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RESEARCH ARTICLE

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Targeting the epithelial–mesenchymal transition (EMT) pathway with combination of Wnt inhibitor and chalcone complexes in lung cancer cells

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Abstract

Non-small cell lung cancer (NSCLC) is the most common type of the lung cancer. Despite development in treatment options in NSCLC, the overall survival ratios is still poor due to epithelial and mesenchymal transition (EMT) feature and associated metastasis event. Thereby there is a need to develop strategy to increase antitumor response against the NSCLC cells by targeting EMT pathway with combination drugs. Niclosamide and chalcone complexes are both affect cancer cell signaling pathways and therefore inhibit the EMT pathway. In this study, it was aimed to increase antitumor response and suppress EMT pathway in NSCLC cells by combining niclosamide and chalcone complexes. SRB cell viability assay was performed to investigate the anticancer activity of drugs. The drugs were tested on both NSCLC cells (A549 and H1299) and normal lung bronchial cells (BEAS-2B). Then the two drugs were combined and their effects on cancer cells were evaluated. Fluorescence imaging and enzyme-linked immunosorbent assay were performed on treated cells to observe the cell death manner. Wound healing assay, real-time quantitative polymerase chain reaction, and western blot analysis were performed to measure EMT pathway activity. Our results showed that niclosamide and chalcone complexes combination kill cancer cells more than normal lung bronchial cells. Compared to single drug administration, the combination of both drugs killed NSCLC cells more effectively by increasing apoptotic activity. In addition, the combination of niclosamide and chalcone complexes decreased multidrug resistance and EMT activity by lowering their gene expressions and protein levels. These results showed that niclosamide and chalcone complexes combination could be a new drug combination for the treatment of NSCLC.

KEYWORDS
chalcone complexes, drug resistance, epithelial–mesenchymal transition (EMT), niclosamide, NSCLC

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1 | INTRODUCTION

Lung cancer is the leading cause of cancer death in both sexes all over the world. It caused an estimated 1.8 million deaths in 2020.[1] According to the American Cancer Society (ACS) estimations, 236 740 people will be diagnosed with lung cancer and about 130 180 deaths from lung cancer in 2022 in the United States. The vast majority of lung cancers are carcinomas, and about 15 percent of lung carcinomas are small cell lung cancer (SCLC) and 85% are non-small cell lung cancer (NSCLC).[2]

Despite improvements in treatment options and substantial improvements in the prognosis of patients in recent years, overall survival rates are still low due to epithelial and mesenchymal transition (EMT) feature of the NSCLC.[3] In the EMT process epithelial cells lose cell–cell adhesion, and gain migratory and invasive properties thereby metastasis occur.[4] EMT pathway is regulated by multiple proteins such as Vimentin and Cadherins, some transcriptional factors including Snail and Twist, and some specific pathways like Wnt/B-Catenin, JAK-STAT pathway.[5]

EMT and drug resistance are interrelated processes. Saxena et al. demonstrated that Snail increased the expression levels of multiple drug-resistance genes and was associated with chemoresistance.[6] In another study, knockdown of Snail by RNA interference increased the sensitivity of A549 lung NSCLC cells to cisplatin.[7] In addition, expression of mesenchymal markers in the EMT process is associated with poor prognosis and response to Tyrosine kinase inhibitor in NSCLC.[8] Targeting the EMT pathway by multiple agents may be effective for increasing the efficacy of therapy and reducing multidrug resistance, thereby better overall survival rates can be obtained in NSCLC.

Niclosamide is an FDA-approved anthelmintic drug for the treatment of parasitic infections (Figure 1A).[9] Beyond this anti-helminthic activity, niclosamide also exhibits anticancer properties by inhibiting oncogenic pathways and activating tumor suppressor signaling pathways.[10] In addition, there are many studies in which niclosamide was combined with different agents.[10] Akgun et al. showed that niclozamide inhibited the Wnt/β-Catenin pathway, which has a role in the EMT process, and that its combinations with histone deacetylase inhibitor valproic acid had a synergistic effect to kill lung cancer cells.[11]

Chalcone complexes are precursors of the flavonoids family and they can be found in a wide range of natural products (Figure 1B,C).[12] In vitro and in vivo studies have demonstrated that chalcone complexes exhibit anticancer activity by targeting multiple biologic pathways including PI3K/Akt, NF-κB, Wnt/B-catenin, mTOR, ROS/MAPK, and effectively kill cancer cells.[13] In the one study, Belinda et al. showed that synthetic chalcone complexes inhibit JAK-STAT signaling pathway and they suggested that these complexes may be useful to treat cancer.[14] In another study showed that chalcone complexes inhibit the EMT pathway by increasing p53 protein expression.[15]

In light of all this information, we hypothesized that the combination of niclosamide and chalcone complexes may show synergistic effects to reduce EMT pathway activity and kill NSCLC cells. Targeting the EMT pathway with combination therapy may improve overall survival and chemoresistance of NSCLC patients. Also, the absence of any combination study on niclosamide

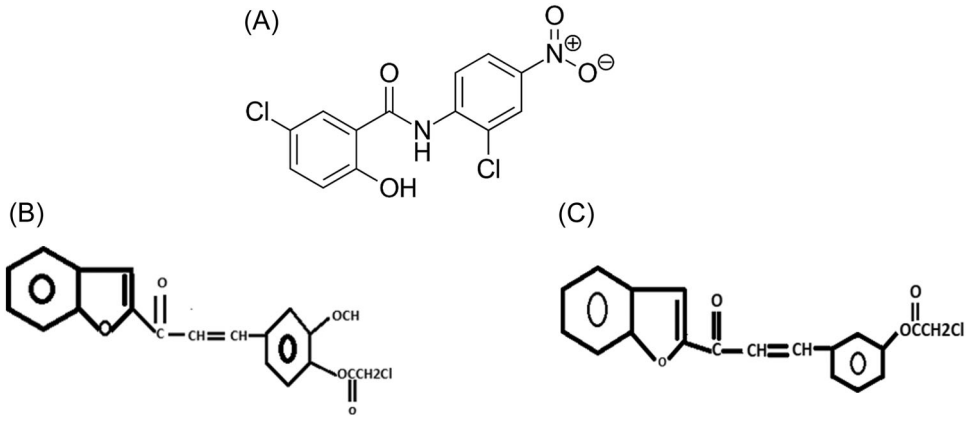


FIGURE 1 (A) Niclosamide (Niclo) molecular structure. (B) Complex 1 molecular structure; [(4)-((1E)-3-(1)-benzofuran-2-yl)-3-oxoprop-1-en-1-yl]-2 methoxyphenyl chloroacetate). (C) Complex 2 molecular structure; [3-[(1E)-3-(1-benzofuran-2-yl)-3-oxoprop-1-en-1-yl)] phenyl chloroacetate).

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and chalcone complexes in the literature constitutes the originality of our study.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Benzofuran substituted chalcone derivatives; complex 1 [(4)-((1E)-3-(1)-benzofuran-2-yl)-3-oxoprop-1-en-1-yl]-2 methoxyphenyl chloroacetate) and complex 2 [3-[(1E)-3-(1-benzofuran-2-yl)-3-oxoprop-1-en-1-yl)] phenyl chloroacetate) were synthesized[16,17] and characterized (Alioglu et al., submitted). Stock concentrations of chalcone complexes (complexes 1 and 2) (50 mM) and niclosamide (20 mM) in dimethyl sulfoxide (DMSO) were prepared. The final DMSO concentration in the concentration of the complexes was determined as 0.2% v/v, which is reported to be nontoxic in the literature.[18] Stock solutions of the complexes were aliquoted and stored at −20°C. Niclosamide was purchased from Sigma (Catalog no: N3510; Sigma-Aldrich). Propidium iodide (PI) was obtained commercially (Sigma-Aldrich). The Hoechst dye 33342 was from Enzo Life Sciences.

2.2 | Cell culture

NSCLC cell lines A549 and H1299 and normal lung bronchial cells BEAS-2B were obtained from ATCC and cultured in Roswell Park Memorial Institute 1640 (with L-glutamine) medium supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum. All cell lines were maintained at 37°C in a humid atmosphere containing 5% CO_2. When the cells reached 70% to 80% confluency, they were passaged by trypsinization.

2.2.1 | The sulforhodamine B (SRB) viability assay

The SRB assay is a method used to assess cell viability based on measurement of cellular protein content.[19] The antiproliferative effect of the combination of complex 1 and complex 2 with niclosamide on A549, H1299, and BEAS-2B cells were evaluated by the SRB method. Cells were seeded at a density of 5×10^3 cells per well of a 96-well plate. Complexes 1 and 2 (0.19–12.5 μM) alone and in combination with a 1.25 μM concentration of niclosamide were administered to the cells. After 48 h of chalcone complexes and niclosamide treatment, SRB viability analysis was performed as in our previous studies.[20] Absorbance was read at 570 nm using a spectrophotometer (ELISA, microplate). Cell viability was calculated with reference to untreated control cells using the following formula:

$$\text{Viability}(\%) = [100 \times (\text{Sample Abs} - \text{Empty Abs}) / (\text{Control Abs} - \text{Empty Abs})].$$

Following the determination of percent viability, the CompuSyn software was utilized to calculate the Combination Index (CI). CompuSyn software, developed by Dr. Dorothy Chou, employs the median-effect principle of the mass-action law to facilitate analytical simulations in pharmacodynamics research.[21,22] The CI value quantitatively assesses the degree of drug interaction at a specific level of effect. The obtained CI values provide insights into whether the combination practice is synergistic, additive, or antagonistic. In general, a CI value of less than 0.9 indicates synergism, a CI value between 0.9 and 1.1 suggests additivity, and a CI value greater than 1.1 indicates antagonism.[23,24]

To calculate IC_{50} values, dose–response data was created. While creating data drug concentration was placed in the x axis and cell viability placed in the y axis. Data fitted with a straight line (linear regression). IC_{50} value is then estimated using the fitted line.

$$Y = a^*X + b, \text{IC}_{50} = (0.5 - b)/a.$$
$$(a = \text{slope}, b = y - \text{intercept}).$$

2.3 | Fluorescence imaging and calculation of apoptotic cell percentage

A549 and H1299 cells were pretreated with complexes 1 and 2 (6.25 μM) for 24 h. After 24 h of chalcone complex treatment, niclosamide (1.25 μM) was added to the medium and a total of 48 h of combination therapy was applied. Cells were incubated with the treatment groups at 37°C in an atmosphere of 5% CO_2. At the end of the treatment, Hoechst 33342 (4 μg/mL), Annexin-V (3 μg/mL), and PI (1 μg/mL) dyes were applied to the wells and cells were examined with fluorescence microscopy. The proportion of early (Annexin-V positive, PI negative) and late apoptotic (Annexin-V positive, PI positive) cells was defined by counting Annexin-V and PI positive cells in five random fields and calculating their percentage relative to the total number of cells (Hoechst positive).

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2.4 | Migration assay

For the migration test, H1299 and A549 cells were seeded at 2×10^5 cells per well in 2 mL of medium and incubated overnight in the incubator to allow time for cells to adhere. Then, in accordance with the purpose of the experiment, 0-h photos were taken by drawing straight lines along the wells with the help of a 1000 μL pipette tip and marking three areas from each well. After, the combination of simultaneously effective chalcone derivatives (complexes 1 and 2) and niclosamide was added to the wells at the determined concentrations and the marked areas were photographed again after 0–36 h for each group. Given the cytotoxic effect, normalization of the proportion of migrating cells relative to the cytotoxic rate at each time interval was performed. Wound closure area for the migration ability of cells at each time interval was calculated using ImageJ software with the following formula.

$$\beta(t) = A(t)/A(0) \times 100.\ \beta(t) \text{ is\%}$$

wound confluence, $A(t)$

is the wound area at time t and $A(0)$

is its initial area.

2.5 | Real-time quantitative polymerase chain reaction (RT-qPCR)

RNA samples from A549 and H1299 cells were obtained using the PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The quality of RNAs was measured using NanoDrop 2000 (Thermo Fisher Scientific). Next, complementary DNA (cDNA) synthesis was performed according to the manufacturer's instructions with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) using 500 ng of RNA for each group. RT-qPCR analysis was performed on the Applied Biosystems Step One Plus Real-Time PCR (Thermo Fisher Scientific) using the primers shown in Table 1. The experiments were made in triplicates and repeated two times. The SYBR Green method used for C_t detection. The graph for gene expression analysis reflects the comparison of the data that we obtained to the negative control (untreated group). We consider at least two–fold increase as statistically significant.

2.6 | Measurement of cleaved caspase 3/7 and cleaved PARP levels

A549 and H1299 cells (1×10^6) were seeded in 25 cm flasks and treated with complex 1, complex 2, and niclosamide for 48 h to detect levels of apoptosis markers cleaved caspase 3/7 and cleaved PARP. After treatment, cleaved PARP levels were calculated using the PARP (Cleaved) [214/215] Human ELISA kit (Cellsignalling, #7262) according to the protocol described in the manufacturer's instructions. Cleaved Caspase 3/7 levels measured by Caspase 3/7 ELISA kit (Cellsignalling, #7190) following the manufacturer's instructions. The absorbance read at 450 nm using an ELISA plate reader (FLASH Scan S12; Analytik Jena).

2.7 | Western blot analysis

A549 and H1299 cells were seeded in six-well cell culture plates. Complexes 1 and 2 (6.25 μM) were combined with

TABLE 1 Primer sets used for RT-qPCR.

	Forward	Reverse
ABCC1	5-GACCGAGGCTACATTCAGATG-3	5-TCCCAGAAAGAGTAGAAGAGGT-3
ABCB1	5-CCCTTGTTAGACAGCCTCATATT-3	5-GCTTTGTCCAGGGCTTCTT-3
FN1	5-CATCGAGCGGATCTGGCCCC-3	5-GCAGCTGACTCCGTTGCCCA-3
VIM	5-TGCAGGAGGCAGAAGAATG-3	5-TCCGGTACTCAGTGGACTC-3
CDH1	5-CCTGCCAATCCCGATGAAA-3	5-CATAGTCAAACACGAGCAGAGA-3
CDH2	5-CCATCAAGCCTGTGGGAATC-3	5-GCAGATCGGACCGGATACTG-3
SMAD2	5-ATGTCGTCCATCTTGCCA-3	5-AACCGTCCTGTTTTCTTTAG-3
SNAII	5-CTCTTTCCTCGTCAGGAAGC-3	5-GCTGGAAGGTAAACTCTGGATTA-3
CTNNB1	5-GTATGAGTGGGAACAGGGATTT-3	5-TGAGCTCGAGTCATTGCATAC-3
WNT3A	5-CCCACTCGGATACTTCTTACTC-3	5-AGCCCAGGGAGGAATACT-3
B-ACTIN	5-CACTCTTCCAGCCTTCCTTC-3	5-GTACAGGTCTTTGCGGATGT-3

Abbreviation: RT-qPCR, real-time quantitative polymerase chain reaction.

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niclosamide (1.25 μM) and treated for 48 h. Protein was extracted using the RIPA Lysing Buffer System (Santa Cruz Biotechnology) according to the manufacturer's protocol. Protein concentration was determined with the BCA Protein Assay Kit (Thermo Fisher Scientific). Equal concentrations of proteins were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was then transferred to a nitrocellulose membrane. Membranes were incubated with the following primary antibodies overnight at 4°C. The following Cell Signaling Technology primary antibodies were used: Glyceraldehyde 3-phosphate dehydrogenase (#5174), Fibronectin/FN1(#26836), Wnt3a (#2721), β-Catenin (#9562), MRP1/ABCC1 (#72202), E-Cadherin (#3195), N-Cadherin (#13116), Vimentin (#5741).

2.8 | Statistical analysis

Experiment results were presented as ±SD (standard deviation) by calculating the mean of at least three independent studies. All statistical analyzes were tested with one-way analysis of variance and evaluated with GraphPad 9.12 program. $p < 0.05$, $p < 0.01$, and $p < 0.001$ values were considered statistically significant.

3 | RESULTS

3.1 | Chalcone complexes exhibits cancer-specific cytotoxic effect

Different concentrations of complexes 1 and 2 (0.19–12.5 μM) were used for 48 h on the A549 and H1299 cancer cell lines and BEAS-2B normal lung bronchial cell lines (Figure 2). It was determined that complex 1 killed A549 and H1299 cancer cells more than BEAS-2B cells at 6.25 and 12.5 μM doses ($p < 0.001$) (Figure 2A). A similar set of experiments was designed for complex 2 (Figure 2B). It was shown that complex 2 killed H1299 cells more than BEAS-2B cells at 3.12, 6.25, and 12.5 μM doses ($p < 0.001$) (Figure 2B). Additionally, complex 2 killed A549 cells more robustly than BEAS-2B cells 12.5 μM doses ($p < 0.001$) (Figure 2B). These results

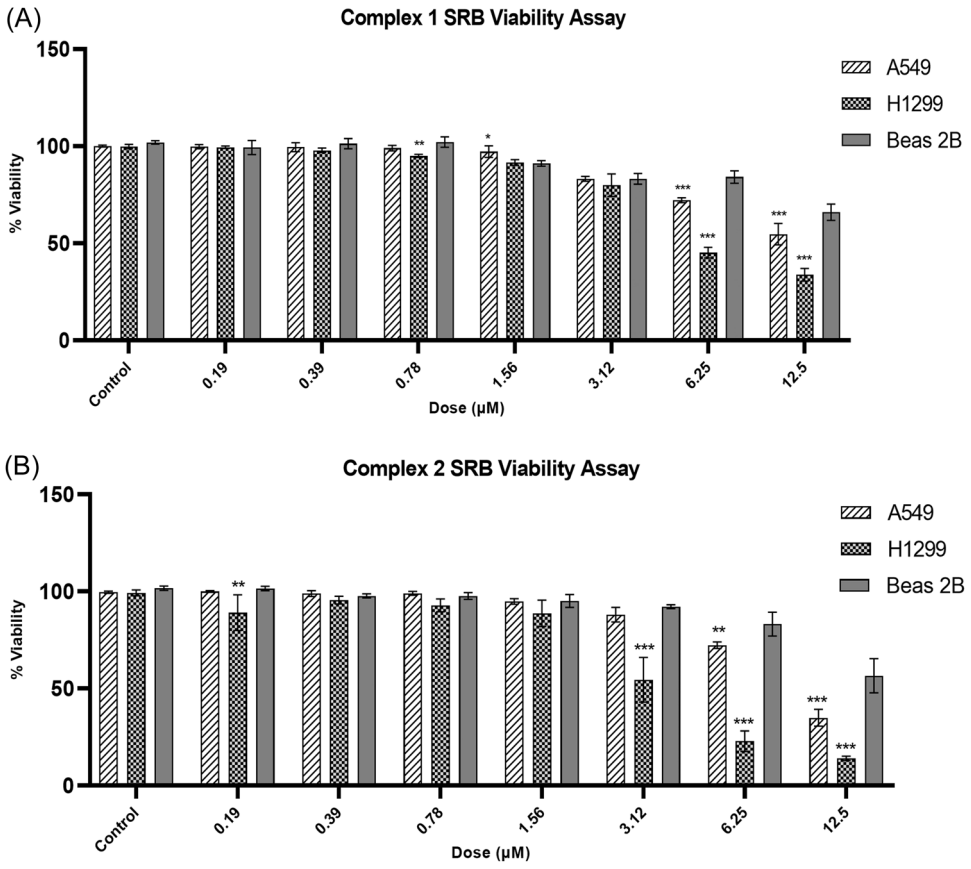


FIGURE 2 Complexes 1 and 2 on lung cancer cells (A549 and H1299) and normal lung bronchial cells (BEAS-2B). 0.19–12.5 μM doses of Complex 1 (A) and Complex 2 (B) were used. Viability measured by SRB viability test. The cells were treated for 48 h. *Denotes statistically significant differences in comparison with control: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are presented as mean ± SD ($n = 3$). SD, standard deviation; SRB, sulforhodamine B.

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showed that complexes 1 and 2 had specific toxic effects on lung cancer cells with less effect on normal lung bronchial cells.

3.2 | Chalcone complexes–niclosamide combination kills lung cancer cells more effectively than single administration of each drugs

Complex 1 (0.19–12.5 μM) and niclosamide (1.25 μM) was applied to A549 and H1299 cells and BEAS-2B normal lung bronchial cell lines for 48 h. Then, in the same doses of complex 1 and niclosamide combined and treated for 48 h on the same cells (Figure 3). Complex 1 and niclosamide combination killed A549 cells more efficiently at 6.25 and 12.5 μM doses compared to the niclosamide and complex 1 cytotoxic effect alone ($p < 0.001$) (Figure 3A). Similarly, complex 1 and niclosamide combination killed H1299 cells more efficiently at 12.5 μM doses compared to the niclosamide and complex 1 cytotoxic effect alone ($p < 0.001$) (Figure 3B). Also complex 1 and niclosamide combination killed BEAS-2B cells more efficiently at 12.5 μM doses compared to the niclosamide and complex 1 cytotoxic effect alone ($p < 0.001$) (Figure 3C). The combination killed H1299 cells more than BEAS-2B cells at each dose in a statistically significant manner ($p < 0.001$) (Figure 3D). Similarly, the combination killed A549 cells more than BEAS-2B cells at 6.25 and 12.5 μM doses ($p < 0.001$) (Figure 3D).

A similar set of experiments was designed for complex 2, niclosamide and combination of these two drugs (Figure 4). Complex 2 and niclosamide combination killed A549 cells more efficiently at 6.25 and 12.5 μM doses compared to the niclosamide and complex 2 cytotoxic effect alone ($p < 0.001$) (Figure 4A). Similarly, complex 2 and niclosamide combination killed H1299 cells more efficiently at 12.5 μM doses compared to the niclosamide and complex 2 cytotoxic effect alone ($p < 0.001$) (Figure 4B). Also complex 2 and niclosamide combination killed BEAS-2B cells more efficiently at each doses except 0.19 μM dose of complex 2 compared to the niclosamide and complex 2 cytotoxic effect alone ($p < 0.001$) (Figure 4C). The combination killed H1299 cells more than BEAS-2B cells at 0.78, 1.56, 3.12, 6.25, and 12.5 μM doses ($p < 0.001$) (Figure 4D). In a similar way, the combination killed A549 cells more than BEAS-2B cells at 6.25 and 12.5 μM dose ($p < 0.001$) (Figure 4D).

These results showed that the combination of 6.25 and 12.5 μM doses of complexes 1 and 2 with niclosamide

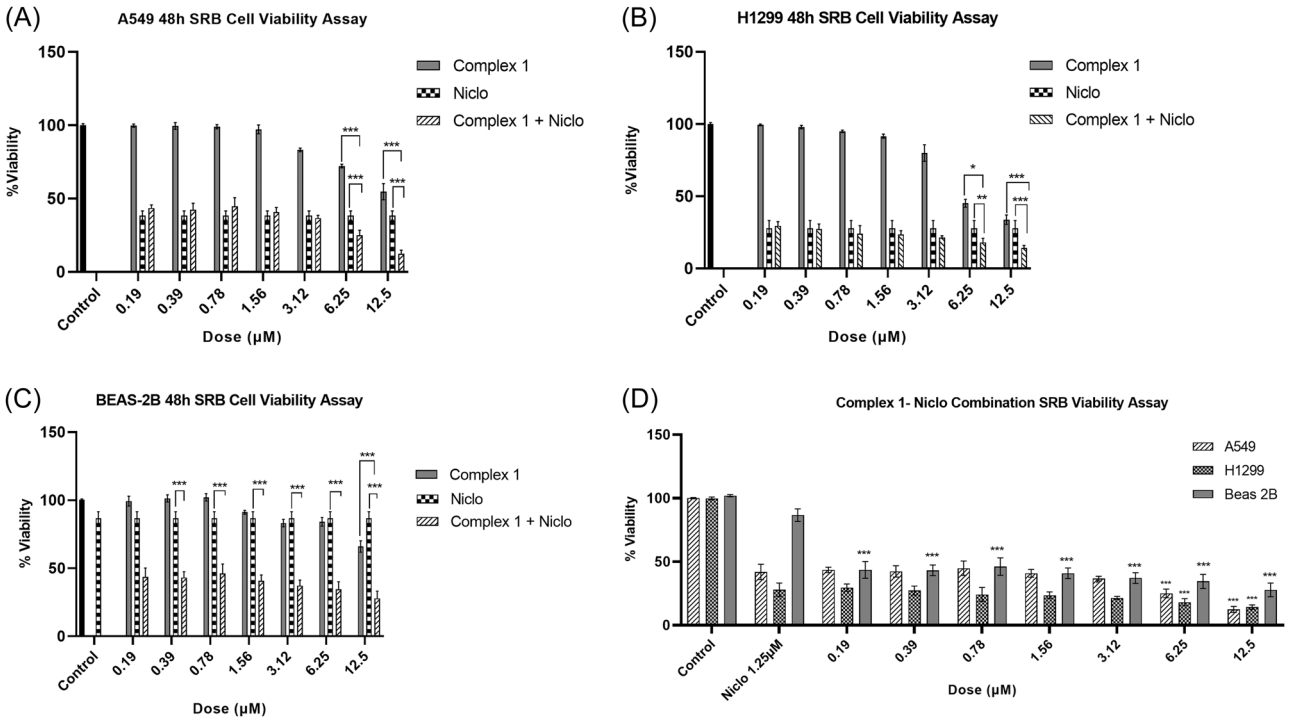


FIGURE 3 Complex 1, Niclosamide (Niclo), and their combination on A549 (A) and H1299 (B) cancer cells and BEAS-2B normal lung bronchial cell lines (C). Complex 1 and Niclosamide combination on all cell line together (D). 0.19–12.5 μM doses of Complex 1 and 1.25 μM of Niclosamide was used. The cells were treated for 48 h. Viability measured by SRB viability test. *Denotes statistically significant differences in comparison with control: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are presented as mean ± SD ($n = 3$). SD, standard deviation; SRB, sulforhodamine B.

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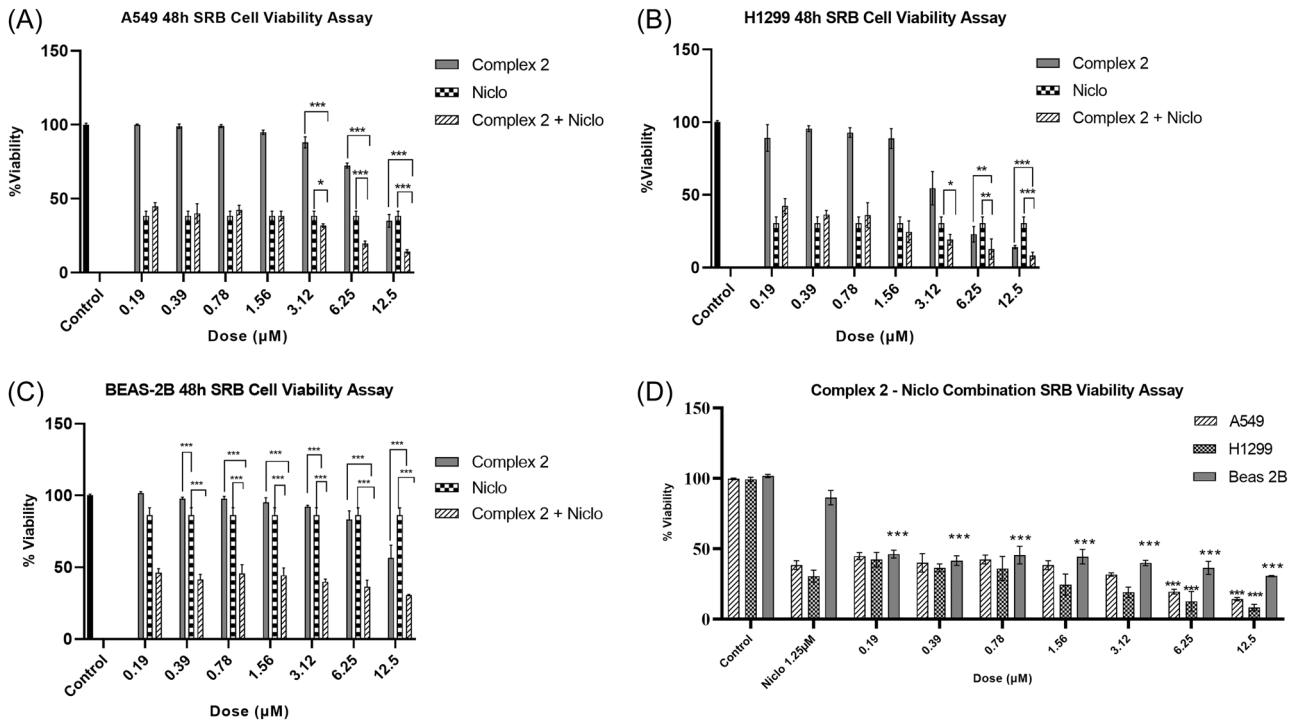


FIGURE 4 Complex 2, Niclosamide (Niclo), and their combination on A549 (A) and H1299 (B) cancer cells and BEAS-2B normal lung bronchial cell lines (C). Complex 2 and Niclosamide combination on all cell line together (D). 0.19–12.5 μM doses of Complex 2 and 1.25 μM of Niclosamide was used. The cells were treated for 48 h. Viability measured by SRB viability test. *Denotes statistically significant differences in comparison with control: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are presented as mean ± SD ($n = 3$). SD, standard deviation; SRB, sulforhodamine B.

TABLE 2 IC_{50} values of 48 h of treatment according to the results of SRB viability administered with niclosamide, complex 1, and complex 2 in A549, H1299, and BEAS-2B cell line.

IC_{50}	A549	H1299	BEAS-2B
Niclosamide, μM	1.17	1.61	>5
Complex 1, μM	>5	>5	>5
Complex 2, μM	>5	>5	>5

showed higher toxicity than single administrations of each drugs. Additionally, these two doses of complexes 1 and 2 with niclosamide showed higher toxicity on A549 and H1299 cancer cells compared to the BEAS-2B normal lung bronchial cells. It was concluded that niclosamide combination of complex 1 and 2 increased antitumor response against cancer cells specifically compared to the single administration of each drugs. Also, according to SRB analysis results, the IC_{50} values (concentration that kills 50% of cells after treatment with the niclosamide and complexes 1–2 compared to control cells) were given in Table 2.

According to our CompuSyn analysis of combination of Complex 1 and 2 (0.19–12.5 μM) with Niclosamide (1.25 μM) shows synergistic effect ($CI < 0.9$). Only 12.5 μM dose of Complex 1 and Niclosamide combination show additive effect ($0.9 < CI < 1.1$) (Figure 5).

3.3 | Chalcone complexes–niclosamide combination induces apoptotic cell death

Fluorescence staining method was used to identify the mode of cell death generated by complex 1, complex 2, and their combination with niclosamide in A549 and H1299 cells (Figure 6). In the experiment, 6.25 μM of complexes 1 and 2, which are the cytotoxic for the cancer cells as a result of SRB cell viability method, were used for 48 h. 6.25 μM dose of complex 1, complex 2 and 1.25 μM dose of niclosamide reduced cell number and formed pycnotic and fragmented nuclei, which is a marker of apoptosis compared to control cells (Figure 6A,B). In combination of complexes 1 and 2 with niclosamide on A549 and H1299 cells, a further decrease in cell density and further increase in Annexin and PI positivity was observed compared to the control and the effect of niclosamide, complex 1 and 2 alone (Figure 6A,B). We also measure the Annexin and PI-positive cell in each cell line Figure 6A1,B1). We saw that combination of complexes 1 and 2 with Niclosamide

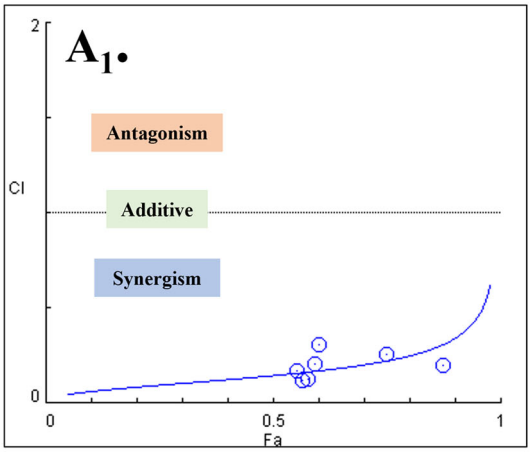
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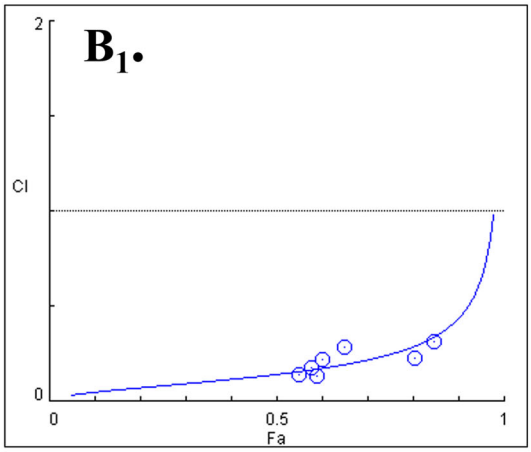
(A)

A549		
Complex-1+Niclo (1.25μM) 48h		
Dose	Fa	CI
C1 0.19 μM +Niclo	0.56	0.117
C1 0.39 μM +Niclo	0.58	0.126
C1 0.78 μM +Niclo	0.55	0.173
C1 1.56 μM +Niclo	0.59	0.203
C1 3.12 μM +Niclo	0.6	0.305
C1 6.25 μM +Niclo	0.75	0.258
C1 12.5 μM +Niclo	0.87	0.197

(B)

A549		
Complex-2+Niclo (1.25μM) 48h		
Dose	Fa	CI
C2 0.19 μM +Niclo	0.55	0.138
C2 0.39 μM +Niclo	0.59	0.135
C2 0.78 μM +Niclo	0.58	0.177
C2 1.56 μM +Niclo	0.60	0.221
C2 3.12 μM +Niclo	0.65	0.284
C2 6.25 μM +Niclo	0.80	0.230
C2 12.5 μM +Niclo	0.85	0.316



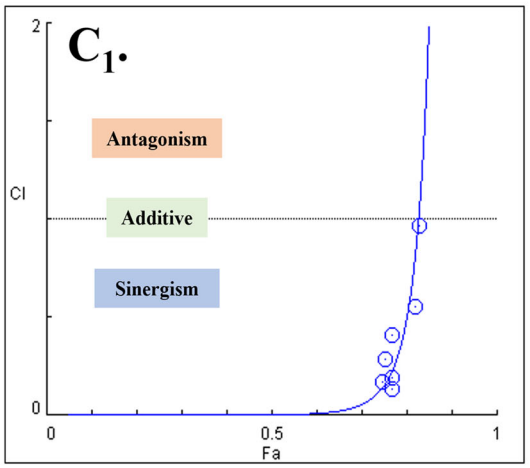


(C)

H1299		
Complex-1+Niclo (1.25μM) 48h		
Doz	Fa	CI
C1 0.19 μM +Niclo	0.77	0.135
C1 0.39 μM +Niclo	0.75	0.170
C1 0.78 μM +Niclo	0.77	0.191
C1 1.56 μM +Niclo	0.75	0.283
C1 3.12 μM +Niclo	0.77	0.413
C1 6.25 μM +Niclo	0.82	0.556
C1 12.5 μM +Niclo	0.83	0.967

(D)

H1299		
Complex-2+Niclo (1.25μM) 48h		
Dose	Fa	CI
C2 0.19 μM +Niclo	0.83	0.175
C2 0.39 μM +Niclo	0.83	0.198
C2 0.78 μM +Niclo	0.83	0.241
C2 1.56 μM +Niclo	0.82	0.356
C2 3.12 μM +Niclo	0.85	0.476
C2 6.25 μM +Niclo	0.94	0.361
C2 12.5 μM +Niclo	0.94	0.634



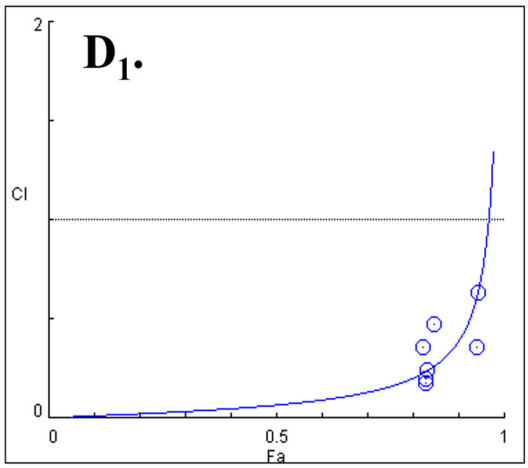


FIGURE 5 CompuSyn CI index results of Complex 1, Complex 2 (0.19-12.5 μM), and Niclosamide (1.25 μM) combinations in A549 and H1299 cell lines. Complex 1 and Niclosamide combination shown in (A) A549, (C) H1299. Complex 2 and Niclosamide combination shown in (B) A549, (D) H1299. CompuSyn CI index results for Complex 1 and Niclosamide combination; (A1) A549, (C1) H1299. CompuSyn CI index results for Complex 2 and Niclosamide combination; (B1) A549, (D1) H1299. ***CI < 0.9 Synergism, 0.9 < CI < 1.1 Additive, CI > 1.1 Antagonism. C1, Complex 1, C1, Complex 1, Niclo, Niclosamide.

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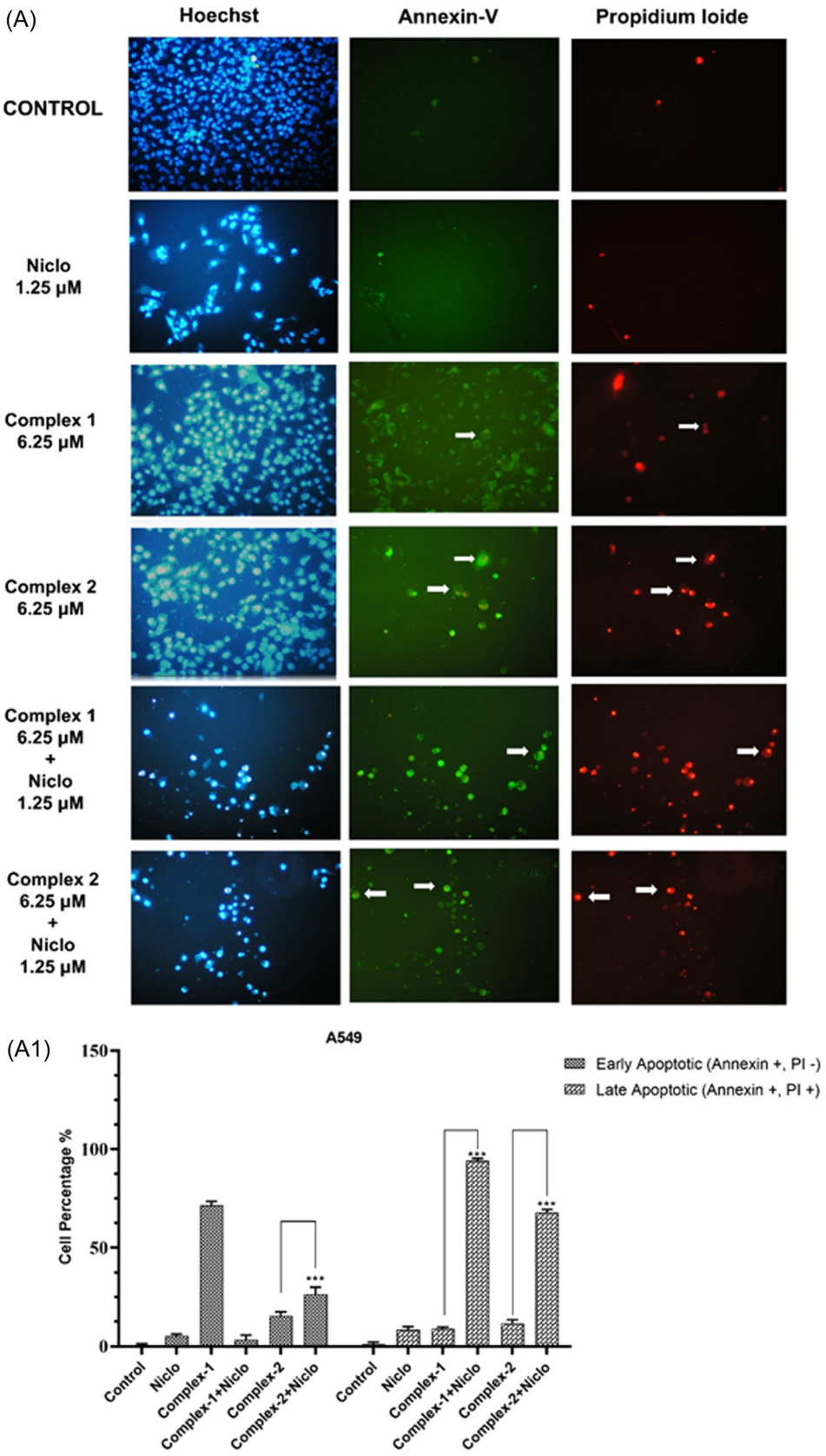


FIGURE 6 Hoechst, Annexin V, and Propidium Iodide (PI) fluorescent staining image of Complex 1, Complex 2 (6.25 μM), and their Niclosamide (Niclo) (1.25 μM) combination after 48 h treatment on A549 (A) and H1299 (B) cells. Early (Annexin+, PI−) and late (Annexin+, PI+) apoptotic cell percentage after treatment with same drugs on A549 (A1) and H1299 (B1) cells. White arrows show pycnotic/fragmented nuclei in apoptotic cells and represent Annexin and PI positive. Microscope objective magnification ×10. Denotes statistically significant differences between complexes 1 or 2 and their combination with niclosamide: ***($p < 0.001$).

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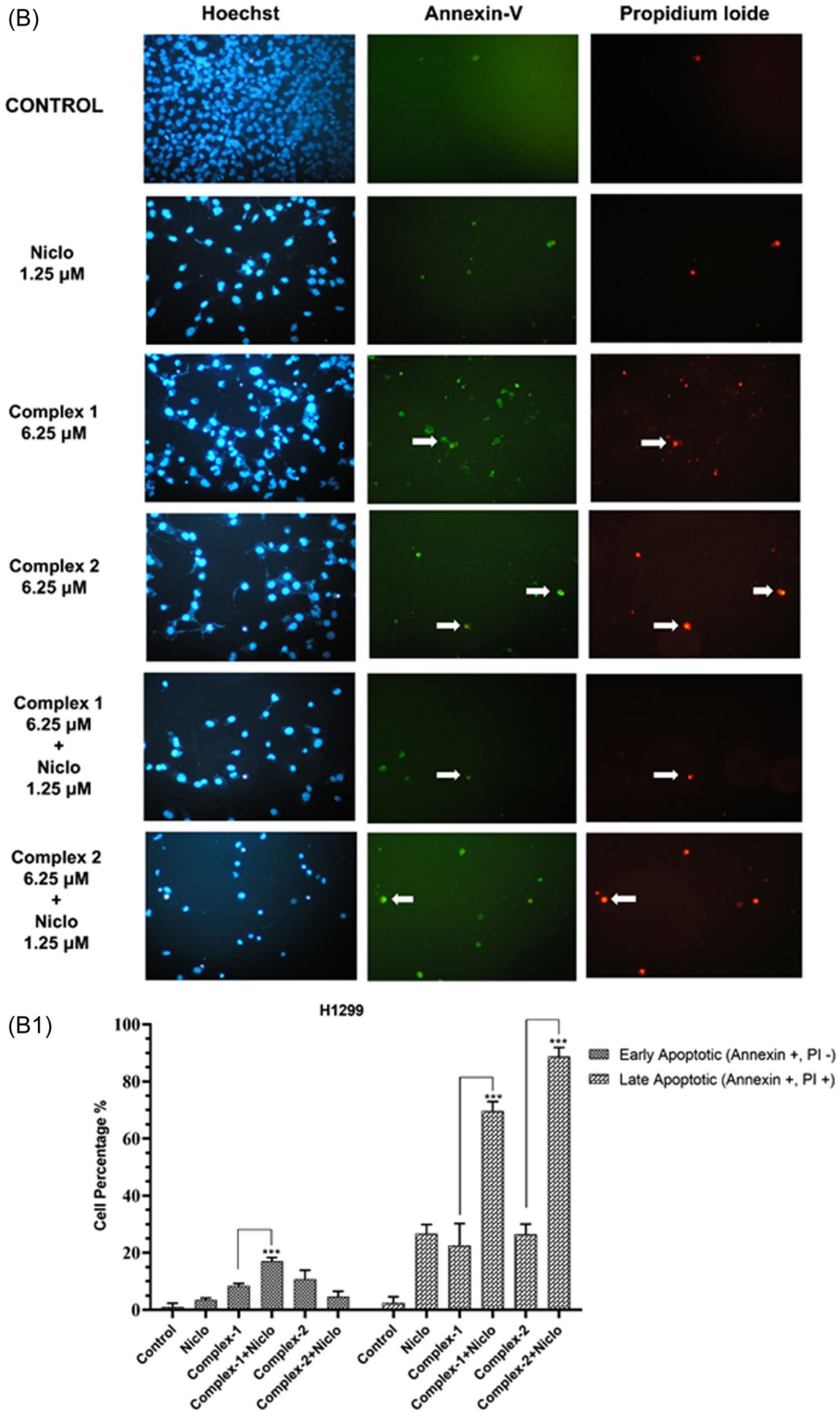


FIGURE 6 (Continued).

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increase late apoptotic cells (both Annexin and PI positive) compared to complexes 1 and 2 alone. It was seen that fluorescent microscope results support our SRB viability test results and combination treatment increase apoptotic activity on A549 and H1299 cells.

Cleaved caspase 3/7 and cleaved PARP are considered to be a hallmark of apoptosis.[25] After the 48 h treatment of complex 1, complex 2 (6.25 μM), and their niclosamide (1.25 μM) combination, the cleaved caspase 3/7 and cleaved PARP levels were measured by ELISA assay to investigate apoptotic activity in both A549 and H1299 cells (Figure 7). In both A549 (Figure 7A,B) and H1299 (Figure 7C,D) cells, combination of the complex 1 and complex 2 with the niclosamide significantly increased the both cleaved Caspase 3/7 and cleaved PARP levels compared to the single administration of complexes 1, 2 and niclosamide alone ($p < 0.001$). Both fluorescent images and ELISA results indicated that the combination treatment induce the apoptotic cell death in A549 and H1299 cells.

3.4 | Chalcone complexes–Niclosamide combination inhibits EMT process

To evalute EMT process in complex 1, 2, and niclosamide-treated cells; wound healing assay, RT-qPCR, and western blot analysis experiments was carried

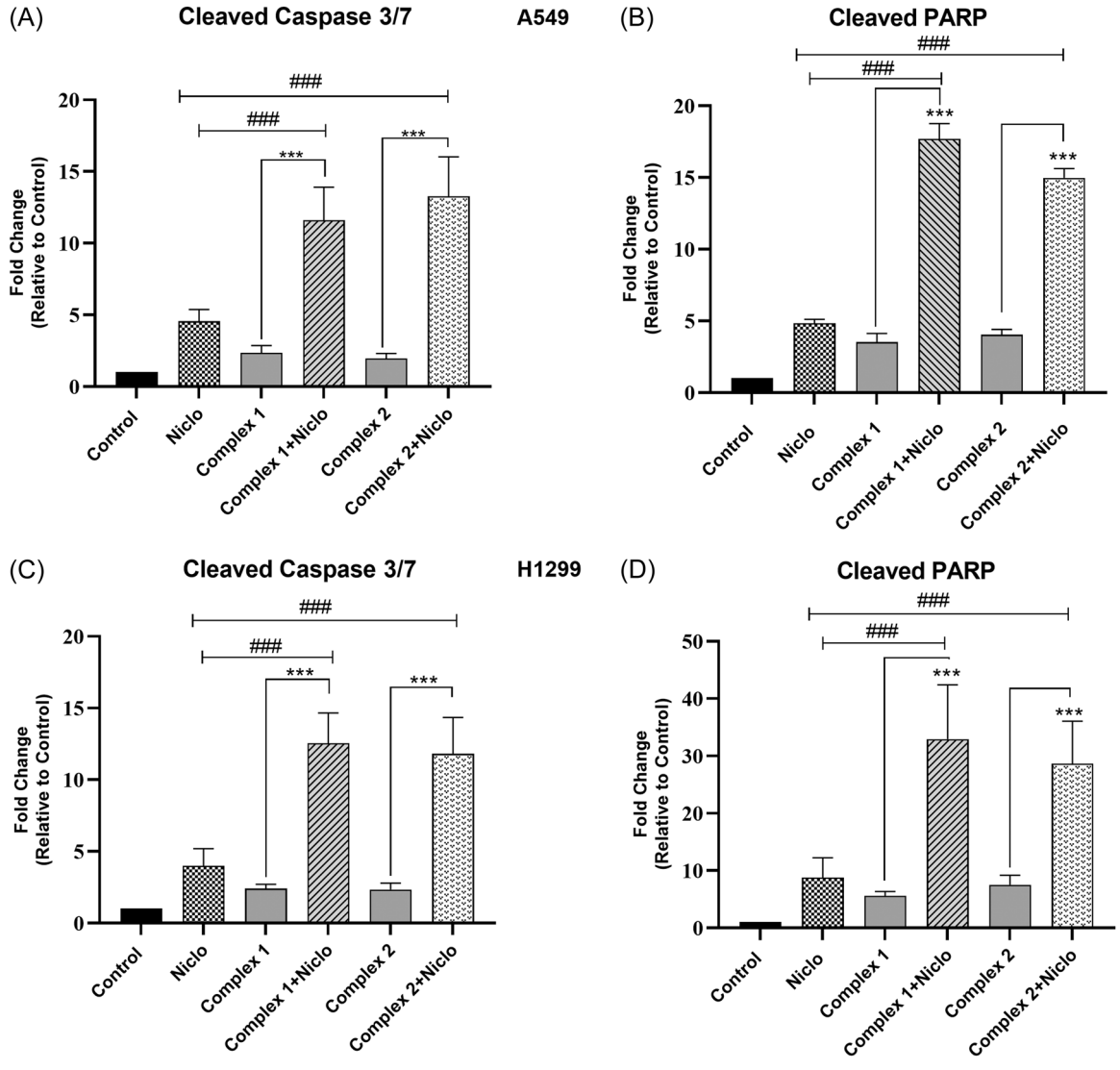


FIGURE 7 Determination of cleaved caspase 3/7 and cleaved PARP levels in A549 (A, B) and H1299 (C, D) cells after treatment with the Complex 1, Complex 2 (6.25 μM), and their Niclosamide (Niclo) (1.25 μM) combination for 48 h by ELISA assay. Denotes statistically significant differences between complex 1 or 2 and their combination with niclosamide: (*$p < 0.05$); (**$p < 0.01$); (***$p < 0.001$) and between niclosamide and their combination with complexes 1 or 2 ($^{\#}p < 0.05$); ($^{\#\#}p < 0.01$); ($^{\#\#\#}p < 0.001$). Data are presented as mean ± SD ($n = 3$). ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

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out. Wound healing assay was performed to investigate the migration potential of both A549 and H1299 cells after complex 1, complex 2 (6.25 μM), and niclosamide (1.25 μM) combination treatment (Figure 8A,B). Compared with the control group and single administration of each drugs, the combination reduced cell migration, leaving a larger scratch area after 36 h in the wound healing experiment. These results implied that the combination of complexes 1 and 2 with niclosamide inhibited the cell migration ability of the NSCLC cells.

Some of the multidrug resistance and EMT pathway genes expressions levels were measured by RT-qPCR analysis (Figure 9). According to our RT-qPCR results, combination of complexes 1 and 2 with niclosamide

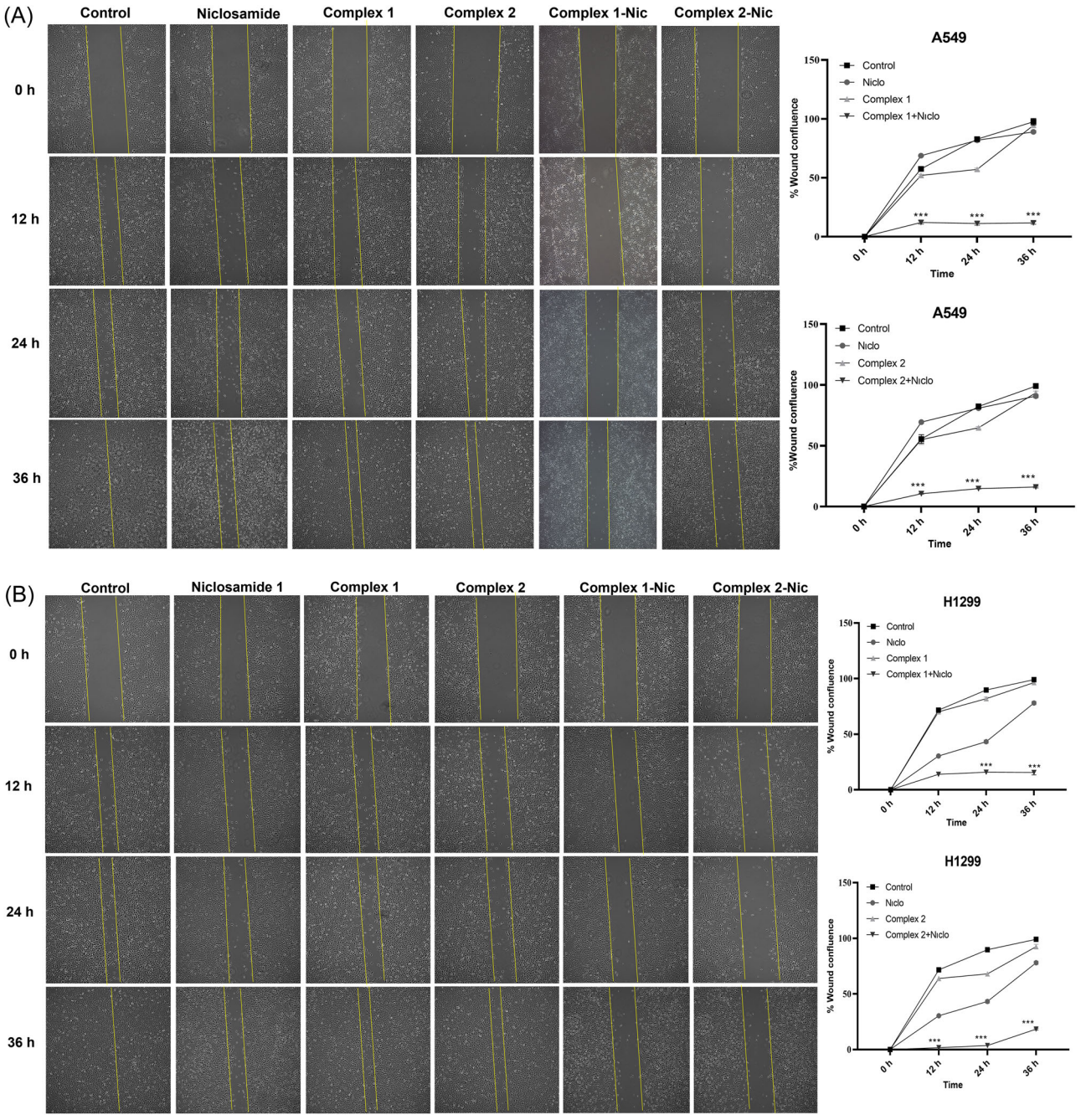


FIGURE 8 Wound healing assay in A549 (A) and H1299 (B) cells after treatment with Complex 1, Complex 2 (6.25 μM), and their combination with Niclosamide (Niclo) (1.25 μM) for 36 h. Combination treatment effect on A549 (A) and H1299 (B) cells migration evaluated by wound-healing assay, the cell images were taken at 0, 12, 24, and 36 h. The wound with of views was measured, and the healing width was calculated by wound with at 0 h time point minus wound with at 36 h time point and normalized by control. Denotes significant difference between control group and combination-treated cells (***$p < 0.001$).

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further decreased multidrug resistance genes ABCB1 and ABCC1 expression levels compared to the single administration of the complexes 1 and 2 in both cells (Figure 9A,B). In addition, combination treatment further reduced the mesenchymal genes VIMENTIN expression in A549 cells (Figure 9A), decreased the N-CADHERIN gene expression in H1299 cells (Figure 9B) and increased epithelial gene expression E-CADHERIN in both cells (Figure 9A,B). Transcriptional activators of the EMT pathway SNAI1 expression levels were further reduced in the combination treatment group in the H1299 cells (Figure 9B) and CTTNB1 gene expression levels further decreased in combination treatment group in both cells (Figure 9A,B). WNT3A, one of Wnt family members also more decreased in combination groups than single administration of complex 1 and 2 in A549 cell line and complex 2 in H1299 cell line (Figure 9A,B). These results stated that combination of complex 1 and 2 with niclosamide suppresses the EMT pathway on gene expression levels.

Western blot analysis demonstrated that, administration of complexes 1 and 2 together with niclosamide reduced the levels of the multidrug resistance protein 1 (MRP-1) in both cells compared to administration of each drug alone (Figure 10). Wnt/β-Catenin pathway proteins β-Catenin decreased in combination groups in both cells and Wnt3a decreased in the combination group in A549 cells (Figure 10A). Mesenchymal marker N-Cadherin was decreased in the combination groups in both cells (Figure 10A,B). Complex 2 and niclosamide combination decreased mesenchymal marker Vimentin and increased E-Cadherin marker in the A549 cells (Figure 10A). The results of all experiments showed us that combination of complex 1 and 2 with niclosamide reduces EMT process at both gene and protein levels.

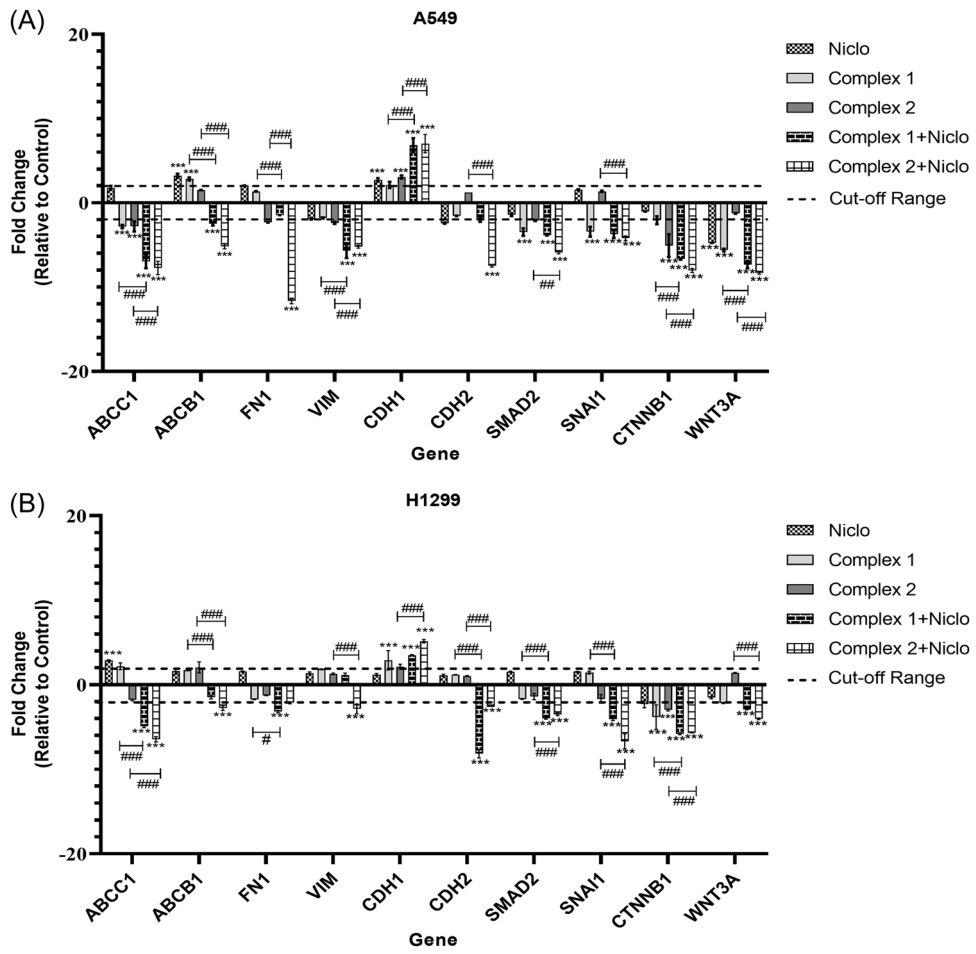


FIGURE 9 RT-qPCR analysis of some of the multidrug resistance and EMT pathway genes in A549 (A) and H1299 (B) cells after treatment with complex 1, complex 2 (6.25 μM), and their combination with Niclosamide (Niclo) (1.25 μM) for 48 h. Genes results denote statistically significant differences in comparison with control (***$p < 0.001$) and in comparison, between complex 1 or 2 and with niclosamide combination ($^{\#}p < 0.05$); ($^{\#\#}p < 0.01$); ($^{\#\#\#}p < 0.001$). Data are presented as mean ± SD ($n = 3$). RT-qPCR, real-time quantitative polymerase chain reaction; SD, standard deviation.

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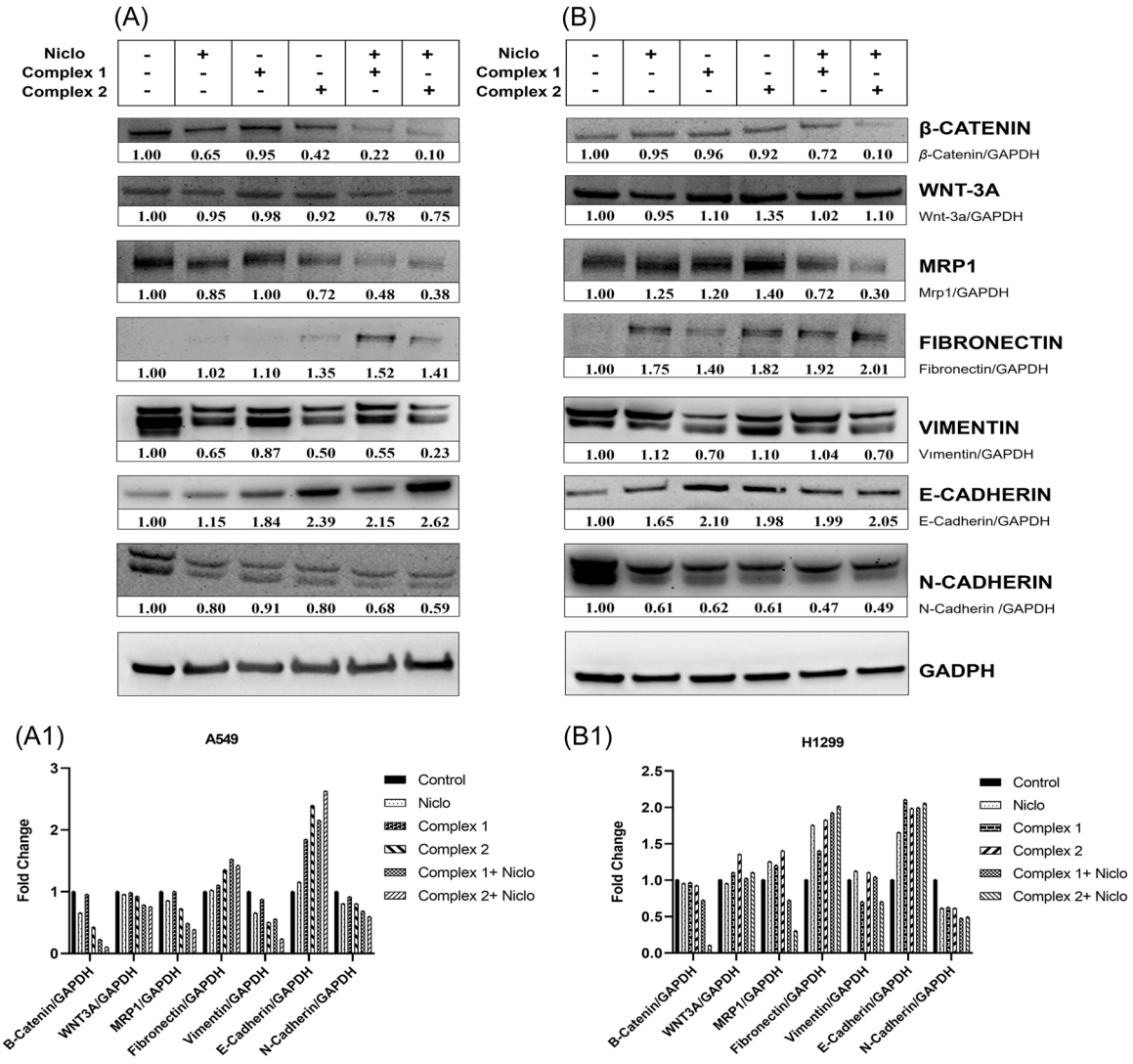


FIGURE 10 Western blot analysis of some of the multidrug resistance and EMT pathway proteins in A549 (A) and H1299 (B) cells after treatment with complex 1, complex 2 (6.25 μM), and their combination with Niclosamide (Niclo) (1.25 μM) for 48 h. Equal protein loading was confirmed by GAPDH. The graphs showed all groups and result together in A549 (A1) and H1299 (B1) cell lines. Densitometry was performed with the ImageJ software and densitometric analysis of the observed bands' intensity normalized to GAPDH and quantified with respect to controls set to 1.0. Data are presented as mean ± SD ($n = 3$). EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.

4 | DISCUSSION

Lung cancer is the leading cause of death from cancer worldwide. Among lung cancers, NSCLC constitute approximately 85% of all lung cancer cases.[26] Although there have been significant improvements in overall survival through screening for early diagnosis and treatment of NSCLC, chemoresistance, rapid relapse, and metastasis is still observed in some patients and this is associated with the exposure of cancer cells to EMT.[26–29] Therefore, there is an urgent need for new, effective therapeutic approaches in lung cancer. Niclosamide is an antihelminthic drug that has shown cytotoxic effects on NSCLC cells. Accumulating evidence has shown that niclosamide has anticancer activity in a variety of cancers, including NSCLC, by modulating multiple signaling pathways, including NFκB, Wnt/β-Catenin, Notch.[30–32] Niclosamide has shown to reduce EMT avtivity by blocking IL-6/STAT3 signaling.[10] Chalcone complexes are compounds that serve as synthetic precursors for different classes of flavonoids and are found in a variety of edible plants. In recent studies, it has been shown that chalcone complexes have many biological activities and are effective in cancer treatment.[13,20,33,34] Chalcone complexes also inhibit the EMT pathway activity by increasing p53 protein

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expression.[15] Considering that both niclosamide and chalcone complexes have anticancer and EMT blocking effects, the present study aimed to evaluate the antitumor response of the combination of niclosamide and chalcone complexes in NSCLC cells.

In the present study, niclosamide and chalcone complex combination was detected to synergistically and specifically kill NSCLC cells compared to the single administration of each drugs. In addition, it was determined that the combination treatment increased apoptotic activity and decreased the multidrug resistance and EMT processes in the lung cancer cells.

The anticancer efficacy of niclosamide has been investigated for many years in various types of cancer, including NSCLC.[10] In one study, niclosamide was shown to reduce the phosphorylation of STAT3, thereby reducing resistance to the estimated glomerular filtration rate inhibitor erlotinib in NSCLC.[35] There are also studies on the inhibition of the Wnt/β-Catenin pathway by niclosamide. Niclosamide increases GSK-3β phosphorylation to promote ubiquitin-mediated degradation of β-Catenin.[36] The Wnt/β-Catenin pathway is also activated during the EMT process of NSCLC.[5] E-Cadherin cleavage during EMT causes the release of β-Catenin, which acts as a transcriptional activator for cell growth.[37] In this study, we found that niclosamide reduced β-Catenin protein expression and its expression was further decreased by adding chalcone complexes in A549 cells. Apart from the Wnt/β-Catenin pathway proteins, there are other proteins has crucial roles in the EMT pathway such as Vimentin, E-Cadherin, N-Cadherin, and Snail. Upregulation of N-Cadherin and Vimentin followed by the downregulation of E-Cadherin is essential for the initiation of the EMT pathway.[38–40] Transcriptional factor Snail represses CDH1 transcription to induce EMT.[41] It was previously shown that niclosamide reduces Snail abundance and induces mesenchymal to epithelial reversion.[42] Our findings showed that Snail gene expression levels were decreased in the combination treatment group in the H1299 cells. In addition, it was shown that N-Cadherin protein expression decreased in the combination groups in both cells. The combination of complex 2 and niclosamide decreased the mesenchymal marker Vimentin and increased the E-Cadherin marker in A549 cells. The obtained results support the literature and showed that the combination therapy inhibited the EMT pathway by decreasing the protein levels of β-Catenin, N-Cadherin, and Vimentin and increasing the protein levels of E-Cadherin.

Drug resistance is one of the biggest hurdle to NSCLC. Different mechanisms that regulate drug resistance are governed by specific cell signaling pathways.[43] It has been stated that the EMT process and drug resistance are both related processes.[6] On the other hand, one of the EMT pathway activator Wnt/β-Catenin signaling is known to be associated with development of resistance against treatment as well.[44] In this current study, we found that combination treatment decreased the expression levels of the multi-drug resistance genes ABCB1 and ABCC1 and decreased MRP-1 protein levels compared to the single administration of the each drug in both cells. These results support the literature findings and suggest that combination therapy reduces drug resistance by inhibiting the EMT pathway.

This study is the first to demonstrate effective antitumor effect of the combination of niclosamide and chalcone complexes in NSCLC. However, in vivo studies are guaranteed to provide stronger evidence of this combination. With in vivo studies, it may be possible to determine the toxicity of each drug and their combination. In addition, basic pharmacokinetic and pharmacodynamic parameters of drugs can be shown. Therefore, the next step in this study may be to test the combination treatment in an animal model.

5 | CONCLUSION

Our results showed that niclosamide and chalcone complexes combination kills cancer cells synergistically and specifically. Combination treatment increase apoptotic activity, decrease EMT pathway and multidrug resistance-related gene expression and protein levels in the NSCLC cells. In this context, the combination of niclosamide and chalcone complex may be a new drug combination for the treatment of the NSCLC.

AUTHOR CONTRIBUTIONS

Ferda Ari supervised the study. Elif Erturk and Ferda Ari designed and organized the research. Omer E. Onur, Ipek Aydin, and Oguzhan Akgun performed the experiments. Demet Coskun performed synthesis and characterization of chalcone complexes. Ferda Ari, Elif Erturk and Oguzhan Akgun analyzed and interpreted the data. Ferda Ari, Elif Erturk, Omer E. Onur, and Ipek Aydin wrote the manuscript. Ipek Aydin, Omer E. Onur, and Elif Erturk prepared the figures and tables. All authors edited and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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