Osteoporosis is characterized by a reduced bone mass, which results in increased bone fragility and fracture risk. Estrogen deficiency which occurs at menopause plays a major role in the development of osteoporosis in elderly women. Therefore, estrogen replacement therapy (ERT) has been widely used for prevention of postmenopausal osteoporosis. However, ERT also shows other undesirable side effects including increased incidence of breast cancer and heart disease. Recently, there is a growing interest in searching for alternatives from dietary natural plant products. Phytoestrogens are naturally occurring polyphenolic plant-derived compounds of nonsteroidal structure that are similar to the mammalian estrogen, such as estradiol. They can bind to estrogen receptor (ER), thus exerting estrogenic activity. Many investigators have reported that phytoestrogens are effective in preventing bone loss in an ovariectomized rats and postmenopausal women. Phytoestrogen includes various families of compounds such as isoflavonoids, flavonoids and lignans. Isoflavonoids, such as daidzein and genistein, which are abundant in soybean, have extensively investigated and have been demonstrated to have beneficial effects against bone loss in a clinical study and various experimental models.

Lignans, another group of phytoestrogens, are widely distributed throughout plants. Especially, cereals, grains, berries and garlic are good dietary sources of lignans. Recently, lignans have received considerable attention for their potential role in prevention of osteoporosis. Secoisolariciresinol diglycoside from flaxseed showed anti-osteoporotic activity in postmenopausal women. Isotaxiresinol from Taxus yunnanensis prevented bone loss in ovariectomized model. We previously isolated lignans from the barks of Machilus thunbergii Sieb. et Zucc (Lauraceae) and evaluated their neuroprotective activity. In the present study, we further tried to elucidate their mechanism using ER antagonist, tamoxifen.

Key words lignans; osteoblast differentiation; estrogen receptor; Machilus thunbergii; isoguaiacin dimethylether

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Fig. 1. Structures of Lignans Isolated from M. thunbergii
MATERIALS AND METHODS

Materials All lignans were isolated from the bark of \textit{M. thunbergii} and their purities were higher than 95.0\%, respectively.\textsuperscript{13} Daidzein was purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.).

Primary Cultures of Mouse Osteoblasts ICR mice (kept at 20–23°C; 12 h light cycle; food, Agribrand Purinar Korea, and water \textit{ad libitum}) were provided by the Laboratory Animal Center, Seoul National University. All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of the Seoul National University. Murine calvarial osteoblasts were obtained from the calvariae of neonatal mice 1—2 d after birth by sequential collagenase digestion method and cultured in DMEM supplemented with 10\% FBS, 100 IU/ml penicillin and 100 \(\mu\)g/ml streptomycin.\textsuperscript{15} After one week, cells were seeded at a concentration of \(1 \times 10^4\) cells/cm\(^2\). Osteoblast differentiation was induced by changing the medium containing 50 \(\mu\)g/ml ascorbic acid.

Test compounds were dissolved in dimethyl sulfoxide (DMSO). Our preliminary study showed that DMSO at a final concentration of 0.1\% in media did not affect the cell viability or differentiation. The cultures were treated with test compounds and maintained for 7 d for the assessment of ALP activity, by changing with fresh medium containing each compound every 3 d. In some experiment, 10 \(\mu\)M tamoxifen was added 1 h before treatment with test compounds to evaluate the involvement of ER in the action of test compounds.

For the assessment of collagen content and calcium deposition, cultures were treated with vehicle or test compounds in the presence of 50 \(\mu\)g/ml ascorbic acid and 10 m\(M\) (\(\beta\)-glycerophosphate, and grown for 2 weeks by changing the medium containing test compounds every 3 d.

Assay of ALP Activity and ALP Staining To assess ALP activity, cells were rinsed with phosphate-buffered saline (PBS) and lysed in 0.01\% sodium dodecyl sulfate in PBS followed by sonication. After clarification by centrifugation, cell lysates were assayed for ALP activity using the Alkaline Phosphate Assay Kit (Youngdong Pharmaceutical Co., Korea). Each value was normalized with the protein content of cell lysate, measured using bicinchoninic acid with bovine serum albumin as a standard.

For ALP staining, cells were rinsed with PBS and fixed in 100\% methanol for 1 h at room temperature. Cells were rinsed with PBS and stained with 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate plus 0.3 mg/ml nitroblue tetrazolium chloride in 0.1 M Tris–HCl, 0.01 N NaOH, 0.05 M MgCl\(_2\) for 2 h in the dark.\textsuperscript{16}

Measurement of Collagen Content Collagen content was quantified by Sirius Red-based colorimetric assay.\textsuperscript{17} Cultured osteoblasts were washed with PBS, followed by fixation with Bouin’s fluid for 1 h. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained by Sirius Red dye reagent for 1 h under mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm against 0.1 N NaOH as a blank.

Measurement of Calcium Deposit The degree of mineralization was determined by measuring the calcium deposition by Alizarin Red staining.\textsuperscript{18} In brief, the cells were rinsed with PBS and fixed with ice-cold 70\% ethanol for 1 h. The ethanol was removed and rinsed with deionized water. The cells were then stained with 40 m\(M\) Alizarin Red S in deionized water (adjusted to pH 4.2) for 10 min at room temperature. The Alizarin Red S solution was removed and rinsed with deionized water and PBS. The stained material was extracted in DMSO and the absorbance was measured at 562 nm.

Statistical Analysis The evaluation of statistical significance was determined by the one-way ANOVA test with a value of \(p<0.05\) considered to be statistically significant.

RESULTS

In the present study, we attempted to evaluate effects of six lignans, such as meso-dihydroguaiaretic acid (MDGA), nordihydroguaiaretic acid (NDGA), machilin A, guaiacin, isoguaiacin and isoguaiacin dimethylether, on osteoblast differentiation employing primary cultures of mouse osteoblasts as an assay system.

The effect of lignans on osteoblast differentiation was first assessed by measuring the ALP activity, one of the major osteoblast differentiation markers.\textsuperscript{19} In our culture system, treatment of primary cultured osteoblasts with ascorbic acid for 7 d increased ALP activity almost ten times, whereas weak increase in ALP activity was observed in non-treated control cells (data not shown). Among the six lignans tested, guaiacin, isoguaiacin and isoguaiacin dimethylether significantly increased the ALP activity at concentrations of 10 and 25 \(\mu\)M (Table 1). Isoguaiacin dimethylether showed the most potent activity, followed by isoguaiacin and guaiacin. At the concentration of 25 \(\mu\)M, isoguaiacin dimethylether increased ALP activity up to 142\% compared to that of control. The activity of isoguaiacin dimethylether was stronger than that of daidzein, a well-known phytoestrogen. MDGA, NDGA, and machilin A, however, showed little effect (Table 1). The effect of guaiacin, isoguaiacin and isoguaiacin dimethylether on ALP activity was also visualized by ALP staining. As

<table>
<thead>
<tr>
<th>Compounds</th>
<th>ALP activity (% of control)\textsuperscript{a}</th>
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<tbody>
<tr>
<td>Control\textsuperscript{b}</td>
<td>1 (\mu)M 10 (\mu)M 25 (\mu)M</td>
</tr>
<tr>
<td>MDGA</td>
<td>104.8(\pm)6.8 108.9(\pm)6.5 106.2(\pm)8.6</td>
</tr>
<tr>
<td>NDGA</td>
<td>102.4(\pm)2.6 95.0(\pm)5.8 82.1(\pm)13.6</td>
</tr>
<tr>
<td>Machilin A</td>
<td>106.5(\pm)2.5 117.0(\pm)14.7 115.5(\pm)12.0</td>
</tr>
<tr>
<td>Guaiacin</td>
<td>111.4(\pm)5.1 122.0(\pm)6.8* 128.2(\pm)8.7*</td>
</tr>
<tr>
<td>Isoguaiacin</td>
<td>117.9(\pm)7.7 131.4(\pm)8.1** 131.0(\pm)6.2**</td>
</tr>
<tr>
<td>Isoguaiacin</td>
<td>125.7(\pm)7.4* 147.8(\pm)4.0** 149.0(\pm)9.4**</td>
</tr>
<tr>
<td>dimethylether</td>
<td>109.1(\pm)8.5 135.1(\pm)5.9** 131.6(\pm)6.7**</td>
</tr>
</tbody>
</table>

\textsuperscript{a} ALP activity of control was 18.9 \(\pm\)1.2 U/mg protein. \textsuperscript{b} ALP activity (%) was calculated as 100/(ALP activity of compound-treated/ALP activity of control). The values are expressed as means\(\pm\)S.D. of triplicate experiments. Mean value is significantly different \((p<0.05, *p<0.01)\) from the control. \textsuperscript{c} Daidzein was used as a positive control.
shown in Fig. 2, the result of ALP staining was in consistent with that of ALP activity.

Since guaiacin, isoguaiacin and isoguaiacin dimethylether significantly increased ALP activity in primary cultures of mouse osteoblasts, we further investigated their effects on collagen synthesis using Sirius Red-based colorimetric assay. As shown in Fig. 3A, isoguaiacin and isoguaiacin dimethylether significantly increased collagen synthesis. At a concentration of 10 μM, isoguaiacin dimethylether increased collagen synthesis up to 141% compared to that of control cells.

Next, we examined the effects of guaiacin, isoguaiacin and isoguaiacin dimethylether on mineralization, another important process in differentiation, by measuring the calcium deposition by Alizarin Red staining. In consistent with the effects on ALP activity and collagen synthesis, isoguaiacin and isoguaiacin dimethylether showed significant stimulatory effect on mineralization (Fig. 3B). At the concentration of 10 μM, about 126% and 138% increase in mineralization was observed by the treatment with isoguaiacin and isoguaiacin dimethylether, respectively, as compared to control.

Phytoestrogens are known to exert stimulatory effects on osteoblast differentiation, in part, via ER-mediated pathway. Therefore, we examined the involvement of ER in the stimulatory effects of guaiacin, isoguaiacin and isoguaiacin dimethylether on osteoblast differentiation, by using tamoxifen, an ER antagonist. As shown in Fig. 4, the addition of 10 μM tamoxifen abolished the stimulatory effects of guaiacin, isoguaiacin and isoguaiacin dimethylether on osteoblast differentiation. These results suggested that guaiacin, isoguaiacin and isoguaiacin dimethylether enhance osteoblast differentiation, in part, via an ER-dependent pathway.

DISCUSSION

Osteoblasts play a crucial role in bone formation through the proliferation and differentiation.20) Especially, osteoblast differentiation, an important process for its function, confers marked rigidity and strength to the bone while still maintaining some degree of elasticity. Thus, stimulation of osteoblast differentiation has been suggested to be an important therapeutic approach for prevention and treatment of bone disease such as osteoporosis.21) Therefore, we tried to search for natural products having stimulatory activity on osteoblast differentiation and our present study demonstrated lignans from M. thunbergii increased osteoblast differentiation in primary cultures of mouse osteoblasts.

During differentiation, osteoblasts exhibit various characteristics in time-dependent manner: increase in ALP activity, followed by extracellular matrix (ECM) synthesis and result in mineralization. ALP, which hydrolyzes the ester bone of organic phosphate compounds under alkaline conditions, plays an important role in the calcification of bone. ALP produces phosphate required for mineralization and, in addition, hydrolyzes substances that inhibit calcification.22) Differentiated osteoblasts also produce collagen, a major constituent of ECM in bone, which is further mineralized by calcium deposition. In the present study, guaiacin, isoguaiacin and isoguaiacin dimethylether increased ALP activity, respectively. Isoguaiacin and isoguaiacin dimethylether also increased col-
Phytoestrogens are known to exert stimulatory effects on osteoblast differentiation, in part, by acting on ER \textsuperscript{23,24}. The structures of phytoestrogens, although they are different each other, are similar to that of 17\(\beta\)-estradiol, in some part. In our study, the activities of arylnaphthalene lignans were more effective in osteoblast differentiation than those of bibenzylbutane lignans. When the structures were compared, the partial structures of arylnaphthalene lignans and 17\(\beta\)-estradiol are similar to each other, which can be one explanation for their stronger activity. The results using tamoxifen supported the involvement of ER in action of arylnaphthalene lignans on osteoblast differentiation.

In conclusion, our present study suggests that lignans isolated from \emph{M. thunbergii} increased osteoblast differentiation in primary cultures of mouse osteoblasts by increasing ALP activity, collagen content and mineralization. We also suggested structure–activity relationship of lignans related to osteoblast differentiation. In agreement to the present study, some lignans are known to stimulate osteoblast differentiation, not only \textit{in vitro} but also \textit{in vivo} study\textsuperscript{11,12}. Thus, it will be of interest to test further whether these lignans increase osteoblast differentiation \textit{in vivo}, for example, in animal models of osteoporosis, to explore their therapeutic potentials for osteoporosis. This will provide further insight into the design of new approaches to osteoporosis.

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**REFERENCES**