



A standardized herbal combination of *Astragalus membranaceus* and *Paeonia japonica*, protects against muscle atrophy in a C26 colon cancer cachexia mouse model

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ABSTRACT

Ethnopharmacological relevance: *Astragalus membranaceus* (Fisch.) and Bunge and *Paeonia japonica* (Makino) Miyabe & H. Takeda have been traditionally used to improve the poor quality of life such as weakness, lack of appetite, fatigue, and malaise which is considered with cachexia condition.

Aim of the study: We investigated anti-cachectic effects of a herbal formula composed of *Astragalus membranaceus* and *Paeonia japonica* (APX) and the molecular mechanisms of APX in C26 cancer-induced cachexia mice and TNF- α -treated C2C12 myotubes. Additionally synergistic anti-cachectic effects of APX were compared to those of individual herbal extracts and megestrol acetate.

Methods and materials: The forty-two BALB/c mice were randomly divided into 6 groups: normal (nontreatment), control (C26 injection), AM (C26 injection with *Astragalus membranaceus*), PJ (C26 injection with *Paeonia japonica*), APX (C26 injection with combination of *Astragalus membranaceus* and *Paeonia japonica* and MA (C26 injection with megestrol acetate). All mice were orally administered DW (normal and control groups) or 100 mg/kg AM, PJ, APX or MA for 10 days. In the animal model, several tissues were weighed, and muscle tissue and blood were used to measure pro-inflammatory cytokines. C2C12 myotubes were exposed to 100 ng/mL TNF- α with or without 10 μ g/mL of AM, PJ, APX or MA for 48 h. The cells were used to immunofluorescence staining and western blot analyses.

Results: C26 injection induced notable body and muscle weight loss while APX administration significantly attenuated these alterations and the decrease of muscle weights and strength. APX also significantly attenuated the abnormal elevations in the concentration of three muscle atrophy-inducible cytokines; serum and muscle TNF- α , muscle TWEAK and IL-6 in C26 tumor-bearing mice. In the TNF- α -treated C2C12 myotube model, TNF- α treatment notably decreased MyH but activated atrophic proteins (MuRF and Fbx32) along with p38 and NF κ B while these molecular alterations were significantly ameliorated by APX treatment. These pharmacological actions of APX were supported by the results of immunofluorescence staining to MyH expression and the translocation of NF κ B into the nucleus in C2C12 myotubes.

Conclusions: Our data indicate the potential of an herbal formula, APX as an anti-cachexia agent; the effect of APX was superior to that of megestrol acetate overall especially for muscle atrophy. The underlying mechanisms of this herbal formula may involve the modulation of muscle atrophy-promoting molecules including p38, NF κ B, TNF- α and TWEAK.

1. Introduction

Cancer cachexia is a multifactorial syndrome characterized by loss of

weight, anorexia, asthenia and anemia that cannot be fully reversed by ordinary nutritional support and leads to progressive functional impairment (Fearon et al., 2011). Fifty percent of all cancer patients and

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80% of patients in the terminal stage experience cachexia (Tisdale, 2002). Cancer cachexia could be a direct cause of death in 20%–60% of patients as well as a main contributor aggravating the side effects of chemotherapy and reducing patient quality of life (von Haehling and Anker, 2014). To date, many therapeutic candidates for cachexia have been discovered but no drug has been approved by the Food and Drug Administration. Only progestin derivatives, such as megestrol acetate (MA) and corticosteroids have been prescribed to patients with cachexia as appetite stimulators (Yavuzsen et al., 2005). However, these drugs have limitations in their clinical use due to side effects such as muscle cramps, fatigue, nausea, and edema (Willemse et al., 1990) and low levels of efficacy (Advani et al., 2018).

The known underlying mechanisms of cancer cachexia involve the systemic influence of tumor-derived inflammatory cytokines, mainly tumor necrosis factor alpha (TNF- α), tumor necrosis factor-related weak inducer of apoptosis (TWEAK) and interleukin 6 (IL-6), which lead especially to skeletal muscle atrophy (Zhou et al., 2016). These muscle-derived pro-inflammatory cytokines induce intense overproduction of those cytokines in muscles, causing the apoptosis of muscle cells (Barbe and Barr, 2006). The ensuing protein degradation in muscles induces muscle atrophy and body weight loss, the typical features of cancer cachexia (Evans, 2010). Accordingly, in addition to treatment with appetite stimulators, targeting muscle atrophy has been an alternative strategy for anti-cachectic interventions by many researchers (Furrer and Handschin, 2019; Argilés et al., 2017).

On the other hand, some clinical and animal studies have shown the anti-cachectic potential of herbal agents such as HY906, TJ-48 and Rikkunshito (Farrell and Kummar, 2003; Niwa et al., 2001; Takeda et al., 2008). To develop anti-cachectic remedies, we screened numerous herbs and combination of herbs. Based on the screening processes, we identified a combination of *Astragalus membranaceus* (Fisch.) Bunge (*A. membranaceus*) and *Paeonia japonica* (Makino) Miyabe & H. Takeda (*P. japonica*) as a strong candidate.

A. membranaceus and *P. japonica* has been traditionally used for “Qi deficiency (气虚)” and “Blood deficiency (血虚)”, respectively (Chinese Pharmacopoeia Commission, 2015), and recently their protective activities against cachexia have been reported (Bae et al., 2020a; Lu et al., 2016). Qi and blood are essential components of whole body as defined by traditional Oriental medicine (Takeichi et al., 1999) and extreme deficiency of both Qi and Blood induce poor quality of life such as weakness, lack of appetite, fatigue, and malaise which is considered with cachexia condition (Chen et al., 2019). Besides, both *A. membranaceus* and *P. japonica* have been used together as the compositional herbs likely *Sijjeondaebo-tang* (*Shi-Quan-Da-Bu-Tang* in Chinese) that is a classical herbal formula treating various cachexia-like conditions in traditional Oriental medicine (Saiki, 2000; Tei, 1996). Furthermore, the synergistic therapeutic effects of the combined formulation consisting of 2 or more medicinal herbs have been practiced over thousands of years and evidenced experimentally (Zhou et al., 2016; Lee et al., 2020).

Thus, herein we compared the anti-cachectic effects of the *A. membranaceus* and *P. japonica* combination to those of megestrol acetate as well as its each herb alone. We also investigated underlying mechanisms in a C26 colon cancer mouse model.

2. Methods

2.1. Preparation of astragali radix and paeonia radix and their combination

Powder of 30% ethanol extracts of *A. membranaceus* (AM, specimen number: CDA-02) and *P. japonica* (PJ, specimen number: CDA-01) were obtained from KGC Yebon, a GMP manufacturing facility (Chung-book, Korea). In Brief, 180 kg of each herb was mixed with 3600 L of 30% ethanol and extracted by circular extraction at 65 °C for 4 h. The extracts were filtered and spray dried. The final yields of both AM and PJ were

22.2%, and the powders were stored at −55 °C until use. An equal ratio of each extract (10 g of AM plus 10 g of PJ) was combined (APX) for the present study.

2.2. Fingerprinting analyses of AM, PL and APX

Fingerprinting analyses of AM, PJ and APX were conducted using high-performance liquid chromatography (HPLC). A total of 50 μ g of AM, PJ and APX and 1 μ g of each reference compounds (albiflorin, paeoniflorin and formononetin) were dissolved in 1 mL of 70% methanol and the solution was filtered (0.2 μ m). A 20 μ L volume of each sample solution was injected into an Agilent 1200 system, and separation was performed using an Agilent Eclipse C₁₈ column (5 μ m, 4.6 $\times$ 250 mm, Agilent Technologies, CA, USA). The column was eluted at a flow rate of 0.1 mL/min at 35 °C and a wavelength of 230 nm using mobile phases A (0.1% formic acid in H₂O) and B (acetonitrile). The gradient flows were applied as follows: 0–60 min, 15–70% B; 60–65 min, 70–90% B; and 65–70 min, 90–90% B.

2.3. Culture of C26 and C2C12 cell lines

Colon-26 adenocarcinoma (C26) cells and C2C12 myoblasts were purchased from Cell Line Service (PA, USA) and ATCC (VA, USA), respectively. C26 and C2C12 cells were incubated in Roswell Park Memorial Institute Medium (RPMI)-1640 medium and Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, WelGENE Inc, Kyeong-book, Korea) at 37 °C with 5% CO₂.

2.4. Animals

A total of 42 BALB/c mice (6 weeks of age, 20–22 g, male) were purchased from Daehan Biolink (Choong-book, Korea) and were housed in a room maintained at 22 $\pm$ 2 °C under a 12-h light/12-h dark cycle (7 mice per cage). Mice were given *ad libitum* access to the diet and distilled water. Diet (Rodent NIH-31 Open Formula Auto) was purchased from Zeigler Co. (PA, USA), which was composed of 72% of carbohydrate, 18% protein and 4% fat (total: 3.97 kcal/g). After one week of acclimation, animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the US National Institutes of Health and procedures approved by the Institutional Animal Care and Use Committee of Daejeon University (DJUAR2019-016).

2.5. In vivo model of cancer cachexia and experimental design

C26 tumor-bearing cancer cachexia model was conducted based on the previous study (Bonetto et al., 2016). The forty-two BALB/c mice were randomly allocated into 6 groups (7 mice per group with equal mean weights): normal, control, AM, PJ, APX and MA. All mice in the 5 groups, except for the 7 mice in the normal group, were subcutaneously injected in the right flank with 100 μ L (5×10^5 cells) of C26 adenocarcinoma cells. On day 5 after C26 cell injection, all mice had developed tumors, as confirmed by palpation of injection site. On day 6 after C26 cell injection, mice in all 6 groups were orally administered DW (normal and control groups) or 100 mg/kg AM, PJ, APX or MA for 10 days. The time point for euthanasia was determined to be 15 days after C26 cell injection based on our pilot experiment, indicated that delaying euthanasia for more 15 days after C26 cell injection could lead to the death of mice. The doses of the tested samples, including MA (the positive control) were calculated according to human-to-mouse dose conversion. The human dose recommended by the manufacturer was 480–625 mg/day. This dose was equivalent to 96–125 mg/kg in mice; thus, we selected 100 mg/kg for this study. For AM, PJ and APX, the same dose (100 mg/kg) was selected based on a previous study (Bae et al., 2020a) and the commonly recommended human dose (500 mg/day per adult).

2.6. Food intake and body and tissue weights

Food intake and body weight were measured daily in the morning (10:00) after injection of C26 adenocarcinoma cells. One hundred gram of food was added to cages, and the reduction in the amount of food was measured at next morning. On the final day of the experiment, the animals were euthanized in a CO₂ chamber (Jeungdo Bio&Plant, Seoul, Korea). The organs (gastrocnemius and rectus femoris, liver, kidney, spleen, heart, lung and epididymal fat) and tumor were removed and weighed. Muscle tissues and organs were stored at -80 °C in a deep freezer for biochemical analysis and measurement of other parameters.

2.7. Grip strength test

The day before sacrifice, the grip strength test was performed using a BIO-GT3 tester (BIOSEB, FL, USA) according to guideline of International Mouse Phenotyping Consortium (Castro and Kuang, 2017). In brief, the four limbs of the mouse were placed in metal a grid mounted at a 45° angle and connected to a force transducer. Then, the tail of the mouse was pulled until the mouse let go of the grid. The test was repeated for a total of 3 sets with a 3 min rest time between each set. The data are presented as an average value of 3 repetitions for each mouse.

2.8. White blood cell counting and serum biochemical analysis

Whole blood was obtained from the inferior vena cava, and a 3-part differential of total white blood cells (WBCs) was immediately obtained using an Exigo-eos hematology analyzer (Exigo, Spanga, Sweden). Serum was separated by centrifugation (3000 × g, 15 min) after blood clotting. The serum levels of aspartate transaminase (AST), lactose dehydrogenase (LDH), glucose, and total protein were measured using an AU680 Auto Chemistry Analyzer (Beckman Coulter, CA, USA).

Determinations of TNF- α , TWEAK/TNFSF12 and IL-6 concentrations in serum and muscle tissue.

Cytokine concentrations were determined for TNF- α (558534, in muscle and serum) using a BD bioscience ELISA kit (NJ, USA) and for TWEAK (DY1237) and IL-6 (DY406) in muscle tissues using R&D System ELISA kits (MN, USA). To measure concentrations in muscles, 100 mg of muscle tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer and centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was used to determine the levels of the three parameters.

2.9. Differentiation of C2C12 myoblasts

We used C2C12 cell for myogenic differentiation in 2% horse serum according to the previous documentation (Portier et al., 1999). In brief, C2C12 myoblasts were seeded in 100 mm dishes (1 × 10⁶ cells/dish) and 6-well plates (1 × 10⁵ cells/well). Then, the C2C12 myoblasts were 80% confluent, the serum was replaced with medium containing 2% horse serum. After 5 days, the C2C12 myoblasts had differentiated into myotubes which were used for western blotting (100 mm dish) and Immunofluorescence staining (6-well plates) to investigate the pharmacological mechanisms of AM, PJ, APX and MA. The concentrations of the drugs were determined through a cell viability assay with C2C12 myoblasts (data not shown).

2.10. Western blot analysis of C2C12 myotubes

Western blot analyses were performed with antibodies against myosin heavy chain (MyH), muscle ring-finger protein 1 (MuRF), f-box only protein 32 (Fbx32), p38 mitogen-activated protein kinases (p38) and its phosphorylated form (pp38), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) and its phosphorylated form (pNF κ B), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (I κ B α), its phosphorylated form (pI κ B α), actin and lamin B1. Western blot analyses of pNF κ B and NF κ B were performed

in respectively nuclear and cytosolic fractions using nuclear and cytoplasmic extraction reagents (#78835, Thermo Fisher Scientific, PA, USA). All primary antibodies were purchased from Abcam (ab124205, ab172417, ab168372 and ab47363, Cambridge, UK), Cell Signaling (#9212, Cambridge, UK), Thermo Fisher Scientific (MA5-16161, MA5-16152 and MA5-11869, PA, USA) or Santa Cruz Biotechnology (sc-374015, TX, USA) and used at the concentration recommended by the data sheets.

C2C12 myotubes were exposed to 100 ng/mL TNF- α (410-MT-50, R&D System, MN, USA) with or without 10 μ g/mL of AM, PJ, APX or MA for 48 h. Cells were lysed in protein extraction solution (PRO-PREP™, iNtRON Biotechnology, Gyeonggi, Korea). The protein concentrations in the samples were measured using Bio-Rad protein assay reagent (Hercules, CA, USA) and adjusted to the same concentration. Samples were separated by 10, 12 or 15% polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking in 5% skim milk or BSA for 1 h, the membranes were probed overnight at 4 °C with primary antibodies against MyH, MuRF, Fbx32, p38, pp38, I κ B α , pI κ B α and actin. Then, the samples were washed three times for 15 min each and incubated for 2 h with HRP-conjugated anti-rabbit or anti-mouse antibodies. Blots were developed using an advanced chemiluminescence (ECL) kit, and immunoreaction visualized using UV fluorescence auto exposure in the Fusion Solo S system (VILBER ROURMAT, France). Semi-quantitative analysis of protein expression was performed using ImageJ software (NIH).

2.11. Immunofluorescence staining in C2C12 myotubes

After exposure of C2C12 myotubes to 100 ng/mL TNF- α with or without 10 μ g/mL AM, PJ, APX or MA for 48 h, the cells were fixed with 4% paraformaldehyde for 2 h at 4 °C. After washing with phosphate buffered saline (PBS) twice for 5 min each, the cells were permeabilized with 1% Triton X-100 in PBS for 10 min and were then blocked in PBS containing 3% bovine serum albumin (BSA) and 0.02% Tween 20 PBS (PBST) for 30 min at room temperature (RT). Cells were washed and incubated with an anti-MyH antibody (MF20, 1:100; R&D Systems, MN, USA) or an anti-NF κ B antibody (ab86299, 1:250, Abcam, Cambridge, UK) diluted in 3% BSA overnight at 4 °C. After washing, cells were incubated with Alexa Fluor 488-conjugated secondary antibodies (ab150077 and ab150113, 1:400; Abcam, Cambridge, UK) at RT. After washing, the cells were incubated with 1 μ g/mL of diaminido-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, MO, USA) for 2 min at RT in the dark. All signals were observed using an IX70 microscope at 400 × magnification (Olympus, Tokyo, Japan).

2.12. Statistical analysis

The results are expressed as the means $\pm$ standard deviations (SD). The statistical significance of differences among the groups was analyzed by one-way ANOVA followed by post hoc multiple comparisons testing (Tukey's HSD test) using Prism ver. 7.0 (GraphPad, CA, USA). For all analyses, # p < 0.05, ## p < 0.01 or ### p < 0.001 compared to the control group and * p < 0.05, ** p < 0.01 or *** p < 0.001 compared to the APX group.

3. Results

3.1. Fingerprinting analyses of AM, PL and APX

The three compounds, namely albiflorin, paeoniflorin and formononetin were detected at retention times of 7.118 min, 7.849 min, and 29.690 min, respectively, in the expected samples. Semi-quantitative analysis using the standard curves of the reference compounds showed that APX contained 14.14 $\pm$ 0.72 μ g/mg albiflorin, 46.81 $\pm$ 2.74 μ g/mg paeoniflorin and 0.69 $\pm$ 0.03 μ g/mg formononetin (Fig. 1).

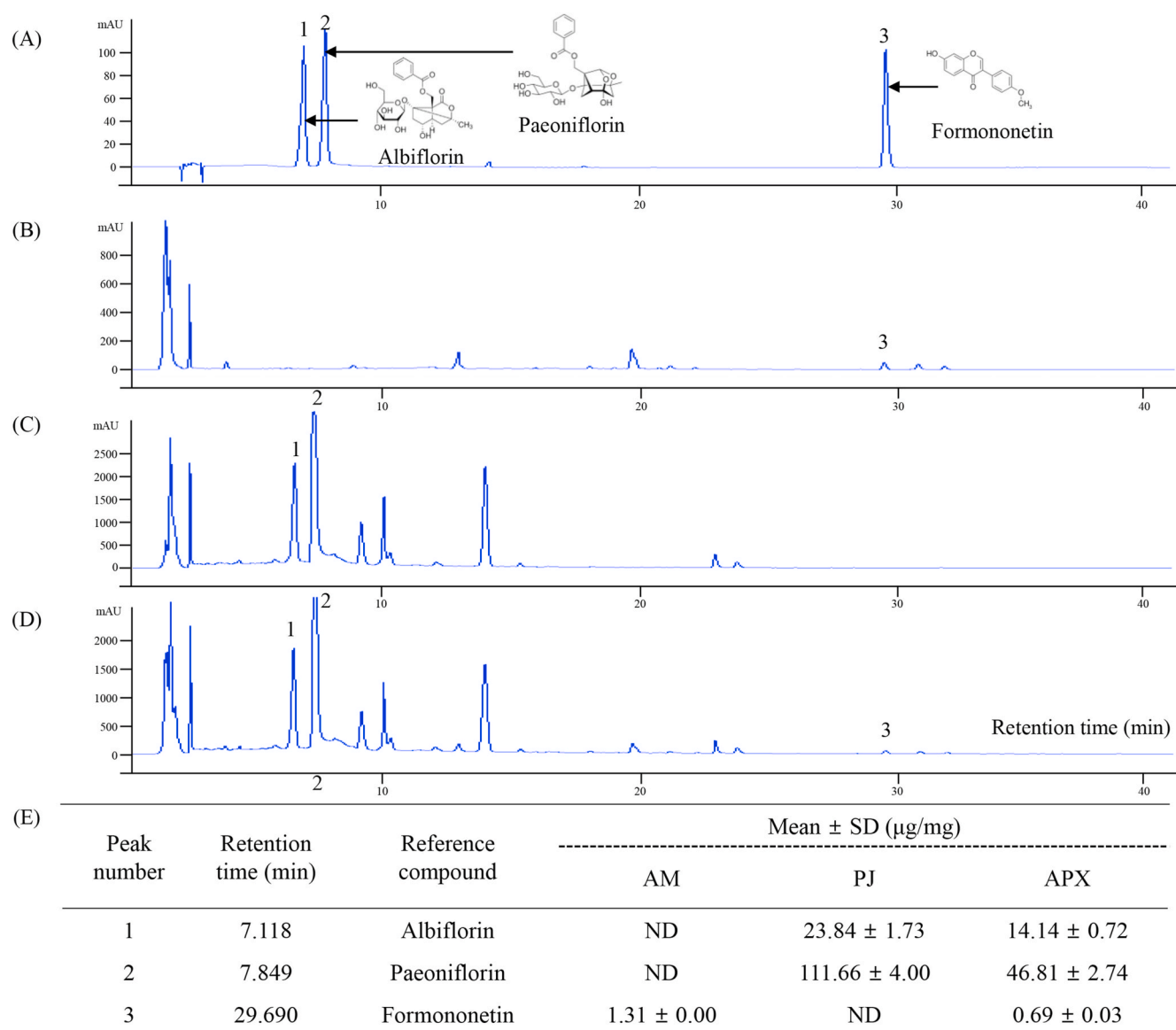


Fig. 1. Fingerprint analysis of APX. (A) Three reference compounds in (B) AM, (C) PJ and (D) APX, were analyzed by HPLC, and (E) semi-quantitative analysis was performed using their standard curves.

3.2. Changes in food intake and body and organ weights

Establishment of the C26 tumor model led to weight loss of 24% with reductions in liver, kidney, heart and epididymal fat weights compared to those in the normal group on the final day (Fig. 2A, C and D; Table 1). The reductions in the body, liver and kidney weights were significantly attenuated by administration of APX ($p < 0.001$), but not by AM or PJ, and furthermore these effects of APX were significantly superior to those of AM or PJ ($p < 0.01$ or $p < 0.001$; Fig. 2A, C and D; Table 1). The relative weights of organs to body however were not different among those 6 groups.

The average food intake was significantly decreased by C26 tumor-bearing condition, and no drug, including MA, significantly affected the food intake compared to that in the control group ($p > 0.05$, Fig. 2B). In addition, no drug significantly reduced tumor growth (Table 1).

3.3. Blood and serum analyses

The peripheral white blood cell counts were dramatically altered in C26 tumor-bearing mice (notable decrease in the lymphocyte count but

an increase in the granulocyte count), however, these alterations were significantly ameliorated by the administration of APX ($p < 0.001$ for lymphocytes and $p < 0.01$ for granulocytes), PJ ($p < 0.01$ for lymphocytes) and MA ($p < 0.05$ for lymphocytes and granulocytes, Table 2). The serum biochemistry results indicated that the levels of AST (1.7-fold) and LDH (3.4-fold) were notably elevated and that of glucose (0.59-fold) was notably decreased in this cachexia model ($p < 0.01$ or $p < 0.001$). Only APX administration significantly ameliorated these alterations, especially for AST ($p < 0.05$) and glucose ($p < 0.05$, Table 2).

3.4. Muscle weight and grip strength

C26 tumor-bearing mice showed the notable loss of muscle weights in both gastrocnemius (23%) and rectus femoris (27%), while this alteration was significantly attenuated by only APX administration ($p < 0.05$ for gastrocnemius and $p < 0.01$ for rectus femoris, Fig. 3A and B). The grip strength was also markedly decreased in the C26 tumor-bearing control group compared to the normal group ($p < 0.001$), and AM, PJ, APX and MA significantly attenuated the decreases in both the absolute ($p < 0.001$) and relative grip strength (except AM, $p < 0.01$ or $p < 0.001$)

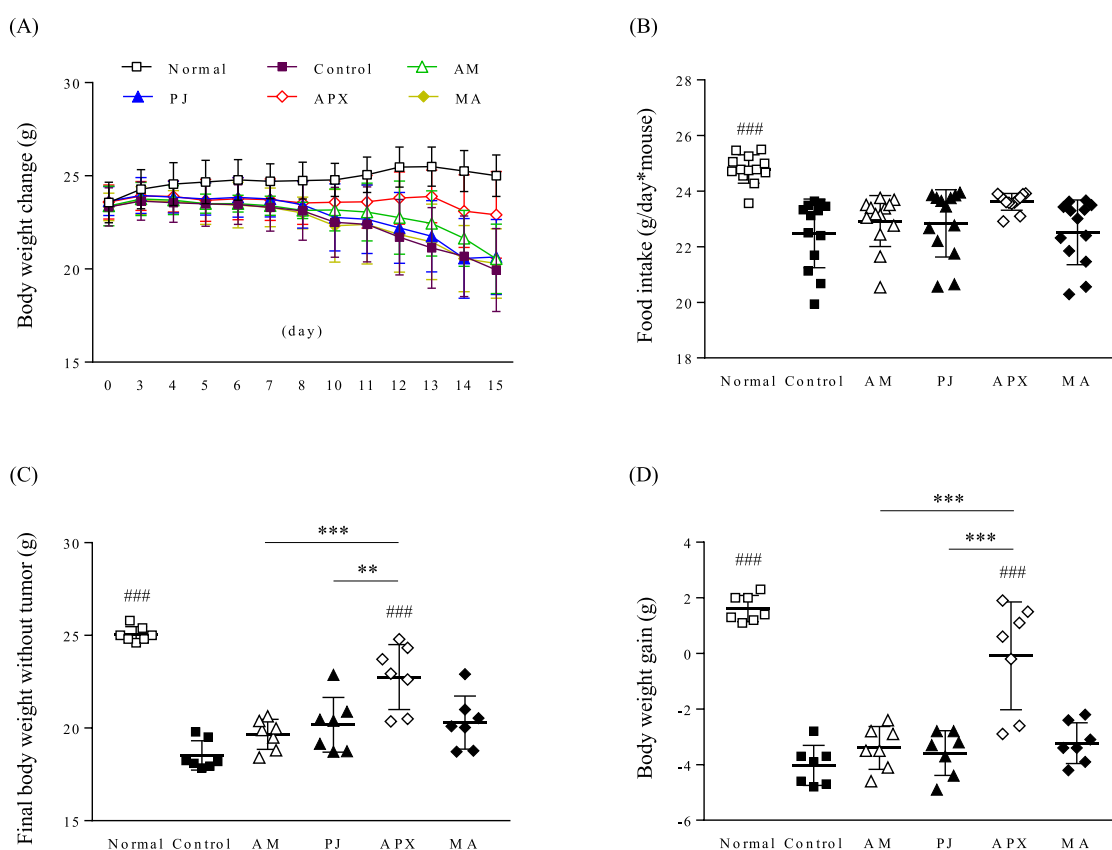


Fig. 2. Changes in body weight and food intake. (A) Body weight and (B) food intake were recorded daily for 15 days. (C) Final body weights not including the tumors and (D) body weight gains were calculated on the final day. The data are expressed as the means $\pm$ SD ($n = 7$ or 13). # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared to the control group; ** $p < 0.01$ and *** $p < 0.001$ compared to the APX group.

Table 1
Tissue weights.

	Group	Normal	Control	AM	PJ	APX	MA
Absolute weights(mg)	Tumor		861.4 $\pm$ 89.7	836.7 $\pm$ 72.3	800.0 $\pm$ 33.1	783.2 $\pm$ 37.2	833.9 $\pm$ 84.7
	Liver	1186.6 $\pm$ 34.2###	765.9 $\pm$ 68.6	789.3 $\pm$ 64.5***	709.7 $\pm$ 41.1***	1018.7 $\pm$ 106.6###	923.6 $\pm$ 83.7##
	Kidney	435.5 $\pm$ 8.18###	279.1 $\pm$ 31.8	282.9 $\pm$ 11.9***	287.1 $\pm$ 13.3**	350.0 $\pm$ 13.3###	328.7 $\pm$ 21.0##
	Heart	141.0 $\pm$ 12.0###	108.3 $\pm$ 15.1	111.6 $\pm$ 9.93	108.9 $\pm$ 4.85	119.1 $\pm$ 12.0	103.9 $\pm$ 9.92
	Lung	131.4 $\pm$ 8.69	119.1 $\pm$ 9.97	122.7 $\pm$ 7.56	119.3 $\pm$ 6.39	122.0 $\pm$ 8.16	134.6 $\pm$ 12.1
	Epididymal Fat	270.1 $\pm$ 34.8###	123.3 $\pm$ 24.7	134.0 $\pm$ 15.6	141.6 $\pm$ 18.3	169.2 $\pm$ 46.3#	165.4 $\pm$ 31.0#
Relative weights to body(mg/g)	Tumor		43.7 $\pm$ 5.75	41.5 $\pm$ 4.32	38.5 $\pm$ 7.98	37.1 $\pm$ 4.66	44.5 $\pm$ 10.2
	Liver	47.4 $\pm$ 2.34	47.0 $\pm$ 10.3	48.9 $\pm$ 4.12	47.5 $\pm$ 6.45	50.3 $\pm$ 4.85	49.9 $\pm$ 7.25
	Kidney	17.5 $\pm$ 0.52	16.4 $\pm$ 3.08	15.2 $\pm$ 0.94	15.5 $\pm$ 1.33	16.7 $\pm$ 1.66	16.7 $\pm$ 1.26
	Heart	5.73 $\pm$ 0.67	5.82 $\pm$ 0.65	5.37 $\pm$ 0.69	5.02 $\pm$ 0.40	5.27 $\pm$ 0.46	5.48 $\pm$ 0.72
	Lung	5.25 $\pm$ 0.31	5.16 $\pm$ 0.55	5.16 $\pm$ 0.67	5.88 $\pm$ 0.77	5.64 $\pm$ 0.98	5.77 $\pm$ 0.11
	Epididymal Fat	10.7 $\pm$ 1.29	6.22 $\pm$ 1.49	6.56 $\pm$ 1.00	6.82 $\pm$ 1.21	7.29 $\pm$ 2.15	7.11 $\pm$ 1.63

$p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to the control group, ** $p < 0.01$, *** $p < 0.001$ compared to the APX group.

compared to that in the control group (Fig. 3C and D). APX was significantly superior to AM in maintaining the absolute grip strength ($p < 0.05$, Fig. 3C).

3.5. Muscle atrophy-related inflammatory cytokines in serum and muscle tissue

C26 tumor-bearing mice exhibited markedly increased concentrations of TNF- α in serum and TWEAK and IL-6 in muscle ($p < 0.001$). These cytokine alterations were significantly attenuated by administration of APX ($p < 0.01$ or $p < 0.001$, Fig. 4A, C and D). Other drugs also showed positive effects on serum concentrations of TNF- α ($p < 0.01$ for AM, PJ and MA) and muscle concentrations of TWEAK ($p < 0.01$ or $p < 0.001$ for AM and PJ). APX was significantly superior to the other drugs in reducing the muscle concentration of TWEAK ($p < 0.01$, Fig. 4A, C and D). Muscle TNF- α concentrations tended to increase appreciably with C26 tumor induction and decrease with drug administration, but the differences were not significant.

3.6. Muscle atrophy-related proteins and immunofluorescence staining of MyH in C2C12 myotubes

TNF- α -treated C2C12 myotubes exhibited notably depleted protein expression of MyH (0.7-fold) but up-regulation of MuRF (1.9-fold) and Fbx32 (1.2-fold), while these alterations were significantly attenuated by APX treatment ($p < 0.01$). These effects of APX were significantly

Table 2
Blood cell counting and serum biochemistry.

Group	Normal	Control	AM	PJ	APX	MA
Total WBC (10^9)	13.5 ± 2.0	12.1 ± 5.1	11.9 ± 2.2	13.5 ± 2.3	15.2 ± 3.1	12.2 ± 1.3
Lymphocyte (10^9)	9.92 ± 0.79 ^{###}	6.0 ± 0.99	6.97 ± 0.92 ^{**}	8.3 ± 1.47 ^{##}	9.98 ± 0.12 ^{###}	7.67 ± 0.59 [#]
Monocyte (10^9)	0.68 ± 0.08 [#]	0.77 ± 0.04	0.73 ± 0.11 ^{**}	0.83 ± 0.13	0.95 ± 0.05 ^{##}	0.78 ± 0.05 [*]
Granulocyte (10^9)	2.9 ± 0.56 ^{###}	5.37 ± 0.26	4.18 ± 0.68	4.32 ± 1.42	4.28 ± 0.97	3.77 ± 0.63 [#]
AST (IU/L)	34.2 ± 4.72 ^{##}	59.8 ± 10.1	58.0 ± 14.6	66.3 ± 17.7 [*]	43.8 ± 10.2 [#]	53.7 ± 10.5
LDH (IU/L)	181.6 ± 35.8 ^{##}	615.2 ± 143.8	522.6 ± 190.9	640.7 ± 327.6	426.6 ± 145.7	659.0 ± 195.4
Glucose (mg/dL)	162.1 ± 30.1 ^{###}	96.4 ± 13.9	108.4 ± 16.0	86.1 ± 22.1 ^{**}	132.2 ± 43.3 [#]	129.0 ± 27.7
Total protein (g/dL)	4.0 ± 0.5	4.2 ± 0.4	4.1 ± 0.4	4.3 ± 0.3	4.1 ± 0.2	4.0 ± 0.5

[#] $p < 0.05$, ^{##} $p < 0.01$, ^{###} $p < 0.001$ compared to the control group, ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ compared to the APX group.

superior to those of AM or PJ ($p < 0.05$, Fig. 5B and C). These positive findings were supported by the results of an immunofluorescence assay for MyH in C2C12 myotubes (Fig. 5A).

3.7. Activation of NFκB pathway molecules and nuclear translocation in C2C12 myotubes

The TNF-α-treated C2C12 myotubes notably promoted the phosphorylation of p38 (1.7-fold) and IκBα (1.3-fold) as well as NFκB (2.0-fold) in the nuclear fraction, while these alterations were significantly ameliorated by APX treatment ($p < 0.05$ or $p < 0.001$). In addition, treatment of PJ significantly attenuated the phosphorylation of p38 and NFκB, while treatment of AM attenuated the phosphorylation of NFκB only ($p < 0.05$ or $p < 0.001$). The effects of APX were significantly superior to that of AM ($p < 0.001$ for pp38 and $p < 0.01$ for pNFκB) or PJ ($p < 0.05$ for pIκB, Fig. 6B and C). These positive findings were supported by the results of the NFκB translocation assay in C2C12 myotubes (Fig. 6A).

4. Discussion

To investigate the anti-cachectic effects of an herbal formula (APX) composed of *Astragalus membranaceus* and *Paeonia japonica*, we adapted a mice colon cancer C26-bearing cachexia model in mice. Severe atrophy of muscle and fat leading to weight loss is well known as a cardinal feature of cancer cachexia (Evans et al., 2008). As we expected, C26 tumor-bearing mice showed a notable decrease in body weight as well as in main organ weights and food intake, while these alterations were significantly reversed by APX treatment (Fig. 2A–D, Table 1).

Previous studies reported that C26 cancer cachexia model mice showed systemic inflammation in various organs via over-activation of pro-inflammatory cytokines, including TNF-α and IL-6, a representative cachectic immunologic condition (Bonetto et al., 2016; Kandarian et al., 2018; Molinari et al., 2017). In our data, C26 injection dramatically

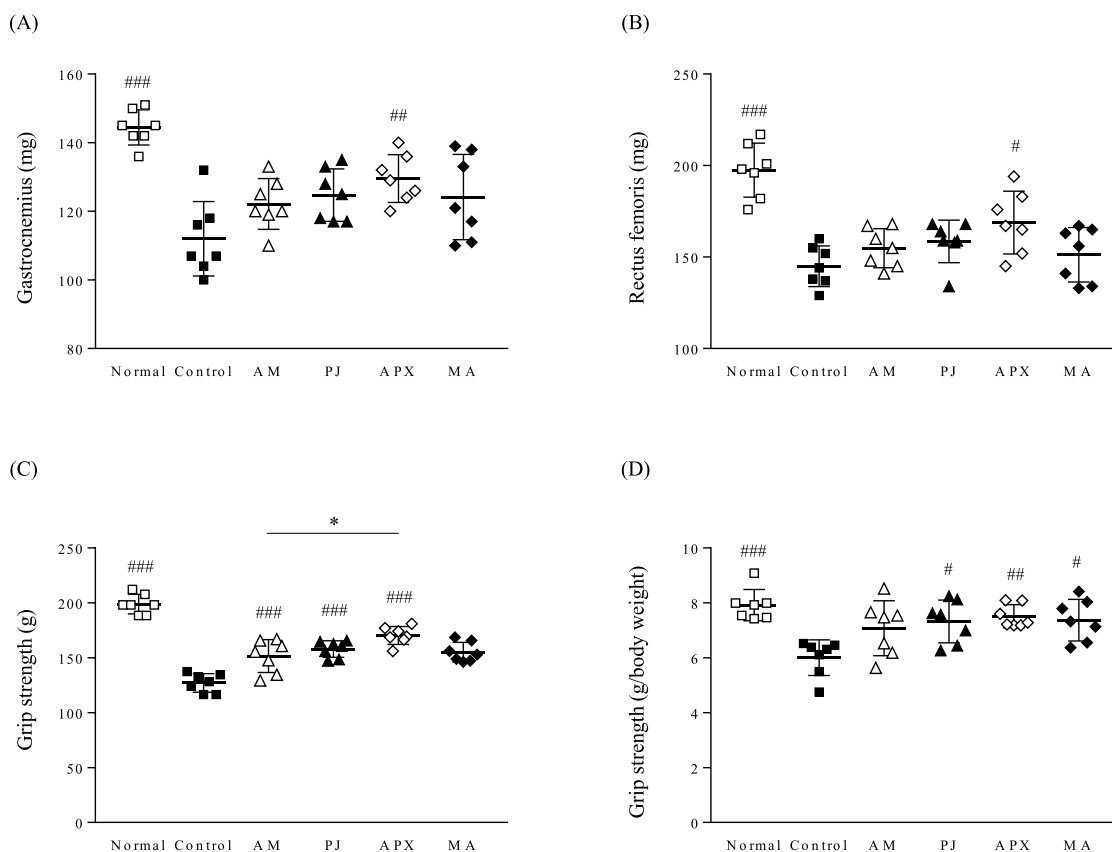


Fig. 3. Muscle weight and grip strength. (A) Gastrocnemius and (B) rectus femoris weights were measured on the final day. (C) Grip strength was measured using a BIO-GT3 tester on the day before sacrifice and (D) its values relative to the body weights were calculated. The data are expressed as the means ± SD ($n = 7$). ^{###} $p < 0.01$ and ^{###} $p < 0.001$ compared to the control group; ^{*} $p < 0.05$ compared to the APX group.

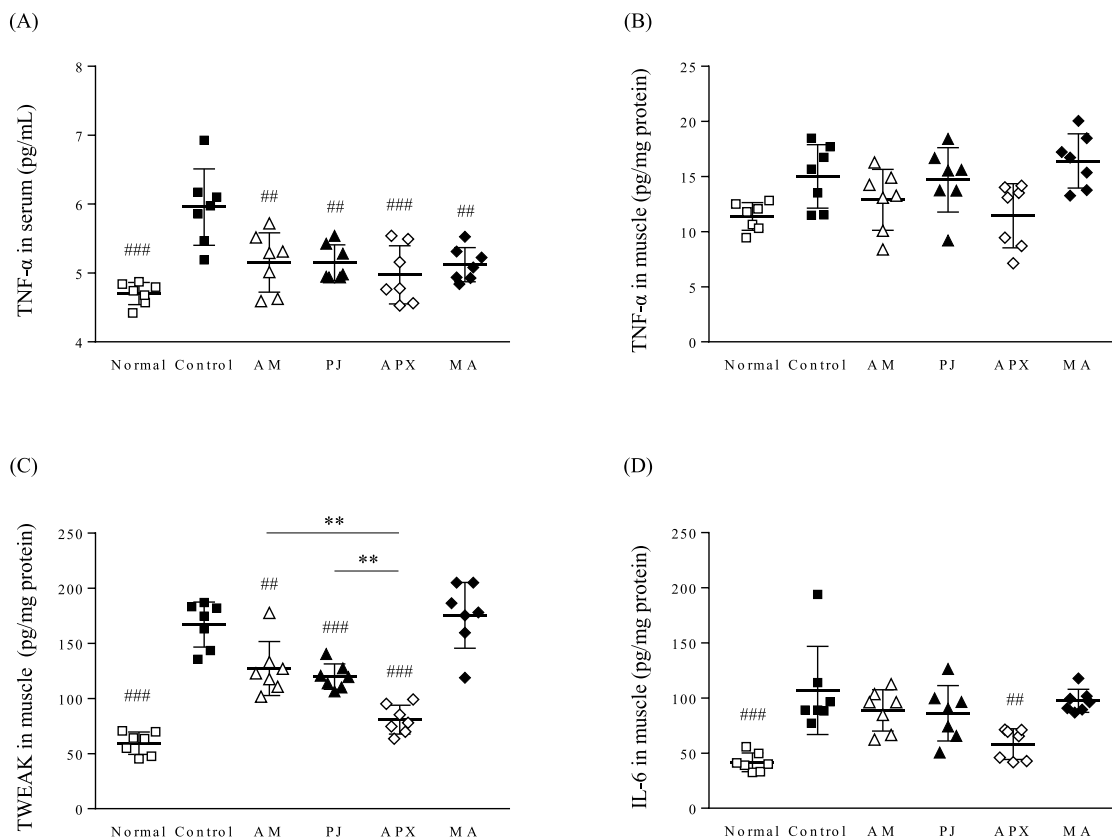


Fig. 4. Determination of pro-inflammatory cytokine concentrations. (A) Serum concentrations of TNF- α were determined using an ELISA kit. Muscle concentrations of (B) TNF- α , (C) TWEAK and (D) IL-6 were determined using ELISA kits. The data are expressed as the means $\pm$ SD (n = 7). #p < 0.05, ##p < 0.01 and ###p < 0.001 compared to the control group; *p < 0.05 and **p < 0.01 compared to the APX group.

elevated the serum concentration of TNF- α , and this increase was significantly attenuated by APX administration (Fig. 4A). The elevated serum TNF- α concentration was consistent with the drastic increase in granulocytes in the peripheral circulation (Table 2). This cachexia-induced systemic inflammation might lead to organ injury via sequentially inflammatory responses in organs such as the liver, kidney, heart and adipose tissue, which result in reductions in the weight of those organs. However, the relative weights (to body weight) of those organs and adipose tissue were not significantly different among 6 groups (Table 1), which indicates the weight loss as a key cause of those alterations. In H&E staining of liver, a little increased infiltration of neutrophils was observed (Supplementary Fig. 1). The results corresponded with the serum levels of AST, LDH and glucose (Table 2). The increased releases of AST and LDH as observed in our study (Table 2), is well known as a byproduct of organ/tissue damage in patients with cancer cachexia (Costelli et al., 1993; Khan et al., 2013).

Moreover, the muscle concentrations of TNF- α , IL-6 and TWEAK were considerably increased in our animal model (Fig. 4B–D). In muscle tissue, inflammatory cytokines such as TNF- α , IL-6 and TWEAK are key players in muscle atrophy and the loss of skeletal muscle, which most commonly develops in cancer cachexia (Dodson et al., 2011; Zhou et al., 2016). TWEAK, as a TNF superfamily member, mediates NF- κ B activation as well as multiple cell death pathways and has recently received attention as a novel therapeutic target for cancer cachexia (Winkles et al., 2006). The increased concentrations of these cytokines were significantly normalized by the administration of AM, PJ and APX. In particular, APX normalized the alteration in muscle TNF- α , IL-6 and TWEAK concentrations more potently than either AM or PJ (Fig. 4B–D). This APX-induced normalization of muscle atrophic cytokines supports the positive findings in muscle weight measurement and grip strength

(Fig. 3A–D). In patients with cancer cachexia, poor quality of life is closely associated with muscle atrophy and reduced activity, and the grip strength test has been broadly used to assess physical activity and muscle function in animal models (Murphy et al., 2012).

To explore the molecular mechanisms underlying the effects of APX against muscle atrophy, we examined the myosin synthesis and NF- κ B pathways in C2C12 myotubes. The NF- κ B/I κ B and p38 signaling pathways have been demonstrated to play instrumental roles in muscle atrophy. These signaling pathways mediate over-expression of E3 ligases such as MuRF and Fbx32 (Bodine et al., 2001). Moreover, MuRF and Fbx32 are key molecules in muscle atrophy via the activation of MyH ubiquitination (Clarke et al., 2007). At least in a cancer cachexia mouse model, inhibition of NF- κ B can significantly protect muscle atrophy by inhibiting the upregulation of MuRF (Cai et al., 2004; Moore-Carrasco et al., 2007). The activation of p38 upregulates Fbx32, which directly induces ubiquitination of eukaryotic initiation factor 3 protein (Zhang et al., 2011; Csibi et al., 2009; Lagirand-Cantaloube et al., 2008). In our cell model, TNF- α treatment dramatically led to a reduction in MyH but a considerable increase in MuRF and Fbx32 as well as the excessive phosphorylation of p38, NF- κ B and I κ B (Fig. 5D and E and 6D and E). The results were in accordance with immunofluorescent staining data on MyH and NF- κ B translocation into the nucleus (Fig. 5A–C and 6A and C). In these results, APX generally showed synergistic activities compared to AM or PJ (p < 0.01, Figs. 5 and 6).

These molecular alterations led to the loss of muscle weight as well as dysfunction of muscle, as shown in our data (Fig. 3A–D). In animal models, the grip strength test has been broadly used to assess physical activity and muscle function (Murphy et al., 2012). APX significantly attenuated both the muscle weights and muscle function (p < 0.01 or p < 0.001, Fig. 3A–D). The above our findings indicate the potential of

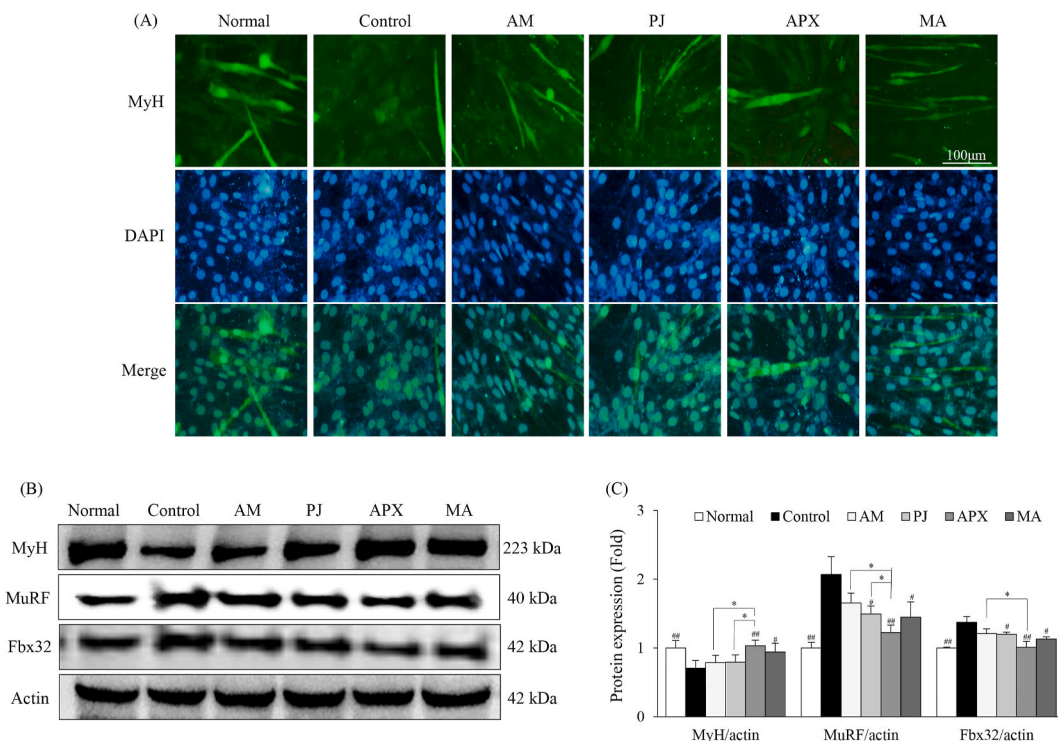


Fig. 5. Muscle atrophy-related proteins and Immunofluorescence staining of MyH in C2C12 myotubes. (A) Immunofluorescence staining of MyH was performed in C2C12 myotubes. Protein levels of (B) MyH, MuRF and Fbx32 in muscle tissue were measured using western blot analysis, and (C) relative quantification with actin was performed by ImageJ software. The data are expressed as the means ± SD (n = 3). #p < 0.05, ##p < 0.01 and ###p < 0.001 compared to the control group; *p < 0.05 compared to the APX group.

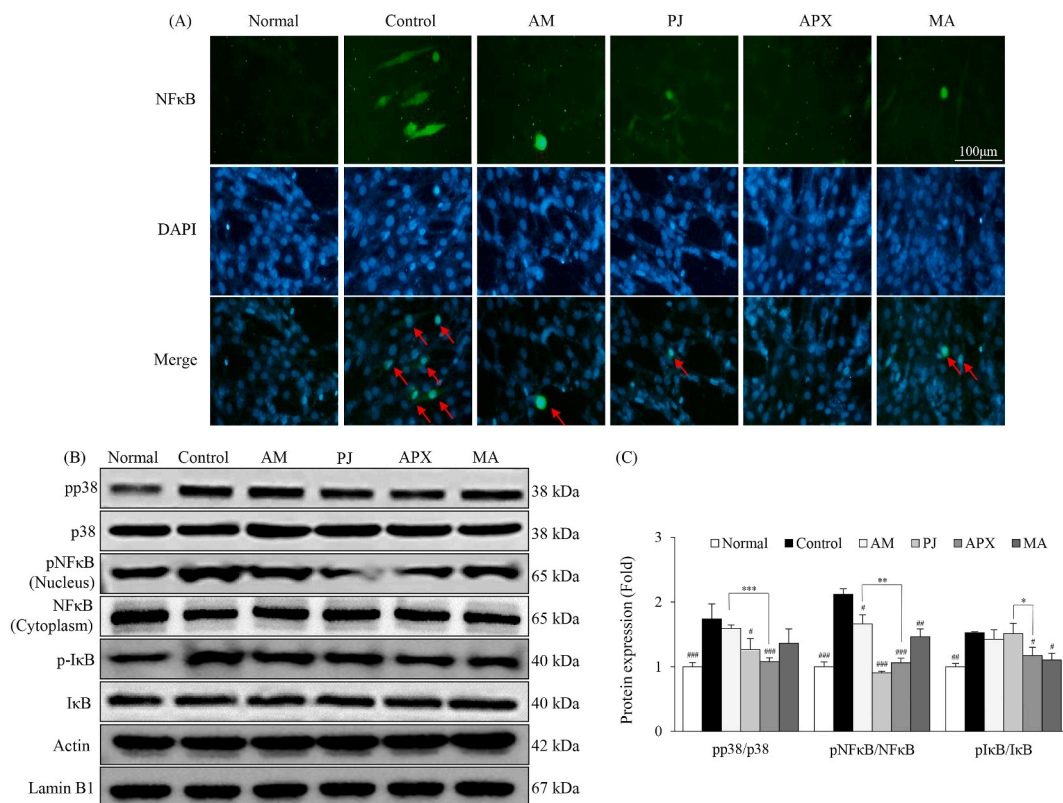


Fig. 6. Activation of NFκB pathway molecules and their translocation in C2C12 myotubes. (A) Immunofluorescence staining to assess translocation of NFκB was performed in C2C12 myotubes. The levels of phosphorylated and inactive forms of (B) p38, NFκB and IκB were measured in C2C12 myotubes using western blot analysis, and (C) the relative quantification of each phosphorylated form with respect to the corresponding inactive form was performed with ImageJ software. The data are expressed as the means ± SD (n = 3). #p < 0.05, ##p < 0.01 and ###p < 0.001 compared to the control group; ***p < 0.001 compared to the APX group.

APX to prevent muscle atrophy. In addition to prevention of muscle atrophy, the amelioration of the whole body weight loss induced by cancer is an important target for further combating cancer using various interventions (Fearon, 2008). Clinically, cancer cachexia is generally diagnosed when more than 5% of the body weight is lost over a 6-month period or 2% of the weight is lost in patients with a BMI of <20 (Wallace and Schwartz, 2002). Our mouse model began to decrease body weight beginning 5 days after injection of C26 cells and finally reduced body weight by approximately 26% (not including tumor) on the last day of the experiment (Fig. 1A and C). Administration of APX (but not AM or PJ) significantly attenuated the whole body weight loss 12 days after injection of C26 cells ($p < 0.05$, $p < 0.01$ or $p < 0.001$, Fig. 1A and C). Anorexia is also a crucial issue associated with weight loss as another major symptom of cancer cachexia (DeWys, 1977). C26 tumor-bearing mice exhibited dramatically suppressed food intake, which was not significantly attenuated by AM, PJ or APX ($p > 0.05$, Fig. 1B). Our data showed synergistic effects of APX on cancer cachexia-induced muscle and body weight loss without a promotive effect on appetite. Many studies have shown improvements in appetite in cancer patients and animal models (Bennani-Baiti and Walsh, 2011; DeWys, 1977); however, unexpectedly, our results did not show a positive effect of MA on food intake (Fig. 1B). In our data, the tumor weight was not affected by any drug (Table 1).

To date, many therapeutic candidates for cancer cachexia have failed. Cancer cachexia is a multifactorial syndrome; however, these candidates generally target specific molecules (Fearon, 2008). Previously, *A. membranaceus* demonstrated the partial anti-muscle atrophy effects in nerve crush and nephrectomy rat models (Lu et al., 2016; Zhou and Mei, 2014). *A. membranaceus* exerted the pharmacological effects on not only cancer but also other disorders likely obesity and oxidative damage (Zhou et al., 2018; Elabd et al., 2020; Huang et al., 2017). Moreover, we found anti-cachectic effects of *P. japonica* in lung and colon cancer-bearing mouse models (Bae et al., 2020b). In the present study, we first showed that APX (a combination of the above two herbs) clearly exhibited synergistic anti-cachectic effects in both a C26 tumor-bearing cachexia mouse model and a TNF- α -treated C2C12 myotube model. As shown in our present study, many studies showed the synergistic actions of poly-herbal formulae similar to the traditional herbal theory (Kim et al., 2017; Lee et al., 2020). Accordingly, we could anticipate the multi-potential applications of this formula composed of two-herbs in the future.

Using HPLC data, we evaluated the main compounds in AM, PJ and APX, for example, formononetin, albiflorin and paeoniflorin (Fig. 1A–E). *A. membranaceus* consists of botanical compounds more than 100 kinds including saponins, flavonoids, polysaccharides and amino acids (Fu et al., 2014), and pharmacological effects of formononetin from used *A. membranaceus* has been reported for anti-tumor and metastasis actions in various cancer cell models (Zhang et al., 2018; Oh et al., 2020). *P. japonica* mostly comprises monoterpene glycosides such as albiflorin and paeoniflorin (Bae et al., 2015). Albiflorin has been reported to attenuate depressive-behavior (Wang et al., 2019) and metabolic syndrome (Zhou et al., 2019). Paeoniflorin is also well known to act as an anti-depressive agent (Li et al., 2020; Mu et al., 2020) along with suppression of NF κ B signaling in gastric carcinoma, oxidative damage and inflammation in rheumatoid arthritis (Jia and He, 2016). Regarding to muscle atrophy, quercetin, another compound of *A. membranaceus*, protected skeletal muscle injury in C2C12 cell and mouse model (Otsuka et al., 2019; Chen et al., 2020). Other studies reported the muscle-related activities of catechin and bisphenol, as two known compounds of *P. japonica*; improvement of skeletal muscle activity in C2C12 cell (Li et al., 2019), and anti-glucose oxidation of skeletal muscle in rats (Indumathi et al., 2013), respectively. We however do not know whether the mentioned compounds above are truly active compounds in our cachexia model yet. Further studies are required to identify the active compounds in APX in the future.

Taken together, our data indicate that APX, a combination of 30%

ethanol extracts of *A. membranaceus* and *P. japonica*, has anti-cachectic effects via the amelioration of body weight loss and muscle atrophy, but not modulation of the appetite. The potential of APX is superior to that of megestrol acetate, and the underlying mechanisms may involve the modulation of muscle atrophy-promoting molecules, including p38, NF κ B, TNF- α and TWEAK.

Author contributions

Sung-Bae Lee designed the overall experiments and wrote the manuscript. Jin-Soek Lee conducted immunofluorescence staining of C2C12 myotubes. Sung-Ok Moon and Hwa-dong Lee performed fingerprint analysis of *A. membranaceus* and *P. japonica* and their combination. Yoo-Sik Yoon measured serum TNF- α concentration. Chang-Gue Son supervised this study and wrote the manuscript. All authors reviewed and approved the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Abbreviations

A. membranaceus	<i>Astragalus membranaceus</i>
AM	30% ethanol extract of <i>A. membranaceus</i>
APX	Combination of AM and PJ
C26	colon-26 adenocarcinoma
DMEM	Dulbecco's modified Eagle's medium
Fbx32	f-box only protein 32
IL-6	interleukin 6
I κ B α	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
MyH	myosin heavy chain
MuRF	muscle ring-finger protein 1
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
p38	mitogen-activated protein kinases
P. japonica	<i>Paeonia japonica</i>
PJ	30% ethanol extract of <i>P. japonica</i>
TNF- α	tumor necrosis factor alpha
TWEAK	tumor necrosis factor-related weak inducer of apoptosis

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2020.113470>.

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