

# Efficacy and toxicity of Anatolian propolis on healthy nasal epithelial cells

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**Abstract. – OBJECTIVE:** In our study we aimed to evaluate the effects of applying propolis topically to epithelial cells of the nasal cells, to discover whether this causes any toxic effect upon the cells.

**MATERIALS AND METHODS:** Samples of healthy human primary nasal epithelium harvested during septoplasty from volunteers were incubated in cell culture. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays may be utilized when assessing cellular damage (toxicity), as evidenced by DNA fragmentation, nuclear condensation, alteration in the outer plasma membrane and cytoskeletal alteration. This was the method used in the study. Cultured epithelial cells were incubated with propolis (Bee&You) for 24 hours at 37°C. The MTT assay was then performed, and the cell morphology was examined by confocal microscopy. In addition, *via* wound healing assay, cellular proliferation was assessed by the artificial scratch method followed by light microscopy.

**RESULTS:** MTT assay results showed that the primary nasal cells were not affected from the topical application of propolis for 24 hours. All of the applied doses not changed significantly the viability of the cells. The agent was not found to cytotoxic to the primary nasal cells in the application time of 24 hours. Our confocal microscopy findings supported the MTT findings. According to the confocal images control cells that were not treated with test agent were with compact morphology and undamaged fusiform cell shape and nucleus. In test group of nasal cells, Propolis found not to be cytotoxic on the cellular morphology and not changed the cells. When evaluating the results from the wound healing assay, the clear area of scratch obtained at the start of incubation (0<sup>th</sup>) was closed totally with the proliferated primary nasal cells after incubation of 24 hours with propolis. These findings are supported by our MTT find-

ings that imply to the slight induce of proliferation of the primary cells by Propolis.

**CONCLUSIONS:** Topically applied propolis did not have a cytotoxic effect on nasal epithelium cells. Considering its antibacterial and antioxidant effects, it has been concluded that topical application in sinonasal inflammatory diseases (e.g., acute and chronic rhinosinusitis) may have an auxiliary effect in treatment. Moreover, there is a slight induce of proliferation of the primary cells by propolis which may help wound healing in septal surgeries and epistaxis.

*Key Words:*

Propolis, Cultured human nasal epithelial cells, Confocal microscopy, Viability, Toxicity.

## Introduction

Propolis is a resin that comes from bees, including the species *Apis mellifera*. It has a complex chemical composition, which is affected by the plants, the bees visit, the climate, atmospheric conditions, and season<sup>1-3</sup>. In dry areas of the world, e.g., the Sonoran Desert in Mexico, the propolis produced is rich in many different molecules of flavonoid and polyphenolic type. This type of propolis may be termed poplar-type. It possesses significant bioactivity, exerting a modulatory effect on the immune response<sup>4</sup>, inhibiting proliferation<sup>5,6</sup>, demonstrating microbicidal abilities<sup>7</sup> and acting as an antioxidant<sup>8</sup>. It has been proposed that poplar-type propolis may be incorporated into silver nanoparticles designed to act as antimicrobial agents. In this role, the propolis is a reducing agent and adds stability to the preparation<sup>9</sup>.

The different varieties of propolis are frequently classified on the basis of color (red, green or brown), the type of bee that made it, the plants visited by the bees (such as poplars, pines or conifers) and the region where the propolis originated. The different types have specific characteristics, such as physical properties, chemical constituents and bioactivity<sup>10</sup>.

Silver nanoparticles containing propolis produced in winter has been shown to be amongst the most potent reducing agents. This effect appears not to result from the principal active ingredients contained in propolis, namely pinocembrin or pinobanksin-3 $\alpha$ -acetate, but from compounds present at lower levels but with higher reductive capacity, such as galangin<sup>8</sup>.

The typical composition of propolis is between 50% and 60% resins or balsams, 30-40% waxy substances, 5-10% essential oils and 5% pollen grains. It also contains certain micronutrient substances, including a low level of the vitamins B1, B2, B6, C and E<sup>11</sup>. The constituents of the resin and the color it takes on are dependent on the type of bee and the plants that the bees feed on<sup>12,13</sup>.

A review article by Silva-Carvalho et al<sup>14</sup> covered the varying chemical composition and bioactivity of different types of propolis. The fact that the constituents of different samples of propolis varies so widely has been extensively discussed, but it remains a hurdle to introducing propolis as a medicinal product without any further processing step. However, even further variety in composition arises from the different materials and methods employed for extracting the active ingredients from the unprocessed propolis.

There is a very high degree of floral diversity in Anatolia thanks to its geographical position, and the range of different habitats, with varying humidity and altitude. The propolis originating from this location enjoys the richest range of phenolic constituents. Most propolis from Anatolia is from bees feeding on trees of the poplar family and has a brown hue.

This type of propolis is prepared for sale by a process that is patented by the Bee and You company (North Bergen, NJ, USA). The resulting product possesses a unique and standardized composition, which contains abundant (over 15 distinct types of) phenols as well as flavonoid compounds. The constituents include caffeic acid phenethyl ester, caffeic acid, quercetin, galantine, chlorogenic acid, linolenic acid, palmitic acid, hydroxytyrosol, coumaric acid, apigenin, chrysin, and pinocembrin.

This study has the objective of assessing potential cytotoxicity to cultured nasal epithelial cells when propolis extract is applied topically.

## Materials and Methods

The study was undertaken jointly by Eskisehir Osmangazi University Department of Ear, Nose and Throat Surgery, and Eskisehir Technical University Faculty of Science, Division of Biology. Nasal epithelial cells for cell culture were harvested from surplus tissue remaining after septorhinoplasty on individuals who had already provided written consent for such scientific use to be made of their tissue. The harvested strips of mucosa were transferred to the Eskisehir Technical University Cell Culture Laboratory in a preservative medium suitable for cell culture.

### Primary Cell Culture

Nasal epithelial cells were obtained from tissue fragments left over following septorhinoplasty. Five patients without any evidence of nasal or sinus inflammation provided these cells. The cells were dissected into smaller pieces on sterile Petri dishes shortly after surgical excision. The tissue culture began in complete DMEM-F12 medium, which was newly prepared for these cultures. The medium also contained fetal bovine serum 10% and penicillin-streptomycin 1%. The temperature of the culture remained at a steady 37°C, with a humid surrounding atmosphere with 5% carbon dioxide. Seven days after the beginning of the cell culture, any excess tissue was removed. The cells adhering to the plate were rinsed with phosphate-buffered saline (PBS) and treated with trypsin prior to transfer to cell culture plates of T25 type. At the point where 85% of the plate was covered by a confluent epithelial surface, the experiment was able to proceed to the following phase<sup>15</sup>.

### MTT Assay for Assessment of Cytotoxicity Resulting from Anatolian Propolis Extract

The nasal epithelial cells were separated by treating them with trypsin, and then transferred to a plate containing 96 wells. The wells each held 5 x 10<sup>3</sup> epithelial cells. The concentration of test reagents was varied in different wells, with a range of 5-50  $\mu$ g/mL. The plates were kept at a steady 37°C in moist air containing 5% carbon dioxide.

The extract of Anatolian propolis was obtained from Bee & You. It was diluted to a concentration of 5 mg/mL using dimethylsulfoxide (DMSO). The concentration of propolis extract was varied between different wells, with a range from 5 up to 50 µg/mL<sup>10</sup>. The period of exposure was timed to last 24 hours.

When this 24-hour exposure was complete, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate-buffered saline (PBS, Invitrogen) at a concentration of 5 mg/mL was added to the wells. The incubation then proceeded for another 4 hours, but no other changes were made to the culture conditions. Finally, the medium was discarded and 200 µL DMSO was added to each well. The 3 plates were then evaluated with an ELISA plate reader measuring at 570 nm wavelength. The percentage viability was calculated on the basis of the absorbance obtained<sup>15</sup>.

#### Confocal Microscopic Assessment

Nasal epithelial cells in a culture maintained at a temperature of 37°C underwent a 24-hour exposure period to an extract of Anatolian propolis at the highest dose.  $3 \times 10^5$  epithelial cells were seeded on coverslips that were placed in 6-well plates before incubation with propolis. Epithelial cells not exposed to propolis were used as controls. After the exposure the medium was washed away, and cells were rinsed in PBS. Then, the cells were placed in glutaraldehyde (2%) at room temperature for 15 minutes. The cells were finally washed once more with PBS then stained with Fluor-488 phalloidin and acridine orange.

A Leica SP5II confocal microscope (Wetzlar, Germany) was utilized for confocal microscopic examination of the epithelial cells, looking for evidence of DNA fragmentation, condensed nuclear morphology, altered cytoskeletal elements or damage to the plasma membrane. These are the features which would point to a cytotoxic effect of propolis.

#### Wound Healing Assay

Nasal epithelial cells were distributed among 6 wells of a culture plate, with each well containing  $3 \times 10^5$  cells. The incubation continued for 24 hours. The plates then contained confluent cells forming a single layer. A scratch was made into this layer in a vertical direction, using the sterile tip of a 20-200 µL pipette. Followingly, the wells were rinsed with PBS. The medium was replaced with 3 mL of either cell culture medium only for control cells

or medium with diluted propolis extract for test cells. The initial appearance following scratching was assessed by light microscopy. The appearances were also examined after 24 hours post-injury, using the same microscope. The control epithelial cells were not treated with propolis extract but were examined at the same intervals<sup>16</sup>.

#### Statistical Analysis

ANOVA (one-way analysis of variance) was utilized for the statistical analysis of multiple comparisons. The software used for this purpose was GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA), running on Windows. A  $p < 0.05$  was considered statistically significant.

## Results

#### Results of the MTT Assay

The MTT assay revealed no alterations in the epithelial cells exposed to propolis, compared to control epithelial cells. The viability of the cells was not affected by propolis. Propolis exposure lasting 24 hours was not associated with any indications of cytotoxicity (Figure 1).

#### Confocal Microscopic Examination Results

Confocal microscopic examination revealed findings in accord with the results of the MTT assay. The control epithelial cells (i.e., unexposed to propolis) had a compact morphological appear-

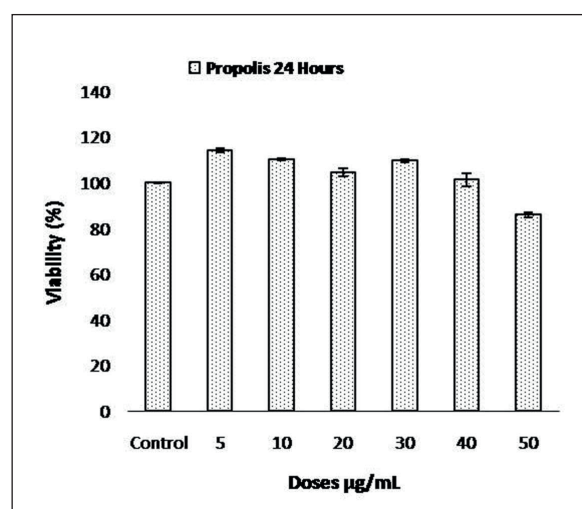
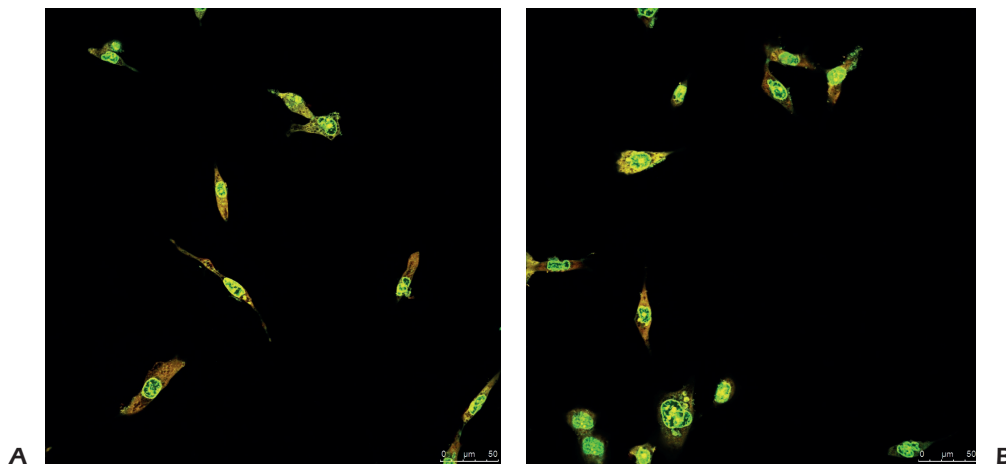


Figure 1. Cell viability results of primary nasal cells exposed to different propolis concentrations for 24 hours obtained by MTT.



**Figure 2.** Confocal microscopy images of primary cultured nasal cells. **A**, Control group and **(B)** Test cells exposed to Propolis for 24 hours.

ance, with a clearly delineated nuclear border and fusiform appearance (Figure 2A). These appearances were the same as the epithelial cells exposed to propolis (Figure 2B). There were no signs of cytotoxicity in the cell cultures examined.

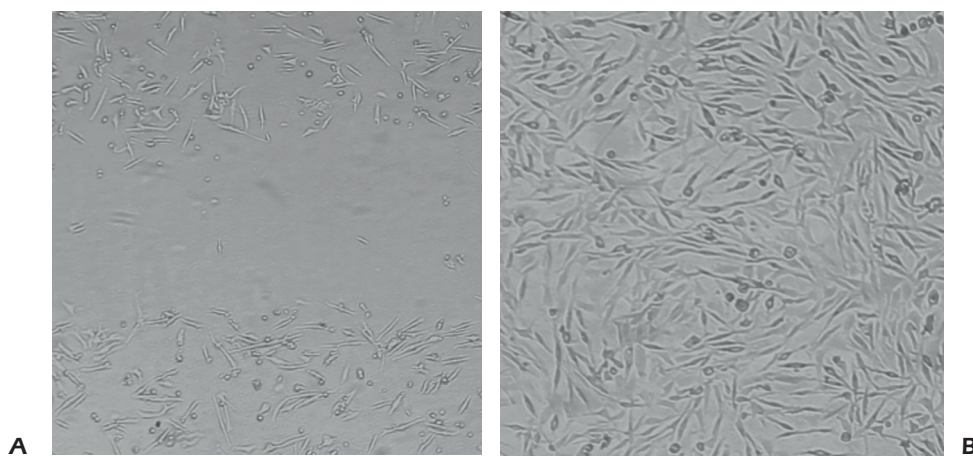
#### **Results from the Wound Healing Assay**

Light microscopy was used to compare the extent of the area denuded of cells by scratching at the onset and 24 hours later. Results of healing assay of control cells at initial stage (0 hours) (Figure 3A) and after 24 hours (Figure 3B) indicated that the wound area is closed after 24 hours. Cells exposed to propolis were compared with control cells (without propolis exposure). Compared to control cells and initial stage of cells treated with propolis (Figure 4A) the cells exposed to propolis extract had completely re-epithelized at 24 hours

after injury (Figure 4B), i.e., the denuded area had been completely re-covered. These findings, in conjunction with the results of the MTT assay, imply that propolis may mildly increase the proliferative rate of primary nasal epithelial cells.

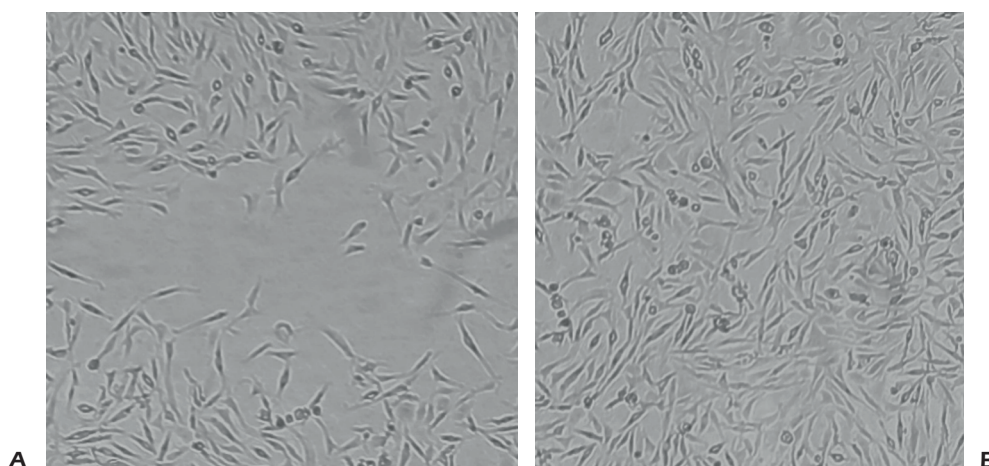
#### **Discussion**

Propolis has been demonstrated to offer benefit through microbicidal activity, inhibition of inflammation, prevention of neoplasia and by acting as an antioxidant. The constituents of propolis differ according to the season when it is produced, the predominant climate and the plants in the geographical area where the bees are living<sup>10</sup>. Around 10% of propolis consists of essential oils and other aliphatic acids, with lesser amounts of pollen, or-



**Figure 3.** Wound healing images of untreated primary nasal cells. **A**, Initial stage (0<sup>th</sup> hour) and **(B)** final stage (24<sup>th</sup> hour). Magnification: 40X.





**Figure 4.** Wound healing images of primary nasal cells exposed to propolis. **A**, Initial stage (0<sup>th</sup> hour) and **(B)** final stage (24<sup>th</sup> hour). Magnification: 40X.

ganic acids, amino acids, some vitamins and minerals<sup>17,18</sup>. A significant percentage of the 300 or so constituents normally found in propolis are flavonoids, flavones, flavanols or phenolic acids<sup>10</sup>.

The objective of the present study was to ascertain how topically applied propolis affects nasal epithelial cells, especially any cytotoxic effects. The MTT assay data indicate that propolis does not cause cytotoxicity within 24 hours of direct application, nor is cellular viability adversely affected. The confocal microscopic examination of propolis-treated epithelial cells produced data which confirmed the impression of no cytotoxicity found from the MTT assay. The unexposed (control) epithelial cells exhibited the same morphology as the cells exposed to propolis, namely a fusiform cellular shape, distinct nuclear outline, and compact overall appearance. Therefore, propolis does not appear to cause any toxic effect on cultured nasal epithelial cells.

For the wound healing assay, light microscopic examination was used to compare the re-epithelialization of the area denuded by scratching in the epithelial cells exposed to propolis with the unexposed controls. The comparison was performed over a 24-hour period after the injury. The researchers observed that the bare area had been completely re-covered by the end of the 24-hour period in the cell cultures where propolis was present. The MTT assay results can also be interpreted as showing that propolis actually mildly increases cellular proliferation. This finding contradicts the results of studies published earlier<sup>5,6,19-21</sup>, which suggested that propolis inhibits the cells' ability to proliferate.

It has already been reported in the literature<sup>22-25</sup> that propolis is an antioxidant and is able to scavenge harmful reactive oxygen species (ROS). It also inhibits inflammation<sup>19,26</sup> and possesses efficacy of antimicrobial agents on parasitic<sup>27</sup>, bacterial<sup>28-31</sup>, fungal<sup>30,32</sup> and viral<sup>33</sup> pathogens. It is neuroprotective<sup>34</sup> and assists in trauma repair<sup>35</sup>. In studies<sup>19-21</sup> of its anti-neoplastic effects, it has been demonstrated to possess selective cytotoxicity, inhibiting proliferation whilst also promoting apoptosis in neoplastic cells. It also reduces tumor metastasis, mutagenesis, invasion and neoangiogenesis. Furthermore, studies<sup>36,37</sup> indicate that propolis is suitable for being packaged in pectin, a polysaccharide substance that is a normal component of plant cell walls. This combination permits a non-toxic drug formulation to be produced. There has already been some development of patch formulations aiming to promote wound healing. These patches were formulated with pectin and propolis<sup>38</sup> or chitosan-pectin plus propolis<sup>39</sup>. In addition to a variety of terpenes, sterols, aldehydes, and tannins, propolis also consists of a number of other phytochemical compounds, such as caffeic acid, caffeic acid phenethyl ester (CAPE), artemillin C, quercetin, myricetin, pinocembrin, pinobanksin, apigenin, luteolin, naringenin, kaempferol, and chrysin<sup>14,21,40-42</sup>. These are not the only compounds of interest so far identified.

Earlier research has already identified propolis originating in the Sonoran Desert as of popular-type. This type of propolis can influence various biological processes, including possessing activity against pathogenic microbes<sup>7,43</sup>. The precise bioactivity possessed by samples of propo-

lis depends on the season it was gathered, which is reflected in differing chemical composition. These differences have been linked to antioxidant activity and effects on cellular proliferation<sup>6,8</sup>.

Botteon et al<sup>44</sup> report on testing Brazilian red propolis formulated as gold nanoparticles against *Escherichia coli*. The hexane fraction of the propolis extract possessed a minimum bactericidal concentration of 50 µg/mL compared to other fractions, in which the MBC was above 198 µg/mL. These fractions included the unrefined extract and the fraction dissolved in ethyl ethanoate and dichloromethane<sup>44</sup>.

According to Lopez et al<sup>45</sup>, red propolis should not be applied at a concentration exceeding 50 µg/mL, as cytotoxicity may occur at levels above this. From studying L929 cultures, these researchers identified evidence of cytotoxic effect at high concentration. It has been shown, using data obtained by Machado et al<sup>24</sup> and Campoccia et al<sup>10</sup> that the IC<sub>50</sub> when applied to L929 cells may be no higher than 30 µg/mL, although this varies according to the type of propolis tested. These results from several studies, reinforce the necessity of testing propolis extracts for cytotoxicity using normal cell cultures, since the precise composition varies from batch to batch, and thus affects the potential for cytotoxicity to occur<sup>10</sup>.

Certain of the constituent compounds in propolis have been demonstrated to trigger apoptosis in neoplastic cells, whilst others are protective against cellular damage and DNA mutations. There are also compounds which act as antioxidants, inhibit bacterial growth, are cytoprotective or dampen inflammation. It is common for propolis extracts to be manufactured from several different varieties of propolis combined, which results in inconsistency in the chemical composition. Despite these limitations, the anti-neoplastic and microbicidal capabilities of certain constituents of propolis make this an area of considerable interest as a potential source of specialized therapeutic agents<sup>10</sup>.

Propolis is a naturally occurring complex chemical mixture produced by a variety of different species of bees, including those that lack a sting. This mixture has demonstrable antioxidant, anti-proliferative and anti-neoplastic actions<sup>12</sup>.

Various phenol-containing substances that are found in propolis are responsible for the beneficial effects seen in neoplasia, namely inhibition of proliferation and death of malignant cells in a variety of different tumors in cell culture, such as renal cell carcinoma<sup>46</sup>, as well as malignancies

of colonic<sup>47</sup>, pancreatic<sup>48</sup>, dermatological<sup>49</sup> or pulmonary<sup>50</sup> origin. Amyrins are naturally occurring phytochemicals with known anti-neoplastic bioactivity. Amyrins can cause apoptosis of human bladder malignancies (NTUB1)<sup>51,52</sup>, in addition to leukemia (HL-60)<sup>12,53</sup>.

Velikova et al<sup>54</sup> examined propolis produced by the bee species *Melipona quadrifasciataanthidoides*, demonstrating its microbicidal activity, whilst Sawaya et al<sup>55</sup> found that propolis produced by *Scaptotrigonadepilis* functioned as an antioxidant.

The constituents of phenolic and flavonoid type appear to be responsible for the antioxidant and anti-neoplastic benefits of propolis<sup>13,47,55-57</sup>. However, the ability of propolis extract to block the action of oncogenic kinase PAK1 is due to other constituents, such as caffeic acid, apigenin and the triterpenes. This enzyme is known to be of significance in the pathogenesis of infection, Alzheimer-type dementia, inflammatory disorders, diabetes mellitus, hypertension and obesity, as well as malignancy<sup>12,58,59</sup>.

## Conclusions

The present study reveals that direct application of a propolis extract does not cause cytotoxicity on nasal epithelial cells in cell culture. The topical use of propolis in disorders involving inflammation of the sinuses and nasal interior has been proposed as a beneficial therapeutic option, given the known propensity of propolis to act as a microbicidal and antioxidant agent. Our study appears to support this approach. Furthermore, our findings contradict previous research, which had concluded that propolis is an inhibitor of cellular proliferation, since we found a mild increase in the proliferative abilities of nasal epithelial cells exposed to the extract<sup>5,6,19-21</sup>. This effect is potentially beneficial following septoplasty or nose bleeds. Rhinapi (Bee & You Nasal Spray-Anatolian Propolis Extract) spray contains 0.3% propolis in hypertonic saline solution 1.7%. It also contains glycerine 0.3% and eucalyptus oil 0.1% as excipients. Despite the fact that the epithelial cells in cell culture were exposed to a high dose of Anatolian propolis, there was no evidence for any cytotoxic effect. It may reasonably be concluded, therefore, that propolis can be safely applied to the nasal interior, for use in either acute or chronic conditions and at the doses suggested by the manufacturer.

### Conflict of Interest

Aslı Elif Tanugur Samanci is the Scientific Director of Bee&You, the company that developed the finished product tested in this study. All other authors declare no conflict of interests.

### Ethics Approval

This is a cell-culture study. Ethics committee approval was not needed.

### Informed Consent

Human primary nasal epithelium was obtained from healthy tissue removed routinely as part of surgery (septorhinoplasty) from individuals who gave written consent for their tissue to be used in scientific research.

### Funding

This study was sponsored by Bee&You. However, Bee&You did not solicit this research project or protocols with the investigators or the institution. Bee&You was not responsible for the management of the study, data processing and reporting.

### Authors' Contributions

Aslı Elif Tanugur Samanci: Planning, designing, literature survey, active intellectual support.

Nuray Bayar Muluk: Planning, designing, literature survey, interpretation of the results, active intellectual support, writing, submission.

Canan Vejselova Sezer: Planning, designing, data collection, literature survey, interpretation of the results, active intellectual support.

Hatice Mehtap Kutlu: Planning, designing, data collection, performing the study, literature survey, interpretation of the results, active intellectual support, writing.

Vedat Topsakal: Planning, designing, literature survey, interpretation of the results, active intellectual support.

Cemal Cingi: Planning, designing, literature survey, interpretation of the results, active intellectual support, English editing.

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