https://doi.org/10.33307/entomon.v47i4.788
ENTOMON 47(4): 353-364 (2022)
Article No. ent. 47401

Effect of stingless bee propolis on the proliferation of human pluripotent stem cells

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ABSTRACT: The effect of stingless bee propolis on the proliferation and differentiation of human stem cells is reported for the first time. Cells (hPSCs) treated with the propolis extracted from *Lisotrigona* sp., *Tetragonula calophyllae* and *T. travancorica* displayed a remarkable difference in their morphology. Gene expression analysis revealed pluripotency markers *OCT4* and *NANOG* to be down-regulated upon treatment with propolis, which confirmed early differentiation of hPSCs. Further investigation on the gene expression of early differentiation markers revealed that propolis supports mesendoderm differentiation, which is a novel finding. The propolis obtained from stingless bees *Tetragonula* spp. probably has more therapeutic value in terms of its effect on hPSCs viz., more tendency of the cells to differentiate into mesoderm and endoderm lineages, compared to the propolis obtained from *Lisotrigona* sp.

KEY WORDS: Gene expression analysis, therapeutic value, hPSCs, cytotoxicity, differentiation

INTRODUCTION

Stingless bees commonly called meliponines are a large group of bees, which belongs to the tribe Meliponini that is widely occurring over the tropical and subtropical areas of the world (Velikova *et al*., 2000). Stingless bees are amongst the longest evolved bees that have been identified in 80 million years old parts of amber, estimated to have 400 to 500 different species but new species are identified every year (Kasote *et al*., 2019). Three new species of stingless bees *Tetragonula travancorica*, *T. calophyllae* and *T. perlucipinnae* were described as new to science from Kerala (Shanas and Faseeh, 2019). Stingless bees use their head gland secretions, plant resins, wax, essential oils, pollen and exudates, including organic and inorganic earth components to produce propolis (Ghisalberti., 1979; Pasupuleti *et al*., 2017). The colour of propolis varies from yellow to dark brown based on the origin of the resin. Propolis is known for its antibacterial, antifungal, antiviral, anti-

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inflammatory, antioxidant, anti-tumoral and tissue generation activities (Bankova and Popova, 2007; Popova *et al*., 2019). Propolis exhibits a complex chemical composition and has been reported to contain more than 300 organic and inorganic compounds (Huang *et al.,* 2014). The chemical composition and pharmacological activities of propolis vary according to the geographical and botanical origin, types of vegetable sources, time of collection and season of the year (Wagh, 2013; Farooqui, 2012; Anjum *et al*., 2011). India, being a vast country, has several different varieties of propolis varying in its chemical compositions and medicinal values. Moreover, the unique geography of Kerala being encroached upon by the Western Ghats provides a variety of propolis differing in chemical composition and medicinal values.

Stem cells are cells that have the potential to develop into different cell types in the body during early life and growth. Induced pluripotent stem cells (iPSCs) are the cells that are reprogrammed from somatic cells to form undifferentiated stem cells having the same properties as Embryonic Stem Cells (ESCs). ESCs are derived from early pre-implantation blastocyst stage embryos, that can self-renew indefinitely in culture and are pluripotent, maintaining the ability to become any cell type in the human body (Takahashi and Yamanaka, 2016). Human PSCs, including hESCs and hiPSCs, hold great promise for drug discovery and regenerative medicine as they can be used for disease modelling, drug screening and understanding of the mechanisms underlying development of tissues and organs (Wu and Hochedlinger, 2011; Robinton and Daley, 2012).

Natural compounds serve as a promising source of alternative medicine for various degenerative diseases. They can recruit stem cells, increase their proliferation and promote their differentiation. Several natural compounds and their combinations can promote the proliferation and differentiation of iPSCs in vitro (Bickford *et al*., 2006). Propolis, a natural compound increased the proliferation rate of bone marrow-derived mesenchymal stem cells (BMMSCs), enhanced the chondrogenic and adipogenic differentiation processes. They also increased the migration capacity of BMMSCs and promoted induced gap closure of cells after osteogenic differentiation in vitro (Elkhenany *et al*., 2019). Another study by using Taiwanese green propolis (TGP) ethanol extract promoted the differentiation of murine mesenchymal stem cells into adipocytes by the activation of the PPARγ (adipogenic transcription factors) dependent pathway. There was also an increase in adiponectin and intracellular triglyceride level in the cells (Chen *et al.,* 2020). One of the important components of propolis extract, caffeic acid phenethyl ester (CAPE), can enhance in vitro expansion of blood derived hematopoietic stem cells (HSPCs) by the upregulation of the expression of genes such as SCF, HIF-1 α, and HO-1 (Liu *et al*., 2014). CAPE was also shown to promote the proliferative capacity of hematopoietic stem cells derived from umbilical cord blood *in vitro* (Ahangari *et al.,* 2012). Studies reported that ethanolic extract of propolis can promote bone regeneration and induce hard tissue bridge formation in pulpotom. Moreover, propolis also displayed acceptable biocompatibility and enhanced the endodontic regeneration process (Elgendy and Fayyaa, 2017). *In vivo* studies in rats suggested that oral administration of propolis enhanced the healing of fractured femur and increased bone mineral density (Guney *et al.,* 2011). Moreover intraperitoneal injection of CAPE, a major component of propolis enhanced bone regeneration in the rat calvarial defect model (Ucan *et al*., 2013).

The effect of stingless bee propolis on stem cells is not very well understood. To our knowledge, there are no reports in the literature that describes the effect of stingless bee propolis on the proliferation of human - induced pluripotent stem cells. In this study, we investigated the effect of propolis extracts at different concentrations towards proliferation, cytotoxicity and lineage - specific differentiation *in vitro*.

MATERIALS AND METHODS

Materials: Propolis samples were collected from managed hives of three different species of stingless bees, viz., *Lisotrigona* sp. (Kollam District) and *Tetragonula* spp. (Thiruvananthapuram District), Kerala. Raw propolis samples were scraped out

from the hives and stored inside refrigerator for further investigations. The samples were named P1, P2 and P3 for propolis collected from *Lisotrigona sp., T. calophyllae* and *T. travancorica* hives respectively for convenience of the study.

Preparation of propolis extract solution: The stock solution was prepared by macerating 3g propolis at room temperature and dissolving it in 10 ml of 95 per cent ethanol. The solution was then incubated at 70°C for 30 min followed by centrifugation at 8800 rpm at 5°C for 10 min. The supernatant was then maintained at 4°C to avoid degradation.

Human Pluripotent Stem Cells (hPSCs): The Human Embryonic Stem Cell (hESCs) (BJNhem19) line was procured from Dr. Maneesha Inamdar, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), India. The human induced Pluripotent Stem Cell (hiPSCs) (D14C2) line, was a kind gift from Dr. R.V. Shaji, Centre for Stem Cell Research, (CSCR) – InStem, India.

The hiPSCs are maintained on Vitronectin (Gibco, A14700) and Essential 8 (E8) medium (ThermoFisher Scientific, A1517001). When the colonies become mature, they are seeded for the treatments. For the MTT assay, the hPSCs are seeded as single cells using Accutase in a Matrigel (Corning, 356234)-coated 96 well plate, at 300 cells/ well density in E8 medium with ROCK inhibitor (ROCK*i*), Y-27632 (Pepro Tech, 1293823) (10 µM). For propolis treatments, the cells are passaged using 0.5mM EDTA (Thermo, life technologies, 15575- 020) and seeded on Matrigel-coated 6-well plates in E8 medium.

For the MTT assay, StemPro Accutase (Thermo Fisher, A1110501) is used for cell dissociation into single cells and seeded with E8 medium supplemented with ROCK*i* on Matrigel-coated 96-well plates (300 cells/well) after counting the viable cells using Trypan blue. After 24 hours, ROCK*i* was withdrawn and the medium is replaced every day with fresh E8 medium. When the cells grown to small colonies, the media was replaced with N2B27 media supplimented with the three different propolis samples, 3LC (P1), TC (P2) and 5TT (P3) with different concentrations (150, 300, 450, 600 and 900µg ml^{-1}) and incubated at 37°C for 24 hours in a CO_2 incubator at 5 per cent CO_2. Next day, MTT assay is carried out using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega- G4000). The absorbance values are recorded at 570 nm wavelength using a plate reader, followed by the calculation of the IC-50 values for each Propolis sample. A reference wavelength of 630nm is used.

For the propolis treatments, the medium is aspirated and washed with DPBS. Then, the cells are incubated with 0.5 mM EDTA for 3-4 minutes at 37°C. After incubation, aspirate 0.5 mM EDTA and dissociate the cell in fresh E8 medium using 1 ml pipette. Seeded the cells on Matrigel-coated 6-well plates in E8 medium. The cells are incubated at 37^0C and 5 per cent CO_2 in a CO_2 incubator (Thermofischer Scientific). Medium is replaced every day with fresh E8 medium. When they are 50-60 per cent confluent, the cells were exposed to the required concentration of propolis (200 µg ml^{-1}) in N2B27 medium.

Trypan blue dye exclusion assay: Seeding density was determined by cell counting by trypan blue dye exclusion assay, for which, 20 µl of the cell suspension is taken in a microfuge tube, to which 30 µl of PBS and 50 µl of 0.4 per cent trypan blue solution are added (creating a dilution factor of 5). With a cover-slip in place, 10 µl of the trypan blue-cell suspension was transferred to the chamber on the hemocytometer. Viable cells are counted (non-viable cells stain blue, viable cells will remain opaque) in the four corner squares.

Cell viability assay: The cell viability test was carried out using 3- (4, 5- dimethyl thiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay. Five different concentrations of the propolis samples were (150, 300, 450, 600 and 900µg ml^{-1}) were taken for the treatment. Cells treated with 95 per cent ethanol and cells alone in the culture medium for blank correction (after MTT assay) are used as controls. After 24 hour treatments, MTT assay was performed using CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega-G4000). 15µl of the Dye Solution is added to each

well and incubated at 37°C for 2 hours 15 minutes at 37°C and 5 per cent CO_2 for 24 hours in a CO_2 incubator. After incubation, 100µl of the solubilization solution/stop mix is added to each well. After 1 hour, the contents of the wells were mixed to get a uniformly coloured solution and absorbance is recorded at 570nm wavelength using a 96-well plate reader (PerkinElmer® EnSpire Multimode Plate Reader).

Gene expression study: Total RNA is isolated by QIAzol Lysis kit (QIAGEN, 79306) The isolated RNA is quantified using NanoDrop Spectrophotometer (ThermoFisher Scientific) and converted to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, 1708891). Quantitative real-time PCR (qRT-PCR) is performed using the PowerUp™ SYBR™ Green Master Mix (2X) (Applied Biosystems, A25776) with gene-specific primers with Tm 58°C (Table 1) in a thermal cycler (Roche Light Cycler 480). The cDNA of control and treatment were subjected to qRT-PCR. Expression of nine genes (*NANOG, OCT 4, ACTB GSC, SOX17, SOX7, MSGN1, PAX6, NCAM1*) were studied. Data analysis is done using the *dd*Ct method, with the house-keeping genes, *GAPDH* or *ACTB*.

Table 1. List of primers and their sequences (5'- 3') used for qRT-PCR

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')
OCT4	GTGGAGGAAGCTGACAACAA	ATTCTCCAGGTTGCCTCTCA
NANOG	CCTGTGATTTGTGGGCCTG	GACAGTCTCCGTGTGAGGCAT
ACTB	TCAAGATCATTGCTCCTCCTGAG	ACATCTGCTGGAAGGTGGACA
GSC	GAGGAGAAAGTGGAGGTCTGG	CGACGTCTTGTTCCACTTCTC
SOX17	ACGTGTACTACGGCGCGATG	CTGGTGCTGGTGCTGGTGTT
MSGN1	CTGCACACCCTCCGGAATT	CTCTGCCGCGGTTAAGGAG
PAX6	CCAGGGCAATCGGTGGTAGT	ACGGGCACTCCCGCTTATAC
NCAM1	TCATGTGCATTGCGGTCAAC	ACGATGGGCTCCTTGGACTC
SOX7	TGCCCACTTCATGCAACTCC	AGGTACCCTGGGTCTTTGGTCA

Supplemental methods

1. MTT Assay

a) Cell plating

The human induced Pluripotent Stem Cells, D14C2 are seeded into 96 well plates.

ROCK inhibition:

- Aspirate the medium from the culture dish with 60 - 70 per cent confluent cells and wash with 1ml DPBS.
- Add 2ml of fresh E8 medium (ThermoFisher Scientific, A1517001) with 10 µM of ROCK inhibitor (ROCKi) (Y-27632, Peprotech – SM-1293823-B).
- Incubate for 1 hour at 37^0C and 5 per cent CO_2 in a CO_2 incubator (ThermoFischer Scientific).

➢ **Cell dissociation:**

- Aspirate the ROCKi containing medium from the culture dish and wash with 1ml DPBS (without Ca and Mg).
- Add 1ml of StemPro Accutase (Thermo fisher- A1110501) and incubate at 37^0C for 25 minutes.
- After incubation, add 1ml of E8 medium

with ROCKi into the dish and gently pipette up and down until cells are in a single cell suspension.

- Transfer the cell suspension to a 15 mL conical tube with 4 ml of E8 medium with ROCKi and centrifuge at 200 xg for 5 minutes.
- Aspirate the supernatant and re-suspend the cells in fresh E8 medium with ROCKi.
- Take a 20 µL sample of the cell suspension to determine viable cells.
- Plate the appropriate number of cells on Matrigel (Corning, 356234)-coated dish(es) and incubate at 37^0C and 5 per cent CO_2 in a CO_2 incubator.

➢ **Cell Counting:**

- Transfer 20 µl of the cell suspension into a 0.5 ml microfuge tube.
- Add 30 µl of PBS and 50 µl of 0.4 per cent trypan blue solution to the cell suspension (dilution factor of 5) in the centrifuge tube.
- Mix thoroughly and incubate for 5 minutes.
- With a cover-slip placed on the chamber on the hemocytometer, transfer 10 µl of the trypan blue-cell suspension to the chamber (by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action).
- Count the viable cells (non-viable cells stain blue, viable cells will remain opaque) in the four corner squares.
- Calculate the total number of cells per ml

Cells per ml = the average count per square x the dilution factor x 10^4

$= (111/4) \times 5 \times 10^4$

$= 138.75 \times 10^4$

Seeding Density = 300 cells per well of 96 well plate

Cells taken per well = $300 / 138.75 \times 10^4 = 0.2$ µl

Therefore, 0.2 µl of D14C2 cells to each well of 96 well plate.

Propolis Treatments for MTT assay

When the cells are 70-80 per cent confluent**,** the cells are treated with the 3 different propolis samples, 3LC (P1), TC (P2) and 5TT (P3) at different concentrations (150, 300, 450, 600 and 900 µg ml^{-1}) in N2B27 medium. Cells treated with 95 per cent ethanol and cells alone in the culture medium for blank correction (after MTT assay) are used as controls. Incubate the plate at 37°C for 24 hours in a CO_2 incubator at 5 per cent CO_2.

b) MTT Assay

After 24 hour of propolis treatment, MTT assay is carried out using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega-G4000). Add 15µl of the Dye Solution to each well. Incubate the plate at 37°C for 2 hours and 15 minutes at 37°C in a CO_2 incubator with 5 per cent CO_2. After incubation, add 100µl of the Solubilization Solution/Stop Mix to each well. Incubate for 1 hour at room temperature. After one hour, the contents of the wells may be mixed to get a uniformly coloured solution. However, care should be taken to avoid bubble formation. Bubbles on the surface may interfere with the accurate recording of absorbance values. Record the absorbance at 570 nm wavelength using a 96-well plate reader (EnSpire Multimode Plate Reader). The use of a reference wavelength will reduce background contributed by cell debris, fingerprints and other non-specific absorbance. A reference wavelength of 630 nm is used. The absorbance values are recorded and cell viability and the IC-50 value for each Propolis sample are calculated.

2. Propolis treatments

Cells are passaged when the hPSCs reach 80-90 per cent confluence by a chemical method using EDTA. Tilt the plate and aspirate the medium and

wash the cells with 0.5 mM EDTA (Thermo, life technologies, Cat. No. 15575- 020) in DPBS (Thermo, life technologies, Cat. No. 14190136). Aspirate the EDTA add 1 ml 0.5mM EDTA and incubate for 3-4 minutes at 37ºC. EDTA is a chelating agent which functions in cell dissociation by blocking cell-cell adhesion by binding to Calcium and Magnesium ions on cell surfaces. Discard EDTA and gently flush the cells from the plate using E8 medium using a micropipette to dislodge the colonies. Make sure that the colony size is neither too big nor too small. Using a micropipette, transfer this solution to Matrigel (Corning, Cat. No- 356264)-coated 6 well plate and 3 cm dish (control) already containing E8 medium drop by drop. Observe under the microscope to ensure adequate colonies and appropriate colony size. Every day medium is replaced with fresh E8 medium.

When the cells reach 50-60 per cent confluence, the medium is aspirated and the wells are washed once with DPBS to remove any contents of E8 medium. The cells are treated with propolis (P1, P2 and P3) at a concentration 200 µg ml^{-1} in N2B27 medium. The cells grown in N2B27 medium with FGF2 (20 ng ml^{-1}) or without ethanol are used as controls. After 24 hours, images are taken using the inverted microscope (Lawrence and Mayo phase contrast inverted microscopy) and the cells are lysed using a lysis buffer and used for RNA isolation (QIAzol Lysis kit: QIAGEN, 79306) and processed for qRT-PCR. The cell lysates and RNA samples may be stored in -80^{0}C.

Matrigel Coating

1. Thaw Matrigel overnight by submerging the entire bottle in ice in a cold room or at 4°C. Use pre-chilled micropipette tips, serological pipettes, and tubes for diluting and aliquoting Matrigel.

2. The protein concentration of Matrigel varies across lots. Calculate the concentration of Matrigel required to coat and the appropriate volume of basal medium (dilution factor) accordingly. The final coating-concentration to be used is 8.7µg cm^{-2}. Once thawed, avoid freeze-thaw cycles. Aliquot working stocks and store at -20°C.

3. For coating the plates, add appropriate volume of ice-cold, serum-free basal medium (DMEM/F12 or DMEM) using a pre-chilled pipette tip to the fresh or frozen Matrigel aliquot. Gently mix by pipetting up and down, while the tube is on ice. Then transfer the diluted Matrigel to the center of the well (1ml/ well of a 6 well plate) and swirl gently to ensure a uniform coating.

4. For later use, wrap the Matrigel-coated plates tightly with parafilm to prevent drying up and store at 2-8°C for a maximum of one week. Prior to use, allow the coated plates to come to room temperature for about 1 hour.

5. For immediate use, incubate the plates at 37°C for an hour for gelation. Tilt the plate and aspirate-off the Matrigel solution. Add 1.5 ml E8 medium to each well and store at 37°C and 5 per cent CO_2 until cells are seeded into them.

Requirements for maintenance of hPSCs

- 0.5 M EDTA. pH 8.0 (Thermo, life technologies, Cat. No. 15575- 020)
- Essential – (E8) complete medium (Thermo, life technologies, Cat. No. A1517001)
- DPBS (Thermo, life technologies, Cat. No. 14190136)
- 6 – well plate (Eppendorf plate Cat. No 0030720016)
- 96-well plate (Nunc - Cat. No 161093)
- N2B27 media
  - DMEM/F12, (Thermo, life technologies, Cat. No. 11330032) - 46.6 ml
  - N-2 Supplement (100X), (Thermo, life technologies, Cat. No. 17502048)–0.5 ml
  - B-27 ®Supplement (50X), (Thermo, life technologies, Cat. No. 12587010)–1.0 ml
  - Bovine Albumin Fraction V (7.5 per cent soln., (Thermo Scientific, Cat. No. 15260037) – 340 µl

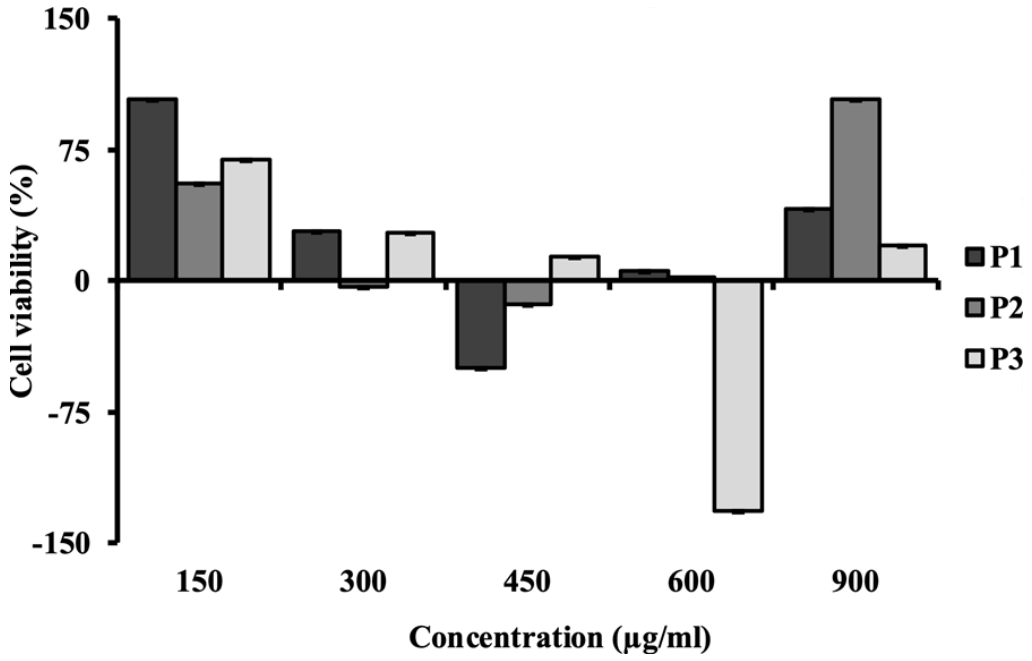

Fig. 1 Comparison of cell viability (MTT assay) of the cells treated with three propolis samples

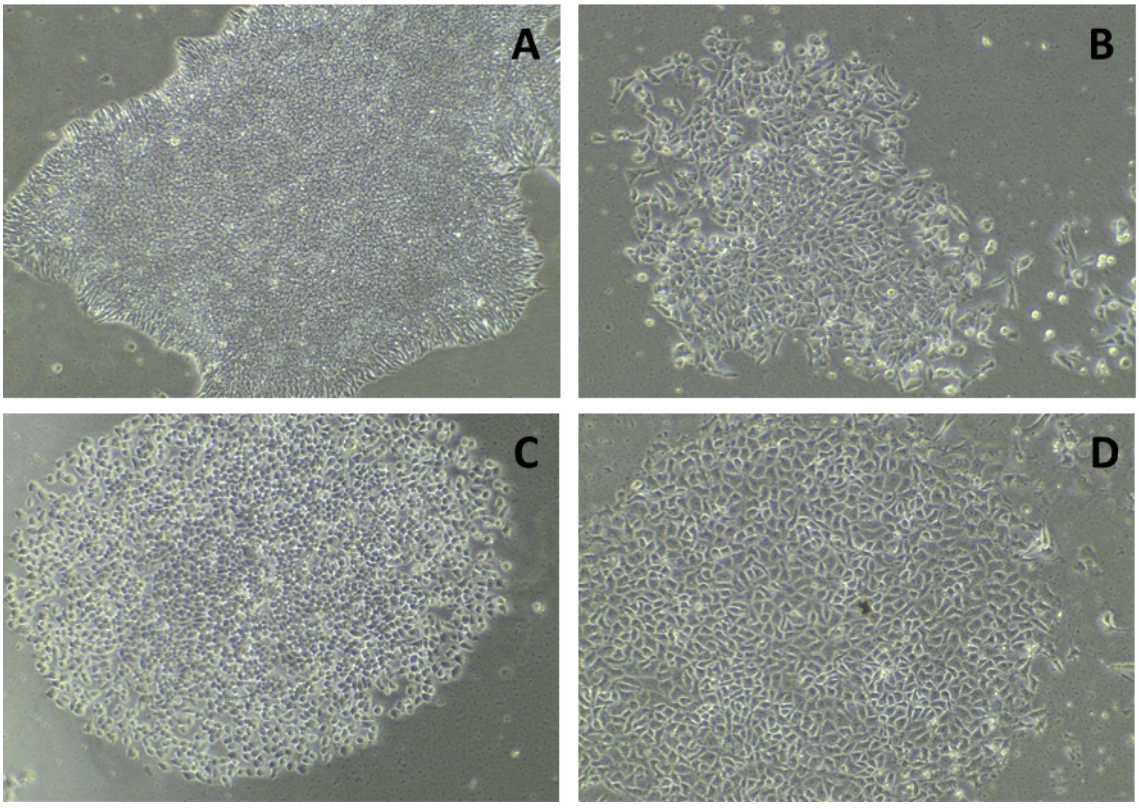

Fig. 2 Morphological changes in hiPSCs after treatment with propolis (A) Day 0 control, (B) P1, (C) P2, (D) P3

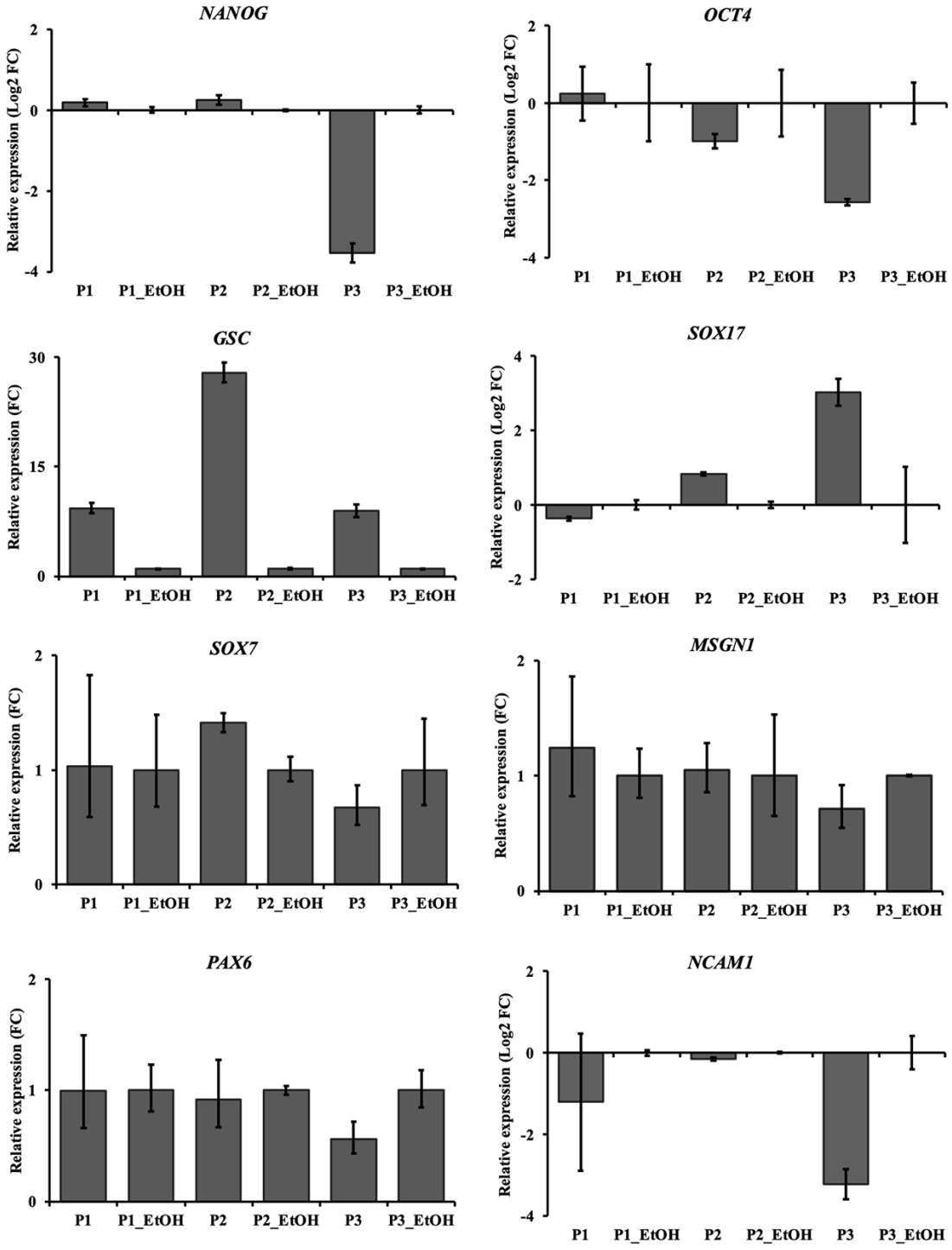

Fig. 3 Gene expression pattern of the mentioned markers after the hPSCs were treated with the propolis samples, P1, P2 and P3. ETOH: Ethanol control

- L-Glutamine (200mM), (Thermo Scientific, Cat. No. 25030149) – 0.5 ml
- Penicillin-Streptomycin, (Thermo Scientific, Cat. No. 15140122) – 0.5 ml
- â-mercaptoethanol, (Thermo, life technologies, Cat. No. 21985023) – 91.0 µl

RESULTS AND DISCUSSION

Propolis promotes cell proliferation and viability:

The influence of propolis on the proliferation and the IC-50 value of propolis was calculated by performing an MTT assay. Five different concentration of propolis - 150, 300, 450, 600 and 900 µg ml^{-1} were respectively added to D14C2 cells for a period of 24 hrs. Cells exposed to complete growth media without any propolis were used as normal control and cells treated with 95 per cent ethanol were taken as control. IC 50 value was determined for the three propolis samples. Cells treated with the propolis extracted from *Lisotrigona* sp. (P1), *T. calophyllae* (P2) and *T. travancorica* (P3) obtained IC50 values of 410.904, 480.097 and 215.157 µg ml^{-1} respectively. The cells displayed a significant proliferation rate after 24 h relative to the control however, higher concentrations were observed to be cytotoxic to cells (Fig. 1). When the cells were treated with propolis, the marked difference in the morphology of cells was observed. Hence it was confirmed that the propolis has an influences on early differentiation of hPSCs.

Morphological changes in hPSCs:

Induced pluripotent stem cells are usually observed as colonies with defined borders and shiny under the microscope. They are seen as a tightly packed cell with high nucleus to cytoplasm ratio, wherein the nucleus practically inhabits the entire cells. Cells when treated with crude propolis showed visible morphological changes compared with ethanol control indicating that propolis has some effect on these cells as they underwent spontaneous differentiation. When cells were treated with propolis, cells lost their border integrity, uniformity and started to migrate from the colonies (Fig. 2).

Early differentiation of pluripotent stem cells:

To study the differentiation potential of human pluripotent stem cells in the presence of propolis *in vitro*, gene expression of pluripotency and early differentiation markers were analysed (Fig. 3). Gene expression analysis revealed that the cells when treated with propolis, lost their pluripotent state. The transcription factors *NANOG* and *OCT4*, required for maintaining pluripotency displayed considerable downregulation in their expressions. Cells treated with propolis collected from the hives of *T. travancorica* showed very low expression of *NANOG* and *OCT4* compared to the other two propolis samples. These pluripotency markers are downregulated upon differentiation indicating that the propolis supported the hPSCs to differentiate. During differentiation, stem cells move into a transition state called primitive streak state or mesendoderm state. Goosicoid (*GSC*), a mesendoderm marker showed high expression in the cells which were treated with propolis. Among the three propolis samples, cells treated with propolis extracted from the hive of *T. calophyllae* displayed high expression of *GSC*. Further, the hPSCs treated with propolis collected from the nest of *T. travancorica* showed more expression of endoderm markers – *SOX17* and *SOX 7*. Propolis did not support the cells to differentiate into mesoderm or neuroectoderm lineage as there was no variation in expression of mesoderm marker (*MSGN1*) and neuroectoderm markers (*PAX6* and *NCAM1*). As per the findings, the propolis sample extracted from the nest of *Tetragonula* spp. supported the cells to differentiate into a mesendoderm lineage

The propolis extracts for the study was collected from live stingless beehives of three different bee species. Many studies have tested the effect of propolis on different cell lines *in vitro*; however, there are no reports on its effect on human induced pluripotent stem cells. Herein, we used *in vitro* experiments to study the cytotoxic effect of propolis and the gene expression of propolis treated cells. Our result revealed that propolis was not cytotoxic at low concentrations, increased the rate of cell proliferation; however, at higher concentrations they hindered cell growth. Cells treated with the propolis

extracted from *Lisotrigona* sp. (P1), *T. calophyllae* (P2) and *T. travancorica* (P3) obtained IC50 values of 410.904 neuroectoderm as there was no variation in the expression of neuroectoderm, 480.097 and 215.157 µg ml^{-1} respectively. These findings agreed with previously published studies, that identified that propolis could enhance the proliferation capacity of BMMSC (Elkheney et al., 2019) and stem cells derived from human exfoliated deciduous teeth (Fung *et al*., 2015).

Propolis, a natural compound is known for its tissue regeneration activities. In the present study, when cells were treated with propolis, the cells lost their pluripotent state and started to differentiate. There was a rapid downregulation in the expression of pluripotency marker *NANOG* and *OCT4*. During embryonic development, the primitive streak initiates the differentiation of pluripotent epiblast cells into germ layers. That is, during differentiation, stem cells move into a transition state called primitive streak state/mesenendoderm state, Hence, transient primitive streak-like mesendodermal state is crucial for the differentiation of stem cells (Takahashi *et al.,* 2014). Goosecoid (GSC) is a mesendoderm marker (Jos *et al*., 1998), cells when treated with propolis showed expression of *GSC*. Cells treated with propolis extracted from the nest of *T. calophyllae,* observed high expression of *GSC* compared to the other two propolis. This indicated that these cells displayed a high tendency to differentiate into meseoendoderm lineage. Endoderm lineage differentiation of cells treated with the propolis was determined by the expression of endoderm markers *SOX17.* However, propolis did not support the cells to differentiate into mesoderm and neuroectoderm as there was no variation in the expression of neuroectoderm markers (*PAX6 and NCAM1*) and mesoderm marker (Mesogenin 1(*MSGN1*)). The propolis extracted from the nest of *Tetragonula* spp. (*T. calophyllae and T. travancorica)* showed more tendency to differentiate into mesoderm and endoderm lineage compared to propolis extracted from *Lisotrigona* sp. Previous studies also reported that propolis enhanced the differentiation of stem cells. Elkhenany in 2019 reported that propolis increased the proliferation rate of bone marrow-derived mesenchymal stem cells (BMMSCs), enhanced the chondrogenic and adipogenic differentiation processes. Intraperitoneal injection of CAPE, a major component of propolis enhanced bone regeneration in the rat calvarial defect model (Ucan *et al.,* 2013) and studies also reported that ethanolic extract of propolis can promote bone regeneration and induce hard tissue bridge formation in pulpotom.

Stem cell therapy has revolutionized modern clinical therapy with the potential of stem cells to differentiate into different cell types which may help to replace different cell lines of an organism (Singh *et al*., 2015). Natural compounds have been used in traditional medicine for the treatment of a wide range of diseases, further investigating their proliferative, differentiation, and cytotoxic effects on stem cells may provide a deeper understanding for curing various diseases. In the present study, propolis, a natural compound, supported the cells to differentiate into a particular lineage. Hence better understanding the chemical composition of propolis, investigating its mechanism and regulatory effects will pave the way as the invaluable candidates in future regenerative medicine research.

ACKNOWLEDGEMENTS

The Authors thank the Kerala Agricultural University. Authors are grateful to Dr. R. V Shaji for his support and the generous gift of the hiPSCs (D14C2). SM.S. acknowledges the financial support from the Indian Council of Medical Research (ICMR) (2019-3008/SCR/ADHOC/BMS), and Council of Scientific and; Industrial Research (CSIR) (09/1108(13739)/2022-EMR-1), India.

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(Received July 31, 2022; revised ms accepted September 31, 2022; published December 31, 2022)