Anti-aging effects of black raspberry extract on cataract, alopecia, skin whitening, and weight loss

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ABSTRACT

Background: To maintain good health, it is important to eliminate extra reactive oxygen generated in the body. Furthermore, ingesting foods containing antioxidants is beneficial. The oxygen radical absorbance capacity (ORAC) values for black raspberry extract (BRE), blueberry extract (BBE), and raspberry extract (RBE) are 62, 66, and 49 µM Trolox equivalents (TE)/g respectively. These values are higher than those for typical antioxidant foods that have been discovered so far (3–30 µM TE/g). Our aim was to find new functionality from the food with the high ORAC value. Therefore, we have prepared these four berry extracts and examined whether they have anti-aging effects and if those effects correlate with their antioxidant activities.

Methods: We studied the following effects of 4 berry extracts: 1) lens cell protective effects; 2) effects against alopecia; 3) induction of uncoupling protein-1 (UCP1), a regulator of fat and energy consumption in adipocytes, and stimulation of irisin secretion from skeletal muscle cells; and 4) inhibitory effects on melanocyte tyrosinase activity. The evaluation method was based on below; 1) α-crystallin, *type 17 collagen, heat shock protein 47 (HSP47), UCP1 and Irisin* - mRNA by qRT-PCR, 2) the amount of the *UCP1 and Irisin* protein by ELISA. 3) Inhibition of tyrosinase activity was measured by dopachrome production using L-tyrosine.

Results: In lens cells, α-*crystallin* mRNA expression was induced 1 hour after treatment of the cell with Blabina (a powdered formulation containing BRE) and BRE. The extracts of all four berry species promoted the growth of follicle dermal papilla cells by 3-20% in a concentration-dependent manner. These berry extracts were also discovered to markedly induce the expression of mRNAs of *type 17 collagen* and *HSP47* in the hair follicle stem cell and elevate the expression levels of *UCP1* mRNA and its protein in adipocytes in a concentration-dependent manner. BRE and Blabina inhibited 5α-reductase in follicle dermal papilla cells and tyrosinase activity in melanocytes at the concentrations which inhibited dopachrome production by at least 50%. Finally, Blabina was discovered to stimulate the irisin secretion from skeletal muscles.

Conclusion: These results suggest that berry extracts, particularly BRE, have anti-aging effects through their higher antioxidant activities.

Keywords: Anti-aging, antioxidant, alopecia, black raspberry, weight loss, oxygen radical, absorbance capacity, skin whitening

INTRODUCTION

Oxygen is essential for most organisms within human beings. A proportion of oxygen inhaled is converted into reactive oxygen species (ROS), which are utilized for protection against invaded bacteria. Additionally, excessive amounts of ROS can be generated by ultraviolet light, smoking, exhaust gas, air pollution and stress, and causes oxidative damages to body tissues [1]. As a result, wide variety of diseases such as lifestyle-related diseases, cancer, and dementia occur. It is estimated that more than 90% of all diseases have a causal relationship with excess ROS production [2]. Therefore, excessive amounts of ROS must be eliminated from the human body

by antioxidant enzymes and exogenous substances to maintain homeostasis and prevent these diseases. Recently, antioxidant substances existing in foods are attracting attention for the potential treatment and prevention of disease and methods to evaluate antioxidant capacity and the oxygen radical absorbance capacity (ORAC) of foods have been established and are widely used [3]. The ORAC is a measurement system developed by the United States Department of Agriculture to evaluate the antioxidant capacity of foods and supplements, and a list of the top 100 of antioxidant foods with high ORAC values including 12 food items containing berries [4]. The relation with ORAC has been considered with Fucoidan, grape wine, apple cider vinegar, Faba bean flour, and more [5-8].

In addition to lifestyle-related diseases, aging-associated events such as the cataract, alopecia, obesity, and skin pigmentation are serious issues for older humans. The cataract results from aging-associated development of opacity of the eye lenses, and occurs in virtually everyone over 80 years of age. It has not yet been elucidated why the lens loses its transparency and becomes opaque. The well-organized network structure of component proteins is involved in the maintenance of lens transparency. Aggregation of α-crystallin, a component protein, causes opacity, reducing lens function and affecting the field and power of vision [9]. Notably, lens proteins are never replaced during a lifetime. In order to prevent opacity, it would be effective to express α-crystallin in the lens.

The alopecia is closely related to the protein levels of type 17 collagen, heat shock protein 47 (HSP47), and 5α-reductase. Proteolysis of type 17 collagen in hair follicle stem cells causes miniaturization of hair follicles, resulting in aging-associated hair loss [10]. High expression of HSP47, which is a collagen-specific chaperone, can suppress this proteolysis, inhibiting alopecia. 5α-reductase binds to testosterone and converts it to dihydrotestosterone (DHT) which promotes hair loss and thinning [11]. These observations suggest that inhibition of 5α-reductase activity and expression of type 17 collagen and HSP47 would suppress alopecia.

The obesity seems to be closely related to expression levels of uncoupling protein-1 (UCP1) and irisin. Higher expression of UCP1 in the mitochondria of adipocytes raises body temperature and increases the number of mitochondria, resulting in the reduction of body fat [12]. Irisin is a peptide hormone secreted from skeletal muscle cells in response to the exercise and induces the differentiation from fat-storing white adipocytes to fat-burning brown adipocytes, leading to weight loss [13]. Therefore, substances that stimulate irisin secretion would have weight loss effects.

Synthesis of melanin, which causes pigmentation of the skin, is promoted by various exogenous and endogenous stimuli. When humans are exposed to the ultraviolet light in sunlight, stress, and air pollution, excess amounts of ROS are generated in the skin surface, and stimulate melanocytes to produce tyrosinase, which reacts with tyrosine to produce melanin. Capturing excess amounts of ROS with antioxidants suppresses melanin production [14].

Our aim was to find new functionality from the food with the high ORAC value. We have previously found that black raspberry extract (BRE) contains a large amount of delphinidin, which can induce the anti-aging-related protein, sirtuin [15]. To evaluate anti-aging effects of berry extracts in this study, we investigated their effects on the induction of α-crystallin expression for anti-aging of the eye, expression of type 17 collagen and HSP47 and inhibition of 5α-reductase for alopecia, induction of UCP1 expression and irisin secretion from skeletal muscle cells for weight loss, and tyrosinase activity in melanocytes. The results suggested that BRE has various anti-aging effects through their antioxidant action.

MATERIALS AND METHODS

Preparation of berry extracts: Frozen Blabina (a powdered formulation containing BRE: trademark registration number 5735928), BRs, blueberries (BBs), and raspberries (RBs) were provided in a frozen state from INNATUS Co. Approximately 100 g of each berry was mixed with 5 volumes of 30% ethanol (approximately 500 mL), blended at 8,000 rpm for 30 min followed by slow stirring at room temperature for 24 hrs. After filtration through mesh with the pore size of 150 nm, a 30% ethanol extract was obtained for ORAC measurements. Blabina was dissolved in 30% ethanol at 9 mg/mL, and processed in the same manner.

Ethanol in the extract was evaporated before use of the extracts in experiments. The dry weights of the 30% ethanol extracts and the ethanol-removed samples were measured after a freeze-dried, and the concentrations of these original liquids were calculated as 9 mg/ml and 11.5 mg/ml respectively. Serial dilutions of ethanol-removed samples were made with ultrapure water and added to the cell culture at 1/100 of the medium volume.

Cell culture: Human follicle dermal papilla cells (C-12071, PromoCell), skeletal muscle cells (C-12530, PromoCell), and juvenile foreskin melanocytes (C-12400, PromoCell) were cultured in Follicle Dermal Papilla Cell Growth Medium Supplement Pack, C-39620, Skeletal Muscle Cell Growth Medium Supplement Pack, C-39360, and Melanocyte Growth Medium Supplement Pack, C-39410, respectively (all from PromoCell), at 37°C in a 5% CO_2 incubator. B-3 cells

(ATCC, CRL-11421), human lens cells transformed with an adenovirus 12-SV40 virus hybrid, and adipocytes differentiated from 3T3-L1 cells as described below were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 incubator. Cells were harvested with 0.25% trypsin and 0.1% EDTA, seeded onto 24- or 96-well plates at $2x10^5$ cells/well, and cultured for 24 hours before use in experiments.

Differentiation of 3T3-L1 cells into adipocytes: 3T3-L1 cells were differentiated into adipocytes according to the method of Rubin et al. [16]. Briefly, 3T3-L1 cells were cultured for 3 days in DMEM containing 10% FCS, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone, and 100 nM insulin (all from Wako Pure Chemical Industry) at 37°C in a 5% CO_2 incubator. After the cell culture medium was exchanged for a DMEM culture medium containing 10% FCS, cells were further cultured for 7 days. Differentiation of 3T3-L1 cells into adipocytes was confirmed by measuring glycerol-3-phosphoric acid dehydrogenase (GPDH) activity in cells, which was increased by about 1000 times. GPDH activity was measured with the GPDH activity assay kit (Code #; 309-06141, Wako Pure Chemical Industry).

ORAC: The ORAC assay was conducted by measuring the fluorescent intensity of fluorescein degraded by ROS over time. When an antioxidant substance is present in this assay system, the decline rate of the fluorescent intensity slows. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard antioxidant substance, and the antioxidant capacities of samples were calculated in comparison to the decline rate of the standard. The results are shown as the amount of Trolox (μM) per 1 gram of the sample extracted with 30% ethanol, in the unit of μM TE (Trolox equivalent)/g.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *type 17 collagen*, *HSP47*, *UCP1*, and *α-crystallin* expression: Total RNA was extracted from cells using TRIzol reagent (Ambion). The extracted RNAs were reverse-transcribed into cDNAs using PrimeScript RT Master Mix (Takara) following the manufacturer's instructions and amplified using SYBR Premix EX Taq II (Takara). Reactions were performed in the final volume of 50 μL (25 μL of 2× SYBR Green Mix, 1 μL cDNA, 2 μL primer pair mix (5 pmol/μL for each primer), and 22 μL H_2O). Each PCR was performed under the following conditions: 95°C for 30 sec, followed by 50 cycles of 95°C for 5 sec and 60°C for 30 sec. Relative gene expression was calculated using the ΔΔCt method, and the housekeeping gene GAPDH was used for

normalization. The primers used in RT-PCR are shown in Table 1. Significant differences were judged when the $\Delta\Delta Ct$ value was >2.

Table 1. Primers for PCR

Gene	Primer
α-Crystallin	Forward; CCT GGT ATG GAC AGG ACC TC
	Reverse; GGA GGA AAT TGC TGT CAA GG
Type17 Collagen	Forward; GAG CTG ACT TTG CTG GAG GT
	Reverse; GTC ACG TTG CTG TAG GCA GA
Hsp47	Forward; CCC TGA AAG TCC CAG ATC AA
	Reverse; GGG AGA GGT TGG GAT AGA GC
UCP-1	Forward; AGG CAC CCA TCA CCA ATG AGG CTT G
	Reverse; TGG TGC AGT TTG TCC ACT GGG CTT C
GAPDH	Forward; AGG GCT GCT TTT AAC TCT GGT
	Reverse; CCC CAC TTG ATT TTG AGG GGA

Inhibitory activity against 5α-reductase: As inhibition of 5α-reductase suppresses DHT production, we measured the amount of DHT produced using a DHT enzyme-linked immunosorbent assay (ELISA) Kit (E18-220, Immunospec) according to the manufacturer's instructions.

Quantitation of UCP1 and irisin by ELISA: To quantify the expression levels of UCP1 and irisin in the cell, UCP1 (MBS101050, MyBioSource) and irisin ELISA Kit (EK-067-52, Phoenix Pharmaceuticals, Inc.) were employed according to the manufacturers' instructions.

Inhibition of tyrosinase activity: Using L-tyrosine (Sigma) as a substrate, we measured the amount of dopachrome produced by tyrosinase. L-tyrosine (33.3 µL of 2.5 mM) was added to 60 µL of 0.1 M phosphate buffer, pH 6.5. After addition of 3.3 µL of L-tyrosine substrate solution, the mixture was stirred, and the enzymatic reaction was started by adding 3.3 µL of tyrosinase (approximately 1300 U/mL). The temperature inside a plate reader was set at 25°C. The inhibitory activity was evaluated using the absorbance of dopachrome (475 nm) synthesized by tyrosinase [17, 18.]. Vitamin C (200 mg/ml) was used as a positive control.

Statistical analysis: Results obtained in this study are presented as mean ± SD. Statistical significance was determined using Student's *t*-test, and the difference was considered statistically significant when $P < 0.05$.

RESULTS

Measurement of antioxidant activity of berry extracts by ORAC: When ORAC values of 3 berry extracts were measured, their values were higher than that of typical antioxidant foods [2] (Table 2). Of 3 berry extracts, BBE had the highest value. Since 0.1% of ethanol affected the growth of various cultured cells (data not shown), ethanol was evaporated from each sample before use with cultured cells in the subsequent experiments.

Table 2. Estimation of ORAC

Sample	ORAC (µM TE / g)
Blackraspberry extract (BRE)	62
Blueberry extract (BBE)	66
Raspberry extract (RBE)	49

Induction of *α-crystallin* expression in lens cells: When human lens cells transformed with an adenovirus 12-SV40 virus hybrid were incubated with serially diluted Blabina and berry extracts, mRNA expression levels of *α-crystallin* in the cell, which was analyzed by qRT-PCR, was markedly increased by Blabina and BRE 1 hour after incubation (Fig. 1). The increase was observed at 100,000-fold dilution and remained at least 12 hours' incubation (Fig. 1). Thus, Blabina and BRE had higher *α-crystallin* induction potency than the other berry extracts.
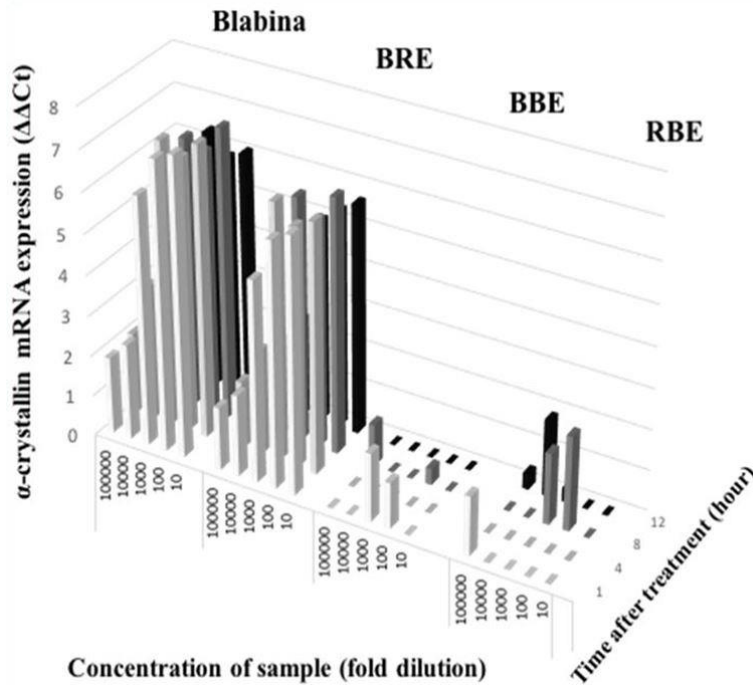
Figure 1. Expression of *α-crystallin* mRNA analyzed by quantitative RT-PCR. Human lens cells transformed with an adenovirus 12-SV40 virus hybrid were incubated with Blabina, BRE, BBE, and RBE for 37 °C for the time indicated, and expression levels of *α-crystallin* mRNA were determined and shown as the ratio to the expression (ΔΔCt) in the control at time 0.

Stimulation of cell growth of follicle dermal papilla cells by berry extracts: When follicle dermal papilla cells were incubated with Blabina and 3 berry extracts, cell growth was increased by 3–20% in a concentration-dependent manner (Fig. 2).
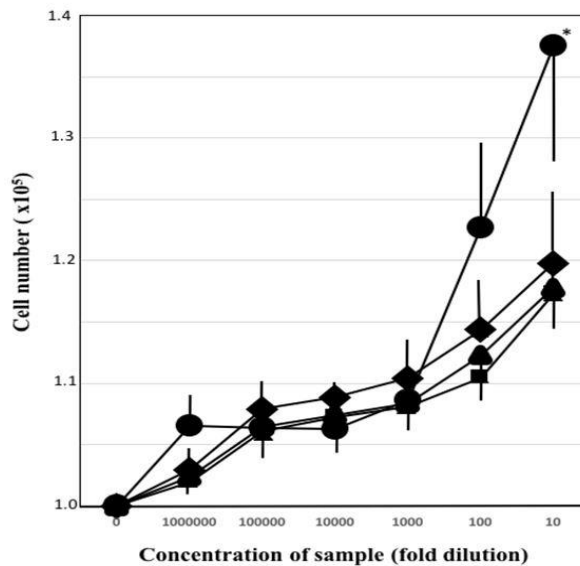
●= Blabina; ▲ = BRE; ■= BBE; ♦= RBE. *P<0.001 vs BRE, BBE, and RBE.

Figure 2. Stimulation of cell growth of follicle dermal papilla cells by berry extracts. Human follicle dermal papilla cells were incubated with berry extracts at 37°C for 48 hours, and cell growth was evaluated by counting cell number with Countess (Invitrogen).

Stimulation of *type 17 collagen* and *HSP47* mRNA expression in follicle dermal papilla cells by berry extracts: Figure 3 shows the effects of BRE on *type 17 collagen* and *HSP47* mRNA expression levels in follicle dermal papilla cells. Both mRNA expression levels were elevated after 1-4 hours of treatment with 1,000-fold diluted BRE and declined by 8-12 hours.
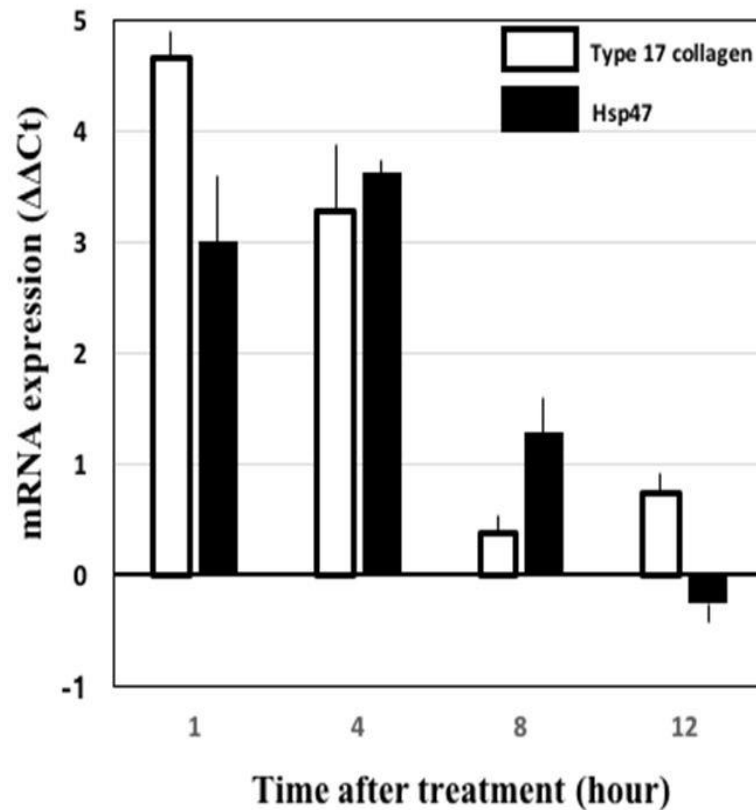

Figure 3. Stimulation of type 17 collagen and HSP47 mRNA expression in follicle dermal papilla cells by BRE. Follicle dermal papilla cells were treated with 1,000-fold diluted BRE for the time indicated, and expression levels of *type 17 collagen* and *HSP47* mRNAs were analyzed by qRT-PCR. White column = *type17 collagen*; black column = *Hsp47*. The elevation of these mRNA expression levels was also observed when the cell was treated with other berry extracts including Blabina (Fig. 4). Blabina and BRE showed higher potency than BBE and RBE.

Inhibition of *5α-reductase* activity by berry extracts: *5α-reductase* catalyzes the conversion of testosterone to DHT that promotes hair loss and thinning [11]. It has been reported that the pharmaceutical products, finasteride and dutasteride, promote hair growth by inhibiting *5α-reductase* activity [24]. These reports suggest that *5α-reductase* is the target molecule to

promote hair growth. Interestingly, Blabina and 3 berry extracts inhibited *5α-reductase* in a dose-dependent manner, and the inhibition was observed within 1 hour after treatment (Fig. 5). Of these 4 extracts, BRE and Blabina showed stronger inhibition than BBE and RBE.
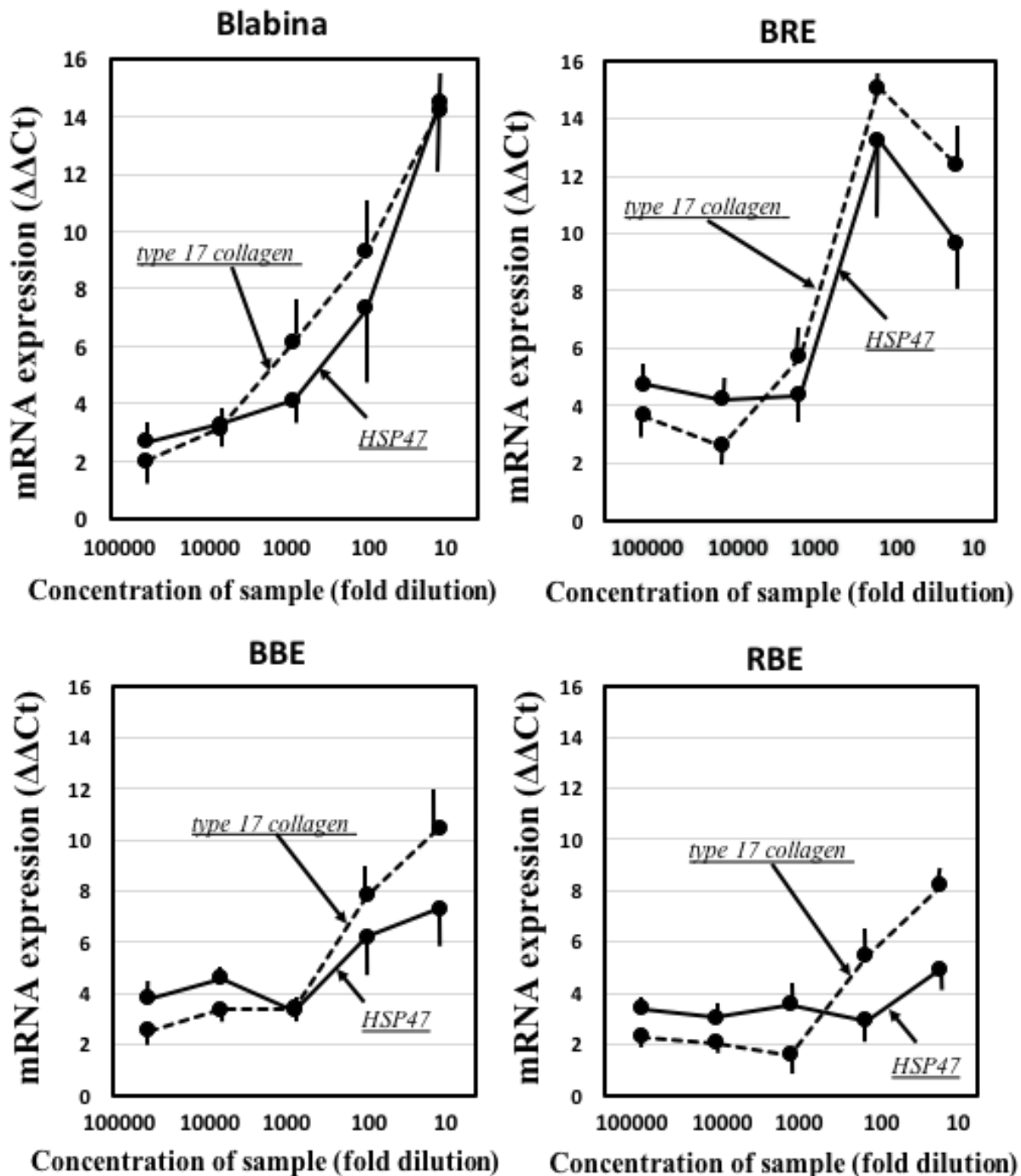

Figure 4. Stimulation of *type 17 collagen* and *HSP47* mRNA expression in follicle dermal papilla cells by berry extracts. Follicle dermal papilla cells were treated with serially diluted Blabina and berry extracts for 1 hour, and expression levels of *type 17 collagen* and *HSP47* mRNAs were analyzed as in Figure 3. mRNA expression levels were calculated relative to the

expression level ($\Delta\Delta Ct$) in the untreated control cells. Solid line= *type 17 collagen*; dotted line= *HSP47*.
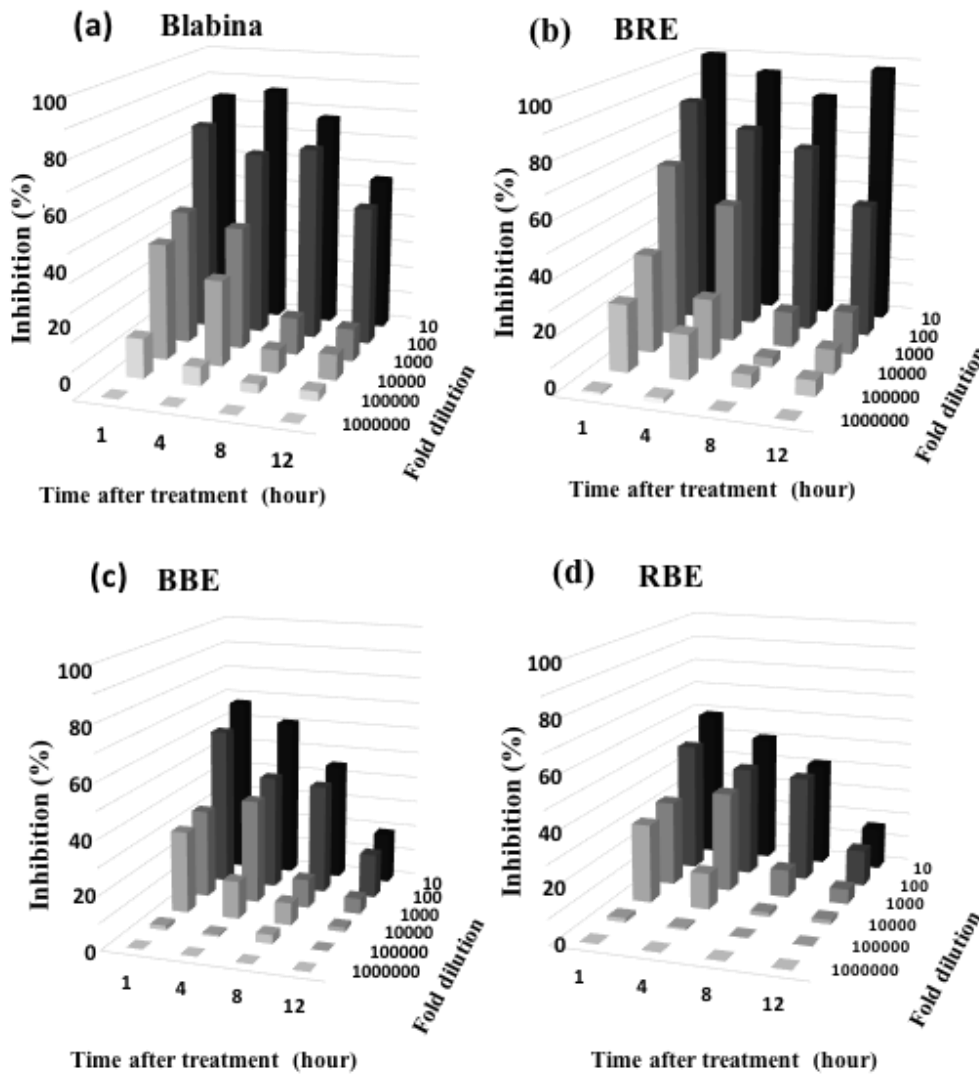

Figure 5. Inhibition of *5α-reductase* in human follicle dermal papilla cells by berry extracts. Follicle dermal papilla cells were treated with serially diluted Blabina and berry extracts for the time indicated, and *5α-reductase* activity was determined as described in the Materials and Methods. The maximum inhibition by BRE (10-fold dilution, at 1 hour), 20 pg/mL, was set as 100% inhibition compared with the activity in control cells, 140 pg/ml.

Induction of *UCP1* mRNA expression in adipocytes: All 4 samples increased *UCP1* mRNA expression levels in a concentration-dependent manner, and Blabina had the strongest effect (Fig. 6). Consistent with this result, the *UCP1* protein secreted from adipocytes was increased by the treatment of the cell with berry extracts in a concentration-dependent manner (Fig. 7).
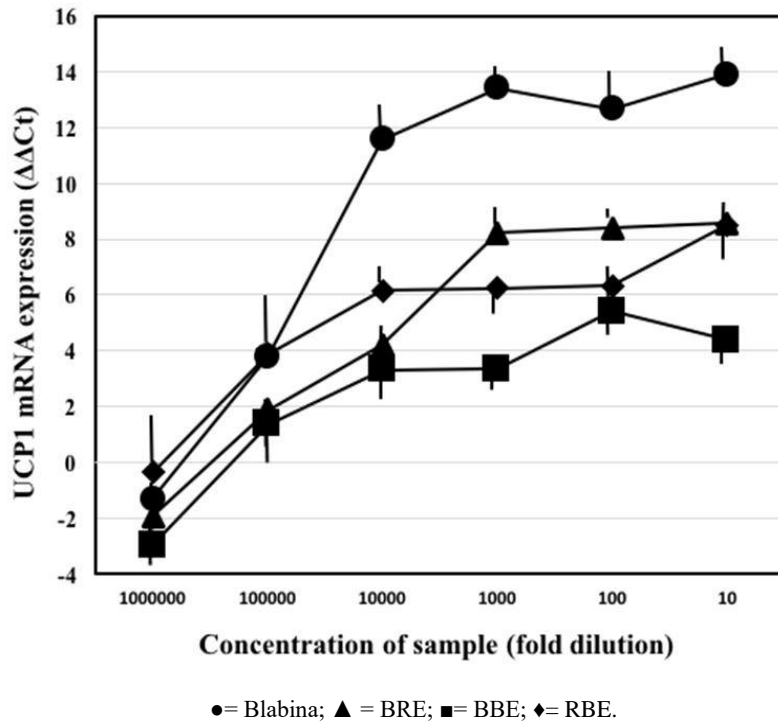
●= Blabina; ▲ = BRE; ■= BBE; ♦= RBE.

Figure 6. Induction of *UCP1* mRNA expression in adipocytes. Adipocytes were treated with Blabina and berry extracts for 1 hour, and expression levels of *UCP1* mRNA were measured by qRT-PCR. The expression levels were calculated relative to the expression level at time 0 (ΔΔCt).
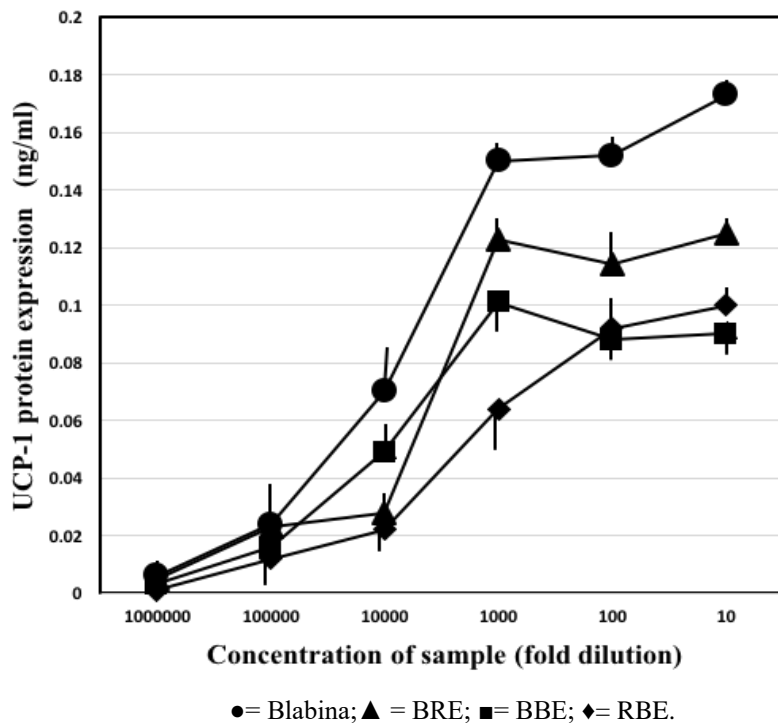

●= Blabina; ▲ = BRE; ■= BBE; ♦= RBE.

Figure 7. Stimulation of *UCP1* protein secretion from adipocytes by berry extracts. Adipocytes were treated with 1,000-fold diluted berry extracts for 1 hour, and the *UCP1* protein secreted was

quantitated by ELISA. The amount of *UCP1* secreted from the non-treated control cells was subtracted from that secreted from berry extract-treated cells.

Stimulation of irisin secretion from skeletal muscle cells: When skeletal muscle cells were incubated with berry extracts, all extracts tested were found to significantly stimulate the irisin secretion from the cell (Fig. 8). Additionally, blabina and BRE demonstrated the stronger effect compared with other berry extracts, suggesting that these berry extracts are effective to prevent obesity.
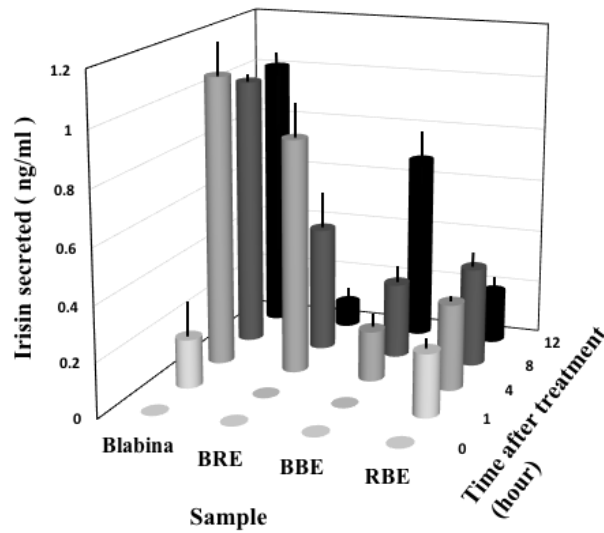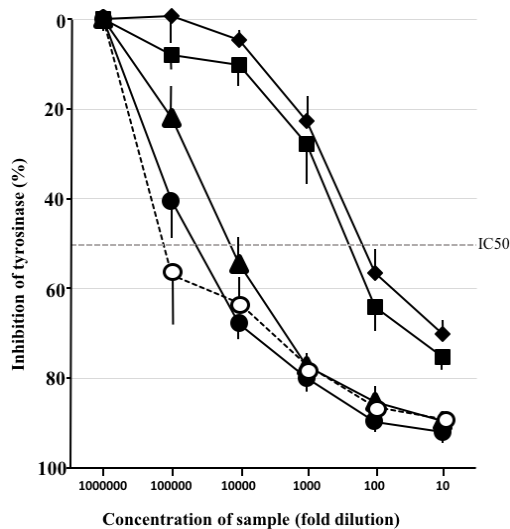

Figure 8. Stimulation of irisin secretion from adipocytes by berry extracts. Adipocytes were treated with berry extracts for 1 hour, and irisin secreted from the cell was quantitated by ELISA. The amount of irisin secreted from the non-treated control cells was subtracted from taht secreted from berry extract-treated cells.

●= Blabina; ▲ = BRE; ■= BBE; ♦= RBE; ◯＝vitamin-C as positive control.

Figure 9. Inhibition of melanocyte tyrosinase activity by berry extracts. Melanocytes were treated with serially diluted berry extracts for 48 hours and tyrosinase activies were determined as described in Materials and Methods. Vitamin C was also tested as a positive control in this experiment. Initial concentration of vitamin C was 200mg/ml.

Inhibition of tyrosinase activity of melanocytes by berry extracts: To evaluate the effect of berry extracts on tyrosinase activity in comparison with that of vitamin C, melanocytes were treated with serially diluted berry extracts and tyrosinase activity was determined. All berry extracts tested, including vitamin C, inhibited the tyrosinase activity in a concentration-dependent manner (Fig. 9). The inhibitory potency of blabina and BRE was higher than that of RBE and comparable to that of vitamin C: IC50s were about 50,000-, 10,000-, 200-, and 100-fold dilutions for Blabina, BRE, BBE, and RBE respectively. Furthermore, maximum inhibition by blabina and BRE was as high as 90%.

DISCUSSION

We examined whether they have anti-aging effects on cataract, alopecia, skin whitening, and weight loss and if those effects correlate with their antioxidant activities.

Various factors are involved in the long-term maintenance of the eye lens function, although the precise mechanisms have not yet been elucidated. The component protein of the lens, α-crystallin, has a chaperone effect preventing the aggregation of β-and γ-crystallin [19]. Moreover, it has been reported that aspirin suppresses lens opacity through the chaperone activity of α-crystallin in a naphthalene-induced cataract rat model [20]. These reports led us to speculate that Blabina and BRE are good candidates to prevent or cure the cataract.

It has been reported that activated human follicle dermal papilla cells secrete fibroblast growth factor 7 (FGF-7), which stimulates the proliferation of hair matrix cells, and vascular endothelial growth factor (VEGF), which promotes angiogenesis, in order to promote hair formation [21, 22]. These reports and our observation raise a possibility that Blabina and berry extracts promote hair formation by stimulating the release of FGF-7 and/or VEGF.   This result is consistent with the report demonstrating that cells missing the HSP47 gene (*SERPINH1*) cannot produce collagen [23].

As shown in Fig. 4, the elevation of *type 17 collagen* and *HSP47* mRNA expression levels was also observed when the cell was treated with other berry extracts including Blabina. Blabina and BRE showed higher potency than BBE and RBE.

Accordingly, it is possible that these berry extracts have potency to stimulate hair growth. As we did not compare the potencies of berry extracts with those of the already approved products, finasteride and dutasteride [25], in this study future experiments should explore this.

It has been reported that the UCP1 expression level is very low in obese animals and increased in animals that do not develop the diet-induced obesity [26]. Additionally, it has been reported that mice in which the UCP1 expression level is artificially lowered induce obesity, while mice in which the UCP1 expression level is elevated lose weight [26, 27.]. Therefore, UCP1 is believed to play an important role in energy consumption and the progress of obesity [27]: UCP1 promotes the metabolism from free fatty acids to heat energy, thereby decreasing the body fat. These reports led us to examine the effects of berry extracts on *UCP1* mRNA expression levels in adipocytes.

Exercise increases irisin secretion from the skeletal muscle [28]. Irisin acts on the white adipose tissue to stimulate the UCP1 expression levels through peroxisome proliferator-activated receptor-α (PPAR-α), and expressed UCP1 stimulates the differentiation of white preadipocytes into brown adipocytes [29, 30]. Importantly, there is a positive correlation between blood irisin concentration and the amount of energy consumption [31]. Moreover, in the elderly, aerobic training elevates irisin levels in the blood, thereby reducing the abdominal visceral fat [32]. Thus, the secretion of irisin from adipocytes is important to prevent obesity.

Vitamin C is applied to prevent melanin production by inhibiting tyrosinase activity in melanocytes [33]. Furthermore, maximum inhibition by blabina and BRE was as high as 90%, which has been reported for kojic acid, arbutin and vitamin C [34].

CONCLUSION

In conclusion, the berry extracts tested in this study were all effective in the anti-aging categories investigated. However, overall blabina and BRE had stronger effects than BBE and RBE. Thus, blabina and BRE are applicable as the anti-aging supplements on cataract, alopecia, skin whitening, and weight loss through their higher antioxidant activities.

List of Abbreviations: BRE, Black raspberry extract; BBE, blueberry extract; RBE, raspberry extract; ORAC, oxygen radical absorbance capacity; UCP1, uncoupling protein-1; DHT, dihydrotestosterone; GPDH, glycerol-3-phosphoric acid dehydrogenase; HSP47, heat shock protein 47; ROS, reactive oxygen species; TE, Trolox equivalents.

Competing Interests: The authors have no financial interest or conflicts of interest.

Authors' Contributions: All authors contributed to this work. H. Banno, Y. Kusumi, K. Dan, and Y. Kanaho designed the research. K. Dan and A. Takada carried out the experiments and analyzed the results. All authors interpreted the results and designed the research strategy. K. Dan, A. Takada and Y. Kanaho prepared the manuscript.

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