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Research Article

AMELIORATIVE EFFECT OF MORIN HYDRATE, A FLAVONOID AGAINST GENTAMICIN INDUCED OXIDATIVE STRESS AND NEPHROTOXICITY IN SPRAGUE-DAWLEY RATS

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ABSTRACT

Objective: Morin Hydrate was recently shown to provide a protection of cultured rat glomerular cells in vitro against oxyradical damage, and as a anticancer agent through inhibition of NF-ǩB pathway. Mainly, we have demonstrated that free radical mechanism mediates, in part in Gentamicin induced nephrotoxicity. The main purpose of the study is to determine the protective effect of Morin Hydrate, mediated through inhibition of free radical mechanism against Gentamicin induced nephrotoxicity in-vivo.

Methods: Fourty two Sprague-Dawley rats were divided into 2 major categories i.e Normal control and Gentamicin treated rats. Normal control rats were again divided into two groups i.e., Vehicle and Morin Hydrate treated (100 mg/kg, p.o, 4th - 18th day). Gentamicin treated animals were divided into 5 groups i.e., prophylactic (treatment was started from 1st day of the study), Gentamicin control, Morin Hydrate treated at 50, 100 and 200mg/kg, p.o. Renal function parameters like serum creatinine (Scr), blood urea nitrogen (BUN) were measured. The levels of antioxidant parameters, and total protein values were determined. Histopathology changes were evaluated.

Results: Morin Hydrate significantly attenuates the effects of Gentamicin induced weight loss and increase in the kidney/body weight ratio in all treated groups. However, it also causes the reduction of Scr, BUN levels and reactive oxygen species (ROS) levels etc in all Morin Hydrate treated groups significantly ($p< 0.05$ and $p< 0.01$). In-case of antioxidant enzyme levels there is a dose dependent increase in its levels in all Morin Hydrate treated groups. In Pre-treated *groups* there is a significant reduction of Scr, BUN levels ($p<0.01$) and there is no significant reduction of ROS, tissue nitrite levels, however there is a significant ($p< 0.05$) increase in levels of antioxidant enzymes like reduced glutathione (GSH), super oxide dismutase (SOD), catalase (CAT), and total protein levels.

Conclusion: Therefore, our present data suggests that Morin Hydrate attenuates the Gentamicin induced nephrotoxicity by intervening the oxidative stress.

Keywords: Aminoglycosides, Morin Hydrate, Nephrotoxicity, Oxidative stress

INTRODUCTION

Gentamicin is a one of the member of aminoglycoside antibiotics obtained from the actinomycetes i.e *micromonospora* species. Gentamicin is used in the treatment of various Gram-positive and Gram-negative infections[1]. However, its usefulness is limited owing to the Nephrotoxicity and Ototoxicity. It has been estimated that up to 30% of patients treated with Gentamicin for more than 7 days show some signs of renal impairment like tubular necrosis[2]. But, the treatment with Gentamicin causes tubular necrosis[3] and apoptosis[4]. Reactive oxygen species (ROS) are one of the plausible reasons for Gentamicin induced nephrotoxicity, also causes, endoplasmic reticulum (ER) stress and unfolded protein response, decreases the cell energy status impairment, and calcium sensing receptor (CasR) stimulation[5]. Up to now various antioxidant agents and Pharmacological agents were evaluated in the amelioration of Gentamicin induced nephrotoxicity[6,7].

Morin Hydrate (3,5,7,2',4'-pentahydroxyflavone) (Fig. 1), is a natural yellow crystalline polyphenolic compound coming from branches of *Morus alba* L (white mulberry) and red Wine , in the family of Moraceae [white mulberry (morus alba)] and in in almond (Prunus dulcis, family Rosaceae), in sweet chestnut (Castanea sativa, family Fagaceae) having antioxidant[8] and Hypouricemic activity[9]. It also protects the rat mesangial glomerular cells against the oxyradical damage[10]. In the present study, an attempt was made to study the effect of Morin Hydrate in Gentamicin induced nephrotoxicity.

MATERIALS and METHODS

Animals

Study scheduled for 18 days, Fourty two Sprague-Dawley rats were taken, randomly divided into 7 groups, each group containing six animals. Rats weighing between 170-210g were obtained from National Institute of Pharmaceutical Education and Research (NIPER), Guwahati, India. The animals were housed in a bio-safe, temperature-controlled environment with a 12-h light/dark cycle with controlled temperature (24±3 °C and relative humidity 55±15%) during the experimental period and they were allowed free access to food and water at all times. The protocol of this study was approved by the Institutional Animal Ethical Committee (IAEC), NIPER, Guwahati, India (Approval. No-MC/32/2012/21) and experimental procedures were conducted in accordance with the CPCSEA guidelines on the safe use and care of experimental animals. Morin Hydrate required for the experimentation was procured from Sigma-Aldrich, Mumbai.

Experimental design

The animals were randomly divided into seven groups and each group containing six rats. Gentamicin was injected subcutaneously at the dose of 100mg/kg for 5 days (4th day to 8th day) to induce nephrotoxicityin rats[11]. Animals were divided into two categories i.e., Normal control and Gentamicin treated rats. Normal control rats were again divided into two groups i.e., Vehicle and Morin Hydrate treated (100 mg/kg, p.o, 4th - 18th day). Gentamicin treated animals were divided into 5 groups i.e., prophylactic (treatment was started from 1st day of the study), Gentamicin control, Morin Hydrate treated at 50, 100 and 200mg/kg, p.o. Treatment was started with Morin Hydrate from 4th day of the study and continued up-to 18th day. At the end of study, animals were sacrificed by Co_2 asphyxiation and kidneys were collected for biochemical analysis and blood was collected from heart puncture.

Biochemical analysis

Estimation of renal total ROS by Maiti *et al* [12], and renal nitrite level by the method of Gonzalez-Barrios *et al* [13]by using Griess Reagent System (Promega technical bulletin, USA).

Tissue Preparations

Kidney homogenate was prepared and estimation of total protein content levels by Lowry e*t al*[14], lipid peroxidation by the method

of Ohkawa *et al*[15], renal antioxidant parameters like reduced glutathione (GSH) by Ellman *et al* [16], Catalase activity (CAT) by Aebiet *et al* [17]and superoxide dismutase (SOD) activity (cytosolic and mitochondrial) was carried out using SOD assay kit (Sigma-Aldrich Co, St Louis, MO, USA) according to manufacturer's instructions.

Histopathological Examination

For histopathological examination of the renal cortical tissues, kidneys were preserved in 10% neutral formalin solution. Kidney tissues were embedded in Paraffin wax and 5µm sections were prepared by using a rotary microtome and stained with haematoxylin and eosin (H&E).

Statistical analysis

All results were expressed as mean ± SEM or as percent activity compared to control rats. The intergroup variation between various groups was measured by one way analysis of variance (ANOVA) using the Graph Pad Prism, version 5.0 and the comparisons between two groups were conducted by unpaired Student's t-test. Results were considered statistically significant when p <0.05.

RESULTS

Effects on body weight and kidney/body-weight ratio

Effect of Morin Hydrate on Body Weight

At the end of the study, a significant weight loss (p<0.01) was observed as a result of Gentamicin administration as compared to the normal control group. Neither Morin Hydrate pre treatment for 3 days before Gentamicin administration nor lowest dose of given therapeutically significantly attenuated the Gentamicin-induced changes in body weight, while treatment (100mg/kg and 200mg/kg body weight) for 15 days significantly attenuated Gentamicin-induced changes in body weight [Table 1]. There was no significant change in body weight between control animals and those received Morin Hydrate alone.

Effect of Morin Hydrate on kidney/body-weight ratio

At the end of the study, a significant increase in kidney/body-weight ratio (p<0.01) was observed as a result of Gentamicin administration when compared to the normal control group. Both Pre-treatment for 3 days of Gentamicin administration and treatment (100mg/kg and 200mg/kg body weight) for 15 days significantly (p<0.01) reduced the Gentamicin-induced changes kidney/body-weight ratio [Table 1]. There was no significant change in kidney/body-weight ratios between control animals and those received Morin Hydrate alone and between pre treated and therapeutic treated 50 mg/kg groups.

Effect of Morin Hydrate on renal dysfunction

Effect of Morin Hydrate on Blood Urea Nitrogen (BUN) level

There was significant difference in serum BUN levels between normal control and Morin Hydrate control (p<0.01) group. Gentamicin administration increased the BUN level in all Gentamicin treated groups compared to the normal control. In all and Gentamicin treated groups, except animals those received 50mg/kg therapeutically, (BUN reduction, compared to Gentamicin control was not significant) BUN levels were found to be reduced significantly (p<0.05) compared to Gentamicin treated group. But, between these treated groups there was no significant difference (Table 1).

Effect of Morin Hydrate on Serum Creatinine (Scr) levels

There was significant difference in Scr levels between normal control and control (p<0.001) group. Gentamicin administration increased the creatinine levels in all Gentamicin treated groups compared to the normal control. In all Morin Hydrate treated and Gentamicin treated groups, except animals those received 50mg/kg therapeutically, (Scr reduction, compared to Gentamicin control was not significant) Scr levels were found to be reduced significantly (p<0.05) compared to Gentamicin treated group. But, between these treated groups there was no significant difference (Table 1).

Effect of Morin Hydrate on total ROS level

Gentamicin significantly (p<0.05) increased the total ROS levels in kidney tissues of *Gentamicin* control group, indicating enhanced oxidative stress compared to normal control group. Therapeutic treatment with Morin Hydrate at all doses (50mg/kg, 100mg/kg and 200mg/kg) for 15 days significantly (p<0.05) attenuated this rise in ROS in kidney tissues of Gentamicin treated rats compared to the Gentamicin control group. Morin Hydrate alone had no effects on the ROS generation and in the pre-treatment case there is no significant reduction of this rise of ROS in the kidney samples (Table 2).

Effect of Morin Hydrate on tissue nitrite level

Renal tissue nitrite levels, which indicate the nitric oxide (NO) levels were significantly (p<0.05) reduced in the kidneys of the Gentamicin group compared to the normal control group. Both pre and therapeutic treatments have opposed this Gentamicin induced NO reduction. But only in therapeutic doses (100mg/kg & 200mg/kg) there was a significant elevation of NO, compared to the Gentamicin control group. However, Morin Hydrate alone treated rats showed a moderately elevated tissue nitrite levels compared to normal control group (Table 2).

Effect of Morin Hydrate on Lipid Peroxidation (MDA) Levels

Kidney tissue MDA levels were increased significantly (p<0.05) by Gentamicin administration as compared to the normal control group. Both pre and therapeutic treatments with Morin Hydrate at all doses significantly attenuated this effect compared to the Gentamicin control group. But there is no significant difference between pre and therapeutic groups at various doses. However, Morin Hydrate alone did not alter MDA level in Morin Hydrate control group (Table 2).

Effect of Morin Hydrate on antioxidant profile

Effect of Morin Hydrate on Glutathione level

A significant reduction (p<0.05) of reduced glutathione was observed in Gentamicin control group compared to the normal control. And in both pre and therapeutic treated groups with Morin Hydrate there was a significant elevation of GSH compared to the Gentamicin control. In therapeutic groups (50, 100, 200 mg/kg) observed there is dose dependent increase in the GSH levels (Table 2).

Effect of Morin Hydrate on Catalase (CAT) activity

A significant (p<0.01) decrease in renal Catalase (CAT) activities was observed in Gentamicin control group compared to the normal control. Both pre and therapeutic Morin Hydrate treatment with Gentamicin administration had significantly higher catalase levels compared to only Gentamicin group. Means a significant increase in the catalase levels were observed in Morin Hydrate & Gentamicin treated groups at all doses and in therapeutic treated animals there was a dose dependent increase from 50mg/kg to 200mg/kg (Table 3).

Effect of Morin Hydrate on superoxide dismutase (SOD) activity

Kidney tissue Superoxide dismutase (SOD) levels decreased significantly (p<0.01) by Gentamicin administration compared to the normal control group. Morin Hydrate pre-treatment for 3 days of Gentamicin administration and treatment of Morin Hydrate (50, 100mg/kg and 200mg/kg body weight) for 15 days produced a significant increase in SOD levels compared to Gentamicin control. Here dose dependence increase was observed in therapeutic groups. Morin Hydrate per se did not alter SOD levels in Morin Hydrate control group (Table 3).

Effect of Morin Hydrate on Total protein content

A significant (p<0.05) decrease in renal total protein content was observed in Gentamicin control group compared to the normal control group. Both in pre and therapeutic groups there was an increase in protein level compared to Gentamicin control group. However significant elevation was there in therapeutic groups (100, 200mg/kg) and there was no change in protein content of normal control and Morin Hydrate control (Table 3).

Histopathological analysis

No death was observed among all groups during the period of this study. Kidney samples were obtained and observed under microscope. As shown in Fig. 2

Gentamicin causes the massive renal congestion, leucocyte infiltrations in proximal tubules and in medullary region, desquamation, degeneration of epithelial cells and hyaline casts in control groups.

Table 1: Effect of Morin Hydrate on body weight, kidney to body weight ratio, BUN and Serum creatinine (Cr) levels (n=6)

	NC	MH	GEN	Pre MH+GEN	GEN+MH 50	GEN+MH 100	GEN+MH 200
% change in body weight (g)	(+)4.6 ±5.6	(+)0.19±2.65	(-)22± 2.65[a]	(-)16 ±2.18	(-)18 ±4.32	(-)12±5.30[b]	(-)10 ±4.55[b]
(Kidney/body weight ratio) x 1000	5.1 ±.0003	5.6 ±0.0003	10.6±0.0002[a]	8.9±0.0002[b]	9.2 ±0.0003[c]	8.3 ±0.0003[d]	7.8±0.0002[d]
BUN (mg/dl)	14.23±0.594	15.42± 0.3	45.49±8.82[a]	22.75±3.65[d]	32.69± 9.12	26.3±3.96[d]	23.65± 6.2[d]
Serum creatinine (mg/dl)	0.54±0.04	0.497±0.017	1.05±0.084[e]	0.59±0.055[b]	0.86± 0.16	0.663±0.05[b]	0.58±0.034[b]

All data are expressed as mean ± S.E.M. MH=Morin hydrate, Gen= Gentamicin, BUN=Blood Urea Nitrogen, Pre=Pre-treatment, n=Number of animals

[a] $p < 0.01$ vs. Normal control group, [b] $p < 0.01$ vs. Gentamicin group, [c] $p < 0.001$ vs. Gentamicin control, [d] $p < 0.05$ vs. Gentamicin group and [e] $p < 0.001$ vs. Normal control group

Table 2: Effect of Morin Hydrate on total ROS, tissue nitrite, MDA and GSH level (n=6)

	NC	MH	GEN	Pre MH+GEN	GEN+MH 50	GEN+MH 100	GEN+MH 200
ROS level (% of Control)	101.2±1.6	98±2.8	131±2.2[a]	126±4.1	114±3.2[b]	112±7.8[b]	103±5.6[c]
Tissue nitrite level (µmol/gm wet tissue)	23.46±2.2	32.5±1.35	14.80±0.85[a]	17.50±2.2	16.82±3.2	19.65±2.4[b]	21.50±3.2[b]
TBARS (MDA level) (nmol/gm wet tissue)	38.45±2.2	38.25±1.6	50.22±1.4[a]	40.62±1.8[b]	42.26±4.2[b]	40.55±2.6[b]	39.22±2.2[b]
GSH level (µmol/gm wet tissue)	0.432±0.04	0.441±0.06	0.38±0.02[a]	0.442±0.01[b]	0.43±0.31[b]	0.46±0.28[b]	0.531±0.09[c]

All data are expressed as mean ± S.E.M. tROS: Total reactive oxygen species, TBARS: Thiobarbituric acid reactive substances, MDA: Malondialdehyde, GSH: Reduced glutathione, MH: Morin hydrate, Gen: gentamicin, Pre: Pre treatment, n: Number of animals

[a] $p < 0.05$ vs. Normal control, [b] $p < 0.05$ vs. Gentamicin control, [c] $p < 0.01$ vs. Gentamicin control

Table 3: Effect of Morin Hydrate on catalase, SOD and total protein levels (n=6)

	NC	MH	GEN	Pre MH+GEN	GEN+MH 50	GEN+MH 100	GEN+MH 200
CAT (k/min)	0.4085±0.007	0.65±0.022	0.232±0.012[a]	0.336±0.006[b]	0.348±0.008	0.369±0.036[c]	0.42±0.004[d]
SOD (% control)	99.75±0.74	99.40±0.54	87.6±1.4[a]	94.2±0.8[b]	91.5±0.66[c]	95.28±1.4[b]	96.55±1.6[c]
Total protein (mg/ml)	7.86±0.38	8.01±0.56	4.08±0.14[a]	5.41±0.38[b]	4.72±0.42[c]	5.82±0.78[d]	6.95±0.22[e]

All data are expressed as mean ± S.E.M. CAT: Catalase, SOD: Superoxide dismutase, MH: Morin hydrate, Gen: Gentamicin, Pre: Pre-treatment, n: Number of animals, k/min: kinetic/minute

[a] $p < 0.01$ vs. Normal control, [b] $p < 0.05$ vs. Gentamicin control, [c] $p < 0.001$ vs. Gentamicin control, [d] $p < 0.05$ vs. Gentamicin control, [e] $p < 0.05$ vs. Gentamicin control

DISCUSSION

Several approaches involve the use of various natural antioxidant compounds that ameliorate the Gentamicin induced nephrotoxicity[18]. Flavonoid compounds from natural sources are known to be having the scavenging effect of reactive oxygen species. Morin Hydrate is a member of flavonoids having free-radical-trapping capacity[19].

Also, it has been demonstrated that treatment of Morin Hydrate in rats significantly protects against mercuric chloride induced nephrotoxicity[20]. Multiple doses of Gentamicin (100 mg/kg b.w) s.c resulted in nephrotoxicity evidenced by significant increase in Scr and BUN levels increased kidney relative weight, reduced body weights of Gentamicin control animals compared to normal rats[11] and may be because of inappropriate GFR, BUN was abnormal. Results of the present study demonstrates that Gentamicin treatment significantly increases the Scr, BUN, decreases the body weight and increases the kidney to body weight ratio levels. On the other hand, Morin Hydrate treatment showed a significant protection against Gentamicin induced weight loss and increase in Scr, BUN levels, kidney to body weight ratio significantly. This weight loss was decreased, but not completely prevented by Pre- and Treatment with Morin Hydrate.

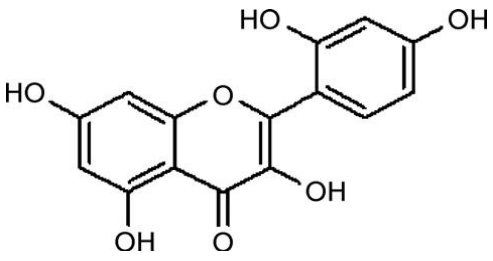

Fig. 1: Morin Hydrate

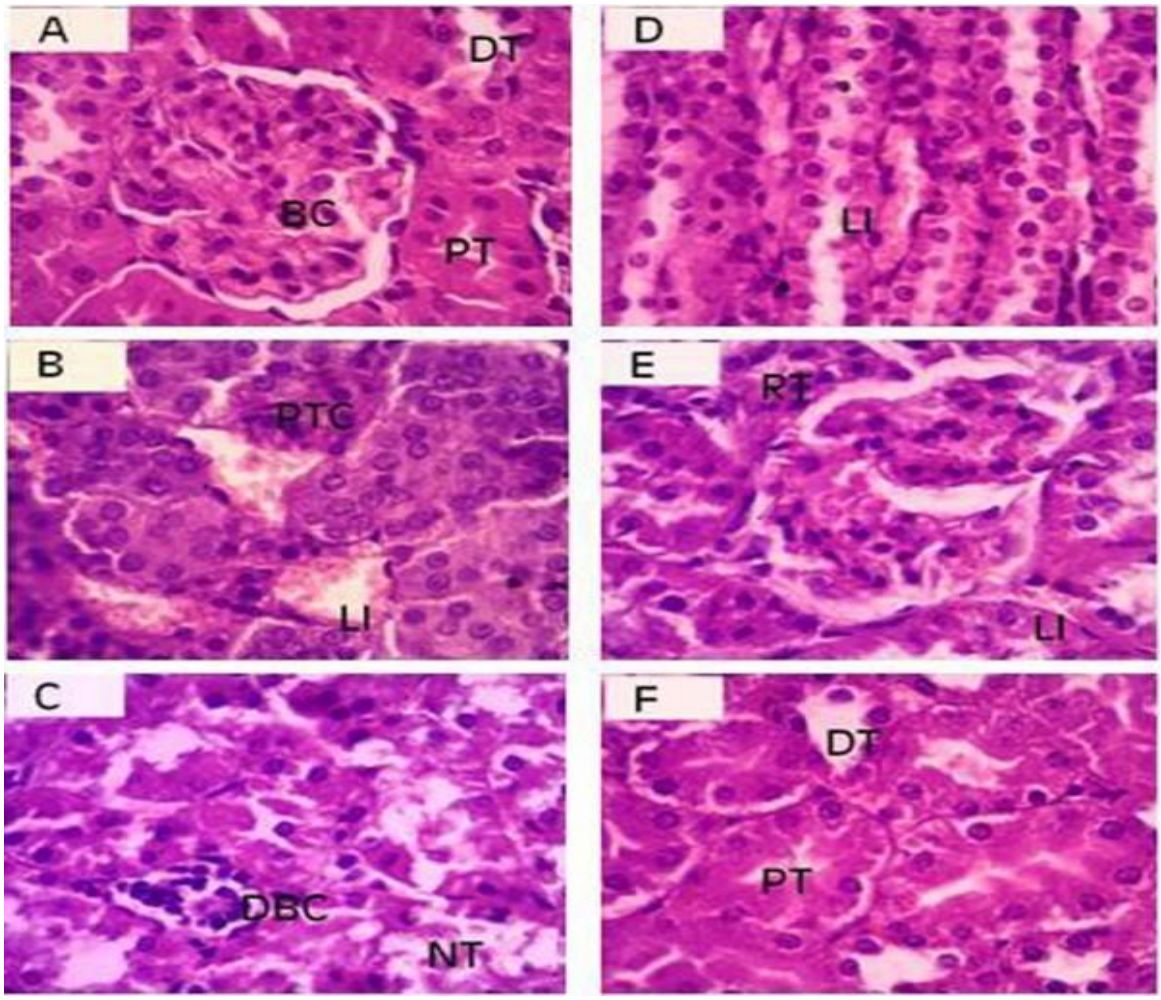

Fig. 2: Photomicrograph of rat kidney section

The results of the present study shows that total ROS levels were increased significantly in the Gentamicin control group, explains the Gentamicin induced nephrotoxicity was caused by increase in ROS levels causing tubular necrosis and impairment of Glomerular Filtration Rate (GFR)[21]. This production of ROS levels would stimulate the inflammatory cascade like increase in intercellular adhesion molecule-1(ICAM) and monocyte chemo-attractant protein, expression of pro-inflammatory mediators, including nuclear factor kappa-β (NF-kβ), Leukocyte adhesion molecule (LAM), and mitogen activated protein kinase (MAPK)[22]. Thus, blockade of ROS system will be an effective approach to blunt the Gentamicin induced nephrotoxicity. Thus, therapeutic doses of Morin Hydrate significantly ($p < 0.05$) attenuate this ROS levels in treated groups.

As expected that tissue nitrite (NO_2^-), and malondialdehyde levels were increased in all Gentamicin treated groups, supporting the previous results and Morin Hydrate significantly reduces it. Along with, the CAT, SOD, and GSH activities were significantly decreased in the Gentamicin treated animals compared to the normal group. In our study, Morin Hydrate treatment (50, 100 and 200 mg/kg/day, p.o) for fifteen consecutive days, from 4th day to 18th day along with Gentamicin administration, provided a significant protection against Gentamicin-induced changes in antioxidant enzyme level by restoring it, suggesting that the protection against Gentamicin induced nephrotoxicity could be attributed to its antioxidant activity and free radical scavenging activity. However, in our study, the total protein levels were decreased in Gentamicin-group compared to the normal group. Reduction of the total protein levels were due to the reactive oxygen species[23]. And all doses of Morin Hydrate prevented this Gentamicin Induced change significantly as it is having anti oxidant activity.

These findings also correlated with the histopathological examination of cortical tissues. In case of Vehicle Control and Morin Hydrate control group, there is no change in glomerular structures and ~~in~~ the Gentamicin control group showed the congestion of proximal tubules, leucocyte infiltration and degeneration of bowman capsule and glomerulus. These alterations could be attributed to its ROS levels and lipid peroxidation levels, same results were also reported by the Kumar *et al* [24], and Stojiljkovic *et al* [25]. Regeneration of glomerular tubules and decreasing in leucocyte infiltration were observed in case of Morin Hydrate treated groups showing the curative effect against Gentamicin induced nephrotoxicity.

Moreover their direct effect on kidney cortical tissues by the reactive oxygen species, also triggers the accumulation of leukocytes, and indirectly activates the neutrophil infiltration. Inflammatory mediators like interleukin-8 (IL-8), and cytokines were recruited, activateing the chemotaxis effect on neutrophil accumulation to the damaged tissue.

CONCLUSION

Morin Hydrate has shown prominent inhibitory effect against all the~~se~~ factors which are mainly involved in Gentamicin induced nephrotoxicity. Our results suggest that the Morin Hydrate, a flavonoid, has a significant therapeutic use against the nephrotoxic complications of Gentamicin chemotherapy and the effect is attributed to its direct strong antioxidant, anti-inflammatory

properties. Hence, Morin Hydrate has a potential to be used as a therapeutic adjuvant in Gentamicin induced nephrotoxicity. So, further aim in future is to find out the molecular mechanisms by which Morin Hydrate protects against Gentamicin induced oxidative stress and nephrotoxicity.

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