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Assessing the anticancer potential of propolis and polyphenolic compounds in combination: An *in vitro* approach

Damla KIRCI[1*], Elif İNCE ERGÜÇ[2]

1 Izmir Katip Çelebi University, Faculty of Pharmacy, Department of Pharmacognosy, Izmir, Türkiye
2 Izmir Katip Çelebi University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Izmir, Türkiye

ABSTRACT

This study aimed to evaluate the in vitro anticancer activity and possible synergism of ethanolic propolis extract combined with quercetin, hesperidin, and hesperetin. Propolis (EEP) was extracted with ethanol and analyzed via high-performance liquid chromatography (HPLC), confirming major phenolic constituents. Quercetin, hesperidin, and hesperetin were tested individually and in combination with the extract. Cytotoxicity was assessed on MCF-7 using the MTT assay. The chemical composition of the EEP was characterized by the identification of 17 phenolic and flavonoid compounds. Among these, caffeic acid phenethyl ester was the most abundant constituent, with a concentration of 5733.58 μg/mL. When applied alone, quercetin reduced MCF-7 cell viability to 59% at 31.25 μg/mL, 38% at 62.50 μg/mL, and 11% at 125.00 μg/mL. Co-administration with EEP significantly enhanced the cytotoxic effect, reducing viability to 28%, 22%, and below 10% at the respective concentrations. The lowest combination index (CI) value, calculated as 1.06 (50 μg/mL propolis + 31.25 μg/mL quercetin), indicated a nearly additive interaction, while higher concentrations resulted in antagonistic effects.

Keywords: polyphenolic compound, propolis, anticancer effect, quercetin, hesperetin

* Corresponding author: Damla KIRCI
E-mail: damla.kirci@ikc.edu.tr
ORCIDs:
Damla KIRCI: 0000-0002-3479-3999
Elif İNCE ERGÜÇ: 0000-0003-0764-7694
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INTRODUCTION

Propolis is a resinous substance collected by bees from plant buds and exudates, enriched with beeswax and enzymes. It has been used since ancient times for wound healing, as an antiseptic, and as an anti-inflammatory agent, with reports of its application in embalming rituals in Ancient Egypt[1]. Today, propolis is widely used in both traditional medicine and pharmaceutical products due to its immunomodulatory, anticancer, antimicrobial, antioxidant, and anti-inflammatory properties[2,3]. These biological activities are attributed to its rich chemical composition, which typically consists of approximately 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and other trace substances[1]. In the beehive, propolis serves as a natural sealant and protective agent, helping prevent microbial contamination and infection within the colony[1,4].

Polyphenols are a large group of plant-derived secondary metabolites widely present in fruits, vegetables, tea, coffee, and red wine[5]. Structurally, they consist of one or more aromatic rings with hydroxyl groups, and over 10,000 distinct compounds have been identified[6]. These natural compounds are mainly classified into flavonoids, phenolic acids, lignans, and stilbenes, with flavonoids being the most abundant in the human diet[7]. Flavonoids are further subdivided into six major classes: flavonols, flavones, flavanones, flavan-3-ols, isoflavones, and anthocyanidins[8].

Polyphenols have attracted considerable scientific interest due to their wide range of biological activities, including antioxidants, anti-inflammatory, antiviral, and anticancer properties. Several studies have demonstrated that polyphenols exert antiproliferative effects on various cancer cells with minimal toxicity to normal tissues. Their anticancer activity is thought to involve modulation of oxidative stress, inflammation, and cell signaling pathways. Moreover, epidemiological data suggest that polyphenol-rich diets may help reduce cancer risk[8,9].

Despite the well-documented biological effects of propolis and various polyphenolic compounds, limited data are available on their combined cytotoxic activity and potential synergistic interactions. Understanding such interactions may offer promising insights for the development of more effective natural compound-based therapies with enhanced efficacy and reduced toxicity. In this study, we aim to evaluate the individual and combined anticancer effects of propolis and selected flavonoids (quercetin, hesperidin, and hesperetin) on MCF-7 using the MTT assay. Furthermore, this study explored the nature of these combinations (synergistic, additive, or antagonistic) through Chou–Ta-

lalay combination index analysis using CompuSyn software. To the best of our knowledge, this is one of the few studies systematically assessing the interaction between a chemically characterized propolis extract and individual flavonoids, offering a novel perspective on the combined therapeutic potential of bee-derived and plant-derived bioactive.

METHODOLOGY

Chemicals and reagents

Quercetin, hesperidin, and hesperetin (purity≥98%) were purchased from Sigma-Aldrich, Alfa-Aesar and Santa Cruz, respectively. All solvents used were of analytical grade. RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] powder was also obtained from Sigma-Aldrich.

Preparation of propolis extract

Raw propolis samples were obtained from Türkiye and stored at 4°C until extraction. Ethanolic extract of propolis (EEP) was prepared by macerating 1 g of ground raw propolis in 3 mL of 70% ethanol at room temperature. The EEP was stored at −20°C for further use.

HPLC analysis of propolis constituents

The identification and quantification of phenolic compounds in the ethanolic propolis extract were performed by high-performance liquid chromatography (HPLC) following the method described, with minor modifications. A Shimadzu HPLC system (LC-20AD/SPD-M20A) equipped with a vacuum degasser, binary pump, autosampler, and diode-array detector (DAD) was employed.

Chromatographic separation was achieved using a C18 reverse-phase column (Inertsil ODS-3, 5 µm, 4.6 × 150 mm) maintained at 30°C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). A gradient elution was applied as follows: 0–3 min, 10–25% B; 3–15 min, 25–30% B; 15–60 min, 30–50% B; 60–70 min, 50–60% B; 70–80 min, 60–90% B; 80–85 min, 90–60% B; 85–90 min, 60–25% B; and 90–95 min, 25–10% B. The column was equilibrated at 30°C for 15 minutes before each injection. The flow rate was 1.0 mL/min, and the injection volume was 5 µL. Each sample was injected twice under the same conditions[10].

Cell culture

Human Breast Cancer Cells (MCF-7) (ATTC HTB-22) were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin under standard conditions (37°C, 5% CO_2, humidified incubator). Cells were subculture every 2–3 days and used for experiments at 70–80% confluency.

Cytotoxicity assay

This method is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases in viable cells, resulting in the formation of purple formazan crystals. To assess the effect of propolis extract and phenolic compounds on cell viability, MCF-7 cells were seeded at a concentration of 6,000 cells per well and incubated for 24 hours at 37°C. Subsequently, the cells were treated with various concentrations of phenolic compounds, either alone or in combination with propolis extract, for 24 hours.

After the treatment period, the wells were washed with PBS and incubated with MTT solution at 37°C for 2 hours. Following incubation, the medium was removed, and the formazan crystals were dissolved in DMSO. The optical density was then measured at 550 nm using the multiplate reader[11].

Combination index (CI) analysis

The interaction between propolis and polyphenolic compounds (quercetin, hesperidin, and hesperetin) was evaluated using the Chou–Talalay method with CompuSyn software (Cambridge, UK). Cells were treated with each compound individually and in combination at fixed doses of propolis (50 µg/mL) and varying concentrations of each polyphenolic compounds. The cytotoxic effect was assessed using the MTT assay, and the percentage of inhibition was converted to fractional effect values (Fa), ranging from 0 (no effect) to 1 (complete inhibition).

Combination index (CI) values were calculated by the software based on the median-effect equation. CI<1 indicates synergism, CI=1 denotes an additive effect, and CI>1 represents antagonism. Statistical analysis[12,13].

Statistical analysis

Statistical analysis was performed using GraphPad Prism9.0 software (GraphPad Software, La Jolla, CA). The data was analyzed using one-way Anova, with post hoc Tukey's multiple comparisons test. P-values<0.05 were considered to indicate significance[14].

RESULTS and DISCUSSION

Chemical characterization of propolis by HPLC

The chemical composition of the ethanolic propolis extract was determined using high-performance liquid chromatography (HPLC), revealing a total of 17 phenolic and flavonoid compounds. The results are summarized in Table 1. These compounds include a wide range of phenolic acids, flavones, flavonols, chalcones, and flavanones, indicating a chemically diverse and biologically active extract.

Table 1. Phenolic compounds identified in ethanolic propolis extract by HPLC analysis

Compound	Amount (μg/mL extract)
Gallic acid	19.70
Epigallocatechin gallate	150.62
Caffeic acid	704.91
p-Coumaric acid	479.46
trans- Ferulic acid	209.65
trans-Isoferulic acid	388.62
3,4-Dimetoxycinnamic acid	675.01
Quercetin	1216.20
trans-Cinnamic acid	38.63
Naringenin	1550.39
Apigenin	447.99
Kaempferol	90.36
Chrystin	2721.28
Pinocembrin	3330.40
Galangin	3645.52
Caffeic acid phenethyl ester	5733.58
trans-Chalcone	1644.20

Among the quantified compounds, caffeic acid phenethyl ester (CAPE) was identified as the most abundant constituent, with a concentration of 5733.58 μg/mL extract. CAPE is a well-known bioactive component of propolis with documented anti-inflammatory and anticancer activities. Other major constituents included galangin (3645.52 μg/mL), pinocembrin (3330.40 μg/mL), and chrysin (2721.28 μg/mL) - all of which are flavonoids known for their cytotoxic and antioxidant potential. These high concentrations suggest that flavonoids major the chemical profile of the extract.

Several phenolic acids were also detected in considerable amounts, particularly caffeic acid (704.91 µg/mL), 3,4-dimethoxycinnamic acid (675.01 µg/mL), and p-coumaric acid (479.46 µg/mL). In addition, several flavones and flavanols such as quercetin (1216.20 µg/mL), naringenin (1550.39 µg/mL), apigenin (447.99 µg/mL), and kaempferol (90.36 µg/mL) were identified.

Cytotoxic effects of propolis, quercetin, hesperidin, and hesperetin on MCF-7

In the present study the cytotoxic effects of propolis extract and phenolic compounds, both individually and in combination, on MCF-7 breast cancer cells were investigated by using the MTT assay (Figures 1 and 2).

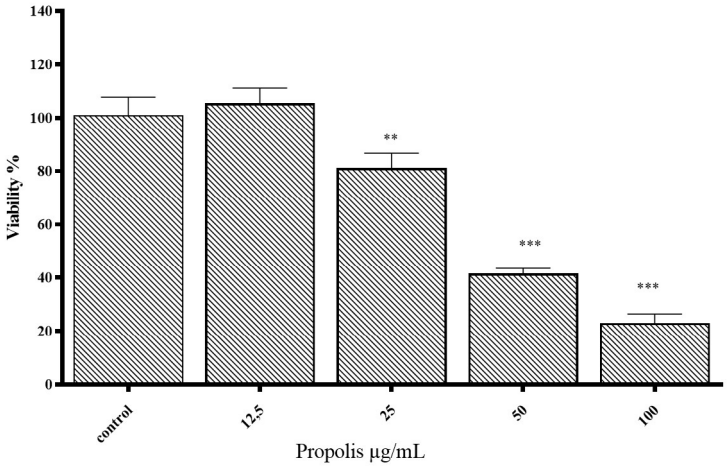

Figure 1. Effects of propolis extract on MCF-7 cell viability as determined by MTT assay

Bars represent the percentage of viable cells relative to the control group, based on three independent experiments. Statistical significance is denoted as follows: *p<0.05, **p<0.01, ***p<0.001.

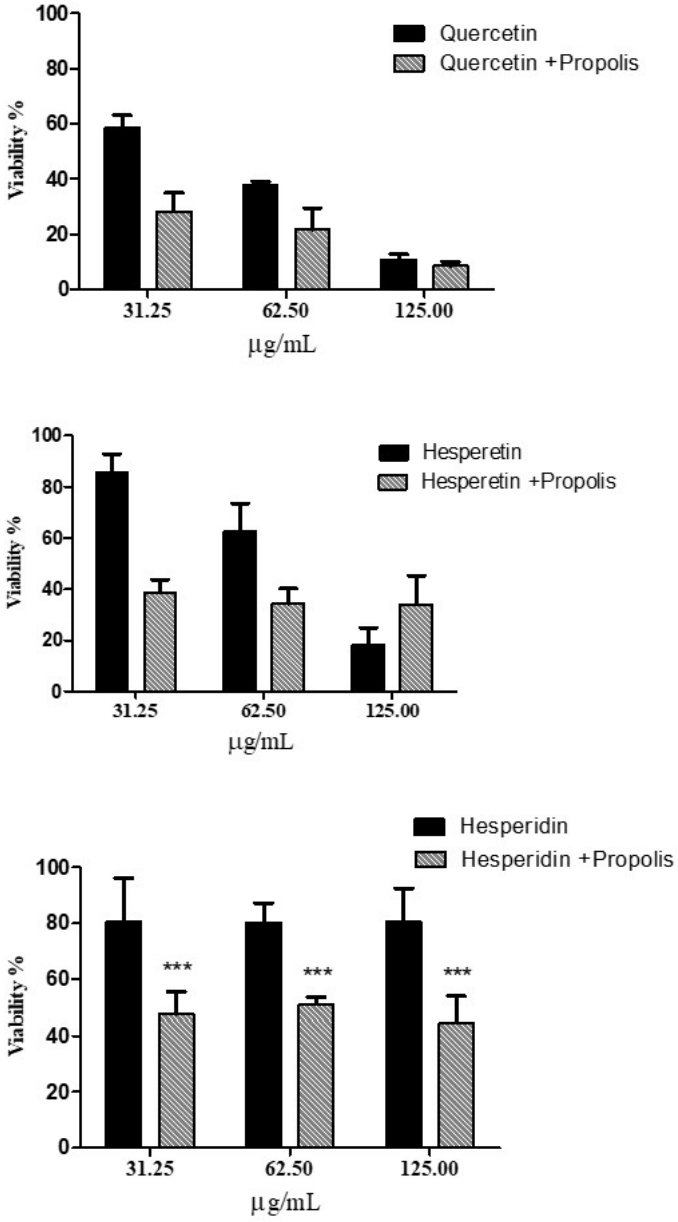

Figure 2. Effects of phenolic compounds on MCF-7 cell viability either alone or combination with 50 µg/mL propolis extract

Bars represent the percentage of viable cells relative to the control group, based on three independent experiments. Statistical significance is denoted as follows: *p<0.05, **p<0.01, ***p<0.001.

The results showed that propolis extract exerted a dose-dependent cytotoxic effect on MCF-7 cells. At the 25 μg/mL concentration, cell viability was reduced to approximately 85% compared to the untreated control group. As the concentration increased to 50 μg/mL, cell viability decreased further to around 50%. A more pronounced reduction was observed at 100 μg/mL, where cell viability dropped to approximately 23%. Statistical analysis indicated that the reduction in cell viability was significant at all concentrations tested, except for 12.5 μg/mL, suggesting a potent cytotoxic effect of propolis extract on MCF-7 cells.

Subsequently, the cytotoxic effects of quercetin, hesperetin, and hesperidin on MCF-7 breast cancer cells were evaluated at three different concentrations (31.25, 62.50, and 125.00 μg/mL), both individually and in combination with 50 μg/mL of propolis extract.

We found that quercetin exhibited the highest cytotoxicity among the tested compounds. When used alone, quercetin reduced cell viability to 59% at 31.25 μg/mL,38% at 62.50 μg/mL, and 11% at 125.00 μg/mL. The combination of quercetin with propolis extract significantly enhanced the cytotoxic effect, resulting in cell viability reductions to 28%, 22%, and below 10% at the respective concentrations.

Hesperetin, in contrast to quercetin, showed relatively high cell viability when used alone, especially at lower concentrations. At the lowest concentration of 31.25 μg/mL, cell viability remained around 90%. As the concentration increased to 62.50 μg/mL, viability dropped to 63%, and at 125.00 μg/mL, viability decreased to about 18%. The combination of hesperetin with propolis extract significantly enhanced the cytotoxic effect only at the highest two concentrations (62.50 and 125.00 μg/mL), where cell viability decreased to 39% and 34%, respectively. At the lowest concentration (31.25 μg/mL), the combination did not significantly reduce cell viability compared to hesperetin alone.

Hesperidin displayed the lowest cytotoxicity among the three compounds, with cell viability remaining around 80% at increasing concentrations. However, the combination with propolis extract significantly reduced viability to 47%, 51%, and 45%, respectively ($p<0.001$), demonstrating a marked enhancement in cytotoxicity compared to hesperidin alone.

Among the three phenolic compounds tested, quercetin exhibited the strongest cytotoxic effect, followed by hesperetin and hesperidin. The addition of propolis extract significantly increased the cytotoxicity of all three compounds, highlighting a potential synergistic or additive interaction.

Combination analysis and synergistic evaluation

The potential synergistic or antagonistic effects of propolis in combination with quercetin, hesperidin, and hesperetin were analyzed using the Chou–Talalay method via CompuSyn software. The results are presented in Table 2 and Figure 3, showing the calculated combination index (CI) values for different dose ratios.

Table 2. Combination index (CI) values of different propolis-polyphenol combinations in MCF-7

Combination	Propolis (μg/mL)	Polyphenolic compound (μg/mL)	Effect (Fa)	CI Value
P+Q	50	31.25	0.72	1.06
P+Q	50	250	0.91	1.94
P+Hd	50	31.25	0.61	1.17
P+Hd	50	125	0.65	2.11
P+Ht	50	31.25	0.53	1.38
P+Ht	50	62.50	0.49	3.19

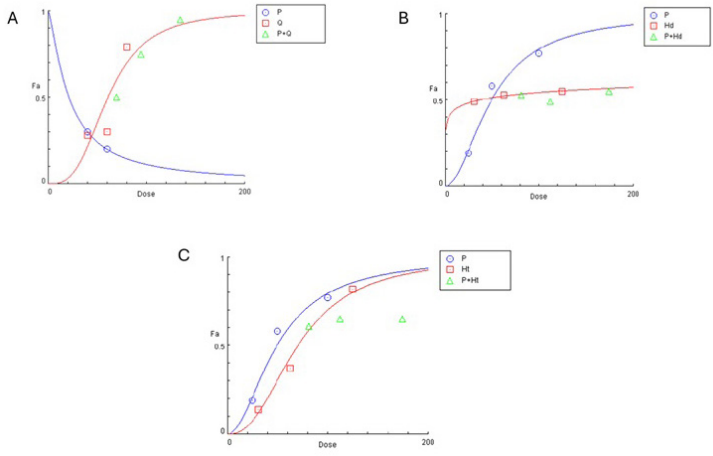

Figure 3. Dose–effect curves in propolis-polyphenol combinations

A: Propolis (P) and quercetin (Q); B: Propolis and hesperidin (Hd); C: Propolis and hesperetin (Ht)

Propolis + quercetin combination

The combination of propolis with quercetin exhibited antagonistic effects at all tested concentrations. The CI values were consistently greater than 1, indi-

cating that the combination was less effective than the individual compounds alone. The lowest CI value was 1.06 at 50 μg/mL Propolis + 31.25 μM Quercetin, suggesting a nearly additive effect. However, as quercetin concentration increased, CI values increased (e.g., CI=1.94 at 50 μg/mL Propolis + 250 μM Quercetin), confirming strong antagonism at higher doses (Figure 3A).

Propolis + hesperetin combination

Similarly, the combination of propolis with hesperetin resulted in antagonistic effects (CI>1 at all tested doses). The highest antagonism was observed at 50 μg/mL Propolis + 125 μM Hesperetin (CI=2.11). A mild reduction in antagonism was noted at lower hesperetin doses, but no synergy was observed (Figure 3B).

Propolis + hesperidin combination

The combination of propolis with hesperidin also failed to exhibit synergistic effects, with all CI values above 1. The strongest antagonistic interaction was observed at 50 μg/mL Propolis + 62.5 μM Hesperidin (CI=3.19). A relatively lower CI value (1.38 at 50 μg/mL Propolis + 31.25 μM Hesperidin) was noted, but the combination remained antagonistic (Figure 3C).

In a recent LC-HRMS-based study, the chemical profiles of seven ethanol–water extracts of propolis collected from different regions of Cyprus were comprehensively characterized. The analysis revealed notable variation in compound composition and abundance among samples. The most prominent flavonoids identified across the samples included isosakuranetin, naringenin, rhamnocitrin, diosmetin, chrysin, and acacetin, while chlorogenic acid and verbascoside stood out among phenolic acids. Isosakuranetin was especially abundant in propolis from Tirmen (102.75 mg/g), and diosmetin was detected at high levels in most samples (18.13–81.91 mg/g), except Tirmen. Compared to the present study, which also employed ethanol-based extraction and HPLC analysis, several overlapping compounds were detected, particularly chrysin, caffeic acid, hesperidin, and quercetin. However, the Cypriot samples showed higher chemical diversity and concentration ranges, which may be attributed to botanical origin, regional flora, or extraction differences. Notably, Tirmen propolis, identified as the richest in flavonoids and phenolics, was suggested to have stronger cytotoxic and antioxidant potential, aligning with the biological relevance of similar compounds evaluated in our study[15].

In comparison to the present study, where the chemical composition and cytotoxic effects of a propolis extract were analyzed along with selected polyphenols, a comprehensive LC-HRMS-based investigation of 39 Turkish propolis

samples revealed a broad chemical diversity. A total of 31 compounds were simultaneously identified, including major flavonoids such as isosakuranetin, diosmetin, chrysin, and naringenin, as well as phenolic acids like caffeic acid and chlorogenic acid. Similarly, our study identified chrysin, quercetin, caffeic acid, and hesperidin as dominant constituents, supporting previous findings. However, the Turkish propolis samples exhibited much wider quantitative ranges, with diosmetin levels reaching over 100 mg/g, while in our sample, such high concentrations were not observed. Additionally, Turkish samples displayed high triterpene content—notably oleanolic and tormentic acids-which were not predominant in our extract. These differences can be attributed to botanical origin, extraction solvent composition, and geographical factors, highlighting the chemical variability of propolis across regions. Despite these compositional differences, the presence of shared bioactive compounds strengthens the rationale for evaluating combined anticancer effects, as explored in the current study[16].

In contrast to the current study, which identified a diverse range of flavonoids and phenolic acids such as chrysin, quercetin, caffeic acid, and hesperidin, the chemical profiling of Brazilian green propolis revealed a more standardized composition. Using UPLC-ESI-QTOF-MS and HPLC, seven phenolic acids—including chlorogenic acid, caffeic acid, and artepillin C—were identified and quantified. Among these, artepillin C was found to be the most abundant compound (2.48 ± 0.94%), while isochlorogenic acid B had the lowest content (0.08 ± 0.04%) (Brazilian study). Unlike the chemical variability observed in our study, particularly in propolis samples from different regions, Brazilian green propolis showed minimal variation in phenolic acid content across samples, which the authors attribute to the use of a consistent plant resin source. Our results, in contrast, revealed noticeable differences in the concentration of major flavonoids depending on sample origin, suggesting a greater influence of regional flora. Furthermore, artepillin C, a characteristic marker of Brazilian green propolis, was not detected in our sample, underscoring botanical and geographical differences in propolis composition[17].

The cytotoxic effect of our propolis extract on MCF-7 cells was found to be dose-dependent, with viability decreasing from ~85% at 25 μg/mL to ~23% at 100 μg/mL. These findings are in line with a study on Moroccan propolis (PNM), which also demonstrated dose-dependent antiproliferative effects in MCF-7 cells, reporting an IC_{50} of 479.22 μg/mL. Notably, the propolis used in our study showed a more pronounced cytotoxic effect at lower concentrations, suggesting possible differences in chemical composition. While both studies

identified chrysin and quercetin as major constituents, the higher potency observed in our extract may be attributed to the presence and concentration of additional active compounds such as hesperidin or pinocembrin. These differences underscore the role of geographical origin and phytochemical variability in the bioactivity of propolis and highlight the necessity of standardizing propolis extracts for therapeutic applications[18].

The cytotoxic activity of the propolis extract on MCF-7 breast cancer cells demonstrated a clear dose-dependent trend. Cell viability decreased from ~85% at 25 μg/mL to ~50% at 50 μg/mL and reached ~23% at 100 μg/mL, indicating a potent antiproliferative effect. These findings are consistent with previous studies demonstrating the anticancer activity of propolis in hormone-dependent breast cancer models. Importantly, fibroblast cells exhibited significantly less sensitivity at equivalent concentrations, suggesting a degree of selectivity toward cancer cells. In comparison to quercetin and paclitaxel, propolis showed a milder but more gradual cytotoxic profile, which may be advantageous in minimizing off-target effects. The observed selective toxicity supports the potential of propolis as a natural, multitarget anticancer agent, particularly when considering its complex composition rich in flavonoids such as chrysin and quercetin. These results highlight the therapeutic relevance of propolis and provide a rationale for further investigation into its use in combination with standard chemotherapeutics[19].

In the current study, hesperetin exhibited moderate cytotoxic activity on MCF-7 cells, with a dose-dependent reduction in cell viability. These findings agree with previous research demonstrating the pro-apoptotic potential of hesperetin in breast cancer models. Palit et al. (2015) reported that hesperetin induces apoptosis in MCF-7 cells via activation of the ASK1/JNK signaling pathway, increasing the Bax/Bcl-2 ratio, promoting cytochrome c release, and subsequently activating caspase-9 and -3. Although the present study did not explore mechanistic pathways, the observed reduction in cell viability upon hesperetin treatment may reflect the activation of similar intrinsic apoptotic cascades. The relatively lower cytotoxicity of hesperetin compared to quercetin observed in our study may be attributed to differences in hydroxylation patterns and cell permeability. Nonetheless, our data support hesperetin's potential as a naturally derived antiproliferative agent, warranting further mechanistic investigations in future studies[20].

Quercetin markedly inhibits the nuclear translocation of Y-box binding protein-1 (YB-1), thereby enhancing the chemosensitivity of both MCF-7 and doxorubicin-resistant MCF-7/dox cells to multiple chemotherapeutic agents[21].

In the referenced study, hesperidin demonstrated significant cytotoxic activity against the MCF-7 breast cancer cell line. At a concentration of 80 μg/mL, cell viability decreased to 11.25% and remained relatively low (15.6%) even at 160 μg/mL, indicating a plateau in response. The calculated IC_{50} was approximately 10 μg/mL, confirming potent antiproliferative effects. These effects were attributed to secondary cytotoxic mechanisms, primarily the induction of apoptosis. The findings are consistent with the analyses conducted and are well-supported[22].

In conclusion, this study demonstrated that an ethanolic propolis extract, along with selected polyphenolic compounds (quercetin, hesperidin, and hesperetin) exerts dose-dependent cytotoxic effects on MCF-7 breast cancer cells. HPLC analysis confirmed a flavonoid-rich chemical profile, aligning with previous findings from both regional and international propolis samples. While each compound displayed significant antiproliferative activity individually, combination index analysis revealed primarily additive or antagonistic interactions, rather than synergistic ones. These results suggest that the complex interplay among bioactive constituents in natural extracts can influence therapeutic efficacy. Moreover, comparisons with propolis from different geographic origins underscored the pivotal role of botanical source and extraction methods in shaping chemical diversity and biological activity. To the best of our knowledge, this is the first study to investigate the combined cytotoxic effects of propolis and individual polyphenols in a cancer model, offering novel insights into natural product-based anticancer strategies. Overall, these findings highlight the need for further mechanistic research to optimize the therapeutic potential of polyphenol-propolis combinations.

STATEMENT OF ETHICS

No need for ethical approval for this study.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the study.

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