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Selective Estrogen Receptor Modulator (SERM) 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 microarchitecture 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].

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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 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

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