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Original Article

Transferulic Acid Suppresses TNF α-induced β-Catenin Activation and Epithelial-Mesenchymal Transition in A549 Lung Cancer Cells

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ABSTRACT

Background and Objectives: Lung cancer is a prevalent malignancy characterized by a high propensity for metastasis and is a rising contributor to mortality. The prognosis and treatment strategies have become challenging due to its metastatic ability. Henceforth, our current work investigates the potential of Transferulic Acid (TRA) in suppressing Tumor Necrosis Factor alpha (TNFα) mediated activation of β-catenin and Epithelial-Mesenchymal Transition (EMT), especially in lung cancer cells (A549). TRA was selected because of its potential to simultaneously modulate inflammatory and oncogenic pathways involved in EMT progression, although evidence in TNFα-driven EMT suppression in lung cancer remains limited. **Materials and Methods:** MTT experiment was performed to demonstrate the cytotoxicity of TNFα-treated cells, TNFα + TRA-treated cells, and TRA alone-treated cells. To study the morphometric deformities of treated and control cells, AO and EtBr staining were carried out. Transwell migration and wound healing assays were conducted to determine the migration and invasion of A549 cells. Clonogenic and cell aggregation assays were carried out to disclose the proliferation capability. Indirect ELISA and qRT-PCR were carried out to investigate the expression of β-catenin in treated and control cells. **Results:** From the results of MTT, the IC_{50} of TRA was found at a concentration of 98.69 µg/mL. Late apoptotic cells with nuclear condensation were observed in the TNFα + TRA group. The findings of transwell migration and wound healing assays revealed that the TNFα + TRA group effectively inhibits cellular migration. Whereas, the results of clonogenic and cell aggregation assays indicate the inability of cells treated with TNFα + TRA to proliferate in an anchorage-independent environment and the inability to form colonies, respectively. Lastly, the gene expression studies indicated that the TNFα + TRA group downregulated beta catenin. **Conclusion:** Transferulic Acid (TRA) inhibits TNFα induced β-catenin activation, suppressing epithelial-mesenchymal transition, cell migration, and proliferation in A549 lung cancer cells. These outcomes indicate that TRA may serve as a promising therapeutic agent towards inflammation-driven lung cancer progression.

Keywords: Epithelial-Mesenchymal Transition, Lung Cancer, Metastasis, Transferulic Acid.

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INTRODUCTION

Lung cancer stands out as one of the notable malignancies in the horizon of respiratory healthcare. During cancer-related morbidity and mortality, lung cancer plays a crucial role and has the most substantial economic cost, in contrast to other tumors. Its overall incidence was greater than that of cervical, colorectal, and breast cancer collectively. Globally, the distribution of lung cancer varies from men to women, demonstrating the commonest cancer in men, while in women, it is the 4th frequently occurring cancer.[1,2] The longstanding disparities between males and females in the rise and fall of cigarette smoking over time are reflected in gender variations in lung cancer mortality rates. Approximately 43% of deaths worldwide are caused by lung cancer in more developed nations, whilst 57% of deaths occur in less developed nations.[2] Each year, lung carcinoma accounts for 25% of all cancer-related fatalities with a high potential of metastasis.[3]

The tumor metastasis comprises a series of events by which tumor-forming cells spread from their initial site and develop into secondary tumors at new sites.[4] Accordingly, Epithelial-Mesenchymal Transition (EMT), a biological process, has become more widely acknowledged and plays significant functions in driving carcinoma invasion and metastasis. The process of EMT originates by setting the framework of embryonic growth and development, where they act as trans differentiation factors that influence pivotal morphogenetic processes. While

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EMT plays a crucial part in the early phases of development, such as gastrulation and formation of neural crest, it also serves as a major factor for metastasis in the majority of human cancer cases, including lung cancer. EMT initiates the advancement of lung cancer through complex and varied signaling networks such as TGF-β/SMAD and non-SMAD, Wnt/β-catenin, Notch and PI3K/AKT/mTOR signaling.[5-7] Furthermore, EMT has been implicated in vital tumorigenic characteristics of lung cancer, including enhanced invasion, angiogenesis, and metastasis. Numerous signaling pathways have been linked to EMT, including Notch, β-catenin, Hedge-hog, and PI3K-AKT.[7] During the initial phases of metastasis, cancer cells go through an EMT-like process, which is regulated by an active β-catenin signaling pathway. In lung cancer, aberrant activation of β-catenin signaling has been shown to promote EMT, tumor cell invasion, stemness, and metastatic dissemination, thereby contributing to disease progression and poor clinical outcomes.[8] The current work focused on the activation of the β-catenin/WNT pathway by TNFα (Tumor Necrosis Factor alpha) in the aspect of inhibiting the metastasis of lung cancer cells.

Despite the fact that there are many options for managing lung cancer, individuals may experience severe side effects with the existing therapeutic methods. Therefore, novel strategies for the treatment of lung cancer are continually needed. Our current study addresses this issue by using Transferulic Acid (TRA), a phenolic phytocompound and an isomer of ferulic acid, comprising a wide array of pharmaceutical attributes, such as antioxidant, anticancer, anti-inflammatory, and anti-aging effects.[9] To date, there is only limited research regarding the anticancer activity of TRA towards lung cancer. Although several established EMT-targeting approaches focus on inhibition of individual signaling pathways, their clinical translation has been limited by pathway redundancy, compensatory mechanisms, concerns regarding toxicity and incomplete therapeutic responses. In contrast, TRA represents a multi-targeted phytocompound with the potential to simultaneously modulate inflammatory and oncogenic signaling networks that contribute to EMT progression. This broader mechanism of action may provide an opportunity to interfere with TNFα-driven β-catenin activation while reducing metastatic and EMT-associated behavior in lung cancer cells. Hence, the present investigation could provide newer insights into targeting EMT by TRA via the influence of TNFα on the activation of β-catenin and lung cancer metastasis.

MATERIALS AND METHODS

Cell culture maintenance and seeding

The A549 (Human lung cancer) cell line was procured from ATCC and maintained in DMEM (Sigma-Aldrich, United States of America). The cell lines were grown in a 25 cm^2 tissue culture flask containing DMEM enriched with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany), and a combination of antibiotics, namely Penicillin (100 U/mL), Streptomycin (100 µg/mL), and Amphotericin B (2.5 µg/mL). The cultured cells were subjected to incubation at 37°C in a humidified 5 % CO_2 incubator (NBS Eppendorf, Germany). The viability was investigated by direct observation using an inverted phase contrast microscope. After 2 days, the confluent monolayer of cells was trypsinized, and the cells were then suspended in growth medium (10%). Thereafter, a 96-well tissue culture plate was seeded with a cell suspension of 100 µL containing $5x10^3$ cells/well, and incubated under humidified conditions.

Compound stock's preparation

1 mg of TRA was purchased from Sigma Aldrich and measured and diluted in 1 mL DMSO of 0.1% by utilizing a cyclomixer. In order to assure sterility, the freshly prepared sample mixture was subjected to a Millipore syringe filter (0.22 µm). Complete dissolution was ensured before use to maintain uniform exposure across experimental groups.

Evaluation of anticancer activity

The growth medium was discarded, TNFα at a concentration of 2 ng/mL was introduced and incubated for 48 hr. Then, newly prepared compounds at the concentrations of 100 µg/mL, 50 µg/mL, 25 µg/ mL, 12.5 µg/ mL, and 6.25µg/ mL of DMEM were introduced. Each dosage was introduced in triplicate to the appropriate wells and kept incubating at 37°C in a humidified 5% CO_2 incubator for 24 hr. Untreated control and only TNFα-treated cells were cultured simultaneously.

Direct microscopic analysis

The whole plate was analyzed under an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) after completion of 24 hr treatment. The observed images were photographed. Morphometric analysis of cells was performed; any evident deformities in cell morphology, including cell rounding and shrinkage, granulation, and vacuolization within the cytoplasm, were deemed markers of cytotoxic effect.

MTT experiment

15 mg of MTT reagent (Sigma, M-5655) was reconstituted in PBS of 3 mL after complete dissolution, and the content was filter sterilized. The sample mixture in the wells was aspirated after 24 hr of treatment, and a reconstituted MTT mixture (30 µL) was introduced into each test well and cell control wells. The plate was shaken gently and kept incubated under humidified conditions for 4 hr. To properly solubilize formazan crystals, the supernatant was carefully removed upon completion of the incubation period. MTT solubilization reagent (DMSO, Sigma Aldrich, United States of America) of 100 µL was incorporated; following this, the wells were gently blended by pipetting from top to bottom. The absorbance was recorded at a wavelength of 540 nm by utilizing a microplate reader.[10]

The formula calculated the % of growth inhibition:

$$\%\text{Growth inhibition} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of treated sample}}{\text{Absorbance of control}}\right) \times 100$$

Analysis of apoptotic activity by AO and EtBr staining

The A549 cells were maintained as mentioned in the protocols stated previously. TNFα (2 ng/mL) was added and incubated for 48 hr. IC_{50} concentration of TRA (98.69 µg/mL) was added further and kept incubating for 24 hr. Untreated control cells, only TNFα-treated, and TRA alone-treated cells were cultured and kept incubated at 37°C in humidified conditions for 24 hr. Following incubation, the cells were exposed to a cold PBS wash and then stained for the duration of 10 min at Room Temperature (RT) using a solution of AO and EtBr at an identical dosage of 100 µg/mL each. A fluorescence microscope attached with a blue filter (Olympus CKX41 with Optika Pro5 camera) was used to examine the stained cells after being twice rinsed with PBS of 1X.[11]

Transwell migration assay

Migration and functional metastatic assays were interpreted alongside cell viability findings to distinguish anti-metastatic activity from nonspecific cytotoxic effects. The A549 cells were cultured and maintained in DMEM under a humidified environment as mentioned earlier. After being trypsinized, the newly formed cells were seeded into a 12-well plate with a population of 200,000 cells per well and kept incubated for 24 hr. After reaching confluency of 90%, TNFα at a dosage of 2 ng/mL was incorporated into the cells and incubated for 48 hr. Following this, TRA at the dosage of 98.69 µg/mL was introduced and incubated for 24 hr. Untreated control cells, only TNFα treated cells, and sample alone treated cells were also cultured similarly and incubated in humidified conditions. After 24 hr of incubation, the cells were eliminated from the tissue culture plate with the help of trypsin-EDTA of 0.25% and subjected to centrifugation, and resuspended in serum-free media. Serum starvation prior to migration analysis was performed to minimize serum-induced cell proliferation and to ensure that the observed changes primarily reflected treatment-mediated effects on cell migration rather than differences in cell growth.

Upon plating a 100 µL aliquot onto a filter membrane in transwell-insert, and kept incubation at 37°C and 5% CO_2 for 10 min. Following the incorporation of DMEM (600 µL) with FBS of 10% to the lower chamber, the cultures were incubated for 24 hr. After completion of the appropriate incubation period, the transwell-insert was removed, and with the aid of a cotton tipped applicator, non-migrated cells were gently eliminated from the membrane. The insert was then placed in 70% ethanol to fix the cells for 10 min and dried for 10-15 min for the removal of ethanol. The membrane experienced staining using crystal violet of 0.2% for a duration of 5-10 min, excess dye was eliminated with water, and the membrane was thereafter dried. An inverted microscope was utilized to count the cells that migrated and adhered to the membrane's underside.[12]

Clonogenic assay

The protocol was followed by the instructions described in the work of Du *et al.*, 2017, with slight alterations.[13] The A549 cell line was cultured and maintained in DMEM under a humidified environment, as performed in earlier assays. Upon achieving adequate confluency, TNFα at a dosage of 2 ng/mL was incorporated into the cells and incubated for 48 hr. TRA at the dosage of 98.69 µg/mL was added and subjected to incubation for 24 hr. Untreated control cells, only TNFα treated cells, and sample alone treated cells were cultured. Following treatment, cells were seeded at a consistent density across all experimental groups to ensure comparability of colony formation outcomes. The transfected cells were trypsinized. The agarose solutions of 1% and 0.8% were made and maintained in a water bath at 56°C. On a 6-well plate, the bottom region was established by combining 2 mL agarose of 1% with an equivalent volume of DMEM and kept for solidification. Following this, 5 mL agarose of 0.8% was combined with DMEM (6 mL). The upper region was created by incorporating a 2 mL agarose mixture of 0.8%. After being trypsinized, the transfected cells were spread on the top region. The plates were subjected to 14 days of incubation. Finally, the colonies were treated with formaldehyde of 4% for 2 hr and subsequently stained in crystal violet of 0.005% for 30 min. Quantification of colony formation was performed across three independent biological replicates, and results are presented as mean±SD.

Migration (wound healing) assay

The A549 cell line was cultured in Ham'sF12 media enriched with 10% FBS, L-glutamine, sodium bicarbonate, and antibiotics (Penicillin-100 U/mL, Streptomycin 100 µg/mL, and Amphotericin B-2.5 µg/mL). The cultured cells were incubated under humidified conditions. The cells were then trypsinized and seeded onto 12-well plate with a population of 200,000 cells per well and subjected to an incubation period of 24 hr. After attaining the appropriate confluency, TNFα at a dosage of 2 ng/mL was incorporated into the cells and kept incubation for 48 hr. A sterilized pipette tip of 1 mL was inserted through a pre-marked line to create the scratch wounds. The debris from 5 linear scratches was removed thoroughly. The monolayer of cells was rinsed thrice with PBS. Following this, the cells were subjected to TRA at a dosage of 98.691 µg/mL and incubated for various intervals of time, including 0 hr, 24 hr, 48 hr, and 72 hr. The wound regions were shown by capturing photographs directly over the intersections of scratched wound regions and pre-marked lines. The impact of the sample on the closure of the wound was assessed with a microscope (4X magnification, Olympus CKX41) following incubation. The sample's impact on

the closure of the wound was estimated on the basis of area by MRI-Image J analysis software.

Cell aggregation assay

The A549 cells were cultured in DMEM and incubated in a similar manner as mentioned earlier. After achieving confluency of 80%, the cells were subjected to TNFα (2 ng/mL) and incubated for 48 hr. TRA (98.691 µg/mL) was introduced and kept incubating for 24 hr. Untreated control cells, only TNFα treated cells, and sample alone treated cells were maintained simultaneously. 50 µL of the agar solution was introduced into each well of a 96-well microtiter plate and positioned on a horizontal surface at 4°C for 1 hr to attain proper solidification. The cells were washed twice in 3 mL of Moscona solution (Into 900 mL of distilled H_2O, 8.0 g of NaCl, 0.3 g of KCl, 0.05 g of $Na_2HPO_4{\cdot}H_2O$, 0.025 g of KH_2PO_4, 1.0 g of $NaHCO_3$, 2.0 g of D (+)-glucose (dextrose) were added and dissolved. The pH was set to 7.0-7.4 using normal HCl and distilled H_2O was added further to reach 1 L. Sterilized by filtration and stored at -20°C. The cells were then detached by trypsinization, and a cell suspension of 100 µL with a population of 20,000 cells was introduced into the agar-coated wells. DMEM of 100 µL was administered to both treated and untreated wells and kept incubated at 37°C in a humidified environment. The aggregation of cells was monitored after 24, 48, and 72 hr of incubation. Formation of large compact aggregates, small loose aggregates, and absence of aggregates were observed using a microscope at the magnification of 4X (Olympus CKX41). The cells were stained using crystal violet after 72 hr. Initially, the aggregates experienced fixation with formaldehyde of 4% for 2 hr followed by staining with crystal violet of 0.005% for 30 min.

Indirect ELISA and Estimation of protein

The cell line culture, maintenance and incubation were similar as described in previous assays. Upon reaching confluency, TNFα at a dosage of 2 pg/mL was introduced to induce toxicity and incubated for 48 hr. TNFα stimulated A549 cells were then subjected to TRA (98.69 µg/mL) and kept incubated for 24 hr under humidified environment. Sample alone treated cells and untreated control cells were maintained in the same manner. Upon completion of incubation, supernatant was gathered, and 100 µL of each was dispensed into the 96-well plate and incubated overnight at 37°C. Following next day, the wells were drained and experienced PBS wash twice. The newly prepared blocking buffer 200 µL (0.2% gelatin in 0.05% Tween 20: Merck; Germany) in PBS was then incorporated and kept incubating for 1 hr at RT, followed by washing twice with PBS-TWEEN. 100 µL of primary antibody (Beta catenin, Santa Cruz, 1:500) was incorporated and kept incubation for 2 hr at RT. Upon completion of the incubatory period, PBS-TWEEN was employed to wash it twice following the addition of secondary antibody of 100 µL (HRP Conjugate, Santacruz, United States of America), the mixture remained at RT for 1 hr. Then, experienced washing with PBS-TWEEN twice. 200 µL of O-dianizdine hydrochloride (1 mg/100 mL methanol, 21 mL citrate buffer pH 5, 60 mL H2O2: Sigma Aldrich, United States of America) was introduced and left for 30 min at RT. Following this, the reaction was halted by the addition of 50 µL

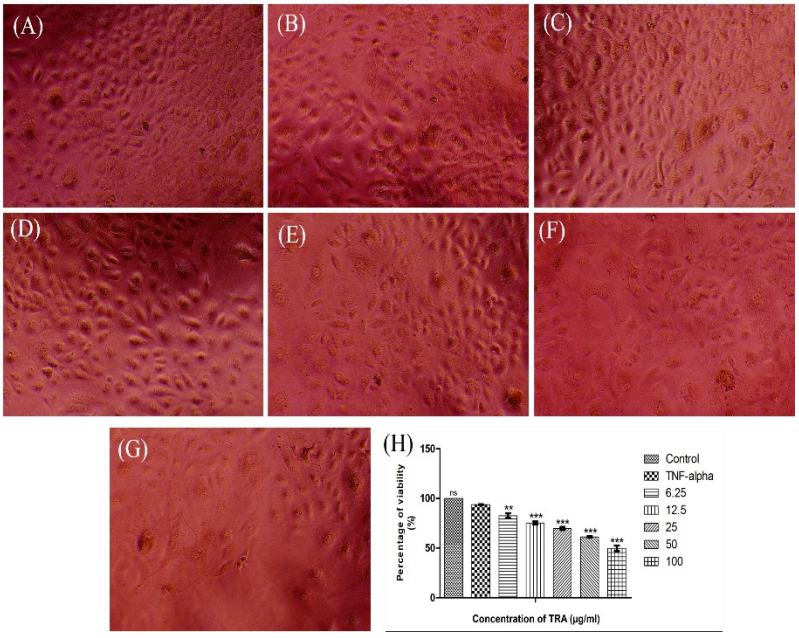

Figure 1: A: Denotes control cells. B: Depicts TNFα induced A549 cells. C-G: Represents TNFα + TRA treated cells at different dosages (6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/ mL, and 100 µg/mL). H: Denotes the graphical representation of MTT assay results. The viability of A549 cells declined on increasing dosages of TRA. The assays were conducted in triplicate, and outcomes were reported as Mean±SD. One-way ANOVA and Dunnett's multiple comparisons test were carried out to analyse the data. ***p<0.0001 denotes highly significant differences compared to the TNFα-induced group, **p<0.001 denotes a very significant difference compared to the TNFα-induced group, and ns represents non-significant compared to the TNFα-induced group.

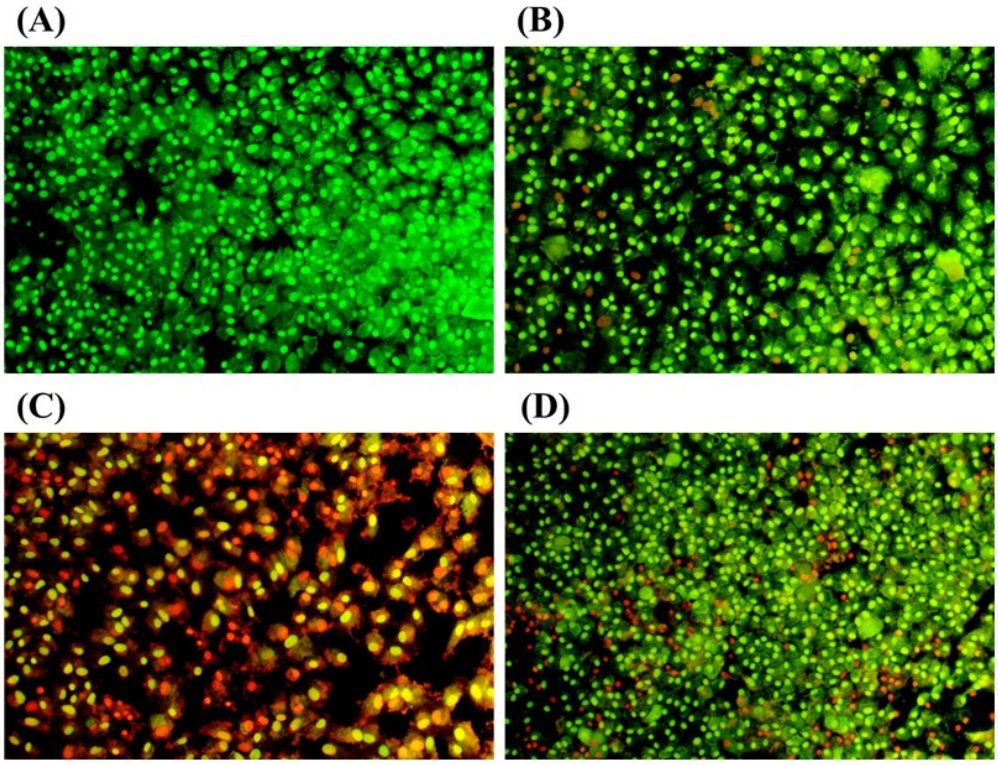

Figure 2: A: Denotes the staining images of control cells. B: Represents the TNFα group, indicating the presence of live cells. C: Depicts TNFα + TRA group revealed the presence of necrotic and late apoptotic cells. D: Represents TRA group, which revealed the presence of live cells and apoptotic cells.

of 5N HCl. The absorbance was read in an ELISA reader at 415 nm.[14]

The protein estimation was quantified by the Bradford method. 100 µL of the samples was combined with 5 mL of diluted dye. The mixture was thoroughly blended and permitted to form color for a minimum of 5 min and a maximum of 30 min. The absorbance was read by utilizing a UV-vis Spectrophotometer (SHIMADZU-UV-1900i) at the wavelength of 595 nm after completion of the incubation period. A standard graph was constructed, and concentration of protein was determined utilizing the standard curve. Bovine serum albumin functioned as the reference standard.

Activity of Antibody = OD Value / Protein Concentration

Gene expression studies

The A549 cell line was cultured as mentioned in standard protocols. Once the appropriate confluency was attained, a non-toxic concentration of TNFα at the dosage of 2 ng/mL was introduced and incubated for 48 hr, followed by exposure of TRA at the concentration of 98.69 µg/mL from the TRA stock of 1 mg/mL and kept incubating for 24 hr. Untreated control cells, TNFα-induced cells, and TRA alone-treated cells were also maintained.

Isolation of total RNA

The Total RNA was extracted by employing a total RNA isolation kit in accordance with the manufacturer's instructions (Invitrogen, Product Code (PC) 10296010). 1 mL of TRIzol reagent was introduced into the culture well-plate and kept incubating for 5 min. The contents were moved to a new sterile Eppendorf tube. 200 µL of chloroform was incorporated at RT, and centrifuged at the rpm of 14000 for 15 min at 4°C. Upon carefully gathering aqueous-layer, isopropanol (100%) of 500 µL was incorporated, and the mixture was allowed to sit at RT for 10 min, followed by centrifugation for 15 min at 4°C at rpm of 14000. After removing the resultant supernatant, of 75% ethanol (Merck) of 200 µL was utilized to wash the pellets. The pellet was then centrifuged for 5 min at 4°C at 14000 rpm in a cooling centrifuge- Remi CM12. Upon drying, TE buffer was utilized to suspend the final RNA pellet.

Synthesis of cDNA

The total RNA was isolated utilizing TRIzol (Invitrogen, United States of America), and its purity was also determined. Template complementary DNA was produced by utilizing cDNA preparation kit (G BIOSCIENCES, PC:786-5019s, 786-5020, master premix for first-strand cDNA production). Into an RNase-free tube, 5 µL of RT Easy mix, 0.5 µL of oligo dT, and 2µL of RNA template (0.5 µg of total RNA) were added. Sterile distilled. H_2O was introduced to raise the total reaction volume

to attain 10 µL. Pipetting upward and downward was used to progressively mix the solution mixture. The thermal cycler (Eppendorf Master Cycler) was set up to perform the synthesis of cDNA. The cycling parameters followed are 20 min at 42°C and 5 min at 85°C (Table 1).

qRT-PCR

The SYBR Green Master Mix (G BIOSCIENCES, PC:786-5062 utilizing Lightcycler 96 Roche) was used for the Real-Time qRT-PCR studies (Table 2). The primer sequences are mentioned in Table 3. All reactions were conducted in triplicate, and the outcomes were evaluated by the ΔΔCt method with Light Cycler 96 SW 1.1 Software. Lastly, agarose (1.5%) gel electrophoresis was performed to visualize the DNA fragments. A gel documentation system (ChemiDoc Imaging system, Biorad) was adopted to visualize the stained gel.

Statistical analysis

The GraphPad Prism version 8.0 was used for statistical evaluation. The assays were conducted in triplicates (biological) and the outcomes were verified by One-way ANOVA. Dunnett's multiple comparisons test was used to compare the treated and control groups. Data are denoted as Mean±SD. $p<0.0001$ was denoted as significant.

RESULTS

MTT experiment

The cytotoxic effect of TNFα induced and TRA treated A549 cells was investigated using MTT reagent. Cell viability declined in a dose-dependent manner. The findings of the MTT experiment (Figure 1) unequivocally indicate that the viability was significantly reduced in TRA treated cells compared to TNFα induced cells. Additionally, the IC_{50} of TRA was noticed at a dosage of 98.69 µg/mL. Further, on treatment with the higher concentration of TRA (100 µg/mL), A549 cells exhibited only 49.78% of viability, strongly suggesting the cytotoxic impact of TRA was higher than at other doses, and TNFα induced A549 cells. Although TRA affected cell viability at higher concentrations, subsequent migration and EMT-related analyses demonstrated molecular and functional changes consistent with suppression of metastatic signaling, suggesting that reduced migration was not solely attributable to cytotoxicity. This interpretation was supported by downregulation of β-catenin activity and reduced EMT-associated responses.

Analysis of apoptotic activity by AO and EtBr staining

Based on the stained cells, they were classified into live cells, which exhibited normal green fluorescence, and early apoptotic cells, which displayed bright green fluorescence, denoting chromatin condensation. Orange fluorescence cells are indicative of late apoptotic and necrotic cells. From the micrographic images of AO and EtBr staining, Figure 2A denotes control cells observed in green color. Whereas Figure B represents cells induced with TNFα, demonstrating green stained cells. Figure C illustrates TNFα induced and TRA treated cells, exhibiting a homogeneous distribution of orange fluorescent cells, which represent apoptotic cells. Conversely, Figure D depicts the cells treated with TRA alone, revealing a predominance of green fluorescence cells alongside a minimal number of orange fluorescent cells.

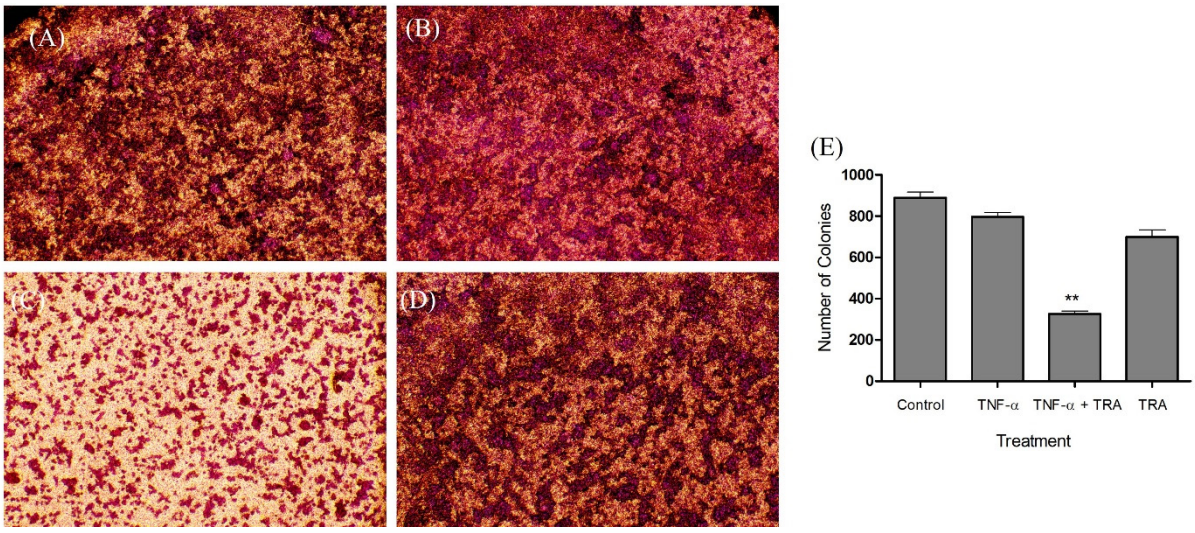

Figure 3: A: Represents control cells. B: Denotes the TNFα group, which revealed the presence of migrated cells. C: Depicts TNFα + TRA group, indicating no signs of migration. D: Represents the TRA group; migrated cells were observed as similar to the TNFα group. E: Represents the graphical data, disclosing that the migrated cells were reduced in the TNFα + TRA group. The assays were conducted in triplicates and the findings were shown as Mean±SD. Data analysis was performed using the one-way ANOVA and the Dunnett's multiple comparisons test. **$p<0.001$ represents significant difference compared to TNFα induced group.

Table 1: Represents the parameters followed in cDNA synthesis.

Steps involved	Temperature °C (Tm)	Time (min)	No. of cycles
cDNA-synthesis	42	20	1
Inactivation step	85	5	1

Transwell migration assay

The transwell migration assay was conducted to assess the migration and invasion of A549 cells. The outcomes revealed that the migrated cells were raised in both the TNFα group and the TRA group. The control groups displayed 894 colonies, while TNFα group and TRA group revealed 892 and 714 colonies, respectively. Furthermore, in the TNFα + TRA group, the population of migrated cells drastically decreased by 327 colonies, indicating that the migration and invasion attributes of A549 cells were hampered Figure 3.

Clonogenic assay

The ability of cells to proliferate independently of anchorage is assessed using the colony formation assay. The TNFα + TRA group had the potential to suppress the capability of A549 cells to form colonies. In the TNFα + TRA group, there are only 36 colonies formed, whereas the TNFα and TRA groups displayed 286 and 178 colonies, respectively. This pattern of formation of colonies proves that A549 cells proliferated independently of anchorage in both TNFα and TRA groups. Yet the cells were unable to proliferate in TNFα + TRA group demonstrating only fewer colonies. The findings (Figure 4) provide proof that the TNFα + TRA group possesses anchorage-independent growth inhibitory effects in A549 cells.

Migration scratch (Wound healing) assay

The migration scratch experiment was conducted to assess the motility of A549 cells, a significant attribute of EMT. As shown in Figure 5, the size of the wound (scratch) analysis was carried out at the time span of 0 hr, 24 hr, 48 hr, and 72 hr in TNFα group, TNFα + TRA group, and TRA group. It can be seen from the findings that, on exposure to TNFα + TRA even after 72 hr, A549 cells exhibit no signs of migration. On the other side, TNFα elicited migration, resulting in the absence of a wound after 24 hr. Similarly, TRA also enhanced migration; the absence of scratch was noticed after 48 hr. Notably, the findings of the migration scratch assay were in correlation with the transwell migration experiment.

Quantitative analysis of wound closure demonstrated increased migratory activity in TNFα-treated A549 cells compared with untreated controls, with approximately 55.6% wound closure at 24 hr and complete closure observed by 48 hr. In contrast, co-treatment with TRA markedly reduced wound closure progression, resulting in only 40.3%, 40.9%, and 54.2% closure at 24, 48, and 72 hr, respectively, compared with the TNFα-induced group. These findings indicate that TRA significantly attenuated TNFα-mediated migratory behavior of A549 cells. Data were expressed as mean±SD from three independent biological replicates and analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test.

Cell aggregation assay

The development of dense aggregates, small loose aggregates, and the absence of aggregates were observed at different time points to evaluate cell adhesion and cancer cell invasiveness. In the TNFα + TRA group, the presence of loosely formed aggregates was observed, indicating loss of cell aggregation and evoking a mesenchymal phenotype. The TNFα group exhibited compact aggregates. In the TRA group, the aggregates are slightly looser (Figure 6).

Indirect ELISA and Gene expression analysis by qRT-PCR

The expression of β-catenin was observed in TNFα group, TNFα + TRA group, and TRA group. In the present investigation, β-catenin was strongly upregulated in the TNFα group. In the TRA group, the upregulation was minimal. Conversely, β-catenin was downregulated in the TNFα + TRA group, as evident from the ELISA findings (Figure 7). In the context of gene expression studies depicted in Figure 8, A549 cells treated with TNFα alone potentially elevated beta catenin expression. This is because TNFα mainly triggers the β-catenin/WNT pathway. In the TNFα + TRA group β-catenin was downregulated, emphasizing that TRA limits the influence of TNFα on beta catenin expression. Likewise, TRA group demonstrated a major drop in β-catenin expression similar to the TNFα + TRA group.

DISCUSSION

Lung cancer ranks as the second most prevalent cancer type all over the world. It presents a significant threat to human health and has been the primary cause of cancer mortality.[3] Tumor progression to metastasis in lung cancer involves a series of stages in which malignant cells disseminate from the primary tumor and invade other tissues.[15] Thus, prevention of metastasis has been a focal point in clinical practice. Transferulic Acid (TRA), a phenolic phytocompound, possesses anti-tumor and anticancer capabilities, along with other notable pharmacological attributes.[9] In the current study, the efficacy of TRA in inhibiting the metastasis of A549 cells was examined. Initially, A549 cells were treated with TNFα, a well-known pro-inflammatory cytokine. Later, A549 cells were categorized into 3 groups: TNFα group, TNFα + TRA group, and TRA group, along with untreated cells. Then, a series

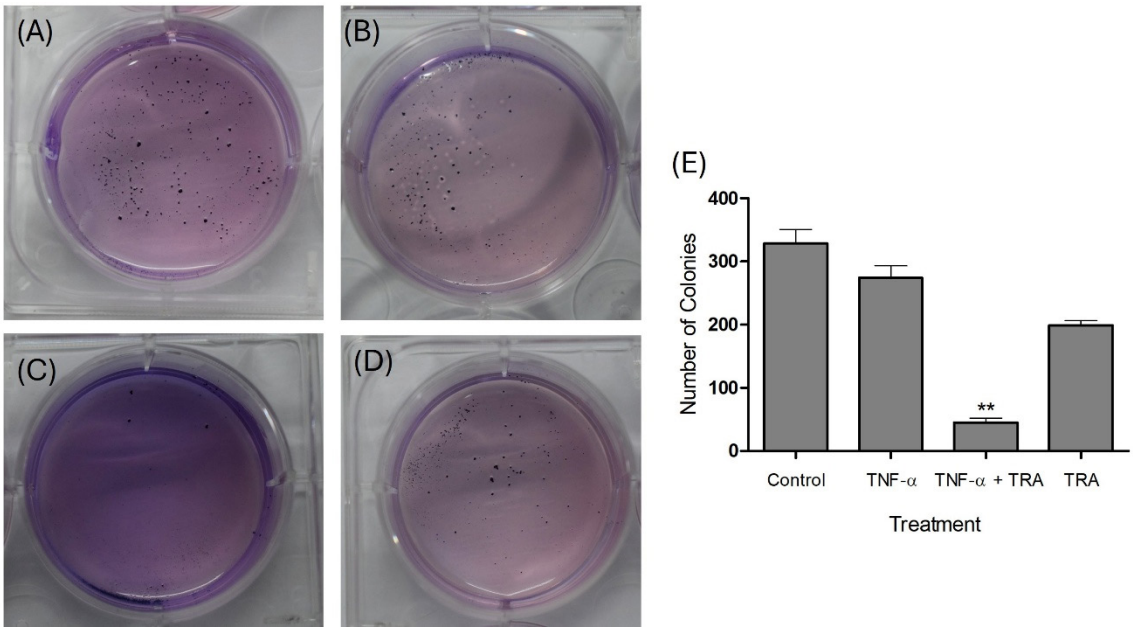

Figure 4: A: Depicts control cells. B: Represents the TNFα group, which disclosed the presence of colonies. C: Denotes TNFα + TRA group, indicating the absence of colonies. D: Denotes the TRA group; the colonies were observed as similar to the TNFα group. E: Represents the graphical data, indicating the colony formation were declined in TNFα + TRA group. The assays were conducted in triplicate, and the findings were depicted as Mean±SD. Data analysis was evaluated by one-way ANOVA and Dunnett's multiple comparisons test. ** $p<0.001$ depicts a significant difference compared to TNFα induced group.

Table 2: Denotes the stages, duration, and temperature of each step in qRT-PCR analysis.

Steps in synthesis	Time	Tm
Initial activation step	2 min	95°C
3-step cycling		
Denaturation	10 sec	95°C
Annealing	1 min	58°C
Extension	1 min/kb	72°C
Cycles count	40 cycles	
Final phase of PCR cycling	Indefinite	4°C

of assays and gene expression studies were performed to evaluate the potential of TRA to impede the metastasis in the presence and absence of TNFα.

TNFα is largely acknowledged as a significant regulator of the tumor microenvironment, which promotes the β-catenin/WNT pathway in several cancer cells, including breast and osteosarcoma.[16,17] Studies revealed TNFα triggers ROS generation, which is closely linked to cancer metastasis.[18] Additionally, TNFα activates the nuclear translocation of β-catenin and stabilizes its protein levels.[19] Alongside, TNFα activates the PI3K/Akt pathway, which causes suppression of Glycogen Synthase Kinase-3 beta (GSK-3β), signifying the degradation of β-catenin.[20] TNFα also sets up signaling cascades that cause GSK-3 β to be phosphorylated. In the end, this will result in the accumulation of free β-catenin in the cytoplasm and proteasomal destruction.[21] This nuclear translocation enables β-catenin to associate with T- Cell Factor/Lymphoid Enhanced Factor (TCF/LEF) transcription factors, thereby promoting the expression of notable oncogenic genes c-Myc, Cyclin D1, and Urokinase-type Plasminogen Activator (uPA).[22,23] Furthermore, TNFα also functions as an activator of the NF-κB pathway. The TNFα/NF-κB pathway triggers the expression of Snail, which interacts with β-catenin to form a favorable feedback mechanism that boosts WNT-dependent transcription.[24-26]

In the context of TRA, it primarily interferes with the mitochondrial function of A549 cells by arresting the cells at G0/G1 phase, enhancing the accumulation of caspase 3, elevates the levels of intracellular ROS,[27,28] inhibits significant pathways namely, PI3K/Akt/mTOR, β-catenin/WNT, and ERK/p38 and promoting apoptosis collectively, causes an impairment of normal metabolic function and proliferation.[29] It can be noted from the MTT assay that there will be a reduction in the conversion of MTT reagent into formazan, indicating a decreasing trend in viable cells, as the MTT assay evaluates the mitochondrial metabolic activity of viable cells.[30] In the same way, late apoptotic and necrotic cells were seen in the micrographs of AO and EtBr staining. As previously stated, TRA induces apoptosis in A549 cells, resulting in compromised membrane integrity and nuclear condensation, which creates a transition from normal fluorescence to orange fluorescence in the cells that can be witnessed clearly in the AO and EtBr staining.

On account of the migration and invasiveness of A549 cells, the TNFα + TRA group hampered the migratory cells in comparison with the other groups. Research indicates that ferulic acid inhibits A549 cell adhesion to the extracellular matrix, thereby obstructing

Table 3: Represents the summarized form of primer sequences.

Oligo Name	Forward sequence		Reverse sequence	
	Sequence (5′->3′)	Tm	Sequence (5′->3′)	Tm
BETA CATENIN	GTACCGGAGCCCTTCACATC	61.4	CTGGCCACACCTTCATTCCTA	59.8
GAPDH	AATGCATCCTGCACCACCAACTGC	64.4	GGAGGCCATGTAGGCCATGAGGTC (24)	67.8

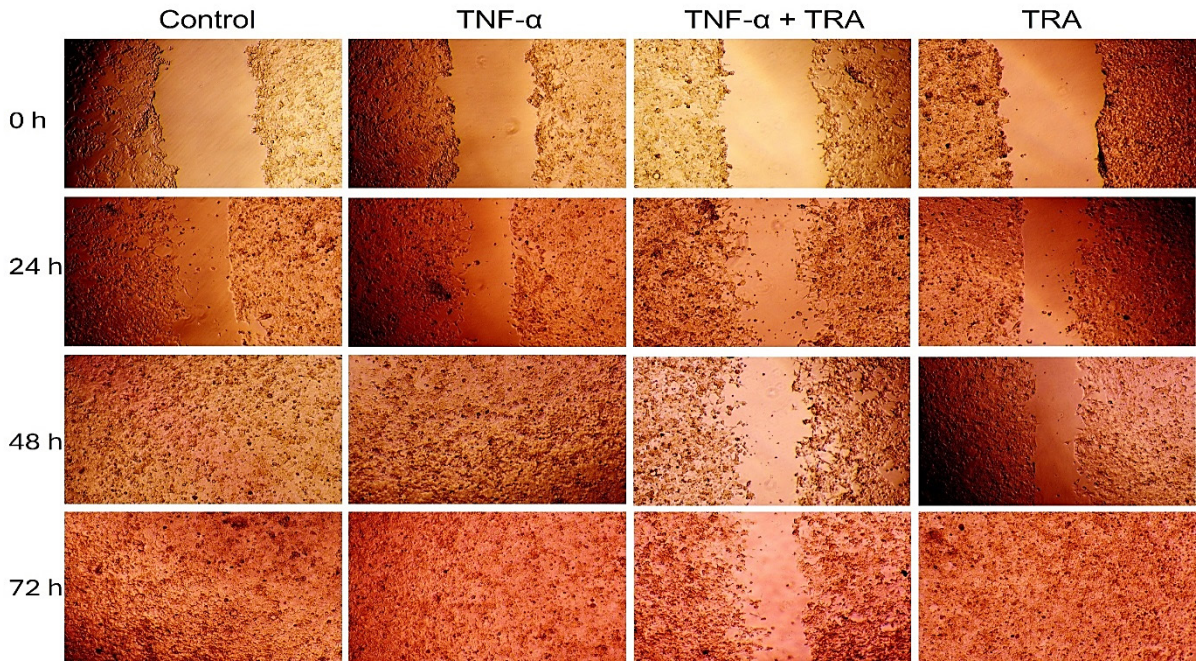

Figure 5A: The represents the findings of the migration scratch (wound healing) assay. TNFα group demonstrated closure of the scratch after 24 hr indicating the presence of migratory cells. TNFα + TRA group depicts the absence of migratory cells after 72 hr. The TRA group demonstrated the absence of a wound after 48 hr.

the early phases of migration. Furthermore, TRA diminishes the MMP-2 and MMP-9 expressions, which are vital for cancer cell invasive properties. Meanwhile, it also induces the inhibition of Akt/mTOR and ERK phosphorylation, which contributes to the loss of cancer cell migration.[31] The transwell migration, wound healing, and clonogenic assays in our study demonstrated that the TNFα + TRA group significantly inhibited A549 cell migration and prevented proliferation and colony formation, thereby attenuating TNFα-induced effects, presumably via the aforementioned strategies. TRA raises E-cadherin expression, promoting cell-to-cell adhesion, while diminishing the activity of significant mesenchymal indicators, such as N-cadherin, Snail, Slug, and Vimentin, which drives EMT.[32] Consequently, this downregulation of mesenchymal markers facilitates the formation of loose aggregates.[16,33] This could be the reason behind the presence of loosely packed aggregates in the TNFα + TRA group, which was observed in the cell aggregation assay. Since cell aggregation represents an indirect indicator of changes associated with metastatic behavior, these findings should be interpreted in conjunction with mechanistic and functional assessments rather than considered direct evidence of EMT regulation.

Meantime, there are certain fascinating traits that were identified in the outcomes of indirect ELISA, especially in the TRA group. The expression of β-catenin was partially found in the TRA group, similar to that of control cells. This is in response to baseline reactions that cause β-catenin to be expressed even in the absence of TNFα induction. The overexpression of β-catenin in the TNFα group in gene expression studies is attributable to the previously explained mechanisms. Our findings show that TRA effectively inhibited the effects induced by the TNFα group, resulting in the downregulation of β-catenin. TRA stimulates β-catenin phosphorylation at crucial N-terminal residues Thr41 and Ser45, activating ubiquitin-proteasome pathways, thus causing proteasomal degradation of cytoplasmic β-catenin and minimizing its protein levels.[34] Likewise, TRA inhibits the NF-κB pathway, thereby preventing the degradation of IκB, eventually reducing the NF-κB p65's phosphorylation, blocking the p65 subunit's nuclear translocation.[35,36] The inhibitory effect of TRA on migratory and metastatic behavior should not be interpreted exclusively as a consequence of reduced cell viability. Rather, concurrent suppression of β-catenin signaling suggests that TRA may interfere with EMT-associated molecular mechanisms contributing to metastatic progression. Collectively, our study

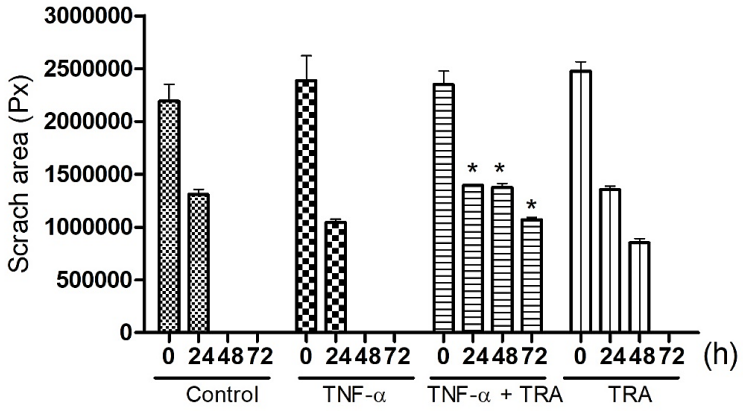

Figure 5B: Denotes the graphical representation of the migration scratch (wound healing) assay. The assays were conducted in triplicate, and the outcomes were denoted as Mean±SD. Data analysis was verified by one-way ANOVA and Dunnett's multiple comparisons test. *p<0.001 representing a statistically significant difference compared TNFα induced group.

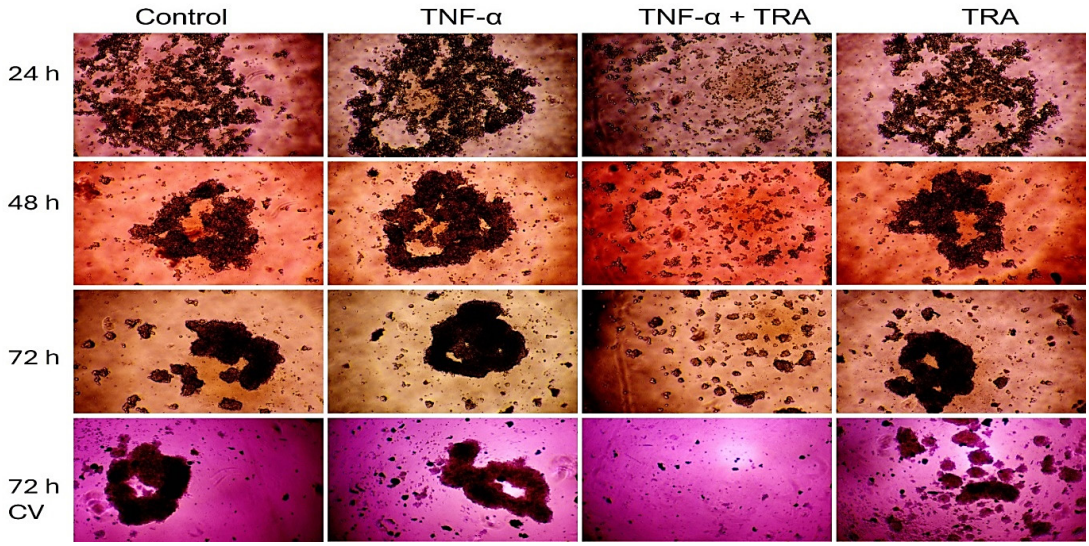

Figure 6: Represents the formation of cell aggregates in the control, TNFα, TNFα + TRA, and TRA groups at different intervals of time. The control, TNFα group, and TRA group demonstrated the presence of dense, tightly packed cell aggregates. The TNFα + TRA group revealed the presence of loosely packed cell aggregates over time.

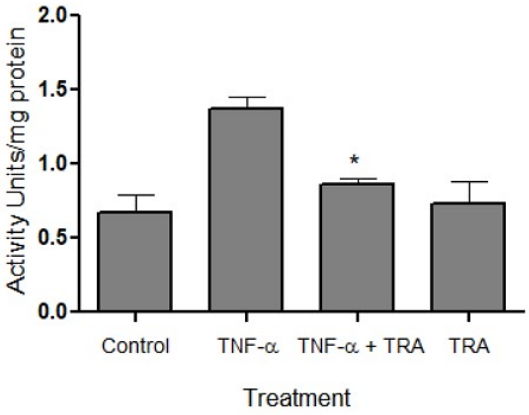

Figure 7: Discloses the graphical representation of indirect ELISA, indicating that the activity of beta-catenin was elevated in the TNFα group. The TNFα + TRA and TRA groups exhibited downregulation of beta catenin activity. The assays were performed in triplicate, and the findings were denoted as Mean±SD. Data analysis was evaluated by one-way ANOVA and Dunnett's multiple comparisons test. *p<0.001 represents a statistically significant difference compared TNFα induced group.

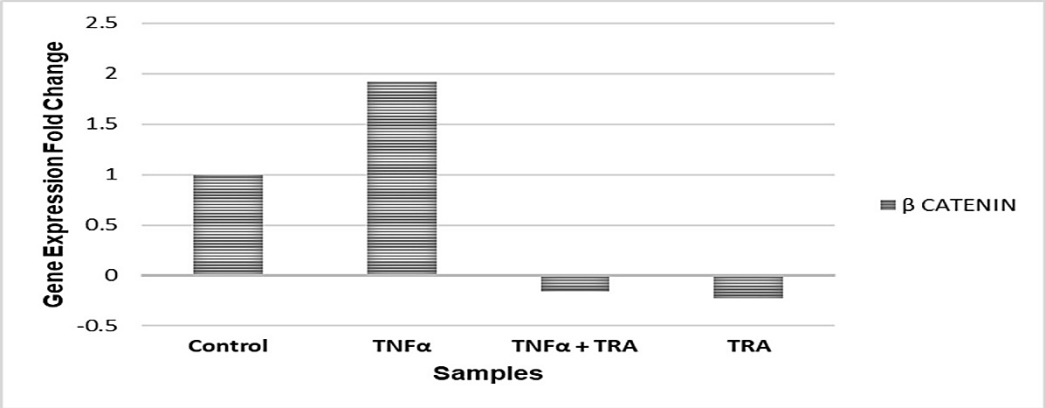

Figure 8: Depicts the graphical data of qRT-PCR outcomes. The expression of beta-catenin was upregulated in the control and TNFα groups. The TNFα + TRA and TRA groups revealed the downregulation of beta catenin.

established that TRA limits the effect of TNFα on β-catenin activity, by inhibiting β-catenin /WNT pathway, a hallmark of cancer progression and metastasis. Additionally, enhancing factors that support cell cycle arrest and apoptosis by upregulating pro-apoptotic factors and downregulating pro-survival factors, ultimately reversing EMT in lung cancer cells. Nonetheless, extensive research, including *in vivo* experiments, is required for complete comprehension of TRA's role in lung cancer cell metastasis.

CONCLUSION

In summary, we verified that TNFα activates the β-catenin/WNT pathway in A549 cells, which subsequently leads to metastasis and EMT. Treatment of TNFα-induced A549 cells with TRA, a phytocompound, declined cell migration and invasiveness, as evidenced by our assays and gene expression analysis. From this, we ruled out the fact that TRA induces its anti-tumor effect in A549 cells through facilitating cell cycle arrest and apoptosis. In a deeper sense, the findings indicated that TRA counteracts the effects induced by TNFα, potentially reversing EMT and thereby eventually hampering the metastasis of lung cancer cells. The mechanism may be involved with the inhibition of β-catenin / WNT, PI3K/Akt, and TNFα/NF-κB pathways. These pathways are crucial for regulating the factors that influence the expression of carcinogenic genes, tumour progression, and EMT.

The present study has a few limitations to acknowledge. This study is limited by the use of a single lung cancer cell line (A549), the absence of *in vivo* validation, and the lack of protein-level confirmation of key signaling molecules. Future studies incorporating multiple cell models, animal validation, and protein expression analyses are warranted to strengthen and validate the mechanistic findings. But the current work robustly establishes a solid basis for the future development of TRA as a promising chemotherapeutic agent.

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ABBREVIATIONS

AO: Acridine Orange; **Akt:** Protein Kinase B; **ANOVA:** Analysis of Variance; **ATCC:** American Type Culture Collection; **β-catenin:** Beta-catenin; **BSA:** Bovine Serum Albumin; **CDNA:** Complementary DNA; **CO_2:** Carbon Dioxide; **Ct:** Cycle Threshold; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl Sulfoxide; **DNA:** Deoxyribonucleic Acid; **ECM:** Extracellular Matrix; **ELISA:** Enzyme-Linked Immunosorbent Assay; **EMT:** Epithelial-Mesenchymal Transition; **ERK:** Extracellular Signal-Regulated Kinase; **EtBr:** Ethidium Bromide; **FBS:** Fetal Bovine Serum; **GAPDH:** Glyceraldehyde-3-Phosphate Dehydrogenase; **GSK-3β:** Glycogen Synthase Kinase-3 Beta; **H_2O_2:** Hydrogen Peroxide; **HCl:** Hydrochloric Acid; **HRP:** Horseradish Peroxidase; **IC_{50}:** Half Maximal Inhibitory Concentration; **IκB:** Inhibitor of κB; **Kb:** Kilobase; **LEF:** Lymphoid Enhancer Factor; **mRNA:** Messenger Ribonucleic Acid; **mTOR:** Mammalian Target of Rapamycin; **MMP:** Matrix Metalloproteinase; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **NF-κB:** Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; **OD:** Optical Density; **PBS:** Phosphate-Buffered Saline; **PI3K:** Phosphoinositide 3-Kinase; **PKA:** Protein Kinase A; **qRT-PCR:** Quantitative Real-Time Polymerase Chain Reaction; **RNA:** Ribonucleic Acid; **ROS:** Reactive Oxygen Species; **RPM:** Revolutions Per Minute; **RT:** Room Temperature; **SD:** Standard Deviation; **SMAD:** Mothers Against Decapentaplegic Homolog; **TCF:** T-Cell Factor; **TE buffer:** Tris-EDTA Buffer; **TGF-β:** Transforming Growth Factor Beta; **TRA:** Transferulic Acid; **UPA:** Urokinase-Type Plasminogen Activator; **UV-vis:** Ultraviolet-visible; **WNT:** Wingless-Related Integration Site.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ETHICAL STATEMENT

There is no Ethical approval required for this project.

AUTHOR CONTRIBUTIONS

All authors contributed equally to Conceptualization, Study design, Methodology, Investigation, Formal analysis, Visualization, Statistical analysis, Writing - original draft, Writing - review and editing, Supervision, Project administration.

SUMMARY

Transferulic Acid (TRA) was evaluated for its anti-metastatic potential in TNFα-stimulated A549 lung cancer cells. TRA exhibited dose-dependent cytotoxicity with an IC_{50} of 98.69 µg/mL and significantly inhibited cell migration, invasion, colony formation, and wound closure. Apoptotic induction was confirmed by AO/EtBr staining. Functional assays demonstrated suppression of anchorage-independent growth and loss of invasive characteristics. Molecular analyses revealed that TRA downregulated TNFα-induced β-catenin expression at both protein and transcript levels, indicating inhibition of the β-catenin/WNT signaling pathway. Overall, TRA effectively counteracted TNFα-driven epithelial-mesenchymal transition and metastatic behavior in lung cancer cells.

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