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Evidence based Osteogenic, anti adipogenic and anti- senescence action of *Centella asiatica* extract on Dental pulp stem cells

Research Article

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Abstract

Aim: *Centella asiatica linn family* of *Apiaceae* has been used as a traditional medicine as anti-aging remedy to minimize the severity of aging problems. However the effects of Centella asiatica on stem cell differentiation and anti-aging activity are not fully understood. In this investigation we tested the effect of an aqueous extract of Centella asiatica on senescence and osteogenic, chondrogenic and adipogenic differentiation of human dental pulp stem cells (hDPSCs). Methods: DPSC (n = 10) from the human pulp was treated with various concentrations of *Centella asiatica*(CA). The cytotoxicity of CA assessed using the MTT. The hDPSCs were then induced to osteogenic, chondrogenic, and adipogenic differentiation for 6 and 21 days using either CA alone or a combination of Centella asiatica with an appropriate induction media. We also evaluated the early and late passage senescence activity of DPSC. Key finding: Our data demonstrate effect of CA extract on adipogenesis, chondrogenesis and osteogenesis, and anti-aging activity. We found that there was initial increase in adipogenesis which was diminished in long-term culture. Similarly, the extract was found to enhance chondrogenesis and osteogenesis and reduced senescence as revealed by β-galactosidase staining. Significance: The present study demonstrated for the first time that the CA extract was able to inhibit adipogenesis, senescence, and accelerated osteogenesis in DPSCs. Overall results show that CA (*Mandukaparni)* extract can be used to treat osteoporosis, delay aging and reduce obesity.

Keywords: Stem cells, Anti-aging, Differentiation, Ayurveda, Obesity.

Introduction

Plants are a natural biological asset that can be used as a medicine because they contain a variety of natural biological and chemical compounds that have pharmacological value. Plants and their products are used around the world to improve health. Plants have provided an endless source of medicine throughout history.

Nowadays, traditional herbal medicines are attracting the attention of researchers around the world. Several ayurvedic herbs and recipes widely used to promote fitness, immunity, and resistance against several of stress. *Centella asiatica* has been reported to have antioxidant, antibacterial, antifungal(1), anti-proliferative(2), wound healing(3,4), anti-cellulite properties in cosmetics. *Centella asiatica* has excellent antioxidant and anti-aging properties, which minimizes stress. These medicines have appealed to support healthy aging and regeneration at the cellular level (5). Their manufacturing process, purpose and use depend on the condition and severity of the disease. Extracts from different parts of the plant can be used for therapeutic purposes e.g. roots, barks, flowers, fruits etc.(6). Traditional medicine, Ayurveda, uses natural products to treat illness. Plant extracts contain bioactive compounds such as polyphenols and flavonoids, as well as many other compounds and chemicals that play important roles to treat autoimmune diseases(7–9).

The various bioactive compounds of *Centella asiatica* extract focus on pharmacological effects such as antioxidant and anti-aging.(10,11) Certain bioactive molecules, asiaticoside and madecassoside, may be effective in treating bone disease..(12,13) For example, madecassoside was active in healing burns, promoted collagen synthesis, and enhance angiogenesis.(14) *C. asiatica* is often used for wound healing, anti-wrinkle, and anti-inflammatory properties. In addition, C. asiatica has shown anti-stress properties in preclinical studies. Due to their health benefits, biological compounds from plants are of great interest and require further scientific research in the pharmaceutical industry. According to the National Institutes of Health database, dietary supplements containing herbal compounds are used in MSCs therapy clinical trials. Herbs have been used clinically for thousands of years and are recommended as a strong source of treatment. In addition, the effects of herbs on stem cells have been tested in recent years. Therefore, dental pulp stem cells

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(DPSC) were used in this study because of their potential for self-renewal and differentiation. (15)

The effectiveness of C. asiatica for anti-aging and osteogenic potential in has not yet been documented. In Ayurveda, the use of whole plants is mentioned. The purpose of this study is to evaluate the effect of Centella asiatica extract on dental pulp-derived human stem cells in terms of anti-aging and osteogenic, chondrogenic and adipogenic potential differentiation.

Materials and Methods

Collection of plant and Authentication

The fresh whole *Mandukaparni (Centella asiatica)* was collected from farm which is located near Dehu road, Pune India. Whole *Mandukaparni* was thoroughly washed with tap water; all the debris and soil were removed and rinsed with distilled water, and dried at normal room temperature. The sample was validated from Botanical Survey of India. Sample voucher specimen number was MMD 01. No. BSI/ WRC/IDEN/.CER./2020/H3184.

Drug sample standardization

Required Analytical tests were done from the dried sample of *Centella asiatica* at Agharkar Research Institute. TLC Analysis, pH, Water soluble ash, Acid insoluble ash, Total ash, Alcohol soluble extractive, Water soluble extractive tests was done.(16)

Preparation of plant material extract

Plant extraction process was performed by modified Soxhlation process. (Figure 1) After removing foreign matter *Centella asiatica* was taken for final extraction procedure. Extraction was carried out by using aqueous extract method. For aqueous extraction temperature was maintained in between 70 to 90^0 C for 27 hours. Extract was filtered with Whatman's filter paper and dry in water bath. The dry extract was rapped with silver foil paper, label it then kept under refrigerated condition until use.

Figure 1: Colonies of Centella asiatica (A) Morphology of Centella asiatica (B) , Extract of *Centella asiatica* (C)

Dental pulp extraction

Third molar tooth was collected at Dr. D. Y. Patil Dental College and Hospital with proper informed consent as per the guideline of institutional stem cells committee. Extraction procedure was carried out in sterile conditions using bur chuck type aerotor hand piece, then pulp was gently removed by using sterile forceps and immersed in tube containing Phosphate buffer solution (PBS) and send instantly to Regenerative medicine laboratory Dr. D. Y. Patil Dental college and hospital.

Table 1: Analytical test of *Centella asiatica*

Sr. No	TEST	RESULTS
1	TLC Analysis	Test solution: Ethanol+ leaf powder Solvent system: Chloroform: GAA (Glacial Acetic Acid), Methanol: Water (60:32:12:8) No. of spots observed: 12 Reference value obtained were: 0.07, 0.12, 0.15, 0.20, 0.31, 0.35, 0.73, 0.74, 0.77, 0.78, 0.83, 0.85
2	pH	6.25 at Temperature 28^{0}C
3	Water soluble Ash	4.6345%
4	Acid insoluble Ash	0.6438%
5	Total Ash	9.4999%
6	Alcohol soluble extractive	34.348%
7	Water soluble extractive	12.206%

Isolation and culture

Dental Pulp tissue minced into 1-2 mm fragments and placed in 60 mm culture dishes. A 20 µl (microliter) of Fetal Bovine Serum (FBS) was introduced on the tissue to cover them entirely. The culture dishes were incubated for 24 hours at 37^0 C with 5% CO2. After incubation, explant was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS with 1% antibiotic at 37^0 C in 5% CO2. When cell reached at 70-80% confluence tissue was washed with PBS and add 0.25% Trypsin-EDTA solution to a flask, and incubated for 1 to 2 min for cell detached. Immediately add FBS solution to deactivate the action of trypsin and cell were centrifuged at 1800 rpm for 5min. The cell pellet obtained was seeded in another flask supplemented with DMEM, FBS and antibiotics 10% up to 4-5passage.

Cytotoxicity analysis of *Centella asiatica*

For cytotoxicity analysis, 1x 10^4 cells/ml human Dental Pulp stem cells (hDPSCs) were seeded in 96 well plates, and they were incubated. After 24hour incubation, cells were treated with different concentrations (3, 6, 15, 20, 25, 30 µg/ml) of aqueous extract of *Centella asiatica* for 24 and 48 hours. All the experiments were carried out in triplicates. The analyses were performed by using MTT Cell Proliferation Assay Kit.

Effect of *Centella asiatica* on Differentiation

DPSCs were cultured in 24 well dishes for tri-lineage differentiation. After the cell acquired 95-100% confluence, they were supplemented with adipogenic, osteogenic and chondrogenic induction medium with and without *Centella asiatica*. Every 4th day the cells were replaced with fresh differentiation media up to 18-21 days. After completion of differentiation, the cells were stained with Oil O red for adipogenesis, Alizarin

Red S for osteogenesis and Alizarin Blue for chondrogenesis. The images were capture underneath light microscope.

Determination of cell senescence activity of CA

Human dental pulp stem cells were treated with aqueous extract of *Centella asiatica* 6µg/ml from passage 9 to 15. Senescence was determined using a βeta-galactosidase staining kit once cells reached 80-90% confluence, plates were fixed in fixative solution and incubated with βeta galactosidase solution at 37^0 C and kept this overnight in dry incubator. Plate was checked for development of blue colouration with bright field phase microscopy.

Results

Isolation and culture of DPSCs

Morphology: Population of HDPSCs shows spindle shaped fibroblast like morphology. (Figure 2D-E).

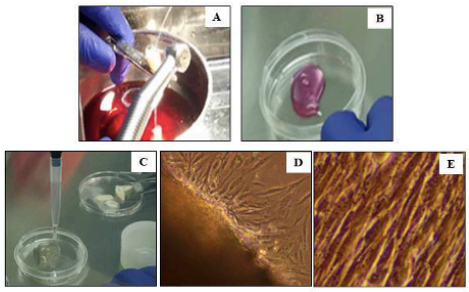

Figure 2. Isolation and Culture of Human Dental Pulp Stem Cells (A) Extraction of dental pulp, (B) Dental Pulp (C) Explant culture (D) cell outgrowth from explant(E) Mesenchymal stem cells

Surface marker analysis for HDPSCs

HDPSCs express mesenchymal stem cell markers CD105 (71.06%), CD90 (97.33%), CD73 (99.12%) and negative for CD34 (3%), CD45(1.5%) and HLA-DR (1.3%) (Figure 3)

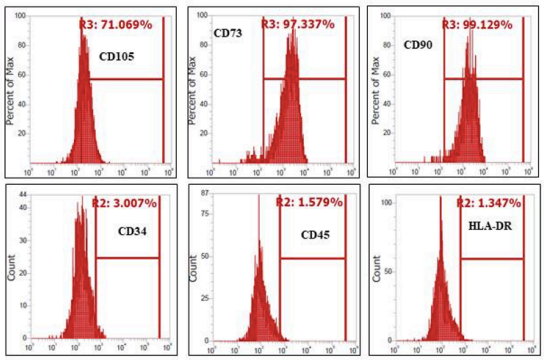

Figure 3. Surface marker analysis of DPSCs (Positive for CD105,CD73,CD90 and negative for CD34,CD45,CD90

Effect of *Centella asiatica* on HDPSCs

MTT assay had been done to investigate rate of cell proliferation and drug toxicity at different concentrations of drug on HDPSCs. HDPSCs showed significant increase in proliferation when treated with aqueous extract of *Centella asiatica* (3, 6, 15, 20, 25, 30 µg/ml). Aqueous extract of CA showed significant increase in proliferation at 6µg/ml on HDPSCs. This concentration were used for all subsequent experiment with the drug. Proliferation rate studied in 24- and 48-hour's interval of time. When drug treated HDPSCs they compare to control group (untreated) show significant results at 6µg/ml showed higher proliferation rate as well as showed no toxic effect on HDPSCs. Hence, this concentration refers safe to the HDPSCs. (Figure 4).

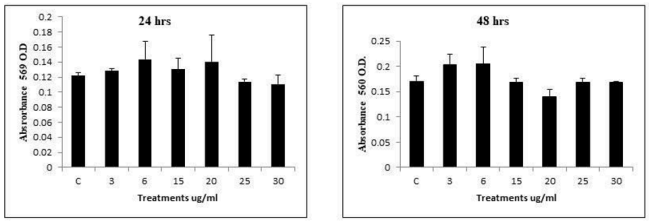

Figure 4: Cell Cytotoxicity assay for Mandukaparni (*Centella asiatica*) on human dental pulp stem cells at 24 hours (A) and 48 hours (B)

Tri-linage differentiation

Stem cells have special characteristic of differentiation. Due to this, we have many opportunities to study in vitro effect of new drugs. When HDPSCs treated with CA, we observed that CA inhibit the process of adipogenesis and increase in osteogenic and chondrogenic properties. Initially, CA triggers the adipogenesis at day 6, after long-term exposer with CA inhibit the adipogenic and accelerate the osteogenic, chondrogenic differentiation.

Adipogenic differentiated cells show oil droplets in the cytoplasm of the cells and observed red after staining with Oil Red O staining. In osteogenic differentiation, the presence of calcium deposits was stained with alizarin red O. Cells were stained with alcian blue for chondrogenic differentiation. (Figure 5).

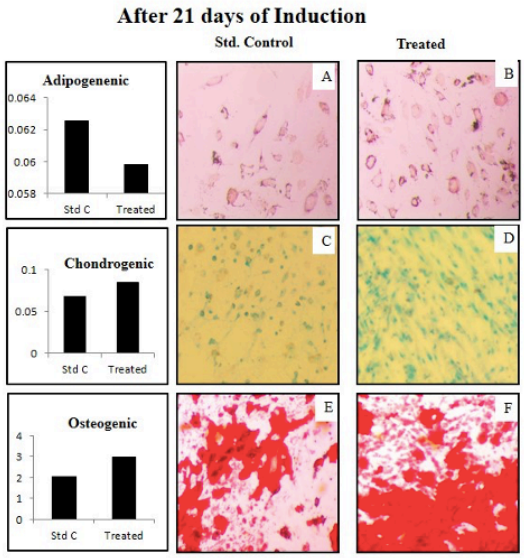

Figure 5: Stem cells differentiation after 21 days of induction Adipogenic (A- Control, B- Treated), Chondrogenic (C-Control, D-Treated), Osteogenic (E- Control, F-Treated)

Role of *Centella asiatica* in cell senescence

Stem cells have self-renewal and differentiate into specialized cells type. During its proliferative stage, they were subjected to different stresses, which lead to cells senescence. Hence, CA was used to evaluate the effect of senescence on hDPSCs with the help of βeta-galactosidase staining. It was clearly observed that the cells which were treated with CA show little positive for the βeta-galactosidase staining. The untreated cells

showed blue staining indicating senescence associated βeta galactosidase staining activity. (Figure 6).

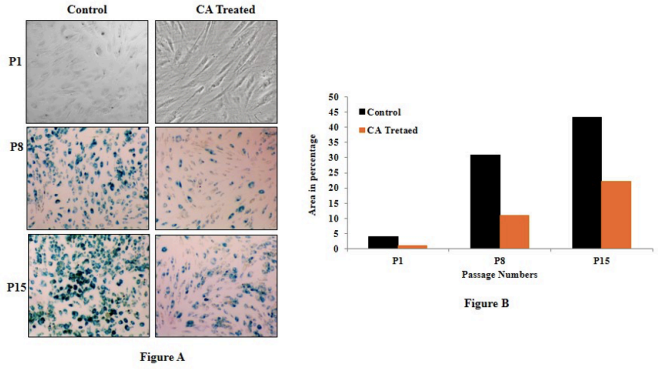

Figure 6. A. Cell Senescence at passage P1, P8, P15 in control and treated DPSCs,(B) Quantitative analysis using imageJ software.

Discussion

Mandukaparni (*Centella asiatica*) could be a one among the traditional herbal plant has therapeutic potential. Ayurveda provides an excellent approach to regulate ageing in a very systematic manner with the help of *Rasayana* therapies.(17) This anti-ageing properties slowing down the ageing process and improve quality of life. The ayurvedic plant *Centella Asiatica* is the anti-ageing aspect in cosmetics.(3) Worldwide, medicinal plant getting attention for the treatment and prevention of several diseases. Numerous studies display those bioactive compounds from plants act as bioactive mediator to regulate the proliferation, differentiation via signalling pathways(18). The use of plant bioactive phytochemicals may also become promising in treating diseases like osteoporosis, neurodegenerative defects (19,20). The *Centella asiatica* have sowed neural differentiation of hWJMSCs (21)

Natural compounds isolated from plant have shown to promote the proliferation and differentiation in mesenchymal stem cell. Mandukaparni (*Centella asiatica)* extract might also become promising in treating condition like osteoporosis, and other tissue degenerative disorders. Mesenchymal Stem cell is promising source for a drug screening, tissue regeneration and other senile conditions.

Our data, hDPSCs treated with *Centella asiatica* extract does not show any toxic effect at higher concentration (Figure 4*)*. In present study, DPSCs treated with crude extracts of *Centella asiatica* shown anti-senescence activity trigger osteogenic, and chondrogenic differentiation. (Figure 5)

Stem cells having self-renewal and differentiated potential properties. However, during its long-term culture, this leads to cell senescence .Therefore, *Centella asiatica* was used to assess the effect of senescence on hDPSCs with the help of βeta-galactosidase staining. It clearly observed that the cells, which were treated with Centella asiatica, show negligible βeta-galactosidase staining. Whereas untreated cells showed blue staining, indicating senescence associated βeta-galactosidase staining activity. Our studies have shown that *Centella asiatica* extracts act as anti-senescence on DPSCs. (Figure 6)

However, its effects on the DPSCs differentiation and senescence still not well documented. Therefore, the present study attempted to evaluate the effect of *Centella asiatica* extract on dental pulp-derived stem cells in terms of anti-aging and osteogenic potential differentiation. Overall results show that *Centella asiatica* extract can be used to treat osteoporosis, delay aging proven by senescence assay, and reduce obesity confirmed by anti-adipogenic activity.

Conclusion

Centella asiatica acts as anti-adipogenic agent accelerate the osteogenic differentiation and delay senescence in DPSCs. This triple action of *Centella asiatica* makes it a drug of choice for treating old patients with osteoporosis.

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Conflict of Interest: All authors declare no conflict of interest

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