

## **The Effect of Fermented Antler Extract in Prevention of Osteoporosis or Reduced Physical Activity in Females during Menopause**

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### **Abstract**

*We was confirmed that the antler extract increases the expression of marker genes expressed in the process of bone formation, and that the effect on the increase in the expression of the gene is further increased by fermentation of the antler extract. In addition, the mouse model in which menopausal was induced by ovary extraction significantly reduced the movement distance and exercise time of mice compared to the control group. But the decrease was somewhat alleviated by the administration of the antler extract, and completely restored when the fermented antler extract was administered. In the menopause-induced mice, the body weight ratio of heart, liver, and spleen weights increased compared to the control group, but the antler extract and the antler ferment extract restored the body weight ratio of various organ weights to the level of the control group in the menopause-induced mice. Consequently, this has led to mitigating changes in the metabolism affected by menopause.*

**Keywords:** *Fermented Antler Extract, Osteoporosis, Physical Fitness, Osteoarthritis, Menopause*

### **1. Introduction**

Menopause in females lasts between one to two years from the abolition of menstruation. When the female's reproductive function is lost, it is the period in which female transitions from sexual maturity to old age. The age of menopause can vary between individuals. It may depend on one's nutritional status or number of children the one has delivered. With extension of life expectancy, the onset of menopause seem to have been slightly delayed, but statistically it is considered to be 45 to 55 years old [1-3]. Before menopause, the secretion of oestrogen and progesterone in the body gradually decreases. Consequently, one experiences symptoms of menopause. Commonly, women can have osteoporosis, which develops from having bone erosion being continued while bone formation being slowed down. Osteoporosis is common in postmenopausal women, but it can also be caused by malnutrition or reduced activity. Other symptoms of menopause include hot flashes, tachycardia, sweating, fatigue, anxiety, depression, memory loss, insomnia, and muscle weakness [4-6]. Hormone replacement therapy is the most efficient way of reducing menopausal symptoms. It is known

that the administration of female hormones increases bone density and reduces fractures. It is also known to be effective in maintaining elasticity and thickness of the skin after menopause. However, if one has a history of endometrial cancer or hormone dependent breast cancer, hormone replacement therapy can increase the risk of recurrence as well as increasing the risk of hypertension, thrombosis, biliary stones and abnormal uterine bleeding. Based on the following problems, studies have been actively conducted to develop plant-derived oestrogen that exhibits the same activity as oestrogen without involving any side-effects. In general, phytoestrogen is a substance that has a structure and function very similar of oestrogen. Isoflavones found in soy and lignans found in linseed are examples of different type of phytoestrogen. However, the direct effect of these phytoestrogens in relieving menopausal symptoms has not yet been clearly elucidated, and phytoestrogens have been reported to potentially cause stroke, heart disease and breast cancer if taken for a long time [7-9].

The deer's antlers fall off and reappear every year around the breeding season. Antler velvet forms between spring and summer. This is a period before calcification. During this time, antler velvet has blood vessels inside and is flexible. As it continues to grow, blood supply reduces and the calcium inside the antler begins to harden. When dry enough, velvet falls off within 24 hours. The nature of antler velvet is warm and non-toxic, and it tastes sweet and salty. It is known for acting on the liver, kidney, heart and pericardium to support basic physiological functions such as reproduction and growth [10-12]. In oriental medicine, antler velvet had been regarded as the best blood tonic since ancient times, and as documented in Donguibogam, antler velvet has tonic action, nutrimentoaction, analgesic action, hematopoietic action, growth promotion action, and heart failure treatment action. In addition, it has been proven to have effects such as recovery from fatigue, enhancement of physical vitality, inhibition of bone resorption and strengthening of renal function. However, antler velvets have side effects such as diarrhoea, and are used for the prevention and treatment of various diseases as herbal medicines removed [13-15]. Accordingly, there is a need for a treatment method capable of increasing functionality while facilitating absorption of the antler extract. In modern society, the standard of living has been improving along with the economic growth. The number of various diseases have been increasing due to stress and dietary imbalances caused by complex social life, and consequently, demand for health functional food to prevent and manage possible diseases have increased. In recent years, health functional food have been developed to be convenient to take and have improved sensuality beyond simply emphasising functionality [15-17]. Antler velvet is sourced from an animal and contains high amount of protein and ash. There were limitations in product development using antler velvet due to its peculiar taste. This study is to inhibit precipitation of antler velvet extract and develop a fermented antler velvet extract using lactic acid bacteria. It is also the aim to maintain its high functionality such as its ability to enhance intestinal absorption and its therapeutic effect on osteoporosis and decreased motility of menopausal women, and have its peculiar taste and smell removed.

## **2. Experiment Materials and Methods**

### **2.1 Preparation of Antler Extract Sample**

Antler of a Korean bred deer, three years old, was obtained in July. The samples were divided into upper, middle and lower parts and cut into thin pieces so that the thickness was less than 5 mm.

### **2.2 Bacteria and Protease Enzymes Materials**

In the fermentation part of this experiment, four of the following products of bacteria, which are licensed from the Ministry of Food and Drug Safety, *Bifidobacterium longum*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, Mixture of eight types of lactobacillus were used. In order to hydrolyse the protein of antlers

before the fermentation, the following product of protease, which was licensed from the Ministry of Food and Drug Safety, Maxazyme NNP DS (*Bacillus amyloliquefaciens*) were used.

### **2.3 Experimental Reagents**

Folin & ciocaltue's phenol reagent (sigma F-9252), aluminium chlorid 6-hydrate (Yakuri Pure Chemical Co., Japn: Test No. 01709), gallic acid (G0448) and quercetin (Sigma Q-0125) were used together with polyphenols and flavonoids in standard quantities. 2,2-Diphenyl-1-picrylhydrazyl (sigma, D9132), linoleic acid (s2240-250 mL) and 2-thiobarbituric acid (T5500-25 g) were used as a reagents for measuring DPPH radical scavenging and TBA antioxidant activities. For other reagents and solvents, extra pure grade reagents were used.

### **2.4 Measurement Equipment**

Microplate reader (Spectrophotometer, Thermo Fisher Scientific: Type 1510), Incubator (SANYO, CO<sub>2</sub> incubator), Evaporator (EYELA, Type N-N), Centrifuge (Hanil, Mega 17R), Shaking incubator (Vision Scientific, VS-8480SF).

### **2.5 Preparation of Antler Water Extract**

Upper, middle and lower parts of the antler, were mixed into a ratio of 1:1:1. 509.7 g of the mixture was put into a stainless steel extractor and extracted at 100 Celsius for 24 hours. The extract filtered using a filtering cloth. It was then concentrated under a low pressure in the evaporator at 70 Celsius into 10 Brix. Then as an incomplete product, the resultant was stored in a refrigerator at 4 Celsius. Before the fermentation, purified water was added into the product, diluting it into 5 Brix. The following product was adjusted to 2940 mL and was used as the substrates for antler water extract. Each 500 mL of the antler water extract substrate was transferred into PP vessels and stored in a freezer at minus 20 Celsius. Before to be used as a sample for the fermentation and experimentation of its protease enzyme activity, it was taken out of the freezer and defrosted in the 50 Celsius water bath.

### **2.6 Evaluation of Cytotoxicity**

The cytotoxicity of antler extract (DA) and fermented antler extract (FDA) against U2OS, a human osteoblast, and RAW264.7, a mouse-derived macrophage, was measured through MTS assay. To this end, U2OS and RAW264.7 cells were aliquoted in a 96-well plate. Fermented antler extract was added at a concentration of 0, 0.13, 0.25, 0.50, 1.00 or 2.00 mg/mL and treated for 24 hours, followed by MTS assay. Cell viability was measured using the method.

### **2.7 Animal Research Design**

To evaluate the effect of antler extract and fermented antler extract on bone formation, U2OS, an osteoblast, was treated with antler extract and fermented antler extract and their effect on the expression of osteogenic differentiation gene was confirmed. The effect of antler extract and fermented antler extract on immune function was determined via inflammatory response index, which was evaluated by treating macrophage RAW 264.7 cells with antler extract and fermented antler extract. Female rats (C57BL/6J) induced with osteoporosis through ovariectomy were provided with a feed mixed with antler extract and fermented antler extract for 12

weeks. Bone density, exercise capacity and blood were analysed to evaluate the effect of antler extract and fermented antler extract on osteoporosis.

### 2.8 Evaluation of the Effect of Fermented Antler Extract on Bone Formation

Human osteoblasts U2OS were treated with the antler extract and fermented antler extract prepared in Examples 1 and 2 at a concentration of 1.0 or 2.0 mg/mL, and cultured for 48 hours. Marker genes expressed in the osteoblastic process of osteoblasts, RUNX2, SP7 and OCN were quantified by rtPCR. The expression level was measured as the mRNA expression level relative to the expression level of GAPDH.

## 3. Result and Discussion

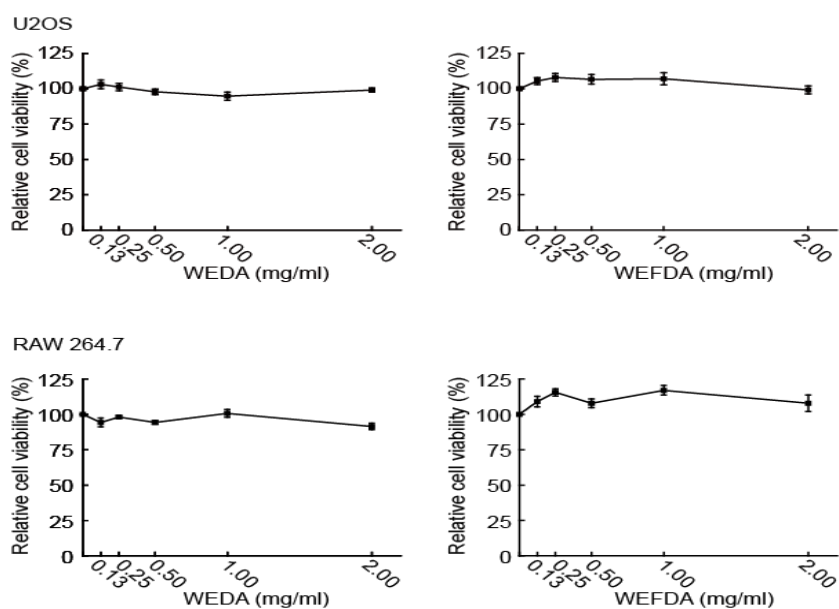
### 3.1 Evaluation of Cytotoxicity of Antler Extract and Fermented Antler Extract

In order to confirm the effect of antler extract (Water extract of deer antler; WEDA) and fermented deer antler (WEFDA) on cellular activity, human-derived osteoblast U2OS and mouse-derived macrophage RAW 264.7 were treated and their activity was measured [17]. MTS is a method of measuring the activity of Intracellular Mitochondrial dehydrogenase (NADH). Cells with high metabolism effectively reduce MTS. At this time, the absorbance of the generated formazan is measured to confirm cell activity and viability. Each cell was dispensed in a 96 well plate and treated with antler extract and fermented antler extract at different concentrations and cultured for 24 hours. Then, cell activity and toxicity were confirmed using MTS reagent. Based on the following results, the concentration for securing the safety of the functional material was confirmed. The concentration for the treatment of cells was determined afterwards (Figure 1).



**Figure 1. Measurement of cell activity by MTS assay.**

Cells were treated with antler extract and fermented antler extract at a concentration of 2.0 mg/mL for 24 hours. As a result, there was no significant change in cell activity. Based on the following results, both substances were evaluated to have no significant cytotoxicity up to a concentration of 2.0 mg/mL. Their effects on bone formation and inflammation were confirmed by treating the cells up to a concentration of 2.0 mg/mL (Figure 2).



**Figure 2. Cytotoxicity evaluation of U2OS osteoblasts and RAW 264.7 macrophages.**

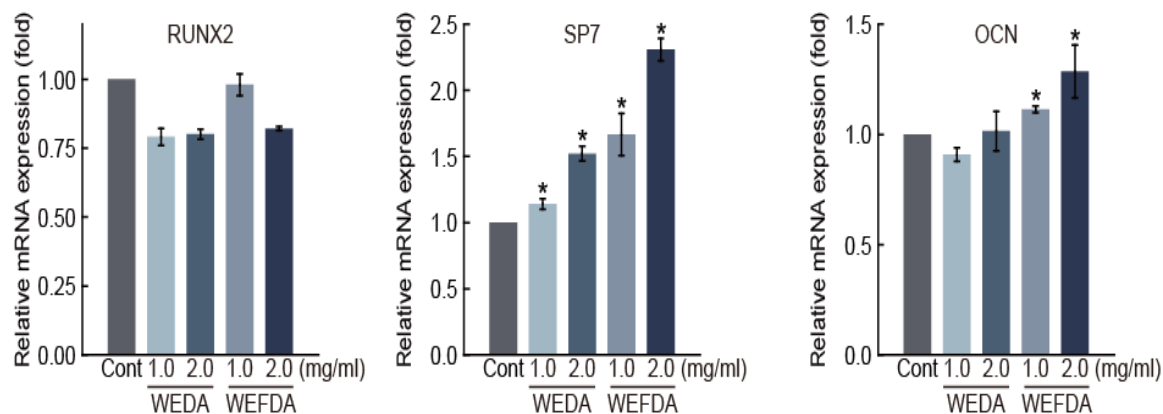
### 3.2 Confirmation of Effects Antler Extract and Fermented Antler Extract on Bone Formation.

Osteoblast U2OS with antler extract and fermented antler extract to evaluate their effect on bone formation. For this purpose, U2OS cells were treated with antler extract and fermented antler extract at different concentrations for 48 hours. The level of gene expression during osteoblastic process of osteoblasts was compared by performing real-time quantitative PCR analysis. In this experiment, relative mRNA expression levels were compared based on GAPDH, and marker genes RUNX2, SP7, OCN, which are known to increase expression levels as osteoblasts differentiate and form a skeleton, were selected and compared. The sequences of primers used for analysis in this experiment are summarised in the table below (Table 1).

**Table 1. Sequences of the Primer to evaluate osteogenicity of U2OS cells**

Gene		sequence (5'→3')
RUNX2	F	TGG ACG AGG CAA GAG TTT CA
	R	TCC CGA GGT CCA TCT ACT GT
SP7	F	GAT CTG GTG CCT AGA AGC CC
	R	CAA GCT CCA GCG GCT TTA AC
OCN	F	GCT CCA GGG GAT CCG GGT A
	R	AAG CCC AGC GGT GCA GAG T
GAPDH	F	TTG AGG TCA ATG AAG GGG TC
	R	GAA GGT GAA GGT CGG AGT CA

While RUNX2 mRNA expression level did not change significantly, SP7 mRNA expression level increased by  $1.52 \pm 0.06$  when treated with 2.0 mg/mL of antler extract. SP7 mRNA expression level increased by  $2.31 \pm 0.08$  when treated with fermented antler extract at 2.0 mg/mL. OCN mRNA expression level increased by  $1.28 \pm 0.01$  when treated with 2.0 mg/mL of fermented antler extract. It is known that expression level of OCN and SP7 increases during bone differentiation. Based on this fact, it was confirmed that bone formation was prompted by antler extract and was enhanced when treated with fermented antler extract (Figure 3).



**Figure 3. Effect of antler extract and fermented antler extract on the expression of osteogenic differentiation genes**

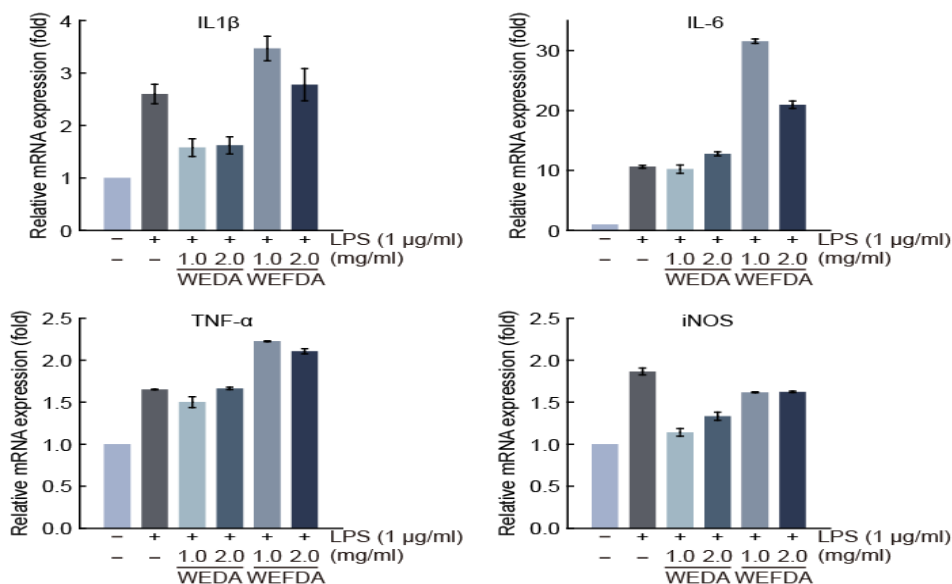
### 3.3 Evaluation of efficacy on inflammatory response

RAW 264.7 cells, a mouse macrophage, was treated with antler extract and fermented antler extract for 24 hours. These were treated with lipopolysaccharide (LPS), a component of the bacterial outer wall, to induce an immune response, then lysed to extract RNA synthesised. In this experiment, mRNA expression levels were compared based on  $\beta$ -actin. The expression levels of IL-1 $\beta$ , IL-6, TNF $\alpha$ , and iNOS, which are inflammatory cytokines expressed when an inflammatory response is induced in macrophages, were also compared to determine the degree of inflammation. The sequences of primers used for analysis in this experiment are summarised in the table below (Table 2).

**Table 2. Primers to evaluate the induction of inflammation in RAW 264.7 cells**

Gene		sequence
IL-1b	F	AGG TCA AAG GTT TGG AAG CA
	R	TGA AGC AGC TAT GGC AAC TG
TNF-a	F	AGG GTC TGG GCC ATA GAA CT
	R	CCA CCA CGC TCT TCT GTC TAC
iNOS	F	CAG CTG GGC TGT ACA AAC CTT
	R	CAT TGG AAG TGA AGC GTT TCG
IL-6	F	GTC CTT CAG AGA GAT ACA GAA ACT
	R	AGC TTA TCT GTT AGG AGA GCA TTG
$\beta$ -actin	F	TCA CCC ACA CTG TGC CCA TCT ACG
	R	CAG CGG AAC CGC TCA TTG CCA ATG

A tendency to increase or decrease the expression level of inflammatory cytokines was not observed significantly in RAW 264.7 cells treated with antler extract. IL-1 $\beta$  and IL-6 expression was observed at 1mg/mL in RAW 264.7 cells but they did not show any change in the expression levels in concentration-dependent manner. Through these results, it was confirmed that the antler extract and the fermented antler extract did not significantly affect the inflammation-inducing response in RAW 264.7 cells under the present experimental conditions (Figure 4).



**Figure 4. Effect of antler extract and fermented antler extract on inflammatory response.**

### 3.4 Effect of Antler Extract and Fermented Antler Extract on Bone Density in Rats

8-week-old C57BL/6 mice was given 1 week stabilisation period before ovarian extraction (OVX) was used to induce menopause and osteoporosis. The effect on bone density was confirmed by providing the mice mixed feed containing antler extract and fermented antler extract. Anaesthesia was carried out by IP injection (10  $\mu$ l/g of body weight) with avertin (2-methy-2-butanol). After a 1 week of recovery period post surgery, the mice was provided with a mixed feed containing antler extract and fermented antler extract. In an environment that is maintained at 25 $^{\circ}$  C and provides a 12-hour day/night cycle and sufficient water, antler extract and fermented antler extract formula feed were provided for 24 hours. The change in body weight according to the feed formulated with antler extract and fermented antler extract was examined for 12 weeks. During this period, the group that consumed the feed formulated with antler extract and fermented antler extract showed significant increase in weight. Total volume, bone volume, volume ratio (BV/TV), bone surface, area to volume ratio (BS/BV), bonemass density, number of trabecular, trabecular thickness and trabecular separation of the left leg skeleton of a rat was examined through CT scan. It was confirmed that bone density decreased through extraction, but no significant change was confirmed by the feed formulated with antler extract and fermented antler extract (Figure 5, 6).

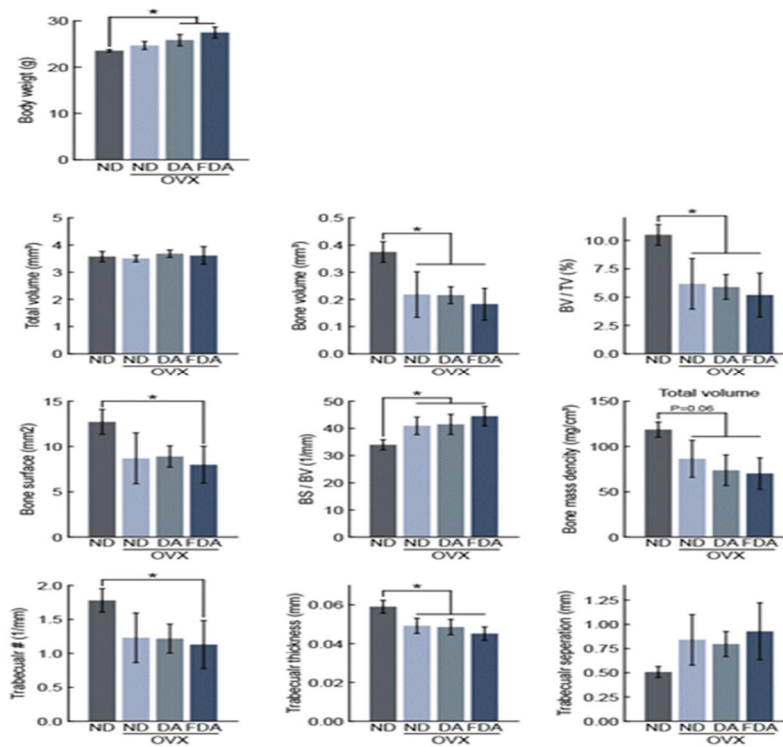


Figure 5. Changes in body weight and bone density by antler extract and fermented antler extract formula.

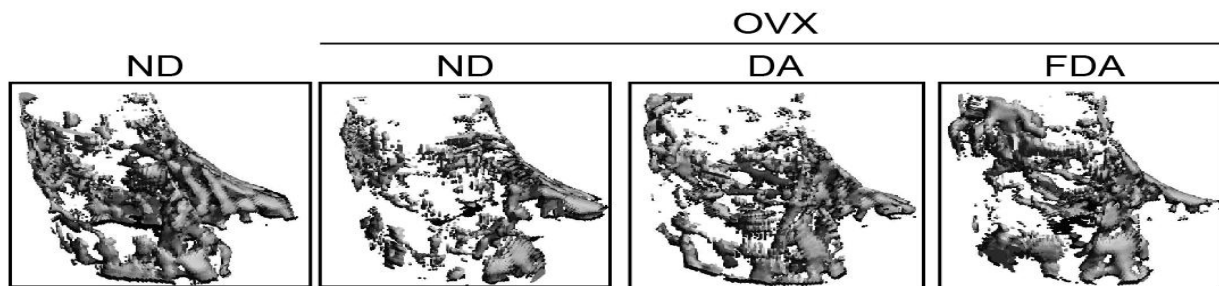
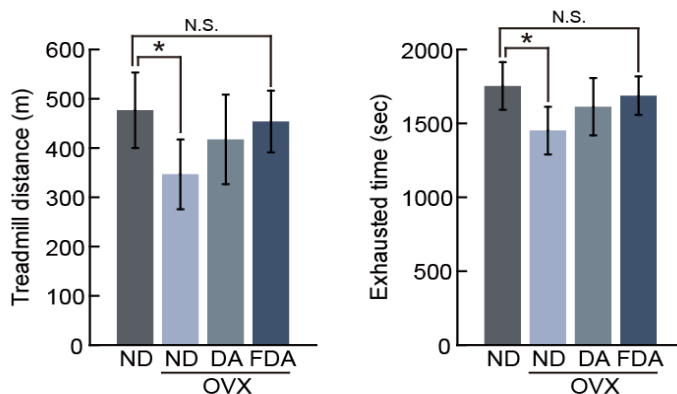


Figure 6. BMD analysis of each group through CT scan.

### 3.5 Effect of Antler Extract and Fermented Antler Extract Formulated Feed on Motility

The treadmill test was conducted to evaluate the motility of mice that received a feed formulated with antler extract and fermented antler extract for 12 weeks. The treadmill test involves a gradual increase of the speed from 10 cm/sec to 45 cm/sec at 0° inclination, and proceeds until it lags behind more than 10 seconds. The motility was compared based on the exercised distance and time. The exercised distance of ovariectomized group was  $346.6 \pm 70.7$  m. This was considered as a decrease in motility compared to the other group that exercised  $476.6 \pm 76.3$  m. The group fed with antler extract exercised  $476.6 \pm 76.3$  m while the other group fed with fermented antler extract exercised  $453.6 \pm 62.6$  m. Based on the following result, it was confirmed that the motility of the mice was reduced through ovariectomy. But it was also confirmed that this decrease in motility was restored by ingesting the antler extract, and that this effect was enhanced by fermenting the antler (Figure 7).

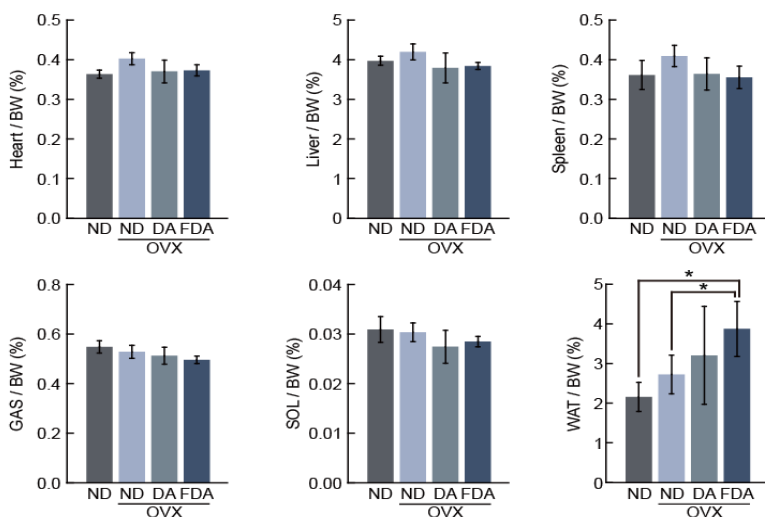




**Figure 7. Mobility enhancement by antler extract and fermented antler extract formula feed.**

### 3.6 Stability Evaluation of Antler Extract and Fermented Antler Extract

After extracting the heart, liver, spleen, gastrocnemius (GAS) and soleus (SOL) muscles and white fat from the mice that received functional cheese formulation for 12 weeks, the weight was recorded to determine what effect of antler extract and fermented antler extract on metabolism make sure you have given. The weight of the rats that received the antler extract increased significantly. Each organ was standardised and compared by the body weight. There was no significant difference in the weight of the organs except for white fat. It was confirmed that the weight of the white fat of rats increased through ovariectomy. But, the increase in weight of the white fat in rats was also observed in the group taking antler extract.

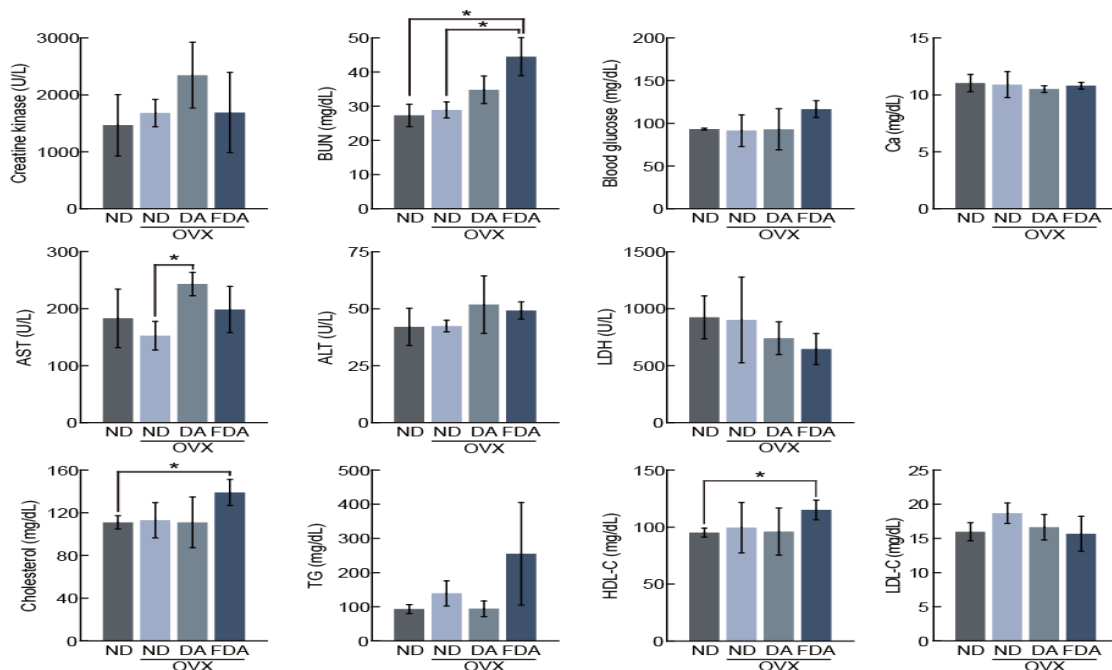


**Figure 8. Comparison of weights of major organs in each group.**

### 3.7 Stability Evaluation of Antler Extract and Fermented Antler

Serum analysis was performed to evaluate the effect of antler extract and fermented antler extract on the metabolism of mice. Serum analysis confirmed the blood levels of creatine kinase, which indicates the degree of tissue damage, blood urea nitrogen (BUN), which indicates the level of renal toxicity, blood glucose level, blood calcium concentration and levels of AST, ALT, and LDH, which are indicators of liver toxicity. Levels of blood triglycerides (TG), HDL and LDL were also analysed to examine the effect of antler extract and fermented antler extract on lipid metabolism of mice. It was confirmed that blood urine protein and cholesterol

HDL levels increased in the group that consumed fermented antler extract. But no significant changes were seen in the levels of other indicators (Figure 9).



**Figure 9. Serum analysis of each group.**

## 5. Conclusion

It was confirmed that the antler extract increases the expression of marker genes expressed in the process of bone formation, and that the effect on the increase in the expression of the gene is further increased by fermentation of the antler extract. In addition, the mouse model in which menopause was induced by ovary extraction significantly reduced the movement distance and exercise time of mice compared to the control group. But the decrease was somewhat alleviated by the administration of the antler extract, and completely restored when the fermented antler extract was administered. In the menopause-induced mice, the body weight ratio of heart, liver, and spleen weights increased compared to the control group, but the antler extract and the antler ferment extract restored the body weight ratio of various organ weights to the level of the control group in the menopause-induced mice removed.

Consequently, this has led to mitigating changes in the metabolism affected by menopause. In addition, the study prevents diarrhoea by facilitating intestinal absorption by hydrolysing the antler extract with proteolytic enzymes and then fermenting with lactic acid bacteria. It also carries less of its unique flavour and taste, and suppresses the formation of precipitates in liquid products. Therefore, this study has shown that antler extract can be utilised more efficiently and with enhanced functional properties via fermentation.

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