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Acacetin and its Combination with Doxorubicin Inhibit Invasive Potential of Lung Cancer Cells

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Abstract—*Cell migration and invasion are integral process in the metastatic progression of cancers, the major cause of cancer related mortality. Current chemotherapeutic drugs or treatment strategies have limited efficacy in preventing or treating lung cancer metastasis. This study considered how to improve the therapeutic intervention of lung cancer which is a leading cause of cancer mortality worldwide. Herein, we demonstrated that combination treatment of acacetin, a flavonoid and doxorubicin, a standard chemotherapeutic drug, effectively inhibit migration and invasion of lung carcinoma cells. For this study, we have used a relative sub-toxic dose of doxorubicin against lung carcinoma cells. Inhibition of migration and invasion was associated with the modulation of EMT markers. Combination treatment of acacetin and doxorubicin increased the transcript levels of E-cadherin which is an epithelial marker. We further demonstrated that combination treatment decreased the levels of mesenchymal marker, vimentin. β-catenin expression was also decreased by the combination treatment. This study provides a framework for the future investigations involving chemotherapy to combine a naturally occurring small molecule towards the understanding of the therapeutic targets in controlling the advanced lung cancer.*

Keywords*: EMT, acacetin, doxorubicin and metastasis.*

Abbreviations
NSCLC, non-small cell lung cancer; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EMT, epithelial-mesenchymal transition.

1. INTRODUCTION

Lung cancer is the most common type of cancer and poses a major public health issue, and reported for approximately 1.82 million cases and almost 1.59 million deaths worldwide in 2012[1]. Chemotherapy still remains a critical therapeutic strategy for the treatment of lung malignancies. Among different chemotherapies, the doxorubicin chemotherapy is commonly used in treatment regimens of lung cancer. Although, it is effective in the treatment of small cell lung cancer (SCLC), it has not proved beneficial in therapeutic regimen of non-small cell lung cancer (NSCLC) which represents almost 80% of all lung cancers [2]. This failure of lung cancer chemotherapy can be associated with toxicity and drug resistance which may be *de novo* or acquired resistance of cancer cells by different mechanisms including the decreased uptake of water soluble drugs inside cancer cells, increased efflux of drugs outside cancer cells, blocked apoptosis, altered drug metabolism, altered cell cycle regulation, etc [3].

Recently, the role of epithelial-to-mesenchymal transition (EMT) which is an integral part of the process of cancer metastasis in chemoresistance to various drugs have come to the fore. Due to tumor heterogeneity or due to the process of metastasis, EMT can elicit the conversion to a cancer stem cell (CSC) -like phenotype which are known to be more resistant to drugs, thus providing an association between the process of metastasis and drug resistance [4]. Therefore, novel strategies to prevent or revert EMT are warranted to improve therapeutic options for lung cancer patients.

The molecular complexity of cancer and its refractory nature limits the use of monotherapy regimen which is profoundly affected by chemoresistance. With perpetual increase in understanding of the molecular mechanisms implicating the failure of the standard monotherapy treatment, there is a paradigm shift toward combination or cocktail therapy involving two or more therapeutic agents [5]. Combination therapy typically acts on multiple therapeutic targets, thus works in a synergistic, additive or antagonistic manner, and thereby may lower therapeutic dosage of each individual drugs [6]. Investigators have shown the inefficiency of TRAIL monotherapy in SCLC cells while the conventional chemotherapeutic drug doxorubicin significantly sensitized SCLC cells to TRAIL induced apoptosis [7]. Although, there are various combinations of chemotherapeutic agents in clinical trials, however, the leading challenge of combination therapy is still to identify an ideal partner, which could attain synergistic efficacy with maximized tumor response [6].

Small molecules such as flavonoids are known for medicinal properties since 1930. They are polyphenolic compounds, which are naturally presents in all the parts of plants like fruits, vegetables etc. They are safe, easily available and cost effective phytochemicals, which have

shown promising therapeutic efficacy against lung cancers [8]. Flavonoids are emerging as chemosensitizers in combination therapy, because of their non-toxic nature. The development of novel chemosensitizer that can augment the cytotoxicity of doxorubicin at low doses by suppressing resistance mechanism in cancer cells remains a big challenge. Acacetin (5,7-dihydroxy-4'-methoxyflavone) is a flavonoid and naturally available in *Cirisium rhinoceros* Nakai, belongs to Compositae family. It is herbaceous perennial and used in folklore medicine [9]. The aim of this study is to explore the efficacy of small molecule acacetin in combination with doxorubicin against lung cancer migration and invasion potential.

2. MATERIALS AND METHODS:

2.1. Cell Lines and Reagents

The human NSCLC cell lines A549 and H1299 were obtained from National Center for Cell Sciences (NCCS, Pune, Maharashtra, India). RPMI and FBS were purchased from Gibco, Invitrogen. DMSO, mitomycin C, and antibody to β-Actin were purchased from Sigma Aldrich. Trypsin-EDTA solution and antibiotic-antimycotic solution were purchased from Himedia Laboratories. Antibodies to E-Cadherin, Vimentin and β-Catenin purchased from Cell Signaling, Inc. Secondary antibody for monoclonal primary antibodies were also from Cell Signaling, Inc. Enhanced chemiluminescence (ECL) detection system was purchased from Millipore, Merck. TRIsoln Reagent was purchased for GeNei, Merck.

2.2 Wound healing assay

H1299 cells were cultured in 6 well culture plate and incubated at 37°C till 95% confluency. Thereafter, cells were pretreated with 5 µg/ml mitomycin C to stop proliferation. After 3 h of treatment, a linear wound was made by scratching the confluent monolayer with a sterile 200 µl tip. The floating cells in media was washed off twice with fresh media. Cells were treated with DMSO vehicle control, 25 µM acacetin, 10 nM doxorubicin and their combination. The size of wound was recorded at regular interval by taking the photograph at 0 h, 12 h, 24 h and 36 h using an inverted microscope equipped with Olympus digital camera at 100x magnification. Three to five random fields along the linear wound was photographed for each time point. The wound size of each treatment group was compared with 0 h control.

2.3 Cell invasion-migration assay

A549 cells were grown on a 60-mm plate and then treated with DMSO vehicle control, 25 µM acacetin, 10 nM doxorubicin and their combination for 48 h. Cells were than trypsinized, centrifuged and washed with serum free media and counted. Total 40,000 live cells in 500 µl serum free media containing respective treatments were seeded in upper chamber of invasion or migration chamber and was allowed to invade or migrate for 12 h towards the lower chamber filled with complete medium. After 12 hours the culture inserts were processed and then counted the invaded or migrated cells at the bottom of the membrane as published [10]. Photographs were taken using an inverted microscope equipped with Olympus digital camera at 400x magnification and the cells were counted. All the treatments were done in duplicate and repeated thrice.

2.4. Western blot analysis

A549 and H1299 cells were seeded in 60 mm plate and incubated with respective drugs and their combination. After 72 hours of treatment cells were lysed on cell culture dish in non-denaturing lysis buffer supplemented with protease inhibitors. The protein concentrations were determined by the Bradford protein assay, and a total of 60 µg of protein was separated by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk in a 0.1% Tris-buffered saline solution for 1 hour at room temperature. Membranes were then incubated with antibody for E-Cadherin, vimentin, β-catenin and β-actin (loading control) for overnight at 4 °C. After washing, blots were incubated with HRP-conjugated secondary antibodies for 2 hours. The immunoreactive proteins were then visualized using the enhanced ECL detection system on a photographic film.

2.5 RNA isolation and Semi-quantitative RT-PCR.

A549 and H1299 cells were seeded in 60 mm culture plates and treated with acacetin, doxorubicin and their combination for 72 hours. Total RNA was extracted from cells with TRIsoln reagent according to manufacturer's instructions. First strand complementary DNA synthesis was carried out using 5µg of total RNA template by MMuLV reverse transcriptase enzyme. This was followed by 30 cycles of regular PCR in a thermocycler using specific forward and reverse primers for E-cadherin, vimentin, N-cadherin and GAPDH. PCR products were analyzed on 1% agarose gel electrophoresis and visualized under UV transilluminator system. Densitometric analysis were done using ImageJ densitometric software (NIH). The gel band intensities were normalized to GAPDH to calculate fold change from control treatment and mentioned below each band.

2.6 Statistical analysis

Statistical significance of the data were performed using SigmaPlot 8.0 software. The statistical significance of difference between control group and treated groups was determined with Student's t-test. $p < 0.05$ was considered as statistically significant.

3. RESULTS

3.1 Combination of acacetin and doxorubicin suppresses A549 cell migration in trans-well migration assay

Acacetin, a phytochemical is known to inhibit the migratory and invasive potential of human NSCLC cells [11]. Doxorubicin is a known chemotherapeutic drug for the treatment of lung cancer. Here in this study we have used very low dose of 10 nM of doxorubicin for our studies as compared to LD_{50} dose of 100nM in A549 cells at 24 hours [12]. In this experiment, cells were treated with 25 μM acacetin, 10 nM of doxorubicin and their combination. Treatment of NSCLC A549 cells with acacetin and doxorubicin inhibited 72% ($P<0.05$) and 5% of migrated cells, respectively at 48 hours as compared to the DMSO vehicle treated cells. However, in the combination treatment of acacetin and doxorubicin, the migratory potential was further decelerated to 83% ($P<0.05$) of control treatment (**Fig. 1A & B**) at 48 hours. These results demonstrated that combination treatment of doxorubicin and acacetin significantly inhibited the migration of A549 lung carcinoma cells.

3.2 Doxorubicin in combination with acacetin inhibits the migratory potential of NSCLC H1299 cells

We further tested the efficacy of the combination treatment of acacetin and doxorubicin in H1299 cells, which was derived from metastatic lymph node and has mesenchymal phenotype. We observed that the low dose of doxorubicin did not cause any significant inhibition on wound healing capacity of H1299 cells as compared to control treated cells. However, combination dose of acacetin and doxorubicin reduced the ability of the H1299 cells to migrate as indicated by the inability of these cells to cover up the denuded zone (**Fig. 2A)**. Quantitative assessment showed that in combination treatment there was 73%, 61% and 45% wound width at 12, 24 and 36 hours, respectively, as compared to control treatment with only 60%, 50% and 32% wound width (**Fig. 2B**). Interestingly, we observed that acacetin induced the highest 53% inhibition in cell migration in 36 hours as compared to control. It can be further noticed that at each time point from 12 hours to 36 hours, combination treatment consistently decelerated the migration of H1299 cells as compared to doxorubicin only treatment. These results further proves that combination dose of acacetin and doxorubicin inhibited the migratory potential in highly metastatic H1299 cells.

3.3 Combination of acacetin with doxorubicin leads to enhanced inhibition of A549 cells invasion

Cancer cell invasion is a key step in the metastasis process, so we further tested the efficacy of the combination of acacetin and doxorubicin against the invasive property of A549 cells using Boyden chamber assay to study invasion. A549 cells were treated with 25 μM acacetin, 10 nM of doxorubicin and their combination. We observed that doxorubicin treatment alone did not impact much on the invasive capability of A549 cells. Whereas, acacetin treatment alone induced a robust 40% ($P\leq 0.05$) decrease in invasive potential of A549 cells. However, the combination treatment of acacetin and doxorubicin further significantly suppressed 58% ($P\leq 0.05$) invasive potential of A549 cells compared to control (**Fig. 3A & 3B**). These results in consistent with the migration assay, demonstrate the effectiveness of the combination treatment of acacetin and doxorubicin to inhibit lung cancer cell migration and invasion and thus may prevent metastasis.

3.4 Doxorubicin in combination with acacetin modulates molecules associated with EMT

EMT is associated with the morphological and phonotypical conversation of epithelial cells into mesenchymal cells. Cancer cells undergo this transition can acquire resistance towards various therapeutic compounds and become unresponsive to therapy. Transition from epithelial to mesenchymal phenotype leads to loss of epithelial marker like E-cadherin and structural changes like loss of cell polarity and cell-cell junction. Acquired mesenchymal phenotype includes increase in invasion and migration potential, up-regulation of vimentin and N-cadherin [4]. Since we observed a significant inhibition of wound healing, invasion and migration capabilities in human lung adenocarcinoma cells by combination treatment, we further investigated the effect of combination on expression of EMT markers.

Effect of combination treatment of acacetin and doxorubicin were analyzed on transcript levels of EMT markers such as E-cadherin, vimentin and N-Cadherin in both A549 and H1299 cells. After 72 h of treatment of acacetin-doxorubicin combination, we observed a moderate increase in the mRNA expression of E-cadherin in both A549 and H1299 cells. No change was observed at mRNA level of vimentin in both cell lines by combination treatment as compared to the control treatment. There was also no change in mRNA levels of N-cadherin by acacetin-doxorubicin combination treatment in both A549 and H1299 cells as compared the vehicle control treated cells **(Fig. 4)**. We further analyzed the protein levels of these EMT markers in NSCLC cells when treated with the combination of acacetin and doxorubicin. In A549 cells, we observed a slight decrease in protein expression of E-cadherin by combination of acacetin-doxorubicin as compared to control treatment. As H1299 cells which is having a mesenchymal phenotype does not express E-cadherin,so, no expression were observed in any treatment group. However, expression of mesenchymal marker, vimentin was down-regulated in both A549 and H1299 cells by acacetin-doxorubicin treatment at 72 h as compared to vehicle control and doxorubicin treatment alone. Beta-catenin is the downstream target of EMT pathway and also known to induce EMT process. It is also a transcriptional activator of Wnt/ β-Catenin signaling and induces chemoresistance in various lung malignancies [13]. Combination of acacetin–doxorubicin down-regulated the protein expression of β-Catenin in H1299 cells, whereas, it didn't affect the expression in A549 cells **(Fig. 5)**.

Based on the overall observations, combination treatment of acacetin and doxorubicin could reverse EMT by downregulating the mesenchymal markers and upregulating epithelial markers in NSCLC cells.

4. ACKNOWLEDGEMENTS

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5. DISCUSSION

Lung malignancies represents an enormous burden on the public health worldwide, ranking among the topmost malignancies in the world. Survival rates of lung cancer is dismal among all cancers [1]. 5 year survival rates is almost 70% for surgically treated lung cancer patient in stage IA, whereas it reduces to almost 2-13% in patients detected with stage IV disease [14]. Lung cancer has already metastasized locally or systemically in these patients, hence, surgery is not an option and they receive only chemotherapy or targeted therapy or radiation therapy [15]. However, owing to tumor heterogeneity, these treatment are fraught with problems of drug resistance and systemic toxicity.

Recent advances in basic and translational cancer research have improved our understandings, in comprehensive detail about the molecular mechanisms of drug resistance. Notably, the nexus between the process of EMT and CSC provide us with the understanding of the development of chemoresistance and their clinical outcome in cancer. In the process of EMT, cancer cells loses its epithelial characteristics to gain mesenchymal traits (similar to the embryogenesis process), thus promoting metastasis [16]. Interestingly, the process of EMT programme enables the conversion of a normal cancer cells to a CSC's [17]. Technical advances in the stem cell biology area has proven that these CSCs are more resistant to the therapeutic drugs as compared to the non-CSC cells or the normal carcinoma cells [18,19]. Thus, the conventional therapeutic strategies are only able to eliminate the bulk of the non CSCs, leaving behind the minor sub-population CSCs which further cause cancer relapse [20]. Thus, therapeutic regimen targeting these process of EMT might be a promising strategy for cancer therapy.

Combination treatment of two or more therapeutic agents as compared to the monotherapy, targets multiple signaling pathways or process has become a common treatment regimen for many cancers [21]. The problems of resistance caused by the process of EMT in CSC could be successfully circumvented by combination therapy approach [22]. In combination therapy, some therapeutic agents may sensitize the cancer cells to lower doses of conventional therapeutic drugs as compared to the normal clinical doses and thus may reduce the side effects of systemic toxicity. Many phytochemical are known to the sensitize the cancer cells to conventional therapeutic drugs and are in clinical trials [23,24].

In this study we evaluated the combination of a standard chemotherapeutic drug, doxorubicin with acacetin, a known phytochemical having inhibitory property against the migratory and invasive potential of lung carcinoma cells. We further used a lower concentration of doxorubicin as compared to the conventional dose used for lung carcinoma cells, thus excluding the possible problem of drug resistance and also systemic toxicity. Acacetin is selectively non-toxic to cancer cells as compared to the normal cells. We observed that doxorubicin slightly enhanced the anti-migratory potential of acacetin in A549 lung carcinoma cells. Also, the combination treatment inhibited the migratory potential of highly invasive H1299 carcinoma cells as compared to doxorubicin alone treatment. Similarly, acacetin also enhanced the anti-invasive potential of doxorubicin in A549 lung carcinoma cells. Further combination treatment also targeted the EMT markers, which are associated with the metastasis of lung cancer cells. We observed that combination treatment decreased the transcript levels of E-cadherin whereas decreased the protein levels of vimentin. The protein level of β-catenin, which activates the EMT programme and increases chemoresistance, was also decreased in lung carcinoma cells.

In conclusion, our results point to the efficacy of the combination treatment of acacetin and doxorubicin in inhibiting the process of cell migration and invasion and thus circumventing the process of metastasis. Combination treatment also targeted the EMT markers in lung carcinoma cells, thus EMT process could be an appealing target for the therapeutic regimen in advanced lung cancers.

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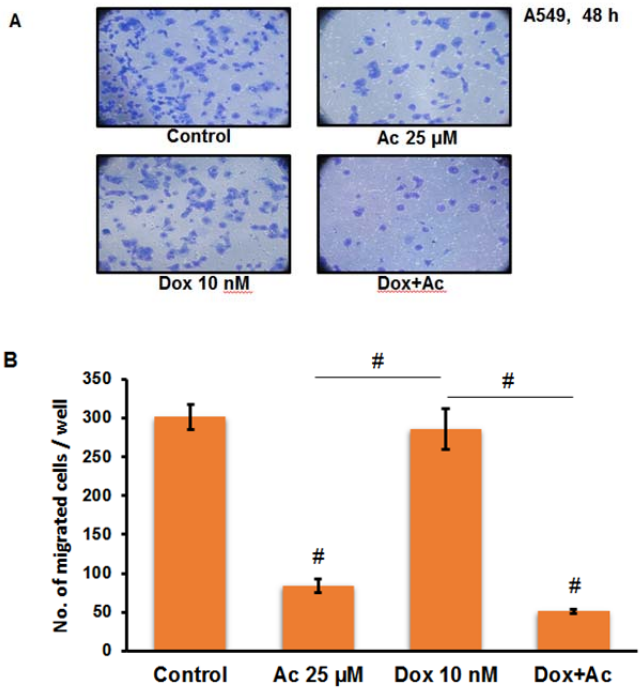
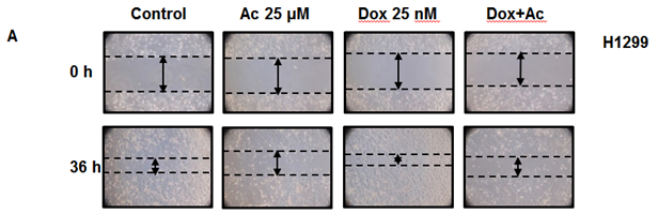


Fig. 1: Effect of combination of acacetin and doxorubicin on migration of A549 cells. A549 cells were grown and treated with 25 µM acacetin, 10 nM doxorubicin and their combination. After 48 h, equal number of live cells from each treatment were seeded into culture inserts. Cells were allowed to migrate for 12 h, processed and counted at 200x magnification, and photographed. Seven independent areas were selected in each sample. (A) is representative images depicting the effect of various treatments on A549 cells migration through Boyden chamber. (B) is quantitative representation of the effect of treatments on migration of A549 cells. Ac, acacetin; Dox, doxorubicin. The quantitative data shown are Mean ± SE of two samples for each treatment. P<0.05 (#).



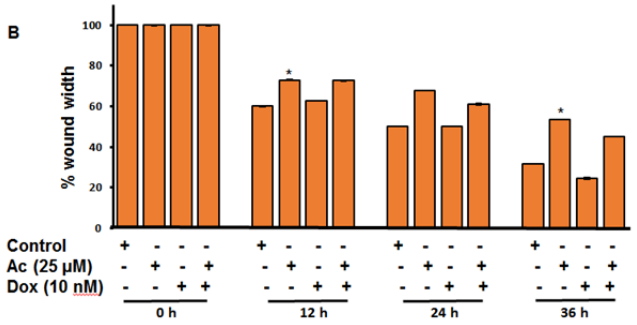


Fig. 2: Effect of combination of acacetin and doxorubicin on cell wound closure in H1299 cells. Cells were grown till 90% confluency, followed by a scratch on cell monolayer by a sterile micropipette tip and immediately treated with 25 µM acacetin, 10 nM doxorubicin and their combination. Therefore, wound healing was visualized by comparing photographs taken at 0 h and then at 12 h, 24 h and 36 h later. (A) is representation images of wound closure taken at different time points by inverted phase contrast microscope. (B) is quantitative data representing the quantitative effect of respective treatment on migration or wound closure of H1299 cells at different time points. Three different area of each wound were measured. Data are shown as percent wound width compared to 0 h control group. Ac, acacetin; Dox, doxorubicin. P< 0.05(*).

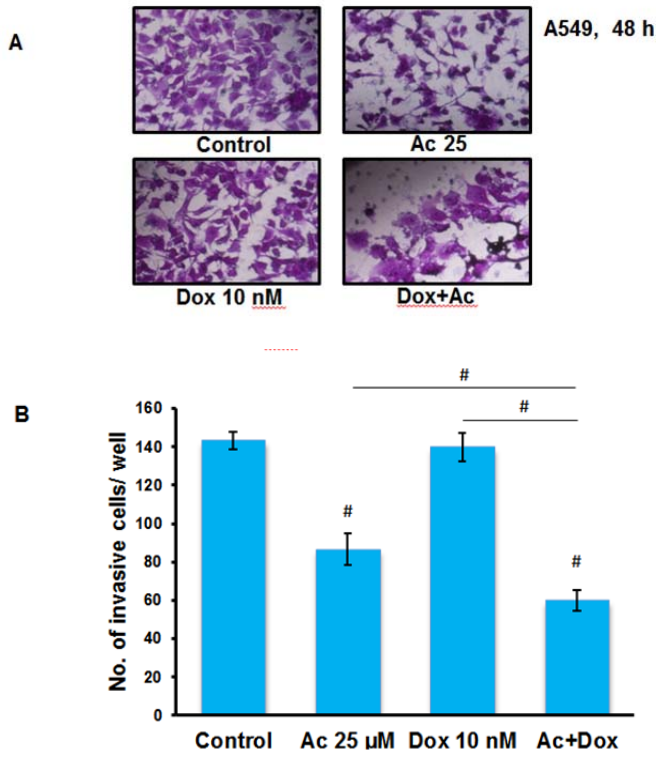


Fig. 3: Combinatorial effect of acacetin and doxorubicin on invasive potential of A549 cells. A549 cells were treated with DMSO vehicle control, 25 µM acacetin, 10 nM doxorubicin and their combination for a period of 48 h. At the end of the treatment, equal number of live cells from each treatment were seeded into the culture inserts. Cells were kept in incubator at 37 °C and allowed to invade. After 12 h, cells were processed and stained with crystal violet. Cells were counted at 200x magnification, and photographed. Five independent areas were selected from each sample. (A) is photographic images showing the effect of various treatments on invasive potential of A549 cells. (B) is quantitative analysis of the effect of various treatments on invasive property of A549 cells. Ac, acacetin; Dox, doxorubicin. The quantitative data shown are Mean ± SE of two samples for each treatment. P<0.05 (#).

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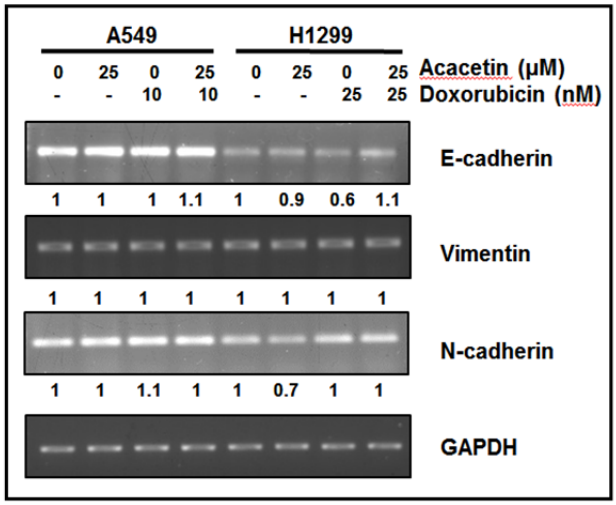

Fig. 4: Combinatorial effect of acacetin and doxorubicin on expression of mRNA involved in EMT process in NSCLC cells. A549 and H1299 cells were treated with DMSO vehicle control and mentioned concentration of acacetin, doxorubicin and their combinations. After 72 h, mRNA was isolated and reverse-transcribed. The resulting cDNA were amplified by regular PCR as indicated in the materials and methods. The reaction products were separated in 1% agarose gel and visualized by EtBr staining.

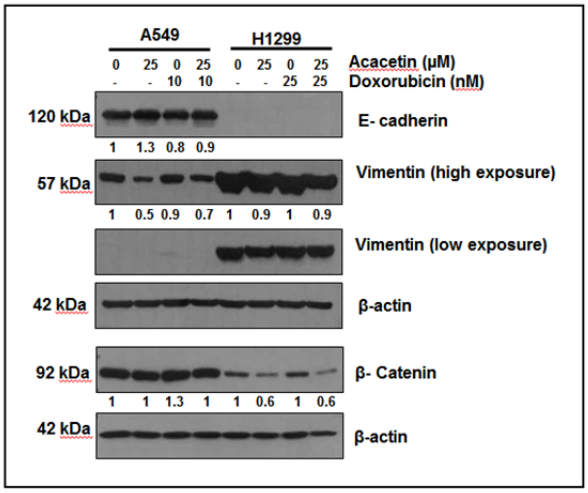

Fig. 5: Effect of combination of acacetin and doxorubicin on EMT markers in NSCLC cells. A549 and H1299 cells were cultured and treated with DMSO vehicle control, 25 μM acacetin, doxorubicin (10 nM for A549 cells and 25 nM for H1299 cells) and their combinations. After 72 h, cell lysate were prepared and subjected to immunoblot analysis with antibodies for the indicated EMT markers. β-Actin was used as internal loading control. Densitometric value of bands are shown as fold change below the band.