

Cytotoxic effects of water and ethanolic extracts of Turkish propolis on human laryngeal epidermoid carcinoma cell lines

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ABSTRACT

Propolis is a natural resinous substance collected by bees from various types of trees and plants and has antibacterial, antiviral and antitumoral features depending on its antioxidant properties. Major aim is to investigate cytotoxic effect of Turkish propolis on human laryngeal epidermoid carcinoma (HEp-2) cells. HEp-2 cells/well were loaded on RTCA (real time cell analysis) system and the cell index was followed up during 48 hours. Water extract of Turkish propolis (WEP) of 250-10.000 µg/mL concentrations and ethanolic extracts of Turkish propolis (EEP) of 10-2.400 µg/mL concentrations were treated with HEp-2 cells and followed by RTCA system. The cell indexes and IC₅₀ values were determined. HEp-2 cells were incubated with WEP and EEP. Proliferation was followed by flow cytometric DNA cycle analysis. WEP and EEP were found to be cytotoxic to HEp-2 cells. When WEP and EEP were incubated with HEp-2 cells during 72 hours, the highest antiproliferative effect was seen by interfering DNA cycles. Turkish propolis extracts were found to be cytotoxic and antiproliferative to HEp-2 cells in the present study, therefore, it was concluded that it may fall within chemotherapy or target therapies for larynx cancers.

Keywords: Antiproliferative effect, Cytotoxic effect, HEp-2 cells, Larynx cancer, Propolis

Introduction

Cancer is still ranked as the second most common cause of death in the world. Head and neck cancers make up 4% of all cancers and 2% of cancer deaths. It has been reported that approximately 12,000 deaths per year in the USA are due to head and neck cancer. According to US national cancer reporting data, approximately 6.6% of all newly diagnosed cancers develop in the head and neck region. Laryngeal cancers are ranked 6th among cancers that cause the most death in men (da Silva Frozza et al., 2013).

As with all diseases, prevention and early diagnosis are very important in cancer. Over 90% of head and neck cancers are preventable. With the application of radical surgeries in patients with cancer, scientists are seeking new treatment modalities because cancer affects the patient physically and mentally and lowers the living standards. New treatment approaches models in laryngeal cancers give new hopes for patients. We aimed to contribute a new approach to treatment of laryngeal cancer in our study.

Propolis is a natural, highly complex resin-like bee product that has been used in traditional medicine for centuries all over the world and produced by bees from trees and plants (Sforcin, 2007). Propolis protects the hive against intruders and bacteria, fungi and virus attacks (Sforcin & Bankova, 2011). The composition of propolis is quite complex and varies according to the plant origin. It usually comprises 50% resin, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other compounds. The main chemical classes found in propolis are flavonoids, phenolic and various aromatic compounds known as antioxidants. In recent years, the use of propolis in food, beverages and cosmetics has increased to improve health and prevent diseases. Therefore, propolis is also called functional food, designed food, or therapeutic food. Antioxidants in the composition of propolis affect the immune system and thus exhibit anti-inflammatory, antibacterial, antiviral and antitumoral effects (Sforcin & Bankova, 2011).

HEp-2 (Human Laryngeal Epidermoid Carcinoma) cell line is produced from laryngeal carcinoma cells of the human laryngeal mucosa. They are in the slow-growing tumor group. Possible cytotoxic effect of propolis on these cells was previously studied with alcoholic extracts of Brazilian propolis (Microscopic cell viability analysis) (Búfalo et al., 2010; Búfalo et al., 2009). Extracts of natural products prepared with alcohol affect biological systems and tissues negatively.

Our aim is to investigate the cytotoxic effect of water and ethanolic extracts of Turkish propolis on HEp-2 cell line with a

different experimental protocol (RTCA, that is, Real-Time Cell Analysis, then Flow Cytometric DNA Analysis).

Materials and Methods

Chemicals

Penicillin (10000U), streptomycin (10 mg), RPMI 1640, sodium pyruvate solution (100 mL), trypsin EDTA solution (100 mL), and Fetal Bovine Serum (500 mL) were purchased from Biological Industries; NaCl, KCl, Na₂HPO₄, and KH₂PO₄ from Merck.

Preparation of Water Extract of Propolis (WEP) and Ethanolic Extract of Propolis (EEP)

Propolis samples were collected from four different regions of Türkiye from Fanus Food Company (Trabzon) and kept in the freezer (-20 °C) until further use. The locations of samples from Türkiye were Trabzon (North of Türkiye), Erzurum (East of Türkiye), Zonguldak (West of Türkiye), and Adıyaman (South of Türkiye). These four different cities of Türkiye were selected since they represent the four separate geographical locations. WEP and EEP were prepared according to our previous work (Bozkuş et al., 2021). Working solutions in various concentrations were prepared by dilution from the stock propolis extract at a concentration of 100 mg/mL prepared in this way.

Planning the Experiment and Passaging Cells

HEp-2 cells kept in the nitrogen tank were slowly dissolved and centrifuged. The medium was removed. About 2 mL of RPMI 1640 was added to it. Cultivation was done in a 25 cm² flask, completed to 5 mL with RPMI 1640. It was incubated in a 5% CO₂ incubator. Flask was divided to 4 flasks of 1 mL. Each flask was completed to 5 mL with RPMI 1640. Flasks were put in a CO₂ incubator. Mediums were replaced at intervals of 3 days.

Determining the Number of Cells to Work and RTCA Analysis

The medium on the confluent cells was removed. Approximately 300 µL of trypsin-EDTA (enough to cover the bottom of the flask) was added to the flask. After waiting and observing under the microscope that the cells were lifted, approximately 3 mL of RPMI 1640 was added to the flask to neutralize the trypsin-EDTA. The cells were transferred to a 15 mL-tube. It was centrifuged at 300g for 5 min. The medium-trypsin mixture in the 15 mL-tube was removed.

Considering the cell density here, the E-plate was inoculated in a manner corresponding to the numbers designed below to

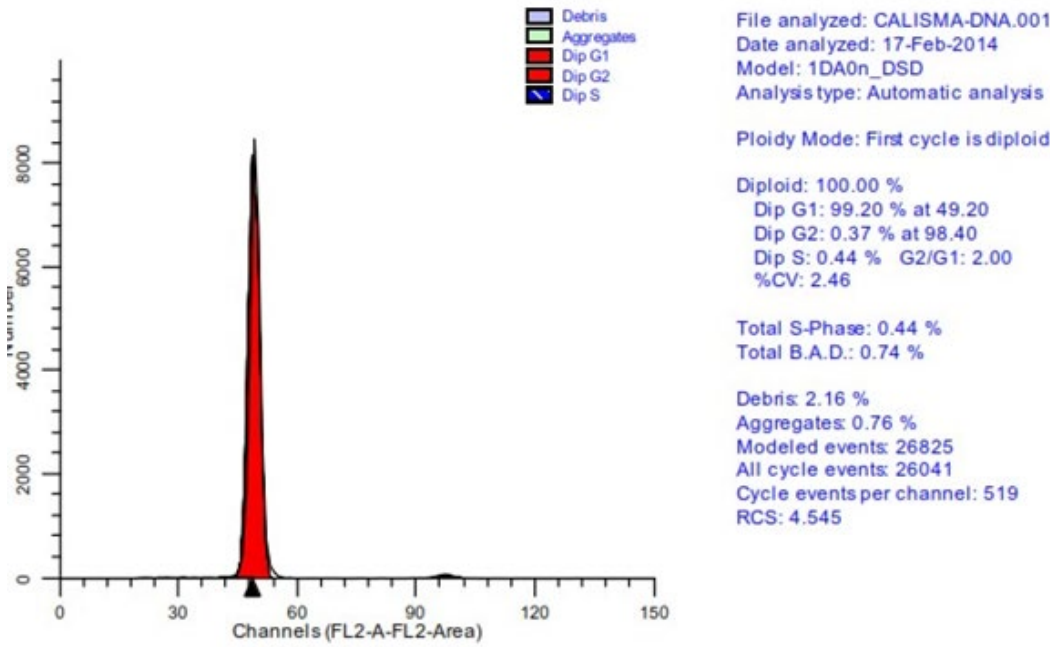


Figure 5. Flow cytometric image of PBMCs after cell culture of 48 hour

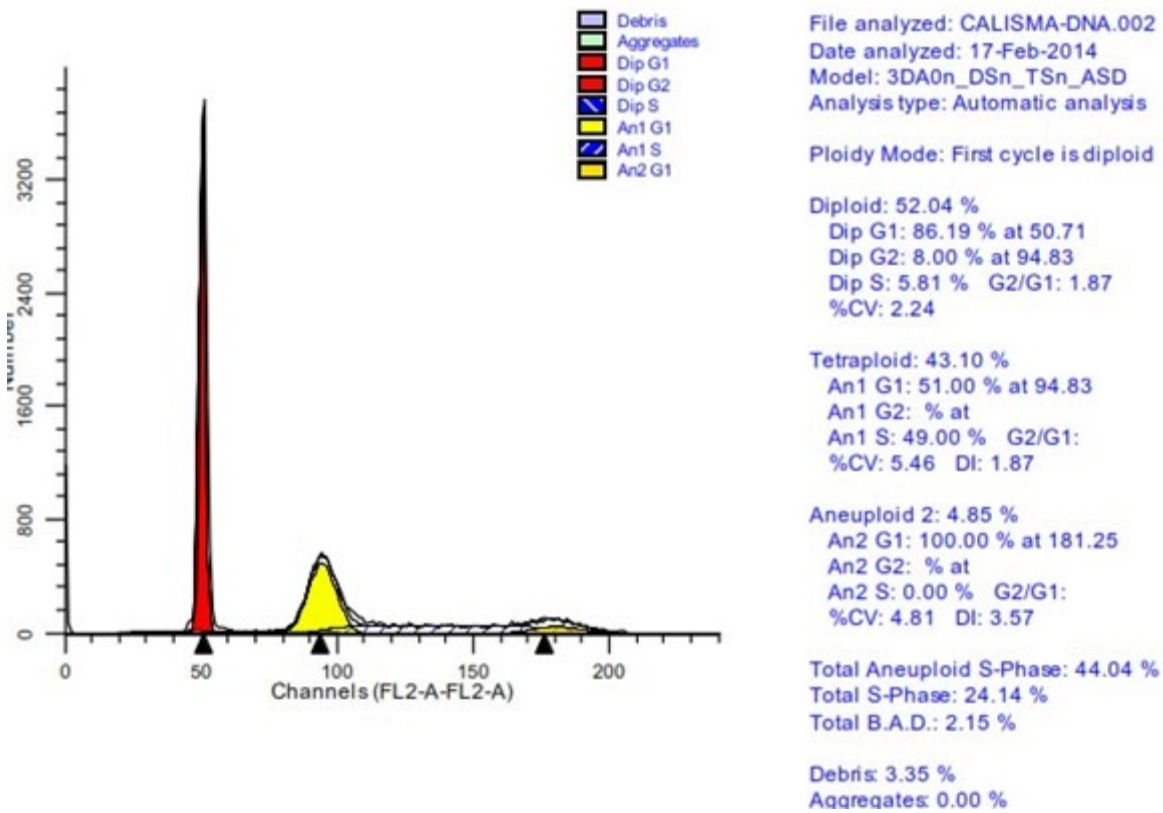


Figure 6. Flow cytometric image of PBMC + Hep-2 cells after cell culture of 48 hour

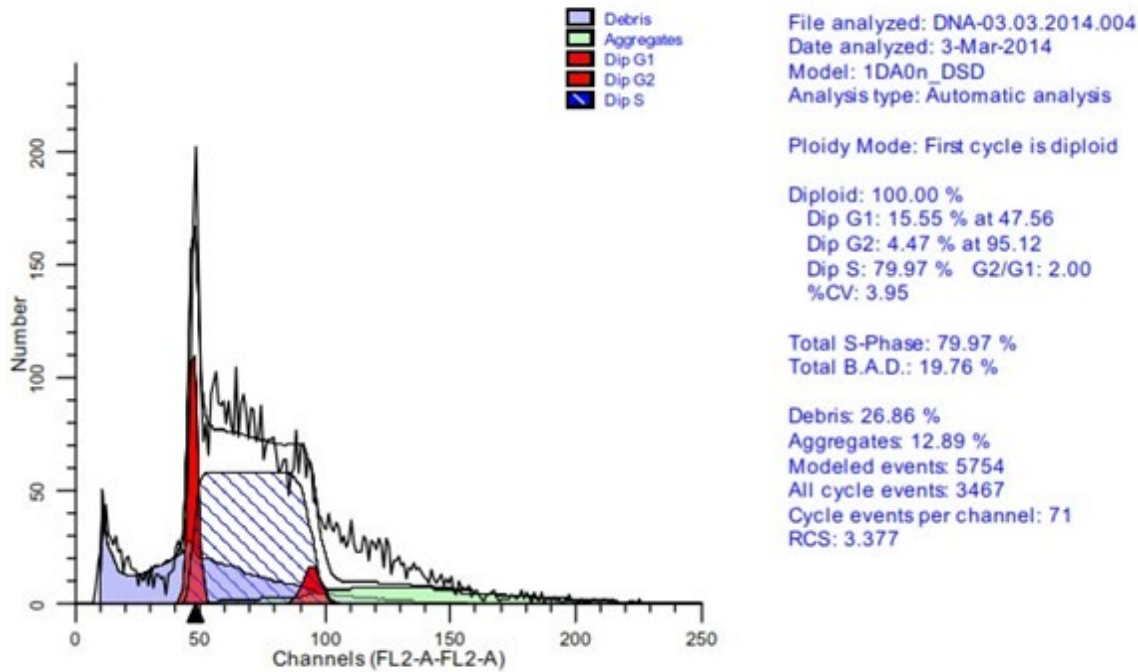


Figure 7. Flow cytometric image of Hep-2 incubated with WEP of 600 µg/mL for 72 hour

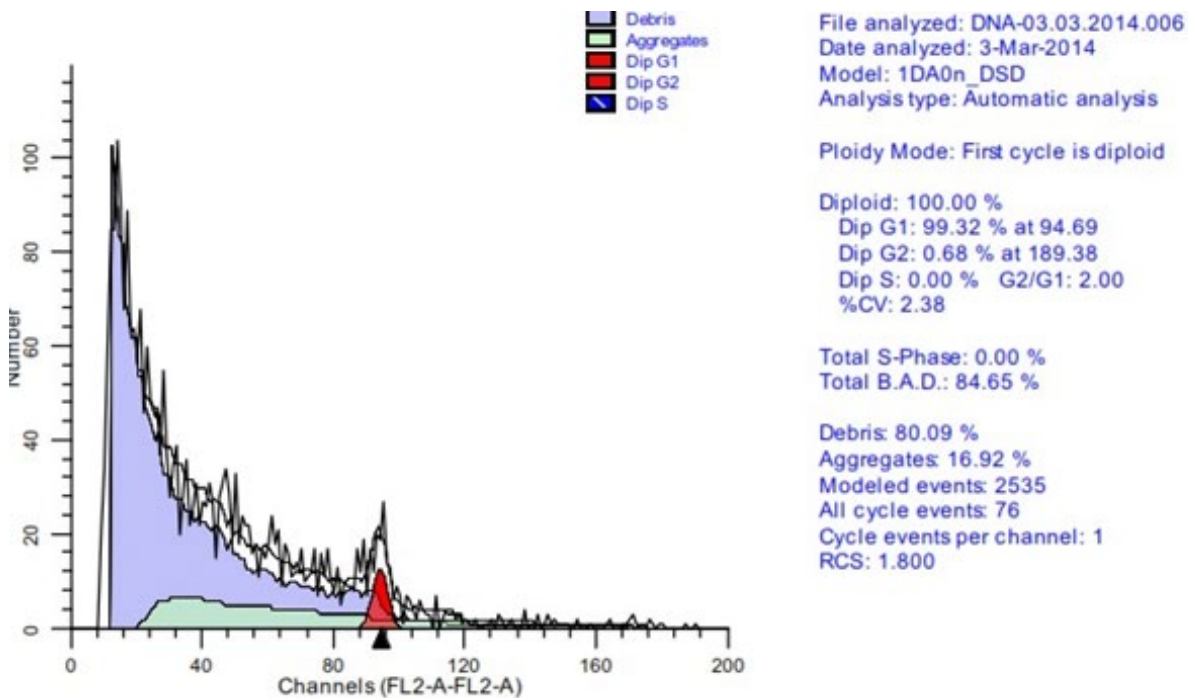


Figure 8. Flow cytometric image of Hep-2 incubated with EEP of 150 µg/mL for 72 hour

It has been understood that the aqueous extract also meets these criteria. Antibacterial and antiviral properties of Turkish propolis have been noted (Barlak et al., 2015). The most important antioxidant mechanism of propolis is that it can repair DNA damage caused by free radicals, and its ability to break the polymer chain reactions that cause lipid peroxidation, and its effect to remove ROS from tissues (Aliyazicioglu et al., 2011).

Studies and evidence on the antitumoral efficacy of propolis are increasing. Some suggest that the primary mechanism of action of propolis on tumor cells is the inhibition of cell proliferation and apoptosis (Sforzin, 2007). Therefore, the view that propolis and its components can be a potential chemotherapeutic or chemopreventive anticancer drug by inhibiting tumor cell progression is getting stronger as the studies increase (Oršolić et al., 2004). The most widely used propolis in research is Brazilian green propolis. It is understood that this propolis shows significant activities against various tumor cells at different concentrations (100, 250, 500, and 1000 µg/mL) (Mishima, et al., 2005a). Flow cytometric analysis has shown that CAPE, the main compound of propolis, also interrupts the cell cycle in oral squamous cell carcinoma cells in the G2/M phase (Lee et al., 2005). Poplar origin propolis (50-150 mg/kg) and some isolated polyphenolic compounds (caffeic acid, CAPE and quercetin) reduced the number of tumor nodules in the lung (Oršolić et al., 2004). In these studies, it is emphasized that the antimetastatic activity of propolis solutions is higher than its components.

Some suggest that propolis, caffeic acid and CAPE (50 mg/kg) are useful tools in the control of tumor cell proliferation and that it provides polyphenolic compounds with a synergistic effect (Oršolic et al., 2005). Carballo et al. reported that propolis had a cytotoxic effect on cancer cells at concentrations of 5-23 µg/mL (IC) in their study with Cuban propolis in various cell lines (colon, ovarian, and prostate carcinoma, neuroblastoma) (Díaz-Carballo et al., 2008). Cuban propolis showed cytotoxic effect at a concentration of 10 µg/mL in PC-3 cells and at a concentration of 12.3 µg/mL in LNCap cells. Since the cancer cells used in this study are independent of immunity, it has been argued that Cuban propolis exerts its anti-tumoral effect by its direct cytotoxic effect rather than its immunomodulating effect. Russo et al. examined the antioxidant properties and antiproliferative capacity of propolis using ethanolic extracts of Chilean propolis in oral epidermoid carcinoma (KB), colon adenocarcinoma (Caco-2) and androgen-insensitive prostate cancer cells (DU-145) (Russo et al., 2004). As a result, in parallel with the increase in the phenolic content of Chilean propolis, its capacity to scavenge free radicals and inhibit the growth of tumor cells increases, it has

been reported that although it protects normal cells from oxidative damage, it decreases the viability of cancer cells by increasing DNA damage in cancer cells.

There are studies on the antitumoral activity of Turkish propolis. Some suggest that Turkish propolis dimethylsulfoxide (DEP) and aqueous extracts (WEP) show antiproliferative activity by changing the protein expression profile in PC-3 prostate cancer cell lines (Barlak et al., 2011). In the same cell lines, the same extracts have been shown to have antimetastatic activity by suppressing the mRNA expression of voltage-gated sodium channels (VGSC Nav 1.5 and 1.7 isoforms), showing the metastatic potential of prostate cancer (Uçar et al., 2016).

In vitro cytotoxic effect of Brazilian green propolis ethyl alcohol extract (50-1000 µg/mL) on HEp-2 Cells (Búfalo et al., 2010; Búfalo et al., 2009). In these studies, the cytotoxic activity of propolis was showed by analyzing cell viability using the simple microscopic staining technique (trypan blue). Some note that the cytotoxic effect is dose-dependent and the best effect is seen at 1000 µg/mL.

In the current study we conducted, there are important differences from the studies of Bufalo et al.:

- i. Turkish propolis was used,
- ii. besides EEP, WEP was also used for the first time,
- iii. instead of viability analysis, which was determined by the cytotoxic effect staining method and the variance of the results was quite high, in the present the kinetic RTCA system was used and the IC₅₀ values were determined. Since this value is the concentration of the substance that prevents the proliferation of half of the cells, it provides how much propolis should be used in the studies. The RTCA system uses a standardized method, with kinetic analysis also providing which cell number should be used in trials. With the kinetic analysis finding in Figure-5 obtained by RTCA, it was decided that 5000 HEp-2 cells were suitable for the study,
- iv. besides the cytotoxic effect, flow cytometric proliferation analysis (cell cycle analysis) was performed to reveal the antitumoral activity.

In the present study, both WEP, at concentrations of 250-4,000 µg/mL (Figure-2), and EEP, at concentrations of 100-2,400 µg/mL (Figure-3) were cytotoxic to HEp-2 cells. It reduced cell viability.

The IC₅₀ value found for WEP is 140 µg/mL and the IC₅₀ value found for EEP is 38.2 µg/mL (Figure 4). Here, EEP appears to be effective at lower concentrations. However, considering the cellular damage from alcohol in direct use by humans, it can be said that WEP can be used safely. Different extraction methods are used in preparing WEP. In the current study, we used the extraction method developed in the laboratory of the K.T.U, Faculty of Medicine, Department of Medical Biochemistry (Bozkuş et al., 2021). Prepared extracts contain highly effective antioxidants. Nagai et al. reported that Brazilian WEP inhibited lipid peroxidation at a concentration of 1-5 mg/mL and completely inhibited superoxide and hydroxyl radical anions at concentrations of 50-100 mg/mL, so these extracts have pharmaceutical potential in cancer, cardiovascular diseases and diabetes (Nagai et al., 2003). Mishima et al. showed that the major component of Brazilian WEP is caffeoylquinic acids (Mishima, et al., 2005b). Nakajima et al. concluded that WEP is better than other bee products (EEP, pollen, royal jelly) in terms of antioxidant capacity; they reported that among the WEP and EEP components, 3,4-di-O-caffeoylquinic acid showed the highest antioxidant capacity against hydrogen peroxide, superoxide and hydroxyl radicals (Nakajima et al., 2009). It is known that the WEP we prepared from Turkish propolis also contains high amounts of caffeoylquinic acids (Bozkuş et al., 2021).

Suzuki et al. suggested that when they used 5-fluorouracil and mitomycin C chemotherapeutics and WEP subcutaneously in experimental Ehrlich carcinoma mice, they significantly increased tumor regression compared to the use of chemotherapeutic alone and minimized the side effects of chemotherapy (Suzuki et al., 2002).

Since propolis has antitumoral effects with apoptotic (Lee et al., 2005) and antiproliferative Mechanisms (Oršolić et al., 2004), the antiproliferative effect of WEP besides EEP has been proven in laryngeal cancer cells in this study, propolis extracts promise a new hope in the treatment of head and neck cancers.

Conclusions

It was concluded that i. In studies of treating Turkish propolis with HEP-2 cells, it was found appropriate to work with 5000 cells per well in RTCA studies, ii. WEP was cytotoxic to HEP-2 cells at concentrations of 250-4,000 µg/mL and EEP at 100-2,400 µg/mL concentrations, iii. WEP at a concentration of 600 µg/mL and EEP at a concentration of 150 µg/mL showed the highest antiproliferative effect by inhibiting the DNA cycle when incubated with HEP-2 cells for 72 hours,

and iv. Turkish propolis extracts were cytotoxic and antiproliferative in HEP-2 cells, so they may be included in chemotherapy or target therapies in laryngeal cancer.

Compliance with Ethical Standard

Conflict of interests: The author declares that for this article they have no actual, potential, or perceived conflict of interests.

Ethics committee approval: The author declares that this study does not include any experiments with human or animal subjects; therefore, no ethics committee approval is needed.

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Disclosure: -

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