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Research Paper

Inhibitory effect of *Ecliptae herba* extract and its component wedelolactone on pre-osteoclastic proliferation and differentiation

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Wedelolactone (PubChem CID: 5281813)
Alendronate (PubChem CID: 2088)
p-nitrophenyl sodium phosphate (PubChem CID: 378)
Naphthol AS-MX phosphate (PubChem CID: 74136)
DMSO (PubChem CID: 679)
Penicillin (PubChem CID: 5904)
Streptomycin (PubChem CID: 19649)
L-glutamine (PubChem CID: 5961)
Acetonitrile (PubChem CID: 6342)
Formic acid (PubChem CID: 284)

ABSTRACT

Ethnopharmacological relevance: Ecliptae herba, also known as "Mo-Han-Lian", has long been used in China to nourish Kidney and thereafter strengthen bones. Accumulating evidence indicates that extracts of Ecliptae herba have antiosteoporotic effect. However, the effective compounds and cellular mode of action are still unclear. To investigate the effect of ethyl acetate extract of Ecliptae herba (EAE) and its component wedelolactone on proliferation and differentiation of preosteoclastic RAW264.7 cells as well as proliferation of bone marrow stromal cells (BMSC).

Materials and methods: RAW264.7 and BMSC were examined for proliferation by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) method. Tartrate-resistant acid phosphatase (TRAP) activity of RAW264.7 was measured by using *p*-nitrophenyl sodium phosphate (pNPP) assay after the cells were treated with 30 ng/ml receptor activator for nuclear factor-κ B ligand (RANKL) plus various concentrations of EAE, wedelolactone or alendronate. The formation of multinucleated TRAP-positive RAW264.7 cells was observed by using a TRAP-staining kit.

Results: Treatment of RAW264.7 cells with EAE at high doses (20 μg/ml and 40 μg/ml) or wedelolactone at 10 μg/ml resulted in a decrease in proliferation of RAW264.7 cells. Low doses of EAE (5, 10 μg/ml) and wedelolactone (2.5 μg/ml) inhibited RANKL-induced TRAP activity by 20.3%, 37.9%, and 48.3%. The inhibitory effect of wedelolactone is more potent than that of alendronate, an anti-resorptive drug. Morphological changes revealed that 5 μg/ml EAE and 2.5 μg/ml wedelolactone reduced the number of multinucleated osteoclast-like cells. At the high doses, EAE (20 μg/ml) and wedelolactone (10 μg/ml) inhibited the growth of BMSC.

Conclusions: EAE and its component wedelolactone inhibited osteoclast RAW264.7 proliferation and differentiation at the low doses, but at the high doses, showed cytotoxic effect on BMSC. These results indicated that EAE and wedelolatone might be potential alternative therapy for osteoporosis.

1. Introduction

Ecliptae herba, also known as "Mo-Han-Lian", is the aerial parts of *Eclipta prostrata* L. (Astraceae), which has been used as "Kidney-nourishing" traditional Chinese medicine for several thousand years. Ecliptae herba is reported to possess hepatoprotective, anti-inflammatory, immunomodulatory, anti-oxidative and hypolipidemic activities (Jayathirtha and Mishra, 2004; Dhandapani, 2007; Kima et al., 2008; Arunachalam et al., 2009). The predominant components isolated from Ecliptae herba are triterpenoid saponins, flavonoids, and coumestans such as wedelolactone (Zhang and Guo, 2001; Wu et al., 2008). Wedelolactone is reported to inhibit 5-lipoxygenase and typsin (Wagner and Fessler, 1986; Syed et al. 2003), antagonize myotoxins (Melo and Ownby, 1999), and induce caspase-dependent apoptosis (Sarveswaran et al., 2012). In China, "Kidney-nourishing" herbal drugs including Ecliptae herba are commonly believed to have the ability of nourishing bones, and therefore are used to treat bone diseases such as osteoporosis. Recently, it was reported that Ecliptae herba extract showed therapeutic effect on disorder of bone metabolism of ovariectomized rats (Zhang et al., 2009). However, the cellular action of Ecliptae herba regulating bone metabolism is still unclear.

Adult bone is continuously remodeled by combinational roles of bone resorbing osteoclasts and bone forming osteoblasts. Osteoporosis, characterized by low bone mass and high risk of fracture, is due to the excess of osteoclastic bone resorption over osteoblastic

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bone formation. Antiresorptive drugs such as alendronate are most frequently used in clinic for curing osteoporosis, but treatment with alendronate for a long time concurrently impaired bone formation (Odvina et al., 2005). This drawback of the current therapies might be attributed to single target on bone resorption, but not on bone formation, which makes the imbalance of bone remodeling lasted. It is thus crucial to identify a new class of agents that can regulate both formation and resorption (Deal, 2009). Recently, a study showed that ethanolic extract of *Ecliptae herba* facilitated proliferation and differentiation of primary osteoblasts (Lin et al., 2010). However, no investigation has yet to elucidate the impact of *Ecliptae herba* on osteoclastic function. In the present study, we aimed to investigate the inhibitory effect of ethyl acetate extract from Ecliptae herba (EAE) as well as its major component wedelolactone on osteoclastic proliferation and differentiation by using a preosteoclastic cell linage, RAW264.7 cells. Simultaneously, the impact of EAE and wedelolactone on BMSC proliferation was evaluated.

2. Materials and methods

2.1. Chemical and drugs

Ecliptae herba is the dried aerial part of *Eclipta prostrata* L. (Astraceae), which was obtained from Sichuan Academy of Chinese Medicine Sciences (Sichuan, China). Plants were identified and a voucher (no. 110406) was lodged in our laboratory. We isolated and chemically characterized wedelolactone in our laboratory and will publish the isolation procedure elsewhere. The purity was reached above 97%.

2.2. Preparation of ethyl acetate extract of Ecliptae herba

30 g Ecliptae herba was powdered and extracted with 500 ml 70% ethanol for 2 h and repeated twice for 1.5 h and 1 h. The ethanol fraction was pool together and dried under reduced pressure. The resulting crude extract was submitted to ultrasonic extraction with 100 ml water. Then liquid/liquid extractions (v/v) were performed with 50 ml petroleum ether for five times. The water fraction was further extracted with 50 ml ethyl acetate for five times. The ethyl acetate fractions were pooled together and concentrated to dryness under reduced pressure. The ethyl acetate extract (EAE) was obtained and was kept at 4 °C in a refrigerator. EAE was redissolved in DMSO (0.1%) with cell culture medium at suitable concentrations for the different assays.

2.3. UPLC analysis

Ultra-performance liquid chromatography (UPLC) was performed with an Agilent ultra-performance liquid chromatography with DAD detector, cooling autosampler, and column oven enabling control of the temperature of the analytical column. Data were collected and processed by Qualitative software. Injections (0.1 μl) of EAE and wedelolactone were made using a 10-μl loop operated in patial-loop mode. UPLC separation was achieved on a 3.0 × 150 mm, 1.8 μm particle size, Zorbax Eclipse plus C18 column (Waters) thermostatted at 50 °C. The mobile phase was a gradient prepared from 0.2% formic acid (component A) and acetonitrile (component B). The amount of B was increased in 15 min, from 20% to 90% and then programmed linearly in 0.1 min to 100%, which was held for 5 min. The total run time was therefore 20 min. The mobile phase flow rate was 0.4 ml/min. The system operating pressure was 7500 psi under the initial gradient conditions. UV detection of the samples was performed at 351 nm.

2.4. Culture of pre-osteoclastic RAW264.7 cells

Mouse pre-osteoclastic RAW264.7 cells were purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% FCS, 0.03% L-glutamine (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

For differentiation, cells were plated at 1×10^3 cells per well of 96-well plates in DMEM supplemented with 30 ng/ml recombinant RANKL. For drug assays, EAE or wedelolactone was added at different concentrations to the culture medium. Then cells were incubated at 37 °C with 5% CO_2 in a humidified incubator, and fed daily with RANKL-supplemented medium for 6 days.

2.5. Isolation and culture of mouse bone marrow stromal cells (BMSC)

BMSC were isolated according to a previously published protocol with some modification (Krebsbach et al., 1999; Zhang et al., 2009). Briefly, MSC were isolated from bone marrow, which aspirated from 8-week old BALB/c mice. BMSC were collected using gradient centrifugation of mesenchymal stem cell-specific gradient solutions (Tianjin Haoyang Biological manufacture Co., LTD., China). A layer of PBS buffered bone marrow cell fraction was placed on the top of gradient solution and centrifuged at 340*g* for 20 min. The cell fraction was collected and washed with PBS. The cell samples were resuspended in Minimum Essential Medium Alpha Medium (α-MEM, Gibco, Paisley, UK), supplemented with 20% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. On day 3, the cell suspension was decanted and it was replaced with fresh complete medium. BMSC was further separated from hematopoietic cells by their differential adhesion to tissue culture plastic and their prolonged proliferation potential. Upon 6–7 days culture, 90% of cell confluence was reached. These cell samples were employed with the experiment.

2.6. MTT assay

RAW264.7 cells or BMSC were plated at 1×10^4 cells per well of 96-well plates. After overnight incubation, various concentrations of EAE and wedelolactone were added to the plates. Following incubation for 3 days, cell growth was measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described with a plate reader (Tecan, Switzerland) (van Meerloo et al., 2011). The percentage of proliferation was calculated as follows:

Proliferation rate (%) = A492 (sample)/A492 (control) × 100%

2.7. Measurement of tartrate-resistant acid phosphatase (TRAP) activity

RAW264.7 cells were fixed with 60% citrate buffered acetone for 30 s. Then the fixed cells were washed with water for three times and were further incubated with 100 μl phosphatase substrate solution containing 10 mM pNPP and 10 mM sodium tartrate in 50 mM citrate buffer (PH 4.6) at 37 °C for 1 h. After incubation, the enzyme reaction mixture was transferred to another plate and the reaction was stopped with 100 μl of 0.1 N NaOH. Absorbance at 405 nm was measured using an ELISA reader (Tecan, Switzerland).

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