Chemical Characterization of the Functional Natural Products from the Fruits of Sea Buckthorn (*Hippophae rhamnoides*) that Control Osteoblast Differentiation

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Abstract

In the screening assay, we found that the ethanolic extract of sea buckthorn (*Hippophae rhamnoides*) fruits showed anti-osteoporosis effects *in vitro* and *in vivo*. A bioassay-guided fractionation and LC/MS-based isolation of the active ethanolic extract resulted to the isolation of potential bioactive compounds (**1-6**), chemical structures of which were identified as four triterpenes including ursolic acid (**1**), uvaol (**2**), oleanolic aldehyde (**3**), and ursolic aldehyde (**4**), and two fatty acids such as methyl linoleate (**5**) and ethyl oleate (**6**) by the interpretation of their spectroscopic NMR data and LC-MS analysis as well as HR-ESI-MS data. The isolated compounds were tested for the efficacy of promoting osteoblast differentiation and the expression of mRNA biomarkers related to osteogenesis. Firstly, the osteogenic effects of the compounds were tested in the mouse mesenchymal stem cell line, C3H10T1/2. As a result of alkaline phosphate staining, ursolic aldehyde (**4**) at 10 μg/mL concentration showed 11.2-fold higher activity than that of negative control (NC). Ursolic aldehyde (**4**) also increased gene expression of bone formation-related biomarkers, such as *Runx2, Osterix, Alp.* and *Osteopontin*. These findings suggest that the fruit extract of *H. rhamnoides* can be a nutraceutical for bone health with ursolic aldehyde (**4**) as an active constituent.

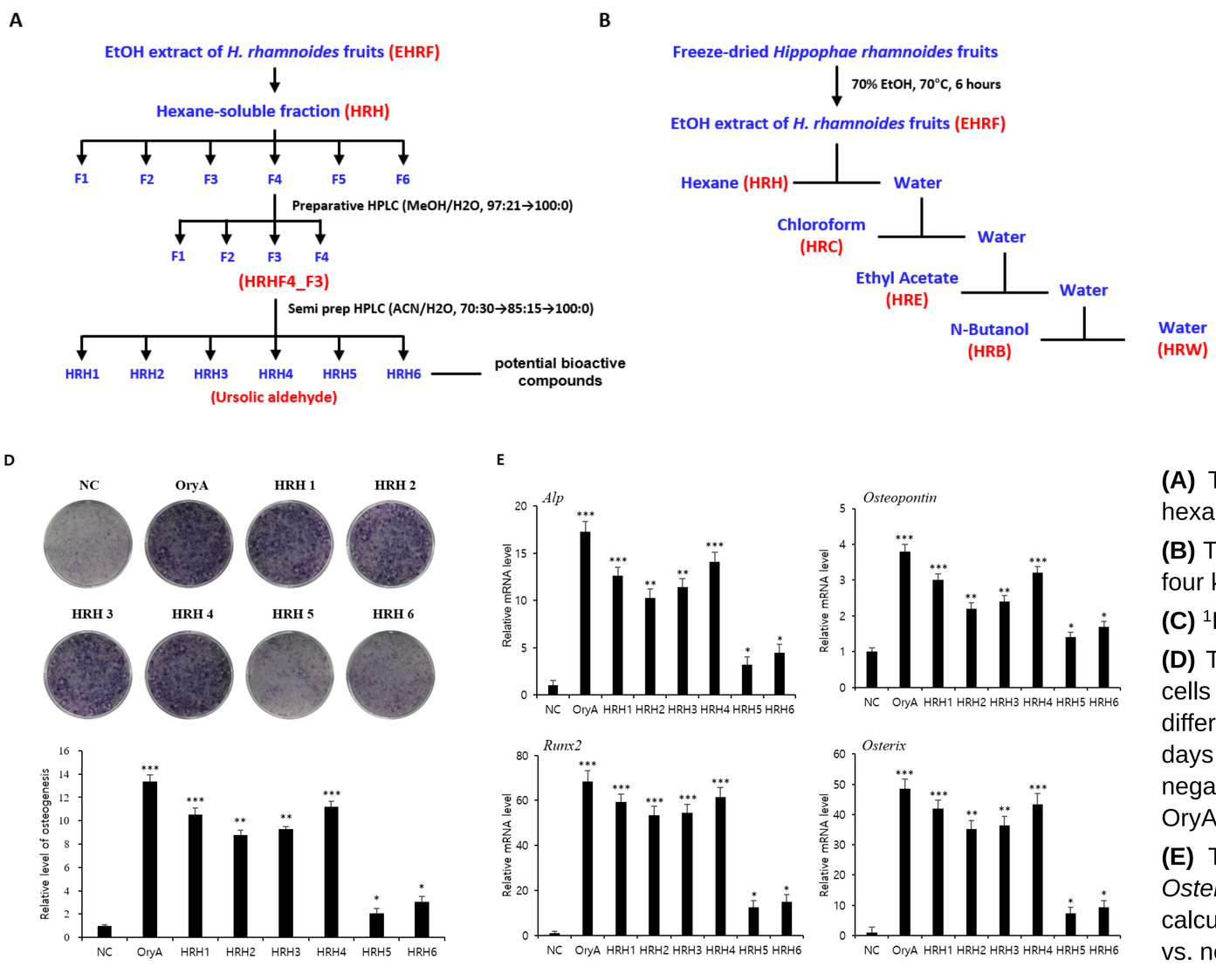
Material & Methods

(A) Procedures for isolation of potential bioactive compounds. The efficacy evaluation was conducted with the six fractions obtained through the results of the prep HPLC fraction, and it was determined that there was an active compound in the HRH4 fraction. The bioactive compound HRH4 was fractionated by semi preparative HPLC (ACN/H_2O, 70:30→85:15 for 70 min and 100% ACN for 10 min) using a Phenyl-Hexyl 100 Å column (10 × 250 mm i.d., 10 μm, Phenomenex) with a flow rate of 2 mL/min to obtain six single compounds (HRH1 ~ HRH4) according on the separation of peaks. Preparative HPLC was performed on a Waters 1525 binary HPLC pump with a Waters 996 photodiode array detector (Waters Corporation, Milford, CT, USA). Nuclear magnetic resonance (NMR) spectra were recorded by using a Bruker AVANCE III (Bruker). HRESIMS spectra were recorded on an Agilent 1290 Infinity II series with a 6545 LC/QTOF mass spectrometer (Agilent Technologies).

(B) Cell culture, osteoblast differentiation, and ALP staining. The C3H10T1/2 cell line, derived from mouse embryonic fibroblasts, was cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 100 μg streptomycin, and 100 U penicillin at 37 °C in an incubator with 5% CO_2. To measure osteoblastic differentiation, C3H10T1/2 cells were seeded at a density of 5 × 10^5 cells/well in 6-well plates and treated with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid for 9 days. The differentiated cells were stained by ALP staining method.

(C) mRNA isolation and real-time polymerase chain reaction. Gene expression of bone formation-related biomarkers was analyzed. The primer sequences were as follows: 36B4, 5'-AGATGCAGCAGATCCGCAT-3' (forward) and 3′-TATGGAGTGCTGCTGGTCTG-5′ (reverse); *Runx2*, 5'-CCCAGCCACCTTTACCTACA-3' (forward) and 3'-TATGGAGTGCTGCTGGTCTG-5' (reverse); *ALP*, 5'- CAAGGATGCTGGGAAGTCCG -3' (forward) and 3'-CGGATAACGAGATGCCACCA-5' (reverse); *Osterix*, 5'-ATCTTCCACTTCGCCTGC-3' (forward) and 3'-AACCAATGGGTCCAGCAC-5' (reverse); and *Osteopontin*, 5'-CTGGCAGCTCAGAGGAGAAG-3' (forward) and 3'-CAGCATTCTGTGGCGCAAG-5' (reverse).

Results



(A) The process of obtaining six bioactive compounds through HPLC separation in hexane soluble fractions from the fruits of *H. rhamnoides.*

(B) The process of extraction with 70% ethanol solution and fractionation process using four kinds of solvents with the fruits of *H. rhamnoides.*

(C) ^{1}H (850 MHz) NMR data of compounds 1, 2, 3, 4, 5 and 6 in CD_3OD.

(D) The effect of compounds from *H. rhamnoides* fruits on osteogenesis. C3H10T1/2 cells were treated with each isolated compound during osteogenic differentiation. Fully differentiated C3H10T1/2 cells were stained with *alkaline phosphatase (ALP)* after 9 days of osteogenic differentiation. The relative levels were calculated by setting the negative control (NC) to 1. Oryzativol A was used as a positive control and marked as OryA.

(E) The mRNA expression of *Alkaline Phosphate (ALP), Osteopontin, Runx2, and Osterix* in C3H10T1/2 osteoblasts was measured by RT qPCR. The relative levels were calculated by setting the negative control (NC) to 1. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. negative control, determined by the least significant difference test.

Conclusion

In conclusion, a bioassay-guided fractionation and LC/MS-based isolation of the active ethanolic extract resulted to the isolation of potential bioactive compounds (1-6), chemical structures of which were identified as four triterpenes and two fatty acids by the interpretation of their spectroscopic NMR data and LC-MS analysis as well as HR-ESI-MS data. The isolated compounds were tested for the efficacy of promoting osteoblast differentiation and the expression of mRNA biomarkers related to osteogenesis, and the physiological effects of ursolic aldehyde (**4**) on osteoblast differentiation of mouse mesenchymal stem cell C3H10T1/2 were the most significant among the six compounds. These findings provide the experimental evidence that the fruit extract of *H. rhamnoides* can be a bone health material and the ursolic aldehyde (**4**) is the active constituent.

References

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