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Protective effect of Naringin flavonoid on cisplatin induced hematotoxicity and hepatotoxicity in wistar rats

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Abstract

Cisplatin is an important anticancer drug used in treatment of wide range of cancers. Even though it has got severe adverse effects which are linked with elevated oxidative stress. The objective of the present study was to investigate the possible protective effect of flavonoid Naringin against the oxidative stress induced by cisplatin (CP) on hematic parameters and hepatic antioxidant biomarkers in *wistar* rats. Animals were randomly divided into four equal groups in separate cages, six rats in each: control (saline 1ml p.o.), Cisplatin (7.5 mg/kg once i.p), combination of cisplatin and Naringin (50mg/kg p.o. for 21 days) and combination of cisplatin and Naringin (100mg/kg p.o. for 21 days). Blood samples were collected and subjected for evaluation of haematological parameters (RBC, WBC, Hb, and Platelets) and serum biomarkers (ALT, AST & ALP). Livers were excised from rats and subjected for antioxidants (GSH, MDA, SOD & Catalase) evaluation and histopathological study. Naringin provided a beneficial ($P<0.001$) role in CP-induced hemotoxicity by increasing RBC, Hb, PCV & platelets. Naringin also showed significant ($P<0.001$) protection against CP induced hepatotoxicity by decreasing ALT, AST, ALP, MDA level and by increasing the GSH, SOD, catalase, due to its ability to reduce free radical-induced oxidative damage in the liver and to scavenge the hydroxyl and peroxyl radicals. The present findings clearly suggest that Naringin protects against cisplatin induced oxidative stress on blood cells and hepatic antioxidant defence system.

Keywords: Naringin, Cisplatin, Haemotoxicity, Oxidative Stress, Hepatotoxicity.

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1. Introduction

Cisplatin is one of salutary anticancer drug extensively used for chemotherapy of different kinds of tumours. Even though, its usage in clinical area is limited because of its toxic effects like renal toxicity, haemotoxicity, ototoxicity, neurotoxicity and liver toxicity[1]. The major mechanisms for cisplatin induced toxicities are development of reactive oxygen species (ROS) and nitrogenous compounds by oxidative stress injury and depletion of antioxidant defence role[2]. For inhibiting the adverse effects caused by cisplatin without affecting its anticancer property several experimental animal models were performed in combination of CP along with various free radical scavengers, enzyme inhibitors, SH- containing antioxidants and natural herbs and plant constituents with antioxidant property [1,3,4].

Presently people receiving diet in the form fruits, flowers, leaves, teas and vegetables contain nutritive and non nutritive constituents which have rich antioxidant property. Flavonoids are secondary metabolites with broad spectrum pharmacological activities such as cardioprotective, anti-inflammatory, antioxidant, antimutagenic, nephroprotective, hepatoprotective, anticarcinogenic, antiallergic, modulation of enzymatic activities etc. It has been already proved that different flavonoids (morin, rutin, hespiridin etc) showed antioxidant properties and protect against some of the adverse effects caused by anticancer drugs. [5,6]

Naringin is a pivot flavanoid belongs to flavones

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group derived from citrus fruits such as grapes, orange and lemon. The bitter principle Naringin has wide pharmacological effects such as antibacterial, antioxidant, anti-inflammatory, anti-diabetic, anti-estrogenic, anti-Alzheimer's disease, antiobesity, anti-allergic, antiviral, anti-thyroid, and anticancer such as lung cancer, breast and cervical cancer.[7`]

The current study was undertaken to investigate the effect of Naringin on Cisplatin induced hematotoxicity and hepatotoxicity in experimental animals.

2. Materials and Methods

2.1 Animals

Albino wistar rats of either sex weighing 170-220g were procured from animal house of Shree Devi College of pharmacy, Mangalore. They were acclimatized to controlled conditions of temperature (23±2^{0}C), 30-70% humidity and 12 hr light-dark cycles. The animals were randomized into experimental and control groups and housed four each in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free assessed to standard pellets as basal diet and *water ad libitum*. All the studies conducted were approved by Institutional Animal Ethical Committee (IAEC), Shree Devi College of pharmacy, Mangalore, Karnataka. According to prescribed guidelines of committee for the purpose of Control and Supervision of Experiments in Animals (CPCSEA), Government of India.

2.2 Chemicals

Cisplatin was procured from Sigma Aldrich, Bangalore and other chemicals used were of analytical grade and purchased from standard companies. Biochemical kits were procured from Robonik India Pvt Ltd, Mumbai.

2.3 Experimental Design

Naringin was extracted from the peels of Citrus fruits by HPLC method[8]. After one week of acclimatization, the rats were randomly divided into four equal groups in separate polypropylene cages, six rats each. Group I was used as normal control & received normal saline 1ml p.o. Group II was used as negative control and received single i.p. dose of Cisplatin (7.5 mg/kg) on 16th day, after successive administration of distilled water (1 ml, orally). Group III & IV were used as test groups with low & high dose and received Naringin 50mg/kg p.o. and Naringin 100mg/kg p.o. respectively for 21 days.[9]

2.4 Samples collection

After 21 day dosing, on 22nd day (6 days after Cisplatin injection), the rats were anesthetized by ether inhalation. Blood was collected by a direct intra-cardiac puncture from each rat. Two blood samples from each rat were collected one sample was collected on EDTA (heparinised tubes) for determination of hematological parameters and the other one left to clot at 37^{o}C and centrifuged at 3000 rpm for 15 min. The serum (supernatant) was collected for biochemical analysis.[9]

2.5 Tissue samples

A vertical midline thoracic and abdominal incision in each animal was made to explore their viscera. Liver of each animal was isolated, cleaned from their surrounding fat and connective tissue, washed with normal saline solution, blotted with filter paper, weighed and rinsed in ice-cold saline. Half of each liver was homogenized for biochemical analysis and the other half was processed for histological examination.

2.6 Hematological parameters estimations

The heparinised blood samples were analyzed for the count of red blood cells (RBCs), white blood cells (WBCs),and platelets, hemoglobin concentration (Hb%), packed cells volume (PCV), mean corpuscle volume (MCV), mean corpuscle hemoglobin (MCH), mean corpuscle hemoglobin concentration (MCHC) using sysmex haemocytometer.

2.7 Biochemical estimations

Hepatic biomarkers assessment: The levels liver biomarkers such as ALT, AST&ALP from one portion of heparinised serum were analyzed by using commercial kits (Robonic) by using semi autoanalyser (Robonik).

2.8 Endogenous antioxidants assessments

Half portion of liver of each rat was homogenized in ice-cold 10%Trichloroacetic acid phosphate buffer, saline (0.05M, pH 7.4). The homogenates were centrifuged for 15000 rpm for 15 minutes. The obtained supernatants were used for estimations of SOD, GSH, MDA & Catalase by colorimetric method.

The lipid peroxides content was determined by estimating MDA using the thiobarbituric acid test. The supernatant (600µL) was mixed with 200µL 1.2% thiobarbituric acid and the mixture was heated at 98°C for 30 min. After cooling to room temperature, the absorbance of the supernatant was measured at 532 nm ($\in = 1.56 \times 105$ M^{-1} cm^{-1}). The results were expressed as micromoles MDA per gram of wet tissue (µmol/g tissue). [10-11]

The liver GSH level was measured by using Ellmanns method. [12] About 3ml of supernatant was added to 2ml phosphate buffer (pH7) & 5ml of water. Then 0.02ml of DTNB solution was added to 3ml of above mixture & absorbance was immediately noted at 412 nm[12]. The amount of glutathione was calculated using absorption co-efficient, $\in = 13.6 \times 104$ $M^{-1}cm^{-1}$.

Catalase activity was measured by the method of Aebi (1974). 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50mM phosphate buffer (pH 77.0). Reaction was started by the addition of 1.0 ml of freshly prepared 30mM H2O2. The rate of decomposition of H2O2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein.[13]

SOD activity of the liver tissue was analyzed by the method described by Kakkar et al. (1984). SOD activity in hepatic tissues was assessed based on the ability of the enzyme to inhibit nitroblue tetrazolium (NBT) reduction by superoxide. The results were expressed as U/mg protein. [14]

2.9 Histopathological Study

Specimens of each liver samples were fixed in 10% buffered formalin and processed with paraffin wax. For histopathological feature examination, 5µm sections were stained with hematoxylin and eosin for the examination using light microscope[15].

3. Statistical analysis

The data obtained by the various parameters was statistically evaluated by one way Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests using Graph Pad InStat software. The mean values ±SEM were calculated for each parameter.

4. Results

4.1 Hematalogical studies

Naringin pre-treated rats of higher & lower dose groups were showed significant (P>0.001) refinement in majority of blood cell parameters compared to CP treated rats. These findings were identified by rise in RBC, HB, Platelets count &PCV. And more over significant decrease (P>0.001) in RBC & platelets and increase (P>0.001) in WBC were observed in CP treated group (Table 1).

4.2 Hepatic biomarkers studies

Naringin pre-treated rats of higher & lower dose groups were showed significant (P>0.001) decrease in the level of ALT, AST & ALP compared CP treated rats. But compared to normal group a significant rise (P>0.001) in the level of ALT, AST & ALP were observed in CP treated rats (Table 2).

Table 1. Effect of Naringin on blood cells parameters in cisplatin induced hemotoxicity in rats

Hematological parameters	Normal	Cisplatin	Naringin (50mg/kg)	Naringin (100mg/kg)
RBC (10^6cell/µL)	7.12±0.24	3.57±0.19###	4.90±0.08***###	6.19±0.12***##
Hb (g/dL)	14.63±0.14	10.16±0.06 ###	12.2±0.17*** ###	14.33±0.15***
WBC (10^3cell/µL)	4.99±0.08	7.43±0.11###	5.66±0.12***##	5.16±0.12***
Platelets (10^5cell/µL)	6.53±0.23	3.05±0.21###	5.16±0.05***###	5.85±0.06***#
PCV (%)	46.24±0.45	25.54±0.89###	32.65±1.15***###	43.65±0.98***
MCV (fL)	65.38±2.59	72.32±3.72	66.61±2.37	70.74±2.53
MCH (pg)	20.64±0.52	28.91±1.68###	24.88±0.15*#	23.20±0.56***
MCHC (g/dL)	31.67±0.55	40.05±1.39###	37.58±1.35 ##	32.89±0.67***

n=6, values are expressed in Mean ± SEM, one way ANOVA followed by Tukey-Kramer multiple comparison test. ***p<0.05, **p<0.01, ***p<0.001** when compared to cisplatin group and **#P<0.05, ##P<0.01, ###P < 0.001** compared to normal group.

Table 2. Effect of Naringin on serum biomarkers in cisplatin induced hepatoxicity in rats

Treatment	Normal	Cisplatin	Naringin (50mg/kg)	Naringin (100mg/kg)
AST (IU/L)	155.16±1.93	511±3.38###	366.33±4.97***###	289.5±1.8***###
ALT (IU/L)	62.5±1.89	167.66±2.95###	110±1.77***###	81.83±2.76***###
ALP (IU/L)	139.66±1.05	270.33±1.76###	224.66±2.02***###	156.66±1.74***###

n=6, values are expressed in Mean ± SEM, one way ANOVA followed by Tukey-Kramer multiple comparison test. ***p<0.05, **p<0.01, ***p<0.001** when compared to cisplatin group and **#P<0.05, ##P<0.01, ###P < 0.001** compared to normal group.

4.3 Endogenous antioxidants assessment

A significant decrease (P>0.001) in MDA level was observed in Naringin pre-treated rats of higher dose group compared to CP treated group and significant rise in MDA level was observed in CP treated rats. Naringin pre-treated rats of higher & lower dose groups were showed remarkable rise (P>0.001) in content of GSH compared to CP treated rats and significant decrease in GSH level was observed in CP treated rats compared to normal group. There was remarkable increase (P>0.001) in SOD & catalase units were observed in Naringin pre-treated rats of higher & lower dose groups compared to CP treated group. But compared to normal group a remarkable depletion (P>0.001) in SOD & Catalase were found in CP treated rats (Table 3).

4.4 Histopathological study

A normal architecture of liver was maintained with central vein, sinusoids and hepatocytes in normal group. CP treated group reveals hepatic architecture was disrupted, showed various degree of degeneration of hepatic cells, fatty cells, and congestion of liver sinusoids and proliferation of kupffer cells. Protective effect of Naringin pre-treated groups on histopathology study showed compared to CP treated group remarkable improvement in hepatic architecture, sinusoids appearance and areas of kupffer cell proliferation (Figure 1).

Table 3. Effect of Naringin on endogenous antioxidants in cisplatin induced Hepatoxicity in rats

Treatment	Normal	Cisplatin	Naringin (50mg/kg)	Naringin (100mg/kg)
GSH (ηM /g wet gland)	19.91.04±0.85	9.86±0.29###	14.11±0.53***###	18.51±0.20***
MDA (ηM /g wet gland)	11.33±0.32	28.46±1.17###	26.46±1.09 ###	13.56±0.23***
CATALASE (U/mg wet gland	48.35±1.04	20.96±0.69###	34.91±0.54***###	43.03±0.44***###
SOD (U/mg wet gland)	89.88±0.45	60.45±0.76###	73.03±0.74***###	83.8±0.44***###

n=6, values are expressed in Mean ± SEM, one way ANOVA followed by Tukey-Kramer multiple comparison test. ***p<0.05, **p<0.01, ***p<0.001** when compared to cisplatin group and **#P<0.05, ##P<0.01, ###P < 0.001** compared to normal group.

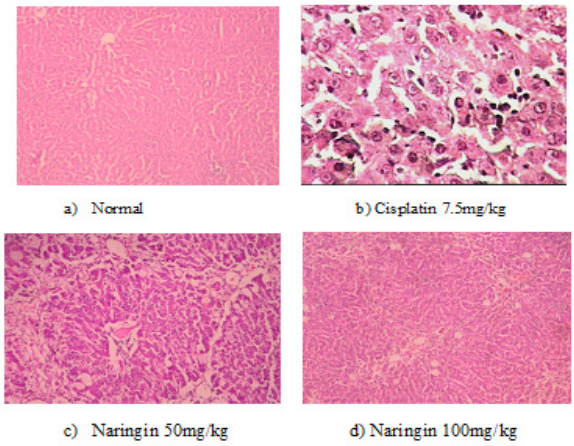


Figure 1. Histological architecture of hepatic tissues in different experimental groups (H and E, X400). H and E = Hematoxylin and Eosin

a) Normal control group (normal texture of hepatocytes and lobules)
b) Cisplatin (7.5mg/kg) control group (degeneration of hepatocytes and hepatic cords, inflammatory infiltration, dilated sinusoids.)
c) Naringin (50mg/kg) low dose group (mild degenerative changes, slight inflammation, dilated sinusoids.
d) Naringin (100mg/kg) high dose group (small focal degeneration, no inflammation, congestion of sinusoids.

5. Discussion

In the present study proposed plan aimed to evaluate the possible protective effect of Naringin on cisplatin induced hemotoxicity and hepatotoxicity in wistar rats.

Cisplatin is an effective anticancer agent widely used against broad spectrum cancers like testicular, ovarian, lung cancer as well as solid tumours. Unfortunately it has several toxicities and side effects like hepatotoxcity, nephrotoxcity, myelosuppression, bone marrow suppression, gastrointestinal toxicity, neurotoxicity, ototoxicity and hypersensitivity reactions.[16]

Varieties of natural compounds and dietary sources have been illustrated as potential protective antioxidant molecules against cisplatin induced hemotoxicity and hepatotoxicty[9]. Flavonoids constitute a group of polyphenolic agents that have free radical scavenging activity & iron chelating properties. Therefore, flavonoids considered as possible protectors against cisplatin induced toxicities.[17]

Naringin is a bitter flavonoid found in citrus fruits and is most common in citrus paradise. Naringin has proved to possess anti-inflammatory, regulation of antioxidant property, antilipoperoxidative activity.[18]

Haemopoietic system is referred to as one of more susceptible system to assess the adverse effects caused by anticancer drugs or other poisons in humans or animals[9]. In the present study administration of single dose of cisplatin showed significant toxic effects in haematological parameters in *wistar* rats six days after treatment and the pre-treatment with Naringin had significantly protected against haematological adverse effects caused by cisplatin. The adverse effects of cisplatin on haematological parameters were identified by considerable decline in count of RBC, PCV, Haemoglobin, Platelets. The results demonstrate an etiological link between anaemia and cisplatin treatment. This could be described by different mechanisms involving destruction of bone marrow cells or higher osmotic fragility of erythrocytes. So, cisplatin treatment might cause anaemia due to suppression of haemopoietic tissue, disturbance in myelosuppression and elevated erythrocytes destruction because of the impaired erythrocyte membrane permeability and defective iron biotransformation.[19]

Furthermore, apart from decline in erythrocytes, chronic administration of cisplatin leads to decrease in platelet count and rise in leucocytes. The decline in thrombocytes could be due to myelosuppression or might be due to thrombocyte aggregation[20]. It was already proved that cisplatin induces oxidative stress in human thrombocytes and lymphocytes leads to apoptosis by affecting their life span. Further, the elevation in leucocyte count might be the consequence of inflammation reactions or infection during cisplatin treatment & its metabolism in wistar rats[21].

The experimental rats pre-treated with Naringin revealed significant protection against CP induced toxic effects on blood cells. Naringin showed beneficial effects by elevating RBC count, Hb%, PCV and rise in platelet count against Cisplatin induced adverse effects on haematological markers. Elevation of erythrocyte count by Naringin might be related to either stimulation of erythropoiesis process or to the capacity of Naringin in reducing membrane rigidity inherent to decreasing cholesterol effects[22]. These findings revealed that Naringin had preventive & protective effects against haematological changes caused by Cisplatin.

The observed depletion in GSH content and rise in MDA levels in addition diminish in units of antioxidant enzymes (SOD & Catalase) were the evident of oxidative injury caused by Cisplatin administration [1,2,3].

The enhancement in lipid peroxidation by rise in MDA value might be due to generation of oxygen free radicals caused by cisplatin. It was already demonstrated that the oxygen free radicals can produce extensive tissue destruction by interacting with membrane lipids, proteins and nucleic acids[23].

The depletion in glutathione content thought to be vital factor in cellular activity and defence against oxidative stress. The decrease in GSH content by cisplatin leads to elevation of lipid peroxide value. Cisplatin in a cell is highly reactive form and immediately react with thiol containing agent; glutathione. Furthermore, inhibition in SOD & catalase enzyme was already demonstrated in the pathogenesis of cisplatin induced liver injury.[23]

Several antioxidants from natural sources were showed beneficial effects against cisplatin induced oxidative stress in liver[1,9]. The pre-treatment with Naringin induced significant elevation in hepatic GSH, Catalase and SOD values along with significant decline in MDA values in Cisplatin administered rats. It was already demonstrated that Naringin showed inhibitory effects on Lipid peroxides generation from ethyl arachidonate and free radical scavenging action in kidney tissues as well as suppression amyloid β-protein induced free radical mediated neurotoxicity. Thus, Naringin considerably ameliorated

lipid peroxidation and oxidative stress injuries in hepatic tissues caused by cisplatin by its antioxidant action[18].

AST, ALT & ALP are considered as more sensitive serum marker enzymes in assessing function and integrity of hepatic cells because these are located in cytoplasmic region and released in circulation after cellular destruction[2,24]. In the present study rats treated with single dose of cisplatin were exhibited significant elevation in the levels AST, ALT & ALP. The rise in the levels of these serum enzymes represents the hepatic dysfunction & cellular disruption followed by necrotic lesions in liver cells. The pre-treatment with Naringin exhibit significant decline in AST, ALT & AST in cisplatin treated rats. The decrease in levels of liver enzymes in Naringin pre-treated rats might be due to its free radical scavenging action that reduces leakage of enzymes into blood stream & stabilizes membrane permeability[18].

The present investigation revealed many histopathological abnormalities in liver like hyperplasia, inflammatory infiltration, periportal fibrosis, elevated disruption of hepatic cords and dilated blood sinusoids in cisplatin induced hepatic injury. It was already reported that alteration in hepatic area, where wide portal ducts, apoptosis, lobular necrotic changes, dilated congested sinusoids and central vein were observed in cisplatin induced hepatotoxicities[25]. The Naringin pre-treated rats were showed significant efficacy in histopathological findings in cisplatin induced hepatic injury.

The improvement revealed in histopathological degenerations of hepatic tissue by Naringin due to its capacity to diminish the free radicals induced oxidative stress in liver and to scavenge the peroxyl and hydroxyl radicals. Moreover, it's already reported that Naringin treatment suppress the liver damage and protects the antioxidant defence system in DMBA-induced hepatic injury[26]. Thus, Oral administration of Naringin revealed significant efficacy in haematological, serum biomarkers and antioxidants and histopathological manifestations induced by Cisplatin through its free radical scavenging and antioxidant defence activities.

6. Conclusion

The current study demonstrates that administration of cisplatin induces hepatic tissue damage through lipid peroxidation and oxidative stress noted by changes in liver biomarkers, lipid peroxidation (MDA), antioxidant enzymes (GSH, catalase & SOD) and histopathological studies. Also, different haematological toxicities were exhibited in cisplatin induced rats. The pre-treatment with Naringin possesses protective role by its antioxidant & free radical scavenging properties in cisplatin induced hematotoxicity and hepatotoxicity in rats. Thus, Naringin may be used as vital supplementary agent to patients treated with anticancer drugs.

7. Conflict of Interest

None Declared

8. References

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