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## Research article

# Protective and Osteogenic Effects of Crude Water Extract from *Cuscuta japonica* Choisy at Gene Expression Level in Human Gingival Cells

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**Abstract** This study focused on observing change of gene expression after long-term treatment with high dose of crude water extract from seeds of *Cuscuta japonica* Choisy (Japanese dodder). The aim of this study was to study the protective and osteogenic effects of dodder seed extract at gene expression level in human gingival cells. In this research, dodder seeds were blended and boiled in water before freeze-drying to preserve as dried powder. Total phenolic content and antioxidant assay were performed. MTT assay was performed with human gingival cells. The concentrations of 100, 250 and 500 µg / mL had no effect on cell viability when cultivated for 48 hours. The extract at the concentration of 250 µg / mL was chosen to treat human gingival cells for many time points to observe the expressions of osteogenic, inflammatory, antioxidant and cancer gene markers by real-time PCR. The results showed that dodder seed water extract could increase the expressions of osteogenic markers; OPN, OCN and Col-I genes. Moreover, dodder seed water extract induced mineralization of human gingival cells cultured in 3D structure. Change of BCL2, CAS3 and LC3 expression indicated involvement in apoptotic process of dodder seed water extract. Significant changes in gene expression of antioxidant markers (GST1, SOD1 and TXNRD1), inflammatory marker (Cox-2) and epithelial cell marker (α-SMA) without change of important cancer genes were observed. These evidences suggested that seeds of *Cuscuta japonica* Choisy could benefit the application in regenerative medicine and alternative medicine.

**Keywords:** *Cuscuta japonica* Choisy, Antioxidant, Osteogenic effects, Gene expression



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## INTRODUCTION

*Cuscutae Semen*, also known as dodder seed or Tu Si Zi, was the seed of a parasitic vine used in Traditional Chinese Medicine. Phytochemical compounds from edible plants were used to prevent degenerative diseases since ancient time. The seeds of *Cuscuta chinensis* (Chinese dodder) were widely used as Chinese herbal remedies (Noureen et al., 2019). Dodder seeds contained various phytochemical agents such as flavonoids, phenolic acids, steroids, hydroquinones, volatile oils, lignins, polysaccharides and glycosides (He et al., 2010). In Taiwan, infertile women were treated with dodder seeds (Wu et al., 2018). Most of the previous studies used alcohol and various types of solvents in the extraction processes and tested with cell lines or animal cells. However, the effects of dodder seeds on gene expression in primary cell culture was not yet performed. In this study, we used simple method by blending and boiling dodder seeds in water which was similar to the real-life use. The extract was tested with human gingival cells. Study of Yang et al. indicated that processing of Chinese dodder was able to alter their chemical constituents and affected their bioactivity profiles. Study of Yang et al. also supported that crude products and processed products should prescribe differently in clinic (Yang et al., 2016). *Cuscuta chinensis*, *Cuscuta australis* and *Cuscuta japonica* Choisy were important species of *Cuscutae Semen* recorded in Chinese Pharmacopoeia (Gao et al., 2017). *C. japonica* Choisy was also widely used as Traditional Chinese Medicine and it was commercially available in Sichuan, China. However, the study about medicinal properties of *C. japonica* Choisy was still limited. The study of Moon et al indicated that *C. japonica* Choisy might improve learning and memory via the enhancement of adult hippocampal neurogenesis by upregulating expression of neurogenic differentiation factor, which was essential for the maturation and differentiation of granule cells in the hippocampus (Moon et al., 2016). The study of Jang et al. indicated that aqueous fraction from *C. japonica* seeds reduced melanin synthesis and tyrosinase activity in  $\alpha$ -MSH-stimulated B16F10 cells (Jang et al., 2012).

This study presented the simple method for crude extraction by blending and boiling *C. japonica* seeds in water which still provided the active components such as phenolic acids and flavonoids that can be used for prevention of diseases. Most of the previous studies employed solvent extractions which were not common in daily life use.

Previous study presented that water extract from dodder seeds prevented death of osteoblastic cell line (MC3T3-E1) from oxidative stress. Dodder seeds water extract could inhibit reactive oxygen species and malondialdehyde generation and increased activity of superoxide dismutase, glutathione reductase, glutathione S-transferase and glucose-6-phosphate dehydrogenase (Gao et al., 2013). Dodder seeds water extract decreased the cytochrome c releasing from mitochondria and increased anti-apoptotic protein expression but reduced apoptotic protein expression (Gao et al., 2013). The aqueous extract of dodder seeds increased alkaline phosphatase activity, collagen synthesis and bone morphogenetic protein 2 expression in the osteosarcoma cell lines (Yang et al., 2009). Previous studies indicated that kaempferol and hyperoside were active compounds in dodder seeds which possessed osteogenic effect (Yang et al., 2011; Zhang et al., 2014).

Seeds of *Cuscuta japonica* Choisy were found in the Southern China and were used as substitutes for *C. chinensis*, which is not readily available (Gao et al., 2017; Oh et al., 2002). No research evidence demonstrated the action of *Cuscuta japonica* Semen as the osteogenic herbal medicine. The aim of this study was to study the protective and osteogenic effects of *Cuscuta japonica* seeds at gene expression level in human gingival cells. In the previous study, biomaterials made from gelatin and tri-calcium phosphate combined with Traditional Chinese Medicine were prepared to cultivate rat bone cells. It was found that addition of *Cuscuta chinensis* extract to the rat bone cell culture clearly promoted proliferation and differentiation of the osteoblasts from their precursor cells. *C. chinensis* extract reduced amount of tartrate-resistant acid phosphatase (TRAP) which indicated that this herb

significantly inhibited osteoclast activities (Yao et al., 2005). In this study, we tried to applied scaffold technology and Traditional Chinese Medicine with human primary cells to prove that dodder seed water extract could enhance bone formation in 3D structure. In this study, we used simple method to prepare 3D-framework filled with gelatin scaffold which was easy for cell culture and treatment (Kantawong et al., 2018). Gelatin scaffold was biocompatible, biodegradable, cost effective and possessed osteogenic (Wang et al., 2016). However, in the real situation, the mechanical properties of gelatin were not matched with bone mechanical properties which lead to the problem with osseointegration when placing the scaffolds to the site of implantation (Wang et al., 2016). In this study, three-dimensional (3D) scaffolds produced with polycaprolactone (PCL) framework filled with gelatin were prepared to solve the problem. PCL was popular in bone tissue engineering because it possessed osteogenic properties and it was not too soft as gelatin (Henkel et al., 2013). The aim of this study is to investigate the osteogenic activity of *Cuscuta japonica Semen*. In the present study, the researchers will observe that the 3D structure could act as a barrier of Traditional Chinese Medicine treatment or not.

The gingiva is the periodontal tissues which originates from the neural crest. The gingiva consists of connective tissue and epithelium. The gingival tissue is a source of stem cells called gingiva-derived mesenchymal stem cells (GMSCs) (Stefanska et al., 2020). GMSCs present stem-cell properties similar to mesenchymal stem cells (MSCs) which possess multipotent differentiation capacities (Kim et al., 2021). Suitable scaffolds are able to support the bone differentiation ability of GMSCs isolated from gingiva tissues which benefit autologous bone tissue regeneration (Cristaldi et al., 2020; Diomedede et al., 2018).

## MATERIALS AND METHODS

### Preparation of dodder seeds water extracts

Dodder seeds were obtained from local trader in Sichuan (Sichuan Shamgracy Pharmaceutical Co., LTD). Commercial *Cuscutae Semen* was *Cuscuta japonica* Choisy as indicated on the package. The voucher specimen of commercial *Cuscuta japonica* Choisy from Sichuan was previously identified by Prof. Yu-lin Lin (Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Science and Peking Union Medical College) and deposited at the herbarium of IMPLAD, Chinese Academy of Medical Sciences and Peking Union Medical College. (Gao et al., 2017) Dried dodder seeds amount of 200 grams were blended in a blender for 2 minutes with 3,000 mL of distilled water before boiling for 5 minutes. After that, the boiled solution was cooled down at 4°C overnight. The next day, the solution was filtered through white gauze. The filtrate was aliquoted in lyophilized flasks and frozen at -20°C before lyophilized into powder for 7 days.

### Total phenolic content (Folin-ciocalteu assay)

Total phenolic content assay was modified from the previous study (Kantawong et al., 2021a). Dried extract was dissolved in distilled water to the concentrations of 5,000 2,500 1,250 625 and 312.5 µg/mL. Then, 50 µL of each concentration was mixed with 2.5 mL Folin ciocalteu reagent and 2.0 mL Na<sub>2</sub>CO<sub>3</sub> was added. The reactions were incubated at 45°C in a water bath for 15 minutes and the optical densities were measured at 764 nm. A standard curve was prepared by diluting gallic acid to the concentrations of 10,000, 5,000, 2,500, 1,250, 625 and 312.5 µg/mL and then the same test as described for the dodder seed extract was performed. 2.5 mL of Folin ciocalteu reagent mixed with 2.0 mL Na<sub>2</sub>CO<sub>3</sub> was used as blank. Triplicate controls and tests were prepared for each concentration.

### Antioxidant capacity (DPPH assay)

DPPH assay was modified from the previous study (Kantawong et al., 2021a). Dried extract was dissolved in distilled water to the concentrations of 5,000, 2,500, 1,250, 625 and 312.5 µg/mL. After that, 50 µL of each concentration was mixed

with 2,950  $\mu\text{L}$  of DPPH reagent (90  $\mu\text{g}/\text{mL}$ ) and incubated in the dark at room temperature for 15 minutes. A standard curve was prepared by diluting ascorbic acid to the concentrations of 10,000, 5,000, 2,500, 1,250, 625 and 312.5  $\mu\text{g}/\text{mL}$  and then performed the same test as described for the dodder extract. Triplicate controls and tests were prepared for each concentration. The absorbances were measured at 515 nm and the optical densities were calculated for % inhibition using the following equation.

$$\% \text{inhibition} = \frac{\text{Absorbance control} - \text{Absorbance test}}{\text{Absorbance control}} \times 100$$

### Human gingival cell culture

Human gingival cells ( $n = 2$ ; passage 3) were seeded into 6-well plate at the density of 40,000 cells/well and incubated in  $\text{CO}_2$  incubator for 24 hours. In the next day, media was discarded and replaced with complete DMEM containing 250  $\mu\text{g}/\text{mL}$  dodder extract. Human gingival cells (passage 3) at the density of 40,000 cells/well cultured in plain complete DMEM were used as control groups. Cultured media was changed every 3 days. Cells were cultured for 3 days, 8 days and 14 days. The experiment was divided into 2 groups: the control group (cells cultured in complete DMEM) and the treated group (cells cultured in DMEM containing 250  $\mu\text{g}/\text{mL}$  dodder seed extract). Triplicate controls and tests were prepared for each time point and independent t-test (SPSS 17.0) was used to determine the difference between groups.

### Cell viability study (MTT assay)

Human gingival tissues were obtained according to a protocol approved by the Ethics Committee, Faculty of Dentistry, Chiang Mai University (70/2019). Human gingival tissues ( $n = 2$ ) were harvested during a surgical procedure and preserved in a sterile phosphate buffer saline. Human gingival tissues were cut with a scalpel in a Petri dish containing complete DMEM before culture in an incubator at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . Complete DMEM was changed every two days until the cells reached 80% confluent. Human gingival cells (passage 3) were seeded in complete DMEM with the density of 20,000 cells/well in the 12-well plates and incubated at  $37^\circ\text{C}$  for 24 hours in the  $\text{CO}_2$  incubator. The next day, media was discarded and replaced with complete DMEM containing dodder extracts dissolved at various concentrations and incubated for 48 hours at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator. Human gingival cells (passage 3) cultured in normal complete DMEM was used as controls. When the incubation time was over, media was discarded and replaced with 2 mL of media containing MTT (1 mg/mL) before incubating for 2 hours in  $\text{CO}_2$  incubator. After the media was discarded, 2 mL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm and 630 nm. The optical densities were used to calculate % cell viability using the following equation. The experiments were repeated 3 times and independent t-test (SPSS 17.0) was used to determine the difference between groups.

$$\% \text{Cell viability} = \frac{\text{Absorbance test}_{570} - \text{Absorbance test}_{630}}{\text{Absorbance control}_{570} - \text{Absorbance control}_{630}} \times 100$$

### Real-time PCR

RNA extraction was performed using NucleoSpin<sup>®</sup> RNA II, according to the protocol provided by the manufacturer (Yin et al., 2020). Total RNA amounts were measured with Nanodrop 2000. The reaction solutions were prepared with 6  $\mu\text{L}$  of RNA, 4  $\mu\text{L}$  of 5x RT Master Mix and 10  $\mu\text{L}$  of DEPC water. The RNA was converted to cDNA in the thermocycler using the following program:  $37^\circ\text{C}$  15 minutes,  $50^\circ\text{C}$  for 5 minutes,  $98^\circ\text{C}$  for 5 minutes. cDNA was stored at  $-20^\circ\text{C}$ . NO-RT control was prepared with the same method using 5x RT buffer. Real-time PCR reaction were

prepared with 8  $\mu$ L RNA, 10  $\mu$ L SYBR Green Mastermix and 2  $\mu$ L primer (the final concentration of each primer was 1.0  $\mu$ M) in the total volume of 20  $\mu$ L. The PCR program was set as following: (1) Pre-incubation at 95°C for 2 minutes. (2) PCR consisted of denature at 95°C, annealing at 60°C and extension at 72°C. Real-time PCR was performed for 40 cycles. Two reference genes; GAPDH and beta-actin were used for normalization. Gene expression was calculated by the relative real-time analysis in LightCycler 1.5 using the Pfaffl analysis method (Pfaffl, 2001). The sequences of primers used in this study was shown in Table 1. The experiment was divided into 2 groups: the control (cells cultured in complete DMEM) and the treatment (cells cultured in DMEM containing 250  $\mu$ g/mL dodder seed extract). Triplicate controls and tests were prepared for each time point and independent t-test (SPSS 17.0) was used to determine the difference between groups. Fold change of gene expressions were presented in the form of mean  $\pm$  SD when  $P < 0.05$  was considered as statistically significant.

**Table 1.** Primer sequences used in this study.

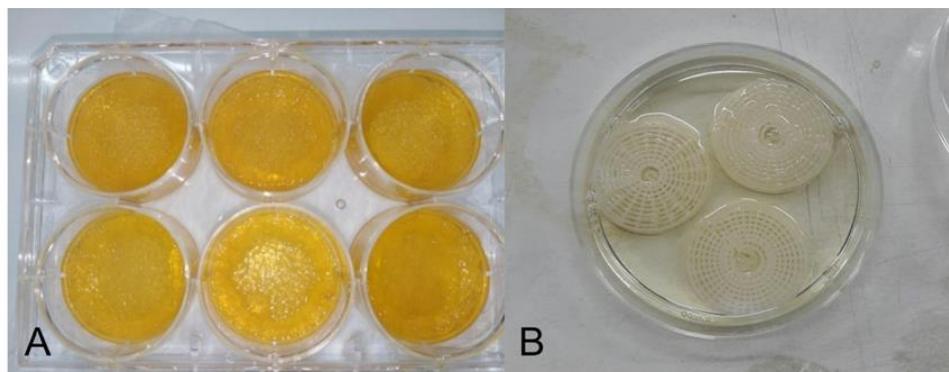
Primer	Sequence (5'→3')	References	Accession number
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	(F) AAATCCCATCACCATCTTCCAGGAGC (R) CATGGTTCACACCCATGACGAACA	(Prins et al., 2014)	NM_001357943.2
Osteopontin (OPN)	(F) GGACAGCCAGGACTCCATTG (R) TGTGGGGACAACCTGGAGTGAA	(Lv et al., 2013)	NM_000582.3
Collagen type -I (ColI)	(F) GATGGATTCCAGTTCGAGTATG (R) GTTTGGGTGCTTGTCTGTTTG	(Yoo et al., 2016)	NM_000088.4
BCL-2	(F) GATGTGATGCCTCTGCGAAG (R) CATGCTGATGTCTCTGGAATCT	(Mollaei et al., 2017)	NM_000633.3
Caspase-9 (CAS9)	(F) ATGGACGAAGCGGATCGGCGGCTCC (R) GCACCACTGGGGTAAGTTTTCTAG	(Krisnamurti et al., 2016)	NM_001278054.2
Cyclooxygenase-2 (COX-2)	(F) CCCTTGGGTGTCAAAGGTAA (R) GCCCTCGTTATGATCTGTC	(Yang and Han, 2014)	NM_000963.4
Glutathione S-transferase 1 (GST1)	(F) GCCACACTCTCCGTCAA (R) TGCCAAGAAGAACGACATTCC	(Spivack et al., 2004)	NM_000853.3
Thioredoxin Reductase 1 (TXNRD1)	(F) GAAGATCTTCCCAAGTCTATGAC (R) ATTTGTTGCCTTAATCCTGTGAGG	(Kahlos et al., 2001)	NM_182743.3
Histone acetyltransferase 1 (HAT1)	(F) CAGATATATAAGGCTGACATGAC (R) GCTGTAATATCAAGAAGTGTAGG	(McGraw et al., 2003)	NM_003642.4
Alpha-smooth muscle actin ( $\alpha$ -SMA)	(F) TGACAATGGCTCTGGGCTCTGTAA (R) TTCGTCACCCACGTAGCTGTCTTT	(Talior-Volodarsky et al., 2012)	NM_001613.4
Transgelin (TAGLN)	(F) CGCGAAGTGCAGTCCAAATCG (R) GGGCTGGTCTTCTTCAATGGGC	(van Tuyn et al., 2005)	NM_003186.5
$\alpha$ -actin	(F) AGAAAATCTGGCACACACACC (R) AGAGGCGTACAGGGATAGCA	(Deng et al., 2013)	NM_001101.5
Osteocalcin (OCN)	(F) GCAAGTAGCGCAATCTAGG (R) GCTTACCCTCGAAATGGTA	(Riksen et al., 2014)	NM_001393915.1
BAX	(F) GGTTGTGCGCCCTTTCTA (R) CGGAGGAAGTCCAATGTC	(Mollaei et al., 2017)	NM_001291430.2
Caspase-3 (CAS3)	(F) TTCAGAGGGGATCGTTGTAGAAGTC (R) CAAGCTTGTCGGCATACTGTTTCAG	(Krisnamurti et al., 2016)	NM_001354783.2
LC3	(F) GAGAAGCAGCTTCTGTTCTGG (R) GTGTCCGTTACCAACAGGAAG	(Peng et al., 2015)	NM_001085481.3
NF- $\kappa$ B	(F) ATGGCTTCTATGAGGCTGAG (R) GTTGTGTTGGTCTGGATGC	(Pekkala et al., 2015)	NM_001243985.2
Superoxide Dismutase 1 (SOD1)	(F) GTAATGGACAGTGAAGGTGTG (R) CAATTACACCACAAGCCAAACG	(Rush et al., 2000)	NM_000454.5
Histone Deacetylase 3 (HDAC3)	(F) TATGCAAGGCTTACCAAGAG (R) TCCGTATTTGTGGAAGGACAC	(McGraw et al., 2003)	NM_001355039.2
Lysine acetyltransferase 2A (Kat2a)	(F) CAGAATGTCTTTCCACCAG (R) GGATTCAGCTCACACTCCATC	(McGraw et al., 2003)	NM_001376227.1
Calponin 2 (CNN2)	(F) CTGCAGAGCGGGTGGACATTGGC (R) GCCGGCCTCTCTGGTAGTAAGG	(Ueda et al., 2002)	NM_001303499.2

### Gelatin preparation for mineralization assay

3 ml of 12% gelatin solution was mixed with 30  $\mu$ l of 25% glutaraldehyde and placed into each well of the 6-well plate. The 6-well plate was left for 24 hours at room temperature to allow gelatin cross-linking. The next day, glutaraldehyde remnant was washed out with distilled water 3 times (30 minutes at a time). The cross-linked gelatins were soaked overnight in distilled water at  $-4^{\circ}\text{C}$  after that distilled water was discarded and polymerized gelatins were frozen for 48 hours before freeze-drying. Before using in cell culture, dried gelatins were sterilized by immersing in 70% ethanol for 30 minutes before rinsing with sterile distilled water 3 times (30 minutes at a time) then polymerized gelatins could be used for further experiments as shown in Figure 1A. Gelatins were equilibrated in complete DMEM at  $37^{\circ}\text{C}$  overnight before cell seeding.

### 3D structure fabrication

3D structure was designed by OpenSCAD program which can adjust the percent of chamber size. The in-houses Fused Deposition Modeling (FDM) machine or 3D printer with 0.4 mm of nozzle size was employed for the scaffold fabrication. The scaffolds with various % of chamber size were print by using polycaprolactone (PCL) filament at layer height = 0.2 mm, % Infill =100, and speed of printing = 10 mm/min. 3 ml of 12% gelatin solution was mixed with 30  $\mu$ l glutaraldehyde and filled into the space of the 3D structures before standing at room temperature for 24 hours to allow gelatin polymerization as shown in Figure 1B. The 3D structures (with polymerized gelatin filler) were placed into 6-well plate (1 piece per well) and sterilized with 70% ethanol before washing with sterilized water 3 times (30 minutes at a time). 3D structures were equilibrated in complete DMEM at  $37^{\circ}\text{C}$  overnight before cell seeding.



**Figure 1. Scaffolds used for mineralization assay.** (A) polymerized gelatins in 6-well plate. (B) 3D structures with gelatin filler.

### Cell culture for mineralization assay

Human gingival cells (passage 3) 40,000 cells were seeded into each well of 6-well plate with complete DMEM then incubated for 24 hours in  $\text{CO}_2$  incubator to allow cell attachment. The next day, complete DMEM was discarded and replaced with 5 mL complete DMEM containing dodder seed extract at the concentration of 250  $\mu\text{g}/\text{mL}$ . Cell culture was maintained for 14 days. Complete DMEM containing dodder seed extract at the concentration of 250  $\mu\text{g}/\text{mL}$  was changed every 5 days.

### Cell staining

4% formaldehyde was added onto cell cultures and left for 10 minutes at room temperature. After that, 4% formaldehyde was discarded and replaced with 1% Alizarin red S solution for 10 minutes. Alizarin red S solution was washed out with tap water overnight and cell morphology was observed under the inverted microscope.

## Statistical analysis

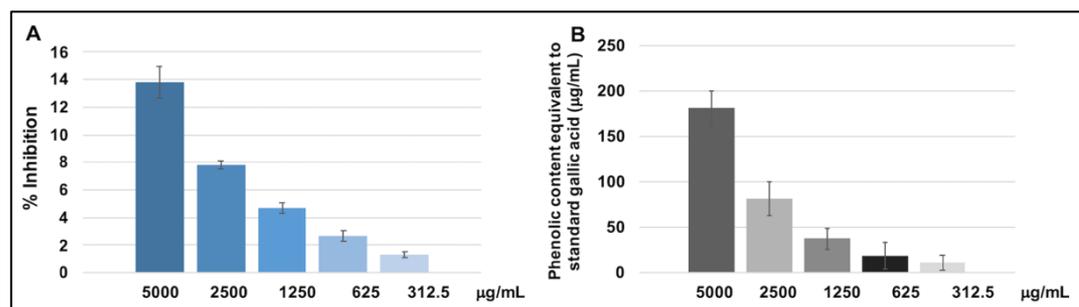
Triplicate controls and tests were prepared and independent t-test was analyzed using SPSS 17.0 to determine the difference between groups.

## RESULTS

### DPPH assay and total phenolic content

Dried powder of dodder seed water extract 14.97 g was obtained when 200 grams of dried dodder seeds were used in the extraction and %yield was equal to 7.5%.

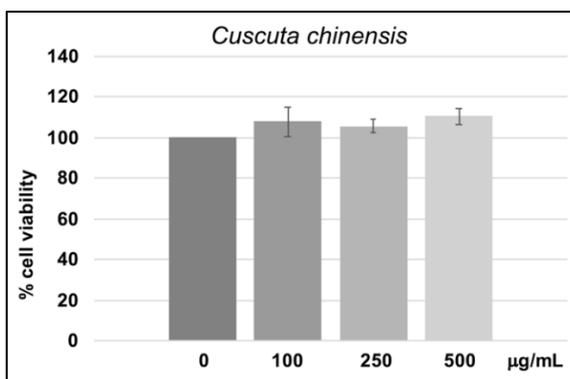
The DPPH radical scavenging of dodder seed water extract at the concentrations of 5,000, 2,500, 1,250, 625 and 312.5  $\mu\text{g}/\text{mL}$  were shown in Figure 2A. It was found that the extract at the concentration of 5,000  $\mu\text{g}/\text{mL}$  showed 13.77% inhibition. The % inhibition of dodder seed extract 5,000  $\mu\text{g}$  was equivalent to ascorbic acid 21.9  $\mu\text{g}$ . When the extract was continuously diluted to various concentration, the % inhibition decreased proportionally as shown in Figure 2A. The extract at the concentrations of 5,000, 2,500, 1,250, 625 and 312.5  $\mu\text{g}/\text{mL}$  contained the total phenolic content equivalent to standard gallic acid were shown in Figure 2B. The total phenolic content of dodder seed extract 5,000  $\mu\text{g}$  was equivalent to gallic acid 181.36  $\mu\text{g}$ .



**Figure 2. (A) The % inhibition of dodder seed extract at various concentrations. (B) The relationship between the amount of phenolic content in the extract at various concentrations equivalent to gallic acid standard.** The DPPH scavenging activity and phenolic content for each concentration were triplicated. Mean and standard deviation were presented for each concentration.

### Study on cell viability using MTT assay

Dodder seed extract at the concentrations of 100, 250 and 500  $\mu\text{g}/\text{mL}$  were employed to test human gingival cell viability. It was found that the concentrations of 100, 250 and 500  $\mu\text{g}/\text{mL}$  had no effect on cell viability when cultivated for 48 hours as shown in Figure 3. The concentration of 250  $\mu\text{g}/\text{mL}$  was therefore chosen for further experiments.

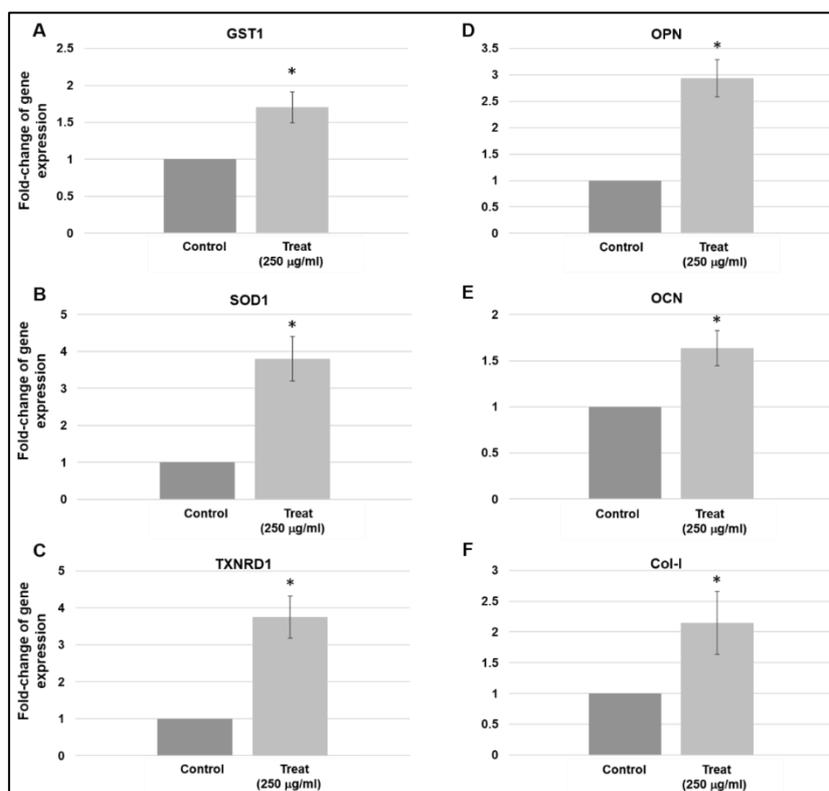


**Figure 3. Dodder extract at various concentrations and % cell viability after 48 hours of culture in the extract compared to controls.** The MTT assay for each concentration was triplicated. Mean and standard deviation were presented for each concentration.

### Antioxidant marker and bone differentiation marker

Antioxidant gene expression in human gingival cells treated with 250 µg/mL for 14 days was shown in Figure 4. GST1 was slightly increased (Figure. 4A). The expression of SOD1 and TXNRD1 increased by 3.8 times (Figure. 4B & 4C).

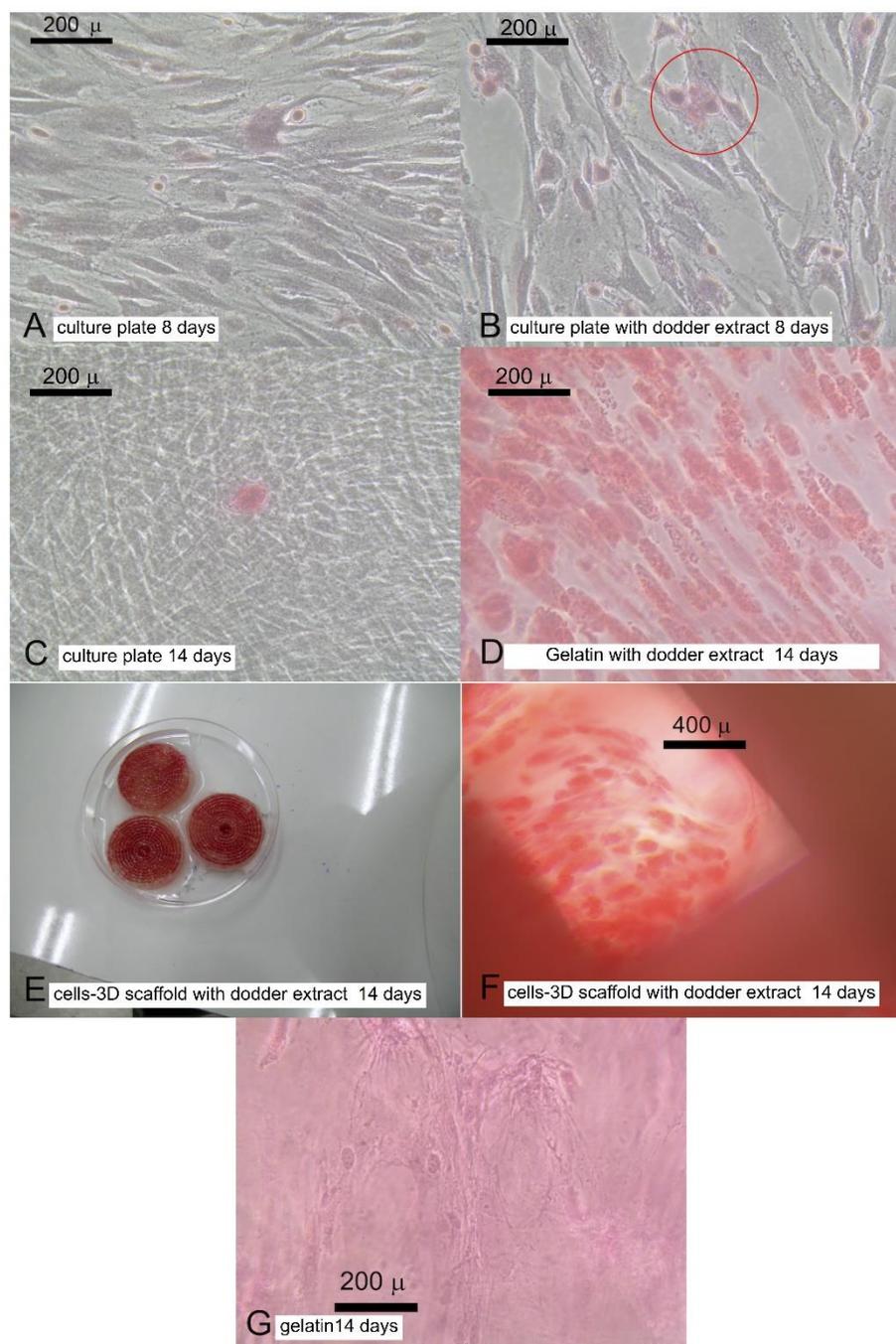
Osteopontin (OPN) gene was 3 times higher than control (Figure. 4D) while osteocalcin (OCN) gene was found 1.8 times higher than control (Figure. 4E). The expression of the Col-I gene increased by 3.8 times (Figure. 4F).



**Figure 4. Gene expression in human gingival cells treated with 250 µg/mL for 14 days.** (A) GST1 expression increased by 1.7 times ( $P = 0.028$ ). (B) SOD1 expression increased by 3.8 times ( $P = 0.015$ ). (C) TXNRD1 gene expression increased by 3.8 times ( $P = 0.014$ ). (D) OPN expression increased by 3 times ( $P = 0.011$ ). (E) OCN gene expression increased by 1.8 times ( $P = 0.028$ ). (F) Col-I gene increased by 3.8 times ( $P = 0.018$ ). Three pairs of control and test samples were investigated. Mean and standard deviation were presented for each concentration.

## Cell staining

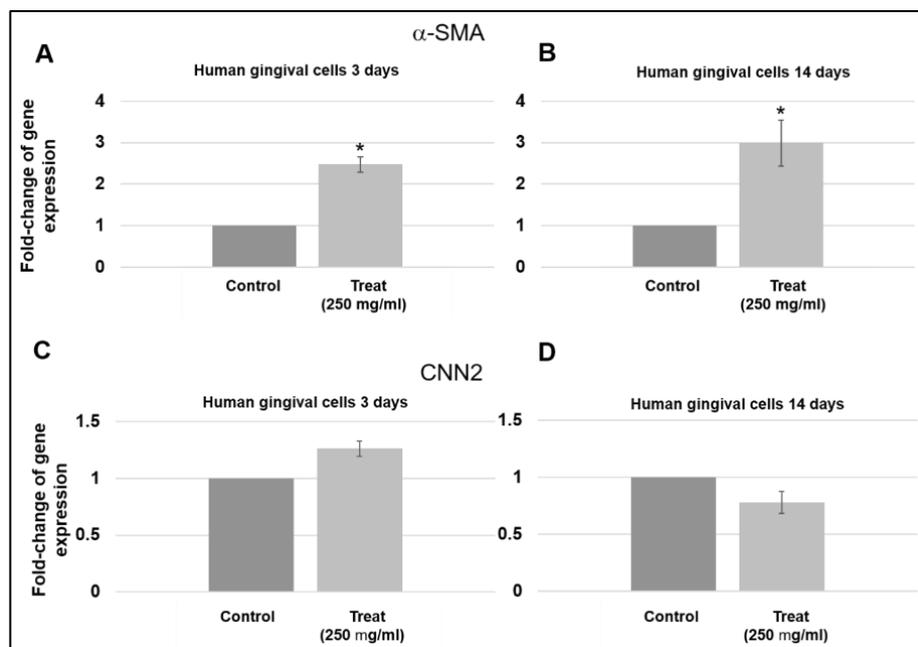
Alizarin red s staining showed calcium accumulation in dodder seed water extract treated group as shown in Figure 5. Mineralized nodule was slightly observed after 1 week (Figure 5B). Mineralization nodules could be clearly observed in gelatin (Figure 5D) and in 3D structure (Figure 5E & 5F).



**Figure 5. Mineralization.** (A) human gingival cells cultured in tissue culture plate and complete DMEM for 8 days. (B) human gingival cells cultured in tissue culture plate and complete DMEM contained dodder seeds water extract for 8 days. (C) human gingival cells cultured in tissue culture plate and complete DMEM for 14 days. (D) human gingival cells cultured in gelatin and complete DMEM contained dodder seeds water extract for 14 days. (E) Cells-3D structures after 14 days treatment in complete DMEM contained dodder seeds water extract. (F) human gingival cells cultured in 3D structures and complete DMEM contained dodder seeds water extract for 14 days. (G) human gingival cells cultured in plain gelatin for 14 days.

## Epithelial markers

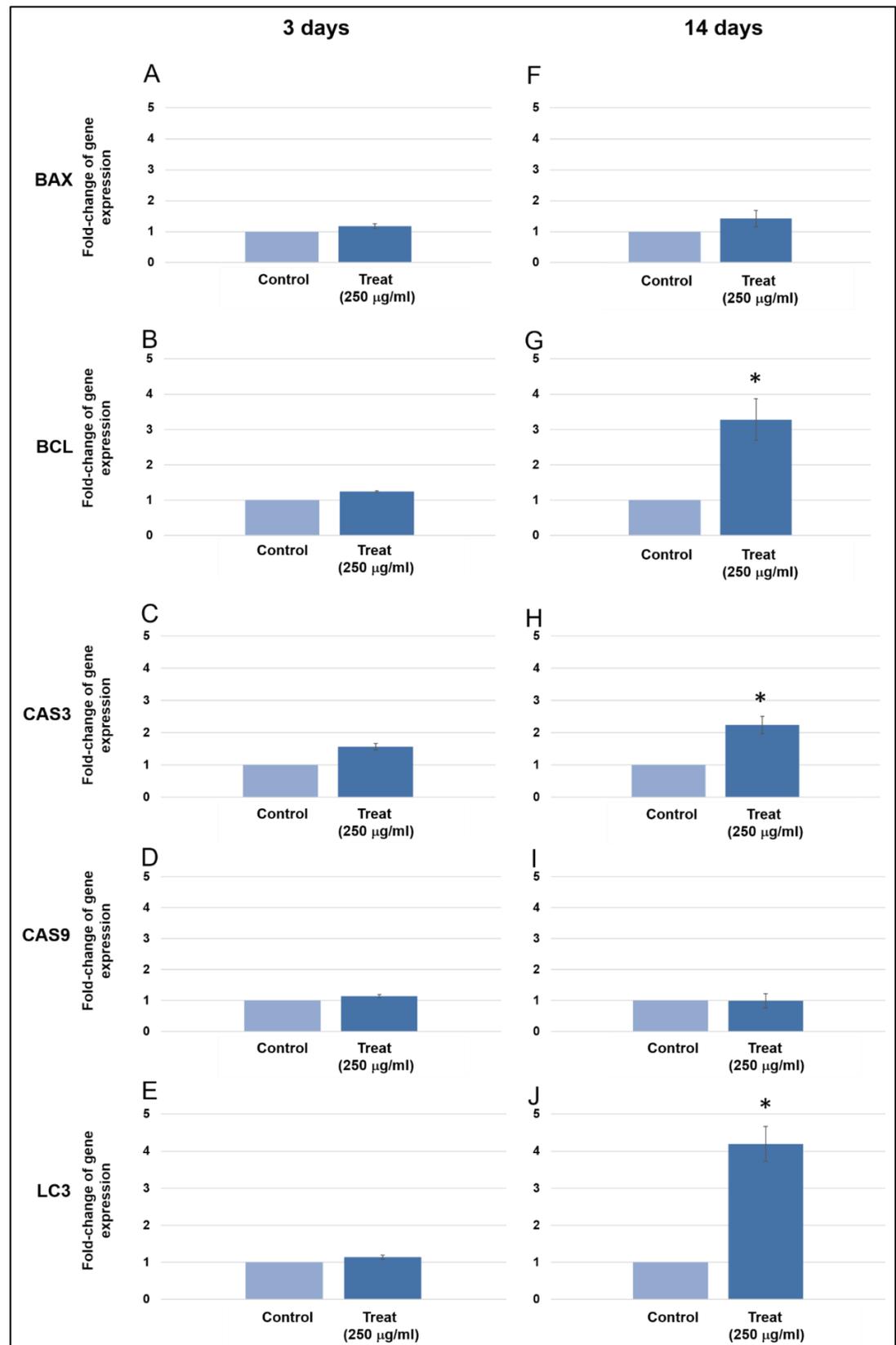
In addition, epithelial marker was also found up-regulated, dodder seed extract increased the expression of Alpha-smooth muscle actin ( $\alpha$ -SMA) in the human gingival cells. The expression of  $\alpha$ -SMA increased by 2.5 and 3 times compared to control at 3 days and 14 days, respectively (Figure. 6A & 6B). When confirmed with other biomarkers such as calponin, no obvious changes were found (Figure 6C & 6D).



**Figure 6. Expression of epithelial markers in human gingival cells treated with 250 µg/mL for 3 days and 14 days.** (A & B)  $\alpha$ -SMA gene expression increased at 3 days ( $P = 0.005$ ) and 14 days ( $P = 0.025$ ). (C & D) Insignificant change in expression of CNN2 gene was found at 3 days and 14 days. Three pairs of control and test samples were investigated. Mean and standard deviation were presented for each concentration.

## Apoptosis and autophagy markers

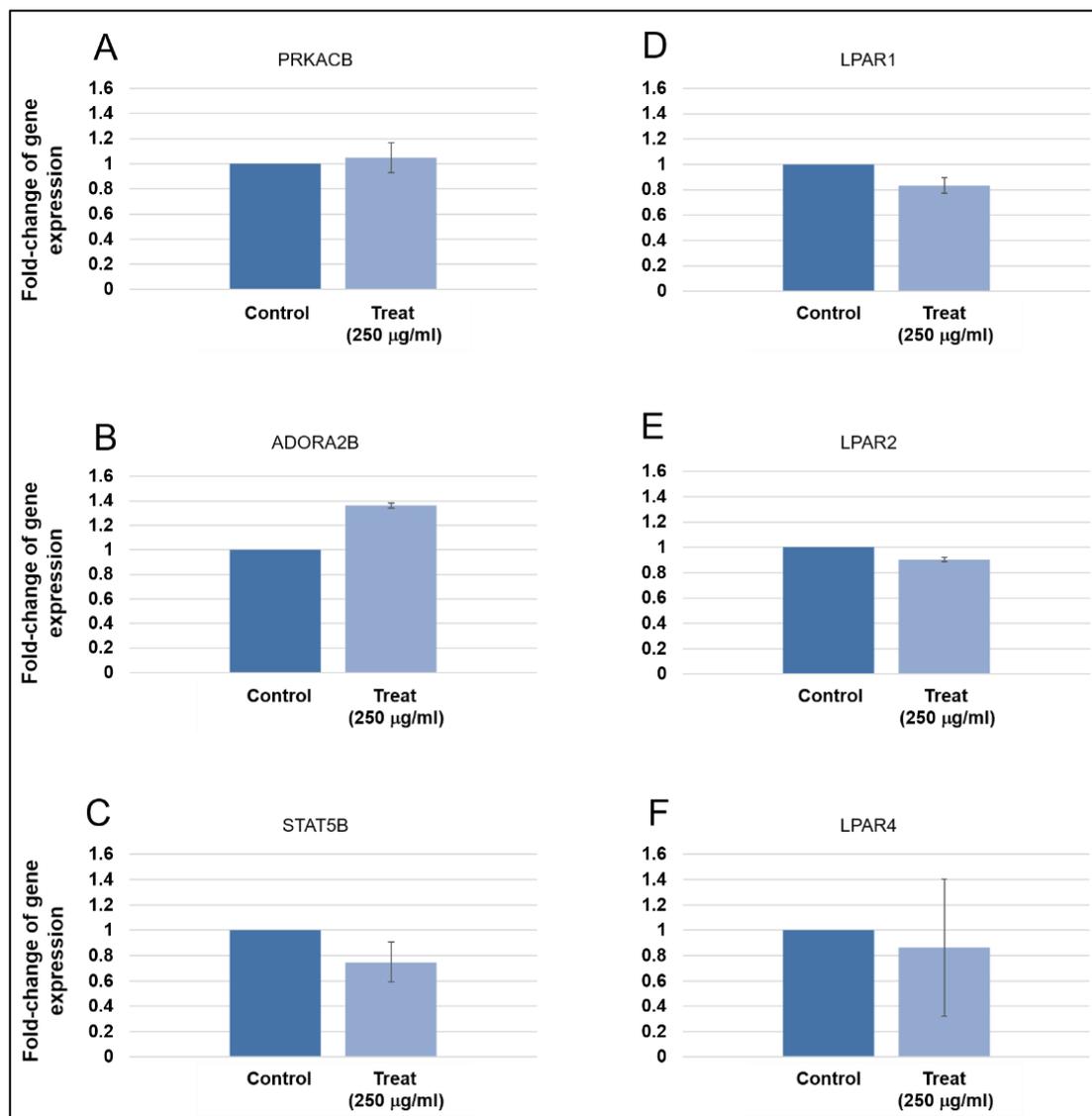
There was no change in expression of BAX gene when cultured in the extract for 3 days and 14 days (Figure 7A & 7F). BCL2 gene expression was not altered at 3 days, but increased more than 3 times when culturing in the extract for 14 days (Figure 7B & 7G). There was no change in expression of CAS3 gene at 3 days but the expression increased more than 2 times when cultured in the extract for 14 days (Figure 7C & 7H). There was no change in expression of CAS9 gene when cultured in the extract for 3 days and 14 days (Figure 7D & 7I). After culturing the human gingival cells in dodder seed extract for 3 days, the expression of the LC3 gene was not much different from the control group. LC3 expression increased more than 4 times when culturing for 14 days (Figure 7E & 7J).



**Figure 7.** The expression of BAX, BCL2, CAS3, CAS9 and LC3 gene after treated human gingival cells with the extracts for 3 days and 14 days. BCL2, CAS3 and LC3 genes were found significantly upregulated at 14 days culture ( $P = 0.022$ ,  $0.015$  and  $0.007$ , respectively) Three pairs of control and test samples were investigated. Mean and standard deviation were presented for each concentration.

## Cancer genes

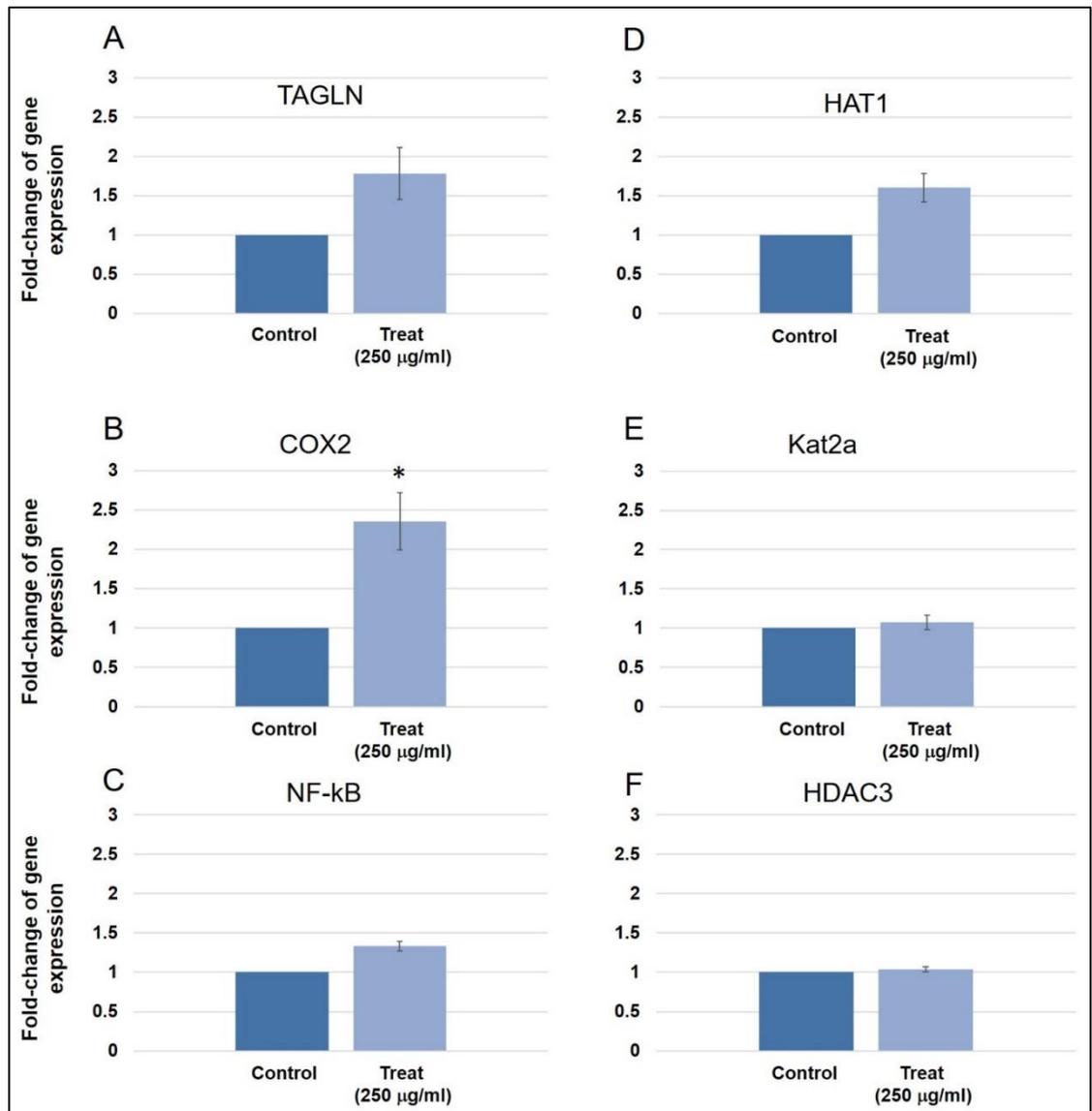
When 250 µg/mL were used to treat the human gingival cells for 8 days, the expression of 6 cancer genes (PRKACB, STAT5B, ADORA2B, LPAR1, LPAR3, LPAR4) were not rendered (Figure 8). None of these genes showed significant alteration of gene expression.



**Figure 8. Expression of tumorigenic gene in human gingival cells at 8 days treatment.** Three pairs of control and test samples were investigated. Mean and standard deviation were presented for each concentration. The expression of PRKACB, STAT5B, ADORA2B, LPAR1, LPAR3, LPAR4 were not rendered.

## Other gene markers

Epigenetic gene markers and inflammatory gene markers were tested to observe other possible effects that could happen after long-term treatment with 250 µg/ml *Cuacuta japonica* water extract (Figure 9). The purpose was to investigate molecular mechanisms associated with inflammation-promoted tumorigenesis. Abnormal epigenetic changes including DNA methylation, histone modification and chromatin remodeling occur during the transformation of chronic inflammation.



**Figure 9. Other gene expression in human gingival cells.** (A) The expression of the Runx2 gene in dodder seed extract for 3 days showed no significant change compared to control. (B) TAGLN gene expression in triplicate experiments increased 1.7 times compared to the control group when cultured for 3 days. (C) Cox-2 gene expression in triplicate experiments at 3 days culture increased 2.4 times compared to control group ( $p = 0.023$ ). (D) NF-kB gene expression in triplicate experiments showed no difference compared to the control group at 3 days culture. (E) ALP gene expression in triplicate experiments found no difference compared to the control group at 14 days culture. (F) HAT1 gene expression in triplicate experiments found increased 1.6 times compared to the control group at 3 days culture. (G) Kat2a gene expression in triplicate experiments found no difference in expression compared to the control group at 3 days. (H) HDAC3 gene expression in triplicate experiments found no differences compared to the control group at 3 days culture. Three pairs of control and test samples were investigated. Mean and standard deviation were presented for each concentration.

## DISCUSSION

The main substances found in dodder seed water extract were polysaccharides, phenolic acids and flavonoids (Kantawong et al., 2021b). The previous study by Kantawong et al. showed the crude extract of *C. japonica*

contained l-arabinose, pinoselinol, 2-pentylfuran, chlorogenic acid, p-coumaric acid, quercetin, rutin, hyperoside, isorhamnetin, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid.

After treating the human gingival cells in dodder seed extract for 14 days, the expressions of bone differentiation genes were detected. Dodder seed water extract induced bone differentiation because the expression of bone differentiation markers (OPN, OCN and collagen type I) increased significantly. OPN, OCN and collagen type I were extracellular matrix proteins responsible for mineral accumulation (Addison et al., 2015; Foster et al., 2018). Recent study reported that OPN plays a crucial role during the formation of the collagen fibrils (Depalle et al., 2020) which correlated with the increased expression of collagen type I. The result from mineralization assay was correlated with the result of gene expression. However, some of our previous studies indicated that the osteogenesis also depended on the quality of human gingival cells because some batches of human gingival cells did not show strong positive bone mineralization even if the OPN and OCN expressions were up-regulated (data not shown).

Pinoselinol and quercetin induced osteoblast differentiation and mineralization by regulating BMP-2/Runx2 signaling because it had similar effects to estradiol (E2) (Jiang et al., 2019; Pang et al., 2018). Chlorogenic acid can promote the osteogenic differentiation of hDPSCs by regulating Wnt signaling (Hu et al., 2021). Rutin affected the osteogenic differentiation and proliferation of PDLSCs via PI3K/AKT/mTOR signal pathway (Zhao et al., 2020). Hyperoside was a plant flavonoid which contained osteogenic effects (Zhang et al., 2014). These evidences suggested the osteogenic effects of dodder seed water extract.

Gelatin provided a biomimetic peptide that promoted cell adhesion and supported tissue regeneration. However, gelatin possessed some disadvantages. Gelatin had poor mechanical properties, lack thermal stability, and presented relatively shorter degradation rate (Bello et al., 2020). Printed framework and a gelatin filler for bone regeneration presented gradient degradation performance to satisfy the uniform adhesion and proliferation of cells in the scaffold at the early stage of implantation, as well as providing space for the subsequent regeneration of bone tissue (Dou et al., 2021). Over swelling and thermal instability made gelatin presented characteristics similar to those of soft tissues which lead to low cell retention and uneven cell distribution as shown in Figure 5G. The printed framework prevented gelatin from over swelling and collapsing. The use of 3D bioprinting technology is shown to have a great potential to fabricate gelatin-based scaffolds for application in the field of bone tissue engineering. The 3D printed scaffolds showed uniform cell distribution and superior bone nodule formation compared with normal gelatin as seen by the alizarin red staining (Figure 5E&5F).

The phenolic acids found in dodder seed extract were chlorogenic acid, caffeic acid, p-coumaric acid, 3, 4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. In the previous studies, phenolic acids showed important roles in oxidative stress prevention and metabolism. 3, 5-Dicaffeoylquinic acid was a potent  $\alpha$ -glucosidase inhibitor which contributed to the antihyperglycemic action of the compound (Simeonova et al., 2019). The beneficial effects of 3,5-dicaffeoylquinic acid support the traditional use of *Cuscuta* spp. for the treatment of diabetes (Noureen et al., 2019). Oh et al. indicated that *C. japonica* contained 3,5-Di-O-caffeoylquinic acid, methyl 3, 5-Di-O-caffeoylquinic acid, 3,4-Di-O-caffeoylquinic acid, and methyl 3,4-Di-O-caffeoylquinic acid which might be responsible for the antihypertensive action (Oh et al., 2002).

In the present study, dodder seed extract affected the expression of antioxidant enzyme SOD1 or copper-zinc superoxide dismutase (Cu/Zn SOD1). This enzyme located in the cytoplasm, transforming superoxide radical into oxygen and hydrogen peroxide (Mondola et al., 2016). Another enzyme that found to increase expression was thioredoxin reductase 1 (TXNRD1). TXNRD1 changed the oxidized form of thioredoxin back into a reduced form which could accelerate reduction of disulfide bond in many proteins to enable their function (Emelyanov and Fyodorov, 2016). GST1, a microsomal glutathione transferase (Schaffert, 2011), showed slightly increased.

Increased expression of  $\alpha$ -SMA might be the advantage for this study because  $\alpha$ -SMA was identified as a biomarker of early osteoprogenitor cells in the periosteum (Matthews et al., 2020). Slightly increase of transgelin (TAGLN) was also observed at 3 days (Figure 9A). Transgelin was expressed in vascular and visceral smooth muscle, and it was an early marker of smooth muscle differentiation (Matsui et al., 2018). Vascularization was important for diffusion of nutrients and regulatory molecules inside the 3D scaffold (Freeman et al., 2019). However, upregulation of the  $\alpha$ -SMA could be used as a marker of the epithelial to mesenchymal transition (EMT) in various types of cancers (Lazarevic et al., 2020). Thus, the investigation of various cancer-related gene expression was also performed in this study (Figure 8). It was found that treatment with 250  $\mu$ g/mL dodder seed water extract longer than 1 week did not significantly affect the expression of 6 cancer genes (PRKACB, STAT5B, ADORA2B, LPAR1, LPAR3, LPAR4). Moreover, dodder seed water extract did not affect the expression of 3 epigenetic genes (HDAC3, HAT1, Kat2a) at 3 days culture (Figure 9D, 9E, 9F).

Quercetin, rutin, hyperoside and isorhamnetin were flavonoids found in dodder seed extract. These substances prevented cancer and oxidative stress. Pinoresinol was a phytoestrogen with antitumor activity, inhibiting breast cancer cells (López-Biedma et al., 2016). 4,5-Dicaffeoylquinic acid maintained prostate cancer cell line in the S-phase (Lodise et al., 2019). These evidences were concordant with the result of cancer gene expression.

The expression of cancer related genes in human gingival cells was observed at 8 days culture. cAMP-dependent catalytic  $\beta$  (PRKACB) proteins involved in many cancers was decreased in non-small cell lung cancer, causing the cancer cells to multiply. Upregulation of PRKACB expression could prevent the progression of non-small cell lung cancer (Chen et al., 2013). In addition, the PRKACB fusions gene was found to be involved in cholangiocarcinoma (Mertens et al., 2018). Signal transducer and activator of transcription (STAT) was a transcription factor known to be involved in cancer progression. Increased expression of STAT5B promoted cell proliferation and resistance in mantle cell lymphoma by activating Akt signaling pathway (Zhang et al., 2019). The expression of STAT5A, STAT5B and STAT6 was used as a prognosis in hepatocellular carcinoma (Dong et al., 2019). STAT5A or STAT5B stimulated T-cell lymphoma development (Maurer et al., 2019). Adenosine A2b receptor (ADORA2B) was associated with oral squamous cell carcinoma, (Kasama et al., 2015) breast cancer and colorectal cancer (Ma et al., 2010). In addition, ADORA2B controlled cellular proliferation via HIF-1 $\alpha$  activation (Lan et al., 2018). Lysophosphatidic acids (LPA) regulated cell processes, including cell cycle progression, viability and wound healing (Llona-Minguez et al., 2015). Lysophosphatidic acid receptors (LPAR) showed increased expression in hepatocellular carcinoma (Mazzocca et al., 2015). In our study, none of these genes showed significant alteration of gene expression.

Various proinflammatory signaling pathways including the NF- $\kappa$ B, IL-6/STAT3, cyclooxygenase-2 (COX-2)/PGE2, and IL-23/Th17 participate in the transformation of inflammation into cancer. Proinflammatory signaling pathways induce the production of inflammatory mediators, upregulate the expression of antiapoptotic genes, stimulate cell proliferation and angiogenesis, and thereby contribute to tumorigenesis (Yang et al., 2019).

BAX gene (Bcl-2 Associated X-protein) was a pro-apoptotic gene (Naseri et al., 2015; Zhu et al., 2015) while BCL2 was responsible for anti-apoptosis (Opferman and Kothari, 2018). CAS3 or caspase3 was an enzyme involved in the apoptosis process and its main function was to drive the process of apoptosis. The upregulation of CAS3 also played other roles in cell functions such as the development into other cells (Nakajima and Kuranaga, 2017; Wang and Luo, 2014). CAS3 played important roles in and autophagy (Wu et al., 2014). Upregulation of BCL2 and CAS3 without change of BAX and CAS9 might imply that dodder seed water extract prevented intrinsic but not extrinsic apoptosis (Voss and Strasser, 2020). The LC3 was a protein involved in the formation of autophagosome fusion with the lysosome. Dodder seed extract might cause autophagy due to the upregulation of LC3. Previously, dodder extract had been reported to increase LC3

expression which caused autophagy in the Jurkat cells (Al-Daghri et al., 2012). However, the increase in LC3 could be found in developing cells such as cell differentiation (Hale et al., 2013). Dodder seed extract induced a noticeable effect on the expression of the COX-2 gene, a prostaglandin inflammatory mediator (Figure 9B).

## CONCLUSION

Dodder seed water extract contained phenolic compounds and possessed antioxidant activity. Dodder seed water extract 250 µg/mL could be used to accelerate bone differentiation in complex tissue engineering constructs which could benefit time-course transplantation. Dodder seed water extract increased antioxidant gene expression without effect on cancer-related and epigenetic genes. Dodder seed water extract prevented intrinsic apoptosis. These findings provided the future benefit for the use of *C. japonica* as supplementation for prevention of bone loss instead of using *C. chinensis*.

## AUTHOR CONTRIBUTIONS

Fahsai Kantawong designed and conducted all of the experiments and wrote the manuscript. Suraiya Sadeeyamoo, Peeraya Wongsit, Montree Tungjai, Phenphichar Wanachantararak, Suruk Udomsom<sup>4</sup> assisted in conducting the experiments. Montree Tungjai performed the data visualization and wrote the manuscript. Jianghua Yang provided *C. Japonica*, Ataya Sathirachinda searched for the information about *C. Japonica*. All authors have read and approved of the final manuscript.

## CONFLICT OF INTEREST

The authors declare no conflicting interests.

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