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Phytoecdysteroid-containing medium component from *Spinacia oleracea* L. improves muscle cell differentiation for cultured meat production

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Running title: Spinach extracts reduce serum use

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Abstract

Cultured meat production remains constrained by the cost, variability, and animal origin of serum supplements. This study evaluated phytoecdysteroid-containing *Spinacia oleracea* L. extracts as partial replacements for fetal bovine serum (FBS) and horse serum (HS) in muscle cell culture. Extracts were prepared from freeze-dried or fresh spinach using methanol or ethanol and characterized for 20-hydroxyecdysone (20-HE), free amino acids, and fatty acids. In C2C12 myoblasts, freeze-dried extracts containing 0.05 μM 20-HE supported proliferation in medium containing 5% FBS at levels comparable to or greater than those obtained with 10% FBS. In bovine myosatellite cells, freeze-dried methanol extract containing 0.5 μM 20-HE with 10% FBS produced proliferation comparable to the 20% FBS control. Selected spinach-derived treatments also maintained myogenic differentiation under reduced-HS conditions. These findings support an approximately 50% reduction in animal-derived serum under the best-performing conditions tested, rather than complete replacement. Responses were concentration-dependent and differed between C2C12 and bovine myosatellite cells, indicating cell-model-specific requirements for serum reduction.

Keywords: Cultured meat; Fetal bovine serum; Horse serum; Phytoecdysteroids; *Spinacia oleracea* L.

1. Introduction

Cultured meat is being developed as an alternative approach to conventional meat production, with potential implications for resource use, animal welfare, and the organization of future food production systems *(1–3)*. Despite rapid technical progress, its feasibility at production scale still depends on reliable expansion of muscle progenitor cells and efficient differentiation under culture conditions that can be reproduced consistently. Culture-medium composition is therefore a central process constraint rather than a secondary cell-culture consideration.

Serum remains widely used in muscle-cell culture because it supplies proteins, growth factors, hormones, lipids, micronutrients, and other components that support survival, proliferation, and differentiation. Fetal bovine serum (FBS) is especially common during cell expansion, while serum-containing formulations are also used during myogenic differentiation *(4, 5)*. In cultured-meat systems, however, FBS is an animal-derived and chemically undefined input that can contribute substantially to process cost and variability. Commercial serum may vary among products or batches and can introduce biological contaminants or other sources of process variation *(6–8)*.

These limitations have motivated serum-reduction and serum-replacement strategies. Complete replacement remains difficult because FBS contains multiple classes of components whose individual and interactive contributions are not fully resolved *(4)*. A practical intermediate strategy is therefore to identify materials that allow serum concentration to be reduced while maintaining proliferation and subsequent differentiation.

Plant-derived compounds are among the candidate inputs for lower-serum media. Phytoecdysteroids are plant-derived polyhydroxylated steroids with diverse reported biological activities *(9)*. Of particular relevance to skeletal muscle, 20-hydroxyecdysone (20-HE) has been reported to induce a rapid intracellular Ca2+ response, Akt activation, and increased

protein synthesis in C2C12 cells *(10)*. These observations provide a mechanistic rationale for examining phytoecdysteroid-containing materials in muscle-cell culture, although pathway activation by a complex plant extract cannot be assumed from the activity of purified 20-HE alone.

Spinach (*Spinacia oleracea* L.) was selected as the plant source because it contains phytoecdysteroids, including 20-HE, and can be processed using conventional solvent extraction. In addition to phytoecdysteroids, the resulting extracts contain amino acids, fatty acids, and other co-extracted compounds that may influence cellular responses. For this reason, the present work compared whole spinach-derived extracts with pure 20-HE and characterized selected compositional features of the extracts rather than attributing all observed activity a priori to a single molecule.

This study aimed to determine how spinach preparation and extraction solvent affect 20-HE recovery and to evaluate whether phytoecdysteroid-containing spinach extracts can reduce FBS or HS requirements during C2C12 and bovine myosatellite cell proliferation and differentiation.

2. Materials and methods

2.1 Extraction of phytoecdysteroid-containing serum substitute from spinach

To obtain the phytoecdysteroid-containing serum substitute, spinach cultivated in open fields in Namhae-gun, Gyeongsangnam-do, was harvested, washed, and used for extraction. Four different extraction methods were employed, utilizing either freeze-dried or fresh spinach with methanol (99.5%; Samchun, Korea) or ethanol (95.0%; Samchun, Korea).

First, 500 g of freeze-dried or fresh spinach was pulverized into a fine powder. Fresh spinach was frozen with liquid nitrogen before grinding. The powdered spinach was subsequently immersed in methanol or ethanol at a 1:10 ratio (w/v) and extracted using an

ultrasonic bath (DAI-HAN Scientific, Wonju, Korea) for 30 min at room temperature. This extraction process, which included stirring at room temperature for 24 h, was repeated three times. The collected extract (10 L) was concentrated under reduced pressure at 45°C using a rotary evaporator (Eyela, Seongnam, Korea).

The concentrated extract was mixed with 20 mL of deionized water and subsequently partitioned twice using 100 mL of n-hexane (95.0%; Samchun, Korea). The separated aqueous and organic phases were collected and further concentrated under reduced pressure at 45°C to remove the organic solvents. Afterward, 100 mL of deionized water was added to recover the final extract. The obtained extract was freeze-dried to determine yield and subsequently sterilized using a 0.1-μm filter (Merck Millipore, MA, USA) before being used in experiments.

2.2 Analysis of spinach-derived serum substitute

2.2.1 Measurement of phytoecdysteroid content

To determine the phytoecdysteroid content of the spinach-derived serum substitute, high-performance liquid chromatography (HPLC) was performed using a modified EcdyBase method *(11)*. The HPLC system was an Agilent 1100 series equipped with a Zorbax Eclipse XDB-C8 column (4.6 × 150 mm, 5 μm). Calibration curves were constructed using 20-hydroxyecdysone (20-HE; H5142, Sigma) and ponasterone A (PonA; P3490, Sigma) standards prepared at 20, 50, 100, and 200 ppm.

2.2.2 Analysis of free amino acids

The free amino acid composition of the spinach-derived serum substitutes and commercial sera was analyzed after protein precipitation and lipid removal. A 1-g sample was mixed with an equal amount of 5% trichloroacetic acid at a 1:1 ratio. The mixture was vortexed for 2 min in two successive cycles (4 min total) and centrifuged at 13,572 × g and 20°C for 20

min. The supernatant was collected, n-hexane was added at twice the original sample volume, and the mixture was shaken vigorously for 15 min. After centrifugation at 1,977 × g and 20°C for 20 min, the lower aqueous phase was collected and subjected to two additional centrifugation steps at 13,572 × g and 20°C for 20 min to remove residual n-hexane. The final extract was left in a fume hood for 20 min to permit volatile solvent evaporation. Free amino acids were analyzed using a HITACHI L-8900 Amino Acid Analyzer (Hitachi High Technologies Corp., Tokyo, Japan) equipped with a HITACHI HPLC Packed Column #2622PF (4.6 × 60 mm, ion-exchange column).

2.2.3 Analysis of fatty acids

For fatty acid analysis, 1 g of sample was combined with 1 g of Folch solution (chloroform:methanol, 2:1, v/v), vortexed for 2 min, and centrifuged at 2,400 × g and 20°C for 5 min. The lower phase was collected, dried, and dissolved in 1 mL n-hexane. For fatty acid methyl ester preparation, 2 mL boron trifluoride-methanol solution was added and the mixture was heated at 80°C for 5 min, then cooled to room temperature. Triple-distilled water (1 mL) was added and the phases were allowed to separate. The upper n-hexane layer was collected and dehydrated using anhydrous sodium sulfate. A 1.5-mL aliquot was transferred to a glass vial and analyzed using an Agilent 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-WAX column (30 m × 0.25 mm × 0.25 μm film thickness).

2.3 Cell line and spinach-derived medium preparation

The effects of the spinach extracts on proliferation and differentiation were evaluated in C2C12 myoblasts and bovine myosatellite cells. The bovine myosatellite-cell culture procedure followed a previously published approach *(12)*. For C2C12 proliferation, DMEM containing

10% FBS served as the control (CTL) and 5% FBS as the reduced-serum negative control (NCTL). For bovine myosatellite-cell proliferation, 20% FBS served as the control, while 5% FBS (NCTL1) and 10% FBS (NCTL2) were used as reduced-serum controls.

For C2C12 proliferation, spinach extracts or pure 20-HE were added to medium containing 5% FBS at final 20-HE-equivalent concentrations of 0.05, 0.1, 0.5, and 1 μM; Fig. 1 presents the 0.05 and 0.5 μM comparisons. For bovine myosatellite-cell proliferation, the same treatment range was evaluated under 5% or 10% FBS and compared with the 20% FBS control; Fig. 2 presents the principal 0.05 and 0.5 μM proliferation comparisons.

For differentiation, 2% HS served as the control (CTL). In the C2C12 experiment, 1% HS served as the reduced-serum negative control (NCTL), and spinach-derived extracts or pure 20-HE were evaluated at 0.05, 0.1, 0.5, and 1 μM as shown in Fig. 3. In the bovine myosatellite-cell experiment, 0% HS (NCTL1) and 1% HS (NCTL2) represented the reduced-serum conditions, with 0.05, 0.5, and 1 μM treatments shown in Fig. 4.

2.4 Cell proliferation analysis

Cell proliferation was assessed using DMEM supplemented with 1% penicillin/streptomycin (P/S) and FBS (10% for C2C12 myoblasts and 20% for bovine myosatellite cells). Cells were cultured in collagen-coated dishes until they reached 60–70% confluence, after which they were passaged with 1X Dulbecco's phosphate-buffered saline (DPBS) containing 1% P/S.

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay, where cells were seeded at 5×10^3 cells/mL in 96-well plates and cultured for 2–3 days. MTT solution was added to the wells and incubated for 4 h, and the formed formazan crystals were dissolved in dimethyl sulfoxide. Absorbance was measured at 540 nm using a SpectraMax® 190 microplate reader. To evaluate cell proliferation,

171 the cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton™
172 X-100 in 1X PBS for 30 min, and stained with Hoechst 33342 for 5 min to visualize the nuclei.
173 Stained nuclei were enumerated to assess proliferation across treatment groups.
174
175 **2.5 Cell differentiation analysis**
176 Plates were washed twice with 1X DPBS. Cells were subsequently fixed by adding 4%
177 paraformaldehyde at the same volume as that of the culture medium and incubating at room
178 temperature for 30 min. Permeabilization was performed using 1X PBS containing 0.2%
179 Triton™ X-100 for 30 min at room temperature.
180 For immunofluorescence staining, cells were blocked with 2% bovine serum albumin
181 (BSA) in 1X PBS for 30 min. Primary antibody against myosin heavy chain (MyHC; MF20,
182 1:800; MAB4470, R&D Systems, Minneapolis, MN, USA) was applied in 1% BSA-containing
183 PBS and incubated overnight at 4°C. Goat anti-mouse immunoglobulin G conjugated to FITC
184 (1:1,000; A90-116F, BETHYL, Montgomery, TX, USA) was then applied for 2 h at 4°C,
185 followed by Hoechst nuclear staining for 5 min at room temperature. Fluorescence images were
186 acquired using an inverted fluorescence microscope. Nuclear count, MyHC-positive area,
187 myofiber area, and fusion/differentiation indices were quantified using ImageJ. Myofiber-area
188 distributions were visualized in RStudio using ggplot2 and dplyr.
189
190 **2.6 Statistical analysis**
191 A minimum of three independent replicates were performed for each experiment. For
192 the cell-based proliferation and differentiation experiments, statistical analyses were conducted
193 using SPSS Statistics 26 (IBM, Armonk, NY, USA). Depending on the comparison, data were
194 analyzed using a t-test or one-way analysis of variance followed by the Student-Newman-Keuls
195 post hoc test. Statistical significance was defined as $p < 0.05$. Compositional data in Tables 1–

3 are presented descriptively as the mean ± standard deviation. Quantification of immunofluorescence images was performed using ImageJ (NIH, Bethesda, MD, USA).

3. Results and discussion

3.1 Characterization of spinach extracts

3.1.1 Determination of phytoecdysteroid content based on extraction method

Phytoecdysteroids are distributed across a range of plant species and include compounds such as 20-HE, PonA, polypodine B, and integristerone *(9)*. In the present study, 20-HE and PonA were used as analytical standards, and the four spinach extracts were compared by HPLC to determine how spinach preparation and extraction solvent affected phytoecdysteroid recovery.

The primary phytoecdysteroid detected in the spinach extracts was 20-HE, whereas PonA was not detected regardless of solvent or spinach preparation. The highest 20-HE content was obtained from freeze-dried spinach extracted with methanol (FM; 87.83 ± 1.40 μg/kg), followed by freeze-dried spinach extracted with ethanol (FE; 55.25 ± 0.19 μg/kg), raw spinach extracted with methanol (RM; 10.96 ± 0.28 μg/kg), and raw spinach extracted with ethanol (RE; 7.06 ± 0.31 μg/kg) (Table 1). Freeze-drying and methanol extraction were associated with higher 20-HE recovery, with FM giving the highest value.

Methanol extraction produced the highest 20-HE recovery, but the use of methanol limits direct application to a food-facing process because residual solvent must be controlled. Accordingly, further work would require either an alternative extraction solvent or an appropriate solvent-removal procedure before such extracts could be considered for cultured-meat production *(13)*.

3.1.2 Comparison of free amino acid and fatty acid compositions between spinach-derived serum substitute and serum

To compare the composition of the spinach-derived materials with animal sera commonly used in cell culture, free amino acid and fatty acid profiles were measured in the four extracts (FM, FE, RM, and RE), commercial FBS from Corning, and commercial HS from Gibco. These analyses were used as compositional context rather than as evidence that the extracts reproduce the complete biochemical complexity of serum.

The free amino acid profiles are presented in Table 2. The spinach-derived extracts were compositionally distinct from both FBS and HS but contained several amino acids relevant to cell metabolism. Glutamine was detected in all four spinach extracts, whereas citrulline, cystathionine, 3-methylhistidine, carnosine, and hydroxyproline were detected in animal sera but not in the spinach-derived extracts. These differences indicate that the extracts do not reproduce the complete amino acid composition of FBS or HS.

The fatty acid profiles are shown in Table 3. Palmitic and stearic acids were abundant in both animal sera and spinach-derived extracts. Linoleic acid was detected in FM, FE, and RE, whereas RM was 0.0 ± 0.0 mg/kg; FE contained the highest linoleic acid value among the spinach extracts. Linoleic acid has been reported to modulate C2C12 proliferation and differentiation under defined conditions *(14)*. Myristoleic acid was detected in FBS and all spinach extracts but not in HS. Overall, the spinach extracts and animal sera remained compositionally distinct.

Leucine was detected in all four spinach extracts, although at lower concentrations than in FBS or HS (Table 2). Leucine availability can influence myogenic differentiation in C2C12 myoblasts and primary satellite cells *(15)*, and intracellular leucine sensing can regulate mTORC1 through leucyl-tRNA synthetase and Sestrin2 *(16, 17)*. These observations provide a plausible nutritional context for the extract composition, but the present study did not

isolate the contribution of leucine or measure mTORC1 activity.

Together, the amino acid and fatty acid analyses show that the spinach-derived materials contain multiple metabolites relevant to cell culture while remaining different from animal serum. The biological effects observed in the cell experiments therefore should be interpreted as responses to the complete extracts rather than as evidence that the extracts reproduce all serum functions.

3.2 Proliferation effects of spinach-derived FBS substitutes

In C2C12 myoblasts, FM and FE adjusted to 0.05 μM 20-HE significantly increased MTT viability relative to the 5% FBS negative control and produced values comparable to or greater than the 10% FBS control (Fig. 1a). Nuclear counts showed the same overall pattern. Under the 0.05 μM condition, FM produced approximately twice the viability and nuclear-count response observed in the 5% FBS group, indicating that the extract recovered a substantial portion of the proliferative response lost after serum reduction.

At 0.5 μM 20-HE equivalent, FM and FE produced lower responses than the 0.05 μM condition but remained broadly comparable to the 10% FBS control. The C2C12 data therefore indicate that the response was concentration dependent and that the lower tested treatment was the most effective among the conditions displayed in Fig. 1.

However, at 0.1 and 1 μM, no treatment group displayed cell-viability and nuclear-count values comparable to or greater than those of the 10% FBS control. These concentrations therefore did not reproduce the response observed at 0.05 μM under the tested conditions.

The complete spinach extracts also differed from pure 20-HE at some matched concentrations. This is consistent with a possible contribution from co-extracted constituents, but the present experiments did not isolate the effects of individual extract components. Previous work has shown that 20-HE can increase intracellular Ca2+, activate Akt, and increase

protein synthesis in C2C12 cells *(10)*; pathway activation was not measured in the present study.

After the proliferation phase, FM- and FE-treated C2C12 cells retained MyHC-positive myotube formation (Fig. 1c). The 5% FBS cultures supplemented with freeze-dried extracts therefore remained capable of subsequent myogenic differentiation rather than showing an obvious loss of differentiation potential. A contribution from 20-HE is biologically plausible because purified 20-HE has been reported to produce rapid Ca2+ signaling, Akt activation, and increased protein synthesis in C2C12 skeletal muscle cells *(10)*. However, the present study did not measure Akt phosphorylation or other pathway intermediates. The signaling explanation should therefore be treated as a mechanistic hypothesis consistent with previous literature, not as a pathway demonstrated by the present experiments.

The bovine myosatellite-cell experiment showed a different serum requirement. All groups maintained with 5% FBS produced lower MTT viability than the 20% FBS control, indicating that the spinach-derived supplements were not sufficient to compensate for this larger reduction in serum. In contrast, the 10% FBS treatment groups generally approached the 20% FBS control, and several were not statistically different from it (Fig. 2a). Among these reduced-serum conditions, pure 20-HE at 0.5 μM and the matched FM treatment showed the strongest responses and were significantly higher than the 10% FBS negative control ($p < 0.001$). These results indicate that bovine myosatellite cells required a higher residual serum concentration than C2C12 cells under the conditions tested.

Nuclear staining supported the same interpretation. Cell counts in the 5% FBS groups remained below the 20% FBS control, whereas the 10% FBS groups showed substantially improved cell numbers, with the 0.5 μM 20-HE and freeze-dried extract conditions among the strongest responses (Fig. 2a,b). The agreement between MTT measurements, nuclear counts, and microscopic observations reduces the likelihood that the observed effect reflects only a change in metabolic activity without a corresponding change in cell abundance.

The selected bovine treatment groups retained differentiation potential after proliferation. Following 3 days of proliferation, the validated treatment groups were differentiated for 10 days in 2% HS differentiation medium. MyHC-positive myotube formation and differentiation indices were comparable to the 20% FBS proliferation control (Fig. 2c,d). Thus, the best-performing proliferation condition differed by cell model: C2C12 cells responded most strongly near 0.05 μM 20-HE equivalent with 5% FBS, whereas bovine myosatellite cells required 10% FBS and showed their strongest response near 0.5 μM.

Taken together, the proliferation experiments support partial rather than complete serum replacement. The spinach-derived extracts were most useful when they supplemented a reduced-serum medium that still retained enough serum to support the baseline requirements of the specific cell type. They also show that the concentration dependence of the response cannot be explained by 20-HE concentration alone. Direct measurements of candidate signaling pathways and fractionation of the extracts would be required to distinguish the contribution of 20-HE from that of other spinach-derived constituents.

3.3 Differentiation effects of spinach-derived HS substitutes

In the C2C12 differentiation experiment, myotube area was evaluated under 1% HS across 0.05, 0.1, 0.5, and 1 μM 20-HE-equivalent treatments (Fig. 3a). The largest measured myotube area was observed in the FE group containing 0.05 μM 20-HE equivalent with 1% HS, which was higher than the 2% HS control ($p < 0.05$). The response was not monotonic across concentrations, indicating that increased treatment concentration did not consistently improve differentiation.

Immunofluorescence imaging confirmed the formation of MyHC-positive myotubes under these reduced-HS conditions (Fig. 3b). Representative FE cultures showed distinct myofiber formation, whereas the response declined at the highest extract concentration. The

overall pattern was therefore non-monotonic: increasing the amount of extract did not continuously increase myotube formation. This result mirrors the concentration dependence observed during proliferation and reinforces the need to optimize the extract as a complete mixture rather than selecting a dose solely on the basis of increasing 20-HE concentration.

In bovine myosatellite cells, spinach-derived extracts were also evaluated under 0% and 1% HS conditions (Fig. 4). In the absence of HS, most extract-treated groups did not show statistically significant improvements over the 0% HS negative control. These results therefore do not support complete replacement of HS by the spinach extract under the tested conditions. The clearer response occurred when HS was reduced to 1% rather than removed entirely.

Among the 1% HS treatments, FM containing 0.05 μM 20-HE equivalent produced a high myotube area. This condition differed significantly from the 1% HS negative control ($p < 0.001$) and from the 2% HS control for myotube area ($p < 0.01$), while the differentiation-index response was not increased to the same extent. At 0.5 and 1 μM, differentiation indices were generally lower or not significantly different among groups. Myotube area and differentiation index should therefore be considered together when interpreting the differentiation response.

The differentiation data are compatible with a contribution from 20-HE and other co-extracted constituents, but the present experiments do not establish a specific molecular mechanism. The observed functional effects should therefore be interpreted at the extract level rather than as direct evidence of PI3K/Akt/mTOR pathway activation.

4. Conclusion

This study shows that phytoecdysteroid-containing spinach extracts can reduce animal-derived serum requirements during muscle-cell culture under the tested conditions. Freeze-dried spinach gave higher 20-HE recovery than fresh spinach, and methanol extraction gave the highest 20-HE concentration. In C2C12 cells, freeze-dried extracts containing 0.05 μM 20-

HE supported proliferation in 5% FBS at levels comparable to or greater than the 10% FBS control. In bovine myosatellite cells, FM containing 0.5 μM 20-HE supported proliferation in 10% FBS at levels comparable to the 20% FBS control. Selected FM and FE conditions also maintained myogenic differentiation when HS was reduced. Together, these findings support approximately 50% serum reduction under the best-performing conditions tested rather than complete serum replacement. The experiments did not identify a single responsible extract component or establish a specific molecular pathway underlying the observed effects.

The methanol-derived FM preparation is not directly suitable for a food-facing process unless residual-solvent safety is demonstrated. Further work should therefore examine alternative extraction solvents or solvent-removal procedures while preserving the serum-reduction effect observed in this study.

References

1. da Silva, B. D. and Conte-Junior, C. A. (2024) Perspectives on cultured meat in countries with economies dependent on animal production: A review of potential challenges and opportunities. Trends Food Sci. Technol. 149, 104551. https://doi.org/10.1016/j.tifs.2024.104551

2. Lee, S. Y., Kang, H. J., Lee, D. Y., Kang, J. H., Ramani, S., Park, S. and Hur, S. J. (2021) Principal protocols for the processing of cultured meat. J. Anim. Sci. Technol. 63, 673–680. https://doi.org/10.5187/jast.2021.e40

3. Rubio, N. R., Xiang, N. and Kaplan, D. L. (2020) Plant-based and cell-based approaches to meat production. Nat. Commun. 11, 6276. https://doi.org/10.1038/s41467-020-20061-y

4. Lee, D. Y., Lee, S. Y., Yun, S. H., Jeong, J. W., Kim, J. H., Kim, H. W., Choi, J. S., Kim, G.-D., Joo, S. T., Choi, I. and Hur, S. J. (2022) Review of the current research on fetal bovine serum and the development of cultured meat. Food Sci. Anim. Resour. 42, 775–799. https://doi.org/10.5851/kosfa.2022.e46

5. Turner, M. C., Brett, R., Saini, A., Stewart, C. E. and Renshaw, D. (2023) Serum concentration impacts myosin heavy chain expression but not cellular respiration in human LHCN-M2 myoblasts undergoing differentiation. Exp. Physiol. 108, 169–176. https://doi.org/10.1113/EP090564

6. Gstraunthaler, G., Lindl, T. and van der Valk, J. (2013) A plea to reduce or replace fetal bovine serum in cell culture media. Cytotechnology 65, 791–793. https://doi.org/10.1007/s10616-013-9633-8

7. Lee, D. Y., Yun, S. H., Lee, S. Y., Lee, J., Mariano, E. Jr., Joo, S.-T., Choi, I., Choi, J. S., Kim, G.-D., Lee, J., Choi, S.-H. and Hur, S. J. (2023) Analysis of commercial fetal bovine serum (FBS) and its substitutes in the development of cultured meat. Food Res.

Int. 174(Pt 1), 113617. https://doi.org/10.1016/j.foodres.2023.113617

8. van der Valk, J. (2022) Fetal bovine serum—A cell culture dilemma. Science 375, 143–144. https://doi.org/10.1126/science.abm1317

9. Arif, Y., Singh, P., Bajguz, A. and Hayat, S. (2022) Phytoecdysteroids: Distribution, structural diversity, biosynthesis, activity, and crosstalk with phytohormones. Int. J. Mol. Sci. 23, 8664. https://doi.org/10.3390/ijms23158664

10. Gorelick-Feldman, J., Cohick, W. and Raskin, I. (2010) Ecdysteroids elicit a rapid Ca2+ flux leading to Akt activation and increased protein synthesis in skeletal muscle cells. Steroids 75, 632–637. https://doi.org/10.1016/j.steroids.2010.03.008

11. Lafont, R., Harmatha, J., Marion-Poll, F., Dinan, L. and Wilson, I. D. (2009) The Ecdysone Handbook. EcdyBase.

12. Yun, S. H., Lee, D. Y., Lee, S. Y., Lee, J., Mariano, E. Jr., Joo, S.-T., Choi, I., Choi, J. S., Kim, G.-D. and Hur, S. J. (2023) Improved culture procedure for bovine muscle satellite cells for cultured meat. Food Res. Int. 174(Pt 2), 113660. https://doi.org/10.1016/j.foodres.2023.113660

13. Tankiewicz, M., Namieśnik, J. and Sawicki, W. (2016) Analytical procedures for quality control of pharmaceuticals in terms of residual solvents content: Challenges and recent developments. TrAC Trends Anal. Chem. 80, 328–344. https://doi.org/10.1016/j.trac.2015.09.008

14. Lee, J. H., Tachibana, H., Morinaga, Y., Fujimura, Y. and Yamada, K. (2009) Modulation of proliferation and differentiation of C2C12 skeletal muscle cells by fatty acids. Life Sci. 84, 415–420. https://doi.org/10.1016/j.lfs.2009.01.004

15. Averous, J., Gabillard, J. C., Seiliez, I. and Dardevet, D. (2012) Leucine limitation regulates myf5 and myoD expression and inhibits myoblast differentiation. Exp. Cell Res. 318, 217–227. https://doi.org/10.1016/j.yexcr.2011.10.015

16. Han, J. M., Jeong, S. J., Park, M. C., Kim, G., Kwon, N. H., Kim, H. K., Ha, S. H., Ryu, S. H. and Kim, S. (2012) Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. Cell 149, 410–424. https://doi.org/10.1016/j.cell.2012.02.044

17. Wolfson, R. L., Chantranupong, L., Saxton, R. A., Shen, K., Scaria, S. M., Cantor, J. R. and Sabatini, D. M. (2016) Sestrin2 is a leucine sensor for the mTORC1 pathway. Science 351, 43–48. https://doi.org/10.1126/science.aab2674

Statements and Declarations

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Competing Interests

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

Author Contributions

J.P. performed the investigation and data curation and wrote the original draft. D.-Y.L., E.J.M., D.H., Y.C., J.S.K., J.W.P., and S.N. contributed to the investigation, data curation, and review and editing of the manuscript. S.J.H. supervised and conceptualized the study and contributed to review and editing of the manuscript. All authors reviewed and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

Not applicable.

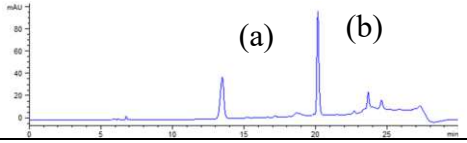
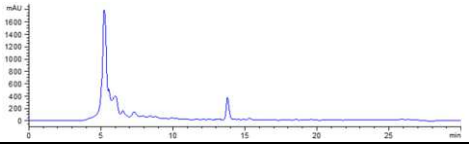
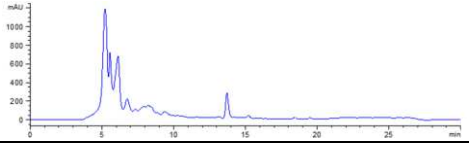
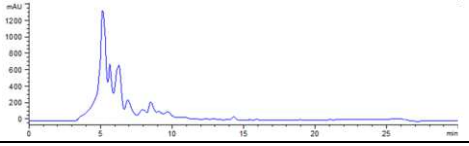
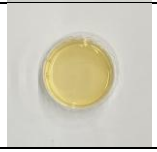
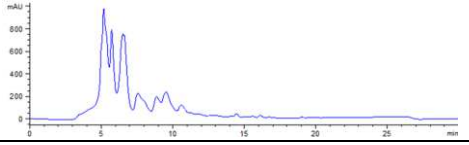
Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Table 1. Variation in phytoecdysteroid content of spinach-derived extracts depending on extraction solvent and method

Sample	Extraction solvent	Extract images	HPLC Chromatogram (100 ppm)	20-HE ($\mu g \cdot kg^{-1}$)
Standard material (20-HE+PonA)	-	-		-
Freeze-dried spinach	MeOH			87.83±1.40
	EtOH			55.25±0.19
Raw spinach	MeOH			10.96±0.28
	EtOH			7.06±0.31

Chromatogram labels: (a) 20-hydroxyecdysone; (b) Ponasterone A. Data are presented as the mean ± standard deviation.

446 **Table 2. Free amino acid profiles**

Compound	Free amino acid profile (mg/kg)					
	FBS	**HS**	**FM**	**FE**	**RM**	**RE**
Phosphoserine	-	-	-	-	-	-
Taurine	22.2 ± 0.0	5.4 ± 0.1	0.1 ± 0.0	-	-	-
Phosphorylethanolamine	-	-	-	-	-	-
Urea	290.0 ± 0.2	178.1 ± 3.8	-	-	-	-
Aspartic acid	14.1 ± 0.0	268.0 ± 1.6	2.3 ± 0.1	0.4 ± 0.0	6.4 ± 0.3	4.2 ± 0.2
Threonine	16.5 ± 0.0	1,389.8 ± 54.9	0.6 ± 0.0	0.7 ± 0.0	1.1 ± 0.0	1.0 ± 0.1
Serine	33.3 ± 0.0	17.1 ± 0.3	1.1 ± 0.1	0.7 ± 0.0	2.1 ± 0.1	1.9 ± 0.1
Asparagine	2.5 ± 0.0	6.2 ± 0.2	1.3 ± 0.0	0.6 ± 0.0	2.3 ± 0.0	1.9 ± 0.0
Glutamic acid	135.7 ± 0.2	26.9 ± 0.5	4.1 ± 0.1	1.0 ± 0.0	6.8 ± 0.1	6.7 ± 0.3
Glutamine	70.8 ± 0.1	14.9 ± 0.4	10.5 ± 0.9	8.9 ± 0.4	13.9 ± 0.2	12.6 ± 0.2
Sarcosine	-	-	-	-	-	-
Glycine	48.6 ± 0.1	32.6 ± 1.6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ±0.0
Alanine	90.3 ± 0.2	20.5 ± 0.6	1.4 ± 0.1	2.0 ± 0.0	1.3 ± 0.0	1.3 ± 0.1
Citrulline	13.1 ± 0.0	340.0 ± 15.8	-	-	-	-
α-amino-n-butyric acid	3.1 ± 0.0	48.2 ± 1.6	-	-	-	-
Valine	40.0 ± 0.1	17.0 ± 0.3	1.0 ± 0.1	2.0 ± 0.1	1.7 ± 0.0	1.2 ± 0.0
Cystine	0.1 ± 0.0	6.8 ± 0.0	-	-	-	0.6 ± 0.0
Methionine	5.02 ± 0.0	284.1 ± 7.0	-	-	-	0.1 ± 0.0
Cystathionine	1.9 ± 0.0	18.5 ± 0.3	-	-	-	-
Isoleucine	18.4 ± 0.0	695.6 ± 7.9	0.7 ± 0.0	1.5 ± 0.0	1.6 ± 0.1	1.5 ± 0.0
Leucine	33.0 ± 0.1	16.6 ± 0.4	1.0 ± 0.0	2.1 ± 0.0	2.5 ± 0.1	2.4 ± 0.1
Tyrosine	17.7 ± 0.0	679.1 ± 26.4	0.4 ± 0.0	0.9 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
Phenylalanine	25.8 ± 0.0	12.7 ± 0.5	0.3 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.0

β-Alanine	-	27.4 ± 0.7	-	-	-	-
β-Amino isobutyric acid	-	-	-	-	-	-
γ-Amino-n-butyric acid	-	-	0.9 ± 0.0	1.3 ± 0.0	3.0 ± 0.1	2.8 ± 0.1
Tryptophan	9.3 ± 0.0	615.1 ± 22.2	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Ethanol amine	0.6 ± 0.0	-	-	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
Ammonia	13.1 ± 0.0	325.8 ± 18.0	2.2 ± 0.2	1.8 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
Hydroxylysine	-	-	-	-	-	-
Ornithine	33.4 ± 0.0	10.5 ± 0.5	-	-	-	-
Lysine	27.3 ± 0.0	15.0 ± 0.8	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
1-Methylhistidine	-	-	-	-	-	-
Histidine	14.4 ± 0.0	942.3 ± 23.2	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
3-Methylhistidine	6.9 ± 0.0	261.4 ± 10.3	-	-	-	-
Anserine	-	-	-	-	-	-
Carnosine	9.2 ± 0.0	194.3 ± 5.8	-	-	-	-
Arginine	4.8 ± 0.0	1,223.6 ± 32.1	0.6 ± 0.0	1.4 ± 0.1	1.9 ± 0.0	2.0 ± 0.0
Hydroxyproline	17.4 ± 0.0	144.7 ± 3.7	-	-	-	-
Proline	24.5 ± 0.0	7.1 ± 0.4	1.7 ± 0.0	3.0 ± 0.1	3.6 ± 0.1	3.9 ± 0.1

FBS: fetal bovine serum; HS: horse serum; FM: methyl alcohol extract of freeze-dried spinach; FE: ethyl alcohol extract of freeze-dried spinach; RM: methyl alcohol extract of raw spinach; RE: ethyl alcohol extract of raw spinach. Data are presented as the mean ± standard deviation.

Table 3. Fatty acid profiles

Compound		Fatty acid profile (mg/kg)					
		FBS	**HS**	**FM**	**FE**	**RM**	**RE**
C12:0	Lauric acid	18.3 ± 0.5	2.8 ± 0.1	7.7 ± 0.1	8.3 ± 0.3	8.3 ± 0.2	8.0 ± 0.7
C14:1n5	Myristoleic acid	11.2 ± 0.5	-	5.5 ± 0.2	6.6 ± 0.1	5.4 ± 0.2	7.6 ± 0.1
C14:0	Myristic acid	21.6 ± 0.4	4.8 ± 0.1	9.8 ± 0.2	10.5 ± 0.1	10.7 ± 0.4	11.2 ± 0.7
C16:1n7	Palmitoleic acid	18.5 ± 0.8	11.9 ± 0.4	5.5 ± 0.2	6.6 ± 0.2	6.8 ± 0.3	6.8 ± 0.4
C16:0	Palmitic acid	1,019.4 ± 23.7	189.6 ± 8.8	977.9 ± 47.1	1,172.6 ±4.6	1,307.7 ± 39.9	1,258.6 ±62.9
C17:0	Margaric acid	15.5 ± 0.3	5.5 ± 0.2	5.7 ± 0.2	6.4 ± 0.6	6.6 ± 0.2	6.2 ± 0.4
C18:2n6	Linoleic acid	25.3 ± 0.4	314.5 ± 6.3	1.8 ± 0.1	5.9 ± 0.0	0.0 ± 0.0	1.6 ± 0.1
C18:1n9	Oleic acid	49.2 ± 2.1	95.4 ± 3.0	7.1 ± 0.2	17.2 ± 0.0	3.2 ± 0.2	2.4 ± 0.2
C18:0	Stearic acid	1,550.6 ± 38.0	271.4 ± 8.4	736.9 ± 0.9	864.0 ± 30.1	1,106.6 ± 56.9	943.8 ± 23.9
C20:0	Arachidic acid	10.3 ± 0.1	2.9 ±0.2	2.8 ± 0.2	3.3 ± 0.2	4.3 ± 0.4	3.9 ± 0.1

FBS: fetal bovine serum; HS: horse serum; FM: methyl alcohol extract of freeze-dried spinach; FE: ethyl alcohol extract of freeze-dried spinach; RM: methyl alcohol extract of raw spinach; RE: ethyl alcohol extract of raw spinach. Data are presented as the mean ± standard deviation.

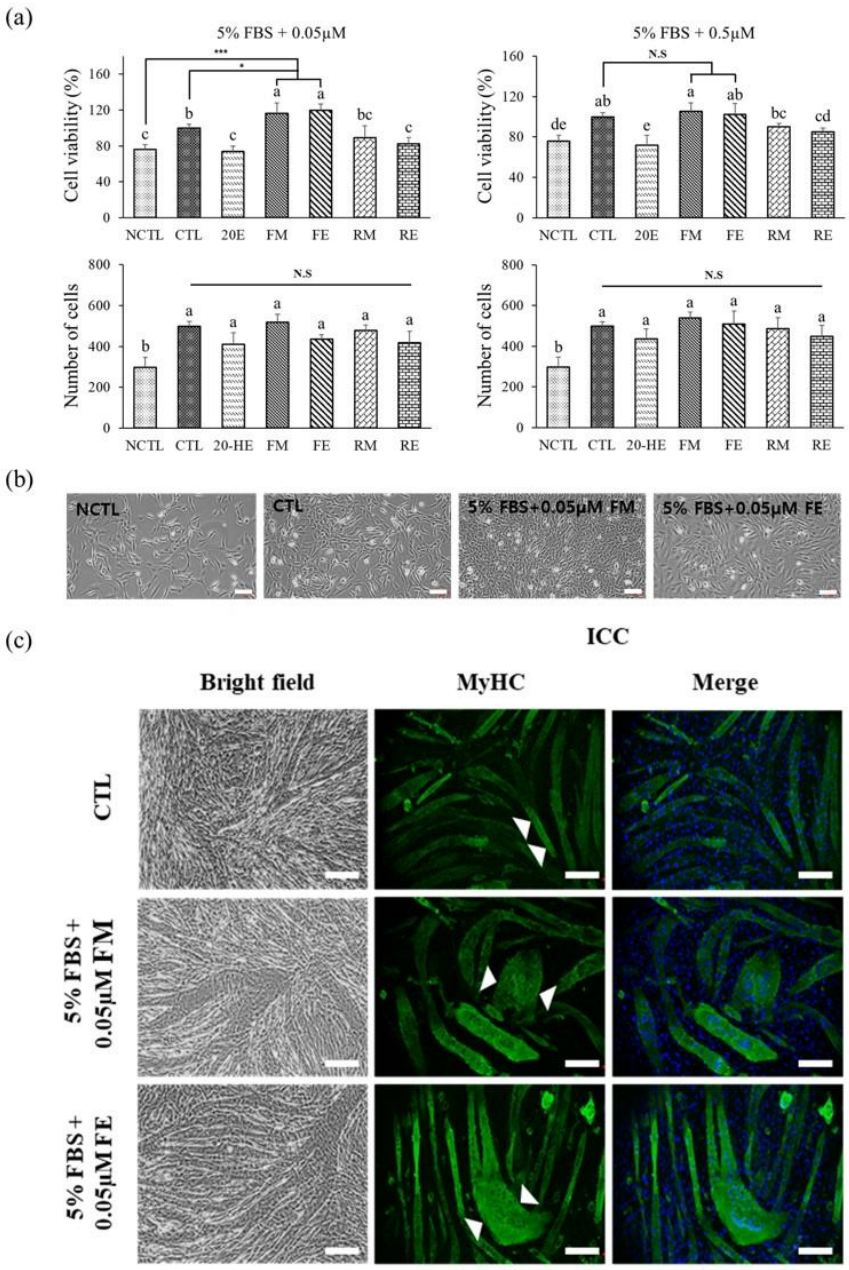


Fig. 1 Effects of spinach extracts on C2C12 myoblast cell viability and proliferation. NCTL: FBS 5%; CTL: FBS 10%; 20-HE: 20-hydroxyecdysone; FM: methyl alcohol extract of freeze-dried spinach; FE: ethyl alcohol extract of freeze-dried spinach; RM: methyl alcohol extract of raw spinach; RE: ethyl alcohol extract of raw spinach. (a) The proliferation effect of the spinach-derived serum substitute containing 0.05 or 0.5 μM 20-HE was investigated using the MTT assay and Hoechst staining. Lowercase letters indicate significant differences ($p < 0.05$). ***$p < 0.001$, *$p < 0.05$, N.S: non-significant ($p > 0.05$); (b) C2C12 myoblast cell morphology on day 3 of proliferation with freeze-dried, spinach-derived serum substitute containing 0.05 μM 20-HE; Scale bar, 100 μm. Data are presented as the mean ± standard deviation; (c) Analysis of myofiber formation in C2C12 myoblasts cultured with spinach extract. MyHC: myosin heavy chain; NCTL: FBS 5%; CTL: FBS 10%; FM: methyl alcohol extract of freeze-dried spinach; FE: ethyl alcohol extract of freeze-dried spinach. Scale bar, 100 μm

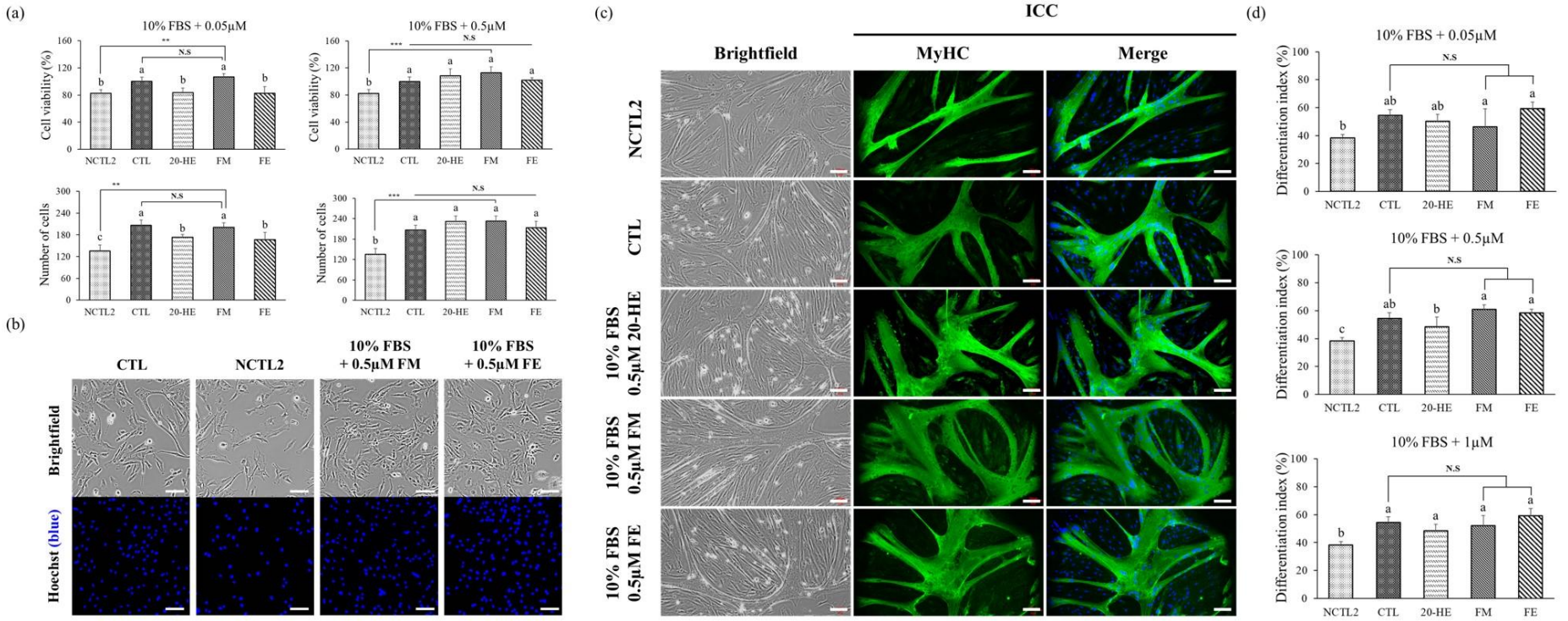


Fig. 2 Effects of spinach extracts on bovine myoblast cell viability and proliferation. NCTL2: FBS 10%; CTL: FBS 20%; 20-HE: 20-hydroxyecdysone; FM: methyl alcohol extract of freeze-dried spinach; FE: ethyl alcohol extract of freeze-dried spinach; (a) The proliferation effect of the spinach-derived serum substitute containing 0.05 or 0.5 μM 20-HE was investigated using the MTT assay and Hoechst staining. Lowercase letters indicate significant differences ($p < 0.05$). ***$p < 0.001$, **$p < 0.01$, N.S: non-significant ($p > 0.05$); (b) Fluorescence micrographs of the nuclei of bovine cells stained with Hoechst (blue); Scale bar, 100 μm. Data are presented as the mean ± standard deviation; (c) Bovine cells cultured for 3 days in spinach-derived serum substitute containing 0.05, 0.5, and 1 μM 20-HE with 10% FBS were transferred to a 2% HS differentiation medium; (d) The differentiation index was calculated for each condition. The differentiation index was calculated based on the total number of MyHC-positive cells versus the total number of nuclei; Scale bar, 100 μm. Data are presented as the mean ± standard deviation. Lowercase letters indicate significant differences ($p < 0.05$). N.S: non-significant ($p > 0.05$)

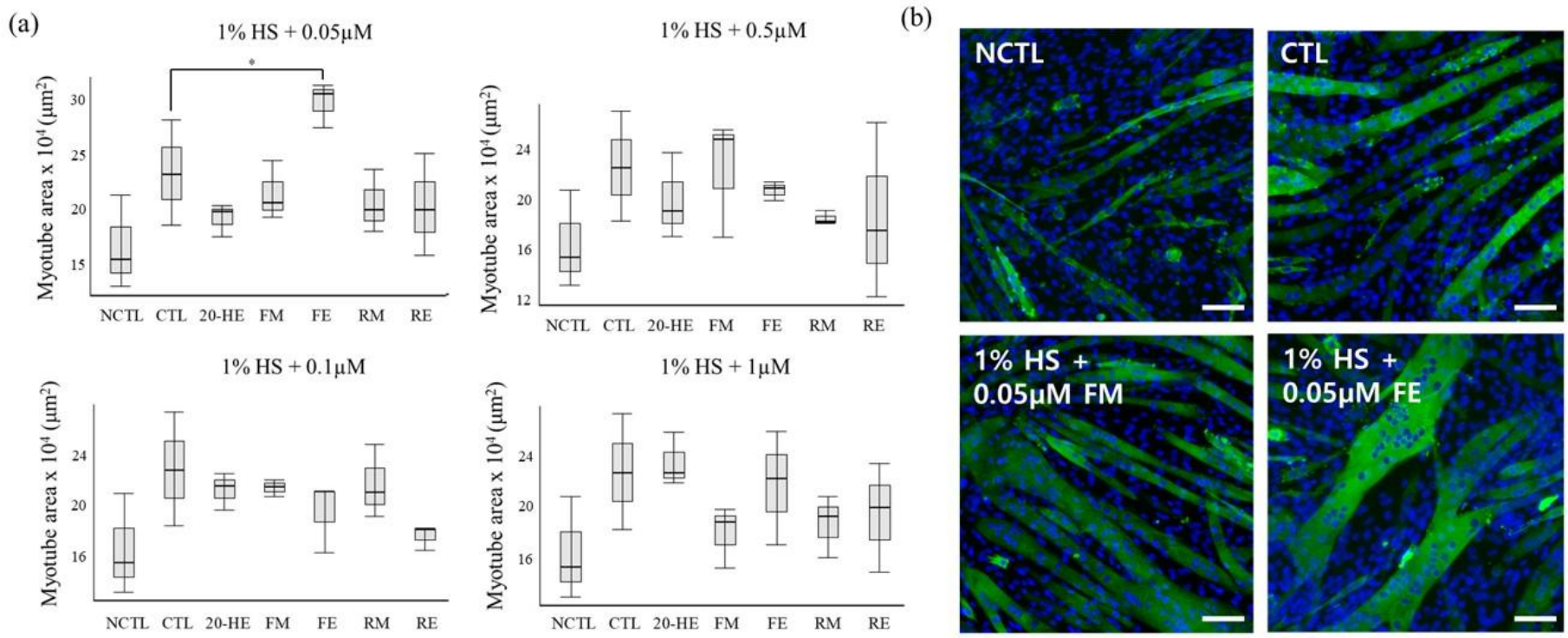
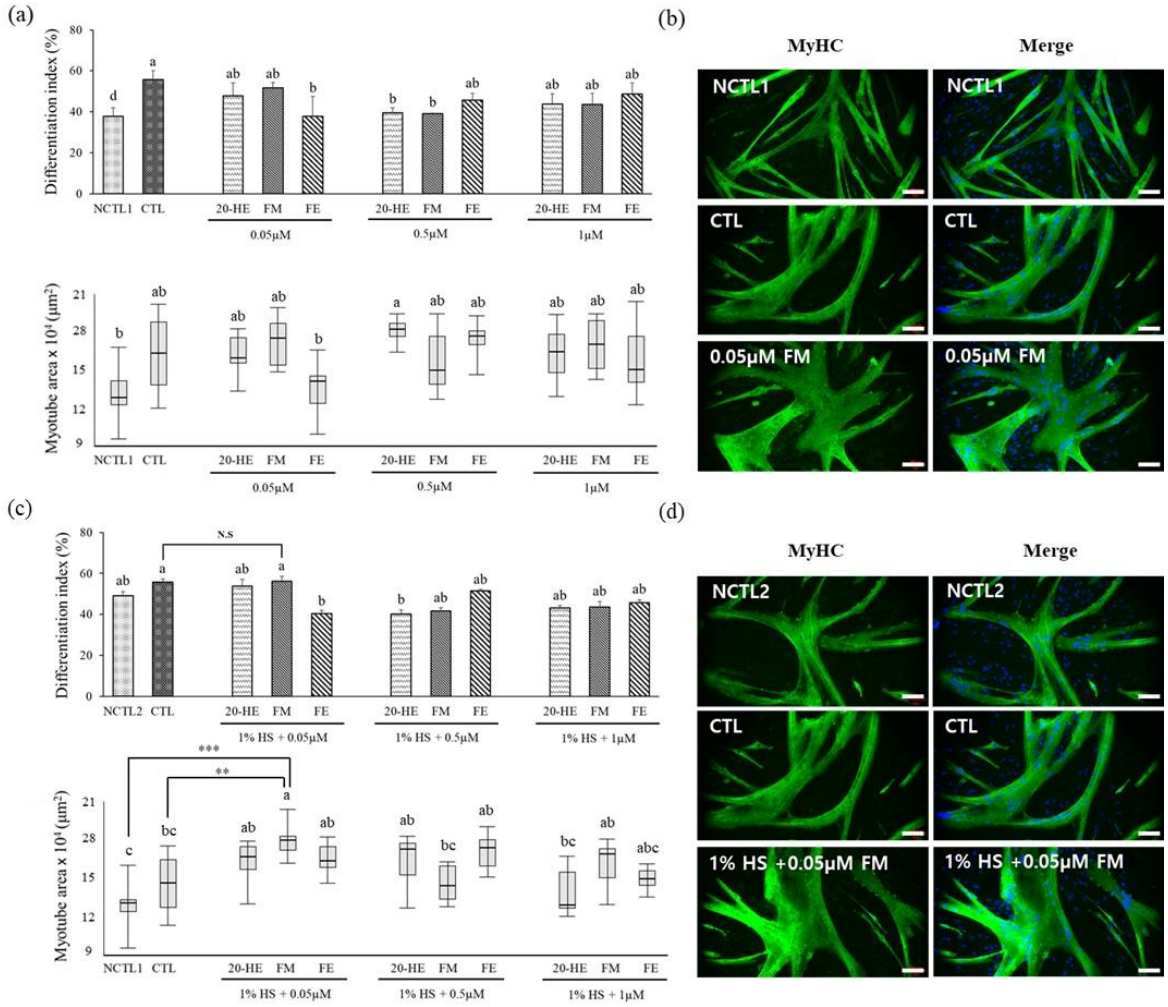


Fig. 3 Immunofluorescent staining (MF20) of the myotube areas of C2C12 myoblasts cultured in different spinach extracts. NCTL: HS 1%; CTL: HS 2%; 20-HE: 20-hydroxyecdysone; FM: methyl alcohol extract of freeze-dried spinach; FE: ethyl alcohol extract of freeze-dried spinach; RM: methyl alcohol extract of raw spinach; RE: ethyl alcohol extract of raw spinach; (a) The differentiation effect of a spinach-derived serum substitute containing 0.05, 0.5, 0.1, and 1 μM 20-HE with 1% HS was investigated based on myotube area, represented by box-and-whisker plots displaying the median, first and third quartiles, and minimum and maximum values for myotube areas in each group; (b) Fixed cells were visualized using anti-MyHC antibody and a fluorescence-labeled secondary antibody (green). Nuclei were stained with Hoechst dye (blue); Scale bar, 100 μm. *p < 0.05



485

486 **Fig. 4** Effects of spinach extracts on bovine myoblast cell differentiation based on fluorescent
487 staining (MF20). MyHC: myosin heavy chain; NCTL1: HS 0%; NCTL2: HS 1%; CTL: HS
488 2%; 20-HE: 20-hydroxyecdysone; FM: methyl alcohol extract of freeze-dried spinach; FE:
489 ethyl alcohol extract of freeze-dried spinach; (a,c) The differentiation index was calculated
490 based on the total number of MyHC-positive cells versus the total number of nuclei. The
491 differentiation effects of spinach-derived serum substitutes containing 0.05, 0.5, and 1 μM 20-
492 HE with 0% or 1% HS were investigated based on myotube area, represented by box-and-
493 whisker plots displaying the median, first and third quartiles, and minimum and maximum
494 values for myotube areas in each group; (b,d) Fixed cells were visualized using anti-MyHC
495 antibody and a fluorescence-labeled secondary antibody (green). Nuclei were stained with
496 Hoechst dye (blue); Scale bar, 100 μm. Data are presented as the mean ± standard deviation.
497 Lowercase letters indicate significant differences ($p < 0.05$). ***$p < 0.001$, **$p < 0.01$, N.S:
498 non-significant ($p > 0.05$)