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A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

The study on the muscle reinforcing materials inducing myogenesis differentiation and growth factors from marine alga

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GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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(Supervised by Professor You-Jin Jeon)

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CONTENTS

국문초록

도핑(Doping)은 운동선수들이 경기력 증가를 위하여 금지된 약물을 복용하거나 금지된 방법을 사용하는 것으로 정의된다. 18세기경 남아프리카의 부족 의식에서 사용되넌 흥분 효과가 있는 음료인 ‘Dop’에서 그 용어가 처음 유래되었다고 한다 (Verroken, 2000; 김은국, 2014).

도핑을 방지하기 위하여 세계도핑방지기구(World Anti-Doping Agency, WADA)에서는 매년 9월에 금지약물 및 금지방법을 발표하여 금지목록 국제표준을 만든다. WADA는 금지목록 국제표준에서 금지한 약물의 복용, 흡입, 주사, 피부 접척 및 혈액제제, 수혈, 인위적 산소 섭취 등 금지된 방법을 사용하거나 사용 행위 은폐, 부정거래를 하는 모든 행위뿐만 아니라 그러한 행위를 시도하는 것까지 도핑방지 규정 위반으로 정의하고 있다.

도핑 금지로 지정되어있는 약물 중 동화작용제(Anabolic steroids)는 남성 호르몬인 테스토스테론의 유도체로써 근육을 강화시키는 anabolic 효과와 남성 호르몬의 역할을 수행하는 androgenic 효과를 가지고 있는 합성 스테로이드 호르몬이다. Anabolic steroids는 체내의 단백질 합성 증가를 통해 근육과 뼈를 강화시켜서 운동선수의 경기력을 향상 시킨다. 그러나 anabolic steroids를 과다하게 복용할 경우 간종양, 내분비 기능 장애, 황달 등의 부작용을 유발시킬 수 있다.

최근에는 천연 화합물을 이용한 근육 성장 소재를 개발하기 위한 연구가 대두되고 있다. 사과껍질 성분인 우르솔산과 인삼 혹은 홍삼 추출액이 근육 성장을 유도한다고 알려져 있다(Kunkel et al., 2012). 특히 해조류는 풍부한 미네랄과 비타민, 다당류 및 폴리페놀 등이 풍부하게 함유되어 있다(Kang et al., 2012a, Kim et al.,

2014). 해조류 중 감태(*Ecklonia cava*)는 다당류와 polyphenol 성분이 주로 함유되어 있고, 그 중 polyphenol 성분인 phlorotannins이 항산화, 항암, 항고혈압, 항당뇨, 항비만 등과 같은 다양한 생리활성효과를 가진다고 보고되어 왔다(Kang et al. 2012b, Lee et al., 2010, Athukorala et al., 2006, Kang et al., 2013, Ko et al., 2013). 하지만 해양생물을 이용한 근육 성장에 대한 연구는 미비한 실정이다. 따라서, 이 연구에서는 제주도 연안에 서식하고 있는 갈조류인 감태로부터 얻은 phlorotannins 계열의 물질로부터 근육의 성장 및 분화를 유도할 수 있는 소재를 밝히고자 했다.

먼저, 감태로부터 얻어진 phlorotannins compound가 근아세포인 C2C12의 성장에 어떠한 영향을 미치는지 현미경과 세포분석기를 통하여 확인하였다. 그 결과, 6가지의 phlorotannins 중 dieckol(DK)과 2,7'-phloroglucino-6,6"-bieckol(PHB)가 세포를 증식하여 과형성(hyperplasia) 시키는 것을 확인하였다. 또한, DK와 PHB가 근육 성장에 어떤 기작을 통해 작용을 하는지 western blot anaylsis를 통해 확인하였다. 그 결과, DK는 Smad 경로를 down regulation 하여 근육성장을 유도시켰고, p-Akt와 p-FKHR (p-FoxO)을 활성화시켜 근육성장을 유도하였다. PHB 또한 Smad 경로를 down regulation 하였고, p-Akt를 활성화시켜 근육성장을 유도하였다. Smad 경로는 그 위에 성장분화인자인 myostatin이 activin type II receptor에 결합을 함으로써 신호를 전달받게 된다. myostatin 신호 전달의 억제인자로는 follistatin, follistatin-like 3 protein, growth and differentiation factor-associated serum protein-1 (GASP-1) 등이 있으며 이러한 인자들은 활성 myostatin이 수용체와 결합하지 못하도록하여 신호전달을 억제하는 것으로 알려져 있다. 따라서, 본 연구에서는 DK와 PHB가 위와 같은 억제인자들과 유사한 작용을 할 수 있는지 molecular docking을 통하여 확인해 본 결과, DK와 PHB는 myostatin과 activin receptor II B가 결합할 수 있는 4가지의

active sites 중 각각 active site 1과 active site 3에 강하게 결합하는 것을 확인할 수 있었다.

반면, 인슐린유사성장인자인 insulin-like growth factor 1 (IGF-1)은 근육 성장의 양성 조절자로 phosphoatidylinositol 3-kinase(PI3K)/Akt 경로의 인산화를 통해 myostatin과 같이 근육 성장을 억제시킬 수 있는 전사 인자들의 신호 전달을 억제시켜서 근육 크기를 조절한다. IGF-1은 IGF-1 receptor에 결합을 함으로써 신호를 전달하게 된다. 수용체와 결합한 IGF-1은 PI3K 경로를 통하여 Akt와 FoxO의 과발현을 유발하여 근육양을 증가시킨다. 따라서, DK와 PHB가 IGF-1과 같이 IGF-1 receptor에 결합하여 Akt와 FoxO의 발현을 증가시킬 수 있는지를 확인하기 위하여 molecular docking을 통하여 DK와 PHB를 IGF-1 receptor에 결합시켜 본 결과, DK와 PHB가 에 결합하는 것을 확인할 수 있었다.

이 모든 결과를 종합해 볼 때, 근육을 성장 및 강화시키는 합성 의약품을 대신하여 천연 소재인 감태를 통하여 DK와 PHB를 자연적으로 섭취하게 됨으로써 근육을 성장시키거나 강화시킬 수 있을 것으로 사료되어진다.

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ABSTRACT

Muscle growth is regulated via a complex signaling pathways associate with myostatin and phophatidylonistiol 3-kinase (PI3K)/Akt. Myostatin is an extracellular growth factor mostly expressed in skeletal muscles and known to play a crucial role in the negative regulation of muscle mass. Upon the biding to activin type IIB receptor, myostatin can initiate several different signalling cascade which is the down regulation of the important for myogenesis genes. Growth and differentiation of the muscle is regulated by a complex interplay of myosatin signalling with the PI3K/Akt pathway responsible for increase in protein synthesis in muscle. Accordingly these findings, in the present study, I evaluated C2C12 myoblasts proliferation and differentiation activities of phlorotannins [dieckol (DK), 6,6'-bieckol (6,6-BK), 2,7'-phloroglucinol-6,6"-bieckol (PHB), phlorofucofuroeckol A (PFFA), phloroglucinol-6,6'-bieckol (PBK), and phloroglucinol (PG)] isolated from *Ecklonia cava*. Among the 6 phlorotannins, DK and PHB showed effect of proliferation of C2C12 myoblasts. In addition, DK and PHB significantly enhanced down-regulation factors such as p-Smad2/3, and Smad4 and up-regualation factors including p-Akt, p-FoxO, and MyoD. Interestingly, DK and PHB were binded with myostatin. Thses findings indicate that DK and PHB isolated from *E. cava* confirmed to specific blocking antibodies of myostatin. Therefore, in this study show the muscle reinforcing materials inducing myogenesis differentiation and grwoth factors from brown alga, *E. cava*.

1. INTRODUCTION

Myostatin (MSTN) known as growth/differentiation factor-8 is a member of transforming and growth factor β (TGF-β) family. MSTN is a potent negative regulator of skeletal muscle growth and maintenance (Jo and Jin, 2012). Mutation of MSTN gene have been reported to cause dramatically increased muscle mass in mice. Futhermore similar effects were observed in the presence of natural mutation of MSTN gene in cattle, sheep, dogs, and humans (Elkina et al., 2011). Active myostatin mostly binds to the ActRIIB receptor and triggers the signalling cascade leading to the inhibition of myoblast differentiation and proliferation. Binding of myostatin to the ActRIIB receptor induces the phosphorylation of two serine residues of Smad2 or Smad3 ar COOH domains. This leads to the assembly of Smad2/3 with Smad4 to the heterodimer that is able to translocate to the nucleus and activate traasncription of target genes (Joulia-Ekaza, and Cabello, 2007; Kolias, and McDermott, 2008). Specific inhibitors can be prevent binding of myostatin to ActRIIB in serum. One of them is follistatin that bids myostatin and inbits its activity by preventing its binding to the receptor (Lee, and McPherron, 2001). Overexpression of follistatin in vivo leads to muscle hypertrophy similar to the one observed in *Mstn* null mice (Lee, and McPherron, 2001). Follistatin-like 3 protein has more than 30% homology with follistatin and was also shown to bind circulating myostatin (Hill et al., 2002). In addition another action of myostatin inhibition of growth and differentation factor-associated with serum protein 1 (GASP-1). Apart from that GASP-1 able

to bind to the myostatin propeptide and possibly regualte the activation of myosatin through proteolytic cleavage (Hill et al., 2003). Moreover, myostatin can be inactivated upon covalent binding to laten TGF- β binding protein 3 (LTBP3). This inactive complex of myostatin/LTBP3 is used for myostatin sotrage in the extracellular matix (Anderson, Goldberg, and Whitman, 2008). Taken together, myostatin is regulated by at least four different inhibitors via binding of the active or latent form. So far, for these proteins have been demonstrated to act effectively as myostatin signalling blockers both *in vitro*, in cell culture, and also in animals (Lee, and McPherron, 2001). In principle blocking of myosatin signalling can be achieved by three different pharmacological strategies. First, there is the posibility to inactivate or neutralise myostatin gene expression. This can be achieved by knocking out the gene and breeding of transgenic animals (Mallory, and Vaucheret, 2004). Second one is inhibition of myostatin signaling pathway by blocking the activation of myostatin protein. Recently, it was shown that inhibitors of metalloproteases increase the fusion of C2C12 myoblasts and provoke myotube hypertrophy (Huet et al., 2001). A third strategy to inhibit myostatin signalling is to block its binding to the receptor. This can be achieved by either expression of the propeptide follistatin or by the sue of specific blocking of antibodies (lee, and McPherron, 2001).

One of the main positive regulators of muscle growth is insulin-like growth factor 1 (IGF-1). The mechanism by switch IGF-1 regulated myostatin signalling includes the inhibition of transcription fafctors responsible for the induction of atrogenes via phosphorylation with

phosphoatidylinositol 3-kinase (PI3K)/Akt pathway (Elkina et al., 2011). Metabolism energy is regulated by Forkhead box O (FoxO) transcription factors as well as the formation of skeletal muscle and adipose tissue consider as organel for energy distribution. FoxOs take part in the formations of skeletal muscle and adipose tissue as major argans for energy distribution (Hribal et al., 2003). During the myoblast proliferation, FoxO1 and FoxO3 are localized in the cytoplasm in the latent, phosphorylated form. Upon initiation of differentiation, FoxO translocates to the nucleus where it binds to DNA and regulates transcription. Akt regulates the activity of FoxO1 and FoxO3 by phosphorylating them and therby retaining them in the cytoplasm (McFarlane et al., 2006). In myotube culture, the level of FoxO1 in the nucleus was reported to increase after treatment with myostatin (Sandri et al., 2004). Expression of constitutively active FoxO1 in transgenic mice led to decrease in myoblast differentiation and reduction in muscle weight (Kamei et al., 2004). In muscle, FoxOs are known to interact with Smad3 and Smad4 inducing the protein degradation. Recently, it was shown that FoxO1 and Smad synergistically increase the expression of myosatin mRNA and its promoter activity in C2C12 myotubes but via different pathways (Allen, and Unterman, 2007).

Theses studies shown that PI3K/Akt pathway, which transcription factors responsible for differentiation of myoblasts and hypertrophy of myotubes are suitable target signal for development of muscle reinforcing.

2. MATERIALS AND METHODS

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal vobine serum (FBS), horse ser um (HS), penicillin-streptomycin, trypsin-EDTA, and Dulbecco's Phosphate Buffered Saline (DPBS) were purchased from Gibco-BRL (Burlington, Ont, Canada). Protein assay kit and ECL detection reagent were bought from Bio-Rad (Richmond, CA, USA) and Amershan Bioscience (Piscataway, NJ, USA), respectively. Antibodies against phosphor-Smad2/3, Smad4, phosphor-Akt, phosphor-FoxO1, MyoD, and β-Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All the other chemicals used were analytical grade.

2.2. Isolation of phlorotannins from *Ecklonia cava*

The marine brown alga E. cava was collected along the coast of Jeju Island, Korea, between March 2013 and June 2013. To remove salt, sand, and epiphytes attached to the surface, the samples were washed three times with tap water and stored in a medical refrigerator at -20ºC. The frozen samples were lyophilized and homogenized with a grinder prior to extraction. The dried *E. cava* powder (500 g) was extracted with 80% aqueous ethanol and filtered. The filtrate was evaporated under reduced pressure at 40ºC to obtain

ethanol extract, which was suspended in water, then extracted with ethyl acetae (EtOAc). The EtOAc extract (46.27 g) was subjected to Shephadex LH-20 column, and then finally purified by reversed-phase HPLC to obtained compound dieckol (DK), 2,7'-phloroglucinol-6,6"-bieckol (PHB), phlorofucofuroeckol A (PFFA), 6,6'-bieckol (6,6-BK), phloroglucinol 6,6'-bieckol (PBK), phloroglucinol (PG). We describe the ligand structure of the myostatin inhibitor candidate in Table 1.

2.3. Cell culture

Mouse C2C12 myoblasts (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS and antibiotics, at 37ºC and 5% CO_2 humified incubator.

2.4. Cytotoxic assessment using MTT assay

Cytotoxicity was assessed using an MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is also a colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). The cells were seeded into 24-well plates at a concentration of 5.0 x 10^4 cells/ml. After 70% confluent of cultures, the cells were treated with isolated phlorotannins (10 nM) from *E. cava*. Then, the cells were incubated for an additional 48 h at 37ºC. MTT stock solution (50 μl; 2 mg/ml in DPBS) was then applied to each of the wells, to a total reaction volume of 250 μl.

After 4 h of incubation, the plates were centrifuged for 5 min at 800 x *g*, and the supernatants were aspirated. The formazan sal was dissolved in DMSO and absorbances was measured via ELISA reader at a wavelength of 540 nm.

2.5. Cell proliferation

The proliferation effect of sample on C2C12 cell was determined by colorimetric MTT assay. C2C12 cells were seeded into a 24-well culture plates at 5.0 x 10^4 cells/ml for 70% confluent of culture. And then, cells supernatant was changed into HS differentiation medium. The cells were treated with 10 nM of dihydrotestosterone (DHT, Sigma-Aldrich, St.Louis, MO), 1 μM ursolic acid (UA, Sigma-Aldrich, St.Louis, MO) and various concentrations of sample (5, 10, 20 and 40 nM) and incubated for 48 h. After incubation, 50 μL (stock concentration 2 mg/mL in DPBS) of MTT solution was added into each well and cells were incuabted at 37ºC for 4 h. The supernatant was aspirated. The formazan was dissolved in DMSO and absorbance was measured at 540 nm. The absorbance of the control was taken as 100% in cell viability. Data are presented as mean percentages of cell proliferation versus respective controls.

Furthermore, the proliferation effect of active phlorotannins on C2C12 cells were determined by cell population. C2C12 cells were seeded into 6-well plate a concentration of 1.0 x 10^5 cells/mL. After 70% confluent cultures of cells, active phlorotannins were treated with various concentrations for 48 h. The cell population was performed with photographs.

Photographs were taken before change to differentiation medium (time = day 0), and after incubation for 48 h (time = day 2) using an Olympus microscope equipped with a CoolSNAP-Pro color digital camera.

2.6. Cell cycle analysis

Cell cycle of sample treated C2C12 cells were analyzed by Flow cytometry. C2C12 cells were placed in a 6-well plate at a concentration of 1.0 x 10^5 cells/mL. After 70% confluent culture of cells, the cells were treated with various concentration (5, 10 and 20 nM) of active phlorotannins. After 48 h incubation, the cells were harvested at the indicated time and fixed in 1 mL 70% ethanol for 30 min at 4ºC. The cells were washed twice with DPBS and incubated in the dark in 1 mL of DPBS containing 100 μg PI and 100 μg RNaseA for 30 min at 37ºC. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect of phlorotannins on cell cycle was assessed by histograms generated by the computer program of Cell Quest and Mod-Fit (New York, USA.) and determined changes in the percentage of cell distribution at S phase of the cell cycle.

2.7. Creatine kinase (CK) activity

CK activity of sample treated cells were determined using the NADPH-coupled assay following the protocol supplied by the manufacture (Bio Vision, Inc., Milpitas, California). In

this assay, the creatine kinase converts creatine into phosphocreatine and ADP. The generated phosphocreatine and ADP reacts with CK Enzyme Mix to form an intermediate, which reduces a colorless Probe to a colored product with strong absorbance at 450 nm. C2C12 cells were placed in a 6-well plate a concentration of 1.0 x 10^5 cells/mL. 70% confluent culture of cells, the cells were treated with various concentration (5, 10 and 20 nM) of active phlorotannins for 48 h. After, cells were homogenized with CK assay buffer. Cell lysates were centrifuged at 12,000 rpm, and the supernatants were determined using the NADPH-coupled assay. The NADPH are generated by creatine kinase reaction which are measured via ELISA reader absorbance at 450 nm.

2.8. Western blot analysis

C2C12 cells (1.0 x 10^5 cells/mL) were treated with 10 nM DHT (positive control), 1 μM UA (positive control) and various concentration of phlorotannins (5, 10 and 20 nM) for 48 h. The cell were washed twice ice-cold phophate-buffered salin (PBS), and harvested by microcentrifugation. The cells were resuspended in lysis buffer [50 mM Tris-Hcl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1 mM EDTA] for 20 min on ice. Cell lysates were centrifuged at 14,000 x *g* for 20 min at 4ºC. And then protein contents in the supernatant was measured using BCA™ protein assay kit. The lysate containing 30 μg of protein was subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrlyamide gel, and the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes

were blocked with 5% non-fat dry milk in TBS buffer containing 0.2% Tween 20 (TBST) for 90 min at room temperature. Then the membrane was incubated with specific primary rabbit polyclonal anti-rabbit p-Smad2/3, p-Akt, p-FoxO1, MyoD Ab (1:500, Santa Cruz Biotechnology, Inc., Texas, USA), or mouse monoclonal anti-mouse Smad4 Ab (1:500, Santa Cruz Biotechnology, Inc.) at 4ºC for overnight. Membranes were washed with TTBS and incubated with goat anti-rabbit or anti-mouse IgG HRP conjugated secondary antibody (1:3,000 dilution) in TBST that contained 5% non-fat dry milk for 90 min at room temperature. After three times washing with TBST for 10 min signals. Signals were developed using ECL western blotting detection kit and exposed to FusionCapt Advance FX7 program (Vilber Lourmat, Australia).

2.9. In silico docking of phlorotannins with Myostatin

Molecular docking is an application wherein molecular modeling techniques are used to predict how a protein (enzyme) interacts with small molecules (ligands) (Perola E., 2006). The ability of a protein to interact with small molecules plays a major role in the dynamics of that protein, which may enhance or inhibit its biological function (Kang et al., 2012c). In the present study, we performed docking of phlorotannin into the active site of the myostatin. The crystal structure of myostatin (PDB ID : 3HH2) was obtained from the Protein Data Bank (PDB, http://www.pdb.org). We performed the docking studies using flexible docking in Accelrys Discovery Studio 3.5 (Accelrys, Inc, San Diego, USA). To prepare for the docking

procedure, we performed the following steps: (1) conversion of the 2D structure into 3D structure; (2) calculation of charges; and (3) addition of hydrogen atoms using the CDOCKER docking program.

2.10. In silico docking of phlorotannins with insulin growth factor-1 (IGF-1) receptor

To determine the docking of phlorotannins with insulin growh factor-1 (IGF-1) receptor, we carried out the docking simulation of phlorotannins into the active site of the IGF-1 receptor. The crystal structure of IGF-1 receptor (PDB ID : 1IGR) was obtained from the PDB. We performed the docking studies using flexible docking program the following steps: (1) converstion of the 2D sturcture into 3D structure; (2) calculation of charges; and (3) addition of hydrogen atoms using the CDOCKIER docking program in Accelrys Discovery Studio 3.5.

2.11. Statistical analysis

All experiments were conducted in triplicate (n=3) and an one-way analyis of variance (ANOVA) test (using SPSS 12.0 statistical software) was used to analyze the data. Significant differences between the means of parameters were determined by using the Duncan and Tukey test to analyze the difference. *P*-values of less than 0.05 ($P < 0.05$) and 0.01 ($P < 0.01$) was considered as significant.

3. RESULTS AND DISCUSSION

3.1. Cytotoxicity of phlorotannins from *E. cava*

The cell toxicity of 6 phlorotannins from *E. cava* on C2C12 myoblasts was evaluated in HS 2% differeniation medium by the MTT colorimetric assay. Among them, 5 phlorotannins (DK, PHB, 6,6'-BK, PBK, PG) except PFFA significantly showed cell viability comparing to non-treatment group (negative control) at 10 nM concentration (Fig 1). Therefore, we evaluated cell proliferation effects of 5 phlorotannins.

Table 1. The list of phlorotannins isolated from *E.cava*

	Sample name
DK	Dieckol
PHB	2,7'-phloroglucino-6,6"-bieckol
PFFA	Phlorofucofuroeckol
6,6-BK	6,6-bieckol
PBK	Phloroglucinol-6,6'-bieckol
PG	Phloroglucinol

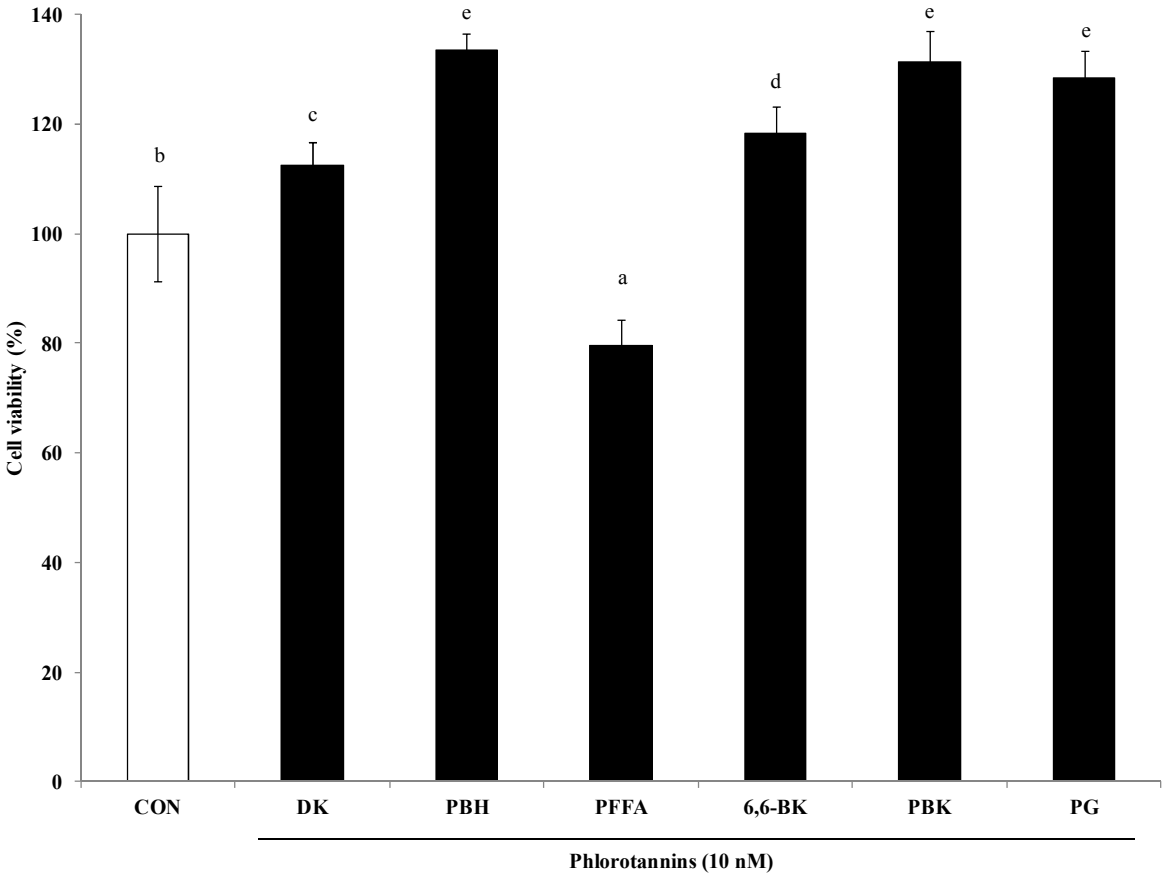

Fig 1. Screening of cell viability of phlorotannins (10 nM) isolated from *E. cava* by a MTT assay on C2C12 myoblasts. Experiments were performed in triplicate and data are expressed as mean ± SD. [a, b, c] Values having different superscirpts are significantly different at $P < 0.05$.

3.2. Cell proliferation

In previous studies, androgen hormone such as DHT (Diel et al., 2008) and ursolic acid isolated from natural products (Kunkel et al., 2012) are introduced as a muscle growth materials. Therefore, we used the DHT and ursolic acid as positive control.

To determine a suitable concentration of positive control, various concentrations of DHT (1 nM – 40 nM) and UA (0.25 μM – 1 μM) was introduced the C2C12 in 2% HS differentiation medium. According the results, DHT dose-dependently increased cell viability of C2C12 myoblasts. Among them, 10 – 40 nM of DHT was significantly increased cell viability comparing to non-treatment group (negative control) (Fig 2). However, 20 nM and 40 nM of DHT was very rapidly increased cell population so that can't accessed cell differentiation. As shown in Fig 3, 0.5 – 1 μM of UA was significantly increased cell viability comparing to negative control. Therefore, the concentration of DHT and UA for further study was optimezed to 10 nM and 1 μM, resepctively.

The cell proliferation effect of 5 phlorotannins from *E. cava* were determined by the MTT assay. As indicated in Fig 4, among the 5 phlorotannins, DK and PHB were significantly increased proliferation of myoblasts compared to non-treated group. And then, the cell populations of DK and PHB treated C2C12 cells were determined with photographs. 70% confluent cultures of C2C12 cells were treated with various concentrations of DK or PHB (5, 10, and 20 nM) and incubated for 48 h. Photographs were taken before changing into

differentiation medium after 48 h incubation period. DK and PHB increased the cell populations (Fig 5 and 6). To determine whether DK and PHB increase the populations of the cell, we performed the proportion of proliferative S-phased cell treated with DK and PHB.

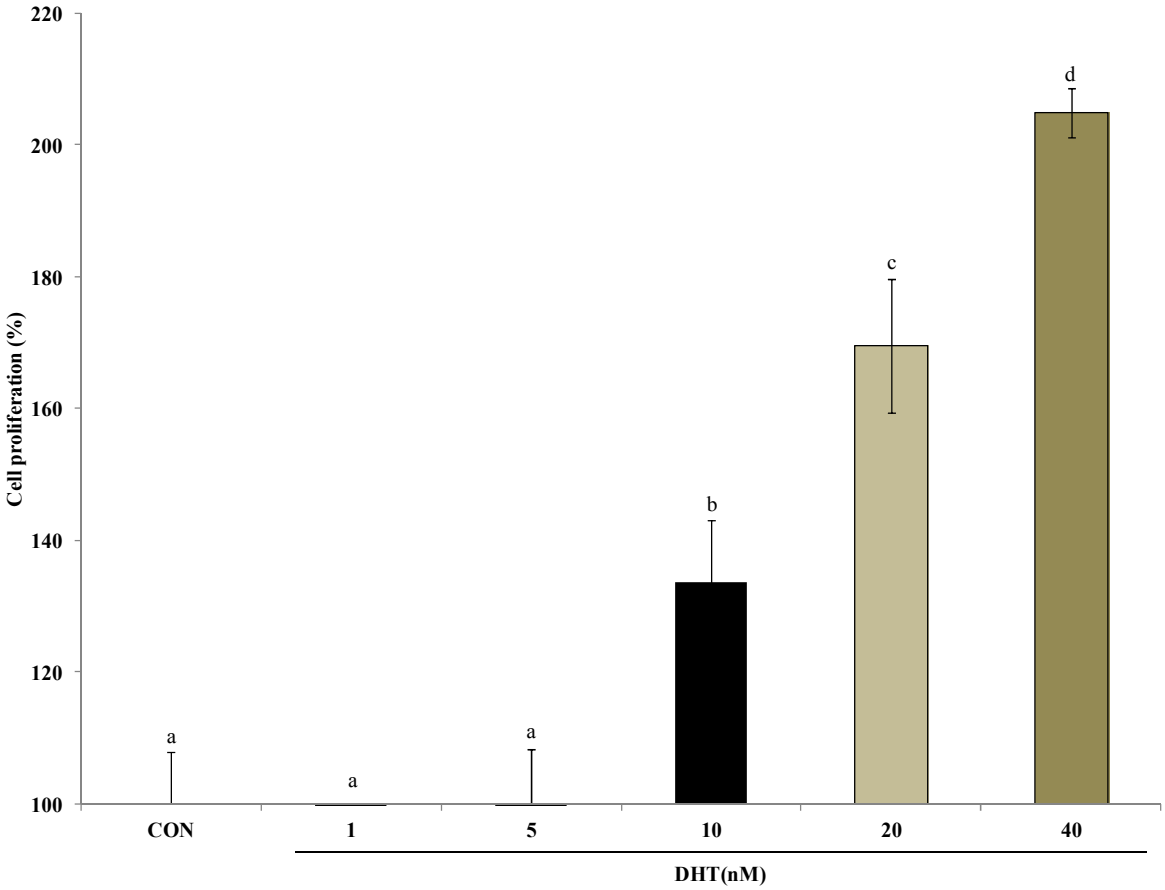

Fig 2. Cell proliferation effects of dihydrotestosterone (DHT) by a MTT assay on C2C12 myoblasts. Experiments were performed in triplicate and data are expressed as mean ± SD. [a, b, c] Values having different superscirpts are significantly different at $P < 0.05$.

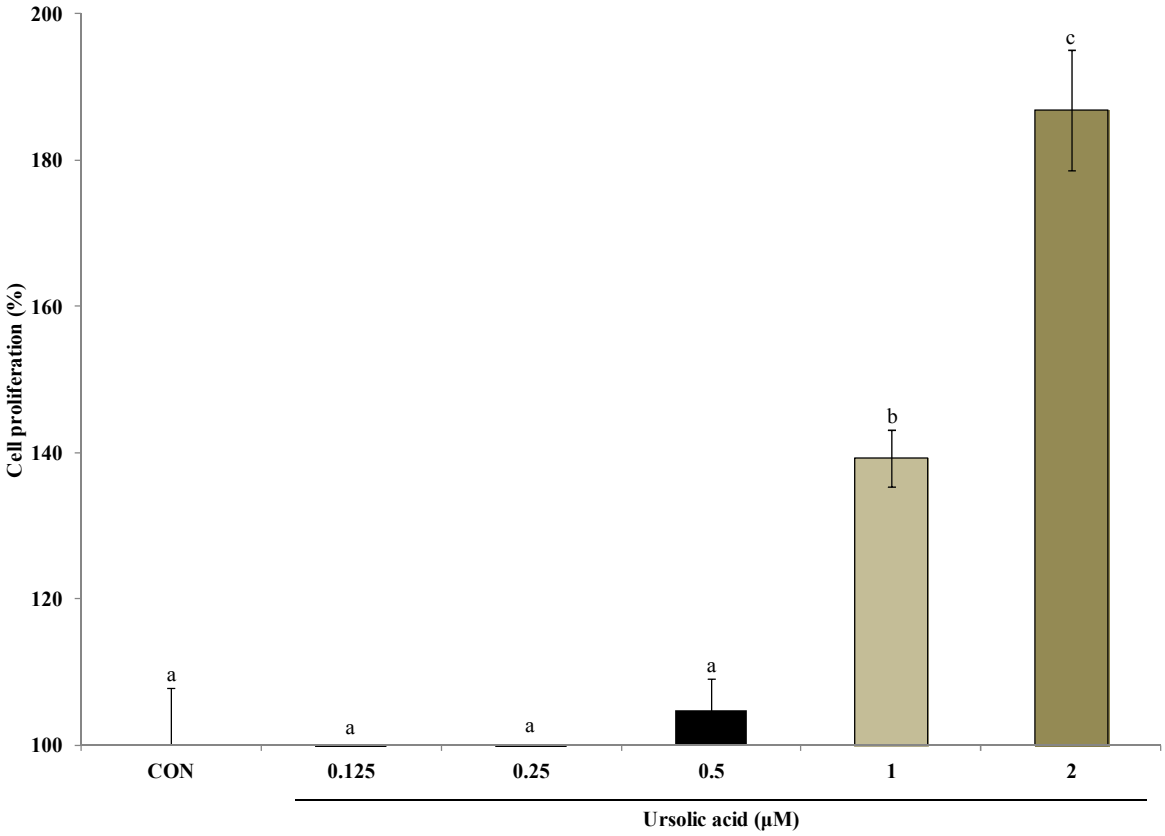

Fig 3. Cell proliferation effects of ursolic acid (UA) by a MTT assay on C2C12 myoblasts. Experiments were performed in triplicate and data are expressed as mean ± SD. [a, b, c] Values having different superscirpts are significantly different at $P < 0.05$.

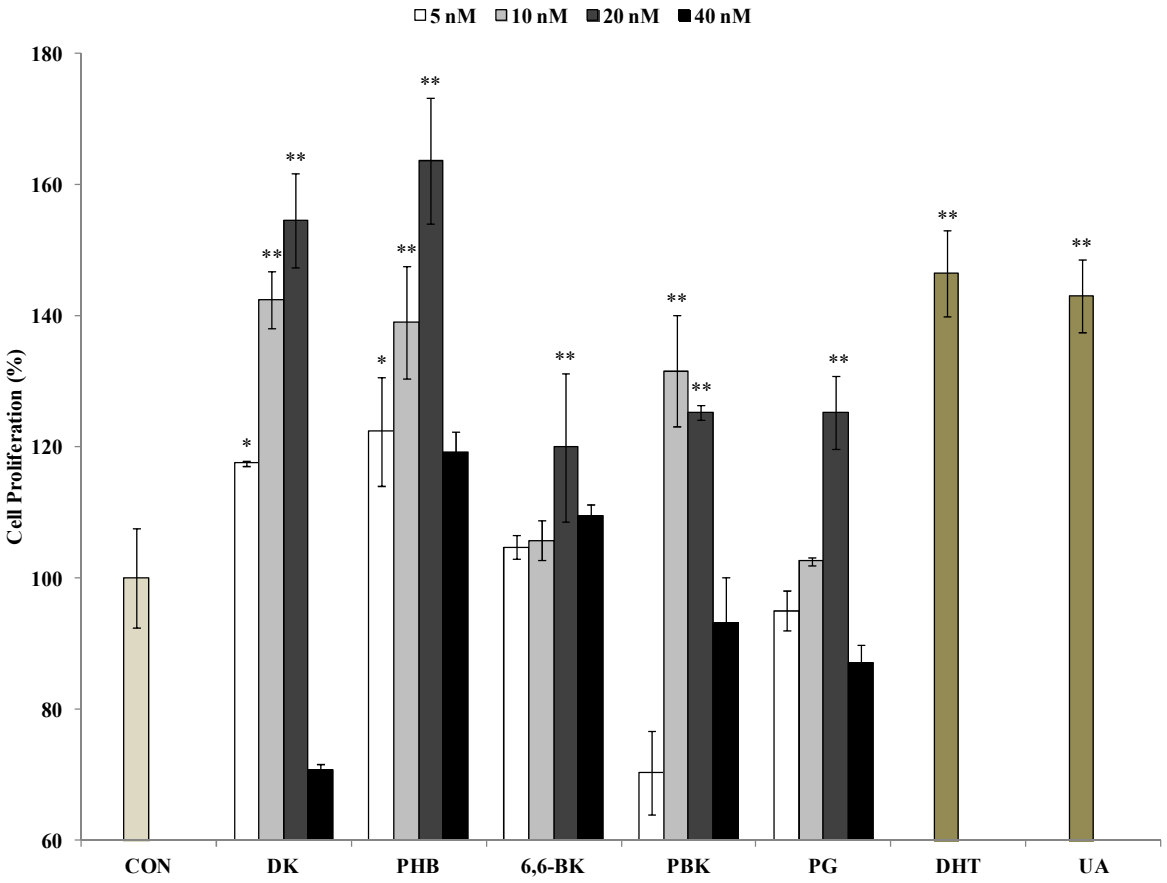

Fig 4. Screening of cell proliferation effects of phlorotannins by a MTT assay on C2C12 myoblasts. The proliferation of non-treated (control), DHT- (10 nM) and UA-treated (1 μM) (positive control) or phlorotannins treated (5, 10, 20 and 40 nM) cells were analyzed. Experiments were performed in triplicate and data are expressed as mean ± SD. * Significantly differenct from only non-treated control ($P < 0.05$), ** significantly different from only non-treated control ($P < 0.01$).

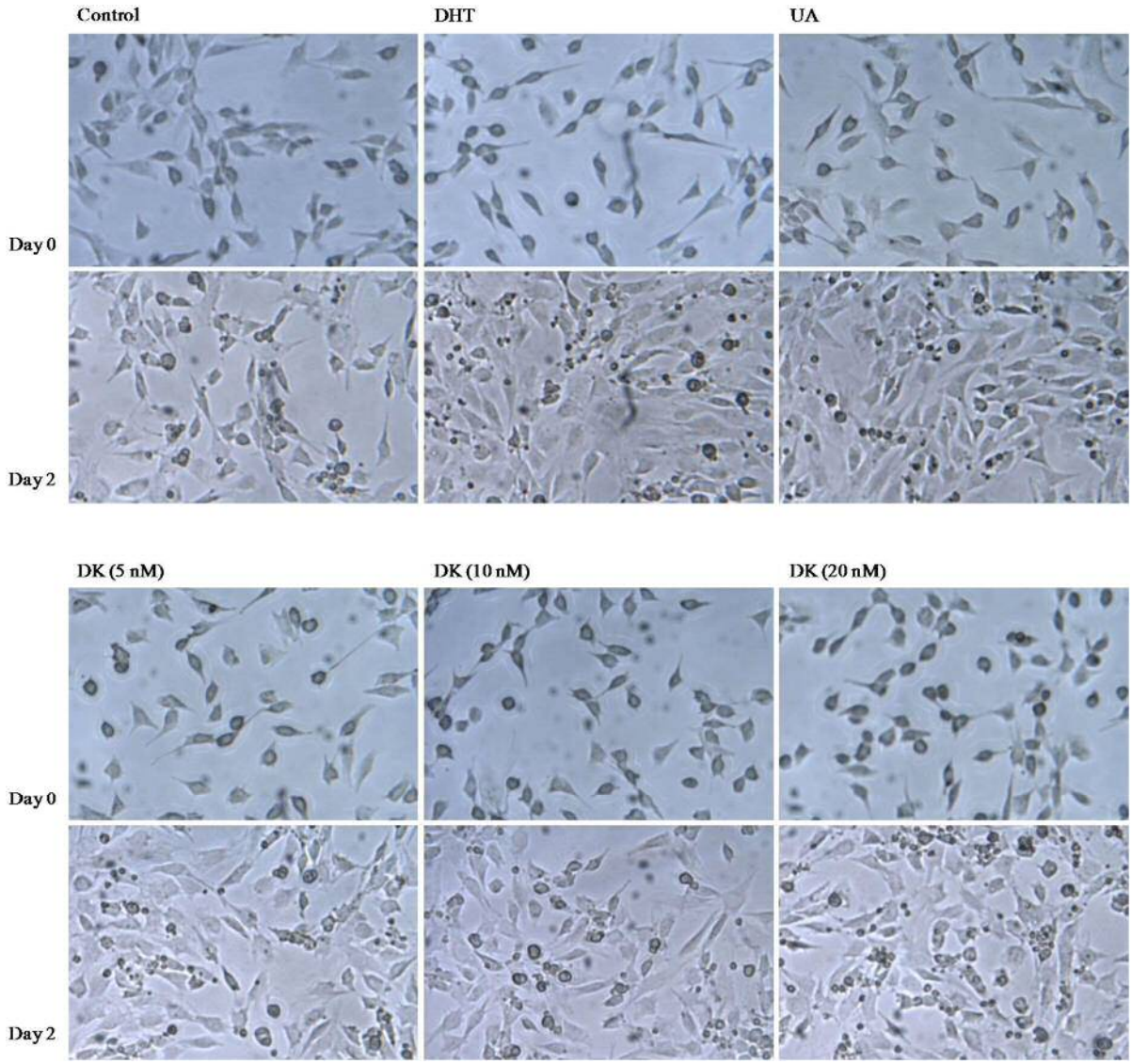

Fig 5. Photographs of cells populaton of DK (5, 10, and 20 nM) on C2C12 myoblasts compared to non-treated cells (negative control) and cells of the treatment of DHT (10 nM) and UA (1 μM) (positive control).

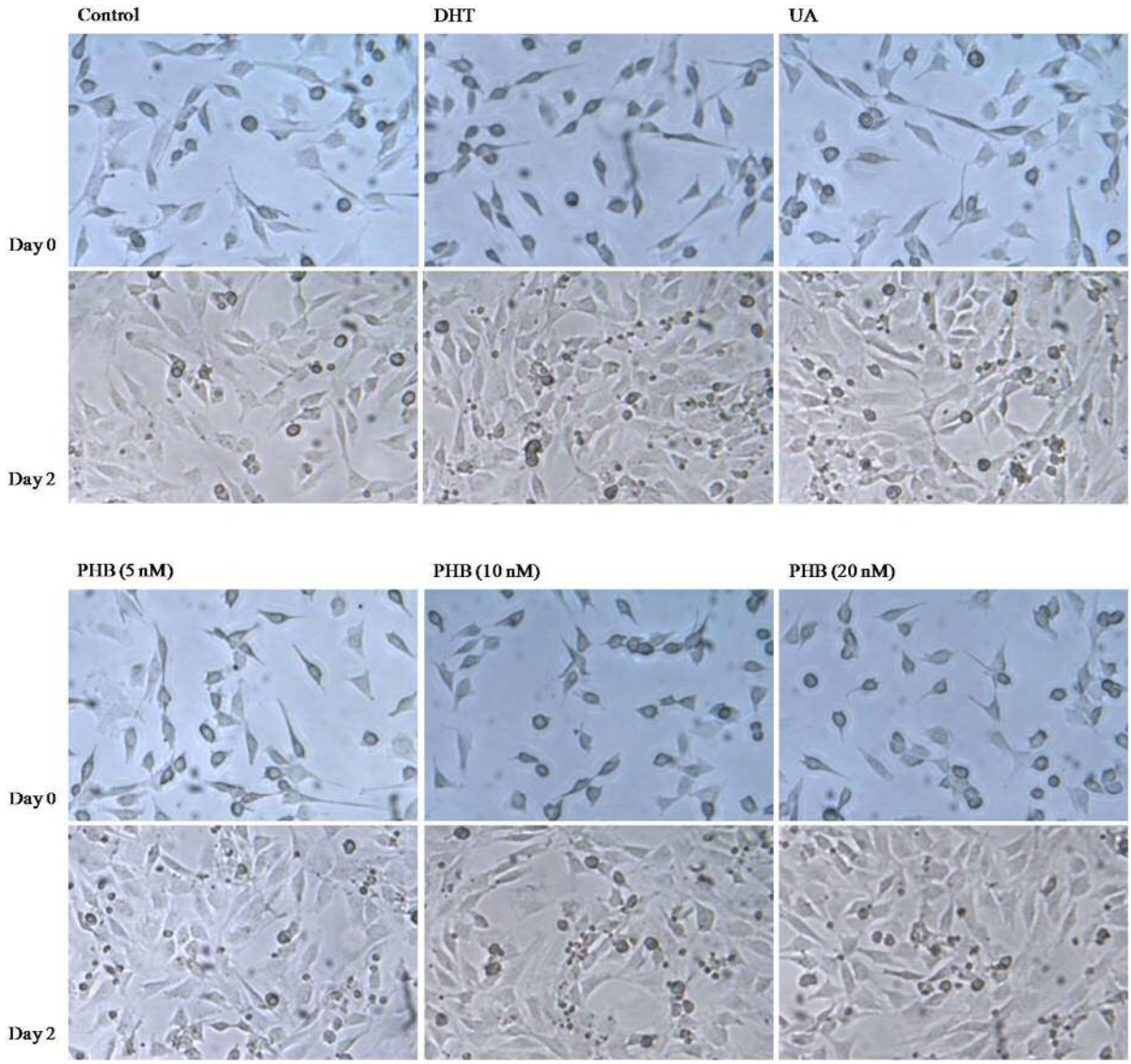

Fig 6. Photographs of cells populaton of PHB (5, 10, and 20 nM) on C2C12 myoblasts compared to non-treated cells (negative control) and cells of the treatment of DHT (10 nM) and UA (1 μM) (positive control).

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3.3. Cell cycle analysis

Flow cytometry was used for further investigation of the underlying mechanisms accounting for the proliferative action of DK and PHB. In vitro DNA detection assay-flow cytometry was carried out to determine whether DK or PHB induce the cell growth. Fig 7-A shows the representative DNA distribution histograms of C2C12 cells incuabted in the absence or presence of DK (5, 10, and 20 nM) for 48 h. Fig 7-B summarizes the relative percentages of cells at S phase of the cell cycle following treatment with different concentrations of DK. The results were reveal that DK treatments (20 nM) for 48 h was significantly caused an increase in the percentage of cells at S phase in cell cycle. In addition, the treatment with various concentrations of PHB (5, 10, and 20 nM) for 48 h were resulted a significant increasment of DNA at S phase of the cell cycle (Fig 8). These results showed that DK and PHB induces myoblast proliferation.

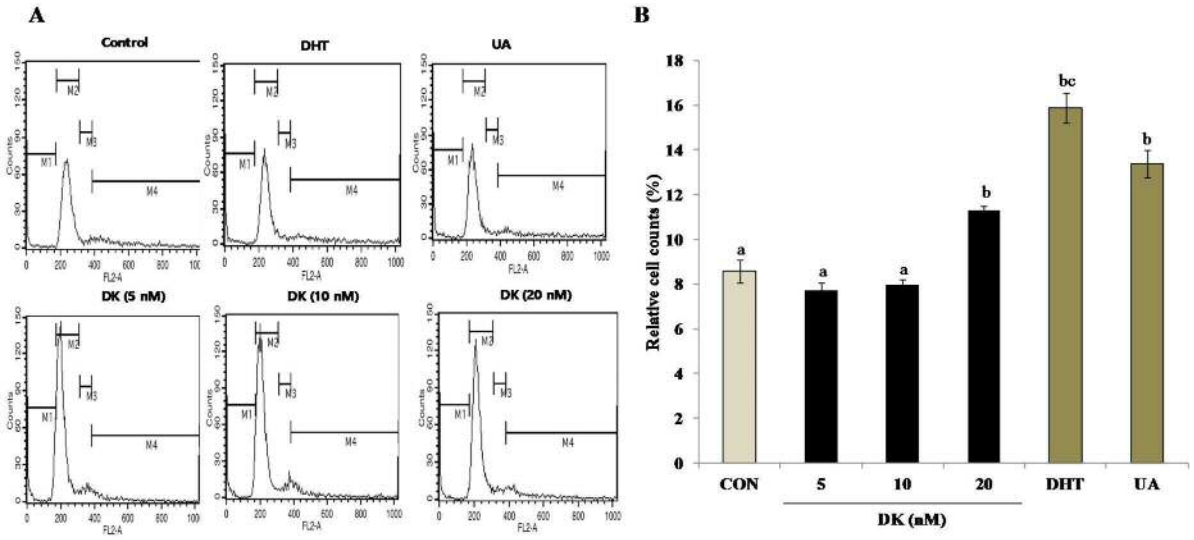

Fig 7. C2C12 cells were seeded at 1.0 x 105 cells/mL and treated with the difference concentrations of DK for 48 h in differentiation medium. The cells were stained with PI and analyzed by flow cytometry. (A) Histogram of cell cycle patterns of C2C12 cells. (B) Bar graph of cell cycle patterns of C2C12 cells. Experiments were performed in triplicate and data are expressed as mean ± SD. $^{a, b, c}$ Values having different superscirpts are significantly different at $P < 0.05$.

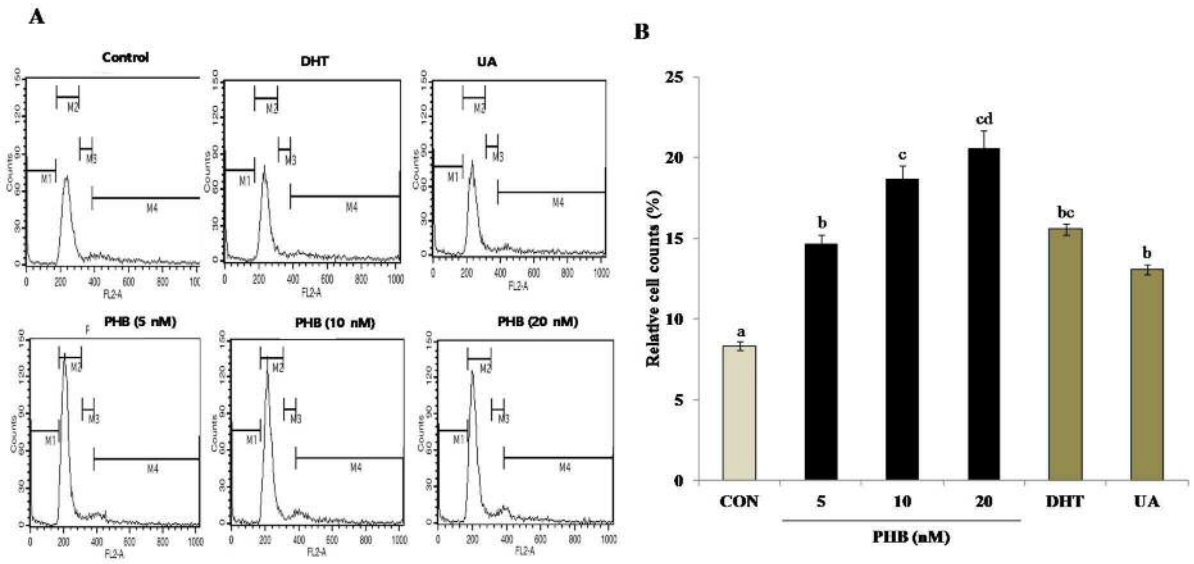

Fig 8. C2C12 cells were seeded at 1.0 x 105 cells/mL and treated with the difference concentrations of PHB for 48 h in differentiation medium. The cells were stained with PI and analyzed by flow cytometry. (A) Histogram of cell cycle patterns of C2C12 cells. (B) Bar graph of cell cycle patterns of C2C12 cells. Experiments were performed in triplicate and data are expressed as mean ± SD. $^{a, b, c}$ Values having different superscirpts are significantly different at $P < 0.05$.

3.4. Creatine kinase activity of the C2C12 cells treated with DK or PHB

The C2C12 cells were seeded as described previously to investigation of the effects of DK and PHB on induction of differentiation and assessed CK activity. CK activity, a well-described marker of C2C12 cell differentiation (Lawson & Purslow, 2000), was measured in the cell lysates. CK is an enzyme (EC 2.7.3.2) expressed by various tissues and cell types. CK catalyses the conversion of creatine and consumes adenosine riphosphate (ATP) to create phosphocreatine (PCr) and adenocine diphosphate (ADP). This CK enzymes reaction is reversible and thus ATP can be generated from PCr and ADP. In tissues and cells that consume ATP rapidly, especially skeletal muscle. Thus, creatine kinase is an important enzyme in such muscle tissue. The cells were treated with various concentration of DK or PHB in 2% HS. After 48 h, CK activity was measured in the cell lysates. As shown in Fig. 9, DK treated cells were increased the CK activity compared to non-treated cells (negative control). Especially, dose-dependent induction of CK activity was observed in PHB treated cells (Fig. 9).

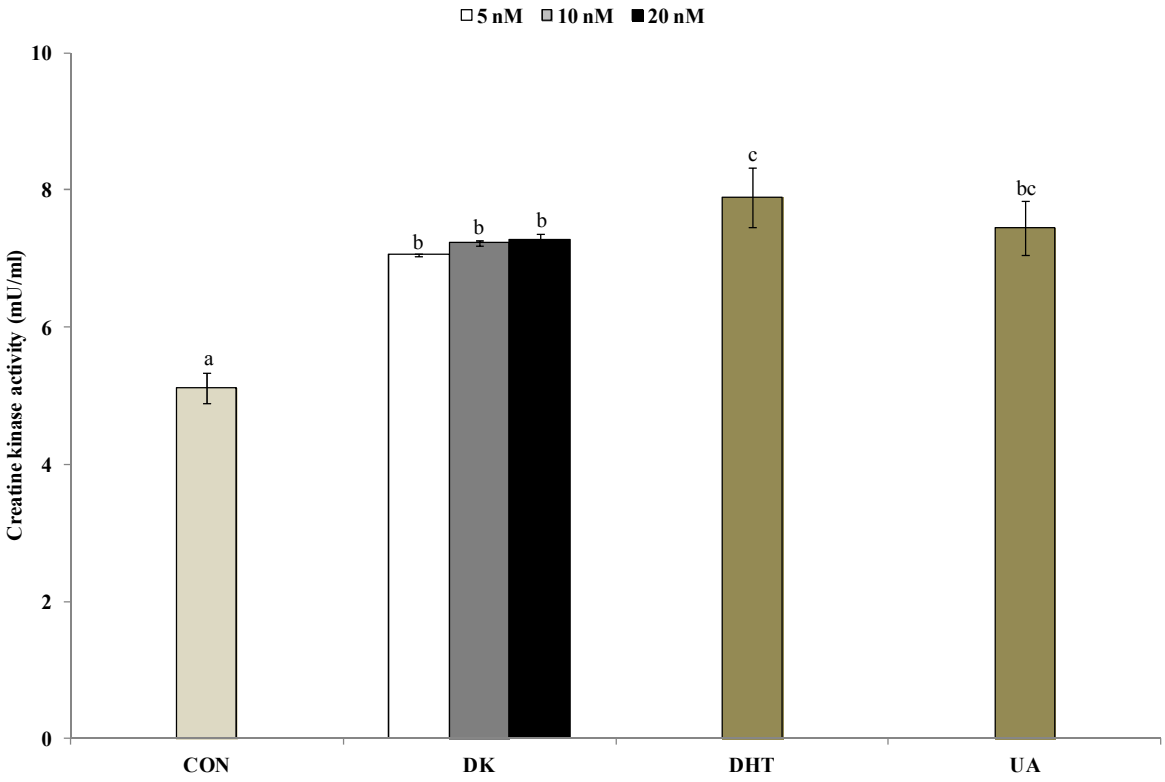

Fig 9. Mouse myoblast C2C12 cells were treated dose-dependently with DK (5, 10, and 20 nM) for analysis the effects of DK on the myoblast differentiation. Experiments were performed in triplicate and data are expressed as mean ± SD. $^{a, b, c}$ Values having different superscirpts are significantly different at $P < 0.05$.

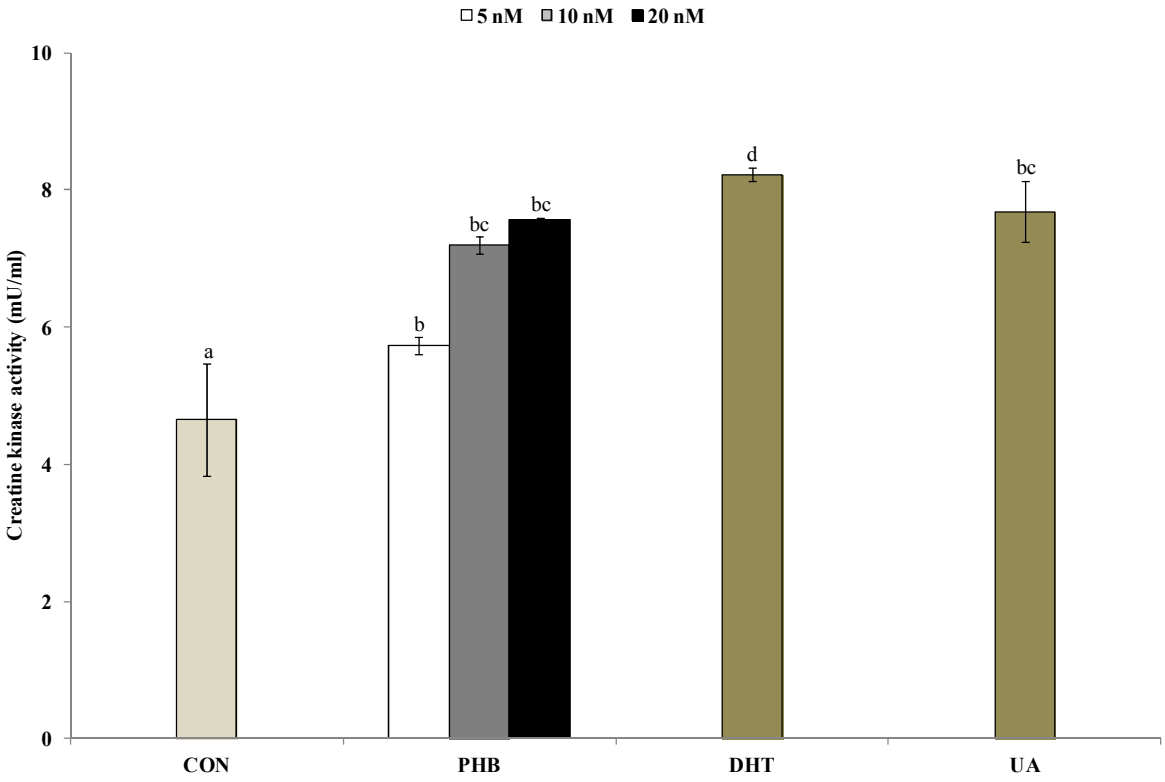

Fig 10. Mouse myoblast C2C12 cells were treated dose-dependently with PHB (5, 10, and 20 nM) for analysis the effects of PHB on the myoblast differentiation. Experiments were performed in triplicate and data are expressed as mean ± SD. $^{a, b, c}$ Values having different superscirpts are significantly different at $P < 0.05$.

3.5. DK promotes C2C12 cell differentiation through the Smad signaling pathways

Myogenesis differentiation and growth mediated intracellular signaling of the TGF-β superfamily and IGF-1. Western blot analysis was carried out to invetigate the TGF-β-mediated Smad proteins expression and PI3K/Akt activation by IGF-1. To determine whether DK can activate the myogenesis differentiation and growth factors in C2C12 myoblast cells, C2C12 cells were incubated with DK for 48 h with 2% HS differentiation medium.

DK was inhibited and the phophorylation of Smad2/3 (p-Smad2/3) in a dose-dependent manner 48 h incubation (Fig 11). Phosphorlyation of Akt (p-Akt) and FKHR (p-FKHR) were slightly induced after 48 with treatment of DK (Fig 11). Furthermore, MyoD, which known to play an important role in the regulation of muscle cell development, which was significantly increase by the treatment of DK for 48 h. These results show that DK promoted the myogenesis diffrentiation through down-regulation of Smad expressiong than PI3K/Akt pathway. Supressed Smad pathway also induce up-regulation of MyoD with DK treated. Therefore, we suggest that DK able to induce the myogenesis of myoblasts thorugh Smad expression.

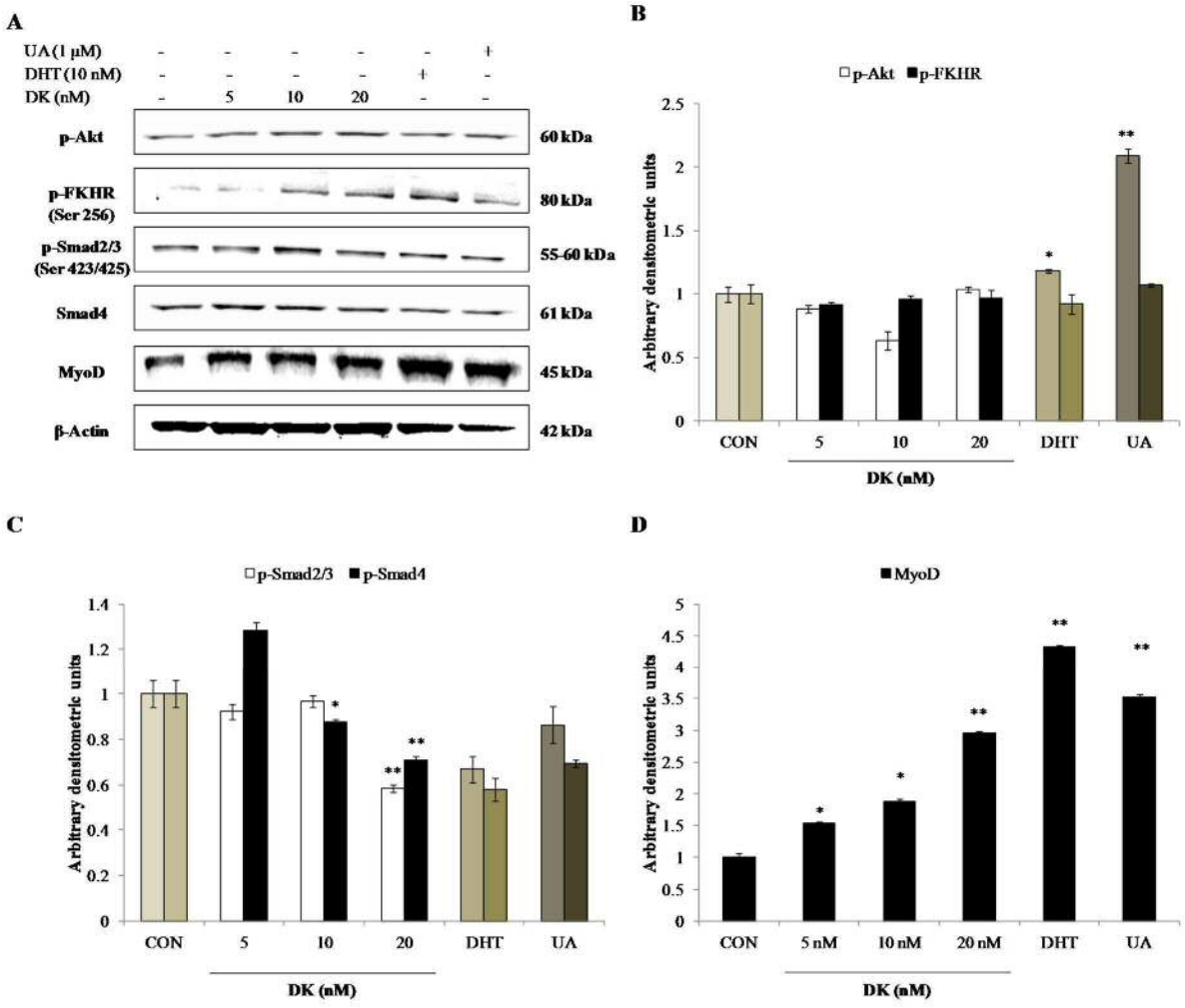

Fig 11. Differentiation activity of DK on the protein level of p-Akt, p-FKHR, p-Smad2/3, Smad 4, and MyoD in C2C12 cells. C2C12 cells (1 x 105 cells/mL) were pre-incubated and the cells were introduced with DK (5, 10, and 20 nM), DHT (10 nM) and UA (1 μM) for 48 h in HS differentiation medium. Experiments were performed in triplicate and data are expressed as mean ± SD. * Significantly differenct from only non-treated control ($P < 0.05$), ** significantly different from only non-treated control ($P < 0.01$).

3.6. PHB elevates C2C12 differentiation through the PI3K/Akt signaling pathways

To verify whether PHB can activate the myogenesis differentiation and growth factors in C2C12 myoblast cells, C2C12 cells were cultured with PHB for 48 h in HS differentiation medium. Western blot analysis was carried out to investigate the Smad and PI3K/Akt signaling expression.

As shown Fig. 12, PHB was induced activation of p-Akt and p-FKHR after 48 h. p-Smad2/3 and Smad4 were also reduced 48 h after PHB treatment. In addition, MyoD were dose-dependently increased by the treatement of PHB for 48 h. Theses results show that PHB more acts through PI3K/Akt pathway than Smad signling. PHB treated C2C12 cells were regulated by myogenesis growth and differentiation factor including p-Akt, pFKHR. These findings indicated that PHB was induced myoblast differentiation through up-regulation p-Akt, p-FKHR.

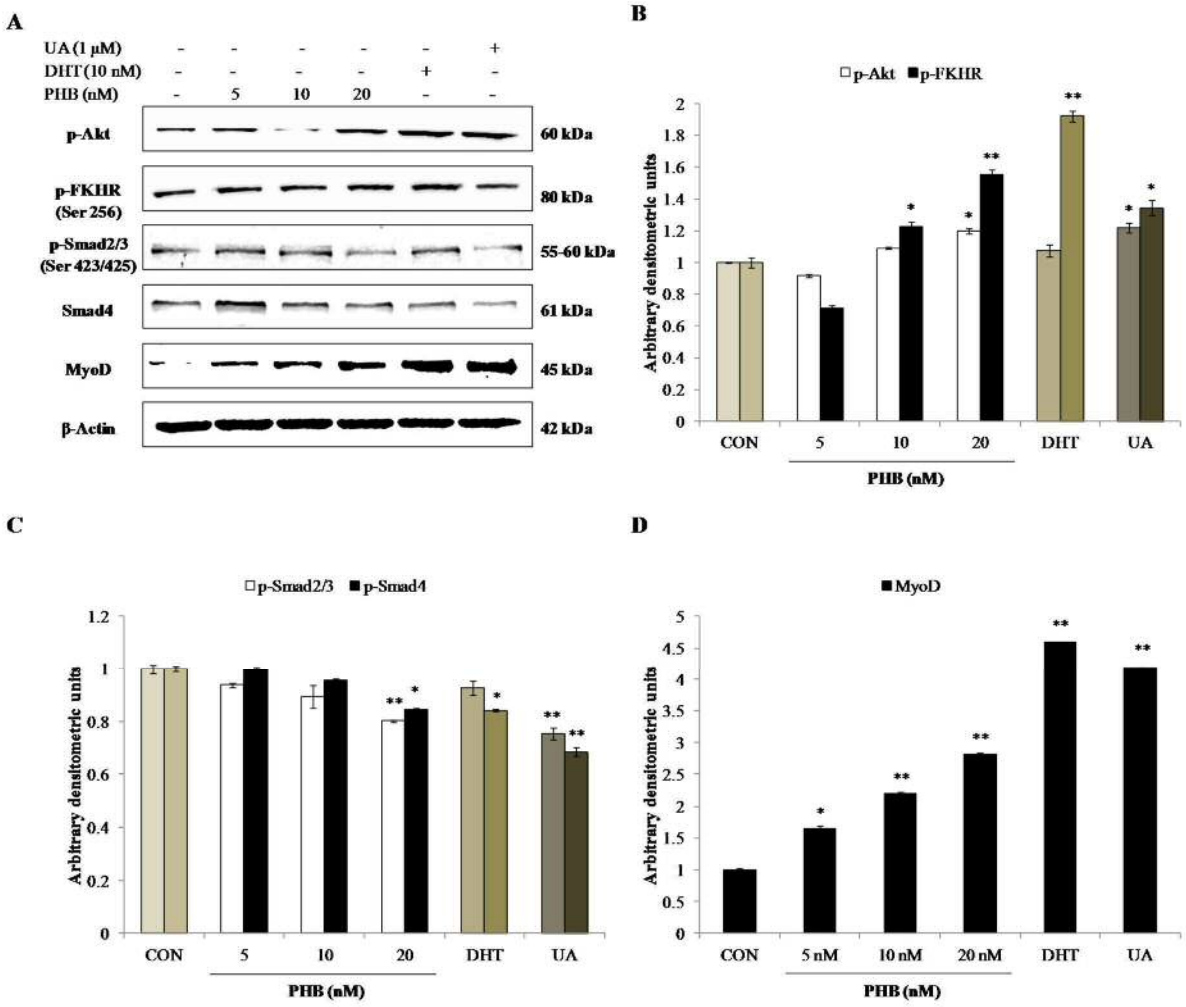

Fig 12. Differentiation activity of PHB on the protein level of p-Akt, p-FKHR, p-Smad2/3, Smad 4, and MyoD in C2C12 cells. C2C12 cells (1 x 105 cells/mL) were pre-incubated and the cells were introduced with PHB (5, 10, and 20 nM), DHT (10 nM) and UA (1 μM) for 48 h in HS differentiation medium. Experiments were performed in triplicate and data are expressed as mean ± SD. * Significantly differenct from only non-treated control ($P < 0.05$), ** significantly different from only non-treated control ($P < 0.01$).

3.7. In silico docking of Myostatin (MSTN)

Myostatin (MSTN) is belong to the activin class of molecules, as it was originally identified to bind both the activin type II receptors, such as ActRIIB and ActRIIA, and the activin inhibitor Fst (Lee and McPherron, 2001).

Ligand signalling is tightly regulated by extracellular antagonists, some of which are broad antagonists that inhibit multiple TGF-β family members, whereas others are more specific and inhibit only a few family members. Myostatin has been shown to be regulated by several antagonists including its propeptide component, decorin, GASP-1, and Fst-type molecules, including Fst-like 3 (Fstl3) and Fst isoformes Fst288 and Fst315 (Lee and McPherron, 2001; Thies et al., 2001; Hill at al., 2002, 2003; Amthor et al, 2004; Sidis et al., 2006).

The MSTN inhibition mode of the DK and PHB were predicted by molecular docking analysis. Docking of the protein-ligand complex, was carried out using Discovery Studio 3.5 (DS). For docking studies, the crystal structure of MSTN was obtained from Protein Data Bank (PDB ID: 3HH2) (Thompson et al., 2005; Groppe et al., 2008).

The docking of the MSTN-ligand complexes was well-performed with DK and PHB stably posed in the MSTN by DS 3.5 (Fig. 14 and 15). Moreover, the results of docking analysis were indicated that the following highest docking binding energy and lowest total binding energy confirmation of the most proposed complex had to be taken into account when using the CDOCKER interaction energy program of DS 3.5 (Table 2).

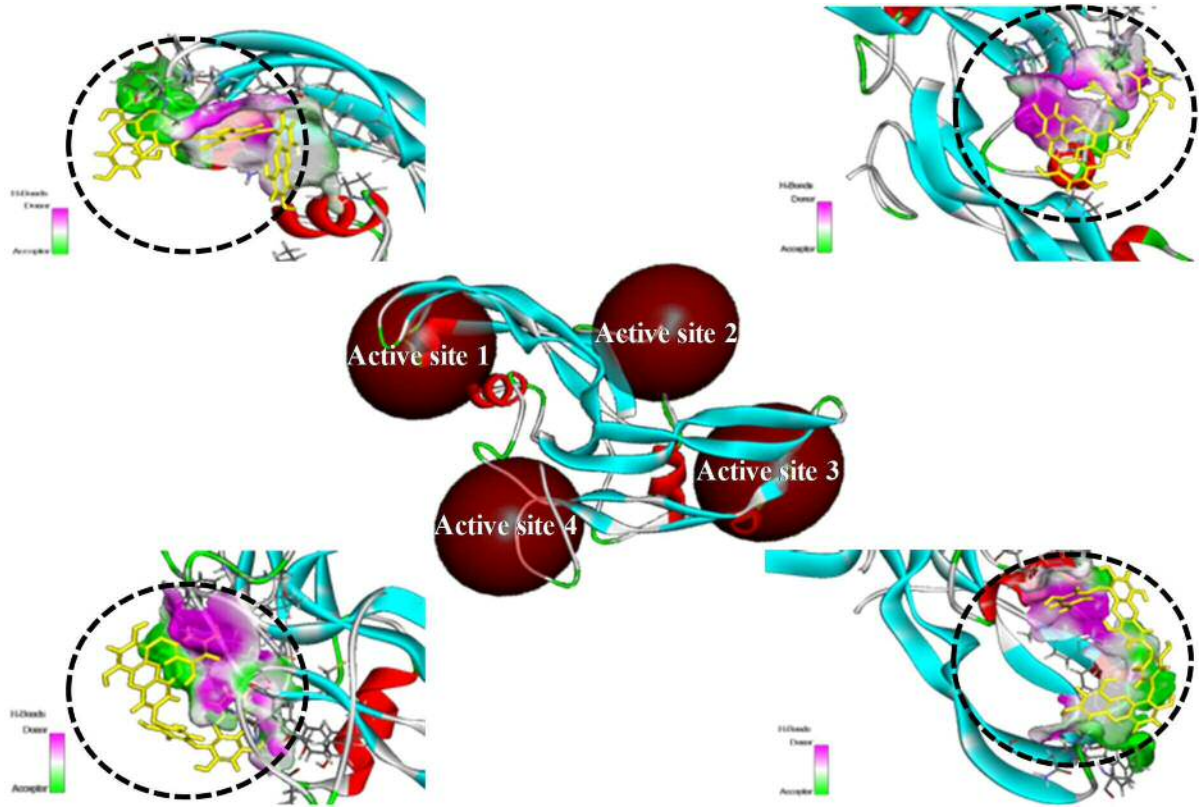

Fig 13. Computational prediction of the structure for MSTN (PDB ID: 3HH2) and docking simulation with DK. Predicted 3D structure of DK with 4 active sites of MSTN.

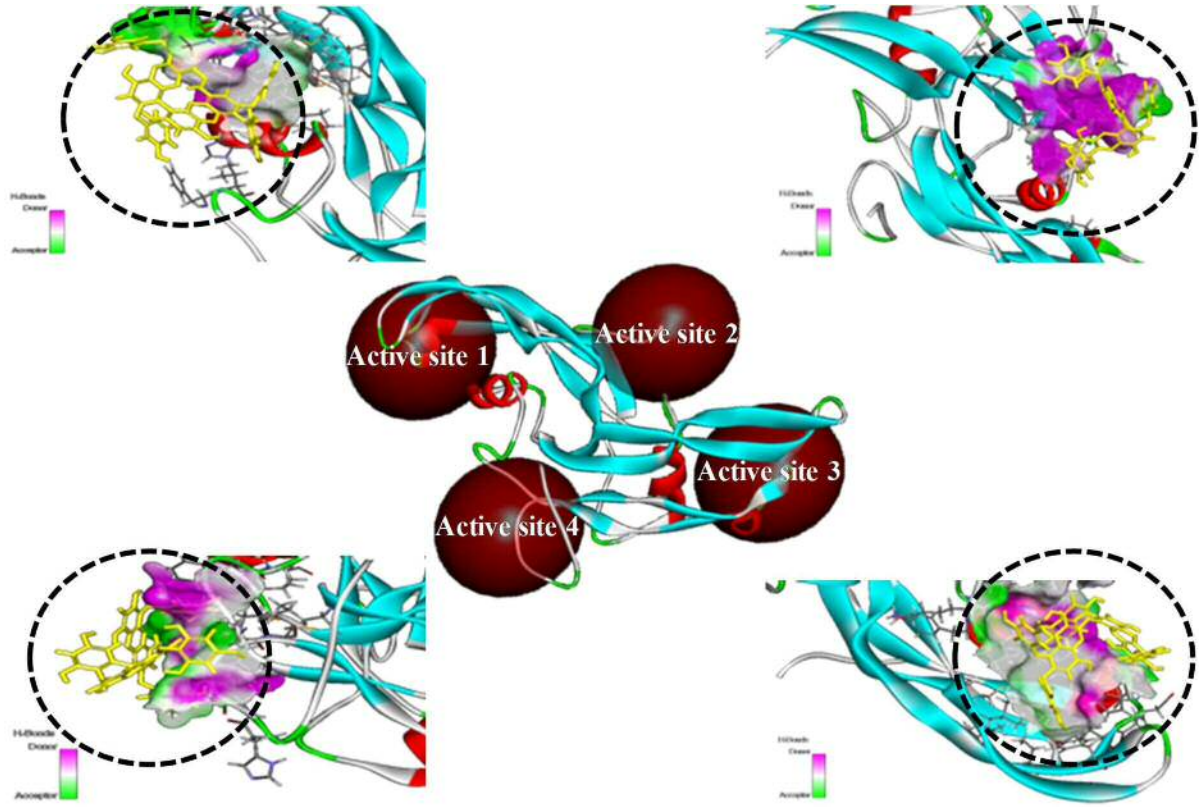

Fig 14. Computational prediction of the structure for MSTN (PDB ID: 3HH2) and docking simulation with PHB. Predicted 3D structure of DK with 4 active sites of MSTN.

Table 2. Results of docking experiments of DK or PHB with the MSTN receptor (PDB ID: 3HH2) compare to DHT and Ursolic acid (positive control)

Ligand	Site position	Binding energy (kcal/mol)	CDOCK interaction energy (kcal/mol)
DK	Active site 1	-146.06	50.77
	Active site 2	-118.99	40.01
	Active site 3	-126.49	47.71
	Active site 4	-108.64	38.27
PHB	Active site 1	-109.90	42.44
	Active site 2	-102.71	49.42
	Active site 3	-143.74	57.70
	Active site 4	-125.90	54.60
DHT	Active site 1	-50.00	24.07
	Active site 2	-48.16	18.28
	Active site 3	-51.51	32.07
	Active site 4	-40.08	18.76
Ursolic acid	Active site 1	-66.12	36.65
	Active site 2	-46.44	30.41
	Active site 3	-69.61	30.64
	Active site 4	-25.74	17.20

3.8. In silico docking of phlorotannins with IGF-1 receptor (IGF-1R)

The insulin receptor family comprises the insulin receptor (IR), the type-I insulin-like growth factor receptor (IGF-1R) and the insulin receptor related receptor (IRR) (Lawrence, et al., 2007). IGF-1 induce an increase in muscle mass by stimulating the phosphatidylonostinol-3 kinase (PI3K)/Akt pathway, resulting in the downstream activation of targets which are requied for protein synthesis (Bodine, Stitt, et al., 2001; Rommel et al., 2001). Overload-induced muscle hypertrophy may lead to activation of the PI3K/Akt pathway by directly inducing muscle expression of IGF-1 (Glass et al., 2005).

IGF-1R binding to DK or PHB was predicted by molecular docking analysis. To simulate docking of the protein-ligand complex, DS 3.5 was utilized. For docking studies, the crystal structure of IGF-1 receptor was allocated rom PDB (PDB ID : 1IGR) (Garrett et al., 1998; Lawrence, et al., 2007).

The docking of the IGF-1R-ligand complexes was well-performed with DK and PHB stably posed in the IGF-1R by DS 3.5 (Fig. 16 and 17). In addition, the docking analysis results indicated that the following highest docking binding energy and lowest total binding energy confirmation of the most proposed complex had to be taken ito account when using the CDOCKER interaction energy program of DS 3.5 (Table 3).

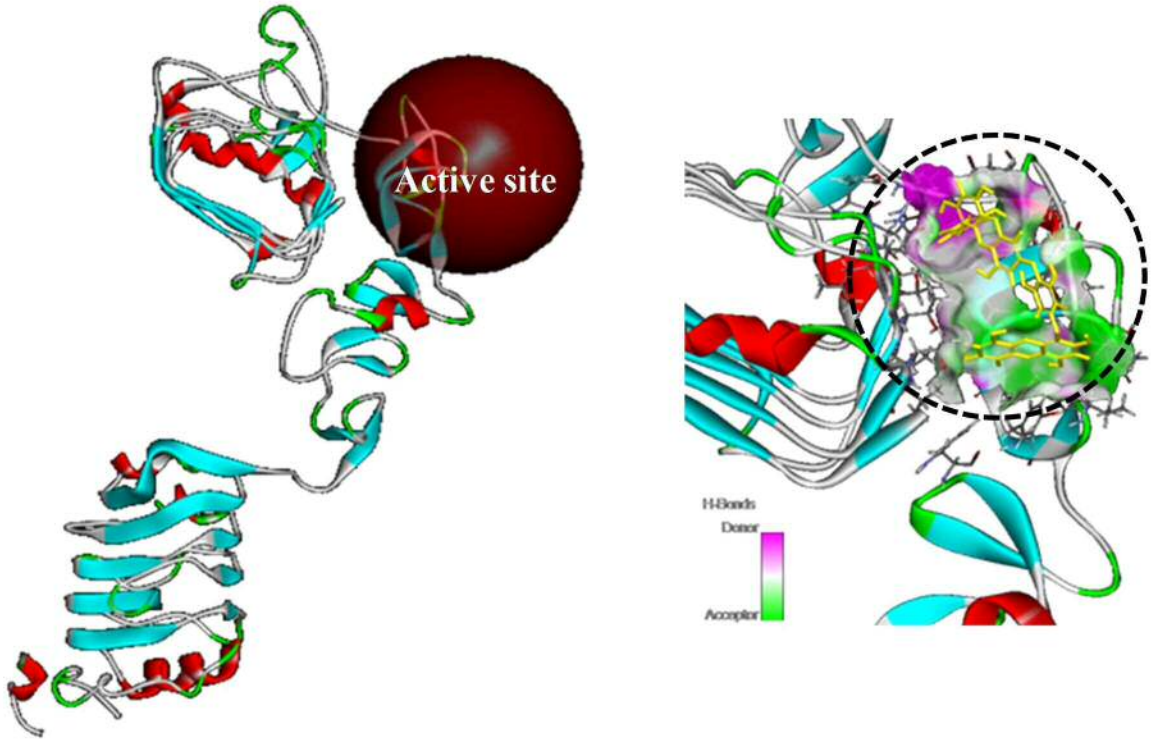

Fig 15. Computational prediction of the structure for IGF-1 (PDB ID: 1IGR) and docking simulation with DK. Predicted 3D structure of DK with active site of 1IGR.

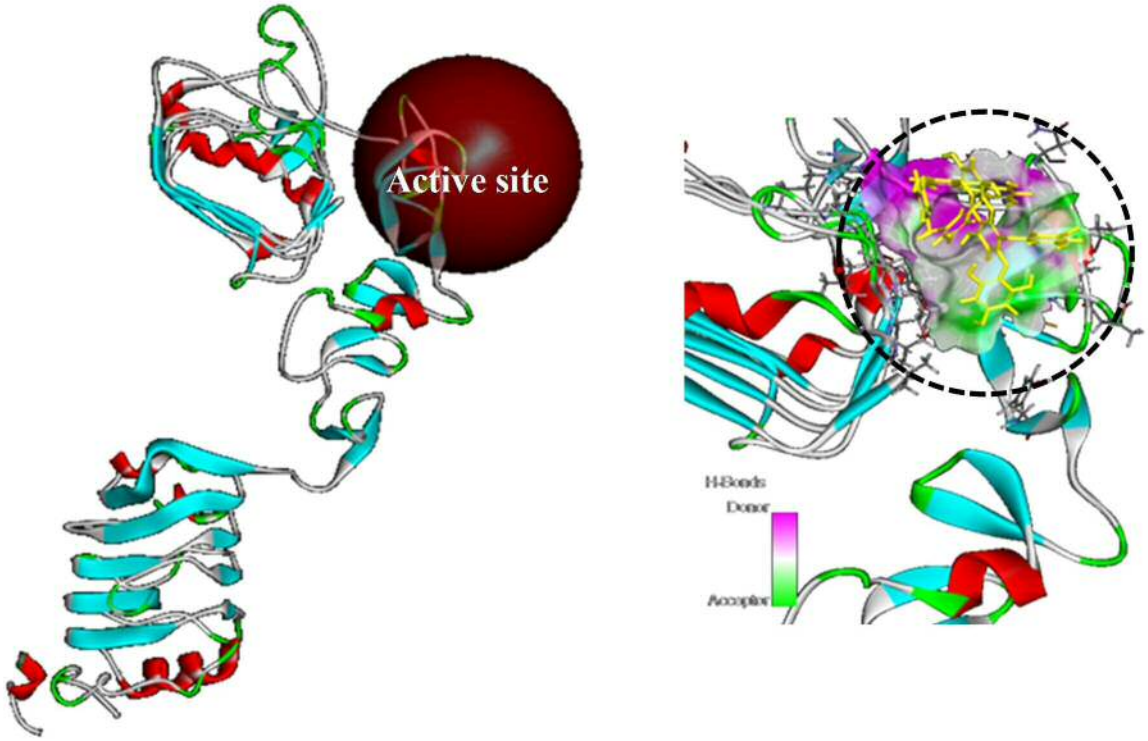

Fig 16. Computational prediction of the structure for IGF-1 (PDB ID: 1IGR) and docking simulation with PHB. Predicted 3D structure of PHB with active site of 1IGR.

Table 3. Results of docking experiments of DK or PHB with the IGF-1 receptor (PDB ID: 1IGR) compare to DHT and Ursolic acid (positive control)

Ligand	Binding energy (kcal/mol)	CDOCK interaction energy (kcal/mol)
DK	-118.71	50.66
PHB	-98.11	60.74
DHT	-60.25	20.33
Ursolic acid	-13.74	24.74

4. CONCLUSION

In this study, we investigated myogenesis activity of DK and PHB isolated from *E. cava*. Our results demonstrate that the profound myogenesis effect of DK and PHB on cell proliferation in C2C12 myoblasts. Proliferation effect of the DK is mediated through the MyoD via phosphorylation of Smad2/3 (Ser 423/425) and Smad4. PHB is mediated thorugh the MyoD via phosphorylation of Akt (p-Akt) and phosphorylation of FKHR (Ser 256). In addition, MSTN inhibition effect of the DK and PHB was predicted through molecular docking program (DS 3.5). Also, Binding thd DK and PHB with IGF-1R was predicted through molecular docking program. Therefore, DK and PHB are considered the muscle reinforcing materials inducing myogenesis differentiation and growth factors

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부족한 저에게 관심을 주시고, 먼 제주까지 와서 연구의 길을 걸을 수 있도록 이끌어주셔서 2년이라는 석사과정을 마치고 본 논문이 있기까지 세심한 가르침과 사랑을 주신 전유진 교수님께 진심으로 감사드립니다. 또한 바쁘신 와중에도 이 논문이 좀 더 나은 방향으로 나아 갈 수 있게 심사해 주시고 관심을 보여주신 여인규 교수님, 이제희 교수님, 허문수 교수님, 정준범 교수님, 이승헌 교수님께도 깊은 감사를 드립니다.

제주에 어떤 연고도 없는 제가 제주에 내려와 실험실 생활을 잘 해나갈 수 있도록 이끌어주시고 좋은 말씀을 해주신 우리 실험실원들에게 감사의 표현을 하려고 합니다. 연구자의 길을 가기 위해서 아낌없이 조언해 주시는 수진오빠, 제 논문이 좋은 방향으로 나아 갈 수 있도록 조언해 주시고 항상 반겨주시는 길남 오빠, 무심한 듯 항상 잘 챙겨주시는 승홍오빠, 석천오빠, 저의 석사학위 연구 기초를 잡아주시고, 함께 운동 다니면서 추억을 쌓은 긴내언니, 항상 좋은 말 많이 해주시는 원우오빠, 제가 제주에 오기 전 강릉에서 처음 만나고, 실험실에서도 많이 챙겨주시고 제가 졸업할 수 있도록 정말 많은 도움을 주신 지혁오빠, 주영언니, 역시 강릉에서 처음 만나고, 처음 실험실에 왔을 때, 세포실험, 동물실험 등 많은 실험과 조언 및 많은 도움을 주셔서 제가 잘 적응할 수 있게 도와주시고 미국에

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