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# Pro-Stimulatory Effects of *Tinospora Cordifolia* (Menispermaceae) on SAOS-2 Osteoblast Cells - Implications on Bone Remodeling and Therapy of Osteoporosis

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**ABSTRACT**

Osteoblasts, the bone forming cells are currently the most studied target for developing therapeutics to treat bone loss associated with various skeletal disorders including osteoporosis. *Tinospora cordifolia* (TC) is used in Ayurveda and other traditional medicinal systems to treat bone fractures. The aim of the present investigation is to evaluate the effects of aqueous and alcoholic extracts of TC on osteogenesis using a widely employed *in vitro* model system for human osteoblasts (human osteoblast like cells SAOS-2), thereby to explore the possibility of its usefulness to stimulate bone formation in osteoporotic conditions. Tests for cell viability and proliferation (crystal violet test, trypan blue dye exclusion test, MTT assay and NBT reduction test) were performed to study the effect of TC on the growth of osteoblasts. It was observed that ethanolic extract of TC stimulated proliferation of osteoblasts at a dosage of 25µg/ml (*P<0.001*) but, the aqueous extract of TC showed no influence on cell proliferation. The effect of alcoholic extract of TC on bone resorption was also studied on functional osteoclasts derived from RANKL activated murine macrophage like cells RAW 264.7. Results of the study implicated that ethanolic extract of TC extract elicits pro-stimulatory effects on osteoblasts, but is devoid of any such effects on osteoclast cells thereby indicating that it has no effect on bone resorption.

**Keywords:** Osteoporosis, *Tinospora cordifolia*, SAOS-2 cell line, Bone formation

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## INTRODUCTION

Bone is a metabolically active and highly organized tissue that undergoes continuous remodeling. Bone Remodeling, a continuous process of resorption and renewal, is essential for calcium homeostasis and to preserve its rigidity and strength in order to withstand various mechanical stresses. In many individuals, bone mass homeostasis starts failing in midlife, leading to excessive bone loss and susceptibility to fractures due to imbalance between bone formation by osteoblasts and resorption by osteoclasts [1]. Osteoporosis is a silent systemic skeletal disorder characterized by reduced bone strength, diminished bone density and debilitating fractures [2]. Most of the available therapies prevent further resorption of bone but there are only few drugs that can replace the lost bone mass and they are also however associated with various strong side effects. Therefore, cost effective and safe anabolic agents that promote the proliferation of osteoblast cells to facilitate bone formation are the need of the hour.

The application of traditional knowledge in search of potential leads for the treatment of osteoporosis is a valuable approach to find a safe and compliant therapy. *Tinospora cordifolia,* commonly known as Guduchi, is used in Ayurveda and other traditional medicinal systems as a rejuvenator and general tonic for vitality. Numerous studies carried out earlier report the chemopreventive, hepatoprotective, anti-tumour, anti-carcinogenic, anti-diabetic, immunomodulatory, anti-inflammatory and anti-arthritic effects of *Tinospora cordifolia* [3]. *Tinospora cordifolia* (TC) is used in various folklore medicines to treat bone disorders and have been referred to have osteoprotective functions [4, 5]. The Muslim tribals of Rajouri, Jammu (Tawi) comprising Gujjar and Backwals used the plant to treat bone fracture [6].

A number of model systems utilizing osteoblastic cells in culture have been developed to study the influence of anabolic agents that can induce proliferation and differentiation of these cells, thereby stimulate bone formation. So the objective of the current study is to investigate the ability to TC to promote proliferation of osteoblasts, thereby induce bone formation *in vitro*. In this study, the osteoblast proliferative potential of aqueous and alcoholic fractions of TC was studied using human osteoblast like cells SAOS-2 and standard assays for cell proliferation like MTT assay and NBT reduction test were carried out to evaluate the proliferative potential of the drug. In addition to tests for cell proliferation, tests for cell viability and cytotoxicity like trypan blue dye exclusion test and crystal violet test were carried out to check for the effect of the drug on osteoblast population and to maintain membrane integrity. In addition to the above, cell proliferation and cytotoxicity studies were carried out on osteoclasts cells derived from RANKL induced RAW 264.7 cells out to find out the effect of TC on bone resorption.

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## MATERIALS AND METHODS

### PLANT MATERIAL

Fresh plant samples of *Tinospora cordifolia (Willd.)Miers* (Family-Menispermaceae) were collected from Dhanwantri vana, Ministry of Forests, Government of Karnataka, JB campus, Bangalore, Karnataka. Then, aerial parts of the plant were cut into small pieces, shade dried and coarsely powdered. A part of the coarse powder was then utilized for the preparation of the ethanolic extract by soxhlation technique. The final extract ratio was 10:1. The ethanolic extract was lyophilized and stored at -20°C in small aliquots. The aqueous extract was prepared using heat distillation method by boiling 10 % w/w of the coarse powder in sterile distilled water for 10 min, then cooled to room temperature overnight and filtered before use. The aqueous extracts were freshly prepared and used for the assays.

### PROCUREMENT AND MAINTENANCE OF SAOS-2 CELLS

Human osteoblast like cells SAOS-2 was procured from National Center for Cell Sciences (NCCS), Pune, India and cultured in ready to use sterile liquid McCoy's 5a medium (AL057S, Himedia, India) supplemented with 1X antibiotic antimycotic solution (A007, Himedia, India) and 10% fetal bovine serum (FBS-RM1112, Himedia, India). Cells were grown under standard growth conditions (temperature 37 ◦C, 5% $CO_2$ and 95% humidity) in a $CO_2$ 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 sub-cultured at a split ratio of 1:3 in 12.5 $cm^2$ volume tissue culture flask (TCG2 – Himedia, India). 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.

### PROCUREMENT AND MAINTENANCE OF MACROPHAGE LIKE CELL LINE - RAW 264.7

Murine macrophage like cell line RAW 264.7 was procured from National Center for Cell Sciences (NCCS), Pune, India and cultured in ready to use sterile liquid Dulbecco's Modified Eagle's Medium - DMEM (AL007S, Himedia, India) supplemented with 1X antibiotic antimycotic solution (A007, Himedia, India) and 10% fetal bovine serum (FBS-RM1112, Himedia, India). Cells were grown under standard growth conditions (temperature 37 ◦C, 5% $CO_2$ and 95% humidity) in a $CO_2$ incubator (Forma Scientific, USA). When a confluent monolayer was formed, cells were detached using cell scrappers(T-001, Himedia, India) and then sub-cultured at a split ratio of 1:3 in 12.5 $cm^2$ volume tissue culture flask (TCG2 – Himedia, India). The media was changed three times a week.

### INDUCTION OF OSTEOCLASTS FROM RAW 264.7

RAW 264.7 cells represent a murine macrophage cell line that has the capability to grow indefinitely as an OC precursor population or can be differentiated by treatment with RANKL into multinucleated osteoclasts. RAW 264.7 cells were treated with soluble recombinant RANKL

(Sigma Aldrich, USA) at a final concentration of 35 ng/mL to initiate osteoclast development as described elsewhere [7]. The multinucleated RAW-OCs appeared after 5-6 days of culture were harvested, purified and used for cell proliferation and cytotoxicity assay [7].

**CELL PROLIFERATION ASSAYS ON OSTEOBLASTS**

After arriving at confluency, the osteoblast cells were plated on to 96 well microtitre plates (initial cell seed of 5 X $10^6$ cells/ml) and were treated with various concentration of both the extracts of TC (1 µg/ml, 7.5 µg/ml, 12.5 µg/ml, 25 µg/ml , 50 µg/ml, 75 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, and 250 µg/ml), incubated for 72 hrs and utilized for cell proliferation assays.

**MTT ASSAY**:

MTT assay was performed following the method described elsewhere [8]. Briefly the cells were plated and treated with different concentrations of the drug allowed to proliferate for 72 hours. After 72 hours of proliferation 25 µl of MTT (5 mg/ml in phosphate buffered saline - pH 7.4) was added to the wells. The plates were then incubated in a $CO_2$ incubator for six hours. After six hours the formazan crystals produced were solubilised by adding 75 µl of DMSO to each well. The intensity of the colour developed was read at 570nm in a micro plate reader (Bio-Tek systems, USA).

**NBT REDUCTION TEST**

NBT reduction test was performed following the method reported earlier [9]. Briefly after the required hours of proliferation, 10 µl of nitro blue tetrazolium chloride (5 mg/ml in phosphate buffered saline – pH 7.4) was added and incubated in a $CO_2$ 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.

**TESTS FOR CELL VIABILITY**

The followings tests were carried out using only five concentrations of aqueous and alcoholic extracts of TC viz 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml and100 µg/ml.

**CRYSTAL VIOLET ASSAY:**

The cells were plated and treated with different concentrations of the drug and allowed to proliferate for 72 hours. After 72 hours of proliferation the cells were fixed in 10% formalin saline for 30 min. (50 µl was added to each well).Then the cells were stained with crystal violet (0.05% w/v) for 30 min. After that the wells were 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 [10].

**TRYPAN BLUE DYE EXCLUSION TEST**

This test was performed following the method described previously [11]. After 72 hrs of proliferation, appropriately diluted cells (treated and untreated 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 was 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 104**

**PHTOCHEMICAL SCREENING OF *TINOSPORA CORDIFOLIA***

The ethanolic extract of TC was subjected to preliminary phytochemical screening to identify various constituents present in it by following standard qualitative methods described elsewhere [12]. The ethanolic extract was tested for the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenolics, proteins, saponins, steroids, tannins and triterpenoids.

**STATISTICAL ANALYSIS OF THE DATA**

All the assays 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 P values were expressed and comparisons were made between the untreated control and the treated groups.

## RESULTS

**EFFECT OF AQUEOUS AND ETHANOLIC EXTRACTS OF TC ON PROLIFERATION OF HUMAN OSTEOBLAST LIKE CELLS SAOS-2 (FIGURE 1):**

The results of the MTT assay and NBT reduction test showed that the treatment with alcoholic extract of TC ranging from 12.5 µg/ml to 100µg/ml induced a statistically significant increase in the proliferation of SAOS-2 cells ($p<0.001$) and maximum increase in cell proliferation was observed at the dosage of 25 µg/ml as compared to control. The lower dosages viz 1 µg/ml, 7.5 µg/ml and dosages 150, 200, and 250 µg/ml also showed statistically significant proliferative effect as compared to control ($p<0.01$ *and* $p<0.05$ ). But, the treatment with aqueous extract of TC (1 µg/ml- 250 µg/ml) exerted no statistically significant proliferative effect on osteoblast cells as compared to control (NS).

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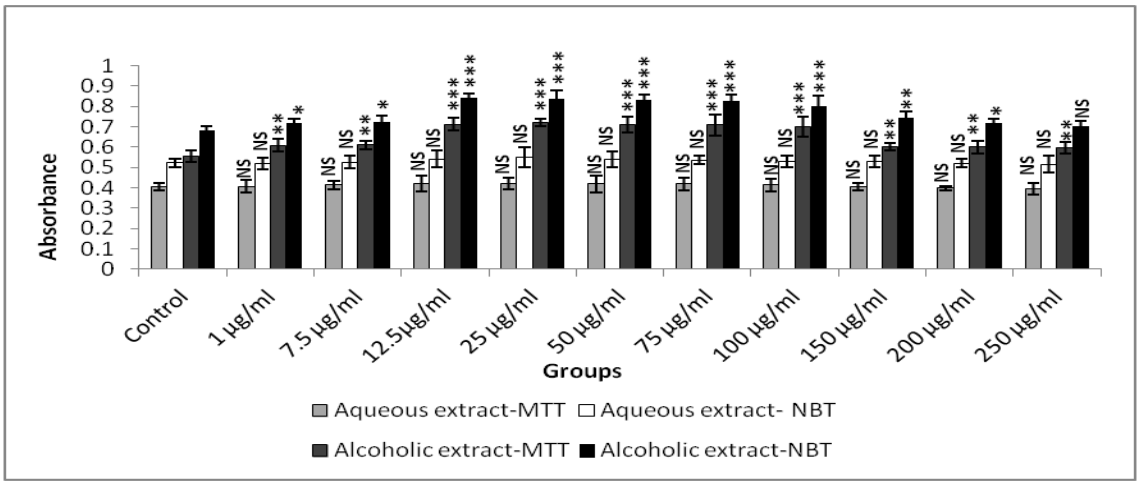
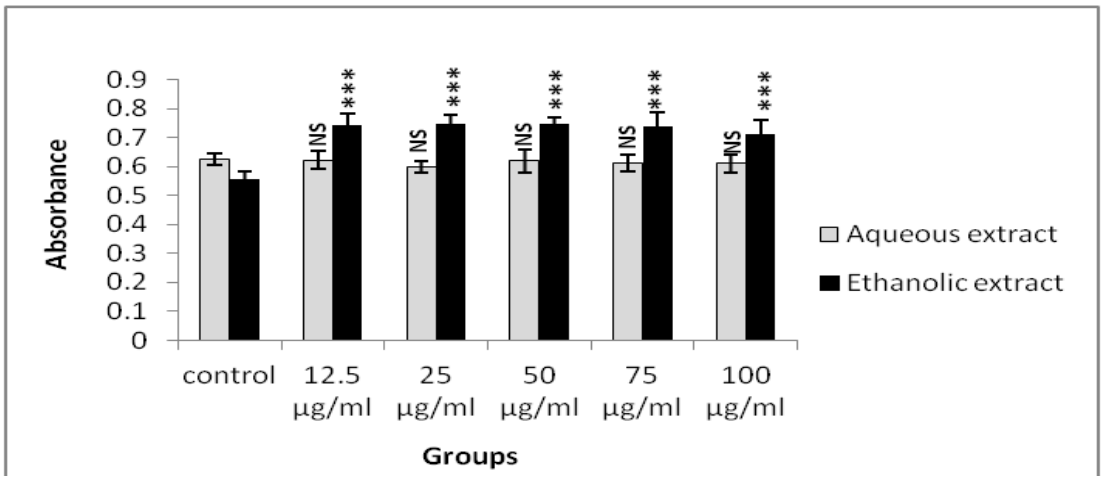
**Figure 1: Effect of Aqueous and Ethanolic Extracts of Tc on Proliferation of Human Osteoblast like cells Saos-2 – Mtt Assay and Nbt Reduction Test**




Statistical analysis of the data was carried out by Student's t-test. Values are expressed as mean ± standard deviation (n=6). Statistical comparisons are made between control Vs other groups. *** *P< 0.001, **P<0.01,*P<0.05 and* $^{NS}$*- Non significant*

## EFFECT OF AQUEOUS AND ETHANOLIC EXTRACTS OF TC ON VIABILITY OF HUMAN OSTEOBLAST LIKE CELLS SAOS-2 (FIGURE 2 AND 3):

**Figure 2: Effect of Aqueous and Ethanolic Extracts of Tc on Viability of Human Osteoblast like Cells Saos-2 – Crystal Violet Test**




Statistical analysis of the data was carried out by Student's t-test. Values are expressed as mean ± standard deviation (n=6). Statistical comparisons are made between control Vs other groups. *** *P< 0.001 and* $^{NS}$*- Non significant*

The crystal violet test and trypan blue dye exclusion test confirmed the results observed with cell proliferation assays that ethanolic extract of TC has the potential to proliferate osteoblasts *in vitro*, but the aqueous extract is devoid of any such effects on the cells. No

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statistically significant increase in cell viability was observed on treatment of osteoblasts with aqueous extract of TC (12.5 µg/ml- 100 µg/ml) as compared to control (NS), while the ethanolic extract showed positive effect on the viability of SAOS-2 cells and maximum number of viable cells was observed at the dosage of 25 µg/ml as compared to control (*P<0.001*).

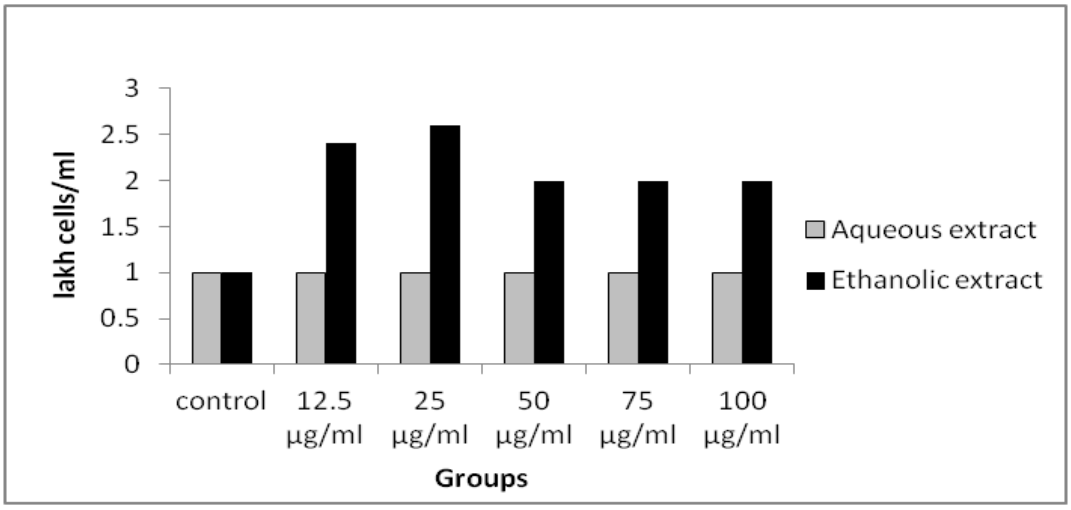
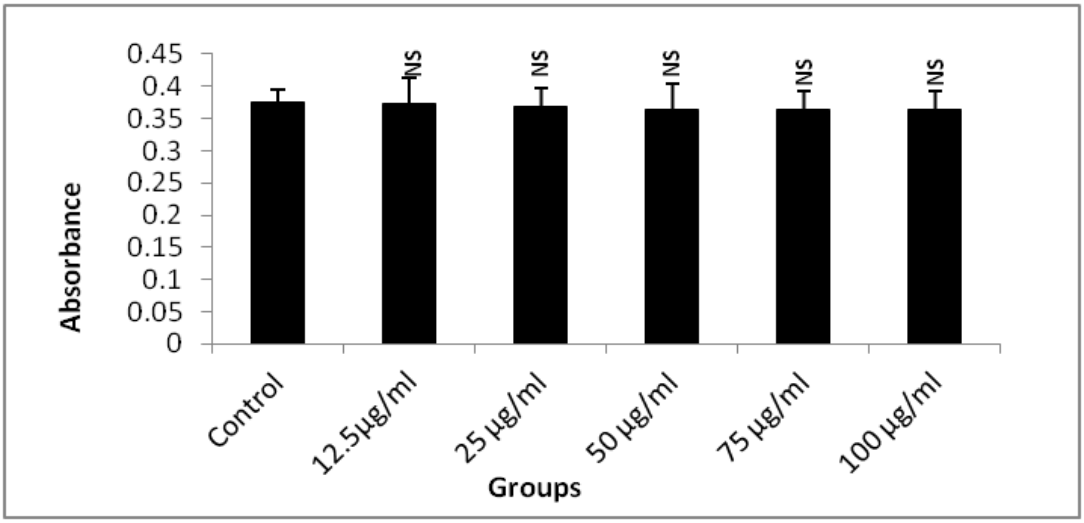



**Figure 3: Effect of Aqueous and Ethanolic Extracts of Tc on Viability of Human Osteoblast like Cells Saos-2 – Trypan Blue Dye Exclusion Test**

**EFFECT OF ETHANOLIC EXTRACT OF TC ON THE VIABILITY OF OSTEOCLASTS DERIVED FROM RANKL ACTIVATED RAW 264.7 MURINE MACROPHAGES (FIGURE 4):**

**Figure 4: Effect of Ethanolic extract of tc on Osteoclast cells – MTT Assay**




Statistical analysis of the data was carried out by Student's t-test. Values are expressed as mean ± standard deviation (n=6). Statistical comparisons are made between control Vs other groups $_{NS}$- *Non significant*

The effect of the ethanolic extract of TC on osteoclast activity was assessed by performing MTT assay. The osteoclasts were treated with five dosages of ethanolic extract of TC viz 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml and100 µg/ml which showed pro-stimulatory effects on osteoblasts. Results of the MTT assay on osteoclasts showed that TC neither exerted any suppressive nor proliferative effect on them. In other words TC did not show any statistically significant effect on osteoclasts derived from RANKL activated RAW 264.7 murine macrophages as compared to control.

**PRELIMINARY PHYTOCHEMICAL SCREENING OF *TINOSPORA CORDIFOLIA* (TABLE 1)*:***

The preliminary phytochemical screening of *Tinospora cordifolia* by standard qualitative methods revealed the presence of alkaloids, carbohydrates, phenolics, proteins, saponins, steroids, tannins and triterpenoids in the ethanolic extract of the plant.

**Table 1: Phytochemical Screening of *Tinospora cordifolia* – Qualitative Analysis by Standard Methods**

| S.no | Name of the chemical constituent | Present/absent |
|---|---|---|
| 1. | Alkaloids | present |
| 2. | Carbohydrates | present |
| 3. | Glycosides | absent |
| 4. | Steroids | present |
| 5. | Triterpenoids | present |
| 6. | Tannins | present |
| 7. | Phenolics | present |
| 8. | Saponins | present |
| 9. | Flavanoids | absent |
| 10. | Proteins | present |

## DISCUSSION

Osteoporosis is now widely recognized as a public health problem throughout the world since this disease is associated not only with high mortality, morbidity but also inflicts enormous economic burden on both the individual and the government. Because osteoporosis occurs frequently in the elderly and as life expectancy around the world continues to increase, the number of people at risk of developing osteoporosis is rising as well. Considering the broad spectrum effect of osteoporosis in the medical system, currently an increasing demand is sought in the alternative system of medicine to design strategies to prevent and cure this debilitating ailment. In Ayurveda, an ancient system of Indian medicine TC is used to treat bone disorders including bone fractures and metabolic disorders with no adverse effects [13]. So the current study is designed to investigate the osteo-protective effect of aqueous and alcoholic extracts of TC on osteoblast model system (viz human osteoblast like cells SAOS-2) *in vitro*.

During the process of bone formation, osteoblasts precursors are recruited at the resorption sites and they undergo differentiation into mature osteoblasts that lay the bone

matrix and facilitate the mineralization of the same. Osteoblasts get trapped into the newly formed extracellular matrix to become osteocytes, the cells that maintain the bone matrix and they also play an important role in osteoclast regulation and initiation of resorption [14]. Thus osteoblasts are primarily responsible for bone formation and crucial player in bone remodeling.

The results indicated that ethanolic extract of TC stimulated growth and proliferation of osteoblasts, but aqueous extract showed no such proliferative effect on the cells, as determined by MTT assay and NBT reduction test. The results of tests for cell viability and cytotoxicity confirmed that the alcoholic extract of TC preserved the membrane integrity and permeability of osteoblast cells, thereby increased number of viable cells in all the treated groups as compared to control ($P<0.001$). No such effect was observed with SAOS-2 cells treated with aqueous extract of TC. This clearly indicates that the ethanolic extract of TC, but not aqueous extract have the ability to induce proliferation and differentiation of the osteoblasts *in vitro* and the maximum proliferative activity of the extract was observed at the dosage of 25µg/ml.

Studies have revealed that a high extracellular calcium concentration enhances DNA synthesis and promotes chemotaxis of osteoblasts. Leaves of *Tinospora cordifolia* are fairly rich in calcium and phosphorus [3]. So, it is hypothesized that the positive effect of ethanolic extract of TC on osteoblast, thereby bone formation could be attributed to the calcium/phosphates content of the drug which could serve as extracellular source of calcium for the osteoblast cells which in turn sense and respond to the signals (enhanced calcium/phosphorus).

As mentioned earlier, bone homeostasis is a delicate balance between osteoclastic bone resorption and osteoblastic bone formation. Osteoclasts are multinucleated giant cells that differentiate from cells of hematopoietic lineage that form monocytes and macrophages and RANKL represents the osteoblasts-derived factor required for osteoclasts formation [15]. Bone loss is primarily caused by excessive osteoclastic activity. The studies on osteoclasts derived from RANKL induced RAW 264.7 macrophages showed that TC has no effect on osteoclast cells thereby do not play any role in bone resorption.

The preliminary phytochemical screening identified various classes of chemical constituents present in TC. The results of the phytochemical tests are considered as the leads useful for the further studies using sub-solvent fractions of ethanolic extract of TC to elucidate bioactive component responsible for its osteoblast proliferative effects. Studies to establish the ability on TC to enhance synthesis and secretion of collagen and non-collagenous matrix proteins by osteoblasts, its role on the regulation of osteogenic genes and elucidate the bio-active component responsible for positive role of TC on bone, are underway in our laboratory to strongly propose TC as an anti-osteoporotic agent.

## CONCLUSION

The current study revealed that the ethanolic extract of *Tinospora cordifolia* has the ability to stimulate osteoblast proliferation thereby may have a positive role on bone

formation. Based on this study, it is suggested that further studies on this topic will help to explore the osteo-protective functions of *Tinospora cordifolia* and to propose it as a potential anti-osteoporotic candidate.

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