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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 IC_{50} 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 5mL 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 300µL 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 3mL 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

load into RTCA (Real Time Cell Analysis System, xCELLigence System Roche Diagnostics GmbH, Mannheim, Germany) with a dilution of 20000, 10000, 5000, 2500, 1250 cells/well. Proliferation was observed in RTCA for 24 hours. (measurement taken every 30 minutes). It was determined that the number of cells suitable for the study was 5000 cells per well.

Cells in continuous exponential phase were preferred considering the duration of the experiment to be run. The number of cells matching this condition was 5000 cells/well. The main experiment was continued with this number of cells. Background reading of E-plates loaded with 100 µL of complete medium was taken. The plates were taken from the incubator and 100 µL of cell suspension were loaded onto these plates. They were kept in a CO2 incubator at 37°C during the experimental periods.

Following the 24-hour plating step, 100 µL of medium was carefully removed from the E-plate wells and the solutions at different concentrations prepared from stock WEP or stock EEP by diluting with RPMI-1640 were added to the plates as 100 µL. WEP concentrations were 250-10000 µg/mL and EEP 10-2400 µg/mL. The medium was added as a control. After the accuracy check-in RTCA, the experiment was started. Proliferation was followed for 48 hours. Propolis concentrations affecting cell proliferation were determined for both EEP and WEP. Cytotoxic concentrations and IC50 levels (50% inhibiting concentration) were calculated using the RTCA instrument software. This value can be interpreted as the concentration that causes the death of 50% of Hep-2 cells.

Evaluation of the Antiproliferative Effect of Propolis on HEp-2 Cells by Flow Cytometric DNA Analysis

100,000 cells from Hep-2 cells were inoculated into 25 cm2 flasks with 4 mL of RPMI 1640 medium. Cell count was done with trypan blue method and hematocytometer. 200, 400, and 600 µg/mL WEP and 75,150 and 300 µg/mL EEP solutions were added to the flasks and allowed to incubate for 48, 72, and 96 hours. All concentrations were run in triplicate. DNA analysis was performed in flow cytometry (Becton Dickinson FACSCalibur) using Cycle Test Plus DNA Reagent Kit (Cat No: 340242).

After 48,72 and 96 hours of Hep-2 /propolis incubations were completed, cell suspensions were placed in 17X100 mm tubes. It was centrifuged at 300xg for 5 minutes. 1 mL of the buffer solution from the kit was added. The same centrifugation was repeated twice.

The cell concentration was adjusted to $1X10^6$ cells/mL with buffer solution by using a hematocytometer. Peripheral Blood Mononuclear Cells (PBMC) were used as control cells in DNA analysis. For this purpose, blood was taken into the hemogram tube and lymphocyte (PBMC) isolation was performed by Ficoll (Biocoll Separating Solution, Biochrom AG, Cat NO: L 6113, Germany) density gradient centrifugation method. 5 mL of buffer was added to the tube and vortexed at low speed. It was centrifuged at 300 xg for 5 min. It was aspirated until 50 µl of liquid remained, and 1.5 mL of buffer solution was added and vortexed at low speed. It was centrifuged at 300 xg for 5 min. It was aspirated until 50 µl of liquid remained, 1 mL of buffer solution was added and vortexed at low speed. Cell count was made and the number of cells was adjusted to $1X10^6$ cells/mL with buffer solution. Incubations were made by adding the solutions in the kit for the staining process, the samples were filtered and analyzed in Flow-Cytometry. Flow cytometry was standardized using a quality control kit (DNA QC Particles, Becton Dickinson, Cat.No: 349523, USA).

Results and Discussion

Evaluation of the Cytotoxic Effect of Propolis on Hep-2 Cells

The optimal number of Hep-2 cells obtained using the RTCA system and software is given in Figure-1. In the RTCA system, the cell index against time is determined. The cell index parameter is related to cell viability, number, morphology, and adhesion. In these studies, a cell index between 0.5-1.5 is ideal. The number of 20,000 and 10,000 cells (top 2 lines on the graph) exceeded this limit, while the number of 2,500 and 1,250 cells (bottom 2 lines on the graph) remained below this limit. The number of cells 5000 (middle line) appears to meet this criterion. The curves obtained by kinetic monitoring of aqueous propolis extracts (WEP) at various concentrations with Hep-2 cells by the RTCA system are given in Figure-2. In the figure, the cell indices of the 1st control (red) (medium) and the 2nd control (purple) (1/2 diluted medium) are high. 10,000 µg/mL (dark green) is a high dose. At concentrations of 4,000 µg/mL (pink), 2,000 µg/mL (orange), 1,000 µg/mL (dark blue), 500 µg/mL (blue), and 250 µg/mL (light green) with cell indexes <1, it was observed that WEP was appeared to be cytotoxic to Hep-2 cells.

The curves obtained by kinetic monitoring of ethanolic propolis extracts (EEP) at various concentrations with Hep-2 cells by RTCA system are given in Figure-3. In the figure, 0.5% ethanol (orange), 10 (pink) and 20 (dark purple) µg/mL concentrations have high cell indices. With cell indices <1, 2,400 µg/mL (light green) is the most cytotoxic concentration. It is seen that concentrations of 800 µg/mL (blue), 200 µg/mL (dark green), 100 µg/mL (dark blue) are cytotoxic, while concentrations of 50 µg/mL (light purple) are less cytotoxic.

The IC50 value (50% inhibiting concentration) found by RTCA analysis of the WEP was 140 µg/mL while that of the EEP was found to be 38.2 µg/mL (Figure-4).

Evaluation of the antiproliferative effect of propolis on Hep-2 cells by flow cytometric DNA analysis

Flow cytometric cell cycle image of PBMCs (lymphocytes), which are control cells, after 48 hours of cell culture is given in Figure-5.

As seen in the figure, all of the PBMCs with control cells are diploid and almost in the G1 cycle. When HEp-2 cancer cells, are added to these cells, the image is as in Figure-6. About half of the two cell groups are diploid (86% G1 phase) and half are tetraploid (51% G1, 49% S phases).

The data obtained in flow cytometry (only diploid data) because of incubation of WEP with HEp-2 cells at various concentrations for 48, 72, and 96 hours are given in Table-1 and those obtained with EEP are given in Table-2. As seen in the tables, 600 µg/mL WEP kept the cells in 80% S phase in 72 hours (also as seen in Fig. 7); It is understood that 150 µg/mL EEP keeps DNA cycle as 99% in the G1 phase in 72 hours (Fig 8). Therefore, EEP in this concentration have an antiproliferative effect on laryngeal cancer cells.

Propolis is a bee product that has been used frequently since ancient times. The Egyptians took advantage of the anti-putrefactive properties of propolis to embalm their dead. Greek and Roman physicians used propolis as an antiseptic and anticicatricial. The Incas used it as an antipyretic agent. In the 17th century London pharmacopoeia, propolis was listed as a medicine. With the development of the pharmaceutical design industry in the last two centuries, interest in natural products has decreased; however, its use as a popular drug still continues. Its use has increased in cosmetic products. Since the 2000s, scientists' interest in propolis has increased and studies on its components, as well as its biological and medicinal properties, have intensified (Russo et al., 2004; J.M. Sforcin, 2007).

Table 1. Data obtained by flow cytometric DNA cell cycle analysis for Hep-2 cells incubated with WEP

Time (h)	Concentration(µg/mL)	Diploid %	G1 %	G2 %	S %	Viability %
48	200	96.6	59.2	8.0	32.8	100
	400	97.6	58.9	8.0	33.1	100
	600	83.9	62.5	8.0	29.5	100
72	200	81.2	63.1	1.4	35.6	100
	400	96.5	59.3	8.0	32.7	100
	600	100	15.6	4.5	80.0	100
96	200	100	55.9	-	44.1	100
	400	98.7	60.7	8.0	31.4	100
	600	90.0	50.0	1.4	48.6	100

Table 2. Data obtained by flow cytometric DNA cycle analysis for Hep-2 cells incubated with EEP

Time (h)	Concentration (µg/mL)	Diploid %	G1 %	G2 %	S %	Viability %
48	75	94.1	32.1	8.0	55.9	90
	150	100	37.2	4.4	58.3	50
	300	100	40.4	16.1	43.3	0
72	75	82.3	50.6	8.0	41.4	74
	150	100	99.3	0.7	-	50
	300	100	48.0	13.6	38.4	0
96	75	88.6	43.5	8.0	48.5	76
	150	89.1	45.3	4.3	50.5	57
	300	100	55.9	-	44.1	0

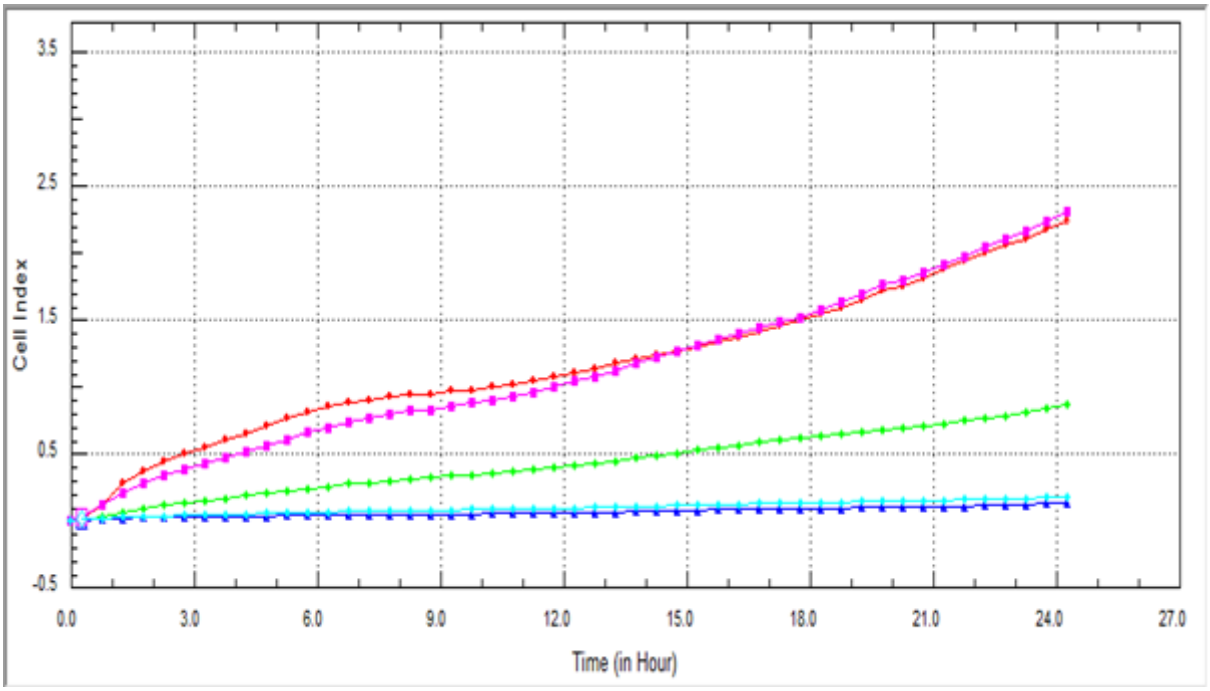

Figure 1. Change in Hep-2 cell number by time obtained with RTCA system [Cell numbers are 1250 (light blue), 2500 (light blue), 5000 (green), 10000 (purple), 20000 (red)]

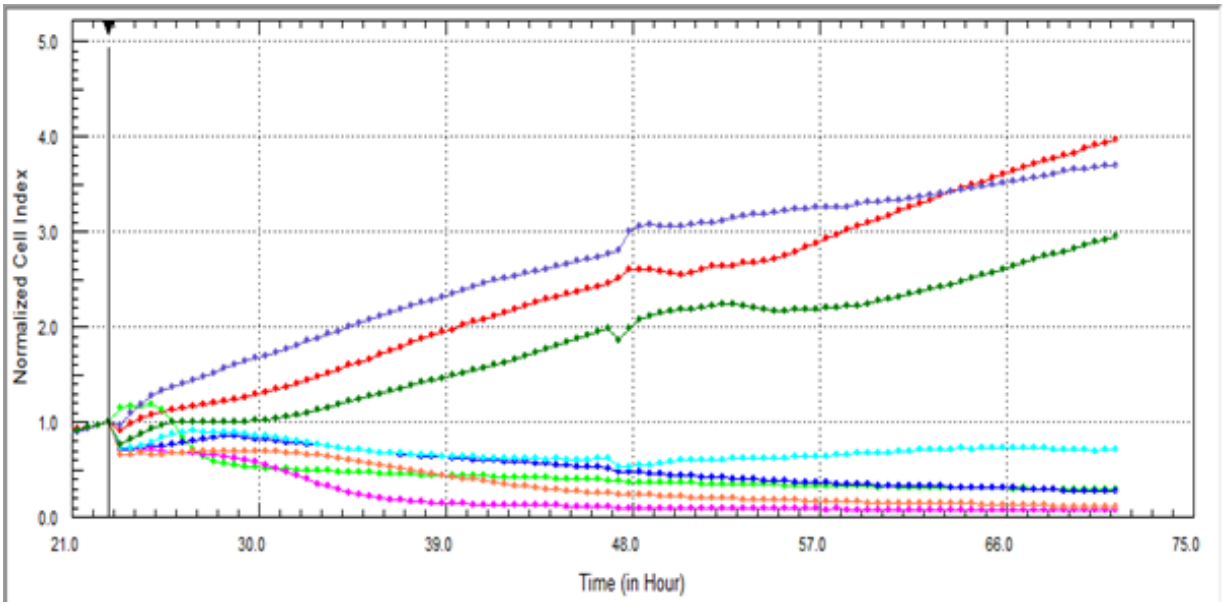

Figure 2. Kinetic montoring of WEPs with RTCA system [Cell indexes are high: red (control 1=medium), purple (control 2), dark green (10000 µg/mL); cell indexes are <1 (cytotoxic): pink (4000 µg/mL), orange (2000 µg/mL), dark blue (1250 µg/mL), light blue (500 µg/mL), light green (250 µg/mL)]

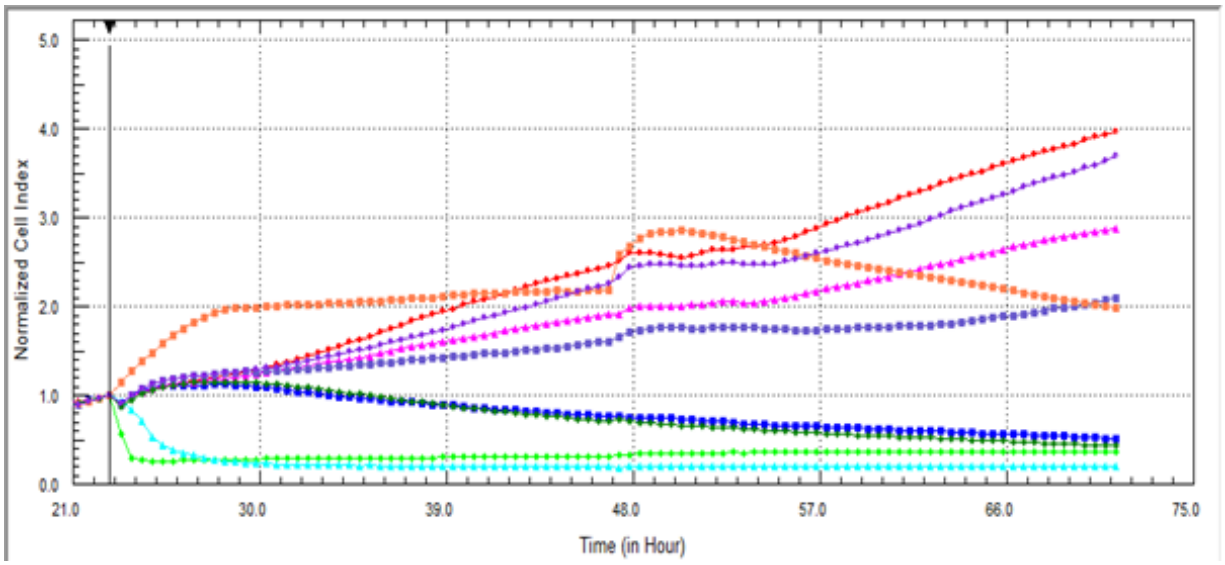

Figure 3. Kinetic montoring of EEPs with RTCA system [Cell indexes are high: orange(%0.5 ethanol),pink (10 µg/mL), purple (20 µg/mL); cell indexes are <1 (cytotoxic): light green (2.400 µg/mL), blue (800 µg/mL), dark green(200 µg/mL), dark blue (100 µg/mL), light purple (50 µg/mL))]

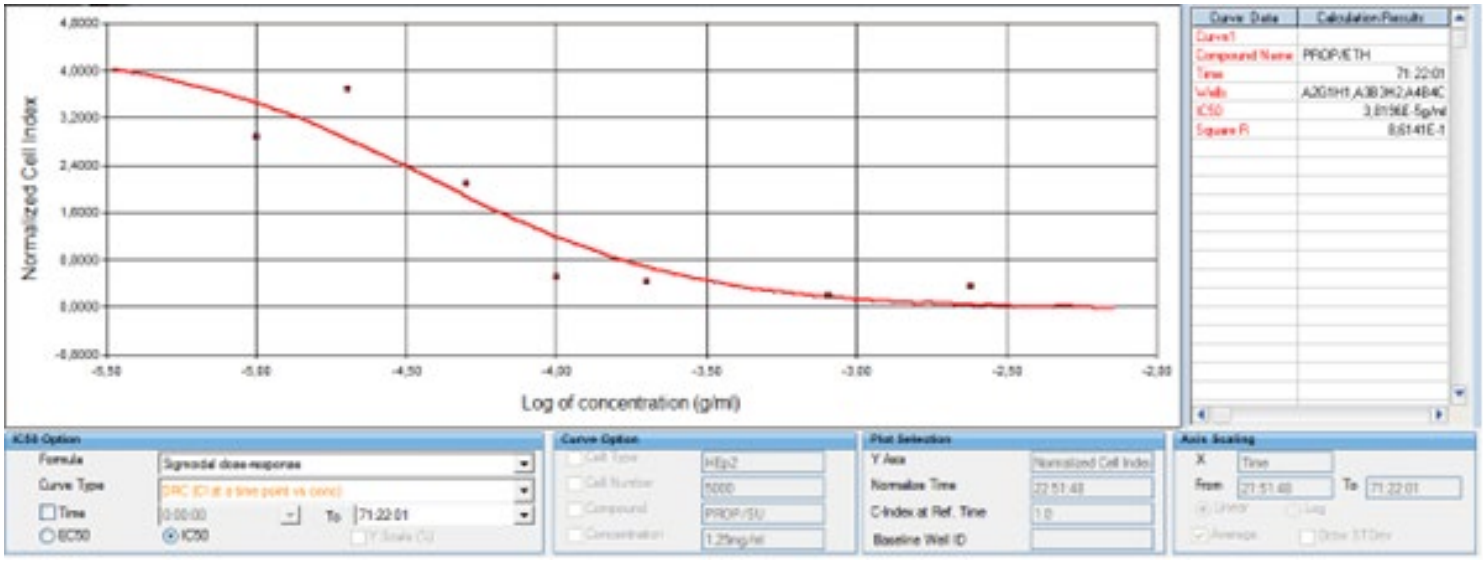

Figure 4. IC50 curve of EEP

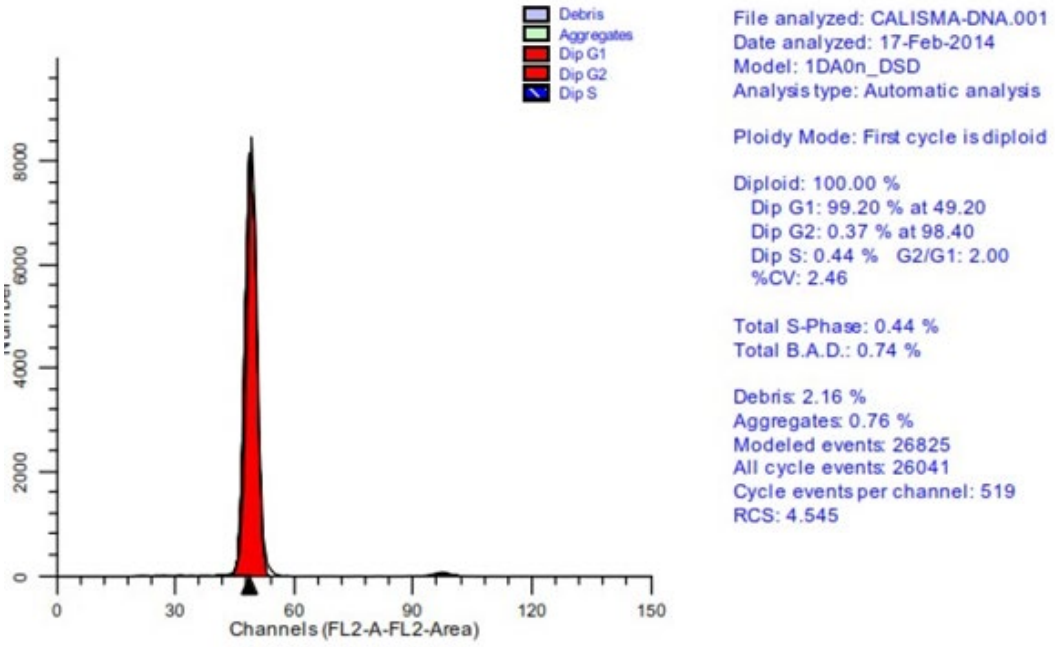

Figure 5. Flow cytometric image of PBMCs after cell culture of 48 hour

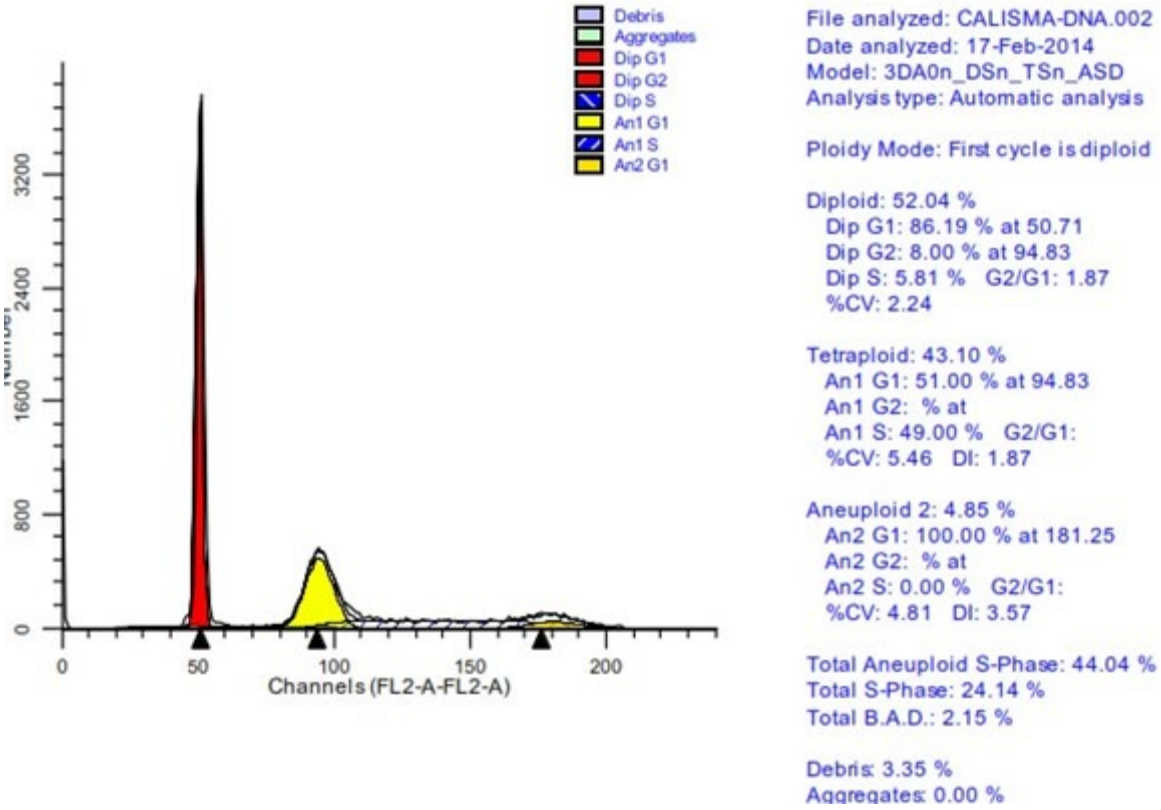

Figure 6. Flow cytometric image of PBMC + Hep-2 cells after cell culture of 48 hour

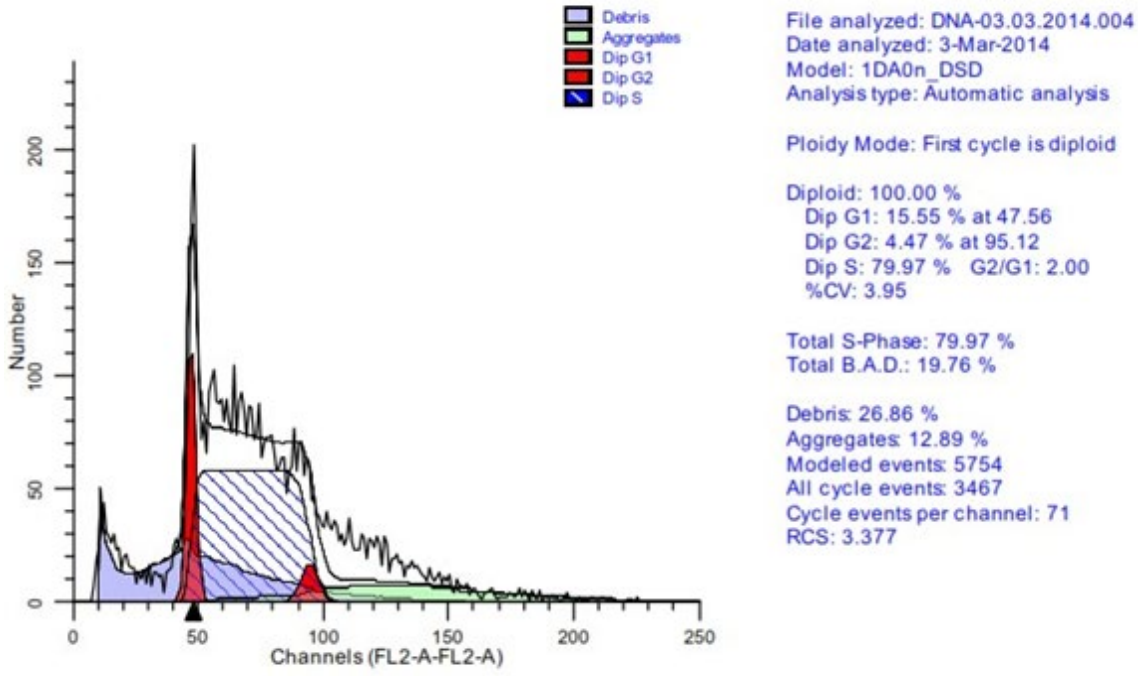

Figure 7. Flow cytometric image of Hep-2 incubated with WEP of 600 µg/mL for 72 hour

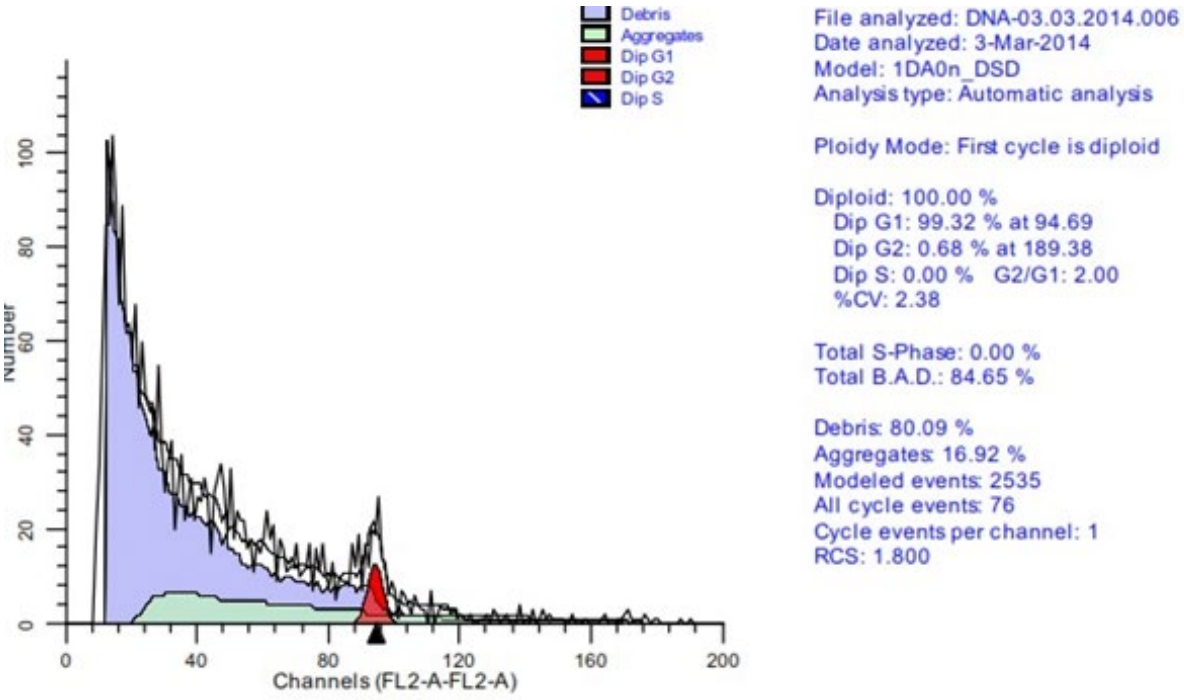

Figure 8. Flow cytometric image of Hep-2 incubated with EEP of 150 µg/mL for 72 hour

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 (Sforcin, 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 5-23µg/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 10µg/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 IC_{50} 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 IC_{50} value found for WEP is 140 µg/mL and the IC_{50} 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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