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NARINGIN AMELIORATES RENAL IMPAIRMENT ON CYCLOSPORINE (A)-INDUCED NEPHROTOXICITY IN RATS: INVOLVEMENT OF iNOS

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ABSTRACT

The therapeutic effecicacy of a flavonoid Naringin (NG) against Cyclosporine A (CsA) induced nephrotoxicity was investigated in this study. Nephrotoxicity was induced in male albino wistar rats by injecting 25mg/kg body weight of CsA for a period of 21 days. CsA induced rats were also cotreated with 40mg of Naringin (NG/kg body weight) orally. After the experimental period the serum levels of urea, uric acid and creatinine were found to be elevated, followed by the decrease in creatinine clearance, whereas the levels of ALP, ACP were increased in serum of CsA induced rats. A significant decrease in LDH levels were observed in CsA induced animals compared to control. Immunohistochemical staining of inducible nitric oxide synthase (iNOS) expression was studied and NG co treatment significantly decreased the expression of iNOS in CsA challenged rats. This data was further evidenced by western blotting expression pattern of iNOS in experimental rats.

KEYWORDS:Renoprotection, CyclosporineA (CsA), Naringin (NG), lactate dehydrogenase, inducible nitric oxide synthase (iNOS)

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INTRODUCTION

Cyclosporine-A(CsA), a cyclic decapeptide obtained from extracts of soil fungus *Tolypocladium inflatum gams*, is the most effective and widely used first-line immunosuppressant in solid organ transplantation and autoimmune diseases[1]. However, initial enthusiasm has been tempered by its propensity to induce nephrotoxicity, in particular a chronic and progressive form culminating in renal failure[2]. The cause for the nephrotoxicity of CsA has not been fully elucidated. Nitric oxide is generated by the enzyme NOS, which exists in 3 isoforms (endothelial, inducible and neural), all of which can be expressed within the kidney[3]. However, during various types of inflammation, increased expression of the inducible form of nitric oxide synthase (iNOS) can dramatically increase the amount of nitric oxide present in tissues. The exact role of NO in the pathogenesis of renal injury appears to depend on the isoform of NOS involved in the production of the NO[4]. Substantial evidence has accumulated to suggest that increased iNOS derived NO production plays an important major role in the pathogenesis of renal injury [5]. Numerous investigations have demonstrated that inhibition of the expression or activity of iNOS, or the absence of iNOS itself, ameliorated or prevented renal injury, suggesting that NO generated by iNOS contributed to renal injury. Furthermore, it has been reported that renal tubules obtained from iNOS knockout mice are not susceptible to hypoxic injury[6]. Naringin has extensive pharmacological activity and may reduce CsA induced nephrotoxicity. In the present study we studied the efficacy of NG on renal marker enzymes and expression of iNOS during the experimental period.

MATERIALS AND METHODS

(i)Animals

Male albino rats of Wistar strain (180±20g) were obtained from the Laboratory Animal Maintenance Unit, Saveetha University, Vellapanchavadi, Chennai, India. The animals were acclimatized to the laboratory conditions for a period of 2 weeks. They were maintained at an ambient temperature of 25±2° C and 12/12 h of light–dark cycle and were given a standard rat feed (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. The experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines.

(ii) Experimental Design

The rats were divided into four groups (n=6 in each group) of same age. Group 1 served as control animals and received olive oil (Vehicle). Group 2 -Rats were administered with Cs (A) (Sandoz Ltd., Switzerland) (25 mg/ kg body weight) dissolved in olive oil orally for a period of 21 days. Group 3 Rats were treated with a single dose with 40 mg/kg body weight of NG (Sigma Aldrich Co., Ltd. St Louis, USA) (based on the effective dosage fixation studies) orally until the end of the experiment, after CsA administration as mentioned in group 2. Group 4 Rats received the same dose of Naringin (40 mg/kg body weight) alone as mentioned in group 3 for a period of 21 days. After the experimental period, the animals were sacrificed and blood was collected and the kidney tissue was cut and placed immediately in phosphate buffered formal saline (pH 7.4) until further use. The serum was collected from the blood samples and used for the biochemical investigations.

(iii) Biochemical Studies

Urea was determined by the method of Geyer and Dabich[7] in the blood. Blood creatinine was estimated by the method of Broad and Sirota[8], using Jaffe's color reaction. Uric acid was estimated according to the method of Caraway[9]. Acid phosphatase was assayed by the method of King (1965a) [10] in serum. Alkaline phosphatase was assayed by the method of King (1965b) [11] in serum. Lactate dehydrogenase was assayed according to the method described by King (1965c) [12]. Immunohistochemistry was performed to identify the protein expression pattern of iNOS, in sections on microscopic slides[13].

Immunoblotting was performed by the method of Towbin[14] to detect the expression pattern of iNOS.

(iv) Statistical analysis

All the data were analyzed using SPSS/10 Student Software. Hypothesis testing methods included one-way analysis of variance (ANOVA). The values are expressed as mean ± S.D., P value of less than 0.05 and 0.001 was considered to indicate statistical significance.

RESULTS

The levels of urea, uric acid and creatinine (Table 1) were assessed in the serum of experimental animals. A significant (P<0.05) increase in their levels were observed in CsA treated animals. Concomitant treatment with NG significantly (P<0.05) altered the levels up to a certain extent compared to control. In this study, CsA induction caused significant decrease in creatinine clearance (Figure 1) and it was prevented by NG co-administration. This may be likely due to the ability of NG to increase the GFR and thereby increasing the creatinine clearance. In the present investigation (Table. 2), the administration of CsA resulted in typical functional disturbances, as evidenced by the marked elevations (P<0.05) of renal marker enzymes ALP, ACP, AST, and ALT in serum. Treatment with NG lowered the levels of these marker enzymes highlighting the nephroprotective role of Naringin. This data is much more evidenced by the fact that there was marked decline (P<0.05) in the activities of these marker enzymes in renal tissues (Figure 2). A significant decreased (p< 0.05) level of LDH was observed (Figure 3) in the renal tissue of CsA administered animals which may be due to the damage of nephrons and the ability of NG to maintain the cell membrane integrity may be the reason for the increased (p< 0.05) activity of LDH in NG treated group of animals. Figure 4, shows the immunohistochemical staining of iNOS in the kidneys of control and CsA induced experimental rats. CsA induced rats (Figure 4, B) shows an increased expression of iNOS in kidney tissues compared with control rats (Figure 4, A). NG treatment was able to reduce the expression of iNOS induced by CsA (Figure 4, C). NG alone treated group of rats showed negligible expression compared to that of control (Figure 4, D). The results were quantified and represented in (Figure 5). Figure 6, shows the Western blot analysis of iNOS in control and experimental group of animals. In CsA-induced (Group 2) rats, increased (Lane 4), expression of iNOS was observed. Treatment with NG (Group 3) showed significant reduction (Lane 3) in the expression of iNOS comparable with control (Lane 1) and Naringin alone treated groups (Lane 2). Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity arbitrary unit (Figure (6a)).

Table1
Effects of CsA and NG on Serum Constituents

Parameters	Control	CsA	CsA+NG	NG
Urea (mg/dL)	17.60±2.39	28.15±5.01*	23.30±3.0**	18.47±2.42
Creatinine (mg/dL)	1.79±0.26	3.15±0.37*	2.25±0.22**	1.75±0.30
Uricacid (mg/dL)	2.27±0.24	5.64±0.38*	4.29±0.63**	2.96±0.38

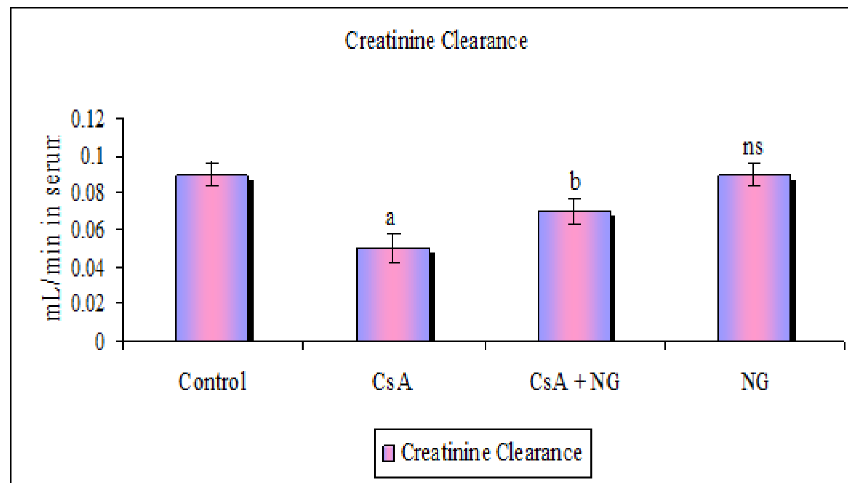
Values are expressed as mean ± SD; n=6
One way ANOVA followed by post hoc test LSD.
Values were statistically significant at P<0.05
Comparisons were made between *Control Vs CsA, **CsA Vs CsA+NG

Table 2
Levels of CsA and NG on the activities of marker enzymes in the serum

Groups	ALP	ACP	AST	ALT
Control	1.87±0.18	1.28±0.26	2.44±0.26	1.28±0.22
CsA	4.32±0.69[*]	3.01±0.44[*]	5.03±0.39[*]	3.08±0.31[*]
CsA + NG	2.65±0.32[**]	2.36±0.31[**]	3.52±0.32[**]	1.17±0.17[**]
NG	1.39±0.33[ns]	1.13±0.29[ns]	2.29±0.15[ns]	1.12±0.14[ns]

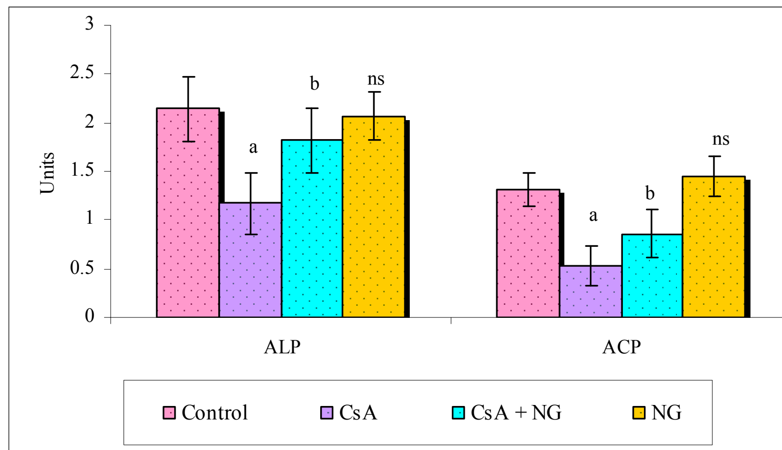
Values are expressed as mean ± S.D for six animals.
Units: ALP, ACP - µ moles of phenol liberated/min/mg protein
AST, ALT - µ moles of pyruvate liberated/min/mg protein
Values were statistically significant at P<0.05
Comparisons were made between [*]Control Vs CsA, [**]CsA Vs CsA+NG, [ns]control VS NG

Figure1
Effects of NG and CsA on Creatinine Clearance



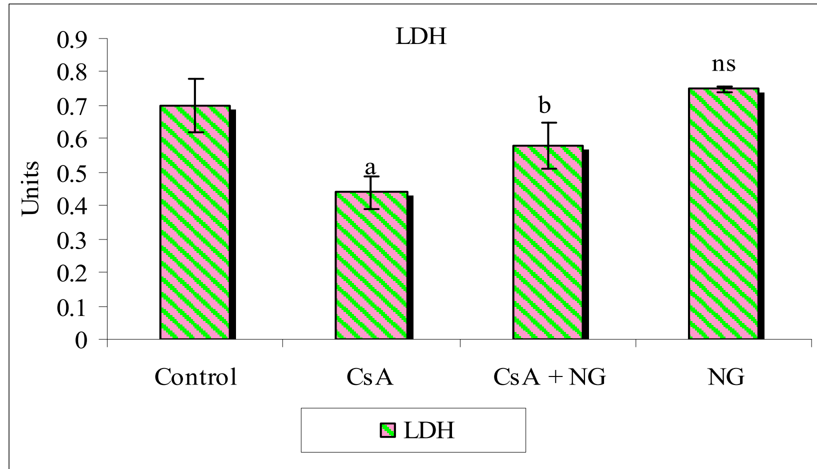
Values are expressed as mean ± SD; n=6
One way ANOVA followed by post hoc test LSD.
Values were statistically significant at P<0.05
Comparisons were made between [a]Control Vs CsA, [b]CsA Vs CsA+NG

Figure 2
The activities of ALP and ACP in renal tissues of CsA and NG treated rats.



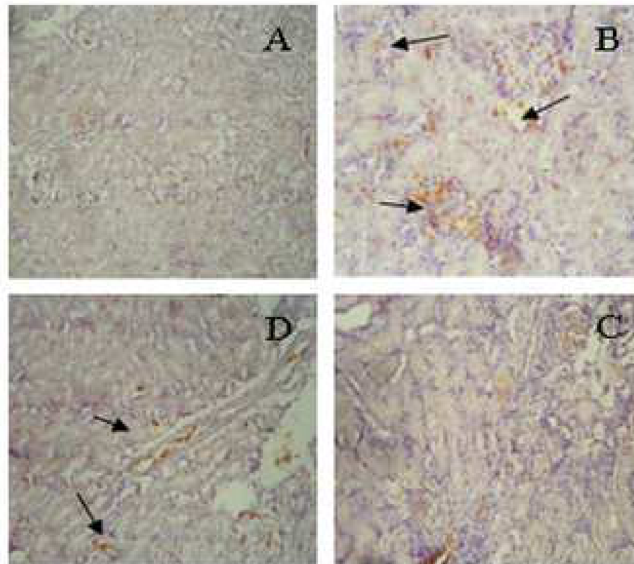
Values are expressed as mean ± S.D. for six animals in each Group.
Units: µ moles of phenol liberated/min/mg protein.
Comparisons are made between [a]Control Vs CsA, [b] CsA Vs CsA+ NG, [ns]control Vs NG
Values were statistically significant at P<0.05

Figure3
LDH activity in renal tissues of CsA and NG treated animals



Values are expressed as mean ± S.D. for six animals in each Group.
Units: µ moles of pyruvate liberated/min/mg protein.
Comparisons are made between [a]Control Vs CsA, [b] CsA Vs CsA+ NG,
Values were statistically significant at P<0.05

Figure 4
Expression of iNOS in Experimental Animals



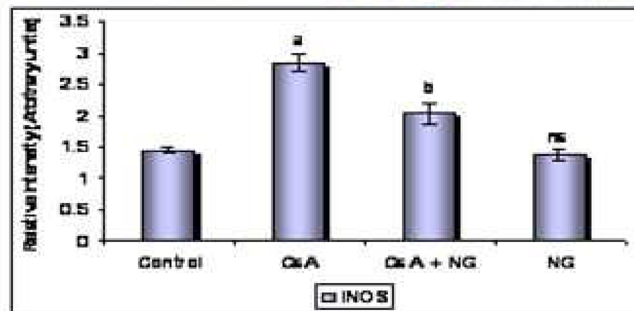
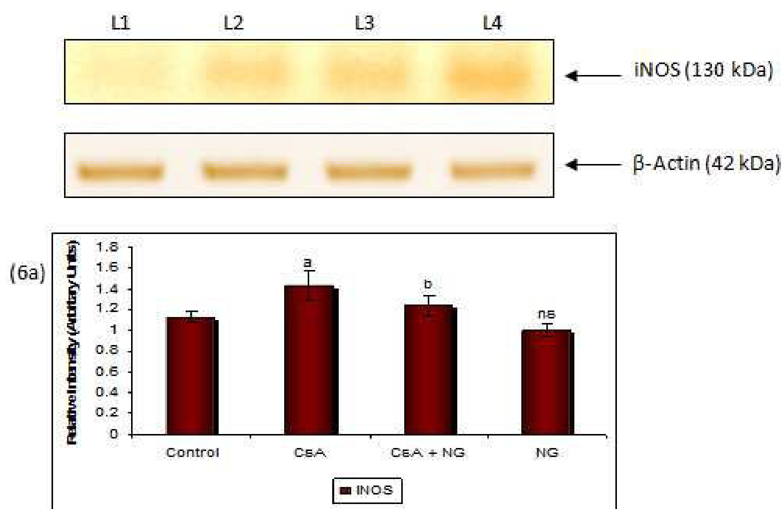


Figure5

Figure 4(A) (Control) Normal expression of iNOS, 4 (B): (CsAinduced) high expression of iNOS (brown color) was observed, 4 (C): (CsA +NG) lesser degree of expression of iNOS was noticed, FigureD: (NG) Expression was similar to that of control. → Figure (5) shows the expression of iNOS. Values are expressed as mean ± S.D. Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA+NG, ns-non significant p< 0.01 (Magnification 40x; Scale bar 50µM).

Figure 6
Western blot analysis of iNOS in control and experimental groups of animals.



(6a)
Lane 1, Lane 2, Lane 3 and Lane 4 correspond to control, NG, CsA+NG and, CsA induced group respectively. Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity arbitrary unit (6a). Values are expressed as mean ± S.D. Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA+NG, ns-non significant, p< 0.01.

DISCUSSION

The alteration in the levels of urea, uric acid and creatinine in experimental animals marks the deterioration of renal function[15]. CsA induced nephrotoxicity is characterized by reduction in glomerular filtration rate and reduction in renal blood flow resulting in elevated serum creatinine levels and decreased creatinine clearance.[16] Serum 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 toxicity[17]. There may be a likely ability of NG to increase the GFR and thereby increasing the creatinine clearance. The administration of CsA resulted in typical functional disturbances, as evidenced by the marked elevations (P<0.05) of renal marker enzymes ALP, ACP, AST, and ALT in serum. Treatment with NG lowered the levels of these marker enzymes highlighting the nephroprotective role of Naringin. This data is much more evidenced by the fact that there was marked decline (P<0.05) in the activities of these marker enzymes in renal tissues. ALP is membrane bound, and its alteration is likely to affect the membrane permeability and produce derangement in transport of metabolites. ACP activity was also found to be declined during cyclosporine administration indicating cellular damage. A decreased activity of tissue ALP and ACP indicates the loss of membrane integrity which might result in the release of these enzymes into the circulation.[18] .This is in agreement with previous findings which showed an increased activity of ALP and ACP in peripheral blood of renal transplant patients.[19]

Lactate dehydrogenase was considered useful for broad detection of damaged nephrons, because of its broad distribution along the nephrons [20]. Decreased levels of cytosolic LDH have been proved to be the most sensitive enzyme in a variety of experimental nephropathies[21]. A significant decreased (p< 0.05) level of LDH was observed in the renal tissue of CsA administered animals which may be due to the damage of nephrons and the ability of NG to maintain the cell membrane integrity may be the reason for the increased (p< 0.05) activity of LDH in NG treated group of animals. Therefore, combinatorial measurement of enzymatic biomarkers may be a powerful tool

for highly effective screening of nephrotoxicity. There is strong evidence from both *in vivo* and *in vitro* studies that the abnormal NO formation and release of proinflammatory mediators play important role in the pathophysiology of renal injury. Numerous investigations have demonstrated that inhibition of the expression or activity of iNOS, or the absence of iNOS itself, ameliorated or prevented renal injury, suggesting that NO generated by iNOS contributed to renal injury. CsA induces an increase in the expression of different factors such as nuclear factor kappa B (NF-kB) that are able to positively regulate the iNOS genes. These genes can produce an increase in iNOS and a high amount of nitric oxide that can combine with superoxide anions and induce lipid peroxidation and oxidative stress[22]. Therefore, the relationship among, iNOS, and nephrotoxicity could be explained by suggesting that their upregulation is linked to renal oxidative stress and fibrosis that impair renal cytoarchitecture. As evidenced by immunohistochemical analysis and western blotting study, Naringin supplementation strongly inhibited the expression of iNOS in this study. Previous findings suggest that NG was found to have blocked the transcriptional activity of NF-kappa B in macrophages[23]. NF-kB activation is upstream of the synthesis of inflammatory mediators. Among the genes positively regulated by NF-kB, there is iNOS and NG could have envisaged iNOS expression by this pathway in the present study.

CONCLUSION

The present study confers that NG co-supplementation significantly decreased the levels of urea, uric acid and creatinine and enhanced the creatinine clearance in the kidneys and restored the levels of renal marker enzymes and lactate dehydrogenase in CsA challenged rats. Further expression of iNOS was greatly reduced during NG treatment which may be the major reason for renoprotection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Padi, S., Chopra, K., (2002). Selective angiotensin II type 1 receptor blockade ameliorates cyclosporine nephrotoxicity. *Pharmacological Research*.45:413-420.
2. Woodward, RS., Kutinova, A., Schnitzler, MA., Brennan, DC., (2005). Renal graft survival and calcineurin inhibitor. *Transplantation*.80:629-633.
3. Kone, BC., (1999). Localization and regulation of nitric oxide synthase isoforms in the kidney. *Semin Nephrol*.19:230–241.
4. Lieberthal, W., (1998). Biology of ischemic and toxic renal tubular cell injury: role of nitric oxide and the inflammatory response. *Curr Opin Nephrol Hypertens*.7:289–295.
5. Weight, SC., Furness, PN., Nicholson, ML., (1998). Nitric oxide generation is increased in experimental renal warm ischaemia-reperfusion injury. *Br J Surg*.85:1663–1668.
6. Ling, H., Edelstein, C., Gengaro, P., *et al,*. (1999). Attenuation of renal ischemia-reperfusion injury in inducible nitric oxide synthase knockout mice. *Am J Physiol*.277: F383–F390.
7. Geyer, JW and Dabich, G (1971). Rapid method for determination of arginase activity in tissue homogenates. *Anal Biochem*.39, 412.
8. Broad, J and Sirota, JH (1948). Renal clearance of endogenous creatinine in man. *J.Clin. Invest*.27: 645.
9. Caraway, WI., (1963). Uric acid. In: Seligson D (ed). *Standard Methods of clinical chemistry*, Vol. 4, Academic Press, New York, 239–247.
10. King, J., (1965a). The hydrolases-acid and alkaline phosphatase. In: *Practical*

clinical enzymology, Van D (ed). Nostrand Company Ltd, London, 191-208.

11. King, J., (1965b). The transferases-alanine and aspartate transaminases. In: *Practical clinical enzymology*, Van D (ed). Nostrand Company Ltd, London, 121-138.
12. King, J., (1965c), The dehydrogenase of oxido-reductase-lactate dehydrogenase. In: Van, D. (Ed.), *Practical Clinical Enzymology*. Nostrand, London, pp. 83-93.
13. Ashokkumar, P., Sudhandiran, G (2011). Luteolin inhibits cell proliferation during azoxymethane-induced experimental colon carcinogenesis via Wnt/beta-catenin pathway. *Investigation New Drugs,* April 2011, 29, 2, 273-284
14. Towbin, H., Stahelin, H., Gordon, J., (1979). Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci*. USA 76: 4350-4354.
15. Nicola Origlia., Massimiliano Migliori., Vincenzo Panichi., Cristina Filippi., Aldo Bertelli., Angelo Carpi., Luca Giovannini., (2006). Protective effect of L-propionylcarnitine in chronic cyclosporine-A induced nephrotoxicity. *Biomedicine and pharmacotheraphy*.60, 77-81.
16. Weir, M.R., Klasen, D.K., Shen, S.Y., (1989). Acute effects of cyclosporine on renal function in healthy humans. *Transplant Proc.*21, 915-917.
17. Paul, L.C., de Fijter, J.H., (2004). Cyclosporine induced renal dysfunction. *Transplant. Proc.*36,224S-228S
18. Amudha, A., Josephine, A., Mythili, Y., Sundarapandiyan, R., Varalakshmi, P., (2007). Therapeutic efficacy of DL-α-lipoic acid on cyclosporine A induced renal alterations. *European journal of Pharmacology*.571:209-214.
19. Rezzani, R., Rodella, L., Bianchi, R., (1999). Early metabolic changes in peripheral blood cells of renal transplant recipients treated with cyclosporine A. *Int.J.Immunopharmacol*.21 (7), 455-462.
20. Yutaka Tonomura., Noriko Tsuchiya., Mikinori Torii and Takeki Uehara., (2010). Evaluation of the usefulness of urinary biomarkers for nephrotoxicity in rats. *Toxicology*. April 30 [Epub ahead of print].
21. Maruhn, M., Paar, D., Bomhard, E., (1983). Diagnostic sensitivity of urinary enzymes in experimental kidney damage in the rat. In: Galteau, M.M.,Siest, G., Henny, J.(Eds.), Biologic perspective: Comptes Rendus Da 5eCollogue' International Do Pont –A-Mousson. Masson, Paris, pp.943-945.
22. Buffoli, B., Pecha´nova,´ O., Kojsˇova,´ S., Andriantsitohaina, R., Giugno, L., Bianchi, R., Rezzani, R., (2005). Provinol prevents CsA-induced nephrotoxicity by reducing reactive oxygen species, iNOS, and NF-kB expression. *J Histochem Cytochem*.53:1459–1468.
23. Kanno, S., Shouji, A., Tomizawa, A., Hiura, T., Osanai, Y., Ujibe, M., Obara, Y., Nakahata, N., Ishikawa, M., (2006). Inhibitory effect of naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages. *Life Sci.*78(7):673-81.

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