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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*.

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

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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 20 ppm. Lead, arsenic and mercury were not more than 3 ppm, 1 ppm and 0.1 ppm respectively. Total bacterial count was 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 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 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 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.

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% 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 subcultured 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.

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 CO_2 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 5×10^6 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.

2.2.4. Studies on cell viability and growth

To determine the cell growth and viability, MTT [24] and crystal violet assays [25] were carried out.

2.3. In vivo model systems

2.3.1. Animals

Adult Sprague–Dawley rats weighing between 100 and 120 g obtained from the approved source of Bangalore University (M/s Raghavendra enterprises, Bangalore, India) were used for the study. The animals were kept in quarantine for a period of two weeks after procurement. After the quarantine period, they were acclimatized to animal house conditions and were fed on commercial pelleted rat chow (M/s Hindustan Lever Limited, Bangalore, India) and water *ad libitum*. For the *in vivo* experiments, *T. cordifolia* extract was administered orally by intra gastric intubation (Feedy intubation tube, India, size 05, Diameter 1.70 mm).

This part of the study which involved the usage of animals was carried out following ethical guidelines specified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest and was approved by IAEC (approval No – BUB/IAEC/MB&BT/Dr.MSP/dated 09.05.11).

2.3.2. In vivo toxicology studies

The animals were segregated into six groups. Five groups of animals were administered with different dosages of *T. cordifolia* extract *per se* (100 mg/kg, 250 mg/kg, 500 mg/kg, 750 mg/kg and 1000 mg/kg body weight/day orally for 30 days). The untreated group served as the control. After the treatment period, the animals were sacrificed by CO_2 asphyxiation.

Biochemical and histopathology analysis: Blood samples collected from different groups of animals were used for the determination of Blood Glucose [26], Serum cholesterol [27], Urea [28], Creatinine [29], activities of aspartate transaminase [30] and alanine transaminase [31,32] after the separation of serum or plasma.

2.3.3. Histopathology analysis

Different tissues like liver, kidney, heart and the bone (femur) was excised from the animals, washed in ice-cold isotonic saline and then blotted to dryness. For histopathological analysis, a section of the organs (heart, kidney, liver, lungs and bone) were fixed in 10% formalin saline [33]. Briefly, the tissue sections were embedded in paraffin wax and after deparaffinising; sections were immersed in the filtered hematoxylin for 3 min and then rinsed with running water until the water became clear. Sections were again immersed in 1% eosin stain for 1–2 min and rinsed until water became clear. There after these were dehydrated in ascending graded alcohol solutions (50%, 70%, 80%, 95% and 100 % × 2 times) and cleared with xylene (3 times) and the architectural changes, if any, were observed under a light microscope.

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 estrogen like substances with breast 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 on 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

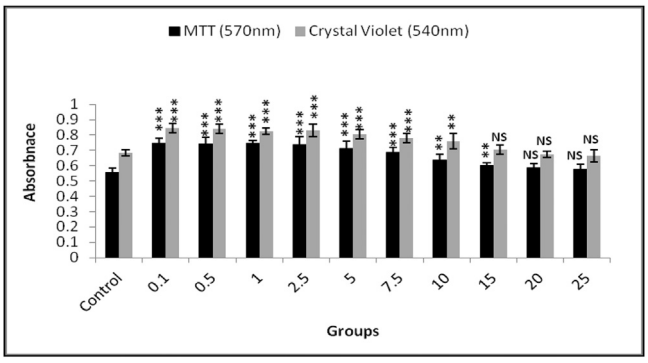

Fig. 2. Effect of β-ecdysone on the growth of MG-63 cells. Figure shows the growth and viability of untreated (control) and β-ecdysone (0.1–25 μg/ml) treated MG-63 cells. Absorbance for MTT assay is 570 nm and for crystal violet is 540 nm. Concentration is expressed as μg/ml of the extract. Values were expressed as mean ± SD (n = 6). Comparisons were made between control Vs other groups. ****P < 0.001, **P < 0.01 and NS – non significant.*

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 SGPT) 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

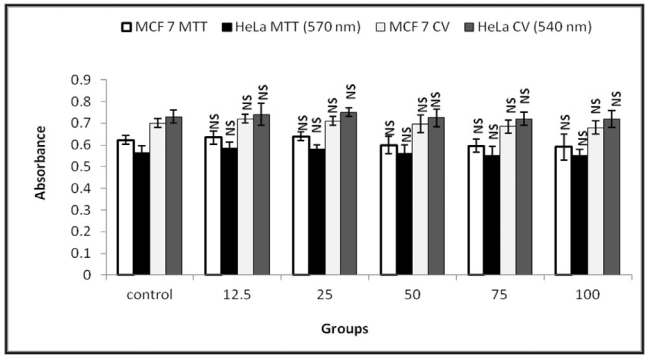

Fig. 1. Effect of *T. cordifolia* on the growth of MCF-7 and HeLa cells. Figure shows the growth and viability of untreated (control) and *T. cordifolia* (12.5–100 μg/ml) treated cells. Absorbance for MTT assay is 570 nm and for crystal violet test is 540 nm. Concentration is expressed as μg/ml of the extract. Values were expressed as mean ± SD (n = 6). Comparisons were made between control Vs other groups. *NS – non significant.*

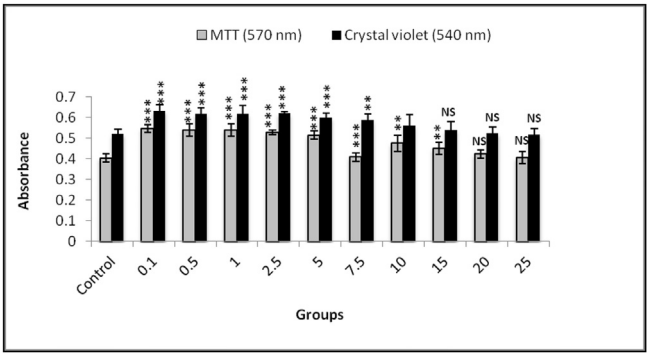

Fig. 3. Effect of β-ecdysone on the growth of primary osteoblasts. Figure shows the growth and viability of untreated (control) and β-ecdysone (0.1–25 μg/ml) treated primary osteoblasts. Absorbance for MTT assay is 570 nm and for crystal violet is 540 nm. Concentration is as μg/ml of the extract. Values were expressed as mean ± SD (n = 6). Comparisons were made between control Vs other groups. ****P < 0.001, **P < 0.01 and NS – non significant.*

Table 1
Effect of ethanolic extract of *T. cordifolia* on biochemical parameters.

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 ± 0.04^{NS}	71.07 ± 5.1^{NS}	9.43 ± 1.32^{NS}	17.67 ± 2.78^{NS}
250	84.24 ± 6.4^{NS}	32.34 ± 4.2^{NS}	0.80 ± 0.02^{NS}	73.65 ± 4.8^{NS}	10.29 ± 0.95^{NS}	17.83 ± 2.04^{NS}
500	84.38 ± 6.3^{NS}	33.77 ± 5.1^{NS}	0.83 ± 0.03^{NS}	75.22 ± 6.8^{NS}	10.66 ± 1.77^{NS}	19.55 ± 2.05*
750	86.12 ± 4.4^{NS}	35.19 ± 5.6^{NS}	0.89 ± 0.02^{NS}	75.56 ± 3.8^{NS}	11.45 ± 1.45^{NS}	20.54 ± 2.33**
1000	87.33 ± 4.1^{NS}	35.55 ± 3.9^{NS}	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.

experimental group of animals. Nonsignificant changes were observed in all the biochemical parameters studied in the group of rats treated with low concentrations of *T. cordifolia* extract (100–250 mg/kg body.wt/day for 30 days) as compared to the untreated control rats. Rats administered with 500 mg/kg body.wt/day of the extract for 30 days also showed non-significant changes when compared with the control rats except for a marginal increase in alanine transaminase activity ($P < 0.05$). But treatment with higher doses viz. 750 mg/kg body.wt/day and 1000 mg/kg body.wt/day for 30 days resulted in statistically significant increase in ALT levels as compared to control ($P < 0.01$). Besides this, treatment with 1000 mg/kg body.wt/day of *T. cordifolia* extract for 30 days, also caused an increase in the activities of AST and levels of creatinine as compared to control ($P < 0.05$). This indicates 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 first report implicating the toxic effects of *T. cordifolia* at higher doses in animals and imply that caution need to be exercised in prescribing higher doses of *T. cordifolia* in humans. There is a possibility that the toxic effects observed could be species specific. Further studies on human hepatocytes and renal cells could throw more light pertaining to the toxic effects of *T. cordifolia*.

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.

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

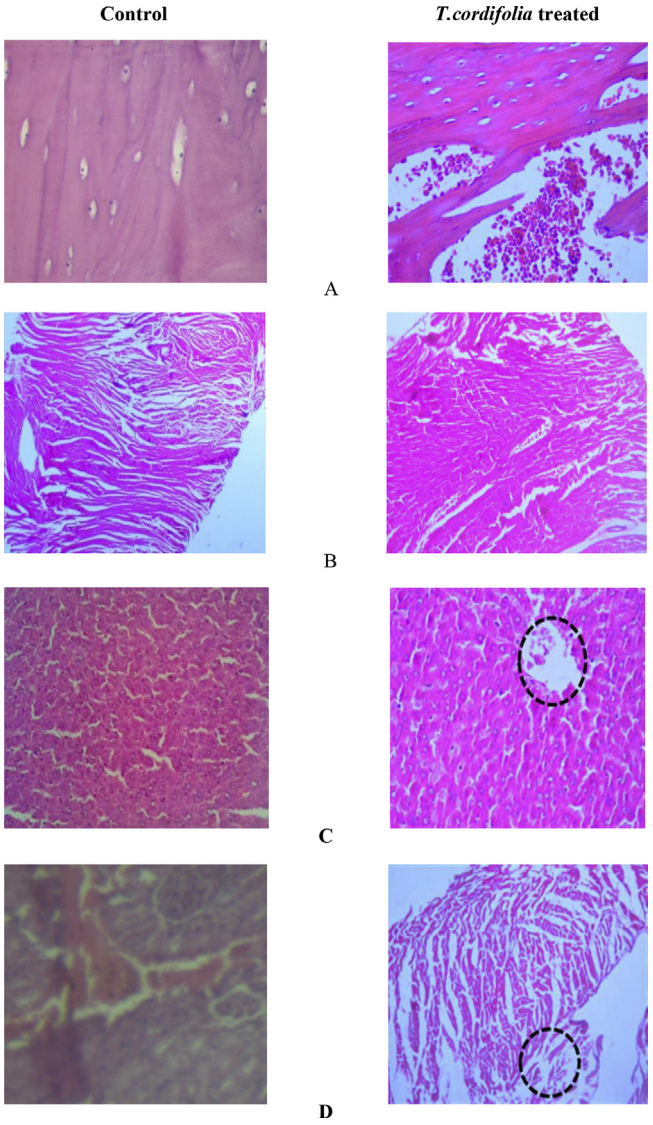

Fig. 4. Effect of *T. cordifolia* on tissue architecture. Fig. A – depicts the hematoxylin and eosin stained sections of bone from control rat and rat treated with 1000 mg/kg bd.wt of *T. cordifolia* showing normal architecture. Fig. B – depicts the hematoxylin and eosin stained section of heart from 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).

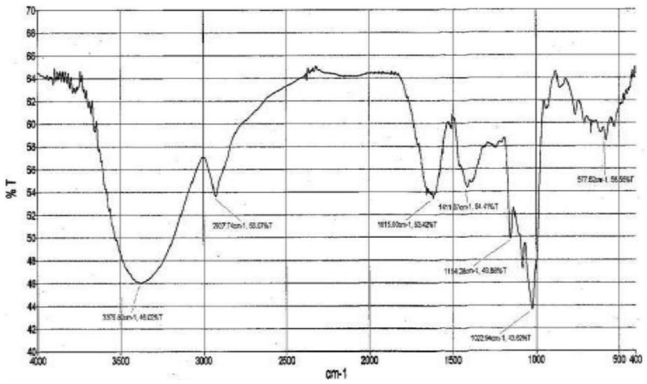

Fig. 5. FTIR analysis. The figure represents the FTIR spectra of the β-ecdysone containing peak of the *T. cordifolia* extract showing the hydroxyl absorption at ~3500 cm and carbonyl absorption at ~1645 cm as compared to the reference spectrum of β-ecdysone retrieved from spectral library.

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

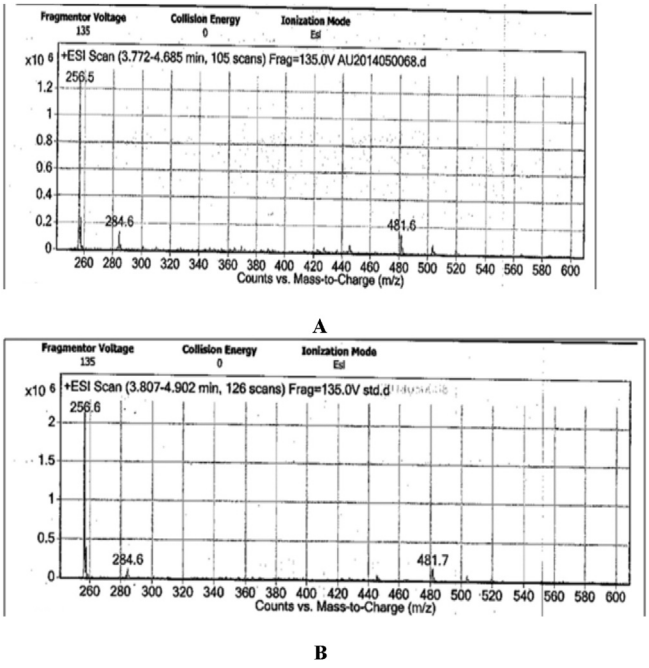

Fig. 6. LC-ESI-MS analysis. The figure A shows the LC-ESI-MS analysis of TLC purified fraction of *T. cordifolia*. The figure B shows the LC-ESI-MS analysis of standard β-ecdysone. Both gave a protonated molecular ion of m/z 481.1, thus confirming that the molecular mass of TLC purified *T. cordifolia* fraction containing β-ecdysone is same as the standard β-ecdysone.

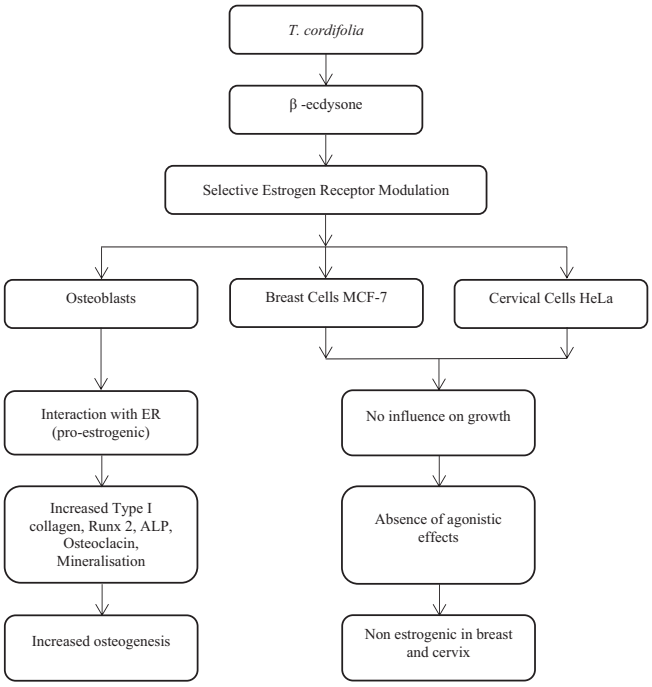

Fig. 7. Probable mechanism of action.

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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