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Coumarins from *Cnidium monnieri* (L.) and their proliferation stimulating activity on osteoblast-like UMR106 cells

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The proliferation of various fractions of alcoholic extracts from fruits of *Cnidium monnieri* (L.) Cuss (umbelifera) were screened using the osteoblast-like UMR106 cells *in vitro*. The chloroform fraction from the crude extract was found to have the most stimulating activity. Three coumarins (osthole, bergapten and imperatorin) were isolated from this fraction by activity-guided assay, and their effects on osteoblastic proliferation were investigated. Osthole, a major pharmacologically active constituent, significantly promoted the cells' activity. Bergapten and imperatorin were less effective than osthole. These results suggested *Cnidium monnieri* (L.) Cuss extracts might have potential activity against osteoporosis, and its chloroform fraction might contain active constituents stimulating osteoblasts.

1. Introduction

The fruits of *Cnidium monnieri* (L.) Cuss (umbelifera) are an important drug in traditional Chinese medicine for the treatment of itchy skin, rashes, eczema and ringworm, typically in the genital area (Chinese Pharmacopoeia 2000). Chemical studies showed that coumarins were the main constitutes in the fruits (Cai 2000; Xiang 1984). Recently, pharmacological studies also showed that it had potential activity against osteoporosis. The crude extracts from fruits of *Cnidium monnieri* (L.) Cuss (total coumarins) inhibited the high bone turnover and reversed the bone loss at early monopause in ovariectomized rats (Li 1994). And the total coumarins could increased the femoral bone density in prednisolone-treated rats (Xie 1994). Additional studies indicated that the total coumarins not only could reverse prednisone-induce bone mass loss, but also increase the anti-torsional strength of femurs in rats (Liao 1997).

However, it is not clear whether the fruits of *Cnidium monnieri* (L.) Cuss affect osteoblast proliferation and what the active constituents responsible for activity against osteoporosis. In this study, the alcoholic extracts from fruits of *Cnidium monnieri* (L.) Cuss and its various fractions on the proliferation of osteoblast-like UMR106 cells were screened *in vitro*. Three coumarins (osthole, bergapten and imperatorin) were isolated from the chloroform fraction by activity-guided assay, and their activities on cell proliferation were investigated.

2. Investigations, results and discussion

As shown in Table 1, when the cells were cultured with 1.0×10^{-2} mg/mL (expressed in the weight of raw material per mL) crude extract, cell proliferation was significantly stimulated (an increase of 33.2% in osteoblastic proliferation). The effect is similar to that of NaF, as the positive control, at a concentration of 1.0×10^{-2} mmol/L which produced an increase of 37.6%. The activities of fractions partitioned from the alcoholic extract were investigated. It was found that the chloroform layer possessed maximum stimulating activity (an increase of 37.7% in osteoblastic proliferation at a concentration of 1.0×10^{-3} mg/mL).

From the chloroform layer three compounds were isolated by a silica gel column with a gradient of petroleum and ethyl acetate. Osthole produced the most significant promoting effect on UMR106 cell proliferation (an increase of 35.3% at a concentration of 1.0×10^{-3} mmol/L). Imperatorin and bergapten also exhibited significant stimulating activities in osteoblastic proliferation (an increase of 29.0% and 24.1% at a concentration of 1.0×10^{-2} mmol/L), but they were less effective than osthole (Table 2).

Osteoblasts play a key role in bone formation and remodeling. Agents stimulating osteoblast activity may be potential drugs for osteoporosis or some other bone diseases. The UMR106 cell line derived from a rat osteogenic osteosarcoma is an osteoblast model and it has stable phenotype and biochemical characteristics (Gray 1989). It has preserved many properties of the osteoblast, including cAMP responsive to PTH, high ALP activity and synthesis of bone collagen (Partridge 1983). Compared with animal *in vivo* experiment, this cell-culture model has advantages such as low dose, short experimental period, and high reproducibility. Therefore, the UMR106 cell line has been widely used as an osteoblast model to study various hormones and factors acting on bone. With this *in vitro* model and activity-guided isolation, active components stimulating bone formation and potentially anti-osteoporosis may be isolated from traditional Chinese medicine.

Table 1: The effects of various extracts of the fruits of *Cnidium monnieri* (L.) Cuss on osteoblast-like UMR106 cell proliferation

Samples	Concentration	A_{Blank} ($\bar{x} \pm SD$)	$A_{595/655nm}$ ($\bar{x} \pm SD$)	Proliferation (%)
NaF (mmol/L)	1.0×10^{-4}	0.186 ± 0.016	0.211 ± 0.018	13.6
	1.0×10^{-3}		0.249 ± 0.015	30.7**
	1.0×10^{-2}		0.257 ± 0.013	37.6**
	1.0×10^{-1}		0.194 ± 0.014	4.0
	1.0		0.146 ± 0.017	−20.3*
Crude extract (mg/mL)	1.0×10^{-5}	0.186 ± 0.016	0.195 ± 0.015	4.6
	1.0×10^{-4}		0.202 ± 0.019	8.4
	1.0×10^{-3}		0.226 ± 0.018	21.4*
	1.0×10^{-2}		0.248 ± 0.016	33.2**
	1.0×10^{-1}		0.189 ± 0.017	1.6
Petroleum ether fraction (mg/mL)	1.0×10^{-5}	0.126 ± 0.012	0.127 ± 0.013	0.5
	1.0×10^{-4}		0.140 ± 0.018	11.4
	1.0×10^{-3}		0.129 ± 0.014	2.4
	1.0×10^{-2}		0.125 ± 0.012	−1.1
	1.0×10^{-1}		0.115 ± 0.019	−9.0
Chloroform fraction (mg/mL)	1.0×10^{-5}	0.126 ± 0.012	0.132 ± 0.012	4.4
	1.0×10^{-4}		0.150 ± 0.015	18.9*
	1.0×10^{-3}		0.174 ± 0.013	37.7**
	1.0×10^{-2}		0.128 ± 0.016	1.9
	1.0×10^{-1}		0.123 ± 0.017	−2.1
Ethyl acetate fraction (mg/mL)	1.0×10^{-5}	0.126 ± 0.012	0.117 ± 0.014	−7.3
	1.0×10^{-4}		0.127 ± 0.012	1.1
	1.0×10^{-3}		0.135 ± 0.017	6.7
	1.0×10^{-2}		0.139 ± 0.013	10.2
	1.0×10^{-1}		0.131 ± 0.018	3.6
n-Butanol fraction (mg/mL)	1.0×10^{-5}	0.133 ± 0.013	0.134 ± 0.016	0.8
	1.0×10^{-4}		0.133 ± 0.016	0
	1.0×10^{-3}		0.128 ± 0.017	−3.8
	1.0×10^{-2}		0.131 ± 0.019	−1.5
	1.0×10^{-1}		0.131 ± 0.018	−1.5
Aqueous fraction (mg/mL)	1.0×10^{-5}	0.133 ± 0.013	0.130 ± 0.014	−2.3
	1.0×10^{-4}		0.131 ± 0.016	−1.5
	1.0×10^{-3}		0.131 ± 0.015	−1.5
	1.0×10^{-2}		0.133 ± 0.015	0
	1.0×10^{-1}		0.131 ± 0.016	−1.5

* $P < 0.05$; ** $P < 0.01$ significant as compared to blank control using Student's t-test

Table 2: The effects of three coumarins from the fruits of *Cnidium monnieri* (L.) Cuss on osteoblast-like UMR106 cell proliferation

Samples	Concentration (mmol/L)	$A_{595/655nm}$ ($\bar{x} \pm SD$)	Proliferation (%)
Blank control	0	0.203 ± 0.015	
Osthole	1.0×10^{-4}	0.232 ± 0.017	14.3
	1.0×10^{-3}	0.275 ± 0.013	35.3**
	1.0×10^{-2}	0.257 ± 0.016	26.8*
	1.0×10^{-1}	0.225 ± 0.014	10.9
Imperatorin	1.0×10^{-4}	0.210 ± 0.018	3.37
	1.0×10^{-3}	0.222 ± 0.016	9.52
	1.0×10^{-2}	0.262 ± 0.012	29.0*
	1.0×10^{-1}	0.235 ± 0.017	15.8
Bergapten	1.0×10^{-4}	$0.219 \pm .017$	8.05
	1.0×10^{-3}	0.225 ± 0.016	10.6
	1.0×10^{-2}	0.252 ± 0.012	24.1*
	1.0×10^{-1}	0.242 ± 0.015	19.1*
NaF	1.0×10^{-2}	0.278 ± 0.015	37.1**

* $P < 0.05$; ** $P < 0.01$ significant as compared to blank control using Student's t-test

The present study showed that *Cnidium monnieri* (L.) Cuss alcoholic extracts promoted the proliferation of osteoblasts-like cells, and its chloroform fraction might contain active constituents stimulating osteoblasts. It also suggested that osthole, a major pharmacologically active substance, might stimulate bone formation or have a therapeutic effect on osteoporosis.

3. Experimental

3.1. Plant material

Raw materials of *Cnidium monnieri (L.)* Cuss was purchased from Tianyitang Chinese drug store (Shenyang, China) in October 2001 and identified by Qishi Sun, professor of pharmacognosy, Shenyang Pharmaceutical University, China. A voucher specimen (no. CM20011003) was deposited in the herbarium of Shenyang Pharmaceutical University for future reference.

3.2. Preparation of the plant extracts

The dried power of fruits of *Cnidium monnieri* (L.) Cuss (1000 g) was refluxed for 2 h with 75% ethanol (2000 ml × 3). The ethanol solution was vacuum concentrated to afford a dark brown extract (145 g). A part (110 g) of the mass was suspended in hot water and then extracted successively with petroleum ether (b.p. 60–90), chloroform, ethyl acetate, and *n*-butanol three times. The solvents and residual water were evaporated to give the petroleum ether fraction (5 g), chloroform fraction (12 g), ethyl acetate fraction (19 g), *n*-butanol fraction (11 g) and residual aqueous fraction (56 g), respectively. The chloroform fraction (10 g) was chromatographed on a silica gel column (8 × 100 cm), eluted with petroleum: ethyl acetate by a stepwise manner, from 10 : 1, 10 : 2, to 10 : 10, 500 ml for each step, affording pure compounds **1** (osthole, 618 mg), **2** (imperatorin, 85 mg), **3** (bergapten, 34 mg) as pure compounds. Their structures were identified by comparison with their physical properties and IR, NMR spectral data with literature values (Xiang 1984, 1986).
The crude extract of *Cnidium monnieri* (L.) Cuss and its fractions were dissolved in ethanol or distilled water to give completely dissolved solutions (10 mg/mL, expressed in the weight of raw materials per mL). The compounds were dissolved in ethanol to give concentrations of 10 mmol/L, and NaF was dissolved in distilled water to give concentrations of 10 mmol/L as positive control. These solutions were sterilized with a 0.2 μm aseptic filter (Gelmann Science), and all sample solutions were diluted with minimum essential medium (MEM) to the required concentration before use. The blank controls contained MEM and the same proportion of ethanol as in the test samples.

3.3. Cells and methods

Osteoblast-like UMR 106 cells were obtained from Beijing Medical University (of origin from the Massachusetts General hospital, Boston, MA, USA). Minimum essential medium (MEM) was obtained from Gibco (USA) and fetal calf serum (FCS) from TBD Bio-engineering Co. (Tianjin, China). Trypsin was supplied by Diffco (USA) and tissue culture materials were provided by Nunc (Denmark). MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was obtained from Sigma (USA). All other chemicals were of analytical grade.
The stimulating proliferation activity of the solutions on osteoblast-like UMR106 cells was assayed in the same procedure as described previously (Li 2001). After UMR106 cells were co-cultured with the prepared sample solutions for 48 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, the medium was removed, 50 μL MTT solution (1 mg MTT/ml PBS) was then added into the wells, and the incubation continued for another 4 h. Finally, MTT solution was removed and 150 μL DMSO (per well) was added. The absorbance was recorded on an enzyme immunoassay plate reader (BIO-RAD, USA) at wavelength of 595 nm with a reference at 655 nm.

3.4. Statistical analysis

Data were expressed as the mean ± standard deviation. Statistical significances were assessed by the Student's t-test. A value of $p < 0.05$ was considered significant. Linear regression analysis was performed by the correlation coefficient. Proliferation ratios were calculated using the following equation: $\text{proliferation\%} = (A_{sample} - A_{blank})/A_{blank} \times 100$, where A is the average absorbance of six wells.

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