

## 枥の実の抗酸化作用と抗遺伝毒性作用

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## Antioxidative and Antigenotoxic Effects of Japanese Horse Chestnut (*Aesculus turbinata*) Seeds

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**ABSTRACT.** Japanese horse chestnut seed extract (HCSE) dose-dependently inhibited the autooxidation of linoleic acid (IC<sub>50</sub>: 0.2 mg/ml), and the inhibition was almost complete at a concentration of 1 mg/ml. The HCSE scavenged DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals and superoxide anions with EC<sub>50</sub>s of 0.65 and 0.21 mg/ml, respectively. However, it had no effect on hydrogen peroxide. The HCSE inhibited the genotoxicities of furylfuramide, N-methyl-N-nitrosourea, methyl methanesulfonate, mitomycin C, 2-aminoanthracene and aflatoxin B1 at a concentration of 1 mg/ml or more. Total polyphenol content of the HCSE was 21 mg/g (13 mg/g-seeds). These results indicate that the Japanese horse chestnut seed is an antioxidative and antimutagenic botanical resource.

**KEY WORDS:** antigenotoxicity, antioxidation, Japanese horse chestnut.

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Horse chestnut (*Aesculus hippocastanum*), which is native to Europe, is a deciduous tree up to 20 m high with seven digitate big leaves. In autumn, this tree bears many fruits containing one or two large shinybrown seed. The seed contains several active chemicals such as aescin, a complex mixture of triterpenoid saponin, coumarin derivatives, flavonoids and tannin. The extract of this seed has been used traditionally for the treatment of chronic venous insufficiency, traumatic edema, hemorrhoids, etc. in Europe [27]. On the other hand, Japanese horse chestnut (*Aesculus turbinata*) seeds were used for foods in old days in Japan. However, we don't make the most of the seeds nowadays.

Recently, the pattern of food consumption has been suggested to associate with the incidence of cancers [5]. Especially, there are many epidemiological evidences that the intake of green tea decreases the risk of cancers, eg. gastrointestinal cancers [11, 30], breast cancers [10, 29] and other cancers [9, 14]. The protective effect of green tea may be caused mainly by the polyphenolic compounds because epigallocatechin gallate, a major polyphenolic component in green tea, is a potent antioxidant and have antimutagenic or antigenotoxic activities [7, 20, 28]. The polyphenol is a common constituent in plants. Antioxidative or antigenotoxic activities have also been examined in other foods or beverages [16, 19], however, it has not been examined whether the Japanese horse chestnut seeds have such activities. In the present study, therefore, antioxidative and antigenotoxic activities of Japanese horse chestnut seeds were examined to search for a novel biochemical function of unutilized bioresources.

Mature seeds of Japanese horse chestnut were gathered from street trees in Morioka city. They were washed with tap water and dried in the sun for several days. Shelled seeds were milled and soaked in 4 volumes of 50% ethanol for two weeks at room temperature. The extract (HCSE) was filtered with a filter paper (No. 5C) and freeze-dried to

apply to the following experiments. All of the experiments were carried out in duplicate or triplicate. Antioxidative activities of the HCSE were compared with those of (+)-catechin.

**Lipid oxidation [4]:** The HCSE and (+)-catechin were dissolved in distilled water. Aliquots of 0.25 ml of the samples were mixed with 1 ml of 1.25% linoleic acid (in 1% sodium dodecylsulfate), and heated at 60°C for 18 hr to induce the autooxidation of linoleic acid. After the heating treatment, 0.25 ml of 0.5% butylated hydroxytoluene (in ethanol) and 1 ml of 0.4% TBA (2-thiobarbituric acid, in 0.2 M PBS, pH 3.0) were added to the test tubes and heated in boiling water for 30 min. After cooling the tubes with cold water, 3 ml of ethanol was added and stirred well to clarify the mixtures. The absorbance was measured at 530 nm with a spectrophotometer to determine the TBA-reactive substances.

**DPPH radicals [1]:** The HCSE and (+)-catechin were dissolved in 50% ethanol. Aliquots of 1 ml of the samples were mixed with 1 ml of 0.1 mM DPPH (1,1-diphenyl-2-picrylhydrazyl, in 50% ethanol) and the absorbance at 520 nm was measured 2 min later.

**Superoxides [2]:** The reaction solution was made by mixing 10 ml of phosphate-borate buffer (65 mM KH<sub>2</sub>PO<sub>4</sub>, 35 mM sodium borate, 0.5 mM Na<sub>2</sub>-EDTA), 10 ml of 0.5 mM xanthine, 5 ml of 10 mM hydroxylammonium chloride, and 10 ml of distilled water just before use. The HCSE and (+)-catechin were dissolved in distilled water. Aliquots of 0.1 ml of the samples were added to 2 test tubes containing 0.7 ml of the reaction solution. Then, 0.2 ml of xanthine oxidase (30 mU/ml) or distilled water (blank) was added and incubated at 37°C for 30 min. After the incubation, 2 ml of color developer (30 µM N-1-naphthylethylenediamine dihydrochloride, 3 mM sulfanilic acid, 25% acetic acid) was added to each test tube, and the absorbance was measured at 550 nm 30 min later.

**Hydrogen peroxides:** A flask containing 40 ml of 10 mM hydrogen peroxide and 10 ml of 50 mM PBS, pH 7.0, was kept at 30°C in a water bath. Just before and 5, 10 and 15 min after the addition of samples (0.1 ml), 10 ml of the solution was deprived into another flask containing 0.5 ml of 20% sulfuric acid. Hydrogen peroxide was determined by titration with 0.025 N potassium permanganate.

**Total polyphenol:** Total polyphenol was determined according to the Folin-Ciocalteu method [25]. One milliliter of HCSE solution (in 50% ethanol) was mixed with 0.5 ml of 1 N phenol reagent and 5 ml of 0.4 M sodium carbonate, and its absorbance was measured at 660 nm after a 30 min incubation at 30°C. The calibration curve was made with (+)-catechin.

**Antigenotoxicity:** Genotoxic chemicals used were furofuramide (AF-2, 0.009 µg/ml), mitomycin C (MMC, 1.0 µg/ml), N-methyl-N-nitrosourea (MNU, 50 µg/ml), methyl methanesulfonate (MMS, 100 µg/ml), 2-aminoanthracene (2AA, 0.3 µg/ml) and aflatoxin B1 (1.0 µg/ml). Antigenotoxic effects of the HCSE were examined with a kit of the umu-test (UMULAC, Japan Immunoresearch Laboratories Co., Ltd.) [17]. The procedure was detailed in the manual of the kit. Statistical analysis was carried out by the method of Dunnett.

Figure 1 shows the results of the antioxidation assays. The HCSE dose-dependently inhibited the autooxidation of linoleic acid ( $IC_{50}$ : 0.2 mg/ml), and the inhibition was almost complete at a concentration of 1 mg/ml. The  $IC_{50}$  of catechin was almost the same as the HCSE. Catechin, however, did not inhibit the oxidation more than 50% when the concentration was increased. DPPH radicals were dose-dependently removed by the HCSE ( $EC_{50}$ : 0.65 mg/ml) and catechin ( $EC_{50}$ : 2.5 µg/ml) and their radical-scavenging capacities were evaluated to be 0.038 mmol/g and 9.9 mmol/g, respectively. The HCSE and catechin also removed superoxide anions with the  $IC_{50}$  of 0.21 mg/ml and 4.6 µg/ml, respectively. Neither HCSE nor catechin scav-

enged hydrogen peroxide.

Total polyphenol content of the HCSE was 21.3 mg/g. Polyphenol content of the Japanese horse chestnut seeds was calculated at 13 mg/g.

Figure 2 shows the results of the umu test. The absorbance indicates the degree of DNA damage. In this figure, the absorbance of blank, which contained no mutagen, had been subtracted from each value to indicate the net genotoxicity. The umu test could detect the genotoxicities of all chemicals used (Fig. 2, Control). The HCSE significantly decreased the genotoxicity of AF-2 (0.3 and 3.0 mg/ml), MNU (3 mg/ml), MMS (3.0 mg/ml), MMC (1.0 and 3.0 mg/ml), 2AA (1.0 and 3.0 mg/ml) and aflatoxin B1 (1.0 and 3.0 mg/ml).

Dietary antioxidants such as ascorbic acid, tocopherol, carotenoids, flavonoids and catechin may act to protect the living system from oxidative damage. Polyphenolic compounds are the most common antioxidants naturally occurring in the botanical resources. This class of plant metabolites contains thousands of known compounds, ranging from simple phenols to highly complexed materials such as tannin. Quantitative estimation of the total polyphenol with HPLC is difficult due to the variety in the structure of carbon skeleton and the pattern of hydroxylation of phenolic rings. Folin-Ciocalteu method is used for a simple determination of the total polyphenol in foods, but this method tends to result in an overestimation if other reducing agents are present in the samples. Ascorbic acid is a major reductant in foods and 1 mg ascorbic acid is equivalent to 0.7 mg catechin [24]. Thus, the polyphenol content obtained by the Folin-Ciocalteu method must be corrected for the amount of ascorbic acid. In the present study, however, ascorbic acid was not determined because the horse chestnut seeds scarcely contain ascorbic acid [18].

The polyphenol contents in polyphenol-rich foods or beverages determined by the Folin-Ciocalteu assay are 1.8 mg/ml for the wine, 0.9–1.0 mg/ml for the coffee and tea, 0.75

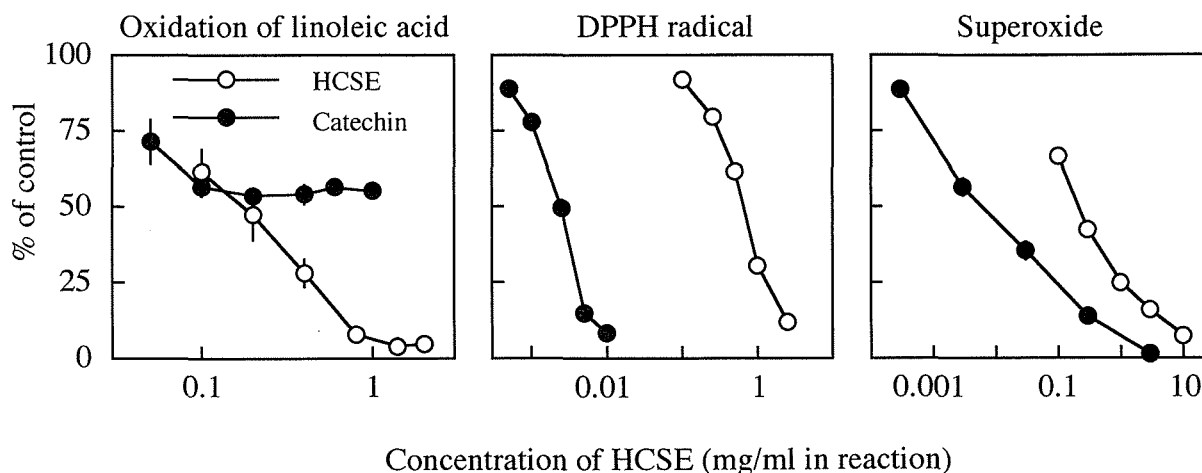


Fig. 1. Antioxidative effects of the Japanese horse chestnut seed extract. Each symbol and vertical bar represents the mean and SD.

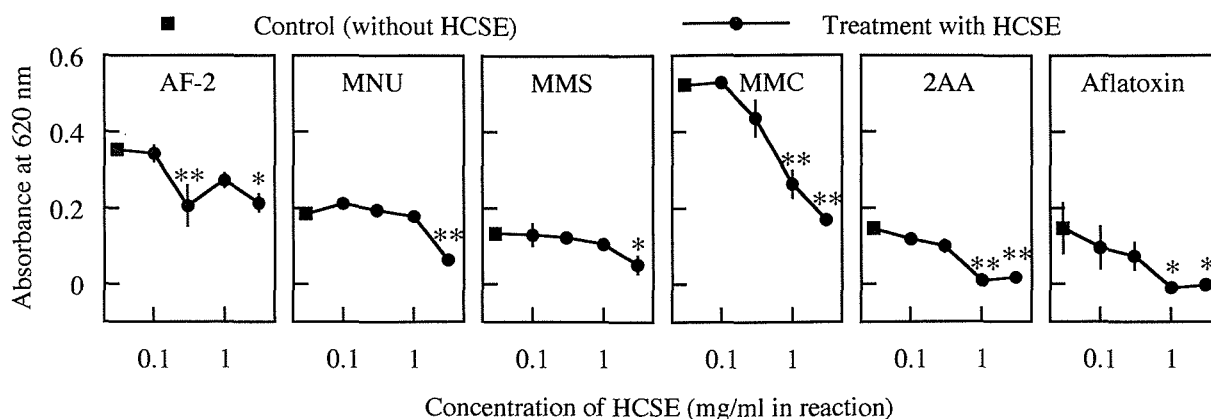


Fig. 2. Antigenotoxic effects of the Japanese horse chestnut seed extract. Each symbol and vertical bar represents the mean and SD. Blank value had been subtracted from each value to indicate the net genotoxicity. \*:  $p < 5\%$ , \*\*:  $p < 1\%$ .

mg/ml for the orange juice, 5.5 mg/g for the cherry and 8.4 mg/g for the chocolate [22]. The Japanese horse chestnut seed was estimated to contain 13 mg/g polyphenols. This suggests that the horse chestnut seed is one of the most polyphenol-rich food materials.

Antioxidative activities of dietary constituents have often been compared with Trolox, a water-soluble derivative of vitamin E. However, we used (+)-catechin as a reference antioxidant because (+)-catechin is a natural polyphenolic compound found in plants and is used for the standard in determining the total polyphenol in foods or beverages. The antioxidative activities of the HCSE were inferior to those of (+)-catechin except for the inhibition of lipid oxidation. The  $EC_{50}$ s of the HCSE on DPPH and superoxide were 260 and 45 times as high as those of (+)-catechin, respectively. This may be caused by the crude nature of the HCSE, which contained polyphenols of only 21.3 mg/g. Therefore, the antioxidative activity of the HCSE was by no means inferior to that of (+)-catechin when compared by the amount of polyphenols. On the other hand, the oxidation of linoleic acid was completely inhibited by the HCSE but not by (+)-catechin. This suggests that antioxidative constituents of the horse chestnut seeds strongly inhibit the lipid oxidation in particular. Although the antioxidative effects may be due to the polyphenolic compounds of the HCSE, other antioxidants may contribute to the effects because the horse chestnut seed contains various chemicals such as saponin.

The umu test detects the SOS response induced by various DNA damage in *S. typhimurium* TA1535/pSK1002. The results of this test coincide well with the Ames test [15], and it has widely been used for screening the genotoxicity of chemicals. In the present study, the HCSE inhibited the genotoxicities of various types of mutagens: a nitrofur derivative (AF-2), an aromatic amine (2AA), alkylating agents (MNU and MMS), a cross-linking agent (MMC), and a carcinogenic micotoxin (aflatoxin B1). The antigenotoxic effect of HCSE may result from the action of polyphenols, because there are many evidences that the polyphenolic

compounds have antimutagenic or antigenotoxic effects *in vitro* and *in vivo* [6, 7, 20, 21, 28]. Several mechanisms have been proposed to explain the antimutagenic effect of polyphenols, eg. scavenging the reactive oxygen species or radicals, decreasing the generation of hydroxyl radicals, modifying the DNA repair pathway after DNA damage, inhibiting the formation of DNA adducts or methylation [3, 13, 26]. Antioxidation may contribute to the antigenotoxic effect of HCSE because both effects appeared at a similar concentration (about 1 mg/ml), however, several other mechanisms may also be implicated in the antimutagenic effect of HCSE.

The Japanese horse chestnut seed was identified as a polyphenol-rich, antioxidative and antigenotoxic botanical resource. Consumption of polyphenol-rich foods or beverages elevates the antioxidative capacity and decreases the oxidation damage in living systems [8, 12, 23]. It may lower the risk of cancers in humans who are exposed potentially to various chemicals through the food, water and air [9–11, 14, 29, 30]. Thus the seed of Japanese horse chestnut might contribute to human health if it were utilized for a food material.

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