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Ethanolic Extract of Pancake Mixture Powder Supplemented with *Helianthus tuberosus* Enhances Antidiabetic Effects via Inhibiting Inflammatory Mediator NO Production

Kyoung-Dong Lee[1], Hyeon-Jin Sun[2], Mina Lee[3], Jiyeon Chun[4], Tai-Sun Shin[5], Kap Seong Choi[4]*, and Sun-Yup Shim[4]*

[1]*Department of Oriental Medicine Materials, College of Oriental Medicine, Dongsin University, Naju 58245, Republic of Korea*
[2]*Subtropical Horticulture Research Institute, Jeju National University, Jeju 63243, Republic of Korea*
[3]*College of Pharmacy, Sunchon National University, Suncheon 57922, Republic of Korea*
[4]*Department of Food Science and Technology, College of Life Science and Natural Resources, Sunchon National University, Suncheon 57922, Republic of Korea*
[5]*Division of Food and Nutrition, Chonnam National University, Gwangju 61186, Republic of Korea*

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Helianthus tuberosus **is perennial plant as Compositae family and is shown various physiological activities such as analgesic, antipyretic, anti-inflammatory, anti-fungal, anti-spasmodic, aperient, cholagogue, diuretic, spermatogenic, stomachic, and tonic effects. In this study, we investigated the antidiabetic and anti-inflammatory effects of pancake mixture powder (PM) supplemented with *H. tuberosus* (PMH) in rat skeletal muscle L6 cells and murine macrophage RAW 264.7 cells, respectively. PM and PMH inhibited *in vitro* α-glucosidase activity. Glucose consumption was increased by PM and PMH without cytotoxicity in rat myoblast L6 cells. Western blot analysis revealed that PM and PMH down-regulated glycogen synthase kinase (GSK)-3β activation in L6 cells. PM and PMH inhibited inflammatory mediator, nitric oxide (NO) production without cytotoxicity in LPS-induced RAW 264.7 cells. The anti-diabetic and anti-inflammatory effects of PMH was more stronger than those of PM. Anti-diabetic and anti-inflammatory effects of PMH would be due to functional characteristics of the supplemented *H. tuberosus* and the presence of garlic and onion used as ingredients of PM. Taken together, our results that addition of functional materials such as *H. tuberosus* in product has synergic effects and PMH is potential candidate for treatment of diabetes through inhibiting inflammation.**

Keywords: ***Helianthus tuberosus,*** **α-glucosidase activity, GSK-3β, rat myoblast L6 cells, nitric oxide, murine macrophage RAW 264.7 cells**

Introduction

Helianthus tuberosus, also known as Jerusalem artichoke, is called earth apple and sunroot. It is a species of sunflower native to central North America. *H. tuberosus* is a perennial plant belonging to the Compositae family. It is widely applied as a medicine owing to its

***Corresponding authors**
K. S. Choi
Tel.: +82-61-750-3257, Fax: +82-61-750-3250
E-mail: chks@scnu.ac.kr
S.-Y. Shim
Tel.: +82-61-750-3250, Fax: +82-61-750-3250
E-mail: shimsy@scnu.ac.kr

physiological activities such as analgesic, antipyretic, anti-inflammatory, anti-fungal, anti-spasmodic, aperient, cholagogue, diuretic, spermatogenic, stomachic, and tonic effects [1–3]. Moreover, *H. tuberosus* contains low-calorie and high content of vitamins, mineral, and dietary fibers and it is a good food material for enhancing intestinal immune response [4]. Regarding bioactive components, *H. tuberosus* has inulin and inulin-derivatives that possess anti-diabetic, anti-carcinogenic, anti-fungistatic, anti-constipation, anti-inflammatory, body mass-reducing, wound-healing and metabolism-improving effects [5–7].

Diabetes mellitus (DM), commonly referred to as diabetes, is one of the most common endocrine metabolic disorders with a significant impact on the health, quality of life, and life expectancy of patients as well as on the health care system [8–10]. Typical DM patients show frequent urination, increased thirst and hunger, and other symptoms. DM is classified into two major types, type I DM which is an autoimmune disease associated with insulin deficiency and type 2 DM, which occurs mainly due to ineffective insulin action. Type 2 DM is one of the major obesity-associated morbidities and the number of patients with type 2 DM has been steadily rising in recent decades [11–14]. If DM is poorly controlled, it can trigger complications that can lead to failures of kidneys, eyes, feet, and other body parts that cannot be cured [15–18]. Diabetes is treated mainly with medication and insulin injection. However, all medication have specific toxic side effects. In addition, long-term use of insulin can decrease insulin receptor sensitivity, resulting in insulin resistance and eventually leading to worsening conditions. Inflammation affects adipose tissues, skeletal muscles, and livers, resulting into insulin resistance and hyperglycemia [19, 20]. It is a non-specific immune response that occurs in reaction to any type of physical injuries [21]. Nitric oxide (NO) plays an important role in various pathological processes as an inflammatory mediator that is generally controlled, that initiate and maintain in inflammation [22].

Many research studies have focused on the development of safer and therapeutic agents and functional foods using natural products or traditional Chinese medicine for diabetes therapy [23–25]. Recently, the diversification of dietary life, the advancement of women to the society, and the increase of the elderly population have led to attention on healthy life. Interest in functional premixes is increasing. To increase the use and discovery of functional food material using of *H. tuberosus*, the present study was to determine anti-diabetic and anti-inflammatory effects of pancake mixture powder (PM) supplemented with *H. tuberosus* (PMH) in rat skeletal muscle L6 cells and murine macrophage RAW 264.7 cells, respectively.

Materials and Methods

PM and PMH extracts preparation

As shown in Table 1, PM contained 92.9% of white flour, 0.8% of onion powder, 0.4% of garlic powder, 0.1% of pepper powder, 2.6% of salt, 2.2% of sugar, and 1.0% of baking powder. PMH had the same composition as PMH. It contained 20% of *H. tuberosus* powder and 72.9% of white flour instead of 92.9% of white flour (Table 1). PM and PMH extracts were added to 10 volumes of ethanol. The extract was filtered, concentrated under vacuum, and dried using nitrogen gas. The extract was then stored at -20℃ and dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA) for experiment.

In vitro α-Glucosidase assay

α-Glucosidase can hydrolyze *p*-nitrophenyl-α-D-glucopyronoside (*p*NPG) (Sigma-Aldrich,), resulting in the formation of a colorimetric product [26]. The activity of α-glucosidase was determined by measuring the release of *p*NPG at 405 nm on a microplate reader (BioTek Instruments, USA) using a α-Glucosidase Assay kit (Sigma-Aldrich) according to the manufactures' instructions.

Table 1. Composition of PM and PMH (%).

	PM	PMH
H. tuberosus podwer	0	20.0
White flour	92.9	72.9
Onion powder	0.8	0.8
Garlic powder	0.4	0.4
Pepper powder	0.1	0.1
Salt	2.6	2.6
Sugar	2.2	2.2
Baking powder	1.0	1.0

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Cell culture, treatment, and stimulation

Rat skeletal muscle L6 cells for anti-diabetic activity and murine macrophage RAW 264.7 cells for anti-inflammatory activity were obtained from Korean Cell Line Bank (KCLB, Korea). They were maintained in α-minimal essential medium (α-MEM) (HyClone, USA) and Dulbecco's modified Eagle's medium (DMEM) (HyClone), respectively, supplemented with 10% fetal bovine serum (FBS) (HyClone, Scientific) and 1% antibiotic and antimycotic (Thermo Fisher, USA) (under an atmosphere of 5% CO_2 at 37℃. For differentiation of myotubes from myoblasts, cells were seeded into 24-well plates at a density of 1×10^4 cells/ml and the medium was changed to α-MEM containing 2% FBS once every 2 days for indicated time [27]. Differentiated myotubes were incubated in serum-free medium for 6 h before any experiment.

Cell viability assay

Cytotoxicity of PM and PMH extracts to L6 and RAW 264.7 cells was assessed by MTS assay using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA). Cells (1×10^4 cells/well) were incubated in a serum-free medium in the presence of PM and PMH extracts at 37℃. After 24 h of PM and PMH treatment, the MTS solution was added to the fresh medium for 1 h according to the manufacturer's instructions. The absorbance at 490 nm was then measured using a microplate reader (BioTek Instruments).

Glucose consumption assay

Glucose consumption was conducted as described previously with slight modifications [28]. In brief, L6 cells were treated with PM extract and 33 mM d-glucose and then cultured with α-MEM supplemented with 2% FBS for 5 days. Glucose concentration in the culture medium was determined by the glucose oxidase method based on absorbance at 540 nm (BioTek Instruments) according to the manufacturer's instruction using a Glucose assay kit (Sigma-Aldrich).

Western blot analysis

Protein expression levels of GSK-3β were examined by Western blot analysis. L6 cells were treated with PM and PMH extract and 33 mM D-glucose and then cultured with α-MEM supplemented with 2% FBS for 5 days, washed with cold PBS, and harvested using a 0.005% Trypsin-EDTA (Thermo Fisher Scientific). Whole cell lysates were used for protein extraction kit (InTRON Biotechnology, Korea). Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Germany). Membranes were blocked with 5% skim milk in a plain buffer (20 mM Tris pH 7.4 and 136 mM NaCl) at RT for 1 h and incubated overnight with primary antibodies GSK 3β (Cell Signaling Technology, USA) at 4℃. Membranes were then incubated with a 500-fold dilution of specific secondary HRP-conjugated antibody (Santa Cruz Biotechnology, USA) at RT for 1 h. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) assay kit (Thermo Fisher) according to the manufacturer's instructions.

Measurement of NO production

Cultured mouse macrophages (1×10^5 cells/well) were treated PM and PMH extracts in a serum-free culture medium for 1 h and then induced with lipopolysaccharide (LPS, 1 μg/ml) (Sigma-Aldrich). After 20 h, the supernatant was harvested and treated with an equal volume of Griess reagent (A [0.1% (w/v) N-(1-naphtyl) ethylenediamine in DW] and B [1% (w/v) sulfanilamide in 5% (v/v) phosphoric]). After incubation at RT for 20 min, the absorbance was measured at 550 nm using a microplate reader (BioTek Instruments). Nitrite production was determined using serum-free culture medium as a control.

Statistical analysis

Comparisons were performed by one-way ANOVA and Duncan's multiple range tests using IBM SPSS Statistics 26. Values are reported as means ± standard error (S.E.), and $p < 0.05$ was considered significant.

Results and Discussion

Effects of PM and PMH extracts on *in vitro* α-Glucosidase activity

Generally, type 2 DM is hyperglycemia that shows abnormally elevated fasting and postprandial blood-glucose levels. α-Glucosidase, which is present in the villi of the small intestine and is involved in the food

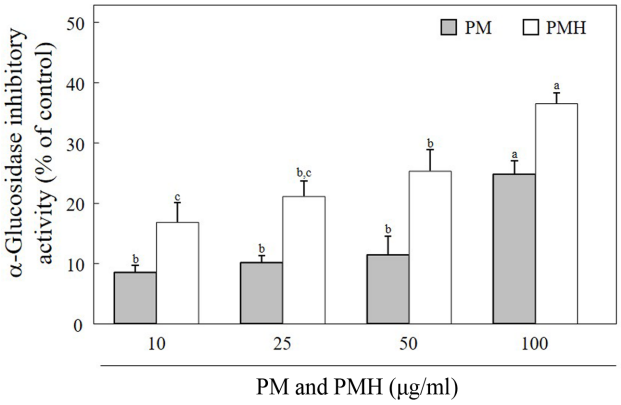

Fig. 1. Effects of PM and PMH on *in vitro* α-glucosidase activity. Data are presented as means ± SD of three independent experiments. $^{*}p < 0.05$ indicates significant differences from the PM and PMH-treated group.

digestion, can degrade oligosaccharides to monosaccharides. Therefore, inhibition of this enzyme activity delays carbohydrate hydrolysis and absorption, and then suppressed increase in blood sugar level [29]. Such hypoglycemia can be controlled by suppressing α-glucosidase activity using various therapeutic food materials. As shown in Fig. 1, PM and PMH extracts inhibited α-glucosidase activity in a dose-dependent manner, with PMH which contained *H. tuberosus* power having a higher inhibitory effect than PM. It has been reported that the extract *H. tuberosus* exhibited powerful inhibitory efficacy against α-glucosidase activity [30]. This result might be basic data for development of anti-diabetic functional therapeutic product using *H. tuberosus*.

Cytotoxicity and effects of PM and PMH extracts on glucose consumption in Rat Skeletal Muscle L6 cells

Recently, type 2 DM as one major obesity-associated endocrine metabolic disorder is increased due to diversified dietary habits and is related to an healthy and imbalanced life [11–14, 31, 32]. Thus, interest in food materials has increased. *H. tuberosus* contains inulin and inulin-derivatives that possess anti-diabetic, anti-carcinogenic, anti-fungistatic, anti-constipation, body mass-reducing, and metabolism-improving effects [5–7, 33]. Our study assessed whether *H. tuberosus*, a food material, had an anti-diabetic activity to develop PM for more attenuating DM. For sample preparation, PM and PMH extracts were prepared with 100% ethanol. L6

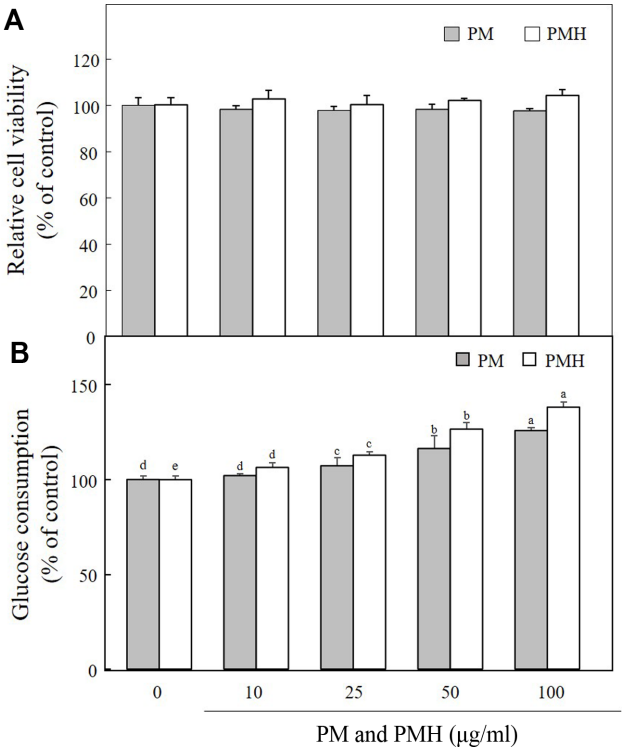

Fig. 2. Effects of PM and PMH on cell viability and glucose consumption in L6 cells. (A) Cell viability, (B) glucose consumption. Data are presented as means ± SD of three independent experiments. $^{*}p < 0.05$ indicates significant differences from the PM and PMH-treated group.

cells were treated with various concentrations of PM and PMH extracts under serum-free condition for 24 h. Cell viability was then measured using MTS solution. As shown in Fig. 2A, PM and PMH extracts did not show any cytotoxic effects at concentrations of 10–100 μg/ml. Therefore, non-toxic levels of PM and PMH extracts were used for further experiments. DM is a disease that causes disorders of glucose and lipid metabolism by decreasing glucose utilization in skeletal muscles through insulin resistance [34]. We examined effects of PM and PMH extracts on glucose consumption in L6 cells. PM and PMH extracts induced glucose consumption in a dose-dependent manner. PMH, which contained *H. tuberosus* power, increased glucose consumption more than PM (Fig. 2B), and we determined anti-diabetic activity of *H. tuberosus*. The glucose transporter, GLUT-4 that is a glucose transporter regulated by the PI3K-AKT signaling pathway in the biological system, is down and fail in glucose metabolism in diabetic condition [35–37].

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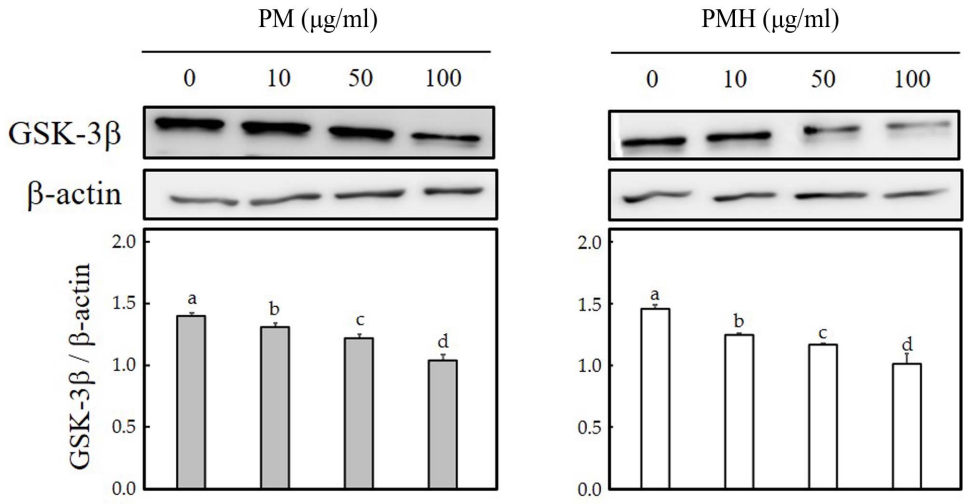

Fig. 3. Effects of PM and PMH on GSK-3β activity in L6 cells. Data are presented as means ± SD of three independent experiments. $^{*}p < 0.05$ indicates significant differences from the PM and PMH-treated group.

Further studies on GLUT-4 and PI3K-AKT signaling mechanism by PM and PMH is necessary to confirm anti-diabetic potential of PM and PMH.

Effects of PM and PMH extracts on protein expression of GSK-3β in L6 cells

GSK-3β plays an important role in inhibiting glycogen synthase which synthesizes glycogen from glucose [38–40]. In the molecular mechanism of DM, GSK-3β might be a target for insulin sensitization and it's inhibitors can improve insulin sensitivity [41, 42]. To examine the ability of PM and PMH extracts as a GSK-3β inhibitor, Western blot analysis was performed using PM and PMH extracts-pretreated L6 cells. As shown in Fig. 3, PM and PMH extracts had inhibitory effect on GSK-3β activity in a dose-dependent manner. PMH extract, which contained *H. tuberosus* powder, inhibited GSK-3β activity more than PM extract. GSK-3β function in diabetes is required for the transcriptional activation of NF-κB in inflammation [38, 39, 43]. Further transcriptional mechanism studies on correlations of diabetes and inflammation are needed to confirm their therapeutic potential.

Cytotoxicity and effects of PM and PMH extracts on NO production in LPS-induced murine macrophage, RAW 264.7 cells

Insulin resistance is involved in the pathogenesis of inflammatory response that is a non-specific immune reaction that occurs in reaction to any type of physical injuries [44]. We evaluated inhibitory effects of PM and PMH extracts using LPS-induced murine macrophage

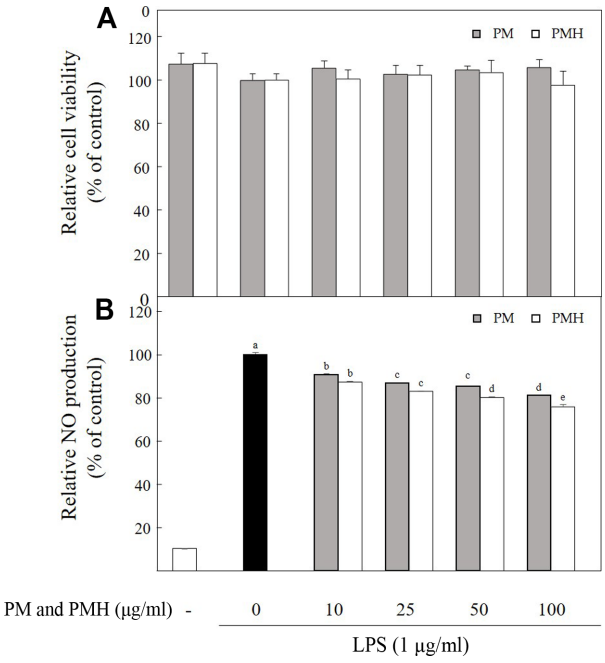

Fig. 4. Effects of PM and PMH on cell viability and NO production in LPS-induced RAW 264.7 cells (A) Cell viability, (B) NO production. Data are presented as means ± SD of three independent experiments. $^{*}p < 0.05$ indicates significant differences from the PM and PMH-treated group.

RAW 264.7 cells. Cells were pretreated with PM and PMH extracts for 1 h and then stimulated with LPS for 24 h. PM and PMH extracts did not show any cytotoxic effects at concentrations of 10–100 μg/ml (Fig. 4A). Therefore, a non-toxic level (~100 μg/ml) of PM and PMH extracts was used for further experiments.

NO is a signaling molecule that plays a key role in the pathogenesis of inflammation. It is released by activated macrophages [21]. As an inflammatory mediator, NO production was inhibited by PM and PMH extracts in a dose-dependent manner in LPS-stimulated RAW 264.7 cells (Fig. 4B). PMH extract, which contained *H. tuberosus* powder, inhibited NO production more than PM extract.

These results demonstrated that PM which contains *H. tuberosus* powder has antidiabetic effects in rat skeletal muscle L6 cells. Our results indicate that PM extracts have anti-diabetic effects by inhibiting α-glucosidase activity, GKS-3β activity, and increasing glucose consumption. It also has anti-inflammatory effects by down-regulating NO production. These results shown that PMH ethanolic extract which contains *H. tuberosus* powder has higher anti-diabetic and anti-inflammatory effects than PM extract. Therefore, the anti-diabetic and anti-inflammatory effects of PM extract is due to functional materials such as garlic and onion that contain bioactive compounds and its' activities can be enhanced by *H. tuberosus* [45–47]. Our results shown for the first time that PM supplemented with functional materials such as *H. tuberosus* enhanced antidiabetic effects. Therefore, PMH could be a therapeutic functional food for protection of diabetes and inflammation.

Taken together, PM and PMH shown anti-diabetic and anti-inflammatory effects. Two extracts suppressed α-glucosidase and GSK-3β activities, and increased glucose consumption in L6 cells. Moreover, PM and PMH extracts shown inhibited production of inflammatory mediator, NO production in LPS-induced RAW 264.7 cells. PMH supplemented with *H. tuberosus* more strong inhibition shown in diabetes and inflammation. Overall, PMH could be a useful functional food for the protection of type 2 DM.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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