Hrana u zdravlju i bolesti, znanstveno-stručni časopis za nutricionizam i dijetetiku (2024) 13 (1) 16-27
*Food in Health and Disease, scientific-professional journal of nutrition and dietetics (2024) 13 (1) 16-27*

# DIETARY EDIBLE FLAVONOID KAEMPFEROL INDUCES APOPTOSIS AND INHIBITS CELL MIGRATION IN PROSTATE CANCER CELLS

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*original scientific paper*

## Summary

Metastatic prostate cancer is the second most common cancer globally, with high mortality and morbidity rates. This study aimed to elucidate the effects of plant-derived kaempferol on the proliferation and migration of human prostate cancer cells at the molecular level. Spectrophotometric analyses proved that kaempferol was stable up to 48 h. Antioxidant properties were investigated by DPPH, CUPRAC, and ABTS methods, and kaempferol possess 3.6 mg/mL DPPH activity. Cytotoxic properties of kaempferol were investigated on various human cancerous cells, including A549(Lung), PC-3(Prostate), NCI-H295R(Adrenal Gland), HUH-7(Liver), HeLa(Cervix) using the Alamar Blue method. Western Blotting and qRT-PCR was employed to analyze Bax, BcL-2, Caspase-3, Caspase-9, Caspase-12, p53, Nf-κB, Smad-4, Kras, APC, MLH-1 expressions. Kaempferol was found the most potent inhibitor against the proliferation of PC-3 cells with an $IC_{50}$ value of 16.9 μM. Confocal microscopy studies proved that kaempferol was primarily localized in the cytoplasm. Besides, PC-3 cells' migration and colony formation rates significantly ($p<0.0001$) inhibited 46% and 68%, respectively. Increased protein expressions of TP53 and Nf-κB due to kaempferol activated the E-cadherin, key protein in the migration process. Kaempferol treatment elevated the early rate of apoptosis by regulating apoptotic and antiapoptotic genes and proteins, including Bax, BcL-2, Caspase-3, Caspase-9, Caspase-12. Protein expression of Bax was increased 2.63-fold ($p<0.001$), while BcL-2 protein expression was decreased 87% ($p<0.05$). Besides, kaempferol modulated PI3K-Akt, TGFβ, and MAPK signaling pathways. mRNA expressions of Smad-4 and Kras were inhibited while APC and MLH-1 mRNA expressions were increased. The low cost and high efficiency of kaempferol used in treating fatal and increased incidence of prostate cancer can reduce and treat prostate cancer by showing a new direction to traditional treatments.

*Keywords:* kaempferol, prostate cancer, cell migration, apoptosis, cytotoxicity

## Introduction

Increased mortality and morbidity rates have made cancer one of the most critical problems worldwide. In 2020, there were an estimated 19.3 million new cancer cases and 10 million cancer-related deaths worldwide (Sung et al., 2021). Prostate cancer is the second most frequently diagnosed noncutaneous cancer among men and the fifth leading cause of cancer deaths, with 1.4 million new cases and 375.000 deaths in 2020 (Carioli et al., 2020). The prostate's malignant transformation follows a multistep process. First, it initiates as prostatic intraepithelial neoplasia followed by localized prostate cancerous cells. Advanced prostate adenocarcinoma starting with local invasion results in metastatic prostate cancer (Jin et al., 2011). The metastatic stage of the prostate is the leading cause of prostate cancer-associated deaths. Even after intensive treatment processes, the chance of success in metastatic prostate cancer is almost negligible (Sumanasuriya & De Bono, 2018). Therefore, clarification of the molecular mechanism of the metastatic process possesses crucial importance in increasing patients' survival rates. P53, tumor suppressor protein, plays a critical role in the regulation of the prostate cancer signaling pathway. Mutation of p53 has been associated with metastatic prostate cancer. Abnormal expression of p53 affects malignant proliferation, metastasis, and differentiation of prostate cancer cells (Meek, 2015; Wan et al., 2018). Hence, alteration of p53 activity is one of the strategies against the proliferation of prostate cancer. Not only p53 but also other genes such as Smad-4, NF-κB, KRAS, APC, MLH-1 were also prognostic factors for metastatic prostate cancer (Ding et al., 2011). NF-κB expression plays a critical role in prostate cancer progression to castrate-resistant and metastatic cancer (Jin et al., 2008).

Many attempts have been developed against prostate cancer; chemotherapy, radiotherapy, or surgery. However, these treatments' effectiveness and severe side effects decrease patients' welfare and increase the mortality rates and leads to the investigation of alternative new drug candidates against prostate cancer. Photosynthetic plants synthesize secondary metabolites to protect them against bacteria, fungi, and insects. Flavonoids, one of the primary-secondary metabolites, are highly preferred in various pharmaceutical and medical applications due to their active side groups (Batra & Sharma, 2013; Panche et al., 2016; Tapas et al., 2008). The yellow color dietary

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flavonoid, kaempferol (3, 4′, 5, 7-tetrahydroxyflavone) found in various plant parts such as seeds and leaves, fruits, flowers, and vegetables (Rajendran et al., 2014). Kaempferol's antioxidant, antimicrobial, anti-inflammatory, neuroprotective, and anti-cancer properties have been demonstrated (Chen & Chen, 2013; Wang et al., 2018). Direct or indirect interference of kaempferol to multiple signaling pathways triggers the cells to apoptosis and inhibits cell proliferation and migration (Ravishankar et al., 2013). Unlike traditional chemotherapeutic drugs used in cancer treatment, kaempferol showed fewer side effects with combinations with different medicines (Chen et al., 2013; Kim & Choi, 2013). Kaempferol targets cell proliferation, tumor growth, apoptosis, and metastasis in most cancer types (Avtanski & Poretsky, 2018; Boam, 2015; Srinivas, 2015). Kaempferol was showed to inactivate Akt signaling pathway and stimulates caspase-dependent apoptosis in human leukemia cells and oral cavity cancer cells (Kang et al., 2010; Marfe et al., 2009). Besides, it inhibits the proliferation and expression of vascular endothelial growth factor (VEGF) in ovarian cancer cells (Luo et al., 2009). Furthermore, it has been found that kaempferol suppresses epithelial-mesenchymal transition and cell migration in lung cancer cells (Han et al., 2018). Also, Kang et al. (2009) proved that kaempferol could cause apoptosis in MCF-7 (breast cancer cells) by activating the intrinsic apoptotic signaling pathway and down-regulating the PLK1 expression. Besides, it has been found that kaempferol induces apoptosis via the CHOP pathway by causing ER stress in HepG2 cells (Guo et al., 2016). Multiple effects of kaempferol lead us to investigate its potential role against human prostate cancer. This study aims to elucidate the effects of kaempferol on cell death, proliferation, and migration of prostate cancer cells at the molecular level.

## Methods

*Materials*

Kaempferol was purchased from Cayman Chemical (USA) and dissolved in DMSO. The growth mediums (DMEM, RPMI-1640, DMEM F12, F12K, EMEM), Fetal Bovine Serum (FBS), L-Glutamine, and Penicillin-Streptomycin were obtained from Biological Industries-BI (USA). QIAzol Lysis Reagent was purchased from QIAGEN (USA). Real-time PCR SYBR Green Master Mix, cDNA Synthesis Kit, ECL Western Blotting Substrates, and Immun-Blot PVDF membranes were obtained from Bio-Rad (USA). The primary antibodies; GAPDH (60004-1-Ig), Bax (50599-2-Ig), P53 (60283-2-Ig), and MLH-1 (11697-1-AP) antibodies were purchased from ProteinTech (USA) and Bcl-2 (ab182858), NF-κB (ab16502) and Smad-4 (ab40759) antibodies were purchased from Abcam (USA).

*Analysis of the Kaempferol*

*Stability of the Kaempferol*

A double beam UV-Vis spectrophotometer (Shimadzu, Japan) was used to determine the stability of kaempferol between 0-48 h. 10 mM of kaempferol was prepared in acetate buffer, and UV spectrum was taken between 200-800 nm.

*Antioxidant Activity of the Kaempferol*

*DPPH Free Radical Scavenging Assay*

The free radical scavenging activity of kaempferol was determined by the DPPH Free Radical Scavenging assay, in which methanol was used as a control (Blois, 1958). The reaction mixture contains 0.4 M of DPPH and various concentrations of methanol diluted kaempferol. The color change was measured spectrophotometrically at 517 nm (Multiskan Go, Thermo Fisher Scientific, USA). DPPH Free Radical Scavenging Assay activity was calculated by GraphPad Prism 8.0.2 as described by Deveci et al. (2019).

*$ABTS^{\bullet+}$ Cation Radical Scavenging Assay*

The cation radical scavenging activity of kaempferol was determined by the $ABTS^{\bullet+}$ Cation Radical Scavenging assay in which methanol was used as a control (Re et al., 1999). The reaction of 7 mM $ABTS^{\bullet+}$ and 2.45 mM potassium persulfate was incubated for 12 h at room temperature in a dark environment. The $ABTS^{\bullet+}$ solution was then added to the kaempferol solution in methanol at different concentrations. After a 10 min incubation, absorbance at 734 nm was measured using a Multiskan Go microplate reader (Thermo Fisher Scientific, USA). $ABTS^{\bullet+}$ Cation Radical Scavenging assay activity was calculated by GraphPad Prism 8.0.2.

*Cupric-Reducing Antioxidant Capacity (CUPRAC) Assay*

The cupric reducing antioxidant capacity of kaempferol was determined by the Cupric Reducing Antioxidant Capacity assay (Apak et al., 2004). The reaction mixture contains 10 mM Cu (II), 7.5 mM neocuproin, 1 M $NH_4Ac$ buffer, and different concentrations of kaempferol, and the mixture were added to each well of

a 96-well plate. After 1 h incubation, the absorbance at 450 nm was measured using a Multiskan Go microplate reader (Thermo Fisher Scientific, USA). Results were given as $A_{0.50}$ that corresponds to the concentration providing 0.500 absorbance. The Cupric Reducing Antioxidant Capacity assay activity was calculated by GraphPad Prism 8.0.2.

*In Vitro Cytotoxicity Studies*

*Cell Lines and Culture*

PC-3 (Human prostate cancer), A549 (Human lung cancer), HUH-7 (Human hepatocarcinoma), NCI-H295R (Human adrenal gland carcinoma), and HeLa (Human cervical cancer) cells were obtained from the ATCC (American Type Culture Collection), and PNT1A (Human healthy epithelia) cells were obtained from Sigma Aldrich. PC-3, HUH-7, A549, NCI-H295R, HeLa, and PNT1A cells were cultured in a DMEM, F12K, DMEM F12, EMEM, and RPMI-1640 medium, respectively, and these media were supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 2 mM L-Glutamine, and 1% with Penicillin-Streptomycin. The cell cultures were maintained in the incubator (BINDER, USA) at 37 °C in a humidified 5% $CO_2$ atmosphere.

*Determination of Cell Viability*

The cell viability was determined using an Alamar Blue method (Karakurt, 2016). Briefly, cells were seeded at $10^4$ cells/well in flat-bottom 96-well plates and allowed to grow for 24 h and then treated with kaempferol (0, 10, 25, 50, 100, 150, 200, and 250 µM) for 48 h. After the incubation, the media were removed, and the cells were washed with PBS and incubated with Alamar Blue (10%) for 3 h. The absorption was measured at 570 nm and 600 nm in an ELISA plate reader (Multiskan Go, Thermo Fisher Scientific, USA). The values of the half-maximum inhibitory concentration ($IC_{50}$) were calculated from the sigmoidal plot of the cell viability.

*Flow Cytometric Analyses*

The effects of kaempferol on the cells' apoptosis were evaluated by flow cytometry using the PE Annexin V Apoptosis Detection Kit (BD, Biosciences, USA). Cells in 6-well plates were treated with an $IC_{50}$ value of the kaempferol for 37 °C for 48 h. The harvested cells were then rinsed with PBS and then incubated with Annexin V-APC and 7-AAD in a binding buffer for 20 min. The stained cells were analyzed using a NovoCyte Flow Cytometer System (Acea, North America).

*Bio-imaging Studies*

To determine the localization of PC-3 cells, the cells were plated at $75x10^3$ cells/well in the glass-bottom 8-well chamber (Ibidi, Munchen, Germany) and incubated at 37 °C for 24 h. Then the media was removed, and cells were washed with 10 mM of PBS. The cells were treated with an $IC_{50}$ value of the kaempferol and incubated at 37 °C for 30 min. Following the incubation, the kaempferol was removed, and the cells were washed with 10 mM of PBS. The fluorescence intensity were monitored with a confocal microscope (Nikon, USA).

*Wound Healing Assay*

*In vitro* wound-healing assay was performed to detect the migration of kaempferol on PC-3 cells. PC-3 cells were seeded into 24-well plates and treated with an $IC_{50}$ value of the kaempferol at 37 °C for 48 h. Then the media was removed, and cells were washed with 10 mM of PBS. Alive cells were trypsinized, and $5x10^5$ of these cells were seeded on both sides of the inserts (Ibidi, Munchen, Germany) that could form a 0.9 mm thick space and photographed. The cells were incubated for 24 h at 37 °C to form a confluent and homogeneous layer and rephotographed. The number of cells migrated to the wound was analyzed by Image J software.

*Cell Colony-Forming Assay*

A soft agar colony-forming assay was performed to evaluate the colony formation and growth abilities of kaempferol on PC-3 cells. A single-cell suspension ($3x10^4$ cells/well) was seeded into 24-well plates and incubated at 37 °C for 48 h at the $IC_{50}$ value of the kaempferol. Base Agar (1%) was mixed with DMEM and added to 6-well plates as the base layer. Then 0.7% top agar was mixed with the cell suspension, and the mixture was added on top of the base agar. Finally, the cell growth medium was added and incubated at 37 °C and 5%$CO_2$ for 15 days. After incubation, cells were stained with 0.01% (w/v) crystal violet, and colony numbers were calculated with Image J software.

*Western Blot Analyses*

To determine the effects of kaempferol on protein expressions, western blot analyses were performed. Total Protein was extracted using RIPA lysis buffer (Danvers, Massachusetts) supplemented 1 mM PMSF, and protein concentration was determined by the BCA method (Karakurt et al., 2016). 15 µg protein lysed were loaded on 7.5–12% SDS/PAGE gel and then transferred onto polyvinylidene difluoride (PVDF)

membranes (Bio-Rad, USA). After transblotting, the membrane was blocked in 5% non-fat dry milk at room temperature (RT) for 1 h, and the blots were probed with primary antibodies with dilutions of 1:1000 overnight at 4 °C (Bax (21 kDa, 1/1000 dilution), BcL-2 (26 kDa, 1/1000 dilution), Nf-κB (60 kDa, 1/1000 dilution), p53 (53 kDa, 1/1000 dilution), Smad-4 (59 kDa, 1/1000 dilution), MLH-1 (85 kDa, 1/1000 dilution)). After washing with TBS-T, the membrane was incubated with secondary antibody (1/5000 dilution) at RT for 1 h and finally with ECL solution (Bio-Rad, USA). Immunoblot analysis was visualized using Syngene chemiluminescent gel documentation systems (Syngene, United Kingdom).

*Quantitative Real-Time PCR Analyses*

To determine the effects of kaempferol on mRNA expressions of the cells, qRT-PCR studies were performed. Total RNA was isolated by the Trizol method (Rio et al., 2010). The concentration and purity of RNA were measured with the Nanodrop (NanoDrop ™ 2000 / Thermo Scientific). Then, cDNA was synthesized using 1 ng of RNA (iScript cDNA Synthesis Kit, Bio-Rad). qRT-PCR analyses were performed using the SYBR Green PCR Master Mix Kit (iTaq™ Unıversal SYBR Green Supermix, Bio-Rad) according to the manufacturer's instructions. Thermal cycling condition was programmed as: an initial denaturation step for 5 min at 95 °C followed by 40 cycles including a denaturation step for 10 sec at 95 °C and annealing step for 30 sec (Gao et al., 2020). The primers used in qRT-PCR were designed using Primer 3 software (Table 1), and gene specificity was checked by NCBI blast. Each sample was analyzed in duplicate, and GAPDH was used as the normalizer. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

**Table 1.** Primary sequences of the genes involved in the apoptosis pathway

| Gene Name | NCBI Code | Forward Primer (F) Reverse Primer (R) | Tm Value (°C) | Product Size (bp) |
|---|---|---|---|---|
| Bax | NM_001291430.2 | F: CAGCTCTTTAATGCCCGTTC<br>R: CTCAGCCCATCTTCTTCCAG | 59 °C | 245 |
| Smad-4 | NM_005359.6 | F: GCAGATGCTGGGATAGGAAG<br>R: GTTGGTAGCCTCCGTTTCAG | 60 °C | 150 |
| TP53 | NM_001276696.2 | F: TTGATGTTCTTCAACGATTTGGR:<br>ACCGTTCTCCTTTCTTCGAT | 60 °C | 158 |
| Bcl-2 | NM_000657.3 | F: GCTCTTGAGATCTCCGGTTG<br>R: AATGCATAAGGCAACGATCC | 58 °C | 186 |
| Nf-κB | NM_006509.4 | F: ACATCAAGGAGAACGGCTTC<br>R: CTCCGTGATGACCAGGTG | 57 °C | 215 |
| Caspase-3 | NM_004346.3 | F: TGTTTGTGTGCTTCTGAGCC<br>R: CACGCCATGTCATCATCAAC | 60 °C | 136 |
| Caspase-9 | NM_001278054.2 | F: CTCACCCTGCCTTATCTTGC<br>R: TGTCGTCAATCTGGAAGCTG | 58 °C | 162 |
| Caspase-12 | NM_001191016.2 | F: ATGGCTGGAAATGGAAACAG<br>R: TGGCAGTTACGGTTGTTGAA | 57 °C | 192 |
| Kras | NM_001369786.1 | F:TCGAGAAATTCGAAAACATAAAGA<br>R: GTCTGCATGGAGCAGGAAA | 60 °C | 201 |
| APC | NM_001127510.3 | F: CTGAGGCACTGCAGAAAGTG<br>R: CCGCATCTCGGTAAGCATAG | 60 °C | 158 |
| MLH-1 | NM_000249.4 | F: GCGAATCGCTTCAGTCTTTG<br>R: AATCATTCCTTTGGTGAAACG | 60 °C | 157 |
| GAPDH | NM_001357943.2 | F: GTCAGTGGTGGACCTGACCT<br>R: TGCTGTAGCCAAATTCGTTG | 60 °C | 82 |

*Statistical Analyses*

Data are presented as mean value ± standard deviation (SD). Differences between groups were assessed using one-way ANOVA and Student's t-test. Statistical significance was expressed as * $p < 0.05$; ** $p < 0.001$ and *** $p < 0.0001$. All calculations, graphs, and statistical analyses were constituted using GraphPad Prism (GraphPad Software 8.0.2).

## Results

The antioxidant activities of kaempferol were tested using DPPH radical scavenging, ABTS cation radical scavenging, and cupric-reducing antioxidant capacity

(CUPRAC) assay systems, and ascorbic acid is used as a standard. The radical scavenging activities of kaempferol were evaluated by DPPH and ABTS. As shown in Table 2, the $IC_{50}$ values of DPPH and ABTS were calculated 3.66 µg/mL and 3.31 µg/mL, respectively. Besides, it was found that kaempferol possesses significant reducing power potency given in Table 2 ($A_{0,50}$=4.84 µg/ml).

**Table 2.** Antioxidant activities of the Kaempferol by DPPH, ABTS, and CUPRAC assays

| | **DPPH assay** | **ABTS assay** | **CUPRAC assay** |
|---|---|---|---|
| | $IC_{50}$ (µg/ml) | $IC_{50}$ (µg/ml) | $A_{0.50}$ (µg/ml) |
| **Kaempferol** | 3.66 | 3.31 | 4.84 |
| **Ascorbic Acid** | - | - | 20.67±0.01 |

Kaempferol was found to have the maximum absorption peak at 226 nm (Figure 1b). spectrophotometric analyses proved that kaempferol was stable for up to 48 h.

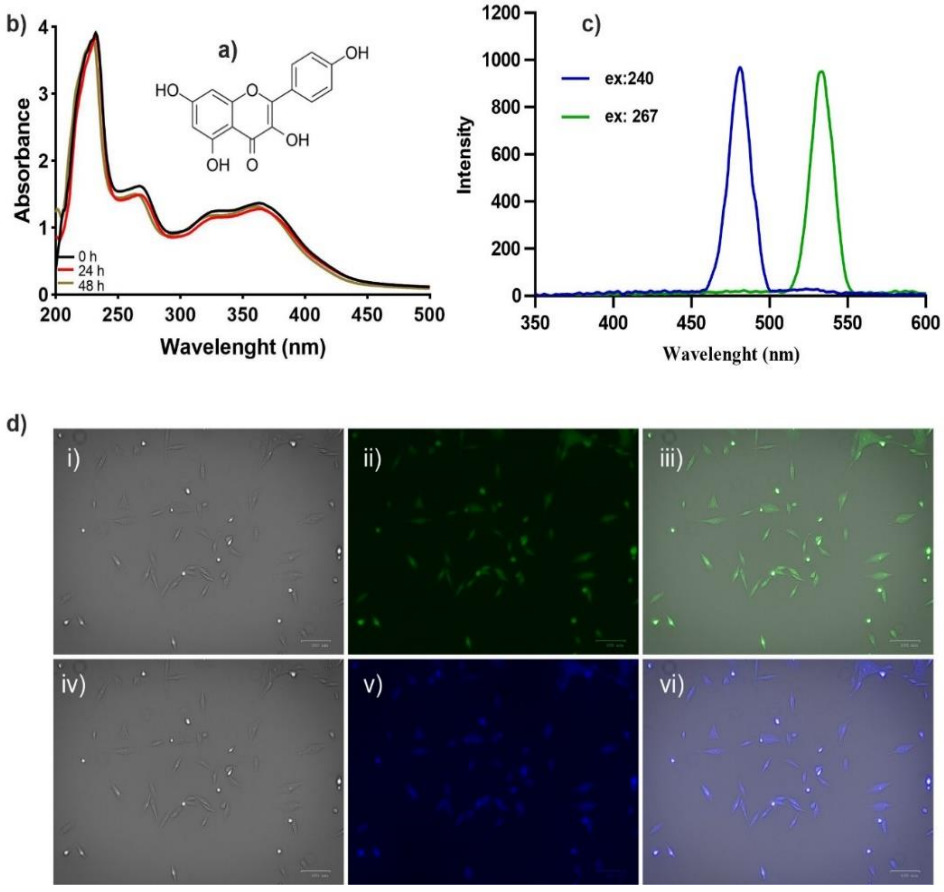


**Figure 1.** Characterization of kaempferol. **a)** Chemical structure of kaempferol. **b)** UV-visible absorption spectra of kaempferol measured at 0 h, 24 h, and 48 h. Kaempferol exhibits maximum absorbance peaks at 220 nm, 266 nm, and 366 nm. **c)** Fluorescence emission spectra of 10 µM kaempferol, with excitation at 240 nm and 267 nm. **d)** Fluorescence cell imaging of kaempferol on PC-3 cells. **i-iv)** Brightfield images, **ii-v)** Fluorescence images, and **iii-vi)** Merged images.

Flourometric analyses showed that when kaempferol was excited at 240 nm and 260 nm, it gave emission peaks at 481 nm and 529 nm (Figure 1c). Confocal studies were also proved that when the cells treated with kaempferol, it mostly localized in cytoplasm and slightly penetrated into the nucleus (Figure 1d).

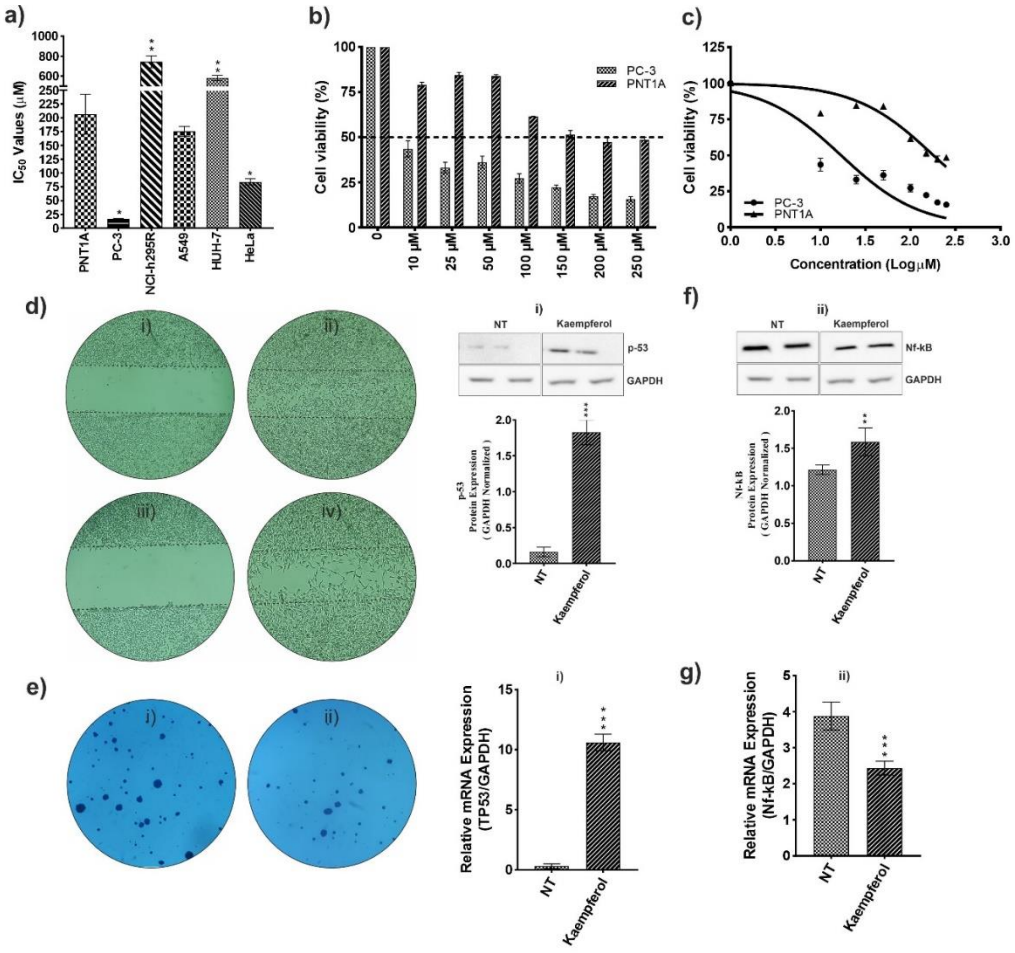


**Figure 2.** Anti-cancer properties of kaempferol. **a)** Cytotoxic effects of kaempferol on various human cancer cell lines. $IC_{50}$ values of kaempferol were determined in PNT1A, PC-3, NCI-h295R, A549, HUH-7, and HeLa cells. **b)** Dose-dependent cell viability of kaempferol on PC-3 and PNT1A cells. **c)** Sigmoidal plot representing % cell viability versus log concentration of kaempferol in PC-3 and PNT1A cells. Kaempferol concentrations ranged from 0 to 250 μM. **d)** In vitro wound healing assay: Images from a scratch assay conducted at different time points. **i)** Image at 0 hours (initial scratch), **ii)** Image at 24 hours for untreated cells, **iii)** Image at 0 hours for the kaempferol-treated group, **iv)** Image at 24 hours for kaempferol-treated cells. **e)** In vitro soft agar colony formation assay. **i)** Colonies formed by untreated (NT) cells and **ii)** Colonies formed by kaempferol-treated cells after 15 days of incubation. **f)** Effect of kaempferol on the protein expression levels of p53 and NF-κB. **g)** Effect of Kaempferol on the mRNA expression levels of TP53 and NF-κB. (Data are presented as mean ± SD. Statistical analysis was performed using Student's t-test, with $p<0.05$ considered statistically significant).

Kaempferol selectively inhibited proliferation of human cancerous cells, and its action was found as dose-dependent manner. Cytotoxic potential of kaempferol was investigate against the viability and proliferation of various human cancerous cells (PC-3, NCI-h295R, A549, HUH-7, HeLa) and healthy epithelial cells (PNT1A) (Figure 2a). No significant cytotoxic effects were observed on HUH-7, NCI-h295R and PNT1A cells (>200 μM). The $IC_{50}$ values were calculated as 787.9 μM (NCI-h295R), 562.2 μM (HUH-7), 182.4 μM (A549), 88 μM (HeLa), 16.9 μM (PC-3), and 206.4 μM (PNT1A), respectively. Kaempferol was found the most potent inhibitor on the viability of PC-3 cells (Figure 2b, 2c). Hence, we further studies were focus on this cell line.

To evaluate the effect of kaempferol in limiting the two dimensions cell migration on PC-3 cells, an *in vitro* wound-healing assay was performed, and gap closure was observed for 24 h (Figure 2d). As a result, the kaempferol-treated group still had a large gap after 24 h compared to the control group, and hence kaempferol was able to slow the cell invasiveness (46%) (Figure 2d-ii, 2d-iv). The colony formation potential of PC-3 cells was clarified by a soft agar colony formation assay. As seen in Figure 2e, colony formation was reduced by 68% in the kaempferol-treated group (Figure 2e-ii)

compared to the control group (Figure 2e-i). p53 (TP53) mRNA (33.6-fold) and protein expressions (19-fold) significantly increased with kaempferol treatment (Figure 2f-i, 2g-i). Kaempferol increased NF-κB protein expression (1.31-fold) while decreasing gene expression (1.59-fold) in PC-3 cells (Figure 2f-ii, 2g-ii).

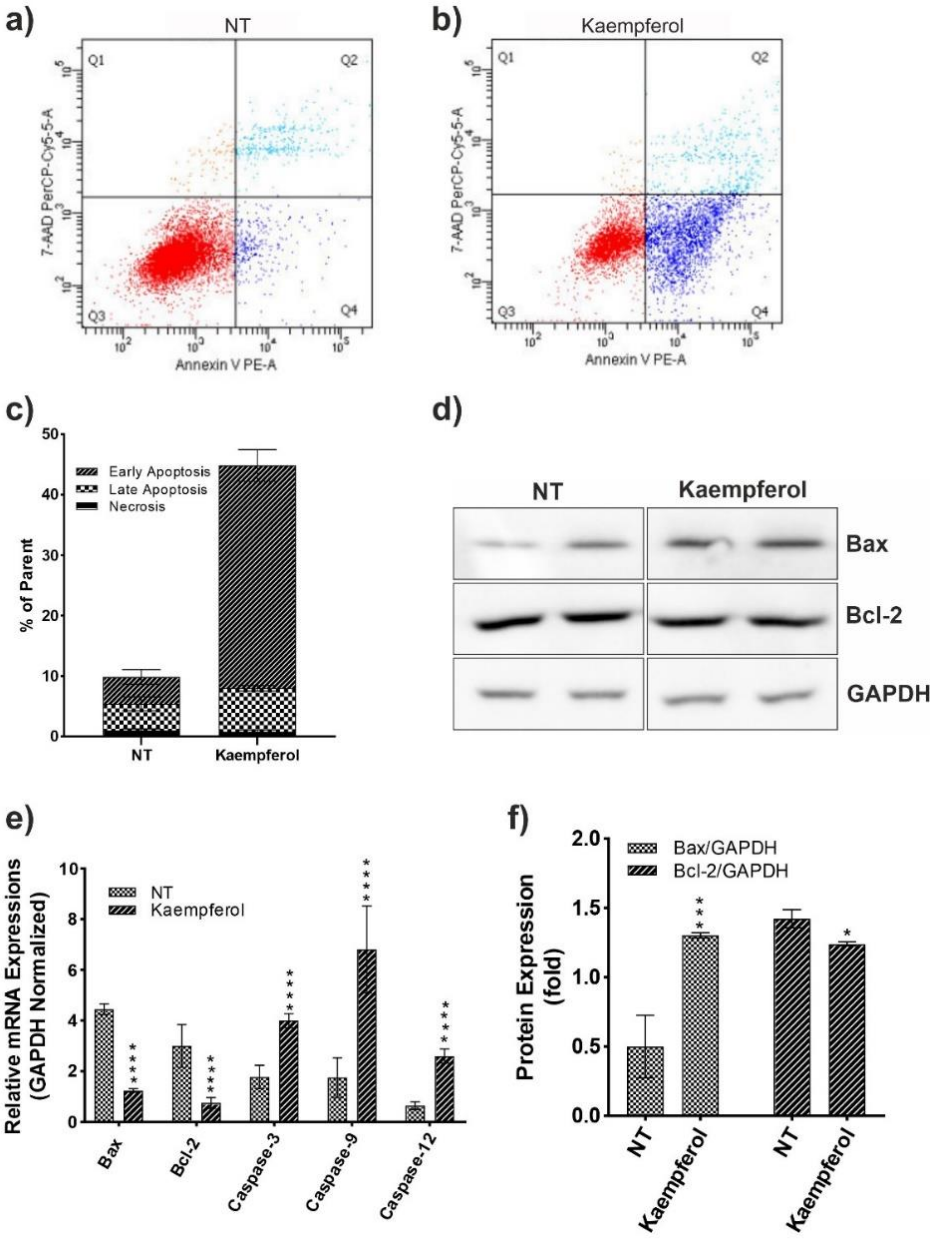


**Figure 3.** The effects of kaempferol on apoptosis of PC-3 cells at the equivalent concentration of $IC_{50}$. a) Flow cytometric plot of the non-treated (NT) group. b) Flow cytometric plot of the kaempferol-treated group. The quadrants represent: Q1: Necrotic cells (Annexin-, 7-AAD+), Q2: Late apoptotic cells (Annexin+, 7-AAD+), Q3: Viable cells (Annexin-, 7-AAD+), Q4: Early apoptotic cells (Annexin+, 7-AAD-). c) Statistical analysis graph from the flow cytometry data, showing the percentage of cells in early apoptosis, late apoptosis, and necrosis stages. d) Representative immunoblot displaying Bax and BcL-2 protein expression, key regulators of the apoptosis pathway. e) Relative mRNA expression levels of Bax, BcL-2, Caspase-3, Caspase-9, and Caspase-12 following kaempferol treatment. f) Relative protein expression levels of Bax and BcL-2 following kaempferol treatment. (Data are presented as mean ± SD. Statistical analysis was performed using Student's t-test, with $p<0.05$ considered statistically significant).

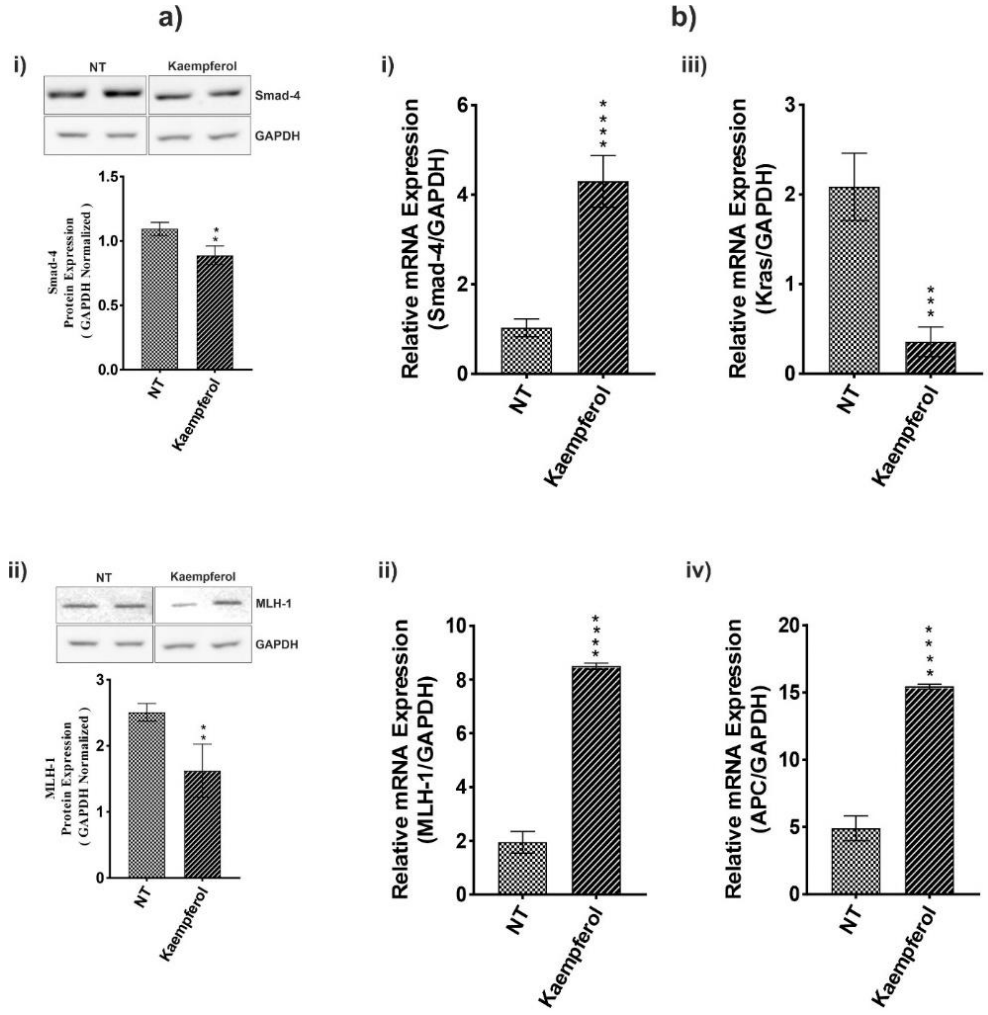


**Figure 4 .**The effect of kaempferol on protein and mRNA expressions in PC-3 cells. Cells were treated with equivalent concentration of $IC_{50}$ value of Kaempferol. **a)** Effect of kaempferol on Protein Expression **i)** and **ii)** represent the immunoblots of Smad-4 and MLH-1 proteins, respectively, in PC-3 cells from the experimental control and kaempferol-treated groups. **b)** Effect of kaempferol on mRNA expressions. Alterations in mRNA expressions of Smad-4 **(i)**, MLH-1 (**ii)** Kras (**iii)** and APC **(iv)** were analyzed by using qRT-PCR. Effects of kaempferol on mRNA levels of the tested genes were normalized to housekeeping GAPDH mRNA. Fold of inhibition was calculated by the following formula: $2^{-\Delta\Delta Ct}$, where ΔΔCt: ΔCt (treated) - ΔCt(control); ΔCt (treated): ΔCt (gene)- ΔCt(GAPDH); ΔCt(control): ΔCt (gene)- ΔCt(GAPDH). Experiments were repeated at least 3 times (n=6). ***, $p<0.0001$ signify a statistically significant difference compared with the control.

To clarify the cell death mechanism, the effects of kaempferol on Apoptosis of the PC-3 cells were investigated by flow cytometer (Figure 3a, 3b). 38% of kaempferol-treated cells progress to early apoptosis (Annexin V-FITC+ / 7-AAD-), 7.2% to late apoptosis (Annexin V-FITC- / 7-AAD+). Thus, the early stage apoptosis rate of kaempferol increased significantly compared to the control group (Figure 3c). Kaempferol significantly increased the expressions of Caspase-9 (3.88-fold), Caspase-3 (2.25-fold), Caspase-12 (4.03-fold), (Figure 3e) and Bax (2.63-fold) (Figure 3f). Besides, kaempferol decreased the expression of the antiapoptotic protein Bcl-2 (0.87-fold) (Figure 3f).

To clarify the molecular mechanism of kaempferol on the proliferation of human metastatic prostate cancer cells, we investigated the protein and gene expression of key genes in signal transduction pathways, including PI3K-Akt, TGFβ, MAPK signaling pathways. Kaempferol caused significant changes in apoptotic and antiapoptotic gene and protein expressions (Figure 4). Kaempferol decreased SMAD-4 protein expression (0.8-fold) while increasing gene expression (4.17-fold) in PC-3 cells (Figure 4a-i, 4b-i). Kaempferol decreased MLH-1 protein expression (0.65-fold) although mRNA expression was increased (4.36-fold) (Figure 4a-ii, 4b-ii). Finally, kaempferol decreased KRAS gene expression 5.87-fold (Figure

4b-iii) and increased APC gene expression 3.15-fold (Figure 4b-iv) in PC-3 cells.

## Discussion

Prostate cancer is one of the leading causes of death among men, and the need for more effective treatments has led to more research (Halimah et al., 2015). Kaempferol, a naturally occurring compound, has high cytotoxicity, making it frequently used in cancer therapy. Recently, the cytotoxicity of kaempferol against various cancer cells has been investigated and remarkable results have been obtained.

Flourometric analyses showed that when kaempferol was excited at 240 nm and 260 nm, it gave emission peaks at 481 nm and 529 nm, which shows that kaempferol has strong fluorescent properties in the blue and green areas. Confocal studies were also proved that when the cells treated with kaempferol, it mostly localized in cytoplasm and slightly penetrated into the nucleus. Aglycone structure of kaempferol led it to pass freely through cell membranes (De Pascual-Teresa et al., 2007; Viskupicva et al., 2008). Kaempferol selectively inhibited proliferation of human cancerous cells, and its action was found as dose-dependent manner. Kaempferol showed the highest toxic effect on PC-3 cells, indicating the selective toxicity of kaempferol on cells. For the specific treatment specific to cancer cells, this was a perfect effect. Kaempferol also increases the level of E-cadherin by increasing the expression of p53 and NF-κB proteins. Thus, the cell's ability to metastasize and colony formation potential is reduced. Also, it has been reported that p53, an independent transcriptional mechanism, migrates to the mitochondria and regulates apoptosis by interacting with members of the Bcl-2 family (Kandasamy et al., 2003; Shankar et al., 2007). Accordingly, our results revealed that p53 increased protein expression in the metastatic prostate cancer cells (PC-3) with kaempferol treatment, which could have a beneficial effect against PCa progression. Analysis of multiple microarray studies where the NF-κB pathway was significantly irregular in metastatic prostate cancer confirmed the opposite effect in our result (Setlur et al., 2007). Increased expression of NF-κB in prostate cancer is associated with a poor prognosis. Therefore, activation of the NF-κB pathway results in prostate cancer progression to androgen independence (Jin et al., 2008). 38% of kaempferol-treated cells progress to early apoptosis. There are death receptors in the external pathway of apoptosis, and they can recognize substances that cause death and enter the internal cytoplasm. Death receptors include tumor necrosis factor (TNF), FAS, and TRAIL (Thorburn, 2004). Kaempferol significantly upregulates TRAIL receptors, suggesting that kaempferol can be an effective factor in treating TRAIL-related diseases (Yoshida et al., 2008). Kaempferol significantly increased the expressions of Caspase-9, Caspase-3, Caspase-12, and Bax. Kaempferol may have synergistically suppressed cell growth by inhibiting the activation of the PI3K/Akt signaling pathway (Li et al., 2019). Besides, kaempferol decreased the expression of the antiapoptotic protein Bcl-2, which is thought to reduce Akt activity (Abotaleb et al., 2018; Ahmed et al., 2019). The proapoptotic and antiapoptotic protein ratios of the Bcl-2 family, specifically the Bax/Bcl-2 ratio, control the resistance or sensitivity of cells to apoptotic stimuli. Moreover, the tumor suppressor TP53 gene manages apoptosis by increasing the expression of several proapoptotic proteins involved in the intrinsic pathway. On the other hand, activation of PI3K/Akt signaling cascade helps cells resist apoptotic triggers (Moradzadeh et al., 2018). That is, the ratio of Bax/Bcl-2 and Caspase-3, Caspase-9, and Caspase-12 were significantly increased in cells treated with kaempferol, suggesting that kaempferol induced Apoptosis both intrinsic and extrinsic pathways in metastatic prostate cancer cells. Kaempferol caused significant changes in apoptotic and antiapoptotic gene and protein expressions. Furthermore, initiation of the MAPK pathway is a key factor in apoptosis, and kaempferol-induced MAPK induction prevents healthy cells from turning into cancer cells (Qattan et al., 2022). Kaempferol decreased SMAD-4 protein expression while increasing gene expression in PC-3 cells. Kaempferol decreased MLH-1 protein expression although mRNA expression was increased. MLH-1 was found to be down-regulated in tumor regions in prostate tissues compared to normal prostate (Chen et al., 2001). MLH-1 protein expression has been shown to be down-regulated in DU145 cells, another prostate cancer cell line, and our results confirm this protein expression deficiency in PC-3 cells (Yeh et al., 2001). This reduction may result from a truncation of the MLH-1 gene caused by the premature stop codon (Chen et al., 2001).

## Conclusion

Given the importance of flavonoids as potential adjuvants or therapeutic agents in cancer treatment, the search for natural products to induce apoptosis of cancer cells could be an excellent strategy for prostate cancer chemoprevention. Kaempferol is a natural flavonoid that inhibits cancerous cells' proliferation while not affect healthy cells; this causes it to be used

frequently in cancer treatment. Prostate cancer is one of the leading causes of cancer-related deaths due to its strong metastatic effect. In this study, low doses of kaempferol highly affected the ability of strong metastatic prostate cancer cells (PC-3) to metastasize and prevented their proliferation. Also, its effects at the protein and gene level in PC-3 cells are quite remarkable. Therefore, the low cost and high efficiency of natural compounds used in treating fatal and increased incidence of prostate cancer can reduce and treat prostate cancer by showing a new direction to traditional treatments. However, some difficulties arise due to the lysosomal degradation and easy excretion of kaempferol from the body. In addition, the poor bioavailability of kaempferol can be increased by developing nano-based formulations. In addition to all these, various animal models can be created to investigate the bioavailability of kaempferol *in vivo* and can be designed as an effective therapeutic agent for preclinical cancer treatments. Therefore, this study will shed light on future studies that can increase the bioavailability of kaempferol and its degradation time.

*Conflict of Interest*

The authors declare no conflict of interest, financial or otherwise.

*Authors' Contributions*

Serdar Karakurt designed the research; Hatice Gul Batur and Irem Mukaddes Bilgiseven performed the experimental study; Sevtap Karakurt analyzed the data; Serdar Karakurt, Hatice Gul Batur and Irem Mukaddes Bilgiseven prepared the manuscript.

*Acknowledgments*

We would like to thank the Research Foundation of Selcuk University [Grant number 19401162].

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