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Fisetin suppresses cigarette smoke extract-induced epithelial to mesenchymal transition of airway epithelial cells through regulating COX-2/MMPs/β-catenin pathway

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ABSTRACT

Cigarette smoke exposure leads to upregulation of cyclooxygenase-2 (COX-2), an inducible enzyme that synthesizes prostaglandin E2 (PGE2) and promotes airway inflammation. COX-2 overexpression is frequently implicated in inflammation, invasion, metastasis, and epithelial-mesenchymal transition (EMT). However, its detailed molecular mechanism in cigarette smoke induced EMT is not clear. Further, fisetin, a bioflavonoid, exhibits antioxidant and anti-inflammatory properties, but its effect in modulating COX-2-mediated inflammation and downstream sequelae remains unexplored. Therefore, we have investigated the mechanism of cigarette smoke-induced COX-2-mediated EMT in airway epithelial cells and examined the role of fisetin in controlling this aberration. MTT, trypan blue staining, gelatin zymography, Western blotting, invasion, wound healing, and tumor sphere formation assays in cigarette smoke extract (CSE) and/or fisetin treated airway epithelial cells, and *in-silico* molecular docking studies were performed. Results revealed that CSE exposure increased the expression and activity of COX-2, MMP-2/9, and β-catenin and also enhanced expression of EMT markers leading to higher migration and invasion potential of airway epithelial cells. A specific COX-2 inhibitor NS-398 as well as fisetin treatment reversed the expression of EMT biomarkers, reduced the activity of MMP-2/9, and blocked the migration and invasion potential induced by CSE. Further, PGE2 also increased MMPs activity, invasion, and migration potential similar to CSE, which were significantly reversed by fisetin. *In-silico* studies showed a high binding affinity of fisetin to key EMT associated proteins, validating its anti-EMT potential. Thus, our study firstly unearths the mechanism of CSE-induced EMT in airway epithelial cells via COX-2/MMP/β-catenin pathway, and secondly, it reveals that fisetin could significantly reverse CSE-induced EMT by inhibiting COX-2, indicating that fisetin could be an effective drug candidate for cigarette smoke-induced lung dysfunction.

1. Introduction

Lung pathologies associated with smoking remained scarce before the start of the 20th century but with a worldwide surge in the use of commercial cigarettes, several respiratory diseases have emerged. These diseases such as chronic obstructive pulmonary disease (COPD) have an inflammatory etiology. Cigarette smoke (CS), a primary hazard for COPD and lung cancer, comprises roughly 7400 compounds that are either carcinogens [1], mutagens [2], or toxicants [3] in nature. These chemical compounds travel in the smoke aerosols with a median aerodynamic diameter of 0.45 μm and interact with the upper and lower respiratory passages and over time they inflict airway injury constituting the respiratory pathogenesis [4]. Generation of a pro-inflammatory milieu due to chronic CS exposure of airway epithelial cells (AECs) results in bronchitis and active epithelial to mesenchymal transition (EMT) in the small airways causing fibrosis of the lungs [5]. The fibrotic lesions of the COPD lungs due to active EMT are reportedly linked with an increased risk of developing lung cancer at a later stage [6]. Although CS-induced pathophysiology of the lungs has been extensively studied, the molecular determinants of CS-induced and inflammation-mediated AEC dysfunction including EMT remain scarcely explored.

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List of abbreviations

CSE	Cigarette smoke extract
COPD	Chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
PGE2	prostaglandin E2
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
EMT	Epithelial-mesenchymal transition
SAEC	Small airway epithelial cells
ECM	Extracellular matrix
DMSO	Dimethyl sulfoxide
SAGM	Small airway epithelial cell growth medium
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
WHO	World Health Organization
FBS	Fetal bovine serum
ATCC	American Type Culture Collection

EMT continues to be a key molecular process for the dissemination of tumor cells from the primary site to distant organs via circulation [7]. The cells undertake an obscure signaling cascade to acquire a mesenchymal phenotype with a change in the morphology and modulation in the expression of EMT-associated proteins and transcription factors. The EMT-resultant myofibroblasts are implicated in several fibrotic diseases [8]. EMT has led to the development of resistance to therapy in non-small cell lung cancer patients (NSCLC) [9]. EMT has also emerged as a central and active player in the pathological airway remodeling in smoker's lungs [10]. Since inflammatory factors are closely associated with EMT [11], it is crucial to understand the role of these factors in mediating the EMT-associated signaling events and highlight the molecular targets for the development of novel and effective therapeutics.

Cyclooxygenase-2 (COX-2), also known as prostaglandin (PG) H synthase, is encoded by PTGS2 gene in humans, expressed in several cells, and mediates inflammatory response via synthesizing prostaglandin E2 (PGE2). Latter binds to specific PGE2 sensitive EP receptors (EP-1 to 4) on the cell surface and exerts its action. COX-2 is overexpressed in macrophages, cancer-associated fibroblasts, and cancer cells [12]. Recently, COX-2 overexpression is also shown to be associated with cancer stem cell-like property which promotes inflammation, proliferation, angiogenesis, invasion, and metastasis [13]. In one such study, CS is shown to induce reactive oxygen species generation and thus induction of TLR4/NADPH oxidase pathway that further mediates airway inflammation in the rodents via COX-2/PGE2 and IL-6 [14]. Similarly, CS is also reported to induce the expression of matrix metalloproteinases (MMPs) which acts on extracellular matrix and participate in the pathophysiology of several respiratory diseases. In the primary nasal fibroblasts, CSE has been shown to significantly induce MMP-2 expression attributing it to the pathological remodeling of rhinosinusitis [15]. Recently, we have also reported the up-regulation of MMP-2 and MMP-9 in the CSE-treated airway epithelial cells, that led to EMT [16].

We aim to develop an efficient and mechanism-based therapeutic approach with little or no side effects for CS-induced lung diseases. Fisetin, a bioactive flavonol present in several fruits and vegetables, has been shown to exert anti-inflammatory [17], antioxidant [18], anti-proliferative, and anticancer effects [19,20]. It has also been exploited for its pharmacologic properties including anti-EMT effects [21]. In a randomized placebo-controlled clinical trial involving colorectal cancer, fisetin supplementation was attributed to a reduced MMP-7 and IL-8 plasma concentration [22], highlighting its anti-tumor and anti-inflammatory effects.

Thus, in the present study, we have investigated if fisetin could modulate cigarette smoke-induced COX-2 upregulation and resultant inflammation-mediated EMT of the airway epithelial cells. We report that CSE treatment up-regulated COX-2 which in turn induced MMP-2/9 mediated EMT and fisetin efficiently downregulated COX-2 expression and thereby EMT. Also, *in-silico* analysis depicted a strong binding affinity of fisetin with key molecular players of EMT.

2. Materials and methods

2.1. Reagents and chemicals

RPMI 1640 media, Fetal bovine serum (FBS), 0.25% Trypsin EDTA solution, MTT dye, Trypan blue, Antibiotic-antimitotic reagents (100X) were bought from GIBCO (Grand Island, NY, USA). HEPES buffer, human primary small airway epithelial cells (SAEC), culture medium, recommended growth supplements were procured from Lonza (Walkersville, MD, USA). COX-2, MMP-2, MMP-9, β-catenin, β-actin, and GAPDH antibodies were from cell signaling technology (Denver, CO, USA), vimentin and cPLA2 antibodies were from Santa Cruz (Dallas, TX, USA) and antibodies for E-cadherin (E-cad) and N-cadherin (N-cad) were procured from BD Biosciences (New Jersey, USA). Fisetin, protease and phosphatase inhibitors, prostaglandin E2, dimethyl sulfoxide (DMSO), and NS-398 were bought from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animal cell culture

A549 cells bought from American type culture collection (ATCC CCL-185) were cultured in RPMI-1640 media, which was fortified with 10% Fetal Bovine Serum (FBS) and 1% PSA. Cells were maintained in a CO_2 incubator at 37 °C. SAECs were maintained as per the manufacturer's instructions in a humidified 5% CO_2 incubator at 37 °C.

2.3. Preparation of cigarette smoke extract (CSE)

CSE was prepared fresh at the time of treatment in each experiment using a modified syringe-driven assembly and diluted as described earlier [16]. Briefly, CSE was prepared using two commercially available Marlboro red cigarettes in 10 mL pre-warmed media (for A549 cells- 1% FBS containing RPMI 1640 media, and for SAECs -SAGM starvation media) in a 50 mL syringe. Each puff was taken in the syringe for 5 s followed by vigorously mixing the smoke in the media. A gap of 30 s was kept between each puff for proper mixing of the smoke into the media and this process was repeated until both the cigarettes were completely consumed. This mixture was sterile filtered using a 0.2 μm syringe filter and pH was adjusted to 7.4. This solution was considered as 100% CSE and was further diluted with culture media to achieve desired concentrations.

2.4. CSE treatment

CSE (100%) solution was diluted to 0.5, 2.5, 5, 10, and 20% using RPMI 1640 or SAEC starvation media for exposing the A549 cells and SAECs as applicable. Cells were exposed to various concentrations of CSE for different time points as per the need of the respective experiments.

2.5. Trypan blue staining

A549 cells (3×10^4/well) in 12-well dishes were grown and starved before treatment with various CSE concentrations (2.5, 5, 10, and 20%) for 12 and 24 h. Cells were harvested using trypsin and resuspended in 1X PBS. Cells were counted on a hemocytometer using 0.5% trypan blue solution as previously described [23].

2.6. MTT assay

A549 cells (8,000/well) were seeded in 96 well dishes, starved and subsequently incubated with or without different concentrations of fisetin (5, 10, 20, and 40 μM) for 24 h to assess its cytotoxicity. In other experiments, serum-starved cells were exposed to various concentrations of CSE (2.5, 5, and 10%) alone or with 10 μM fisetin. After incubation period of 24 h, MTT assay was performed as described previously [16]. Absorbance was taken at 570 nm using a multimode reader (Winooski, VT, USA).

2.7. Gelatin zymography

MMP-2/9 activation in media supernatant was measured using gelatin zymography. A549 cells were seeded and starved as described earlier, and cells were exposed to different CSE concentrations (2.5, 5, and 10%) or prostaglandin E2 (10 μM) with or without fisetin (10 μM), or COX-2 inhibitor NS-398 (10 μM) for 24 h. Pre-treatment of fisetin and NS-398 was done 4 h before exposing cells with CSE or prostaglandin E2. Similarly, SAECs (50,000/well) seeded in 12 well plates were starved as described, and exposed to CSE (0.5 and 2.5%) for 24 h with or without 10 μM fisetin. Gelatin zymography was done using cell culture media on 8% SDS-PAGE gel containing 0.1% gelatin. Gel was rinsed in renaturing buffer and kept in developing buffer at 37 °C overnight, dyed with Coomassie blue R-250, de-stained, and images were acquired [24].

2.8. Western blotting

A549 cells and SAECs were seeded, starved, and stimulated with different CSE concentrations or PGE2 as described in the earlier section (2.7) and incubated for 24 h. Cell lysates were prepared and an equal amounts of protein from different groups were electrophoresed on 8–10% polyacrylamide gels. The proteins were transferred onto the PVDF membrane followed by 1 h blocking and overnight incubation with primary antibodies against COX-2, MMP-2, MMP-9, cPLA2, E-cad, vimentin, N-cad, and β-catenin at 4 °C. Next day, membranes were incubated with respective secondary antibodies followed by developing using ECL reagent [25]. Image J software was used to analyze the protein bands.

2.9. Wound healing assay

A549 cells (1×10^5/well) were grown in 6 well dishes till confluency followed by starvation and wound was created as explained earlier [16]. Next, cells were subjected to CSE with or without fisetin and NS-398, or prostaglandin E2 with or without fisetin for various time intervals. Pretreatment with fisetin for 4 h was done before CSE/prostaglandin E2 treatment. The photomicrographs of the wounds were acquired at the time of treatment and after the incubations using a digital camera fitted on a phase-contrast microscope.

2.10. Matrigel invasion assay

CSE and prostaglandin E2 induced invasion potential of A549 cells was analyzed by Matrigel-coated 8.0-μm pore size transwells (BD Biosciences, NJ, USA). A549 cells (1×10^5/well) were grown in 6 well plates, starved and exposed to CSE or PGE2 with or without fisetin or NS-398 and incubated for 24 h. Pre-treatment of fisetin/NS-398 was given 4 h before the CSE/prostaglandin E2 addition to media. Once the incubation was complete, Matrigel invasion assay was performed as explained earlier [16].

2.11. Tumor sphere formation

A549 cells (1000/well) were grown in 96 well ultra-low attachment dishes at 37 °C. After 24 h, cells were subjected to CSE with or without fisetin or NS-398. CSE containing media was replaced every alternate day. The formation of spheroids/tumor sphere, was continuously observed under a phase-contrast microscope without disturbing the cells. The treatment was continued up to 7 consecutive days and photomicrographs of spheroids were taken at various time intervals [26].

2.12. In-silico molecular docking studies

The three-dimensional structures of human MMP-2 (gelatinase A) (PDBID 1CK7), COX-2 (PDB ID: 6COX), and MMP-9 (gelatinase B) (PDB ID 1L6J) were obtained from X-ray data with 2.80 Å, 2.80 Å, 2.50 Å resolutions, respectively and the protein coordinates were downloaded in PDB format from RCSB protein data bank (https://www.rcsb.org). Structural coordinates of compound fisetin were retrieved from the PubChem database under code 5281614 [27]. PyRx 0.8, a virtual screening software, was used for docking study of fisetin against human MMP-2, COX-2, and MMP-9 [28]. PyRx uses AutoDock Vina as docking software and exploits Lamarckian genetic algorithm as well as empirical free energy scoring function [29]. The macromolecular structures of MMP-2, COX-2, MMP-9, and Fisetin were prepared, and docking was carried out without defining any specified binding sites against whole surface of the proteins. Once docking was complete, best conformation with lowest docked energy was chosen. Further, AutoDock Vina was used for determining ligand structure, and the best pose was saved. The outcomes of docking results were presented in the form of binding energy. Interaction between targeted protein and ligands was studied using software for hydrogen and hydrophobic bond interaction analysis using Discovery Studio Visualizer 4.0.

2.13. Statistical analysis

Statistical analysis was done using the student's t-test in GraphPad Prism software version 6 to examine the differences between mean values (mean ± SEM). P value less than 0.05 was taken as significant.

3. Results

3.1. Effect of CSE and fisetin treatment on viability of airway epithelial cells

The viability of A549 cells was studied after exposure to different concentrations (2.5, 5, 10, and 20%) of CSE for 12 and 24 h. CSE abated the total cell number of the cells to 79.8%–55.7% after 12 h and 73.3%–42.1% ($p<0.05$-$p<0.001$) after 24 h compared to the control cells. CSE exposure also increased cell death by 1.9–2.2-fold after 12 h, and 2.1–2.9-fold ($p<0.05$-$p<0.001$) after 24 h (Fig. 1A). We also investigated if fisetin affected cell viability. Although at higher concentrations (20 and 40 μM) fisetin decreased the cell viability to 93.1–67.2% ($p<0.05$-$p<0.0001$) as compared to untreated control groups (Fig. 1B), at lower concentrations (5 and 10 μM) no significant loss of cell viability was observed. Further, at a lower concentration (10 μM) we observed that fisetin did not cause additional toxicity to the cells when used in combination with different concentrations of CSE (Fig. 1C). Thus, we decided to use 2.5, 5.0, and 10.0% of CSE in A549 cells and 0.5 and 2.5% of CSE in SAECs for further experiments with or without fisetin (10 μM) [16]. The difference in CSE concentration for the two types of cells may be due to differences in their origin, nature, and growth pattern in culture.

3.2. Effect of fisetin on CSE induced COX-2, MMP-2 and MMP-9 expression/activation

The modulatory effect of CSE on the expression of COX-2 and MMP-2 was investigated using Western blotting and to assess the activity of MMPs in CSE-treated cells gelatin zymography was performed. The cells were exposed to CSE with or without fisetin (10 μM) for 24 h. The

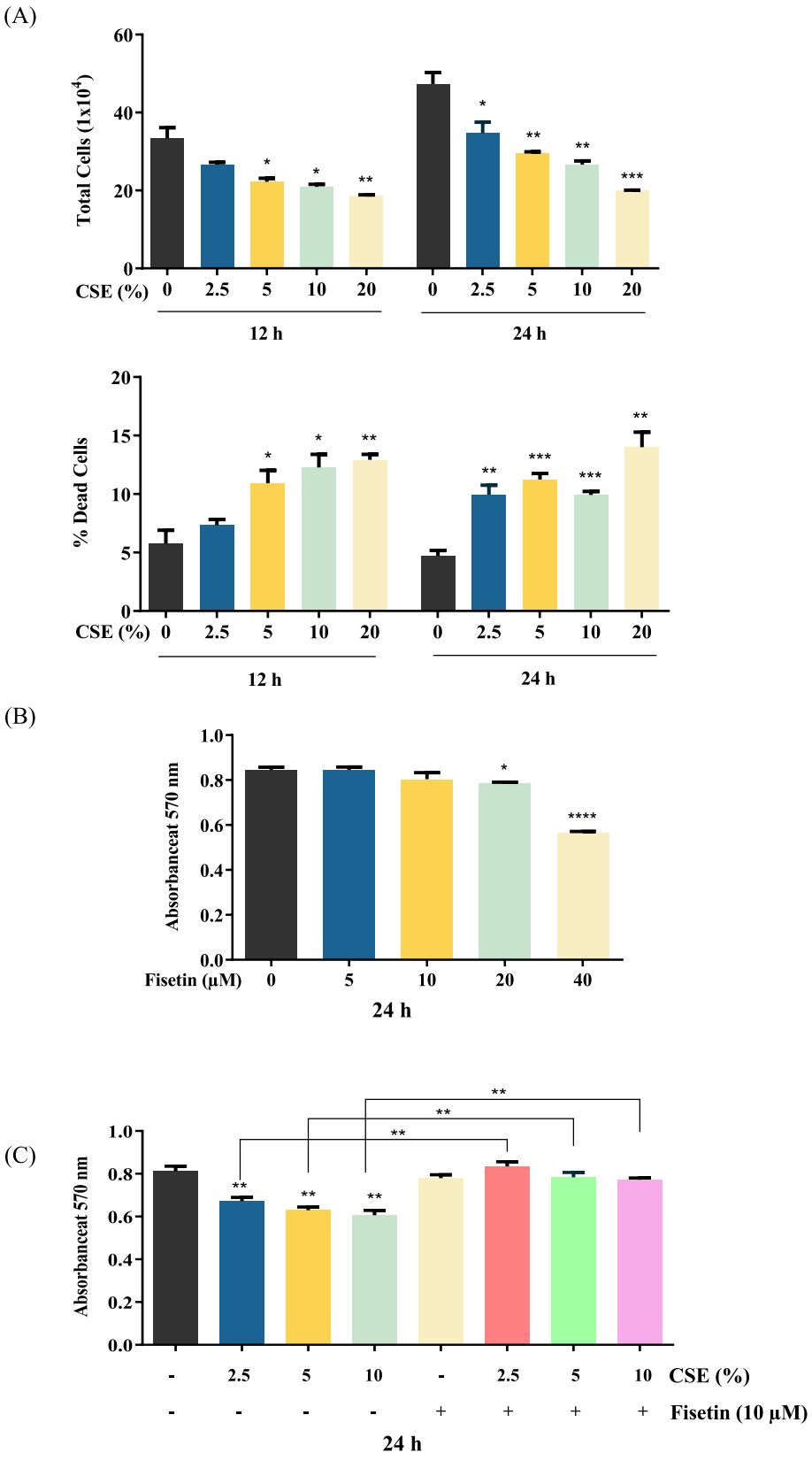


Fig. 1. Growth and viability of A549 cells affected by CSE and fisetin: (A) CSE (2.5%, 5%, 10% and 20%) treatment was given to A549 cells and trypan blue assay was performed. (B) A549 cells were incubated with different concentration of fisetin (5, 10, 20, and 40 μM) for 24 h and MTT assay was performed. (C) MTT assay was performed on CSE and/or fisetin (24 h) treated A549 cells. The data are presented as mean ± SEM (n = 3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs control group.

expression of COX-2 and MMP-2 increased in a dose-dependent manner after 24 h of CSE (5%) treatment in A549 cells to 3.5- and 2.3-fold, respectively. In SAECs, COX-2 expression increased to nearly 1.5-fold after CSE (0.5 and 2.5%) treatment. In fisetin treated groups COX-2 and MMP-2 expression in A549 cells (1.2- and 0.4-fold) and that of COX-2 in SAECs (0.7-fold at 0.5% CSE) was reversed to near control values (Fig. 2A and B). CSE treatment also induced the expression of MMP-9 in A549 cells, which was reduced when fisetin was given before

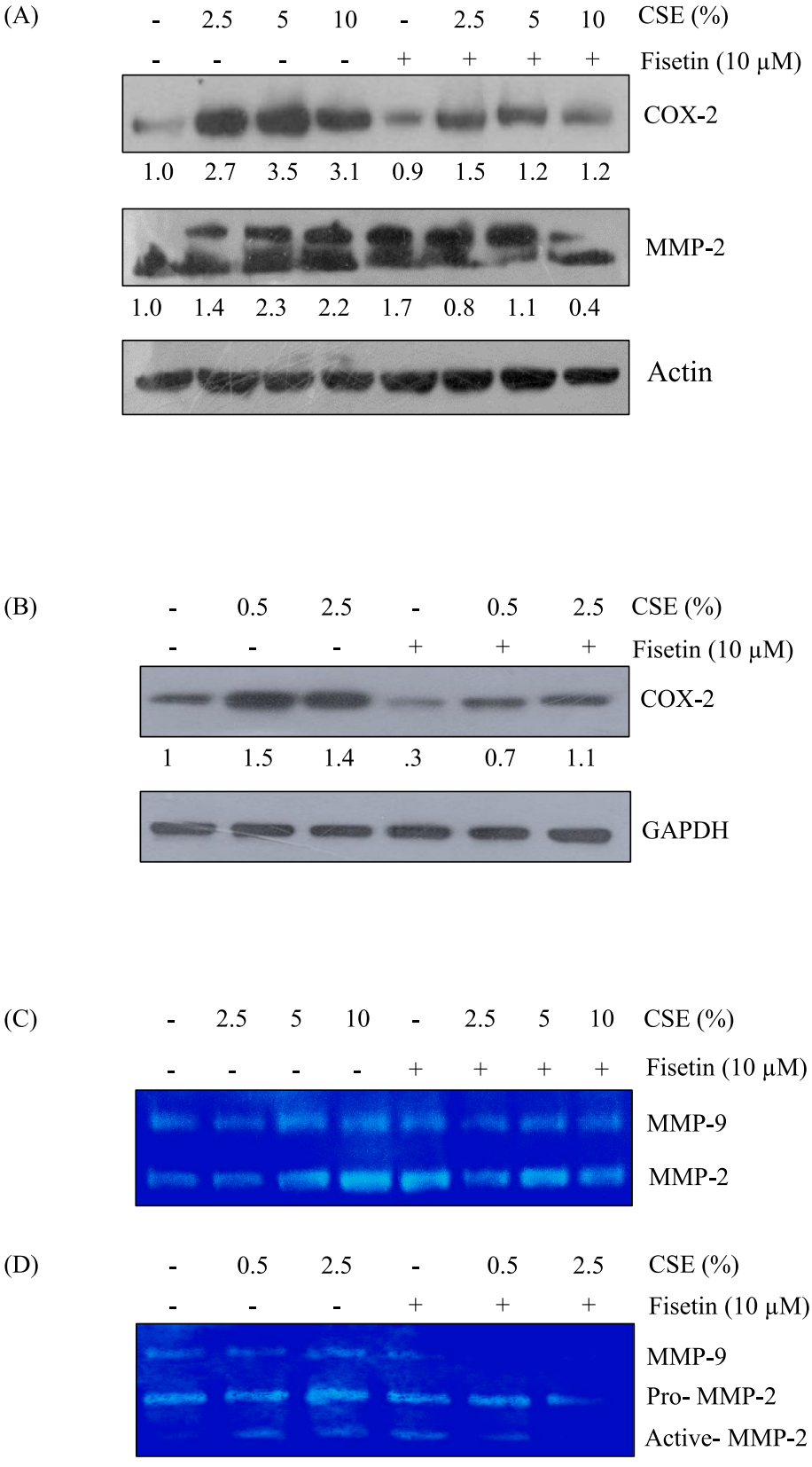


Fig. 2. CSE-induced expression of COX-2 and MMPs reversed by fisetin: COX-2 and MMPs expression in CSE-stimulated (A) A549 cells and (B) SAECs with or without fisetin (10 μM) was analyzed by Western blotting. Cell culture media of similarly treated (C) A549 cells and (D) SAECs were subjected to gelatin zymography. Representative images shown (n = 3). The numbers below Western blot images indicate fold changes.

CSE (Supporting Fig. 1). The expression of cPLA2, which lies upstream of COX-2, increased after CSE exposure, which decreased when fisetin was given before CSE (Supporting Fig. 2). Next, MMP-2/9 activities, assessed by gelatin zymography, increased in both cell types after CSE exposure, and in fisetin treated cells CSE-induced activation of MMP-2/9 decreased significantly (Fig. 2C and D).

3.3. Fisetin and NS-398 reversed CSE induced MMP-2/9 activation and EMT in A549 cells

COX-2 overexpression is observed in various malignancies and implicated in cancer progression, metastasis, and EMT [30]. We have previously reported that CSE could induce MMP-2/9 activities and expression in airway epithelial cells and further modulated the signaling cascade leading to EMT [16]. Here, our goal was to analyze the role of COX-2 (by using its specific inhibitor NS-398) in modulating MMP-2/9 and the overall EMT process in CSE exposed cells and to assess and

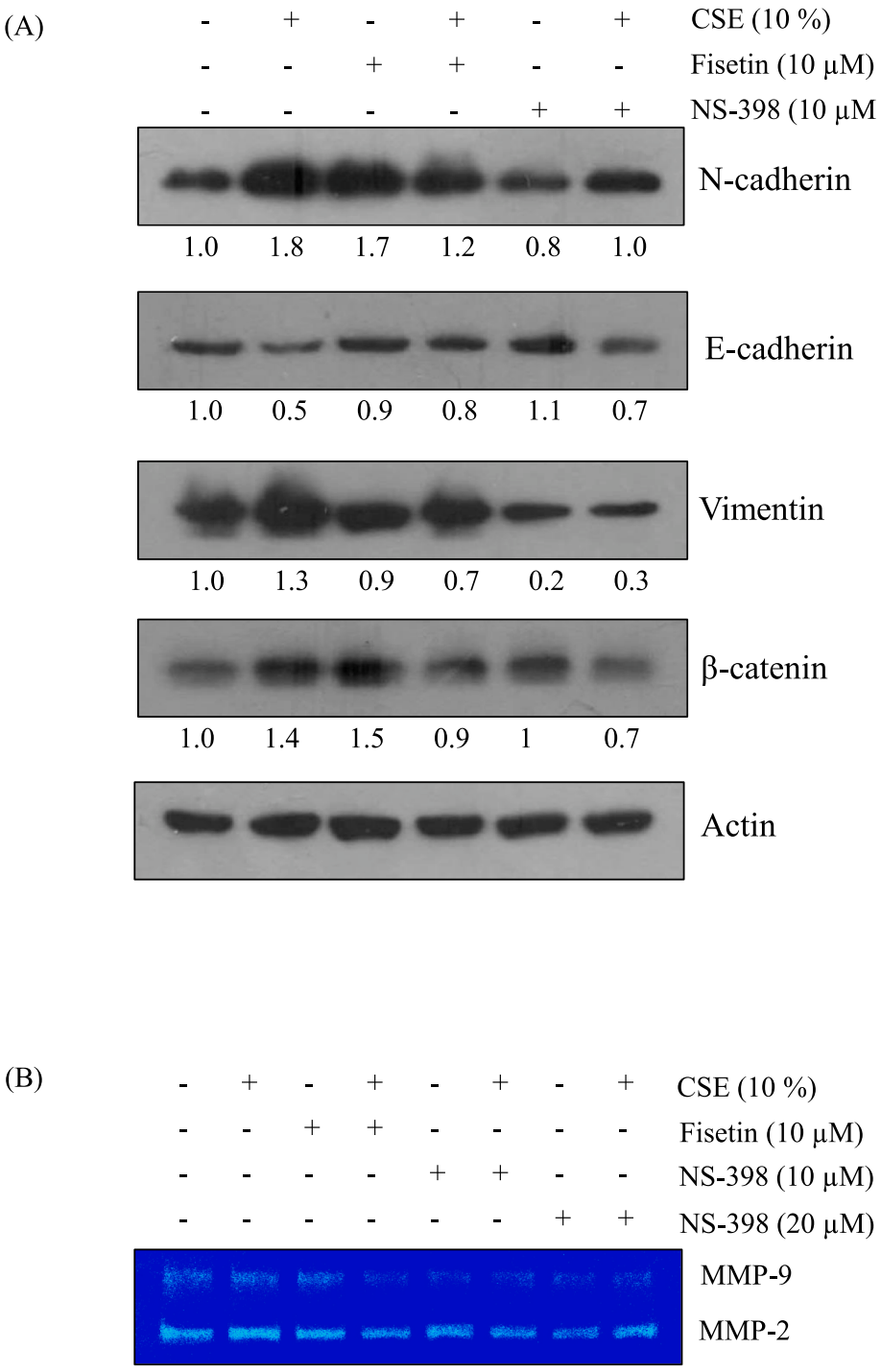


Fig. 3. CSE -induced expression of EMT markers and MMPs activation reversed by fisetin and NS-398: CSE exposed A549 cells with or without fisetin or NS-398 were examined for expression of EMT markers using (A) Western blotting and activation of MMPs using (B) Gelatin zymography. Representative images shown (n = 3). The numbers below Western blot images indicate fold changes.

compare the effect of fisetin. CSE exposure of A549 cells with or without fisetin or NS-398 for 24 h was followed by measurement of MMP-2/9 activity and expression of various EMT regulators. As depicted in Fig. 3B, CSE increased MMP-2/9 activation, and fisetin and NS-398 reduced the activity. Further, upon CSE exposure the expression of mesenchymal markers like N-cad, vimentin and β-catenin enhanced to 1.8, 1.3 and 1.4-fold, respectively. Fisetin treatment reduced these values to 1.2, 0.7 and 0.9-fold, respectively. COX-2 inhibitor NS-398 also decreased their expression to 1.0, 0.3, and 0.7-fold, respectively suggesting the role of COX-2 in upregulation of mesenchymal markers by CSE. CSE also abated the expression of E-cad to 0.5-fold which reverted to 0.8- and 0.7-fold in fisetin and NS-398 treated groups, respectively (Fig. 3A). These results indicate that COX-2, MMPs and β-catenin signaling axis may be involved in CSE-induced EMT and fisetin could be an effective anti-EMT molecule.

3.4. Fisetin and NS-398 suppress migration and invasion potential of CSE-induced A549 cells

Numerous studies suggest the involvement of EMT in cell invasion, migration, and cancer progression [31,32]. Similarly, COX-2 and MMP-2/9 have also been implicated in EMT in various cancers [30,33,34]. Thus, we next analyzed CSE-induced migration potential of A549 cells by wound healing assay and whether presence of NS-398 (COX-2 specific inhibitor) could inhibit it. As shown in Fig. 4A, wound gap was significantly filled in CSE exposed cells after 48 h in comparison to control cells while in fisetin and NS-398 treated groups migration of A549 cells was inhibited and a significant wound gap remained ($p<0.05$-$p<0.0001$). Further, CSE increased the invasion of cells by 2-fold after 24 h. Fisetin and NS-398 treatment in CSE exposed cells reversed CSE-induced invasion of cells ($p<0.01$) (Fig. 4B). These results suggest an important role of COX-2 in CSE-mediated EMT and indicate the ability of fisetin as an anti-EMT molecule in CSE-induced lung pathologies.

3.5. Effect of prostaglandin E2 on A549 cells morphology and MMP-2/9 activation

COX-2-derived prostaglandin E2 has been shown to induce tumor growth via regulating various cellular processes including inflammation, proliferation, migration, metastasis, and angiogenesis [35]. To analyze the role of prostaglandin E2 in MMPs activation, we stimulated A549 cells with prostaglandin E2 for 24 h in the presence or absence of fisetin. Upon morphological examination, prostaglandin E2 treated cells were fibrous and elongated in shape as in the CSE group (Fig. 5A). Further, prostaglandin E2 exposure increased MMP-2 and MMP-9 activity and fisetin decreased these changes significantly (Fig. 5B) suggesting the role of COX-2 catalytic product prostaglandin E2 in regulating the activation of MMP-2/9 in airway epithelial cells.

3.6. Fisetin suppresses prostaglandin E2 induced migration and invasion

Since COX-2 was involved in migration and invasion, we used its catalytic product prostaglandin E2 to ascertain it further. A549 cells were exposed to prostaglandin E2 for 24 h with or without fisetin, and the migration and invasion capacities were analyzed. The results showed that prostaglandin E2 stimulated cells had increased migration in comparison to control cells as the wound gap had filled significantly more. Fisetin, which was given as pretreatment and remained present till the end of the experiment, decreased the migration of cells and a significant wound gap remained in this group ($p<0.01$-$p<0.0001$) (Fig. 6A). Further, prostaglandin E2 increased the invasion by 1.7-fold which was reversed in presence of fisetin ($p<0.01$) (Fig. 6B). This suggests the crucial role of COX-2-derived prostaglandin E2 in inducing migration and invasion of A549 cells which could be reversed in the presence of fisetin.

3.7. CSE increases the size and number of three-dimensional spheroid formation

Spheroid forming cancer stem cells with self-renewal properties are responsible for metastasis, invasion, and drug resistance. Thus, sphere formation assay was performed to examine the sphere forming capacity of CSE exposed A549 cells. The results showed that CSE-exposed A549 cells had increased spheroid perimeter by 2.2-fold ($p<0.05$-$p<0.0001$) in comparison to control groups after 7 days and fisetin and NS-398 treatment significantly decreased the spheroid perimeter (in comparison to CSE group). Also, the number of spheroids was increased by 1.5-fold ($p<0.05$-$p<0.01$) after CSE exposure for 7 days (in comparison to control), and fisetin and NS-398 treatment resulted in a significant reduction in the number of spheroids (Fig. 7A, B and C).

3.8. In-silico analysis of fisetin

Protein-ligand docking was carried out to examine the molecular details of association of MMP-2, COX-2, and MMP-9 with fisetin as a molecular level approach. After successful docking between fisetin and binding sites of human MMP-2, MMP-9, and COX-2 docking scores of ligands were noted. For detailed interaction studies, best-ranked poses having lowest docking scores were selected. Fisetin showed a high binding affinity with MMP-2 (−10 kcal/mol), COX-2 (−9.1 kcal/mol), and MMP-9 (−8 kcal/mol). Binding energy and details about the amino acid residues that are involved in hydrogen bond interaction are mentioned in the figure in detail (Fig. 8A and B). These results further corroborate anti-EMT potential of fisetin obtained in *in-vitro* studies.

4. Discussion

Smoking cigarettes (actively or passively) is one of the prime factors responsible for various chronic lung diseases including COPD and lung cancer affecting the health of millions of people worldwide [36]. Cigarette smoke-induced inflammation plays a prominent role in driving lung cancer and tumor-associated inflammation has been implicated at every stage of cancer development such as cancer initiation, promotion, metastasis, and malignant phenotype acquisition. In addition, inflammation is believed to induce EMT during cancer progression and in turn, EMT can also drive inflammatory response during cancer [26].

EMT is an evolutionarily conserved process, and it is associated with tissue repair, fibrosis, and cancer progression. Recent studies have established that EMT is a central player and at the core of COPD pathophysiology and lung cancer. Tissue fibrosis (EMT-type-2) and cancer associated EMT (EMT-type-3) have been identified to be present in majority of lung cancer and COPD patients [37,38]. EMT is a cascade of molecular events that is driven by several transcription factors. The Zeb1/2, SNAIL and TWIST are some of the transcription factors that play a vital role in EMT of lung cancer and COPD [39,40]. Upregulation of these transcription factors is associated with invasiveness, metastasis, and poor prognosis of lung cancer. Molecular markers involved in inflammation such as COX-2 and PGE-2 have been shown to induce the expression of EMT transcription factors such as ZEB1/2 and SNAIL which indicates that there is crosstalk between inflammation and EMT [41]. Considering how CS can induce inflammation, and that nearly half of the lung cancer patients are active smokers [38], it is essential to understand how CS-induced inflammation may play a role in EMT-driven lung cancer progression and COPD.

COX-2 is an inducible inflammatory enzyme that catalyzes the conversion of arachidonic acid into prostaglandins. Increased expression of COX-2 is associated with cancer invasion, angiogenesis, immune modulation, and apoptosis resistance [13]. MMPs, zinc-dependent proteases, are mainly involved in ECM degradation and play a pivotal role in tumor invasion and metastasis. They also mediate EMT through regulating various cell surface receptors and other downstream signaling molecules [16]. Various studies correlate the overexpression of COX-2 with

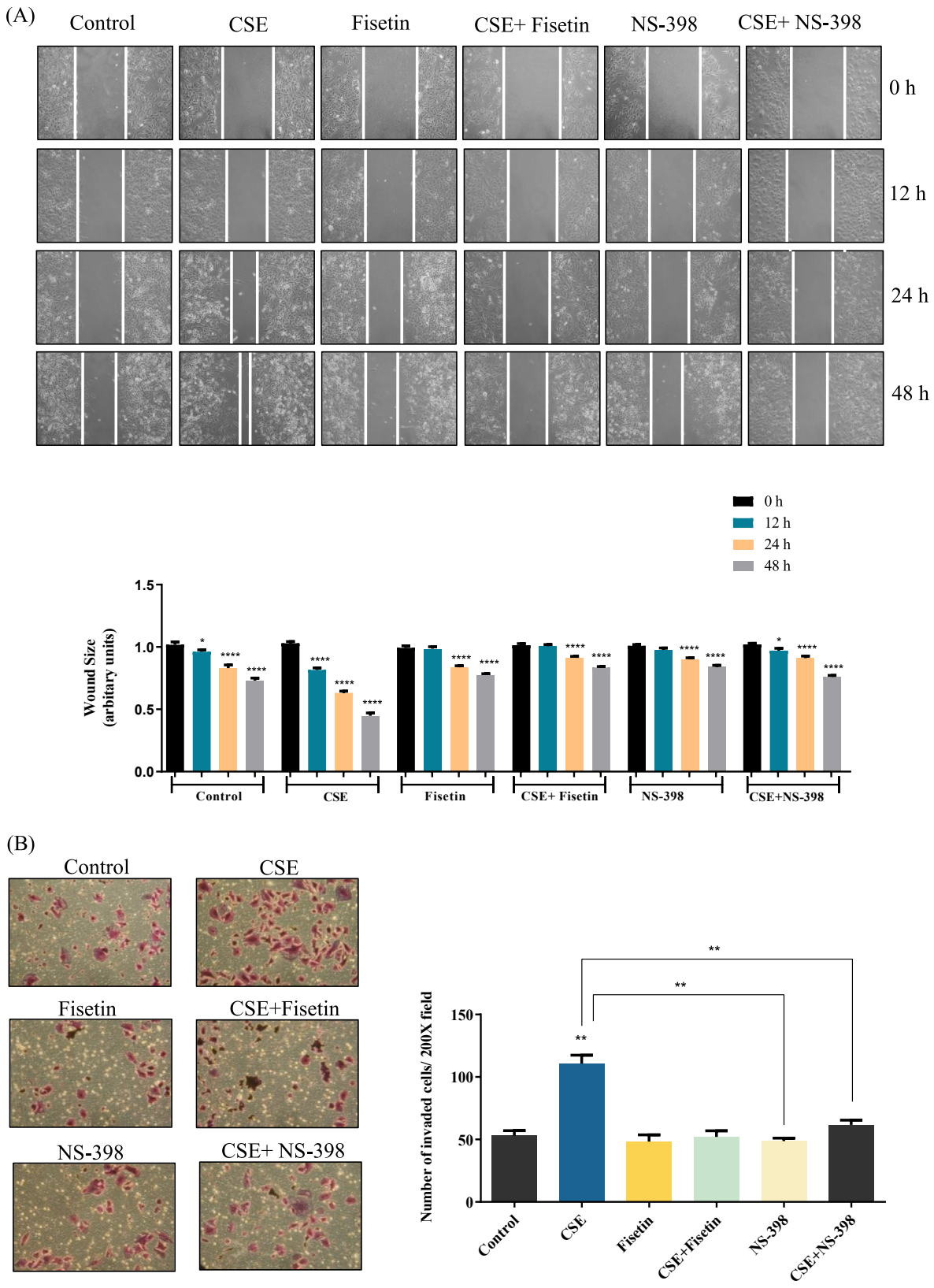


Fig. 4. CSE-induced invasion and migration inhibited by NS-398 and fisetin: (A) Confluent monolayer of cells were scratched using 200 μl tip and exposed to CSE with or without NS-398 and fisetin for 0, 12, 24 h and 48 h and migration potential was investigated through wound healing assay. *p<0.05; **p<0.01, ****p<0.0001 vs control (n = 3). (B) CSE treatment was given to A549 cells with or without fisetin and NS-398 and Matrigel invasion assay was performed. Phase-contrast microscope was used to measure the cell invasion. **p<0.01 vs control (n = 3).

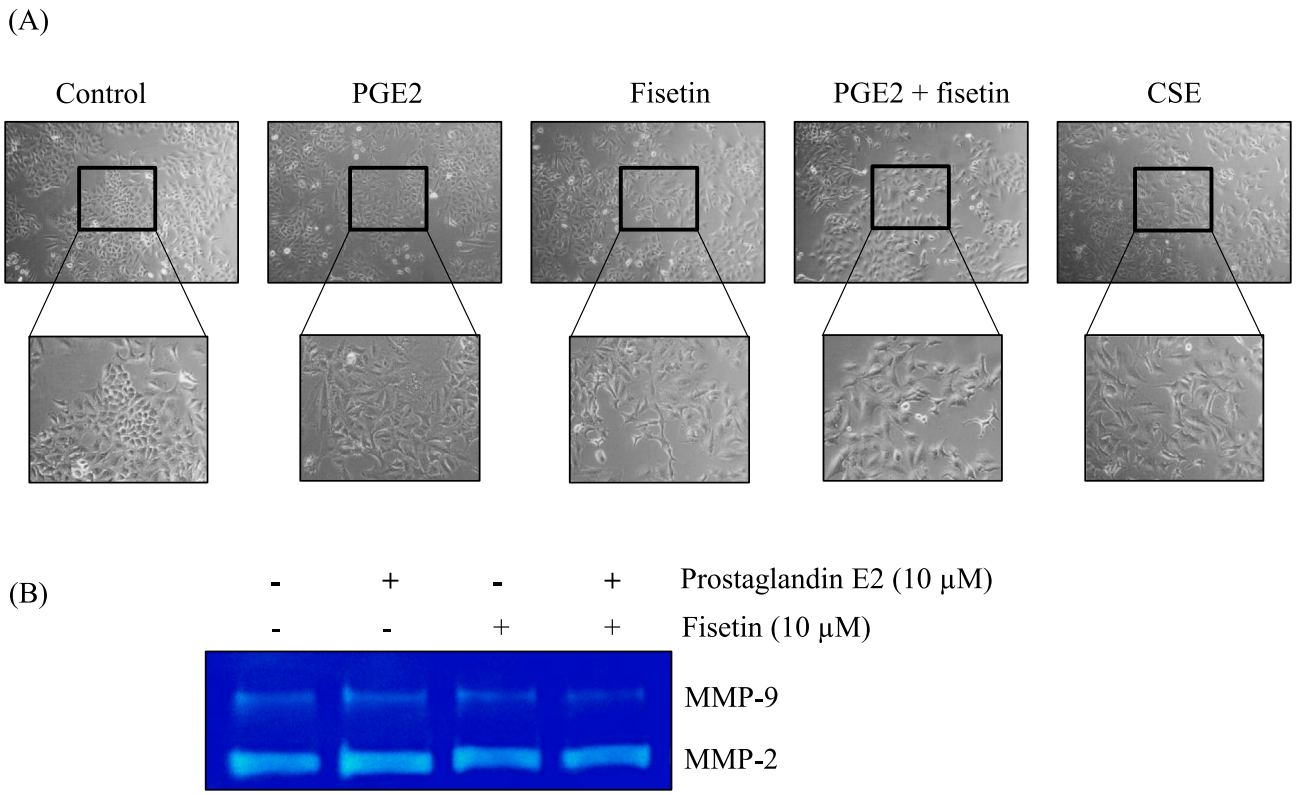


Fig. 5. Effect of prostaglandin E2 and fisetin on morphological features and MMPs activity of A549 cells: (A) A549 cells were treated with prostaglandin E2 (10 μM) with or without fisetin (10 μM) for 24 h and morphological changes were observed under a phase-contrast microscope. (B) A549 cells were treated with prostaglandin E2 (10 μM) with or without fisetin (10 μM) for 24 h and activation of MMP-2/9 was examined by gelatin zymography.

increased levels of MMPs and increased invasion of cancer cells [42,43].

Intake of protective phytochemicals having antioxidant or anti-inflammatory properties alleviate the damage caused by toxicants present in cigarette smoke and improves lung function [44]. Fisetin, a natural flavonoid has been extensively studied for its anti-cancer, anti-oxidant, immunosuppressing, and anti-inflammatory properties. A study showed that fisetin prevented LPS-mediated acute lung injury through inhibiting TLR4-mediated NF-κB signaling pathways [45]. Fisetin has also been shown to protect airway epithelial cells against tumor necrosis factor-alpha (TNF-α)-induced and interleukin (IL)-8 -mediated injuries [46]. Another study showed that fisetin inhibited cigarette smoke-induced inflammation and oxidative stress through upregulating Nrf2-mediated antioxidant gene expression [47]. Considering this evidence, we aimed to understand the protective role of fisetin against CSE-induced damage and asked if it could be through inhibition of COX-2/MMP-2/9 signaling axis.

Towards this aim, we first studied the effect of CSE on airway epithelial cell viability. CSE exposure decreased the cell viability in a time- and dose-dependent manner and fisetin were non-toxic at lower concentrations (up to 10 μM). Also, when fisetin was given along with CSE exposure (up to 10%) it did not cause any additional cytotoxicity to the cells, rather it offered protection to the cells. Further, CSE induced the expression of COX-2 and enhanced the activation and expression of MMP-2/9 in airway epithelial cells, and fisetin could revert these changes significantly. Since COX-2 overexpression is associated with increased cancer invasion and motility [48], we reasoned that increased MMP-2/9 activation and expression could be through COX-2 -mediated MMPs regulation which would further regulate CSE-induced EMT.

Thus, we analyzed the effect of COX-2 inhibition on CSE-induced EMT and activation status of MMP-2/9. During EMT, epithelial cells lose their adherent junctions, detach from the matrix and gain mesenchymal cell-like migratory and invasive properties [49]. We measured these markers and found that expression of E-cad was reduced, and levels of vimentin and N-cad was increased in response to CSE exposure for 24 h. As anticipated, COX-2 specific inhibitor NS-398, when given along with CSE, restored the expressions of E-cad as well as N-cad and vimentin in A549 cells. This signifies the potential of COX-2 in EMT regulation by CSE exposure of airway epithelia. Fisetin also reverted these changes significantly showing its anti-EMT potential in CSE-treated cells.

Aberrant activation of Wnt/β-catenin signaling has been linked with EMT and subsequent cancer progression [50]. The β-catenin and E-cad complex play an important role in EMT. When WNT ligands are not present, β-catenin interacts with cytoplasmic chain of E-cad and other proteins like adenomatous polyposis coli (APC), AXIN and glycogen synthase kinase 3 beta (GSK3β). This is followed by phosphorylation and subsequent degradation of β-catenin through ubiquitination. In the presence of WNT ligands, GSK3β dissociates from this protein complex and makes it inactive thus allowing the accumulation of β-catenin in the nucleus, and in turn, it modulates the expression of EMT associated transcription factors [51,52]. Thus, we next examined the involvement of β-catenin in CSE-induced and COX-2 -mediated EMT and tested the effect of fisetin in reverting these changes. We observed that CSE enhanced the expression of β-catenin which was reversed by fisetin treatment suggesting an important role of fisetin in regulating CSE-induced EMT. COX-2 specific inhibitor NS-398 also reverted changes in β-catenin expression signifying that CSE-induced EMT is mediated via COX-2 in A549 cells.

Next, we aimed to understand the effect of COX-2 inhibition on activation of MMP-2/9 as MMPs are implicated in altered expression of various EMT-associated biomarkers and in turn metastasis. We found that CSE -induced changes in the activities of MMP-2/9 were decreased in presence of NS-398 and fisetin, suggesting the importance of COX-2 in the regulation of MMPs-mediated EMT. These results thus indicate that CSE-induced EMT could be COX-2/MMP-2/9/β-catenin mediated and inhibition of COX-2 with NS-398 or fisetin could impede these EMT-

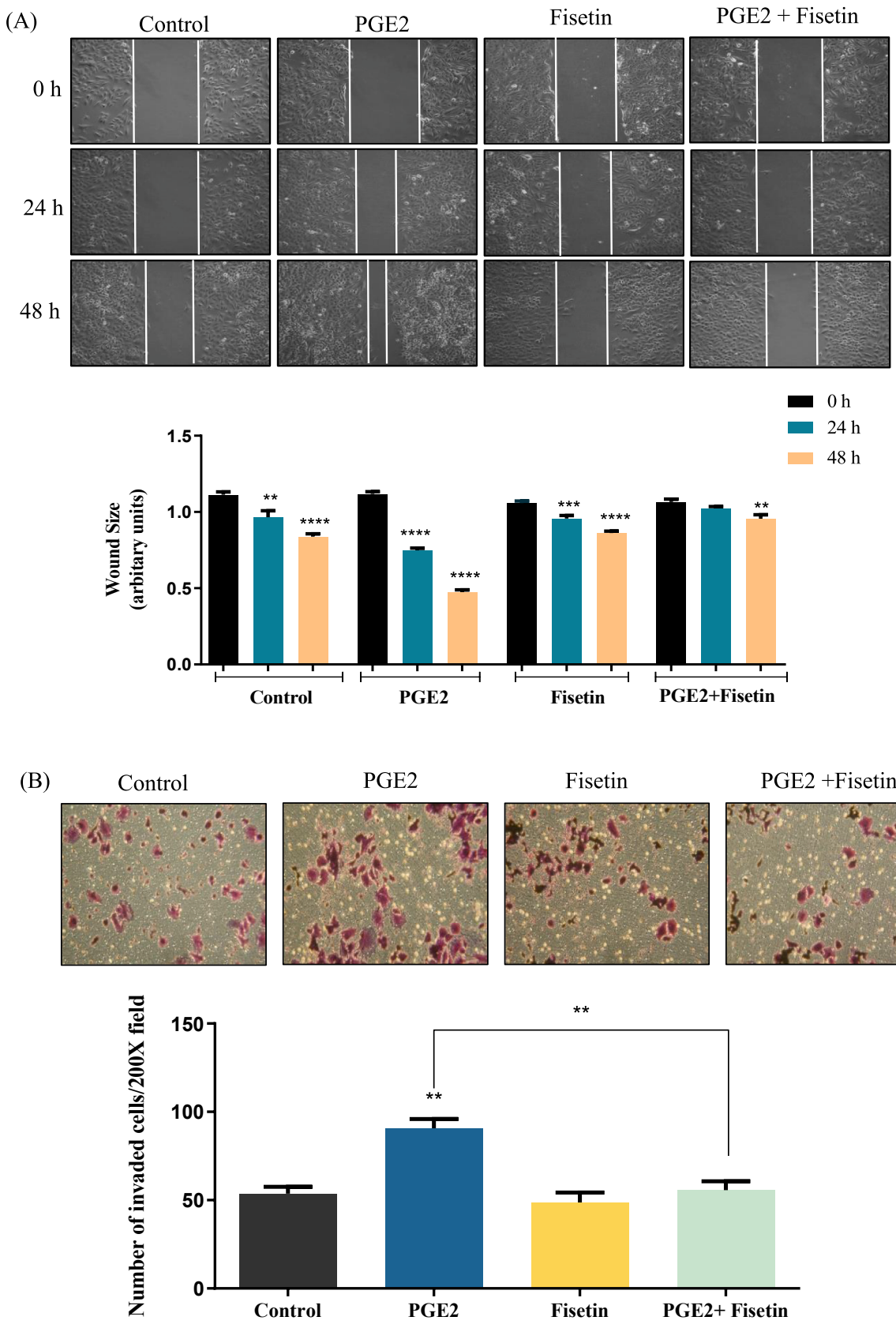


Fig. 6. Modulation of A549 invasion and migration potential by prostaglandin E2 and reversal by fisetin: (A) The confluent cells were scratched using 200 μl tip to make wound and exposed to prostaglandin E2 (10 μM) with or without fisetin (10 μM) and migration assay was performed. *p<0.05; **p<0.01, ****p<0.0001 vs control, (n = 3). (B) A549 cells were treated prostaglandin E2 with or without fisetin and a Matrigel invasion assay was performed. Phase-contrast microscope was used to measure the number of invaded cells **p<0.01 vs control, (n = 3).

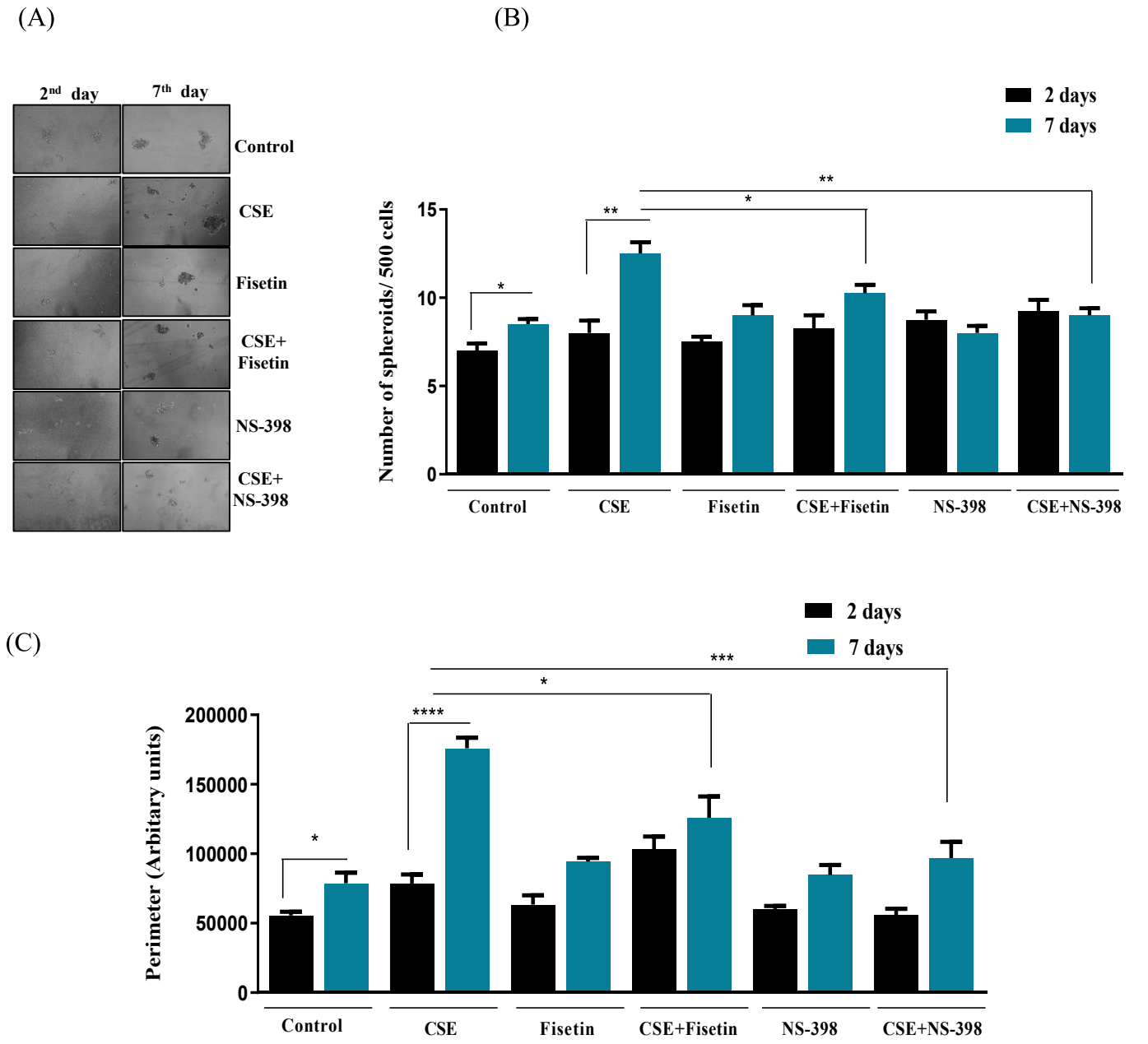


Fig. 7. CSE induced tumor sphere formation potential: CSE with or without NS-398 (10 μM) or fisetin (10 μM) was given to A549 cells for 7 days and (A) size, (B) number spheroid forming cells and (C) Perimeter were analyzed. All experiments were performed in triplicates and the data are presented as mean ± SEM.

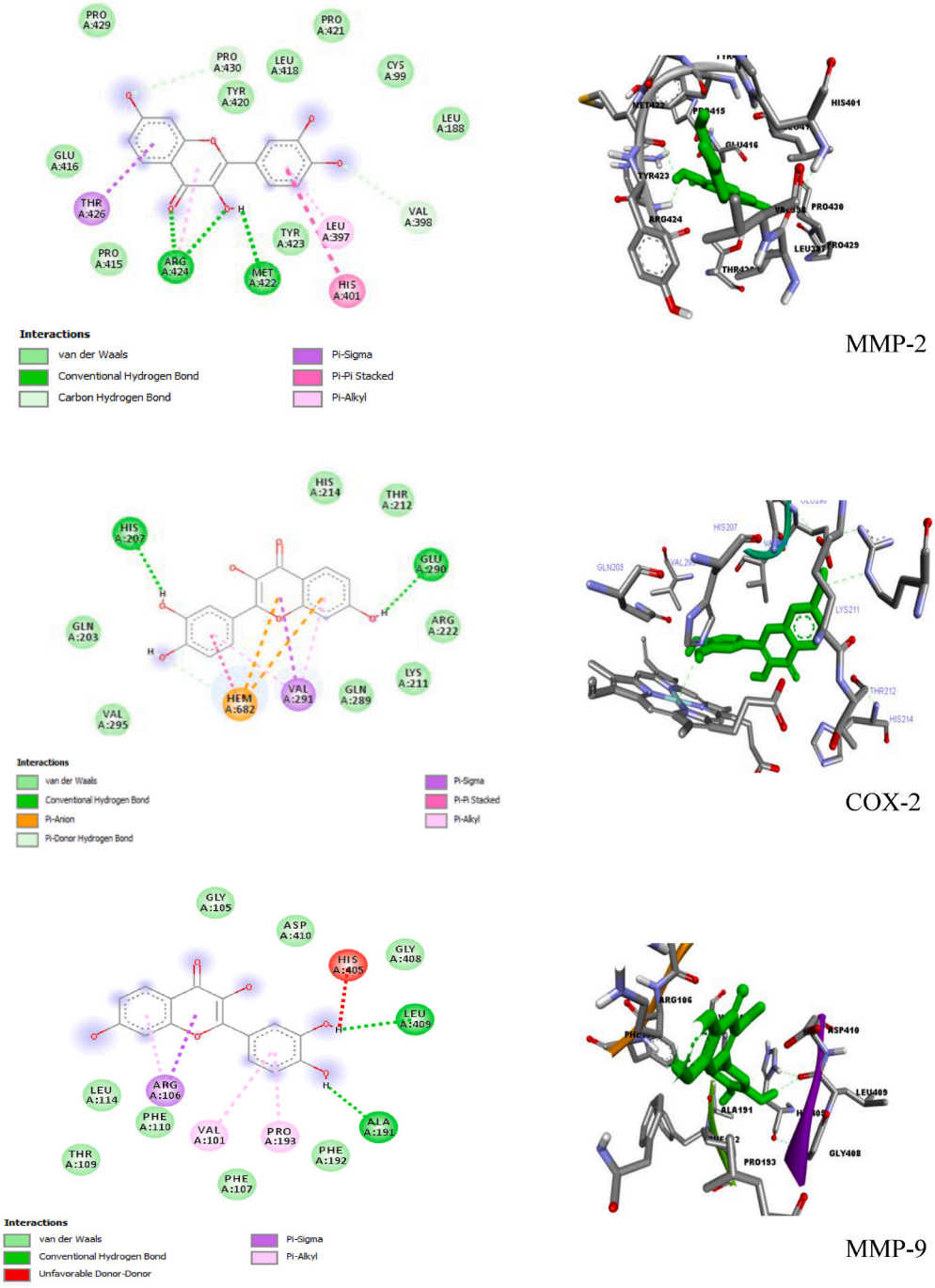
associated changes.

Metastasis is a term associated with the cascade of events in which the cancer spreads from the primary site to other parts of the body. Cell invasion is one of the crucial phenomena during metastasis and involves abrupt activation of various oncogenic proteins which are linked with EMT-inducing signaling cascades [31]. Studies have shown that COX-2 overexpression is associated with increased migratory and invasive properties of cancer cells [53,54]. Thus, we examined the role of CSE-induced COX-2 in elevating the invasion and migration of cells. CSE exposed A549 cells acquired increased invasion and migration capabilities and fisetin or NS-398 reduced it significantly. These results suggest the important role of COX-2 in CSE-mediated EMT and indicate that fisetin could revert these changes at the cellular level by inhibiting COX-2.

Prostaglandin E2, a catalytic product of COX-2, is a subclass of eicosanoid that aids in cancer progression by binding to prostanoid receptors on cell surface and regulates cell migration, proliferation, angiogenesis, and invasion [55,56]. Since in our study, COX-2 overexpression was correlated with EMT, we examined whether prostaglandin E2 can manifest the similar phenomenon and fisetin could be effective against EMT induced by it. Prostaglandin E2 changed the morphology of cells to elongated and fibrous type akin to mesenchymal phenotype. It also increased the activity of MMP-2/9 after 24 h of treatment analogous to CSE exposure suggesting the strong connection between COX-2 overexpression and subsequent EMT-associated changes. Prostaglandin E2 also increased the migratory and invasive capabilities of A549 cells and fisetin reverted these changes suggesting its potential as an effective anti-EMT molecule in cancers with high levels of COX-2 and prostaglandin E2.

Since EMT is conceived as a central player for stemness in the transformed cells [57], we next performed an assay to examine the sphere-forming ability of A549 cells in the presence of CSE and whether

(A)



(B)

Name of the protein	PDB ID	Binding Affinity ($K_{cal/mol}$)	Interaction	Amino acid residue involved
MMP-2	1CK7	-10	Hydrogen Bond	ARG 424 MET 422
COX-2	6COX	-9.1	Hydrogen bond	HIS 207 GLU 290
MMP-9	1L6J	-8	Hydrogen bond	ALA 191 LEU 409

Fig. 8. *In-silico* molecular docking studies between fisetin and MMP-2, COX-2 and MMP-9: (A) and (B) Molecular docking between fisetin and MMP-2, COX-2 and MMP-9 was performed and hydrogen bond interactions of fisetin is presented as 2D and 3D images.

fisetin could inhibit this ability. A549 cells exposed to CSE for 7 days showed increased size and number of spheroids, and NS-398 (COX-2 inhibitor) and fisetin treatment significantly reduced both size and number of spheres. These results demonstrate that fisetin can reduce sphere-forming capacity similar to specific COX-2 inhibitor, NS-398, and could be used in other 3D *in-vitro* models to further validate EMT-associated changes. Thus, further investigation would be needed to confirm the potential of fisetin in controlling the self-renewal and stemness properties of CSE-exposed airway epithelial cells.

We also performed an *in-silico* docking analysis of fisetin with key players like COX-2, MMP-2, and MMP-9. The computational results suggest that fisetin binds with these proteins with high binding affinity and thus could be a potential small molecule to be used as a lead therapeutic candidate. However, more extensive screening along with *in-vivo* and patient data may further support these results.

5. Conclusion

This study shows that fisetin could reverse CSE-induced and COX-2 -mediated EMT through regulating the expression and activities of MMP-2/9/β-catenin signaling axis in airway epithelial cells (Fig. 9). It is a continuation of our previous work, and one of the very few reports that show that fisetin could revert CSE-induced, and inflammation-associated EMT, and thus, fisetin could be used as a potential drug candidate for the treatment of various lung pathologies such as COPD and lung cancer that have cigarette smoke as the common risk factor.

Author's contribution

Hina Agraval: Conceptualization. Investigation, Data curation, Writing- original draft, visualization. Jiten R. Sharma: Investigation, Writing- Review & Editing. Nutan Prakash: Formal analysis - in-silico analysis. Umesh C. S. Yadav: Conceptualization, Supervision,

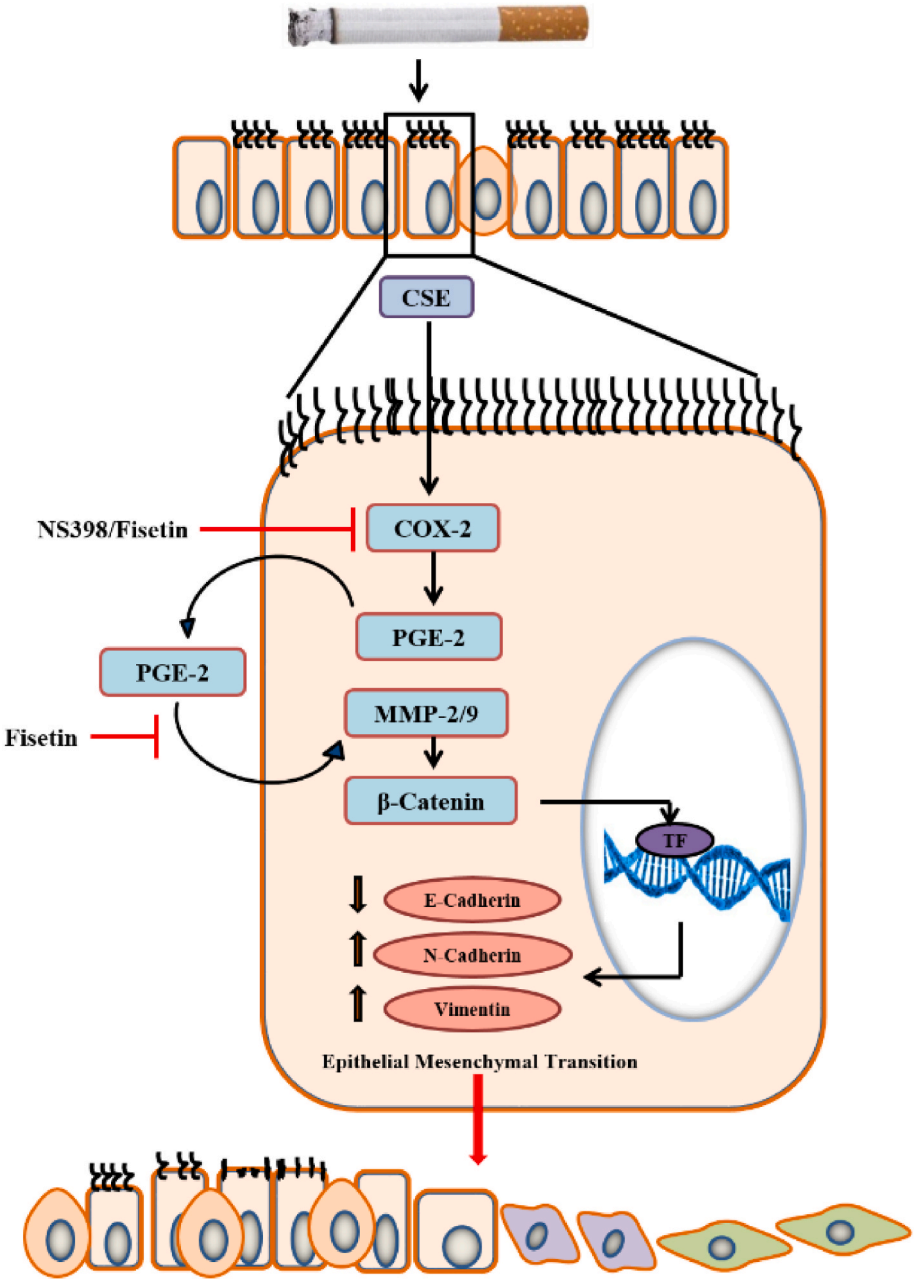


Fig. 9. Schematic representation of plausible mechanism of CSE-induced EMT involving COX-2/PGE2/MMPs axis.

Resources, Writing- Reviewing and Editing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2021.109771.

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