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Amla (*Emblica officinalis* Gaertn.) Attenuates Age-Related Renal Dysfunction by Oxidative Stress

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To investigate the effects of amla on renal dysfunction involved in oxidative stress during the aging process, we employed young (2 months old) and aged (13 months old) male rats and administered SunAmla (Taiyo Kagaku Co., Ltd., Japan) or an ethyl acetate (EtOAc) extract of amla, a polyphenol-rich fraction, at a dose of 40 or 10 mg/kg body weight/day for 100 days. The administration of SunAmla or EtOAc extract of amla reduced the elevated levels of serum creatinine and urea nitrogen in the aged rats. In addition, the tail arterial blood pressure was markedly elevated in aged control rats as compared with young rats, while the systolic blood pressure was significantly decreased by the administration of SunAmla or EtOAc extract of amla. Furthermore, the oral administration of SunAmla or EtOAc extract of amla significantly reduced thiobarbituric acid-reactive substance levels of serum, renal homogenate, and mitochondria in aged rats, suggesting that amla would ameliorate oxidative stress under aging. The increases of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression in the aorta of aging rats were also significantly suppressed by SunAmla extract or EtOAc extract of amla, respectively. Moreover, the elevated expression level of bax, a proapoptotic protein, was significantly decreased after oral administration of SunAmla or EtOAc extract of amla. However, the level of bcl-2, an antiapoptotic protein, did not show any difference among the groups. The expressions of renal nuclear factor-κB (NF-κB), inhibitory κB in cytoplasm, iNOS, and COX-2 protein levels were also increased with aging. However, SunAmla or EtOAc extract of amla reduced the iNOS and COX-2 expression levels by inhibiting NF-κB activation in the aged rats. These results indicate that amla would be a very useful antioxidant for the prevention of age-related renal disease.

KEYWORDS: Amla; aging; kidney; TBA-reactive substance; NF-κB; iNOS; COX-2; bax; rat

INTRODUCTION

Emblica officinalis Gaertn., commonly known as amla, is a member of the small genus of *Emblica* (Euphorbiaceae). It grows in tropical and subtropical parts of China, India, Indonesia, and the Malay Peninsula. It is an important dietary source of vitamin C, minerals, and amino acids and also contains phenolic compounds, tannins, phyllembelic acid, phyllemblin, rutin, curcuminoides, and emblicol. All parts of the plant are used for medicinal purposes. Especially, the fruit has been used in Ayurveda as a potent rasayana and in traditional medicines for the treatment of diarrhea, jaundice, and inflammation. In addition, the pulp of the fruit is smeared on the head to dispel headache and dizziness. Recently, amla extract has been tested for various pharmacological activities. The fruit extract was reported to have hypolipidemic (*1*), antidiabetic (*2*), and antiinflammatory activities (*3*) and to inhibit retroviruses such as HIV-1 (*4*), tumor development (*5*), and gastric ulcer (*6*). Moreover, amla extract exhibits antioxidant properties (*7*), and it has been reported that the aqueous extract of amla is a potent inhibitor of lipid peroxide formation and scavenger of hydroxyl and superoxide radicals in vitro (*8*). In a previous study, we also demonstrated the antioxidative property of amla using Cu^{2+}-induced oxidized human low density lipoprotein (*9*).

Aging is defined as a multifactorial phenomenon characterized by a time-dependent decline in physiological function, and it is well known that the aging process involves the accumulation of oxidative damage to cells and tissues. Oxidative stress, such as via an increased production of free radicals and other reactive species and decreased antioxidative defenses, is known to occur during aging (*10*, *11*). Numerous studies show that this oxidative stress causes structural and functional alterations to molecular, cellular, tissue, and organ systems. In particular, structural and

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functional changes also occur in the kidney with aging (*12, 13*). The glomerular filtration rate (GFR) declines with age, along with renal blood flow, while the filtration fraction and renal vascular resistance increase (*14*). These changes reduce the capacity to respond to pathological stress and renal function. On the other hand, some reports showed that an antioxidant, a nuclear factor-κB (NF-κB) inhibitor, and selective inducible nitric oxide synthase (iNOS) inhibitor attenuate age-related renal dysfunction and increasing blood pressure by inhibiting a pathophysiologic function of iNOS expression by activating NF-κB (*15, 16*).

Thus, we hypothesized that amla could be an effective modulator against oxidative stress-related alterations under aging and investigated the preventive effects of amla on the reduction of renal function by increasing oxidative stress during the aging process.

MATERIALS AND METHODS

Materials. The following reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan): 4,6-dihydroxy-2-mercaptopyrimidine [2-thiobarbituric acid (TBA)], bovine serum albumin (BSA), 2-amino-2-hydroxymethyl-1,3-propanediol [tris (hydroxymethyl) aminomethane], Tween 20, phenylmethyl sulfonyl fluoride (PMSF), protease inhibitor cocktail, and skim milk powder. Precision plus protein standards and the Bio-Rad protein assay kit were purchased from Bio-Rad Laboratories (Japan). Rabbit antihuman bax, mouse antimouse bcl-2, rabbit antihuman NF-κB p65 polyclonal antibody, rabbit antihuman inhibitor binding protein κB-α (IκB-α) polyclonal antibody, mouse antimouse NOS2 monoclonal antibody (primary antibody for iNOS), mouse antihuman cyclooxygenase-2 (COX-2) monoclonal antibody, goat antirabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody, and goat antimouse IgG HRP-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antimouse β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). ECL Western blotting detection reagents were purchased from Amersham Bioscience (Piscataway, NJ).

Preparation of Amla Extract. Two grades of amla extract have been used. The first one is a commercial product of amla; an enzymatic amla fruit juice extract, SunAmla (Taiyo Kagaku Co., Ltd., Japan), and the other one is a polyphenol-rich fraction of amla fruits derived from ethyl acetate (EtOAc) extraction. SunAmla is a dry powder of a water extract of amla fruit hydrolyzed by treatment with pectinase. The enzyme was reacted for 2 h at 55 °C, and then, the inactivated mixture was centrifuged. The spray-dried supernatant is the product named SunAmla. The EtOAc extract was prepared by extracting the air-dried amla fruit pieces in H_2O:EtOAc (1:4) at room temperature for 24 h. The extract was evaporated under low pressure followed by lyophilization. The yield of the extract was 12% by weight of the original material. The total polyphenol content was 80.4%. The composition of original fresh fruit was 81.2% moisture, 14.1% carbohydrate, 3.4% fiber, 0.63% mineral, 0.5% protein, 0.1% fat, 0.05% calcium, and 0.02% phosphorus.

Total Polyphenols and Vitamin C Contents of Amla Extract. Total polyphenol contents of SunAmla and EtOAc extract of amla were measured by a colorimetric method using gallic acid as a standard. The vitamin C content was measured using high-performance liquid chromatography (HPLC).

HPLC Analysis of Polyphenol Components in Amla Extract. The HPLC system (Waters Co., United States) was used for analysis of polyphenol components of amla extract. Samples were analyzed by the reverse phase column C18 Cosmosil AR II (25 cm $\times$ 0.4 cm, particle size; 5 μm, Nakarai Tesque Inc., Japan) using 50 mM phosphoric acid (A) and CH_3CN (B) as the solvent at a flow rate of 0.8 mL/min. The gradient used was 5% B in A solvent to reach 30% B during the first 39 min, and 75% B in A solvent at 54 min. Chromatograms were detected by 280 nm UV.

Animals and Treatments. The Guidelines for Animal Experimentation approved by the University of Toyama were followed during these experiments. Male Wistar rats (Japan SLC Inc., Hamamatsu, Japan) were used in this experiment. Aged rats were 10 months old and weighed 476 $\pm$ 15 g. Rats were kept in wire-bottomed cages under a conventional light regimen with a dark night at a room temperature of about 23 °C and a humidity of about 60%. Animals were given laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan; comprising 24.0% protein, 3.5% lipid, and 60.5% carbohydrate) and water ad libitum. Food consumption was measured daily, and body weight was recorded weekly. Twenty-one rats were divided into three groups and matched for their body weight and serum creatinine (Cr). The rats were fed a basal diet (CE-2) and treated with SunAmla extract or EtOAc extract of amla. The amla extract was dissolved in water and given orally to rats at a dose of 40 mg/kg body weight/day SunAmla and 10 mg/kg body weight/day EtOAc extract of amla for 100 days using a stomach tube. The contents of polyphenols were 11.8 and 8.0 mg/kg body weight/day, respectively. Control rats were given access to water alone. The oral dose was determined by the effective dose in our previous study (*9*). Six hours after the last dose, the rats were decapitated, their blood was drawn, and serum was collected by centrifuging the blood at 1000*g* for 15 min at 4 °C. The kidney and aorta were removed, dried on tissue paper, weighed, and stored at −80 °C until analysis. Two month old rats (n = 5) weighing 135 $\pm$ 3 g were used as a young group.

Measurement of Renal Function. The serum Cr and urea nitrogen levels were determined using the commercial reagents CRE-EN Kainos and BUN Kainos (Kainos Laboratories, Tokyo, Japan), respectively.

Measurement of Blood Pressure. At the end of the experiment, the blood pressure was measured by the tail-cuff method using an automatic blood pressure monitoring system (UR-5000, UETA, Tokyo, Japan). In brief, a tail cuff was used to constrict caudal artery flow, and photoelectric sensors detected the tail pulses, as the cuff pressure was reduced (*17*).

Isolation of Renal Mitochondria and Measurement of TBA-Reactive Substance Levels. Kidneys were homogenized with a nine-fold volume of ice-cold 0.9% NaCl solution. Mitochondria were prepared from renal homogenates by differential centrifugation (800*g* and 12000*g*, 4 °C, 15 min) according to the methods of Johnson and Lardy (*18*) and Jung and Pergande (*19*), respectively, with slight modifications. Each pellet was resuspended in preparation medium, and the TBA-reactive substance concentration was determined by the method of Buege and Aust (*20*). Briefly, 250 μL of resuspended pellet or working standard was added to 750 μL of TBA–TCA–HCl solution (0.4% of TBA, 15% of TCA, and 2.5% HCl), heated at 95–100 °C for 20 min, and cooled on an ice bath. Then, samples were centrifuged at 1000*g* at room temperature for 10 min to transfer supernatants from the denatured protein precipitate. TBA-reactive substance levels were determined by measuring the absorbance at 532 nm. The TBA-reactive substance value was expressed in nmol malondialdehyde (MDA)/mg protein by a calibration curve constructed from MDA (0–25 nmol/mL) in 1,1,3,3-tetramethoxypropane. The protein level was evaluated by the method of Itzhaki and Gill (*21*) with BSA as the standard.

Homogenization and Isolation of Cytosol and Nuclear Extracts. The kidney and aorta were homogenized at 4 °C in homogenization buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 1 mM dithiothreitol] and supplemented with protease inhibitor cocktail that consisted of 100 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 5 mM bestatin, 1 mM pepstatin A, and 1.5 mM E-64 (Sigma-Aldrich). Homogenates were incubated for 15 min on ice, added to 10% Nonidet P-40, and centrifuged at 4000*g*, 4 °C, for 5 min. For Western blot analysis, each sample (20 μg protein/lane) was denatured by boiling in Laemmli sample buffer and stored at −80 °C until the assay (*22*).

Nuclear extracts were isolated using the method of Sakurai et al. (*23*). Briefly, renal tissue was weighed and homogenized by a Potter Elvehjem homogenizer in 4 volumes (w/v) of buffer A containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and protease inhibitors. Homogenates were incubated for 15 min on ice, added to 10% Nonidet P-40, and centrifuged at 4000*g*, 4 °C, for 5 min. Supernatants were discarded, and pellets were resuspended in 2 volumes of buffer B containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors. Homogenates were kept for 15 min at 4 °C and then centrifuged at 14000*g* for 5 min at 4 °C. Nuclear extracts were collected in microcentrifuge tubes and stored in aliquots at −80 °C. The protein concentration of the nuclear extracts was determined by the Bio-Rad protein assay.

Table 1. Total Polyphenols and Vitamin C Contents of Amla Extract[a]

item	total polyphenols (%)	vitamin C (%)
SunAmla	29.4 ± 2.2	2.0 ± 0.2
EtOAc extract of amla	80.4 ± 4.3	

[a] Values are presented as the means ± SE of 10 replicate samples.

Western Blot Analysis. For Western blot analysis, the homogenates (30 μg for iNOS, COX-2, bax, and bcl-2), cytosol extract (30 μg for I-κBα), and crude nuclear extract (30 μg for NF-κB) from the kidney and homogenates (30 μg for iNOS, COX-2) from the aorta were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% wt/vol). The separated proteins were blotted onto nitrocellulose (Bio-Rad, Hercules, CA). Blots were blocked overnight at 4 °C with 5% nonfat dry milk in TBS-T (25 mM Tris-Cl, pH 8.3, 140 mM NaCl, 2 mM KCl, and 0.1% Tween 20). Membranes were then incubated for 3 h at 4 °C with the primary polyclonal antibody raised against NF-κB, I-κBα, bax (dilution 1:1000), and monoclonal antibodies against iNOS, COX-2, bcl-2 (dilution 1:1000), and β-actin (1:5000) (antibodies from Santa Cruz Biotechnology). After extensive washing, they were incubated with the second antibody (rabbit polyclonal or mouse monoclonal antibody) at a dilution of 1:1000 (Santa Cruz Biotechnology) for 40 min at room temperature. The specific protein was detected using the ECL chemiluminescence kit for horseradish peroxidase, evaluated by densitometry (Molecular Dynamics), and quantified by a Phosphor Imager (Bio-Rad Laboratories).

Statistical Analysis. Results are expressed as means ± standard errors (SE). The effect on each parameter was examined using one-way analysis of variance. Individual differences between groups were evaluated using Dunnett's test, and those at $p < 0.05$ were considered to be significant.

RESULTS

Total Polyphenols and Vitamin C Contents of Amla Extract. The total polyphenol content of SunAmla was about one-third of the EtOAc extract. SunAmla contained vitamin C at 2%, but it was not present in the EtOAc extract (**Table 1**).

Polyphenol Components of Amla Extract. Chromatograms of SunAmla and EtOAc extract of amla are shown in **Figure 1A**,**B**, respectively. Major components were gallic acid (peak A), ellagic acid (peak I), and ellagitannins. Ellagitannins were comprised of four compounds: corilagin, geraniin, elaeocarpusin, and chebulagic acid (peaks E, F, G, and H, respectively). Elaeocarpusin (peak G) could scarcely be detected in the EtOAc extract. Components of polyphenol compounds in SunAmla were as follows: 3.458% gallic acid, 0.656% corilagin, 0.880% geraniin, 0.659% elaeocarpusin, 1.240% chebulagic acid, and 0.220% ellagic acid, while components of polyphenol compounds in EtOAc extract were 5.847% gallic acid, 1.187% corilagin, 4.303% geraniin, 5.547% chebulagic acid, and 1.603% ellagic acid. Furthermore, several peaks besides the above components were detected; that is, mucic acid mono- and digallate and their mixtures including their isomers (eluted before peak A) were as follows: mucic acid 1,4-lactone 3-*O*-gallate (peak B), mucic acid 1,4-lactone 2-*O*-gallate (peak C), 1,6-di-*O*-galloyl-β-D-glucose (peak D), and furosin (peak J), as reported by Zhang et al. (*24*).

Body, Kidney Weights and Food Intake. Table 2 shows the changes in body and kidney weights via the oral administration of amla extract in the aged rats for 100 days. Body weight gain was slightly decreased in rats administered amla extract as compared with aged control rats but was not significantly different between the groups. Also, kidney weight and food intake showed no differences among groups.

Renal Function Parameters. The renal function parameters are summarized in **Figure 2**. The serum Cr and urea nitrogen levels were significantly increased in aged as compared with young rats ($p < 0.001$), whereas those levels were significantly reduced by amla extract administration, especially in the EtOAc extract of amla 10 mg/kg body weight/day ($p < 0.001$).

Blood Pressure. As shown in **Figure 3**, the systolic blood pressure was significantly elevated with age ($p < 0.001$). However, the systolic blood pressure levels in rats given SunAmla or EtOAc extract of amla were significantly decreased as compared with aged control rats. In addition, the mean blood pressure was significantly higher in aged control rats than in young rats ($p < 0.05$), whereas the levels were lower in rats given the EtOAc extract of amla as compared with aged control rats ($p < 0.01$). However, no significant change in the diastolic blood pressure was observed.

TBA-Reactive Substance Levels of Serum, Renal Homogenate, and Mitochondria. TBA-reactive substance levels of serum, renal homogenate, and mitochondria were significantly increased with age (**Table 3**). However, the administration of SunAmla or EtOAc extract of amla significantly reduced the TBA-reactive substance levels by 22 or 37% in the serum and by 25 or 13% in the renal homogenate as compared to aged control rats, respectively. The TBA-reactive substance levels in the renal mitochondria of rats administered SunAmla were markedly reduced.

Expression of iNOS and COX-2 Proteins in Aorta. Figure 4 shows the effects of amla on iNOS and COX-2 proteins in the aorta of aged rats administered amla extract. The iNOS protein level in aged control rats increased by 293% as compared with young rats ($p < 0.001$), whereas the oral administration of amla extract significantly decreased the level to 34% with SunAmla and 17% with EtOAc extract of amla as compared with aged control rats, respectively ($p < 0.001$). The COX-2 protein in aged control rats was not significantly changed as compared with young rats ($p < 0.01$), whereas the oral administration of EtOAc extract of amla significantly decreased the COX-2 protein level to 25% as compared with aged control rats ($p < 0.01$).

Expression of Bax and Bcl-2 Proteins in Kidney. To investigate the effects of amla extract on apoptosis in the kidney of aged rats, levels of bax and bcl-2, the apoptosis-specific proteins, were determined using Western blot analysis (**Figure 5**). Bax protein in aged control rats significantly increased by 11% as compared with young rats ($p < 0.001$). However, the administration of SunAmla 40 mg/kg body weight/day or EtOAc extract of amla 10 mg/kg body weight/day significantly decreased the bax protein level by 26 or 34% as compared with aged control rats, respectively ($p < 0.001$). However, bcl-2 protein was not significantly different among the groups.

Expression of Nuclear NF-κB and Cytoplasmic I-κBα Proteins in Kidney. Figure 6 represents the effects of amla extract on NF-κB and I-κBα protein levels in the kidney of aged rats. NF-κB protein in aged control rats significantly increased by 26% as compared with young rats ($p < 0.001$), whereas the oral administration of SunAmla or EtOAc extract of amla significantly decreased the NF-κB protein level as compared with aged control rats. The level of I-κBα protein in aged control rats was significantly elevated as compared with young rats. On the other hand, it was significantly decreased by the administration of SunAmla or EtOAc extract of amla.

Expression of iNOS and COX-2 Proteins in Kidney. As shown in **Figure 7**, the iNOS protein level in the renal homogenate of aged control rats increased by 45% as compared with young rats ($p < 0.001$), but it was significantly decreased

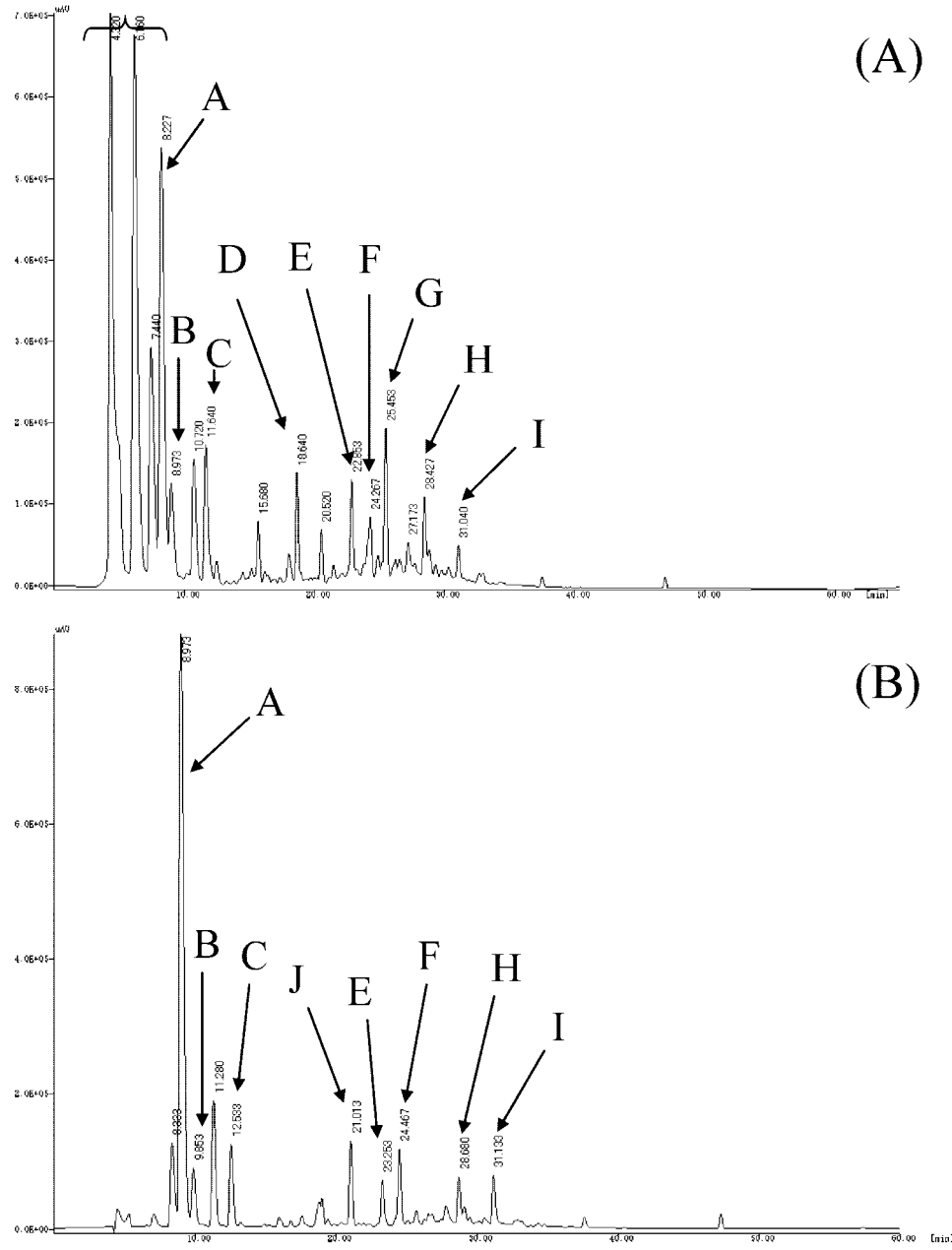

Figure 1. HPLC analysis of polyphenol components in SunAmla (**A**) and EtOAc extract of amla (**B**). Peaks: A, gallic acid; B, mucic acid 1,4-lactone 3-*O*-gallate; C, mucic acid 1,4-lactone 2-*O*-gallate; D, 1,6-di-*O*-galloyl-β-D-glucose; E, corilagin; F, geraniin; G, elaeocarpusin; H, chebulagic acid; I, ellagic acid; and J, furosin.

by the oral administration of SunAmla to 29% or the EtOAc extract of amla to 35% as compared with aged control rats, respectively ($p < 0.001$). The COX-2 protein level in aged control rats did not show significant differences as compared with that of young rats, while rats given the EtOAc extract of amla showed a significant decrease in the COX-2 protein level to 14% as compared with aged control rats ($p < 0.01$).

DISCUSSION

Kidney failure is predominantly a disease of older people. In humans and laboratory animals, the normal aging process is associated with progressive structural and functional alterations in the kidney, including glomerulosclerosis, tubulointerstitial fibrosis, decreases in renal blood flow and GFR, and changes in tubular functions that lead to defects in urinary concentration, dilution, acidification, and phosphate transport (*12*). In addition, the incidence of acute renal failure also increases with age (*25*). Several factors, including angiotensin II, advanced glycosylation end products, oxidative stress, and nitric oxide, may contribute to age-related renal diseases (*26, 27*). Leibovitz and Siegel (*28*) reported that free radical generation and lipid peroxidation are important factors in causing age-related renal dysfunction and injury. In addition, de la Lastra and Villegas (*29*) proposed that the antiaging action of antioxidants could retard the age-related increase of free radicals and renal dysfunction. An active extract and polyphenolic compounds from amla have been widely used to elicit antioxidant effects in a wide range of diseases. Thus, this study was conducted to investigate the effect of amla extract on renal dysfunction by oxidative stress in the aging process.

To examine renal function related to age, we measured serum Cr and urea nitrogen, which are used as indicators of renal dysfunction in clinical practice. GFR is also assessed

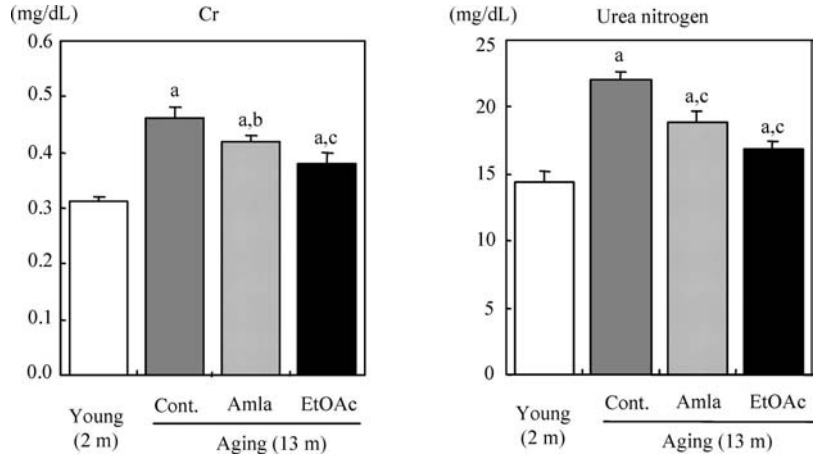

Figure 2. Renal function parameters. Values are the means ± SE. Significance: [a]$p < 0.001$ vs young rats; [b]$p < 0.01$ and [c]$p < 0.001$ vs aging control rats.

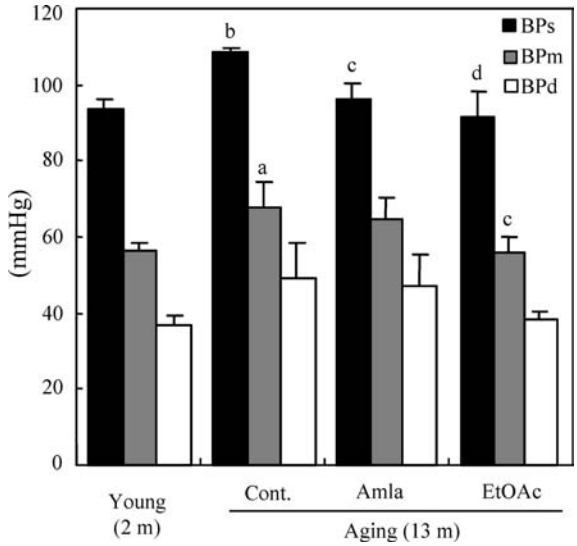

Figure 3. Blood pressure. Values are the means ± SE. Significance: [a]$p < 0.05$ and [b]$p < 0.001$ vs young rats; [c]$p < 0.01$ and [d]$p < 0.001$ vs aging control rats.

by measuring serum Cr. Our results showed an age-related increase of serum Cr and urea nitrogen. However, the oral administration of amla extract significantly decreased serum Cr and urea nitrogen levels as compared with aging control rats, especially with the EtOAc extract of amla (**Figure 2**). These results suggest that amla may ameliorate renal dysfunction in the aging process.

Hypertension is an important factor in the development and progression of renal dysfunction in the elderly. In addition, it is a well-established cause of chronic kidney disease and its prevalence increases with falling GFR. Kannel et al. (*30*) reported that systolic blood pressure and pulse pressure also increase with age. Thus, tight control of blood pressure is the most important intervention to slow the progression of chronic kidney disease. Furthermore, recent studies have shown that blood pressure control by the angiotensin-converting enzyme inhibitor, quinapril, or the AT1 receptor antagonist, losartan, decreases the degree of renal injury in animal models (*31*). Therefore, we determined the systolic blood pressure in this study. Our results also showed that systolic blood pressure was significantly elevated with age ($p < 0.001$). However, the systolic blood pressure levels in rats given SunAmla or EtOAc extract of amla were significantly decreased as compared with aged control rats (**Figure 3**). These results suggest that amla may be an effective blood pressure-lowering agent and protect against renal diseases resulting from hypertension.

It is well-known that reactive oxygen species (ROS)-mediated lipid peroxidation increases with age (*32*). In the kidney, the local synthesis of ROS, at least in experimental animals and cultured cells, seems to increase with age (*33*), and antioxidant treatment could prevent the morphological and functional aging-related renal changes (*34*). As shown by our present study, TBA-reactive substance levels of serum and renal mitochondria in aged rats were also significantly increased, whereas the administration of SunAmla or EtOAc extract of amla significantly decreased the TBA-reactive substance level as compared with aged control rats, respectively (**Table 3**). This reduction of the TBA-reactive substance level by amla extract could be explained as an antioxidant effect of amla extract, which contains polyphenols and vitamin C (**Table 1**).

iNOS and COX-2, proinflammatory enzymes, are known to be involved in the pathogenesis of many chronic diseases associated with oxidative stress. It has been reported that nitric oxide synthesis is markedly increased in spontaneously hypertensive rats (SHR) (*35*). In particular, several papers demonstrated that the expression of iNOS in the aorta was significantly greater in SHR (*36*). Vaziri et al. (*37*) reported that antioxidant therapy significantly ameliorates hypertension and partially reverses the upregulation of NOS isotypes in various tissues of hypertensive rats. In addition, peroxynitrite ($ONOO^-$) is known

Table 2. Body, Kidney Weights, and Food Intake[a]

group	dose (mg/kg BW/day)	body weight			kidney weight (g/100 g BW)	food intake (g/day)
		initial (g)	final (g)	gain (g/days)		
aging rats						
control		476.0 ± 17.6	489.9 ± 19.3	13.6 ± 4.8	0.51 ± 0.0	19.0 ± 0.4
SunAmla	40	476.3 ± 8.9	484.7 ± 22.2	8.4 ± 4.6	0.52 ± 0.0	20.0 ± 0.4
EtOAc extract of amla	10	476.3 ± 14.3	479.0 ± 23.8	11.0 ± 1.4	0.51 ± 0.0	19.9 ± 0.5

[a] Values are the means ± SE.

Table 3. Serum and Renal TBA-Reactive Substance[a]

group	dose (mg/kg BW/day)	TBA-reactive substance		
		serum (nmol/mL)	homogenate (nmol/mg protein)	mitochondria (nmol/mg protein)
young (2 months)		2.045 ± 0.096	42.41 ± 1.88	0.761 ± 0.001
aging (13 months)				
control		2.882 ± 0.443[b]	48.43 ± 2.47[c]	0.920 ± 0.010[d]
SunAmla	40	2.239 ± 0.219[e]	36.43 ± 1.40[c,g]	0.815 ± 0.032[f]
EtOAc extract of amla	10	1.827 ± 0.189[f]	42.18 ± 3.10[f]	0.840 ± 0.071[b,e]

[a] Values are the means ± SE. Significance: [b] $p < 0.05$, [c] $p < 0.01$, and [d] $p < 0.001$ vs young rats; [e] $p < 0.05$, [f] $p < 0.01$, and [g] $p < 0.001$ vs aging control rats.

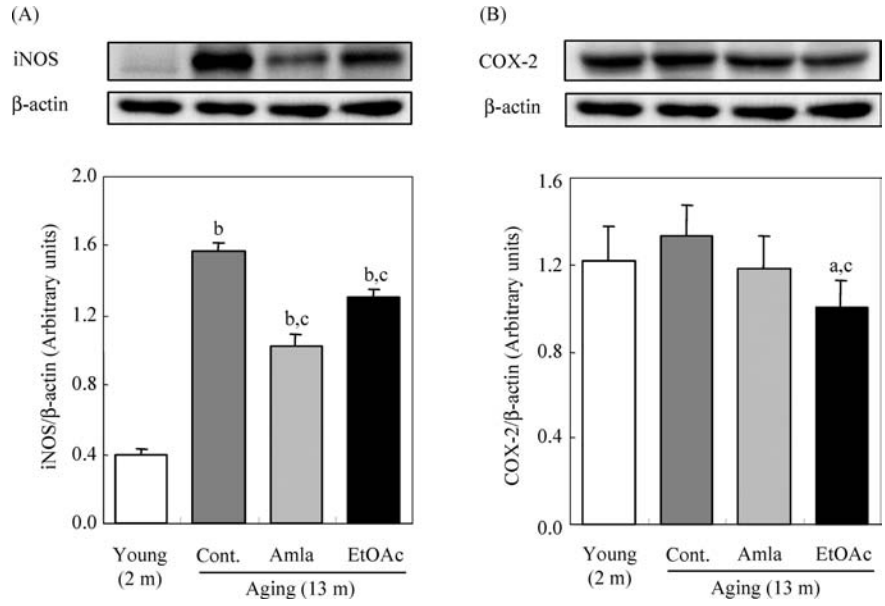

Figure 4. Western blot analysis of expressions of iNOS (**A**) and COX-2 (**B**) in the aorta. Values are the means ± SE. Significance: [a] $p < 0.05$ and [b] $p < 0.001$ vs young rats; [c] $p < 0.001$ vs aging control rats.

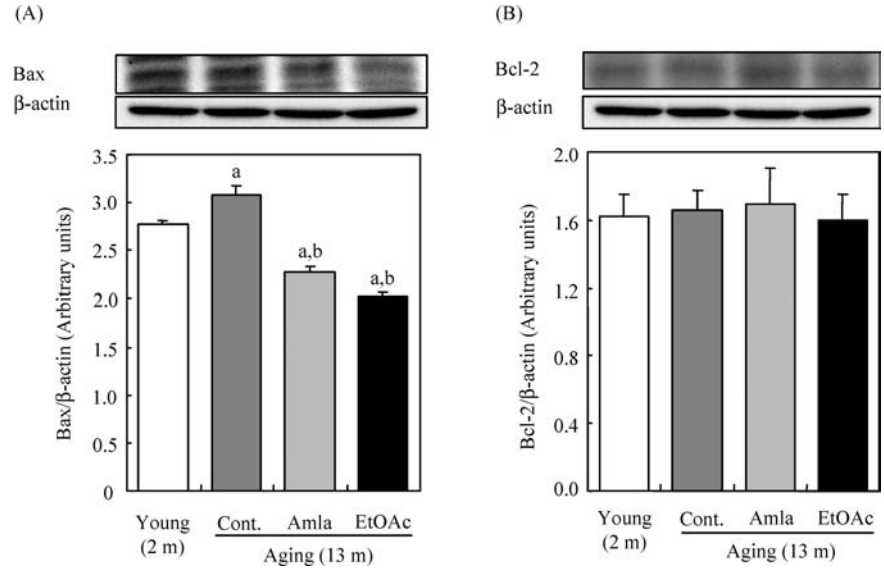

Figure 5. Western blot analysis of expressions of bax (**A**) and bcl-2 (**B**) in the renal homogenate. Values are the means ± SE. Significance: [a] $p < 0.001$ vs young rats; [b] $p < 0.001$ vs aging control rats.

to have important actions in cellular cytotoxicity and may also play an important role in mediating lipid peroxidation associated with systemic blood pressure and renal function in aging (*38*). Some reports showed that an antioxidant, an NF-κB inhibitor, aminoguanidine, and a selective iNOS inhibitor can prevent hypertension development (*15, 16, 39*). As shown by our present study, iNOS and COX-2 protein expressions in the aortas of aged rats were increased, and these expression levels were inhibited by the oral administration of 40 mg/kg body weight/day SunAmla or 10 mg/kg body weight/day EtOAc extract of amla, respectively, as compared with aged control rats (**Figure 4**). Particularly, amla extract markedly inhibits not only iNOS expression in aortic tissues but also increases in blood pressure (**Figures 3** and **4**). On the basis of these results, we found that iNOS and COX-2 levels in the aorta are increased in old animals, while amla extract decreased the elevated hypertension

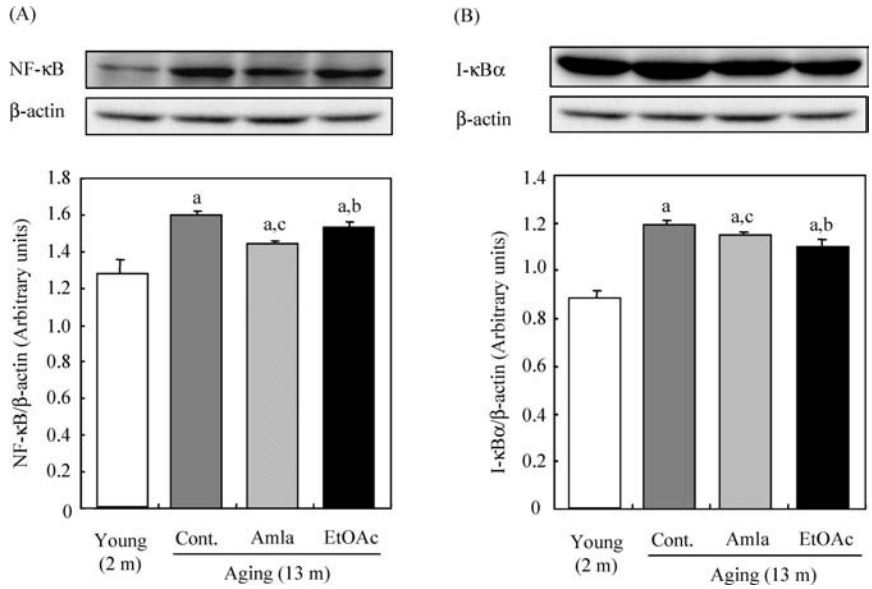

Figure 6. Western blot analysis of expressions of NF-κB (**A**) and I-κBα (**B**) in the renal nucleus and cytoplasm. Values are the means$\pm$ SE. Significance: $^{a}p < 0.001$ vs young rats; $^{b}p < 0.05$ and $^{c}p < 0.001$ vs aging control rats.

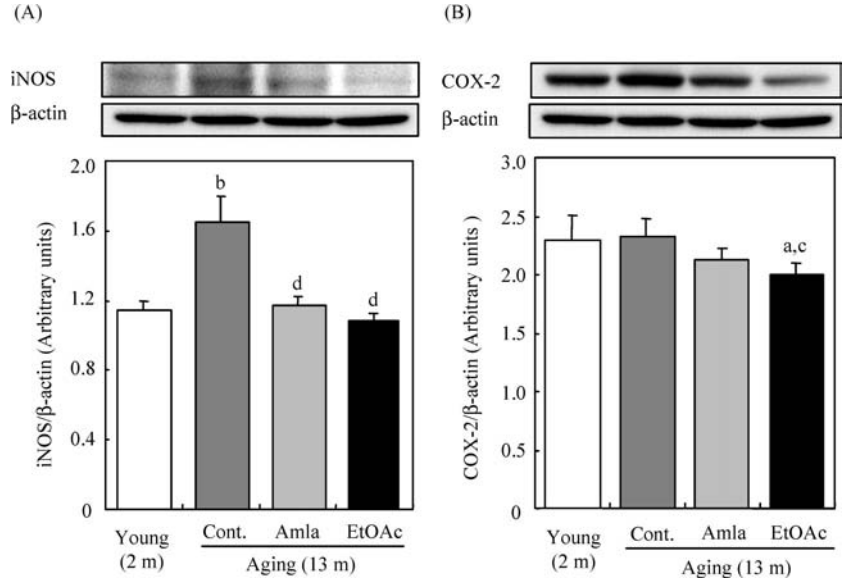

Figure 7. Western blot analysis of expressions of iNOS (**A**) and COX-2 (**B**) in the renal homogenate. Values are the means $\pm$ SE. Significance: $^{a}p < 0.05$ and $^{b}p < 0.001$ vs young rats; $^{c}p < 0.01$ and $^{d}p < 0.001$ vs aging control rats.

level in aged rats by inhibiting iNOS and COX-2 levels. Thus, our results provide evidence that amla extract suppresses the increase of blood pressure in aging rats by the inhibition of both aortic iNOS and COX-2.

Bcl-2 family proteins, antiapoptotic proteins, protect against cell death by acute oxidative stress. It has been demonstrated that bcl-2 overexpression preserves viability and lowers lipid peroxidation in cells exposed to oxidative stress (*40*). Conversely, some proapoptotic proteins such as bax, a mammalian cell death protein that targets mitochondrial membranes, can induce mitochondrial damage and cell death even when caspases are inactivated (*41*). However, several workers have reported that antioxidants slow down or block the apoptotic process by stabilizing mitochondrial functions (*42*). In our results, the bax protein level was significantly inhibited in the SunAmla and EtOAc extract-treated rats, while bcl-2 protein did not show any significant difference between aged rat groups (**Figure 5**). These results indicate that SunAmla and EtOAc extract of amla have protective effects against cell death by age-related oxidative stress in the kidney.

Studies have shown that the activation of redox-sensitive NF-κB plays a pivotal role in modulating the cellular signaling mechanism for oxidative stress-induced inflammation during aging. NF-κB is an ubiquitous transcription factor known to be activated by a wide variety of stimuli including infection, inflammation, and oxidative stress (*43*). Chronic oxidative stress and inflammatory reactions also lead to many age-associated diseases, and the aging process enhances the activation of NF-κB by downregulating I-κBα (*44*). Under resting conditions, NF-κB exists in the cytoplasm as a dimer bound to the inhibitory protein I-κBα. Inducers of NF-κB, such as inflammatory cytokines, ROS, and viral products, activate a dimeric I-κBα kinase complex, causing the phosphorylation and ubiquitination of I-κBα and its release from NF-κB. Free NF-κB dimers translocate to the nucleus, where they regulate target gene transcription such as iNOS, COX-2, interleukin (IL)-6, IL-12,

and tumor necrosis factor-α (*45*). Transcription factors that are directly influenced by ROS and proinflammatory cytokines include NF-κB, AP-1, and other members of the nuclear receptor superfamily involved in aging (*46*). In the present study, we also measured NF-κB and I-κBα protein levels in the kidney during aging. Nuclear NF-κB protein in the kidney of aged control rats increased as compared with young rats, whereas the oral administration of amla extract decreased the elevated NF-κB protein level as compared with aged control rats. However, the cytoplasmic I-κBα level was not reduced with aging, and also, the oral administration of amla extract decreased the protein level, indicating that there might be another mechanism regulating the I-κBα protein level during aging (**Figure 6**). These results imply that amla protects against oxidative stress by inhibiting NF-κB activation during aging.

Modulation of this process may prevent or at least attenuate the age-related changes in the kidney and oxidative stress. Many investigators have shown that antioxidants slow the aging process and prevent age-related disease. Thus, we assessed the contributory role of iNOS and COX-2 to investigate the effect of amla on oxidative stress in the aging process. In our present study, old age induced the overexpression of iNOS protein, and this age-related change was inhibited by the oral administration of amla extract. In addition, the COX-2 protein level did not show any significant difference between young and aged control rats, but it was significantly decreased by the administration of EtOAc extract of amla, implying that the polyphenol-rich extract of amla is more effective than SunAmla (**Figure 7**). Therefore, these findings suggest that amla extract can act as an antioxidant to alleviate age-related oxidative stress by the inhibition of both iNOS and COX-2.

Our data also show that the inhibitory effects against age-related renal dysfunction and blood pressure-lowering effect of amla were stronger with EtOAc extract of amla, a polyphenol-enriched extract, than SunAmla. Moreover, polyphenolic compounds found in various plants are involved in the downregulation of inflammatory responses through inhibiting iNOS and COX-2 via their inhibitory effects on NF-κB or AP-1 in antiinflammatory and antiaging mechanisms (*29*). From these results, we speculate that the attenuating activity of renal dysfunction and hypotensive activity of amla in this study could be due to its antioxidant effect derived from the polyphenolic compounds. The polyphenolic portion of amla extract is comprised of gallic acid, ellagic acid, and ellagitannins. Ellagitannins are divided into corilagin, geraniin, elaeocarpusin, and chebulagic acid. The bioactivity of each compound could be further investigated.

In conclusion, our results demonstrated that age-related renal dysfunction is mediated by increases of oxidative stress and blood pressure in aging rats. However, the administration of amla extract to aged rats attenuates this age-related renal dysfunction by inhibiting the blood pressure rise due to the inhibition of iNOS and COX-2 expression and oxidative stress due to the inhibition of NF-κB transcriptional pathways. These findings suggest that amla may be beneficial in preventing age-related kidney diseases.

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