Journal of Chromatography B, 904 (2012) 115–120

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Combining human periodontal ligament cell membrane chromatography with online HPLC/MS for screening osteoplastic active compounds from *Coptidis Rhizoma*

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ARTICLE INFO

Article history:
Received 3 December 2011
Accepted 25 July 2012
Available online 4 August 2012

Keywords:
Human periodontal ligament cell (hPDLC)
Cell membrane chromatography (CMC)
High performance liquid chromatography/mass spectrometry (LC/MS)
Coptidis Rhizoma
Berberine
Simvastatin

ABSTRACT

We have developed an online analytical method that combines human periodontal ligament cell membrane chromatography (hPDLC/CMC) with high-performance liquid chromatography and mass spectrometry (LC/MS) for recognizing and identifying osteoplastic active components from *Coptidis Rhizoma*. Retention fractions on hPDLC/CMC were enriched onto an enrichment column and the components were directly analyzed by combining a 10-port column switcher with an LC/MS system for separation and preliminary identification. Using simvastatin (SIM) as a positive control, berberine from *Coptidis Rhizoma* was identified as the active component which could act on the hPDLC. The MTT colorimetric assay, alkaline phosphatase (ALP) activity, and staining tests revealed that berberine could promote hPDLC growth, increase the secretion of ALP in the culture medium, and enhance the formation of mineralized nodule, thus it is a potential osteoplastic ingredient. This hPDLC/CMC–online-LC/MS method can be applied for screening active components acting on hPDLC from traditional Chinese medicines exemplified by *Coptidis Rhizoma* and will be of great utility in drug discovery using natural medicinal herbs as a source of leading compounds.

1. Introduction

Periodontopathy (periodontitis) is an infectious disease which can lead to the destruction of periodontal tissue, including periodontal ligaments (PDL), cementum, and alveolar bone. In the present day, periodontitis is a main cause of tooth loss in adults and has become a substantial public-health burden worldwide [1,2]. The major goal of periodontal therapy is to reconstruct and maintain healthy periodontium destroyed by periodontopathy. In periodontal tissues, PDL not only has an important role in maintaining and supporting the teeth in situ, but also contributes to teeth nutrition, tissue homoeostasis, and repair of damaged tissues [3,4]. Moreover, PDL contains heterogeneous cell populations named human periodontal ligament cells (hPDLCs) [5], which can differentiate into either cementoblasts or osteoblasts [6,7], form mineralized nodules and express some key osteogenesis factors [8]. Thus, hPDLCs could educe an important efficacy for the regeneration of periodontal tissue [9–11].

Coptidis Rhizoma (the Chinese name is Huanglian), which roots have a variety of isoquinoline alkaloids such as coptisine, berberine, jateorhizine [12,13], already have studies on bone metabolism and oral diseases in foundational and clinical studies [14,15]. But up to now, no study has investigated which component of *Coptidis Rhizoma* plays a role in treating periodontitis and the relationship between bone regeneration and periodontal after intervention with *Coptidis Rhizoma*.

Since the establishment of cell membrane chromatography (CMC), there has been some research about the basic methodology [16,17] and drug–receptor interactions [18–20], and up to now some active components from some TCMs they have been screened using CMC [21–25].

In this study, firstly we established an hPDLC/CMC combined with an HPLC/MS system for screening the active osteoplastic components from *Coptidis Rhizoma*, and an online column-switching technique was used to combine the two systems for the direct recognition–separation–identification of the active components. Finally berberine was the targeted component which could act on the hPDLC specificity. Further the in vitro proliferation and osteoplastic activity tests confirmed that berberine acts on hPDLCs' osteogenesis in a dose-dependent manner. In short, we draw preliminary that berberine is the effective osteogenic

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http://dx.doi.org/10.1016/j.jchromb.2012.07.031

component acting on hPDLC, through drug–receptor interactions for the first time. We believe that these study results can be used to provide a new and better therapeutic drug for the treatment of periodontitis.

2. Experimental

2.1. Chemicals and materials

Coptidis Rhizoma was collected in the Qinba Mountains area (Shaanxi Province, China) and authenticated by the Department of Pharmacognosy, Xi'an Jiaotong University (Xi'an, China). A reference sample has been deposited at the Specimen Laboratory, Research and Engineering Center for Natural Medicine, Xi'an Jiaotong University (Xi'an, China). Silica gel (ZEX-II, 5 μm, 200 Å) was obtained from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China). Simvastatin (SIM) and berberine (BER) were purchased from the National Institutes for Food and Drug Control of China (Beijing, China). hPDLCs were cultured in Research Center, College of Stomatology, Xi'an Jiaotong University. DMEM F12 medium, fetal calf serum, and trypsin were purchased from Sigma (Saint Louis, MO, USA). Collagenase Type I (Worthington Biochem, Freehold, NJ, USA) and ascorbic acid 2-phosphate (WAKO, Tokyo, Japan), glutamine, penicillin, and streptomycin were ordered. HPLC-grade methanol was purchased from Burdick & Jackson (Morris, NJ, USA). Other reagents used were of analytical grade.

2.2. Instruments

The HPLC mass spectrometry system (LC/MS, Shimadzu Corporation, Kyoto, Japan) included three LC-20AD pumps, a DGU-20A3 degasser, a SIL-20A autosampler, a CTO-20A column oven, a SPD-20A UV/VIS detector, a SPD-M20A diode array detector, a LCMS2010EV mass spectrometer, and an LC/MS solution workstation.

A VICIAG 10G-0911V 10-port 2-pos valve (Valco Instrument Co, Inc., Houston, USA) was used as the column switcher and two Shim-pack VP-ODS pre-columns (10 mm × 2.0 mm i.d., 5 μm, Shimadzu Corporation, Kyoto, Japan) were used as the enrichment columns. An hPDLC/CMC column (10 mm × 2.0 mm i.d.) was used as the first-dimension column and a Shimadzu Shim-pack VP-ODS column (150 mm × 2.0 mm i.d., 5 μm, Kyoto, Japan) as the second-dimension column.

2.3. Preparation of standard solutions

Standard stock solutions of SIM and BER (1 mg/mL each) were separately prepared in methanol. Standards solution (0.01 mg/mL) of SIM and BER were prepared in 5 mmol/L ammonium acetate water solutions.

2.4. Sample preparation

As is showed in Fig. 1, 50 g sample from *Coptidis Rhizoma* roots was ultrasonically extracted with methanol. The supernatant were filtered twice, concentrated by rotary evaporation and dryness to yield the total alkaloid extraction. Then dissolved it in water and ultrasonically extracted with petroleum ether, ethyl ether, chloroform, and ethyl acetate by turns thrice to obtain the 5 different solvent part extractions. Sample solutions of all 6 extractions (1 mg/mL) were prepared in methanol and stored at 4 °C in the dark. Working solutions (0.1 or 0.01 mg/mL) were diluted with a mobile phase on the day of the experiment.

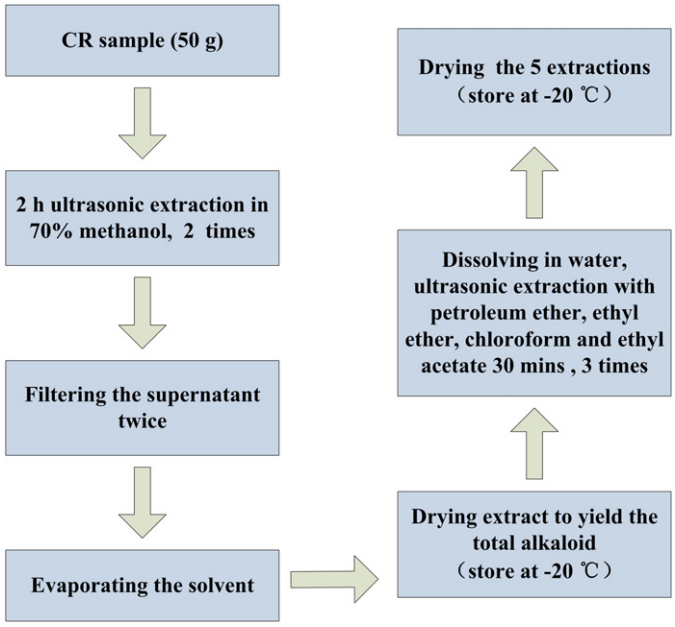

Fig. 1. The preparation of total alkaloid and 5 solvent parts extraction samples of CR.

2.5. Cell culture of hPDLCs and preparation of stationary phase

Extraction premolar teeth ($n = 15$) were collected from six individuals (aged 15–29 years) at the Stomatological Hospital of Xi'an Jiaotong University (Xi'an, China), following approved guidelines set by the National Institutes of Health Office of Human Subjects Research. The PDL was gently separated from the surface of the 1/3 root and then digested in a solution of 3 mg/mL Collagenase Type I for 30 min at 37 °C. The cell suspensions were seeded into dishes with DMEM/F12 supplemented with 15% fetal calf serum, 100 μmol/L ascorbic acid 2-phosphate, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, and then incubated at 37 °C in a humidified atmosphere with 5% carbon dioxide. Cells from exponentially growing cultures (approximately 80% coverage) were used in all experiments. Cells were harvested using 0.25% trypsin and resuspended to a single cell suspension. Cell counting was performed to ensure the amount of cells was no less than 1×10^7. Then the cells were washed three times and a Tris–HCl (50 mM, pH 7.4) hypotonic solution was added to produce a cell suspension. The cells were then ruptured by ultrasonication for 30 min. The resulting homogenate was centrifuged at 1000 × *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 12,000 × *g* for 20 min at 4 °C. The precipitation was suspended in 10 mL Tris–HCl (50 mM, pH 7.4) and the suspension was centrifuged again at 12,000 × *g*. A cell membrane suspension was then obtained by adding 5 mL physiological saline solution to the precipitate and the suspension was reserved.

According to previously reported methods [16], 0.05 g silica was activated at 105 °C for 30 min and used as a carrier. It was then homogenized with the cell membrane suspension, by adding the mixture slowly to it under vacuum and with agitation at 4 °C to obtain the hPDLCs cell membrane stationary phase (CMSP). The CMSP was then packed into the column (10 mm × 2.0 mm i.d.) using a wet-packing procedure to yield the hPDLC/CMC column.

2.6. Chromatography and mass spectrometry conditions

The hPDLC/CMC model (the first dimension): mobile phase (M_1) of 5 mmol/L phosphate buffer with a flow rate of 0.2 mL/min at the column temperature of 37 °C; UV detection, 345 nm.

The HPLC system (the second dimension): with the VP-ODS column (150 mm × 2.0 mm i.d., 5 μm), mobile phase (M_2) of acetonitrile–water–glacial acetic acid–triethylamine (40:60:0.3:0.8, v/v) with a 0.2 mL/min flow rate and column temperature of 37 °C, diode array detector (DAD), 345 nm.

Mass spectrometry (MS) conditions were as follows: nebulizer gas (N_2, purity >99.999%), flow rate 1.5 L/min, drying gas (N_2, purity >99.999%), pressure 0.1 MPa, interface temperature 250 °C, heat block temperature 200 °C, detector voltage 1.5 kV, *m*/*z* scan range, 50–1000 *m*/*z*; scan mode: positive ionization mode.

VP-ODS enrichment columns: The first enrichment column (EC_1) and the second enrichment column (EC_2) were combined by means of an online 10-port column switcher. EC_1 and EC_2 can be alternately in either the first- or the second-dimension system. Alternation of enrichment and elution can be achieved by this parallel connection of two enrichment columns, which enables the online enrichment of the eluent from the first dimension.

2.7. Application of hPDLC/cell membrane chromatography–online-HPLC/MS

The hPDLC/CMC model was combined with the HPLC/MS system by means of an online 10-port column switcher. A 1 μL injection was performed after 2 h of chromatographic system balance. Any retention fraction which was "recognized" on the first-dimension hPDLC/CMC model was enriched onto an ODS enrichment column (EC_1 or EC_2), and the enriched fraction was then alternately eluted into the second-dimension HPLC/MS system for "separation and identification." This hPDLC/CMC–online-HPLC/MS method was used to screen active ingredients using SIM as a positive control. Standard solutions and total alkaloids of *Coptidis Rhizoma* were analyzed separately.

2.8. Competitive displacement assay

To confirm that SIM and BER (the active compound screened from *Coptidis Rhizoma*) were both active on the same site of the hPDLC cell membrane receptor, we performed the competitive displacement test [26]. BER was injected into hPDLC/CMC with SIM of gradient concentration dissolved in mobile phase (0, 1×10^{-7}, 2×10^{-7}, 4×10^{-7}, 8×10^{-7}, and 16×10^{-7} M). Phosphate buffers (5 mmol/L) were used as a dissolvent of the mobile phase. Other chromatographic condition parameters were the same as in Section 2.6. The variation of retention time was recorded for analysis.

2.9. Cell growth assay

The effects of SIM and BER on hPDLC viability and proliferation were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures the metabolic reduction of MTT to formazan [27,28]. Cells were treated with SIM and BER for 1, 3, 5, 7, and 9 days then the absorbance values were determined using a microplate reader (Bio-RAD instruments, USA) at 490 nm.

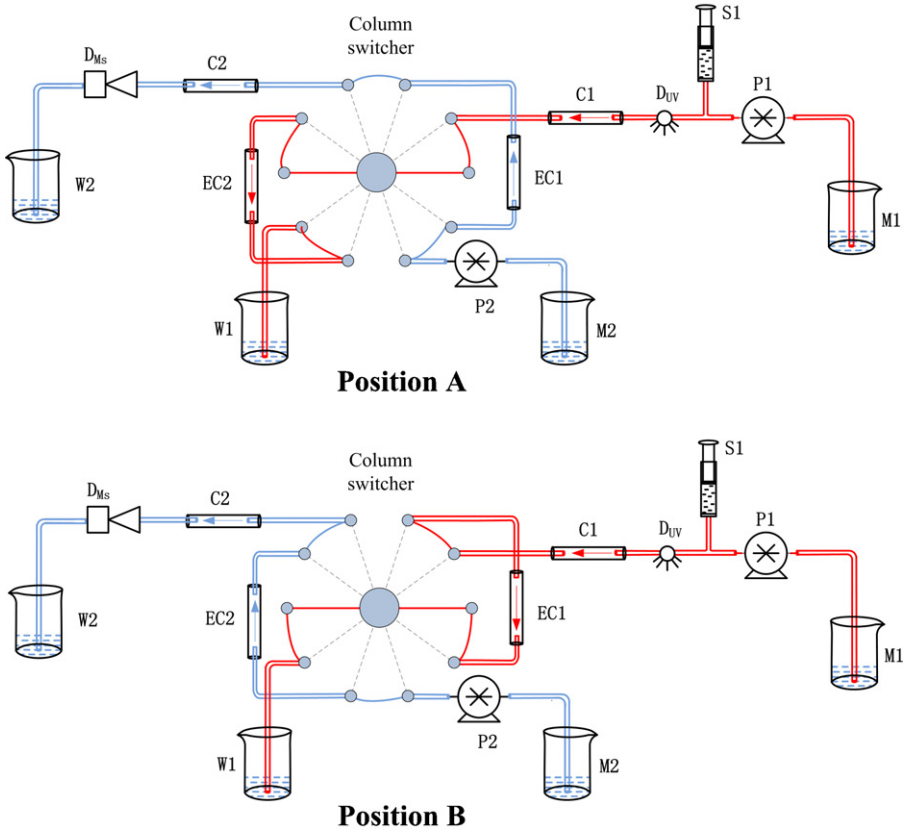

Fig. 2. Brief scheme of the hPDLC/CMC–online-HPLC/MS method. Position A: Affinity-recognition procedure using the hPDLC/CMC model with the first retention fraction enriched onto an ODS pre-column (EC_1) after the hPDLC/CMC column (C_1) from a complex sample (S_1); Establishing the equilibrium procedure of the HPLC/MS system from another ODS pre-column (EC_2) to an analytical column (C_2). Position B: Analytical identification procedure using the HPLC/MS system with the first extracted fraction analyzed using C_{ODS}-HPLC/MS to identify their chemical structures; the second retention fraction (if there was one) was enriched onto another extraction column (EC_2) after the hPDLC/CMC column (C_1) from the same sample (S_1). *Note*: D_{UV}: ultra-violet detector, D_{MS}: mass spectrographic detector, P_1 and P_2: pumps.

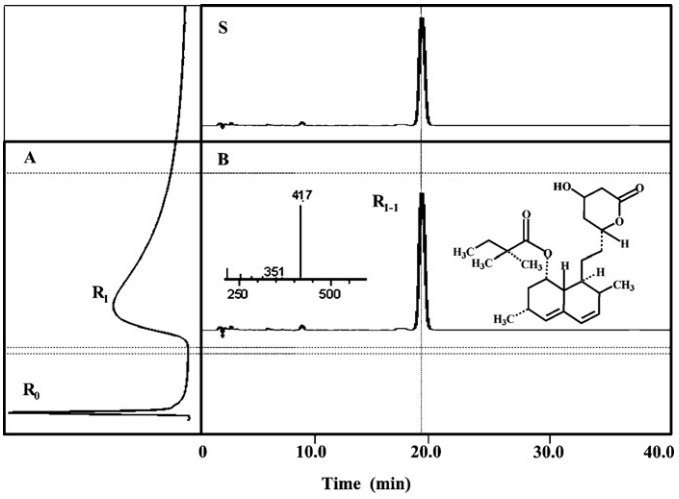

Fig. 3. Chromatograms of SIM using the hPDLC/CMC–online-HPLC/MS method. (A) hPDLC/CMC chromatogram of SIM; (B) HPLC/MS chromatogram of the corresponding fraction (between two dotted lines in hPDLC/CMC chromatogram) extracted onto the EC_1, and identified as SIM; (S) HPLC/MS chromatogram of SIM standard solution.

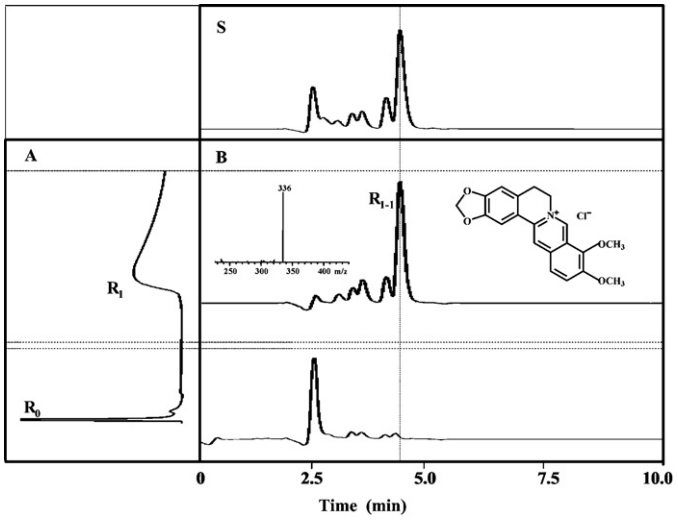

Fig. 4. Chromatograms of CRAE using hPDLC/CMC–online-HPLC/MS method (A) hPDLC/CMC chromatogram of CRAE including R_0 and R_1 fractions (between two dotted lines); (B) HPLC/MS chromatogram of R_1 fraction with a main retention peak ($R_{1\text{-}1}$) identified as BER; (S) HPLC/MS chromatogram of CRAE.

2.10. Medium ALP activity

Medium ALP activity was measured treated with different concentrations of SIM and BER at day 3, 5, 7, and 9 after cell seeding. Media were collected for the measurement of the secreted ALP. The supernatant was preserved at −20 °C until analysis. The ALP activity was measured by absorbance at 520 nm using *p*-nitrophenyl phosphate as the substrate as previously described [28,29].

2.11. Cell matrix ALP staining

To confirm the induction and activity of ALP enzyme in hPDLCs, ALP activity on the cell layers was measured using ALP staining. Briefly, cultured cells were rinsed with PBS and fixed in 70% ethanol. The cells were stained using Naphthol As-Mx phosphate disodium salt as the substrate, N,N-dimethyl formamide and hematoxylin as dye for 3 min at 37 °C. After washing with PBS, the cells were photographed. The products of ALP activity were stained for black mineralization tubercles as an indicator of bone nodule area.

2.12. Statistical analyses

Statistical analysis was performed using software SPSS13.0. Data were analyzed using 1-way analysis of variance (ANOVA). The probability level (P) at which differences were considered significant was at $P < 0.05$. Absorbance values for cell proliferation from the MTT assay are presented as the mean ± SE.

3. Results and discussion

3.1. Combined hPDLC/CMC–online-HPLC/MS

The hPDLC/CMC system was combined with online HPLC/MS analysis by the use of a column-switching device. Thus, the method developed in this study was suitable for qualitative analysis of active components from the complex samples. As shown in Fig. 2, at position A, the first retention fraction recognized in the hPDLC/CMC model was extracted onto an ODS pre-column (EC_1), and then at position B, the extracted components were pumped into an ODS analytical column (C_{ODS}) for qualitative analysis. At the same time, the second retention fraction was pumped into another ODS pre-column (EC_2) and into the C_{ODS} for analysis, alternately. C_{18} VP-ODS pre-column has better retentive ability than the hPDLC/CMC column. Therefore, the eluted fraction from the hPDLC/CMC system could be well reserved when it was used as the enrichment column in the first dimension. The retention fraction was easy to wash out and pump into the analytical column for analysis in the second dimension. In this alternative mode, online enrichment of retentive components could be achieved and the sensibility was improved correspondingly.

SIM standard solution was used to verify the suitability and reliability of the hPDLC/CMC–online-HPLC/MS system for "recognizing" and "identifying" target components from *Coptidis Rhizoma*. As shown in Fig. 3, SIM was specifically retained by the hPDLC/CMC model and the retention time was 12 min (Fig. 3A), the selected fraction R_1 (between the two dotted lines) was extracted onto EC_1, and then switched into C_2 with a 10-port valve for chromatographic separation and MS identification

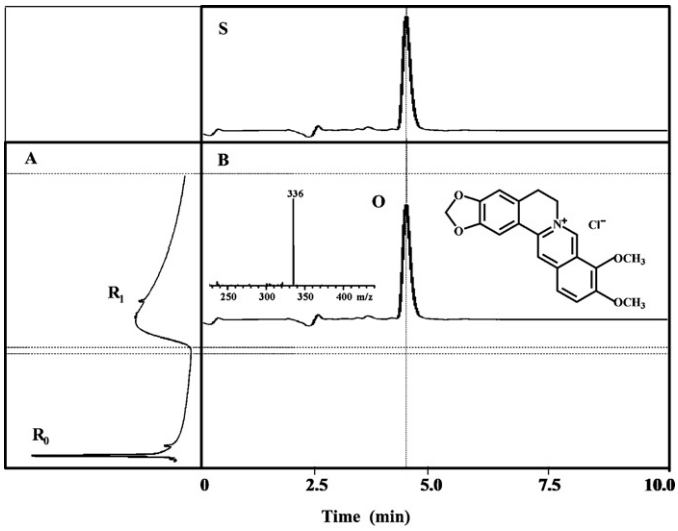

Fig. 5. Chromatograms of the BER standard solution using an hPDLC/CMC–online-HPLC/MS method. (A) hPDLC/CMC chromatogram of the BER standard solution including R_1 fraction (between two dotted lines); (B) HPLC/MS chromatogram of R_1 fraction with solely one retention peak (O) identified as BER; (S) HPLC/MS chromatogram of the BER standard solution.

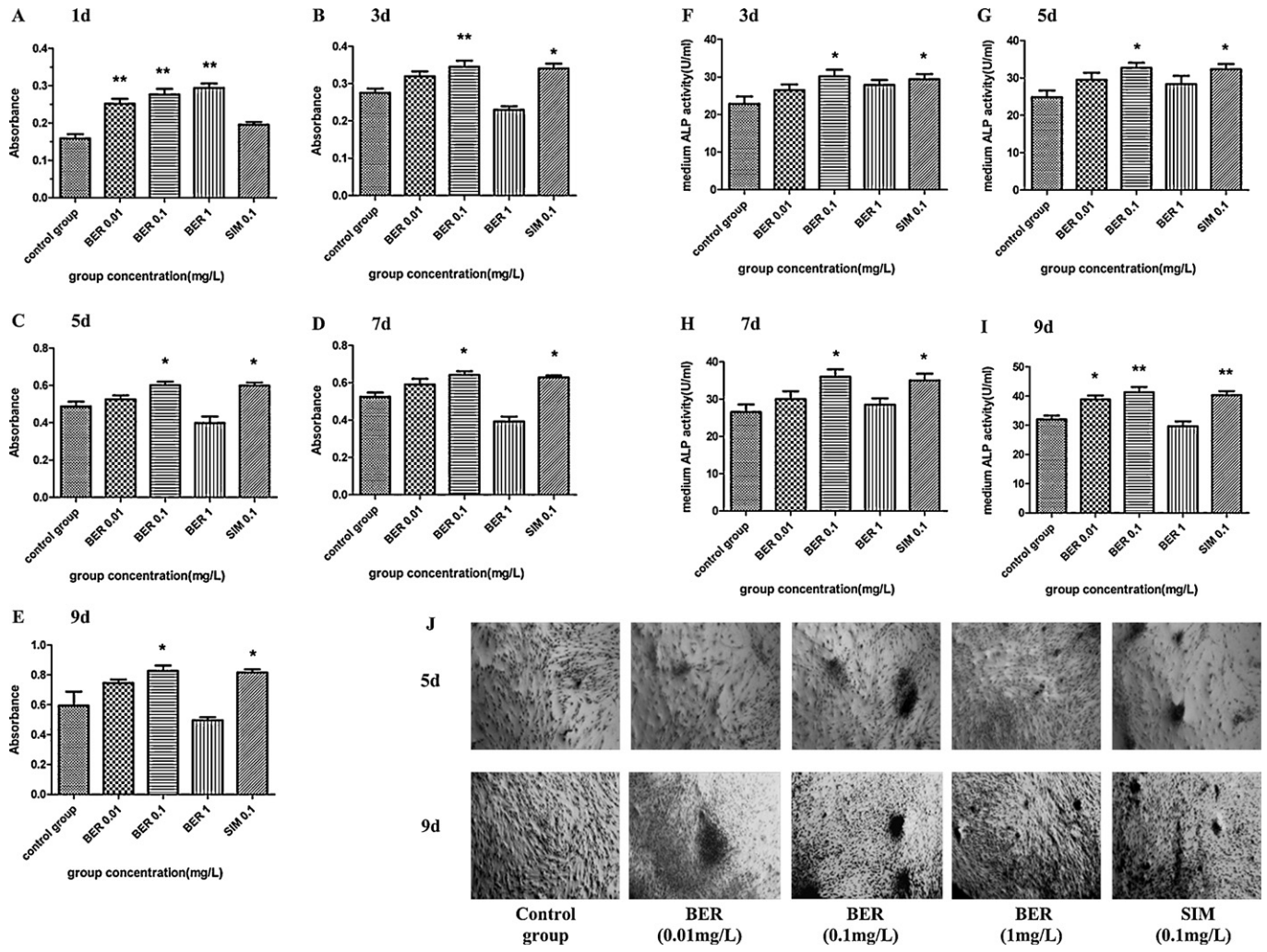

Fig. 6. Effect of BER and SIM on the proliferation, ALP activity and staining in hPDLCs (A–E) The proliferation of hPDLCs by BER and SIM in the whole period (1, 3, 5, 7, and 9 days) was determined using the MTT assay, with the DMEM/F12 with 10% FBS used as the negative control group. hPDLC proliferation increased by BER and SIM at lower concentration (0.1 mg/L); (F–I) secreted medium ALP activity was increased by the BER treatment within the range of 0.01–0.1 mg/L and 0.1 mg/L SIM during the period (3, 5, 7, and 9 days), as compared to control group. Data represent mean ± SE and statistical comparisons were made using a 1-way ANOVA followed by Tukey's test as a post hoc comparison. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group; (J) the effect of BER on extracellular matrix ALP activity was measured by staining the products of enzyme activity and presented as the image of the cellular layer on phase-contrast microscopic morphology. For the cell layer ALP staining, the cells were cultured in 12-well plates and incubated in As-Mx phosphate solution as substrate with hematoxylin as stain. Matrix ALP activity shown that there were some mineralization tubercles in 0.1 mg/L BER and SIM at 5 and 9 days.

(Fig. 3B). Data from the second-dimensional chromatography and mass spectrometry confirmed that R_1 was SIM. Therefore, SIM could be recognized, captured, and identified by this online-coupled method. From this, we could draw the conclusion that the hPDLC/CMC–online-HPLC/MS system established by the primary cultured hPDLCs could be used for recognition, analysis, and identification of active components from TCMs acting on the hPDLC receptor.

3.2. Practical application of the hPDLC/CMC-HPLC/MS system (screening target components from Coptidis Rhizoma)

The hPDLC/CMC-HPLC/MS system was applied for screening the active component from TCM *Coptidis Rhizoma*. The total alkaloid and five different solvent extracts were studied on the hPDLC/CMC system. The total alkaloid (TACR) and *Coptidis Rhizoma* aqueous extract (CRAE) were significantly retained on hPDLC/CMC. *Coptidis Rhizoma* petroleum ether extract (CRPEE) and ethyl ether extract (CREEE) had slight retention, and chloroform and ethyl acetate extracts (CRCE and CREAE) had no retention. These results indicated that CR was able to interact with the cell membrane receptor of hPDLC, and the chromatographic retentions were associated with the component difference among the different extract parts as well. Because an apparent retention of CRAE, we choosed it as the sample in the next part of the experiment.

Chromatograms of CRAE obtained using the hPDLC/CMC–online-HPLC/MS method are shown in Fig. 4, in which R_0 was the non-retentive fraction and R_1 was the retentive fraction on hPDLC/CMC (Fig. 4A). Each fraction was assessed by capturing and switching on the HPLC/MS system online for further separation and identification, as shown in Fig. 4B. From UV and MS data, the peak $R_{1\text{-}1}$, a main component of the R_1 fraction, was identified as BER. Comparison with Fig. 4S indicated that BER was indeed present in the total CRAE preparation, and was the major retained component from it at the same conditions. Peak $R_{1\text{-}1}$ was assigned to BER in the chromatography of CRAE. The chemical structures of BER are shown in Fig. 4B. Accordingly, we drew the conclusion that this hPDLC/CMC–online-HPLC/MS system could be used to screen compounds samples that had complicated constitutions.

In order to further verify the screening results above, the standard solutions of BER were analyzed using this hPDLC/CMC–online-HPLC/MS method. As shown in Fig. 5, the main retention fractions on hPDLC/CMC model were BER (Fig. 5A, B, and S). Through this system, we could consider that CRAE is the active part which interacted with the hPDLC receptor and BER is the active ingredient in it, but determining its efficacy would require further research.

3.3. Interactions between BER and cell membrane receptors

The interaction between BER and cell membrane receptors was quantified by competitive displacement experiments. As the concentration of SIM in the mobile phase increased (from 0 to 16×10^{-7} M), the retention time of BER decreased. It could be inferred that the more the active points of hPDLC cell membrane receptor on the solid phase were occupied by SIM in the mobile phase, the weaker the binding between BER and receptor would become. This variation tendency of retention characteristic suggested that BER acted on the same site as positive medicine SIM on the hPDLCs receptor, both of which could have parallel effects for hPDLCs.

3.4. The in vitro promotion activity of BER and SIM on hPDLCs

As shown in Fig. 6A–E, both BER and SIM had promoted hPDLC growth. The retention time of BER in hPDLC/CMC was about 35 min, compared with 10 min of SIM, which indicated a stronger interaction of BER with hPDLCs. This illustrates that BER screened from CRAE could promote the proliferative viability of hPDLCs in a way which is similar to SIM in the appropriate concentration rage, and the retention behaviors of the two were correlated to their pharmacological activities.

3.5. The medium (secreted) ALP activity on hPDLCs

ALP is the most widely recognized biochemical marker for osteoblastic activity, and also has a stimulatory effect for matrix calcification in bone tissue [29,30]. From Fig. 6F–I, the medium ALP activity was increased by BER within the concentration of 0.01–0.1 mg/L and by SIM at a concentration of 0.1 mg/L, as compared to negative controls. The findings supported the hypothesis that the active ingredient BER could increase the osteogenic effect in hPDLCs and these stimulated osteogenic effects are mediated by increasing activity of ALP in matrix.

3.6. The extracellular matrix ALP activity

In contrast to the control, BER in the concentration of 0.1 mg/L could increase the matrix mineralization in hPDLCs from Fig. 6J. Therefore, the increased ALP activity in the extracellular matrix was also confirmed from ALP staining, which indicates stimulation of matrix maturation and mineralization of BER.

As reported [31,32], berberine, the major medically important isoquinoline alkaloid in *Coptidis Rhizoma*, could affect bone metabolism, inhibit osteoclast formation, and promote osteoblast differentiation through different pathways. We also screened berberine from the CRAE by our online-coupled screening system as the osteoplastic active constituents on our hPDLCs, which was supported by the result of in vitro bone formation test. Findings from this study show that berberine, within the concentration range of 0.01–0.1 mg/L, increased hPDLCs proliferation and matrix mineralization in bone formation.

4. Conclusion

In brief, we have developed an hPDLC/CMC–online-HPLC/MS method for screening the osteoplastic active part and ingredient from complex mixtures of traditional Chinese medicine *Coptidis Rhizoma*. Next, we considered using the active part and ingredient to treat the periodontitis directly, which provides the same or better efficiency as *Coptidis Rhizoma*, but more conveniently. The specific bone metabolism mechanism between the active part and ingredient and hPDLCs will be our future research direction. This method can efficiently drive the screening process by combining specific recognition via the hPDLC/CMC model with accurate identification via the HPLC/MS online system. It will be a useful method in drug discovery with natural medicinal herbs as the leading compounds.

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant number 81173311) and Shaanxi Province "13115" Science and Technology Innovation Engineering Major Projects (Grant number 2009ZDKG-77).

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