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Evaluation of growth inhibitory response of Resveratrol and Salinomycin combinations against triple negative breast cancer cells

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ABSTRACT

Resveratrol (RSVL) a dietary phytochemical showed to enhance the efficacy of chemotherapeutic drugs. Recently, Salinomycin (SAL) has gained importance as cancer therapeutic value for breast cancer (BC), however, its superfluxious toxicity delimits the utility. Taking the advantage of RSVL, the therapeutic efficacy of RSVL and SAL combination was studied *in vitro* and *in vivo* system. Firstly, the synergistic combination dose of RSVL and SAL was calculated and further, the efficacy was examined by wound healing, and Western blots analysis. Further, *in vivo* study was performed to confirm the effect of colony formation and apoptosis detection by flow cytometry based assays. Further, the molecular mode of action was determined at both transcript and translational level by quantitative Real Time PCR combination in Ehrlich ascitic carcinoma model.The combination of IC20 (R20) of RSVL and IC10 (S10) dose of SAL showed best synergism (CI < 1) with ~5 fold dose advantage of SAL. Gene expression results at mRNA and protein level revealed that the unique combination of RSVL and SAL significantly inhibited epithelial mesenchymal transition (Fibronectin, Vimentin, N-Cadherin, and Slug); chronic inflammation (Cox2, NF-kB, p53), autophagy (Beclin and LC3) and apoptotic (Bax, Bcl-2) markers. Further, i *n vivo* study showed that low dose of SAL in combination with RSVL increased life span of Ehrlich ascitic mice. Overall, our study revealed that RSVL synergistically potentiated the anticancer potential of SAL against triple negative BC.

1. Introduction

Breast cancer is the most dreadful diseases among women worldwide [1]. In which, United States of America, India and China account for almost one third of the global breast cancer burden [1]. Triple negative breast cancer (TNBC) is one of the most aggressive intrinsic subtype, which accounts for more than 10% among all breast cancer subtypes [2]. The aggressiveness of TNBC is usually known due to the absence of hormonal receptor expressions [2], and high metastatic potential compared to receptor positive breast tumors [3]. Hence, the treatment of TNBC by receptor mediated conventional therapies doesn't act for therapeutic intervention in this breast cancer subtype [4]. Beside these, TNBC also possess high epithelial mesenchymal transition (EMT) ability of cancer cells, which may significantly enhance metastatic potential.

It has been recently demonstrated that salinomycin (SAL), an antibiotic reduces the proportion of breast cancer stem cells (BCSCs) at least 100 times more efficiently than Paclitaxel, a well known chemotherapeutic drug used in cancer treatment [5,6]. *In vivo* studies also showed that the SAL treatment increased epithelial differentiation of the tumor cells and inhibited mammary tumor growth [5]. Kai et al. showed that SAL act as promising drug for the eradication of TNBC cells in combination with histone deacetylase inhibitor (LBH589) [7]. It is also showed that SAL acts as a promising agent to inhibit EMT [8]. In the recent studies, SAL has reported as potent anticancer agent, because it inhibits *p*-glycoprotein transporters and thus, limits drug resistance in several types of cancer [8,9]. SAL also sensitizes cancer cells towards radiation or cytostatic drugs, such as etoposide or doxorubicin [10,11]. Despite the high anticancer effects of SAL against many cancer types, organ toxicity or other side effects limits its uses as promising anticancer agent [12,13]. Thus, to utilize the anticancer potency of SAL, it is very important to reduce the

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dose of this drug to minimize toxicity without altering its anticancer efficacy.

The natural dietary agents, including phytochemicals proved as novel agents for cancer chemoprevention [14,15]. In the recent past, phtyochemical emerged as an associated molecules, which is not only synergistically enhance anticancer property of chemotherapeutic drugs but also minimize their side effects [16,17]. Resveratrol (RSVL) is a well known naturally occurring compound, that has been suggested to have marked chemopreventive and chemotherapeutic properties [17]. RSVL reduces cell proliferation and tumor growth, chemical carcinogen-induced epithelial cell transformation, cell migration, invasion, metastasis and angiogenesis, and induces cell cycle arrest [18,19].

RSVL also known to potentiate anticancer effects of many anticancer drugs including doxorubicin, paclitaxel and cisplatin [20–22]. Inspired by these studies, the combination of both SAL and RSVL was taken to analyze their anticancer efficacy against TNBC cells. In the present study, *in vitro* experiments were performed to determine synergy between SAL and RSVL in TNBC cells to establish the clinical utility of this novel combination against TNBC cases. We also analyzed the expression of EMT, inflammatory and autophagy associated marker modulated by this novel combination. Moreover, we also evaluated the effect of this combination in the Ehlrich ascetic mice model.

2. Material and methods

2.1. Chemicals

Salinomycin, Resveratrol, 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1680 medium and RNA isolation kit (Cat No. 83913-1EA) were purchased from Sigma-aldrich (St Louis, MO, USA). Medium 171 (Cat no. M-171-500), Mammary epithelial growth supplement (MEGS) (Cat No.S-015-5), 0.25% Trypsin-EDTA (1X) Phenol Red (Cat No. 25200-072), Fetal Bovine Serum, certified, heat inactivated, US origin (Cat No.10082-147), 100X antibiotic-antimycotic (Cat No. 15240-062) were purchased from Gibco/Thermofisher scientific, FITC Annexin V Apoptosis kit I (Cat No.556547) purchased from BD Biosciences (San Diego, CA, USA). The polyvinylidene fluoride membrane (PVDF) and Chemoluminescent HRP substrate (Cat No. WBKLS0500) were sourced from Millipore (Bedford, MA, USA). The entire antibodies used were purchased from Cell Signaling Technology (Beverly, MA, USA). Other chemical used were procured locally of analytical grade.

2.2. Cell culture

MDA-MB-231 cell lines were procured from National centre for cell sciences, Pune, India. Monolayer culture was maintained in RPMI1640 supplemented with 10% FBS, 1% antimycotic/antibiotic and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Stock solutions of RSVL and SAL were prepared in dimethylsulphoxide (DMSO) and diluted with the same medium until <0.1% concentration of DMSO was achieved.

2.3. MTT assay and synergistic analysis

To determine the effect of RSVL and SAL combination we performed the MTT assay. 1×10^4 MDA-MB-231 Cells were seeded in 96 well plate with dose treatment of 16 different combinations (below IC_{50} of each concentration of RSVL and SAL) of drugs for 48 h (Table 1). After that MTT assay was done. The Absorbance was taken at 570 nm using FLUOstar Omega plate reader (BMGLABTECH). Further, drug interactions in both of cells were analyzed by Chau Talalay method (CompuSyn Sofware) and data were visualized in the isobologram [23].

Table 1
Combination index of RSVL and Sal combination in MDA-MB-231 treated cells.

Combination Dose	RSVL (μM)	SAL (μM)	Combination Index (CI)
R10S10	36.0	1.34	0.54839
R10S20	36.0	2.68	0.54450
R10S30	36.0	4	0.65292
R10S40	36.0	5.34	0.76220
R20S10	72.0	1.34	0.52184
R20S20	72.0	2.68	0.48956
R20S30	72.0	4	0.66231
R20S40	72.0	5.34	1.00750
R30S10	108.0	1.34	0.22378
R30S20	108.0	2.68	0.37569
R30S30	108.0	4	0.24814
R30S40	108.0	5.34	0.20227
R40S10	144.0	1.34	0.19710
R40S20	144.0	2.68	0.21626
R40S30	144.0	4	0.24955
R40S40	144.0	5.34	0.24955

Abbreviations: R10, R20, R30 and R40 are indicated IC10, IC20, IC30, IC40 values of RSVL and S10, S20, S30 and S40 are indicated IC10, IC20, IC30, and IC40 values of SAL, respectively.

2.4. Tumorosphere culture

Monolayer culture of actively dividing MDA-MB-231 cells was grown on T75 flask till 90% of cell confluency. Cells were scratched and spin at 2500× rpm. Cells pellet was washed 3× with culture grade 1× PBS. Further, 2×10^5 cells were counted in automated cell counter (Invitrogen) and grown in tumorospheres forming Medium 171, supplemented with mammary epithelial growth supplement (MEGS) and 1% antibiotic and antimycotic. Cells were exposed to drug for 48 h and then removed. Further, fresh media was added to each well and left for at least 14 days. The images of tumorospheres were captured in bright field (20X) with Olympus IX 51 microscope (Olympus America Inc., USA.)

2.5. Wound healing and Colony formation assay

Wound healing assay was performed as described previously [24]. For colony formation assay actively growing MDA-MB-231 cells were equally seeded (1000 cells/well) in six well plate and exposed to drugs for 48hr, and then, drugs were removed and fresh media was added to each well. Further, cells were incubated for 14 days with media changed at every 3rd days. After 14 days, media were removed and wells were washed gently with 1× PBS. Cells were fixed with 70% methanol and stained with crystal violet for 5 min, further wells were washed with PBS and images were acquired. The experiment was performed in triplicates.

2.6. Determination of apoptosis

The cellular apoptosis by the effect of drugs was determined by DNA fragmentation and chromatin condensation as marked after staining with DAPI (10ng/ml) and fluorescence emitted was capture by microscope Olympus IX 51 (Olympus America Inc., USA.) after excitation at 350 nm. Further apoptosis was quantified by sub G1 population and Annexin-V/PI (+) population in flow cytometry study. The procedure for flow cytometric studied was followed as described earlier [24].

2.7. RNA extraction and quantitative Real time PCR

Total RNA was isolated from monolayer and tumorosphere cultures of MDA-MB-231 cells as described in RNA isolation kit and

Table 2
Effects of RSVL and/or SAL on survival of Ehrlich carcinoma mice.

Groups	Dosing schedule of treated drugs	Mean Survival time[a] (days)
Group1	Control	–
Group 2	EAC tumor-bearing untreated mice	20.5±3.86
Group 3	RSVL (10 mg/kg b.wt.)	35.33±6.10
Group 4	SAL (0.5 mg/kg b.wt.)	40.67±10.06
Group 5	RSVL (10 mg/kg b.wt.) & SAL (0.5 mg/kg b.wt.)	55±6.61

[a] Data represented as mean ± S.D of n = 6 animals.

stored in aliquots at −80 °C until analysis. RNA yield and purity were determined using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The expression of marker genes associated with EMT, inflammation, autophagy, and apoptosis done by SYBR Green qRT-PCR (Applied Biosystem) as described according to manufacturer instructions. Data were normalized to GAPDH Ct values from the same sample and the fold-changes in gene expression were calculated by using the delta–delta Ct method. The sequences of forward and reverse primers used are given in Supplementary Table S2.

2.8. Western blots analysis

Western blotting was performed by earlier described method of Rai et al. [24]. Proteins were separated on 10% SDS polyacrylamide gel, transferred to PVDF membranes, blocked by 1× blocking buffer (Sigma Aldrich) and probed with primary antibodies (E-cadhrin, Fibronectin, Vimentin, N-cadhrin, Slug, Cox-2, NF-kB, P53, Beclin, LC3, Bax, Bcl-2 and β-actin) in blocking buffer overnight at 4 °C. Subsequently, membranes were incubated with HRP (horse-radish peroxidase) conjugated secondary antibodies and signals were visualized with enhanced chemiluminescence on VersaDoc 4000 MP system (Bio-Rad). Membranes were reprobed with β-actin (Cell Signaling Technology) antibodies for taking as the loading control.

2.9. Animals and treatments

Swiss albino mice (male, 25–30 g body weight) were obtained from the animal breeding colony of CSIR-Indian Institute of Toxicology Research, Lucknow, prior ethical approval for the experiment was obtained from Institutional Animal Ethical Committee (IAEC, CSIR-IITR). Animals were maintained at standard conditions. Further, exponentially grown 1×10^6 EAC cells collected from peritoneal cavity from ascitic mice (after the 10th day of ascites induction in healthy mice), and were inoculated into the peritoneal cavity of fresh Swiss Albino mice as described earlier [25]. Prior injection of EAC cells, these cells were separated from macrophage cells and counted by trypan blue exclusion test. Swiss albino mice were categorized into five groups comprising 6 animals in each group (Detail of group mentioned in Table 2) and

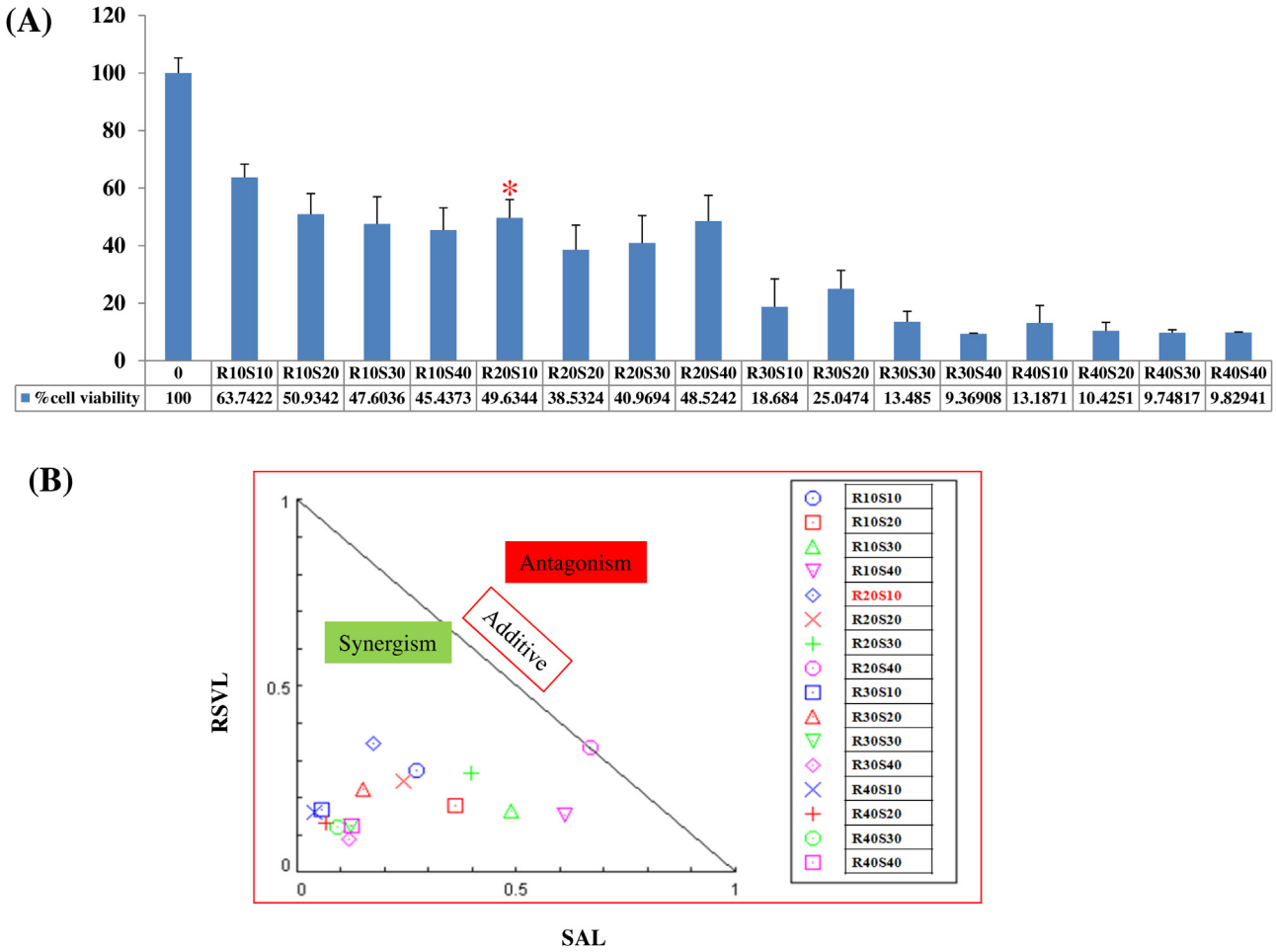

Fig. 1. Effects of RSVL and SAL combination through Cell viability assay, (A) Bar diagram represent percentage cell viability after exposure with 16 different combination of RSVL and DOX treatment in MDA-MB-231 cells, (B) Isobologram data were taken for the selection of best combination with synergistic effects in MDA-MB-231 cells. *R20S10 combination showed best synergistic combination based on CI value (Table 1) and Isobologram analysis.

treated with different doses of SAL (0.5 mg/kg b.wt.), RSVL (10 mg/kg b.wt.) and RSVL (10 mg/kg b.wt) + SAL (0.5 mg/kg b.wt.) and changes in body weight was observed up to 21 days from the day of treatment. After 24 h of injecting EAC cells, animals were treated with different doses (mentioned in Table 2) of drug and phytochemical, two times in a week through intraperitoneal (*i. p.*) route. Animals of group 1 and 2 were treated with saline (0.9% NaCl) solution. All the animals were monitored regularly for ascitic tumor growth and their overall survival till death. Mean survival time and percentage increase in life span were calculated as follows:

$$\text{Mean survival time(MST)} = \frac{\sum \text{Survival time(days) of each mouse}}{\text{Total number of mice}}$$

$$\text{And, \%Increase in Life Span(\%ILS)} = \frac{T - C}{C} \times 100$$

Where, T is the number of days the treated animals survived and C is the number of days the control animals survived.

2.10. Statistical analysis

One-way ANOVA was performed for the significance (SPSS package ver.10) and p value <0.05 was considered as significant.

3. Results

3.1. Combination of RSVL and SAL synergistically inhibit the proliferation of TNBC (MDA-MB-231) cells

Cell viability assay of both RSVL and SAL was performed on MDA-MB-231 cells to compute dose of inhibitory concentration (IC) values (Data not shown). Following the IC values calculation, 16 different combinations of RSVL and SAL were selected below their IC50 values to evaluate combination index in MDA-MB-231 cells by exposing these combination for 48 h (Fig. 1A & B, Table 1). The synergism was calculated for the effective dose analysis using Chau Talay method by CalcuSyn software [23]. The analyzed data showed that the combination of IC20 of RSVL (72 μM) and IC10 (1.34 μM) dose of SAL were effective and showed best synergism ($CI < 1$). This combination also showed potent growth inhibition with ~ 5-fold dose advantage of SAL.

3.2. Synergistic dose of RSVL and SAL effectively reduced wound healing, colony and tumorosphere forming capability in TNBC cells

Wound healing capability in MDA-MB-231 cells exposed to synergistic dose of RSVL (IC20) and SAL (IC10) was calculated in both time and dose dependent manner. As compared to untreated control, the percentage wound healing capacity in

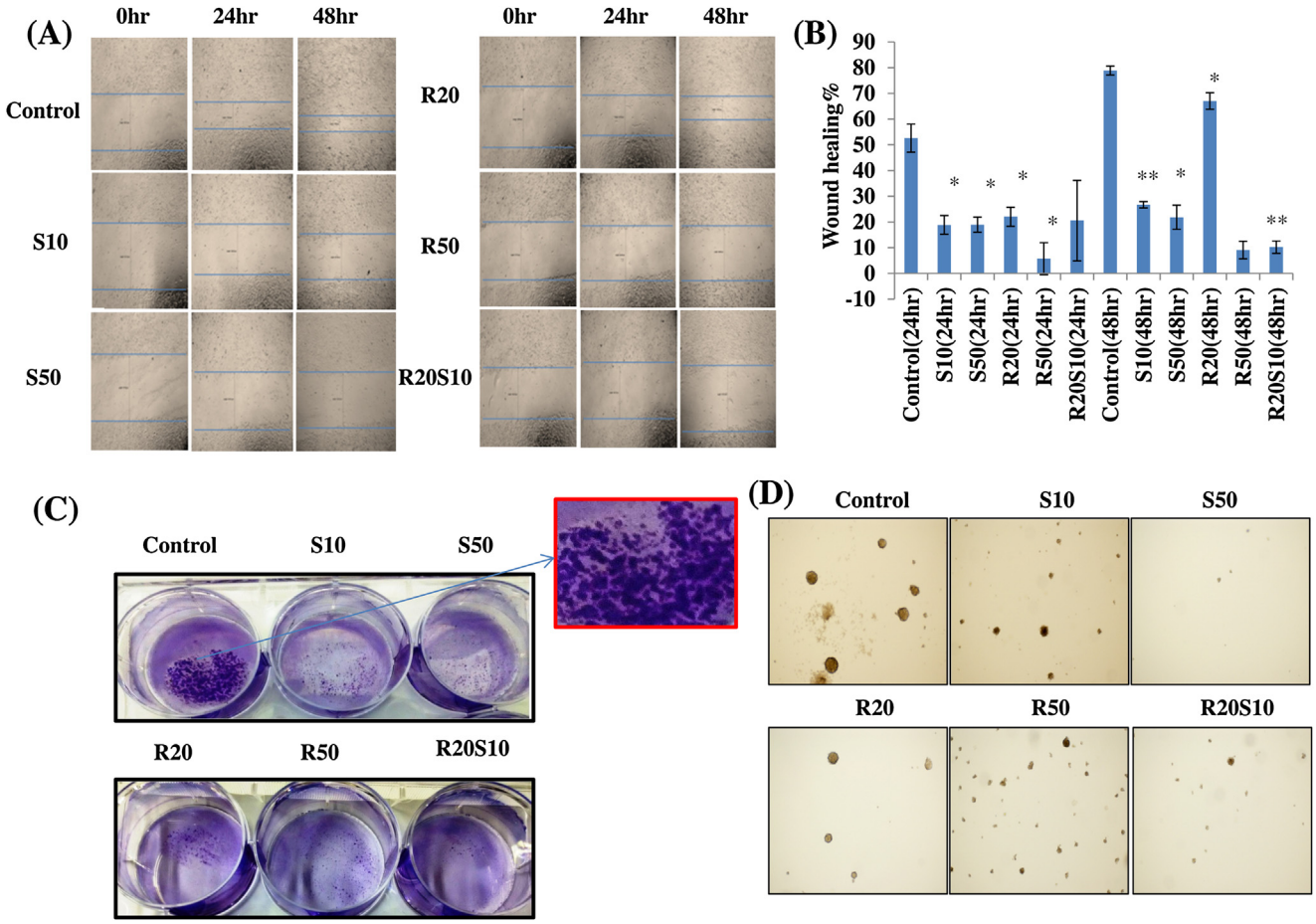

Fig. 2. (A) Dose and time dependent effects of RSVL and SAL in alone and combination on wound healing ability in MDA-MB-231 cells (4x Microscopic image) (B) Bar diagram represents wound healing ability in MDA-MB-231 cells reduced effectively by combination dose of RSVL and SAL after 48hr. exposure. (*$p < 0.05$). (C) Sensitivity of MDA-MB-231 Cells to alone and combination doses of RSVL and SAL in colony formation ability. Colony formation assay was performed in triplicate (D) Effects on sphere forming ability in MDA-MB-231 cells.

combinatorial exposure was found to be reduced after 24 h scratch generation by 20.5% (~4.18 folds), while in alone treatment with corresponding doses of SAL and RSVL reduced it by 18.8% (~3.58 folds), and 22.03% (~3.84 folds), respectively. However, after 48 h of time interval, wound healing capability in combinatorial dose was much more reduced 10.2% (~7.7folds) and corresponding alone treated dose of SAL and RSVL showed% wound healing of 26.69% (~2.95 folds) and 67.04% (~1.18 folds) (Fig. 2A & B, Supplementary Table S1). Colony formation and tumorosphere forming capacity was also found to be significantly reduced in combinatorial dose as compared to alone doses of RSVL and SAL (Fig. 2C & D).

3.3. Synergistic dose of RSVL and SAL induced apoptosis in TNBC cells

Chromatin condensation and DNA fragmentation reported to be key hall mark of terminal stages of apoptosis [26] were found to be significantly enhanced in MDA-MB-231 cells treated with combined dose of RSVL and SAL as compared to their corresponding alone doses (Fig. 3A). Moreover, It was further confirmed by increased Annexin V (+) and sub-G1 population in RSVL+SAL treated MDA-MB-231 cell as compared to control and alone doses (Fig. 3B–D)

3.4. RSVL+SAL modulated the mRNA expression level of apoptotic, autophagy, inflammatory and EMT associated markers in monolayer and tumorospheres culture

To explore the molecular pathways modulated by combinatorial treatment of RSVL and SAL in TNBC cells, the differential expression of genes associated with apoptotic, autophagy, inflammatory and EMT markers were evaluated by qRT-PCR analysis in monolayer and tumorospheres cells. In this study, the expression of AKT was decreased in both monolayer and tumorospheres cells as compared to alone treated cells. Combinatorial treatment of RSVL and SAL was also found to induce a significant upregulation of PTEN gene in both cultures as compared to untreated and alone treated TNBC cells. On the other hand, the synergistic combination was found to decrease the expression of P21 and P53 in both culture conditions in comparison to all other groups. Moreover, synergistic combination of RSVL and SAL was showed to induce apoptosis in both culture conditions by significant upregulation of Bax with decreased Bcl-2 expression as comparison to untreated and alone drug treatments. Beside these, the synergistic combination of RSVL and SAL was also found to significantly suppress the net autophagic flux (LC3 and Beclin) with low inflammatory response (COX-2) in MDA-MB-231 cells as

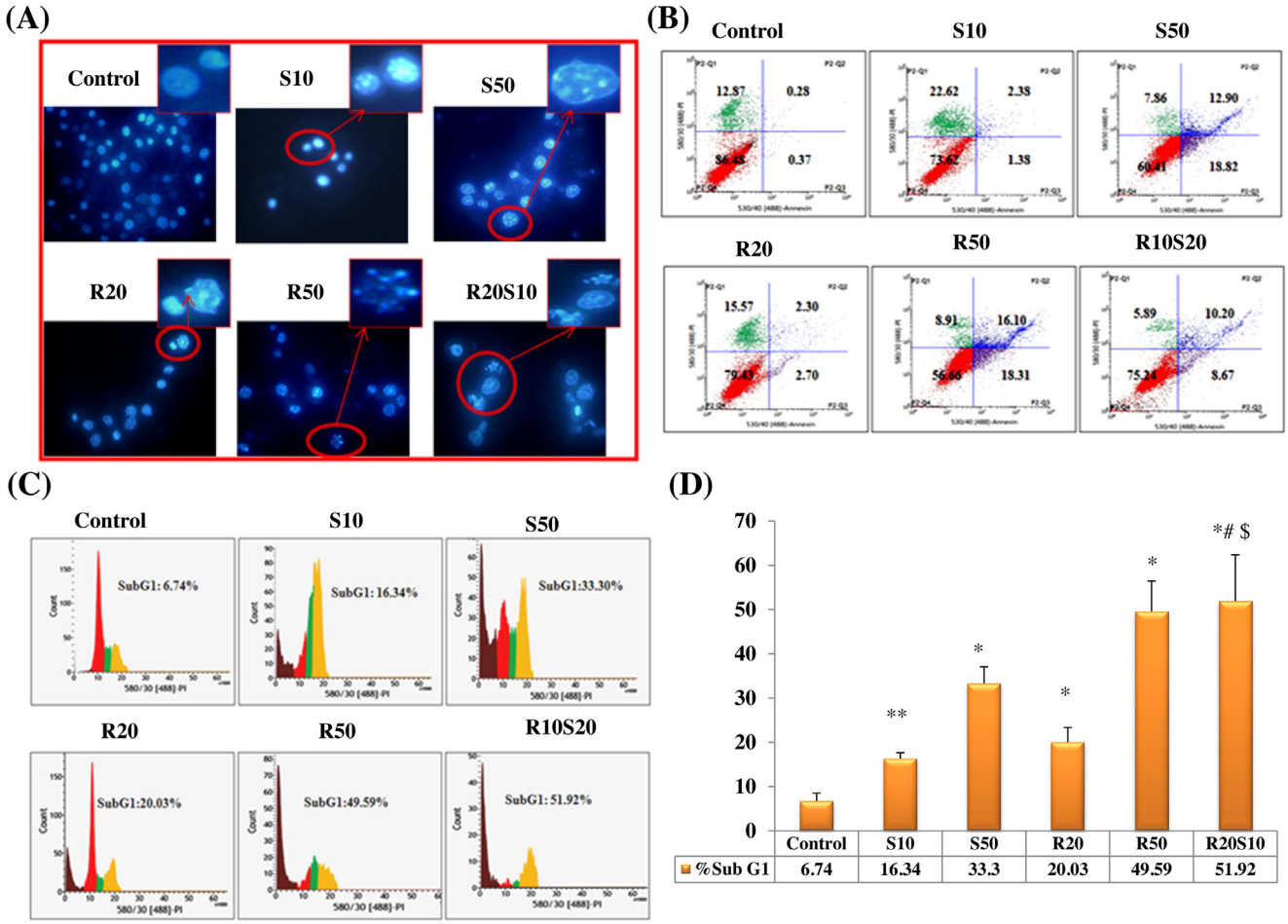

Fig. 3. Induction of apoptosis by alone and combination doses of RSVL and SAL (A) DNA fragmentation and chromatin condensation was observed by staining with DAPI after 48 h exposure with RSVL and SAL in alone and combination (40× magnifying image). (B) Flow cytometric analysis of PI and Annexin-FITC stained apoptotic population after 48 h exposure with RSVL and SAL (C) Flow cytometry measured DNA of hypodiploid population(Apoptotic population) and (D)Bar diagram showing%sub-G1 cell fraction after propidium iodide staining (* $P < 0.05$; ** $P < 0.001$; Control vs. Dose, $^{\#}P < 0.05$;S10 vs. R20S10, $^{\$}P < 0.05$; R20 vs. R20S10).

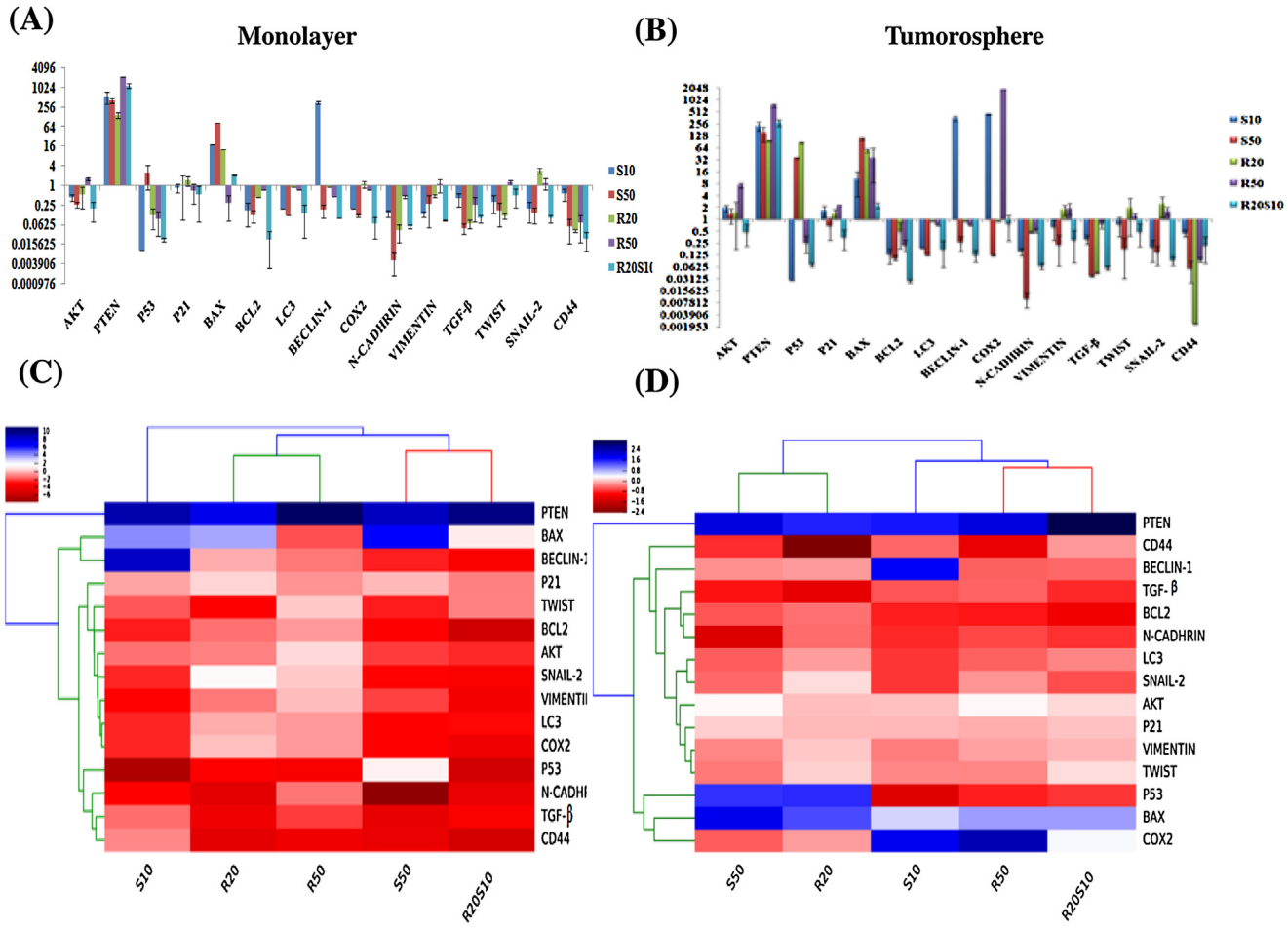

Fig. 4. Bar diagram represents the effects of RSVL and SAL either alone or in combination on differential expression of genes associated with different signaling pathways in (A) Monolayer culture (MDA-MB-231 cells) and, (B) Tumorospheres culture. The hierarchical clustering (heat map) analysis of differentially expressed genes associated with different signaling pathways in (C) Monolayer culture (MDA-MB-231 cells) and, (D) Tumorospheres culture were also evaluated. Fold changes were normalized to Ct value of GAPDH gene and data were represented as mean ±SD of three independent experiments ($P < 0.05$).

compared to untreated and alone treated TNBC cells. Above all, the synergistic doses of RSVL and SAL were found to inhibit the epithelial scattering (EMT) as well as stemness of adherent MDA-MB-231 cells and tumorospheres forming TNBC cells as compared to untreated and alone treated groups. These effects were found to be mediated by the decreased expression of EMT (N-cadherin, Vimentin, TGFB1, TWIST1, and SNAIL-2) and stemness (CD44) markers in these two culture conditions (Fig. 4A & B).

The heat map analysis of these differentially expressed genes in both culture conditions also showed that the synergistic combination of RSVL and SAL was much more effective in modulating the diverse cancer cell signaling pathways associated with breast cancer pathogenesis as compared to untreated and alone treated groups (Fig. 4C & D).

3.5. RSVL and SAL suppress EMT, inflammatory, autophagy and apoptotic associated protein markers

To examine the effect of RSVL and SAL on EMT associated markers, we analyzed the expression of EMT related proteins (E-cadherin, Fibronectin, Vimentin, N-cadherin, and Slug) by western blotting. It was observed that compared to untreated control, E-cadhrin was upregulated by SAL in dose dependent manner and was further upregulated by combination dose of RSVL and SAL. Fibronectin and Vimentin were downregulated by alone doses of SAL and RSVL in dose dependent manner, however, it was highly upregulated in combinatorial treatment. N-cadherin and Slug were also highly downregulated in combination dose in comparison to alone doses of RSVL and SAL (Fig. 5A & C). Further, we analyzed the expression of inflammation-associated markers (COX2, NF-kB, P53) in different treatment groups (Fig. 5A & C). COX2 and NF-kB were found to be downregulated in dose dependent manner. However, in combinatorial dose COX2 was highly decreased than alone doses of RSVL and SAL. Further, the expression of NFk-B was slightly more downregulated in combination in comparison to untreated control and alone treated doses. p53 is reported to be dominant mutant version in TNBC and acts as oncogene, mediates the inflammatory pro-survival environment in TNBC growth [27,28] in the present study, it was found to be highly down-regulated on cotreated MDA-MB-231 cells as compared to untreated control and corresponding alone dose of SAL. We also examined the effect of combination dose on prosurvival autophagy pathway associated markers (Beclin, LC3) and Bax/Bcl-2 expression. The analysis of data showed a slight down regulation of Beclin expression in combination treatment as compared to corresponding alone doses of RSVL and SAL, while, in comparison to untreated control LC3I and LCII level were also highly down regulated in combination treated as compared to untreated control (Fig. 5B & D) and alone doses of RSVL and SAL. Finally Bax/Bcl-2 ratio as indicator of apoptosis was found to be highly upregulated (by ~8 fold) in combination treatment (Fig. 5B & D).

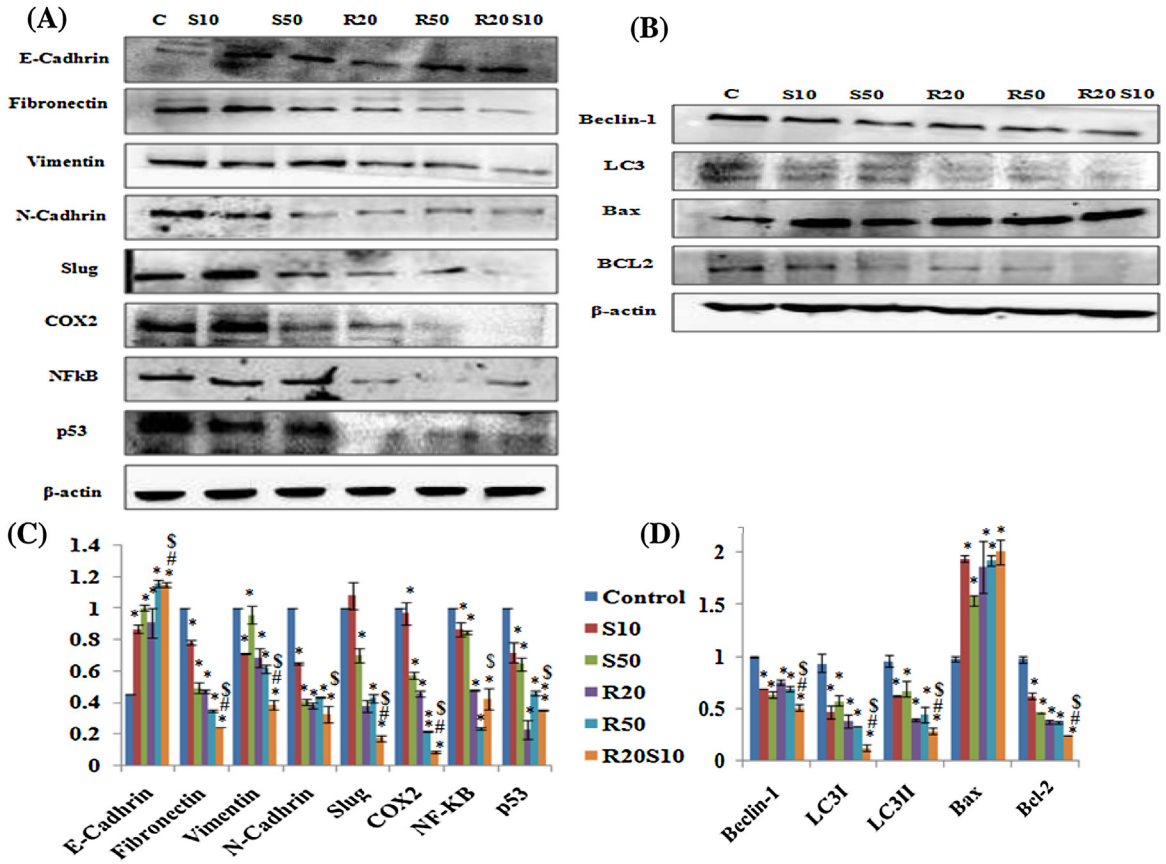

Fig. 5. Representative western blots showing the effect of RSVL and SAL on genes expression associated with (A) EMT(E-cadherin, Fibronectin, Vimentin, N-cadherin and Slug) Inflammation (COX2, NF-kB, P53), (B) autophagy (LC3B, Beclin-1) and apoptosis (Bax, Bcl-2) in MDA-MB-231 cells after the exposure of 48 h.(C&D) Bar diagram showing the fold changes of proteins normalized to β-actin. Data represented as mean ± SD of three independent experiments (*$P < 0.05$) Control vs. treatment, (**$P < 0.001$) Control vs treatment, ($^{\$}P < 0.05$) S10 vs. R20S10, ($^{\#}P < 0.05$) R20 vs. R20S10.

3.6. Effects of combination on survival of tumor bearing Swiss albino mice

Morphological observation showed that there was apparent regression in the tumor volume after combined exposure. It was further evaluated that tumor bearing untreated control mice (Group 2) showed mean survival time of ∼20 days, whereas, treatment of single dose of RSVL (Group 3) and SAL (Group 4) enhanced the mean survival time to ∼35days/∼1.72 fold and ∼40days/∼2 fold respectively. However, combinatorial treatment, (Group 5) significantly increased the MST by ∼55 days/∼2.75fold. Moreover, evaluation of percentage increase in life span showed that combination treatment increased the life span of tumor bearing mice by ∼168% while alone treatment with RSVL and SAL increased the life span by ∼72%and∼ 98.5% respectively (Fig. 6A–D, Table 2).

4. Discussion

Triple negative breast cancer (TNBC) was reported as the most refractory subtype and possessing high drug resistivity among all breast cancer subtypes [29]. In the present study, we aim to explore various cancer-associated pathways in TNBC after treatment with RSVL and SAL in MDA-MB 231 cells. Initially, the doses of RSVL and SAL were screened for calculating IC50 by using cell viability assay (data not shown). Further, 16 different combinations of RSVL and SAL were used to analyze the synergistic effect of both compounds through *in-vitro* studies. Chau Talalay method was employed to calculate the synergistic interaction of the combination. The analysis of combination index (CI) values of different combinations was done and the values of CI < 1, synergism; CI = 1, additive effects; CI > 1, antagonism, were considered for different modes of drugs interactions [23,30] (Table 1). It was found that all the different combinations showed synergism except R20S40, (CI = 1.007) combination.

It was found that the combination of IC20 of RSVL and IC10 dose of SAL was an effective and best synergistic (CI < 1) dose among all different analyzed combination doses (Fig. 1A & B, Table 1). This combination showed a potent growth inhibition with ∼5 fold dose advantage of SAL. To validate the effect over long term period colony formation assay was performed and it was found that combination dose of RSVL and SAL (R20S10) significantly reduced the colony forming ability of MDA-MB-231 cells as compared to corresponding alone doses and untreated control (Fig. 2C). TNBC cells are reported to be highly invasive form of breast cancer cells because of having high epithelial mesenchymal transition (EMT) capabilities [29]. Thus, to investigate the effect of this novel combination, wound healing assay was performed, which showed that this novel combination reduced the wound healing capability in MDA-MB-231 cells more significantly as compared to alone doses of RSVL and SAL (Fig. 2A & B). Tumorosphere represent the exact architect of solid tumor in *in vivo* in 3D space, which mimics the tumor microenvironment as observed in different tumor heterogenic cells [31]. Furthermore, the combinatorial treatment also reduced the tumorospheres forming ability as compared to alone treatment These data clearly demonstrate that the novel

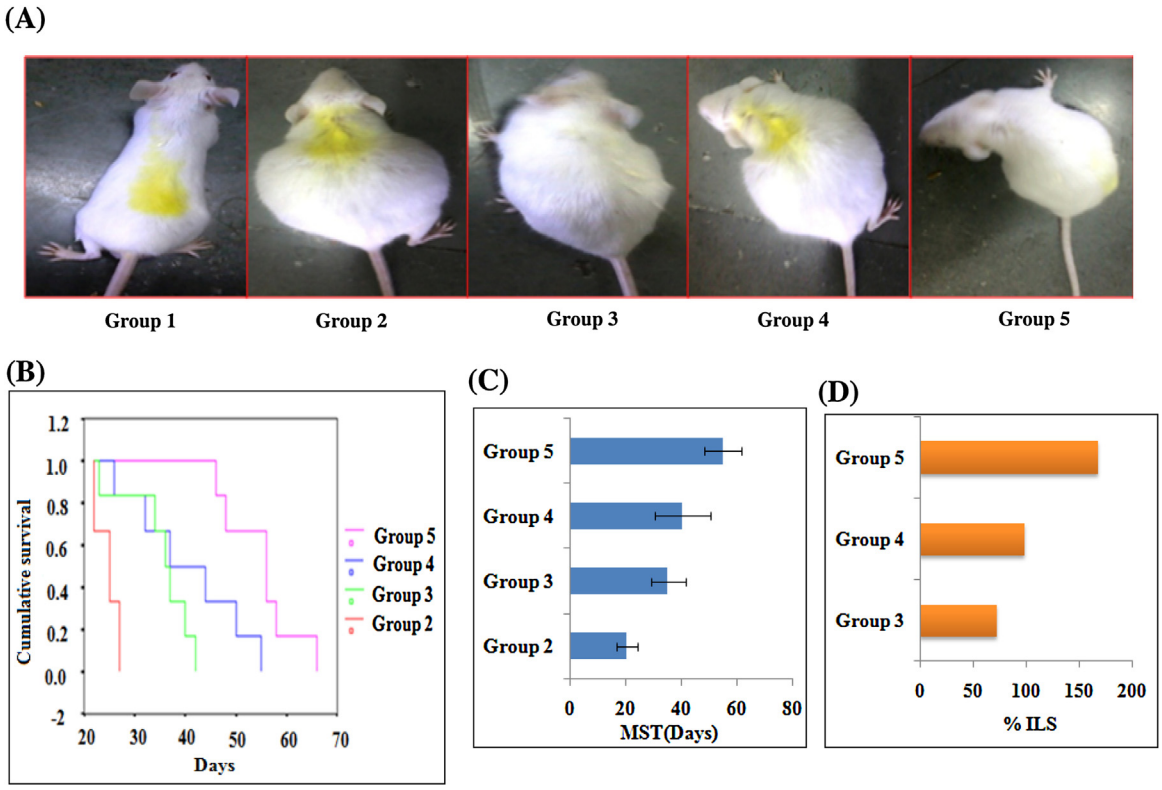

Fig. 6. (A) Morphology of EAC mice showing change in body weight in (i) Control, (ii) untreated EAC, (iii) RSVL treated (10 mg/kg b. wt.), (iv) SAL treated (0.5 mg/kg b.wt.), (v) RSVL + SAL treated (10 mg/kg + 0.5 mg/kg b.wt.), (B) Kaplan- Meier Survival Curve of mice treated with RSVL and SAL alone and in combination. Bar diagrams showing the (C) Mean survival time (MST) and (D) Percentage increase in life span (%ILS) of treated EAC mice as compared to untreated EAC group ($p < 0.05$).

combination of drugs was not only effective against monolayer culture of TNBC but also in 3D tumorospheres.

The ultimate goal of anticancer drug is to kill the cancer cells in programmed way *i.e.* apoptosis. To investigate the effect of novel combination, on apoptosis induction, we performed flow cytometric based study and DAPI staining (Fig. 3A). The result showed a significant apoptosis induction in MDA-MB-231 cells by combination treatment as evidenced by increased hypodiploid sub-G1 and annexin V/PI positive population as well as marked chromatin condensation and DNA fragmented population in combination treatment. So, it is our first report showing that this novel combination of RSVL and SAL can synergistically inhibit cellular viability and can further impact on cellular proliferation through apoptosis induction in TNBC cells. Besides, revealing anti proliferative effect of RSVL and SAL combination, we further delineated the mechanistic pathways modulated by this novel combination. To figure out some of the key signaling pathways in cancer progression, we analyzed EMT, chronic inflammation, apoptosis, and autophagy associated pathways by effect of alone and combinatorial treatments.

The process of EMT has been drawn much attention towards cancer research. Because, EMT phenomena is highly associated with aggressiveness as well as in recurrence of breast cancer [32,33]. Some of EMT associated markers, which were highly upregulated in aggressive form of tumors are Fibronectin, Vimentin, N-cadherin, ZEB1, and ZEB2 [34–36]. It has also been reported that chronic inflammatory environment in the breast cancer vicinity also promoted EMT as well as tumor recurrence [37–39]. P53 is a key regulator of chronic inflammatory signaling pathway, known to be tumor suppressor but in TNBC, P53 dominant mutant version is known to act as an oncogene [27,40]. Accounting the above facts, we investigated the effect of RSVL and SAL in alone and combination on the modulation of EMT and inflammatory associated markers. It was observed that compared to untreated control, epithelial marker E-cadhrin (epithelial marker) was up-regulated by SAL, in dose dependent manner and was further upregulated by combination dose of RSVL and SAL. Fibronectin and Vimentin (mesenchymal marker) expressions were downregulated by alone doses of SAL and RSVL in dose dependent manner. However, it is highly upregulated after combined treatment on MDA-MB-231 cells. Also, the expressions of N-cadherin and Slug (mesenchymal marker) were down regulated in alone doses of RSVL and SAL, however, the combination dose further decreased the expression of each of these EMT associated markers in MDA-MB-231 cells. P53 and Cox-2 were downregulated in co-treatment, which confirm that this novel combination inhibits the growth of TNBC cells by modulation of inflammatory environment and thus EMT associated markers.

Autophagy is known to play a dual role in carcinogenesis, because in early stage, it helps to prevent tumorogensis, while, in advance stage it promotes the survival of cancer cells by reliving stressful environment [41,42]. Recent studies showed that autophagic flux in the mitochondria of the metastatic triple-negative MDA-MB-231cells is more as compared to the less metastatic MDA-MB-468 and noncancerous MCF7–10A cells [43]. It has been also showed that in nutrient rich conditions, autophagy supplements to the growth and proliferation of MDA-MB-231 cells [44]. Studies also showed that autophagy assists the survival of TNBC cells by enhancing the rate of glycolysis [44,45]. Thus, we analyzed the status of autophagy-associated markers (Beclin, LC3I, and LC3II) by the effect of treatment of both SAL and RSVL in MD-MB-231 cells. Results showed that the expression of Beclin was apparently down regulated after the combined treatment of RSVL and SAL as compared to corresponding alone doses, but it was

highly downregulated in both combination and alone treatment compared to untreated control. LC3I and LCII level were also highly down regulated in combination treated MDA-MB-231cells as compared to untreated control and alone doses of RSVL and SAL. Thus, our results suggested that the combination dose of RSVL and SAL might revert the protective autophagy by modulation of key autophagy regulators. Further, a significant fold upregulation in Bax/Bcl-2 ratio in combined treated dose of SAL and RSVL in MDA-MB-231 cells showed that this combination might synergistically act against cancer cell proliferation by induction of apoptosis.

To support the results of *in- vitro* studies, we used a transplantable murine EAC tumor model to investigate the antitumor activity of SAL in alone and in combination with RSVL. Low dose of SAL in combination with RSVL significantly reduced tumor volume with an increased mean survival time (MST) and percentage increase of life span (% ILS) in EAC-bearing mice. These *in vivo* observations also showed the better therapeutic efficacy of novel combination. Prolongation of survival in tumor bearing animals is a well established method of evaluates the anticancer potential of a drug [46,47]. Further, the reduction in the number of ascitic tumor cells is an indication of the effect of a drug on peritoneal macrophages and other components of the immune system [46]. In this respect, it is very much convincing to consider that the increased killing of tumor cells by our novel combination might be happening in the same manner in an EAC model as well as *in vitro* system and therefore, slow tumor growth and enhanced survival of animals were observed in groups treated with combination dose as compared to alone treated groups.

5. Conclusions

Data of our study revealed that combination of RSVL and SAL has synergistic inhibitory effect on growth and proliferation of TNBC cells in both monolayer culture and tumorosphere model. Besides, this novel combination has powerful synergistic inhibitory effect against metastatic or invasive properties of TNBC cells by modulating the EMT and chronic inflammation regulatory signalling cascade. Furthermore, using RSVL with SAL in combination may also alter the pro-survival autophagic cascade and thus, extensively enhanced the apoptosis in TNBC cells. Although, this combination of RSVL with SAL is novel and our study strongly suggest that combination of dietary phytochemical like RSVL with SAL could be recommend a new therapeutic window for TNBC cases.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2017.02.110.

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