



## Action of *Berberis aristata* on various aspects of bone remodeling

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### Abstract

**Context:** Osteoporosis is an age-related bone disease in which bone mineral density is decreased which may lead to fracture with minimal trauma or without severe stress to bone. Several herbs have been used in Ayurveda for treatment of bone fracture. *Berberis aristata* (BA) stem bark, traditionally known as *Daruharidra*, is one such drug used in Ayurveda for bone-related disorders.

**Aim:** The aim of the study was to examine the effects of *B. aristata* stem bark on various aspects of bone-remolding using *in-vitro* cell-based assays.

**Materials and Methods:** The effect of alcoholic and aqueous extracts of *Berberis aristata* on proliferation of primary bone marrow cells (mesenchymal stem cells) was studied using MTT assay. The stimulation of differentiation of primary bone marrow cells into osteoblasts was assessed by measuring ALP activity, which is marker of osteoblast activity. The inhibition of osteoclast formation by the extracts was assessed by quantifying the TRAP activity. The deposition of calcium in extracellular matrix by osteoblasts was observed under microscope after formation of an orange-red complex of calcium with alizarin red S.

**Statistical analysis used:** Statistical analysis of the data was performed by one- way ANOVA followed by Tukey's multiple comparison test

**Results:** The aqueous extract of stem bark of *Berberis aristata* was found to stimulate the differentiation of primary bone marrow cells to osteoblasts, to promote the deposition of calcium in extracellular bone matrix and to inhibit the formation of osteoclasts.

**Conclusions:** *Berberis aristata* stem bark acts on various aspects of bone remodeling, justifying its ability to restore equilibrium between bone formation and resorption in osteoporosis.

**Keywords:** *Berberis aristata*, bone-remodeling, osteoblast, osteoclast, mesenchymal stem cells

### 1. Introduction

Osteoporosis causes progressive bone loss, which renders the bones susceptible to fractures and is popularly known as "the silent disease" because early symptoms are absent [1]. According to a WHO report, osteoporosis has been recognized as an established and well-defined disease that affects more than 75 million people in the United States, Europe and Japan [2]. In osteoporosis, fractures can occur with minimal trauma. These fractures are common and have extensive medical and personal burden on individuals and also on the nation. Osteoporosis can be prevented, diagnosed and treated before any fracture occurs and effective treatments can be used to decrease the risk of further fractures even after first fracture has been occurred [3].

Plants have been the rich source of medicines throughout the world from thousands of years and still provide new remedies to mankind. Natural products for the management of osteoporosis are largely phytoestrogens which include isoflavones, lignins, flavonoids, and coumestans that share structural and functional similarities with naturally occurring or synthetic estrogens [4]. *Berberis aristata* (BA) which belongs to the family Berberidaceae, is one of the Indian medicinal plants used in Ayurveda for several remedies. *Berberis aristata* is spinous herb native to sub-

Himalayan regions and Nilgiri Hills of southern India [5]. It is also known as *Daruharidra* or Indian beriberi [6]. It is included in pharmacopoeia of many countries such as Britain, India, China and in several other Homeopathic Materia Medica [7]. It is used as a tonic, alternative, demulcent, diaphoretic and diuretic, and in the treatment of diarrhea, jaundice, skin diseases, syphilis, chronic rheumatism and urinary disorders [8]. Its roots, stem, bark, leaves, rhizomes and fruits are used in many classical Ayurvedic preparations. Extract of BA is used in pharmaceuticals, nutraceuticals and cosmeceutical preparations. Various pharmacological activities of BA have been explored and scientifically documented [6, 8] BA has been reported to exhibit anti-osteoporotic activity in ovariectomized (OVX) rat model. Aqueous-methanol extract of BA possesses potent antiosteoporotic activity in OVX rats and substantiates the ethnic use of BA in the treatment of postmenopausal osteoporosis [9]. Till date, no detailed *in vitro* scientific investigation was carried out to explore the influence of BA on bone remodeling. In the present study, BA extract was evaluated for its ability to promote the proliferation of osteoblast, mineralization of bone matrix, conversion of primary bone marrow cells to mature osteoblast, and to inhibit osteoclast formation *in vitro*. Results of this study reveal the potential usefulness of

BA as an anti-osteoporotic agent and justify its use in the Indian systems of medicine in diseases related to bones and joints.

## 2. Materials and Methods

### 2.1 Chemicals, apparatus and equipments

Sterile liquid Dulbecco's minimum essential medium (HiMedia-DMEM AL007), Fetal bovine serum (HiMedia-RM1112), Trypsin-EDTA solution (HiMedia-TCL007), Dulbecco's phosphate buffered saline-PBS (HiMedia-TL1006), Trypan Blue Dye 0.4% w/v (HiMedia-TCL046), 1X antibiotic-antimycotic solution (10000 U Penicillin and 10 mg Streptomycin/ml) (HiMedia-A003), Alendronate as positive control, Dexamethasone (HiMedia-TC 277), Glycerol phosphate (HiMedia-GRM665), L- Ascorbic acid (HiMedia-TC094), Tris HCL (HiMedia-TC073), Triton X, ALP Kit- Span diagnostic (Code: 75DP200-20), disodium 4-nitrophenyl phosphate, potassium tartrate, 1,25-Dihydroxy Vitamin D<sub>3</sub>, Paraformaldehyde (HiMedia-GRM3660), Alizarin Red S, cetylpyridinium chloride, 70% (v/v) Iso-propyl alcohol.

CO<sub>2</sub> Incubator (Thermo Scientific), Inverted microscope, Bio- Safety Cabinet, Centrifuge (Eltek – Research Centrifuge TC 1400 F), Autoclave, Tissue Culture Flask (T-25 cm<sup>2</sup>) (HiMedia-TCG-4), Micro-pipettes (Tarsons India Pvt. Ltd.), Microtips (200-1000, 2-200 µl) (Tarsons India Pvt. Ltd. - 521010, 521020), Centrifuge tubes (15ml and 50 ml) (Tarsons India), ALP Kit (Span diagnostics; Code: 75DP200-20).

### 2.2 Procurement and authentication of drug

The stem bark of *Berberis aristata* was purchased from a local herb supplier in Ahmedabad, India. The stem bark was authenticated by its morphological and microscopical characteristics and physicochemical parameters, which were compared with the parameters mentioned in the pharmacopoeial monograph of The *Ayurvedic Pharmacopoeia of India*. A voucher specimen (Number: KBIPER/2017/V/02) of stem bark was deposited in the Pharmacognosy department of K. B. Institute of Pharmaceutical Education and Research, Gandhinagar, India. The dried bark was ground to a coarse powder using pulverizer and then stored in airtight container in a dry place till further use.

### 2.3 Extract preparation

The aqueous and alcoholic extract of BA were prepared by macerating 100 g aliquots of powdered stem bark with 1000 ml of distilled water and ethanol, respectively, at 70°C under reflux for 6 h. The mixture was 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. Percentage yields of aqueous and ethanolic extracts were 10% and 5.5%, respectively.

### 2.4 Primary cell culture

Three to four-week-old adult Sprague Dawley rats of either sex weighing about 120–140 g were used for the isolation of primary osteoblasts from the femur. The animals were housed at temperature of 25±1°C, relative humidity 55±5% and 12/12 hr light/dark cycle with normal chow diet and acclimatized for one week. After the quarantine period, the

animals were used for the experiment. The study was approved by the institutional animal ethics committee of K. B. Institute of Pharmaceutical Education and Research, Gandhinagar (approval No: KBIPER/2016/577). The rats were euthanized by cervical dislocation under ether anesthesia, the femurs were removed aseptically and the soft tissue and muscles attached were cleaned-off from the femur. Femurs were cut from both ends and bone marrow was flushed with 2 ml DMEM with help of a syringe with needle (27 gauge). Bone marrow cell suspension was filtered through sterile filter to remove cell debris and collected cell suspension was centrifuged at 1000 rpm for 5 min. The MSCs were seeded in T-25cm<sup>2</sup> tissue culture flask. Primary cells (MSCs) were in cultured DMEM supplemented with the 1X antibiotic-antimycotic solution and 10% FBS. MSCs were allowed to grow under standard growth conditions. The media was changed at every alternate day until the full confluence was achieved. Cells were trypsinized with 0.25% trypsin, 0.2% EDTA in Dulbecco's phosphate buffered saline and either subcultured at a split ratio of 1:2 in 25 cm<sup>2</sup> volume tissue culture flask or seeded in microtitre plates (96 well and 24 well plates) for different cell-based assays [10-12].

### 2.5 Cell viability assay

The viability of cells was determined by trypan blue dye exclusion assay. Primary cells were trypsinized and the cell suspension was prepared by mixing 20 µL of cell suspension with an equal volume of 4% trypan blue prepared in PBS. The mixture was loaded on a hemocytometer and the unstained (viable) cells and blue stained (nonviable) cells were counted separately. Percentage viability and the cell count (total number of cells) were calculated as follows, % viability = (viable cells/total cells) × 100.

Cultures having 90% viability were used for further assays [13].

### 2.6 Osteoblast proliferation assay

The primary bone marrow cells were plated into 96-well plate at 1×10<sup>4</sup> cells/ml in DMEM containing 10% FBS and incubated for 24h at 37°C and in 5% CO<sub>2</sub>. After 24h, the old medium was discarded and the cells were treated with 3 different concentrations (1000µg/ml, 100µg/ml, and 10µg/ml) of aqueous and ethanolic extracts of BA and allowed to proliferate for 48 h and Alendronate was used as a positive control. After 48 h of incubation with drug extracts, the 20 µl of MTT (final concentration 0.5mg/ml) was added to each well and incubated for 4h at 37°C in dark place. Media was carefully pipetted out without disturbing the cell monolayer and 200 µl of DMSO was added to each well, the plate was shaken carefully and incubated for 1h in 37°C in dark place. Optical density (OD) of each well was measured at 570nm in microwell plate reader. Percentage of cell viability was determined as, % Cell Proliferation = (Avg. OD of treated cells - Avg. OD of control cells) \* 100 / Avg. OD of control cells [14].

### 2.7 Osteoblast differentiation assay

Primary bone marrow cells were plated at the concentration of 2×10<sup>4</sup> cells/ml with DMEM in 96-well plates and incubated for 48h at 37°C and in 5% CO<sub>2</sub>. The spent media was then discarded, cells were washed with ice-cold PBS and treated with 3 different concentrations (1000µg/ml, 100µg/ml and 10µg/ml) of aqueous and ethanolic extracts of

BA. Osteogenic medium (10mM  $\beta$ - glycerol phosphate,  $10^{-8}$  M dexamethasone and 50 $\mu$ M- Ascorbic acid in final concentration in Basal media) was taken as positive control and basal media was considered as a negative control. Drug treatment was given on every alternate day, for 6 days. After completion of drug treatment, cell monolayer was washed with ice-cold PBS and cells were lysed by treatment with 200  $\mu$ l 0.5% Triton X for 20 min in an ice bath [15-17]. Alkaline phosphates activity in each well was measured using ALP assay kit. Optical density was measured at 405 nm after 30 min incubation of 50 $\mu$ l of cell lysate and 150 $\mu$ l working ALP reagent (prepared according to kit protocol).

### 2.8 Osteoclastic inhibition assay (Tartarate resistant acid phosphatase-TRAP assay)

Primary bone marrow cells were plated at a concentration of  $2 \times 10^4$  cells/ml in 96-well plates; cells were allowed to adhere to the wall for 48 hours for complete adherence. Next day spent media was discarded and cells washed with ice-cold PBS. Freshly prepared osteoclastic media (10nM 1,25-Dihydroxy Vitamin D<sub>3</sub> and  $10^{-7}$  M Dexamethasone in the final concentration in Basal media) [18] was added an alternate day for 6 days, to convert primary cells to osteoclasts. Then the cells were treated with 3 different concentrations (1000 $\mu$ g/ml, 100 $\mu$ g/ml, and 10 $\mu$ g/ml) of aqueous and ethanolic extracts of BA, for 48 hrs. Alendronate (1000 $\mu$ g/ml, 100 $\mu$ g/ml, and 10 $\mu$ g/ml) was used as positive control. After completion of the drug treatment, cells were washed twice with ice-cold PBS and the lysed by treating with 20  $\mu$ l of 0.1% TritonX-100 for 15min on an ice bath. To the cell lysate, 100  $\mu$ l of substrate solution (0.4g disodium 4-nitrophenyl phosphate and 2.0g potassium tartrate, dissolved in 200ml of deionized water, adjusting pH to 3.5 with 1M hydrochloric acid solution) was added and the plate was incubated at 37 $^{\circ}$  C for 30min. The 100  $\mu$ l of 1M NaOH was added to each well to stop the reaction. Optical density was measured at 405nm [19].

### 2.9 Matrix mineralization assay

Alizarin Red Staining was used to identify calcium content in osteoblasts according to a reported method [18] with slight modification. Approximately  $1 \times 10^4$  cells/well was plated in a 24 well plate. Primary cells were allowed to adhere for 48 h and thereafter they were treated with 4 different concentrations (1000 $\mu$ g/ml, 500 $\mu$ g/ml, 250 $\mu$ g/ml and 100 $\mu$ g/ml) of aqueous and ethanolic extracts of BA for 21 days, while the media was changed every alternate day. Dexamethasone ( $10^{-7}$  M) was used as positive control. After drug treatment, cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 15 min and stained for 30 min with 40 mM Alizarin Red-S (pH 4.5). Cells were again washed with distilled water and observed under inverted phase contrast microscope. Calcified nodules appeared as bright red color. Matrix mineralization was quantified by adding 100 mM cetylpyridinium chloride solution and incubating for 1 h. A 100  $\mu$ l aliquot of the supernatant from each well was transferred in a 96 well plate and the absorbance of each well was measured at 570 nm.

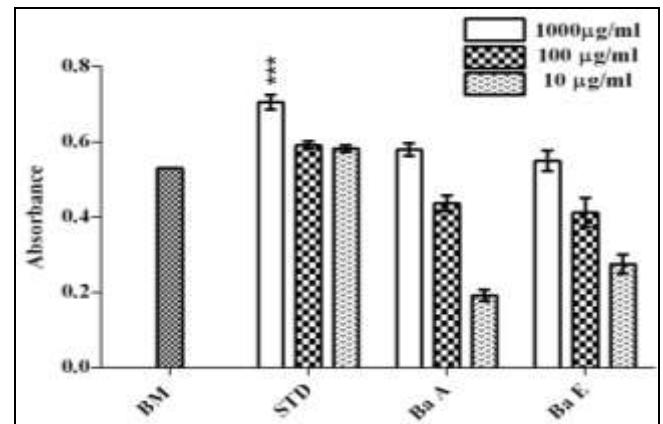
### 2.10 Statistical analysis

All the experiments were carried out in triplicate. Values were expressed as mean  $\pm$  SEM. Statistical analysis of the data was performed by one- way ANOVA followed by

Tukey's multiple comparison test, using Graph Pad Prism (version 5.3) software.

### 3. Results

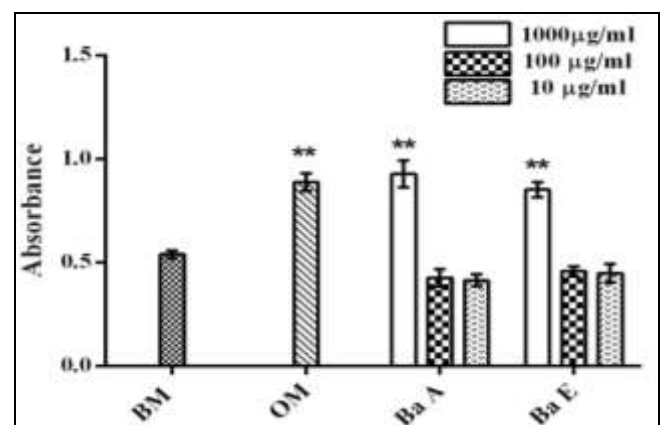
Cell proliferation activity of aqueous and ethanolic extracts of *Berberis aristata* (Ba A) and (BaE) on the primary mesenchymal stem cells was studied. BaA and BaE extracts were found to increase the proliferation rate of undifferentiated MSCs (Fig. 1). Standard Alendronate was taken as a positive control and basal media (BM) was taken as a negative control. Alendronate induced significant proliferation of MSCs at higher concentration (1000  $\mu$ g/ml). BaA and BaE showed dose-dependent cell proliferation activity.



**Fig 1:** Cell proliferation activity of aqueous and ethanolic extract of *Berberis aristata* on the primary Mesenchymal stem cells.

(BaA) aqueous extract of *Berberis aristata*, (BaE) ethanolic extract of *Berberis aristata*, (BM) Basal media, (STD) Alendronate as positive control. Values are expressed in mean  $\pm$  S.E.M. (n=3). \*\*\*p < 0.001 compared with negative control (BM).

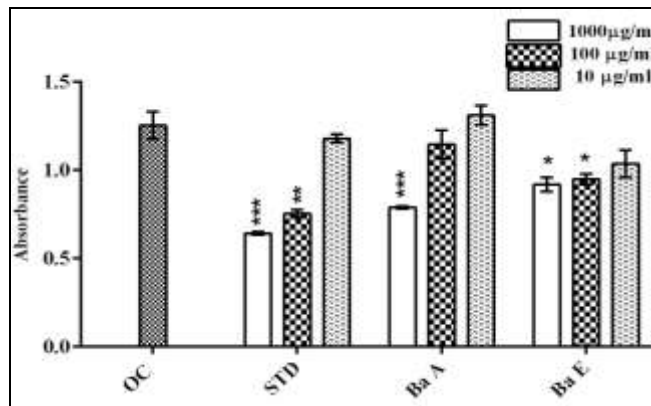
Mature osteoblasts produce large amounts of alkaline phosphatase, a phosphate-splitting enzyme that is released into the osteoid to initiate the deposition of minerals. BaA and BaE induced differentiation of MSCs into osteoblasts. MSCs grown in basal media (DMEM) alone showed less significant differentiation of MSCs into osteoblast. BaA and BaE both showed significant differentiation as compared to osteogenic media (OM), which was taken as a positive control (Fig. 2).



**Fig 2:** Effect of aqueous and ethanolic extract of *Berberis aristata* on alkaline phosphatase activity in osteoblasts in *in vitro* experiment.

(BaA) aqueous extract of *Berberis aristata*, (BaE) ethanolic extract of *Berberis aristata*, Cells grown in basal medium (BM) were taken as untreated control. ALP activity of both extracts is as significant as osteogenic medium (OM) taken as a positive control. Data represent the mean  $\pm$  SEM of 3 replicates. \*\* P<0.01 vs. untreated control (BM).

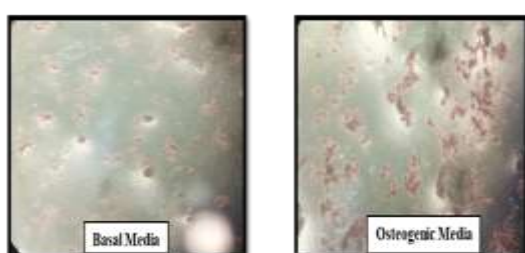
The anti-osteoclast activity of the BaA and BaE was compared with that of alendronate, which is taken as a standard inhibitor of bone resorption. BaA exhibited significant dose-dependent anti-osteoclast activity, which was measured in terms of TRAP activity. MSCs grown in osteoclastic medium (OC) were taken as a negative control. BaE also showed moderate anti-osteoclastic activity compared to cells grown in OC medium (Fig. 3).



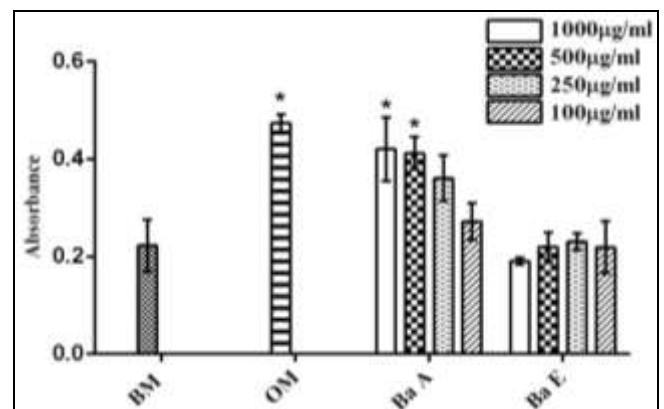
**Fig 3:** Anti-osteoclast activity of aqueous and ethanolic extract of *Berberis aristata* determined through inhibition of TRAP activity.

(BaA) aqueous extract of *Berberis aristata*, (BaE) ethanolic extract of *Berberis aristata* \*p<0.05, \*\*p<0.01, compared with OC as negative control. Values are expressed as mean  $\pm$  S.E.M. (n=3). STD-Alendronate standard used as positive control

Alizarin red-s staining indicates the presence of calcium in the bone nodules by forming an orange red colored alizarin red-calcium complex. Bright orange red colored crystals, indicative of matrix mineralization, were observed in the cells after 21 days of treatment with BaA and BaE. Calcium deposition was found to be more in the cells grown in different concentrations of BaA and BaE(Fig.4) than those grown in BM and OM, taken as negative and positive control, respectively. BaA showed more calcium deposition in bone matrix as compared to control at higher concentrations. The extent of calcium deposition was quantified colorimetrically after dissolving the calcium-alizarin red complex in cetylpyridinium chloride (Fig. 5). The extent of calcium deposition in extracellular matrix was significantly higher in the osteoblasts treated with 1000 and 500 µg/ml of BaA. BaE did not cause a significant increase in the deposition of calcium in the extracellular matrix as compared to that in the untreated cells.



**Fig 4:** Mineralization of extracellular bone matrix (seen as bright red coloured calcium-alizarin red complex) after treatment of osteoblasts with different concentrations of aqueous and ethanolic extract of *Berberis aristata*.



**Fig 5:** Effect of aqueous and ethanolic extract of *Berberis aristata* on mineralization of extracellular bone matrix.

Y-axis denotes absorbance of calcium-alizarin red complex extracted with cetylpyridinium chloride. High absorbance indicates presence of more calcium in extracellular bone matrix. (BaA) aqueous extract of *Berberis aristata*, (BaE) ethanolic extract of *Berberis aristata*, (BM) cells grown in Basal media (OM) cells grown in osteogenic media. Data represent mean  $\pm$  SEM of 3 replicates. \*P < 0.05 vs control.

**4. Discussion**

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 [20].

Certain plant compounds, which have been characterized as phytoestrogens, have shown a weak estrogenic effect on bone in human and animal studies [21-25]. Some herbal drugs, including *Berberis aristata* stem bark, have been traditionally used in *Ayurveda* to accelerate the healing of bone fractures and to strengthen the bones [26]. Exploring the effects of these traditional drugs on various aspects of bone-remodeling could provide an insight into the mechanism of action of these plants.

*Berberis aristata* (Ba) stem bark was selected for this study due to its anti-inflammatory activity demonstrated in *in vivo* animal models [27, 28]. It is reported that plants with anti-inflammatory activity can be potent osteo-protective agents [29]. Moreover, Ba has been reported to exhibit osteo-protective activity in ovariectomy-induced osteoporosis in rats [30]. A combination of anti-osteoporotic and anti-inflammatory activity makes it a promising therapeutic agent for osteoporosis. An attempt was made to explore the mechanism of action of Ba using cell-based assays targeting different aspects of bone remodeling.

The bone undergoes continuous turnover throughout life, due to the activity of two types of cells; osteoblasts, which lead to formation of bones and osteoclasts, which lead to resorption of bones. Equilibrium between these two processes, viz. bone formation and resorption, maintains the normal architecture of the bone. In old age and after menopause in females, the bone resorption by osteoclasts outperforms bone formation by osteoblasts, leading to osteoporosis [19, 31]. The bone metabolism may be shifted back towards equilibrium by enhancing the proliferation and activity of osteoblasts or by inhibiting the activity of osteoclasts.

With an aim to understand the mechanism through which Ba mitigates osteoporosis, the alcoholic and aqueous extracts of *Berberis aristata* were studied for their effect on differentiation of mesenchymal stem cells into osteoblasts, proliferation of osteoblasts, osteoclast activity and mineralization of bone matrix.

All the studies were carried out using mesenchymal stem cells aseptically isolated from the rat femur. Both the extracts, BaA and BaE, were found to affect the proliferation of primary bone marrow cells. However, they significantly stimulated the differentiation of primary bone marrow cells into osteoblasts, as compared to the untreated cells. ALP, which is a specific marker for osteoblasts, was quantified to assess the extent of differentiation of primary bone marrow cells to osteoblasts. The effect of both the extracts was comparable to that exhibited by osteogenic medium, which was used as a positive control.

Apart from stimulating formation of osteoblasts from primary bone marrow cells, both the extracts also caused increased deposition of calcium in the extracellular bone matrix when the cells were incubated in the medium for a longer period of time. Alizarin-red S which forms an orange-red coloured complex with calcium was used to visualize the deposition of calcium in extracellular matrix. In order to quantify the extent of calcium deposition in the matrix, the alizarin red-calcium complex was dissolved in cetylpyridinium chloride and the optical density of the

solution was measured. BaA significantly promoted calcium deposition in extracellular matrix.

Osteoclasts are characterized by the presence of TRAP enzyme. Hence, TRAP activity was determined to estimate the number of osteoclasts in osteoclast inhibition assay. The basal medium supplemented with 1,25-Dihydroxy Vitamin D<sub>3</sub> promoted formation of osteoclasts from osteoblasts [32]. Both the extracts of Ba significantly inhibited osteoclast activity, which was revealed by the reduction in TRAP activity. However, the aqueous extract was found to inhibit the osteoclast activity to a greater extent as compared to the alcoholic extract. Moreover, the osteoclast inhibition activity of aqueous extract was comparable to that of Alendronate, an anti-osteoporotic drug known to act by inhibiting the osteoclast activity [33].

## 5. Conclusion

In summation, the aqueous extract of stem bark of *B. aristata* was found to stimulate the differentiation of primary bone marrow cells to osteoblasts, to promote the deposition of calcium in extracellular bone matrix and to inhibit the formation of osteoclasts.

The aqueous extract of stem bark of *B. aristata* has been reported to contain saponins, tannins, and alkaloids, mainly berberine [34, 35]. Contrary to the earlier reports (20-24), phytoestrogen types of compounds are not reported to be present in this extract. Hence, further research towards finding the bioactive molecules of *B. aristata* acting on the above processes of bone remodeling may lead to novel compounds acting on the bone metabolism.

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