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Abstract

Chinese ant extract preparations (CAEP) are a Chinese traditional medicine which is mainly used as a health food or drink for the treatment of rheumatism, rheumatoid arthritis, chronic hepatitis, sexual hypofunction, and antiaging in China. The effects on free radicals were examined by electron spin resonance spectrometry using the spin trapping agent 5.5-dimethyl-1-pyrroline-1-oxide (DMPO). Superoxide radicals (3.35 x 10(15) spins/ml) were quenched 50% by the extract at 0.5 mg/ml. The CAEP extract at 0.7 mg/ml inhibited 50% of hydroxyl radicals (52.0 x 10(15) spins/ml) generated by the Fenton reaction. Against DPPH radical, the scavenging action of CAEP was observed at 1.8 mg/ml of the extract and 50% of the DPPH radicals (8.14 x 10(15) spins/ml) were quenched. In vitro tests showed that CAEP inhibited the production of thiobarbituric acid-reactive substances, an index of lipid peroxidation, in rat brain homogenate.

KEYWORDS: free radical scavenger, antioxidant, hydroxyl radical, superoxide radical, lipid peroxidation

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Antioxidant Activity of Chinese Ant Extract Preparations

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Chinese ant extract preparations (CAEP) are a Chinese traditional medicine which is mainly used as a health food or drink for the treatment of rheumatism, rheumatoid arthritis, chronic hepatitis, sexual hypofunction, and antiaging in China. The effects on free radicals were examined by electron spin resonance spectrometry using the spin trapping agent 5.5-dimethyl-1-pyrroline-1-oxide (DMPO). Superoxide radicals (3.35×10^{15} spins/ml) were quenched 50% by the extract at 0.5 mg/ml. The CAEP extract at 0.7 mg/ml inhibited 50% of hydroxyl radicals (52.0×10^{15} spins/ml) generated by the Fenton reaction. Against DPPH radical, the scavenging action of CAEP was observed at 1.8 mg/ml of the extract and 50% of the DPPH radicals (8.14×10^{15} spins/ml) were quenched. *In vitro* tests showed that CAEP inhibited the production of thiobarbituric acid-reactive substances, an index of lipid peroxidation, in rat brain homogenate.

Key words; free radical scavenger, antioxidant, hydroxyl radical, superoxide radical, lipid peroxidation

Ants as a health food (drink) or medicine have a long history in China, dating back about 3,000 years. It was first recorded in Zhou-Li, a historical book which talks about the popularity of greetings in China, and ants were mentioned as an excellent food for the king (1). Li Shizhen, a famous pharmacologist during the Ming dynasty, edited Ben-Cao-Gang-Mu a Compendium of Materia medica in which ants were recorded as a food and as a medicine as well (2). At present, there are some areas in China such as Guangxi, where people eat ant powder or drink ant wine to maintain their health. In several other countries, such as Argentina, Burma, Colombia, Mexico, Africa, and America, ants are also used in folk medicine (3).

As a Chinese traditional medicine, Chinese ant extract preparation (CAEP) is mainly used as a health food or drink for the treatment of rheumatism, rheumatoid arthritis, chronic hepatitis, sexual hypofunction, and for antiaging in China (4). Some pharmacological effects and clinical uses of ants were reported (5, 6), but the results were preliminary.

Free radicals have been implicated in many disorders such as epilepsy (7), aging (8), ischemia (9), trauma (10), and rheumatoid arthritis (11), and even in cancer. Among the reactive oxygen species (ROS), hydroxyl radicals are the most reactive and they damage proteins, break DNA, and promote lipid peroxidation (7). Therefore, we investigated CAEP to determine whether or not it has the ability to quench free radicals.

Materials and Methods

CAEP

CAEP was prepared from *Polyrhachis vicina R.*, a kind of black ant. Ant was rinsed with ethanol-water several times and powdered. Further ant powder was infusioned for 24 h with 5 volumes of ethanol 40% (w/v) (500 ml). After filtration with two layer gauzes, ant powder was centrifugated (3,000 rpm, 15 min) and filtrated with quantitative filter paper. After removal of ethanol, the remaining ant extracts water solution was evaporated and concentrated to 50% (Fig. 1). The extract solution (CAEP) prepared by this method was equivalent to 100 mg dried ants/ml.

Chemical

Hypoxanthine (HPX), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and diethylenetriaminepentaacetic acid (DETAPAC) were obtained from Sigma Chemical Co.

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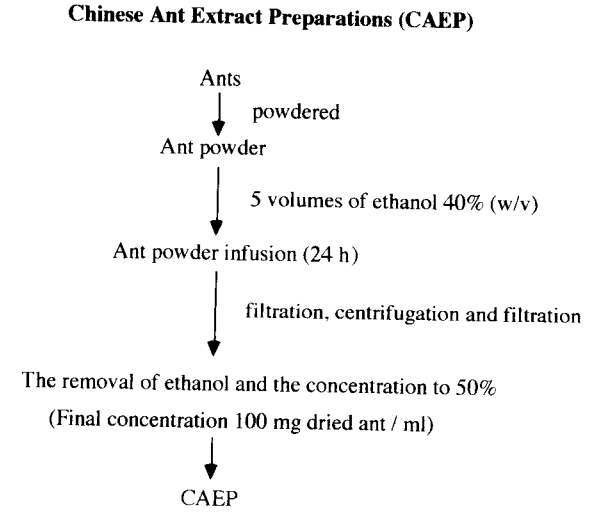

Fig. 1 Method of Chinese ant extract preparations.

(St. Louis, MO); xanthine oxidase (XOD) was from Boehringer Mannheim (Mannheim, Germany); 5.5-dimethyl-1-pyrrolyne-1-oxide (DMPO) was from Labotec Co. (Tokyo, Japan), and ferrous sulfate, hydrogen peroxide and other ordinary reagents were of the highest quality available from Wako Pure Chemical Industries or Katayama Chemical Co. (Osaka, Japan).

Free Radical Analysis

Free radicals were analyzed by electron spin resonance (ESR) spectrometer (JES-FE1XG, JEOL Co., Japan) according to the method as described by Liu *et al.* (8, 9).

DPPH radical analysis. For the DPPH radical analysis, 100 μl of 50 mM DPPH ethanol solution and 100 ml of CAEP solution were mixed for 5 sec and then transferred to a special flat cell in which the amount of DPPH radical was estimated by ESR spectrometry exactly 40 sec after the addition of CAEP. Signal intensities were evaluated by taking the height of the third peak of the five-peak DPPH radical signal. The ESR settings were as follows: microwave power 8 mW, modulation frequency 100 kHz, modulation width 0.08 mT, scan time 1 min/360 mm, response 0.1 s, magnetic field intensity 335.6 $\pm$ 10 mT and amplitude 4.0×10^2.

Superoxide radical analysis. For the superoxide radical analysis, the following reagents were added in a test tube in the following order: 50 μl of 2 mM HPX, 35 μl of 11.0 mM DETAPAC, 50 μl of the CAEP solution, 50 μl of XOD (0.326 units/ml) and 15 μl of 9.2 M DMPO. The solution was mixed and then placed in a flat ESR cell (volume; 160 μl) and DMPO-OH spin adduct was analyzed by ESR spectrometry. The ESR settings were as follows: microwave power 8 mW, modulation frequency 100 kHz, modulation width 0.08 mT, scan time 1 min/360 mm, response 0.1 s, magnetic field intensity 335.6 $\pm$ 5 mT and amplitude 3.2×10^3.

Hydroxyl radical analysis. For the hydroxyl radical analysis, 75 μl of 1 mM $FeSO_4$ and DETAPAC solution, 50 μl of the extract solution, 20 μl of 0.092 M DMPO and 75 μl of 1 mM hydrogen peroxide were put into a test tube and mixed. The amount of DMPO-OH spin adducts formed was estimated exactly 40 sec after adding the DMPO. The ESR settings were as follows: microwave power 8 mW, modulation frequency 100 kHz, modulation width 0.08 mT, scan time 1 min/360 mm, response 0.1 s, magnetic field intensity 335.6 $\pm$ 5 mT and amplitude 1.0×10^2.

Measurement of Lipid Peroxides

Tissue homogenate was prepared from the brains and livers of 15-week-old male Sprague-Dawley rats. Thiobarbituric acid- reactive substances (TBARS) was measured as described by Ohkawa *et al.* (10) using malondialdehyde as the standard. Briefly, 1.5 ml of 1 % thiobarbituric acid (TBA) was added to each sample, followed by 1.5 ml of 20 % acetic acid (pH 3.5) and 0.2 ml of sodium dodecyl sulfate. After heating for 60 min at 100 °C, the samples were extracted into 3 ml of n-butanol and measured spectrofluorimetrically.

Statistical Analysis

The statistical significance of the differences was examined by two-way analysis of variance.

Results

The scavenging action of CAEP on DPPH radicals, superoxide radicals and hydroxyl radicals were showed (Fig. 2-A, Fig. 3-A and Fig. 4-A). Against DPPH radical, the scavenging action of CAEP was observed at 1.8 mg/ml of the extract and 50 % of the DPPH radicals (8.14×10^{15} spins/ml) were quenched (Fig. 2-B). Superoxide radicals (3.53×10^{15} spins/ml) were quenched 50 % by the extract at 0.5 mg/ml (Fig. 3-B). The CAEP extract at 0.7 mg/ml inhibited 50 % of hydroxyl radicals (52.0×10^{15} spins/ml) generated by the Fenton reaction (Fig. 4-B).

As shown in Fig. 5, at the concentrations of 0.3 and 0.63 mg/ml of CAEP, there was a decrease in the TBARS level compared to the control (7.26 $\pm$ 1.15; n =

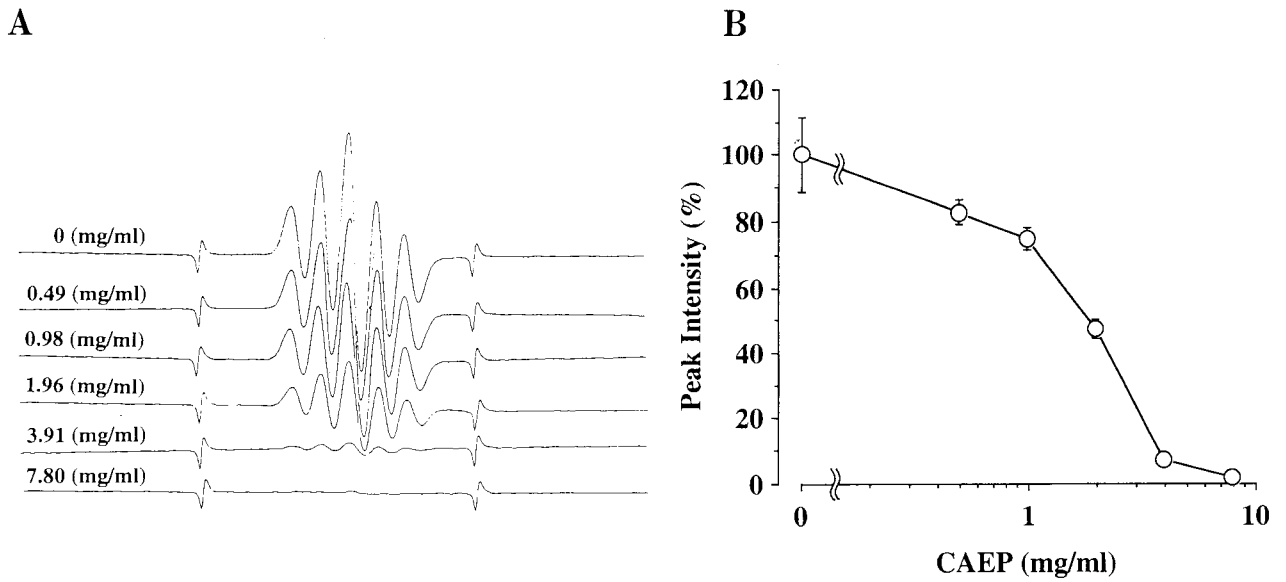

Fig. 2 Effects of Chinese ant extract preparation (CAEP) on DPPH radicals.
A. ESR spectra obtained in the reaction mixture containing 0–7.8 mg/ml concentrations of the extract. **B**. Dose-dependent effects on DPPH radicals. Each point is the mean ± SD of 5–6 experiments.

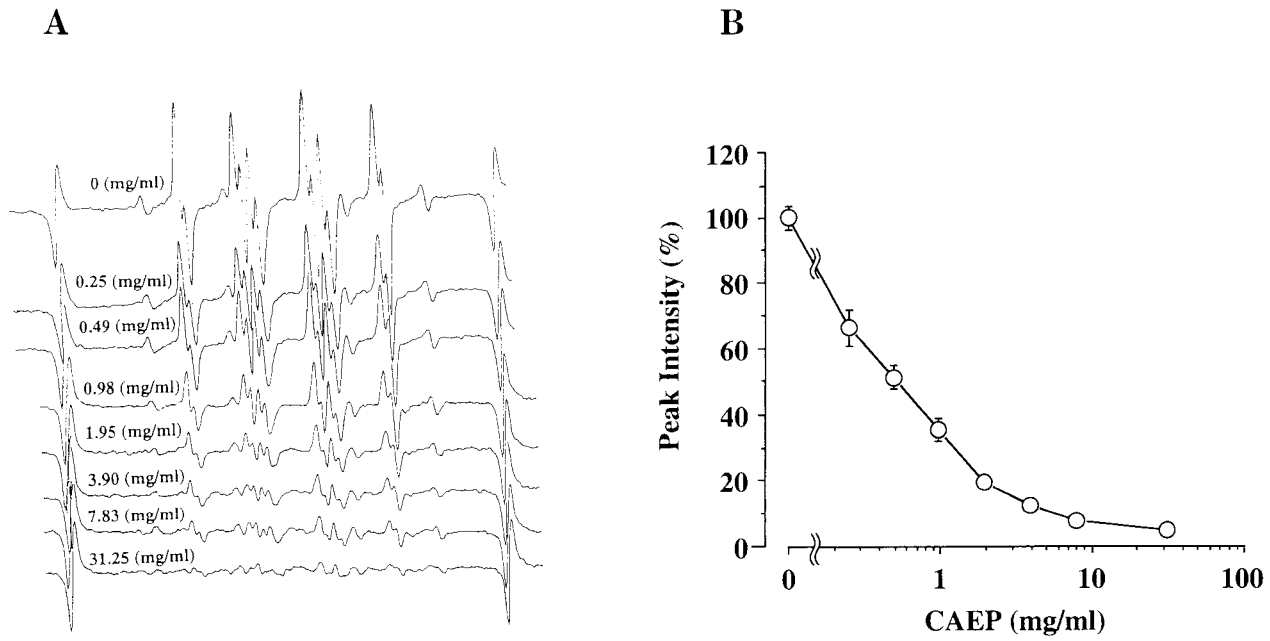

Fig. 3 Effects of Chinese ant extract preparation (CAEP) on superoxide radicals, generated by the hypoxanthine-xanthine oxidase system, measured as DMPO-OOH spin adducts.
A. ESR spectra obtained in the reaction mixture containing 0–31.25 mg/ml concentrations of the extract. **B**. Dose-dependent effects on superoxide radicals. Each point is the mean ± SD of 5–6 experiments.

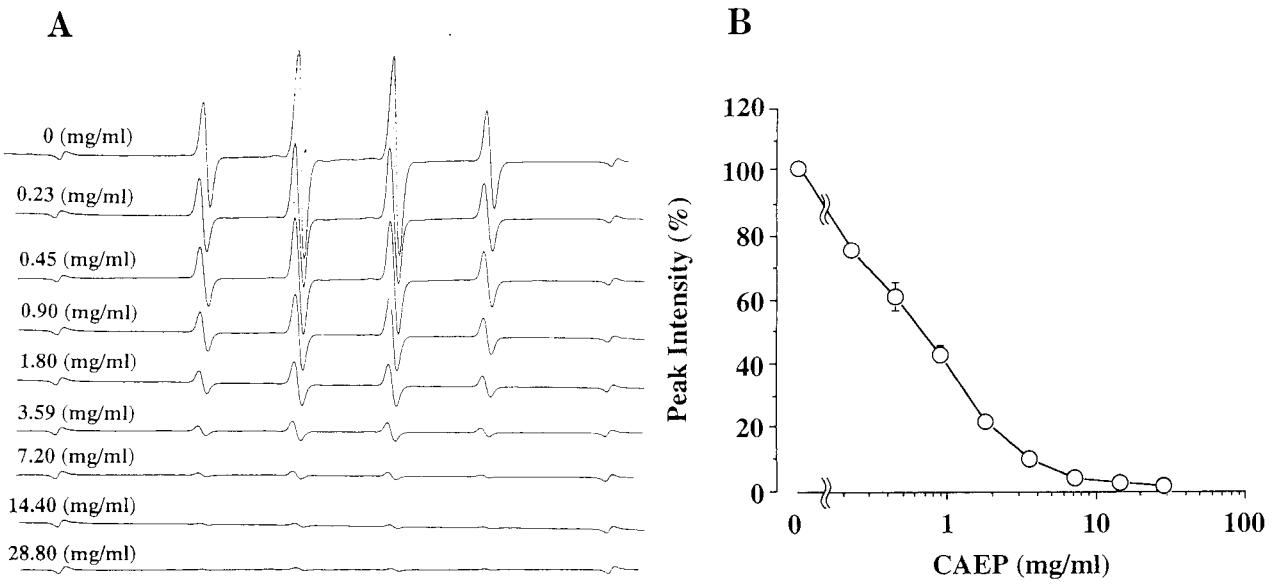

Fig. 4 Effects of Chinese ant extract preparation (CAEP) on hydroxyl radicals, generated by Fenton reagents, measured as DMPO-OH spin adducts.
A. ESR spectra obtained in the reaction mixture containing 0–28.8 mg/ml concentrations of the extract. **B**. Dose-dependent effects on hydroxyl radicals. Each point is the mean ± SD of 5–6 experiments.

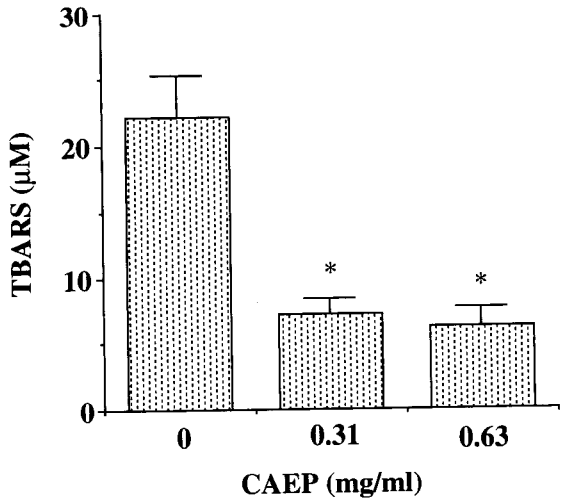

Fig. 5 TBARS level in brain homogenate incubated with Chinese ant extract preparation (CAEP).

6; $P < 0.001$ *vs.* control, 6.23 ± 1.55; n = 7; $P < 0.001$ *vs.* control)

Discussion

Although the antioxidants, or free radical scavengers, in plants have been studied extensively (11–13), the antioxidants in insects and animals have received little attention (14). In the present study, we studied the free radical scavenging effect of CAEP and found that it is a potent scavenger of superoxide, hydroxyl and DPPH radicals. Furthermore, the IC_{50} of superoxide radical scavenging activity by CAEP was almost the same as of TJ-960, which is a Japanese herbal medicine contained seven herbs (Table 1).

CAEP has been widely used as a detoxifying agent to eliminate swelling and facilitate recovery (17). It has been shown to have beneficial effects on both rheumatism and chronic hepatitis (18–20). Most recently, clinical and animal experiments showed that CAEP can regulate the immune system by activating certain enzymes, stimulating the division of immune cells, and promoting the growth of immune organs, and also that CAEP can strengthen resistance to disease by regulating the physiological equilibrium of the whole immune system (20 and

Table I Concentration of antioxidants required to quench 50% (IC_{50}) of the free radicals

Antioxidant/ scavenger	IC_{50} of antioxidant		Reference
	Hydroxyl radicals (μg/ml)	Superoxide radicals (μg/ml)	
Vitamin C	35	24	15
Vitamin E	37	——	15
TJ-960	——	700	16
Soybean paste miso	4000	5000	15
CAEP	700	500	——

unpublished data). Although the mechanisms are not clearly understood, it seems very likely that the effectiveness of CAEP is at least partly attributable to its free radical scavenging activity. Therefore, the beneficial effects of CAEP on free radical related diseases such as rheumatism, rheumatoid arthritis and chronic hepatitis, may be based, at least in a part, on this mechanism. Chemical analysis revealed that *Polyrhachis vicina R.* (PVR) contains aliphatic hydrocarbons in its stomach, formic acid in its toxic saliva, and more than 17 kinds of free amino acids such as, 2-amino-6-hydroxypteridine and biopterin (14). Therefore, it is quite interesting and important to further identify the biologically active components in the extract and to study the relationship between their structure and activity. It is also important to identify the mechanism of the synergistic effects, by isolating the pure natural products from the extract and by experimenting with chemical substances of known structure.

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