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Inhibitory effects of herbal teas and herb extracts on the mutagenicity of 1-methyl-1, 2, 3, 4-tetrahydro-β-carboline-3-carboxylic acid upon treatment with nitrite in the presence of ethanol

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Summary

The mutagenicity of 1-methyl-1, 2, 3, 4-tetrahydro-β-carboline-3-carboxylic acid (MTCCA), a major mutagen precursor in soy sauce, upon treatment with nitrite and ethanol was considerably reduced by the addition of herbal tea or herb extracts in the reaction mixture when it was treated with 50 mM nitrite at pH 3, 37 ℃ for 60 min in the presence of 7.5% ethanol. Among the herbal teas tested, *Banaba* and *Tencha* teas showed strong mutagenicity-reducing activity, and *Kakinoha*, *Kakidooshi* and *Yomogi* teas, and *Tochu* and *Senna* teas also showed moderate and weak antimutagenicity, respectively, in the Ames *Salmonella* mutagenicity test. Abundant amounts of typical polyphenols such as catechins were detected in the highly antimutagenic herbal teas. The antimutagenicity and the reducing power of herbal teas were positively correlated. Among the herb extracts tested, *Jiou* extract showed strong antimutagenicity and *Touki* extract was mildly antimutagenic. *Oubaku* extract showed strong bactericidal activity because of its high content of alkaloid berberine. Diluted *Oubaku* extract showed dose-dependent antimutagenicity. These results suggest that the mutagenicity of MTCCA upon treatment with nitrite in the presence of ethanol is decreased by mixed fractions containing polyphenols such as catechins, which have strong reducing power, and other compounds such as derivatives of catechins, which have little reducing power.

Keywords : antimutagenicity, herb, nitrosation, soy sauce, ethanol

Abbreviations : MTCCA, 1-methyl-1, 2, 3, 4-tetrahydro-β-carboline-3-carboxylic acid ; EC, (−)-epicatechin ; ECG, (−)-epicatechin gallate ; EGC, (−)-epigallocatechin ; EGCG, (−)-epigallocatechin gallate ; FIA, flow-injection analysis ; HPLC, high-performance liquid chromatography.

Introduction

Carcinogenic *N*-nitroso compounds are known to be easily produced under acidic conditions in the stomach and are suspected to cause stomach cancer (Magee and Barnes, 1967 ; Hartman, 1982). When various Japanese foodstuffs were treated with nitrite, soy sauce was found to have the highest mutagenicity and is therefore very important in the relationship between consumption of Japanese food and the high gastric cancer mortality rate in Japan (Wakabayashi et al., 1983). Tyramine is a major mutagen precursor in soy sauce treated with nitrite, and 1-methyl-1, 2, 3, 4-tetrahydro-β-carboline-3-carboxylic acid (MTCCA) is a minor one (Ochiai, et al., 1984 ; Higashimoto et al., 1988). However, MTCCA becomes the most potent mutagen precursor in soy sauce when it is treated with nitrite in the presence of ethanol, while the

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Table 1 Recommended medicinal applications of herbs used in Japan

Japanese name	English name	Applications[a]
Banaba	banaba leaf[b]	obesity, diabetes
Hatomugi	Job's tears	obesity, liver spots, acne, skin eruption, wart, neuralgia
Juyaku	houttuynia herb	obesity, constipation, acne, chapping, skin eruption, feeling of cold, arteriosclerosis
Kakidooshi	ground ivy	edema, obesity, nephropathy
Kakinoha	persimon leaf[b]	hypertension, arteriosclerosis, heart failure, chapping
Senna	senna leaf	constipation, piles, skin eruption
Sugina	horsetail	constipation, edema, skin trouble, cystitis, urethritis, nephropathy, asthma
Tencha	tian cha tea[b]	pollinosis, allergic disease, asthma
Tochu	eucommia leaf[b]	obesity, feeling of cold, hypertension, chapping
Yomogi	mugwort	feeling of cold, shoulder discomfort, low back pain, abdominal pain
Jiou	rehmannia root	anemia, hematemesis, nephropathy, heart disease, women's disease
Ninjin	ginseng	coronary arteriosclerosis, angina pectoris, myocardial infarction, hypertension
Oubaku	phellodendron bark	gastritis, enteritis, myalgia
Touki	Japanese angerica root	loss of vitality, women's disease, pain
Yokuinin[c]	coix seed	same as Hatomugi

[a] Mitsuhashi (1988), Hotta (1989), Uno (1997).

[b] named by Yamada Yakken Co., Ltd..

[c] Chinese medicinal name of the seed of Hatomugi (Job's tears).

mutagenicity induced by nitrite-treated tyramine is strongly decreased in the presence of ethanol (Higashimoto et al., 1995, 1996). It is very likely that a large amount of mutagens may be produced in the stomach of a person who consumes alcoholic beverages while eating food cooked with soy sauce, whereas the results of epidemiological studies have shown that habitual drinking does not necessarily increase the risk of stomach cancer (Hirayama, 1977 ; Kato et al., 1990). We therefore speculated that some anticarcinogens in the diet (Hayatsu et al., 1993) reduce the mutagenicity produced by nitrite-treated MTCCA in the presence of ethanol.

It has been shown that green, black and oolong teas derived from *Camellia sinensis* inhibit *N*-nitrosation (Wu et al., 1993 ; Tanaka et al., 1998) and have antimutagenic and antitumor activities (Jain et al., 1989 ; Hayatsu et al., 1993 ; Yang and Wang, 1993 ; Weisburger et al., 1996 ; Chung, 1999). The antimutagenic and anticarcinogenic activities are mainly due to the actions of polyphenols such as catechins in the teas (Kuroda and Hara, 1999a, 1999b). In addition, much attention has been given in recent years not only to green, black and oolong teas but also to many kinds of herbal teas for their various health-promoting effects (Uno, 1997 ; Craig, 1999). We previously reported that the mutagenicity of MTCCA treated with nitrite in the presence of ethanol was decreased by the addition of citrus fruits (Higashimoto et al., 1998) and by the addition of green, black or oolong teas (Higashimoto et al., 2000) to the reaction mixture. We concluded in our previous papers that the main antimutagens in the citrus fruits and teas are dietary fibers and polyphenols, respectively. In the present study, we found that the mutagenicity of nitrite-treated MTCCA in the presence of ethanol was considerably decreased by the addition of some herbal teas or herb extracts to the nitrosation reaction.

Materials and methods

Herbal teas and chemicals

Ten commercial dried herb products prepared from *Banaba* (*Lagerstroemia speciosa* Pers.), *Hatomugi* (*Coix lacryma-jobi* L. *var. ma-yuen* Stapf), *Juyaku* (*Houttuynia cordata* Thunb.), *Kakidooshi* (*Glechoma hederacea* L. *var. grandis* Kudo), *Kakinoha* (*Diospyros kaki* Thunb.), *Senna* (*Cassia angustifolia* Vahl.), *Sugina* (*Equisetum arvense* L.), *Tencha* (*Rubus suavissimus* S. Lee), *Tochu* (*Eucommia ulmoides* Oliv.) and *Yomogi* (*Artemsia princeps* Pampan.) were provided by Yamada Yakken Co., Ltd. (Osaka, Japan). Loose tea leaves were packed in commercial tea bags (Tokiwa Industry Co., Ltd., Ehime, Japan), and teas in bags were used as such. The tea leaves were weakly boiled for 10 min in one liter of water per 5 g and then removed. The herbal teas were used for experiments after cooling.

Herb extracts were prepared from 330 g of each herb with 2.2 liters of 50% 1,3-butylene glycol aqueous solution at room temperature by Yamada Yakken Co., Ltd.. Bottled herb extracts from *Jiou* (*Rehmannia glutinosa* Libosch.), *Ninjin* (*Panax ginseng* C. A. Meyer), *Oubaku* (*Phellodendron amurense* Rupr.), *Touki* (*Angelica acutiloba* Kitagawa) and *Yokuinin* (*Coix lacryma-jobi* L. *var. ma-yuen* Stapf) were used as such.

The 15 herbs used in the present study are all popular folk medicines in Japan and are used for various applications (Table 1).

MTCCA (CAS No. 5470-37-1), (−)-epicatechin (EC ; CAS No. 490-46-0), (−)-epicatechin gallate (ECG ; CAS No. 1257-08-5), (−)-epigallocatechin

(EGC ; CAS No. 970-74-1) and (−)-epigallocatechin gallate (EGCG ; CAS No. 989-51-5) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). (+)-Catechin (CAS No. 154-23-4) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Gallic acid (CAS No. 149-91-7) and berberine chloride (CAS No. 633-65-8) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The other chemicals were of reagent grade.

Nitrite treatment

Chemicals were dissolved or suspended in sterilized water. One milliliter of aqueous solution containing 0.6 mg of MTCCA, 0.15 m*l* of ethanol and 0 - 0.4 m*l* of one of the herbal teas or herb extracts was mixed in a brown tube with 1 m*l* of 0.1 M sodium nitrite and adjusted to pH 3.0 with 6 N HCl and by monitoring using a pH meter equipped with a small electrode. The reaction mixtures were incubated at 37 ℃ for 60 min in the dark. Nitrosation was stopped by the addition of 1 m*l* of 0.1 M ammonium sulfamate to decompose the residual nitrite. All nitrite treatments were performed in triplicate. Each nitrite-treated sample was immediately used for the mutation assay.

Mutation test

The mutation test with *Salmonella typhimurium* strain TA100 was conducted in duplicate by the preincubation procedure of Maron and Ames (1983) in the absence of S9 mix. A mixture containing 0.1 m*l* of a nitrite-treated sample, 0.5 m*l* of buffer solution (pH 7.4) and 0.1 m*l* of an overnight culture of strain TA100 was preincubated at 37 ℃ for 20 min, mixed with 2 m*l* of soft agar, and plated on an agar plate. The His^+ revertant colonies were counted after incubation at 37 ℃ for 48 hr. The mutation was assayed in brown tubes under a yellow lamp in a dark room. The numbers of spontaneous revertants (118 ± 10) were subtracted from all the data on mutagenicity. A positive control, 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2, 10 ng), generated 431 ± 36 His^+ revertants.

High-performance liquid chromatography

Six catechin monomers (EGCG, EGC, ECG, EC, catechin and gallic acid) in the herbal teas and herb extracts were detected by high-performance liquid chromatography (HPLC). HPLC was performed using a Shimadzu LC-6A equipped with a UV monitor set usually at 270 nm. The column oven was maintained at 50 ℃. Separation was conducted with a Shim-pack ODS column (6 mm i.d. × 150 mm) using a mobile phase (1 m*l*/min) of 10 mM phosphate buffer containing acetonitrile, whose content was linearly increased from 0 to 30 % over a period of 45 min.

Reducing power

Polyphenols such as catechins reduce Fe (III) to Fe (II), which subsequently reacts with 1,10-phenanthroline to form a colored complex (Tomàs et al., 1993). The intensity of the absorbance by the colored product, namely a reducing power, reflects the polyphenol contents in herbal teas. The reducing power of each of the herbal teas was determined according to the flow-injection spectrophotometric method of Tomàs et al. Each tea extract to which 0.01 M iron (III) chloride had been added was mixed with 0.5 M acetic acid and subsequently mixed with 0.05 M 1, 10-phenanthroline in the flow-injection apparatus. The difference between the maximal absorption (510 nm) and zero absorption (680 nm) derived from the colored complex iron (II)-1, 10-phenanthroline was monitored. The data were processed with a computer system to calculate the corresponding analytical concentrations. A calibration was constructed from standards containing gallic acid.

Statistical analysis

The data were analyzed statistically by analysis of variance. The correlation coefficient was calculated by the formula of Pearson.

Results

Mutagenicity-reducing activity of herbal teas

The mutagenicity of MTCCA treated with nitrite in the presence of ethanol was strongly decreased by the addition of 0.1 - 0.4 m*l* of *Banaba* or *Tencha* teas in 2 m*l* of the nitrosating reaction mixture as shown in Fig. 1. The mutagenicity was significantly and dose-dependently decreased by the addition of *Kakinoha*, *Kakidooshi* and *Yomogi* teas at almost all the dose levels tested. *Tochu* and *Senna* teas also showed significant antimutagenicity at high dose levels (0.3 - 0.4 m*l*). The addition of maximum doses of *Banaba*, *Tencha*, *Kakinoha*, *Yomogi*, *Kakidooshi*, *Tochu* and *Senna* teas reduced the levels of mutagenicity to 3.5, 4.5, 18.3, 36.0, 45.4, 51.1 and 58.7 % of the levels in the absence of those teas.

Reducing power of herbal teas

The correlation between the reducing power and the antimutagenicity of the ten herbal teas is shown in Fig. 2. The antimutagenicity of the tea extracts showed a significant positive correlation with the reducing power as a whole ($r = 0.824, p = 0.01$).

Catechin contents in the herbal teas

Six catechin monomers (EGCG, EGC, ECG, EC, catechin and gallic acid) in the ten herbal teas were detected by HPLC. Among the herbal teas, *Banaba*, *Kakidooshi*, *Kakinoha*, *Tencha* and *Yomogi* teas, which had strong antimutagenicity against the nitrite-treated

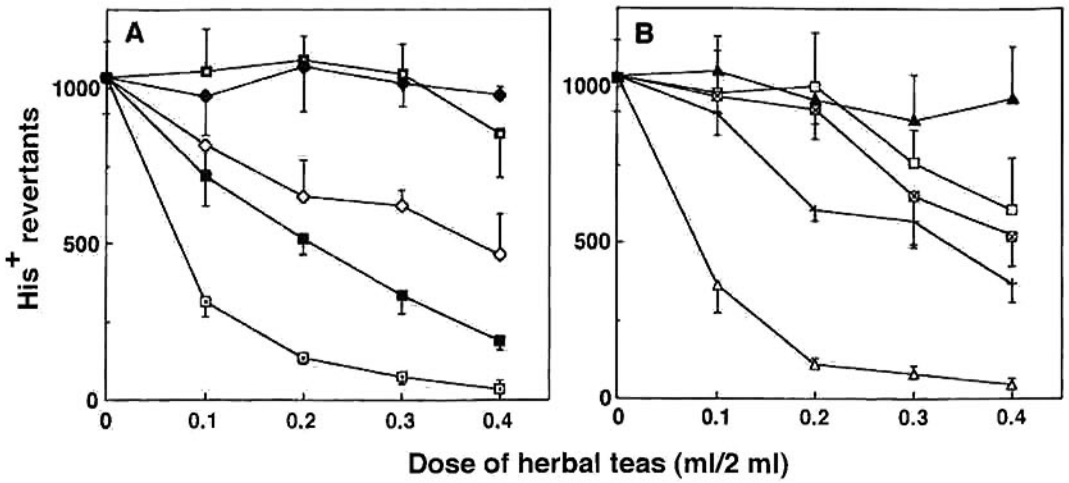

Fig. 1 Antimutagenicity of herbal teas against the mutagenicity of MTCCA upon treatment with nitrite in the presence of ethanol
(A) —⊡— *Banaba*, —◆— *Hatomugi*, —⊡— *Juyaku*, —◇— *Kakidooshi*, —■— *Kakinoha*
(B) —□— *Senna*, —▲— *Sugina*, —△— *Tencha*, —⊠— *Tochu*, —+— *Yomogi*
A 0.1 - 0.4 m*l* aliquot of herbal tea was mixed in 2 m*l* of the nitrosating reaction mixture

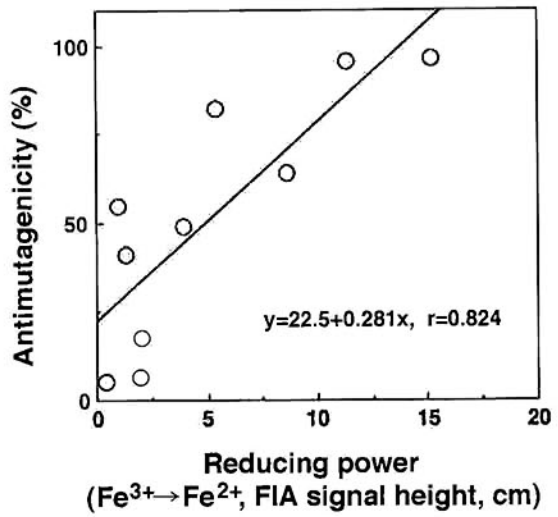

Fig. 2 Correlation between antimutagenicity and reducing power of herbal teas.
FIA : flow-injection analysis

Table 2 Catechin contents detected in typical herbal teas (μg/m*l*)

Herbal tea	EGCG	EGC	ECG	EC	catechin	gallic acid
Banaba	115.2	11.5	1.3	9.9	18.1	2.0
Kakidooshi	0.8	1.9	14.4	11.9	4.1	0.2
Kakinoha	71.0	27.2	14.8	6.0	4.4	1.6
Tencha	13.2	14.5	19.8	18.5	10.2	n.d.[a]
Yomogi	7.7	10.6	7.0	n.d.[a]	n.d.[a]	4.2

[a] not detected.
HPLC conditions ; HPLC, Shimadzu LC-6A ; Detector, UV 270 nm ; column, Shim-pack ODS, 6.0 mm i.d. × 150 mm ; mobile phase, 10 mM phosphate containing acetonitrile (linear gradient from 0 to 30% in 45 min) ; flow rate, 1 m*l*/min.

MTCCA in the presence of ethanol, contained considerably high levels of the six typical catechins listed in Table 2. The other herbal teas contained very low levels of the catechins (data not shown).

Mutagenicity-reducing activity of herb extracts

As shown in Fig. 3, herb extract prepared from *Jiou* strongly decreased the mutagenicity of MTCCA treated with nitrite and ethanol, and *Touki* extract had mild antimutagenicity. The strong decrease in the mutagenicity of nitrite-treated MTCCA in the presence of *Oubaku* extract was a false one because *Oubaku* extract had strong bactericidal activity. When fivefold-diluted *Oubaku* extract was added to the reaction mixture, it showed dose-responsive antimutagenicity without

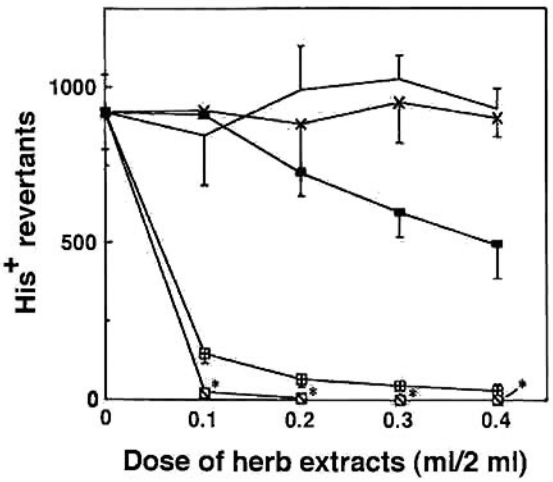

Fig. 3 Antimutagenicity of herb extracts against the mutagenicity of MTCCA upon treatment with nitrite in the presence of ethanol
—⊞— *Jiou*, —✕— *Ninjin*, —⊠— *Oubaku*, —■— *Touki*, —— *Yokuinin*
* Bacterial growth in the background culture was found to be inhibited to a great extent
A 0.1 - 0.4 m*l* aliquot of herb extract was mixed in 2 m*l* of the nitrosating reaction mixture

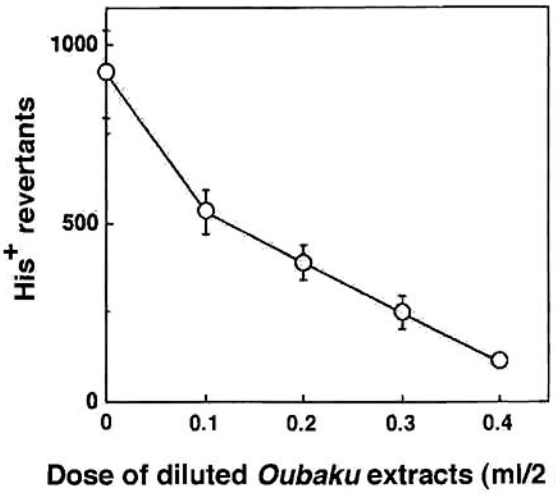

Fig. 4 Antimutagenicity of fivefold-diluted *Oubaku* extract against the mutagenicity of MTCCA treated with nitrite in the presence of ethanol
A 0.1 - 0.4 m*l* aliquot of fivefold-diluted *Oubaku* extract was mixed in 2 m*l* of the nitrosating reaction mixture

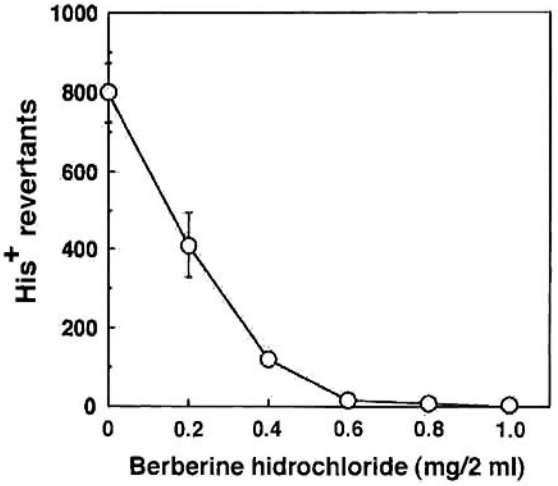

Fig. 5 Antimutagenicity of berberine against the mutagenicity of MTCCA treated with nitrite in the presence of ethanol
A 0.2 - 1.0 mg berberine hydrochloride was mixed in 2 m*l* of the nitrosating reaction mixture

bactericidal activity (Fig. 4). The antimutagenicity of *Oubaku* extract was thought to be a result of the high content (3.15%) of berberine, which had strong antimutagenicity for MTCCA treated with nitrite in the presence of ethanol as shown in Fig. 5.

Catechin contents of herb extracts

Catechin monomers were not detected because of the high contents of unknown ingredients in the *Jiou* extract. *Touki* and the other herb extracts contained less than the detection limits of catechins.

Discussion

The mutagenicity of MTCCA treated with nitrite in the presence of ethanol was strongly and dose-dependently decreased by the addition of some herbal teas to the reaction mixture. It is reported that the antimutagenicity of teas is closely related to their polyphenols such as catechins, namely, reducing components in the teas (Kada et al., 1985 ; 1989 ; Hayatsu et al., 1993 ; Yang and Wang, 1993 ; Apostolides et al., 1996). The antimutagenicity of the herbal teas used in the present study was positively correlated with their reducing power (Fig. 2) as reported previously (Yen and Chen, 1995 ; Higashimoto et al., 2000). The green tea polyphenol fraction containing six major catechins (49% EGCG, 14% ECG, 11% EGC, 6% EC, 2% catechin and 0.3% gallic acid) mainly accounts for the antimutagenicity of green tea (Ho et al., 1994). Although each herb is a different species of plant, as described above, the five herbal teas, which showed strong mutagenicity-reducing activity against MTCCA treated with nitrite in the presence of ethanol, were also found to contain abundant

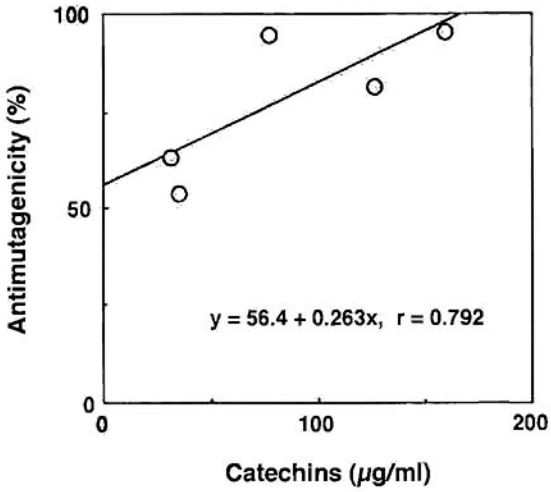

Fig. 6 Correlation of antimutagenicity and the contents of six catechins in five typical herbal teas

amounts of the six catechins. The antimutagenicity and the contents of the six catechins in the five herbal teas were correlated well as shown in Fig. 6, although about half of the antimutagenicity was associated with another factor(s). Among the herbal teas, *Banaba* and *Tencha* teas showed strong antimutagenicity. They have been used as folk medicines for treating obesity and diabetes, and pollinosis, allergic disease and asthma, respectively (Table 1) .

All of the herb extracts used in this study are products from Chinese medicinal plants. Among the herb extracts, *Oubaku* and *Jiou* extracts showed strong antimutagenicity. *Oubaku* is known as a bacteriostatic herb because it contains a large amount of berberine, an antibiotic alkaloid. The original *Oubaku* extract, which

had strong bactericidal activity (Fig. 3), showed dose-responsive antimutagenicity when it was diluted fivefold (Fig. 4), while the antimutagenicity was considerably moderate compared with that of the authentic berberine hydrochloride (Fig. 5). *Oubaku* extract is therefore more suited for natural medicinal or cosmetic use than as a food ingredient. Catechin monomers in herb extracts could not determined well by our HPLC system. The antimutagenicity of *Jiou* and *Touki* extracts may be due to some polyphenol species such as catechin derivatives and flavonoids. Further work is needed to clarify the antimutagens in them.

Tea is one of the most popular beverages consumed worldwide. Epidemiological studies have indicated that habitual drinking of green tea may diminish the risk of stomach cancer (Oguni et al., 1989 ; Fujiki et al., 1998). In recent years, various herbal teas, as well as green, black and oolong teas, have become popular due to their beneficial health effects such as prevention of allergic disease, obesity, diabetes, nephrosis and arteriosclerosis (Table 1). Although there is still a lack of scientific proof (Winslow and Kroll, 1998 ; Craig,1999), habitual drinking of herbal teas over a long period of time does seem to have beneficial health effects. Our results also suggest that some herbal teas have health-promoting activity due to their antimutagenic activity in an acidic environment such as that inside the stomach.

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