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Short communication

Synergistic inhibitory effect of Icariside II with Icaritin from *Herba Epimedii* on pre-osteoclastic RAW264.7 cell growth

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ABSTRACT

Increasing evidence shows the therapeutic superiority of herbal extracts in comparison to isolated single constituents. One of the reasons may be attributed to the synergy effect of compound combinations. Flavonoids from Herba Epimedii have been shown to have therapeutic effect against bone loss. Our previous study showed that Icariside II inhibited pre-osteoclast RAW264.7 growth. The aim of this study was to investigate whether the activity of Icariside II is synergized by other components of Herba Epimedii. The inhibitory activity of Icariside II was significantly enhanced in the presence of the extract of Herba Epimedii (EHE) at the ratio of 1:1, 1:5 and 1:10. Icaritin, another flavonoid constituent, was shown here to inhibit RAW264.7 growth in a dose-dependent manner. Further, we found that Icariside II, together with Icaritin, synergistically inhibited RAW264.7 growth. The synergistic effect is significant when the ratio of Icariside II and Icaritin was 10:1, 5:1, 1:1, 1:2, and 1:5, respectively. In conclusion, Icaritin were an active component. The inhibitory activity of Icariside II on pre-osteoclast RAW264.7 growth was synergized by Icaritin, which maybe contribute to the efficiency of Herba Epimedii extract on curing bone-related diseases, such as osteoporosis

Introduction

The efficacy of plant extracts used for centuries has been verified in many cases by clinical studies. It is believed that synergy effect of the mixture of bioactive constituents from either several types of herbal extracts or mono-extract preparations could be responsible for the improved therapeutic efficacies. However, most herbal extracts contain many chemical constituents, resulting in the synergistic action of the mixture remaining to be addressed. *Herba Epimedii* is a commonly used Chinese medicine for "strengthening kidney" for thousands of years. It contains active components such as flavonoids and phytosteroids (Wu et al., 2003; Zhao et al., 2008). Total flavonoids of *Herba Epimedii* are suggested to be potential candidates for treating osteoporosis (Zhang et al., 2009; Zhang et al., 2010). It includes Icariin, Epimedin A, Epimedin B, Epimedin C, Icariside II, Icaritin, etc. The anti-osteoportic effect of total flavonoids is shown to be more potent than its single compound (Meng et al., 2005; Zhang et al., 2008). Although Icariin is the principal flavonoid glycoside in *Herba Epimedii*, Icariside II exerted higher bone resorption activity of osteoclasts (Huang et al., 2007). Our previous study showed that Icariside II inhibited pre-osteoclast RAW264.6 growth (Yang et al., 2012). In the present study, we investigate whether the active component Icariside II exerts synergistic effect with other flavonoids from *Herba Epimedii* on osteoclastic growth.

Osteoclasts, originated from monocyte/macrophage precursors, mediate bone resorption of old bone tissue, which was coupled by osteoblast-mediated bone formation of new bone tissue. This process, called bone remodeling, maintains bone homeostasis (Matsuo and Irie, 2008). However, under certain pathological conditions, such as osteoporosis, there is an abnormally high bone turnover, with enhanced osteoclastic bone resorption (Ralston and Uitterlinden, 2010; Ohlsson 2013). Therefore, inhibition of osteoclast proliferation and activation is the target for the therapeutic intervention of pathological bone loss. Previously, *Herba Epimedii* extract and its component including icariin were shown to inhibit osteoclast differentiation (Huang et al. 2007). In this study, we investigated the inhibitory effect of Icariside II and Icaritin on preosteoclastic RAW264.7 cell growth. We further investigate

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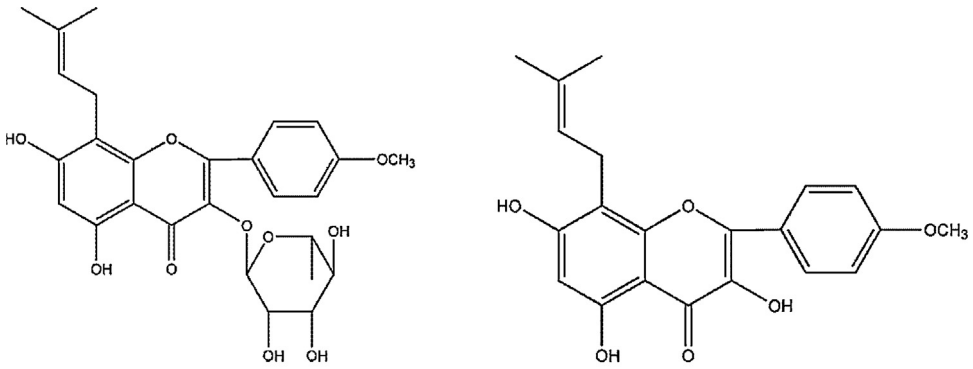

Fig. 1. Chemical structures of Icariside II and Icaritin.

whether the inhibitory action of Icariside II is synergized by other components in the Herba Epimedii extract.

Materials and methods

Materials

The extracts of *Epimediums* (EHE) were prepared and characterized by HPLC analysis as our previously reported (Yang et al., 2012). Icariside II was purchased from Shanghai Winherb Medical Science Co., Ltd. (Shanghai, China). The purity of Icariside II is 99%. Icaritin was prepared in our lab. The purity is above 98%. The chemical structures of Icariside II and Icaritin are shown as Fig. 1. Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Grand Island, NY, U.S.A.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Dimethyl sulfoxide (DMSO) was from Ameresco (U.S.A.).

Cell culture

Murine preosteoclast RAW264.7 cells were obtained from the Chinese Academy of Sciences (Shanghai, China). The RAW 264.7 cell line was established from the ascites of a tumor induced in a male BAB/14 mouse by the intraperitoneal injection of A-MuLV (Raschke et al., 1978; Ralph and Nakoinz, 1977). The cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

MTT assay

RAW264.7 cells were seeded into 96-well plates at 1×10^5/ml density. After overnight incubation with 10% FCS, various concentrations of extract, Icaritin and Icariside II were added to the plates. Following incubation, cell growth was measured at different time points by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described with a plate reader (Tecan, Switzerland). The percentage of inhibition was calculated as follows:

$$\text{Inhibitory effect } (\%) = \frac{\left[A_{492\,(\text{control})} - A_{492(\text{sample})}\right]}{A_{492(\text{control})}} \times 100\%$$

Drug assays

To test the synergistic activity of Icariside II with EHE, Icariside II was added at the concentrations of 0.1, 1, 10, 20 μg/ml. According to 10-fold concentration of Icariside II, the combined concentrations of EHE were 1, 10, 100, 200 μg/ml. According to five-fold concentrations of Icariside II, The combined concentrations of EAE were 0.5, 5, 50, 100 μg/ml. According to equal concentrations of Icariside II, the combined concentrations of EAE were 0.1, 1, 10, 20 μg/ml. The concentrations of Icaritin were determined according to the ratio of Icaritin and Icariside II, which was chosen to be 10:1, 5:1, 2:1, 1:1, and 1:2, respectively.

Dose-inhibitory curve methods were used to evaluate the synergistic activities of Icariside II alone or in combination with EHE or Icaritin. Synergistic activity of Icariside II with Icaritin was evaluated according to the "isobol method" reported by Berenbaum (1989). Synergy was defined when combined index (CI) was less than 1; while additive in which CI was equal to 1; and antagonistic in cases which CI was greater than 1.

Statistical analysis

Assays were set up in triplicate. Statistical analysis and significance were performed by using CalcuSyn software (Biosoft, Cambridge, UK).

Results and discussion

The growth inhibitory effect of Icariside II alone was moderate at 10 μg/ml, but significant synergistic activities against RAW264.7 cell growth were observed when combinations of 10 μg/ml Icariside II with 10 μg/ml EHE (Fig. 2a). The inhibitory rate reached 47.2%, which was higher than the inhibitory rate (23.8%) of 10 μg/ml Icariside II treatment alone. EHE was ineffective in inhibiting the growth of RAW264.7 cells below the dose of 200 μg/ml. The increased inhibitory rate was more potent when 20 μg/ml Icariside II and 20 μg/ml EHE were combinated, in contrast with 20 μg/ml Icariside II treatment alone. To further confirm the synergistic activity of Icariside II with EHE, the different combinated ratios of Icariside II to EHE were used. When the ratio of Icariside II to EHE is 1:5, the inhibitory effect of Icariside II at 10 μg/ml combined with EHE at 50 μg/ml was significantly enhanced, in relevant to Icariside II alone, with the increased inhibitory rate from 23.8% to 47% after 24 h incubation (Fig. 2b). Moreover, the synergism of Icariside II with EHE at the ratio 1:10 against RAW264.7 cell growth was stronger than that of the combination at the ratio 1:5. The inhibitory