Enhanced antitumor activity of doxorubicin by naringenin and metformin in breast carcinoma: an experimental study

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Abstract
Breast cancer is the most common malignancy in women worldwide. Strategies for cancer chemotherapy commonly require the use of combination therapy for better outcomes of results. The present work is aimed to evaluate the potential of naringenin and metformin concomitant addition with doxorubicin chemotherapy against experimental breast carcinoma. The antitumor potential of drugs under the study was evaluated against methylnitrosourea (MNU)-induced breast cancer in rats and 4T1 cells–induced orthotopic breast cancer mouse model. Parameters like tumor growth, body weight, survival rate, blood glucose, hematology, and histology were determined. There was a marked reduction in tumor weight and an observed decrease in tumor multiplicity by naringenin and metformin concomitant addition with doxorubicin against MNU-induced breast carcinoma. Likewise, naringenin and metformin with doxorubicin showed a significant reduction of tumor volume and tumor weight \( p < 0.01 \) in 4T1-induced orthotopic mouse model as compared to the same dose of doxorubicin alone, suggesting combination treatment enhanced antitumor activity in vivo. Furthermore, histology of tumor biopsies presented the improved antitumor activity of doxorubicin via increasing tumor necrosis. Hematological parameters, body weight, and survival data presented remarkable safety of combination treatment without compromising efficacy using 50% lower dose of doxorubicin as compared to the large dose of doxorubicin alone. These results demonstrate that naringenin and metformin enhanced the antitumor effect of doxorubicin in animal models of breast carcinoma, and therefore can be useful as an adjunct treatment with doxorubicin to increase its effectiveness at the lower dose level for the treatment of cancer.

Keywords Naringenin · Metformin · Doxorubicin · Breast carcinoma

Introduction
Breast cancer is the most common malignancy in women around the world. Early-stage and locally advanced disease is curable in 70–80% of patients but advanced metastatic breast cancer is incurable (Harbeck et al. 2019). Unless some urgent action is taken, the number of females diagnosed with breast cancer in the world would almost double to 3.2 million a year by 2030 (Noori et al. 2020). Development of resistance and toxicity to normal cells are major dose-related limitations of chemotherapeutic agents in the present scenario. The use of combination therapy in breast cancer potentially provided advantages for better efficacy and safety at the lower dose and reduced or delayed development of drug resistance (Fisusi and Akala 2019). Therefore, it is an unmet need to evaluate the currently available medicines and some plant-based active components for their potential role in the treatment of breast carcinoma.

Doxorubicin is one of the most frequently used anticancer agents in the treatment of human malignancies and acts by DNA intercalation as well as the disruption of topoisomerase II-mediated DNA repair. It is used to treat both early-stage and metastatic breast cancer. Doxorubicin can kill cancer cells at every point in their life cycle, and it has not required specific receptor expression, therefore an advantage over some other breast cancer drugs like hormone therapy, trastuzumab, and checkpoint inhibitors. Unfortunately, its use
is associated with the development of severe cumulative dose-related cardiotoxicity, myelosuppression, and treatment resistance due to its oxidative stress action. Therefore, it is preferred to combine with other compounds to reduce its dosage without compromising its efficacy (El-Ashmawy et al. 2017). Naringenin, a naturally occurring flavonoid has shown anti-inflammatory, anti-atherogenic, anti-mutagenic, hepatoprotective, antidiabetic, cardioprotective, and anticancer potential in many non-clinical studies (Salehi et al. 2019). Naringenin is reported to block transforming growth factor (TGF)-β1 secretion from breast cancer cells and suppress pulmonary metastasis by inhibiting protein kinase C (PKC) activation (Zhang et al. 2016). It also regulates the mitochondrial-mediated apoptosis cell signaling pathway and reveals anti-inflammatory potential in an animal model of breast cancer (Zhao et al. 2019). In combination with doxorubicin, naringenin increases the cellular doxorubicin accumulation by inhibiting doxorubicin efflux and enhancing antitumor activity with a reduction in systemic toxicity (Zhang et al. 2009). Also, naringenin is a promising co-chemotherapeutic agent for cancer that has inactivated p53 gene (Kanno et al. 2005). Metformin is a well-known orally effective and safe medicine for type II diabetes. It has shown antitumor activity in non-clinical studies (Viollet et al. 2012; Aljofan and Riethmacher 2019). Metformin has been reported to improve the overall survival of several cancers (Kaszniki et al. 2014; Lee et al. 2020; Ugwueze et al. 2020). The anticancer effect of metformin is mediated by the down-regulation of cyclin D1 and increased levels of the tumor suppressor gene p53. It activates the AMP-activated protein kinase (AMPK) pathway for tumor suppression effects, and reduces the mammalian target of rapamycin (mTOR) signaling pathway and protein synthesis in cancer cells (Zadra et al. 2015; El-Ashmawy et al. 2017).

Despite available information for naringenin, metformin, and doxorubicin for their use against cancers, there is no literature available for their concomitant use in the treatment of breast cancer. We have therefore chosen naringenin and metformin under the study as both the drugs have literature evidence for anticancer effects as well as protective effects against doxorubicin-induced toxicity (Subburaman et al. 2014; Sheta et al. 2016; Liu et al. 2017; Ajzashokouhi et al. 2020). The doses for the naringenin and metformin have been selected based on previous studies (Subburaman et al. 2014; Grossmann et al. 2015; Li et al. 2015; Liu et al. 2017). Our previous in vitro study on breast cancer cells (MDA-MB-231 and 4T1) revealed enhanced expression of the sensitivity of doxorubicin using combination of naringenin and metformin (Pateliya et al. 2021). Besides, in vivo study against the MDA-MB-231 xenograft model showed improvement in the efficacy and safety of doxorubicin using combination treatment (Pateliya et al. 2021). The current study is aimed to investigate the adjuvant anticancer activity of naringenin and metformin with a lower dose of doxorubicin chemotherapy against another different in vivo experimental breast carcinoma models to explore the further scope of treatment for breast cancer.

Materials and methods

Culture condition and reagents

The mouse breast cancer cell line 4T1 (CRL-2539™) was purchased from the American Type Culture Collection (ATCC; Bangalore, India). Cells were cultured with RPMI-1640 medium (Sigma-Aldrich, Merck) supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. MethylNitrosourea (MNU) and naringenin were purchased from Sigma-Aldrich (Bangalore, India). Metformin was procured from Parth Medicine (Vadodara, India). Liposomal doxorubicin was obtained from Sun Pharmaceutical Industries Limited (Vadodara, India).

Animals

Female Sprague Dawley rats (80–120 g) and female Balb/c mice (18–22 g) were used for the studies. Animals were housed in the group (2 to 3 animals/cage) maintained 12 h light and 12 h dark condition. Animals received water and rodent diet ad libitum. Animal experiments were conducted according to the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA). The project proposal was approved by the Institutional Animal Ethics Committee of Sardar Patel College of Pharmacy, Gujarat Technological University, Gujarat, India (IAEC No. SPCP/IAEC/RP-03/2017).

MNU-induced rat model (chemical-induced breast carcinoma)

Breast tumor was induced in SD rats by injecting 50 mg/kg MNU intraperitoneally as described earlier (Thompson and Adlakha 1991). After the development of the tumor, i.e., 90 days from the injection, animals were divided into eight different treatment groups (n = 6/group) based on tumor occurrence and body weight as shown in the study design (Table 1). Naringenin (50 mg/kg) or/and metformin (100 mg/kg) were administered orally to their respective treatment groups of animals for 28 days. Intravenous injections of liposomal doxorubicin (2 mg/kg or 4 mg/kg) or saline were administered on days 0, 7, 14, and 21 to the respective group of animals as shown in Table 1. Tumor development and body weights were recorded weekly up
to day 28. Blood glucose was measured on day 21 for each animal to check any hypoglycemic effect of treatments given under the study. The animals were checked daily throughout the study for any mortality. All the animals were sacrificed on day 28, their tumors were isolated and weighed. Anti-tumor activities of a single drug or combination of drugs were assessed based on tumor parameters (Parvathaneni et al. 2014; Karia et al. 2018) like tumor incidence, the total number of a tumor, tumor multiplicity, and tumor weight (Table 2).

**4T1-induced mouse model (orthotopic breast carcinoma)**

Breast tumors were induced using the orthotopic injection of $1 \times 10^6$ 4T1 breast carcinoma cells at mammary fat pad of Balb/c mice as described earlier (Paschall and Liu 2016; Zhang et al. 2018). Animals were divided in to eight groups ($n=6$) based on tumor volume and body weight (tumor volume 50 to 100 mm$^3$ and had a body weight 18–24 g at the time of treatment initiation) as shown in Table 1. Naringenin (50 mg/kg) or/and metformin (100 mg/kg) were administered orally to their respective treatment groups of animals for 28 days. Intravenous injections of liposomal doxorubicin (3 mg/kg or 6 mg/kg) or saline were administered on days 0, 7, 14, and 21 to their respective group of animals as shown in Table 1. Tumor diameter using digital vernier caliper and body weights were recorded twice weekly up to 28$^{th}$ day. For ethical reasons, animals were euthanized when tumor volume reached >4000 mm$^3$. The animals were checked daily throughout the study for mortality. On day 28, blood was withdrawn for the estimation of hematology parameters. Animals were sacrificed, tumors were isolated and weighed. Tumor volume ($V$) was calculated using the formula of a sphere as under:

\[\frac{V}{\text{mm}^3} = \frac{(D_1 + D_2)^3}{2} \times 0.5236\]

where $D_1$ and $D_2$ were the largest and smallest diameters of tumor respectively.

### Table 1 Study design for in vivo efficacy in animal models of breast carcinoma

| Group No | Treatment groups | Dose and schedule |
|----------|------------------|-------------------|
| 1        | Disease control  | 2 ml/kg water (po) + 3 ml/kg saline (iv) |
| 2        | Naringenin      | 50 mg/kg/po/day for 28 days |
| 3        | Metformin       | 100 mg/kg /po/day for 28 days |
| 4        | Liposomal doxorubicin | 4 or 6 mg/kg/iv/week for 4 weeks* |
| 5        | Liposomal doxorubicin | 2 or 3 mg/kg/iv/week for 4 weeks** |
| 6        | Naringenin + Liposomal doxorubicin | 50 mg/kg/po/day for 28 days |
| 7        | Metformin + Liposomal doxorubicin | 100 mg/kg /po/day for 28 days |
| 8        | Naringenin + Metformin + Liposomal doxorubicin | 50 mg/kg/po/day for 28 days |

$n=6$; *4 mg/kg/iv/week for rats and 6 mg/kg/iv/week for mice; **2 mg/kg/iv/week for rats and 3 mg/kg/iv/week for mice; breast carcinoma was induced using single intraperitoneal injection of MNU (50 mg/kg) in rats; and injection of 4 T1 cells in mammary fat pad of mice. *intravenous; po, per oral

### Table 2 Tumor parameters in MNU-induced breast carcinoma in rats

| Groups        | Tumor incidence (%) | Total number of tumor (n) | Tumor multiplicity | Tumor weight (g) *$
|---------------|---------------------|---------------------------|--------------------|----------------|
|               |                     |                           |                    |                 |
| Disease control | 100                 | 17                        | 2.8                | 8.6±2.48        |
| Nar 50        | 83                  | 8                         | 1.3                | 5.7±2.73        |
| Met 100       | 100                 | 11                        | 1.8                | 4.9±2.21        |
| Lipo-dox 4    | 17                  | 2                         | 0.3                | 0.40±0.37**     |
| Lipo-dox 2    | 67                  | 9                         | 1.5                | 3.4±1.36        |
| Lipo-dox 2 + Nar | 67                 | 6                         | 1.0                | 1.9±1.09*       |
| Lipo-dox 2 + Met | 50                 | 5                         | 0.8                | 1.1±0.58*       |
| Lipo-dox 2 + Nar + Met | 33              | 3                         | 0.5                | 0.7±0.46*       |

*Data were expressed as mean±SEM, n=6. The data were analyzed using one-way ANOVA followed by Dunnett’s test. $^*p<0.05$, $^{**}p<0.01$ compared to disease control group; Nar, naringenin; Met, metformin; Lipo-dox, liposomal doxorubicin
Percentage test/control (%T/C) was calculated as follows:

\[
\%T/C = \frac{\text{Mean tumor volume of drug treated group on day } X}{\text{Mean tumor volume of control group on day } X} \times 100
\]

where \( X \) was the day of observation.

The optimal %T/C value for each group was the minimal %T/C ratio, thus reflecting the maximal tumor growth inhibition. According to National Cancer Institute (NCI) standard criteria, %T/C \( \leq 42\% \) indicates acceptable antitumor activity; %T/C \( \leq 20\% \) indicates moderate antitumor activity; %T/C \( \leq 10\% \) indicates high antitumor activity (Bisser and Gueritte-Voegelein 1991; Kruczynski and Hill 2002; Burade et al. 2017).

Animal body weight changes were calculated as follows:

\[
\text{Body weight change (\%) = } \frac{\text{Mouse weight on Day } X - \text{mouse weight on Day } 0}{\text{Mouse weight on Day } 0} \times 100
\]

where \( X \) was the day of observation.

A dose producing a mean weight loss \( \geq 15\% \) of initial body weight was considered toxic (Kruczynski and Hill 2002; Burade et al. 2017).

**Hematology estimation**

The blood was collected from the mice at the end of the study (day 28) and processed for hematology parameters analysis using the ADVIA 120 hematology system.

**Histology**

Histology was performed using hematoxylin and eosin (H&E) staining for the tumor sections of MNU-induced rat model as well as the orthotopic mouse model. Tissue samples were fixed in 10% formalin and embedded in paraffin, cut in 4-mm sections, stained with H&E, and then observed for tumor necrosis based on morphology under a bright field microscope (Alyahya et al. 2015; Liu et al. 2017).

**Statistical analysis**

Tumor volume data were analyzed using two-way ANOVA followed by Bonferroni’s test. Body weight, hematology, and blood glucose parameters were analyzed using one-way ANOVA followed by Dunnett’s test. The Kaplan–Meier method, the log-rank test was used to estimate survival differences. Statistical analysis was carried out using GraphPad Prism 9.0 (GraphPad Prism Software, LLC) and \( p \) values \(< 0.05\) were considered statistically significant.

### Results

**Effect of naringenin, metformin and doxorubicin on MNU-induced breast carcinoma in rats**

In MNU-induced breast carcinoma model of rats, a significant reduction (\( p < 0.05\)) in tumor weight was observed in metformin + 2 mg/kg lipo-dox, naringenin + 2 mg/kg lipo-dox and concomitant treatment of naringenin, metformin, and 2 mg/kg lipo-dox treated groups when compared with the disease control group (\( p < 0.05\)). A higher dose of lipo-dox (4 mg/kg) also showed a significant reduction in tumor weight as compared to the disease control group (\( p < 0.01\)). Besides, combination of treatment (lipo-dox 2 mg/kg + naringenin + metformin) showed marked reduction in tumor weight when compared with the lipo-dox 2 mg/kg alone treatment (Table 2). The combination of lipo-dox 2 mg/kg with naringenin and metformin showed a reduction in tumor incidence, the total number of tumors as well as tumor multiplicity as compared to lipo-dox 2 mg/kg alone and showing effects closer to a higher dose of lipo-dox (4 mg/kg) treated rats. At the same time, reduction in mean tumor weight was not found significant in individual drug treatments viz. naringenin or metformin or lipo-dox 2 mg/kg as compared to a disease control group. A maximum decrease in body weight was observed in the lipo-dox 4 mg/kg (12%) when compared with their respective body weight on day 0 (Fig. 1A); although other treatments showed less than 5% body weight loss in the rat model of MNU-induced breast carcinoma. Besides, no mortality was observed in any treatment group throughout the study (Fig. 1C).

**Effect of naringenin, metformin and doxorubicin on 4T1-induced orthotopic breast carcinoma in mice**

Significant reduction in tumor volume was observed in both the 3.0 and 6.0 mg/kg lipo-dox treated, metformin alone, and combinations of naringenin and/or metformin with 3 mg/kg lipo-dox-treated animals, as compared to the disease control group. Moreover, combination treatment (lipo-dox 3 mg/kg + naringenin + metformin) showed significant reduction in tumor volume as compared to lipo-dox 3 mg/kg alone treatment establishing the synergistic effect (\( p < 0.05\); Fig. 2A). Tumor weight at the end of the study (day 28) showed a similar pattern with
Fig. 1 Effect of single or combination treatment of naringenin, metformin and liposomal doxorubicin in MNU-induced breast carcinoma in rats. A body weight, B glucose levels and C survival chart. Body weight data were expressed as % change in body weight from initial body weight. n=6. The data was analyzed using one-way ANOVA followed by Dunnett’s test. \( p < 0.05 \) compared to initial body weight of same group. A dose producing a weight loss \( \geq 15\% \) of initial body weight was considered toxic. No significant change in body weight was observed in any treatment group. Survival was estimated using the Kaplan–Meier method, and differences were analyzed by log-rank test. No mortality was observed in any treatment group. Nar, naringenin; Met, metformin; Lipo-dox, liposomal doxorubicin.
tumor volume data and thereby confirming the synergistic effect of concomitant treatments (Fig. 2B and C). However, no significant change was seen in tumor volume in naringenin-treated mice throughout the study. Antitumor activities have been compared based on %T/C (Table 3). Acceptable antitumor activity (%T/C ≤ 42, based on NCI criteria) was observed in lipo-dox 3 alone. Tumor weight data were analyzed using one-way ANOVA followed by Dunnett’s test. \( p < 0.05 \), \( **p < 0.01 \), \( ***p < 0.001 \) compared to control group. \( $$p < 0.01 \) compared to Lipo-dox 3 alone group. Groups (a) Nar 50, (b) Met 100, (c) Lipo-dox 6, (d) Lipo-dox 3, (e) Lipo-dox 3 + Nar, (f) Lipo-dox 3 + Met and (g) Lipo-dox 3 + Nar + Met; Nar, naringenin, Met, metformin, Lipo-dox, liposomal doxorubicin, q7d*4, total 4 dose at weekly interval. qd*28, daily for total 28 days and metformin with lipo-dox 3 mg/kg showed highly significant antitumor activity (optimal %T/C values on day 28 were 7.2) indicating the synergistic effect of combination treatments. In addition, the efficacy of combination of naringenin and metformin with lipo-dox 3 mg/kg was comparable with that of 6 mg/kg lipo-dox findings (optimal %T/C values on day 28 was 2.8) (Table 3).

In 4T1-bearing mice, body weight loss from the baseline value was not found significant in any of the treatment groups, except for the higher dose of lipo-dox (i.e., 6 mg/kg) (Fig. 3A). Looking at the...
survival data, mortality was observed in each treatment group indicating the highly aggressive nature of 4T1 cells on mouse survival. However, the maximum mice survival (83%) was found in control, lipo-dox 3 mg/kg + naringenin + metformin concomitant treatment, lipo-dox 3 mg/kg + naringenin combination, and metformin alone treatment groups at the end of the study. Furthermore, 67% survival was observed in naringenin, lipo-dox 3 mg/kg, and lipo-dox 3 mg/kg + metformin combination group. However, 50% survival was noticed at a higher dose of lipo-dox 6 mg/kg (Fig. 3B).

**Effect of naringenin, metformin and doxorubicin on hematology parameters**

The hematological parameters in the whole blood of mice showed significant reduction (\(***p<0.001\)) in total WBC and neutrophils counts in lipo-dox alone and combination treatment groups as compared to the control group (Table 4). Also,

| Groups              | Day 0  | Day 3  | Day 7  | Day 10 | Day 14 | Day 17 | Day 21 | Day 24 | Day 28 |
|---------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Nar 50              | 98.2   | 108.3  | 77.8   | 67.8   | 78.5   | 95.6   | 83.3   | 86.4   | 90.1   |
| Met 100             | 101.6  | 112.9  | 84.7   | 66.7   | 71.9   |        | 68.4   | 69.0   | 76.3   |
| Lipo-dox 6          | 101.4  | 87.5   | 36.5a  | 25.4a  | 15.9b  | 12.0b  | 6.7c   | 2.9c   | 2.8c   |
| Lipo-dox 3          | 99.1   | 63.4   | 38.9a  | 38.6a  | 27.7a  | 25.8a  | 20.3a  | 19.1b  | 22.1a  |
| Lipo-dox 3 + Nar    | 92.0   | 75.6   | 38.2a  | 36.5a  | 29.5a  | 25.0a  | 18.6b  | 15.2b  | 16.2b  |
| Lipo-dox 3 + Met    | 98.0   | 79.2   | 49.1   | 32.4a  | 21.6a  | 18.3b  | 16.2b  | 13.5b  | 12.6b  |
| Lipo-dox 3 + Nar + Met | 101.9 | 88.5   | 41.3   | 26.8a  | 17.0b  | 14.1b  | 9.7c   | 7.8c   | 7.2c   |

Percentage test/control (%T/C) was calculated from tumor volume data. NCI criteria for anticancer activity, 
\(^{a}%\text{T/C} \leq 42\%\) indicates acceptable antitumor activity; \(^{b}%\text{T/C} \leq 20\%\) indicates moderate antitumor activity; \(^{c}%\text{T/C} \leq 10\%\) indicates highly significant antitumor activity. Nar, naringenin; Met, metformin; Lipo-dox, liposomal doxorubicin.
Fig. 3 Effect of single or combination treatment of naringenin, metformin and liposomal doxorubicin in 4T1-induced orthotopic breast carcinoma in mice. A body weight and B survival chart. Body weight data were expressed as % change in body weight from initial body weight. *p < 0.05 compared to initial body weight of same group. A dose producing a weight loss ≥ 15% of initial body weight was considered toxic. No significant change in body weight was observed in any treatment group except Lipo-dox 6 survival was estimated using the Kaplan–Meier method, and differences were analyzed by log-rank test. No statically significant difference was observed in survival data.

Table 4 Hematology parameters in 4T1-induced orthotopic breast carcinoma in mice

| Treatment | WBC ($\times 10^3$) | Neutrophils ($\times 10^3$) | Platelets ($\times 10^3$) | HB (g/dl) | RBCs ($\times 10^6$) | Lymphocyte ($\times 10^3$) |
|-----------|---------------------|-----------------------------|--------------------------|-----------|----------------------|--------------------------|
| Control   | 574.4±146.2         | 544.5±130.4                 | 1069.4±320.4             | 11.5±1.1  | 6.7±0.8              | 10.2±2.6                 |
| Nar       | 493.1±85.7          | 470.1±88.4                  | 714.3±119.5              | 10.5±1.2  | 7±0.5                | 12.3±1.6                 |
| Met 100   | 546±136.1           | 517.7±124                   | 920.5±107.5              | 10.5±1.1  | 6.8±0.6              | 12.4±2.5                 |
| Lipo-dox 6| 53***±12.4          | 45.6***±10.9                | 998.7±278.4              | 11.6±1    | 7.8±0.5              | 4.7±0.9                  |
| Lipo-dox 3| 178.5***±71.8       | 166.1***±70.5               | 942.2±174.5              | 11.5±1.8  | 7.8±1                | 8.2±2.4                  |
| Lipo-dox 3+Nar | 185.9***±82.5       | 170.7***±80.4               | 1052±77.8                | 12.7±0.6  | 8.6±0.5              | 7.6±2.9                  |
| Lipo-dox 3+Met | 191.7***±58        | 1801.1***±62.7             | 901.5±283.9              | 11.8±2.3  | 7.9±1.1              | 6.9±1.6                  |
| Lipo-dox 3+Nar+Met | 170.2***±77.4     | 158.2***±80.8               | 876.5±137.1              | 12.6±0.6  | 8.4±0.2              | 6.3±1.4                  |

Blood samples (K$_2$EDTA) were analyzed with the ADVIA 120 hematology system; data were expressed as mean±SD and analyzed using one-way ANOVA followed by Dunnnett’s test. *p < 0.05 compared to control. Nar, naringenin; Met, metformin; Lipo-dox, liposomal doxorubicin.
the higher dose of lipo-dox receiving animals showed a marked reduction of WBC and neutrophils counts. This indicates the use of a lower dose of lipo-dox along with naringenin and metformin is helpful to minimize toxicity without compromising efficacy. Although, there was no significant difference in platelets, hemoglobin, RBC, and lymphocyte counts following various treatment groups under the study (Table 4).

**Effect of naringenin, metformin, and doxorubicin on histopathological examination**

Histopathological examination of tumor sections showed that naringenin or metformin alone treatment was not able to affect tumor necrosis in MNU-induced rat model and 4T1-induced mouse breast carcinoma.
model. However, concomitant treatment of naringenin, metformin, and lipo-dox showed a remarkable increase in the tumor necrotic area when compared with the vehicle control group in both the models (Fig. 4A and B). These findings have further established the efficacy of combination treatment against experimentally induced breast carcinoma.

**Discussion**

The therapeutic dose of doxorubicin is frequently insufficient in cancer treatment, and the use of its higher dose produces dose-related toxicity (Hanušová et al. 2011). Moreover, long-term use of doxorubicin chemotherapy develops its resistance and toxicity that can be attenuated by increasing
its effectiveness using adjuvant therapy. In the present study, we focused on the in vivo efficacy and safety of concomitant use of naringenin and metformin with doxorubicin by reducing its dose and subsequently dose-related toxicities of doxorubicin against experimental breast cancer models.

MNU is the most reliable carcinogen, mutagen, and teratogen to induce breast carcinogenesis in experimental rats (Faustino-Rocha et al. 2015). 4T1-induced orthotopic breast carcinoma mouse model is frequently used for the evaluation of breast carcinoma study. We have included both the above-mentioned models in the present study to evaluate the treatment effect on different subtypes like luminal-like and basa-like breast cancer. Wang and coworkers reported that naringenin has a chemoprotective effect in breast cancer cells via inhibition of caspase-3 and caspase-9 activities (Wang et al. 2019). Recently, it has been observed that naringenin improves the anticancer effect of cyclophosphamide in breast carcinoma, whereas other reports have shown the synergistic effect of 5-fluorouracil combined with naringin in MDA-MB-231 human breast cancer cells (Muthusamy et al. 2020; Noori et al. 2020). In addition to anticancer effects, naringenin has shown cardioprotective effects. Also, the protective effects of naringenin have been reported against doxorubicin-induced cardiac, hepatic, and renal toxicities in rats (Shabanah et al. 2019).

Hirsch and coworkers have reported that metformin selectively targets cancer stem cells and helps chemotherapy to block tumor growth and delay remission (Hirsch et al. 2009). It has been reported that metformin synergistically inhibits tumor growth and reverses resistance with doxorubicin in both MCF7/ADR cells and xenograft study (Li et al. 2018). Researchers have assessed the effect of metformin and sitagliptin on doxorubicin-induced cardiotoxicity in rats and reported their protective effect via reducing oxidative stress, inflammation, and apoptosis pathways (Sheta et al. 2016). Furthermore, metformin is also reported to prevent doxorubicin resistance in MCF-7 and MDA-MB-231 through oxidative stress and modulation of cell adaptation genes (Marinello et al. 2019).

The present study of MNU-induced breast carcinoma in rats wherein a combination of naringenin, metformin, and lipo-dox 2 mg/kg showed a marked reduction in tumor weight as compared to lipo-dox 2 mg/kg single treatment and the effects were closer to the higher dose of lipo-dox 4 mg/kg treatment. Also, histology revealed higher necrosis in those concomitant treatment groups. Other findings such as blood glucose levels, body weight, and survival observations did not change remarkably suggesting no significant toxicity with any of the treatment groups under the study. Our earlier in vitro data demonstrated that the combination of naringenin and metformin enhanced the sensitivity of breast cancer cells (MDA-MB-231 and 4 T1) to doxorubicin (Pateliya et al. 2021). Also, we have shown improved efficacy and safety of doxorubicin combination with naringenin and metformin against in vivo study using MDA-MB-231 xenograft model (Pateliya et al. 2021). Parallel to above, the present study of 4T1-induced orthotopic breast carcinoma in mice showed a statistically significant reduction in tumor volume as well as tumor weight in the animals receiving concomitant treatment of naringenin and metformin with lipo-dox 3 mg/kg compared to lipo-dox 3 mg/kg alone. Also, this antitumor effect exhibited a comparable efficacy to that of the higher dose of lipo-dox (6 mg/kg).

In conclusion, our results demonstrated that the use of naringenin and metformin together with doxorubicin chemotherapy has good potential as candidates for co-chemotherapy agents for the treatment of breast cancer. Hence, our study provides an opportunity and the scope for the use of naringenin and metformin with doxorubicin chemotherapy for reducing its dose and subsequently associated toxicity in patients. However, further in-depth studies are needed to fully understand the role of naringenin and metformin in combination with chemotherapeutic agents for the treatment of human cancers.

Data availability Data is available in the form of tables and figures.

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Author contributions The authors declare that all data were generated in-house and that no paper mill was used. Bharat Pateliya (BP): designed and performed the experiments, contributed to the data analysis and writing original draft. Vinod Burade (VB): reviewed and performed supervision. Sunita Goswami (SG): reviewed, results interpretation and work supervision. All the authors read and approved the final manuscript.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate This article does not contain any studies with human participants performed by any of the authors. All animal studies were carried out with prior approval from the Institutional Animal Ethics Committee (IAEC) and care of animals complied according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India.

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication Not applicable.
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