ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

EVALUATION OF RENOPROTECTIVE EFFECTS OF *EUCLEA DIVINORUM* HIERNS AGAINST GENTAMICIN-INDUCED NEPHROTOXICITY IN RATS.

TADIWOS FEYISSA

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A Thesis Submitted to Department of Pharmacology and Therapeutics, School of Pharmacy, Addis Ababa University in Partial Fulfillment of the Degree of Master in Experimental Pharmacology

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DEPARTMENT OF PHARMACOLOGY AND THERAPEUTICS

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

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Abbreviations

ARF:	acute renal failure
ATL:	ascending thin limb
BUN:	blood urea nitrogen
CAT:	catalase
DCT:	distal convoluted tubule
DPPH:	1,1-diphenylpicrylhydrazyl
DTL:	descending thin limb
GFR:	glomerular filtration rate
GSH:	glutathione
GSH-Px:	glutathione peroxidase
GSSG:	glutathione disulfide
IC_{50}:	50% inhibition
JG:	juxtaglomerular
JGA:	juxtaglomerular apparatus
LH:	loops of Henle
MDA:	malondialdehyde
ROS:	reactive oxygen species
SOD:	superoxide dismutase
TAL:	thick ascending limb
TBA:	thio barbituric acid

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Abstract

Despite their nephrotoxicity and ototoxicity, aminoglycoside antibiotics (such as gentamicin and amikacin) are still considered to be important agents against life-threatening infections owing to their bactericidal efficacy, synergism with ß-lactam agents, low cost, limited bacterial resistance and a post-antibiotic effect. The goal of reducing or protecting against their nephrotoxicity will increase the safety of the drugs and significantly improve the quality of life of patients taking aminoglycosides. In this study, the renoprotective effects of the crude extract and solvent fractions of *Euclea divinorum* H. leaves was evaluated against gentamicin-induced nephrotoxicity in rats as the dried roots of the plant is crushed and swallowed for some kidney problems in some regions of Ethiopia. For the assessment of gentamicin nephrotoxicity biochemical and morphologic parameters were measured.

Rats were divided into seven experimental groups of 6 (three male and three female) each. The first group served as a control (CON, group I) and given vehicle (Tween 80, 2%, v/v) in water orally. The second group (GEN, group II) were treated with normal saline intraperitonally for two days and afterwards gentamicin (100 mg/kg/day) for eight days. The rest of the groups received crude extract at three different doses 200 mg/kg (C200, group III), 150 mg/kg (C150, group IV) and 100 mg/kg (C100, group V), methanol fraction (M100, group VI) and aqueous fraction (W100, group VII) of *E. divinorum* orally for two days before and eight days concomitantly with gentamicin (100 mg/kg/day, intraperitonally).

Gentamicin produced changes in renal indices, including increase in blood urea nitrogen (BUN) (111.46%, $p<0.001$) and creatinine (183.26%, $p<0.001$) levels compared to CON. Moreover, it also caused alterations in redox parameters such as increase in lipid peroxidation (83.89%, $p<0.01$) and decrease in catalase (CAT) activity (81.8%, $p<0.05$), superoxide dismutase (SOD) activity (40.3%, $p<0.01$) and glutathione (GSH) levels (38.06%, $p<0.01$) when compared to CON. Morphologic pathologic analysis also revealed a decrease in body weight ($p<0.001$), an increase in normalized kidney weight (42.34%, $p<0.001$) and more extensive/marked tubular necrosis, inflammation, hyaline

casts in tubular lumen, hydropic degeneration and disintegrated nucleus in GEN group compared to CON. Pre- and co-treatment with the crude extract and solvent fractions *E. divinorum* leaves reversed alterations caused by gentamicin as evidenced by decrease in normalized kidney weight ($p<0.05$ in C150, C100 and W100; $p<0.001$ in M100), BUN ($p<0.05$ in C200; $p<0.001$ in C150, C100, M100 and W100), serum creatinine ($p<0.05$ in C200; $p<0.01$ in C150; $p<0.001$ in C100, M100 and W100), lipid peroxidation ($p<0.05$ in all cases) and increase in body weight ($p<0.05$ in C200 and C150; $p<0.01$ in C100, M100 and W100), CAT activity ($p<0.05$ in C200 and C150; $p<0.01$ in W100; $p<0.001$ in C100 and M100), SOD activity ($p<0.05$ in C200, C150, C100 and W100; $p<0.01$ in M100) and GSH level ($p<0.05$ in all cases). Further, crude extract and solvent fractions of the plant were observed to mitigate the histological renal changes associated gentamicin.

Based on the study findings, it could be concluded that gentamicin induces generation of free radicals leading to oxidative damage to kidneys and acute renal failure. *E. divinorum* might act in the kidney as a potent scavenger of free radicals to prevent the toxic effects of gentamicin with a maximal nephroprotection by the methanol fraction followed by aqueous fraction and then by crude extract. In conclusion, *E. divinorum* might be a potential protective agent against drugs-induced nephrotoxicity besides its traditional uses.

Key terms: gentamicin, gentamicin-induced nephrotoxicity, nephroprotection, Euclea divinorum, Dedeho and Mi'essa.

1. Introduction

1.1. Anatomy and physiology of kidney

The renal system, consisting of kidney, ureter, bladder and urethra as well as associated blood vessels, is responsible for the regulation of fluid, electrolytes and arterial blood pressure and the elimination of toxins (Bissinger, 1995). The kidney is covered by a fibrous capsule which is further surrounded by perinephric fat and then by the perinephric (perirenal) fascia, which also enclose the adrenal gland (O'Callaghan, 2009).

The kidney is composed of the cortex, medulla, renal sinus and pelvis. The cortex, the outermost part contains the glomeruli, proximal and distal tubules, cortical collecting ducts, and peritubular capillaries of the nephrons. The middle part of the kidney, the medulla contains the renal pyramids, straight portions of the tubules, loops of Henle (LH), vasa recta, and terminal collecting ducts. The renal sinus and pelvis compose the innermost portion of the kidney (Bissinger, 1995).

The nephron, the basic urine forming unit of the kidney, consists of an initial filtering component called the renal corpuscle and a long tubular portion that extends out from the renal corpuscle, reabsorbing and conditioning the filtrate. Each kidney has over a million nephrons (Vander *et al.*, 2001; Jackson, 2006). Each renal corpuscle contains a compact tuft of interconnected capillary loops called the glomerulus (plural, glomeruli) and a fluid-filled capsule, Bowman's capsule, into which glomerulus protrudes. As blood flows through the glomerulus, a portion of the plasma filters into Bowman's capsule that is separated from the fluid in Bowman's space by a filtration barrier (Vander *et al.*, 2001).

The renal tubule is a very narrow hollow cylinder made up of a single layer of epithelial cells that differ in structure and function along the tubule's length. It consists of proximal tubule, LH and distal tubule (Vander *et al.*, 2001). The proximal tubule is contiguous with Bowman's capsule and takes a tortuous path until finally forming a straight portion that dives into the renal medulla. The tubular cells are tall, columnar epithelial cells with many microvilli, a high surface area, and a well developed luminal endocytic apparatus. Normally, approximately 65% of filtered Na^+ is reabsorbed in the proximal tubule, and

since this part of the tubule is highly permeable to water, reabsorption is essentially isotonic (Jackson, 2006; O'Callaghan, 2009).

Between the outer and inner strips of the outer medulla, the tubule abruptly changes morphology to become the descending thin limb (DTL), which penetrates the inner medulla, makes a hairpin turn, and then forms the ascending thin limb (ATL). At the juncture between the inner and outer medulla, the tubule once again changes morphology and becomes the thick ascending limb (TAL). Together the proximal straight tubule, DTL, ATL and TAL form the LH. The DTL is highly permeable to water, yet its permeability to NaCl and urea is low. In contrast, the ATL is permeable to NaCl and urea but is impermeable to water. The TAL actively reabsorbs NaCl but is impermeable to water and urea. Approximately 25% of filtered Na^+ is reabsorbed in the LH, mostly in the TAL, which has a large reabsorptive capacity (Jackson, 2006).

Near its end, the ascending limb of each LH passes between the afferent and efferent arterioles of that loop's own nephron contacting with the afferent arteriole *via* a cluster of specialized columnar epithelial cells known as the macula densa. The wall of the afferent arteriole contains secretory cells known as juxtaglomerular (JG) cells. The combination of macula densa and JG cells is known as the juxtaglomerular apparatus (JGA). The macula densa is strategically located to sense concentrations of NaCl leaving the LH and regulate renin release (Vander *et al.*, 2001; Jackson, 2006).

Approximately 0.2 mm past the macula densa, the tubule changes morphology once again to become the distal convoluted tubule (DCT). Like the TAL, the DCT actively transports NaCl and is impermeable to water. Since these characteristics impart the ability to produce dilute urine, the TAL and the DCT are collectively called the diluting segment of the nephron (Jackson, 2006). Several distal tubules empty into each collecting tubule and the collecting tubules join to form collecting ducts. The collecting tubule has two different cell types: the principal cells, which reabsorb Na^+ and secrete K^+ *via* sodium and potassium channels and the intercalated cells, which are involved mainly in H^+ secretion. In this portion of the nephron, the movement of ions and water is regulated by the mineralocorticoid aldosterone and antidiuretic hormone, respectively (Rang *et al*., 2006).

The kidneys play the central role in regulating the water concentration, inorganic-ion composition, and volume of the internal environment. It is also involved in the excretion of metabolic waste products such as urea, uric acid, creatinine as well as some foreign chemicals, such as drugs, pesticides, and food additives. During prolonged fasting, the kidneys synthesize glucose from amino acids and other precursors and release it into the blood. Further, the kidneys act as endocrine glands, secreting at least three hormones: erythropoietin, renin, and 1,25-dihydroxyvitamin D3 (Vander *et al*., 2001).

1.2. Renal pharmacology of aminoglycosides

The antibiotic aminoglycoside group includes amikacin, arbekacin, dibekacin, framycetin, gentamicin, isepamicin, kanamycin, neomycin, netilmicin, paromomycin, sissomicin, streptomycin, tobramycin and others. They belong to the group of antibiotics that act on ribosome and classified as protein synthesis inhibitors (Noone, 1978; Chambers, 2007). They also alter integrity of the bacterial cell membrane (Kadurugamuwa *et al*., 1993). In contrast to most inhibitors of microbial protein synthesis, which are bacteriostatic, aminoglycosides are bactericidal (Wagan *et al*., 2004).

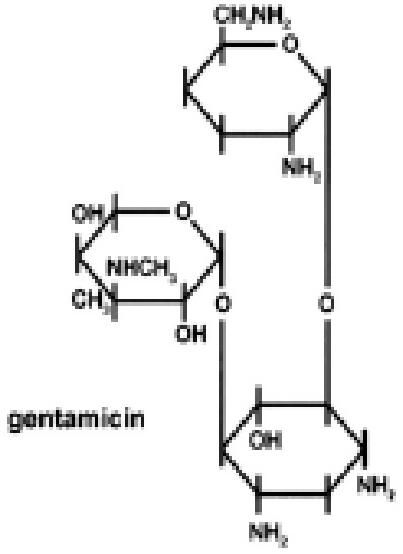

Fig 1: Chemical structure of gentamicin.

Clinically significant aminoglycosides have a heterocyclic structure formed by two or more aminosugars linked by glycoside bonds to an aminocyclitol ring (Fig 1), which is highly-conserved and usually located in a central position (Chamber, 2006, Martínez-Salgado *et al*., 2007). This hexose nucleus comprised primarily of 2-deoxystreptamine and has 1,3-diamino functionality and three or four hydroxyl groups that provide anchoring points for aminosugars (Chittapragada *et al*., 2009).

This chemical structure is responsible for polycationic nature as well as polarity of aminoglycosides that inturn is accountable in part for pharmacokinetic properties shared by all members of the group (Martínez-Salgado *et al*., 2007). Aminoglycosides are not appreciably absorbed from oral administration and must be administered by other routes to treat systemic infections (Noone, 1978). They do not penetrate into most cells. However, they are well distributed throughout the extracellular water compartment (Noone, 1978; Chambers, 2007). Except for streptomycin, there is negligible binding of aminoglycosides to plasma albumin (Chambers, 2006).

Aminoglycosides are not metabolized and are essentially eliminated by glomerular filtration. The half-lives of the aminoglycosides in plasma are similar and vary between 2 and 3 h in patients with normal renal function. When renal function is impaired, serum and tissue concentrations may readily increase to toxic levels necessitating dosage adjustment in such patients (Nagai and Takano, 2004; Chambers, 2007).

Since their introduction into therapeutic practice in 1944, aminoglycosides are used most widely against gram-negative enteric bacteria, especially in bacteremia and sepsis, in combination with vancomycin or penicillin for endocarditis, in eradication of facultative gut flora (neomycin and kanamycin), cystic fibrosis, meningitis, ophthalmological infection, gonococcal urethritis and for treatment of tuberculosis (streptomycin) (Belknap, 1999; Martínez-Salgado *et al*., 2007). Although aminoglycosides are widely used and important agents, serious toxicity limits their usefulness. All members of the group share the same spectrum of toxicity, most notably nephrotoxicity and ototoxicity that dominated attempts to rationalize dosing (Begg and Barclay, 1995; Wagan *et al*., 2004; Martínez-Salgado *et al*., 2007).

1.2.1. Renal concentration of aminoglycosides

The first step in the renal elimination of aminoglycosides involves glomerular filtration, which is quantitatively the most important elimination pathway as their plasma clearance is nearly equal to the glomerular filtration rate (GFR) (Kaloyanides and Pastoriza-Munoz, 1980). A specific high-capacity transport mechanism has been identified in proximal tubule epithelial cells, which accumulate aminoglycosides at higher levels than those detected in plasma (Humes, 1988; Martínez-Salgado *et al*., 2007).

Several studies evidenced that aminoglycosides are transported across the apical membrane of proximal tubular cells by pinocytosis after binding of the aminoglycosides to receptors located there (Just *et al*., 1977; Silverblatt and Kuehn, 1979). The initial points of attachment are the acidic phospholipids mainly phosphatidylserine, an abundant acidic phospholipid of brush borders, since modulation of the membrane content in these phospholipids results in commensurate changes in uptake (Molitoris and Simon, 1985).

Quickly thereafter as a second step, aminoglycosides are transferred to the transmembrane protein megalin, with which they become internalized in endosomes (Moestrup *et al*., 1995). Megalin, a giant endocytic receptor abundantly expressed at the apical membrane of renal proximal tubules, plays an important role in binding and endocytosis of aminoglycosides in proximal tubule cells. Megalin antagonists have been developed such as cytochrome c, which hold promise as prospective therapeutic agents for preventing or minimizing the iatrogenic tubular damage induced by gentamicin (Nagai and Takano, 2004).

Although the available evidence unequivocally supports the intralysosomal accumulation of aminoglycosides, it remains unclear whether pinocytosis is the sole or even the major route by which these drugs gain entry into proximal tubular cells (Kaloyanides and Pastoriza-Munoz, 1980). Thus, the renal accumulation of aminoglycosides reflects transport of the drugs to renal proximal tubular cells (Fig 2) *via* pinocytosis, apical membrane transport by some other mechanism, and basolateral membrane transport (Pastoriza-Munoz *et al*., 1979; Senekjian *et al*., 1981; Bennett, 1989).

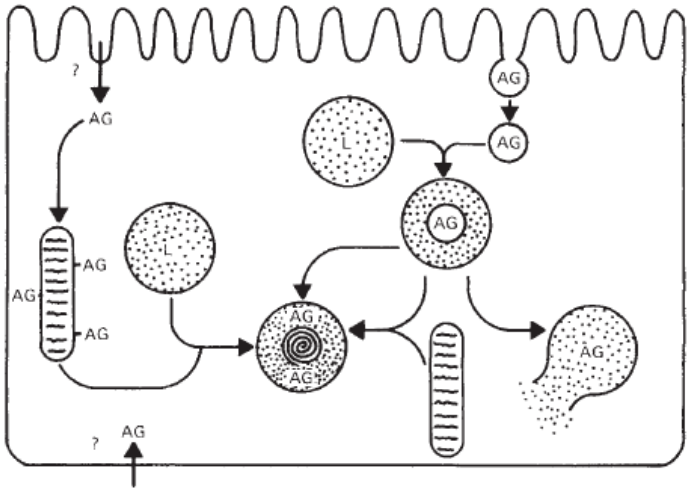

Fig 2: Pathways of aminoglycoside-induced cellular injury. On the right the aminoglycoside is shown entering the cell by pinocytosis and subsequently fusing with a primary lysosome. The aminoglycoside may interfere with normal lysosomal digestion giving rise to myeloid body formation, and the aminoglycosides may labilize lysosomes leading to the release of potent acid hydrolases into the cytosol. If aminoglycosides gained entry into the cell by other pathways as depicted on the left, then the aminoglycosides could cause direct injury to intracellular organelles. AG: aminoglycoside, L: lysosome (copied from Kaloyanides and Pastoriza-Munoz, 1980).

The basolateral membrane transport, quantitatively less important than apical membrane transport, is critical as it exposes critical sites in key organelles such as mitochrondria and microsomes (Williams *et al*., 1984; Weinberg *et al*., 1985). Thus, it is apparent that the pathway(s) by which aminoglycosides gain entry into proximal tubular cells holds important implications for the pathogenesis of aminoglycoside induced nephrotoxicity as well as possible strategies for preventing aminoglycoside nephrotoxicity (Kaloyanides and Pastoriza-Munoz, 1980).

Even though brush border receptor-mediated binding and cellular uptake of aminoglycosides is well established experimentally (Williams *et al*., 1987), cell necrosis can be dissociated from intracellular aminoglycoside concentrations as pharmacologic agents which reduce nephrotoxicity and cell necrosis don't necessarily reduce cellular concentrations of aminoglycosides. Thus, while binding and intracellular uptake of aminoglycosides is probably necessary, it alone is not sufficient to produce cell necrosis (Schentag *et al*., 1978; Quarum *et al*., 1984).

1.3. Aminoglycosides-induced nephrotoxicity

Kidney is a common target for toxic xenobiotics, due to its capacity to extract and concentrate toxic substances to its large blood flow share (about 20% of cardiac output) and an unequal intrarenal distribution of drug metabolizing enzymes (Werner and Costa, 1995; Kacew and Bergeron, 1990; Leehey *et al*., 1993; Sandhu *et al*., 2007). Nephrotoxic substances damage different nephron cell types leading to tubular epithelial cell necrosis as well as glomerular injuries and functional alterations (Kohn *et al*., 2002). Accordingly, nephrotoxics may cause direct tubular injury, interstitial nephritis, decreased renal perfusion, primary glomerulopathy and obstructive nephropathy (Werner and Costa, 1995; Martínez-Salgado *et al*., 2007).

Acute renal failure (ARF), sudden and usually reversible loss of renal function, can result from damage to any part of the nephron, and is the most obvious and immediately dangerous form of drug nephrotoxicity (Werner and Costa, 1995). Acute tubular necrosis, the most common causes of ARF accounting for 85% of the incidence, occurs due to ischemia or nephrotoxins like cisplatin and gentamicin (Shirwaikar *et al*., 2004). Despite its advancement, modern medicine offers no remedial measures for nephrotoxic induced renal impairment turning mankind toward alternative systems of medicine for solutions (Annie *et al*., 2005).

Aminoglycoside produce nephrotoxicity in 10–25% of therapeutic courses, despite rigorous monitoring of serum drug concentration and adequate fluid volume control. The most common clinical presentation is non-oliguric ARF, which is manifested functionally by decreased urine concentrating capacity, tubular proteinuria, lysosomal enzymuria, mild glucosuria, decreased ammonium excretion, and depression of GFR. Associated alterations in electrolytes levels include hypomagnesaemia, hypocalcaemia and hypokalaemia (Kaloyanides and Pastoriza-Munoz, 1980; Mingeot-Leclercq and Tulkens, 1999; Rougier *et al*., 2003).

Nephrotoxicity associated with aminoglycoside is dependent on dose and the number of free amino groups on their surface. Streptomycin with only two free amino groups has limited, if any, nephrotoxicity. Gentamicin, tobramycin, amikacin, kanamycin and netilmicin, which have each five free amino groups, are potentially nephrotoxic, but can all be used systematically. Neomycin with six free amino groups is too nephrotoxic for parenteral use and has been administered only for irrigation, topically and as a bowel-sterilizing drug (Werner and Costa, 1995). Thus, nephrotoxicity of various aminoglycosides in decreasing order is neomycin > gentamicin ≥ tobramycin ≥ amikacin ≥ netilmicin > streptomycin (Sandhu *et al*., 2007).

1.3.1. Mechanisms of renal cell death

While extensive necrosis of tubular cells is ultimately observed with high doses of aminoglycosides, specific effects on subcellular organelles can be observed well before any other changes in cell function (Bennett, 1989). At cellular level, several hypotheses have been suggested to elucidate the possible mechanism(s) of nephrotoxicity (Fig 3).

i) Generation of hydroxyradicals

Hydroxyradicals are strong mediators of tissue injury as they are involved in oxidation of a wide variety of biomolecules leading to cell membrane injury and protein degeneration (Ali, 1995). Gentamicin has been shown to enhance the generation of superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) by renal cortical mitochondria. The interaction between $O_2^{\cdot -}$ and H_2O_2 in the presence of metal catalyst can lead to the generation of hydroxyl radical ($OH^{\cdot -}$) (Walker *et al*., 1999).

In vivo studies have shown that gentamicin leads to release of iron from renal cortical mitochondria and enhance generation of $OH^{\bullet}$ (Walker and Shah, 1987). Similarly, others also reported that iron supplementation can aggravate gentamicin nephrotoxicity as it plays a critical role in initiating free radical oxidation process leading to lipid peroxidation (Kays *et al*., 1992; Ben-Ismail *et al*., 1994). On the other hand, hydroxyl scavengers and iron chelators were shown to prevent gentamicin induced ARF (Yang *et*

al., 1991; Ali, 2003; Derakhshanfar *et al*., 2007). Thus taken together, it appears that reactive oxygen metabolites are one of the important mediators responsible for gentamicin nephrotoxicity (Walker *et al*., 1999).

ii) Lysosomal dysfunction

Due to the accumulation of aminoglycoside in the proximal tubule lysosomes, impaired function of these organelles is suggested to be an important mechanism of nephrotoxicity (Kaloyanides and Pastoriza-Munoz, 1980). Aminoglycosides lead to an extensive dysfunction of these organelles through inhibition of the activities of the enzymes and the alteration of the properties of the lysosomal membrane permeability (Morin *et al*., 1980).

Animal and clinical observations support the hypothesis of the mechanism of lysosomal damage is inhibition of phosphatidylinositol phospholipase C, causing a phospholipidosis within the proximal tubular lumen and eventually an enrichment of lipid material in the lysosomes themselves (Hostetler and Hall, 1982; Laurent *et al*., 1982; Powell and Reidenberg, 1982). At this latter stage, electron microscopy shows lysosomal swelling together with an accumulation of myeloid bodies (Houghton *et al*., 1976). The latter are not specific for aminoglycoside administration, and their presence does not indicate clinical toxicity (Jao *et al*., 1983; Werner and Costa, 1995). It results in cell injury and death either by depriving the cell of critically important substrate or promoting (mechanically as well as chemically) labilization of lysosomes with the release of potent acid hydrolases intracellularly that could attack other intracellular organelles leading to cell injury and necrosis (Werner and Costa, 1995).

It has been reported that gentamicin causes significant alterations in other lysosomal enzymes as gentamicin per se decreased cathepsin B and L activities in proximal tubule segments due to either enzyme inhibition or reduced generation. Since cathepsin B and L are proteolytic activators of other lysosomal enzymes, their reduced activity may also decrease the activities of other lysosomal enzymes (Olbricht *et al*., 1991).

iii) Mitochondrial injury

Simmons *et al* (1980) showed that mitochondrial injury is involved in the pathogenesis of gentamicin nephrotoxicity as the drug compromises oxidative phosphorylation impairing cellular energy production (Fig 3). Besides, gentamicin competes with magnesium for inner mitochondrial membrane sites, where magnesium limits membrane permeability to monovalent cations. This leads to increased mitochondrial membrane permeability resulting in mitochondrial swelling and alterations in mitochondrial respiratory function (Weinberg and Humes, 1980; Weinberg *et al*., 1980). In addition, gentamicin limits mitochondrial calcium uptake and cytochrome a synthesis (Sastrasinh *et al*., 1982).

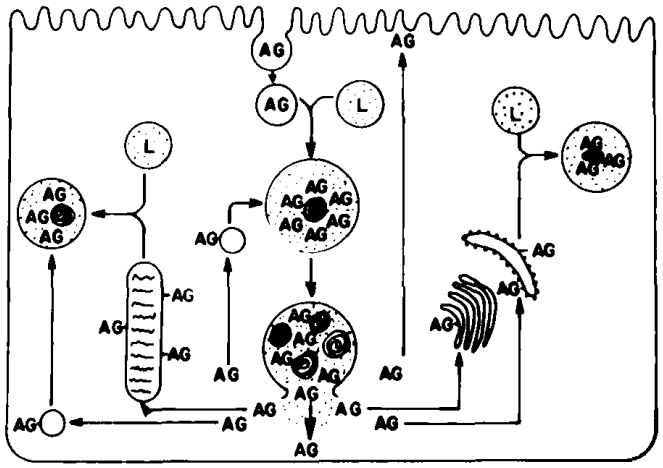

Fig 3: Model depicting the postulated pathogenesis of aminoglycoside nephrotoxicity. The polycationic aminoglycosides interact electrostatically with anionic sites on the apical membranes of renal proximal tubular cells, following which they are transported across the membrane by endocytosis and sequestered within the lysosomal compartment where they accumulate in high concentration. Within the acidic lysosomal compartment the drugs bind to anionic phospholipids and inhibit the activity of phospholipases and possibly other enzymes, which leads to the accumulation of phospholipid in the form of myeloid bodies. The lysosomes become progressively distended and eventually become leaky and rupture, resulting in the release of acid hydrolases and high concentrations of aminoglycoside antibiotics into the cytoplasm. This redistribution of drugs to other cellular membranes and organelles perturb a wide variety of metabolic processes including mitochondrial respiration and microsomal function that initiates the irreversible injury cascade (copied from Kaloyanides, 1992).

iv) Others factors

In addition to these major tubular alterations, gentamicin induced renal failure was suggested to be due to the involvement (directly or indirectly) of other alterations including increase in the renal cortical phospholipidosis (Laurent *et al*., 1982; Marche *et al*., 1987; Sundin *et al*., 2001), inhibition of Na^+/K^+ ATPase (Fukuda *et al*., 1990; Ali, 1995), inhibition of microsomal protein synthesis (Buss and Piatt, 1985; Bennett, 1989; Sundin *et al*., 2001), involvement of vascular factors (Fernandez-Repollat and Fantauzzi, 1994; Hishida *et al*., 1994), stimulation of calcium-sensing receptors (Ward *et al*., 2005; Martínez-Salgado *et al*., 2007), apoptosis (Servais *et al*., 2006; Martínez-Salgado *et al*., 2007), Jun-Kinase activation and Jun–AP-1 expression (Martínez-Salgado *et al*., 2005) and increased in nitric oxide synthesis (Martínez-Salgado *et al*., 2007).

1.4. Protection against aminoglycoside nephrotoxicity

Despite their nephrotoxicity and ototoxicity, aminoglycoside antibiotics are still considered to be important agents against life-threatening infections owing to their bactericidal efficacy, synergism with ß-lactam agents, low cost, limited bacterial resistance and a post-antibiotic effect (Sandhu *et al*., 2007). Therefore, reducing or protecting against their toxicity will increase the safety of the drugs as well as significantly improve the quality of life of patients taking aminoglycosides.

In humans, identification of patient and treatment related risk factors such as age, sex, nutrition, hepatic and renal disease, dose and duration of therapy, effective monitoring procedures, the use of once a day schedule especially in high risk individuals and avoiding combination of aminoglycosides with other potential nephrotoxins have all helped in reducing the possibility of the occurrence of gentamicin nephrotoxicity (Bennett *et al*., 1979; Zhanel *et al*., 1992; Swan, 1997; Wagan *et al*., 2004; Ali, 2003; Sandhu *et al*., 2007). However, once developed the initial therapy is basically supportive, consisting of stopping the causative drug and other nephrotoxic agents, maintaining fluid and electrolyte balance. Renal dysfunction is usually reversible after stopping

aminoglycoside therapy, though hemodialysis may be required in some cases (Guo and Nzerue, 2002).

Several approaches, utilizing different mechanisms, have been attempted to reduce the nephrotoxicity of gentamicin and related aminoglycoside antibiotics. These mechanisms include decreasing or preventing drug accumulation by the kidneys (Fujita *et al*., 1983; Kikuchi *et al*., 1991), competition or decreasing aminoglycoside binding to brush border membrane (Chiu *et al*., 1979; Humes *et al*., 1984), protection against vascular and glomerular effects (Hishida *et al*., 1994; Rodriguez-Barbero *et al*., 1997), protection against Ca^{2+} influx (Stojiljković *et al*., 2008), pre-treatment with potassium chloride (Thompson *et al*., 1990), co-administration of different polyanions and polyamines such as inositol hexasulphate (Kojima *et al*., 1990) and polyaspartic acid (Gibert *et al*., 1989; Kishore *et al*., 1990) with aminoglycosides, and concurrent penicillin therapy (Hayashi *et al*., 1988).

In addition and more importantly, the use of antioxidant agents consistently ameliorated or protected against gentamicin nephrotoxicity in rats (Mingeot-Leclercq and Tulkens, 1999). Ascorbic acid (Ben-Ismail *et al*., 1994), carvedilol (Kumar *et al*., 2000), dimethyl sulphoxide (Ali and Mousa, 2001), L-arginine (Can *et al*., 2000), α-lipoic acid (Sandhya *et al*., 1995), melatonin (Sener *et al*., 2002), methimazole (Elfarra *et al*., 1994), probucol (Kumar *et al*., 2000), superoxide dismutase (SOD) (Ali and Bashir, 1996) and vitamin E (Derakhshanfar *et al*., 2007) are some of the antioxidants that offer protection against gentamicin nephrotoxicity in rats. Extracts of several plants endowed with free radical scavenging activity have also showed a consistent reduction of gentamicin nephrotoxicity (Shirwaikar *et al*., 2004; Ali *et al*., 2005; Annie *et al*., 2005; Harlalka *et al*., 2007; Khan *et al*., 2009).

1.5. The experimental plant (*Euclea divinorum* Hierns)

1.5.1. The genus *Euclea*

E. divinorum Hierns belongs to *Euclea* genus of the family Ebenaceae. The Ebenaceae family are pantropical in distribution and encompass the genera *Diospyros* and *Euclea* with about 500-600 species. Main centers of diversity are in South East-Asia, Madagascar, tropical Africa, and South America. The genus *Euclea* is well presented in Eastern and Southern Africa and Southern Arabia (Mebe *et al*., 1998; Wallnöfer, 2001). It comprises about 12 species confined to Africa, Arabia, Socorta and the Comoro islands (Friis and White, 2003). In Ethiopia, the two *Euclea* species namely; *Euclea divinorum* and *Euclea racemosa* (Friis and White, 2003) are distributed mainly in hot dry regions and usually at the bottom of mountains/hills (Abate, 1989).

1.5.2. *Euclea divinorum* Hierns

E. divinorum is an evergreen, dioecious shrub or small tree up to 9-15 m tall (Fig 4) and widespread from Ethiopia and Sudan to South Africa (Friis and White, 2003). It occurs in grassland with scattered trees and open bushland, often on termite mounds, but also in secondary forest, margins of evergreen forest and on stony slopes, from sea-level up to 2700 m altitude (Njuguna, 2005).

Fig 4. Photographs of *E. divinorum* Hierns.

The bark of *E. divinorum* is collected from the wild and used to produce fast reddish-brown dyes, and for tanning hides and skins. In East Africa an infusion of the bark is used as a condiment in the preparation of a fatty meat and milk soup. The thin fleshy part of the fruits is edible, but not very palatable as it consists of a variety of emetic compounds. The roots and twigs are popular as toothbrushes and roots are chewed as a disinfectant and to colour the lips and mouth red. The wood is used for tool handles and carving, and as firewood (Mebe *et al*., 1998; Wallnöfer, 2001; Njuguna, 2005; Bussmann *et al*., 2006*)*.

In traditional medicine, root extracts and dried powdered roots are applied for the treatment of diarrhoea, convulsions, cancer, skin diseases, ulcers, wounds, headache, arthritis, miscarriage, jaundice, bleeding, snakebites and gonorrhea (Mebe *et al*., 1998; Njuguna, 2005; Cheikhyoussef, 2011). The root is also used for oral care, tooth ache, fungal diseases, sores and abscesses (Mothana *et al*., 2009). In Kenya, the hot root decoction is drunk for malaria, fevers and anaplasmosis and venereal diseases (Nanyingi *et al*., 2008). In South Africa, the leaves are used for management of noisy stomach, headache, general cleansing, and tooth ache (Samie *et al*., 2010).

In Ethiopia, the dried powdered leaves of these perennial shrubs commonly known by their vernacular names Dedeho (Amharic) and Mi'essa (Oromiffa) are used to treat scabies, inflammation of the skin, eczema, abdominal pain, gonorrhoea and constipation (Abate, 1989). In some regions of Ethiopia, the dried roots of these plants are also crushed and swallowed for some kidney problems (Wondimu *et al*., 2007*)*.

The application of *Euclea* spp in Ethiopia is not limited to their medicinal uses only; the fresh leaves of *Euclea* spp are placed under ‘enjera’, a traditional Ethiopian bread made out of teff flour, to prevent the development of molds. It is not uncommon to find the plants in some areas being used for ornamental and shade purpose. They have been also used in order to prevent milk from curdling by treating the pots in which milk is kept with the smoke of *Euclea* spp (Neuwinger, 1996). In South-Western region, the branches of the *E. divinorum* are put into the water gourds and left there for several hours to attract dirt and purify the water (Abbink, 2002).

Several phytochemical studies on *E. divinorum* and other *Euclea* species evidenced the presence of some naphthoquinones and napthoquinone dimers and trimers (7-methyljuglone), terpenoids (lupanes, lupenes, lupeols, ursanes, oleananes, taraxeranes and 3-Fl-(5-hydroxyferuloyl) lup-20(30)-ene), betulin, isodiospyrin, shinalone, benzopyrones, polyphenols, flavonoids ((+)-catechin, glycosides of aromadendrin, quercetin and myricetin), tannins, steroids, naphtalene-based aromatics, hydrocarbons, lipids, amino acids, carotenoids and sugars (Ferreira *et al*., 1974, 1977; Dagne *et al*., 1993; Mebe *et al*., 1998; Wallnöfer, 2001; Njuguna, 2005).

These phytoconstituents are in part responsible for the biological activities of *E. divinorum*. Naphthoquinones, which occur in several organs are active against fungi, bacteria, mollusks, insects, worms, termites (Neuwinger 1998; Wallnöfer, 2001). Some evidence exists that twigs of *E. divinorum* used as a toothbrush not only clean the teeth but also inhibit growth of teeth-attacking bacteria because of their high polyphenolic content. One of the triterpenoid compounds and 7-methyljuglone showed cytotoxicity, accounting for the anticancer activities (Njuguna, 2005*).*

More *et al* (2008) reported that the ethanolic extract of *E. divinorum* has antimicrobial effect against several bacterial strains. In another study, the methanolic extract of the plant showed positive inhibitory activity against Gram-positive, Gram-negative and multiresistant bacterial strains. *E. divinorum* also showed antifugal activity against *Candida maltosa* (Mothana *et al*., 2009).

The fact that root of *Euclea* spp are crushed and swallowed for kidney problem (Wondimu *et al*., 2007) and methanol extracts of the same plant possess a free radical scavenging abilities (Mothana *et al*., 2009) prompted us to evaluate the renoprotective effect of these plants against gentamicin-induced nephrotoxicity in rats. Furthermore, to the best of our knowledge, there is no any scientific justification whether the renal use of the plants is applicable to drug induced ARF. Thus, this study is done in order to fill this knowledge gap.

2. Objective

2.1. General objective

- To investigate the protective effects of *E. divinorum* against gentamicin-induced nephrotoxicity in rats.

2.2. Specific objectives

- To conduct acute toxicity study of the crude extracts of *E. divinorum* leaves
- To asses the biochemical changes associated with gentamicin-induced nephrotoxicity by measuring serum creatinine and blood urea nitrogen (BUN) levels.
- To asses the oxidative stress associated with gentamicin-induced nephrotoxicity by measuring oxidative markers, including catalase (CAT) and SOD activity as well as gluthatione (GSH) and malondialdehyde (MDA) levels.
- To investigate the renoprotective effects of the crude extract as well as aqueous and methanolic fractions of *E. divinorum* leaves by monitoring renal and oxidative markers.
- To examine renal histopathological changes associated with gentamicin-induced nephrotoxicity and its reversal by the crude extract as well as aqueous and methanolic fractions of *E. divinorum* leaves.
- To propose a possible mechanism for the renoprotective effect of the plant

3. Materials and Method

3.1. Chemicals

Analytical grade chemicals and solvents were used for this study. Absolute ethanol (Changshu Yangyuan Chemical, China), acetone (Reagent Chemical Services Limited, USA), chloroform (Research-lab Fine Chem Industries, Mumbai), DPPH (Sigma Aldrich, Germany and USA), diethyl ether (BDH laboratory supplies, England), formalin (El Nasr Pharmaceutical Chemicals Co., Egypt), gentamicin (SPCL, China), glacial acetic acid (Fisher Scientific limited, UK), methanol (Reagent Chemical Services Limited, USA), sodium chloride (BDH laboratory supplies, England) and Tween 80 (Research-lab Fine Chem Industries, Mumbai) were purchased from local markets. The assay kits for serum creatinine and BUN were acquired from Roche-Cobas, Switzerland. CAT, GSH, MDA and SOD assay kits were imported from Nanjing JianChen Bioengineering Institute, China.

3.2. Experimental animals

For this study, 42 (21 male and 21 female) Sprague Dawley rats (200-300 g) and 10 female Albino mice (25-30 g) bred in the Animal House of School of Pharmacy, Addis Ababa University were used. The animals were maintained under natural lighting conditions (12 h light and 12 h dark cycle) with temperature of 22-25°C and relative humidity of approximately 50%. Six rats (three males and three females) were housed in a single polypropylene transparent cage (Erdem *et al*., 2000). The rats were fed on standard pellet diet and water *ad libitum*. All animals were handled according to internationally accepted guidelines (ILAR, 1996) and the protocol was approved by the School of Pharmacy Ethics committee.

3.3. Plant collection

The leaves of *E. divinorum* were collected from one of its natural habitats in the surrounding area of Dire; a place located about 15 km south east of Debre Zeit town (50 km east of Addis Ababa), Ethiopia. The plant material was identified by a taxonomist and a voucher specimen (FT 001) was deposited at the National Herbarium, College of Natural Sciences, Addis Ababa University.

3.4. Plant extraction

3.4.1. Crude extract

The extraction was made following the methods stated by Wube *et al.* (2005), with slight modification. The air-dried leaves (350 g) of *E. divinorum* was grounded and extracted with 80% methanol by maceration for 72 h. The extractant were filtered with Whatman No.4 (Whatman international Ltd. England) filter paper, and then dried using an oven. The dried powder of the extract was weighed to calculate percentage yield and kept in air-tight containers wrapped with the aluminum foil and stored in refrigerator until used.

3.4.2. Methanol and aqueous fractions

The solvent fractions were prepared following the method described by Asres *et al.* (2006), with some modifications. Powdered air-dried leaves (180 g) of *E. divinorum* was extracted sequentially with chloroform, acetone and methanol in a Soxhlet apparatus. The left over marc was then macerated with distilled water for 72 h and filtered with Whatman No.4 filter paper to get the aqueous fraction. The extractant of chloroform, acetone and methanol fractions as well as the filtrate of the aqueous fraction were then dried using an oven maintained at 40°C. The dried powders of the extracts were weighed and percentage yields were calculated based on the weight of dried plant material. The dried powders were kept in air-tight containers wrapped with the aluminum foil and stored in refrigerator until used.

3.5. Acute toxicity testing

Acute toxicity study was conducted according to the OECD 425 guideline in order to evaluate the potential toxicity of the extracts as well as to select safe doses for evaluation of renoprotective activity. Accordingly, five female mice were used and fasted for 4 h before and 2 h after extract administration. The mice were weighed and received 2000 mg/kg of the extracts of *E. divinorum* orally by gavage. The animals were then observed individually for toxic symptoms at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter, for a total of 14 days. Finally, the number of survivors was noted at the end of the 14 days (OECD, 2001).

3.6. Nephroprotective activity testing

3.6.1. Grouping and dosing of animals

The rats were divided into seven experimental groups of 6 each. The first group served as a control (CON, group I) and the rats were given the vehicle, Tween 80 (2%, v/v) in water orally. The second group was treated with normal saline intraperitonally for two days and afterwards gentamicin (GEN, group II) for eight days, at a dose of 100 mg/kg/day, intraperitonally (Parlakpinar *et al*., 2005). The rest of the groups received crude extract or methanol or aqueous fraction of *E. divinorum* at different doses orally for two days before and eight days concomitantly with gentamicin (100 mg/kg/day, intraperitonally) (Kuhad *et al*., 2006). For the evaluation of renoprotective effect, a high safe dose of crude extract was chosen, which was one-tenth of the acute toxicity test dose (Annie *et al*., 2005; Adeneye *et al*., 2006; Deshmukh *et al*., 2009).

The crude extract, prepared in an aqueous solution containing Tween 80 (2% v/v), was administered orally in a once-daily regimen at three different doses 200 mg/kg (C200, group III), 150 mg/kg (C150, group IV) and 100 mg/kg (C100, group V) for 10 days. The methanol (M100, group VI) and aqueous (W100, group VII) fractions, prepared similarly, were given orally in a once daily regimen at a dose of 100 mg/kg for 10 days. The doses for the plant extracts were chosen after acute toxicity study and pilot study

were performed. The rats were weighed on alternate days and the last known weight was used for dose calculations. The final day body weight was used for the calculation of body weight change and expression of kidney weight (Annie *et al*., 2005; Harlalksa *et al*., 2007).

3.6.2. Sample collection

Twenty-four hour after the last treatment (Parlakpinar *et al*., 2005), the animals were slightly anesthetized with diethyl ether for blood collection by punching the vein plexus of the retro-orbital sinus into polyethylene tubes (Derakhshanfar *et al*., 2007). The samples were left at room temp for 30 min for coagulation and then centrifuged at 3000 rpm for 15 min at 4^{o}C (Centurion scientific ltd K240R, UK) to separate serum. The serum was stored at -20°C for 48 hours (Aftron AFF 545, Denmark) until subjected for analysis of creatinine and BUN levels.

Animals were then killed by cervical dislocation under ether anesthesia. The abdominal cavity was immediately opened and right and left kidneys were removed and processed for antioxidant as well as histological examinations (Al-Majed *et al*., 2002; Parlakpinar et al., 2006). One of the isolated kidneys was rinsed in chilled saline, decapsulated, blotted on a filter paper and quickly weighed. Then, it was homogenised in ice-cold saline in volume of nine times of its weight to yield 10% (w/v) tissue homogenate and stored at -20°C for 14 days until it was analyzed for CAT and SOD activity as well as GSH and MDA levels. The other kidney was fixed in 10% formalin for histopathological examination (Al-Majed *et al*., 2002).

3.6.3. Biochemical analysis

Serum creatinine and blood urea nitrogen

The concentration of serum creatinine and BUN were measured by Cobas integra 400 (Roche, Switzerland) using commercial kits according to the manufacturer's protocol. Creatinine level was determined by Jaffe' reaction without deproteinization where the samples were subjected to react with picrate in alkaline pH forming a yellow-red

complex with maximum absorbance at 512 nm. For measurement of BUN level kinetic test with urease and glutamate dehydrogens was used. Urea in the sample was hydrolyzed by urease forming ammonia that in turn reacts with 2-oxoglutarate in the presence of glutamate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) to produce L-glutamate. The rate of decrease in the NADH concentration is directly proportional to the urea in sample that can be determined by measuring the absorbance at 340 nm. The BUN was calculated from the urea using a formula: BUN (mg/dl) = urea (mg/dl) * 0. 467.

Determination of catalase activity

CAT activity was measured based on the provided manufacturer procedure that rely on the reaction of the enzyme in the presence of an optimal concentration of H_2O_2. The rate of dismutation of H_2O_2 to H_2O and O_2 is proportional to the concentration of CAT. In short, 50 ul of 10% renal homogenates of rats were mixed well with a known concentration of H_2O_2 on a vortex mixer (Labnet International Inc. Labnet S0100-230V, USA) and incubated in 37°C water bath (Oakton Stable Temp® WD-12501-15, USA) for exactly 1 min. Then, ammonium molybdate was added to the mixture to quench the reaction and react with the remained H_2O_2 forming a stable colored complex. The absorbance of the complex was measured at 405 nm (Unic Model 2100 spectrophotometer). Finally, the CAT catalytic activity of the tissue samples was calculated and expressed as U/mg protein. One unit of CAT catalytic activity is defined as the amount of enzyme that will decompose of 1 µmol H_2O_2 per second at 37°C in 1 mg protein of tissue homogenate.

Determination of total super oxide dismutase activity

The total SOD activity was determined using a reaction system consisting of xanthine and xanthine oxidase that produces $O_2^{\cdot-}$. The $O_2^{\cdot-}$ oxidizes hydroxylamine forming nitrite, which colors amaranth by the color developer and can be assayed at 550 nm. During assay, 50 µl of the 10% tissue homogenates were mixed well with the reaction system on a vortex mixer and incubated in 37°C water bath for 40 min. The formation of $O_2^{\cdot-}$ and

nitrite was inhibited by SOD in the samples reducing the intensity of the amaranth color as well as the absorbance upon the addition of the color developing agent. The total SOD activity in the samples were calculated and expressed as U/mg protein. One unit of SOD activity is defined as the number of SOD that will produce 50% inhibition of oxidation of hydroxylamine induced by xanthine and xanthine oxidase at 37°C in 1 mg/ml protein concentration of tissue homogenate.

Determination of gluthatione levels

The renal GSH level was analyzed using the procedure provided by the manufacturer. Initially, the supernatants of 10% tissue homogenates were deproteinized by mixing 0.5 ml of the samples with 2 ml of the deproteinizing reagent on vortex mixer. Then, the mixture was allowed to stand at room temprature for 5 min and centrifuged under reduced temprature (4°C) at 4000 rpm for 10 min. Finally, the supernatant was carefully collected without disturbing the precipitate and stored at -20°C for 14 days till used for GSH assay.

Briefly, 1 ml of deproteinized samples were allowed to react with 5,5-dithiobis (2-nitrobenzoic acid), a reagent developed to detect thiol compounds. After 5 min reaction at room temprature, 2-nitro-5-thiobenzioc acid and glutathione disulfide (GSSG) were generated. The absorbance of 2-nitro-5-thiobenzioc acid, a yellow colored product, at 420 nm was used to determine the concentration of GSH in the samples and the levels of GSH were expressed as µmol/g protein.

Determination of lipid peroxides levels

The amount of lipid peroxides was calculated as thiobarbituric acid reacting substance (TBARS) such as MDA, formed from the breakdown of polyunsaturated fatty acids, and considered as an index for the peroxidation reaction. The level of MDA in renal homogenates was assayed based on thio-barbituric acid (TBA) method where MDA undergoes condesation reaction with TBA generating red product that has a maximum absorption peak at 532 nm.

Tissue homogenates (10%) were well mixed with TBA reaction system in test tubes on vortex mixer and then test tubes were sealed with aluminum foil with a hole stung with a needle. The mixtures were incubated in 95°C water bath for 40 min, cooled with flowing water and centrifuged at 4000 rpm for 10 min. The supernatants were carefully pippeted into quartz cuvete (Exactaoptech, Germany) to read the absorbance of the red color at 532 nm.

3.6.4. Morphologic pathology

Body and kidney weight change

The body weights of all animals before and after the experiments were taken and their difference was expressed as body weight change. The weight of the kidney of each rat was measured at the end of treatment after scarifying the animal. For standardization, total kidney weight was normalized as kidney/body-weight ratio [kidney weight/ 100 g body weight] (Erdem *et al*., 2000).

Histopathology examination

The kidney tissues were cleared in xylene, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin for slide preparation. Then, the slides were coded and examined by a histopathologist, who was blinded to the treatment groups, via light microscopy for assessment of histopathological changes (Ulutas *et al*., 2006).

3.7. Antioxidant activity

The antioxidant activities of the extracts of *E. divinorum* were tested using 1,1-diphenylpicrylhydrazyl (DPPH) assay that rely on the disappearance of purple coloured methanol solution of DPPH, a stable free radical reagent (Blois, 1958). Briefly, 50 µl of various methanolic dilutions (2000, 1000, 500, 250 and 125 µg/ml) of the crude extract, methanolic and aqueous fractions were mixed with 5 ml of a 0.004% methanol solution of DPPH. Each test was done in triplicate. After a 30 min incubation period at 37^{o}C, the

absorbance of each dilution was measured against a blank at 517 nm. Inhibition of free radical, DPPH, in percent (% scavenging) was calculated as follows:

$$\% \text{ Scavenging} = A_{blank} - A_{sample} / A_{blank} \times 100;$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting percentage inhibition against concentration of the tested plant extract (Blois, 1958).

3.8. Statistical analysis

All data were presented as mean ± standard error of mean. The analysis was performed by one way ANOVA followed by Tukey's multiple comparison test. Level of significance was at $p<0.05$ and the analyses were performed by computerized GraphPad Prism version 4.0 (Graph pad software, USA) was used.

4. Results

4.1. Extract yield

Extraction of *E. divinorum* leaves with 80% methanol provided a percentage yield (w/w) of 18.86% while the yields of the methanol and aqueous fractions were 10.36% and 4.29%, respectively.

4.2. Acute toxicity

The acute toxicity test result of this study documented that the crude extract of *E. divinorum* leaves was safe by oral route at a dose of 2000 mg/kg. After 72 h animals were found to tolerate the administered dose and there were no significant changes in behaviour such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions, coma and appearance of the animals. There was no mortality within 14 days of observations and lethal dose 50 (LD_{50}) is assumed to be greater than 2000 mg/kg.

4.3. Biochemical changes

4.3.1. Effect on serum creatinine and blood urea nitrogen levels

GEN group exhibited a significantly greater creatinine levels (183.26%, $p<0.001$) compared to CON animals. Treatment with extract attenuated increased creatinine levels caused by gentamicin treatment. Gentamicin induced elevation of creatinine levels were significantly decreased by C200 ($p<0.05$) and C150 ($p<0.01$) but these doses were unable to bring values back to control levels. In addition, pre- and concomitant treatment with C100, M100 and W100 significantly ($p<0.001$) inhibited rise in serum creatinine levels compared with GEN group. Compared to CON, C200 and C150 showed a significantly ($p<0.01$) elevated serum creatinine, while C100, M100 and W100 groups showed insignicant elevation. The percentages increase in serum creatinine in C100, M100 and W100 groups compared to CON were 63.35%, 29.96% and 49.77%, respectively. There was no a significant difference in serum creatinine concentration among plant extract treated groups (Table 1).

Table 1: Effect of crude extracts and solvent fractions of *Euclea divinorum* leaves on gentamicin induced increase in serum creatinine levels.

Treatment	Serum creatinine (mg/dL)
CON	0.4420 ± 0.0074
GEN	1.252 ± 0.1618***
C200	0.8780 ± 0.0398**†
C150	0.8380 ± 0.0356**††
C100	0.7220 ± 0.0682† † †
M100	0.570 ± 0.0420†††
W100	0.662± 0.0523†††

Values are mean ± SEM; **P < 0.01, ***P < 0.001 (compared to control); †P < 0.05, ††P < 0.01, †††P < 0.001 (compared to gentamicin group). (CON: control; GEN: gentamicin 100 mg/kg; C200: crude extract 200 mg/kg; C150: crude extract 150 mg/kg; C100: crude extract 100 mg/kg; M100: methanol fraction 100 mg/kg; W100: aqueous fraction 100 mg/kg).

Fig 5 depicts a very significant (111.46%, p<0.001) increase in BUN levels in GEN groups compared to CON group. Pre- and co-treatment with extract inhibited gentamicin-induced rise of BUN. Compared to GEN, the reduction of BUN levels was significant (p<0.05) in C200 group, while it was very significant (p<0.001) in C150, C100, M100 and W100 groups. Significant elevation of BUN levels were observed in C200 (p<0.001) and C150 (p<0.05) groups when compared to controls. The difference in BUN levels was insignificant among CON and C100, M100 and W100 groups. The elevation (with respect to control groups) of BUN levels in C100, M100 and W100 groups were 31.13%, 7.93% and 23.35%, respectively. Crude extract and solvent fractions treated groups did not show a significant difference in terms of serum BUN concetration.

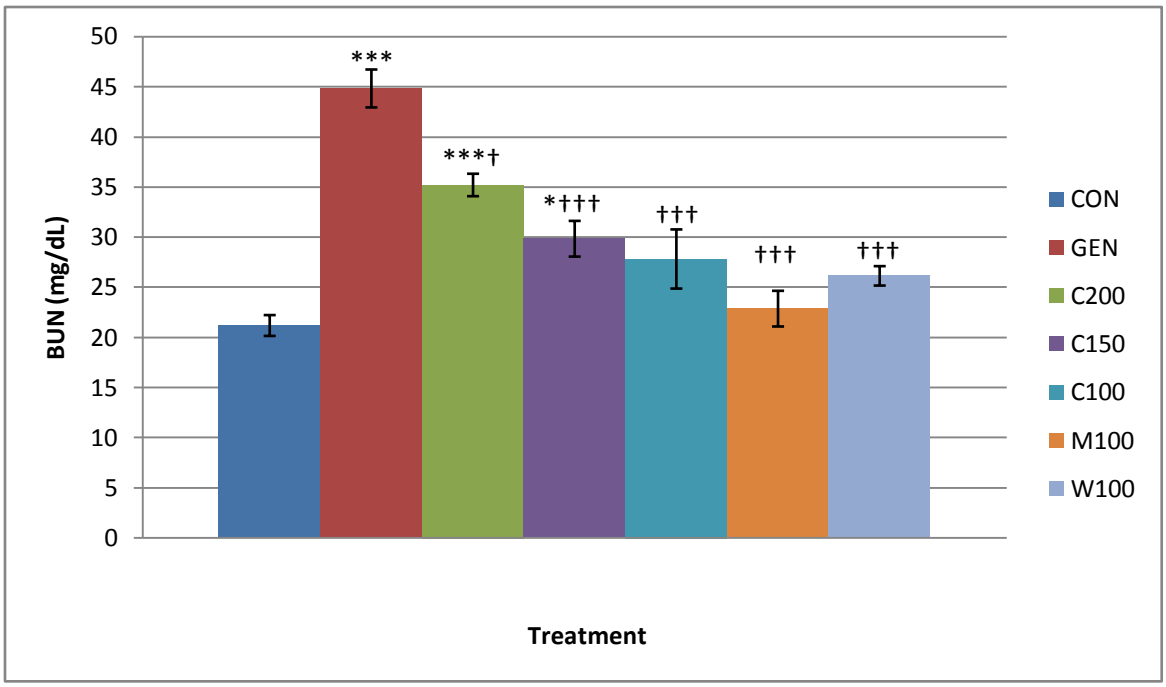

Fig 5: Effects of crude extract and solvent fractions of *Euclea divinorum* leaves on blood urea nitrogen levels of rats treated with gentamicin. Data are expressed as mean±SEM; *p < 0.05, ***p < 0.001 (compared to control group); †p < 0.05, †††p < 0.001 (compared to gentamicin group). (CON: control; GEN: gentamicin 100 mg/kg; C200: crude extract 200 mg/kg; C150: crude extract 150 mg/kg; C100: crude extract 100 mg/kg; M100: methanol fraction 100 mg/kg; W100: aqueous fraction 100 mg/kg).

4.3.2. Effects on renal catalase and superoxide dismutase activity and glutathione levels

Compared to control, gentamicin markedly (81.80%, $p<0.05$) decreased renal activity of CAT. Gentamicin associated reduction of CAT activity was attenuated by plant extract treatment. C200 and C150 significantly ($p<0.05$) ameliorated the reduced activity of CAT when compared to GEN group. The attenuation of gentamicin induced fall in CAT activity was very significant in C100 ($p<0.001$), W100 ($p<0.01$) and M100 ($p<0.001$) groups compared to GEN group. Decreased renal CAT activity was insignificant (compared to control) in C200, C150, C100, M100 and W100 groups with a reduction percentage of 39.64%, 32.74%, 16.73%, 8.06% and 22.28%, respectively. There was no significant difference in renal CAT activity amongst crude extract and solvent fractions treated groups (Table 2).

Table 2: Effect of crude extract and solvent fractions of *Euclea divinorum* leaves on catalase and superoxide dismutase activity and glutathione levels following injection of gentamicin (100 mg/kg, ip) for eight days.

Treatment	CAT (U/mg protein)	SOD (U/mg protein)	GSH(µmol/g protein)
CON	13.150 ± 1.679	263.7 ± 12.01	29.19 ± 2.018
GEN	2.394 ± 0.200*	157.4 ± 23.66**	18.79 ± 1.145**
C200	7.938 ± 0.623†	239.7 ± 15.24†	26.54 ± 1.281†
C150	8.845 ± 1.184†	242.9 ± 17.47†	26.56 ± 1.882†
C100	10.950 ± 1.769†††	249.4 ± 9.83†	26.91 ± 1.726†
M100	12.090 ± 1.074†††	260.0 ± 17.71††	27.32 ± 1.269†
W100	10.220 ± 1.095††	250.9 ± 23.07†	26.96 ± 1.961†

Values are mean ± SEM; *p<0.05, **p<0.01 (compared to control); †p<0.05, †††p<0.01, †††p<0.001(compared to gentamicin group). (CON: control; GEN: gentamicin 100 mg/kg; C200: crude extract 200 mg/kg; C150: crude extract 150 mg/kg; C100: crude extract 100 mg/kg; M100: methanol fraction 100 mg/kg; W100: aqueous fraction 100 mg/kg).

Gentamicin significantly (40.3%, p<0.01) decreased renal activity of SOD when compared to CON groups. Ten days treatment with plant crude extract prevented the fall in renal activity of SOD associated with gentamicin treatment. Compared to GEN group, C200, C150, C100 and W100 significantly (p<0.05) attenuated gentamicin-induced fall in renal activity of SOD. Inhibition of fall of renal activity of SOD was very significant (p<0.01) in M100 group compared to GEN group. Despite the insignificant variations in the renal SOD activity against the control group, there were percentage (9.10%, 7.89%, 5.42%, 1.40% and 4.85%) reductions in C200, C150, C100, M100 and W100 groups, respectively (Table 2).

As shown in Table 2, gentamicin considerably (38.06%, p<0.01) decreased renal GSH levels in GEN group compared to control. Treatment with C200, C150, C100, M100 and W100 significantly (p<0.05) prevented reduction of GSH levels resulted from gentamicin

administration. There was no significant difference in the kidney GSH content in plant extract treated compared to the controls. The corresponding percentage fall (with respect to control) in the renal levels of GSH in C200, C150, C100, M100 and W100 were 9.08%, 9.00%, 7.81%, 6.41% and 7.64%, respectively.

4.3.3. Effects on lipid peroxides levels

GEN group exhibited a significantly greater MDA levels (83.89%, $p<0.01$) compared to CON animals (Fig 6). Treatment with *E. divinorum* ameliorated increased MDA levels associated with gentamicin administration. C200, C150, C100, M100 and W100 produced a significant ($p<0.05$) reduction in MDA levels compared to GEN group. Compared to CON, the plant extract-treated groups showed insignificant variations in the extent of lipid peroxidation with slight increase in MDA levels by 21.87%, 17.91%, 14.90%, 9.98% and 14.57% in C200, C150, C100, M100 and W100, respectively. There was no significant difference in MDA levels among crude extract and solvent fractions treated groups.

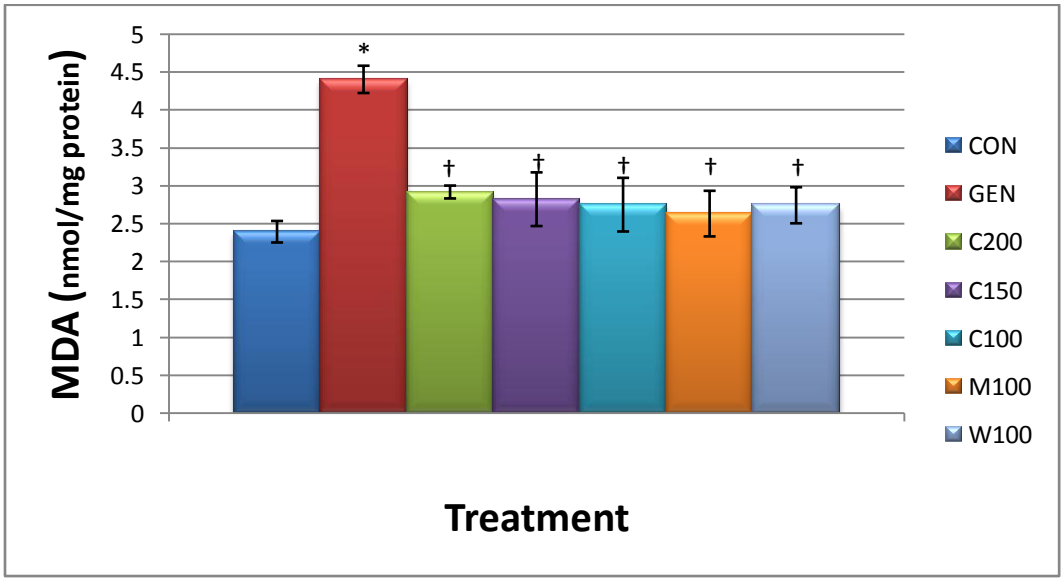

Fig 6: Effect of crude extract and solvent fractions of *Euclea divinorum* leaves on malondialdehyde levels of rats treated with gentamicin. Data are expressed as mean±SEM; *p < 0.05 (compared to control group); †p < 0.05 (compared to gentamicin group). (CON: control; GEN: gentamicin 100 mg/kg; C200: crude extract 200 mg/kg; C150: crude extract 150 mg/kg; C100: crude extract 100 mg/kg; M100: methanol fraction 100 mg/kg; W100: aqueous fraction 100 mg/kg).

4.4. Morphologic pathology

4.4.1. Effects on body and kidney weight

At the end of the experiment, gentamicin caused a severe loss in body weight ($p<0.001$) compared to control. Treatment with plant extract inhibited the weight loss caused by gentamicin administration. Compared to GEN, C200 and C150 significantly ($p<0.05$) attenuated the weight loss caused by gentamicin administration. The attenuation of weight loss was very significant ($p<0.01$) in C100, M100 and W100 groups. At the end of the experiment, slight weight loss in C200 and C150, and weight gain in CON, C100, M100 and W100 was observed (Fig 5).

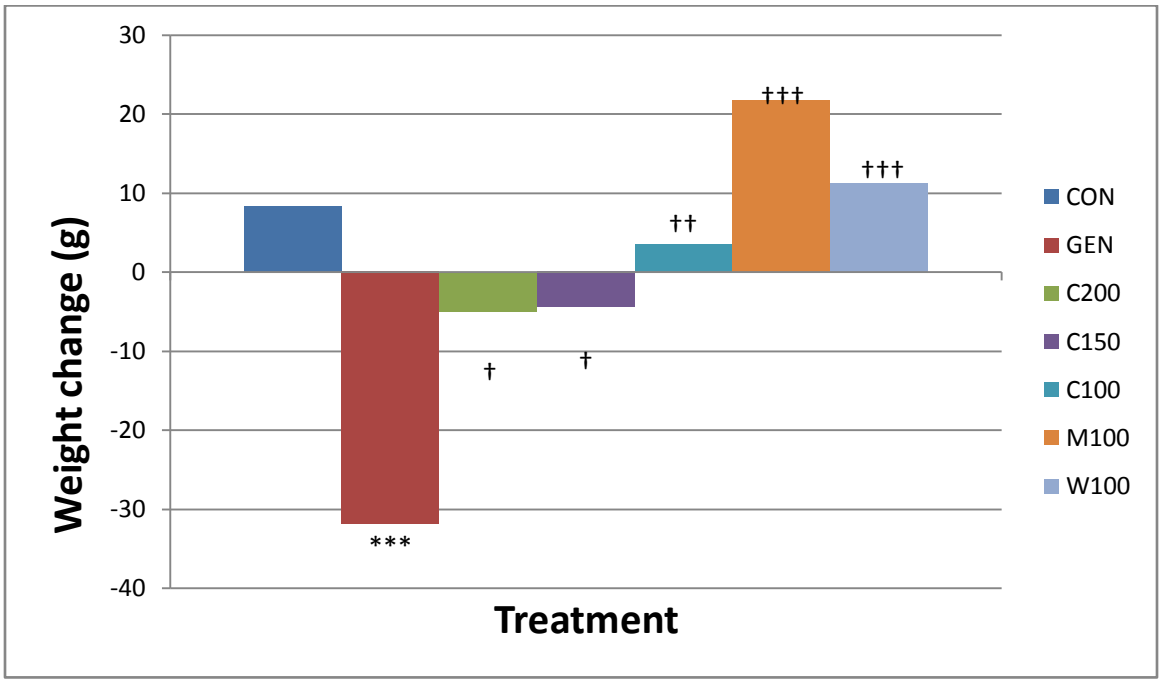

Fig 7: Effects of crude extracts and solvent fractions of *Euclea divinorum* on body weight of rats with gentamicin induced nephrotoxicity. Data are expressed as mean±SEM, ***$p < 0.001$ vs. control group, †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$ (compared to gentamicin group). (CON: control; GEN: gentamicin 100 mg/kg; C200: crude extract 200 mg/kg; C150: crude extract 150 mg/kg; C100: crude extract 100 mg/kg; M100: methanol fraction 100 mg/kg; W100: aqueous fraction 100 mg/kg).

As shown in Table 3, treatment with gentamicin resulted in a significant (42.34%, $p<0.001$) increase in normalized kidney weight compared to CON. Treatment with plant extract prevented an increase in kidney weight gain caused by gentamicin treatment. C200 reduced (12.53%) renal weight increase, although it was not able to bring values back to control levels. On the other hand, C150, C100, W100 significantly ($p<0.05$) ablated gentamicin associated renal weight gain. Inhibition of gentamicin induced kidney weight gain was very significant ($p<0.001$) in methanol fraction treated groups. Compared to CON, C200 significantly ($p<0.05$) increased the normalized kidney weight. Conversely, renal weight gain was insignificant (compared to control) in C150, C100, M100 and W100 groups with the corresponding percentage of 14.07%, 14.80%, 0.80% and 14.84%. There was no significant difference in normalized kidney weight among the plant treated groups.

Table 3: Effect of crude extract and solvent fractions of *Euclea divinorum* on gentamicin induced increase in kidney weight.

Treatment	Kidney wt (g)/100 g body wt
CON	0.274 ± 0.00534
GEN	0.390 ± 0.0117***
C200	0.341 ± 0.007160*
C150	0.313 ± 0.02077†
C100	0.315 ± 0.009276 †
M100	0.277 ± 0.01357†††
W100	0.315 ± 0.01496†

Values are mean ± SEM; *P < 0.05, ***P < 0.001 (compared to control); †P < 0.05, ††P < 0.01, †††P < 0.001 (compared to gentamicin group). (CON: control; GEN: gentamicin 100 mg/kg; C200: crude extract 200 mg/kg; C150: crude extract 150 mg/kg; C100: crude extract 100 mg/kg; M100: methanol fraction 100 mg/kg; W100: aqueous fraction 100 mg/kg).

4.4.2. Histopathology studies

The histological changes in kidneys were evaluated and the result were graded and scored as described under materials and methods section. And the results are depicted in Fig 8. The kidneys of the control group showed normal renal parenchyma with normal histoarchitecture (Fig 8A). On the other hand, GEN group showed more extensive and marked tubular necrosis, inflammation, hyaline casts in tubular lumen, hydropic degeneration and disintegrated nucleus (Fig 8B).

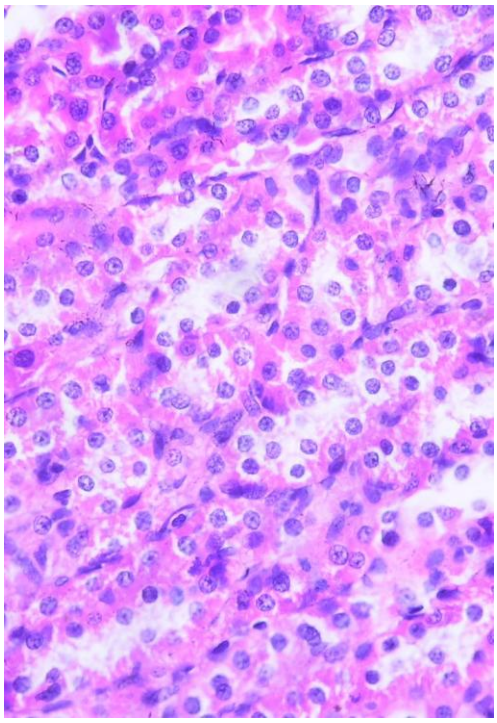

Fig 8A: Photomicrograph of renal tissue of control rats showing normal kidney cells with intact nucleus

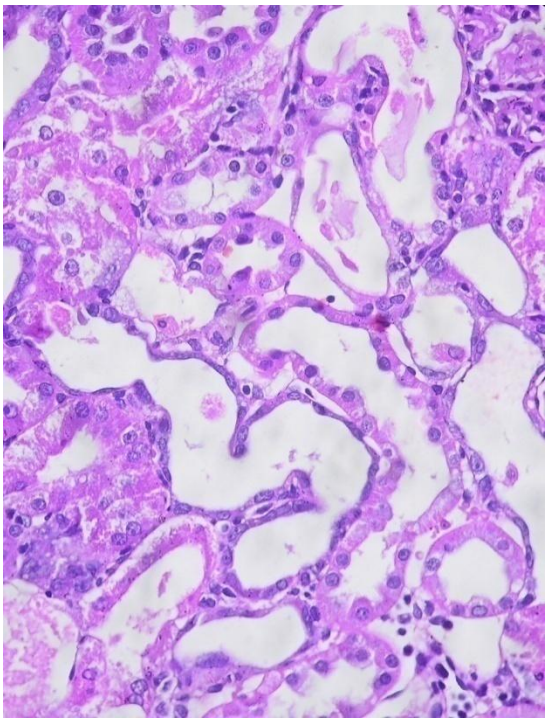

Fig 8B: Photomicrograph of renal tissue of rats treated with gentaimicin showing a considerable tubular necrosis with a disintegrated nucleus

As shown in Fig 8C-G, pre- and simultaneous treatment with *E. divinorum* induced regression these changes. Accordingily, there were no marked microscopical differences among the control and plant extract treated groups.

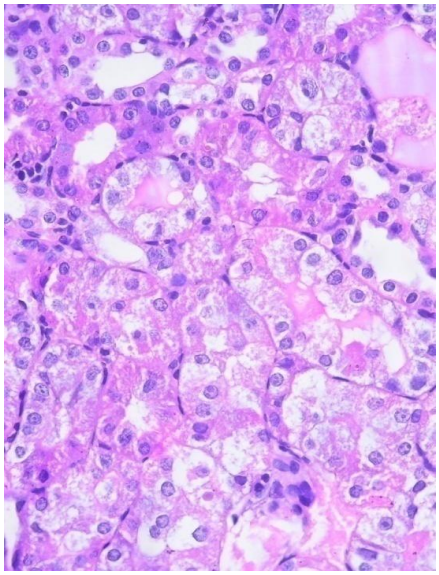

Fig 8C: Photomicrograph of renal tissue of rats treated with the crude extract of *Euclea divinorum* (200 mg/kg + gentamicin) showing kidney cells with mild to moderate necrosis.

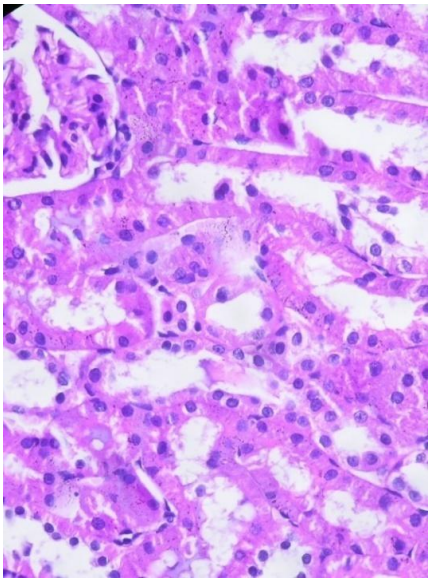

Fig 8D: Photomicrograph of renal tissue of rats treated with the crude extract of *Euclea divinorum* (150 mg/kg + gentamicin) showing kidney cells with mild necrosis.

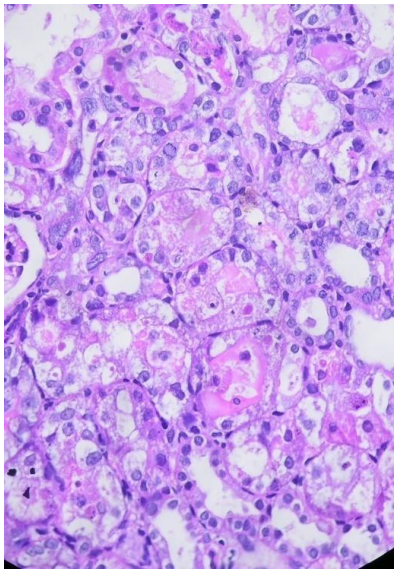

Fig 8E: Photomicrograph of renal tissue of rats treated with the crude extract of *Euclea divinorum* (100 mg/kg + gentamicin) showing normal kidney cells.

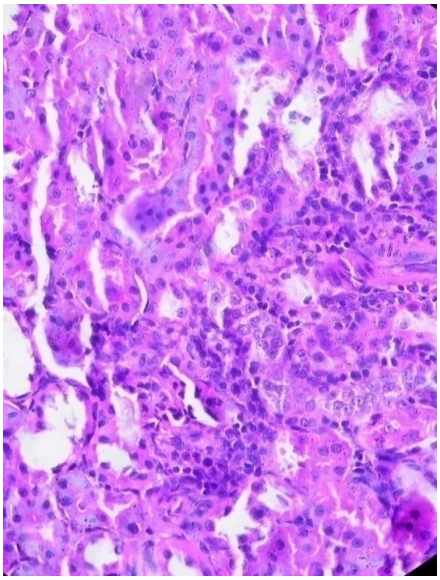

Fig 8F: Photomicrograph of renal tissue of rats treated with the methanol fraction of *Euclea divinorum* (100 mg/kg + gentamicin) showing normal kidney cells.

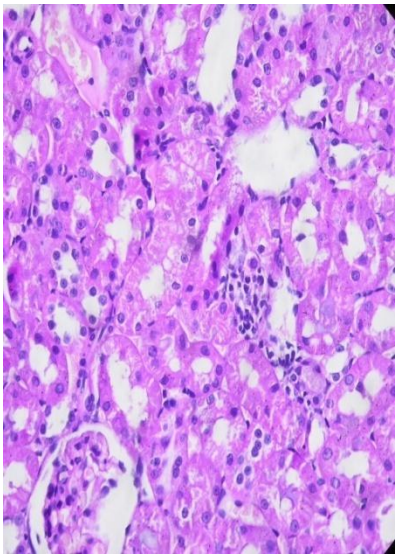

Fig 8G: Photomicrograph of renal tissue of rats treated with the aqueous fraction of *Euclea divinorum* at a dose of 100 mg/kg (+ gentamicin) showing normal kidney cells.

4.5. Antioxidant activity

Fig 9 shows the concentration dependent free radical scavenging activity of the crude extract, methanolic and aqueous fractions of *E. divinorum*. The maximum percentage inhibitions of DPPH at a concentration of 2000 μg/ml were 82.5, 74.5 and 62.5% for the methanol fraction, aqueous fraction and crude extract, respectively. Thus, it appeared that the methanolic fraction of *E. divinorum* had the highest antioxidant activity when compared to the aqueous fraction and crude extract.

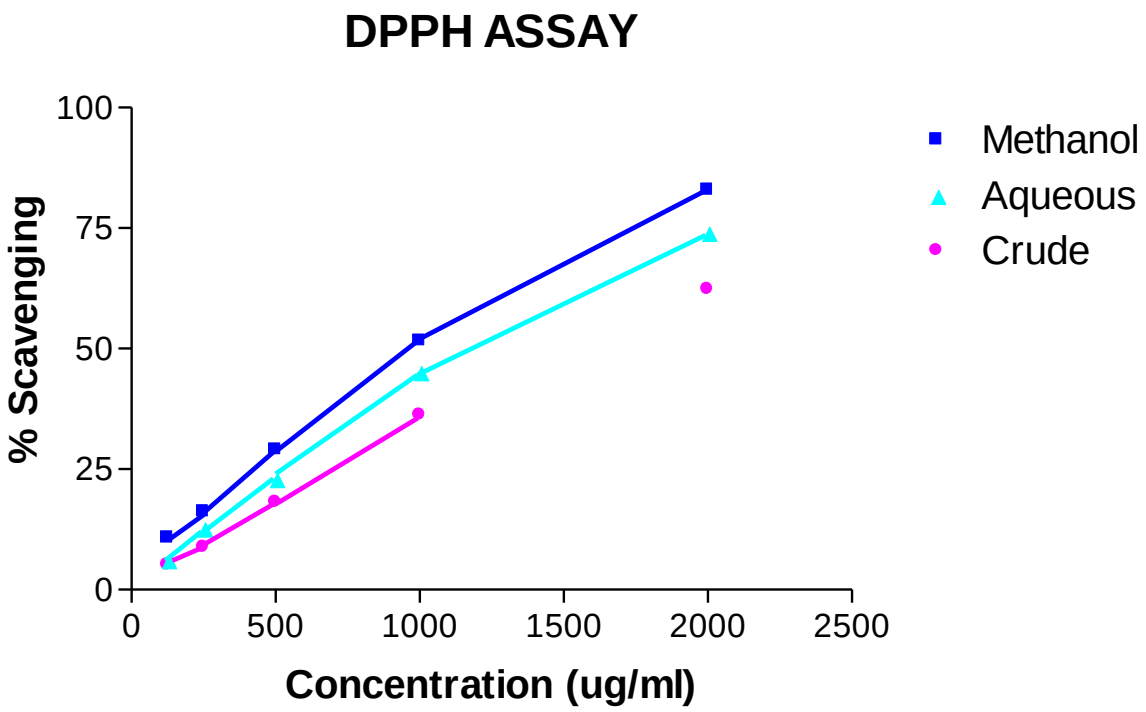

Fig 9: Antioxidant activity of the crude extract as well as the methanol and aqueous fractions of *Euclea divinorum.*

5. Discussion

In this study, the effect of the crude extract and fractions of *E. divinorum* in gentamicin-induced nephrotixicity was investigated. Antioxidant and nephroprotective properties were given an emphasis owning to their contribution in protection against this side effect of aminoglycosides improving their therapeutic indices. The solvent fractions were selected based on the pilot study result that showed significant nephroprotection of methanol and aqueous extracts and less renoprotective activity of acetone and chloroform fractions. Thus, methanol and aqueous fractions were selected for the main experiment. Further, the pilot study revealed insignificant renal alterations in rats treated with the plant extracts without concomitant administration of gentamicin.

Results of this study confirmed that gentamicin at a dose of 100 mg/kg/day for 8 days in rats produced significant nephrotoxicity as evidenced by increase in normalized kidney weight, BUN, serum creatinine, lipid peroxidation and decrease in body weight, SOD activity, CAT activity and GSH level.

5.1. Biochemical changes

In the present study, rats treated with gentamicin (100 mg/kg, i.p.) showed reduction in GFR which was substantiated by an increase in serum creatinine and BUN levels. In addition, gentamicin induced increase in serum creatinine and BUN levels were significantly halted by *E. divinorum* bringing about marked recovery in kidneys. Similar findings were also observed in other studies (Karahan *et al*., 2005, Parlakpinar, *et al*., 2005, Parlakpinar *et al.*, 2006).

Serum creatinine and BUN are commonly used to assess GFR, concentrating and diluting capacity of tubular function of kidneys. An increase in the values of these markers may indicate development and extent of renal tubular damage (Kakadiya and Shah, 2010). Creatinine, the major constituents of muscle, is derived from creatine and phosphocreatine as a waste product depending on individual's muscle mass (Gowda *et al*., 2010). It is a more reliable indicator of renal function than BUN as it is less directly affected by exogenous factors (Schwartz and Garrison, 2009). Serum creatinine

concentration increases in impaired renal function (mainly), muscular dystrophy paralysis, anemia, leukemia and hyperthyroidism (Kakadiya and Shah, 2010).

Urea, the major nitrogenous end product of protein and amino acid catabolism, is produced by liver and excreted by kidney (Kakadiya and Shah, 2010). It does not sufficiently quantify the extent of renal disease as its level depends on several non renal factors, including high protein intake, hydration status, increased protein catabolism and urea cycle enzymes (Schwartz and Garrison, 2009; Gowda *et al*., 2010; Kakadiya and Shah, 2010). Increased BUN may be associated with kidney disease or failure, blockage of the urinary tract by a kidney stone, congestive heart failure, dehydration, fever, shock and bleeding in the digestive tract (Gowda *et al*., 2010; Kakadiya and Shah, 2010).

Enhanced ROS generation shown to play a major role in gentamicin-induced impairement of glomerular functions (Pedraza-Chaverri *et al*., 2000; Karahan *et al*., 2005; Bello and Chika 2009). Excess ROS stimulate the mesangial cells contraction altering the filtration surface area and modifying the ultrafiltration coefficient, which inturn decreases the GFR without alternating the glomerular structure. It was also observed that increased $O_2^{\bullet-}$ could react with nitric oxide (NO), a vasodilator, to form peroxynitrite, a cytotoxic oxidant radical specie, which decreases the GFR (Guidet and Shah, 1989; Yang *et al*., 1995; Pedraza-Chaverri *et al*., 2003; Parlakpinar *et al*., 2005). Thus, it is evident that potent natural or synthetic agents capable of scavenging or interfering with ROS production successfully ameliorate gentamicin mediated impairement of glomerular function (Al-Majed *et al*., 2002; Cuzzocrea *et al*., 2002).

In the present study, nephroprotection by *E. divinorum* was relatively better at the lower doses (100 mg/kg and 150 mg/kg) compared to the higher dose (200 mg/kg). This might be ascribed to increase in (unprotective) phytoconstituents in a ratio that might compromise the level of protective constituents. There are several reports describing the cytotoxic properties of naphthoquinones isolated from *Euclea* species (*Euclea racemosa*) on a panel of cancer cell lines (Wube *et al*., 2005).

Mebe *et al.* (1998) reported cytotoxicity of 7-methyljuglone, a naphthoquinone derived from *E. divinorum*. Isodiosprin, on the other hand, has been reported to show cytotoxicity via inhibition of DNA topoisomerase I (Ting *et al*., 2003). Therefore, as the dose of crude plant extract increases toxic naphthoquinones might impose further insult to the kidney and abolish protective effect of *E. divinorum*. Thus, it appears that as the dose of the crude extract increases from 100 mg/kg to 200 mg/kg, the nephroprotective effect decreases. This finding was in contrary to Sener *et al.* (2002) finding in which *Withania somnifera* dose dependently inhibited the rise of serum creatinine and BUN associated with gentamicin treatment.

In solvent fractions study, 100 mg/kg pre- and co-treatment with methanol or aqueous fraction showed a considerable nephroprotection evidenced by a significant reduction of high levels of serum creatinine and BUN associated with gentamicin. The maximum renoprotective effect of *E. divinorum* was observed with methanolic fraction when compared to the aqueous fraction or the crude extract. This effect could be attributed to the different abilities of the solvent to extract protective phytoconstituents from the plant which inturn might lead to variation in antioxidant effect to improve the renal hemodynamics.

In agreement with previous observations (Karadeniz *et al*., 2008; Khan *et al*., 2009), the present study also indicated gentamicin induced oxidative stress, as shown by significant decrease in kidney GSH level, CAT and SOD activities. Exhaustion of enzymatic renal oxidative defense mechanisms along with enhanced ROS generation could aggravate the oxidative damage in gentamicin treated rats (Karahan *et al*., 2005). In this study, pre- and concomitant treatment with *E. divinorum* extracts attenuated gentamicin mediated oxidative stress and renal damage evidenced by a marked rise in renal GSH content, CAT and SOD activities.

SOD, the first line of defense against free radicals, catalyse the dismutation of $O_2^{\bullet -}$ to H_2O_2 that inturn removed by CAT or glutathione peroxidase (GSH-Px) (Maritim *et al*., 2003). There are three forms of SOD in mammalian tissues: copper zinc SOD, manganese SOD and extracellular SOD, togher contributing to the total SOD activities

(Young and Woodside, 2001). CAT decomposes H_2O_2 to H_2O and O_2 by two stage reactions. CAT first reacts with H_2O_2 to form an intermediate species that in turn reacts with extra H_2O_2 converting it to H_2O and O_2 (Young and Woodside, 2001; Maritim *et al*., 2003). The GPx enzyme removes H_2O_2 by oxidizing GSH into oxidized glutathione, GSSG (Ursini *et al*., 1982; Pham-Huy *et al*., 2008).

It has been shown that gentamicin generates ROS depleting GSH (Khan *et al*., 2009) that disturbs the delicate balance between oxidant-antioxidant and causes oxidative damages (Sandhya *et al*., 1995; Parlakpinar *et al*., 2004; Karahan *et al*., 2005). GSH, the major soluble antioxidant in cells, is synthesized from glutamate, cysteine, and glycine (Fang *et al*., 2002). It is reversibly oxidized to GSSG that represents two GSH molecules with a disulphide bond (Valko *et al*., 2007; Hamid *et al*., 2010). The ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Jones *et al*., 2000).

The attenuation of gentamicin-induced oxidative stress by *E. divinorum* might be attributed to its antioxidant property and was in harmony with the earlier studies (Sener *et al*., 2002; Ali *et al*., 2005; Dhanarajan *et al*., 2006). In correlation with the serum biochemical results, the renal antioxidant results revealed maximal protection in rats treated with the methanolic fraction of *E. divinorum*, which is ascribed to its greater free radical scavenging and antioxidant activity. Further, the results of this study strongly indicate that the crude extract and the solvent fractions of *E. divinorum* are beneficial in breaking the vicious circle by increasing the activities of SOD, CAT and GSH contents. This supports the prominent role of antioxidants in protecting the kidney from gentamicin-induced renal damage. Antioxidants maintain the concentration of reduced GSH restoring the cellular defense mechanisms and blocking lipid peroxidation (Babu *et al*., 1995).

The decrease in the amount of intracellular GSH and the accumulation of H_2O_2 and hydroxyl radicals are the triggering factors in gentamicin nephrotoxicity. Fall in intracellular GSH level inhibits GSH-Px activity, H_2O_2 inactivating enzyme, leading to vicious circle that makes the prognosis more severe (Erdem *et al*., 2000). The main protective roles of GSH against oxidative stress include: acting as a cofactor for several

detoxifying enzymes against oxidative stress; participating in amino acid transport through the plasma membrane; scavenging hydroxyl radical and singlet oxygen directly, detoxifying H_2O_2 and lipid peroxides by the catalytic action of GSH-Px and regenerating important antioxidants back to their active forms (Pastore *et al*., 2003; Masella *et al*., 2005; Valko *et al*., 2007).

In accordance with the previous reports (Ramasammy *et al*., 1985; Yamada *et al*., 1995), the results of this study demonstrate that gentamicin treated rats show accelerated lipid peroxidation in the renal tissue as reflected by an increase in MDA. Gentamicin-induced increment in renal MDA content was significantly prevented by *E. divinorum* treatment in the present study. Similar observations were made with oral Arabic gum (Al-Majed *et al*., 2002), caffeic acid phenethyl ester (Parlakpinar *et al*., 2005), probucol (Kumar *et al*., 2000), spirulina (Kuhad *et al*., 2006), vitamin E (Derakhshanfar *et al*., 2007) and *Nigella sativa* (Yaman and Balikci, 2010).

The peroxidation of lipids gives rise to a number of secondary products, MDA being the principal and most studied one. This aldehyde is a highly toxic molecule and has been considered as more than just a marker of lipid peroxidation (Rio *et al*., 2005). The rationale of MDA as a biomarker relies on that it is (solely) derived from lipid peroxides and changes in MDA concentration reflects changes in lipid oxidation level (Lykkesfeldt *et al*., 2007). Lipid peroxidation and the subsequent product MDA are typical examples of oxidation indicating reaction in nephrotoxicity causing irreversible cell damages (Kumar *et al*., 2000; Derakhshanfar *et al*., 2007).

Baliga *et al.* (1999) documented that gentamicin causes lipid peroxidation in the kidneys via ROS generation. Besides, a depletion of renal GSH, which is caused by gentamicin administration, may result in lipid peroxidation and increases MDA levels (Karadeniz *et al*., 2008). Thus, antioxidants can block lipid peroxidation and protect against the toxicity of a wide variety of nephrotoxic chemicals by maintaining the concentration of reduced GSH (Babu *et al*., 1995). In this study, a significant decrease in MDA levels in rats treated with the crude extract as well as solvent fractions was an indication of attenuation of lipid peroxidation and probably due to less damage by oxygen-free radicals in the

presence of *E. divinorum*. The result was in rapport with the biochemical and antioxidant findings in which pre- and co-treatment of rats with the methanolic fraction showed a maximum inhibition of gentamicin mediated lipid peroxidation.

5.2. Morphologic pathology

As shown in figure 7, eight day treatment of animals with gentamicin (100 mg/kg, i.p.) resulted in severe weight loss and treatment with plant extract inhibited the weight loss. These findings were consistent with other reports (Ali *et al*., 1992; Erdem *et al*., 2000; Ali *et al*., 2005; Harlalka *et al*., 2007; Jeyanthi and Subramanian, 2009; Lakshmi and Sudhakar, 2010).

Gentamicin-induced weight loss may be related to direct renal tubules injury and/or increased catabolism. Injury of the renal tubules leads to subsequent loss of the tubular cells that take part in renal water reabsorbtion. This is accompanied by loss of water, leading to dehydration and loss of body weight (Ali *et al*., 2005). On the other hand, increased catabolism associated with gentamicin-induced ARF causes acidosis. Acidosis results in anorexia that in turn decreases oral food intake and causes body weight loss (Ali *et al*., 1992). The increase in the normalized kidney weight of gentamicin-treated rats probably resulted from the edema that was caused by drug-induced acute tubular necrosis (Erdem *et al*., 2000).

The fact that renal injury by gentamicin involves ROS and *E. divinorum* has antioxidant properties may rationalize the alleviation of the gentamicin-induced body weight reduction and normalized kidney weight gain. Further, it may be a reflection of the general palliative effect of the plant on nephrotoxicity.

The histopathological results in this study were paralleled by the biochemical, antioxidant and lipid peroxidation findings. In this experiment, rats treated with gentamicin revealed extensive and marked renal tubular necrosis, in line with several reports indicating the aforementioned changes (Kumar *et al*., 2000; Al-Majed *et al*., 2002; Yaman and Balikci, 2010). The extensive histopathological changes in the renal tubules of rats treated with gentamicin were mitigated by concomitant treatment with *E. divinorum.* As a result, there

were mild histopathological changes in the groups treated with both crude and solvent extracts of *E. divinorum.* The histopathological results in connection with protective effects of plant extract against gentamicin induced nephrotoxicity were in agreement with other reports (Karahan *et al*., 2005; Parlakpinar *et al*., 2005; Kuhad *et al*., 2006).

5.3. Antioxidant activity

The *in vitro* antioxidant assay highly corroborates the *in vivo* results revealing the free radical scavenging activities of *E. divinorum* leaves. The assay documented the DPPH scavenging activities of crude and solvent fractions. Accordingily, the methanolic fraction has the highest antioxidant activity among the plant extracts tested which could justify the maximal nephroprotection observed in rats. The plausible reason for this effect will be the superior capability of methanol to extract the protective phytoconstituents from the leaves of *E. divinorum.* Thus, rats administered with the methanolic fraction might have received relatively a concentrated amount of protective phytoconstituents and have enhaced antioxidant defense.

Based on preliminary phytochemical reports, the presence of flavonoids and polyphenols in the extract are partly responsible for the free radical scavenging activity of and nephroprotection exerted by *E. divinorum*. Flavonoids are well known potent antioxidant and free radical scavengers. Shirwaikar *et al.* (2004) documented and suggested that the nephroprotective effect of *Aerva lanata* is attributed to the antioxidant and free radical scavenging property of flavonoids of the plant. The same documentation and suggestion was made by Annie *et al.* (2005) while evaluating the effect of *Cassia auriculata* root extract on cisplatin and gentamicin-induced renal injury. Similarily, the protective effect of *Zingiber officinale* on gentamicin induced nephrotoxicity was ascribed to its flavonoids which could enhance renal mitochondrial antioxidant system (Lakshmi and Sudhakar, 2010).

The strong antioxidant activity of polyphenols was well documented. Rodrigo and Bosco (2006) suggested that the renoprotective effects of polyphenols could be partly attributed to their properties causing an enhancement of the antioxidant defense system and to an

increased release of NO by endothelial cells. Consistently, Khan *et al.* (2009) reported that green tea polyphenols ameliorated gentamicin elicited nephrotoxicity and oxidative damage by improving antioxidant defense, tissue integrity and energy metabolism. It was also revealed that the protective effects of phenolic extract of soyabean on gentamicin mediated nephropathy to be related to the polyphenolic content (Ekor *et al.*, 2006).

6. Conclusion

The results of the present study show that gentamicin induces generation of free radicals that causes oxidative damage to kidneys of rats leading to ARF. Moreover, the results provide convincing evidence that pre- and co-administration of *E. divinorum* along with gentamicin prevents both functional and histological renal changes elicited by gentamicin in rats. Based on our *in vitro* and *in vivo* findings, it could be proposed that *E. divinorum* acts in the kidney as a potent scavenger of free radicals to prevent the toxic effects of gentamicin. The methanol fraction showed superior *in vivo* nephroprotection followed by the aqueous fraction and then by the crude extract in line with their *in vitro* free redical scavenging activity. Although the protective effect of *E. divinorum* appears to be due to its inhibition of gentamicin induced oxidative stress, further investigations are essential to elucidate the exact mechanism of protection and potential usefulness of *E. divinorum* as a protective agent against drugs or xenobiotics toxicity.

7. Recommendations

The following are recommended for further work:

- Further studies usings longer times, varied animal models and more detailed analysis of the pathways to elucidate the exact mechanisms of nephroprotection;
- Evaluation of the effect of *E. divinorum* on the levels of mRNA of renal antioxidant enzymes;
- Subchronic and chronic toxicity tests to have the whole toxicological profile of the plant; and
- Isolation and structure elucidation of renoprotective compound(s).

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