Studies on the safety and functionality of bioactive substances derived from Wasabi (*Eutrema japonicum* (Miq.) Koidz.)

Isao Okunishi

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Abbreviations

6-MSITC	6-(methylsulfinyl) hexyl Isothiocyanate
4-MSITC	sulforaphane (4-methylsulfinylbutyl isothiocyanate)
WS	wasabi sulfinyl, a wasabi rhizome extract powder containing 6-MSITC
WLE	wasabi leaf extract
ITC	isothiocyanate
AITC	allyl isothiocyanate
UCP-1	uncoupling protein
AGA	androgenic alopecia
AA	alopecia areata
VEGF	vascular endothelial growth factor
DPCs	dermal papilla cells
FGF-7	fibroblast growth factor 7
DMSO	dimethyl sulfoxide
AD-MSCs	adipose-derived mesenchymal stem cells
SVFs	mesenchymal vascular differentiated cells
HFSCs	human hair follicle stem cells
SUR	sulfonylurea receptor
VFA	visceral fat area
SFA	subcutaneous fat area
TP	total protein
Alb	albumin
TB	total bilirubin
D-B	direct bilirubin
I-B	indirect bilirubin
AST [GOT]	aspartate-aminotransferase
ALT [GPT]	alanin-aminotransferase
γ-GT	γ-glutamyltransferase
LDH	lactate dehydrogenase
ALP	alkaline phosphatase
UN	blood urea nitrogen
Cr	creatinine
UA	uric acid
TC	total cholesterol
HDL-C	high density lipoprotein-cholesterol

LDL-C	low density lipoprotein-cholesterol
TG	triglycerides
CL	chloride
GLU	glucose
HbA1c	hemoglobin A1c
Hb	hemoglobin
PLT	platelets
BMI	body mass index
WBC	white blood count
RBC	red blood cell count
Ht	hematocrit

Abstract

Wasabi, a plant native to Japan, has been known as a medicinal herb from ancient times to modern times. It is used as a basic condiment in Japanese cuisine. Its root parts and stems are used as a condiment, but most of its leaves are discarded. In addition, the number of farmers engaging for cultivating wasabi, and the amount of wasabi consumed in Japan are decreasing. In order to create new value for revitalizing the wasabi industry and utilization, in present study, I conducted to investigate the safety and functionality of wasabi.

First, the safety evaluation of the wasabi leaf (WL) was conducted with the assays of the mutagenicity, acute and sub-acute toxicity, and human safety trial. The results from toxicity test showed that WL extracts (WLE) did not show mutagenicity up to 5,000 μg/plate. WLE were not observed that acute toxicity in mice administered 5,000 mg/kg/day and sub-acute toxicity in rats administered 2,500 mg/kg/day. Twelve healthy subjects, aged 20–64 years and mildly obese (BMI 23.0 to 30.0 kg/m^2), were enrolled in the clinical trial, and ingested 200 mg/day WLE for 12 weeks. Ingestion of 200 mg/day of WLE was demonstrated to be safe. These data provide the first standard references for wasabi leaf supplement application. Additionally, ingestion of 200 mg/day of WLE powder had effects in the reduction of visceral fat although the number of subjects in the present clinical trial was small.

Next, 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) [Fig.1a] has recently been reported to have antioxidant and brain function improvement effects, and its use is expected to expand in the future. However, its safety has not been reported. The safety evaluation of 6-MSITC and Wasabi sulfinyl (WS), a wasabi rhizome extract powder containing 6-MSITC, was conducted by a toxicity test and human clinical trial. Our data showed that no mutagenicity was observed and Lethal dose 50 (LD_{50}) values of synthetic 6-MSITC were estimated to be 451.2 mg/kg in male rats and 400.7 mg/kg in female rats. No sub-acute toxicity was observed in rats administrated WS at 2,500 mg/kg/day. In human trial, ingestion of 500 mg/day of WS was demonstrated to be safe at the least. Our data on the safety evaluation of 6-MSITC and WS provide the first standard references for wasabi rhizome supplement application.

Finally, WL and isosaponarin [Fig.1b], a flavonoid contained in the WL, was found to enhance the proliferation of human follicle dermal papilla cells (DPCs) in culture cells. Moreover, isosaponarin increased the amount and activities of mitochondrial and the productions of vascular endothelial growth factor (VEGF). Furthermore, the data from human clinical trials by applying isosaponarin-rich WLE to 18 subjects who has scalp essence for 8 weeks showed that hair loss was reduced about 60% and scalp redness and itching were also suppressed.

These results indicate that the roots and leaves of wasabi are safely used in the general range of now consumption and have various functional properties. Moreover, these data will provide standard references for safe utilization of wasabi rhizome and leaves, and will help to revitalize wasabi industry.

概要

日本原産の植物であるわさびは、古くから薬草として使われていたことが知られているが、現代では和食の薬味として広く使われている。また、根茎や茎の部分は薬味として使われているが、その葉はほとんど廃棄されている。さらに、日本国内で消費されるわさびの量もわさび農家も共に減少しており、わさびを取り巻く環境は厳しいものとなっている。そこで、わさびの価値を高め、わさび産業を活性化するために、わさびの安全性と機能性について研究を行った。

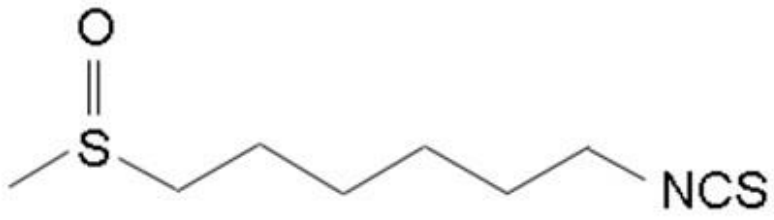
まず、わさび葉エキス（WLE）の安全性評価として、変異原性、急性毒性、亜急性毒性試験とヒト臨床試験を行った。毒性試験の結果、5,000 μ g/plate まで変異原性は示されず、急性毒性では 5,000mg/kg/日まで、亜急性毒性では 2,500mg/kg/日まで毒性は見られなかった。臨床試験では、20〜64 歳の軽度肥満(BMI 23.0 to 30.0 kg/m^2)の健常者 12 名を対象に、WLE を 1 日 200mg、12 週間摂取させた。その結果、1 日 200mg までの WLE 摂取は安全であることが確認できた。以上のように、WLE の変異原性、急性および亜急性毒性、ヒト試験の安全性に関するデータは、わさび葉サプリメントの応用に関する最初の標準的な参考資料となる。さらに、今回の臨床試験は被験者数が少なく、統計的に有意な差は認められなかったが、WLE 粉末は内臓脂肪の減少に効果がある可能性が示唆された。

次に、6-メチルスルフィニルヘキシルイソチオシアネート（6-MSITC、図 1a）は、近年、抗酸化作用や脳機能改善作用が報告され、今後の使用拡大が期待されている。しかし、その安全性に関する報告はまだない。そこで、6-MSITC と、わさびの根茎エキス末「わさびスルフィニル（WS)」の安全性を、毒性試験とヒト臨床試験によって評価した。その結果、変異原性は観察されず、急性毒性試験では、合成 6-MSITC の LD_{50} 値は雄ラットで 451.2 mg/kg、雌ラットで 400.7 mg/kg であった。また、WS による亜急性毒性試験では 2,500 mg/kg/日まで毒性は認められなかった。ヒト試験では WS を 500mg/日摂取しても、少なくとも安全であることが確認された。以上のように、6-MSITC と WS の安全性評価に関するデータは、わさび成分の応用に関する最初の標準的な参考資料となる。

最後に、わさびの葉（WL）に含まれるフラボノイドである「イソサポナリン、図 1b」に毛乳頭細胞を活性化させる働きがあることを発見した。培養した毛乳頭細胞にわさび成分を添加し WST 法で評価したところ、イソサポナリンに有効性があった。さらに、イソサポナリンの作用を解明するために、ELISA による分析を行ったところ、ヘアサイクルの維持に関係すると考えられているアデノシンレセプターや血管内皮細胞増殖因子(VEGF)の増加が認められた。そこで、イソサポナリンを含むスカルプエッセンスを 18 名の被験者に 8 週間塗布したところ、抜け毛が約 60%減少し、頭皮の赤みやかゆみも抑制された。

これらの結果は、わさびの根と葉が、現在消費されている一般的な範囲で安全に使用でき、様々な機能性を持っていることを示している。さらに、これらのデータは、わさびの根や葉を安全に利用するための標準的な参考資料となり、わさび産業の活性化につながるものと期待される。

a) 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC)



b) isosaponarin

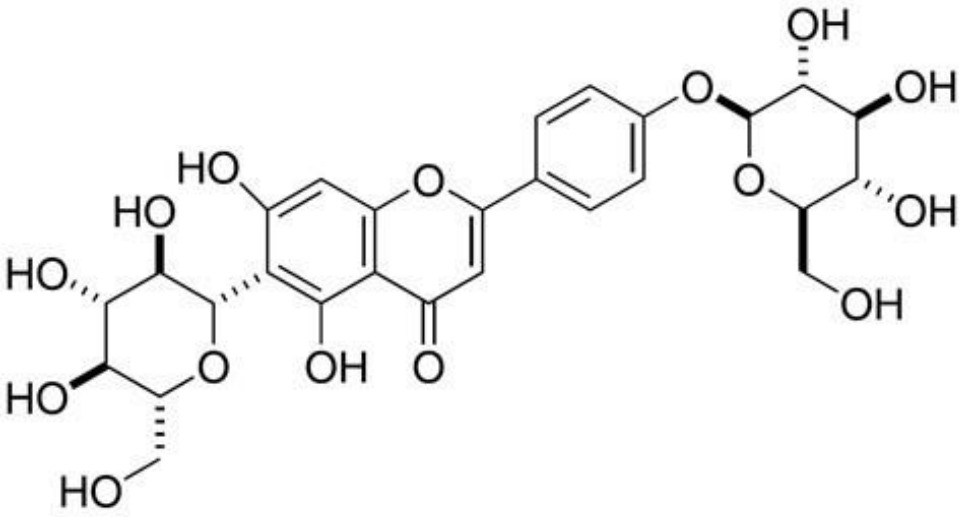


Fig.1 Chemical structure of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) and isosaponarin

Chapter 1. Introduction

1.1. The situation facing Wasabi industry

Wasabi originating in Japan is the plant of Brassicaceae Wasabia. “Wasabi” written on wooden strips was found in the ruins of a medicinal herb garden in Asuka-Kyo that existed about 1,300 years ago (Hasegawa 2004), and was recorded in “Honzo Wamyo,” the oldest Japanese encyclopedia of medicinal plants, about 1,100 years ago. Wasabi was traditionally used for medicine at this time, and then used as a food condiment about 800 years ago (Hasegawa 2004). In 2020, about 2,000 tons of wasabi will be consumed annually, while 4,600 tons were produced in 2005 (Ministry of Agriculture, Forestry and Fisheries, Special Forest Products Production Statistics 2020). The reason for the large decline over the past 15 years is thought to be the inability to maintain production area due to the aging of farmers and the fact that wasabi consumption is declining due to food diversification and changing tastes. In addition, the processed wasabi industry is facing a major problem in that more and more young people are unfamiliar with the taste of wasabi because of the widespread use of inexpensive tubular products that use a low percentage of wasabi.

In order to increase the consumption of wasabi and maintain the production area of wasabi, we focus on its medicinal properties and conduct research to see if we can come up with new uses for it.

1.2. Research on wasabi leaves, an underutilized resource

Wasabi leaf is used for wasabi paste, but most wasabi leaves are discarded because they are less pungent and more bitter than the rhizome. On the other hand, wasabi leaf extract (WLE) is reported to have anti-obesity activity (Yamasaki 2013, Oowatari 2016, Misawa 2018). Animal experiments further demonstrated that the body weight of rats fed a high-fat diet supplemented with wasabi leaf was significantly reduced compared to the control (Yamada-Kato 2016). Regarding the active ingredients, it has been reported that wasabi leaf contains many flavonoid glycosides (Hosoya 2005) and relatively low isothiocyanate content. Isosaponarin, one of flavonoids in wasabi leaf, is reported to have antioxidant (Hosoya 2016, Sekiguchi 2010) and anti-inflammatory (Yoshida 2015) activities. Moreover, isosaponarin could promote collagen production (Nagai 2010) and showed increased expression of β-3 adrenergic receptors and the enzyme UCP-1, which is related to fat β-oxidation. Isovitexin is another flavonoid that has been isolated from wasabi leaf (Mashima 2019), and has been reported to have some bioactive functions, e.g., antioxidative (Ramarathnam 1989) and renoprotective (Liu 2020) effects. Isovitexin

also reduced postprandial blood glucose levels in sucrose-loaded normoglycemic mice (Choo 2012).

Some flavonoids have also been reported to activate hair papilla cells, suggesting that the flavonoids in wasabi leaves may also have hair growth effects (Bassino 2020, Madaan 2017, Kwon 2007, Fei 2018).

Hair growth and fat burning were considered to be high needs in the market, so we focused on these effects of WLE and conducted detailed studies. To make it industrially viable, we also worked to confirm its safety and conducted mutagenicity, acute toxicity and subacute toxicity tests. In addition, we conducted small-scale trials in humans to confirm hematological and biochemical parameters, adverse events, and effects on lipid metabolism.

1.3. The safety of the rhizome component 6-(methylsulfinyl) hexyl ITC (6-MSITC)

Wasabi contains 21 kinds of isothiocyanates (ITCs) (Ina 1982, Etoh 1990). Allyl ITC (AITC), a pungent ingredient, is the most well-known compound. In recent years, 6-MSITC has attracted attention due to various functional reports. Many physiological functions of 6-MSITC, including detoxification (Morimitsu 2002), antioxidation (Hou 2000, 2011, Lee 2010), anti-inflammation (Uto 2007, 2012, Yamada-Kato 2012), blood flow improvement (Morimitsu 2000), anticancer functions (Fuke 2014), and the protection of neuronal cells (Trio 2016), have been reported. Recently, 6-MSITC has been reported to improve outcomes in mouse models of Parkinson's and Alzheimer's diseases (Morroni 2014, 2018). In addition, the data from a human clinical intervention including 37 healthy males and females who ingested Wasabi sulfinyl (WS), a wasabi extract powder containing 0.8 % 6-MSITC, for eight weeks revealed that the attention and judgment of the clinical intervention volunteers were improved (Okunishi 2019).

Although 6-MSITC has been reported to have benefits to health, there are few data on their safety assessment. Thus, confirming the safety of these materials is very important for their use in industry.

No-observed-adverse-effect levels have been reported for AITC, benzyl ITC, phenethyl ITC, and 3-methylthiopropyl ITC at 12, 5, 5, and 30 mg/kg body weight/ day, respectively (Valerio 2006). Additionally, sulforaphane (4-methylsulfinylbutyl isothiocyanate (4-MSITC)) found in broccoli has been investigated by several clinical trials for its internal metabolism and functionality (Shapiro 2001, Kensler 2005). No clinical trial on 4-MSITC safety has been performed even though it was used in a perfume (JECFA No. 1892, FEMA No. 4414). On the other hand, there are no reports on the safety of 6-MSITC.

Based on this information, we estimated the safety of 6-MSITC by the Ames test, acute toxicity test, and sub-acute toxicity test. Finally, human clinical trials were also carried out for WS containing 0.8 % 6-MSITC. According to the previous results that middle-aged and older adults who ingested 100 mg WS for eight weeks daily showed improved cognitive performance (Okunishi 2019), a five-time overdose was designed in the present trial to examine the safety of 500 mg WS intake for four weeks in healthy adults.

Chapter 2. Assessment of mutagenicity, acute and sub-acute toxicity and human trial safety of wasabi leaf extract powder

2.1. Introduction

Although wasabi leaf has rarely been used industrially, it has been found to have useful functional ingredients, such as hair growth and fat burning effects and so on (Yamasaki 2013, Oowatari 2016, Misawa 2018, Yamada-Kato 2016, Hosoya 2005, 2016, Sekiguchi 2010, Yoshida 2015, Nagai 2010, Mashima 2019, Ramarathnam 1989, Liu 2020, Choo 2012). Although these studies have suggested that wasabi leaf has health benefits, there are minimal data on its safety. As research progresses, it is expected to increase consumption largely which functional foods and cosmetics made from wasabi leaves.

However, when it is used as a food product, there is a fear of adverse events due to overconsumption as wasabi leaves have not been consumed in large quantities. Therefore, in order to expand the use of wasabi leaves in a safe and effective manner, we evaluated the safety of WLE by using the Ames test, as well as its acute toxicity in mice and sub-acute toxicity in rats. In addition, to enable future large-scale clinical trials, small-scale human trials were conducted to evaluate the safety of WLE, in which adverse events as well as hematological and biochemical parameters were investigated. We also evaluated the effect of the extract on visceral fat metabolism, among others, for future large-scale studies.

2.2 Material and methods

2.2.1. Preparation of WLE powder, the Ames test, and acute and sub-acute toxicity tests

Wasabi leaves were harvested from Hokkaido by Kinjirushi Co., Ltd., a manufacturer of wasabi products, and were then grated into a paste. WLE used in this study was extracted from the paste using 50 % ethanol, which was shown to be an efficient solvent for the extraction of many kinds of polyphenols in plants in our previous in-house test and is also used in the food industry. The ethanol extracts were concentrated in an evaporator and then lyophilized to a powder. The yield of extract from raw wasabi leaves was 2.8 %. The WLE concentration was adjusted with distilled water to prepare samples for each experiment. The flavonoids were analyzed by high performance liquid chromatography, and total polyphenol content was measured by the Folin-Ciocalteau method with gallic acid as a standard (Gabriel 2014). WLE contained three major flavonoids, isovitexin, isosaponarin, and isoorientin, at concentrations of

0.272, 0.012, and 0.030 mg/g, respectively, as well as a total polyphenol content of 2.28 mg GAE/g.

The Ames test and acute/sub-acute toxicity tests were carried out by TTC Co., Ltd. (Tokyo, Japan). *Salmonella typhimurium* TA100, TA1535, TA98, TA1537, and Escherichia coli WP2 uvrA strains were used for the Ames test. The test was performed using the S9 (±) pre-incubation method (37 °C, 20 min) with a culture time of 48 h, a maximum volume of 5,000 μg/plate, a common ratio of 4, and seven volumes of two plates each. The acute toxicity test consisted of single oral gavage administration of WLE (adjusted to 1,250, 2,500, and 5,000 mg/kg in 20 mL of water) to 20 male and female Slc:ICR mice (SPF) (5 weeks old). Administration was started on day 0 in all cases, and the general condition of mice was monitored for 14 days.

Furthermore, rats were used for the sub-acute toxicity test because various organs, blood, and urine were required to assess symptoms of toxicity. Eighteen male and 18 female Crl:CD (SD) rats (5 weeks old) were administrated by repeated oral gavage with WLE for 28 days at a dose of 500 mg/kg/day or 1,500 mg/kg/day with water. The general condition of rats was observed before and after administration. During the study period, solid feed CRF-1 (Oriental Yeast Co., Ltd.) was provided, and drinking water was freely available. Each animal was subjected to urinalysis, necropsy, organ weight measurement, hematology, and blood biochemical analysis after 28 days.

2.2.2. Ethics

The toxicity tests adhered to the Good Laboratory Practices (GLP, Ministry of Health, Labour and Welfare Ordinance No. 21 of 1997) and the Organization for Economic Co-operation and Development Guidelines for the Testing of Chemicals; however, the assays were performed under non-GLP conditions.

All animal experiments adhered to the Guidelines for the Proper Implementation of Animal Experiments (Science Council of Japan, 2006), Act on Welfare and Management of Animals (Amendment of Act No. 68 of 2005) and Standards Relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Notice of the Ministry of the Environment No. 88 of 2006). Sub-acute toxicity tests were conducted according to the Ministerial Ordinance on Good Laboratory Practices for Nonclinical Safety Studies of Drugs (Ordinance of the Ministry of Health and Welfare No. 21 of 1997) and partial revision of the guidelines for repeated dose toxicity studies (PMSB/ELD Notification No. 902).

The human clinical trial adhered to all guidelines set forth in the Declaration of Helsinki (revised by WMA Fortaleza General Assembly, Brazil, in October 2013) and

Ethical Guidelines for Medical Research on Humans (partially revised December 22, 2014/February 28, 2017). The human rights of the subjects were respected and the trial protocol was confirmed with the approval of the Ethics Review Committee. The trial was carried out under the supervision of a doctor. This study was conducted following review and approval by the Medical Association Hakusuikai Suda Clinic Investigational Review Board. The study was registered with the University Hospital Medical Information Network Clinical Trials Registry, study ID UMIN000036597.

2.2.3. Participants

Of 22 subjects initially screened, 12 healthy male and female subjects, aged 20–64 years and mildly obese (BMI 23.0 to 30.0 kg/m^2), were enrolled in this study. Individuals were excluded if they were (1) undergoing treatment with any drug at the time of the study, including drugs used to treat obesity, hyperlipidemia, or lipid metabolism; (2) on a diet or exercise regimen under the supervision of a physician; (3) unable to stop taking health foods or supplements during the study; (4) unable to refrain from drinking alcohol on the day before the test; (5) illness or history of serious illness; (6) food allergy or drug dependence; (7) history of alcohol dependence; (8) metal in the body due to surgery; (9) presence of a cardiac pacemaker or another medical device implanted in the body; (10) claustrophobia; (11) shift worker or working night shift; (12) pregnancy, nursing, or wishing to become pregnant during the study period; (13) current participation in another clinical trial; (14) participation in another clinical trial within 1 month of the present study; and (15) judged by the investigator to be inappropriate for participation in the clinical trial on vitals (blood pressure/pulse) were excluded.

2.2.3. Study design and procedure

The study was a randomized, placebo-controlled, double-blind, parallel-design, twelve-week treatment study [Table 1]. A flow chart detailing the selection and grouping of study subjects is presented in Figure 2. The study was designed to assess the efficacy and safety of WLE powder intake for improved body composition. After medical screening, each eligible subject was randomly assigned to one of two groups: WLE powder group, who received two capsules containing 100 mg WLE powder/capsule embedded in dextrin daily, and the placebo group, who received two capsules containing 100 mg dextrin/capsule daily. Tablets were taken with water before going to bed. As a restriction, during the test period, the subjects were required to record specified items, sleep time, activities and physical condition changes, in an electronic

Table 1. Human clinic trial schedule

		Screening	0 week	8 weeks	12 weeks
Subject visit		●		●	●
Obtain consent		●			
Interview		●		●	●
Vital signs		●		●	●
Physical measurement	Height *1	●			
	Weight and waist circumfe	●		●	●
	Body fat percentage	●		●	●
	BMI	●		●	●
Subject diary			←	→	
Nutrition survey *2		←		→	
Test food intake			←	→	
CT scan		●			●
Blood Inspection	Hematology	●		●	●
	General biochemical	●		●	●
	Lipids(T-cho、HDL-C、LD	●		●	●

*1: Height is measured only for screening. *2: Nutrition survey (recorded 3d before week 0 (ingestion starting date), 8 weeks after ingestion, 3d before visiting doctor at 12 weeks of intake).

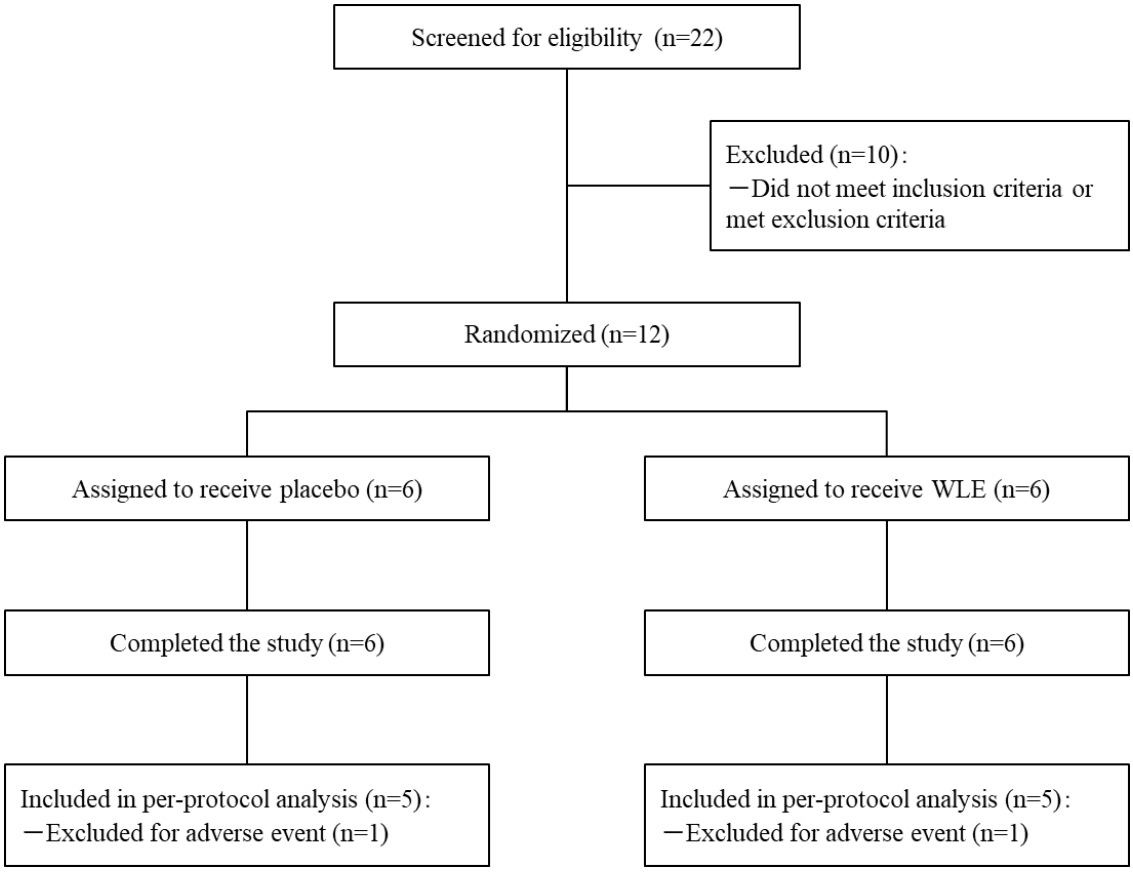


Fig. 2. Flow chart for the selection and grouping of subjects in the human clinic trial

diary every day, and to keep a food diary for 3 days before the start of WLE powder intake and for 3 days before visiting the clinic. The ingestion of medicines and all health foods and supplements that potentially affect body fat and lipid metabolism was prohibited (if normally taken, these were discontinued from the date of consent to participate). Subjects were instructed to not make any changes to their lifestyle habits including diet, exercise, smoking, and administration of medicines, before participating in the trial. The Examination Consultation Service was used to notify the subjects to avoid any lifestyle changes. Any event including excessive exercise that greatly deviated from their normal daily range, more or less food intake than usual, temporary illnesses, or the intake of any medication was recorded in the electronic diary.

On the day before visiting the clinic, subjects were instructed not to consume anything except water, and the intake of food and drink must be completed by 21:00. Additionally, no excessive exercise or drastic change in caloric intake was allowed. On the day of the test, subjects could not smoke from the time they woke up until the end of the test. They also did not eat breakfast or consume WLE powder on that morning.

2.2.4. Evaluation/observation items

Doctor interviews, physical measurements, and blood examinations were performed three times, at the initial screening and at the following 8 and 12 weeks. Abdominal VFA, abdominal SFA, and total abdominal fat area were calculated using PC software (FAT scan) with image data obtained from the CT scans (Brivo CT385; GE Healthcare). The V/S area ratio was determined from the VFA and SFA. Body weight, BMI, waist circumference, body fat percentage, total cholesterol (T-Cho), HDL cholesterol, and LDL cholesterol were also evaluated.

2.2.5. Safety assessment

Safety was evaluated by the incidence and severity of WLE powder ingestion-related adverse events in subjects during the research period. An adverse event was defined as any undesirable or unintended injury or symptom, including abnormalities in clinical laboratory test values, occurring in subjects after the medical screening.

The incidence of side effects, vital signs (blood pressure and pulse), and other symptoms were monitored. Blood tests were performed to measure white blood cell count, red blood cell count, platelets, hematocrit, hemoglobin, total protein, alkaline phosphatase, aspartate transaminase, alanine transaminase, γ-glutamyltransferase, lactate dehydrogenase, uric acid, urea nitrogen, total bilirubin, albumin, creatinine, creatine phosphokinase, glucose, and hemoglobin A1c.

2.2.6. Data analyses

In this study, the null hypothesis that each measured value would not differ before and after ingestion of WLE or between groups was tested. The significance level was 5 % in a two-sided test, and the null hypothesis was rejected if a significant difference was found. A significance level of 5–10 % was considered to indicate a tendency. The normality of evaluation items obtained was examined using the Shapiro-Wilk test for continuous data; a paired t-test was performed if normality was shown, and the Wilcoxon signed-rank test was performed if normality was not shown. For unpaired data, if normality was indicated, a test of variance (F-test) was performed, followed by the Student's t-test (for equal variance) or Aspin-Welch's t-test (for unequal variance). The Mann–Whitney U-test was performed for unpaired data when normality was not shown. For ordered data with many stages, corresponding data were subjected to the Wilcoxon signed-rank test, and uncorrelated data were subjected to the Mann-Whitney U-test. For ordinal data and nominal data with few steps, the corresponding data were subjected to McNemar’s test, and the uncorrelated data were analyzed by Fisher's direct method or the $\chi 2$ test according to the number of data points. When multiple comparisons were required, Bonferroni’s, Tukey’s or Dunnett’s method was used according to the format of the data. Variance and correlation coefficient analyses were also performed as necessary. For safety evaluation items, in principle, only intra-group comparison of values before and after intake was performed, and comparison between groups before and after intake was not performed. Statistical analysis was performed using IBM SPSS Statistics (ver. 25).

2.3 Results

2.3.1. Results of the Ames, acute and sub-acute tests

The Ames test was conducted to investigate the mutagenicity of WLE in the concentration range of 1.2–5,000 μg/plate. Growth inhibition was observed in all strains at 1,250–5,000 μg/plate. In the case of +S9, growth inhibition was only observed at 5,000 μg/plate for the TA98 strain. On the other hand, no mutagenicity was observed at any concentration of WLE [Table 2].

The acute toxicity of WLE was tested in male and female Slc:ICR mice (SPF). No mortality was observed at 5,000 mg/kg. Compared to the control group, no significant increase or decrease in body weight after 14-day administration was observed [Fig. 3]. At necropsy, liver discoloration was observed in two male rats administered 2,500 mg/kg WLE; however, it was clarified that the effect was not due to the test substance, as no dose-dependency was observed in the administration groups.

Table 2. Results of the Ames test (revertant colony number)

		Base pair substitution type						Frame shift type			
		TA100		TA1535		WP2 uvrA		TA98		TA1537	
		- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
Negative control		152	151	12	15	24	30	17	32	8	17
Wasabi leaf extract	1.2 μg/plate	143	178	11	18	29	47	17	33	11	11
	4.9 μg/plate	173	172	16	6	30	35	23	37	7	7
	20 μg/plate	173	181	15	10	36	30	13	34	6	9
	78 μg/plate	171	174	15	13	33	35	21	27	5	9
	313 μg/plate	160	169	12	9	25	33	25	31	5	11
	1250 μg/plate	144	208	13 *	17	30	21	15 *	29	5 *	14
	5000 μg/plate	178 *	224	12 *	14	26 *	33	22 *	36 *	3 *	7
Positive control		628 +	1063 +	550 +	326 +	142 +	1363 +	464 +	331 +	2465 +	154 +
reagent		AF-2	2-AA	NaN_3	2-AA	AF-2	2-AA	AF-2	2-AA	ICR-191	2-AA
concentration(μg/plate)		0.01	1.0	0.5	2.0	0.01	10	0.1	0.5	1.0	2.0

*: Growth inhibition was observed, +: More than 2 times back mutations were seen.
The strain -S9 or +S9 was pre-incubated at 30 ° C for 20 minutes, and further cultured for 48 hours after addition of test substance. The solvent used was dehydrated DMSO, and no precipitation of the test substance was observed. Positive control reagents. AF-2; 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, 2-AA; 2-Aminoanthracene, NaN3; Sodium azide, ICR-191; 6-Chloro-9-[3-(2-chloroethylamino)-propylamino]-2-methoxyacridine dihydrochloride.

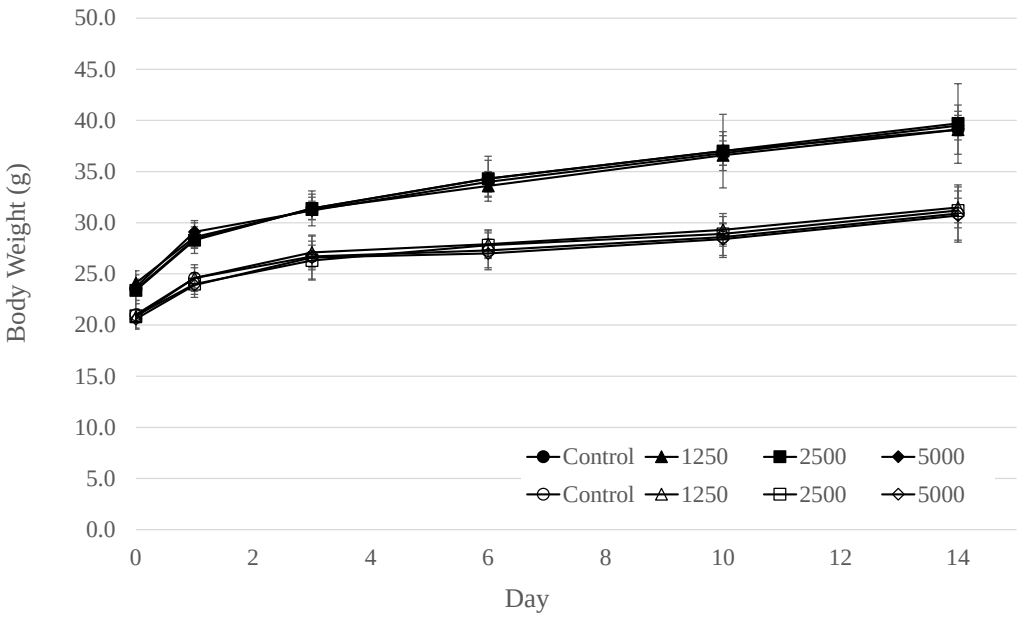


Fig. 3. Effect of single dose administration of WLE on body weight of male and female rats

The administration method was adjusted to 20 ml / kg each using a disposable syringe and an oral sonde. Control group was administered water in the same manner. Body weight was measured using an electronic balance (PM2000, METTLER TOLEDO Co., Ltd.). Values are expressed as mean ± SD (n = 5).

From these data, the non-toxic equivalent of WLE was estimated as 5,000 mg/kg.

The sub-acute toxicity of WLE was further evaluated by repeated oral administration to five-week-old Sprague-Dawley rats for 28 days. No mortality was observed in any case throughout the observation period. No significant changes were observed in the general observation states and body weight as compared with the control group [Fig. 4], and no significant changes were observed in water intake, food consumption, urinalysis, and hematological indexes [Table 3-7].

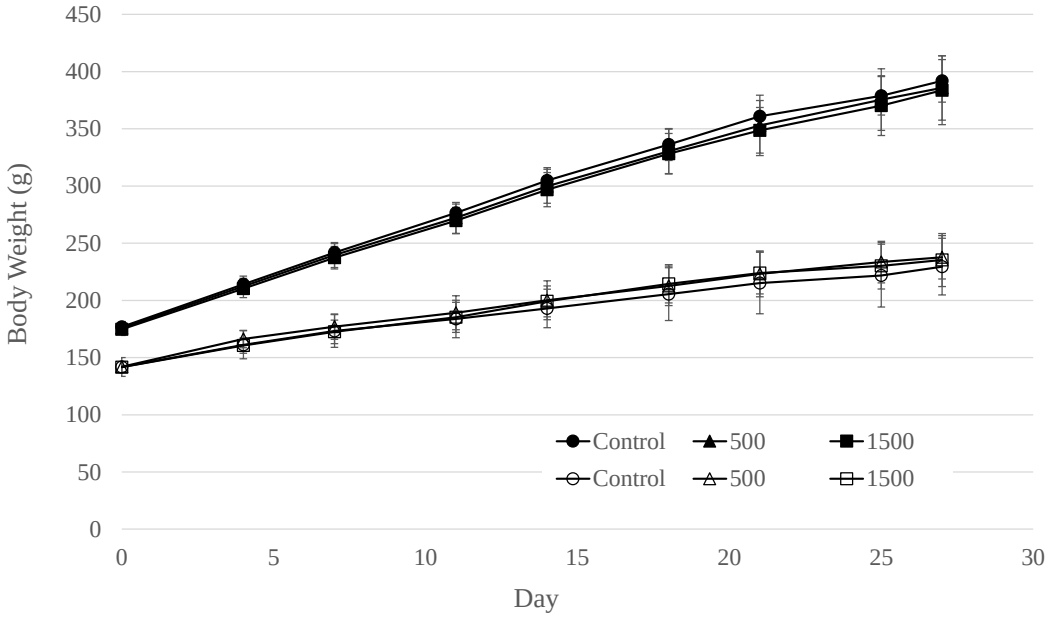


Fig. 4. Effect of 28-day treatment with WLE on body weight of male and female rats

All males and females in each group were measured using an electronic balance (EW-300G, A&D Company, Limited) twice a week during the administration period before administration of the test substance. Values are expressed as mean ± SD (n = 6).

Table 3. Effect of WLE on mean feed consumption (g) in rats

Sex	Group	Dose (mg/kg)		Day 0	4	7	11	14	18	21	25	27
Male	Control	0	Mean	21.7	24.2	24.3	26.2	27.7	26.3	28.2	27.3	26.8
			SD	1.4	1.2	2.3	1.9	1.8	2.1	2.3	2.0	2.1
	WLE	500	Mean	22.2	23.5	25.0	26.5	27.5	28.8	29.2	30.7	28.3
			SD	2.3	1.0	3.2	2.9	2.3	2.6	3.5	3.8	2.0
		1500	Mean	22.5	23.5	24.0	25.8	27.0	28.0	27.5	29.7	27.8
			SD	1.2	2.2	1.7	1.9	2.0	2.9	2.5	4.6	4.6
Female	Control	0	Mean	17.7	17.8	17.7	18.0	19.0	19.0	18.0	20.0	21.0
			SD	1.2	1.0	1.5	2.2	2.1	1.4	2.5	3.7	1.5
	WLE	500	Mean	17.8	18.7	17.7	18.0	18.2	19.0	17.3	17.2	19.3
			SD	1.8	2.0	3.4	2.2	1.5	2.1	3.7	2.1	2.9
		1500	Mean	17.5	17.7	18.5	16.7	16.5	17.2	19.0	18.8	21.2
			SD	2.4	2.1	1.9	3.1	2.1	2.5	4.2	2.7	2.8

Each value represented mean ± SD (g/day, n = 6). Solid feed CRF-1 (lot numbers 060411B1 and 060511B1, manufactured by Oriental Yeast Co., Ltd.) was freely fed throughout the test period.

Table 4. Effect of WLE on average water consumption (mL) in rats

Sex	Group	Dose (mg/kg)		Day								
				0	4	7	11	14	18	21	25	27
Male	Control	0	Mean	26.7	25.2	28.3	28.8	30.5	31.3	32.0	29.8	30.2
			SD	1.5	1.7	2.4	3.3	3.8	3.6	3.3	3.7	3.4
	WLE	500	Mean	25.8	25.2	27.5	27.7	31.0	29.7	29.7	28.2	26.0
			SD	1.9	1.6	3.7	3.3	3.3	5.0	5.0	3.6	2.8
		1500	Mean	27.5	28.5	30.2	32.8	32.7	32.5	32.0	32.3	32.7
			SD	2.0	2.8	2.7	4.6	4.4	4.8	4.9	5.2	6.5
Female	Control	0	Mean	22.5	20.3	20.8	20.0	22.2	19.5	20.5	20.3	21.8
			SD	1.4	2.2	3.4	3.1	5.6	3.6	4.0	3.1	2.5
	WLE	500	Mean	22.7	22.7	24.3	21.8	24.5	22.7	22.7	23.3	22.7
			SD	1.9	2.9	3.4	4.4	2.9	2.5	2.5	5.1	4.2
		1500	Mean	22.8	20.5	23.3	20.5	20.7	19.8	19.8	21.2	22.3
			SD	1.5	2.3	2.4	3.6	3.4	4.0	4.0	4.8	3.9

Each value represents mean ± SD (g/day, n = 6). Tap water was freely ingested by a water absorbing bottle.

Table 5. Urinalysis in rats treated orally with WLE for 28 days (3hr stock urine)

Sex			Control	WLE (mg/kg B.W.)	
				500mg/kg	1500mg/kg
Male	pH	7.5	1	0	0
		8.0	5	3	5
		8.5	0	3	1
	Protein	—	6	6	5
		+	0	0	1
	Glucose	—	6	6	6
	Ketones	—	6	6	6
	Occult blood	—	6	6	6
	Urobilinogen	0.1	6	6	6
	Bilirubin	—	6	6	6
Female	pH	7.5	0	1	1
		8.0	4	3	3
		8.5	2	2	2
	Protein	—	6	6	6
	Glucose	—	6	6	6
	Ketones	—	6	6	6
	Occult blood	—	6	6	6
	Urobilinogen	0.1	6	6	6
	Bilirubin	—	6	6	6

At 4 weeks after administration, all males and females in each group were housed in cages after the end of administration on the day, and livestock urine was collected and measured for up to 3 hours under fasting and water intake. Number of rats (N = 6), —; Negative, +(Protein); 30mg/dL, Urobilinogen; Ehrlich unit/dL.

Table 6. Urinalysis in rats treated orally with WLE for 28 days (21hr stock urine)

Sex			Control	WLE (mg/kg B.W.)	
				500mg/kg	1500mg/kg
Male	Volume	(mL/21hr)	10.8 ± 2.5	12 ± 2.8	13.9 ± 4.2
	Specific gravity		1.054 ± 0.016	1.055 ± 0.013	1.048 ± 0.014
	Na	(mEq/21hr)	1.565 ± 0.619	1.787 ± 0.230	1.587 ± 0.229
	K	(mEq/21hr)	2.739 ± 0.616	3.143 ± 0.205	3.298 ± 0.475
	Cl	(mEq/21hr)	1.86 ± 0.553	2.004 ± 0.173	1.877 ± 0.204
Female	Volume	(mL/21hr)	9.2 ± 4.9	7.2 ± 2.8	10.3 ± 4.4
	Specific gravity		1.043 ± 0.014	1.055 ± 0.015	1.034 ± 0.010
	Na	(mEq/21hr)	0.832 ± 0.572	0.851 ± 0.307	0.781 ± 0.444
	K	(mEq/21hr)	1.451 ± 0.796	1.51 ± 0.445	1.411 ± 0.824
	Cl	(mEq/21hr)	0.957 ± 0.614	0.927 ± 0.294	0.812 ± 0.469

At 4 weeks after administration, all males and females in each group were housed in cages after the end of administration on the day, and livestock urine was collected and measured for up to 21 hours under fasting and water intake. Each value represents mean ± SD.

Table 7. Effect of WLE on hematological parameters in rats

Sex	Organ		Control	WLE (mg/kg B.W.)	
				500mg/kg	1500mg/kg
Male	Erythrocytes	($\times10^4$/μL)	750 ± 37	749 ± 31	742 ± 41
	Reticulocytes	(‰)	26 ± 3	31 ± 3	29 ± 3
	Hemoglobin	(g/dL)	16.0 ± 0.8	16.0 ± 0.5	15.9 ± 0.7
	Hematocrit	(%)	44.6 ± 2.1	44.8 ± 1.6	44.2 ± 2.8
	MCV	(fL)	59.4 ± 0.5	59.8 ± 1.8	59.6 ± 2.1
	MCH	(pg)	21.3 ± 0.6	21.4 ± 0.8	21.4 ± 0.8
	MCHC	(%)	35.9 ± 1.0	35.8 ± 0.8	35.9 ± 0.7
	Leukocytes	($\times10^2$/μL)	97 ± 35	123 ± 29	106 ± 23
	Differential count of leukocytes (%)				
	Eosinophils		0.5 ± 1.2	0.8 ± 0.8	0.3 0.8
	Neutrophils	Stab	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Segment	11.8 ± 9.0	10.0 ± 4.6	8.3 ± 2.7
	Basophils		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Monocytes		0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4
	Lymphocytes		87.7 ± 8.9	89.2 ± 5.0	91.2 ± 3.3
	Plateles	($\times10^4$/μL)	113.9 ± 13.0	111.9 ± 8.6	99.4 ± 11.5
Female	Erythrocytes	($\times10^4$/μL)	764 ± 43	751 ± 46	770 ± 43
	Reticulocytes	(‰)	23 ± 3	21 ± 3	22 ± 2
	Hemoglobin	(g/dL)	16.1 ± 0.7	16.3 ± 0.8	16.3 ± 0.4
	Hematocrit	(%)	43.8 ± 2.4	44.1 ± 2.3	44.9 ± 1.3
	MCV	(fL)	57.3 ± 0.9	58.8 ± 1.6	58.4 ± 1.8
	MCH	(pg)	21.1 ± 0.5	21.7 ± 0.7	21.2 ± 0.7
	MCHC	(%)	36.8 ± 0.7	36.9 ± 0.5	36.4 ± 0.4
	Leukocytes	($\times10^2$/μL)	73 ± 37	74 ± 10	81 ± 11
	Differential count of leukocytes (%)				
	Eosinophils		0.8 ± 1.2	1.2 ± 0.8	0.5 ± 0.8
	Neutrophils	Stab	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
		Segment	12.8 ± 5.3	12.3 ± 4.3	8.5 ± 2.7
	Basophils		0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0
	Monocytes		0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4
	Lymphocytes		86.3 ± 6.4	86.3 ± 4.4	90.8 ± 2.4
	Plateles	($\times10^4$/μL)	111.7 ± 16.4	116.4 ± 6.0	113.4 ± 6.0

Blood was collected before pathological autopsy after fasting for 18 hours under water intake from the evening of the last administration day. Each value represents mean ± SD (N = 6).

In blood biochemical indexes, GOT values were 64 ± 4 IU/L in the control group, 76 ± 10 IU/L in the 500 mg/kg/day group, and 75 ± 6 IU/L in the 1,500 mg/kg/day group of male rats. A significant increase in GOT was observed in the 500 and 1,500 mg/kg WLE groups, and a low A/G ratio was observed in the 500 mg/kg WLE group of female rats [Table 8].

Table 8. Effect of WLE on serum biochemical parameters in rats

Sex	Organ		Control	WLE (mg/kg B.W.)	
				500mg/kg	1500mg/kg
Male	AST(GOT)	IU/L	64 ± 4	76 ± 10**	75 ± 6*
	ALT(GPT)	IU/L	24 ± 2	27 ± 4	25 ± 1
	ALP	IU/L	704 ± 184	662 ± 71	658 ± 149
	γ-GTP	IU/L	0.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
	Total ptotein	g/dL	5.9 ± 0.1	5.9 ± 0.1	5.9 ± 0.2
	Albumin	g/dL	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.2
	Globulin	g/dL	3.4 ± 0.2	3.3 ± 0.2	3.3 ± 0.3
	A/G ratio		0.75 ± 0.07	0.79 ± 0.06	0.77 ± 0.07
	Glucose	mg/dL	149 ± 22	155 ± 29	135 ± 6
	Triglyceride	mg/dL	59 ± 19	60 ± 33	40 ± 10
	Total cholesterol	mg/dL	49 ± 5	60 ± 8	49 ± 6
	BUN	mg/dL	12 ± 1.0	13 ± 2.1	11 ± 1.4
	Creatinine	mg/dL	0.21 ± 0.01	0.27 ± 0.06	0.24 ± 0.04
	Total bilirubin	mg/dL	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
	Na	mEq/L	143.0 ± 1.0	142.9 ± 1.3	143.3 ± 0.9
	K	mEq/L	4.62 ± 0.24	4.54 ± 0.13	4.48 ± 0.28
	Cl	mEq/L	104.7 ± 1.6	103.9 ± 1.6	103.9 ± 1.8
	Inorganic P	mg/dL	8.6 ± 0.4	9.0 ± 0.5	8.9 ± 0.3
	Ca	mg/dL	10.6 ± 0.3	10.5 ± 0.2	10.5 ± 0.2
Female	AST(GOT)	IU/L	71 ± 11	82 ± 14	87 ± 10
	ALT(GPT)	IU/L	21 ± 3	22 ± 3	23 ± 2
	ALP	IU/L	411 ± 62	418 ± 84	408 ± 65
	γ-GTP	IU/L	1.2 ± 0.4	1.0 ± 0.6	1.0 ± 0.0
	Total ptotein	g/dL	6.0 ± 0.3	6.0 ± 0.2	6.0 ± 0.2
	Albumin	g/dL	2.8 ± 0.1	2.6 ± 0.1	2.9 ± 0.2
	Globulin	g/dL	3.2 ± 0.2	3.4 ± 0.2	3.1 ± 0.2
	A/G ratio		0.89 ± 0.04	0.79 ± 0.06*	0.92 ± 0.12
	Glucose	mg/dL	134 ± 22	117 ± 19	121 ± 12
	Triglyceride	mg/dL	25 ± 9	23 ± 11	26 ± 20
	Total cholesterol	mg/dL	64 ± 19	66 ± 7	66 ± 15
	BUN	mg/dL	15 ± 1.0	13 ± 1.1	15 ± 1.7
	Creatinine	mg/dL	0.33 ± 0.05	0.31 ± 0.05	0.27 ± 0.05
	Total bilirubin	mg/dL	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
	Na	mEq/L	142.1 ± 1.4	142.4 ± 0.7	141.3 ± 1.6
	K	mEq/L	4.61 ± 0.08	4.37 ± 0.26	4.72 ± 0.07
	Cl	mEq/L	105.7 ± 1.9	106.3 ± 1.4	105.4 ± 2.5
	Inorganic P	mg/dL	7.8 ± 0.7	7.7 ± 0.5	7.5 ± 0.7
	Ca	mg/dL	10.3 ± 0.3	10.3 ± 0.2	10.3 ± 0.3

Blood was collected before pathological autopsy after fasting for 18 hours under water intake from the evening of the last administration day. Each value represents mean ± SD (N = 6). $p < 0.01$**, $p < 0.05$*.

Necropsy results showed an enlarged thymus gland and increased lung weight in females fed 500 mg/kg WLE compared to the control group [Table 9]. On the other hand, there were no significant differences in other organs and in absolute weight. The changes in females fed 500 mg/kg WLE did not show dose-dependency and may have been a random change, as these changes were not observed in the 1,500 mg/kg WLE group.

Table 9. Relative organ weights of rats treated with WLE for 28 days

Sex	Organ	Weight (/100g B.W.)	Control	WLE (mg/kg B.W.)	
				500mg/kg	1500mg/kg
Male	Brain	g	0.55 ± 0.02	0.53 ± 0.05	0.54 ± 0.02
	Thymus	g	0.18 ± 0.03	0.19 ± 0.04	0.16 ± 0.02
	Heart	g	0.34 ± 0.01	0.36 ± 0.03	0.36 ± 0.02
	Lung	g	0.33 ± 0.02	0.37 ± 0.03	0.34 ± 0.01
	Liver	g	2.9 ± 0.1	3.1 ± 0.2	2.9 ± 0.1
	Spleen	g	0.20 ± 0.03	0.19 ± 0.04	0.21 ± 0.01
	Kidneys	g	0.77 ± 0.06	0.76 ± 0.07	0.78 ± 0.05
	Adrenals	mg	16 ± 3	15 ± 3	15 ± 2
	Testes	g	0.89 ± 0.07	0.86 ± 0.10	0.89 ± 0.12
Female	Brain	g	0.83 ± 0.09	0.86 ± 0.04	0.84 ± 0.08
	Thymus	g	0.21 ± 0.03	0.24 ± 0.05	0.20 ± 0.03
	Heart	g	0.38 ± 0.07	0.39 ± 0.03	0.37 ± 0.03
	Lung	g	0.43 ± 0.05	0.44 ± 0.04	0.44 ± 0.03
	Liver	g	2.9 ± 0.2	2.8 ± 0.1	2.8 ± 0.1
	Spleen	g	0.20 ± 0.03	0.21 ± 0.02	0.22 ± 0.03
	Kidneys	g	0.79 ± 0.09	0.78 ± 0.03	0.81 ± 0.05
	Adrenals	mg	30 ± 4	32 ± 4	26 ± 4
	Ovaries	mg	35 ± 8	40 ± 6	35 ± 4
	Uterus	g	0.21 ± 0.05	0.2 ± 0.05	0.23 ± 0.08

After the organs were removed, the weight of each organ was measured, and the relative weight per 100 g was calculated from the body weight at the time of autopsy. Each value represents mean ± SD (N = 6).

2.3.2. Characteristics of clinical trial subjects

Of the 22 subjects who participated in the screening test, 12 met the selection criteria. The subjects' backgrounds are shown in Table 10. Visceral fat obesity with a VFA and SFA area ratio (V/S) was estimated. As a result, the V/S ratio was 0.57 in the placebo group (mean 0.57 ± 0.23) and 0.56 in the WLE group (mean 0.56 ± 0.27) (p = 0.91). The final basic data of the 10 subjects are presented in Table 11. Initial BMI was significantly higher in the WLE powder group; however, there were no significant differences in VFA, SFA, body weight, body fat percentage, or waist diameter between the groups. Although there was a significant difference in BMI, there was no significant

Table 10. Baseline characteristics of subjects at the beginning of the trial

	Placebo (n=6)	WLE powder (n=6)	*P*-value
Age (years)	50.3 ± 4.9	50.8 ± 6.6	0.89
Male/female (number)	2/4	3/3	1.00
BMI (kg/m^2)	25.5 ± 1.0	27.7 ± 1.4	0.01
Blood pressure (mmHg) :			
Systolic	119.2 ± 5.9	125.2 ± 8.1	0.18
Diastolic	85.8 ± 6.8	82.5 ± 8.0	0.70
Pulse rate (beats/min)	70.0 ± 4.9	74.7 ± 12.3	0.82
Body fat percentage(%)	33.3 ± 5.5	32.2 ± 6.8	0.76
Waist diameter(cm)	94.7 ± 3.9	98.2 ± 3.2	0.11
Abdominal visceral fat area(VFA)	137.5 ± 49.3	132.0 ± 47.8	0.85
Abdominal subcutaneous fat area(SFA)	245.5 ± 33.9	248.0 ± 47.3	0.92
VFA/SFA raio(V/S)	0.57 ± 0.23	0.56 ± 0.27	0.91

Each value represents mean ± standard deviation (other than the number of people).

Table 11. Baseline characteristics of subjects at the end of the trial

	Placebo (n=5)	WLE powder (n=5)	*P*-value
Age (years)	51.6 ± 4.3	50.6 ± 7.2	0.82
Male/female (number)	2/3	3/2	1.00
BMI (kg/m^2)	25.5 ± 1.1	27.7 ± 1.6	0.06
Blood pressure (mmHg) :			
Systolic	120.2 ± 5.8	126.6 ± 8.1	0.19
Diastolic	86.6 ± 7.3	84.0 ± 7.9	0.60
Pulse rate (beats/min)	68.4 ± 3.2	71.8 ± 11.2	0.53
Body fat percentage(%)	32.3 ± 5.6	31.1 ± 7.0	1.00
Waist diameter(cm)	94.9 ± 3.7	98.2 ± 3.8	0.15
Abdominal visceral fat area(VFA)	145.5 ± 50.5	141.9 ± 46.1	1.00
Abdominal subcutaneous fat area(SFA)	246.7 ± 37.7	253.3 ± 50.8	0.69
VFA/SFA raio(V/S)	0.61 ± 0.24	0.59 ± 0.28	1.00

Each value represents mean ± standard deviation (other than the number of people).

difference in visceral fat, subcutaneous fat, etc. Therefore, the difference in BMI was proposed to be a result of differences in other aspects such as muscle mass, etc.

2.3.3. Evaluation of safety

Results of adverse events are summarized in Table 12. A total of 16 events occurred in five of the twelve subjects. All were mild, except for bruises and fractures, and were randomly distributed in both the placebo and WLE groups. No significant change was observed in blood biochemical indexes. The medical investigator concluded that WLE as the test food showed no clinical issues according to the judgment standards.

Table 12. Adverse events reported by one or more subjects in the trial

	Placebo (n=6)	WLE powder (n=6)
Total subjects reporting one or more adverse event(s)	3	2
Types of adverse events reported :		
Gastrointestinal complaints	5	
Headache	2	4
Toothache/Gingivitis		1
Rhinitis	2	
Bruise		1
Fracture	1	
Total of adverse events reported	10	6

Although there are physical condition changes (cold, runny nose/nasal congestion, headache, abdominal pain, constipation, abdominal bloating, toothache/gingivitis, menstrual cramps, etc.), unless otherwise specified, it will not be treated as an adverse event.
Abdominal bloating, abdominal pain, constipation: Those who had symptoms for 3 consecutive days or more were treated as adverse events. Patients who had symptoms for 1-2 days without taking the test foods were not treated as adverse events. However, when the test food was taken, it was treated as an adverse event even if the symptom was only for one day.

2.3.4. Evaluation of body composition

As shown in Tables 13 and 14, no significant differences were observed in the initial endpoint of VFA and in the secondary endpoints of SFA, V/S area ratio, body weight, BMI, TG, T-Cho, HDL-C, LDL-C, waist circumference, or body fat percentage. However, in the placebo group, the mean visceral fat at the start was 145.5 cm^2, and increased to 165.0 cm^2 (by 19.6 cm^2, 13.4 %) at the end of the study. On the other hand, in the WLE group, the mean visceral fat at the start was 141.9 cm^2, and increased to 145.3 cm^2 (by 3.4 cm^2, 2.4 %) at the end of the test. No statistically significant difference was observed, probably due to the small numbers, i.e., only five subjects per group.

Table 13. Measurement results of body fat-related indexes

	treatment (week)	group	Average		SD	P-value	
						Innergroup comparison	Intergroup comparison
Abdominal visceral fat area(VFA)	Screening	WLE powder	141.9	±	46.12		1
		Placebo	145.46		50.54		
	12 weeks	WLE powder	145.32	±	64.73	0.686	0.548
		Placebo	165.04		55.08	0.043	
	12 weeks - screening	WLE powder	3.42	±	23.88		0.151
		Placebo	19.58		18.39		
Abdominal subcutaneous fat area(SFA)	Screening	WLE powder	253.32	±	50.82		0.69
		Placebo	246.7		37.71		
	12 weeks	WLE powder	245.04	±	54.18	0.08	0.841
		Placebo	232.46		37.14	0.08	
	12 weeks - screening	WLE powder	-8.28	±	7.18		0.841
		Placebo	-14.24		20.87		
Abdominal total fat area(TFA)	Screening	WLE powder	395.22	±	50.42		1
		Placebo	392.16		54.97		
	12 weeks	WLE powder	390.36	±	67.01	0.345	0.841
		Placebo	397.5		46.81	0.893	
	12 weeks - screening	WLE powder	-4.86	±	23.09		0.548
		Placebo	5.34		28.99		
VFA/SFA ratio	Screening	WLE powder	0.59	±	0.28		1
		Placebo	0.61		0.24		
	12 weeks	WLE powder	0.63	±	0.36	0.686	0.69
		Placebo	0.74		0.3	0.043	
	12 weeks - screening	WLE powder	0.04	±	0.12		0.151
		Placebo	0.13		0.13		
Body Weight(Kg)	Screening	WLE powder	76.62	±	12.47		0.31
		Placebo	66.88		11.37		
	8 weeks	WLE powder	76.44	±	11.16	1	0.31
		Placebo	66.14		11.86	1	
	12 weeks	WLE powder	77.5	±	12.18	0.446	0.31
		Placebo	66.96		11.83	1	
	8 weeks - screening	WLE powder	-0.18	±	1.49		0.841
		Placebo	-0.74		2.43		
	12 weeks - screening	WLE powder	0.88	±	1.43		0.69
		Placebo	0.08		1.86		
BMI	Screening	WLE powder	27.71	±	1.56		0.056
		Placebo	25.49		1.11		
	8 weeks	WLE powder	27.7	±	1.39	1	0.032
		Placebo	25.17		0.99	1	
	12 weeks	WLE powder	28.06	±	1.6	0.45	0.032
		Placebo	25.49		0.99	1	
	8 weeks - screening	WLE powder	-0.01	±	0.56		0.69
		Placebo	-0.32		0.98		
	12 weeks - screening	WLE powder	0.34	±	0.52		0.69
		Placebo	0		0.73		
Body fat percentage(%)	Screening	WLE powder	31.12	±	6.98		1
		Placebo	32.34		5.6		
	8 weeks	WLE powder	31.76	±	6.62	1	0.841
		Placebo	31.64		4.86	0.45	
	12 weeks	WLE powder	31.02	±	7.54	1	1
		Placebo	31.36		4.91	0.084	
	8 weeks - screening	WLE powder	0.64	±	1.72		0.421
		Placebo	-0.7		1.49		
	12 weeks - screening	WLE powder	-0.1	±	0.9		0.151
		Placebo	-0.98		1.16		
Waist diameter(cm)	Screening	WLE powder	98.18	±	3.75		0.151
		Placebo	94.89		3.69		
	8 weeks	WLE powder	98	±	3.44	1	0.31
		Placebo	93.14		5.3	0.16	
	12 weeks	WLE powder	98.27	±	4.18	1	0.222
		Placebo	94.89		3.61	1	
	8 weeks - screening	WLE powder	-0.18	±	1.14		0.151
		Placebo	-1.75		2		
	12 weeks - screening	WLE powder	0.09	±	1.45		1
		Placebo	0		0.85		

Each value represents mean ± standard deviation (SD). Comparison Innergroup: Wilcoxon signed-rank test (Bonferroni correction). Comparison Intergroups: Mann-Whitney's U test. Although twelve subjects completed the study, efficacy was evaluated in ten subjects because two subjects who had the abnormal values in the blood test during the study period and a fracture during the study period were excluded.

Table 14. Results of blood lipid measurement

	treatment (week)	group	Average		SD	P-value	
						Innergroup comparison	Intergroup comparison
Triglyceride(TG)	Screening	WLE powder	109.6	±	57.53		1
		Placebo	96.8		36.52		
	8W	WLE powder	93.4	±	20.84	1	0.841
		Placebo	101.2		39.24	1	
	12W	WLE powder	103.6	±	34.8	1	0.69
		Placebo	123.4		76.05	0.446	
	8W-screening	WLE powder	-16.2	±	50.77		0.69
		Placebo	4.4		35.5		
	12W-screening	WLE powder	-6	±	57.25		0.548
		Placebo	26.6		50.36		
Total cholesterol(T-CHO)	Screening	WLE powder	238.2	±	42.61		0.421
		Placebo	227.4		56.77		
	8W	WLE powder	206	±	21.71	0.276	0.548
		Placebo	225		53.16	1	
	12W	WLE powder	211	±	24.23	0.156	0.69
		Placebo	221.6		35.17	1	
	8W-screening	WLE powder	-32.2	±	33.41		0.222
		Placebo	-2.4		11.48		
	12W-screening	WLE powder	-27.2	±	23.91		0.31
		Placebo	-5.8		26.49		
HDL-cholesterol	Screening	WLE powder	66.4	±	10.14		0.31
		Placebo	59.8		14.81		
	8W	WLE powder	59.8	±	11.65	0.136	0.69
		Placebo	58.8		17.99	0.992	
	12W	WLE powder	61.6	±	8.59	0.136	0.31
		Placebo	58.8		21.61	0.984	
	8W-screening	WLE powder	-6.6	±	4.51		0.095
		Placebo	-1		3.39		
	12W-screening	WLE powder	-4.8	±	3.27		0.421
		Placebo	-1		7.55		
LDL-cholesterol	Screening	WLE powder	153.8	±	31.11		0.421
		Placebo	148.4		52.86		
	8W	WLE powder	129.4	±	11.28	0.276	0.69
		Placebo	144		53.83	0.45	
	12W	WLE powder	134.4	±	18.45	0.45	1
		Placebo	136.6		27.79	0.69	
	8W-screening	WLE powder	-24.4	±	30.49		0.31
		Placebo	-4.4		7.23		
	12W-screening	WLE powder	-19.4	±	24.97		0.841
		Placebo	-11.8		25.25		

Each value represents mean ± standard deviation (SD). Comparison Innergroup: Wilcoxon signed-rank test (Bonferroni correction). Comparison Intergroups: Mann-Whitney's U test.

Moreover, in the placebo group, VFA was increased in all subjects, whereas in the WLE group, VFA was decreased in three of five subjects [Fig. 5] and increased in one

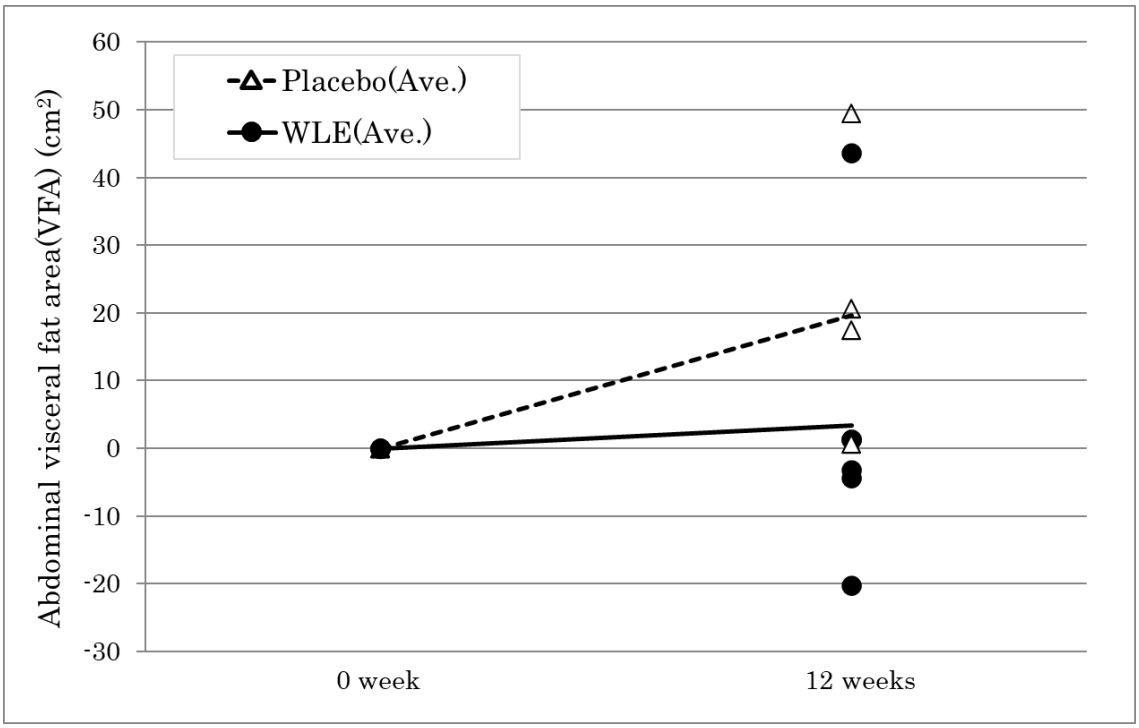


Fig. 5. Changes in visceral fat area by individual

Equipment: Brivo CT385 (GE Healthcare). The abdominal visceral fat area (VFA), abdominal subcutaneous fat area (SFA), and abdominal total fat area (TFA) are calculated from the image data obtained by the CT scan using PC software (FAT scan). The V/S area ratio was calculated from the VFA and SFA.

subject (by 43.6 cm^2). Although the cause of this large increase is unknown, it may be due to external factors such as stress. Blood lipid-related indexes in the WLE group tended to decrease compared with the placebo group. The values of triglycerides, total cholesterol, LDL cholesterol also decreased significantly in the WLE group compared with the placebo group. The results of blood hematological and biochemical analyses showed no significant differences between the two groups [Tables 15, 16].

Although no statistically significant differences were found in this study, possibly due to the small number of subjects, WLE may have an effect in the reduction of blood cholesterol and VFA.

2.4 Discussion

In this study, the mutagenicity, acute and sub-acute toxicity and human trial safety of WLE were investigated. In the acute and sub-acute toxicity tests of WLE, a significant increase in blood GOT value was observed in the 500 (76 ± 10 IU/L) and 1,500 mg/kg (75 ± 6 IU/L) WLE groups of male rats, and a low A/G ratio was observed in the 500 mg/kg WLE group of female rats. Since the population mean ± acceptable

Table 15. Results of hematology examinations

	treatment (week)	group	Average		SD	P-value	
						Innergroup comparison	Intergroup comparison
White blood cell count	Screening	WLE powder	5400	±	1425		0.59
		placebo	5533		432		
	8weeks	WLE powder	5067	±	1506	0.68	0.70
		placebo	5100		797	0.80	
	12weeks	WLE powder	5400	±	1325	1.00	1.00
		placebo	5400		858	1.00	
Red blood cell count	Screening	WLE powder	482	±	61.3		0.70
		placebo	464		32.5		
	8weeks	WLE powder	471	±	71.3	0.23	0.94
		placebo	456		27.6	0.23	
	12weeks	WLE powder	472	±	68.8	0.23	0.94
		placebo	459		33.5	0.92	
Hemoglobin	Screening	WLE powder	14.1	±	2.6		0.94
		placebo	14.2		2.4		
	8weeks	WLE powder	13.8	±	2.4	0.29	1.00
		placebo	13.9		2	0.14	
	12weeks	WLE powder	13.9	±	2.4	0.69	1.00
		placebo	14		2	1.00	
Hematocrit value	Screening	WLE powder	43.7	±	6.6		0.59
		placebo	42.9		5.5		
	8weeks	WLE powder	43.9	±	6.5	1.00	0.94
		placebo	43.5		5.2	0.50	
	12weeks	WLE powder	43.7	±	6.1	1.00	1.00
		placebo	43.8		5.2	0.50	
Platelet count	Screening	WLE powder	28.8	±	11		0.82
		Placebo	30.8		13		
	8 weeks	WLE powder	30.4	±	10.2	1.00	0.82
		Placebo	29.4		10.9	0.69	
	12 weeks	WLE powder	29.6	±	8.4	1.00	1.00
		Placebo	30		10.4	1.00	

The hematology tests were evaluated by all subjects to judge the safety. Each value represents mean ± standard deviation (SD). Comparison Intergroup: Wilcoxon signed-rank test (Bonferroni correction). Comparison Intergroups: Mann-Whitney's U test.

range of blood GOT value of Sprague-Dawley rats is reported as 86 ± 18 IU/L (n = 135), the change in GOT values in this study could be considered to be toxicologically insignificant. Regarding the change in A/G value, no significant change was observed in albumin and globulin values. Moreover, this change did not show dose-dependency, since it was not observed in the high-dose group (1,500 mg/kg of WLE), indicating that this may be a random observation. Based on these data, the non-toxic equivalent for administration of WLE for 28 days is estimated to be 1,500 mg/kg/day for both males and females.

In the human clinical trial, dietary intake of 200 mg WLE was determined to be a safe concentration. Although a total of 16 adverse events occurred in five of the twelve subjects, all adverse events were randomly distributed in both the placebo and WLE groups. No significant change was observed in blood biochemical indexes, and

Table 16. Results of blood biochemical indexes examinations

	treatment (week)	group	Average		SD	P-value	
						Innergroup comparison	Intergroup comparison
Total protein (TP)	SCR	WS	7.4	±	0.2		1.00
		placebo	7.4		0.5		
	8weeks	WS	7.3	±	0.3	0.83	0.94
		placebo	7.3		0.4	0.13	
	12weeks	WS	7.5	±	0.4	1.00	0.39
		placebo	7.3		0.4	0.49	
Albumin (ALB)	SCR	WS	4.3	±	0.2		0.82
		placebo	4.2		0.3		
	8weeks	WS	4.3	±	0.3	1.00	0.70
		placebo	4.2		0.2	0.36	
	12weeks	WS	4.3	±	0.2	1.00	0.24
		placebo	4.1		0.2	0.08	
CK(CPK)	SCR	WS	138.5	±	60.1		0.24
		placebo	104.8		29.8		
	8weeks	WS	139.8	±	62.9	0.92	0.39
		placebo	105.8		20.2	1.00	
	12weeks	WS	257.0	±	288.9	1.00	0.07
		placebo	94.5		12.4	0.93	
AST(GOT)	SCR	WS	20.2	±	2.6		0.82
		placebo	20.5		2.9		
	8weeks	WS	18.2	±	2.3	0.28	0.70
		placebo	18.2		2.6	0.14	
	12weeks	WS	21.2	±	5.5	1.00	0.59
		placebo	19.2		3.4	0.83	
ALT(GPT)	SCR	WS	21.3	±	4.7		0.24
		placebo	19.2		12.3		
	8weeks	WS	19.0	±	1.8	0.68	0.07
		placebo	14.5		3.8	0.92	
	12weeks	WS	19.2	±	4.5	0.34	0.70
		placebo	16.7		6.8	1.00	
LD(LDH)	SCR	WS	199.0	±	12.6		0.49
		placebo	187.2		28.3		
	8weeks	WS	195.5	±	16.6	0.59	0.24
		placebo	177.3		25.7	0.05	
	12weeks	WS	254.8	±	104.8	0.09	0.09
		placebo	189.5		23.2	1.00	
Alkaline phosphatase (ALP)	SCR	WS	220.2	±	34.7		0.39
		placebo	194.3		40.0		
	8weeks	WS	216.5	±	19.6	1.00	0.13
		placebo	187.3		43.7	1.00	
	12weeks	WS	210.2	±	26.7	0.23	0.70
		placebo	198.2		28.9	1.00	

Each value represents mean ± standard deviation (SD).

	treatment (week)	group	Average	SD	P-value	
					Innergroup comparison	Intergroup comparison
γ-GT(γ-GTP)	SCR	WS	34.5	18.7		0.04
		placebo	18.2	7.7		
	8weeks	WS	31.7	18.4	0.69	0.07
		placebo	15.3	4.9	0.13	
	12weeks	WS	30.8	16.1	0.49	0.24
		placebo	19.7	11.9	0.93	
Urea nitrogen (BUN)	SCR	WS	13.3	4.7		1.00
		placebo	13.2	3.8		
	8weeks	WS	13.8	4.0	1.00	0.82
		placebo	14.1	2.6	0.69	
	12weeks	WS	14.7	3.3	0.35	0.94
		placebo	14.3	2.4	0.69	
Creatinine	SCR	WS	0.8	0.2		1.00
		placebo	0.7	0.2		
	8weeks	WS	0.8	0.2	0.45	0.49
		placebo	0.7	0.1	0.34	
	12weeks	WS	0.8	0.2	0.69	0.94
		placebo	0.8	0.2	0.34	
uric acid (UA)	SCR	WS	4.9	1.8		0.59
		placebo	5.8	1.2		
	8weeks	WS	4.8	1.5	1.00	0.24
		placebo	5.8	1.2	1.00	
	12weeks	WS	4.9	1.7	1.00	0.82
		placebo	5.7	1.5	1.00	
Total bilirubin(T-BIL)	SCR	WS	0.6	0.2		0.24
		placebo	0.8	0.3		
	8weeks	WS	0.5	0.2	0.05	0.18
		placebo	0.8	0.3	0.63	
	12weeks	WS	0.6	0.2	1.00	0.82
		placebo	0.7	0.5	0.67	
glucose	SCR	WS	89.5	4.8		0.09
		placebo	94.0	5.7		
	8weeks	WS	84.2	15.7	1.00	0.82
		placebo	89.7	8.9	0.49	
	12weeks	WS	90.7	3.6	1.00	0.24
		placebo	94.3	8.4	1.00	
HbA1c(NGSP)	SCR	WS	5.8	0.4		0.07
		placebo	5.5	0.1		
	8weeks	WS	5.8	0.5	0.31	0.01
		placebo	5.4	0.1	0.28	
	12weeks	WS	5.7	0.4	0.63	0.03
		placebo	5.4	0.2	0.28	

Each value represents mean ± standard deviation (SD).

the medical investigator concluded that WLE (as the test food) showed no clinical issues within the designed concentration and intake period according to the judgment standards.

Interestedly, the WLE group showed lower VFA ($p = 0.15$) at the end of the 12 week treatment period. The V/S ratio was increased 0.13 in the placebo group (mean 0.13 ± 0.13) and increased 0.04 in the WLE group (mean 0.04 ± 0.12) ($p = 0.15$) [Table 13]. Although the number of subjects in the present clinical trial was small and no statistically significant differences were observed, we obtained interesting information regarding the effects of WLE on regulation of lipid metabolism. In the placebo group, the mean visceral fat was increased from 145.5 to 165.0 cm^2 (13.4 %) at the end of the trial. Meanwhile, in the WLE group, the mean visceral fat was increased from 141.9 to 145.3 cm^2 (2.4 %). These data suggest that WLE powder may have effects in the reduction of visceral fat. Our data are consistent with the results from other animal experiments. For example, WLE could attenuate liver lipid accumulation and white adipose tissue weight induced by a high-fat diet in C57J/BL mice (Yamasaki et al., 2013). Additionally, WLE was reported to reduce fat hypertrophy of adipose tissue by suppressing PPARγ expression in rats (Oowatari 2016).

Regarding the bioactive compounds in WLE responsible for the visceral fat reduction, we analyzed the bioactive components in WLE, and detected at least three major flavonoids, isovitexin, isosaponarin, and isoorientin, at concentrations of 0.272, 0.012 and 0.030 mg/g, respectively. The total polyphenol content in WLE was estimated as 2.28 mg/g. Isosaponarin in wasabi leaf has been reported to have antioxidant (Hosoya 2008, Sekiguchi 2010)) and anti-obesity (Yamasaki 2013, Oowatari 2016, Misawa 2018)) activities. Specifically, animal experiments demonstrated that the body weight of rats fed a high-fat diet supplemented with WLE was significantly reduced compared to the control (Yamada-Kato 2016). The increase in β3 adrenergic receptors in adipocytes and enhancement in the activity of UCP-1 by WLE could stimulate fat beta-oxidation (Yamada-Kato 2016). Moreover, some acid esters and polyphenols derived from wasabi leaf have also been reported to regulate lipid metabolism. For example, 5-hydroxyferulic acid ester could modulate PPARγ and AMPK to suppress 3T3-L1 cell differentiation to adipocytes (Misawa 2018). Caffeic acid 2-phenylethyl ester and luteolin inhibit fat accumulation in 3T3-L1 cells (Juman 2010, Nishina 2015)). Thus, isosaponarin, other flavonoids as well as acid esters present in WLE may contribute to the regulation of lipid metabolism. Confirmation of this will be required in future studies using a larger population, following detailed identification of the active ingredient(s).

2.5 Abstract

Wasabi leaf has been reported to show human health benefits without assessment of its safety. This study aims to investigate the mutagenicity, acute and sub-acute toxicity and human trial safety of wasabi leaf extract (WLE). The Ames test was used to assess mutagenicity, while acute and sub-acute toxicity were assessed by oral administration in five-week-old Slc:ICR mice (SPF) and five-week-old Sprague-Dawley (SD) rats, respectively. Human trial safety was further determined in a clinical trial. Twelve healthy subjects, aged 20–64 years and mildly obese (BMI 23.0 to 30.0 kg/m^2), were enrolled in the clinical trial, and participants ingested 200 mg WLE daily for 12 weeks. The effect of WLE on fat metabolism was evaluated by visceral fat area (VFA), subcutaneous fat area (SFA), VFA/SFA(V/S) area ratio, body weight, BMI, TG, T-Cho, HDL-C, LDL-C, waist circumference, and body fat percentage. In the Ames test, WLE did not show mutagenicity in the range of 1.2-5,000 μg/plate. No acute toxicity was observed in Slc:ICR mice (SPF) administered 5,000 mg/kg/day WLE, and no sub-acute toxicity was observed in Crl:CD (SD) rats administered 2,500 mg/kg/day WLE. In the human clinical trial, there were no significant differences between the WLE and placebo groups for any outcome measure assessed. Thus, ingestion of 200 mg/day of WLE was demonstrated for the first time to be safe. Taken together, our data on the mutagenicity, acute and sub-acute toxicity and human trial safety of WLE provide the first standard references for wasabi leaf supplement application.

Chapter 3. Safety Evaluation of 6-(Methylsulfinyl) Hexyl Isothiocyanate (6-MSITC) and Wasabi Sulfinyl, a 6-MSITC-containing Supplement

3.1 Introduction

In recent years, 6-(methylsulfinyl) hexyl ITC (6-MSITC) has attracted attention due to various functional reports. Many physiological functions of 6-MSITC have been reported to have benefits to health (Morimitsu 2002, Hou 2000, 2011, Lee 2010, Uto 2007, 2012, Yamada-Kato 2012, Morimitsu 2000, Fuke 2014, Trio 2016, Morroni 2014, 2018, Okunishi 2019). Therefore, WS, a wasabi extract powder containing 0.8% 6-MSITC, has recently been developed as a health supplement. Although WS is expected to be beneficial to health, there are few data on their safety assessment. Thus, safety confirmation for WS is considered to be crucial and important.

No-observed-adverse-effect levels have been reported for AITC, benzyl ITC, phenethyl ITC, and 3-methylthiopropyl ITC at 12, 5, 5, and 30 mg/kg body weight/ day, respectively (Valerio 2006). Additionally, sulforaphane (4-methylsulfinylbutyl isothiocyanate (4-MSITC)) found in broccoli has been investigated by several clinical trials for its internal metabolism and functionality (Shapiro 2001, Kensler 2005).

Based on this information, in this study, we estimated the safety of 6-MSITC by the Ames test, acute toxicity test, and sub-acute toxicity test. Finally, a human clinical trial was also carried out for WS containing 0.8 % 6-MSITC. According to the previous results that middle-aged and older adults who ingested 100 mg WS for eight weeks daily showed improved cognitive performance (Okunishi 2019), a five-time overdose was designed in the present trial to examine the safety of 500 mg WS intake for four weeks in healthy adults. It is calculated to take 4.0 mg of 6-MSITC per day.

3.2 Material and methods

3.2.1. Preparation of 6-MSITC, acute toxicity and sub-acute toxicity tests

Synthetic 6-MSITC (SS) was synthesized at Ogawa & Co., Ltd. (Tokyo, Japan) through the oxidation of 6-(methylthio) hexyl ITC, which is an analog of 6-MSITC (Murata 2004). WS containing 0.1 % 6-MSITC was extracted from rhizome.

The Ames tests were carried out at the Koei Techno Service Co., Ltd. (Osaka City, Osaka, Japan) by a reverse mutation assay with two strains of Salmonella typhimurium (TA100 and TA98). The assay was conducted for SS with seven doses from 20 μg to 1,250 μg/plate for each strain, and for WS with seven doses from 78 μg to 5,000 μg/plate for each strain.

The acute toxicity tests were performed at the Japan Bio Science Laboratory

Co., Ltd. (Kaizu-cho, Gifu, Japan) by oral administration once to both sexes of Sprague-Dawley (SD) rats [Crj:CD(SD)IGS, SPF]. The dosage levels were designed for seven doses from 125 mg/kg to 759 mg/kg, individually based on the body weight at the first day of administration (Day 0). Body weight was measured at Day 0 and at Days 1, 3, 5, 7, 10 and 14 (day of autopsy). The Lethal dose 50 (LD_{50}) value was calculated according to the 14-day cumulative mortality with the probit method.

The sub-acute toxicity tests were done at TTC Co., Ltd. (Tokyo, Japan) by repeated administration to both sexes of SD rats [Crl:CD(SD)] for 28 days. The dosage levels were designed for 500 mg/kg/day and 2,500 mg/kg/day, individually based on the body weight at Day 0. The toxicity was evaluated based on the 28-day observation data, including the general condition, confirmation of life and death, hematology, blood chemistry, urinalysis, and necropsy.

3.2.2. Ethics

The toxicity tests adhered to the Good Laboratory Practices (GLP, the Ministry of Health, Labour and Welfare Ordinance No. 21 of 1997) and the Organization for Economic Co-operation and Development Guidelines for the Testing of Chemicals as a non-GLP test.

All of the animal experiments adhered to the Guidelines for the Proper Implementation of Animal Experiments (Science Council of Japan, 2006), Act on Welfare and Management of Animals (Amendment of Act No. 68 of 2005) and Standards Relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Notice of the Ministry of the Environment No. 88 of 2006). Sub-acute toxicity tests were conducted according to the Ministerial Ordinance on Good Laboratory Practice for Nonclinical Safety Studies of Drugs (Ordinance of the Ministry of Health and Welfare No. 21 of 1997) and Partial Revision of the Guidelines for Repeated Dose Toxicity Studies (PMSB/ELD Notification No. 902).

The human clinical trial adhered to all guidelines set forth in the Declaration of Helsinki (amended in October 2013), the Ethical Guidelines for Medical Research in Humans (observed by the Ministry of Education and Science and the Ministry of Health, Labour and Welfare, the Japanese Government, in December 2014 [amended in February 2017]), and the guidance of the Ethical Guidelines for Medical Research in Humans (enacted by the Ministry of Education and Science and the Ministry of Health, Labour and Welfare, the Japanese Government, in February 2015 [amended in March 2017]). The protocol of the study was approved by the Aisei Hospital Ueno Clinic Research Ethics Committee, Tokyo, Japan (IRB number: 12000071) on April 27 2017

before starting the clinical trial. All volunteers received a full explanation of the aims and procedures. Written informed consent was obtained from all participants before starting the study. All records were classified with an anonymous subject identification number to ensure privacy.

The study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR; study ID UMIN000027402).

3.2.3. Participants

Of the 26 subjects who participated in the screening, 11 healthy male and female subjects, between the ages of 20 to 64 years, were initially enrolled in this study. Individuals were excluded if: (1) they were allergic to Wasabi or likely to get allergies by participating in this research; (2) they were under treatment for a disease; (3) they had a severe disease, such as diabetes, liver disease, kidney disease, heart disease or cancer, or had a history of a severe disease; (4) their physical measurement, physical examination and clinical laboratory values before the start of ingestion significantly deviated from the reference range; (5) they had lifestyle habits that would affect the research results, such as working day and night shifts, or were scheduled to go on a shift day and night or overseas during the research period; (6) they had participated in another clinical research within one month before obtaining acquisition, or were planning to participate in another clinical study after acquiring consent to participate in this study; (7) they were pregnant or nursing, or planned to get pregnant during the research period; (8) they were unsuitable as subjects from the questionnaire on lifestyle habits; and (9) they were judged unsuitable for this study by the medical investigator.

3.2.4. Study design and procedure

The study was an open-label trial. It consisted of a screening test, a four-week WS ingestion period, and a two-week follow-up period without WS ingestion. The trial schedule is shown in Table 17.

After the screening test, each eligible subject received one capsule that contained 100 mg WS (0.8 mg 6-MSITC) adsorbed on α-cyclodextrin and a 110 mg vehicle (consisting of starch, silicon dioxide and calcium stearate). The WS capsules were provided by Kinjirushi Co., Ltd. (Nagoya, Japan). The nutritional components of the test foods (per daily intake; five capsules) are shown in Table 18. During the trial period, a medical investigator instructed all subjects on the following points: (1) avoid excessive exercise or eating too little or too much; (2) take five capsules per day, according to the safety test standards for food for specified health uses (Precautions for

Table 17. Human clinical trial schedule

Inspection date	Screening	Selection	Ingestion Period (4 weeks)		After a 2-week observation
			2nd week	4th week	
Selection*1		●			
Lifestyle questionnaire	●				
Physical condition check · Physical measurement ·Physical examination	●		●	●	●
Clinical examination	●		●	●	●
Test food ingestion			←	→	
Daily report			←	→	→

* 1: The selection of the subjects was evaluated within four weeks from screening test and distributed test food. ●: Item on the examination date: During the research period, subjects were required to perform the indicated daily. During the ingestion period, the test food ingestion and diary were descripted daily. Subjects had no eating for 12 h prior to the day of the examination.

Table 18. Nutritional components of the test food (daily intake of five capsules)

Item	Amount
Energy (kcal)	5.04
Protein (mg)	257.8
Fat (mg)	78.6
Carbohydrate (mg)	826.3
Sodium (mg)	0.57

Analysis was carried out in Japan Food Research Laboratories by standard methods.

Preparing Application Forms Pertaining to Food Applications for Specified Health Uses, Consumer Affairs Agency, Japan, April 2020), and record a daily report; (3) maintain a healthy daily lifestyle; (4) do not ingest other health foods (foods for a specified health use, functional display foods, health supplements, or other supplements); (5) on the day before the examination, go to bed before midnight to ensure enough sleep; (6) on the day before the examination, do not eat or drink anything other than water after 10 pm until the end of the examination.

This study was conducted from May to August 2017 in the Medical Station Clinic (Tokyo, Japan) and the Public Interest Incorporated Foundation Aiseikai Aisei Hospital

Ueno Medical Clinic (Tokyo, Japan).

3.2.5. Safety assessment

Safety was evaluated by the incidence and severity of WS ingestion-related adverse events in subjects during the research period. An adverse event was defined as an undesirable or unintended injury or symptom, including an abnormality in clinical laboratory test values for the subjects after the preliminary examination, regardless of whether or not there was a causal relationship with the conducted study. A safety evaluation was also conducted through physical examinations, measurements of the subjects' weights and vital signs (resting systolic and diastolic blood pressures, and heart rate), and laboratory assessments, including biochemical, hematological and urinal analysis. The laboratory biochemical indexes included total protein (TP), albumin (Alb), total bilirubin (TB), direct bilirubin (D-B), indirect bilirubin (I-B), aspartate-aminotransferase, alanine-aminotransferase, γ-glutamyltransferase (γ-GT), lactate dehydrogenase (LDH), alkaline phosphatase, blood urea nitrogen, creatinine (Cr), uric acid, lipid profile (total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), triglycerides, sodium, potassium, chloride, glucose (GLU), and hemoglobin A1c (screening only). Hematological indexes consisted of the white blood cell count, red blood cell count, hemoglobin (Hb), hematocrit and platelets (PLT). The urinalysis consisted of the occult blood reaction, protein, and GLU in urine.

3.2.6. Data analyses

An analysis of adverse events was conducted for all subjects, and the number of adverse event occurrences was counted. The values at each time point before and after ingestion were compared using the paired t-test. A p-value <0.05 was considered statistically significant. No statistical adjustment for multiple testing was performed. Microsoft Excel 2013 was used for these analyses, and the results are expressed as the mean ± standard deviation.

3.3. Results

3.3.1. Results of the Ames test, acute test, and sub-acute test

The Ames test was conducted to investigate the mutagenicity of SS (purity: 95 % or more) and WS in the concentration ranges of 20–1,250 μg/plate and 78–5,000 μg/plate, respectively. In the case of SS, growth inhibition of TA100 (-S9) and TA98 (both –S9 and +S9) was observed at a concentration of 313 μg/plate. In the case of WS, growth inhibition of TA100 (-S9) was observed in the concentration range of 156–5,000 μg/plate. On the other

hand, no mutagenicity was observed at any concentration of either SS or WS [Table 19].

The results of the acute toxicity test for SS performed in male and female SD rats are shown in Table 20. No death was observed in rats administrated WS at the dose of 2,000 mg/kg, but death was observed with SS at the dose of 308 mg/kg or more. The LD_{50} value was estimated to be 451.2 mg/kg in males (95 % confidence limit: 357.5–573.8 mg/kg) and 400.7 mg/kg in females (95 % confidence limit: 322.1– 507.6 mg/kg) for SS. Necropsy of dead rats at 1–3 days after administration revealed signs of erosion due to irritant substances, ulceration and/or reddening of the forestomach and glandular mucosa. No changes in the other organs were observed. Autopsies were performed for all surviving rats. Secondary symptoms, such as an increase in forestomach thickening, were observed in the rats fed 169 mg/kg or more.

The sub-acute toxicity test for WS (containing 0.1 % 6-MSITC) was further evaluated by repeated oral administrations to five-week-old male and female SD rats for 28 days. No animal died even when 2,500 mg/kg WS was administered, and no change was observed in the general observations, including body weight, food consumption, water rehydration, urinalysis, hematological examination, and pathological anatomical examination.

Table 19. Results of the Ames test

	SS				WS			
	TA100 (Base pair substitution type)		TA98 (Frame shift type)		TA100 (Base pair substitution type)		TA98 (Frame shift type)	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
20 μg/plate	—	—	—	—	／	／	／	／
39 μg/plate	—	—	—	—	／	／	／	／
78 μg/plate	—	—	—	—	—	—	—	—
156 μg/plate	—	—	—	—	—	—	— *	—
313 μg/plate	— *	—	— *	— *	—	—	— *	—
625 μg/plate	N	N	N	N	—	—	— *	—
1250 μg/plate	N	N	N	N	—	—	— *	—
2500 μg/plate	／	／	／	／	—	—	— *	—
5000 μg/plate	／	／	／	／	—	—	— *	—
Positive Control	+	+	+	+	+	+	+	+

*: Growth inhibition was observed, N: No growth was observed, －:No more than 2 times back mutation, ＋:More than 2 times back mutation were seen, ／: No data. The strain -S9 or +S9 was pre-incubated at 30 ° C for 20 minutes, and further cultured for 48 hours after addition of test substance. The solvent used was dehydrated DMSO, and no precipitation of the test substance was observed.

Table 20. Results of the acute and sub-acute test

	Acute		Sub-acute
	LD_{50}		NOAEL
	SS	WS	WS
SD-rats(male)	451.2 mg/kg	—	2500 mg/kg
SD-rats(female)	400.7 mg/kg	—	

※—: No deaths were observed at 2,000 mg/kg, NOAEL: No Observed Adverse effect level. The SD rats were bred in a stainless-steel wire hanger cage in a barrier breeding room with a temperature of 20 to 26 ° C., a relative humidity of 40 to 70%, a ventilation rate of 10 to 17 times / hour under controlled light (12-h light/day), where they had free access to water and feed.

3.3.2. Characteristics of the subjects in the human clinical trial

Of the 26 subjects who participated in the screening test, 11 met the selection criteria and did not fall under the exclusion criteria. The subjects' backgrounds are shown in Table 21. Some subjects whose values were outside of the reference range in the clinical examination were also included in the study after confirming no problems for participating in the study by a medical investigator. All 11 subjects completed this study with a 100.0 % ingestion rate of the test food.

3.3.3 Evaluation of safety

The results on the adverse events are summarized in Table 22. Three adverse events occurred in 2 of 11 subjects (18.2 %), including nausea, stomachache, heavy stomach, and anorexia. As causes of the adverse events, there were self-reports of excessive drinking of cold beverages and poor physical condition due to high temperatures and high humidity levels. Therefore, the adverse events were not caused by the test food. The medical investigator concluded that the test food had no clinical problem.

The results of the blood biochemical examination, hematological examination and urinalysis are shown in Tables 23, 24 and 25, respectively. Significant fluctuations after ingestion were observed in the Hb (female), TP and Alb values at the second week, Hb (female), PLT, γ-GT (female), Cr (female) and HDL-C (male) values at the fourth week, and TB, I-B, γ-GT (male), LDH, Cr (female), and CL after two weeks of observation. These fluctuations were within the reference ranges, and the medical investigator concluded that there was no clinical significance. The physical examination values are shown in Table 26. When compared to the screening results significant fluctuations after ingestion were observed in the body weight at the second week, and in the body weight (female) and body mass index

Table 21. Subjects' background

Item	All examples	Male	Female
Numbers	11	6	5
Age (years)	45.2 ± 13.0	45.0 ± 13.0	45.4 ± 14.5
Height (cm)	169.11 ± 7.85	173.95 ± 6.86	163.30 ± 4.21
Body weight (kg)	59.52 ± 8.06	65.47 ± 5.41	52.38 ± 2.98
BMI (kg/m^2)	20.72 ± 1.29	21.60 ± 0.78	19.66 ± 0.92
Systolic blood pressure (mmHg)	108.9 ± 9.4	108.7 ± 7.8	109.2 ± 12.1
Diastolic blood pressure (mmHg)	63.6 ± 8.5	61.5 ± 6.7	66.2 ± 10.5
Pulse rate (beat/minute)	67.6 ± 10.4	69.5 ± 13.4	65.4 ± 5.7

Each value represents mean±standard deviation (other than the number of people). Men and women from age 20s to 60s were incorporated in human clinical trial in order to assess the safety of food in a wide range of ages and genders. All of subjects were required to have medical check to confirm their health background.

Table 22. The total adverse events

Items	Number
Subjects to safety analysis	11
Persons expressing secondary effects	0
Incidence rate of side effects (%)	0
Occurrences of secondary effects	0
Adverse events	2
Incidence of adverse events (%)	18.2
Occurrences of adverse events	3
Adverse events by symptom:	
Nausea, stomachache	1
Heavy Stomach	1
Anorexia	1

If the same adverse event occurred multiple times in the same subject, it was accounted as one event. Subject's subjective symptoms and adverse events were judged by the responsible physician with objective findings.

Table 23. Blood biochemical examinations

Item	Reference values	sex	n	Screening	2nd week	4th week	After a 2-week observation
TP (g/dL)	6.7–8.3		11	7.22 ± 0.49	7.45 ± 0.43*	7.29 ± 0.46	7.23 ± 0.43
Alb (g/dL)	3.8–5.2		11	4.34 ± 0.27	4.48 ± 0.26*	4.43 ± 0.24	4.39 ± 0.28
TB (mg/dL)	0.2–1.2		11	0.62 ± 0.15	0.74 ± 0.20	0.71 ± 0.19	0.75 ± 0.21*
D-B (mg/dL)	0.0–0.2		11	0.10 ± 0.04	0.13 ± 0.05	0.13 ± 0.05	0.13 ± 0.05
I-B (mg/dL)	0.2–1.0		11	0.52 ± 0.13	0.61 ± 0.20	0.58 ± 0.15	0.62 ± 0.19*
AST (GOT) (U/L)	10–40		11	20.3 ± 6.8	20.8 ± 6.5	18.6 ± 6.4	21.4 ± 6.3
ALT (GPT) (U/L)	5–45		11	18.0 ± 10.0	18.2 ± 8.5	16.2 ± 9.5	18.7 ± 9.8
γ-GT (U/L)	M : 0–80	male	6	24.2 ± 6.6	23.5 ± 5.4	23.2 ± 6.4	22.3 ± 6.4*
	F : 0–30	female	5	14.8 ± 5.0	16.0 ± 6.0	16.0 ± 4.5*	16.2 ± 8.1
LDH (U/L)	120–240		11	156.5 ± 20.0	166.2 ± 28.7	162.6 ± 17.6	168.3 ± 19.0*
ALP (U/L)	100–325		11	193.2 ± 48.8	196.5 ± 60.6	188.9 ± 60.3	189.8 ± 58.9
UN (mg/dL)	8.0–20.0		11	12.83 ± 3.51	12.63 ± 3.01	13.58 ± 3.17	12.82 ± 3.80
Cr (mg/dL)	M : 0.61–1.04	male	6	0.785 ± 0.079	0.830 ± 0.072	0.798 ± 0.081	0.807 ± 0.085
	F : 0.47–0.79	female	5	0.602 ± 0.036	0.666 ± 0.052	0.672 ± 0.067*	0.652 ± 0.047**
UA (mg/dL)	M : 3.8–7.0	male	6	5.50 ± 0.47	5.37 ± 0.37	5.12 ± 0.35	5.50 ± 0.59
	F : 2.5–7.0	female	5	4.28 ± 0.63	4.56 ± 1.10	4.52 ± 0.87	4.32 ± 1.11
TC (mg/dL)	120–219		11	191.8 ± 29.7	197.0 ± 31.8	192.5 ± 36.6	194.5 ± 35.6
HDL-C (mg/dL)	M : 40–85	male	6	60.7 ± 5.2	58.0 ± 8.9	53.8 ± 9.7*	56.2 ± 13.5
	F : 40–95	female	5	66.4 ± 15.4	71.0 ± 21.9	70.6 ± 24.3	70.8 ± 27.3
LDL-C (mg/dL)	65–139		11	112.9 ± 25.1	114.5 ± 27.1	111.0 ± 27.4	111.1 ± 27.2
TG (mg/dL)	30–149		11	72.0 ± 26.7	78.1 ± 26.4	73.0 ± 34.5	86.1 ± 26.2
Na (mEq/L)	137–147		11	142.3 ± 1.9	142.6 ± 1.0	142.0 ± 1.9	141.5 ± 1.8
K (mEq/L)	3.5–5.0		11	4.44 ± 0.32	4.35 ± 0.27	4.36 ± 0.33	4.30 ± 0.23
CL (mEq/L)	98–108		11	105.6 ± 2.1	105.6 ± 2.2	105.3 ± 1.7	104.4 ± 1.9*
GLU (mg/dL)	70–109		11	86.5 ± 7.0	85.5 ± 8.4	87.9 ± 6.9	85.5 ± 9.4
HbA1c (%)	4.6–6.2		11	5.20 ± 0.36			

Each value represents mean±standard deviation. $p<0.01$**, $p<0.05$*. Blood biochemical examinations were carried out in LSI Medience Corporation by standard methods. The adverse events were judged by responsible physician according to the criteria for abnormal changes (antimicrobial safety evaluation criteria) established by the Japanese Society of Chemotherapy, Common Terminology Criteria for Adverse Events v4.0, and the Japan Ningen Dock Society criteria (revised April 2017).

Table 24. Hematological examinations

Item	Reference values	sex	n	Screening	2nd week	4th week	After a 2-week observation
WBC (/μL)	3300–9000		11	5290.9 ± 1164.0	5409.1 ± 1432.8	5290.9 ± 1116.7	5418.2 ± 1050.5
RBC ($\times10^4$/μL)	M : 430–570	male	6	475.0 ± 40.2	481.3 ± 42.7	466.2 ± 37.0	479.3 ± 43.8
	F : 380–500	female	5	440.0 ± 29.3	451.0 ± 26.4	447.4 ± 25.2	444.0 ± 11.6
Hb (g/dL)	M : 13.5–17.5	male	6	14.08 ± 1.06	14.53 ± 0.80	14.18 ± 0.73	14.52 ± 0.78
	F : 11.5–15.0	female	5	13.12 ± 0.86	13.50 ± 0.60*	13.60 ± 0.71*	13.46 ± 0.40
Ht (%)	M : 39.7–52.4	male	6	45.13 ± 2.44	46.00 ± 2.33	44.10 ± 1.98	45.95 ± 2.28
	F : 34.8–45.0	female	5	42.14 ± 2.35	43.26 ± 1.44	42.88 ± 1.71	42.88 ± 0.82
PLT ($\times10^4$/μL)	14.0–34.0		11	26.04 ± 5.30	26.47 ± 6.00	23.90 ± 5.28*	26.86 ± 6.37

Each value represents mean ± standard deviation. $p<0.05$*. Hematological examinations were carried out in LSI Medience Corporation by standard methods.

Table 25. Urinalyses

Item		n	Screening			2nd week			4th week			After a 2-week observation		
			(-)	(±)	(+)	(-)	(±)	(+)	(-)	(±)	(+)	(-)	(±)	(+)
Protein	total	11	10	1	0	10	1	0	10	0	1	11	0	0
	male	6	6	0	0	6	0	0	5	0	1	6	0	0
	female	5	4	1	0	4	1	0	5	0	0	5	0	0
Glucose	total	11	11	0	0	11	0	0	11	0	0	11	0	0
	male	6	6	0	0	6	0	0	6	0	0	6	0	0
	female	5	5	0	0	5	0	0	5	0	0	5	0	0
Occult blood	total	11	11	0	0	11	0	0	11	0	0	11	0	0
	male	6	6	0	0	6	0	0	6	0	0	6	0	0
	female	5	5	0	0	5	0	0	5	0	0	5	0	0

The numerical data is a summary of the measurement results. Urinalyses including urine protein, glucose and occult blood were carried out in LSI Medience Corporation by standard methods.

(female) at the fourth week. These fluctuations were minor, and the medical investigator concluded that there was no clinical significance.

3.4. Discussion

Wasabi is widely consumed in many areas of the world as a spice, and it is indispensable for Japanese food. Its ingredient, 6-MSITC, has been reported to have many functions. However, there are few reports concerning its safety or toxicity. Therefore, in this study, we estimated the safety of pure SS and an extract from Wasabi rhizome, WS, containing 0.8 % 6-MSITC, by examining its mutagenic activity in bacteria, acute and sub-acute toxicities in SD rats, and side effects in a human clinical trial.

In the Ames test, no mutagenicity was observed for SS and WS. However, growth inhibition was observed at 313 μg/plate or more for SS, and 156 μg/plate or more in the TA98 strain (-S9) for WS. No growth inhibition was observed up to 500 μg/ plate in the TA98 strain

Table 26. Physical examinations

Item	sex	n	Screening	2nd week	4th week	After a 2 week observation
Body weight (kg)	total	11	59.52 ± 8.06	59.03 ± 8.36*	59.06 ± 8.49	59.05 ± 8.36
	male	6	65.47 ± 5.41	65.28 ± 5.35	65.37 ± 5.53	64.98 ± 6.11
	female	5	52.38 ± 2.98	51.52 ± 3.13	51.50 ± 3.31*	51.94 ± 3.44
BMI (kg/m^2)	total	11	20.72 ± 1.29	20.55 ± 1.48	20.55 ± 1.45	20.56 ± 1.42
	male	6	21.60 ± 0.78	21.57 ± 0.77	21.58 ± 0.86	21.47 ± 0.98
	female	5	19.66 ± 0.92	19.32 ± 1.13	19.32 ± 0.94*	19.48 ± 1.06
Systolic blood pressure (mmHg)	total	11	108.9 ± 9.4	105.1 ± 5.9	107.0 ± 9.3	104.5 ± 11.0
	male	6	108.7 ± 7.8	106.3 ± 5.0	107.7 ± 10.8	104.0 ± 11.1
	female	5	109.2 ± 12.1	103.6 ± 7.2	106.2 ± 8.3	105.2 ± 12.0
Diastolic blood pressure (mmHg)	total	11	63.6 ± 8.5	62.9 ± 6.3	62.6 ± 7.4	60.5 ± 7.4
	male	6	61.5 ± 6.7	61.3 ± 6.9	60.3 ± 6.4	58.0 ± 6.2
	female	5	66.2 ± 10.5	64.8 ± 5.5	65.4 ± 8.3	63.6 ± 8.4
Pulse rate (beat/minute)	total	11	67.6 ± 10.4	69.0 ± 11.8	68.1 ± 12.0	69.3 ± 9.8
	male	6	69.5 ± 13.4	70.5 ± 14.9	70.3 ± 15.2	71.3 ± 10.9
	female	5	65.4 ± 5.7	67.2 ± 8.1	65.4 ± 7.6	66.8 ± 8.6

Each value represents mean ± standard deviation. $p<0.05$*. Physical examinations were carried out in Medical Station Clinic by standard methods.

(+S9) for WS. Therefore, the components metabolized by the TA98 strain (-S9) might be involved in the growth inhibition of the bacterium. It has been reported that ITCs, including allyl ITC, ethyl ITC, methyl ITC, sulforaphane and benzyl ITC, accumulate in bacteria as GSH, small thiol-like BSH or thioredoxin-dithiocarbamate conjugates, and they attack the active center of enzymes by binding to thiol or amine groups to affect enzymatic activities such as respiration, metabolism and the transcription of genes. The reactivity of ITC sulfhydryl groups was demonstrated to be a major player for these conjugates, and it disturbs the biochemical processes of bacteria to lead to growth inhibition (Dufour 2015). Thus, 6-MSITC might also inhibit the growth of TA98 strain (-S9) through similar kinds of conjugates, because 6-MSITC is a representative ITC, although direct data are needed for this to be proved in future studies.

In the acute toxicity test, the LD_{50} value of SS in rats was estimated to be 451.2 mg/kg in males and 400.7 mg/kg in females. It has been reported that the LD_{50} values of AITC were 339–490 mg/kg body weight, and that the LD_{50} values of 3-methylthiopropyl ITC were 490 mg/kg body weight in males and 540 mg/kg body weight in females (Jenner 1964). Although the molecular weights of these ITCs differ by more than two-fold, their toxicity doses are close. It is considered that the NCS group of ITCs has higher reactivity, which principally contributes to the toxicity. Wasabi generally contains approximately 0.2 % AITC and about 0.04 % 6-MSITC (Murata 2004, Sultana 2003). Based on these data and our evaluation results for

toxicity, even if 6-MSITC is concentrated five-fold, the dose is within the safe range, because the AITC content is five times higher than 6-MSITC in the natural state.

In the acute toxicity test and sub-acute toxicity test of WS, there were no deaths at doses of 2,000 mg/kg and 2,500 mg/kg/ day, respectively, and also, no other obvious changes were observed. In the overdose test of WS, the intake was set to be 500 mg WS containing 0.8 % 6-MSITC/day for 4 weeks, which was five times higher than the effective intake in the previous clinical studies on brain function (Okunishi 2019). Although three adverse events, including nausea, stomach pain, stomach slack and anorexia occurred in 2 of 11 (18.2 %) subjects, the medical investigator confirmed that all adverse events had a clear cause and were not related to WS intake.

Although the body weight and body mass index of the female group at the fourth week were significantly reduced, no subject lost a large amount of weight, and the difference was about 0.02 kg on average, which was a slight difference when compared to the second week. Considering that 2 of 5 women were constipated, it is possible that there was a significant difference depending on the timing of defecation. Therefore, this weight loss was not evaluated as toxicity.

Moreover, significant changes in some of the laboratory test values, anthropometric measurements, and physical test values were also observed; however, these changes were mild and were judged to be not clinically relevant to WS intake. Thus, it is considered that there is no safety problem with the consumption of WS within 500 mg/day.

Additionally, a human clinical trial of sulforaphane found in broccoli was previously performed (Shapiro 2001). Twenty-five μmol (4.43 mg) was consumed three times a day for 7 days, and a total of 525 μmol (93 mg) was consumed by three subjects. As a result, increases in alanine-aminotransferase and thyroid-stimulating hormone were observed, but it was confirmed that these changes had no clinical significance for safety problems. It is clear that sulforaphane has the same backbone chemical structure as 6-MSITC (Hou 2000). There is only a small structural difference in the length of the methyl chain: sulforaphane has four methyl groups and 6-MSITC has six methyl groups. In our previous studies, we demonstrated that 6-MSITC and sulforaphane have the same molecular mechanisms for their antioxidant (Hou 2000) and anti-inflammatory activities (Uto 2007), although 6-MSITC showed lightly stronger activities than sulforaphane. Therefore, our 6-MSITC data from the human clinical trial are consistent with the results for sulforaphane.

Wasabi contains 21 kinds of ITCs, and most of these ITCs have been reported to have a wide range of effects, such as antioxidant effects (Yamada-Kato 2016, Morimitsu 2002, Lee 2010, Uto 2012, Morimitsu 2000, Fuke 2014, Trio 2016, Morroni 2014, 2018, Okunishi 2019,

Valerio 2006). To establish precision safety, it is necessary to further examine differences in susceptibility between races and differences in sensitivity between different age groups.

3.5. Abstract

The safety of 6-MSITC and Wasabi sulfinyl (WS), a 6-MSITC-containing extract powder from Wasabi rhizome, was evaluated by a toxicity test and human clinical trial. Synthetic 6-MSITC (SS) and WS were used in the Ames test to clarify their mutagenic activity, and then used in acute and sub-acute toxicity tests via oral administration in five-week-old Sprague-Dawley rats. WS was further estimated by a human clinical trial. Eleven healthy subjects, aged 20–64 years, were enrolled in the clinical trial; they ingested five WS capsules daily for four weeks. The incidence and severity of WS ingestion-related adverse events were estimated by physical examination, weight and vital sign measurements (resting systolic and diastolic blood pressures, and heart rate), blood analysis, and urinalysis. In the Ames test, no mutagenicity of SS was observed in the range of 20-1,250 μg/plate. LD_{50} values of SS were estimated to be 451.2 mg/kg in male rats and 400.7 mg/kg in female rats. No sub-acute toxicity was observed in Sprague-Dawley rats administrated WS at 2,500 mg/kg/day (containing 2.5 mg 6-MSITC /kg/day). In the human clinical trial, three adverse events occurred in 2 of 11 (18.2 %) subjects, which were classified not to be side effects from WS ingestion by clinical physicians. Although some fluctuations were observed in clinical laboratory values and physical measurement values, these fluctuations were within the reference ranges without clinical significance. Thus, ingestion of 500 mg/day of WS was demonstrated for the first time to be safe at the least. Taken together, our data on the safety evaluation of 6-MSITC and WS provide the first standard references for Wasabi supplement application.

Chapter 4. Effect and potential compounds of Wasabi leaves extract on the growth promotion of hair dermal papilla cells and suppression of hair loss in humans

4.1 Introduction

Hair grows with a hair cycle including anagen (growing phase), catagen (regressing phase), and telogen (resting phase) (Greco 2009). The hair growth is affected by various growth factors, cytokines, and the keratinocyte growth factors such as interleukin-1 and TGF-β2. It is considered that one of the causes of thinning hair and hair loss is that the hair root begins to regress or deteriorate, and unable to undergo a sufficient period (Marlon 2008).

Hair loss is a symptom of different forms of alopecia including androgenic alopecia (AGA), alopecia areata (AA), and telogen effluvium (Claudia 2015). Although drugs such as Minoxidil have been developed, there are some side effects. Some ingredients from plant and herb extracts are reported to have few side effects (Kanti 2018, Famenini 2014). Especially, some flavonoids have been suggested as stimulating substances for hair care (Bassino 2020). For example, naringenin and hesperetin are reported to increase cell proliferation and the level of vascular endothelial growth factor (VEGF) in dermal papilla cells (DPCs) (Madaan 2017). Epigallocatechin-3-gallate is reported to stimulate the growth of DPCs by increasing the phosphorylation of Erk and Akt, and the ratio of Bcl-2/Bax ratio (Kwon 2007). Baicalin from a traditional Chinese herbal medicine is reported to increase hair follicle development by upregulating canonical Wnt/β-catenin signaling and activating DPCs proliferation (Fei 2018). Dicerocaryum senecioides, a common herb in Zimbabwe, is reported to have hair growth promoting effect by upregulating canonical Wnt/β-catenin signaling in the hair follicles of DPCs (Rambwawasvika 2019).

Wasabi is a uniquely pungent vegetable in Japan. Its rhizome is generally used as a spice, and has been reported to have many biological functions because it contains 21 kinds of isothiocyanates (ITC) (Ina 1982, Etoh 1990). On the other hand, Wasabi leaf is almost discarded due to its less pungent and bitter than the rhizome. However, Wasabi leaf contains several flavonoids (Hosoya 2005) including apigenin, isosaponarin and isovitexin, as well as phenylpropanoids such as 5-hydroxyferulic acid methyl ester. These compounds have been reported to have antioxidant activity (Hosoya 2008, Sekiguchi 2010), anti-inflammatory activity (Yoshida 2015), enhancing collagen production (Nagai 2010). Those bioactivities are suggested to link to the hair care effect. Therefore, in the present study, we first screened the effects of Wasabi leaf extract and its major compound isosaponarin, companying other known compounds, on the proliferation of DPCs. Then, we investigated the expressions of proteins concerned to hair growth in isosaponarin-treated DPCs by ELISA. The effect on hair growth promoting effects including hair dermal papilla cells activation and hair loss inhibition were

further confirmed in human clinical trials.

4.2 Material and methods

4.2.1. Reagents

Wasabi leaves cultivated in Hokkaido were harvested by Kinjirushi Co., Ltd., a manufacturer of wasabi products. Washed wasabi leaves in water, cut into small pieces and divided into 108 g, 106 g and 106 g. Distilled water, 50% ethanol, and 100% ethanol were added to make 400 g each. Each was ground in a mixer and stirred with a stirrer for 1 hour. Then filtered by suction through filter paper to obtain 365 g, 402 g and 424 g of the extract and they were concentrated in an evaporator and freeze-dried to obtain 2.9 g, 2.4 g and 2.0 g of powders, respectively. Those powders were diluted to various concentrations before use. Isosaponarin is purified from Wasabi leaves. Briefly, the Wasabi leaf extract with 50% ethanol was dissolved in water, fractionated with 30% methanol using DIAIONTM HP-20 resin (Mitsubishi Chemical), and then purified with an octadecylsilane column (Nomura chemical; mobile phase: 20% methanol plus 0.05% formic acid, flow rate: L/min). The isosaponarin fraction obtained was concentrated by an evaporator and lyophilized to a powder (isosaponarin purity was 79%).

The reagents used in the experiments include trans-ferulic acid (128708-5G; Sigma-Aldrich), *p*-coumaric acid (C9008-1G; Sigma-Aldrich), caffeic acid (C0002; Tokyo Chemical Industry), apigenin (012-18913; FUJIFILM Wako Pure Chemical), trans-sinapic acid (93878; Sigma-Aldrich), methyl caffeate (3843-74-1; Alfa Aesar). Minoxidil was purchased from Sigma (St. Louis, MO, USA). Hair follicle DPC growth medium (TMTPGM-250) was purchased from Toyobo Co., Ltd. (Osaka, Japan). Cell count reagent SF was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

4.2.2. Cell Culture

Human hair follicle DPCs (CA60205a) were purchased from Cell Applications, Inc. (San Diego, CA, USA). DPCs with subculture number 2 or less were used in this study. DPCs were cultured on type I collagen-coated flasks with DPC growth medium at 37°C in a 5% CO_2 incubator.

4.2.3. Cell Viability and Cell Proliferation Assay

To determine the concentration range of each test substance, the cell viability and cell proliferation were determined by the WST assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5- (2,4-disulfonyl)-2H-tetrazolium (WST-8) (Ishiyama,1997). First, DPCs were suspended at 1.2~5.0 × 10^4 cells/mL in DPC growth medium. The cell suspension was

transferred to type I collagen-coated multi-well plates (100 μL/well) and incubated at 37°C for 1 day in a 5% CO_2 incubator. The medium was then changed by DPC growth medium with test substance and incubated at 37°C for 3 days in a 5% CO_2 incubator. After removing the medium, the cells were then washed with phosphate-buffered saline (PBS), and then treated by 100 μL of the hair follicle DPC growth medium with Cell Count Reagent SF (final concentration 10%) at 37°C in a 5% CO_2 incubator. After 30 and 90 min, absorption at 450 nm was measured with the reference wavelength at 595 nm using a plate reader (Precision microplate reader; Molecular Devices Corporation, San Jose, CA, USA). The cell viability and cell proliferation were determined by calculating the change in absorption per unit of time. Effect on cell proliferation was calculated as: % cellular proliferation = {(X-Y)/Y} x 100; where X = absorbance of cells treated with compounds, Y= absorbance of control cells.

4.2.4. Mitochondrial Activity Evaluation

DPCs were suspended at 5.0×10^4 cells/well in DPC growth medium, and then transferred to type I collagen-coated 96-well plates and incubated at 37°C for 1 day in a 5% CO_2 incubator. A test substance (isosaponarin: 0.1, 10, 1,000 μM) was added to the test medium. An additive-free test medium was used as a negative control, and 100 μM adenosine and 30 μM minoxidil-added medium were used as positive control. The medium containing 0.1% DMSO was used as a vehicle control for minoxidil. The cells were cultured for 24, 48 and 72 hours and the culture supernatant were removed. A medium containing Hoechst (reagent for nuclear staining) diluted 1,000-fold and MitoTracker (reagent for mitochondrial staining) diluted 2,000-fold were added, and the mixture was cultured at 37° C for 30 minutes. Then, each fluorescence intensity was measured with a fluorescence plate reader (Hoechst: excitation wavelength 356 nm, fluorescence wavelength 465 nm, MitoTracker: excitation wavelength 579 nm, fluorescence wavelength 599 nm) (Samudio,2005). Furthermore, the cells were photographed for nuclear staining (live cells) and mitochondrial staining images under a fluorescence microscope, finally, the cells medium was replaced with a medium containing 0.5 mg/mL MTT (100 μL/well), and incubated at 37° C for 4 hours. After the reaction, 100 μL of 0.01 M HCl 10% SDS was added to each well and incubated at room temperature for 24 hours to dissolve MTT formazone. Then, the absorbance (measurement wavelength 550 nm, reference wavelength 660 nm) was measured with a plate reader. The experiment was performed with five times. In addition, the number of living cells was also measured by the WST-8 method under the same conditions. In brief, the medium containing 10% of the viable cell count reagent SF was prepared and incubated in a CO_2 incubator (5% CO_2, 37°C), and the absorbance at 450 nm was measured for 60 minutes with a plate reader.

4.2.5. Quantitative ELISA

DPCs cells were plated in 48-well culture plates (1.2×10^4 cells/well) in 5% CO_2 at 37°C for 1 day. After sera-starvation, cells were treated with isosaponarin or Minoxidil Sulphate for 1 day to 3 days. Secretions of VEGF and FGF-7 into extracellular medium were determined by ELISA (abcam and RandD systems, respectively). Effects on VEGF and FGF-7–secretions were calculated as: % Increase in VEGF = {(C-D)/D} x 100; where C = Concentrations of FGF-7 and VEGF (pg/ml) in cells treated with compounds, D = Concentrations of VEGF and FGF-7 ((pg/ml) in control cells.

4.2.6. Human Clinical Trial

4.2.6.1. Ethic

The human clinical trial adhered to all guidelines set forth in the Declaration of Helsinki (revised by WMA Fortaleza General Assembly, Brazil, in October 2013) and Ethical Guidelines for Medical Research on Humans (partially revised December 22, 2014/February 28, 2017).

The protocol of the study was approved by the DERMAPRO Ltd., and validated by receiving a “QUALITY MANAGEMENT SYSTEM CERTIFICATE” (KS Q ISO 9001:2009 / ISO 9001:2008; Certificate No.5855) from the KTR Certification Center for providing contract research and consulting services on human skin safety and efficacy (IRB number: 1-22077-A-N-02-DICN18211) on Nov. 2, 2018, before starting the clinical trial. All volunteers received a full explanation of the aims and procedures. The written informed consents were obtained from all participants before starting the study. All records were classified with an anonymous subject identification number to ensure privacy.

4.2.6.2. Protocol

Eighteen female participants were enrolled in open trial study to investigate the safety and effect of isosaponarin on hair loss and scalp for 8 weeks.

Product: WL Scalp Essence (Ethanol: 12.0%, Wasabi flavone 1.0%, some based materials under 1.0% each, remaining ingredients: water), which is contained 0.336μM of isosaponarin. Formulation type: toner.

Procedure: The product was applied once a day in night time after shampooing at home. The assessments were performed under the controlled environmental conditions in the center. All subjects were washed their hair using the test product at baseline, 3, 7 days, 4 and 8 weeks after treatment and the fallen hair after shampooing was collected through a filter and counted. The photograph of fallen hair was taken using the digital camera (Nikon D-300, Japan) and the scalp scale and erythema images were taken using Aramo TS® (magnify 60 times, AramHuvis,

Korea) at 20 minutes after shampooing. Then, two researchers were assessed clinical images on a 5 to 7-point scales. An adverse event was defined as any unusual event, which in the researcher's opinion, related to the study.

Measurement: The scalp hydration was measured by DermaLab® (Cortex, Denmark) at 40 minutes after shampooing. The instrument makes use of the conductance measurement principle, which was known to measure the water binding capacity of the stratum corneum. The 8 pins probe, minimizing moisture accumulation, was ideal for scalp applications and in hairy areas. The scalp hydration on middle line of parietal region (8cm behind the hairline) was measured. The scalp sebum was measured by Sebumeter® SM815 (C+K, Germany) at 60 minutes after shampooing. The measurement was based on grease spot photometry. The mat tape was brought into contact with skin. It becomes transparent in relation to the sebum on the surface of the measurement area. The tape was then inserted into the aperture of the device, and the transparency was measured by a photocell. The light transmission represents the sebum content. The scalp sebum output level on middle line of parietal region (10cm behind the hairline) was measured.

4.2.6.3. Subjects

Healthy asian female subjects, aged from 20 to 59 years old, who worried about hair loss and provided informed consent after having the purpose and protocol of the study explained, were selected except for subjects who are pregnant, breastfeeding or planning to become pregnant, subjects who are using skin diseases, drug treatment or hair restorer, subjects with hypersensitivity or atopic dermatitis, etc. As further, subjects judged by the doctor were excluded.

The subject could be withdrawing at any time for any reason, and the researcher could exclude subjects who were non-compliance with test conditions, side effects.

4.2.7. Statistical Analysis

The results from *in vitro* test are expressed as mean ± standard deviation. The statistical analysis was performed by an ANOVA, followed by Turkey's test to identify significant differences. A level of $p < 0.05$ was considered statistically significant.

In Human trial, statistical analysis was performed using IBM SPSS Statistics. To determine whether variables followed a normal distribution or not, the Shapiro-Wilks test or Kurtosis and Skewness was used for normality test. For the photographic grade assessment of hair cuticle, if the intraclass correlation coefficient (ICC) values of the two investigators exceeded 0.8, the mean value was used for the analysis. Statistical analysis for variables for parametric was conducted using the paired *t*-test or RM ANOVA. If value was non-parametric,

all of them were initially compared by the Wilcoxon's Signed Rank test (Bonferroni correction used to counteract the problem of multiple comparisons). A statistically significant difference was set at $p< 0.05$. Change from baseline (%) was defined as | (Baseline –After treatment) |/ Baseline* 100

4.3 Results

4.3.1. WL and Isosaponarin Stimulated DPCs Proliferation

To screen the effect of Wasabi leaf components on DPCs proliferation, three kinds of Wasabi leaf extractions were used with water, 50% ethanol, and isosaponarin, a major flavonoid compound in Wasabi leaf extracts (Hosoya 2005, 2008). Minoxidil which is a drug showing hair growth promoting effect (Villez 1987) and adenosine which is reported to promote DPCs growth by inducing VEGF production (Li 2001) were used as positive controls.

The stimulation effects on the proliferation of DPCs were shown in Table 27. In the extracts of Wasabi leaf, water extract showed significant stimulation effect with 114.4% of control at the addition of 0.01%. On the other hand, 50% ethanol extracts of Wasabi leaf showed significant inhibition of DPCs proliferation. Isosaponarin showed significant stimulation effect on the proliferation of DPCs in the concentration range of 0.1-1,000 μM. The maximum activity was 1.23 times higher than that of the control, and 1.12 times higher than that of Minoxidil (30 μM).

Since the stimulation effect of isosaponarin on proliferation of DPCs was significantly observed, we next confirmed whether this effect was due to an increase in number of mitochondria. The number of mitochondria was measured by Mito Tracker staining. After 72 hours of culture, the number of mitochondrial increased 1.07 to 1.17 times from 0.1 μM to 1,000 μM treatment of isosaponarin [Fig. 6].

4.3.2. Isosaponarin Enhanced the Expressions of Hair Growth-Related Proteins

It is known that human VEGF induces the proliferation of DPCs (Li 2001, Li 2012), and forms capillaries around hair follicles which are disappeared during the resting period (Liachgar 1996). Minoxidil is reported to increase hair volume with upregulated VEGF mRNA and hair follicle angiogenesis (Villez 1987, Han 2004, Lachgar 1998). FGF-7 of DPCs is also required for the proliferation of hair mother cells (Han 2004, Lachgar 1998). Therefore, we investigated the effect of isosaponarin on the production of VEGF and FGF-7 in DPCs by ELISA. The cells were cultured in a medium containing the test substance for 3 days, the supernatant was collected to measure VEGF and FGF-7 protein by ELISA.

The results showed that VEGF was significantly increased by treatment with 10 and 1,000μM, similar to the results with positive Minoxidil (30μM) treatment [Fig. 7a]. However,

Table 27. The effect of Wasabi leaf extracts and its compounds on proliferation of DPCs

Extracts (%)	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
WL Water Extracts	101.5±0.8	102±1.2	103±1.4	104.4±0.4*	114.4±0.7*
WL 50% EtOH Extracts	98.2±1.1	96.9±1.0	93.0±1.0*	62.7±2.8*	-

Compounds (μM)	0.001	0.01	0.1	10	100	1000
Adenosine	-	-	-	-	129.9±3.5*	-
Minoxidil (30μM)	-	-	-	112.2±3.7*	-	-
Isosaponarin	99.5±3.2	109.9±3.4	123.3±2.0*	122.5±0.8*	-	118.3±2.7*

The effect of Wasabi leaf extracts and isosaponarin on the proliferation of DPCs. DPCs were treated with indicated concentrations of Wasabi leaf extracts and compounds as well as positive controls (adenosine and Minoxidil) for 3 days at 37°C, 5% CO_2 incubator. The cell proliferation was determined by WST assay as described in Materials and Methods section. Each value represents mean ± SD of 5 replicates. Asterisk (*) indicates significant difference to control ($p < 0.05$).

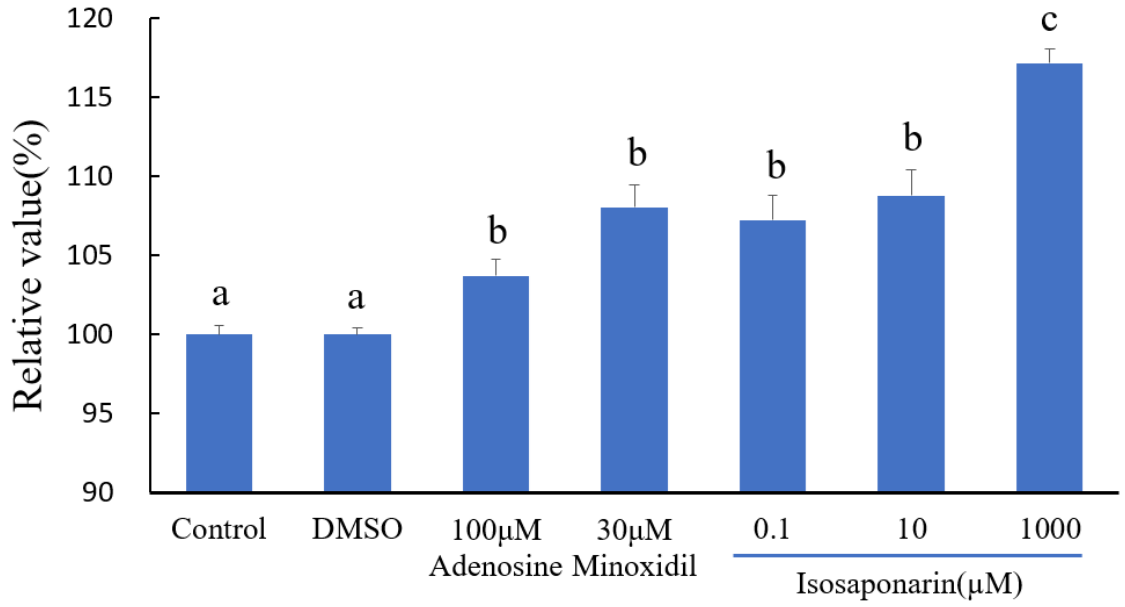


Fig.6. The effect of isosaponarin on amounts of mitochondria in DPCs.
DPCs were cultured for 72 hours with indicated concentrations of isosaponarin, solvent control (DMSO) and positive controls (adenosine and Minoxidil). The number of mitochondria was measured by Mito Tracker staining. Cellular nuclei were stained by Hoechist staining and used to evaluate the number of cells. The data from 5 replicates was represented as relative value (%) to control. Differently lettered superscripts differ significantly at $p < 0.05$.

FGF-7 was significantly decreased by treatment with 0.1 and 1,000μM although the positive control adenosine (100 μM) significantly increased FGF-7 level [Fig. 7b].

These results suggest that isosaponarin may prolong hair anagen by inducing VEGF but not EGF-7.

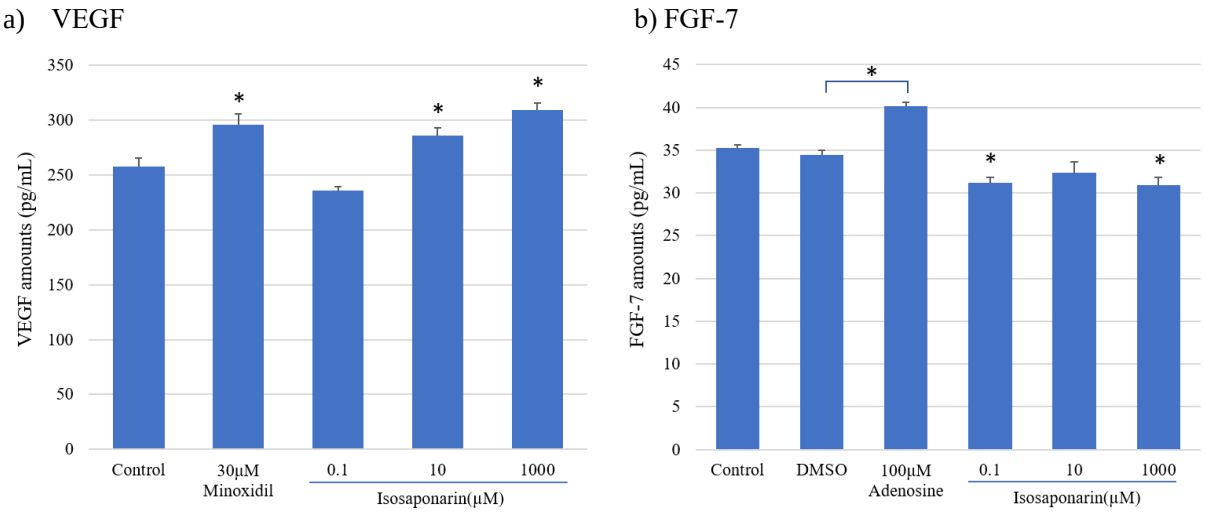


Fig. 7. The effect of isosaponarin on levels of hair growth-related proteins
The effect of isosaponarin on the productions of VEGF (a) and FGF-7 (b). DPCs were cultured for 72 hours with indicated concentrations of isosaponarin and positive controls (Minoxidil or adenosine). The levels of VEGF and FGF-7 were measured by ELISA as described in Methods section. Each value represents mean ± SD of 5 replicates. Asterisk (*) indicates significant difference to control ($p < 0.05$).

4.3.3. The Preventive effects of isosaponarin-involved WL on hair loss in human clinical trial

To estimate the hair growth-promoting effect, we conducted human clinical trials as described in Material and Methods. Eighteen female subjects who has scalp scale and hair loss were recruited and divided into three groups (30-39, 40-49 and 50-59 age) according to ages. The parameters of hair thickness, scalp hydration, scalp sebum and hair loss counts were measured as baseline before trail [left in Table. 28] and hair loss counts were further estimated after 3 days, 7days, 4weeks and 8 weeks after trial beginning [right in Table. 28]. As shown in Table 28, hair loss counts were reduced in time-dependent manner, and significant effects were observed at 30-39 age and 40-49 age after 8 weeks ($p<0.05$).

Next, we also measured the scalp erythema and scalp itching. As shown in Table 29, scalp erythema was decreased in time-dependent manner after treatment, and significant effects were observed at 4-8 weeks for 40-49 age ($p<0.05$). The typical images of scalp erythema were shown in Figure 8. The scalp itching was also decreased in time-dependent manner after treatment, and significant effects were observed at 4-8 weeks for 30-39 and 40-49 age ($p<0.05$) [bottom in Table 29].

Table 28. Hair and scalp characteristics of subjects and hair loss counts by age

Ages	N		Before				After (Hair loss counts)			
			Hair thickness	Scalp hydration	Scrab sebum	Hair loss counts	3 days	7 days	4 weeks	8 weeks
30～39	6	AVE	1.83	2.83	1.83	60.0	52.5	22.0	28.2	16.7
		± SD	± 0.75	± 0.41	± 0.75	± 49.4	± 39.0	± 10.2	± 14.1	± 16.7
		p-Value	-	-	-	-	0.77	0.07	0.19	0.03*
40～49	8	AVE	1.50	2.75	2.00	53.9	55.0	31.3	36.4	26.0
		± SD	± 0.76	± 0.46	± 0.76	± 34.9	± 35.8	± 12.4	± 21.2	± 16.1
		p-Value	-	-	-	-	0.92	0.07	0.24	0.03*
50～59	4	AVE	1.50	2.25	2.00	53.8	30.5	46.0	30.0	34.5
		± SD	± 1.00	± 0.50	± 0.82	± 24.8	± 3.1	± 24.2	± 8.2	± 11.4
		p-Value	-	-	-	-	0.16	0.05	0.13	0.26

Hair and scalp characteristics as well as hair loss counts of subjects and by age. Eighteen of female subjects were divided into three groups of 30-39, 40-49, 50-59 according to age. Hair thickness, scalp hydration and scalp sebum were measured before the study and quantified as points according to the following criteria. Hair thickness: Thin as 1, Normal as 2, Thick as 3; Scalp hydration: Sufficient as 1, Normal as 2, Dry as 3; Scalp sebum: Glossy as 1, Normal as 2, Deficient as 3. Hair loss counts were measured before the study as baseline and after 3 days, 1 week, 4 weeks, 8 weeks of study. Each value represents mean ± SD of each group. Asterisk (*) indicates significant difference to Baseline ($p < 0.05$).

In addition, we investigated the correlation between hair loss and hair thinning or scalp hydration. The results showed that the “Thin” and “Normal” grade of hair thickness had significant effects on the prevention for hair loss at 8 weeks after treatment than “Thick” grade [Table 30]. Moreover, the hair loss in dry scalp subjects was significantly inhibited than those with normal scalp [Table 31].

4.4 Discussion

Isosaponarin is unique flavonoid glycoside presenting in Wasabi leaf c (Mashima 2019), and easy dissolved in water. Our data showed that water extract of Wasabi leaf and isosaponarin compound could enhance the proliferation of DPCs. In addition, isosaponarin showed wider cytotoxicity. On the other hand, 50-100% ethanol extract of Wasabi leaf showed significant inhibitory effect on the proliferation of DPCs. These data suggested the components between water and ethanol extract are different, we are planning to perform a detail chemical analysis in next work. Taken together, our data suggested that isosaponarin might be an active ingredient for the proliferation effect of Wasabi leaf extract.

It has been suggested that the function of mitochondria in dermal papilla cells is also important for hair growth (Mifude 2015). Therefore, we investigated the effects of isosaponarin

Table 29. Statistical analysis of scalp redness and itching by age

	Ages	N		Before	After 3 days	After 7 days	After 4 weeks	After 8 weeks
Scalp erythema	30～39	6	AVE ± SD	0.00	-0.33 ± 0.52	-0.33 ± 0.53	-0.33 ± 0.54	-0.33 ± 0.55
			p-Value	-	0.17	0.17	0.17	0.17
	40～49	8	AVE ± SD	0.00	-0.31 ± 0.59	-0.31 ± 0.46	-0.50 ± 0.53	-0.63 ± 0.74
			p-Value	-	0.18	0.09	0.03*	0.05*
	50～59	4	AVE ± SD	0.00	-0.25 ± 0.56	-0.38 ± 0.52	-0.38 ± 0.54	-0.38 ± 0.60
			p-Value	-	0.39	0.22	0.22	0.22
Scalp itching	30～39	6	AVE ± SD	2.83 ± 0.75	2.17 ± 1.47	1.67 ± 1.51	1.33 ± 1.37	1.33 ± 1.21
			p-Value	-	0.17	0.06	0.02*	0.03*
	40～49	8	AVE ± SD	1.88 ± 0.83	2.00 ± 0.93	1.50 ± 0.76	0.88 ± 0.83	0.50 ± 0.53
			p-Value	-	0.35	0.28	0.00*	0.00*
	50～59	4	AVE ± SD	2.00 ± 1.41	1.50 ± 0.58	1.75 ± 0.50	0.75 ± 0.96	0.50 ± 1.00
			p-Value	-	0.39	0.72	0.08	0.25

Eighteen of female subjects were divided into three groups of 30-39, 40-49, 50-59 according to age. The assessments of scalp erythema and scalp itching were performed before the study as baseline and after 3 days, 1 week, 4 weeks, 8 weeks of study. The status was quantified as points according to the criteria of the following criteria. Scalp erythema: Extreme decrease as -3, Moderate decrease as -2, Slight decrease as -1, No change as 0, Slight increase as 1, Moderate increase as 2, Extreme increase as 3; Scalp itching: No as 1, Mild as 2, Moderate as 3, Severe as 4, Very severe as 5. Each value represents mean ± SD of each group. Asterisk (*) indicates significant difference to Baseline ($p < 0.05$).

on mitochondria in dermal papilla cells. Firstly, we observed the effect of isosaponarin on the proliferation using WST-8 method. However, since this intracellular metabolic activity is evaluated by the dye-redox method, it is not possible to distinguish whether the cell activation effect is due to mitochondrial proliferation or increase. Therefore, we further evaluated the mechanism of isosaponarin's effect by Hoxst (reagent for nuclear staining) and MitoTracker (reagent for mitochondrial staining). As a result, the mitochondrial amount increased in a concentration-dependent manner in the range of 0.1 μM and 1,000 μM. This data suggested

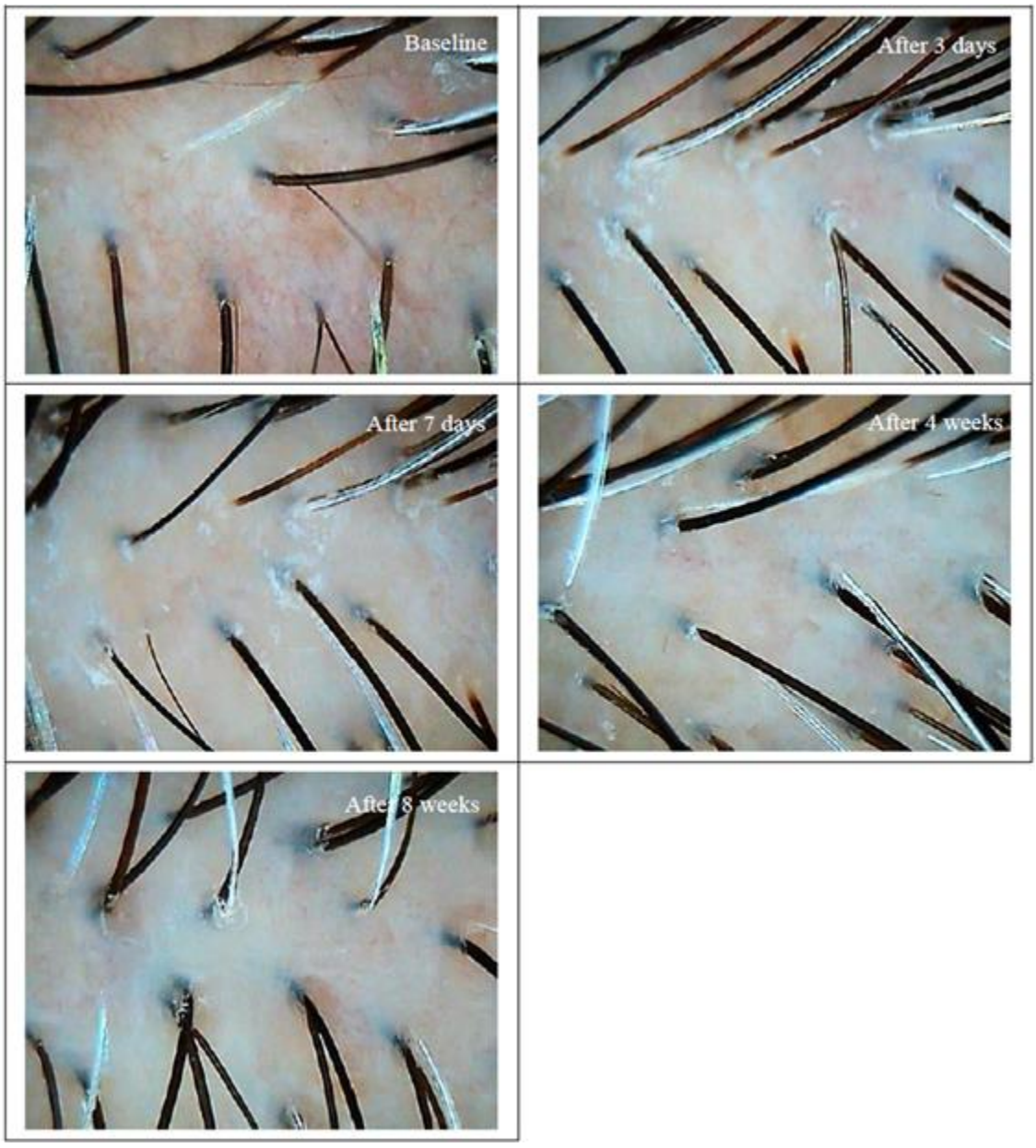


Fig. 8. Typical images of scalp erythema after treatment of wasabi leaf extract

WL Scalp Essence containing contained 0.336μM of isosaponarin was applied once per day for night times after shampooing at home. The assessments were performed under the controlled environmental conditions in the center. The scalp scale and erythema images at start (Baseline) and after 3 days, 7days, 4weeks and 8weeks were taken using Aramo TS® (magnify 60 times, AramHuvis, Korea) at 20 minutes after shampooing, and then quantified.

that the effect of isosaponarin on mitochondrial is mainly due to the mitochondrial proliferative action, and at high concentration, the mitochondrial activity itself is also activated.

Activation of dermal papilla cells was decreased at 1,000 μM, while mitochondria showed the highest activity at 1,000 μM. This may be due to the fact that the activity of the papilla cells is measured by the WST-8 method, which evaluates the metabolic activity of the

Table 30. Statistical analysis of hair loss counts by hair thickness

Hair thickness	N		Baseline	After 3 days	After 7 days	After 4 weeks	After 8 weeks
Thin	10	AVE ± SD	53.2 ± 43.0	40.4 ± 21.9	33.6 ± 20.4	33.6 ± 16.0	23.4 ± 12.9
		p-Value	-	0.42	0.14	0.23	0.03*
Normal	5	AVE ± SD	42.7 ± 33.1	69.0 ± 51.7	29.4 ± 13.4	35.4 ± 19.5	24.4 ± 20.6
		p-Value	-	0.99	0.01*	0.06	0.00*
Thick	3	AVE ± SD	42.7 ± 9.5	42.7 ± 14.8	27.7 ± 9.5	22.3 ± 14.2	30.0 ± 23.3
		p-Value	-	1.00	0.06	0.27	0.36

Statistical analysis of hair loss counts by hair thickness. Eighteen of female subjects were divided into three groups of Thin, Normal and Thick according to hair thickness. The assessments of hair loss counts were performed before the study as baseline and after 3 days, 1 week, 4 weeks, 8 weeks of study. Each value represents mean ± SD of each group. Asterisk (*) indicates significant difference to Baseline ($p < 0.05$).

Table 31. Statistical analysis of hair loss counts by scalp hydration

Scalp hydration	N		Baseline	After 3 days	After 7 days	After 4 weeks	After 8 weeks
Normal	6	AV ± SD	44.0 ± 25.6	30.3 ± 13.4	39.5 ± 21.3	28.0 ± 8.1	30.0 ± 12.0
		p-Value	-	0.27	0.75	0.18	0.25
Dry	12	AVE ± SD	61.8 ± 40.6	57.9 ± 36.2	27.4 ± 13.1	34.3 ± 19.3	22.2 ± 17.6
		p-Value	-	0.81	0.01*	0.05*	0.01*

Statistical analysis of hair loss counts by scalp hydration. Eighteen of female subjects were divided into two groups of Normal and Dry according to scalp hydration. The assessments of hair loss counts were performed before the study as baseline and after 3 days, 1 week, 4 weeks, 8 weeks of study. Each value represents mean ± SD of each group. Asterisk (*) indicates significant difference to Baseline ($p < 0.05$).

entire cell, while the mitochondrial activity is evaluated only for the mitochondria by the MTT method. It suggested that the appropriate concentration may differ between the concentration that promotes the activity of whole cell and the concentration that promotes the activity of mitochondria alone.

Furthermore, we investigated the effect of isosaponarin on the production of proteins concerned to hair growth, VEGF and FGF-7, in DPCs by ELISA. It is interesting that

isosaponarin treatment significantly increased the productions of VEGF, but not FGF-7. Although the reason why isosaponarin decreased the level of FGF-7 is not unclear, isosaponarin possibly interferes with FGF-7 production cascade in part, which has been reported to be activated by the SUR extracellular ATP excretion pathway (Li 2001). Our data suggest that isosaponarin may prolong hair anagen by inducing VEGF but not EGF-7.

Hair loss was most severe in summer, while the trial period was winter (11.12.2018--01.15.2019) and relatively stable. Therefore, we did not place a control as a pre-test to observe the effect on hair loss (Kunz 2009).

The concentration of isosaponarin in the lotion used in the clinical test was 0.336 μM. The reason for this concentration is that the EC_{50} value of isosaponarin is 0.01 μM and cytotoxicity is seen at 5 mM (Data not shown). Thus, the concentration of isosaponarin in the lotion was set to 0.336 μM, concerning both the absorption efficient and dose safety of isosaponarin in skin. Moreover, the inhibitory effect of hair loss was observed higher in dry skinned individuals than skinned individuals [Table 31], suggesting the deterioration of scalp environment was improved to reduce the number of hair loss.

Our clinical studies revealed that WL extract not only suppressed hair loss, but also had an anti-inflammatory effect and suppressed itching and redness of the scalp. These effects are thought to be due to flavonoids in WL extract, which are known to have antioxidant and anti-inflammatory effects.

Any adverse reaction on scalp was not observed during the observation periods.

Recently, some advanced biotechnologies in hair regeneration therapies have been reported. For example, using stem cell therapy and biotechnology, each platelet-rich fraction is purified from the patient's own blood, and then injected into the scalp with a syringe. The hair regeneration is promoted by the various cell growth factors and anti-apoptotic factors contained in the blood (Pietro 2020). In addition, autologous growth factors such as adipose-derived mesenchymal stem cells (AD-MSCs), mesenchymal vascular differentiated cells (SVFs) and human hair follicle stem cells (HFSCs) have been used in various clinical applications in patients with AGA. Vascular endothelial growth factor is also considered as one of important factors. Treatment with platelet-rich plasma (PRP) has been reported to increase hair density and thickness by approximately 40% (Gupta 2017), AD-MSCs by 31% and hair thickness by 29% (Gentile 2019), and HFSCs by 29% (Gentile 2017). In our clinical trial, hair loss was inhibited by 63% at 8 weeks although these numbers are not comparable to placebo. The effect of isosaponarin may be more modest. We also noticed that this clinical trial had not a comparable placebo group, the number of subjects were small and female only. A randomized controlled trial study is further required to verify the suppressive effect of hair loss of Wasabi leaf extract.

4.5 Abstract

Wasabi (*Eutrema japonicum* (Miq.) Koidz.) is a uniquely pungent spice in Japan since its rhizome contains several flavoring isothiocyanates. On the other hand, its leaves are almost discarded although it contains several bioactive flavonoid glycosides, which are suggested to have hair growth stimulating effects. Thus, we first investigated the effects and mechanisms of Wasabi leaf (WL) components on the proliferation of human follicle dermal papilla cells (DPCs) and then confirmed the effects on hair growth by human clinical trials. The cell culture data revealed that WL and isosaponarin, one of major flavonoids in WL, enhanced the proliferation of DPCs. Especially, isosaponarin increased the amount and activities of mitochondrial. ELISA data revealed that isosaponarin increased the productions of vascular endothelial growth factor (VEGF) proteins. Furthermore, the data from human clinical trials by applying isosaponarin-rich WL to 18 women subjects who has scalp essence for 8 weeks showed that hair loss was reduced about 60%, and scalp redness and itching were also suppressed. These results suggested that Wasabi leaf extract rich with isosaponarin could improve hair loss by activating the proliferation of dermal papilla cells and modulating the productions of VEGF.

Chapter 5. General discussion and conclusion

5.1. General discussion about wasabi leaf

We worked on research to increase the value of wasabi leaves, most of which were discarded. As it has recently been found to contain many flavonoids and polyphenols, it is expected to focus on its functional aspects when utilized. In particular, it is expected to be taken in high concentrations as a supplement due to its reported effects on hair growth and fat burning in line with societal needs. Therefore, in this study, mutagenicity and toxicity tests were performed and no adverse events were observed even with a daily intake of 200 mg of wasabi leaf extract. This study can be considered as a reference for the safe use range of wasabi leaf extract, which is useful for industrial applications.

As a functional study of wasabi leaves, it was reported that it activated hair papilla cells and the mechanism was elucidated. The results concluded that isosaponarin, a flavonoid in the composition of wasabi leaves, activates hair papilla cells and enhances the production of vascular endothelial growth factor and other substances, thereby inhibiting hair loss. The human clinical trial was conducted without a placebo and in small numbers, so its effectiveness needs to be further verified.

In addition, several effects of ingesting wasabi leaf extract have been reported to improve lipid metabolism (Yamasaki 2013, Oowatari 2016). The activation of UCP-1 in cell tests and the activation of brown adipocytes, which are involved in weight loss and fat burning, have been reported in animal tests (Yamada-Kato 2016). In addition, several components related to lipid metabolism have been identified, and are expected to have functional properties when ingested (Misawa 2018). Since there have been no reports of human intake studies, we evaluated the safety of the product in this clinical study, and also evaluated its effects on visceral fat. Although no significant difference has been obtained, there is a tendency for visceral fat to decrease with the intake of wasabi leaves, and significant results may be obtained by conducting a study with a sufficient size. Several flavonoids and low-molecular-weight compounds are possible candidates for the beneficial components, but given the low absorption rate of flavonoids in the intestinal tract, further studies are needed to identify the functional components.

However, it is believed that the utility of wasabi leaves has been further enhanced through this study, and the increased use of wasabi leaves as a functional material is expected to lead to the revitalization of wasabi production areas, making this study highly relevant.

5.2. General conclusion about 6-MSITC

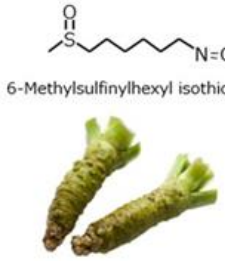
Wasabi rhizomes contain various types of isothiocyanates, many of which are volatile and can be enjoyed for their flavor. Among them, 6-MSITC is not volatile and has many functional properties. Among its functions, cancer inhibition, antioxidant effects and detoxification have been reported. In recent years, it has also been elucidated to improve brain function and hair growth, and its use as a supplement is expanding. The clinical trial that confirmed the improvement in cognitive function was designed with 100mg/day of wasabi rhizome extract (containing 0.8mg of 6-MSITC), which is about 5-10g of wasabi. This is slightly more than the amount eaten as a condiment for sashimi or sushi, but not an inedible amount. However, because those products are usually made by mixing many ingredients, furthermore, by using mainly parts other than rhizome, wasabi products containing enough 6-MSITC are not available in the market. That is, it is difficult to take 6-MSITC on a daily basis. Therefore, because it is expected that the use of 6-MSITC as a supplement will increase, it will be meaningful to confirm the effects of excessive intake of 6-MSITC.

In this study, mutagenicity and toxicity tests were performed and no adverse events were observed even with an excess intake of five times the confirmed function in humans. This study can be considered as a reference for the scope of safe use of wasabi extracts, which is useful for industrial applications.

5.3. General conclusion about wasabi

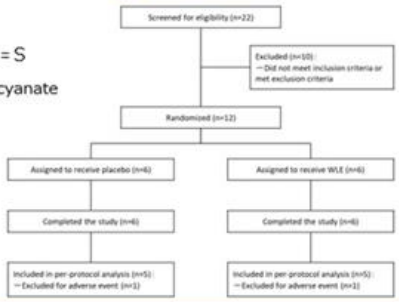
To date, wasabi is primarily known for its mechanistic elucidation and fundamental efficacy assessment in cellular and animal studies. This research has confirmed its safety and efficacy in humans and can now be considered as a material that can be used in industry. In other words, considering the stage of industrial use, product development will be carried out only after the efficacy and safety in humans have been confirmed. At present, basic research is beginning to progress not only in Japan but also around the world, and clinical research on wasabi is expected to increase in the future. In addition, as awareness of the functionality of wasabi increases, research on each part of wasabi is expected to advance, which will ultimately lead to increased demand for wasabi and revitalization of the wasabi industry. It is hoped that this research will be the first step in this direction.

The studies on the functionality and safety of bioactive substances derived from wasabi



6-Methylsulfinylhexyl isothiocyanate

Over dose test



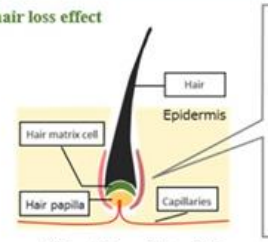
Toxicity

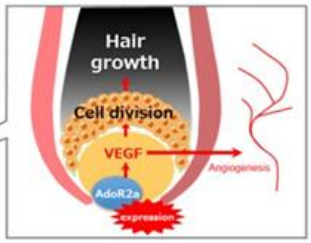
	Acute		Sub-acute
	LD_{50}		NOAEL
	SS	WS	WS
SD-rat(male)	481.2 mg/kg	—	2000 mg/kg
SD-rat(female)	400.7 mg/kg	—	

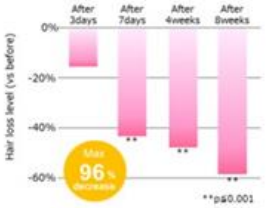
※ —: No deaths were observed at 2000 mg/kg. NOAEL: No Observed Adverse Effect Level

The safety of 6-(methylsulfinyl) hexyl isothiocyanate (6-MSITC) and Wasabi sulfinyl (WS), a 6-MSITC-containing supplement from Wasabi rhizome, was evaluated by a toxicity test and human clinical trial. Synthetic 6-MSITC (SS) and WS were used in the Ames, in acute and sub-acute toxicity. Our data on the safety evaluation of 6-MSITC and WS, a 6-MSITC-containing supplement, provide the first standard references for Wasabi supplement application.

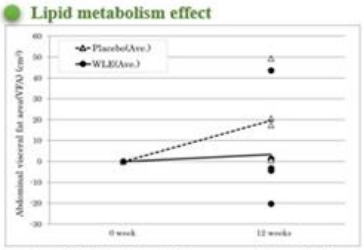
Anti-hair loss effect







Lipid metabolism effect



Wasabi leaf extract (WLE) rich with isosaponarin could improve hair loss by activating dermal papilla cells and modulating the productions of VEGF. In addition, acute and subacute toxicity tests of WLE were conducted to confirm its safety. Furthermore, the safety of WLE was confirmed in an ingestion study of 200 mg/day of WLE in humans, and its effect on lipid metabolism was evaluated. The results provide a first standard reference for future use of horseradish leaf supplements.

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