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Extracellular matrix calcification in human dental pulp stem cells treated with ethanol extract of *Cissus quadrangularis* – An *in vitro* study

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ABSTRACT

Objectives: To induce osteoblastic differentiation in HDP-SC using ethanolic extract of *C. quadrangularis* and evaluate extracellular matrix calcification using von Kossa staining.

Materials and Methods: An alcoholic extract of *C. quadrangularis* extract was prepared using the Soxhlet apparatus. HDP-SCs were procured from HiMedia laboratories in Mumbai and subcultured. On confluence, the cells were subjected to 100, 200, 300, 400 and 500 µg/mL of ethanolic extract of *C. quadrangularis*. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) assay was used to ascertain cell proliferation and cell cytotoxicity. The cells were then treated with the desired concentration of *C. quadrangularis* extract and incubated for 5 days. The presence of matrix calcification was determined using von Kossa staining on the 5th, 10th and 15th day.

Results: The MTT assay revealed that at concentrations of 100, 200 and 300 µg/mL, the percentage of viable HDP-SCs were 99.1%, 98.3% and 95.7%, respectively. The comparison of mean value between all the concentrations (100, 200, 300, 400 and 500 µg/mL) was established to be significant, showing $P < 0.05$. The calcification assay showed extracellular matrix calcification as brown to black nodules on the 10th and 15th day for 100 µg/dl concentration of *C. quadrangularis* extract, on the 5th, 10th and 15th day for 200 and 300 µg/dL concentration of *C. quadrangularis* extract.

Conclusion: The current research is an attempt to evaluate the regenerative potential of plant-based products on HDP-SC. *C. quadrangularis* has shown potential for osteoblastic differentiation on HDP-SC and subsequent matrix formation and calcification and, hence, can be utilised as a therapeutic agent in bone regeneration therapies.

Keywords: Dental pulp stem cells, *Cissus quadrangularis*, Bone, Calcification

INTRODUCTION

Regenerative medicine is a promising field of science that aims to restore the architecture and function of damaged tissues and organs. Regenerative periodontal therapy is an offset of regenerative medicine that serves as a boon in the regeneration of periodontal tissues, including gingiva, periodontal ligament, cementum and bone.[1]

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'Human dental pulp stem cells' (HDP-SCs) possess several qualities, including capacity for self-renewal, multi-lineage differentiation and clonogenic efficiency, and have the capability to function as primordium for the generation of any cell type and thus an invaluable entity for regenerative medicine in dentistry. [2-4]

Cissus quadrangularis, belonging to the *Vitaceae* family, is an ancient medicinal plant native to the warmer regions of India and China. It is believed to have bone healing properties and can be utilised in managing an array of conditions involving bone, including periodontitis, augmentation of surgical and implant sites with bone loss, traumatic and pathological bone loss and so on. [5]

At present, research is directed toward regenerating defaced dentin, pulp revitalisation, periodontal tissue, as well as whole tooth regeneration.[6] However, the possibility of regenerating bone using herbal concoctions has not been explored to a large extent. The aim of the present study dictates the need for a standardised protocol to stimulate HDP-SC for osteoblastic differentiation using plant derivatives. The endpoint of the study is to detect the presence of extracellular matrix calcification in HDP-SCs treated with ethanol extract of *C. quadrangularis* – A preliminary step.

Hence, the current research aims to induce osteoblastic differentiation in HDP-SC using ethanolic extract of *C. quadrangularis* and study the potential of *C. quadrangularis* extract to induce extracellular matrix calcification using von Kossa stain.

MATERIALS AND METHODS

The current research was started following approval from the Institutional Review Board and Ethical Committee of SRMDC, IRB Approval number – IRB/2017/MDS/No.606, dated 8.8.17. As the current research was performed *in vitro*, ethical consent was not a mandate as per the guidelines of the institutional committee.

Preparation of extract

C. quadrangularis (stem) was obtained from the Villages of Hesaraghatta Region, Bangalore. The stems procured were washed 3 times with double distilled water, chopped into 1 cm sized small pieces and kept for air drying under room temperature for nearly 4–9 days. Once the stems were completely dried, they were finely ground into powder. The fine powder was then shifted to a round flask and extracted employing the Soxhlet apparatus. The heating cycle was performed for 48 h. The alcoholic extract thus formed was filtered and transferred to a container till further use.

Institution of cell culture

HDP-SCs were obtained from HiMedia laboratories in Mumbai. The HDP-SCs were maintained in Dulbeco media supplemented with 15% foetal bovine serum at 37°C at 5% carbon dioxide (CO_2). These cells were then trypsinised and subcultured, and the third passage of cells was used in the study.

Assessment of cell proliferation and cytotoxicity

MTT assay was used to ascertain cell proliferation and cell cytotoxicity. Two hundred microlitres of cell suspension were seeded in a 96-well plate (20,000 cells/well). Following an incubation period of 24 h, ethanolic extract of *C. quadrangularis* was added at concentrations of 100, 200, 300, 400 and 500 μg/mL, respectively. Once the test drug was added, the culture plates were once again incubated at 37°C at 5%CO_2 for 24 h. Following incubation, MTT reagent was added and incubated for 3 h. Subsequently, the MTT reagent was removed, and 100 μL of solubilisation solution (DMSO) was added. The absorbance of the sample was read at 570 nm. Each of the experiments was done in triplicates and the mean value for each concentration was used to calculate the cell viability percentage and to assess the difference in cell viability among the concentrations.

Calcification assay

A cutoff value of 95% viability was set to ensure that sufficient replicative viable cells would be available to participate in the hard tissue formation. The cells were cultured in a 6-well plate at a density of 5 × 105 cells/2 mL and incubated overnight at 37°C for 24–48 h until they reached 70–80% confluency. The cells were then treated with *C. quadrangularis* extract and incubated for 5 days. After the incubation period, the medium was removed and phosphate buffer wash was done. The cells were then fixed in 4% paraformaldehyde at room temp for approximately 20 min. For staining with von Kossa stain, the cells were washed in phosphate buffer solution again and then treated with 100 μL of 5% silver nitrate solution and exposed to ultraviolet light for 1 h. The cells were then washed with sterile distilled water and then treated with 2.5% sodium thiosulphate for 5 min and washed again with distilled water. The cells were counterstained with 500 μL of methyl green per well. Observations were made using an inverted microscope with ×20 magnification using MICAM software. The same procedure was repeated for two more periods, that is, on the 10th day and the 15th day, to check for matrix calcification.

Statistical analysis

Mean and standard deviation values are calculated as descriptive statistics. Since the data did not follow normal distribution, the Kruskal–Wallis test was used to determine

the mean difference between each of the concentrations used. A checklist for reporting in vitro studies guidelines-CRIS Guidelines have been followed.

RESULTS

Cell cytotoxicity/proliferation study of *C. quadrangularis* extract against HDPSCs by MTT assay

The MTT assay revealed that at concentrations of 100, 200 and 300 µg/mL, the percentage of viability of HDP-SC was 99.1%, 98.3% and 95.7%, respectively. These observations showed that the *C. quadrangularis* extract does not show any significant cytotoxicity against HDP-SCs with concentrations ranging from 100 to 500 µg/mL following 24-h incubation. [Table 1]. The comparison of mean value between all the concentrations (100, 200, 300, 400 and 500 µg/mL) was found to be statistically significant with $P < 0.05$ [Table 2].

Extracellular matrix calcification of *C. quadrangularis* extract against HDP-SCs by von Kossa staining

The cutoff of 95% viability was set to ensure that sufficient replicative viable cells were available to participate in hard tissue formation. Hence, the concentrations of 100, 200 and 300 µg/dL were subject to extracellular matrix calcification assay. There was a dose-dependent and time-dependent increase in the calcium salt deposition of *C. quadrangularis* extract groups when compared to the control one. The extracellular matrix calcification was observed as brown to black nodules on the 10th and 15th day for 100 µg/dL concentration of *C. quadrangularis* extract, on the 5th, 10th and 15th day for 200 and 300 µg/dL concentration of *C. quadrangularis* extract [Table 3 and Figure 1].

DISCUSSION

Tissue engineering and regenerative medicine have become one of the most fascinating areas of science which has opened up a wide scope for research. Bone is known to have the internal capacity to regenerate throughout life.[7] However, an adequate supply of stem cells is required for effective regeneration to take place in conditions involving large bone defects, tumour resection or any skeletal abnormality where self-healing is not sufficient to cause repair and regeneration. Under inductive conditions, *in vitro* studies have publicised

Table 1: Mean values, standard deviation, standard error and percentage of cell viability for (100, 200, 300, 400 and 500 µg/mL) concentration.

Concentration Unit: µg	Untreated	Standard (PHA) (5 ug/mL)	100	200	300	400	500
Reading 1	0.95	0.98	0.95	0.94	0.92	0.87	0.79
Reading 2	0.95	0.99	0.943	0.94	0.92	0.87	0.80
Reading 3	0.96	0.99	0.947	0.94	0.91	0.88	0.79
Mean	0.95	0.98	0.95	0.94	0.92	0.87	0.79
Mean OD-Mean B	0.92	0.95	0.91	0.90	0.88	0.84	0.76
Standard deviation	0.001	0.002	0.001	0.001	0.002	0.003	0.004
Standard error	0.00	0.00	0.002	0.001	0.001	0.002	0.002
Viability %	100	103	99.17	98.37	95.72	91.32	82.21

OD: Optical density, Mean B: Mean blank (0.036), PHA: Phytohaemagglutinin

Table 2: Comparison of mean values between each concentration.

Concentration	*n*	Mean	Mean difference	*P*-value	Significance
100	3	0.946667 (A)	A-B=0.007334 A-C=0.031667 A-D=0.072 A-E=0.155667	0.009	Significant
200	3	0.939333 (B)	B-C=0.024333 B-D=0.064666 B-E=0.148333	0.009	Significant
300	3	0.915000 (C)	C-D=0.040333 C-E=0.124	0.009	Significant
400 500	3 3	0.874667 (D) 0.791000(E)	D-E=0.083667	0.009	Significant

Table 3: Presence and absence of extracellular matrix calcification observed in varying concentrations of *Cissus quadrangularis* extract on the incubation period of 5, 10 and 15 days.

Concentration-	5th day	10th day	15th day
Untreated	-	-	-
100 µg/dL	-	+	+
200 µg/dL	+	++	++
300 µg/dL	++	+++	+++

- Calcification absent, + Mild Calcification seen, ++ Moderate calcification seen, +++ Profound calcification seen

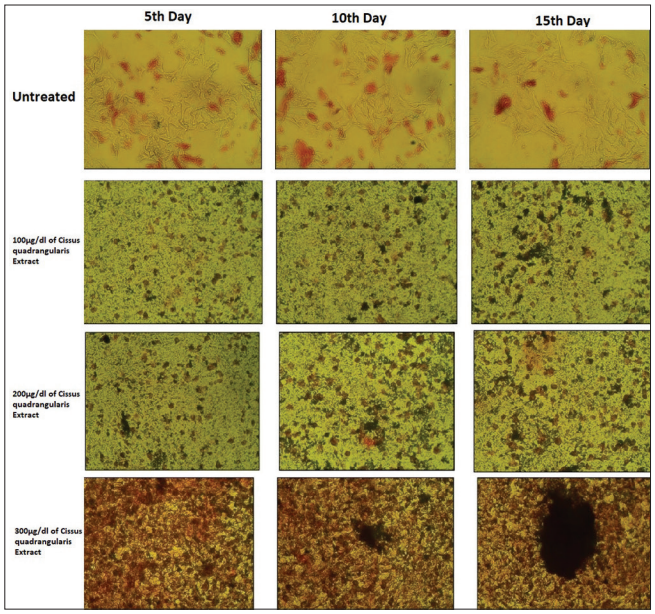

Figure 1: Extracellular matrix calcification observed in human dental pulp stem cells with 100, 200 and 300 µg/dL of *Cissus quadrangularis* extract, respectively, after incubation period of 5, 10 and 15 days in comparison to control.

that HDP-SCs have the capacity to differentiate into bone and cartilage-forming cells under specific impulses.

A previous study done by Parisuthiman *et al*. established that the active constituents present in *C. quadrangularis* help to induce the growth and differentiation of mesenchymal stem cells and also help in stimulating the formation of bone.[8] In the present research, extracellular matrix calcification was observed in HDP-SC on treatment with *C. quadrangularis* after an incubation period of 5, 10 and 15 days. This observation proved that *C. quadrangularis* extract, along with HDP-SC, may have a possible therapeutic role in regenerative medicine without any side effects.

A study by Potu *et al*. evaluated the effectiveness of *C. quadrangularis* extract in the differentiation of mesenchymal stem cells procured from the bone marrow and its subsequent calcification during the 7th, 15th and 28th days. It was observed that, after incubating the cells with 100, 200 or 300 µg/mL of *C. quadrangularis* extract, there was an increase in the differentiation of bone marrow mesenchymal stromal cells into 'Alkaline phosphatase positive' bone-forming cells. An increase in the formation of extracellular matrix was also noted in the study similar to the present research.[9] In the present study, similar results were obtained by inducing HDP-SC to differentiate into bone-forming cells.

Several other studies have also tried to induce HDP-SC to differentiate into bone-forming cells and induce matrix calcification using potential osteogenic herbs. Mendi *et al*., in their study, assessed the presence or absence of extracellular matrix calcification in HDP-SC treated with C. zeylanicum extract using Alizarin red staining in osteogenic media. It was observed that after 21 days, there was a dose-dependent increase in the presence of calcium nodules in the treated group when compared to the control group, which was also in harmony with the present study.[10] In the present study, extracellular matrix calcification was achieved using *C. quadrangularis* extract after an incubation period of 5, 10 and 15 days.

In another study, Jittapiromsak *et al*. used Acemannan, a herbal extract procured from aloe vera, to assess the extracellular matrix inception and mineralisation on HDP-SC after 15 days using Alizarin red staining. Based on the observations, it was concluded that the amount of extracellular matrix calcification and mineralisation significantly increased in all the herbal extract-treated groups when compared to the untreated group.[11]

A study by Soumya *et al*. to evaluate the physio-chemical and biological properties of the herbal scaffolds showed that the *C. quadrangularis* extract with natural biopolymers could serve as an osteoinductive scaffold which can be used for bone tissue engineering therapeutics.[12] Liao *et al*., in their study, integrated *C. quadrangularis* extract with gelatin and pectin polymers using β-tricalcium phosphate bioceramic. Their study concluded that the so-formed composite matrix provided a 92.0% favourable environment for mesenchymal stem cell viability, showed positive expression of bone markers on reverse transcription-polymerase chain reaction and could thus serve as a potential biomaterial for bone tissue regeneration.[13] In contrast, in the present study, scaffolds were not utilised due to financial constraints.

A pilot study by Managutti *et al*. to assess pain relief and bone repair following implant placement demonstrated that the ingestion of *C. quadrangularis* capsules after implant placement showed a remarkable effect on pain and swelling. Formation of new bone was also evident thus helping in osseointegration.[14] Another study by Brahmkshatriya *et al*. evaluating the clinical worth of *C. quadrangularis* capsules following maxillofacial fracture reduction found a significant reduction in pain, swelling and fragment mobility in the treatment group when compared to controls.[15] A

recent study by Altaweel *et al*. for evaluating the osteogenic inductive property of *C. quadrangularis* on alveolar ridge distraction found radiological as well as histological evidence of bone formation in the study group who were given *C. quadrangularis* capsules when compared to the controls.[16] In contrast to these studies, our study was purely an *in vitro* trial, which could, in the future, serve as a stepping stone for targeted alveolar bone regeneration using HDP-SCs.

Limitations and future direction

The present study is not without limitations**.** HDP-SC were procured from an outside source and induced to undergo differentiation under laboratory conditions, and hence, it may not entirely mimic the oral environment. Furthermore, confounding factors that could occur in the oral environment could not be assessed as the study was conducted *in vitro*. The study was done to assess the presence of matrix calcification; however, the quantity and quality of the bone matrix formed were not assessed in the study. Although limitations exist, the present study unravels new avenues for investigation and provides scope for further research in this area. Strong levels of evidence derived from such basic science research in developing a standardised protocol in the differentiation of HDP-SC may eventually justify its use in clinical trials especially in autogenic osteoinductive scaffold for tissue regeneration.

CONCLUSION

The present study is an attempt to evaluate the regenerative potential of plant-based products on dental pulp stem cells. *C. quadrangularis* is a traditional herb native to the Indian subcontinent which has been proven to have regenerative potential. In the current research, we have appraised the ability of *C. quadrangularis* to stimulate hard tissue formation in dental pulp stem cells. The results of the study on further validation can serve as a natural regenerative substitute to conventional grafts, as the use of HDP-SC in tissue regeneration carries a lesser stigma than the use of embryonic cells or tissues.

Ethical approval: The research/study was approved by the Institutional Review Board and Ethical Committee of SRMDC, IRB Approval number – IRB/2017/MDS/No.606, dated 8.8.17.
Declaration of patient consent: Patient's consent was not required as there are no patients in this study.
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