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Original Article

The therapeutic approach to fibrocystic breast disease in the MCF-10A cell culture model: Striking efficacy of polyphenols

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ABSTRACT

Background and Aims: Standard treatment regimens for fibrocystic breast disease (FBD) do not provide a permanent cure and have undesirable side effects. This study aims to investigate the therapeutic potential of different honey and propolis, as well as some important polyphenols, on breast epithelial cells (MCF-10A).

Materials and Methods: The effects of five honey, two propolis extracts and seven polyphenol samples on the cell viability were assessed the WST-1 assay. Content analysis of the propolis samples was performed using high performance liquid chromatography (HPLC).

Results: Chestnut and cedar honey had antiproliferative effects on MCF-10A cells at all doses (1-10 µg/mL), as well as pine honey at the highest dose. However, multifloral honey had no similar effect. Chinese propolis had significant antiproliferative effects on MCF-10A cells at doses of 50-250 µg/mL and on the human periodontal ligament (hPDL) control cells at a dose of 5 µg/mL. Türkiye propolis only had an antiproliferative effect on MCF-10A cells at the highest dose ($p = 0.0013$). Higher levels of ferulic acid, kaempferol, caffeic acid, pinocembrin and quercetin were detected in Türkiye propolis, while Chinese propolis was rich in pinostrobin. Ferulic acid, pinostrobin and galangin showed antiproliferative properties on MCF-10A cells ($p < 0.0001$), whereas the remaining four polyphenols had no significant effect on cell viability ($p > 0.05$).

Conclusion: The findings of the study highlight the antiproliferative effects of pinostrobin, ferulic acid and galangin on MCF-10A cells and has also confirmed the antiproliferative effects of honey and propolis samples to be due to their polyphenolic properties. Therefore, this study suggests that polyphenolic substances may have both preventive and therapeutic potential in FBD.

Keywords: Fibrocystic breast, honey, MCF-10A, polyphenol, propolis

INTRODUCTION

Fibrocystic breast disease (FBD) is the most common type of benign breast disease, occurring in 30% to 60% of women worldwide, often in the 30-50 age group (Gopalani et al., 2020). The term fibrocystic describes benign breast diseases including a variety of non-malignant lesions such as nipple discharge, trauma, mastalgia, and benign tumors. While these benign lesions are not associated with an increased risk of malignancy, they are associated with up to a 50% risk of developing breast cancer under certain histopathological and clinical conditions. Some sex steroid hormones such as estrogen and progesterone have effects on this disease's progression, evaluation and treatment (McMullen, Zoumberos, & Kleer, 2019; Tu et al., 2019). FBD has clinical findings and symptoms such as a palpable mass, skin dimpling, thickening, pain and nipple discharge (Vorherr, 1986). Although fibrocyst formation is associated with high estrogen levels, more research is needed to elucidate the exact pathophysiology of FBD (Vorherr, 1986; Brkić et al., 2018). Cystic mastitis, cystic hyperplasia and adenosis were reported in histological examinations (Greenblatt, Samaras, Vasquez, Nezhat, 1982).

Various treatment methods are applied to cure FBD or alleviate its symptoms, especially breast pain (Alipour, et al., 2021; Sasaki et al., 2018). While mechanical support of the breast, vitamin supplements, dietary restrictions, hormonal manage-

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ment, non-steroidal anti-inflammatory drugs, danazol and tamoxifen have frequently been used for treatment, no completely efficient and noncontroversial option is found regarding FBD, and surgical intervention is recommended for unresponsive and recurrent cases (Brkic et al., 2018; Alipour et al., 2021; Irgebay et al., 2017; Yadav, Sharma, Singh, Gupta, 2020). Studies on progressive cases of FDB have reported the use of danazol at doses of 200 and 400 mg/day, despite its serious side effects, eases the symptoms of the disease (Yadav et al., 2020; Gateley, Miers, Mansel, Hughes, 1992). Additionally, afimoxifen gel, which is a tamoxifen metabolite, has promising effects on FBD without serious side effects (Mansel et al., 2007). The effects of different herbal products on treating FBD have also been investigated as an alternative to existing therapeutics that do not provide a permanent cure and that have undesirable side effects. Among the herbal supplements recommended for treatment, evening primrose oil, flaxseed oil and vitamin E have been reported to relieve symptoms without side effects (Gateley et al., 1992; Godazandeh, Ala, Motlaq, Sahebnasagh & Bazi, 2021).

Apart from being a nutrient, honey produced by *Apis mellifera* (honeybee) has also been used as a herbal medicine, along with other bee products such as propolis, pollen, and royal jelly, to treat various diseases throughout history (Lusby, Coombes, & Wilkinson, 2005). The amount and different types of phenolic substances that give honey its antioxidant properties vary according to the flora used (Jaganathan, & Mandal, 2009; Mandal & Mandal., 2011). Propolis is a natural product honeybees make from various plants' leaves, stems and buds to repair and protect their hives. Propolis contains phenolic acids, tannins, polysaccharides, terpenes, aromatic acids, aldehydes and many other chemicals, and has important pharmacological properties (Silici, & Kutluca, 2005). These chemicals, called phytochemicals, are produced as secondary metabolites in plants and turned into bioactive compounds with beneficial health effects when consumed as nutrients (Naeem & Ugur., 2020). Phenolic compounds are characterized as natural sources of the antioxidant requirement of metabolism. They show their antioxidant activity by binding free radicals or chelating with metals (Verma, Hucl, & Chibbar, 2009). Based on the chemical structures, phenolic compounds are generally classified as flavonoids (e.g., anthocyanidins, flavones and flavonols, flavanones, flavanols and isoflavones), phenolic acids, stilbenes and lignans (Khan, & Dangles, 2014).

Previous studies have reported the effects of honey and propolis on malignant diseases of the mammary gland may vary depending on their polyphenol content (Seyhan et al., 2017; Seyhan et al., 2019). When considering the individual effects of polyphenolic substances on breast cancer cells, different effects of these polyphenols have been reported (Yang et al., 2014; Omene et al., 2013; Hung., 2004; Abbas Momtazi-Borojeni, Behbahani, Sadeghi-Aliabadi, 2013; Serafim et al., 2011). Although bee products and polyphenolic compounds have been studied extensively in malignant cell lines, there is a limited number of in vitro research focusing on FBD in the literature.

Therefore, this study aims to investigate the potential antiproliferative effects of honey and propolis samples, whose therapeutic effects have been shown on malignant breast cell lines in previous studies, on MCF-10A cells modeling FBD and on human periodontal ligament fibroblast (hPDL) control cells, together with the bioactive polyphenols found in the samples' contents.

MATERIALS AND METHODS

Sample Preparation

Honey and propolis samples were obtained from Altıparmak Gıda Inc. (Istanbul, Türkiye) and stored at +4°C. These samples were produced for consumption and verified to be free of biological or heavy metal contamination through detailed chemical analysis. Polyphenols (ferulic acid, kaempferol, caffeic acid, pinocembrin, pinostrobin, galangin and quercetin) were purchased from Merck (Darmstadt, Germany) and stored at +4°C. All samples were dissolved in 60% ethanol (60% ethanol; 40% water) and stored at -20°C.

HPLC Analysis

The amounts of the phenolic compounds in propolis were determined by high performance liquid chromatography (HPLC, Waters 600 controller and Waters 996 PDA detector). Prior to analysis, propolis extracts were prepared by dissolving 0.1 g of propolis in 25 mL of 60% ethanol and introduced into the HPLC system. A C18 column was used for the analysis of phenolic compounds. As mobile phases, 0.1% trifluoroacetic acid (TFA) prepared in distilled water and 0.1% TFA prepared in acetonitrile were used. During the analysis, the flow rate was set at 1 mL/min, and the phenolic compounds in propolis were measured at wavelengths of 280 nm, 312 nm, and 360 nm (Bino et al., 2005; Ahn, Kumazawa, Hamasaka, Bang, & Nakayama, 2004).

Cell Culture

MCF-10A breast epithelial cells, a model of fibrocystic cell disease, were purchased from ATCC (ATCC, Rockville, MD). These MCF-10A cells were maintained in a suitable growth medium DMEM F12 containing 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin-streptomycin (all obtain from Gibco, Thermo Fisher Scientific, Waltham, MA, USA) , and a bullet kit in humidified air at 37ºC and 5% CO_2. Normal hPDL cells were purchased from the Lonza Group (Basel, Switzerland). The hPDL cells were cultured using the Stromal Cell Growth Medium BulletKit and ReagentPack Subculture Reagents (Lonza, Basel, Switzerland) following the

manufacturer-recommended protocol at 37ºC in humidified air with 5% CO_2. The study used hPDL as a control cell line.

WST-1 Analysis

All cell proliferation analyses of this study were performed using the Cell Proliferation Reagent water-soluble tetrazolium salt-1 (WST-1; Roche, Manheim, Germany). WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt), a tetrazolium salt, reacts with mitochondrial dehydrogenases in the mitochondria of living cells to form a yellow formazan crystal. The yellow colour is correlated with the presence of living cells and this change in formazan formation can be measured spectrophotometrically at a wavelength of 450nm. Briefly, after the number of cells were counted on the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Brea, CA), 1×10^4 cells were seeded in each well of a 96-well plate (Sarstedt, Nümbrecht, Germany) in 3 replicates. The cells were allowed to adhere for 24 h, and the medium was aspirated. Then, intended doses of samples (for honey samples 1, 2.5, 5, 7.5, 10 µg/mL doses; for propolis samples 2.5, 5, 50, 100, 250 µg/mL doses; for phenolic substances 2.5, 5, 7.5, 10, 15, 20, 22.5, 25, 30, 37.5 µg/mL) and medium alone as negative control were applied within a fresh medium with 3% FBS (Seyhan et al., 2017; Seyhan et al., 2019). After 46 h of incubation, 10 µL WST-1 was applied to each well in the dark. 2 h later, the absorbances of the wells at 450 nm (with the reference wavelength at 600nm) were measured using Multiscan enzyme-linked immunosorbent assay reader (Thermo Fisher Scientific, Massachusetts, USA).

In the experiments, all doses were performed as triple biological replicates. Untreated cells were used as control group (negative) (DMEMF12 for MCF10A cells and Bulletkit hPDL cells) and the values of the groups are given as mean±standard deviation (X±SD). Viability of control cells was set to 100%. Cell viability is given by the following formula:

Viability (%) = OD of treated group / OD of negative control group ×100.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Prism Software, San Diego, CA, USA). The equality of means of independent groups was tested by one-way analysis of variance (ANOVA), and Dunnet's test was used as the post-hoc test following ANOVA to calculate the difference between the mean of each dose group with the mean of the control group. Values of $p < 0.05$ are considered significant.

RESULTS

HPLC Analysis of the Propolis Samples

The amounts of important phenolic substances in the Chinese propolis and Türkiye propolis were measured using the HPLC analysis. Accordingly, Türkiye propolis had higher ferulic acid (3.12 mg/g vs. 1.03 mg/g), kaempferol (1.13 mg/g vs. 0.15 mg/g), caffeic acid (7.98 mg/g vs. 0.21 mg/g), pinocembrin (9.84 mg/g vs. 0.15 mg/g) and quercetin (0.73 mg/g vs. 0.14 mg/g) levels compared to the Chinese propolis. However, the pinostrobin level in the Chinese propolis was higher than in the Türkiye propolis (17.99 mg/g vs. 10.85 mg/g). Galangin content was similar in both propolis samples (13.90 mg/g vs. 13.80 mg/g; Figure 1).

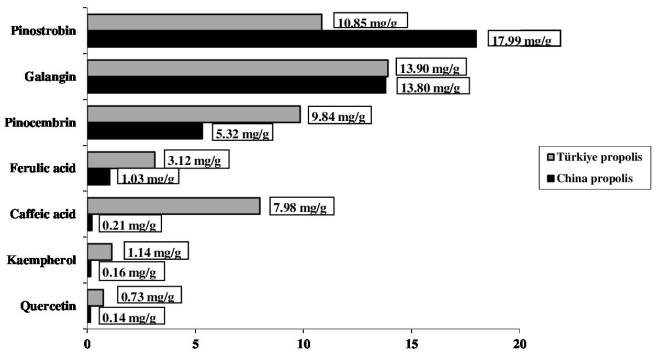

Figure 1. HPLC analysis in China and Türkiye propolis samples.

Cell Viability and Proliferation

The effects of chestnut, pine, cedar, multifloral and artificial honey on the viability of MCF-10A cell lines were investigated at doses of 1-10 µg/mL (Figure 2). Accordingly, both chestnut and cedar honey were observed to have an antiproliferative effect on the MCF-10A cell line at all doses (1-10 µg/mL, $p < 0.0001$) and pine honey to have the same effect at its highest dose (10 µg/mL, $p < 0.0001$). Multifloral and artificial honey had no cytotoxic effects on MCF-10A cell lines (Table 1).

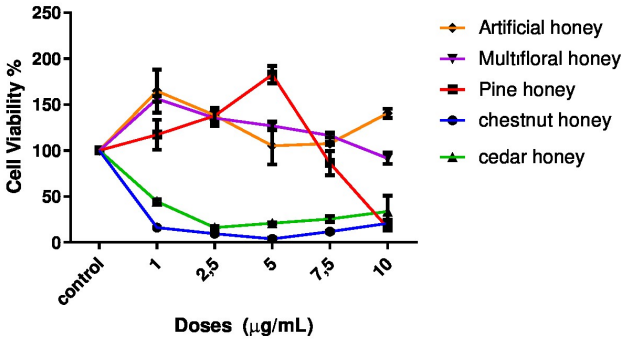

Figure 2. 48th-hour cell viability data of chestnut, pine, cedar, multifloral, and artificial honey samples in MCF-10A cell line.

Table 1. *The effects of Chestnut, Pine, Cedar, Multifloral, and Artificial honey on the MCF-10A cell line using the WST-1 Assay*

Doses (µg/mL)	Chestnut Honey	Pine Honey	Cedar Honey	Multifloral Honey	Artificial Honey
	X ± *SD*	X ± *SD*	X ± *SD*	X ± *SD*	X ± *SD*
1	16.16 ± 1.01 *	117.20 ± 16.20	44.52 ± 2.15 *	156.26 ± 3.31 *	164.64 ± 23.45
2.5	9.56 ± 0.92 *	137.87 ± 7.47 **	16.14 ± 1.90 *	135.22 ± 8.44 *	138.38 ± 7.70 ****
5	3.78 ± 1.54 *	182.65 ± 9.44 *	21.01 ± 0.53 *	126.82 ± 4.66 *	105.02 ± 20.15
7.5	11.73 ± 0.75 *	86.31 ± 13.10	25.59 ± 3.04 *	116.33 ± 2.07 **	107.66 ± 1.31
10	20.66 ± 4.04 *	16.25 ± 1.78 *	33.57 ± 17.25 *	91.48 ± 6.17	140.50 ± 5.04 ****

Data are expressed as percentage of absorbance values compared to negative control wells (mean standard deviation, X ± SD). Control cell viability was set at 100%. For each dose, comparisons were made with medium-only controls. *, p<0.0001; **, p< 0.01; ***, p< 0.001; ****, p< 0.05.

The antiproliferative effects of propolis originating from Türkiye and China on the MCF-10A and hPDL cells were analyzed using the WST-1 test at doses of 1-250 µg/mL (Figure 3). The effects of Chinese propolis on MCF-10A cells at high doses (50, 100, 250 µg/mL) were antiproliferative (p < 0.001). Chinese propolis also had a significant antiproliferative effect on the hPDL cell line at moderate-to-high doses (5–100 µg/mL). Meanwhile, the Türkiye propolis had no cytotoxic effect on either cell line up to 100 µg/mL (p>0.05). It had an antiproliferative effect in the MCF-10A cells, but only at the highest dose (250 µg/mL; p = 0.0013; Table 2).

Antiproliferative effects of ferulic acid (20-30 µg/mL), galangin (15-30 µg/mL), and pinostrobin (5-30 µg/mL) were observed on the MCF-10A cell line at 48 h (p < 0.0001). Kaempferol was also found to have a moderate cytotoxic effect on the MCF-10A cells at high doses (for 20 µg/mL, p < 0.05, for 30 µg/mL, p = 0.0004). Meanwhile, pinocembrin, caffeic acid and quercetin had no significant effects on the MCF-10A cell line (p > 0.05; Figure 4).

DISCUSSION

FBD is the most common benign breast disease in women, and in some cases requiring a differential diagnosis from breast cancer. This study investigated the antiproliferative effects of multifloral, cedar, pine, chestnut and artificial honey samples, as well as two propolis samples (Chinese and Türkiye) and seven polyphenols with known cytotoxic effects, on MCF-10A cells modeling FBD.

Different results have been obtained from studies that examined the effects of bee products on viability in MCF-10A cells based on the type and geographical origin of the product. In a study in which MCF-10A cells were treated with 1-10% doses of honey produced by Asian giant bees (*Apis dorsata*) from Tualang trees in Malaysian, no significant antiproliferative effect was reported even after 72 hours of incubation (Fauzi, Norazmi, & Yaacob, 2011). Similarly, Manuka honey was reported to cause no significant loss of viability in MCF-10A cells at low concentrations (0.6% and 1.25%) after 72 hours of incubation and to have a cytotoxic effect at high concentrations (2.5% and 5%; Aryappalli et al., 2017). While the present study observed no cytotoxic effect for multifloral honey originating from the Southeastern Anatolia region of Türkiye origin on MCF-10A cells, tree honey (cedar, pine, and especially chestnut) had significant antiproliferative effects on these cells (Figure 2). Previous study has determined the total phenolic contents and antioxidant capacities of the honey samples included in this study to rank from highest to lowest as chestnut, cedar, pine and multifloral honey (Seyhan et al., 2017). Taken together, the cytotoxic effects of honey samples on MCF-10A cells can be said to parallel their total phenolic content and antioxidant activities.

Studies investigating the effects of propolis on the MCF-10A cell line have mostly focused on the flavonoids and other bioactive components in the contents of propolis. Mohamed et al. (2020) reported that IC50 values for propolis extracts produced by *Tetrigona apicalis* bees on the Malay Peninsula regarding MCF-10A cells at 24 h, 48 h and 72 h to be 49.55 µg/mL, 56.05 µg/mL and 72.10 µg/mL, respectively. The Chinese propolis in

Table 2. *The effects of Chinese Propolis and Türkiye Propolis in MCF-10A and hPDL cell line Using the WST-1 Assay*

MCF-10A			
Chinese propolis		**Türkiye propolis (from Kartal)**	
Doses (µg/mL)	**X ± *SD***	**Doses (µg/mL)**	**X ± *SD***
2.5	209.639 ± 19.666 *	**2.5**	128.061 ± 19.453
5	157.37 ± 0.654 **	**5**	130.208 ± 4.951
15	127.1 ± 18.806	**15**	114.508 ± 62.133
50	11.5613 ± 1.877 *	**50**	98.2276 ± 1.131
100	7.99492 ± 0.454 *	**100**	87.8683 ± 38.127
250	9.50964 ± 0.571 *	**250**	1.777± 0.148 **
hPDL			
Chinese propolis		**Türkiye propolis (from Kartal)**	
Doses (µg/mL)	**X ± *SD***	**Doses (µg/mL)**	**X ± *SD***
1	105.925 ± 6.889	**5**	116.933 ± 8.638
2.5	92.098 ± 5.989	**10**	121.476 ± 15.942
5	51.789 ± 2.352 ***	**25**	134.334 ± 7.313 ****
15	7.196 ± 0.245 ***	**50**	132.540 ± 15.176
50	11.121 ± 0.303 ***	**100**	137.541 ± 24.515 ****
100	51.242 ± 0.623 ***		

Data are expressed as percentage of absorbance values compared to negative control wells (mean standard deviation, X ± SD). Control cell viability was set at 100%. For each dose, comparisons were made withmedium-only controls.
*, p< 0.001; **, p< 0.01; ***, p< 0.0001; ****, p< 0.05.

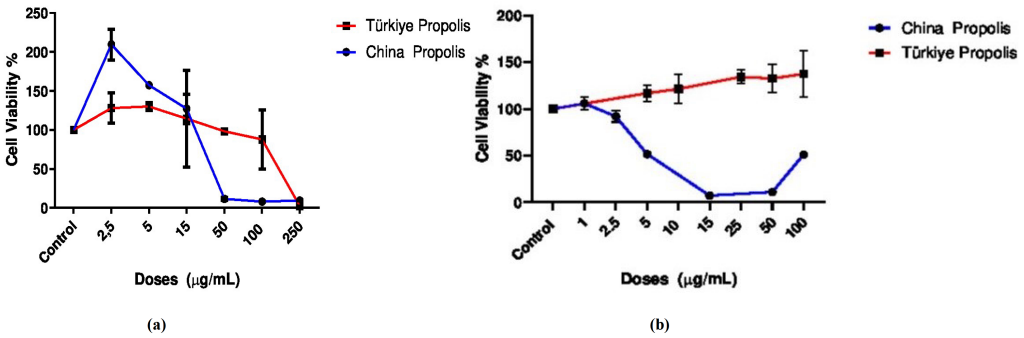

Figure 3. 48th-hour cell viability data of Türkiye (Istanbul/Kartal) and China propolis samples in MCF-10A (a) and hPDL (b) cells.

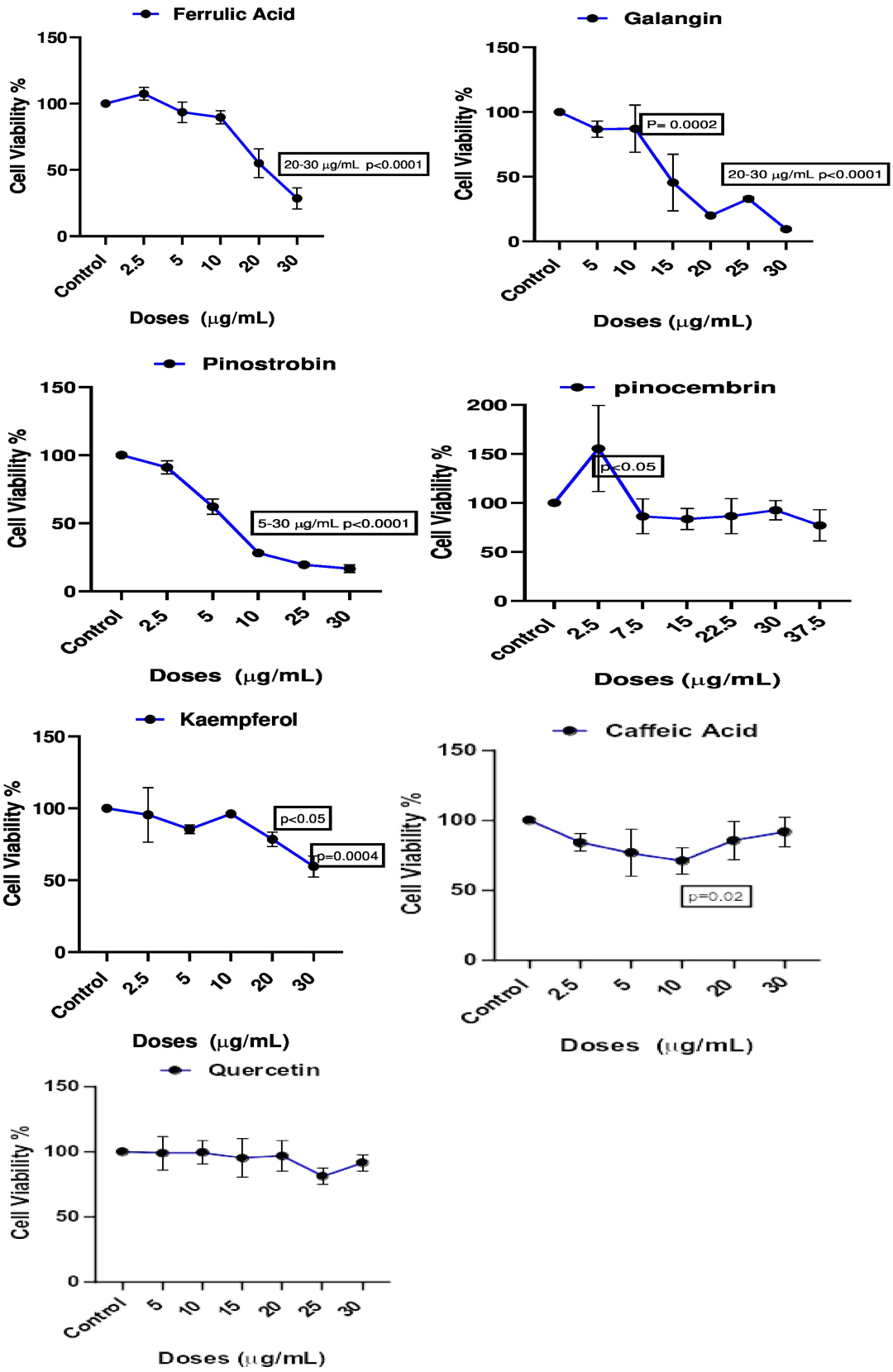

Figure 4. 48th hour cell viability data of ferrulic acid, galangin, pinostrobin, pinocembrin, kaempferol, caffeic acid quercetin samples in MCF-10A cell line.

the current study showed antiproliferative effects on MCF-10A cells at high doses (50-250 µg/mL) and on the hPDL control cell line at medium and high doses (5–100 µg/mL). Additionally, the Türkiye propolis had no significant effect on cell viability in both the MCF-10A and hPDL cell lines at the 1-100 µg/mL doses but it had an antiproliferative effect only at the 250 µg/mL dose on the MCF-10A cells (Table 2, Figure 3). These results indicate that propolis samples from distant regions affect the viability of the cells differently.

When considering how the Türkiye propolis showed no cytotoxic effects in the control hPDL cell line, this propolis type can be suggested as being protective of normal cells. A previous study examined the different cytotoxic effects of propolis extracts from different geographical origins on breast cancer cell lines (Seyhan et al., 2019) and found that, while the cytotoxic effect of Chinese propolis extract was observed starting from 15 µg/mL on MCF-10A cells ($p < 0.01$), the Türkiye propolis was only found to be cytotoxic at high doses (250-500 µg/mL, $p < 0.01$). Previous studies have also shown the cytotoxic effects of honey (chestnut, cedar and pine) and propolis (Chinese and Türkiye) samples to be apoptotic in breast cancer cell lines and MCF-10A cells (Seyhan et al., 2017; Seyhan et al., 2019).

The present study performed polyphenolic content analyses of propolis samples using HPLC to determine whether the different effects of Türkiye and Chinese propolis on cell viability in MCF-10A cell lines depend on the polyphenolic compounds in their contents. The results from the HPLC analysis revealed the Türkiye propolis to be rich in ferulic acid, kaempferol, caffeic acid, and pinocembrin and the Chinese propolis to be rich in pinostrobin.

Meanwhile, only a limited number of studies have so far investigated the effects of these polyphenolic substances on MCF-10A cell viability. Song, Yan, Zhou & Zhen, (2017) reported galangin at 5.4 and 10.8 µg/mL doses and at 24 h to have no significant effect on viability in MCF-10A cells. Still, the current study showed the antiproliferative effects of galangin treatment on these cells at 48 h and a dose range of 10-20 µg/mL ($p = 0.0002$; Figure 4). This observed difference may stem from the application of galangin to the cells at different doses and/or the evaluation of its effects over different time periods. Therefore, the effects of galangin on MCF-10A cells need to be evaluated over wider dose and time ranges to obtain more precise results.

In a recent study examining the effects of kaempferol on the MCF-10 cell line at 72 hours and at a dose range of 0.003–14.31 µg/mL, it was reported that the IC50 value of this polyphenolic substance was 10.6 µg/mL (37 µM) (Pham, Sakoff, Vuong, Bowyer, & Scarlett, 2018). On the other hand, Der Medizin and Abutayeh (2014) suggested that kaempferol (0.05–100 µg/mL) and quercetin (0.05–100 µg/mL) show no cytotoxic effect on MCF-10A cells at 24 h. The current study confirmed that kaempferol has a mild antiproliferative effect on MCF-10A cells only at high doses (for 20 µg/mL, $p < 0.05$; for 30 µg/mL, $p = 0.0004$). However, this study found no remarkable effect of quercetin ($p > 0.05$; Figure 4).

A recent study reported cell proliferation not to be affected when MCF-10A cells are cultured with pinostrobin at doses of 0.003 µg/mL-5.40 µg/mL for 24 h (Jones & Gehler., 2020). In contrast, this study showed that pinostrobin at doses of 5–30 µg/mL over 48 h had a very strong antiproliferative effect on these cells ($p < 0.0001$; Figure 4). Therefore, this study suggests that the effect of pinostrobin on MCF-10A cells should be investigated over wider dose and time intervals. Furthermore, pinocembrin was also previously reported to have no effect on MCF-10A cell viability (doses of 0.26 to 12.81 µg/mL over 72 h; Aiello et al., 2017). Consistent with this finding, no significant effect of pinocembrin at doses of 7.5-37.5 µg/mL (48 h) on MCF-10 cell viability was observed in the present study, but a mild cytotoxic effect was present at the low dose (2.5 µg/mL) (Figure 4).

To the best of the authors' knowledge, this study is also the first in the literature to investigate the effects of ferulic acid and caffeic acid on MCF-10A cells. Ferulic acid showed a significant antiproliferative effect on the cells at high doses (20–30 µg/mL, $p < 0.0001$), whereas caffeic acid did not. When evaluating the Türkiye and Chinese propolis extracts alongside their polyphenol contents and effects the Türkiye propolis is rich in ferulic acid and kaempferol, which showed antiproliferative effects only at high doses, and caffeic acid, quercetin and pinocembrin polyphenols, which did not affect viability in the MCF-10A cell line at the given doses. Therefore, a part of the cytotoxic effect of Türkiye propolis on the MCF-10A cells can be attributed to its ferulic acid content. Meanwhile, the Chinese propolis was found to be rich in pinostrobin, which had a strong antiproliferative effect even at low doses. Therefore, the cytotoxic effect of China propolis can be associated with its pinostrobin content.

Individual analyses of the polyphenolic content of honey samples could not be performed. Therefore, this study was unable to evaluate the effects of honey samples on the viability of MCF-10A cells in terms of their polyphenol content. However, the study did obtain findings that tree honey (cedar, pine and especially chestnut) may be effective against FBD. We aim to reach more precise results in future studies evaluating the effects of tree and multifloral honey samples regarding content analysis, as well as the apoptotic effects of polyphenolic substances.

CONCLUSION

In conclusion, the study's findings highlight the potential effects of pinostrobin, ferulic acid and galangin on the fibrocystic breast disease model and investigating their combined effects may

be rewarding in terms of discovering new prophylactic and complementary therapies with no serious side effects.

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