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Proliferative Effect of Malaysian Propolis on Stem Cells from Human Exfoliated Deciduous Teeth: An *In vitro* Study

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ATH and AA designed the study. Authors CSF and HM managed the literature searches and wrote the protocol. Authors SNMH, CSF and HM performed the experiments, analyzed the results and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To investigate the effect of Propolis on viability of stem cells from human exfoliated deciduous teeth via 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay.
Study Design: Cell based experimental study.
Place and Duration of Study: The Craniofacial Science Laboratory located at the School of Dental Sciences, University of Science, Malaysia (USM) Health Campus, between June 2014 and September 2014.
Methodology: Different concentrations of Malaysian Propolis (groups 1, 2 and 3) diluted in culture media were used to treat on culture of stem cells from human exfoliated deciduous teeth. Pure culture media and culture media with Ethanol solvent were used as controls. MTT assay was done after 72 hours and the cell viability indices for Propolis-treated stem cells and respective controls were read via absorbance-rays 4 hours afterwards.
Results: Cell viability indices in Group-1 that has high Propolis concentrations showed significant

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reduction from control. In Group-2, stem cell viability in the lowest concentration of Propolis significantly increased. Cell viability in Group-3 all showed significant increase except for one concentration. One Way ANOVA was used for determination of significant difference in tested concentrations of Propolis. Differences were considered significant at (P = .05). The cell viability indices were significantly higher in culture media with lower concentrations of Propolis in comparison to control.

Conclusion: Propolis at low concentrations maintained and caused a significant increase in stem cell proliferation. Additional studies are necessary to investigate the optimal concentration of Propolis to enhance stem cell proliferation and its differentiation effect.

Keywords: Propolis; stem cell; viability; proliferation; stem cell from human exfoliated deciduous teeth.

1. INTRODUCTION

Propolis is a resinous substance in the beehives. It has been used in folk medicine for centuries. Bees collect it from exudates of plants so Propolis mainly composed of resin, wax, essential oils, pollen and other organic compounds. A lot of external factors such as geographical location, including the season and bee species, type (green, brown and red Propolis) and form (crude or purified) of Propolis itself will affect the efficiency of Propolis in practical usage [1]. Its antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anti-tumoral and tissue generation activities have been widely analyzed in research society.

Although Propolis has been well tested upon normal cells, inflammatory cells and a lot of cancer cell lineage, we scarcely know its effect on stem cells. Stem cells are being widely examined in recent years for their countless potential to be able to cure many different degenerative diseases, especially under so-called regenerative medicine. While different sources and types of stem cells continuously emerge, stem cells from human exfoliated deciduous teeth (SHED) are immature, unspecialized cells in the teeth that are able to develop into specialized ones. SHED have been characterized as multi-potent cells, which hold the potential to arise into a wide variety of tissue types [2]. If researchers can effectively expand stem cells while preserving the self-renewal capacity, the track to their clinical applications would become more efficiently feasible.

Propolis, in previous studies on normal and malignant cell lines, can affect cell survival and proliferation. The aim of this study was to investigate the effect of Propolis on proliferation of stem cells from human exfoliated deciduous teeth (SHED).

2. MATERIALS AND METHODS

This experimental study was carried out between June to September in 2014, at the Craniofacial Science Laboratory of the School of Dental Sciences, University of Science, Malaysia (USM) Health Campus in Malaysia.

2.1 Propolis Preparation

Propolis samples collected from different locations in Malaysia was used in this study. Solid form of Propolis was cut, weighed and crushed before putting into 1.5ml centrifuge-tubes and mixed with 2.5% Ethanol (Merck, Germany) as Propolis solvent. The tubes were inverted twice daily and after 2 weeks, Propolis solution was sterilized using filter sterilization technique. The dissolved Propolis was mixed with culture media to make it into different concentrations (Table 1). The acidity of the Propolis solutions was tested using a pH meter.

Table 1. Experimental propolis concentrations (mg/ml) in culture media

Group 1 (mg/ml)	Group 2 (mg/ml)	Group 3 (mg/ml)
0.5	0.05	0.005
1.25	0.125	0.025
2.5	0.25	0.125
25	2.5	0.25
50	5.0	0.5

2.2 Culture and Seeding of SHED

The frozen commercial SHED purchased from AllCells (USA) was retrieved and cultured in alpha minimal essential media (α-MEM) (Gibco, Invitrogen, USA) supplemented with 15% Fetal Bovine Serum (FBS) (Gibco, Invitrogen, USA) and 1% Penicillin-Streptomycin (Gibco, Invitrogen, USA). Cells were passaged upon 70-80% confluency and de-attached using TrypLE Express (Gibco, Invitrogen, USA). Confluent

SHED of passage 10-15 were used in this study. 5, 000 cells per cell well were seeded in 96-well plates. After cell attachment, the cells were treated using different concentrations of Propolis in three groups.

2.3 Treatment of SHED with Propolis Solutions

100 µL of Propolis solution was added into each well in which SHEDs were seeded. 100 µL of culture media was used for control. To compensate the potential cytotoxic effect of the alcoholic solvent, 0.0625% Ethanol (Merck, Germany) added to culture media was also used as a control. After 96-cell well has been filled with the 100µL of different solutions (12 cell-wells per each concentration of Propolis solutions) (Fig. 1), the plate was incubated for 72 hours in 37ºC, humidity >80% and CO_2 level of 5%.

2.4 Cell Viability Test

The cell viability test was carried out using 3-(4`, 5`-dimethylthiazol-2`-yl)-2, 5-dipheyltertazolium bromide (MTT) assay. After incubation of 72 hours, 10 µL MTT (Life Technologies, USA) was added and incubated for 4 hours. The content of 96-cell well plate was discarded and replaced with 100 µL DMSO. The absorbance was read at 570 nm by using the Sunrise Elisa Reader (Tecan, Japan).

2.5 Data Analysis

The raw data of Propolis-treated cell wells and control cell wells were obtained from the absorbance readings. The average of raw data in each 12-cell wells is divided by the average of the control (readings from stem cells in culture media without Propolis) using Microsoft Office Excel 2007 to get the cell viability index.

All cell viability indices were analyzed with one-way ANOVA (SPSS software version=19) and repeated once for every concentration for consistency. For determination of significant difference in the tested concentrations of Propolis, it was considered significant at (P = .05).

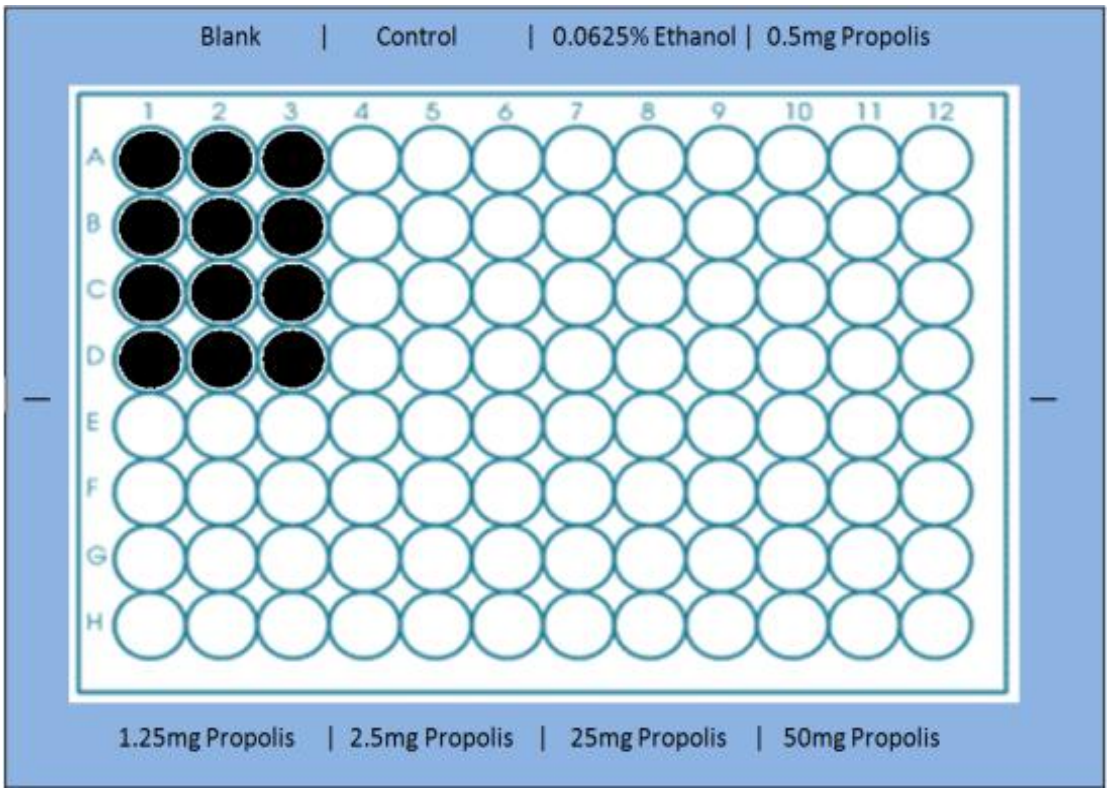

Fig. 1. Schematic presentation of a 96-cell well palate for the test in group 1

3. RESULTS

A one-way (within cell viability indices) ANOVA was conducted to compare the effect of Propolis concentrations on cell viablity in SHED culture.

In Group-1, cell viability indices in Propolis concentrations of 1.25 mg/ml, 2.5 mg/ml, 25 mg/ml and 50 mg/ml showed significant reduction while 0.5 mg/ml showed no significant difference from control (Fig. 2).

Cell viability of Group-2 showed significant reduction for 2.5 mg/ml and 5.0 mg/ml concentrations of Propolis while 0.125 mg/ml and 0.25 mg/ml were not significantly different from the control. However, 0.05 mg/ml showed significant increase in cell viability (Fig. 3).

Cell viability in Group-3 all showed significant increase in cell viability (0.005 mg/ml, 0.125 mg/ml, 0.25 mg/ml and 0.5 mg/ml) except for concentration 0.025 mg/ml (Fig. 4).

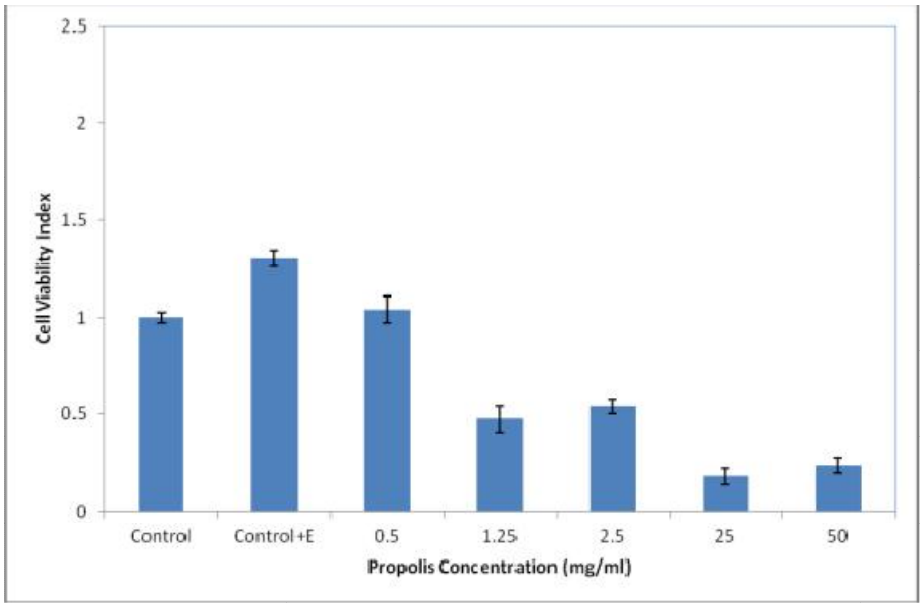

Fig. 2. Cell viability indices in group-1 propolis solutions

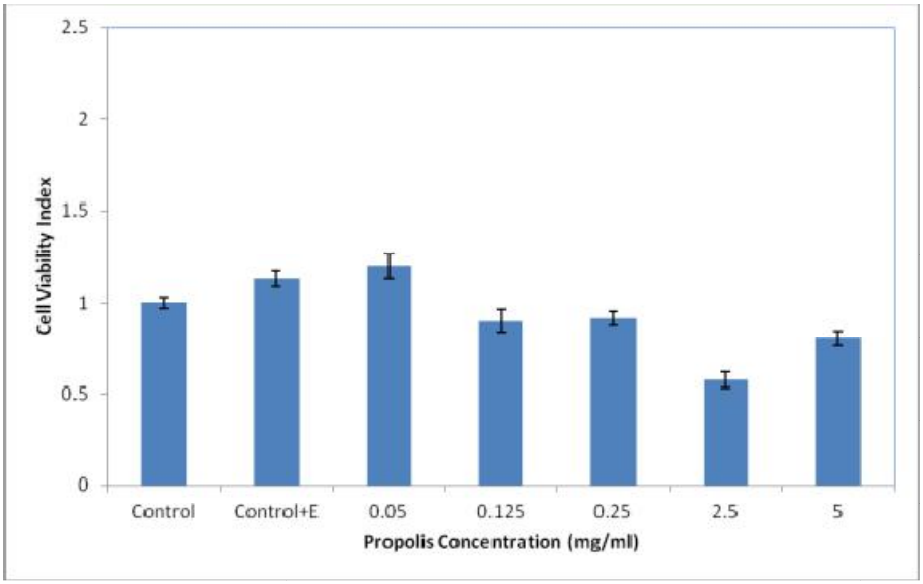

Fig. 3. Cell viability indices in group-2 propolis solutions

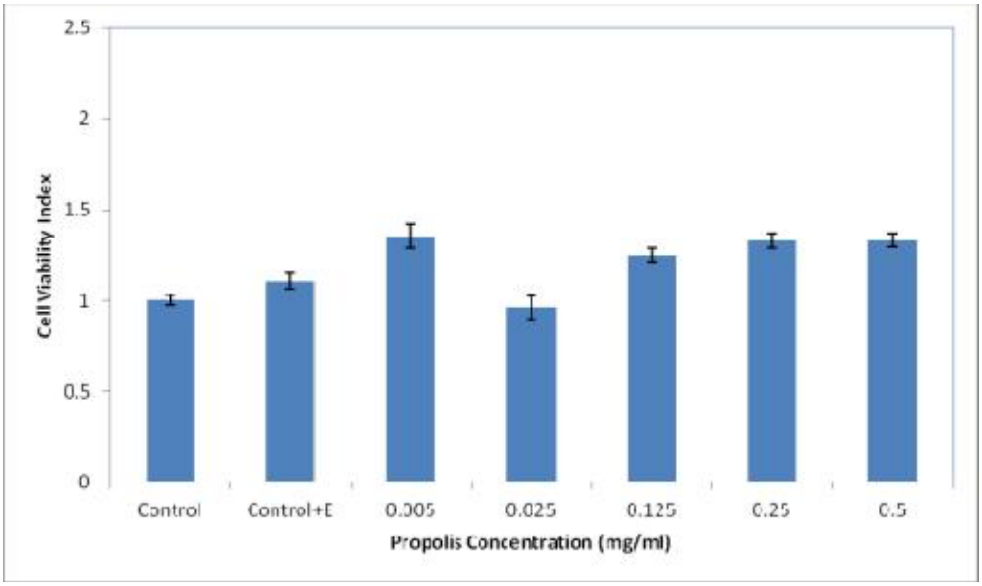

Fig. 4. Cell viability indices in group-3 propolis solutions

Acidity or pH value of tested Propolis solutions shows that, the higher the concentration, the lesser its pH value (Table 2).

Table 2. pH value of propolis in different concentrations

Concentration (mg/ml)	pH value
0.005	8.05
0.5	8.08
2.5	8.13
5	8.06
12.5	7.86
25	7.66
50	7.46

4. DISCUSSION

Propolis is a natural product of plant resins that is used by bees to seal and protect their hives. It is composed of resin (55%), essential oils and wax (30%), pollen (5%) and other constituents (10%) such as amino acids, minerals, in addition to some vitamins and bioflavonoid [3]. A recent study showed most of the chemical compounds in extracts of Malaysian Propolis are different from Turkish and Brazilian red Propolis [4]. Individual constituents of Propolis and their possible synergistic activity will affect towards the experimentation. In other words, differences in the chemical composition of the Propolis samples will lead to a variety of biological activities [5]. The crude Propolis can be advantageous by allowing for any synergistic effects of different compounds, whilst the purified compounds may allow the standardization of bioactive effects among different types of Propolis [1].

In vitro and *in vivo* studies have reported cytotoxicity of Propolis against cancer cells for both crude and some of its purified components. Propolis can significantly reduce cervical tumour cell growth [5] and markedly increase the apoptosis of human laryngeal epidermoid carcinoma cells [6]. It also inhibited human leukemic cell proliferation [7] and human breast carcinoma cell lines [8]. Hence, these studies concluded that Propolis has antitumor effect by increasing apoptosis. Malignant cells were more sensitive to Propolis constituents than normal cells for growth inhibition [9,10]. Propolis has exhibited even selective toxicity against malignant cells compared to normal cells in cultures [10]. Another study pointed out the interestingly weak cytotoxic effect of Propolis extracts on normal human blood cells when compared to that induced on tumor cells [11]. One recent study supported this finding, testing cytotoxicity of Indian Propolis on malignant cells, but the effect spared on normal cells [12].

Many studies on Propolis were conducted focusing on anti-bacterial, anti-inflammatory and anti-tumoral activities. However, it was scarcely done on stem cells. One study [13] has documented the possible stimulation of dental pulp stem cells (DPSCs) differentiation by

Propolis. In their study of dental pulp healing in guinea pigs, Propolis induced to bring better quality dentin that is secreted by odontoblasts. Although these odontoblasts were believed to be differentiated from dental pulp stem cells, no written report has ever revealed yet the direct stimulation effect of crude Propolis on stem cells. Recently, an *in vitro* study tested a bioactive compound of Propolis extract, caffeic acid phenethyl ester, which was found to promote the expansion of hematopoietic stem and progenitor cells [14].

A natural exfoliating deciduous tooth contains stem cells: SHED. SHED represent a population of postnatal stem cells capable of extensive proliferation and multi-potential differentiation. It can be the ideal stem cells to be used in regenerative medicine to treat degenerative diseases [2]. Depending on its cellular environment, stem cells can proliferate without differentiation or it may differentiate into local functional cell types [15]. So far, no experiment has documented the effect of Propolis towards SHED that is regarded to be an important source in the field of regenerative medicine.

We addressed the cytotoxicity of Propolis to SHED culture by lowering the concentration to a level that favoured cell growth. Group-1 in which Propolis concentrations are relatively higher than other groups can be viewed as a pilot study since no reference concentration of Propolis was available on viability of SHEDs. It was found that they are not viable in high concentrations of Propolis. SHEDs were only viable when treated with the lowest concentration in this group but this finding is not significant to the control of the study. This is relatable to a study of Al-Shaher which showed that lower concentrations of Propolis increased viability of human fibroblasts [16]. A similar cell viability study using MTT assay on astroglia cell line in different concentrations of Propolis showed reduced viability of the cells [17]. Propolis was able to induce an apoptotic cell death [18]. Dose-dependent inhibitory effect of Propolis on osteoclast cells has also been studied, but it did not preclude cell growth and survival [19].

To know a desirable concentration that would not be toxic to the SHEDs, Propolis concentrations in our experiment were reduced in Group-2. SHEDs were viable in this group with lower concentrations of Propolis; there is no significant difference to the control. However, in a study by Anjanette W. Gjertsen, Propolis not only increased the metabolic activity and proliferation, but also decreased apoptosis of periodontal ligament fibroblast cells [20]. Ahangari, in their study also on periodontal ligament cells, pointed out that the cells are more viable in Propolis solutions than HBSS, milk, or tap water [21]. Some other studies also documented Propolis as a biocompatible compound with the periodontal ligament cells [22,23].

Hence, the last group (Group-3) of Propolis with fewer concentrations to the Group-2 was tested for the treatment of SHEDs. SHEDs were viable significantly in all concentrations of Propolis in this group except one solution. All results in this group can be referred that low concentrations of Propolis solutions caused increased stem cell viability (up to 18%) and it is significantly different to the control.

From our experiment with different concentrations of Propolis solutions, it was found that the higher the Propolis concentration, the lesser the SHEDs viability. This could be related to the acidity of the Propolis solutions where it was tested using a pH meter and tabulated in results (Table 2). Our data showed that the pH of the culture medium was altered by the amount of Propolis and the medium became alkaline at low concentrations. In addition, a highly concentrated Propolis solution could produce a hyperosmotic culture medium that will induce apoptosis in SHEDs.

The molecular mechanism of Propolis-induced cytotoxicity has not been well documented until now. The underlying mechanism for the increased viability in our study could be linked to the active compounds present in Propolis. One major component of Propolis; flavonoids are believed as the most pharmacologically active constituent and potent antioxidant [24,25,21]. An alternative extraction method using non-alcoholic solvent should be applied in experiments with live cells as alcoholic extracts showed cytotoxicity in previous studies. Further studies need to investigate the optimal concentration of Propolis for the best stimulation of proliferation of SHEDs.

Overall, our data imply that Propolis is bioactive and biocompatible in optimal concentration. It could be utilized for enhancement of stem cell proliferation in culture media. Further studies will need to ascertain whether the SHED undergoes simple proliferation or differentiation into other types of cells. *In vivo* studies on usage of

Propolis should be implemented as Propolis can positively influence healing by enhancing the proliferation of stem cells.

5. CONCLUSION

Effect of Propolis on stem cells viability and proliferation was studied using MTT assay. Our study reinforced the fact that the concentration of Propolis is important to maintain cell viability. Proliferation of stem cells (SHED) was significantly increased in low concentrations of Propolis. The results from this study can be used as a baseline data in finding the optimal concentration of Propolis to enhance stem cell proliferation. Its differentiation effect on stem cells needs further investigations.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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