PHARMACIA

Pharmacia 71: 1–10
DOI 10.3897/pharmacia.71.e122171

Research Article

# Combination of resveratrol and piperine to target doxorubicin resistance in breast cancer: An *in vitro* and *in vivo* study

Mallak J. AlHur[1], Nadezhda Barakova[2], Wamidh H. Talib[3]

1 *Department of Clinical Pharmacy and Therapeutics, Applied Science Private University, Amman, Jordan*
2 *FoodTech, Faculty of Biotechnology, ITMO University, 191002 St. Petersburg, Russia*
3 *Faculty of Allied Medical Sciences, Applied Science Private University, Amman 11931-166, Jordan*

Corresponding author: Wamidh H. Talib (altaei_wamidh@yahoo.com)

**Received** 3 March 2024 ♦ **Accepted** 22 July 2024 ♦ **Published** 22 August 2024

**Citation:** AlHur MJ, Barakova N, Talib WH (2024) Combination of resveratrol and piperine to target doxorubicin resistance in breast cancer: An *in vitro* and *in vivo* study. Pharmacia 71: 1–10. https://doi.org/10.3897/pharmacia.71.e122171

## Abstract

One of the biggest causes of death worldwide is cancer, which poses a serious threat to healthcare systems all over the world. Triple-negative breast cancer (TNBC), in particular, presents substantial clinical challenges because it does not express Human Epidermal Growth Factor Receptor 2 (HER2) or hormone receptors. Despite concerns about early recurrence and drug resistance, chemotherapy, particularly anthracyclines like Doxorubicin (DOX), remains the mainstay of treatment for TNBC patients. This research investigates the possibility of using natural products, especially piperine (PIP) and resveratrol (RES), to overcome DOX resistance. PIP and RES have shown anticancer effects via various pathways, such as inducing apoptosis and controlling the cell cycle. In this research, *in vitro* experiments showed that RES and PIP inhibit cell growth in a dose-dependent manner using triple-negative parent mouse mammary breast cancer cells and DOX resistance cells (EMT-6/P and EMT-6/DOX). RES in EMT-6/P cells showed an IC50 value of 146.511 ± 5.35 μM, and in EMT-6/DOX cells, it was 88.635 ± 29.507 μM. In the same way, PIP was found to have IC50 values of 148.819 ± 14.317 and 9.375 μM in the triplicate trials in EMT-6/P and EMT-6/DOX, respectively. Furthermore, on both cell lines, the combination demonstrated very strong synergistic effects; noticeably lower doses were needed for the combined treatment to reduce cell viability by 50%. The IC50 values for the combination treatments of RES and PIP were found to be < 2.289 μM and < 2.325 μM in EMT-6/P cells, respectively, with values < 0.348 μM and 0.243 ± 0.142 μM in EMT-6/DOX cells. The *in vivo* experiment conducted on *Balb/C* females indicated that mice bearing EMT-6/Dox cells and treated with the combination of RES and PIP had the highest cure percentage; however, this treatment showed mild toxicity. The study clarifies the potential synergistic activity between PIP and RES in combating DOX-resistant TNBC cells. These findings highlight the significance of investigating natural products as supplemental therapies in cancer treatment and provide insights into new treatment approaches. While the combination shows promise as a therapeutic option for treating breast cancer, especially in cases of DOX resistance, further investigation is needed.

## Keywords

overcoming drug resistance, natural products, alternative anticancer therapies, combination therapy

## Introduction

As a major cause of death worldwide and a significant obstacle to extending life expectancy globally, cancer is a severe global health concern. According to data from the World Health Organization (WHO), among individuals under 70 years old, cancer was either the primary or secondary cause of death in 112 out of 183 countries in 2019 AD. Additionally, it ranked third or fourth in 23 other countries (Sung et al. 2021). As the most frequently diagnosed cancer and the world's fifth leading cause of cancer-related deaths, breast cancer has surpassed lung cancer. In 2020 AD, breast cancer ranked as the highest in the majority of the world's countries for both incidence and mortality among women, accounting for about 24.5% of all cancer cases and 15.5% of cancer deaths (Lei et al. 2021). Jordan is a country in the Eastern Mediterranean region (EMR). Of its 10.20 million citizens, 12% of deaths in 2016 AD were related to cancer (Mousa et al. 2021). Because TNBC lacks the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), it presents a substantial problem in clinical practice. Chemotherapy is the cornerstone of the systemic treatment of TNBC; anthracyclines are essential agents and the current standard of care. DOX, an antibiotic based on anthracyclines, is currently one of the most effective medications for treating TNBC. Medication resistance and DOX-induced cardiotoxicity are the two main obstacles to DOX therapy (Al-Malky et al. 2020). DOX cardiotoxicity may manifest as either acute toxicity determined two or three days after the drug is administered or chronic cardiotoxicity discovered weeks or even months later. Arrhythmias, cardiomyopathies, left ventricular dysfunction, and congestive heart failure are possible examples of cardiotoxicity (Renu et al. 2018). Apart from the early development of drug resistance that results in treatment failure, physicians have identified additional significant concerns regarding the efficacy of DOX, such as the early recurrence of the disease, which can have serious consequences for cancer patients (Luo et al. 2022). Anticipating drug resistance, which makes chemotherapy drugs ineffective, accounts for 90% of cancer-related deaths. A deeper understanding of these mechanisms holds significant potential for clinical applications and opens the door to the creation of novel drugs that use cutting-edge targeting strategies (Talib et al. 2021). Natural products offer accessible and affordable sources of chemotherapeutic agents with multiple mechanisms of action that effectively impede the development of drug resistance (Talib et al. 2021). RES, also known as trans-3,4,5-trihydroxystilbene, is a naturally occurring phytoalexin belonging to the stilbene class. RES exists in two primary isomers, cis and trans, which often coexist (refer to Fig. 1. for chemical structures). The trans isomer exhibits greater biological activity compared to the cis form (Talib et al. 2022). RES exerts its anticancer effects by influencing multiple cell-signaling molecules involved in regulating cell cycle progression, inflammation, proliferation, apoptosis, invasion, metastasis, and angiogenesis in tumor cells (Gupta et al. 2011). Between 1990 and 2021, there were 30 research reports examining the impact of RES on cancer cells that had developed resistance to various drugs. These studies covered a range of cancer types along with their resistance to multiple chemotherapeutic agents, including bladder, breast, colon, gastric, leukemia, lung, melanoma, oral, ovarian, and prostate cancer cells (Choi et al. 2022). The 'King of Spices,' Piper nigrum, and its main ingredient, PIP, are well known for their therapeutic qualities. This black pepper, which is a member of the Piperaceae family, is popularly used as a spice in homes due to its unique pungency (refer to Fig. 2 for chemical structure) (Chopra et al. 2016; Turrini et al. 2020). PIP effects on apoptotic signaling activation and cell cycle progression inhibition have been reported to limit the growth and survival of various cancer cell types. In addition to these effects, PIP has been shown to influence redox homeostasis, prevent cancer stem cell (CSC) self-renewal, and modify autophagy and ER stress. PIP inhibits invasion, metastasis, and angiogenesis by altering the activity of numerous enzymes and transcription factors (Rather and Bhagat 2018). Interestingly, PIP has also shown antimutagenic properties and has been effective in inhibiting the activity and expression of multidrug resistance transporters such as P-glycoprotein and multidrug resistance proteins (Manayi et al. 2018). Moreover, PIP effectively increases the bioavailability of several chemotherapeutic drugs due to its inhibitory effect on P-gp activity, considered to be the first bioavailability enhancer in history to receive scientific validation (Rather and Bhagat 2018).

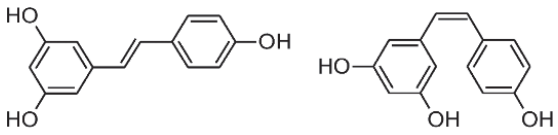


**Figure 1.** Chemical structure of RES: (a) Trans RES and (b) Cis-RES (Talib et al. 2022).

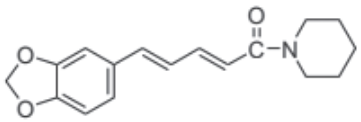


**Figure 2.** PIP chemical structure (Chopra et al. 2016).

## Materials and methods

### Cell line and culture conditions

The European Collection of Cell Cultures (ECACC; Salisbury, United Kingdom) provided the parent (EMT-6/P) and DOX-resistant (EMT-6/DOX) triple-negative mouse mammary breast cancer cell lines that were used in this research. The cells were cultured in minimum essential medium (MEM) (500 ml bottle) supplemented with 0.1% non-essential amino acids (0.5 ml), 10% fetal bovine

serum (50 ml), 1% L glutamine (5 ml), 0.1% gentamycin (0.5 ml), and 1% penicillin-streptomycin (5 ml) solution as supplied. Incubation conditions for cultured cells included 5% CO2, 95% humidity, and 37 °C.

## Preparation of resveratrol and piperine working solutions

The working solution was composed of pure RES (99.5% Trans, CurEase) and PIP (Sigma, USA), which were dissolved immediately before use in dimethyl sulfoxide (DMSO) (Extra PURE, Pharmpur, ph EUR, USP, and Scharlab). The working solution was then diluted in MEM to produce a working solution with concentrations of 2000 and 1200 μM for RES and PIP, respectively.

To prepare a working solution with a concentration equal to the IC50 values obtained from a single treatment, RES and PIP were directly dissolved in DMSO and 70% ethanol and then diluted in the MEM right before use. This solution was then applied to both cell lines for the combination treatment.

Since DOX (Ebewe Pharma, Austria) is already prepared as a medication on the market, as a stock solution of 50 mg per 25 mL (2 mg/mL), 100 μg/ml equivalent to 183.99 μM was used as a working solution from the available stock as the positive control for both cell lines.

## Antiproliferative assay (MTT)

The overnight culture was performed for both cell lines, EMT-6/P and EMT-6/DOX. The trypsinization technique was used to collect the cells, and the trypan blue exclusion method was used to count the cells that were growing exponentially. Subsequently, the cells were incubated for 24 hours at 10,000 cells per well in 96-well tissue culture flat-bottom microplates. Following seeding, both cell lines were exposed for 48 hours to PIP (600–9.375 μM) and RES (1000–7.8125 μM). Additionally, they were exposed to DOX (183.99–5.75 μM) for 48 hours, with three replicates for every concentration.

In combination treatment, PIP (74.4–2.325 μM) with a fixed concentration of RES (146.510 μM) and RES (73.25–2.289 μM) with a fixed dose of PIP (148.819 μM) were applied to EMT-6/P cells. For the EMT-6/DOX cell line, the cells were exposed to RES at varying concentrations (44.318–0.348 μM), while PIP was kept at a constant concentration (9.375 μM) and PIP at varying concentrations (4.688–0.037 μM) with a fixed concentration of RES (88.635 μM) for 48 hours, and three replicates were used for each concentration as well.

Following the incubation period of 48 hours, 200 μL were removed, followed by washing each well with 100 μL of phosphate buffered saline (PBS). Then 100 μL of the medium was added to every well along with 20 μL of the MTT solution (the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (AK Scientific, Union City, USA), that had been dissolved in PBS). Using MTT, the antiproliferative activity was identified. This test finds the reduction of MTT by mitochondrial dehydrogenase to a blue formazan product, indicating both cell viability and normal mitochondrial function.

The microplate was then incubated for an additional 3 hours in a CO2 incubator, and then 100 μL of DMSO were added and incubated for another hour. Using a microplate reader (Biotek, Winooski, VT, USA), the absorbance was then measured at 550 nm.

To prevent interference of DOX with MTT, after treating the cells with DOX and allowing a 48-hour incubation period, 200 μL of the solution will be removed from each well, followed by washing with 100 μL of PBS. Then 200 μL of PBS was added to each well, along with 20 μL of MTT. The mixture was then incubated for 3 hours at 37 °C. Following the incubation period, the violet formazan crystals were solubilized by adding 200 μL of DMSO after the solution had been carefully removed. After allowing the plates to fully dissolve for five minutes on a horizontal shaker, the absorbance was measured at 550 nm using a microplate reader (Biotek, Winooski, VT, USA) (Luis et al. 2019).

## Calculation of inhibitory concentration ($IC_{50}$)

The drug concentration required to kill or inhibit cells by 50% relative to untreated cells is referred to as "$IC_{50}$," which implies that the inhibitory substrate only exerts 50% of its maximum inhibitory action at that concentration (Caldwell et al. 2012). The Statistical Package for the Social Sciences, IBM SPSS, version 27, was utilized in this research to calculate and assess $IC_{50}$ values. The information was subjected to a nonlinear regression test to ascertain the $IC_{50}$ values for both single and combination treatments.

## Calculation of the combination index

The following formula was used to calculate the combination index (CI) for RES and PIP against EMT-6/P and EMT-6/Dox cells to evaluate their interaction (Ichite et al. 2009).

$$CI = \frac{D_1}{Dx_1} + \frac{D_2}{Dx_2} + \alpha \frac{D_1 D_2}{Dx_1 Dx_2}$$

Where:
(Dx) 1 = $IC_{50}$ of RES alone.
(D) 1 = $IC_{50}$ of RES in combination with PIP.
(Dx) 2 = $IC_{50}$ of PIP alone.
(D) 2 = $IC_{50}$ of PIP in combination with RES.
a = 0 for mutually exclusive interaction or 1 for mutually non-exclusive interaction.

According to the literature review, RES and PIP each have a unique strategy for combating cancer. As a result, the mutually non-exclusive model was used to calculate CI with α = 1.

An explanation of the CI results is provided below:

CI < 0.1 Very Strong Synergism, CI = 0.1–0.3 Strong Synergism, CI = 0.3–0.7 Synergism, CI = 0.7–0.85 Moderate Synergism, CI = 0.85–0.90 Slight Synergism,

CI = 0.9–1.10 Nearly Additive, CI = 1.10–1.20 Slight Antagonism, CI = 1.20–1.45 Moderate Antagonism, and CI = 1.45–3.3 Antagonism (Chou 2006).

## Calculation of the resistance index

When comparing resistant cell lines to sensitive ones, the resistance index indicates the difference in concentration needed to kill 50% of the cells. In this research, the resistance index was calculated by comparing the $IC_{50}$ values of the resistant EMT-6/DOX and the sensitive EMT-6/P cell lines using the following formula (Wang et al. 2010):

$$\text{Resistance Index (RI)} = (IC_{50} \text{ of Resistant Cell Line} / IC_{50} \text{ of Parental Cell Line}) * 100\%$$

## *In vivo* experiment

### *Mice*

The Institutional Review Board (IRB) in the Faculty of Pharmacy, Applied Science Private University, approved all the experimental protocols used in this research (Approval Number: 2023-PHA-44) that were conducted following accepted ethical standards.

Thirty-six *Balb/C* female mice, weighing between 21 and 25 g per mouse, at the age of four to six weeks, were used in this research. The mice were housed in individual cages with bedding made of wooden shavings. The conditions included constant air ventilation, a temperature of about 25 °C, a humidity of between 50 and 60 percent, and alternating cycles of light and dark lasting 12 hours.

### *Tumor inoculation*

Trypsinization was used to extract exponentially growing EMT-6/P and EMT-6/Dox cells, which were then cleaned and resuspended in MEM at a density of 1.5 × 10^6 cells/ml. After that, the trypan blue exclusion method was used to determine viability. Each female *Balb/C* mouse had a subcutaneous injection of 1.5 × 10^5 cells in 0.1 ml medium as a tumor induction dose, which was left to grow and form new tumors for ten days.

### *Mice groups, treatment, and antitumor activity*

The average tumor volume for each group was roughly matched, and tumors of similar sizes were selected. Each mouse received an injection on the right side of EMT-6/DOX and the left side of EMT-6/P. The treatment started ten days after the tumor was injected. Due to their limited solubility in water, olive oil was utilized as a non-toxic solvent compared to other synthetic solvents to yield the treatment doses for RES and PIP (Koul and Kapil 1993; Balata et al. 2016). Six groups (n = 6 for each group) of 36 tumor-bearing mice were used in this research (as mentioned in Fig. 3). Treatment groups were assigned randomly as follows: Group 1 received intraperitoneal injections of RES at a dose of 50 mg/kg/day (Alobaedi et al. 2017). Group 2 received intraperitoneal injections of PIP at a dose of 25 mg/kg/day (Talib 2017). Group 3 received

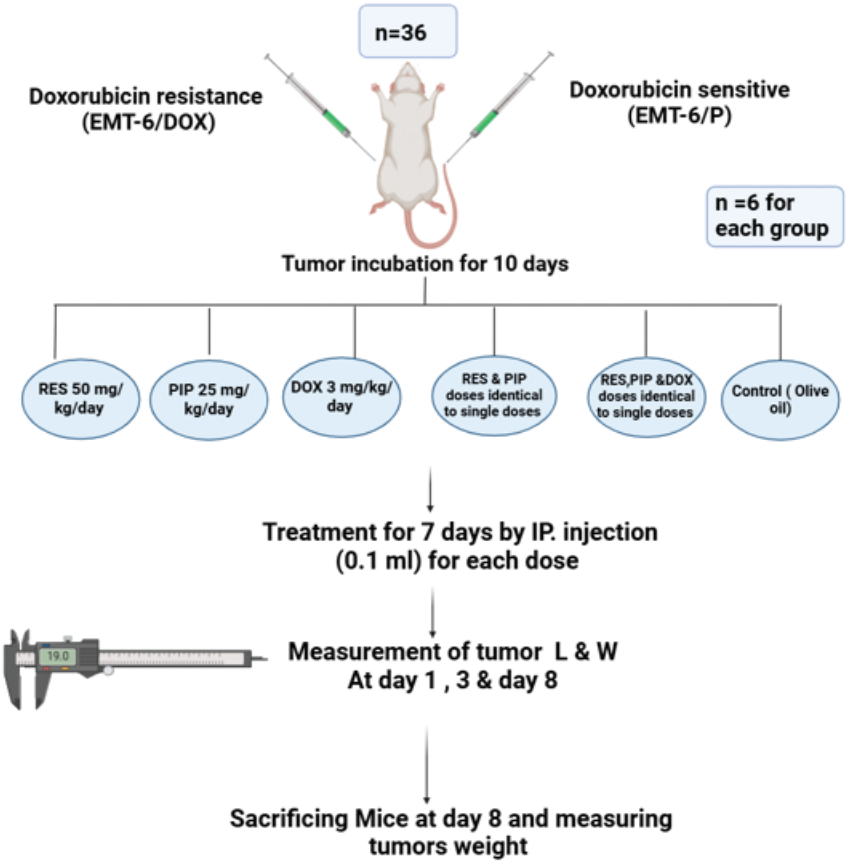


**Figure 3.** *In vivo* study details.

DOX (3 mg/kg/day, diluted in PBS) (Aston et al. 2017). RES and PIP were administered in combination for Group 4. DOX, RES, and PIP as a triplicate therapy were administered to Group 5. The doses of RES, PIP, and DOX in the combination-treated groups were the same as those in the single-treated groups. Group 6 was given intraperitoneally 0.1 mL of vehicle (olive oil) each day as a negative control.

Blood samples were collected to evaluate liver and kidney function in the treated mice after the seven-day course of treatment. A digital calliper, a non-invasive instrument, was used to measure the tumor's dimensions three times during the treatment: on days 1, 3, and 8 (the sacrificing day). The tumor volume was then computed using the following equation:

$$\text{Tumor volume} = A \times B2 \times 0.5$$

Where:
A = the length of the longest aspect of the tumor.
B = the length of the tumor aspect perpendicular to A.

The percent change in tumor volumes between the initial and final volumes was calculated using the following equation:

$$\% \text{ Tumor change} = ((F - I) / I) \times 100\%$$

where I stands for the initial tumor volumes and F stands for the final tumor volumes.

At last, cervical dislocation proved fatal to mice. To preserve the morphology of the tumors, they were removed, weighed, and then preserved in 10% formalin.

## Evaluation of liver and kidney function

The following parameters were measured from serum levels for each group, including the healthy mice group, using commercial kits (DiaSys Diagnostic Systems GmbH, Germany): alanine aminotransferase (ALT), alkaline phosphatase (AP), aspartate aminotransferase (AST), creatinine, gamma-glutamyl transferase (GGT), uric acid, and urea.

## Statistical analysis

The statistical analyses were carried out using IBM SPSS (Statistical Package for the Social Sciences) version 27. Nonlinear regression was used to analyze IC50 values, which were reported as mean values with a standard error of mean (SEM). To assess treatment toxicity for the *in vivo* part, comparisons were conducted for each treated group with the placebo and healthy mouse groups. The independent sample T-test for normally distributed variables was used to determine statistically significant differences. When normality was not assumed, the comparison was conducted using the Mann-Whitney U test, considering that normality was checked by the Shapiro-Wilk test. The statistically significant difference between groups was indicated by a significance level of $p \leq 0.05$. Six mice were used for *in vivo* experiments in each group, and n = 3 was used for all statistical tests to assess treatment toxicity. To determine the statistical significance of the percentage change in tumor volume between groups, IBM SPSS was used as well. The Kruskal-Wallis H test was chosen because the assumptions for one-way ANOVA were not met. Upon finding a significant difference in the Kruskal-Wallis test, further investigations were conducted using the Mann-Whitney U test and the independent samples t-test, with n = 6 for all tests. To control the increased risk of Type I errors (false positives) associated with multiple comparisons, the Bonferroni correction method was applied. This adjustment involved dividing the original significance level (0.05) by the number of comparisons (6), resulting in a new significance level of 0.0083. This correction helps maintain the overall Type I error rate at 0.05, despite multiple comparisons.

# Results

## *In vitro* results

The inhibition of cell growth and proliferation in the EMT-6/P and EMT-6/Dox cell lines in response to different concentrations of RES and PIP is shown in Figs 4, 5. The RES in EMT-6/P cells showed an IC50 value of 146.511 ± 5.35 μM, while in EMT-6/Dox cells, it was 88.635 ± 29.507 μM. This inhibition showed a dose-dependent relationship. In the same way, PIP was found to have IC50 values of 148.819 ± 14.317 μM and 9.375 μM (as the survival percentage for both the average and single trials was around 50% at a concentration of 9.375 μM) in EMT-6/P and EMT-6/DOX, respectively.

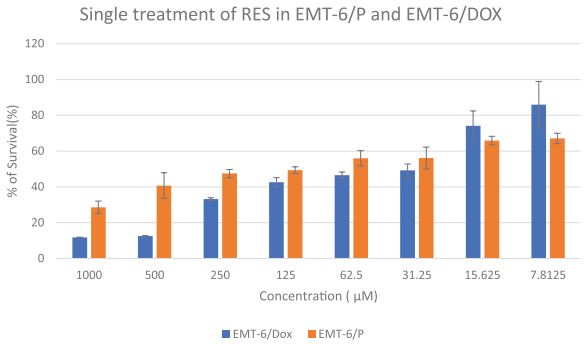


**Figure 4.** Anti-proliferation effect of RES in single treatment against EMT-6/P and EMT-6/DOX cell lines.

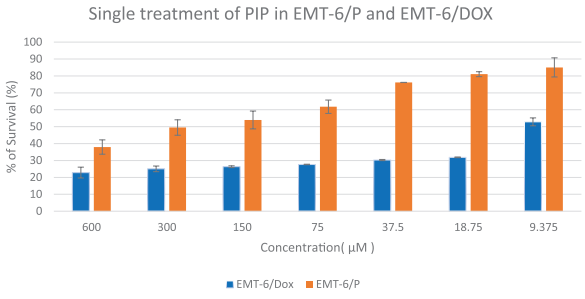


**Figure 5.** Anti-proliferation effect of PIP in single treatment against EMT-6/P and EMT-6/DOX cell lines.

The EMT6/P and EMT6/Dox cell lines were used to study the relationship between RES and PIP. Various PIP concentrations and fixed RES concentrations were tested on both cell lines, and vice versa. Figs 6–9 show the inhibition of cell growth and proliferation, which also follow a dose-dependent relationship. Furthermore, noticeably lower doses were needed for the combined treatment to reduce cell viability by 50%. Comparing EMT-6/P cells to EMT-6/DOX cells, it was found that the latter were more sensitive to combination therapy at lower dosages. The combination treatment's 50% inhibitory concentrations ($IC_{50}$) for RES and PIP were found to be < 2.289 μM (as the survival percentage for both the average and single trials was below 50% at a concentration of 2.289 μM) and < 2.325 μM (as the survival percentage for both the average and single trials was below 50% at a concentration of 2.325 μM) in EMT-6/P cells, respectively, with values < 0.348 μM (as the survival percentage for both the average and single trials was below 50% at a concentration of 0.348 μM) and 0.243 ± 0.142 μM in EMT-6/DOX cells. In addition, the sensitivity of DOX was also assessed on both cell lines, as shown in Fig. 10, with a result of IC50 > 183.99 μM (as the survival percentage for both the average and single trials ranged from 90% to 113% at a concentration of 183.99 μM) for EMT-6/Dox cells and 68.58 ± 9.50 μM for EMT-6/P cells. All results are summarized in Table 1, including IC50 values, combination index, and resistance index for RES, PIP, their combinations, and DOX.

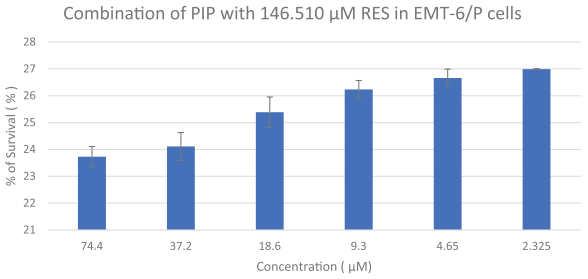


**Figure 6.** The anti-proliferative effects of different concentrations of PIP in combination with 146.510 μM RES against the EMT-6/P cell line.

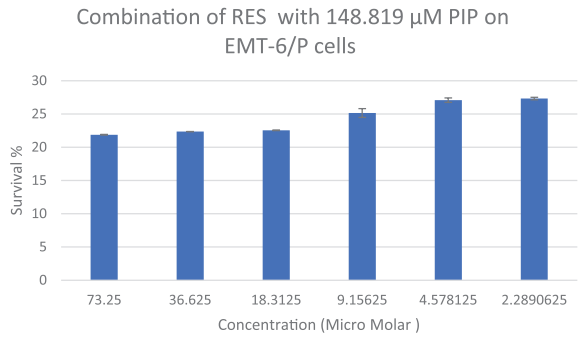


**Figure 7.** The anti-proliferative effects of different concentrations of RES in combination with 148.819 μM PIP against the EMT-6/P cell line.

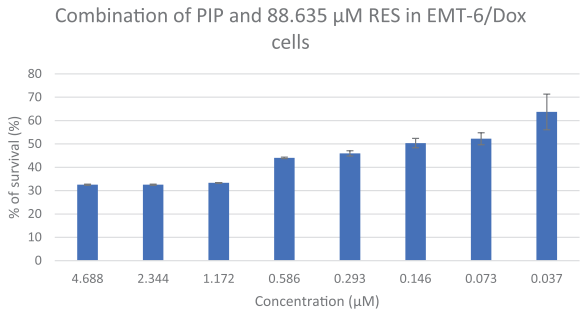


**Figure 8.** The anti-proliferative effects of different concentrations of PIP in combination with 88.635 μM RES against the EMT-6/DOX cell line.

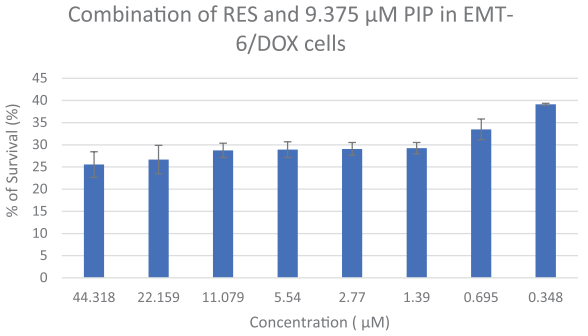


**Figure 9.** The anti-proliferative effects of different concentrations of RES in combination with 9.375 μM PIP against the EMT-6/Dox cell line.

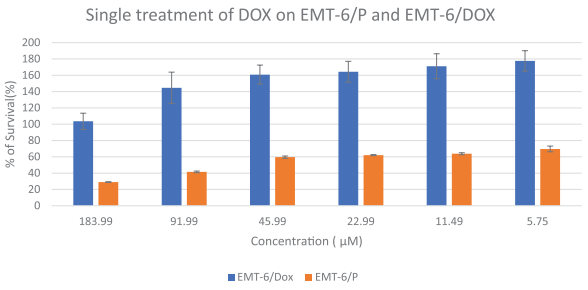


**Figure 10.** The sensitivity of EMT-6/P and EMT-6/DOX cell lines to DOX at various doses was evaluated using the anti-proliferative assay (MTT).

**Table 1.** The IC50 values of RES, PIP, their combination, and DOX on EMT-6/P and EMT-6/DOX cell lines, along with the combination index (CI), Interpretation, and resistance index.

| Cell line | $IC_{50}$ of RES (μM) | $IC_{50}$ of PIP (μM) | $IC_{50}$ of DOX (μM) | $IC_{50}$ of RES combined with PIP (μM) | $IC_{50}$ of PIP combined with RES (μM) | CI | Interpretation |
|---|---|---|---|---|---|---|---|
| EMT-6/P | 146.511 | 148.819 | 68.58 | < 2.289 | < 2.325 | < 0.031 | very strong synergism |
| EMT-6/Dox | 88.635 | 9.375 | > 183.99 | < 0.348 | 0.243 | < 0.029 | very strong synergism |
| Resistance Index | 60.5 | 6.3 | > 268 | < 15.2 | < 10.4 | | |

## *In vivo* results

Following 10 days of tumor inoculation and 7 days of mice treatment, the mice were sacrificed, and the tumor size was assessed. The volume change (in $mm^3$) and the percentage change in tumor volume for each group were calculated as mentioned in Table 2. In EMT-6/P, the triplicate therapy involving RES, PIP, and DOX, along with individual treatments of PIP and DOX, achieved the highest cure percentage (66.67%) among all treated groups. This was followed by the group treated with a combination of RES and PIP, as well as the group treated with RES as a single treatment, both showing a 50% cure percentage. Additionally, Fig. 11 illustrates the reduction in average tumor volume during the treatment period for all groups except the control group. The statistically significant differences in the percentage change in tumor volume in mice bearing EMT-6/P cells were assessed by using IBM SPSS. The Kruskal-Wallis H test was chosen because the assumptions for one-way ANOVA were not met with n = 6. The test resulted in a P value of 0.062, indicating no significant difference between the groups when compared to a significance level of p-value ≤ 0.05.

Therefore, the same treatment approaches were used for EMT-6/DOX, as Table 3 illustrates. The combination therapy involving RES and PIP achieved the highest cure percentage (66.67%) among all treated groups. This was followed by the group treated with a triplicate therapy of RES, PIP, and DOX, as well as the group treated with PIP as a single treatment and DOX as a single treatment, all showing a 50% cure percentage. Additionally, Fig. 12 illustrates the change in average tumor volume during the treatment period for all groups. The combination treatment of RES and PIP showed the largest change in average tumor volume.

To determine the statistical significance of the percentage change in tumor volume in EMT-6/DOX cells between groups by using IBM SPSS. The Kruskal-Wallis H test was chosen because the assumptions for one-way ANOVA were not met with n = 6 for each group. The test resulted in a P value of 0.003, indicating a significant difference between the groups when compared to a significance level

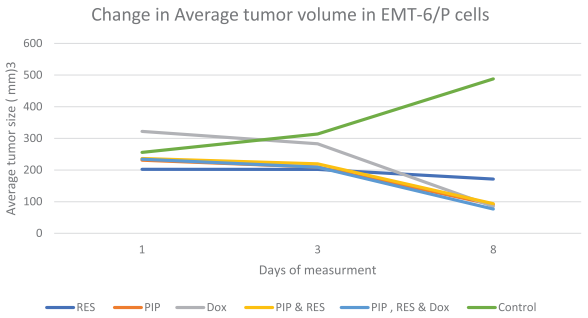


**Figure 11.** Change in average tumor volume in EMT-6/P cells during treatment days.

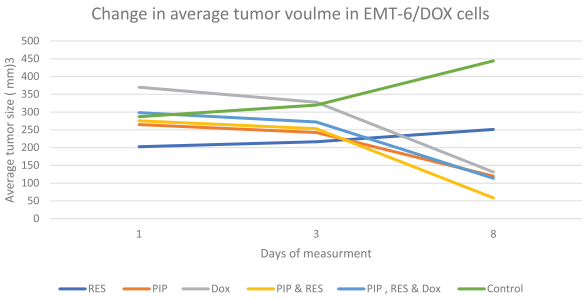


**Figure 12.** Change in average tumor volume in the EMT-6/DOX cells during treatment days.

**Table 2.** In the EMT-6/P cell line (n = 6), the effects of RES, PIP, their combinations, and DOX on changes in tumor volume, percentages of change in tumor volume, and average tumor weight are investigated.

| Treatment group EMT-6/P | Av. initial tumor volume ($mm^3$) | Av. final tumor volume ($mm^3$) | %Change in tumor volume (%) | Mice with no detectable tumor (Cure %) | Av. tumor weight (mg) |
|---|---|---|---|---|---|
| RES | 202.23 | 171.40 | -15.25 | 50 | 109.28 |
| PIP | 231.05 | 90.32 | -60.91 | 66.67 | 109.98 |
| DOX | 322.07 | 84.76 | -73.68 | 66.67 | 69.13 |
| RES and PIP | 236.43 | 93.63 | -60.40 | 50 | 107.92 |
| RES, PIP, and DOX | 234.20 | 76.67 | -67.30 | 66.67 | 81.65 |
| Control | 255.72 | 488.04 | 90.85 | 16.67 | 514.07 |

Abbreviations: Av. = average, $mm^3$ = cubic millimeter.

**Table 3.** EMT-6/DOX cell line (n = 6), the effects of RES, PIP, their combinations, and DOX on changes in tumor volume, percentages of change in tumor volume, and average tumor weight are investigated.

| Treatment group EMT-6/DOX | Av. initial tumor volume ($mm^3$) | Av. final tumor volume ($mm^3$) | %Change in tumor volume (%) | Mice with no detectable tumor (Cure %) | Av. tumor weight (mg) |
|---|---|---|---|---|---|
| RES | 202.49 | 251.18 | 24.05 | 16.67 | 219.68 |
| PIP | 264.84 | 119.69 | -54.81 | 50 | 110.73 |
| DOX | 369.96 | 131.22 | -64.53 | 50 | 121.10 |
| RES and PIP | 275.37 | 58.42 | -78.79 | 66.67 | 74.30 |
| RES, PIP, and DOX | 298.30 | 113.64 | -61.90 | 50 | 181.13 |
| Control | 286.94 | 444.14 | 54.78 | 0 | 351.60 |

Abbreviations: Av. = average, $mm^3$ = cubic millimeter.

of p-value ≤ 0.05. Upon finding a significant difference with the Kruskal-Wallis test, further investigations were conducted using the Mann-Whitney U test and the independent samples t-test, with n = 6 for all tests. To control for the increased risk of Type I errors (false positives) associated with multiple comparisons, the Bonferroni correction method was applied. This adjustment involved dividing the original significance level (0.05) by the number of comparisons (6), resulting in a new significance level of 0.0083. This correction helps maintain the overall Type I error rate at 0.05, despite multiple comparisons. From the six comparisons, the following were considered significant when compared to the new significance level of 0.0083: a single-treated group with PIP compared to a control group with p value = 0.006, a combination treatment of RES and PIP compared to a control group with p value = 0.003, and a single-treated group with DOX compared to a control group with p value = < 0.001, and no significant differences were found for the following comparisons: combination-treated group with PIP and RES compared to the triple treated group with RES, PIP, and DOX with p value = 0.475, and the single treated group with DOX compared to the combination-treated group with RES, PIP with p value = 0.475, and the single treated group with PIP compared to the combination-treated group with RES and PIP with p value = 0.284.

### Evaluation of liver and kidney function

To evaluate liver toxicity in each treatment group, including the negative control group (placebo) and a group of healthy mice. The levels of alanine aminotransferase (ALT), alkaline phosphatase (AP), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) were measured. In addition to that, kidney toxicity was also evaluated by measuring the levels of urea, creatinine, and uric acid.

All treatment groups, the negative control group, and a group of healthy mice showed consistent results for the serum GGT levels, which were less than 3 U/L.

When comparing each treatment group to the negative control group, no significant differences were found in ALT, creatinine, urea, and AP levels. Significant differences in AST levels were found in the following treated groups: the DOX single-treated group and the RES and PIP combination-treated group. Significant differences in uric acid levels were found in all treated groups. These significant differences are considered mild toxicity.

When each treated group was compared with healthy mice in the second comparison, no significant differences were found in the levels of ALT and AP. The following significant differences were found:

Significant differences in uric acid and creatinine levels were found in all treated groups.

Significant differences were observed in the levels of urea in the following treated groups: The RES and PIP combination, the triple combination of RES, PIP, and Dox, in addition to the single treated group with DOX. Also, significant differences were observed in the levels of AST in the following treated groups: The RES and PIP combination, the triple combination of RES, PIP, and DOX, the single treated group with DOX, and the single treated group with RES. These significant differences are considered mild toxicity.

## Discussion

One of the most common primary treatment approaches for TNBC is chemotherapy. Chemoresistance, or the emergence of tumor resistance to chemotherapy, is a major obstacle to cancer treatment (Gupta et al. 2011). The use of combination approaches in cancer therapy offers the advantage of targeting different pathways in a synergistic or additive manner (Talib et al. 2022). Various approaches have been examined to overcome drug resistance in cancer, with natural products emerging as a promising strategy. The specific targets of each natural product have been identified, and their mechanisms of action have been thoroughly investigated through experimental and clinical studies (Talib et al. 2021). As RES exhibits potent anti-cancer properties through various mechanisms, the most extensively documented mechanism of action for its anti-cancer properties is its capacity to trigger apoptosis in cancer cells through multiple pathways associated with the control of cell death and survival (Udenigwe et al. 2008). Additionally, numerous mechanisms have been proposed to explain piperine's chemopreventive effect. PIP effects on apoptotic signaling activation and cell cycle progression inhibition have been reported to limit the growth and survival of various cancer cell types (Rather and Bhagat 2018). In this research, RES and PIP showed inhibition of cell growth and proliferation, and this inhibition follows a dose-dependent relationship in EMT-6/P and EMT-6/Dox cell lines. These results align with previous studies on both compounds. In this research, EMT-6/DOX cells were more sensitive to RES and PIP than the sensitive cell line. This could be explained, as a previous *in vitro* study showed that treatment with a combination of RES and DOX on DOX-resistant breast cancer cells markedly increased the cellular accumulation of DOX by downregulating the expression levels of the ATP-binding cassette (ABC) transporter genes, MDR1, and MRP1, which is considered one of the mechanisms for developing treatment resistance (Kim et al. 2014). Additionally, the higher sensitivity of PIP in resistance cells can be explained by *in vitro* studies that have demonstrated PIP's ability to re-sensitize P-gp, MRP1, and BCRP as one of the mechanisms for developing treatment resistance in multidrug-resistant cancer cells (Li et al. 2011). Moreover, when used in combination, PIP and RES demonstrated strong synergistic effects in both cell lines, suggesting the potential for overcoming DOX resistance. Regarding *in vivo* experiments, one of the previous studies revealed that upon administering RES intraperitoneally to rats, analysis of rat hepatocytes detected two prominent peaks identified as resveratrol-3-glucuronide and resveratrol-3-sulfate 71 (Wenzel and Somoza 2005). The metabolism of RES

could account for the lower cure percentage observed in the single RES-treated group compared to the combination-treated group in this research, as well as the discrepancy between *in vitro* and *in vivo* results regarding RES treatment. In addition, as mentioned in the literature, combining RES with PIP leads to the inhibition of various ABC transporters, including resveratrol-excreting ABCB1, MRP2, and ABCG2, which has been demonstrated to increase the bioavailability of RES (Schaafsma et al. 2016). Considering that breast cancer cells develop resistance to DOX through multiple pathways, including the up-regulation of multiple transporter genes (AbuHammad and Zihlif 2013), it is notable that both RES and PIP specifically target the resistance mechanisms involved in resistance proteins and plasma membrane transporters, as previously mentioned. The combination of RES and PIP significantly inhibits the growth of tumors in both animal and cell culture models, demonstrating enhanced anti-cancer effects. To determine the precise synergistic activity of both compounds at the cellular level and to evaluate their effect on cancer metastasis, more research is necessary. However, these results indicate that RES and PIP could be promising candidates for combination cancer therapies, with the potential to provide better efficacy and fewer side effects than conventional therapies.

Notably, this study stands out as the first of its kind to investigate the potential synergy between PIP and RES in the DOX resistance cell line. By examining the combined impact of these agents, this research aims to shed light on a previously unexplored aspect of their interaction, opening the door to possible breakthroughs in cancer treatment approaches.

## Conclusion

Chemotherapy is still an essential part of treating TNBC. The combination of RES and PIP demonstrates promise in simultaneously addressing multiple pathways to fight off cancerous cells. Based on the available data, it can be concluded that the combination of PIP and RES has a very strong synergistic anticancer effect against the parent (EMT-6/P) and resistant (EMT-6/Dox) cell lines, better than each treatment alone *in vitro*. Groups treated with PIP as a single treatment or a combination of RES and PIP have a significant reduction in tumor volume *in vivo* experiments compared to the control group in the resistant cell line. These results highlight the potential for PIP and RES to work synergetically, opening exciting new possibilities for creating more effective cancer treatments. A DOX-resistant cell line was used to test the novel combination of RES and PIP, which showed a mild toxicity profile. Investigating such out-of-the-ordinary pairings might broaden research and offer insight to improve breast cancer treatment.

## Recommendations

Research and development is an ongoing process that aims to produce important and innovative results that advance our understanding of cancer prevention and treatment.

As a result, the following recommendations are made for additional research in this area:

- Investigating the use of PIP, RES, and DOX in triple therapy to treat mice implanted with DOX-resistant breast cancer.
- Use VEGF and caspase-3 assays to further investigate the mechanisms underlying this combination's synergistic activity.
- Examining this combination's effectiveness in conjunction with different chemotherapeutic agents for various cancer types.
- Assess the effects of RES, PIP, and their combined administration on DOX-induced cardiotoxicity.

## References

AbuHammad S, Zihlif M (2013) Gene expression alterations in doxorubicin resistant MCF7 breast cancer cell line. Genomics 101(4): 213–220. https://doi.org/10.1016/j.ygeno.2012.11.009

Al-Malky HS, Al Harthi SE, Osman A-MM (2020) Major obstacles to doxorubicin therapy: Cardiotoxicity and drug resistance. Journal of Oncology Pharmacy Practice 26(2): 434–444. https://doi.org/10.1177/1078155219877931

Alobaedi OH, Talib WH, Basheti IA (2017) Antitumor effect of thymoquinone combined with resveratrol on mice transplanted with breast cancer. Asian Pacific Journal of Tropical Medicine 10(4): 400–408. https://doi.org/10.1016/j.apjtm.2017.03.026

Aston WJ, Hope DE, Nowak AK, Robinson BW, Lake RA, Lesterhuis WJ (2017) A systematic investigation of the maximum tolerated dose of cytotoxic chemotherapy with and without supportive care in mice. BMC Cancer 17(1): 1–10. https://doi.org/10.1186/s12885-017-3677-7

Balata GF, Essa EA, Shamardl HA, Zaidan SH, Abourehab MA (2016) Self-emulsifying drug delivery systems as a tool to improve solubility and bioavailability of resveratrol. Drug Design, Development and Therapy 2016: 117–128. https://doi.org/10.2147/DDDT.S95905

Caldwell WG, Yan Z, Lang W, Masucci JA (2012) The IC50 concept revisited. Current Topics in Medicinal Chemistry 12(11): 1282–1290. https://doi.org/10.2174/156802612800672844

Choi CY, Lim S-C, Lee T-B, Han SI (2022) Molecular basis of resveratrol-induced resensitization of acquired drug-resistant cancer cells. Nutrients 14(3): 699. https://doi.org/10.3390/nu14030699

Chopra B, Dhingra AK, Kapoor RP, Prasad DN (2016) Piperine and its various physicochemical and biological aspects: A review. Open Chemistry Journal 3(1). https://doi.org/10.2174/1874842201603010075

Chou T-C (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacological Reviews 58(3): 621–681. https://doi.org/10.1124/pr.58.3.10

Gupta SC, Kannappan R, Reuter S, Kim JH, Aggarwal BB (2011) Chemosensitization of tumors by resveratrol. Annals of the New

York Academy of Sciences 1215(1): 150–160. https://doi.org/10.1111/j.1749-6632.2010.05852.x

Ichite N, Chougule MB, Jackson T, Fulzele SV, Safe S, Singh M (2009) Enhancement of docetaxel anticancer activity by a novel diindolylmethane compound in human non–small cell lung cancer. Clinical Cancer Research 15(2): 543–552. https://doi.org/10.1158/1078-0432.CCR-08-1558

Kim TH, Shin YJ, Won AJ, Lee BM, Choi WS, Jung JH, Chung HY, Kim HS (2014) Resveratrol enhances chemosensitivity of doxorubicin in multidrug-resistant human breast cancer cells via increased cellular influx of doxorubicin. Biochimica et Biophysica Acta (BBA)-General Subjects 1840(1): 615–625. https://doi.org/10.1016/j.bbagen.2013.10.023

Koul IB, Kapil A (1993) Evaluation of the liver protective potential of piperine, an active principle of black and long peppers. Planta Medica 59(05): 413–417. https://doi.org/10.1055/s-2006-959721

Lei S, Zheng R, Zhang S, Wang S, Chen R, Sun K, Zeng H, Zhou J, Wei W (2021) Global patterns of breast cancer incidence and mortality: A population-based cancer registry data analysis from 2000 to 2020. Cancer Communications 41(11): 1183–1194. https://doi.org/10.1002/cac2.12207

Li S, Lei Y, Jia Y, Li N, Wink M, Ma Y (2011) Piperine, a piperidine alkaloid from Piper nigrum re-sensitizes P-gp, MRP1 and BCRP dependent multidrug resistant cancer cells. Phytomedicine 19(1): 83–87. https://doi.org/10.1016/j.phymed.2011.06.031

Luis C, Castaño-Guerrero Y, Soares R, Sales G, Fernandes R (2019) Avoiding the interference of doxorubicin with MTT measurements on the MCF-7 breast cancer cell line. Methods and Protocols 2(2): 29. https://doi.org/10.3390/mps2020029

Luo M, Fu A, Wu R, Wei N, Song K, Lim S, Luo KQ (2022) High expression of G6PD increases doxorubicin resistance in triple negative breast cancer cells by maintaining GSH level. International Journal of Biological Sciences 18(3): 1120. https://doi.org/10.7150/ijbs.65555

Manayi A, Nabavi SM, Setzer WN, Jafari S (2018) Piperine as a potential anti-cancer agent: a review on preclinical studies. Current Medicinal Chemistry 25(37): 4918–4928. https://doi.org/10.2174/0929867324666170523120656

Mousa RH, Melhem JM, Hammad EA (2021) Epidemiology of women diagnosed with breast cancer in Jordan: A 5-year survival analysis and patients' characteristics from 2 public hospitals. Saudi Medical Journal 42(7): 776. https://doi.org/10.15537/smj.2021.42.7.20210112

Rather RA, Bhagat M (2018) Cancer chemoprevention and piperine: molecular mechanisms and therapeutic opportunities. Frontiers in Cell and Developmental Biology 6: 10. https://doi.org/10.3389/fcell.2018.00010

Renu K, Abilash V, PB, TP, Arunachalam S (2018) Molecular mechanism of doxorubicin-induced cardiomyopathy–An update. European Journal of Pharmacology 818: 241–253. https://doi.org/10.1016/j.ejphar.2017.10.043

Schaafsma E, Hsieh T-C, Doonan BB, Pinto JT, Wu JM (2016) Anticancer activities of resveratrol in colorectal cancer. Biology and Medicine 8(5): 1000317. https://doi.org/10.4172/0974-8369.1000317

Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a Cancer Journal for Clinicians 71(3): 209–249. https://doi.org/10.3322/caac.21660

Talib WH (2017) Regressions of breast carcinoma syngraft following treatment with piperine in combination with thymoquinone. Scientia Pharmaceutica 85(3): 27. https://doi.org/10.3390/scipharm85030027

Talib WH, Alsayed AR, Barakat M, Abu-Taha M, Mahmod AI (2021) Targeting Drug Chemo-Resistance in Cancer Using Natural Products. Biomedicines 9(10): 1353. https://doi.org/10.3390/biomedicines9101353

Talib WH, Awajan D, Hamed RA, Azzam AO, Mahmod AI, Al-Yasari IH (2022) Combination anticancer therapies using selected phytochemicals. Molecules 27(17): 5452. https://doi.org/10.3390/molecules27175452

Turrini E, Sestili P, Fimognari C (2020) Overview of the anticancer potential of the "king of spices" piper nigrum and its main constituent piperine. Toxins 12(12): 747. https://doi.org/10.3390/toxins12120747

Udenigwe CC, Ramprasath VR, Aluko RE, Jones PJ (2008) Potential of resveratrol in anticancer and anti-inflammatory therapy. Nutrition Reviews 66(8): 445–454. https://doi.org/10.1111/j.1753-4887.2008.00076.x

Wang X-B, Wang S-S, Zhang Q-F, Liu M, Li H-L, Liu Y, Wang J-N, Zheng F, Guo L-Y, Xiang J-Z (2010) Inhibition of tetramethylpyrazine on P-gp, MRP2, MRP3 and MRP5 in multidrug resistant human hepatocellular carcinoma cells. Oncology reports 23(1): 211–215. https://doi.org/10.3892/or_00000625

Wenzel E, Somoza V (2005) Metabolism and bioavailability of trans-resveratrol. Molecular Nutrition & Food Research 49(5): 472–481. https://doi.org/10.1002/mnfr.200500010