Selective Estrogen Receptor Modulator and prostimulatory effects of phytoestrogen β-ecdysone in *Tinospora cordifolia* on osteoblast cells

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**Abstract**

Background: Indian ethnomedicine acclaims *Tinospora cordifolia* as a bone strengthening agent and prescribes it for the treatment of bone fractures, gout and other inflammatory diseases of the bone.

Objective: (a) To understand the potential of *T. cordifolia* to act as a Selective Estrogen Receptor Modulator (SERM) on in vitro models, (b) To understand the toxic effects (if any) of *T. cordifolia* in vivo, (c) To understand the effects of β-ecdysone (proposed osteoprotective principle of *T. cordifolia*) on the growth of human osteoblast-like cells MG-63 and rat primary culture of osteoblasts, (d) To conduct phytochemical analysis on *T. cordifolia* extract to confirm the presence of β-ecdysone.

Materials and Methods: The role of *T. cordifolia* as SERM was analyzed by investigating the effect of the extract on the growth of MCF-7 and HeLa cells. The effects of *T. cordifolia* in vivo was studied by biochemical (Liver function and renal function tests) and histopathological (Hematoxylin/Eosin staining) analysis. Phytochemical analysis of *T. cordifolia* was carried out by performing FT-IR and LC-ESI-MS analysis.

Results: (a) *T. cordifolia* extract exerted non-estrogenic effects on MCF-7 and HeLa cells implicating its role as SERM. (b) High doses of *T. cordifolia* extract (750 and 1000 mg/kg body wt.) showed impairment of hepatic and renal function, induced pathological alterations in hepatic and renal architecture in albino rats. (c) β-ecdysone an ecdysteroid proposed as the osteoprotective principle of *T. cordifolia* exhibited significant prostimulatory effects on osteoblast cells and rat primary osteoblasts. (d) Phytochemical analysis confirmed the presence of β-ecdysone in alcoholic extract of *T. cordifolia* extract substantiating its role as the osteoprotective principle of *T. cordifolia*.

Conclusion: (a) *T. cordifolia* could function as SERM and can have applications in the therapy of osteoporosis. (b) β-ecdysone is the osteoprotective principle of *T. cordifolia*.

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1. Introduction

Osteoporosis is a progressive, systemic skeletal disease that is characterized by compromised bone strength, bone micro-architecture degradation, increased bone fragility culminating in increased risk of fractures. Post-menopausal osteoporosis (PMO) is the most prevalent form of osteoporosis caused due to estrogen deficiency in elderly women [1,2]. Estrogen replacement therapy (ERT) has been the gold standard treatment in the prevention and management of PMO [3–5]. Unfortunately, Estrogen replacement therapy (ERT) is associated with several side effects including increased risk of breast and endometrial cancers [6]. Hence research on estrogen mimicking compounds which is devoid of the side effects of estrogen but possess the beneficial effects of estrogen on the bone has gained considerable attention in the recent years.

Selective Estrogen Receptor Modulators (SERMs) are a class of compounds that interact with intracellular estrogen receptors in target organs as estrogen agonists and antagonists. They include chemically diverse molecules that lack the steroid structure of estrogens, but possess a tertiary structure that allows them to bind to ERα and/or ERβ [7,8]. Over the past decade, different compounds that possess a SERM profile have been intensely studied and have proven to be a highly versatile group for the treatment of different conditions associated with aging, including hormone-responsive cancer and osteoporosis [9].

*Tinospora cordifolia*, commonly known as Guduchi belonging to the family Menispermaceae, is used in Ayurveda and other traditional Indian medicinal systems as a rejuvenator and general tonic...
for vitality. Its health benefits were described in various classical texts of Ayurvedic Medicine, viz. Charaka samhita, Sushruta samhita, Ashtang Hridaya and other treaties like Bhava Prakasha and Dhanvantari Nighantu [10]. Several medicinal properties of T. cordifolia have already been reported. It is used in Indian folklore medicine to treat bone disorders and has been referred to have osteoprotective functions [11]. Being a rejuvenator, its use is indicated in the treatment of several diseases causing debility including bone fractures. Plants benefit bones through different pathways: some have agents that decrease systemic levels of the pro-inflammatory cytokines associated with bone loss; others contain high levels of calcium, while some others act in the gastrointestinal tract to enhance calcium absorption [12]. It is well known that T. cordifolia is an anti-inflammatory agent and its leaves are reported to contain high amounts of calcium [13,10]. Elevated external calcium in the resorption lacunae acts as a negative feedback on osteoclasts, inhibiting their resorptive capacity [14]. In contrast, a high calcium concentration enhances DNA synthesis and promotes chemotaxis of osteoblasts [15,16].

Previous studies from the laboratory [17] implicate the prostimulatory effects of T. cordifolia extract on human osteoblasts and rat primary osteoblasts. Studies have also confirmed the beneficial effects of treatment with T. cordifolia extract in preventing bone loss against experimentally-induced estrogen deficiency caused by ovariectomy in animals [18]. Till date, there are no reports pertaining to the role of T. cordifolia as a selective estrogen receptor modulator. To the best of knowledge, current study is the first attempt to investigate the role of T. cordifolia as SERM. Also, in the current study the prostimulatory effects of β-ecdysone, the proposed osteoprotective principle of T. cordifolia on osteoblast growth was analyzed. Detailed phytochemical analysis to substantiate the presence of β-ecdysone in T. cordifolia extract was performed. Although generally assumed to be safe, a complete toxicological study on T. cordifolia comprising biochemical and histopathological studies on suitable in vivo models are lacking. Hence the effect of sub-acute exposure of animals with T. cordifolia extract was studied by biochemical and histopathological analysis.

2. Materials and methods

2.1. Plant material

Ready to use commercially available ethanolic extract of aerial parts of wild crafted T. cordifolia (Guduchi) was procured from Sami labs limited, Peenya industrial area, Bangalore, India (Product code no: 2020; Batch No: C81644). The percentage yield of the extract as specified by the commercial source was 10%.

2.1.1. Standardisation of the plant extract — quantitative standards

The following standardization procedures for identity and purity were performed (as reported in the certificate of analysis of the commercial source from which the extract is procured). Loss on drying not more than 6%, w/w (dried at 105 °C). Residue on ignition not more than 15%, w/w. Tapped bulk density between 0.5 g/ml and 0.80 g/ml and loose bulk density is between 0.30 g/ml and 0.60 g/ml. Content of bitter principles on dry basis by gravimetry not less than 2.5%, w/w and not more than 3.5%, w/w. Total heavy metals not more than 5000 CFU/g. Yeast and moulds not more than 100 CFU/g. Identification of the plant was based on the presence of marker compound berberine.

2.1.2. Procurement of β-ecdysone

Commercially available β-ecdysone was procured from Sigma aldrich, USA (Catalog no. H5142) and was used as reference standard compound in all studies related to phytochemical analysis of T. cordifolia extract. The purity of the substance as mentioned by the commercial source is <93%.

2.1.3. Preparation of drug stock

For the in vitro assays, a stock solution of the test compound (1 mg/ml of the extract or β-ecdysone as the case may be) was prepared by dissolving 1 mg of the extract in 1 ml of incomplete culture media for the plant extract and 1 mg of the β-ecdysone in 1 ml of DMSO. From the stock solution, appropriate dilutions were carried out to prepare various concentrations of the plant extract. The final concentration of DMSO in culture (when used as a vehicle in the stock solution of β-ecdysone) was less than 0.1%. The stock solution was freshly prepared every time the assays were performed.

2.2. In vitro model systems

2.2.1. Procurement and maintenance of MCF-7 and HeLa cells

The human breast adenocarcinoma cells MCF-7 and cervical adenocarcinoma cells HeLa were procured from National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained under standard conditions following the procedure mentioned by Dwivedi et al. [19]. The cells were cultured in ready to use sterile liquid Dulbecco’s minimum essential medium-Eagle (DMEM AL0075, 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% 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 1:3 in 12.5 cm2 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.

2.2.2. Procurement and maintenance of human osteoblast cells MG-63

Human osteoblast cells MG-63 was procured from National Center for Cell Sciences (NCCS), Pune, India and cultured in ready to use sterile liquid minimum essential medium–Eagle (MEM AL075A, 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% 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 1:3 in 12.5 cm2 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.

2.2.3. Isolation of osteoblasts 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 approved animal source of Bangalore University (M/S Raghavendra enterprises, Bangalore) and were kept under quarantine for a period of two weeks. After the quarantine period,
the animals were used for the experiment. This part of the study which involved the usage of animals was carried out following ethical guidelines specified by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest and was approved by the Institutional Animal Ethics Committee (IAEC) (Approval No – BUB/IAEC/MB&BT/Dr.MSP/dated 09.05.11). The animals were sacrificed by CO2 asphyxiation and the primary osteoblast cells were isolated from rat femur as described earlier [20–22].

The viability of the cells and the cell count was calculated following trypan blue dye exclusion method [23] and subsequent counting of the cells in a hemocytometer. Only cell preparations having greater than 90% viability were used for the different assays. After arriving at confluency, the cells (initial cell seed of 5 × 10^5 cells/ml) were plated on to 96 well microtiter plates and allowed to attach. After 24 h, they were treated with various doses of test compound (plant extract or β-ecdysone as the case may be) and utilized for the assays. Untreated cells were used as control.

2.4. Phytochemical analysis of T. cordifolia

2.4.1. FTIR analysis

FTIR spectra of T. cordifolia extract was measured at room temperature using a Perkin–Elmer Spectrum GX FT-IR equipped with a high-purity dried potassium bromide (KBr) beam splitter at scan range of 4000 to 400 cm⁻¹.

2.4.2. LC-ESI-MS analysis

The LC-ESI-MS analysis was carried out in the LC instrument 1200 from Agilent Technologies (Agilent, Waldbronn, Germany). An Agilent 6410 triple quadruple tandem mass spectrometer coupled to electrospray ionization (ESI) interface was used for mass analysis and quantification of target analytes. The chromatographic separation was carried out using an Agilent Zorbax column (4.6 × 250 mm, 5 μm). Mobile phase A was water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. The gradient started with 20% of component B: acetonitrile (0.1% formic acid) for 0.5 min and then increased to 95% within 0.1 min. This composition was kept till 4.2 min, and then decreased to 20% of component B within 0.1 min. The total run time was 10 min, and an equilibration step of 5.7 min was included. The flow rate of the mobile phase and the column temperature were set at 0.3 ml/min (injection volume: 5 μL) and 30 °C, respectively. In order to avoid carryover, the auto sampler needle was rinsed automatically with the mixed solution (80% component A and 20% component B) for 3 s among a series of calibration standards, and control samples. The mass spectrometer was operated in the positive ion mode. The tuning parameters were optimized for TLC purified T. cordifolia fraction containing β-ecdysone and standard β-ecdysone (gas temperature: 3500 °C, drying gas flow: 6 L/min, nebulizer pressure: 35 psi, V cap voltage: 3500 V, sheath gas temperature: 3500 °C, sheath gas flow: 9 L/min, Nozzle voltage: 1000 V). The full-scan MS spectra were acquired by scanning the mass spectrometer in the m/z range of 200–2000 at a unit mass resolution.

2.5. Statistical analysis

Statistical analysis of the data was carried out by Student’s t-test. For the in vitro experiments on osteoblast growth and viability, 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 (n = 6). For in vitro and in vivo studies, comparisons were made between control Vs other groups.

***p < 0.001, **p < 0.01, *p < 0.05 and NS – non significant.

3. Results and discussion

3.1. Effect of T. cordifolia on growth of breast and cervical carcinoma cells

Cytotoxicity assays were carried out to assess the effect of T. cordifolia on human breast and cervical adenocarcinoma cells.
MCF-7 human breast cancer cells provide a useful in vitro model system to study the interactions between estrogen and ecdysone like substances with breast cancer cells as they contain receptors for the same. Estrogen induces the synthesis of specific proteins in these cells and increases their rate of proliferation [34]. HeLa cells were employed because of their easy availability and the difficulty in obtaining primary cultures representative of cervix. Also, these two cell types have the advantage that they are rapidly growing cells. Hence, the experiments can be easily replicated and large number of data can be easily generated.

Results show the effect of T. cordifolia extract on the viability and growth of breast adenocarcinoma cell line MCF-7 and cervical adenocarcinoma cell line HeLa as determined by MTT assay and crystal violet test (Fig. 1). No significant changes in growth was observed in MCF-7 and HeLa cells treated with the plant extract (12.5, 25, 50, 75 and 100 μg/ml) as compared to control in both assays. This indicates that T. cordifolia is devoid of any pro proliferative or anti-proliferative effects on breast and cervical cells and hence cannot be an endocrine disruptor. This also implies that the active compound in T. cordifolia which contributes for its osteoprotective effect might act as a Selective Estrogen Receptor Modulator (SERM’s) exhibiting prostimulatory agonistic effects on the bone and non-stimulatory to reproductive organs.

A detailed literature search for the presence of estrogenic compounds in T. cordifolia indicated that it possess an ecdysteroid 20 hydroxyecdysone also called as β-ecdysone which has been reported to exhibit pro estrogenic effects on the bone [35]. To confirm the prostimulatory estrogenic effects of β-ecdysone on osteoblast cells, MTT assay and crystal violet test were performed.

Figs. 2 and 3 shows the effect of β-ecdysone on proliferation of osteoblast model systems in vitro as assessed by MTT assay and crystal violet test. In both the assays, doses of β-ecdysone ranging from 0.1 μg/ml – 5 μg/ml showed a statistically significant increase in the proliferation of osteoblasts as compared to control (P < 0.001) and treatment with 0.1 μg/ml of β-ecdysone resulted in maximum proliferation of osteoblasts in both the model systems used. The higher doses (12.5 μg/ml – 25 μg/ml) showed less significant or non-significant effect on the growth of osteoblasts as compared to control. Hence, it was concluded that β-ecdysone induced proliferation of osteoblasts in vitro on MG-63 and primary osteoblasts at low doses. The observed results are in agreement with the earlier studies which demonstrated that β-ecdysone exhibited osteoprotective effects [36,37] induced the osteogenic differentiation in mouse mesenchymal stem cells [38] and in human periodontal ligament stem cells [39].

3.2. Effect of T. cordifolia on biochemical parameters in vivo

Liver function tests viz. assay of aspartate transaminase (AST or SGOT) and alanine transaminase (ALT or SCGT) activity are routinely monitored in preclinical assessment of test compounds. These parameters serve as an index to assess the degree of hepatocellular injury and resultant hepatic dysfunction inflicted on the model system by the test substances. In addition, biochemical tests like estimation of urea and creatinine levels provide a precise idea about the influence of such test substances on renal function. Estimation of glucose and cholesterol levels indicate any disturbance in carbohydrate and lipid metabolism respectively. Hence, in the current investigation, assay of activities of AST, ALT and determination of the levels of urea, creatinine, glucose and total cholesterol was carried out in the control and experimental groups of animals to have an idea about the effects of T. cordifolia extract in vivo.

Table 1 shows the effect of administration of T. cordifolia extract on the biochemical parameters analyzed in the control and
3.3. Effect of T. cordifolia on tissue architecture — histopathology analysis

Fig. 4 shows the result of histopathology analysis of different tissues in the control and experimental groups of rats. The results of histopathology studies confirmed the results obtained with biochemical studies. Bone and heart sections from rats treated with 1000 mg/kg body.wt of T. cordifolia showed no signs of abnormality and toxicity. Tissue sections of liver and kidney of rats treated with 1000 mg/kg body.wt for 30 days showed signs of hepatotoxicity and nephrotoxicity (inflammatory changes) respectively. This again confirmed that sub-acute administration of T. cordifolia extract did not exert any undesirable toxic effects on the animals at low doses but induced nephrotoxicity and hepatotoxicity at higher doses. This is the desirable toxic effects on the animals at low doses but induced nephrotoxicity and hepatotoxicity at higher doses.

3.4. Phytochemical analysis of T. cordifolia

The positive results on osteogenesis obtained with T. cordifolia extract on in vitro models prompted to explore the phytochemical constituents present in the extract. This was to have a better understanding about the mechanisms of action of the plant. Generally, it is presumed that compounds which have estrogen mimicking actions might possess a steroidal backbone in its structure. It is well

| Groups (mg/kg bd.wt) | Glucose (mg/dl) | Urea (mg/dl) | Creatinine (mg/dl) | Cholesterol (mg/dl) | AST (IU/L) | ALT (IU/L) |
|----------------------|----------------|-------------|-------------------|--------------------|------------|------------|
| Control              | 90.26 ± 5.1    | 35.14 ± 5.1 | 0.85 ± 0.07       | 71.11 ± 4.2        | 10.13 ± 1.44 | 16.39 ± 1.95 |
| 100                  | 82.78 ± 6.5*   | 29.17 ± 4.5*| 0.79 ± 0.04NS     | 71.07 ± 5.1NS      | 9.43 ± 1.32NS | 17.67 ± 2.78NS |
| 250                  | 84.24 ± 6.4NS  | 32.34 ± 4.2NS| 0.80 ± 0.02NS     | 73.65 ± 4.8NS      | 10.29 ± 0.95NS | 17.83 ± 2.04NS |
| 500                  | 84.38 ± 6.3NS  | 33.77 ± 5.1NS| 0.83 ± 0.03NS     | 75.22 ± 6.8NS      | 10.66 ± 1.77NS | 19.55 ± 2.05NS |
| 750                  | 86.12 ± 4.4NS  | 35.19 ± 5.6NS| 0.89 ± 0.02NS     | 75.56 ± 3.8NS      | 11.45 ± 1.45NS | 20.54 ± 2.33** |
| 1000                 | 87.33 ± 4.1NS  | 35.55 ± 3.9NS| 0.94 ± 0.07*      | 78.73 ± 7.1*       | 12.71 ± 1.64* | 20.77 ± 2.56** |

Values were expressed as mean ± SD (n = 6). Comparisons were made between control Vs other groups. **P < 0.01, *P < 0.05 and NS — non significant.

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Fig. 4. Effect of T. cordifolia on tissue architecture. Fig. A — depicts the hematoxylin and eosin stained sections of bone from control rat and rat treated with 1000 mg/kg bd.wt of T. cordifolia showing normal architecture. Fig. B — depicts the hematoxylin and eosin stained section of heart from control rat and rat treated with 1000 mg/kg bd.wt showing normal architecture. Fig. C — depicts the hematoxylin and eosin stained section of liver from control rat and rat treated with 1000 mg/kg bd.wt showing signs of hepatotoxicity (shown in circle). Fig. D — depicts the hematoxylin and eosin stained section of kidney from control rat and rat treated with 1000 mg/kg bd.wt showing signs of nephrotoxicity (shown in circle).
known that β-ecdysone also known as 20-hydroxyecdysone reported [35] to be present in T. cordifolia is a steroid and hence is highly soluble in chloroform. The chloroform soluble fraction of T. cordifolia was prepared to solubilize β-ecdysone and tested qualitatively by Salkowski’s test. Thin-layer chromatography (TLC) analysis showed the presence of green spots with the same Rf value (0.72) in the samples corresponding to standard β-ecdysone and chloroform soluble fraction of T. cordifolia respectively thereby confirming the presence of β-ecdysone in the extract.

Fig. 5 shows the results of FT-IR analysis of T. cordifolia extract. The FT-IR spectrum of the main eluting peak for the plant extract also showed a strong absorption at ca. 1645 cm and 3500 cm, with similarities in the fingerprint region to 20-hydroxyecdysone (β-ecdysone). The quality of the spectrum obtained enabled the identification of the material in the extract as β-ecdysone when searched against a reference spectral library, giving a very good match of 92% to the reference spectrum for β-ecdysone.

Fig. 6 shows the results of LC-ESI-MS analysis. The calculated molecular mass of β-ecdysone is 480. The optimization of the LC-ESI-MS conditions was carried out by infusion of the individual standard solutions at the concentration levels of 1 µg/ml for TLC purified T. cordifolia fraction containing β-ecdysone and 2 µg/ml for β-ecdysone in electrospray ionization (ESI) positive mode. Both gave a protonated molecular ion of m/z 481.1, thus confirming that the molecular mass of TLC purified T. cordifolia fraction containing β-ecdysone is same as the standard β-ecdysone. Hence, put together, the results of phytochemical analysis confirmed the presence of β-ecdysone in T. cordifolia.

Estrogen replacement therapy (ERT) is recommended for postmenopausal women primarily for reduction of menopausal symptoms and prevention of osteoporosis. However, only 35%—40% of women ever start ERT, and many do not continue it. Conventional ERT drugs, especially diethylstilbestrol, have been shown to cause serious side effects including stroke, gallbladder disease and certain types of cancer. Because of this, there is increasing interest in the use of plant-derived estrogens, also known as phytoestrogens.

In the current study, the results of phytochemical screening indicated the presence of a phytoestrogen β-ecdysone in T. cordifolia. MTT assay and crystal violet test carried out on MG-63 cells and primary osteoblasts proved that β-ecdysone has prostimulatory effects on osteoblast cells. The absence of
prostimulatory effects of *T. cordifolia extract* on MCF-7 and HeLa cells indicate that the osteoprotective principle of *T. cordifolia* might act as a selective estrogen receptor modulator exerting growth stimulatory effects only on the bone and not on the reproductive organs.

Therefore, based on the results obtained, β-ecdysone an ecdysteroid is proposed as the active principle in *T. cordifolia* extract which is responsible for triggering the proliferation/differentiation of osteoblasts, modulating the expression of genes regulating osteoblastogenesis and thereby probably accounts for its anti-osteoporotic effects. It is proposed for the first time that *T. cordifolia* extract could act as a Selective Estrogen Receptor Modulator (SERM) (Fig. 7). *T. cordifolia* extract containing the phytoestrogen β-ecdysone has immense potential to be developed into a promising anti-osteoporotic drug in postmenopausal women.

4. Conclusion

*T. cordifolia* could act as a selective estrogen receptor modulator eliciting prostimulatory effects on the bone and not on the reproductive organs like breast or cervix. Hence *T. cordifolia* could be a potential/probable anti-osteoporotic candidate. Low doses of *T. cordifolia* is devoid of any undesirable toxic effects in animal models whereas high doses were found to be both hepatotoxic and nephrotoxic. Also, it is proposed that the phytoestrogen β-ecdysone could be the active component responsible for the bone protective properties of *T. cordifolia*.

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Conflict of interest

None.

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