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Study on Osteopotential activity of *Terminalia arjuna* bark extract using UMR 106 cells

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Abstract
Bark extract of *Terminalia arjuna* (TA) possesses potent medicinal properties and therefore, used to cure various ailments in Ayurveda for over 2,500 years. We analyzed the phytochemicals contained in the methanol extract by common and available standard tests. The phytochemical screening revealed that the major bioactive components of the plants are terpenoids, polyphenols, saponins, tannins, alkaloids, flavonoids, carbohydrates, proteins, steroids and glycosides. Cytotoxicity, alkaline phosphatase activity and mineralization potential were assessed using UMR 106 cells. Increased alkaline phosphatase and calcium release were observed in TA extract compared with control. The observed osteoblastogenic induction effect of TA extract holds promise for its development as a bio supplement in bone tissue engineering research.

Keywords: *Terminalia arjuna*, phytochemical profile, MTT assay, osteoporosis, mineralization, UMR 106 cells

Introduction
Herbal drugs are produced from plant kingdom. Different parts of the plants such as seeds, berries, leaves, bark, root or flowers with significant medicinal value are used as phyto medicines. Day by day awareness about the importance of medicinal plants is increasing. Generally, herbal drugs are easily available, safe, less expensive, more efficient, and rarely have side effects when compared to synthetic drugs. Medicinal plants contain some bioactive substances such as tannins, alkaloids, proteins, carbohydrates, terpenoids, saponins, glycosides, steroids, flavonoids and phenols and these may provide definite physiological action on the human body. The chances of prevention of various degenerative diseases and other diseases associated with increased reactive oxygen species are observed to be high with the medicinal plants containing phytochemical constituents with high antioxidant property [1].
Osteoporosis (OP) is a progressive systemic disease characterized by bone mass loss and the deterioration of bone micro architecture, which leads to bone fragility and increased risk of fractures. It is now widely recognized as a public health problem since this disease is associated with high mortality, morbidity and medical expenses throughout the world. Osteoblast inhibition or osteoclast breakdown in the case of osteoporosis results in reduced bone mineral density. Osteoporosis may be either primary or secondary. Primary osteoporosis are subdivided into type I (postmenopausal) and II (senile) and secondary osteoporosis is also referred as type III osteoporosis. Type I, also known as postmenopausal, is characterized by rapid bone loss and affects women shortly after the start of menopause, mainly in the trabecular bone and is associated with the vertebrae and distal radio fractures.
Chronic deficiency of calcium with aging, increase in parathormone activity and decrease in bone formation leads to Type II or senile osteoporosis. It occurs in individuals over the age of 70 years, in a 2:1 ratio of women to men. Both trabecular and cortical bones are affected and a causal association with hip fractures has been observed. The Secondary or type III osteoporosis results from an inflammatory process, endocrine changes, multiple myeloma, sedentariness and the use of drugs such as heparin, corticoids and alcohol.
Terminalia arjuna is found ubiquitously in India, a deciduous tree belonging to *Combretaceae* family. This tree is an exotic tree in India and is one of the most versatile medicinal plants having a wide spectrum of biological activities. The bark of *T. arjuna* is anti-dysentric, antipyretic, astringent, cardiotonic, lithotriptic, anticoagulant, hypolipidemi, antimicrobial [2] and antiuremic [3] agent. Various phyto-constituents have been isolated from *T. arjuna*, which included triterpenoids for cardiovascular properties, tannins and flavonoids for its anticancer, antimicrobial properties and so on [4].

Materials and Methods

Plant Collection and preparation of plant extracts

The *Terminalia arjuna* bark was collected from the nearest Ayurvedic herbal center. A simple hot percolation method using hot water bath was used for extraction. About 10g of dried powder of the bark was dissolved in 50 mL of methanol (1:5) in 500 mL beaker and was covered with aluminum foil. Then the beaker was kept on hot water bath at 50 ºC for 4 h. After the incubation period the extract was filtered with Whatman No.1 filter paper and the filtrate was collected in separate 50 mL beaker. Residue present over the filter paper was discarded and the filtrate was taken for further use. Then the filtrate was kept at 50 ºC in the water bath until extract solvent gets completely evaporate and turn into a semi solid form. The % of total yield of the obtained crude extract was calculated as follows:

Extract yield %= (W1/W2) X 100

Where, W1 is the net weight of TA extract in grams after extraction and W2 is the total weight of bark in grams taken for extraction.

Phytochemicals analysis

Phytochemical analysis of the TA extract was carried out according to standard methods [5-7].

Salkowski reaction test for phytosterols

To 0.5 mL TA extract taken in a test tube, 1 mL of concentrated H_2SO_4 was added from the sides of the test tube. Reddish brown color in the chloroform layer indicated the presence of phytosterols.

Liebermann-Burchard's test for triterpenoids

Few drops of acetic anhydride were added to TA extract, boiled and cooled. From the sides of the test tubes concentrated H_2SO_4 was added. The formation of deep red color and the brown ring at the junction of two layers indicated the presence of triterpenoids.

Foam test for saponins

Small amount of TA extract was taken along with little quantity of water and shaken vigorously. The presence of saponins was confirmed when the appearance of foam was persisted for 10 min.

Dragendroff's test for alkaloids

Initially, the TA extract was dissolved in chloroform. The residue left behind after chloroform evaporated was acidified by adding a few drops of Dragendroff's reagent (Potassium bismuth iodide). Presence of alkaloids was confirmed upon appearance of orange red precipitate.

Molisch's test for carbohydrates

The TA extract was mixed with Molisch reagent, and conc. H_2SO_4 along the sides of the test tube was added. The presence of carbohydrates was confirmed after the appearance of a reddish violet ring.

Lead acetate test for flavanoids

Appearance of yellow precipitate indicated the presence of flavonoids when a few drops of 10% lead acetate solution were added to TA extract.

Test for phenolic compounds and tannins

Ferric chloride solution was added drop by drop to 2 mL of TA extract. The appearance of a bluish black precipitate confirmed the presence of phenolic compounds and tannins.

Ninhydrin test for proteins

The appearance of blue color indicated presence of amino acid when a few drops of ninhydrin were added to TA extract.

Keller-Killiani test for glycosides

Few drop of ferric chloride solution and conc. H_2SO_4 (slowly through the sides of the test tube) and were 1 mL of glacial acetic acid added to the TA extract. Appearance of the reddish brown ring at the junction of the liquids indicated the presence of deoxy-sugars.

Cell culture

In the present study UMR 106 cell line was obtained from the American Tissue Culture Collection (ATCC, Cryosite, Lane Cove, NSW, Australia) and were cultured in Dulbecco's modified Eagle's medium (DMEM); supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 µg/mL), l-glutamine (4.5 mg/mL), and glucose (4.5 mg/mL). Cells were incubated at 37 ºC in a humidified atmosphere containing 5% CO_2 and 95% air. The cells were subcultured every time they reached 80–90% of confluency.

Cell viability

Cell viability was determined calorimetrically using the 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay. UMR 106 cells were seeded at $1X10^4$ cells/well in 96-well plate. After 2 h, the TA extract (50, 100 and 200 µg/mL) was added to the cells and later incubated at 37 ºC for 18 h. MTT solution of 20 µL (5 mg/mL) was added to each well, and the plates were incubated for 4 h at 37 ºC. The formazan crystals in each well were dissolved in 200 µL of dimethyl sulfoxide (DMSO) and incubated for 30 min at 37 ºC after removing the supernatant. The optical density was read on a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm wavelength. The EC50 values were calculated using Graphpad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) after 5days of treatment.

Alkaline phosphatase (ALP) enzyme assay using UMR106 cells

The cell count was adjusted to 1.0 X 10^5 cells/mL using DMEM media containing 10% FBS after monolayer cell culture was trypsinized. Each well of the 96 well microtiter plate was added with 100 µL of the diluted cell suspension (50, 000 cells/well). The supernatant was removed from the partial monolayer formed after 24 hrs, and was washed once with the medium and then 100 µL of different concentrations of test drugs were added on to the micro titer plate. The plates were then incubated at 37 ºC for 24 hrs in 5% CO_2 atmosphere. After incubation the test solutions in the wells were collected. To these collected 20 µL of test solutions, 1000 µL working reagents prepared by mixing 4 volumes of R1 with 1 volume of R2 from ALP activity assay kit (Elabscience Biotechnology Inc. USA). Optical density was taken at 405 nm for every 1min. ALP activity was calculated later using the formula.

ALP activity (IU/L) = ΔA/min*2720

Evaluation of mineralization by Alizarin red- S staining in UMR 106 cells

About $1X10^6$ UMR 106 cells were cultured in a p-35 dishes containing 2 mL of complete DMEM media. After 24 hrs of incubation, cells were treated with 40 and 80 µg/mL samples. Cells in one p-35 dish was treated as control (DMEM media without test sample) and incubated for 24 hrs. On every third day media was changed in a p-35 dishes containing test samples for 21 days. After 21 days, the cell monolayer was washed with 1X Phosphate Buffered Saline (PBS) without disrupting the cell monolayer. The PBS was carefully aspirated and discarded. Cells were then fixed with 10% formalin and incubated for 30 min. The formalin was carefully aspirated after incubation and the cell monolayer was washed without disturbing the monolayer using distilled water.

Distilled water was carefully aspirated and discarded. Enough amount of Alizarin Red S stain was added to cover the monolayer cells and was incubated in the dark for 45 min at room temperature (RT). Alizarin Red S staining solution was carefully aspirated and the cell monolayer was washed four times with 1 mL distilled water. Distilled water was carefully aspirated and PBS was added. The cells were observed under microscope for their staining property.

Results

Percent of yield determination

The obtained yield (%) of the *T. arjuna* bark methanolic extract was 0.3%.

Preliminary phytochemical screening

The preliminary phytochemical analysis showed the presence of high concentration of active compounds, such as phytosterol, lactones, flavonoids, phenolic compounds, tannins and glycosides and also, the active compounds presence in low concentration, such as triterpenoids, saponins, alkaloids, carbohydrates and proteins in methanolic extract *T. arjuna* bark (Table 1).

Table 1: Qualitative phytochemical analysis of methanolic extracts of *T. arjuna*,

Phytoconstituents	Tests	Conclusion
Alkaloids	Dragendroff's	+
Phenolic compounds and Tannins	Ferric chloride test	++
Glycosides	Keller-Killiani test	++
Flavanoids	Lead acetate test	++
Carbohydrates	Molisch's test	+
Proteins	Ninhydrin test	+
Polysterols	Salkowski reaction test	++
Triterpenoids	Liebermann-Burchard's test	+
Saponins	Foam test	+

+: Present in low concentration; ++: Present in high concentration.

Effect of TA extract on UMR 106 Cell proliferation using MTT assay

The cell proliferation study of UMR 106 cell lines was carried out using MTT assay. TA extract showed EC50 value of 301 µg/mL dose dependent proliferation in UMR 106 cells and is highly active (Fig. 1).

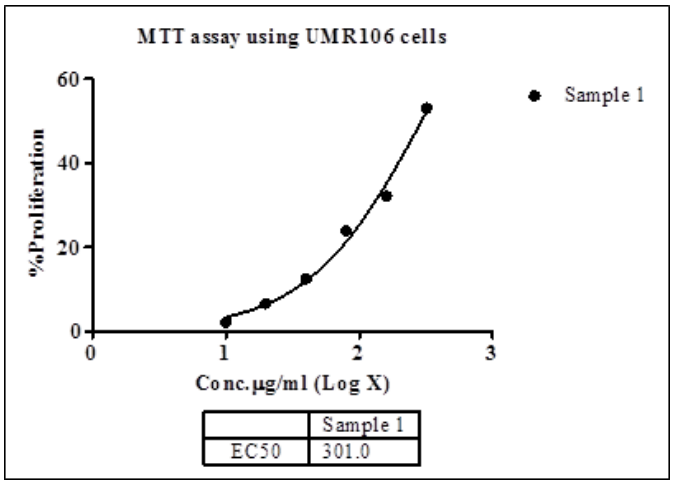

Fig 1: Effect of TA extract on proliferation of UMR106 cells

Alkaline phosphatase enzyme assay using UMR106 cells

The optical density of control and TA extract were measured at 405 nm at the 1st and 3rd minute. The TA extract showed the maximum ALP activity of 90.67 U when compared to control indicating the increased activity of ALP enzyme.

Evaluation of mineralization by Alizarin red- S staining of UMR 106 cells

The UMR-106 cell lines treated with TA extract at 40 and 80 µg/mL induced mineralization when compared to control. Mineralized osteoblasts with extracellular calcium deposits are bright orange-red in color, whereas cells without extracellular calcium deposits are slightly reddish in color. Increased TA extract concentration resulted in increased calcium deposits in UMR-106 cells (Fig. 2).

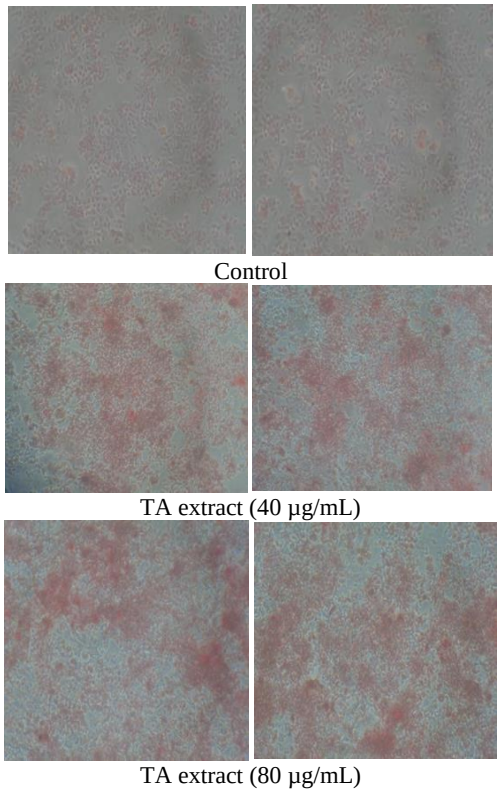

Fig 2: Microscopic view of UMR 106 cells after Alizarin Red S staining

Discussion

A variety of chemical constituents present in the medicinal plants that differ from each other regarding polarity and other chemical properties. Isolation of chemical compounds from plants through solvents of different polarity is frequently practiced in phytochemistry. The presence of phytochemicals, such as proteins, carbohydrates, phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids and alkaloids was revealed after analysis of plant extracts. The various degenerative diseases are addressed by *T. arjuna* [4]. The preliminary phytochemical analysis of methanol extract of *T. arjuna* bark revealed the presence a large amount of phenolic compounds, flavonoids, phytosterol, glycosides and tannins.

A number of investigating studies on chemical treatment such as selective estrogen receptor modulator (SERM), vitamin D3 and parathyroid hormone (PTH), have been reported for osteoporosis. Currently, Biphosphanates (BPs) are widely used as a therapeutic medicine for the OP, as well as bone-metastatic cancers, since they effectively inhibit bone resorption. Despite of great pharmacological and clinical advantages of BPs Yoneda [8] reported serious side effects. This suggests that there is an urgent need for the identification and development of novel medicines. A variety of herbal extracts have been studied for their therapeutic effects and reported worldwide. Among these, curcumin is one of the most investigated herbal extracts [9], isolated from the rhizome of *Curcuma longa*. Curcumin has been reported to exhibit anti-cancer, anti-viral, anti-arthritic, anti-amyloid, anti-oxidant, and anti-inflammatory properties, and is considered a potent therapeutic agent in the prevention and/or treatment of various diseases *viz*., malignant diseases, arthritis, allergies, Alzheimer's disease, inflammation and osteoporosis [10-13].

By the investigation on the osteopotential activity of *Terminalia arjuna* bark extract by Krithiga *et al*. [14], it has been determined that on treatment of MG 63 cell lines with the bark extract has shown a significant increase in calcium content in *T. arjuna* extract treated cells compared to control on 14th day. Here, mineralization potential studied by treating the UMR 106 cells with methanolic bark extract of *T. arjuna* at a concentration of 40 µg/mL and 80 µg/mL for a longer period of 21 days, has revealed its capacity of deposition of calcium inside the cells. The longer duration taken from the sample for mineralization depends on various environmental factors influencing the plant material. This activity plays a major role in reducing the pores present in the cell likewise decreasing the bone fragility.

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