

## Antioxidant Activities of Uyaku (*Lindera Strychnifolia*) Leaf Extract: A Natural Extract Used in Traditional Medicine

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**Summary** Uyaku (*Lindera strychnifolia*, Sieb. et Zucc.) is used in traditional Asian medicine to treat stomach and renal diseases, neuralgia, rheumatism, and aging. In this study, the effects of lyophilized extracts on hydroxyl ( $\cdot\text{OH}$ ) and superoxide ( $\text{O}_2^{\cdot-}$ ) radicals were examined using an electron spin resonance (ESR) spectrometer with the spin trap, 5,5'-dimethyl-1-pyrroline-N-oxide. Inhibitory effects were assessed using the following reagents: for nitric oxide ( $\text{NO}\cdot$ ), the Griess reagent; for ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2$ )-induced lipid peroxidation, 2-thiobarbituric acid; for ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2$ )-induced protein carbonyl, 2,4-dinitrophenylhydrazine. Analysis of ESR data of the extracts indicated the direct  $\cdot\text{OH}$  and  $\text{O}_2^{\cdot-}$  scavenging. The extracts scavenged  $\text{NO}\cdot$  in a dose-dependent manner, inhibited lipid peroxidation of linolenic acid, and protein carbonyl formation in bovine serum albumin. In conclusion, the Uyaku leaf hot-water extract has potent scavenging activity against reactive oxygen species and reactive nitrogen species, and effectively inhibited lipid peroxidation. These results might contribute to understanding age-associated or free radical-related diseases induced by excess reactive oxygen and also nitrogen species.

**Key Words:** Uyaku, natural antioxidant, lipid peroxidation

### Introduction

Uyaku is the common name for *Lindera strychnifolia* (Sieb. et Zucc.) F. Villar in the Lauraceae family [1–5]. The use of Uyaku roots is especially important in the traditional phamacopeia of China and Japan for treating kidney deficiencies such as pollakisuria or urinary incontinence [2, 3]. Other well-known uses are; as an astringent, carminative, stomachic, tonic; and as protection against asthma, cholera, congestion, dyspepsia, dysmenorrhea, fluxes, gonorrhoea, hernia, malaria, menorrhagia, stomach ache, stroke, and

urinary difficulties; as treatment for apoplexy, backache, cancer, gastritis, gastric ulcers, leg ache, polyuria, rheumatism [5]. Uyaku is also thought to have beneficial effects for prevention of aging in general in legendary, though it is not yet clarified.

Moreover, its fruits are used for treating abdominal distention, dysuria, edema, fungus infections, scabies, and worms; and its seeds are used for antipyretics [5]. Clearly this plant has many potential uses that are worthy of modern scientific investigation.

The mechanisms of Uyaku's effectiveness are not known. In the 1990's, many compounds were isolated from roots of Uyaku, and their structures were determined. These include: linderol (borneol), linderane, linderasure [6–11], lindesterene, linderene acetate [12], isolinderoxide [13], linderoxide [14], sesquiterpene lactones [15], bisquiterpene

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[16], strychnilactone [17], strychnistenolide and its acetate [18], and alkaloids [19, 20]. The well-known Uyaku compounds mentioned above, and other sesquiterpenes such as isolinderalactone, neolinderalactone, lindestrenolide lindenone, linderazulene, chamazulene, laurilitsine, isogermafirene have been summarized in previous other studies [2–5]. Recent studies demonstrated that Uyaku extract has antiviral activity [21], an inhibitory effect on prolyl endopeptidase [22], and is associated with anticancer activity in lungs [23]. It has also a potentially protective effect against post-ischemic myocardial dysfunction [24], and diabetic nephropathy [25]. In a previous study, we examined whether the water-soluble components of Uyaku leaves have antioxidant activity, and found that root and leaf extracts by hot-water extraction have specific and potent superoxide anion radical scavenging activity as well as several other natural extracts [26]. This is a potentially rich line of research that should be pursued.

In the present study, we examined the effects of hot-water extract of Uyaku leaves on reactive oxygen species (ROS), reactive nitrogen species (NOS), lipid peroxidation, and protein oxidation.

## Materials and Methods

### Sample preparation

Uyaku (*Lindera strychnifolia*, Sieb. et Zucc.) leaves were obtained from Shingu-City, Japan. The leaves were freshly harvested through July to October and raw leaves were stored at 4°C in a sealed plastic bag to eliminate exposure to air; i.e., the leaves were not artificially dried. Uyaku leaves were minced or ground using a porcelain mortar and pestle. The leaves were boiled in water (1:20, w/v) for 10 min, and the sample suspension was filtered (Whatman filter paper No. 2). The filtrate was condensed by a rotary evaporator *in vacuo* and freeze-dried. Freeze-dried samples were stored at 4°C until use. For analysis, the sample solution was prepared just before use. The freeze-dried sample was dissolved in 0.1 M potassium phosphate buffer (pH 7.4) just before use.

### Reagents

Xanthine oxidase (1 U/mg, from cow milk) was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). The spin trap, 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Labotec Co. (Tokyo, Japan). 1-Hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) was obtained from Alexis Corp. (San Diego, CA). Sodium dodecyl sulfate was obtained from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl-2H-1-benzopyran-6yl hydrogen phosphate)] potassium salt (EPC-K<sub>1</sub>), of which compound represents phosphate diester linkage of vitamin E and vitamin C, was a kind gift from Dr. Kazumi Oga

(Oga-Research, Osaka, Japan). All other chemicals used were the highest grade available. Linolenic acid was used as a standard reagent for lipid peroxidation. Bovine serum albumin (BSA; M.W. 66,430, Sigma #A-7030) was used for protein carbonyl formation.

### Electron spin resonance (ESR) spectrometry

An ESR spectrometer (JES-FR30; JEOL, Tokyo, Japan) equipped with manganese oxide (MnO) as an internal standard was used for measuring  $\cdot\text{OH}$  and  $\text{O}_2^{\cdot-}$  scavenging activities.

### ESR settings

The conditions for ESR were as follows: magnetic field:  $335.6 \pm 5$  mT; power: 4 mW; modulation frequency: 9.41 GHz; modulation amplitude:  $1 \times 0.1$  mT; response time: 0.1 sec; amplitude:  $1 \times 200$ ; sweep width: 5.000 mT; sweep time: 2 min; temperature: 23°C. Methods for the spin trapping of  $\cdot\text{OH}$  or  $\text{O}_2^{\cdot-}$  radicals were based on previous studies [27–29].

### Hydroxyl radical ( $\cdot\text{OH}$ ) scavenging activity

All solutions except for  $\text{FeSO}_4$  were dissolved in 0.1 M potassium phosphate buffer (pH 7.4);  $\text{FeSO}_4$  was dissolved in distilled water. Fifty microliters each of the sample solution, 18 or 1.8 mM DMPO (final concentration: 4.5 or 0.45 mM), 2 mM  $\text{H}_2\text{O}_2$ , and 0.2 mM  $\text{FeSO}_4$  were mixed and placed into the ESR quartz flat cell (200  $\mu\text{l}$  capacity). Exactly 30 s after the addition of  $\text{FeSO}_4$ , the ESR spectra of the DMPO-OH spin adducts were recorded [30, 31].

### Superoxide anion radical ( $\text{O}_2^{\cdot-}$ ) scavenging activity (SOD-like activity)

All solutions except dimethyl sulfoxide (DMSO) were dissolved in 0.1 M potassium phosphate buffer (pH 7.4) just before use. Fifty microliters of 4 mM hypoxanthine, 30  $\mu\text{l}$  of DMSO, 50  $\mu\text{l}$  of sample solution, 20  $\mu\text{l}$  of 4.5 or 0.45 M DMPO (final concentration: 450 or 45 mM), and 50  $\mu\text{l}$  of xanthine oxidase (0.4 U/ml) were mixed and transferred into the ESR quartz flat cell. In this assay, DMSO was included because it diminish the DMPO-OH signal for  $\cdot\text{OH}$ , but not the DMPO-OOH signal for  $\text{O}_2^{\cdot-}$ . Exactly 30 s after adding xanthine oxidase, the ESR spectra of the DMPO-OOH spin adducts were recorded [30, 31].

### Flow injection analysis (FIA) of the inhibitory effect of Uyaku leaf extract on nitric oxide ( $\text{NO}$ ) generation

Various concentrations of sample solution (480  $\mu\text{l}$ ) were transferred into 1.5-ml microcentrifuge tubes, and 0.1 M potassium phosphate buffer (pH 7.4; 600  $\mu\text{l}$ ) and 0.2 mM NOC-7 (120  $\mu\text{l}$ ) in 0.1 M NaOH were added to the each tube. All microcentrifuge tubes were rinsed three times with pure water just before use. The pH of the solution was measured using pH test paper. Immediately after adding

NOC-7, the mixture was incubated with gentle shaking for up to 45 min at 23°C. At each time point (0, 15, 30, and 45 min), an aliquot (200  $\mu$ l) was injected into a flow injection analyzer equipped with a cadmium-copper reduction column (TCI-NOX 1000, Tokyo Kasei Kogyo, Tokyo) to measure the reaction product of the Griess reagent and total  $\text{NO}_2^-$ , which was converted by a reduction column from the stable end product of  $\text{NO}^\bullet$  oxidation (545 nm) [32]. Non-specific absorbance was corrected by measurement of the reaction mixture in the absence of NOC-7 for each concentration of sample solution, because the color of the sample solution itself slightly increased and affected the absorbance as concentration increased.

#### *Inhibitory effect of Uyaku leaf extract on ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2$ )-induced lipid peroxidation in linolenic acid*

Linolenic acid was used as a standard. The measurements were obtained according to Knight *et al.* [33, 34] with slight modification. Briefly, 2 % (w/v) sodium dodecylsulfate (400  $\mu$ l), 2 mM linolenic acid dissolved in 2 % sodium dodecylsulfate (200  $\mu$ l), the sample solution in buffer (pH 7.4; 200  $\mu$ l), and 1 mM  $\text{FeSO}_4$  in distilled water (200  $\mu$ l) were put into a test tube. The control solution comprised linolenic acid and the sample. The samples were incubated for 24 h at 37°C with gentle shaking. After the incubation, diluted  $\text{H}_3\text{PO}_4$  (1:2000, v/v; 3 ml) and 0.75 % (w/v) 2-thiobarbituric acid (1 ml) were added to the sample, and the reaction mixture was heated in boiling water for 1 h. After cooling to room temperature, an aliquot (1 ml) was added to a NaOH-methanol solution (1 ml) to neutralize, and the absorbance of thiobarbituric acid reactive substances (TBARS) was measured at 532 nm using a spectrophotometer [33–35].

#### *Inhibitory effect of Uyaku leaf extracts on ( $\text{Fe}^{2+} + \text{H}_2\text{O}_2$ )-induced protein carbonyl formation in bovine serum albumin (BSA)*

Bovine serum albumin was used as the standard. The levels of protein carbonyl was measured by the reaction of protein carbonyl in BSA with 2,4-dinitrophenylhydrazine [36, 37] with slight modification. Briefly, bovine serum albumin in buffer (protein content: 5 mg; 400  $\mu$ l), the sample solution (10  $\mu$ l), 42 mM  $\text{H}_2\text{O}_2$  (10  $\mu$ l), and 4.2 mM  $\text{FeSO}_4$  (10  $\mu$ l) were incubated in a 2-ml microcentrifuge tube for 15 min at 37°C. The control solution comprised bovine serum albumin and the sample. After incubation, 215 mM EDTA solution (10  $\mu$ l) and 10 mM 2,4-dinitrophenylhydrazine (10  $\mu$ l) or 2 M HCl (control) were added, and incubated for 30 min at 23°C. The reaction was terminated by adding 30% (w/v) trichloroacetic acid (400  $\mu$ l), and the suspension was centrifuged (11,000  $\times$  g for 10 min). The pellet was washed twice with ethanol-ethyl acetate (1:1, v/v; 1 ml) by centrifugation and recovered. The pellet was dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate,

adjusted to pH 2.3 with trifluoroacetic acid (1 ml) using a bath-type ultrasonic generator, and the protein carbonyl content was measured using a spectrophotometer (370 nm; a molar absorption coefficient: 22,000  $\text{M}^{-1} \text{cm}^{-1}$ ) [36, 37].

## Results

The yields of freeze-dried extracts (percentages of freeze-dried extract to raw material of leaves) were approximately 13% to 15% for hot-water extracts.

#### *Hydroxyl radical ( $\text{OH}^\bullet$ ) scavenging activity*

The leaf extract showed dose-dependent  $\text{OH}^\bullet$  scavenging activity. A representative dose-response curve for hot-water extract is shown in Fig. 1. To examine the radical scavenging activity, 4.5 mM DMPO (final concentration) and a 10-fold lower concentration of DMPO (0.45 mM, final concentration) were used. The  $\text{ID}_{50}$  value of hot-water extract decreased 10-fold when a 10-fold lower concentration of DMPO was used. The  $\text{ID}_{50}$  value was 1.0 mg/ml when 4.5 mM DMPO (final concentration) was used. In comparison, under the same experimental condition, the  $\text{ID}_{50}$  value of Trolox (water-soluble vitamin E) was 0.3 mg/ml (1.2 mM) and the  $\text{ID}_{50}$  value of ascorbate was 2  $\mu$ g/ml (11  $\mu$ M).

#### *Superoxide anion radical ( $\text{O}_2^{\bullet-}$ ) scavenging activity (SOD-like activity)*

The hot-water extract scavenged  $\text{O}_2^{\bullet-}$  in a dose-dependent manner. The  $\text{ID}_{50}$  value of hot-water decreased 10-fold when

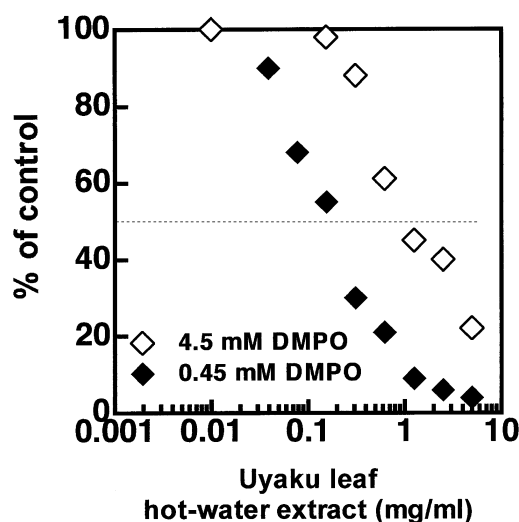


Fig. 1. Representative dose-response curve of hydroxyl radical scavenging activity for Uyaku leaf hot-water extract. The data were obtained by two separate experiments. The symbols of the opened diamond and the closed diamond represent the data points obtained by using 4.5 mM and 0.45 mM DMPO (each final concentration), respectively.

a 10-fold lower concentration of DMPO was used. The typical ESR spectra and dose-response curve for the hot-water extract are shown in Fig. 2a and b, respectively. The  $ID_{50}$  value of hot-water extract was 0.01 mg/ml when 450 mM DMPO (final concentration) was used.

#### *Inhibitory effect on $NO\cdot$ generation*

The hot-water extract showed a dose-dependent  $NO\cdot$  scavenging activity (Fig. 3). The sample solution itself was slightly yellow at higher concentrations (up to 10 mg/ml),

and color varied in a dose-dependent manner; therefore, the observed absorbance was corrected by subtracting the control values (for each concentration of the sample). The  $ID_{50}$  values were approximately 2 to 3 mg/ml.

#### *Inhibitory effect on $(Fe^{2+} + H_2O_2)$ -induced lipid peroxidation in linolenic acid*

The inhibitory effects of hot-water extracts on lipid peroxidation are summarized in Fig. 4. The hot-water extract inhibited lipid peroxidation in a dose-dependent

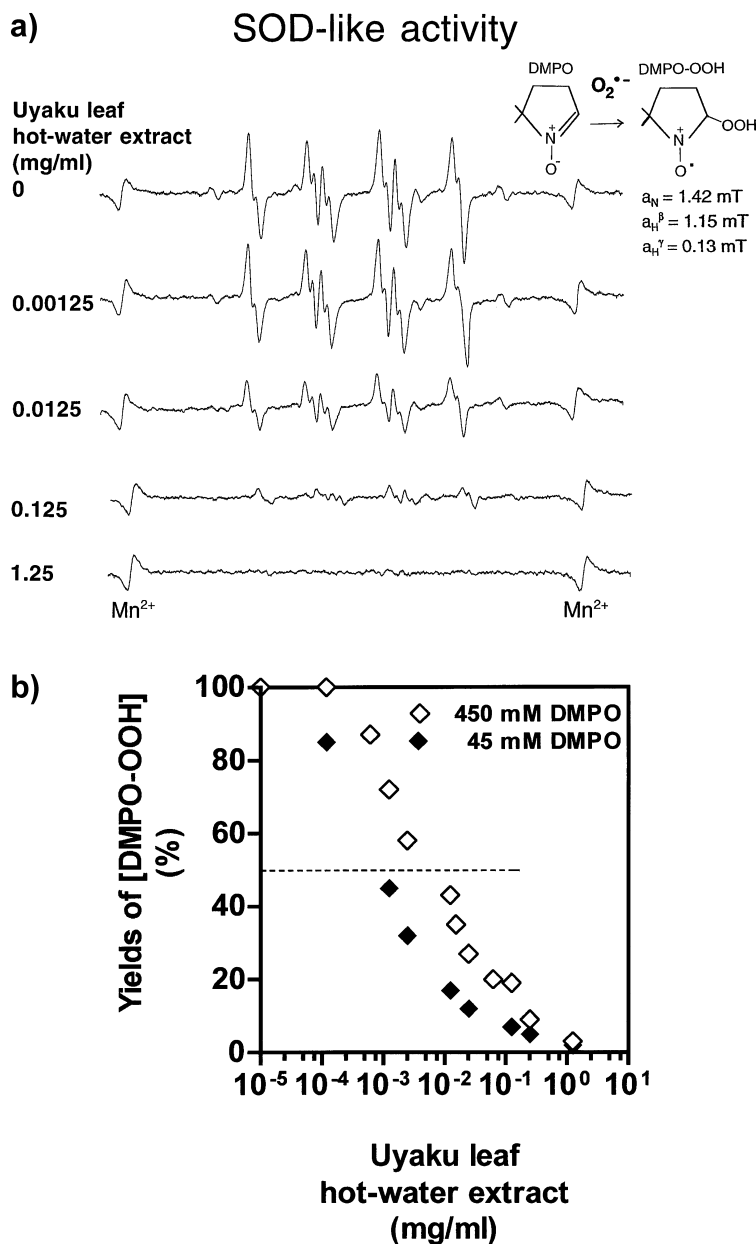


Fig. 2. a) Typical ESR spectra of DMPO-OOH at the various concentration of Uyaku leaf extract. The hyperfine coupling constants of spin adducts, DMPO-OOH were  $a_N = 1.42 \text{ mT}$ ,  $a_{H\beta} = 1.15 \text{ mT}$ , and  $a_{H\gamma} = 0.13 \text{ mT}$ . b) A dose-dependent curve of SOD-like activity for Uyaku leaf hot-water extract. The data were obtained by two separate experiments. The symbols of the open diamond and the closed diamond represent the data points obtained by using 450 mM and 45 mM DMPO (each final concentration), respectively.

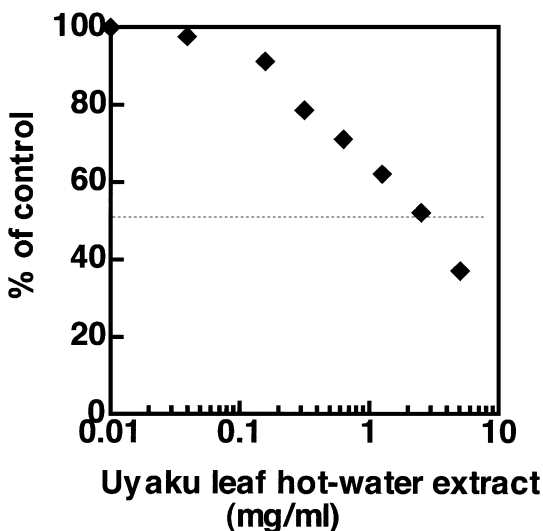


Fig. 3. Inhibitory effect of Uyaku leaf hot-water extract on NO generation. The data points represent 30 min after addition of NOC-7 (20  $\mu$ M). The reproducibility was confirmed by two separate experiments.

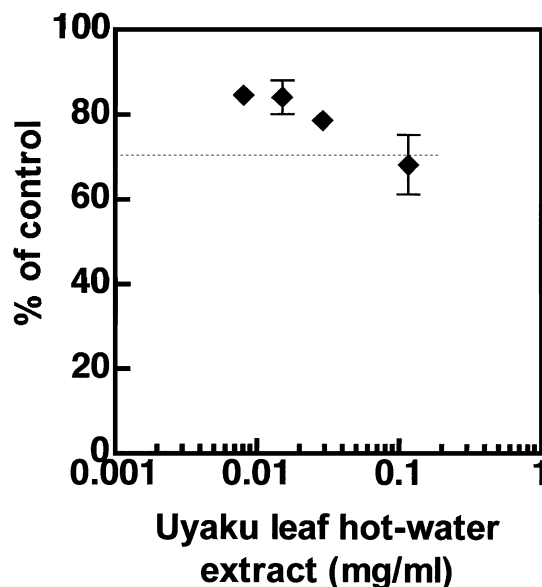


Fig. 5. Inhibitory effect of Uyaku leaf hot-water extract on protein carbonyl formation in bovine serum albumin. The data was obtained from three separate experiments.

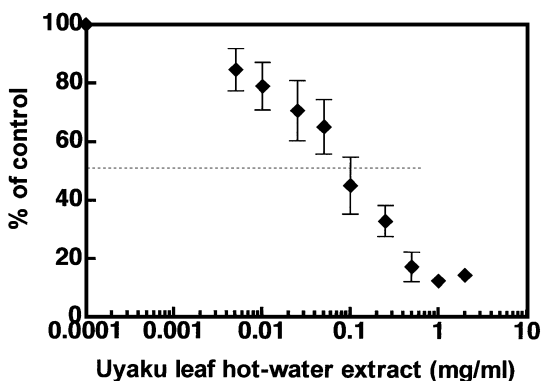


Fig. 4. Inhibitory effect of Uyaku leaf hot-water extract on lipid peroxidation in linolenic acid. The data was obtained from six separate experiments.

manner. The  $ID_{50}$  value was 0.08 mg/ml. In our experimental condition, for comparison, known antioxidants were also examined; the  $ID_{50}$  value of EPC-K<sub>1</sub> was 0.09 mg/ml (0.13 mM).

#### Inhibitory effect on ( $Fe^{2+} + H_2O_2$ )-induced carbonyl formation in bovine serum albumin (BSA)

The hot-water extracts slightly inhibited protein carbonyl formation in BSA in a dose-dependent manner (Fig. 5). Therefore, the precise  $ID_{50}$  value could not be obtained in this assay. At a dose of 0.12 mg of hot-water extract/ml during the oxidation using ( $Fe^{2+} + H_2O_2$ ) system, the percentage value of inhibition was 30% when compared to the control (without Uyaku). The protein carbonyl formation in BSA

(control: without Uyaku extract) was approximately 3.6 n moles of carbonyl groups/mg of protein. The sample solution was slightly yellow; therefore, the values of absorbance were corrected by subtracting the control values (without 2,4-dinitrophenylhydrazine for each concentration of the sample).

#### Discussion

The Uyaku hot-water extracts scavenged  $\cdot OH$  in a dose-dependent manner. When a 10-fold lower DMPO concentration (0.45 mM, final concentration) was used, the  $ID_{50}$  values also decreased 10-fold, indicating that these extracts directly scavenge  $\cdot OH$  when the extract encounters  $\cdot OH$  radicals; i.e., due to the direct scavenging activity but not to be due to the inhibition of  $\cdot OH$  generation system, for example, not by chelation with iron ions.

The superoxide anion radical ( $O_2^{\cdot -}$ ) scavenging activity of hot-water extract prepared from fresh wet Uyaku leaves was similar to the scavenging activity of dry Uyaku leaves when the activity is expressed in units of weight of the freeze-dried extract.

The Uyaku extracts showed strong SOD-like activity. The direct scavenging activity was confirmed by analyzing ESR spectra with different concentrations of the spin trapping agent; i.e., when a 10-fold lower concentration of DMPO (45 mM, final concentration) was used, the  $ID_{50}$  values decreased 10-fold [38].

The scavenging activity of the hot-water extract corresponded to approximately 100 SOD-equivalent U/mg of

freeze-dried extract. In preliminary experiments, Uyaku leaves were minced into small pieces. For comparison, the minced leaves were ground using a porcelain mortar and pestle, and both preparations were used to produce hot-water extracts. Extracts from both preparations had similar  $O_2^{\cdot-}$  scavenging activity, though these activities may vary depending upon other conditions, such as season of harvest, and preservation.

Our previous study demonstrated that relatively smaller molecular components (M.W.<10000) contribute to the strong  $O_2^{\cdot-}$  scavenging activity [26]. The hydrophilic compounds that contribute to the strong activity have not been identified.

The results of the present study, together with the earlier findings described above strongly suggest that the most productive focus for further research would be a comprehensive analysis of the chemical compounds in the Uyaku hot-water extract.

In our preliminary experiments,  $NO^{\cdot}$  scavenging activity was examined by an ESR with the spin trapping  $[(MGD)_2Fe^{2+}]$ -complex. In this case, however, the extract itself affected the ESR signal, although the reason is not yet clarified; apparently enlarged the relative peak height of  $[(MGD)_2Fe^{2+}]-NO^{\cdot}$ , so that the activity could not be evaluated. Therefore, a flow injection analysis with the Griess reagent was used to determine the  $NO^{\cdot}$  scavenging activity of the extract.

Uyaku leaf hot-water extract inhibited  $(Fe^{2+} + H_2O_2)$ -induced lipid peroxidation in linolenic acid in a dose-dependent manner. Taken together with our ESR analysis, these results suggest that the inhibitory effect against lipid peroxidation would most likely be due to the scavenging capacity against  $^{\cdot}OH$ , which were generated by Fenton reaction and known to be potent oxidant to initiate lipid peroxidation, because in our ESR analysis the hot-water extract showed direct  $^{\cdot}OH$  scavenging activity upon encounter to  $^{\cdot}OH$  radicals.

Uyaku leaf hot-water extract showed  $(Fe^{2+} + H_2O_2)$ -induced protein carbonyl formation in this system; i.e., metal  $(Fe^{2+}/Fe^{3+})$ -catalyzed oxidation of protein by reactive oxygen species mainly such as  $^{\cdot}OH$  and maybe some other active oxygen (ferryl; perferryl ion) [37] at metal-binding sites such as lysine residue ( $-CH_2NH_2$ ) to produce the end product of protein oxidation, carbonyl ( $RC=O$ ) derivatives [37]. Under this experimental condition, this inhibitory effect probably, at least in part, due to the scavenging capacity against  $^{\cdot}OH$  rather than iron-chelating effect.

In summary, the hot-water extract of Uyaku leaves has potent scavenging activity of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and inhibits lipid peroxidation and protein oxidation. Further studies are needed to identify the compounds responsible for the antioxidant activities. Uyaku has long been used for the treatment of stomach and renal diseases, uralgia, rheumatism, and aging

as folk or traditional medicine, although little is known about the chemical properties of its leaves. The present experimental results suggest that, because of its free radical scavenging activity, Uyaku extract might be beneficial for protection against diseases related to the free radicals.

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