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Original article

Investigation of herbal extracts that have both OPH activity enhancing action and AGE crosslink cleaving activity

Hiroshige Kawai [1)], Naoki Matsuo [1)], Eiji Yuasa [1)], Kaori Ishizaki [2)], Masayuki Yagi [2)]

1) Karada Lab, Inc. (Arkray Group), Kyoto, Japan
2) Anti-Aging Medical Research Center/Glycative Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Abstract

***Objective*:** Accumulation of advanced glycation end products (AGEs) produced by glycative stress promotes the progression of age-related diseases. The potential methods to reduce already accumulated AGEs include enhancing the oxidized protein hydrolase (OPH) that originally exists in the body to promote the decomposition of the AGE-modified protein, and directly acting on the AGE crosslinks to cleave them. Therefore, this study investigated herbs that work to enhance OPH activity as well as to cleave AGE crosslinks.

***Methods*:** Hot water (80 °C) extracts of 6 kinds of herbs were used as the samples.

1) N-acetyl-L-alanine p-nitroanilide (AAPA) was used as a substrate for the OPH activity. The herbal extracts, OPH solution, AAPA solution and Tris-HCl buffer solution were mixed at a ratio of 1: 1: 2: 21 and left to react for 24 hours at 37 °C. Then, the absorbance of p-nitroaniline, which is produced by enzymatic degradation, at 405 nm was measured.

2) 1-Phenyl-1,2-propanedione (PPD), which has an α-diketone structure as a substrate, was used as the AGE crosslink cleaving model. The herbal extracts, PPD solution, and phosphate buffer solution were mixed at a ratio of 5 : 1 : 4 and left to react at 37 °C for 8 hours. Then, hydrochloric acid was added to the solution and the solution was centrifuged to obtain a supernatant. The benzoic acid in the supernatant, which was produced upon decomposition of PPD by the herbal extracts, was measured using the HPLC method.

***Results*:** The OPH activity enhancing action was found to be stronger in the order of fenugreek (*Trigonella foenum-graecum*) seeds (106.9 %), fennel (*Foeniculum vulgare*) seeds (81.8 %), hibiscus (*Hibiscus sabdariffa*) calyxes and bracts (63.0%). The AGE crosslink cleaving action was stronger in the order of fennel seeds (39.0 %), lemon balm (*Melissa officinalis*) leaves (29.6 %), rosemary (*Rosmarinus officinalis*) leaves and stems (26.6 %). In combination, OPH activity was on the attenuated side when even one herbal extract that attenuates the OPH activity was included.

***Conclusion*:** Fennel, fenugreek and hibiscus were found to have both OPH activity enhancing action and AGE crosslink cleaving activity. It was also suggested that depending on the combination of these herbs and their ratio, it may optimize both effects.

***KEY WORDS*:** oxidized protein hydrolase (OPH), advanced glycation end products (AGEs), crosslink cleaving activity, herb

Introduction

Advanced glycation end products (AGEs) are involved in aging and age-related diseases such as skin aging, Alzheimer's disease, hypertension, arteriosclerosis, and osteoporosis [1)]. Potential methods of reducing AGEs that have already accumulated in the body include 1) improving the metabolism of the body, that is, enhancing oxidized protein hydrolase (OPH) in the body to promote the decomposition of AGE-modified protein, and 2) directly cleaving the AGE crosslink in the body [2)].

Therefore, we examined of the effects of 6 types of herbs on the OPH activity enhancing action and the AGE crosslink cleaving activity.

Contact Address: Hiroshige Kawai
Karada Lab, Inc. (Arkray Group)
Yousuien-nai, 59 Gansuin-cho, Kamigyo-ku, Kyoto 602-0008 Japan
TEL: +81-50-5830-1040 FAX: +81-75-431-1253 e-mail: kawaih@arkray.co.jp
Co-authors: Matsuo N, matsuo.hv@arkray.co.jp; Yuasa E, yuasa.al@arkray.co.jp;
Ishizaki K, ko-sei12@mail.doshisha.ac.jp; Yagi M, myagi@mail.doshisha.ac.jp

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Methods

Reagents

As OPH, acylamino-acid releasing enzyme (AARE) (Takara Bio Inc., Kusatsu, Shiga, Japan) was used. *N*-acetyl-L-alanine *p*-nitroanilide (AAPA; Bachem AG, Bubendorf, Switzerland) was used as the enzyme substrate for OPH. 1-Phenyl-1,2-propanedione (PPD; Sigma-Aldrich Japan K.K., Meguro-ku, Tokyo, Japan) was used as a substrate for the AGE crosslink cleaving model. As a positive control for AGE crosslink cleaving activity, *N*-phenacyl thiazolium bromide (PTB; Fujifilm Wako Pure Chemical Corporation, Chuo-ku, Osaka, Japan) was used. Special grade reagents (Fujifilm Wako Pure Chemical Corporation and/or Nacalai Tesque, Inc., Nakagyo-ku, Kyoto, Japan) were used for the other regents.

Preparation of herbal extracts

Six types of dried herbs were used: thyme (*Thymus vulgaris*) leaves and stems, rosemary (*Rosmarinus officinalis*) leaves and stems, lemon balm (*Melissa officinalis*) leaves, fennel (*Foeniculum vulgare*) seeds, hibiscus (*Hibiscus sabdariffa*) calyxes and bracts and fenugreek (*Trigonella foenum-graecum*) seeds (***Table 1***). Two grams of each dried herb were soaked in 40 mL of distilled water at 80°C for 1 hour for extraction. The extract was cooled to room temperature and filtered. The obtained filtrate was used in measurements as an herbal extract. Further, in addition to the examination of a single herbal extract, mixtures of an equal amount of multiple herbal extracts were also examined in the same manner.

Measurement of solid concentration

The solid concentration of each herbal extract was calculated by placing 5 mL of herbal extract in an aluminum tray, drying it in an incubator at 110°C for 4 hours and measuring its residual weight.

Measurement of OPH activity enhancing action

According to the previous report [3], the measurements were carried out as follows:

The OPH solution of 0.5 U/mL was prepared using the sodium phosphate buffer included in the AARE kit. The AAPA solution of 0.05 mol/L was prepared using a 50% ethanol aqueous solution.

Ten µL of herbal extract, 10 µL of OPH solution, 20 µL of AAPA solution and 210 µL of 0.2 mol/L Tris-HCl buffer (pH 7.4) were mixed in a 96-well microplate (PerkinElmer, MA, USA). For each blank sample, 0.2 mol/L of Tris-HCl buffer (pH 7.4) was added instead of OPH solution to make the total amount 250 µL. Therefore, the herbal extract was diluted 25-fold for the measurements.

After that, the microplate was set in a microplate reader (SpectraMax Paradigm Multi-Mode Microplate Reader; Molecular Devices, CA, USA) and let the solution react at 37°C for 48 hours. The absorbance of *p*-nitroaniline, which is produced by enzymatic degradation, at 405 nm was measured continuously (***Fig. 1***). The OPH activity was evaluated based on the results after the reaction for 24 hours, and the activation rate was defined as the rate of increase or decrease from the OPH activity of the sample blank (distilled water was used instead of the herbal extract),

Table 1. Herbs.

General name	Japanese name	Family	Site of use	Scientific name
Thyme	Tachijakousou	Lamiaceae	Leaves, stems	*Thymus vulgaris*
Rosemary	Mannenrou	Lamiaceae	Leaves, stems	*Rosmarinus officinalis*
Lemon balm	Kousuihakka	Lamiaceae	Leaves	*Melissa officinalis*
Fennel	Uikyou	Apiaceae	Seeds	*Foeniculum vulgare*
Hibiscus	Hibiscus	Malvaceae	Calyx, bract	*Hibiscus sabdariffa*
Fenugreek	Koroha	Fabaceae	Seeds	*Trigonella foenum-graecum*

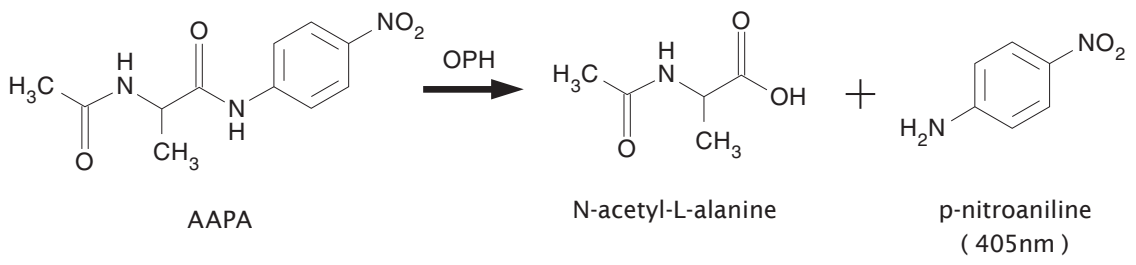

Fig. 1. **Measurement principle of OPH activity.**

OPH, oxidized protein hydrolase; AAPA, *N*-acetyl-L-alanine p-nitroanilide.

which was set as 100% as shown in the following formula. The measurements were performed 3 times and the results are shown as mean ± standard deviation.

OPH activation rate (%) = (A – B) / (C – D) x 100 – 100
A: sample solution + OPH + AAPA, B: sample solution + AAPA, C: OPH + AAPA, D: AAPA

Measurement of AGE crosslink cleaving activity

The evaluation was carried out based on the modified method[4] of Vasan *et al.*[5]. Briefly, 1-phenyl-1,2-propanedione (PPD), dissolved in 50% acetonitrile, was used as a reactive substrate in the AGE crosslink model that contains an α-diketone structure. For the measurement of AGE crosslink cleaving activity, 500 µL of the 10-fold diluted herbal extracts were mixed with 100 µL of 10 mmol/L PPD and 400 µL of 0.2 mol/L phosphate buffered saline (PBS: pH 7.4), and then incubated at 37°C for 8 hours. The reaction was stopped by adding 200 µL of 2 mol/L hydrochloric acid (HCl), then, it was centrifuged at 10,000 rpm (9,170 g) for 2 minutes. The benzoic acid amount in the supernatant was measured by high performance liquid chromatography (HPLC). An LC-10A analyzer (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) was used for HPLC. The analytical conditions were as follows:

Column: Cadenza CD-C18 75 x 4.6 mmID (Imtakt Corporation, Shimogyo-ku, Kyoto, Japan)
Eluate: 0.2% acetic acid/acetonitrile (70/30) containing 2 mmol/L ethylenediamine N, N, N', N'-tetraacetic acid-disodium salt (EDTA-2Na) -dihydrate
Flow rate: 1.0 mL/min
Column temperature: 40°C
Detection wavelength: UV 270 nm
Injection volume: 50 µL

When 1 mol of PPD breaks down, 1 mol of benzoic acid is formed (***Fig. 2***). The AGE crosslink cleaving activity was calculated using the following formula. The measurement was conducted three times and the results were expressed as mean ± standard deviation.

AGE crosslink cleaving activity rate (%) = ((A – B) / C) x 100
A: amount of benzonic acid after reaction, B: amount of benzonic acid in the herbal extract, C: amount of PPD added (mol)

Statistics analysis

The measurement values were expressed as mean value ± standard deviation. The statistics analysis was performed using IBM SPSS Statistics V23 (IBM Japan, Ltd. Chuo-ku, Tokyo, Japan), a statistics software. Tukey's multiple comparison test was conducted. The significance level was set to less than 5% in the two-tailed test.

Results

Solid concentration

The solid concentration of each herbal extract is shown in ***Table 2***.
Hibiscus (22.1 mg/mL) was the highest, with a concentration that was 1.5 to 2.0 times higher than the other 5 herbal extracts (11.0 to 14.5 mg/mL).

Table 2. Solid concentration of herbal extracts.

	Solid content (mg/mL)
Thyme	14.5
Rosemary	11.1
Lemon balm	13.2
Fennel	11.5
Hibiscus	22.1
Fenugreek	11.0

OPH activity enhancing action

The OPH activity enhancing action of each herbal extract is shown in ***Fig. 3*** and ***Table 3***.

As these are the values where the activity (100%) of the sample blank, which used distilled water instead of the herbal extracts, is assumed to be 0%, fenugreek, fennel, and hibiscus enhanced the OPH activity in this order while rosemary, thyme, and lemon balm impaired the OPH activity in this order

The results of the mixtures of an equal amount of multiple herbal extracts are shown in ***Table 4***. We also

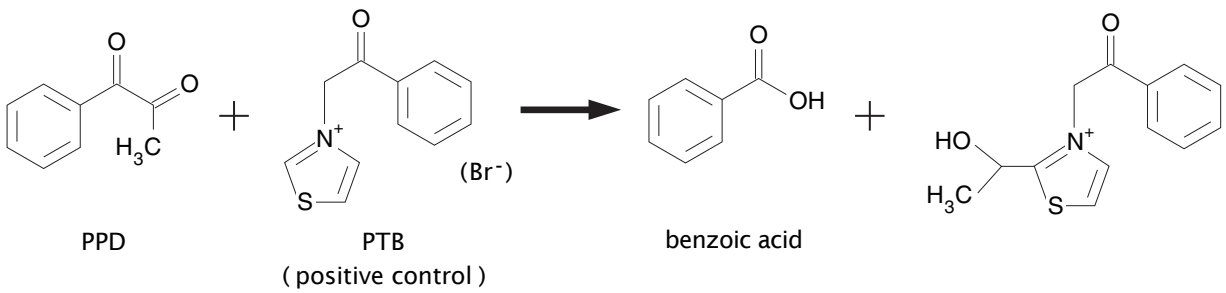

Fig. 2. **Measurement principle of AGE crosslink cleaving activity.**
AGE, advanced glycation end product; PPD, 1-Phenyl-1,2-propanedione; PTB, *N*-phenacyl thiazolium bromide.

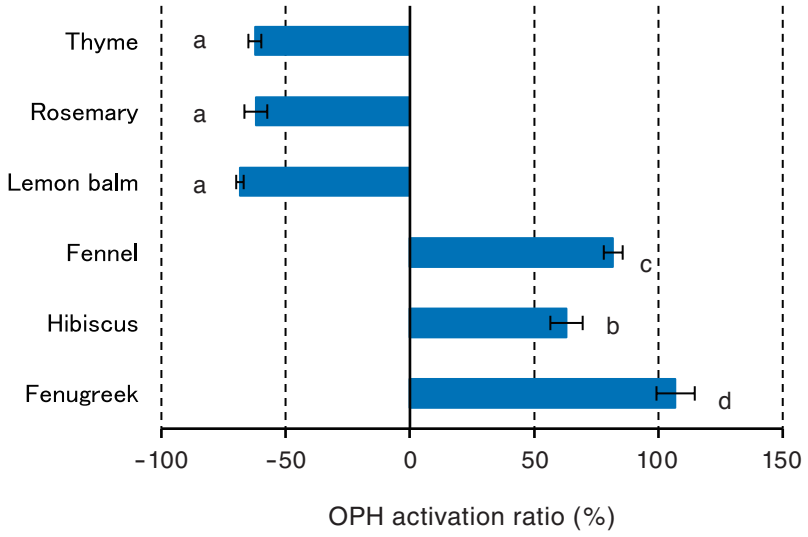

Fig. 3. **OPH activation ratio.**

Results are expressed as mean ± SD; n = 3; Tukey's multiple comparison test. Values with different letters are significantly different at $p < 0.05$. OPH activation ratio, Activation ratio of OPH to reference; OPH, oxidized protein hydrolase; SD, standard deviation.

Table 3. OPH activation ratio.

	Concentration (mg/mL)	Activation ratio [1] (%)
Thyme	0.58	-62.4 ± 2.6
Rosemary	0.44	-62.0 ± 4.6
Lemon balm	0.53	-68.4 ± 1.5
Fennel	0.46	81.8 ± 3.8
Hibiscus	0.88	63.0 ± 6.5
Fenugreek	0.44	106.9 ± 7.7

1) Activation ratio: mean ± SD, n = 3. OPH, OPH, oxidized protein hydrolase; SD, standard deviation.

Table 4. OPH activation ratio in combination of herbal extracts

	Concentration (mg/mL)	Activation ratio [1] (%)	Theoretical value [2] (%)
Mixing the same amount of thyme, rosemary and lemon balm extract	0.52	-47.7 ± 1.8	-64.3
Mixing the same amount of fennel, hibiscus and fenugreek extract	0.59	78.8 ± 12.2	83.9
Mixing the same amount of all 6 extracts	0.56	-67.0 ± 5.8	9.8
Mixing the same amount of lemon balm and hibiscus extract	0.71	-60.7 ± 2.4	-2.7
Mixing the same amount of thyme and fenugreek extract	0.51	-45.2 ± 4.1	22.3

1) Activation ratio, mean ± SD, n = 3. OPH, oxidized protein hydrolase; SD, standard deviation.
2) The theoretical values are a mean of the measured values of each individual herbal extract.

compared the following with the theoretical value of the average of the measured values for each of the single herbal extracts. Regarding the mixture of the 3 extracts with impairing effects, the measured value was −47.7% as opposed to the theoretical value of −64.3%. Meanwhile, regarding the mixture of the 3 extracts with enhancing effects, the theoretical value was 83.9% as opposed to the measured value of 78.8%. Therefore, both theoretical and measured values were relatively close to each other. However, when the extracts with impaired and enhanced effects were combined, the measured values were −67.0 to −45.2% against the theoretical values of −2.7 to 22.3%, which was a substantial shift to the impaired side.

AGE crosslink cleaving activity

The AGE crosslink cleaving activity of each herbal extract is shown in ***Fig. 4*** and ***Table 5***.

The average AGE crosslink cleaving activity rate was highest in fennel, followed by lemon balm, rosemary, thyme, fenugreek, and hibiscus.

Discussion

It has been reported that rosmarinic acid [6, 7] and carnosic acid [7] have the AGE crosslink cleaving activity. It is considered that rosmarinic acid and carnosic acid contained in rosemary, and rosmarinic acid contained in lemon balm and thyme contributed to this action.

In general, compounds that have a high AGE crosslink cleaving activity have high reactivity, and thus it is considered that they tend to inhibit enzymes conversely. In one example, catechins such as epigallocatechin gallate and epigallocatechin, which are contained in green tea (tea plant, *Camellia sinensis*) have been confirmed to have a high AGE crosslink cleaving activity compared to other typical plant components [7]. On the other hand, it has been reported that all 6 types of green tea have an inhibitory effect on OPH activity [3].

In this study, we tried to find herbal extracts that have both OPH activity enhancing action and AGE crosslink cleaving activity. This is because simultaneous action of these two actions in the living body is expected to have a

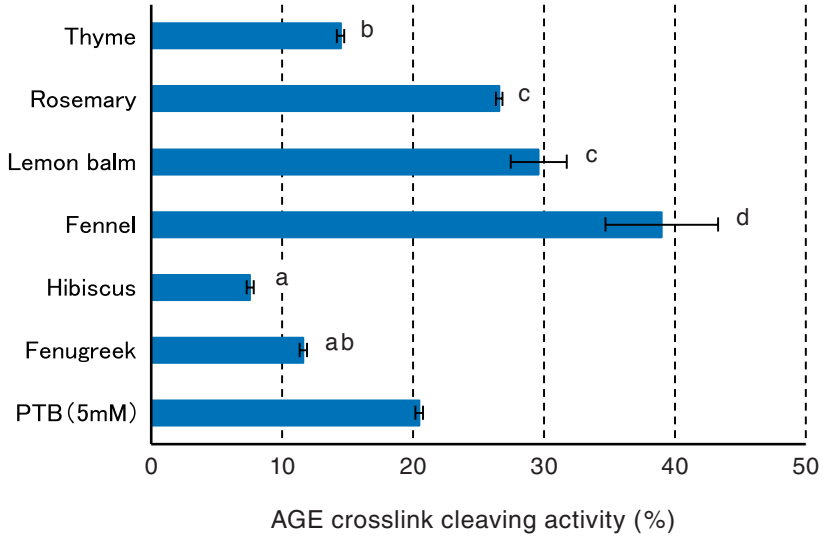

Fig. 4. **AGE crosslink cleaving activity.**

Results are expressed as mean ± SD; n = 3; Tukey's multiple comparison test. Values with different letters are significantly different at $p < 0.05$. AGE, advanced glycation end product; SD, standard deviation.

Table 5. AGE crosslink cleaving activity

	Concentration (mg/mL)	Cleaving activity [1] (%)
Thyme	1.45	14.5 ± 0.3
Rosemary	1.11	26.6 ± 0.3
Lemon balm	1.32	29.6 ± 2.1
Fennel	1.15	39.0 ± 4.3
Hibiscus	2.21	7.6 ± 0.3
Fenugreek	1.10	11.6 ± 0.3
PTB (5mmol/L)	–	20.5 ± 0.3

1) Cleaving ratio, mean ± SD, n = 3. AGE, advanced glycation end product; PTB, *N*-phenacyl thiazolium bromide; SD, standard deviation.

higher AGEs degradation effect.

As a result, both effects were observed in fennel, fenugreek and hibiscus. Fennel contains anethole, anisaldehyde, *etc*.[8], fenugreek contains trigonelline, diosgenin, *etc*.[9], and hibiscus contains citric acid, anthocyanins, *etc*.[10]. However, it is currently unknown which components contribute to the 2 actions. Melatonin has been reported to have an AGE crosslink cleaving activity [7], and it is also contained in fennel and fenugreek, yet, its contents are at quite a low level of 28 ng/g and 43 ng/g, respectively [11]. The melatonin concentrations were different by more than 10,000-fold when comparing their experimental conditions, therefore, it was suggested that its contribution was low.

Furthermore, the examination of how combinations of herbs affect the OPH activity enhancing action revealed that combinations that include even one herb that impairs the OPH activity also impaired the activity of the combined herbs regardless of the types and number of herbs as far as these combination methods were concerned. This was consistent with the above-mentioned assumption that the enzyme was inhibited by the inclusion of highly reactive compounds.

The only combination with enhanced activity this time was a mixture that contained equal amounts of fenugreek, fennel and hibiscus. However, it is considered to be possible that even higher activity could be obtained by changing the mixing ratio.

Conclusion

Fennel, fenugreek and hibiscus were found to have both OPH activity enhancement and AGE crosslink cleaving actions. It was also suggested that different combinations of these herbs and the ratios may optimize both effects.

Conflict of interest statement

This research was funded by Arkray, Inc.

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