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Protective effects of plumbagin against folic acid-induced oxidative stress in *Swiss albino* mice

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Abstract
An experimental study was conducted to evaluate the pharmacological activity of plumbagin against Folic acid-induced acute kidney injury/renal fibrosis. A total of 30 male *Swiss albino* mice were procured and acclimatized for one week before experimentation. Mice were randomly assigned to five groups of 6 animals each. Group 1 (control group) was administered normal saline. Group 2 was given Folic acid @ 250mg/kg BW IP given on 1st day of the experiment, Group 3 was kept as plumbagin *per se* group (10mg/kg IP). Groups 4 and 5 were kept as treatment groups with plumbagin @ 5 mg/kg BW IP and 10mg/kg IP, respectively, daily, along with folic acid on 1st day of the experiment. The experiment was conducted for a period of 14 days. On the 15th day the mice were sacrificed and kidneys were collected for the analysis of anti-oxidant parameters.
The present study revealed a significant alteration in anti-oxidant profile (Nitrite assay, TBARS, SOD, GSH and Catalase) of mice treated with folic acid (group 2) when compared to sham (group 1) and there was a significant amelioration of alterations in all the parameters in group 4 and 5 when compared to Folic acid control group 2. The values of group 1 and 3 were comparable without any significant differences.
In conclusion, plumbagin was found to possess the ameliorating action against folic acid-induced oxidative stress. The results revealed a greater significance of amelioration in group 5 compared to group 4, as seen through restoration of anti-oxidant enzyme levels Hence, plumbagin can be used as a protective strategy for renal injury.

Keywords: plumbagin, acid-induced, oxidative, *Swiss albino*

Introduction
Kidneys are vulnerable to acute kidney injury (AKI) and chronic kidney disease (CKD), caused by numerous risk factors such as ischemia, sepsis, drug toxicity and drug overdose, exposure to heavy metals and diabetes mellitus. AKI is a common clinical complication with abrupt glomerular filtration rate (GFR) decrease. Proteinuria, and increase in creatinine and blood urea nitrogen.
A high dose of FA is very toxic to the kidneys by damage occurring mainly to the proximal tubular epithelial cell, while a low dose can be useful for therapeutic purposes. There is evidence that high doses of folic acid can cause renal injury through crystal-induced obstruction of the tubules and direct damage to the epithelial cells of renal tissue, thereby causing ischemia-reperfusion and tubulointerstitial inflammation leading to renal fibrosis. The molecular mechanism involved in the development of fibrosis still unknown, but putatively oxidative stress could be involved in the pathogenesis of fibrosis.
Since ancient times, natural products (NPs) have been considered useful, safe, and readily available to treat various diseases. Some NPs, believed to have renal protective effects, are still commonly used against kidney diseases in modern medicine worldwide. Plumbagin is one of the secondary plant metabolites that comes under the naphthoquinones group. It is derived from three prominent phylogenic families *viz*. Plumbaginaceae, Droseraceae and Ebenceae. Plumbagin exhibits highly potent biological activities, including anti-oxidant, anti-inflammatory, anticancer, antifungal and antibacterial activities (Roy and Bharadvaja, 2018) [17]. Herein we investigate the protective role of plumbagin against folic acid induced AKI/renal fibrosis.

Materials and Methods

An experimental study was conducted in thirty (30) adult *Swiss albino* mice (20-25 g) that will be procured from M/s Jeeva life sciences, Hyderabad (CPCSEA: 2085/PO/Rc.Bi.Bt/S/19/CPCSEA). They were reared under uniform environmental conditions with a temperature range of 22±2 °C for about 3 weeks with an automatically controlled photoperiod (12hr light-12hr dark cycle). Mice were provided with a standard diet *ad libitum* and free access to clean drinking water throughout the experiment. Prior to the beginning of the trial, the animals were acclimatized for a period of 15 days.

Table 1: Experimental Design

Group	Treatment	No. of Animals
Group-1:	Normal Control (Saline @ 0.2 mL/mice /single dose)	6
Group-2:	Folic acid control @250mg/kg i.p single dose on the first day (Disease control-DC)	6
Group-3:	Plumbagin per se @10mg/kg i.p daily dose for 14 days	6
Group-4:	Folic acid (single dose @250mg/kg on first day) + Plumbagin LD (@5mg/kg i.p daily dose for 14 days)	6
Group-5:	Folic acid (Single dose @250mg/kg on first day) + Plumbagin HD (@10mg/kg i.p daily dose for 14 days)	6

Preparation of Tissue Homogenates

Mice were euthanized using CO_2 chamber after the 14th day, and kidney tissues were removed immediately and made into four parts for analysis of various parameters like oxidative stress, ELISA, immunohistochemistry, and histopathology. The tissue samples used for oxidative stress were homogenized in a solution of ice-cold PBS buffer (pH 7.4) at 4 °C.

Antioxidant enzymes: The antioxidant enzymes activities were measured *viz*., Thiobarbituric Acid Reacting Substances (TBARS): (Balasubramanian *et al*., 1988) [5] Protein concertation (Lowry *et al*., 1951) Reduced Glutathione (GSH) (Moron *et al.,* 1979), Catalase activity (Asru, 1972), Super Oxide Dismutase (SOD) (Madesh and Balasubramanian, 1998) [5] and Nitric Oxide Levels (Miranda *et al.,* 2001).

Results and discussion

The activity of SOD (U/mg protein) and catalase (U/mg protein) and the activity of GSH (µM/mg protein) in FA treated group 2 were significantly decreased (p<0.01) than in the NC group 1. In contrast, group 5 exhibited significant (p<0.001) improvement over group 2, and group 4 also revealed significant improvement. However, non-significant variation in the values between NC and *per se* was observed. The activity of TBARS (nM/mg protein) and Nitrite (µM/mg protein) in FA-treated group 2 revealed a significant (p<0.001) increase in the NC group 1, whereas *plumbagin* treatment groups 4 and 5 showed a significant decrease in TBARS (p<0.01 and p<0.001, respectively) and Nitrite (p<0.001 and p<0.001, respectively), which indicated a dose-dependent reduction in their concentrations over group 2. No significant difference in the nitrite content was observed between per se and NC (Values are presented in table 2).

Oxidative stress refers to a severe imbalance between ROS production and the anti-oxidant defence system (Haritha *et al.,* 2015; Kumar and Reddy 2012.) [10, 13], which leads to cause "a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage". Multiple biomolecules, including proteins, DNA, lipids and other macromolecules, are attacked by ROS (Jiang *et al*., 2014). Ultimately, the lipids are broken down to produce MDA (Tsikas, 2017), indicative of an increase in lipid peroxidation besides a decrease in intracellular glutathione levels, activities of CAT and GSH-Px are accompanied reduction in SOD activity during the stess (Gupta *et al.,* 2012) [9]. An increased MDA level can therefore serve as a sign of oxidative stress. The kidney has a very active oxidative metabolism because of its transport functions, which produce ROS as well as rich in antioxidant enzymes like GSH, which can scavenge free radicals, as it's an essential component of the cellular anti-oxidant defence system and is vital for maintaining cellular integrity. It is accomplished by immediately donating one hydrogen atom and neutralising free radicals. The overconsumption of GSH and inhibition of anti-oxidant enzymes are caused by excessive lipid peroxide production in tissues. SOD protects against ROS by catalysing the dismutation of superoxide anion free radicals into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Younus, 2018) [20]. CAT is a crucial enzyme that mitigates oxidative stress by converting H_2O_2 into water and oxygen (Nandi *et al*., 2019 Anilkumar *et al.,* 2010) [15, 2]. Renal GSH levels decreased following the folic acid injection, and the nuciferine treatment effectively inhibited the reduction in GSH. A few researchers reported the role of oxidative stress in folic acid-induced nephrotoxicity. Under physiological conditions, oxidative stress leading to disturbances in redox balance is restored by an anti-oxidant defence system, which includes both enzymatic (SOD, CAT) and non-enzymatic (GSH) mechanisms. However, studies have demonstrated that FA-induced GSH level insult was subsequently increased profoundly with concomitant ameliorative agents (Ezzat *et al.,* 2020) [7]. The present study showed a significant increase in renal malondialdehyde (MDA) levels concomitant with significant depletion of GSH and anti-oxidant enzymes CAT and SOD. The current study results corroborate the findings of Zaghloul *et al*. (2019) [21].

Folic acid also triggers nitrosative stress in the kidneys through iNOS (inducible nitric oxide synthase) expression and nitric oxide (NO) production. The study observed a significant increase in iNOS mRNA levels after folic acid treatment (Ezzat *et al.,* 2020) [7]. Excess NO reacts with superoxide radicals to generate peroxynitrite ($ONOO^-$) radicals capable of causing cellular damage, increased lipid peroxidation and cellular apoptosis. Further, excess NO depletes intracellular GSH, increasing cell susceptibility to oxidative stress (Jung *et al*., 2019) [12]. In the current study, a considerable increase in NO levels was observed in the FA-treated group (2) compared to the control group (1) and the findings were consistent with other studies (Plotnikov *et al*., 2007; Takahashi *et al*., 2014) [16, 18] who reported a similar elevation in NO production in renal tissues.

The prevention of free radical generation by therapeutic intervention could effectively prevent oxidative stress-induced renal damage (Ahmed *et al*., 2020 Anilkumar *et al*., 2013) [1, 3]. Our findings revealed that *plumbagin*

administration increased GSH levels, decreased nitrosative stress and, restored other anti-oxidant enzyme levels, suppressed the increase in MDA concentration. A similar effect of *plumbagin* against oxidative stress was demonstrated in a neuropathic pain model of rats (Arruri *et al*., 2017) [4] and inhibition of malaria pathogenesis by inhibiting oxidative stress and inflammation. (Gupta *et al*., 2018) [8].

Table 2: Oxidative stress parameters in different groups of mice

Group	CAT (U/mg protein)	SOD (U/mg protein)	TBARS (nM/mg protein)	GSH (µM/mg protein)	Nitrite (µM/mg protein)
G1	6.84±0.30	55.04±1.74	2.18±0.21and	6.12±0.42	35.68±1.10
G2	2.65±0.24	24.88±0.96	5.96±0.81	2.58±0.41	83.88±2.02
G3	52.76±1.21	6.09±0.13	2.07±0.17	5.40±0.30	42.28±1.06
G4	39.90±0.73	4.27±0.48	3.30±0.41	4.29±0.22	62.41± 1.57
G5	50.76±1.39	5.06±0.45	2.46±0.15	4.86±0.26	46.66 ±1.63

Conclusion

The treatment of mice with a high dose of plumbagin (10 mg/kg BW) has exerted appreciable preventive effects against FA-induced renal injury with its anti-oxidant potential.

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