Inhibitory Effects of Triterpenes Isolated from Chuling (Polyporus umbellatus FRIES) on Free Radical-Induced Lysis of Red Blood Cells

Nobuyasu SEKIYA,∗,a Hiroaki HIKIAMI,a Yoichiro NAKAI,∗ b Iwao SAKAKIBARA,∗ Kazuya NOZAKI,a Kazufumi KOUTA,a Yutaka SHIMADA,a,c and Katsutoshi TERASAWA∗

a Department of Japanese Oriental Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University; 2630 Sugitani, Toyama 930–0194, Japan; b Pharmacognosy & Medicinal Resources Laboratory, Tsumura & Co.; 3586 Yoshiwara, Aminachi, Ibaraki 300–1192, Japan; and c 21st Century COE Program, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930–0194, Japan.

Received December 9, 2004; accepted February 7, 2005; published online February 9, 2005

Chuling, sclerotia of Polyporus umbellatus FRIES, has long been used for urological disorders in traditional medicine. In this study, we demonstrated that Chuling in vitro protects red blood cells from 2,2-azo-bis(2-amidinopropane)dihydrochloride (AAPH)-induced hemolysis. The inhibitory effect was dose-dependent at concentrations of 50 to 1000 μg/ml. Moreover, tests were carried out to identify the main ingredient of Chuling with scavenging effect on free radicals. Triterpen carboxylic acids isolated from the methanol extract of Chuling, namely, polyisorsterone A and polyisorsterone B, were found to have inhibitory activities against AAPH-induced lysis of red blood cells. The anti-hemolytic effect was significantly stronger in polyisorsterone B compared with polyisorsterone A. Furthermore, the ingestion of 150 mg of Chuling was associated with a significant increase in free-radical scavenging effect of plasma in rats.

Key words Chuling; Polyporus umbellatus; triterpene; hemolysis; anti-oxidative activity

Since anti-oxidative activity may play an important role in the mechanisms of atherogenesis, inflammation and aging, we have searched for new anti-oxidative substances among natural products. We have previously reported that Hoelen (Poria cocos Wolf) was found to have remarkable inhibitory effect on hemolysis induced by 2,2-azo-bis(2-amidinopropane)dihydrochloride (AAPH), an azo free-radical initiator.1

Polyporus umbellatus FRIES is a saprophytic fungus growing on withered beech and maple trees roots. Its sclerotium, properly called Chuling, is a crude drug commonly used because of its diuretic effect, like Hoelen. Moreover, Chuling belongs to Basidiomycetes in Eumycetes, also like Hoelen.

In this study, we examined the effect of Chuling on the membrane oxidative process. For that purpose, water extract powder was assayed and fractionated in order to isolate the principles responsible for the activity. The effects of Chuling on red blood cells (RBC) hemolysis induced by an azo free-radical initiator in healthy volunteers, and on scavenging reactive oxygen species (ROS) was examined by means of electron spin resonance (ESR) in the present study.

Furthermore, we hypothesized that ingestion of Chuling would strengthen the antioxidative effect of plasma if the responsible compounds in Chuling could be absorbed and circulated in blood. Therefore, the present study was to examine the effect on plasma antioxidative activity after a gavage-dose of Chuling in rats.

MATERIALS AND METHODS

Extraction and Fractionation of Chuling Dried, ground sclerotia (1.0 kg) of Polyporus umbellatus FRIES (Chuling) were commercially purchased in China and extracted with water (8 l) under reflux for 2 h. The extract was filtered using a stainless steel filter (pore size 150 μm) and lyophilized to afford an aqueous extract (18.5 g). An aliquot of the extract (9.59 g) was first dissolved in water (100 ml), and ethanol was gradually added to a final concentration of 80%. After centrifugation, the precipitate was collected and dried under reduced pressure (P-fraction). The supernatant was fractionated using Diaion HP20 (500 ml, Mitsubishi Chemical Co., Japan), and eluted with 21 of water (W-fraction), 50% aqueous methanol (50M-fraction), and then methanol (M-fraction), successively. These eluates were concentrated under reduced pressure to yield three fractions. The yields of P-, W-, 50M-, and M-fraction from 9.59 g of aqueous extract were 5.14, 3.40, 0.68, and 0.36 g, respectively.

Analytical Methods of the M-Fraction of Chuling The M-fraction was characterized by an HPLC analytical system with LC-10AD pumps, a SPD-M10AVP photodiode-array...
washed three times with 5 volumes of PBS (pH 7.4). The UV data of effluent from the column ranging from 200 to 400 nm were collected and the peak analysis and assignment were performed using the system analysis software, CLASS-LC10 (Shimadzu, Kyoto, Japan). The flow rate and the column temperature were 1.0 ml/min and 40 °C, respectively. The analytical conditions was as follows: a liner gradient of 90% X and 10% Z changing over 30 min to 45% X and 55% Y, and immediately change over 30 min to 0% X and 100% Y. The HPLC profile of the M-fraction and chemical structures of polyporusterone A and polyporusterone B are shown in Fig. 1.

Anti-hemolytic Activity of Chuling (Water Extract, Fractions and Triterpenes) Whole blood obtained from eight healthy male volunteers was analyzed in this experiment. Blood was drawn from an antecubital vein into heparinized tubes. RBC were separated from plasma by centrifugation at 1500 g for 20 min. Crude RBC was then washed three times with 5 volumes of PBS (pH 7.4). Packed RBC was thereafter suspended in 4 volumes of PBS solution.

In the present study, the previously reported method was used to determine the hemolysis of RBC mediated by AAPH.1,5–9) The addition of AAPH (a peroxyl radical initiator) to the RBC suspension causes the oxidation of lipids and proteins in cell membrane and thereby induces hemolysis. It is known that AAPH-induced hemolysis in RBC is a function of incubation time and is proportional to the concentration of free radicals.1,10) The inhibitory effect on RBC hemolysis is also proportional to the concentration of antioxidants in the incubation mixture.

One milliliter of RBC suspension was mixed with 1 ml of PBS solution containing various amounts of Chuling, its fractions or triterpenes. One milliliter of RBC suspension mixed with 1 ml of PBS solution alone was used as control. One milliliter of 200 mM AAPH in PBS solution was then added to the mixture. The incubation mixture was shaken gently in a water bath at 37 °C for 3 h. After incubation, 4 ml of PBS solution was added to the reaction mixture, and this was followed by centrifugation at 1000 g for 10 min. The absorbance (A) of the supernatant at 540 nm was recorded with a UV-visible spectrophotometer (UV-265FS, Shimadzu, Kyoto, Japan). Percentage inhibition was calculated by the following equation:

\[
\% \text{ inhibition} = \frac{A_{\text{AAPH}} - A_{\text{CHU}}}{A_{\text{AAPH}}} \times 100
\]

where \(A_{\text{CHU}}\) is the absorbance of the sample containing Chuling, its fractions or triterpenes, and \(A_{\text{AAPH}}\) is the absorbance of the control sample.

We used the l-ascorbic acid again as a reference antioxidant compound for comparison purpose.

ESR Assay for \(O_2^\cdot\) and \(HO^\cdot\) Scavenging Activities of Chuling Measurement of \(O_2^\cdot\) (superoxide anion) and \(HO^\cdot\) (hydroxyl radical) scavenging activities was performed as described previously3) with slight modification. Chuling extract was diluted to 0.2, 2.0, and 20.0 mg/ml with phosphate buffered saline (PBS). The superoxide anion and hydroxyl radical scavenging activities of each sample (50 µl) were assessed by the ESR technique,4) and then the IC50 values (inhibition concentration 50%) were calculated. Superoxide anion was generated from a hypoxanthine (HPX)—xanthine oxidase (XOD) reaction system in PBS.5) Briefly, 15 µl of 9.2 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (LABOTEC, Tokyo, Japan), 50 µl of 2 mM HPX (Sigma, St. Louis, MO, U.S.A.), 35 µl of 5.5 mM diethylenetriamine-N,N',N''-pentaacetic dianhydride (DETPAC; Wako Pure Chemical Industries, Tokyo, Japan) and 50 µl of prepared sample were put into a test tube. After adding 50 µl of 0.4 U/ml XOD Roche, Indianapolis, IN, U.S.A.) and quick mixing, 200 µl of the mixture was transferred to a flat quartz ESR cuvette, which was fixed to the cavity of an ESR spectrometer (JES-FR30, JEOL, Tokyo, Japan). Recordings of the spectra were made at 24 °C and started at 1 min after the mixing of XOD; each scan took 1 min. Data were expressed as the ratio of the peak of the DMPO–OOH signal to the peak of the intrinsic standard, MnO (S/M). Scavenging activity (Scv) was calculated by the following equation:

\[
\text{Scv (\%)} = \frac{(S/M_{\text{blank}} - S/M_{\text{SAMPLE}})}{S/M_{\text{blank}} \times 100}
\]

where \(S/M_{\text{blank}}\) is the intensity of the ESR spectrum of DMPO–OOH spin adduct in PBS as a blank, and \(S/M_{\text{SAMPLE}}\) is the intensity of the ESR spectrum of DMPO–OOH spin adduct in the sample.

Hydroxyl radical was generated by the Fenton reaction6) consisting of 75 µl of 0.1 mM H2O2, 50 µl of each sample,
20 μl of 92 mM DMPO and 75 μl of 0.1 mM FeSO₄. The spectrum of DMPO–OH was measured at 1 min after the addition of H₂O₂. Scavenging activity was calculated as described above.

We used the L-ascorbic acid (Wako, Osaka, Japan) as a reference water-soluble antioxidant compound for comparison purposes.

**Superoxide Anion and Hydroxyl Radical Scavenging Activities of Rat Plasma after Chuling Ingestion** Fifteen Wistar male rats (300 g) were fed commercial feed (type CE-2, CLEA Japan Inc., Tokyo, Japan) for one week and randomly allotted into three groups. After overnight fasting, group A rats (n=5) were gavage-dosed with 1 ml of distilled water containing 150 mg of Chuling extract, group B rats (n=5) were gavage-dosed with 1 ml of distilled water containing 75 mg of L-ascorbic acid, and the control rats (group C; n=5) were gavage-dosed with 1 ml of distilled water only. Thirty minutes after administration, blood from the heart was collected into heparinized tubes. Then, plasma was immediately separated by low-speed centrifugation at 1500g for 20 min. After removal of white blood cells and platelets, the remaining RBC was mixed with the volume of plasma beforehand. The reconstituted blood (1 ml) was then used for the hemolysis assay by adding 1 ml of AAPH solution and 1 ml of PBS followed by incubation at 37 °C for 2, 3 or 4 h. PBS solution (4 ml) was added to the incubation mixture followed by centrifugation at 1000g for 10 min. Absorbance (A) of the supernatant at 540 nm was measured. The percentages of inhibition of a gavage dose of Chuling and L-ascorbic acid were calculated in the same way as in the in vitro study.

Furthermore, the separated plasma was diluted with 7 volumes of saline. The O₂•- and HO• scavenging activities (Scv) of rat plasma were determined by ESR method and calculated by the same equation as that for the in vitro assay.

\[
Scv(%) = \frac{S/M_{\text{BLANK}} - S/M_{\text{PLASMA}}}{S/M_{\text{BLANK}}} \times 100
\]

where S/M_{BLANK} is the intensity of the ESR spectra of DMPO–O2•OH or DMPO–OH spin adduct in PBS as a blank, and S/M_{PLASMA} is the intensity of the ESR spectra of DMPO–O2•OH or DMPO–OH spin adduct in the diluted plasma sample. This study conformed to the guidelines for the care and use of animals as approved by the Ethics Committee for Animal Experiments of Toyama Medical and Pharmaceutical University.

**Statistical Analysis** Data are reported as mean±standard deviation. Kruskal–Wallis test was used for statistical analysis of the in vivo ESR study. Repeated measures ANOVA and repeated measures one-way ANOVA followed by Fisher's PLSD test were used for the assay of anti-hemolytic activity. A value of p<0.05 was accepted as statistically significant.

**RESULTS**

The anti-hemolytic activities of the water extract of Chuling and L-ascorbic acid are shown in Fig. 2. The water extract of Chuling and L-ascorbic acid in vitro inhibited the hemolysis of rat RBC due to AAPH-induced peroxynitric radicals in a dose-dependent manner.

Figure 3 shows the anti-hemolytic activities of four fractions obtained from the water extract of Chuling. The inhibitory activities of M-fraction and 50 M-fraction were significantly stronger than those of P-fraction, W-fraction. Furthermore, inhibitory activity of M-fraction was considerably stronger than that of 50 M-fraction.

The M-fraction of Chuling is composed of triterpenes. In this study, two triterpenes, polyisorsterone A and polyisorsterone B, were isolated and assayed. Both triterpenes inhibited the free radical-induced lysis of RBC dose-dependently (Fig. 4).

The anti-hemolytic effect was significantly stronger in...
and B rat plasma (Chuling and L-ascorbic acid groups) were determined by the ESR method. The radical scavenging activities of groups A and B were more resistant to AAPH-induced hemolysis than that obtained from group C rats significantly (Fig. 5). Chuling inhibited the free radical generation dose-dependently by means of the ESR method. The IC_{50} values of the water extract of Chuling for superoxide anion and hydroxyl radical were 0.88 and 1.32 mg/ml, respectively. This suggests that Chuling as its major component and has been approved for prescription as a Kampo formulation for urolithiasis patients in Japan, exhibited apparent stone prophylaxis in an animal model.

Some years ago, alkali-soluble polysaccharide obtained from Chuling was found to have an anti-tumor activity against Sarcoma 180. In addition, it was reported that the combined administration of Chuling and mitomycin C increased the life span of tumor-bearing (Sarcoma 180-induced liver tumor) mice by inhibiting the synthetic rates of DNA, RNA and protein in tumor cells. Moreover, ergosterol isolated from Chuling reportedly provides significant protection against bladder tumor in rats. These findings in the literature point to the potential usefulness of Chuling as an anti-cancer agent.

So far, only the hair regrowth effect has been reported in regard to polyporusterone A and polyporusterone B. Direct protection of the RBC membrane from free radical attack as observed in the present study would provide an important pathophysiological basis for putting to use the helpful hemorrheological, anti-atherosclerotic and anti-inflammatory effects of polyporusterone A, polyporusterone B and Chuling.

**REFERENCES**


