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Article

Renal protective effects of vicenin-2 and scolymoside in a mouse model of sepsis

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This study was initiated to determine whether 2 structurally related flavonoids found in *Cyclopia subternata*—vicenin-2 (VCN) and scolymoside (SCL)—could modulate renal functional damage in a mouse model of sepsis, and to elucidate the relevant underlying mechanisms. The potential of VCN and SCL treatment to reduce renal damage induced by cecal ligation and puncture (CLP) surgery in mice was measured via assessment of serum creatinine, blood urea nitrogen (BUN), lipid peroxidation, total glutathione, glutathione peroxidase activity, catalase activity, and superoxide dismutase activity. Treatment with either VCN or SCL resulted in elevated plasma levels of BUN and creatinine, and of protein in the urine of mice with CLP-induced renal damage. Moreover, both VCN and SCL inhibited nuclear factor κB activation and reduced the induction of nitric oxide synthase and excessive production of nitric acid. VCN and SCL treatment also reduced the plasma levels of interleukin-6, and tumor necrosis factor-α, reduced lethality due to CLP-induced sepsis, increased lipid peroxidation, and markedly enhanced the antioxidant defense system by restoring the levels of superoxide dismutase, glutathione peroxidase, and catalase in kidney tissues. The present results suggest that VCN and SCL protect mice from sepsis-triggered renal injury.

Keywords: Vicenin-2. Scolymoside. Sepsis, Antioxidant, Renal injury, Renal toxicity.

INTRODUCTION

Sepsis is a serious infection that causes severe inflammatory responses and is associated with a high level of mortality despite recent advances in critical care (Russell, 2006). Cytokine activation is part of the host defense system against infection, but the excessive production of cytokines can cause extensive damage and multiple organ failure (MOF) (Chaudhry *et al*., 2013). Sepsis induces the activation of inducible nitric oxide synthase (iNOS) and upregulates the production of nitric oxide (NO) and reactive oxygen species (ROS), leading to cellular toxicity (Parratt, 1998; Symeonides and Balk, 1999). Sepsis-induced MOF may be reduced by inhibiting inflammatory cytokine production and iNOS activity (Draisma *et al*., 2010; Parratt, 1998; Symeonides, Balk, 1999). In addition, the overproduction of ROS can cause oxidative stress, as indicated by a decrease in the endogenous antioxidant defense system and lipid peroxidation. Therefore, new drug candidates that inhibit the production of ROS and inflammatory cytokines may help in the management of severe sepsis or septic shock (Cadenas, Cadenas, 2002; Vincent *et al*., 2000).

Tea and herbal infusions are natural beverages containing compounds that are of particular interest to the health sciences owing to their potential *in vivo* biological properties (Prior, Cao, 1999; Warren, 1999). Health-promoting properties such as antioxidant, anti-inflammatory, and memory-enhancing activities

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have been documented for active compounds found in Cyclopia subternata, Peperomia blanda, Ocimum sanctum, Perilla frutescens, Urtica circularis, and Potentilla discolor (Islam *et al*., 2014; Joubert *et al*., 2011; Leiro *et al*., 2003; Pardo Andreu *et al*., 2010; Sanchez *et al*., 2000). C. subternata is known to contain abundant flavonoids, particularly vicenin-2 (VCN) and scolymoside (SCL) (Kazuno *et al*., 2005). Furthermore, previous reports have shown that VCN exerts anti-diabetic, anti-glycation, and anti-inflammatory activities (Islam *et al*., 2014; Marrassini *et al*., 2011). However, the beneficial effects of VCN and SCL on sepsis-induced renal damage have not yet been studied. Therefore, in this study, we investigated the renal protective effects of VCN and SCL in an animal model of sepsis.

MATERIAL AND METHODS

Animals and sepsis mouse model

Before cecal ligation and puncture (CLP) surgery, male C57BL/6 mice (average weight 27 g; Sungnam, Republic of Korea) were anesthetized using Rompum (xylazine, 10 mg/kg) and Zoletil (30 mg/kg). The CLP-induced sepsis mouse model was developed as previously described (Bae *et al*., 2014; Wang *et al*., 2004), and sham-operated animals were used as controls. Animals were randomly divided into the following groups (n = 10 each): sham-operated control; VCN or SCL only (1.2 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA), administrated in 0.5% dimethyl sulfoxide (DMSO); CLP surgery only; and CLP + VCN or SCL (0.3, 0.6, or 1.2 mg/kg body weight). VCN and SCL were intravenously injected 12 h after CLP and again 50 h after CLP. Blood and organ samples were collected 4 days after VCN or SCL injection for functional assays. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2017-102).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bio Science (Charles City, IA, USA), maintained as previously described (Jung *et al*., 2016; Kim, Bae, 2016), and used after three to five passages.

Sample preparation and evaluation of nephrotoxicity and lactate dehydrogenase (LDH)

Four days after VCN or SCL injection, blood, urine, and kidney samples were collected and prepared as previously described (Lee *et al*., 2017). Renal dysfunctional markers, such as BUN and creatinine, and a tissue injury marker (LDH) were measured as previously described (Lee *et al*., 2017).

Plasma nitrite/nitrate determination

Levels of nitrite and nitrate in the plasma were determined using Griess reagents and vanadium solution (VCl3), as previously described (Lee *et al*., 2017; Miranda, Espey, Wink, 2001). Briefly, each sample (100 μL) was mixed with VCl3 (100 μL) and incubated with Griess reagents. After 30 min, the optical density was measured at 540 nm (OD540nm) and a change in color was monitored. Sodium nitrite standards were used to prepare a standard curve to measure the final nitrite/nitrate concentrations.

Determination of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and renal myeloperoxidase (MPO) activity via enzyme-linked immunosorbent assays (ELISA)

Levels of IL-6, TNF-α (R&D Systems, Minneapolis, MN, USA), and MPO (Abcam, Cambridge, UK) in plasma were determined using commercially available ELISA kits.

Evaluation of oxidative stress markers

Lipid peroxidation, malondialdehyde (MDA), total glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase activities (CAT) were measured as described previously (Beutler, Duron, Kelly, 1963; Lee *et al*., 2017).

Western blotting

Kidney samples were prepared and western blotting using primary antibodies for iNOS, inhibitor of kappa B (IκB), nuclear factor-kappa B (NF-κB), and β-actin was conducted as previously described (Lee *et al*., 2017).

Cell viability assay

Cellular viability was measured via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay, as previously described (Lee *et al*., 2017). HUVECs were incubated with VCN or SCL for 48 h.

RESULTS

VCN and SCL reduced CLP-induced renal tissue injury and plasma nitrite and nitrate production

CLP surgery increased nephrotoxic markers levels (Table I), such as BUN and creatinine in blood, and protein concentrations in the urine compared to those in the sham-operated group, all of which were reduced by administration of VCN and SCL (0.3, 0.6, or 1.2 mg/kg, 12 h and 50 h after CLP). Levels of LDH, a marker of general tissue injury, were also reduced by VCN and SCL treatment in CLP-operated mice (Table I). In addition, the effects of VCN and SCL treatment on the inflammatory response in kidney tissue were investigated *in vivo* by measuring plasma nitrite and nitrate levels (stable end products of NO). NO production in CLP-operated mice was significantly increased (7.3-fold) compared to that in the control group (Table II), and was reduced by treatment with VCN or SCL (Table II).

TABLE I - Effects of VCN or SCL treatment on plasma levels of BUN and creatinine and urine level of protein in CLP-operated micea

	BUN (mg/dL)	Creatinine (mg/dL)	Urine protein (mg/12 hour)	LDH (U/dL)
Sham	14.6 ± 0.6	0.118 ± 0.02	2.3 ± 0.21	256 ± 15.27
VCN (1.2 mg/kg)	14.9 ± 0.5	0.125 ± 0.01	2.1 ± 0.25	265 ± 14.9
SCL (1.2 mg/kg)	15.2 ± 0.4	0.123 ± 0.02	2.2 ± 0.27	259 ± 20.3
CLP	78.9 ± 4.3	0.425 ± 0.03	13.9 ± 0.57	3570 ± 150.8
CLP + VCN (0.3 mg/kg)	77.5 ± 3.5	0.431 ± 0.02	13.6 ± 0.42	3380 ± 210.4
CLP + VCN (0.6 mg/kg)	55.3 ± 4.7*	0.289 ± 0.02*	7.2 ± 0.52*	2250 ± 150.5*
CLP + VCN (1.2 mg/kg)	30.2 ± 2.5*	0.215 ± 0.02*	3.9 ± 0.23*	1280 ± 125.3*
CLP + SCL (0.3 mg/kg)	75.6 ± 4.3	0.422 ± 0.03	13.2 ± 0.31	3480 ± 250.9
CLP + SCL (0.6 mg/kg)	59.2 ± 3.9*	0.275 ± 0.03*	6.9 ± 0.47*	2580 ± 120.4*
CLP + SCL (1.2 mg/kg)	32.5 ± 3.1*	0.202 ± 0.02*	3.5 ± 0.31*	1350 ± 102.2*

[a]Each value represents the mean ± SD (n = 10).
Sham, sham-operated mice; VCN or SCL, mice treated with VCN or SCL (1.2 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; VCN or SCL + CLP, mice treated with PEL at 12 and 50 h after CLP surgery.
* $p < 0.05$ as compared to CLP.

TABLE II - Effects of VCN or SCL treatment on NO, TNF- , IL-6 levels and renal MPO activity in CLP-operated micea

	NO (mM)	TNF-a (pg/mL)	IL-6 (pg/mL)	MPO (U/g tissue)
Sham	30.8 ± 2.5	111.3 ± 8.6	0.69 ± 0.06	0.59 ± 0.04
VCN (1.2 mg/kg)	31.5 ± 3.1	121.5 ± 8.9	0.72 ± 0.05	0.58 ± 0.05
SCL (1.2 mg/kg)	30.9 ± 2.8	123.8 ± 10.2	0.71 ± 0.04	0.52 ± 0.06
CLP	218.9 ± 15.3	528.4± 41.9	75.42 ± 6.15	3.42 ± 0.32
CLP + VCN (0.3 mg/kg)	220.2 ± 18.7	535.6 ± 35.3	79.71 ± 5.83	3.59 ± 0.41
CLP + VCN (0.6 mg/kg)	156.4 ± 12.5*	301.7 ± 21.3*	43.25 ± 4.11*	2.37 ± 0.25*
CLP + VCN (1.2 mg/kg)	85.8 ± 7.3*	185.9 ± 14.7*	22.64 ± 2.32*	1.19 ± 0.17*
CLP + SCL (0.3 mg/kg)	215.4 ± 12.9	531.5 ± 31.4	78.52 ± 6.12	3.47 ± 0.39
CLP + SCL (0.6 mg/kg)	170.3 ± 13.1*	310.4 ± 25.7*	45.19 ± 3.15*	2.41 ± 0.31*
CLP + SCL (1.2 mg/kg)	82.9 ± 6.2*	175.4 ± 11.8*	23.57 ± 2.18*	1.25 ± 0.11*

[a]Each value represents the mean ± SD (n = 10).
Sham, sham-operated mice; VCN or SCL, mice treated with VCN or SCL (1.2 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; VCN or SCL + CLP, mice treated with PEL at 12 and 50 h after CLP surgery.
* $p < 0.05$ as compared to CLP.

VCN and SCL inhibited TNF-α, IL-6, MPO, and MDA levels induced by CLP

Next, we determined the effects of VCN and SCL on inflammatory markers such as TNF-α and IL-6. Treatment with VCN and SCL inhibited CLP-induced production of TNF-α and IL-6 in plasma (Table II). Next, we tested the effects of VCN and SCL on the infiltration of neutrophils by measuring MPO levels (an indicator of renal infiltration by neutrophils) after CLP surgery. We identified a marked increase in MPO levels after CLP surgery (Table II), which was associated with nephritis. Post-surgical treatment with VCN or SCL resulted in significantly lower MPO concentrations in renal tissues than in CLP-operated mice. We also determined the effects of VCN and SCL on MDA, an indicator of lipid peroxidation levels. Increased MDA levels induced by CLP were reduced by treatment with VCN or SCL (Table III).

TABLE III - Effects of VCN or SCL treatment on MDA level and the activities of renal antioxidant enzymes in CLP-operated micea

	MDA (nM/mg protein)	GSH (nM/mg protein)	SOD (U/mg protein)	GSH-Px (U/mg protein)	CAT (U/mg protein)
Sham	187.6 ± 12.3	26.9 ± 2.3	1.02 ± 0.07	2.39 ± 0.15	4.12 ± 0.37
VCN (1.2 mg/kg)	182.9 ± 13.4	27.1 ± 1.9	1.09 ± 0.08	2.41 ± 0.22	4.31 ± 0.32
SCL (1.2 mg/kg)	184.5 ± 12.7	27.5 ± 1.8	1.06 ± 0.11	2.43 ± 0.27	4.25 ± 0.36
CLP	312.5 ± 25.2	16.8 ± 1.4	0.72 ± 0.06	1.39 ± 0.12	2.58 ± 0.21
CLP + VCN (0.3 mg/kg)	308.4 ± 22.7	17.1 ± 1.2	0.75 ± 0.07	1.44 ± 0.14	2.62 ± 0.31
CLP + VCN (0.6 mg/kg)	275.4 ± 13.5*	19.7 ± 1.4*	0.87 ± 0.08*	1.75 ± 0.14*	3.28 ± 0.29*
CLP + VCN (1.2 mg/kg)	212.7 ± 18.2*	23.5 ± 2.0*	0.98 ± 0.06*	2.02 ± 0.17*	3.85 ± 0.24*
CLP + SCL (0.3 mg/kg)	311.3 ± 21.8	17.2 ± 1.7	0.73 ± 0.06	1.49 ± 0.21	2.67 ± 0.25
CLP + SCL (0.6 mg/kg)	283.7 ± 14.7*	20.4 ± 1.7*	0.82 ± 0.06*	1.86 ± 0.15*	3.31 ± 0.21*
CLP + SCL (1.2 mg/kg)	223.7 ± 21.4*	24.9 ± 2.2*	0.95 ± 0.08*	2.11 ± 0.14*	3.92 ± 0.34*

[a]Each value represents the mean ± SD (n = 10).
Sham, sham-operated mice; VCN or SCL, mice treated with VCN or SCL (1.2 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; VCN or SCL + CLP, mice treated with PEL at 12 and 50 h after CLP surgery.
* $p < 0.05$ as compared to CLP.

VCN and SCL increased total GSH and antioxidant enzyme activity in the kidney tissues

We determined whether VCN and SCL could control CLP-induced oxidative stress by measuring antioxidant GSH levels and activities of the oxidative stress-associated enzymes SOD, GSH-Px, and CAT. Total GSH levels and the activities of SOD, GSH-Px, and CAT were similar to those in the VCN only, SCL only, and sham-operated groups. In contrast, the total GSH levels and renal activities of all three enzymes were reduced in CLP-operated mice and were increased by treatment with VCN or SCL (Table III).

VCN and SCL reduced renal proteins and iNOS levels, and inhibited NF-κB activity

We investigated the levels of iNOS, IκB, and NF-κB to define the underlying renal protective effects of VCN and SCL. Elevated protein levels of iNOS by CLP were dramatically reduced by treatment with VCN or SCL (Figure 1). Next, we determined the effects

of VCN and SCL on IκB degradation by CLP and translocation of NF-κB in the cell. CLP-induced IκB degradation was significantly lowered by treatment with VCN or SCL and decreased levels of NF-κB p65 in the cytosol were reversed by treatment with VCN or SCL (Figure 1A). In addition, the cellular toxicity of VCN and SCL was determined via MTT assay, showing that neither VCN nor SCL affected cellular viability when administered at concentrations up to 100 μM (Figure 1B).

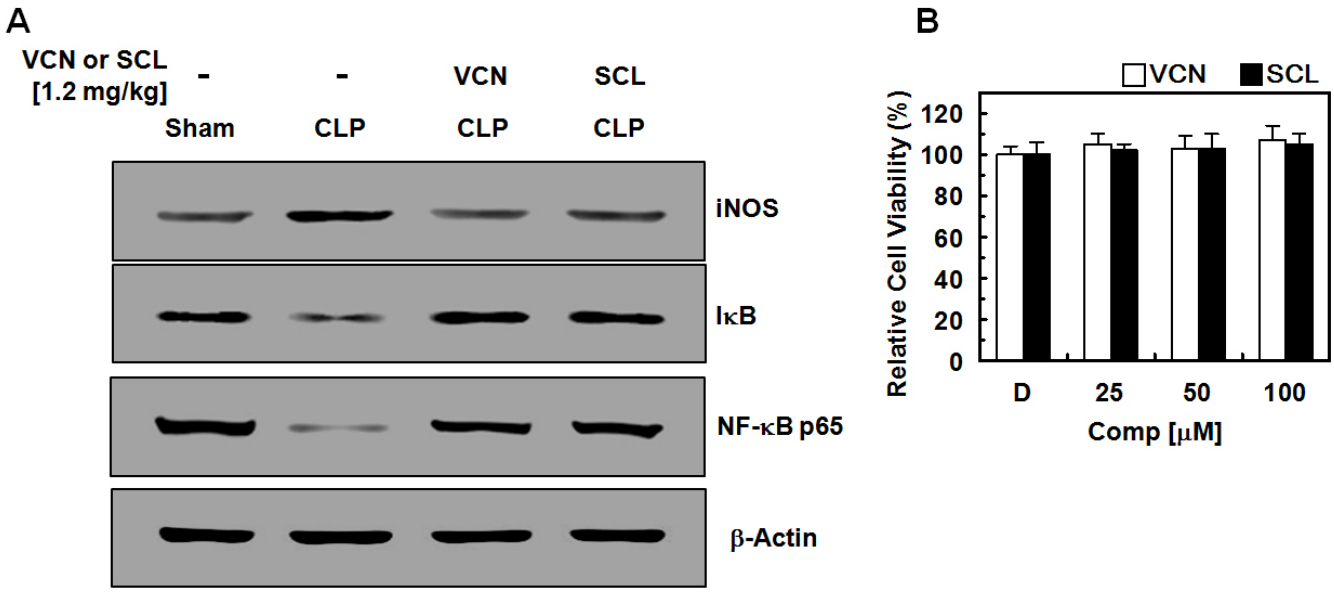

FIGURE 1 - Effects of vicenin-2 (VCN) and scolymoside (SCL) treatment on renal inducible nitric oxide synthase (iNOS), inhibitor of kappa B (IκB), and nuclear factor-kappa B (NF-κB) expression in cecal ligation and puncture (CLP)-operated mice. (A) Sham-operated mice; CLP-operated mice; mice treated with VCN or SCL (1.2 mg/kg body weight) 12 h and 50 h after CLP surgery (from left line). Western blots of iNOS, IκB, NF-κB, and β-actin. The image is representative of results obtained from three different experiments. (B) The effects of VCN and SCL on cellular viability were measured via MTT assay. D = 0.2% dimethyl sulfoxide (DMSO), used as the vehicle control. The results are shown as means ± SD from three separate experiments on different days performed in triplicate.

VCN and SCL reduced CLP-induced septic lethality

To confirm the renal protective effects of VCN and SCL, we tested their effects on CLP-induced lethality. Data showed that injection with VCN or SCL (1.2 mg/kg, 12 h and 50 h after CLP) increased the survival rate (50%) of CLP-operated mice ($p < 0.0001$, Figure 2).

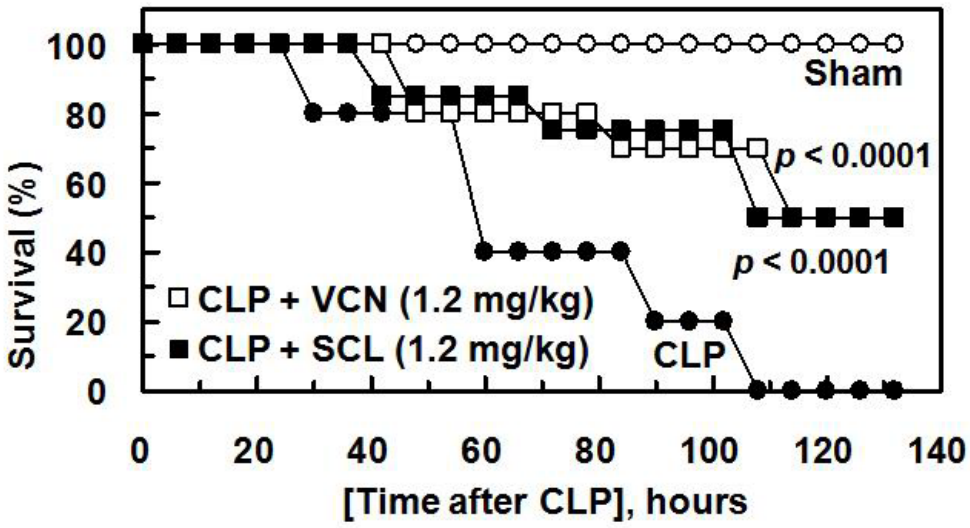

FIGURE 2 - Effects of VCN and SCL on CLP-induced septic lethality. Male C57BL/6 mice (n = 20) were administered VCN or SCL at 1.2 mg/kg (intravenous, i.v.) 12 h and 50 h after CLP. Animal survival was monitored every 12 h for 132 h after CLP. Control CLP-operated mice ( ) and sham-operated mice ( ) were administered sterile saline (n = 20). Kaplan-Meier survival analysis was used to determine the overall survival rates versus treated CLP-operated mice.

DISCUSSION

In this study, we report that: (1) the expression of plasma BUN and creatinine and urine protein levels were increased by CLP, but decreased by VCN and SCL treatment; (2) CLP increased renal tissue injury inflammatory protein markers, and treatment with VCN and SCL reduced these levels; (3) CLP-operated mice showed increased NO production in the blood and iNOS expression, which was significantly attenuated by treatment with VCN or SCL; and (4) VCN and SCL increased GSH and antioxidant enzyme activity. Therefore, the renal protective effects of VCN and SCL were mediated by reducing NO production and inflammatory protein levels, and increasing antioxidant enzymes.

It is well known that levels of TNF-α, IL-6, and NF-κB are increased under severe inflammatory conditions (Chaudhry *et al*., 2013; Stearns-Kurosawa *et al*., 2011). Based on the current findings that VCN and SCL inhibited the upregulation of TNF- α and IL-6, and NF-κB by CLP, we concluded that the renal protective effects of VCN and SCL were mediated by suppressing inflammatory gene expression. When cells are activated by inflammatory mediators, NF-κB dissociates from IκB and translocates to the nucleus (Oeckinghaus, Ghosh, 2009). NF-κB is a potential primary target for inflammatory diseases, as the renal protective mechanisms of VCN and SCL were mediated by inhibiting the degradation of IκB and the CLP-mediated activation of NF-κB.

Genes encoding three antioxidant enzymes (SOD, CAT, and GSH-Px) are regarded as the primary oxidative defense genes (Birben *et al*., 2012). CLP surgery lowered the expression levels of SOD, CAT, and GSH-Px, but these were significantly increased by VCN and SCL treatment, indicating that these exert cytoprotective effects on CLP-induced oxidative damage in renal tissues. CLP also increased renal MDA concentrations, a major lipid peroxidation product and oxidative stress marker (Qiao *et al*., 2011; Xiao *et al*., 2012; Zhang *et al*., 2011), and these were reduced by VCN and SCL administration. Therefore, VCN and SCL protected against CLP-induced oxidative stress and injury through suppression of lipid peroxidation and activation of antioxidant enzymes.

Intravenously injected VCN or SCL (1.2 mg/kg) showed renal protective functions against CLP-induced responses. Considering that the average weight of mice used in this study was 27 g and the

average weight of an adult human is 70 kg, we would require 84 mg of VCN or SCL to achieve renal protective effects if the compound is administrated intravenously in humans. However, if the compounds are ingested through herbal tea, an amount greater than this would be needed for the following reasons: 1) large differences exist between intravenous injection and the oral route; 2) after ingesting the tea, several pharmacological processes are required for the compound (VCN or SCL) to reach the bloodstream, such as absorption, distribution, metabolism, and excretion (ADME); and 3) the entire content of the compound in the herbal tea is not absorbed. In order for a consumed compound to be effectively utilized by the body's vascular system, these four processes (ADME), which represent the distribution of a pharmaceutical compound within an organism, must be optimized. These properties influence the compound levels and the kinetics of exposure of the tissues to the compound; hence, these criteria influence the performance and pharmacological activity of the compound.

Collectively, our data have demonstrated the renal protective effects of VCN and SCL against sepsis. The beneficial activities of VCN and SCL were accompanied by lower levels of TNF-α, IL-6, iNOS, and NO, via inhibition of the NF-κB signaling pathway, which is associated with increased antioxidant defenses and decreased lipid peroxidation *in vivo*. Therefore, VCN and SCL show potential for therapeutic use in the treatment of renal inflammatory diseases.

ADDITIONAL INFORMATION

Competing financial interests: the authors declare no competing financial interests.

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