

## Original Article

# Kang Ru enhances paclitaxel's efficacy against breast cancer progression

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**Abstract:** Breast cancer and its associated drug resistance present significant clinical challenges. From a translational medicine perspective, Traditional Chinese Herbal Medicine (TCHM) offers promising integrative approach to bridge the experimental findings with clinical application, enhancing cancer treatment outcomes and mitigate drug resistance. In this study, we evaluated a TCM herbal formulation, Kang Ru (KR), comprising *Artemisia argyi*, *Ohwia caudata*, and *Scoparia dulcis* Linn, each traditionally and experimentally recognized for their potential therapeutic properties. We aimed to evaluate the anti-cancer effects of KR on breast cancer and its potential to enhance the efficacy of the chemotherapeutic agent paclitaxel. Using breast cancer cell lines we evaluated the effects of KR, both alone and in combination with paclitaxel on cell growth, viability, migration, and invasion. We further evaluated the potential of KR in enhancing paclitaxel to reduce tumor progression using a breast cancer xenograft model in mice. Our findings demonstrated that KR significantly inhibited cell growth and migration in breast cancer cell lines. Moreover, the combination of KR with paclitaxel demonstrated synergistic effects, effectively reducing cell viability, inducing cell apoptosis and inhibiting cell migration. In an animal model, the combination of KR and paclitaxel significantly enhanced breast cancer suppression, corroborating the in vitro findings. In conclusion, KR is a novel Chinese herbal formulation, with significant anti-cancer potential, enhancing paclitaxel's efficacy in inhibiting breast cancer progression. These findings suggest that KR could be a promising adjunctive strategy to improve breast cancer treatment and warrant further translational research to facilitate its clinical development as a complementary therapeutic option.

**Keywords:** Breast cancer, drug efficacy, herbal formulation, Kang Ru, paclitaxel

## Introduction

Breast cancer is one of the most common and first ranked among cancers affecting women

worldwide [1-3]. With a high mortality rate, breast cancer accounted for an estimated 11.6% of all cancer incidences by the year 2022 [4]. Treatment of breast cancer depend

on clinical assessment and may include radiotherapy, chemotherapy, endocrine therapy, and immunotherapy. Current treatment strategies rely on clinical assessment and may include radiotherapy, chemotherapy, endocrine therapy, and immunotherapy. Chemotherapeutic agents such as doxorubicin, docetaxel, and paclitaxel remain essential components of breast cancer management [5]. A significant challenge in treating breast cancer is the development of drug resistance and inefficacy. Drug resistance rates can be high, particularly in aggressive types like Triple Negative Breast Cancer (TNBC), where conventional hormone therapies and human epidermal growth factor receptor 2 (HER2)-targeted therapies are ineffective [2, 6]. This necessitates ongoing research and development of new treatment strategies to improve survival outcomes.

Paclitaxel (thereon to be referred to as TAX) is a widely used anticancer drug, particularly effective against various malignant diseases. Its primary mechanism involves microtubule stabilization, which disrupts multiple cellular processes, including programmed cell death [7]. TAX is commonly employed as a first-line treatment for breast cancer, either as a monotherapy or in combination with other agents [8, 9]. However, clinical applications frequently encounter treatment failure due to the development of resistance or recurrences by breast cancer cells post-treatment [9, 10].

Traditional Chinese Medicine (TCM) encompasses pharmaceutical products and practices used for prevention and treatment of diseases. As a holistic healthcare approach practiced for over 5,000 years, TCM includes various modalities such as acupuncture, herbal medicine, massage, exercise, and dietary therapy [11-14]. Among these, Chinese herbal medicines (CHM) - the largest category of TCM [15, 16] - comprise Chinese crude drugs, decoction pieces, and Chinese patent medicines. These have attracted considerable attention for their potential as alternative anticancer treatments. Numerous natural products and formulations derived from CHM have demonstrated direct or indirect anticancer effects [16-18]. Additionally, translational studies have demonstrated that certain TCM formulations, such as PHY906 based on Huang-Qin-Tang (a classic TCM prescription containing multiple herbs, traditionally used for gastrointestinal health and inflam-

mation, among other purposes), can enhance patient quality of life and exhibit synergistic effects with conventional therapies [19]. This attribute is particularly significant in the context of diseases like cancer, where treatment efficacy often presents challenges. Therefore, it is imperative to explore and formulate TCM compounds that may either improve treatment outcomes or augment the efficacy of the existing therapeutic regimens. In the context of breast cancer, TCM has demonstrated efficacy in inhibiting tumor cell growth and proliferation, reducing tumor recurrence and metastasis after surgery, and alleviating adverse reactions [3].

To identify potential TCM formulation that could enhance breast cancer treatment, either alone or in combination with existing clinical regimens, we embarked on the exploration of a formulation, Kang Ru (KR). This formulation combines extracts of *Artemisia argyi*, *Ohwia caudata*, and *Scoparia dulcis* Linn. Each of these extracts has individually demonstrated beneficial health and therapeutic effects in various conditions [20-22]. For instance, *Ohwia caudata* extract has demonstrated attenuation of doxorubicin-induced mitochondrial dysfunction in Wharton's jelly-derived mesenchymal stem cells as well as senescence in aging-derived mesenchymal stems cells [23, 24]. In other studies, *Artemisia argyi* extract has been shown to induce apoptosis in gemcitabine-resistant human lung cancer cells and overcome resistance to lapatinib in breast cancer [25, 26]. Additionally, *Scoparia dulcis* Linn, exhibits potent cytotoxic activity against MCF-7 and T47D breast cancer cells [27]. Furthermore, *Scoparia dulcis* Linn extract demonstrated protective effects on high glucose-induced injury in human retinol pigment epithelial cells [28]. Following these previous findings, we investigated the anticancer effects of this formulation and its potential to enhance the therapeutic efficacy of TAX in breast cancer.

### Materials and methods

#### Cell culture

Breast cancer cell lines, MDA-MB-231 and MCF-7 were maintained in RPMI1640 containing 10% Fetal Bovine Serum (FBS), with 1%-Penicillin-Streptomycin solution. The media was replaced every three days.

## Formulation of Kang Ru

The healthy, mature, and fresh leaves of *Artemisia argyi*, *Ohwia caudata*, and *Scoparia dulcis* Linn were locally collected in Taiwan. The collected plants were washed, air-dried, finely powdered, and mixed in the following proportions: 4 g of *Artemisia argyi* (40%), 4 g of *Ohwia caudata* (40%), and 2 g of *Scoparia dulcis* Linn (20%). The final mixture was dissolved in 600 mL of water and then concentrated into a 60 mL stock solution.

## Cell viability assay

Cell viability of MDA-MB-231 and MCF-7 cells treated with various concentrations and combinations was determined using the MTT assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma). Briefly, 12-well plates were seeded with  $1 \times 10^5$  cells per well and incubated for 24 hours. Subsequently, the cells were treated with different concentrations of KR, TAX, or their combinations. At designated time points, the cells were washed with PBS and then treated with MTT solution in each well, followed by incubation for 2 hours at 37°C in a 5% CO<sub>2</sub> incubator. Finally, the absorbance of each well was measured at 570 nm using a microplate reader.

## Flow cytometry

For apoptosis detection, Annexin V and propidium iodide (PI) double staining using the BD Biosciences in-situ apoptosis detection kit was performed according to the manufacturer's protocol. Briefly, approximately  $2 \times 10^5$  cells were seeded per well in a 6-well plate. After treatment, the cells were trypsinized, collected by centrifugation, and diluted with staining buffer in 5 mL tubes. Annexin V-FITC and propidium iodide in binding buffer were then added, followed by flow cytometry analysis using the BD FACSLytic™.

## Western blot

Briefly, 30-40 µg of protein extracted from cells or tumors were separated using 8-12% SDS-PAGE (Bio-Rad). The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked in 5% skimmed milk in Tris-buffered saline containing

1% Tween 20 (TBST) for 1 hour at room temperature. After washing with TBST, the membranes were incubated with the appropriate primary antibodies at 4°C overnight. Next, the membranes were washed again with TBST and then incubated with the appropriate secondary antibodies. Following this, the membranes were washed with TBST once more. Finally, a 1:1 ratio of solutions A and B from the chemiluminescent HRP (Millipore) was applied to visualize band images using iBright 1500 (Invitrogen).

## Wound healing assay

Cells were seeded at a density of  $1 \times 10^6$  cells per well. The plates were then incubated for 24 hours at 37°C with 5% CO<sub>2</sub>. A scratch wound was created using a 200 µL pipette tip, after which the cells were treated accordingly. At various time points, photographs were taken to compare the wound sizes. Wound healing area was measured and quantified using ImageJ 1.4t software.

## Transwell migration assay

Cell migration assays were conducted using 24-well plates equipped with an 8-µm pore membrane (EMD Millipore, Billerica, MA, USA) coated with matrigel. Briefly, cells were treated with KR, TAX, and their combinations for 24 hours. The treated cells were then suspended in culture medium without fetal bovine serum (FBS). 200 µL of the cell suspension, containing approximately  $2 \times 10^4$  cells, was placed in the upper chamber, while 600 µL of medium supplemented with FBS was added to the lower chamber. The chambers were incubated for 24 hours at 37°C in a 5% CO<sub>2</sub> atmosphere. After incubation, the upper surface of the membrane was gently wiped to remove the cells not migrated. The migrated cells on the lower surface were fixed with 4% paraformaldehyde for 20 minutes. Staining was performed using 0.1% crystal violet diluted in methanol for 30 minutes at room temperature. Images of the migrated cells were captured and number of cells per field counted using an inverted microscope (Olympus BX53) at 100× magnification. Images of migrated cells were captured, and the number of cells per field was counted using an inverted microscope (Olympus BX53) at 100× magnification.

## Animal model

Four-weeks-old nude mice were randomly divided into five groups (n=5 per group) and housed in well-ventilated cages at the Animal Center of Tzu Chi University Laboratory. All procedures were conducted in compliance with ethical guidelines and the principles of the three Rs (Replacement, Reduction, and Refinement), with approval from the Institutional Animal Care and Use Committee (IACUC) (Approval Number: 111-62). The mice were monitored daily for health parameters, including food intake, air circulation, and light-dark cycles. Tumor xenografts were established by subcutaneously injecting cells into the right flanks of each mouse. Tumor growth was monitored regularly until the tumors reached a volume of approximately 100 mm<sup>3</sup>, at which point the mice were randomly assigned to five treatment groups: control, 10 mg/kg TAX, 100 mg/kg KR, 200 mg/kg KR, and combination treatments of 10 mg/kg TAX with either 100 mg/kg KR or 200 mg/kg KR, as described in Section 3.4. During the experiment, any mouse with a tumor volume exceeding 1,500 mm<sup>3</sup> was scheduled for humane termination in accordance with ethical guidelines. At the conclusion of the study, all mice were humanely euthanized following the induction of anesthesia using 1-3% isoflurane administered via inhalation.

## Statistical analysis

All statistical analyses were performed using Graph Pad Prism 8 software and Excel 2016. Data are presented as the mean  $\pm$  standard deviation from at least three independent experiments. Two-sided statistical comparisons between groups were conducted using a Student's t-test or two-way ANOVA, depending on the distribution of the samples. A *p*-value of  $< 0.05$  was considered significant, with \**P*  $\leq 0.05$ , \*\**P*  $< 0.01$ , and \*\*\**P*  $< 0.001$ .

## Results

### *Kang Ru (KR) reduced breast cancer cells' viability and induced cell apoptosis dose-dependently*

Cancer cell viability is one of the indicators of cancer survival and aggressiveness. In this study, we first aimed to investigate the effect of

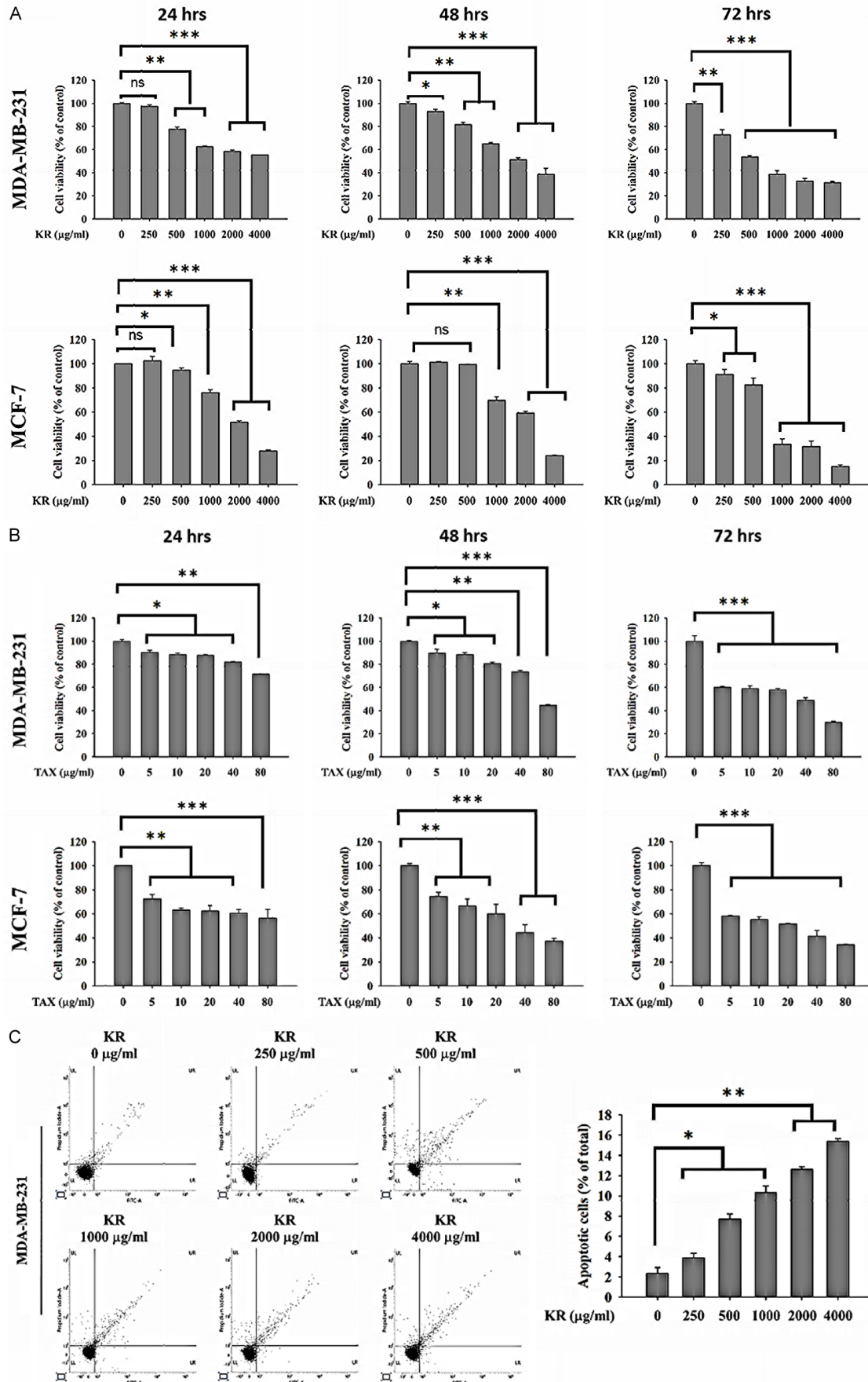
KR on breast cancer cell viability by treating the less aggressive MCF-7 cells and the highly aggressive MDA-MB-231 cells with various concentrations of KR, assessing the effects at 24, 48, and 72 hours. Our findings show that KR reduced cell viability in both cell lines in a dose- and time-dependent manner, with differing degrees of efficacy between the two cell types (**Figure 1A**). Secondly, we evaluated the effect of TAX by treating MCF-7 and MDA-MB-231 cells with different concentrations of TAX, also assessing the impact at 24-, 48-, and 72-hour intervals. TAX similarly reduced cell viability in a dose- and time-dependent manner, with varying degrees of response between the two cell lines (**Figure 1B**). To investigate if this reduction in cell viability could translate to programmed cell death, apoptosis, we treated these cells with the same concentrations of KR and TAX and stained with Annexin V and PI to determine apoptosis effect at 48 hours. Consistently, the results exhibited pro-apoptotic effect of KR in a dose dependent manner in both cell lines. Notably, the percentage of apoptotic cells was higher in MCF-7 cells than in MDA-MB-231 cells at each concentration tested (**Figure 1C**). The same effect and trend of results were also observed when these cells were treated with TAX (**Figure 1D**).

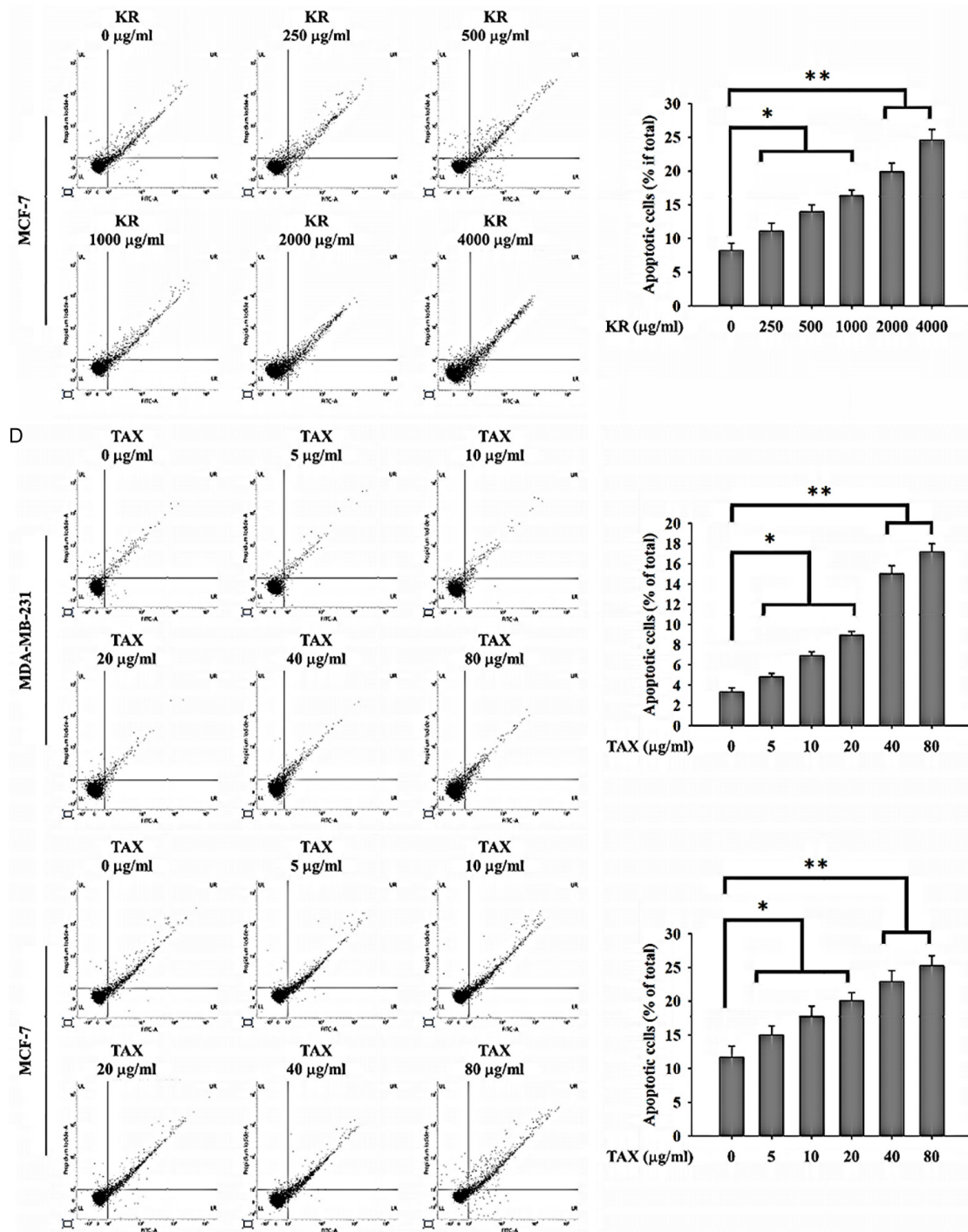
These findings suggest that KR possesses anti-cancer potential by reducing cell viability and inducing apoptosis in both less and more aggressive breast cancer cell phenotypes.

### *Combination of Kang Ru (KR) and paclitaxel (TAX) enhanced the induction of apoptosis in breast cancer cells*

We aimed to investigate whether the combination of KR and TAX could enhance the effects observed when these compounds are administered individually. To assess this, we combined TAX at 10  $\mu\text{g/mL}$  and 40  $\mu\text{g/mL}$  with a single concentration of KR. Based on preliminary findings showing that 1000  $\mu\text{g/mL}$  of KR reduced cell viability to slightly above its IC<sub>50</sub> at 48 hours, we selected this concentration as a candidate to combine with the two TAX doses (10 and 40  $\mu\text{g/mL}$ ). To evaluate the effect on apoptosis, cells were subjected to double staining with PI and Annexin V for apoptotic assessment. Consistently, the results at 48 hours indi-



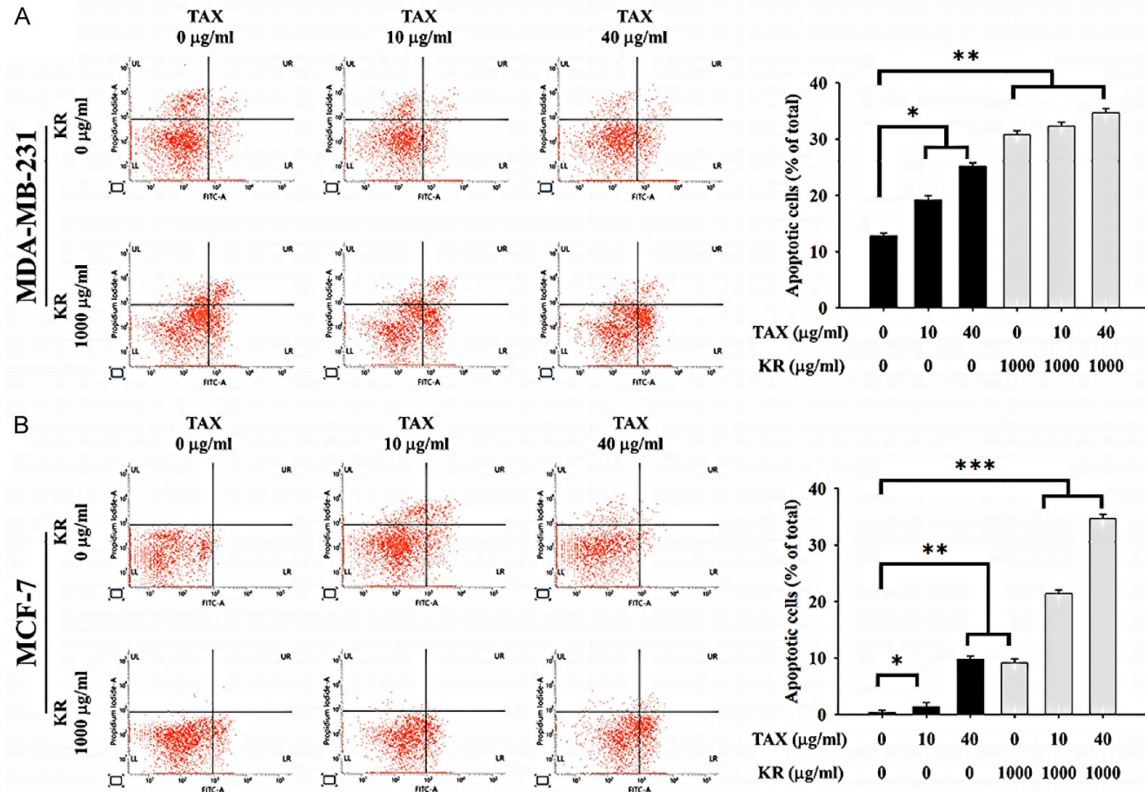




**Figure 1.** Kang Ru (KR) reduced breast cancer cells' viability and induced cell apoptosis dose-dependently. Breast cancer cell viability and apoptosis were assessed following treatment with KR and TAX. Both KR and TAX significantly reduced cell viability (A, B) and induced apoptosis (C, D). Results are presented as the mean  $\pm$  SD from three independent experiments.

cated that the KR and TAX combination significantly enhanced apoptosis induction compared to each compound alone, with a TAX dose-dependent response. These effects were

observed in both cell lines tested (**Figure 2A, 2B**), suggesting that KR potentiates the apoptotic effect of TAX in co-treatment conditions.



**Figure 2.** Combination of Kang Ru (KR) and paclitaxel (TAX) enhanced the induction of apoptosis. Breast cancer cell apoptosis was evaluated in response to combined treatment with KR and TAX. Combinations of 1000 µg/mL KR with either 10 µg/mL or 40 µg/mL TAX were used to assess the apoptotic effect. The combined treatments exhibited a significantly enhanced induction of apoptosis compared to individual treatments (A, B). Results are expressed as mean  $\pm$  SD from three independent experiments.

#### Combination of Kang Ru (KR) and paclitaxel (TAX) enhanced the inhibition of breast cancer cell migration

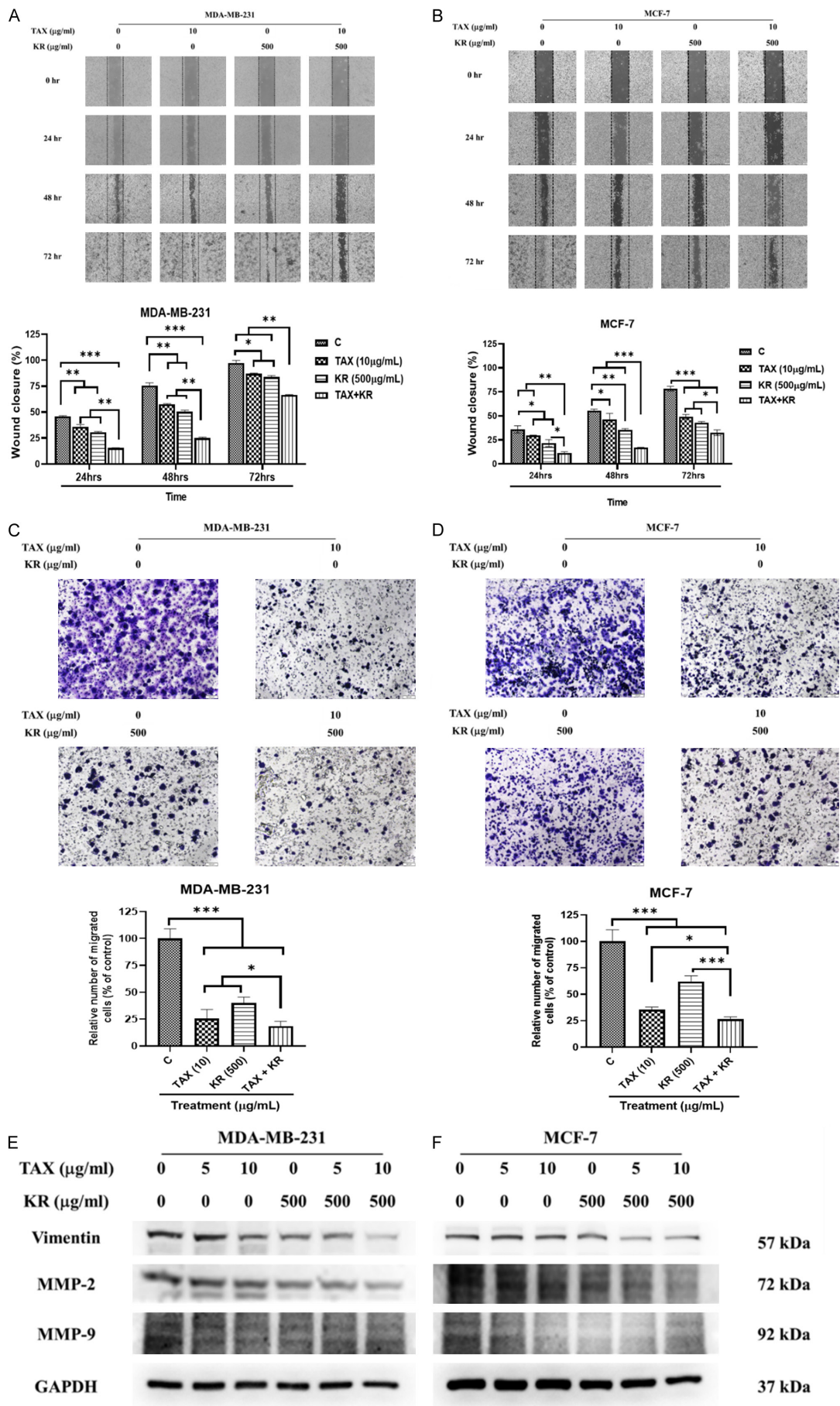
Cell migration and invasion are critical characteristics of cancer, often indicative of tumor aggressiveness and potential for metastasis to distant organs. This study aimed to investigate whether the combined effect of KR and TAX, as observed in previous sections (Section 3.2), could also extend to the inhibition of migration and invasion. To test this, a wound healing assay was performed, treating MDA-MB-231 and MCF-7 cells with a combination of 500 µg/mL KR and 10 µg/mL TAX. The results demonstrated that, in both cell lines, the combination treatment significantly enhanced the inhibition of cell migration compared to treatment with either 10 µg/mL TAX or 500 µg/mL KR alone (Figure 3A, 3B).

To assess the combination treatment's effect on cell invasion, a transwell invasion assay was

conducted following treatment, allowing us to determine the invasive capacity of the cells. Results indicated that the combination treatment more effectively inhibited invasion in both cell types than either TAX or KR alone (Figure 3C, 3D). Given that cell migration and invasion are associated with the dysregulation of epithelial-mesenchymal transition (EMT) markers and matrix metalloproteinases (MMPs), we supplemented the wound healing and invasion assays with western blot analysis to examine the expression of Vimentin and matrix metalloproteinases MMP-2 and MMP-9. Western blot results indicated that the combination treatment of KR and TAX enhanced the downregulation of Vimentin, MMP-2, and MMP-9 compared to individual treatments with KR or TAX (Figure 3E, 3F).

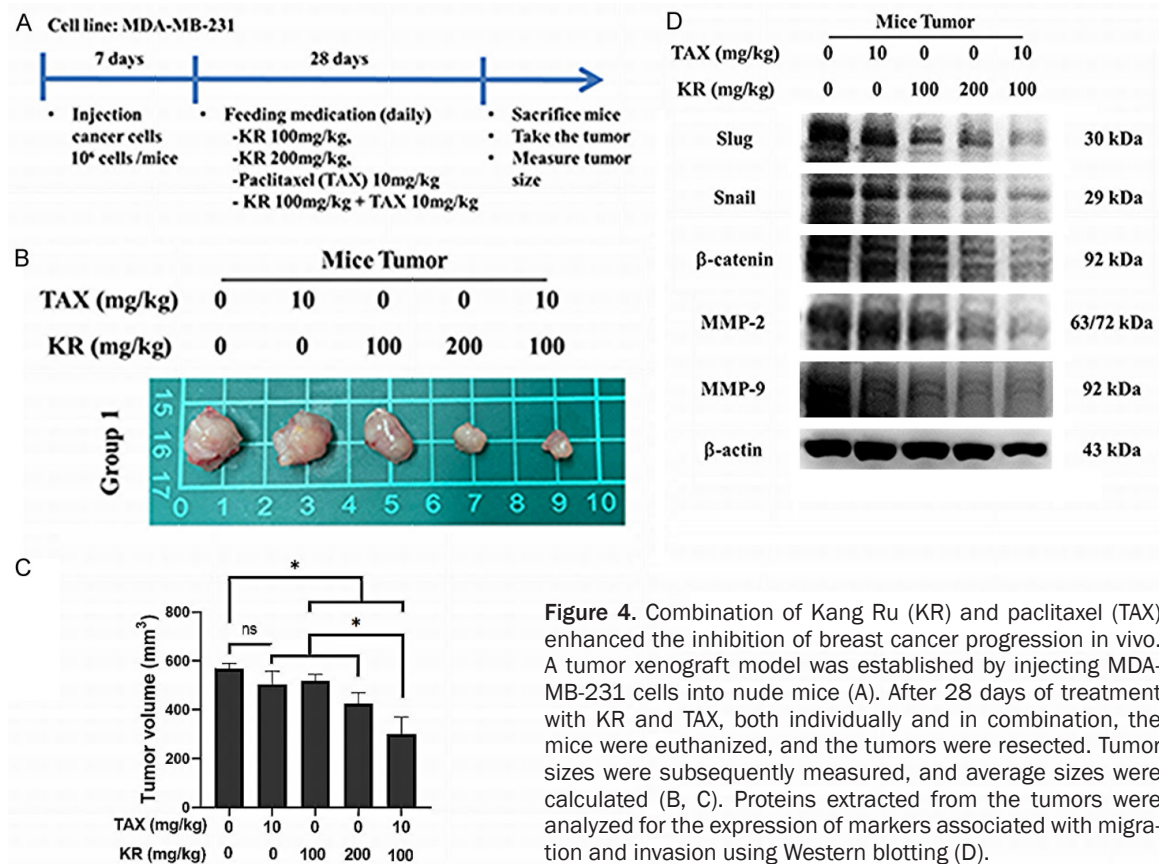
Collectively, these findings suggest that the potential anticancer effects of KR include the inhibition of cancer cell migration and invasion. Moreover, combining KR with the established







**Figure 3.** Combination of Kang Ru (KR) and paclitaxel (TAX) enhanced the inhibition of breast cancer cell migration and invasion. Breast cancer cell migration and invasion were evaluated in response to the combination treatment of KR and TAX. The combination of KR and TAX significantly enhanced the inhibition of breast cancer cell migration (A, B) and invasion (C, D). Dysregulation of migration-associated markers was assessed by Western blot analysis (E, F).



**Figure 4.** Combination of Kang Ru (KR) and paclitaxel (TAX) enhanced the inhibition of breast cancer progression in vivo. A tumor xenograft model was established by injecting MDA-MB-231 cells into nude mice (A). After 28 days of treatment with KR and TAX, both individually and in combination, the mice were euthanized, and the tumors were resected. Tumor sizes were subsequently measured, and average sizes were calculated (B, C). Proteins extracted from the tumors were analyzed for the expression of markers associated with migration and invasion using Western blotting (D).

breast cancer drug TAX enhances these inhibitory effects.

*Combination of Kang Ru (KR) and paclitaxel (TAX) enhanced the inhibition of breast cancer progression in vivo*

Finally, we aimed to evaluate the anticancer effects of KR in combination with TAX in a pre-clinical mouse model. To achieve this, an in vivo tumor model was established by injecting MDA-MB-231 breast cancer cells into mice, a highly aggressive cell line. Mice were then treated daily for 28 days with either vehicle, 100 mg/kg KR, 200 mg/kg KR, 10 mg/kg TAX, or a combination of 10 mg/kg TAX and 100 mg/kg KR (Figure 4A). At the end of the treatment period, mice were ethically euthanized, and tumors were excised. Tumor sizes were measured, recorded, and averaged. We observed that the

combination treatment of KR (100 mg/kg) and TAX (10 mg/kg) significantly reduced tumor size compared to KR or TAX alone (Figure 4B, 4C).

When the tumors were homogenized, proteins were extracted for Western blot analysis of markers associated with cell migration and invasion. The results revealed a downregulation of Slug, Snail,  $\beta$ -catenin, MMP9, and MMP2 proteins when KR and TAX were combined in treatment, compared to treatment with KR and TAX alone (Figure 4D).

Collectively, these results suggest that KR has the potential to inhibit breast cancer progression, and its combination with TAX enhances treatment efficacy. The downregulation of EMT markers and metalloproteinases suggest that the combination of KR and TAX may improve the inhibition of breast cancer migration and invasion.

## Discussion

Exploring TCM formulations for anticancer properties and integration with western medicine through in vitro, preclinical model experiments and clinical trials promises improved alternative and adjunct treatment efficacies [29-31]. By combining the existing anticancer drugs with novel formulations, the current challenges of drug ineffectiveness and acquisition of resistance might be effectively contained. In our study, KR, is a formulation derived from combining three different compounds *Artemisia argyi*, *Ohwia caudata*, and *Scoparia dulcis* Linn. We investigated this formulation with the aim to explore its effectiveness in improving breast cancer treatment. The quest in exploring novel compounds with therapeutic potential through the formulations of TCM and HCM in particular is increasingly gaining attention among researchers [32, 33]. The fact that TCM and CHM in particular has become an accepted part in clinical management alongside western medicine in china and has demonstrated beneficial therapeutic effects against various diseases with low side effects [16] emphasizes the same. For instance, the isolation of artemisinin and the discovery of its therapeutic effects on Malaria was inspired by TCM [14, 34]. Moreover, CHM has been described to enhance life quality and survival rate in none small-cell lung cancer (NSCLC) patients, with low side effects [16]. Furthermore, during the previous pandemics of SARS (Severe Acute Respiratory Syndrome)-CoV in 2003 and MERS (Middle East respiratory syndrome)-CoV in 2012, in China, TCM was used as preventive and treatment strategies which had been shown to result in shorter hospitalization, reduced side effects from steroid treatment, and relief from dyspnea and malaise [34]. Additionally, these compounds have a characteristic of multiple targeting [18, 35] which is advantageous against the conditions with multiple survival pathways such as cancer. With all being said, the increasing incidences and mortalities in cancer makes it imperative to embark on exploring potential compounds or herbal formulations to improve the current treatment efficacies. Our formulation of KR was inspired based on the two important factors. First, the individual compounds used in the formulation have demonstrated significant health benefits and therapeutic potential against specific diseases, as evidenced by prior studies. For instance, *Ohwia caudata* has been shown

to attenuate doxorubicin-induced mitochondrial dysfunction in Wharton's jelly-derived mesenchymal stem cells and mitigate senescence in aging-derived mesenchymal stem cells, as well as in adipose-derived cells [23, 24], *Artemisia argyi* have been reported to induce apoptosis in gemcitabine-resistant lung cancer cells and overcome lapatinib resistance in breast cancer cells [25, 26]. Furthermore, *Scoparia dulcis* Linn has exhibited cytotoxic effects against MCF-7 and T47D breast cancer cell lines and protective effects against high glucose-induced injury in human retinal pigment epithelial cells [27, 28]. The anti-inflammatory effects of *Ohwia caudata* in synovio-cytes and the antidiabetic and antioxidant activities of *Scoparia dulcis* Linn have also been explored in studies by Lu, C.Y., et al., and Mishra et al., respectively [36, 37].

Second, it is well documented that combining natural compounds possessing health and therapeutic benefits improves treatment efficacy of the existing drugs [38]. We aimed to investigate the effect of this compound in breast cancer owing to increased incidences and mortalities despite the existences of different drugs. Such being said, our formulation, KR, demonstrated anti-cancer properties in breast cancer cell lines. The reduction of cell viability and induction of apoptosis are the two of the several indicators for characterizing the anti-cancer properties of drugs and compounds [39]. In this study, KR reduced cell viability in both less, and highly aggressive breast cancer cell lines, MCF-7 and MDA-MB-231 respectively, in time and dose dependency. In addition, KR induced apoptosis in similar fashion in both cell lines emphasizing on its potential. Considering the potential of breast cancer to acquire resistance to TAX and recur, period after treatment [10], the addition of novel compounds might potentiate TAX to neutralize this effect [40]. We assessed the enhancement effect of KR to TAX by combination treatment. KR enhanced the reduction of cell viability and the induction of apoptosis in both cell lines. Furthermore, this enhancement effect was observed on the inhibition of cell migration and invasion. The downregulation of mesenchymal markers and metalloproteinase emphasized on the same. This suggest that, the aggressiveness of breast cancer through migration and invasion might be attenuated if KR is adjunctively administered with TAX. Finally, we investi-

gated the effect of KR in vivo. KR inhibited tumor progression demonstrated by the reduction in tumor size. Although we did not investigate the inhibition of tumor metastasis in vivo, the reduced expressions of mesenchymal markers and metalloproteinase in tumors derived from the combined treatment of KR and TAX compared to control and individual treatment might suggest this effect. Collectively, the data indicates potential anti-breast cancer and adjunctive treatment of KR with paclitaxel for the improvement of breast cancer treatment.

## Conclusion

In our study, KR reduced cell viability, induced apoptosis and inhibited cell migration and invasion. Additionally, KR enhanced TAX-induced reduction of cell viability, induction of cell apoptosis and inhibition of cell migration and invasion. Furthermore, KR demonstrated the enhancement of TAX-induced inhibition of breast cancer progression in vivo. These findings suggest KR as a potential adjunct therapy to paclitaxel, highlighting its translational relevance for improving breast cancer treatment outcomes.

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## Disclosure of conflict of interest

None.

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