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

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DMSO (PubChem CID: 679)
Penicillin (PubChem CID: 5904)
Streptomycin (PubChem CID: 19649)
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**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 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) 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 wedelolactone might be potential alternative therapy for osteoporosis.

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1. Introduction

*Ecliptae herba*, also known as "Mo-Han-Lian", is the aerial parts of *Eclipta prostrata* L. (*Asteraceae*), 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). 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 trypsin (Wagner and Fessler, 1986; Syed et al. 2003), antagonize myotoxins (Melo and Ownby, 1999), and induce caspase-dependent apoptosis ( Saraswaran 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
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 prolifera-
tion 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 proes-
toelastic cell lineage, 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 Chi-

nese 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 formic acid 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% CO2 in a humidified atmosphere.

For differentiation, cells were plated at 1 × 105 cells per well of 96-well plates in DMEM supplemented with 30 ng/ml recombi-
nant 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% CO2 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 pro-
tocol with some modification (Krebbsbach 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 340g 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% CO2 in a humidified atmosphere. On day 3, the cell suspension was decanted and it was replaced with fresh complete medium. BMSC was further sepa-
rated 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 × 104 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:

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\text{Proliferation rate (％)} = \frac{A_{492}}{A_{492}^\text{control}} \times 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 sub-
strate solution containing 10 mM pNPP and 10 mM sodium tar-
trate 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).
2.8. Staining of TRAP

Cells were fixed with 60% citrate buffered acetone for 30 s. Then the cells were stained for TRAP with 0.1 M acetate solution (PH 5.0) containing 6.76 mM sodium tartrate, 0.12 mg/ml naphthol AS-MX phosphate, and 0.07 mg/ml of fast Garnet GBC solution as described in the manufacturer’s instruction (sigma). Photomicrographs were obtained using an Olympus microscope at 200 × magnification.

2.9. Statistical analysis

All statistical analyses were performed using Student’s t-test. Results were represented as the mean ± S.E.M. Values were considered to be significant at *p < 0.05, and **p < 0.01.

3. Results and discussion

3.1. Inhibitory effect of EAE and its major component wedelolactone on preosteoclastic RAW264.7 cell proliferation

To investigate the effects of Ecliptae herba on osteoclast proliferation, EAE and its component wedelolactone as shown in Fig. 1 are used. The content of wedelolactone in EAE was determined to be 25.65% by UPLC analysis. After treatment of 20 μg/ml EAE and 5 μg/ml wedelolactone, the proliferation of RAW264.6 was respectively reduced by 75% and 15.1%, in contrast to untreated control. Alendronate, a known anti-resorptive drug, was used here as a positive control, which inhibited RAW264.7 proliferation by 41.5% at the concentration of 5 μg/ml (Fig. 2).

Ecliptae herba and other tonic traditional Chinese herbs have, for many years, been widely used in China to strengthen bones. A mixture of Ecliptae herba and Fructus Ligustri Lucidi, named as Er-Zhi-Wan, has been reported to possess antosteoporotic effect (Cheng et al., 2011). Zhang et al. (2013) observed that the aqueous extract of Ecliptae herba increased bone mass in ovariectomized rats by inhibiting RANKL expression in tibiae and IL-6 level in serum. However, to the best of our knowledge, the direct impact of Ecliptae herba on osteoclasts has not been investigated. The murine monocyte cell line RAW264.7, which can differentiate into osteoclast-like cells on the exposure to RANKL, represent a widely used in vitro model of osteoclast precursors (Pederson et al., 2009). In this study, we demonstrated that EAE inhibited preosteoclast RAW264.7 proliferation, indicating that Ecliptae herba has the ability to directly target osteoclastogenesis. From 10 μg/ml to 20 μg/ml EAE triggered the sharp drop in proliferation, which might be attributed to some more proliferation inhibitory constituents in EAE or insoluble EAE triggering the cytotoxic response. Ecliptae herba is reported to contain many chemical constituents, including triterpenoid sapo-nins, flavonoids, and coumestans (Zhang and Guo, 2001). Wedelolactone is a marker for the authentication of Ecliptae herba (Lee et al., 2009). Wedelolactone is indicated here to be an active component in EAE, which reduced RAW264.7 proliferation.

3.2. EAE and wedelolactone inhibits osteoclast differentiation of RAW264.7 cells

The effect of EAE and wedelolactone on RANKL-induced osteoclast differentiation of RAW264.7 was examined. Osteoclast-like cells were characterized by monitoring the formation of multinucleated cells and by measuring the activity of TRAP, a marker enzyme of osteoclasts. Compared to the RANKL-treated control, the TRAP activity of RAW264.7 cells treated with RANKL plus EAE reduced in a dose-dependent manner by 2.37%, 14.5%, 20.3%, 37.9%, 57%. Wedelolactone at the concentration of 2.5 μg/ml inhibited RANKL-induced TRAP activity by 48.3% (Fig. 3). The inhibitory effect of wedelolactone is more potent than the positive control alendronate.

In correlation with TRAP activity, the number of TRAP staining-positive RAW264.7 cells and multinucleated osteoclast-like cells were also observed in response to EAE and wedelolactone. When RANKL was added to RAW264.7 cell culture, the TRAP-staining positive RAW264.7 cells increased. However, addition of EAE or wedelolactone resulted in a significant decrease in the number of TRAP-staining positive cells. Simultaneously, the RANKL-induced formation of multinucleated osteoclast-like cells was reduced by EAE or wedelolactone (Fig. 4). These results demonstrate that EAE and wedelolactone effectively suppressed both the activity of TRAP and the number of multinucleated TRAP-positive cells, indicating that EAE and wedelolactone are potent inhibitors of osteoclast differentiation.

Under pathological conditions such as osteoporosis, excessive differentiation and activation of osteoclasts induce bone lose. Therefore, inhibition of osteoclast differentiation becomes a potential target for drug development. It is well established that RANKL/RANK plays a central role in regulating osteoclast differentiation and function (Khosia, 2001). RANK is a receptor located on osteoclast surface. Ligands of RANK (RANKL) are secreted from osteoblasts and bone marrow stromal cells. When RANK is activated by RANKL, a signaling cascade is initiated, causing osteoclast differentiation. The importance of this system in bone metabolism is demonstrated by the fact that pharmacological blockage of RANKL/RANK cascade is an effective treatment for osteoporosis (Lewiecki, 2007). In this study, we for the first time demonstrated that EAE as well as its major component wedelolactone inhibited differentiation of preosteoclast RAW264.7 into osteoclasts stimulated by RANKL. This inhibitory effect is not due to direct cytotoxic activity of EAE because a low dose of EAE (5 μg/ml) significantly reduced RANKL-induced TRAP activity but a high dose of EAE (20 μg/ml) started to generate cytotoxic response. Also, we
observed that wedelolactone at 2.5 μg/ml dramatically inhibited osteoclast differentiation but did not cause cytotoxicity. The inhibitory effect of wedelolactone on osteoclast differentiation is more potent than that of alendronate. Alendronate is known to be an inhibitor of farnesyl pyrophosphate synthase (Russell et al., 2007), which has been demonstrated to reduce osteoclast number and resorption in vivo and in vitro (Ott, 2001). The fact of relatively high activity of differentiation inhibition by wedelolactone, in contrast to alendronate, indicates that it is valuable for studying the molecular mechanism of action of wedelolactone in the future.

3.3. The impact of EAE and wedelolactone on proliferation of bone marrow stromal cells (BMSC)

Since EAE and wedelolactone have the ability to inhibit preosteoclast proliferation and differentiation, we further test the impact of...
EAE and wedelolactone, at the same concentration, on proliferation of BMSC, the osteoblast precursors. At the concentrations of 0.25, 2.5, 5 μg/ml, EAE had no cytotoxic effect on BMSC, while slightly increased the proliferation instead this inhibitory effect is slighter than that of EAE on osteoclast RAW264.7 proliferation. Wedelolactone at the concentrations of 0.6, 1.25, 2.5, 5 μg/ml was capable of suppressing TRAP activity, but did not show significantly cytotoxic effect on BMSC. At the high dose of 10 μg/ml, wedelolactone showed proliferation inhibitory effect on BMSC. According to a concentration of about 26% of wedelolactone in EAE, 20 μg/ml EAE contained about 5.2 μg/ml wedelolactone. Although 20 μg/ml EAE inhibited BMSC proliferation by 32.5%, 5 μg/ml wedelolactone had no influence on BMSC proliferation (Fig. 5). These results indicated that EAE might contain some more proliferation inhibiting constituents.

BMSC are the osteoprogenitors, which are capable to differentiate into mature osteoblasts (Jaiswal et al., 1997). The deficiency in osteogenesis from BMSC resulted in various bone diseases (Rodríguez et al., 2008). In recent years, the tendency in applying BMSC to cure bone-related diseases is raising since they have shown promising results for treating bone fractures (Egermann et al., 2005). The effect of Ecliptae herba on proliferation of osteoblasts has been studied, but there is no report about the impact of Ecliptae herba on osteoprogenitors derived from bone marrow. It was reported that ethanolic extract from Ecliptae herba stimulated the proliferation of primary osteoblasts (Lin et al., 2010). Er-Zhi-Wan containing serum, however, did not facilitate proliferation of primary osteoblasts and UMR106 cells (Zhang et al., 2009). Our results showed that low doses of EAE, at which doses EAE inhibited preosteoclast RAW264.7 differentiation, failed to show cytotoxic effect on BMSC, but slightly increased proliferation of BMSC instead. Also, wedelolactone below the dose of 5 μg/ml did not elicit cytotoxic response in BMSC. From the high dose of 10 μg/ml, wedelolactone showed sharp drop in proliferation of BMSC, indicating that insoluble wedelolactone might trigger cytotoxic response. These results indicate that wedelolactone at low doses has anti-osteoclastogenic effect, possibly through RANKL/RANK-induced NF-κB pathway (Yuan et al., 2013), but no side effect on osteoblastogenesis (Fig. 6).

Fig. 4. Morphological changes of RAW264.7 cells. The cells were treated with 30 ng/ml RANKL for 6 days in the presence of EAE or wedelolactone. Then the cells were stained for TRAP by using a TRAP staining kit and imaged at 200×.

Fig. 5. The effect of EAE and wedelolactone on BMSC proliferation. EAE and wedelolactone were exposed to the RAW264.7 cells for 3 days, and then MTT assay was performed. Values are mean ± SEM, n=3.
In conclusion, this is the first report to show that EAE and its component wedelolactone inhibited osteoclast proliferation and differentiation. The inhibitory effect on RANKL-induced differentiation included the inhibition of TRAP activity as well as reduction of the number of TRAP-positive multinucleated RAW264.7 cells. Additionally, wedelolactone at the doses of which TRAP activity was suppressed did not show cytotoxic effect on BMSC. Taken together, our data suggest that EAE as well as its component wedelolactone have the potential to serve as therapeutic intervention for skeletal diseases associated with bone loss.

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