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ORIGINAL ARTICLE

Curculigo orchioides polysaccharides extraction, characterization, and their protective effects against femoral head necrosis

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Abstract The use of steroid drugs such as dexamethasone in long-term treatment poses a challenge called femoral head necrosis. To reduce the destructive effects of dexamethasone, the use of herbal suppressants such as *Curculigo orchioides* polysaccharides (COPs) is recommended despite the ambiguities in their chemical composition and the effects of each component on the anti-necrosis activity of the femoral head. In this study, after separation of COPs through hot water–ethanol precipitation method and purification through DEAE-Sepharose fast flow column, their properties were explained by FTIR, NMR, methylation and chromatography. Moreover, the COPs biocompatibility and its inhibitory effects against dexamethasone-induced cytotoxicity were evaluated by MTT assay and gene expression on human primary osteoblasts cells. The results exhibit that the 4756 Da molecular weight COPs is generally composed of Rha, Ara, Fru, Xyl, Man, Glc, Gal, Glu A and Gal A. Also, MTT results recognized high biocompatibility of COPs and suppressive effect on dexamethasone. Also, COPs significantly reduced dexamethasone-induced intracellular levels of apoptosis and ROS. Furthermore, the use of COPs has significantly increased ALP activity, collagen content and mineralization, which are very effective in differentiating HPOS cells to repair bone tissue. Meanwhile, the gene expression outcomes indicate an increase in Bcl-2 expres-

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sion gene and decrease in BAX, Caspase-9 and Caspase-3 gene expression in the presence of COPs, which are important in controlling apoptotic activity and bone regeneration. Overall, this study revealed that COPs can lead to the treatment of femoral head necrosis.

1. Introduction

Curculigo orchioides is a small native plant in China, India, Malaya, Japan and Australia that is widely found in East and Southeast Asia. Although this plant has been used in Chinese and Indian medicine for restorative activities, treatment of arthritis, and diuretics (Nie et al. 2013); But reports of antibacterial (Singh and Gupta 2008), anti-osteoporosis (Wang et al. 2019), antitumor (Xia et al. 2016) and even estrogenic (Vijayanarayana et al. 2007) activities are seen based on the glycosidic and alcoholic structures in the plant extract. However, the identification of the active ingredient of the extracts obtained in medical activities has been challenged due to the high diversity of chemical compounds, especially in the field of polysaccharides.

Femoral head necrosis is a serious and common challenge among patients taking corticosteroids (Liu et al. 2017). However, the use of these drugs such as dexamethasone are widely used in the control of inflammatory diseases and immunity. Prolonged use of these drugs, in addition to necrosis of the femoral head, causes permanent deformation of the bone and even stops the process of bone regeneration and fracture (Luo et al. 2018; Sandberg and Aspenberg 2015). Despite many efforts to reduce the effects of glucocorticoid drugs, bone necrosis problems are still abundant in patients (Jin et al. 2020; Peng et al. 2021). For example, despite the use of glucocorticoid occlusive ointments (McLean et al. 1995), necrosis of the femoral head was still observed in patients taking corticosteroids. Nowadays, the use of herbal medicines to reduce the complications of femoral head necrosis has received much attention. For example, in the cellular model, Wang et al. (2019) and revealed that the use of *Curculigo orchioides*-derived polysaccharides (COPs) not only increased alkaline phosphatase activity and mineralization, but also increased the proliferation rate and differentiation of primary osteoblasts cells to repair bone tissue. In this regard, it was found that *Agrimonia pilosa*-derived polysaccharide by increasing the expression of BMP2, Runx2, Bcl-2, OSX and OCN, and decrease in the Wnt3, β-catenin and c-Myc protein has provided good results in reducing femoral necrosis (Huang et al. 2020). On the other hand, Wang et al. (2017) exhibited that COPs significantly increased the proliferation rate of MC3T3-E1 cells. Nonetheless, it is still unclear which of the compounds in the plant extract has a direct effect on the regeneration of femoral head tissue.

In this literature, an attempt was made to control and stop the activity of femoral head necrosis in the presence of dexamethasone by isolating and purifying the COPs and structural analysis using FTIR and NMR methods. Also, an attempt was made to explain the biocompatibility of polysaccharides using the MTT method on osteoblasts cells. The mechanism of action of COPs on the control of femoral head necrosis was explained by studying BAX, Bcl-2, Caspase-9 and -3 genes. Finally, Finally, the effect of COPs on femoral bone tissue was described by controlling morphological changes and bone density in the presence of dexamethasone.

2. Material and methods

2.1. Extraction and purification of COPs

Curculigo orchioides rhizome obtained from Beijing Tong Ren Tang Chinese Medicine Co., Ltd. After washing and drying in the oven, the rhizomes were ground. Then, the polysaccharide was extracted by 4 kg of rhizomes ground in distilled water in a ratio of 1:10 (w:v, kg/L) at 90 °C for 4 h. The resulting solution was then boiled to increase the concentration at 60 °C for 4 h. For precipitation of COPs, the concentrated solution with 95% ethanol was concentrated to 50% and kept in an incubator at 23 °C for 24 h. The solution was re-concentrated to 70% anhydrous ethanol and kept in the incubator at 23 °C for 24 h. Finally, after the alcohol was determined by an alcohol meter, the precipitate was removed and dialyzed using the Sevag technique for 4 times.

For fractionation and purification of polysaccharide, 300 mg of the COPs was dissolved in 15 mL of distilled water and centrifuged at 8000g for 10 min. The supernatant (5 mL) was loaded onto the Diethylaminoethyl cellulose-52-cellulose column (Channel Size: 2 μm, Column Tube Length: 110 mm, Connectors: TC 1 in, Inner Diameter: 15 mm, Outer Diameter: 34 mm, and Recommended Operating Temperature: 4 to 40 °C.) after filtration. Then use distilled water as a mobile phase (distilled water) to move the solution through the column at a rate of 1.2 mL per min. After 5 h, the effluent was examined by phenol–sulfuric acid, and the washed portion was concentrated with sodium chloride and dialyzed again with distilled water. Then, the results were concentrated in a Sephadex G-75 gel filtration column at a flow rate of 18 mL/h with the help of purified distilled water, and finally lyophilized.

2.2. Molecular weight and chemical composition of COPs

High-performance liquid chromatography (HPLC) with TSK-G4000 PWXL column (7.8 × 300 mm) was used to assess the molecular weight of COPs. A 0.45 μm membrane was used to purify the COPs solution at a concentration of 1% (w/v). The mobile phase in the molecular weight examination was deionized water with a flow rate of 0.5 mL/min. Dextran T-series standards were applied for calibrating the columns.

Gas chromatography was applied to survey the composition of monosaccharides in COPs. For this purpose, 3 mg of COPs was hydrolyzed with 1.5 mL of trifluoroacetic acid (2 M) for 6 h at 120 °C. Afterwards, 100 μL of methanol was added to the solution to remove trifluoroacetic acid. Then, hydroxylamine hydrochloride (5 mg) and pyridine (0.35 mL) were added to the mixture and the mixture was incubated

for 25 min at 95 °C. Next, acetic anhydride (0.5 mL) was added to the cold mixture and incubated again at 95 °C for 25 min. The sample was dried and then mixed with chloroform (0.5 mL). Finally, 0.2 μL of the final sample was used for gas chromatography-mass spectrometry analysis. 9 standard monosaccharide types were considered for graph analysis including rhamnose, arabinose, fructose, xylose, mannose, glucose, galactose, glucuronic acid, and galacturonic acid.

2.3. FTIR, methylation investigation and NMR

To explore the polysaccharide FTIR spectrum, 2 mg of COPs was assorted with 170 mg of ground dry KBr powder. The mixture was then entirely compacted in a 1 mm pellet. The FTIR spectra of samples were recognized by applying a Nicolet 5700 FTIR spectrometer in a wave number range of 400–4000 cm^{-1} using the KBr way.

Methylation analysis was used to evaluate glycosidic bonds in COPs. For this purpose, based on the report of Pettolino et al. (2012), COPs was methylated. COPs' methylation can be tracked by removing the O-H band in the range of 3200–3700 cm^{-1}. The 3 mg of methylated sample was then hydrolyzed with 1 mol per L trifluoroacetic acid (3 mL) for 2 h at 120 °C, reduced with 50 mg NaBH4, and acetylated with acetic anhydride-pyridine (1:1) for 1 h. Finally, it was analyzed by gas chromatography–mass spectrometry.

In order to evaluate the COPs by NMR method, 30 mg of the sample (for 5 days) was dried in vacuum. Then, the COPs was impregnated with 1 mL of D_2O and the sample was exchanged 3 times with D_2O in freeze drying mode. Afterwards, the COPs was dissolved in 0.5 mL of D_2O. Finally, the ^1H and ^{13}C NMR spectra were recorded on a Bruker AV-500 spectrometer, respectively. Chemical changes were explained with acetone as an internal standard.

2.4. MTT assay

Human primary osteoblast (HPOS) cells were cultured at a density of 3×10^4 cells per well in 96-well plate with DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and then incubated for 12 h at 37 °C with 5% CO_2. Afterwards, the HPOS cells were incubated for 24 h at concentrations of 0.5, 1, 2 and 4 μM dexamethasone and concentrations of 50, 100, 150 and 200 μg/mL COPs, separately. To determine the effect of dexamethasone and COPs on HPOS cells, the old culture medium containing 1 μM dexamethasone was transformed to new medium containing 50, 100, 150 and 200 μg/mL COPs and incubated for 24 h at 37 °C with 5% CO_2. Subsequently, all media were replaced with PBS and 0.5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was distributed in their medium. The samples were then incubated again for 4 h (50 μL per well). Then, the culture medium was drained from the wells and the crystals formed in each well were dissolved by 100 μL of dimethyl sulfoxide by shaking for 2 min. Ultimately, the cell absorbance at $\lambda = 490$ nm was calculated

2.5. Apoptosis and reactive oxygen species (ROS) assays

Flow-cytometry was used to estimate the level of COPs-induced apoptosis. After culturing HPOS cells (3×10^5 cells per well) in a 6-well plate, the cells were treated with 1 μM of dexamethasone and 150 μg/mL of COPs and incubated for 24 h at 37 °C. The HPOS cells were then centrifuged at 3000g for 3 min at 4 °C. The samples were then washed with PBS. Afterwards, the HPOS cells were distributed in 100 μL of Annexin V binding buffer (HEPES buffer containing 0.1 M, NaCl 1.4 M, $CaCl_2$ 25 mm, pH 7.4). Next, 2 μL of Alexa Fluor 488 conjugated with Annexin V was augmented to the solution and saved in the dark at 21 °C for 15 min. Subsequently, 400 μL of the binding buffer and 5 μL of 50 μg/mL propidium iodide were added to the solution and saved in ice. Ultimately, samples were considered by FACscan (BD Bioscience).

In order to characterize the level of intracellular ROS at concentrations of 100 μg/mL COPs and 1 μM of dexamethasone, 3×10^5 cells per well were cultured for 24 h in 6 well plates. The culture medium was replaced with phenol red free DMEM containing H2DCFDA (10 μM) 30 min. Then, cells were supplemented by GYY4137 (100 μM) for 30 min. Next being washed-out twice, cells were incubated with H_2O_2 (400 μM) for 2 h. The ROS generation was evaluated with a fluorescence reader (Safire2, Tecan Group Ltd.).

2.6. Lactate dehydrogenase release

Lactate dehydrogenase (LDH) release method was applied to evaluate Dexamethasone-induced cytotoxicity. HPOS were supplemented with dexamethasone (1 μM) and then incubated with concentrations of 50, 100, 150 and 200 μg/ml COPs for 24 h in an incubator maintained (at 37 °C with 5% CO_2). The cell culture was harvested and LDH release was examined in accordance with the manufacturer's instructions (Beyotime, China).

2.7. Alkaline phosphatase (ALP) activity assay and collagen content

In order to investigate the ALP activity in the HPOS cells, the HPOS cells (3×10^4 each well) were cultured into 6-well plates which were supplemented with different COPs concentrations (50, 100, 150 and 200 μg/ml) and 1 μM dexamethasone. After 3 days, the cells were washed and harvested, then the HPOS cells were lysed with 100 μL lysis buffer (Beyotime Institute of Biotechnology). An ALP reagent kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) was applied to evaluate the ALP activity.

The culture medium of the HPOS cells treated with concentrations of 50, 100, 150 and 200 μg/ml COPs, and 1 μM of dexamethasone was removed after 72 h and the HPOS cells were washed twice with PBS. Then fixed with Bouin's fluid for 1.30 h. The fixation fluid was then removed and all the HPOS cells were washed three times with distilled water. The well was stained with 120 μL of 0.1% Sirius red dye (weight/ volume) for 1.30 h at 37 °C with gentle shaking. The plates were washed again three times with 200 μL of 10 mM HCl solution. Then, 0.1 N NaOH was added to the plate to dissolve the collagen for 30 min. Ultimately, UV absorption was evaluated at 550 nm using an ELISA reader.

2.8. Mineralization

Staining density was used to evaluate the mineralization of osteoblast cells. In this regard, the amount of mineralization

was determined using the Alizarin Red S method after 24 h of HPOS cell treatment with COPs and dexamethasone. After incubation of the cells according to section 3, the cells were collected and washed with a cold frozen PBS buffer. Then, the HPOS cells were fixed in 70% cold frozen ethanol at 4 °C for 30 min and stained with 0.1% Alizarin Red S-Tris-HCl solution with pH 8.3. After 20 min of staining, the dye medium was deleted and the plates were washed four times with distilled water. Ultimately, 10% (w/w) cetylpyridinium chloride solution was used to dissolve the remaining dye in the HPOS cells and was measured by UV absorption at 560 nm using an ELISA reader.

2.9. Analysis of BAX, Bcl-2, Caspase-3 and Caspase-9

The quantitative real-time PCR (qPCR) method was used to evaluate the expression level of BAX, Bcl-2, Caspase-3 and Caspase-9 genes. Trizol reagent was used to isolate the total RNA according to the manufacturer's protocol in the control and COPs at concentrations of 50, 100, 150 and 150 μg/mL. To remove the contaminant genome, RNA samples were treated with DNAse I, and a cDNA of 1 μg RNA was generated using revert Aid First Strand cDNA Synthesis Kit (Fermentas). The primers include of BAX: forward 5′-ATG GACGGGTCCGGGGAG-3′, reverse 5′-ATCCAGCCCAA CAGCCGC-3′; Caspase-9: forward 5′-GTTTGAG GACCTTCGACCAGCT-3′, reverse 5′-CAACGTACCAG GAGCCACTCTT-3′; Bcl-2: forward 5′-ATCGCCCTGTG GATGACTGAGT-3′, reverse 5′-GCCAGGAGAAATCAAA CAGAGGC-3′; Caspase-3: forward 5′-GGTATTGAGACA GACAGTGG-3′, reverse 5′-CATGG GATCTGTTTCTTTGC-3′; and β-actin: forward 5′-AATTC CATCATGAAGTGTGA-3′, reverse 5′-ACTCCTGCTTGCT GATCCAC-3′.

The qPCR was done by an ABI 7500 real-time PCR system (ABI, USA) using a SYBR Premix Ex Taq Reagent Kit (Takara) according to the manufacturers' protocols. The β-actin amplification was used as a housekeeping gene.

2.10. Statistical analysis

The data were investigated with statistical analysis software SPSS 16.0 (SPSS Inc., Chicago, IL, US). Probabilities less than 0.05, 0.01 and 0.001 were considered significant, and statistical differences among groups were gained by analysis of variance (ANOVA) following Dunnett's tests.

3. Results and discussion

3.1. Isolation, molecular weight and composition of COPs

The extraction results show a 15.98% efficiency of the extraction method (water extraction, ethanol precipitation and deproteinization) in the production of COPs from the prototypes. Also, Fig. 1A exposes that the highest concentrations of COPs extracted in the tubes are 0.73, 0.45 and 0.35 mg/mL, respectively. The molecular weight of the COPs was determined to be approximately 4756 Da based on the standard dextran curve. Finally, Fig. 1B and 1C illustrated the presence of Rha, Ara, Fru, Xyl, Man, Glc, Gal, Glu A and Gal A in the extracted samples, of which the most abundant among monosaccharide were Man and Gal.

3.2. Characterization of COPs

Investigations of the properties of COPs extracted by FTIR in Fig. 1D show that the higher the tensile peak at 3350 cm^{-1}, which refers to the vibration of the O-H bond. While vibrations in the range of 2900 cm^{-1} indicate the C-H band, in the range of 1700 cm^{-1} and 1420 cm^{-1} infer the C = O and the symmetric C-H bands, in the range of 1200 cm^{-1} and 1060 cm^{-1}, respectively indicate the C-H band and pyranoside, and in the range of 850 cm^{-1} represents the mannose pyranose ring. After COPs methylation, it was revealed that the O-H band was removed in the range of 3200–3700 cm^{-1}, indicating the success of the methylation process. The methylation outcomes, in order to determine the main and lateral structure of COPs according to retention time and ions fragments, demonstrates the presence of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-mannitol, 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-mannitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol, and 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol in the COPs. These outcomes are consistent with the NMR consequences, which demonstrated the main residue linkage kinds of COPs were → 4)-D-Man*p*-(1→, →4)-D-Glc*p*-(1→, →3,6)-D-Man*p*-(1 → and D-Gal*p*-(1 →).

The results of Fig. 1E shows the greater accumulation of ^{1}H NMR and ^{13}C NMR spectra in the region of 3.2–5.8 and 58–112 ppm, respectively. In addition, the presence of the O-acetyl group in COPs is confirmed based on carbon signals at 20 ppm and proton signals at 2.1 ppm. Analysis of ^{1}H NMR and ^{13}C NMR spectra indicates the presence of seven peaks in confirmation of FTIR outcomes, which indicate the bands and chemical components of monosaccharides in the COPs structure. The provided signals demonstrate the presence of β-D-Man*p* (4.70 to 4.80 ppm and 60.5 to 61.1 ppm in the ^{1}H and ^{13}C NMR spectrum, respectively), β-D-Gal*p* (4.82 to 4.93 ppm and 99.54 to 100.43 ppm in the ^{1}H and ^{13}C NMR spectrum, respectively), α-L-Rha*p* (3.94 to 4.16 ppm and 71.3 to 71.8 ppm in the ^{1}H and ^{13}C NMR spectrum, respectively), β-D-Gal*p*A (3.46 to 3.50 ppm and 73.4 to 74.7 ppm in the ^{1}H and ^{13}C NMR spectrum, respectively), α-D-Glc*p*A (3.68 to 3.80 ppm and 76.4 to 77.1 ppm in the ^{1}H and ^{13}C NMR spectrum, respectively), and α-L-Ara*f* (5.12 to 5.20 ppm and 69.4 to 71.2 ppm in the ^{1}H ^{13}C NMR spectrum, respectively) (Fig. 1F). Definitely, the chemical shifts of anomeric protons below 4.77 ppm and carbon resonance above 100.45 ppm display β-linked-anomeric of the Gal*p* residues, which is in line with the methylation results.

3.3. Cell viability, apoptosis and ROS analysis

The results of Fig. 2A displays that the use of dexamethasone at concentrations of 0.5 to 4 μM significantly reduces the viability of HPOS cells compared to the control group. Moreover, concentrations of COPs 50, 100, and 150 μg/mL increased cell viability by 9.9%, 12.2%, and 11.4%, respectively, compared with control (Fig. 2B). Whereas the concentration of 200 μg/mL did not show a significant difference in the survival of HPOS cells compared to the control. Although increasing dex-

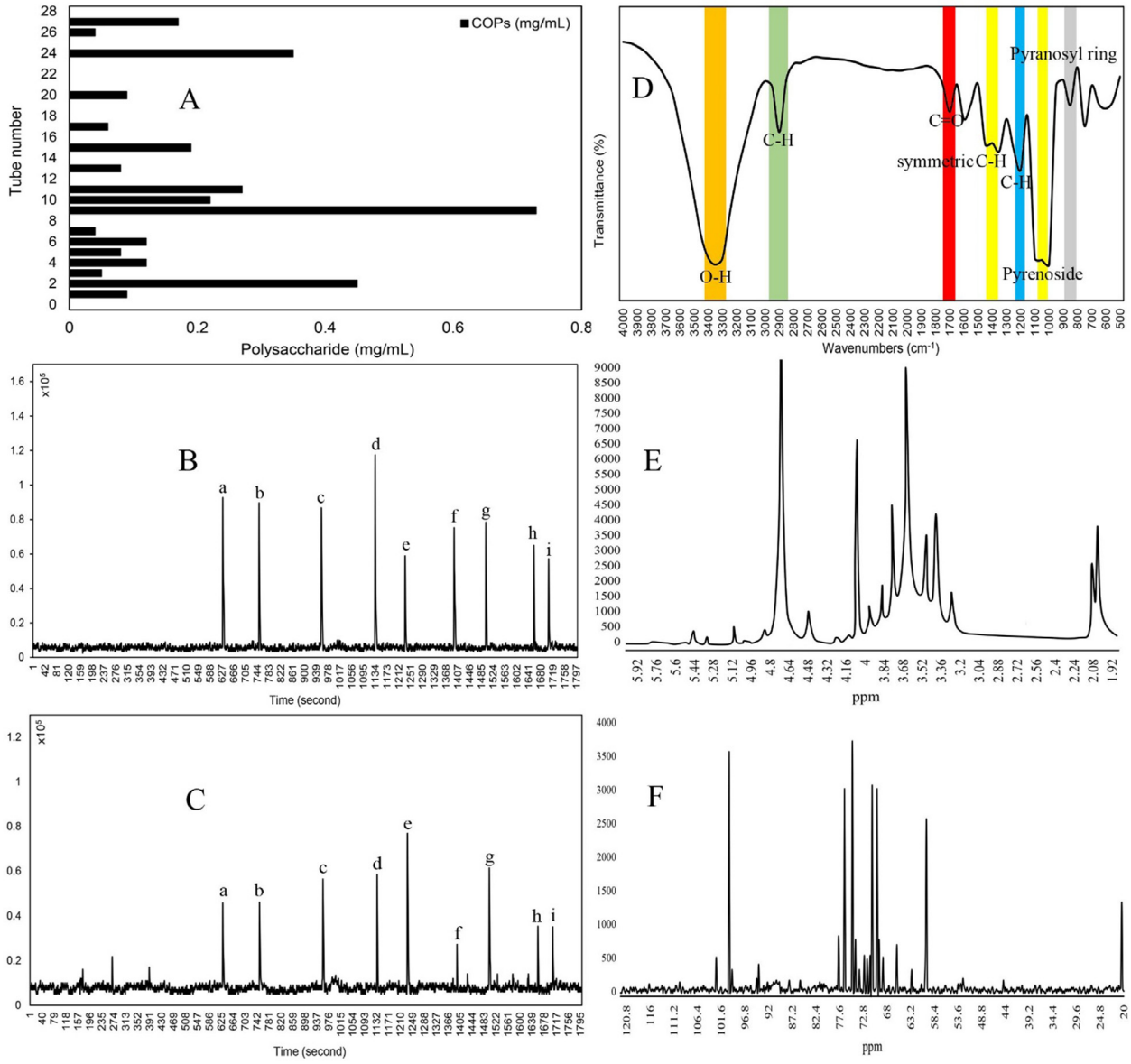

Fig. 1 (A) The elution profile of the *Curculigo orchioides* polysaccharide (COPs) on a cellulose DEAE sepharose fast flow chromatography eluted with stepwise gradient of NaCl aqueous solutions (0, 0.1, 0.2 and 0.3 M) at a flow rate of 1.0 mL/min. GC–MS analysis of standard (B) monosaccharide (a: Rha, b: Ara, c: Fru, d: Xyl, e: Man, f: Glc, g: Gal, h: Glu A and i: Gal A) and (C) *Curculigo orchioides* polysaccharide. (D) Infrared Spectrum of *Curculigo orchioides* polysaccharide. (E) ^{1}H NMR spectrum of *Curculigo orchioides* polysaccharide. (F) ^{13}C NMR spectrum of *Curculigo orchioides* polysaccharide.

amethasone concentration decreases cell viability, concomitant use of dexamethasone with COPs significantly reduces the effect of dexamethasone on cell viability (Fig. 2C). The results illustrate that the use of 100 μg/mL COPs is appropriate to enhance cell viability in the presence of dexamethasone.

As shown in Fig. 2D and 2E, the use of dexamethasone increases necrosis and apoptosis in HPOS cells significantly compared to the control group by increasing the level of necrosis (15.11% vs 5.21%), and apoptosis in Q2 (1.02% vs. 25.60%) or Q3 (2.85% vs. 32.02%). Conversely, the application of 150 μg/mL COPs simultaneously with dexamethasone reduced necrosis (10.37% vs. 15.11%) and apoptosis by 42 and 31% in Q2 (19.46%) and Q3 (20.15%), respectively (Fig. 2F). Based on the results of apoptosis, as expected, the use of COPs in the presence of dexamethasone significantly reduced intracellular ROS levels from 499 to 274 (Fig. 2G). Nonetheless, compared with the control group, the use of COPs in the presence of dexamethasone could not stop intracellular ROS levels optimally. Thus, the use of COPs can have an inhibitory effect on the generation of intracellular ROS and prevent the development of femoral necrosis activity.

3.4. LDH assay

The results of LDH in Fig. 3A shows that the use of dexamethasone increases LDH by 4.3 times compared to the control

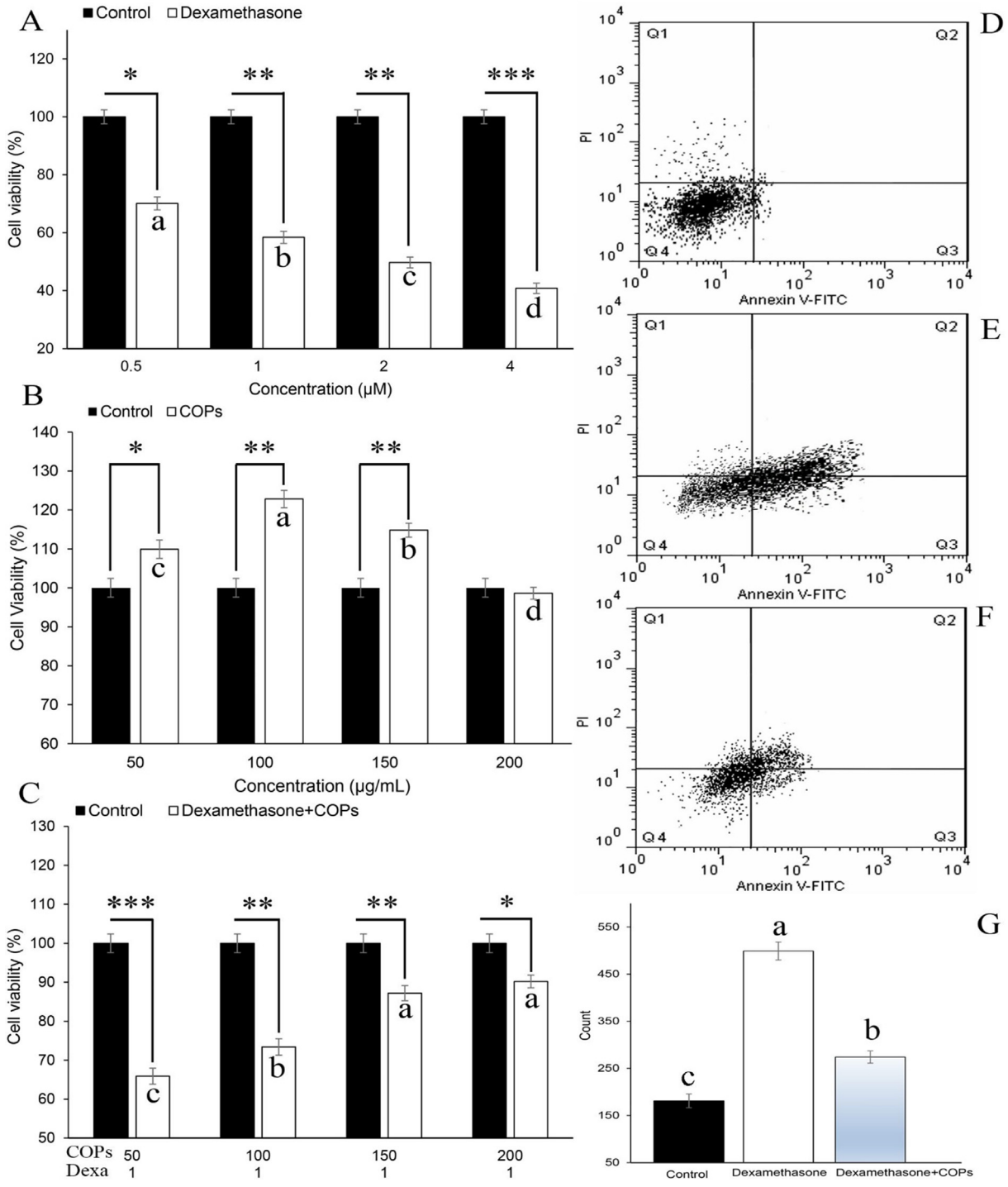

Fig. 2 (A) Effects of dexamethasone on cell viability *in vitro*. (B) Effects of *Curculigo orchioides* polysaccharide (COPs) on cell viability *in vitro*. (C) Effect of COPs on cell viability pre-treated with dexamethasone *in vitro*. Two-dimensional contour density plots of osteoblasts cells determined by flow cytometry assay. (D) Control (Q1: 5.21%, Q2: 1.02%, Q3: 2.85% and Q4: 90.92%), (E) Dexamethasone (1 μM) (Q1: 15.11%, Q2: 22.60%, Q3: 29.02% and Q4: 32.81%), (F) *Curculigo orchioides* polysaccharide (150 μg/mL) + dexamethasone (1 μM) (Q1: 10.37%, Q2: 19.46%, Q3: 20.15% and Q4: 50.02%). Cell death measured using propidium iodide (PI) and Annexin V-FITC staining, (G) the effects of dexamethasone and *Curculigo orchioides* polysaccharide + dexamethasone on the ROS production. Statistical differences were measured at level of $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

group. However, the results indicate a decrease in LDH levels in the use of COPs along with dexamethasone. This means that with increasing COPs levels, LDH concentration decreases significantly from 23.6% in the dexamethasone group to 22.1% (at a concentration of 50 μg/mL of COPs), 17.2% (at a concentration of 100 μg /mL of COPs), 12.3% (at a concentration of 150 μg /mL of COPs) and 10.7%. (at a concentration of 200 μg /mL of COPs). Despite the decrease in LDH level, LDH con-

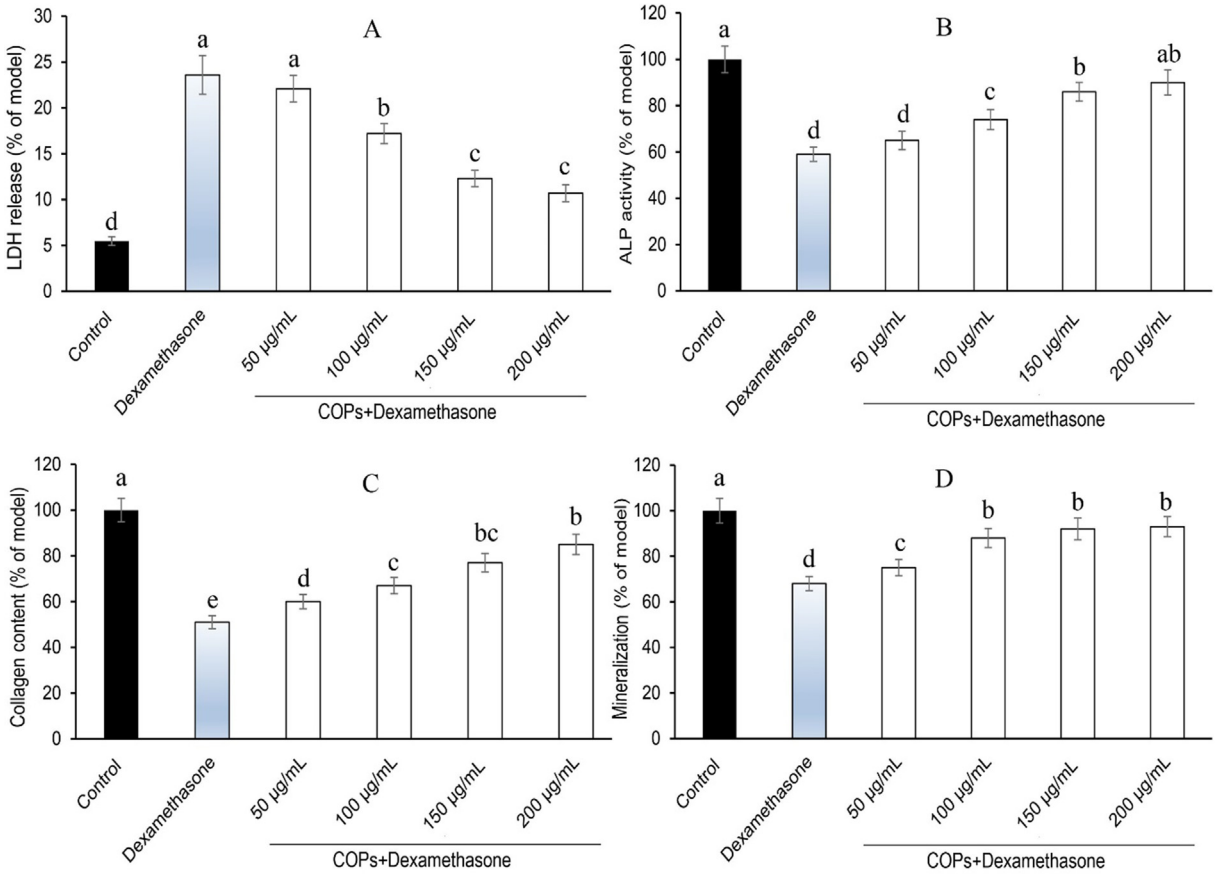

Fig. 3 Effects of *Curculigo orchioides* polysaccharide on (A) lactate dehydrogenase (LDH), (B) alkaline phosphatase (ALP) activity, (C) collagen (COL: measured by a Sirius Red-based assay) and (D) mineralization of human primary osteoblasts cells *in vitro*. [a,b,c,d,e] Least square means with different letters in superscripts are different at $*P < 0.05$.

centration is still higher than the control group in the presence of COPs and dexamethasone.

3.5. Alkaline phosphatase (ALP) activity, collagen content and mineralization

Because ALP activity is a reliable marker for early detection of osteoblastogenesis, its evaluation is suitable for controlling femoral necrosis activity, and repairing the damaged area. As shown in Fig. 3B, ALP activity in dexamethasone-treated HPOS cells were significantly reduced compared with the control group. However, the use of COPs effectively reduces the negative effects of dexamethasone. Concentrations of 150 and 200 μg/mL of COPs will have the greatest effect on the suppression of femoral head necrosis by reducing the negative activity of dexamethasone. On the other hand, the results of the study of collagen content in HPOS cells revealed that the increase in collagen levels by COPs is similar to the increase in ALP activity (Fig. 3C).

As shown in Fig. 3D, intracellular calcium levels are reduced with the dexamethasone. Also, the use of COPs in comparison with the dexamethasone group effectively increases intracellular calcium levels. Among the different concentrations of COPs, the greatest effect is observed on concentrations of 150 μg /mL and 200 μg /mL.

3.6. Effects of COPs on genes expression

The expression of BAX (Fig. 4A), Bcl-2 (Fig. 4B), Caspase-3 (Fig. 4C), and Caspase-9 (Fig. 4D) were assessed by qPCR assay.

As shown in Fig. 4, despite a 1.39-fold reduction in Bcl-2, the production and expression of BAX, Caspase-9 and Caspase-3 genes increased 1.5-fold, 2.24-fold and 2.44-fold in the presence of dexamethasone, respectively. While, the application of COPs with different concentrations in the presence of dexamethasone significantly reduced the expression of BAX, Caspase-9 and Caspase-3 genes. Also, the use of COPs significantly improved the expression of the Bcl-2 gene. Despite a significant decrease in the expression of BAX, Caspase-9 and Caspase-3 genes at a concentration of 100 μg/mL COPs, the results show that the most effective decrease in the expression of BAX and Caspase-9 genes is related to a concentration of 150 μg/mL COPs with 40.9% and 29.7% reduction compared to dexamethasone, respectively. While the greatest decrease in the expression Caspase-3 gene refers to the concentration of 200 μg/mL of COPs with 58.1% reduction compared to dexamethasone. On the other hand, the use of tetrad with a concentration of 150 μg/mL causes a 2.46-fold and 1.66-fold increase in Bcl-2 compared to the dexamethasone and the control groups. Since increasing Bcl-2 can reduce the level of

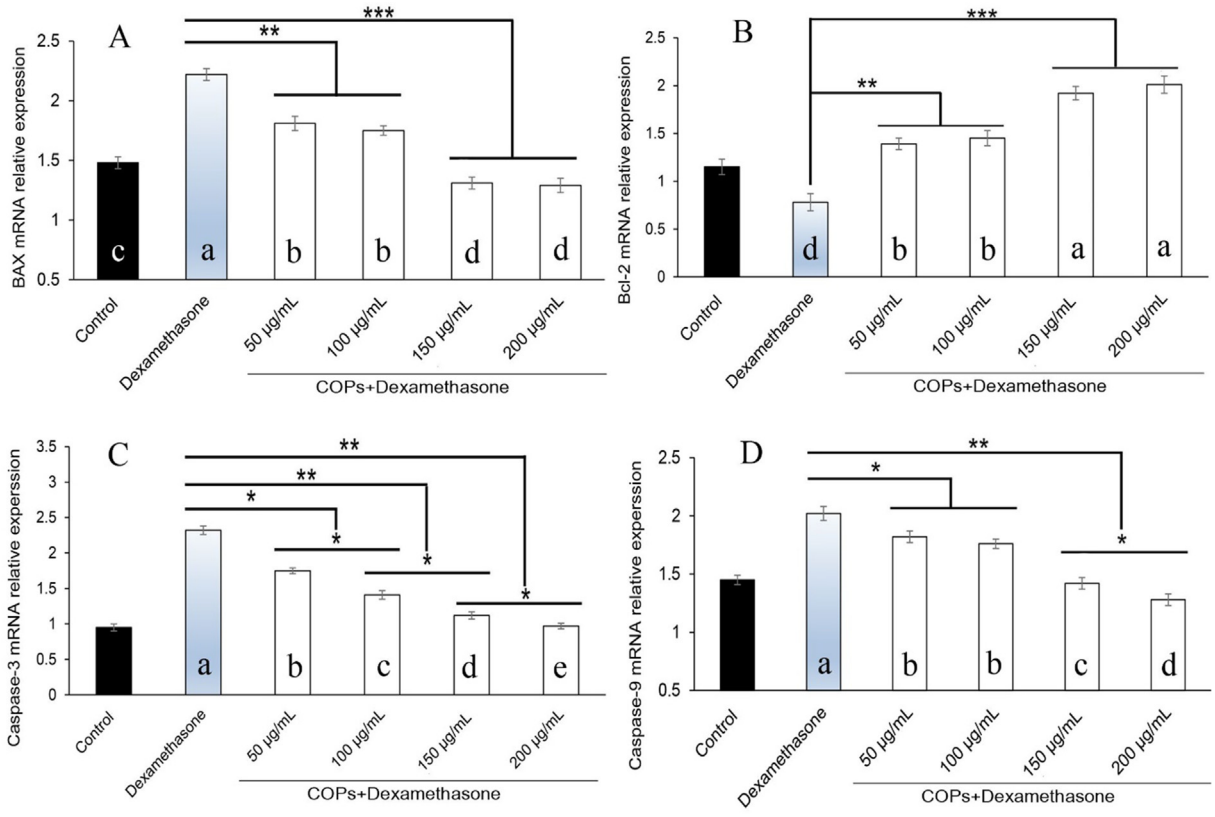

Fig. 4 Effects of *Curculigo orchioides* polysaccharide on (A) BAX, (B) Bcl-2, (C) Caspase-3, and (D) Caspase-9 expression of human primary osteoblasts cells induced by dexamethasone (1 µM). Statistical differences were measured at level of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.^{a,b,c,d,e} Least square means with different letters in superscripts are different at $^{*}P < 0.05$.

apoptosis in HPOS cells, increasing Bcl-2 will increase the hope of bone tissue regeneration. Overall, the inhibitory effect of polysaccharides on Caspase-3 gene was greater than that of BAX and Caspase-9, whereas, similar to MTT results, the best concentration of COPs to reduce apoptosis-inducing genes is 150 µg/mL.

4. Discussion

Femoral head necrosis is a multifactorial disorder that faces ambiguities such as the causes of formation and treatment methods (Samy 2016). However, there is strong evidence that long-term use of glucocorticoid compounds such as dexamethasone may increase the incidence of this disorder (Qi and Zeng 2015). In this regard, it is thought that the use of dexamethasone inhibitor compounds is desirable to prevent the occurrence of this disease (Hao et al. 2016). Because, the apoptotic activity detected in this method is increased based on the use of dexamethasone (Ma et al. 2020). However, depending on the causes of this disorder in patients, different types of treatment can be followed. For example, one of the methods that can be used to prevent and treat this disorder is the use of herbal polysaccharides (Cui et al. 2018; Huang et al. 2019).

In this study, part of the plant polysaccharide was isolated and examined by FTIR, methylation and NMR methods. In agreement with the results of Wang et al. (2017) and Wang et al. (2019) it was revealed that the extracted COPs has a normal structure of carbohydrates, but contrary to the results of Wang et al. (2017) who showed that the predominant sugar of polysaccharide is glucose and mannose, the predominant monosaccharides in this study are mannose and galactose. Nevertheless, there still seems to be a challenge in more accurately and clearly diagnosing COPs status based on differences in composition and monosaccharide ratios in the literature. In this regard, the results of several articles show that the effective components in herbal polysaccharides to control femoral head necrosis disorder are more than one hundred active compounds (Chhikara et al., 2020; Faizi et al. 2014). In the following, the results of this study indicate the existence of seven vibration peaks in the FTIR and NMR diagrams. While the findings of Wang et al. (2017) and Wang et al. (2019) identified 6 and 13 peaks, respectively. Differences in identified vibration peaks can be attributed to differences in plant age, place of cultivation, plant components used, and even plant subspecies. However, the peaks provided in this study are consistent with the vibration peaks in the above findings.

Previous articles have shown that an imbalance between osteoblasts and osteoclasts causes osteoporosis (Ivanova et al. 2016; Tanaka et al. 2005). Therefore, increasing the level of apoptosis in osteoblasts in the presence of dexamethasone can impair the proliferation and repair of femoral head necrosis (Deng et al. 2019). The results of this study showed that the use of COPs significantly improves the proliferation and cell viability in the presence of dexamethasone. Also, LDH release demonstrated that COPs could drastically ameliorate dexamethasone-induced HPOS cells death in a

concentration-dependent profile. In this regard, earlier in a cellular model, Wang et al. (2017) exhibited that the use of COPs not only increases the cells viability, but also significantly improves the proliferation and differentiation of osteoblasts cells. As well, recently Wang et al. (2019) revealed that in addition to high biocompatibility of COPs, their use increases the proliferation and differentiation of osteoblasts cells. In this field, Shen et al. (2013) using COPs in addition to increasing the proliferation of osteoblasts cells improved their differentiation in restorative activities. On the other hand, the results of this study show that the concentration of 100 μg/mL was not only non-toxic to osteoblasts cells, but also significantly reduced the level of dexamethasone-induced apoptosis and intracellular ROS. In this line, studies by Huang et al. (2019) and Cui et al. (2018) showed that the use of *Agrimonia pilosa* and *Safflower* polysaccharides reduces the level of apoptosis and intracellular ROS. Numerous results show that increased intracellular ROS causes tissue damage and destruction of femoral head necrosis (Deng et al. 2018; Peng et al. 2021).

The presence of active monosaccharides such as galactan and mannan, which are abundant in this polysaccharide based on methylation and NMR outputs, can be effective in reducing inflammation due to reduced intracellular ROS. In this regard, Barbosa et al. (2020) exposed that the presence of galactan significantly reduces the level of intracellular ROS. Furthermore, the use of galactan-containing polysaccharides in LPS-induced macrophage cells has been shown to reduce the expression of inflammatory genes (Cui et al. 2019), which play an important role in increasing the level of inflammation (Li and Wang 2019). In line with the results of this study, Huang et al. (2019) used *Agrimonia pilosa* polysaccharide to reduce the necrosis of the femoral head to stop the apoptotic pathway. Similarly, Yamamoto et al. (1999), Zhang et al. (2017) and Cui et al. (2018) by polysaccharides were able to reduce the level of inflammation by reducing the expression of Caspase-3 gene, which can prevent femoral head necrosis. Also, the results of Nie et al. (2019) show that the use of dexamethasone, in addition to reducing mitochondrial transmembrane potential, increases the activity of Caspase-9 and then Caspase-3. Loss of mitochondrial transmembrane potential occurs with the release of cytochrome *c* from the mitochondria to the cytosol and activation of Caspase-3 (Sharifi et al. 2019). On the other hand, increasing the BAX/Bcl-2 ratio can cause the loss of mitochondrial transmembrane potential (Giménez-Cassina and Danial 2015). Therefore, in accordance with these findings and the results of the present study, it exhibits that the use of COPs can be dose-dependent in suppressing the signaling pathway of the femoral head necrosis.

Since osteoblasts are responsible for the formation of bone tissue during complex processes including proliferation and differentiation, the study of osteoblast differentiation signals include of ALP activity and collagen content can be effective in understanding the effect of polysaccharides on femoral head necrosis control and its tissue repair (Tabatabai et al. 2021). In the process of differentiation, the two primary markers of osteoblastogenesis, including ALP and collagen, are expressed. Then, in the next stage, calcium deposition is visible (Guerreiro et al. 2021). The results of this study, in line with the study of Cui et al. (2018) demonstrate that ALP activity and collagen content are increased by plant polysaccharides such as COPs and safflower, which is effective in regenerating necrotic femoral head tissue by long-term use of dexamethasone. Increasing the concentration of polysaccharides can not only reduce the toxicity of dexamethasone based on MTT testing, but also increase bone repair by increasing the level of ALP activity and collagen content. Also, increased mineralization of polysaccharide-based HPOS cells along with increased ALP and collagen levels could be associated with increased osteoblastogenesis, which is an important factor in the regeneration of necrotic femoral bone.

Overall, the findings of this study suggest that polysaccharides may prevent dexamethasone-induced femoral head necrosis by reducing the level of apoptosis in osteoblasts.

5. Conclusions

Overall, this study showed that the COPs extracted by hot water–ethanol deposition method have high biocompatibility. The results also revealed that the use of COPs could play an important role in the proliferation of HPOS cells. However, COPs face serious challenges such as the lack of sufficient information about the chemical components and the effects of each of them on anti-necrosis activities of the femoral head. Despite the identification of the mechanism of action of COPs on reducing apoptosis by reducing the expression of BAX, Caspase-9, and Caspase-3 genes and raising the Bcl-2 expression gene, more molecular mechanisms are needed to evaluate the multifaceted nature of COPs. Finally, dexamethasone-inhibiting COPs capable of suppressing apoptotic activity could provide a viable pathway for the treatment of femoral head necrosis.

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