

Examination of the nitric oxide production-suppressing activity in *Sanguisorbae Radix*

Takako Yokozawa

Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

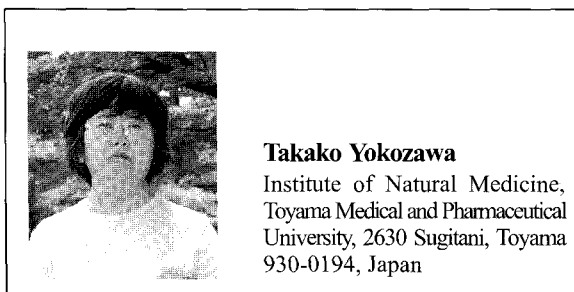
Nitric oxide (NO) is an interesting and important molecule with diverse functions, exerting a variety of both beneficial and detrimental effects in different tissues, including the kidney. It has been demonstrated that NO plays an important role in renal function by participating in the regulation of renal hemodynamics, sodium excretion and renin release, tubuloglomerular feedback, pressure natriuresis, and tubule function (Galle and Wanner, 1996; Lahera *et al.*, 1997). However, excessive NO produced under certain pathological conditions may also produce cytotoxic effects and aggravate renal dysfunction. It still remains debatable whether NO is beneficial or harmful in acute and chronic renal failure or other renal diseases, and whether NO or NO synthase inhibitors should be used to regulate these conditions. Conflicting results have been reported in studies on humans, and also in animal experimental models (Fujihara *et al.*, 1995; Noiri *et al.*, 1996; Thorup and Persson, 1996).

Lipopolysaccharide (LPS) releases many mediators and produces the condition known as endotoxemia. These mediators include interleukins, tumor necrosis factor, oxygen free radicals, toxic eicosanoids,

platelet activating factor and NO (Wolkow, 1998). Endotoxemia gives rise to a multiple organ dysfunction syndrome involving failure of the circulatory system, liver, lungs and kidney. An increase in the production of NO due to the induction of inducible NO synthase (iNOS) has been proposed to be responsible for this multiple organ dysfunction (Thiemermann *et al.*, 1995), and selective iNOS inhibition has been shown to attenuate or prevent the syndrome (Liaudet *et al.*, 1998).

On the other hand, NO, which has an enormous range of beneficial functions in organisms, including regulation of vascular tone, ventilation, hormone secretion, inflammation, immunity and neurotransmission, is also suspected to be cytotoxic or cytostatic to host cells, and to act as a toxic radical (Moncada *et al.*, 1991; Nathan, 1992; Gross and Wolin, 1995; Harrison, 1997). In addition, recent experiments have revealed that the toxicity and damage caused by NO in tissues and cells is multiplied enormously if it reacts with the superoxide radical (O_2^-) to yield peroxynitrite (ONOO⁻), an extremely reactive radical (Radi *et al.*, 1991; Akaike *et al.*, 1998).

Sanguisorbae Radix is an oriental medicine that is rarely used in Japan, and is not used in any of the oriental medical prescriptions that are in current clinical use. In China, however, it is used in internal medications for hemostasis and for the treatment of hematemesis, hemoptysis, melena and hypermenorrhea, and in external medications for the treatment of dermatitis, eczema and incised wounds. On the other hand, experimental pharmacological data related to this medicine are insufficient; there are only a few reports on its anti-burn, hemostatic and antiemetic effects in rabbits and pigeons, and on its antibacterial effects on *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Jiangsu New Medical College, 1987). In a previous study, to investigate the effects of some oriental medicines on diseases



*Correspondence: Dr Takako Yokozawa, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan.
Fax: 81-76-434-4656; E-mail: yokozawa@ms.toyama/mpu.ac.jp

that are attributable to excessive NO, we carried out a screening test using the NO donor sodium nitroprusside in an *in vitro* evaluation system, and reported the newly found NO-suppressive activity of *Sanguisorbae Radix* (Chen *et al.*, 1999a ; Yokozawa *et al.*, 1999).

In this study, we investigated whether *Sanguisorbae Radix* has a protective effect against pathological conditions involving NO and ONOO. The present study was also designed to determine which component of *Sanguisorbae Radix* is responsible for NO-suppressive activity, using LPS-stimulated macrophages, and then investigated the mechanism by which sanguin H-6 acts, with special reference to iNOS.

EFFECTS OF SANGUISORBAE RADIX IN RENAL DYSFUNCTION INDUCED BY LPS

NO is produced from L-arginine by the action of NO synthase. In the kidney, three isoforms of NOS, located in different regions of the kidney and expressing different functions, have been found. The neuronal isoform (nNOS) is found in macula densa cells and the epithelium of Bowman's capsule; epithelial NOS (eNOS) is present in the endothelium of the glomerular capillaries, the afferent and efferent arteriole, the intrarenal arteries and the medullary vasa recta, and iNOS is found in the proximal tubule and glomerulus (Kone and Baylis, 1997). NO generated by these NOS isoforms has been shown to exert different effects on renal physiology and pathology. Both nNOS and eNOS release a low and constant amount of NO, which plays a major role in the modulation of renal vascular tone and sodium excretion. Under normal conditions, iNOS also generates a physiological level of NO, which may participate in the modulation of vascular tone by an indirect mechanism in mesangial cell relaxation. However, as iNOS is induced by certain cytokines and hypoxia, it generates NO in large quantities and for a prolonged period (Bachmann and Mundel, 1994). Excessive NO has been shown to be strongly cytotoxic, injuring cells and tissues. In freshly isolated rat proximal tubules, it has been demonstrated that NO, and its metabolic product ONOO, mediate tubular hypoxia-reperfusion injury (Yu *et al.*, 1994).

Table 1. Effect of *Sanguisorbae Radix* extract on urea nitrogen and Cr levels in serum.

Group	Urea nitrogen (mg/dl)	Cr (mg/dl)
Normal	21.3 ± 2.0	0.36 ± 0.01
LPS-treated		
Control	38.1 ± 2.9 ^a	1.20 ± 0.08 ^a
<i>Sanguisorbae Radix</i> extract (50 mg/kg/d)	33.8 ± 3.1 ^{ab}	0.78 ± 0.10 ^{ad}
<i>Sanguisorbae Radix</i> extract (100 mg/kg/d)	31.6 ± 2.3 ^{ad}	0.68 ± 0.08 ^{ad}
LPS-treated		
Control	37.8 ± 1.6 ^a	1.18 ± 0.06 ^a
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	32.4 ± 2.8 ^{ac}	0.66 ± 0.14 ^{ad}

Statistical significance: ^a*p*<0.001 vs. normal values, ^b*p*<0.05, ^c*p*<0.01, ^d*p*<0.001 vs. LPS-treated control values.

Cattell *et al.* (1990) also provided experimental evidence for the effects of NO in accelerated nephrotoxic nephritis using isolated rat glomeruli. In our study, LPS-treated rats showed a rapid decline in renal function, which was indicated by large increases in two renal function parameters, blood urea nitrogen and creatinine (Cr) (Table 1). The serum nitrite/nitrate level, an indicator of NO formation, was also seen to be markedly increased in LPS-treated rats compared with that seen in normal rats (Table 2). As a more direct indicator, we monitored the activity of iNOS in renal homogenate using the method of Suh *et al.* (1998). As shown in Table 3, LPS treatment resulted in an approximately 1.9-fold increase in iNOS activity, suggesting the possible association of additional induction of iNOS with renal dysfunction *in situ*.

Table 2. Effect of *Sanguisorbae Radix* extract on nitrite/nitrate level in serum.

Group	Nitrite/nitrate (μM)
Normal	1.78 ± 1.02
LPS-treated	
Control	6.50 ± 1.35 ^b
<i>Sanguisorbae Radix</i> extract (50 mg/kg/d)	4.39 ± 1.82 ^{bc}
<i>Sanguisorbae Radix</i> extract (100 mg/kg/d)	3.72 ± 0.89 ^{ac}
LPS-treated	
Control	6.39 ± 1.24 ^b
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	3.13 ± 1.28 ^c

Statistical significance: ^a*p*<0.01, ^b*p*<0.001 vs. normal values, ^c*p*<0.001 vs. LPS-treated control values.

Table 3. Effect of *Sanguisorbae Radix* extract on iNOS activity in kidney.

Group	iNOS (pmol/mg protein/min)
Normal	1.94 ± 0.11
LPS-treated	
Control	3.67 ± 0.27 ^b
<i>Sanguisorbae Radix</i> extract (50 mg/kg/d)	2.69 ± 0.10 ^{a,c}
<i>Sanguisorbae Radix</i> extract (100 mg/kg/d)	2.58 ± 0.06 ^{a,c}
LPS-treated	
Control	3.64 ± 0.29 ^b
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	2.02 ± 0.18 ^c

Statistical significance: ^a $p < 0.01$, ^b $p < 0.001$ vs. normal values, ^c $p < 0.001$ vs. LPS-treated control values.

Various inhibitors of NO or NOS have been used in attempts to improve or attenuate the pathology involved in excessive generation of NO, but conflicting results have been obtained. Using isolated renal proximal tubules, Yu *et al.* (1994) reported that the NOS inhibitor, *N*-nitro-*L*-arginine methyl ester, protected the renal tubular epithelium against hypoxic injury. Weinberg *et al.* (1994) demonstrated that oral administration of *N*^G-monomethyl-*L*-arginine prevented the development of glomerulonephritis and reduced the intensity of inflammatory arthritis in MRL-*lpr/lpr* mice. In contrast to these observed beneficial effects, NOS inhibitors have been shown to aggravate renal dysfunction in several *in vivo* models of acute renal failure (Schwartz *et al.*, 1994; Mashiach *et al.*, 1998). It is speculated that these contradictory results were attributable to a lack of selective NOS inhibitors.

As excessive generation of NO in renal disease is mainly associated with the induction of iNOS, the therapeutic strategy has concentrated on developing effective iNOS inhibitors. During systematic studies of the traditional drugs used in the treatment of renal diseases related to free radical injury, we found that *in vitro* *Sanguisorbae Radix*, a traditional crude drug which contains a large amount of tannin as its major constituent, has a strong scavenging effect on NO induced by sodium nitroprusside, an NO donor (Yokozawa *et al.*, 1999). We also recently observed that *Sanguisorbae Radix* extract effectively inhibited the activity of iNOS in activated

macrophages induced by LPS (data not shown). These findings prompted us to see if *Sanguisorbae Radix* extract could improve impaired renal function related to excessive generation of NO *in vivo*. We therefore conducted the present experiment and found that *Sanguisorbae Radix* extract significantly improved the impairment of renal function caused by LPS. As shown in Table 1, the raised levels of serum urea nitrogen and Cr were markedly reduced in the two groups treated with different doses of *Sanguisorbae Radix*. Reduced serum nitrite/nitrate levels and renal iNOS activity demonstrated a protective action against the renal dysfunction caused by LPS, although these effects were weaker than those produced by aminoguanidine, a selective iNOS inhibitor (Tables 2 and 3).

EFFECTS OF SANGUISORBAE RADIX AGAINST OXIDATIVE DAMAGE CAUSED BY ONOO⁻

ONOO⁻ is a new focus of interest in studies of free radicals, particularly those related to the detrimental effect of NO. The potential pathophysiological effects of ONOO⁻ include activation of poly (ADP-ribose) synthetase, inhibition of mitochondrial respiration, and activation of caspase-3, as well as alteration of the lipid-aggregatory properties of surfactant protein A, which may play an important role in tissue damage and organ dysfunction (Haddad *et al.*, 1996; Virag *et al.*, 1999). Although ONOO⁻ is generated in a large number of pathophysiological conditions, few *in vivo* models are available that produce enough ONOO⁻ for evaluating its cytotoxicity and its contribution to NO cytotoxicity as well as to tissue injuries. As ONOO⁻ is generated via a reaction between NO and O₂⁻, we decided to treat the rats with LPS and ischemia-reperfusion, the former providing abundant NO and the latter causing elevated O₂⁻.

It is important to know whether ONOO⁻ is elevated in this model. Until now, it has been very difficult to detect ONOO⁻ directly in biological fluids and tissues because it is unstable and decomposes rapidly. However, ONOO⁻ can oxidize the tyrosine in protein into nitrotyrosine, which is stable and detectable. The presence of nitrotyrosine has been considered as evidence for *in vivo*

Table 4. Effect of Sanguisorbae Radix extract on 3-nitrotyrosine levels in plasma.

Group	3-Nitrotyrosine (pmol/ml)
Sham treatment	N.D.
LPS	482.9 ± 18.9
Ischemia-reperfusion	N.D.
LPS + ischemia-reperfusion	
No pretreatment	699.6 ± 67.1
Sanguisorbae Radix extract (100 mg/kg/d)	329.3 ± 30.6 ^a
Sanguisorbae Radix extract (200 mg/kg/d)	N.D.
LPS + ischemia-reperfusion	
Saline	708.8 ± 38.9
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	259.6 ± 14.5 ^a

Statistical significance: ^a*p*<0.001 vs. LPS + ischemia-reperfusion without pretreatment. N.D., not detectable.

formation of ONOO⁻ (Herce-Pagliai *et al.*, 1998; Di Stasi *et al.*, 1999). In the present experiment, we observed a high level of nitrotyrosine in plasma from LPS+ischemia-reperfusion rats. In comparison, the nitrotyrosine level induced by LPS treatment alone was lower, and nitrotyrosine was not detected in rats subjected to a sham operation and ischemia-reperfusion (Table 4). On these grounds, we were unable to conclude that nitrotyrosine is not formed in ischemia-reperfusion injury because several studies have demonstrated that ONOO⁻ is an important mediator of ischemia-reperfusion injury (Yasmin *et al.*, 1997; Eliasson *et al.*, 1999), and the method we used - HPLC with UV detection - has limited sensitivity. However, our results suggest that ONOO⁻ formation increases in the presence of excessively generated NO and O₂⁻.

To evaluate the effect of elevated ONOO⁻ on impaired renal function, we measured urea nitrogen and Cr in plasma. Treatment of the rats with LPS+ischemia-reperfusion resulted in significantly higher urea nitrogen and Cr levels than either LPS treatment or ischemia-reperfusion alone, which suggested that ONOO⁻ aggravates the impairment of renal function (Table 5).

We have shown previously that Sanguisorbae Radix extract ameliorates renal dysfunction in an LPS-challenged rat model, and that this effect

Table 5. Effect of Sanguisorbae Radix extract on urea nitrogen and Cr levels in plasma.

Group	Urea nitrogen (mg/dl)	Cr (mg/dl)
Sham treatment	17.6 ± 1.5	0.33 ± 0.09
LPS	43.9 ± 1.8 ^a	0.71 ± 0.05 ^a
Ischemia-reperfusion	57.9 ± 2.6 ^a	1.47 ± 0.21 ^a
LPS + ischemia-reperfusion		
No pretreatment	86.7 ± 2.0 ^a	2.03 ± 0.10 ^a
Sanguisorbae Radix extract (100 mg/kg/d)	77.9 ± 4.0 ^{a,b}	1.77 ± 0.12 ^{a,b}
Sanguisorbae Radix extract (200 mg/kg/d)	68.2 ± 1.9 ^{a,c}	1.24 ± 0.05 ^{a,c}
LPS + ischemia-reperfusion		
Saline	86.5 ± 1.7 ^a	2.03 ± 0.14 ^a
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	72.1 ± 3.5 ^{a,c}	1.52 ± 0.12 ^{a,c}

Statistical significance: ^a*p*<0.001 vs. normal values, ^b*p*<0.01, ^c*p*<0.001 vs. LPS + ischemia-reperfusion without pretreatment.

involves the inhibition of NO generation and iNOS activation. On the other hand, in a recent preliminary *in vitro* experiment, we found that this extract inhibited ONOO⁻ directly. Hence, it is possible that Sanguisorbae Radix extract ameliorates renal dysfunction by inhibiting excessive generation of NO and/or ONOO⁻ or by scavenging O₂⁻. Using the LPS+ischemia-reperfusion, we have clarified this effect. Our results showed that administration of Sanguisorbae Radix extract significantly ameliorated increases in plasma urea nitrogen and Cr, which corresponded to a significant decrease in the ONOO⁻ level (Tables 4 and 5). This suggested that inhibition of ONOO⁻ may be attributable to the improvement of renal dysfunction. However, the protective action against renal dysfunction was weaker than that of the ONOO⁻ level, which implied that some other factors are also involved in the renal dysfunction. Indeed, the pathogenesis of renal dysfunction in a living system is extremely complex. We intend to carry out further detailed investigations on this aspect.

ONOO⁻ is a highly cytotoxic oxidant, which oxidizes proteins and inhibits cellular respiration. The resulting changes in mitochondrial function lead to activation of caspase, and subsequently induce DNA fragmentation and enterocyte apoptosis (Virag *et al.*, 1999). We have previously reported that ischemia-reperfusion can induce DNA ladder formation (Chen *et al.*, 1999b). In the present study, rats subjected to LPS+ischemia-reperfusion had more distinct DNA ladders than rats subjected to

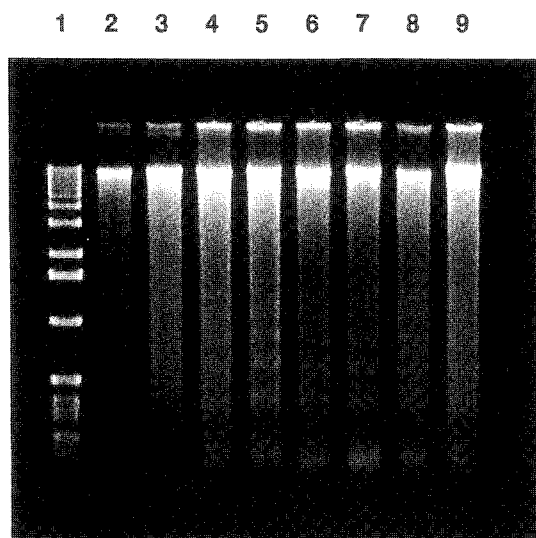


Fig. 1. Agarose gel electrophoresis of DNA. Lane 1: 1-kb marker DNA; lane 2: normal; lane 3: LPS; lane 4: ischemic and reperfusion; lane 5: LPS plus ischemic and reperfusion control (water drink); lane 6; LPS plus ischemic and reperfusion control (saline infusion); lane 7: ischemic and reperfusion aminoguanidine-treated; lane 8: LPS plus ischemic and reperfusion Sanguisorbae Radix extract-treated (100 mg/kg/day); lane 9: LPS plus ischemic and reperfusion Sanguisorbae Radix extract-treated (200 mg/kg/day).

ischemia-reperfusion alone, suggesting that ONOO⁻ is a strong cytotoxic agent. The ladders we observed had intervals of about 180 bp, which is characteristic of apoptosis. LPS treatment did not result in ladder formation, and instead a 'smear' pattern was observed, indicating that necrosis was a major pathway of cell death in endotoxin shock. Pretreatment with *Sanguisorbae Radix* extract significantly ameliorated DNA laddering. At doses of 100 and 200 mg/kg body weight/day *Sanguisorbae Radix* extract, the DNA ladders were indistinct (Fig. 1). A semiquantitative method showed that the DNA fragmentation level was markedly decreased in the groups treated with *Sanguisorbae Radix* extract. On the other hand, infusion of saline did not affect DNA fragmentation, whereas treatment with aminoguanidine (a specific iNOS inhibitor (Southan and Szabo, 1996)) reduced it significantly. The effect of aminoguanidine was weaker than that of

Table 6. Effect of *Sanguisorbae Radix* extract on DNA fragmentation.

Group	Fragmentation level (%)
Sham treatment	-
LPS	3.9 ± 0.5
Ischemia-reperfusion	15.1 ± 1.7
LPS + ischemia-reperfusion	
No pretreatment	25.2 ± 2.2
<i>Sanguisorbae Radix</i> extract (100 mg/kg/d)	14.7 ± 1.9 ^a
<i>Sanguisorbae Radix</i> extract (200 mg/kg/d)	13.3 ± 1.6 ^a
LPS + ischemia-reperfusion	
Saline	24.7 ± 1.7
Aminoguanidine (5 mg/kg plus 5 mg/kg/h)	18.4 ± 2.5 ^a

Statistical significance: ^a*p*<0.001 vs. LPS + ischemia-reperfusion without pretreatment.

200 mg/kg body weight/day *Sanguisorbae Radix* extract (*p*<0.001), as shown in Table 6. This effect of aminoguanidine may be due to inhibition of NO and to blocking of ONOO⁻ formation. These results suggest that ONOO⁻ mediates, at least partially, cell death, and that *Sanguisorbae Radix* extract protects renal cells against such injury.

STUDY ON THE COMPONENTS OF SANGUISORBAE RADIX WITH NO PRODUCTION-SUPPRESSING ACTIVITY

The active components of an aqueous extract of *Sanguisorbae Radix*, which possesses NO production-suppressing activity, were determined using macrophages that were activated by the addition of LPS. Mice given LPS showed greatly increased amounts of NO in macrophages, with increased iNOS and NADPH-diaphorase activities and decreased cell viability. In contrast, in macrophages treated with a stepwise dose of *Sanguisorbae Radix* aqueous extract, the level of NO and the iNOS and NADPH-diaphorase activity were suppressed in a dose-dependent manner, while cell viability was increased (Table 7). *Sanguisorbae Radix* was then examined to establish the component responsible for the activity found in its aqueous extract.

Since saponins and tannins are known to be major components of *Sanguisorbae Radix* (Nonaka

Table 7. NO production, iNOS activity, NADPH-diaphorase activity and cell viability of macrophages incubated with LPS.

Group	NO (μM)	iNOS ($\text{pmol/mg protein/min}$)	NADPH-diaphorase (nmol/mg protein)	Cell viability (%)
None	4.43 \pm 0.13	6.83 \pm 1.59	23.72 \pm 0.75	100.0 \pm 2.1
LPS-treatment				
Control	51.50 \pm 0.30 ^e	26.42 \pm 1.64 ^c	47.05 \pm 4.60 ^c	69.8 \pm 4.6 ^c
Extract (25 $\mu\text{g/ml}$)	42.00 \pm 0.46 ^{ef}	22.88 \pm 1.52 ^{cd}	44.93 \pm 3.88 ^c	72.8 \pm 2.1 ^c
Extract (50 $\mu\text{g/ml}$)	32.51 \pm 0.25 ^{ef}	20.54 \pm 1.09 ^{cf}	35.17 \pm 2.04 ^{cf}	74.4 \pm 1.3 ^c
Extract (100 $\mu\text{g/ml}$)	21.55 \pm 0.22 ^{ef}	16.81 \pm 1.93 ^{cf}	30.45 \pm 2.52 ^{af}	77.9 \pm 0.5 ^{cf}
LPS-treatment				
T-A (50 $\mu\text{g/ml}$)	43.45 \pm 0.30 ^{ef}	23.32 \pm 3.26 ^c	36.14 \pm 3.70 ^{cf}	68.1 \pm 2.3 ^c
T-B (50 $\mu\text{g/ml}$)	22.80 \pm 0.16 ^{ef}	15.21 \pm 1.31 ^{cf}	22.09 \pm 2.00 ^f	79.1 \pm 1.0 ^{ce}
T-C (50 $\mu\text{g/ml}$)	21.62 \pm 0.07 ^{ef}	12.37 \pm 1.07 ^{bf}	20.02 \pm 1.62 ^f	87.0 \pm 3.0 ^{cf}
Non-tannin fraction (50 $\mu\text{g/ml}$)	42.89 \pm 0.36 ^{ef}	21.04 \pm 2.90 ^c	35.23 \pm 3.20 ^{cf}	83.1 \pm 2.3 ^{cf}
LPS-treatment				
Control	50.13 \pm 0.38 ^c	26.28 \pm 1.87 ^c	49.93 \pm 3.88 ^c	71.0 \pm 3.1 ^c
TB-2 (50 $\mu\text{g/ml}$)	23.35 \pm 0.86 ^{ef}	14.75 \pm 1.03 ^{cf}	15.44 \pm 1.26 ^{cf}	81.0 \pm 3.4 ^{cf}
TB-3 (50 $\mu\text{g/ml}$)	13.99 \pm 0.32 ^{ef}	11.19 \pm 1.65 ^{bf}	13.03 \pm 1.88 ^{cf}	84.6 \pm 2.0 ^{cf}
TB-4 (50 $\mu\text{g/ml}$)	17.50 \pm 1.48 ^{ef}	13.97 \pm 2.28 ^{ef}	18.54 \pm 1.07 ^{bf}	81.9 \pm 1.2 ^{cf}
TB-5 (50 $\mu\text{g/ml}$)	22.13 \pm 1.63 ^{ef}	15.33 \pm 1.03 ^{cf}	22.86 \pm 1.00 ^f	76.3 \pm 2.2 ^{ce}
TC-1 (50 $\mu\text{g/ml}$)	26.74 \pm 2.50 ^{ef}	13.96 \pm 2.25 ^{cf}	28.24 \pm 4.50 ^{af}	84.5 \pm 2.3 ^{cf}
TC-2 (50 $\mu\text{g/ml}$)	22.41 \pm 2.00 ^{ef}	13.47 \pm 1.29 ^{cf}	17.63 \pm 0.84 ^{bf}	86.1 \pm 1.4 ^{cf}
TC-3 (50 $\mu\text{g/ml}$)	14.41 \pm 2.05 ^{ef}	11.18 \pm 0.44 ^{bf}	16.82 \pm 2.30 ^{cf}	92.1 \pm 3.2 ^{cf}
TC-4 (50 $\mu\text{g/ml}$)	11.67 \pm 1.80 ^{ef}	10.09 \pm 2.09 ^{af}	12.82 \pm 1.30 ^{cf}	93.1 \pm 2.8 ^{cf}
LPS-treatment				
Control	47.80 \pm 0.33 ^c	26.08 \pm 1.52 ^c	50.01 \pm 3.09 ^c	69.5 \pm 3.2 ^c
Sanguin H-6 (50 $\mu\text{g/ml}$)	9.03 \pm 0.27 ^{ef}	8.74 \pm 1.59 ^f	11.44 \pm 1.56 ^{cf}	92.4 \pm 0.9 ^{bf}
Sanguin H-11 (50 $\mu\text{g/ml}$)	11.27 \pm 0.89 ^{ef}	10.41 \pm 1.67 ^{bf}	16.71 \pm 1.64 ^{cf}	89.5 \pm 3.5 ^{cf}
1,2,3,4,6-Penta-O-galloyl- β -D-glucose (50 $\mu\text{g/ml}$)	10.74 \pm 0.45 ^{ef}	9.32 \pm 0.42 ^f	13.02 \pm 1.45 ^{cf}	82.8 \pm 0.9 ^{cf}
Eugenin (50 $\mu\text{g/ml}$)	12.87 \pm 0.55 ^{ef}	15.73 \pm 1.26 ^{cf}	25.10 \pm 2.40 ^f	81.1 \pm 2.4 ^{cf}
Polymeric proanthocyanidin (50 $\mu\text{g/ml}$)	14.85 \pm 0.85 ^{ef}	19.29 \pm 1.67 ^{cf}	29.70 \pm 1.10 ^{cf}	83.7 \pm 4.2 ^{cf}
Aminoguanidine (100 μM)	8.99 \pm 0.10 ^{ef}	8.98 \pm 0.53 ^f	10.91 \pm 0.89 ^{cf}	73.7 \pm 1.3 ^c

Statistical significance: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs. non-treatment values, ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$ vs. LPS-treatment control values.

et al., 1982a, 1982b, 1984; Tanaka *et al.*, 1983, 1984), Sanguisorbae Radix was extracted with water-acetone to assure a high extraction efficacy by making use of the characteristic features of these components, and the extract was divided into the saponin (non-tannin) and tannin fractions (Fig. 2). As a result, the non-tannin fraction and fraction T-A, which contain mainly sugars with some proportions of lower-molecular-weight tannin and glucosides of phenol compounds, demonstrated

weaker activity than the extract. In contrast, high activity was found in one fraction, T-B, which is composed mainly of gallic acid, catechin, procyanidin B-3 and its gallates, and lower-molecular-weight hydrolyzable tannin, and in another fraction, T-C, which contains lower- and higher-molecular-weight hydrolyzable tannin and high-molecular-weight procyanidin. These fractions were also associated with decreased cytotoxicity (Table 7). The active T-B and T-C fractions were further fractionated

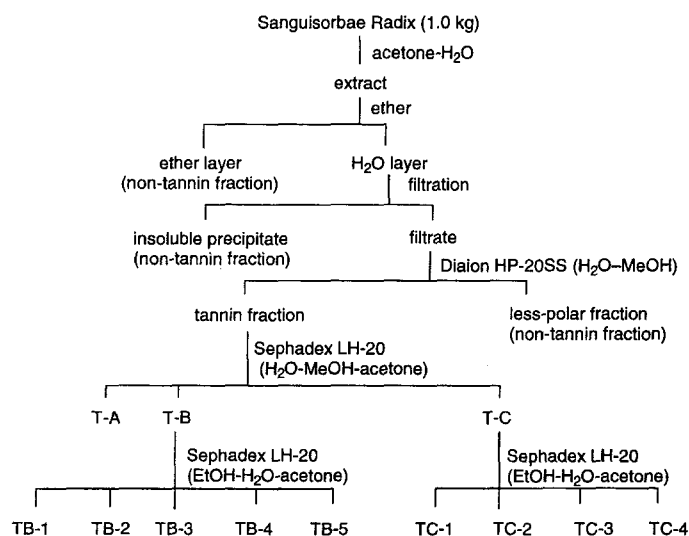


Fig. 2. Purification and isolation of the active component from *Sanguisorbae Radix*.

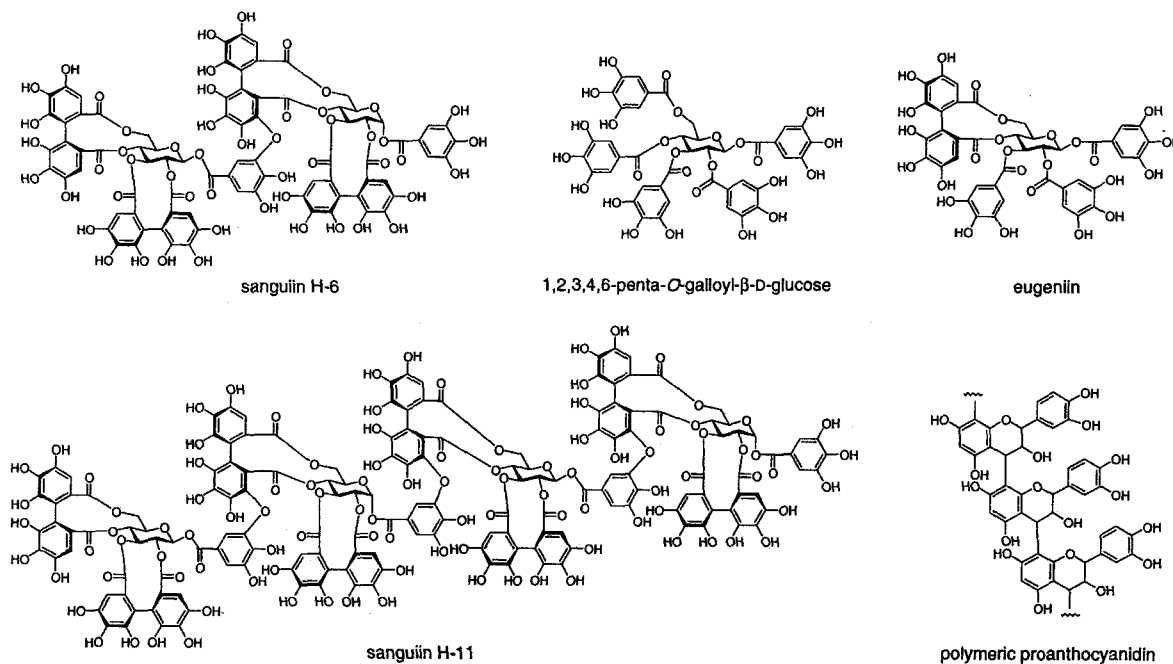


Fig. 3. Chemical structures of components isolated from *Sanguisorbae Radix*.

into five and four subfractions, respectively, and these subfractions were examined for the presence of anti-NO activity. Among the subfractions of T-B, fraction TB-3, which is composed mainly of procyanidin B-3 and its galloyl ester, showed the highest activity. The TC-4 subfraction showed the highest activity among the subfractions of T-C, and was highest among all nine subfractions of T-B and T-C (Table 7). HPLC analysis of this subfraction

revealed that its principal components are sanguiniin H-6 and sanguiniin H-11, with small amounts of 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose, eugenin and condensed polymeric proanthocyanidin (Fig. 3). These five components were examined for the presence of NO-inhibitory activity, and sanguiniin H-6 was found to have the highest activity, followed by 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose and sanguiniin H-11 (Table 7). Therefore, it was apparent

that the NO production-suppressing action of *Sanguisorbae Radix* is attributable to these tannin components. Sanguin H-6, which has the highest activity, has previously been described by Nonaka *et al.* of our group (Nonaka *et al.*, 1982a). The present study revealed a new effect of this component, the suppression of NO production by macrophages, in parallel with the suppression of iNOS and NADPH-diaphorase activities. This anti-NO activity was comparable to the effect of 100 μ M aminoguanidine, a specific inhibitor of iNOS.

The iNOS expressed in inflammatory cells produces a large amount of NO, and as Moncada and Higgs (1993) suggested, may not only act as an effector for the non-specific defense mechanism, but may also serve as an effector for self-cell destruction in autoimmune disease. Therefore, an ideal NOS inhibitor should maintain or enhance the desirable action of NO while blocking its harmful action. However, in actuality, as Vallance *et al.* (1992) reported, increased concentrations of endogenous NOS inhibitors in chronic renal failure induce hypertension and, consequently, the deterioration of renal function. Although the exact effects of sanguin H-6 and other tannin components on the defense mechanism of the body remain to be elucidated by future investigation, we have already reported that *Sanguisorbae Radix* extract decreases the LPS-induced high levels of serum urea nitrogen, Cr and nitrite/nitrate, proving its suppressive effect on iNOS activity in renal tissue (Chen *et al.*, 1999a).

NADPH-diaphorase activity is used as a histochemical marker of nNOS (Norris *et al.*, 1995). However, it remains unclear whether these activities are directly correlated in all tissues. Tracey *et al.* (1993) demonstrated that purified NOS from brain (nNOS), macrophages (iNOS) and endothelium (eNOS) all show NADPH-diaphorase activity; the relative activities were: macrophage > endothelium > brain. These data indicate that all known NOSs are NADPH-diaphorase. Mitchell *et al.* (1992) have also published data showing that in macrophages, both NADPH-diaphorase and NOS activity can be induced by LPS. Moreover, NADPH-diaphorase activity appeared in peritoneal macrophages of LPS-pretreated mice after the induction of pancreatitis, and the elevated NO was inhibited by

N^G-nitro-L-arginine (Kikuchi *et al.*, 1996). In our study we have shown that the inducible NADPH-diaphorase activity was approximately two-fold that in the vehicle alone, but was less than that seen with iNOS. This observation is in good agreement with the findings of Tracey *et al.* (1993). In contrast, *Sanguisorbae Radix* extract inhibited NADPH-diaphorase activity dose-dependently. The most active compound of *Sanguisorbae Radix* extract is sanguin H-6, consistent with those of NO production and iNOS (Table 7). Although further

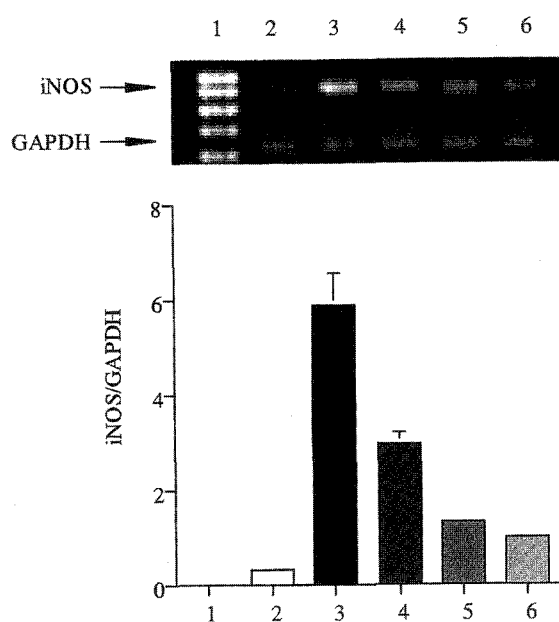


Fig. 4. Effect of sanguin H-6 on iNOS mRNA expression in activated macrophages. (1) 50 bp marker DNA; (2) no treatment (control); (3) LPS-treated control; (4) LPS-treated sanguin H-6 (12.5 μ M); (5) LPS-treated sanguin H-6 (25 μ M); and (6) LPS-treated sanguin H-6 (50 μ M).

Table 8. Effect of sanguin H-6 on iNOS enzyme activity.

Group	iNOS (pmol/mg protein/min)
None	5.87 \pm 0.96
LPS-treatment	
Control	25.98 \pm 3.65 ^b
Sanguin H-6 (12.5 μ M)	19.98 \pm 2.72 ^{b,c}
Sanguin H-6 (25 μ M)	9.80 \pm 0.75 ^{a,c}
Sanguin H-6 (50 μ M)	7.01 \pm 1.10 ^f
Aminoguanidine (50 μ M)	9.75 \pm 0.61 ^{a,c}

Statistical significance: ^a $p < 0.05$, ^b $p < 0.001$ vs. non-treatment values, ^c $p < 0.001$ vs. LPS-treatment control values.

Table 9. Effect of sanguin H-6 on NO production in macrophages.

Group	Nitrite (μM)	Cell viability (%)
None	4.55 \pm 0.34	100.0 \pm 1.3
LPS-treatment		
Control	49.86 \pm 1.44 ^c	74.9 \pm 2.4 ^c
Sanguin H-6 (12.5 μM)	15.60 \pm 0.50 ^{bc}	82.9 \pm 3.6 ^{cd}
Sanguin H-6 (25 μM)	12.08 \pm 0.96 ^{bc}	94.7 \pm 1.3 ^{bc}
Sanguin H-6 (50 μM)	7.75 \pm 0.49 ^{bc}	107.6 \pm 3.9 ^{bc}
Aminoguanidine (50 μM)	11.72 \pm 0.53 ^{bc}	76.0 \pm 2.7 ^c

Statistical significance: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs. non-treatment values, ^d $p < 0.01$, ^e $p < 0.001$ vs. LPS-treatment control values.

studies are needed to elucidate the mechanism by which sanguin H-6 exerts its inhibitory actions, we hypothesize that its actions may be partially attributable to its ability to target the multifunctional signaling molecule NO, which is known to exert similar effects in many pathological conditions.

EFFECTS OF SANGUIN H-6 ON NO PRODUCTION

The present study found that sanguin H-6 not only inhibited the expression of iNOS mRNA in a dose-dependent manner and but also inhibited iNOS activity as well, demonstrating for the first time that a compound of this kind can inhibit the iNOS activity mediated by iNOS mRNA (Fig. 4, Table 8). However, it still remains unclear whether sanguin H-6 inhibits the induction of iNOS mRNA by a direct action on LPS, or indirectly through the production/release of cytokines, where it could act on the signal transduction pathways involved in cytokine production by tyrosine kinases, or alternatively inhibit the phosphorylation of proteins induced by the cytokines themselves. Although the

expression of iNOS mRNA and the iNOS activity were suppressed more as the concentration of sanguin H-6 increased, the production of NO was markedly suppressed even at a low concentration of this agent (Table 9), suggesting the possibility that sanguin H-6 directly eliminated NO. In another experiment using the NO donor sodium nitroprusside, sanguin H-6, even at a low concentration, was found to eliminate NO, as shown in Table 10. These findings suggest that sanguin H-6 has the capacity to directly eliminate NO and to suppress the iNOS gene-mediated system. However, the precise mechanism needs to be clarified by further study.

The efficacy of aminoguanidine, an iNOS-selective inhibitor, in endotoxin shock has been reported (Wolff and Lubeskie, 1995). Sanguin H-6 at a concentration of 25 μM showed an effect equivalent to that of 50 μM aminoguanidine. Aminoguanidine resulted in no improvement in the cell viability which decreased in the presence of LPS, whereas sanguin H-6 improved the cell viability in a dose-dependent manner, reducing the toxicity of LPS (Table 9). Moncada *et al.* (1991) have shown that the iNOS expressed in inflammatory cells produces a large amount of NO, and this not only acts as an effector for the non-specific defense mechanism, but also possibly damages normal cells, serving as an effector for autocytoclasia in autoimmune disease. Therefore, the ideal NOS inhibitor should not effect the favorable actions of NO and possibly enhance them, but block the harmful actions alone. Currently the available findings on sanguin H-6 suggest that this agent has such an ideal activity. Although the exact mechanism has not been clarified, it may be a promising approach for the development of a safe selective iNOS inhibitor.

Table 10. Effect of sanguin H-6 on NO generation from sodium nitroprusside.

Sample	Concentration (μM)	NO (μM)	Inhibition %
Sanguin H-6	2.5	8.29 \pm 0.07 ^a	37.0
	5	8.16 \pm 0.09 ^a	37.9
	12.5	8.07 \pm 0.10 ^a	38.6
	25	7.69 \pm 0.07 ^a	41.5
	50	6.91 \pm 0.10 ^a	47.5
	100	4.78 \pm 0.05 ^a	63.7
Control	-	13.15 \pm 0.11	

Statistical significance: ^a $p < 0.001$ vs. control values. Inhibition % = $A/B \times 100$ (A: individual value of NO production obtained in each group; B: control value).

CONCLUSION

According to Narita *et al.* (1995) in a renal injury model mediated by the immune reaction, NO is the mediator of mesangial fusion, and inhibition of NO production can reduce glomerular injury which leads to subsequent glomerulosclerosis and tubular interstitium damage. Thus, it has been suggested that in renal injury mediated by the immune reaction, inhibition of the L-arginine/NO system at the initial stage may be a useful

therapeutic measure. Although clarification of the effects of sanguin H-6 on renal failure requires further investigation, it has become apparent that Sanguisorbae Radix extract significantly improves the high levels of NO and deteriorated renal function after LPS administration, and in addition inhibits the iNOS activity in renal tissue. It is also known that Sanguisorbae Radix extract not only eliminates ONOO⁻ but also reduces oxidative injury in the kidney (Chen *et al.*, 1999a, 2001), suggesting the importance of sanguin H-6 in the treatment of renal injury.

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