



ORIGINAL RESEARCH

Medicine Science 2019;8(3):557-62

In vitro investigation of anti-cancer activity of propolis on hepatocellular carcinoma cells**Kurtulus Gokduman***Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA**Institute of Biomedical Engineering, Bogazici University, Istanbul, Turkey*Received 23 January 2019; Accepted 11 February 2019
Available online 20.03.2019 with doi:10.5455/medscience.2019.08.9013

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Abstract

The aim of the current study is to investigate the anti-cancer activity of propolis on liver cancer, with a mechanistic approach. Ethanol extract of the collected propolis was prepared using maceration technique and anti-cancer activity of the extract was investigated on liver hepatocellular carcinoma cells (HepG2 and Hep3B cells) by focusing on viability, intracellular ROS level, total antioxidant status and apoptosis through caspase-3 activity. Total flavonoid content, total phenolic content and ferric reducing antioxidant potential values of the propolis were determined as 505.12 ± 10.08 (mg QE/g), 308.72 ± 5.33 (mg GAE/g) and 247.64 ± 7.27 (mg Tro/g), respectively. Ethanol extract of the propolis induced statistically significant ($p < 0.01$) decreases in viability of hepatocellular carcinoma cells, HepG2 and Hep3B cells, as evidenced by IC₅₀ values (e.g. IC₅₀ values of 72 h treated HepG2 and Hep3B cells were 25.62 ± 1.50 and 31.74 ± 2.72 μ g/mL, respectively). These powerful cytotoxic effects are caused by statistically significant ($p < 0.01$) decrease in intracellular ROS level by natural antioxidants such as flavonoids and phenolic acids, as well as statistically significant ($p < 0.01$) increase in apoptosis induced by caspase-3 activity. These results suggest the great potential of propolis as a potent anti-tumor compound in liver cancer treatment for further researches.

Keywords: Hepatocellular carcinoma, propolis, flavonoid, polyphenols, ROS, caspase-3**Introduction**

It is expected in this century that cancer will be the most important reason of death and the leading obstacle to rise in life expectancy; over 18 million new cancer cases with over 50 % mortality has been mentioned in 2018 estimates [1]. In spite of the progresses in the treatment of liver cancer, it continues to be one of the most challenging cancers to treat [2]. With over 840,000 new cases and over 780,000 deaths annually, among 36 cancers, liver cancer is forecasted to be the sixth and fourth in terms of diagnosis and death rates, respectively, in 2018; in terms of death percentages of new cases, liver cancer is forecasted as the second (~93%) after pancreas cancer (~94%) among 36 cancers [1]. For patients in early stage, recurrence is an important issue after remedial treatment, besides, liver cancer is generally detected at later stages, which is not appropriate for the remedial treatments; in addition, conventional chemotherapy has lack of effectiveness and low survival rates [2]. Therefore, new strategies with minimal side effects are crucial for liver cancer.

Propolis, which is a bee adhesive, is a dark colored, cohesive resinous material, and gathered from leaf sprouts, branches,

trees and bole wounds, to cover and sanitize the inner surface of the colony nest [3,4]. Accumulated evidence demonstrates that propolis has very complex chemical structure; different propolis samples have more than 300 compounds [5,6]. Collection time, geographical origin, local flora and tree species determine the chemical structure and color of propolis; for example, while the main ingredients of European and Chinese propolis are flavonoids and esters, the main ingredients of the Brazilian propolis are the terpenoids and coumaric acids [5]. Due to its vast variety of therapeutic (biological/pharmacological) properties, propolis has long been used to prevent and treat variety of diseases [4,5,7].

Thus, propolis can be involved in alternative treatment strategies for liver cancer. However, although numerous studies have reported on the efficacy of propolis on many types of cancer cells, a limited number of studies have investigated the anti-cancer activity of Turkish propolis [4,6,8], and these limited studies have lack of mechanistic investigation of Turkish propolis on liver cancer. In this context, the aim of the current study is to investigate the anti-cancer activity of propolis from Central Anatolia region of Turkey on liver cancer, with a mechanistic approach. To this end, ethanol extract of the collected propolis (the most common and the most effective formulation [6,7]) was prepared using maceration technique, and anti-cancer activity of the prepared extract was investigated on liver hepatocellular carcinoma cells (HepG2 and Hep3B cells) (hepatocellular carcinoma accounts for great

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majority (~90%) of primary liver cancer cases [9]) by focusing on viability, intracellular ROS level, total antioxidant status and apoptosis through caspase-3 activity.

Material and Methods

Materials

Cell lines (HepG2 and Hep3B) and culture media components (Eagle's Minimum Essential Medium (EMEM), Dimethylsulfoxide (DMSO), Fetal Bovine Serum (FBS), Trypsin-EDTA Solution) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All chemicals (analytical grade), Folin-Ciocalteu reagent, Dichloro-dihydro-fluorescein diacetate (DCFH-DA), MTT assay kit and Caspase 3 Assay Kit were purchased from Sigma Aldrich (Saint Louis, MO 63103, USA). The Cell Death Detection ELISA Kit was purchased from Roche Diagnostic GmbH (Mannheim, Germany).

Propolis Raw Material and Preparation of Propolis Extracts

The propolis samples were produced by honey bees (*Apis mellifera L*) in Kayseri (Central Anatolia region of Turkey), and were provided from the local beekeepers. Preparation of propolis extracts was carried out as described previously [6]. Briefly, 1 g propolis in 20 mL absolute ethanol was incubated at 60 °C and 150 rpm for 24 h; using filter paper and 0.22 µm filters, supernatants were filtrated after centrifugation at 4000 rpm for 10 minutes.

Total Flavonoid and Polyphenolic Contents

Using aluminum chloride spectrophotometric assay [10] total flavonoid content of the extracts was detected. Briefly, ethanol extract of propolis (0.5 ml) was mixed with 2% AlCl₃-ethanol solution (0.5 ml); the absorbance measurement was carried out at 420 nm following incubation at room temperature for 1 h. The values were expressed as mg quercetin equivalents per g of sample dry weight. Three different experiments were performed in triplicate in three different weeks.

Using the method of Singleton & Rossi [11] total polyphenol content of the extracts was detected spectrophotometrically by the Folin-Ciocalteu reagent. Briefly, ethanol extract of propolis (0.5 ml) was mixed with the Folin-Ciocalteu reagent (0.5 ml) and 10% Na₂CO₃ (0.5 ml); the absorbance measurement was carried out at 760 nm following incubation at room temperature for 1 h. The values were expressed as mg of gallic acid equivalents per g of sample dry weight. Three different experiments were performed in triplicate in three different weeks.

Ferric Reducing Antioxidant Potential

Using ferric to ferrous ion reduction method at low pH [12], the reduction power of the extracts was detected. The values representing antioxidant potentials of propolis extracts were expressed as mg trolox equivalents per g of sample dry weight. Three different experiments were performed in triplicate in three different weeks.

Cell Culture and Maintenance

The liver hepatocellular carcinoma cells (HepG2 and Hep3B cells) were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 100 UI/mL penicillin G and 100 µg/mL streptomycin, 20 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), adjusted to pH 7.4 with 1 mol/L sodium bicarbonate. The cells were grown in poly-

L-lysine-coated flasks at 37°C, 5% CO₂, 95% air in a humidified incubator and confirmed free of mycoplasma infection through regular testing. Sub-culturing was performed at intervals of 48 h, and cell growth was monitored using an inverted microscope; to ensure stability of cell line, in the experiments, cells were used within eight passages.

Cytotoxicity Assay

Both HepG2 and Hep3B cells were seeded in culturing plates and divided into three groups: I) Blank group containing medium without HepG2 or Hep3B cells; II) Control group containing medium with HepG2 or Hep3B cells that are not treated with the ethanol extract of propolis; III) Test group containing medium with HepG2 or Hep3B cells that are treated with different concentrations of the ethanol extract of propolis (0- 100 µg/ml).

HepG2 and Hep3B cells in the logarithmic phase of growth were seeded in 96-well plates at a density of 10000 cells/well. 24 h after seeding, the cells were exposed to the ethanol extracts of propolis (10, 25, 50, 75 and 100 µg/ml) for 24 h or 48 h or 72 h (the control group received medium only) at 37 °C and 5% CO₂/95% air in a humidified incubator, followed by incubated with 50 µL of MTT (5 mg/ml) for another 4 h. The supernatant was removed after centrifugation (1250 rpm at 4 °C for 5 minutes); then, 100 µL of DMSO was added and absorbance at 550 nm wavelength was measured using an ELISA reader. Three different experiments were performed in triplicate in three different weeks.

Intracellular ROS Level

Oxidative stress was measured by the Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay as described previously [13]. 2 x 10⁴ cells were seeded on 96 well/plates and incubated with DCFH-DA probe for 40 min. Then, medium was removed and cells were exposed to the ethanol extracts of propolis (10, 25, 50, 75 and 100 µg/ml), (the control group received medium only); the cells were incubated at 37 °C for 24 h and fluorescence of the samples was read at 485 nm (excitation) and 527 nm (emission) wavelengths using a microplate reader. Three different experiments were performed in triplicate in three different weeks.

Apoptosis/Necrosis

The Cell Death Detection ELISA Kit (Roche Diagnostic GmbH, Germany) was used to detect apoptosis/necrosis in HepG2 and Hep3B cells exposed to the ethanol extracts of propolis, as described previously [14]. Briefly, cells (1 x 10⁴) were seeded in 96-well plates; after 24 h of treatment with the ethanol extracts of propolis (10, 25, 50, 75 and 100 µg/ml) (the control group received medium only) the supernatants and lysate of cells were extracted and incubated in the microtiter plate modules coated with streptavidin. Subsequently, to detect immobilized histone/DNA fragments followed by color development with ABTS substrate for peroxidase, a mixture of anti-histone-biotin and peroxidase-conjugated anti-DNA antibody was used. Finally, the results were analyzed spectrophotometrically after measuring absorbance values at 405 nm using an ELISA reader. The apoptosis and necrosis were expressed as fold change of absorbance value over that of control group. Three different experiments were performed in triplicate in three different weeks.

Caspase-3 Activity

Using a Caspase 3 Assay Kit, Colorimetric (Sigma Aldrich, Saint Louis, MO 63103, USA), the activity of CPP32/caspase-3 was

determined in cell extracts, as described previously [15]. Briefly, the HepG2 and Hep3B cells exposed to the ethanol extracts of propolis (10, 25, 50, 75 and 100 µg/ml) (the control group received medium only) for 24 h were collected and lysed with cell lysis buffer. Following incubation of cell lysate in reaction buffer that contains peptide substrate (DEVD-pNA, 5 µL) for 2 h at 37 °C, absorbance at 405 nm wavelength was measured using a microtiter plate reader. The activity was expressed as fold change of absorbance value over that of control group. Three different experiments were performed in triplicate in three different weeks.

Statistical Analyses

To determine whether there were any statistically significant differences between the means of the groups, one-way analysis of variance (ANOVA) with Tukey HSD test was used. Data were expressed as the mean ± standard deviation (SD) from three independent experiments with three replicates. For all the statistical analyses, as the threshold for significance, $p < 0.05$ was used.

Results

Total Phenolic Contents and Antioxidant Potentials of Propolis Extracts

Total flavonoid and polyphenolic contents, and ferric reducing antioxidant potential of the ethanol extract of propolis were determined as mg quercetin (Q)/g propolis, mg gallic acid (GA)/g propolis and mg trolox (Tro)/g propolis, respectively, and the results were presented in Table 1.

Table 1. Total flavonoid content, total polyphenolic content and ferric reducing antioxidant potential of the ethanol extract of propolis

Total flavonoid content (mg QE/g)	Total phenolic content (mg GAE/g)	Ferric reducing antioxidant potential (mg Tro/g)
505.12 ± 10.08	308.72 ± 5.33	247.64 ± 7.27

Results were expressed as the mean ± SD

Cytotoxicity Assay

Cytotoxic effects of the ethanol extract of propolis were tested on liver hepatocellular carcinoma cells (HepG2 and Hep3B cells). Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in propolis treated (0-100 µg/ml) cancer cells after 24 h, 48 h, and 72 h treatment, and the results were presented in Figure 1A (HepG2 cells) and Figure 1B (Hep3B cells).

Using MTT cell viability assay results, time dependent half-maximal (50%) inhibitory concentration (IC 50) values of the ethanol extract of propolis were determined for hepatocellular carcinoma cells (HepG2 and Hep3B cells), and the results were presented in Table 2.

Table 2. Time dependent IC 50 (µg/mL) values of the ethanol extract of propolis for hepatocellular carcinoma cells (HepG2 and Hep3B cells)

Cancer Cells	24h	48h	72h
HepG2	51.66 ± 0.48	36.57 ± 2.01	25.62 ± 1.50
Hep3B	71.74 ± 2.76	50.02 ± 3.79	31.74 ± 2.72

Results were expressed as the mean ± SD

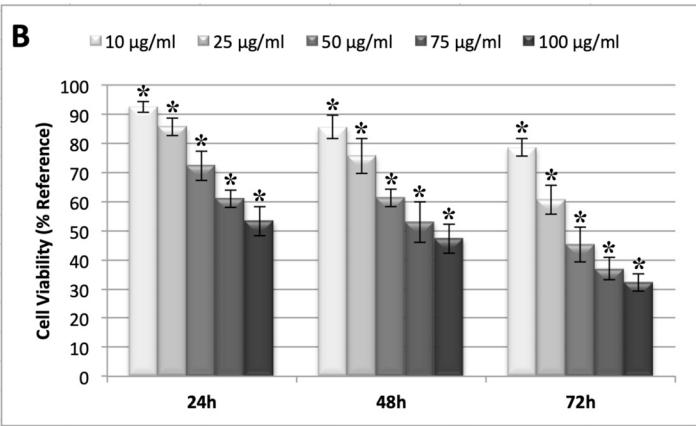
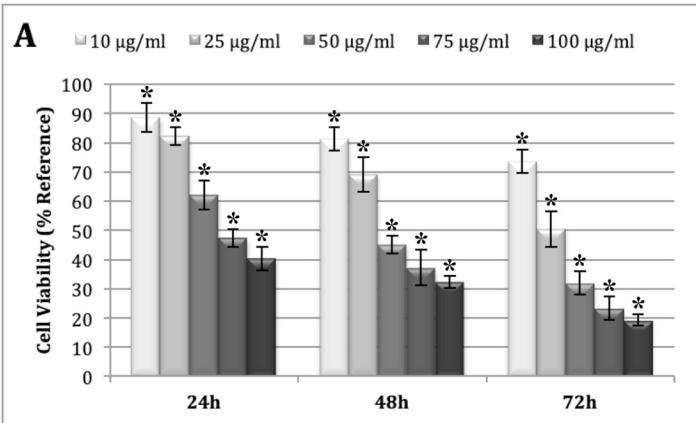


Figure 1. MTT assay results for HepG2 cells (A) and Hep3B cells (B). Significant difference with respect to control is denoted as * p value < 0.01

Intracellular ROS Level

It was analyzed that whether the cytotoxicity of the ethanol extract of propolis is correlated with the intracellular ROS levels of the hepatocellular carcinoma cells (HepG2 and Hep3B cells), and the results were presented in Figure 2.

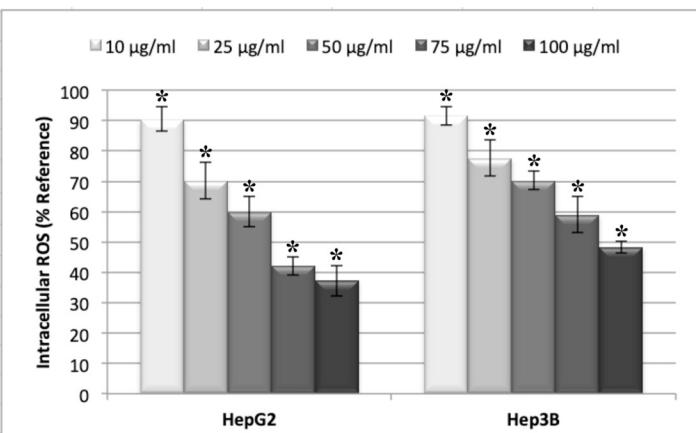


Figure 2. Effect of ethanol extract of the propolis on intracellular ROS level of the hepatocellular carcinoma cells (HepG2 and Hep3B cells) detected after 24 h of treatment. Significant difference with respect to control is denoted as * p value < 0.01

Apoptosis/Necrosis

It was analyzed that whether the cytotoxicity of the ethanol extract of the propolis is due to apoptosis or necrosis,. In terms of necrosis, no statistically significant differences ($p > 0.05$) were found as compared to controls as well as among test groups. The

apoptotic effect of the ethanol extract of the propolis was presented in Figure 3.

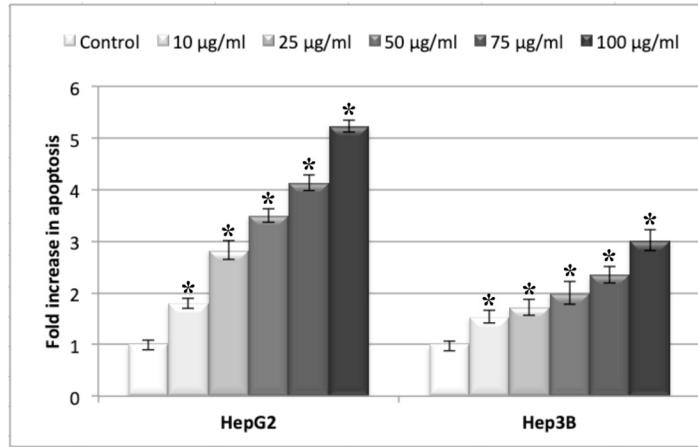


Figure 3. The apoptotic effect of ethanol extract of propolis on HepG2 and Hep3B cells detected by cell death ELISA assay after 24 h of treatment. Significant difference with respect to control is denoted as *p value < 0.01

Caspase-3 Activity

The role of caspase-3 activity in apoptosis of the hepatocellular carcinoma cells (HepG2 and Hep3B cells) was investigated, and the results were presented in Figure 4.

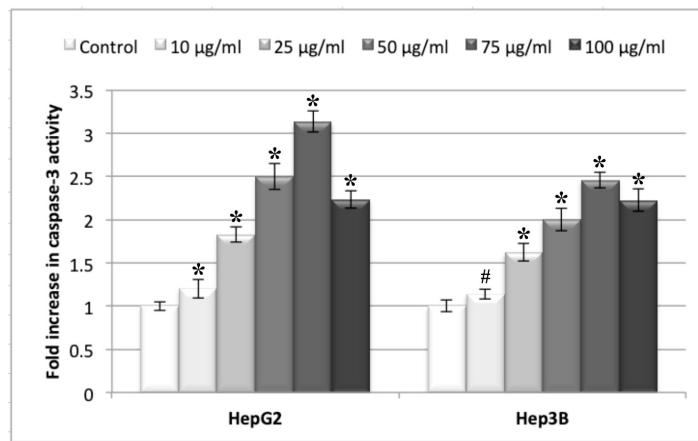


Figure 4. Effect of the ethanol extract of propolis on caspase-3 activity in the hepatocellular carcinoma cells (HepG2 and Hep3B cells) detected after 24 h of treatment. Significant difference with respect to control is denoted as #p value < 0.05, *p value < 0.01

Discussion

The previous comprehensive study [16] examined propolis samples from various regions in the world, and reported total phenolic content as in the range of 31.2–299 mg GAE/g and total flavonoid content as in the range of 2.5–176 mg QE/g. Total phenolic and total flavonoid content values of the collected propolis (Table 1) can be accepted as high values. These values are consistent with the previous comprehensive study [17] containing propolis samples from 54 distinct areas of Turkey (the study does not contain the region from which propolis samples were collected in the current study). Thus, in the light of accumulating evidence, it can be inferred that Turkish propolis is characterized by high total phenolic and flavonoid contents.

The FRAP value representing antioxidant potential of the collected propolis samples was measured as 257.64 ± 7.27 mg

Tro/g sample dry weight. The value is consistent with the previous studies reporting FRAP values belong to Turkish propolis as 246.8 ± 0.01 mg Tro/g sample dry weight [18] as well as other studies containing propolis from close region of Turkey, e.g. it has been reported that propolis samples from distinct areas of Iran have FRAP values in the range of 31.5 - 1650 mg Tro/g sample dry weight [6,12]. It is well known that the antioxidant capacity of bee propolis is dependent on its content; some studies [19,20] have shown a strong positive correlation between the total content of phenolic compounds and antioxidant capacity of bee propolis, whereas others [21] found no considerable relationships [22]. In addition, both content and properties of bee propolis are dependent on the kind of its plant source, the time of harvesting as well as the conditions of the plants growing like soil or climate [22].

HepG2 and Hep3B cell lines have crucial advantages for *in vitro* studies of hepatocellular carcinoma; they are the most widely accessible and decent defined liver cancer cell lines, in addition, they have a lot of common features, thus they allow parallel testing [23]. Therefore, after determining favorable total flavonoid content, total phenolic content and antioxidant potential characteristics of the collected propolis samples, their anti-cancer potential was investigated on hepatocellular carcinoma cells using HepG2 and Hep3B cell lines.

MTT assay illustrated that highly significant differences ($p < 0.01$) in viability values of the hepatocellular carcinoma cells were found between control and test groups as well as among test groups with some exceptions: For HepG2 cells 24 h: $p < 0.05$ between 10 µg/ml and 25 µg/ml treated cells as well as between 75 µg/ml and 100 µg/ml treated cells, 48 h: $p < 0.05$ between 50 µg/ml and 75 µg/ml treated cells, non significant ($p > 0.05$) difference between 75 µg/ml and 100 µg/ml treated cells, 72 h: non significant ($p > 0.05$) difference between 75 µg/ml and 100 µg/ml treated cells; For Hep3B cells 48 h: $p < 0.05$ between 50 µg/ml and 75 µg/ml treated cells, non significant ($p > 0.05$) difference between 75 µg/ml and 100 µg/ml treated cells, 72 h: non significant ($p > 0.05$) difference between 75 µg/ml and 100 µg/ml treated cells.

From Figure 1 representing dose and time dependent toxicity of ethanol extract of the propolis on HepG2 and Hep3B cells and Table 2 representing time dependent IC 50 (µg/mL) values of the ethanol extract of propolis for HepG2 and Hep3B cells, it can be inferred that ethanol extract of the propolis is more effective on HepG2 cells compared to Hep3B cells. HepG2 cells are wild type p53-containing cells, while Hep3B cells are p53-deficient cells [23]; and thus, accumulating evidence strongly suggests that generally, HepG2 cells are highly susceptible to xenobiotics compared to Hep3B cells [24-26]. Therefore, the observed differences between viability values of these cells after treatment of ethanol extract of the propolis (Figure 1 and Table 2) may result from the differences between HepG2 and Hep3B cells in terms of p53. Significant toxicity on p53-deficient Hep3B cells in a dose and time dependent manner illustrates that cytotoxicity of ethanol extract of the propolis on hepatocellular carcinoma cells contain p53 independent pathways.

On the other hand, IC 50 < 30 µg/ml as the cytotoxic activity of an extracted compound is stated within acceptable limits by the American National Cancer Institute (NCI) [17,27]. Accordingly, the propolis extract in the current study can be evaluated as an anti-

tumor compound in terms of 72 h treatment for the hepatocellular carcinoma cells (the IC 50 value of 72 h treatment for Hep3B cells is at the boundary). 48 h treatment of the propolis extract also has significant anti-proliferative effects on HepG2 cells; the IC 50 value is on the upper limit.

ROS assay illustrated that highly significant differences ($p < 0.01$) in intracellular ROS levels of the hepatocellular carcinoma cells were found between control and test groups as well as among test groups with some exceptions (non significant ($p > 0.05$) difference between 75 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ treated HepG2 cells; $p < 0.05$ between 25 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$ treated Hep3B cells).

In cancer cells, free radicals can cause: At low levels, activation of signaling pathways and induction of cell reproduction; at moderate levels, promotion of stress responsive genes and cell survival; at high levels, damages in macromolecules and organelles that results in senescence or apoptosis [28, 29]. Due to its natural antioxidants such as flavonoids and phenolic acids, ethanol extract of the propolis decreased intracellular ROS level significantly ($p < 0.01$) with the anti-radical activity (Figure 2). Accumulating evidence strongly suggests that cancer cells are more sensitive to ROS level compared with healthy cells [30, 31]; thus, in correlation with the decrease in ROS level, cytotoxicity of the ethanol extract of the propolis increased in a dose and time dependent manner.

All concentrations of ethanol extract of the propolis caused highly significant ($p < 0.01$) increases in apoptosis rate in comparison with controls in hepatocellular carcinoma cells. Besides, highly significant differences ($p < 0.01$) in apoptosis rate of the hepatocellular carcinoma cells were found among test groups with some exceptions (non significant ($p > 0.05$) difference between 10 $\mu\text{g}/\text{ml}$ and 25 $\mu\text{g}/\text{ml}$ treated Hep3B cells; $p < 0.05$ between 25 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$ treated Hep3B cells) (Figure 3). On the other hand, the number of necrotic cells in cell line supernatants was determined; no statistically significant differences ($p > 0.05$) were found as compared to controls as well as among test groups. Thus, the cytotoxicity of the ethanol extract of the propolis on hepatocellular carcinoma cells (HepG2 and Hep3B cells) contains apoptosis rather than necrosis.

Caspase-3 assay illustrated that highly significant differences ($p < 0.01$) in caspase-3 activity of the hepatocellular carcinoma cells were found between control and test groups as well as among test groups with some exceptions ($p < 0.05$ between control cells and 10 $\mu\text{g}/\text{ml}$ treated Hep3B cells).

Due to being an executioner caspase in apoptotic pathways, caspase-3 activation is a very important step inducing apoptosis; thus, this activation is extensively used as an apoptotic marker [32-34]. Therefore, the activity of caspase-3 in hepatocellular carcinoma cells (HepG2 and Hep3B cells) was measured to evaluate the role of caspase signal cascade in apoptosis. And, it was found that ethanol extract of the propolis significantly induced the activation of caspase-3 in a dose-dependent manner (Figure 4).

Conclusion

The results revealed by the current study suggest the great potential of propolis as a potent anti-tumor compound in liver cancer treatment for further researches. Due to their potential

as therapeutic agents against various disease conditions, natural products including propolis have been used in medicine for many years; on the other hand, partly due to their low bioavailability, they have less impressive success in clinical trials [35-37]. Therefore, the incorporation of nanoparticles into natural product delivery systems will be a notable progress to enhance their therapeutic efficacy [35-39]. In this context, nanoparticles (e.g. superparamagnetic iron oxide nanoparticles (SPION) in safe concentrations [38-40]) will be very efficient nano carrier system for extracted bioactive molecules of the propolis from Central Anatolia region of Turkey, to increase the mentioned therapeutic effects. The studies to increase the therapeutic potential of the propolis through SPION-based nanotechnology approaches are ongoing in our laboratory.

Acknowledgements

The author would like to thank local beekeepers in Kayseri, Turkey for supplying samples of propolis.

Competing interests

The author confirms that this article content has no conflict of interest.

Financial Disclosure

The author declares no financial support.

Ethical approval

Consent of ethics was approved by the local ethics committee.

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References

- Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394-424.
- Liu C, Chen K, Chen P. Treatment of liver cancer. Cold Spring Harb Perspect Med. 2015;5:a021535.
- Sorkun K, Suer B, Salih B. Determination of chemical composition of Turkish propolis. Z Naturforsch. 2001;56:666-8.
- Vatansever SH, Sorkun K, Gurhan SID, et al. Propolis from Turkey induces apoptosis through activating caspases in human breast carcinoma cell lines. Acta Histochem. 2010;112:546-56.
- Farooqui T, Farooqui AA. Molecular mechanism underlying the therapeutic activities of propolis: A critical review. Curr Nutr Food Sci. 2010;6:186-99.
- Turan I, Demir S, Misir S, et al. Cytotoxic effect of Turkish Propolis on liver, colon, breast, cervix and prostate cancer cell lines. Trop J Pharm Res. 2015;14:777-82.
- Kubiliene L, Laugaliene V, Pavilonis A, et al. Alternative preparation of propolis extracts: comparison of their composition and biological activities. BMC Complement Altern Med. 2015;15:156.
- Gunduz C, Biray C, Kosova B, et al. Evaluation of Manisa propolis effect on leukemia cell line by telomerase activity. Leuk Res. 2005;29:1343-46.
- Llovet JM, Zucman-Rossi J, Pikarsky E, et al. Hepatocellular carcinoma. Nat Rev Dis Primers. 2016;2:16018.
- Ahn MR, Kumazawa S, Usui Y, et al. Antioxidant activity and constituents of propolis collected in various areas of China. Food Chem. 2007;101:1383-92.
- Singleton VL, Rossi JR. Colorimetry of total phenols with phosphomolybdate-phosphotungstic acid reagents. Am J Enol Vitic. 1965;16:144-58.
- Mohammadzadeh S, Sharriatpanahi M, Hamed M, et al. Antioxidant power of Iranian propolis extract. Food Chem. 2007;103:729-33.

13. Aranda A, Sequedo L, Tolosa L, et al. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: a quantitative method for oxidative stress assessment of nanoparticle-treated cells. *Toxicol In Vitro*. 2013;27:954-63.
14. Orangi M, Pasdaran A, Shanelbandi D, et al. Cytotoxic and apoptotic activities of methanolic subfractions of *Scrophularia oxysepala* against human breast cancer cell line. *Evid Based Complement Alternat Med*. 2016;2016:8540640.
15. Ucar M, Deger O, Gerigelmez AY, et al. Effect of Turkish pollen and propolis extracts on caspase-3 activity in myeloid cancer cell lines. *Trop J Pharm Res*. 2016;15:2445-49.
16. Kumazawa S, Hamasaki T, Nakayama T. Antioxidant activity of propolis of various geographic origins. *Food Chem*. 2004;84:329-39.
17. Ozdal T, Sari-Kaplan G, Mutlu-Altundag E, et al. Evaluation of Turkish propolis for its chemical composition, antioxidant capacity, anti-proliferative effect on several human breast cancer cell lines and proliferative effect on fibroblasts and mouse mesenchymal stem cell line. *J Apic Res*. 2018;57:1-12.
18. Misir S, Aliyazicioglu Y, Demir S, et al. Antioxidant properties and protective effect of Turkish propolis on t-BHP-induced oxidative stress in foreskin fibroblast cells. *Indian J Pharm Educ*. 2018;52:94-100.
19. Kalaycioglu Z, Kaygusuz H, Döker S, et al. Characterization of Turkish honeybee pollens by principal component analysis based on their individual organic acids, sugars, minerals, and antioxidant activities. *LWT-Food Sci Technol*. 2017;84:402-8.
20. Freire KRL, Lins ACS, Dórea MC, et al. Palynological origin, phenolic content, and antioxidant properties of honeybee collected pollen from Bahia, Brazil. *Molecules*. 2012;17:1652-64.
21. Negri G, Teixeira EW, Alves ML, et al. Hydroxycinnamic acid amide derivatives, phenolic compounds and antioxidant activities of extracts of pollen samples from Southeast Brazil. *J Agric Food Chem*. 2011;59:5516-22.
22. Kocot J, Kielczykowska M, Luchowska-Kocot D, et al. Antioxidant potential of propolis, bee pollen, and royal jelly: Possible medical application. *Oxid Med Cell Longev*. 2018;2018:7074209.
23. Qiu GH, Xie X, Xu F, et al. Distinctive pharmacological differences between liver cancer cell lines HepG2 and Hep3B. *Cytotechnology*. 2015;67:1-12.
24. Murakami Y, Hayashi K, Hirohashi S, et al. Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res*. 1991;51:5520-5.
25. Chi TY, Chen GG, Lai PB. Eicosapentaenoic acid induces Fas-mediated apoptosis through a p53-dependent pathway in hepatoma cells. *Cancer J*. 2004;10:190-200.
26. Choi E, Kim G. Antioxidant and anticancer activity of *Artemisia princeps* var. *orientalis* extract in HepG2 and Hep3B hepatocellular carcinoma cells. *Chin J Cancer Res*. 2013;25:536-43.
27. Žižić JB, Vuković NL, Jadranin MB, et al. Chemical composition, cytotoxic and antioxidative activities of ethanolic extracts of propolis on HCT-116 cell line. *J Sci Food Agric*. 2013;93:3001-9.
28. Sainz RM, Lombo F, Mayo JC. Radical decisions in cancer: redox control of cell growth and death. *Cancers (Basel)*. 2012;4:442-74.
29. Feitelson MA, Arzumanyan A, Kulathinal RJ, et al. Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. *Semin Cancer Biol*. 2015;35:S25-54.
30. Liu J, Wang Z. Increased oxidative stress as a selective anticancer therapy. *Oxid Med Cell Longev*. 2015;2015:294303.
31. Kaushik N, Uddin N, Sim GB, et al. Responses of solid tumor cells in DMEM to reactive oxygen species generated by non-thermal plasma and chemically induced ROS systems. *Sci Rep*. 2015;5:8587.
32. Cohen GM. Caspases: The executioners of apoptosis. *Biochem J*. 1997;326:1-16.
33. Janicke RU, Sprengart ML, Wati MR, et al. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem*. 1998;273:9357-60.
34. Eom HS, Lee EJ, Yoon BS, et al. Propolis inhibits the proliferation of human leukaemia HL-60 cells by inducing apoptosis through the mitochondrial pathway. *Nat Prod Res*. 2010;24:375-86.
35. Watkins R, Wu L, Zhang C, et al. Natural product-based nanomedicine: recent advances and issues. *Int J Nanomedicine*. 2015;10:6055-74.
36. Bilia AR, Piazzini V, Guccione C, et al. Improving on nature: The role of nanomedicine in the development of clinical natural drugs. *Planta Med*. 2017;83(5):366-81.
37. Bilia AR, Piazzini V, Risaliti L, et al. Nanocarriers: A successful tool to increase solubility, stability and optimise bioefficacy of natural constituents. *Curr Med Chem*. 2018;[Epub ahead of print]
38. Gokduman K, Demir AS. Camptothecin anticancer drug loaded iron oxide micro and nanoparticles: Tuning targeted and sustained release of the drug in bioactive form. *Curr Nanosci*. 2013;9:704-10.
39. Gokduman K. Strategies targeting DNA topoisomerase I in cancer chemotherapy: Camptothecins, nanocarriers for camptothecins, organic non-camptothecin compounds and metal complexes. *Curr Drug Targets*. 2016;17:1928-39.
40. Gokduman K, Bestepe F, Li L, et al. Dose-, treatment- and time-dependent toxicity of superparamagnetic iron oxide nanoparticles on primary rat hepatocytes. *Nanomedicine*. 2018;13:1267-84.