

Protective Effect of Ferulic Acid Against Cyclophosphamide-Induced Nephrotoxicity and Urotoxicity

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Abstract

Cyclophosphamide (CYP) is a widely used chemotherapeutic agent known for its efficacy in treating cancer and other conditions. However, its clinical use is limited by nephrotoxicity and urotoxicity, which can significantly affect patient quality of life. These toxicities are primarily attributed to the accumulation of acrolein, a toxic metabolite of CYP that induces oxidative stress and inflammation. To mitigate these side effects, natural products such as ferulic acid (FA), a phenolic compound with antioxidant and anti-inflammatory properties, have gained attention. This study aimed to evaluate the nephroprotective and uroprotective effects of FA in an in vivo model of CYP-induced toxicity in male Wistar albino rats.

Rats were divided into four groups: control, CYP-treated, and two groups receiving FA at doses of 50 mg/kg and 100 mg/kg. The animals were treated for 14 days, followed by CYP administration on Day 14. Parameters assessed included urine output, serum urea and creatinine levels, antioxidant enzyme activities, bladder weight, nociceptive behavior, and histopathological changes in kidney and bladder tissues.

Results showed that CYP treatment significantly reduced urine output, increased serum urea and creatinine levels, and elevated oxidative stress markers in both kidneys and bladder tissues. FA treatment, particularly at the higher dose (100 mg/kg), restored urine output, reduced renal dysfunction, enhanced antioxidant enzyme activities, and decreased oxidative stress markers. Additionally, FA significantly reduced bladder weight, alleviated nociceptive behavior, and improved tissue architecture, with the 100 mg/kg dose showing superior protective effects.

Histopathological analysis confirmed the protective effects of FA, with a marked reduction in CYP-induced damage to kidney and bladder tissues. The findings suggest that FA, through its antioxidant and anti-inflammatory properties, offers significant protection against CYP-induced nephrotoxicity and urotoxicity. This study highlights the potential of FA as a therapeutic adjunct to mitigate the adverse effects of CYP in clinical settings, warranting further research into its clinical applicability.

Keywords; Cyclophosphamide; Nephrotoxicity; Urotoxicity; Ferulic acid; Oxidative stress.

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Introduction

Cancer comprises a range of distinct diseases marked by unregulated cell proliferation, dysregulated growth, the ability to invade nearby tissues, and the potential to spread to distant organs ie metastasis[1].

Cyclophosphamide (CYP) is a powerful cytotoxic agent commonly used to treat a variety of conditions, including cancer, organ transplants, and inflammatory diseases[2]. However, its clinical utility is limited by severe adverse effects, particularly nephrotoxicity and urotoxicity, which impair renal function and damage the urinary bladder, thereby diminishing the quality of life for cancer patients[3].

Nephrotoxicity is one of the leading causes for the kidney problems in the form of acute and chronic renal failure which in turn causes anaemia, bone disorders, CVS risk, dyslipidaemia and nephritis when the body is exposed to a drug or toxic substances [4]. Though cancer treatment success rates are rising due to newer therapies, high-dose combination therapies etc concerns about the long-term side effects of these therapies are also increasing[5]. These toxicities are primarily attributed to the production of acrolein, a toxic metabolite of CYP, which induces oxidative stress, inflammation, and cellular injury in renal and bladder tissues[6].

The pathophysiology of cyclophosphamide (CYP)-induced nephrotoxicity is primarily associated with its metabolite, acrolein, which promotes the generation of reactive oxygen species (ROS). This leads to glutathione (GSH) depletion and compromised antioxidant defenses, resulting in oxidative stress, inflammation, and vascular dysfunction. These events collectively contribute to a reduction in glomerular filtration rate (GFR) and structural tubular injury [7]. The alkylating agent cyclophosphamide (CPA) belongs to the oxazaphosphorine class. An alkylating agent

is a type of drug that binds to the cell's nucleophilic DNA and prevents it from replicating. Although previous studies have suggested that CPA does not exert direct nephrotoxic effects—attributing its toxicity largely to the urotoxic nature of acrolein—histopathological findings have demonstrated significant renal alterations, such as acute inflammatory cell infiltration in the cortex, tubular cell edema, loss of the brush border, and pyknotic nuclear changes [8]. Several studies have been reported that oxidative stress mediated disruption of redox balance after CP exposure generates biochemical and physiological disruptions [9].

The clinical utility of cyclophosphamide is further constrained by its potential to cause bladder toxicity, most notably hemorrhagic cystitis (HC) [10,11]. HC is characterized by damage to the bladder urothelium, edema, and the presence of blood clots [12]. The underlying mechanism of CYP-induced urotoxicity also involves acrolein, which generates ROS and nitric oxide, leading to lipid and protein oxidation, DNA damage, and necrotic cell death. These processes culminate in bladder inflammation, overactive bladder symptoms, and, in severe cases, hemorrhage [11].

To reduce the urotoxic effects of cyclophosphamide (CYP), detoxification can be achieved through aggressive diuresis and the administration of Mesna. However, Mesna is not always fully effective in preventing hemorrhagic cystitis and may cause allergic reactions ranging from mild skin issues to severe anaphylaxis. As a result, researchers are investigating alternative treatments, with increasing interest in natural products to mitigate CYP-induced HC.[13][14]

Ferulic acid (FA), a naturally occurring phenolic compound, has garnered significant attention for its potent antioxidant, anti-inflammatory, and cytoprotective properties. Found abundantly in cereals, fruits, and vegetables, FA has been shown

to neutralize free radicals, stimulate endogenous antioxidant enzyme activity, and alleviate tissue damage caused by oxidative stress[15][16]. Ferulic acid is most often found in anti-aging skin creams, where it is believed to neutralize free radicals that damage and age cells. [17]

The present study evaluates the nephroprotective and uroprotective potential of FA in an in vivo model of cyclophosphamide-induced nephrotoxicity and urotoxicity in male Wistar albino rats. By assessing functional, biochemical, and histopathological parameters. The findings could pave the way for the development of adjunctive therapies to improve the therapeutic outcomes of CYP-based chemotherapy regimens, enhancing patient safety and quality of life.

Materials and Methods

Chemicals and Reagents

Ferulic acid and cyclophosphamide (CYP) were obtained from Sisco Research Laboratories Pvt. Ltd. (SRL), Maharashtra, India. The dosages and routes of administration for CYP and Ferulic acid were selected based on previously published literature [18] [19].

Animals and Experimental Conditions

Adult male Wistar rats (180 ± 5 g) were used for the experiments. The animals were housed individually in standard cages under controlled environmental conditions: temperature (24–28°C), relative humidity (60–70%), and a 12-hour light/dark cycle. The rats were provided with ad libitum access to a standard pellet diet and water. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India

Experimental design

The rats were divided into four groups (n = 6 per group) and treated as follows:

- Group 1 (Control Group): Received sterile water (1 mL/kg) via oral gavage for 14 days.
- Group 2 (CYP Group): Administered Cyclophosphamide (150 mg/kg) via intraperitoneal injection on Day 14.
- Group 3 (CYP + FA 50 Group): Received Ferulic acid (50 mg/kg) orally daily for 14 days and Cyclophosphamide (150 mg/kg) intraperitoneally on Day 14.
- Group 4 (CYP + FA 100 Group): Received Ferulic acid (100 mg/kg) orally daily for 14 days and Cyclophosphamide (150 mg/kg) intraperitoneally on Day 14.

Behavioral studies

Nociceptive behavior was assessed using the open field test, a validated method for evaluating pain-related behavior in animal models of hemorrhagic cystitis (urotoxicity). Nine square boxes were arranged in a grid, and each rat was placed individually in one box. The number of squares crossed was recorded over 10 minutes. Other parameters that can be studied include the frequency of crossing square, total numbers of squares crossed, center square entries, etc. [20]. [21], [22], [23].

Urine analysis

Following behavioral assessment on Day 14, the rats were housed in metabolic cages to collect urine samples. Circadian diuresis was measured over the next 24 hours.

Serum biochemistry

Blood samples were collected via cardiac puncture under anesthesia and centrifuged at 3000 rpm for 15 minutes to separate serum. Serum urea and creatinine levels, markers of renal function, were analyzed using commercially available assay kits [24].

Bladder weight assessment

After euthanasia, the urinary bladders and kidneys were excised, cleaned of excess fluid, and

weighed. Increased bladder wet weight was considered indicative of edema [25].

Tissue homogenization

The kidneys and urinary bladders were rinsed with ice-cold phosphate buffer and homogenized in a 10-fold (w/v) volume of ice-cold sodium phosphate buffer (KH_2PO_4 and Na_2HPO_4). The homogenates were centrifuged at 3000 rpm for 15 minutes at 4°C , and the supernatants were collected for biochemical assays.

Oxidative stress markers

Malondialdehyde (MDA): LPO was estimated colorimetrically by measuring malondialdehyde (MDA) formation as described by Nwanjo and Ojiako, 2005. In brief, 0.1 ml of homogenate was treated with 2 ml of a 1:1:1 ratio of TBA–TCA–HCl (TBA 0.37%, TCA 15%, HCl 0.25 N) and placed in water bath at 65°C for 15 min, cooled, and centrifuged at 5,000 rpm for 10 min at room temperature. The optical density of the clear supernatant was measured at 535 nm against a reference blank. The MDA formed was calculated by using the molar extinction coefficient of thiobarbituric acid reactants (TBARS; 1.56×10^5 l/mole cm^{-1}). The product of LPO was expressed as nmol of MDA formed per g of tissue. [26]

Superoxide Dismutase (SOD): Superoxide dismutase (SOD) activity was measured using a colorimetric assay that tracks the rate of autoxidation of hematoxylin. The presence of SOD inhibits this autoxidation, with the extent of inhibition correlating with the concentration of SOD in the sample. SOD activity was calculated by comparing the rates of autoxidation with and without the presence of the sample and expressed as units of SOD per gram of tissue.

Catalase (CAT): Catalase activity in tissue homogenates was assessed using a colorimetric assay that measures the decomposition of hydrogen peroxide at 240 nm. Since water and oxygen do not absorb at this wavelength, we can directly measure the absorbance associated with hydrogen peroxide. Catalase accelerates the

breakdown of hydrogen peroxide, so the reaction rate increases with higher catalase activity. Superoxide dismutase (SOD) activity was determined using a colorimetric assay that tracks the rate of autoxidation of hematoxylin. The presence of SOD inhibits this autoxidation, with the extent of inhibition correlating with the concentration of SOD in the sample. SOD activity was calculated by comparing the rates of autoxidation with and without the presence of the sample and expressed as units of SOD per gram of tissue. Results were expressed as units of catalase per gram of tissue[20].

Histopathological examination

Kidney and urinary bladder tissues were fixed in 10% formalin, processed, and embedded in

Groups	Urine Volume (ml)
Normal	8.26±0.38****
Control (CYP)	4.03±0.55
Ferulic acid 50	6.06±0.35**
Ferulic acid 100	6.73±0.25***

paraffin. Thin sections (5 μm) were cut and stained with hematoxylin and eosin (H&E) for microscopic examination. The histological assessment was conducted in a blinded manner.

Statistical Analysis

Data were analyzed using GraphPad Prism software (version 10.3.0, 507). One-way ANOVA followed by Dunnett's multiple comparison test was employed to compare treatment groups with the control group. Results are presented as mean $\pm$ SEM, with a significance level of $P < 0.05$.

Table 1 : Effect of Ferulic Acid (FA) on Urine Output

Results

Values are expressed as mean $\pm$ SEM * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $P < 0.0001$ when compared with positive control group.

Effect of Ferulic Acid (FA) on Urine Output

Cyclophosphamide (CYP)-treated animals demonstrated a significant reduction in urine volume compared to the control group ($P < 0.001$). Pretreatment with FA at a dose of 50 mg/kg significantly increased urine output in comparison to the CYP-treated group ($P < 0.01$). Moreover, a higher dose of FA (100 mg/kg) further enhanced urine output when compared to the 50 mg/kg dose

GROUPS	BLADDER WEIGHT(mg)
Normal	65.16±3.18****
Control (CYP)	125.0±2.50
Ferulic Acid 50	99.33±4.60***
Ferulic acid 100	74.16± 2.70****

($P < 0.001$).

Effect of FA on Renal Function (Urea and Creatinine Levels) Intraperitoneal administration of cyclophosphamide resulted in a significant increase in serum creatinine and urea levels ($P < 0.001$) in the cyclophosphamide-treated group. Treatment with ferulic acid notably reduced the elevated serum levels of these markers in cyclophosphamide-induced nephrotoxic rats. However, the reduction was even more pronounced in the FA 100 group compared to those treated with FA 50 Group ($P < 0.01$).

Table: 10, Effect of FA on Renal Function (Urea and Creatinine Levels)

Param eters	Normal	control(CYP)	Ferulic acid 50	Feruli c acid 100
Urea(mg/dl)	20.85±1. 29****	35.01±2 .04	27.15± 0.74**	24.52 ±1.53* **
Creati nine (mg/dl)	0.28±0.0 2****	2.38±0. 18	0.83±0. 03**	0.74±0 .04**

Values are expressed as mean ± SEM * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $P < 0.0001$ when compared with positive control group

Effect of FA on Antioxidant Enzyme Activity in Kidney Homogenates

CYP treatment significantly reduced the activities of superoxide dismutase (SOD) and catalase (CAT) ($P < 0.001$) and increased malondialdehyde (MDA) levels ($P < 0.01$) in kidney homogenates compared to the control group. Pretreatment with FA markedly enhanced SOD and CAT activities ($P < 0.001$) and lowered MDA levels ($P < 0.05$) compared to the CYP group. Furthermore, the FA 100 mg/kg group demonstrated a more significant increase in SOD and CAT activities and a greater reduction in MDA levels compared to the FA 50 mg/kg group ($P < 0.01$).

Table:12, Effect of FA and FANC on antioxidant enzymes in kidney homogenates

Values are expressed as mean ± SEM * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $P < 0.0001$ when

Paramet ers	Normal	Contr ol (CYP)	Ferulic Acid 50	Ferulic Acid 100
SOD(U/g tissue)	4.20±0.2 5***	1.19± 0.10	2.83±0.2 2***	3.29±0.0 7***
CAT(U/g tissue)	8.52±0.2 1****	3.17± 0.13	5.34±0.0 5***	7.08±0.7 9****
MDA nm ol/ml	0.90±0.0 2**	1.85± 0.22	1.31±0.1 2**	1.21±0.2 1**

compared with positive control group

Effect of FA on Bladder Weight

CYP treatment significantly increased bladder weight ($P < 0.001$) compared to controls. Pretreatment with FA significantly reduced bladder weight ($P < 0.001$), with the FA 100 mg/kg group showing a greater reduction compared to the FA 50 mg/kg group.

Table: 13 Effect of FA on Bladder Weight

Parameter s	Normal	control (CYP)	Ferulic acid 50	Ferulic acid 100
SOD (U/g tissue)	47.96±0.99****	20.06±0.77	88.02±0.27****	47.54±1.08****
CAT (U/g tissue)	56.47±0.38****	27.18±0.51	44.98±0.15****	47.73±0.63****
MDA (nmol/100g)	0.70±0.104***	1.97±0.23	1.25±0.20**	0.94±0.11**

Values are expressed as mean $\pm$ SEM * P<0.05, ** p<0.01, *** p<0.001, **** P<0.0001 when compared with positive control group

Assessment of Nociception Using the Open Field Test

In the open field test, CYP-treated animals displayed a significantly lower number of box crossings (9.0 ± 0.77) compared to the control group (23 ± 1.26). Pretreatment with FA improved locomotor activity, as evident by an increased number of crossings in the FA 50 mg/kg (15 ± 1.97) and FA 100 mg/kg (18 ± 0.51) groups ($P < 0.05$).

Table:14 Assessment of Nociception Using the Open Field Test

Values are expressed as mean $\pm$ SEM * P<0.05, ** p<0.01, *** p<0.001, **** P<0.0001 when compared with positive control group.

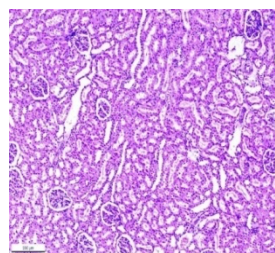
Effect of FA on Antioxidant Enzymes in Urinary Bladder Homogenates

CYP treatment led to a significant increase in MDA levels ($P < 0.01$) and a reduction in SOD and CAT activities ($P < 0.001$) in bladder homogenates compared to controls. Pretreatment with FA significantly reduced MDA levels ($P < 0.01$) and improved SOD and CAT activities ($P < 0.001$) in a dose-dependent manner, with the FA 100 mg/kg group showing superior effects compared to the FA 50 mg/kg group.

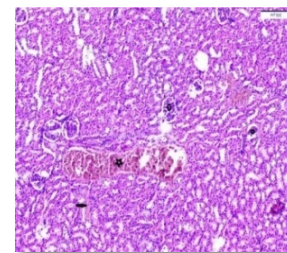
Table:16 Effect of FA on antioxidant enzymes in urinary bladder homogenates

Values are expressed as mean $\pm$ SEM * P<0.05, ** p<0.01, *** p<0.001, **** P<0.0001 when compared with positive control group.

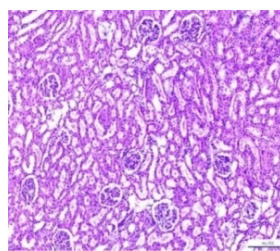
Histopathology of Kidney



(a)



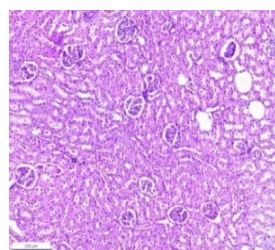
(b)



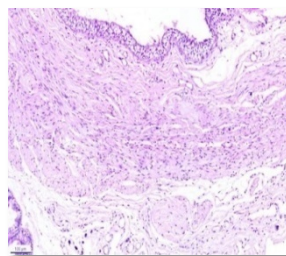
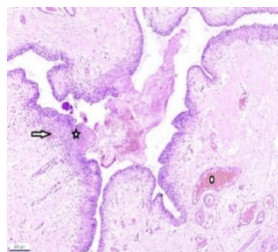
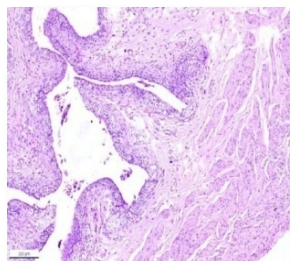
(c)

(d)

a) Kidney section showing normal histological features of glomeruli and tubules. b) Kidney section showing glomerular degeneration (round), tubular necrosis, degeneration and desquamation of tubular epithelium with interstitial hemorrhage (arrow) and vascular congestion (star). c) Kidney section shows improvement were noticed, however some glomeruli were degenerated, and tubular necrosis also evident. d) Kidney section Remarkable improvement were noticed, normal histological features of glomeruli and only focal areas of tubular necrosis were evident.



Histopathology of urinary bladder



a)-Bladder section showing normal Urothelium, sub mucosa and muscularis layer

b)-Bladder section showing -Urothelial ulceration and infiltration of inflammatory cells (star), hemorrhage (arrow), congestion (round) present at the submucosal layer and submucosal edema

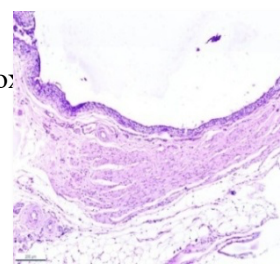
c)- Bladder section showing Normal Urothelium, sub mucosa and muscularis layer .

d)-Bladder section showing normal urothelium, sub mucosa and muscularis layer.

Discussion

Cyclophosphamide (CYP) has been utilized in clinical practice since the late 1950s for treating both cancerous diseases, such as solid tumors and lymphomas, as well as non-cancerous conditions like rheumatoid arthritis and systemic lupus erythematosus. However, CYP and its metabolites are widely recognized for causing acute urinary bladder inflammation and potential renal damage [27]. The primary mechanisms of CYP toxicity include the direct detrimental effects of acrolein on the tubular and bladder epithelium and the oxidative stress induced

by phosphoramidate mustard, its major alkylating metabolite [28],[29].



Ferulic acid (FA), a derivative of cinnamic acid found in fruits, vegetables [30]. and medicinal herbs [31]exhibits significant biological activity. It functions as an inhibitor of lipid peroxidation, and acts as a scavenger of various free radicals, such as hydroxyl and peroxy radicals [32],[33]. This study investigated the therapeutic effects of FA on CYP-induced nephrotoxicity and urotoxicity.

In the current study, CYP treatment significantly reduced urine output compared to the normal control group, the result consistent with previous findings that associate CYP with renal dysfunction due to oxidative damage [34]. Pretreatment with FA at doses of 50 mg/kg and 100 mg/kg restored urine output in a dose-dependent manner, with the higher dose exhibiting a more pronounced effect.

Renal function biomarkers, creatinine and urea, were also evaluated. These waste products are eliminated by the kidneys, and their elevated plasma levels indicate impaired renal function [35].CYP administration significantly increased serum creatinine and urea levels, aligning with earlier findings on CYP-induced renal dysfunction [24] .FA pretreatment significantly reduced these biomarkers, with the 100 mg/kg dose demonstrating superior efficacy. These results suggest that FA enhances renal filtration efficiency and mitigates CYP-induced renal impairment.

Oxidative stress and elevated reactive oxygen species production play a pivotal role in CYP-induced urototoxicity and nephrotoxicity. CYP-generated free radicals, damage lipids and alter membrane structure and function [36]. Additionally, CYP conjugates acrolein with glutathione (GSH), reducing intracellular GSH levels and increasing malondialdehyde (MDA), a marker of oxidative stress. This process leads to

lipid degradation and membrane damage [27]. FA pretreatment significantly restored superoxide dismutase (SOD) and catalase (CAT) activities while reducing MDA levels in kidney and urinary bladder homogenates. These findings align with FA's antioxidant properties, including free radical scavenging and upregulation of antioxidant defenses [37]. The dose-dependent efficacy observed further supports FA's role as an effective antioxidant in mitigating oxidative damage.

CYP-induced urototoxicity was evident from the increased bladder weight, likely caused by inflammation and edema due to CYP metabolites [38]. FA pretreatment significantly reduced bladder weight, with the 100 mg/kg dose showing superior efficacy.

Additionally, CYP-treated animals exhibited reduced locomotor activity in the open field test, indicative of pain and discomfort linked to cystitis [39]. FA treatment improved locomotor activity in a dose-dependent manner, suggesting anti-inflammatory and analgesic effects. These results align with previous studies highlighting FA's efficacy in reducing pain and inflammation [40].

Histopathological analysis of kidney and bladder tissues corroborated the biochemical findings. CYP-induced structural damage, including tubular necrosis and inflammatory infiltration, was markedly alleviated by FA treatment. The 100 mg/kg dose demonstrated better tissue preservation compared to the 50 mg/kg dose, emphasizing the dose-dependent protective effects of FA.

The protective effects of FA can be attributed to its robust antioxidant and anti-inflammatory properties. FA's ability to reduce oxidative stress, as evidenced by increased SOD and CAT activities and decreased MDA levels, underscores its role in mitigating CYP-induced damage. Additionally, FA's modulation of inflammatory pathways likely contributes to its therapeutic effects on renal and bladder dysfunction. These findings suggest that FA is a promising candidate

for managing CYP-induced toxicity, warranting further exploration in clinical settings.

Conclusion

Based on the results of this study, ferulic acid (FA) emerges as a potent protective agent against cyclophosphamide (CYP)-induced nephrotoxicity and urotoxicity. By restoring renal function, reducing oxidative stress, and alleviating inflammation, FA significantly mitigates the damaging effects of CYP on kidney and bladder tissues. The dose-dependent efficacy, particularly at 100 mg/kg, highlights its potential for clinical applications. These results underscore FA's dual antioxidant and anti-inflammatory mechanisms as a promising strategy to combat CYP-induced toxicity, paving the way for further exploration in therapeutic settings.

Conflicts of interest: The authors declare that they have no conflict of interest in the publication.

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