Research Communication

Different Propolis Samples, Phenolic Content, and Breast Cancer Cell Lines: Variable Cytotoxicity Ranging from Ineffective to Potent

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ABSTRACT

Researchers have started focusing on investigating the anticarcinogenic effects of natural products with the slightest side effects possible, because current breast cancer treatment approaches are unable to achieve absolute success especially on aggressive subtypes. Propolis is among these products with its antimicrobial, antifungal, anti-inflammatory, and anticancer effects. Therefore, seven different samples were collected from different regions (Argentina, China, and Istanbul-Turkey) and applied on nonaggressive breast cancer cell line (BCCL) MCF-7 and aggressive cell lines SK-BR-3, and MDA-MB-231. Initially, the phenolic/flavonoid constituents of the propolis ethanol extracts were investigated by liquid chromatography-mass spectrometry–mass spectrometry (LS-MS/MS) and high-performance liquid chromatography (HPLC) analyses. Then, the anticarcinogenic effects of the propolis samples on MCF-7, SK-BR-3, MDA-MB-231 were evaluated by WST1 analysis and only selected ones on MCF-10A and hPdLF. According to the LS-MS/MS and HPLC analysis, Turkey originated propolis (Turkey3) were found to be richer than the other propolis samples in terms of phenolic/flavonoid compounds. Turkey propolis significantly inhibited cell proliferation in both nonaggressive and aggressive BCCL ($P < 0.01$). Therefore, Turkey3 propolis was selected for further evaluation using Annexin V-PI apoptosis detection assays. In addition, selected compounds among the propolis contents such as galangin, caffeic acid, apigenin, quercetin, and ferulic acid were applied to the MCF-7 cell line to detect cytotoxic and apoptotic effects. Galangin, caffeic acid, apigenin, and quercetin remarkably induced cell proliferation inhibition at all time intervals, whereas ferulic acid was found non efficient on the MCF-7 cell line. Annexin V-PI assay clarified that all cell proliferation inhibitions were markedly apoptotic. Our findings indicated that the inhibition effect of propolis on breast cancer cell proliferation was in a propolis type-, dose- and time-dependent fashion. Turkey3 propolis showed statistically significant cytotoxic effects on both the nonaggressive and aggressive BCCL. These findings were consistent with the effects of its rich phenolic and flavonoid contents, in terms of variety. © 2018 IUBMB Life, 71(5):619–631, 2019

Keywords: propolis; breast cancer; cell line; flavonoid; LS/MS–MS; cytotoxicity; apoptosis

Abbreviations: *BCCL, breast cancer cell lines; CAPE, caffeic acid phenethyl ester; EECP, ethanol extract of Chinese propolis; ER, estrogen receptor; FBS, fetal bovine serum; HER2, human epithelial growth factor receptor 2; HPLC, high-performance liquid chromatography; IC50, the half maximal inhibitory concentration.; LC–MS/MS, liquid chromatography-mass spectrometry–mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; PI, propidium iodide; PR, progesterone receptor; WST-1, water soluble tetrazolium-1*

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INTRODUCTION

Since ancient times, physicians have been using propolis in folk medicine due to its many potent characteristics. It has been shown that propolis possesses a wide spectrum of biological properties such as anticancer, antioxidant, anti-inflammatory, antibiotic, and antifungal activities (1). Recently, propolis is being widely used in foods and beverages by virtue of preventing or ameliorating various illnesses including heart disease (2), diabetes (3), inflammatory diseases (4) and cancer (5–7). Propolis is a natural and adhesive substance produced by honeybees, *Apis melifera*, by collecting various constituents from tree exudates, leaf buds, and other parts of plants (8). It comprises mostly bee wax (20–30%) and resin (40–80%). Moreover, propolis has shown to contain more than 300 compounds such as alcohols, aromatic acids and esters, aldehydes, fatty acids, terpenoids, steroids, carbohydrates, and most importantly flavonoids and phenolic acids (9).

Due to the rich bioactive content of propolis, it found a place for itself as a promising agent in cancer research. Therefore, its various effects on breast cancer, the most common cancer among women, have been investigated in vitro and in vivo. (10). Breast cancer is classified into three subgroups according to their receptor status, among which the estrogen receptor positive (ER+) group is the most common type with an incidence of 50–70%. Human epidermal growth factor receptor 2, HER2/*neu* (ERBB2), amplification or overexpression is a very valuable marker, because it can be the main object of targeted therapy. The HER2 (+) group comprises 15–20% of all breast cancers. Moreover, the group lacking ER, progesterone (PR) or HER2 receptors, which is identified as triple negative breast cancer, is also named basal-like breast cancer (11). So far, an effective and successful treatment, especially for the aggressive breast cancer types, has not been developed. Therefore, there is a growing need for new anticancer agents that may be effective in prevention and/or treatment of breast cancer subtypes (12). Natural products such as propolis and its bioactive compounds can be useful in the prevention or treatment of breast cancer. Thus, the number of in vivo and in vitro studies investigating the anticancer effects of natural products is increasing for the treatment of breast cancer (13, 14).

Recently, in vitro cell culture studies on the cytotoxic effects of propolis extracts from different regions have shown noteworthy findings (15–22). In this context, the anticarcinogenic effects of propolis originated from diverse geographies were studied on breast cancer cell lines (BCCLs) (23–29). Lately, the chemical compositions, biological properties, and therapeutic activities of Turkey propolis have been analyzed using chromatographic and molecular biological techniques (30–34) and the typical Turkey poplar propolis samples displayed high phenolic and flavonoid contents (32). However, the anticarcinogenic effects of Turkey propolis have only been investigated in a limited number of studies (7, 35, 36). According to the best of our knowledge, there are only two previous studies that investigated the anticarcinogenic effects of Turkey propolis on BCCLs (24, 37). It has been shown that the apoptosis induction effect of propolis increases relative to dose.

In the present study, to clarify the potential anticarcinogenic effects of eight propolis samples collected from different regions (China, Argentina, and Turkey) on BCCLs, modeling aggressive and hormone sensitive breast cancer types were evaluated. For this purpose, we investigated the cytotoxic and apoptotic effects of these propolis samples on cell lines representing ER/PR(+), HER2/*neu*(−) (MCF-7), ER/PR(−), HER2/*neu*(+) (SK-BR-3) and triple negative (MDA-MB-231) breast cancers. In addition, it was also aimed to reveal the anticarcinogenic activities of some of the active phenolic and flavonoid contents of the propolis samples, individually. Therefore, the cytotoxic and apoptotic effects of galangin, caffeic acid, apigenin, quercetin, and ferulic acid on MCF-7 BCCL were studied in vitro.

MATERIAL AND METHODS

Propolis Extracts

Eight different propolis samples from different geographic locations were obtained from Altiparmak Inc. (Istanbul, Turkey). Prior to any application, the propolis samples were dispersed in 60% ethanol and all were filtered in 0.22 μm for sterilization. Biochemical and microbiological analysis of propolis samples were conducted in Altiparmak Apilab Laboratories (Istanbul, Turkey) and no heavy metals such as mercury, cadmium, lead, tin, or arsenic were detected in seven propolis samples. However, one of the Argentina-originated propolis samples was not included in the following studies due to the high level of heavy metal content. Moreover, no pathogens such as bacterial or yeast contamination or pesticides or antibiotics were detected in any of the studied propolis samples.

The Chemical Characterization of Ethanol Extract of Propolis

LS/MS–MS Experiment Chemicals. Stock solutions were prepared as 10 mg/L in methanol, which were prepared as 0.1 mg/L and 5 mg/L, respectively, in the same solvent. High-performance liquid chromatography (HPLC) grade methanol was purchased from Merck (Darmstadt, Germany). Calibration solutions were prepared in methanol in a linear range (Table 1). Dilutions were performed using automatic pipettes and glass volumetric flasks (A class), which were stored at −20 °C in glass containers. A 100 mg/L curcumin solution was freshly prepared, from which 50 μL was used as an Internal Standard (IS) in all the experiments. Compounds given in Table 2 were used as standards in liquid chromatography-mass spectrometry–mass spectrometry (LC–MS/MS) analysis and were purchased from Sigma-Aldrich, St. Louis, MO (Merck), ExtraSynthese (Genay-France).

Preparation of Test Solution. Of the extracts, 100 mg were dissolved in 25 mL ethanol-water (60:40 v/v) in a volumetric flask, were refluxed in ethanol-water (60:50 v/v) for 1 h, from which 400 μL was transferred into a 1 mL of volumetric vial.

TABLE 1 *LC–MS/MS parameters of selected compounds*

	Compounds	Parent ion	Daughter ion	Collision energy (V)
1	Kaempferol	287	152.3	30
2	Fumaric acid	115	71	8
3	Pyrogallol	125	80	16
4	p-OH benzoic acid	136.7	92.6	12
5	vanillin	150.7	135.4	12
6	p-coumaric acid	163.2	118.7	14
7	Caffeic acid	179	135	10
8	t-ferulic acid	193	133	15
9	Apigenin	269	151	22
10	Quercetin	301	178.5	16
11	Ellagic acid	301	228.3	25
12	Isorhamnetin	315	300	15
13	Quercetagetin-3,6-dimethylether	345.1	329.5	16
14	Chlorogenic acid	353	191	14
15	Rosmarinic acid	359.2	160.5	15
16	Kaempferol-3-rutinoside	593	284.4	18
17	Rutin	609	301	16
18	Gallic acid	168.6	124	13
19	Salvigenin	329	295.8	15
20	Penduletin	345.2	311	25
21	Curcumin[a]	369.3	176.9	20

[a] *Used as internal standard.*

TABLE 2 *Validation and Uncertainty parameters*

	Compounds	Linear regression equation	R^2	LOD (mg/L)	LOQ (mg/L)	RSD (%)
1	Kaempferol	y = 0.022x + 0.016	0.9898	0.002	0.008	5.47
2	Fumaric acid	y = 0.056x + 0.018	0.9912	0.003	0.010	5.44
3	Pyrogallol	y = 0.039x + 0.014	0.9876	0.001	0.002	5.47
4	p-OH benzoic acid	y = 0.129x + 0.020	0.9979	0.002	0.007	4.78
5	Vanillin	y = 0.098x + 0.016	0.9983	0.019	0.064	6.57
6	p-coumaric acid	y = 0.289x + 0.153	0.9873	0.006	0.021	6.39
7	Caffeic acid	y = 0.351x-0.022	0.9981	0.028	0.093	8.04
8	t-ferulic acid	y = 0.086x + 0.015	0.9922	0.047	0.158	5.21
9	Apigenin	y = 0.181x + 0.078	0.9920	0.150	0.501	4.01
10	Quercetin	y = 0.111x + 0.069	0.9810	0.001	0.002	0.11
11	Ellagic acid	y = 0.024x + 0.005	0.9951	0.020	0.068	0.11
12	Isorhamnetin	y = 0.251x + 0.115	0.9812	0.088	0.294	3.67
13	Quercetagetin-3,6-dimethylether	y = 0.019x + 0.013	0.9876	0.022	0.074	0.10
14	Chlorogenic Acid	y = 0.262x-0.0004	0.9981	0.445	1.483	5.45
15	Rosmarinic acid	y = 0.177x + 0.014	0.9934	0.022	0.072	3.73
16	Kaempferol-3-O-Rutinoside	y = 0.108x + 0.013	0.9978	0.014	0.045	8.15
17	Rutin Hydrate	y = 0.023x + 0.001	0.9966	0.010	0.034	7.90
18	Gallic acid	y = 0.401x-0.020	0.9986	0.030	0.561	5.45
19	Salvigenin	y = 0.095x + 0.023	0.9948	0.036	0.119	5.21
20	Penduletin	y = 0.151x + 0.034	0.9949	0.089	0.297	9.47

Then, 50 μL of curcumin was added as an IS and diluted to the volume with 550 μL of mobile phase and vortexed for 20 s. Then, 10 μL of sample was injected to LC. Samples in the autosampler were kept at 15 °C during the experiment.

Instruments and Chromatographic Conditions. LC–MS/MS analyses were conducted using a Zivak® HPLC and Zivak® Tandem Gold Triple Quadrupole (Istanbul, Turkey) mass spectrometer, equipped with a Synergy Max C18 column (250 × 2 mm i.d., 5 μm particle size). The mobile phase was composed of water (A, 0.1% formic acid) and methanol (B, 0.1% formic acid), and the gradient program was 0–1.00 min 55% A and 45% B, 1.01–20.00 min 100% B and finally 20.01–23.00 55% A and 45% B. The flow rate of the mobile phase was 0.25 mL/min, and the column temperature was set to 30 °C. The injection volume was 10 μL.

Optimization of the LC/MS/MS Procedure. The optimum mobile phase solution was determined to be a gradient of acidified methanol and water system and good ionization of small and relatively polar antioxidants was obtained by the ESI source using triple quadrupole mass spectrometry system, details of experimental parameters are also given in our previous studies (38–40). For the MS analysis of samples, the following settings were used: CID gas pressure of 2.40 mTorr, EIS needle voltage of 5,000 V, EIS shield voltage of 600 V and drying gas temperature of 300 °C, API housing temperature of 50 °C, Nebulizer gas pressure of 55 psi and drying gas pressure of 40 psi. Detailed information on experiment parameters are given in Table 1.

Validation of Experiments and Uncertainty Evaluation. The validation parameters of the developed method were determined as linearity, repeatability, limit of detection (LOD), and limit of quantification (LOQ). Curcumin was used as an IS in all experiments. The linearity for each compound for the reported method was determined by analyzing the standard solution. Correlation coefficients were found to be higher than 0.99. Linear regression equations of the reported compounds are also presented in Table 2, where y is the peak area and x is the concentration.

The precision of the reported method was evaluated by repeating the measurements with three different concentrations. A good precision was determined and the results were implemented to the uncertainty budget. Finally, a LOD and LOQ for the reported method of the above compounds were calculated to be 0.5–50 mg/L. The LODs were determined to be 3 times bigger than the LOQ. The concentration of each analyte within the linear range and concentration of the reported method were obtained from the calibration curve. Finally, the calculated concentrations were converted to mg/kg of crude sample by the equation given as follows:

$$\text{Amount}(\text{mg/kg}) = \frac{C_a \times V_{final}}{m \times V_{initial}} \times 1000$$

where C_a is the concentration of compound (in mg/L), V_{final} is the final diluted volume, m is amount of extract as gram, and $V_{initial}$ is the initial sample volume.

Sources and quantification of the uncertainty of LC–MS/MS method were determined using EURACHEM/CITAC Guide, 2000. The maximum contribution comes from the calibration curve. Detailed procedures of uncertainty evaluation are previously reported in the literature (38, 39, 41).

HPLC Analysis. In the HPLC analysis, propolis extracts were prepared to 0.1 g propolis sample by adding 25 mL of 60% ethanol. The prepared extracts were filled in 1.5 mL vials and applied to the HPLC system. The HPLC system consisted of the Waters 600 control unit and the Waters 996 PDA detector. The C18 column was used to examine the phenolic materials. Flow rate was 1 mL/min and **A** (0.1% TFA containing pure water) and **B** (0.1% TFA containing acetonitrile) solvent system were used. The phenolic substance profiles were determined in the HPLC/PDA unit of the samples. Identification and quantification of the peaks of the phenolic compounds were carried out at the wavelengths at which the compounds gave maximum absorbance and measurements were made at 280, 312, and 360 nm (42, 43).

Cell Culture. All cell culture materials were purchased from (Biochrome, Berlin, Germany) and the ER/PR(+), HER2(–) human BCCL MCF-7; ER/PR(–) and HER2(+) human BCCL SK-BR-3; ER/PR(–), HER2(–) human BCCL MDA-MB-231; and fibrocystic breast tissue MCF-10A from American Type Culture Collection (ATCC), (ATCC, Rockville, MD) and human periodontal ligament cell line hPdLF from Lonza (Basel, Switzerland). All cell lines were maintained in their recommended mediums and all were supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin (all obtained from Biochrome, Berlin, Germany). All cell lines were maintained at 37 °C in a 5% $C0_2$ humidified atmosphere.

Cell Proliferation Analysis. The Cell Proliferation Reagent WST-1 (Roche, Manheim, Germany) was used for the cell proliferation analysis. Cell counting process was performed on Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Brea, CA, USA). Cells were seeded in 96 well plates (Greiner Bio-one, Kremsmünster, Austria) with a density of 1×10^4 cell/ well. They were permitted to adhere overnight, then the medium was replaced with fresh medium supplemented with 3% FBS. Afterward, doses (1, 2.5, 5, 50, 100, 250, 750 μg/mL) of propolis were applied to the cells to determine the effects at different time intervals (24th, 48th, and 72nd h). Also, following flavonoids were applied to the MCF-7 cell line as 5, 15, and 30 μg/mL of galangin, caffeic acid, and apigenin; 10, 25, 70 μg/mL of quercetin; 5, 20, 50 μg/mL doses of ferulic acid at 24th, 48th and 72nd h. At these time intervals, 10 μL/well WST-1 was applied and then plates were incubated for 2 h at 37 °C in 5% CO_2. Subsequently, the effects of WST-1 were monitored via measuring their absorbance at 450 nm with the reference wavelength set at 620 nm using Multiscan ELISA reader

(Thermo Fisher Scientific, Waltham, MA, USA). The viability of the control cells was set as 100%. Data are expressed as percentages of absorbance readings compared to control wells on a relative proliferation index scale (Mean ± Standard deviation). The IC50 values were calculated using an approximated sigmoidal curve. All tests were performed in triplicate.

Analysis of Apoptosis. All cell lines were cultured in six-well plates (Greiner Bio-one, Kremsmünster, Austria) at the density of 5×10^5/well. The cells were permitted to adhere overnight, then the medium was replaced with fresh medium supplemented with %3 FBS. Afterward, 100 and 250 μg/mL of propolis were selected as the most effective doses and these doses were applied to the cells. The effects of propolis on apoptosis were monitored at the 48th and 72nd h. Subsequently, their apoptotic state was evaluated using Annexin V-FITC and propidium iodide (PI) (BD, Bioscience Pharmingen, San Jose, CA) according to the manufacturer's protocol. Thereafter, cells were applied with Annexin V-FITC/PI were detected using FACSCalibur (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Statistical evaluations were performed using One-Way Anova (Dunnet) and Multiple T-tests. Values of $P < 0.05$ were considered significant. All values are expressed as mean ± standard deviation. All statistical analysis and IC50 value calculations were performed using GraphPad Prism 6 (GraphPad Prism Software, San Diego, CA).

RESULTS

LS-MS/MS Analysis of the Propolis Samples

The HPLC and LS-MS/MS analyses of the propolis samples are given in Table 3. All the analyzed flavonoid compounds were

TABLE 3 ***LS-MS/MS and HPLC Analysis of Propolis Samples***

LS-MS/MS		*China1*	*China2*	*China 3*	*Argentina*	*Turkey 1*	*Turkey 2*	*Turkey 3*
	Compounds							
1	Kaempferol	0.913	0.158	1.260	0.166	1.979	1.462	1.135
2	Fumaric acid	–	3.256	3.439	–	1.250	0.613	4.975
3	Pyrogallol	0.390	3.605	1.107	0.566	6.123	3.216	5.625
4	p-OH benzoic acid	0.069	–	–	–	0.608	2.673	0.478
5	Vanillin	–	–	–	–	0.656	2.568	–
6	p-coumaric acid	–	–	–	1.547	6.140	16.197	4.227
7	Caffeic acid	0.479	0.211	0.185	1.354	7.691	12.566	7.978
8	*t*-ferulic acid	4.839	1.032	1.107	5.024	3.572	14.617	3.121
9	Quercetin	1.579	0.141	0.563	1.715	0.682	0.494	0.734
10	Ellagic acid	0.106	–	–	0.080	–	–	0.052
11	Isorhamnetin	1.852	0.298	1.281	0.713	1.420	0.243	0.571
12	Quercetagetin-3,6-dimethyl ether	–	–	–	–	–	–	1.819
13	Chlorogenic Acid	0.284	0.272	0.275	0.283	0.272	0.271	0.301
14	Rosmarinic acid	0.134	0.139	0.134	0.134	0.140	0.135	0.139
15	Kaempferol-3-O-Rutinoside	–	–	–	–	306.28	–	–
16	Rutin Hydrate	0.056	0.059	0.060	0.084	0.119	0.071	1.136
17	Gallic acid	0.189	2.038	2.283	0.193	0.273	0.211	0.249
18	Salvigenin	–	–	–	–	–	–	2.597
19	Penduletin	–	–	–	–	–	–	0.698
	HPLC							
	Compounds							
20	Galangin	42.464	13.796	21.424	20.024	11.887	35.815	13.903
21	Chrysin	19.666	11.352	17.531	14.169	20.689	70.686	15.595
22	Pinobanksin	11.286	4.295	13.362	10.407	13.916	24.929	7.135
23	Pinocembrin	33.813	5.323	9.640	32.255	12.009	26.097	9.844
24	Pinostrobin	81.493	17.989	24.473	81.350	21.512	68.703	10.848
25	Apigenin	1.896	1.068	1.338	1.129	0.473	0.883	0.698
26	Luteolin	0.088	–	–	0.077	–	0.189	0.049
27	*t*-cinnamic acid	13.146	2.394	1.645	15.247	0.660	3.458	1.007
28	Quercetin dihydrate	0.146	0.210	0.522	0.128	0.423	0.314	0.250

Values are given as mg/g.

detected in Turkey-originated propolis samples. Moreover, vanillin, quercetagetin-3, 6-dimethyl ether, kaemferol-3-O-rutinoside, salvigenin, and penduletin were only found in Turkey-originated propolis samples. In Turkey-originated propolis samples, pyrogallol, p-OH benzoic acid, p-coumaric acid, caffeic acid, and chrysin compounds were also found in higher levels compared to the China and Argentina originated propolis samples. However, Gallic acid was found at the highest level (approximately 10 times) in China 2 and 3 propolis samples compared to the others. Pinostrobin and t-cinnamic acid in China 1 and Argentina propolis samples were detected at a considerably higher level than Turkey-originated propolis samples.

Cell Proliferation Assay

Effects of Propolis Extracts on Cell Proliferation of Cell Lines. Cell proliferation experiments were analyzed using the WST-1 assay. The MCF-7, MDA-MB-231, and SK-BR-3 BCCLs were treated with different doses (2.5, 5, 15, 100, 250, 500, 750 μg/mL) of propolis extracts for the 24th, 48th and 72nd h. To decide on the concentrations of propolis and its compounds preliminary assays were performed with a wide range of concentrations, which demonstrated that concentrations of propolis between 2.5 μg/mL and 750 μg/mL inhibit the proliferation of the investigated human cancer cell lines. Cell viability is represented as the percentage of relative absorbance by comparison with untreated cells (% of the control).

Cell proliferation plots of all WST-1 assays were normalized via the R statistical language program. Following the normalization of the WST-1 data, statistical analysis was performed. The WST1 plots of the four most effective of the tested seven propolis samples are given in Figure 1 (China2, Argentina, Turkey2, and Turkey3).

In the **MDA-MB-231** cell line, the most aggressive BCCL, China 2 propolis was cytotoxic only at high doses (500–750 μg/mL) at the 24th ($P < 0.0001$) and 72nd ($P < 0.01$) h. However, propolis samples from Argentina showed significant effects on these cells ($P < 0.001$). A mild but not statistically significant proliferative effect of Turkey 2 propolis was observed ($P > 0.05$). On the other hand, Turkey3 propolis demonstrated the most potent cytotoxic effect at early periods (24th h) starting from 100 μg/mL ($P < 0.01$) and late time intervals (48th and 72nd h) starting from 5 μg/mL dose ($P < 0.0001$).

On **SK-BR-3** cells cytotoxic effects of administered propolis were observed at lower doses compared to MDA-MB-231 cells. The China2 propolis extract was observed to be cytotoxic at moderate to high doses starting from 100 μg/mL at all time intervals (24th, 48th, and 72nd h) on SK-BR-3 cells ($P > 0.05$). Argentina propolis has been found to have a significant cytotoxic effect from lower doses (50 μg/mL) ($P < 0.01$), at all of the time intervals in this cell line. Turkey2 propolis did not show a significant cytotoxic effect on SK-BR-3. The effect of Turkey3 propolis at early periods was observed starting from the dose of 100 μg/mL ($P < 0.001$). Moreover, its effect was similar to that

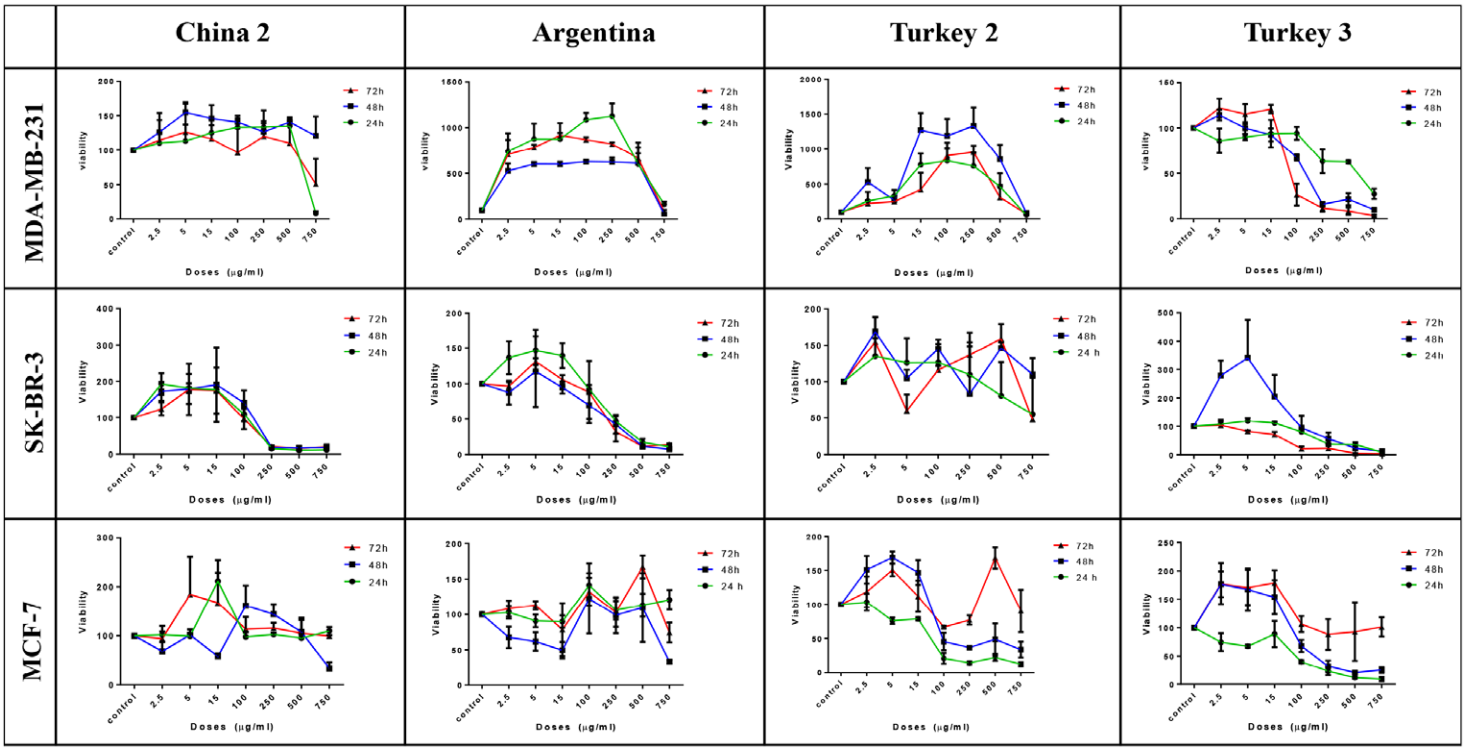

FIG 1 *The WST1 plots of the China2, Argentina, Turkey2, and Turkey3 propolis samples in aggressive and nonaggressive BCCLs.*

of MDA-MB-231 cell line and was cytotoxic at low doses (2.5 μg/mL) at the 72nd ($P < 0.001$).

On the **MCF-7** cell line, China2 and Argentina propolis showed no significant toxicity. Turkey2 propolis showed a strong cytotoxic effect at the early periods (24th h) starting from the 2.5 μg/mL dose; however, this effect fades at later periods. On the MCF-7 cell line, Turkey3 propolis caused progressive cytotoxicity from the 2.5 μg/mL dose at the 24th h. At the 48th h, cell proliferation inhibition was observed starting from the 5 μg/mL dose ($P < 0.0001$). Similar with Turkey 2 this effect was not observed at later periods (Fig. 1).

Since Argentina and Turkey2 did not show reliable cytotoxicity patterns and yet China2 and Turkey3 were the two most effective propolis extracts selected in WST-1 cytotoxicity analysis, they were also applied to the non-tumorigenic *fibrocystic* mammary epithelial cell line, **MCF10A**. China2 showed cytotoxicity starting from the 5 μg/mL dose ($P < 0.01$), whereas the cytotoxic effect of Turkey3 started from 100 μg/mL dose in this cell line at all time points ($P < 0.01$) (Fig. 2). Based on the MCF10A findings, only Turkey3 was applied on the healthy Human Periodontal Ligament Fibroblast (**hPdLF**) cell line, which was found non cytotoxic. This selective inhibitory effect is the main purpose in cancer treatment (Fig. 3).

Effects of the Flavonoid Compounds on the MCF-7 Cell Line.. Cell viability/cytotoxicity effects of five bioactive compounds individually, which were found in all studied propolis samples mutually that have also been widely studied in the literature, were only studied on the MCF-7 cell line in this study, because it represents the most common breast cancer type. MCF-7 cells were treated with five flavonoids with a range of different doses (caffeic acid and galangin: 5, 15, 30 μg/mL; ferulic acid: 5, 20, 50 μg/mL; apigenin: 5, 15, 30 μg/mL; quercetin: 10, 25, 70 μg/mL) at 24th, 48th, and 72nd h. Galangin showed a remarkable cytotoxic effect in all doses and all time points ($P < 0.0001$). Cytotoxic effect of caffeic acid was detected mostly at the 24th h in all doses ($P < 0.0001$); however, it showed

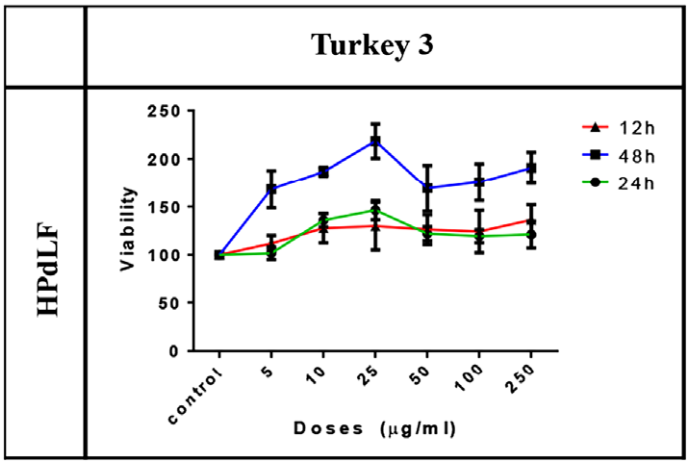

FIG 3 *The WST1 plots of the Turkey3 propolis sample in the human periodontal ligament fibroblast (hPdLF) cell line.*

cytotoxicity merely at the 48th and 72nd h starting from 15 μg/mL ($P < 0.0001$). The cytotoxic effect of Quercetin was determined at a mild-to-moderate dose at the 24th h ($p < 0.05$), whereas at the 72nd h, it was detected starting from the lowest doses ($P < 0.0001$). A remarkable cytotoxicity of apigenin was detected at the 48th and 72nd h in all doses ($P < 0.0001$). Cytotoxicity was increasing in the order of quercetin < apigenin < caffeic acid < galangin on the MCF-7 BCCL. Hovewer, ferulic acid did not show any cytotoxicity and statistical significance on MCF-7 ($P > 0.05$). The detailed WST1 results are given in Table 4.

Annexin V-PI Assay

Apoptotic Effects of the Propolis Extracts. Considering the results of the Annexin V-PI assay (Fig. 4), Turkey3 induced cell death as concordant with increasing doses. Treatment of 100 μg/mL and 250 μg/mL doses of the Turkey3 propolis on MCF-7 induced apoptosis at the 48th and 72nd h. Furthermore,

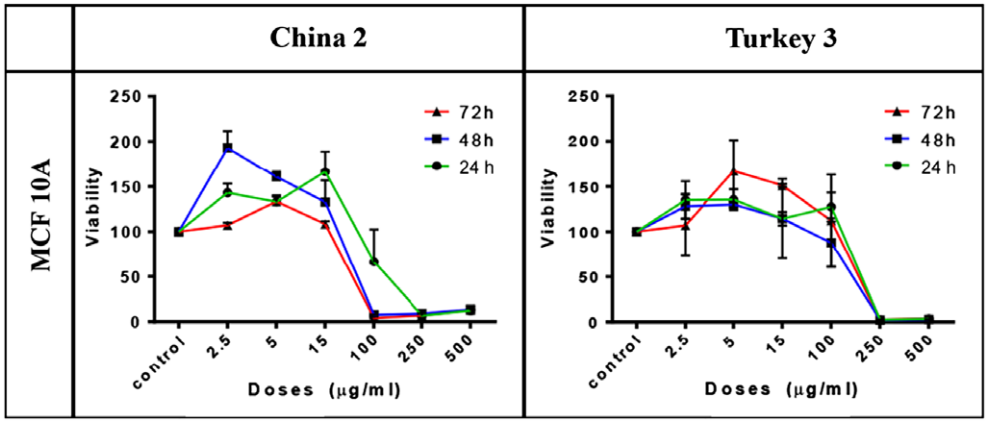

FIG 2 *The WST1 plots of the China2 and Turkey3 propolis samples in nontumorigenic* fibrocystic *mammary epithelial cell line, MCF10A.*

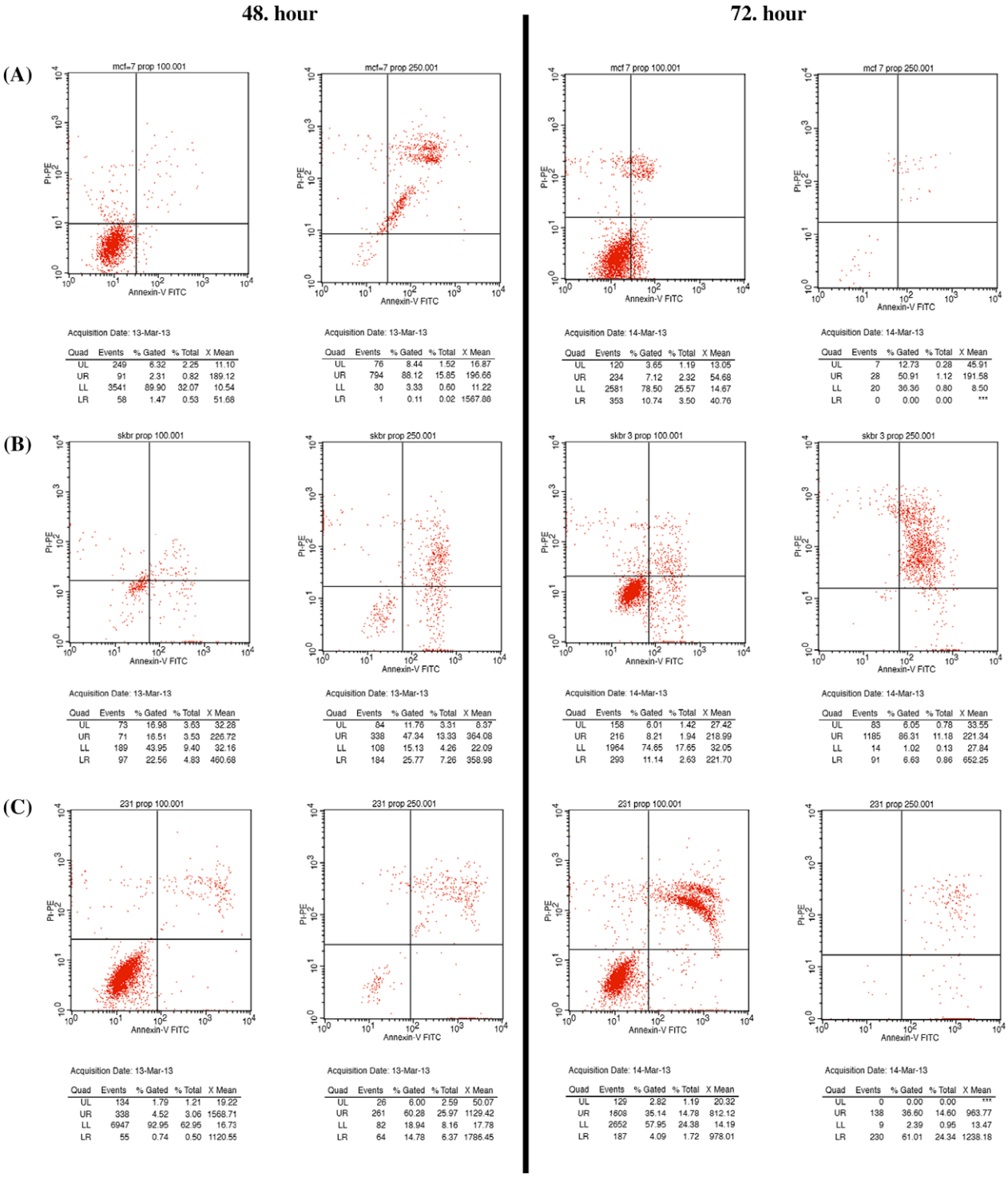

FIG 4 *Annexin V-PI results. Turkey3 Propolis induced apoptosis of MCF-7 (A), SK-BR-3 (B) and MDA-MB-231 (C) cells. Cells were treated with selected concentrations (100 and 250 μg/mL) of propolis for 48 h and 72 h double staining with annexin-V FITC and propidium iodide. Quadrant locations for dot plot graphics: lower-left (LL) indicates negative immunofluorescence (living cells); lower-right (LR) indicates annexin V positive (early apoptotic cells); upper-right (UR) indicates double positive annexin V and PI (late apoptotic cells); upper-left (UL) indicates negative annexin-V and positive PI (necrotic cells).*

Turkey3 lead SK-BR-3 cells to apoptosis starting from the 100 μg/mL dose and especially dramatically with the 250 μg/mL dose at the 48th and 72nd h. Similarly, after MDA-MB-231 cells were exposed to Turkey3, apoptotic cells were observed starting from the 100 μg/mL and 250 μg/mL doses at the 48th and even more remarkably at the 72nd h. These SK-BR-3 and

TABLE 4 *WST1 results of selected flavonoid compounds*

		Hours					
		24th		48th		72nd	
		$X \pm SD$	P *value*	$X \pm SD$	P *value*	$X \pm SD$	P *value*
Propolis Flavonoid Compounds	*Doses (μg/ml)*						
Galangin	5	43.74 ± 2.578	<0.0001	45.33 ± 3.440	<0.0001	77.99 ± 3.679	0.0010
	15	15.36 ± 1.542	<0.0001	18.15 ± 0.853	<0.0001	13.67 ± 2.011	<0.0001
	30	11.92 ± 0.467	<0.0001	8.528 ± 0.829	<0.0001	14.45 ± 1.299	<0.0001
Caffeic acid	5	42.40 ± 3.395	<0.0001	70.19 ± 3.964	0.0017	86.83 ± 3.565	0.0209
	15	35.66 ± 9.464	0.0024	66.71 ± 10.10	0.0300	52.15 ± 3.274	0.0001
	30	11.19 ± 5.550	<0.0001	29.23 ± 12.72	0.0051	18.84 ± 3.688	<0.0001
Apigenin	5	96.62 ± 3.348	0.281	69.23 ± 12.30	0.030	71.88 ± 10.49	0.023
	15	53.53 ± 5.259	0.0001	14.37 ± 4.772	0.0001	25.66 ± 5.026	0.0001
	30	88.81 ± 15.29	0.491	21.95 ± 0.268	0.0001	8.74 ± 0.69	0.0001
Quercetin	10	94.77 ± 2,\.444	0.0991	78.47 ± 4.808	0.0110	69.64 ± 2.174	0.0002
	25	62.30 ± 2.415	<0.0001	79.34 ± 7.142	0.0445	32.71 ± 1.619	<0.0001
	70	35.16 ± 6.085	0.0004	46.08 ± 3.404	<0.0001	16.70 ± 0.886	<0.0001
Ferulic acid	5	80.07 ± 3.292	0.0009	144.4 ± 7.648	0.0011	139.3 ± 8.104	0.0022
	20	72.28 ± 5.500	0.0024	114.5 ± 8.844	0.1527	105.7 ± 1.246	0.0028
	50	68.03 ± 4.500	0.0004	93.79 ± 12.48	0.6365	106.1 ± 3.232	0.0752

Values are given as Mean ± Standard deviation.

MDA-MB-231 Annexin V-PI findings showed compatible results with the WST-1 data of the same cell lines.

Apoptotic Effects of the Flavonoid Compounds on MCF-7. Following the application of 5, 15, and 30 μg/mL doses of Apigenin on MCF-7 at all time points, we detected nearly 25% apoptotic cells in Annexin V-PI assay at the 24th h. The highest level of apoptotic death was observed at the 72nd h with more than 60%. Following the caffeic acid treatment of 5, 15, and 30 μg/mL on MCF-7, nearly 25% apoptotic cells were detected at all time points, most prominently at the 24th h with more than 40% apoptotic cells. In contrast, at the 48th and 72nd h, the apoptotic cell rate was decreased to approximately 10%.

When 5, 20, and 50 μg/mL of ferulic acid were applied on MCF-7; the apoptotic cell death ratio was low as 10.60%, 8.44%, and 16.19%, respectively, at the 24th h. Application of 5, 15, 30 μg/mL of galangin on MCF-7 showed that the 15 μg/mL dose lead the cells to apoptosis, in which more then 35% apoptotic cells were detected at the 48th and 72nd h. In addition, following the exposure of 10, 25, 70 μg/mL of Quercetin moderate apoptotic effect was observed on MCF-7 (10–30% apoptotic cells). Detailed Annevin V-PI assay results are given in Table 5.

Shortly, the Annexin V-PI assay exhibited dramatical findings in MCF-7. Although all doses of caffeic acid induced apoptotic death at the 24th h, 30 μg/mL dose of apigenin and galangin showed a remarkable apoptotic affect at the 72nd h.

DISCUSSION

Propolis has been used for medicinal purposes throughout history (44). Today, the positive contribution of propolis to health, which are via its antimicrobial, anti-inflammatory, regenerative, immunomodulatory, antioxidant and antimutagenic effects have been proven by scientific studies (1–4, 6). One of the most important biological attributes of propolis has been pointed out by various studies to be the anticarcinogenic effects (5, 7, 15–28).

Many efforts have been made in the search for effective and safe treatment, and prevention of breast cancer (12, 13). In this context, propolis and its active compounds hold promise as an adjunct to breast cancer treatment and prevention with its numerous effects.

The ethanol extracts of Brazilian red propolis has been reported to significantly reduce the viability of estrogen receptor positive (ER+) MCF-7 breast cancer cells through the induction of mitochondrial dysfunction and DNA fragmentation (26). The antiproliferative activities of the ethanol extract of brown Cuban propolis (BCP) were also investigated on human BCCLs.

TABLE 5 *Annexin V-PI results of flavonoids*

		Percentage of apoptotic cells		
Flavonoids	*Doses (μg/ml)*	*24th h*	*48th h*	*72nd h*
Apigenin	5	23	25	30
	15	19	26	36
	30	26	26	63
Caffeic acid	5	44	4	12
	15	42	8	12
	30	46	18	18
Ferulic acid	5	11	9	12
	20	8	14	15
	50	16	19	41
Galangin	5	37	37	13
	15	15	24	36
	30	26	35	72
Quercetin	10	20	10	23
	25	29	11	32
	70	23	32	27

Percentage of apoptotic cells were given by rounding off.

They reported that BCP exhibit a significant antiproliferative activity on MCF-7 rather than triple negative MDA-MB 231 ($P < 0.005$), in a dose (1–25 μg/mL) and time-dependent (24th–48th h) manner. This inhibition of cell growth in the cell cycle G1 phase by BCP application was suggested to be apoptotic (23).

Moreover, the anticancer/cytotoxic effects of the ethanol extract of Chinese propolis (EECP) were studied (25, 50, 100, and 200 μg/mL) on MCF-7 and MDA-MB-231. Treatment of EECP induced dose- and time-dependent cytotoxic/apoptotic effects on both cells. However, it was shown that EECP inhibited migration in MDA-MB-231 cells in a dose-dependent manner at the 48th h ($P < 0.01$), whereas this inhibitory effect did not reach significance on MCF-7 (27). Moreover, later Nemorosone, which is found in the content of Brown Cuban propolis, was also individually studied and it inhibited the cell viability of ERα(+) MCF-7, but not of ERα(–) MDA-MB-231 and LNCaP (25).

Furthermore, the cytotoxic effects of CAPE and Ashland propolis on different BCCLs were investigated and its inhibitory effects were reported on SK-BR3 for the first time (29). They noted decreased MCF-7, MDA-MB-231, and SK-BR-3 viabilities in a concentration and time-dependent manner by CAPE (IC_{50} of 20 μM on MCF-7, SK-BR3 and 35 mM on MDA-231) and propolis (IC_{50} of about 10 μM for all the cell lines) at the 72nd h.

The cytotoxic/apoptotic effect of propolis extracts from Turkey on the caspase pathway in MCF-7 was investigated by Vatansever et al.(24). Seven different propolis extracts were collected from Ankara, Turkey. Propolis extracts exhibited a dose-dependent inhibition of cellular growth and induction of apoptosis, which was suggested to be via the activation of caspase-8,-9, and – 6.

The present study is the first to evaluate the anticarcinogenic effect of different propolis, including Argentina, China, and Turkey, on cell lines that represent all the most common breast cancer types. Moreover, the phenolic/flavonoid content of the different propolis samples were determined via LS-MS/MS and HPLC analysis. Following the content analysis, proliferative and apoptotic effects of the most effective propolis samples were investigated. To cover the most common breast cancer types, ER/PR(+), HER2/neu (–) MCF-7, ER/PR(–), HER2/neu(+) SK-BR-3, and triple negative MDA-MB-231 cell lines were selected. In addition, it was aimed to understand the effect of cell viability/cytotoxicity of some phenolic/flavonoid constituent of the propolis samples including apigenin, caffeic acid, ferulic acid, galangin, and quercetin only on MCF-7 cells. It was observed that propolis samples from different regions have different effects on cell proliferation/cytotoxicity on different types of breast cancer depending on dose and time. The effects of the flavonoids individually was only investigated on the MCF-7 cell line, because it is represents the most common breast cancer type and due to limited resources.

Turkey2 and Turkey3 propolis extracts had similar significant cytotoxic effects from relatively moderate doses on MCF-7 at the 24th–48th h. Consistently, Annexin V-PI results of the Turkey3 propolis extract confirmed these findings with the high apopototic cell presence especially with the dose of 250 μg/mL at the 48th h (approximately 90%). Argentina propolis was slightly effective on cell proliferation inhibition on MCF-7 where China2 propolis was ineffective.

Also for the first time, we studied the inhibitory effects of Turkey propolis on SK-BR-3. The Turkey3 propolis extract induced the most outstanding effect on SK-BR-3. It caused dramatical cytotoxicity at a low dose (2.5 μg/mL) especially at the 72nd h ($P < 0.001$) and starting from the 15 μg/mL dose at the 24th h. On SK-BR-3, China2 propolis was cytotoxic at moderate to high doses (100–750 μg/ml) at all time periods, whereas Argentina propolis from lower doses (15 μg/mL). Turkey2 propolis had no significant cytotoxic effect on SK-BR-3.

Furthermore, on MDA-MD-231, Turkey3 showed potent cytotoxicity from moderate doses like 15–100 μg/mL at all time intervals, yet more distinctively at the 72nd h. China2 propolis was relatively ineffective on MDA-MD-231, and the effects of the Argentina and Turkey2 propolis samples were found even in favor of cell proliferation.

Therefore, it can be seen that propolis samples from different regions demonstrated a certain cytotoxicity but with inconstant doses. Contents and their concentrations alter among regions and even annually due to climatory changes (45). In addition, different cell lines are diversely affected by these varying compositions. Thus, it can be conceived that the main determinant of the value of propolis in cancer treatment is the diversity of its content and concentrations.

Several components isolated from propolis have been shown to have anticancer activity. It is thought that caffeic acid

phenethyl ester (CAPE), chrysin, galangin, quercetin, nemorosone, ellagic acid, apigenin, ferulic acid, and so on are important active ingredients of propolis responsible for its anticarcinogenic effects (14). Past several years, anticarcinogenic effects of these propolis bioactive compounds on breast cancer cells have also been investigated intensively.

Caffeic acid is the main phenolic constituent of coffee, which is thought to be responsible for its anticarcinogenic effect. These effects have been demonstrated in various cancer types (46–48). In this study, caffecic acid showed a moderate but noteworthy anticarcinogenic effect on MCF-7, especially in the early time period. On the other hand, although it was not investigated in this study, a derivate of caffeic acid, CAPE is considered a major pharmaceutical component of propolis (49) and evaluated more extensively in the literature. CAPE was reported to inhibit the growth of the BCCLs, MDA-MB-231 (28, 29), Hs578T (28), MCF-7 (29), and SK-BR-3 (29) in a dose-dependent and exposure time-dependent manner (28). It was also reported that CAPE reduces expression of growth and transcription factors, including NF-κB (50) and induces cell cycle arrest and apoptosis (51). Furthermore, CAPE has shown a more potent migration inhibion effect on MCF-7 and MDA-MB-231cell lines than Caffeic acid (refs. 52, 53).

Ferulic acid is another chemical content of propolis. Park et al. reported that ferulic acid reduced the S-phase post to UV treatment in MDA-MB-231 (54). On the other hand, in the study conducted by Hao et al., they did not observe the growth-inhibiting effect of ferulic acid administration on the MDA-MB-231 (55). Similarly, Serafim et al. investigated the cytotoxic effects of caffeic and ferulic acid compounds (25 μM and 75 μM) on MCF-7, HS578T, and MDA-MB-231, and on a nontransformed human fibroblast cell line (56). Caffeic and ferulic acid did not inhibit the proliferation of any of the four cell lines included in that study, whereas caffeic acid derivatives, hexyl caffeate, and caffeoylhexylamide showed an overall inhibition of proliferation on all the cell lines. Hovewer, ferulic acid derivatives hexyl ferulate (HF) and feruloylhexylamide (HFA) showed diverse effects according to the cell line tested. It was shown that MCF-7 was particularly susceptible to both compounds, especially for longer incubation periods, while HS578T appeared to be largely insensitive, with the exception of a 25% inhibition of proliferation observed when HFA was incubated for 3 days. Finally, effects of ferulic acid derivatives were most potently observed on MCF-7 cells. No cell growth inhibitory effect was observed in our study on MCF-7 at doses ranging between 1 and 50 μg/mL. Our results on ferulic acid are consistent with those of Serafim et al. Interestingly, we only observed a mild apoptotic effect of ferulic acid at the 24th h.

Galangin (3, 5, 7-trihydroxyflavone) is another flavonoid content of propolis (7). In a study, it was shown that galangin is a strong inhibitor of the Hs578T cell proliferation and it was suggested that it may be useful as a chemotherapeutic in combination with agents that target other components of the tumor cell cycle and in situations where ER-specific therapeutics are ineffective (57). Liu et al. reported that galangin-induced cytotoxicity on MCF-7 and cell apoptosis via the mitochondrial pathway and PI3K/Akt inhibition as well as cell cycle arrest (58). In the present study, gGalangin showed the most compelling cytotoxicity on MCF-7 ($P < 0.0001$) at all time points, which was confirmed to be apoptotic with the Annexin V-PI assay.

Apigenin is a flavonoid commonly found in fruits, vegetables, and propolis. Bai et al. investigated the effect of apigenin on MCF-7. They showed that the growth inhibition induced by apigenin was in a dose-dependent manner (59). Tseng et al. observed that apigenin induced G2/M arrest in MDA-MB-231 in vitro and in vivo. In addition, apigenin had an inhibitory effect on histone deacetylase (HDAC) activity and induced histone H3 acetylation to promote p21 expression. Histone acetylation is a valuable mechanism of epigenetic regulation. Therefore, Tseng et al. suggested that apigenin inhibited breast cancer cell growth through the modulation of epigenetic regulation (60). In the present study, a remarkable cytotoxic effect of apigenin on MCF-7 was detected. We also found that cytotoxic effects of apigenin on MCF-7 cells are considerably apoptotic.

Furthermore, quercetin is another flavonoid that is found in propolis and one of the most investigated. Chou et al. applied quercetin with a 10–175 μM dose interval on MCF-7 and reported efficiency at the 24th and 48th h. Moreover, they detected that quercetin induced significant cell cycle arrest at the GO/G1 phase (61). In another study, doses of 25, 50, 100 μM Quercetin were applied on MCF-7 and the effect was monitored for 3 days (62). At the end of the 24th h, DNA synthesis was approximately 35% decreased with 50 μM of quercetin compared to the control cells. In the present study, 5–90 μg/mL of quercetin were applied on MCF-7 and even more prominent inhibition was observed in later periods. Consistent with our results, Deng et al. (63) reported that quercetin the cell viability inhibition effect with a dose of 40 mg/mL. Moreover, they noted that the antiproliferative effect of Quercetin on MCF-7 was via survivin gene expression. They reported that quercetin inhibited the growth of the MCF-7 cells, promoted apoptosis by inducing G0/G1 phase arrest, and regulated the expression of survivin mRNA. They also suggested that quercetin may be capable of improving the sensitivity of breast cancer cells to chemotherapy by decreasing the expression level of survivin mRNA in MCF-7 cells (63). We also observed that cytotoxic effects of Quercetin on MCF-7 cells are considerably apoptotic.

In this study, it was observed that the inhibition effect of propolis on breast cancer cell proliferation was in a propolis type-, dose- and time-dependent fashion. Our results strongly suggest that Turkey3 propolis inhibits MCF-7, SK-BR-3, and MDA-MB-231 human breast cancer cell growth via its apoptotic effects. According to the LS-MS/MS and HPLC analysis results, Turkey3 propolis did not have the total phenolic/flavonoid content with the highest concentration. However, it had the richest phenolic/flavonoid content diversity among evaluated propolis types. It has been observed that Turkey3 propolis inhibited growth and induced apoptosis in both non-aggressive and aggressive cell lines, which was confirmed by the Annexin V-PI experiments. We suggest that the prominent cytotoxic effect of

Turkey3 propolis on BCCLs, particularly on the aggressive types, is due to the rich bioactive phenolic/flavonoid components in its content. Therefore, a possible synergistic impact or high bioavailability may arise. We also analyzed the cytotoxicity of China2 and Turkey3 propolis on the nontumorigenic MCF10A epithelial cell line. China2 propolis showed cytotoxicity relatively from lower doses than Turkey3 propolis. Impressively, Turkey3 propolis had no cytotoxic effect on the hPdLF normal cell line. Therefore, Turkey3 propolis and its ingredients merit further investigation with respect to breast cancer chemoprevention or therapy due to its selective effect. Based on our findings, with a standardized propolis content and further studies, a more eminent success may be achieved in breast cancer treatment. However, we have also observed that the effects of different propolis samples on different BCCLs are diverse. This can be explained by the fact that each of the propolis collected from different regions has different phenolic/flavonoid content due to their unique geographical diversity. Supporting these results, in our previous study, we have shown that different anatolian honey samples produced different cytotoxic effects on MCF-7, SK-BR-3, and MDA-MB-231 BCCLs (64).

In addition, galangin and apigenin were found to be inhibitors of cell proliferation on MCF-7. However, quercetin and caffeic acid also showed antiproliferative effects on MCF-7, but at higher doses. These compounds, which are found within propolis, are worthy for further analyses and may be promising in the development of novel complementary approaches in the treatment of breast cancer.

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AUTHOR CONTRIBUTIONS

M.F.S, E.Y., Ö.T-K., N.S., S. G., and A.C.G. performed the experiments; A.B.C. and H.Y-A. analyzed the data; M.F.S, H.Y-A., and A.P.E. wrote the article; O.Ö. and T.Ö. conceived and designed the study and reviewed the manuscript. All the authors read and approved the manuscript.

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