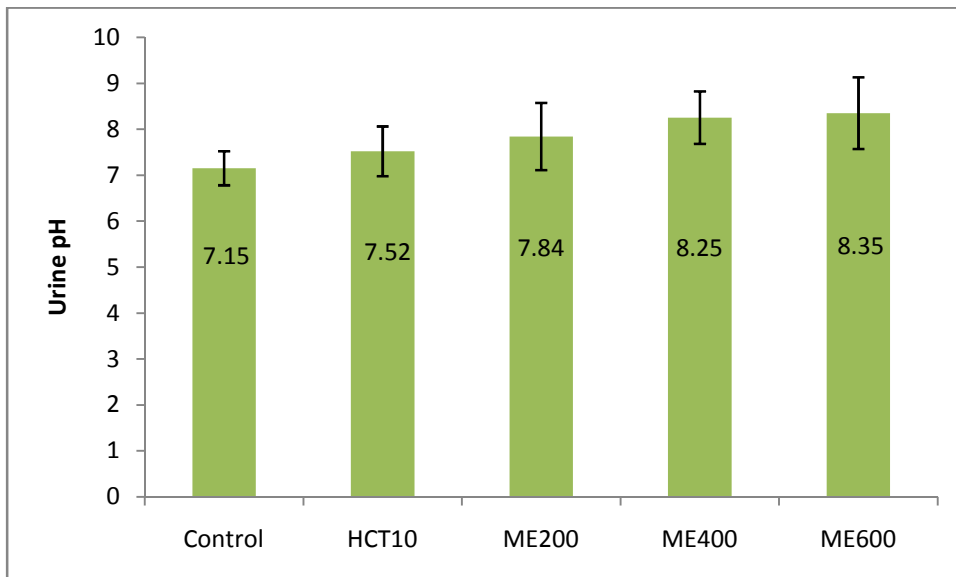


Figure 4: Urinary pH of rats treated with hydro-alcoholic extract of the root of *Withania somnifera*. ME, methanol extract of *Withania somnifera*; HCT10, hydrochlorothiazide 10 mg/kg; control received distilled water

#### 4.5.2. Effects of solvent fractions on urinary pH

The urinary pH measurement of the groups treated with aqueous and butanol fractions was shown to increase with dose while the chloroform fraction only the highest dose revealed an increased urine pH as shown in figure 5. For the aqueous fraction, the pH increased from AF200 (7.72) to AF600 (8.28). Similarly, the pH for the butanol fraction increased from BF200 (7.50) to BF600 (8.12), while the chloroform only CF600 was slightly alkaline. The control showed relatively neutral, where as the standard drug was slightly alkaline. The difference between the groups was found to be statistically insignificant.

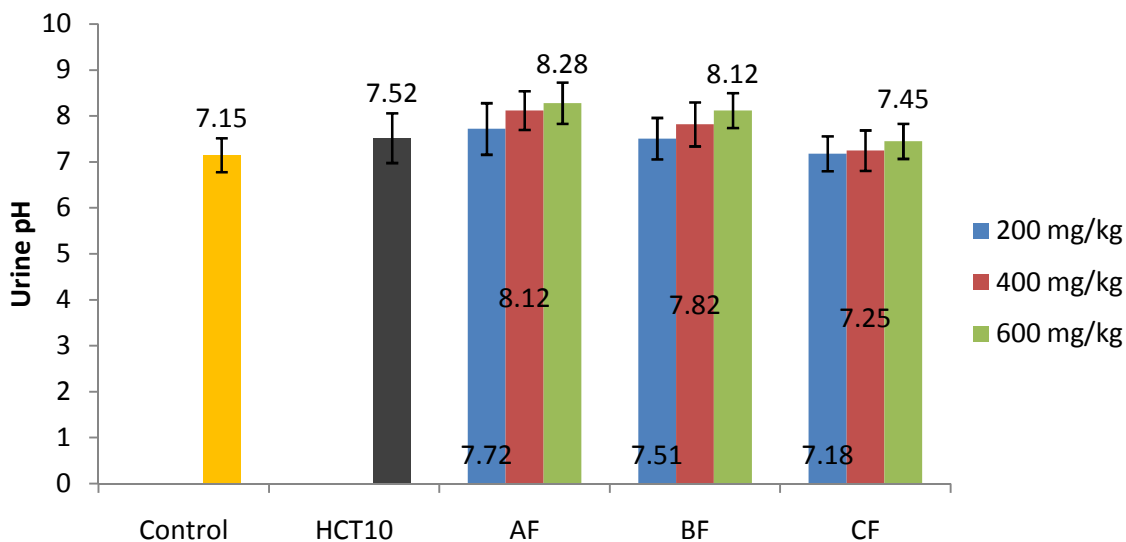


Figure 5: Urinary pH of rats treated with aqueous, butanol and chloroform fractions of the roots of *Withania somnifera*. AF, aqueous fraction; BF, butanol fraction; CF, chloroform fraction; HCT10, hydrochlorothiazide 10 mg/kg; and control received for the vehicle for reconstitution.

#### 4.6. Preliminary phytochemical screening

The result of phytochemical screening test was shown in Table 7. According to the result of qualitative phytochemical study, the crude extract of the roots of *Withania somnifera* was found to be positive for the presence of all of the tested secondary metabolites where as the aqueous fraction was positive for the presence of alkaloids, tannins, saponins, flavonoids, glycosides, and the butanol fraction was confirmed for the presence of alkaloids, tannins, saponins, glycosides, while the chloroform fraction was present by tannins, saponins, steroids, and terpenoids.

Table 7: Preliminary phytochemical screening of 80% methanol extract and solvent fractions of the hydro-alcoholic roots of *Withania somnifera*.

Secondary metabolite	Crude extract (80% methanol)	Solvent fractions			Vehicle for reconstitution	
		Aqueous	Butanol	Chloroform	Distilled water	Tween 80
Alkaloids	+++	++	+	-	-	-
Tannins	++	+	+	+	-	-
saponins	++	++	+	-	-	-
terpenoids	+	-	-	++	-	-
steroids	+	-	-	++	-	-
flavonoids	+	++	-	-	-	-
glycosides	+	+	+	-	-	-
anthraquinons	+	-	-	-	-	-

+ = present    - = absent    (+ = mild    ++ = moderate    +++ = high)

## 5. DISCUSSIONS

Diuretics alter physiologic renal mechanisms to increase the flow of urine together with greater excretion of electrolytes in the urine. Hence, diuresis is the sum of increase in urine volume and a net loss of electrolytes in the urine (Jackson, 2006). They act either by increasing the glomerular filtration rate (or) by decreasing the rate of reabsorption of fluid from the tubules (Kumar *et al.*, 2010). In the present study, therefore, both volume and electrolyte parameters were measured to evaluate the diuretic effect of 80% methanol extract and solvent fractions of *Withania somnifera* in rats. Thiazide diuretics such as hydrochlorothiazide can increase the urinary flow rate, although it's a moderate diuretic but its duration of action is longer compared to other diuretics like furosemide and as much as it increases urinary sodium and chloride excretion was used as positive control. 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 (Nedi *et al.*, 2004). Thus, the saline was administered to simulate edema. In view of urine output, the 80% methanol extract and solvent fractions showed an increase in diuresis that appeared to vary with dose and time. Compared to the solvent fractions, the 80% methanol extract produced a better diuretic effect. This difference in their diuretic activity could be seen in different doses used in the experiment. The lower doses of 80% methanol extract and solvent fractions did not produce an effect throughout the experiment, but the middle dose of 80% methanol extract and aqueous fraction was able to produce significant effect beginning from the 1<sup>st</sup> h, but the middle dose of butanol fraction produce significant effect starting from

The 3<sup>rd</sup> h, while the chloroform fraction only the highest dose produce significant effect beginning from the 1<sup>st</sup> h. This could probably suggest that the lower doses of both extract might represent minimum doses that cannot elicit diuresis, 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. Increasing the dose did affect the diuretic effect produced by the extract. For example the diuretic effect produced by ME600 was higher than that achieved by ME400 ( $5.01 \pm 0.06$  vs  $4.00 \pm 0.05$ ) after 5 h (Table 1). Moreover, the diuretic activity (0.90) of ME400 was lower than that of ME600 (1.02). on the other hand, AF600, BF600 and CF600 produced a diuretic effect of ( $4.66 \pm 0.12$ ), ( $4.20 \pm 0.11$ ) and ( $3.15 \pm 0.16$ ) respectively, which was still lower than the diuretic effect of ME600 after 5 h. its therefore, possible to suggest that the active principles of the plant responsible for the diuretic effect could probably be more polar and semi polar that synergies in the 80% methanol extract.

The diuretic activity of the extracts of *W. somnifera* at their higher corresponding doses was a moderate type for the 80% methanol extract and mild for the solvent fractions as their values were 1.02, 0.98, 0.94 and 0.86 for ME600, AF600, BF600 and CF600 respectively. The diuretic activity is considered to be good if the diuretic activity value is greater than 1.50, moderate if the value is between 1.00 and 1.50, mild if the value lies between 0.72 and 1.00, and nil if the value is  $<0.72$  (Vogel., 2007).

The effect of the extract had shown an increased electrolyte excretion in proportion to water excretion. Thus it is reasonable to propose that the diuretic effect of the fractions of the plant *W. somnifera* was a saluretic type, in contrast to aquaretic type typical of most phytodiuretic agents (Martin-Herrera *et al.*, 2008; Praveen *et al.*, 2013).

The middle and higher doses of the 80% methanol extract as well as aqueous and butanol fractions produced significant increase in  $\text{Na}^+$  and chloride excretion while only the highest dose of chloroform fraction resulted in significant difference in  $\text{Na}^+$  and  $\text{Cl}^-$  excretion compared to control. The ratio  $\text{Na}^+/\text{K}^+$  was calculated as an indicator for natriuretic activity. Values greater than 2.0 indicate a favorable natriuretic effect, while ratios greater than 10.0 indicate a potassium-sparing effect (Tirumalasetty *et al.*, 2013).  $\text{Na}^+/\text{K}^+$  values for the highest doses of the 80% methanol extract, aqueous, butanol and chloroform fractions were 1.66, 1.60, 1.58 and 1.52, respectively. This indicates that the extracts increased sodium excretions compared to control groups.

Regarding,  $\text{K}^+$  excretion. ME400, ME600, AF400, AF600, BF600 and CF600 resulted significant  $\text{K}^+$  excretion compared to control. This suggests that the components in the methanol extract and solvent fractions are not acting as a potassium sparing agents. This finding is in line with the aqueous and 80% methanol extracts of leaves of *Costus speciosus* (Prabhu *et al.*, 2014) and solvent fractions of the plant *Holarrhena antidysenterica* (Khan *et al.*, 2012). Which demonstrated to be devoid of  $\text{K}^+$ -sparing effect. Some herbal diuretics induce diuresis by stimulating the thirst center in the hypothalamus and thereby enhancing the fluid intake (Jayakody *et al.*, 2011). Such mode of action is unlikely to work in this study, since the rats had no access to fluid intake during the 5 h experimental period. Thus, the crude and fractions exerted their diuretic activity possibly by inhibiting tubular reabsorption of water and accompanying electrolytes; as such action has been hypothesized for some other plant species (Bhavin *et al.*, 2011; Khan *et al.*, 2012). The possibility of direct action of the potassium content of the extract on diuresis is ruled out as the  $\text{K}^+$  concentration of the fraction was very low

compared to salt content obtained in other plants with diuretic effect (Sripanidkulchai *et al.*, 2001). Instead it could be due to the intrinsic ability of the phytochemicals of *Withania somnifera* to exert a diuretic activity.

The ratio  $\text{Cl}^-/\text{Na}^+ + \text{K}^+$  was calculated to estimate CA inhibition. CA inhibition can be excluded at ratios between 1.0 and 0.80. With decreasing ratios slight to strong CA inhibition can be assumed (Vogel, 2007; Tirumalasetty *et al.*, 2013). The  $\text{Cl}^-/\text{Na}^+ + \text{K}^+$  value for the highest doses of the hydro-alcoholic extract, aqueous, butanol and chloroform fractions was 0.62, 0.60, 0.59 and 0.58, respectively. Therefore, it is reasonable to suggest that one of the possible mechanism of action of the extract is CA inhibition. Alkalanization of the urine is also one of the possible effect resulted from inhibition CA enzyme. Therefore, measurement of urinary pH indicated a relatively higher pH for the treatment rat than the negative controls. Components which act on the collecting tubule could increase urinary pH (Ives, 2012). However, the potassium wasting effect of the crude and solvent fractions ruled out the possibility of having similar mechanism to that of the potassium sparing diuretics. Thus, this rise in urinary pH supports the notion that CA inhibition could be one possible mechanism of action of the extracts.

On the other hand, the extracts have rapid onset of diuretic action of the middle and higher doses of the methanol extract and aqueous fraction while only the highest doses of butanol and chloroform fractions resulted in rapid onset of diuretic action, this indicates a fast absorption from the GIT, in addition it had a long duration of action as it produced significant effect from the 1<sup>st</sup> h ( $p < 0.001$ ) to 24<sup>th</sup> h ( $p < 0.001$ ) of the experiment.

Such a fast onset of action is therapeutically desirable as it would result rapid response and the long duration of action is important to decrease the frequency of administration.

The active principle/s responsible for the diuretic effects of the 80 % methanol and solvent fractions of the plant *W. somnifera* is/are, so far, not known, so it is not identified which compounds are exactly responsible for the diuretic, natriuretic and kaliuretic activities of *W. somnifera*. Previous studies have demonstrated that there are several compounds which could be responsible for the diuretic effect of medicinal plants such as, flavonoids, terpenoids, glycosides (Rohini *et al.*, 2009; Swain *et al.*, 2008; Gasparroto *et al.*, 2011). Saponins (Meera *et al.*, 2009). Anthraquinones (Freitas *et al.*, 2011), alkaloids and steroids (Bhavin *et al.*, 2011). 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 (Martin-Herrera *et al.*, 2008; Abdala *et al.*, 2012). Preliminary phytochemical analysis done on the crude extract and solvent fractions revealed the presence of the above phytochemicals distributed in the crude and fractions. The crude extract showed to present all the bioactive secondary metabolites. Aqueous fraction was revealed to contain phytochemicals like alkaloids, tannins, saponins, flavonoids, and glycosides. The butanol fraction was tested positive for the presence of alkaloids, tannins, saponins and glycosides and the chloroform fraction was found to be positive for tannins, saponins, steroids and terpenoids. As a result, it is reasonable to suggest that these phytochemicals mentioned above may be responsible acting individually or synergistically, for the diuretic activity of *Withania somnifera*.

Flavonoids and saponins appeared to be responsible for the better diuretic activity of the crude extract and aqueous fraction. Flavonoids produce an increase in water excretion and lower blood pressure by promoting vasodilation in the afferent arterioles of the renal vasculature and thus increasing GFR, in addition to promoting GFR, these compounds have an ability to increase renal water and solute excretion by reducing tubular reabsorption of water and accompanying anions (Jouad *et al.*, 2001). Saponins are known to have several physiologic activities, depending on their chemical structures, such as hemolytic properties, alteration of membrane permeability and, in particular the modulation of renal sodium excretion (Amuthan *et al.*, 2012; Bhusan *et al.*, 2012).

Studies have showed that saponins isolated from various plants inhibit the furosemide-sensitive Na<sup>+</sup>-ATPase that has been demonstrated in the basolateral membrane of PCT of the kidney which is responsible for trans-cellular sodium reabsorption (Suoza *et al.*, 2004; Dinizet *et al.*, 2012), showing why the methanolic extract and aqueous fraction had a better saluretic activity.

In summary, the present study revealed that the methanolic extract and aqueous fraction possessed a comparable diuretic activity with that of the standard hydrochlorothiazide. Therefore, this study supports the ethno medical use of *W.somnifera* for its diuretic effect. Finally, results obtained from the different fractions revealed that there was an increase in the volume of urine output as the polarity of the fractions increased; therefore, this data seems to indicate that the diuretic effect of the plant is distributed to more polar bioactive principles contained in the aqueous and butanol fractions. So, this result is concordant with some similar studies that showed more polar compounds for the observed diuretic activity (Khan *et al.*, 2012)

## 6. CONCLUSION

The present study provide support for the traditional use of *W. somnifera* as a diuretic agent, and the crude as well as the fractions had possessed varying degree of diuretic activity, with the methanol extract being the most effective extract followed by the aqueous fraction. The most nonpolar chloroform fraction was associated with the least diuretic and saluretic activity. This difference in the diuretic effect of the methanol and the fractions might be related due to their difference in the secondary metabolites. Although the bioactive components which is responsible for the observed effect is not known, polar or semi polar constituents singly or in synergy may act by multiple mechanisms to produce the observed effect. Finally, the data from the experiment indicate that the methanol extract and solvent fractions of *W. somnifera* roots have showed an increase in urine and electrolyte excretion as well as safe nature of the plant supports its traditional use for dropsy and urine retention.

## 7. RECOMMENDATIONS

- ➡ 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/fractions
- ➡ *In vitro* studies of the fractions on isolated tissue preparations should be done to support the *in vivo* methods
- ➡ Further studies should be done to isolate and identify pharmacologically active principle (s) responsible for the diuretic activities of the plant
- ➡ Understanding the precise site(s) of action, the molecular and cellular mechanism(s) by which the active compounds in the plant *W. somnifera* affect diuresis and urinary electrolyte excretion are necessary.

## 8. REFERENCES

- Abdala S, Martin-Herrera D, Benjumea D, Gutierrez S. (2012). Diuretic activity of some *Smilax canariensis* fractions. *Journal of Ethnopharmacology*; 140:277-281.
- Abdala, S., Martin-Herrera, D., Benjumea, D., Perez-Paz, P., (2008), Diuretic activity of *Smilax canariensis*, an endemic Canary Island species. *J.Ethnopharmacology* 119; 12–16.
- AdilMT, Mishra G.(2013). Ethnobotany and diuretic activity of some selected Indian medicinal plants: A scientific review. *The Pharma Innovation Journal*. Vol. 2(3)
- Aditya S, Rattan A. (2012) Vaptans: A new option in the management of hyponatremia. *Int. J. Appl. Bas. Med Res*; 2(2): 77-83
- Amuthan A, Chogtub B, Bairy LK, Sudhakar S, Prakash M (2012). Evaluation of diuretic activity of *Amaranthus spinosus* Linn. Aqueous extract in Wistar rats. *Journal of Ethnopharmacology*; 140:424-427.
- Anthony L. Mescher, 2010. Junqueira's basic histology, 12<sup>th</sup> edition. Bloomington, Indiana, McGraw-Hill. pp.
- Ariel, J. Reyesa, T., Leary, W.P., Crippac G., Mario, F.C. Maranhão, Hernandez R.H., (2005), The aldosterone antagonist and facultative diuretic eplerenone; A critical review, *European Journal of Internal Medicine* 16 :145– 153..
- Asare K. (2009) Management of loop diuretic resistance in the intensive care unit. *Am J Health-Syst Pharm*; 66:1635-1640
- Asif M, Jabeen Q, Majid AM, Atif M. (2015) Diuretic Activity of aqueous extract of *Nigella sativa* in albino rats. *Acta Poloniae Pharm- Drug Res*; 72 (1): 129-135

- Atherton JC, (2012). Renal blood flow, glomerular filtration and plasma clearance. *Anesthesia and Intensive Care Medicine* **13**(7): 315-319.
- Ayoola GA, Coker HA, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, *et al* (2008). Phytochemical screening and antioxidant activities of some selected medicinal Plants used for malaria therapy in Southwestern Nigeria. *Trop J Pharm Res* **7**(3): 1019-1024.
- Bansal, S., Lindenfeld, J.A., Schrier, R.W., (2009). Sodium retention in heart failure and cirrhosis: potential role of natriuretic doses of mineralocorticoid antagonist. *Circ Heart Fail*; **2**:370-6.
- Baraldi GP, Tabrizi A, Gessi S, Borea AP. (2008) Adenosine receptor antagonists: Translating medicinal chemistry & pharmacology into clinical utility. *Chem. Rev*; **108**(1): 238-263.
- Basu S.K., Arivukkarasu R., (2006), Acute toxicity and diuretic studies of *Rungia repens* aerial parts in rats; *Fitoterapia*, **77**; 83–85.
- Benjumea D, Abdala S, Hernandez-Luis, F, Perez-Paz, P, Martin-Herrera D. (2005) Diuretic activity of *Artemisia thuscula*, *J Ethnopharmacol*; **100**: 205–209.
- Bhavin VA, Ruchi VB, Shrikant JV, Payal SD and Dev SD, (2011). Effect of aqueous extract of *Pergulariadaemia* on urine production. *Der Pharmacia Lettre* **3**(4): 207-214.
- Bhusan HS, Kumar A, Ashish TF, Lal MK (2012). Evaluation of polyherbal formulation for diuretic activity in albino rats. *Asian Pacific Journal of Tropical Disease*; **S442-S445**.
- Bhushan Le, T., V., Sochat, M. 2013. First Aid for the USMLE STEP 1 2014, New York, McGraw Hill Medical.
- Bruyne DM. (2003). Mechanisms and management of diuretic resistance in congestive heart failure. *Postgraduate Medical Journal*; **79**:268-271.

- Bugaj V, Pochynyuk O and Stockand JD (2009). Activation of the epithelial Na<sup>+</sup> channel in the collecting duct by vasopressin contributes to water reabsorption. *American Journal of Physiology Renal Physiology* 297(5): F1411-F1418.
- Costantini F and Kopan R (2010). Patterning a Complex Organ: Branching Morphogenesis and Nephron Segmentation in Kidney Development. *Developmental cell* 18: 698-712.
- Deb L, Panchawat S, Jain A, Gupta VB. (2006). Acute diuretic activity of *Withaniasomnifera* (L.) Dunal leaves in normal rats. *An Indian journal*. Vol. 2, issue 3-4.
- Debella A (2002). Manual for phytochemical screening of medicinal plants. Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia: 35-47.
- Desta, B., 1994. Ethiopian traditional herbal drugs. Part III: Anti-fertility activity of 70 medicinal plants. *Journal of Ethnopharmacology* 44, 199-209.
- Diniz LRL, Portella VG, Cardoso FM, Souza AM, Caruso-Neves C et al (2012). The effect of saponins from *Ampelozizyphusamazonicus* Ducke on renal Na<sup>+</sup> pumps' activities and urinary excretion of natriuretic peptides. *Bio Med Central Complementary and Alternative Medicine* 12(40): 1-7.
- Dipiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG and Posey LM (2008). Cardiovascular and renal disorders. In: pharmacotherapy a pathophysiologic approach 7<sup>th</sup> edition. New York, McGraw-Hill.
- Ellison DH (2013). Physiology and pathophysiology of diuretic action. In: Alpern RJ, Caplan MJ and Moe OW (eds). Seldin and Giebisch's the kidney physiology and pathophysiology 5<sup>th</sup> edition. New York, Elsevier: 1353-1404.
- Ellison, D.H., (1997). Adaptation to Diuretic Drugs. In: Seldin, D.W, Giebisch, G.H. Diuretic Agents: Clinical Physiology and Pharmacology, Academic Press. p209-232.

- Ernst ME and Gordon JA (2010). Diuretic therapy: key aspects in hypertension and renal disease. *Public health and preventive nephrology* 23(05): 487-493.
- Ernst, M.E., Mann, S.J., (2011). Diuretics in the Treatment of Hypertension. *SemNephrol.*, 31(6): 495-502.
- Faris, R., Flather, M., Purcell, H., Henein, M., Poole-Wilson, P., (2002), Current evidence supporting the role of diuretics in heart failure: a meta-analysis of randomised controlled trials; *International Journal of Cardiology*: 82,149–158.
- Freda, B.J., Slawsky, M., Mallidi, J., Braden, G.L (2011). Decongestive treatment of acute decompensated heart failure: Cardiorenal Implications of Ultrafiltration and Diuretics. *Am J Kidney Dis.*; 58(6):1005-1017.
- Freitas PC, Puccib LL, Vieiraa MS, Lino RS, Oliveirad MC et al. (2011). Diuretic activity and acute oral toxicity of *Palicourea coriacea* (Cham) K Schum. *Journal of Ethnopharmacology* ; 134:501-503.
- Garwood S (2009). Osmotic Diuretics. In: Ronco C, Bellomo R and Kellum JA (eds). *Critical Care Nephrology* 2<sup>nd</sup> edition. New York, Elsevier
- Gasparotto A.J., Boffo M.A., Botelho E.L. (2009), Natriuretic and diuretic effects of *Tropaeolum majus* (Tropaeolaceae) in rats; *Journal of Ethnopharmacology*.122; 517–522.
- George CRP (2011). Mercury and the kidney. *Societaltaliana di Nefrologia*24(S17): S126-S132.
- Gheorghiade, M., Orlandi, C., Burnett, J.C., et al. (2005) Rationale and design of the multicenter, randomized, double-blind, placebo-controlled study to evaluate the efficacy of vasopressin antagonism in heart failure: Out- come study with tolvaptan (EVEREST). *Journal of Cardiac Failure*, 11, 260-269.
- Gilani AH, Atta-ur-Rahman (2005). Trends ethnopharmacol. *J. Ethnopharmacol.*, 100: 43-49.

- Givertz MM, Massie BM, Fields TK, Pearson LL, Dittrich HC. (2007). The effects of KW-3902, an adenosine  $A_1$ R antagonist, on diuresis and renal function in patients with acute decompensated heart failure and renal impairment. *J Am Cardiol*; 50 (6):1551–1560.
- Goldsmith, S.R. and Gheorghide, M. (2005), Vasopressin antagonism in heart failure. *Journal of the American College of Cardiology*, 46, 1785-1791.
- Greenberg A. (2000). Diuretic complications. *Am J Med Sci*; 319:10-24.
- Harlan, I. (2007), The diuretic agents. In: Katzung et al (eds.), Basic Clinical Pharmacology. 10<sup>th</sup> ed. New York.
- Hassan S, Dwivedi V, Misra M, Prashant KS, Hashmi F, Ahmed T. (2012). Antiepileptic activity of some medicinal plants. *International journal of aroma plants*. Vol. 2(2): 354-360
- Imtiyaz S, Ali J, Aslam M, Tariq M, Shahid SC. (2013). *Withaniasomnifera*: a potent unani aphrodisiac drug. *International research journal of pharmaceutical and applied sciences*. Vol. 3(4): 59-63.
- Ives HE (2012). Cardiovascular-renal drugs. In: Katzung BG. Masters SB and Trevor AJ (EDS). Basic and clinical pharmacology 12<sup>th</sup> edition. New York, McGraw-HILL: 251-270.
- Jackson EK, Cheng D and Mi Z (2012). Endogenous adenosine contributes to renal sympathetic neurotransmission via postjunctional  $A_1$  receptor-mediated coincident signaling. *American Journal of Physiology Renal Physiology* 302(4): F466-F476.
- Jackson EK. 2006. Drugs affecting renal and cardiovascular function. In: Brunton LL, Lazo JS, Parker KL (Eds.), Goodman and Gilman's the Pharmacological Basis of Therapeutics, 11th Ed, McGraw-Hill Companies, New York, pp. 737-766
- Jagadeesh JS, Muthiah NS, Muniappan M. (2014) Vasopressin receptors and drugs: A brief perspective. *Global J. Pharmacol*; 8 (1): 80-83

- Jan FG (2011). Nutritional and elemental analyses of some selected fodder species used in traditional medicine. *Afr. J. Pharm. Pharmacol.*, 5: 1157-61.
- Jayakody R, Ratnasooriya WD, Fernando W, Weerasekera KR. (2011) Diuretic activity of hot water infusion of *Rutagraveolens* L. in rats. *J.Pharmacol.Toxicol*; 6(5): 525-532
- Jouad H., Lacaille-Dubois M.A., Eddouks M., (2001), Chronic diuretic effect of the water extract of *Spergularia purpurea* in normal rats; *Journal of Ethnopharmacology* 75 ;219–223.
- Kellum JA. (1997). Systematic review: The use of diuretics and dopamine in acute renal failure: a systematic review of the evidence. *Kellum Critical Care*; 1:1-8.
- Khan A, Bashir S, Gilani AH.(2012) An in vivo study on the diuretic activity of *Holarrhena antidysenterica*. *Afr. J. Pharm. Pharmacol*; 6(7): 454-458
- Knepper MA, Kleyman T, Gamba G.(2005) Diuretics: Mechanisms of Action. In: Hypertension: a companion to Brenner and Rector's the kidney, 2<sup>nd</sup> ed. Philadelph: *Elsevier*, pp638-652.
- Kumar BN, VrushabendraBN, Swamy A, Murali A. (2010). A review of natural diuretics. *Research journal of pharmaceutical, biological and chemical science*. ISSN: 0975-8585.
- Lahlou S, Tahraoui A, Israili Z, Lyoussi B. (2007) Diuretic Activity of the aqueous extracts of *Carum carvi* and *Tanacetum vulgare* in rats. *J Ethnopharmacol*; 110:458–463
- Lehrich RW, Ortiz-Melo DI, Patel MB, Greenberg A. (2013) Role of vaptans in the management of hyponatremia. *Am J Kidney Dis*; 62(2):364-376.
- Marti'n-Herrera, D., Abdala, S., Benjumea, D., Perez-Paz, P., (2007). Diuretic activity of *Withania aristata*; an endemic Canary Island species. *Journal of Ethnopharmacology* 113, 487–491.
- Martin-Herrera D, Abdala S, Benjumea D, Gutierrez-Luis J. (2008) Diuretic activity of some *Withania aristata* Ait. fractions. *J Ethnopharmacol*; 117: 496–499.

- Marvin M, Peter UF. (2009) Fifty years of thiazide diuretic therapy for hypertension. *Arch Intern Med*; 169(20):1851-1856.
- Matthew R. W. and Moser M., (2000), Diuretics and  $\beta$ -blockers: Is there a risk for dyslipidemia?; *American Heart Journal* Volume 139, Number 1, Part 1,;139:174-84.
- Meera R, Devi P, Muthumani P, Kameswari B and Eswarapriya B (2009). Evaluation of Diuretic activity from *Tylophoraindica* leaves extracts. *Journal of pharmaceutical sciences and research* 1(3): 112-116.
- Mehta R, Pascual M, Soroko S, Chertow G. (2002). Diuretics, mortality and nonrecovery of renal failure. *JAMA*; 288:2547-2553.
- Mielniczuk L.M., Tsang S.W., et al., (2008), The Association Between High-Dose Diuretics and Clinical Stability in Ambulatory Chronic Heart Failure Patients, *Journal of Cardiac Failure* Vol. 14 No. 5.
- Mukherjee P.K., (2002), Evaluation of diuretic agents. In: Quality control of herbal drugs; *Business Horizon*; 2:123-129.
- Mullera CE, Jacobson KA. (2011) Recent developments in adenosine receptor ligands and their potential as novel drugs. *BiochemBiophysActa*; 1808(5): 1290–1308
- Narayan G, Mandal SN. (2012) Vasopressin receptor antagonists and their role in clinical medicine. *Indian J EndocrinolMetab*; 16(2): 183-191
- Nedia T., Mekonnena N., Urga K., (2004), Diuretic effect of the crude extracts of *Carissa edulis* in rats; *Journal of Ethnopharmacology* 95: 57–61
- Nielsen S, Kwon TH, Christensen BM, Promeneur D, Frokiaer J and Marples D (1999). Physiology and Pathophysiology of Renal Aquaporins. *Journal of the American Society of Nephrology* **10**: 647-663.

- Nigwekar SU, Waikar SS (2011). Diuretics in a kidney injury. *Sem in Nephrology*; 31: 523-534.
- OECD (2008) Organization of Economic Corporation and Development guideline for testing of chemicals: Acute oral toxicity-Fixed dose procedure (425)
- Odigie, I.P., Ettarh, R.R., Adigun, S.A.,(2003), Chronic administration of aqueous extract of *Hibiscus sabdAAiffa* attenuates hypertension and reverses cardiac hypertrophy in 2K-1C hypertensive rats; *J. Ethnopharmacol.* 86:181–185.
- Okusa M.D. and Ellison D.H., (2008), Physiology and Pathophysiology of Diuretic Action; In Seldin and Giebisch's .The Kidney 4<sup>th</sup> ed ,pages 1051-1094.
- Opie, L.H., Kaplan, N.M., (2010). Diuretics. In: Opie, L.H., Gersh, B.j., editors. Drugs for the heart. 7<sup>th</sup> ed. Philadephia: Elsvier/WB Saunders; p130-167.
- Palmer B.F. and Naderi A.S.A., (2007), Metabolic complications associated with use of thiazide diuretics; *Journal of the American Society of Hypertension*:1(6) 381–392.
- Perez A, Ocotero M, Castaneda G. (2007). Alkaloids in *Solanum torvum* Sw (Solanaceae). *International Journal of Experimental Botany* 76: 39-45.
- Prabhu MN, Pai PG, Kumar SV, Gaurav, D'Silva, Ullal SD et al. (2014) Diuretic activity of aqueous ethanolic extract of leaves of *Costusspeciosus* in rats. *RJPBCS*; 5(3): 127-132
- Praveen KD, Suchita M. (2013) A comprehensive review on herbal remedies of diuretic potential. *IJRPS*; 3(1): 41-51
- Rajalakshmy MR, Sindhu A, Geethu G. (2013). Phytochemical investigation on *withaniasomnifera* root marc generated in ayurvedic industry. *International Research Journal of Pharmacy*. Vol. 4(8)
- Rang HP, Dale MM, Flower RJ, 2007. Rang and Dale's Pharmacology, 6<sup>th</sup> Ed, Elsevier Inc., Churchill Livingstone, pp. 368-380

- Reddy KY, Sandya L, Sandeep D, Salomi K, Nagarjuna S and Reddy PY. (2011) Evaluation of diuretic activity of aqueous and ethanolic extracts of *Lawsoniainermis* leaves in rats. *Asian J. Plant Sci. Res*; 1(3):28-33
- Rohini MR, Nayeem N, Das KA. (2009). Diuretic effects of *Trigonellafoenumgraecum* seed extracts. *The internet journal of alternative medicine*; 6(2):83-88.
- Roopesh C, Salomi RS, Nagarjuna, Y, Padmanabha R.(2011). Diuretic activity of methanolic and ethanolic extracts of *Centellaasiatica* leaves in rats. *International research journal of pharmacy*. Vol. 2(11):163-165
- Saidulu C, Venkateshwar S, Rao G. (2014). Morphological studies of medicinal plant of *Withaniasomnifer* (L.) Dunal grown in heavy metal treated contaminated soil. *Journal of Pharmacognosy and Phytochemistry*; 3 (1): 37-42.
- Sands J.M.,Verlander J.W.,Comprehensive toxicology, (2010), Functional Anatomy of the Kidney, chap.7.01, pp 1-22.
- Sands JM., (2013). Urea transporter inhibitors: en route to new diuretics. *Chem Biol.*; 20: 1201-1202.
- Sayana SB, Khanwelkar CC, Nimmagadda VR, Dasi JB, Chavan VR, Kutani A et al. (2014) Evaluation of diuretic activity of alcoholic extract of roots of *Cissampelospareira* in albino rats. *J. CliniDiagnos Res.*; 8(5): HC01-HC04.
- Schetz, M., (2004). Should we use diuretics in acute renal failure? *Best Prac Res ClinAnaesthesiol.*; 18(1): 75-89.
- Schrier, R.W., (2011). Use of Diuretics in Heart Failure and Cirrhosis. *SemNephrol.*; 31:503-512.

- Shankar BN, Chandra S, Ellaiah P. (2013). Evaluation of diuretic activity of *Gmelinaarborescens*. Fruit extracts. *Asian journal of pharmaceutical and clinical research*, vol. 6(1): 111-113.
- Sheth S, Brito R, Mukherjea D, Rybak, LP, Ramkumar V. (2014) Adenosine receptors: Expression, function and regulation. *Int. J. Mol. Sci*; 15: 2024-2052
- Shirley DG, Unwin RJ. (2010). Renal Physiology. In: comprehensive clinical nephrology, 4<sup>th</sup> Ed. *Elsevier*, pp15-28.
- Sica, D.A., Carter, B.I., (2011). Diuretic Therapy in Cardiovascular Disease. In: Oparil, S., Weber, M.A., editors, Hypertension: A Companion to Braunwald's Heart Disease. 2<sup>nd</sup> ed. Philadelphia: Elsevier Saunders. Pp160-171.
- Smith H. (2014) Diuretics: A review for the pharmacist. *S Afr Pharm J*;81(7):18-21
- Somova LI, Shode FO, Ramnandan P, Nadar, A. (2003) Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from *Olea europaea*, subspecies *Africana* leaves. *J Ethnopharmacol*; 84, 299–305
- Souza MA, Lara SL, Previatoa OJ, Lopes GA, Caruso-Neves C et al. (2004). Modulation of Sodium Pumps by Steroidal Saponins. *Z.Naturforsch*; 59:432-436.
- Sripanidkulchai, B., Wongpanich, V., Laupattarakasem, P., Suwansaksri, J., Jirakulsomchok, D., (2001), Diuretic effects of selected Thai indigenous medicinal plants in rats; *Journal of Ethnopharmacology* 75, 185–190.
- Sterns RH. (2011). *Diuretics. Seminars in nephrology*; 31(6):473-74.
- Subodh D, Verma VK, Sahu AK, Jain AK and Akash T (2010). Evaluation of diuretic activity of aqueous and alcoholic rhizomes extracts of *Costus speciosus* Linn. In Wistar albino rats. *International Journal of Research in Ayurveda and Pharmacy* 1(2): 648-652.

- Swain RS, Sinha NB, Murthy NP (2008). Anti-inflammatory, Diuretic and Antimicrobial Activities of *Rungiapectinata* Linn. And *Rungiarepens* Nees. *Indian Journal of Pharmaceutical Sciences*; 70(5): 679-683.
- Tirumalasetty J, Chandrasekhar N and Naveen A (2013). Evaluation of diuretic activity of ethanol extract of *Benincasahispida* stem in Swiss albino rats. *Journal of Chemical and Pharmaceutical Research* 5(3): 91-97.
- Tisher, C. C. Brenner, B.M., (1989), In *Renal Pathology with Clinical and Functional Correlations*; 1st ed: Philadelphia, PA, Vol. 1, pp 92–110.
- Vallon V, Miracle C, Thomson S. (2008) Adenosine and kidney function: Potential implications in patients with heart failure. *Eur.J. Heart Failure*; 10: 176–187
- Vogel HG. (2007) Diuretic and saluretic activity, In: *Drug Discovery and Evaluation: Pharmacological Assays*; 3rd Ed, pp 513-517.
- World Health Organization, (2003), International Society of Hypertension Writing Group, *Journal of hypertension*; 21:1 983-992.
- Wright, C.I., Van-Buren, L., Kroner, C.I., Koning, M.M.G., (2007), Herbal medicines as diuretics: A review of the scientific evidence. *Journal of Ethnopharmacology* 114; 1–31.
- Yang B. (2015) Urea transporter inhibitors as novel diuretics. *Ann Med Chem Res*; 1(2): 1-7
- Yu H, Meng Y, Wang LS, Jin X, Gao LF, Zhou L, et al., (2009). Differential protein expression in heart in UT-B null mice with cardiac conduction defects. *Proteomics.*; 9: 504-511.
- Zhuo JL and Li XC (2013). Proximal Nephron. *Comprehensive Physiology* 3(3): 1079-1123.

