

A network pharmacology approach to assess Reno-protective and -curative effects of methanolic extract of *Malva neglecta* Wallr in gentamicin induced renal toxicity rat model

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The aim of present study was to explore protective and curative effects of *Malva neglecta* on kidneys. *In silico* study with network pharmacology was performed to find out potential target organs, genes and cellular cell lines which confirmed kidneys as target organ of phyto-constituents present in *Malva neglecta* extract. Gentamicin (40 mg/kg, i.p) was given to induce renal toxicity. Prophylactic study was performed with 300-, 600- and 900 mg/kg doses to find out nephro-protective and -curative effects and curative potential was evaluated at 900 mg/kg dose. Renal function biomarkers, blood urea, BUN, serum creatinine and uric acid, and oxidative stress measuring biomarkers, SOD, CAT, GSH and MDA levels in kidney homogenate were quantified at the end of study. Treatment groups showed decrease in blood urea, BUN, serum creatinine and uric acid levels dose dependently and curative group also showed decline in these biomarkers. SOD, CAT, GSH levels were increased and MDA level decreased in treatment groups significantly as compared to toxic control which revealed the role of oxidative stress in renal damage and anti-oxidant power of *MN*. Data suggested that use of *MN* along with drugs causing renal toxicity may prove beneficial due to its nephro-protective and curative effects.

Keywords: *Malva neglecta*. Network Pharmacology. Gentamicin. Silymarin. Anti-oxidant property.

INTRODUCTION

The kidney is a vital organ of the body to perform many necessary functions such as the maintenance of homeostasis in blood vessels, extracellular environment regulation, such as detoxification, elimination of drugs and toxic metabolites (Kim, Moon, 2012). Renal failure is a potentially fatal condition resulting in irreversible and progressive loss of kidney function. When the kidneys fail to remove the waste products properly from blood, finally dialysis may be needed or transplantation of kidney

to prevent the high level of urea, creatinine and nitrogen in blood, multiple organ failure and ultimately death (De Zeeuw, Hillege, De Jong, 2005).

A number of antibiotics such as aminoglycosides, amphotericin B, sulphonamides, rifampicin, ciprofloxacin, levofloxacin, tetracycline, and vancomycin cause nephrotoxicity but gentamicin has prominent side effect in the form of nephrotoxicity (Geetha, Ramarao, 2014). Gentamicin generates hydrogen peroxide in renal cortex mitochondria of rat and it can also enhance the generation of reactive oxygen species (ROS) which may induce cellular injury by damaging to macromolecules (Geetha, Ramarao, 2014; El-Tantawy, Mohamed, Al Haleem, 2013; Megraj, 2011; Lakshmi, Sudhakar, 2010).

Malva neglecta (*MN*) belongs to *Malvaceae* family and most commonly known by different names such

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as, 'button weed', 'common mallow', 'cheese weed', 'cheese plant', 'round leaf mallow', 'dwarf mallow', 'toluk', 'sonchal', 'khabasi', and 'berberu' (Dalar, Türker, Konczak, 2012; Ghanati, Khatami, 2011; Rahim *et al.*, 2016). Literature showed presence of phenolic compounds named quinic acid, malic acid, tr-aconitic acid, protocatechuic acid, tr-caffeic acid, p-coumaric acid, 4-hydroxy benzoic acid and salicylic acid. 4-hydroxy benzoic acid was found to be most abundant among all (amount phenolic compounds) quantification of phenolic compounds (Hasimi *et al.*, 2017). Traditionally, *MN* is used in food as vegetable and fruit (Ghanati, Khatami, 2011; Dalar, Turker, Konczak, 2012; Rahim *et al.*, 2016; Saremi, Kargar, Pourahmadi, 2015). The leaf part used to promote maturation of abscess, abdominal pain, infertility, wound healing, mouth pain, stomachache, colds, menstrual disorders, beneficial for intestines, cancers, gynecological disorders, hemorrhoids, diabetes. The aerial parts of plant are used in diarrhea, kidney stones, inflammation of respiratory tract, urinary inflammation. Stem part is used as abortifacient & tension. Root part is used as abortifacient. Fruit part is used in diarrhea & abdominal pain (Dalar, Konczak, 2012). The present study was designed to determine nephron-protective and-curative effects of methanolic extract of *Malva neglecta Wallr* against gentamicin induced renal failure.

MATERIAL AND METHODS

Plant Material

Fresh plant of *Malva neglecta Wallr* was collected from fields of Gujarat, Pakistan and was authenticated by Dr. Mansoor (Botanist) from Department of Botany, University of Agriculture Faisalabad, Pakistan.

Preparation of plant extract

Plant was washed to remove dust and superfluous material with distilled water and shade dried for two weeks. Then it was crushed into powder form. The coarse powder (1kg) was subjected to maceration with 1500 mL of 70% methanol for seven days in air tight container with

occasional shake at room temperature. The macerate was passed through muslin cloth and then filtered through whatman filter paper no. 1. Excess solvent was evaporated with rotary evaporator at 40 °C and semisolid extract was stored at 4°C in amber colored glass container. The dried extract was placed in desiccator for storing and weighed amount was triturated with Tween 80 freshly before administration.

Animals

Healthy wistar adult male albino rats weighing about 200-220 gm were housed in polypropylene cages in Animal house of Govt. College University, Faisalabad. The temperature of animal house maintained at 23± 2 °C under 12 hour light/dark cycle. They were fed with standard rat pellet diet and water *ad libitum*.

Ethical approval

Institute of Laboratory Animal Resources, National Research Council rules for animal study was followed and experimental protocol was approved by the Institutional Review Board of Govt. College University Faisalabad (Ref no. GCUF/ERC/1982).

Phytochemical screening

Qualitative phytochemical analysis was performed by adopting Prabhavathi *et al.*, (2016) methods.

Network pharmacology for prediction of potential drug targets

Phytoconstituents detected with HPLC were studied in protein data bank to find out target genes which were inserted to human protein atlas software to predict effects on cellular levels.

Experimental protocol

Fifty six male wistar rats were used for the study and animals were divided into seven groups containing eight animals in each. Study design is as below:

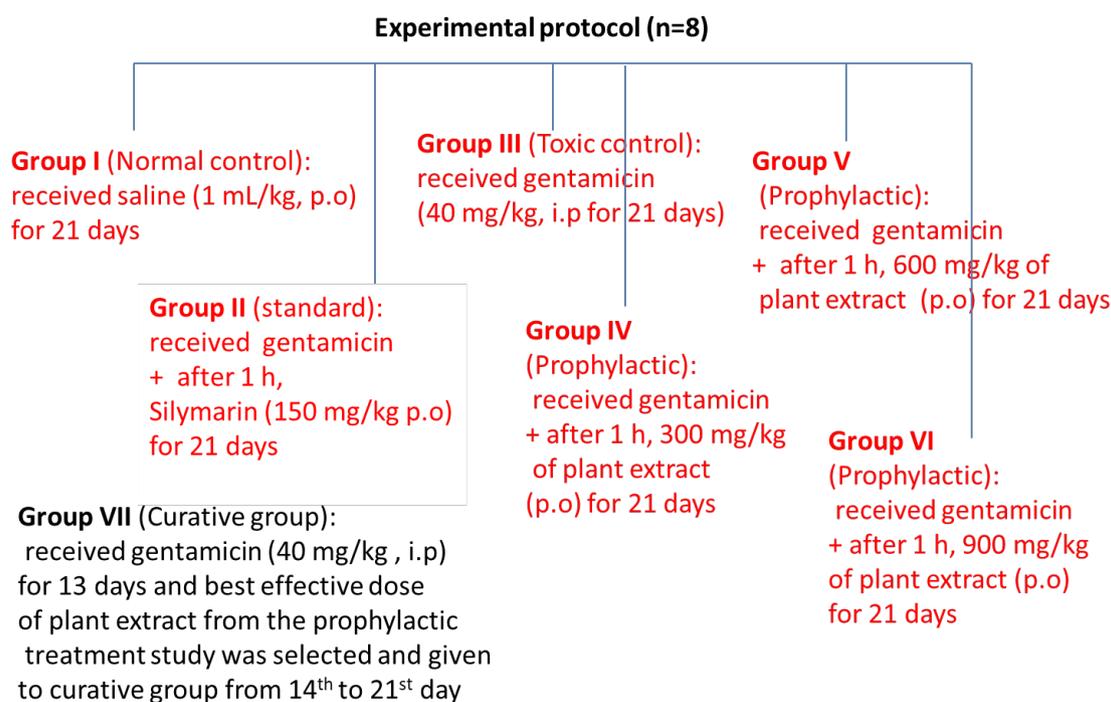


FIGURE 1 - Experimental design.

Animals were sacrificed on 22nd day of the study after collecting blood by cardiac puncture. Urine was collected in metabolic cages. Biochemical analysis (Uric acid, urea, creatinine and BUN levels) was performed on serum and urine. Kidneys were isolated after humanly killing of animals for histopathological and *in-vivo* antioxidant analyses. Kidneys were preserved in 10% formalin for further processing.

Parameters assessed

Biochemical analysis in blood/serum

Uric acid, urea, creatinine and BUN levels were estimated in serum by using Randox diagnostic kits on a chemistry analyzer (Evolution 3000).

Biochemical analysis in urine

Uric acid, urea and creatinine were measured in urine by using Randox diagnostic kits on a chemistry analyzer (Evolution 3000). Urine volume was estimated in all the groups during last 24 hours of the experiment.

Preparation of kidney homogenate

Excised kidneys were pierced with phosphate buffer saline (1g organ: 10 mL buffer) in homogenator then mixture was centrifuged at 6000 rpm for 10 minutes. Supernatant was collected to perform *in-vivo* anti-oxidant assays.

In-vivo anti-oxidant assays performed on kidneys homogenate

Glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) levels were measured by following Saleem *et al.*, 2014 methods.

Histopathological studies

Animals were dissected and rat's kidney removed and preserved in 10% formalin solution, kidney was sectioned longitudinally into two parts and dehydrated in graded alcohol, then sectioned part fixed in paraffin wax. Sample fixed in paraffin sectioned at 5µm in thickness and stained with standard hematoxylin and eosin stain.

Renal tubular necrosis was observed under the microscope (Mayer *et al.*,2002).

Statistical analysis

The data were expressed as mean \pm SEM. One way ANOVA followed by Dunnett's multiple comparison test was applied by using graphpad prism version 5. $p < 0.05$ was set as statistical significance.

RESULTS

Phytochemical screening

Qualitative phytochemical screening confirmed the presence of lipids, proteins, carbohydrates, alkaloids, flavonoids, polyphenolics and glycosaponins (Table I).

TABLE I - List of phytochemical constituents present in plant extract

Phytochemicals	Qualitative results
Lipids	+
Proteins	+

(continues on the next column...)

Phytochemicals	Qualitative results
Carbohydrates	+
Alkaloids	+
Flavonoids	+
Polyphenolics	+
Glycosaponins	+
Tannins	-
Steroids	-
Phytosterols	-

Present (+), Absent (-)

Network pharmacology for prediction of potential drug targets

The *in silico* study performed by employing protein db software showed phytoconstituents identified in HPLC (quercetin, gallic acid, M. cumaric acid, ferulic acid, cinnamic acid) are controlling kidney function through four targets. The human protein atlas database indicated target genes ALOX5, CA2, DAO, MAOB (Table II). Kidneys as target organ provide us base to work on kidney problem.

TABLE II - Network pharmacology results predicting potential targets

Compound Name	Target Protein	Target Gene	Tissue Represent the Gene	Examples of target RNA cell line
Quercetin	Lipoxygenase	ALOX5	Brain, Skin, Kidney, pancrease, Female tissues	RT4, HMC-1, Daudi, U266/70
Gallic Acid	Carbonic anhydrase	CA2	GIT, Kidney, Liver, Brain, Bone marrow	HEK 293, THP-1, HaCaT
M. Coumaric acid	D amino acid oxidase	DAO	Brain, Kidney, Liver	Hep-G2
Ferulic acid	Trysoniase	TYR	Skin	SK-MEL-30
Cinnamic acid	Amine Oxidase B	MAOB	Brain, Skin, Kidney. Liver, GIT	SiHa, Hep G2

Biochemical analysis in blood/serum

Table III showed the levels of urea, serum creatinine, uric acid, and BUN increased significantly in toxic control group when compared to normal control group. In prophylactic study groups, the levels of urea, serum creatinine, uric acid, and BUN were dose

dependently reduced to a significant degree ($p < 0.05$) with reference to toxic control group. The curative group dose was selected from prophylactic studies showing the best outcome i.e. 900mg/kg, p.o. The data showed that the values of all the parameters were decreased significantly ($p < 0.05$) as compared to toxic control group.

TABLE III - Biochemical analysis of *Malva neglecta* methanol extract in blood/serum

S. No.	Groups	Blood urea (mg/dL)	Serum creatinine (mg/dL)	Uric acid (mg/dL)	BUN (g/dL)
1	Normal control	23.21 ± 2.60	0.58 ± 0.07	3.91 ± 0.21	30.87 ± 0.90
2	Standard	48.2 ± 3.68*	1.64 ± 0.10*	21.17 ± 1.34*	55.90 ± 2.40*
3	Toxic control	85.6 ± 4.10#	2.8 ± 0.02#	36.13 ± 2.52#	90.78 ± 1.50#
4	Prophylactic (300 mg/kg)	44.08 ± 3.13*	1.28 ± 0.01*	12.27 ± 0.59*	48.53 ± 0.40*
5	Prophylactic (600 mg/kg)	39.86 ± 3.43*	0.95 ± 0.09*	09.25 ± 0.50*	41.10 ± 4.50*
6	Prophylactic (900 mg/kg)	32.76 ± 2.51*	0.61 ± 0.05*	05.61 ± 0.49*	37.66 ± 6.07*
7	Curative (900 mg/kg)	36.44 ± 1.51*	0.77 ± 0.09*	06.15 ± 0.50*	40.71 ± 2.20*

Values are expressed as Mean ± SEM, One way ANOVA followed by Dunnett's t' Posttest was applied for analysis.

* $p < 0.05$ as compared to toxic control, # $p < 0.05$ as compared to normal control

Biochemical analysis in urine

Urea, creatinine and uric acid values were raised significantly ($p < 0.05$) in toxic control groups as compared to normal control values. There was little change in the urine volume in all the treated groups but the change did not reach the significance level statistically. Prophylactic treatments in all the groups, in comparison to toxic control, seemed to

exhibit significant ($p < 0.05$) nephroprotective effect dose dependently; maximal effect at 900 mg/kg dose. The curative group also showed significant ($p < 0.05$) reduction in urine biomarkers to assess renal toxicity, meaning by that *Malva neglecta* extract could be effective in the cure of renal toxicity. Urine volume (mL) was decreased in toxic control group whereas other treated groups had urine volume (mL) near to the value of normal control group (Table IV).

TABLE IV - Biochemical analysis of *Malva neglecta* methanol extract in urine

S. No.	Groups	Urine volume (mL)	Urea (mg/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)
1	Normal control	8.22 ± 0.07	61.55 ± 2.07	1.67 ± 0.01	3.94 ± 0.21
2	Standard	8.0 ± 0.05	97.31 ± 6.65*	2.03 ± 1.52*	21.10 ± 1.3*
3	Toxic control	5.13 ± 0.09#	132.4 ± 9.23#	3.81 ± 1.00#	36.63 ± 2.52#
4	Prophylactic (300 mg/kg)	6.05 ± 0.09	70.25 ± 7.02*	2.17 ± 0.47*	9.27 ± 0.59*
5	Prophylactic (600 mg/kg)	7.40 ± 0.07	66.45 ± 3.11*	1.89 ± 0.02*	8.05 ± 0.90*
6	Prophylactic (900 mg/kg)	8.10 ± 0.08	62.93 ± 0.57*	1.67 ± 0.70*	6.11 ± 0.41*
7	Curative (900 mg/kg)	6.93 ± 0.08	64.17 ± 2.15*	1.70 ± 0.33*	6.70 ± 1.43*

Values are expressed as Mean ± SEM, One way ANOVA followed by Dunnett's 't' Posttest was applied for analysis. *p<0.05 as compared to toxic control, #p<0.05 as compared to normal control.

In-vivo anti-oxidant assays performed on kidneys homogenate

SOD, CAT and GSH levels significantly (p<0.05) decreased whereas MDA level increased significantly (p<0.05) in toxic control group with reference to normal control values which is indicative of nephrotoxicity. Significant (p<0.05) dose dependent increase appeared

in levels of SOD, CAT and GSH in prophylactic treatment groups and curative group as compared to toxic control group. Hence, the MDA level significantly (p<0.05) declined in prophylactic treatment groups and curative group as compared to toxic control group (Table V). These results are providing scientific evidence to nephro-protective and -curative effects of *Malva neglecta*.

TABLE V - Evaluation of kidney homogenate for in-vivo anti-oxidant activity after treatment of rats with *Malva neglecta* methanol extract for 21 days

S. No.	Groups	SOD (µg/mg of kidney tissues)	CAT (µg/mg of kidney tissues)	GSH (µg/mg of kidney tissues)	MDA (µg/mg of kidney tissues)
1	Normal control	15.37 ± 0.49	42.02 ± 1.80	104.07 ± 1.44	14.30 ± 1.95
2	Standard	6.52 ± 1.40*	16.22 ± 1.82*	49.01 ± 2.73*	32.59 ± 2.36ns
3	Toxic control	9.6 ± 2.16#	21.98 ± 2.06#	66.75 ± 1.90#	37.33 ± 2.36#
4	Prophylactic (300 mg/kg)	11.16 ± 2.45*	29.89 ± 2.63ns	73.95 ± 2.95*	26.33 ± 1.34*
5	Prophylactic (600 mg/kg)	12.77 ± 1.50*	33.97 ± 1.06*	81.11 ± 1.34*	21.23 ± 2.09*
6	Prophylactic (900 mg/kg)	14.12 ± 1.40*	38.43 ± 2.09*	95.05 ± 1.65*	18.65 ± 1.29*
7	Curative (900 mg/kg)	11.98 ± 0.90*	32.21 ± 1.75*	88.56 ± 1.05*	20.19 ± 1.50*

Values are expressed as Mean ± SEM, One way ANOVA followed by Dunnett's 't' Posttest was applied for analysis.

*p<0.05 as compared to toxic control, #p<0.05 as compared to normal control, ns = non significant with reference to toxic control

Histopathological examination

Histopathological examination of sections of rat kidney treated with gentamicin showing moderate to severe degree of congestion was present in the renal parenchyma. Nuclei were condensed. Tubular epithelial cells showed moderate degree of necrotic changes indicating by pyknotic nuclei in the tubular epithelial cells. These changes were present throughout the renal parenchyma. In the lumen of tubules, renal casts were also present. It indicated moderate degree of pathological changes induced by oral administration of gentamicin whereas normal control group showed normal glomerulus and tubules with regular morphology but group treated with silymarin showed mild degree of

congestion. Prophylactic (300 mg/kg) group showed that in few places, pyknotic nuclei were present in tubular epithelial cells. Mild to moderate degree of congestion was also present. Prophylactic (600 mg/kg) group showed predominant normal kidney morphology with only occasional degenerating tubules. Prophylactic-900 mg/kg- group showed predominant normal kidney morphology with only mild degree of congestion. Curative group showed predominant normal kidney morphology with only occasional degenerating tubules (H&E staining X 10) (Figure 2). Dose dependent effect was seen in all the parameters, 900 mg/kg dose of methanolic extract of *Malva neglecta Wallr* had shown best reno-protective and -curative effects against gentamicin induced renal toxicity.

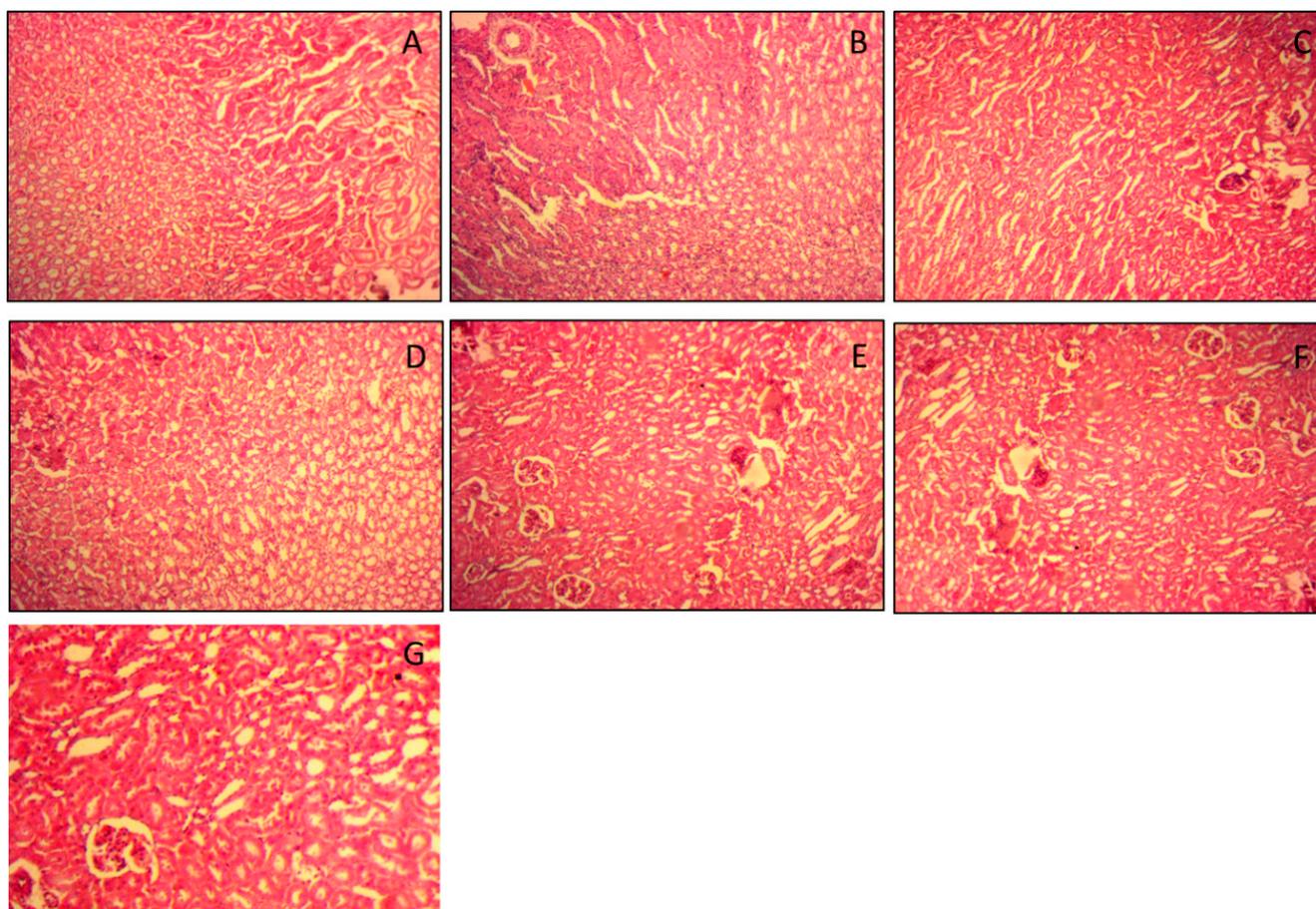


FIGURE 2 - Histopathological analysis. A: Normal control, B: Standard, C: Toxic control, D-F: Prophylactic groups (300-, 600-, & 900 mg/kg respectively), G: Curative group (900 mg/kg)

DISCUSSION

In present study, gentamicin was used to induce renal toxicity. Gentamicin induced oxidative stress at high dose that leads to renal toxicity due to accumulation of reactive oxygen species in renal cortex (Acharya *et al.*, 2013; Goldstein, Hewitt, Hook, 2013). Blood urea, serum creatinine and uric acid, synthesized in the body as a result of various metabolic processes, levels were measured as biomarkers of renal toxicity. BUN was also measured as it is more specific indicator of renal damage as compared to urea clearance test (Gowda *et al.*, 2010). Prophylactic treatment groups and curative group values displayed decrease in these biomarkers as compared to disease control group values which is indicating protective and curative role of *MN* in kidneys.

To prove the role of oxidative stress in renal damage, SOD, CAT, GSH and MDA levels were quantified in kidney homogenate. The levels of SOD, CAT, GSH were decreased and MDA value was increased in disease control group whereas as treatment groups showed rise in SOD, CAT, GSH levels and decline in MDA level.

The nephro-protective and curative effects of *Malva neglecta* may be attributed to quercetin, gallic acid, m-coumaric acid, cinnamic acid. Literature also supported it and *in-silico* network pharmacology approach also predicts the kidney as potential target of quercetin, gallic acid, m-coumaric acid, cinnamic acid.

CONCLUSION

Data suggested nephron-protective and – curative effects of *MN* that may be attributed to its phytoconstituents (quercetin, gallic acid, m-coumaric acid, cinnamic acid) as they have kidney as target organ.

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