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RETRACTED: Myricetin Abrogates Cisplatin-Induced Oxidative Stress, Inflammatory Response, and Goblet Cell Disintegration in Colon of Wistar Rats

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Abstract: Cisplatin [cis-diamminedichloroplatinum II] is an extensively prescribed drug in cancer chemotherapy; it is also useful for the treatment of diverse types of malignancies. Conversely, cisplatin is associated with a range of side effects such as nephrotoxicity, hepatotoxicity, gastrointestinal toxicity, and so on. Myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4chromenone) is a very common natural flavonoid found in fruits, tea, and plants. It has been found to have high-value pharmacological properties and strong health benefits. To examine the role of myricetin in colon toxicity induced by cisplatin, we conducted a concurrent prophylactic study in experimental animals that were treated orally with myricetin for 14 days at two doses—25 and 50 mg/kg of body weight. On the 14th day, a single intraperitoneal injection of cisplatin (7.5 mg/kg body weight) was administered in all groups except control. The effects of myricetin in cisplatin-induced toxicity in the colon were assessed in terms of antioxidant status, phase-II detoxification enzymes, the level of inflammatory markers, and goblet cell disintegration. Myricetin was found to restore the level of all the antioxidant enzymes analyzed in the study. In addition, the compound ameliorated cisplatin-induced lipid peroxidation, increase in xanthine oxidase activity, and phase-II detoxifying enzyme activity. Myricetin also attenuated deteriorative effects induced by cisplatin by regulating the level of molecular markers of inflammation (NF-κB, Nrf-2, IL-6, and TNF-α), restoring Nrf-2 levels, and controlling goblet cell disintegration. The current study reinforces the conclusion that myricetin exerts protection in colon toxicity via up-regulation of inflammatory markers, improving anti-oxidant status, and protecting tissue damage.

Keywords: myricetin; cisplatin; colon toxicity; inflammation; Nrf-2; NF-κB

1. Introduction

Cisplatin [cis-diamminedichloroplatinum (II) or cisplatium] is the first generation of anti-cancer drug that possesses the platinum coordination complex with a square planar geometry. Cisplatin is a solid white crystalline powder that is stable at ambient temperature [1]. It was approved for clinical use as the drug of choice for cancer chemotherapy by the US-FDA (United States Food and Drug Administration) in 1978 [2]. Cisplatin is regarded as the major antiproliferative drug, and has been extensively used for the treatment of cervical, bladder, ovarian, testis, head and neck, gastrointestinal, esophageal, and lung cancers for the last 40 years, with a success rate of more than

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90% [2–4]. The nephrotoxicity of cisplatin is its major side effect, as well as its dose-limiting factor with respect to its effective anti-cancer action. Increased and frequent doses of cisplatin have been reported to cause other serious complications, such as hepatotoxicity, gastrointestinal toxicity, neurotoxicity, myelosuppression, ototoxicity, and spermiotoxicity [2,5,6]. Cisplatin toxicity as a whole involves the generation of free radicals, peroxidation of membranes, mitochondrial dysfunction, and damage to DNA and protein synthesis inhibition [7]. The most widely accepted mechanism of cisplatin toxicity involves generation of a massive amount of reactive oxygen species (ROS) and induction of oxidative stress. These ROS generated by cisplatin interact with cellular molecules like proteins, DNA and lipids [6,8,9]. Basu and Krishnamurthy (2010) [10] reported that cisplatin acts on the sulfhydryl (-SH) groups of cellular proteins; however, the primary target of cisplatin toxicity is the DNA, as it has the ability to create both intra- and inter-strand cross-linking between N7 and O6 of nearby guanine molecules, which hampers the repair mechanism and causes damage to DNA, interferes with replication and transcription mechanisms, and induces apoptosis [7]. In addition, cisplatin gives rise to the platinum-DNA (Pt-DNA) adduct formation, which affects DNA synthesis and cell division. In mitochondria, cisplatin also leads to adduct formation in mitochondrial DNA (mtDNA) and inhibits the replication of mtDNA and gene transcription [1].

Many reports support the notion that cisplatin is not specific in action at the site of cancers, but affects rapidly dividing cells elsewhere in the body, e.g., the epithelial cells of intestines. It does so by massively producing free radicals and generating oxidative and nitrosative stress [2,6,11,12]. With regard to anticancer drug toxicity, there is a huge amount of published evidence that supports naturally occurring nutraceuticals/compounds possessing antioxidant and anti-inflammatory potential, which could counter anticancer drug toxicity [6,11,13–15]. What is needed now is to explore natural compounds that efficiently counter cisplatin toxicity to improve their chemotherapeutic efficacy. Natural dietary supplements like vegetables, fruits and so on that have pharmacological properties are now increasingly being used for the benefit of human health and to counter anticancer drug toxicity [6,11,14,15].

Flavonoids are an abundantly found, naturally occurring class of polyphenolic phytochemicals found in the human diet [16,17]. Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) is one of the most commonly found naturally occurring flavanones (Figure 1), and is predominantly but not exclusively found in berries, tea, vegetables, and herbs [18]. Myricetin is found in abundance in red wine and grapes, with several varieties of grape containing as much as 24 mg of myricetin per kilogram of fresh weight [19,20]. It was first discovered by Perkin and Hummel from the bark of *Myrica nagi* in 1896. Later, its structural configuration was observed by the same authors [21,22]. Usually, myricetin is poorly soluble in water (16.6 µg/mL); nevertheless, after deprotonation in basic aqueous solution, it dissolves rapidly. It is also easily soluble in some organic solvents (acetone, tetrahydrofuran, etc.) [23]. Myricetin is an important component of the human diet, and has been shown to have major iron-chelating and anti-oxidant roles [24]. In addition, it was observed and proven to have multiple biological activities, including anti-oxidant, anti-inflammatory, neuro-protective, anti-diabetic, ant-arthritic, and anticancer activities [24–34]. Therefore, this study was undertaken to check the prophylactic effect of myricetin in cisplatin-induced colon toxicity by blocking colonic damage, an doxidative and inflammatory processes in Wistar rats.

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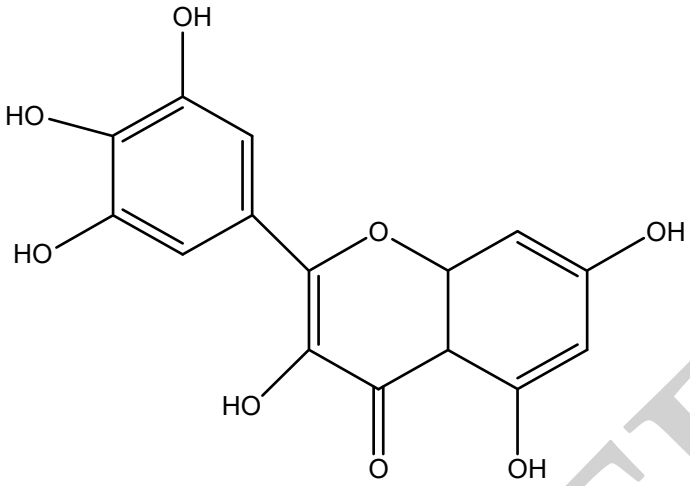


Figure 1. Chemical structure of Myricetin (molecular Formal: C_{15} H_{10} O_5).

2. Material and Methods

2.1. Chemicals

Bovine serum albumin (BSA), reduced glutathione (GSH), ethylene diamine tetra-acetic acid (EDTA), oxidized glutathione (GSSG), xanthine, poly L-lysine, glucose-6-phosphate, dichlorophenolindophenol (DCPIP), nicotinamide adenine dinucleotide phosphate reduced (NADPH), glutathione reductase, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithio-bis-[2nitrobenzoic acid] (DTNB), thiobarbituric acid (TBA), folin ciocalteau reagent (FCR), nicotinamide adenine dinucleotide phosphate oxidized (NADP), flavin adenine dinucleotide (FAD) and myricetin were purchased from Sigma (Sigma Chemical Co., St Louis, MO). Sodium dihydrogen phosphate, sodium potassium tartrate, pyrogallol, sodium hydroxide, and di-sodium hydrogen phosphate were obtained from E. Merck Limited, India. Cisplatin was procured from Dr. Reddy's Laboratories, India.

2.2. Treatment

To study the cisplatin-induced oxidative burst and inflammatory response in the colon, a prophylactic treatment with myricetin was conducted. In this study, 4 groups with 6 Wistar male rats in each group were arbitrarily allocated. Myricetin was suspended in 5% sodium carboxymethyl cellulose (CMC-Na) [35]. The first group served as vehicle control and the animals were treated with CMC-Na for only 14 days. Groups II, III, and IV received a single intraperitoneal injection of cisplatin (7.5 mg/kg body weight) on 14th day in accordance with earlier reports [11,36,37]. Groups III and IV were treated with myricetin 25 and 50 mg/kg body weight, respectively. All the animals were sacrificed by cervical dislocation under light anesthesia 24 h after cisplatin injection.

2.3. Post-Mitochondrial Supernatant (PMS)

Colon tissues free of any extraneous material were detached rapidly from the euthanized rats and were perfused in ice-cold saline (0.85% sodium chloride), then homogenized by means of in ice-cold phosphate buffer (0.1 M, pH 7.4). After filtering the homogenate through a muslin cloth, it was centrifuged for 10 min at 1744× *g* with temperature set at 4 °C for the separation of nuclear debris. The supernatant obtained was centrifuged for 20 min at 15,520× *g* at 4 °C to obtain Post-mitochondrial Supernatant (PMS), which was also used as the source of various enzymes.

2.4. Staining for Goblet Cell Analysis

Formalin-fixed and paraffin-embedded colonic sections of size 4 µm from tissue blocks were mounted on poly L-lysine finished glass slides. The tissues were de-waxed in xylene and rehydrated

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through a graded sequence of water and ethanol. Slides were then stained for 30 min in Alcian blue (1%: pH 2.5) with 3% acetic acid solution. To remove non-specific staining, the glass slides were rinsed in 3% acetic acid for 60 s after staining and then washed with distilled water. The tissue sections were then counterstained using neutral red (0.5% aqueous solution) for 20 s and then dehydrated in ethyl alcohol. The sections were then covered using mounting media and the slides were examined under the microscope.

2.5. Immunohistochemical Staining (Nrf-2, NF-κB)

Sections of 4 µm each from the tissue blocks which were formalin-fixed and paraffin-embedded were mounted on poly L-lysine coated glass slides. The sections, after dewaxing them in xylene and then rehydrated through graded series of ethanol and water, went through a process of antigen retrieval in sodium citrate buffer (10 mM, pH 6.0); after that, slides were cooled for 15 min. Then they were washed with tris-buffered saline (TBS) 3 times each for 5 min. To reduce the activity of endogenous peroxidase, the slides were incubated in 3% H_2O_2 in methanol for 10 min and then were subjected to power block for 10 min to block non-specific binding. The sections were rinsed in TBS and incubated at 4 °C overnight inside a humidified chamber with the primary antibody, then washed in TBS. The tissue sections were incubatedwith biotinylated goat anti-polyvalent secondary antibody for 20 min and rinsed in TBS. The sections were incubated again with streptavidin peroxidase plus for 30 min. The sections were washed in TBS and developed with 3,3′-Diaminobenzidine (DAB) solution until they became brown. The sections were counterstained with Mayer's hematoxylin and then mounted using the mounting media. Finally, slides were evaluated under the microscope. Primary antibodies were rabbit anti-Nrf-2 (dilution 1:200), and rabbit NF-κB (dilution 1:100).

2.6. Measurement of MDA

Assay for lipid peroxidation (LPO) was carried out as per the protocol of Wright et al. The assay mixture consisted of 800 µL phosphate buffer (0.1 M, pH 7.4) and 200 µL PMS, making the final volume of the assay mixture 1 mL. The reaction mixture was incubated at 37 °C in a water bath for 60 min. The addition of 1 mL trichloroacetic acid (10%) stopped the reaction. Furthermore, 1 mL thiobarbituric acid (TBA) (0.67%) was added, and all the tubes were placed in a boiling water bath for a period of 20 min. The tubes were then shifted to an ice bath and centrifuged at 2500× *g* for 10 min. Optical density was measured at 535 nm to assess the quantity of malondialdehyde (MDA) formed. The units of the results obtained were for nmol TBA formed/h per g tissue at 37 °C [38].

2.7. Measurement of NO

The production of NO was evaluated by measuring the level of nitrite (an indicator of NO) in the supernatant using a colorimetric reaction with Griess reagent. NO levels were measured by checking the levels of nitrite (indirect indicator of NO) in the assay mixture by colorimetric reaction in the form of Griess reagent. 100 µL of reaction mixture and 100 µL of Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H3PO4] were incubated together for 10 min in the dark at room temperature. Nitrites were measured at 540 nm with spectrophotometer. The level of nitrites was derived by drawing a standard curve of $NaNO_2$ [39].

2.8. Assay for Myloperoxidase Activity, Antioxidant, and Prooxidant Enzymes

The neutrophil quantification is measured as a level of myloperoxidase (MPO) activity and was carried using the Bradley et al. method (1982) [40].

The quinone reductase enzyme activity was assayed by the method of Benson et al. (1980) [41]. Marklund and Marklund (1974) method of analyzing the activity of Hydrogen Peroxidase (H_2O_2) was implemented [42]. The activity of xanthine oxidase was assayed by the Stripe and Della Corte method (1969) [43]. The activity of catalase enzyme was assayed by Claiborne method (1985) [44]. The glutathione peroxidase activity was measured by the Mohandas et al. method (1984) [45]. The

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reduced glutathione levels were determined by the method of Jollow et al. (1974) [46]. GST activity was assayed by the Habig et al. method (1974) [47]. The enzyme activity of glutathione reductase was determined by following the Carlberg and Mannervik method (1975) [48].

G6PD activity was assayed by the method of Zaheer et al. (1965) [49]. TNF-α levels were determined by rat TNF-α kit (eBioscience, San Diego, USA: Cat No. 88-7340-22). In addition, IL-6 levels were determined by rat IL-6 kit (eBioscience, San Diego, USA: Cat # 88-7066-22). Both methods are based on enzyme linked immunosorbent assay (ELISA).

2.9. Statistical Analysis

The data from the individual groups are presented as the mean ± standard error of the mean (SEM). The differences between the groups were analyzed using analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparisons test. The minimum criterion for statistical significance was set at $p < 0.05$ for all comparisons.

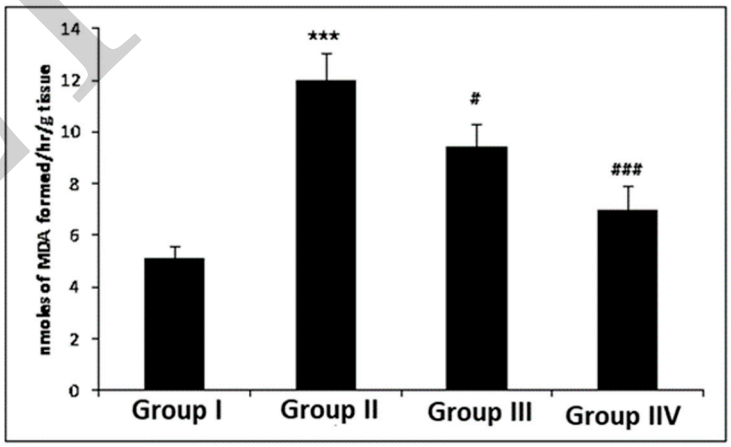
2.10. Statement of Ethical Approval

All procedures for using experimental animals were checked and proper permission was obtained from the "Institutional Animal Ethical Committee (IAEC)" (Approval No: Au/FVS/PS-57/9713) that is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA), New Delhi, India.

3. Results

3.1. Effect of Myricetin and Cisplatin Treatment on Lipid Peroxidation, MPO, and Nitrite Levels

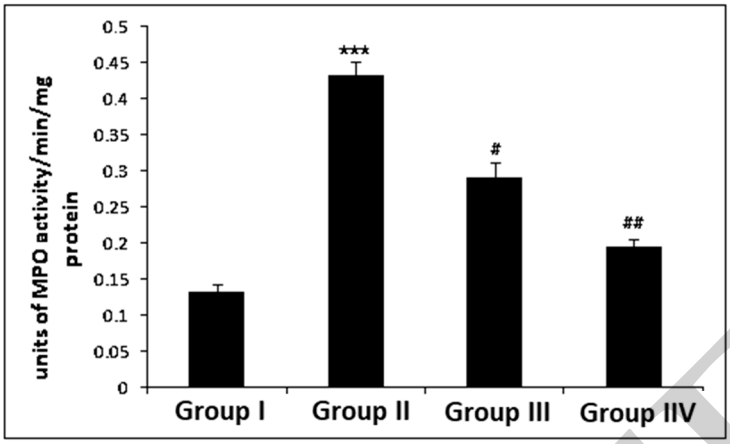
The levels of MDA were markedly enhanced ($p < 0.001$) in cisplatin-induced group II as compared to group I. Myricetin pre-treatment noticeably decreased the level of MDA in group III ($p < 0.05$) and group IV ($p < 0.001$), respectively, when compared to group II (Figure 2a). Treatment with cisplatin resulted in a significant increase ($p < 0.001$) in the MPO activity in group II when compared to control group I. Myricetin for both the doses in group III ($p < 0.05$) and group IV ($p < 0.01$) was instrumental in bringing back the unusual levels of MPO to normal (Figure 2b). In addition, a significant increase ($p < 0.001$) was observed in nitrite levels in group II when compared to control group I. Myricetin for both the doses (25 and 50 mg/kg body weight) in group III ($p < 0.01$) and group IV ($p < 0.001$) was effective in normalizing nitrite levels (Figure 2c).



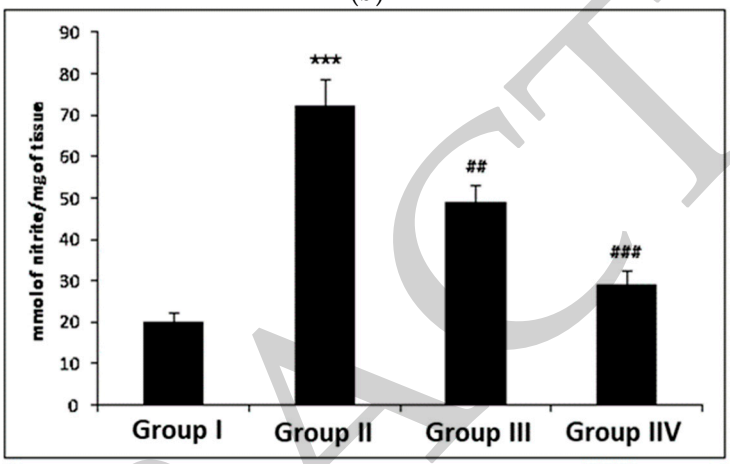
(**a**)

Figure 2. *Cont.*

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(**b**)



(**c**)

Figure 2. Effects of myricetin and cisplatin on MDA, MPO and nitrite levels. (**a**) In cisplatin-treated group II, the MDA level increased significantly (*** $p < 0.001$) as compared to control group I. Pre-treatment with and myricetin (25 and 50 mg/kg b. wt.) significantly attenuated the MDA level in group III (# $p < 0.05$) and group IV (### $p < 0.001$) when compared to group II. (**b**) In cisplatin-treated group II, the MPO level (Leukocyte infiltration) increased significantly (*** $p < 0.001$) as compared to control group I. Pre-treatment with myricetin (25 and 50 mg/kg b. wt.) significantly attenuated the MPO level in group III (# $p < 0.05$) and group IV (## $p < 0.01$) as compared to group II. (**c**) In cisplatin-treated group II, the nitrite levels were significantly increased (*** $p < 0.001$) as compared to control group I. Pre-treatment with myricetin significantly (25 and 50 mg/kg b. wt.) attenuated the nitrite levels in group III (## $p < 0.01$) and group IV (## $p < 0.001$) as compared to group II.

3.2. *The Effect of Myricetin and Cisplatin on the Activities of Glutathione and Dependent Enzymes in the Colonic Tissue*

Reduced glutathione levels were depleted in cisplatin-treated group II ($p < 0.01$) as compared to control group I. Myricetin pre-treatment revealed a visible increase in reduced glutathione level in group III ($p < 0.05$) and group IV ($p < 0.05$) when compared with group II. Cisplatin administration caused a decrease in the activities of GPx ($p < 0.001$), GST ($p < 0.05$), G6PD ($p < 0.001$) and GR ($p < 0.001$) in group II as compared to group I. Myricetin supplementation for both doses (25 and 50 mg/kg body weight) showed remarkable improvement in restoring levels of all the above-mentioned enzymes. Myricetin at higher dose showed higher potential ($p < 0.001$) in restoring the glutathione-dependent enzyme activity (Tables 1 and 2).

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Table 1. Effect of cisplatin and myricetin on glutathione dependent and other antioxidant enzymes.

	Group I	Group II	Group III	Group IV
Reduced glutathione (GSH; nmol mg protein)	343.87 ± 18.2	159.46 ± 14.2 ***	181.76 ± 11.7 #	321.08 ± 16.1 ###
Oxidized glutathione (GSSG; nmol mg protein)	42.01 ± 4.76	81.32 ± 7.27 ***	67.31 ± 5.01 #	40.32 ± 4.34 ###
GSH/GSSG Ratio	8.18 ± 0.82	1.96 ± 0.21 ***	2.70 ± 0.33 #	7.96 ± 0.61 ###
GPx (nmol/min/mg protein)	211.81 ± 20.1	87.17 ± 9.24 ***	117.62 ± 11.3 ##	179.53 ± 16.3 ###
GR (nmol min/min/mg protein	247.26 ± 19.8	98.32 ± 8.34 ***	147.81 ± 10.3 ##	213.53 ± 19.2 ###
G6PD (nmol NADP reduced/min/mg protein)	234.27 ± 21.9	96.81 ± 9.51 ***	149.90 ± 11.7 ##	227.12 ± 20.1 ###
SOD (units/min/mg protein	13.17 ± 1.34	4.87 ± 0.49 ***	5.97 ± 0.91 #	11.32 ± 2.23 ###
Catalase (nmol H_2O_2 consumed/min/mg protein	10.37 ± 0.91	3.32 ± 0.12 ***	5.32 ± 0.89 #	9.11 ± 0.61 ###
H_2O_2 (nmol of H_2O_2/g tissue)	164.2 ± 15.6	390.1 ± 32.9 ***	319.1 ± 19.3 #	210.7 ± 12.9 ###

Values of these parameters were expressed as mean ± S.D. (n = 6). Significant differences were indicated by *** $p < 0.001$ when compared group II, and, # $p < 0.01$, ## $p < 0.01$ and ### $p < 0.001$ when compared with group II.

Table 2. Effect of cisplatin and myricetin on levels of GST, QR and XO.

	Group I	Group II	Group III	Group IV
GST (µmol CDNB conjugate formed/min/mg protein)	4.09 ± 0.79	2.87 ± 0.89 ***	3.01 ± 0.62 ##	3.89 ± 31.9 ##
QR (µmol DCPIP reduced/min/mg protein)	1.98 ± 0.22	1.12 ± 0.16 ***	1.63 ± 0.19 #	1.45 ± 0.13 ##
XO (µg uric acid formed/min/mg protein)	15.43 ± 3.21	39.31 ± 4.02 ***	21.21 ± 3.83 ##	31.24 ± 3.12 ##

Values of these parameters were expressed as mean ± S.D. (n = 6). Significant differences were indicated by *** $p < 0.001$ when compared group I and, # $p < 0.01$, ## $p < 0.01$ and ### $p < 0.001$ when compared with group II.

3.3. Effect of Myricetin and Cisplatin on Antioxidant Enzymes Activity in Colonic Tissue

The activities of quinone reductase (QR) and catalase were drastically lowered ($p < 0.001$) in group II when compared to group I. In addition, the activity of superoxide dismutase (SOD) increased significantly ($p < 0.001$) after cisplatin treatment in group II as compared to group I. Treatment with myricetin at a dose of 25 mg/kg body weight augmented the activities of QR ($p < 0.05$) and catalase ($p < 0.05$) in group III as compared to group II, while the activity of SOD was markedly lower ($p < 0.05$) in group III as compared to group II. The higher dose of myricetin (50 mg/kg b. wt.) also showed a significant increase in the activities of catalase ($p < 0.001$) and QR ($p < 0.01$) in group IV as compared to group II, while the activity of SOD decreased ($p < 0.001$) in group IV as compared to group II (Tables 1 and 2).

3.4. Effect of Myricetin and Cisplatin on Xanthine Oxidase (XO) Activity in Colonic Tissue

The activity of XO was significantly enhanced ($p < 0.001$) in group II as compared to group I. Myricetin treatment markedly lowered the level of XO at both the doses in group III ($p < 0.01$) and group IV ($p < 0.01$), as shown in Table 2.

3.5. Effect of Myricetin on the Cisplatin-Induced Colonic Immunohistochemical Expression of Nrf-2

The colonic section of cisplatin-treated group II has reduced immunopositive staining of Nrf-2 as indicated by brown color as compared to control group I. Pretreatment with myricetin in group III and Group IV enhanced the immunopositive staining of Nrf-2 as compared to Group II. For

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immune-histochemical analysis, Nrf-2 specific immunostaining is indicated by a brown color and hematoxylin staining by a light blue color (Figure 3).

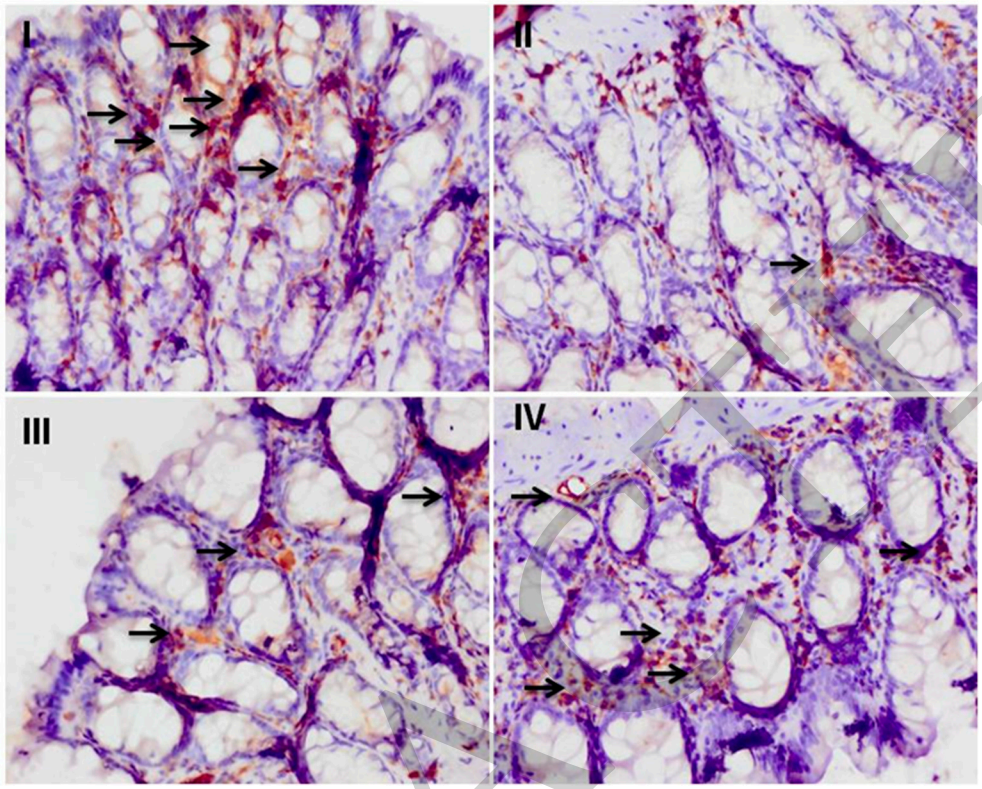


Figure 3. Effect of myricetin treatment on cisplatin-induced Nrf-2 expression. Photomicrographs of colonic sections depicting immunohistochemical analyses, with brown color indicating specific immunostaining of Nrf-2 and light blue color indicating counterstaining by nuclear hematoxylin. The colonic section of cisplatin-treated group-II has the least Nrf-2 immunopositive staining, as indicated by the much less brown color as compared to control group I, while pretreatment with myricetin (25 and 50 mg/kg b. wt.) in groups III and IV increased Nrf-2 immunostaining (check arrows) as compared to group II. Original magnification: 40×.

3.6. Effect of Myricetin and Cisplatin Treatment on the Expression of NF-κB and Cox-2

Some sections of the colon treated with cisplatin in group II have intense NF-κB immuno-positive staining, shown by a brown color, when compared to group I. Myricetin pre-treatment in groups III and IV decreased NF-κB and Cox-2 immunostaining when compared to group II, as indicated by the less intense staining. For immune-histochemical analysis, NF-κB specific immunostaining is indicated by a brown color and hematoxylin staining by a light blue color (Figure 4).

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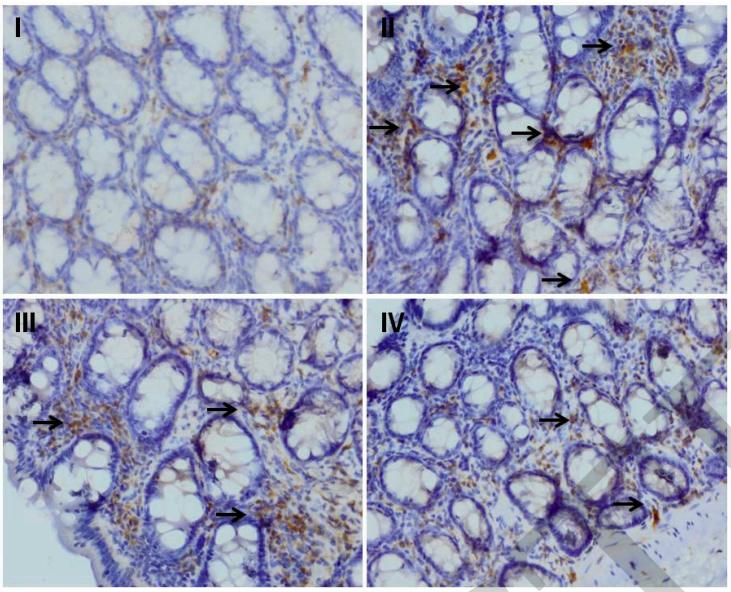


Figure 4. Effect of myricetin treatment on cisplatin-induced NF-κB expression. Photomicrographs of colonic sections depicting immunohistochemical analyses, with brown color indicating specific immunostaining of NF-κB (arrows) and light blue color indicating counterstaining by nuclear hematoxylin. The colonic section of cisplatin-treated group II has more NF-κB immunopositive staining, as indicated by brown color as compared to control group I, while pre-treatment of myricetin (25 and 50 mg/kg b. wt.) in groups III and IV reduced NF-κB immunostaining when compared to group II. Original magnification: 40×.

3.7. Effect of Treatment of Myricetin and Cisplatin on TNF-α and IL-6 Levels

Quantitative production of TNF-α was assessed in colon via cisplatin treatment. We observed that there was a marked difference in the level of pro-inflammatory cytokines in control group I ($p < 0.001$) as compared to the cisplatin-treated group II. Pre-treatment with myricetin markedly inhibits the production of TNF-α in the groups III and IV when compared to the cisplatin-treated group II (Figure 5a). The effect of cisplatin treatment on IL-6 levels in the colon was assessed. We observed that there was a marked difference in the level of pro-inflammatory cytokine in control group I ($p < 0.001$) as compared to cisplatin-treated group II. Pre-treatment with myricetin markedly inhibited the production of IL-6 in groups III ($p < 0.01$) and IV ($p < 0.001$) when compared to the cisplatin-treated group II (Figure 5b).

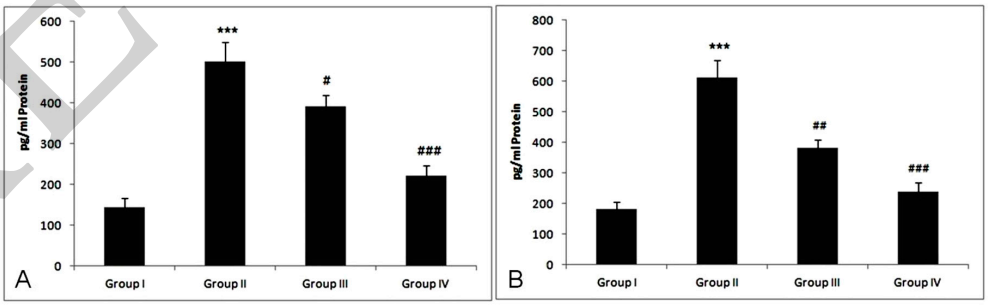


Figure 5. Effect of myricetin and cisplatin on TNF-α and IL-6 level. (**A**) In the cisplatin-treated group, the level of TNF-α was markedly increased (*** $p < 0.001$) as compared to group I. Treatment with myricetin significantly attenuated the level of TNF-α in group III ($^{\#}$ $p < 0.05$) and group IV ($^{\#\#\#}$ $p < 0.001$) as compared to group II. (**B**) In the cisplatin-treated group, the level of IL-6 was markedly increased (*** $p < 0.001$) as compared to group I. Treatment with myricetin (25 and 50 mg/kg b. wt.) significantly attenuated the level of IL-6 in group III ($^{\#\#}$ $p < 0.01$) and group IV ($^{\#\#\#}$ $p < 0.001$) as compared to group II.

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3.8. Effect of Myricetin and Cisplatin on Goblet Cell Disintegration

Colonic sections treated with cisplatin in group II showed a massive disintegration of the goblet cells compared to the control group I. Myricetin supplementation at both the doses (25 and 50 mg/kg body weight) prevented the disintegration of the goblet cells and prevented colonic damage (Figure 6).

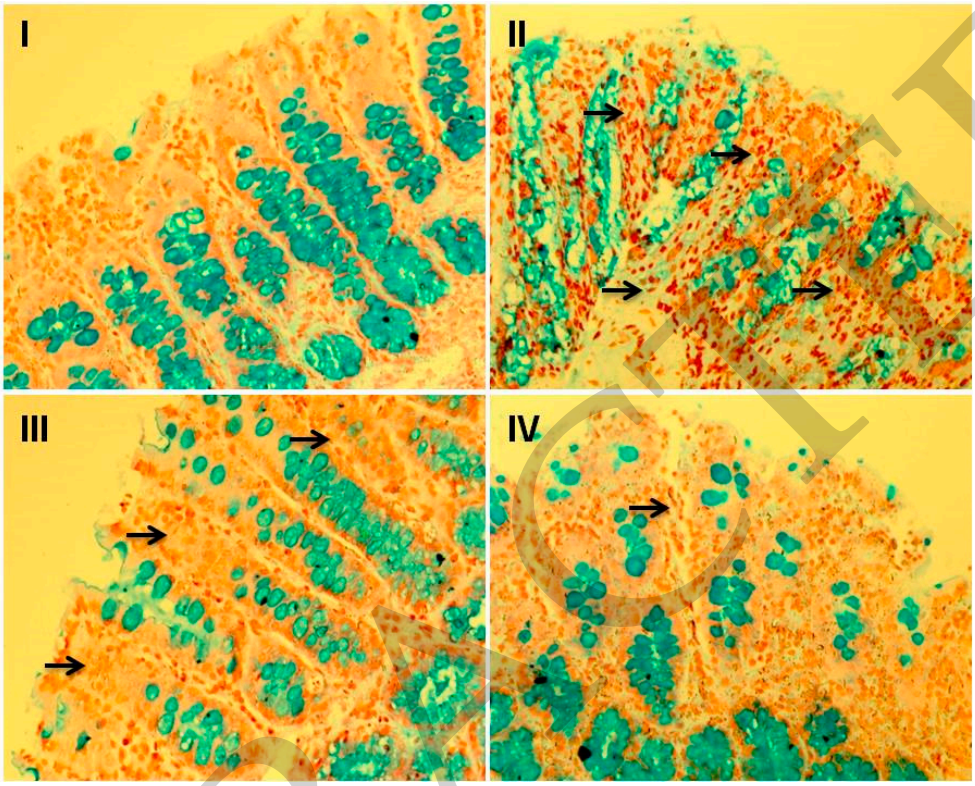


Figure 6. Effect of myricetin treatment on cisplatin-induced goblet cell disintegration. Photomicrographs of staining of histological sections of colon depicting different experimental groups: group I indicates goblet cells stained blue due to acidic mucin secreted by these cells and exhibited the normal integrated goblet cells along the colonic sections. Group II shows extensive disintegration of goblet cells, which is the hallmark of cisplatin toxicity, as represented by arrows. In groups III and IV, myricetin pre-treatment showed protection against cisplatin-induced goblet cell disintegration. Both the doses of myricetin (25 and 50 mg/kg b. wt.) maintained the integrity of goblet cells. magnification: 40×.

4. Discussion

The current study was undertaken to analyze the protective effects of myricetin in cisplatin-induced colon toxicity in an experimental model. Cisplatin-induced colon toxicity is extensively documented in the literature [6,11]. Emesis and diarrhea are recognized as the two most severe side effects of cisplatin therapy [50,51]. Chemotherapy based on cisplatin involves intravenous or intraperitoneal administration of cisplatin [52]. Animal studies set up for exploring cisplatin toxicity have utilized intra-peritoneal or intravenous routes for administration [53], while studies favoring the oral route have found similar toxicological effects as other routes [54]. After administration, cisplatin is distributed to almost all tissues and organs, with increased uptake in liver, kidney, and intestines [2,55,56].

Many mechanisms have been put in place to explain the actual phenomenon involved in cisplatin toxicity in colon/intestines, but the most accepted one involves reactive oxygen species (ROS) generated by cisplatin [2,57]. Accumulation of ROS as a result of cisplatin administration creates a condition called oxidative stress [11]. Since cisplatin and other anti-cancer therapies are associated with numerous side effects, natural phytochemicals become a relatively good choice for patients in chemotherapy. Natural compounds with tremendous antioxidant activity are noticed and explored for their role

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in mitigating and countering cisplatin-induced colon toxicity [6,11,58]. In our study, the protective properties of myricetin are speculated to be linked with mitigating oxidative and inflammatory response in the colon of experimental animals. Cisplatin administration creates hydroxyl radical (•OH), superoxide anion (O2•−), and hydrogen peroxide (H_2O_2), which play a crucial role in the instigation of lipid peroxidation [59]. Chang et al. (2002) [60] reported that cisplatin treatment initiates an autocatalytic lipid peroxidation reaction that amplifies the levels of fatty acids in the mucosal layer of the intestine. The long-chain free fatty acids are speculated to be responsible for functional and structural abnormalities in subcellular and plasma membranes [61]. One of the key markers of oxidative stress, malondialdehyde (MDA), is an intermediate product for lipid peroxidation, and it has been found to increase after cisplatin treatment [6,11,60,62]. Our results showed a similar pattern of increased lipid membrane peroxidation as reported previously, and treatment with myricetin reduced the abnormal levels of MDA [63] (Figure 2).

Enzymatic and non-enzymatic antioxidants act as the most important defense to counter free radicals in any biological system [64,65]. Glutathione, both in reduced and oxidized state, is the main redox buffer system inside the cell. The oxidized state of glutathione as co-enzyme or co-factor is directly involved in enzymatic detoxification reactions for ROS, or it can act as non-enzymatic antioxidant by ensuring a direct interaction of the –SH group with ROS [6,64,65]. The Thiol (–SH) groups, known to be sensitive to oxidative damage, decreased with cisplatin treatment. Myricetin treatment enhanced the thiol (–SH) content, proving that myricetin helped in restoring the complete thiol pool. This effect of myricetin on total thiol is attributed to its antioxidant effect and potential to enhance biosynthesis of GSH [63,66]. Our data is in agreement with past studies, showing similar trend in cellular GSH levels for treatment with antioxidants (Table 1) [6,11,34,66]. Xanthine oxidase (XO) is a key cellular enzyme that reduces oxygen to superoxide anion radical and, as a result, produces oxidative burst [67]. XO levels were enhanced after the cisplatin treatment, which is in agreement with previous reports. Myricetin was found instrumental in reducing XO levels [11,68]. Also, the antioxidants in intestinal tissue is drastically decreased in the cisplatin administered animals due to the decreased activity of antioxidant enzyme pool, viz., Catalase (Cat), glutathione peroxidase (GPx), glutathione reductase (GR), and Glucose-6-phosphate dehydrogenase (G6PD) and a phase-II detoxifying enzyme, namely, Glutathione-S-Transferase (GST), and quinone reductase (QR). In addition, the levels of Superoxide dismutase (SOD) showed a steep increase [51,62]. Myricetin treatment restored the normal level of these antioxidant enzymes and improved the level of phase-II detoxifying enzymes in accordance with previous published reports [2,68] (Tables 1 and 2).

As reported earlier by many groups, and as discussed above, cisplatin-induced toxicity was closely associated with the generation of ROS [2,65]. Cisplatin causes extensive cellular damage and forms a platinum-based DNA adduct (Pt-DNA), which activates the p38 mitogen-activated protein kinase (MAPK) pathway and inflammatory pathway [69]. Rehman et al. (2014) and a few others have reported an increase in the tissue levels of the inflammatory mediators, along with the infiltration of cells, highlighting the role of inflammation in cisplatin-induced organ injury [70–72]. Nuclear factor kappa-B (NF-κB) is a well-known redox-sensitive transcription factor, and its activation is important for downstream inflammatory mediators such as TNF-α, IL-6, and so on, playing an important role in acute inflammatory processes and conditions linked to oxidative stress [73,74]. The dormant form of NF-κB is present in the cytoplasm attached to the inhibitory protein subunit IkB, which belongs to the transcriptional activator proteins of Rel family. ROS or other external stimuli facilitate the phosphorylation of IkB in the presence of IkB kinases and causes its disassociation leading to translocation from cytoplasm into nucleus [14,73,75].

Inflammation and oxidative insult are together considered crucial factors responsible for cisplatin-induced intestinal toxicity. Nuclear factor erythroid 2-related factor 2 (Nrf-2) has been shown to up-regulate the expression of the various antioxidants while role of ROS in inflammation in NF-kB mediated inflammation has been studied in cisplatin-induced organ injury [50]. Nuclear factor erythroid 2-related factor 2 (Nrf-2) is a basic leucine zipper transcription factor present in inactive form

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and associated with cytoskeleton-associated protein, Keap1, in cytoplasm [76]. The activation of this transcription factor is regarded as a crucial molecular target of several chemopreventive agents [77,78]. The function of Nrf-2 is to prevent oxidative stress via antioxidant response element (ARE)-mediated inductions of numerous phase-2 detoxifying enzymes, specifically HO-1 [78,79]. Generally, the activation of the transcription factor Nrf-2 is induced by cellular oxidative stress and acts as a marker of pro-oxidant stressors and electrophiles [76,80]. Many research groups have shown strong interaction between Nrf-2 and NF-κB [81,82]. Thimmulappa et al. (2006) [83] revealed that Nrf-2-deficient mice showed enhanced NF-κB activation with treatment with lipopolysaccharides. In addition, the disturbance in Nrf-2 was shown to enhance the levels of NF-κB and other pro-inflammatory cytokines after tissue injury [84,85]. We observed increased expression in NF-κB transcription factor in intestinal tissues after cisplatin treatment (Figure 3), while Nrf-2 showed a marked decrease in expression after cisplatin treatment (Figure 4). Myricetin was instrumental in normalizing the expression of both Nrf-2 and NF-κB in intestinal toxicity (Figures 3 and 4). Our claims are supported by the previous reports of Kilic et al. (2013), Sahin et al. (2010), and Youn et al. (2017) [78,79,86].

Cisplatin exposure is reported to be linked with increased levels of neutrophils and macrophages [6,87]. These activated neutrophils have been shown to provoke tissue injury via the accumulation and discharge of cytotoxic proteins (e.g., myeloperoxidase) and ROS into the extracellular fluid [88]. The infiltration of neutrophil is an important marker of acute inflammatory response, and is measured indirectly as myeloperoxidase activity (MPO). An increase in MPO due to cisplatin toxicity is responsible for tissue damage and inflammation [88]. Federico et al. (2007) [89] and a few others have reported that an increased nitrite level is exhibited in many pathological conditions [14,15]. Further reaction of the superoxide anion with nitric oxide (NO) results in the formation of peroxynitrite in large quantities. Peroxynitrite is a very aggressive cellular oxidant and gives rise to increased 3-nitroL-tyrosine [90]. Cisplatin toxicity has been reported to be associated with increased NO and MPO levels, with similar patterns being observed in our experiments [91,92], as shown in Figure 2. Myricetin treatment attenuated this abnormal level of NO and MPO in accordance with previous reports [93,94] (Figure 2).

The pro-inflammatory cytokines (IL-6 and TNF-α) have been shown to play a crucial role in cisplatin-induced organ injury [6,11,75]. TNF-α is a key inflammatory cytokine actively involved in many pathophysiological conditions involving inflammation [15]. TNF-α causes the local generation of reactive nitrogen species (RNS) via nitric oxide synthase induction and hence enhances the oxidative stress leading to organ injury. Inhibitors of TNF-α have been extensively used in the recent past as a strategy to counter anti-cancer drug-induced inflammatory conditions [95]. Recent studies have revealed elevated levels of TNF-α in animal model of cisplatin-induced organ toxicity [6,11,74,96]. TNF-α, as one of the major players responsible for inflammatory responses, directly affects the production of other chemokines and cytokines, leading to injury in the colon [96,97]. In our experiment, there was also a similar trend in the levels of TNF-α after cisplatin treatment was observed. This established the role of TNF-α in the patho-physiology of cisplatin-induced colon toxicity [96,98]. With the activation of NF-κB by cisplatin, cytokine-like TNF-α can mediate the release of a considerable amount of IL-6, leading to inflammatory conditions. Naturally occurring compounds, which are the inhibitors of IL-6 and TNF-α, have been used to counter anticancer drug toxicity [73,99,100]. Myricetin treatment was effective in attenuating cisplatin-induced levels of TNF-α and IL-6 (Figure 5). Similar reports that support the role of flavonoids in countering anti-cancer drug toxicity have been published by Rehman et al. (2014a), Khan et al. (2012), Semwal et al. (2016), and Hassan et al. (2017) [6,11,24,66].

In the colon, mucosal barriers have multi-pronged defense mechanisms that specifically respond to oxidative insults in order to maintain constant homeostasis [58]. Many preclinical and clinical groups have publicized that anticancer drugs cause severe damage to the intestinal microflora [101,102]. The goblet cells, which represent the first line of defense of the mucosal surface, are highly advanced exocrine cells of colonic crypts that mainly synthesize mucin and are enriched with threonine and serine. The actual building blocks that give the mucus its properties are highly glycosylated proteins

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called mucins; they form a protective gel-like covering in the gastrointestinal lumen and are secreted by goblet cells [103]. The normal functions of the goblet cells are necessary for countering oxidative insults, inflammatory disorders, and intestinal infections [58]. In accordance with previous findings cisplatin caused an extensive disintegration of the goblet cells in the crypts of the colon [11]. Myricetin treatment showed promising activity in restoring the damaged crypts and also prevented mucin depletion (Figure 6).

5. Conclusions

The actual mechanism of action of myricetin in preventing cisplatin toxicity has not been revealed yet. The findings from the current study show that myricetin demonstrated a protective effect in cisplatin-induced toxicity by controlling oxidative stress, tissue damage and inflammation. A combination of myricetin and cisplatin as combinational therapy offers a better alternative for reducing the side effects of cisplatin and improving chemotherapeutic value. Further pre-clinical and clinical studies should be undertaken for the above combinations to determine the actual mechanisms and effects in humans.

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