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# Osteoblastic differentiation bioassay and its application to investigating the activity of fractions and compounds from *Psoralea corylifolia* L.

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A cell differentiation bioassay measuring alkaline phosphatase activity was developed using osteoblast-like UMR 106 cell line as a model. The effect of fractions and compounds of *Psoralea corylifolia* L. extract on osteoblastic differentiation was investigated. The fractions or compounds were co-cultured with cells for 48 h, the cellular ALP activity was then measured. The crude ethanol extract of *Psoralea corylifolia* L. increased ALP activity by 39.5% at a concentration of 0.1 mg/ml, and the activity was concentrated in the ethyl acetate fraction, which produced a maximum ALP activity increase of 38.2%. A significant activity was observed for a flavonoid, corylin, providing 30.0% increment in ALP. These effects suggest that *Psoralea corylifolia* L. extract, its ethyl acetate fraction and corylin would stimulate bone formation.

## 1. Introduction

Osteoblasts play an important role in bone growth and remodeling. Agents stimulating osteoblast activity may be potential drugs for osteoporosis and/or other bone diseases. The osteoblast-like UMR 106 cell line has been used widely as an osteoblast model [1, 2]. In our previous studies [3–6], a proliferation assay using osteoblast-like UMR 106 cells was developed and employed in screening medicinal herbs from traditional Chinese medicine and other plants and their active constituents stimulating bone formation. However, it is well known that the maturation of osteoblasts involves two phases, proliferation and differentiation. It is therefore worth developing a bioassay to investigate the effect on osteoblastic differentiation. During differentiation of osteoblasts, alkaline phosphatase (ALP) is expressed and secreted. ALP plays an important role in bone formation, particularly in bone mineralization. The ALP level reflects the status of osteoblastic differentiation [7]. It is therefore selected as the marker for evaluating the differentiation of osteoblast-like UMR 106 cells.

Non-polar fractions of the acetone extract of *Psoralea corylifolia* L. seeds were reported to elevate serum inorganic phosphorous levels and improve bone calcification in rats [8]. However, *Psoralea corylifolia* L. is traditionally used as a decoction in water or alcohol. It is therefore more important to investigate the activity of its alcohol or aqueous extract. In a study of screening potential anti-osteoporostic agents from medicinal herbs and other plants, an alcohol extract of *Psoralea corylifolia* L. significautly stimulated osteoblastic proliferation. This activity of its two flavonoids has been investigated in detail [6]. However, it is not known whether *Psoralea corylifolia* L. extract and its fractions or components are effective in promotion of osteoblastic differentiation. The aim of the present study was to develop a method for investigating the effects of herbal medicines on osteoblastic differentiation using the osteoblast-like UMR 106 cell line, to evaluate the osteoblatic activity on stimulating differentiation produced by various fractions and components of alcohol extracts of *Psoralea corylifilia* L. fruits.

## 2. Investigations and results

### *2.1. Optimization of differentiation assay*

During method development, conditions including cell density in the culture medium, exposure time, organic solvents used in the preparation of samples and selection of positive control were investigated. A linear range up to an ALP activity of 1000 U/l was established in this assay.

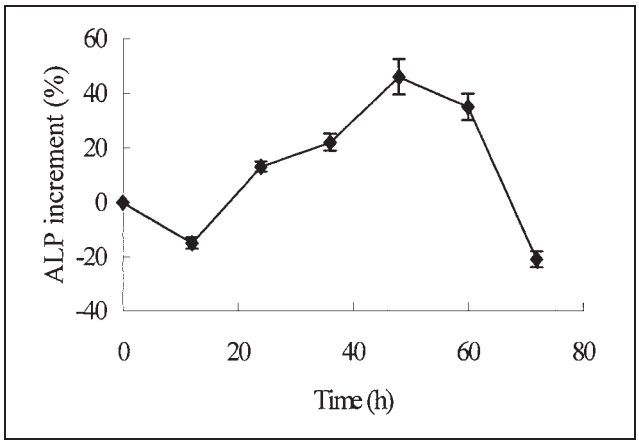


Fig.: Effect of exposure time to *Psoralea corylifolia* extract on ALP activity of osteoblast-like UMR 106 cells (n = 4)

In the cell density range of $2-10 \times 10^4$ cells/ml tested, the density of $6-8 \times 10^4$ cells/ml was found optimal for all assays. As shown in the Fig., the elevation of cell ALP activity started at 24 h after co-cultured with the extract, reached a maximum at 48 h and declined rapidly after 60 h. Therefore the assay was carried out by culturing the cells in a medium containing extract, fractions or compounds for 48 h.

Since ethanol or dimethyl sulfoxide (DMSO) may be employed in dissolving fractions or compounds, the effect of ethanol and DMSO on cell differentiation was examined. No obvious interference was observed at concentrations up to 0.75% of ethanol and 1.0% of DMSO. The final concentration of ethanol and DMSO in culture medium was not higher than 0.75% and 1.0% respectively, and blank controls were set up containing the same concentration of organic solvent.

The reproducibility of this method was investigated. The RSD of the ALP activity of cells co-cultured with MEM for 48 h was 10.1% ($n = 5$) and that of the ALP increment with 1.0 μM dexamethasone was 10.3% for assay at 5 separate days.

### 2.2. Selection of positive control

Some agents were tested in order to choose a positive control in this assay. Sodium fluoride (NaF) and calcitonin increase bone formation and bone cell proliferation *in vitro* [5, 6, 9], but they do not promote the differentiation of UMR 106 cells. Dexamethasone was reported to elevate the ALP of osteoblast-like cells [10]. In this *in vitro* model, it showed some effect on cell differentiation in the concentration range of 0.1–100 μM. Its optimal concentration in culture medium was 1.0 μM. Significant ALP promotion (ALP increment of 42.9%, $n = 4$) was observed from dexamethasone after being exposed to UMR 106 cells for 48 h. Dexamethasone (1.0 μM) was therefore used as the positive control in this *in vitro* model of osteoblastic differentiation.

### 2.3. Identification of the isolated compounds

The isolation, purification and identification of two flavonoid compounds, corylin and bavachin, from *Psoralea corylifolia* L. were reported previously [6]. Isopsoralen (needle crystalls) was isolated from sub-fraction 2 and purified by recrystalization in hot methanol. It was identified by m.p. (137–139 °C), co-TLC with reference compound, $^1$H NMR and the $^{13}$C NMR spectra in comparison with literature data [11]. Psoralen (needle crystalls) was isolated from sub-fraction 3 and purified by recrystallization in chloroform-cyclohexane after washed with methanol. It was identified by m.p. (161–163 °C), co-TLC with reference compound, the $^1$H NMR and $^{13}$C NMR spectra in comparison with literature data [11].

### 2.4. Effect of fractions and compounds on osteoblastic differentiation

The effect of *Psoralea corylifolia* L. fruit on the osteoblastic differentiation of UMR 106 cells was first examined in its crude ethanol extract. When the cells were co-cultured with the extract at a concentration of 0.1 mg/ml (expressed in weight of raw herbal material per ml), the ALP activity was significantly ($P < 0.05$) increased. The stimulating effect (an average ALP increment of 39.5%) was similar to that of dexamethasone (42.9%), a positive control, at a concentration of 1.0 μM. Based on these results and those previously published [6], it can be concluded that *Psoralea corylifolia* L. fruit has an osteoblastic stimulating activity in both proliferation and differentiation.

**Table 1: The effect of three fractions partitioned from *Psoralea corylifolia* (PC) ethanol extract on UMR 106 cell differentiation**

| Samples | Concentration (mg/ml) | ALP activity (U/l) | ALP increment (%) |
|---|---|---|---|
| Blank | 0 | 105.28 ± 28.17 | |
| Ether fraction | $1 \times 10^{-3}$ | 106.75 ± 16.25 | 1.4 |
| | $1 \times 10^{-2}$ | 109.28 ± 20.92 | 3.8 |
| | $1 \times 10^{-1}$ | 122.02 ± 24.84 | 15.9 |
| Dexamethason | $5 \times 10^{-4}$ | 142.97 ± 14.32* | 35.8* |
| Blank | 0 | 124.12 ± 33.55 | |
| EtOAc fraction | $1 \times 10^{-3}$ | 145.60 ± 19.08 | 17.3 |
| | $1 \times 10^{-2}$ | 171.53 ± 28.23* | 38.2* |
| | $1 \times 10^{-1}$ | 119.23 ± 32.50 | – |
| Dexamethason | $5 \times 10^{-4}$ | 175.01 ± 26.34* | 41.0* |
| Blank | 0 | 118.31 ± 30.71 | |
| BuOH fraction | $1 \times 10^{-3}$ | 129.07 ± 18.29 | 9.1 |
| | $1 \times 10^{-2}$ | 128.13 ± 23.07 | 8.3 |
| | $1 \times 10^{-1}$ | 138.66 ± 16.57 | 17.2 |
| Dexamethason | $5 \times 10^{-4}$ | 168.24 ± 20.86* | 42.2* |

The activity is expressed as mean ± standard deviation ($n = 4$), * $P < 0.05$, significant as compared to blank by t-test.

The crude extract was partitioned successively with petroleum ether, ethyl acetate and *n*-butanol. The yielded fractions were assayed for activities promoting cell differentiation. The results indicate that the activities were concentrated in the ethyl acetate fraction, which produced an ALP activity increase of 38.2% at a concentration of 0.01 mg/ml. No significant effects were observed in either the petroleum ether or the *n*-butanol fraction at the tested concentrations (Table 1). The residual aqueous fraction was also tested and showed no activity at any concentration level.

Eight sub-fractions were tested in the cell-culture assay. Sub-fractions were assayed for ALP activity increment in concentration range of 0.1–10 μg/ml. Sub-fractions 2 and

**Table 2: The effect of compounds from the ethyl acetate fraction of *Psoralea corylifolia* (PC) ethanol extract on UMR 106 cell differentiation**

| Compounds | Concentration (μM) | ALP (U/l) | Increment (%) |
|---|---|---|---|
| Blank | 0 | 131.04 ± 21.14 | |
| Isopsorlean | 0.54 | 148.86 ± 13.90 | 13.6 |
| (from sub-fr.2) | 5.4 | 132.72 ± 30.56 | – |
| | 54 | 114.08 ± 12.98 | – |
| Dexamethasone | 1.0 | 181.32 ± 34.08* | 38.4* |
| Blank | 0 | 120.76 ± 11.70 | |
| Psorlean | 0.54 | 140.32 ± 24.82 | 16.2 |
| (from sub-fr.3) | 5.4 | 127.64 ± 25.65 | 5.7 |
| | 54 | 106.79 ± 32.64 | – |
| Dexamethasone | 1.0 | 164.11 ± 38.34* | 35.9* |
| Blank | 0 | 102.85 ± 18.36 | |
| Corylin | 0.3 | 114.46 ± 19.38 | 11.3 |
| (from sub-fr.5) | 3 | 126.17 ± 23.22 | 22.7 |
| | 30 | 133.55 ± 13.37* | 30.0* |
| Dexamethasone | 1.0 | 139.90 ± 20.31* | 36.0* |
| Blank | 0 | 120.31 ± 19.60 | |
| Bavachin | 0.3 | 157.02 ± 30.95 | 20.5 |
| (from sub-fr.7) | 3 | 132.10 ± 31.57 | 9.8 |
| | 30 | 122.23 ± 33.90 | 1.6 |
| Dexamethasone | 1.0 | 162.54 ± 32.47* | 35.1* |

* $P < 0.05$, significant as compared to blank by t-test.

3 produced significant increment in cellular ALP activity. Isolated compounds at concentrations of 0.3–54 μM were assayed with UMR 106 cells, the results are listed in Table 2. Psorlean and isopsorlean, the two major coumarins in *Psoralea corylifolia* L., produced slight stimulation of ALP activity at lower concentrations, although sub-fractions 2 and 3 from which the compounds were isolated showed significant effects. The flavonoid, corylin and bavachin exhibited stronger activity, particularly, corylin produced significant osteoblastic differentiation stimulation.

## 3. Discussion

Although many *in vitro* assays have been developed for screening active agents against osteoporosis and for studying the mechanism of its occurrence and mediation, a bioassay using cell lines is the most convenient method. It provides advantages of high sensitivity, high speed, high reproducibility, low dose and low cost. UMR 106 cell line is a useful model in studying the mechanism of antiosteoporostic drugs on osteoblasts [12, 13]. It has been successfully used in cell proliferation assay [3–6, 14]. In order to further study the two phases of osteoblastic, proliferation and differentiation, a cell differentiation assay was developed.

During osteoblastic differentiation, various special proteins such as osteocalcin and alkaline phosphatase are expressed and secreted into the bone matrix. Alkaline phosphotase is a key enzyme for calcification of the bone. The cellular ALP value is a measure of the differentiation of osteoblastic cells and an increase in ALP levels by agents such as herbal medicines or their components would indicate positive effects on osteoblastic differentiation. ALP activity is an index of cell differentiation of bone formation [7, 15] and is therefore used in this assay. With this test system and the proliferation assay, agents stimulating both osteoblastic differentiation and proliferation may be found. Such agents would be much more beneficial to bone formation and osteoporosis than those active in cell proliferation only.

Previous studies have shown that dexamethasone characterized by increasing receptor contents of 1,25-dihydroxyvitamine $D_3$ in culture rat osteoblast-like cells [16] is an important early differentiation marker of osteoblasts *in vivo*, and of osteoblasts and also osteoblast-like cells in culture [17]. It has been reported to increase ALP levels in the osteoblastic osteosarcoma cell line ROS 17/2.8 [10] and in rat osteogenesis of osteoblasts [18]. In this study, dexamethasone showed a maximum activity at a concentration of 1.0 μM, therefore dexamethasone of 1.0 μM in MEM was used as the positive control.

By means of the bioassay developed in this study, fractions and some components isolated from a *Psoralea corylifolia* L. alcohol extract were investigated for their effects on osteoblastic differentiation. It was found that the alcohol extract significautly promoted UMR 106 cell differentiation. When the alcohol extract was partitioned using solvents of various polarities, osteoblastic stimulation activity was mainly distributed in the ethyl acetate fraction. As regards to the effect of single components, it was observed that two coumarines, psorlean and isopsorlean, had insignificant effects on differentiation of UMR 106 cells. This suggests that the two coumarines would not be active principles in stimulating bone formation, although an anti-osteoporosis effect was reported to be produced by total coumarines of *Cnidium monnieri* (L.) Cuss, another commonly used traditional Chinese medicinal plant [19, 20]. However, flavonoid compounds of *Psoralea corylifolia* L., particularly corylin, significantly increased ALP activity of UMR 106 cells at concentration of 30 μmol/L. The results of this study together with those in our previous report [6], indicated that the ethanol extract of *Psoralea corylifolia* L., its further separated ethyl acetate fraction and corylin stimulates not only proliferation but also differentiation of UMR 106 cells. This suggests an osteoblastic promotion effect in bone formation.

In conclusion, for the first time an *in vitro* bioassay for determining effect of agents on cellular ALP activity was developed using osteoblast-like UMR 106 cell line. This method was successfully employed in the activity investigation of the ethanol extract of *Psoralea corylifolia* L., its fractions and compounds. It was found that the ethanol extracts, its ethyl acetate fraction and a flavonoid compound, corylin, promoted osteoblastic differentiation significantly. The combination of osteoblastic differentiation assay with proliferation assay would provide a more reliable test system for screening active agents promoting bone formation and anti-osteoporosis.

## 4. Experimental

### *4.1. Cell culture materials and apparatus*

An osteoblast-like UMR 106 cell line was employed as the *in vitro* bioassay model. The cells of origin from the Massachusetts General Hospital (Boston, MA, USA) were obtained from Beijing Medical University (Beijing, China). Minimum essential medium (MEM) from Gibco (Grand Island, NY, USA) and fetal calf serum (FCS) from Tianjin Chuanyie Bio-engineering Product CO. (Tianjin, China) were used in the bioassay. NP40 (nonylphenoxy-polyethoxy-ethanol) was provided by Fluka (USA) and ALP kits by Beijing Zhong Sheng Bio-med Co. Ltd (Beijing, China). The cell culture plates were provided by Nunc (Denmark).

A homoeothermic culture box (Heraeus, Germany) and an invert microscope (Olympus, Japan) were used during the cell culture. An ToshiBA AerosET autobioanalizer (Abbott, USA) was used to determine the cellar ALP activity.

### *4.2. Plant materials and reagents*

Raw materials of the fruit of *Psoralea corylifolia* L. was purchased from Tianyitang Chinese drug store (Shenyang, China) and identified by Sun Q, Professor of Pharmacognosy, Shenyang Pharmaceutical University (China). Ethanol, petroleum ether, ethyl acetate, *n*-butanol and other chemicals of analytical grade were from Shenyang Chemical Reagent Factory (China).

### *4.3. Preparation of fractions and compounds*

The extraction and fractionation procedure was described previously [6]. Briefly, dry powdered raw material was refluxed 3 times for 2 h with 95% ethanol. The crude extract was successively fractionated with petroleum ether, ethyl acetate and *n*-butanol. The yielded fractions were assayed for the activity on stimulating osteoblastic differentiation. Only the active fraction (ethyl acetate fraction in this case) was further subjected to column chromatography eluted with petroleum ether-ethyl acetate gradient. Eluates with the same constitutes indicated by $GF_{254}$ TLC were combined as one sub-fraction. Pure compounds were further isolated from sub-fractions exhibiting significant activity.

### *4.4. Cell culture and differentiation assay*

UMR 106 cells were cultured with MEM containing 100 U penicillin, 100 μg streptomycin supplemented with 7% heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$.

Alkaline phosphatase (ALP) induction was assayed as follows. UMR 106 cells were seeded in 24-well plate at a density of $6–8 \times 10^4$ cells/ml (500 μl per well) and cultured for 24 h. The cells were first washed with phosphate buffered saline (PBS) (pH 7.4) and incubated overnight in fresh serum-free MEM medium. The UMR 106 cells were exposed to various concentrations of fractions and compounds of *Psoralea corylifolia* L. extract in serum-free medium. The medium was changed daily and the cells were harvested for ALP activity at 48 h after exposure. At the end of the culture period, the medium was removed and the cells were rinsed 3 times with ice-cold PBS (pH 7.4) and extracted with a solution of 500 μl

0.5% NP40 in 50 mmol/L tris-HCl (pH 7.4). The cells were allowed to bath on ice for 20 min and store at −70 °C for measurement. The supernatant of thawed cell suspensions was used for the determination of enzyme activity with ALP kits by measuring the release of *p*-nitrophenol from *p*-nitrophenol phosphate using an autobioanalizer at 405 nm. Dexamethasone was used as the positive control.

### *4.5. Statistics*

All ALP data were expressed as mean ± standard deviation from 4 wells in cell plate. Differences between pairs of means were statistically analyzed using Student's t-test. The P value less than 0.05 was considered as significant. ALP increment$\% = 100 \times (A_{sample} - A_{blank})/A_{blank}$, where A is the average of ALP activity in four wells.

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