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Nephroprotective effect of the hydromethanolic extract and fractions of *Viola serpens* Wall. Histological and hematological evidence

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ABSTRACT

The current study was planned to examine the nephroprotective effect of the crude extract and its various fractions of *Viola serpense* Wall against paracetamol-induced toxicity in rabbits. The serum creatinine levels of all fractions, as well as the crude extract, were found to have a greater effect. The effect on urine urea by the n-hexane, ethyl acetate, n-butanol and aqueous fraction in high doses (300 mg/kg b.wt.) and crude extract and chloroform in low doses (150 mg/kg bwts.) were comparatively more effective and comparable to silymarin. The creatinine clearance of the fractions except for chloroform, aqueous at 300 mg/kg and the hydro-methanolic extracts at both doses were highly significant. The histological structures of kidneys in crude extract and chloroform-treated groups showed more improvement at the lower doses. The fractions n-hexane, ethyl acetate and n-butanolic exhibited an inverse dose relationship in the histology of the kidney. However, the aqueous fraction showed a dose-dependent nephroprotective effect. Finally, the crude extract and fractions significantly improved paracetamol-induced nephrotoxicity in rabbits.

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Introduction

Renal disorders are alarming issues of the day so new technologies have been developed like transplantation, surgery, chemotherapy and haemodialysis etc. The mentioned technologies are expensive and unaffordable, especially to common people. The modern system of medicine has ineffective treatment for hepatic diseases/failure. The solution was, therefore, to be sorted out in the traditional system of medicine, being comparatively inexpensive with minimum side effects and useful (1, 2).

V. serpense Wall. an important medicinal plant that belongs to the Violaceae family. It consists of twenty-three genera and 930 species (3). Out of the total 930 species, about 111 were identified and distributed in China and 17 in Pakistan in different localities (4). Height is around 800-3000m, mostly in the mountains of Northern areas from the sea level (5). It is also distributed in Afghanistan, India, Bhutan, Indonesia, Kashmir, Thailand, Malaysia, Sri Lanka, Myanmar, China and Nepal (6). The plant and its various species are used for treating widespread diseases such as hepatoprotective, Laxative (7), emollient and for the treatment of jaundice, hepatitis, pneumonia, HIV and anticancer, bronchitis, urinary infections and kidney diseases (4, 8-10).

The phytochemistry of *V. serpens* showed that it contains glycosides, flavonoids, alkaloids, coumarins and tannins (11). It also contains methyl salicylate, sugar, mucilage, gum, violin and saponins (8). Ascorbic acid, ascorbate oxidase, peroxidase and catalase are its antioxidant constituents (12). *V. serpens* is traditionally used for the treatment of kidney diseases (10). In light of its great therapeutic potential, the current study was designed to explore the nephroprotective potentials of the crude extract/fractions of *V. serpens* in paracetamol-induced nephrotoxicity rabbits via evaluation of various biomarkers and histological changes.

Materials and Methods

Plant collection

During the month of April 2011, the entire plant (10 kg dry weight) of *V. serpens* Wall. was collected from the Shangla district of Khyber Pakhtunkhwa, Pakistan. Dr. Mohmmad Ibrar, a taxonomist at the Department of Botany, University of Peshawar, Peshawar, identified the plant and placed voucher No.Bot.20158 (PUP) in the department's herbarium.

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Sample preparation

The freshly collected shade-dried plant was powdered and macerated in 80% methanol for 10 days (3x50 L). The methanolic extract was filtered with a muslin cloth, evaporated, and concentrated by a rotary evaporator under a vacuum (at 40°C). The viscous extract for fractionation was dissolved in water and partitioned between n-hexane, ethyl acetate, chloroform, and butanol using a separating funnel with a capacity of 5000 ml. The fractions of n-hexane (27g), ethyl acetate (22.7g), chloroform (17g), butanol (35g) and aqueous (25g) were obtained and investigated along with the crude extract in the schemed protocol.

Animals and experimental layout

Sixty (60) domestic rabbits (Oryctolagus cuniculus), both sexes purchased from the local market, were acclimatized and maintained under optimal conditions at the University of Malakand, Pakistan. The rabbits were fed chaw pellets, fresh green vegetables and grasses and had unlimited access to fresh water. For two weeks, the animals were acclimatised. The research protocols were approved by the Research Ethics Committee, Department of Pharmacy, the University of Malakand on May 24, 2016, with Rec. Ref. No: DREC / 20160524-1.

Animals grouping and dosing

The rabbits were divided into fifteen groups of four animals each. For the crude extract and each fraction, two doses were tested: low (150mg/kg body weight) and high (300mg/kg bwt). During the experiment, paracetamol (PCM) (Glaxo Smith Kline) 2 g /kg body weight, while silymarin was at 50 mg/kg body weight (13).

Group 1 administered with normal saline (5%), served as control,

Group 2 was treated with paracetamol only,

Group 3 received paracetamol on day 0, followed by silymarin.

Groups 4 and 5 received paracetamol followed by crude hydro-methnolic extract at low and high doses, respectively

Groups 6 and 7 received paracetamol followed by n-hexane fraction at low and high doses, respectively

Groups 8 and 9 received paracetamol followed by ethyl acetate fraction at low and high doses, respectively

Group 10 and 11 received paracetamol followed by chloroform fraction at low and high doses, respectively

Group 12 and 13 received paracetamol followed by butanol fraction at low and high doses, respectively

Group 14 and 15 received paracetamol followed by an aqueous fraction at low and high doses, respectively

The animal treatment/dosing was continued for 8 days.

Samples collection and processing

On the day 9th, the animals were anesthetized by chloroform inhalation. Blood was directly collected from the heart and transferred to EDTA tubes. Centrifugation was used to separate the serum, which was then stored at -20°C until further use. Kidney functions were accessed by determining the serum levels of urea and creatinine by using commercially available kits.

Analysis of urine

At the end of the experiment, 24 h urine samples were collected from each animal in each group. After measuring volume, these urine samples were used for the determination of creatinine and urea. These parameters were estimated through COBAS chemistry automation using Roche Diagnostic kits.

Determination of glomerular filtration rate

The urea and creatinine clearance tests were used to estimate the glomerular filtration rate. For this purpose, the following formulas were applied;

Urea clearance

GFR = [Urine urea x Urine volume]/Serum urea.

Creatinine clearance

GFR = [Serum creatinine x Urine volume]/Serum creatinine.

Histopathology

To record the protective role of the crude extract and fractions of *V. serpens* against the paracetamol-induced tissue damage, samples from kidneys were collected immediately after killing the animals and preserved in 10% buffered formalin. Tissues were dehydrated in ethanol in increasing concentrations, cleared with xylene, and embedded in paraffin (14). After making thin slices (3-5µm), samples were stained with Hematoxlin and Eosin (H&E). Representative areas were selected for photography using camera fitted microscope.

Statistical analysis

The data is presented in the form of means and standard deviations (SD). The data were subjected to the Tukey Test of Post Hoc Multiple Comparisons in One Way ANOVA to compare means. SPSS 16.0 software was used for all of these analyses.

Results

Effects of extract/fraction in hematology

The results of the extract/fractions in hematological parameters are presented in table 1. In kidney-related blood parameters, blood urea of some of the fractions and crude extract were non-significant in comparison with the PCM values. Whereas, aqueous fraction, both in low and high doses (150 and 300 mg/kg), crude methanolic extract and chloroform in low doses (150 mg/kg), n-hexane, ethyl acetate and n-butanol in high doses (300 mg/kg) were comparatively more effective than the PCM values and closer to the standard drug (silymarin).

Serum creatinine values of all the fractions, along with the crude methanolic extract, are significant in comparison with the PCM, silymarin and normal saline values. Creatinine clearance reading has been reduced to a low level than the normal value by the PCM dose at 1 mg/kg body weight for 8 days. Chloroform and aqueous fractions at high doses (300 mg/kg) showed highly significant values. The aqueous fraction at a low dose (150 mg/kg) is also comparatively significant. The creatinine clearance values of all the other fractions, along with crude methanolic extracts both at low and high doses are closer to silymarin values, being nephroprotective.

Histopathology

The histological sections of the kidneys of rabbits trea-

Table1. Effect of different solvents extracts of V. serpens Wall. on the kidney function and clearance of the rabbits

Groups	Dose mg/kg	Kidney related parameters with % change values		
		Blood urea	Serum Creatinine	Creatinine Clearance
Normal saline		12.0±2.6	0.3±0.12	4.7±2.8
PCM Control	1000	24.3 ± 2.3	1.5± 0.29	0.36 ± 1.3
Standard silymarin	50	14.5±3.4	0.65±0.3	1.7±0.8
Hydro-Methanolic	150	15.3 ±1.3	0.6± 0.04**	1.5 ±0.29
	300	21± 2.5	0.5±0.00 **	1.35±0.26
n-hexane	150	25±2.6	0.05±0.03***	1.1± 0.21
	300	19.8± 3	0.52±0.02**	1.36±0.27
Chloroform	150	18.5±1.5	0.5 ±0.11**	2.0±0.6
	300	22.3±2.3	0.4±0.03***	4.0±0.9 ***
Ethyl Acetate	150	24 ±3.1	0.6 ±0.12**	0.84±0.18
	300	19.8 ±4.5	0.6± 0.06-1**	0.93 ± 0.24
n-Butanol	150	23.3 ± 3.1	0.62±0.04**	1.26± 0.12
	300	18.8±3.3	0.6± 0.04**	1.5± 0.4
Aqueous	150	17.7± 2.0	0.7±0.08*	2.5±_ 0.59*
	300	14.3±2.0	0.5± 0.08**	4.7 ±1.0***

*P<0.05, **P<0.01 ***P<0.001 when compared with PCM treated group % change = extract treatment value-PCM toxic value/extract treatment value X100.

ted with normal saline showed normal tissue structure with normally placed glomeruli and tubules. The size of glomerular cells and urinary spaces was normal. The tubular epithelial cells were normal in size and adhered to basement membranes. No vascular disturbance was observed (Figure 1).

The histological sections of the kidney of the rabbits treated with paracetamol alone showed a widespread sign of toxicities. The most obvious ones were degeneration changes in the tubules, where the tubular epithelial cells were swollen (most probably hydropic change) and in some places, fatty change. The sloughing of tubular epithelial cells from the basement membrane and accumulation in the tubular lumen was another prominent lesion in the tubular cells. The glomeruli showed shrinkages and increased urinary spaces. No histological observable difference was noted in the sections (Figure 2). The *n*-hexane extract at 150 mg/kg showed normal architecture of distal convoluted tubules and lined by cuboidal epithelial cells (Figure 3), while at 300 mg/kg, normal renal corpuscules with mild dilatation of proximal and distal convoluted tubules with more significant architecture have been observed (Figure 4). The chloroform fraction treatment at 150 mg/kg caused normal renal corpuscules, proximal-distal and convoluted tubules (Figure 5), which further improved at 300 mg/kg. It is very clear from Figures 5 and 6 that there is a direct relation between dose on the significance level of the kidney architecture. With increasing the

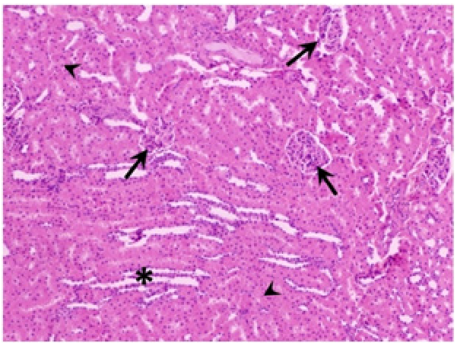

Figure 1. Photomicrograph (100X H&E) of a section of kidney from a rabbit treated with normal saline showing a normal histological appearance of the renal cortex. The cortex contains renal corpuscles (large arrows) embedded among proximal (arrow heads) and distal (asterisk) convoluted tubules.

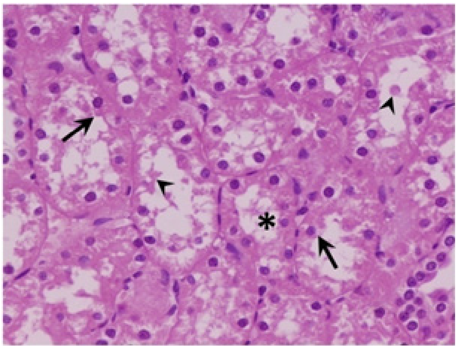

Figure 2. Photomicrograph (100X H&E) of a section of kidney from a rabbit treated with PCM showing necrosis of cuboidal epithelial cells (large arrows) of proximal convoluted tubules with exfoliation of their brush border. The lumen (asterisk) of tubules contains numerous cellular casts (small arrows).

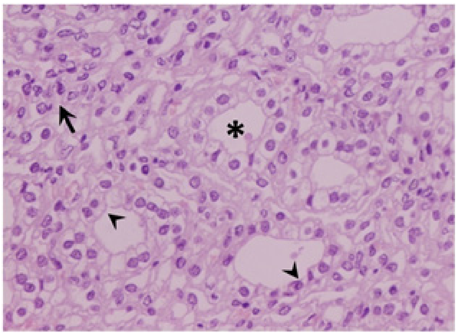

Figure 3. Photomicrograph (100X H&E) of a kidney section from a rabbit treated with *n*-hexane soluble fraction 150 mg/kg showing normal histo-architecture of distal convoluted tubules with a wider lumen (asterisk) and lined by cuboidal epithelial cells (arrow heads). Numerous loops of Henle tubules are also visible (large arrows).

dose (from 150-300 mg/kg) the normality of the kidney architecture improved (Figure 6). Figure 7 shows the kidney slide of a rabbit treated with chloroform fraction at a dose of 300 mg/kg. It shows the clear architecture of renal corpuscles (large arrows) and proximal convoluted tubules (arrow heads). The distal convoluted tubules (asterisk) exhibited mild tubular necrosis of the cuboidal epithelial cells. Figure 8 shows a kidney section of a rabbit treated with ethyl acetate fraction at a dose of 300 mg/kg. It shows normal proximal convoluted tubules (large arrows) with numerous loops of Henle tubules (asterisk). The interlobular blood vessels (arrow heads) among the renal tubules exhibited mild congestion with red blood cells.

Figure 9 shows a kidney section of a rabbit treated with aqueous fraction at a dose of 300 mg/kg. it shows normal renal corpuscles (large arrows). The renal tubules exhibited dilatation (arrow heads) with exfoliation of the brush border lining the proximal convoluted tubules into their lumen. Figure 10. shows a kidney slide, treated with aqueous soluble fraction at a dose of 100mg/kg. It shows mild congestion of the renal corpuscles (large arrows) with severe dilatation of the renal tubules (asterisk). Numerous cellular casts (arrow head) are also visible in the lumen of renal tubules.

In the group of rabbits given silymarin after treatment with paracetamol, a significant protective role was noted in the kidneys histology. The tubular lesions and glomerular structure showed much improvement in the histologi-

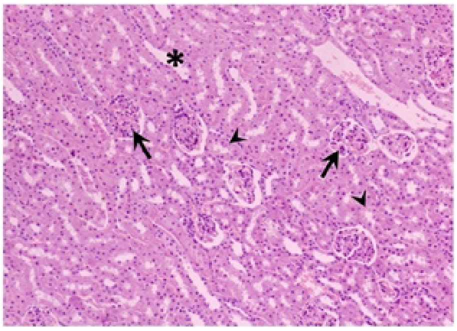

Figure 4. Photomicrograph (100X H&E) of a section of kidney from a rabbit treated with *n*-hexane soluble fraction 300 mg/kg showing normal renal corpuscles (large arrows) with mild dilatation of proximal (arrow heads) and distal (asterisk) convoluted tubules.

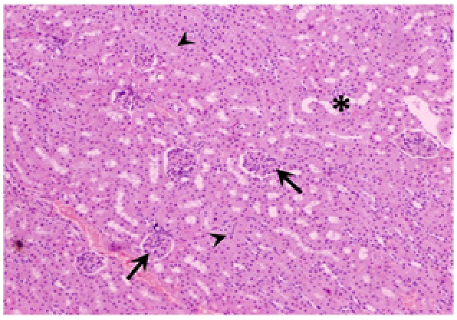

Figure 5. Photomicrograph ((100X H&E)) of a section of kidney from a rabbit treated with chloroform soluble fraction 150 mg/kg showing normal renal corpuscles (large arrows), proximal (arrow heads) and distal (asterisk) convoluted tubules.

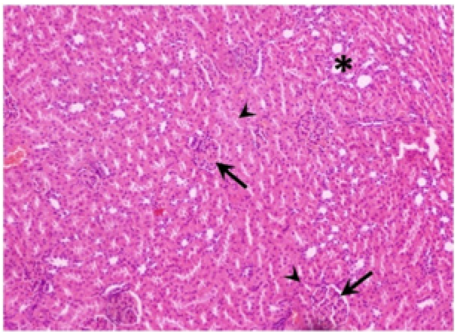

Figure 6. Photomicrograph (100X H&E) of a section of kidney from a rabbit treated with ethyl acetate soluble fraction 150 mg/kg showing normal renal corpuscles (large arrows) with mild dilatation of proximal (arrow heads) and distal (asterisk) convoluted tubules.

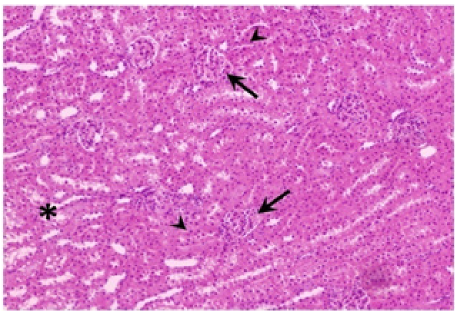

Figure 7. Photomicrograph ((100X H&E)) of a section of kidney from a rabbit treated with chloroform soluble fraction 300 mg/kg showing normal renal corpuscles (large arrows) and proximal convoluted tubules (arrow heads). The distal convoluted tubules (asterisk) exhibited mild tubular necrosis of the cuboidal epithelial cells.

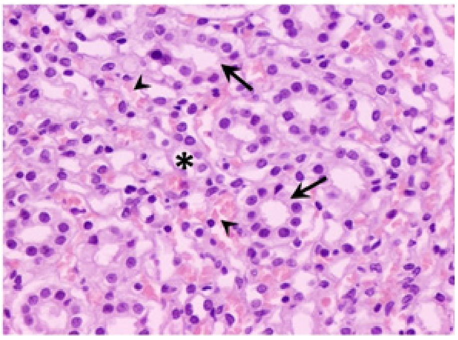

Figure 8. Photomicrograph (100X H&E) of a section of kidney from a rabbit treated with ethyl acetate soluble fraction 300 mg/kg showing normal proximal convoluted tubules (large arrows) with numerous loops of Henle tubules (asterisk). The interlobular blood vessels (arrow heads) among the renal tubules exhibited mild congestion with red blood cells.

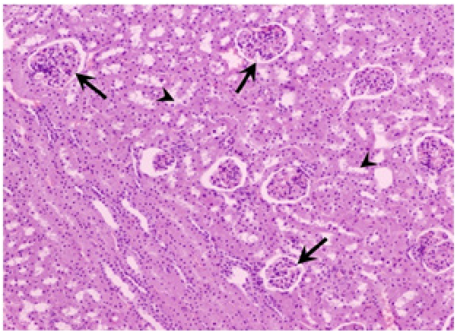

Figure 9. Photomicrograph (100X H&E) of a section of kidney from a rabbit treated with aqueous soluble fraction 300 mg/kg showing normal renal corpuscles (large arrows). The renal tubules exhibited dilatation (arrow heads) with exfoliation of the brush border lining the proximal convoluted tubules into their lumen.

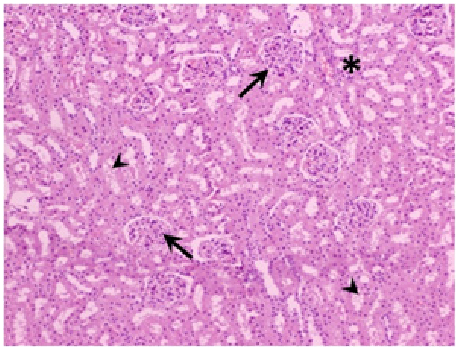

Figure 10. Photomicrograph (100X H&E) of a section of kidney from a rabbit treated with an aqueous soluble fraction (100mg/kg) showing mild congestion of the renal corpuscles (large arrows) with severe dilatation of the renal tubules (asterisk). Numerous cellular casts (arrow head) are also visible in the lumen of renal tubules.

cal structure. The protective role of plant material extracted with methanol and chloroform was obvious by kidney histology. Administering the higher doses of plant extract (300 mg/kg) showed lesser improvement in the histological structure than the lower dose group. In contrast to liver histology, the groups of rabbits given *n*-hexane, ethyl acetate and *n*-butanolic factions showed an inverse dose-related relationship in the kidney's histology. The improvement in the lesions was lesser in groups given a higher dose of plant extract as compared to the lower-dosed group. However, the aqueous fraction showed a dose-dependent response.

Discussion

Exposure of kidneys to drug or their active metabolites may result either in direct toxicity or certain immunological reactions (15). Toxic metabolites are the results of about 62% of administered drug withdrawal. The 90-95% of hepatic metabolites paracetamol metabolism is hepatic which is excretion by the kidney (16, 17). In the body various reactive radicals like hydroxyl radicals, hydrogen peroxide, superoxide anions, nitric oxide, nascent oxygen, and lipid oxides generation occur due to certain internal and external factors resulting in disorders like hepatic ailment (18). Paracetamol is a commonly used analgesic and antipyretic drug results in acute centrilobular necrosis and centrizonal heamorrhagic. In therapeutic doses of paracetamol only 5% of the drug is converted to N-acetyl-p-benzoquineimine (NAPQI) (19). However, it's toxic doses are mostly oxidized by cytochrome p-450 enzymes to highly reactive NAPQI. Decreased glutathione stores or metabolites NAPQI covalently binds to vital proteins, hepatocyte membrane's lipid bilayer and raises the lipid peroxidation (20), which are responsible in mediating cellular damages, and liver and renal toxicity. Drugs-include nephrotoxicity reliable parameters involve increased levels of serum electrolytes, creatinine and urea (21). Low-level Creatinine clearance in the blood circulation is an indication of kidney/s toxicity.

In the present study, the kidney biomarkers, blood urea, and serum creatinine were significantly elevated and the creatinine clearance level was decreased by the paracetamol doses (2 g/kg body weight, orally) as compared to the controlled and the treated groups of the plant extract along with the fractions. The blood urea and serum creatinine values decreased whereas the level of creatinine clearance increased in the crude extract and the fractions intoxicated with paracetamol as compared with the pure paracetamol treated group. The values of the tested parameters were closer to the standard drug silymarin, suggesting the role of the crude extracts and the fractions in repairing kidney injuries and restoring cellular permeability. The free radical scavenging mechanisms may be involved in intercepting the radicals involved in paracetamol metabolism by microsomal enzymes. Anti-oxidants are agents that can neutralize the deleterious effects of free radicals. Exogenous support is taken to keep a balance between oxidants and antioxidants. Plants with anti-oxidant properties are becoming more and more popular all over the world (22). There is a strong relationship between phenols and antioxidant activity (23). The antioxidant constituents and the phenolic compounds showed the potential to prevent the oxidative degradation of cellular components (24). *V. serpens* also contain antioxidant constituents such as ascorbic acid, ascorbate oxidase peroxidase and catalase (12) along with the phenolic contents, which can be on the reason behind its nephroprotection activities against hepatotoxins and nephrotoxins respectively. Another important reason may be the total phenolic contents and antioxidant capacities. Linear positive correlations were found in the plant of *V. serpens* (25). Nephroprotective activity may be another additional mechanism that involves the presence of phytochemicals like flavonoids, glycosides, alkaloids, tannins and coumarins in *V. serpens* (11). Scientific reports also indicate the role of certain flavonoids, triterpenoids and steroids in toxicity (26).

The histological sections of the kidney of the rabbits treated with paracetamol alone showed widespread signs of toxicities like degeneration of tubular epithelial cells, swelling and fatty changes, shrinked glomeruli and increase urinary spaces. It is clear from the histological slides of the groups treated with methanol and chloroform along with the toxic paracetamol doses the presence of certain biochemical constituents secures the kidneys against toxicity. The rabbit groups of n-hexane, ethyl acetate and n-butanolic factions showed inverse dose-related relationships in the kidney's histology.

In ancient medicine, drugs were obtained from plants. Herbal chemicals determine their therapeutic effect according to their action in the human body. Therefore, medicinal plants are classified into certain groups according to their radius of action (27-30). A medicinal plant does not always have a specific effect, and the spectrum of its effects may increase or decrease. This means that one plant may be effective in treating several diseases; on the contrary, a mixture of several plants is often prepared to multiply their effect to strengthen their therapeutic effect (31-34).

Altogether, in a paracetamol-induced nephrotoxicity test in rabbits, the crude extract as well as the different solvent fractions of *V. serpens* exhibited a significant nephroprotective effect. It is due to the presence of antioxidant activity constituents such as flavonoids, glycosides, alkaloids, tannins and coumarins, as well as phenolic compounds. As a result, the most likely mechanism is antioxidant activity, which protects against cellular damage caused by paracetamol toxicity. However, more mechanistic research is needed to precisely elucidate the underlying mechanism for the effects.

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