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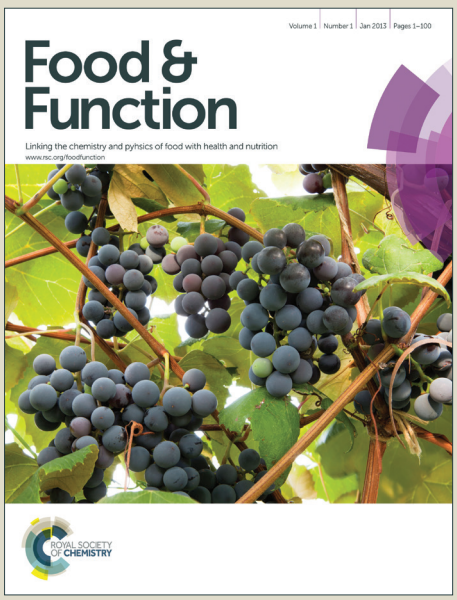

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Ashitaba (*Angelica keiskei*) extract prevent adiposity in high-fat diet-fed C57BL/6 mice.

Tianshun Zhang[1], Yoko Yamashita[1], Michiko Yasuda[2], Norio Yamamoto[3], Hitoshi Ashida[1]*

[1]*Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan*

[2]*Organization of Advanced Science and Technology, Kobe University, Kobe 657-8501, Japan*

[3]*Food Science Research Center, House Wellness Foods Corporation, Imoji 3-20, Itami, Hyogo 664-0011, Japan*

*Corresponding author: Hitoshi Ashida, Ph.D.

Address: Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, Nada-ku, Kobe 657-8501, Japan

E-mail: ashida@kobe-u.ac.jp Tel and Fax: +81-78-803-5878

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Abbreviations used: ACC, acetyl CoA carboxylase; ACOX1, acy-CoA carboxylase X1; AMPK, AMP-activated protein kinase; C/EBP, CCAAT/enhancer-binding protein; CPT-1A, carnitine palmitoyltransferase-1A; FAS, fatty acid synthase; 4HD, 4-hydroxyderricin; HF, high-fat; PPAR, peroxisome proliferator-activated receptor; SREBP1, sterol regulatory element binding protein 1; UCP-2, uncoupling protein-2; XAG, xanthoangelol

ABSTRACT

Two main chalcones, 4-hydroxyderricin and xanthoangelol, from ashitaba, a food ingredient and folk medicine in Asia, have been demonstrated to modulate lipid metabolism in 3T3-L1 and HepG2 cells. In this study, we investigated the effects of ashitaba extract on adiposity in mice fed a high-fat (HF) diet and its underlying mechanisms based on adipose tissue and hepatic lipid metabolism. C57BL/6 mice were fed a normal or HF diet supplemented with ashitaba extract (0.01 % and 0.1 %, *w/w*) for 16 weeks. Ashitaba extract: suppressed HF diet-induced body weight gain and fat deposition in white adipose tissue; reduced plasma cholesterol, glucose, and insulin levels; increased adiponectin level; lowered triglyceride and liver cholesterol content; increased phosphorylation of AMP-activated protein kinase (AMPK) in adipose tissue and liver; inhibited lipogenesis in adipose tissue by down-expression of peroxisome proliferator-activated receptor (PPAR) γ, CCAAT/ enhancer-binding protein α and sterol regulatory element-binding protein 1 (SREBP1); inhibited lipogenesis in the liver by down-expression of SREBP1 and its target enzyme fatty acid synthase; and promoted fatty acid oxidation by up-expression of carnitine palmitoyltransferase-1A and PPARα. In conclusion, ashitaba extract can possibly prevent adiposity through modulating lipid metabolism though phosphorylation of AMPK in adipose tissue and liver.

INTRODUCTION

Ashitaba, a Japanese herb, is drunk as a tea and used as a vegetable as well as the folk medicine for diuretic, laxative, analeptic and galactagogue. Several attractive compounds have been identified from this plant for health promoting effects including coumarins, flavanones and chalcones.[1] Among them, 4-hydroxyderricin (4HD) and xanthoangelol (XAG) are considered to be the major active compounds for various biofunctions including anti-tumor,[2,3] anti-inflammatory[4] and anti-diabetes[5,6] activities. Our previous report[7] demonstrated that 4HD and XAG inhibited the differentiation of preadipocytes into adipocytes via down-regulating expression of C/EBPβ, C/EBPα and PPARγ involving in the activation of AMPK signaling pathway. Moreover, we also found that 4HD and XAG prevented free fatty acids-induced impairment of lipid metabolism though the activation of liver kinase B1/AMPK pathway in HepG2 cells.[8] However, the *in vivo* effect of Ashitaba extract on the HF diet-induced adiposity is still unclear.

Recently, the rates of obesity have increased dramatically.[9] Obesity usually results from an energy imbalance:[10] Excessive energy storage and insufficient energy expenditure induced lipid accumulation in both adipose tissue and liver leading to type II diabetes, cardiovascular diseases, no-alcoholic fatty liver disease and other metabolic disorders.[9,11,12] Dietary fat is considered to be one of the most important factors in the pathophysiology of obesity. C57BL/6 mice are obesity-prone strain and develop obesity, hyperglycemia, and hyperlipidemia when feed a high-fat (HF) diet. Thus, C57BL/6 mice are commonly used for research on obesity and obesity-related diseases.[13,14]

Lipid accumulation and energy metabolism are tightly controlled in the adipose tissue and liver. AMP-activated protein kinase (AMPK) is a key modulator to maintain the cellular as well as whole-body energy balance. There increased an interest in developing AMPK activators as potential therapies for prevention of amelioration of obesity, diabetes and hepatic

steatosis[15,16] The activation of AMPK interacted with sterol regulatory element binding protein 1 (SREBP1) and inhibited target molecules for SREBP1 including fatty acid synthase (FAS) expression, leading to reduced lipogenesis and lipid accumulation.[17,18] Moreover, the activation of AMPK also leads to phosphorylation of acetyl CoA carboxylase (ACC) and inhibits its activity.[19] Inactivation of ACC reduces the synthesis of malonyl-CoA, which in turn activates carnitine palmitoyltransferase-1A (CPT-1A) and increases fatty acid oxidation.[20] In addition, AMPK also increases fatty acid oxidation by up-expression of peroxisome proliferator-activated receptor (PPAR) α.[21] It is also reported that the activation of AMPK inhibits differentiation of adipocytes through down- expression of CCAAT/enhancer-binding protein (C/EBP) α and PPARγ.[22]

In this study, Ashitaba extract was given to C57BL/6 mice fed the control or HF diet for 16 weeks to examine whether the extract prevent HF diet caused adiposity. We further clarified the underlying molecular mechanisms based on lipid metabolism in the adipose tissue and liver.

MATERIAL AND METHODS

Materials

Ashitaba extract was prepared from Ashitaba Chalcone Powder supplied by Japan Bio Science Laboratory (Osaka, Japan) as previously described.[6] The powder (10 g) was extracted with ethyl acetate (100 mL × 3 times) at room temperature, and obtained extract was dried *in vacuo* (The yield was 17%). Antibodies for PPARγ, C/EBPα, PPARα, ACC horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG and anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), β-actin, p-AMPK, AMPK and p-ACC were from Cell Signaling Technology (Beverly, MA), SREBP-1 CPT-1A and acy-CoA carboxylase X1 (ACOX1) were from Abcam (Cambrige, MA) and uncoupling

protein-2 (UCP-2) was from BioLegend Inc. (San Diego, CA). All other reagents used were of the highest grade available from the commercial sources.

Measurement of 4HD and XAG in Ashitaba extract by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Detection and quantification of 4HD and XAG were performed with LC-MS/MS (4000 Q TRAP, AB Sciex, Foster City, CA, USA) using electrospray ionization. Chalcone was used as an internal standard compound. HPLC separation was done with a gradient system using solvent A (0.1% formic acid) and solvent B (acetonitrile) equipped with a L-column-2 ODS (2.1×150 mm) column (Chemicals Evaluation and Research Institute, Tokyo, Japan) at a flow rate of 0.2 mL/min. The column oven was maintained at 40 °C. The gradient program was: 0-2 min, 45% A; 2-7 min, linear gradient to 0% A; 7-8 min, 0% A hold; 8-8.1 min, linear gradient to 45% A; and 8.1-15 min, 45% A hold. The chalcones were detected by multiple reaction monitoring as follows: 4HD 339.2/163.1 $[M + H]^+$, XAG 393.2/131.0 $[M + H]^+$, chalcone 209.1/131.0 $[M + H]^+$. For quantification, standard curves of Ashitaba chalcones from 0.05 to 500 fmol/5 μL injection were generated as described in the previously study.[23] Concentrations of 4HD and XAG were corrected by the comparison between a peak area of these compounds and that of the internal standard.

Animal treatment

All animal experiments were approved by the Institutional Animal Care and Use Committee (Permission #25-04-02) and were carried out according to the guidelines for Animal Experiments at Kobe University. Male C57BL/6 mice (5 weeks old, n=36) were obtained from Japan SLC (Shizuoka, Japan) and maintained in a temperature-controlled room (22°C). The mice had free access to tap water and an AIM-93M laboratory purified diet

(Oriental Yeast, Tokyo, Japan), and were acclimatized for 7 days before the experiments. They were then randomly divided into six groups of six mice each and fed a control (AIN-93M) or HF diet containing 30% (w/w) lard for 16 weeks. The compositions of the diets and energy densities are shown in Table 1. The diets were supplemented with 0% (C-0 and HF-0 groups), 0.01% (C-0.01 and HF-0.01 groups) or 0.1% (C-0.1 and HF-0.1 groups) Ashitaba extract. Food and water intake were measured, and the diets replaced every 2 days. Body weight was measured weekly. After 16 weeks of feeding, the mice were fasted for 18 h and sacrificed under anesthesia with sodium pentobarbital. Blood was collected from cardiac puncture using a heparinized syringe. The liver, white adipose tissues (subcutaneous, epididymal, mesenteric and retroperitoneal adipose tissues) and brown adipose tissue were also collected. Tissue samples were washed with 1.15% (w/v) KCl, weighed, immediately frozen using liquid nitrogen, and kept at −80°C until use.

Measurement of plasma parameters related to lipid and glucose metabolism

Plasma triglyceride, total cholesterol, non-esterified fatty acid (NEFA) and glucose levels were measured using corresponding commercial assay kit according to the manufacture's instruction (Triglyceride-E test, Cholesterol-E test, NEFA-C test, and Glucose CII-test, respectively, Wako Pure Chemical). Plasma insulin and adiponectin levels were measured by the commercial ELISA assay kits according to the manufacturer's instructions (mouse insulin ELISA kit and mouse/rat adiponectin ELISA kit, Shibayagi, Shibukawa, Japan). The index of the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from the values of the plasma glucose and insulin levels according to the following formula:[24]

HOMA-IR = fasting glucose (mg per 100 mL) × fasting insulin (μU per mL)/405.

Measurement of hepatic lipid levels

An aliquot of 100 mg of liver was homogenized with 0.35 mL of distilled water, and the homogenate was extracted three times with 0.7 mL of chloroform–methanol (2/1, *v/v*) mixture. The chloroform layer was collected after centrifugation at 1800 × *g* for 10 min, and washed with a 1/4 volume of 0.88% (*w/v*) KCl. The obtained chloroform layer was dried *in vacuo*, and measured the weight of the residue as total lipids. After the residue was dissolved in isopropanol containing 10% (*v/v*) Triton-X, triglyceride and cholesterol levels were measured using respective commercial kit as described above.

Western blotting

Preparation of the cell lysate was performed according to the previous study.[25] Proteins in the cell lysate of the adipose tissue and liver were separated by the SDS-polyacrylamide gels and transferred to the polyvinylidene difluoride membranes. After blocking with commercial Blocking One solution (Nacalai Tesque), The membranes were incubated with primary antibodies for PPARγ (1:20000), C/EBPα (1:10000), SREBP-1 (1:10000), PPARα (1:20000), CPT-1A (1:5000), ACOX1 (1:20000), UCP-2 (1:20000), β-actin (1:20000), AMPK (1:10000), p-AMPK 1:5000), ACC (1:10000) or p-ACC (1:5000) overnight at 4 °C, followed by the corresponding HRP-conjugated secondary antibody (1:50000) for 1 h at room temperature. Specific immune complexes were detected with the ATTO Light-Capture II Western Blotting Detection System. The density of specific bands was calculated using the ImageJ image analysis software.

Statistical analysis

Data are represented as the means ± SD (n=6). The statistical significance of experimental observations was determined using the Dunnett's test (Fig.1) or the Tukey-Kramer multiple comparison test (other Tables and Figs.). The level of significance

was set at $p < 0.05$.

RESULTS

Effect of Ashitaba extract on body weight and adipose tissue weight

Ashitaba extract contained 64.89 and 84.86 mg/g of 4HD and XAG, respectively, determined by LC-MS/MS analysis. During the feeding period, body weight of mice was significantly lowered in the HF-0.01 and HF-0.1 groups compared with the HF-0 group from week 10 to 16, respectively (Fig. 1). At the end of experiment (week 16), we confirmed that the body weight of the mice was significantly higher in the HF-0 group than that in C-0 group, and that in the HF-0.01 and HF-0.1 groups significantly lowered compared with the HF-0 group (Table 2). The weight of white adipose tissues (epididymal, mesenteric, retroperitoneal, and subcutaneous adipose tissue) was greater in the HF-0 group than that in C-0 group. Supplementation of Ashitaba extract suppressed HF-increased adipose tissue weight compared with the HF-0 group. In the groups given the control diet, Ashitaba extract neither affect body weight nor adipose tissue weights (Table 2). Ashitaba extract has the potential to reduce body weight and white adipose weight gain induced by the HF diet.

Effects of Ashitaba extract on lipid metabolism

Total plasma cholesterol level was significantly increased in the HF-0 group compared with the C-0 group. HF-0.01 and -0.1 groups significantly lowered plasma cholesterol level to almost the same level as the control diet-fed groups. On the other hand, Ashitaba extract did not affect plasma triglyceride and NEFA levels in both the control and HF diet-fed groups (Fig. 2). Intake of a HF diet is reported to induce hepatic lipid accumulation, which are involved in systemic insulin resistance.[26,27] As shown in Fig. 3, the hepatic total lipids, triglyceride and cholesterol levels in HF-0 group were significantly higher than those in the

C-0 group. The hepatic total lipid, triglyceride and cholesterol levels were significantly lower in HF-0.01 and HF-0.1 groups than those in the HF-0 group. In the control-diet fed-mice, Ashitaba extract did not affect hepatic total lipid triglyceride and cholesterol levels. These results indicate that Ashitaba extract has the ability to prevent HF diet-induced lipid accumulation in the liver.

Effect of Ashitaba extract on plasma glucose, insulin and adiponectin levels.

The plasma glucose level at the end of the experiment was significantly higher in the HF-0 group compared with the C-0 group (Fig. 4A). Supplementation of Ashitaba extract to the HF diet significantly reduced the plasma glucose levels. The plasma insulin level in the HF-0 group was also higher than that in the C-0 group, and supplementation of Ashitaba extract also reduced the HF-diet increased insulin level to almost the same level as that in the control-diet fed groups (Fig. 4B). Neither glucose nor insulin levels changed among the control diet groups. HOMA-IR, predictor of total insulin sensitivity, was significantly higher in the HF-0 group than that in the C-0 group (Fig. 4C). Supplementation of the HF diet with Ashitaba extract significantly attenuated the HF diet-induced increase in HOMA-IR.

White adipose tissue is a major endocrine tissue that releases various adipocytokines into the bloodstream. Because adiponectin is one of the major adipocytokines associated with maintaining glucose homeostasis,[28] we measured the plasma level of adiponectin (Fig. 4D). The plasma adiponectin level was lower in the HF-0 group than that in control-diet fed-groups. Ashitaba extract helped to retain the decreased adiponectin level in the HF diet groups in a dose-dependent manner: HF-0.1 group revealed significantly higher adiponectin level compared with HF-0 group with almost the same level to the control diet groups.

Effect of Ashitaba extract on expression of adipogenesis- and energy metabolism-related

proteins in the adipose tissue

AMP-activated protein kinase (AMPK) is a key modulator to maintain the cellular as well as whole-body energy balance.[15] It has been known that HF diet suppresses AMPK activation.[29,30] We found Ashitaba extract supplementation restored AMPK phosphorylation in HF-diet fed-mice (Fig. 5). Since PPARγ, C/EBPα, and SREBP-1 are associated with adipogenesis,[31] the expression of these proteins levels in white adipose tissue were determined in Fig. 5, the expression of PPARγ, C/EBPα and SREBP-1 were significantly increased in HF-0 group compared with those in the control diet groups. In the HF diet group, supplementation with ashitaba extract significantly decreased the expression of these adipogenesis-related proteins. In the groups given the control diet, Ashitaba extract did not affect the expression of these proteins. UCP-2, a member of UCP family, is involved in energy metabolism in adipose tissue and liver.[32,33] However, Ashidaba extract did not affect the expression UCP-2 in both control diet and HF diet groups.

Effect of Ashitaba extract on expression of adipogenesis- and energy metabolism-related proteins in the liver

To investigate whether the reduction of fat mass in Ashitaba extract-dosed mice is accompanied by modulation of AMPK activation and lipid metabolism in the liver, western blotting was performed. As shown in Fig. 6, supplementation of Ashitaba extract in HF diet groups increased in phosphorylation of AMPK and ACC compared with HF-0 group. HF diet significantly increased the expression of SREBP-1 and FAS and supplementation of Ashitaba extract significantly decreased HF diet-induced expression of these proteins, though their expressions in the control-diet fed-mice were remained unchanged. Then, we investigated the expression of protein responsible for fatty acids oxidation and thermogenesis in the liver. HF diet tended to decrease the expression of CPT1, ACOX1 and PPARα and supplementation of

Ashitaba extract significantly increased HF diet-depressed expression of these proteins. Ashitaba extract did not alter the expression of these proteins in the control diet groups. Similar to the adipose tissue, Ashitaba extract did not affect UCP-2 expression in the liver.

DISCUSSION

In this study Ashitaba extract lowered HF diet-induced body weight and body fat (Table 2 and Fig. 1), accompanied by the prevention of hyperglycemia and hyperlipidemia effects, as estimated by reducing serum levels of cholesterol, glucose, insulin and enhancing adiponectin (Figs. 2 and 4). Ashitaba extract decreased the hepatic contents of triglyceride and cholesterol, thereby protected the liver from HF diet-induced dysfunctions (Fig. 3). It was noteworthy that Ashitaba extract restored HF diet-induced inactivation of AMPK in both adipose tissue and liver (Figs. 5 and 6). As the downstream events: Ashitaba extract decreased expression of PPARγ, C/EBPα and SREBP-1, which were involved in adipogenesis in the adipose tissue (Fig. 5); and the extract also decreased the expression of SREBP-1 and FAS, while increased the hepatic expression of CPT-1, ACOX1 and PPARα (Fig. 6).

AMPK is a key modulator to maintain the cellular as well as whole-body energy balance. AMPK is activated in response to an increase in the AMP:ATP ratio within the cell and therefore acts as a sensor for cellular energy regulation. Binding of AMP with AMPK allosterically phosphorylates and activates AMPK,[34] which in turn shuts down anabolic pathways and supports catabolic pathways through regulating the expression of several proteins of energy metabolism. The activation of AMPK interacted with SREBP-1 and inhibited SREBP-1 target including FAS expression, leading to reduced lipogenesis and lipid accumulation.[17,18] Moreover, the activation of AMPK also leads to the phosphorylation and inhibition of ACC activity, resulting in an increase of fatty acids oxidation:[19] i.e., Inactivation of ACC reduces the synthesis of malonyl-CoA, which leads to derepression of CPT-1A and

ACOX1, and activates fatty acid oxidation.[20] In addition, AMPK also stimulates fatty acids oxidation by up-regulating expression of PPARα.[21] The activation of AMPK inhibits the differentiation of adipocytes by down-regulating the expression of C/EBPα and PPARγ.[22] It has been known that HF diet suppresses AMPK activation.[29,30] Noticeably, Ashitaba extract treatment restored AMPK phosphorylation in HF-diet fed-mice in both adipose and liver tissues (Fig. 5 and 6). These results indicated that Ashitaba extract regulated lipid metabolism in adipose and liver through the activation of AMPK.

Ashitaba extract significantly suppressed lipid accumulation in the white adipose tissue, including visceral adipose tissue (Table 2). Furthermore, we found that Ashitaba extract significantly decreased the expression of C/EBPα, PPARγ and SREBP-1 in HF-diet fed-mice (Fig. 5). PPARγ and C/EBPα are considered to be the master regulators or the crucial determinants of adipocyte fate and play an important role in adipogenesis.[31,35] For instance, immortalized fibroblasts lacking PPARγ lose the potential for differentiation to mature adipocytes.[36] C/EBPα functions were revealed to be a principal player in adipogenesis from gain-of-function studies in cultured cells[37] as well as studies establishing appropriate knockout mice-whole-body C/EBPα-knock mice, which die shortly after birth owing to liver defects and hypoglycemia because they fail to accumulate lipid in the white or brown adipocytes.[38] SREBP-1 regulates lipid metabolism and plays an essential role in the regulation of lipogenesis in fatty acids and triglyceride synthesis.[17] Our previous report showed that 4HD and XAG, two main chalcones of Ashitaba, inhibited adipocytes differentiation by down-regulating C/EBPs and PPARγ expression.[7] The present results indicate that Ashitaba extract suppresses the lipid accumulation in the white adipose tissue by decreasing adipocyte differentiation and lipogenesis.

Ashitaba extract normalized hepatic lipid content in the HF-diet groups (Fig. 3), Furthermore, we found that Ashitaba extract not only significantly decreased SREBP-1 and

FAS expression, but also increased CPT-1A, ACOX1 and PPARα expression in the liver of HF-diet treated mice (Fig. 6). Lipid accumulation in the liver is caused by enhancing *de novo* lipogenesis, and lowing of lipid catabolism. It is known that 25% of liver triglyceride is derived from increased *de novo* lipogenesis.[39] *De novo* lipogenesis is mediated by SREBP-1 that is a key lipogenic transcription factor and nutritionally regulated by glucose and insulin.[40,41] SREBP-1 preferentially regulates the lipogenic process by activating genes including FAS, involved in fatty acids and triglyceride synthesis, which contribute to hepatic steatosis. CPT-1, ACOX1 and PPARα were critically associated with the process of fatty acids oxidation. [42-45] CPT-1 regulates the transport of fatty acids from the cytoplasm to the mitochondrial matrix across the membrane,[42] while ACOX1 is the initial enzyme in the peroxisomal β-oxidation system.[43,44] PPARα-mediated responses have been well studied in the liver. It has been reported PPARα agonist normalized fatty liver in fat-fed rats[45] and markedly improved lipid accumulation in the liver of rats.[46] Our previous report showed that 4HD and XAG inhibited the fatty acids-mixture induced lipid accumulation by down-regulating SREBP-1 and up-regulating PPARα expression.[8] These results indicated that Ashitaba extract modulated lipid metabolism in the liver by decreasing lipogenesis and increasing fatty acids oxidation.

Visceral adipose tissue is an important predictor of insulin resistance, hyperglycemia and other metabolic risk factors.[12,47] Increased adipose tissue weight is accompanied by the induction of inflammatory cytokines involved in insulin resistance.[48,49] In addition, visceral adipose tissue has been correlated with intrahepatic triglyceride content, and an increase in intrahepatic triglycerides is associated with the metabolic abnormalities.[26,27,50] The inhibition of fat accumulation in the white adipose tissue and liver by Ashitaba extract may also contribute to its prevention of hyperglycemia and insulin resistance. We found that Ashitaba extract decreased plasma glucose and insulin levels and increasing adiponectin level (Fig. 4).

Adiponectin is one of the major adipocytokines associated with maintaining glucose homeostasis.[28] Previous study showed that Ashitaba extract suppressed acute hyperglycemia in oral glucose tolerance test of mice, and 4HD and XAG, major polyphenols in Ashitaba extract stimulate glucose uptake in skeletal muscles cells.[6] These results indicate that Ashidaba extract should be effective material for inhibition of hyperglycemia and insulin resistance.

4HD and XAG are considered to be the major polyphenols in Ashitaba extract. LC-MS/MS results also showed that Ashitaba Chalcone Powder contained 64.89 and 84.86 mg/g of 4HD and XAG. These two compounds modulate lipid metabolism in 3T3-L1 and HepG2 cells.[7,8] These results indicate that 4HD and XAG should be the effective compounds for modulating lipid metabolism. The preventive effects of other compounds in Ashitaba extract are negligible. It has been reported that Ashitaba contains other chalcones, coumarins and flavanones.[51] Among them, pteryxin also showed anti-obesity activity.[52] However, the preventive effects of other compounds in Ashitaba on adiposity are still not clear. It is needed to clarify this issue in the future study.

CONCLUSION

We found that administration of Ashitaba extract reduced HF diet-induced adiposity, because the extract lowered body weight gain, serum levels of cholesterol, glucose, insulin and enhanced the level of adiponectin, and inhibiting deposition of lipid in both adipose tissue and liver. These effects are mainly regulated by the activation of AMPK. Together, these findings show that Ashitaba extract should be of benefit to improve HF diet-induced adiposity.

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437 **Figure legends**

438 Fig. 1. Changes in body weight of mice fed the control- and HF-diets containing Ashitaba
439 extract for 16 weeks. Open symbols represent control diet-fed groups, while closed symbols
440 HF diet-fed groups. Values are the mean ± SD (n=6). *p<0.05 when compared with 0%AE,
441 (Dunnett's test).

442

443 Fig. 2. Effect of Ashitaba extract on the levels of plasma lipid levels. Mice were fed the
444 control- or HF-diet containing Ashitaba extract for 16 weeks. At the end of the experiment,
445 the plasma levels of cholesterol (A), triglyceride (B) and NEFA (C) levels were measured.
446 Values are the mean ± SD (n=6). The same letters represent no significant differences
447 according to the Tukey-Kramer multiple comparison test. p< 0.05 was considered significant.

448

449 Fig. 3. Effects of Ashitaba extract on the hepatic lipid levels. Mice were fed the control- or
450 HF-diet containing Ashitaba extract for 16 weeks. At the end of the experiment, total lipid (A),
451 cholesterol (B) and triglyceride (C) levels were measured. Values are the mean ± SD (n=6).
452 The same letters represent no significant differences according to the Tukey-Kramer multiple
453 comparison test. p < 0.05 was considered significant.

454

455 Fig. 4. Effects of Ashitaba extract on plasma glucose, insulin and adiponecitn levels. Mice
456 were fed the control- or HF-diet containing Ashitaba extract for 16 weeks. At the end of the
457 experiment, the glucose (A) and insulin (B) levels were measured and the homeostasis model
458 assessment of insulin resistance index (HOMA-IR) was calculated (C). The plasma
459 adiponectin was also measured (D). Values are the mean ± SD (n=6). The same letters
460 represent no significant differences according to the Tukey-Kramer multiple comparison test.
461 p< 0.05 was considered significant.

462

463 Fig. 5. Effects of Ashitaba extract on expression of adipogenesis- and energy
464 metabolism-related proteins in adipose tissue. Mice were fed the control- or HF-diet
465 containing Ashitaba extract for 16 weeks. The expression of p-AMPK, AMPK, C/EBPα,
466 PPARγ, SREBP-1 and UCP2 in white adipose tissue was evaluated by western blotting.
467 Densitometric analysis of specific bands for p-AMPK was also shown after normalization by
468 AMPK expression, C/EBPα, PPARγ, SREBP-1 and UCP-2 was shown after normalization by
469 β-actin expression. Values are the mean ± SD (n=6). The same letters represent no significant
470 differences according to the Tukey-Kramer multiple comparison test. $p< 0.05$ was considered
471 significant.

472

473 Fig. 6. Effects of Ashitaba extract on expression of adipogenesis- and energy
474 metabolism-related proteins in liver tissue. Mice were fed the control- or HF-diet containing
475 Ashitaba extract for 16 weeks. The expression of p-AMPK, AMPK, p-ACC, ACC, SREBP-1,
476 FAS, CPT-1A, ACOX1, PPARα and UCP-2 in the liver tissue was evaluated by western
477 blotting. Densitometric analysis of specific bands for p-AMPK, p-ACC was shown after
478 normalization by AMPK and ACC expression, respectively. SREBP-1, FAS, CPT-1A,
479 ACOX1, PPARα and UCP-2 were also shown after normalization by β-actin expression.
480 Values are the mean ± SD (n=6). The same letters represent no significant differences
481 according to the Tukey-Kramer multiple comparison test. $p< 0.05$ was considered significant.

482

Table 1. Composition of the control- and HF-diets

	Control	HF
Ingredients	(g/100 g diet)	
Casein	14	14
L-Cystin	0.2	0.2
Cornstarch	46.6	16.6
Dextrin	15.5	15.5
Sucrose	10	10
Soybean oil	4	4
Cellulose	5	5
Mineral mixture	3.5	3.5
Vitamin mixture	1	1
Choline bitartate	0.3	0.3
Tertiary butyl hydroxyl quinone	0.0008	0.0014
Lard	0	30
	(kcal/100 g diet)	
Energy density	348	518

488 Table 2. Effects of Ashitaba extract on body weight, and adipose tissue weights of mice fed control- and HF-diet for 16 weeks

	Group					
	Control			HF		
Ashitaba extract(%)	0	0.01	0.1	0	0.01	0.1
Final body weight (g)	30.8±2.2[a]	32.8±1.1[ab]	29.2±2.21[a]	35.9±3.34[b]	31.5±2.11[a]	31.5±1.64[a]
Tissue weight (g per 100 g body weight)						
Liver	3.79±0.36[a]	3.71±0.33[a]	3.88±0.47[a]	3.74±0.27[a]	3.91±0.32[a]	3.62±0.56[a]
White adipose tissue weight						
Total	10.91±4.06[a]	13.98±1.18[a]	9.75±2.60[a]	19.90±4.79[b]	11.99±2.8[a]	12.25±1.48[a]
Epididymal	3.22±0.65[a]	4.31±0.49[ab]	2.94±0.74[a]	5.50±1.26[b]	3.60±1.26[a]	3.85±0.56[a]
Mesenteric	1.47±0.45[ab]	2.10±0.32[ab]	1.36±0.24[ac]	2.40±0.30[b]	1.71±1.03[ab]	1.31±0.21[ac]
Retroperitoneal	1.62±0.61[a]	2.22±0.32[ab]	1.59±0.42[a]	3.10±1.00[b]	1.72±0.69[a]	2.10±0.73[ab]
Subcutaneous	4.59±2.47[a]	5.53±0.53[ab]	3.85±1.61[a]	8.90±3.37[b]	4.97±1.71[a]	4.98±0.87[a]
Brown adipose tissue	0.73±0.16[a]	0.72±0.09[a]	0.55±0.12[a]	0.47±0.11[a]	0.56±0.20[a]	0.70±0.19[a]

489 Mice were fed the control- or HF diet containing Ashitaba extract for 16 weeks. At the end of the experiment, body weight and adipose tissue weights were measured after 18 hours fasting. Values are the mean ± SD (n=6). Values without a common letter in a row differ significantly among groups (p<0.05) by the Tukey-Kramer multiple comparison test.

Asitaba extract

C57BL/6 mice

p-AMPK

White adipose

lipogenisis

C/EBPα

PPARγ

SREBP-1

Liver

lipogenisis

SREBP-1

FAS

Fatty acid oxidation

p-ACC

CPT-1A

ACOX1

PPARα

Plasma

Cholesterol
Glucose
Insulin

Adiponectin

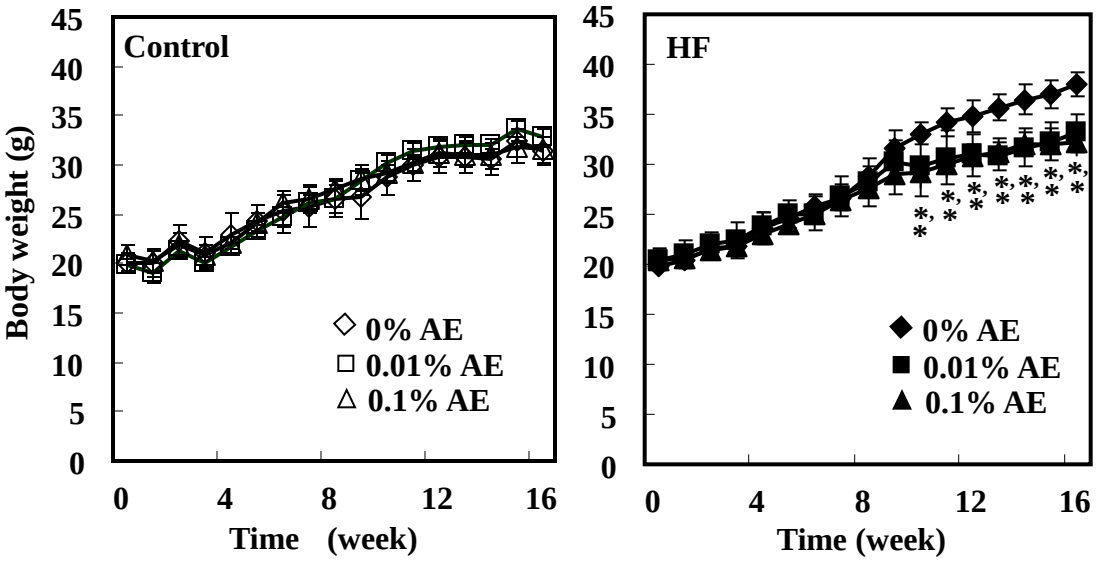


Fig. 1 Zhang *et al.*

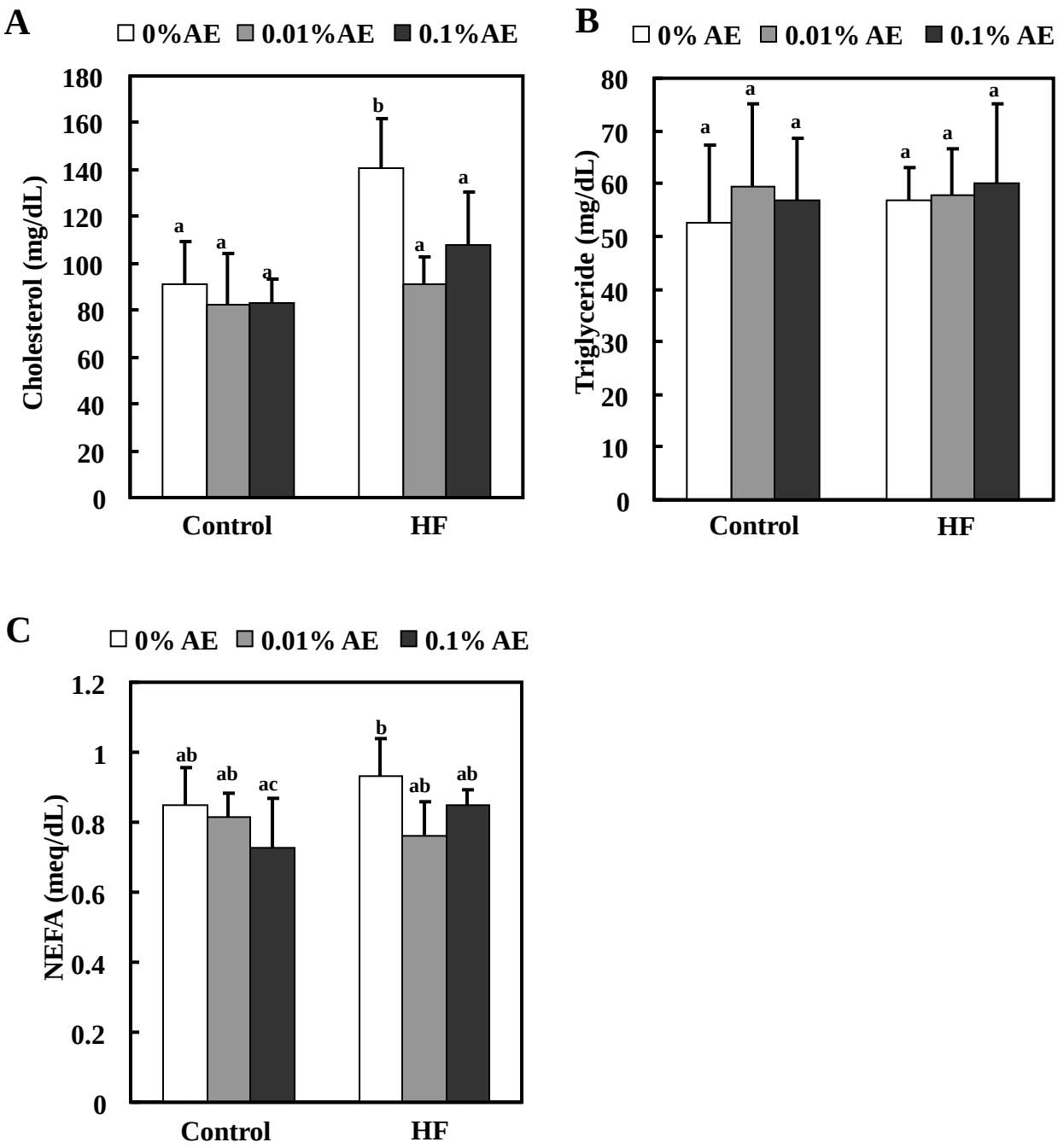
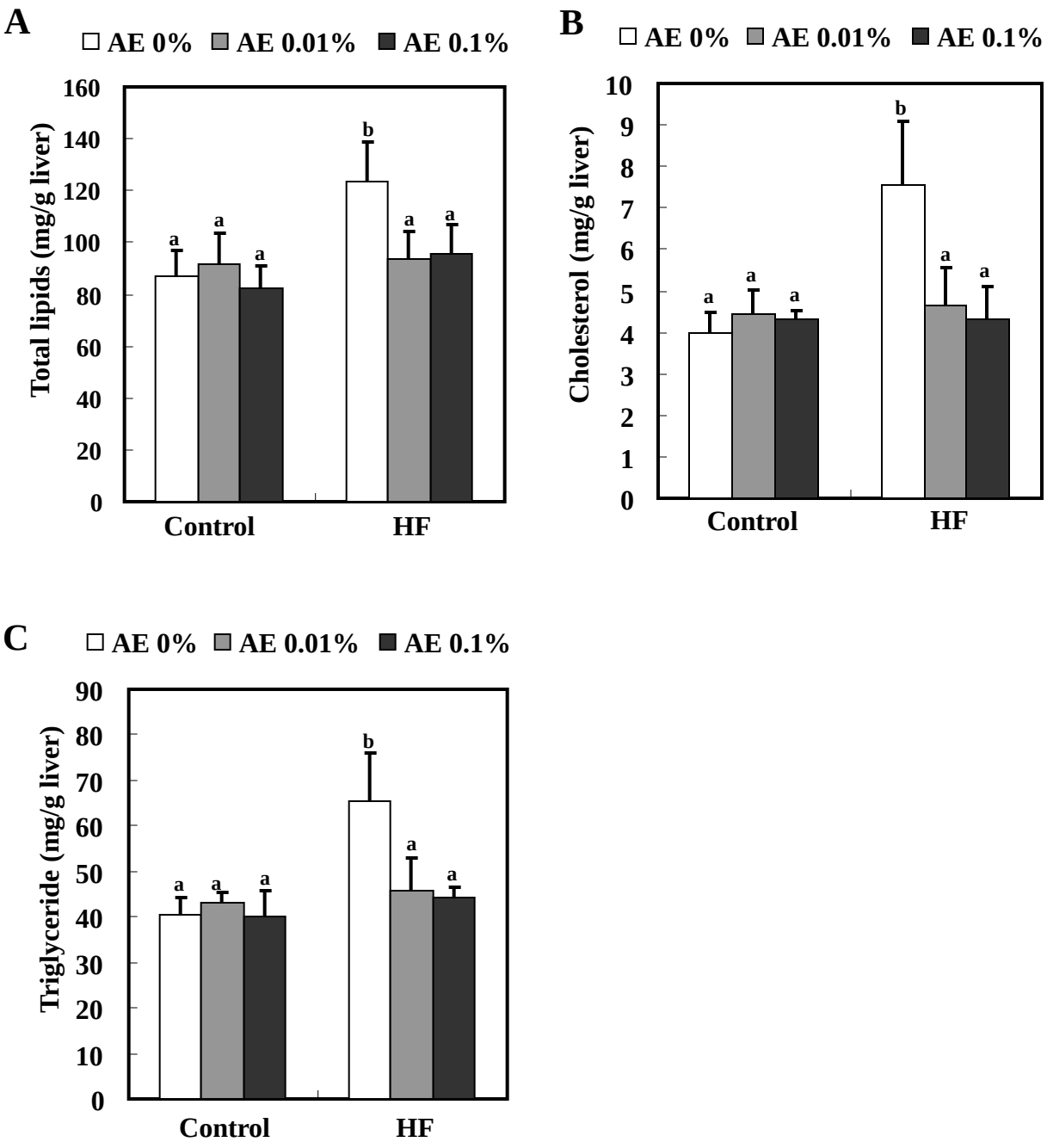


Fig. 2 Zhang *et al.*



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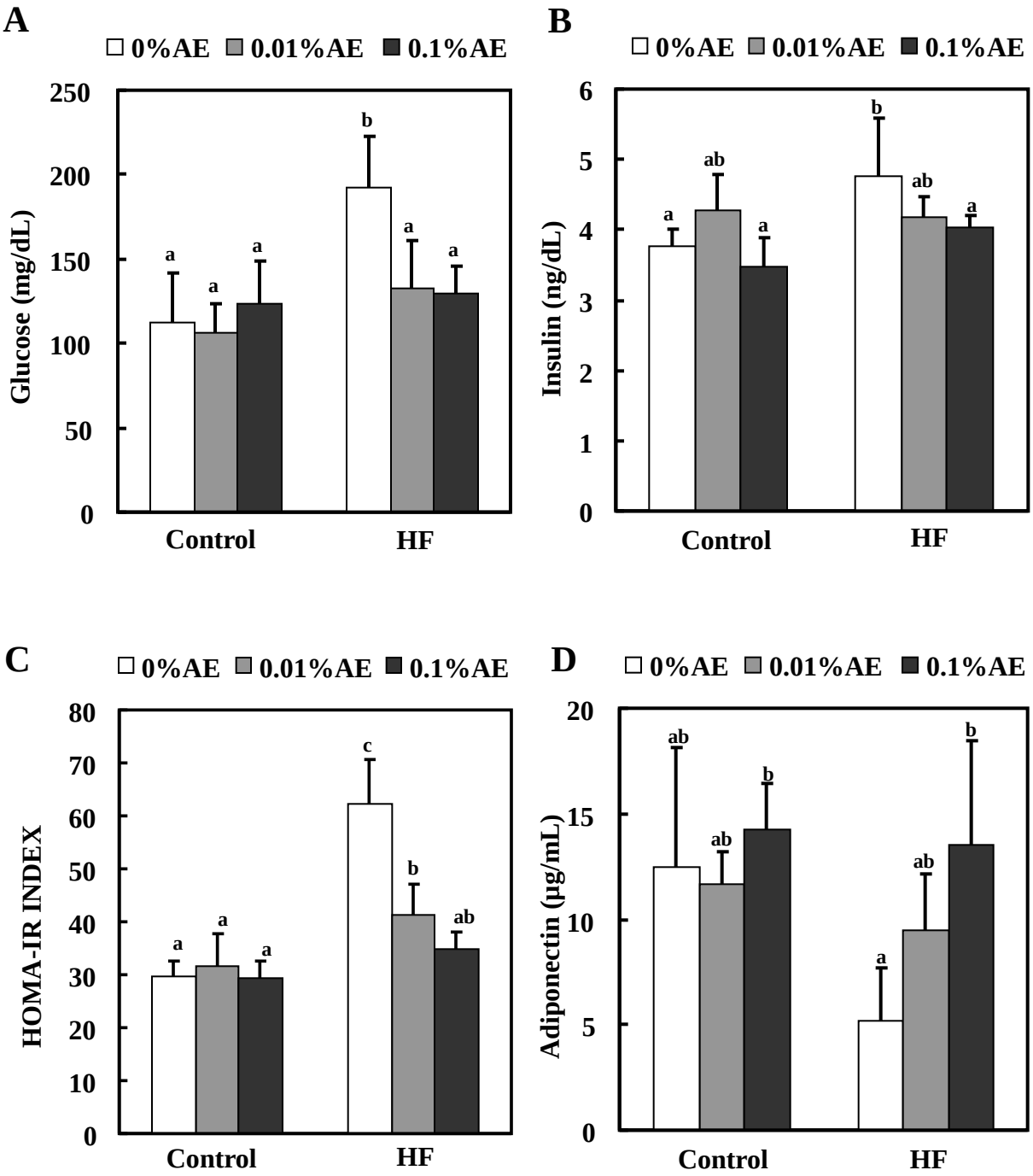
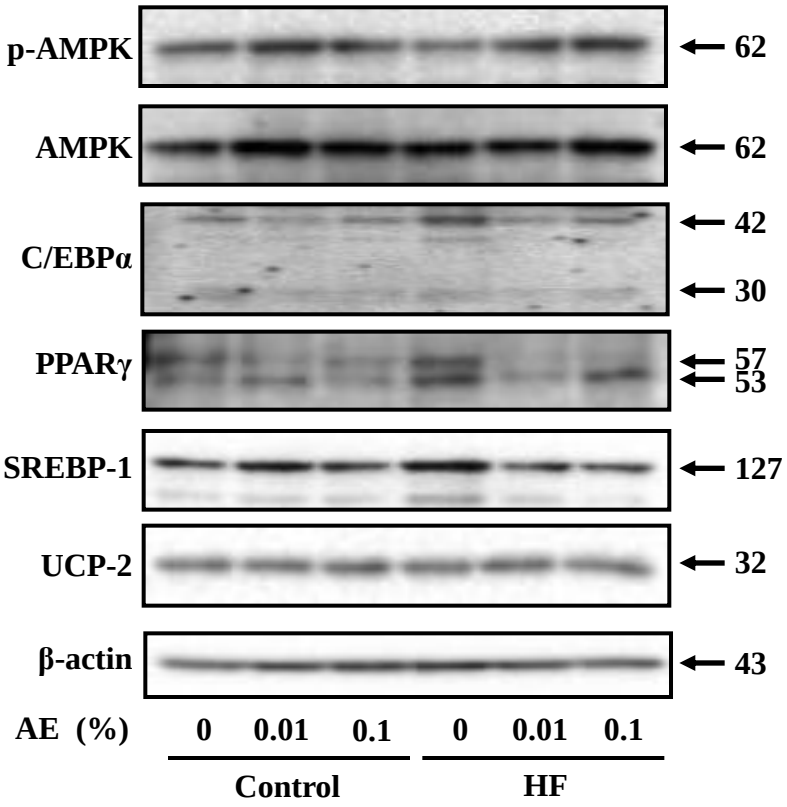
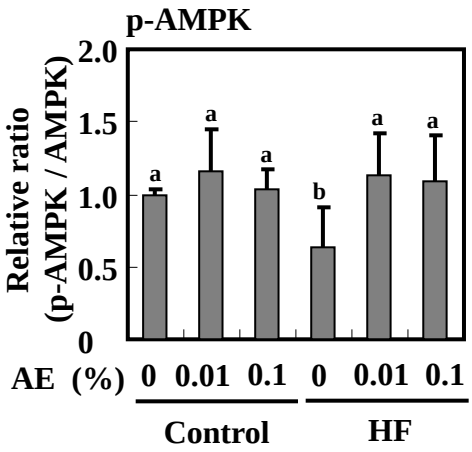
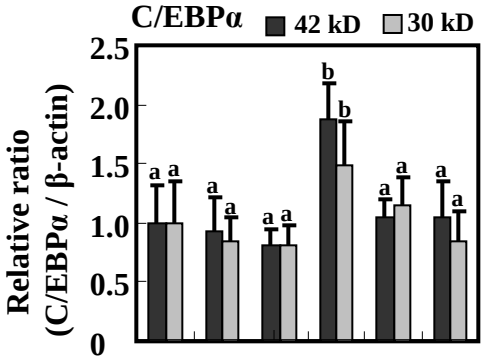
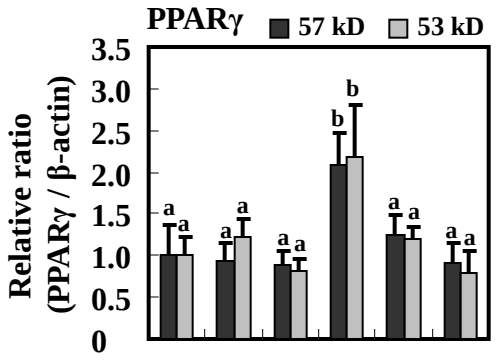


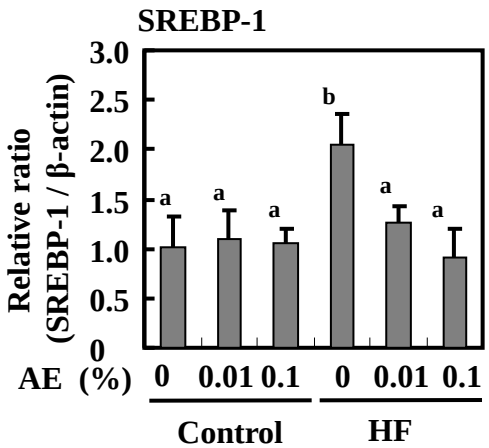
Fig. 4 Zhang *et al.*











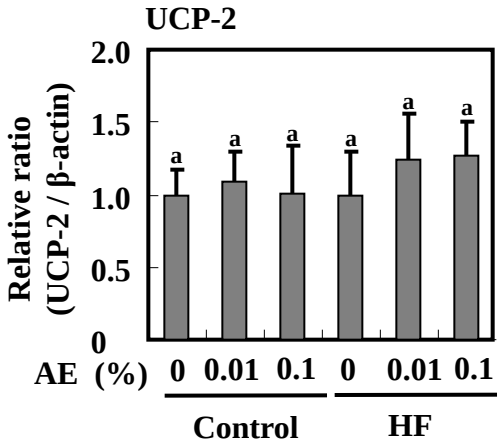


Fig. 5 Zhang *et al.*

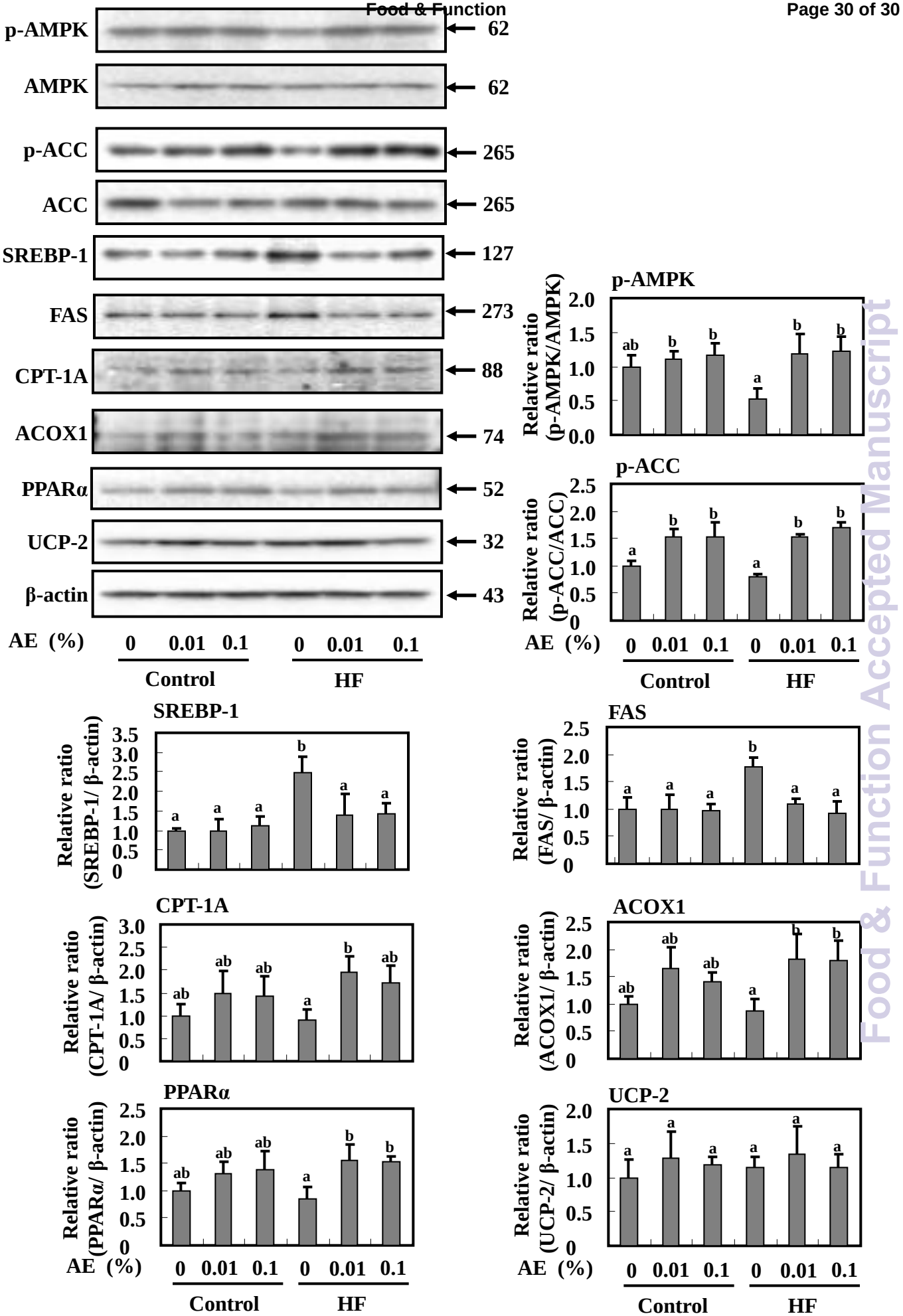


Fig. 6 Zhang *et al.*