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**Research Article**

# Synergistic and Dose-Dependent Effects of Pinostrobin, Pinocembrin and Pinobanksin on Different Breast Cancer Cell Lines

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**Abstract**

In this study, the cytotoxic and apoptotic effects of three phenolic compounds highly found in the poplar type propolis; pinostrobin (PS), pinocembrin (PC) and pinobanksin (PB), were investigated individually and in combination on hormon-positive (MCF-7) and triple negative (MDA-MB-231) breast cancer cell lines and fibrocystic breast epithelium (MCF-10A) as control. Assessment of cytotoxicity and apoptosis were performed with WST-1 and Annexin V-7AAD assays, respectively. All statistical analyses including the two-way ANOVA and multiple-t-test were performed using GraphPad Prism software. Individually, PB ($P<0.0001$), PS ($P<0.0001$), and PC ($P<0.05$) demonstrated potent cytotoxic effects at moderate to high doses and late time intervals on MCF-7. PB and PS have been found to have a significant proliferative effect at low doses ($P<0.0001$), however, this effect disappeared in higher doses in this cell line. Dual combinations of PB+PC and PB+PS were toxic on MCF-10A, however, dual combination of PS+PC and the triple combination (PB+PS+PC) showed no cytotoxicity until high doses at late time intervals ($P>0.05$). On MCF-7, the triple combination induced cytotoxic/apoptotic effects even with the 25% dose and 50% dose on MDA-MB-231($p<0.0001$). Our findings clearly showed that different combinations of these phenolic substances can have synergistic cytotoxic effects and even hormetic effects in non-tumorogenic cells.

**Keywords:** Breast cancer cell line, Cytotoxicity, Pinobanksin, Pinocembrin, Pinostrobin, Propolis

## Introduction

Breast cancer (BC), a heterogeneous disease, comprises 4 main molecular subtypes with different gene expression profiles and clinical outcomes [1,2]. Luminal A and B subtypes are characterized with estrogen receptor (ER) positivity, HER2+ with the over amplification (over-expression) of HER2 and triple-negative with the lack of ER, progesterone receptor (PR) expressions and HER2 overexpression [3]. The hormone receptor positive luminal types are the most commonly diagnosed types with making up 70-75% of all BCs [4]. The main medical treatment approach for ER+ BCs is endocrine treatment [5], yet recurrences occur in 20-30% of the cases [6]. Moreover, the most aggressive type, triple negative BCs constitute 10-20% of all invasive BCs. There is no targeted therapy available against triple negative BCs, thus, only the conventional chemotherapy is used, which has a poor response rate in these cases [7]. Therefore, supporting approaches are investigated in the treatment of BC.

The lack of targeted therapy and high recurrence rates made many researches focus on different complementary approaches. Propolis, one of the most focused substances among these, is a natural product produced by honeybees from substances collected from various plants. It is

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widely used since the ancient times, generally owing to its anti-bacterial, anti-viral properties, and more recently further for its anti-oxidant, anti-inflammatory and anti-tumoral properties [8-10]. Among the active components of propolis, bioflavonoids emerge as the most prominent. Flavonoids, secondary metabolites of plants, are highly found in the poplar type propolis, including pinocembrin and pinobanksin [11-14]. Pinostrobin (PS), which was discovered in pine *(Pinus strobus)* wood content 60 years ago, is a flavanon, a subtype of flavonoids. It can be found in galangal, honey and propolis. It has been demonstrated that pinostrobin shows significant biological activities such as anti-proliferative [15,16], anti-inflammatory [17], anti-oxidant [18], and anti-aromatase [19] effects on various cancer cell lines, inhibits HIV-1 protease [20], and shows anti-ulcerative activity [21]. Moreover, by decreasing the Alzheimer's disease related amyloid peptide activity and calcium overload inhibits the mitochondrial pathway related to apoptosis [22]. The cytotoxic effect of pinostrobin may be related to topoisomerase inhibition [23] and its anti-aromatase effect in hormone-dependent cancers [19]. However, like many other flavonoids, pinostrobin also shows low water-solubility which creates a significant limitation for its use in pharmaceutical applications.

Pinobanksin (PB), a member of the dihydroflavonol subtype of flavonoids, is found in Pinus resinosa tree trunk [24], sunflower honey and propolis [25]. It has been shown that besides reducing Fe (II)- induced lipid peroxidation, it also shows antioxidant activity by inhibiting mitochondrial membrane permeability transmission [26]. Some PB derivatives are shown to be apoptosis inducing compounds [27], however, their apoptotic effects vary among cancer cell lines. While pinobanksin-3-acetate (PB3A) application to HCT-116 colon cancer cell lines have shown to induce apoptotic and anti-carcinogenic effects [28], it showed no effect on A549 lung adenocarcinoma cells [24].

Application of pinocembrin (PC), a member of the flavanon subtype, induced Bax-dependent apoptosis in HCT-116 colon cancer cell line [29]. PC showed protective effects against LPS related inflammation both *in vitro* and *in vivo* [30]. Furthermore, in a study conducted on diabetic mice, it was demonstrated that it reduces reactive oxygen radical and inflammatory agent levels [31]. Moreover, it was found to be a BC resistance protein 2 (ABCG2) inhibitor [32]. Lastly, it was shown to inhibit TGF-β1 induced cell invasion and migration in retinoblastoma cells in non-cytotoxic concentrations [33]. In the same study, it was reported that PC decreased vimentin, N-cadherin and integrin expressions, and inhibited focal adhesion kinase (FAK) and p38α signal activation via inducing the down-regulation of pTGF-β1 induced metalloproteinase enzyme mRNA and protein levels.

Cancer may relapse or cells may generate multiple drug resistance, therefore, current treatment approaches are frequently inadequate to achieve a complete cure. To overcome, novel, less aggressive approaches are needed. In many studies, various plant-based active substances attracted attention and were found promising. Although propolis is one of the most focused substances for this purpose, the propolis content varies according to seasonal and regional flower diversities, thus the cytotoxic and biological properties of propolis differ depending on its content.

Therefore, in this study, it was aimed to determine the cytotoxic and apoptotic effects of PS, PB and PC, substances found in poplar type propolis in varying levels, individually and in combination on a human breast cancer cell line MCF-7 and a human fibrocystic breast epithelial cell line, MCF-10A as a control. Moreover, in order to evaluate the effects of the triple-mixture on the most aggressive BC type, the MDA-MB-231, triple negative BC cell line was utilized.

## Material and Methods

### Cell Culture

In this study, MCF-7 cell line representing hormone positive (ER/PR(+)), triple negative (ER/PR(−), HER2(−)) MDA-MB-231 breast cancer cell lines and MCF-10A cell line representing fibroadenoma breast epithelial as control were used. All cell lines purchased from the American Type Culture Collection (ATCC) (Rockville, Maryland, USA) were maintained in their recommended mediums and all supplemented with 1% glutamine and 1% penicillin and 10% fetal bovine serum (FBS) (Biochrome, Berlin, Germany).

### Preparation of Test Solutions of Phenolics

The main stock solutions were formed by dissolving the HPLC >95% purity PC (No: PHL80061), PB (No: 68530) and PS (No: 38790) in 60% ethanol purchased from -Aldrich (Sigma-Aldrich, Merck, KGaA, Darmstadt, Germany). Then the doses to be applied in the experiment were diluted with the medium containing 3% FBS. Doses of pinobanksin (1.25, 2.5, 5, 7.5, 12.5, 15, 20, 25 µg/mL), pinostrobin (1.25, 2.5, 5, 7.5, 12.5, 15, 20, 25, 37.5, 50 µg/mL) and pinocembrin (2.5, 7.5, 15, 22.5, 30, 37.5 µg/mL) were applied to the cells to determine the effects at different time intervals (24$^{th}$, 48$^{th}$, and 72$^{nd}$ h). The substances dissolved with the sonicator were filtered through a 0.22 µm diameter filter before application.

First of all, the individual half-maximal inhibitory concentration (IC50) values of the substances were determined, then, in order to prepare the dual combinations and triple mixtures, they were brought together as in the final total mixture, the concentrations of every single

substance were similar to their IC50 values (MCF-7), which is stated as 100%. After that, the 25%, 50% and 75% doses were prepared with following dilutions. All four doses of the dual combinations and triple mixture were applied to MCF-7 and MCF-10A, in addition, only the triple mixture was applied to MDA-MB-231.

### Cell Viability Assay

*In vitro* cytotoxicity studies were performed using the WST-1 test, which is more sensitive and easier than other tetrazolium salt based cell viability tests. Cells were trypsinized and plated in 96 well plates $1x10^4$ cells per well. Determined doses of PS, PB and PC were administered to cells both individually and in combination after 24 h for cell attachment and differentiation. WST-1 reagent was applied and the plates were incubated for 2 h in the dark prior to 24, 48 and 72 h following substance administration. Plate absorbances were measured with a multi-plate reader (Thermofisher Sci., Waltham, MA, USA) at 450 and 620 nm wavelengths at the 24$^{th}$, 48$^{th}$, and 72$^{nd}$ h.

### Apoptosis Assay

Annexin V and Dead Cell Assay was performed utilizing Muse™ Cell Analyzer (Millipore Corporation, Merck, KGaA, Darmstadt, Germany). Per well, 250,000 cells were seeded into 6 well plates and 24 h were allowed for cells to attach and differentiate. Cells were treated with the triple combination mixture containing PB, PS and PC with the specified dose, and at the following 48$^{th}$ h they were treated with Annexin V and Dead Cell Reagent (7-AAD) (Merck, KGaA, Darmstadt, Germany) incubated for 20 min. After incubation, each sample was analyzed utilizing the Muse™ Cell Analyzer device. Dead, late apoptotic, early apoptotic and viable cells were determined using 3 biological replicates.

### Statistical Analysis

Power analysis was performed using PS Power and Sample Size Calculation (PS) program *(http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize)* [34]. The sample size was estimated using the data published in the article by Wiyono et al.[35], regarding the two groups, MCF-7 and MDA-MB-231, treated with the same doses of pinostrobin. Assuming the difference of 9.948 between the two groups and an standart deviation of 3.366, the sample size estimated for a t-test (P<0.05 and power=0.8) was in a total of 6 samples (3 samples per group).

WST-1 finding were evaluated utilizing Dunnet's multiple comparisons test and calculations of the IC50 values were performed on the GraphPad Prism version 9.5.0 software (San Diego, CA, USA). Moreover, Annexin-PI evaluations were conducted using two-way ANOVA Sidak's multiple comparisons test. A P-value less than 0.05 was considered significant. The values represent the mean±standart deviation of three independent experiments, each carried out in duplicate.

## Results

### The Individual Proliferative Effects of PB, PS and PC on the MCF 7 and MCF 10A Cell Lines

Cytotoxic effects of PB, PS and PC on MCF-10A and MCF-7 are given in *Fig 1*. PB was found ineffective on MCF-7 at the 24$^{th}$ and 48$^{th}$ h (P>0.05), but on the 72$^{nd}$ h starting from the dose of 15 µg/mL cytotoxicity was observed (P<0.0001). On MCF-10A, a statistically significant proliferation was observed at the 24$^{th}$ and 72$^{nd}$ h with the doses of 1.25 and 2.5 µg/mL (P<0.0001). Starting from 5 µg/mL anti-proliferative effect was observed at the 48$^{th}$ and 72$^{nd}$ h (P<0.05) *(Fig. 1)*.

In the MCF-10A cells, the IC50 values of PB at 24 and 48 h were undetectable as it did not show more than 50% inhibition in this cell line at all doses, while the 72$^{nd}$ h value was determined as 17 µg/mL.

PS was found ineffective with low doses (1.25 and 2.5 µg/mL) on MCF-7 (P>0.05), while it showed cytotoxic effects at all the time intervals starting from the dose of 12.5 µg/mL (P<0.0001). At lower doses (1.25, 2.5, 5 and 7.5 µg/mL) a proliferative effect was observed on MCF-10 at all 3 h (P<0.0001), however, this proliferative effect disappeared after the 12.5 µg/mL dose *(Fig. 1)*. The IC50 values were determined as 20.15 µg/mL, 20.22 µg/mL and 19.53 µg/mL at the 24$^{th}$, 48$^{th}$, and 72$^{nd}$ h respectively.

Although no significant cytotoxicity was observed in MCF-10A in all hours with the PC treatment (P<0.05), an increased anti-proliferative effect with the dose was found in MCF-7 starting from the dose of 15 µg/mL at the 48$^{th}$ and 72$^{nd}$ h (P<0.05) *(Fig. 1)*. The IC50 values were determined as 20.74 µg/mL, 20.67 µg/mL and 19.24 µg/mL at the 24$^{th}$, 48$^{th}$, and 72$^{nd}$ h respectively.

### Effects of Combined Applications of PS, PB and PC on Cell Proliferation in MCF-7 and MCF-10A Cells

WST-1 assay demonstrated that PB and PC in combination have an anti-proliferative effect on MCF-10A at all doses at all of the time intervals (P<0.0001), but only at the 48$^{th}$ and 72$^{nd}$ h a significant cytotoxic effect was observed on MCF-7 (P<0.0001). Cytotoxic effects were similar in MCF-10A cells treated with PB and PS at all doses and all three h (P<0.0001). In MCF-7 cells, no significant cytotoxicity was observed at the 24$^{th}$ h (P>0.05), but approximately 80% of the cells (P<0.0001) were dead at the 48$^{th}$ h at every 4 doses and 50% at the 72$^{nd}$ h (P<0.0001). As a result of the combined application of PS and PC, a significant proliferation was observed in MCF-10A at a dose of 25% at the 24$^{th}$ h (P=0.0066) and a dose-dependent cytotoxic

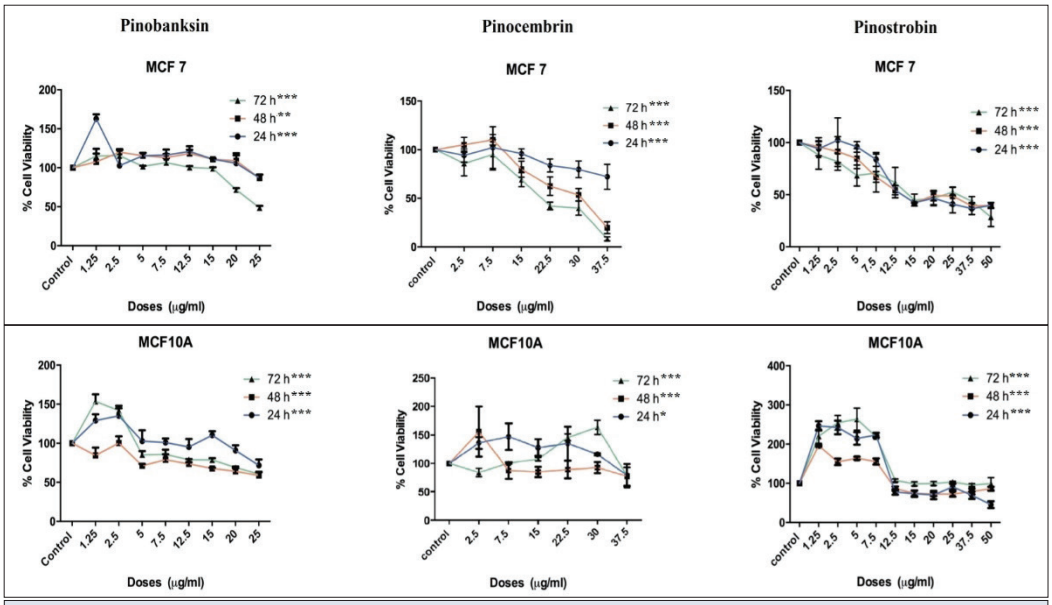


**Fig 1.** Cellular cytotoxicity of all three substances individually on MCF-10A and MCF-7. Detailed statistical findings are presented in results section. *P<0.05, **P< 0.01, ***P< 0.0001 compared to the untreated group

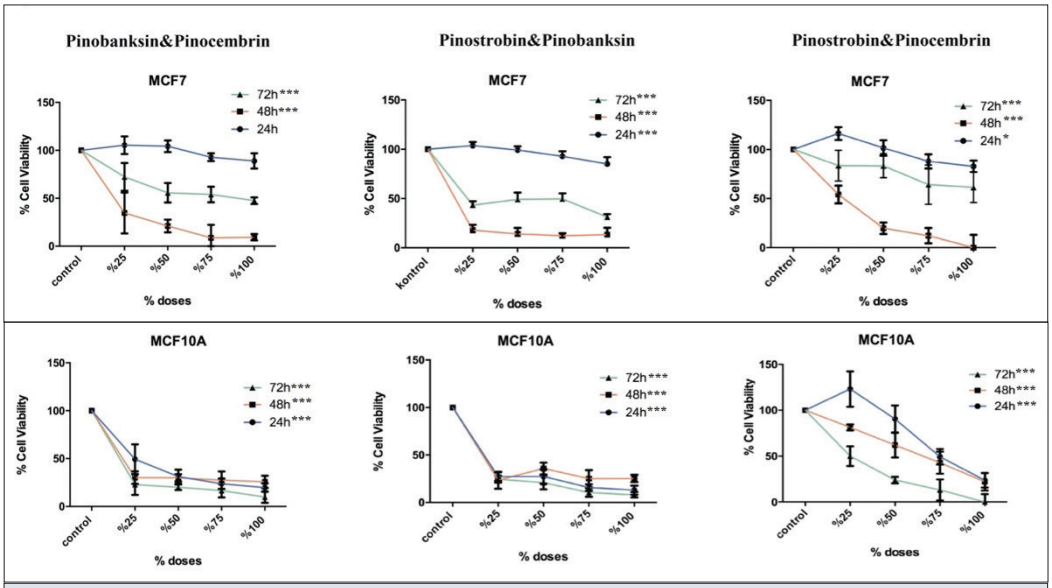


**Fig 2.** Cellular cytotoxicity of all dual combinations on MCF-10A and MCF-7. Detailed statistical findings are presented in results section. *P< 0.05, **P<0.01, ***P< 0.0001 compared to the untreated group

effect was observed starting from this dose. In MCF-7, dose-dependently increased cytotoxicity was observed at all doses at the 48$^{th}$ h, while a significant cytotoxic effect was only observed at 75% and 100% doses at the 72$^{nd}$ h (P<0.0001) *(Fig. 2)*.

PB, PS and PC triple mixture induced a significant dose dependent cytotoxic effect on MCF-7 cells at all doses starting from 25% at all 3 h and became severe with the 50% dose (P<0.0001). On MCF-10A cells, a dose dependent cytotoxicity was observed, with a milder effect at the 24th h (P<0.0001), and more dramatic effect at higher doses at the 75% and 100% doses at late time intervals (48$^{th}$ and 72$^{nd}$ h) (P<0.0001). Moreover, on MDA-MB-231, a significant cytotoxicity was detected starting from the 50% dose at all 3 h (P<0.0001). A preferred toxicity was observed with the 50% dose of the triple mixture being more effective in MCF-7 cell line and MDA-MB-231 than MCF-10A. Therefore, these conditions were selected for the determination of apoptotic and necrotic death *(Fig. 3)*.

## Flow Cytometric Analysis

Fourty-eight h following the treatment of the 50% dose of the triple mixture on MCF-7 and MCF-10A cells, each cell line was compared with its own untreated controls. Annexin V-7AAD analysis findings are given in *Table 1*

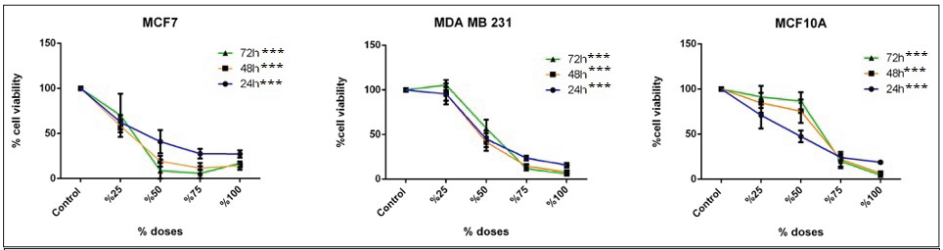


**Fig 3.** Cellular cytotoxicity of the triple mix on MCF-10A, MCF-7 and MDA-MB-231. Detailed statistical findings are presented in results section. *P<0.05, **P<0.01, ***P<0.0001 compared to the untreated group

***Table 1.*** *Statistical Analysis of the Annexin-V/7-AAD of the triple mixture on MCF-10A, MCF-7 and MDA-MB-231*

| Cell Types | MCF-7 | | MDA- MB- 231 | | MCF-10A | |
|---|---|---|---|---|---|---|
| | Mean+SD | P Value | Mean+SD | P Value | Mean±SD | P Value |
| Live (%) | 58.24±2.86 | < 0.0001 | 63.41±2.63 | < 0.0001 | 84.13±2.65 | 0.0112 |
| Early Apoptotic (%) | 19.52±1.75 | < 0.0001 | 24.92±1.56 | < 0.0001 | 10.54±1.36 | 0.0011 |
| Late Apoptotic (%) | 21.26±2.22 | < 0.0001 | 10.04±1.09 | 0.0001 | 4.14±1.46 | 0.8349 |
| Debris (%) | 0.99±0.07 | 0.9985 | 1.63±0.79 | > 0.9999 | 1.19±0.39 | > 0.9999 |
| Total Apoptotic (%) | 40.77±2.85 | < 0.0001 | 34.96±2.04 | < 0.0001 | 14.68±2.5 | 0.0125 |

*Percentage of apoptotic cells post treatment with 50 % doses of triple mixture for 48 h. Values were mean ± SD from three independent experiments (n = 3)*

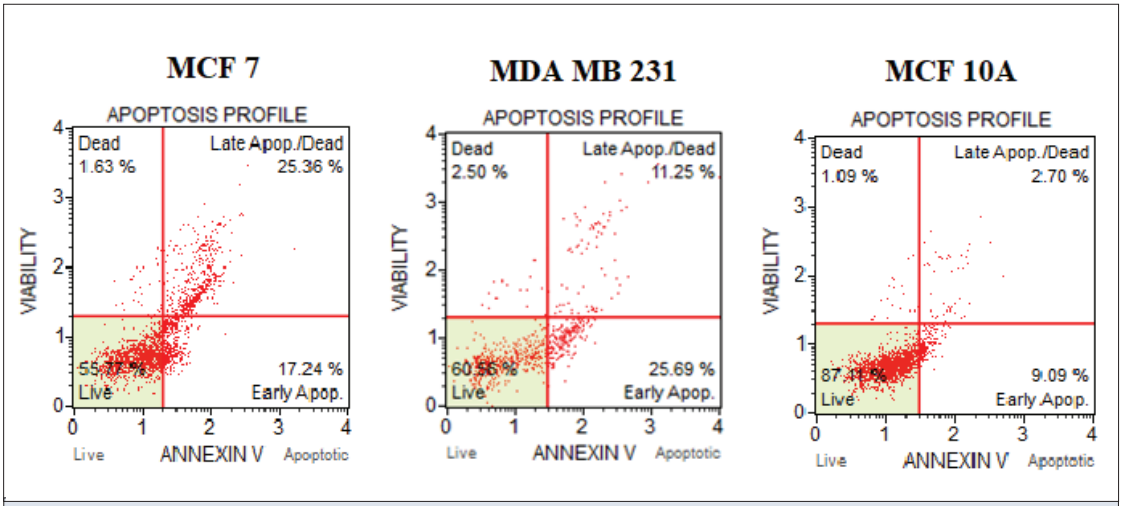


**Fig 4.** Annexin-V/7-AAD analysis of the triple mixture on MCF-10A, MCF-7 and MDA-MB-231

and *Fig. 4*. The triple mixture induced significant apoptotic death on MCF-7 and MDA-MB-231 cells but not on MCF-10A cells.

## Discussion

Propolis is produced by honeybees basically to protect and sterilize their hives from different plant exudates, therefore contains a variety and high levels of phenolic compounds, which attracted many researchers especially this past decade [1,9]. The content, types and efficiency on different diseases have been the main focus. Many studies demonstrated its numerous significant biological effects such as anti-inflammatory, anti-bacterial, anti-viral, anti-fungal, anti-oxidant anti-cancer effects and cardioprotective and hepatoprotective activity of this amazing bee product [8-10]. Studies have shown the anticancer effects of propolis on BC, however, as has been shown before that not every type of propolis may show these effects. The anticancer activity of propolis on BC can generally vary depending on the propolis content and breast cancer type [10,36]. In the presented study, based on the fact that this difference is related to the content of propolis, the effects of PB, PC and PS, compounds highly found in especially poplar type propolis [37], was studied individually and in combination on the most common BC type, hormone responsive (MCF-7) and fibrocystic breast epithelium (MCF-10) as control, *in vitro*. In addition, the triple combination was also investigated on the most aggressive BC type, triple negative (MDA-MB-231)

in order to see its cytotoxic effects, since its treatment approach and efficiency is very limited.

PB was only found effective on MCF-7 at the late time period (72nd h). On the other hand, it showed a proliferative effect with low doses on MCF-10A. In the literature, this incidence is called '*hormesis*' and defines the biphasic behavior with in most cases stimulating effects on proliferation at low doses and proliferation inhibitory effects at high doses. Stress inducers such as heat, dietary restrictions, radiation and various phytochemicals are known to possibly generate the hormetic effect [38,39]. The molecular mechanism of the hormetic effect is thought to be as cells that undergo such stress use all the proteins available to proliferate before being unable to produce more vital proteins. At this point, it can be interpreted as a response of cancer cell against the apoptotic effect of the substance, first by stimulating growth but then not being able to overcome the cytotoxicity/stress. This study is the first to demonstrate the hormetic behavior of PB on MCF-10A cells.

In the study conducted by Xuan et al.[40], PB isolated from Chinese propolis was ineffective at concentrations up to 160 µM (43 µg/mL) on MCF-7 cells at the 24th and 48th h, while in the same study, PC has been shown to be more effective on MCF-7 cells at a dose of 160 µM compared to PB at the 48th h. Our findings also confirmed the cytotoxic effect of PC on MCF-7 starting from the dose of 15 µg /mL at the 48th and 72nd h.

A significant proliferation was observed in MCF10A cells with a low dose administration of PS, also known as pinocembrin-7-methyl ether. A recent study, on the other hand, demonstrated that the maximum dose of 20 µM (5.4 µg/mL) of PS had no effect on proliferation on MDA MB 231 and MCF 10A cells [41]. However, it was observed that PS decreased migration in MDA-MB-231. According to our findings starting from 12.5 µg/mL PS is cytotoxic on MCF-7 cell lines. Moreover, in a previous study, the IC50 value of a pinostrobin derivative, pinostrobin-chalcone, isolated from *Alpinia mutica* rhizomes in MCF-7 cell line was determined as 7.3 µg/mL [42]. In addition, our study is the first in which PB and PC were applied in fibrocystic breast epithelium MCF 10A. The significant cytotoxic effect was observed at a dose of 25% on MCF-10A cells treated with a combination of PB and PC may indicate that administration in combination inhibited the proliferative effect observed with the PB administration alone, suggesting that these two compounds behave differently in a way that PC may be eliminating the growth stimulating activity of PB. With a dose of 25% more than half of MCF-7 cells were dead at the 48th h. Interestingly, PS and PB, which have little or no cytotoxic effect when applied on MCF 10A cells alone, showed a significant and strong cytotoxicity at all doses and hours when administered in combination. This also underlines the synergistic effect of these two compounds. In other words, although when applied individually, PS showed a dramatic hormetic effect on MCF-10A, PB showed a milder hormetic effect. On the other hand, the combination of PS and PB induced a high cytotoxic effect, which indicates a synergistic effect of these substances on MCF-10A. On MCF-7, only PS was cytotoxic among these two, yet together they show cytotoxic effect on later intervals.

PS and PC combination demonstrated the hormetic effect on the 24th h at low doses on MCF-10A. The severe proliferative effect of PS at early doses seems to be inhibited when applied together with PC. Their combination showed dose dependent cytotoxicity on MCF-7. Generally, in the case of the MCF-7 cell line, in all two-substance combinations, dose dependent cytotoxic effects were observed rather at later time periods; no significant toxicity was observed at the 24th h, and effects were more prominent at the 72nd h. On the other hand, PS-PC combination had at all hours cytotoxic effects on MCF-10A. Therefore, when two-substance combinations are taken into account, they were all toxic on MCF-10A, which means that they do not have a selective anti-carcinogenic effect.

The mixture containing all these flavonoids showed cytotoxicity on MCF-10A at higher doses, however, on both MCF-7 and MDA-MB-231 a cytotoxic effect was observed with 50% doses at all 3 h, which is preferable in terms of cancer treatment. In all substance treatments, Annexin V analyses confirmed the cytotoxic effects as apoptotic.

In conclusion, in this study in which the individual, dual or triple combined effects of PB, PS and PC flavonoids were evaluated, we demonstrated that pinobanksin and pinocembrin had a significant synergistic effect in breast cancer cell lines, as well as the possible hormetic effect of PB on MCF10A. Our results show how important the presence of these substances in combination and different levels, which are present in large amounts in poplar-type propolis. It is clearly demonstrated that different combinations may have different cytotoxic effects, sometimes even proliferative effects. It has been observed that when a compound that is proliferative alone in lower doses when administered in combination may show inhibitory effects in the cell and impairs its effect. In addition, some combinations seem to increase their own effect by showing synergistic effects. Therefore, these studies emphasize the importance and need of analyzing the content and standardizing propolis as a natural treatment approach.

### Availability of Data and Materials

The datasets generated during and analyzed during the current

study are available from the corresponding author (O. Öztürk) on reasonable request.

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### Conflict of Interest

The authors declare that they have no conflict of interest.

### Author Contributions

Conception/Design of Study- O.O., A.M.; Data Acquisition- A.B.C., A.P.E.; A.M.; Data Analysis/Interpretation- A.B.C., F.P., T.O., O.O.; Drafting Manuscript- A.B.C., A.P.E.; Critical Revision of Manuscript- T.O., H.Y.A.; Final Approval and Accountability- O.O., A.M.

### Informed Consent

Not applicable.

### Ethical approval

The study does not require ethics committee approval.

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