ORIGINAL ARTICLE e-ISSN: 2349-0659 p-ISSN; 2350-0964

Amelioration of Rhabdomyolysis-Induced Myoglobinuric Acute Renal Failure by *Citrullus lanatus* Seeds

Rupali A. Patil*, Amit Tiwari, Sunil V. Amrutkar

Abstract

Citrullus lanatus (Thunb.) (Cucerbitaceae) is a trailing annual herb native to India, Nigeria, and Africa, commonly known as Matsum and Nakai. It thrives in all tropical, subtropical, and warm temperate areas with hot summers. In India, seeds have long been used to treat hypotensive and diuretic effects, as well as kidney stones and urinary passages. This study looked at the effects of a methanol extract of *C. lanatus* seeds (MCL) on rhabdomyolysis-induced myoglobinuric acute renal failure (ARF) in Wistar rats. Five groups (n = 5) of male Albino Wistar rats weighing 150–200 g were formed. A single intramuscular injection of glycerol (GL) (8 ml/kg) was used to induce ARF. Following GL injection, all animals were sacrificed and blood was collected. Renal function tests utilizing blood urea nitrogen (BUN) and creatinine were performed on freshly separated serum. The right kidney was stored in 10% buffered formalin for histological sectioning, and the amount of lipid peroxidation, superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) activity were all measured. The data were analyzed using a one-way ANOVA and Dunnett's test. *$P > 0.05$ was considered statistically significant. Animals pre-treated with MCL (100 and 300 mg kg^{-1}, p.o.) for 7 days before GL dramatically altered and restored serum creatinine, BUN, creatinine clearance, urea clearance, and renal morphology in comparison to the GL-treated group. Oxidative stress markers such as lipid peroxidation, SOD, CAT, and GSH were also dramatically improved. The findings of this study suggest that *C. lanatus* seed has a possible anti-GL-induced ARF effect, verifying its ethnomedicinal use.

Keywords: Acute renal failure, *Citrullus lanatus*, Glycerol, Myoglobinuria

Asian Pac. J. Health Sci., (2022); DOI: 10.21276/apjhs.2022.9.4.41

Introduction

Rhabdomyolysis is the disintegration of striated muscles that cause the release of potentially dangerous intracellular components like myoglobin into the bloodstream.[1] Intrinsic muscular dysfunction (due to trauma, burns, intrinsic muscle illness, or excessive physical activity), metabolic diseases, hypoxia, medicines, toxins, infections, temperature extremes, and idiopathic disorders are all causes of rhabdomyolysis.[2] Rhabdomyolysis can cause abrupt renal failure, hyperkalemia, acute cardiomyopathy, and disseminated intravascular coagulation, among other consequences. The key processes implicated in myoglobinuric ARC caused by rhabdomyolysis are vasoconstriction, intraluminal cast formation, and direct myoglobin-induced cytotoxicity.[1,3] Acute renal failure (ARF) accounts for 8–16% of hospitalizations, with rhabdomyolysis accounting for 10% of ARF.[4]

Glycerol (GL)-induced renal injury in rats is a well-established model of ARF.[5] GL-induced ARF can be caused by a reduction in renal blood flow, the release of myoglobin from damaged muscle, or an increase in reactive oxygen species (ROS).[6]

The breakdown of ATP to hypoxanthine by vasoconstriction/hypoperfusion produces superoxide radical (O^{2-}).[7] Myoglobin's catabolized porphyrine rings could engage in Fenton and Haber-Wesis chemistry, producing hydroxyl radicals (OH-) from O^{2-} and hydrogen peroxide (H_2O_2), which are collectively known as ROS.[8] Oxidative stress[9] and the inflammatory process found in GL-treated rats can activate the nuclear factor-kappa β (NFB) system. GL-induced changes in renal function and structure are also influenced by the inflammatory process.[10]

Hypovolemia and metabolic acidosis enhance tubular myoglobin precipitation. Myoglobin also leads to tubular cell damage, most likely by heme-iron-mediated lipid peroxidation.[8]

Citrullus lanatus (Thunb.) (Cucerbitaceae) is abundantly available in countries such as India, Nigeria, Africa, and many other tropical and warmer zones of the world. Native people use various plant parts to get relief from cholera, diarrhea, dysentery, chronic bronchitis, etc. Its seeds are one of the safest diuretics; seeds are used in high blood pressure, scanty urination, burning menstruation, and many other uses are reported in various studies. It shows analgesic and anti-inflammatory activity[11], *in vitro* antioxidant activity.[12,13] It also shows usefulness in cardiac disorders.[14]

The *C. lanatus* seeds contain Vitamin C and B_2, riboflavin, minerals, fat, carbohydrates, and protein.[15] Seeds contain various essential amino acids (mainly *L*-arginine)[16] and non-essential amino acid citrulline (an endogenous precursor for L-arginine synthesis), a broad class of polyphenolic compounds, flavonoids.[12]

The present study investigated the effects of a methanol extract of *C. lanatus* seeds (MCL) on renal function, renal structure, and renal oxidative stress in rats with GL-induced myoglobinuric ARF.

GES's Sir Dr. M. S. Gosavi College of Pharmaceutical Education and Research, Nasik 422005, Maharashtra, India.

Corresponding Author: Dr. Rupali A. Patil, Associate Professor and Head-Pharmacology, GES's Sir Dr. M. S. Gosavi College of Pharmaceutical Education and Research, Nasik - 422005, Maharashtra, India. E-mail: ruupalipatil@gmail.com

How to cite this article: Patil RA, Tiwari A, Amrutkar SV. Amelioration of Rhabdomyolysis-Induced Myoglobinuric Acute Renal Failure by *Citrullus lanatus* Seeds. Asian Pac. J. Health Sci., 2022;9(4):209-214.

Source of support: Nil

Conflicts of interest: None.

Received: 14/04/2022 **Revised:** 07/05/2022 **Accepted:** 26/05/2022

Materials and Methods

Animals

Bharat Serum and Vaccines Ltd., Thane, India, provided male albino Wistar rats weighing 150–200 g. They were housed in an air

conditioned room with 12 h light-dark cycles and a temperature of 25°C ± 0.5°C. The Institutional Animal Ethical Committee (IAEC) at MGV's Pharmacy College, Nashik approved the study protocol.

Drugs and Chemicals

GL was purchased from Merck. Standard kits for blood urea nitrogen (BUN) (Reckon Diagnostics, Baroda, India) and creatinine (Vital Diagnostics (P) Ltd., Thane, India) were used.

Experimental Design

The research was divided into five groups, each with five animals. Before being injected with GL, the animals were given free access to food but were not allowed to drink water for 24 h. Group 1 (C) was a control group that received the same quantity of CMC dosage as advised by the CMC dosage calculation. Hypertonic GL injection of 8ml/kg was given intramuscularly in a split dosage to the hind limbs of Group 2 (GL) animals. The animals in the third and fourth groups were given MCL (100 and 300 mg/kg p.o., respectively) 7 days before the GL injection. After the GL injection, all animals were slaughtered under light anesthesia for 24 h. Through a cardiac puncture, blood was collected in heparinized centrifuge tubes. Renal function tests were performed using freshly extracted serum. Both kidneys were removed and stored in 10% buffered formalin for histological sectioning, with the left kidney being deep frozen for enzymatic analysis and the right kidney being stored in 10% buffered formalin for enzymatic analysis.

Lipid Peroxidative Indices Estimation

Two ml 0.37% TBA: 15% TCA: HCl (0.25 N) (1:1:1) was added to 0.1 ml homogenate. The mixture was immersed in hot water at 80°C for 15 min, then cooled to room temperature for 10 min before centrifuging at 1000 rpm. At 535 nm, the absorbance was measured. The results were reported in nanomoles per milligram of moist tissue.[17]

Antioxidant Enzymes Estimation.

Superoxide Dismutase (SOD)

By comparing the change in optical density at 480 nm every minute to a blank at pH = 10.2, auto-oxidation of adrenaline (3×10^{-4} M) to adrenochrome was determined.[18]

Catalase (CAT)

For 1 min, the absorbance was measured at 240 nm every 10 s. Units of CAT, U/g of wet tissue were used to express the results.[19]

Reduced Glutathione (GSH)

At 412 nm, the color developed was measured. The results were reported in nanomoles of GSH per milligram of moist tissue.[20]

Histology of the Kidneys

After the animal was sacrificed, the right kidney was removed and cleaned in cold saline. The kidney was immediately fixed in 10% buffered formalin following washing. Serial histological slices of 5 m thickness were cut from the paraffin blocks and stained with hematoxylin and eosin. Under a light microscope, the sections were viewed, and photomicrographs were produced at a magnification of 10× using Motic, USA. In all treatments, the renal segment was evaluated for hemorrhagic cast, tubular necrosis, and apical blebbing.

Total Phenol Content Estimation

Gallic acid solutions at concentrations of 10, 20, 30, 40, 50, 75, and 100 g/ml in ethanol were used to create the calibration curve. One ml of Folin-Ciocalteu reagent was added to 0.1 ml of fraction (1 mg/ml in distilled water). *C. lanatus* extract was made in the same concentrations as the C. *lanatus* extract. After shaking the mixture for 2 h at room temperature, the absorbance was measured at 760 nm using a Shimadzu UV-2450 spectrophotometer. All of the tests were done three times. Total phenol concentrations were measured in milligrams per gram of dry mass (mg/g dry mass).[21]

Total Flavonoid Content Estimation

The calibration curve was made by diluting 10 mg of rutin in 80% ethanol to 100, 200, 300, 400, and 500 g/ml. Separately, 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were combined with the diluted standard solutions (0.5 mL). *C. lanatus* extract was made in the same concentrations as the *C. lanatus* extract. The absorbance of the reaction mixture was measured at 415 nm after 30 min at room temperature.[22]

C. lanatus Extract HPTLC Analysis

The characterization of L-argenine in *C. lanatus* seeds extract was done using HPTLC.[23]

Results

GL-induced Renal Impairment and the Effect of MCL Seeds

GL administration resulted in a considerable increase in serum BNU and creatinine levels, as well as a significant decrease in urea and creatinine clearance, as compared to control rats. In GL-treated rats, pre-treatment with MCL (100 and 300 mg/kg) significantly changed blood BUN, creatinine, urea clearance, and creatinine clearance [Table 1].

Effect of MCL Seeds on Lipid Peroxidation Induced by GL

The concentration of thiobarbituric acid reactive substances (TBRS) was considerably higher in the GL treatment group (7.4980.5811) than in the control group. In the 100 and 300 mg/kg groups, pre-treatment with MCL significantly reduced TBRS levels in GL-administered rats [Figure 1].

Effect of MCL Seeds on GL-Induced Antioxidant Pool Changes

In GL-treated rats, the enzymatic activity of SOD, CAT, and reduced GSH was considerably changed. Pre-treatment with MCL seeds (100 and 300 mg/kg) substantially and dose-dependently reduced these modifications. SOD levels were significantly maintained

Table 1: Effect of *C. lanatus* extract on kidney function test

Treatment group (mg/kg)	*BUN (mg/dl)*	*Creatinine (mg/dl)*	*Urea clearance (ml/min)*	*Creatinine clearance (ml/min)*
Vehicle	12.56±0.29	1.105±0.097	0.46±0.011	0.55±0.047
GL (8)	28.02±0.43*	3.43±0.36*	0.044±0.0061*	0.051±0.013*
MCL (100) + GL	17.78±0.60**	1.70±0.35**	0.34±0.017**	0.42±0.040**
MCL (300) + GL	13.92±0.15**	1.30±0.44**	0.41±0.010**	0.46±0.029**
MCL (300)	12.06±0.18	1.0±0.89	0.47±0.0083	0.57±0.028

All values are expressed as mean±SEM at n=5 (one-way ANOVA followed by Dunnett's test). *P<0.05 when compared to vehicle. **P<0.05 when compared to GL. MCL: Methanol extract of *C. lanatus*, GL: Glycerol, BUN: Blood urea nitrogen

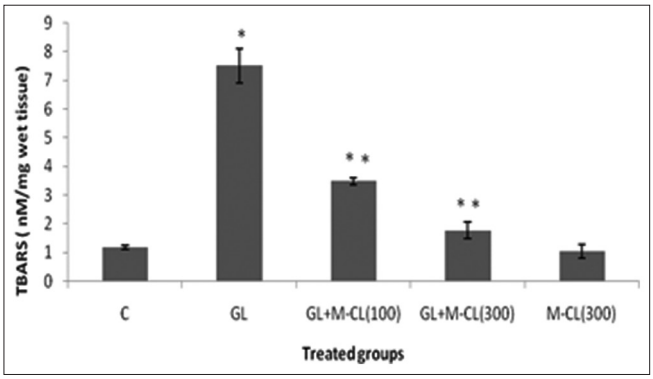

Figure 1: Effect of methanol extract of *Citrullus lanatus* extract (100, 300 mg/kg/day, p.o., for 7 days) on TBARS in glycerol (GL) treated rats. All values are expressed as mean ± scanning electron microscope at n = 5 (one-way ANOVA followed by Dunnett's test). *P < 0.05 when compared to vehicle. **P < 0.05 when compared to GL

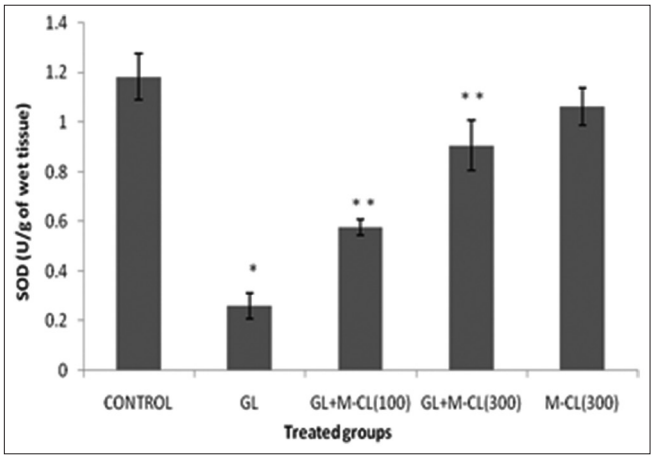

Figure 2: Effect of methanol extract of *Citrullus lanatus* extract (100, 300 mg/kg/day, p.o., for 7 days) on superoxide dismutase enzyme in glycerol (GL) treated male rats. All values are expressed as mean ± scanning electron microscope at n = 5 (one-way ANOVA followed by Dunnett's test). *P < 0.05 compared to vehicle and **P < 0.05 compared to GL

when doses of 100 and 300 mg/kg MCL extract were given [Figure 2]. Animals treated with 300 mg/kg MCL extract only had significantly higher levels of CAT and GSH [Figures 3 and 4].

GL-induced Alterations in Renal Structure and the Effect of MCL Seeds

The control group showed no morphological alterations. Rats treated with GL, on the other hand, revealed significant histological changes in the cortex and outer medulla. Severe hyaline casts, tubular necrosis, and hemorrhagic casts were found in the renal sections. In GL-treated mice, MCL (100 mg/kg) provided no significant morphological protection, but MCL (300 mg/kg) kept the normal kidney morphology. When normal animals are given 300 mg/kg of MCL, their morphology is practically normal [Figure 5].

MCL Extract Total Phenol Content

The phenol content of MCL was found to be 8.69±0.7539 mg/g in terms of Gallic acid equivalent (the standard curve equation: y = 0.0191x + 0.0637 and regression (R^2) = 0.9962).

MCL Extract Total Flavonoid Content

The flavonoid content of MCL was found to be 1.825±0.48 mg/g in terms of rutin equivalent (the standard curve equation: y = 0.0042x + 0.0578 and regression (R^2) = 0.9925).

HPTLC Analysis of MCL Extract

Densitometric evaluation in visible region of *C. lanatus* extract and standard L-argenine, Rf values were found 0.486 and 0.50,

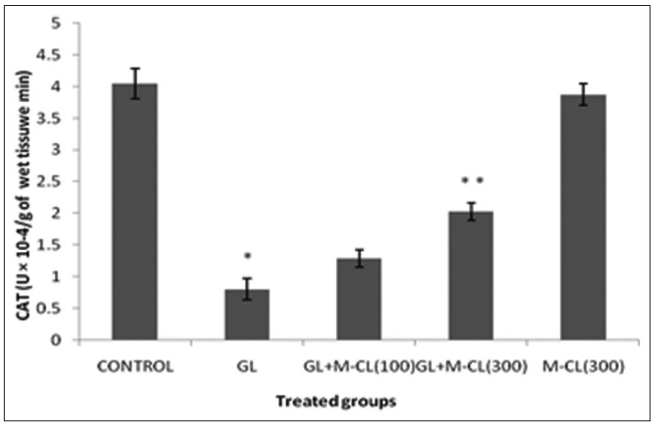

Figure 3: Effect of methanol extract of *Citrullus lanatus* extract (100, 300 mg/kg/day, p.o., for 7 days) on catalase enzyme in glycerol (GL) treated rats. All values are expressed as mean ± scanning electron microscope at n = 5 (one-way ANOVA followed by Dunnett's test). *P < 0.05 compared to vehicle. **P < 0.05 compared to GL

respectively. This confirms presence of L-arginine as a constituent in *C. lanatus* extract.

Discussion

C. lanatus seeds are one the safest and best diuretics which can be used with grafting results in the treatment of diminished urination,

kidney, and bladder stones. Its seeds are used in medicines from ancient times as a base in many unani and ayurvedic tonic preparations, seeds are used in high blood pressure, scanty urination, burning micturation, tuberculosis, loss of weight, and reduce blood cholesterol as folk medicines.

The most commonly utilized model for myogolbinuric ARF research is an intramuscular hypertonic injection.[8] In an animal model of GL-induced ARF, the pathophysiology of myogolbinuric ARF was investigated.[1] The key pathophysiologic processes of rhabdomyolsis caused by myoglobinuric ARF include vasoconstriction, intraluminal cast formation, and direct myoglobin-induced cytotoxicity.[1] Muscle nacrosis results in significant fluid third spacing, which leads to intravascular depletion, hypovolemia, and hypertension, all of which contribute to decreased renal perfusion.[24] Due to the biochemical features of myoglobin, acidic urine causes myoglobin casts to form, which obstruct the renal tumbles and prevent urine flow. The iron-containing heme molecule can combine with oxygen to form free radicals, which can cause lipid peroxidation within biological membranes. Heme proteins scavenge nitric oxide (NO), a powerful renal vasodilator, resulting in renal vasoconstriction and reduced renal blood flow.[1] Vasoactive agents such as platelet activating factor, endothelins, and prostaglandin F2 may be elevated in rhabdomyolysis, causing renal arteriole constriction and a reduction in GFR.[25]

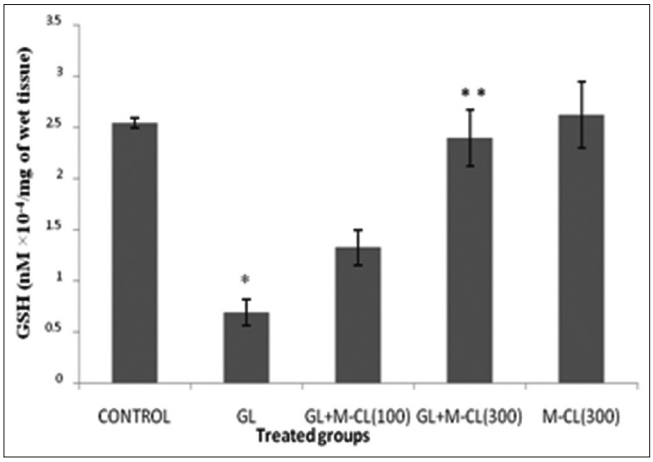

Figure 4: Effect of methanol extract of *Citrullus lanatus* extract (100, 300 mg/kg/day, p.o., for 7 days) on reduced glutathione enzyme in glycerol (GL) treated rats. All values are expressed as mean ± scanning electron microscope at $n = 5$ (one-way ANOVA followed by Dunnett's test). *$P < 0.05$ compared to vehicle and **$P < 0.05$ compared to GL

Although the pigments (hemoglobin and myoglobin) are unlikely to cause ARF on their own, their presence in the systemic circulation during acidosis, dehydration, shock, or other conditions involving decreased renal perfusion may induce both direct toxic and hemodynamic abnormalities, resulting in ARF.[24]

For 24 h, animals in this study were dehydrated. Dehydration before the onset of renal injury allows for the complete manifestation of renal injury, including the formation of a cast.[26] During the dehydration period, the animals lost an average of 5–8% of their body weight. After GL injection, only dehydrated rats exhibited oliguria or anuria.[27]

In this investigation, intramuscular GL injection resulted in significantly higher serum creatinine and BUN levels, as well as decreased creatinine and urea clearance. GL-induced ARF was linked to renal lipid peroxidation and had a significant impact on anti-oxidant enzyme pools, as seen by lower levels of SOD, GSH, and Cat enzymes. Furthermore, the GL-treated rats' histological pattern revealed typical hemorrhagic and hyaline cast deposits, as well as tubular necrosis. By disrupting intracellular heme-containing proteins, oxidative stress releases even more free heme.[28]

In GL-treated rats, both tested dosages of M-CL seed extract as pretreatments inhibited lipid peroxidation and protected

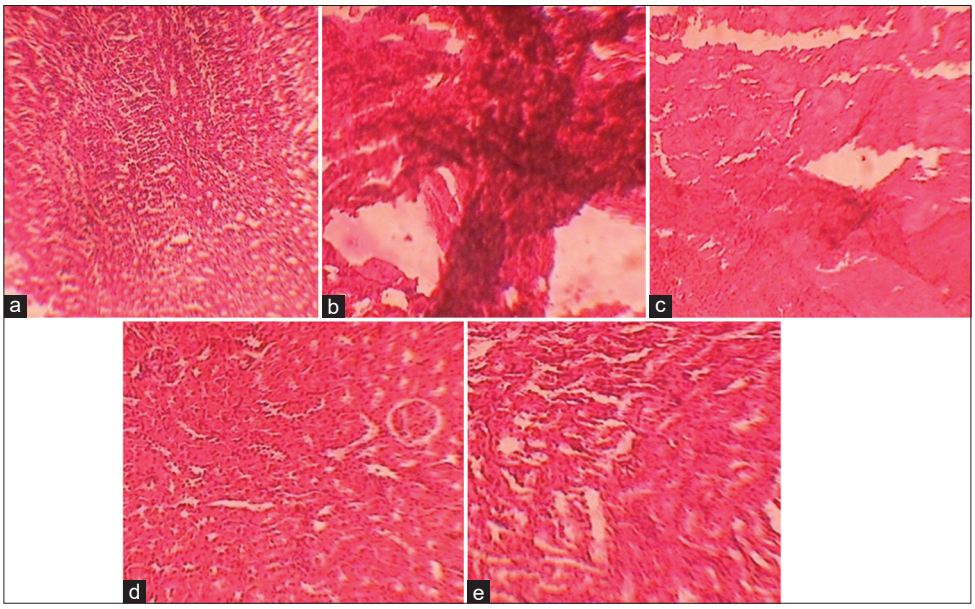

Figure 5: Histopathological examination (10×) of rat kidney from various groups. Kidney section of control rats (a) shows normal architecture. Glycerol (GL) (8 ml/kg i.m) treated animals (b) shows severe tubular necrosis and cast formation in their cellular population of kidney cells. Less tubular necrosis and cast formation are occurred in GL and methanol extract of *Citrullus lanatus* (MCL) 100 mg/kg treated animals (c). Partial restoration of cellular population from tubular necrosis and cast formation in GL and MCL 300 treated animals (d). Less normal cellular population in kidney cells of MCL 300 treated animals (e)

the severe depletion of antioxidant enzyme pools (GSH, SOD, and Cat); however, the best results were achieved with the 300 mg/kg doses. A marked reduction in renal oxidative stress coupled with significant improvement in renal function as well as renal morphology.

Polyphenols and different amino acids (mostly L-arginine) were found in MCL.[12] Polyphenols' antioxidant properties may be responsible for protection against GL-induced ARF, based on phytochemicals found in MCL. The major variables influencing phenolic compounds' antioxidant activity are their redox properties: Reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelation potential.[12] MCL polyphones, like other polyphenols, have antioxidant properties.[29]

Another mechanism may involve the protective effect of exogenous L-arginine against toxic or ischemic injury to the kidneys that have been shown in several studies.[30] However, the molecular processes underlying these positive effects are unknown, but L-arginine is thought to be a substrate for NO synthesis (NOS)[31] through NOS.[32] This mechanism is responsible for metabolizing about 1% of daily L-arginine consumption. The kidney expresses all three NOS isoforms.[32] Endothelial NOS (eNOS) is required for maintaining renal blood flow, glomerular filtration rate, and regional vascular tone.[33] L-arginine is a substrate for arginases, a group of enzymes involved in tissue healing processes that convert L-arginine to L-ornithine. The liver shows presence of arginase I, while the kidney and macrophages contain arginase II.[34]

Another L-arginine metabolizing route has just been discovered. The enzyme L-arginine decarboxylase (ADC) is involved in the production of agmatine in this route. Imidazol-guanidine receptors and α-1 adrenoreceptors are both activated by adamantine[35] and increases glomerular filtration and tubular reabsorption when injected into the renal interstitium.[36] As ADC activity is high in healthy kidneys,[37] agmatine could be regulating some of the physiologic effects of L-arginine supplementation in renal illness. All of the aforementioned L-arginine metabolites may have a role in the development of renal illness. This may be rational in altering L-arginine metabolism as an approach to treat kidney disease makes sense.

Endotoxins and cytokines are released into the systemic circulation as a result of muscle injury, which promotes vasoconstriction.[38] Myoglobins scavenge NO, the most potent endogenous vasodilator, which is generated by dead muscle cells. Finally, the above-mentioned process causes renal vasoconstriction, renal ischemia, and decreased ATP production due to reduced oxygen supply in the renal tubular cells, as well as epithelial cell necrosis, causing renal blood flow reduction one of the most important, and early events following GL injection.[39]

Administration of MCL before GL injection normalizes the renal function, renal oxidative stress and damage to renal structure, may be by normalizing the NO level due to the present L-arginine. L-arginine has been shown to restore NOS activity and zinc – MT balance in the kidneys of animals that have been chronically exposed to inorganic mercury.[40] *C. lanatus* is also a source of various amino acids and proteins, which may be involved in the protective effect of *C. lanatus* against GL-induced ARF. This mechanism may be supported by studies of Roberts *et al*. (1997).[41] Results of this study are in accordance with the previous studies.[42-44]

Conclusion

C. lanatus seed showed promising preventive effect against GL-induced ARF in this investigation, substantiating its ethnomedicinal use.

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