Effect of ethnomedicinal plants on osteoblast proliferation using cell line based assay method

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Abstract

Osteoporosis reduced bone strength and it is responsible for bone fractures. Worldwide, approximately one in three women and one in five men over the age of 50 will suffer an osteoporotic fracture in their remaining lifetime. Since many decades, herbal plants are utilized to increase bone health, however many of them are not scientifically evaluated. We selected herbal plants on the basis of their ethnobotanical use of scientific evidence that suggests a potential efficacy in the treatment of bone-loss diseases. This study was aimed to evaluate the cell proliferation activity of selected ethnomedicinal plants on MG 63 cell line. Aqueous and ethanolic extracts of Asparagus racemosus (Ar), Berberis aristata (Ba), Emblica officinalis (Eo), Hemidesmus indicus (Hi) and Nigella sativa (Ns) were screened for osteoblast cell proliferation study using MTT assay method. Three different ranges of concentration were selected for each extracts and percentage proliferation was analyzed using MTT assay. In conclusion, Ns have a highest cell proliferation capacity amongst selected plant. Ar and Ba cause inhibition effect on cell. Wherever, Eo and Hi cause moderate stimulatory effect on MG 63 cell line.

Keywords: Asparagus racemosus, Berberis aristata, Emblica officinalis, Hemidesmus indicus, Nigella sativa, fracture, osteoporosis

Introduction

Since last few decades, it has been observed that fractures due to osteoporosis is a major cause of disability and morbidity in the established nations and it will lead to increased significant socio-economic burden and cause sacrifice health related quality of life of patient. In India, the burden of fracture is drastically increased due to the life style modification and other risk factors. Although several synthetic drug therapies has been shown to be effective in prevention and treatment of osteoporosis, substitutes are constantly being searched because of its definite or possible side effects, or contraindications limiting their use, and poor adherence to treatment of patients. Herbal treatment for osteoporosis would be advantageous because natural plants have the potential of having fewer side effects, making them more suitable for long-term use. Today, it is estimated that about 80% of individuals in the developing countries still rely on traditional medicine-based largely on plants and animals for their primary health care. Herbal medicines are currently in demand, and their popularity is growing day by day.[1-3]. Since ancient time, many herbal plants are utilized for the healing of fracture. They also utilized in other co-morbid conditions of bones like rheumatoid arthritis, osteoarthritis, osteoarthritis and osteomalacia. Based on this literature information, there are total five drugs (Asparagus racemosus (Ar), Berberis aristata (Ba), Emblica officinalis (Eo), Hemidesmus indicus (Hi) and Nigella sativa (Ns)) selected for their effect on osteoblast proliferation. During the selection of the plant, we also focused on its ethnombotanical uses as an anti-inflammatory action and its phytoconstituent because of the established link between the inflammation, estrogen and bone loss. These herbal plants might have an effect on bone cells, which may leads to improve the bone disease condition but scientifically it is not evaluated till the date. So, the purpose of our study is to identify and analyze the effect of aqueous and methanolic herbal plants extracts on osteoblast proliferation using MG 63 cell line based MTT assay method.

Materials and Methods

Collection and authentication of selected plants

The roots of Asparagus racemosus and fruits of Emblica officinalis were collected from the Botanical garden of Gandhinagar. The stem bark of Berberis aristata, roots of Hemidesmus indicus and seeds of Nigella sativa were purchased from Lallu Vrajlal Gandhi (LVG) herbal store, Ahmedabad, Gujarat, India. The voucher specimen and herbarium for the same were deposited and authenticated by expert of department of Pharmacognosy, K. B. Institute of
Pharmaceutical education and research, Gandhinagar. The collected plant material was washed, dried under shadow and then pulverized to coarse powder using pulverizer and kept in airtight container in cool, dark and dry place till further use.

Preparation of extracts for in vitro cell proliferation activity
Authenticated plant material was powdered and aqueous extract (ArA, BaA, EoA, NsA, HiA) was prepared using hot maceration technique. Hundred gm of powder was mixed with 1000 ml of distilled water and then it was heated on boiling waterbath for six hours and allowed to stand overnight. The mixture was then filtered and the marc was extracted twice again in the same manner. The filtrates from each extraction step were pooled and concentrated to dryness (% Yield: ArA (33.7 %), BaA (10 %), EoA (11.3 %), NsA (8 %), HiA (9.6 %)). For ethanolic extract (ArE, BaE, EoE, NsE, HiE), 100 gm of powder were extracted separately with 1000 ml of methanol by heating under reflux on waterbath for 6 hours at 55°C. The mixture was then filtered and the marc was extracted twice again in the same manner. The filtrates from each extraction step were pooled and concentrated under vacuum using a rotary vacuum evaporator. The concentrate was evaporated to dryness at temperature not exceeding 60°C (% Yield: ArE (11.4 %), BaE (5.5 %), EoE (10 %), NsE (11.7 %), HiE (8.3 %)).

Phytochemical Analysis of plant extracts
Extracts were subjected to various qualitative tests to detect the presence of phytoconstituents like alkaloids, flavonoids, saponins, carbohydrates, sterols and terpenoids, anthraquinone glycosides, coumarins and tannins.

Procurement of MG 63 cell line and it’s Maintenance
For this study, the human bone osteosarcoma cell line, MG-63, was used as the test system and used for the study of different plant extracts effects on cell proliferation. It was procured from NCCS, Pune. This MG 63 cell line was obtained from 14 years human male bone with osteosarcoma. Cell line was maintained as per ATCC protocol throughout the project work.

MG 63 cell line was cultured in minimum essential medium-Eagle supplemented with 1X antibiotic antimiycotic solution (A007, Himedia, India) and 10% fetal bovine serum(FBS-RM1112, Himedia, India). 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 (T-001, Himedia, India) and then subcultured at a split ratio of 1:2 in 25 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 96well microtitre plates and were utilized for various cell proliferation assays. All the chemicals used in this experiment were analytical grade.

Trypan blue dye exclusion assay for cell viability
Trypan Blue is acid dye that has two azo chromophore groups. Trypan blue will not enter into the cell wall of living cells. Trypan Blue is an essential dye, use in estimating the number of viable cells present in a population. Make a cell suspension in a fixed volume of cells. Take 50μL of cell suspension and mix it with an equal volume of trypan blue. Mix solution carefully and load on haemocytometer and count the live cell as clear form and dead blue cell under inverted microscope. Calculate the number of cells/ ml, and the total number of cells, using the following formula. Calculate percent viability by using formula:

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% \text{ Viability} = \frac{\text{live cell count}}{\text{total cell count}} \times 100
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MTT- A cell proliferation assay
Trypsinized the MG 63 cells and perform cell count by trypan blue assay. Prepare cell dilution accordingly for 96 well plates. Cells (5000-10000 cells/ well) were seeded in a flat-bottomed 96-well plate and incubated for 24 hour at 37°C and in 5% CO₂. Cells were treated with plant extracts at 3 different concentrations (100μg/ml, 100μg/ml and 10μg/ml) for 48 hours. Alendronate was used as a Positive control. Add 20μl of MTT reagent (0.5 mg/ml) to cells for 4 h at 37°C in dark. All media and MTT reagent was removed and 200 μl DMSO was added to each well to dissolve the formazan crystals. The optical density (OD) was recorded at 570 nm in a microplate (ELISA) reader. Percentage of cell viability was determined using standard formula.

Statistical analysis
The data was expressed as mean ± standard error of the mean (SEM). Statistical calculations were performed by applying one-way analysis of variance (ANOVA) followed by Tukey Test, using Graph Pad Prism software. The results were considered statistically significant if the P<0.05.

Result:
All the macroscopic characters were comparable with those mentioned in literature review. In the preliminary phytochemical screening it was found that aqueous extracts mainly contain carbohydrate, saponins and alkaloids wherever, ethanolic extract mainly contain saponin, tannin, steroids and flavonoids.

MTT assay
MTT assay was used to check the proliferation of the cell and which indicates the viability of the cell. Alendronate has a positive effect on the bone cells and used as a standard for the current assay. In our study we observed that, alendronate cause remarkably increase the proliferation of MG 63 cells. From the literature review, as previously noted we selected five herbal plants for the MTT assay. One of the drugs is Asparagus racemosus, commonly known as a shatavari also studied for their effect on osteoblast proliferation. ArA in higher concentration leads to inhibitory effect on the cells, and it is a dose dependent inhibition, wherever ArE cause not much stimulation as compare to basal media and it may inhibit osteoblast proliferation rather than the stimulation.
Berberis aristata, locally recognized as a daruharida also exhibited the same effect like Ar. BaA and BaE both decrease the percentage viability of the cells in all concentration, which revealed that BaA and BaE instigate the osteoblast inhibition. Emblica officinalis, a well-known anti-oxidant plant trigger the osteoblast cells in the higher concentration of EoA only. However, EoE did not show any stimulation in the all three concentrations. Hemidesmus indicus, vernacular name is anatmool was cause stimulation in higher concentration only in both the extracts. Lower concentration does not show any effect on cells. The UV absorbance of MTT assay, in STD and treated group in different doses and % Proliferation is reported in figure 1 and 2 respectively.

![Fig 1: Proliferation activity of selected ethnomedicinal plants using MTT assay.](image)

Fig 1: Proliferation activity of selected ethnomedicinal plants using MTT assay. BM- Basal media, ALD-Alendronate standard, NsA and NsE- aqueous and ethanolic extract of Nigella sativa, ArA and ArE- aqueous and ethanolic extract of Asparagus racemosus, BaA and BaE- aqueous and ethanolic extract of Berberis aristata, EoA and EoE- aqueous and ethanolic extract of Emblica officinalis, HiA and HiE- aqueous and ethanolic extract of Hemidesmus indicus. All values represent mean ± S.E.M; n=3; ***p<0.001, ** p<0.01, *p<0.05 compared with BM.

![Fig 2: Percentage proliferation of selected ethnomedicinal plants.](image)

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Discussion

Osteoporosis, a silent epidemic has become a major health hazard in recent years, afflicting over 2000 million people worldwide [6]. Frailty fractures due to osteoporosis currently is a major public health burden throughout the Asia Pacific. Due in large part to the dramatic aging of the region’s population, there is an exorbitant increase in the number of fracture patients in the coming decades [7]. The number of women with osteoporosis, ie, with reduced bone mass and the disruption of bone architecture, is increasing in India. While data on the prevalence of osteoporosis among women in India come from studies conducted in small groups spread across the country, estimates suggest that of the 230 million Indians expected to be over the age of 50 years in 2015, 20%, ie, ~46 million, are women with osteoporosis. Thus, osteoporosis is a major public health problem in Indian women [8]. Though ovarian hormone deficiency is a major risk factor for osteoporosis in the postmenopausal women, hormone replacement therapy (HRT), perhaps the most effective treatment, is not preferred as it increases the risk of breast cancer and of cardiovascular diseases. The other available therapeutic agents are also associated with certain adverse effects. In this context, phytoestrogens are believed to play a role in maintaining or improving skeletal health. The advantages of the natural drugs are their easy availability, and negligible side effects but on the other side there is the disadvantage that they do not have consistent quality. A significant factor which can add to the consistent quality of the medicinal plant is to have satisfactory standardization. Due to the resurgence of interest in herbal drugs demand and hence the supply of herbal drugs has increased. In order to maintain trust in herbal medicines, it is also important to ensure that only quality products enter the market.

Certain plant compounds, which have been characterized as phytoestrogens, have shown a weak estrogenic effect on bone in human and animal studies [9]. It is also reported that plants with anti-inflammatory can be potent candidates as an osteoprotective agent [10]. From many years herbal drugs have been traditionally used in Ayurveda to accelerate the healing of bone fractures and to strengthen the bones [11].

In the MTT assay, the viable cells convert the yellow colored MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a purple colored formazan crystals which can be quantified using a spectrophotometric method. In osteoporosis there is deterioration of equilibrium between bone synthesis and bone resorption. So for treatment of osteoporosis either increase bone synthesis by osteoblast proliferation and decrease bone resorption by Antiosteoclastic activity. The extracts which shows more proliferation of osteoblast like cell MG 63, can act on one of the mechanism for treatment of osteoporosis. Both extracts of Nigella sativa show more than 100% of cell proliferation at moderate concentration while fruits of Emblica officinalis and roots of Hemidesmus indicus show dose dependent cell proliferation activity but there is not as makeable as Nigella sativa.

Conclusion

From this MTT assay, we can conclude that NsA and NsE have the drug, which cause significant stimulation in the MG 63 cell line amongst all five-selected drug. It will be potential treatment for the osteoporotic patient. This drug can further be use for the preparation of herbal formulation for the treatment of osteoporosis.
References