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Reactive Oxygen Species Scavenging Activity of *Jixueteng* Evaluated by Electron Spin Resonance (ESR) and Photon Emission

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Jixueteng, the dried stem of *Spatholobus suberectus* Dunn (Leguminosae), is a traditional Chinese herbal medicine that is commonly classified as a herb that promotes blood circulation and can be used to treat blood stasis. The aim of this study was to examine the reactive oxygen species (ROS) scavenging activity of *Jixueteng* and other herbal medicines. The ROS scavenging activities of the water extracts of *Jixueteng*, *Cnidium officinale* and *Salvia miltiorrhiza* were examined using an electron spin resonance (ESR) technique and faint luminescence measurement. The ESR signal intensities of the superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical (HO$^\bullet$) were reduced more by *Jixueteng* than the other herbal medicines we tested. High photon emission intensity to hydrogen peroxide (H_2O_2) and HO$^\bullet$ was observed in *Jixueteng* using the XYZ chemiluminescence system that was used as faint luminescence measurement and analysis. The results of the present study revealed that the ROS scavenging activity of 8% *Jixueteng* was the strongest among the herbal medicines we tested. It has been reported that *Jixueteng* includes various polyphenols. In the ROS scavenging activity by *Jixueteng*, it is supposed that the antioxidant activity caused by these polyphenols would contribute greatly. In conclusion, a water extract component of *Jixueteng* had potent free radical scavenging activity and an antioxidative effect that inhibited the oxidative actions of $O_2^{\bullet-}$, H_2O_2 and HO$^\bullet$. Therefore, *Jixueteng* represents a promising therapeutic drug for reactive oxygen-associated pathologies.

Keywords: *Jixueteng*, Herbal medicine, Reactive oxygen species scavenging activity, XYZ chemiluminescence system, Electron spin resonance

Jixueteng is a herbal medicine that possesses pharmacological properties, such as improving the circulation, analgesia, and increasing the number of white and red blood cells, and is composed of the dried stems of *Spatholobus suberectus* Dunn and *Millettia dielsiana* Harms, both family Leguminosae [1-5]. We recently reported the potent local anti-infection effects of *Jixueteng* on oral indigenous bacteria and also demonstrated that it inhibited alveolar bone loss [6,7]. These findings suggested that *Jixueteng* may be used as a safe and effective therapeutic agent for periodontal disease through various approaches because of its anti-bacterial and immune activities, in addition to its ability to improve the circulation. However, *Jixueteng* has not yet been clinically applied to the oral field, and its effects on reactive oxygen species (ROS) in inflamed regions and the detailed mechanisms underlying these pharmacological actions remain unclear. ROS play key roles in various physiological and pathological events. The overproduction of ROS can cause oxidative damage to biomolecules, (lipids, proteins, and DNA), which ultimately results in many chronic diseases in humans such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke, septic shock, aging, and other degenerative diseases [8,9]. In the present study, we measured the ROS scavenging activity of *Jixueteng* using X-band electron spin resonance (ESR) spectroscopy and the XYZ chemiluminescence system. This XYZ chemiluminescence system is an assay that is used to detect the energy that is emitted as ultra-weak luminescence when ROS are eliminated by a scavenger. This chemiluminescence system suggests that the two species of a hydrogen donor (Y) and a mediator or accelerator (Z) are important and necessary to complete the elimination of ROS (X) through light energy transformations. In this system, the mediator (Z) represents the ability of a compound to mediate the transfer of hydrogen or an electron between X and Y [10]. This system is characterized by a photon detection method that uses fixed XY reagents to search for Z species, XZ reagents for Y species, and YZ reagents for X species. ROS scavenging activity correlates with luminescence intensity; therefore, this luminescence system differs from chemiluminescence, which is characterized by a reaction between luminol and hydrogen peroxide (H_2O_2), and also other methods such as the ORAC and DPPH assays. Based on this evidence, the chemiluminescence system has been applied to screen for ROS scavenging activity and the freshness of foods and agricultural products [11-13]. The ROS scavenging activity of *Jixueteng* has also been confirmed by X-band ESR spectroscopy. The aim of this study was to investigate the ROS scavenging activity of *Jixueteng* and the possibility of this herbal medicine being an effective therapeutic agent for ROS-related pathologies.

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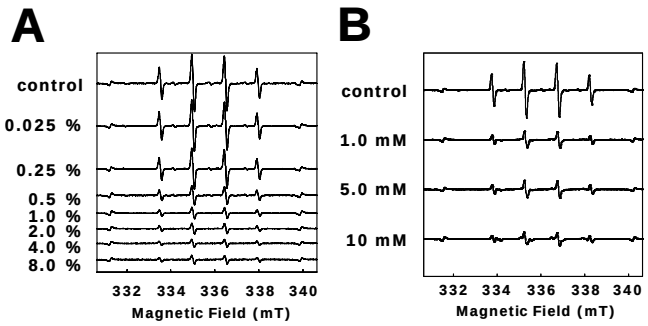

Figure 1: Effects of 0.025-8% *Jixueteng* water extracts on the ESR spin adduct of HO• (A) and the effects of deferoxamine on the ESR spin adduct of HO• (B). X-band ESR was used to determine the signal decay of DMPO-OH.

Evaluation of ROS scavenging activities of Jixueteng and the other herbal medicines by ESR: The *Jixueteng* water extract markedly reduced the ESR signal intensity of HO• in a concentration-dependent manner from 0.025% to 2% (Figure 1A). The maximum scavenging activity was observed at 2%, and was equivalent to the activity of 10 mM deferoxamine (Figure 1B).

The HO• scavenging activities of the water extracts of *Cnidium officinale*, *Salvia miltiorrhiza* and CoQ10 were also concentration-dependent, while that of *Jixueteng* was significantly higher than the 2% ($P < 0.001$) and 4% ($P < 0.05$) water extracts of CoQ10 (Figure 2).

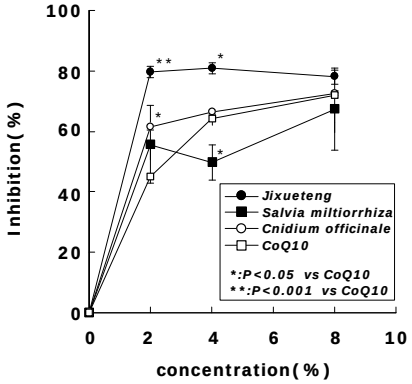

Figure 2: Relative inhibition rate of various herbal medicines to the HO• signal intensity. *Jixueteng*: closed circle, *Cnidium officinale*: open circle and *Salvia miltiorrhiza*: closed square. Data are presented as the mean ± SD. (n = 5 in each group). $^{**}P < 0.001$, $^{*}P < 0.05$ vs CoQ10 (as control data: open square).

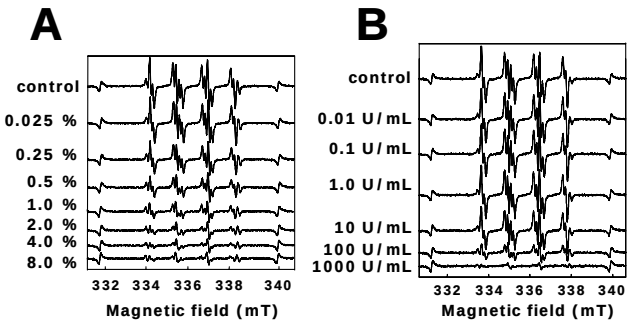

Figure 3: Effects of 0.025-8% *Jixueteng* water extracts on the ESR spin adduct of $O_2^{\bullet-}$ (A) and the effects of 0.01-1,000 U/mL SOD on the ESR spin adduct of $O_2^{\bullet-}$ (B). X-band ESR was used to determine the signal decay of DMPO-OOH.

The *Jixueteng* water extract also markedly reduced the ESR signal intensity of $O_2^{\bullet-}$ (Figure 3A) in a concentration-dependent manner from 0.25 to 8%. The maximum scavenging activity was noted in the 2% *Jixueteng* water extract, and was equivalent to the activity of 100 U/mL SOD (Figure 3B). The $O_2^{\bullet-}$ scavenging activities of the water extracts of *Cnidium officinale*, *Salvia miltiorrhiza* and CoQ10 were also concentration-dependent. The $O_2^{\bullet-}$ scavenging activities of *Salvia miltiorrhiza* and *Jixueteng* were significantly higher than those of CoQ10 ($P < 0.001$). The scavenging activity level of the 8% water extract of *Salvia miltiorrhiza* was similar to that of the 8% water extract of *Jixueteng* (Figure 4).

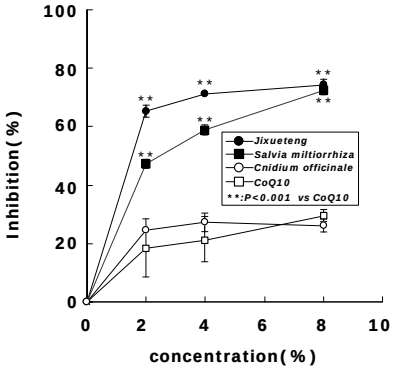

Figure 4: Relative inhibition rate of various herbal medicines to the $O_2^{\bullet-}$ signal intensity. *Jixueteng*: closed circle, *Cnidium officinale*: open circle and *Salvia miltiorrhiza*: closed square. Data are presented as the mean ± SD. (n = 5 in each group). $^{**}P < 0.001$, $^{*}P < 0.05$ vs CoQ10 (as control data: open square).

Evaluation of ROS scavenging activities of Jixueteng and other herbal medicines using XYZ chemiluminescence: H_2O_2 and HO• scavenging activities were detected in the water extracts of *Jixueteng*, *Cnidium officinale* and *Salvia miltiorrhiza*; gallic acid was used as a reference compound of an antioxidative reagent on XYZ chemiluminescence (Figures 5, 6). The water extracts of each herbal medicine exhibited potent H_2O_2 and HO• scavenging activities in a concentration-dependent manner (A of Figures 5 and 6). The ROS scavenging activity of the 8% water extract of *Jixueteng* was the strongest among the herbal medicines we tested (B of Figures 5 and 6).

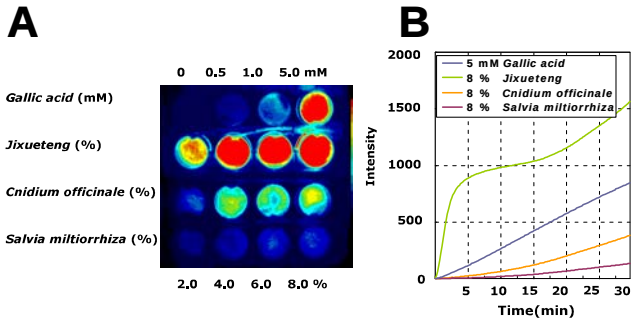

Figure 5: Effects of herbal medicines on photon emission (A) and photon emission intensities (B) in the presence of the oxidant H_2O_2. The reaction mixture contained X (250 μL), Y (100 μL) and Z (250 μL) reagents in a 12-well plate (A). The color bar in (A) shows light emitting intensity that is antioxidant activity, and the red in top of color bar shows the highest antioxidant activity. Luminescence was initiated by adding the concentration-fixed XZ mixture into various concentrations of the Y solution. These experiments were performed in the presence of the oxidant H_2O_2 (X) + a hydrogen donor, an 8% water extract of a herbal medicine (*Jixueteng*, *Cnidium officinale* and *Salvia miltiorrhiza*) or 5 mM gallic acid (Y) + mediator (Z) (antioxidant activity increased as the emission intensity increased).

Authentic *Jixueteng*, which is listed in the Chinese Pharmacopoeia, is the dried stem of *Spatholobus suberectus* Dunn of the family

Leguminosae. However, more than 30 plant species have been used under the general name ''*Jixueteng*'' in traditional Chinese and folk medicine [1,2]. *Jixueteng* is a traditional Chinese medicine that

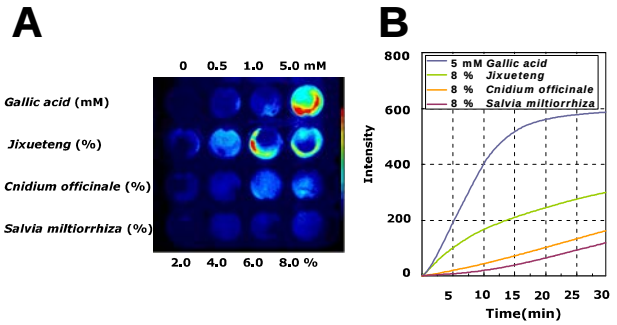

Figure 6: Effects of herbal medicines on photon emission (A) and photon emission intensities (B) in the presence of the oxidant HO˙. The reaction mixture contained X (250 μL), Y (100 μL) and Z (250 μL) reagents in a 12-well plate (A). The color bar in (A) shows light emitting intensity that is antioxidant activity, and the red in top of color bar shows the highest antioxidant activity. Luminescence was initiated by adding the concentration-fixed XZ mixture into various concentrations of the Y solution. These experiments were performed in the presence of the oxidant HO˙(X) + a hydrogen donor, an 8% water extract of a herbal medicine (*Jixueteng*, *Cnidium officinale* and *Salvia miltiorrhiza*) or 5 mM gallic acid (Y) + mediator (Z) (antioxidant activity increased as the emission intensity increased).

improves, increases, and tonifies blood circulation [3], and is also used to treat irregular menstruation, blood deficiencies, and rheumatalgia [4,5]. The chemical profile of *Jixueteng* was investigated extensively by Tang *et al*. [14]. *Jixueteng* also contains various components that are predominantly polyphenols, including flavonoids [15, 16]. Previous chemical and pharmacological investigations have indicated that flavonoids are the main ingredients of *Spatholobus suberectus* [17, 18]. The other herbal medicines that were used as a reference for *Jixueteng* in the present study, *Salvia miltiorrhiza* and *Cnidium officinale*, have been used to tonify the blood and increase its circulation, relieve rigidity of muscles and joints, and promote menstruation. Therefore, these herbal medicines improve blood circulation and invigorate blood in a similar manner to *Jixueteng*.

Several assays have been frequently used in clinical studies to estimate antioxidant capacities, including the oxygen radical absorption capacity (ORAC) [19, 20] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays [21, 22]. The ORAC assay determines fluorescence decay resulting from the oxidation of fluorescein by the peroxy radical. An antioxidant suppresses fluorescence decay. Thus, the ORAC assay is an indicator of antioxidant activity. The DPPH assay measures changes induced in color by the antioxidant material using the stable radical DPPH. Previous studies using these methods demonstrated that the antioxidant activity of *Jixueteng* (*Spatholobus suberectus*) was high [20, 22]. Methods that are frequently used to evaluate antioxidant activity only determine the antioxidant effect for Y in the XYZ system, namely the antioxidant substances. However, in the XYZ photon emission system, Z (a mediator or accelerator) facilitates Y activity. In the absence of Z, Y (antioxidant substance) generates no weak luminescence by photons as energy release. It may alternatively be released as another form of energy such as heat or may accumulate in Y. Therefore, tissue and cell functions may be affected under conditions by which the known antioxidant substance continues receiving energy from ROS. If this antioxidant substance has the effect of Y as the hydrogen donor, the antioxidant activity of Y will be more stable in the presence of Z [10]. In other words, an evaluation of antioxidant activity using this XYZ system considers not only antioxidant activity, but also the stability of the antioxidant substance.

In the present study, we investigated the antioxidative effects of *Jixueteng* and other herbal medicines, *Cnidium officinale* and *Salvia miltiorrhiza*, using ESR spectroscopy and ultra-weak photon detection based on the XYZ chemiluminescence system. This system does not employ chemiluminescence reagents such as luminol, which reacts with hydrogen peroxide to produce luminescence, the intensity of which is dependent on ROS scavenging activity [10]. Okubo *et al.* recently reported that natural radical scavengers produce ultra-weak light emission (photon emission) in the presence of active oxygen species and acetaldehyde [10]. This photon emission occurred nonenzymatically without photon enhancement, such as that observed with luminol. We demonstrated that photon emission was observed due to the antioxidant activities of several traditional Chinese medicines in the presence of ROS and acetaldehyde after the ROS scavenging activities and antioxidant activities of these medicines had been confirmed by X-band ESR spectroscopy. As stated above, this ultra-weak photon emission of the XYZ chemiluminescence system is thought to involve the transformation of energy. Although the ROS (X) has strong reactivities, it did not exhibit photon emission. Therefore, ROS is an intermediate state of energy. By adding an ROS scavenger (Y), for example polyphenols such as flavonoids, anthocyanins, and catechins and also traditional Chinese medicines extracted from leaves, root, and bark, an excited-state compound, a Y'-radical, will be produced. Y'-radicals accumulate at this point because the X and Y mixture does not exhibit photon emission. The release of energy is observed following the addition of a receptive species, a mediator or accelerator (Z), such as acetaldehyde and saponins. This energy release represents photon emission in the XYZ system. The Y'-radical changes to a stable state with the oxygenation or degradation of Z by a radical reaction. Therefore, the mechanism responsible for the scavenging of ROS by flavonoids can be deduced from the photon emissions observed using this system composed of X, Y and Z [10-13].

We demonstrated that the *Jixueteng* water extract had strong H_2O_2, $HO^{\bullet}$ and $O_2^{\bullet-}$ scavenging activities. These ROS are known to be closely associated with various pathologies in the body (inflammatory reactions, ischemic cytopathy, adverse effects of drugs and cancer) and aging. The ROS scavenging activity of *Jixueteng* was markedly stronger than that of the other herbal medicines, and the effects of the 8% water extract of *Jixueteng* were similar to those of an iron chelator, 10 mM deferoxamine, and 100 U/mL of the $O_2^{\bullet-}$ scavenger, SOD. When its antioxidative effects on H_2O_2 were evaluated using the XYZ theory, the antioxidant activity of the 8% water extract of *Jixueteng* was higher than that of 5 mM (0.085%) gallic acid, and was also the strongest among the herbal medicines we tested. Gallic acid is a polyhydroxyphenolic compound that can be found in various natural products, such as green tea, grapes, strawberries, bananas, and many other fruits [23]. This compound is widely used as a natural antioxidant for the oils and fats of food, cosmetics, and pharmaceutical products. Gallic acid has also been shown to inhibit significantly cell proliferation, induce apoptosis in a series of cancer cell lines, and exhibit selective cytotoxicity against tumor cells with higher sensitivity than normal cells [24].

Many herbal medicines are known to possess some degree of antioxidant activity, and phenolic compounds mainly contribute to this activity [20]. Although we did not investigate the involvement of phenolic compounds, there is no doubt that certain water-soluble components involve polyphenolic compounds [14-16]. Therefore,

the water extract components involved in this reactive oxygen scavenging activity needs to be identified and their relationship with the pharmacological effects of *Jixueteng* investigated; this will form part of our future studies. In the present study, the ROS scavenging activity of *Jixueteng* was almost constant at concentrations of more than 2%. Therefore, further antioxidative effects would not be obtained. However, since phenolic compounds, including those in *Jixueteng*, may possess the properties of both antioxidants and prooxidants under some conditions [25], cytotoxicity should be evaluated at concentrations of more than 8%. The water extract component of *Jixueteng* was shown to have potent free radical scavenging activity and antioxidative effects that inhibited the oxidative actions of H_2O_2, $O_2^{\bullet-}$ and $HO^{\bullet}$. Therefore, *Jixueteng* represents a promising therapeutic drug for reactive oxygen-associated pathologies.

Experimental

Preparation of herbal medicine water extracts: The dried vines of *Spatholobus suberectus* Dunn, as well as the herbal medicines, *Salvia miltiorrhiza* and *Cnidium officinale* (Tochimototenkaido, Osaka, Japan), were used in the present study. Each sample (200 g) was extracted by heating at 95°C in 1 L of distilled water for 3 h, followed by filtration (Toyo filter paper No.2) to remove debris. All samples were finally designed as 20% water extracts. Each extract solution was cooled to room temperature, diluted with phosphate-buffered saline (PBS, pH 7.0), and subjected to experiments. To compare the ROS scavenging activities of the herbal medicines, either antioxidative reagents, gallic acid (Wako Pure Chemical Industries, Osaka, Japan), or ROS scavengers, deferoxamine (Novartis Pharma AG, Basel, Switzerland), SOD (Sigma-Aldrich, St. Louis, MO, USA), and coenzyme Q10 (CoQ10: Sigma-Aldrich, St. Louis, MO, USA), were used.

Determination of ROS scavenging activities of Jixueteng and other herbal medicines using ESR: ESR spin trapping was conducted with a ROS generating system containing 5,5-dimethyl-1-pyrriline-*N*-oxide (DMPO: Dojindo Laboratories, Kumamoto, Japan).

The hydroxyl radical ($HO^{\bullet}$) was generated by the Fenton reaction (H_2O_2 plus $FeSO_4$), as described previously [26, 27]. To measure $HO^{\bullet}$, 160 µL of phosphate-buffered saline (PBS, pH 7.0), 10^{-4} M $FeSO_4$ (Sigma-Aldrich, St. Louis, MO, USA), 25 µL of the water extract of the test herbal medicine at specific concentrations (0.02, 0.2, 2, 4, or 8 %) (*Jixueteng*, *Cnidium officinale*, *Salvia miltiorrhiza*) or PBS for the condition without herbal medicine, and 15 µL of 8.8 M DMPO were mixed and reacted with 25 µL of 10^{-4} M H_2O_2 (Wako Pure Chemical Industries, Osaka, Japan). This reaction solution was aspirated into a 130 µL ESR flat cell after 1 min, and ROS scavenging activities were measured using ESR. Deferoxamine and CoQ10 were used as reference compounds for $HO^{\bullet}$ scavengers.

Superoxide anion ($O_2^{\bullet-}$) was generated by the hypoxanthine + xanthine oxidase (XO) system using a previously described method [28]. To measure $O_2^{\bullet-}$, 135 µL of PBS, 0.01 U/mL of XO (Roche Diagnostics GmbH, Mannheim Germany), 25 µL of the water extract of the herbal medicine (*Jixueteng*, *Cnidium officinale*, *Salvia miltiorrhiza*) or PBS for the condition without herbal medicine, 25 µL of 0.2 mM DTPA (Sigma-Aldrich, St. Louis, MO, USA), and 15 µL of 8.8 M DMPO were mixed and reacted with 25 µL of 10^{-5} M hypoxanthine (Sigma-Aldrich, St. Louis, MO, USA). The reaction solution was aspirated into a 130 µL ESR flat cell after 1 min, and ROS scavenging activities were measured using ESR (JES-RE 3X X-band, Tokyo, Japan). The $O_2^{\bullet-}$ scavengers, SOD (0.01, 0.1, 1, 10, 100, and 1,000 U/mL) and CoQ10 were used as reference compounds.

All ESR observations were performed with a JES-RE 3X, X-band spectrometer (JEOL, Tokyo, Japan) connected to a WIN-RAD ESR Data Analyzer (Radical Research, Tokyo, Japan) at the following instrument settings: microwave power, 8.00 mW; magnetic field, 335.638 ± 5 mT; field modulation width, 0.079 mT; receiver gain, 400; sweep time, 1.0 min; and time constant, 0.03 s. Hyperfine coupling constants were calculated based on the resonance frequency, measured with a microwave frequency counter, and resonance field, measured with a JEOL ES-FC5 field measurement unit. We obtained ESR spectra for the manganese oxide standards in order to quantify the spin adducts detected. After the ESR spectra were recorded, the signal intensity, expressed as relative height, was normalized against the signal intensity of the manganese oxide standard [26, 27]. All experiments were repeated a minimum of 4 times.

Determination of ROS scavenging activities of Jixueteng and other herbal medicines using the XYZ chemiluminescence method: The ROS-generating substances, 0.6% H_2O_2 (Wako Pure Chemical Industries, Osaka, Japan) and/or $HO^{\bullet}$ (0.6% H_2O_2 + 25 mM $FeSO_4$) were used for X. Herbal medicine water extracts from 2 to 8% were used as ROS scavengers: Y and 10% acetaldehyde/saturated potassium bicarbonate were used as the mediator: Z. Chemiluminescence was detected using a photon counting charge-coupled device (CCD) camera (C2400-30, Hamamatsu Photonics, Japan) connected to an imaging PMT (a photocathode and microchannel plates), and a position-sensitive detector coupled to an ARGUS-20 (Hamamatsu Photonics, Japan) and image processor, for further image enhancement and quantitative analysis (AQUACOSMOS, Hamamatsu Photonics, Japan). The wave range of the detector was 360-650 nm. The reaction mixture was composed of X (250 µL), Y (100 µL), and Z (250 µL) reagents. Luminescence was initiated by adding the concentration-fixed XZ mixture into various concentrations of the Y solution in a 12-well plate. The ultra-weak light that was emitted was measured and accumulated every 10 sec, and this was repeated 200 times. These results were expressed as the emission intensity. ROS scavenging activity was evaluated by analyzing the emission intensity (ROS scavenging activity became stronger as the emission intensity increased). Gallic acid was used as a reference compound for the antioxidative reagents. This substance has been used in similar chemiluminescent studies as a reference for antioxidative effects [10-13]. All experiments were repeated 3 times.

Statistical analysis: An analysis of variance and multiple comparison tests using the Tukey's method were applied to determine differences among the herbal medicine groups. The Student's unpaired *t*-test was used when comparing one pair only. Data are expressed as the mean ± SD.

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