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Original article

Screening and selection of anti-glycative materials: Kuromoji (*Lindera umbellata*)

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Abstract

***Aim*:** We participated in the Cross-ministerial Strategic Innovation Promotion Program (SIP). SIP aims to find materials made from agricultural crops which have new functions, and to realize the social implementations to launch new products into markets. This study selected, from plant-derived materials, materials which have strong inhibitory activities against the formation of advanced glycation end products (AGEs) and high quality of intestinal absorption. Furthermore, we examined whether or not these materials exerted anti-diastatic actions in experiments with diabetic animal models.

***Methods*:** From extracts of 536 types of plant-derived materials, this study selected materials with strong inhibitory activities against fluorescent AGE formation ("AGE inhibition"), which were produced in the reaction between human serum albumin (HSA) and glucose (comparison in each IC_{50}). Screening was conducted for the examination of AGE inhibition in culture fluid which passed through intestinal epithelial cells (Caco-2), and furthermore, the AGE inhibition in serum of rats following oral administration. Aminoguanidine was used as a positive control. Test materials were administered to streptozotocin (STZ)-induced diabetic rats for 8 weeks. Subsequently, this study examined the metabolic index of glycolipids, diabetic nephropathy index and cataract stage.

***Results*:** Kuromoji (*Lindera umbellata*) and yomogi (*Artemisia indica* var. *maximowiczii*) were selected by screening, as AGE inhibition following an absorption test was the same level as aminoguanidine. STZ treated rats (control) showed, in comparison with rats without STZ treatment, increases in blood glucose, glycoalbumin (GA), triglyceride (TG) and free fatty acid (FFA), increases in renal tissue TNF-α/IL-6 and decreases in creatinine clearance (CCr). Administration of kuromoji extract significantly improved TG and FFA. TNF-α/IL-6 in renal tissues and CCr were also significantly improved. Kuromoji and yomogi extracts both showed effects on the prevention of onset and progression of cataracts.

***Conclusion*:** This study concluded that kuromoji extracts, which were screened and selected in terms of high anti-glycative activity and characteristic of intestinal absorption, had the effects of prevention of onset and progression of nephropathy and cataracts in the diabetic rat model. Further examination of kuromoji has been decided to continue for the purpose of social implementations.

***KEY WORDS*:** kuromoji (*Lindera umbellata*), Japanese mugwort (yomogi; *Artemisia indica* var. *maximowiczii*), advanced glycation end products (AGEs), diabetic nephropathy, cataract

Introduction

We participated in the Cross-ministerial Strategic Innovation Promotion Program (SIP). We are a member of the Development of Functional Agricultural and Food Products for the Next Generation Program, Technologies for Creating Next-Generation Agriculture, Forestry and Fisheries of SIP. The purpose of this project is to contribute to the vitalization of agriculture, forestry and fishery, acquiring new next-generation functionality in agricultural, forest and fishery products and providing added value to the products. The budget for this program is as much as 2.5 billion yen for five years, which is the largest scale in Japan as a program for the development of functional food and the survey of medical evidence. Glycative stress has been positioned as a risk factor for both. It is highly expected that new functional

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food with anti-glycative activity should be developed.

Our research center has examined more than 500 types of food materials. Anti-glycative materials have been explored [1-3] to measure the inhibitory activities against the formation of advanced glycation end products (AGEs), where glycation reaction model *in vitro* was employed with human serum albumin (HSA) as a target protein. Therefore, this study has identified more than 100 types of materials with inhibitory activities against the AGE formation. The identified materials had the same as or higher activities than aminoguanidine than the positive control in the HSA glycation model. The SIP requires, other than experimental outcomes, the performance of the social implementation to develop and launch new products into markets. Therefore, for the development of commercial products created from these materials, this study examined the absorption property and the preventive effect of nephropathy and cataracts in experiments with animal models of diabetes mellitus.

Methods

1. Screening of anti-glycative materials

(1) Materials

Dried materials of 536 types of plants were diluted at a weight ratio of 10 : 1 (water: dried material) and were extracted by heating at 95°C for 60 minutes. These materials were filtrated and then were dried under reduced pressure by an evaporator. These extracts were examined with positive control arm using aminoguanidine (Wako Pure Chemical Industries, Ltd. Chuo-ku, Osaka, Japan).

(2) Methods of Examination

1) Human serum albumin-glucose (HSA-Glu) test system [1, 2]

Into the glycation solution consisting of 0.1 mol/L phosphate buffer (pH 7.4), 8.0 mg/mL HSA and 0.2 mol/L glucose, plant extracts or aminoguanidine of a positive control, which were at the stipulated concentration, and were added to reach 1/10 of volume concentration. Solutions of plant extracts and aminoguanidine were incubated at 60°C for 40 hours. For a control arm, the solution was distilled with water instead of plant extracts. After the completion of the glycation reaction, fluorescent AGEs, which were produced in the reaction fluid, were measured using a microplate reader (excitation wavelength: 370 nm, fluorescent wavelength: 440 nm). For analysis of the inhibition rate of each AGE formation, A is the measurement value of the reaction liquid where sample solution was added to glycative reaction system, B is the measurement value of the reaction liquid where distilled water was added instead of glucose solution, C is the measurement value of the reaction liquid where distilled water was added instead of extracts or aminoguanidine, and D is the measurement value of the control liquid where distilled water was added instead of glucose solution. IC_{50} (inhibitory concentration 50%) was calculated in the following formula:

The inhibitory concentration of AGE formation (%) = $\{1 - (A-B) / (C-D)\} \times 100$

The strength of activity was evaluated by the relative values in the following formula, as IC_{50} of aminoguanidine was designated as one.

The activity value of extracts = aminoguanidine (IC_{50}) / plant extracts (IC_{50})

2) Permeability test in intestinal epithelium absorption model

The permeability test in the intestinal epithelium absorption model employed POCA® intestinal absorption kit (DS Pharma Biomedical Co., Ltd., Suita, Osaka, Japan), where cells derived from human colon cancer (Caco-2) [4-6] were disseminated on the insert side of a transwell plate. Culture solution of D-MEM with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) in the amount of 100 µL, which contained extracts (5 mg/mL) and aminoguanidine (5 mg/mL), was added to the insert side of the plate. After the solution was incubated for 3 hours, the inhibitory rate of the AGE formation in HSA-Glu test system was measured for culture solution which was permeated to the side of multiwell. The strength of activity was evaluated by relative values in the following formula, as the inhibitory rate of aminoguanidine was designated as 1.

The activity value of extracts = inhibitory rate of plant extracts / inhibitory rate of aminoguanidine

3) Test using blood serum of rats which extracts were administered to

SD male rats at 5 week-old (Charles River Laboratories Japan, Inc. Kohoku-ku, Yokohama, Kanagawa, Japan) were kept for one-week to be prepared for the test. Then, plant extracts (2 g/kg weight) and aminoguanidine (1 g/kg weight) were administered to the rats after 16-hour fasting. Blood sampling from jugular vein was conducted with an injector heparin sodium treated 1 hour pre, 1 hour after and 3 hours after administrations. Blood sampling was centrifuged to obtain blood plasma of 500 µL. This blood plasma was deproteinized using Nanosep® Centrifugal Devices (Pall Corporation, Port Washington, NY, USA) to obtain samples. Furthermore, the AGE formation inhibitory rate was measured in HSA-Glu test system. The strength of activity was evaluated by relative values in the following formula, as AUC (area under curve) of aminoguanidine was designated as one.

The activity value of extracts = AUC of plant extracts / AUC of aminoguanidine

2. Streptozotocin (STZ)-induced diabetic rats

(1) Materials

Dried and ground plants of trunks and branches of *Lindera umbellata* (kuromoji) and leaves of *Artemisia indica* var. *maximowiczii* (yomogi) were diluted at a 10 : 1 ratio (distilled water: dried plants), which were extracted by heating at 95°C for 60 minutes. After filtrated, extracts were condensed using continuous vacuum evaporator and then were lyophilized, which were obtained as subject materials.

(2) Animal experiments

1) Animals

SD rats at 6-week old (Japan SLC, Inc., Nishi-ku, Hamamatsu, Shizuoka, Japan) arrived and were reared in a barriered facility under the following environment: temperature: 24 ± 2°C, humidity: 50 ± 10%, lighting time: 12 hours a day (7:00-19:00). After one week of preliminary period rearing, SD rats were examined in experiments.

2) Rearing management

Through the periods of both preliminary rearing and administrating test material, SD rats were kept separately in a breeding cage, which was made from polycarbonate (W26 × D42 × H18 cm). In the cage, animal bedding made of fir tree (bedding for experimental animals soft wood chips: Japan

SLC, Inc.) was used.

3) Feed and drinking water

Labo MR Stock Powder (Nosan Corporation, Nishi-ku, Yokohama, Kanagawa, Japan) or Labo MR Stock Powder added to test materials were supplied to be freely consumed. Also, drinking water, which was well water disinfected with chlorine, was free to drink using water supply bottles.

4) Production of diabetic animal models and construction of groups

After the period of rearing for preparation, seven-week-old rats were given intraperitoneal injections of STZ with 60 mg/kg weight. One week later, a partial blood sample was collected from the caudal vein under non-fasting conditions, followed by the measurement of blood glucose level (Simple blood sugar measuring instrument, One-touch Ultra: Johnson & Johnson, Chiyoda-ku, Tokyo, Japan). Animals with blood glucose level 300 mg/dL or more were divided into groups without deviation of blood glucose level and weight (Statlight Grouping Soft: Yukms Co. Ltd., Asao-ku, Kawasaki, Japan). The grouping structure was as follows:

Group without treatment: untreated rats were given Labo MR Stock Powder (n = 8).

Control group: STZ administered rats were given Labo MR Stock Powder (n = 8).

Group with the administration of yomogi: STZ administered rats were given Labo MR Stock Powder combined 2% extracts of leaves of yomogi (n = 8).

Group with the administration of kuromoji: STZ administered rats were given Labo MR Stock Powder combined 2% extracts of kuromoji (n = 7).

Rats were maintained for 8 weeks with free access to subject materials.

5) Method of measurement

i) Weight and intake amount

Weight and intake amount were measured once a week.

ii) Ophthalmological test

Eight weeks after the beginning of administration, cataract stage examinations were conducted to observe corneas and crystalline lenses of both eyes under isoflurane anesthesia using a portable slit lamp (SL-5: Kowa Company. Ltd., Naka-ku, Nagoya, Aichi, Japan). Assessment of cataract stage was as follows:

Stage 0: Normal

Stage 1: Early cataract (discrete anterior and posterior cortical opacities)

Stage 2: Immature cataract (development of opaque lenses from the cortex to the inner core)

Stage 3: Mature cataract (severely opaque lenses and white pupils)

iii) Blood sample collection, hemoglobin Alc (HbA1c) measurement and biochemical examination

On the completion day of the 8-week administration, 16-hour fasting was conducted from the evening to the next morning. Then, whole blood collection was performed from abdominal aorta under isoflurane anesthesia followed by HbA1c measurement for some portion of blood (HbAlc measurement device: Siemens Healthcare Diagnostic, Shinagawa-ku, Tokyo, Japan). Blood serum sampling was separately obtained from the remainder of the blood and was kept frozen. At a later time, measurements were performed by enzymatic method in blood glucose (Glu,) glycoalbumin (GA), urea nitrogen (BUN), creatinine (CRE), amylase (AMY), triglyceride (TG), and free fatty acid (FFA).

iv) Renal function

After whole blood collection, severance of the abdominal aorta and the caudal vena cava was performed as euthanasia. Right renal gland was collected and was measured by weight, to which was added phosphate buffer saline (PBS) 4 times as much as the weight, homogenated and centrifuged. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in supernatant were measured using Rat OptEIA ELISA Set (BD Pharmingen [Becton, Dickinson and Company], Franklin Lakes, NJ, USA) and protein was measured using BCA Protein Assay Kit (Takara Bio INC., Kusatsu, Shiga, Japan), which was shown as a value per protein mg. For the assessment of creatinine clearance (CCr,) urine sampling was performed (V) for 16 hours at the 8 week after administration. Cr concentration value in urine was measured (uCr.) From serum creatinine concentration (serum Cr concentration) (sCr.) CCr was calculated in the following formula and CCr was shown as a value per one minute:

$CCr = (uCr / sCr) \times V$

6) Ethical review on animal experiments

This research abided by "Act on Welfare and Management of Animals" (Act No. 105 of October 1, 1973, Revision June 22, 2005) and "Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals" (the Ministry of Environment Notification No. 88 of April 28, 2006.) This study was given review and approval based on Guide for the Care and Use of Laboratory Animals.

7) Data analysis

Results were shown as mean ± standard deviation (SD). To provide a statistical processing system, student's t-test was employed for comparison between two groups and multiple comparison tests of Bonferroni corrections was employed for comparison among multiple groups; $p < 0.05$ is regarded as a significant difference.

Results

1. Screening of anti-glycation materials

Materials which proceeded to the next step of screening from 536 types of plant extracts were shown in ***Table 1***, as results of examinations in inhibitory actions (IC_{50}) to inhibit the fluorescence AGE formation in HSA-Glu reaction system *in vitro*. Data which had already been published was excluded.

Sample materials with stronger inhibitory effects against the formation of AGEs than aminoguanidine, which were more than 5 times larger than aminoguanidine in IC_{50}, were the following: ([ ] shows IC_{50} rate to aminoguanidine).

Forsythia koreana A [11.11], *Syzygium aromaticum* A [9.33], *Aspalathus linearis* [9.33], *Camellia sinensis* (L.) *Kuntze* [7.64], *Camellia sinensis* A, [7.64], *Rhus javanica* [6.74], *Camellia sinensis* [6.46], *Cinnamomum verum* [6.31], *Forsythia suspense* B [6.03], kuromoji_branch [5.80], kuromoji_branch and leaf [5.69] , *Origanum majorana* [5.60], *Castanea crenata*_shell [5.60], *Spatholobus suberectus Dunn* [5.09], *Rubus suavissimus* A [5.08].

The second screening assessed the inhibitory actions against the AGE formation after permeating intestinal

Table 1. The sample list of vegetables and herbs with high anti-glycative activity: ratio against aminoguanidine.

Engilish name	Japanese name	Chinese character	Scientific name	HSA-Glu model (n = 3)	Caco-2 test (n = 1)	Rat serum test (n = 2～3)
Japanese mugwort	Yomogi	蓬	*Artemisia indica* var. *maximowiczii*	1.20	0.33	1.16
Clove [A],	Choji [A]	丁子	*Syzygium aromaticum*	9.33	1.55	1.08
Gallnut	Fushi, Gobaishi	五倍子	*Rhus javanica*	6.74	0.55	0.78
Lindera, Kuromoji [twigg]	Kuromoji [eda]	黒文字	*Lindera umbellata*	5.80	0.91	0.75
Udo	Udo	独活	*Angelica pubescens* Maxim.f. biserrata Shan et Yuan	1.98	0.14	0.75
Eucommia leaf	Tochu ha	杜仲葉	*Eucommia ulmoides*	0.56	0.27	0.75
Bay leaf, Laurel	Gekkeiju, Laure	月桂樹	*Cinnamomum tamala*	3.82	0.65	0.72
Pu-erh tea [A]	Puaru-cha [A]	普洱茶	*Camellia sinensis*	7.64	0.63	0.59
Tsukikusa	Tsukikusa	鴨跖草	*Commelina communis* L.	1.86	0.45	0.50
Awa bancha	Awa bancha	阿波番茶	*Camellia sinensis*	6.46	0.51	0.50
Fatsia sprouts	Tara no me	楤芽	*Aralia elata*	2.58	0.35	0.47
Star anise	Dai-uikyo	大茴香	*Illicium verum* Hook. f.	1.06	0.43	0.46
Smartweed	Tade	蓼	*Polygonum hydropiper*	1.17	1.41	0.42
Xiangru, Chinese mosla	Koju	香需	*Mosla chinensis*	1.71	0.74	0.41
Sichuan pepper	Kasho	花椒	*Zanthoxylum bungeanum*	2.60	0.39	0.34
Banaba tea	Banaba cha	巴拿巴茶	*Lagerstroemia speciosa*	1.39	0.98	0.25
Sunflower seeds	Himawari no tane	向日葵種子	*Helianthus annuus*	2.29	1.05	0.22
Lindera, Kuromoji [foliage]	Kuromoji [edaha]	黒文字 枝葉	*Lindera umbellata*	5.69	1.21	0.18
Spearmint	Spearmint [A]	緑薄荷 [A]	*Mentha spicata*	3.50	0.40	0.15
Wild strawberry	Wild strawberry	蝦夷蛇苺	*Fragaria vesca*	–	0.46	0.09
Cinnamon	Keihi, Cinnamon	桂皮	*Cinnamomum verum*	6.31	0.23	0.03
Coarse tea	Ban-cha	番茶	*Camellia sinensis*	0.87	0.56	0.02
Plantain	Shazen-so	車前草	*Plantago asiatica*	2.04	0.34	0.01
Persimmon leaves	Kaki no ha	柿葉	*Diospyros kaki*	0.75	1.00	0.00
Turmeric	Turmeric, Ukon	鬱金、欝金	*Curcuma longa*	0.89	0.72	0.00
Marjoram	Marjoram, Mayorana	茉夭剌那	*Origanum majorana*	5.60	0.69	0.00
Ajowan	Ajowan	アジョワン	*Trachyspermum ammi*	2.15	0.54	0.00
Houttuynia [B]	Dokudami [B]	蕺草 [B]	*Houttuynia cordata*	2.15	0.46	0.00
Allspice	Allspice	三香子	*Pimenta dioica*	0.70	0.46	0.00
Walnut seed	Onikurumi [seed], hú táo rén	胡桃仁	*Juglans regia* L.	0.95	0.44	0.00
Mulberry leaves	Kuwa no ha	桑葉	*Morus alba* L.	1.50	0.42	0.00
Buckwheat [shell]	Soba [kara]	蕎麦 [殻]	*Fagopyrum esculentum*	1.56	0.38	0.00
Dipsacus japonicus root	Zokudan, nabena	続断	*Dipsacus asperoides* C. Y. Cheng et T. M. Ai	1.91	0.33	0.00

Engilish name	Japanese name	Chinese character	Scientific name	HSA-Glu model (n = 3)	Caco-2 test (n = 1)	Rat serum test (n = 2～3)
Lotus leaf	Kayo	荷葉	*Nelumbo nucifera*	1.79	0.29	0.00
Green tea	Ryoku-cha	緑茶	*Camellia sinensis*	1.37	0.35	–
Japanese pepper	Sansho	山椒	*Zanthoxylum piperitum*	1.58	0.30	–
Darjeeling tea	Darjeeling tea	大吉岭紅茶	*Camellia sinensis* (L.) Kuntze	3.82	0.27	–
Plum [flesh]	Ume [kaniku]	梅 [果肉]	*Prunus mume*	–	0.27	–
Chrysanthemum	Kiku [hana]	菊花	*Chrysanthemum morifolium* Ramat.	1.53	0.26	–
Black pepper	Kuro-kosho	黒胡椒	*Piper nigrum*	3.00	0.24	–
Lemongrass	Lemongrass	檸檬草	*Cymbopogon citratus*	4.20	0.21	–
Savory	Savory	木立薄荷	*Satureja montana*	1.68	0.20	–
Jew's mallow	Molokheiya	縞綱麻	*Corchorus olitorius*	2.10	0.18	–
Oregano	Oregano, hana-hakka	花薄荷	*Origanum vulgare*	1.00	0.17	–
Milettia reticulata	Keiketto	鶏血藤	*Spatholobus suberectus Dunn*	5.09	0.17	–
Bracken	Warabi	蕨	*Pteridium aquilinum*	2.79	0.16	–
Tien-cha, Chinese blackberry tea	Ten-cha [B]	甜茶 [B]	*Rubus suavissimus*	4.20	0.14	–
Olive leaf	Olive [leaf]	阿列布葉	*Olea europaea*	–	0.14	–
Sissoko, fruit of purple perilla	Shisoko	紫蘇子	*Perilla frutescens*	–	0.12	–
Araliaceae	Ukogi, okogi	五加木	*Acanthopanax sieboldianus*	4.29	0.11	–
Peppermint	Hakka	薄荷	*Mentha x piperita* L.	4.24	0.09	–
Artichoke	Artichoke	朝鮮薊	*Cynara scolymus*	–	0.09	–
Chinese alehoof	Rensen-so, kakidoshi	連銭草	*Glechoma longituba* (Naka) Kupr.	1.41	0.08	–
Apple fuji	Ringo fuji	林檎 富士	*Malus domestica*	0.06	0.08	–
Golden bell flower leaf [B]	Rengyo-ha [B]	連翹葉 [B]	*Forsythia suspensa*	6.03	0.07	–
Japanese horse chestnut [shell]	Tochi [kara]	栃 [殻]	*Aesculus turbinata*	2.14	0.07	–
Centipeda, diet flower	Gafushoku-so, ebushicao.	鵝不食草	*Centipeda minima* (L.) A. Braun et Aschers.	1.13	0.06	–
Peppermint	Peppermint	胡椒薄荷、西洋薄荷	*Mentha* × *piperita*	1.35	0.05	–
Rooibos tea [B]	Rooibos tea [B]	南非国寶茶 [B]	*Aspalathus linearis*	9.33	0.05	–
Beautiful sweetgum fruit, formosa sweetgum	Rorotu	路路通	*Liquidambar formosana* Hance	2.11	0.05	–
Callaway	Callaway, Hime-uikyo	姫茴香	*Carum carvi*	1.14	0.04	–
Fennel	Fennel	茴香	*Foeniculum vulgare*	1.83	0.03	–
Rosemary	Rosemary	迷迭香	*Rosmarinus officinalis*	1.56	0.03	–
Elderberry	Elderberry	庭常属	*Sambucus sieboldiana*	–	0.02	–
Ryûgesô	Sentsurukusa	仙鶴草	*Agrimonia pilosa* Ledeb.	1.62	0.01	–
scarlet runner bean [sheath]	Hanamame_[saya]	花豆 [鞘]	*Phaseolus coccineus*	2.05	0.01	–

Engilish name	Japanese name	Chinese character	Scientific name	HSA-Glu model (n = 3)	Caco-2 test (n = 1)	Rat serum test (n = 2 ~ 3)
Scutellaria barbata	Hanshiren	半枝蓮	*Scutellaria barbata*	2.25	0.00	–
Gôkanpi,	Gokanhi	合歓皮	*Albizia julibrissin* Durazz.	0.99	0.00	–
Dragon's blood, xuè jié	Kekketsu	血竭	*Daemonorops draco* Bl.	0.80	0.00	–
Cibotium rhizome	Kuseki	狗脊	*Cibotium barometz* (L.) J.Sm.	0.71	0.00	–
Golden bell flower leaf [A]	Rengyo-ha [A]	連翹葉 [A]	*Forsythia koreana*	11.11	0.00	–
Lemon balm [A]	Lemon balm [A]	香水薄荷 [A]	*Melissa officinalis*	2.47	0.00	–
Rhubarb	Rhubarb	食用大黄	*Rheum rhabarbarum*	0.00	0.00	–
Apple orin	Ringo orin	林檎 王林	*Malus domestica*	0.01	0.00	–
Apple Shinano-gold	Ringo Shinano-gold	林檎 信濃金	*Malus domestica*	–	0.00	–
Japanese quince	Boke	木瓜	*Chaenomeles speciosa*	1.09	0.00	–
Blue mallow	Usubeni-aoi	薄紅葵	*Malva sylvestris*	–	0.00	–
Butterbu	Fuki	蕗	*Petasites japonicus* (Siebold et Zucc.) Maxim.	3.64	0.00	–
Saffron	Safuran	番紅花	*Crocus sativus*	2.50	0.00	–
Passion fruit	Passion fruit	果物時計草	*Passiflora edulis*	0.28	0.00	–
Broccoli sprout	Hatsuga broccoli	発芽芽花野菜	*Brassica oleracea* var. *italica*	0.83	0.00	–
Basil	Bajiru	目箒，羅勒	*Ocimum basilicum*	1.02	0.00	–
Citrus peel	Chinpi	陳皮	*Citrus unshiu*	–	0.00	–
Celery [leaf]	Celery [ha]	和蘭三葉 [葉]	*Apium graveolens* var. *dulce*	0.70	0.00	–
Celery [stalk]	Celery [kuki]	和蘭三葉 [茎]	*Apium graveolens* var. *dulce*	0.21	0.00	–
Watermelon [peel]	Suika [kawa]	西瓜 [皮]	*Citrullus lanatus*	–	0.00	–
Edible chrysanthemum 「A」	Shokuyo-kiku [A]	食用菊 [A]	*Chrysanthemum morifolium*	0.88	0.00	–
Ginger [A]	Shoga [A]	生姜 [A]	*Zingiber officinale*	4.20	0.00	–
Lollo rosso, Red-leaf lettuce	Sunny lettuce [A]	葉苣 [A]	*Lactuca sativa* var. *crispa*	1.12	0.00	–
Ostrich fern	Kogomi [A]	屈，草蘇鉄 [A]	*Matteuccia struthiopteris*	1.29	0.00	–
Cumin	Cumin	馬芹	*Cuminum cyminum*	0.53	0.00	–
Persimmon	Kaki [A]	柿 [A]	*Diospyros kaki*	0.06	0.00	–
Ginkgo biloba	Icho ha	銀杏葉	*Ginkgo biloba*	–	0.00	–

HSA-Glu model: inhibitory actions of each sample were examined in the reaction model between HSA and glucose. The results are expressed as ratio of IC_{50} when assumed aminoguanidine as 1.0. Caco-2 test: inhibitory actions of each sample which has been passed through the Caco-2 cell layer, using the HSA-Glu model. The results were expressed as ratio of percentage inhibition when assumed aminoguanidine as 1.0. Rat serum test: inhibitory actions of rat serum was examined in the HSA-Glu model after each sample has been orally applied and the serum obtained at 0-, 1- and 3-hour. Results are expressed as ratio of AUC of percentage inhibition for 3 hours when assumed aminoguanidine as 1.0. IC_{50}, 50%inhibitory concentration; HSA, human serum albumin, Glu, glucose, AUC, area under curve.

epithelial cells, using intestinal epithelial absorption models. In consideration of potential commercial products, 11 sample materials were newly added: *Sambucus sieboldiana*, *Prunus mume*_flesh, *Perilla frutescens*, *Olea europaea*, *Malva sylvestris*, *Malus domestica* Shinano Gold, *Ginkgo biloba*, *Fragaria vesca*, *Cynara scolymus*, *Citrus unshiu*, and *Citrullus lanatus*_peel. The total number of sample materials in the second screening was 93 types of plants. Among these samples, 5 types had smaller IC_{50} than aminoguanidine: *Syzygium aromaticum* A [1.55], *Polygonum hydropiper* [1.41], kuromoji_branch and leaf) [1.21], *Helianthus annuus*_seed) [1.05], and *Diospyros kaki*_leaf [1.003]. *Forsythia koreana* showed strong *in vitro* activity to inhibit the AGE formation but extremely weak inhibition activity after permeating intestinal epithelial cells.

The third screening was the examination where 34 types of sample materials were assessed, taking into consideration absorption and metabolism of extracts in rats which test materials were administered to. Only 2 types, yomogi [1.16] and *Syzygium aromaticum-Choji*_A [1.08] had stronger AGE inhibition than aminoguanidine of the materials which were absorbed and metabolized inside rat serum. Next, *Rhus javanica* [0.78], kuromoji_branch [0.755], *Angelica pubescens* Maxim.f. *biserrata* Shan et Yuan [0.754] and *Eucommia ulmoides* [0.748] were shown. Kuromoji_branch and leaf showed relatively strong activity [1.21] in intestinal epithelium absorption models but low activity [0.18] in the rat absorption test.

2. *Evaluation of streptozotocin (STZ)-induced diabetic rats*

To contemplate future application of functional food and the results of the screenings, yomogi and kuromoji were selected to examine the effectiveness for glycative-stress-induced disorders.

Fig. 1, 2 show the transitions of weight and intake volume following STZ treatment. There was no significant difference

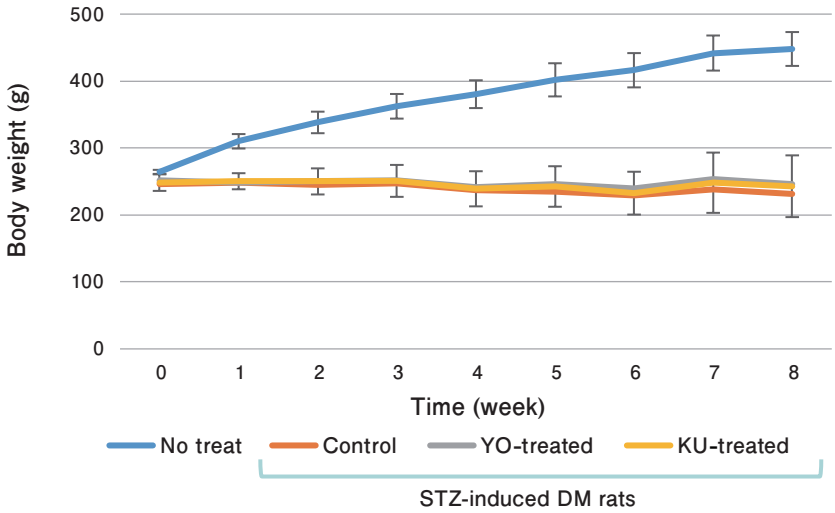

Fig. 1. **Change of body weight in the STZ-induced diabetic rats.**

No treatment group (n = 8), STZ-treated group (n = 8), STZ & YO-treated group (n = 8) and STZ & KU-treated group (n =7). Results are expressed as mean ± SD (shown in STZ-treated group and STZ & LU-treated group). STZ, streptozotocin; YO, yomogi (Japanese mugwort; *Artemisia indica* var. *maximowiczii*); KU, kuromoji (*Lindera umbellata*); SD, standard deviation.

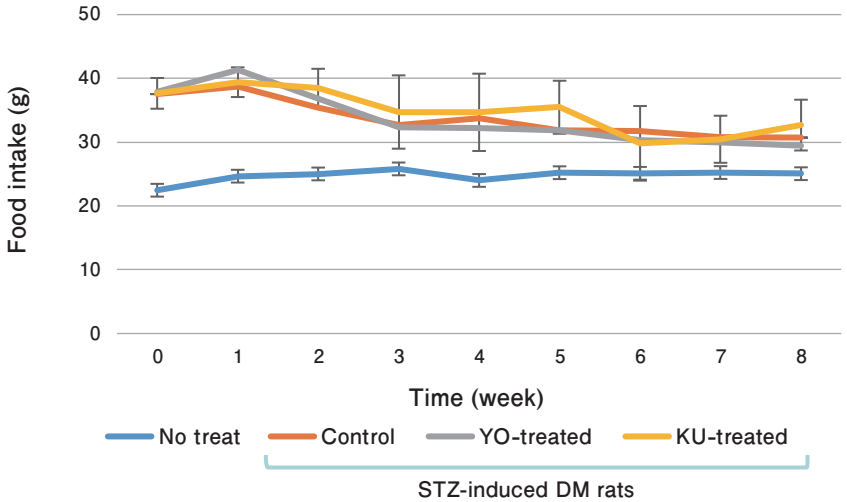

Fig. 2. **Change of food intake amount in the STZ-induced diabetic rats.**

No treatment group (n = 8), STZ-treated group (n = 8), STZ & YO-treated group (n = 8) and STZ & KU-treated group (n =7). Results are expressed as mean ± SD (shown in STZ-treated group and STZ & KU-treated group). STZ, streptozotocin; YO, yomogi (Japanese mugwort; *Artemisia indica* var. *maximowiczii*); KU, kuromoji (*Lindera umbellata*); SD, standard deviation.

in weight and intake amount among three groups: the control group, the group with the administration of yomogi and the group with the administration of kuromoji.

Fig. 3 shows the results of blood tests eight weeks after the administration of test materials. There was no significant difference in Glu, GA and HbA1c between the control, the yomogi group and the kuromoji group. As for the serum AMY, the kuromoji group (1,224 ± 176 IU/L) showed significantly higher than the control (1,009 ± 134 IU/L, $p < 0.05$). As for the serum TG, the kuromoji group (217 ± 95 mg/dL) showed significantly lower than the control (379 ± 150 mg/dL, $p < 0.01$). As for serum FFA, the kuromoji group (512 ± 114 μEq/L) showed significantly lower than the control (811 ± 126 μEq/L, $p < 0.05$).

Fig. 4 shows the results of the renal function tests (BUN, CRE and CCr) and TNF-α and lL-6 in renal tissues, which were conducted 8 weeks after administration of test materials. As for renal weight, the kuromoji group (0.91 ± 0.10 g/100g body weight) was significantly lighter than the control (1.02 ± 0.06 g/100g body weight, $p < 0.05$). As for the serum TNF-α and 1L-6 in renal tissues, the kuromoji group (TNF-α: 0.72 ± 0.20 ng/mg protein, IL-6: 5.68 ± 1.60 ng/mg protein) showed significantly lower than the control (TNF-α:

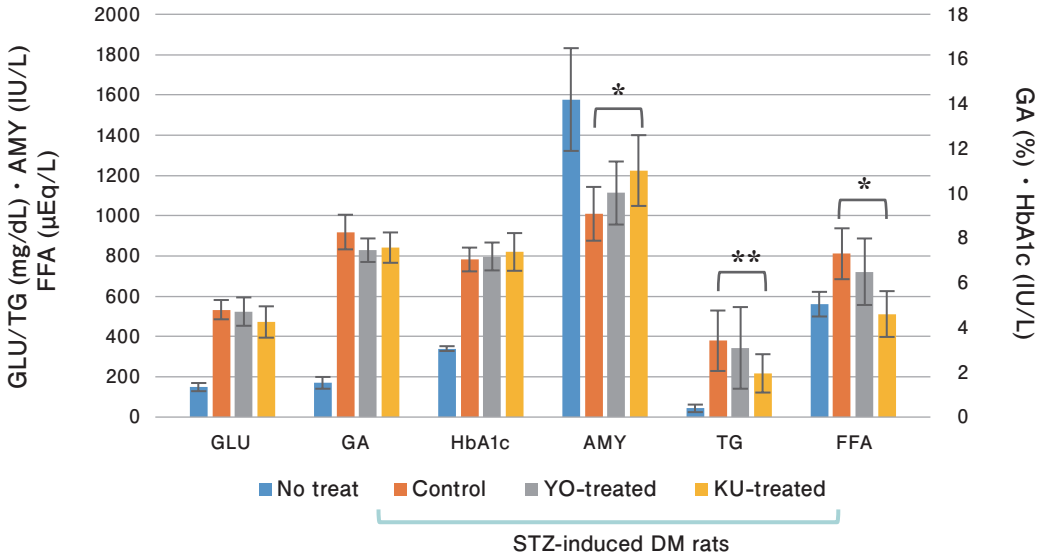

Fig. 3. **The values of blood markers after 8 week treatment by kuromoji and yomogi in the STZ-induced diabetic rats.**

Results are expressed as mean ± SD. No treatment group (n = 8), STZ-treated group (n = 8), STZ & YO-treated group (n = 8) and STZ & KU-treated group (n =7). GLU, glucose; GA, glycoalbumin; AMY, amylase; TG, triglyceride; FFA, free fatty acid; STZ, streptozotocin; YO, yomogi (Japanese mugwort; *Artemisia indica* var. *maximowiczii*); KU, kuromoji (*Lindera umbellata*); SD, standard deviation.

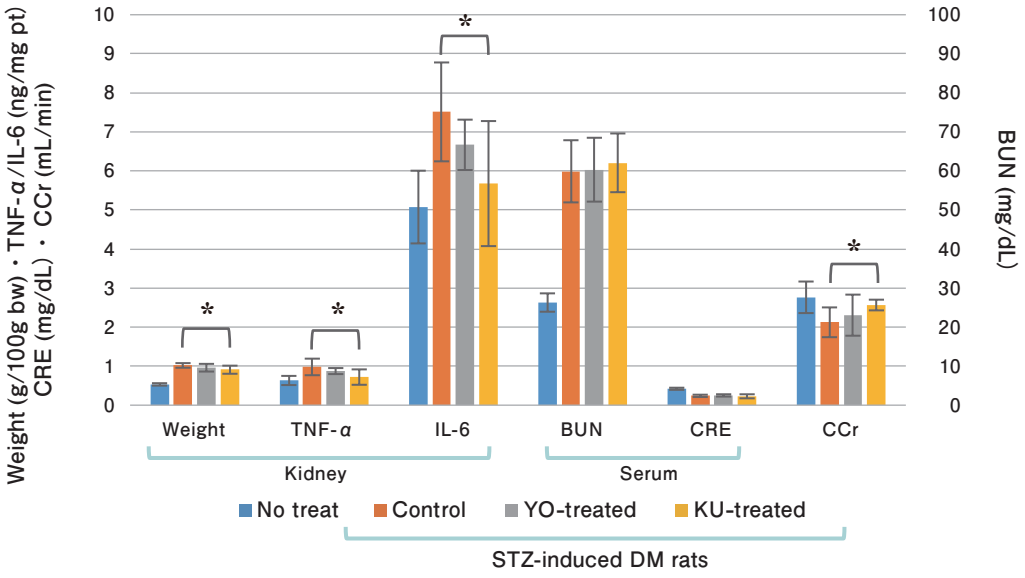

Fig. 4. **The renal function index after 8 week treatment by kuromoji and yomogi in the STZ-induced diabetic rats.**

Results are expressed as mean ± SD. No treatment group (n = 8), STZ-treated group (n = 8), STZ & YO-treated group (n = 8) and STZ & KU-treated group (n =7). TNFα, tumor necrosis factor-α; IL-6, interleukin-6; BUN, blood urea nitrogen; CRE, creatinine; CCr, creatinine clearance; STZ, streptozotocin; YO, yomogi (Japanese mugwort; *Artemisia indica* var. *maximowiczii*); KU, kuromoji (*Lindera umbellata*); SD, standard deviation.

0.98 ± 0.21 ng/mg protein, IL-6: 7.51 ± 1.27 ng/mg protein, $p < 0.05$) and inflammatory cytokine in the tissue was kept low. There was no significant difference in serum BUN and CRE. The kuromoji group showed a significantly higher CCr value (2.57 ± 0.14 mL/min) than that of the control (2.12 ± 0.38 mL/min, $p < 0.05$). The renal function was maintained.

There was no significant difference in the above-mentioned index between the yomogi group and the control.

Fig. 5 shows assessments of the cataract stage 8 weeks after the administration of test material. The control had a higher ratio of cataract stage 2 (immature cataract), while the kuromoji group and the yomogi group had a higher ratio in cataract stage 0 (normal) and stage 1 (early cataract), which indicates that cataracts were light, slight or mild.

Discussion

Kuromoji

Kuromoji tree belongs to the *Lauraceae*, or laurel family and is indigenous to Japan, which is widely distributed from Kyushu to Hokkaido in Japan. The trunks contain an aromatic essential oil component and the branches are used as herbal medicine called *Usho*. *Usho* has various beneficial drug effects to improve the alimentary system under the following conditions: *kakuran* which means ictus solis or illness in summer, *senki*, which means abdominal pain, *fukucho*, which means sense of distension, and *shukujiki*, which means maldigestion. Also, the branches and leaves emit a refreshing fragrance and are used as a material for high-end toothpicks. Kuromoji essential oil, which is extracted by steam distillation from branches and leaves, is used as perfume and soap. Kuromoji essential oil contains linalool, cineol and geraniol [7,8]. These oil components also contribute to forest bath effect [9,10].

It has been reported that kuromoji essential oil provides diversified effects, such as anti-tumor activity [11], anti-inflammatory activity [12], mentally and physically relaxing activity [10,13,14], stress-relief activity [15,16], anti-bacterial activity [17] on various bacteria, periodontal disease bacteria, and candida. Kuromoji essential oil has a less potent anti-oxidative activity [18] than the essential oil of cypress leaves, cypress foliage and cryptomeria leaves. It has been reported that analysis of electro cardiogram examinations revealed an increase in parasympathetic nerve system activities, a decrease in sympathetic nervous system activities, an increase in salivary secretion [14] and a significant decrease in ratio of α wave of electroencephalogram [13], which enhances the dominance of parasympathetic nerve system activities.

Kuromoji leaves contains linderatin [18] of the dihydrochalcone strain and linderatone [19] of flavanones. Kuromoji branches contain kaempferol, quercetin, afzerin, avicularin, hyperin, isoquercitrin and rutin as flavanones [20]. Tannin contained by kuromoji extracts is reported to have anti-pepsin activity and anti-tumor activity [21]. Hexahydrodibenzofuran derivative of kuromoji branch extracts has functions to control the production of melanin pigments [22].

This study confirmed that kuromoji has, other than the activities mentioned above, inhibitory activities against the formation of *in vitro* AGEs in the HSA-Glu reaction model. Furthermore, it was shown that these activities were exerted even after the permeation of intestinal epithelium and after digestion and absorption in rats. Also, kuromoji extracts exerted inhibitory activities against the AGEs formation and showed effects resulting in prevention and progression of nephropathy and cataract formation after kuromoji extracts were administered to streptozotocin-induced diabetic rats.

It has been reported that cataract formation deeply relates

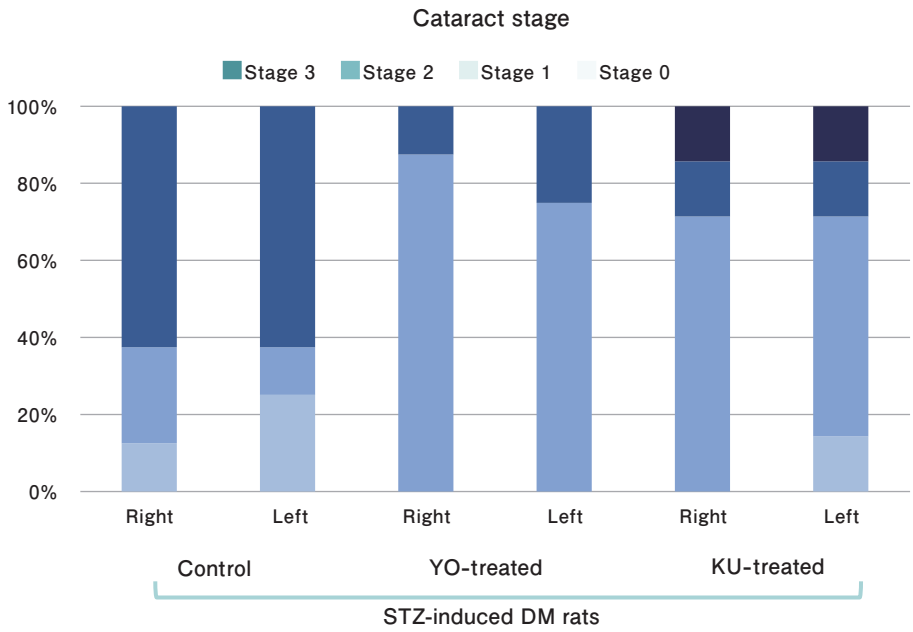

Fig. 5. **Cataract prevention by kuromoji and yomogi (Japanese mugwort).**

The graph shows the ratio of each stage of cataract in the STZ-treated rats. STZ-treated group (n = 8), STZ & YO-treated group (n = 8) and STZ & KU-treated group (n =7). Stages are classified as follows: Stage 0, normal; Stage 1, early phase; Stage 2, inmatured phase; Stage 3, matured phase.

to glycative reactions of crystallin protein in the crystalline lens. *Trapa japonica* extracts are reported to inhibit glycation of crystalline by glyoxal treatment. The data of kuromoji extracts showed the inhibitory effects on cataract formation and there is a possibility that kuromoji may have a similar mechanism to inhibit the glycation of crystallin protein [23]. Kuromoji administration elevated amylase values, which had been reduced by STZ treatments. Pancreatic amylase reflects pancreatic function. Thus, it was assumed that the administration of kuromoji could improve pancreatic functions, which were lowered by STZ treatment.

Glycative stress shows the physical states where aldehyde is likely to be produced [24,25]: diabetes, dyslipidemia, metabolic syndrome, excessive alcohol intake and chronic kidney disease (CKD). STZ-induced rats showed high values in Glu, TG and FFA. Aldehyde, which resulted from these conditions, reacts with protein in the body and initial indexes of glycation reaction such as GA and HbA1c became high, which induces the formation and accumulation of AGEs. Furthermore, AGEs bond with RAGE (Receptor for AGEs) on the cell surface of macrophage system. Signal activation of AGEs/RAGE mediates the production of inflammatory cytokine. Thus, tissue injuries, such as nephropathy and cataracts, could be improved. Pocyanidin B2, which is contained in kuromoji extracts, inhibits the production of IL-6 and TNF-α in macrophage culture stimulated by lipopolysaccharide [12]. This finding and view supports the results of examination where IL-6 and TNF-α were significantly lowered in the renal glands of diabetic rats of the kuromoji group. In addition of kuromoji inhibitory activities against the formation of AGEs, there was a possibility that kuromoji also inhibited the activation of AGEs/RAGE signal. Further examinations need to be conducted in the future.

Yomogi

Yomogi is a perennial of the *asteraceae* family and is used diversely. The leaves are used as *yomogi-mochi*, glutinouns rice dumpling with a green color and a characteristic flavor, and dried leaves are made into moxa for *kyu*, moxibustion [26,27]. Yomogi is used as *shoyaku*, a type of herbal medicine. The herbal medicine is called *gaiyo*, which has the functions of a blood coagulant and improves problematic symptoms during pregnancy. *Kyukikyogaito* has functional effects in the cases of continuous vaginal bleeding, hematemesis after a miscarriage and abdominal pain during pregnancy. *Hakuyoto* is administered for hematemesis and hemorrhage. It is reported that skin external agents containing yomogi extracts have anti-pruritic effects [28-38] and mitigating atopic dermatitis [39-42]. Yomogi extracts are widely used for skin external products such as soap [43] and cosmetics.

The chemical components of yomogi were essential oil, flavonoid, polyphenol and others [27]. The main components in the flavor are the following: cineol, thujone, β-caryophyllene, borneol and camphor (these 5 components are terpenoids [44]), and in addition, nonacosane, and hentriacontane (these two are hydrocarbons [45]). Apart from that, yomogi contains tannin, palmitic acid, oleic acid, and linoleic acid (these are fatty acids), and vitamin A, B1 and B2 [46].

Yomogi leaf extracts have been reported to have choleretic action [47], growth stimulation of vascular endothelial cells [48], inhibitory activity against angiotensin-converting enzyme (ACE) [49], reductive activity on age-related body odor and uraroma [50], and anti-inflammatory activity [51]. Our research center has confirmed that polyphenol contained in yomogi extracts has inhibitory effect against the formation of AGEs [3,52]. Caffeic acid and chlorogenic acid, which are isolated as tannin from yomogi extracts, have inhibitory activities against lipid peroxidation [53-55], improving actions for lipometabolism (decrease in TG and decrease in lipid peroxide) [53,54] and inhibitory activities against histamines disengagement [53,55]. These stabilization activities are characteristic to yomogi, which is the reason that yomogi is used for the purpose of inhibiting atopic dermatitis and skin itching.

In comparison between yomogi and kuromoji, both have the same level of inhibitory effect against the formation of AGEs. However, yomogi has an advantage in stabilizing mast cells and kuromoji exerted an anti-inflammatory effect against macrophage. It is assumed that these differences produced the results in this examination where kuromoji significantly decreased cytokine in renal tissues but yomogi did not show a significant difference.

Conclusion

As a part of the SIP Program, this study performed screenings and selections from five hundred and 36 agricultural, forest and fishery materials to evaluate plant-derived materials, focusing on the inhibitory effects against the formation *in vitro* fluoresce AGEs, permeability in intestinal epithelium and absorption activities in model rats. Selection of kuromoji extracts and yomogi extracts was performed to identify materials, focusing on domestic availability of materials and application to functional food production. The resulting data of the examination of STZ-induced diabetes mellitus model rats revealed that kuromoji extracts had effects on improving TG and FFA and preventing the onset and progression of diabetic nephropathy. Furthermore, effects to prevent the onset and progression of cataracts were also recognized in kuromoji and yomogi extracts. We have decided to continue further examination of kuromoji, taking it into consideration to prepare for social implementation of commercialized products.

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Conflict of Interest Statement

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