

Evaluation of the effects of *Bambusa tulda* on osteogenic differentiation and mineralization of human stem cells.

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Abstract

Bambusa tulda has been used for various Purpose and is considered as one of the most useful bamboo species. This study aimed to evaluate the effects of *Bambusa tulda* extract (BBT) on the osteogenic differentiation and mineralization of human mesenchymal stem cells. Stem cells obtained from gingivae were cultured in an osteogenic medium in the presence of BBT at concentrations ranging from 0.001% to 1%. Evaluations of cell morphology and cellular viability were done at days 1, 3, 5 and 7. Alkaline phosphatase activity assays and Alizarin red S staining were performed to evaluate the osteogenic differentiation of stem cells. The morphology of stem cells in the presence of BBT at final concentrations of 0%, 0.001%, 0.01%, 0.1%, and 1% did not show any noticeable changes when compared with the untreated control group. The treatment of BBT (from 0.001% to 1% groups) showed decrease in alkaline phosphatase activity. The results of the Alizarin Red S staining showed a significant decrease with application of BBT. Conclusively, *Bambusa tulda* had influenced the osteogenic differentiation of the stem cells derived from the gingiva. Thus, the use of *Bambusa tulda* may produce adverse effects onto oral tissues. The concentration and application time of *Bambusa tulda* should be meticulously controlled to minimize the adverse effects.

Keywords: Bambusa, Cell differentiation, Cell survival, Herbal medicine, Medical plants, Stem cells.

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Introduction

Herbal medicines are widely used for treating the various kinds of diseases and generally regarded as safe due to their extensive use in Traditional Medicine practice for thousands of years [1-3]. Many plants are shown to possess antimicrobiological and anti-inflammatory functions and they have been applied for the the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic [4]. More recently, potential role of herbal medicine on stem cell have been tested *in vitro* and *in vivo* experiments [5]. One of the traditional Chinese herbal medicine, *Sarcandra glabra* is reported to protect mesenchymal stem cells from oxidative stress [6]. Icaritin, derived from *Epimedium* is shown to effectively alleviate nephrectomy induced chronic renal failure through increasing renal stem cells [7]. However, the traditional Chinese medicine formula, Pien Tze Huang inhibited the proliferation, and induced the apoptosis and differentiation of cancer stem cells *via* suppression of the Notch1 pathway [8].

Bambusa tulda Roxburgh has been widely used and it is considered one of the most useful bamboo species [9]. It is

native to Indian subcontinent, Indochina, Tibet, and Yunnan [10]. *Bambusa tulda* has nutritive shoots and it is widely consumed as edible bamboos in Asia [11]. However, within the authors' knowledge, the effects of extracts of *Bambusa tulda* on differentiation of stem cells have not been revealed yet. This study aimed to evaluate the effects of *Bambusa tulda* extract (BBT) on the osteogenic differentiation and mineralization of human mesenchymal stem cells.

Material and Methods

Preparation of plant materials

Bambusa tulda Roxburgh was collected by Vashkar Chowdhury from Amki village, Sonaimuri Upazilla, Noakhali District, Bangladesh. A voucher specimen recoded as PB022073 was deposited in the herbarium of the Korea Research Institute of Bioscience and Biotechnology. After drying and grinding leaves of *Bambusa tulda*, the powder (63 g) was added to 500 mL of methanol. The method of repercolation at room temperature was used for the extraction method. The extract was filtered and concentrated by rotavapor

under reduced pressure, thereby obtaining 2.75 g of *Bambusa tulda* methanolic extract (BBT).

Stem cells isolated from human gingiva

The gingivae were obtained from healthy patients visiting the Department of Periodontics, Seoul St. Mary's Hospital, College of Medicine, the Catholic University of Korea. The approval number of the Institutional Review Board is KC11SISI0348 and informed consents were obtained from the participants. This study was performed in accordance with the relevant guidelines and regulations. De-epithelialization of the gingivae was done and fragmentation of the tissue was done into 1-2 mm fragments. Dispase (1 mg/ml; Sigma-Aldrich Co., St. Louis, MO, USA) and collagenase IV (2 mg/mL; Sigma-Aldrich Co.) were used for the digestion of fragmented gingivae [10]. Unattached cells were removed from the plates. The media was changed every 2 to 3 days and cells were incubated with 5% CO₂ and 95% O₂ in 37°C incubator.

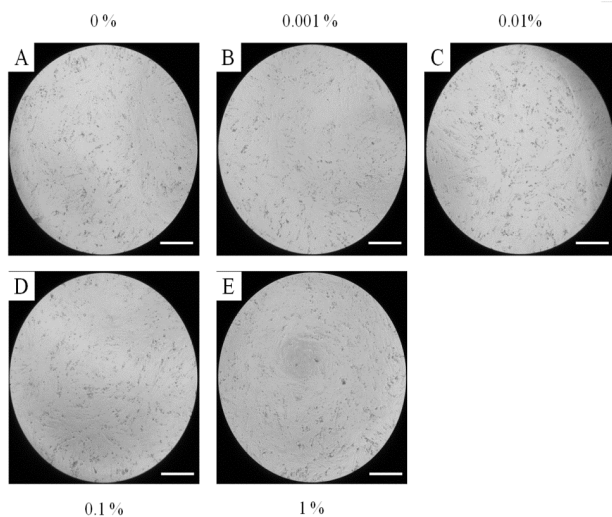


Figure 1. Evaluation of cell morphology on day 1 using inverted microscopy following treatment with different concentrations of BBT in osteogenic media. A) Control group (original magnification 200X); B) 0.001% group (original magnification 200X); C) 0.01% group (original magnification 200X); D) 0.1% group (original magnification 200X); E) 1% group (original magnification 200X). The bar indicates 200 μ m.

Evaluation of cellular morphology

Stem cells were plated at a density of 2.0×10^3 cells/well and cultured in an osteogenic medium (alpha-minimal essential medium (α -MEM, Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS, Gibco), 200 mM L-glutamine (Sigma-Aldrich Co.), 10 mM of ascorbic acid 2-phosphate (Sigma-Aldrich Co.), 38 μ g/ml of dexamethasone, 2 mg/ml of glycerophosphate disodium salt hydrate, and 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma-Aldrich Co.)). The final concentrations of BBT were 0%, 0.001%, 0.01%, 0.1%, and 1%, respectively. On days 1, 3, 5 and 7, the morphological evaluation was performed using inverted microscopy (CKX41SF, Olympus Corporation, Tokyo, Japan).

Evaluation of cellular viability

On days 1, 3, 5, and 7, the evaluation of cellular viability was performed according to the previous report using the Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) assay [11]. In short, cells were incubated with tetrazolium monosodium salt for 2 h at 37°C. Spectrophotometric absorbance at 450 nm was done using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

Alkaline phosphatase activity assays

On days 3 and 7, alkaline phosphatase activity assays were done using a commercially available kit (K412-500, BioVision, Inc., Milpitas, CA, USA). A microplate reader (BioTek Instruments Inc.) was used to measure the spectrophotometric absorbance of the samples.

Evaluation of Alizarin Red S staining

On days 3, 7, and 14, Alizarin Red S staining was performed. The cells were washed, fixed, and stained with 2% Alizarin Red S Solution (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) and evaluated with a microscope (CKX41SF, Olympus Corporation). Ten percent cetylpyridinium chloride (Sigma-Aldrich Co.) was used to solubilize the bound dye, and spectrophotometric quantification was performed at 560 nm (BioTek Instruments Inc.).

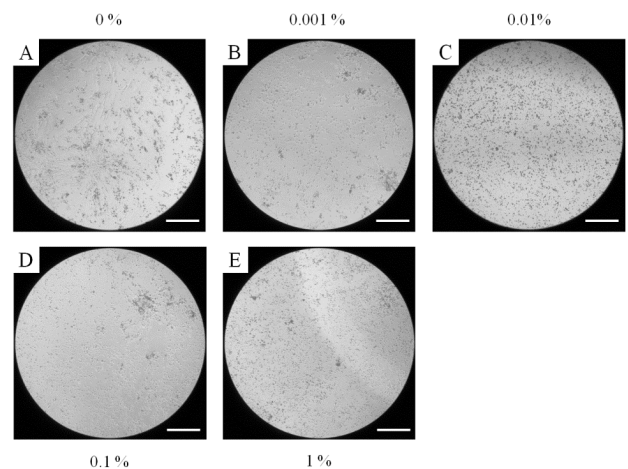


Figure 2. Evaluation of cell morphology on day 3 using inverted microscopy following treatment with different concentrations of BBT in osteogenic media. A) Control group (original magnification 200X); B) 0.001% group (original magnification 200X); C) 0.01% group (original magnification 200X); D) 0.1% group (original magnification 200X); E) 1% group (original magnification 200X). The bar indicates 200 μ m.

Statistical analysis

The data were shown as mean \pm standard deviation of the experiments. A test of normality was performed, and a one-way analysis of variance with post hoc Tukey's test was performed to determine the differences between the groups using a commercially available program (SPSS 12 for

Windows, SPSS Inc., Chicago, IL, USA). The level of significance was considered 0.05.

Results

Evaluation of cell morphology

The morphology of stem cells treated with BBT at final concentrations of 0%, 0.001%, 0.01%, 0.1%, and 1% on day 1 is shown in Figure 1. Stem cells in the control group showed fibroblast-like morphology on day 1 (Figure 1). The morphology of stem cells in the presence of BBT at final concentrations of 0%, 0.001%, 0.01%, 0.1%, and 1% did show noticeable changes when compared with the untreated control group (Figures 2-4).

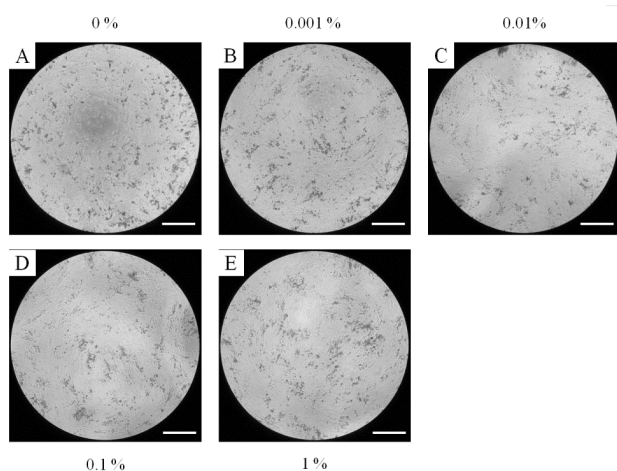


Figure 3. Evaluation of cell morphology on day 5 using inverted microscopy following treatment with different concentrations of BBT in osteogenic media. A) Control group (original magnification 200X); B) 0.001% group (original magnification 200X); C) 0.01% group (original magnification 200X); D) 0.1% group (original magnification 200X); E) 1% group (original magnification 200X); The bar indicates 200 μ m.

Cellular viability

Results from the CCK8 assay revealed cellular viability on days 1, 3, 5 and 7 which are shown in Figure 5. The relative values of CCK-8 on day 1 for 0.001%, 0.01%, 0.1% and 1% are $72.2\% \pm 8.2\%$, $81.5\% \pm 7.4\%$, $79.2\% \pm 1.2\%$, $74.1\% \pm 6.2\%$, and $68.5\% \pm 8.7\%$, respectively, when the control (0%) group on day 1 is considered 100% ($100.0\% \pm 5.7\%$) ($P < 0.05$) (Figure 5A). The relative values of CCK-8 on day 3 for 0.001%, 0.01%, 0.1% and 1% are $81.2\% \pm 7.1\%$, $65.2\% \pm 9.5\%$, $77.6\% \pm 4.5\%$, $71.0\% \pm 4.1\%$, and $90.6\% \pm 35.3\%$, respectively, whereas the control (0%) group on day 3 is considered 100% ($100.0\% \pm 1.7\%$) ($P < 0.05$) (Figure 5B). The relative values of CCK-8 on day 5 for 0.001%, 0.01%, 0.1% and 1% are $91.4\% \pm 1.9\%$, $97.7\% \pm 5.7\%$, $92.5\% \pm 0.1\%$, $92.5\% \pm 0.7\%$, and $94.5\% \pm 1.1\%$, respectively, whereas the control (0%) group on day 5 is considered 100% ($100.0\% \pm 3.9\%$) (Figure 5C). The relative values of CCK-8 on day 5 for 0.001%, 0.01%, 0.1% and 1% are $47.9\% \pm 0.5\%$, $44.5\% \pm 0.7\%$, $45.9\% \pm 0.2\%$, $42.1\% \pm 0.7\%$, and $42.8\% \pm 0.2\%$,

respectively, whereas the control (0%) group at day 7 is considered 100% ($100.0\% \pm 1.5\%$) ($P < 0.05$) (Figure 5D).

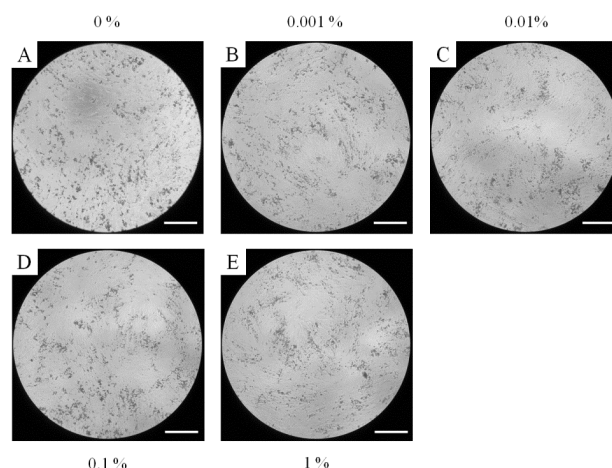


Figure 4. Evaluation of cell morphology on day 7 using inverted microscopy following treatment with different concentrations of BBT in osteogenic media. A) Control group (original magnification 200X); B) 0.001% group (original magnification 200X); C) 0.01% group (original magnification 200X); D) 0.1% group (original magnification 200X); E) 1% group (original magnification 200X). The bar indicates 200 μ m.

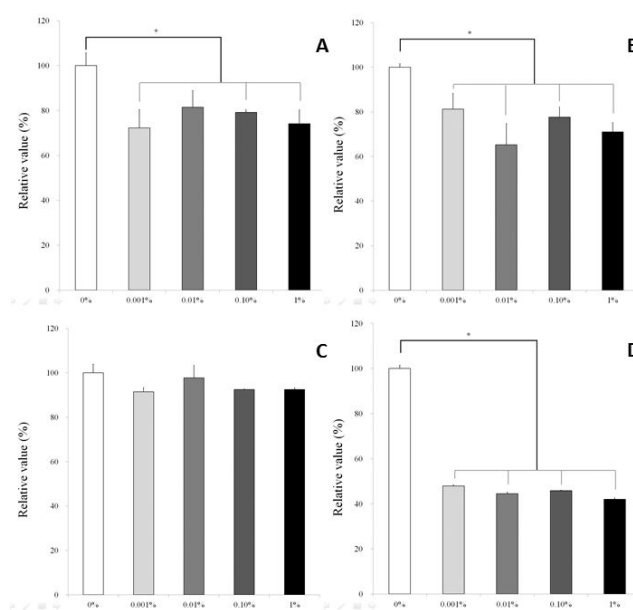


Figure 5. The CCK-8 assay results cultured with osteogenic media. A) Day 1; B) Day 3; C) Day 5; D) Day 7.

Alkaline phosphatase activity assays

The alkaline phosphatase activity treated with BBT at days 3 and 7 is shown in Figure 6. The absorbance values of cells on day 3 cultured with 0%, 0.001%, 0.01%, 0.1%, and 1% BBT were 0.083 ± 0.001 , 0.089 ± 0.006 , 0.076 ± 0.000 , 0.085 ± 0.001 , 0.091 ± 0.001 , and 0.076 ± 0.001 , respectively. The absorbance values of cells on day 7 cultured with 0%, 0.001%, 0.01%, 0.1%, and 1% BBT were 0.102 ± 0.001 , 0.095 ± 0.000 , 0.096 ± 0.001 , 0.098 ± 0.001 , 0.089 ± 0.003 , and $0.087 \pm$

0.001, respectively. The treatment of BBT (from 0.001% to 1% groups) showed decrease in alkaline phosphatase activity but this did not reach statistical significance at day 7.

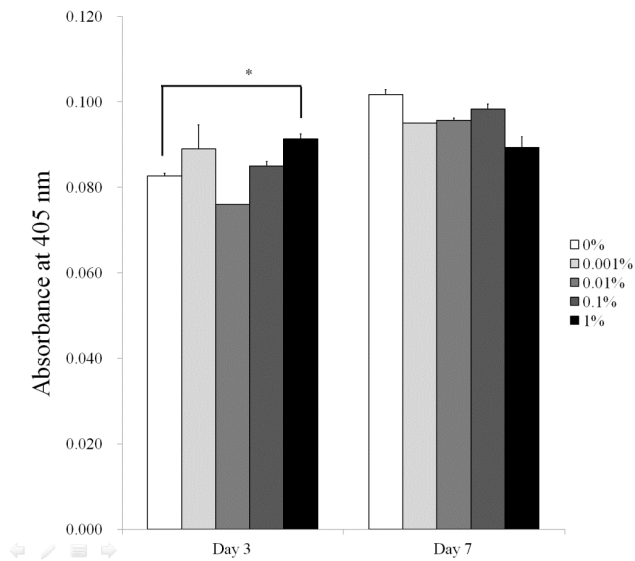


Figure 6. Alkaline phosphatase activity on days 3 and 7.

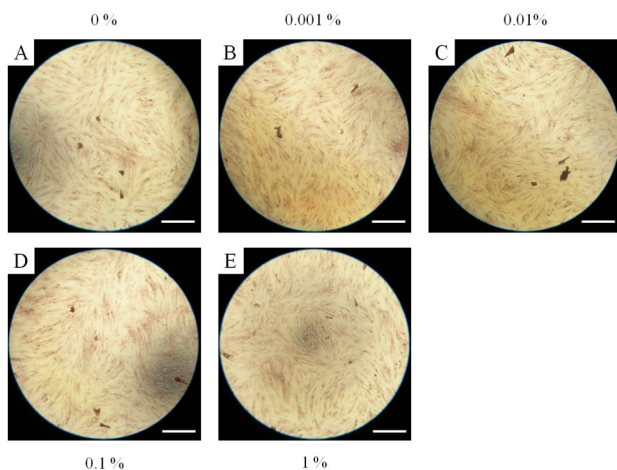


Figure 7. Results of Alizarin Red S staining on day 3 following treatment with different concentrations of BBT on osteogenic supplement. A) Control group (original magnification 100X); B) 0.001% group (original magnification 100X); C) 0.01% group (original magnification 100X); D) 0.1% group (original magnification 100X); E) 1% group (original magnification 100X). The bar indicates 400 μ m.

Mineralization assay

The results of the Alizarin Red S staining on days 3, 7 and 14 following treatment with different concentrations of BBT in an osteogenic supplement are shown in Figures 7-9. The absorbance of cells on day 3 cultured with 0%, 0.001%, 0.01%, 0.1%, and 1% BBT was 0.053 ± 0.002 , 0.064 ± 0.009 , 0.060 ± 0.005 , 0.074 ± 0.017 , and 0.051 ± 0.003 , respectively (Figure 10). The absorbance of cells on day 7 cultured with 0%, 0%, 0.001%, 0.01%, 0.1%, and 1% BBT was 0.107 ± 0.001 , 0.102 ± 0.004 , 0.096 ± 0.005 , 0.094 ± 0.013 , and 0.102 ± 0.001 ,

respectively (Figure 10). The absorbance of cells on day 14 cultured with 0%, 0.01%, 0.1%, and 1% BBT was 0.250 ± 0.004 , 0.098 ± 0.003 , 0.085 ± 0.001 , 0.096 ± 0.003 , and 0.100 ± 0.011 , respectively. A statistically significant decrease of absorbance was noted with application of BBT on day 14 ($P < 0.05$) (Figure 10).

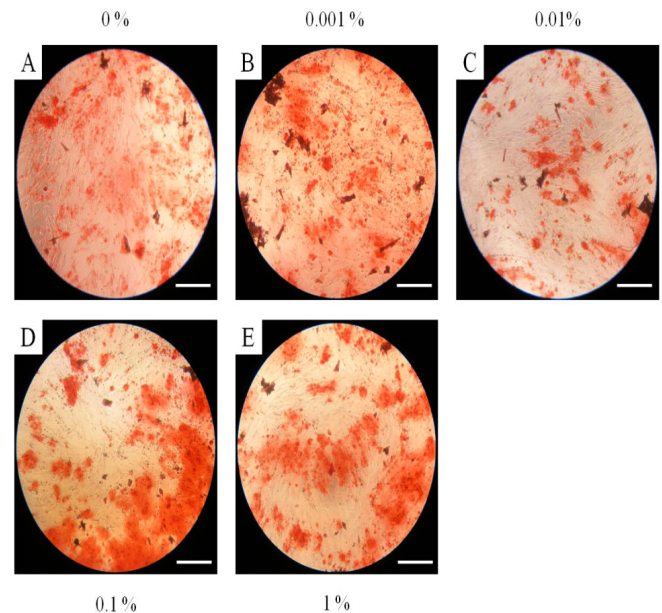


Figure 8. Results of Alizarin Red S staining on day 7 following treatment with different concentrations of BBT on osteogenic supplement. A) Control group (original magnification 100X); B) 0.001% group (original magnification 100X); C) 0.01% group (original magnification 100X); D) 0.1% group (original magnification 100X); E) 1% group (original magnification 100X). The bar indicates 400 μ m.

Discussion

This report discusses the effects of different concentrations of BBT on the osteogenic differentiation of stem cells derived from human gingival tissues. This study clearly showed that the application of *Bambusa tulda* decreased mesenchymal stem cells. In previous reports, mesenchymal stromal cells are treated with individual and mixtures of crude herbal extracts, as well as with purified compounds from herbal extracts, to investigate the mechanisms and effects of differentiation of stem cells [5,12-15]. Er-Xian Decoction, a traditional Chinese herbal formula widely used for postmenopausal osteoporosis treatment is reported to stimulate osteoblastic differentiation of bone mesenchymal stem cells in ovariectomized mice by rescuing several gene expressions that were dysregulated including osteocalcin [12]. BuShen NingXin Decoction, a traditional Chinese medicinal compound modulated mesenchymal stem cell differentiation into osteoblasts in a postmenopausal osteoporotic mouse model with enhanced expression of osteoblastogenesis-related genes [14]. Similarly, *Ginkgo biloba* extract promotes osteogenic differentiation of human bone marrow mesenchymal stem cells in a pathway involving Wnt/ β -catenin signaling [13].

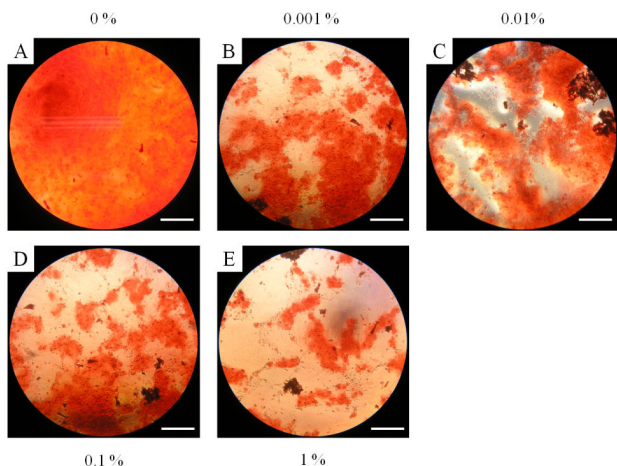


Figure 9. Results of Alizarin Red S staining on day 14 following treatment with different concentrations of BBT on osteogenic supplement. A) Control group (original magnification 100X); B) 0.001% group (original magnification 100X); C) 0.01% group (original magnification 100X); D) 0.1% group (original magnification 100X); E) 1% group (original magnification 100X). The bar indicates 400 μ m.

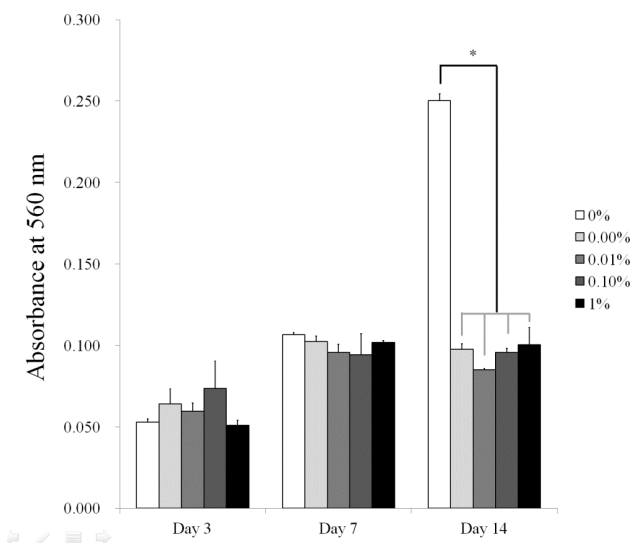


Figure 10. Quantitative results of Alizarin Red S staining on days 3, 7 and 14.

Extract of beet roots, which is widely used in folk medicine as wound healing medicine have been applied with culture of mesenchymal stem cells for tissue engineering [15]. Herbal medicine has been considered as an alternative due to various reasons including antibiotic overuse and misuse [4]. Herbal medicines are considered very safe but there have been some concerns regarding the long-term risk of herb-induced liver injury in patients with liver dysfunction [3]. In this report, the decreased osteogenic differentiation of mesenchymal stem cells derived from gingival has been applied irrespective of different concentration of BBT. Previously, differentiation effects of individual herbal extracts have been tested on human mesenchymal stem cells mainly derived from bone marrow and

elaborated on the plausible underlying mechanisms of action [5]. Mesenchymal stem cells may be isolated from various tissues including gingival, as used in this study [16-20]. Gingiva can be easily accessible and obtainable [10,21-25]. This procedure can be performed under local anesthesia and the obtained tissues are usually discarded in routine periodontal surgery [11,26-31].

Conclusion

Bambusa tulda had influenced the osteogenic differentiation of the stem cells derived from the gingiva. Thus, the use of *Bambusa tulda* may produce adverse effects onto oral tissues. The concentration and application time of *Bambusa tulda* should be meticulously controlled to minimize the adverse effects.

Acknowledgement

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Conflict of Interests

The authors report no conflicts of interest related to this study. The author does not have any financial interest in the companies whose materials are included in the article.

Ethics Statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consents were received from the participants.

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