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In vitro study of the effect of *Benincasa hispida* seed extract on neural differentiation of human dental pulp stem cells

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Benincasa hispida (BHS) is traditionally recognized for its *Medhya* and *Rasayana* properties, while its potential to influence stem cell-associated neural differentiation has received limited experimental attention. The present *in vitro* study evaluated the effect of aqueous and ethanolic seed extracts of BHS on human dental pulp stem cells (hDPSCs). BHS seeds were extracted separately with distilled water and ethanol using Soxhlet extraction, and the extracts were subjected to physicochemical evaluation. The hDPSCs were isolated by explant culture and characterized by flow cytometry using CD73, CD90, CD105, CD34, CD45 and HLA-DR markers. The cytocompatibility of both extracts was assessed by MTT assay at concentrations ranging from 2 to 25 µg/mL. For neural differentiation assessment, hDPSCs were cultured in neural basal medium supplemented with aqueous or ethanolic BHS extract at 10 µg/mL, and morphological changes were monitored for 10 days by phase-contrast microscopy. Nestin gene expression was evaluated in the extract-treated cells using real-time RT-PCR, with GAPDH used as the housekeeping gene. Both BHS extracts showed high cell viability at concentrations up to 10 µg/mL and were associated with morphological changes suggestive of neural lineage development during the 10-day culture period. These findings indicate that BHS seed extracts possess *in vitro* potential to support early neural progenitor-associated changes in hDPSCs. Further studies using additional neuronal and glial markers and appropriate positive controls are required to confirm lineage-specific differentiation and clarify the underlying mechanisms.

Keywords: *Benincasa hispida*, Dental pulp stem cells, Mesenchymal stem cells, Neurodifferentiation, Regenerative medicine

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Neurological disorders constitute one of the most significant challenges in healthcare globally, primarily due to their chronic characteristics, increasing prevalence, and the scarcity of regenerative treatments. In India, approximately 30 million people are affected by neurological conditions, including Alzheimer's disease, Parkinson's disease, epilepsy, and dementia[1]. These conditions progressively degrade cognitive abilities, memory, motor skills, and overall quality of life, creating a considerable socioeconomic burden. Current therapeutic strategies mainly concentrate on managing symptoms and offering neuroprotection; however, the potential for neural regeneration is extremely restricted[2].

Traditional Knowledge systems, particularly *Ayurveda*, provide a comprehensive approach to neurological disorders by emphasizing both structural and functional recovery of the nervous system. In *Ayurveda*, neurological conditions are generally categorized into *Unmada*, *Apasmara*, *Pakshaghata*, and *Smritibhramsha*. *Benincasa hispida* (*Kushmanda*) is traditionally recognized as *Medhya*, *Rasayana*, and *Balya*, highlighting its role in promoting intelligence, rejuvenation, and enhancing neurological strength[3]. Classical texts suggest using its seeds for conditions like *Unmada*, *Apasmara*, and *Smritibhramsha*, indicating a specific connection to the central nervous system[4].

Recent phytochemical research indicates that the seeds of *Benincasa hispida* (BHS) contain alkaloids, flavonoids, tannins, glycosides, phenolic compounds, triterpenoids, and saponins[5,6]. Notably, polyphenolic compounds display strong antioxidant properties and safeguard neurons from oxidative stress, which significantly contributes to the development of neurodegenerative diseases such as Alzheimer's and Parkinson's disease[7]. Various *in vitro* and *in vivo* studies have substantiated the neuroprotective, anxiolytic,

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anticonvulsant, and memory-enhancing effects of this plant[8].

Although *Benincasa hispida* has been investigated for several pharmacological activities relevant to neurological health, including neuroprotective, anticonvulsant, antioxidant and cognitive-related effects, the existing literature has predominantly focused on pharmacological outcomes, behavioural responses, oxidative stress and the identification of bioactive constituents. Phytochemical investigations have also demonstrated the presence of diverse bioactive constituents in BHS seeds, providing a basis for further exploration of their biological effects. However, the direct influence of BHS seed extracts on stem-cell-associated neural lineage development has received comparatively limited attention. Thus, there remains a need to investigate whether BHS seed extracts can influence cellular responses associated with neural differentiation in an appropriate stem-cell model[9,10]

Human dental pulp stem cells (hDPSCs) are mesenchymal stem/stromal cells with neural crest-associated developmental characteristics and have been investigated as an accessible cell source for neural differentiation and neuro-regenerative research. Previous studies have demonstrated the neural differentiation potential of hDPSCs using defined neural induction conditions, growth factors or chemical inducers, with neural progenitor and neuronal markers being used to assess lineage-associated changes[11-13]. In contrast, the use of a *B. hispida* seed extract as a botanical stimulus for neural lineage-associated changes in hDPSCs has not been adequately characterized. The present study therefore explores the effects of aqueous and ethanolic *B. hispida* seed extracts on hDPSCs by integrating assessment of cell viability, cellular morphology and Nestin gene expression. This approach extends the investigation of *B. hispida* from its previously reported neuroprotective and cognitive-related effects toward a cell-based neuroregenerative context.

Materials and Methods

Ethical approval

The Institutional Committee for Stem Cell Research at Dr. D. Y. Patil Dental College, Pimpri, Pune granted approval for the experiment, under Ref. No. IC-SCR/RM/68/23. An *in vitro* study was conducted on human dental pulp stem cells at the Regenerative Medicine Laboratory of Dr. D. Y. Patil Dental College, Pimpri, Pune.

Collection, identification, and authentication of BHS

BHS were obtained from anauthentic vendor and verified for their authenticity by the Agharkar Research Institute, Pune. The extract was formulated and evaluated for its physico-chemical properties in a GMP-certified pharmacy, adhering to the standards outlined in the Ayurvedic Pharmacopoeia of India.

Preparation and physico-chemical analysis of aqueous and ethanolic extract of BHS

The extraction was performed using a Soxhlet apparatus. In this setup, 250 mL of distilled water was added to a round-bottom flask, while 50 g of the BHS coarse powder of mesh size 36 was placed in a thimble. A continuous flow of water was maintained for the apparatus, and the flask was heated to a stable temperature of 50°C for six hours. Once the extraction was finished, the Soxhlet apparatus was detached from the heat source, and the thimble containing the extracted material was carefully removed. The extract was filtered using ashless filter paper. The aqueous extract was then transferred to a petri dish and placed in a water bath at 60°C for two hours to yield a dry extract. The sequential steps involved in aqueous extraction are shown in (Fig. 1). The same procedure was repeated using 250 mL of ethanol as the solvent instead of distilled water to prepare the ethanolic extract of BHS.

The physico-chemical analysis of the raw herb and both extracts was conducted according to the standard protocols specified in the Ayurvedic Pharmacopoeia of India[14], which include total ash, acid-insoluble ash, pH, water-soluble extractive, and alcohol-soluble extractive, loss on drying.

Isolation and cultivation of mesenchymal stem cells from human dental pulp

Human dental pulp was utilized to extract the multipotent mesenchymal stem cells. The donor, who had no medical history, provided consent prior to the extraction of the tooth. Initially, the sample was immersed in a povidone and iodine solution. Following this, it was transferred to a 15 mL tube containing 1% antibiotic and antimycotic solution in phosphate-buffered saline (PBS) after being rinsed in saline solution. The pulp was retrieved by cutting the tooth with a sterile Aerotor handpiece. The extracted pulp sample was placed in a clean petri dish filled with PBS. An adequate amount of Fetal bovine serum (FBS) was added, and the sample was then incubated in a CO_2 incubator at 37°C for 15 min. The tissue was

finely cut into 1 mm pieces using a sterile blade, rinsed with PBS, and moved to a T25 flask that contained FBS, along with 1% antibiotic and antimycotic solution.

The flask was incubated at 37°C with 5% CO_2 for twenty-four hours. After this period, the flask was supplemented with 10% FBS, 1% antibiotic, and Dulbecco's Modified Eagle Medium (DMEM), and the incubation continued at 37°C with 5% CO_2. The sample was cultured in a CO_2 incubator for five to seven days, with the medium being replaced every two to three days.

Cell growth was monitored with an inverted phase-contrast microscope. Once the cells reached 80% confluence, they were treated with two millilitres of a 0.25% trypsin EDTA solution and incubated for one minute in the CO_2 incubator. The mixture was then transferred to a sterile 15 mL tube and centrifuged at 1800 rpm and 20°C for 5 min. After removing the supernatant, the cell pellet was placed into a sterile T25 flask. Passage numbers were assigned to the cells in the flask starting from zero. The process was repeated until 80% confluence of the cells was achieved.

Analysis of human dental pulp stem cells (hDPSCs) using surface marker assessment via fluorescence-activated cell sorting (FACS)

Confluent hDPSCs at passage 4 were evaluated using flow cytometry and an analysis of cell surface markers. The antibody panel included FITC-labelled CD34, CD45, and HLA-DR, as well as PE-labelled CD90, CD73, and CD105. Each of the four Eppendorf tubes received 200 µL of a cell suspension containing 2×10^6 cells. The cells were fixed at room temperature for half an hour with 4% formaldehyde. After fixation, the cells were washed with PBS. Following the addition of 3 µL of specific antibodies, the tubes underwent FACS analysis using Cell Quest Pro software.

Cytotoxic effect of BHS extract on hDPSCs

Cytotoxicity of the aqueous and ethanolic extracts of BHS was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). A stock solution of the aqueous extract of BHS was prepared at a concentration of 10 mg/ml. A total of 140 hDPSCs per millilitre were plated into a 96-well plate. Cytotoxicity was investigated at BHS concentrations of 2 µg/mL, 3 µg/mL, 4 µg/mL, 5

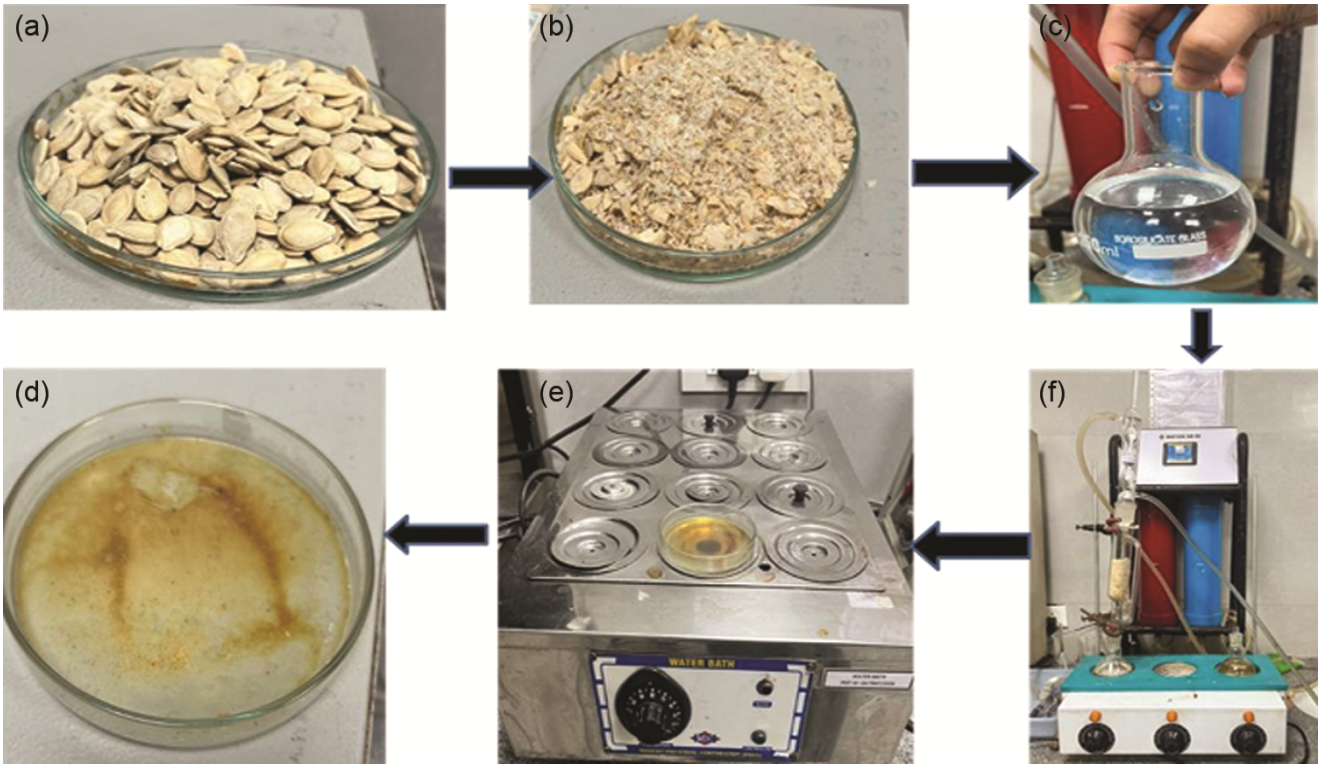


Fig. 1 — Sequential steps involved in the preparation of aqueous extract of BHS: (a) raw seeds, (b) BHS powder, (c) Distilled water in round-bottom flask, (d) Extraction in Soxhlet apparatus, (e) Extract drying on water bath, and (f) Aqueous extract of BHS

µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, and 25 µg/mL. After achieving 80% confluence, 20 µL of MTT was added to each well, and the cells were incubated at room temperature. After three hours, the MTT was removed, and 100 µL of Dimethyl sulfoxide (DMSO) was introduced into each well. Absorbance was measured at 570 nm using an ELISA reader. This procedure was repeated to evaluate the cytotoxic effects of the ethanolic extract of BHS.

Impact of BHS extracts on neural differentiation of hDPSCs

The neural differentiation medium was prepared by combining 5 mL of neural basal media, 5 mL of DMEM, 100 µL of FBS, 100 µL of B27, 200 µL of N_2 supplement, 200 µL of neural supplement, and 50 µL of antibiotic solution. A total of 500 µL of this complete medium was dispensed into 12 wells of a 24-well plate, and 200 µL of cell suspension containing 2 × 10^4 cells was added. The plate was incubated at 95% humidity and 5% CO_2 at 37°C for 24 h. The medium was refreshed every 2 to 3 days, and the cells were examined under an inverted phase contrast microscope. The incubation continued in a CO_2 incubator at 37°C, maintaining 5% CO_2 and 95% humidity for a period of 10 days. Media from the control group was routinely refreshed, and observations were made under the inverted phase contrast microscope, capturing photographs at intervals of 1, 3, and 10 days. The induced cells were also observed with the inverted phase contrast microscope. Proliferation and differentiation of stem cells were noted across all three groups over the ten-day period. A conventional positive control based on an established chemical or growth-factor-induced neural differentiation protocol was not included in the present experimental design. The groups designated for the neural differentiation of hDPSCs were as follows, as presented in (Table 1).

Table 1 — Groups used for neural differentiation of hDPSCs

Sr No	Group	Cells	Components
1	Control group	hDPSCs	Neural basal medium + B27 + N2
2	Aqueous BHS extract group	hDPSCs	50% neural basal medium + B27 + N2 + 50% aqueous extract of BHS (10 µg/mL)
3	Ethanolic BHS extract group	hDPSCs	50% neural basal medium + B27 + N2 + 50% ethanolic extract of BHS (10 µg/mL)

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Real-time

The process of Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) includes essential steps to convert RNA into complementary DNA (cDNA) and amplify specific DNA sequences. A gene expression analysis was conducted on cells that received treatment with 10 µg/mL of an aqueous extract of BHS. RNA extraction was carried out using the TRIZOL method (Ambion Life Technologies), followed by cDNA synthesis with the high-capacity cDNA Reverse Transcription Kit (Thermofisher). The components included RNase inhibitor, a 25X dNTP mix, a 10X random primer, and a 10X reverse transcription buffer. Additional materials included Multiscribe TM Reverse Transcriptase (50 U/µL) and DEPC-treated water (Himedia). For five reactions, the total volume of reagents was 34 µL, while the volume for a single reaction was 6.8 µL. A total of 2 µL of the isolated RNA was mixed with 6.8 µL of the master mix. The genes selected for the RT-PCR analysis included GAPDH as the housekeeping gene and Nestin as the target gene. The RT-PCR was performed using the Quant Studio 5 Real-Time PCR System. The primer sequences used for gene expression analysis are presented in (Table 2).

Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD), wherever applicable. The MTT assay findings for aqueous and ethanolic *Benincasa hispida* seed extracts were evaluated using descriptive statistics across the tested concentration range. Nestin gene expression was expressed as fold change relative to the control group and presented as mean ± SD. The morphological observations were assessed descriptively based on phase-contrast microscopic examination at the specified time points. As the present study was exploratory in nature, the quantitative findings were interpreted primarily through descriptive comparison. No retrospective inferential statistical testing was applied where such analysis was not documented in the original experimental record. Accordingly, p-values and formal statistical significance thresholds were not assigned to the reported findings. Confidence intervals were not calculated for the descriptive analyses.

Table 2 — Primers used for gene expression analysis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GAPDH	TCCCTGAGCTGAACGGGAAG	GGAGGAGTGGGTGTCGCTGT
Nestin	GAGGAAACCGAAGAGGAGG	GGGGTGTTGTTCTCATCCAG

Results

Physicochemical evaluation of BHS and extracts

The physicochemical evaluation of raw BHS powder, aqueous extract and ethanolic extract revealed that all tested parameters complied with the standards prescribed in the Ayurvedic Pharmacopoeia of India. The raw seed powder exhibited a creamy-brown colour, sweet taste and characteristic odour. The aqueous extract was white in colour with a sweet taste, whereas the ethanolic extract appeared yellowish with a characteristic alcoholic odour.

The pH of the aqueous extract was found to be slightly acidic, while the ethanolic extract showed near-neutral pH, indicating chemical stability of both extracts. Losses on drying values were within the acceptable range, indicating minimal moisture content and good shelf stability. Totalash values reflected the overall inorganic content of the drug, while acid-insoluble ash values confirmed the absence of excess siliceous impurities. The water-soluble extractive values were higher in the aqueous extract, whereas alcohol-soluble extractive values were predominant in the ethanolic extract, confirming efficient extraction of both polar and semi-polar phytoconstituents[5,6]. Taken together, these results established the purity, identity, quality and pharmaceutical suitability of BHS extracts for biological experimentation. The physicochemical characteristics of raw BHS seed powder and the aqueous and ethanolic extracts are summarized in (Table 3).

Isolation and morphological characterization of hDPSCs

Human dental pulp stem cells were successfully isolated using the explant culture technique[15,16]. Within the first 48–72 h of incubation, tissue adherence to the surface of the culture flask was observed. Gradual migration of cells from the explant edges was noted. By the 5th to 7th day of incubation, the cells showed elongated spindle-shaped morphology with fibroblastic appearance, which is a characteristic feature of mesenchymal stem cells[15] (Fig. 2).

On the 8th day, the culture attained approximately 80% confluency. These cells were subsequently detached using 0.25% trypsin–EDTA solution and sub cultured for further experiments. The proliferative ability and uniform morphology of the cultured cells confirmed successful isolation and expansion of hDPSCs with stable growth characteristics.

Flow cytometric characterization of hDPSCs

Flow cytometry was performed to confirm the mesenchymal nature of the isolated stem cells. The hDPSCs exhibited strong positive expression for the mesenchymal markers CD90, CD105 and CD73. More than 95% of the cell population showed positivity for CD90 and CD105, indicating high purity of the cultured MSC population[17].

The cells were negative for the hematopoietic and immunogenic markers CD34, CD45 and HLADR. The absence of these markers confirmed that the isolated cells were not of hematopoietic lineage and were immunologically stable. These results validated the mesenchymal identity and suitability of the isolated hDPSCs for neural differentiation studies. (Fig. 3).

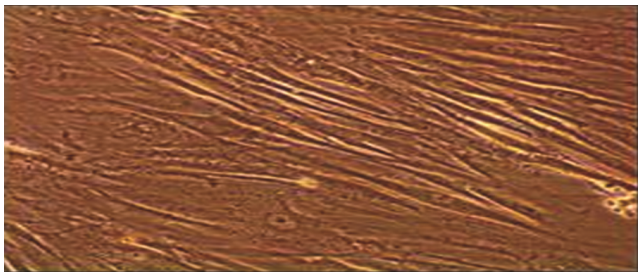


Fig. 2 — Fibroblastic morphology of human dental pulp stem cells (hDPSCs) observed under inverted phase-contrast microscopy, showing spindle-shaped cells migrating and proliferating from the explant culture

Table 3 — Physicochemical analysis, aqueous extract and ethanolic extract of BHS

Sr No	Parameter	Raw BHS seed powder	Aqueous extract of BHS	Ethanolic extract of BHS
1	Description	Colour: creamy brown; taste: sweet; odour: characteristic	Colour: white; taste: sweet; odour: characteristic	Colour: yellow; taste: ethanolic; odour: characteristic
2	Solubility	Soluble in water and alcohol	Soluble in water	Soluble in ethanol
3	pH	6.78	6.09	6.75
4	Loss on drying (%)	0.96	3.48	1.48
5	Total ash (%)	1.94	1.78	1.84
6	Acid-insoluble ash (%)	0.24	0.56	0.65
7	Water-soluble extractive (%)	25.98	28.13	23.67
8	Alcohol-soluble extractive (%)	31.51	23.17	27.58

Cytotoxicity assessment of BHS extracts using MTT assay

The cytotoxicity of aqueous and ethanolic extracts of BHS was evaluated on hDPSCs using the MTT assay[18]. The cells were exposed to different concentrations ranging from 2 µg/mL to 25 µg/mL.

At lower concentrations (2–10 µg/mL), both aqueous and ethanolic extracts showed high cell viability (>90%), indicating excellent cellular compatibility. The results for the aqueous and ethanolic extracts are presented in (Fig. 4 & Fig. 5), respectively. At concentrations beyond 20 µg/mL, a mild reduction in cell viability was observed, suggesting a dose-dependent cellular response. However, no gross cytotoxic effects were noted even at the higher concentrations tested.

Based on these findings, 10 µg/mL was selected as the optimal, non-cytotoxic therapeutic concentration for subsequent neural differentiation experiments. These findings confirm the biocompatibility of BHS extracts with mesenchymal stem cells.

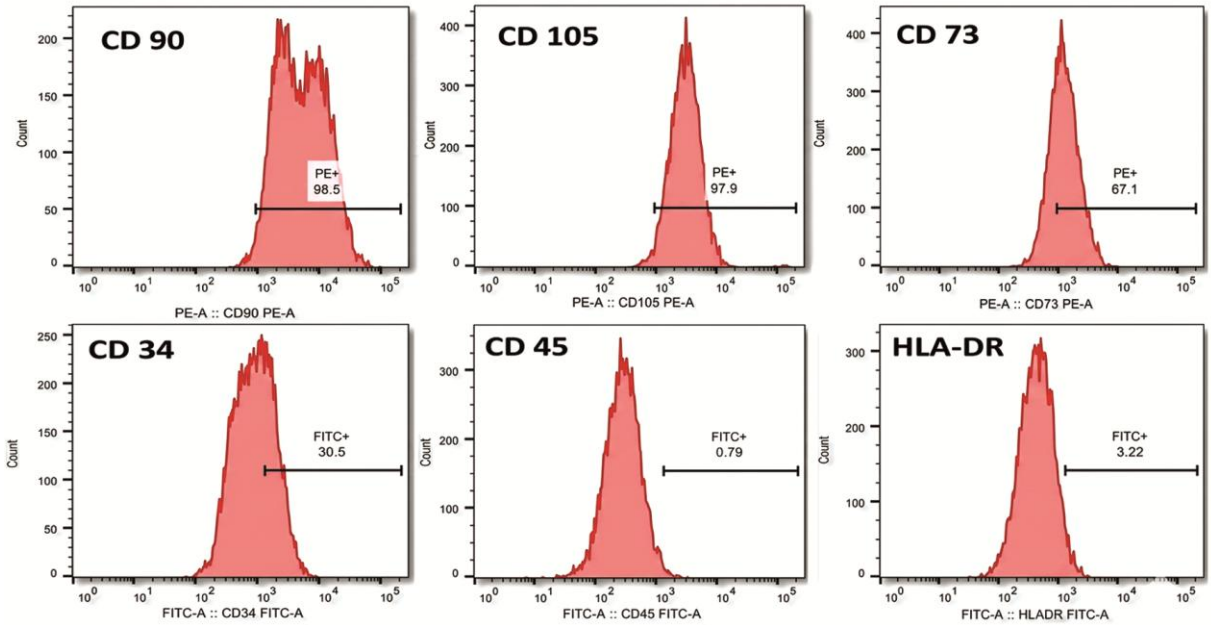


Fig. 3 — Flow cytometric analysis of human dental pulp stem cells (hDPSCs) showing positive expression of mesenchymal stem cell surface markers CD73, CD90, and CD105 (upper panel) and negative expression of hematopoietic markers CD34, CD45, and HLA-DR (lower panel)

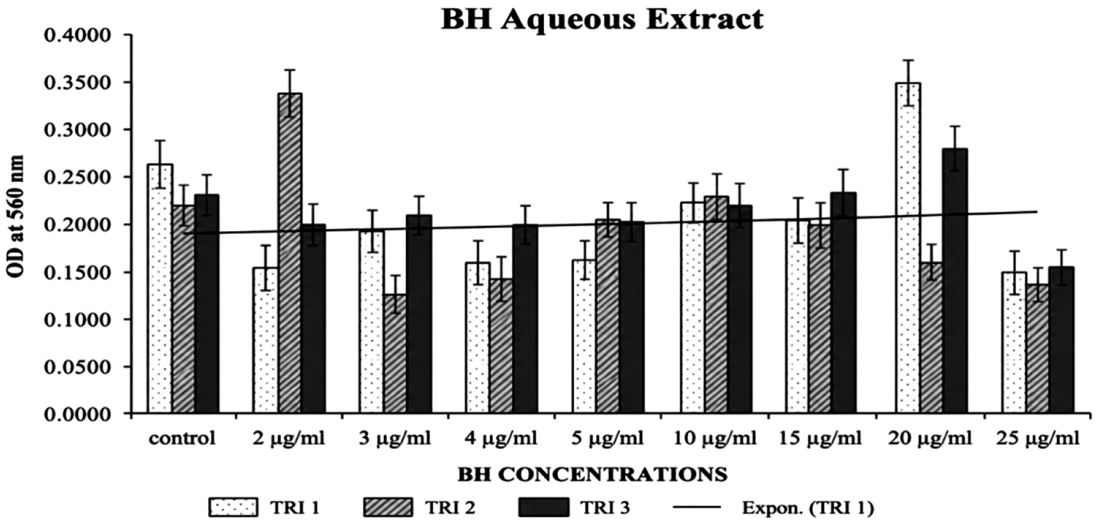


Fig. 4 — Effect of aqueous extract of *Benincasa hispida* seeds (BHS) on the viability of human dental pulp stem cells (hDPSCs) at different concentrations as assessed by the MTT assay. Results are presented descriptively across the tested concentration range

Neural differentiation of hDPSCs induced by BHS extracts

Neural differentiation of hDPSCs was assessed using neural induction medium supplemented with aqueous and ethanolic extracts at a concentration of 10 µg/mL. On day 1 of induction, the cells retained their original spindle-shaped fibroblastic morphology. By day 4, elongation of cytoplasmic processes was evident, indicating initiation of neuronal lineage commitment.

By day 7, cells exhibited prominent morphological changes characterized by the development of bipolar and multipolar neuron-like shapes. Distinct neurite-like extensions were observed. By day 10, the treated cells displayed stellate (star-shaped) morphology with well-defined neurite-like extensions and cellular processes[13] (Fig. 6).

In contrast, control cells maintained in neural basal media without *Benincasa hispida* extract showed

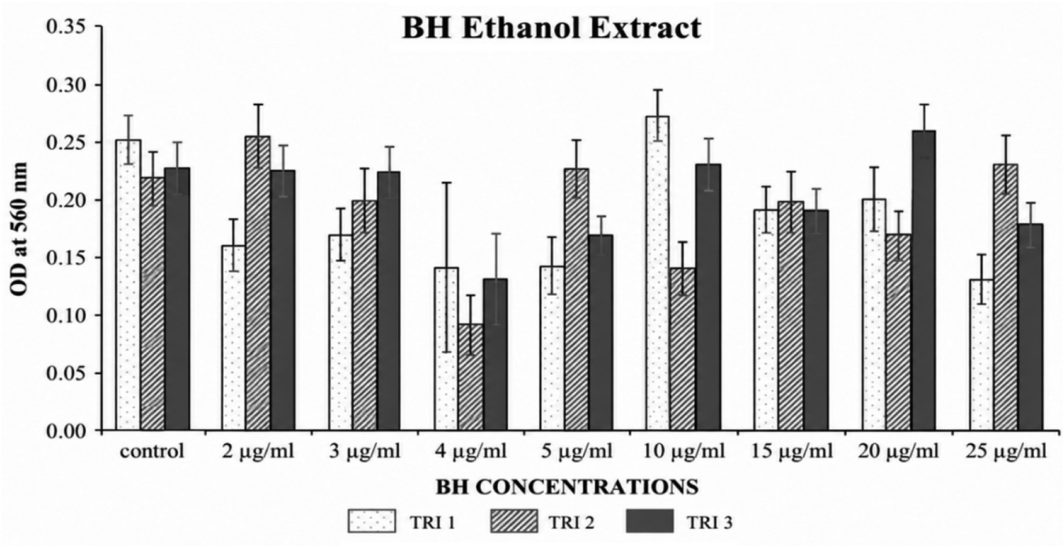


Fig. 5 — Effect of ethanolic extract of *Benincasa hispida* seeds (BHS) on the viability of human dental pulp stem cells (hDPSCs) at different concentrations as assessed by the MTT assay. Results are presented descriptively across the tested concentration range

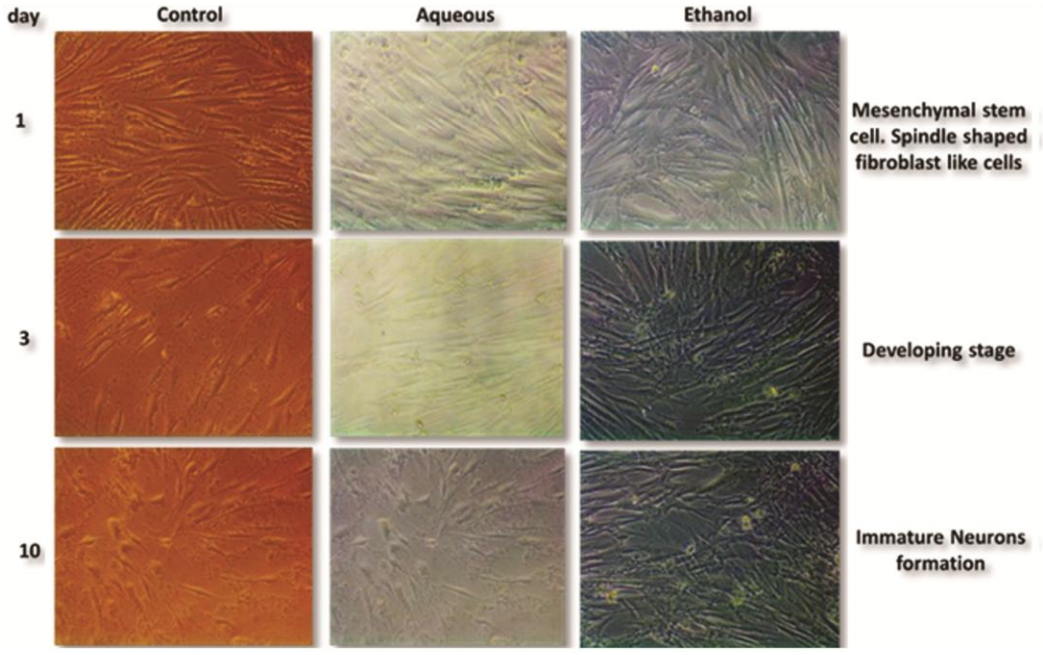


Fig. 6 — Neural differentiation-associated morphological changes in human dental pulp stem cells (hDPSCs) observed under phase-contrast microscopy. The left panel shows the control group, while the middle and right panels show cells treated with aqueous and ethanolic extracts of *Benincasa hispida* seeds (BHS), respectively. Morphological changes were recorded at 1, 3, and 10 days

minimal differentiation and retained predominantly spindle-shaped morphology. These morphological changes suggest that BHS extracts were associated with neural lineage-associated cellular changes in hDPSCs.

Assessment of Nestin gene expression

Nestin gene expression was evaluated as an early neural progenitor-associated marker following treatment with aqueous BHS extract. Real-time RT-PCR was performed using GAPDH as the housekeeping gene, and gene expression was expressed as fold change relative to the control group. The control group was assigned a relative expression value of 1.0, whereas the aqueous BHS-treated group showed an approximately 0.6-fold relative expression compared with the control (Fig. 7). The results are presented as mean ± SD. The observed difference between the control and aqueous BHS-treated groups is represented by the significance marker shown in (Fig. 7). These findings, together with the morphological observations, suggest an early neural lineage-associated response in hDPSCs. However, because Nestin was the only molecular marker assessed, the findings should be interpreted as preliminary evidence of neural progenitor-associated changes rather than definitive confirmation of mature neuronal differentiation.

Discussion

Neurodegenerative disorders are progressive, irreversible conditions characterized by neuronal loss, synaptic dysfunction and cognitive decline. Alzheimer's disease remains the most common cause of dementia worldwide and poses a major public health challenge[19]. Despite significant advances in pharmacological management, currently available drugs mainly provide symptomatic relief and fail to reverse neuronal degeneration[20]. Hence, regenerative strategies aimed at neural restoration and repair are urgently required.

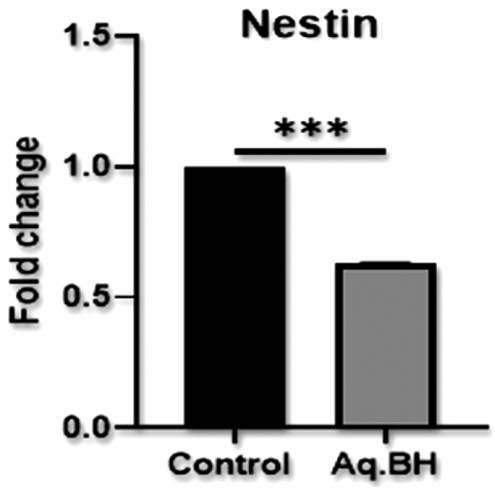


Fig. 7 — Nestin gene expression in human dental pulp stem cells (hDPSCs) treated with aqueous extract of *Benincasa hispida* seeds (BHS), expressed as fold change relative to the control group. Data are presented as mean ± SD. Asterisks indicate the level of statistical significance between the control and aqueous BHS-treated groups

Ayurveda describes several *Medhya Rasayana* drugs that promote intellect, memory and higher mental functions through rejuvenation of the nervous system. *Benincasa hispida* (*Kushmanda*) is one such classical *Medhya* drug widely used in traditional medicine for the management of *Unmada*, *Apasmara* and *Smritibhramsha*[3,4]. The present study provides direct experimental evidence supporting these classical claims at a cellular and molecular level.

Oxidative stress and neuroinflammation play a central role in the pathogenesis of neurodegenerative disorders[7]. Excessive production of reactive oxygen species leads to lipid peroxidation, mitochondrial dysfunction and neuronal apoptosis. The phytochemical constituents of *Benincasa hispida*, particularly polyphenols, flavonoids and triterpenoids, possess potent antioxidant and anti-inflammatory properties[5-7,21]. These phytochemicals likely created a favourable microenvironment for stem cell survival, proliferation and neural differentiation observed in the present study. Earlier pharmacological studies have also demonstrated the anxiolytic, anticonvulsant and cognitive-enhancing effects of *Benincasa hispida*[21,22].

Human dental pulp stem cells are an excellent experimental model for neuroregenerative research due to their neural crest origin, high plasticity and ability to differentiate into neuron-like cells[15]. In the present study, hDPSCs showed rapid morphological transformation into neuron-like cells within ten days of treatment with BHS extracts. These morphological changes suggest that BHS extracts may influence neural lineage-associated cellular responses in hDPSCs. However, because a conventional positive control was not included in the present study, the extent of this response cannot be directly compared with that produced by an established chemical or growth-factor-induced neural differentiation protocol[13].

Nestin is a well-established neural progenitor-associated marker expressed during early neurogenesis[21,22]. In the present study, Nestin gene expression was assessed to obtain molecular information regarding the early neural lineage-

associated response following aqueous BHS extract treatment. The observed Nestin expression, together with the morphological changes, provides preliminary evidence of a cellular response associated with neural progenitor characteristics. However, the observed expression pattern should be interpreted cautiously, as Nestin alone does not establish mature neuronal differentiation and the present study did not include additional neuronal or glial markers. Furthermore, because the present analysis was interpreted descriptively, inferential statistical significance was not established.

An important limitation of the present study is that the assessment of neural differentiation was based on morphological observations and Nestin gene expression. Nestin is widely associated with neural stem and progenitor cells; however, its expression alone does not establish differentiation into mature neuronal or glial cells. Additional lineage-specific markers such as βIII-tubulin/TUBB3 and MAP2 for neuronal differentiation, NeuN for more mature neuronal phenotypes, and GFAP for glial differentiation can provide a more comprehensive assessment of lineage specification and maturation. Therefore, the absence of these additional markers limits the extent to which the present findings can be interpreted as definitive neuronal or glial differentiation. Future studies should incorporate a broader panel of neuronal and glial markers together with appropriate positive differentiation controls to confirm lineage specificity and maturation.

The preliminary morphological changes and Nestin expression observed following BHS treatment provide a basis for considering possible intracellular mechanisms underlying the cellular response. Natural plant-derived compounds have attracted considerable interest in regenerative medicine because of their potential to influence stem-cell behaviour and intracellular signalling pathways involved in cell fate determination. Among these, the Wnt/β-catenin, MAPK and Notch pathways are recognized as important regulators of neural progenitor maintenance, proliferation and differentiation. The phytochemical profile of *Benincasa hispida* seeds includes flavonoids, phenolic compounds, triterpenoids, alkaloids and other bioactive constituents, several of which may possess antioxidant and signalling-modulatory properties. In the context of the present findings, it is plausible that one or more constituents of BHS extract could influence intracellular pathways associated with neural lineage development, including MAPK-, Wnt/β-catenin- or Notch-related signalling. However, these pathways were not directly investigated in the present study; therefore, this mechanistic interpretation remains hypothetical. Future studies incorporating pathway-specific molecular markers, protein-expression analysis and functional pathway assays will be required to determine whether these signalling mechanisms contribute to the neural lineage-associated response observed following BHS treatment.

From an Ayurvedic perspective, *Medhya Rasayana* drugs enhance higher mental faculties by nourishing the brain tissue and strengthening *Prana*, *Ojas* and *Majja Dhatu*. According to *Chakrapani*, *Medhya* enhances retention of knowledge and sustains intellectual functions[23]. The present cellular findings provide a scientific basis for this classical *Medhya* concept by demonstrating the ability of *Benincasa hispida* to initiate neural differentiation at the stem cell level.

Thus, the study successfully bridges Traditional Knowledge with contemporary regenerative medicine and provides a novel cellular mechanism for the neuroprotective and cognitive-enhancing effects of *Benincasa hispida*.

Conclusion

In conclusion, the present study demonstrates that aqueous and ethanolic BHS seed extracts were cytocompatible with hDPSCs at the tested concentrations and were associated with morphological changes suggestive of neural lineage development. The findings provide preliminary evidence of neural progenitor-associated responses following BHS extract exposure. However, confirmation of neuronal or glial differentiation requires assessment of additional lineage-specific markers and appropriate positive differentiation controls. Inclusion of a standardized positive neural differentiation control in future studies would further strengthen the interpretation and validation of these findings.

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Author Contributions

ND – Supervision, Writing, Review & Editing, MK – Methodology, Data curation, GR – Editing, AK – Conceptualization, Investigation.

Conflict of Interest

The authors declare that there is no conflict of interest related to this research or its publication.

Ethics Approval

The study was approved by the Institutional Committee for Stem Cell Research, Dr. D. Y. Patil Dental College and Hospital, Pimpri, Pune, Maharashtra, India (Ref. No. IC-SCR/RM/68/23).

Informed Consent

Informed consent was obtained from the donor prior to collection of the human dental pulp sample used in the study.

Use of Artificial Intelligence (AI)

No artificial intelligence (AI) tools were used in the preparation, analysis, interpretation, or writing of the original manuscript.

Data Availability

The data generated and/or analysed during the present study are available from the corresponding author on reasonable request.

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