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Evaluation of *Argyreia speciosa* (Linn.) sweet activity on human dental pulp stem cells

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Indian traditional medicine specifically defines a class of substances including *Argyreia speciosa* (Linn.) sweet (AS) that modulates the central nervous system. Nevertheless, the precise mechanism of action of AS remains unknown. The objective of this investigation was to determine the neural lineage and gene expression by utilizing Nestin and beta-tubulin. A standardized aqueous extract was produced by processing an authentic sample of AS. Stem cells were identified and characterized. The drug's viability was assessed using the MTT assay. We utilized AS to stimulate neural differentiation in human dental pulp stem cells (hDPSCs). We employed RTPCR to evaluate gene expression in hDPSCs using markers such as Nestin and beta-tubulin. The MTT assay demonstrated that the 10 µg/mL concentration facilitates cell differentiation into trilineage differentiation. Neural differentiation was observed in the treated group, which received AS and neurogenic media. The neurogenic potential was assessed by RT-PCR, which revealed significantly greater Nestin expression in the experimental group comparable to the control group. The aqueous extract of AS met the previously stated pharmacokinetic requirements. Neural differentiation was observed within seven days. The sole indicator of gene expression was the Nestin neural marker. *A. speciosa* may be employed as a complementary medication to address a diverse array of neurological disorders.

Keywords: *Argyreia speciosa* (Linn.) sweet, Gene expression, Human dental pulp stem cells, Nestin, Neural differentiation, β-tubulin

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Neurological disorders, both fatal and non-fatal, contribute significantly to India's noncommunicable and communicable disease burden[1]. Neurological disorders involve the brain, spinal cord, cranial nerves, peripheral nerves, nerve roots, autonomic nervous system, neuromuscular junctions, and muscles[2-4]. Alzheimer's disease is the leading cause of dementia and may account for 60-70% of cases. The world's elderly population is growing which are most vulnerable to neurological disorders. Therefore, solutions to combat Alzheimer's and Parkinson's diseases (P. D.) and Dementia is necessary. The mental impulses of greed, fear, wrath, depression, vanity, hatred, harsh speech, and wicked ideas are as harmful to the body as they are to the mind, and are the primary causes of neurological disorders[5,6]. Because of the serious adverse effects, psychiatrists are cautious about prescribing Donepezil-like medication. As a result, scientists are looking for effective herbal remedies to treat neurological disorders. Ayurveda defines a unique class of central nervous system (CNS) modifying medications, known as Medhya drugs[7]. *Argyreia speciosa* (Linn.) sweet (Family: Convolvulaceae) is a significant Medhya plant that is widely utilized as an adaptogen in the Ayurvedic system of medicine[8,9]. Understanding neural lineage pathways is essential for evidence-based Ayurveda[10]. Stem cell research in Ayurveda is still in its early stages. More scientific data is required to prove a theory.

This research was conducted with the intention of determining the influence that AS has on human embryonic stem cells (hDPSCs) in relation to neuronal differentiation.

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Materials and Methods

Authentication and characterization of raw material

During the month of December, 2022 fresh roots of *A. speciosa* (Linn.) Sweet, which is part of the Convolvulaceae family were meticulously gathered from a home garden that is a specialized cultivation site from Latur, Maharashtra. This garden was cultivated through genuine and organic farming techniques, meticulously adhering to the principles established by Good Agricultural Practices (GAP). These methodologies guaranteed the application of non-chemical inputs, sustainable agricultural practices, and appropriate management of medicinal flora to maintain their phytochemical integrity, therapeutic efficacy, and ecological sustainability. The careful observance of GAP principles ensures traceability, quality assurance, and the safety of the harvested plant material, rendering it appropriate for subsequent pharmacological and research endeavours (Fig. 1). Analysis of raw, unprocessed roots, leaves, and flowers of AS has been conducted and authenticated (AUTH 22 - 97) at the Agharkar Research Institute, Pune. This raw material was microscopically evaluated by using a LYNX (XSZ-N207) binocular microscope. This fresh roots weighing 4 kg were dried in a well-illuminated shaded area. After three months, the desiccated roots had a mass of two kilograms. The desiccated roots underwent a meticulous pulverization process in a 2-HP pulveriser (Ajanta, India), adhering to the rigorous ISO 9001:2008 standards, utilizing a 60 μm sieve for optimal granulation.

Preparation of extract

In order to prepare the aqueous extract, 50 g of coarsely powdered plant material were meticulously weighed and positioned within a Soxhlet apparatus, utilizing 250 millilitres of distilled water as the solvent for extraction. Upon the conclusion of the extraction cycle, the thimble housing the residual plant matter was meticulously extracted. The resultant extract was subsequently subjected to filtration using Whatman Ashless filter paper, thereby removing any residual solid particles or impurities. The transparent filtrate was transferred into a petri dish and positioned in a water bath regulated at 60°C. The extract underwent a process of sustained heating for roughly 2 h, culminating in the total evaporation of the solvent and resulting in a dry residue of the aqueous extract.

Physicochemical analysis

The powder was subjected to an examination of its *sparsha* parameters (consistency and texture), *rupa* parameters (color), *rasa* parameters (taste), and *gandha* parameters (odor). A series of physico-chemical analyses of the powder and its raw material were performed utilizing recognized pharmacopoeia methodologies. The process involved quantifying the proportion of foreign matter through the use of a digital balance, assessing loss on drying and total ash content via a hot air oven and digital balance, and filtering acid-insoluble ash combined with dilute HCl utilizing Watman's filter paper, which was also measured with a digital balance.

Isolation and culture of mesenchymal stem cells from human dental pulp

Multipotent mesenchymal stem cells were isolated from human dental pulp obtained from a tissue donor devoid of any medical history pertaining to dental complications. Prior to the extraction of the tooth, informed consent was duly acquired. A series of treatments was administered to the tooth pulp sample, commencing with immersion in a povidone-iodine

Fig. 1 — Different parts of *Argyreia speciosa* (Linn.) Sweet plant, (a) Leaves and flower, (b) Stem, (c) Roots

solution, followed by submersion in a saline solution, and concluding with transfer to a 15 mL tube containing phosphate-buffered saline (PBS) enriched with a 1% antibiotic and antimycotic solution. An aseptic Airotor hand piece was employed to segment the tooth into distinct sections and extract the pulp. The obtained pulp sample was subsequently and carefully sectioned into minute fragments, approximately 1mm in diameter, utilizing a sterile blade. Subsequently, the specimens were carefully rinsed with PBS and transferred into a T25 flask, accompanied by a 1% solution of antibiotics and antimycotic agents, as well as fetal bovine serum (FBS). The combination was subsequently incubated at 37°C in an atmosphere of 5% CO_2 for a period of 24 h. Thereafter, the flask was supplemented with Dulbecco's modified eagle medium (DMEM), 10% fetal bovine serum (FBS), and 1% antibiotic, maintaining the incubation parameters at 5% carbon dioxide (CO_2) and 37°C. To promote cell proliferation, the medium was substituted every 2 to 3 days[11,12]. Cellular proliferation was observed utilizing an inverted OLYMPUS CKX53 phase contrast microscope. Upon reaching 80% confluence, trypsinization was conducted utilizing a 0.25% trypsin EDTA solution. Subsequently, the cells were transferred to T-25 flasks, marking the initiation of passage 0 (P0). The procedure was reiterated until P4, following which these cells were employed for further explorations.

Characterization of human dental pulp stem cells

Cell surface antigen expression analysis

The identification of isolated and cultivated stem cells was conducted through flow cytometry, employing specific cell surface markers including PE-labelled CD90, CD73, and CD105, alongside FITC-labelled CD34, CD45, and HLA-DR[13]. A 200-microliter cell suspension, comprising 2 x 10^6 cells, was meticulously distributed among four Eppendorf tubes. Cell fixation was conducted at room temperature for duration of 30 min using a 4% formaldehyde solution. Subsequent to fixation, the cells underwent a washing procedure utilizing PBS. Subsequently, 3 microliters of specific antibodies were administered to the cells and allowed to incubate at room temperature for duration of 30 min. Subsequently, the tubes were subjected to FACS analysis utilizing Cell Quest Pro software.

Trilineage differentiation of human dental pulp stem cells

To evaluate the development of mesenchymal stem cells into osteogenic, chondrogenic, and neuronal lineages, the cells were exposed to specialized induction media and then stained with suitable dyes after a period of 13 days. Cells were experimentally exposed to Dexamethasone at a dose of 0.1 µM, glycerophosphate at 10 mM, and Ascorbic acid at 2 mM in order to assess osteogenic differentiation. Colouring was carried out with a 2% solution of Alizarin Red S. To induce chondrogenic differentiation, cells were cultured in a media supplemented with Dexamethasone at a concentration of 0.1 µM, Sodium pyruvate at 100 µg/mL, ITS at a concentration of 1X, Ascorbic acid at 2 mM, L-proline at 40 µg/mL, and TGF-ε3 at 10 ng/mL. The staining process was conducted using a 0.1% solution of Alcian blue. Observation of neurogenic differentiation was conducted using neurogenic induction medium. The medium was replaced every 2 to 3 days and allowed to incubate for 7 days.

Viability assay

A cell viability study was conducted using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) technique. A stock solution of MTT was produced at a concentration of 10 mg/mL for the aqueous extract of aerial stem bark. Hematopoietic stem cells (HDPSCs) were cultivated at a concentration of $1x10^4$ cells per milliliter in a standard 96-well plate. Viability was assessed at several concentrations (1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 25 µg/mL, and 50 µg/mL) of AS. Following 24 h of drug treatment, 20 µL of MTT were applied to each well and allowed to incubate at ambient temperature. At the 3 h mark, the MTT solution was extracted and replaced with 100 µL of DMSO. Measurement of the absorbance at 560 nm was conducted using an ELISA reader.

Real-time polymerase chain reaction (RT-PCR)

Genomic expression study was conducted on cells treated with a 10 µg/mL aqueous extract of *A. speciosa* (AS). The TRIzol method established by Ambion Life Technologies was employed for RNA extraction, while the High-Capacity cDNA Reverse Transcription Kit from Thermofischer was used for cDNA synthesis. Included in the kit were a 10X Random primer, a 10X Reverse transcription buffer, a 25X dNTP mix, an RNase inhibitor (20 U/µL), DEPC-treated water (Himedia), and a 50 U/µL Multiscribe TM Reverse transcriptase. The RT-PCR analysis focused on the genes Beta-Tubulin and Nestin, with GAPDH specified as the housekeeping gene. The primer specifications

are described in Table 1. The RT-PCR in this study was performed using the Quant Studio 5 Real-Time PCR System instrument.

Ethical approval

The present study was approved by Institutional Ethical Committee (DPU/RM/29/20). An *in- vitro* experiment on human dental pulp stem cells was carried out at the Regenerative Medicine Laboratory.

Analysis of statistical data

The experiments were conducted in triplicate, with results expressed as the mean ± standard deviation (SD). Data analysis was performed utilizing GraphPad Prism 8. "Two-way ANOVA" was conducted, followed by the appropriate post-hoc tests which was determined and recommended by the software at ****$p<0.0001$, ***$p<0.001$, and **$p<0.01$ to assess the statistical significance among the groups in comparison to the control.

Results

Raw material analysis

The authentication procedure for the raw material of *A. speciosa* (Linn.) sweet included a meticulous analysis of their structure using a LYNX (XSZ-N207) binocular microscope. This analysis is essential for verifying the identification and quality of the listed medication. The microscopic analysis included tracking the morphology of plant tissues to identify cells, trichomes, vascular bundles, and other unique features unique to AS, such as pith, pericyte, and xylem (Fig. 2).

Physico-chemical analysis of *A. speciosa* (Linn.) sweet

According to the results of the physicochemical examination, all of the parameters were within the normal range, and there was no evidence of any adulteration or authentication present (Table 2).

Isolation and culture of human dental pulp stem cells (hDPSCs)

Following a 24 h incubation period (day 1), the tissue adhered to the surface of the T25 flask. Every two to three days, the culture medium of the explant was refreshed, and meticulous observations were carried out utilizing an inverted phase contrast microscope until the nineteenth day. No cellular proliferation surrounding the explant was observed until the second day. By the third day, the proliferation of the explant became apparent, showcasing cells exhibiting an elongated, fibroblastic morphology. By the nineteenth day, the cells had attained a confluence of 75-80% (Fig. 3).

Characterization of human dental pulp stem cells (hDPSCs)

Mesenchymal stem cells derived from human dental pulp demonstrated a commendable expression of the CD73, CD90, and CD105 markers. Consequently, the percentages of favorable expression were recorded at 98.400%, 98.420%, and 99.380%, respectively

Table 1 — Primers used for the Neural gene expression

Name of gene	Forward primer	Reverse primer
GAPDH	TCCCTGAGCTGAACGGGAAG	GGAGGAGTGGGTGTCGCTGT
Beta-Tubulin	GACCGGACCCTCGCCAGTGCT	AATCGACGTGGGTGGGTGGGG
Nestin	GAGGAAACCGAAGAGGAGG	GGGGTGTTGTTCTCATCCAG

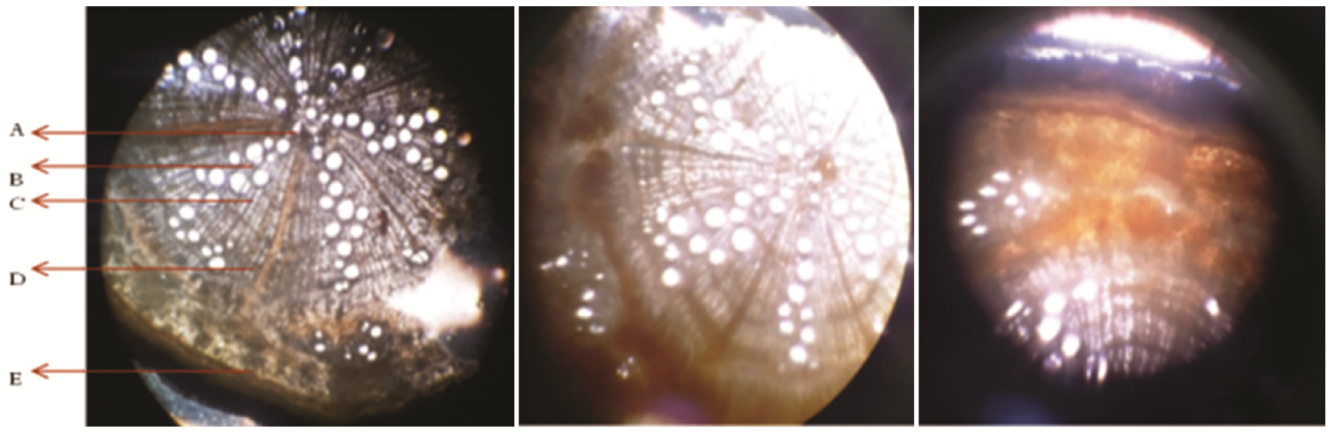

Fig. 2 — Microscopic examination: Transverse section of raw drug of AS showing various components. (100x), (a) Pith, (b) Cortex, (c) Xylem, (d) Phloem, (e) Cork

(Fig. 4). Conversely, Histograms D, E, and F demonstrated negative quantification for CD45, HLADR, and CD34, with proportions falling below 10% (0.290%, 4.690%, and 8.600%, respectively) Figure 4. The evidence delineated above suggests that the isolated cells are indeed stem cells.

Table 2 — Physicochemical analysis of AS

Parameter	Raw drug
Foreign matter (%)	0.89
Loss on drying	9.7
Total ash (%)	9.7
Acid insoluble ash (%)	0.8
Water soluble extract (%)	11
Alcohol soluble extract (%)	8.1
PH	6.8
TLC	Rf – 2 spots; 0.77, 0.85

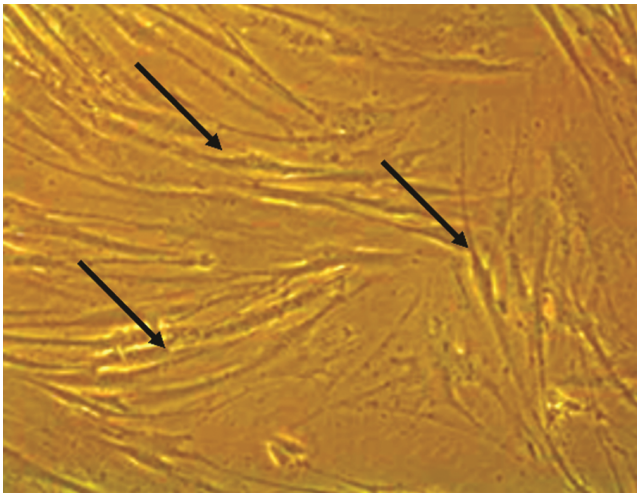

Fig. 3 — Fibroblastic morphology (long, thin, slender cells – black arrows) of human dental pulp stem cells under inverted microscope on 17th day

Viability assay

The viability of human induced pluripotent stem cells (hDPSCs) was assessed through a viability assay conducted in triplicate following a 24 h treatment with AS. Upon conducting a thorough calculation of the optical density (OD) values and deriving the averages from the measurements, a concentration of 10 µg/mL was deemed appropriate for the ensuing experiment (Fig. 5).

Trilineage differentiation

The cells cultivated in osteogenic medium exhibited deposits of a black hue encircling them. The observation of spherical cell morphology coincided with the presence of cartilage-specific components and extracellular matrix in media generated through chondrogenesis. Cells induced by neurogenic medium displayed elongated, spindle-like, and intricately branching structures. On the thirteenth day, cells cultured in Osteogenic and Chondrogenic medium underwent staining with a 2% solution of Alizarin Red S and a 0.1% solution of Alcian blue, respectively (Fig. 6).

Neural differentiation of hDPSCs with AS

The process of neural differentiation in Dental Pulp Stem Cells (DPSCs) is accomplished by guiding these cells towards a neural cell fate through the application

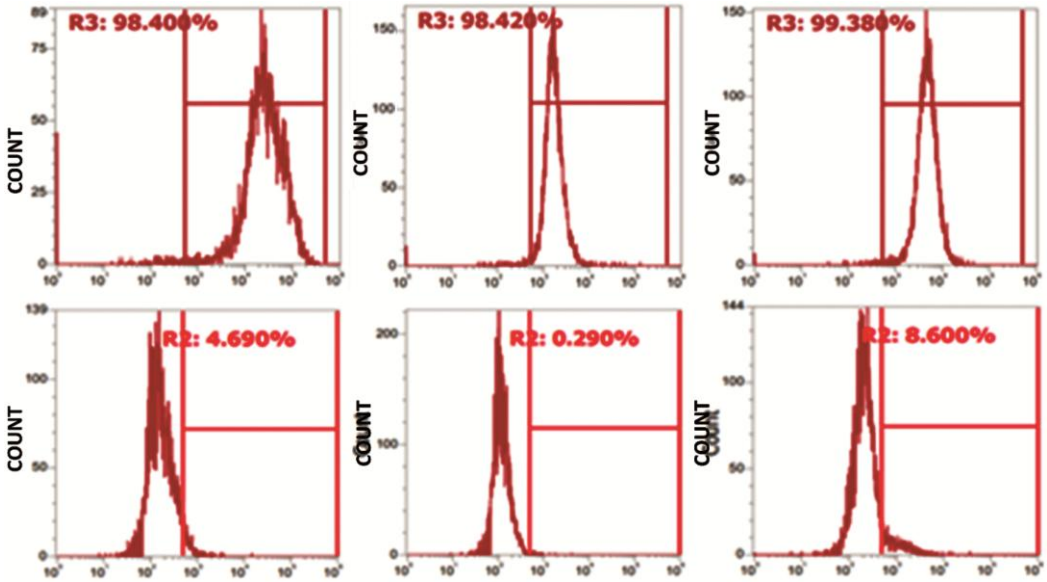

Fig. 4 — Surface markers expression for human dental pulp stem cells: (a-c) are positive for CD90-PE-H, CD73-PE-H, CD105-PE-H surface markers and (d-f) negative for CD45-FITC-H, HLADR-FITC-H, CD34-FITC-H surface markers

of a test drug. This approach illustrates their potential to evolve into neural lineages tailored for

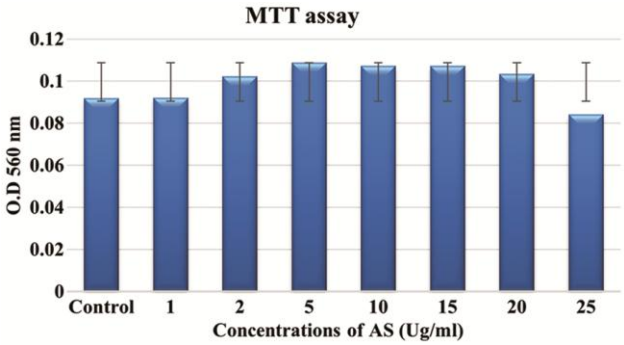

Fig. 5 — MTT assay showing various concentrations of aqueous extract of AS on X-axis and different OD values according to the concentration on Y-axis that are read at 560 nm for 24 h

neural differentiation. Throughout the initial week, the progression of neuronal differentiation was meticulously observed through various methodologies, including microscopy, which was later complemented by molecular analysis. An observed morphological alteration was the emergence of elongated, slender cells featuring branched, neurite-like extracellular structures (Fig. 7).

Neural specific gene expression by AS in *hDPSCs*

A RT-PCR was employed to conduct gene expression profiling to evaluate the over expression of neural-specific genes. This provides molecular evidence that the cells have embraced a neuronal lineage that they have assimilated. The target genes underwent assessment in each sample, and the relative

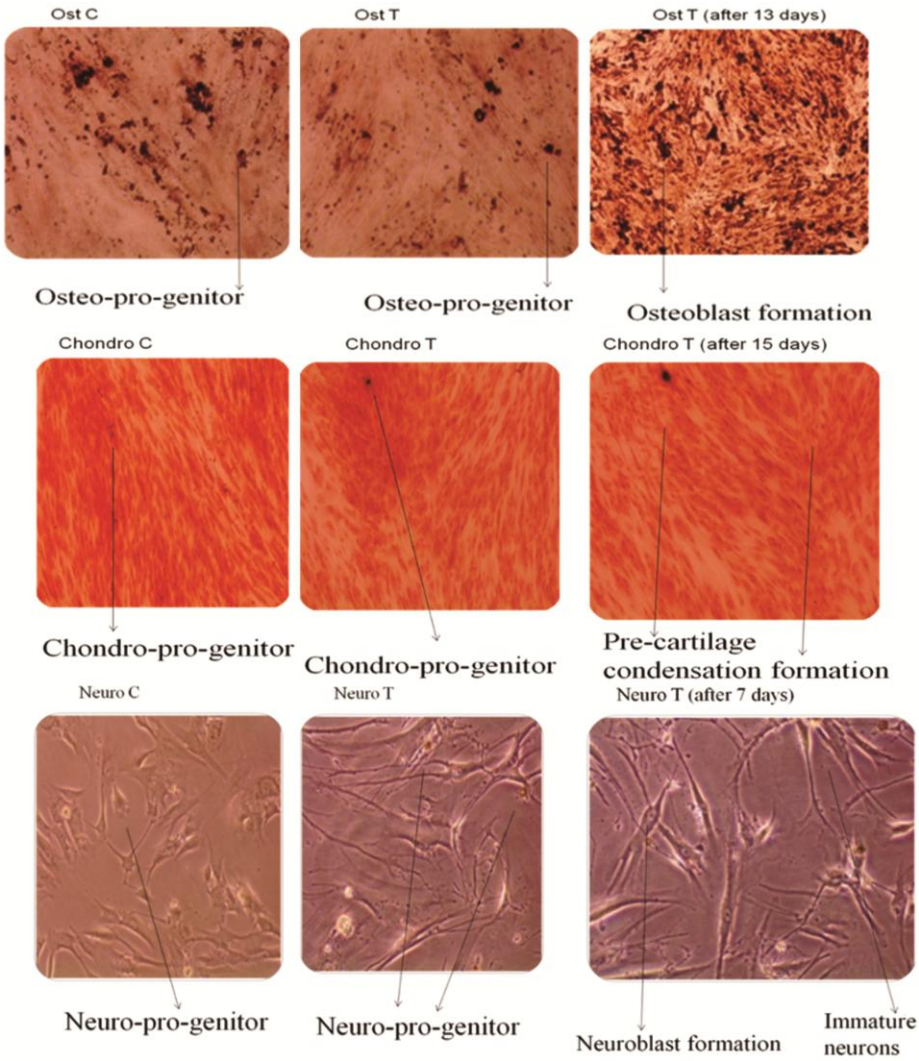

Fig. 6 — Differentiation of hDPSCs: (a) shows differentiation of hDPSCs into bone with black deposits with different stages in bone formation, (b) shows chondrogenic differentiation with cells positive for Alizarin Red, (c) exhibits morphological changes of hDPSCs into neuronal cells.

fold changes of Beta Tubulin and Nestin were calculated for each sample employing the comparative fold change formula [2^(-ΔΔCT)]. This procedure was executed for each of the samples. Following treatment with AS, the expression of Nestin was found to be similar to that of the control, suggesting that AS possesses neuro-differentiating properties. Conversely, Beta Tubulin exhibited diminished expression (Fig. 8).

Discussion

Alzheimer's disease constitutes the foremost cause of dementia, accounting for 60-70% of instances. Individuals with neurological impairments often require support in managing their daily activities, a necessity that is intensified by the growing global demographic of older adults. The management of conditions such as Alzheimer's, Parkinson's disease, and dementia holds significant importance.

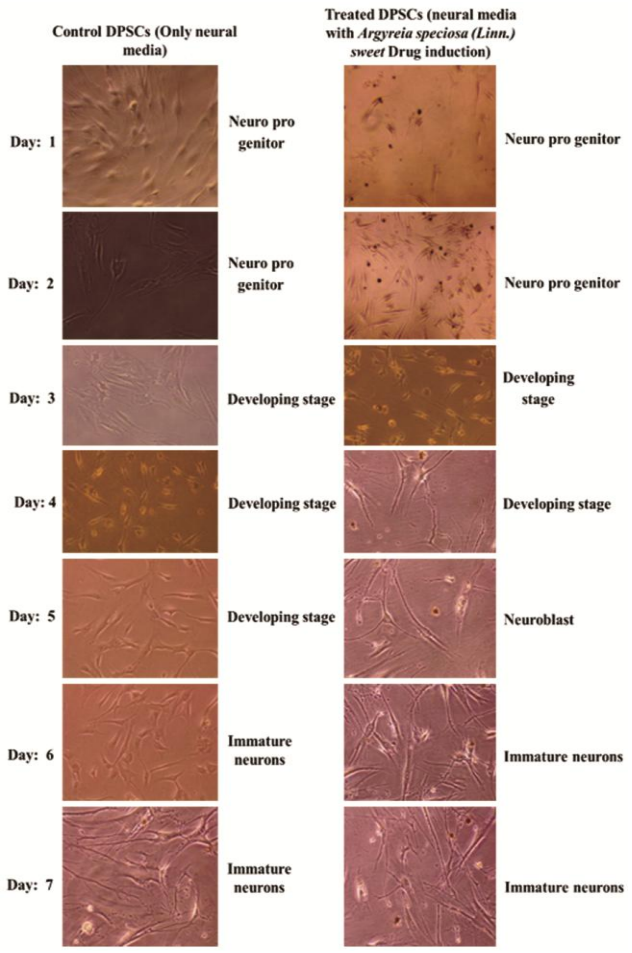

Fig. 7 — Neural differentiation of hDPSCs: (a) shows differentiation of hDPSCs supplemented with neurogenic media, (b) shows neural differentiation of cells supplemented with AS

While conventional herbal treatments have been employed and substantiated in clinical contexts, there exists a necessity for a deeper comprehension of the fundamental mechanisms associated with neurological disorders[5,6].

Chakrapani posits that Medha embodies a form of intellect characterized by its capacity to safeguard knowledge. Medhya medications are celebrated for their beneficial effects on the capacity to retain information, a faculty inherently associated with the cellular processes and evolution of the human brain. A comprehensive understanding of these mechanisms at the cellular and molecular level is essential for formulating targeted therapies for neurological disorders[14,15].

A stem cell model was considered advantageous for clarifying the genetic mechanisms that underpin these disorders. The analysis of physico-chemical characteristics substantiates the authenticity of the selected species, ensuring it is devoid of adulteration, with all results falling within the permissible range. This verification not only confirms the consistency of the medicine but also underscores its capacity for replication.

The retention factor (Rf) value of *A. speciosa* (Linn.) sweet remains inadequately characterized, prompting the execution of a thin-layer chromatography (TLC) analysis to ascertain this value. Nonetheless, the observed pattern of spots exhibited a remarkably low intensity. The observed spot pattern exhibited a notable similarity to the API (Active Pharmaceutical Ingredient) parameter, suggesting that, during the present study, the spot pattern in the thin-layer

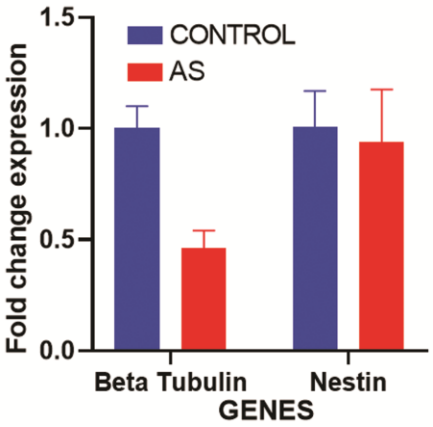

Fig. 8 — Effect of AS on Gene expression of Beta Tubulin and Nestin. The bar graph shows fold change expression in the gene expression levels of Nestin and Beta Tubulin in control and AS treated group. Nestin shows equal expression of Nestin in control and AS treated group. Error bar shows mean with standard deviation. *p<0.05

chromatography (TLC) of *A. speciosa* (Linn.) Sweet remains uncharacterized.

A plethora of researchers have thoroughly examined the safety of this specific species, as evidenced by studies like "Central Nervous System Activity of *Argyreia speciosa*" carried out by SV Neeraj[16]. Moreover, P Kashyap highlighted the neuroprotective attributes of scopoletin, a naturally occurring compound found in the roots of *A. speciosa*, proposing its potential to safeguard cells from damage during their growth, proliferation, and differentiation[17]. With regards to the neuroprotective properties of *A. speciosa* (Linn.) Sweet as a whole extract, there is no previous work that has been carried out.

This research provides the first documentation of the neural differentiation process utilizing *A. speciosa* (Linn.) sweet in conjunction with DPSCs. The findings indicate that neural differentiation transpires over a span of 7 days. This finding aligns with prior research on neural differentiation of dental pulp stem cells to neuron-like cells in dopaminergic and motor neuronal inductive media[18].

The present study revealed that AS exhibited notable expression of Nestin in DPSCs, with a statistically significant p-value of 0.02 ($p<0.05$). These findings align with those of previous research[19,20]. Nonetheless, it was demonstrated that there was no statistical significance for β-Tubulin, which was indicated to play a role in brain development according to the study conducted by Yasuhiro Nakamura *et al*.[21]. Consequently, one may deduce that AS could play a significant role in cellular functions, including cell division, movement, and specialization, thereby facilitating the transmission of information through the communication pathways of the human brain. The findings hold significant promise for enhancing our nuanced comprehension of the extract's mechanism of action across various neurological disorders.

Conclusion

The findings from the physicochemical analysis indicate that AS stands as a noteworthy and pure candidate for drug development. The influence of AS on hDPSCs demonstrated both viability and neural differentiation, suggesting that this may serve as a noteworthy complementary therapeutic approach in the treatment of neurological disorders. While the mechanism of action of AS requires further investigation, it is essential that all findings related to toxicity be conducted *in vivo*.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

SB & VSL facilitated the procurement of resources and formal analysis, VSL & RB aided the procurement of resources. SB, AW, RB, AD contributed to the Writing - Review & Editing, Supervision, and SB & VSL contributed to the Conceptualization, Methodology, Formal analysis, Investigation, Resources & Writing -Original Draft.

Ethics Approval

The current investigation received approval from the Institutional Ethical Committee (DPU/RM/29/20).

Informed Consent

Human dental pulp stem cells were obtained and studied.

Data Availability

The article itself presents the analytical data that substantiates the outcomes of our study. Nevertheless, the data can be obtained from the authors upon a reasonable request.

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