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Evaluation of osteogenic activity of Plaksha (*Ficus lacor* Buch. Ham.) stem bark on human dental pulp derived mesenchymal stem cells

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The adverse effects and lower efficacy of synthetic drugs have increased scientists' interest in traditional medicine for managing chronic diseases like osteoporosis and osteoarthritis, which raise the risk of bone fractures. Herbal medications are increasingly used for joint disorders due to their potential benefits in reducing pain, inflammation, and stiffness, as well as their safety when used correctly. *Plaksha* (*Ficus lacor Buch. Ham*) possess a great potential in treating bone disorders. The purpose of the study is to determine the osteogenic effect of stem bark extract of Plaksha on mesenchymal stem cells. Aqueous extract of *Plaksha* obtained *via* Soxhlet Apparatus. Mesenchymal stem cells were obtained from human dental pulp; Characterized using Cell Surface Antigen Expression Analysis *via* Fluorescence-Activated Cell Sorting (FACS). Cytotoxicity assessed with MTT. Osteogenic potential evaluated through trilineage differentiation and gene expression analysis (RT-PCR) for Alkaline Phosphatase (ALP) and Osteonectin (OSN). Viability assay revealed that the 10 µg/mL concentration helps for trilineage differentiation of cells. Upregulation of ALP and OSN genes *via* RT-PCR in treated group showed the osteogenic differentiation of cells. This demonstrates the aq. Extract of FL promotes the osteogenic differentiation of MSCs and facilitate the osteogenesis. Hence the drug can be used in various bone disorders.

Keywords: Alkaline phosphatase, *Ficus lacor*, Human dental pulp stem cells, Mesenchymal stem cells, Osteogenesis, Osteonectin, *Plaksha*

An increasing prevalence of bone disorders is a major concern throughout every phase of life. Conditions like osteoarthritis, osteoporosis, osteomyelitis, various fractures and injuries are significantly affecting large no. of patients. In modern medicine, treatment modalities for such conditions typically include NSAIDs, DMARDs, and corticosteroids which gives only symptomatic relief and do not address the root cause of the conditions[1]. In case of fracture, different allografts, xenografts, ceramics including calcium-based substitutes are used rather than bone surgeries[2]. These substitutes may lead to central osteonecrosis[3]. Hence, the utilization of traditional medicine can contribute to the management of bone disorders and the prevention of diseases by promoting bone health. *Plaksha* (*Ficus lacor Buch. Ham*) a 20-meter-tall evergreen tree from Moraceae family is available all over the India and hasa huge osteogenic potential along with an anti-inflammatory, wound healing, anti- bacterial and blood clotting properties[4]. Although, these properties of *Ficus lacor* (FL) have been evaluated, the bone healing property has not been evaluated. The extract of stem bark was reported to contain bergenin, caffeic acid, saponin, flavonoids and β- sitosterol[5]. There are many drugs having above properties but their overuse led to depletion and also created a risk of an adulteration. This research aims to demonstrate the impact of *Ficus lacor* on promoting bone formation in mesenchymal stem cells derived from dental pulp. Mesenchymal stem cells (MSCs) have become increasingly popular in clinical settings for the management of bone diseases, owing to their significant capacity to undergo differentiation into osteoblasts. The biological effects of traditional medicines can be studied using this *in vitro* system.

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Abbreviations: ALP, Alkaline phosphatise; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethyl Sulfoxide; FBS, Fetal Bovine Serum; FL, Ficus lacor; HDPSCs, Human dental pulp stem cells; MSCs, Mesenchymal stem cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphyltetrazolium bromide; OSN, Osteonectin; PBS, Phosphate Buffer Saline

Hence osteogenic potential of FL was studied on human dental pulp derived mesenchymal stem cells.

Material and Methods

Ethical approval

The experiment was approved by both Institutional Ethical Committee with Ref. no. DPU/924/13/2021at D. Y. Patil College of Ayurved and Research Centre Pimpri, Pune and Institutional Committee for Stem Cell Research at Dr. D. Y. Patil Dental College Pimpri, Pune with Ref. No. IC-SCR/RM/43/21. An In- vitro experiment on human dental pulp stem cells was carried out at the Regenerative Medicine Laboratory Dr. D. Y. Patil Dental College, Pimpri, Pune.

Preparation of extract

The *Ficus lacor* stem bark was collected from the nearby region of Pimpri. Identification of drug was done at Botanical Survey of India, Pune. The identification no. Is BSI/WRC/Iden.Cer. /2022/ 1703220003407. Standardization of drug was done at *Sudhatatva* Pharmacy of Dr. D. Y. Patil College of Ayurved and Research Centre Pimpri, Pune, Maharashtra. The aqueous extract of stembark of FL was obtained through the Soxhlet apparatus using 50 g of dried powder of stembark[6].

Isolation and culture of human dental pulp stem cells

Human dental pulp was used to obtained the multipotent mesenchymal stem cells. The tooth donor had no any medical history. And consent was obtained prior to extraction of tooth. Initially, the sample was immersed into the povidone-iodine solution. Next submerged in saline solution and transferred to 15mL tube having (PBS) phosphate buffered saline with 1% antibiotic and antimycotic solution.

Sterile Airotor hand piece was used to cut the tooth and the pulp was obtained. The pulp sample was placed on a clean petri dish with PBS. The FBS was added in sufficient amount and incubated for 15 min in CO_2 incubator at 37°C. The tissue was cut into small pieces of approximately 1mm with the help of sterile blade. The pieces were again cleaned with PBS and transferred to T25 flask with 1% antibiotic and antimycotic solution and FBS. The flask was kept for incubation at 37°C and 5% CO_2 for 24 h. After 24 h the flask was supplemented with Dulbecco's modified eagle medium (DMEM), 10% FBS and 1% Antibiotic. Again, kept for incubation at 5% CO_2 and 37°C. The new medium was added at every 2 to 3 days. The plate was incubated for 5 to 7 days in CO_2 incubator[7].

The out -growth of cells was observed under the inverted phase contrast microscope. When the cells attained 80% confluence, the trypsinization was done using 2 mL of 0.25% trypsin EDTA solution and kept in CO2 incubator for 1 min. To stop the trypsinization 3 mL of culture media was added to the flask. This mixture was then transported to the 15 mL sterile tube and centrifuged for 5 min at 1800 rpm and 20°C. The floating solution was removed and the pellet was transferred to the sterile T25 flask. The cells in the flask were passage zero cells. The procedure was carried out till the cells reach 80% confluence. Passage no. 4 cells were used for the further experiment.

Characterization of human dental pulp stem cells using Fluorescence-activated cell sorting (FACS)

The isolated and cultured stem cells were identified using the flow cytometry with specific cell surface markers. The antibody panel includes a variety of markers, comprising PE labelled CD90, CD73, and CD105 as well as FITC-labelled CD34, CD45, and HLA-DR. A 200 µL of cell suspension containing 2×10^6 cells was transferred to the 4 Eppendorf tubes. The fixation of cells was carried out at room temperature (35°C) for 30 min with 4% formaldehyde. After the fixation the cells were cleaned with PBS. A 3 µL of specific antibodies were added to the cells and kept for incubation at room temperature for 30 min[8]. The tubes were used for FACS analysis with the help of Cell Quest Pro software.

MTT assay

Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The stock solution for the aqueous extract of FL stem bark was produced by dissolving 10 mg of FL in 1 mL of Dimethyl Sulfoxide (DMSO). A 96-well plate was utilized to seed the HDPSCs at a density of 1×10^4 cells per millilitre. FL concentrations of 2 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL, and 50 µg/mL were employed for 24 h after the cells reached 80% confluence. Then, 20 µL of MTT was applied to each well and incubated at room temperature. After 3 h, remove the MTT and add 100 µL of DMSO[9]. The absorbance was measured using an ELISA reader at 560 nm.

Trilineage differentiation of human dental pulp stem cells

Differentiation of mesenchymal stem cells into osteogenic, chondrogenic and adipocyte was evaluated by providing specific induction medium and stained using appropriate stain after 13 days. To assess the osteogenic differentiation cells were incubated using the reagents Dexamethasone at 0.1 µM, glycerophosphate at 10mM and Ascorbic acid at 2 mM concentration and stained with 2% Alizarin Red S. For the chondrogenic differentiation, cells were incubated using the media containing Dexamethasone at 0.1 µM, Sodium pyruvate at 100 µg/mL, ITS at 1X, Ascorbic acid at 2mM, L- proline 40 µg/mL, TGF-β3 at 10 ng/mL and stained using 0.1% Alcian blue. The adipogenic differentiation was observed providing the adipogenic induction media containing dexamethasone, 3-isobutyl-5-methyl xanthine, Indomethacin and insulin and observed using 0.3% Oil Red O stain[10]. The media was changed after every 2 to 3 days and incubated for 13 days.

Real - Time reverse transcriptase - Polymerase chain reaction (RT-PCR)

Cells treated with 10 µg/mL aq. Extract of FL were used for the gene expression. The reagents require for RNA extraction are TRIzol method (Ambion Life Technologies), The kit used for cDNA coding was High-Capacity cDNA Reverse Transcription Kit (Thermofischer). 10X Random primer, 10X Reverse transcription buffer, 25X dNTP mix, RNase inhibitor (20 U/µL), DEPC treated water (Himedia), Multiscribe TM Reverse transcriptase (50 U/µL). The volume for one reaction was 6.8 µL and volume of reagents for 5 reactions was 34 µL. The 6.8 µL of master mix was mixed with 13.2 µL of RNA extracted[11]. The genes used for RT-PCR are Alkaline phosphatase (ALP) and osteocalcin (OSN) and GAPDH as housekeeping gene. The primers are enlisted in the (Table 1). The RT-PCR was done using QuantStudio 5 Real-Time PCR System instrument.

Results

Aqueous extract of FL obtained through Soxhlet apparatus

The final aqueous extract of FL obtained was 50.22 g including the wt. of empty petri dish (47.55 g) and the extractive value is 2.67 g in sticky form. Total time required for extract formation was 13 h.

Isolation and culture of human dental pulp stem cells

After 24 h of incubation (day1) tissue adhered to the surface of T25 flask. The media of explant culture was changed at the interval of 2 to 3 days and observed under an inverted phase contrast microscope till day 19. No outgrowth around the explant was seen up-to day2. On the day 3 out-growth of explant was seen. The cells had elongated structure and were fibroblastic in nature. On the 19th day cells achieved 75- 80% confluency. Subsequently, the cells were detached using 0.25% trypsin EDTA and transferred to a T25 flask for further incubation. After culture the cells acquired the fibroblastic morphology with 75- 80% confluency these cells were transferred toT75 flask for further assay (Fig. 1).

Cell characterization revealed stemness of the HDPSC

The human dental pulp derived mesenchymal stem cells used in this study exhibited positive expression of markers CD73, CD90, CD105 and tested negative for the CD45, HLADR, CD34. Histogram A, B and C shows presence of more than 90% of cells *i.e.* 90.227%, 98.989%, and 99.278% showing thatthe markers are positive for, CD90, CD105, CD73, respectively. On the other hand, histograms D, E, and F do not show the presence of stem cells, as they

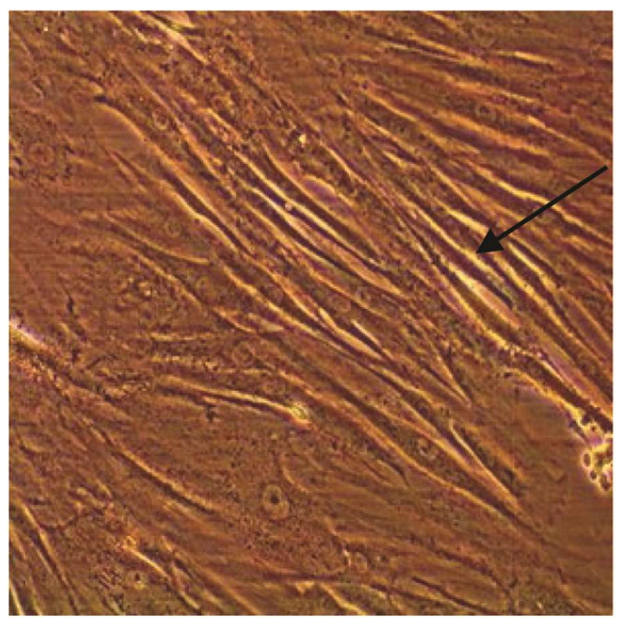

Fig. 1 — Thin slender Fibroblastic morphology of human dental pulp stem cells under inverted phase contrast microscope on 19th day (100X magnification). (Black arrow)

Table 1 — primers used for the osteogenic gene expression

Name of gene	Forward primer	Reverse primer
GAPDH	TCCCTGAGCTGAACGGGAAG	GGAGGAGTGGGTGTCGCTGT
ALP	GACCGGACCCTCGCCAGTGCT	AATCGACGTGGGTGGGTGGGG
OSN	GAGGAAACCGAAGAGGAGG	GGGGTGTTGTTCTCATCCAG

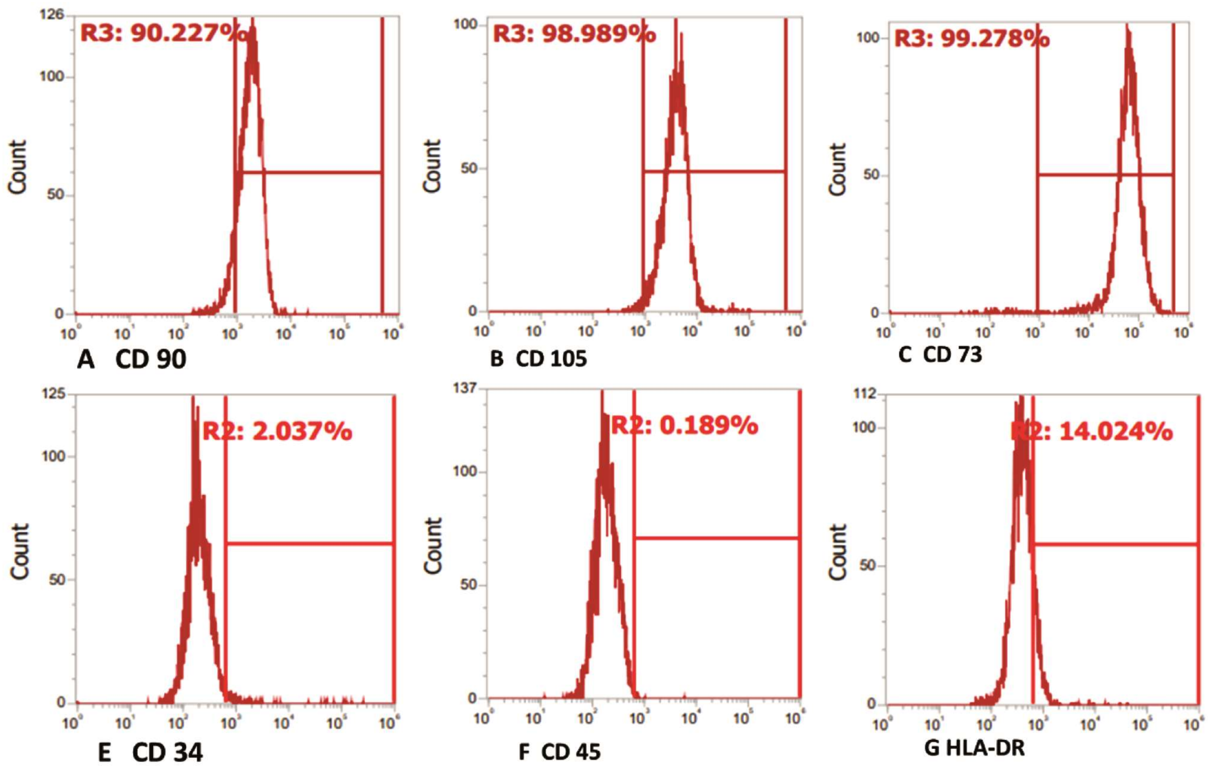

Fig. 2 — surface markers expression for human dental pulp stem cells are positive for (A-C) CD90-PE-H, CD73-PE-H, CD105-PE-H; and (D-F) negative for stemness CD45-FITC-H, HLADR-FITC-H, CD34-FITC-H for stemness

exhibit less than 14% of cells *i.e*. 2.037%, 0.189%, and 14.024% indicating the markers are negative for CD34, CD45 and HLADR, respectively (Fig. 2).

MTT assay

The viability of FL was tested by MTT assay in triplicate with concentrations of 2 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL, and 50 µg/mL. The 10 µg/mL concentration was estimated using Microsoft Excel and this concentration (10 µg/mL) was used in the subsequent experiment (Fig. 3).

Trilineage differentiation

The cells induced by osteogenic media showed black coloured deposits around them. Spherical morphology of cells was observed along with the cartilage specific molecules and extracellular matrix in chondrogenic induced medium. Small oil droplets were seen around the edges of cells in those induced with adipogenic media. On day 13 cells were stained using 2% Alizarin Red S, 0.3% Oil Red O and 0.1% Alcian blue stain in Osteogenic, adipogenic and chondrogenic media cells, respectively, (Fig. 4).

***Ficus lacor* (FL) showing the osteogenic differentiation**

A 10 µg/mLconcentration of FL was used along with the control group which showed the osteogenic

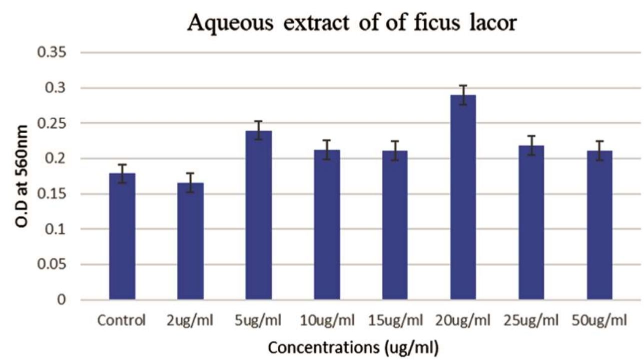

Fig. 3 — Analysis of MTT assay: Aqueous extract of FL with various concentrations 2 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL, and 50 µg/mL used for 24 hrs. X - axis indicates various concentrations of Fl and Y – axis indicates OD at 560 nm

differentiation in the form of osteogenic components (osteoblasts) which was stained using alizarin red S stain (Fig. 5).

Osteogenic Gene expression by FL in hDPSCs

In each sample, the target genes osteonectin and alkaline phosphatase were analysed, and by applying the comparative ΔΔCt method, the relative fold changes of osteonectin and alkaline phosphatase were calculated for the respective samples. Following

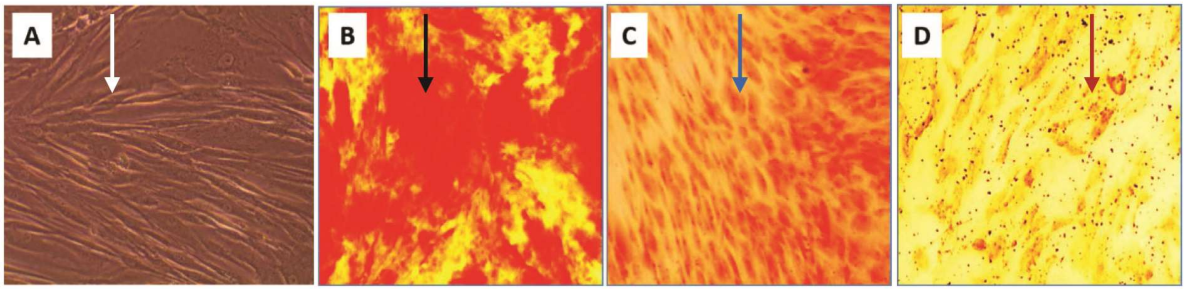

Fig. 4 — Trilineage differentiation of hDPSCs using aq. extract of FL. (A) Fibroblastic morphology of hDPSCs supplemented with complete media (DMEM+FBS + antibiotic and antimycotic) (white arrow); (B) hDPSCs differentiated into osteocytes stained with Alizarin Red S and red colour indicates calcium deposits (black arrow); (C) hDPSCs differentiated into chondrocytes stained with Alcian blue (blue arrow); and (D) hDPSCs differentiated into adipocytes stained with Oil red O stain where yellow dots indicate oil deposits (Red Arrow)

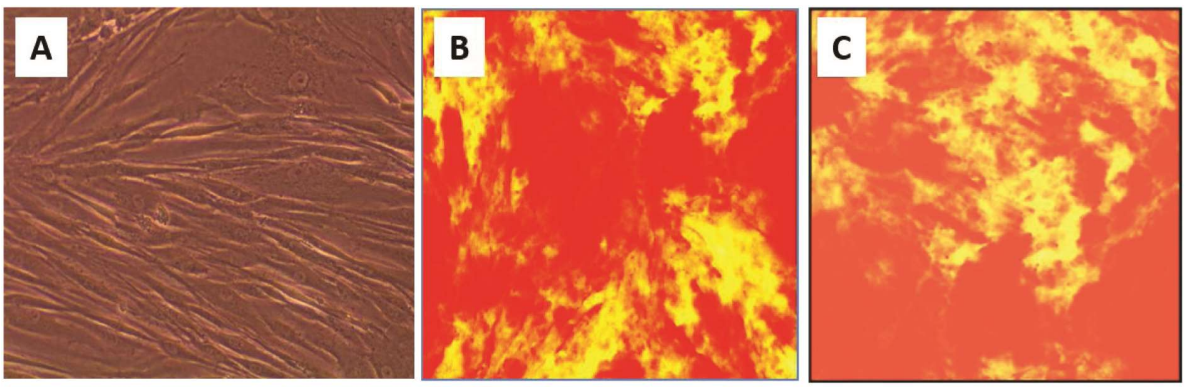

Fig. 5 — Osteogenic differentiation of hDPSCs using aq. extract of FL. (A) fibroblastic morphology of hDPSCs supplemented with complete media (white arrow); (B)osteogenic differentiation of hDPSCs supplemented with osteogenic media stained with Alizarin Red S stain (Black arrows); and (C) osteogenic differentiation of hDPSCs treated with aq. Extract of FL with osteogenic media stained with Alizarin Red S Stain, where red colour indicates calcium deposits (Black arrows)

Table 2 — Comprehensive analysis of alp and osn genes in hdpscs cells treated with ficus lacor byreal time qrt-pcr. The *gapdh* gene is used as a housekeeping gene

	Mean Fold Expression Value		
	Control	*Ficus lacor*	P Value
ALP	1.00541	23.01000646	0.0011**
OSN	1.0037559	8.782906517	0.049*

treatment with FL, there was an increase in the levels of osteonectin and alkaline phosphatase associated with osteogenesis. Based on the gene expression analysis and the proliferation data, calcium deposition and the alkaline phosphatase (ALP) level were observed in the cells (Table 2). The increased expression of OSN indicated an augmentation in the mineralization of the bone matrix.

The values are presented as mean fold expression in control and treated group. The results were subjected to Two-way ANOVA followed by Tukey post hoc tests as $^{**}P<0.01$ and $^{*}P<0.05$ to determine the statistical significance when the tested group is compared with the control group.

Discussion

Bones are essential for protecting organs, producing blood cells, and regulating hormones. Their dynamic balance is influenced by intrinsic and extrinsic factors like diet, mechanical stress, hormones, and genetics. Disruptions in these factors can lead to bone disorders such as osteoporosis, osteoarthritis, osteomyelitis, and fractures, which affect people of all ages, from paediatric to geriatric[12].

Modern management for such conditions includes calcium supplements, vitamin D, hormone replacement therapy, bisphosphonates, selective oestrogen receptor modulators, a human monoclonal antibody targeting the receptor activator of NF-κB ligand (RANKL) and strontium ranelate (SR) which leads to development of gallstone, cervical cancer, breast cancer, endometrial Ca, gastroesophageal reflux, osteonecrosis of jaw, suppression of bone turnover and adversely affect the bone stability. Also, NSAID's and opioids causes nausea and vomiting and acts as gastro-irritant[13-18].

Herbs and natural substances with osteogenic activity have garnered interest for enhancing bone health and treating bone disorders. Ginkgo biloba extract has been shown to induce osteogenic differentiation in bone marrow-derived mesenchymal stem cells at 150 µg/mL, increasing expression of osteogenic genes like BMP-2, RUNX-2, and Colla-1[19]. Cissus quadrangularis extracts (hexane and aqueous) promote bone tissue regeneration, while the ethanolic extract of Piper sarmentosum stimulates osteoblast differentiation and osteogenesis[20]. Additionally, *Ficus lacor* aerial roots exhibit antiarthritic activity in rats, likely due to the presence of phenols, alkaloids, glycosides, steroids, and flavonoids[21].

The research demonstrated that mesenchymal stem cells, when exposed to aqueous extract of FL at 10 µg/mL and osteogenic media, exhibited an increased capability to transform into osteoblasts. This transformation was confirmed using Alizarin Red S staining, as well as the assessment of ALP and OSN gene expressions. The significant increase in the ALP and OSN genes implies that FL may play a regulatory role in proliferation of mesenchymal stem cells by promoting the formation of osteoblast and facilitate osteogenesis. Thus, the drug can be used in the low-density bone disorders.

The stem bark of FL contains caffeic acid and bergenin, which support bone health. Caffeic acid inhibits osteoclastogenesis induced by RANKL and TNF-α[23], while bergenin enhances collagen synthesis and ALP activity, promoting osteoblastic differentiation and bone matrix development[24]. Increased gene expression and ALP protein levels serve as early markers of osteogenic differentiation, with ALP playing a crucial role in bone turnover[25]. Osteonectin, a glycoprotein from osteoblasts, binds collagen type I and hydroxyapatite, linking organic and mineral components in bone tissue[26].

The presentstudy suggests the osteogenic action of stem bark extract of FL on human dental pulp derived mesenchymal stem cells as the drug is abundantly available and its osteogenic potential is remained undiscovered.

Conclusion

The upregulation of ALP and OSN genes proves that the aqueousextract of FL promotes the osteogenic differentiation of hDPSCs and facilitate the osteogenesis. Given these findings, the aqueous extract of *Ficus lacor* could be considered a valuable candidate for developing therapies aimed at promoting bone regeneration and treating bone disorders. Its use could help mitigate the adverse effects commonly associated with modern pharmacological treatments, offering a safer, more holistic approach to managing conditions like osteoporosis, bone fractures, and other degenerative bone diseases. Further research and clinical trials would be necessary to fully understand its efficacy and safety profile in humans.

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Conflict of interests

All authors declare no conflict of interest.

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