i) Lipid peroxidation

Lipid peroxidation is considered as the main molecular mechanism involved in the oxidative damage to cell structure and in the toxicity process that lead to cell death. Lipid peroxidation is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers (Dianzani & Barrera, 2008). In pathological situations the reactive oxygen and nitrogen species are generated at higher than normal rates, and as a consequence, lipid peroxidation occurs with α-tocopherol deficiency. In addition to containing high concentrations of polyunsaturated fatty acids and transition metals, biological membranes of cells and organelles are constantly being subjected to various types of damage (Pallavi et al., 2012). The mechanism of biological damage and the toxicity of these reactive species on biological systems are currently explained by the sequential stages of reversible oxidative stress and irreversible oxidative damage. Oxidative stress is understood as an imbalance situation with increased oxidants or decreased antioxidants. The concept implies the recognition of the physiological production of oxidants (oxidizing free-radicals and related species) and the existence of operative antioxidant defenses. The imbalance concept recognizes the physiological effectiveness of the antioxidant defenses in maintaining both oxidative stress and cellular damage at a minimum level in physiological conditions (Boveris et al., 2008).

Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids (PUFA). Since polyunsaturated fatty acids are more sensitive than saturated ones, it is obvious that the activated methylene (RH) bridge represents a critical target site. The presence of a double bond adjacent to a methylene group makes the methylene C-H bond weaker and therefore the hydrogen in more susceptible to abstraction. This leaves an unpaired electron on the carbon, forming a carbon-centered radical, which is stabilized by a molecular rearrangement of the double bonds to form a conjugated diene which then combines with oxygen to form a peroxyl radical. The peroxyl radical is itself capable of abstracting a hydrogen atom
from another polyunsaturated fatty acid and so of starting a chain reaction (Alzoghaibi, 2013).

**Initiation step of lipid peroxidation process**

Molecular oxygen rapidly adds to the carbon-centered radicals (R’) formed in this process, yielding lipid peroxyl radicals (ROO’). Decomposition of lipid peroxides is catalyzed by transition metal complexes yielding alcoxyl (RO’) or hydroxyl (HO’) radicals. These participate in chain reaction initiation that in turn abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The formation of peroxyl radicals leads to the production of organic hydroperoxides, which, in turn, can subtract hydrogen from another PUFA. This reaction is termed propagation, implying that one initiating hit can result in the conversion of numerous PUFA to lipid hydroperoxides. In sequence of their appearance, alkyl, peroxyl and alkoxy radicals are involved. The resulting fatty acid radical is stabilized by rearrangement into a conjugated diene that retains the more stable products including hydroperoxides, alcohols, aldehydes and alkanes. Lipid hydroperoxide (ROOH) is the first, comparatively stable, product of the lipid peroxidation reaction (Frankel, 1995).
Initial phase of the propagation step of lipid peroxidation process indicating the oxygen uptake.

Reduced iron complexes (Fe\(^{2+}\)) react with lipid peroxides (ROOH) to give alkoxy radicals, whereas oxidized iron complexes (Fe\(^{3+}\)) react more slowly to produce peroxyl radicals. Both radicals can take part in the propagation of the chain reaction. The end products of these complex metal ion-catalyzed breakdowns of lipid hydroperoxides include the cytotoxic aldehydes and hydrocarbon gases such as ethane. The free radical chain reaction propagates until two free radicals conjugate each other to terminate the chain.

In conditions in which lipid peroxidation is continuously initiated it gives non-radical products destroying two radicals at a time. In the presence of transition metal ions, ROOH can give rise to the generation of radicals capable of re-initiating lipid peroxidation by redox-cycling of these metal ions (Abraham, 2014). Lipid peroxidation causes a decrease in membrane fluidity and in the barrier functions of the membranes. The many products of lipid peroxidation such as hydroperoxides or their aldehyde derivatives inhibit protein synthesis, blood macrophage actions and alter chemotactic signals and enzyme activity. Lipid peroxidation or reaction of oxygen with unsaturated lipids produces a wide variety of oxidation products. The main primary products of lipid peroxidation are lipid hydroperoxides (LOOH). Among the many different aldehydes which can be formed as secondary products during lipid peroxidation, malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) (Esterbauer et al., 1991). MDA appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most toxic (Esterbauer et al., 1990).

a) Malondialdehyde (MDA)

MDA has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its facile reaction with thiobarbituric acid (TBA) (Esterbauer & Cheeseman, 1990). The TBA test is predicated upon the reactivity of TBA toward MDA to yield an intensely colored chromogen fluorescent red adduct; this test was first used by food chemists to evaluate autooxidative degradation of fats and oils (Sinnhuber et al., 1958). However, the thiobarbituric acid reacting substances test (TBARS) is notoriously nonspecific
which has led to substantial controversy over its use for quantification of MDA from in vivo samples. Several technologies for the determination of free and total MDA, such as gas chromatography-mass spectrometry (GC-MS/MS), liquid chromatography-mass spectrometry (LC-MS/MS), and several derivatization-based strategies, have been developed during the last decade. Because MDA is one of the most popular and reliable marker that determine oxidative stress in clinical situations (Giera et al., 2012), and due to MDA’s high reactivity and toxicity underlying the fact that this molecule is very relevant to biomedical research community.

MDA Production by Enzymatic Processes

MDA can be generated in vivo as a side product by enzymatic processes during the biosynthesis of thromboxane A₂ (Tsikas et al., 2012). TXA₂ is a biologically active metabolite of arachidonic acid formed by the action of the thromboxane A₂ synthase, on prostaglandin endoperoxide or prostaglandin H₂ (PGH₂) (Ricciotti et al., 2011). PGH₂ previously is generated by the actions of cyclooxygenases on AA (Yang et al., 2008).

MDA Production by Nonenzymatic Processes

A mixture of lipid hydroperoxides is formed during lipid peroxidation process. The peroxyl radical of the hydroperoxides with a cis-double bond homoallylic to the peroxyl group permits their facile cyclization by intramolecular radical addition to the double bond and the formation of a new radical. The intermediate free radicals formed after cyclization can cyclize again to form bicycle endoperoxides, structurally related to prostaglandins, and undergo cleavage to produce MDA. Through nonenzymatic oxygen radical-dependent reaction, arachidonic acid (AA) is the main precursor of bicyclic endoperoxide, which then undergoes further reactions with or without the participation of other compounds to form MDA (Milne et al., 2008). However, it should be possible that other eicosanoids that can also be generated by nonenzymatic oxygen radical-dependent reaction (Brooks et al., 2008) may be precursor of bicyclic endoperoxide and MDA. Recent review has addressed the pathways for the nonenzymatic formation of MDA under specific conditions (Onyango & Baba, 2010).
MDA Metabolism

Once formed MDA can be enzymatically metabolized or can react on cellular and tissular proteins or with DNA to form adducts resulting in biomolecular damages. Early studies showed that a probable biochemical route for MDA metabolism involves its oxidation by mitochondrial aldehyde dehydrogenase followed by decarboxylation to produce acetaldehyde, which is oxidized by aldehyde dehydrogenase to acetate and further to CO₂ and H₂O (Esterbauer et al., 1991). On the other hand, phosphoglucone isomerase is probably responsible for metabolizing cytoplasmic MDA to methylglyoxal (MG) and further to D-lactate by enzymes of the glyoxalase system by using GSH as a cofactor (Agadjanyan et al., 2005). A portion of MDA is excreted in the urine as various enaminals (RNH-CH-CHO) such as N-epsilon-(2-propenal) lysine, or N-2-(propenal) serine (Esterbauer).
MDA can be generated in vivo by decomposition of arachidonic acid (AA) and larger PUFAs as a side product by enzymatic processes during the biosynthesis of thromboxane A₂ (TXA₂) and 12-l-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (blue pathway), or through nonenzymatic processes by bicyclic endoperoxides produced during lipid peroxidation (red pathway). Once formed MDA can be enzymatically metabolized (green pathway). Key enzymes involved in the formation and metabolism of MDA: cyclooxygenases (1), prostacyclin hydroperoxidase (2), thromboxane synthase (3), aldehyde dehydrogenase (4), decarboxylase (5), acetyl CoA synthase (6), and tricarboxylic acid cycle (7).

Results and Discussion:

In the current study malondialdehyde (MDA) content was estimated in kidney tissues of all the experimental groups such as Normal control (NC), Xanthium indicum treated (Xi)t, α-tocopherol treated (Tpt), Diabetic control (DC), Glibenclamaide treated diabetic (Di + Glbt), α-tocopherol treated diabetic (Di + Tpt) and Xanthium indicum treated diabetic (Di + Xi)t rats. The increased lipid peroxidation caused by the increased oxidative stress in the kidneys induced by STZ was assessed and investigated. It was found that the lipid peroxidation products, in terms of MDA, increased in renal tissue. Treatment with Xanthium indicum extract, its bioactive compound α-tocopherol and Glibenclamide decrease the MDA levels in diabetic group of rats and the effect was more prominent in the diabetic group of rats treated with α-tocopherol (Table-10, Figure-18 and 19).

Diabetes-induced oxidative stress generates reactive oxygen species and an imbalance among antioxidants. Bhor et al., (2004) reported altered activity in primary antioxidant enzymes (e.g., catalase, superoxide dismutase, and glutathione peroxidase) and an increase in lipid peroxidation and carbonyl protein content, thus ensuring the occurrence of oxidative stress in diabetic rats. Lipid peroxidation may bring about protein damage and inactivation of membrane bound enzymes either through direct attack by free radicals or through chemical modification by its end products, malondialdehyde and 4-hydroxynonenal (Halliwell & Gutteridge, 1999). The accompanying increase in the transport of oxidizable compounds such as glucose, amino acids (Fedorak, 1990), lipids (Staprans et al., 1993) along with the increased synthesis of cholesterol and triglycerides (Feingold et al., 1990) and decreased
utilization of glucose within the enterocyte (Madsen et al., 1995) can lead to transient increases in the intracellular concentrations of these compounds. The free radicals generated by autoxidation of these compounds may have been responsible for the elevation in lipid peroxidation and protein oxidation. In addition, glucose is known to induce lipid peroxidation through activation of the lipoxygenase enzymes (Rajeswari et al., 1991). pro-oxidative conditions in the cell lead to production of reactive aldehydes such as malondialdehyde (MDA). Among these, oxidative stress has been suggested extensively as a potential mechanism for diabetic kidney disease because oxidative stress promotes the formation of AGE as well as protein kinase C – mitogen activated protein kinase (PKC-MAPK) activation (Brownlee, 2001). Indeed, involvement of oxidative stress has been indicated by the presence of lipid peroxidation products in the kidney from streptozotocin (STZ)-induced diabetic rats (Horie et al., 1997). Increased formation of ROS may occur in diabetes for reasons possibly related to an increase in glucose concentrations in plasma and tissues (Brownlee, 2001) and may have a role in the pathogenesis of diabetic nephropathy. However, the functional and pathophysiologic role of excessive oxidative stress in diabetic kidney disease was indicated merely by the presence of increased levels of MDA in the kidney of STZ-induced diabetic rats (Horie et al., 1997). These mechanisms may explain the increased lipid peroxidation associated with hyperglycaemia. Alternatively, increased ROS-mediated lipid peroxidation in diabetes may result from disturbances in antioxidant defense. In the present investigation MDA levels were increased drastically in diabetic rats (52%) than the normal controlled rats.

α-tocopherol seems to be a very important agent in providing protection against oxidation of cellular lipids by free radicals that are potentially damaging byproducts of cellular metabolism (Halsted, 2000). It is a lipid soluble vitamin responsible for prevention of lipid peroxidation. The most attributed function of tocopherols is their involvement in various mechanisms in protecting poly unsaturated fatty acids (PUFA) from oxidation (Ledford and Niyogi, 2005). α-Tocopherols scavenge and quench various ROS and lipid oxidation products, stabilize membranes, and modulate signal transduction (Noctor, 2006). It is likely that vitamins C and α-tocopherol act in a potentiation with each other, where α-tocopherol is mostly being oxidized to the tocopheroxyl radical and subsequently reduced to tocopherol in
presence of vitamin C and glutathione (Vijay & Vimukta, 2014). α - Tocopherols also function as recyclable chain reaction terminators of polyunsaturated fatty acid (PUFA) radicals generated by lipid oxidation (Hare et al., 1998). α-Tocopherols scavenge lipid peroxy radicals and yield a toopheroxyl radical that can be recycled back to the corresponding α-tocopherol by reacting with ascorbate or other antioxidants (Igamberdiev & Hill, 2004). α-tocopherol is generally assumed to protect the unsaturated lipid bilayer of vital cellular and sub cellular membrane against endogenous or exogenous oxy- free radicals, which initiate or propagate nonenzymatic lipid peroxidation damage (Chisdm et al., 1992). However, the antioxidant property of α-tocopherol is not only to scavenge ROS, but also to up-regulate antioxidant enzymes through regulation of the gene expression or activity of antioxidant enzymes (Hajiani et al., 2008). Supplementation with α-tocopherol decreased the level of MDA in both the normal and diabetic groups, confirming the role of α-tocopherol as a powerful antioxidant as well as anti diabetic agent. Jorge et al. reported that a minor oxidative stress was observed in type 2 diabetic patients after α-Tocopherols treatment interfered from the reduced level of erythrocyte MDA and the increase total antioxidant status. In animal studies, the cyclosporine-induced rise in renal malondialdehyde was prevented by α-tocopherol supplementation (Parra et al., 1998). The present study reveals that MDA content was raised to 1% in α-tocopherol treated rats and 10% increase in α-tocopherol treated diabetic rats than the normal controlled rats, but in α-tocopherol treated diabetic rats nearly 43% decreased levels were observed than the diabetic controlled rats.

Herbal medicines are popularly used remedies for many diseases by a vast majority of the world's population, because allopathic medicines have large number of side-effects. Thus, herbal formulations have attained widespread acceptability as therapeutic agents in many developing countries as anti-diabetics and lipid lowering agents (Atawodi, 2011; Abolfathi et al., 2012). The ability of the hydromethanolic extract of Xanthium indicum to manage dyslipidemia is a potential beneficial effect on diabetic complications. In the present study, MDA levels in diabetic rat kidneys were higher than non-diabetic rats. The tissue oxidative damage as reflected by high amount of lipid peroxidation products (MDA) occurs in the kidney of diabetic rats. The decreased levels of MDA following Xanthium indicum leaf extract treatment could also be due to reduced level of free radicals as observed from in vitro radical
scavenging effect of the leaf extract. In the current research work, MDA levels were increased to 2% in *Xanthium indicum* treated rats and 27% increase in *Xanthium indicum* treated diabetic rats when compared to normal controlled rats. A decrease of 25% MDA content in *X. indicum* treated diabetic rats than the diabetic rats was observed in these findings.

ii. Kidney biomarkers

a) Microalbumin

The kidneys are responsible for removing waste products from the blood and regulating the water fluid levels. When damage to the kidneys occurs, this pair of organs may fail to filter out wastes. Additionally, damaged kidneys may fail to retain nutrients and proteins from the body that are essential to health. Such is the case with albumin. Albumin is a protein that is used by the body for normal cell growth and tissue repair. If the kidneys become damaged, they may not retain this protein within bloodstream causing it to be excreted with the urine. When this occurs you may experience serious health complications. Maintaining normal kidney function is vital to ensuring that albumin remains in the bloodstream. The microalbuminuria test is a urine test that measures the amount of albumin in your urine. If kidney damage has occurred, albumin will leak into the bloodstream and will be present in the urine. Albumin is a protein. Albuminuria is having too much protein in the urine. This is referred to as "microalbuminuria" which indicates a slightly high level of protein in the urine. Overt proteinuria or "macroalbuminuria" indicates more than 300mg of albumin in the urine per day (Timothy & Peter, 2000). Microalbuminuria may progress over a span of a number of years to overt nephropathy characterized by the presence of larger amounts of the protein albumin leaking through the kidneys’ filter mechanism into the urine. This is called macroalbuminuria (urinary albumin > 300 mg/24 hours) (Justesen et al., 2006). Microalbuminuria is characterized by increased prevalence of arterial hypertension, proliferative retinopathy, and peripheral neuropathy. Studies in the Western literature have documented the linear relationship of degree of microalbuminuria with body mass index (BMI), blood pressure, and duration of diabetes. Gender correlation of microalbuminuria was not seen in type-2 diabetes mellitus (Ruilope & Segura, 2006).
The reduction in albuminuria leads to reduced risk of adverse renal and cardiovascular events (Ibsen et al., 2005). Microalbuminuria which is an early marker of diabetic nephropathy may be present to diagnose the type 2 diabetes (American Diabetes Association, 2003). An increase in albumin excretion rate leading to microalbuminuria is widely acknowledged as the earliest index of diabetic nephropathy and as a risk factor for the development of overt diabetic renal disease (Mogensen et al., 1995) and macrovascular disease (Boyle, 2007). The mechanisms underlying the increase in albumin excretion rate have both functional and structural changes in the kidney have been proposed as possible contributors. These include early alterations in glomerular filtration rate, intraglomerular pressure, glomerular size (Melissa et al., 1998), glycosylation of membrane proteins (Meyer, 1990) and circulating albumin (Daniels & Hauser, 1992), and changes in the composition of glomerular extracellular membrane material leading to glomerular basement membrane thickening (Østerby, 1995), loss of heparin sulphate and alterations in epithelial slit pores may also contribute to increases in albumin excretion rate in diabetic nephropathy (Pegtalunan et al., 1997).

Results and discussion:

In the present study changes in albumin levels in urine was estimated in all the experimental groups such as Normal control (NC), Xanthium indicum treated (Xi t), α-tocopherol treated (Tpt), Diabetic control (DC), Glibenclamaide treated diabetic (Di + Glbt), α-tocopherol treated diabetic (Di + Tpt) and Xanthium indicum treated diabetic (Di + Xi t) rats. The increased albumin levels in the urine would be caused by the increased oxidative stress in the kidneys due to induction by STZ. Treatment with Xanthium indicum leaf hydromethanolic extract, its bioactive compound α-tocopherol and Glibenclamid decreased the urine microalbumin levels in the diabetic group of rats and the effect was more prominent in α-tocopherol treated diabetic group of rats (Table-11, Figures-20 and 21).

Albumin is heavily degraded during renal passage by post-glomerular cells. These fragments, which are most probably produced in lysosomes of the proximal tubule, are regurgitated back into the tubular lumen with subsequent excretion in the urine within a matter of minutes. Studies both in vivo and in the isolated perfused kidney system have demonstrated that albumin is degraded by the kidney to peptides
that are exclusively excreted in the urine (Osicka & Comper, 1997). Albumin is normally excreted as a mixture of intact protein and fragments that are produced during renal passage (Melissa et al., 1998). Increase in albumin excretion rate in early diabetes due to the disproportionate increase in the excretion of intact albumin. This means that the appearance of fragments in the urine is a direct result of the renal passage of albumin. This indicates that the changes seen in the ratio of intact versus fragmented albumin are not the result of a change in the perm-permselectivity of the glomerular capillary wall but an alteration in a post-glomerular site that does not allow backflux into the circulation. In any case, recent studies have demonstrated that these changes would not be associated with biophysical alterations in glomerular transport. The influence of glomerular charge on transglomerular transport of albumin has now been shown to be far smaller than originally thought (Osicka & Comper, 1998). The fact that glomerular charge selectivity is essentially negligible invalidates the concept of large pores or ‘shunts’ that are thought to allow non-selective passage of albumin across the capillary wall (Blouch et al., 1997). Albumin is size selected as a 36-AI radius molecule which would mean that its fractional clearance would be unchanged or reduced in diabetic states (Scandling & Myers, 1992). The change in the ratio of intact versus degraded excreted albumin that accompanies the increase in albumin excretion rate in early diabetes is due to the inhibition of degradation of albumin at a post-glomerular site; that is, after the albumin has passed the glomerular filtration barrier (Burne et al., 1998).

Diabetic nephropathy (DN) is the most common single cause of renal damage in the world that often progresses to end-stage renal disease (Navarro-Gonzalez et al., 2008). The proteinuria that develops after induction of diabetes is mainly due to an increased excretion of low molecular weight proteins. About 20%-30% of patients with type 2 diabetes, accompanied by renal insufficiency, showed normoalbuminuria (Rigalleau et al., 2007; Kramer et al., 2007). Microalbuminuria and proteinuria typically reflect the presence of moderate and advance lesions, respectively in kidney disease. Progressive renal function decline in diabetes is an early event that occurs in a proportion of patients without increased albumin excretion rate (Perkins et al., 2007). There are various mechanisms of albuminuria which involve abnormalities of the glomerular endothelial barrier (Stehouwer et al., 2004), causing excessive filtration as well as reduction of renal tubular cell albumin degradation and
reabsorption. Glomerular hypertension, inflammation, and oxidative stress worsen albuminuria, with angiotensin-II (Coresh et al., 2003) and mechanical stress factors contributing to these processes. Microalbuminuria is the strong predictor of diabetic nephropathy, which is the main cause of morbidity and mortality in patients with diabetes mellitus. Microalbuminuria is also characterized by increased prevalence of arterial hypertension, proliferative retinopathy, and peripheral neuropathy. Studies in the Western literature have documented the linear relationship of degree of microalbuminuria with body mass index (BMI), blood pressure, and duration of diabetes (Ruilope et al., 2006). Increased urinary excretion of low molecular weight proteins generally is because of decreased tubular re-absorption of filtered plasma proteins. This is important because elevated microalbumin in urine is indicator of renal disease, especially in diabetics (Pazdro & Burgess, 2010). We examined in the present research work the albumin levels in urine were increased to drastically 7 fold in diabetic rats than the normal controlled rats.

Studies have shown that diabetes and diabetic complications are associated with greater oxidative stress and reduced levels of antioxidants (Yorek, 2003). Recently, a unifying mechanism has been proposed suggesting that both macrovascular and microvascular (including nephropathy) diabetic complications are all mediated via an intracellular increase in reactive oxygen species (Brownlee, 2005). Treatment with α-tocopherol has all been shown to significantly reduce urinary albumin excretion ratio among diabetics (Gaede et al., 2001 & Hirnerova et al., 2003). α-tocopherol reduce the serum levels of C-reactive protein (CRP) and advanced glycation end products, expression of cell adhesion molecules and inflammatory mediators (Prasad, 2011). Koya et al. (1997) reported that hemodynamic abnormalities in diabetic rats were normalized by treatment with α-Tocopherol. The hypertrophic response usually found in diabetes was prevented by α-tocopherol administration; tubular acidification, which was significantly changed by diabetes. α-tocopherol is able to protect, at least in part, against the harmful effects of diabetes on renal function (Gomes et al., 2005). Diabetic rats showed increased urinary protein excretion compared to controls. The treatment of diabetic rats with D-α-tocopherol caused a small decrease in protein excretion, an effect that may be secondary to the decrease in glomerular filtration rate (GFR) caused by α-tocopherol treatment. Albuminuria was prevented by treatment with α-tocopherol (Koya et al.,
The present work reveals that urine albumin levels were raised to 28% in α-tocopherol treated rats and 1 fold increase in α-tocopherol treated diabetic rats than the normal controlled rats. But in α-tocopherol treated diabetic rats nearly 71% decrease in microalbumin levels were observed than the diabetic controlled rats.

*Xanthium indicum* possess diuretic properties (Gladys et al., 2004; Lesly et al., 1999). The *Xanthium indicum* extract exerted its diuretic activity possibly by inhibiting tubular reabsorption of water and accompanying anions, as such action has been hypothesized for some other plant species (Bevevino et al., 1994). Therefore *Xanthium indicum* extract significantly increased the GFR due to (a) A detergent like interaction with structural components of glomerular membranes. (b) A decrease in renal perfusion pressure, attributable to decrease in the resistance of the afferent arteriole and/or an increase in the resistance of the efferent arteriole and/or. (c) The direct effect on the arteriole wall affecting glomerular blood flow (Abderahim et al., 2008). In the current research work, urine albumin levels were increased to 12% in *Xanthium indicum* treated rats and a 2 fold increase in *Xanthium indicum* treated diabetic rats when compared to normal controlled rats, while the *X. indicum* treated diabetic rats shown 68% decrease in microalbuminuria when compared to the diabetic controlled rats.

**b) Urine creatinine:**

Creatinine is a breakdown product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body. Creatine is synthesized primarily in the liver from the methylation of glycocyamine (guanidino acetate, synthesized in the kidney from the amino acids arginine and glycine) by S-adenosyl methionine. It is then transported through blood to the other organs, muscle, and brain, where, through phosphorylation, it becomes the high-energy compound phosphocreatine. During the reaction, creatine and phosphocreatine are catalyzed by creatine kinase, and a spontaneous conversion to creatinine may occur (Allen, 2012). Creatinine (molecular mass 113 Da, molecular radius 30 nm) fulfills most of the requirements for a perfect filtration marker. It is not protein bound; it is freely filtered; it is not metabolized by the kidney; and it is physiologically inert. Creatine is reabsorbed by the kidney, and urinary creatine excretion is usually <10 mg/24 h.
Urinary excretion of creatine increases in diseases associated with abnormal muscle metabolism (Brian, 2001).

Creatinine is removed from the blood chiefly by the kidneys, primarily by glomerular filtration, but also by proximal tubular secretion. Little or no tubular reabsorption of creatinine occurs. If the filtration in the kidney is deficient, creatinine blood levels rise. Therefore, creatinine levels in blood and urine may be used to calculate the creatinine clearance (CrCl), which correlates with the GFR. Blood creatinine levels may also be used alone to calculate the estimated GFR (eGFR). The GFR is clinically important because it is a measurement of renal function. However, in cases of severe renal dysfunction, the CrCl rate will overestimate the GFR because hyper secretion of creatinine by the proximal tubules will account for a larger fraction of the total creatinine cleared. Creatinine is the most widely used biomarker of kidney function. It is inaccurate at detecting mild renal impairment, and levels can vary with muscle mass and protein intake (Ronald et al., 1992). Formulas such as the Cockcroft and Gault formula and the MDRD formula try to adjust for these variables.

The major problem with measuring creatinine clearance is that the collection may be incomplete; often urine is passed into the toilet rather than into the collection bottles. This results in an underestimation of renal function, and has led to some commentators to recommend alternative measures such as calculated creatinine clearance or an isotopic GFR. In hospital, especially when the patient is catheterised, creatinine clearance provides an accurate estimate of GFR. Overestimation of the GFR occurs at low levels of renal function, due to tubular secretion of creatinine. This can be corrected by collecting the urine while the patient is taking cimetidine or by averaging a urea and creatinine clearance in a single 24-hour collection. To accurately define the GFR at low levels of renal function, an isotopic GFR is recommended. Ketoacids, cimetidine, and trimethoprim reduce creatinine tubular secretion and, therefore, increase the accuracy of the GFR estimate, in particular in severe renal dysfunction (In the absence of secretion, creatinine behaves like inulin).

Creatinine clearance has been used for many decades to estimate GFR. It involves a 24-hour urine collection to measure creatinine excretion. As the same sample can be used to measure the protein excretion rate, creatinine clearance is often used for the initial evaluation of renal diseases, such as glomerulonephritis (GN).
can also be used to monitor the progression of chronic renal failure, the response to therapy or to help decide when to start dialysis in patients with declining renal function. However, because a small amount of creatinine is released by the filtering tubes in the kidneys, creatinine clearance is not exactly as same as the GFR. In fact, creatinine clearance usually overestimates the GFR, particularly in patients with advanced kidney failure. Normal clearance values are: Male: 97 to 137 mL/min; Female: 88 to 128 mL/min (Bazari, 2007). There are several factors that may interfere with the accuracy of the test. These include: (1) Incomplete urine collection; (2) Pregnancy; and (3) Vigorous exercise. Creatinine clearance measurements can also be affected by drugs, such as: cimetidine, trimethoprim, and drugs that can damage the kidneys (cephalosporins).

**Results and discussion:**

In the present study changes in creatinine levels in urine were estimated in all the experimental groups such as Normal control (NC), *Xanthium indicum* treated (*Xit*), α-tocopherol treated (Tpt), Diabetic control (DC), Glibenclamide treated diabetic (Di + Glbt), α-tocopherol treated diabetic (Di + Tpt) and *Xanthium indicum* treated diabetic (Di + Xit) rats. In diabetic conditions, urine creatinine levels were decreased due to inefficient glomerular filtration and formation of advanced glycation end products (AGE) in glomerular proteins. Treatment with *Xanthium indicum* leaf hydromethanolic extract, its bioactive compound α-tocopherol and Glibenclamide increase the urine creatinine levels in diabetic group of rats and the effect was more prominent in plant extract treated diabetic group of rats (Table-12, Figures-22 and 23).

Renal disease in diabetes is found to be associated with abnormalities of vasodilatation and generates reactive oxygen species mediated by endothelial derived nitric oxide (NO), suggesting linkage between vascular and metabolic abnormalities. Angiotensin II and aldosterone, interacting with pulse pressure and increased systolic blood pressure, activate NADP oxidase, which acts as mediator of oxidative stress. Angiotensin II increases metabolism of NO to peroxynitrite (Coresh et al., 2003), which further impairs endothelial-derived vasodilation. (Hill et al., 2003). Charles Heilig et al., (2006) discussed the relationship of renal glucose transporter expression in relation to the development of diabetic nephropathy. Expression of GLUT1, the
major mesangial glucose transporter, regulates extracellular matrix production. Mesangial cells overexpressing GLUT1 show increased production of both types I and IV collagen, as well as increased fibronectin and laminin production, leading to a phenotype similar to that of diabetes. In animal models, GLUT1 overexpression in glomeruli creates a nephropathy phenotype resembling that of diabetic renal disease with increased mean glomerular volume, mesangial expansion, and sclerosis (Heilig et al., 2006). As the kidneys fail and glomerular filtration is severely compromised, the level of protein excretion declines. The onset of diabetes is associated with hemodynamic changes in the renal circulation that lead to increased renal plasma flow (RPF), glomerular capillary hyperperfusion, and an increased glomerular transcapillary hydraulic pressure gradient (Parving et al., 1992). These hemodynamic alterations are hypothesized to cause functional and structural damage to the glomeruli that result in defects of selective glomerular capillary permeability, proteinuria, protein extravasation into the glomerular mesangium, expansion of mesangial matrix, and glomerulosclerosis (Lebovitz & Palmisano, 1990).

Distinct metabolic renal alterations are demonstrable in experimental diabetes, leading to a negative nitrogen balance, enhanced proteolysis and lowered protein synthesis (Bhavapriya et al., 2001). Changes in protein metabolism include a reduced uptake of amino acids by tissues, a higher rate of proteolysis and a fall in protein synthesis, leading to an increase in the production of urea by the liver (Felig & Bergaman, 1995). The overload of urea, glucose and other compounds in the kidney, together with renal vascular changes arising from the increased glycosylation of blood proteins, can damage the kidney and thus promote a loss of protein in the urine (Viberti et al., 1994). Creatinine is the major waste product of creatine metabolism. In the kidney, it is filtered by the glomerulus and actively excreted by the tubules. Under normal circumstances, creatine is eliminated solely by renal excretion. A creatinine-based estimate of GFR seems to be good markers of renal function decline in advanced stages of diabetic renal disease. The exogenous creatinine clearance decreased as the concentration of creatinine in the blood was acutely increased 10-fold by creatinine infusion (Edelstein, 2000). This decrease was thought to be due to saturation of the tubular secretory mechanism. Creatinine reabsorption during low rates of urine flow is thought to result from its passive back-diffusion from the lumen to the blood. Thus, when passive reabsorption of creatinine might result in a lower
creatine clearance and a higher concentration of serum creatinine. The maximum effect of passive creatinine reabsorption probably amounts to only a 5-10% decrease in creatinine clearance (Ronald et al., 1992). From the present investigation, it was revealed that urine creatinine levels were decreased to 27% in diabetic rats when compared to the normal controlled rats.

α-tocopherol could exert a beneficial effect on renal function by reducing hyperfiltration and possibly decreasing hemodynamically mediated renal injury, furthering the beneficial effect of improved glycemic control. α-tocopherol is a scavenger of free radicals, and has an important role in the defense against oxidative stress. It maintains concentrations of reduced glutathione, and the activities of the antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase in the glomeruli (Branden et al., 2002; Shang et al., 2003). Craven et al., (1997) reported that biochemical markers and histologic features of diabetic nephropathy were attenuated in streptozotocin rats that received dietary supplementation of α-Tocopherol. Increased α-tocopherol concentrations in the renal cortex, and was associated concomitantly with a reduction in glomerular expansion. Its supplementation is able to abrogate specific hemodynamic and biochemical abnormalities associated with renal disease in man (Saran et al., 2003; Sato et al., 2003). Diabetic patients with better creatinine clearances and poorest glycemic control showed the most marked normalization in response to α-tocopherol treatment (Bursell et al., 1999). The present data reveals that urine creatinine levels were raised to 10% in α-tocopherol treated rats but 7% decrease in α-tocopherol treated diabetic rats than the normal controlled rats. However in α-tocopherol treated diabetic rats, nearly 28% increased levels were observed than the diabetic controlled rats.

*Xanthium indicum* leaves have been shown to ameliorate hyperglycemia in streptozotocin-induced diabetic rats. It significantly decreases high blood glucose level in diabetic rats and improved insulin production. Diabetic-dependent alterations in urinary albumin excretion, creatinine clearance, kidney hypertrophy and basement membrane thickening may attenuated by plant extract. Ching-Yuang (2005), determined that the effects of methanolic crude extract of *Xanthium indicum* herb on proliferation and cytokine gene expression and production in human mesangial cells. *Xanthium indicum* controls raised liver enzymes, elevated blood urea nitrogen (BUN) and creatinine levels and coagulopathy (Hossein & Mani, 2009). In particular,
increased creatinine clearance (Cr) in STZ-diabetic rats was obviously observed after eight weeks of ruscogenin or rosiglitazone treatment. Treatment with hydromethanolic leaf extract of *Xanthium indicum* (200 mg/kg/day) for four weeks caused 8% and <1% elevated levels of urine creatinine in *Xanthium indicum* treated rats and *Xanthium indicum* treated diabetic rats respectively when compared to normal controlled rats. An increase of 38% urine creatinine levels in *X. indicum* treated diabetic rats was observed in these findings.

c) **Serum creatinine:**

Serum creatinine is primarily a metabolite of creatine, almost all of which is located in skeletal muscle. The normal level of creatinine is 0.8 to 1.4 mg/dL. Females usually have a lower creatinine (0.6 to 1.2 mg/dL) than males, because they usually have less muscle mass (Molitoris, 2007). The amount of creatine per unit of skeletal muscle mass is consistent and the breakdown rate of creatine is also consistent. Thus, serum creatinine concentration is very stable and a direct reflection of skeletal muscle mass (Martin, 2003). It (a blood measurement) is an important indicator of renal health because it is an easily measured byproduct of muscle metabolism that is excreted unchanged by the kidneys. Creatinine itself is produced via a biological system involving creatine, phosphocreatine (also known as creatine phosphate), and adenosine triphosphate (ATP, the body's immediate energy supply). The synthesis of creatine, the precursor of creatinine, takes place primarily in the liver. After its release into the circulation, creatine is actively taken up by muscle and other tissues. Muscle contains 98% of the total body creatine pool (i.e., 120 g in a 70 kg man), of which 60-70% exists as phosphocreatine, and the remainder as free creatine. Small amounts of creatine are measurable in liver, kidney, brain, and body fluids. The serum concentration in adults ranges from 1.6 to 7.9 mg/L (Ronald *et al.*, 1992). Serum creatinine is commonly used to screen for renal disease or to investigate urinary sediment abnormalities, hypertension or non-specific symptoms such as tiredness. It is also used to monitor renal function after transplantation, in chronic renal disease, and in patients with glomerulonephritis (GN) taking disease-modifying therapy. Serum creatinine can also be used to monitor the effects of nephrotoxic drugs such as gentamicin or anticancer drugs.
Serum creatinine is mainly produced by the metabolism of creatine in muscle, but also originates from dietary sources of creatinine such as cooked meat. Creatinine generation from the muscles is proportional to the total muscle mass and muscle catabolism. In people with a relatively low muscle mass, including children, women, the elderly, malnourished patients and cancer patients, the serum creatinine is lower for a given glomerular filtration rate (GFR). There is a danger of underestimating the amount of renal impairment in these patients, as their serum creatinine is also relatively lower. For example, the GFR may be reduced as low as 20-30 mL/min in a small elderly woman, while her serum creatinine remains in the upper range of normal. Creatinine is an imperfect filtration marker, because it is secreted by the tubular cells into the tubular lumen, especially if renal function is impaired. When the GFR is low, the serum creatinine and creatinine clearance overestimate the true GFR. Some drugs (such as cimetidine or trimethoprim) have the effect of reducing tubular secretion of creatinine. This increases the serum creatinine and decreases the measured creatinine clearance paradoxically, when these drugs are used, a more accurate measurement of GFR is obtained as it is largely free from the error contributed by the physiological tubular secretion of creatinine.

As serum creatinine is so highly dependent on age, sex and body size, a number of corrections and formulae have been developed to estimate the muscle mass and assumed creatinine production. The most well-known formula is the Cockcroft-Gault formula, which is relatively simple to use and reasonably accurate. It is given as:

$$\text{Creatinine clearance (mL/min)} = \frac{(140 - \text{age (yrs)}) \times \text{weight (kg)}}{\text{serum creatinine (µmol/L)}}$$

Multiply result x 1.22 for male patients

This is a good estimate of GFR, but it becomes inaccurate when a patient's body mass is significantly outside the normal range (for example, morbid obesity or severe malnutrition) or when renal function is very impaired (i.e. GFR <20 mL/min). In these circumstances an isotopic method can be used if the GFR needs to be accurately measured.
Errors in measurement of renal function using creatinine

<table>
<thead>
<tr>
<th>Assay interference</th>
<th>Effects on creatinine clearance</th>
<th>Effects on serum creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketosis</td>
<td>Nil</td>
<td>↑</td>
</tr>
<tr>
<td>Hyperbilirubinaemia</td>
<td>Nil</td>
<td>↑</td>
</tr>
<tr>
<td>Cephalosporin</td>
<td>Nil</td>
<td>↑</td>
</tr>
</tbody>
</table>

**Inhibition of tubular secretion of creatinine**

cimetidine or trimethoprim            ↓*          ↑

**Alteration of creatine/creatinine load**

eating cooked meat                   ↑        ↑

low protein diet                      ↓         ↓

body building                         Nil       ↑

muscle wasting                        Nil       ↓

Renal disease                         ↓         ↑

* becomes more accurate at low levels of GFR when increased tubular secretion of creatinine is blocked

**Results and discussion:**

In the present study changes in serum creatinine levels were estimated in all the experimental groups such as Normal control (NC), *Xanthium indicum* treated (Xi), α-tocopherol treated (Tpt), Diabetic control (DC), Glibenclamide treated diabetic (Di + Glbt), α-tocopherol treated diabetic (Di + Tpt) and *Xanthium indicum* treated diabetic (Di + Xit) rats. In diabetic conditions, serum creatinine levels were increased due to glomerular hyperfiltration, renal hypertrophy, tubular function and then progress to proteinuria and reduction of glomerular filtration rate. Treatment with *Xanthium indicum* leaf hydromethanolic extract its bioactive compound α-tocopherol and Glibenclamide decreased the serum creatinine levels in diabetic group of rats and the effect was more prominent in α-tocopherol treated diabetic group of rats when compared to others (Table-13, Figures-24 and 25).
Increased oxidative stress in diabetes is postulated to promote the development of nephropathy (Kowluru et al., 2007). Diabetic nephropathy is a leading cause of end stage renal disease. It is characterized functionally by proteinuria and albuminuria and pathologically by glomerular hypertrophy, mesangial expansion and tubulointerstitial fibrosis; these findings are closely related to the loss of renal function (Eun et al., 2007). This damage occurs as a result of increased reactive oxygen species production. The diabetic hyperglycemia induces elevation of serum levels of creatinine which are considered as significant markers of renal dysfunction (Shind & Goyal, 2003). Serum creatinine is primarily a metabolite of creatine, almost all of which is located in skeletal muscle. The amount of creatine per unit of skeletal muscle mass is consistent and the breakdown rate of creatine is also consistent. Thus, serum creatinine concentration is very stable and a direct reflection of skeletal muscle mass (Martin, 2003). However, serum creatinine also depends on creatinine production, extrarenal elimination and tubular handling (Stevens & Levey, 2005). Moreover, tubular involvement may precede glomerular involvement because several tubular proteins and enzymes are detectable even before the appearance of microalbuminuria and a rise in serum creatinine (Uslu et al., 2005). Harita et al., (2009) hypothesized that; lower serum creatinine is associated with an increased risk of type 2 diabetes, which might reflect a lower volume of skeletal muscle. Skeletal muscle is a major target tissue of insulin and a lower volume of skeletal muscle would mean fewer target sites for insulin which causes increase in insulin resistance. This leads to the development of type 2 diabetes (Dabla, 2010). The abnormally high levels of serum creatinine were consistent with the impaired kidney function (Ronco et al. 2010). An increased creatinine concentration in diabetic rats is associated with greater protein catabolism. Ceriello et al., (2000) demonstrated a positive correlation between hyperglycaemia and the development of nephropathy. It has been demonstrated that the metabolic abnormalities observed in uncontrolled diabetes result in gluconeogenesis (Punithavathi et al., 2008) and consequently accumulation of creatinine in blood, which is substantially enhanced in diabetes. From the present work, it was revealed that serum creatinine levels were elevated drastically in diabetic rats (more than one fold) when compared to the normal controlled rats.
Extensive investigation has evaluated the ability of antioxidants like α-tocopherol to ameliorate complications of diabetes (Robert et al., 2010). The action of α-tocopherol was able to decreased renal oxidative stress, and kidney damage, and increased renal hemodynamics. In addition, α-tocopherol improves vascular function and structure, and prevents progression of diabetic complications. α-tocopherol treatment, from a young age, prevented increase in interlobar artery wall thickness and renal vascular resistance. Glomerular hypertrophy could be related to increase blood pressure transmitted to glomerular capillaries as demonstrated by Ofstad and Iversen (2005), and/or could be a consequence of increased postglomerular vascular resistance. Treatment with α-tocopherol also prevented glomerular hypertrophy (Pletiskaitz et al., 2012). Administration of α-tocopherol decreased urinary protein and improves renal damage (El-hadjela et al., 2013). α-tocopherol administration reduced the ischemia-reperfusion injury (Marubayashi et al., 1986). In the kidney, Demirbas et al., demonstrated that the addition of α-tocopherol to the commercial Euro-Collins solution improved renal function and decreased lipid peroxidation rate caused by oxygen free radicals in a canine kidney autotransplantation model (Demirbas et al., 1993). On the other hand, the injection of α-tocopherol before renal ischemia had a mild effect on the MDA content in rats (Kirpatovskii et al., 1993). Moreover, dietary enrichment of rats with α-tocopherol was effective in suppressing the renal epithelial lipid peroxidation in a rat model of bilateral renal ischemia-reperfusion (Salahudeen et al., 1996). The high dosages of α-tocopherol found to be effectively protective of the renal function from oxidative damage in rat models have been demonstrated in several studies (Naziroglu et al., 2004). Although α-tocopherol reduced the occurrence of glomerular membrane damage and prevented the increases in serum creatinine level (Almeida et al., 2012). Mc Ginley et al., (2009) reported that α-tocopherol reduced creatine kinase activity, enzyme that catalyzed the conversion of creatinine in phosphoceratine. Results of the present study imply that high α-tocopherol provide a beneficial effect against STZ induced renal outcomes. The present data reveals that creatinine levels in serum were increased to 69% in α-tocopherol treated rats, but <1% decrease in α-tocopherol treated diabetic rats than the normal controlled rats, where as α-tocopherol treated diabetic rats shown nearly 57% decreased levels when compared to the diabetic controlled rats.
In the present investigation the changes in STZ diabetic rats is associated with significant increase in the levels of serum creatinine, indicating impaired renal function of diabetic rats. The diabetic hyperglycemia induces elevation of the serum levels of creatinine and are considered as significant markers of renal dysfunction (Bhuvaneswari and Krishnakumari, 2012). Plant extract treatment significantly decreased the levels of creatinine in diabetic rats, which could be due to the prevention of protein and nucleic acid degradation. Similar reduction in the levels of blood urea and serum uric acid and creatinine were observed in the glibenclamide treated rats. Treatment of STZ-diabetic rats with hydromethanolic leaf extract of Xanthium indicum (200 mg/kg/day) for four weeks caused 30% down regulation of serum creatinine levels than in the diabetic rats. 68% and 62% elevated levels of serum creatinine in Xanthium indicum treated rats and Xanthium indicum treated diabetic rats respectively when compared to normal controlled rats.

d) Serum Cystatin C:

Cystatin C (CysC) has been described as a promising endogenous marker of GFR for both adults and children. It has a low molecular weight (13 kDa) as a chain of 120 amino acids in length and is a member of the superfamily of cysteine protease inhibitors. Cystatin C or cystatin 3 a protein encoded by the CST3 gene, is mainly used as biomarker of kidney function. The gene for Cys C is expressed in all nucleated cells, has the characteristics of a housekeeping gene and is thought to be produced and secreted at a constant rate. CysC is a nonglycosylated protein that is freely filtered at the glomerulus level and almost completely reabsorbed and catabolized, but not secreted, by the tubular epithelial cells. Since only small amounts are excreted into the urine, its urinary clearance cannot be measured. Therefore, the blood concentration of CysC depends almost entirely on the GFR and is not substantially affected by diet or nutritional status (Filler et al., 2005). The function of CysC seems to be to protect connective tissue from destruction by intracellular enzymes. It may also have an antibacterial or antiviral function (Randers et al., 1999). It is removed from the bloodstream by glomerular filtration in the kidneys. It is found in virtually all tissues and body fluids. It is a potent inhibitor of lysosomal proteinases (enzymes from a special subunit of the cell that break down proteins) and probably one of the most important extracellular inhibitors of cysteine proteases (it prevents the breakdown of proteins outside the cell by a specific type of protein degrading
enzymes). If kidney function and glomerular filtration rate decline, the blood levels of cystatin C rise. Serum levels of cystatin C are a more precise test of kidney function (as represented by the glomerular filtration rate, GFR) than serum creatinine levels (Dharnidharka et al., 2002). Cystatin C levels are less dependent on age, sex, race and muscle mass compared to creatinine. Cystatin C measurements alone have not been shown to be superior to formula-adjusted estimations of kidney function (Stevens et al., 2008). Cystatin C levels have been reported to be altered in patients with cancer (Nakai et al., 2008), (even subtle) thyroid dysfunction (Manetti et al., 2005) and glucocorticoid therapy (Risch et al., 2001) in some but not all situations (Bökenkamp et al., 2002). Other reports have found that levels are influenced by cigarette smoking and levels of C-reactive protein (Knight et al., 2004). Cystatin C levels seem to be increased in HIV infection, which might or might not reflect actual renal dysfunction (Odden et al., 2007).

Cystatin C has been proposed as a good marker of GFR, particularly in patients with mild to moderate renal impairment (Uzun et al., 2005). The production is not altered by inflammatory conditions, is not related to lean muscle mass. Because of its low molecular mass (approx. 13000 kpa) and its positive charge at physiological pH, cystatin C easily crosses the glomerular filter, after filtration, the proximal tubular cells reabsorb and catabolize virtually all of the filtered cystatin C. It was demonstrated that the renal clearance of cystatin C is closely related to GFR, measured as $^{51}$Cr-EDTA clearance. Cystatin C does not have a blind area will therefore show a positive reaction of GFR. One of most significant advantages of cystatin C in comparison with traditional markers of renal impairment is that very small reductions in GFR cause significant increase in cystatin C serum levels (Uzun et al., 2002). Cystatin C is a more sensitive indicator of mild renal impairment and may better estimate the GFR than serum creatinine (Sarnak et al., 2005). Moreover, concentrations of Cystatin C are not affected by sex, age, or muscle mass (Coll et al., 2000). There is supportive evidence that the reciprocal of Cystatin-C correlates more closely with isotopic GFR than the CG or MDRD equations in subjects with mild renal impairment (Dharnidharka et al., 2002).

Cystatin C determination does not have any interfering factors, except excessively elevated rheumatoid factor in vitro (Lamb and Stowe, 2003). In healthy children, Cys C concentration stabilizes from the second year of life and the reference
range is identical to that of adults. Higher Cys C concentrations reflect maturation of glomerular filtration in neonates and are related to decreased kidney function in the elderly (Filler et al., 2005). Cys C may identify the gradient of kidney function among persons who do not meet conventional definitions of clinical kidney disease. Therefore, the term preclinical kidney disease” was proposed to represent persons with estimated GFR >60 mL/min, but who have abnormal concentrations of CysC (≥1.0 mg/L) (Shlipak et al., 2006). Several cross-sectional studies have shown that CysC has greater sensitivity in detecting mildly reduced GFR than creatinine and other low molecular weight proteins (Roos et al., 2007). Serum CysC concentration increases already with mildly reduced GFR of 70 to 90 mL/min, i.e., in the “creatinine-blind range” (Hoek et al., 2003). In addition, recent longitudinal studies have reported that CysC concentrations increase earlier in acute kidney injury in the intensive care unit, in cardiac surgery, coronary angiography, after liver transplantation, cisplatin chemotherapy, following uninephrectomy, and in progressive diabetic nephropathy (Herget-Rosenthal et al., 2007). The performance of CysC as an estimator of GFR has been evaluated in large diverse populations such as geriatric patients (Burkhardt et al., 2002), pediatric patients with various renal diseases (Filler et al., 2005), renal transplantrecipients, type I and type II diabetics, patients with mild to moderate CKD of non-diabetic origin, patients with severe liver or neuromuscular disease, cancer patients, and women with preeclampsia (Herget-Rosenthal et al., 2007).

Results and discussion:

In the present study changes in Cystatin C levels in serum was estimated of all the experimental groups such as Normal control (NC), Xanthium indicum treated (Xi t), α-tocopherol treated (Tpt), Diabetic control (DC), Glibenclamaide treated diabetic (Di + Glbt), α-tocopherol treated diabetic (Di + Tpt) and Xanthium indicum treated diabetic (Di + Xi t) rats. The diabetic hyperglycemia induces elevation of the serum cystatin C levels which is significant marker of renal dysfunction and reflecting a decline in the glomerular filtration rate. Treatment with Xanthium indicum leaf hydromethanolic extract, its bioactive compound α-tocopherol and Glibenclamide decrease the serum cystatin C levels in diabetic group of rats and the effect was more prominent in α-tocopherol treated diabetic group of rats (Table-14, Figures-26 and 27).
Diabetic nephropathy (DN), one of the most serious micro vascular complications of diabetes, is a major cause of end stage renal disease (Yan et al., 2007). It occurs approximately in one third type 2 diabetic patients (Rehman et al., 2005) and is on rise. Kidney excretes the metabolic wastes, which includes urea, uric acid and creatinine and other ions. By the removal of these metabolic wastes it maintains the optimum balance in the body fluids. In the renal damage associated with diabetes, the increased levels of these metabolites were observed (Rajkiran et al., 2011). Due to the uncontrolled blood glucose levels, these metabolites may deposit in the vital organs such as kidneys, the toxic concentration of blood sugar damages the kidney tissue. This leads to altered kidney function causing Diabetic nephropathy. Diabetes causes renal damage due to abnormal glucose regulation, including elevated glucose and glycosylated protein tissue levels, haemodynamic changes within the kidney tissue and increased oxidative stress (Aurell & Bjorck, 1992). DM is also grossly reflected by profound changes in protein metabolism and by a negative nitrogen (N) balance and loss of nitrogen from most organs. Increased urea nitrogen production in diabetes may be accounted for by enhanced catabolism of both liver and plasma proteins (Singh et al., 2012). Oxygen free radicals (OFR) were shown to contribute to the cellular damage induced by ischemia-reperfusion, probably due to their lipidic oxidative characteristics, and several agents have been used to minimize the OFR action in renal ischemia-reperfusion (Rhoden et al., 2001). The development of diabetic nephropathy is characterized by a progressive increase in urine protein particularly albumin and a late decline in glomerular filtration rate leading eventually end stage of renal disease (Salah et al., 2004). The pathophysiology involves glucose that binds irreversibly to proteins in kidney and circulation to form advanced glycolation end products (AGEs). AGEs can form complex crosslinking over years of hyperglycemia and can contribute to renal damage by stimulation of growth and fibrotic factors via receptors for AGEs. Increased glomerular capillary pressure occurs early in diabetes and is associated with hyperfiltration at the glomerulus. The glomerular mesangium expands, initially by cell proliferation and then by cell hypertrophy. Increased mesangial stretch pressure can stimulate this expansion, as can high glucose levels. Mediators of proliferation and expansion include platelet-derived growth factor and transforming growth factor and transforming growth factor β (TGF-β). TGF-βs are particularly important in the mediation of expansion and later fibrosis via the stimulation of collagen and fibronectin. Angiotensin-II (AT-II) also
contributes to the progression of diabetic nephropathy. AT-II preferentially constricts the efferent arteriole in the glomerulus, leading to higher glomerular capillary pressure. In addition to its hemodynamic effects, AT-II also stimulates renal growth and fibrosis through AT-II type-1 receptors, which secondarily upregulate TGF-β and other growth factors (Soleimani et al., 2007).

The routine classical evaluation of diabetic nephropathy includes appearance of microalbuminuria, decreased creatinine clearance and increased serum creatinine (Hong and Chia, 1998). But, it has been reported that a decline in the renal function of patients with diabetes was not always accompanied by an increased Albumin - Creatinine ratio (ACR) (Lane et al., 1992 & Tsalamandris et al., 1994). A large percentage of individuals with type 2 diabetes pass through a period of pre-diabetes and may experience early renal dysfunction, e.g. a GFR > 60 mL/min per 1.73 m². Serum creatinine has been found to be deficient to detect mild renal impairment, even when used with prediction equations (Poggio et al., 2005 & Nielsen et al., 1999). Cystatin-C is a non-glycosylated basic protein, as a potential endogenous filtration marker of GFR. Cystatin-C is a cysteine protease inhibitor that is produced by virtually all nucleated cells and released into the bloodstream. It is entirely filtered by the kidney glomerulus and metabolized by the proximal tubule (Randers et al., 1998). Serum cystatin C might meet the need for detecting trends in renal function over time when GFR is normal or elevated. Various formulae have been used to measure serum cystatin C levels by different methodologies. Recent estimations were done using a particle-enhanced immune nephelometric assay or immune turbidimetric assays (Premaratne et al., 2008). In all formulae, Cys C is serum cystatin-C (in milligrams per liter). Serum cystatin C might meet the need for detecting trends in renal function over time when GFR is normal or elevated (Perkins et al., 2005). Cystatin C might be a better marker, not only in detecting of chronic kidney disease, but also in early prediction of diabetic kidney disease. Cystatin C, a cationic low-molecular weight protein (13 kD), is freely filtered across the glomerular membrane and is almost completely reabsorbed in proximal renal tubular cells (Tenstad et al., 1996). Unlike serum creatinine, for which the serum concentration is strongly affected by muscle mass, cystatin C is produced by all nucleated cells at a constant rate and its serum level is relatively unaffected by age, sex, body composition, diet, and exercise (Abrahamson et al., 1990 & Price and Finney, 2000). Based on these characteristics,
cystatin C has been extensively studied as a potential alternative for creatinine as a serum marker of kidney function. Many studies have been shown that serum cystatin C level is superior to serum creatinine as an estimate for GFR, and can be used to detect mild renal function impairment and rapid changes in GFR. Also, cystatin C level is superior to creatinine for the estimation of GFR under conditions in which there is an altered creatinine production (Bokenkamp et al., 1998). Like creatinine, the elimination of cystatin C via routes other than the kidney increase with worsening GFR (Sjöström et al., 2005). In some conditions like diabetes with ischemic nephropathy, nodular glomerulosclerosis and renal failure, serum cystatin C levels were significantly increases (Shimizu et al., 2003). From the present experiment, it was observed that nearly 7 fold serum cystatin C levels were increased in diabetic rats when compared to the normal controlled rats.

α-tocopherol showed a reduction in blood pressure together with reduced urinary thiobarbituric acid reactive substances (TBARS) excretion, suggesting that the reduction in blood pressure occurs as consequence of reduced oxidative stress. These results are in agreement with data from Newaz & Nawal (1998), suggests that α-tocopherol supplement showing reduction of lipid peroxides in plasma and blood vessels and enhancement of total antioxidants, in parallel to reduced blood pressure. Thus, it is possible that α-tocopherol, by improving the action of antioxidant enzymes (Lin et al., 2005; Lexis et al., 2006), reduces ROS and restores NO levels to within normal range, resulting in vasodilatation and reduced blood pressure. In the kidney, effects of ANG II and other vasoconstrictors are modulated by NO (Herman et al., 2006; Patzak & Persson, 2007). Increasing oxidative stress by reducing NO availability can exacerbate the renal vasculature response to vasoconstrictors. Hence, chronic use of α-tocopherol can enhance antioxidant enzymes, resulting in vasodilatation of afferent and/or efferent arterioles, decreased pre and/or post-glomerular resistances, and reduced changes observed in renal morphology of glomerular and vascular hypertrophy. It is known that glomerular hypertrophy is related to the development of glomerulosclerosis (Fogo, 2000) where the prevention of this alteration can contribute toward preventing the development of kidney disease. Increased renal vascular resistance is also related to the reduced levels of renal plasma flow (RPF) and Glomerular filtration rate (GFR). Treatment with α-tocopherol appears to improve renal hemodynamics since it significantly increased RPF and GFR.
levels (Pletiskaitz et al., 2012). α-tocopherol can also prevent the activation of DAG-PKC pathway induced high glucose levels (Kunisaki et al., 1995). Results from the present study suggest that α-tocopherol treatment could prevent the onset of glomerular hyperfiltration and increases the serum cystatin C levels induced by diabetes. The current experiment reveals that cystatin C levels in serum were increased to 2 fold in α-tocopherol treated rats and 3 fold increase in α-tocopherol treated diabetic rats than the normal controlled rats. Whereas in α-tocopherol treated diabetic rats exhibited nearly 49% decreased levels when compared to the diabetic controlled rats.

*Xanthium indicum* having a high level of diuretic efficacy (Snigdha et al., 2013). Shravani et al., (2010) studied the diuretic activity of *Xanthium indicum* in albino rats. The petroleum ether extract in normal saline showed significant increase in diuresis, natriuresis, kaliuresis, glomerular filtration rate. All extract causes increase in urine elimination and increase in Na⁺, K⁺, Cl⁻ excretion compared to normal saline. They reported that the diuretic activity of the extract may be due to the presence of Flavanoides, Saponins, and Organic acid. It is also possible that defect of water could be due to other secondary active metabolites; or it may be indirect change of some physiological parameters before filtration step. The present study demonstrated the protective effect of *Xanthium indicum* leaf extract on diabetic renal injury through reduction of serum cystatin C levels. Treatment of hydromethanolic leaf extract of *Xanthium indicum* (200 mg/kg/day) for four weeks causes an increase of 96% and 3 fold elevated levels of serum cystatin C in *Xanthium indicum* treated rats and *Xanthium indicum* treated diabetic rats respectively when compared to normal controlled rats but these levels were down regulated to 42% in *Xanthium indicum* treated diabetic rats when compared to STZ rats.
Table-10: Changes in MDA content in the Kidney tissue of Normal control (NC), *Xanthium indicum* (200mg/kg) (Xi*t), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+Glbt), α-tocopherol treated diabetic (Di+ Tpt) and *Xanthium indicum* (Di+Xi*t) treated diabetic male albino rats. The values are expressed in μ moles of malondialdehyde formed / gram wet weight of the tissue.

<table>
<thead>
<tr>
<th>MDA</th>
<th>NC</th>
<th>Xi*t</th>
<th>Tpt</th>
<th>DC</th>
<th>Di+Glbt</th>
<th>Di+ Tpt</th>
<th>Di+Xi*t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>45.416</td>
<td>47.244</td>
<td>46.270</td>
<td>97.312***</td>
<td>66.192**</td>
<td>55.179**</td>
<td>72.161**</td>
</tr>
<tr>
<td>±SD</td>
<td>(±1.635)</td>
<td>(±2.256)</td>
<td>(±4.493)</td>
<td>(±3.196)</td>
<td>(±3.666)</td>
<td>(±4.312)</td>
<td>(±4.12)</td>
</tr>
<tr>
<td>Percent change (%)</td>
<td>(1.828)</td>
<td>(0.854)</td>
<td>(51.896)</td>
<td>(20.776)</td>
<td>(9.763)</td>
<td>(26.745)</td>
<td></td>
</tr>
</tbody>
</table>

All the values are mean ± ±SD of six individual observations

Values in the parentheses denote per cent change over normal control

Groups that share **, *** represent significant difference between the sets of data (P<0.01 & P<0.001) when compare to the control.

**One way ANOVA**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Between Treatment</th>
<th>Within treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>df (a)</td>
<td>Sum of squares (x)</td>
<td>Mean Squares</td>
<td>df (b)</td>
</tr>
<tr>
<td>Kidney</td>
<td>6</td>
<td>13610.999</td>
<td>2268.500</td>
</tr>
</tbody>
</table>

Degrees of freedom (df): number of values in the final calculation of a statistic that are free to vary.

A big value of variance ratio (F) means to reject the null hypothesis (P).
**Figure-18:** Bar diagram shows changes in MDA content in the Kidney tissue of Normal control (NC), *Xanthium indicum* (200mg/kg) (Xit), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glbt), α-tocopherol treated diabetic (Di+ Tpt) and *Xanthium indicum* (Di+Xit) treated diabetic male albino rats. Groups having * * symbols (P<0.01) *** (P<0.001) are significant when compared to the control.

**Figure-19:** Bar diagram shows changes percent change in MDA in kidney experimental rats over the control.
Table-11: Table shows changes in Microalbumin levels in urine of Normal control (NC), Xanthium indicum (200mg/kg) (Xit), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glbt), α-tocopherol treated diabetic (Di+ Tpt) and Xanthium indicum (Di+Xit) treated diabetic male albino rats. The values are expressed micro albumin levels in mg/l.

<table>
<thead>
<tr>
<th>Urine Microalbumin levels</th>
<th>Experimental Groups (Treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NC</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>2.466 (±0.156)</td>
</tr>
<tr>
<td>Percent Change (%)</td>
<td>(+11.922)</td>
</tr>
</tbody>
</table>

All values are mean ±SD of six individual observations.

Values in parentheses denote per cent change over respective normal control.

Groups that share *** represent significant difference between the sets of data (P<0.001) when compare to the control.

**One way Annova**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Between Treatment</th>
<th>Within treatment</th>
<th>a+b</th>
<th>x+y</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Df (a)</td>
<td>Sum of squares (x)</td>
<td>Mean Squares</td>
<td>df (b)</td>
<td>Sum of squares (y)</td>
<td>Mean Squares</td>
</tr>
<tr>
<td>Kidney</td>
<td>6</td>
<td>1194.602</td>
<td>199.100</td>
<td>33</td>
<td>23.795</td>
<td>0.721</td>
</tr>
</tbody>
</table>

Degrees of freedom (df): number of values in the final calculation of a statistic that are free to vary.

A big value of variance ratio (F) means to reject the null hypothesis (P).
Figure-20: Bar diagram shows changes in Microalbumin levels in the urine of Normal control (NC), *Xanthium indicum* (200mg/kg) (Xit), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glbt), α-tocopherol treated diabetic (Di+ Tpt) and *Xanthium indicum* (Di+Xit) treated diabetic male albino rats. Groups having *** symbols are more significant (P<0.01) when compared to the control.

![Microalbumin Bar Diagram](image)

Figure-21: Graph shows percent change in Microalbumin levels in urine in experimental rats over the control.

![Microalbumin Graph](image)
Table-12: Changes in Creatinine levels in urine of Normal control (NC), Xanthium indicum (200mg/kg) (Xi t), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glb), α-tocopherol treated diabetic (Di+ Tpt) and Xanthium indicum (Di+Xi t) treated diabetic male albino rats. The values are expressed creatinine levels in mg/dl.

<table>
<thead>
<tr>
<th>Urine Creatinine levels</th>
<th>Experimental Groups (Treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NC</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>5.625 (±0.150)</td>
</tr>
<tr>
<td>Percent change (%)</td>
<td>(+8.248)</td>
</tr>
</tbody>
</table>

All values are mean ±SD of six individual observations.

Values in parentheses denote per cent change over respective normal control.

Groups that share *** represent significant difference between the sets of data (P<0.001) when compare to the control.

One way Anova

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Between Treatment</th>
<th>Within treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df (a)</td>
<td>Sum of squares (x)</td>
<td>Mean Squares</td>
</tr>
<tr>
<td>Kidney</td>
<td>6</td>
<td>17.966</td>
<td>2.994</td>
</tr>
</tbody>
</table>

Degrees of freedom (df): number of values in the final calculation of a statistic that are free to vary.

A big value of variance ratio (F) means to reject the null hypothesis (P).
**Figure-22:** Bar diagram shows the changes in **Creatinine levels in urine** of Normal control (NC), *Xanthium indicum* (200mg/kg) (Xit), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glbt), α-tocopherol treated diabetic (Di+ Tpt) and *Xanthium indicum* (Di+Xit) treated diabetic male albino rats. Groups having *** symbols are more significant (P<0.001) when compared to the control.

**Figure-23:** Bar diagram shows percent change in **creatinine levels in urine** in experimental rats over the control.
**Table-13:** Changes in **Creatinine levels in serum** of Normal control (NC), *Xanthium indicum* (200mg/kg) (Xit), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glbt), α-tocopherol treated diabetic (Di+ Tpt) and *Xanthium indicum* (Di+Xit) treated diabetic male albino rats. The values are expressed creatinine levels in mg/dl

<table>
<thead>
<tr>
<th>Serum Creatinine levels</th>
<th>NC</th>
<th>Xit</th>
<th>Tpt</th>
<th>DC</th>
<th>Di+Glbt</th>
<th>Di+Tpt</th>
<th>Di+Xit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SD</td>
<td>0.637 (±0.204)</td>
<td>1.067** (±0.103)</td>
<td>1.077** (±0.659)</td>
<td>1.468*** (±0.163)</td>
<td>1.103*** (±0.077)</td>
<td>0.633 (±0.218)</td>
<td>1.032** (±0.128)</td>
</tr>
<tr>
<td>Percent Change (%)</td>
<td>(+67.503)</td>
<td>(+69.073)</td>
<td>(+130.455)</td>
<td>(+73.155)</td>
<td>(-0.627)</td>
<td>(+62.009)</td>
<td></td>
</tr>
</tbody>
</table>

All values are mean ±SD of six individual observations.

Values in parentheses denote per cent change over respective normal control.

Groups that share **, *** represent significant difference between the sets of data (P<0.05 & P<0.001) when compare to the control.

### One way ANOVA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Between Treatment</th>
<th>Within treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df (a)</td>
<td>Sum of squares (x)</td>
<td>Mean Squares</td>
</tr>
<tr>
<td>Kidney</td>
<td>6</td>
<td>3.047</td>
<td>0.508</td>
</tr>
</tbody>
</table>

Degrees of freedom (df): number of values in the final calculation of a statistic that are free to vary.

A big value of variance ratio (F) means to reject the null hypothesis (P).
**Figure-24:** Bar diagram shows changes in **Creatinine levels in serum** of Normal control (NC), *Xanthium indicum* (200mg/kg) (Xit), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glbt), α-tocopherol treated diabetic (Di+ Tpt) and *Xanthium indicum* (Di+Xit) treated diabetic male albino rats. Groups having *** symbols are more significant (P<0.01) when compared to the control.

**Figure-25:** Bar diagram shows percent change in **Creatinine levels in serum** in experimental rats over the control.
Table-14: Changes in Cystatin C levels in serum of Normal control (NC), Xanthium indicum (200mg/kg) (Xit), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glbt), α-tocopherol treated diabetic (Di+ Tpt) and Xanthium indicum (Di+Xit) treated diabetic male albino rats. The values are expressed in mg of Cystatin C /l.

<table>
<thead>
<tr>
<th>Serum Cystatin C levels</th>
<th>Experimental Groups (Treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NC</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>0.072 (±0.031)</td>
</tr>
<tr>
<td>Percent change (%)</td>
<td>(+95.833)</td>
</tr>
</tbody>
</table>

All values are mean ±SD of six individual observations.

Values in parentheses denote per cent change over respective normal control.

Groups that share *** represent significant difference between the sets of data (P<0.001) when compare to the control.

One way Annova

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Between Treatment</th>
<th>Within treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df (a) Sum of squares (x) Mean Squares</td>
<td>df (b) Sum of squares (y) Mean Squares</td>
<td>a+b</td>
</tr>
<tr>
<td>Kidney</td>
<td>6</td>
<td>0.804</td>
<td>0.134</td>
</tr>
</tbody>
</table>

Degrees of freedom (df): number of values in the final calculation of a statistic that are free to vary.

A big value of variance ratio (F) means to reject the null hypothesis (P).
**Figure-26:** Bar diagram shows changes in **Cystatin C** levels in serum of Normal control (NC), *Xanthium indicum* (200mg/kg) (Xit), α-tocopherol (100mg/kg) (Tpt), Diabetes control (DC) Glibenclamaide (20mg/kg) (Di+ Glbt), α-tocopherol treated diabetic (Di+ Tpt) and *Xanthium indicum* (Di+Xit) treated diabetic male albino rats. Groups having *** symbols are more significant (P<0.01) when compared to the control.

![Cystatin C Bar Diagram](image)

**Figure-27:** Bar diagram shows percent change in **Cystatin C** levels in serum in experimental rats over the control.

![Cystatin C Percent Change Bar Diagram](image)