



BIOLOGICAL EFFECT OF EGYPTIAN PROPOLIS ON BHK CELL LINE AND ON DENTAL PULP TISSUE (AN IN VITRO AND IN VIVO STUDY)

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ABSTRACT

Background: Propolis is a natural material with different biological activities such as anti-inflammatory, antibacterial and antifungal action. **The aim** of the present work was to evaluate biocompatibility of Egyptian Propolis by using two methods of assessment: the invitro test using Baby hamster kidney cell (BHK) cell line and the invivo study on dental pulp of dogs. **Materials & Methods:** For the invitro study: ethanol extract of propolis (EEP) was prepared at a concentration of 1mg/ml. A suspension of the cultured BHK cells was placed in 96 multi-well dishes and divided into two groups : control group containing BHK cells without EEP and propolis group containing BHK cells with EEP at a concentration of 1mg/ml. Cell counting was performed at 24 hours, 72 hours and at the seventh day. Mean values were then calculated for each group. The results were then statistically analyzed using ANOVA test. Scanning Electron Microscopy (SEM) was done for both groups at the seventh day. For the invivo study, 2 young healthy adult dogs, of comparable age, 1-1.5 years old and complete set of permanent dentition were used in this study. The canine and first molar of each quadrant were used in this study and second upper molars of each dog was also prepared, forming a sum of 10 sound teeth. A standardized class V cavity was prepared on the facial surface of the selected teeth. All the cavities were restored with resin modified glass ionomer while in half of them Propolis was used as lining material under resin modified glass ionomer. At the end of experimental period after 6 months, dogs were sacrificed. Microsections with 5 μ m width were prepared and stained with hematoxylin and eosin. **Results:** invitro study results showed a significant increase in number of BHK cells of propolis group at 72 hours and at the seventh day when compared to the control group. Scanning electron microscopic examination of BHK cells showed increase in density of BHK cells of propolis group as compared to cells of the control group. Both groups showed uniform spreading and attachment of cells to root surface. Examination of the decalcified stained sections of teeth of propolis group revealed no inflammation, normal tubular pattern of dentin and arrangement of odontoblasts in multiple layers . Presence of line of demarcation between primary and secondary dentine. **Conclusion :** Egyptian propolis stimulated growth and proliferation of BHK cell in vitro and showed no inflamatory effect on pulp tissue invivo. These results collectively proved the biocomptability and good biological properties of Egyptian propolis and support the application of Egyptian propolis in numerous fields of dentistry.

KEYWORDS: Egyptian propolis, Baby hamster kidney (BHK) cell line, ethanol extract of propolis (EEP), dental pulp.

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INTRODUCTION

Propolis is a beehive product rich in phenolic compounds. More than 300 compounds, such as phenolic compounds, flavonoids and aromatic compounds, terpenes, cinnamic acid, caffeic acid, several esters and essential oils have been detected in propolis. Chemical composition of propolis is mainly dependent on the collection location, time and plant source.^(1,2)

Numerous research have demonstrated that ethanol extract of propolis (EEP) exerts various biological effects, including anti-inflammatory, tumoricidal, immunomodulatory and antidiabetic effects⁽³⁻⁶⁾.

Biological activity of propolis is related to flavonoids compounds⁽⁷⁾. Flavonoids are well-known plant compounds having anti-inflammatory, antioxidant, antibacterial, antifungal and antiviral^(8,9). Biocompatibility of propolis is the outcome of its anti-inflammatory properties.⁽¹⁰⁾ . Because of its biological properties, propolis have been applied in numerous fields of dentistry , particularly in cariology, endodontics, periodontics and oral surgery.^(8, 11,12).

The biological response of the materials used for dental treatment are of particular concern, because damage and irritation could cause degeneration to pulp or periapical tissue and cause delayed wound healing⁽¹³⁾ .

Many studies of pulp capping materials are carried out for selection of materials with the best treatment outcome. The best treatment outcome results depend on the ability of the material to control infection , its ease of handling, prevention of micro leakage and promotion of hard tissue formation⁽¹⁴⁾.

Various cell lines are commonly used for evaluation of the biological response of the materials and is recommended by the ISO 7405 guidelines^(15,16)

Baby hamster kidney cell (BHK) is an established cell line from a clone 13. BHK cells are isolated from a rapidly growing primary culture of newborn Syrian hamster kidney tissue. BHK cell line has been widely used as a viral host in studies of oncogenic transformation, cell physiology, growth of countless viruses and the study of many biological processes BHK cell line is one of the most widely used cell lines for biological processes.⁽¹⁷⁾. BHK cells have fibroblastic morphology and express muscle intermediate filament protein desmin.^(17, 18)

Previous studies on the biological effect of propolis had revealed that Propolis is not toxic to pulpal fibroblasts and periodontal ligament fibroblasts⁽¹⁹⁾. Other researchers found that propolis inhibit osteoclastogenesis and osteoclast activation in tissue culture⁽²⁰⁾

Martin and Pileggi found that Propolis can maintain periodontal ligament cell viability after avulsion and storage .The results of their research showed that 75% of cells of the periodontal ligament were kept viable after 20 hour maintenance in propolis solution⁽²¹⁾

Propolis occludes dentinal tubules and control dentin hypersensitivity⁽²²⁾. Propolis showed regeneration of pulp when it is directly applied to injured dental pulp.^(23,24).

Formation of reparative dentin when propolis is applied as pulp capping material was reported by many researches.^(25,26).

Little data is available about the biological effect of Egyptian propolis and its effect on dental pulp tissue. The aim of present study was to evaluate invitro biocompatibility of Egyptian propolis on of baby hamster kidney (BHK) cell line and investigate its biological effect as a lining material on dental pulp tissue of dogs.

MATERIALS AND METHODS

1-Invitro experimental procedure

BHK Culturing procedure

BHK cell line (Invitrogen, Carlsbad, California, USA) was used in this study. Cells were washed with phosphate buffer saline (PBS). The monolayer of the cells was covered with 1–2 ml 0.05% trypsin and 0.02% EDTA mixture for 2 min at 37°C. BHK-21 cells initially growing in Eagle's modified minimum essential medium (EMEM) containing 10% newborn bovine serum 2–3% as maintenance media and antibiotics (10 000 µg penicillin-G + 10 000 µg streptomycin and 25 µg amphotericin B). Sodium bicarbonate solution was prepared as 75% solution in de-ionized distilled water and used for adjustment of the required pH of cell culture solution. Cells were collected at the walls of sterile falcon tubes, centrifuged for 5 min and then washed twice with fresh complete medium.

The cell suspension was transferred into two sterile cell flasks labeled with cell type and date, then 10 ml fresh, growth medium was then added to the culture flasks to dilute the cell concentration to reach 10^4 viable cells/ml. The cells were incubated at an atmosphere of 5% CO₂ and 95% air at 37°C. The cells were observed daily by inverted phase-contrast microscope to check cell growth and morphological changes. The culture media was periodically changed every 24 hours. Cells were incubated until confluent monolayers of cells were obtained for the study.

Preparation of ethanol extract of propolis (EEP)

Addition of 10 grams of the Propolis Powder to 90 mL of 70% Ethanol. 70% ethanol is the best concentration as some components of the propolis dissolve in the alcohol, while small amounts dissolve in water. Ethanol was evaporated using a rotary vacuum evaporator to obtain the purified propolis extract. The EEP concentrations were measured and diluted to a concentration of 1mg/ml.

(Propolis Powder: flavonoids, phenolic, aromatic compounds, amino acids, minerals and vitamins supplied by Imtenan health shop)

Application of ethanol extract of propolis to BHK cells :

Group I (control group): included 96 multi-well dish containing only the culture media covered by a suspension of the cultured BHK cells.

Group II (propolis group): 96 multi-well dish containing the culture media covered by a suspension of the cultured cells with ethanol extract of propolis (EEP) at a concentration of 1mg/ml.

All the steps were done under laminar flowhood under strict aseptic condition to avoid infection of the cultured cells. Incubation was continued for 7 days in the presence of a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Cell counting:

Cell counting was carried out by first trypsinization the walls of wells to detach the cells, then a haemocytometer was used to count viable cells according to the method of Aleo et al. ⁽²⁷⁾

By putting one drop of the cell suspension on a haemocytometer slide. Cells were then stained by trypan blue vital stain and counted in five separate squares and the average was obtained. Multiplying this average by 10^4 gave the viable cell count in 1ml of suspension.

The process of cell counting was performed at 24 hours, 72 hours and the seventh day. Mean values were then calculated for each group. The results were then statistically analyzed using ANOVA test. Significance level was set at $P \leq 0.05$

Scanning Electron Microscopy (SEM):

Extracted teeth were obtained and their roots were fragmented, washed with Hank's balanced salt solution (HBSS) and soaked in trypsin for an

hour, then washed again with HBSS. The fragments were sterilized by autoclaving under atmospheric pressure; 1 lb/mm³ and temperature 120°C for 20 minutes. root fragments were divided into 2 groups, each group included 10 root fragments:

Group I (control group): included 10 wells of a multi-well dish containing only the culture media covered by a suspension of the cultured BHK cells with root fragments and this acted as control group.

Group II (propolis group): Included 10 wells of a multi-well dish containing the culture media covered by a suspension containing the cultured cells and the root fragments placed with ethanolic extract of propolis (EEP) at concentration of 1mg/ml.

Each cell culture plate was given a number and the date was recorded. Incubation was continued for 7 days in the presence of a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

At the seventh day, the culture medium was removed by suction and the specimens were gently washed with HBSS.

Specimen preparations for electron microscopic scanning were carried out as follows:

Each specimen were washed twice in phosphate buffered saline and fixed in cacodylate buffer containing 3% glutaraldehyde at 4°C for 3 hours. This was followed by post fixation in 1% Osmium tetroxide (OSO₄) in 0.1 M cacodylate buffer for 30 minutes followed by rinsing in distilled water. The specimens were then dehydrated through ascending grads of ethanol (alcohol) starting from 30-50% for 10 minutes, followed by 75-85% for 10 minutes then 90-95% for another 10 minutes and finally 100% concentration for half an hour. Each specimen was coated with gold palladium and thus became ready for scanning. Electron microscopic scanning was carried out utilizing Energy Dispersive X-ray (EDAX), whereby line scan analysis was performed on selected fields of the surface, and then photomi-

crographs were taken for the samples with different magnifications. Scanning electron microscope was performed in the National research center.

In vivo experimental procedure

Materials:

The materials used in the study were:

- a) Propolis, (bee glue): It is supplied in the form of jars of brown powder. Propolis was used in the current study as a lining material. (Propolis Powder: flavonoids, phenolic, aromatic compounds, amino acids, minerals and vitamins supplied by Imtenan health shop).
- b) Resin modified glass ionomer: used in the current study as a restorative material.

Experimental Animals:

2 young healthy adult dogs, of comparable age, 1-1.5 years old and complete set of permanent dentition were used in this study. The canine and first molar of each quadrant were used in this study and second upper molars of each dog was also prepared, forming a sum of 10 sound teeth. Dogs were housed separately and allowed to live in optimal condition under supervision. The experimental procedures on dogs were made in the Animal House Department, Faculty of Medicine, Cairo University and lasted for 6 months.

Premedication and Anesthesia:

The experimental animals were anaesthetized by intramuscular injections with ketamine hydrochloride: 7mg/kg body weight and xlazine hydrochloride: 1mg/kg body weight. The level of anesthesia was maintained by a solution of 2.5% thiopental sodium. The animal was fixed on the operating table. The operating field was disinfected with antiseptic solution. Teeth were cleaned with sterile cotton rolls. The selected teeth were examined for presence of any cracks or defects, cleaned and

isolated with cotton rolls. Operative procedures were done following standard aseptic techniques.

Cavity preparation

A template was used to prepare a standardized class V cavity preparations on the facial surface of the selected teeth. In the prepared cavities, the occlusal margin was placed in enamel and the gingival margin was placed in dentin. All the cavity margins were left unbeveled. A carbide round bur was used to gain access through the enamel, the cavities were completed by using a carbide fissure bur, the bur was changed every five cavities. The dimensions of the cavity were 2mm width and 2mm depth. Following the manufacturer's instructions, the resin modified glass ionomer was placed in the prepared cavities. All cavities were restored with resin modified glass ionomer while in half of them Propolis was used as lining material under resin modified glass ionomer. Finishing and polishing were done after light curing.

Grouping of teeth:

The prepared teeth were divided into two main groups, according to the use of Propolis lining material

Control group : the teeth were restored with resin-modified glass ionomer without using Propolis lining material .

Propolis group: the teeth were restored with resin-modified glass ionomer with Propolis lining material .

The Propolis powder was dissolved in alcohol solvent (70% Ethanol) until being a paste and left for 5 minutes to allow volatilization of alcohol then applied as a lining material inside the cavity by amalgam carrier and adapted to the cavity walls by small amalgam condenser .

Histological Assessment: At the end of experimental period after 6 months, dogs were sacrificed

and then the teeth were separated from the jaw and stored in saline until tested. The specimens were removed from the saline and dried. The teeth of both groups were fixed in 10% neutral formalin, decalcified in 10% EDTA , dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax. Microsections with 5 μ m width were prepared and stained with hematoxylin and eosin. ⁽²⁸⁾

RESULTS

Invitro study results:

A) BHK Cell count results:

At 24 hour: propolis group showed slight non significant increase in BHK cell count. At 72 hours and at the seventh day : there is a significant increase in number of cells of propolis group as compared to number of cells of control group. (figure 1)

B) Scanning electron microscopic results

Scanning electron microscopic examination of BHK cells of the control group showed uniform spreading and attachment of cells to root surface. Some cells showed polyhedral morphology and other cells had spindle shape. Cells had long numerous anastomosing cytoplasmic extensions extending on the root surface .(figure 2 a&b).

Scanning electron microscope of BHK cells of propolis group showed increase in density of cells as compared to control group. Cells showed good spreading and attachment to root surface. Cells exhibited normal polyhydral and spindle morphology with long numerous overlapping lamellopodia (cytoplasmic extensions).Through these lamellopodia anastomosis between cells was achieved. (Figure 2c&d).

Invivo study results:

Examination of the decalcified stained sections of teeth of the control group revealed tubular pattern of dentin .Odontoblasts are lining the pulpal surface of dentin and attached to predentin. (figure 3a).

Examination of the decalcified stained sections of teeth of propolis group revealed no signs of inflammation, normal tubular pattern of dentin with odontoblasts attached to predentine layer. Line of demarcation is present between primary and secondary dentine (figure 3 b).

Odontoblasts are arranged in multiple layers and condensation of cells of the pulp was also evident. (figure 3 c&d).

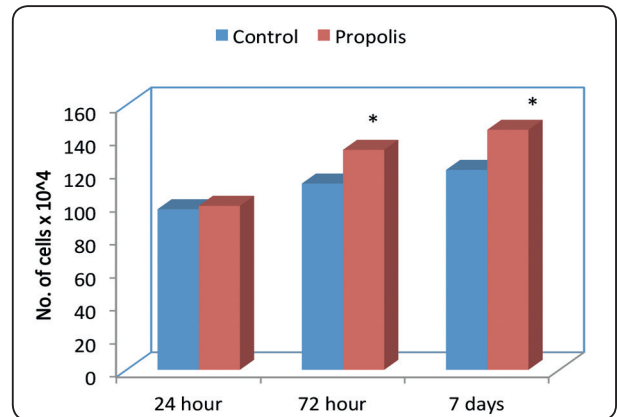


Fig. (1) Bar chart showing non significant increase in BHK cell count of propolis group at 24 hours, a significant increase in BHK cell count of propolis group at 72 hours and at the seventh day. *means significant difference ($P \leq 0.05$)

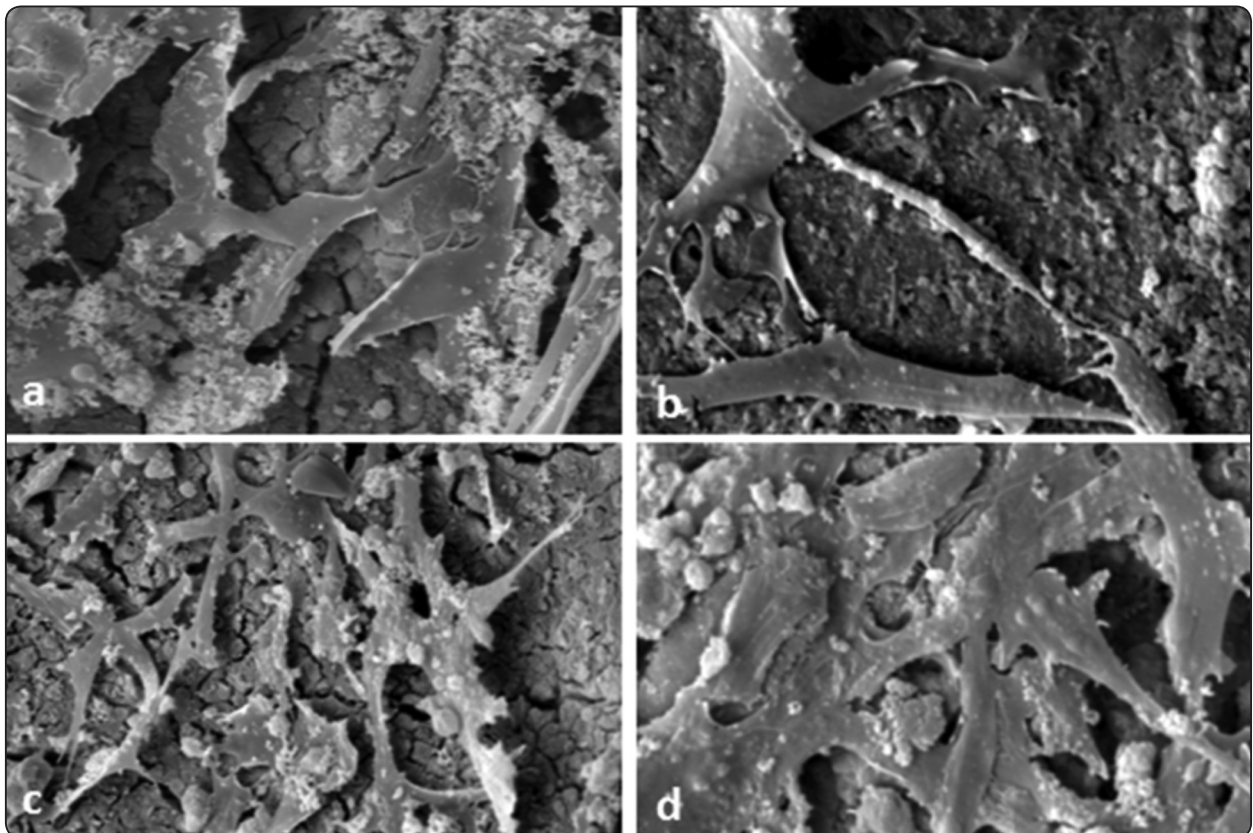


Fig. (2) a: scanning electron micrograph of BHK cells of control group showing cells with polyhedral and spindle morphology, anastomosing cell processes. (orig. mag. 1000). Figure 2b: Higher magnification showing polyhedral, spindle cell morphology with anastomosing cell processes. (orig. mag. 2200). Fig. 2 c: scanning electron micrograph of BHK cells of propolis group showing increase density of BHK cells than control group. Cells have polyhedral and spindle cell morphology with anastomosing cell processes. (orig. mag. 1000). Fig. 2d: Higher magnification of the previous figure showing overlapping anastomosing cell processes. (orig. mag. 2000).

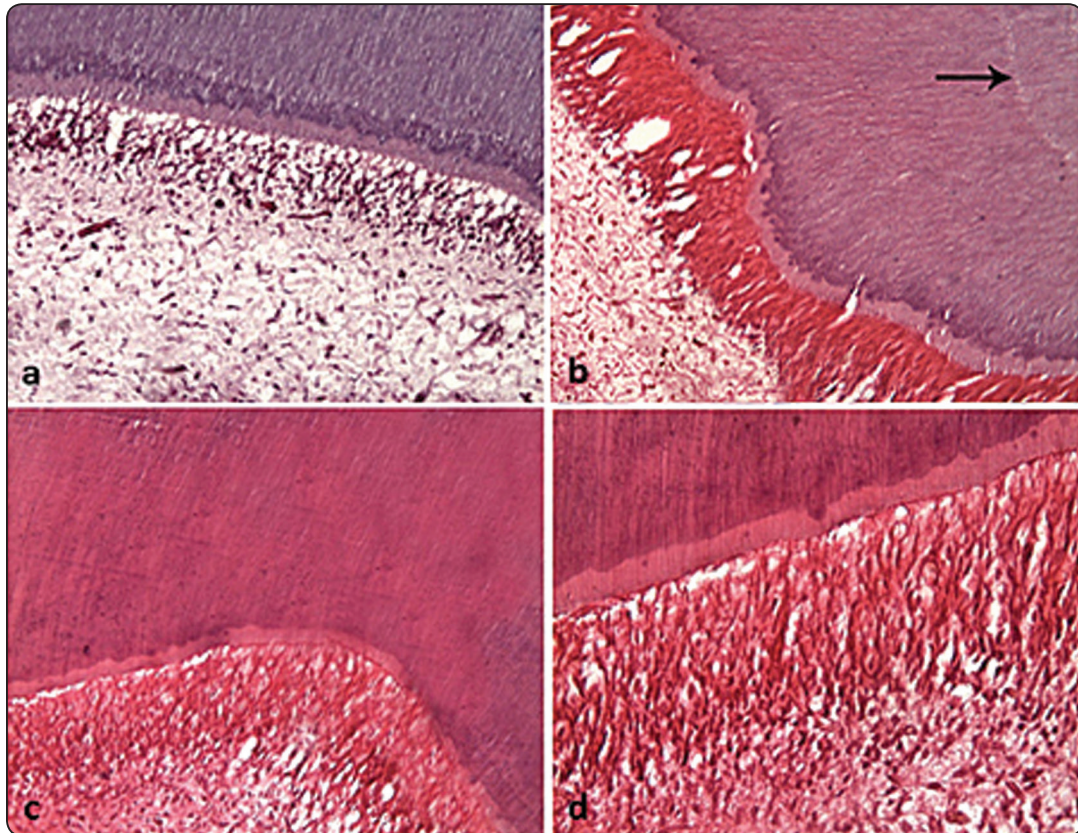


Fig. (3) a: a photomicrograph of control group revealed tubular pattern of dentin. Odontoblasts are lining the pulpal surface of dentin and attached to predentin. (H&E. orig. mag.200). Figure 3b: a photomicrograph of propolis group showing normal tubular pattern of dentid, line of demarcation (black arrow), arrangement of odntoblasts in multiple layers. (H&E. orig. mag.200). figure 3c&d: photomicrographs of propolis group showing arrangement of odontoblasts in multiple layers and condensation of cells of the pulp . (H&E. orig. mag.400).

DISCUSSION

Natural substances in medical treatment, complementary and alternative therapies has been used extensively in the present time. ⁽²⁹⁾

When a new dental material is developed or an existing material is proposed for novel application in human subjects, it must first meet certain standard of biocompatibility. The biocompatibility is assessed using three steps approach. First step is an *in vitro* study. When material is determined to be compatible *in vitro*, local tissue reaction is evaluated through implantation of the biomaterial in subcutaneous tissue or muscle. Finally, the *in*

vivo reaction of the target tissue versus the tested material is done in experimental animals.

The advance of this sequence is lowering the chances for the use of incompatible substances , elimination of unsuitable materials and reduction of the *in vivo* experimermental procedure⁽³⁰⁾.

Numerous investigators have conducted biocompatibility studies *in vitro* using cell cultures and *in vivo* in laboratory animals. The objective of the present work was to evaluate biocompatibility of Egyptian Propolis by using two methods of assessment : the *in vitro* test using BHK cell line and the *in vivo* study on dental pulp of dogs .

In the present study, BHK cells were selected because of their great rate of proliferation and sensitivity⁽³¹⁾.

In the present work, ethanol extract of propolis (EEP) was used to allow evaluation of the effect of the materials on the surrounding as well as distant cells. The extract of the material permits monitoring the effect of chemical components leaching out of the materials and avoids the limitation of the direct contact of the materials with the cells. Elimination of direct contact of the materials with the cells which affect the proliferation of the cells.⁽¹⁵⁾

In the present study, propolis group showed slight non significant increase in BHK cell count at 24 hours, but there was a significant increase in number of BHK cells of propolis group at 72 hours and at the seventh day as compared to control group. The stimulation of proliferation could be linked to the effect of bioactive material dominated by Caffeic acid phenethyl ester (CAPE). CAPE (one of the chemical components of flavonoids) is lipophilic and can easily infiltrate the cell. CAPE inhibits the release of pro-inflammatory cytokines (IL-1, IL-6 and TNF- α) and increases production of anti-inflammatory cytokine (TGF- β , IL-10 and IL-4) promoting cell proliferation.⁽³²⁻³⁴⁾

Our in vitro results are in accordance with other researchers who concluded that propolis extract at a concentration of 1.5 mg / ml is not toxic to BHK cell⁽³⁵⁾.

Our in vivo results showed no inflammatory reaction in pulp tissue of propolis group. The anti-inflammatory properties of Egyptian propolis proved by our results could be attributed to flavonoids and caffeic acid present in Propolis. Flavonoids and caffeic acid play an important role in reducing the inflammatory response by inhibiting the lipoxigenase pathway of arachidonic acid⁽³⁶⁾.

In the present work, the pulp tissue of propolis group showed arrangement of odontoblasts in multiple layers and condensation of cells of the pulp. This can be linked to the ability of Propolis to stimulate the production of transforming

growth factor TGF- β 1 which is important for the differentiation of odontoblasts⁽³⁷⁾. Propolis also induces the synthesis of collagen by dental pulp cells⁽²⁵⁾. Zinc present in propolis promotes cell proliferation and differentiation⁽³⁸⁾. This explains the results of our research

In the present work, line of demarcation was present separating primary from secondary dentin. Our results are in accordance with a histologic study showed secondary dentin development after application of a paste made from an alcoholic solution of propolis and zinc oxide for indirect pulp capping of cavities.⁽³⁹⁾

CONCLUSION

Under the limitations of the present investigation the following conclusion can be drawn:

Propolis stimulated growth and proliferation of BHK cell in vitro and showed no inflammatory effect on pulp tissue in vivo. These results collectively proved the biocompatibility and good biological properties of Egyptian propolis and support the application of Egyptian propolis in numerous fields of dentistry.

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