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Comparative studies on protective efficacy of gentisic acid and 2-pyrocatechuic acid against 5-fluorouracil induced nephrotoxicity in Wistar rats

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Nephrotoxicity is a frequent and severe side effect of 5-fluorouracil (5-FU) chemotherapy which limits its use clinically regardless of being one of the most promising chemotherapeutic agents. Here, we assessed the nephroprotective activity of two structurally related phenolic acids 2-pyrocatechuic acid (2,3 dihyroxybenzoic acid) and gentisic acid (2,5 dihyroxybenzoic acid) against 5-FU induced nephrotoxicity in Wistar rats. Intraperitoneal administration of 5-FU at a dose of 20 mg/kg once a day for 5 days produced a significant elevation in serum parameters of the kidney such as uric acid, urea, creatinine, sodium and potassium levels along with severe histopathological changes in renal tissues of rats indicating severe nephrotoxicity. Administration of 2-pyrocatechuic acid (2-PCA) at 10, 30 and 100 by oral route for 9 days and additional 5 days with 5-FU resulted in an amelioration of altered serum parameters in a dose-dependent manner. Moreover, 2-PCA attenuated the renal damage produced by 5-FU demonstrating its efficacy as a nephroprotective agent for the prevention as well as amelioration of 5-FU induced nephrotoxicity. None of the doses of gentisic acid (GA) were found to be effective in this posology when given orally.

Keywords: Chemotherapy, Dihyroxybenzoic acid, Renal toxicity

Chemotherapy is an extensively used, efficacious cancer treatment modality that includes the use of cytotoxic drugs to control or eliminate cancer. The uracil derivative 5-fluorouracil (5-FU), a broadly used antimetabolite chemotherapeutic drug, particularly in the management of several cancers such as pancreatic, gastrointestinal, breast, cervical, colorectal, bladder, esophageal, head and neck cancers by inhibiting both the DNA and RNA synthesis causing cytotoxicity and cell death[1,2]. It has an indiscriminate mechanism of action; its cytotoxicity destroys not only the existing tumors but also other rapidly dividing normal cells in the body as well. Its non-targeted action results in severe toxicities, amongst which one of the most serious adverse effects is nephrotoxicity which restricts its wide clinical usage[3]. Several studies have documented the nephrotoxicity produced by 5-FU chemotherapy. Mechanisms of nephrotoxicity usually comprise of a variable extent of pre-renal hypoperfusion, oxidative stress, obstruction of renal tubules, intrinsic damage to renal tissue and microvascular renal structure leading to renal toxicities including tubule-interstitial damage, glomerular disease, abnormalities in the electrolyte and renal biomarker levels[4].

Phytochemicals, including specific plant phenolic compounds, are essential dietary components[4] because of their potent antioxidant effect, ability to reduce oxidative stress-induced damage to the tissues and their potent chemopreventive and chemoprotective activities[5]. In this line, 2-pyrocatechuic acid (2,3-dihydroxybenzoic acid) is a phenolic acid and natural siderophore occurring in various medicinal plants such as *Boreava orientalis* and rosy periwinkle, fruits such as batoko plum, avocados and cranberries. It possesses wide-ranging pharmacological properties such as free radical scavenging, anti-inflammatory, antiplatelet, antioxidant, antiseptic, antibiotic and iron-chelating activities[6-8]. It is also documented for attenuating ototoxicity produced by aminoglycosides such as gentamicin and kanamycin and for ameliorating vancomycin induced nephrotoxicity[9].

Similarly, gentisic acid (2,5-dihydroxybenzoic acid) is another phenolic acid having widespread occurrence in citrus fruits, grapes, artichoke, sesame, gentians, red sandalwood, rose gum, saxifrage, and

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olives[10]. It has been scientifically proven for its potential analgesic, anti-inflammatory, anti-mutagenic, anti-rheumatic, antiparkinsonian, anti-arthritic and cytostatic activities[10]. Gentisic acid (GA) has been also documented for its crucial role in the anticarcinogenic activity of *Hibiscus rosa* Sinensis extract[11]. GA has recently exhibited fibroblast growth factor (FGF) inhibitory effect[12] and ameliorative action against cyclophosphamide-induced genotoxicity and hepatotoxicity[13].

Here, we did a comparative study of these two naturally occurring structurally related phenolic acids, 2-PCA and GA, for their protective role against 5-FU induced nephrotoxicity.

Materials and Methods

Chemicals and kits

Gentisic acid and 2-pyrocatechuic acid were procured from Sigma Aldrich Chemicals Co., St. Louis, MO, USA. 5-fluorouracil (Fiveflurd) was obtained from GlaxoSmithKline Pharmaceuticals Ltd. Other solvents and chemicals used for the study were procured from standard vendors and suppliers. Standard commercial diagnostic kits for biochemical estimations were procured from Kiran Enterprises, Pune, India.

Animals

Six weeks old Swiss albino mice (25-30 g) and 6 weeks old Wistar albino rats (200-250 g) of both sexes were used for the study. They were grouped in respective groups, caged and housed under standard environmental conditions (relative humidity 55±5%, temperature 23±10°C and 12 h light/dark cycle). The rats and mice were fed on a pelleted diet (Nutrivet Lifesciences, Pune, India) and water ad libitum. All experimental procedures were conducted during the day (08:00-16:00 h). The rats were shifted to the laboratory one hour before the start of the experiment[14]. All the studies were carried out in accordance with the guidelines given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India) and the Institutional Animal Ethical Committee (IAEC) approved the study (Approval No.: 1036/RE/S/2007/CPCSEA/16-17/F-6)[14].

Preparation of drug solutions

Accurately weighed quantities of 2-PCA, GA and 5-FU were dissolved individually in distilled water for preparation of the stock solutions of the drugs i.e. 3, 10, 30 and 100 mg/mL for 2-PCA and GA, respectively and 20 mg/mL for 5-FU. The respective doses were selected for administration to the animals from the respective stock solution[15].

Preliminary acute oral toxicity testing

Healthy adult albino mice were subjected to acute toxicity studies as per guidelines (AOT 425) suggested by the organization for economic cooperation and development (OECD-2000). The mice were continuously observed for two hours for autonomic and behavioural alterations and any sign of morbidity or mortality for the duration of seven days[14-17].

Treatment protocol[14-16]

Wistar albino rats of either sex were divided into 10 groups with six animals in each group (n=6) as follows: Gr. I (Normal control), Rats received distilled water @1.0 mL/kg body wt./day orally throughout 14 days study period; Gr. II (5-FU control group), Rats first received distilled water @1.0 mL/kg body wt./day orally for 9 days and subsequently received 5-FU (20 mg/kg body wt.) with normal saline once daily intraperitoneally for additional 5 days; Gr. III-VI (5-FU + 2-PCA treated) Rats first received 2-PCA @ 3, 10, 30 and 100 mg/kg body wt./day alone orally for 9 days and subsequently received 5-FU @20 mg/kg body wt./day) intraperitoneally with 2-PCA @ 3, 10, 30 and 100 mg/kg body wt./day for additional 5 days, respectively; and Gr. VII-X (5-FU + GA treated), Rats first received GA @ 3, 10, 30 and 100 mg/kg body wt./day alone orally for 9 days and subsequently received 5-FU @20 mg/kg body wt./day) intraperitoneally with 2-PCA @ 3, 10, 30 and 100 mg/kg body wt./day for additional 5 days, respectively[14-16].

Sample collection and biochemical estimations

At the end of the study, blood was collected from retro-orbital plexus under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia. The collected blood was subjected to centrifugation at 3000 rpm for 20 min for separation of serum which was then stored into Eppendorf tubes at –20°C to be used for the determination and assessment of biochemical estimations. Serum creatinine, urea, uric acid, sodium (Na^+) and potassium (K^+) levels were estimated by spectrophotometry using commercially available kits[15,16].

Histopathological studies

After blood sample collection, rats were sacrificed by decapitation method and the whole intact specimens of kidneys were rapidly excised by dissection. The kidneys were fixed by placing them in 10% v/v formalin solution for 24 h and were then embedded in paraffin. The coronal sections (thickness of 5 µm) of kidneys were incised using a rotary microtome (Biocraft) and stained successively, first with hematoxylin for 8 min and then with eosin for 3 min[21]. The thin sections were then prepared into permanent slides and observed under magnification power of 45X through a digital trinocular microscope (Olympus CX-21-TR) with photographic facility. Photomicrographs were captured using Magnuspro eyepiece camera software[15,16].

Statistical analysis

The results were expressed as mean ± SEM. Comparison between the groups was made by one-way analysis of variance (ANOVA) followed by Tukey's Kramer multiple comparison test. $^{*,\#}P$ <0.05; $^{**,\#\#}P$ <0.01; and $^{***,\#\#\#}P$ <0.001; *Normal control group with 5-FU induction control group; # GA and 2-PCA treated groups against 5-FU induction control group[14-16].

Results

Preliminary acute oral toxicity testing

The mice treated with 2-PCA acid and GA were found to be safe and devoid of any toxicity up to 2000 mg/kg dose and showed normal behaviour. From this data, four different doses of 2-PCA and GA i.e. 3, 10, 30 and 100 mg/kg were selected for further study by the geometrical method.

Estimation of biomarkers of renal functions

Results of renal function test revealed that the treatment with 5-FU produced a significant elevation in the serum levels of urea, uric acid, creatinine as well as Na^+ and K^+ ion concentrations showing diminished renal function due to the damage caused to the nephrons indicating marked nephrotoxicity of 5-FU in 5-FU control group rats in comparison with normal control group rats. These changes in the renal biomarkers were significantly and dose-dependently reversed to near about the normal levels by the pretreatment with 2-PCA at doses 10, 30 and 100 mg/kg indicating its potential nephroprotective activity against 5-FU induced toxicities (Table 1). But these alterations in the renal biomarkers were not ameliorated by the co-treatment with GA at all the doses in oral administration indicating its inefficiency in showing protective activity against 5-FU induced nephrotoxicity.

Histopathological studies of renal tissue

The treatment with 5-FU produced histopathological alterations like swelling, tubular atrophy, cytoplasmic vacuolization and desquamation or necrosis in kidneys of 5-FU control group rats indicating severe damage of the renal tissue. These alterations were markedly ameliorated by co-treatment with 2-PCA (10, 30 and 100 mg/kg) in a dose-dependant manner showing mild, moderate and marked improvement respectively in histopathological alterations in renal tissues (Fig. 1 A-I). Co-treatments with none of the doses of GA were able to alleviate these alterations indicating its inefficiency towards protective activity against 5-FU induced nephrotoxicity (Fig. 1).

Discussion

Nephrotoxicity refers to structural and/or functional damage to the renal tissues caused due exposure of renal tissue to any ischemic or toxic factor[18]. The 5-FU

Table 1 — Estimations of biomarkers of renal functions

Groups	Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)	Na+ (mEq/L)	K+ (mEq/L)
Normal Control	0.58±0.05	45.67±2.31	2.64±0.83	132.84±5.31	6.45±1.02
5-FU Control	1.40±0.16***	115.33±4.0l***	7.80±0.95***	148.06±3.34***	8.22±0.63***
2-PCA 3+5-FU	1.02±0.24	108.67±5.32	6.16±0.77	145.98±4.21	8.10±0.52
2-PCA 10+5-FU	0.94±0.56#	91.89±5.75#	5.24±0.51#	138.43±6.09#	7.16±0.60#
2-PCA 30+5-FU	0.81±0.09##	83.64±3.05##	4.66±0.61##	135.96±7.05##	6.91±0.49##
2-PCA 100+5-FU	0.66±0.04###	70.21±3.44###	3.89±0.42###	133.68±6.01###	6.55±0.53###
GA 3+5-FU	1.51±0.32	119.54±7.15	7.44±0.70	151.22±5.76	8.54±0.66
GA 10+5-FU	1.38±0.47	110.73±7.25	7.25±0.67	147.51±7.15	8.28±0.69
GA 30+5-FU	1.27±0.36	105.23±4.77	7.32±0.77	148.37±6.05	8.17±0.62
GA 100+5-FU	1.26±0.43	106.61±5.12	7.19±0.56	145.36±7.20	7.98±0.74

[The results were expressed as mean ± SEM. Comparison between the groups was made by one way analysis of variance (ANOVA) followed by Tukey's Kramer Multiple Comparison test. $^{*,\#}P$ <0.05; $^{**,\#\#}P$ <0.01; and $^{***,\#\#\#}P$ <0.001. *Normal control group with 5-FU induction control group; #GA and 2-PCA treated groups against 5-FU induction control group]

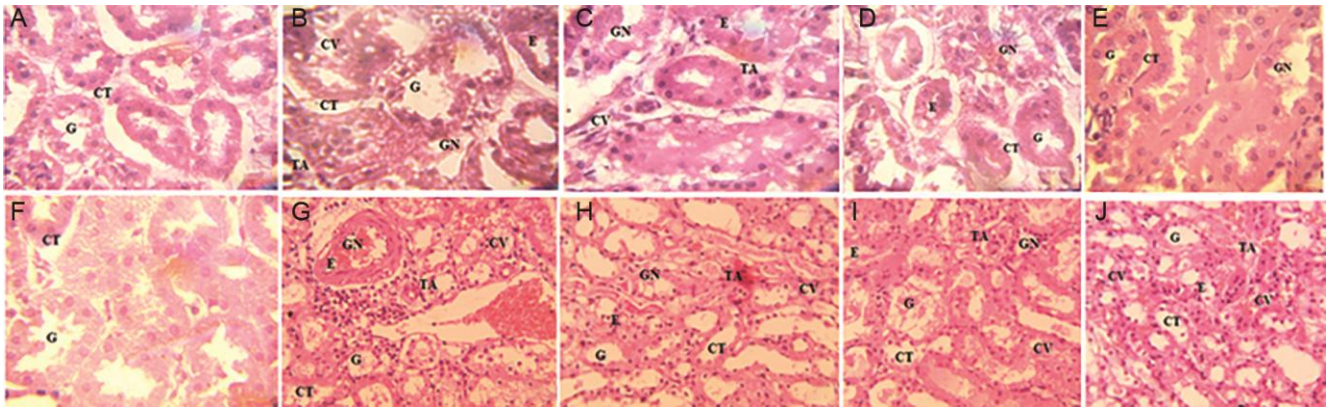

Fig. 1 — Effect of 2-PCA and GA on 5-FU induced histopathological changes in the renal tissue. Representative photomicrographs (H & E stain) of kidney sections of: (A) Normal control rat (Gr. I) showing normal histological structure with normal glomeruli (G) and convoluted tubules (CT); (B) 5-FU control rat (Gr. II) showing marked reactive changes indicative of glomerular necrosis (GN) edema (E), tubular atrophy (TA), cytoplasmic vacuolization (CV); (C) 2-PCA + 5-FU treated rat (Gr. III) showing similar reactive changes as 5-FU control with no improvement; (D) 2-PCA + 5-FU (Gr. IV) showing mild improvement in the reactive changes caused by 5-FU; (E) 2-PCA + 5-FU (Gr. V) showing moderate reduction in the reactive changes caused by 5-FU; (F) 2-PCA + 5-FU (Gr. VI) showing marked amlelioration of histological alterations caused by 5-FU; (G) GA + 5-FU (Gr. VII); (H) GA + 5-FU (Gr. VIII); (I) GA + 5-FU (Gr. IX); and (J) GA + 5-FU treated rat (Gr. X) showing similar reactive changes as 5-FU control with no improvement. [Co-treatment with GA did not attenuate these reactive changes caused by 5-FU at all the doses. Photographs taken through under high magnification power 45X using trinocular microscope (Olympus CX-21-TR) with camera (Magnuspro eyepiece camera software)]

induced renal toxicity remains a serious adverse reaction restricting its extensive clinical usage. It is characterized by increasing tubular or glomerular dysfunction, reduction in renal perfusion or impairment of endocrine and metabolic aspect of renal function, supplemented by gross morphological alterations and microscopic structural lesions[19].

Several approaches have been suggested for the amelioration of 5-FU induced nephrotoxicity using some popular synthetic drugs since there is no specific treatment available for nephrotoxicity. However, these drugs are associated with several side effects[20], hence there is a need for natural alternatives of plant origin devoid of any side effect. Phenolic acids are secondary plants metabolites that are widely distributed in plant-derived foods, such as vegetables, nuts, fruits, cereals, legumes and in beverages such as fruit juices and black or green tea[21]. The 2-PCA (2,3-dihyroxybenzoic acid) and GA (2,5-dihyroxybenzoic acid) are two common phenolic acids that are structurally related. Significant amounts of experimental data on the antioxidant potential of both 2-PCA and GA have been reported. They have also been documented to possess several pharmacological activities including anticarcinogenic activities[11,22]. Wide-ranging studies have been conducted in context with the protective effect of co- and post-treatment of phenolic acids against 5-FU induced nephrotoxicity[23,24]. Hence, the present study was conducted to assess and compare the effect of administration of both 2-PCA and GA at the same doses i.e., 3, 10, 30 and 100 mg/kg on 5-FU induced nephrotoxicity in rats. The elevations in key biomarkers of renal function such as urea, uric acid, creatinine, Na+ and K+ have been advocated to be suggestive of decreased renal functioning[25]. Thus, in the present investigation, the estimation of these parameters was employed as a key test to assess kidney function.

Urea is the principal nitrogen-containing product of end protein catabolism produced by the detoxification of the ammonia derived from the deamination of amino acids in the liver. It is found dissolved in the blood and is excreted by the kidney as a component of urine. Serum urea levels are important indicators of renal function applicable in the differential diagnosis of the pre-renal condition and acute renal failure. Increased serum urea has been reported to be associated with kidney disease or failure[26]. Uric acid is considered as the final metabolic product of dietary and endogenously-synthesized purine nucleotides, adenosine and guanosine. Uric acid undergoes both reabsorption and excretion in the proximal convoluted tubules of the nephron. The elevation in serum uric acid concentration is an indicator of impaired renal function since the kidney principally excretes urea through urine. The elevation in serum uric acid levels is associated with a decrease in glomerular filtration rate, indicating renal injury and suggesting the role of uric acid in the development and advancement of kidney diseases[27].

Creatinine, an anhydride of creatine is a catabolic end product of phosphocreatine formed by spontaneous and irreversible reactions during skeletal muscle metabolism. It is excreted by the kidneys with minimum tubular reabsorption. It accumulates in the blood when the glomerular filtration rate decreases due to renal impairment. An increase in serum creatinine is a biomarker for renal damage[28].

Electrolyte level estimation is commonly used to assess acid-base or electrolyte imbalance and to monitor the effectiveness of drugs in the management of imbalance affecting functions of vital organs. Retention of excessive amounts of sodium in serum is an indication of a reduction in urinary sodium excretion due to glomerulotubular damage[29]. Potassium levels are considered as the most substantial electrolyte marker of kidney failure. The combination of reduced potassium filtration and secretion in the distal tubule during renal failure and enhanced leakage of intracellular potassium into the bloodstream as a result of lesions in reno-tubular epithelium cause enhanced plasma potassium concentration. Hyperkalemia is the most significant and life-threatening consequence of renal failure[30].

The treatment with 5-FU was found to produce substantial renal damage biochemically exhibited through a significant elevation in serum urea, uric acid, creatinine, sodium and potassium levels in the 5-FU control group in comparison with the normal control group indicative of the nephrotoxicity produced by 5-FU. The findings were in harmony with previously documented investigations[31].

The protective effects of co-treatment with four different doses of 2-PCA and GA were compared and the results revealed that the alterations in the aforementioned renal biomarkers were significantly and dose-dependently ameliorated by the pretreatment with 2-PCA at doses 10, 30 and 100 mg/kg indicating its potential nephroprotective activity. But surprisingly none of the doses of GA were able to normalize these parameters indicating its inefficiency in counteracting the nephrotoxicity produced by 5-FU.

In the evaluation of the potency and efficacy of the nephroprotective drug, the necessity for the assessment of the drug effects on the histological parameters of renal injury has always been emphasized. In the present study, the histopathological changes seen in the kidneys section in 5-FU treated group in comparison with the normal group supported the concept of 5-FU induced renal toxicity and were concomitant with the significant alteration in kidney function tests found in this study. The histopathological changes included swelling, tubular atrophy, cytoplasmic vacuolization and necrosis in kidneys were similar to the previous findings[32]. These histopathological changes were alleviated by pretreatment with 2-PCA at doses 10, 30 and 100 mg/kg exhibiting its nephroprotective activity but GA was found to be ineffective in these regards too.

The present findings suggest the palliative effect of 2-PCA on functional deficits and structural alterations in renal tissues produced by 5-FU. The nephroprotective activity of 2-PCA may be attributed to its antioxidant potential revealed in the previous reports as one of the mechanisms underlying 5-FU-induced renal toxicity includes severe oxidative stress-induced damage in normal tissue. It has also been documented to possess nephroprotective activity against vancomycin induced nephrotoxicity by enhancing urinary alpha-glutamyl-transferase (GGT), reducing serum LDH, urea and uric acid levels which may be added to possible mechanisms of 2-PCA[12]. Nephrotoxic effects of 5-FU were not overcome by GA at any doses showing its inefficiency as the nephroprotective agent. These results may be attributed to insufficiency of the present doses of GA to achieve the therapeutic levels in the blood to produce nephroprotective activity. Higher doses of GA may show expected pharmacological actions which need further investigations.

Conclusion

In this study, we investigated the protective effect of two structurally related phenolic acids, namely 2-PCA (2,3-dihyroxybenzoic acid) and Gentisic acid (GA) (2,5 dihyroxybenzoic acid) against 5-fluorouracil (5-FU) induced nephrotoxicity in rats. The results of the study revealed that chemotherapy with 5-FU caused severe nephrotoxicity in rats confirmed by the virtue of severe alterations in renal biomarkers and histology. Co-treatment with 2-PCA showed marked protection against 5-FU induced renal injury as, abnormal architecture and increased levels of markers renal function were brought to near normal levels by 2-PCA. The nephroprotective activity exhibited by 2-PCA suggested its usefulness as an adjuvant to 5-FU chemotherapy to reduce its nephrotoxicity. The conclusions of the study can form the basis for the design of suitable clinical

studies. GA even though having structural similarity with 2-PCA was found to be inefficient in exhibiting its nephroprotective activity in the oral intervention. Further studies are required to understand the inability of GA in this regard.

Conflict of interest

Authors declare no competing interests.

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