Methanolic extract of tubers of *Pueraria tuberosa* Linn. ameliorates glycerol induced acute kidney injury in rats

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ABSTRACT

Rhabdomyolysis is very common in traumatic cases and its later implications include acute kidney damage. It is a challenging task for medical doctors, so there is a need to develop newer therapeutic measures. Glycerol induced rhabdomyolysis is a standard experimental model for acute kidney injury. There is breakdown of muscles, resulting to release of excess myoglobin in the blood which induces kidney injury. Oxidative stress and local inflammation are reported to be involved in the pathogenesis. Here the protective effect of methanolic extract of Pueraria tuberosa Linn. (PT) has been investigated against glycerol (35%) induced AKI. Glycerol was injected in thigh-muscles and after 48 h; serum urea and creatinine were assessed. Catalase and superoxide dismutase activities along with lipid peroxidation were determined in the kidney homogenates. Treated group had significant changes in the lipid peroxidation, superoxide dismutase and catalase activity. Histological picture showed lesser accumulation of hyaline casts and lesser degree of tubular necrosis. Thus, it could be concluded that polar fraction of PT tuber could be an effective treatment for rhabdomyolysis induced kidney injury. Its mechanism of action involves maintenance of antioxidant activity in the target tissue.

Keywords: Rhabdomyolysis, oxidative stress, *Pueraria tuberosa*.
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INTRODUCTION

Rhabdomyolysis is associated with excess muscle breakdown and is a severe form of myositis. [1, 2] It is common in road accidents, injuries during war, burns, intrinsic muscle diseases, excessive physical exertion, metabolic disorders, hypoxia, drug-toxicity and severe infections. In general, 10-40% of the cases with rhabdomyolysis develop AKI. [3] The excess release of heme from muscle-degradation causes ischemic injury, oxidative stress and excess release of inflammatory cytokines. [4] The degree of rhabdomyolysis manifests itself by increasing the concentration of creatinine kinase in blood by the breakdown of striated muscles. There is enhanced generation of hydrogen peroxide in renal cortex leading to acute renal failure and then turns into an acute kidney injury. Both the oxidant and antioxidant mechanisms are involved in the complex pathophysiology of myoglobin mediated kidney injury. [5, 6, 7] In normal body conditions, heme-oxygenase of epithelial cells of proximal tubule degrades this heme, but in case of excess myoglobin, this protection is failed resulting to hematuria. Statin (hypolipidemic drug) like drugs also induce rhabdomylosis. It is a novel concept that enhanced protein degradation via ubiquitin proteosome pathway may represent a key mechanism underlying statin myalgia.[8] Its effect is more severe in case of hypothyroidism.[9] These traumatic conditions lead to the acute kidney injury (AKI), which accounts for 10% of all reported cases of AKI in hospitals.[10] Glycerol induced rhabdomylosis is its standard animal model. Intramuscular injection of glycerol in rats resembles acute renal failure by massive release of myoglobin in crush

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syndrome in humans. [11] There is high serum creatinine and urea along with low activity of antioxidant enzymes. Evidences suggest that transcriptional activation of heme oxygenase (HO)-1, the rate-limiting enzyme in heme degradation, participates in its defenses.[12] Thus, drugs which could activate HO-1 expression or scavenge free radicals could be effective in preventing the rhabdomyolysis mediated kidney damage. Currently there is no effective method to cure acute kidney injury, so it is important to find an effective therapy for the improvement of the kidney function. Medicinal plants have shown significant antioxidant and anti-inflammatory properties. Antioxidants play a noteworthy role in ameliorating the effect of toxicity produced by rhabdomyolysis by inhibiting the lipid and protein peroxidation and redox cycling between ferric and ferryl myoglobin.[13, 14]. Underground roots of *Pueraria tuberosa* Linn. (PT) had shown sufficiently important antioxidant and anti-inflammatory potential.[15] It is used as medicine in Ayurveda (Indian system of Medicine) to manage cough and cold.[16] Its rich content of glycosides and polyphenolic compounds are responsible for its antioxidant property. It is recommended as general health promoting drug, diuretic, galactagogue and aphrodisiac.[17] It has shown nephroprotection against cisplatin induced kidney damage.[18] Here we have explored the protective role of methanolic extract of PT on glycerol induced AKI model. Changes in the activity of antioxidant enzymes in blood hemolysates, kidney tissues and renal function tests in serum were observed which gives an outlook about the condition of kidney cellular homeostasis.

EXPERIMENTAL SECTION

Glycerol and NBT (nitrobluetetrazolium chloride) were purchased from E. Merck (India) Pvt Limited and Himedia, Mumbai. All other chemical and staining materials were of analytical grade. Male adult albino rats (100-200 g) of Charles Foster strain were purchased from central animal house of our Institute (IMS, BHU). Experimental animal protocols were approved by Ethical committee on Experimental Animal Resources, IMS, BHU by reference no. Dean/2012-13/192. Authentications of the herbs were done by Prof. K.N. Dwivedi of Department of Dravyaguna, IMS, BHU by letter no. Da/13-14/139 by comparing the characteristics of the plant mentioned in botanical texts[19] and other floras.[20] Animals were kept in a room on 12 h light/dark cycle at a room temperature of 25°C with free access to food and water *ad libitum.*

Preparation of the methanolic extract (PTME)
One hundred grams of coarse powder of the PT plant was taken, sample put in thimble; was made to fit in the soxhlet apparatus. Pure methanol was added and set up was made to run for 24 h. All the components were come in the solvent making the coloured solution and filtered. Solution was heated on distilled unit to obtain the pure extract of *Pueraria tuberosa* and distilled methanol was separated. Oil and various components were found to be present in the extraction.

Experimental protocol
Rats were randomly divided into four groups having six animals in each. Six h fasted rats were given deep intramuscular injection of glycerol (35%) in both the thighs of hind limbs equally and then transferred to metabolic cages for urine collection. Urine was analysed by instrument urometer (Agappe Diagnostics Ltd, India) for pH, glucose, proteins and ketones. Urine was collected on 12 h interval for 48 h for calculating the creatinine clearance for the estimation of GFR. [21] Creatine clearance was calculated by using the formula:

Creatinine clearance = [urinary creatinine (mg/ml) x 24 h urine volume (ml)]/ {serum creatinine (mg/ml) x [1000/body weight (g)] x [1/86,400 s]}.

In an untreated group, animals were pretreated i.e. 2 h before with drug vector (20% Tween 20) followed by continuity for 2 days. In the drug treated group, PTME was given orally in doses of 20 mg/100g BW and 40mg/100g BW for 2 consecutive days.

After 48 h, animals were sacrificed under anaesthesia pentabarbital to collect kidney and blood in heparinized or plain tubes. Kidneys were served in -20ºC freezer for biochemical analysis and in 10% formaldehyde solution for histological studies. Serum urea [22] and creatinine[23] were analyzed spectrophotometrically by using commercial kits provided by Accurex Biomedical Pvt Limited, Thane, India. Red blood cells were separated by centrifugation at 3000 rpm from heparinized blood to prepare blood hemolysate. Its Hb content was estimated by cyanmethaemoglobin method [24], catalase by Aebi's method [25] and superoxide dismutase (SOD) activity as standardized in our laboratory conditions.[26] One kidney was homogenized in (50 mM

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Tris-HCl, pH 7.4) and clear supernatant was used for assay of catalase, superoxide dismutase, protein and lipid peroxidation.

In brief, catalase activity was determined by measuring the decomposition of H_2O_2 at 240 nm in a UV-V Spectrophotometer (SL 210, ELICO, India). The enzymatic activity was expressed in Units/mg protein (one unit decomposes 1mMole of H_2O_2 per minute at pH 7 and 25°C). SOD activity was determined by measuring the concentration of formazone formation after NBT reaction at 540 nm. Lipid peroxidation was assayed by thiobarbituric acid (TBA) reactive substances method. Tetra ethoxypropane (TEP) was used as standard to draw the standard curve and ascorbic acid was used as a positive control.[27] Protein peroxidation was determined by DNPH assay protocol for measuring protein carbonyl and reading was taken at 340 nm. Proteins in the homogenates were estimated by Bradford reagent and standard curve was drawn with BSA.

For histological examination, fixed kidney was processed by dehydration for making wax-blocks; transverse sections of 3-6 µm thickness were cut on microtome and stained with hematoxylin and eosin (H&E). Stained slides were monitored under the Nikon microscope by using NIS element software.

RESULTS AND DISCUSSION

Dose standardization of glycerol for induction of AKI
Animals of Group I (untreated) received normal saline (8 ml/kg BW). Animals of Group II (A, B, C, D, and E) received different concentrations of glycerol 25%, 30%, 35%, 40% and 50%. Rats of Group D and E having higher dose of glycerol (40% and 50 %), died within 3h of glycerol administration, without any urine output showing severe decrease in the creatinine clearance rate. However, rats with lower doses (25%, 30%, 35%) showed significant hematuria in the first 2-4 h. Degree of hematuria increased with increasing concentration of glycerol. Initially urine had reddish brown appearance for 2 h and after that it attained normal colour. Renal function tests after 48h showed concentration dependent increase in serum urea and creatinine. In normal group, serum urea was 36 mg/dl and it rose to 71.3% in 35% glycerol. Similarly serum creatinine was 0.34 mg/dl in untreated group and rises to 112.96% at 35% (Fig 2 mentioned later part). Thus 35% glycerol dose was considered as an optimum dose in our experimental conditions as there were greater chances of survival of rats with AKI for the study.
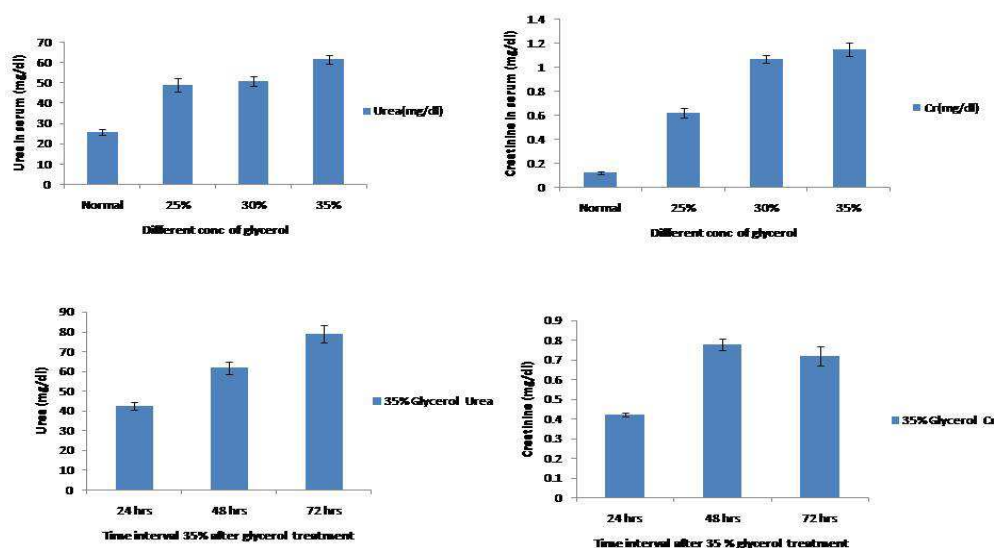
Graphical presentation of renal parameters

Figure 1: Effect of different concentration of glycerol on blood urea and creatinine and 35% glycerol treatment after 72 h

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Although creatinine level was higher than normal control but it was gradually decreasing by lapse of time as checked after 72 h. This indicated the process of natural recovery and considered as conservative approach of therapy for rhabdomyolysis. Hence 35% glycerol administration was considered for 48 h in this study for further investigation (Fig 1).

Effect of PTME on glycerol induced acute kidney injury (AKI)
Effect of PTME on glycerol induced changes in urine biochemistry (Table 1)
In an untreated group urine had alkaline pH with presence of protein and ketone bodies while in the PTME treated group pH of the urine was found to be acidic ie normal pH of the urine. Further there were no traces of the protein. Creatinine clearance improved significantly in the drug treated group as compared to the untreated group showing the rise in glomerular filtration rate.

Table 1: Effect of PTME on the biochemical parameters of urine

S. No	Parameters	Normal	Only glycerol	Glycerol+PTME (20mg)	Glycerol+ PTME (40mg)
1	Glucose	-ve	2+	-ve	-ve
2	Protein	-ve	3+, >=300mg/dl	-ve	trace
3	Ketones	-ve	+ve	-ve	-ve
4	pH	6.3	8.75	6.5	5.6
5	*Creatinine clearance (ml/min/kg BW)	19.56 ± 1.23	6.78 ± 2.21	14.56 ± 2.05	17.54 ± 2.06

Data was generated by repeating it three times (n=6). *

Effect of PTME on glycerol induced changes in serum urea and creatinine (Table 2)
PTME treatment significantly prevented the rise in serum urea and creatinine. Its 20 mg/100g BW dose brought down serum creatinine to 0.85 mg /dl (44.09%) which was significantly lower than the experimental control in 48 h. In similar conditions, PTME at 40 mg/100g BW dose showed dose dependent reduction in the values suggesting nephroprotective property.

Table 2: **Effect of PTME on glycerol induced changes on kidney function after 48 hrs**

Parameters	Groups			
	Normal	Glycerol treated	Treated group	
			20 mg/100 g BW	40 mg/100 g BW
Serum urea (mg/dl)	31.67 ± 2.65	51.52 ± 8.04	39.57 ± 3.39	32.57 ± 2.52
Serum creatinine (mg/dl)	0.59 ± 0.21	1.52 ± 0.44	0.85 ± 0.24	0.58 ± 0.19

*Data are expressed as mean $\pm$ SE. *P<0.001 normal control vs untreated group; **P<0.05 drug treated vs untreated group (glycerol).*

Effect of PTME on glycerol induced changes in activity of CAT and SOD in blood hemolysate (Table 3)
Activity of CAT and SOD was significantly higher in untreated group. In glycerol treated group, SOD and CAT activity was raised to several folds from normal control but PTME (40 mg) treatment prevented this rise. This indirectly suggested lower generation and accumulation of FRs in the system, indicating the FR- scavenging potential of PTME. Therefore lower activity of SOD and CAT could be the secondary response to lower concentration of free radicals in the system. Dose of 20 mg/100g BW also showed speedy response.

Table 3: Effect of different doses of PTME on antioxidant enzymes and oxidative stress in blood hemolysates

S.No	Parameters	Antioxidant status		Oxidative stress	
		CAT (U/mg Hb)	SOD (U/mg Hb)	LPO (nmol/mg protein)	Protein carbonyl (nmol mg protein)
1	Normal	35.47 ± 3.45	8.23 ± 2.13	0.25 ± 0.02	20.21 ± 3.29
2	Untreated	$76.32 \pm 5.98*$	18.98 ± 2.87	0.46 ± 0.01	$53.60 \pm 3.89*$
3	Treated 20 mg/100g BW	$46.37 \pm 6.43**$	13.19 ± 2.65	0.27 ± 0.02	$28.46 \pm 2.37**$
4	Treated 40 mg/100g BW	$37.23 \pm 5.86**$	9.57 ± 2.24	0.25 ± 0.04	$21.67 \pm 2.37**$

*Data are expressed as mean $\pm$ SE. *P<0.05 normal vs untreated group; **P<0.05 drug treated vs untreated group.*

Effect of PTME on antioxidant enzymes in kidney homogenate (Table 4)
Activity of CAT and SOD enzymes were significantly higher in untreated group than the normal group. PTME treatment showed a significant decrease in both the enzymes in dose dependent manner. The LPO and the protein carbonyl levels were also lower in PTME treated animals.

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<p align="center">**Table 4: Effect of PTME on activity of antioxidant enzymes and oxidative stress in the kidney homogenates**</p>

S.No	Groups	Antioxidant status		Oxidative stress	
		CAT (U/mg protein)	SOD (U/mg protein)	LPO (nmol/mg protein)	PPO (nmol/mg protein)
1	Normal	27.21 ± 8.89	7.28 ± 0.02	0.11 ± 0.01	16.89 ± 3.76
2	Untreated	44.12 ± 5.67*	16.48 ± 0.02*	0.63 ± 0.02	39.14 ± 3.89*
3	Treated 20 mg/100 g BW	33.23 ± 9.67**	11.12 ± 0.12	0.31 ± 0.01	25.76 ± 2.67
4	Treated 40 mg/100 g BW	29.86 ± 11.78**	7.54 ± 0.02**	0.21 ± 0.01	20.84 ± 3.31**

<p align="center">*Data were expressed as mean ± SE. *P<0.05 normal vs untreated;**P< 0.05 untreated vs treated group.*</p>

Histological studies
In an untreated group there was prominent distortion in the glomerular morphology along with tubular necrosis and hyaline casts deposition within the tubular lumen. There was dilatation in the tubular structure also, with cloudy appearance across the epithelial lining (Fig-2). Necrosis was prominent throughout the cortico-medullary region. In drug administrated group tubular damage was highly minimised with lesser apical blebbing.
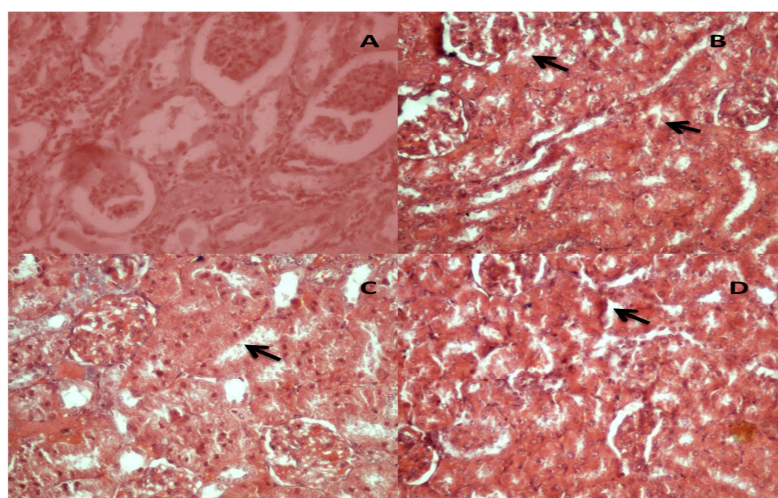
<p align="center">**Fig 2: Effect of PTME on histopathological changes**</p>

Fig-2: Effect of PTME on histological changes in TS of Kidney in glycerol induced rhabdomyolysis. TS of kidney H and E staining , original magnification was 400 X- A) normal ; B) glycerol induced damage; Arrow mark was showing the severe epithelial lining damage and cells of glomerulus showed shrinkage in the structure C) 20 mg/100g BW ; threre was appearance of cloudiness. D) 40 mg/100g BW. Here cloudiness diappeared and epithelial lining was slowly regenerated.

Elevation in plasma creatinine reflects transient rhabdomyolysis-induced renal impairment.[28, 29] It may be associated with lactic acidosis. It is evident with acidic pH of urine in an untreated group. Rhabdomyolysis result from various factors, namely trauma, exercise, medications, infections, endocrine disorders, congenital myopathies, and metabolic diseases.[30] Defect in the mitochondrial fatty acid β-oxidation (FAO) has been reported in rhabdomylosis associated with metabolic disorders. Tricyclic antidepressives and statins have side effect of rhabdomylosis.[31] It is secondary to simvastatin and phenofibrate. PTME had shown significant protection against glycerol induced AKI by improving renal function tests. Various antioxidants have shown renoprotection in this model, such as L-citrulline [31], N-acetylcysteine (NAC) [32], 7-O-galloyl-D-sedoheptulose (GS) isolated from Corni – Fructus [33], etc. Since FRs are basically involved in pathogenesis of heme mediated AKI, which is released during disruption of muscles after glycerol injection, therefore protection could be sought either by using antioxidants or by induction of heme oxygenase-1 (HO-1). It is a critical enzyme for heme/hemin degradation and detoxification and such report has been published earlier. [34] We have earlier reported the FR scavenging potential of PTME. [16] Thus its protective action described above, could be due to FR scavenging role. This has been further supported by decrease in raised activity of CAT and SOD in blood hemolysates and kidney homogenates, as the rise in these enzymes in an untreated group could be a secondary response to high concentration of free radicals in the tissue.

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Role of inflammation has also been documented in the pathogenesis of glycerol induced AKI, as reported by high expression of inducible nitric oxide synthase (iNOS), IL-6, interleukin-10, TNF-α, and Cox-II. [35] We have also earlier reported that PTME significantly prevents the expression of LPS induced iNOS in macrophage culture. [16] Thus it could be suggested that PTME might be inhibiting glycerol induced inflammatory process in the kidney tissue. Kidney damage is associated with accumulation of tubular casts, damages epithelia and tubular necrosis along with increase in urinary total protein, kidney-injury molecule-1 (KIM-1) and clusterin. [36] PTME treatment had prevented these changes. PTME significantly decreased the urine volume and fractional excretion of sodium, but it decreased the urine osmolality, suggesting the protective role against renal dysfunction. Urine osmolality is the concentration of urine; a large value indicates concentrated urine while small value indicates dilute urine. Different metabolites alter the concentration of the urine. There was decreased urine volume and hence urine osmolality increases significantly with the single injection of glycerol. While in drug treated rats urine osmolality decreased in comparison to experimental control rats.

Interestingly, PTME has beneficial role over other existing herbal preparation such as 7-O-galloyl-D-sedoheptulose (GS), as it also significantly prevented the rise in creatinine clearance, which was not seen in other drug. [37] It improved the glycerol induced renal dysfunction and damage. Glycerol injection also induces the apoptosis in muscle resulting to muscular degeneration. [30] Thus antiapoptotic drugs could also be an effective agent in preventing AKI in this situation. p53 inhibitor (pifithrin-α) had already shown protection in this AKI model. [38] Although, we do not have any direct evidence for anti-apoptotic role of PTME, but its antioxidant and anti-inflammatory role might be associated with its anti-apoptotic property as indicated in other reports. [39] Although diagnostic markers for AKI, include plasma creatinine, blood urea nitrogen (BUN), presence or absence of urinary casts, fractional excretion of sodium (FENa) and urinary protein, but these markers are of limited use for early detection of AKI[40] 60% of kidney get damaged till there is marked rise in serum creatinine. There is a need of early biomarkers other than serum urea and creatinine which will show promising results in early diagnosis. Herbal treatment is a holistic approach to the curable therapy which needs further study for improvement of health of the mankind.

DATA AND STATISTICAL ANALYSIS
All data were expressed as mean ± SD. The significance of differences between group means was established by using student's t test. To establish the significance of differences between more than two group means, we used One-Way ANOVA followed by Tuckey's and Dunnett's type multiple comparision tests by using IBM SPSS 20 software. $P < 0.05$ and $P < 0.001$ were regarded as significant.

CONCLUSION

Thus, it could be suggested that PTME has significant role in protection against rhabdomyolysis-induced acute kidney injury. Mechanism of action could be through its antioxidant and anti-inflammatory property, polyphenols and flavones, present in PTME might be responsible for this activity. Since kidney is very miraculous organ and we need to preserve its function by any possible means.

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