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EVIDENCE SUGGESTING A NITRIC OXIDE-SCAVENGING ACTIVITY FOR TRADITIONAL CRUDE DRUGS, AND ACTION MECHANISMS OF SANGUISORBAE RADIX AGAINST OXIDATIVE STRESS AND AGING

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ABSTRACT

In this series of experiments, we found that Sanguisorbae Radix extract possesses strong free radical-scavenging activity *in vitro* and *in vivo*. This crude drug protected against renal disease, which is closely associated with excessive generation of reactive oxygen species. We also showed that Sanguisorbae Radix extract can suppress lipid peroxidation and stimulate an antioxidant defense ability in SAM, suggesting that this crude drug may be an effective agent for ameliorating the pathological conditions related to excessive generation of free radicals and oxidant damage, particularly in the aging process.

Aging is associated with a progressive decline in the ability to respond to the stresses of a dynamic environment. Several theories have been proposed to explain the aging process, among which the free radical theory has received particular attention in recent years (1,2). Physiologically, the formation and consumption of free radicals, particularly oxygen radicals, in the body are balanced by antioxidant defense systems; when the antioxidant ability provided by this defense system is decreased by various physiopathologic causes, reactive free radicals accumulate, and this in turn induces lipid peroxidation, which consequently leads to oxidative stress or damage. It is widely accepted that oxidative stress plays an important role in degenerative senescence, and various studies have revealed increased oxidative modification of proteins and changes in plasma membrane lipids in association with aging (3,4).

The free radical theory of aging proposes that intervention designed to retard the intrinsic aging process would be possible by the administration of antioxidants or free-radical scavengers. A number of dietary antioxidants have been administered to different organisms in attempts to increase life expectancy, and very interesting results have been obtained (5-8).

Traditional crude drugs, derived from nature and employed for thousands of years in Chinese medicine, continue to play important roles in promoting health and the treatment of various diseases. They are also considered to be potential sources of new therapeutic agents and medicines because of their distinctive biological activity associated with low toxicity. However, details of the mechanisms by which crude drugs exert their effects are not well understood.

In recent years, powerful evidence has emerged to suggest that lipid peroxidation and free radicals, such as reactive oxygen species, are involved in the pathogenesis of a number of processes including carcinogenesis, aging, cardiovascular disease, ischemia and inflammation (9-12). Numerous crude drugs or their constituents have shown, both *in vivo* and *in vitro*, remarkable suppression of lipid peroxidation and the scavenging of reactive free radicals; activities which are thought to contribute to their pharmacological effects (13-19).

On the other hand, nitric oxide (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 (20-23). In addition, recent experiments 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 (24,25).

The ability of traditional medicines or their constituents to scavenge the O_2^- radical, which obstructs the formation of $ONOO^-$ and thus alleviates the injury caused by NO, has been extensively confirmed (26,27). However, little research has investigated the possible regulatory effects of traditional drugs or their constituents on NO to date. We therefore conducted the experiments to investigate the direct NO-scavenging effect of 31 traditional crude drugs using sodium nitroprusside as a NO donor, in an attempt to search for more potent NO regulators and to provide a scientific explanation for their biological activities and effects. Several pure tannins and alkaloids were also examined (28). In addition, we examined whether Sanguisorbae Radix extract protects against oxidative stress using rats either treated with lipopolysaccharide (LPS) or injected with LPS and subjected to renal ischemia followed by reperfusion, and senescence-accelerated mice (SAM) (29-31). The purpose of this article is to review the functional significance of traditional crude drugs, based mainly on our series of studies.

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NO-Scavenging Effect of Traditional Crude Drugs

Sodium nitroprusside is widely used as both a vasoactive substance in clinical situation and as a NO donor *in vivo* and *in vitro* (32,33), releasing NO spontaneously in aqueous solution at physiological pH. Furthermore, NO reacts with oxygen to produce nitrite ions, which can be estimated using Griess reagent (34). We utilized this donor to elucidate the direct NO scavenging effect of traditional crude drugs.

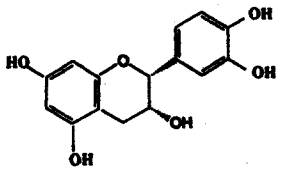
The crude drugs used in this experiment were as follows: Aconiti Tuber *(Aconitum japonicum* Thunberg*)*, Angelicae Radix *(Angelica acutiloba* Kitagawa*)*, Artemisiae Capillaris Flos *(Artemisia capillaris* Thunb.*)*, Asiasari Radix *(Asiasarum sieboldii* F. Maekawa*)*, Astragali Radix *(Astragalusmembranaceus* Bunge*)*, Atractylodis Lanceae Rhizoma *(Atractylodes lancea* DC.*)*, Bupleuri Radix *(Bupleurum falcatum* L.*)*, Carthami Flos *(Carthamus tinctorius* L.*)*, Caryophylli Flos *(Eugenia caryophyllata* Thunb.*)*, Chebulae Fructus *(Terminalia chebula* Retzus*)*, Cinnamomi Cortex *(Cinnamomum cassia* Blume*)*, Cnidii Rhizoma *(Cnidium officinale* Makino*)*, Coptidis Rhizoma *(Coptis japonica Makino)*, Ephedrae Herba *(Ephedra sinica* Stapf*)*, Gallae Rhois *(Rhus javanica* L.*)*, Gambir *(Uncaria gambir* Roxb.*)*, Ganoderma *(Ganoderma lucidum* Karst.*)*, Ginseng Radix (*Panax ginseng* C.A. Meyer*)*, Glycyrrhizae Radix *(Glycyrrhiza glabra* Linn. var. *glandulifera* Regel et Herder*)*, Granati Cortex *(Punica granatum* L.*)*, Hoelen *(Poria cocos* Wolf*)*, Moutan Cortex *(Paeonia suffruticosa* Andrews*)*, Paeoniae Radix *(Paeonia albiflora* Pallas var. *trichocarpa* Bunge*)*, Perillae Herba *(Perilla frutescens* Britt. var. *acuta* Kudo*)*, Pinelliae Tuber *(Pinellia ternata* Breit.*)*, Polygoni Multiflori Radix *(Polygonum multiflorum* Thunb.*)*, Rhei Rhizoma *(Rheum officinale* Baillon*)*, Salviae Miltiorrhizae Radix *(Salvia miltiorrhiza* Bunge*)*, Sanguisorbae Radix *(Sanguisorba officinalis* L.*)*, Scutellariae Radix *(Scutellaria baicalensis* Georgi*)* and Zingiberis Rhizoma *(Zingiber officinale* Roscoe*)*. One hundred grams of each crude drug was boiled gently in 1 L of water for 60 min. The extract was then concentrated under reduced pressure to leave a residue. The yields of the crude drugs ranged from 11-32%. Ten tannins and eight alkaloids were isolated from Sanguisorbae Radix, Rhei Rhizoma and Coptidis Rhizoma, as reported previously (35-41). The chemical structures of these compounds are shown in Figs. 1 and 2.

The present results showed that many crude drugs are potential scavengers of NO, effectively scavenging over different ranges of concentration. The scavenging effects were concentration-dependent, and there was an obvious relationship between the activity and the constituents of the crude drugs examined. As summarized in Table 1, eight crude drugs (Sanguisorbae Radix, Caryophylli Flos, Gambir, Coptidis Rhizoma, Granati Cortex, Gallae Rhois, Rhei Rhizoma and Cinnamomi Cortex) effectively scavenged NO, yielding 50% when their concentrations were below 1000 μg/ml. The most potent scavenger was Sanguisorbae Radix, which had an IC_{50} value (concentration required to inhibit NO formation by 50%) of 112.8 μg/ml. Almost all of the crude drugs that exhibited strong scavenging activity (IC_{50} <1000 μg/ml) were found to contain tannins as their major constituent: Sanguisorbae Radix, Caryophylli Flos, Gambir, Granati Cortex, Gallae Rhois, Rhei Rhizoma and Cinnamomi Cortex. Other tannin-containing drugs such as Chebulae Fructus and Polygoni Multiflori Radix also showed effective activity against NO at high concentrations, the former reducing NO production by 33.8% compared with the control value and the latter by 29.3% at 250 μg/ml.

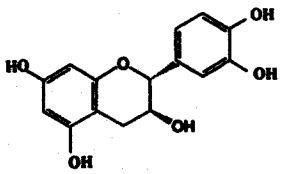
Table 1: IC_{50} values of crude drugs tested against NO production.

Crude drugs	*IC_{50} (μg/ml)*
Sanguisorbae Radix	112.8± 1.5
Caryophilli Flos	152.0± 1.7
Gambir	181.9± 2.4
Coptidis Rhizoma	182.9± 3.8
Granati Cortex	270.9± 2.3
Gallae Rhois	286.4± 6.0
Rhei Rhizoma	394.4± 1.4
Cinnamomi Cortex	759.3±12.9

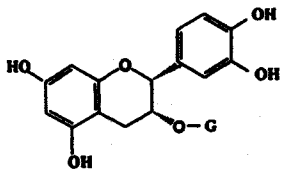
Hong et al. (42) reported that tannins have been widely shown to protect lipids from peroxidation. Tannins also scavenge reactive free radicals such as reactive oxygen species and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (13,19,43). In order to evaluate whether tannins are the active principles responsible for scavenging NO, we also examined 10 tannin compounds mainly contained in Sanguisorbae Radix and Rhei Rhizoma, and found that they were strong NO scavengers (Table 2). Moreover, it was observed that the scavenging activity was obviously stronger when the number of galloyl groups in the molecule increased. Among the condensed tannins, (-)-epicatechin and (+)-catechin, the optical isomer, showed similar activity, having IC_{50} values of 240.7 and 257.4 μg/ml, respectively; however, as one of the hydroxyl groups of epicatechin was galloylated, the IC_{50} of (-)-epicatechin 3-*O*-gallate decreased to 120.9 μg/ml. Similarly, the IC_{50} of procyanidin B-3 was 221.9 μg/ml, whereas those of procyanidin B-2 3,3'-di-*O*-gallate and procyanidin C-1 3,3',3"-tri-*O*-gallate were 59.2 and 41.2 μg/ml, respectively. In addition, galloyl groups were also shown to have a similar influence on hydrolyzable tannins. For example, pentagalloyl glucose showed higher activity than tetragalloyl glucose, having IC_{50} values of 179.1 and 326.3 μg/ml, respectively. The difference in the potency of the above compounds against NO was attributed mainly to the influence of the galloyl groups, although increased molecular weight may also have been a contributory factor. Further evidence for this was provided by eugeniin and sanguiin H-6, the former being a monomer with an IC_{50} value of 123.1 μg/ml, and the latter a dimer with an IC_{50} of 72.1 μg/ml (Table 2).



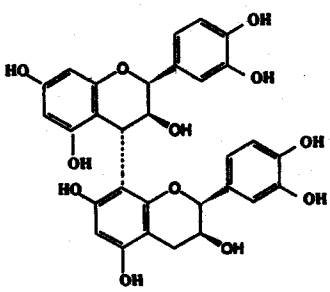
(-)-Epicatechin



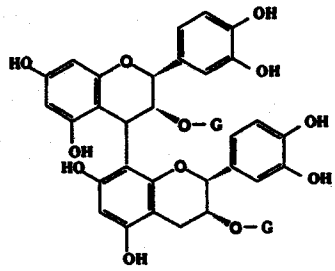
(+)-Catechin



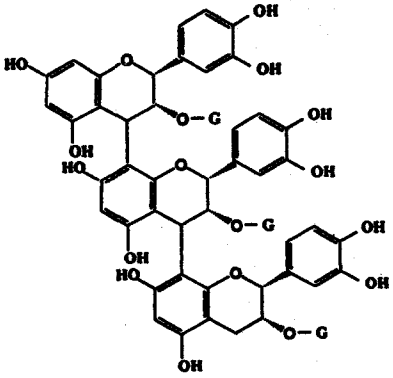
(-)-Epicatechin-3-*O*-Gallate



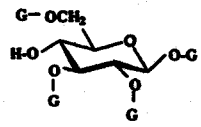
Procyanidin B-3



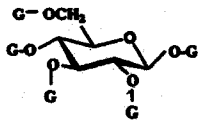
Procyanidin B-2 3,3'-di-*O*-gallate



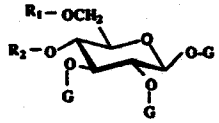
Procyanidin C-1 3,3',3"-tri-*O*-gallate



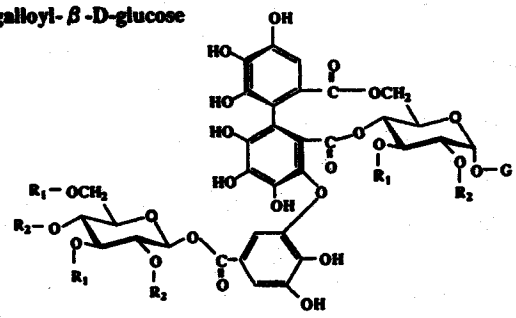
1,2,3,6-Tetra-*O*-galloyl-*β*-D-glucose



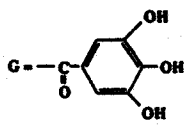
1,2,3,4,6-Penta-*O*-galloyl-*β*-D-glucose



Eugeniin



Sanguiin H-6



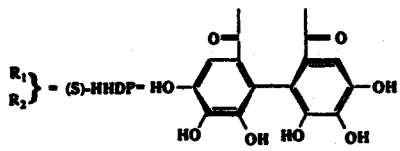
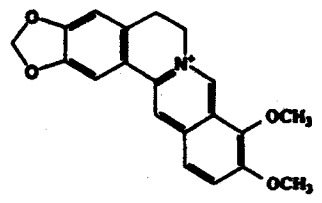
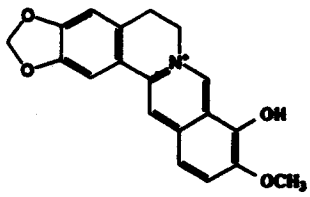


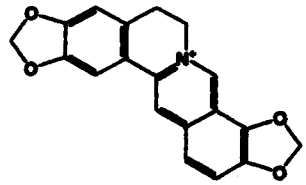
Fig. 1



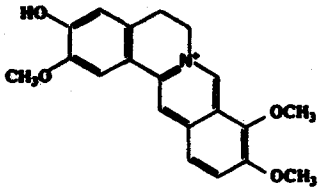
Berberine



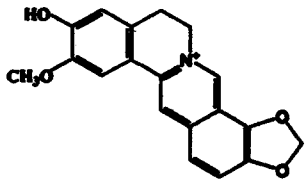
Berberrubine



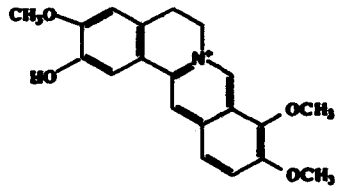
Coptisine



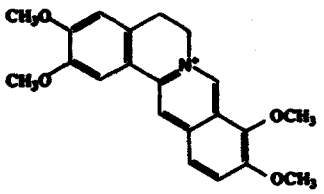
Jatrorrhizine



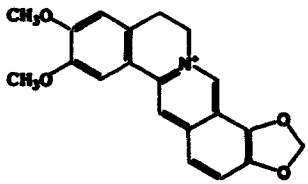
Groenlandicine



Columbamine



Palmatine



Epiberberine

Fig. 2

Table 2. IC_{50} values of tannins and alkaloids tested against NO production.

Family	*Compound*	*IC_{50} (μg/ml)*
Tannins	Procyanidin C-1 3,3',3"-tri-*O*-gallate	41.2± 0.7
	Procyanidin B-2 3,3'-di-*O*-gallate	59.2± 1.7
	Sanguiin H-6	72.1± 1.4
	(-)-Epicatechin 3-*O*-gallate	120.9± 1.5
	Eugeniin	123.1± 3.4
	1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose	179.1± 1.5
	Procyanidin B-3	221.9± 5.9
	(-)-Epicatechin	240.7±12.6
	(+)-Catechin	240.7± 5.1
	1,2,3,6-Tetra-*O*-galloyl-β-D-glucose	326.3±13.9
Alkaloids	Berberrubine	411.5± 0.5
	Jatrorrhizine	107.9± 3.4
	Coptisine	234.2± 3.4
	Epiberberine	287.9± 4.9
	Palmatine	382.8±11.2
	Columbamine	455.4±26.9
	Berberine	>1000
	Groenlandicine	>1000

Recent research indicates that NO acts as part of the host defense mechanism, and is generated in considerably increased amounts during infection and inflammation due to immunological stimulation, and shows cytotoxic or cytostatic activity against viruses and invasive organisms (25). However, such an increase in NO may also adversely affect host cells, resulting in tissue injury (20). Therefore, the production of NO must be tightly regulated. Traditional crude drugs are effective for the treatment of infection and inflammation, and their constituent compounds such as alkaloids, flavonoids, anthraquinones and essential oils have been clinically employed as anti-infective and anti-inflammatory agents. The exact mechanism behind their therapeutic effects remains unclear, but it has been proposed that the anti-inflammatory properties of flavonoids result from diminished formation of proinflammatory mediators (prostaglandins, leukotrienes, reactive oxygen species and NO) (15). In our present experiment, some crude drugs known to have anti-infection or anti-inflammatory properties effectively reduced the production of NO, suggesting that a direct scavenging effect against NO might contribute to their actions. For example, Coptidis Rhizoma, one of the most well known and widely used bacteriostatic, antipyretic and antiphlogistic medicines, and its anti-inflammatory active constituents berberine, coptisine, jatrorrhizine and palmatine (44,45) demonstrated a strong NO scavenging effect (IC_{50}=182.9 μg/ml).

To investigate the possible relationship between the anti-inflammatory effect and NO scavenging, we tested

the NO scavenging activity of its anti-inflammatory active principles, which are 8 quarternary protoberberine-type alkaloids. These alkaloids, except for berberine and groenlandicine, were found to scavenge NO effectively, having IC_{50} values of less than 455.4 μg/ml (Table 2). Similarly, Scutellariae Radix, whose flavone derivatives including baicalin, baicalein and wogonin are reported to suppress the development of secondary lesions in rats with adjuvant-induced arthritis (46) also reduced the level of NO by 32.8% compared with the control value at 250 μg/ml. NO production was comparably reduced by 34.1% and 28.7% at 250 μg/ml during previous research by other crude drugs such as Artemisiae Capillaris Flos and Astragali Radix, whose effective anti-inflammatory constituents are flavone and a coumarin derivative (47,48), respectively.

In addition, crude drugs containing essential oils displayed considerable NO-scavenging activity. Several crude drugs, such as Caryophylli Flos, Granati Cortex and Perillae Herba, demonstrated marked activity, reducing the NO level by 57.2%, 49.4%, and 34.6%, respectively, in comparison with the control value at a concentration of 250 μg/ml; Angelicae Radix, Asiasari Radix, Cnidii Rhizoma and Zingiberis Rhizoma showed scavenging effects of between 15.2% and 26.6% at the same concentration. To date, although the phenolic hydroxyl group is confirmed as the most active functional group for antioxidation and scavenging of free radicals such as reactive oxygen species and the DPPH radical (13,17,19,27,43,49,50), it has not been shown to be essential for NO-scavenging activity. A diketone system was suggested to play an important role in NO scavenging (51). In addition, in the present study, the galloyl group strengthened the NO scavenging activity of tannins, although an increase in molecular weight also seems to exert a similar influence. The structural requirements necessary for NO scavenging activity remain to be elucidated further.

In summary, traditional crude drugs are potent NO scavengers that can protect tissues and cells from injury caused by excess levels of NO and $ONOO^-$ through a direct scavenging effect. They can also alleviate peroxidant damage mediated by reactive free radicals including NO, and provide a novel therapy for regulating pathological conditions related to excessive generation of NO. Tannins and alkaloids appear to be the most effective constituents, while flavonoids and essential oils also appear to act against NO production.

Beneficial Effects of Sanguisorbae Radix in Renal Dysfunction Caused by Endotoxin

NO plays an important role in the kidney, both under normal and pathological conditions. NO is produced from L-arginine by the action of NO synthase (NOS). Three isoforms of NOS have been detected in different regions of the kidney, expressing different functions: 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 vasarecta; and inducible NOS (iNOS) is found in the proximal tubule and the glomerulus (52). NO generated by these NOS isoforms exerts 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 mesangical cell relaxation. However, iNOS generates NO in a large quantities and for a prolonged period, since it is induced by certain cytokines and hypoxia (53). Excessive NO is strongly cytotoxic, and thus injures cells and tissues. In freshly isolated rat proximal tubules, NO and its metabolic product, $ONOO^-$, mediate tubular hypoxia-reperfusion injury (54). Cattell et al. (55) also provided experimental evidence for the mediatory 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 3). The serum nitrite/nitrate level, an indicator of NO formation, was also markedly increased in LPS-treated rats compared with that in normal rats (Table 4). As a more direct indicator, we monitored the activity of iNOS in renal homogenate using the method of Suhet al. (56). LPS-treatment resulted in an approximately 2-fold increase in the activity of iNOS, suggesting the possible association of additional induction ofiNOS with renal dysfunction *in situ*, as shown in Table 5.

Table 3. 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]
Sanguisorbae Radix extract (50 mg/kg/d)	33.8±3.1[a,b]	0.78±0.10[a,d]
Sanguisorbae Radix extract	31.6±2.3[a,d]	0.68±0.08[a,d]
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[a,c]	0.66±0.14[a,d]

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

Table 4. 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]
Sanguisorbae Radix extract (50 mg/kg/d)	4.39±1.82[b,c]
Sanguisorbae Radix extract (100 mg/kg/d)	3.72s±0.89[a,c]
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.

Various inhibitors of NO or NOS have been used in attempts to improve or attenuate the pathology involved in excessive generation of NO. However, conflicting results have been obtained. Using isolated renal proximal tubules,Yu et al. (54) reported that the NOS inhibitor, N-nitro-L-arginine methyl ester, protected the renal tubular epithelium against hypoxic injury. Weinberg et al. (57) 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 beneficial effects, NOS inhibitors aggravated renal dysfunction in several in vivo models of acute renal failure (58,59). We speculate that these contradictory results are attributable to a lack of selective NOS inhibitors.

Table 5. 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]
Sanguisorbae Radix extract (50 mg/kg/d)	2.69±0.10[a,c]
Sanguisorbae Radix 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]<p0.01, [b]p<0.001 vs. normal values, [c]p<0.001 vs. LPS-treated control values.

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 described in the previous section that in vitro Sanguisorbae Radix (Fig. 3), a traditional crude drug that contains a large amount of tannin as its major constituent, has a strong scavenging effect on NO induced by sodium nitroprusside, a NO donor. We also recently observed that Sanguisorbae Radix extract effectively inhibited the activity of iNOS in activated macrophages induced by LPS (60). These findings prompted us to investigate whether 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 3, 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 actively protected against the renal dysfunction caused by LPS, although these effects were weaker than those produced by aminoguanidine, a selective iNOS inhibitor (Tables 4 and 5).

Fig. 3: Sanguisorbae Radix.

Protective Effect of Sanguisorbae Radix against Peroxynitrite-mediated Renal Injury

NO reacts rapidly with O_2^- to form a potentially more toxic agent, $ONOO^-$. $ONOO^-$ is highly reactive, oxidizes a large number of bio-molecules and initiates a wide range of toxic reactions, including tyrosine nitration, lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inhibition of membrane sodium/potassium ATPase activity, inactivation of membrane sodium channels, and other oxidative modifications of proteins (20,24,61-64). It has been suggested that $ONOO^-$ may contribute to the toxic effect of NO and, therefore, may be a major factor in oxidative tissue injury.

Although $ONOO^-$ is generated in numerous pathophysiological conditions, few in vivo models produce sufficient amounts of $ONOO^-$ for the evaluation of its cytotoxicity, or its contribution to NO cytotoxicity and tissue injury. As $ONOO^-$ is generated via a reaction between NO and O_2^-, we decided to treat the rats with LPS and ischemia-reperfusion, the former providing abundant NO and the latter causing elevated O_2^-. However, 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. $ONOO^-$ can oxidize the tyrosine in protein into nitrotyrosine,

which is stable and detectable. Nitrotyrosine has been taken as evidence for in vivo formation of $ONOO^-$ (65,66). 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 6). We were unable to conclude, on these grounds, that nitrotyrosine is not formed in ischemia-reperfusion injury because several studies demonstrated that $ONOO^-$ is an important mediator in ischemia-reperfusion injury (67,68), and the HPLC method with UV detection that we used, has limited sensitivity. However, our results suggest that $ONOO^-$ formation increases in the presence of excessive generation of NO and O_2^-.

To evaluate the effect of elevated $ONOO^-$ on impaired renal function, we measured urea nitrogen and creatinine in plasma. As shown in Table 7, treatment of the rats with LPS + ischemia-reperfusion resulted in significantly higher urea nitrogen and creatinine levels than either LPS-treatment or ischemia-reperfusion alone, which suggests that $ONOO^-$ aggravates the impairment of renal function.

Table 6. 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.

In a previous section, we reported that Sanguisorbae Radix extract ameliorates renal dysfunction in an LPS-challenged rat model, and that this effect involves the inhibition of NO generation and iNOS activation. On the other hand, we found in a recent preliminary in vitro experiment that this extract inhibited $ONOO^-$ directly. Hence, it is possible that Sanguisorbae Radix extract ameliorated renal dysfunction by inhibiting excessive generation of NO and/or $ONOO^-$, or by scavenging O_2^-. We clarified this effect using the LPS + ischemia-reperfusion model. Our results showed that administration of Sanguisorbae Radix extract significantly ameliorated increases in plasma urea nitrogen and creatinine, which corresponded with a significant decrease in the $ONOO^-$ level. In particular, the effects of 200 mg/kg body weight/day Sanguisorbae Radix extract were stronger than the effects of aminoguanidine, a specific iNOS inhibitor (Tables 6 and 7).

Table 7. Effect of Sanguisorbae Radix extract on urea nitrogen and Cr levels in plasma.

Group	*Urea nitrogen (mg/dl)*	*Creatinine (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]$p<0.001$, [c]$p<0.001$ vs. LPS + ischemia-reperfusion without pretreatment.

$ONOO^-$ 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 (69). We have previously reported that ischemia-reperfusion can induce DNA ladder formation (70). In the present study, rats subjected to LPS + ischemia-reperfusion had more distinct DNA ladders than rats subjected to ischemia-reperfusion alone, suggesting that $ONOO^-$ is a strong cytotoxic agent (Fig. 4). 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, which indicates 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. 4). A semiquantative method showed that the DNA fragmentation level was markedly decreased in the Sanguisorbae Radix extract-treated groups. On the other hand, infusion of saline did not affect the formation of DNA ladders, whereas aminoguanidine treatment reduced DNA fragmentation. The effect of aminoguanidine was weaker than that of Sanguisorbae Radix extract. This effect of aminoguanidine may be due to inhibition of NO and to blocking of $ONOO^-$ formation. These results suggested that $ONOO^-$ mediates, at least partially, cell death, and that Sanguisorbae Radix extract protects renal cells against such injury.

Antioxidant Defense Ability of Sanguisorbae Radix in SAM

Tannins are a family of bioactive natural compounds that have been extensively studied and are reported to possess distinctive antioxidant and free-radical-scavenging properties (71-77). These properties are associated with several of the bioactivities and pharmacological effects of tannins, but their possible anti-aging effects have received less attention. In view of the

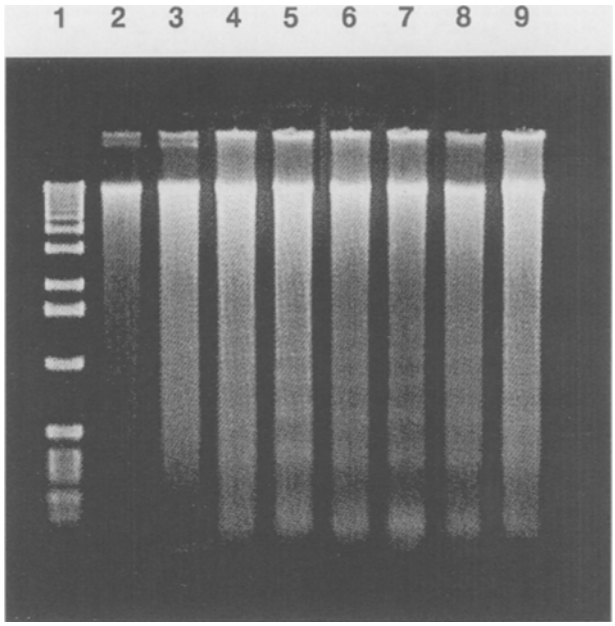


Fig. 4: Agarose gel electrophoresis of DNA. Lane 1: 1-kb marker DNA; lane 2: sham treatment; lane 3: LPS; lane 4: ischemia-reperfusion; lane 5: LPS + ischemia-reperfused no treatment (water drink); lane 6: LPS + ischemia-reperfused no treatment (saline infusion); lane 7: LPS + ischemia-reperfused aminoguanidine treatment; lane 8: LPS + ischemia-reperfused Sanguisorbae Radix extract treatment (100 mg/kg/d); lane 9: LPS + ischemia-reperfused Sanguisorbae Radix extract treatment (200 mg/kg/d).

effects of free radicals and lipid peroxidation in accelerating senescence, we hypothesized that tannins would probably be beneficial to the attenuation of oxidative damage, thereby retarding the aging process. In this chapter, using SAM, experiments were performed whether Sanguisorbae Radix extract, which contains a large quantity of both hydrolyzable and condensed tannin, acts against oxidative stress related to the aging process.

The antioxidant defense system, which includes antioxidant enzymes and non-enzymatic low-molecular-weight antioxidant molecules such as glutathione(GSH), is present in the living body and plays a critical role in maintaining the balance between prooxidant and antioxidant to protect cells and tissues against the potentially harmful effects of reactive free radicals and peroxidation. The system formed by superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), glutathione reductase and GSH plays a major role in scavenging reactive oxygen radicals, including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical, which have been extensively implicated in pathogenic mechanisms associated with aging (78). Among these, GSH is considered to be the most important antioxidant molecule in cells, possibly reacting directly with reactive oxygen species and also serving as a substrate in the enzymatic reduction of detoxified H_2O_2 and lipid hydroperoxides (LOOH), thus protecting cells against oxidative damage. The GSH/glutathione disulfide (GSSG) ratio is considered to be a sensitive measure of both tissue GSH status and oxidative stress (79-81). As the kidney and liver are the most important organs involved in the regulation of the glutathione redox cycle, we examined the alterations of antioxidant defense related to aging in these two organ tissues.

In the present study, SAM showed a lower GSH level and a higher GSSG level. Compared with normal AKR/N Slc mice, the GSH/GSSG ratio was a 30% lower in the kidney and 22% lower in the liver, suggesting that GSH is excessively consumed for direct and indirect scavenging of H_2O_2 or LOOH, and that the oxidative product accumulates in SAM. In contrast, oral administration of Sanguisorbae Radix extract significantly elevated GSH and decreased GSSG, consequently increasing the GSH/GSSG ratio in both the kidney and liver (Tables 8 and 9). These effects may be associated with increased glutathione reductase activity in the liver, although this activity was not detected in the kidney because of the smaller amount present (Tables 10 and 11).

Table 8. Effect of Sanguisorbae Radix extract on glutathione in kidney.

Group	GSH (μmol/g kidney)	GSSH (μmol/g kidney)	GSH/GSSG
AKR/N Slc mice	3.15±0.04	0.50±0.03	6.44±0.77
SAM			
Control	2.66±0.01[b]	0.62±0.08[a]	4.50±0.55[a]
Sanguisorbae Radix extract	2.99±0.01[b,e]	0.48±0.01[c]	6.39±0.44[d]

Statistical significance: [a]p<0.01, [b]p<0.001 vs. AKR/N Slc mice values, [c]p<0.05, [d]p<0.01, [e]p<0.001 vs. SAM control values.

Table 9. Effect of Sanguisorbae Radix extract on glutathione in the liver.

Group	GSH (μmol/g liver)	GSSH (μmol/g liver)	GSH/GSSG
AKR/N Slc mice	5.64±0.01	0.97±0.06	5.94±0.40
SAM			
Control	5.05±0.01[b]	1.09±0.02[a]	4.63±0.29[b]
Sanguisorbae Radix extract	6.25±0.03[b,d]	0.99±0.03[c]	6.33±0.34[d]

Statistical significance: [a]p<0.01, [b]p<0.001 vs. AKR/N Slc mice values, [c]p<0.05, [d]p<0.001 vs. SAM control values.

We also examined the activities of several antioxidant enzymes. SOD is the most important enzyme providing defense against the deleterious effect of reactive oxygen species, and can rapidly scavenge O_2^- by converting it toH_2O_2. In addition, SOD activity is associated with life span in various species and strains (82,83). In the present study, SOD did not show significant alteration among the three test groups. However, the activities of catalase and GSH-Px, two enzymes responsible for scavenging H_2O_2, were significantly depressed in SAM, whereas Sanguisorbae Radix extract reversed the decrease in enzyme activities (Tables 10 and 11). These results suggest that the antioxidant defense system in SAM was partly impaired, and that Sanguisorbae Radix extract can in part stimulate antioxidant defense ability.

Lipid peroxidation has been proposed as a major mechanism by which free radicals induce tissue injury. The reactive free radicals attack polyunsaturated fatty acids, initiate lipid peroxidation in biological membranes, and consequently alter membrane structure and function. Furthermore, accumulated lipid peroxides leak

Table 10. Effect of Sanguisorbae Radix extract on renal enzyme activities involved in the glutathione redox cycle.

Group	SOD (U/mg protein)	Catalase (U/mg protein)	GSH-Px (U/mg protein)
AKR/N Slc mice	19.73±2.65	190.3±7.0	167.7±3.4
SAM			
Control	16.20±4.39	147.8±4.3[a]	151.2±2.8[a]
Sanguisorbae Radix extract	16.68±2.01	157.8±2.9[a,b]	163.0±3.2[b]

Statistical significance: [a]p<0.001 vs. AKR/N Slc mice values, [b]p<0.001 vs. SAM control values.

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Table 11. Effect of Sanguisorbae Radix extract on hepatic enzyme activities involved in the glutathione redox cycle.

Group	SOD (U/mg protein)	Catalase (U/mg protein)	GSH-Px (U/mg protein)	Glutathione reductase (nmol/min/mg protein)
AKR/N Slc mice	27.38±3.17	285.4±3.5	170.6±7.4	18.26±0.36
SAM				
Control	26.45±4.78	264.5±4.3[a]	157.5±3.9[a]	17.20±0.65
Sanguisorbae Radix extract	29.38±5.97	274.7±2.3[a,b]	159.3±6.0[a]	19.43±0.76[a,c]

Statistical significance: [a]p<0.05 vs. AKR/N Slc mice values, [b]p<0.05, [c]p<0.01 vs. SAM control values.

Table 12. Effect of Sanguisorbae Radix extract on malondialdehyde.

Group	Serum MDA (nmol/ml)	Kidney MDA (nmol/mg protein)	Liver MDA (nmol/mg protein)
AKR/N Slc mice	2.13±0.13	0.16±0.01	0.59±0.04
SAM			
Control	2.90±0.20[a]	0.18±0.01a	0.92±0.12[a]
Sanguisorbae Radix extract	2.34±0.09[c]	0.16±0.01[c]	0.70±0.07[b]

Statistical significance: [a]p<0.001 vs. AKR/N Slc mice values, [b]p<0.01, [c]p<0.001 vs. SAM control values.

from organs and tissues into the blood system, increasing their level in blood lipoproteins and promoting atherogenesis and cardiovascular disease, which are major factors in morbidity and mortality among the elderly (84). The level of malondialdehyde (MDA), as a lipid peroxidation product, was measured in serum, and the kidney and liver. As predicted, MDA levels were markedly increased in SAM, being 36% higher in serum,13% higher in the kidney and 56% higher in the liver, thus providing direct evidence of peroxidative conditions in SAM. Sanguisorbae Radix extract suppressed the lipid peroxidation mediated by these reactive free radicals, and lowered MDA formation markedly in serum, and the kidney and liver (Table12).

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