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Beneficial Role of Ellagic Acid on Cyclosporine A Induced Nephrotoxicity and Oxidative Stress in Rats

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ABSTRACT: *The present study was investigate to evaluate the possible beneficial effect of ellagic acid on Cyclosporine A induced nephrotoxicity using biochemical and histopathological approaches. Adult male albino rats of Wistar strains divided into six groups. Four groups received CsA by oral garvage (25mg\kg\body weight) for 21 days to induce nephrotoxicity, three of which simultaneously received ellagic acid treatment (12.5mg\kg, 25mg \kg, 50mg\kg\body weight) for 21 days. A vehicle (0.2% DMSO) and ellagic acid on cyclosporine A induced nephrotoxicity were evaluated by plasma creatinine, urea and creatinine clearance; kidney tissue TBARS, lipid hydroperoxides, enzymic antioxidants (Superoxide dismutase, catalase and glutathione –S-transferase), non enzymic antioxidants (reduced glutathione, vitaminC and vitaminE) and histopathological examinations. Administration of cyclosporine A to rats induced a marked renal failure, characterized by significant increases in plasma creatinine and urea with significant decrease in creatinine clearance. Cyclosporine A also induced oxidative stress, as shown by increased kidney tissue concentration of TBARS, lipid hydroperoxides and reduced activities of enzymic and non enzymic antioxidants. Histopathological observations were also agreement with above abnormal changes. Ellagic acid reverted these abnormal biochemical changes, and ameliorated cyclosporine a induced pathological changes. These results indicate that the antioxidant ellagic acid might have a protective role against cyclosporine A induced nephrotoxicity and oxidative stress in rats.*

I. INTRODUCTION

Cyclosporine A is the drug most frequently used for the management of organ transplantation and has been increasingly applied with significant clinical benefits in the treatment of autoimmune diseases [1]. However, recipients must maintain therapy for the rest of their lives. Unfortunately, the extensive use of this drug is cautionable due to its adverse effect including, nephrotoxicity in 40–90% of patients [2]. Cyclosporine A causes unique structural and functional nephrotoxicity, which leads to loss of proximal tubular epithelial integrity, tubular atrophy, vacuolization and micro calcification [3]. Lysosomal enzymes are also found to play a major role in the pathogenesis of kidney damage induced by cyclosporine A [4]. It has been speculated that different mechanisms are responsible for CsA-induced nephrotoxicity, with oxidant stress, according to recent studies, playing a central role as pathogentic factor [5-6]. Previous studies have shown that the toxicity of cyclosporine A may be mediated by an increase in free radicals, which affect the endothelium and ion transport mechanisms. These highly reactive species may attack the soluble cellular components as well as the membranes eventually leading to the impairment of cellular functioning and cytolysis [7]. Furthermore, antioxidants have shown to be protective in CsA dependent renal toxicity, indicating functional relevance and a causative role for oxygen radicals in this drug related disorder [8]. Flavanoids, which are polyphenolic antioxidants, occur naturally in vegetables and fruits. They are widely recognized as a naturally occurring antioxidant that can inhibit lipid oxidation in biological membrane. Ellagic acid 2,3,7,8 tetrahydroxy - benzopyrano [5,4,3, -cde] benzopyran –5-10-dione] is a naturally occurring phenolic constituent in certain fruits and nuts, such as raspberries, strawberries, walnuts, longan seed, mango kernel, pomegranate, etc [9]. Ellagic acid has a wide variety of biological activities including potent antioxidant [10], anti inflammatory [11], anti carcinogenic and antifibrosis in bacterial and mammalian systems [12]. It is believed that ellagic acid either by countering the negative effects of oxidative stress by directly acting as an antioxidant or by activating or inducing cellular antioxidant enzyme systems.

In the light of above information, the present study was carried out to investigate the protective influence of ellagic acid on cyclosporine A induced nephrotoxicity and oxidative stress in rats.

II. MATERIALS AND METHODS

Animals. Adult male albino Wistar rats (8 weeks), weighing 200 - 220 g bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, were used in this study. Throughout the study, the animals were housed a six animals per each polypropylene cage and were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian council of Medical Research, Hyderabad, India, and approved by the Institutional ethical committee (Vide No: 419, 2007) Annamalai university. The animals were fed on a pellet diet (Lipton India Ltd., mumbai, India) and drank ad libitum.

Chemicals. Ellagic acid was a supplied by Sigma chemicals company, USA. Cyclosporine A was purchased from Panacea Biotech Ltd, New Delhi. All other chemicals and biochemicals were of analytical grade obtained from local firms. The organic solvents were distilled before use.

Experimental design. The animals were randomly divided into six groups of six rats in each group. Group I. Control rats orally treated with Dimethyl sulphoxide (0.2%). Group II. Rats orally received ellagic acid (50 mg /kg body weight) dissolved in dimethyl sulphoxide (0.2%) for 21 days using intragastric intubation. Group III. Rats orally received cyclosporine A (25 mg/kg body weight) dissolved in olive oil for 21 days[13]. Group IV. Rats orally received cyclosporine A (25 mg/kg body weight) followed by oral administration of ellagic acid (12.5 mg/kg body weight) in dimethyl sulphoxide (0.2%) for 21 days. Group V. Rats orally received cyclosporine A (25 mg/kg body weight) followed by oral administration of ellagic acid (25 mg/kg body weight) in dimethyl sulphoxide (0.2%) for 21 days. Group VI. Rats orally received cyclosporine A (25 mg/kg body weight) followed by oral administration of ellagic acid (50 mg/kg body weight) in dimethyl sulphoxide (0.2%) for 21 days.

At the end of experimental period, animals in different groups were sacrificed by cervical decapitation. Blood was collected in two different tubes i.e. one is heparinised for plasma and another without heparin for serum separation by centrifugation and used for various biochemical estimations.

Biochemical determination. The levels of urea and creatinine were estimated spectophotometrically according to the standard procedures using commercially available diagnostic kits (Sigma Diagnostics (I) Pvt Ltd, Baroda, India). Creatinine clearance was calculated from the values of urinary and serum creatinine, time (last 24 h), and body weight.

In kidney homogenate, lipid peroxidation was assayed by estimating the levels of thiobarbituric acid reactive substances in tissues were estimated by the method of [14]. In this method, malondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid (TBA) in acidic condition to generate a pink coloured chromophore, which was read at 535 nm and tissue lipid hydroperoxides was estimated by the method of [15]. In this method, oxidation of ferrous ions (Fe^{2+}) under acidic conditions in the presence of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560 nm.

The following analysis was also carried out in kidney homogenate: Reduced glutathione was estimated by the method of [16]. This method was based on the development of yellow colour when dithionitrobis benzoic acid (DTNB) was added to the compounds containing sulphydryl groups; Ascorbic acid was estimated by the method of [17]. The ascorbic acid was oxidised by copper to form dehydroascorbicacid and diketoglutaricacid. These products when treated with 2,4 dinitrophenylhydrazine (DNPH) form the derivative, bis-2, 4-dinitrophenyl hydrazone which undergoes rearrangement to form a product with an absorption maxima at 520nm. α-Tocopherol was estimated by the method [18] , in which the reduction of ferric ion to ferrous ion by α-tocopherol and the formation of a red coloured complex with 2,2′-dipyridyl. Absorbance of the chromophore was measured at 520nm.

Superoxide dismutase (SOD) activity was determined by the method of [19]. Superoxide radicals react with nitroblue tetrazolium in the presence of NADH and produce formazan blue. SOD removes the superoxide radicals and inhibits the formation of formazan blue. The intensity of colour is inversely proportional to the activity of the enzyme. The activity of Catalase was determined by the method of [20]. Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H_2O_2. The chromic acetate formed was measured at 620nm. Glutathione –S-Transferase (GST) was determined spectrophotometrically by the method of Habig et al in which 1-chloro-2, 4-dinitrobenzene was used as substrate [21].

Histopathological investigation. The kidney samples fixed for 48h in 10% formal-saline were dehydrated by passing successfully in different mixture of ethyl alcohol – water, cleaned in xylene and embedded in paraffin. Sections of liver (4-5 mm thick) were prepared and then stained with hematoxylin and eosin dye, which mounted in neutral DPX medium for microscopic observations.

Statistical analysis. All the data were expressed as mean ± SD of number of experiments (n=6). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 9.05 (SPSS, Cary, NC, and USA) and Duncans' Multiple Range Test (DMRT) obtained the individual comparisons. Values were considered statistically significant when $P< 0.05$.

III. RESULTS

The changes in the level of serum creatinine, urea, and creatinine clearance in control and experimental animals are shown in (table I). A significant increase in the levels of serum urea and creatinine and decrease in the levels of creatinine clearance in cyclosporine A treated rats. Treatment with ellagic acid at 50 mg/kg significantly decreased the levels of serum urea and creatinine and significantly restored the creatinine clearance in cyclosporine A treated rats when compared to other doses such as (12.5 and 25 mg/kg). Based on these finding 50 mg/kg of ellagic acid was fixed as a dose for further biochemical studies.

The levels of TBARS and lipid hydroperoxides were significantly increased in rats treated with Cyclosporine A when compared with control rats (table2). Table 2 also depicts the levels of nonenzymatic antioxidants namely vitamin C, vitamin E and GSH that were significantly decreased in cyclosporine A treated rats. Administration of ellagic acid to cyclosporine A treated rats significantly decreased the levels of lipid peroxidation with significantly increased levels of nonenzymatic antioxidants. The changes in the activities of enzymatic antioxidants such as SOD, CAT, and GST in renal tissue of control and experimental rats are illustrated in (table 3) A significant decrease in the activities of enzymatic antioxidants was observed in cyclosporine A treated rats. Treatment with ellagic acid significantly increased the activities of enzymatic antioxidants in cyclosporine A treated rats. The kidney of control (fig. 1) and ellagic acid alone (fig. 2) treated rats showed a normal structure of kidney. Cyclosporine A exposure caused changes in kidney architecture characterized by tubular damage and inflammatory cells (fig 3). Cyclosporine A along with ellagic acid (fig. 4) significantly reduced the tubular damage.

IV. DISCUSSION

Nephrotoxicity is a major problem of drug therapy, frequently leading to acute renal failure and nephritic syndrome [22]. Kidney dysfunction can result in decreased drug elimination and consequent toxicity. In the reduced state of cardiac output, aging, and disease condition glomerular filtration rate and tubular secretion has been reduced, resulting in higher serum drug concentration [23]. Oxidative stress and the generation of oxygen free radicals are increasingly considered as important factor involved in CyclosporineA nephrotoxicity. In the present study the levels of serum urea and creatinine were significantly increased, and in the levels of creatinine clearance and hemoglobin were significantly decreased in Cyclosporine A treated rats. Treatment with ellagic acid significantly decreased the levels of serum urea and creatinine and significantly restored the levels of creatinine clearance and hemoglobin in Cyclosporine A treated rats.

Cyclosporine A induced nephrotoxicity is characterized by renal dysfunction and renal morphological change with interstitial fibrosis [24]. It is well documented that Cyclosporine A induced nephrotoxicity has been characterized by 20-30% reduction in glomerular filtration rate and upto 40% reduction in renal blood flow resulting in elevated serum creatinine levels and decreased creatinine clearance [25]. Alterations in renal haemodynamics, glomerular or tubular structures, including inflammation have all been reported to play an important role in Cyclosporine A induced renal dysfunction [26]. Urea is transported into blood from kidney where it is filtered out. It has been observed in oxidative stress and other factors could induce increase protein catabolism, to be responsible for the significant increase in serum urea level in Cyclosporine A toxicity [4]. Creatinine clearance is the most widely accepted methods for non-invasive estimation of glomerular filtration rate in clinical practice to diagnose patients with Cyclosporine A [27], and elevations in serum creatinine observed in the present study are often the initial reason of renal complication [28]. Administration of ellagic acid significantly decreased the levels of serum urea and creatinine with normalization of creatinine clearance indicating that ellagic acid restore the renal functions from Cyclosporine A induced damage. In this context, the previous report showed that ellagic acid has protective effect against cisplatin induced oxidative stress in rats [29].

Lipid peroxidation induced oxidative alteration of polyunsaturated fatty acid is a mechanism generally recognized as most important in the pathogenesis of tissue injury by a number of toxic compounds [30]. Cyclosporine A inhibit mitochondrial β-oxidation and disrupts the respiratory chain that can produce more super oxide anion, which plays a crucial role in production of more reactive oxygen and nitrogen species such as peroxy nitrite and hydroxy radical. Such free radicals attack the cell membrane and leads to destabilization and disintegration of cell membrane as a result of lipid peroxidation [31]. The elevated levels of lipid peroxidation indices such as TBARS and hydroperoxides in kidney of Cyclosporine A treated rats is clearly indicate the

excessive formation of free radicals and activation of lipid peroxidation system resulting in tissue damage [32]. The induction of lipid peroxidation due to Cyclosporine A toxicity via active intermediate, and increased the concentration of lipid peroxide in tissues, which resulting in the breakdown of lipid constituents of cell membrane and eventually leading to alteration of membrane integrity and function [33]. The increase in plasma lipid peroxide has been found to have some deleterious effects on intact tissues and organs [34] and therefore their increase could be the reflection of membrane damage.

In our study, administration of ellagic acid significantly decreased the lipid peroxidative indices and DNA damage, and improved the antioxidant status. Phenolic compounds can act as free radical scavengers by virtue of their hydrogen donating ability, forming phenoxy radical that can rearrange to form quinone methide radical intermediate, which is excreted via bile. Existing reports have shown that polyphenols exhibit antioxidative effect both in vitro and in vivo [35]. Ellagic acid, being a polyphenol, can scavenge the superoxide and hydroxy anion [36] and can terminate the propagation of lipid peroxidation. Thus treatment with ellagic acid might have significantly decreased the levels of TBARS, HP showing its antioxidant property [11]. They have also shown that ellagic acid attenuates the tissue damage by decreasing lipid peroxidation. In our study, administration of ellagic acid significantly decreased the lipid peroxidative indices and DNA damage, and improved the antioxidant status.

GSH being the most important biomolecule against chemically induced toxicity can participate in the elimination of reactive intermediate by reduction of hydroperoxide in the presence of GSH dependent enzymes [37]. The levels of GSH also found to have some positive relation with vitamins C and E. Vitamins C and E plays a crucial role in scavenging excessively generated reactive oxygen species. Under pathological conditions, lowered levels of these antioxidants were reported in human and experimental rats [38]. In our study shows that these non-enzymatic antioxidant levels significantly lowered in plasma and kidney tissues. It may be due to their utilization by the damaged tissues to scavenge excessively generated reactive oxygen species.

It is well documented that ellagic acid acts as a free radical scavenger and chain breaking antioxidant [10]. In this regard ellagic acid acts as a powerful antioxidant, which minimizes the consumption of endogenous antioxidants and improve the levels of these non-enzymic antioxidants in kidney tissues of Cyclosporine A treated rats. Previous reports also showed that ellagic acid increased concentration glutathione in cisplatin-induced nephrotoxicity [29].

SOD is a metalloprotein and is the first enzyme involved in the antioxidant enzyme by lowering the steady state level of O_2^- [39]. CAT is a heme protein, which catalyses the decomposition of hydrogen peroxide to water and oxygen and thus protecting the cell from oxidative damage by hydrogen peroxide and OH^- [40]. The GST is a group of isoenzyme is capable of detoxifying various endogenous and exogenous substances by conjugating GSH [41] reported that toxic manifestations of Cyclosporine A is known to undergo biotransformation by hepatic glutathione S-transferase and form aldehyde derivatives, which inturn may generate free radical species due to oxidation by xanthine and aldehyde oxidases. The ability of aldehydes to free radicals to generate free radicals capable of inactivating enzyme proteins is well recognized. Hence, Cyclosporine A or its active metabolites might alter the native form of antioxidant enzyme and impair their function, which indicate the decreased activity of these enzymes in Cyclosporine A treated rats.

In our study, decline in the activities of these enzymes in cyclosporine A administered rats revealed that lipid peroxidation and oxidative stress is elicited by cyclosporine A intoxication. Administration of ellagic acid attenuated the lipid peroxidation and reduced the accumulation of radicals generated by cyclosporine A, which might lead to increased activities of SOD, CAT, and GST in cyclosporine A treated rats. In this context, ellagic acid [29] and resveratrol of grape extract[41] also improved the superoxide dismutase and catalase activity in cisplatin and cyclosporine A induced nephrotoxicity respectively.

The present study suggest that the tubular damage and inflammatory cells in cyclosporine Atreated rats reflected by accumulation of free radical as well as increased oxidative stress as a basis for cellular damage. Previous reports suggest that after exposure to cyclosporine A kidney showed several pathological changes including tubular epithelial damage[13]. Administration of ellagic acid reduced the histological alteration caused by cyclosporine A, which may be due to antioxidant property of ellagic acid and therefore be important in protecting cell against toxicity. Recent reports showed that ellagic acid reduced the tubular damage in cisplatin induced renal damage [29].On the basis of above results, we can infer that ellagic acid possesses an antioxidant activity, which may be responsible for the protection against cyclosporine A induced oxidative stress and nephrotoxicity.

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Table 1 Changes in the levels of kidney functional markers in control and experimental rats

Groups	Control	Control +EA (50 mg/kg)	Normal + CsA (25 mg/kg)	CsA (25 mg/kg) +EA (12.5 mg/ g)	CsA (25 mg/kg) +EA (25 mg/ kg)	CsA (25 mg/kg) +EA (50 mg/ kg)
Urea (mg/dl)	33.41 ± 1.48[a]	34.66 ± 2.05[ad]	57.08 ±3.51[b]	54.00 ± 3.71[b]	46.58± 3.12[c]	38.83 ± 2.77[d]
Creatinine (mg/dl)	0.44 ± 0.04[a]	0.45 ± 0.04[a]	0.80 ± 0.06[b]	0.76 ± 0.05[b]	0.67± 0.05[c]	0.52 ±0.02[d]
Creatinineclearance (mg/min)	0.41 ± 0.06[2a]	0.40 ± 0.03[a]	0.22± 0.02[b]	0.28 ± 0.03[b]	0.33 ± 0.03[c]	0.31 ±0.02[d]

CsA –Cyclosporine A; EA –Ellagic acid. Values are given as mean ± S.D from 6 rats in each group.
Values not sharing common superscripts letters (a-d) differ significantly at p<0.05 (DMRT).

Table 2. Changes in the levels of lipid peroxidation and nonenzymic antioxidant status in kidney of control and experimental rats

Parameters	Normal	Normal + EA (50mg/kg)	Normal + CsA (25mg/kg)	CsA + EA (50mg/kg)
TBARS (mM / 100g tissue)	1.66 ± 0.10[a]	1.68 ± 0.12[a]	2.77 ± 0.16[b]	1.90 ± 0.18[c]
Hydroperoxides (mM /100g tissue)	60.64 ± 3.63[a]	58.44 ± 4.78[a]	92.07 ± 7.65[b]	68.74± 7.94[c]
Vitamin C (mole / mg tissue)	0.95 ± 0.08[a]	1.04 ± 0.09[a]	0.60 ± 0.05[b]	0.83 ± 0.07[c]
Vitamin E (mole / mg tissue)	0.48 ± 0.05[a]	0.50 ± 0.05[a]	0.28± 0.02[b]	0.46 ± 0.03[c]
GSH (mg/100g tissue)	2.84 ± 0.39[a]	2.97± 0.26[a]	1.52 ± 0.19[b]	2.521 ± 0.33[ac]

CsA-Cyclosporine A; EA- Ellagic acid. Values are mean ± SD for 6 rats in each group
Values not sharing a common superscript letter differ significantly at p < 0.05 (DMRT)

Table 3. Changes in the activities of kidney SOD, CAT and GPx in normal and experimental rats

Groups	SOD (units[#]/ mg protein)	CAT (units[#]/ mg protein)	GST (units[#]/ mg protein)
Normal	11.43 ± 0.92[a]	47.24 ± 3.88[a]	5.60 ± 0.30[a]
Normal + EA (50mg/kg)	12.47 ± 1.06[a]	50.10 ± 2.85[a]	5.73 ± 0.48[a]
Normal + CsA (25 mg/kg)	6.53 ± 0.50[b]	30.43 ± 1.62[b]	4.20 ± 0.34[b]
CsA + EA (50mg/kg)	9.83 ± 1.81[c]	42.91 ± 2.28[c]	5.40 ± 0.44[c]

CsA-Cyclosporine A, EA- Ellagic acid. Values are mean ± SD for 6 rats in each group
Values not sharing a common superscript letter differ significantly at p < 0.05 (DMRT)

[#] Units of enzyme activities are expresses as:
SOD - One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute.
CAT - μ moles of hydrogen peroxide consumed / minute.
GST- μg of 1-chloro2,4-dinitrobenzene-GST conjugate formed/min/mg protein.

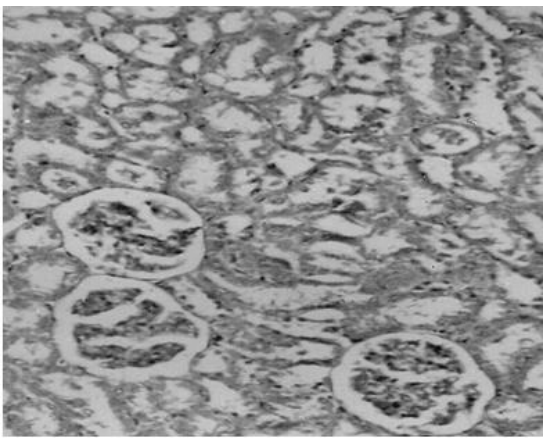


Fig. 1.

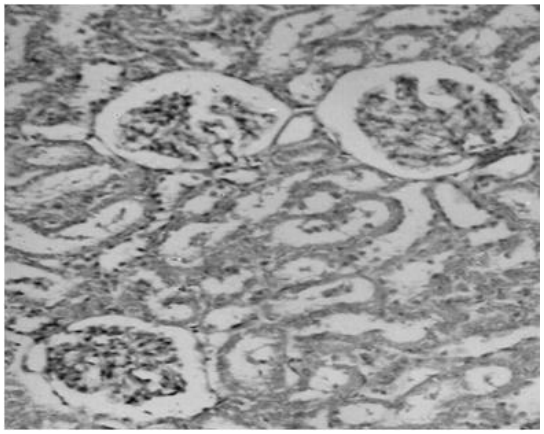
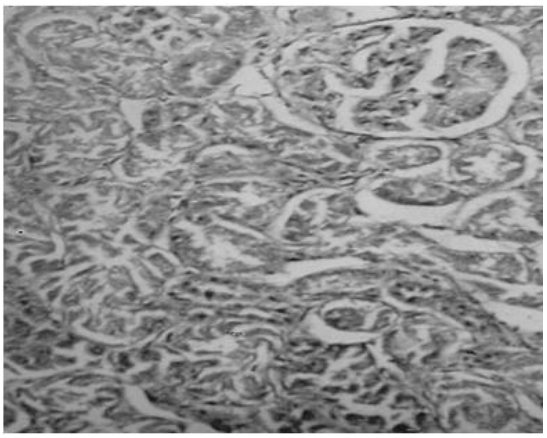


Fig. 2.



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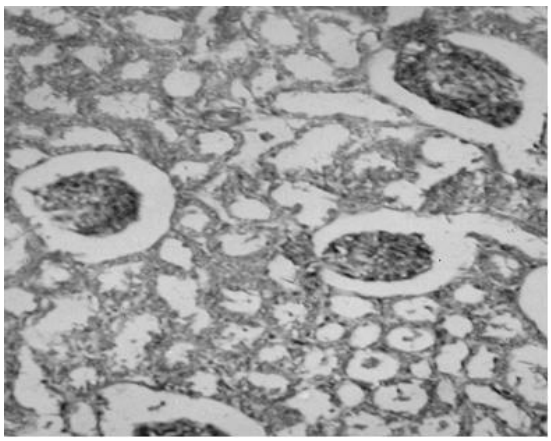

Fig. 4.

LEGENDS

Fig. 1: Control rat kidney H&E x 20. Normal architecture of kidney.
Fig. 2: Normal + Ellagic acid (50mg)-treated rat kidney H&E x 20. Normal glomeruli with tubules.
Fig. 3: Normal + Cyclosporine A (25mg) treated rats kidney H&E x 20 Tubular damage and inflammatory cells.
Fig. 4: Cyclosporine A + Ellagic acid treated rat kidney H&E x 20. Near normal.