Evaluation of Cell Proliferative Potential of Ethanomedicinal Plants by MG-63 Cell Line and Primary Cell Culture

Kunjal Vegad*[1], Divyakant Patel[1], Ekta Patel[1], Dhwani Shah[1], Yogesh Patel[1], Nimisha Kakadia[2], Niranjan S. Kanaki[2]

1. ShardaSchool of Pharmacy, gandhinagar, Gujarat, India
2. K. B. Institute of Pharmaceutical Education & Research Centre, Gandhinagar, Gujarat, India

Corresponding Author: Dr. Kunjal L. Vegad
Email: kunjalvegad11@gmail.com

ABSTRACT

Osteoporosis is a silent disease until it is complicated by fractures. Osteoporosis is a progressive bone disease that is characterized by a decrease in bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk. There is continuous remodeling of bone throughout the life. Osteoblasts, which cause bone formation and osteoclasts, the cause bone resorption work in equilibrium to maintain the bone mass and bone mineral density; the in equilibrium leading to osteoporosis. Many traditional plants or phytoconstituents are used in bone fractures as potential therapy for reducing fracture healing period. However, these phytoconstituents or herbs require scientific study to validate their use for the treatment in bone related disorders. Aim of the present study is to identify the plant extracts acting on bone remodeling process by using *in vitro* cell culture models (MG 63 cell line and primary mesenchymal stem cells (MSCs) isolated and from bone marrow of rat femur.Several plant based drugs have been used in *Ayurveda* for various bone related disorders. Based on the Ayurvedic literature, *hadjod* - stems of *Cissus quadrangularis* (Cq), *kali mushali* - rhizomes of *Curculigo orchioides* (Co),*arjuna* - bark of *Terminalia arjuna* and *guduchi*- stem of *Tinospora cordifolia* were selected for the study. The aqueous and ethanolic extracts of these plants were evaluated for their ability to potentiate proliferation of osteoblasts using MG-63 osteosarcoma cell line and MSCs by MTT assay. All the plant extracts, more or less, stimulated the proliferation of cells. However, the potentiation of cell proliferation was more significant in case of MSCs as compared to that of MG-63 cell line.

Key words: *Cissus quadrangularis*, *Curculigo orchioides*, *Terminalia arjuna*, *Tinospora -cordifolia*, osteoporosis, osteoblast

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1. INTRODUCTION

Osteoporosis is a very common metabolic disorder of the skeleton, in which the bone mineral density (BMD) is reduced, the bone microarchitecture is disrupted, and the amount and variety of noncollagenous proteins in bone is altered, leading to increased risk of fracture[1]. Osteoporosis is associated with estrogen deficiency following menopause in women. In osteoporosis there is progressive bone loss, and lead them susceptible to fractures with minimal trauma without early signs. Hence, it is popularly known as "silent disease"[2, 3]. Osteoporotic fractures also associated with high mortality, morbidity and high medical expenses throughout the world[4].

Current therapy available in the market include, biphosphonates, Vitamin D and calcium supplementation, hormone replacement

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therapy, and selective estrogen receptor modulators etc.[5]. These all treatments were associated with several adverse effects; oral biphophonates cause gastrointestinal side effects such as abdominal pain, esophagitis, osteonecrosis of jaw and musculoskeletal pain. Long term use of calcium leads to deposition of calcium in blood vessels and increased risk of cardiovascular diseases[6], and estrogen therapy increases the risk of cancer[7]. Hence, to avoid the adverse effects of available synthetic therapy there is need to search for relatively safer medicines, or 'green medicines' for the treatment of osteoporosis. The herbs are in the great demand due to their efficacy, safety and lesser side effects as compared to synthetic molecules. Pharmacognosy has always been a translational or multidisciplinary science, most recently emphasized in the discussion of modern pharmacognosy. In the last few decades there has been an exponential growth in the field of herbal medicine.

These Plants have been a potential source for discovery of new therapeutic agents. The knowledge of traditional systems of medicine is an added advantage, since it helps to identify the plants having a particular therapeutic activity. Isolation of therapeutically active compounds from traditionally used plants is a very rational approach of discovering new chemical entities. Several plant based drugs have been used in *Ayurveda* for various bone related disorders. Based on the *Ayurvedic* literature, hadjod - stems of *Cissus quadrangularis* (Cq)[8], kali mushali - rhizomes of *Curculigo orchioides* (Co)[9], arjuna - bark of *Terminalia arjuna* (Ta)[10], and guduchi - stem of *Tinospora cordifolia* (Tc)[11]were selected for the study. The ability of these drugs to increase proliferation of osteoblasts was studied using two *in vitro* osteoblast model systems, viz., primary osteoblast cells isolated from rat femur and human osteoblast-like cells MG-63.

2. MATERIAL AND METHODS

2.1. Collection and authentication of selected plants

Fresh stems of hadjod and guduchi were collected from the open field of Gandhinagar. Arjuna and kali mushali were procured from local herbal store in Ahmedabad, Gujarat, India. The plant materials were authenticated by comparing their morphological and microscopical characters with those mentioned in the reference literature. The herbarium and voucher specimen of drugs were submitted to the Pharmacognosy department of K.B. Institute of Pharmaceutical Education and Research, Gandhinagar. Freshly collected crude drugs were cleaned, dried and coarsely powdered. Crude drug powders were evaluated for ash values (Total ash and acid insoluble ash), extractive values (alcohol and water extractive) and loss on drying for their authentication.

2.2 Preparation of plant extracts

For the preparation of ethanolic extract 20g of dried powdered drug was taken, and extracted with ethanol (100ml x 2) by heating under reflux below 50˚C and filtered. Aqueous extract of each drug was prepared in same manner and solvent were evaporated to dryness on water bath temperature not exceeding 60˚C. The dried extracts were stored at 2-8°C till further use.

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2.3 Procurement and maintenance of Cell line

Complete growth medium for culturing primary mesenchymal stem cells (MSCs) and MG-63 cells was prepared by supplementing the sterile liquid Dulbecco's minimum essential medium DMEM with 10 % Fetal Bovine Serum and 1% Antibiotic solution (10000 U Penicillin and 10 mg Streptomycin/ml). The medium, henceforth denoted as basal medium (BM), was stored in refrigerator at 8^{o}C.

MG-63 cell line, which is a human bone osteosarcoma cell line, was used for the screening of extracts to assess their cell proliferative activity. MG-63 cell line was procured from NCCS, Pune and was maintained as per ATCC protocol.

As per ATCC protocol, the cell line was allow to grow in DMEM basal media under standard growth conditions (37°Ctemperature, 5% CO_2 and 95% humidity). Media was changed every alternate day until a confluent monolayer of cells was formed. The cells were, then, dislodged from the flask using 0.25% trypsin–0.2%EDTA in Dulbecco's phosphate buffered saline (DPBS) and transferred to new flask to obtain enough number of cells for proliferation assay.

2.4 Determination of cell viability[12]

The number of viable cells in the tissue culture was determined by Trypan Blue dye exclusion assay[14, 15]. Cell suspension and 0.4% trypan blue dye solution were mixed gently in a micro centrifuge tube in 1:1 proportion. Haemocytometer and the cover slip were sterilized with 70% (v/v) iso-propyl alcohol and were allowed to air-dry. The cell suspension mixed with trypan blue dye was loaded on the haemocytometer and allowed to set. The haemocytometer was observed under the microscope and the live cells which appeared colourless, and the dead cells which were stained blue with the trypan blue dye were counted. Cell viability was calculated using the formula mentioned below,

% cell viability = (number of living cells/ total cell count)*100

2.5 Isolation of mesenchymal stem cells (MSCs) from rat femur[13, 14]

The animal study was approved by the institutional animal ethics committee of K. B. Institute of Pharmaceutical Education and Research, Gandhinagar (KBIPER/ approval No: KBIPER/IAEC/ANIM/2016/577). The rats were euthanized by cervical dislocation under ether anesthesia, the femurs were separated aseptically and the soft tissue and muscles attached were cleaned-off. Isolated femurs were cut from both ends and bone marrow was flushed with help of 2 ml DMEM using syringe with needle (27 gauge). Bone marrow was filtered through sterile filter to remove cell debris and filtered cell suspension was centrifuged for 5 minutes at 1000 rpm. Supernatant was discarded carefully and cell pellet was carefully resuspended in fresh basal media and MSCs were seeded in T-25cm^2 tissue culture flask. Primary cells (MSCs) were maintained in a similar as that used for MG-63 cells.

2.6 Cell Proliferation activity by MTT assay[9]

Cell proliferative potential of each prepared extracts was evaluated by primary MSCs and osteoblast like cells of MG-63 cell line. For the MTT assay, 100 µl of cell suspension prepared in basal medium was plated in each well of a 96-well plate to get a cell concentration of 1×10^4 cells/well. After 24 hours, used media was

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discarded carefully and the adhered cells were treated with aqueous and ethanolic extracts of each drug in triplicate at different concentrations (1000µg/ml, 100µg/ml and 10µg/ml) for 48 hours. After 48 hours of drug treatment, 20 µl of MTT (final concentration 0.5mg/ml in the well) was added to each well and further incubated for 4h at 37°C in dark place. Spent media was carefully discarded without disturbing the cell monolayer at the bottom of well and 200 µl of DMSO was added to each well. The plate was shaken carefully and incubated for 1h at 37°C in a dark place. Optical density (OD) of each well was measured at 570 nm .

2.7 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test, using Graphpad Prism software. The results are expressed as mean ± standard error of the mean (SEM). The results were considered statistically significant if *p* value was less than 0.05.

3 RESULTS

3.1 Authentication of selected plants

Collected plants were validated by comparing their morphological, microscopical and physicochemical parameters with their monographs and literature[15, 16].Physicochemical parameters are as shown in the Table 1.

Table 1: Physicochemical parameters of selected plants

Parameters / Name of Plant	Ash value (%w/w)		Loss on Drying (%w/w)	Extractive value (%w/w)	
	Total ash	Acid insoluble ash		Water soluble extractive	Alcohol soluble extractive
Stems of ***Cissus quadrangularis***	18.2 ± 0.76	0.9 ± 0.05	5.2 ± 0.28	27.4 ± 0.81	5.4 ± 0.27
Rhizomes of ***Curculigo orchioides***	7.92 ± 0.53	0.75 ± 0.03	7.98 ± 0.37	18.5 ± 0.53	2.39 ± 0.18
Bark of ***Terminaliaarjuna***	21.1 ± 0.57	0.87 ± 0.08	4.3 ± 0.53	17.2 ± 0.68	21.6 ± 0.68
Stems of ***Tinospora cordifolia***	6.12 ± 0.58	0.61 ± 0.05	4.7 ± 0.68	13.7 ± 0.68	6.6 ± 0.22

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3.2 Preparation of extracts

Ethanolic and aqueous extract of four selected plants were prepared. The % yield obtained for each extract is mentioned in Table 2.

Table 2 : Percentage yield of extracts

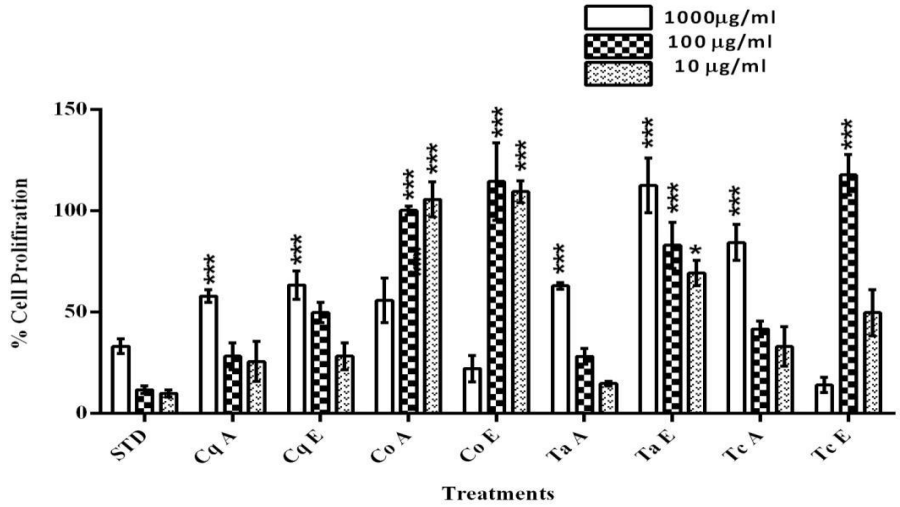
Name of Plant	Extract	% Yield of Extract (% w/w)
Stems of ***Cissus quadrangularis***	Aqueous (Cq A)	27.1
	Ethanolic (Cq E)	5.3
Rhizomes of ***Curculigo orchioides***	Aqueous (Co A)	18.7
	Ethanolic (Co E)	12.2
Bark of ***Terminalia arjuna***	Aqueous (Ta A)	17.2
	Ethanolic (Ta E)	21.3
Stems of ***Tinospora cordifolia***	Aqueous (Tc A)	13.6
	Ethanolic (Tc E)	6.61

3.3 Determination of cell viability

The viability of the cultured cells was determined by trypan blue dye exclusion assay, before using them for bioassay. The total viable cell count and % cell viability of MG-63 cell line was found to be 2.28 x 10^6 cells/ ml and 98.21%, respectively. Total viable cell count and % cell viability of primary mesenchymal stem cell was found to be 3.16 x 10^6 cells/ ml and 96.19%, respectively.

3.4 Cell proliferation assay

MTT assay was used to evaluate the ability of the plant extracts to stimulate proliferation of MSCs and MG63 cells. The results of the assay are represented in figure 1 and 2. Basal medium was taken as a negative control and alendronate treatment acted as a positive control.



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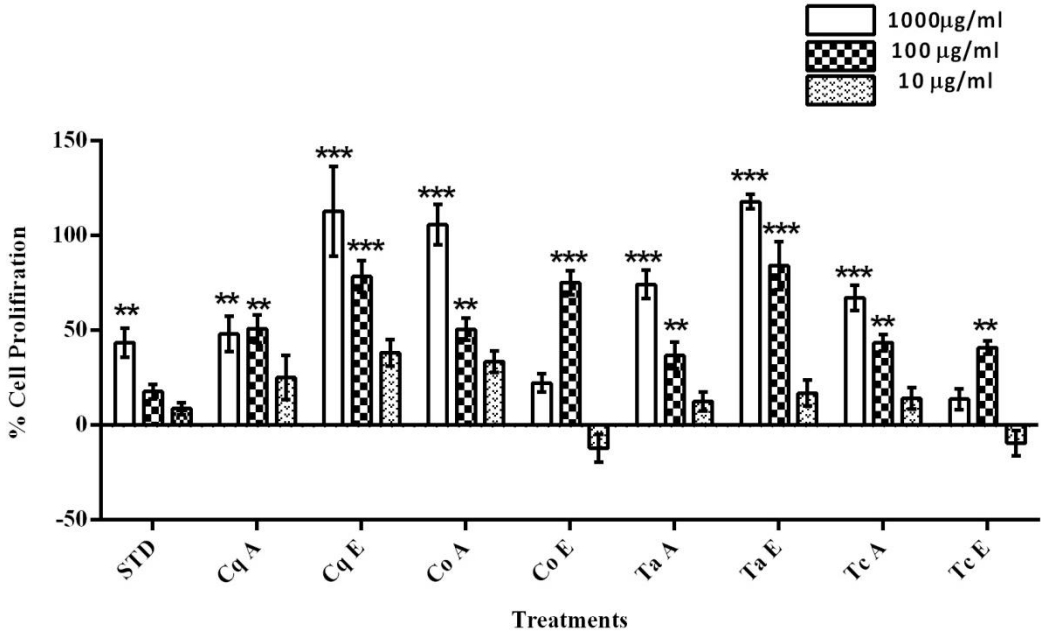
*The values depicted in the graph are from higher to lower concentrations (i.e. 1000 µg/ml, 100µg/ml, and 10µg/ml) for each drug extract. STD = Alendronate standard Cq A = Aqueous extract of Cissus quadrangularis Cq E = Ethanolic extract of Cissus quadrangularis Co A = Aqueous extract of Curculigo orchioides Co E = Ethanolic extract of Curculigo orchioides Ta A = Aqueous extract of Terminalia arjuna Ta E = Ethanolic extract of Terminalia arjuna Tc A = Aqueous extract of Tinospora cordifolia Tc E = Ethanolic extract of Tinospora cordifolia Values are expressed in mean ± SEM. n=3. Significantly different from *P < 0.05 **P < 0.01 ***P < 0.001 Vs Control (Basal media)*

Figure 1: Cell proliferation activity of selected plant extracts on MG-63 cell line



*The values depicted in the graph are from higher to lower concentrations (i.e. 1000 µg/ml, 100µg/ml, and 10µg/ml) for each drug extract. **STD** = Alendronate standard **Cq A** = Aqueous extract of Cissus quadrangularis **Cq E** = Ethanolic extract of Cissus quadrangularis **Co A** = Aqueous extract of Curculigo orchioides **Co E** = Ethanolic extract of Curculigo orchioides **Ta A** = Aqueous extract of Terminalia arjuna **Ta E** = Ethanolic extract of Terminalia arjuna **Tc A** = Aqueous extract of Tinospora cordifolia **Tc E** = Ethanolic extract of Tinospora cordifolia Values are expressed in mean ± SEM. n=3. Significantly different from *P < 0.05 **P < 0.01 ***P < 0.001 Vs Control (Basal media)*

Figure 2: Cell proliferative potential of selected plant extracts on primary mesenchymal stem cells

Alendronate caused moderate increase in proliferation of MSCs ($p < 0.01$) in a dose-dependent manner but not in osteoblast like cells of MG-63 cell line as compared to the cells grown in the basal medium alone. Aqueous and ethanolic extracts of all the four plants resulted in moderate to excellent proliferation of MSCs and MG63 cells.

CqA and CqE caused significant increase in proliferation of MG63 cells ($p< 0.001$) at concentration of 1000 µg/ml, and an increase in

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proliferation of MSCs at 1000 µg/ml and 100µg/ml concentrations.

CoA and CoE resulted in more prominent increase in proliferation of MG63 cells ($p< 0.001$) at concentrations of 100 µg/ml and 10 µg/ml, but at higher concentration, the effect was reversed. However, the proliferation of MSCs was significantly potentiated by CoA in a dose dependent manner. CoE did not cause significant increase in proliferation of MSCs.

TaA caused significant increase in proliferation of MG63 and MSCs ($p< 0.001$) at concentration of 1000 µg/ml, whereas TaE increased proliferation both the types of cells ($p< 0.001$) in dose dependent manner.

Treatment with TcA and TcE remarkably increased proliferation of MG-63 and MSCs ($P < 0.01$) at higher concentrations (1000 µg/ml).

3. DISCUSSION

Osteoporosis constitutes a major public health problem, contributing significantly to morbidity, mortality and healthcare spending[17]. Though osteoporosis is a senile disease, it will be started in young age and showed in adulthood[18]. With increasing life expectancy the prevalence of osteoporosis is on rise and it poses a major public health issue[19]. Natural menopause in women, making sponataneous cessation of estrogen has potent effect on the development and integrity of skeleton. Lifestyle changes, modifications in consumption of food, regular exercise have beneficial effects on bone health. The bone undergoes continuous turnover throughout life. Bone mass decreased due to activation of osteoclast which enhance bone resorption. Currently available treatments for osteoporosis mostly include antiresorptive agents. Antiresorptive agents inhibit osteoclastic bone resorption and slow down loss of bone mass[20]. As estrogen deficiency in postmenopausal women is a major risk factor for osteoporosis, hormone replacement therapy (HRT), is the effective treatment, but less preferred due to the risk of breast cancer and cardiovascular diseases. All other synthetic therapeutic agents for osteoporosis have some adverse effects, which necessitates the search for newer therapeutic agents[21].Nature has been a source of medicinal agents since years ago, it comprises of compounds that are highly diverse and provide specific biological activities. The study of herbal medicines proved the proper use and source for newer drug[22]. Plants with anti-inflammatory active constituents can act as a potent candidates for the treatment of osteoporosis [23]. Several herbs with phytoestrogens, have shown a estrogenic effect on bone in human and animal studies[24]. Few drugs have been used in *Ayurveda* to accelerate the healing of bone fractures and to strengthen the bones. Four such drugs, stem of Cissus quadrangularis (Cq), rhizomes of Curculigo orchioides (Co), bark of Terminalia arjuna (Ta), and stem of Tinospora cordifolia (Tc) were selected for the study based on their use in bone disorder in Ayurveda.

There is continuous remodeling of bone throughout the life. Osteoblast, a bone forming cells and osteoclast, a resorption cells are involved in bone metabolism. There is equilibrium between bone formation and bone resorption which helps to maintain the bone mass and bone mineral density, imbalance leading to osteoporosis[34]. Hence, there are two approaches for the prevention of osteoporosis; one is to decrease the activity of osteoclast to decrease bone resorption, and another is to increase the activity of osteoblast to increase the bone formation[25]. Based on the later approach, the extracts of selected crude drugs were evaluated for their ability to potentiate proliferation of MG-63 osteoblast like cells and primary bone marrow cells (mesenchymal stem cells).

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According to study, cell proliferation ability of extracts may differ in normal cells, i.e. primary mesenchymal stem cells and cancerous cells, i.e. cells form osteosarcoma cell line such as MG-63 cell line [26]. So, the plant extracts were studied using cells of MG-63 cell line and primary mesenchymal stem cells, which ultimately give rise to osteoblasts in bones[27].

In MTT assay, mitochondrial succinate dehydrogenase of viable cells reduce yellow 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to an insoluble, dark purple coloured formazan crystal which can be quantify spectrophotmetrically by dissolving them in to an organic solvent (eg. DMSO, isopropanol).

Results of the current study suggested that plant hadjod, kali mushali, arjuna chhal and guduchi which are selected on the basis of their ethnomedicinal use, have good cell proliferative potential on normal cells. Some of selected plants also showed cell proliferative activity on cells of MG -63 cell line. But both extract of hadjod, aqueous extract of kali mushali and ethanolic extract of arjuna chhal have shown remarkable cell proliferation on osteoblast like cells obtained from MG -63 cell line.

4. CONCLUSION

The experiments lead to a conclusion that all the selected plants can stimulate the proliferation of primary bone marrow cells, less so in case of MG63 cells. Hence these plants have the potential to be used for the treatment of osteoporosis. Cell proliferation is not only the target for the treatment, so, these plants further need to evaluate for the different targets of bone remodelling which evaluate the use of selected extracts for prevention of bone loss.

5. CONFLICT OF INTEREST

There is no conflict of interest.

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