Protective effects of Allium macrostemon extract on adipose tissue and liver dysregulation induced by high-fat diet and bisphenol A in C57BL/6 mice

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Research Article

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Abstract

Exposure of humans to high-fat (HF) diet and bisphenol A (BPA) has increased in recent times, and co-exposure to these causes harmful tissue toxicity. Here, the preventive effect of *Allium macrostemon* (AM) extract against dysfunction of adipose tissue and the liver under co-exposure to BPA and HF diet was examined. C57BL/6 mice were fed control (CON) diet, HF diet, HF diet with an oral injection of BPA (HF + BP), or HF diet with an oral co-injection of BPA and AM extract (HF + BP + AM). While HF feeding increased body weight gain compared to CON feeding, BP + HF suppressed body weight gain. BP + HF with lower body weight than HF had similar epididymal fat mass and inflammatory stress as HF, but significantly higher serum triglyceride and free fatty acid levels. In the liver, altered endoplasmic reticulum (ER) stress response and decreased autophagy were observed in HF + BP, as shown by increased t-IRE1α, p-JNK, and p62 levels and decreased beclin-1 protein levels, compared to those in HF. HF + BP + AM reduced HF and BPA-induced pro-inflammatory responses in adipose tissue, ER stress in liver, and total cholesterol level in serum. In addition, ecdysterone 25-O-β-D-glucopyranoside and 6-gingerol were firstly identified in AM extract by mass spectrometry and molecular networking analysis. In summary, the AM extract diminished liver and adipose tissue dysregulation, including hepatic ER stress and adipose tissue inflammation while a HF diet and BPA co-exposure induced harmful health outcomes in adipocytes and the liver. Therefore, AM has the potential to alleviate the harmful effects of a HF diet and BPA via anti-ER stress and anti-inflammatory activities.

Introduction

Exposure of humans to a combined high-fat (HF) diet and bisphenol A (4, 4’-isopropylidenediphenol, BPA) has recently increased. One reason for this is the surge in food delivery services due to the coronavirus disease 2019 (COVID-19) pandemic and social distancing (Nguyen & Vu 2020). Representative delivery menus include foods containing high amounts of fat, which are frequently consumed in Westernized diets. The fat in foods reportedly eases the migration of BPA particles to food, resulting in increased exposure of humans to BPA (Manzoor et al. 2022). In keeping with this trend, several preclinical studies with co-exposure have demonstrated the negative effects of a combined HF diet and BPA exposure on health.

The elevation of inflammation in adipose tissue is reported to be a consequence of BPA exposure when added to HF conditions (Luo et al. 2017). The release of inflammatory adipokines and excessive amounts of free fatty acids (FFAs) are characteristics of dysfunctional adipose tissue (Cusi 2010). Interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), F4/80, and monocyte chemoattractant protein-1 (MCP-1) are examples of pro-inflammatory adipokines. Immune cell infiltration, histologically detected by a crown-like structure (CLS), is also a prominent feature of dysfunctional inflamed adipose tissue (Guzik et al. 2017). FFAs promote ectopic fat deposition and lipotoxicity, of which a representative molecular mechanism is endoplasmic reticulum (ER) stress in the liver. In addition to this secretory function, adipose tissue has been suggested as a potential site of toxicant accumulation (Jackson et al. 2017). Consistently, susceptibility of adipose tissue to BPA has resulted in its being reported as the site containing the highest
amount of free BPA concentrations, followed by the liver and brain, as per a human study (Geens et al. 2012).

The synergistic harmful effects of BPA with HF on the liver have also been reported as chronic ER stress-mediated steatosis progression (Figueiredo et al. 2020) and immune-metabolic dysfunction (Pirozzi et al. 2020). The role of ER stress in hepatic dysfunction has been well demonstrated, as BPA exposure increases ER stress-associated apoptosis or hepatocellular injury in *in vitro* as well as *in vivo* models (Asahi et al. 2010, Figueiredo et al. 2020). During ER stress, the unfolded protein response involves activation of transmembrane ER-resident stress sensors. One sensor is inositol-requiring kinase 1 (IRE1), which subsequently activates Jun N-terminal kinase (JNK), an inducer of cell apoptosis and inflammation (Malhi & Kaufman 2011). Another sensor, protein kinase RNA-like endoplasmic reticulum kinase (PERK), is activated by the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) and translation of activating transcription factor 4 (ATF4). The eIF2α/ATF4 pathway has also been reported to induce the transcription of genes involved in autophagy, such as *Becn 1* and *Ma1lc3b*, which encode autophagy marker proteins beclin 1 and LC3 (B'Chir et al. 2013).

The therapeutic properties of many nutraceuticals against the adverse health effects of BPA *per se* have been investigated (D’Angelo et al. 2019). Resveratrol (Rameshrad et al. 2019), lycopene (Elgawish et al. 2020), curcumin (Geng et al. 2018), and thymoquinone (Fadishei et al. 2021) ameliorate BPA-induced metabolic disorders including hyperlipidemia, upregulated inflammation, and insulin resistance. In addition to single compounds, food extracts from olive leaves, green tea, grape seeds, and ginger have been reported to possess a protective role against BPA-induced damage, including liver injury and lipid metabolism disturbance (Mahmoudi et al. 2018), vascular toxicity (Kazemi et al. 2016, Mohsenzadeh et al. 2021), and metabolic syndrome (Rameshrad et al. 2019). In line with these findings, it has been reported that plants have an intrinsic detoxication potential for reducing BPA content by oxygenation to quinones using polyphenol oxidase (Yoshida et al. 2002). Thus, plant extracts and/or plant-derived compounds can alleviate BPA toxicity. However, compared to those of BPA exposure, nutraceutical studies targeting combined HF diet and BPA conditions have not been well investigated.

*Allium Macrostemon* (AM), also known as long-stamen chive and commonly grown in East Asia, is used in medicine and as a dietary ingredient because of its beneficial effects, including lipid-lowering, antioxidant, and anti-obesity activities (Jia et al. 2020, Lee et al. 2020, Wu et al. 2020, Yao et al. 2016). Due to these health benefits, it is believed that AM has the potential to attenuate the harmful effects of a HF diet with BPA co-exposure. Since main BPA- and HF-targeting organs are the adipocyte tissue and liver, respectively, the present study aimed to evaluate the effect of AM on adipose tissue and liver dysregulation, focusing on inflammation and ER stress, respectively.

**Materials and methods**

**Preparation of AM extract**
AM was purchased from a commercial market (Sacheon, Gyengnam Province, Korea) in April, 2021. As previously reported (Lee et al. 2010), the dried part of the AM was extracted using a high-pressure/high-temperature reactor at 118 kPa/121°C for 20 min. After filtration, the solvent was removed and the extracts were concentrated using a rotary evaporator and lyophilized. The yield was 54.8%. The obtained AM extract was stored at −20°C until use.

**Animals and experimental design**

Three-week-old male C57BL/6N mice were obtained from Central Lab Animal (SLC, Osaka, Japan) and housed under optimal environmental conditions at a temperature of 21 ± 2°C and a humidity of 50 ± 5% under a 12:12 light: dark cycle. The mice were acclimatized for two weeks, and then randomly divided into four groups of 6–7 mice each, including mice fed the control diet (CON; group 1) or HF diet (group 2–4) for 9 weeks. The control diet (containing 10 kcal% fat, 20 kcal% protein, and 70 kcal% carbohydrate, D12450B) and HF diet (containing 60 kcal% fat, 20 kcal% protein, and 20 kcal% carbohydrate, D12452) were purchased from Research Diet, Inc. (New Brunswick, NJ, USA). Among the mice fed the HF diet, the HF + BP group was treated with 50 µg/kg/day of BPA (n = 7). Lastly, the HF + BP + AM group was treated with 50 µg/kg/day of BPA plus 400 mg/kg/day of AM extract simultaneously (n = 7). The dose of BPA (50 µg/kg/day) was set based on the dose considered safe for tolerable daily intake by the U.S. Environmental Protection Agency and the dose was also tested in previous mouse studies (Ding et al. 2016, Yang et al. 2016). The AM extract dose was also tested in a previous mouse study with no side effects (Lee et al. 2010). BPA and/or AM extracts were dissolved in distilled water containing 0.01% dimethyl sulfoxide (Sigma-Aldrich Co., St. Louis, MO, USA) and administered daily via oral gavage. All mice were fed a diet thrice a week and treated with BPA and/or AM daily (10 µL/g body weight) for 9 weeks. The mice in the CON (n = 6) and HF (n = 6) groups were also administered an equal volume of vehicle solution by oral gavage. During the experimental period, body weight and food intake were measured twice per week and three times per week, respectively. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of Yeungnam University (approval number: #2021-033).

**Serum and tissue isolation**

After 9 weeks of the experimental period, all mice were fasted for 16 h and anesthetized with isoflurane (Isotroy 250; Troikaa Pharmaceuticals, Gujarat, India). Before tissue harvesting, blood was collected by cardiac puncture, transferred into serum separation tubes (BD Microtainer ® tube, #365967, BD Biosciences, Franklin Lakes, NJ, USA), clotted for 30 min at RT, and centrifuged at 15,000 rpm at 4°C for 90 s. Isolated serum was collected and stored at −70°C until further analysis. After collecting blood, epididymal, perirenal, and retroperitoneal fat pads as well as liver tissue were rapidly removed from the mice and weighed immediately. The tissues were snap-frozen in liquid nitrogen and stored at −70°C until analysis.

**Serum analysis**
Serum samples were analyzed for liver injury biomarkers [glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT)], kidney injury biomarkers [blood urea nitrogen (BUN)], as well as lipid profiles including triacylglycerol (TG), total cholesterol (TC) (Asan Pharmaceutical Co., Seoul, Korea), free fatty acid (Shinyang Chemical, Seoul, Korea), and glycerol (Sigma-Aldrich Co., USA), using commercial kits according to the manufacturer’s instructions.

**Histological analysis**

Epididymal white adipose tissue was fixed in 10% neutral formalin solution (#HT501128; Sigma-Aldrich Co.) and embedded in paraffin. Tissues were cut to a thickness of 6 µm and stained with hematoxylin and eosin (H&E). To quantify CLS, the stained sections were analyzed using a light microscope (Eclipse Ni-U, Nikon, Tokyo, Japan) and an image analysis program (NIS-Element BR, Basic Research software, Nikon).

**RNA extraction, cDNA synthesis, and real-time polymerase chain reaction**

Adipose tissue was homogenized using the TissueLyser system (#85300, Qiagen, Venlo, Netherlands), and total RNA was isolated using TRlzol reagent (#15596018, Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA content and purity were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One microgram of total RNA was reverse transcribed into cDNA using the AMPIGENE® cDNA synthesis kit (#END-KIT106, Enzo Life Sciences, Farmingdale, NY, USA) and a SimpliAmp Thermal Cycler (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. Real-time PCR was performed using AMPIGENE® qPCR Green Mix Hi-ROX (#ENZ-NUC104, Enzo Life Sciences) on a StepOne Plus real-time PCR system (Applied Biosystems). The thermal cycles were as follows: 94°C for 3 min, followed by 40 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. Mouse 18s rRNA was used as a reference gene, and relative gene expression levels were analyzed using the $2^{-\Delta\DeltaCt}$ method. The primers used for the real-time PCR analysis are listed in Table 1.
Table 1
Effects of *Allium macrostemon* (AM) extract on body weight gain, energy intake, and organ weights

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CON</th>
<th>HF</th>
<th>HF + BP</th>
<th>HF + BP + AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>6.3 ± 0.5</td>
<td>16.6 ± 0.9</td>
<td>12.8 ± 0.7</td>
<td>12.9 ± 0.7</td>
</tr>
<tr>
<td>Energy intake (kcal/d/mouse)</td>
<td>8.94 ± 0.00</td>
<td>11.76 ± 0.72</td>
<td>11.47 ± 0.46</td>
<td>11.06 ± 0.21</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>0.58 ± 0.06</td>
<td>2.09 ± 0.11</td>
<td>2.00 ± 0.06</td>
<td>1.57 ± 0.11</td>
</tr>
<tr>
<td>Perirenal and retroperitoneal fat</td>
<td>0.29 ± 0.04</td>
<td>1.12 ± 0.07</td>
<td>0.87 ± 0.06</td>
<td>0.70 ± 0.11</td>
</tr>
<tr>
<td>Liver</td>
<td>0.74 ± 0.03</td>
<td>0.80 ± 0.02</td>
<td>0.77 ± 0.02</td>
<td>0.74 ± 0.01</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.26 ± 0.01</td>
<td>0.31 ± 0.02</td>
<td>0.27 ± 0.01</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.06 ± 0.00</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *Significantly different from the control group (independent *t*-test). Different letters indicate significant differences at *p* < 0.05, as determined by one-way ANOVA with Tukey’s multiple comparison test among HF, HF + BP, and HF + BP + AM groups. CON, control diet group; HF, high-fat diet group; HF + BP, high-fat diet treated with BPA group; HF + BP + AM, high-fat diet treated with BPA plus *Allium macrostemon* extract group.
Table 2
Primer sequences used in quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′→3′)</th>
<th>Reverse (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammation markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4/80</td>
<td>GGC CAT TGC CCA GAT TTT C</td>
<td>CGG TTG AGC AGA CAG TGA ATG A</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GCA ACT GTT CCT GAA CTC AAC T</td>
<td>ATC TTT TGG GGT CCG TCA ACT</td>
</tr>
<tr>
<td>NOS2</td>
<td>CAG CTG GGC TGT ACA AAC CTT</td>
<td>CAT TGG AAG TGA AGC GTT TCG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TGG CCT CCC TCT CAT CAG TT</td>
<td>CAG GCT TGT CAC TCG AAT TTT G</td>
</tr>
<tr>
<td><strong>Antioxidant enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>TGG ACA AGT ACA ACG CTG AGA AG</td>
<td>AGC CGG CCT GCG TGT AG</td>
</tr>
<tr>
<td>GPX</td>
<td>GGC TCA CCC GCT CTT TAC C</td>
<td>GGG TCG TCA CTG GGT GTT G</td>
</tr>
<tr>
<td>SOD1</td>
<td>GGC CCG GCG GAT GA</td>
<td>GTC CTT TCC AGC AGT CAC ATT G</td>
</tr>
<tr>
<td>SOD2</td>
<td>GCT GCA CCA CAG CAA GCA</td>
<td>CTC GGT GGC GTT GAG ATT G</td>
</tr>
<tr>
<td>TRX2</td>
<td>CAG CCT CTG GCA CAT TTC CT</td>
<td>TGT TCG GCT TCT GGT TTC CT</td>
</tr>
<tr>
<td><strong>Endogenous control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18s rRNA</td>
<td>AAC CCG TTG AAC CCC ATT</td>
<td>CCA TCC AAT CGG TAG TAG CG</td>
</tr>
</tbody>
</table>

1 F4/80, adhesion G protein-coupled receptor E1; IL-1β, Interleukin-1β; NOS2, Nitric oxide synthase 2, inducible; TNF-α, Tumor necrosis factor-α; CAT, Catalase; GPX, Gluthatione peroxidase 1; SOD1, Superoxide dismutase 1, soluble; SOD2, Superoxide dismutase 2, soluble; Trx2, Thioredoxin 2; 18s rRNA, 18s ribosomal RNA.

Protein extraction and western blotting analysis

Liver tissue or epididymal fat depots were homogenized in ice-cold lysis buffer (100 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 0.5% Triton X-100) containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, and a protease inhibitor cocktail (#P3100, GenDEPOT, Katy, TX, USA) using the TissueLyser system (#85300, Qiagen). The liver homogenates were incubated at 4°C for 20 min with gentle rotation and centrifuged at 10,000 × g for 30 min at 4°C. The epididymal fat homogenates were incubated at 4°C for 1 h with gentle rotation and centrifuged three times at 20,000 × g for 15 min at 4°C to remove excess lipids. The final supernatant from each tissue sample was collected and stored at −70°C until further analysis. The protein content of the lysates was determined using the Bradford protein assay kit (Bio-Rad, USA).

Equal amounts of protein were loaded onto the lanes of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, separated, and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk (BD Biosciences) or bovine serum albumin...
(Bovostar, Bovogen, Victoria, Australia) in a Tris-buffered saline solution containing 0.05% Tween-20 (pH 7.5), the membrane was probed with a specific primary antibody at 1:1,000 dilution of one of the following: ATF4 (#11815, Cell Signaling Technology, Denver, MA, USA), beclin-1 (#3495, Cell Signaling Technology), phosphorylated eIF2α (#3398, Cell Signaling Technology), eIF2α (#9722, Cell Signaling Technology), HSC70 (#sc-7298, Santa Cruz Biotechnology, Dallas, TX, USA), phosphorylated IRE1α (#NB-100-2323, Novus Biologics, USA), IRE1α (sc-390960, Santa Cruz Biotechnology), phosphorylated JNK (#4668, Cell Signaling Technology, USA), JNK (#9252, Cell Signaling Technology), LC3B (#2775, Cell Signaling Technologies), and SQSTM1/p62 (#5114, Cell Signaling Technologies). The membrane was then incubated with anti-mouse or anti-rabbit horseradish peroxidase-linked secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). The protein expression signal was detected using the Amersham ECL western blotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA). Immunoreactive bands were visualized and analyzed using Amersham ImageQuant 800 (Cytiva, Marlborough, MA, USA). The expression of HSC70 was used as a control to monitor equal protein loading in each lane.

Mass spectrometry data acquisition

UHPLC-MS/MS data acquisition was conducted on a Waters SYNAPT G2-Si quadrupole time-of-flight (TOF) mass spectrometer with MassLynx 4.1 software (Waters, MA, USA). Separation of the AM extract was achieved on an Agilent Zorbax Eclipse Plus C18 column (2.1 × 150 mm, 1.8 µm, Agilent Technologies, Santa Clara, CA, USA). The mobile phase was water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient elution was as follows: 0-2.5 min, 5% B; 2.5–21 min, 5-100% B; 21–26 min, 100% B; 26.1–30 min, 5% B. The column oven was set to 40°C and the autosampler temperature was 15°C. The flow rate was 0.4 mL/min and the injection volume 1 µL.

Comprehensive mass spectra information was acquired using a negative MSE mode. The parameters of the MSE mode were set as follows: mass range, 100–1200 Da; capillary voltage, 2.2 kV; sampling cone voltage, 50 eV; source offset voltage, 30 eV; source temperature, 120°C; desolvation temperature, 450°C; cone gas flow rate, 50 L/h; and desolvation gas flow rate, 800 L/h. Nitrogen and argon were applied as cone and collision gases, respectively. The collision energy was 20–40 eV for high energy function, and the scan time was 0.5 s. The data were calibrated in real time using a leucine-enkephalin solution (m/z 554.2615 [M-H]-) as an external reference (LockSpray”) at a flow rate of 5 µL/min.

Feature-based molecular networking analysis

The acquired UPLC-Q-TOF-MSE data was analyzed for feature-based molecular networking (FBMN) based on the online FBMN-Progenesis QI workflow (https://ccms-ucsd.github.io/GNPSDocumentation/featurebasedmolecularnetworking-with-progenesisQI/) (Nothias et al. 2020). Raw LC-MS/MS data was processed in the Progenesis QI software (v3.0) for alignment and peak-picking (intensity > 10,000; spectral width > 0.1 min). Both feature quantification table (CSV file) and MS/MS spectral fragment summary (MSP file) of the raw LC-MS/MS data were exported from the Progenesis QI and submitted to the GNPS platform (https://gnps.ucsd.edu) using WinSCP (https://winscp.net). The configurations of network parameters were as follows: Precursor ion mass tolerance and fragment ion mass tolerance, 0.02; minimum cosine score, 0.70; minimum matched
fragment ions, 6; maximum number of neighbor nodes for one single node, 10; and maximum size of a spectral family, 100. Cytoscape (ver. 3.9.1) was used for network visualization and analysis.

**Statistical analysis**

Data are presented as mean ± standard error of the mean (SEM). For serum parameters, outliers identified using Grubbs’ outlier test (Grubbs 1969) were excluded from the analyses. Comparisons between the CON and HF groups were performed using the independent *t*-test. For multiple-group comparisons among the HF, HF + BP, and HF + BP + AM groups, analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was used. SPSS 25 (IBM, Chicago, IL, USA) was used for all statistical analyses. Statistical significance was set at *p* < 0.05.

**Results**

**Effects of AM supplementation on body weight gain, energy intake, and organ weights in HF diet-fed and BPA-treated mice**

During the 9-week experimental period, HF feeding induced a significant increase in body weight compared to that in the CON group (Table 1). The body weight gain in the HF + BP group was significantly lower by 1.3-fold than that in the HF group. No further effect of AM supplementation on body weight gain was observed. Energy intake was 1.3-fold higher in the HF group than that in the CON group (Table 1). However, there was no difference in the energy intake between the groups. Similar to the body weight changes, the weights of epididymal fat, kidneys, and spleen were 3.6-fold, 1.2-fold, and 1.2-fold, respectively, higher than those of the CON group (Table 1). Notably, the epididymal fat weight of the HF + BP + AM group was significantly decreased by 1.3-fold compared to that of the HF + BP group. A similar pattern was observed in the case of the sum of perirenal and retroperitoneal fat weights. Spleen weight was lower in the HF + BP group than in the HF group, with no further effect of AM supplementation.

**Effects of AM supplementation on serum biochemistry in HF diet-fed and BPA-treated mice**

The results of lipid parameter analysis are shown in Fig. 1. Serum levels of triglycerides, free fatty acids, and glycerol in the HF + BP group increased by 1.53-fold, 1.73-fold, and 1.70-fold, respectively, compared to those in the HF group. The HF + BP + AM group had a 1.13-fold lower serum total cholesterol level compared to that of the HF + BP group (HF + BP group: 135.7 ± 1.9 vs. HF + BP + AM group: 120.1 ± 3.7 g, *p* = 0.003 by independent *t*-test), indicating the cholesterol-lowering activity of AM supplementation. Kidney and liver injury biomarkers were also evaluated as indicated in Fig. 2. Although there were no significant alterations in biomarker levels among all groups, we observed a 1.51-fold increase in GPT levels in the HF + BP group compared to that in the HF group (*p* = 0.099 by independent *t*-test).

**Effects of AM supplementation on adipose tissue inflammation and oxidative stress in HF diet-fed and BPA-treated mice**
Since there were significant changes in adipose tissue weight among the groups (Table 1), histological and molecular changes in adipose tissue were examined by H&E staining and qRT-PCR analysis. The number of CLS, a hallmark of macrophage infiltration, was markedly increased in the HF group by 13.5-fold compared to that in the CON group (Fig. 3a-b). Among the HF diet-fed groups, no significant changes were detected between the HF and HF + BP groups, whereas the HF + BP + AM group showed significantly lower levels of CLS than the HF + BP group by 2.86-fold. Similar to histological observations, the mRNA expression levels of pro-inflammatory markers, including *Il-1β* and *Tnf-α*, and macrophage markers, including F4/80 and MCP-1, were higher in the HF group than in the CON group. Similarly, slightly lower levels of these pro-inflammatory markers were observed in the HF + BP group than in the HF group (Fig. 3c). Lower levels of *Il-1β* and *Tnf-α* were detected in the HF + BP + AM and HF + BP groups compared to the CON group by 3.90-fold and 3.96-fold, respectively. Changes in the expression of antioxidative enzymes were also evaluated, as shown in Fig. 3d. The gene expression levels of catalase (CAT), superoxide dismutase 2 (SOD2), and thiredoxin2 (TRX2) were higher in the HF group than in the CON group. The HF + BP group showed a similar or lower tendency of expression levels compared to those in the HF group. AM supplementation lowered the gene expression levels of SOD2 and TRX2 by 1.56-fold and 1.95-fold, respectively, compared to those of the HF + BP group, suggesting an antioxidant role of AM supplementation.

**Effects of AM supplementation on hepatic ER stress and autophagy in HF diet-fed and BPA-treated mice**

As increased levels of serum free fatty acids induce hepatic toxicity by upregulating ER stress (Estadella et al. 2013), we further examined ER stress-related protein expression. Phosphorylated JNK and eIF2α levels were significantly increased by the HF diet. The protein levels of activated JNK and its upstream transmembrane ER-resistant stress sensor IRE1α were higher in the HF + BP group than in the HF group by 1.36-fold and 1.95-fold, respectively. Downstream members of other transmembrane ER-resistant stress sensors, eIF2α and ATF4, showed lower expression levels in the HF + BP group than in the HF group. Notably, AM supplementation resulted in lower levels of t-IRE1α/HSC70, p-JNK/t-JNK, p-eIF2α/t-eIF2α, and ATF4/HSC70 by 2.43-fold, 1.85-fold, 1.61-fold, and 1.41-fold, respectively, compared to those of the CON group (Fig. 4a). In addition, the expression of autophagy-related proteins was examined. Although HF feeding did not alter autophagy-related protein expression, 1.30-fold higher levels of p62, as well as 1.45-fold and 1.22-fold higher levels of LC3-II/LC3-I ratio and beclin-1, respectively, were detected in the HF + BP group compared to those in the HF group. In response to AM supplementation, p62 expression levels were decreased compared to those in the HF + BP group, but further effects on other proteins were not observed (Fig. 4b).

**Identification of compounds in AM extract by UHPLC-MS/MS and molecular network analysis**

As illustrated in Fig. 5, 162 precursor ions were organized into a molecular network, which included 12 clusters (nodes ≥ 2) and 162 single nodes. Two compounds from the AM extract were identified as ecdysterone 25-O-β-D-glucopyranoside (1) in Cluster 2 and 6-gingerol (2) in Cluster 11 by the FBMN
analysis and more details can be found on the GNPS website (https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=d827b40931454be6bb67f9521e2a64c8). Compound 1 have been found in *Pfaffia iresinoides* (Kunth) Spreng. for the first time (Nishimoto et al. 1988), and 2 have been known as a major bioactive component in ginger (Semwal et al. 2015). Compound 1 and 2 have never been identified in *Allium macrostemon*.

**Discussion**

The primary aim of the present study was to evaluate the preventive effect of AM supplementation against the endocrine-disrupting chemical BPA in combination with an HF diet-altered adverse health effects. One of the major findings was that exposure to BPA exacerbated dysfunction in mice fed a HF diet. Although BPA suppressed HF diet-induced body weight gain, there was no difference in epididymal fat mass between the HF + BP and HF groups. These results indicated that BPA may exert toxicity by inhibiting body weight gain. This result agrees with the results of a previous study in which 8-week-old HF/ high-cholesterol/high-cholic acid diet (HFCCD)-fed C57BL/6 male mice were treated with 50 mg/kg/day BPA for 8 weeks (Lee et al. 2022). BPA coupled with HFCCD promoted the development of fatty liver by enhancing reactive oxygen species production in hepatocytes. The authors explained body weight loss as a result of severe liver damage, as shown by the significantly increased level of liver injury serum marker, GPT, whose level was increased by 2.23-fold compared to that in the HFCCD group. Here, we also determined serum liver injury marker levels, revealing a modest increase in GOT levels in the HF + BP group compared to those in the HF group. In addition, no additional impact of BPA administration on epididymal fat mass in HF-fed mice indicates that lower body weight gain is not derived from lower adipose tissue weight. Previous studies have demonstrated that BPA-induced adverse health outcomes include bone loss (Thent et al. 2018). It would be interesting to clarify the mechanism by which body weight loss occurs in future studies.

Hyperlipidemic conditions were also observed in the HF + BP group compared the HF group, as shown by the increased serum levels of TG, FFA, and glycerol (Fig. 1). In accordance with our data, an increase in serum TG levels by combined exposure to HF and BPA has been reported earlier, and previous studies have reported BPA-amplified promotion of fatty liver by enhancing reactive oxygen species production and hepatic lipid accumulation (Lee et al. 2022, Pirozzi et al. 2020). Activation of ER stress is a key pathological mechanism of steatosis (Zhang et al. 2014). Our data showed that HF + BP activated one ER branch, the IRE/JNK pathway, and inhibited the other branch, p-eIF2α/ATF4 level. Interestingly, ATF4 expression was significantly positively correlated with autophagy marker protein beclin-1 (r = 0.762, p = 0.004) and LC3-II/LC3-I (r = 0.611, p = 0.035) levels. These associations suggest that decreased autophagy in the HF + BP group may be linked to changes in the eIF2α/ATF4 pathway (B'Chir et al. 2013). In a previous study using Neuro-2a cells, autophagy was decreased by BPA, as determined by the reduction in beclin-1 and LC3-II/LC3-I levels, suggesting BPA-induced neurotoxicity (Li et al. 2022). A macrophage study also showed a BPA-induced reduction in LC3-II/LC3-I levels and an increase in p62 levels, contributing to BPA-induced immune cell injury (Wu et al. 2022). Taken together, the results of the increase in serum lipid levels, altered ER stress, and inhibition of autophagy might contribute to combined
HF and BP exposure-induced mild liver damage, although the measured GOT levels were not remarkably different between the experimental groups.

In this study, a notable protective effect of AM against HF and BPA co-exposure was demonstrated by significant reduction in epididymal fat mass and serum TC levels (Table 1 and Fig. 1b). These results are consistent with those of previous preclinical and clinical AM studies. In HF diet-fed mice, daily administration of a steroidal saponin, macrostemonoside A, isolated from the bulbs of AM at a concentration of 4 mg/kg for 30 days showed a decrease in serum TC levels and visceral fat accumulation (Xie et al. 2008). In human subjects with hyperglyceridemia, daily administration of Chinese medicine extract of AM Xuezhitong at 2,700 mg for 12 weeks induced a superior reduction in TC levels by 14.18% compared to 3.89% in the placebo group (Jia et al. 2020). Our data support a reduction in TC levels, with only a statistically significant difference between the HF + BP and HF + BP + AM groups by independent t-test (p = 0.003), but not by three-group ANOVA analysis. This weak improvement in TC changes in our model could be derived from the relatively low feed amount of AM (400 mg/kg/d for 9 weeks) compared to that of the previous study or from different experimental conditions, such as the amount or source of fat as well as BPA. In addition, similar to the present results, a previous study demonstrated that administration of AM-derived saponins reduced visceral fat accumulation but was not accompanied by changes in body weight (Xie et al. 2008). The lack of body weight reduction by AM may be explained by changes in body composition, including other fat depot alterations, as well as muscle weight, which warrants further study.

AM supplementation improved adipocyte tissue dysfunction due to the HF diet and BPA exposure. Adipose tissue analysis revealed that AM supplementation not only decreased epididymal fat pad weight, but also reduced inflammation, as determined by CLS and pro-inflammatory gene expression (Fig. 3a-c). In line with our data, a previous study investigating lycopene supplementation in a BPA-treated rats showed loss of adipose tissue weight and reduction of pro-inflammatory cytokine levels, explaining its antioxidant potential (Elgawish et al. 2020). The HF diet increases cardiac CAT mRNA expression and activity in mice (Rindler et al. 2013). Consistently, mice fed a HF and high-fructose diet for two to eight weeks also showed upregulation of mRNA levels and enzymatic activity of SOD, CAT, and glutathione peroxidase (Jarukamjorn et al. 2016). Similarly, the HF group showed significantly higher levels of antioxidant enzymes than those in the CON group, and AM treatment reduced the expression of these genes in the adipose tissue (Fig. 3d). The increased gene expression levels in the HF group in the present study can be interpreted as a response to oxidative stress, and the lower levels in the HF + BP + AM groups can be explained by the antioxidant activity of AM. Previous studies have suggested that AM exhibits antioxidant and anti-inflammatory properties (Wu et al. 2020, Yao et al. 2016).

It is believed that polyphenols in AM extract are responsible for its biological function. We previously reported the results of HPLC analysis showing that the AM contained several phenolic compounds including ellagic acid, protocatechuic acid, catechin, ferulic acid, chlorogenic, p-coumaric acid, and caffeic acid which had anti-obesity, antioxidant and anti-inflammatory activities (Kim et al. 2023). Herein, we further analyzed AM by UHPLC-mass spectrometry and molecular networking analysis. As shown in
Fig. 5, the analysis identified two compounds named ecdysterone 25-O-β-D-glucopyranoside and 6-gingerol which were not reported to have before. Taken together, various phytochemicals in AM extract which identified by two different analysis methods might explain the beneficial effects, including antioxidant and anti-inflammatory properties, in HF + BP + AM group compared HF + BP group.

Conclusion

Co-exposure to a HF diet and BPA suppressed HF diet-induced body weight gain, hyperlipidemia, and hepatic ER stress induction. Although not all perturbations caused by co-exposure were recovered by AM supplementation, AM supplementation reduced adipose tissue weight, serum total cholesterol level, hepatic ER stress levels, and adipose tissue inflammation. In the context of BPA-exaggerated metabolic perturbations induced by HF diet, the antioxidant potential of AM may play an important role in counteracting BPA-induced adipose tissue inflammation. AM is a potential functional ingredient candidate against HF diet- and BPA-induced adipose tissue and liver dysregulation and can be used to counteract the dietary issues in modern life.

Declarations

**Ethical Approval**

The animal study protocol was approved by the Institute Institutional Animal Care and Use Committee of Yeungnam University (Approval NO. 2021-033).

**Consent to Participate**

'Not applicable'

**Consent to Publish**

All authors approved the final version of the manuscript to be published.

**Authors Contributions**

Choon Young Kim contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juhae Kim, Isoo Youn and Eun Kyoung Seo. The first draft of the manuscript was written by Juhae Kim and Isoo Youn and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Competing Interests**
The authors have no relevant financial or non-financial interests to disclose.

**Availability of Data and Materials**

The data and materials that support the findings of this study are available from the corresponding author, Choon Young Kim, upon reasonable request.

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**References**


Figures
Figure 1

Effects of *Allium macrostemon* (AM) extract supplementation on serum lipid parameters in high-fat diet and BPA-treated mice. (a) triglycerides (TG), (b) total cholesterol (TC), (c) free fatty acids (FFA), and (d) glycerol in serum are shown. Data are expressed as mean ± SEM. Asterisk indicates significant differences between CON and HF groups (*p* < 0.05). Different letters indicate significant differences at *p* < 0.05, as determined by one-way ANOVA with Tukey’s multiple comparison test among HF, HF + BP, and HF.
Effects of *Allium macrostemon* (AM) extract supplementation on serum kidney and liver injury parameters in high-fat diet and BPA-treated mice. (a) Blood urea nitrogen (BUN), (b) glutamic oxaloacetic transaminase (GOT), and (c) glutamic pyruvic transaminase (GPT) are shown. Data are expressed as mean ± SEM. Asterisk indicates significant differences between CON and HF groups ($p < 0.05$). Different letters indicate significant differences at $p < 0.05$, as determined by one-way ANOVA with Tukey's multiple comparison test among HF, HF + BP, and HF + BP + AM groups. CON, control diet group; HF, high-fat diet group; HF + BP, high-fat diet treated with BPA group; HF + BP + AM, high-fat diet treated with BPA plus *Allium macrostemon* extract group.
Figure 3

Effects of *Allium macrostemon* (AM) extract supplementation on adipose tissue inflammation and oxidative stress in high-fat diet and BPA-treated mice. (a) Representative hematoxylin and eosin (H&E) staining of epididymal adipose tissue (200× magnification, scale bar = 50 μm) are shown. Crown-like structures (CLS) are depicted with arrows. (b) The numbers of CLS were counted under one field in 100× magnification. (c) Relative mRNA levels of pro-inflammatory genes were measured by real-time PCR. (d) Relative mRNA levels of antioxidant enzyme genes were measured by real-time PCR. Data are expressed as mean ± SEM. Asterisk indicates significant differences between CON and HF groups (p < 0.05). Different letters indicate significant differences at p < 0.05, as determined by one-way ANOVA with Tukey’s multiple comparison test among HF, HF + BP, and HF + BP + AM groups. CON, control diet group;
HF, high-fat diet group; HF + BP, high-fat diet treated with BPA group; HF + BP + AM, high-fat diet treated with BPA plus *Allium macrostemon* extract group.

**Fig. 4**

**a**

Effects of *Allium macrostemon* (AM) extract supplementation on hepatic endoplasmic reticulum (ER) stress and autophagy in high-fat diet and BPA-treated mice. (a) Protein expression of ER stress-related mediators including total IRE1α, phosphorylated and total JNK, phosphorylated and total eIF2α and ATF4.

**b**

Figure 4

Effects of *Allium macrostemon* (AM) extract supplementation on hepatic endoplasmic reticulum (ER) stress and autophagy in high-fat diet and BPA-treated mice. (a) Protein expression of ER stress-related mediators including total IRE1α, phosphorylated and total JNK, phosphorylated and total eIF2α and ATF4.
was measured by western blot analysis. (b) Protein expression of autophagy-related mediators including p62, beclin-1, and LC3 was determined by western blot analysis. Data are expressed as mean ± SEM. Asterisk indicates significant differences between CON and HF groups ($p < 0.05$). Different letters indicate significant differences at $p < 0.05$, as determined by one-way ANOVA with Tukey's multiple comparison test among HF, HF + BP, and HF + BP + AM groups. CON, control diet group; HF, high-fat diet group; HF + BP, high-fat diet treated with BPA group; HF + BP + AM, high-fat diet treated with BPA plus *Allium macrostemon* extract group.

**Fig. 5**

Feature-based molecular networking of the AM extract. Total of 162 nodes and 12 clusters (nodes $\geq 2$) were organized into a network and ecdysterone 25-O-β-D-glucopyranoside (1, Cluster 2) and 6-gingerol (2, Cluster 11) were identified.