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ORIGINAL ARTICLE

Renoprotective Effects of the Hydroethanolic Extract of *Senecio serratuloides* against N_w-Nitro L-arginine Methyl Ester-induced Oxidative Stress in Wistar Rats

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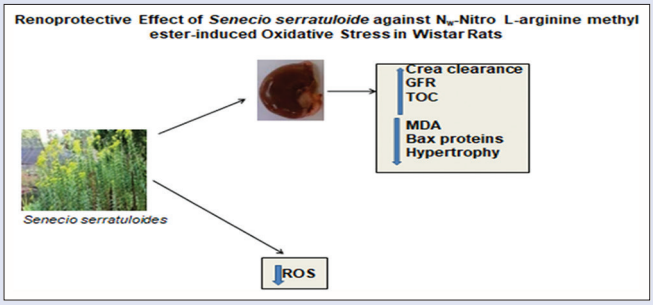
ABSTRACT

Background: Oxidative stress is implicated in the pathogenesis of many diseases. Proper management of oxidative stress requires antioxidants from external sources to supplement that of the body. Plants are considered as a major source of antioxidants because of their natural origin and therapeutic benefits. **Objectives:** This study was aimed at investigating the renoprotective and antioxidant capacity of hydroethanolic extract of *Senecio serratuloides* (HESS). **Materials and Methods:** *In vitro* and *ex vivo* antioxidant capacity of the extract was investigated. Female Wistar rats were treated with N_w-nitro L-arginine methyl ester (L-NAME) (40 mg/kg) for 4 weeks and then cotreating with L-NAME (20 mg/kg) and extract (HESS150 or HESS300 mg/kg) for 2 weeks and finally with plant extract or normal saline only for 2 weeks making a total of 8 weeks. Twenty-four-hour urine samples were collected during the study, and at the end of the study; blood and kidneys were harvested for biochemical and histological assays. **Results:** HESS exhibited high antiradical activity against 1,1-diphenyl-2-picrylhydrazyl and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radicals with IC_{50} values of 0.1 and 0.4 mg/ml, respectively. HESS significantly ($P < 0.01$) augmented L-NAME-induced decrease in creatinine clearance, glomerular filtration rate, and serum calcium concentration. HESS significantly increased *in vivo* antioxidant capacity ($P < 0.01$), decreased malondialdehyde ($P < 0.01$), and Bax ($P < 0.001$) concentration. It showed renoprotection and significantly ($P < 0.01$) prevented collagen deposition in the kidneys. **Conclusion:** *S. serratuloides* has renoprotective and free radical-scavenging properties and therefore maybe important in combating oxidative stress-mediated diseases in the kidneys and other parts of the body.

Key words: Antioxidants, Nw-nitro L-arginine methyl ester, oxidative stress, renoprotection, *Senecio serratuloides*

SUMMARY

- This study was aimed at examining the renoprotective and antioxidant capacity of the hydroethanolic extract of *Senecio serratuloides*. Oxidative stress was induced in female Wistar rats using N_w-nitro L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor. *In vitro* and *ex vivo* antioxidant capacity of the extract, as well as biochemical and histological assays of samples harvested from the rats treated with L-NAME and the plant extract, was carried out. Therefore, *S. serratuloides* has renoprotective and free radical-scavenging properties and therefore maybe important in combating oxidative stress-mediated diseases.



Abbreviations used: ABTS: 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); ATPase: adenosine triphosphatase; BH_4: 4-tetrahydrobiopterin; Crea: Creatinine; CPT: Captopril; cGMP: Cyclic guanosine monophosphate; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ECs: Endothelialcells; eNOS: Endothelial nitric oxide synthase; FRAP: Ferric reducing antioxidant power; GAE: Gallic acid equivalent; GFR: Glomerular filtration rate; HESS: Hydroethanolic extract of *Senecio serratuloides*; L-NAME (LN): N_w-nitro L-arginine methyl ester; NO: Nitric oxide; PKGI: Protein kinase GI; ROS: Reactive oxygen species; TBA: Thiobarbituric acid; TBARS: Thiobarbituric acid reactive substance; TCA: Trichloroacetic acid; TPTZ: 2,4,6-tripyridyl-s-triazine; TOC: Total antioxidant capacity; UP/UCr: Urine protein-to-urine creatinine ratio; VSMCs: Vascular smooth muscle cells.

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INTRODUCTION

Oxidative stress (imbalance between oxidants and antioxidants) is implicated in the pathogenesis of many diseases.[1-3] In normal physiological conditions, the production of reactive oxygen species (ROS) is tightly regulated by endogenous cellular antioxidants; hence, their rate of generation is counterbalanced by the rate of elimination. However, in pathological conditions, either due to increased ROS production or diminished antioxidant levels, the

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presence of ROS outweighs the usual protective antioxidant mechanisms employed by the cells, leading to a state of oxidative stress.[4] Increased production of ROS decreases nitric oxide (NO) bioavailability by direct inactivation through formation of peroxynitrite[3] or by inhibition of endothelial nitric oxide synthase (eNOS) activity through oxidation of 4-tetrahydrobiopterin (BH_4) leading eNOS uncoupling.[5]

ROS also stimulate increased deposition of extracellular matrix (ECM) proteins such as collagen while modulating matrix metalloproteinases, which are responsible for degrading collagen and other ECM proteins. In addition, ROS increase intracellular free Ca^{2+} concentration in vascular smooth muscle cells and endothelial cells (ECs) by mobilization from reticular stores and activation of Ca^{2+} channels.[6,7]

Medicinal plants are a major reservoir of phytochemicals which can play a great role in reducing occurrences of many diseases by acting as antioxidants and supplying the body with necessary nutrients.[8] Examples of these phytochemicals include tannins, saponins, phenols, terpenoids, alkaloids, and flavonoids.[9] Sterol, flavonoid, saponin, tannin, phenol, alkaloid, and cardiac glycoside have been proven to have antioxidant activities.[10,11] These phytochemicals exhibit their antioxidant effects through; scavenging ROS or suppressing their function and upregulating or protecting antioxidant defenses[12] *Senecio serratuloides* is a herb which is used in traditional medicine for treating, skin disorders, sores, rashes, burns, and wounds.[13] Based on these ethnomedicinal reports, the objective of this study was to investigate the renoprotective effects of the hydroethanolic extract of *S. serratuloides* (HESS) by determining its antioxidant effects, its effect on renal parameters, and renal histology.

MATERIALS AND METHODS

Materials

Creatinine reagent (CR510), urea reagent (UR221), calcium reagent (CA590), potassium reagent (PT1600), and total protein reagent (UP1571) (Randox Laboratories Ltd., UK); Bradford reagent, FastCast acrylamide kit, Turbo transfer kit (170–4270), and Clarity Western ELC substrate (170–5060) (Bio-Rad Laboratories, USA); anti-Bax antibody (ab32503), anti-ß-actin antibody (ab8227), and goat anti-rabbit IgG HandL (HRP) (ab205718) (Abcam Laboratories Inc., USA); protease inhibitor (S8820-20TAB), RIPA buffer (R0278), Nω-nitro-L-arginine methyl ester, and ß-mercaptoethanol (Sigma, USA); stains (Harris hematoxylin, eosin, and picro-sirius red); and solvents (ethanol, glacial acetic acid, and methanol xylene) were of analytical grade.

Plant material and extraction

S. serratuloides whole plant was supplied by Mr Fikile Mahlakata of Lusikisiki, Eastern Cape, South Africa. It was authenticated by Dr Immelman in the KEI Herbarium of Walter Sisulu University where a voucher specimen (Tata 1/13967) was deposited. The plant was air-dried in the laboratory and pulverized and thoroughly extracted in 70% ethanol. The ethanol was recovered using a rotary evaporator (Heidolph Laborota 4000, Germany) at 35°C, and the extract was dried in an oven at the same temperature. The plant extract was then stored in a refrigerator and dissolved in distilled water before use.

Animal handling

Thirty female Wistar rats weighing 200–240 g were lodged in cages in the animal room at Walter Sisulu University which is maintained at 24°C. The room was lit by daylight and dark at night. The rats were allowed free access to rat chow and water. The ethical clearance for the study was approved the Faculty of Health Sciences Ethical Clearance Committee, Walter Sisulu University, South Africa. All animal procedures were carried out in line with the South African National Standards: The Care and Use of Animals for Scientific Purposes.[14]

Phytochemical screening

Phytochemical screening of HESS for the presence of various phytochemical constituents was done following standard procedures as described by Balamuniappan *et al.*[15] Total phenolic content was quantified using Folin's reagent with gallic acid as standard, whereas flavonoid content was determined using quercetin as standard.[16]

Antioxidant capacity of extract

1,1-diphenyl-2-picrylhydrazyl assay

The 1,1-diphenyl-2-picrylhydrazyl assay (DPPH) assay is a method for determining the ability of extracts to trap free radicals. The test was done following the method described by Irshad *et al.*[16] with modifications. DPPH radical solution was prepared in a dark room by dissolving 0.01 g of DPPH radical in 500 mL of methanol to get an absorbance of 1.5 units at 517 nm. The stock solution of the standard was prepared by dissolving 0.1 g ascorbic acid in 100 mL distilled water. 640 µL of the stock solution was added to 3360 µL distilled water to yield a concentration of 160 µg/mL working solution which was double diluted serially to several concentrations (160, 80, 40, 20, 10, 5, 2.5, and 1.25 µg/mL). The working solution of the extracts was prepared by adding 2 mL distilled water to 2 mL extract stock yielding a concentration of 5 mg/mL which was then double diluted serially to various concentrations (5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04 mg/mL). In a dark room, 3 mL DPPH solution was added to 1 mL of the working solutions (standard or extract) and incubation was done at room temperature for 30 min. Absorbance was measured using a spectrophotometer using a ultraviolet–visible spectrophotometer (Phoenix-2000V, UK) at 517 nm. The percentage DPPH radical-scavenging activity was calculated using the following equation:

$$\%\text{DPPH radical scavenging} = ([A_{blank} - A_{std}/_{extract}]/A_{blank}) \times 100$$

The percentage inhibition was plotted against concentration, and IC_{50} values were extrapolated from plots.

2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) assay

The 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation was used to screen the radical-scavenging abilities of some phytochemicals in the crude extract. The procedure was done following the methods as described by Thaipong *et al.*[17] with modifications. ABTS stock solution was prepared from 7.4 mM ABTS and 2.6 mM potassium persulfate solution that is 0.4 g ABTS was dissolved in 100 mL distilled water and 0.07 g of potassium persulfate was dissolved in 100 mL distilled water. The ABTS and potassium persulfate solutions were mixed in the ratio 1:1 and incubated for 12 h in a dark room at room temperature. ABTS working solution was prepared by mixing 1 mL ABTS stock and 60 mL methanol to get an absorbance of 1.17 units at 734 nm. The working solution of the extracts was prepared by adding 1.6 mL distilled water to 0.4 mL extract stock to give a concentration of 2 mg/mL which was then double diluted serially (2, 1, 0.5, 0.25, 0.13, 0.06, and 0.03 mg/mL). The stock of the standard was prepared by dissolving 0.5 g Trolox in a few drops of dimethyl sulfoxide and making up the volume to 100 mL with ethanol. Trolox working solution was prepared by adding 1960 µl distilled water to 40 µL Trolox stock solution to give a concentration of 100 µg/mL which was double diluted serially (100, 50, 25, 12.5, 6.25, 3.13, and 1.56 µg/mL). 2850 µL ABTS working solution was added to 150 µL extract or standard and incubated for 1 h in a dark room. Absorbance was read at 734 nm using a spectrophotometer and IC_{50} values calculated as was done with DPPH assay.

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay measures the total reducing power of electron-donating antioxidants found in a reaction mixture. It was done using method as described by Irshad *et al.*[16] with modifications. The stock solutions (300 mM acetate buffer [3.1 g $C_2H_3NaO_2$ $3H_2O$ and 16 mL glacial acetic acid ($C_2H_4O_2$), 1 L distilled water], 10 mM 2,4,6-tripyridyl-s-triazine [TPTZ] solution in 40 mM HCl) was made by dissolving 0.156 g TPTZ in 50 ml HCl and then 1.1 g $FeCl_3$ in 200 mL dH_2O solution. Fresh FRAP reagent was prepared by combining 100 mL acetate buffer, 10 mL TPTZ, and 10 mL $FeCl_3$. The standard stock was prepared by dissolving 0.1 g ascorbic acid in 100 mL distilled water to yield a concentration of 1 mg/mL. 320 µL stock solution of the standard was added to 3380 µL distilled water to give a concentration of 80 µg/ml which was double diluted serially (80, 40, 20, 10, 5, 2.5, and 1.25 µg/mL). 0.2 ml extract stock added to 0.8 mL distilled water forming a working solution of 1 mg/mL. The assay was done in triplicate; the freshly prepared FRAP reagent was added to 100 µL extract or standard and incubated in a water bath at 40°C for 4 min. Absorbance was measured at 593 nm. Results were extrapolated from standard curve and expressed in µg ascorbic acid equivalents.

Treatment with N_w-nitro L-arginine methyl ester and extract

Animals were randomly assigned to five treatment groups with six rats per group ($n = 6$): NT (normal saline only) group, LN (L NAME) group, CPT (captopril (20 mg/kg)) group, HESSLN150 (HESS (150 mg/kg)) group and HESSLN300 (HESS (300 mg/kg)) group.

Rats were treated with L-NAME (40 mg/kg) for 4 weeks and then cotreated with normal saline or captopril or extract and L-NAME (20 mg/kg) for 2 weeks and finally with the normal saline or captopril or extract only in the past 2 weeks.[18] Throughout the 8 weeks, the NT control group received normal saline only. Necropsy was performed at the end of the study.

Urine collection

Urine was collected in graduated cylinders by placing rats singly in metabolic cages for 24-h urine collection on day 0 before treatment and then weekly after daily oral administration of assigned treatment. Collected urine was kept in a minus 20 freezer for later analysis. The quantity of water consumed was also monitored. Urine flow was calculated using the formula:

Urine flow = 24 h urine volume/1440 ml/min

where 1440 = 24 h × 60 min.

Termination of treatment

Two days to the end of the experiment, the rats were not treated, and on the evening, they were fasted for 16 h, weighed, and sacrificed.[19] Blood was collected by cardiac puncture for serum preparation. Kidneys were harvested, and one kidney from each rat was fixed in 10% buffered formalin[20] and the other was stored in a minus 20 freezer for later analysis.

Urine analysis

Urine samples were analyzed for protein, urea, creatinine, and calcium using commercial kits (Randox Co., UK), and procedure was followed as described by manufacturer. Urine protein-to-creatinine ratio, creatinine clearance, and glomerular filtration rate (GFR) were calculated using the following equations:[21]

Urine protein-to-urine creatinine ratio (UP/UCr) = urine protein/urine creatinine

Crea clearance = (urine Crea [mg/dl] × 24 h urine [ml])/(serum Crea [mg/dl] × 1440)

GFR = (Urine Crea × Urine volume × 1000)/(serum Crea × body weight × 1440)

Where Crea: creatinine, 1440: min in 24 h, GFR: glomerular filtration rate.

Determination Bax protein concentration in kidney

Identification of Bax protein was by western blotting using commercial kits (Bio-Rad Laboratories, USA) following the method described by Singh *et al.*[22] with modifications. In brief, proteins in kidney homogenate were quantified using Bradford reagent. The proteins were separated by electrophoresis and blotted unto nitrocellulose membrane. The membrane was then incubated with anti-Bax antibody (ab32503), followed by goat anti-rabbit IgG HandL (HRP) (ab205718). Bound antibodies were detected by chemiluminescence using Clarity Western enhanced chemiluminescence substrate (170–5060), and imaging was done using ChemiDoc Touch Imaging System (Bio-Rad Laboratories, USA). Analysis of images was performed using Image Lab software (Bio-Rad Laboratories, USA), and Bax protein bands were normalized using housekeeping proteins (ß-actin, ab8227).

Determination of total antioxidant capacity in serum and kidney tissues

Total antioxidant capacity (TOC) of serum and tissue homogenates from treated animals was determined by FRAP assay following standard procedure[23] with modifications. Briefly, 3 ml freshly prepared FRAP reagent was added 100 µl of sample (serum, heart, or kidney homogenates), and the solutions were incubated for 15 min at room temperature. Absorbance was read at 593 nm. Antioxidant activity was extrapolated from the standard curve and expressed as ascorbic acid equivalent per milliliter of serum and per milligram of tissue.

Determination of lipid peroxidation

Thiobarbituric acid (TBA) reactive substance assay was used to evaluate lipid peroxidation. Malondialdehyde (MDA), which is an end product of polyunsaturated fatty acid peroxidation, reacted with TBA to form a pink complex, whereas trichloroacetic acid (TCA) in hydrochloric acid extracted lipids and proteins to prevent interference.[24] Briefly, 0.392 g TBA was dissolved in 75 mL of 0.25 M HCl. 5 g TCA was added to the mixture, and the volume was made up to 100 ml using 0.25 M HCl forming a TBA-TCA mixture. 500 µL of TBA-TCA mixture was then added 200 µL serum or homogenates (heart or kidney), and 150 µL PBS solutions were mixed, covered with aluminum foil, and boiled in a water bath at 100°C for 15 min. The mixtures were then allowed to cool to room temperature and centrifuged at 3000 rpm, 20°C for 10 min. 200 µL supernatant was transferred into microplate wells, and absorbance was read at 540 nm using a microplate reader (Bio-Rad model 680, USA). The concentration of MDA in micrometer was calculated using a molar extinction coefficient of 1.56×10^5/M/cm

MDA concentration (µM) = Absorbance at 540 nm × 0.156.

Renal histology

Kidney sections were fixed in 10% buffered formalin. The fixed sections were embedded in paraffin wax,[20] sectioned to 5-µm slices, and stained with hematoxylin/eosin[25] and picro-sirius red stain.[26] The sectioned slices were examined by light microscopy at ×20 and ×40, whereas semi-quantification of collagen was done using scientific image analysis software (ImageJ.NIH.gov/ij/).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). One-way analysis of variance, followed by Tukey's test for multiple comparisons, was performed to determine differences between treatment groups. Results were expressed as mean ± standard error (SEM). $P < 0.05$ was considered statistically significant.

RESULTS

Phytochemical constituents of hydroethanolic extract of *Senecio serratuloides*

Phytochemical analysis of HESS showed that it contains; alkaloids, phenols, steroids, tannins, saponins, flavonoids, and terpenes. The total phenolic content of HESS was 172.7 ± 1.5 µg GAE/mg of extract, whereas the total flavonoid content was found to be 36.3 ± 0.2 µg quercetin equivalent/mg of extract.

Antioxidant capacity of hydroethanolic extract of *Senecio serratuloides*

From the ABTS assay, HESS had an IC_{50} value of 0.4 mg/ml compared to the standard Trolox with IC_{50} of 0.07 mg/ml. HESS showed a scavenging effect on DPPH radical with an IC_{50} of 0.1 mg/mL compared to standard ascorbic acid of IC_{50} 0.03 mg/ml. HESS equally had a strong reducing power with a value of 151 ± 0.8 µg in GAE/mg of the extract.

Effect of hydroethanolic extract of *Senecio serratuloides* on total antioxidant capacity

In kidney homogenates, HESS300 (44.39 ± 5 µgGAE/mg kidney tissue) had significantly ($P < 0.05$) higher TOC compared to the NT (27.5 ± 1 µgGAE/mg kidney tissue) and LN (28.2 ± 3 µgGAE/mg kidney tissue) control groups [Figure 1]. There was no significant difference in TOC in serum and heart samples of all treatment groups [Table S1].

Effect of hydroethanolic extract of *Senecio serratuloides* on malondialdehyde concentration

The concentrations of MDA in serum were different among treatment groups; LN (2.29 ± 0.2 µM/ml serum; $P < 0.01$) and CPT (3.82 ± 0.7 µM/ml serum; $P < 0.01$) were significantly higher compared to the NT group (0.49 ± 0.1 µM/ml serum). On the other hand, HESS150 (0.6 ± 0.2 µM/ml serum; $P < 0.05$) and HESS300 (0.31 ± 0.1 µM/ml serum; $P < 0.01$) significantly prevented the L-NAME-induced increase in MDA concentration [Figure 2]. In heart homogenate, HESS300 (0.28 ± 0.03 µM/mg heart tissue) and CPT had significantly ($P < 0.01$) lower MDA concentration compared to the LN (1.22 ± 0.2 µM/mg heart tissue) group [Figure 2]. In kidney homogenate, there was no significant difference in MDA concentration although HESS300 (3.99 ± 0.1 µM/mg kidney tissue) had slightly lower MDA concentration compared to the LN (4.81 ± 0.3 µM/mg kidney tissue) group.

Effect of hydroethanolic extract of *Senecio serratuloides* renal function

Results from renal function tests showed that L-NAME significantly increased urine output and hence urine flow; it decreased creatinine, urea, and calcium excretion in urine and induced proteinuria with higher protein-to-creatinine ratios in the LN group compared to the NT group. HESS150 and HESS300 significantly decreased urine output, increased urea, and creatinine excretion, whereas HESS300 increased calcium excretion and decreased proteinuria compared to the LN control group [Table 1].

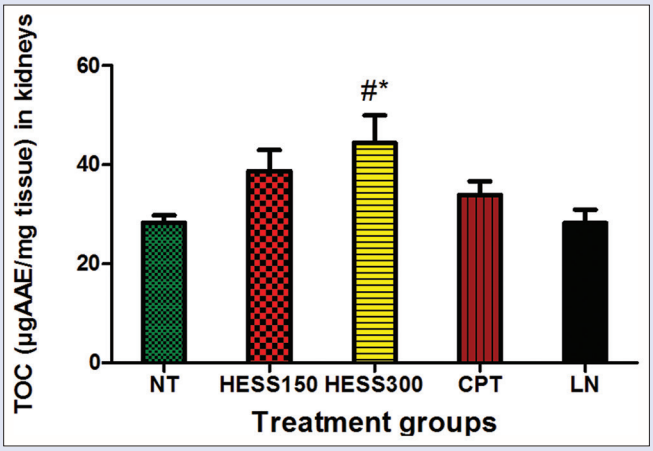

Figure 1: Total antioxidant capacity in kidney homogenates. Values are expressed as mean ± standard error, $n = 6$; NT: Normotensive control; LN: L-NAME control; CPT: Captopril; HESS150 and HESS300: Hydroethanolic extract of *Senecio serratuloides* at 150 and 300 mg/kg, respectively. $^{*}P < 0.05$ compared to L-NAME (LN) control group; $^{\#}P < 0.05$ compared to normotensive control group

In serum, L-NAME significantly increased calcium and creatinine concentrations in the LN group compared to the NT group. It was also observed that creatinine clearance and GFR in the LN group were lower compared to all treatment groups; meanwhile, HESS150 and HESS300 significantly increased creatinine clearance and GFR compared to the LN control group [Table 2]. There was, however, no significant difference in blood urea nitrogen and potassium concentration between the treatment groups in serum.

Effect of hydroethanolic extract of *Senecio serratuloides* on Bax concentration in the kidney

Figure 3 shows blots of Bax and graph with normalized concentrations of Bax in kidneys. L-NAME significantly ($P < 0.05$) increased the expression of Bax in the kidney compared to the NT control. HESS150, HESS300, and CPT had significantly ($P < 0.001$) lower Bax concentration compared to the LN control group.

Effect of hydroethanolic extract of *Senecio serratuloides* on renal tissue

L-NAME caused glomerular degeneration and shrinkage that was observed with both stains. HESS prevented these degeneration and shrinkages in a dose-dependent manner. There was a significant deposition of collagen in kidney samples from the LN group (0.84 ± 0.1%) compared to the NT group (0.15 ± 0.02%). This L-NAME-induced fibrosis was significantly ($P < 0.01$) attenuated by HESS150 (0.31 ± 0.03%), CPT (0.15 ± 0.03%), and HESS300 (0.27 ± 0.03%) [Figure 4].

DISCUSSION

Results from this study showed that HESS exhibited high antioxidant capacity which was evident in its ability to improve TOC and prevent lipid peroxidation. *In vivo* HESS prevented L-NAME-induced proteinuria, increased creatinine clearance, GFR, and serum calcium concentration while decreasing the concentration of Bax proteins in kidneys and protecting the kidneys.

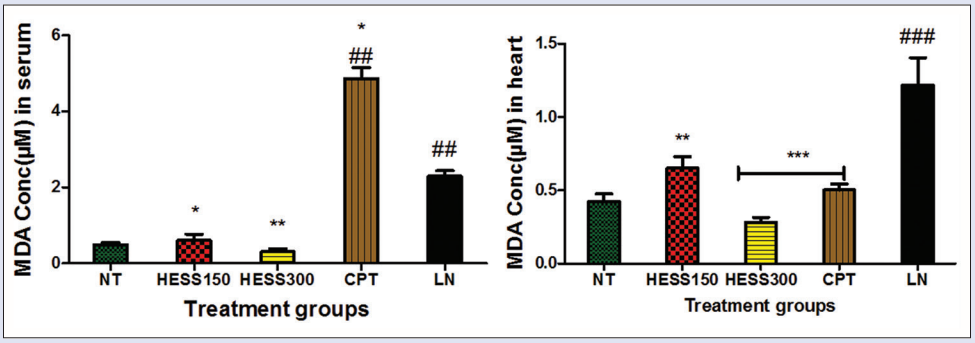

Figure 2: Concentration of malondialdehyde in serum and heart homogenates. Values are expressed as mean ± standard error, $n = 6$; NT: Normotensive control; LN: L-NAME control; CPT: Captopril; HESS150 and HESS300: Hydroethanolic extract of *Senecio serratuloides* at 150 and 300 mg/kg, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to L-NAME control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to normotensive control group

Table 1: Effect of hydroethanolic extract of *Senecio serratuloides* on markers of renal function

Parameters	Treatment groups				
	NT	SS150	SS300	CPT	LN
Urine output (ml)					
Week 5	10±1	24±5$^{\#}$	17±2	13±1	21±2
Week 6	9±1	31±6$^{\#}$	13±4	16±1	25±2$^{\#}$
Week 7	11±1	20±2	16±2**	10±0***	29±4$^{\#\#\#}$
Week 8	10±1	17±1$^{\#\#,**}$	16±1$^{\#,***}$	11±1***	25±2$^{\#\#\#}$
Urine flow (ml/min)					
Week 5	$6.9\times10^{-3}\pm0.001$	$16.7\times10^{-3}\pm0.004^{\#\#}$	$11.7\times10^{-3}\pm0.002$	$8.7\times10^{-3}\pm0.004$	$14.6\times10^{-3}\pm0.002^{\#}$
Week 6	$6.3\times10^{-3}\pm0.001$	$21.5\times10^{-3}\pm0.001^{\#\#\#}$	$8.7\times10^{-3}\pm0.003$	$11.1\times10^{-3}\pm0.001$	$17.5\times10^{-3}\pm0.002^{\#\#}$
Week 7	$7.4\times10^{-3}\pm0.001$	$13.9\times10^{-3}\pm0.001$	$10.7\times10^{-3}\pm0.001^{**}$	$6.9\times10^{-3}\pm0.001^{***}$	$20\times10^{-3}\pm0.003^{\#\#\#}$
Week 8	$6.8\times10^{-3}\pm0.001$	$11.9\times10^{-3}\pm0.001^{\#\#,**}$	$10.9\times10^{-3}\pm0.001^{\#,***}$	$7.5\times10^{-3}\pm0.001^{\#,***}$	$17.5\times10^{-3}\pm0.001^{\#\#\#}$
Creatinine (mg/dl)					
Week 6	72.7±10	48.67±3	59.02±7	57.73±9	32.24±3$^{\#\#}$
Week 7	70.42±4	54.49±6	61.5±10	81.3±7**	33.54±8$^{\#\#}$
Week 8	74.12±8	64.7±7*	68.77±9**	73.86±7**	29.96±5$^{\#\#}$
Protein (mg/24 h)					
Week 6	10.75±1	39.94±2$^{\#\#}$	19.34±7	28.66±9	34.63±7$^{\#}$
Week 7	9.86±1	34.03±2$^{\#\#}$	20.2±4**	10.2±2***	43.49±6$^{\#\#\#}$
Week 8	10.11±1	32.6±4$^{\#}$	21.05±6	11.62±3**	45.42±12$^{\#\#}$
Protein: creatinine ratio					
Week 6	1.13±0.2	3.75±0.4	1.5±0.3*	1.83±0.2	4.06±1$^{\#}$
Week 7	1.01±0.2	2.46±0.3	1.67±0.3	0.80±0.1**	5.05±2$^{\#}$
Week 8	1.08±0.1	2.66±0.2$^{\#}$	1.48±0.4	0.89±0.1**	5.22±1$^{\#}$
Urea (mg/dl)					
Week 6	1688±139	1519±906	1959±415	2614±1348	825±173
Week 7	1581±265	1538±246	1539±270	1869±128*	760±136
Week 8	1526±101	1802±111**	2320±298$^{***,\#}$	1979±109***	801±33$^{\#}$
Calcium (mg/dl)					
Week 6	17.79±2	16.44±2	19±3	19.48±1	14.4±1
Week 7	17.35±2	22.3±2*	21.42±1	23.21±2*	13.34±2
Week 8	15.84±2	17.6±1	20.2±0.4$^{*\#}$	17.86±2	14.78±1

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to LN control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared to normotensive control group. Values are expressed as mean±SEM, $n=6$. NT: Normotensive control; LN: L-NAME control; CPT: Captopril; HESS150 and HESS300: Hydroethanolic extract of *Senecio serratuloides* at 150 and 300 mg/kg, respectively; SEM: Standard deviation

The high antioxidant capacity of HESS was due to the presence of a wide range of phytochemicals such as polyphenols (phenols, flavonoids, tannins, and lignins) and terpenoids. Oxidative stress may hasten renal injury development through cytotoxicity[27] as seen with increase in both plasma and renal MDA levels, which suggests that the levels of ROS in plasma could reflect its production in the kidney. The antioxidants found in HESS could have prevented oxidative stress by either suppressing ROS formation, scavenging of ROS, or by upregulating or protecting antioxidant defenses.[12] The *in vitro* antioxidant capacity of HESS was translated *in vivo* as it showed higher TOC and prevented lipid peroxidation.

The protective role of HESS and CPT against oxidative stress may have been through scavenging of free radicals or preventing of free radical formation. Captopril has the ability to scavenge free radicals due to the presence of a sulfhydryl group; thus, it is capable of preventing oxidant-induced cell injury.[28] HESS may also have been capable of preventing oxidation of NO to peroxynitrite or oxidation of BH_4 by ROS, thus preventing eNOS uncoupling and oxidative stress.

The HESS extract was found to prevent proteinuria and increase creatinine clearance, urea excretion, and GFR. The function of HESS in renal protection was further reinforced by its ability to prevent

Table 2: Effect of hydroethanolic extract of *Senecio serratuloides* on markers of renal function

Parameters	Treatment groups				
	NT	SS150	SS300	CPT	LN
Serum potassium (mmol/l)	5.31±0.3	4.71±0.8	5.32±0.7	4.14±0.6	5.86±0.3
Serum calcium (mg/dl)	10.52±0.2	9.77±0.2***	10.94±0.4***	10.58±0.2***	4.91±0.6###
BUN (mg/dl)	23.23±1	20.95±3	23.97±1	19.85±3	25.14±1
Serum creatinine (mg/dl)	0.63±0.1	0.78±0.1**	0.53±0.04***	0.74±0.04**	1.44±0.2###
Creatinine clearance (ml/min)	0.85±0.1	1.22±0.3*	1.32±0.2**	0.75±0.1	0.39±0.1
GFR	3.55±0.5	4.68±1*	5.7±1**	3.13±0.3	1.55±0.2

*P<0.05, **P<0.01, ***P<0.001 compared to LN control group; ###P<0.001 compared to normotensive control group. Values are expressed as mean±SEM, n=6. NT: Normotensive control; LN: L-NAME control; CPT: Captopril; HESS150 and HESS300=hydroethanolic extract of *Senecio serratuloides* at 150 and 300 mg/kg, respectively; GFR: Glomerular filtration rate; BUN: Blood urea nitrogen

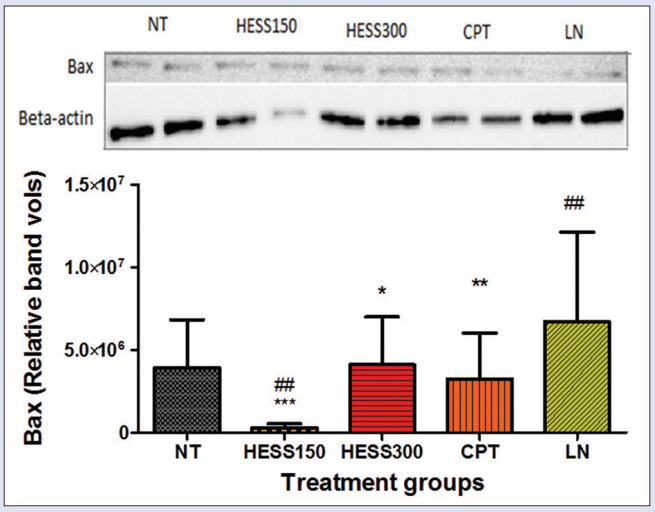

Figure 3: Western blot analysis to measure the effect of L-NAME treatment on the expression of Bax in kidneys. The volumes of Bax bands were evaluated and their ratios to β-actin were measured. Results are represented as mean ± standard error. *P < 0.05; **P < 0.01; ***P < 0.001 different from LN control group, ##P < 0.01 different from NT control group. L-NAME: N_w-nitro L-arginine methyl ester; LN: L-NAME; NT: Normotensive control

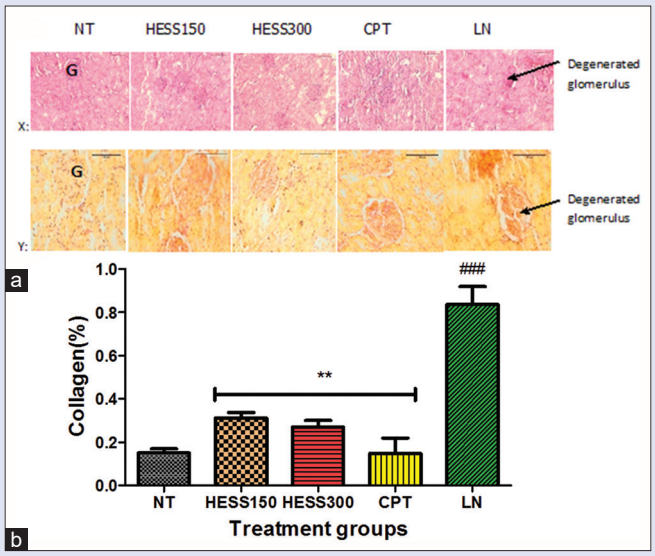

Figure 4: Representative photomicrographs (a) of renal tissue and graph (b) of standard error i-quantitative analysis of collagen in renal tissue.(x)-micrographs of renal tissue stained with hematoxylin and eosin (×20); (y)-micrographs of renal tissue stained with picro-sirius red stain (×40). NT: normotensive control; HESS: hydroethanolic extract of *Senecio serratuloides* at 150 and 300 mg/kg; CPT: captopril group; LN: L-NAME group. Values are expressed as mean ± standard error. **P < 0.01 versus LN control; ###P < 0.001 versus NT control

glomerular degeneration/shrinkage which is generally observed with L-NAME treatment. Oxidative stress has a role in the induction of cardiac dysfunction in renal patients.[29] In fact, cardiovascular morbidity and mortality occurs throughout renal dysfunction progression even in patients with moderate renal insufficiency,[27] and most of the patients with renal dysfunction die of cardiovascular reasons rather than progression to complicated renal disease.[27] Given that HESS had the ability to prevent oxidative stress in the heart and kidneys and previous studies have reported its ability to prevent cardiac hypertrophy,[30] it suggests that it may be important in combating renal dysfunction as well as associated cardiovascular disorders.

It also prevented L-NAME-induced release of Bax in the kidney. The renoprotective effect of HESS could also have been through inhibiting inflammation and oxidative stress. Increased glomerular capillary wall pressure and oxidative stress may have accounted for the elevated levels of Bax and hence apoptosis revealed by glomerular degeneration. Studies done in some cell lines have proposed the cytoprotective role of NO through inactivation of caspase-3 through S-nitrosylation; thus, decreased NO production in L-NAME model can promote apoptosis.[31]

The decreased concentration of calcium in serum induced by L-NAME was prevented by HESS. This suggested that the protective role of HESS may have been by preventing calcium influx through its upregulation of NO availability. Iyú *et al.*[32] investigated calcium signaling in platelets from L-NAME-treated rats. They found that reduction of NO altered the regulation of platelet calcium levels such that calcium entry from the extracellular space and mobilization from the internal stores were enhanced.[32] This can be explained by the fact that NO is an important regulator of intracellular calcium level, and it activates soluble guanylyl cyclase which leads to increase cyclic guanosine monophosphate (cGMP). cGMP inhibits voltage-gated calcium channels and activates protein kinase GI which phosphorylates calcium-dependent potassium channels, inositol trisphosphate receptor-associated cGMP kinase substrate, and sarcoplasmic reticulum adenosine triphosphatase.[33] Thus resulting in the sequestration of calcium in the sarcoplasmic reticulum and thereby decreasing intracellular calcium flux.[34] NO also directly inhibits calcium-dependent potassium channels preventing calcium influx.[33] This mechanism supported the proposal that L-NAME-induced decrease in serum calcium may be due to increased calcium influx due to decreased bioavailability of NO.

CONCLUSION

The HESS had free radical-scavenging properties and hence renoprotective properties. Therefore, it may serve as a potential source of antioxidants which can be used for treating oxidative stress-induced diseases.

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Conflicts of interest

There are no conflicts of interest.

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