



Analysis of anti-obesity and anti-diabetic effects of acacia bark-derived proanthocyanidins in type 2 diabetes model KKAY mice

Mayumi Kashiwada¹ · Saho Nakaishi¹ · Ayumi Usuda¹ · Yumi Miyahara¹ · Kenta Katsumoto¹ · Kyoko Katsura¹ · Izumi Terakado¹ · Makiko Jindo¹ · Shihori Nakajima¹ · Sosuke Ogawa² · Kiyoshi Sugiyama³ · Wataru Ochiai¹

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Abstract

The acacia bark extract derived from *Acacia mearnsii* De Wild is rich in proanthocyanidins, whose constituent units are robinetinidol, fisetinidol, catechin, and gallicocatechin. In this study, we examined the effect of proanthocyanidins on obesity and diabetes using KKAY mice, a type 2 diabetes model. KKAY mice were fed either a low-fat diet, a high-fat diet, or a high-fat diet mixed with an acacia bark extract, a proanthocyanidins fraction, and other fraction for 7 weeks. Monitoring the changes in the body weight revealed that acacia bark extract and proanthocyanidins fraction could prevent excessive weight gain resulting from a high-fat diet. In addition, increases in the fasting blood glucose level due to high-fat diet intake were found to be suppressed by acacia bark extract and proanthocyanidins fraction. Furthermore, proanthocyanidins derived from acacia bark were found to increase the expression of adiponectin in white adipose tissue, which enhances the action of insulin. In addition, acacia bark-derived proanthocyanidins suppressed gluconeogenesis and fatty acid synthesis in the liver, as well as suppressing the decrease in energy production under pathological conditions in skeletal muscle. In addition, acacia bark-derived proanthocyanidins showed AMPK activation and DPP-4 inhibitory action. Therefore, it was suggested that acacia bark-derived proanthocyanidins lowered fasting blood glucose levels through the above mechanism. These results suggest that proanthocyanidins derived from acacia bark are the active ingredients of the anti-obesity and anti-diabetic effects of acacia bark extract.

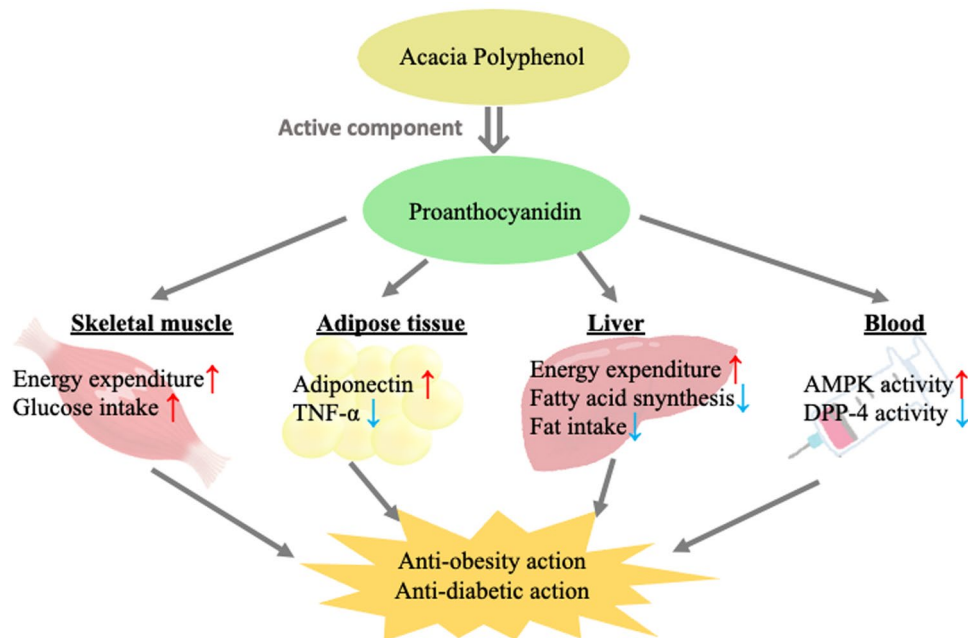
✉ Wataru Ochiai
w-ochiai@hoshi.ac.jp

¹ Department of Pharmacokinetics, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

² Acacia-No-Ki Co., Ltd., 4291-1, Miyauchi, Hatsukaichi-shi, Hiroshima 738-0034, Japan

³ Department of Functional Molecule Kinetics, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

Graphic abstract



Keywords Acacia bark · Proanthocyanidin · Diabetes · Obesity · KKAY mouse

Introduction

Acacia mearnsii De Wild is an evergreen tree belonging to the genus *Acacia* of the leguminous family and is widely distributed in the continents of Australia and Africa.

In recent years, various pharmacological effects, such as blood pressure lowering effects [1], and anti-inflammatory effects have been attributed to acacia bark extract [2, 3].

Furthermore, feeding acacia bark extract (5% mixed diet) to type 2 diabetic KKAY mice with high-fat diet-induced obesity has been found to suppress excessive weight gain and significantly lower fasting blood glucose levels [4]. It has also been clarified that the action of these acacia bark extracts occurs in a dose-dependent manner [4]. However, the active components of the anti-obesity and anti-diabetic effects of acacia bark extract have yet to be identified.

Acacia bark extract is rich in proanthocyanidins derived from acacia bark, which have a molecular weight of about 300–3000 kDa, such as unique flavan-3-ol oligomers composed of robinetinidol, fisetinidol, catechin, and gallocatechin, and tetramers and pentamers [5].

In recent years, among the thousands of types of polyphenols existing in nature, those classified as proanthocyanidins have been attracting attention due to their various pharmacological effects. In particular, proanthocyanidins in acacia bark extract have a characteristic structure. In previous studies, we found that acacia bark extract suppress postprandial

sugar absorption and moderate postprandial blood glucose elevation [6]. Therefore, in the present study, we investigated whether proanthocyanidins derived from acacia bark lowers fasting blood glucose levels.

Furthermore, we also examined whether proanthocyanidins derived from acacia bark have anti-obesity and anti-diabetic effects. In this study, KKAY mice, a type 2 diabetes model, were fed a high-fat diet to induce severe obesity and diabetes. We then used these KKAY mice to analyze the effects of proanthocyanidins derived from acacia bark on obesity and diabetes. In addition, to clarify the mechanism of the inhibitory effect of acacia bark extract on obesity and diabetes, the changes in the expression levels of genes related to glycolipid metabolism in white adipose tissue, liver, and skeletal muscle were analyzed.

Lastly, we examined AMPK and DPP-4 activity, which plays an important role in the regulation of fasting blood glucose, in the blood of KKAY mice.

Materials and methods

Preparation of hot water extract from acacia bark

Acacia mearnsii De Wild bark extract (lot; D18/2) was provided by Acacia-No-Ki Co., Ltd. (Hiroshima, Japan). This acacia bark extract was prepared according to the method

of Cutting et al. (J. Soc. Leather Technol. Chem. 1997, 81, 89–93). Briefly, the powdered bark of South African *Acacia mearnsii* De Wild was crushed, extracted with hot water (100 °C), and dried with a spray dryer. The total proanthocyanidins content in the extract was quantified using the Folin-Ciocalteu method. The acacia bark hydrothermal extract used in this study contained 77.5% proanthocyanidins [7]. The acacia bark extract was then fractionated into a proanthocyanidins fraction and other fraction, predominantly sugar-containing, by chromatography using a Diaion HP20 column. The proportions of proanthocyanidins derived from acacia bark and predominantly sugar-containing fraction in the acacia bark extract were 71.7 and 28.3%, respectively.

Preparation of mixed feed

The mixed diets used in the experiments were a low-fat diet (10 kcal%) (D12450J; Research Diets, Inc., New Brunswick, NJ, USA) and a high-fat diet (60 kcal%) (D12492; Research Diets, Inc., New Brunswick). In addition, a high-fat diet (60 kcal%) was mixed with acacia bark extract, proanthocyanidins, and sugar fractions at 5%, 3.6% (5% × 71.6% of acacia bark extract), and 1.4% (5% × 28.3% of acacia bark extract), respectively. Mixing of each test substance into a high-fat diet was outsourced to Research Diets, Inc.

Animal handling

KKAy mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were kept at room temperature (24 ± 1 °C) and 55 ± 5% humidity with 12 h of light (artificial illumination; 08:00–20:00). Food and water were available ad libitum. Mice were bred one per cage throughout the preliminary breeding period and the experimental period. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Animal Care Committee of Hoshi University (Tokyo, Japan). All procedures using animals were carried out according to protocols approved by the Animal Care Committee of Hoshi University, approval number 19-115.

Blood analysis

Blood samples were centrifuged (1000 g for 15 min at 4 °C), and plasma stored at 80 °C until assay. Plasma glucose concentrations were enzymatically quantified using a Glucose CII Test (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Plasma insulin concentrations were measured according to the protocol described by the manufacturer of the Mouse Insulin ELISA Kit (Shibayagi, Gunma, Japan). HbA1c levels were measured by Oriental Yeast Co., Ltd. (Tokyo, Japan). Homeostasis model assessment of insulin resistance (HOMA-IR), an index of insulin resistance, was calculated from plasma

glucose concentrations. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were quantified using Transaminase CII Test (FUJIFILM Wako Pure Chemical Corporation). Plasma AMPK activities were measured according to the protocol described by the manufacturer of the CycLex AMPK Kinase Assay Kit (MEDICAL & BIOLOGICAL LABORATORIES Co. Ltd., Nagoya, Japan). Plasma DPP-4 activities were measured according to the protocol described by the manufacturer of the DPP4 Activity Fluorometric Assay Kit (BioVision, Inc., CA, USA).

Quantitative PCR

Total RNA was extracted from the mouse tissue using TRI reagent (Sigma-Aldrich Corp., St. Louis, MO, USA). A high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA from 1 µg of RNA. Target gene expression was analyzed with real-time PCR using the primers listed in Table 1. The mRNA gene expression levels were normalized to β-actin gene expression levels.

Hematoxylin–eosin staining and oil red O staining

The epididymal white adipose tissues and livers isolated from the mice were immersed in 10% neutral buffered formalin to fix the tissues. The tissues sectioning and staining was performed by Biopathology Institute Co., Ltd. (Ota, Japan).

Measurement of liver triglyceride/cholesterol content

Liver triglyceride and cholesterol content were enzymatically quantified using a Triglyceride E-Test and Cholesterol E-Test (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Statistical analysis

Numerical data are expressed as the mean ± standard deviation (SD). The significance of differences was examined using analysis of variance (ANOVA), followed by Tukey's test. $P < 0.05$ was considered significant. Outliers were calculated using the Smirnov–Grubbs outliers test.

Results

Food intake and weight change

Each diet (i.e., low-fat diet (LF), high-fat diet (HF), high-fat diet + acacia bark extract (AP), high-fat

Table 1 Primer sequence of target gene

Target gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)	Accession number
ACC	ATGGGCGGAATGGTCTCTTTC	TGGGGACCTTGCTTTCATCAT	148	NM_133360
ACO	GTGCAGCTCAGAGTCTGTCCAA	TACTGCTGCGTCTGAAAATCCA	112	NM_015729
Adiponectin	GAGATGCAGGTCTTCTTGGTC	GCTCTCCTTTCCTGCCAG	10	NM_009605
CPT1	GTGACTGGTGGGAGGAATAC	GAGCATCTCCATGGCGTAG	83	NM_013495
FAS	AGCCTGTGTAGCCTTCGAGA	GCCCGGTAGCTCTGGGTGTA	129	NM_007988
GLUT4	GGAAGGAAAAGGGCTATGCTG	TGAGGAACCGTCCAAGAATGA	115	NM_009204
LPL	CCACAGCAGCAAGACCTTC	CCACATCATTTCCACCAG	128	NM_008509
PPAR α	GTACGGTGTGTATGAAGCCATCTT	GCCGTACGCGATCAGCAT	76	NM_011144
PPAR γ	ATGGAAGACCACTCGCATT	CATTGGGTGAGCTCTTGTGA	111	NM_011146
PPAR δ	GCCATATTCCCAGGCTGTC	CAGCACAAGGGTCATCTGTG	102	NM_011145
SREBP-1c	GCCTGGGATCAAAGAGGAG	TAGATGGTGGCTGCTGAGTG	197	NM_011480
TNF- α	AAGCTGTAGCCCACGTCGTA	GGCACCAGTGTGGTTGCTTTG	122	NM_013693
UCP3	CCGGTGGATGTGGTAAAGAC	CTCCTGAGCCACCATCTTC	93	NM_009464
β -Actin	GAGCGCAAGTACTCTGTGTG	CGGACTCATCGTACTCTCTG	97	NM_007393

diet + proanthocyanidins fraction (Pro), and high-fat diet + sugar fraction (S)) was freely ingested by 7-week-old KKAY mice for 7 weeks. The acacia bark extract (5% mixed diet) was used as a positive control [4]. Food intake was measured weekly during the administration period to clarify that the change in body weight was not due to differences in food intake. No significant difference in food intake was observed between the group of mice that consumed the mixed diet containing the test substance (Fig. 1a).

In addition, the weight of the mice in each group was measured weekly during the test period. The weights of the mice in the HF and S groups increased rapidly immediately after the start of administration and were significantly higher than those in the LF group from the 4th day after the start of administration. On the other hand, the weights of the mice in the AP and Pro groups were slightly lower or similar to those in the LF group (Fig. 1b).

Effect of proanthocyanidins derived from acacia bark on type 2 diabetes model KKAY mice

We evaluated the state of diabetes at the time of dissection in each group using the fasting blood glucose level, fasting insulin level, HbA1c, and HOMA-IR as indicators. The fasting blood glucose levels of the HF and S groups were significantly higher than those of the LF group. On the other hand, the AP and Pro groups were significantly lower than those in the LF and HF groups (Fig. 2a).

The insulin levels in the HF and S groups tended to increase compared to those in the LF group. In addition, the Pro group was significantly lower than that in the HF group. The AP group also tended to decrease compared to the HF group (Fig. 2b). The HbA1c levels in the AP and Pro groups were significantly lower than those in the LF and HF groups. On the other hand, there was no change between the other groups (Fig. 2c).

Fig. 1 Food intake and weight change. Seven-week-old male KKAY mice were fed each diet for 7 weeks: LF, low-fat diet; HF, high-fat diet; AP, high-fat diet + acacia bark extract; Pro, high-fat diet + proanthocyanidin fraction; S, high-fat diet + sugar fraction. The mean food intake (a) and body weight change (b) during the dosing period were measured. The results of the analysis are displayed as mean \pm SD ($n = 8 - 10$)

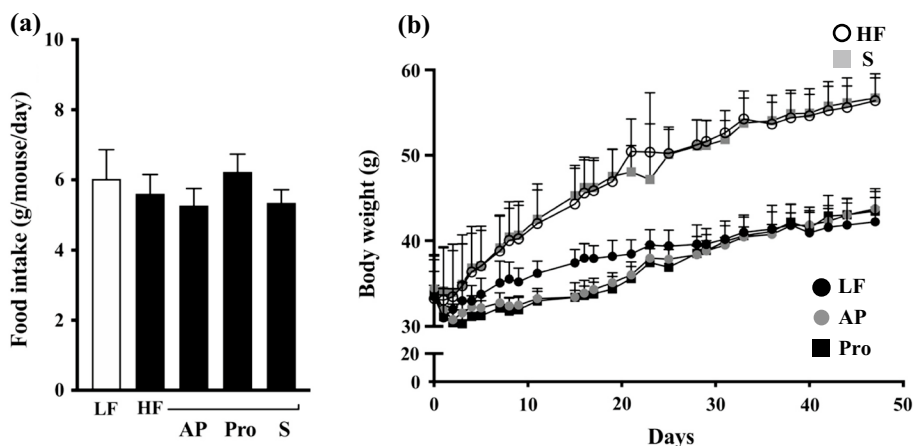
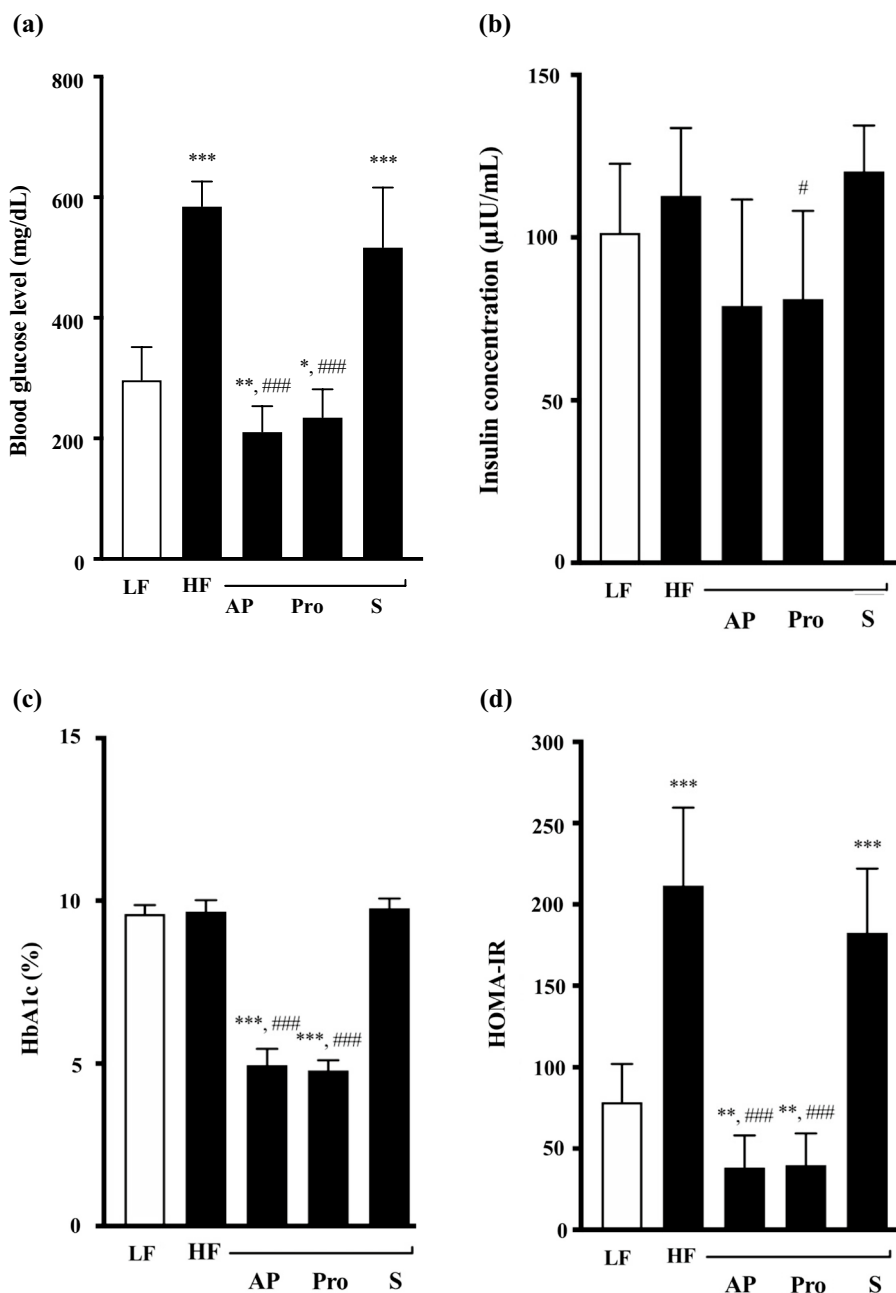


Fig. 2 Evaluation of diabetes after the end of the administration period. After the end of the administration period, the blood glucose level (a), insulin level (b), and HbA1c level (c) of each group were measured. HOMA-IR (d) was calculated from the blood glucose level and the amount of insulin. The results of the analysis are displayed as mean ± SD (*n* = 8~10). (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001 vs. LF, #*p* < 0.05, ##*p* < 0.01 and ###*p* < 0.001 vs. HF)



HOMA-IR was calculated from the fasting blood glucose level and fasting insulin level. It was found that the HOMA-IR of the HF and S groups was significantly higher than that of the LF group. On the other hand, the AP and Pro groups were significantly lower than those in the LF and HF groups (Fig. 2d).

Adipose tissue weight and its morphological changes

We evaluated the degree of obesity in each group using the weight of white adipose tissue (WAT) as an index

(Fig. 3a). In this study, WAT was analyzed separately for the epididymal, retroperitoneum, and kidneys (Fig. 3b-d). It was revealed that the fat weights of the HF and S groups were significantly higher than those of the LF group in all of the total fat weight and each WAT (epididymal, retroperitoneum, and perirenal) (Fig. 3a-d). On the other hand, the fat weights of the AP and Pro groups were not significantly different from those of the LF group at sites other than the retroperitoneum (Fig. 3c).

The fat weights of the AP and Pro groups in the retroperitoneum were significantly higher than those of the LF group, but significantly lower than those of the HF group (Fig. 3c).

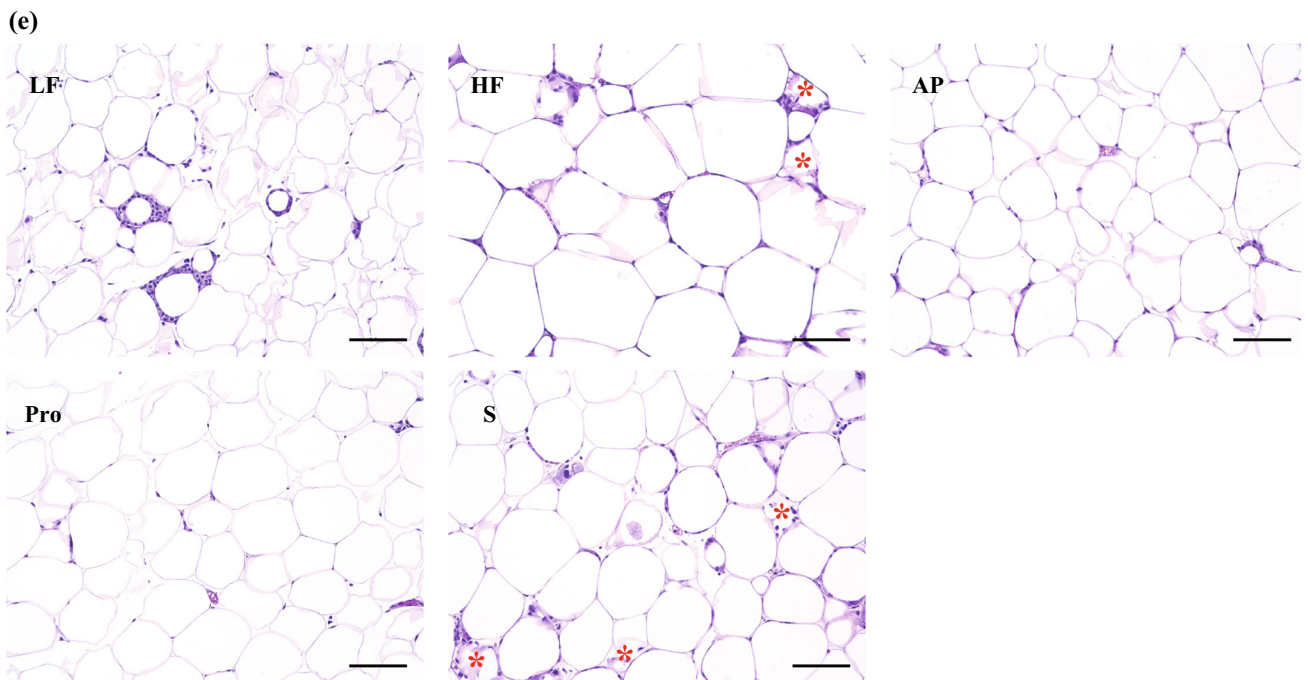
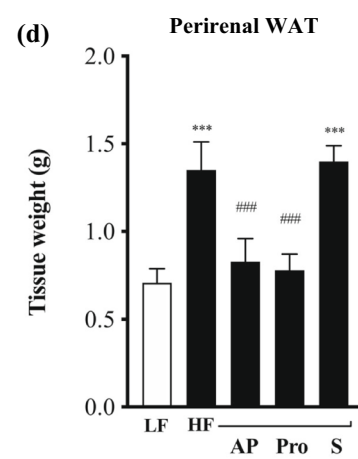
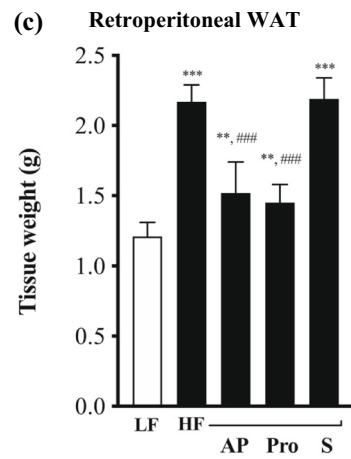
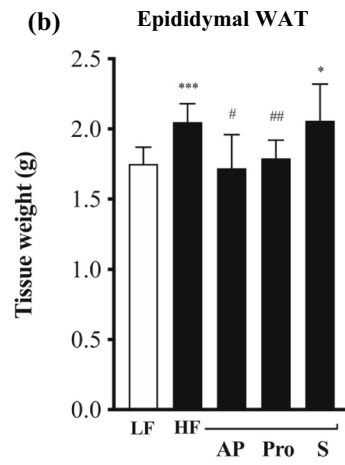
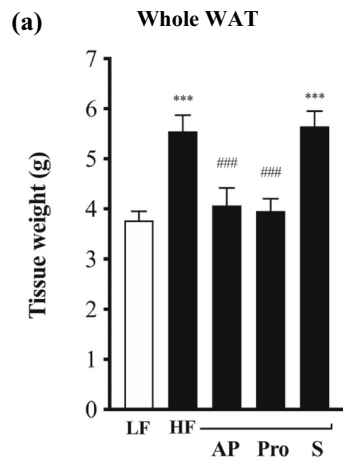


Fig. 3 Weight and morphological evaluation of adipose tissue in each group. After the end of the administration period, the fat weight of each group was measured (**a** total amount, **b** epididymal, **c** retroperitoneum, **d** perirenal). The results of the analysis are displayed as mean \pm SD ($n=8-10$). WAT: white adipose tissue. (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. LF, # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ vs. HF). Each tissue section of the adipose tissue around the excised testis was prepared and stained with HE (**e**). *immature adipocytes

In this study, the morphological changes in the WAT of each test group were analyzed by HE staining. Immature adipocytes transformed into monocellular cells during maturation. In addition, storing new fat leads to the enlargement of the entire adipose tissue. Only unicellular mature adipocytes were found in the LF, AP, and Pro groups. On the other hand, in the HF group and S group, in addition to mature adipocytes, multilocular immature adipocytes were observed (Fig. 3e).

Effect of proanthocyanidins derived from acacia bark on the expression level of each gene in adipose tissue

The accumulation of excess lipids in adipose tissue changes the expression level of cytokines, such as adiponectin and TNF- α in adipose tissue, causing insulin resistance. Therefore, in this study, the expression levels of adiponectin and TNF- α in adipose tissue and the expression level of PPAR γ , which controls the expression of adiponectin, were analyzed by quantitative PCR. We found that the expression levels of adiponectin in the HF and S groups were significantly lower than those in the LF group. On the other hand, no significant difference was observed between the AP and Pro groups compared with the LF group (Fig. 4a). The expression level of TNF- α was significantly lower in the AP and Pro groups than in the HF group (Fig. 4b).

In addition, the expression level of PPAR γ was significantly lower in the HF and S groups than in the LF group. However, the decrease in the expression level of PPAR γ in the AP group was not as significant as the decrease in the HF and S groups. The expression level of PPAR γ in the Pro group was significantly higher than that in the HF group (Fig. 4c).

Effect of proanthocyanidins derived from acacia bark on the liver

As obesity progresses, lipids accumulate in the liver, causing the liver to become enlarged. Therefore, we removed the livers of the mice in each group and weighed them. We calculated the ratio of liver weight to body weight (liver/body weight ratio). As a result, both the liver weight and the liver/body weight ratio were found to be significantly higher

in the HF and S groups than in the LF group. On the other hand, in the AP and Pro groups, the liver weight and liver-to-body weight ratio were significantly lower than those in the LF group (Fig. 5a, b).

Since the progression of fatty liver may cause liver damage, we evaluated the levels of AST and ALT in the blood, which are markers of liver damage. As a result, the HF and S groups were found to have significantly higher activity levels than the LF group. On the other hand, the AP and Pro groups did not change compared with the LF group (Fig. 5c, d).

In this study, the morphological changes in the liver of each group were analyzed by HE staining. Oil Red O staining, in which lipids were stained, was performed in the same manner, and the amount of lipids accumulated in the liver of each group was analyzed. As a result, no fatty liver was observed in the HE staining of the LF, AP, and Pro groups. In addition, the lipids were stained red by Oil Red O staining, but no such findings were observed in the LF, AP, and Pro groups. On the other hand, in the HE staining of the HF and S groups, unstained areas were scattered throughout the tissue. This finding is attributed to the cytoplasm not being stained red by eosin as a result of the accumulation of lipids in the cells. In addition, in the Oil Red O staining of the HF and S groups, many cells were stained red as a result of lipid accumulation in the cytoplasm (Fig. 5e).

Next, we quantified the amounts of triglyceride and cholesterol in the liver. As a result, the amount of triglycerides in the liver of the HF and S groups was found to be significantly higher than that in the LF group. On the other hand, the amount of triglycerides in the liver of the AP and Pro groups was higher than that in the LF group, but the rate of increase was much smaller than that in the HF and S groups (Fig. 5g). Furthermore, the amount of cholesterol in the liver was significantly changed in the S group compared with the LF and HF groups. However, there was no significant change in the AP and Pro groups compared with the LF and HF groups (Fig. 5h).

Effect of proanthocyanidins derived from acacia bark on the expression level of genes involved in the suppression of fat uptake into the liver

In the liver, fatty acid synthase (sterol regulatory element-binding protein (SREBP) -1c, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and genes involved in the degradation and uptake of fatty acid into the liver (PPAR α , the expression levels of PPAR γ and Lipoprotein lipase (LPL)) were analyzed by quantitative PCR. As a result, the expression level of SREBP-1c involved in cholesterol synthesis was significantly different between the AP and the Pro groups compared with the LF group. The expression level of ACC was significantly decreased or tended to decrease in all groups compared with the LF group. In addition, the

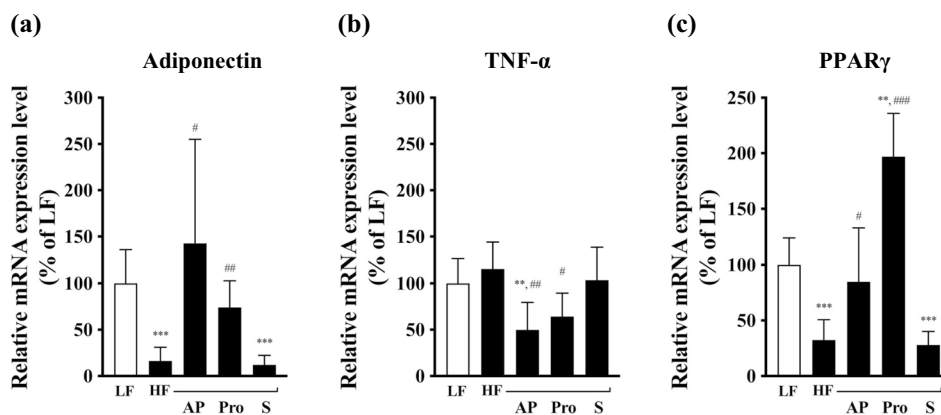


Fig. 4 Effect of proanthocyanidins on the expression level of each gene in adipose tissue. After the end of the administration period, adipose tissue was removed from the testis. Total RNA was prepared from the excised adipose tissue, and the expression level of each

gene (**a** adiponectin; **b** TNF- α ; **c** PPAR γ) was analyzed by quantitative PCR. The results of the analysis are displayed as mean \pm SD ($n = 8 \sim 10$). (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. LF, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. HF)

AP and Pro groups were significantly reduced. FAS was significantly increased in the HF and S groups compared with the LF group. On the other hand, in the AP and Pro groups, it was significantly lower than that in the LF and HF groups (Fig. 6).

The expression level of PPAR α , which is involved in the decomposition of fatty acids, was significantly lower in all groups compared with the LF group. The expression level of PPAR γ involved in the uptake of fatty acids into hepatocytes was significantly increased in the HF group compared with the LF group. On the other hand, there was no change in the AP and S groups compared with the LF group. In addition, it was significantly lower in the Pro group than in the LF group. Furthermore, the expression level of LPL was significantly lower in the Pro group than in the HF group (Fig. 6).

Effect of proanthocyanidins derived from acacia bark on the expression level of genes related to energy consumption in skeletal muscle

In skeletal muscle, the expression level of the GLUT4 gene, which transports *in vivo* molecules and glucose, which are important in consuming energy and producing heat, was analyzed by quantitative PCR.

The expression level of PPAR α did not change significantly in any of the groups. The expression level of PPAR δ was significantly lower in the HF and S groups than in the LF group. On the other hand, there was no change in the AP and Pro groups compared with the LF group. In addition, The expression level of CPT1 was elevated in AP and Pro groups compared to group HF, but there was no significant difference. The expression level of ACO was significantly lower in the HF group than in the LF group. On the other hand, in the AP and Pro groups, there was a

significant increase compared to the LF and HF groups. The expression level of UCP3 was significantly decreased in all groups compared with that in the LF group. However, the decrease in the UCP3 expression level in the AP and Pro groups was smaller than that in the HF and S groups, and was significantly higher than that in the HF group (Fig. 7).

Effect of proanthocyanidins derived from acacia bark on AMPK and DPP-4 activity in blood

AMPK acts as an energy sensor in the body, and its activation leads to energy consumption. Therefore, it has attracted attention as a therapeutic target for diabetes and obesity. Therefore, in this study, we measured the level of AMPK activity in the blood. As a result, the activity of AMPK in the AP and Pro groups was significantly higher than that in the LF and HF groups (Fig. 8).

In addition, dipeptidyl peptidase-IV (DPP-4) degrades GLP-1, a hormone that promotes insulin secretion from pancreatic islet β cells. Therefore, when the activity of DPP-4 increases, GLP-1 is decomposed and the amount of insulin secreted decreases.

Therefore, when the activity of DPP-4 increases, GLP-1 is decomposed and the amount of insulin secreted decreases. As a result, DPP-4 is currently used as a target molecule for diabetes treatment. Therefore, in this study, we evaluated the effect of proanthocyanidins derived from acacia bark on the activity of DPP-4. As a result, the increase in DPP-4 activity observed in the HF group was suppressed by the administration of acacia polyphenols or proanthocyanidins derived from acacia bark (Fig. 8).

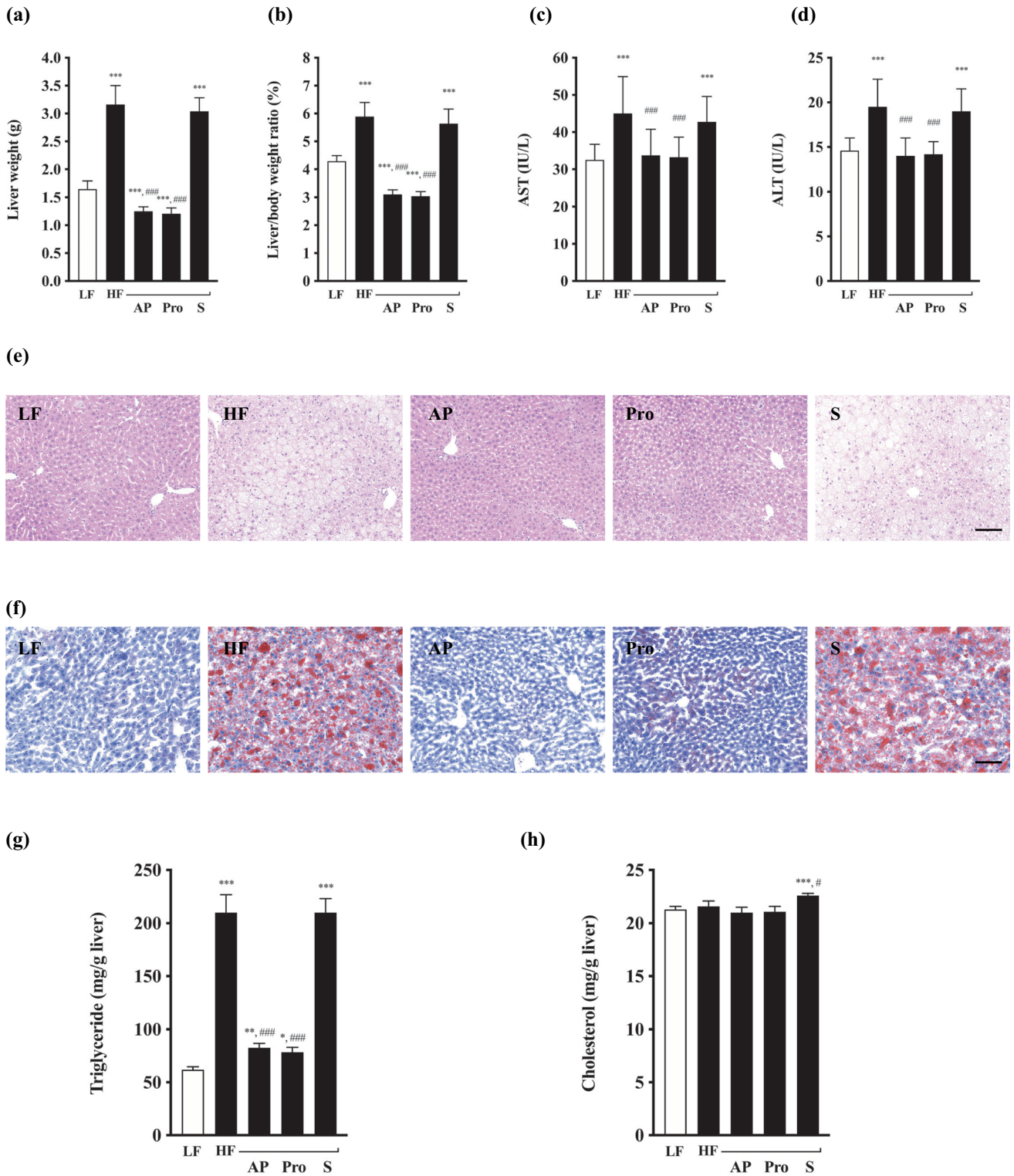
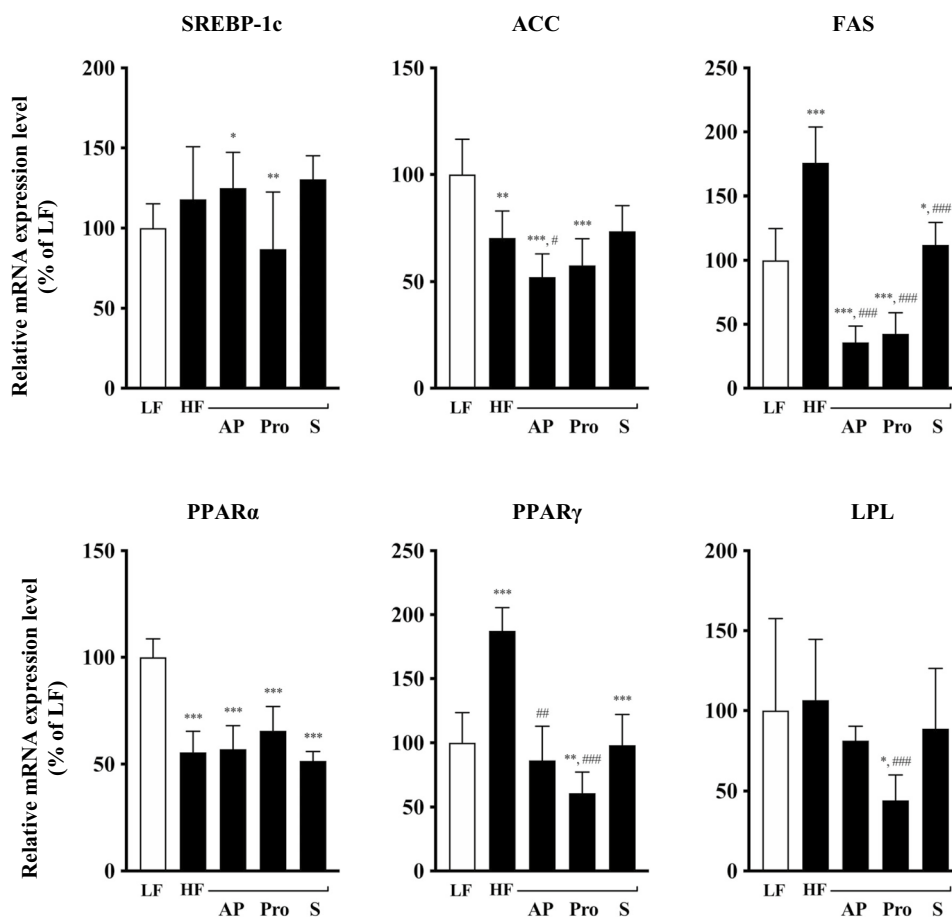


Fig. 5 Effect of proanthocyanidins on the liver. After the end of the administration period, the liver weight was measured and the liver/body weight ratio was calculated (**a** liver weight, **b** liver/body weight ratio). Plasma was prepared from the blood, and the levels of plasma AST and ALT activity were measured (**c** AST, **d** ALT). Tissue sec-

tions of the excised liver were prepared and stained with HE (**e**) and Oil Red O (**f**). The amount of triglyceride (**g**) and cholesterol (**h**) in the liver was quantified. The results of the analysis are displayed as mean ± SD ($n=8-10$). (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. LF, # $p<0.05$ and ### $p<0.001$ vs. HF)

Fig. 6 Effect of proanthocyanidins on the expression level of each gene in the liver. Total RNA was prepared from the livers of mice in each group, and the expression levels of each gene (SREBP-1c, ACC, FAS, PPAR α , PPAR γ , and LPL) were analyzed by quantitative PCR. The results of the analysis are displayed as mean \pm SD ($n = 8 \sim 10$). (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. LF, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. HF)



Discussion

In this study, we investigated the anti-obesity and anti-diabetic effects of proanthocyanidins in acacia bark extract using KKAY mice, which spontaneously develop type 2 diabetes upon obesity via a high-fat diet. The weight of the HF group 4th day after the start of feeding a high-fat diet was significantly higher than that of the LF group (Fig. 1). In addition, the fasting blood glucose level in the HF group increased significantly, rising to approximately twice that of the LF group. The HOMA-IR level, which is an index of insulin resistance, also increased significantly, reaching about threefold that of the LF group (Fig. 2). In this study, the KKAY mice developed severe diabetes with obesity. We used this mouse model to evaluate the anti-obesity and anti-diabetic effects of acacia bark extract (AP group: positive control) [4] and acacia bark-derived proanthocyanidins (Pro group) and acacia bark-derived sugars fraction (S group).

The proanthocyanidins derived from acacia bark were found to have anti-obesity effects, with weight gain and the increase in white adipose tissue weight being significantly suppressed by the administration of acacia bark extract and proanthocyanidins to KKAY mice. On the other hand, the

body weight and white adipose tissue weight of the mice (S group) to which the sugar fraction derived from the acacia bark extract was administered were the same as those of the HF group (Figs. 1 and 3). In addition, we also analyzed the accumulation of fat in the liver. As a result, acacia bark extract and proanthocyanidins were found to significantly suppress the increase in the amount of triglycerides in the liver caused by ingestion of a high-fat diet (Fig. 5). This effect was observed only when acacia bark extract and proanthocyanidins were administered. Furthermore, in the HF group, the liver weight ratio, AST value, and ALT value were significantly increased, indicating liver damage due to fatty liver. On the other hand, in the AP and Pro groups, the liver/weight ratio, AST, and ALT values did not change compared with the LF group. Therefore, acacia bark extract and proanthocyanidins appear to suppress the onset of liver damage resulting from fatty liver (Fig. 5).

As mentioned above, KKAY mice develop severe type 2 diabetes when fed a high-fat diet that results in obesity. In this study, the fasting blood glucose level in the HF group was found to be significantly higher than that in the LF group. On the other hand, the fasting blood glucose levels of the AP and Pro groups were significantly lower than those

Fig. 7 Effect of proanthocyanidins on the expression level of each gene in skeletal muscle. Total RNA was prepared from the skeletal muscle of each group of mice, and the expression level of each gene (PPAR α , PPAR δ , CPT1, ACO, UCP3, and GLUT4) was analyzed by quantitative PCR. The results of the analysis are displayed as mean \pm SD ($n=8 \sim 10$). (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. LF, # $p < 0.05$, ## $p < 0.01$ vs. HF)

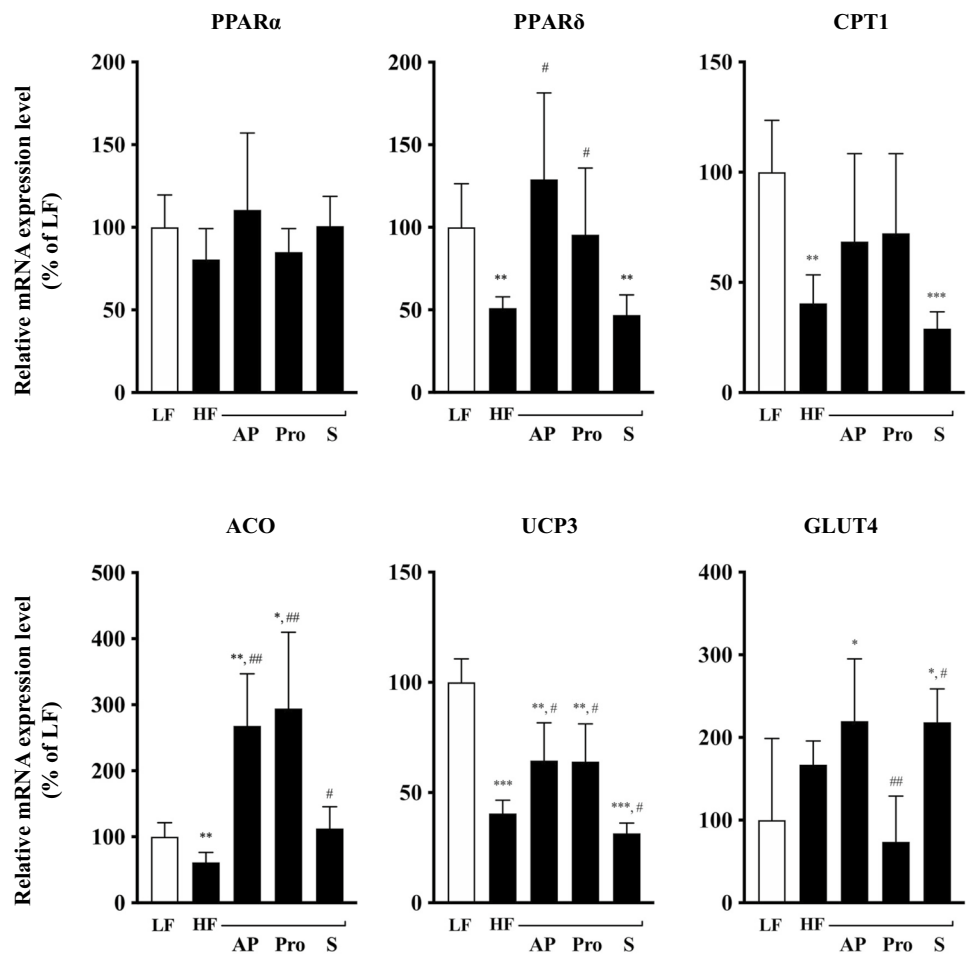
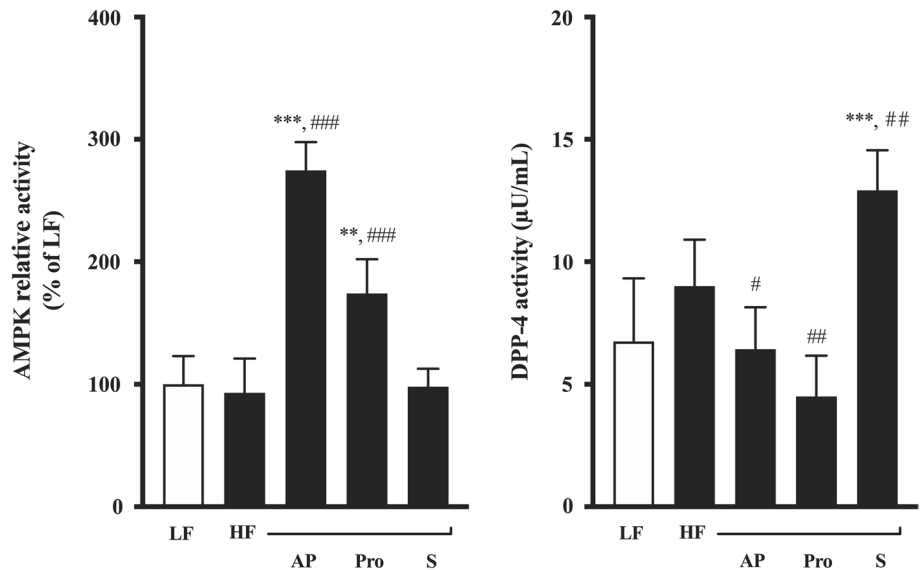


Fig. 8 Effect of proanthocyanidins on blood AMPK and DPP-4 activity. Blood was collected from each group of mice, and plasma AMPK and DPP-4 activity were measured. The results of the analysis are displayed as mean \pm SD ($n=8 \sim 10$). (** $p < 0.01$ and *** $p < 0.001$ vs. LF, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. HF)



in the HF group. Furthermore, the fasting blood glucose levels of the AP and Pro groups were significantly lower than those in the LF group. In addition, the S group showed

a significantly higher value than the LF group. In addition, S group did not change compared to HF. As such, no effect of lowering the fasting blood glucose level was observed

(Fig. 2). From the above, it was considered that the active component that lowered the fasting blood glucose level was proanthocyanidins in the acacia bark extract.

When the HbA1c value was measured, it was found to fall within the normal human range (4.6–6.2%) in the AP and Pro groups, but was significantly higher in the other groups. In humans, HbA1c is used as an index of recent blood glucose levels (past 1–2 months). However, human erythrocytes have a lifespan of approximately 120 days, whereas in mice it can be as short as 20–30 days. Therefore, the HbA1c level in mice is an index of blood glucose level only for the past 1–2 weeks. In this study, it is probable that blood glucose levels were normal in the AP and Pro groups from the 5th week after the start of administration to the end of the study. On the other hand, although the blood glucose level in the LF group was significantly lower than that in the HF group, the HbA1c level was similar. This may be due to the fact that the blood glucose level in the LF group increased temporarily 1–2 weeks before the end of the experiment.

We also calculated HOMA-IR from the fasting blood glucose and fasting insulin levels. HOMA-IR is a simpler marker for measuring insulin resistance, and is commonly used in clinical and animal studies [8, 9]. In the AP and Pro groups, there was a significant decrease compared to the HF group, demonstrating that the acacia bark extract and proanthocyanidins improved insulin resistance (Fig. 2). Insulin resistance is caused by a decrease in adiponectin production and an increase in TNF- α secretion in white adipocytes [10, 11]. Acacia bark extract and proanthocyanidins were found to enhance the production of adiponectin by increasing the expression level of PPAR γ in white adipocytes (Fig. 4). In addition, acacia bark extract and proanthocyanidins were also found to reduce the expression level of TNF- α in white adipose tissue. These results suggest that acacia bark extract and proanthocyanidins improve insulin resistance by increasing adiponectin production and decreasing TNF- α production. Therefore, it is considered that improving insulin resistance led to a decrease in fasting blood glucose level.

We analyzed the anti-obesity and anti-diabetic effects of proanthocyanidins derived from acacia bark on skeletal muscle and the liver. Obesity is generally caused by reduced energy expenditure, increased fatty acid synthesis from carbohydrates, and accumulation of fat in organs. In this context, we analyzed the effect of proanthocyanidins derived from acacia bark on the expression of genes related to energy consumption in skeletal muscle with high-energy consumption. We also analyzed the effect of proanthocyanidins on the expression of genes related to fatty acid synthesis and fat uptake in the liver.

In addition, acacia bark extract and proanthocyanidins derived from acacia bark reduced mRNA expression in FAS, the rate-limiting enzyme for fatty acid synthesis in the liver [12] (Fig. 6). In addition, proanthocyanidins reduced the

mRNA expression of PPAR γ and LPL associated with fat uptake into the liver (Fig. 6). The onset of obesity and fatty liver was suppressed by the decreased expression of FAS, PPAR γ [13, 14], and LPL [15] in the liver.

In skeletal muscle, the administration of acacia bark extract or proanthocyanidins increased the ACO mRNA expression levels compared to the HF group. In addition, the decrease in the mRNA expression levels of CPT1 (β -oxidizing enzyme) and UCP3 (uncoupling protein) due to high-fat diet intake was suppressed (Fig. 7). Furthermore, the administration of acacia bark extract or proanthocyanidins suppressed the decrease in the expression level of PPAR δ mRNA. PPAR δ regulates the expression levels of genes related to energy consumption, such as CPT1, ACO, and UCP3 [16–19]. Therefore, it was suggested that acacia bark extract and proanthocyanidins suppress the decrease in energy expenditure in skeletal muscle due to the decreased expression of PPAR δ and show anti-obesity action.

AMPK regulates glucose and lipid metabolism via the phosphorylation of PPAR and cAMP responsive element-binding protein (CREB) [20, 21]. AMPK inhibits gluconeogenesis by inhibiting several transcription factors, such as hepatocyte nuclear factor 4 (HNF4) and CREB regulated transcription coactivator 2 (CRTC2), that promote the expression of gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [21, 22]. In this study, we investigated the effects of acacia bark extract and proanthocyanidins on AMPK activity. The levels of AMPK activity in the blood were found to be significantly increased after the administration of acacia bark extract or proanthocyanidins compared with mice fed a low-fat or high-fat diet (Fig. 8). From these results, it is inferred that ingestion of AP and Pro suppresses gluconeogenesis by increasing AMPK activity. Therefore, it is considered that decreasing the fasting blood glucose level of AP and Pro groups referable to the suppression of gluconeogenesis by increasing AMPK activity.

Furthermore, Acacia bark extract and proanthocyanidins improved the progression of obesity by inactivating ACC by phosphorylating it through the activation of AMPK and suppressing fatty acid synthesis. In other words, AMPK activation is considered to be one of the mechanisms responsible for the anti-obesity and anti-diabetic effects of acacia bark extract and proanthocyanidins.

DPP-4 inhibitors promote the action of insulin by suppressing the degradation of GLP-1, a hormone that promotes insulin secretion from pancreatic islet β cells. DPP-4 inhibitors have a lower risk of hypoglycemia than SU and glinide drugs. The clinical needs of patients with diabetes are increasing, and foods that exhibit DPP-4 inhibitory activity are considered to be extremely beneficial in terms of their therapeutic efficacy and safety. In this context, in the present study we investigated whether acacia bark extract and proanthocyanidins exert an

inhibitory effect on DPP-4 activity. As a result, acacia bark extract and proanthocyanidins were found to inhibit the activation of DPP-4 (Fig. 8). Taken together, these results suggest that the inhibition of DPP-4 activity is one of the mechanisms responsible for lowering fasting blood glucose by the administration of acacia bark extract and proanthocyanidins.

In conclusion, we attempted to identify the active fractions of the anti-obesity and anti-diabetic effects of acacia bark extract using KKAY mice, as well as their mechanism of action. This study is the first to identify the active fraction of the anti-obesity and diabetic effects of acacia bark extract as proanthocyanidins. In fact, acacia bark-derived proanthocyanidins were demonstrated to lower fasting blood glucose levels through the activation of AMPK and the inhibition of DPP-4 activity. Furthermore, acacia bark-derived proanthocyanidins were also found to increase the expression of adiponectin and decrease TNF- α production, which enhances the action of insulin, by enhancing the expression of PPAR γ in white adipose tissue. Proanthocyanidins were also found to reduce the expression level of FAS, the rate-limiting enzyme for fatty acid synthesis, in the liver. Furthermore, proanthocyanidins lowered the mRNA expression of LPL, which are related to fat intake by the liver (Fig. 6). Reduced expressions of FAS, PPAR γ [19] and LPL [12] in the liver suppress the onset of obesity and fatty liver. In addition, they were found to also suppress the decrease in energy consumption by suppressing the decrease in the expression level of CPT1 and UCP3 in skeletal muscle, even in diabetes with obesity. These findings suggest that acacia bark-derived proanthocyanidins lower the fasting blood glucose levels by exerting these effects on the adipose tissue, skeletal muscle and liver.

The findings present in this study provide new scientific evidence on the anti-obesity and anti-diabetic effects of acacia bark extract. From the above results, it was clarified that proanthocyanidins are active component. Thus, due to its anti-obesity and anti-diabetic effects, acacia bark extract is expected to have an even higher commercial value as a health supplement in the near future.

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Declarations

Conflict of interest Financial interests: W. Ochiai has received research support from Company Acacia-No-Ki Co., Ltd.

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