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Original Research

Flavonoids, bioactive components of propolis, exhibit cytotoxic activity and induce cell cycle arrest and apoptosis in human breast cancer cells MDA-MB-231 and MCF-7 – a comparative study

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Abstract: Breast cancer is one of the most common causes of mortality in women. Flavonoids, among other compounds, are bioactive constituents of propolis. In this comparative study, we investigated the effects of flavonoids apigenin (API), genistein (GEN), hesperidin (HES), naringin (NAR) and quercetin (QUE) on the proliferation, apoptosis, and cell cycle of two different human cancer cells - MDA-MB-231, estrogen-negative, and MCF-7, estrogen-positive receptor breast carcinoma cells. Many cytotoxic reports of flavonoids were performed by MTT assay. However, it's reported that MTT is reduced in metabolically active cells and yields an insoluble purple formazan, which indicates that obtained cytotoxic results of flavonoids could be inconsistent. Cell viability was measured by NR, neutral red assay, while the percentage of apoptotic cells and cell cycle arrest were determined by flow cytometry and Muse cell cycle assay, respectively. The results showed a high dose-dependent effect in cell viability tests. IC_{50} values were as follows (MCF-7/MDA-MB-231, for 48 h, in μM): 9.39/50.83 for HES, 25.19/88.17 for API, 40.26/333.51 for NAR, 49.49/47.50 for GEN and 95.12/130.10 for QUE. Flavonoid-induced apoptosis was dose- and time-dependent, for both cancer cell lines, though flavonoids were more active on MCF-7 cells. The flavonoids also induced cell cycle arrest in cancer cells.

Key words: Flavonoids; Propolis; Breast cancer.

Introduction

Breast cancer is one of the most common causes of mortality among women. Worldwide, this malignancy ranks as the fifth leading cause of cancer related deaths. Among inhabitants of less developed countries it is the leading cause of death (324,000 deaths, 14.3% of cancer mortality). In more developed regions of the world breast cancer ranks second just after lung cancer as to cancer related deaths (198,000 deaths, 15.4% of cancer related mortality). In the next 10 years, the number of breast cancer patients are expected to exceed an additional 20,000 per year (1).

Breast cancer is a heterogeneous group of tumors. Recently, numerous personalized therapies against breast cancer have been implemented which have proven effective (2). Among the molecular subtypes of breast cancer, recently much attention has been placed on triple-negative breast cancer (TNBC), derived from cells characterized by lack of expression of both steroid receptors (estrogen receptors - ER, and progesterone receptors-PR), as well as the human epidermal growth factor-2 (HER-2) receptor (3-6). TNBC totals approximately 9% of total breast cancers treated surgically (7). Many patients with this type of cancer have an extremely poor prognosis due to low remission during adjuvant therapy and in cases of metastases, a short survival time and high resistance to chemotherapy (8-13). Hormonal and targeted therapy against TNBC is highly problematic, depending on the specific cell signaling pathways (14-19).

Recently, an increased interest in natural chemopreventive agents has been observed (20). Natural products, rich in bioactive compounds are now being used for both cancer chemoprevention and chemotherapy. Presently, over 70% of anticancer drugs used are derived from natural compounds. The progress of nano-targeted therapy also increases the efficacy of natural products by a combination of monoclonal antibodies or polymeric carriers (21).

Propolis, a naturally occurring antibiotic is processed by bees. Its composition and properties are variable and

depend on the geographical origin of the substance (22-24). Research has shown that propolis possesses antimicrobial, anti-inflammatory, immune-stimulatory, antiviral and hepatoprotective activities. These properties have been mostly ascribed to flavonoids, a group of natural compounds contained in propolis (25-32). It is noteworthy that both *in vitro* and *in vivo* studies have recently documented the anticancer activity of propolis, as well as its mechanism of action in relation to the phytochemical composition (33-36). These studies have demonstrated both the anticarcinogenic properties of propolis and its biologically active compounds (including flavonoids) against different cancer cell lines (37, 38).

Flavonoids are polyphenols which contain fifteen carbon atoms. Their carbon skeleton consists of two benzene (A and B) rings connected by a short three carbon chain (C6-C3-C6). One of the carbons in this chain is connected to a carbon atom in one of the benzene rings, either through an oxygen bridge or directly, which produces a third heterocyclic middle (C) ring. The main classes of flavonoids (flavanones, flavones, flavonols, flavanols, isoflavonoids and anthocyanidins) differ in their level of oxidation and saturation of the C ring, while individual compounds within this class vary in their substitution pattern of the A and B rings. Flavonoids are powerful antioxidants, with the ability to scavenge free radicals, chelate transition metal ions and inhibit the enzymes responsible for this chain reaction initiation (39-43). Both preclinical and clinical studies have reported antimicrobial, antioxidant, anti-inflammatory, anticancer, antiatherosclerotic, antiallergic, anti-platelet and anti-hypertensive activities of flavonoids (44-47). Aside from this antioxidant ability, the anticancer potential of flavonoids also depends on their ability to interact with Phase I and II enzymes involved in procarcinogen biotransformation, as well as their ability to inhibit the enzymes involved in DNA replication. Additionally, flavonoids modulate cyclin activity, thus arresting cell cycle (phases G1/S or G2/M), inhibiting cancer cell proliferation and inducing apoptosis (48).

Our research has compared the cellular response of human breast adenocarcinoma MDA-MB-231 and MCF-7 cells to five flavonoids, which normally occur in propolis: apigenin (API), genistein (GEN), hesperidin (HES), naringin (NAR) and quercetin (QUE), all of which are representatives of different subclasses (Table 1). The chemical structures of the flavonoids tested are shown in Figure 1.

Table 1. Flavonoids existing in propolis, and their activities.

Flavonoid	Flavonoid class	Activities	References
Apigenin	Flavones	antiallergic	(49)
		anticancer	(50, 51)
		anti-inflammatory	(52)
		proapoptotic	(53)
Quercetin	Flavonols	proapoptotic	(54, 55)
		anticancer	(55, 56)
		anti-inflammatory	(56, 57)
		antioxidant	(57)
		proapoptotic	(58)
Naringin Hesperidin	Flavanones	anticancer	(59)
		antioxidant	(59, 60)
		anti-inflammatory	(60)
		chemopreventive	(61, 62)
Genistein	Isoflavones	anticancer	(63-65)
		proapoptotic	

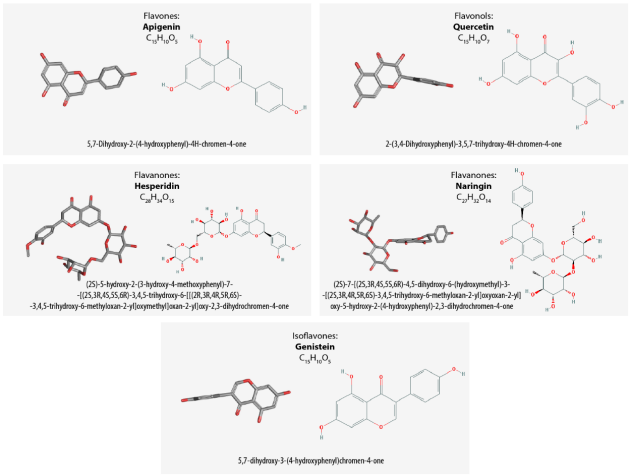

Figure 1. Chemical structures of flavonoids (66-70).

To the best of our knowledge, this is the first time such comparative research has been performed. In this study, we investigated cytotoxic and proapoptotic activities of selected flavonoids, representative of different subclasses, as well as the induction of cell cycle arrest. Flavonoids, which occur in propolis and other honey bee products is the subject of our specific research interest. In earlier studies, we compared two other components of propolis: caffeic acid (CA) and caffeic acid phenethyl ester (CAPE), which inhibited migration rate, induced apoptosis and cell cycle arrest in the breast cancer MDA-MB-231 cells; in particular, CAPE exhibited better activity against TNBC (38, 71).

Materials and Methods

Cell lines and reagents

Breast cancer cell cultures

Two breast adenocarcinoma cell lines, from Caucasian females were used: MCF-7 and MDA-MB-231, the latter being a model of human triple-negative breast cancer (TNBC). MCF-7 cells (human breast adenocarcinoma, no. 86012803 SIGMA from Sigma-Aldrich, Poznań, Poland) were cultured on Dulbecco's modified Eagle's medium, with 10% inactivated fetal bovine serum (Sigma-Aldrich, Poznań, Poland) and 0.01 mg·mL−1 bovine insulin (Sigma-Aldrich, Poznań, Poland). Cells were incubated at 37 °C in 5% CO2 atmosphere. MDA-MB-231 cells (human breast adenocarcinoma, TNBC, no. 92020424 SIGMA from Sigma-Aldrich, Poznań, Poland) were cultured on Leibovitz's L-15 medium with 10% inactivated fetal bovine serum (Sigma-Aldrich, Poznań, Poland), at 37 °C without CO2. Both cell lines were supplemented with antibiotics: 100 U·mL^{-1} penicillin, 100 µg·mL^{-1} confluence of 80%–90%. All manufacturer's recommendations for preparation were carefully followed.

Flavonoids

Apigenin (No. 01760595 SIGMA), quercetin (No. 1592409 SIGMA), naringin (No. Y0001378 SIGMA), hesperidin (No. Y0001203 SIGMA), genistein (No. 05360590 SIGMA) were purchased from Sigma-Al-

drich, Poznań, Poland, and they were all stored, collected, and used strictly according to the manufacturer's instruction.

Cytotoxicity by lyzosomal activity, NR test assay

NR is a weak cationic dye that, relatively easily, penetrates the cell membrane and accumulates intracellularly in lysosomes (lysosomal pH < cytoplasmic pH), where it binds to the lysosomal matrix anionic sites. Therefore, this assay can be used to measure cell viability.

The sensitivity of the lysosome and other effects that gradually become irreversible are due to cell surface changes and lysosomal membrane sensitivity. Such changes caused by xenobiotics result in a reduction in NR uptake binding. Therefore, using this method can distinguish viable, damaged or dead cells (72). The NR test was obtained from Xenometrix AG, Allschwil, Switzerland. We used flavonoids at concentrations of 50 µM and 100 µM, with 24 h and 48 h of incubation. The quantity of dye incorporated into cells was measured by spectrometry at 540 nm, and is directly proportional to the quantity of cells of an intact membrane. Procedure of the test was performed exactly in accordance to the manufacturer's instruction and protocol.

Apoptosis detection: Muse® annexin V and dead cell assay

Briefly, both cell lines were seeded in 6 well plates, with an amount of 5 × 105 cells/well, grown for 72 h to obtain logarithmic growth. The cells were incubated in a complete culture medium containing flavonoids (50 and 100 µM) for 24 h and 48 h. Then, 1 × 106 cells in suspension were transferred to a new tube and incubated with 100 µL of Annexin V & Dead Cell Reagent (Merck Millipore, Warsaw, Poland) for 20 min. at room temperature. Pure medium was used as a control. Apoptosis was determined by Muse® Cell Analyzer, Millipore, Billerica, MA, USA (emission max: yellow-red 576 and 680 nm; excitation max: 532 nm).

Muse® cell cycle assay

This assay was performed according to the manufacturer's instructions. Briefly, cells were plated at 1 x 104 cells/well and incubated in a medium containing 10% FBS (fetal bovine serum) at 37°C. Then, the cells were incubated in a medium containing flavonoids, at measures of 50 and 100 µM, for 24 h and 48 h. Finally, the cells were washed with PBS (phosphate-buffered saline) and centrifuged, then cold, freshly prepared 70%-ethanol was poured and samples were maintained at 20°C until analysis. Afterwards, cells were washed with PBS and further processed for cell cycle analysis in accordance with the manufacturer's instructions.

Statistical analysis

All results were expressed as means ± SD obtained from three separate experiments and performed in quadruplicates (n=12). The results were performed with independent sample t-tests. The experimental means were compared to the means of untreated cells harvested in a parallel manner. Differences between control and 24 or 48 h incubated samples, were tested for significance using the one-way Friedman ANOVA test. A p-value less than 0.05 were considered statistically significant.

Results

Cytotoxic effects of selected flavonoids on MDA-MB-231 and MCF-7 cells

Cytotoxicity of the tested flavonoids was measured by NR (Neutral Red) assay. The results are presented in Figure 2. A strong dose-dependent trend is clearly indicated.

For the MDA-MB-231 cells, only mild cytotoxic activity was observed for 24 h (Figure 2a). Using a dose of 5 µM, only HES exposed significant activity on the MDA-MB-231 cells, reaching a viability of 88.4%. Increasing doses of all flavonoids resulted in viability inhibition of MDA-MB-231 cells, though QUE, even at a dose of 10 µM, showed no significant cytotoxic activity against MDA-MB-231 cells after 24 h. No flavonoids were below the 65% line (HES reached a minimum value of 68.2%).

Increasing incubation time to 48 h resulted in an evident viability decline from the lowest doses for MDA-MB-231 samples (Figure 2b). The values of cell viability decreased proportionally as the doses were increased. With a dose of 100 µM the following values, ordered from lowest to highest, were: HES – 39.5%, GEN – 42.2%, and API – 48.4%, QUE - 48.7% and NAR: 60.5%

Against the MCF-7 cell line, almost all flavonoids exhibited higher cytotoxicity than on MDA-MB-231 cells after 24 h (Figure 2c). The viability of the MCF-7 cells decreased in a dose-dependent manner. All doses, of all tested flavonoids, resulted in a significant viability inhibition. QUE exhibited slightly higher cytotoxic activity against MCF-7 than MDA-MB-231 cells, reaching its minimum of 62.3% at a dose 100 µM, compared to 71.1% against MDA-MB-231 cells, after 24 h with the same dosage. The highest cytotoxic activity was seen with HES, at 19.4% with a 100 µM dose after 24 h.

The viability values of MCF-7 cells were the lowest at 48 h (Figure 2d). HES showed strong activity even with a 5 µM dose, with a value of 62.9%. The dose-dependent trend remained. With the highest 100 µM dosage, the flavonoids reached the following viability

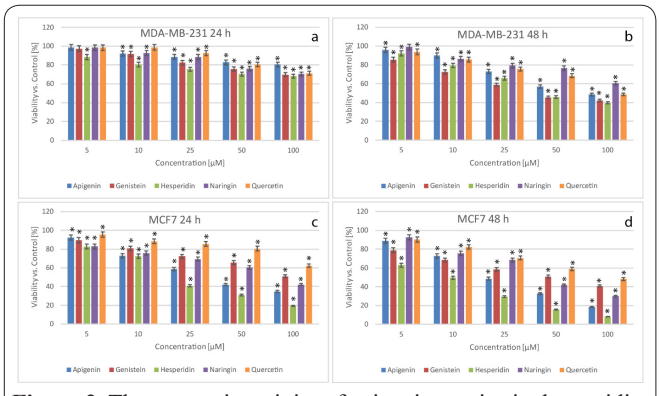

Figure 2. The cytotoxic activity of apigenin, genistein, hesperidin, naringin and quercetin were tested using concentrations from 5 to 100 µM with 24 (**a**, **c**) and 48 (**b**, **d**) h incubation times, on the breast cancer cell lines, MDA-MB-231 (**a**, **b**) and MCF-7 (**c**, **d**). Cell viability was analyzed by NR assay. The results were presented as mean and standard deviation of three independent experiments, with 12 wells each (* p <0.05; Friedman ANOVA test).

Table 2. IC_{50} (μM) values of flavonoids on MDA-MB-231 and MCF-7 breast cancer cell lines.

Flavonoids	MDA-MB-231 cell line Time of incubation	
	24 h	**48 h**
Apigenin	>1000	88.17
Genistein	802.64	47.50
Hesperidin	>1000	50.83
Naringin	913.99	333.51
Quercetin	>1000	130.10
Flavonoids	**MCF-7 cell line**	
	24 h	**48 h**
Apigenin	38.84	25.19
Genistein	139.43	49.49
Hesperidin	22.14	9.39
Naringin	82.54	40.26
Quercetin	661.07	95.12

values: API: 18.43%, GEN: 40.8%, HES: 8.4%, NAR: 30.2% and QUE: 48.2%.

For all flavonoids, IC_{50} was calculated on both breast cancer cell lines. The 50%-mortality results are shown in Table 2.

It can be clearly observed that the tested flavonoids showed higher cytotoxic activity against MCF-7 cells and displayed a dose- and time dependent trend.

For the MDA-MB-231 line, the flavonoids ordered according to their decreasing cytotoxic activity are as follow: GEN > HES > API > QUE > NAR. For the MCF-7 line, the cytotoxic activity of the tested flavonoids decreased as follow: HES > API > NAR > GEN > QUE.

Apoptotic effects of selected flavonoids on MDA-MB-231 and MCF-7 Cells

For subsequent experiments, only flavonoids with the highest concentrations, *i.e.* 50 and 100 μM, were used.

For the MDA-MB-231 cells, all tested flavonoids significantly decreased the percentage of live cancer cells and an apoptotic effect was recorded after 24 h of incubation (Figure 3a). All flavonoids induced apoptosis in a dose-dependent manner, except HES, although its increased dosage resulted in an increased amount of dead cells. For doses of 50 μM, the percentage of MDA-MB-231 apoptotic cells were: API - 9.52%, QUE - 12.29%, NAR - 12.64%, GEN - 14.46%, with the highest value displayed by HES - 22.89%. In the same cells, after 24 h, 100 μM flavonoids induced apoptosis as follows: API - 15.40%, NAR - 16.78%, GEN - 16.89%, QUE - 21.62% and HES - 21.99%.

During the 48-h experiment (Figure 3b), the percentage of MDA-MB-231 cells with live phenotype decreased significantly and all flavonoids induced apoptosis. Apart from HES and GEN, other flavonoids triggered apoptosis with a dose-dependent trend. Indeed, HES and GEN were more active at 50 than 100 μM, though, again, the number of dead cells significantly increased at the highest concentration. Therefore, after 48 h of incubation, the percentages of MDA-MB-231 apoptotic cells were: NAR - 18.37%, GEN - 18.46%,

Figure 3. Apoptotic effect of flavonoids on MDA-MB-231 at 50 μM and 100 μM, after 24 h (**a**) and 48 h (**b**) of incubation. Apoptosis was measured by Muse® Annexin V and Dead Cell Assay. All values are presented as means, and vertical bars represent the standard deviation of means (SD) (n = 3 experiments); *p < 0.05.

QUE - 20.47%, API - 25.70% and HES - 38.37% for doses of 50 μM. In the same cells, for the dose of 100 μM, the values were: GEN - 6.55%, NAR - 22.23%, API - 28.82%, HES - 32.90% and QUE - 36.29%.

All results of apoptosis in the MDA-MB-231 cells are shown in Figure 3.

All tested flavonoids showed an apoptotic effect against MCF-7 cells, both at 24 h and 48 h of incubation (Figure 4). In particular, after 24 h treatment, all flavonoids decreased the percentage of live cancer cells and significantly induced dose-dependent apoptosis, except for HES, which again resulted in an increased amount of dead cells with increased dosage (Figure 4a). For doses of 50 μM, the percentages of MCF-7 apoptotic cells were as follow: QUE - 12.26%, GEN - 26.36%, NAR - 35.86%, API - 36.15% and HES - 57.10%. Apoptosis

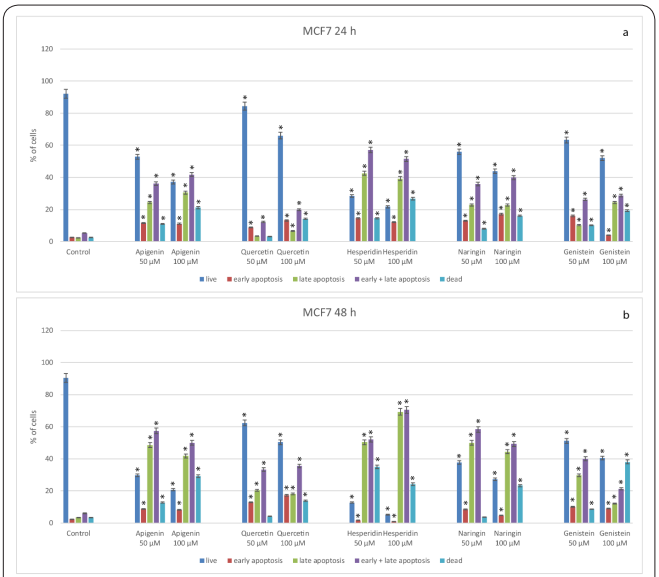

Figure 4. Apoptotic effect of flavonoids on MCF-7 cells at 50 μM and 100 μM, after 24 h (**a**) and 48 h (**b**) of incubation. Apoptosis was measured by Muse® Annexin V and Dead Cell Assay. All values are presented as means, and vertical bars represent the standard deviation of means (SD) (n = 3 experiments); *p < 0.05.

was also induced in the same cells treated with flavonoids at 100 µM, after 24 h incubation: QUE - 19.79%, GEN - 28.64%, NAR - 39.95%, API - 41,71% and HES - 51.48%.

During the 48-h experiment (Figure 4b), the percentage of MCF-7 cells with live phenotype decreased and apoptosis was significantly induced by all flavonoids. At these conditions, only QUE and, above all, HES dose-dependently activated apoptosis. All other flavonoids exhibited apoptosis induction with a slight dose-dependent trend. Percentages of apoptotic MCF-7 cells were: QUE - 33.39%, GEN - 40.11%, HES - 52.15%, API - 57.45% and NAR - 58.39% for doses of 50 µM. In the same cells, 24 h after incubation with the highest flavonoid concentration, percentages of apoptotic cells were: GEN - 21.30%, QUE - 35.66%, NAR - 49.28%, API - 50,01% and HES - 70.47%.

Effects of selected flavonoids on cell cycle in MDA-MB-231 and MCF-7 Cells

In MDA-MB-231 cells, after 24 h of incubation (Figure 5a), 50 µM API induced cell cycle arrest in the G2/M phase in a dose-dependent manner, reaching values of 28.00% for S-phase and 14.57% for G2/M. At 100 µM of API, the value of S-phase declined to 17.30%, while G2/M increased to 22.50%. The values of the G0/G1 phase showed slight change. 50 µM QUE decreased the number of cells in the S-phase to 24.17%, while the G2/M-phase increased to 17.43%. 100 µM QUE only slightly affected cell cycle: 27.90% and 19.40% cells were in S-phase and G2/M, respectively. Only 100 µM HES significantly changed cell cycle progression: the percentage of cells in G0/G1 phase declined to 34.40%, while increasing to 39.67% and 25.77% in S-phase and G2/M, respectively. 50 µM NAR and 50 µM GEN resulted in only slight changes of the cell cycle: NAR showed 44.20% for the S-phase and 11.97% for the G2/M, while GEN showed 26.73% for the S-phase and 20.03% for the G2/M. At 100 µM, no significant changes were recorded: NAR was 45.03% for the S-phase and 9.90% for G2/M, while GEN was 26.87% for the S-phase and 16.10% for the G2/M phase. However, 100 µM GEN arrested cell cycle at the G0/G1 checkpoint (56.90%).

In MDA-MB-231 cells, after 48 h of incubation (Figure 5b), 50 µM API exhibited slight effects on cell cycle progression, with 53.73%, 31.53% and 14.63% of cells in the G0/G1 phase, S-phase and G2/M. QUE induced a weak decrease of the number of cells in the G0/G1 phase (54.83% at 50 µM, and 53.40% at 100 µM) and cell cycle arrest in the S-phase (36.63% at 50 µM, and 34.97% at 100 µM). The cells in G2/M phase increased from 8.47% for 50 µM QUE to 11.53% for 100 µM QUE. For HES, the number of cells in the S-phase declined from 46.67% (50 µM) to 39.57% (100 µM). Cells in G2/M phase were 11.07% after incubation with 50 µM HES, whereas the highest concentration of this flavonoid significantly increased the percentage of cells in phase G0/G1 (50.77%). Only 100 µM NAR significantly affected cell cycle, with 59.17%, 33.67% and 6.63% of cells in G0/G1 phase, S-phase and G2/M phase, respectively. For GEN as well, the cell cycle was arrested at the G0/G1 checkpoint, with the values of 56.37% for 50 µM and 64.60% for 100 µM.

In MCF-7 cells, after 24 h of incubation (Figure 6a), API induced cell cycle arrest in the G0/G1 phase, in a dose dependent manner, with 53.47% and 63.90% of cells at 50 µM and 100 µM, respectively. A dose-depen-

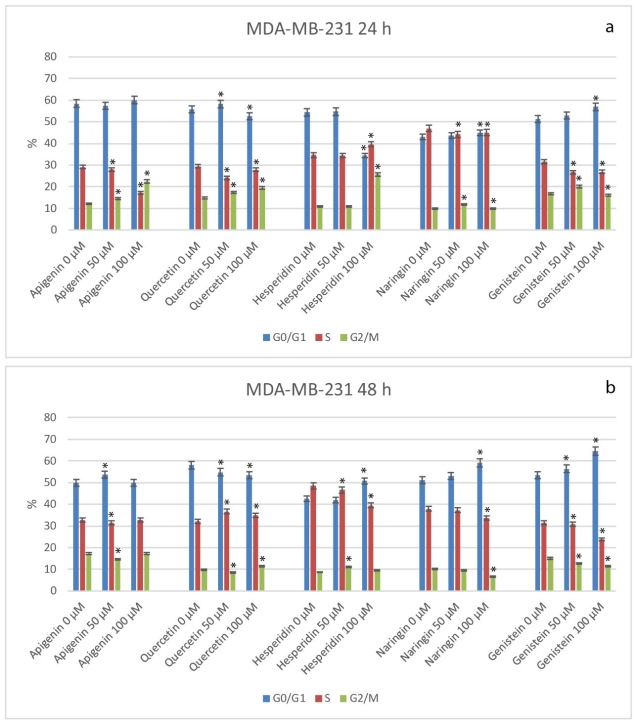

Figure 5. Effects of flavonoids at 50 µM and 100 µM on MDA-MB-231 cell cycle arrest after 24 h (**a**) and 48 h (**b**) of incubation. Cells were stained with Muse® Annexin V and Dead Cell kit and analyzed by flow cytometry with 10,000 events collected. The vertical bars represent the standard deviation of means (SD) (n = 3 experiments); *p < 0.05 value.

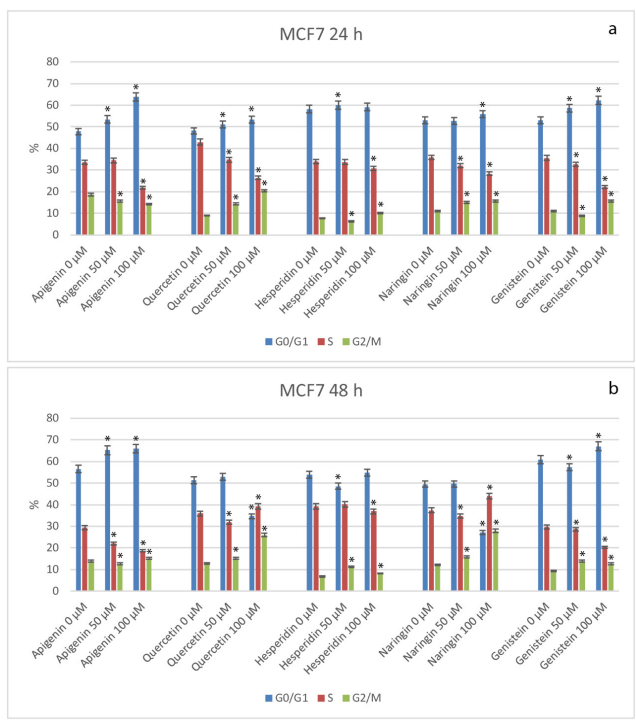

Figure 6. Effects of flavonoids at 50 µM and 100 µM on MCF-7 cell cycle arrest after 24 h (**a**) and 48 h (**b**) of incubation. Cells were stained with Muse® Annexin V and Dead Cell kit and analyzed by flow cytometry with 10,000 events collected. The vertical bars represent the standard deviation of means (SD) (n = 3 experiments); *p < 0.05 value.

dent depletion of cells in G2/M was also observed. QUE treatment resulted in an increase of percentages of cells in the G0/G1 and G2/M phases, in a dose-dependent manner. The values were: G0/G1 - 51.00% and 53.17%, G2/M - 14.23% and 20.40%, for 50 μM and 100 μM, respectively. For HES, with a dose of 50 μM, cells in the G0/G1 phase increased to 60.00% with a slight depletion of cells in G2/M to 6.20%. Increasing the dose to 100 μM, cells in the S-phase decreased to 30.77% and cells in G2/M increased to 9.97%. Treatment with NAR decreased the cells in the S-phase, compared with control, to 32.03% and 28.23%, for dosages of 50 and 100 μM, respectively. Cells in the G2/M phase reached a value of 15.0% for 50 μM NAR and remained stable at 100 μM NAR (15.73%). Once more, GEN resulted in cell cycle arrest at the G0/G1 checkpoint, in a dose-dependent manner, with 58.63% and 62.20% of cells at 50 μM and 100 μM, respectively. The S-phase decreased to 32.60% and 22.17% for 50 μM GEN and 100 μM GEN respectively.

In MCF-7 cells, after 48 h of incubation (Figure 6b), API induced a dose-dependent cell cycle arrest in the G0/G1 phase, with 65.17% and 65.90% of cells at 50 μM and 100 μM, respectively. A dose-dependent depletion of cells in S-phase was also observed. QUE treatment dose-dependently increased the number of cells in the G2/M phase and S-phase, namely G2/M - 15.13% and 25.93%, S - 31.77% and 39.27%, for doses of 50 and 100 μM, respectively. For HES, with a dose of 50 μM, the cells in the G0/G1 phase increased to 48.50%. Increasing the dose to 100 μM, the cells in the S-phase decreased to 36.87%, whereas cells in the G0/G1 increased to 54.80%.

Treatment with 100 μM NAR decreased the cells in the S-phase to 27.13%, whereas the cells in the G2/M phase dose-dependently increased to 15.73% and 27.90% at 50 μM and 100 μM, respectively. Finally, 100 μM GEN resulted in significant cell cycle arrest at the G0/G1 checkpoint, with 67.00% of cells. The S-phase decreased to 28.63% and 20.30%, at 50 μM and 100 μM, respectively.

Discussion

Constituents of honey bee propolis may vary depending on the region of origin. In this comparative study, we investigated flavonoids naturally occur in propolis, *i.e*. API, GEN, HES, NAR and QUE on the proliferation, apoptosis as well as cell cycle of MDA-MB-231 (estrogen receptor negative) and MCF-7 (estrogen receptor positive) human breast carcinoma cell lines. Recently, we compared other bioactive flavonoid agents also found in propolis: caffeic acid (CA) and its ester (CAPE) (38). Flavonoids exhibit a broad spectrum of biological activity, including multitarget anticancer and cancer preventive properties (73). *In vitro* studies on flavonoids have encouraged the research for their use in chemoprevention, though their bioavailability needs to be further investigated. The cytotoxic activity of dietary flavonoids on different human cancer types was reviewed by Sak (74). Bai et al. (75) showed API cytotoxicity on MCF-7 cells, assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), although using higher API concentrations and incubation times (up to 72 h). Noteworthy, as MTT is reduced in metabolically active cells and yields an insoluble purple formazan, the reported cytotoxic effects of flavonoids measured by MTT assay could be inconsistent and need of further study (76). Uifălean et al. (77) reported the cytotoxic activity of GEN on MDA-MB-231 and MCF-7 cells, after 72 h while we achieved similar effects after 48 h of GEN treatment. The dose-dependent cytotoxicity of HES was demonstrated by Febriansah et al. (78) on MCF-7 cells. Noteworthy, they measured 0 % cell viability after treatment with 50 μM of HES, after 24 h, while we determined the lowest cell viability with 100 μM at 48 h. QUE exhibited a dose-dependent growth inhibition both on MCF-7 and MDA-MB-231 cells after 24 and 48 h. Furthermore, according to Ranganathan et al. (79), and in line with our results, QUE was less effective on MDA-MB-231 cells compared with MCF-7 cells. Finally, NAR was protective for neuronal PC12 cells against hydrogen peroxide-induced cytotoxicity (80) in a dose dependent manner.

Bai et al. (75) showed similar results on MCF-7 cells, after 24 h of API treatment. Particularly, at 100 μM, the flavonoid increased late apoptotic cells by 17%, less than in our experimental conditions, while the number of early apoptotic cells was similar to our measurements with 50 μM of API. Tsuboy et al. (81) reported that doses of 10 and 25 μM of GEN did not induce apoptosis in the MCF-7 cell line which may well be due to the lower concentrations of flavonoid they used in their experiments.

In experimental colon carcinogenesis, HES induced both apoptotic and autophagic cell death (82). This flavonoid also exhibited proapoptotic activity in the human hepatocellular carcinoma HepG2 cell line (83). Moreover, Febriansah et al. (78) detected apoptosis in MCF-7 cells by acridine orange/ethidium bromide double staining after treatment with 3.5 μM of HES, which is a concentration far below the doses we used. Our results are in agreement with these reports and confirm that HES induces apoptosis in breast cancer cells. Our results are also in line with Li et al. (84); the authors who also used NAR, to induce apoptosis in MDA-MB-231 cells. After QUE treatment, Choi et al. (85) detected apoptosis in MDA-MB-453 cells, another TNBC line, similar to research by Rivera et al. (86) and in line with our own results on different breast cancer cells.

In agreement with our results, a time- and dose-dependent end cell cycle arrest of MDA-MB-231 cells at G2/M phase after API treatment was reported by Tseng et al. (87), though they used lower concentrations of the flavonoid. Similarly, Lin et al. (88) demonstrated that API induced arrest at the G2/M phase and G0/G1 phase in MDA-MB-231 and MCF-7 cells, respectively. Tsuboy et al. (81) showed that 10 and 25 μM of GEN arrested MCF-7 cells at the G0/G1 phase, as we also observed, however with higher concentrations. Interestingly, GEN enhanced the radiosensitivity of both MCF-7 and MDA-MB-231 cells via G2/M cell cycle arrest (85). Dourado et al. (89) didn't observed any significant change in cell cycle progression on leukemia cells treated with HES. A dose-dependent cell cycle arrest in the G0/G1 phase was induced by NAR in urinary bladder cancer cells (90), and similarly, a cell cycle arrest in G0/G1 phase was induced by QUE in HT-29 colon cancer cells (91).

In the review by Nabavi et al. (92), API suppressed MAPK (mitogen-activated protein kinases) in breast cancer, while NF-κβ and VEGF (vascular endothelial growth factor) as well as PI3K/AKT (phosphatidylinositol-4,5-bisphosphate 3-kinase / Protein kinase B) and ErbB2 expression were all down-regulated. Long et al. (93) showed that API (> 10μM) downregulated ERα and AIB1 expression levels, and also inhibited multiple protein kinases, including p38, PKA, MAPK and AKT, in MCF-7 cells. The antiproliferative proapoptotic effect of API was mediated by ERβ, in MDA-MB-231 cells (Mak et al.) (94).

Fang et al. (95) suggested that GEN induced activation of ATR signaling in MDA-MB-231 cells. Interestingly, GEN activated the BRCA1-A and -B complexes via the ATR signaling pathway. GEN induced changes of 23 signaling molecules, in ATM/ATR-mediated DNA damage response. Zhang et al (96) indicated that high levels of GEN promoted changes in DEG (degenerin) expression. Forty-seven DEGs are involved the cell cycle, including CDC20, BUB1, MCM2 and cyclin B1, thus indicating as the cell cycle pathway represents a relevant target in breast cancer treatment.

Febriansah et al. (78), showed that HES exhibited a synergism in Pgp-expression through inhibition of MCF-7/Dox cells (doxorubicin-resistant), as well as increased sensitivity of these cells to doxorubicin, a widely used chemotherapeutic agent.

Kaur et al. (97) recognized QUE and NAR, as inducers of BCRP, the breast cancer resistance protein. Schindler et al. (98) showed that NAR inhibited the release of VEGF from breast cancer cells. Ranganathan et al. (79) reported that, in the MCF-7 cells, NAR suppressed Twist, p16 and p21 via the p38MAPK pathway. QUE also increased FasL mRNA expression and p51, p21 and GADD45 signaling activities, as well as Foxo3a protein levels and nuclear translocation in MDA-MB-231 cells (99). Generally, we observed, that MCF-7 cells were more sensitive on flavonoids treatment, it is probably due the estrogen receptor. Further research in hormone dependent therapy should be conducted.

In conclusion, a diet rich in flavonoids from natural sources (plant foods and bee products) may represent a protective lifestyle in (breast) cancer chemoprevention as well as promising anticancer phytotherapeutics might be developed from food flavonoids.

This comparative *in vitro* study assessed the effects of selected flavonoids found in propolis on MDA-MB-231 and MCF-7 breast cancer cells. In particular, a correlation was demonstrated between flavonoids and their anticancer properties. Indeed, flavonoids induced a dose-dependent cytotoxicity as well as apoptosis and cell cycle arrest in cancer cells. However, as previously mentioned, the composition of propolis varies, depending on the geographic origin and other environmental factors, and, therefore, the actual content of these bioactive components is variable. In the future, nano-formulation of flavonoids will certainly improve their delivery and targeting, while further studies are needed to fully ascertain their in human bioavailability, efficacy and safety.

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Conflict of interest

Authors declare no conflict of interest.

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