



Contents lists available at ScienceDirect

## Journal of Ethnic Foods

journal homepage: <http://journalofethnicfoods.net>

## Original article

# Function of Korean black ginseng: Improvement of andropause symptoms by a complex extract of black ginseng and fenugreek in TM3 Leydig cells and aged rats



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## ARTICLE INFO

## Article history:

Received 19 July 2016

Received in revised form

9 August 2016

Accepted 19 August 2016

Available online 26 August 2016

## Keywords:

andropause symptoms

black ginseng

fenugreek

leydig cell

testosterone

## ABSTRACT

**Background:** Korean ginseng (*Panax ginseng* C.A. Meyer), the native ginseng of Korea, has traditionally been widely used for thousands of years in Korea. Various biological functions of Korean ginseng have been reported. However, there have not been many studies on its effect on andropause. As women reach menopause, they show a sudden decrease in female hormone levels; men also undergo similar endocrinologic changes. Andropause is also referred to as late-onset hypogonadism, male hormone deficiency syndrome, and male climacteric syndrome. It is linked to increasing age among men and serum testosterone deficiency and specific clinical and biochemical symptoms that accompany it. To improve such andropause symptoms, male hormone supplementation therapy has been attempted. However, owing to various adverse effects, the development of improved therapeutic agents that are safe, both psychologically and physically, is in demand. The present study examined the andropause symptom-alleviating effects of black ginseng and fenugreek complex extract to develop a natural therapeutic agent with minimal adverse effects.

**Methods:** In the present study, we measured the anti-oxidant effect of black ginseng and fenugreek complex extracts using Cell viability of TM3 mouse leydig cells. The mechanism of apoptosis was assessed Erk and Akt kinase activity. We also investigated whether these complex extracts could affect on male hormone and muscle endurance. After administrating black ginseng and fenugreek complex extracts for 4 weeks. The aged rats were sacrificed and checked the testosterone hormone and forced swimming test.

**Results:** The extract increased cell viability, which had been reduced after oxidative stress, confirming the antioxidant effects of the complex, and this effect involved regulation of Erk kinase activation. Moreover, in a hormone-deficient animal model, after oral administration of the complex extract, the group that received 100 mg/kg showed significantly increased total and bioavailable testosterone levels. Besides the levels of sex hormones, those of luteinizing hormone and follicle-stimulating hormone that promote spermatogenesis were also elevated in a forced swimming test for verification of muscle endurance and motor functions, the group that received 100 mg/kg of the complex extract showed improved motor function and increased muscle endurance.

**Conclusion:** Thus, it is believed that the complex extract of black ginseng and fenugreek may be used in the future as a preventive and therapeutic agent for alleviating the symptoms of andropause.

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## 1. Introduction

Korea ginseng (*Panax ginseng* C.A. Meyer), the native ginseng of Korea, has traditionally been widely used for thousands of

years in Korea, as well as China and Japan, as an *elixir* mostly for promoting good health. Currently, the outstanding efficacies of ginseng and its components are also being reported. However, studies on overall aspects of ginseng, including its history of origin, are still lacking. The present study aims to take a brief look at ginseng and black ginseng, one of its processed products. We believe that an additional comprehensive review of ginseng,

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its processed products, and fenugreek will be needed in the future.

### 1.1. History of Korean ginseng

For over 2,000 years, Korean ginseng (*P. ginseng* C.A. Meyer), a native plant of Korea, has been used in Northeast Asia as an important medicinal herb for protecting vitality. In Shen-Nung Pharmacopeia (神農本草經), the oldest herbal pharmacopeia in Asia, it is written that ginseng protects the five viscera and supplements vitality [1]. Ginseng has the scientific name of *Panax ginseng* C.A. Meyer (1843), and, looking at its word origin, *pan* means all and *axos* means medicine, which combined gives the meaning *panacea*. The first record of ginseng being discovered by an alchemist as a tonic or elixir is in a Chinese book from the pre-Han era (BCE 33–48), about 2,000 years ago. In Hanwon (翰苑), there is a record of ginseng being found in Dah-Jang Mountain (長多山) in Koryo (probably Gae Jang Mountain) and record of “Koryo ginseng packaging and transport method...” is found in Gookjungbaekrok (國定百錄). Moreover, Myungibyugok (名義別錄) (456–536) contains a record of Baekje (白帝) ginseng being gifted as a tribute when Song (梁) was the emperor (武帝).

More than that, considering that there were frequent travels between Goguryeo (高句麗) and Cao Wei (魏; CE 220–265), and that there were 92 records of Goguryeo's bringing tribute (健使朝貢), it can be surmised that the ginseng from Goguryeo was also gifted as tribute [2]. The first record of such ginseng being cultivated can be found in *Dohkyungchobon* (圖經草本, CE 1061), which was written during the time of Song (梁) of China. Furthermore, there are records of it in *Sanrimgyeongje* (山林經濟) by Hong of Joseon dynasty as well, while the first record of *gah-sam*, which was the term given to ginseng cultivated in Korea, was in the *Annals of the Joseon Dynasty, the Veritable Record of King Chongjo* [3,4]. In these records, cultivation of Korean ginseng began as early as CE 1000 or so during the Koryo Dynasty, and sharing of ginseng cultivation technology began as writing and distribution of books happened throughout the King Sejong (CE 1419–1450) and King Sukjong (CE 1675–1720) periods during the Joseon Dynasty, when the expression *san-sam* (wild ginseng) was used [5]. Such ginseng is cultivated not only in Korea, but also in China, Northeast Asia, and far eastern regions of Russia. In addition to Korean ginseng (*P. ginseng* C.A. Meyer), there is also American ginseng (western ginseng, *hwa-gi-sam*), *Panax quinquefolius* L., which are being cultivated in the USA and Canada, as well as China in recent times. *Panax notoginseng* (Burk.) F.H. Chen cultivated in China and *Panax japonicus* D.A. Meyer cultivated mainly in Japan are being used commercially.

### 1.2. Types of Korean ginseng

Types of ginseng can be categorized largely into fresh ginseng in its natural form and its processed forms: white, red, and black ginseng. White ginseng can be subcategorized as straight, curved, and semicurved ginseng, depending on its dried form, and the names refer to the ginseng processing methods that involve drying by heat or sunlight. Red ginseng can be subcategorized as heaven-, earth-, and good-grade ginseng, which refer to ginseng that has been dried after being steam cooked or by other methods using raw ginseng, or fresh ginseng with its skin retained. These ginseng processing methods increase ginsenoside content in ginseng and thus such processing methods are being studied. Particularly in Korea, red ginseng has been developed into a variety of products, such as candy, jelly, powder, and extract, which are being enjoyed by not only Koreans but also people worldwide, and, as such, studies related to its processing are being actively

pursued. In addition to red ginseng processing methods, ginseng processed by steaming it nine times, then drying it nine times to take on an exterior color close to black is referred to as black ginseng, which has been reported to have increased new ginsenosides. Unlike active studying of red ginseng, the effects of black ginseng are being studied gradually, while the known effects of black ginseng include anticancer, antithrombin, and immune boosting effects. However, there have not been many studies on its effect on andropause.

### 1.3. Fenugreek (*Trigonella foenum-graecum* L.)

Fenugreek, also used in the present study, is an annual plant belonging to the Fabaceae family, which reaches a height of 0.7–1 m and has pod-shaped fruit. Its leaves have a nutty scent, and it is rich in carotene and consumed as a vegetable. Throughout history, it has been used as a medicinal plant, especially its seeds, in China, India, and Egypt, and has been used as a medicinal ingredient and spice in curries, while it is known to have a bitter taste, its warm temperament is believed to help protect vitality [6]. Pharmacologically, fenugreek is known to possess a variety of effects, such as antidiabetic and anticholesterolemic. However, even though a recent study reported it being effective against male infertility, there have been no studies on its alleviating effects on andropause symptoms [7,8].

Accordingly, the present study aimed to investigate what effects a complex extract comprised of black ginseng and fenugreek (BF) has on andropause.

### 1.4. Andropause symptoms

It is known that each year, men experience decreases in total and free testosterone by 0.4% and 1.2%, respectively, and such decrease in male hormone levels has an impact on sexual function, bone metabolism, muscle and body fat distribution, mood, and cognitive function. As such, decrease in male hormones associated with aging is referred to as partial androgen deficiency in the aging male. Moreover, various symptoms that fall under the category of andropause symptoms caused by decreased sex hormone level are collectively referred to as late onset hypogonadism in men. Typical symptoms include clinical and biochemical manifestations such as nervousness, emotional instability, depression, vertigo, sleep disorder, hypodynamia, memory impairment, diminished work performance, hyposexuality, and reduced muscle strength [9,10].

Andropause symptoms can cause significant damage to quality of life and have negative impacts on various bodily functions [11].

Various therapeutic modalities are being studied to alleviate and treat such andropause symptoms, and the most commonly used therapy is a male hormone replacement method using oral medications, injections, and transdermal agents. However, caution should be exercised when using hormone replacement therapy, and such therapy should only be administered based on accurate diagnosis since it may result in various adverse effects, such as enlarged prostate, growth in prostate cancer tumor size, exacerbation of sleep apnea, gynecomastia, polycythemia, and increased risk of cardiovascular diseases [12]. Therefore, it is believed that if treatments to increase testosterone hormone levels can be developed from foods and natural products, then the adverse effects can be prevented and alleviated in the treatment of andropause. Accordingly, there is an urgent need to develop foods and natural products as therapeutic agents with fewer adverse effects that can prevent and improve andropause symptoms.

In the present study, BF were used to produce the complex extract of BF with an optimal ratio of the two for investigating their efficacy in alleviating andropause symptoms. As a result, the

present study identified its efficacy for alleviating serum testosterone decline, which is known to cause andropause syndrome, as well as its efficacy for suppressing oxidative stress in Leydig cells and improving muscle endurance and motor performance.

## 2. Materials and methods

### 2.1. Fenugreek complex material selection

The complex extracts of BF used in the present study were provided by Kwang Dong Pharmaceutical Co., Ltd. (Seoul, Korea). After screening for the efficacies of BF extract, the extracts were mixed by optimal ratio and used in the study. Black ginseng used in the present study underwent primary extraction for 12 hours at 70°C in 70% ethanol and secondary extraction using the same method with 30% ethanol. Finally, it was extracted at 100°C using 100% purified water, and dried by spray dry method for use. Fenugreek seeds were extracted for 4 hours at 75°C using 70% ethanol, and dried by spray dry method for use. The dried extracts were used as a complex extract with optimal ratio which have most effective in cell level screening of black ginseng and fenugreek.

### 2.2. Cell culture

TM3 cells, mouse-derived Leydig cells, are cells found in the interstitial tissue within the testes and they were procured from Korean Cell Line Bank. TM3 cells were cultured at 37°C and 5% CO<sub>2</sub>, using Dulbecco's modified Eagle medium (Life technology, St Louis, MO, USA) containing 10% fetal bovine serum and 1% penicillin–streptomycin. After inoculating  $1 \times 10^5$  cells/well onto a 96-well plate and cultivating for 24 hours.

### 2.3. Cell viability measurement

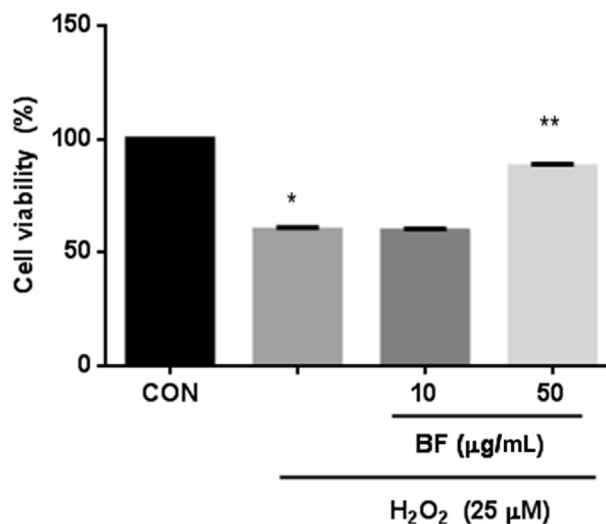
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was performed to test for a cytotoxicity of the complex extract. After inoculating the cells onto a 96-well plate, the complex extracts were treated by concentrations after 24 hours. Then, after additional culturing for at least 16 hours, they were treated with 100  $\mu$ L of 5 mg/mL MTT after the medium was collected. After culturing for at least 2 hours, MTT crystals were dissolved with dimethyl sulfoxide, and a microplate reader was used to measure the absorbance at 570 nm.

### 2.4. Efficacy verification on oxidative stress

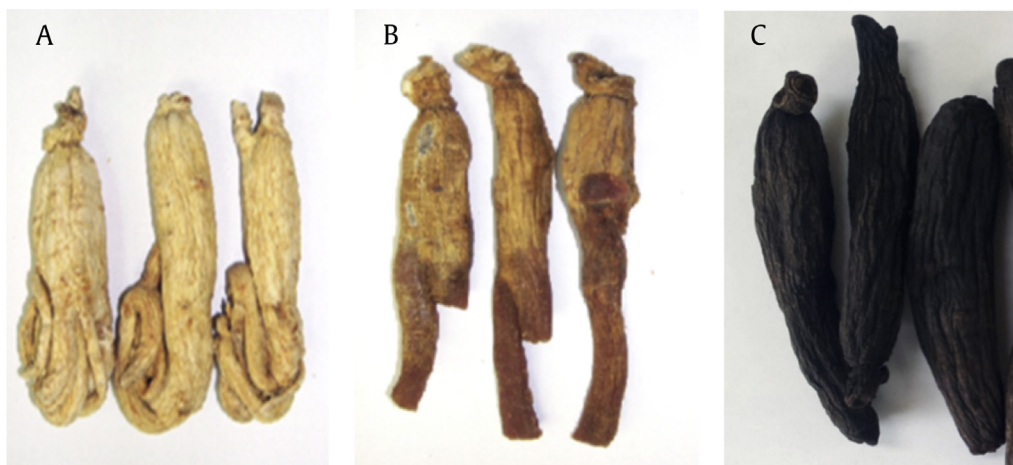
Reproductive cells, including spermatogonia, react sensitively to oxidative stress and they show endogenous antioxidant mechanism, such as antioxidant enzymes, to facilitate recovery of cells damaged by external oxidative stress. When intracellular antioxidant capabilities that protect the cells from oxidative stress are degraded, cell apoptosis occurs. The present study aimed to verify whether the complex extract had antioxidant effects by inducing oxidative stress in TM3 cells. After inoculating  $1 \times 10^5$  cells/well onto a 96-well plate and culturing for 24 hours. After the additional culturing, MTT assay was performed to measure cell viability.

### 2.5. Test on mechanism involved in cell apoptosis inhibition

Extracellular signal-regulated kinase (ERK), a signaling molecule, is activated by active oxygen and external stimulus to be involved in cell survival and proliferation. The present study aimed

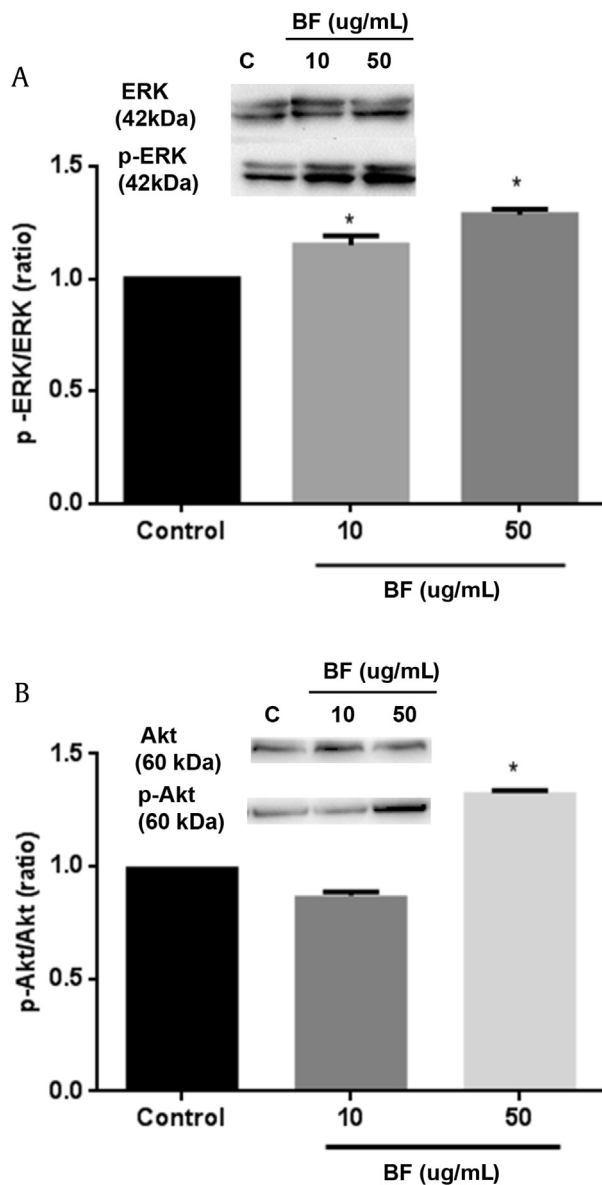


**Fig. 2.** Protective effect of black ginseng and fenugreek (BF) extracts against H<sub>2</sub>O<sub>2</sub> induced TM3 Leydig cells. Cell viability was confirmed by the MTT assay. Values indicate the mean  $\pm$  standard error of the mean and presents the results obtained from three independent experiments. \*  $p < 0.001$  and \*\*  $p < 0.05$  indicate that the mean value was significantly different from the treated group.



**Fig. 1.** Korean ginseng. (A) White ginseng, (B) red ginseng that has been dried after being steam cooked or by other methods using raw ginseng, and (C) black ginseng that has been dried nine times after being steam cooked nine times to take on an exterior color close to black.

to investigate whether fenugreek complex extract can play a role in activation of ERK in TM3 cells. TM3 cells were inoculated onto a six-well plate, and after 24 hours, the cells were treated with extract mixed with BF at ratios of 1:9 and 3:7 (black ginseng:fenugreek). After 30 minutes, the cells were collected and proteins extracted. The extracted proteins were quantified via Bradford technique and 50  $\mu$ g of the proteins were loaded into SDS-PAGE and separated by electrophoresis. After transferring to a nitrocellulose membrane, the primary antibodies anti-ERK (1:1,000), antiphospho ERK (1:1,000) anti-Akt, and phosphor anti-Akt (Cell Signaling Technology, Danvers, MA, USA) were used for blotting. After reacting with the secondary antibodies, the proteins were exposed to light with enhanced chemiluminescence to check for their level of expression.



**Fig. 3.** Effect of black ginseng and fenugreek (BF) extracts on p-Erk phosphorylation in TM3 cells. After administering the complex extract, proteins were extracted 30 minutes later and their level of expression was measured via Western blot method. Values indicate the mean  $\pm$  standard error of the mean and presents the results obtained from three independent experiments. \*  $p < 0.05$  indicate that the mean value was significantly different from the control group.

## 2.6. Establishment of animal model

Because the andropause model is used in a similar manner as the male sexual function enhancement model, there is no specific model that has been pre-designated. The present study chose a model that could naturally exhibit symptoms of reduced sex hormones, rather than an artificial sexual dysfunction model. Rats were procured from Central Lab Animals, Inc. (Seoul, Korea). After allowing the 26-week-old rats to be acclimated for 1 week, the complex extract was administered orally, every day at 9 AM for 4 weeks. The rats were divided into control, 100 mg/kg, and 500 mg/kg groups with eight rats per group, and fed accordingly. In the 3rd week, a forced swimming experiment was performed to assess improvements in muscle endurance and motor functions. In the 4th week, blood was collected and organs were harvested for tests on sex hormones, tissue weight, and liver toxicity.

## 2.7. Verification of complex extract testosterone increase efficacy

It has been reported that, after the age of 40 years, men started to experience a decrease in male hormone levels of 2.5% per annum, with 50% of men aged 40–49 years and 70% of those 70 years or older showing signs of male hormone deficiency. Such a decrease in male hormone levels is the cause of various diseases and sexual dysfunction. The present study used aged rats in investigating whether fenugreek complex extract could elevate male hormone levels that have been reduced. After administering fenugreek complex extract for 4 weeks, the rats were sacrificed and their blood collected. Serum was separated from the blood collected and the level of testosterone in serum was measured. Bioactive testosterone level was calculated from measurements of serum albumin and sex hormone binding globulin (SHBG) levels.

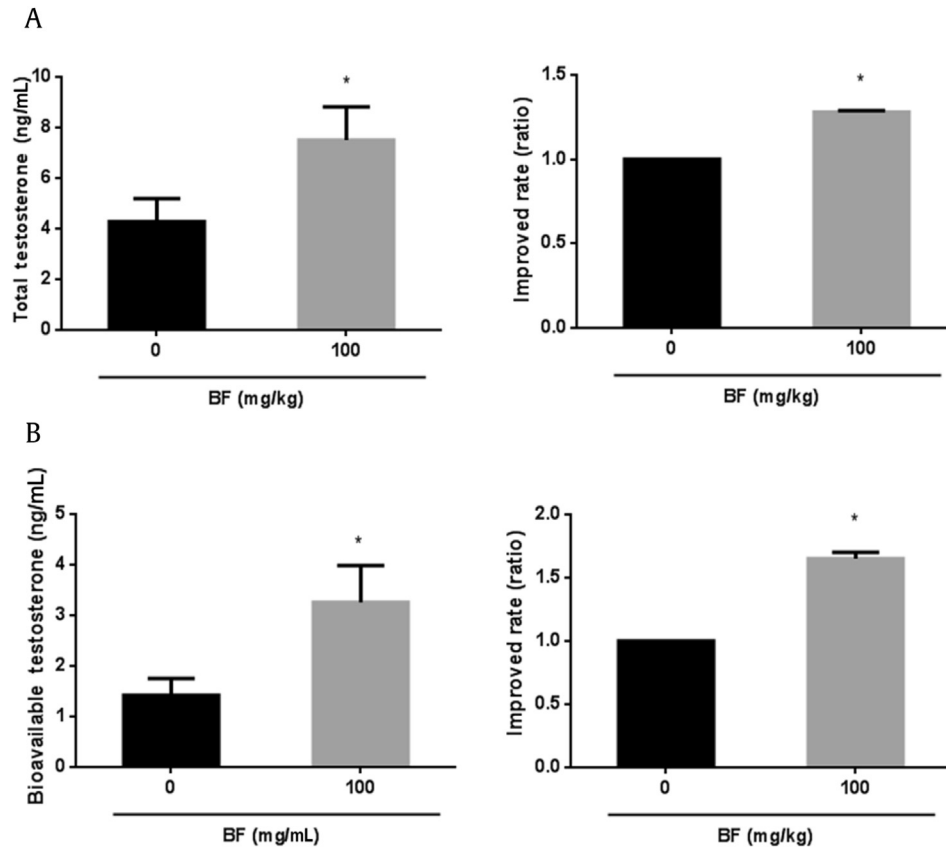
## 2.8. Verification of indicators related to liver function test and lipid metabolism- using complex extract

Liver function test includes liver enzyme test, as both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the most useful indicators of liver cell damage. In the stage right before complete destruction of liver cells, as a supply of energy to the liver cells decreases, AST and ALT are released from the cells, and these enzymes spread quickly in the blood, which makes it easy to measure their levels. In the present study, for investigation of what effects fenugreek complex extract administration has on liver function of animal models, blood was collected and serum was separated for measurements using an ALT/AST kit (Roche, Mannheim, Germany).

Ageing-related testosterone decline has been reported to regulate lipid metabolism-related markers to reduce high-density lipoprotein cholesterol. In the present study, the effects of fenugreek complex extract on increase or decrease in low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were examined using the respective measurement kit (Roche).

## 2.9. Assessment of muscle endurance and motor performance via forced swimming experiment

During the 3rd week of complex extract administration, a forced swimming experiment was performed to assess muscle endurance. A clear plexiglass cylinder (50 cm  $\times$  20 cm) was filled with water (25–27°C) up to a volume where the rat's tail would not touch the bottom. Then, a rat from each group was placed into a cylinder and forced to swim for 12 minutes. Immobility time, when the rat showed little movement or was floating after swimming actively,



**Fig. 4.** Increased serum total and bioavailable testosterone by the black ginseng and fenugreek (BF) extract. Serum testosterone levels after complex extract administration were measured and significant effect was exhibited when 100 mg/kg was administered. \*  $p < 0.05$  indicates significance.

and high activity time, when the rat was swimming actively, were measured blindly, and this was measured using Smart v3.0 video tracking system (Panlab Harvard Apparatus, Barcelona, Spain).

### 2.10. Statistical analysis

Data were statistically analyzed using GraphPad Prism statistics (GraphPad Software, San Diego, CA, USA) and significance was analyzed using *t* test and ANOVA.

## 3. Results and discussion

### 3.1. Protective effects of each extract against oxidative stress

To investigate the protective effects of complex extracts of BF against oxidative stress, TM3 cells were cultured and treated with 20  $\mu\text{g/mL}$  hydrogen peroxide and 50  $\mu\text{L}$  each of concentrations equivalent to  $2\times$  the concentration of each extract (20  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ , and 200  $\mu\text{g/mL}$ ) to yield final concentrations of 10  $\mu\text{g/mL}$  hydrogen peroxide and 10  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , and 100  $\mu\text{g/mL}$  BF complex extract. The cells were then cultured for an additional 24 hours. After culturing, supernatant was removed and the cells were treated with 100  $\mu\text{L}$  MTT solution diluted in phosphate-buffered saline at 5 mg/mL concentration. The cells were cultured for at least 2 more hours and absorbance was measured at 570 nm. The results showed a trend of cell viability that had been reduced by 40% from hydrogen peroxide increasing in a concentration-dependent manner when treated with black ginseng (Fig. 1). When treated only with BF extract, cytotoxicity was not observed (data not shown).

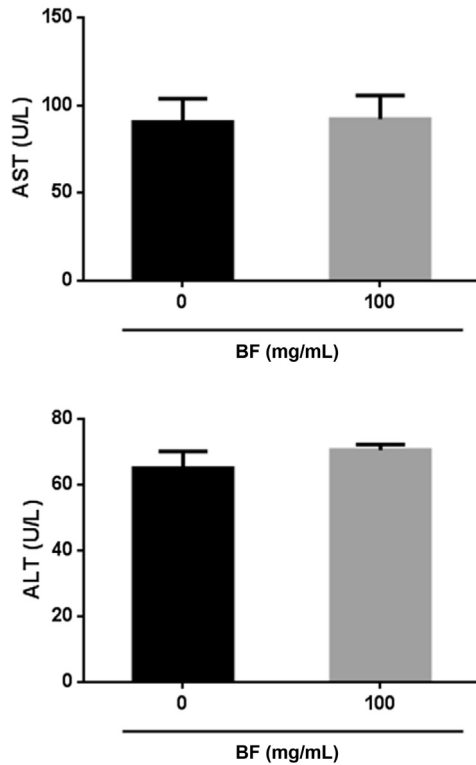
### 3.2. Activation of cell survival mechanism by complex extract

ERK, a signaling molecule involved in cell survival and proliferation, is activated by active oxygen and external stimuli. The present study aimed to investigate whether BF complex extract can play a role in activation of ERK in TM3 cells. TM3 cells were inoculated onto a six-well plate, and after 24 hours, the cells were treated with the complex extract. After 30 minutes, the cells were collected and proteins were extracted. Western blot was performed on the extracted proteins and the results indicated that the 50  $\mu\text{g/mL}$  treatment group showed significantly higher activation, 1.3 times greater, than the control group, and the results were more significant than that of the complex used as the positive control (Fig. 2).

### 3.3. Efficacy of increasing testosterone levels in animal model

In the human body, regulation of testosterone secretion is achieved through the hypothalamic–pituitary–gonadal axis, and the gonad stimulating hormones, luteinizing hormone (LH) and follicle-stimulating hormone, are regulated by testosterone, while LH acts upon the LH receptors inside Leydig cells in the gonads to regulate testosterone synthesis inside the gonads. Biologically useful testosterone undergoes a significant decrease after age 55 years, which is known to occur from aging-related increase in physiologic concentration of SHBG, which binds strongly to hormones, such as testosterone, to reduce hormone activities [13,14].

Testosterone has a direct proportional relationship with muscle strength and bone density, and as such, a decrease in testosterone



**Fig. 5.** Assessment of effects on each tissue and liver toxicity after complex extract administration. Liver tissues were harvested for investigation of whether the complex extracts exhibit *in vivo* toxicity after administration. When 100 mg/kg was administered, there was no difference with the control group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BF, black ginseng and fenugreek.

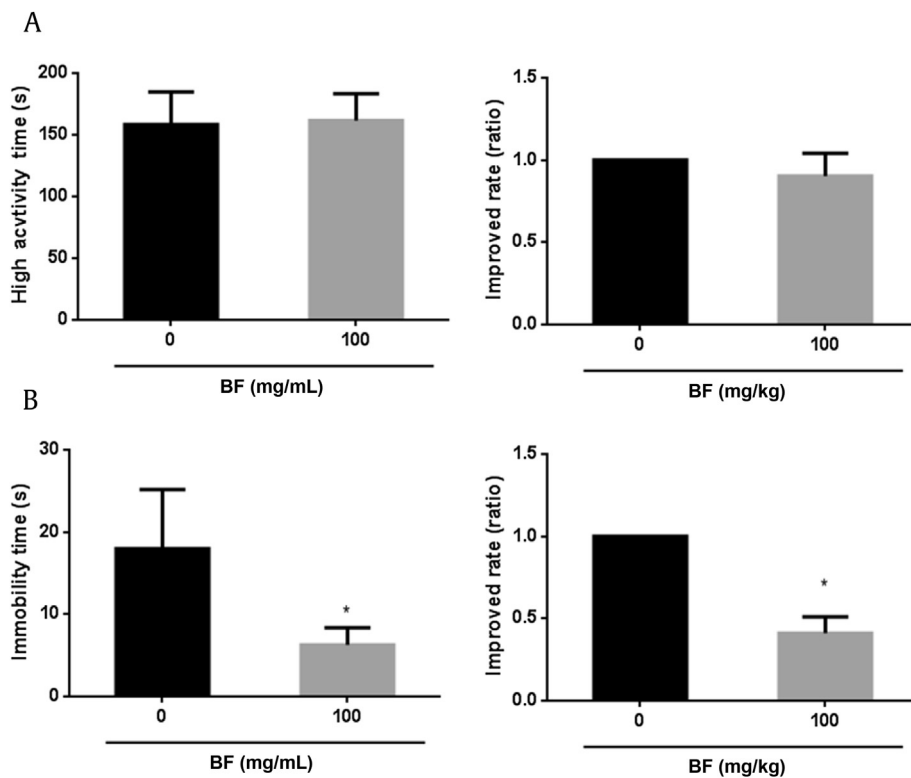
content results in diminished motor function or reduced muscle endurance from decreased muscle strength.

In men, testosterone is an important circulating steroid, that is produced mostly in the testes. The diagnosis of late onset hypogonadism uses free and bioavailable levels calculated from total testosterone and SHBG levels measured from blood collected between 7 AM and 11 AM. The present study also used the same method as above to collect blood and separated the serum for each measurement.

In order to investigate whether the complex extract can show increased hormone levels in aged rat andropause model, 26-week-old male rats were orally administered 100 mg/kg and 500 mg/kg of the complex extract for 4 weeks. The complex extract was administered every day at 9 AM and after 4 weeks, tissues were harvested and blood was collected. Serum was separated from the collected blood and testosterone, SHBG, and albumin levels were measured. The results showed that total testosterone in the 100 mg/kg group had increased by 1.2 times, as compared with the control group, while bioavailable (free) testosterone in the 100 mg/kg group increased significantly by 1.7 times (Fig. 3).

### 3.4. Liver toxicity of complex extract

While investigating whether sex hormones had increased in the animal model, the present study also examined the levels of ALT and AST, liver function markers, and weight of each tissue to determine whether the complex extract had an abnormal effect on liver function or caused tissue damage. The results showed that there were no changes in weight and phase of each tissue for all groups and changes in ALT and AST, which appear during liver dysfunction, also was not observed, based on which it was determined that the complex extract was not toxic in the animal model (Fig. 4).



**Fig. 6.** Assessment on enhancement of muscle endurance and motor performance using a forced swimming experiment for black ginseng and fenugreek (BF) extract. After allowing the rats to practice a swimming for 10 days, (A) swimming time and (B) retention time were measured for 30 seconds without any stoppage in movement. \*  $p < 0.05$  indicates significance.

### 3.5. Verification of enhanced in muscle endurance and motor performance of complex extract

After allowing the 26-week-old rats to be acclimated for 1 week, 100 µg/mL and 500 µg/mL of the complex extract was orally administered every day for 4 weeks. In the 3rd week, a forced swimming experiment was performed to assess muscle endurance and motor performance. After 4 weeks, blood was collected and tissues were harvested for use in the experiment. Decreased agility and muscle endurance, which are physical symptoms of andropause syndrome, may be the outcome of aging, but muscle endurance may decline from decreased testosterone level as well. In the present study, a forced swimming experiment was applied to determine what effects the complex extract had on muscle endurance in the aged rat model. The results showed that, as compared with the control group, the 100 mg/kg group had increased swimming time (Fig. 5). Moreover, immobility time measured from not moving before becoming exhausted or just floating in water was also decreased. This demonstrates that the complex extract administration enhanced muscle endurance and motor performance (Fig. 6).

Andropause syndrome from stress, as well as aging, is increasing among middle-aged men. Andropause can cause not only male sexual dysfunction, but also other psychological and physical problems, and thus, prevention of andropause is important from social and economic perspectives. Men often end up receiving hormone therapy without being aware of the fact that they were experiencing andropause. In Korea, an estimated 15–20% of men aged 40 years or older exhibit the symptoms of male hormone deficiency, and, worldwide, the market for male hormone supplements is growing at a rapid pace and the current market size for Korea is estimated to be approximately 12 million dollars. However, because of various adverse effects associated with current supplements, a development of functional health foods that can address these problems is expected to play an important role in preventing andropause. It is believed that the fenugreek complex extract developed in the present study will play an important role as a novel natural ingredient that can alleviate the symptoms of andropause by elevating testosterone hormone level, as well as

preventing andropause through its antioxidant effect and by increasing muscle strength and endurance.

### Conflicts of interest

The authors declare no conflicts of interest.

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