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Phytoestrogens Formononetin and Biochanin A stimulate osteoblast proliferation and influences osteogenesis *in vitro.*

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ABSTRACT

Hormone replacement therapy (HRT) with estrogen is the gold standard treatment for postmenopausal osteoporosis, but is limited by the side effects that is caused in the elderly population. Phytoestrogens possess structural similarity to estradiol and may possess estrogen receptor agonistic or antagonistic effects depending on the target tissue and hence can be selective estrogen receptor modulators(SERMs). There is a considerable interest in the development of antiosteoporotic SERMs that will possess estrogen agonistic effects on the bone and estrogen antagonistic effects in the reproductive tissues. In the current study, the effects of two isoflavone phytoestrogens Formononetin and Biochanin A on osteoblast growth was evaluated *in vitro* on human osteoblast-like cells MG-63 and rat primary culture of osteoblasts isolated from femur. Cell growth and viability assays like MTT assay, neutral red dye uptake test, NBT reduction test and Sulphorhodamine B assay were carried out to understand the effect of phytoestrogens on osteoblast growth. Cytomorphological studies involving phase contrast microscopy analysis and crystal violet staining of the cells were carried out to support the cell viability assays. Results of the study implicate the positive influence of phytoestrogens Formononetin and Biochanin A on osteoblast growth and their ability to influence bone remodeling*in vitro*. The osteoblast stimulatory effects of Formononetin was found to be superior to that of Biochanin A at the dose employed and in the model systems used in the study.

Keywords: Phytoestrogens, Formononetin, Biochanin A, Osteoblast, Cell viability

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INTRODUCTION

Post menopausal osteoporosis in women is caused due to the decline in the levels of estrogen (1) that contributes significantly to an increased rate of bone remodeling and leads to an imbalance between bone resorption and bone formation. Increased bone resorption leads to bone loss, enhanced bone fragility, increased susceptibility to fractures and culminate in osteoporosis (2).Hormone replacement therapy(HRT) is the first line therapy for post menospausal osteoporosis (3). But it causes an array of painful side effects that in majority of the patients the therapy is either abandoned or advised to be withdrawn (4). Hence prevention and management of osteoporosis need to focus on improved safety and tolerability in patients especially elderly women. This stimulated considerable interest in the clinical development of Selective Estrogen Receptor Modulators (SERMs) as antiosteoporotic agents (5). SERMs can have estrogen receptor agonistic or antagonistic activity depending on the target tissue (6).

Phytoestrogens are natural plant derived compounds that has structural similarties to estradiol and can have estrogen mimicking effects (7). They are considered to be safe alternatives to HRT with estrogen. Formononetin and Biochanin A are o-methylated isoflavones- phytoestrogens, produced by red clover (*TrifloiumPratenese*) and few other leguminous plants. Formononetin and Biochanin A show structural similarity to estrogens(8) and hence can bind estrogen receptors thereby resulting in downstream signalling effects.The aim of the present study is to evaluate the prostimulatory effects of Biochanin A and Formononetin on osteoblast model systems *in vitro.*

MATERIALS AND METHODS

Chemicals and Reagents

Formononetin, Biochanin A and 17 β estradiol were purchased from Sigma Aldrich Company (St Louis, USA). A stock solution of the test compounds was prepared by dissolving 1 mg of the drug in dimethyl sulfoxide (DMSO) and final concentration (0.1%) the test compound was prepared and frozen at -20ºC in small aliquots. From the stock solution, appropriate dilutions were carried out to prepare various concentrations of the test compounds for different analysis.

In vitro model systems

Procurement and maintenance of human osteoblast-like cells MG-63

Human osteoblast-like cells MG-63 was procured from National Centre for Cell Sciences (NCCS), Pune, India and cultured in ready to use sterile liquid Minimum essential Medium-Eagle supplemented with 1X antibiotic antimycotic solution and 10% fetal bovine serum. Cells were grown under standard growth conditions (temperature 37◦C, 5% CO2 and 95% humidity) in a CO2 incubator (Forma Scientific, USA). When a confluent monolayer was formed, cells were detached with 0.25% trypsin–0.2% EDTA in Dulbecco's phosphate buffered saline and then subcultured at a split ratio of 1:3 in 12.5 cm2 volume tissue culture flask. The media was changed three times a week. The cells were grown in growth medium containing 10% FBS or maintained in maintenance medium containing 5% FBS. After arriving at confluency, the cells were seeded on to 96 well microtiter plates and were utilized for various cell proliferation, cytotoxicity and bone mineralization assays.

Isolation of osteoblast from rat femur and maintenance of primary culture

Adult female Sprague-Dawley rats weighing about 120–140 g were used for the isolation of osteoblasts from femur. The animals were procured from authentic animal sources of the University and were subjected to a quarantine period of two weeks. After the quarantine period, the animals were used for the experiment. This part of the study which involves the usage of animals was carried out following ethical guidelines and is approved by the institutional animal ethics committee (Approval No. Dr MSP-SK/DST WOS-A/IAEC/BUB dt 17/01/2015). The animals were sacrificed by cervical dislocation under ether anaesthesia and osteoblasts isolated from femur by following the method of Bard et al (9) and as modified by (10,11).

Assays for cell growth and proliferation

MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed following the method (12). Briefly the cells were plated and treated with different concentrations of test compounds and allowed to proliferate for 72 hours. After 72 hours, 25µl of MTT (5 mg/ml in phosphate buffered saline - pH 7.4) was added to the wells. The plates were then incubated in a CO2 incubator for six hours. After six hours the formazan crystals produced were solubilised by adding 75µl of DMSO to each well. The intensity of the colour developed was read at 570nm in a micro plate reader (Bio-Tek systems, USA).

Crystal violet test

To determine the cell viability, crystal violet assay was carried out following the method (13). The cells were plated and treated with different concentrations of test compound and allowed to proliferate for 72 hours. After 72 hours of proliferation the cells were fixed in 10% formalin saline (50µl/well) for 30 min. Then the cells were stained with crystal violet (0.05% w/v) for 30 min. The wells were then washed thoroughly with distilled water to remove any unbound dye and destained with Sorenson's buffer (0.1 M sodium citrate in 50% ethanol, pH 4.2). The absorbance of the extracted stain was measured at 540 nm.

NBT reduction test

NBT (Nitrobluetetrazolium) reduction test was performed following the method (14). Briefly, after 72 hrs of proliferation, 10µl of nitro blue tetrazolium chloride (5 mg/ml in phosphate buffered saline – pH 7.4) was added to the cultured cells and incubated in a CO2 incubator at 37ºC for 5 hrs. The cells were then washed three times with saline and the formazan crystals were solubilised by adding 100µl of isopropanol. The optical density was measured at 570nm in a microplate reader.

Neutral red dye uptake test

Neutral red dye uptake test was performed by the method (15). Briefly, after 48 hrs of incubation, the medium was removed and viable cells were stained for 45 minutes with 5 µl of neutral red (0.1%). The stain was then removed and the wells were washed three times with 0.9% saline. Neutral red was then released by the addition of 1:50 mixture of 100mM acetic acid and ethanol. The optical density was measured at 570nm in a microplate reader.

SRB (Sulfarhodamine B) dye uptake test

Cell growth was assessed by SRB assay as previously reported (16). Briefly cell suspension containing $1X10^6$ cells /ml were plated onto 96 well plates and allowed to attach for 24h at 37^0 C in a 5 % CO_2 atmosphere. The cells were then exposed to test compounds for 48h, cells were washed with PBS and fixed with TCA at 4^0 C for 1h. After washing with water, cells were stained with SRB (0.4%w/v SRB in 1% v/v acetic acid). Protein bound was solubilized with unbuffered 10mM tris buffer. Intensity measured at 540 nm.

Trypan blue dye exclusion test

This test was performed following the method (17). After 48 hrs of incubation, appropriately diluted cells (treated, untreated and control groups) were mixed with trypan blue (0.1%) and the suspension was charged on to a haemocytometer. The number of viable cells present in the treated and non-treated groups were counted under an inverted microscope and the cell count was determined by employing the formula

Cell count/ml = Mean cell count X dilution factor X 10^4

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Light microscopic analysis of the cells

Giemsa/crystal violet staining

After 48 hrs, the cells were fixed using Carnoy's fixative (Carnoy's Solution is composed of 1g of ferric chloride (FeCl3) dissolved in 24 mL of absolute alcohol, 12 mL of chloroform and 4 mL of glacial acetic acid) and subsequently stained with 0.5% Giemsa / crystal violet. The stained cells were examined under microscope and photographed. The change in the morphology of treated cells, if any, was compared with that of the untreated control cells.

Phase contrast microscopic analysis of the cells

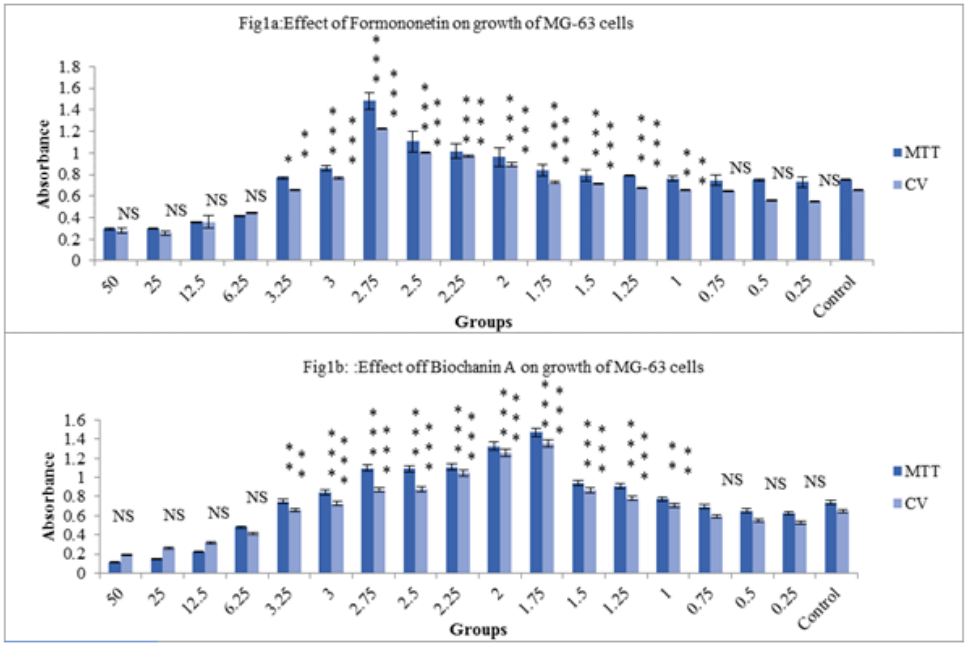
Briefly, the cells (5 X 10^6 cells/ml) were cultured on six well plates. After 24 hrs of proliferation, cells were treated with the plant extract and the plates were observed under a phase contrast microscope and the cells were photographed (Olympus, Japan). The change in the morphology of treated cells, if any, was compared with that of the untreated control cells.

Statistical analysis

All the experiments were carried out in triplicate on at least three different occasions and the mean of replicate values were taken. Values were expressed as mean± SD. Statistical analysis of the data was determined by Student's t-test and comparisons were made between the control and the test groups.

RESULTS

Evaluation of the effect of Formononetin and Biochanin A on cell growth and viability on osteoblast model systems in vitro (Figs. 1a, 1b and 2a, 2b)-Pilot Studies



In both the assays, treatment of MG-63 cells and primary osteoblast cells with lower concentrations (0.25, 0.50, 0.75µg/ml) of test compounds showed no influence on the proliferation of the osteoblasts cells as compared to control. Maximum increase in the cell growth was observed at high concentrations (at 2.75µg/ml of Formononetin and 1.75 µg/ml of Biochanin A) on MG-63 cells and (at 2 µg/ml of Formononetin, 1.5 µg/ml of Biochanin A) on primary osteoblast cells. Higher concentrations (6.25-50µg/ml) failed to elicit any profound influence on growth of the cells.

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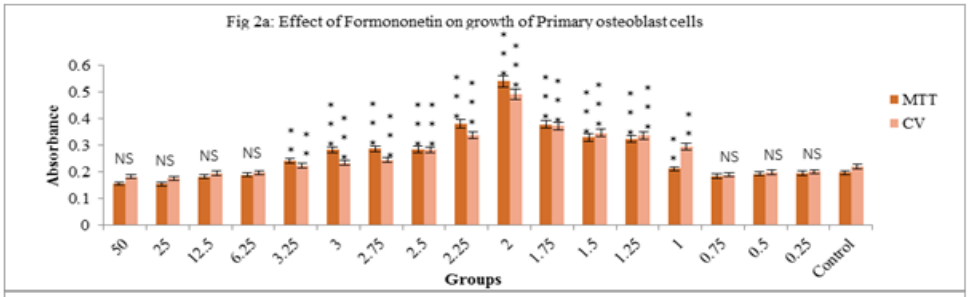
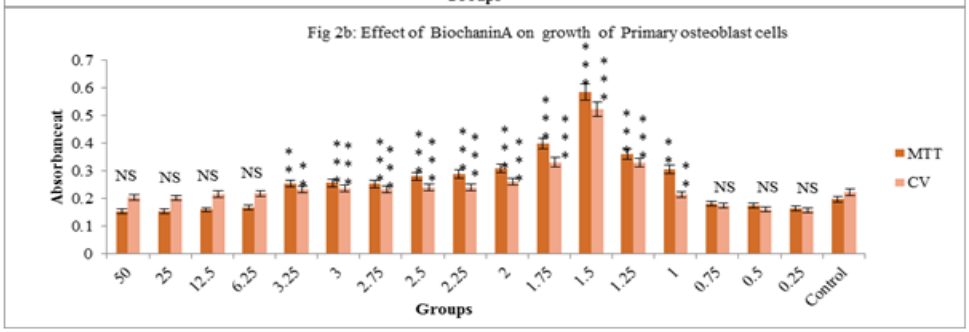
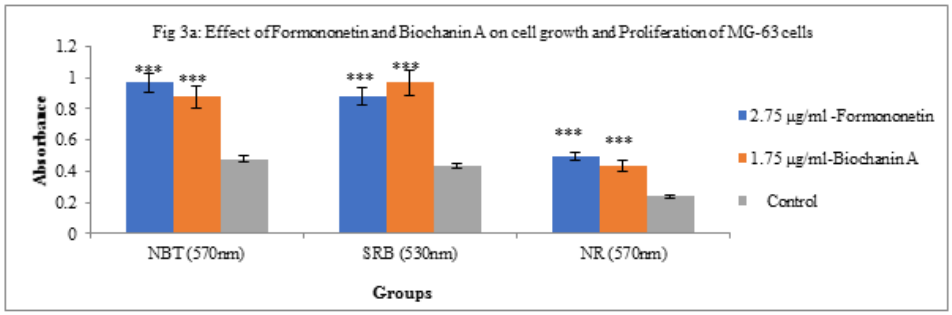
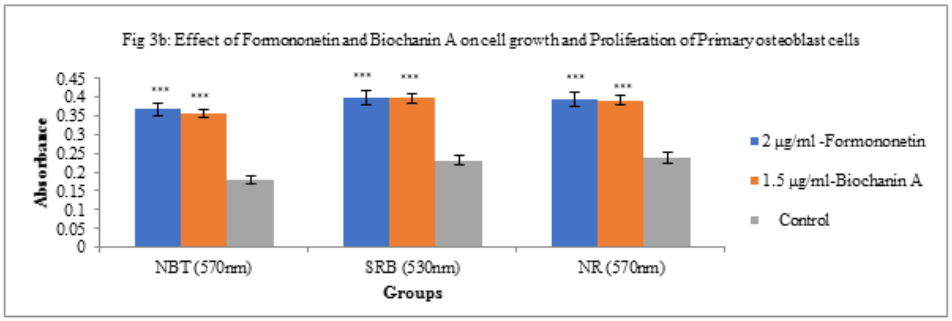




Evaluation of the effect of Formononetin and Biochanin A on cell survival on osteoblast model systems *in vitro* (Fig 3a, 3b)





Hence based on MTT and Crystal violet assays concentrations of Formononetin (2.75 µg/ml) and Biochanin A(1.75 µg/ml) on MG-63 cells and Formononetin (2 µg/ml) and Biochanin A (1.5 µg/ml) on primary cells were used for all subsequent assays.

These concentrations were selected after preliminary pilot studies. A battery of short term *in vitro* assays (comprising neutral red dye uptake, NBT reduction test, crystal violet test, SRB uptake test) were performed to confirm the results obtained with MTT assay. The results of these tests were in tandem with the results of MTT assay and based on all these tests it was concluded that treatment with the specified concentrations of tests compounds resulted in statistically significant increase in cell viability, growth, survival and proliferation of MG-63 cells and primary culture of osteoblasts ($P < 0.001$).

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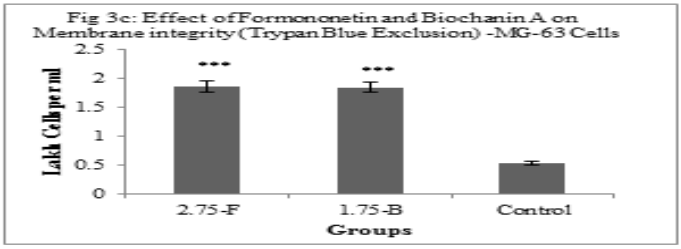
Effect of Formononetin and Biochanin A on osteoblast morphology-cytopathological and cytochemical analysis

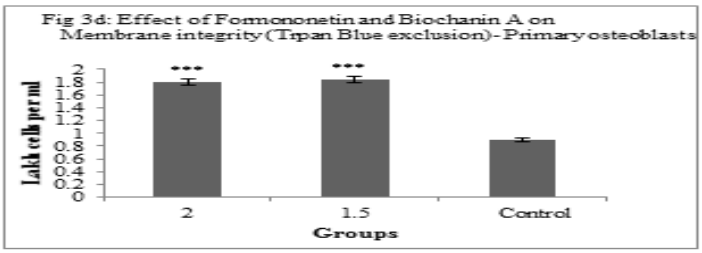
Giemsa/crystal violet staining (Plate 3)

The results of Giemsa/crystal violet staining was in line with the results obtained from cell viability assays showing increased cell numbers in the group of cells treated with various concentrations of the extract as compared to the control cells. No significant change or alteration in the morphology was noticed in the treated groups as compared to control group.

Phase contrast microscopy (Plate 4)

The human osteoblast-like MG-63 cells, with the typical spindle shaped fibroblastic appearance, were mononucleated when observed under a trinocular inverted phase contrast microscope. The groups of cells treated with different concentrations of test compounds showed faster growth but did not show any undesirable change in morphology and were comparable to the untreated control cells. The primary culture of osteoblasts treated with test compounds showed quicker fusiform appearance and increased cell numbers as compared to the control cells. No undesirable change in morphology was noticed as compared to control.





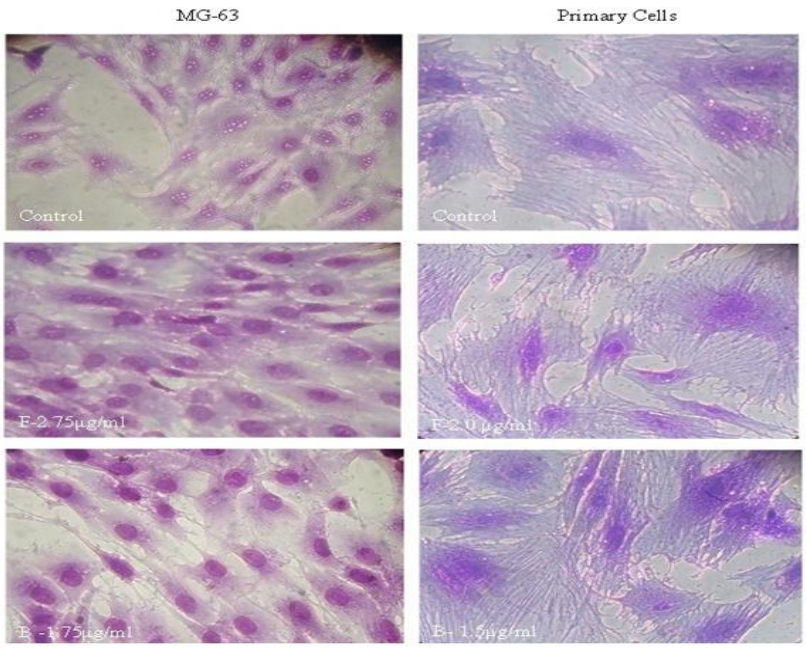


Plate 1-Effect of Formononetin and Biochanin A on Morphology of Osteoblasts-Crystal Violet Staining

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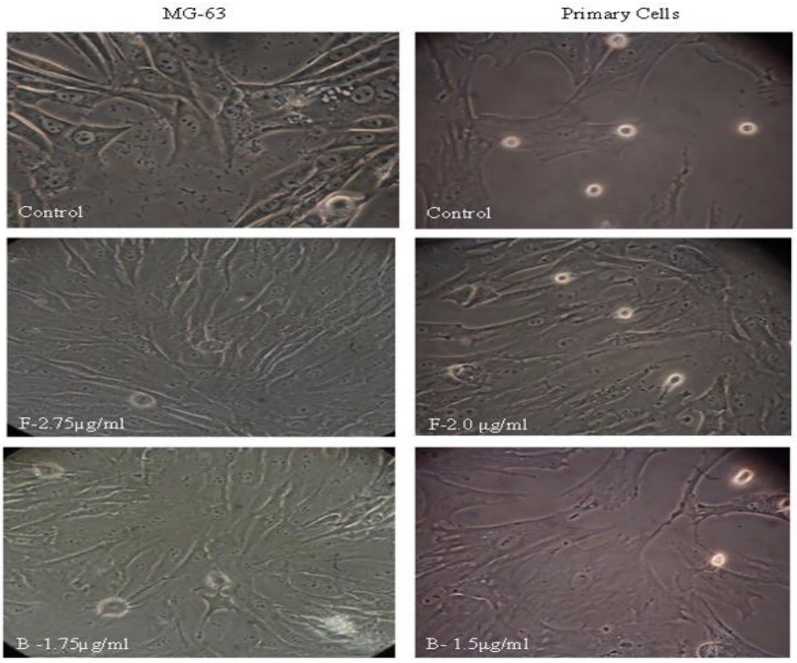

Plate 2-Effect of Formononetin and Biochanin A on Morphology of Osteoblasts- Phase Contrast Microscopy

DISCUSSION

Phytoestrogens are plant derived compounds with estrogenic activity found in natural diet (18). They can act as selective estrogen receptor modulators with agonistic effects on the bone and antagonistic effects on the reproductive organs, which could be beneficial in the treatment of osteoporosis (19). Globally several research groups have made an attempt to understand the beneficial effects of phytoestrogen supplementation to combat against postmenopausal bone loss in experimental animals(20). Most of such studies were limited to soy isoflavonesgenistein, and daidzen. Formononetin and Biochanin A are o-methylated isoflavones - phytoestrogens, produced by leguminous plants (21) andare reported to have estrogen mimicking actions. Till date, no detailed investigation was carried out on the phytoestrogens Formononetin and Biochanin A with respect to osteoprotective properties.Hence in the current study an attempt was made to understand the influence of Biochanin A and Formononetin on human osteoblast-like cells and rat primary osteoblasts by both cell based biochemical assays and cytomorphological analysis.

MTT assay is a standard reliable assay to study the influence of test compounds on cell growth and viability and serve as a precise indicator of cytostimulatory or cytotoxic effects. Increased numbers and viability of osteoblast cells upon treatment with Formononetin and Biochanin A implicate the prostimulatory effects of these compounds on osteoblast growth. The results obtained with other cell growth and viability assays like neutral red dye uptake, NBT reduction, crystal violet and sulphorhodamine B assay clearly indicate the ability of these compounds to positively affect osteogenesis*in vitro*.This was again confirmed by cytomorphology and cytochemical analysis wherein increased cell numbers were observed upon treatment with the compounds by both phase contrast microscopy analysis and by cytochemical staining with crystal violet. The absence of any adverse change in the morphology of the cells when treated with the phytoestrogens indicate their nontoxic effects on osteoblasts. Interestingly, both the phytoestrogens did not induce any growth stimulatory effects at lower doses of <1µg/ml and at higher doses > 6µg/ml up to 50µg/ml. This clearly indicate that the observed prostimulatory effects of Formononetin and Biochanin A on both MG-63 cells and rat primary osteoblasts are strictly dose dependent. Further studies are in progress in the laboratory to understand whether these phytoestrogens can act as selective estrogen receptor modulators and thereby could be used as potential alternatives to synthetic estrogen in the treatment of postmenopausal osteoporosis.

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