Evaluation of the diuretic activity of the aqueous and 80% methanolic extracts of the leaves of Medicago sativa L.(Fabaceae) in mice

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A thesis submitted to the Department of Pharmacology and Clinical Pharmacyin partial fulfillment for the requirement of the Degree of Master of Science in Pharmacology

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This is to certify that the thesis prepared by Amare Temesgen, entitled: Evaluation of the diuretic activity of the aqueous and 80% methanolic extracts of the leaves of *Medicago sativa* L.(Fabaceae) in miceand 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.

Signed by the Examining Committee:

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ABSTRACT

Evaluation of the diuretic activity of the aqueous and 80% methanolic extractsof the leaves of *Medicago sativa* L.(Fabaceae) in mice.

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Addis Ababa University, 2015

Diuretics are therapeutic agents that are used to increase the rate of urine flow and sodium excretion in order to adjust the volume and composition of body fluids or to eliminate excess of fluids from tissues. Most diuretic agents available in the market present a wide range of problems such as efficacy and undesired effects. These limit their clinical usefulness and remain to be solved, leaving an open door for new and better compounds. *Medicago sativa*L.(Fabaceae)is used as diuretic in traditional medicine practice, but the plant has not been scientifically evaluated for the claimed activity.The present study was therefore conducted to evaluate thediuretic activity of the aqueous and 80% methanolextracts of the leaves of *Medicago sativa* L.(Fabaceae) in mice.Extraction of the leaves of *Medicago sativa* was performed using successive maceration. Male mice were treated with vehicle (distilled water or 2% tween 80), standard (hydrochlorothiazide 10 mg/kg) and three doses (250 mg/kg, 500 mg/kg, and 1000 mg/kg,) of aqueous, and 80% methanol extract. Parameters, including urine volume, electrolyte concentration, and pH were measured and analyzed. The aqueous and80% methanolextracts significantly increased ($p<0.01$) diuresis starting from 500 mg/kg. Regarding electrolyte excretion, the aqueous extract produced significant natriuresis at 500 mg/kg ($p<0.001$) and significant kaliuresis at 250 mg/kg ($p<0.001$), and the80% methanolic extract showed significant natriuresisat 500 mg/kg ($p<0.001$)and kaliuresisat 250 mg/kg ($p<0.001$).Preliminary phytochemical screening of the extracts revealed the presence of secondary metabolites, including alkaloids terpenoids, flavonoids and saponins, which could be the responsible component(s) for the diuretic activity.

The two extracts were also found to be safe at 2000mg/kg. The findings collectively indicate that the plant extract do have diuretic activity as it is claimed by the traditional healers and activity increased with increasing dose(R^2=0.98).

Keywords: *Medicago sativa* ,diuresis, natriuresis and kaluresis.

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ACRONYMS

AIRA1 Adenosine Receptor

ADHAntidiuretic Hormone

AQPAquaporin

CACarbonic Anhydrase

DCTDistal Convoluted Tubule

ECFExtracellular Fluid

EPHI Ethiopian Public Health Institute

FDA Food and Drug Administration

GFR Glomerular Filtration Rate

ISEIon Selective Electrode

NCCSodium/Chloride Cotransporter

OECDOrganization for Economic Co-Operation and Development

PCTProximal Convoluted Tubule

SEM Standard Error of the Mean

SIADHSyndrome of Inappropriate Antidiuretic Hormone

SPSSStatistical Package for Social Sciences

TALThick Ascending Loop

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1. INTRODUCTION

1.1 Definition and History of Diuretics

Diuretics are therapeutic agents that are used to increase the rate of urine flow and/or sodium excretion in order to adjust the volume and composition ofbody fluids or to eliminate excess of fluids from tissues (Jackson, 2006). They are used for the treatment of various diseases and syndromes, including hypertension, heart failure, liver cirrhosis, renal failure, kidney and lung diseases, as well as a more general reduction of the adverse effects of salts and/or water retention (Jackson, 2006). Naturally occuring diuretics include caffeine in coffee, tea and cola, which inhabits Na^+ reabsorption and alcohol in beer, wine and mixed drinks which inhibit secretion of antidiuretic hormone (ADH) (Koti and Purnima,2008).

The ability of mercurial antisyphiliticus to affect diuresis was discovered by VogelIn 1919 . This observation led to the development of effective organic mercurial diuretics, drugs that were used commonly until the 1960s (Eknoyal et al .,1975). In 1937 sulfanilamide was found to cause metabolic acidosis in patients. Carbonic anhydrase (CA) had been discovered in 1932. Soon more potent sulfonamide based carbnonic anhydrase inhibitors (CAIs) were discovered, but these drugs suffered from multiple side effects and low potency (Eknoyal et al .,1975).

First modern, orally active diuretic ,chlorothiazide was discovered in 1957, and by early 1960s its congeners (thiazide diuretics). The search for more potent classes of diuretic continued, led to the development of furosemide and ethacrynic acid (Jackson and Chabbers,2001).

Although all these compounds proved to be very effective in promoting sodium excretion, they all caused potassium loss, and this prompted the search for potassium sparing diuretics.

Aldosterone antagonists such as spironolactone were, introduced in 1962. Numerous compounds screened and eventually amiloride and triameterene had emerged (Rang et al.,1999).

Diuretics were first banned in sport (both in competition and out of competition) in 1988 because they can be used by athletes for two primary reasons. First, their potent ability to remove water from the body can cause a rapid weight loss that can be required to meet a weight category in sporting events. Second, they can be used to mask the administration of other doping agents by reducing their concentration in urine primarily because of an increase in urine volume. The urine dilution effect of diuretics also allows them to be classified as masking agents and precludes their use both in and out of competition. Some diuretics also cause a masking effect by altering the urinary pH and inhibiting the passive excretion of acidic and basic drugs in urine (Ventura and Segura, 1996; Goebel *et al*., 2004; Trout and Kazlauskas, 2004; Furlanello*et al*., 2007).

1.2. Renal Anatomy and Physiology

As shown in Figure 1, the kidneys are a pair of bean-shaped organs found along the posterior wall of the abdominal cavity. The left kidney is located slightly higher than the right kidney because the right side of the liver is much larger than the left side. The kidneys, unlike the other organs of the abdominal cavity, are located posterior to the peritoneum and touch the muscles of the back. The kidneys are surrounded by a layer of adipose that holds them in place and protects them from physical damage. The kidneys filter metabolic wastes, excess ions, and chemicals from the blood to form urine (Sands and verlander, 2010). In sagittal section, the kidneys have three main regions: the outer region called cortex, the central region called medulla and the innermost tip of the inner medulla called papilla (Guyton and Hall, 2006; Saladin, 2008). Blood

enters each kidney via a renal artery and leaves through a small veins into the renal vein, which drains into the inferior venacava (Saladin, 2008).

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 (Chmielewski, 2003; Zhuo and Li, 2013). Nephron is the basic urine-forming unit of the kidney (Jackson, 2006). Each adult kidney contains around 1-1.5 million nephrons (Kardasz, 2009). A nephron consists of two principal parts: a renal corpuscle, which filters the blood plasma, and a long renal tubule, which converts the filtrate to urine. The renal tubule is divided into four major regions: the proximal convoluted tubule (PCT), nephron loop, distal convoluted tubule (DCT), and collecting duct (Saladin, 2008). Urine formation begins when a large amount of fluid that is virtually free of protein is filtered from the glomerular capillaries into the Bowman's capsule. After it passes into the renal tubule, its composition is quickly modified by tubular reabsorption and tubular secretion (Guyton and Hall, 2006; Costantini and Kopan, 2010). PCT which arises from the glomerular capsule is responsible for reabsorbing approximately 65% of the filtrate. $NaHCO_3$ reabsorption by the PCT is mediated by the action of a Na^+/H^+ exchanger, which allows Na^+ to enter the cell from the tubular lumen in exchange for H^+ from inside the cell. 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 (Jackson, 2006; Ives, 2012).

From PCT, the fluid dives deep into the loop of Henle, which is a long U-shaped portion of the renal tubule found mostly in the medulla (Saladin, 2008). The loop of Henle consists of three functionally distinct segments: the thin descending segment, the thin ascending segment, and the

thick ascending segment. The thick ascending loop of Henle (TAL) which is virtually impermeable to water is responsible to reabsorb about 25% of the filtered loads of Na^+, Cl^- and K^+ (Guyton and Hall, 2006). Na^+ transport in this nephron segment is mediated by $Na^+/K^+/2Cl^-$ symport (Friedman and Berndt, 1997). Although the $Na^+/K^+/2Cl^-$ transporter is itself electrically neutral, the action of the transporter contributes to excess K^+ accumulation within the cell. Back diffusion of this K^+ into the tubular lumen causes a lumen-positive electrical potential that provides the driving force for reabsorption of cations, including Mg^{2+} and Ca^{2+}, via the paracellular pathway (Ives, 2012). The TAL empties into the DCT, where 5-10% of the filtered sodium and chloride ions are reabsorbed (Reilly and Ellison, 2000; Saladin, 2008). It is relatively impermeable to water (Barrett *et al*., 2010). The entry of Na^+ across the apical cell membrane is mediated by Na^+/Cl^- cotransport (Friedman and Berndt, 1997).

Finally, the urine reaches at the collecting tubule system, which is responsible for only 2-5% of NaCl reabsorption by the kidney (Ives, 2012). The connecting tubule, which coalesces to form the collecting ducts, performs the final adjustment of renal excretion (Range *et al.,* 2012; Staruschenko, 2012). It is the site at which mineralocorticoids exert a significant influence (Ives, 2012). Collecting tubules include principal cells, which reabsorb Na^+ and secrete K^+, and two populations of intercalated cells, α and β, which secrete acid (H^+) and base (bicarbonate), respectively (Ives, 2012; Range*et al.,* 2012). Unlike other segments of the nephron, Principal cell membranes exhibit separate ion channels for Na^+ and K^+. Since these channels exclude anions, transport of Na^+ or K^+ leads to a net movement of charge across the membrane. Because Na^+ entry into the principal cell predominates over K^+ secretion into the lumen, a 10-50 mV lumen negative electrical potential develops. This lumen-negative electrical potential drives the transport of Cl^- back to the blood via the paracellular pathway and draws K^+ out of cells through the apical

membrane K^+ channel. Thus, there is an important relationship between Na^+ delivery to the collecting tubule system and the resulting secretion of K^+. That is, Na^+ delivery to this site enhances K^+ secretion (Ives, 2012). 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_2) into the apical membrane of the principal cells. In the absence of ADH, the collecting tubule (and duct) 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 (Friedman and Berndt, 1997; Ives, 2012).

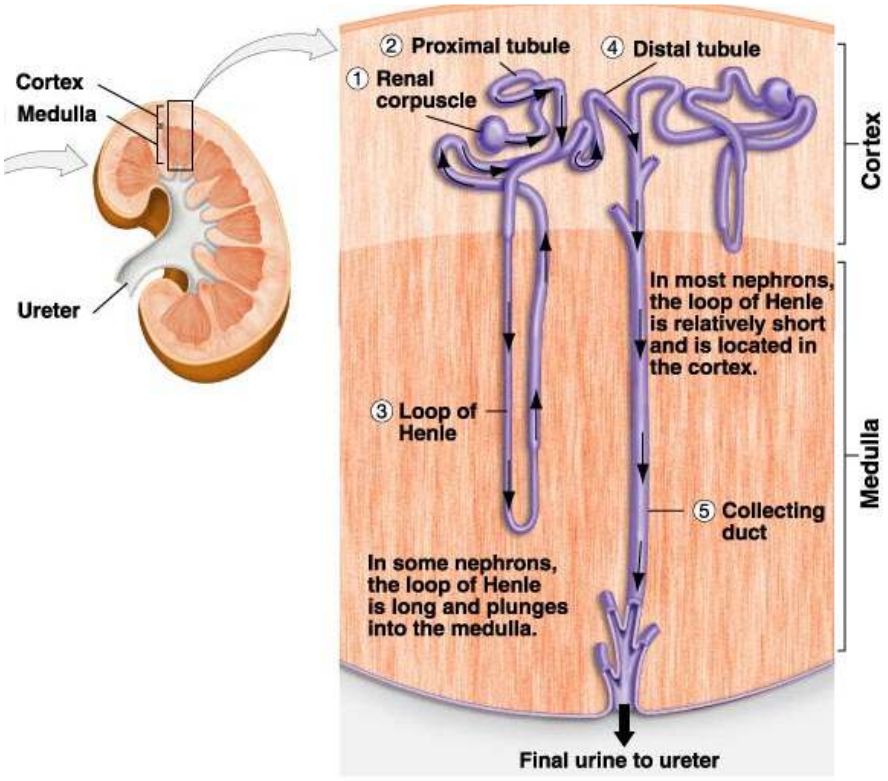

Fig 1: Diagram showing the anatomy of kidney and nephron (Guyton,2006)

1.3.Mechanism and site of action of Conventional diuretics

Many diuretics exert their effects on specific membrane transport proteins in renal tubular epithelial cells. Other diuretics exert osmotic effects that prevent water reabsorption (mannitol), inhibit enzymes (acetazolamide), or interfere with hormone receptors in renal epithelial cells (aldosterone receptor blockers).

Osmotic diuretics are substances that are freely filtered at the glomerulus, poorly reabsorbed and are relatively inert pharmacologically (Jackson, 2006). 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 (Beckman et al ., 1999) . Other agents considered in this class include urea, glycerin and isosorbide (Garwood, 2009; Ellison, 2013).

Carbonic anhydrase inhibitors (e.g., acetazolamide and methazolamide) act by inhibiting the enzyme CA in the PCT to interfere with bicarbonate (HCO_3-) reabsorption which results in increased loss of Na^+, bicarbonate and water in the urine (Ives, 2012; Snigdha *et al*., 2013).Loop diuretics (e.g., furosemide, torsemide, azosemide, bumetanide and ethacrynic acid) act by blocking the $Na^+/K^+/2Cl^-$ cotransporter in the thick ascending loop of Henle resulting in decreased Na^+ and Cl^- reabsorption from the urine and subsequent natriuresis and diuresis (Felker, 2011; Musini *et al*., 2012; Shchekochikhin *et al*., 2013).

Thiazides achieve their diuretic action via inhibition of the apical Na^+/Cl^- cotransporter (NCC) in the DCT. Thiazide or thiazide-like diuretics include hydrochlorothiazide, chlorthalidone, indapamide, metolazone and chlorothiazide (Duarte and Cooper-DeHoff, 2010; Musini *et al*., 2012).

Potassium sparing diuretics act primarily at the cortical part of the collecting duct and to a lesser extent in the late distal and collecting 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).

1.4.Therapeutic importance of diuretics

Diuretics are important therapeutic tools to induce negative sodium and fluid balance. They are used to reduce blood pressure in hypertension, mobilize fluid for removal in patients with renal disease and other volume-retaining states, making them essential components of therapy for heart failure, kidney diseases and cirrhosis (Ernst and Gordon, 2010). Fluid retention, which is manifested as pulmonary and peripheral edema, is a consistent finding in almost all acute and most chronic heart failure patients (Shah *et al*., 2004).

Diuretics are the only drugs used to adequately control fluid retention in heart failure. Appropriate use of diuretics is also a key element in the success of other drugs used for the

treatment of heart failure (Yancy *et al*., 2013). Diuretics are also among the most effective blood pressure lowering drugs in hypertensive patients (Brewster and Seedat, 2013). Thiazide and thiazide-like diuretics are among the most commonly used antihypertensive drugs and have been available for over 50 years (Duarte and Cooper-Dehoff, 2010).

1.5.Diuretic resistence

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 (rebound) ,and some increase sodium and water retention in the long term (the braking phenomenon) . When one dose diuretic worn off, the kidneys may respond aggressively retaining sodium. The rebound effect can last for long hours and may even counteract the diuretic effect of the previous dose. The complex process, called the braking phenomenon is the adaptation to the drug that is due to change in the structure and function of the kidney itself, activation of the sympathetic nervous system and changes in the several hormone pathways (Wilcox *et al* ., 1983; Asare,2009) .

When treating diuretic resistance, it is important for the patient to restrict daily consumption of fluid intake, follow a low-sodium diet and avoid taking non-steroidal anti inflammatory drugs (NSAIDS). The other approach that is remarkably effective for managing diuretic resistance is sequential blockade of the nephron. This is done by combining diuretics that act in different segments of the nephron, usually a loop and a thiazide diuretic, resulting in inhibition of reabsorption at multiple sites(Asare,2009) .

1.6.Adverse effects

The major concerns about their use arise from their tendency to cause hypomagnesemia(Ryan, et al 1984),hyperurecemia(Ljunghall, et al 1982),Hyperglycemia(Karakurt, Kasikci ,2012, Chrostowska, Narkiewicz ,2010,lancet,1981, Furman,1981),hyperlipidemia (Ruppert, et al ,1993, Mantel-Teeuwisse, et al ,2001, Lakshman, et a,1999, Kasiske, et al ,1995),Impotence(Chrostowska, et al ,1991, Wassertheil-Smoller, et al.,1991),Photosensitivity dermatitis(Addo et al ,1987),Allergic interstitial nephritis(Magil, et al ,1980),Stevens-Johnson syndrome(Strom, et al,1994).

1.7.Novel diuretics

1.7.1. Adenosine A1 receptor antagonists

In addition to vasoconstrictive and vasodilatory effects, adenosine is intrinsic to the tubuloglomerular feedback which occurs when an acute increase in sodium levels in the proximal tubule feeds back to decrease glomerular filtration. Adenosine works via both adenosine A1 and A2 receptors. A1-receptor antagonists decrease afferent arteriolar pressure, and increase urine flow and sodium excretion. Studies suggest that A1-receptor antagonists cause a diuretic effect not by a change in the renal haemodynamics, but by the inhibition of water and sodium reabsorption in tubular sites secondary to direct tubuloglomerular feedback. Less consistent has been the occasional finding of increased glomerular filtration rate despite the lack of improved renal plasma flow. A1-receptor antagonist cause diuresis while maintaining or improving glomerular filtration, it is therefore a useful adjunct in the treatment of severe heart failure. It has been evaluated the effects of the A1-receptor antagonist CVT-124 (BG-9719) in heart failure patients. CVT-124 increased sodium excretion without decreasing glomerular

filtration rate. These findings suggest that adenosine might be an important determinant of renal function in patients with heart failure (Gottlieb ,2001).

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1.7.2.Antidiuretic hormone (ADH) receptor antagonists

Arginine vasopressin or ADH, a cyclic peptide (9 amino-acids) with a disulfide bridge,is released from the brain and known to work in the kidney , suppressing the diuresis .Well known ADH receptors are :V1a,V1b (also known as V3) and V2. They belong to G protein coupled receptor super family. Non peptide vasopressin antagonists (i.e vaptans) are mozavaptan, lixivaptan, conivaptan, satavaptan, solvaptan.vaptans are particularly useful to treathypervolemic hyponatremia associated with severe congestive heart failure or chronic liver failure, as the only other treatments currently available, such as fluid restriction and diuretics, are slow-acting and minimally effective. Vaptans are also useful for treating euvolemic hyponatremia associated with the syndrome of inappropriate antidiuretic hormone (SIADH), at least when it is chronic and/or minimally symptomatic (Inomata*et al.,*2011;D' Auria et al ., 2012).

1.8.Herbal Diuretics

There is increasing interest in the health and wellness benefits of herbs and botanicals. This is with good reason as they might offer a natural safeguard against the development of certain conditions and be a putative treatment for some diseases. One such area may be the lowering of blood pressure in those where it is elevated (i.e., hypertension). There are a growing number of studies purporting diuretic effects with traditional medicines(Wright et al.,2007). Pharmacological studies done on some Ethiopian traditional medicinal plants have supported their folkloric use as diuretic agents. Some of the promising plants include: *Carissa edulis* (Nedi

et al., 2004), *Rumex abyssinicus* (Mekonnen *et al*., 2010) and Ajuga *remota* (Hailu and Engidawork, 2014).

1.9. *Medicago sativa Linn* (Fabaceae)

Medicago sativaLinn (Kingdom: Plantae; Division: Magnoliophyta; Class:Magnoliopsida; Order: Fabaces; Family: Fabaceae;Subfamily: Faboideae; Tribe: trifolieae; Genus:Medicago;Species:sativa; Binomial name:*M. sativaLinn*)(Heywood & Ball, 1968) ,commonly known as "the father of all foods "(al-fal-fa) ,is a perennial herbaceous leguminous plant species that originated in Asia (Ehsanpour & Razavizadeh, 2005;Duke, 1985;BHMA, 1996).*M. sativa*has been growing for a variety of purposes such as soil improvement, animal feed and medicinal uses (Steppler, 1987).

Fig 2: Photo of *Medicago sativa*

M. sativa is a cool season perennial legume living from three to twelve years.The general morphology of *M. sativa* plant was considered by Teuber and Brick (1988), and Barnes and Sheafer (1995). The mature*M. sativa* plant is characterized by a strong taproot. This taproot may eventually surpass 6 m or more in length with several to many lateral roots con-nected at the

crown when grown in deep. The crown, a complex structure near the soil surface, has perennial meristem activity, producing buds that develop into stems. tri- or multi-foliateleaves form alternately on the stem, and secondary and tertiary stems can develop from leaf axils. A plant in atypical forage production field has between 5 and 15 stems and can reach nearly 1 m in height (Figure3). Flowers vary incolor yet purple, variegated, yellow, cream and white are the most common. After pollination, these flowers most commonly produce spiral-shaped seed pods (Duke, 1985).

Traditionally, *M. sativa* is used to improve memory, to cure kidney pain, cough, sore muscles, as a rejuvenator,antidiabetic, antioxidant, anti-inflammatory, antifungal,anti-asthmatic, antimicrobial, diuretic, galactagogue and in central nervous system (CNS) disorders (Finkler, 1985;BHMA, 1996;Inamul, 2004;DerMarderosian et al., 2005). For diuretic use people initially collect the arial part of the plant, cut in to small pieces and mixed with water in a cup before drinking it(Finkler, 1985). Several studies indicate that*M. sativa*reduces cholesterol absorption and atherosclerotic plaque formation in animals (Wilcox& Galloway, 1961;Malinow et al., 1977a, 1981a;Cohen et al., 1990),Reduce the level of hyperglycemia in streptozotocin-induced diabetes (Swanston et al., 1990), M. sativa has been also shown sig-nificant estrogenic activity using an estrogen-dependent MCF-7 breast cancer cell proliferation assay. (Boue et al., 2003).Anti-dopaminergic action without side effects (Minerva, 1998),immune potentiating (Zhao et al., 1993), antifungal(Avato et al., 2006) ,inhibit the activities of reverse transcriptase of HIV and protease of HIV (Zhang et al., 2006)

*M. sativa*has been reported to contain a variety of phytochemicals,Alkaloids: (Duke, 1985;Mills, 1994;Tamsyn et al., 2009),Amino acids (Worthington & Breskin, 1983;Fushiya et al., 1984;Lihu & Pedak, 1979;Mego & Erdersky, 1977).Carotene(Gupta et al., 1981). Coumarins: (Duke, 1985;El-Khrisy et al., 1994;Orr et al., 1993).

2. OBJECTIVES

2.1. General objective

- To evaluate the diuretic activity of aqueous and 80% methanolcrude extract of *Medicago sativa* (Fabaceae) in mice

2.2. Specific objectives

- To determine effect on urine out put of ***Medicago sativa*** extracts
- To determine urine Na^{+},K^{+},Cl^{-} concentration.
- To determineLD_{50} of the extracts of***Medicago sativa***.
- To determine effect on urine P^{H}
- To perform preliminary phytochemical analysis

3. MATERIALS AND METHODS

3.1.Drugs and chemicals

The drug and chemical reagents used in this study include, Absolute methanol (EPFSA, Addis Ababa, Ethiopia), Distilled water, normal saline, Tween 80 ,hydrochlorothiazide (Esidrex-Novartis Pharma AG, Basle Switzerland), and Methanol. Other chemicals and reagents used for phytochemical tests were obtained from Department of Pharmaceutical Chemistry and Pharmacognosy of School of Pharmacy, College of Health Sciences, Addis Ababa University. All reagents used were analytical grade.

3.2. Experimental animals

Male (for diuretic activity test) and female (for acute toxicity test) Swiss albino mice, weighing 25–35 gram and aged 6–8 weeks, were purchased from Ethiopian Public health Institute (EPHI). The animals were kept in plastic cage in the animal house of the School of Pharmacy, Addis Ababa University, with 12 hour/light dark cycle. The animals were allowed free access to standard laboratory pellet and tap water. Prior to the start of the experiment all animals were fasted overnight with water allowed ad libtum. The care and handling were in accordance with the internationally accepted guidelines for use of experimental animals (Vogel, 2007; OECD, 2008).

3.3. Collection of the plant

Arial parts of *Medicago sativa* were collected from a place called Bishoftu 40 km east of Addis Ababa inDecember 2013 . The plant was identified and authenticated by a taxonomist (voucher specimen: MS 001) and was deposited at the National Herbarium of College of Natural and Computational Sciences, Addis Ababa University, Ethiopia for future reference.

3.4. Extraction of the plant

The leafl part of *M.sativa*were dried under shade for two weeks. The dried *M.sativa* then coarsely powdered and extracted as follows.

3.4.1. Aqueous extraction

Dried, powdered *M.Sativa*of mass 500gm was cold macerated in an Erlenmeyer flask with distilled water and allowed to stand at room temperature for a period of 72h with occasional shaking using mini orbital shaker (Stuart, UK). It was then filtered with gauze. Counting from intial maceration of first maceration,the first residue was remacerated after three days and the second residue was was remaceratedafter 6 days in order to obtain better yield. The filtrate was freeze dried in a lyophilizer (Operon, Korea vacuum limited, Korea) to remove water. After drying, percentage yield was calculated(Gakunga *et al*., 2013) . Then reconstituted in distilled water for oral administration.

3.4.2. 80 % methanol extraction

Dried, powdered *M.sativa*of 500gmwas cold macerated in an Erlenmeyer flask with 80% 80 % methanol and allowed to stand at room temperature for a period of 72h with occasional shaking using mini orbital shaker (Stuart, UK). It was then filtered with gauze. The residue was remacerated two times for a total of 6 days in order to obtain better yield. The filtrate was put in rotary evaporator to remove methanol and freeze dried in a lyophilizer (Operon, Korea vacuum limited, Korea) to remove water. After drying, percentage yield was calculated(Gakunga *et al*., 2013) . Then reconstituted in2% Tween 80 for oral administration.

3.5. Acute toxicity study

Acute oral toxicity test was carried out as per the Organization for Economic Co-operation and Development (OECD) guidelines for Testing of Chemicals number 425 (Kane *et al*., 2009 and Ancy *et al*., 2013). Female Swiss albino mice weighing 25g-30g were fasted for 12 hour prior to the experiment and were administered with single dose (2000 mg/kg) of hydroalcoholic and aqueous fractions orally using oral gavage. Immediately after dosing, the animalwas observed continuously for the first 4 hours for any behavioral changes and if no death observed after a day it is administered for the rest four mice. Thereafter, they were then kept under observation up to 14 days after drug administration to find out the mortality if any.

3.6. Grouping and dosing of animals

Animals were randomly assigned into five groups each consisting of 8 animals for diuretic test. Negative controls were treated with the vehicle used for reconstitution (1ml/100gm of body weight). Positive controls were treated with standard drug, hydrochlorothiazide 10mg/kg. Four treatment groups in each test were treated with 250mg/kg (MS250), 500mg/kg (MS500) and 1000mg/kg (MS1000) doses of the two different extracts. Doses were determined using data from acute toxicity and pilot test. Route of administration for all groups was orally using oral gavage.

3.7. Diuretic activity

Diuretic activity was determined following the methods used by Lahlou *et al* (2007). Each male mouse was placed in an individual metabolic cage 24 h prior to commencement of the experiment for adaptation and then fasted overnight with free access to water. The animals were pre-treated with physiological saline (0.9% NaCl) at an oral dose of 0.15 ml/10 g body weight (BW), to impose a uniform water and salt load (Nedi *et al*., 2004). Then, the animals were treated with vehicle, standard or the fractions as described in section 3.7orally by gavage.

Immediately after administration, the mice were individually placed in a metabolic cage provided with a wire mesh bottom and a funnel for collecting the urine. Urine was then collected and measured for total of 5 h at 1, 2, 3, 4 and 5h after dosing. The urine was then stored at -20^{o}C for further analysis. The following parameters were calculated in order to compare the effects of the fractions and hydrochlorothiazide on urine excretion. The urinary excretion independent of the animal weight was calculated as ratio of total urinary output by total liquid administered (formula-1). The ratio of urinary excretion in test group to urinary excretion in the control group was used as a measure of diuretic action of a given dose of a drug (formula-2). To obtain diuretic activity, the diuretic action of the extracts was compared to that of the standard drug in the test group (formula-3) (Mukherjee, 2002).

$$\text{Urinary excretion} = \frac{\text{total urinary output}}{\text{total liquid administered}} \times 100\% \qquad \text{(formula-1)}$$

$$\text{Diuretic action} = \frac{\text{urinary excretion of treatment groups}}{\text{urinary excretion of control group}} \qquad \text{(formula-2)}$$

$$\text{Diuretic activity} = \frac{\text{diuretic action of test group}}{\text{diuretic action of standard drug}} \text{ (formula-3)}$$

3.8. Analytical procedures

Electrolyte level of the urine and the different extracts were determined at EPHI.Sodium, potassium and chloride level of the urine and the different extracts was determined. The pH of fresh urine was also measured. Na^{+} and K^{+} concentrations were measured using an Ion Selective Electrode (ISE) analyzer (AVL 9180 electrolyte analyzer, Roche, USA). The instrument was automatically calibrated with standard solutions containing different concentrations of sodium, potassium and chloride ions. pH was measured with a pH meter (Sigma-Aldrich, USA) on fresh urine sample.

3.9. Phytochemical screening

Phytochemical screening tests were carried out on each of the hydroalcoholic and aqueous extracts of the *M. sativa* using standard procedures to identify the constituents as described below.

3.9.1 Test for terpenoids (Salkowski test)

To 0.5 g of each solvent fraction of *M.sativa* , 2 ml of chloroform was added. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids (Ayoola et al., 2008) .

3.9.2. Test for Saponins

To 0.5 g of 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 (Ayoola et al., 2008) .

3.9.3.Test for tannins

About 0.5 g of each fraction was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added. A brownish green or a blue-black precipitate indicated the presence of tannins.

3.9.4 Test for flavonoids

About 10 ml of ethyl acetate was added to 0.2gram of each fraction and heated on a water bath for 3 min. The mixture was cooled and filtered. Then, About 4 ml of the filtrate was taken and shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammoniacal layer indicated the presence of flavonoids (Oloyinka,2010).

3.9.5. Test for cardiac glycosides (Keller-Killiani test)

To 0.5 g of each fraction diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml 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 (Harbone,1998).

3.9.6. Test for steroids

Two ml of acetic anhydride was added to 0.5 g fraction 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 (Ayoola et al., 2008) **.**

3.9.7. Test for alkaloids

0.5 g of extract was diluted to 10 ml with acid alcohol, boiled, and filtered. To 5 ml of the filtrate, 2 ml of dilute ammonia and 5ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions.

Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids (Trease,1989)

4.0.Statistical analysis

The experimental results wereanalyzed using the Statistical Package for the Social Sciences (SPSS), version 16.0 software. Results are expressed as mean ± standard error of the mean(SEM), and statistical analysis was carried out by employing one way analysis of variance (ANOVA) followed by Tukey post Hoc test. $P < 0.05$ was considered as statistically significant.

4. RESULTS

4.1. Acute toxicity study

Mice were observed for two weeks to see if the aqueousand 80% methanol extracts had toxiceffect. Both extracts of the plant did not produce any visiblesigns of toxicity up to the dose of 2 g/kg. This was evidencedby absence of tremor, loss of weight, lethargy,paralysis, stress or adverse behaviors. In addition, therewas also no sign of diarrhea and none of the treated micedied, suggesting the LD50 is greater than 2000 mg/kg.

4.2.Urinary pH

The urinary pH was measured. And the different treated groups of both aqueous and hydro alcoholic extracts had resulted in the production of alkaline urine (Figure3).

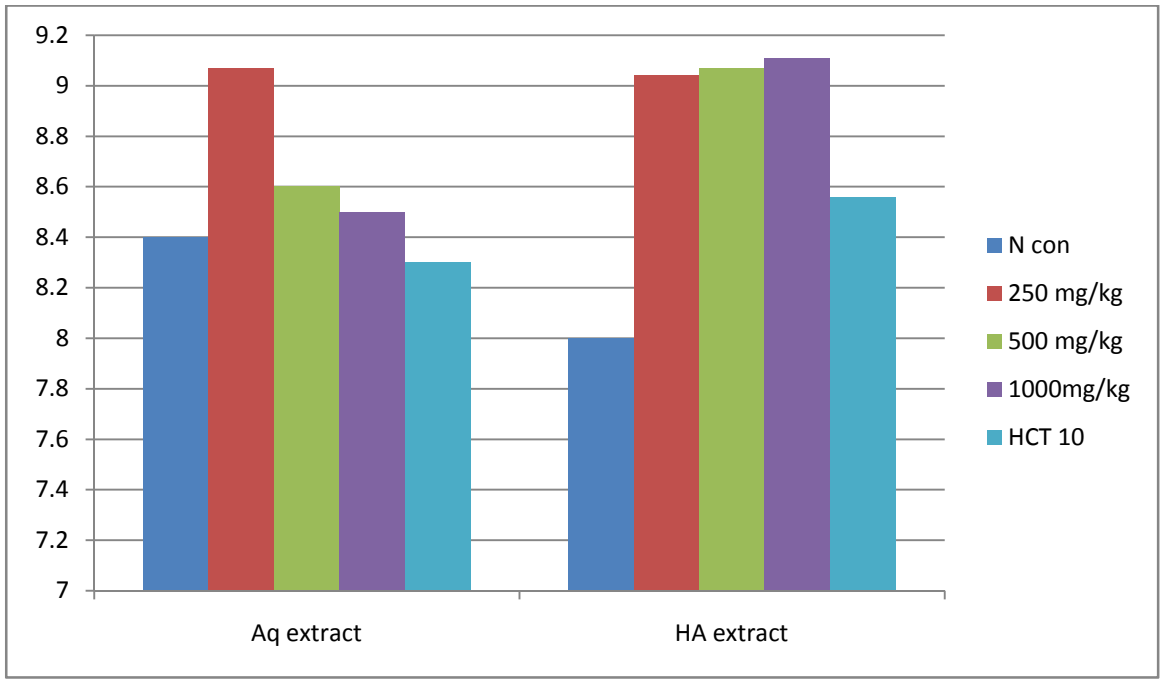

Fig 3: Effect of different extracts on urine pH of mice. N con : negative control; HCT 10 :positive control 10mg/kg; Aq extract: aqueous extract ;HA extract: hydro alcoholic extract; 250mg/kg :250mg/kg dose;500mg/kg :500mg/kg dose :1000mg/kg:1000mg/kg dose

As it is shown from figure 4 the maximal pH value was produced by 1000mg/kg dose of hydro alcoholic extract. And the leastpH was produced by HCT 10.

4.3. Phytochemical screening

The experimental plant was explored for the composition of different classes of secondary metabolites and both extracts were found to be positive foralkaloids,steroids,Saponins andTanins. WhileFlavonoids and Cardiac glycosides found only in aqueous extract (Table 1) .

Table 1: Phytochemical screening of different extracts of *M.sativa*

Secondary metabolites	Aqueous extract	80% Methanol extract
Steroids	+	+
Alkaloids	+	+
Terpenoids	-	-
Saponins	+	+
Flavonoids	+	-
Tanins	+	+
Cardiac glycosides	+	-

+= present - = absent

4.4. Diuretic activity: Effect on urine volume

4.4.1. Aqueous extract

The aqueous extract of the plant produced diuresis which appeared to bedosedependent (Table 2). MA250 did not produce better diuresis than the vehicle in the first and second hour, but starting from the third hour a slight increase inurine volume was produced and then an increased diuresis by about 10.5% had been recorded at the fifth hour,which was not found to be significant. Mice treated with MA500 had an increased diuresis startingfrom the second hour of urines collection but a significant diuresis was produced starting from the third hour (151%, $p<0$, 01) when compared with CON animals. However the highest dose of MA1000 produced diuresis which was significant (457 %,$p<0.01$) starting from the very first hour compared to control.

Hydrochlothiazide treated mice produced diuresis which was significant as compared to CON group, starting from the first hour (457%,$p<0.01$) and continued until the end of the fifth hour (287%, $p<0.01$) so that the onset of diuresis is almost one hour (Table 2).The standard drug HCT10 had a significant diuretic effect than that of MA250 ($p<0.001$) but had comparable effect with MA1000 at the end of the fifth hour. This could be revealed from the diuretic activity of MA1000 and HCT10 which were 0.97, and 1.00 respectively (Table 2).When the different doses of the aqueous extracts compared each other, the highest dose, MA1000, produced diuresis which was significant starting from the first hour ($p<0.01$) and continued till the end of the fifth hour ($p<0.01$) as compared with MA250. Indeed, as time went by the doses appeared to exhibit similar effectiveness, as they had comparable diuretic action (3.58 and 4.04 for MA500 and MA1000 respectively) (Table 1).

Table 2: effect of aqueous extracts of M. sativa on diuresis in mice

Group	Volume of urine(ml)					Diuretic action	Diuretic activity
	1h	2h	3h	4h	5h		
CON	0.14±0.09	0.25±0.09	0.39±0.06	0.40±0.06	0.40±0.06	1.0	
HCT 10	0.86±0.16^{a2}	1.41±0.12^{a1}	1.56±0.1^{a3c3d2}	1.6±0.11^{a3c2}	1.6±0.11^{a3c2}	4.13	1.0
MA250	0.41±0.08	0.72±0.19	0.76±0.14	0.76±0.14	0.79±0.13	2.49	0.60
MA500	0.58±0.16	0.84±0.14	0.98±0.13^{a2}	1.15±0.17^{a2}	1.15±0.17^{a2}	3.58	0.86
MA1000	0.78±0.11^{a1}	1.04±0.09	1.40±0.09^{a3c2}	1.55±0.1^{a3c2}	1.55±0.1^{a3c2}	4.04	0.97

Values are expressed as Mean ± S.E.M (n=8); analysis was performed with One-Way ANOVA followed by Tukey test; ^{a}against control, ^{b}against standard drug, ^{c}against MA250, ^{d}against MA500, ^{e}against MA1000; $^{1}P<0.05$, $^{2}P<0.01$, $^{3}P<0.001$; Diuretic Action = mmol of electrolyte of test group/mmol of electrolyte of control group; HCT10 refers to standard and MA refers to aqueous fraction of *Medicago sativa*; control received distilled water where as standard received hydrochlorothiazide; Numbers refer to dose in mg/kg.($R^2=0.97$).

4.4.2. Hydro alcoholic Extract

For the hydro alcoholic extract, diuresis was not as effective as that of aqueous extract. The effect was significant at fourth and third for two doses (MM500 and MM1000) compared to CON group. Although MM 500 resulted in an increased diuresis starting from the first hour, It failed to reach statistical significance. However, increase in diuresis started to become significant at the fourth hour (110%, $p<0.05$), when compared with the CON (Table 3). HCT10 on the other hand produced a significant diuresis starting from the first hour.

As compared to that of HCT10, MM250 ($p<0.05$) produced a lower diuretic effect, while the maximum dose (MM1000) had an effect which was comparable to that of HCT10 *i.e.* With diuretic action of (1.79 vs. 2.23) (Table 3).Among the different doses of treatment groups, the maximum dose of MM1000 produced an increased diuresis which was significant at the third , fourth and fifth hour ($p<0.05$), when compared with MM250.

Table 3: Effect of Hydro alcoholicextracts of the leaves of *M. sativa* on diueresis in mice

Group	Volume of urine(ml)					Diuretic action	Diuretic activity
	1h	2h	3h	4h	5h		
CON	0.15±0.01	0.37±0.05	0.47±0.02	0.48±0.02	0.48±0.02	1.0	
HCT10	0.41±0.08^{a1c1}	0.78±0.09^{a2c2}	1.10±0.1^{a2c1}	1.3±0.1^{a2c2}	1.3±0.12 ^{a3c2}	2.23	1.0
MM250	0.13±0.02	0.40±0.03	0.57±0.05	0.57±0.05	0.57±0.05	1.12	0.50
MM500	0.31±0.05	0.50±0.07	0.83±0.13	1.01±0.15^{a1}	1.01±0.15^{a1}	1.43	0.64
MM1000	0.27±0.06	0.62±0.09	1.0±0.16^{a2c1}	1.1±0.2^{a2c1}	1.13±0.18^{a2c1}	1.79	0.80

Values are expressed as Mean ± S.E.M (n=8); analysis was performed with One-Way ANOVA followed by Tukey test; ^{a}against control, ^{b}against standard drug, ^{c}against MM250, ^{d}against MM500, ^{e}against MM1000; $^{1}P<0.05$, $^{2}P<0.01$, $^{3}P<0.001$; Diuretic Action = mmol of electrolyte of test group/mmol of electrolyte of control group; HCT10 refers to standard and MM refers to alcoholic fraction of *Medicago sativa*; control received 2% tween 80 where as standard received hydrochlorothiazide; Numbers refer to dose in mg/kg.($R^2=0.98$).

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

4.5.1. Aqueous Extract

The urine samples collected over the five hours were analyzed for the electrolyte content (Na^+, K^+, and Cl^-) and presented in Table 4, Whilst MA250 tended to decrease sodium loss by 14.9%, MA500 increased by 39.9% compared to CON group. By contrast, MA1000 significantly increased sodium loss by 70.5% ($p<0.001$).

Urinary K^+ excretion was measured for all treatment groups, and HCT10 had shown significant K^+ loss with 129.4% ($p<0.001$) compared to the CON group. MA250 showed a slight kaliuresis (42.3%) and that of MA500 even showed more increased K^+ excretion (80.2%) also found to be significant at both doses when compared to CON group. But the maximum dose of the aqueous extract showed relatively lower amount of potassium excretion even when compared with that of the CON group. In the case of CI^- the extract doses produced an increased excretion in the urine which was 16.9%, 20.8% and 83.9% ($p<0.001$) forMA250, MA500 and MA1000, respectively (Table 4).

The first two doses of the extract showed a lowered excretion of urinary Na than HCT10 while the highest dose (MA1000) had a higher excretion effect compared with the standard. However, K^+ Excretion of HCT10 significantly exceeded all the three doses MA250 ($p<0.001$) ,MA500 ($p<0.001$) and MA1000 ($p<0.001$). For CI^-, however, both HCT10 and MA1000 are significant to the other two doses of MA250 and MA500 (Table 4). Table 4 also shows that the Saluretic indices of $Na^+$and CI^- of the extract at the highest dose and HCT10 were comparable (1.70, 1.83 vs 1.59, 1.63), while the saluretic index of K^+ for the highest dose was smaller than HCT10 (0.99 vs 2.29). In addition, the Na^+/K^+ ration of MA1000 was higher than HCT10. $CI^-/Na^{+/}K^+$was also calculated and MA500 had the lowest value (0.35).

Comparing the different doses of the extract, MA1000 and MA500 had produced a significant natriuretic as compared with MA250 with $p<0.001$, but with regard to K^+ excretion the medium dose (MA500) had the highest kaliuresis as compared to MA1000 ($p<0.001$). MA1000 had significant excretion of CI compared with MA500 and MA250.

Table 4:Effect of aqueous extract of the leaves of *M. sativa* on 5h urinary electrolyte excretion in mice

Group	Urinary electrolyte excretion (mmol/L)			Saluretic index			Na^+/K^+	Cl^-/Na^++K^+
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-		
CON	58.50±2.58	39.87±2.97	44.37±3.33				1.46	0.45
STND 10	93.37±2.45^{a3c3d1}	91.5±2.65^{a3c3d3e3}	72.6±3.30^{a3c3d2}	1.59	2.29	1.63	1.02	0.39
MA250	49.75±2.54^{b3d3e3}	56.7±3.34^{a3b3d2e2}	51.87±2.82^{b3e3}	0.85	1.42	1.17	0.88	0.48
MA500	81.87±3.44^{a3b1c3e3}	71.8±3.30^{a3b3c2e3}	53.62±4.45^{b2e3}	1.4	1.8	1.2	1.14	0.35
MA1000	99.75±2.26^{a3c3d3}	39.50±3.84^{b3c3d3}	81.62±3.09^{a3c3d3}	1.7	0.99	1.83	2.50	0.59

Values are expressed as Mean ± S.E.M (n=8); analysis was performed with One-Way ANOVA followed by Tukey test; [a]against control, [b]against standard drug, [c]against MA250, [d]against MA500, [e]against MA1000; [1]P<0.05, [2]P<0.01, [3]P<0.001; Saluretic Index = mmol of electrolyte of test group/mmol of electrolyte of control group; HCT10 refers to standard and MA refers to aqueous fraction of *Medicago sativa*; control received distilled water where as standard received hydrochlorothiazide; Numbers refer to dose in mg/kg.

4.5.2. Hydroalcoholic Extract

Similarlythe five hours urine obtained from the hydroalcoholic extracts were analyzed for the same electrolytes. The urinary Na+excretion showed an increasing pattern as it was 4.2%, 53.8%($p<0.001$) and 70.5% ($p<0.001$), for the respective dosed of MM250, MM500 and MM1000 when compared with CON group. On the other hand, K^+ excretion had shown a decreasing order from MM250 to MM1000 .In case of Cl^- excretion,MM1000had showed highest record ofCl^-excretion while MM500 showed the lowestCl^-excretion .

Sodium excretion of MM250 and MM500 was lesser as compared to HCT10, but MM1000 have had comparable excretory effect. On the contrary K^+ excretions for the first two doses were higher than that of HCT10 but there was not significant relationship in between the extracts andthe standard drug. In the case of Cl^- , excretion there was notany significantdifference between the standardand the MM1000 dose of the extract. The saluretic indices had also been calculated similarly and closer results were obtained for Na+and Cl^- between the highest dose of the extract and HCT10(1.70, 1.73 Vs 1.59, 1.63). $Cl^-/Na^+ + K^+$value also had been calculated and the MM500 provided the least value (0.29). (Table 5).For the different doses of hydroalcoholic extract, both MM1000 and MM500 had resulted in a significant Na^+ excretion when compared with MM250 i.e. $p<0.001$. In the case of K^+ excretion, MM250 had the highest kaliuretic effect when compared with MM1000 ($p<0.001$). In Cl^- excretion MM1000showed a highest excretion difference.

Table 5:Effect ofhydroalcoholic extract of the leaves of *M. sativa* on 5h urinary electrolyte excretion in mice

Group	Urinary electrolyte excretion (mmol/L)			Saluretic index			Na^+/K^+	Cl^-/Na^++K^+
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-		
CON	58.5±2.58	39.871±2.92	44.37±3.33				1.46	0.45
HCT 10	93.37±2.45^{a3C3}	91.50±2.65^{a3e3}	72.6±3.3^{a3c1d2}	1.59	2.29	1.63	1.02	0.39
MM250	61.00±2.97^{b3d3e3}	99.25±4.15^{a3e3}	58.50±3.19^{a1b1e1}	1.04	2.48	1.32	0.61	0.36
MM500	90.00±2.69^{a3c3}	98.62±3.13^{a3e3}	55.12±3.71^{b2e3}	1.53	2.47	1.24	0.91	0.29
MM1000	99.75±2.26^{a3c3}	52.50±2.71^{b3c3d3}	77.12±1.90^{a3d3c2}	1.70	1.31	1.73	1.90	0.51

Values are expressed as Mean ± S.E.M (n=8); analysis was performed with One-Way ANOVA followed by Tukey test; [a]against control, [b]against standard drug, [c]against MM250, [d]against MM500, [e]against MM1000; [1]P<0.05, [2]P<0.01, [3]P<0.001; Saluretic Index = mmol of electrolyte of test group/mmol of electrolyte of control group; HCT10 refers to standard and MM to methanol fraction of *Medicago sativa*; control received 2% tween80; Numbers refer to dose in mg/kg.

4.6. Electrolyte Content of the Extracts.

The electrolyte content of both aqueous and 80 % methanolextracthad been explored so as to rule out the interference of the result. The result showed that $Na^+$and Cl^-amounts were below detectionlevel. And K^+ content was found to be 25.1, 50.5 and 57.2 mmol/lit for MA250, MA500 and MA1000, respectively, in the case of aqueous extract. And lower values of 14, 23 and 26 mmol/lit for MM250, MM500 and MM1000, respectively, in case of hydroalcholic extract.

5. DISCUSSION

Diuresis has two components increase in urine volume and a net loss of electrolytes in the urine (Jackson, 2006). These processes result from suppression of renal tubular reabsorption of water and electrolyte in to the blood stream. In the present study, therefore, both volume and electrolyte parameters were measured to evaluate the diuretic activity of the plant extracts.

The plant was prepared as maceration in order to simulate the way it is traditionally used, by macerating the arial part for about three days in water. And with the assumption that the active ingredient (s) responsible for the claimed diuretic activity might not be soluble in water adequately; hydroalcoholic extract of the plant was also included in the study.Previous studies on diuretic agents have found it to be advantageous to 'pre-treat' or'prime' the test animals with various fluids. Since diuretics are employed clinically in the treatment of edema, it would be highly important to demonstrate effectiveness in the presence of electrolyte and water (Nedi et al, 2004). Thus, the saline was administered to simulate edema.In view of urine output, both aqueous and 80% methanol extracts of the leaves of the plant *M.sativa* showed an increased diuresis as compared to the control group. Compared to hydroalcoholic extract, the aqueous extract produced better diuretic effect. This difference in their effect could be seen in the different doses used in this study. The minimum doses of both extracts did not produce an effect and this could be accounted by the lack of enough concentration of active components which were responsible for the diuretic activity at these lower doses. But in case of the medium doses, while the aqueous extract MA500, was able to produce significant diuresis starting from third hour (Table2), the same dose of the hydroalcoholic extract was able to produce significant diuresis starting from forth hour (Table 3). Increasing the dose did affect the diuretic effect produced especially by the aqueous extract. For e.g., the diuretic effect produced by MA1000

was higher than that achieved by MA500 (1.55± 0.12 vs 1.15± 0.17) (table 2). Moreover, the diureticactively (0.86) of MA 500, was lower than MA1000 (0.97). On the other hand, MM1000 produced a diuretic effect of (1.13±0.18), which was yet lower than both the medium and maximum doses of aqueous extract. It is therefore possible to suggest that the ingredient (s) of the plant material responsible for the diuretic effect could probably be more polar and hence better extracted in water than80%methanol.

Comparison of the two extracts of *M.sativa* indicate that both extracts showed an increase in diuresis which appeared to be dose- dependent. The diuretic activity of *M.sativa*at their highestrespective was closer to HCT10. Since their values were 0.97 and 0.80 for MA1000 and MM1000 respectively.According toGujral et al, diuretic activity is good if it is greater than 1.5, moderate if it is between 1.0 and 1.5,little if between 0.72 and 1.0 and nil if it is less than 0..The diuretic action of the plant extracts, particularly, at the highestdoses was high and quantitatively similar to that of HCT10. Although the lower doses of the extracts produced diuretic effect significantly lower than HCT10, both MA1000 and MM1000 of the aqueous and hydroalcoholic extract, respectively, wereable to produce effects comparable to that of HCT10 at the fifth hour (Table 2 and 3) which clearly shows that the extracts have a potential to induce diuresis markedly as those of known synthetic diuretics.

Furthermore, the onset of diuretic action of the most effective dose of the extracts such as MA1000 sufficiently rapid and had a fairly long duration of action as it produced its significant effect from the first hour ($p<0.01$) to the fifth hour ($p<0.001$) (Table 2). The difference in the time of onset of the diuretic action of the different doses and between extracts may be related to the gastrointestinal absorption characteristics of the active principle (s) which are responsible for

the evidenced diuretic activity. This is an appealing diuretic profile as it would curtail the frequency of administration, in addition to a decreased risk of hypokalemia.

The effect of the extracts on water excretion was accompanied by urinary electrolyte excretion effect, since there appeared to be an increased salt excretion as compared to the control group, which supports the idea that the diuretic effect of *M.sativa* was of the saluretic type in contrast to aquaretic type which is feature of most diuretic agents (Martin-Herrera *et al*., 2007). In the case of Na^+ and Cl^-, all doses of both extracts showed significant effect when compared to the CON group. It is therefore highly beneficial in different edematousconditions. The ratio Na^+/K^+ was calculated as indicator of natriuretic activity. Values greater than 2.0 indicate a favorable natriuretic effect, if the ratio exceeds 10.0, it would have potassium-sparing effect (Vogel, 2007). The Na^+/K^+ values were calculated and showed the natriuretic effect to be, 2.50 and 1.90 for MA1000 and MM1000, respectively, which further strengthens the higher natriuretic effect of the extracts at these doses. So the aqueous extract at the dose of MA1000 had the best natriuretic effect and its value was greater than 2.0 and the hydroalcoholic extracts (MM1000) also had a value of 1.90 which was around the acceptable value to have natriuretic effect.

Regarding K^+ excretion, it is easy to observethat *M.sativa* at doses of 1000 mg/kg (aqueous) and 1000 mg/kg (80 % methanol) showed an interesting k^+sparing effect, whose values were nearer tothose of the CON group and as loop diuretic, furosemide by acting in the TAL resulted in hypokalemia. But in the case of *M.sativa*extracts of MA1000 and MM1000, there happened to be a K^+-savingeffect,when compared to HCT10 group and the difference found to be significant both extracts (MA1000 and MM1000). This fact may point out that the extract at higher peak

doses exhibited advantageous effect with respect to hypokalemia, one of the potential adverse effects of hydrochlorothiazide. But still even higher doses are needed tobe investigated to definitely assure if this K^+- saving effect would show a dose dependent manner henceforth.

In contrast with the previous assays carried out comparing aqueous and methanol extracts of some plants which showed an interesting K^+- saving effect at low and intermediate doses (Mart'In-Herrera *et al*.,2007), in the present study of*M.sativa* for both extracts the above K^+- saving effects were observed only at maximum doses (i.e. MA1000 and MM1000). It is probable that at low dosages of the aqueous and methanol extracts of *M.sativa*, the substances responsible for the K^+- sparing effect were not found in sufficient concentrations as occurred with the maximum dose. And this could be highly associated with the amount of the active principle that should be reached to the site of action, so at higher doses there would be a sufficient amount of active component to distribute and reach the receptors, so as to produce the effect, in addition the interaction between active principles probably have been highly found at lower doses at sufficient amount. And this seems to account for the existence, at leastwith very strong diuresis with a sparing of potassium (Mari' in-Herrera *et al*., 2008).

In view of the mechanistic study of the plant extracts, it is possible that *M.sativa* extracts exerted diuretic effect by inhibiting tubular reabsorption of water and direct action of potassium content of *M.sativa* extract on diuretic effect is not considered particularly in case of hydro alcoholic extract, since the K^+ content of the extract was very low in comparison with the salt concentrationobtained from other plants (Sripanidkulchai et al., 2010). Regarding to the 80 % methanol extract, it should be pointed outthat, in contrast to the aqueous extract in whose water preparation it occurs a removal of salts, with the hydro alcohol this salts removal does not

generate. Thus the notable diuretic effect produced bythe 80 % methanol extract at the maximum dose reformed the concept that the diuretic activity of *M.sativa*was not solely due to its content of potassium salts. So it is possible to rule out the osmotic mechanism of action that could occur due to the higher salt content of the plant at least for the hydroalcholic extract. Even if, this difference in potassium content might contribute for a discrepancy in diuretic effect between the aqueous and hydroalcholic extracts, since the potassium content was relatively higher in the case of aqueous extract.

Loop diuretics like furosemide increases urinary flow rate and urinary excretion of sodium, potassium and chloride, by inhibiting Na^+-K^+-2CI' symporter in the TAL and inhibiting CA enzyme (Bevevino et al., 1994). Aqueous and hydroalcoholic extracts of *M.sativa* produced diuresis and saluresis, but at the highest doses Na^+ and CI^- excretion were similar with that of hydrocholorothiazide, however with regard to $K^+$the extracts had a lower K^+ excretion at these doses nothing like loop diuretics which significantly produced kaluresis, so the mechanism was unlikely to be the loop diuretics type. It is also possible to exclude the thiazide like type mechanism either, as these diuretics relatively increase the urinary out put more and alters the urinary Na^+/K^+ ratio, but the extracts from plant *M.sativa* showed a K^+-saving effect at their maximal doses. The $Cl^-/ Na^+ +K^+$ ratio was calculated and showed the extent of CA inhibitory effect. CA inhibition can be excluded at ratios between 1.0 and 0.8. With decreasing ratios slight to strong CAI can be assumed (Vogel, 2007). The $Cl^-/ Na^+ + K^+$ amount was strongest CA inhibitory effectwith values of 0.38 and 0.29 respectively. And still the maximum doses produced the highest diuresis, even though the medium doses had the lowest $CI^-/Na^+ + K^+$ ratio, thus there ought to be another mode of action which manifested at the higher doses.

Indetermination of the urinary pH, the extracts showed a relative increase in the pH values as compared to the controls, so this strengthens that CA inhibition as one of the mechanisms of action of the plant. Thus, these reductions of potassium excretion at the maximum doses of the extracts along with the resulted alkalization of the urine might give clue on the probability of the plant acting as modest potassium-saving diuretics.The active principle/s responsible for the diuretic effects of the 80 % methanol and aqueous extracts of this spices is/are, so far, not known, so it is not identified which compounds are exactly responsible for the diuretic, natriuretic and kaliureticactivities of *M.sativa.* But preliminary phytochemical analysis carried out with the 80 % methanoland aqueousextracts revealed the presence of polar compounds such as alkaloids and steroids. One can suppose that these substances might be responsible, at least in part, for the observed diuretic activity and that they may act individually or synergistically. Previous studies have demonstrated also that there are several compounds which could be responsible for the plants diuretic effects such as flavonoids, saponins or organic acids (Maghrani*et al*., 2005). The effect may be produced by stimulating regional blood flow or initial vasodilatation, or by producing inhibition of tubular reabsorption of water and anions. With the result in both cases being diuresis (Mart'in-Herrera *et al*., 2008)

The present study supports the ethno medical use of *M.sativa* for its diuretic effect. Although, the active component (s) remained un known, based on the pattern of excretion of water, sodium and potassium it appears that the plant could possibly have more than one mechanism of action which contributes to the potassium- saving and natriuretic effect especially at the maximal

doses. Multiple mode of action had been reported with some herbal medications (Jaykody*et al.,* 2011). Thus, adding up to the predicted CA inhibitory effect there must be another active component (s which reaches effective concentration at maximum doses of the extracts that contributes to the potassium saving and highest diuretic effect of the plant *M.sativa*.

6. CONCLUSIONS

Looking at the data shown in the results from both aqueous and hydroalcholic extracts of *M. sativa* there had been a very interesting Saluretic diuresis noted especially in case of aqueous extract. The diuretic action of the plant extracts especially at the higher doses had been

comparable to the standard drug. And the maximum doses of both extracts had a little diuretic activity, since their values were near 1.0. Diuretic activity increased with increasing dose.

From the electrolytes analyzed andurinary pH it was possible to assumethat the plant could have multiple mode of action, CAI mechanism beingone of them.

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

The safe nature of the plant in addition to the evidenced diuretic effect from both extracts in the present study provides further support to explain the traditional folk-medicine use of *M.sativa* to treat edematous conditions.

7. RECOMMENDATIONNS

- Investigation of specific component (s) responsible for the diuresis should be analyzed from the different extracts of the crudes extracts.

- Further investigations are necessary to determine the precise mechanism by which the extracts of Medicago Sativa affect diuresis and urinary electrolytes excretion especially to confirm the evidenced in-vivo CAI effect on mice though further in-vitro test.

- The chronic toxicity profile of the plant also should be performed so as to prove the safety in long term use.

REFERENCES

Addo HA, Ferguson J, Frain-Bell W. Thiazide-induced photosensitivity: a study of 33 subjects. *Br J Dermatol.* 1987;116:749–760.

Aditya S and Rattan A (2012). Vaptans: A new option in the management of

hyponatremia. *International Journal of Applied and Basic Medical Research* **2**(2): 77-83.

Armentero MT, Pinna A, Lanciego JL, Müller CE and Franco R (2011).Past, present and future of A_{2A} adenosine receptor antagonists in the therapy of Parkinson's disease.*Pharmacology & Therapeutics***132**(3): 280-299.

Aronow WS. Optimal Blood Pressure Goals in Patients With Hypertension at High Risk for Cardiovascular Events. Am J Ther.2013 (epub ahead of print).

Asare K (2009). Management of loop diuretic resistance in the intensive care unit.*American Journal of Health-System Pharmacy* **66**: 1635-1640.

Atherton JC(2012). Renal blood flow, glomerular filtration and plasma clearance. *Anesthesia and Intensive Care Medicine***13**(7): 315-319.

Atta AH, Nasr SM, Mouneir SM, Al-Wabel NA and Essawy SS (2010).Evaluation of the diuretic effect of *Conyza dioscorides* and *Alhagi maurorum*.*International journal of pharmacy and pharmaceutical sciences* **2**: 162-165.

Auria DD, Marinosci GZ, Benedictis GD and Piazza O (2012).Vaptans and hyponatremia in critical patients.*Translational Medicine* **3**(1): 1-14.

Avato P, Bucci R, Lava A, Vitali C, Rosato A, Bialy Z, Jurzysta M. (2006). Antimicrobial activity of saponins fromMedicagospp. structure-activity relationship.Phytother Res, 20, 454–457

Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K and Ezennia EC *et al* (2008). Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in south western

Nigeria. *Tropical Journal ofPharmaceutical Research***7**(3): 1019-1024.

Bansal S. Sexual dysfunction in hypertensive men.A critical review of the literature.*Hypertension.*1988;12:1–10.

Barnes DK, Sheafer CC. (1995). Alfalfa. In: Barnes RF, Miller DA,Nelon CJ, eds.

Barone S, Churchill PC and Jacobson KA (1989). Adenosine Receptor Prodrugs: Towards Kidney-Selective Dialkylxanthines. *Journal of Pharmacology and ExperimentalTherapeutics***250**(1): 79-85.

Barrett KE, Brooks HL, Boitano S and Barman SM (2010).Renal function and micturition. In: Ganong's Review of Medical Physiology 23th edition. New York, McGraw-Hill: 639-663.

Beevers DG, Hamilton M and Harpur JE (1971).The long-term treatment of hypertension with thiazide diuretics.*Postgraduate Medical Journal* **47**: 639-643.

Benjumea, D., Abdala, S., Hermandez-Luis, F., Perez-Paz, P., Martin-Herrea, D., 2005. Adverse reactions to bendrofluazide and propranolol for the treatment of mild hypertension.Report of Medical Research Council Working Party on Mild to Moderate Hypertension.Lancet.1981;2:539–543.

Berl T, Quittnat-Pelletier F, Verbalis JG, Schrier RW, Bichet DG and Ouyang J *et al* (2010). Oral tolvaptan is safe and effective in chronic hyponatremia. *Journal of the American Society of Nephrology* **21**(4): 705-712.

Bevevino,L.H.,Vieira,F.S.A.,Cassola,A.C.,Sanioto,S.M.L.,1994.Effectofcrudeextractof roots of Bredemeyera floribunda Willd I. Effect on arterial blood pressure andrenal excretion in the rats. Journal of Ethnopharmacology 43, 197–201.

Beyer KH (1982).Chlorothiazide.*British journal of clinical pharmacology* **13**: 15-24.

Bhavin V, Ruchi V and Santani DD (2011).Diuretic Potential of Whole Plant Extracts of *Pergularia daemia* (Forsk.).*Iranian Journal of Pharmaceutical Research* **10**(4): 795-798.

Bhavin VA, Ruchi VB, Shrikant JV, Payal SD and Dev SD (2011).Effect of aqueous extract of *Pergularia daemia* on urine production. Der Pharmacia Lettre **3** (4): 207-214.

BHMA (1996).British Herbal Pharmacopoeia(BHP). Bournemouth:British Herbal Medical Association.

Biagioni M, Alpi A, Piccia relli P. (1990). Aminopeptidases (EC.3.4.11) in alfalfa (Medicago sativaL.) leaves. J Plant Physiol , 135, 559–564

Blair GS and Allie ND (2011).Spironolactone Use for Women with Acne or Hirsutism.*Journal of the Dermatology Nurses' Association* **10**: 374-376.

Boue SM, Wiese TE, Nehls S, Burow ME, Elliott S, Carter-Wientjes CH,Shih BY, McLachlan JA, Cleveland TE. 2003. Evaluation of the estrogenic efects of legume extracts containing phytoestrogens. J Agr Food Chem, 51, 2193–2199.

Bowman BT and Rosner MH (2013). Lixivaptan - an evidence-based review of its clinical potential in the treatment of hyponatremia. *Core evidence* **8**: 47-56.

Brewster LM and Seedat YK (2013). Why do hypertensive patients of African ancestry respond better to calcium blockers and diuretics than to ACE inhibitors and β-adrenergic blockers? A systematic review.*Biomed central Medicine* **11**(141): 1-16.

Bugaj V, Pochynyuk O and Stockand JD (2009). Activation of the epithelial Na^+ channel in the collecting duct by vasopressin contributes to water reabsorption.

American journal of Physiology Renal Physiology **297**(5): F1411-F1418.

Chang SW, Fine R, Siegel D, et al. The impact of diuretic therapy on reported sexual function. *Arch Intern Med.* 1991;151:2402–2408.

Chen JF, Eltzschig HK and Fredholm BB (2013).Adenosine receptors as drug targets- what are the Challenges?*Nature Reviews Drug Discovery***12**(4): 265-286.

Chmielewski C (2003). Renal anatomy and overview of nephron function. *Journal of theAmerican Nephrology Nurses' Association***30**(2): 185-90.

Chrostowska M, Narkiewicz K. Improving patient compliance with hypertension treatment: mission possible? Curr Vasc Pharmacol. 2010;8:804–7.

Clayton JA, Rodgers S, Blakey J, Avery A and Hall IP (2005).Thiazide diuretic prescription and electrolyte abnormalities in primary care.*British journal of clinical pharmacology* **61**(1): 87-95.

Cohen BI, Mosbach EH, Matoba N, Suh SO, McSherry CK. (1990).The effect of alfalfa-corn diets on cholesterol metabolism and gallstones in prairie dogs.Lipids, 25, 143–148

Costantini F and Kopan R (2010).Patterning a Complex Organ: Branching Morphogenesis and Nephron Segmentation in Kidney Development.*Developmental cell* **18**: 698-712.

Debella A (2002). Manual for phytochemical screening of medicinal plants. Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia: 35-47.
Delhi.

DerMarderosian A, Liberti L, Beutler JA, Grauds C, Tatro DS,Cirigliano M, DeSilva D Jr. (2005). Alfalfa. Review of Natural Products: Facts and comparisons. Alphen aan den Rijn, theNetherlands: Wolters Kluwer Health.

Diniz LRL, Portella VG, Cardoso FM, Souza AM, Caruso-Neves C *et al* (2012). The effect of saponins from *Ampelozizyphus amazonicus* Ducke on the renal Na^+ 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 7th edition. New York, McGraw-Hill.

Duarte JD and Cooper-DeHoff RM (2010). Mechanisms for blood pressure lowering and

Duke J. (1985).Handbook of Medicinal Herbs. Boca Raton, FL: CRC Press.

Duke, James A., Handbook of medicinal herbs / James A. Duke, with Mary Jo Bogenschutz-Godwin,

Ehsanpour AA, Razavizadeh R. (2005). Efect of UV-C on drought tolerance of alfalfa (Medicago sativa) callus. Am J Biochem Biotechnol , 1, 107–110.

El-Khrisy EAM, Abdel Hafez OM, Khattab AA, Ahmed KM. (1994).Chemical constituents of alfalfa

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 5th edition. New York, Elsevier: 1353-1404.

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 ME and Moser M (2009).Use of Diuretics in Patients with Hypertension.*The New England Journal of Medicine* **361**(22): 2153-2164.
Ethnopharmacology 100, 205–209.

Evans WC (2002). A taxonomic approach to the study of medicinal plants and animal derived drug. In: Trease and Evans Pharmacognosy 15th edition. London, Elsevier.

Felker GM (2012). Loop diuretics in heart failure. *Heart failure reviews***17**(2): 305-311.

Fenton M and Burch M (2007).Understanding chronic heart failure.*Archives of Disease inChildhood***92**: 812-816.

Finkler K. (1985). Spiritualist Healers in Mexico. South Hadley, MA:Bergin& Garvey. Forages: An Introduction to Grassland . Ames: IowaState University, pp.205–216.

Friedman PA and Berndt WO (1997).Drugs affecting the cardiovascular system. In: Craig CR and Stitzel RE (eds). Modern Pharmacology with Clinical Applications 5th edition.239-254.

Furman BL. Impairment of glucose tolerance produced by diuretics and other drugs.*Pharmacol Ther*. 1981;12:613–649.

Fushiya S, Tamura T, Tashiro T, Nozoe S. (1984). Structure andsynthesis oF medicanine, a new amino acid FromMedicago sativa.Heterocycles, 22, 1039–1040.

Garwood S (2009). Osmotic Diuretics. In: Ronco C, Bellomo R and Kellum JA (eds). Critical Care Nephrology 2nd edition. New York, Elsevier.

George CRP (2011). Mercury and the kidney.*SocietàItaliana di Nefrologia* **24**(S17): S126-S132.

Ghani A. Medicinal plants of Bangladesh. Chemical constituents and uses. Dhaka: The Asiatic Society of Bangladesh; 2003. pp. 500–508.

Gowda SKP, Satish S, Mahesh CM and kumar V (2009). Study on the diuretic

activity of *Cynodon dactylon* root stalk extract in albino rats. *Research Journal of Pharmacy and Technology* **2**(2): 338-340.

Grimm RH Jr, Grandits GA, Prineas RJ, et al. Long-term effects on sexual function of five antihypertensive drugs and nutritional hygienic treatment in hypertensive men and women. Treatment of Mild Hypertension Study (TOMHS).*Hypertension.*1997;29:8–14.

Gupta BK, Nandra KS, Chopra AK. (1981). Chemical and mineral composition on diferent cuttings of lucerne (Medicago sativa). J Res(Punjab Agricultural University), 18, 345–348.

Guyton AC, Hall JE (2006). The body fluids and kidneys. In: textbook of medical physiology 11th edition. Philadelphia, Elsevier. 325-359.

Hailu W and Engidawork E (2014). Evaluation of the diuretic activity of the aqueous and 80% methanol extracts of *Ajuga remota* Benth (Lamiaceae) leaves in mice. *Biomed central Complementary and alternative medicine***14**(135): 1-8.

Hebert, S.C. Molecular mechanisms.*Semin.Nephrol.*, **1999,** *19*:504-523.

Heywood VH, Ball PW. (1968). Leguminosae. In: Tutin TG, Heywood VH, Burges NA, Moore DM, VaLentine DH, WaLters SM, Webb DA,Eds.Flora Europaea, Vol II. Cambridge: Cambridge University Press, pp. 245–260.

Inamul H. (2004). Safety of medicinal plants.Pakistan J Med Res, 43, 1–8.

Isenring, P., and Forbush, B., III. Ion and bumetanide binding by the Na-K-Cl cotransporter: Importance of transmembrane domains. *J. Biol. Chem.,* **1997,** *272*:24556-24562

Ives HE (2012).Cardiovascular-renal drugs. In: Katzung BG,Masters SB and Trevor AJ (eds). Basic and clinical pharmacology 12th edition. New York, McGraw-Hill: 251-270.

Jackson EK (2006). Drugs affecting renal and cardiovascular function. In: Brunton LL (ed). Goodman & Gilman's the pharmacological basis of therapeutics 11th

edition. New York, McGraw-Hill.

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 K. Persistence of fixed versus free combination with valsartan and HCTZ for patients with hypertension. Value Health Suppl. 2006;9:A363

Jayakody RJ, Ratnasooriya WD, Fernando WA and Weerasekera KR (2011). Diuretic activity of leaves extract of hot water infusion of Ruta graveolens L. in rats.*Journal of pharmacology and toxicology* **6**(5): 525-532.

Johnson RJ, Feig DI, Nakagawa T, Sanchez-Lozada G and Rodriguez-Iturbe B (2008).Pathogenesis of essential hypertension: historical paradigms and modern insights. *Journal of Hypertension* **26**(3): 381-391.

Jouad H, Lacaille-Dubois MA, Lyoussi B & Eddouks M (2001). Effects of the flavonoids extracted from *Spergularia purpurea* Pers. on arterial blood pressure and renal function in normal and hypertensive rats. *Journal of ethnopharmacology***76**(2): 159-163.

Judi duCellier, Peggy-Ann K. Duke.-- 2nd ed. 1929

Kalra OP and Aggarwal A (2012).Rational use of diuretics and pathophysiology of edema.*Medicine Update* **22**: 601-610.

Karakurt P, Kasikci M. Factors affecting medication adherence in patients with hypertension. Journal of vascular nursing: official publication of the Society for Peripheral Vascular Nursing 2012;30:118–26.

Kardasz S, 2009. The function of the nephron and the formation of urine.*Anesthesia &Intensive Care Medicine***10**(6): 265-270.

Kasiske BL, Ma JZ, Kalil RS, et al. Effects of antihypertensive therapy on serum lipids.*Ann Intern Med.* 1995;122:133–141.

Khan A, Bashir S and Gilani AH (2012).An in vivo study on the diuretic activity of *Holarrhena antidysenterica*.*African Journal of Pharmacy and Pharmacology* **6**(7): 454-458.

Kleyman, T.R., Sheng, S., Kosari, F., and Kieber-Emmons, T. Mechanisms of action of amiloride: A molecular prospective. *Semin.Nephrol.*, **1999,** *19*:524-532.

Koechel DA (2004). Diuretics. In: Block JH and Beale JM (eds). Wilson and Gisvold's text book of organic medicinal and pharmaceutical chemistry 11th edition. Baltimore, Lippincott: 596-598.

Koti BC, Purnima A. Diuretic activity of extracts of *Centratherum anthelminticum*. international journal of green pharmacy 2008;2:228- 231.

Kumar BNS, Swamy BMV, Swamy A and Mural A (2010).A Review on Natural Diuretics.*Research Journal of Pharmaceutical, Biological and Chemical Sciences* **1**(4): 615-634.

LaFleur J, Oderda GM. Methods to measure patient compliance with medication regimens. J Pain Palliat Care Pharmacother. 2004;18:81–7.

Lahlou S, Tahraoui A, Israili Z and Lyoussi B (2007). Diuretic activity of the aqueous extracts of *Carum carvi* and *Tanacetum vulgare* in normal rats. *Journal ofEthnopharmacology* **110**: 458-463.

Lakshman MR, Reda DJ, Materson BJ, et al. Diuretics and beta-blockers do not have adverse effects at 1 year on plasma lipid and lipoprotein profiles in men with hypertension. *Arch Intern Med.* 1999;159:551–558.

Lant AF (1987). Evolution of diuretics and ACE inhibitors, their renal and antihypertensive actions-parallels and contrasts.*British journal of clinical pharmacology* **23**: 27S-41S.

Leone A. The Economic Costs for the Control of Cardiovascular Risk: An Overview. Current Pharmaceutical Design. 2013;19(13):2447–53.

Levi TM, Rocha MS, Almeida DN, Martins RTC, Silva MDC, Santana NCP *et al* (2012). Furosemide is associated with acute kidney injury in critically ill patients. *Brazilian Journal of medical and biological research* **45**(9): 827-833.

Liamis G, Milionis H and Elisaf M (2008). A review of drug-induced hyponatremia.*American Journal of Kidney Diseases* **52**(1): 144-153.

Lihu M, Pedak E. (1979). Chemical composition of alfalfa (Medicago sativaL.) in diferent stages of growth. Teaduslike Toode Kogumik ,49, 104–109.

Ljunghall S, Backman U, Danielson BG, et al. Effects of bendroflumethiazide on urate metabolism during treatment of patients with renal stones.*J Urol*. 1982;127:1207–1210.

Lueder TG, Atar D and Krum H (2013).Diuretic use in heart failure and outcomes.*Clinical Pharmacology & Therapeutics* **94**(4): 490-498.

Magil AB, Ballon HS, Cameron EC, et al. Acute interstitial nephritis associated with thiazide diuretics. Clinical and pathologic observations in three cases.*Am J Med.* 1980;69:939–943.

Malinow MR, Connor WE, McLaughlin P, Staford C, Lin DS,Livingston AL, Kohler GO, McNulty WP. (1981b). Cholesterol and bile acid balance inMacaca fascicularis.Efects of alfalfa saponins. J Clin Invest , 67, 156–162

Mantel-Teeuwisse AK, Kloosterman JM, Maitland-van der Zee AH, et al. Drug-Induced lipid changes: a review of the unintended effects of some commonly used drugs on serum lipid levels. *Drug Saf.*2001;24:443–456.

Maramag RP (2013). Diuretic potential of *Capsicum frutescens*Linn.,*Corchorus oliturius* Linn., and *Abelmoschus esculentus Linn.Asian journal of natural & applied sciences* **2**(1): 60-69.

Martin-Herrera D, Abdala S, Benjumea D and Perez-Paz P (2007). Diuretic activity of *Withania aristata*: An endemic Canary Island species. *Journal of Ethnopharmacology* **113**: 487-491.

Martin-Herreraa D, Abdalaa S, Benjumeaa D and Gutierrez-Luis J (2008). Diuretic activity of some *Withania aristata* Ait.Fractions.*Journal of Ethnopharmacology* **117**: 496-499.

McDonnell PJ, Jacobs MR. Hospital admissions resulting from preventable adverse drug reactions. The Ann Pharmacother. 2002;36:1331–6. Medicago sativaL.Bull Nat Res Cent (Egypt), 19, 117–122.

Meera R, Devi P, Muthumani P, Kameswari B and Eswarapriya B (2009). Evaluation of Diuretic activity from *Tylophora indica* leaves extracts. *Journal of pharmaceutical sciences and research* **1**(3): 112-116.

Mego V, Erdelsky K. (1977). Free amino acids in alfalfa (Medicago sativa) L.Physiol Plantarum, 13, 49–55.

Mehta RL, Pascual MT, Soroko S and Chertow GM (2002).Diuretics, mortality, and non-recovery of renal function in acute renal failure.*Journal of American medical association***288**(20): 2547-2553.

Mekonnen T, Urga K and Engidawork E (2010). Evaluation of the diuretic and analgesic activities of the rhizomes of *Rumex abyssinicus* Jacq in mice. *Journal ofEthnopharmacology* **127**: 433-439.

Mekonnena T, Urga K, Engidawork E , Evaluation of the diuretic and analgesic activities of the rhizomes of Rumex abyssinicus Jacq in mice

Melis MS, Rocha ST and Augusto A (2007). Steviol effect, a glycoside of *Stevia rebaudiana*, on glucose clearances in rats. *Brazilian Journal of Biology* **69**(2): 371-374.

Metabolic effects of thiazide and thiazide-like diuretics. *ExpertReview* of *Cardiovascular Therapy* **8**(6): 793-802.

Mills S. (1994).Te Complete Guide to Modern Herbalism. Wellingborough:Torsons.

Min B and White CM (2009). A review of critical differences among loop, thiazide, and thiazide-like diuretics.*Hospital Pharmacy***44**(2): 129-149.

Minerva G. (1998). Treatment of neurovegetative menopausal symptoms with a phytotherapeutic agent.Universita degli Studi-Siena, 50, 207–211.

Miracle CM, Rieg T, Blantz RC, Vallon V and Thomson SC (2007).Combined Effects of Carbonic Anhydrase Inhibitor and Adenosine A_1 Receptor Antagonist on Hemodynamic and Tubular Function in the Kidney.*Kidney and Blood Pressure Research***30**: 388-399.

Monroy, A., Plata, C., Hebert, S.C., and Gamba, G. Characterization of the thiazide-sensitive Na^+-Cl^- cotransporter: A new model for ions and diuretics interaction. *Am. J. Physiol. Renal Physiol.*, **2000,** *279*:F161-F169.

Mukherjee PK (2002). Evaluation of diuretic agents. In: Quality Control of Herbal Drugs. Business Horizons, New Delhi.

Mukherjee, P.K., 2002. Quality Control of Herbal Drugs. Business Horizons, New

Musini VM, Rezapour P, Wright JM, Bassett K and Jauca CD (2012). Blood pressure lowering efficacy of loop diuretics for primary hypertension (Review)

Issue 8. Newjersy, John Wiley & Sons: 1-58.

Naveen KB, Ahad AH, Thippeswamy M, Sreeramulu NS, Anuradha CM and Anil KK (2013).Evaluation of diuretic activity with different concentrations of *Allium cepa on* Albino rats.*International Journal of Chemistry and Pharmaceutical Sciences* **1**(1):56-62.

Nedi T, Mekonnen N and Urga K (2004).Diuretic effect of the crude extract of *Carissa edulis* in rats.*Journal of Ethnopharmacology* **95**: 57-61.

Nielsen S, Kwon TH, Christensen BM, Promeneur D, Frøkiær J and Marples D (1999).Physiology and Pathophysiology of Renal Aquaporins.*Journal of the American Societyof Nephrology***10**: 647- 663.

Nielsen S, Kwon TH, Fenton RA, Prætorious J (2012). Anatomy of the kidney. In: Taal MW, Chertow GM, Marsden PA, Skorecki K, Yu ASL, and Brenner BM (eds). Brennerand Rector's the Kidney 9th edition. Elsevier: 31-93.

Nonaka H, Ichimura M, Takeda M, Kanda T, Shimada J and Suzuki F (1996). KW-3902, a selective high affinity antagonist for adenosine A_1 receptors. *British Journal of Pharmacology* **117**: 1645-1652.

OECD (2008). Guidelines for Testing of Chemicals: Guideline 425: Acute Oral Toxicity. Paris, the Organization of Economic Co-operation and Development.

OECD 423 Acute Oral toxicity - Acute Toxic Class Method (Updated Guideline, adopted 20th December 2001)

Orr JD, Sumner LW, Edwards R, Dixon RA. (1993). Determination of cinnamic acid and 4-coumaric acid in alfalfa (Medicago sativaL.)cell suspension cultures by gas

chromatography.Phytochem Anal polysaccharides to the activities of reversetranscriptase of HIV and protease of HIV. Zhongguo Shipin Xuebao,6, 59–62.

Phillipson JD (2001). Phytochemistry and medicinal plants.*Phytochemistry* **56**: 237-243. Preethi GP, Gopalakrishna HN, Rathnakar UP, Durga P, Vishnu and Shenoy J (2012). Acute diuretic activity of alcoholic extracts of *Hygrophila auriculata* seeds in normal wistar albino rats. *International Journal of Pharma and Bio Sciences* **3**(1): 283-289.

Rahman A, Hasan SN, Sampad KS and Das AK (2011).Ant nociceptive, anti-diarrheal and cytotoxic activities of *Rhizophora mucronata* Lamk.*Pharmacology online* **1**: 921-929.

Rang HP, Dale MM, Ritter JM, Flower RJ and Henderson G (2012). Drugs affecting major organ systems; In Rang & Dale's Pharmacology 7th edition.Elsevier.347-359.

Rangarajan B, Binoy V, Hingmire SS and Noronha V (2014).Tolvaptan.*South Asian Journal of Cancer***3**(3): 182-184.

Rankin GO (2008). Drugs affecting the cardiovascular system. In: Lemke TL, Williams DA, Roche VF and Zito SW (eds). Foye's principles of medicinal chemistry 6th edition.Philadelphia, Lippincott: 722-735.

Reddy KY, Lakshmi SM and Kumar AS (2012). Evaluation of diuretic activity of methanolic extract of *Oxalis corniculata* L. in rats. *International Journal of Phytopharmacology* **3**(1): 61-65.

Reilly RF and Ellison DH (2000). Mammalian Distal Tubule: Physiology, Pathophysiology, and Molecular Anatomy. *Physiological reviews* **80**(1): 277-313.

Rochefort CM, Morlec J and Tamblyn RM (2012). What differentiates primary care physicians who predominantly prescribe diuretics for treating mild to moderate hypertension from those who do not? A comparative qualitative study.*Biomed central Family Practice* **13**(9): 1-10.

Ruppert M, Overlack A, Kolloch R, et al. Neurohormonal and metabolic effects of severe and moderate salt restriction in nonobese normotensive adults. *J Hypertens.*1993;11:743–749.

Ryan MP, Devane J, Ryan MF, et al. Effects of diuretics on the renal handling of magnesium.*Drugs.*1984;28(suppl 1):167–181.

Saladin KS (2008). The urinary system. In: Human Anatomy 2nd edition. Boston, McGraw-Hill: 717-135.

Shah SU, Anjum S and Littler WA (2004). Use of diuretics in cardiovascular disease: hypertension. *Postgraduate Medical Journal* **80**: 271-276.

Shah SU, Anjum S and Littler WA (2004). Use of diuretics in cardiovascular diseases: (1) heart failure. *Postgraduate Medical Journal* **80**: 201-205.

Shao H, Dixon RA, Wang X. (2007). Crystal structure of vestitonereductase from alfalfa (Medicago sativaL).J Mol Biol , 369, 265–276

Sharma VK, Tiwari M, Chauhan NS and Nema RK (2012).Phytochemical investigation on the ethanolic extract on the leaves of *Zizyphus xylopyrus* (Retz.)Willd.*International journal of Agronomy and Plant Production* **3**(1): 26-37.

Shchekochikhin D, Al Ammary F, Lindenfeld J and Schrier R (2013).Role of Diuretics and Ultrafiltration in Congestive Heart Failure.*Pharmaceuticals* **6**: 851-866.

Sherrill B, Halpern M, Khan S, Zhang J, Panjabi S. Single-pill vs free-equivalent combination therapies for hypertension: a meta-analysis of health care costs and adherence. J Clin Hypertens. (Greenwich) 2011;13:898–909

Simpson FO. Long-term cost and life-expectancy consequences of hypertension.J Hypertens. 1998;16:1099–100.

Singh S, Singh SK, Srivastava S, Singh P, Trivedi M, Shanker P *et al* (2013). Experimental evaluation of diuretic activity of *Aegle marmelos* in rats.*International Journal of Pharmacy and Biological Sciences* **3**(1): 98-102.

Snigdha M, Kumar SS, Jaya Y and Kasana B (2013).A review on "how exactly diuretic drugs are working in our body".*Journal of Drug Delivery & Therapeutics* **3**(5): 115-120.

Solomon, R., Werner, C., Mann, D., DiElia, J., and Silva, P. Effects of saline, mannitol, and furosemide to prevent acute decreases in renal function induced by radiocontrast agents. *New Engl. J. Med.,* **1994,** *331*:1416-1420

Souzaa AM, Lara LS, Previato JO, Lopes AG and Caruso-Neves C *et al* (2003).Modulation of Sodium Pumps by Steroidal Saponins.*Zeitschrift für Naturforschung***59c**: 432-436.

Sripanidkulchai B, Wongpanich V, Laupattarakasem P, Suwansaksri J and Jirakulsomchok D (2001).Diuretic effects of selected Thai indigenous medicinal plants in rats.*Journal of Ethnopharmacology* **75**: 185- 190.

Staruschenko A (2012). Regulation of transport in the connecting tubule and cortical collecting duct.*Comprehensive Physiology***2**: 1541-1584.

Steppler HA.(1987). ICRAF and a decade of agroforestry development.In: Steppler HA, Nair PKR, eds.Agroforestry: A Decade of Development . Nairobi, Kenya: International Council for Researchin Agroforestry, pp. 13–24.

Strom BL, Schinnar R, Apter AJ, et al. Absence of crossreactivity between sulfonamide antibiotics and sulfonamide nonantibiotics. *N Engl J Med.* 2003;349:1628–1635.

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 Wister albino rats. *International Journal of Research in Ayurveda and Pharmacy***1**(2): 648-652.

Swanston SK, Day C, Bailey CJ, Flatt PR. (1990). Traditional plant treatments for diabetes.Studies in normal and streptozotocin diabetic mice.Diabetologia, 33, 462–464.

Tamsyn SA, Tring PH, Declan PN. (2009). Anti-collagenase anti-elastase and anti-oxidant activities of extracts from 21 plants.BMC Complement Altern Med , 9, 27–37.

Teuber LR, Brick MA. (1988). Morphology and anatomy. In: Hanson AA,Barnes DK, Hill Jr RR, eds.Alfalfa and Alfalfa Improvement .Madison, WI, 125–162

Thomson GE (2007). The health benefits of traditional Chinese plant medicines: weighing the scientific evidence. Australian government **06**(128).

Tirumalasetty J, Chandrasekhar N and Naveen A (2013). Evaluation of diuretic activity of ethanol extract of *Benincasa hispida* stem in Swiss albino rats. *Journal of Chemical and Pharmaceutical Research***5**(3): 91-97.

Van der Vorst MMJ (2007). Optimal Furosemide Therapy in Critically Ill infants. Erasmus MC: University Medical Center Rotterdam.

Varma GG, Mathai BK, Das K, Gowda G, Rammohan S and Einstein JW (2014). Evaluation of antiepileptic activity of methanolic leaves extract of *Tragia*

involucrata Linn. In mice.*International Letters of Natural Sciences* **12**(2): 167-179.

Vazir A and Cowie MR (2013). The use of diuretics in acute heart failure: Evidence based therapy? *World Journal of Cardiovascular Diseases* **3**: 25-34.

Vogel HG (2007). Guidelines for the Care and Use of Laboratory Animals. In: Drug Discovery and Evaluation: Pharmacological Assays 3rd edition. Berlin, Springer-Verlag: 2023-2029.

Wassertheil-Smoller S, Blaufox MD, Oberman A, et al. Effect of antihypertensives on sexual function and quality of life: the TAIM study. *Ann Intern Med.* 1991;114:613–620.

Wilcox EB, Galloway LS. (1961). Serum and liver cholesterol total lipidsand lipid phosphorus levels of rats under various dietary regimes.Am J Clin Nutr , 9, 236–243.

Worthington RB, Breskin MF. (1983). A pharmacists guide to controversial "nutrition products" Am Pharm, 23, 30–42.

Wright CI, Van-Buren L, Kroner CI and Koning MM (2007). Herbal medicines as diuretics: a review of scientific evidence. *Journal of ethnopharmacology* **114**(1): 1-31.

Xiaoqiang W, Xianzhi H, Jianqiao L, Hui S, Zhenzhan C, Richard AD. (2006). Crystal structure of isoflavone reductase from alfalfa(Medicago sativaL.). J Mol Biol , 358 1341–1352.

Yadav R, Kharya MD, Yadav N and Savadi R (2011). Diuretic activity of *Spilanthes acmella*, Murr. Leaves extract in rats. *International journal of research in pharmacy and chemistry* **1**(1): 57-61.

Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE and Drazner MH *et al* (2013).ACCF/AHA Guideline for the Management of Heart Failure.*Journal of the AmericanCollege of Cardiology* **62**(16): 147-239.

Zhang L, Zhang D, Feng K. (2006). Inhibition of refined components of Medicago sativa

Zhao WS, Zhang YQ, Ren LJ, Zhang L, Yang J. (1993).Immunopotentiating efects of polysaccharides isolated fromMedicago sativaL. Zhongguo Yao Li Xue Bao, 14, 273–276.

Zhuo JL and Li XC (2013).Proximal Nephron.*Comprehensive Physiology***3**(3): 1079-1123.