Original Article

Liposomal Nanoparticles Reduce Dose-Dependent Behavior of Paclitaxel against MDA-MB 468 Breast Cancer Cells

*Toofan Aghazadeh[1], Nuredin Bakhtiari[2], Isa Abdi Rad[2], Fatemeh Ramezani[4]**

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Abstract

Background & Aims: Owing to its anti-cancer and anti-oxidant properties, Kaempferol (KAE) has become an ideal candidate to be more welcome into clinical practice. However, Due to its low water solubility and bioavailability, we aimed to design and address a new liposomal formulation with KAE and evaluate its anti-cancer activity against MDA-MB 468 breast cancer cells.

Materials & Methods*:* To characterize the physicochemical features, pharmaceutical parameters such as nanoparticle size, morphology of particles under scanning electron microscopy (SEM), and zeta potential were measured. The optimum liposomal formulation along with paclitaxel was incubated to investigate their biological activity against breast cancer cells. Furthermore, molecular mechanisms related to program cell death (apoptosis) and their gene expression were measured by flowcytometric and real-time PCR, respectively.

Results*:* SEM images showed narrow distributed and scattered particles with the size of 80.3 nm (KAE) formulated in liposomes. IC_{50} values for KAE and paclitaxel were determined to be as 44 ± 0.52 µM and 1.75 ± 0.36 nM, respectively. Cell proliferation averaged from 44 ± 3.9% to 56 ± 26.8% ($p < 0.05$) after treatment with KAE-loaded liposomes. Co-administration of nanoparticles containing KAE and paclitaxel in cancer cells significantly increased the percentage of apoptosis ($P < 0.05$).

Conclusion*:* Taking our data into consideration, we suggest that insertion of KAE into liposomal carriers not only improved the bioavailability of this flavonoid but also surged the anti-cancer efficacy of paclitaxel.

Keywords: Apoptosis, Breast Cancer, Kaempferol, Liposome, Paclitaxel

Address**:** Department of Molecular Medicine, School of Advanced Medical Science, Tabriz University of Medical Sciences, Tabriz, Iran

Tel**:** +984133355789

Email**:** framezani82@gmail.com

Introduction

Cancer is one of the main causes of death and remains an inspiring medical complication that affects millions of people all around the world. As a serious menace in the healthcare system, cancer led to about 9 million deaths annually (1). Among conventional chemotherapy agents, paclitaxel is considered as most common anti-cancer agent for triple negative breast cancer patients, however, the use of paclitaxel is accompanied with several side effect in normal cells along with chemo-resistance and tumor recurrence (2). So, there is an urgent to use the other components as adjuvant in combination with paclitaxel to diminish dose-dependent behavior of one and to provide

[1] *Department of Biochemistry, Faculty of Life Sciences, Tehran North Branch, Islamic Azad University, Tehran, Iran*
[2] *Department of Biochemistry, Faculty of Life Sciences, Tehran North Branch, Islamic Azad University, Tehran, Iran*
[3] *Cellular and Molecular Research Center, Cellular and Molecular Medicine Institute, Urmia University of Medical Sciences, Urmia, Iran*
[4] *Department of Molecular Medicine, School of Advanced Medical Science, Tabriz University of Medical Sciences, Tabriz, Iran (Corresponding Author)*

satisfactory therapeutical outcome as well as to enhance the quality of patient's life with keeping normal cells from its severe side effects (3). There are many anti-cancer compounds which belong to flavonoids because they are safe, and exist in natural products such as vegetables, tea, and fruits (4, 5). One of the best-recognized members of this family is Kaempferol (3,4%, 5,7-tetrahydroxyflavone) which is extensively distributed under therapeutic experiments. This therapeutic feature is associated with anti-cancer, anti-microbial, anti-inflammatory, and other biological effects of KAE (6). To understand and uncover the molecular mechanism behind this compound, many different signal transduction pathways have been investigated(7). However, water instability along with weak pharmaceutical properties such as low bioavailability and high biodegradability has confined KAE as a potential component to entrance any therapeutic application procedure (8). Various drug delivery systems have attempted to tackle this issue by finding safe and stable formulations to address KAE limitations in cancer programs. One of these delivery systems is liposomes which have many superiorities to other systems such as being compatible with lipophilic compounds to intravenous delivery, nontoxic and high loading capacity along with the controlled release of the drug (9). Moreover, liposomes are categorized as a real model of biological membrane, similarity to cell membrane structures, because of bilayer phospholipid assembling, size as well as flexible modulating of surface charge. Thus, to improve the bioavailability of orally insoluble drugs, liposomes have attracted the attention of scientists to a promising strategy (10). The ability of liposomes to improve oral absorption has been demonstrated in studies of curcumin and rapamycin (11-13). However, inhibition of its absorption in the intestine is affected by electrostatic repulsions between the negatively charged surface of the lipid bilayer. Recently, complex surface-modified liposomes have been designed to achieve specific functions by increasing oral bioavailability and intestinal absorption (14, 15).

The ability to open tight connections in intestinal epithelial cells and adhere to mucosal surfaces are two factors that increase uptake of components after corporation to the new formulation (16). In terms of drug delivery science, lecithin is a great opportunity to absorb lipid nanoparticles including SLN, NLCs, and liposomes (17, 18). However, the cellular and molecular properties of Kaempferol containing lecithin nanoparticle systems have not yet been reported. Therefore, we designed a new formulation of KAE to overcome its low bioavailability and to prepare an optimum dosage form for in-vitro administration. Our formulation can be considered as the real and ideal candidate for application in chemotherapy protocols, especially in triple-negative breast cancers where we have no molecular to target. In this work, we formulated KAE in liposome nanoparticles and investigated the role of this formulation in induction of apoptosis in MDA-MB 468 breast cancer cells. Furthermore, we evaluated the expression levels of apoptosis-mediated genes including KI-67, BAD, Bcl2 and Mcl-1 under incubation with KAE incorporated into liposome nanoparticles at the same breast cancer cells.

Materials & Methods

Kaempferol, paclitaxel, cholesterol, dimethyl sulfoxide, PBS and L-α-Phosphatidylcholine were purchased from sigma Aldrich (Darmstadt, Germany). Apoptosis Detection kit was provided by eBioscience (USA). TRIzol reagent was provided by sigma Aldrich (Darmstadt, Germany). Primers were provided from Takapouzist, Co. (Tehran, Iran).

Formulation of kaempferol -loaded liposome:

The modified Thin-Film Hydration (TFH) method was used to formulate KAE-loaded liposomes based on

the following instructions; the L-α-Phosphatidylcholine and cholesterol is dissolved in a beaker containing 10 mL of dichloromethane and slightly stirred for 2 min by a magnetic stirrer. Then kaempferol was added to a mixture and gently stirred for 2 min until a homogeneous combination was attained. The subsequent reaction mixture was held on a hot plate at 70 C◦ for 30 min to evaporated solvent completely for the preparation of thin layer (19). In the ending step, hydrated the lipid film applying distilled water or normal (phosphate) saline buffer at pH 7.4. In the hydration phase, the lipid becomes inflamed and hydrated, resulting in the construction of a MLV (multi-lamellar vesicles) suspension that is exceedingly assorted in size and lamellarity (20). In order to reduce the size, firstly the sample was exposed to homogenization (15 min/60°C) (Silent Crusher M homogenizer, Germany). Then the formulations mixture was exposed to a sonicator (Vibra Cell - Sonics & Material, 130 W, 20 kHz, USA) at 70% sonication strength in ice bath for 5 min (21).

Optimization of kaempferol -Loaded liposome:

Particle size and KAE-liposomes polydispersity index were identified by the dynamic light scattering technique. To evaluate the zeta potential of kaempferol-loaded liposomes, Malvern zeta analyzer was applied. The ultra-filtrated water was utilized to dilute the samples for repeated measurements three times. The size and morphology of the nanoparticles were examined using SEM scanning electron microscope (Kyoto, Japan).

Investigation of cell proliferation with MTT assay:

The 96-well microplates were applied to culture MDA-MB-468 in triplicate with different concentrations including 1-16 nM of paclitaxel and 12-150 µM of kaempferol for 24 and 48 hours. To replace the medium in each well, 200 µl of fresh medium containing 20 µl of MTT solution (2 mg/ml) was used. Then, the cells were incubated for 3-4 hours at 37 ° C and then the medium / MTT mixture was changed with 200µl of dimethyl sulfoxide plus 25µl of glycine Sorenson buffer in each well. After 20 minutes of shaking the plate, the reading was performed using the ELISA Reader 3200 to assess the absorbance at 570 nm (21).

Detection of apoptosis by flow cytometry:

Cell death diagnostic kit (Annexin V / FITC) was employed to evaluate early, late death and necrosis of cancer cells. MDA-MB-468 cells were seeded in Six-well plates (3×10^5) after that, they were treated with 1.75 nM paclitaxel and 45 µM KAE for 24 hours at 37 ° C. After detaching MDA-MB-468 cells from the plate, washed once in PBS and washed once in 1X binding buffer and then resuspended in binding buffer. Finally, Annexin V / Propidium iodide (PI) was utilized to stain cells for 15 minutes at room temperature in the dark, and the percentage of apoptotic cells were determined using the FACS Caliber flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and Flowjo 7.6 Software (FlowJo LLC, Ashland, OR, USA).

Investigation of expression of genes using Real-time qPCR method:

After culturing of MDA-MB 468 cell lines in six-well plates, they were exposed to KAE-loaded liposomes for 24 hours. Total RNA isolation was performed using a TRIzol reagent and a NanoDrop device was used for evaluating quantity and quality of RNA. One microgram of total RNA was used for cDNA synthesis by cDNA synthesis kit and reverse transcriptase enzyme (Thermo Fisher Scientific, Inc.). To detection of gene expression by Quantitative Real-time PCR, primer pairs for each gene and SYBR green PCR Master Mix Kit were applied. The primers sequence is as follow: Ki-67 Forward: GAAAGAGTGGCAACCTGCCTTC Reverse: GCACCAAGTTTTACTACATCTGCC BAD Forward: GGAAGACGCTAGTGCTACAGA Reverse GAGCCTCCTTTGCCCAAGTTT Bcl-2 Forward:

TACCGTCGTGACTTCGCAGAG Reverse: GGCAGGCTGAGCAGGGTCTT MCl-1 Forward: AAC AAA GAG GCT GGG ATG Reverse: ATT GCA CTT ACA GTA AGG CTA TC β-actin Forward: TGCCCATCTACGAGGGGTATG Reverse: CTCCTTAATGTCACGCACGATTTC. A housekeeping gene was used to normalize the relative expression of each gene.

Statistical analysis:

Data are considered as the mean ± SD. IC50 values were evaluated applying Graph Pad V8.3 Software. One-way ANOVA with Tukey's test was accomplished to determine significant variances between test and control groups. $P < 0.05$ was reflected to signify a statistically significant difference.

Results

The characterization of the specificity of liposome Kaempferol formulation:

The Thin Film Hydration technique was used to collect Kaempferol within the liposome system. In size analysis, the particles showed an almost controlled size when the liposome nanoparticles were dispersed with the value of 30-95 (Figure 1a), which was confirmed by the SEM image (Figure 1 c). The stability of the nanoparticles was confirmed with a zeta potential of approximately +30 and the limited size dispensation with a scattering index (PDI) of 0.26 (Figure 1b).

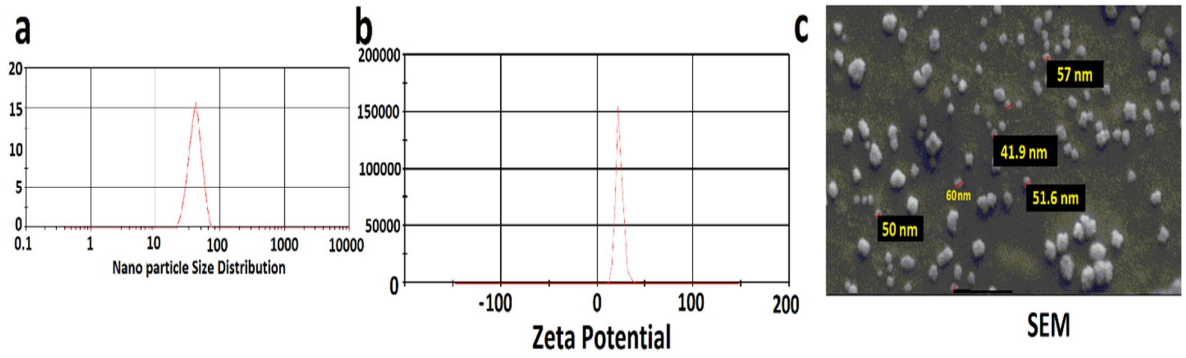

Fig 1. (a) Nanoparticle size distribution diagram (b) Zeta potential distribution histogram (c) Kaempferol scanning electron microscope loaded on liposome nanoparticles in aqueous solution.

MTT assay was performed to evaluate the antiproliferative effects of Kaempferol:

After incubation with different concentrations of each drug for 24 and 48 hours, the anti-proliferative behavior of Kaempferol, paclitaxel, and formulation on MDA-MB468 breast cancer cells was investigated. The IC_{50} values for Kaempferol and paclitaxel were 44± 0.52 µM and 1.7 ± 0.36 nM, correspondingly. Kaempferol-loaded liposomes inhibited MDA-MB-468 cell proliferation more effectively than unformulated Kaempferol ($P < 0.05$).

Assessment of apoptosis of MDA-MB468 breast cancer cells after treatment with Kaempferol loaded on liposome nanoparticles:

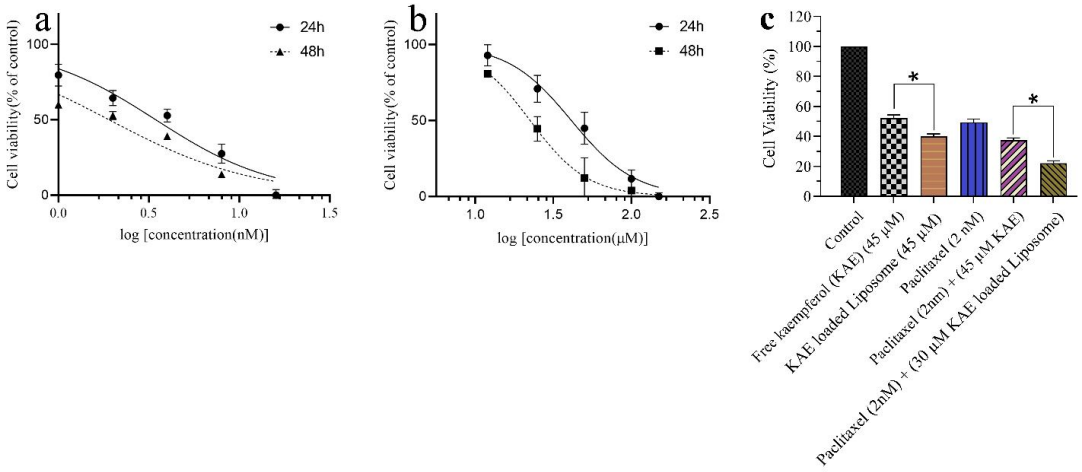

Fig 2. MDA-MB468 breast cancer cells were treated with concentrations of 1-16 nM of paclitaxel 12-150 μM of Kaempferol. Growth inhibition rate of these cells between different treatments (paclitaxel, Kaempferol, and Kaempferol loaded with liposome) Comparison Combined treatment with Kaempferol loaded on liposome nanoparticles with paclitaxel showed greater toxicity in inhibiting cancer cell growth than treatment with KEA-liposome and paclitaxel alone (1.75 nM) ($P < 0.05$). In addition, treatment with Kaempferol loaded on liposome nanoparticles inhibited the growth of MDA-MB468 cells more than Kaempferol alone (45 μM) ($P < 0.05$). The results are shown as the mean standard deviation from the standard (n = 3).

Compared to control cells, no significant change was observed in the cancer cell population when treated with liposomes (Figure 3).

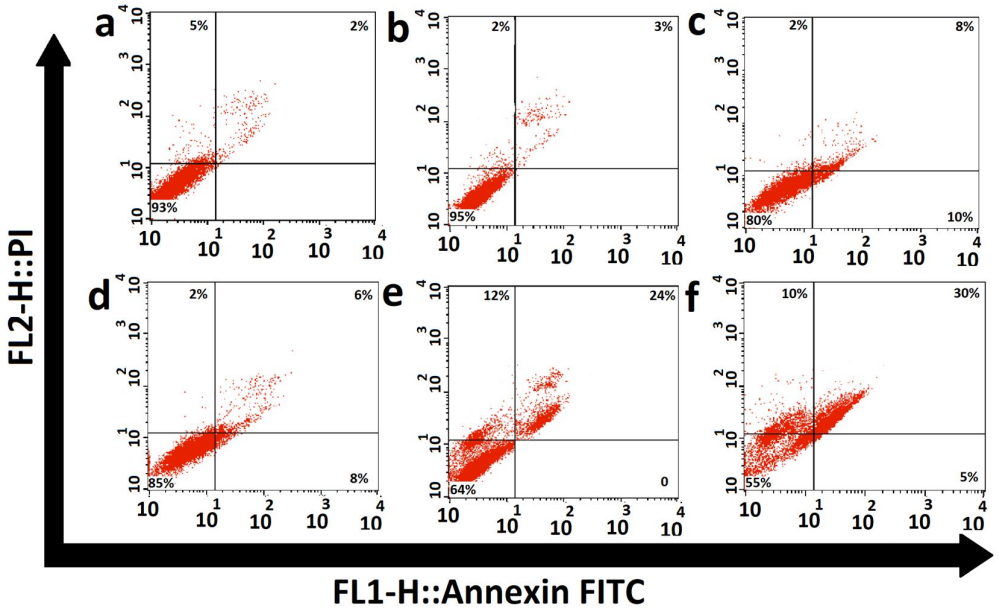

Fig 3. KEA-liposome increased premature cell death in MDA-MB468 breast cancer cells. Cells were treated with IC50 values of Kaempferol, paclitaxel and KEA-liposomes. (A) Control group (b) Nano-blank (c) Paclitaxel (d) Kaempferol (e) Kaempferol loaded in liposome (i) Kaempferol loaded in liposome with paclitaxel. The results are shown as the mean standard deviation from the standard (n = 3).

Treatment of MDA-MB468 breast cancer cells with paclitaxel only, augmented the rate of apoptotic cells by 10.4%. In addition, after treating the cells with Kaempferol-loaded liposomes, the fraction of apoptotic cells increased to 24.2%, while combination therapy of breast cancer cells with paclitaxel plus Kaempferol-loaded liposomes, showed a 35% increase in cell death.

The expression of anti-apoptotic and pro-apoptotic genes by real-time PCR:

The anti-apoptotic and pro-apoptotic pathway genes were examined to shed more light on the evidence that nanoparticle-loaded Kaempferol was involved in the apoptotic pathway of breast MDA-MB468 cells. The liposomal kaempferol significantly reduced the expression levels of Ki-67, MCl-1, and BCL-2 compared to Kaempferol alone. However, compared with the control group, the expression level of proapoptotic BAD gene raised significantly following therapy with KAE-liposomes as well ($P < 0.05$) (Figure 4).

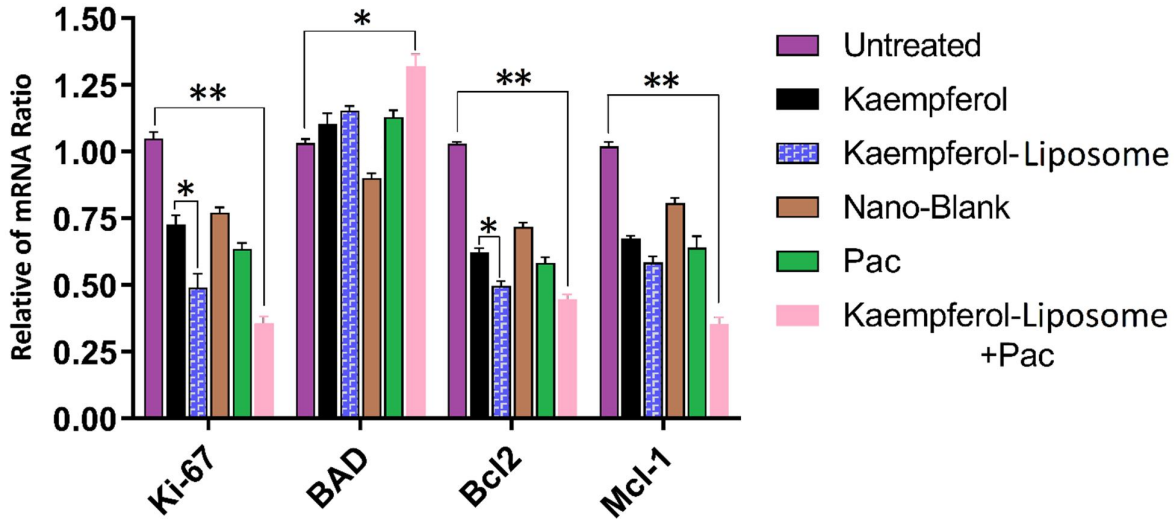

Fig 4. Changes in Bcl2, Mcl1, ki-67, Bad gene expression levels in comparison with changes in cell growth inhibition in response to different doses of the drug. The results are shown as the mean standard deviation from the standard (n=3).

Discussion

Cancer be counted among the most dominant human health complications, relying on chemoprevention procedures as a method to moderate both occurrence and mortality. The study of kaempferol astonishing list of cancer-fighting properties greatens its full potential. These investigations are full of hope, particularly because kaempferol selectively inhibits cancerous cells without having adverse effect on healthy tissues (22).

Because nanoparticles enter the cell wall of cancer cells through increased permeability and retention mechanism (EPR), particle size plays an important role in determining anticancer activity (23). Therefore, the increase in carrier uptake into cancer cells with smaller particle sizes increases. Another factor that plays an important role in the inhibitory effect of nanoparticles is the surface charge of particles, as positively charged nanoparticles interact more strongly with negatively charged cancer cell membranes (24). Based on our test conditions, the average size of nanoparticles was obtained using Kaempferol in the formulation 30-150 nm (Figure 1a). The particle size distribution of Kaempferol saved the formulation from aggregation and showed a high PDI (0.24). The present study showed

that high concentrations of Kaempferol reduce the distance between nanoparticles and thus prevent repulsive interaction between nanoparticles and their accumulation. The results of this study were consistent with previous studies (25, 26). The surface charge of nanoparticles, which is determined by the zeta potential, affects the stability of nanoparticles due to the electrostatic interaction between the particles (Figure 1b). The surface charge of the colloidal particles was in the range of +30 mV, which was caused by high concentrations of chitosan. These values are suitable for a stable nanoparticle system because the range of +30 mV is sufficient to prevent the accumulation of nanoparticles. The morphology of the nanoparticles was examined by scanning electron micrographs. According to Figure 1c, KAE-LC nanoparticles in the optimal formulation had a spherical, uniform shape and dispersion sizes from 30 to 150 nm with an average size of 80 nm. Also, almost a small accumulation of particles was observed during the drying process of nanoparticles. No significant changes were observed between liposomes-treated breast cancer cells alone and untreated cells, indicating that the nanoparticles are safe and biocompatible with minimal toxicity (Figure 2). In order to compare Kaempferol alone and in the form of intra-particle formulation, the anti-proliferative effect of Kaempferol against MDA-MB-468 cells alone and in nanoparticle loaded was investigated. It was observed that in comparison with the standard anticancer drug paclitaxel with an inhibitory dose of 1.75 nM, the inhibitory dose of flavonoid Kaempferol was 45 µM. Based on the results of the present study, Kaempferol loaded on nanoparticles not only enhanced the anti-cancer behavior of paclitaxel on MDA-MB468 cell proliferation but also significantly reduced the dose of paclitaxel required to inhibit cancer cells. Kaempferol, paclitaxel, and Kaempferol loaded on liposomes had anti-tumor behaviors against the structure of tumor cells, such as spherical deformity and deformity of cancer cells into compressed and crushed form, which led to nuclear disintegration and the onset of planned cell death. In one study, Kaempferol showed a concentration-dependent antiproliferative effect on SiHa cells (27). In another study, Kaempferol showed significant tumor effects by stopping the cell cycle and inducing apoptosis in HeLa cells (28). Kaempferol reduced the survival of HeLa cervical cancer cells by inducing apoptosis through the PI3K / Akt and hTERT pathways (29).

Other studies have shown that Kaempferol inhibits the growth of prostate, lung, and bladder cancer cells by increasing the expression of caspases (9, 8, and 3) based on the PTEN activation mechanism (30, 31). In one study, apoptotic death was induced by Kaempferol through activation of PARP-activating failure in renal cancer cells (32). Therefore, the present study investigated the mechanism of programmed cell death (apoptosis) after treatment of MDA-MB 468 breast cancer cells with Kaempferol-loaded nanoparticles to determine whether the formulation could enhance the effect of Kaempferol. As shown in Figure 3, the nanoparticles alone had no cytotoxic effect on cancer cells, indicating that these drug carriers are safe and biocompatible.

In addition, treatment of cancer cells with nanoparticles containing Kaempferol increased primary apoptosis by 0.1 to 6% compared with Kaempferol alone. Kaempferol loaded on nanoparticles and paclitaxel showed the highest percentage of apoptosis compared to Kaempferol with paclitaxel, indicating the synergistic effect of nanoparticles on drug delivery to cancer cells. Treatment of cancer cells with nanoparticles loaded with Kaempferol showed 11% apoptosis, which was higher than Kaempferol and paclitaxel. Because different mechanisms are involved in the phenomenon of apoptosis in different tumor cells, identifying the different pathways that cause cell death needs further investigation. Kaempferol has a high

potential for cancer prevention due to its bioavailability, low cost, and safety. Real-time PCR was used to confirm which genes were involved in initiating the apoptotic pathway treated with Kaempferol-loaded nanoparticles. Our results showed that nanoparticles containing KAE decreased the expression of MCL-1 and bcl2 genes and increased BAD as proapoptotic genes. The level of Ki-67 as an indicator of proliferation in cells after incubation with different conditions was studied and it was found that the present formulation was able to significantly reduce the expression of ki-67 levels compared to other groups. Therefore, a natural, non-toxic agent can be useful when loaded into effective drug delivery systems against cancer cells, either alone or in combination with first-line chemotherapy agents.

Conclusion

Kaempferol-containing liposomes enhanced the cytotoxicity of paclitaxel against MDA-MB 468 breast cancer cells. Our data also showed that the combined treatment of breast cancer cells with paclitaxel and camphor-filled nanoparticles could inhibit apoptotic messaging, stop the cancer cell cycle in the Sub G1 phase and also reduce the expression of anti-apoptotic genes of the Bcl-2 family, Have synergistic effects on anti-tumor behavior. Considering the results of the present study, treatment with paclitaxel along with liposomes containing Kaempferol as adjuvants may be able to provide more effective treatment for breast cancer.

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Conflicts of Interest

There is no conflict of interest

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