Evaluation of Combination Therapy of Naringenin and Metformin in Doxorubicin Chemotherapy against *in vitro* and *in vivo* Models of Breast Carcinoma

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1. Title of the thesis and abstract

Title of the thesis: "Evaluation of Combination Therapy of Naringenin and Metformin in Doxorubicin Chemotherapy against *in vitro* and *in vivo* Models of Breast Carcinoma"

Abstract:

Introduction: Breast cancer is the most common malignancy in women around the world. The present work is aimed to evaluate the potential of naringenin and metformin concomitant addition with doxorubicin chemotherapy against *in vitro* and *in vivo* breast carcinoma models.

Methods: Cell viability was measured by MTT assay using breast cancer cell lines (MDA-MB-231 and 4T1). The antitumor potential of drugs under the study was evaluated against methylnitrosourea (MNU)- induced breast carcinoma in rats, MDA-MB-231 xenograft model in athymic nude mice and finally 4T1 cells- induced orthotopic mouse model of breast cancer. The efficacy and safety of single-drug treatment and combination treatment were evaluated using different parameters in selected animal models of breast carcinoma.

Results: The use of naringenin and metformin together with doxorubicin, have shown a significant increase in cytotoxicity when compared with the same concentration of doxorubicin alone in MDA-MB-231 cells and 4T1 cells *in vitro*. 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 as compared to the same dose of doxorubicin alone in MDA-MB-231 xenograft model and 4T1- induced orthotopic mouse model, suggesting combination treatment enhanced antitumor activity *in vivo*. Besides, H&E and Ki-67 staining of tumor biopsies showed enhancement of the antitumor activity via increasing necrosis and inhibiting cell proliferation respectively. Hematological parameters, body weight, survival data, and cardiac biomarker levels presented remarkable safety of combination treatment without compromising efficacy using 50% lower dose of doxorubicin, compared to the large dose of doxorubicin alone.

Conclusion: These results demonstrate that naringenin and metformin enhanced the antitumor effect of doxorubicin in the *in vitro* as well as 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 breast cancer.

2. Brief description on the state of the art of the research topic

Breast cancer is the most common cancer in women worldwide (1). Advanced breast cancer with distant organ metastases is considered incurable with currently available therapies (2).

Doxorubicin acts by DNA intercalation as well as the disruption of topoisomerase II-mediated DNA repair. 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. Naringenin, a naturally occurring flavonoid has shown anti-inflammatory, anti-atherogenic, anti-mutagenic, hepatoprotective, antidiabetic, cardioprotective and anticancer potential in many non-clinical studies (3). 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 (4). It also regulates the mitochondrial-mediated apoptosis cell signaling pathway and reveals anti-inflammatory potential in an animal model of breast cancer (5). In combination with doxorubicin, naringenin increases the cellular doxorubicin accumulation by inhibiting doxorubicin efflux and enhancing antitumor activity with a reduction in systemic toxicity (6). Metformin has shown antitumor activity in non-clinical studies (7,8). Metformin has been reported to improve the overall survival of several cancers in humans (9–11). 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 (12,13).

3. Definition of the problem

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 (14). 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. 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 (15–18).

4. Objectives and scope of work

4.1 Objectives

- To evaluate the *in vitro* cell cytotoxicity of combining naringenin and metformin with doxorubicin using breast carcinoma cell lines.
- To evaluate the efficacy of combining naringenin and metformin with doxorubicin chemotherapy using various *in vivo* models of breast carcinoma
- To evaluate the safety of combination therapy of naringenin and metformin with the lower dose of doxorubicin chemotherapy.

4.2 Scope of work

The current research work throws light on the efficacy and safety of concomitant treatment of naringenin and metformin with the lower dose of doxorubicin chemotherapy using *in vitro* as well as animal models of breast carcinoma. This study also provides an opportunity and the scope for using a combination of naringenin and metformin with doxorubicin chemotherapy for reducing the dose and subsequently toxicity of chemotherapy in humans. Additionally, the use of metformin and naringenin may help in improving the quality of life of diabetic patients suffering from cancer. However, further in-depth studies including clinical trials are recommended to fully establish the role of naringenin and metformin in combination with chemotherapeutic agents for the treatment of human cancers.

5. Original contribution by the thesis

The current study provides scientific data regarding the potential of naringenin and metformin with doxorubicin chemotherapy treatment options in animal models of breast carcinoma. This investigation is useful for further evaluation of breast cancer treatment option in clinical studies.

6. Methodology of Research, Results / Comparisons

6.1 Methods

6.1.1 Experimental animals & ethical approval

Animal experiments were conducted according to the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), India. The project proposal was approved by the Institutional Animal Ethics Committee (IAEC No. SPCP/IAEC/RP-03/2017 and IAEC/448).

6.1.2 Cell culture and reagents

The human breast cancer cell line MDA-MB-231 (HTB-26™) and murine breast cancer cell line 4T1 (CRL-2539™) were purchased from the American Type Culture Collection (ATCC; Bangalore, India). Naringenin was purchased from Sigma-Aldrich (Bangalore, India). Doxorubicin

injection (Dox) and metformin were purchased from Parth Medicine (Vadodara, India). Lipo-dox was received from Sun Pharma, Vadodara.

6.1.3 *In vitro* studies

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay as previously described (6,19). Cell viability was calculated as follow:

$$\text{Viability (\%)} = \frac{\text{Absorbance of sample wells}}{\text{Absorbance of control wells}} \text{ x } 100$$

Concentrations for combination treatment assay were determined based on % cell viability following single-drug treatment. The different concentration of each drug for combination treatment assay was selected based on initial separate experiments, using various concentration of single-drug treatment in both the cell lines.

6.1.4 *In vivo* studies

Three different animal models for breast carcinoma were selected for *in vivo* evaluation of the effectiveness of combination treatment. Breast carcinoma was developed using chemical (MNU) or cell lines (MDA-MB-231 and 4T1). After the development of the tumor, a single drug and combination treatment was initiated as per the study design (Table 1). The doses for the naringenin and metformin have been selected based on previous studies (16,18,20,21).

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 2 or 3 mg/kg/iv/week for 4 weeks[##]
7	Metformin + Liposomal doxorubicin	100 mg/kg /po/day for 28 days 2 or 3 mg/kg/iv/week for 4 weeks[##]
8	Naringenin + Metformin + Liposomal doxorubicin	50 mg/kg/po/day for 28 days 100 mg/kg /po/day for 28 days 2 or 3 mg/kg/iv/week for 4 weeks[##]

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 or subcutaneous injection of MDA-MB-231cells in backside of athymic nude mice or subcutaneous injection of 4T1 cells in the mammary fat pad of Balb/c mice. iv- intravenous, po- per oral

6.1.4.1 Chemical induced breast carcinoma model

Breast tumor was induced in SD rats by injecting 50 mg/kg MNU intraperitoneally as described earlier (22). Treatment was given to the respective group of animals as shown in Table 1. Tumor development and body weights were recorded weekly up to the 28th day. Additionally, 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. Antitumor activities of a single drug or combination of drugs were assessed based on tumor parameters (23,24) like tumor incidence, the total number of a tumor, tumor multiplicity and tumor weight (Table 2).

6.1.4.2 Xenograft mouse model of breast carcinoma

Breast tumors were induced by subcutaneous injection of human origin breast carcinoma cells (5 × 10^6 MDA-MB-231 cells) at the right flank region of the backside of athymic nude mice as described earlier (25,26). Animals were divided into different treatment groups based on tumor volume and body weight and treatment was given as shown in Table 1. Tumor size (using digital vernier caliper) and body weights were recorded twice weekly till 28 days of the study period. Additionally, blood glucose was measured on day 22. On day 28th, blood was withdrawn for cytokines (TNF-α and IL-1β) and cardiac biomarker (cTnI) levels estimation using ELISA kits. Major organs (spleen, kidney, heart, lung and liver) and tumor were also isolated, weighted and observed for gross morphology. Hearts were also prepared for histology. Tumors were subjected to histology (H&E) for tumor necrosis evaluation (16,27) for Immunohistochemistry for Ki-67 expression evaluation (28).

6.1.4.2.1 Experimental outcomes

Tumor volume (V) was calculated using the formula of a sphere as follows:

$$V\ (mm^3) = \left[\frac{(D1 + D2)}{2}\right]^3 \text{ x } 0.5236$$

where D1 and D2 were the largest and smallest diameters of tumors respectively.

Percentage test/control (%T/C) was calculated as follows:

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

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 NCI standard criteria, %T/C ≤ 42% indicates acceptable antitumor activity; %T/C ≤ 20% indicates moderate antitumor activity; %T/C ≤ 10% indicates high antitumor activity (29–31).

Mouse body weight changes were calculated as follows:

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

where X was the day of observation.

A dose producing a mean weight loss ≥15% of initial body weight was considered toxic (30,31).

The relative necrotic area (%) of tumor tissues was calculated using a given formula as under.

$$\text{Relative necrotic area (\%)} = \frac{\text{Necrotic area in tumor section}}{\text{Total area of tumor section}} \text{x } 100$$

The relative Ki-67 expression (%) of tumor tissues was calculated using a given formula as under.

$$\text{Ki-67 expression level (\%)} = \text{Average score x } 100$$

6.1.4.3 Orthotopic mouse model of breast carcinoma

Breast tumors were induced using the orthotopic injection of 1 x 10^6 4T1 breast carcinoma cells at the mammary fat pad of Balb/c mice as described earlier (32,33). Animals were divided and treated as shown in Table 1. Tumor diameter using digital vernier caliper and body weights were recorded twice weekly up to 28th day. On 28th day, blood was withdrawn for the estimation of hematology parameters. Animals were sacrificed and tumors were weighed after isolation. Tumor volume, %T/C, body weight and histology data were evaluated as described earlier in section 6.1.4.2.1 (Experimental outcomes).

6.2 Statistical methods

All the data are expressed as mean ± standard deviation. Cell cytotoxicity data were analyzed using one-way ANOVA, followed by Tukey's test. Tumor volume data were analyzed using two-way ANOVA followed by Bonferroni's test. Body weight, organ weights, serum cytokines, biomarker, blood glucose, hematology, histology and immune histology data 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 and $p<0.05$ was considered statistically significant.

6.3 Results

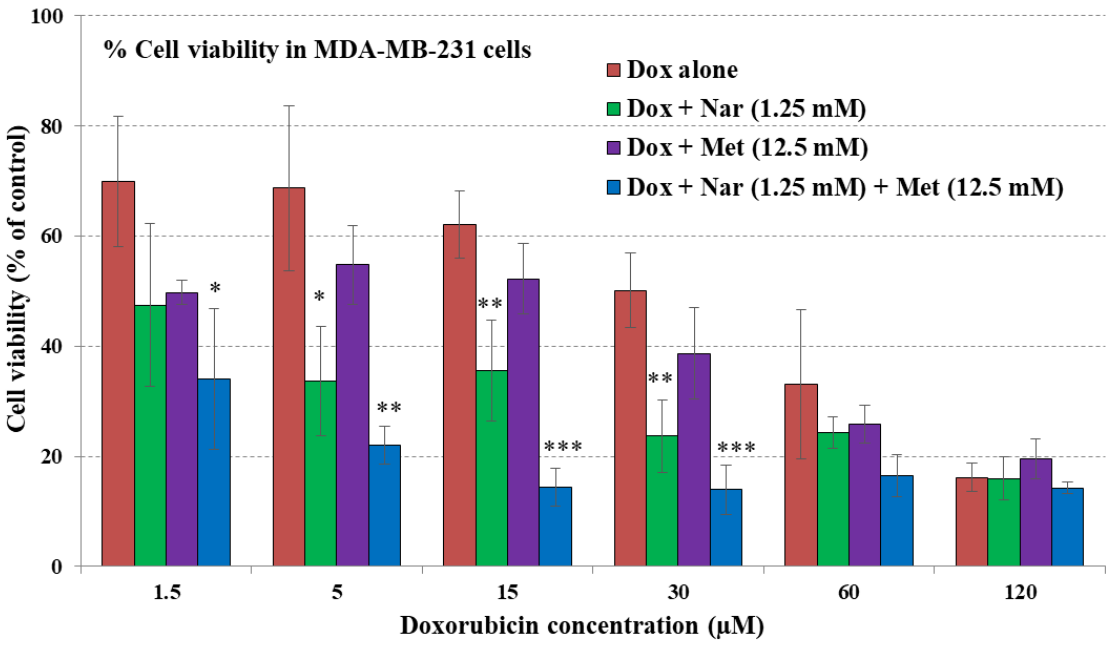
6.3.1 Effect on cell viability using MTT assay (*in vitro*)

The effectiveness of doxorubicin in different combinations was studied in breast cancer cell lines (MDA-MB-231 and 4T1) by treating cells with various concentrations of doxorubicin, keeping constant concentrations of naringenin and metformin. The proliferation of MDA-MB-231 cells was not significantly affected by co-treatment with 12.5 mM metformin and 1.5 to 120 µM doxorubicin when compared with the same concentration of doxorubicin alone. However, co-treatment of naringenin 1.25mM offered improved sensitivity of MDA-MB-231 cells to doxorubicin at 5, 15 and 30 µM ($p<0.01$). Interestingly, the concomitant use of metformin (12.5 mM) and naringenin

(1.25 mM) with doxorubicin at the concentration of 1.5 to 30 µM showed a significant ($p<0.001$) decrease in the cellular viability as compared to doxorubicin alone, suggesting an increased sensitivity of doxorubicin to MDA-MB-231 cells (Fig. 1A). The combination of metformin and naringenin showed a significant increase in efficacy (1.7 to 2.5 fold) of doxorubicin at the concentration of 1.5 to 30 µM but did not show any remarkable increase in efficacy at the concentration 60 µM and 120 µM. This might be because doxorubicin itself has high cell toxicity and therefore, no further scope for the combination treatment at a higher concentration of doxorubicin.

The proliferation of 4T1 cells was not significantly ($p>0.05$) affected by co-treatment with either 6.25 mM metformin or 0.625 mM naringenin with different concentrations of doxorubicin (0.47, 1.88, 3.75, 7.50, 15 and 30 µM, except 0.94 µM) when compared with the doxorubicin alone. Moreover, the combination of metformin (6.25 mM) and naringenin (0.625 mM) together with doxorubicin at the concentration of 0.47 to 30 µM showed significantly ($p<0.001$) lower cellular viability compared to doxorubicin alone, suggesting an increased sensitivity of doxorubicin to 4T1 cells (Fig. 1B).

1A



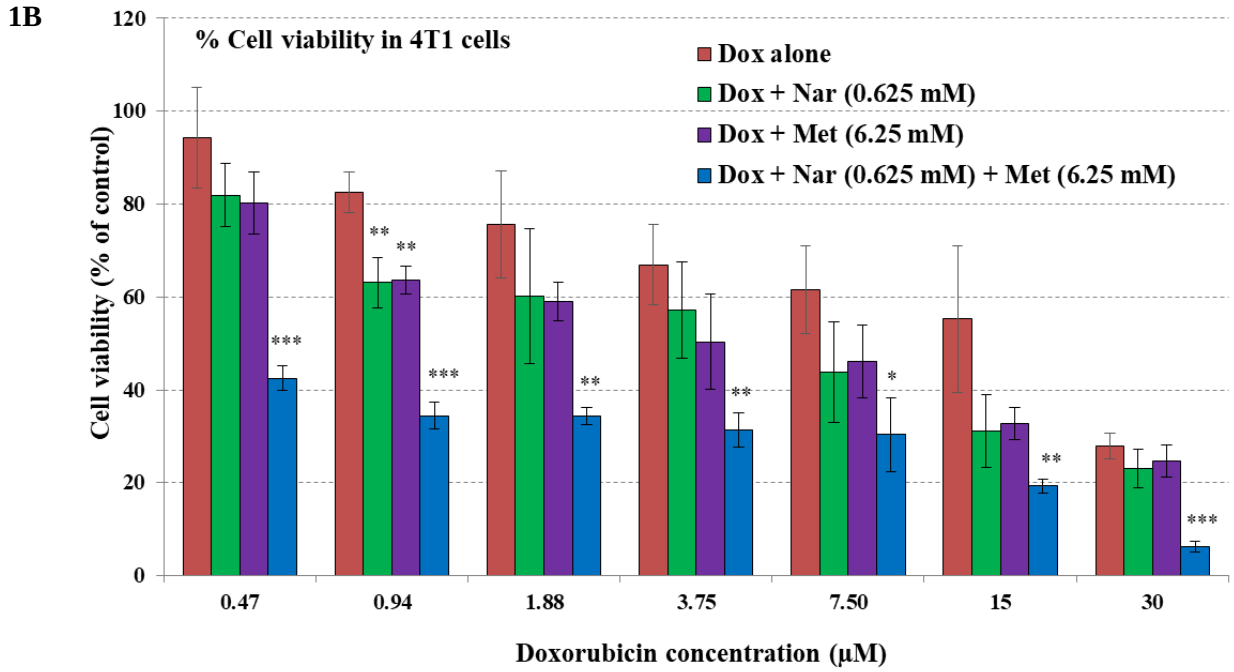


Figure 1: Effect on cell viability (%) after concomitant treatment of naringenin or/and metformin with doxorubicin in breast cancer (A) MDA-MB-231 cells and (B) 4T1 cells

Data are expressed as mean ± S.D. The data were analyzed using one-way ANOVA, followed by Tukey's test (n=3 replicates); Differences were considered to be statistically significant where $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ as compared to the same concentration of doxorubicin alone treatment. Nar- Naringenin, Met- Metformin, Dox- Doxorubicin

6.3.2 Effect on MNU- induced breast carcinoma in rats (*in vivo*)

In MNU- induced breast carcinoma model of rats, a significant reduction ($p<0.05$) in tumor weight was observed in combination treatment groups when compared with the disease control group (Table 2). A higher dose of lipo-dox (4 mg/kg) also showed a significant reduction in tumor weight as compared to the disease control group. At the same time, reduction in mean tumor weight was not found significant in individual drug treatments like naringenin or metformin or lipo-dox 2 mg/kg as compared to a disease control group. The combination of lipo-dox 2 mg/kg with naringenin and metformin showed a marked 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. Besides, concomitant treatment of naringenin, metformin and lipo-dox showed a remarkable increase in the tumor necrotic area when compared with the vehicle control group further supported the efficacy of combination treatment.

Further, 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, although other treatments showed less than 5 % body weight loss in the rat model of MNU-induced breast carcinoma. Besides, no change in blood glucose levels observed in any of the treatment groups may be due to the euglycemic effect

of metformin on non-diabetic animals. In addition to the above, no mortality was observed in any treatment group throughout the study.

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

6.3.3 Effect on xenograft mouse model of breast carcinoma (*in vivo*)

Significant reduction ($p<0.05$) in tumor volume was observed from day 14 onwards in 3 mg/kg lipo-dox treated groups, 6 mg/kg lipo-dox treated group and combinations of naringenin and/ or metformin with 3 mg/kg lipo-dox treated groups as compared to vehicle control in MDA-MB-231-bearing mice. Moreover, combination of treatment (lipo-dox 3 mg/kg + naringenin + metformin) showed significant reduction in tumor volume as compared to lipo-dox 3 mg/kg alone treatment showing synergistic effect ($p<0.01$; Fig. 2A). Tumor weight at the end of the study (day 28) was in line with tumor volume data and thus confirmed the synergistic effect of combination treatment (Fig. 2B & 2C). Antitumor activities of single-drug or combination of drugs were assessed and compared based on %T/C and NCI criteria. In MDA-MB-231- bearing mice, acceptable antitumor activity was observed in lipo-dox 3 mg/kg (%T/C $\leq$ 42). However, a combination of lipo-dox 3 mg/kg with naringenin or a combination of lipo-dox 3 mg/kg with metformin showed moderate antitumor activity (optimal %T/C values were 17.3 and 19.7 respectively). In addition, concomitant use of naringenin and metformin with lipo-dox 3 mg/kg showed highly significant antitumor activity (optimal %T/C values were 9.8) suggesting a synergistic effect of combination treatments. Also, the efficacy of a combination of naringenin and metformin with lipo-dox 3 mg/kg was comparable with highly significant antitumor activity of 6 mg/kg lipo-dox (optimal %T/C values was 5.9). Although, no significant biologically antitumor activity was seen in the naringenin or metformin single-drug treatment throughout the study.

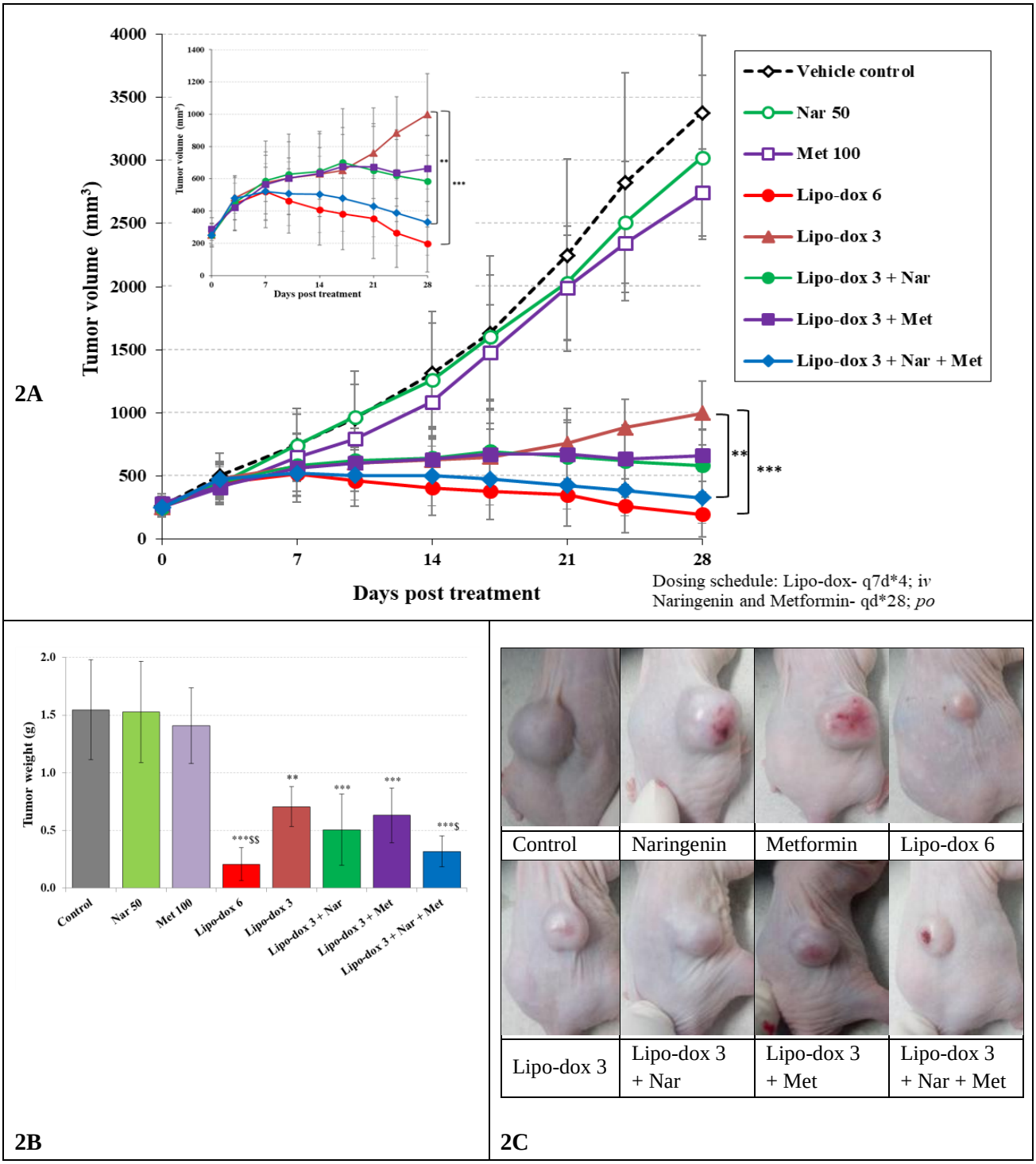


Figure 2: Effect on tumor volume and weight following single or concomitant treatment in MDA-MB-231 xenograft mouse model.

(A) Change in tumor volumes (B) tumor weights and (C) representative figures of xenograft bearing athymic nude mice. Data are expressed as mean ± S.D. n=6. The tumor volumes were measured throughout the study and data were analyzed using two-way ANOVA followed by Bonferroni's test. $^{**}p<0.01$, $^{***}p<0.001$ compared to lipo-dox 3 alone. The tumor weights were taken at the end of the study and data were analyzed using one-way ANOVA followed by Dunnett's test. $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p < 0.001$ compared to the vehicle control group. $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ compared to lipo-dox 3 alone group. Nar- Naringenin, Met- Metformin, Lipo-dox- Liposomal doxorubicin, iv- intravenous, po- per oral, q7d*4- total 4 dose at weekly interval. qd*28- daily for total 28 days.

Further, in MDA-MB-231-bearing mice, no significant decrease in body weight was observed when compared with the baseline (day 0) weight at any dose of the study treatment groups. The maximum decrease in body weight was observed in the lipo-dox 6 mg/kg treated group (8 %); whereas all other treatment groups showed body weight reduction less than 4 %. There was no significant change found in major organ weights and blood glucose levels at selected doses in athymic nude mice. Additionally, mortality didn't observe in any of the treatment groups throughout the study suggesting no significant difference in toxicity profile following mono-therapy and combination of different treatments.

6.3.3.1 Effect on serum cytokines levels and biomarker estimation

Serum TNF-α (**$p<0.01$) and IL-1β (***$p<0.001$) increased significantly in lipo-dox 6 mg/kg as compared to the vehicle control group. Also, TNF-α (*$p<0.05$) and IL-1β (**$p<0.01$) increased in lipo-dox 3 mg/kg compared to the vehicle control group revealing an increase in the pro-inflammatory cytokines in the lipo-dox treatment group. Paradoxically, TNF-α was significantly decreased in lipo-dox 3 mg/kg + naringenin treatment group; while IL-1β significantly decreased ($^{\$}p<0.05$) in lipo-dox 3 mg/kg + naringenin group as well as lipo-dox 3 mg/kg + metformin group. Further, combined treatment of naringenin and metformin with lipo-dox 3 mg/kg produced significant inhibition of both TNF-α ($^{\$\$}p<0.01$) and IL-1β ($^{\$\$\$}p<0.001$) when compared with the lipo-dox 3 mg/kg alone treated group (Fig. 3A & 3B). In addition to the above, no significant change in cardiac Troponin I (cTnI) levels was observed in any of the study treatment groups (Fig. 3C).

6.3.3.2 Histology and Immunohistochemistry findings

The tumor necrosis was determined using ZEN 2 software of H&E stained sections. The necrotic area of combination treatment groups was remarkably higher when compared with the control group. Based on histology (H&E) of the heart, no myocardial abnormality was observed in any of the treatment groups. The expression of Ki-67 in single and different combination treatment groups showed the weakest positive signal in the nucleus in the combination treatment group which indicated that the combination treatment effectively inhibited tumor cells proliferation *in vivo*. To further analyze Ki-67 expression in tumor tissues quantitatively, the parts of proliferating cells were counted carefully. The percentage of proliferating cells of combination treatment groups was reduced compared to single-drug treatment. In contrast, neither naringenin nor metformin alone was able to affect Ki-67 expression in tumors. Finally, the combination of the lipo-dox 3+naringenin+metformin showed significant increase in necrotic area and significant ($p<0.01$) reduction in Ki-67 expression compared to lipo-dox 3 mg/kg alone treatment, further supported the effectiveness of combination treatment.

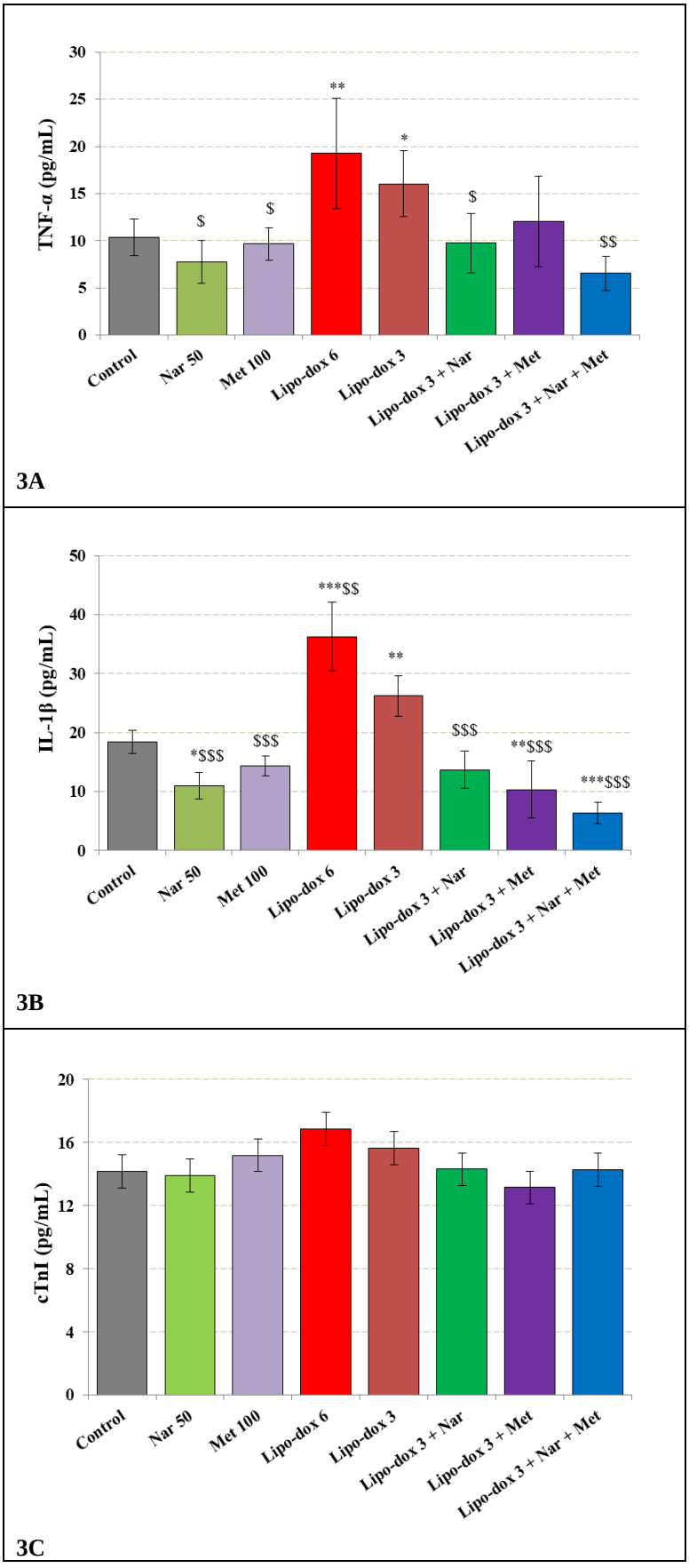


Figure 3: Effect on pro-inflammatory cytokines and cardiac biomarker levels following single or concomitant treatment in xenograft mouse model.

(A) TNF-α (B) IL-1β (C) Troponin I (cTnI) levels. Data were expressed as Mean ± S.D. and analyzed using one-way ANOVA followed by Dunnett's test. n=6. Differences were considered to be statistically significant where $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to vehicle control. $^{\$}p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ compared to lipo-dox 3.

6.3.4 Effect on orthotopic mouse model of breast carcinoma (*in vivo*)

Significant reduction in tumor volume was observed in lipo-dox treated, metformin alone treated 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. 4A). Tumor weight at the end of the study (day 28) showed a similar pattern with tumor volume data and thereby confirming the synergistic effect of concomitant treatments (Fig. 4B & 4C). 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. Acceptable antitumor activity (%T/C $\leq$ 42, based on NCI criteria) was observed in lipo-dox 3 mg/kg in 4T1- bearing mice. A combination of lipo-dox 3 mg/kg either with naringenin or metformin showed moderate antitumor activity (optimal %T/C values were 16.2 and 12.6 respectively). Further, a combination of naringenin and metformin with lipo-dox 3 mg/kg showed highly significant antitumor activity (optimal %T/C values was 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 was 2.8). Besides, concomitant treatment of naringenin, metformin and lipo-dox showed a remarkable increase in the tumor necrotic area when compared with the vehicle control group further supported the efficacy of combination treatment.

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. 5A). 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. Further, 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. 5B).

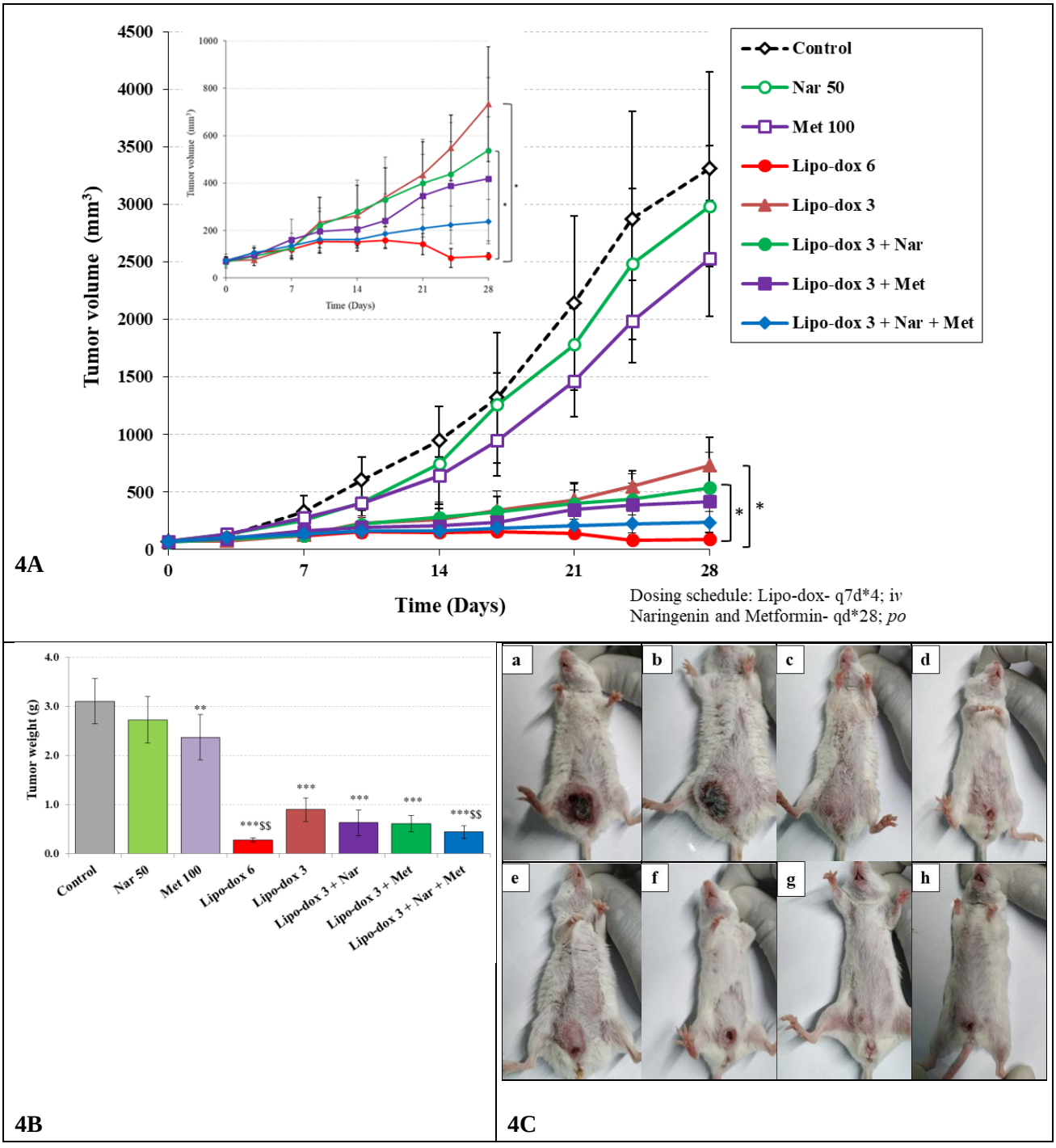


Figure 4: Effect on tumor volume and weight following single or concomitant treatment in 4TI-induced orthotopic mouse model

(A) tumor volume (B) tumor weight (C) representative images tumor bearing mice. Data were expressed as mean ± SD. n=6. Tumor volume data were analyzed using two-way ANOVA followed by Bonferroni's test. $^{*}p<0.05$ compared to 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.

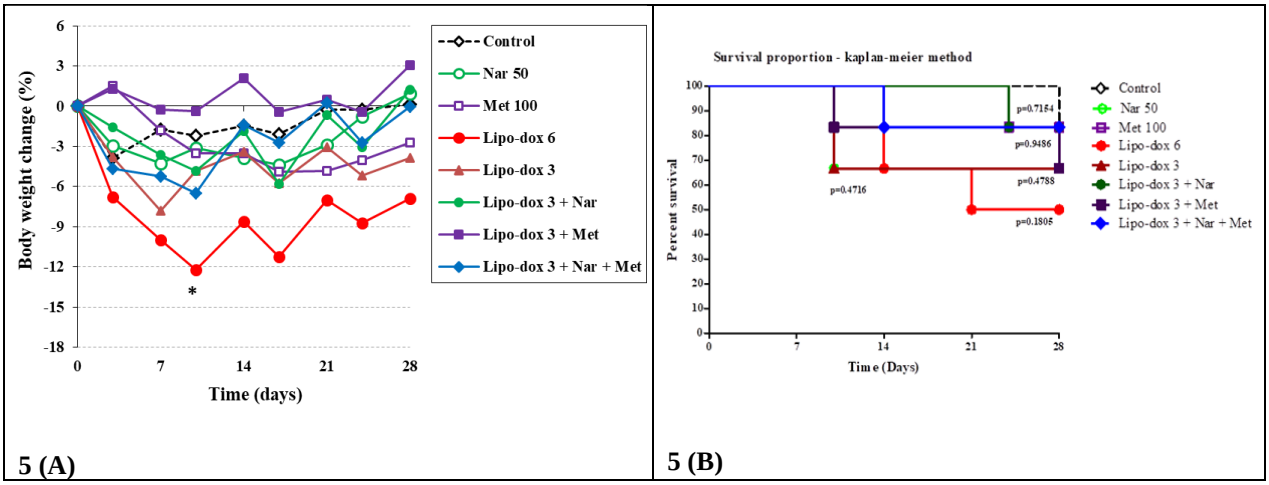


Figure 5: Effect on body weight and animal survival following single or concomitant treatment on orthotopic mouse model

Body weight data were expressed as % change in body weight from initial body weight. The data was analyzed using one-way ANOVA followed by Dunnett's test. $^{*}p<0.05$ compared to initial body weight of same group. 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.

6.3.4.1 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 3). Also, 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 3: Hematology parameters in orthotopic mouse model

Treatment	WBC (x10*3)	Neutrophils (x10*3)	Platelets (x10*3)	HB (g/dl)
Control	574.4 + 146.2	544.5 + 130.4	1069.4 + 320.4	11.5 + 1.1
Nar	493.1 + 85.7	470.1 + 88.4	714.3 + 119.5	10.5 + 1.2
Met 100	546 + 136.1	517.7 + 124	920.5 + 107.5	10.5 + 1.1
Lipo-dox 6	53^{***} + 12.4	45.6^{***} + 10.9	998.7 + 278.4	11.6 + 1
Lipo-dox 3	178.5^{***} + 71.8	166.1^{***} + 70.5	942.2 + 174.5	11.5 + 1.8
Lipo-dox 3 + Nar	185.9^{***} + 82.5	170.7^{***} + 80.4	1052 + 77.8	12.7 + 0.6
Lipo-dox 3 + Met	191.7^{***} + 58	180.1^{***} + 62.7	901.5 + 283.9	11.8 + 2.3
Lipo-dox 3 + Nar + Met	170.2^{***} + 77.4	158.2^{***} + 80.8	876.5 + 137.1	12.6 + 0.6

Blood samples were analyzed with the ADVIA 120 hematology system; Data were expressed as Mean ± SD and analyzed using one-way ANOVA followed by Dunnett's test. n=6. Differences were considered to be statistically significant where $^{***}p<0.001$ compared to control. Nar- Naringenin, Met- Metformin, Lipo-dox- Liposomal doxorubicin

7. Achievements with respect to objectives

In the present *in vitro* studies, we found that combination of naringenin and metformin decreased the cellular viability of breast cancer cells (MDA-MB-231 and 4T1 cells) with doxorubicin, suggesting increased the cytotoxic effects and potentiation of the sensitivity of doxorubicin.

In the present *in vivo* studies, we observed that naringenin alone and metformin alone have no significant anti-tumor effect in animal models of breast carcinoma. However, based on tumor volume and tumor weight data, the combination of naringenin and metformin exhibited synergistic antitumor effects with liposomal doxorubicin. Also based on survival, body weight, hematology, cardiac biomarkers (cTnI), cytokines levels, we observed that chemotherapy using liposomal doxorubicin showed dose-dependent toxicity in the *in vivo* models and which was overcome by the use of a lower dose of liposomal doxorubicin.

8. Conclusion

Based on result, the combination of naringenin and metformin enhanced the anti-tumor activity of doxorubicin in both *in vitro* and *in vivo* models of breast carcinoma. It may serve as a new approach for the treatment of human breast cancers for reducing the dose and subsequently toxicity of chemotherapy.

9. Copies of papers published and a list of publications arising from the thesis

Sr. No.	**Details**
Papers published	
1	Pateliya B, Burade V, Goswami S (2021) Combining naringenin and metformin with doxorubicin enhances anticancer activity against triple-negative breast cancer in vitro and in vivo. Eur J Pharmacol 891:173725. https://doi.org/10.1016/j.ejphar.2020.173725
2	Pateliya B, Burade V, Goswami S (2021) Enhanced antitumor activity of doxorubicin by naringenin and metformin in breast carcinoma: an experimental study. Naunyn Schmiedebergs Arch Pharmacol 16:32:16Z https://doi.org/10.1007/s00210-021-02104-3
Paper presented	
1	Oral presentation titled "Naringenin and metformin enhance the antitumor effect of doxorubicin against experimental models of breast carcinoma" in prize session of ISCOMS (International Student Congress Of bio Medical Sciences) on 8th June 2021.

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