

Study of the antitumor activity of the combination baicalin and epigallocatechin gallate in a murine model of vincristine-resistant breast cancer

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Abstract

Background: Breast cancer is one of the primary causes of death in women worldwide. Yearly, a quarter of cancer cases in women are breast cancer. The problem is aggravated by the emergence of chemotherapeutic resistance. One of the practices to overcome this problem is using natural products along with chemotherapy. Epigallocatechin Gallate (EGCG) is the main efficient catechin in green tea. Baicalin is the main active chemical constituent of *Scutellaria baicalensis*. Both natural products have anti-cancer and improving resistance activities. Therefore, a combination of EGCG and baicalin may afford a possible way out to overcome chemotherapeutic resistance.

Methods: EGCG and baicalin were tested on Vincristine-sensitive (EMT-6/P) and Vincristine-resistant (EMT-6/V) mouse mammary cell lines. Antiproliferative and apoptotic effects were assessed for EGCG, baicalin, their combination, and Vincristine *in vitro* using MTT and caspase-3 assays. An *in vivo* study was conducted to assess the effect of EGCG, baicalin, their combination, vincristine, vincristine along with the combination in both EMT-6/P and EMT-6/V mouse mammary cell lines. The safety profile was also considered using liver enzymes and creatinine assays.

Results: *in vitro*, EGCG and baicalin had synergistic effects in both cell lines. The combination of baicalin and EGCG as 140 and 100 μ M respectively, caused an apoptotic effect higher than the single treatment of baicalin 175 μ M or EGCG 125 μ M, in vincristine-resistant cell lines. *In vivo*, the combination along with vincristine significantly enhanced the reduction of tumour size in mice implanted with EMT-6/P and EMT-6/V cell lines. According to the safety profile, the combinations of EGCG and baicalin are safe.

Conclusion: The combination of baicalin and EGCG can be optimistic in improving vincristine-resistant cells to treatments.

Keywords

cancer resistance, breast cancer resistance, baicalin, EGCG, vincristine resistance

Introduction

Breast cancer is the highest occurring cancer in women (Druesne-Pecollo et al. 2012). In 2020 there were 19.3 million new cases of cancer (Ferlay et al. 2021). Additionally, it has been estimated that the most common cancer in 2040 will be breast cancer (Rahib et al. 2021). Moreover, the estimated topmost four cancers in 2040 for male and female individuals, taking into consideration that demographic changes only are: breast, lung, prostate, and colorectal cancer (Rahib et al. 2021). By 2050 it is estimated that the incidence of female breast cancer will reach approximately 3.2 million new cases each year (Hortobagyi et al. 2005). Moreover, Sung et al. has reported that female breast cancer has exceeded lung cancer as the most frequently diagnosed cancer in 2020, with an estimated 2.3 million new cases (11.7%) followed by lung (11.4%), colorectal (10%), prostate (7.3%), and stomach (5.6%) cancers (Sung et al. 2021). These statistics indicate that breast cancer has a great impact on society worldwide and the need for urgent treatments will continually be arising. Several advances have been utilized for breast cancer management such as surgery, radiation treatment, endocrine treatment, and chemotherapy (Lukaszewicz et al. 2010). However, the efficacy of some advances such as chemotherapy has been reduced. As a result of continuous use of chemotherapy, drug resistance, which is a well-known consequence of the tolerance to the medication will be developed. This concept was first noticed when bacteria developed resistance to certain antibiotics, however, later alike mechanisms have been noticed to occur in other diseases, such as cancer and resulting in 90% breakdowns with the chemotherapy in invasion and metastasis cancers (Mansoori et al. 2017). Therefore, constant research and treatment modification should always be updated to accommodate such emerging resistance. One of the most used drugs in advanced breast cancer management is vincristine. Nevertheless, its continuous use resulted in the development of many mechanisms of resistance such as the expression of exosome-mediated transfer of chloride intracellular channel 1 (Zhao et al. 2019), overexpression of survivin *via* activation of the extracellular signal-regulated kinase 1/2 (ERK1/2), Akt strain transforming (Akt), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Tsubaki et al. 2015).

One of the most well-known practices that are used as resistance modifiers is using natural products along with chemotherapy to enhance their activity, many natural products have been studied such as epigallocatechin gallate (EGCG) which is the main polyphenol in *Camellia sinensis* or green tea, baicalin which is extracted from *Scutellaria baicalensis* and many others (Demain and Vaishnav 2011). Several studies have evaluated the anti-cancer mechanisms for both EGCG and baicalin. EGCG was reported to inhibit cancer cell proliferation by encouraging apoptosis and cell cycle arrest. In addition, EGCG can reduce tumour cell metastasis and angiogenesis (Aggarwal et al. 2022). baicalin also has an important role in the induction of apoptosis (Ma et al. 2019).

Furthermore, it has a role in the inhibition of migration, invasion, and metastasis of cancer (Wang et al. 2013).

Besides EGCG and baicalin have a starring role in decreasing the resistance of many chemotherapeutic drugs such as doxorubicin (Chen et al. 2014; Lin et al. 2021), cisplatin (Zhang et al. 2015; Yin et al. 2022) and vincristine (Chen et al. 2013b; Chen et al. 2020).

Although many studies have proved the role of using EGCG or baicalin in decreasing vincristine or other chemotherapeutics resistance, no previous studies have studied the effect of using both natural products as a combination in reducing vincristine resistance in cancer. As EGCG and baicalin have proven anti-cancer effects, we hypothesize that using them together may enhance the ultimate activity and helps in reducing vincristine resistance. In this study, we aim to evaluate the potential synergistic effect of using EGCG with baicalin in reducing vincristine resistance in breast cancer.

Materials and methods

Chemicals, cell lines and culture conditions

According to the natural products that were used, baicalin was supplied from Sigma, (St. Louis, MO, USA), and EGCG was supplied from Biosynth Carbosynth (Compton, England). Mice mammary cell line that is sensitive (EMT-6/P) and resistant to vincristine (EMT-6/V) were obtained from the European Collection of Cell Cultures (Salisbury, UK). cells were cultivated in a minimum essential (MEM) (Caisson, USA), supplemented with 50 ml of 10% fetal calf serum (Sigma, USA), 5 ml of 1% L-glutamine (Eurobio, France), 5 ml of 1% penicillin-streptomycin solution (Eurobio, France), 0.5 of 0.1% gentamycin (Sigma, USA), 0.5 ml of non-essential amino acids solution which includes: Glycine, L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, L-Proline, L-Serine (Caisson, USA). Components were added to a 500 mL bottle of MEM media (as supplied). Cells were incubated at 37 °C, with 5% carbon dioxide, and 95% humidity.

To disengage the adherent cells from the walls of the cell culture flask, trypsinization was done by using trypsin ethylene diamine tetra acetic acid (trypsin EDTA) (Eurobio, France) with phosphate buffer saline (PBS) (Eurobio, France). Cell counting was accomplished using trypan blue (0.4%) (Sigma, USA). Dimethyl sulfoxide (DMSO) (Alpha Chemika, India) was used in the MTT assay. Buffered formalin (10%) (S.D. Fine-Chem Ltd, India) was used for conserving tumours after vivisection *in vivo*.

Commercial kits

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) assay kit (Sigma, USA) was carried out to determine the percentage of cell viability. To detect apoptosis, Caspase-3 Assay (Colorimetric) Kit (Invitrogen Thermo Fisher Scientific, USA) catalog number: BMS2012INST

(128 tests) was done. Alanine transaminase (ALT) and Aspartate transaminase (AST) were measured by using ALAT and ASAT (GPT) FS* (IFCC mod.) with Pyridoxal-5-Phosphate FS (P-5-P) kit, (DiaSys Diagnostic Systems GmbH, Germany). However, creatinine was detected using Creatinine FS* (DiaSys Diagnostic Systems GmbH, Germany).

Preparation of epigallocatechin Gallate, baicalin, and vincristine working solutions

To prepare treatments for the MTT assay, baicalin (0.089 gm/ml), and EGCG (0.058 g/ml) were dissolved in 1ml medium to generate a concentration of 2000 μM as a working solution in the interest of producing a serial concentration of (1000–7.8125 μM) for single treatments in EMT-6/P and EMT-6/V cells. By reducing concentration by 50% in each serial dilution.

The positive control (vincristine) (Hospira, UK) was supplied as a stock solution of 1 mg/mL used in a single treatment experiment for both cell lines *in vitro*.

In a single treatment, in both EMT-6/P and EMT-6/V, baicalin and EGCG were prepared at a concentration of 2000 μM in a 50% reduction in serial dilution of (1000 to 7.81 μM).

In combination with treatment in EMT-6/P, EGCG was prepared at a concentration of 300 μM with a 50% reduction in serial dilution of (150 to 1.17 μM) with a fixed dose of baicalin 114 μM .

According to combination treatment in vincristine resistant cell line, EGCG was prepared at a concentration of 100 μM in 50% reduction in serial dilution of (50–0.39 μM) with a fixed dose of baicalin 70 μM .

Antiproliferative assay

The number of viable cells was detected by the exclusion method using trypan blue dye. Firstly, cells were disconnected from the wall of the cell culture flask using trypsin-EDTA and 1 mL 1X PBS. Then cells were washed with MEM, moved to a sterile centrifuge tube, and centrifuged at 1000 rpm, 4 °C for 10 min, succeeding, the supernatant was removed, and the cells were resuspended in MEM, then homogenized using the vortex. To count the cells, 100 μL of the cells sample added to 100 μL of trypan blue in Eppendorf tube, then mixed. Using hemocytometer, sample was loaded to both sides of the chamber. Later, the sample was shown under a light microscope. Just clear intense cells were counted. Calculations were employed to count the number of viable cells in the whole sample. In the end, the volume that included the desired count of cells was drawn out from the sample for proceeding with seeding.

The antiproliferative activity was detected using MTT (the tetrazolium salt, 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), a colorimetric reduction assay (Sigma, USA).

The mouse mammary cell lines (EMT-6/P and EMT-6/V) have been counted as mentioned earlier, then seeded at 10,000 cells/well into 96-well microplates supplied with growth medium for 24 h incubation. Afterward seeding

and the development of a monolayer, the media was removed, and cells were treated by increasing concentrations of EGCG (7.81–1000 μM) and different concentrations of baicalin (7.81–1000 μM). In addition, they were also exposed to increasing concentrations of vincristine (3.90–500 $\mu\text{g/ml}$) for 48 h in a single treatment experiment.

In combination treatment, EMT-6/P cells were exposed to different concentrations of EGCG (1.17–150 μM) with fixed concentrations of baicalin 114 μM .

The same procedure was done to the vincristine-resistant cells, in single treatments, the cells were treated with increasing concentrations of EGCG (7.81–1000 μM) and with different concentrations of baicalin (7.81–1000 μM). They were also exposed to vincristine (3.90–500 $\mu\text{g/ml}$) for 48 h in a single treatment experiment.

In combination treatment, EMT-6/V cells were treated addition with increasing concentrations of EGCG (0.39–50 μM), and fixed concentration of baicalin 70 μM .

Then, the media was taken out after the incubation, and a new 100 μL of the medium was added with 10 μL of the MTT solution in each well and incubated in a CO₂ incubator for 3 h, then, 100 μL of DMSO was added with incubation for 1 h, and lastly, the reduced MTT was assayed at 550 nm using a microplate reader (Biotech, Winooski, VT, USA).

Stock solutions of the compounds were solvated directly in the medium (MEM).

IC₅₀ values were calculated as the average of three replicates. Cell viability (% survival) was calculated for all treatments and compared with the negative control cells which contain only tissue culture media (untreated cells).

Calculation of inhibitory concentration (IC₅₀)

IC₅₀ is defined as the concentration of a drug needed for 50% inhibition or killing of cells compared to the untreated cells, and it is usually calculated by molar concentration. In our study, IC₅₀ values were calculated and analysed via the statistical package for the social sciences (SPSS) version 25 (Chicago, IL, US). A nonlinear regression test was applied to the data to obtain IC₅₀ values for single and combination treatments.

The combination index (CI) was calculated for EGCG and baicalin against the two cell lines (EMT-6/P and EMT-6/V) by using an equation published previously (Ichite et al. 2009b).

$$CI = \frac{(D) 1 / (Dx) 1 + (D) 2 / (Dx) 2 + a (D) 1 (D) 2}{(Dx) 1 (Dx) 2}$$

Where: (Dx) 1 = IC₅₀ of EGCG alone, (D) 1 = IC₅₀ of EGCG in combination with baicalin, (Dx) 2 = IC₅₀ of baicalin alone, (D) 2 = IC₅₀ of baicalin in combination with EGCG. a = 0 for mutually exclusive or 1 for mutually non-exclusive interaction, depending on the literature review, both EGCG and baicalin exert their anticancer effect by different mechanisms of action. Hence, we applied the mutually nonexclusive model, where $\alpha = 1$, in CI calculations.

CI values are explained according to the following:

If $CI > 1.3$ that indicates antagonism, $CI = 1.1-1.3$ indicates moderate antagonism, $CI = 0.9-1.1$ represents additive effect, $CI = 0.8-0.9$ means slight synergism, $CI = 0.6-0.8$ means moderate synergism, $CI = 0.4-0.6$ reveals synergism, and $CI = 0.2-0.4$ represents strong synergism (Ichite et al. 2009a).

Calculation of resistance fold

Resistance fold is a term used to determine the times of change in the needed concentration to obtain 50% killing in the resistant cell line in contrast with the sensitive cell line. In this study, we calculated the resistance fold by comparing the values of IC_{50} between the resistance EMT-6/V and the sensitive EMT-6/P cell lines using the following formula (Wang et al. 2010):

$$\text{Resistance fold} = \frac{IC_{50} \text{ of Resistant Cell Line}}{IC_{50} \text{ of Parental Cell Line}}$$

MTT assay was carried out to find IC_{50} values of baicalin, EGCG, their combination, and vincristine for both cell lines.

Colorimetric assay of caspase-3 activity in EMT-6/V cells

The examined cell line (EMT-6/V) was cultured. Afterward, the cultured cells were incubated at 37 °C, 5% CO_2 , and 95% humidity to permit the formation of confluent layers overnight. Cells were disconnected from the walls of the flasks using trypsin-EDTA and PBS and incubated for 2–3 min. After that, cells were washed with MEM, transferred to sterile centrifuge tubes, and centrifuged at 1000 rpm at 4 °C for 10 min. Cells (pellets) were resuspended in 5 mL MEM and cell counting was conducted. Seeding of the cells was performed in 75 cm^2 flask, at a concentration of 100,000 cells/mL. Five flasks were prepared, and they incubated for 24 h for proper adhesion and proliferation. At that instant, the media was removed and replaced with the treatments dissolved in new media. EMT-6/V flasks were treated with a single treatment of EGCG 125 μM , a single treatment of baicalin 175 μM , a combination treatment of baicalin and EGCG as 140 and 100 μM respectively, vincristine 643 $\mu g/ml$, and control with only MEM as the negative control. Following, the flasks were incubated for 48 h. After the incubation period, the old media have been removed from all flasks and discarded, then the flasks were washed by using PBS, followed by adding trypsin to each flask to detach the cells. Then we added 4 ml of fresh media to the trypsin and the whole 5 ml was transferred to centrifugation type. and centrifuged at 1000 rpm at 4 °C for 10 min. Cells (pellets) now the pellets are ready for the Caspase-3 activity assay.

The lysis buffer was prepared depending on the kit instruction, followed by adding 1ml of the lysis buffer to every 5×10^6 cells. Then the tubes were incubated for 1 hour

at room temperature with gentle shaking. Then the tubes were centrifuged at 1000 \times for 15 minutes. Afterward, distilled water was added to all wells including blank, standard, and samples as 100 μL , 100 μL , and 140 μL respectively. After that, 10 ml of each sample was added to the wells in duplicates, then the plate was covered and incubated at room temperature for 3 hours on the microplate shaker. After three hours, the microwell strips were washed 6 times by using the washing buffer, which was previously prepared referring to the kit instruction, each wash was done by adding 140 μL to each well including blank, standard, and samples. After the last wash, the microwell strip was tapped on a paper towel to remove the excess buffer. Next, 100 μL of substrate solution (tetramethyl-benzidine) was added to all wells. After that, the microplate was incubated at room temperature for nearly 10 minutes until the highest standard developed a dark blue colour. Subsequently, 100 μL of the stopping solution (1M phosphoric acid) was added to each well. Then the plate was directly read by a Spectro-photometer using 450 nm wavelength.

Mice

In this study, 82 female mice of Blab/C strain aged from 4–6 weeks with weight ranging from 21 to 25 g/mouse weight were used. Mice were provided by the animal house in the Applied Science Private University, Amman, Jordan. Animals were kept in separate cages with bedding of wooden shavings. The provided conditions in the animal house were stable temperature at 25 °C, 50% to 60% humidity, continuous air ventilation, and alternating light/dark cycles of 12 h. Mice were handled gently by the base of tail during the conduction of the experiments, routinely monitored for their health status, and finally euthanized by cervical dislocation and disposed appropriately after the end of the experiments.

Establishing regimens for the *in vivo* experiment

We accomplished the *in vivo* part on female Balb/C mice bearing EMT-6/P and EMT-6/V tumors. Doses of baicalin, EGCG, and vincristine were chosen depending on studies available in the literature. Baicalin, EGCG, and vincristine doses were used based on previous literature.

The baicalin dose was 75 mg/kg/day for 10 days (Peng et al. 2015; Gao et al. 2017). While the EGCG dose was 50 mg/kg/day for 10 days (Luo et al. 2017; Gu et al. 2018). On the other hand, the vincristine dose was 2mg/kg/every other day prepared as 0.5 mg/ml (1 ml of the stock with 1 ml PBS) every other day for 10 days (Talib 2020). Baicalin, EGCG, and vincristine were given intraperitoneally.

Tumor inoculation and antitumor activity *in vivo*

EMT-6/V and EMT-6/P cells were thawed, cultured, detached, counted, and seeded using MEM then they were

left to grow for 24 h. Exponentially growing EMT-6/P and EMT-6/V cells were harvested by trypsinization and were washed and resuspended in MEM, at a density of 1.5×10^6 cells / mL. After that, viability was spotted using the trypan blue exclusion method. A tumor induction dose of 1.5×10^5 cells in 0.1 mL medium was injected into the abdominal area of each female BALB/C mouse subcutaneously. Then injected cancer cells were left for 14 days to grow and form new tumors, then at day 15 tumor-bearing mice received the treatments. A digital caliper was used to measure tumor dimensions and the following formula was used to calculate tumor volumes (Wang et al. 2010):

$$V = (L * W * W) / 2$$

Where L acts as the length of the longest aspect of the tumor and W acts as the length of the aspect perpendicular to L.

Tumors of similar sizes were chosen and the average tumor volume for all groups approximately matched. 42 mice were inoculated with EMT-6/P and other 42 mice incubated with EMT-6/V. The treatments started 14 days after tumor inoculation. 82 tumor-bearing mice were used in this investigation, and mice were divided into six groups (n = 7 for each group) for each cell line, except for two groups (n = 6) because two mice died during the tumor inoculation Fig. 1.

Group 1 was used as a negative control and exposed to intraperitoneal injection of vehicle (10 ml phosphate-buffered saline (PBS), and 10 drops of tween (20%) and injected

as 0.1 mL daily for 10 days. Group 2 was treated with Baicalin (75 mg/kg/day) by intraperitoneal injections. Group 3 was treated with EGCG with a daily dose of (50 mg/kg/day) administered intraperitoneally. Group 4 was exposed to a combination of baicalin and EGCG at a dose (75 and 100 mg/kg/day respectively) injected intraperitoneally. Group 5 was treated with vincristine at a dose (2 mg/kg/every other day) injected intraperitoneally. Group 6 was treated with the combination therapy of EGCG (50 mg/kg/day), baicalin (75 mg/kg/day), and vincristine (2 mg/kg/every other day) intraperitoneal injection. The treatment lasted for 10 days, and blood samples were collected at three-time points over the treatments on days 0, 3, and 6 Fig. 1. Tumour volumes were measured at three-time points: days 0, 3, and 6 during the treatment period. The percentage of change in tumour volumes comparing initial and final volumes was calculated using the following equation:

$$\% \text{ Tumour change} = ((F-I) / I) * 100\%$$

Where: F, I represent the final and initial tumor volumes, respectively. On days 0, 3 and 6 of treatment, blood samples were withdrawn by retroorbital method for each group, and on day ten mice were sacrificed by cervical dislocation. Tumors were removed, weighed, and stored in 10% formalin to preserve their morphology. Blood samples were centrifuged at 5000 rpm for 10 min. The resulting serum was transferred to a new pre-labelled Eppendorf tube for each sample. Serum samples were stored at -20 °C for the next experiments.

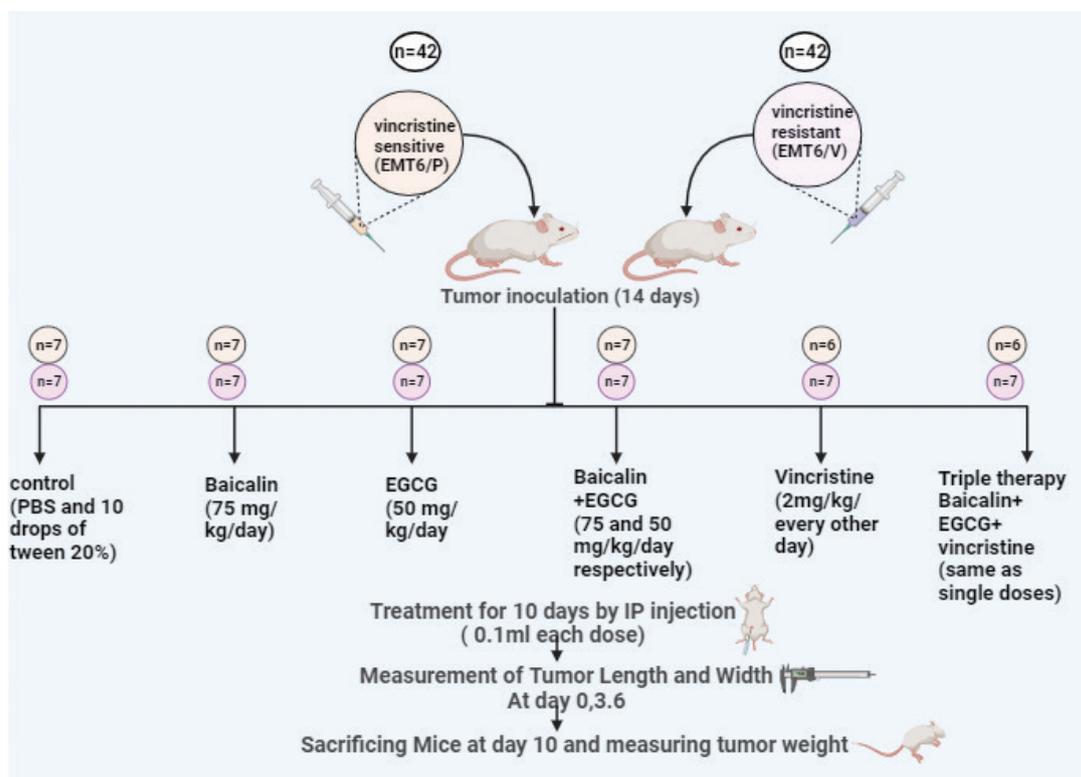


Figure 1. *In vivo* experiment: groups of EMT-6/P and EMT-6/V inoculated mice, Treatment approaches were: baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), and vincristine (2 mg/kg/every other day). The same concentrations were used in the combination treatment and triple therapy.

Evaluation of liver function in treated mice

Level of toxicity exerted on the liver by the used treatments was measured, serum levels activity of the liver enzymes alanine transaminase (ALT) and aspartate aminotransferase (AST), were evaluated for baicalin, EGCG, their combination, vincristine, combination with vincristine, in addition to the negative control group and normal healthy group. After serum samples collection, ALT and AST were statistically analysed.

According to the kit mentioned earlier, reagents were mixed following the protocol's directions to produce functional reagents. Working reagents were then incubated at 37 °C to bring the reaction's optimal temperature to a close. A single-use cuvette was then filled with 100 l of each sample and 1 mL of the working reagent. The initial absorbance at time zero was noted after one minute of incubation. The absorbance was measured at 0, 1, 2, and 3 minutes. The spectrophotometer was synchronized to read absorbance at $\lambda = 340$ nm and calibrated to zero absorbance using distilled water. Additionally, working reagents were utilized as blanks.

Evaluation of kidney function in treated mice

levels of toxicity exerted on the kidney by the used treatment were measured, serum levels of creatinine were evaluated for baicalin, EGCG, their combination, vincristine, the combination with vincristine, in addition to the negative control group and normal healthy group. After serum samples collection, creatinine was analysed by Statistical analysis. The method behind creatinine measurement is Kinetic test without deproteinization, according to the Jaffé method Creatinine forms a coloured orange-red complex in an alkaline picrate solution. The difference in absorbance at fixed times during conversion is proportional to the concentration of creatinine in the sample. According to the kit mentioned earlier, standard (S) was ready to be used, reagents were mixed following the instructions to produce functional reagents. The working reagent was then incubated for a further period at 37 °C to precisely determine the reaction temperature. In a disposable cuvette, 100 l of each sample was combined with 1 ml of the working solution, and 30 and 90 seconds later, absorbance readings were collected. The spectrophotometer was calibrated to read absorbance at 505 nm and set to zero absorbance using distilled water. Working reagents were further used as blank samples.

Data analysis

The data created was managed in Microsoft Excel, and statistical analysis was carried out using GraphPad Prism 8 software and SPSS (Statistical Package for the Social Science, Chicago, IL, USA 25). SPSS had been used using nonlinear regression to calculate the IC_{50} values for baicalin, EGCG, their combination, and vincristine, in

EMT-6/P and EMT-6/V cell lines. While GraphPad Prism 8 software had been used to determine the statistical significance between groups, a one-way analysis of variance (ANOVA) was utilized for *in vivo* results, kidney, and liver results, if the data were normally distributed. If not, the Kruskal Wallis test was done. For caspase activity analysis, GraphPad Prism 8 was also used to draw the standard curve and to predict the concentrations. P-value less than 0.05 considered statistically significant. Results were expressed as (mean \pm SEM).

Results

In vitro results

Anti-proliferative effect of Epigallocatechin Gallate and Baicalin single treatment on both EMT-6/P and EMT-6/V cell lines

To evaluate the effect of a single treatment of baicalin and EGCG, an MTT assay was accomplished on vincristine-sensitive parent EMT-6 breast cancer cells (EMT-6/P) and EMT-6 vincristine-resistant breast cancer cell line (EMT-6/V). In comparison with the vehicle control, baicalin and EGCG attenuated cell proliferation in a concentration-dependent fashion in both cell lines as single treatments. Our results showed that EMT-6/P presented higher survival rates in comparison with EMT-6/V when it was subjected to similar concentrations of baicalin and EGCG shown in Figs 2, 3 respectively. For baicalin Values of IC_{50} were 228.02 ± 7.47 μ M and 143.70 ± 2.46 μ M in EMT-6/P and EMT-6/V, respectively. While in EGCG, the IC_{50} were 300.00 ± 12.39 and 109.29 ± 2.45 μ M in EMT-6/P and EMT-6/V, respectively.

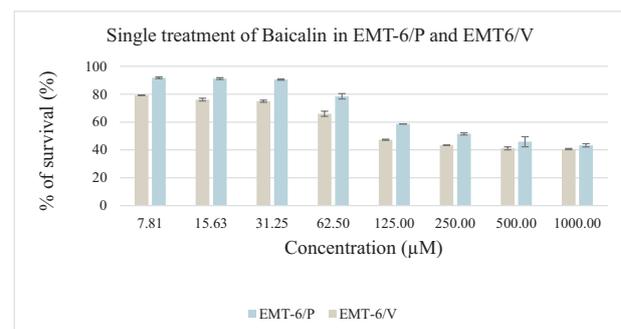


Figure 2. Anti-proliferative effect of baicalin in single treatment against EMT-6/P and EMT-6/V cell lines.

Anti-proliferative effect of vincristine on EMT-6/P and EMT-6/V cell line

The anti-proliferative assay (MTT) was conducted to evaluate the vincristine-mediated anti-proliferative effect on EMT-6/P and EMT-6/V cell lines. We studied the sensitivity of these paired cell lines to vincristine by exposing them to a range of vincristine concentrations (500–3.9 μ g/ml) for 48 h. Then, the vincristine concentration required for the 50% inhibition of cell growth (IC_{50}) was calculated,

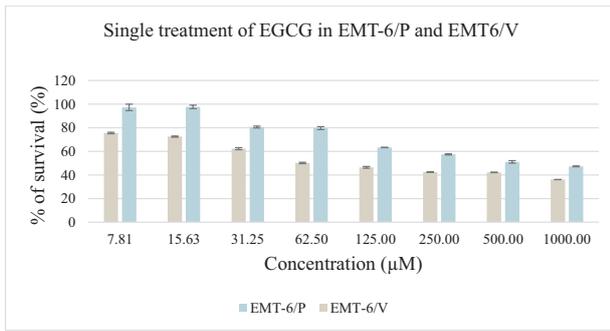


Figure 3. Anti-proliferative effect of EGCG in single treatment against EMT-6/P and EMT-6/V cell lines.

as detected, it was 177.58 μg/ml for the EMT-6/P cell line and 449.33 μg/ml for EMT-6/V cell line. After that, the resistance fold was calculated using the previously mentioned equation and results showed that EMT-6/V was (2.53) times more resistant to vincristine against EMT-6/P cells, Fig. 4.

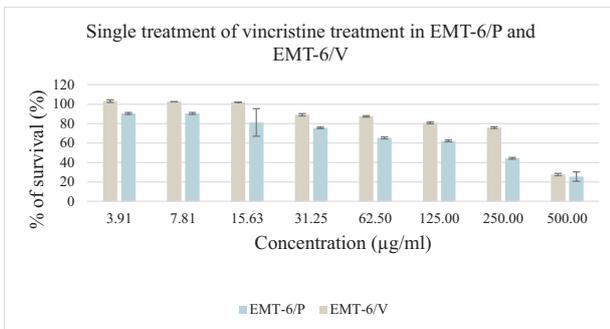


Figure 4. The sensitivity of EMT-6/P and EMT-6/V cell lines to vincristine was measured by testing decreasing concentrations of vincristine using the anti-proliferative assay (MTT).

Anti-proliferative effect of Epigallocatechin Gallate and Baicalin combination treatment on EMT-6/P, EMT-6/V cell lines.

To examine the inhibitory effect of the combined treatment of EGCG and baicalin, EMT-6/P and EMT-6/V cell lines were treated with different concentrations of EGCG and a fixed concentration of baicalin for 48 h. MTT results exposed that baicalin combined with EGCG significantly reduced cell viability in a concentration-dependent manner in both cell lines. Shown in Figs 5, 6. Moreover, surprisingly lower doses were required to acquire 50% killing in combination treatment. Based on observations, it was noticed that EMT-6/V cells displayed greater susceptibility to the combination treat-

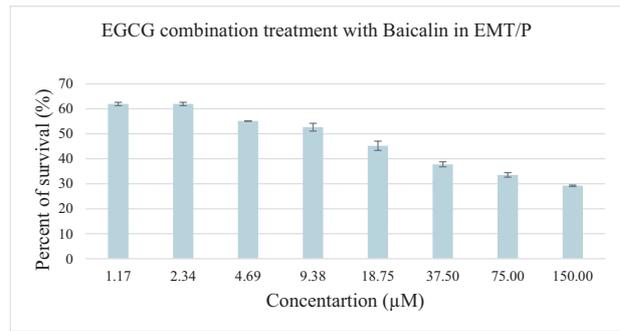


Figure 5. Anti-proliferation effect of EGCG and baicalin in combination treatment against EMT-6/P different doses of EGCG with a fixed dose of baicalin (114 μM).

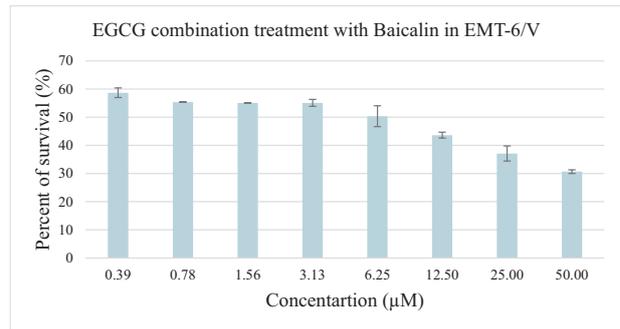


Figure 6. Anti-proliferation effect of EGCG and baicalin in combination treatment against EMT-6/V different doses of EGCG with a fixed dose of baicalin (70 μM).

ment at lower doses compared to EMT-6/P cells. The (IC₅₀) values for EGCG in the combination treatment were 11.6 ± 1.47 μM and 11.36 ± 2.83 μM for EMT-6/P and EMT-6/V cells, respectively. These findings indicate that EMT-6/V cells were more responsive to the combination treatment than EMT-6/P cells.

Combination index (CI) calculations

Combination therapy is frequently used nowadays to lessen drug side effects, prevent the development of drug resistance, and improve favourable benefits while using lower doses.

Several doses of baicalin, EGCG, their combination, and vincristine are administered to both cell lines using the MTT test. using a dose-effect experiment to acquire the IC₅₀ values that allow for the calculation of the CI using the aforementioned equation and the explanation that follows. The combined therapy exhibited a strong synergistic impact on EMT-6/P and EMT-6/V cells, with a CI of (0.52) and (0.63), respectively, according to the analysis of the combination test, Table 1.

Table 1. IC₅₀ values for EMT-6/P and EMT-6/V cell lines along with the combination index, related interpretation, and resistant folds.

Cell line	IC ₅₀ of Baicalin (μM)	IC ₅₀ of EGCG (μM)	IC ₅₀ of vincristine (μg/ml)	EGCG in combination with Baicalin (μM)	Baicalin IC ₅₀ in combination (μM)	CI	Interpretation
EMT-6/P	228.02	300.00	177.58	11.60	111.5	0.52	Synergism
EMT-6/V	143.70	109.29	449.33	11.36	70	0.63	Moderate Synergism
Resistance folds	0.63	0.36	2.53	0.98	0.63		

Resistance fold

MTT assay was carried out to compare IC₅₀ values of baicalin, EGCG, their combination, and vincristine treatments for both cell lines, to evaluate the resistance of EMT-6/V to these treatments. The mean IC₅₀ values were reported in Table 1, known here as the concentration of drug needed for 50% inhibition of viable cell number *in vitro*.

The concentration of baicalin required to achieve 50% of inhibition was 228.02 μ M and 143.70 μ M for EMT-6/P and EMT-6/V, respectively, with a resistance fold of 0.63, which means that EMT-6/V cells were 0.63 times more resistant to baicalin than EMT-6/P. While EGCG IC₅₀ values were 300.00 μ M and 109.29 μ M for EMT-6/P and EMT-6/V, respectively. With a resistance fold of 0.36, it means that EMT-6/V cells were 0.36 times more resistant to the EGCG than EMT-6/P. The IC₅₀ values of vincristine were 177.58 μ g/ml and 449.33 μ g/ml for EMT-6/P and EMT-6/V, respectively, and this means that EMT-6/V cells were 2.53 times more resistant to vincristine in contrary to EMT-6/P.

While for the combination, when used different concentrations of EGCG and fixed doses of baicalin, were EGCG, the resistant fold increased from 0.36 to 0.98.

The resistance fold was calculated by dividing the IC₅₀ value of the baicalin, EGCG, and vincristine in the resistant EMT-6/V cell line by the IC₅₀ value of the extract for the sensitive EMT-6/P cell line.

Effect of Epigallocatechin Gallate, Baicalin, their combination, and vincristine on caspase-3 levels in EMT-6/V cells.

The caspase concentrations resulting from the treatment of baicalin, EGCG, their combination, vincristine, and positive control in EMT-6/V were measured. The total volume of treatment was 13 ml. Media has been used to complete the volume of treatments. The concentrations used in treatment were 175 μ M for baicalin single treatment, 125 μ M for EGCG single treatment, 140 for baicalin μ M, and 100 μ M for EGCG in combination treatment, vincristine 643 μ g/ml, and the positive control was treated only with 13 ml MEM. Caspase concentrations is shown in Figs 7, 8.

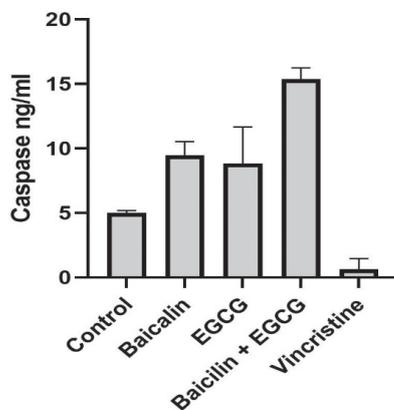


Figure 7. Caspase concentrations in (ng/ml) for each treatment group in EMT-6/V. Concentrations used were: baicalin 175 μ M, EGCG 125 μ M, baicalin and EGCG 140 and 100 μ M respectively, vincristine 643 μ g/ml, and positive control treated with MEM.

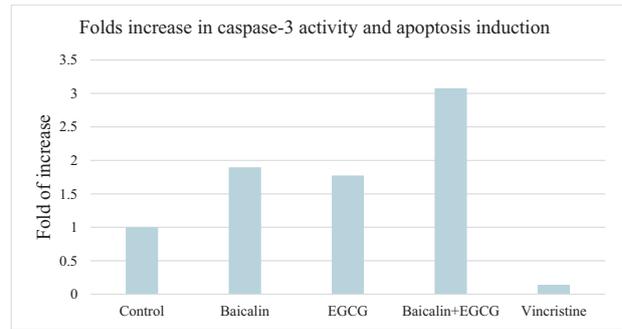


Figure 8. Folds increase in caspase-3 activity and apoptosis induction, concentrations used were: baicalin 175 μ M, EGCG 125 μ M, baicalin, and EGCG 140 and 100 μ M respectively, vincristine 643 μ g/ml and positive control treated with MEM in EMT-6/V.

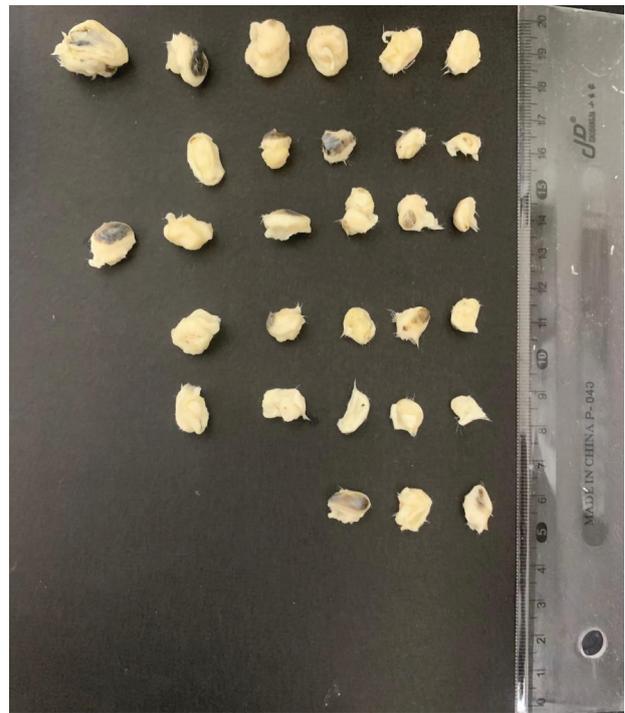


Figure 9. Tumor sizes of EMT-6/P after dissection at day 10 in all groups compared to each other, n = 7 except for the last two groups n = 6.

In vivo results

Antitumor effects of Baicalin, Epigallocatechin Gallate, their combination, vincristine, and vincristine together with the combination on tumor size implanted in mice on EMT-6/P and EMT-6/V

After tumour inoculation for 14 days, and mice treatments for 10 days, mice were sacrificed, and the tumour size was detected. The change in volume (mm³) and % of the change in tumor volume (%) were calculated for each treatment group, then analysed for the statistical significance shown in Figs 13, 14. All groups were compared to the positive control group also all groups were compared to each other. Only statistically significant associations are shown in the figures.

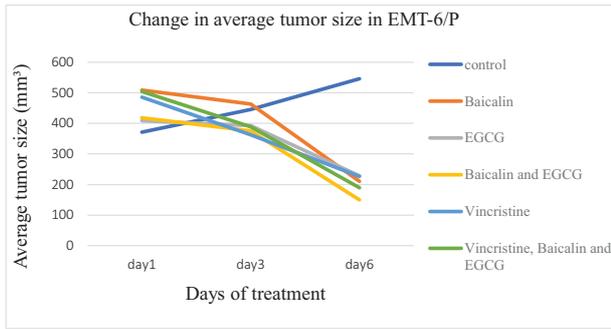


Figure 10. A plot of change in average tumor size (mm³) vs. time in (days) of treatment in the EMT-6/P cell line.

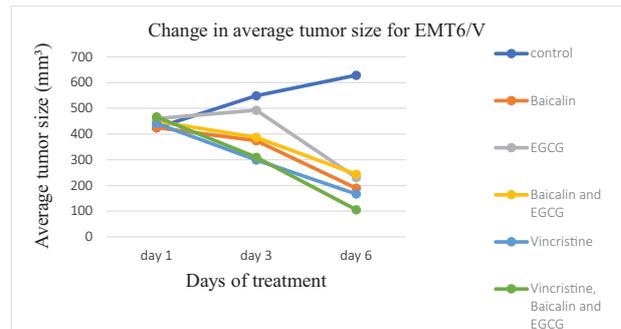


Figure 12. A plot of change in average tumor size (mm³) vs. time in days of treatment in EMT-6/V cell line.



Figure 11. Tumor sizes in EMT-6/V after dissection at day 10 in all groups compared to each other, n = 7.

In EMT-6/P, using the % change in tumour size as an indicator, the treatments using baicalin, baicalin along with EGCG, and the triple therapy which includes baicalin, EGCG, and vincristine showed a significant difference (p-value < 0.05), Fig. 14. Almost the same results were detected when the change in volume indicator was used, all the previous groups showed a significant difference in addition to the vincristine treatment Fig. 13.

Table 2. Results of baicalin, EGCG, their combination, vincristine, and vincristine together with the combination on tumour size changes, percentages of change in tumour size and average tumour weight in EMT-6/P cell line (n = 7 except last two groups were 6).

Treatment group ENT-6/P	Av. initial tumor size (mm ³)	Av. final tumor size (mm ³)	%-Change in tumor size (%)	Mice with no detectable tumor (%)	Av. tumor weight (gm)
Positive control	371.30	546.0018	47.04	0	1.02
Baicalin	508.83	210.44	58.64	28.57	0.22
EGCG	408.87	228.45	-44.12	28.57	0.36
Baicalin and EGCG	418.27	149.82	-64.18	42.85	0.43
Vincristine	485.77	226.43	-53.38	33.33	0.46
Vincristine, Baicalin and EGCG	504.20	189.51	-62.41	50.00	0.20

Abbreviations: Av. = Average, mm³ = cubic millimetre.

The triple therapy using baicalin, EGCG, and vincristine accomplished the highest percentage of cured tumors (50%) compared to other treatment groups. Followed by the group that was treated with a combination of baicalin and EGCG with a 42.85% cured percentage. Shown in Table 2. As shown in Fig. 10 the tumour size had decreased during the treatment except for the positive control group.

As such, the same treatment approaches were applied to EMT-6/V, as observed in Table 2.

Using the change in tumour size as a parameter, baicalin, baicalin and EGCG, and the triple therapy which includes baicalin, EGCG, and vincristine, reduced the tumour size in a considerable way (P-value < 0.05) compared to the positive control, represented in Fig. 13.

While only the triple therapy showed a significant reduction if the parameter percent change in tumour size was used in Fig. 14.

The triple therapy showed the highest curable percentage 57.14% among all the other groups. Table 2.

Liver toxicity evaluation in mice

We conducted an ALT and AST assay since these enzymes are thought to be indicators of liver injury, hence, blood levels of liver enzymes were assessed in all treated groups in both cell lines. Serum samples of mice treated with baicalin, EGCG, their combination, vincristine, and the combination with vincristine were collected. In addition, samples were collected from negative (untreated) and normal-untreated mice which did not have any tumour.

The average serum ALT for the treatments in EMT-6/P and EMT6/V is shown in Figs 15, 16 respectively. The average AST for the treatments in EMT-6/P and EMT6/V is shown in Figs 17, 18 respectively.

Table 3. Results of baicalin, EGCG, their combination, vincristine, and vincristine together with the combination on tumor size changes, percentages of change in tumour size and average tumour weight in EMT-6/V cell line (n = 7).

Treatment group ENT-6/V	Av. initial tumor size (mm ³)	Av. final tumor size (mm ³)	%Change in tumor size (%)	Mice with no detectable tumor (%)	Av. tumor weight (g)
Positive control	424.15	628.58	48.19	14.28	0.70
Baicalin	425.16	189.53	-55.42	42.85	0.33
EGCG	459.89	231.12	-49.74	42.85	0.64
Baicalin and EGCG	449.34	243.89	-45.72	28.57	0.31
Vincristine	441.41	166.91	-62.18	42.85	0.30
Vincristine, Baicalin and EGCG	466.55	105.87	-77.30	57.14	0.18

Abbreviations: Av. = Average, mm³ = cubic millimetre.

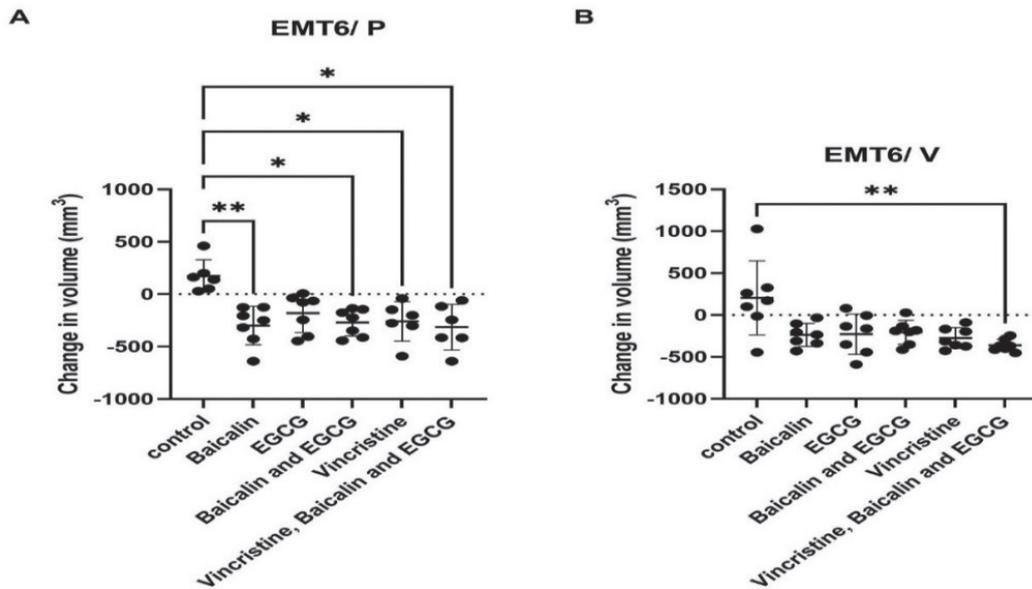


Figure 13. The volume change (mm³) for treatment groups *in vivo* for EMT-6/P and EMT-6/V. Only statistically significant associations are shown. Positive control (PBS and tween 20%), baicalin 75 mg/kg/day, EGCG: 50 mg/kg/day, baicalin and EGCG combination 75 and 50 mg/kg/day respectively, vincristine 2mg/kg/every other day, and same doses were used in the triple therapy.

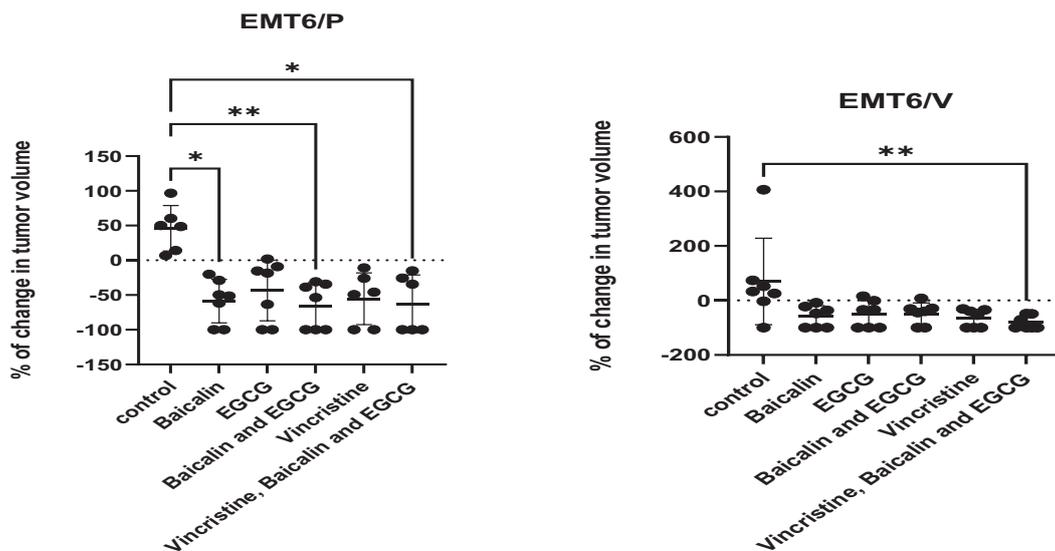


Figure 14. The volume change (mm³) for treatment groups *in vivo* for EMT-6/P and EMT-6/V. Only statistically significant associations are shown. Positive control (PBS and tween 20%), baicalin 75 mg/kg/day, EGCG: 50 mg/kg/day, baicalin and EGCG combination 75 and 50 mg/kg/day respectively, vincristine 2mg/kg/every other day, and same doses were used in the triple therapy.

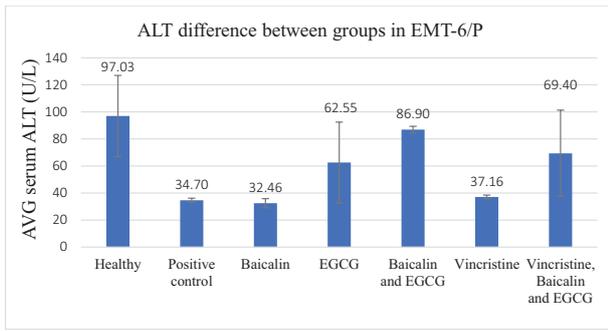


Figure 15. Effect of baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2 mg/kg/every other day) for vincristine, positive control (PBS and tween 20%), and healthy group on serum ALT level measured by (IU/L) in EMT-6/P.

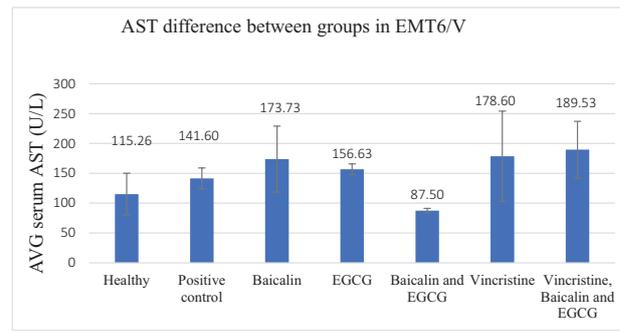


Figure 18. Effect of baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2 mg/kg/every other day) for vincristine, Positive control (PBS and tween 20%), and healthy group on serum AST average level measured by (IU/L) in EMT-6/V.

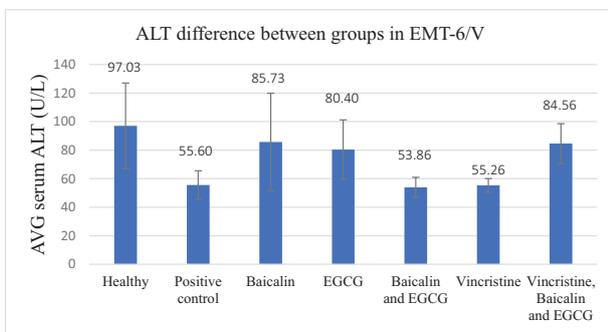


Figure 16. Effect of baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2 mg/kg/every other day) for vincristine, positive control (PBS and tween 20%), and healthy group on serum ALT level measured by (IU/L) in EMT-6/V.

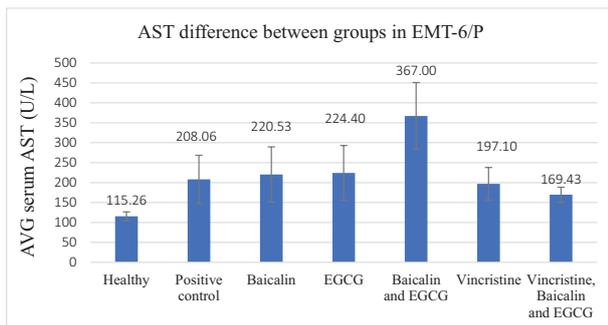


Figure 17. Effect of baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2 mg/kg/every other day) for vincristine, Positive control (PBS and tween 20%), and healthy group on serum AST average level measured by (IU/L) in EMT-6/P.

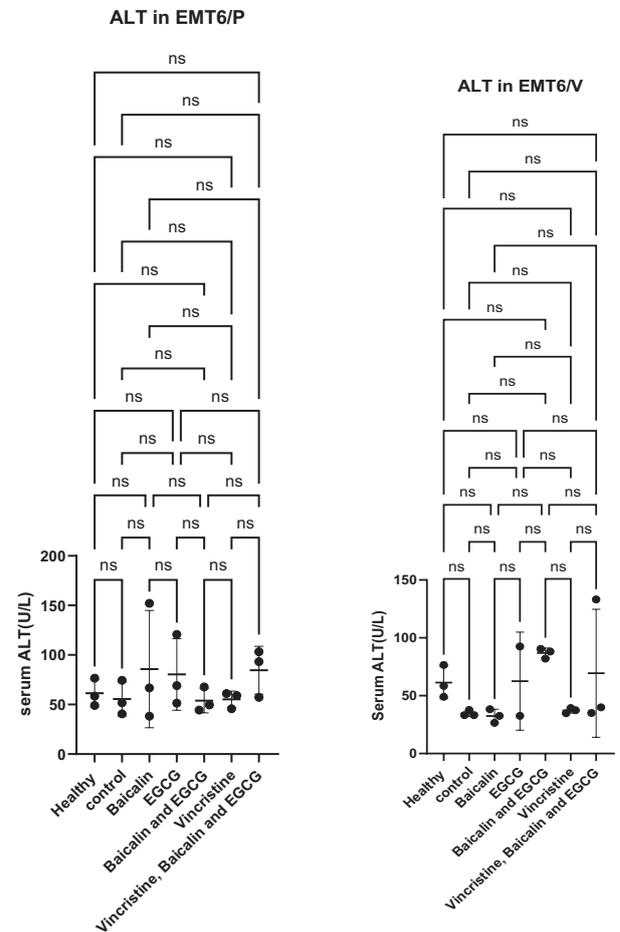


Figure 19. ALT serum level (U/L) in different treatment groups. Concentrations used baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2 mg/kg/every other day) for vincristine, positive control (PBS and tween 20%), and healthy group in EMT-6/P and EMT-6/V. Including the statistical correlations between groups.

The results of the treatment groups were compared to each other and compared to positive control mice which has a tumour but were not treated also with healthy mice which did not have any tumours. All treatments' ALT and AST serum levels were non-significant compared to the positive control group and healthy group (P -value > 0.05), Figs 19, 20.

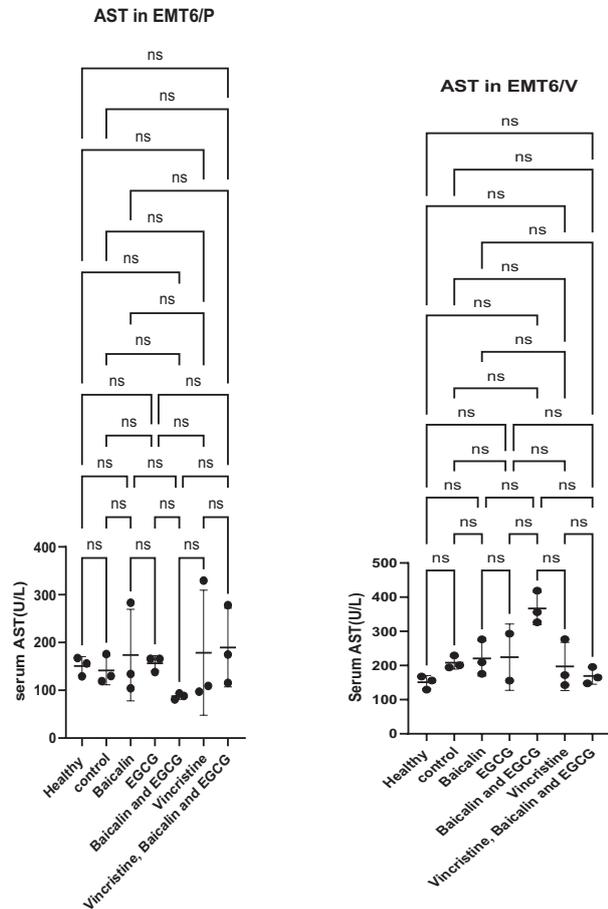


Figure 20. AST serum level (U/L) in different treatment groups. Concentrations used baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2 mg/kg/every other day) for vincristine, positive control (PBS and tween 20%), and healthy group in EMT-6/P and EMT-6/V. Including the statistical correlations between groups.

Kidney toxicity evaluation in mice

Creatinine levels were detected in the treatment groups as mentioned above in addition to positive control and healthy groups of mice. To evaluate the toxicity of treatments on the kidneys. The average serum creatinine for both cell lines, EMT-6/P and EMT-6/V are shown in Figs 21, 22.

All groups were compared together, including between the treatment groups with positive control and healthy mice. All treatment groups were non-significantly correlated to the positive control and healthy groups (p -value > 0.05), Fig. 23.

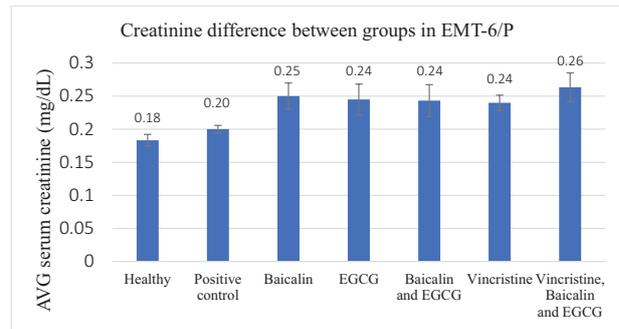


Figure 21. Effect of baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2mg/kg/every other day) for vincristine, positive control (PBS and tween 20%), and healthy group on average creatinine measured by (mg/dl) in EMT-6/P.

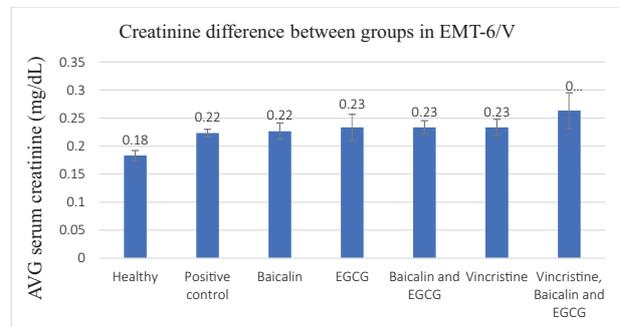


Figure 22. Effect of baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2 mg/kg/every other day) for vincristine, positive control (PBS and tween 20%), and healthy group on average creatinine measured by (mg/dl) in EMT-6/V.

Table 4, 5 summarise the treatment's effects on ALT, AST, and creatine levels in EMT-6/P and EMT-6/V respectively.

Discussion

Breast cancer is the most common cancer diagnosed worldwide, also, it has replaced lung cancer as the most detected cancer worldwide (Arnold et al. 2022). There were 2.3 million new cases and 685.000 deaths in 2020, and it is estimated that by 2040, there will be 3 million new cases and 1 million deaths (Arnold et al. 2022). Also, according to the American Cancer Society (ACS), breast cancer represents one in three of all new female cancers annually. Furthermore, ACS also reported that there is a 13% average risk for women in the USA to develop breast cancer in their lifetime, based on these statistics, it is always important to discover new treatments to keep pace with this burden.

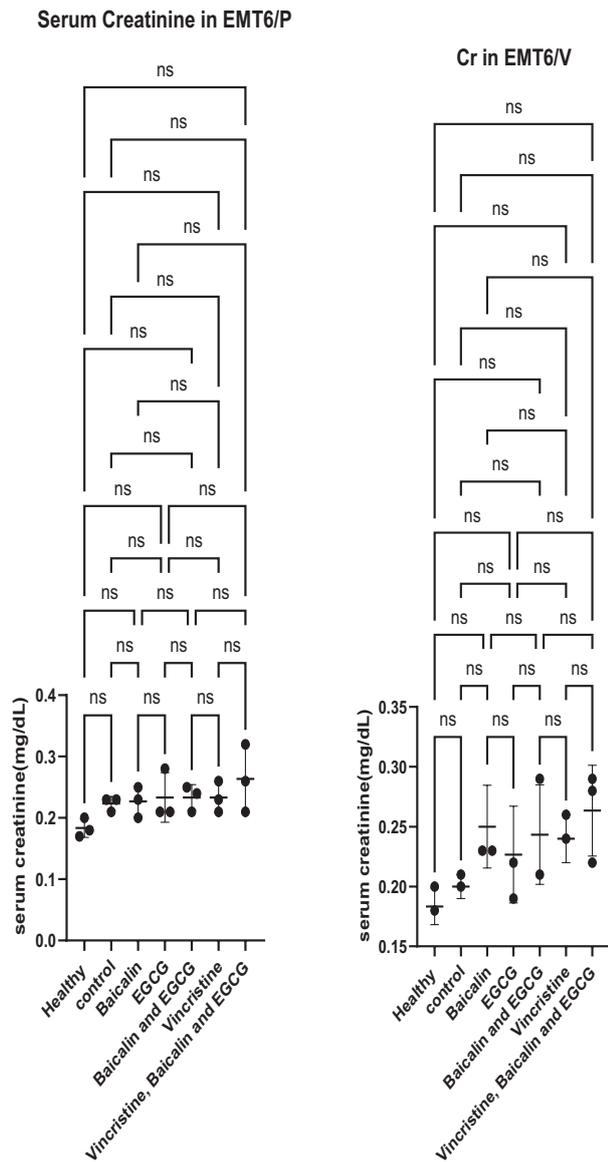


Figure 23. Creatinine serum levels (mg/dl) in different treatment groups. Concentrations used baicalin (75 mg/kg/day), EGCG (50 mg/kg/day), a combination of baicalin and EGCG (75 and 50 mg/kg/day) respectively, vincristine 2 mg/kg/every other day, combination with vincristine (75 and 50 mg/kg/day) for combination and (2 mg/kg/every other day) for vincristine, positive control (PBS and tween 20%), and healthy group in EMT-6/P and EMT-6/V. Including the statistical correlations between groups.

Using natural products in cancer treatment is not a new idea, natural products were and still an important source for cancer treatment. It was reported that 60% of the present anticancer drugs were obtained in some manner from natural sources (Newman and Cragg 2012). This high percentage is attributed to natural product diversity and safety (Cragg and Pezzuto 2016). Various natural products had showed a surprising effect in fighting cancer, especially the resistant ones (Kumar and Jaitak 2019). Many studies reviewed the effect of various natural products on different types of cancers (Talib et al. 2022b). Furthermore, it goes

Table 4. Serum ALT, AST, and Cr levels for groups with different treatments, control treated with only PBS and tween 20%, and healthy mice in EMT-6/P.

Treatment groups	ALT (IU/L)	AST (IU/L)	Cr (mg/dl)
Healthy	97.03 ± 29.99	115.26± 34.88	0.18± 0.008
Positive control	34.70 ± 1.50	208.06 ± 60.37	0.20 ± 0.005
Baicalin	32.46 ± 3.40	220.53 ± 69.02	0.25 ± 0.02
EGCG	62.55 ± 29.95	224.40 ± 68.60	0.22 ± 0.02
Baicalin and EGCG	86.90 ± 2.48	367.00 ± 84.00	0.24 ± 0.02
Vincristine	37.16 ± 1.30	197.10 ± 40.81	0.24 ± 0.01
Baicalin, EGCG and Vincristine	69.40 ± 31.93	169.43 ± 18.92	0.26 ± 0.02

Note: No statistical significance was reported.

Table 5. Serum ALT, AST, and Cr levels for groups with different treatments, positive control treated with only PBS and tween 20%, and healthy mice in EMT-6/V.

Treatment groups	ALT (IU/L)	AST (IU/L)	Cr (mg/dl)
Healthy	97.03 ± 29.99	115.26± 34.88	0.18± 0.008
Positive control	55.60 ± 9.94	141.6 ± 17.41	0.22 ± 0.006
Baicalin	85.73±34.18	173.73 ± 55.36	0.22 ± 0.01
EGCG	80.40 ± 20.82	156.63 ± 9.16	0.23± 0.02
Baicalin and EGCG	53.86 ± 7.07	87.50 ± 3.74	0.23 ± 0.01
Vincristine	55.26 ± 4.81	178.60 ± 75.57	0.23 ± 0.01
Baicalin, EGCG and Vincristine	84.56 ± 13.97	189.53 ± 47.54	0.26 ± 0.03

Note: No statistical significance was reported.

beyond using natural products alone, besides many studies reviewing combination therapy in cancer treatment (Talib et al. 2022a). Two of the most well-studied natural products in cancer treatments are baicalin and EGCG (Wang et al. 2013; Wang et al. 2018a; Yang et al. 2020; Singh et al. 2021). These two natural products also showed astonishing activity in reducing the resistance of many anti-cancer drugs (Xu et al. 2017; Wang et al. 2023). As these two natural products have multiple activities in cancer, it is expected that baicalin and EGCG may potentiate each other if they were used as a combination therapy. Although many studies have proven their role as an anticancer, this is the first study to test them as a combination therapy in cancer, particularly breast cancer. Moreover, this is the first study to test this combination on decreasing the resistant specifically, vincristine-resistant cancer cells. In this study, we evaluated the effect of baicalin, EGCG, and the combination, on EMT-6/P and EMT-6/V breast cancer cell lines, *in vitro* and *in vivo*. The results showed a promising effect in reducing the cancer burden.

In vitro, baicalin showed a noticed effect in inhibiting cancer cell proliferation, in both sensitive and vincristine-resistant cells, these results are quite like what was mentioned in previous literature.

Particularly in breast cancer, baicalin was discovered to inhibit the migration, metastasis, and invasion of MDA-MB-231 breast cancer cells through the interruption of the p38MAPK signaling pathway (Wang et al. 2013). Also in another study, it was reported that baicalin had a role in inhibiting cell proliferation, invasion, and migration

and suppressing anti-apoptotic factors *in vitro* and *in vivo* using MCF-7 and MDA-MB-231 cells (Gao et al. 2018). Also, baicalin had a role in orthotopic breast cancer, it was noticed that baicalin reserved the bone mass in a bone metastasis model and reduced the metastasis growth of MDA-MB-231 cells (Wang et al. 2020). While in prostate cancer, baicalin showed anti-cancer effects on DU145, PC-3, LNCaP, and CA-HPV-10 cell lines (Chan et al. 2000). Moreover, in ovarian cancer, baicalin affected the viability and proliferation of ovarian cancer cells (Chen et al. 2013a). In addition to the baicalin effect in bladder cancer, it was reported that baicalin had exerted its anticancer activity by inducing ferritin-heavy chain-dependent ferroptosis in both 5637 and KU-19-19 cells (Kong et al. 2021). Also, in colon cancer, baicalin inhibited colon cancer *in vitro* and *in vivo* by apoptosis and senescence in HCT116 and SW480 colon cancer cells (Dou et al. 2018). Likewise, in hepatic cancer, baicalin inhibited hepatic cancer cell growth and survival both *in vitro* and *in vivo* in Hep G2 and SMMC-7721 cells (Yu et al. 2015). Furthermore, in lung cancer, baicalin selectively suppresses lung carcinoma and lung metastasis in A549 and mouse Lewis lung cancer (LLC) cells (Du et al. 2010).

Baicalin also has a role in sensitizing cancer cells to anti-cancer treatments, for example, baicalin improved the response of hepatocellular carcinoma cells to 5-FU and Epirubicin (Li et al. 2018). In addition, it was identified that baicalin sensitized lung cancer cells to cisplatin by XRCC1-mediated DNA repair (Yin et al. 2022). Besides, it was uncovered that baicalin sensitized triple-negative breast cancer MDA-MB-231 cells to Doxorubicin by encouraging the autophagy-mediated down-regulation of Cyclin-Dependent Kinase 1 (CDK1) (Hua et al. 2022).

Baicalin also plays a role in battling cancer resistance. In breast cancer, baicalin re-sensitized tamoxifen-resistant breast cancer cells by decreasing aerobic glycolysis and withdrawing mitochondrial dysfunction (Chen et al. 2021). A study reported that baicalin reduced cisplatin resistance in lung cancer through the downregulation of protein expression of MARK2 and p-Akt (Xu et al. 2017). Also, in gastric cancer, baicalin diminished 5-FU resistance in AGS cells by the suppression of glycolysis via adjustment of the PTEN/Akt/HIF-1 α signaling pathway (Chen et al. 2015).

In vitro, EGCG showed dose-dependent anti-cancer activity whether on the sensitive or vincristine-resistant cell line. Our results were similar to what was detected in the literature. EGCG has a proven anti-cancer role in breast and other types of cancer. In cervical cancer, EGCG has capabilities in the anti-proliferation, anti-metastasis, and pro-apoptosis of cervical cancer cells (Wang et al. 2018a). In addition, EGCG has a role in fighting prostate cancer as it inhibits the growth, invasion, angiogenesis, and metastasis of this cancer (Shankar et al. 2008). Moreover in bladder cancer, EGCG showed significant inhibition of cell proliferation (Luo et al. 2017). According to breast cancer, a study reported that EGCG inhibits breast cancer proliferation in the MCF-7 cell line (Zan et al. 2019).

In addition, EGCG was noticed to suppress the growth, migration, and invasion of Hs578T human breast cancer cells (Braicu et al. 2013). Similar results were detected on MDA-MB-231 breast cancer cell lines (Sen et al. 2010).

In addition to what was mentioned, EGCG also has an improving effect on anti-cancer drugs. It was reported that EGCG sensitized lung adenocarcinoma cells A549 cells towards etoposide by producing G2/M arrest and overcoming the multi-drug resistance (Datta and Sinha 2019). Moreover, EGCG decreased the toxicity of Arsenic trioxide (ATO) by increasing the sensitivity of cells toward it, specifically in Acute promyelocytic leukemia (APL) using HL-60 cells (Lee et al. 2011). Furthermore, EGCG enhanced the effect of cisplatin and tamoxifen in glioma cells (Shervington et al. 2009). In another study, it was also reported that EGCG enhances sensitivity to cisplatin in various types of cancer cells by restraining the activity of the nucleic acid repair enzymes ERCC1/XPF (Heyza et al. 2018). In cholangiocarcinoma cells, EGCG sensitized cells to gemcitabine (GEM), mitomycin C, and 5-fluorouracil *in vitro* (Lang et al. 2009). In addition, EGCG had lowered the IC50 of many chemotherapeutic drugs such as cisplatin, 5-FU, doxorubicin, and tamoxifen (Wang et al. 2023).

According to the role of EGCG in cancer resistance, as detected in our study, EGCG is reported to sensitize multidrug-resistant oral carcinoma nude mice xenografts inoculated with KBV200 cells to vincristine sulfate, by inhibiting tumor growth and inhibiting angiogenesis (Chen et al. 2020). Besides vincristine, it was reported that EGCG reversed cisplatin resistance in human lung cancer by the downregulation of Axl and Tyro 3 Expression (Kim and Lee 2014). Likewise, EGCG re-sensitized non-small-cell lung cancer (NSCLC) A549 cisplatin-resistant cells to cisplatin through candidate gene (Zhang et al. 2015).

In our study, it was noticed that a combination of baicalin and EGCG has shown a synergistic effect in both cell lines, EMT-6/P and EMT-6/V. This combination was never tested before. In literature combinations of baicalin and EGCG with other natural products were reported to affect cancer. For baicalin, a study reported that scutellarin and baicalin inhibited the proliferation of human breast cancer cells (Franek et al. 2005). EGCG combination therapy was more popular than baicalin. EGCG combinations with curcumin (Zhou et al. 2013), 6-gingerol (Rahman et al. 2014), Quercetin (Wang et al. 2012), panaxadiol (Du et al. 2013), ascorbic acid (Wei et al. 2003), pterostilbene (Kostin et al. 2012), and others (Roomi et al. 2005; Papi et al. 2013; D'Angelo et al. 2014) all proved anticancer effects on different types of cancer whether *in vitro* or *in vivo*. In particular, EGCG combination with flavones (Baicalin class) also showed proven anticancer as with luteolin, the combination proved its efficacy in prostate cancer (Gray et al. 2014).

To investigate the possible mechanism for baicalin and EGCG combination in targeting vincristine resistance, we detected the caspase enzymes in treated groups as an indicator for apoptosis activity. Caspase enzyme was detected when cancer cells were treated with baicalin, EGCG,

and their combination. The combination of baicalin and EGCG resulted in higher caspase activity rather each one alone. The apoptotic effect for baicalin or EGCG alone was detected in many previous studies. baicalin was reported to induce apoptosis in hepatic cancer cells (Yu et al. 2015). Also, it was reported that baicalin caused apoptosis in SW620 human colorectal cancer (Chen et al. 2012). Furthermore, baicalin encouraged apoptosis in H1299 and H1650 lung cancer cells (Sui et al. 2021). Moreover in osteosarcoma, baicalin also induced apoptosis through ROS-mediated mitochondrial pathway (Wan and Ouyang 2018). Besides, in T-cell acute lymphoblastic leukemia (T-ALL), baicalin induced apoptosis by Bcl-2 dependent pathway (Shieh et al. 2006). Correspondingly, baicalin improved chemosensitivity to Doxorubicin through the Up-regulation of oxidative stress-mediated mitochondria-dependent apoptosis (Lin et al. 2021). As baicalin, EGCG was also reported to induce apoptosis in different cancer types. In a study, it was reported that EGCG triggered apoptosis in MCF-7 breast cancer cell lines (Huang et al. 2017). Also, EGCG encouraged apoptosis in H1299 lung cancer cells by inhibiting the activation of the PI3K/Akt signaling pathway (Gu et al. 2018). Besides in laryngeal epidermoid carcinoma Hep2 cells, EGCG triggered apoptosis by the release of apoptosis-inducing factor and endonuclease G (Lee et al. 2010). Moreover, in CaSki cervical cancer cells, EGCG induced apoptosis significantly (Ahn et al. 2003). Also, EGCG provoked apoptosis in laryngeal squamous cell carcinoma cells by telomerase suppression (Wang et al. 2009). EGCG also stimulated apoptosis in cisplatin-resistant cell lines (Zhang et al. 2015).

Succeeding the *in vitro* experiments, *in vivo*, results showed that baicalin and EGCG combination along with vincristine has efficiently reduced tumour size in both cell lines, EMT-6/P and EMT-6/V. Also, it was shown that the combination of baicalin and EGCG improved vincristine-resistant cells to vincristine treatment.

In the vincristine-sensitive cell line, baicalin, the combination of baicalin and EGCG, and the triple therapy showed a significant reduction in the percent change of tumour size. The percentages of tumour change were: -58.64, -64.18, and -62.41 respectively.

These results are consistent with many studies. It has been previously reported the effect of baicalin *in vivo* on different types of cancer. In a study, using female nude mice inoculated with MDA-MB-23 breast cancer cells, when received 100 and 200 mg/kg i.p. injections of baicalin, for 25 days showed suppression of breast tumour growth (Gao et al. 2018). In addition, when female BALB/c mice were injected with 4T1 cells and were treated with 100 mg/kg baicalin in an intraperitoneal injection every 3 days for 6 weeks, showed the numbers of metastatic nodules on the surface of the liver and lung in the baicalin-treated group were less than these numbers in the positive control (Zhou et al. 2017). Also, it was reported that when baicalin (100 mg/kg) was administered to MCF-7 to female BALB/c-null every day for 2 weeks, the tumour volumes were smaller than those in the positive

control group (Liu et al. 2020). Also, it was reported that oral administration of baicalin in nude mice bearing A549 tumour xenografts in a daily dose of 100 mg/kg for 24 days resulted in 35.01% tumour inhibition rates (Hou et al. 2020). Furthermore, when SW620 human Colorectal cancer cells were injected into nude mice and then treated with i.p. baicalin (50 mg/kg), resulted in significant inhibition of tumour growth by 55% (Chen et al. 2012).

EGCG also has a significant role in battling cancer. It was proved that (1 mg/0.1 ml/mouse) of EGCG when administered by oral gavage in athymic nude mice inoculated with MDA-MB-231 cells, for 10 weeks, 10% of mice did not develop tumours and the rest of the tumours reduced in volume significantly by 45% (Thangapazham et al. 2007). In bladder cancer, it was established when Female BALB/c mice with SW780 cells and then treated with EGCG high-dose (100 mg/kg), i.p. injected every day, tumour volume was decreased, and significant differences were shown from day 22, tumour weight decreased significantly only in high-dose EGCG group by 68.4%, while the groups which were treated in low (25 mg/kg) and medium (50 mg/kg) doses did not show any significance. This may explain our results that using EGCG in higher doses and longer periods may produce significant results (Luo et al. 2017).

The insignificant results of EGCG *in vivo* in EMT-6/P may be also related to peracetate activity on the reactive hydroxyls of EGCG, as it was proved in research that pro-EGCG which is a prodrug of EGCG and has the peracetate-protecting groups to the reactive hydroxyls of EGCG, resulted in converting to its parent compound EGCG that was then accumulated. Pro-EGCG showed inhibition for breast tumour growth and proteasome (Landis-Piwowar et al. 2007).

The combination of baicalin and EGCG also showed a significant reduction in the percent change in tumour size equal to 64.18% in EMT-6/P, this result is not shocking, as baicalin and EGCG belong to the flavonoids class, and this class of natural products is well-known for their anti-cancer activities (Ponte et al. 2021).

Furthermore, vincristine showed a significant reduction in the percent change in tumour size, the percent was -53.38% in EMT-6/P. The effect of vincristine on breast cancer was established in much research. It was found that the co-encapsulation of Doxorubicin and vincristine as pegylated liposomal dramatically increased *in vitro* and *in vivo* therapeutic efficacy against triple-negative breast cancer cells (Ghosh et al. 2021). In another study, it was also reported that liposomal delivery of quercetin and vincristine enhanced estrogen-receptor-negative breast cancer treatment (Wong and Chiu 2010).

The triple therapy of baicalin, EGCG, and vincristine showed a noteworthy impact on EMT-6/P. The percent change in tumour size was - 62.41%. it is very close to the combination treatment. The little lower percentage between these two groups was non-significant correlated.

While in the vincristine-resistant cell line, only the triple therapy showed a significant reduction in the

percent change in tumour size equals - 77.30% and it was the highest percentage among all groups. These results prove that this combination significantly alters vincristine-resistant cells to vincristine. These results may be explained as baicalin may have better transportation through carrier-mediated transporters since baicalin is very polar and has limited transportation through the lipid bilayer via simple diffusion. Therefore, carrier-mediated transport is essential to baicalin's distribution. Transporters include multidrug-resistant protein (MRP) 3, MRP4, MRP2, and the breast cancer resistance protein (BCRP). The affinities of baicalin to transporters were found to be in the order BCRP > MRP3 > MRP2 > MRP1 (Huang et al. 2019). So it could be that more baicalin had been transported to cells by multidrug-resistant protein. For EGCG, it was shown that it increases the anti-angiogenic effect when used along with vincristine (Chen et al. 2020) also EGCG sensitizes vincristine-resistant cells to vincristine treatment (Hu et al. 2017).

According to vincristine, despite the cells being resistant it showed an insignificant higher percentage change in tumour volume than expected, this result may be justified as two mice had been cured, and this cure may be due to spontaneous regression, or the immune system was efficient enough to eradicate the tumour. So, the primary reason for recovery was not vincristine itself.

According to kidney and liver toxicity, none of the treatment groups show a significant difference in Cr, ALT, and AST compared to the healthy mice group.

It was reported that EGCG had a beneficial effect on kidney and liver functions. It was shown that EGCG lowered liver and kidney function damage and enhanced age-associated inflammation and oxidative stress (Niu et al. 2013). In addition to EGCG, it was noticed that

Baicalin protected against liver and kidney cell apoptosis and attenuates acute injury symptoms (Wang et al. 2018b).

For vincristine, our results are consistent with other study discovered that weekly therapy with vincristine (0.025 mg/kg IV) as a single agent for 28 days does not induce renal injury in female dogs in most cases (de Albuquerque Souza et al. 2022). In contrast, another study showed that sub-chronic administration of vincristine Sulphate (50 mg/kg) at a single dose/day (i.p.) for succeeding 30 days, induces renal damage and apoptosis in rats through the induction of oxidative stress but this dose and interval much higher and longer than our study (Shati 2019). For liver toxicity, it was perceived that Vinca alkaloids have not often been associated with significant hepatic toxicity. Vinca alkaloids are correlated with momentary and symptomless elevations in serum AST levels in 5% to 10% of patients (National Institute of et al. 2012).

Conclusion

Based on the data resulting from this research, we proved that baicalin and EGCG combination has a synergistic

anticancer effect against the parent (EMT-6/P) and the resistant (EMT-6/V) cell lines *in vitro* through apoptosis induction and caspase-3 activation. In addition, a combination of baicalin, EGCG, and vincristine established tumour size reduction in both cell lines. Also, we conclude that using baicalin and EGCG along with vincristine has significantly increased the sensitivity of vincristine-resistant cell lines *in vivo* and has superior tumour size reduction. This combination is safe for the liver and kidneys. The combination of baicalin and EGCG holds the potential to significantly amplify research opportunities, leading to increased knowledge and broader applications in breast cancer treatment.

Recommendations

Research is an ongoing process of inquiry and discovery, and it should always be in progress. To enhance human health and well-being. Research is also the leading light to the development of new treatments for diseases. For these purposes, here are some recommendations regarding the future work of this study:

- To further explore the mechanisms by which these combinations impose their synergistic effect.
- To manipulate these combinations on other types of cancer and with other chemotherapies.
- To optimize the regimens of baicalin with EGCG with vincristine in triple therapy for the best outcomes.

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Author contributions

DA contributed to study conduction, analysis and interpretation of data, manuscript writing, and revision. AHAA contributed to data analysis and interpretation of data. WT contributed to conceive and design the experiments also revision.

Ethical approval

All protocols of animal experiments were validated by the Research and Ethical Committee of Applied Science Private University with Standard ethical guidelines. Tumour tissue samples was obtained based on protocols approved by the Institutional Review Board (IRB) committees at the IRB (Approval number:2023-PHA-11). In accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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