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Research paper

Suppressing effects of green tea extract and Epigallocatechin-3-gallate (EGCG) on TGF-β- induced Epithelial-to-mesenchymal transition via ROS/Smad signaling in human cervical cancer cells

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ABSTRACT

Background: Transforming growth factor-β (TGF-β)-induced Epithelial-to-mesenchymal transition (EMT) process is a fundamental target for preventing cervical cancer cells' progression and invasion. Green tea and its principal active substance, Epigallocatechin-3-gallate (EGCG), demonstrate anti-tumor activities in various tumor cells.
Methods: The cell viability of two cervical cancer cell lines, Hela and SiHa, in the experimental groups was examined employing the MTT method, and ROS generation was probed applying 2′,7′-dichlorofluorescein diacetate-based assay. The Smad signaling and EMT process was evaluated utilizing western blot analysis and quantitative real-time polymerase chain reaction (qRT-PCR). Chromatin immunoprecipitation (ChIP) and Smad binding element (SBE)-luciferase assays were employed to measure Smad-DNA interaction and Smad transcriptional activity, respectively.
Results: EGCG (0–100 μmol/L) and green tea extract (0–250 μg/ml) suppressed the viability of cancer cells in a dose-dependent manner ($p < 0.01$). Our conclusions affirmed that pre-incubation with green tea extract (80 μg/ml) and EGCG (60 μmol/L) significantly reversed the impacts of TGF-β in Hela and SiHa cells by decreasing Vimentin, ZEB, Slug, Snail, and Twist and increasing E-cadherin expression. The molecular mechanism of green tea extract and EGCG for TGF-β-induced EMT inhibition interfered with ROS generation and Smad signaling. Green tea extract and EGCG could significantly decrease ROS levels, the phosphorylation of Smad2/3, the translocation, DNA binding, and activity of Smads in cervical cancer cell lines treated with TGF-β1 ($p < 0.01$).
Conclusion: EGCG and green tea extract suppressed TGF-β-induced EMT in Hela and SiHa cells, and the underlying molecular mechanism may be related to the ROS generation and Smad signaling pathway.

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1. Introduction

Cervical cancer is one of the most frequent malignant tumors observed in women worldwide, with 200,000 deaths each year (Faghihloo et al., 2017). However, poor prognosis and high mortality of patients with cervical cancer are principally due to tumor invasion and metastasis to other organs of the body (Fan et al., 2015; Qureshi et al., 2015). Epithelial-to-mesenchymal transition (EMT) is associated with cervical cancer progression and metastasis (Fan et al., 2015; Lv et al., 2013). E-cadherin is an imperative factor in maintaining the cell–cell adhesion and epithelial features, and failure of this molecule is one of the characteristics of the EMT mechanism and is regularly found in cervical cancer. Also, mesenchymal markers such as Vimentin may be upregulated in the EMT process (Lamouille et al., 2014). Transforming growth factor-β (TGF-β) induces EMT process in cancer cells through the ligand binding to cell surface-TGF-β receptor I and TGF-β receptor II, which leads to downstream phosphorylation of Smad2 and Smad3 transcription factors (Gasior et al., 2019). Then, Smad2 and Smad3 bind to Smad4 and translocate into the nucleus, where Smad complexes regulate the expression of target genes, including those associated with EMT (Ji et al., 2015; Mohamed et al., 2019).

Reactive oxygen species (ROS) have been monitored to mediate diverse TGF-β signaling forces during tumorigenesis (Krstić et al., 2015; Costa et al., 2014; Abazari et al., 2020). Some thoughts have previously described that TGF-β induces EMT in various tumor cells through ROS and is effectively inhibited by antioxidants (Krstić et al., 2015). Due to TGF-β/Smad signaling's indispensable role in promoting tumor progression and invasion, papers suggest that this signaling pathway may be an imperative target for tumor treatment. Traditional herbal medicine has been identified as capable anti-cancer agents in cancer prevention and treatment (Miyata et al., 2019; Sen and Chakraborty, 2017; Sharifat et al., 2017). Green tea is a natural polyphenolic substance elicited from the *Camellia Sinensis* plant's leaves, widely consumed worldwide (Chu et al., 2017). Epigallocatechin-3-gallate (EGCG) is the most active polyphenolic compound in green tea, which has been broadly scrutinized to review its actions, including antioxidants, antiproliferative and anti-neoplastic activity (Miyata et al., 2019; Panji et al., 2021; Fattah et al., 2021). There are some basic and clinical considerations about the anti-neoplastic properties of green tea extract and its chief compound, EGCG, which modulates diverse decisive signal transduction pathways in cancer cells (Wang et al., 2018; Rady et al., 2018; Abazari et al., 2016; Abbasi and Abazari, 2018). Previous inquiries have detailed that incubation of tumor cells with green tea extract and EGCG leads to inhibit TGF-β signaling in various types of tumors; however, their actions on TGF-β signaling-induced EMT mechanism in cervical cancer cells remains to be examined. The present inquiry's principal objective was to investigate the consequences of green tea extract and EGCG on TGF-β-induced EMT and underlying molecular mechanisms in the human cervical cancer cell line, Hela cells.

2. Materials and methods

2.1. Green tea extraction

The fresh leaves of green tea were afforded from a tea garden in Lahijan (northern Iran). First, the fresh leaves of green tea were dried at room temperature and powdered. Next, green tea extract was performed from 30 g green tea leaves using 600 ml of boiling water in a 1: 20 ratio stirring for 1-hour. A filtration paper filtered the retrieved extraction sample, and the pure solution was concentrated in a rotary evaporator and then freeze-dried.

2.2. Detection of total tea polyphenols

According to the method, the Folin detected the total green tea polyphenol amounts- Ciocalteu's reagent (Merck company), and all data were computed employing a gallic acid curve as standard (Singleton et al., 1999).

2.3. Cell line and culture

Two human cervical cancer cell lines, Hela and SiHa, were acquired from the Institute of Pasteur (Iran, Tehran). These cancer cells were cultivated and maintained in RPMI 1640 (Bioidea Company, Iran) culture medium supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Bioidea Company, Iran) and incubated at 37 °C in a humidified incubator containing 5% CO_2 atmosphere.

2.4. Cell treatment with green tea extract and EGCG

A stock solution of green tea extract and EGCG (Sigma-Aldrich Chemical Co, USA) was prepared in distilled water to concentrate 10 mg/ml and 100 mmol/L, respectively, and then stored at − 80 °C. For investigating the toxicity impressions of green tea extract and EGCG on the cervical cancer cell lines, the HeLa and SiHa cells were exposed to 0–250 μg/ml of green tea extract and 0–100 μmol/L of EGCG for 24 h, and cell viability was ruled by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The cells of the control group were exposed to the culture medium. For examining the impacts of green tea extract and EGCG on TGF-β (Cell Signaling, USA)-induced EMT, cancer cells (~80% confluence) were pre-incubated with 20 μmol/L of LY2109761 (Sigma-Aldrich Chemical Co, USA) as a TGF-β receptor inhibitor, 10 mmol/L of N-acetylcysteine (NAC) (Sigma-Aldrich Chemical Co, USA) as a ROS scavenger, 80 μg/ml of green tea extract, and 60 μmol/L of EGCG for 1 h followed by the incubation with 10 ng/ml TGF-β for 24 h according to the purpose of the essay.

For evaluating the effects of ROS production on TGF-β-induced EMT, cancer cells (~80% confluence) were pre-incubated with 400 U/mL of catalase (CAT) (Sigma-Aldrich Chemical Co, USA) as a ROS scavenger, 10 mmol/L of NAC, 80 μg/ml of green tea extract, and 60 μmol/L of EGCG for 1 h followed by the incubation with 250 μM H2O2 for 24 h.

2.5. MTT assay

Hela and SiHa cells were plated in 96-well plates at a density of 10^4 cells/well and incubated overnight to allow cells attachment. Subsequently, cells were incubated with 0–250 μg/ml of green tea extract and 0–100 μmol/L of EGCG for 24 h, and the viable cells were determined using MTT assay (Sigma-Aldrich Chemical Co, USA) according to the manufacturer's protocol. After incubation, MTT solution (5 mg/mL) was added to each well and incubated for the next 4 h at 37 °C. The medium was carefully discarded, and then dimethyl sulfoxide (DMSO) (Cinnagen, Iran) was added to all experimental groups to dissolve the formazan complexes, and absorbance was read at 492 nm.

2.6. Intracellular ROS assay

After incubation time, according to the experiment's purpose, intracellular ROS was monitored applying 2′,7′–dichlorofluorescein diacetate (also known as H2DCFDA) assay (Sigma-Aldrich Chemical Co, USA). In brief, cancer cells (~80% confluence) were pre-incubated with 20 μmol/L of LY2109761, 10 mmol/L of N-acetylcysteine, 80 μg/ml of green tea extract, and 60 μmol/L of EGCG for 1 h followed by the incubation with 10 ng/ml TGF-β for 24 h. After the incubation period, Hela and SiHa cells were exposed to serum-free RPMI 1640 containing H2DCFDA (10 μmol/L) for 1 h at 37 °C. Finally, the cells were washed and resuspended in 1 ml of phosphate buffer saline (PBS; Bioidea Company, Iran). A spectrofluorometer (Cary, Australia) at 485 nm (an excitation wavelength) and 535 nm (an emission wavelength) was used to detection of fluorescence intensity.

2.7. Nuclear fractionation

HeLa and SiHa cells were pre-incubated with 80 μg/ml of green tea extract and 60 μmol/L of EGCG for 1 h, followed by incubation with 10 ng/ml TGF-β for 24 h. After incubation time, cells were scraped, and cell lysates were centrifuged at 4 °C at 3000 rpm for 10 min. The cytosolic fraction was discarded, and the remaining pellet was resuspended in PBS.

2.8. Western blot analysis

According to the experiment's purpose, Hela and SiHa cells were collected 24 h after incubation, and then whole and nuclear protein concentrations were estimated using Bradford assay (Bradford, 1976). Next, 50 μg of protein samples were subjected to SDS-PAGE, followed by transfer onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA). For blocking non-specific binding sites, the membranes were exposed to 5% non-fat milk (Sigma-Aldrich Chemical Co, USA) for 1 h at room temperature. In the next step, the membrane was exposed to specific primary antibodies against total Smad3 (#9513, Cell Signaling Technology, USA) or phosphorylated Smad3 (Ser423/425) (#9520, Cell Signaling Technology, USA), total Smad2 (#5339, Cell Signaling Technology, USA) or phosphorylated Smad2 (Ser465/467) (#3108, Cell Signaling Technology, USA), Smad4 (#38454, Cell Signaling Technology, USA), E-cadherin (#3195, Cell Signaling Technology, USA), and Vimentin (1: 1000 dilution) (#5741, Abcam, Cell Signaling Technology, USA) at 4 ^{0}C overnight, followed by incubation with horseradish peroxidase-labeled secondary antibody (1: 10,000 dilution) (Sigma-Aldrich Chemical Co, USA). Finally, the blots were reprobed with a specific antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1: 5000 dilution) (Sigma-Aldrich Chemical Co, USA) and histone H3 (#9715, Cell Signaling Technology, USA) as a loading control. GAPDH was used as a loading control for whole-cell lysates, and histone H3 was used as a nuclear loading control. The protein bands were visualized, adopting an enhanced chemiluminescence detection system (Bio-Rad, USA) through the Chemi Doc image analysis system (Bio-Rad, USA). The final intensity of interest bands was evaluated by normalizing the interest bands' density using loading control.

2.9. Quantitative Real-time polymerase chain reaction (q-RT-PCR) analysis for EMT-related markers at mRNA levels

According to the experiment's purpose, total RNA was isolated from cancer cells using Trizol reagent (Invitrogen, Karlsruhe, Germany). 1 μg of total RNA was converted to First-strand cDNA using reverse transcriptase according to the protocols of the Takara kit (Japan). QRT-PCR analysis of E-cadherin, Vimentin, ZEB, Slug, Snail, Twist, and β-actin mRNA levels was done in an ABI 7500 Real-Time PCR System using SYBR Green PCR kit (Takara, Japan) and appropriate primers synthesized by Yekta Tajhiz (Iran). The related primer pairs were as follows: E-cadherin (forward primer 5′- TGCCCAGAA AATGAA AAAGG-3′ and reverse primer 5′- GTGTATGTGGCA ATGCGTTC-3′), Vimentin (forward primer 5′- CTTCGCCAACTACATCGACAA-3′ and reverse primer 5′- CGCATTGTC AACATCCTGTC-3′) (Sun et al., 2017), Snail (forward primer 5′- CCTCAA GATGCACATCCGAAG-3′ and reverse primer 5′- ACATGGCCTTGTAGCAGCCA-3′), Slug (forward primer 5′- GAGCATA-CAGCCCCATCACT-3′ and reverse primer 5′- GGGTCTGAAAGCTTG-GACTG-3′) (Ko et al., 2013), Twist (forward primer 5′- TCTGGAGGACCTGGTAGAGG-3′ and reverse primer 5′- GGAGTCCG-CAGTCTTACGAG-3′) (Pai et al., 2013), ZEB (forward primer 5′- CCCAGGACAGCACAGTAAAT-3′ and reverse primer 5′- GATGGTG-TACTACTTCTGGAACC-3′) (Jia et al., 2016), and β-actin (forward primer 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ and reverse primer 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′). β-actin was used as a housekeeping gene. The fold change in mRNA expression was analyzed by the delta-delta cycle-thresh-old (ΔΔCt) method.

Ct values for all samples were recorded and normalized against β-actin.

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP is a very widely used technique in the evaluation of protein-DNA interactions. A ChIP assay was executed in two cervical cancer cells using a commercial kit (Millipore, Darmstadt, Germany).

Briefly, after cell treatment, 10^{7} cells were cross-linked with 1% formaldehyde for 10 min at 37 °C. Following sonication and centrifugation, an equal amount of the chromatin-DNA complexes was incubated with protein G magnetic beads and antibodies against Smad4 or IgG (negative control) (#sc-2358, Santa Cruz) overnight at 4 °C. An aliquot of chromatin that was not incubated with an antibody was used as the input control sample. Antibody-bound protein-DNA complexes were eluted and subjected to PCR, which amplified ZEB2 and TWIST promoter regions containing Smads-binding sites. Specific ChIP primers used for PCR were:

Snail: 5′-GCTGTCACACCCGGCACCAAG-3′ and 5′-GGCGGCTTGAAATGCCACGG-3′,

ZEB2: 5′-TACGCCTGCGCTGTGACCTA-3′ and 5′-ACTCACTG-GACCCGCCTCAG-3′ (Sakamoto et al., 2016).

2.11. Smad binding element (SBE)-Luciferase assay

The cancer cells were transiently cotransfected with SBE-Luciferase reporter construct and Renilla reporter (internal control) plasmids for 6 h using Lipofectamine 2000 reagent (#11668; Invitrogen, Carlsbad, CA, USA). To measure Smad transcriptional activity, HeLa and SiHa cancer cells were exposed to TGF-β1 with or without EGCG (60 μmol/L) and green tea extract (80 μg/ml) for 24 h. The cervical cancer cells were harvested, and dual luciferase and Renilla activities were measured by a Dual-Luciferase Reporter Assay System (#E1910, Promega, Madison, USA).

2.12. Statistical analysis

All results are presented as mean ± SD for three independent tests. One-way ANOVA and LSD were employed for statistical analyses. $P < 0.05$ and $P < 0.05$ were considered statistically significant.

3. Results

3.1. The content of green tea polyphenols

Polyphenols are fundamental components of green tea and may have a direct relation to anti-cancer properties. The content of total green tea polyphenols measured by the Folin- Ciocalteu's reagent was about 20.15 ± 4.2% according to milligrams of gallic acid equivalents per g of dry tea sample.

3.2. The cytotoxicity effects of green tea extract and EGCG on cervical cancer cells

We explored the cytotoxic impression of green tea extract and EGCG on cervical cancer cells utilizing the MTT test. As manifested in Fig. 1A and B, Hela and SiHa cells were exposed to 0–250 μg/ml of green tea extract and 0–100 μmol/L of EGCG for 24 h. Our results pointed out that green tea extract inhibited the viability of cervical cancer cell lines in a dose-dependent manner. To authenticate the impact of green tea polyphenols on blocking Hela and SiHa cells' viability, we used pure EGCG to inhibit cell viability. Additionally, we stated that EGCG could also repress the viability of both cervical cancer cell lines in a dose-dependent manner. After 24 h of treatment with 60 μg/ml EGCG and 80 μmol/L green tea extract, cell viability was hindered by approximately 50%, which indicated the higher concentrations of green tea

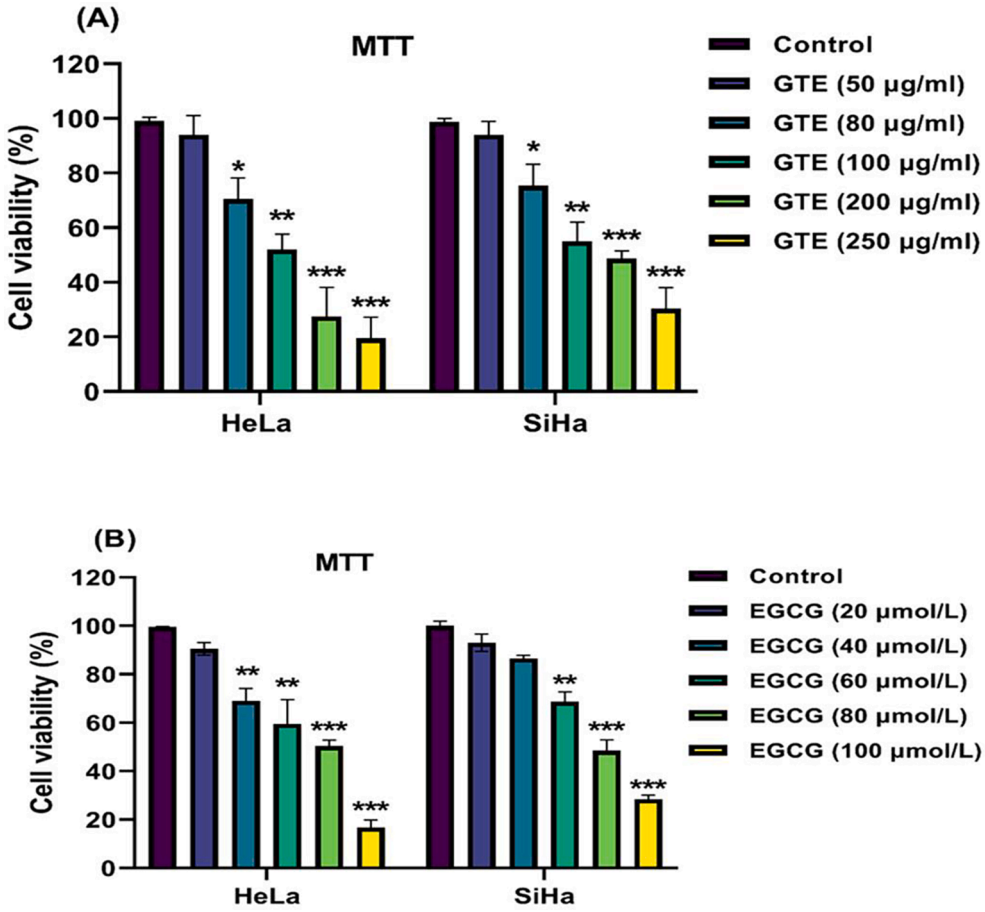


Fig. 1. Inhibition effect of green tea extract (GTE) and EGCG on cell viability of human cervical cancer cell lines. A) Effect of GTE (0, 50, 80, 100, 200 and 250 µg/ml) and B) effect of EGCG (0, 20, 40, 60, 80 and 100 µmol/L) on cell viability of Hela and SiHa cell lines was determined using MTT test after 24 h' exposure. The results represented the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the control group. EGCG = Epigallocatechin-3-gallate;TGF-β = transforming growth factor-beta; MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extract (60 µg/ml) and ECGC (80 µmol/L) had a high cytotoxic rule on the viability of Hela and SiHa cells ($P < 0.01$).

3.3. Green tea extract and EGCG decrease TGF-β-induced EMT markers in vitro

Previous readings have illustrated that TGF-β1 induces EMT in cultured cervical cancer cells (Xu et al., 2017). Here, for investigating the influence of green tea extract and EGCG in the TGF-β1-EMT process, Hela and SiHa cells were pre-incubated with EGCG (60 µmol/L) and green tea extract (80 µg/ml) for 1 h followed by stimulation with TGF-β1 (10 ng/ml) for 24 h and the expression of E-cadherin and Vimentin were quantified by qRT-PCR and western blotting methods. In response to TGF-β1 incubation (10 ng/ml; 24 h), the mRNA and protein expressions of E-cadherin were significantly down-regulated ($p < 0.05$, $p < 0.01$) (Fig. 2A and C), while the mRNA and protein expression of Vimentin was significantly upregulated ($p < 0.05$, $p < 0.01$) (Fig. 2B and D). However, pre-incubation with green tea extract and EGCG could return cadherin and Vimentin levels to prior levels ($p < 0.05$, $p < 0.01$). Also, When the cells were pre-incubated with TGFβRI/II inhibitor, the mRNA and protein expression of E-cadherin and Vimentin returned to previous levels ($p < 0.01$) (Fig. 2).

3.4. Green tea and EGCG repressed the mRNA expression of EMT-associated transcription factors

In the EMT process, increased Snail, ZEB, Slung, or Twist transcription factors expression is associated with tumor cell metastasis through E-cadherin suppression (Jia et al., 2016). Here, we examined whether cell incubation with EGCG (60 µmol/L) or green tea extract (80 µg/ml) impaired the mRNA expression of Slug, Snail, ZEB, and Twist in TGF-β1-stimulated Hela and SiHa cells. The expression of Slug, Snail, ZEB, and Twist was detected by qRT-PCR. As revealed in Fig. 3A–D, TGF-β1 (10 ng/ml, 24 h) treatment significantly upregulated Snail ($p < 0.01$), ZEB ($p < 0.01$), TWIST ($p < 0.01$), and Slug ($p < 0.05$, $p < 0.01$) mRNA expression in Hela and SiHa cells. Pre-treatment with EGCG or green tea for 1 h significantly attenuated the gene expression of Snail, Slug, ZEB, and Twist in TGF-β1-stimulated cervical cancer cells ($p < 0.05$, $p < 0.01$). When the TGFβRI/II inhibitor was added, all markers' levels returned to former levels ($p < 0.01$). These results suggest that green tea extract and EGCG decreased TGF-β1-induced EMT-associated transcription factors.

3.5. Green tea extract and EGCG repressed TGF-β1–induced Smad2/3 phosphorylation in cervical cancer cells

We examined whether cell incubation with EGCG or green tea blocked the Smad signaling pathway in TGF-β1-stimulated Hela and SiHa cells. To validate the impact of green tea on inhibiting the TGFβ signaling pathway, we used LY2109761. Additionally, the LY2109761 could specifically inhibit the phosphorylation of Smad2 and Smad3 induced by TGFβ1 in cervical cancer cells ($p < 0.05$, $p < 0.01$). To examine the inhibitory ability of green tea on the activation of R-Smads, we examine the phosphorylation of Smad2 and Smad3 using western blotting in cancer cells. Not surprisingly, we unveiled that the phosphorylation of Smad2 and Smad3 was upgraded in TGF-β-treated Hela and SiHa cells (10 ng/ml, 24 h). When green tea (80 µg/ml, 1 h) or EGCG (60 µmol/L, 1 h) were added, the phosphorylation levels of Smad2 and Smad3 return to the prior levels without any treatment ($p < 0.05$, $p < 0.01$) (Fig. 4A and B).

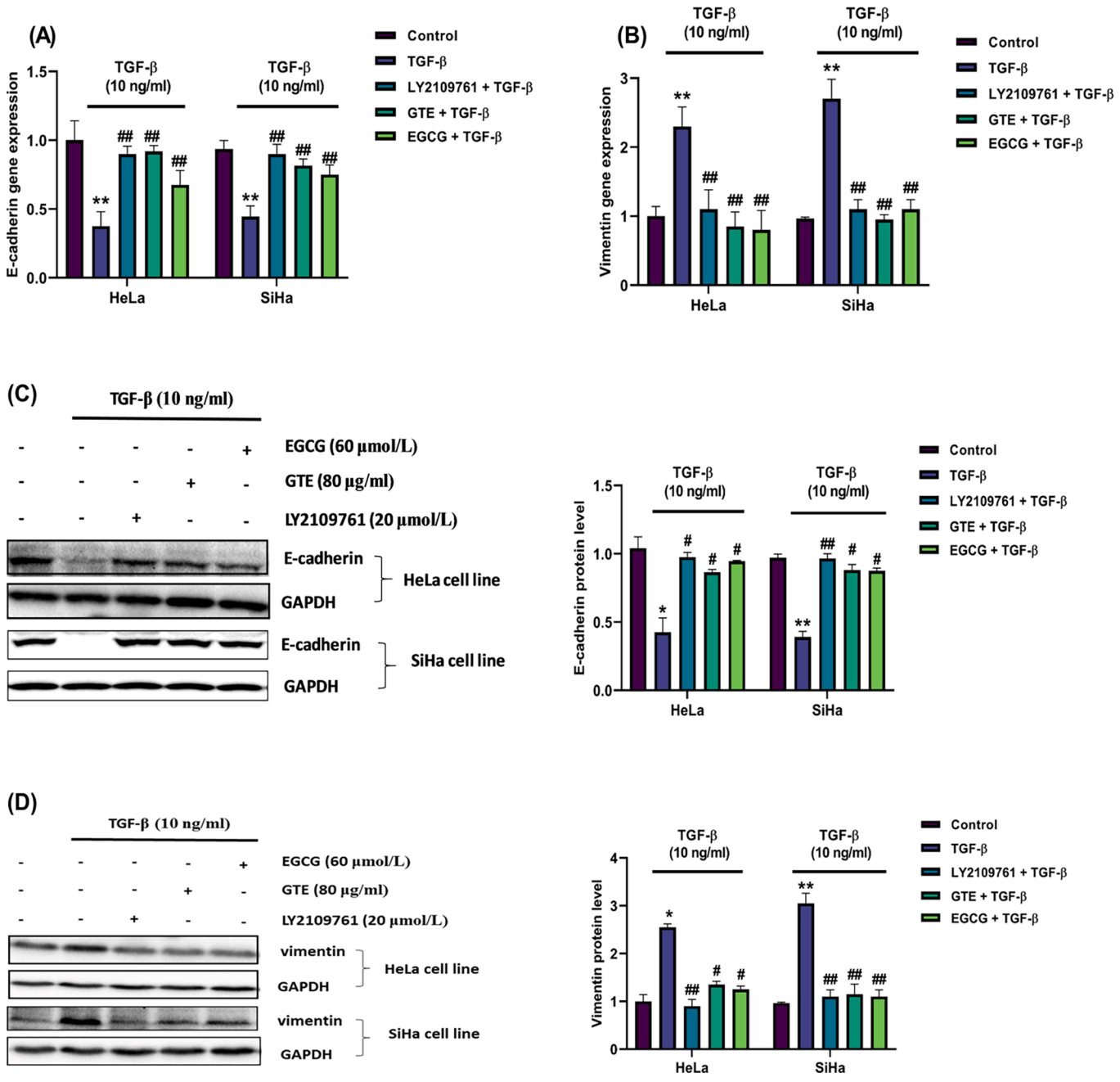


Fig. 2. Green tea extract (GTE) and EGCG suppress TGF-β-induced E-cadherin and vimentin mRNA and protein expression in cervical cancer cells. HeLa and SiHa cells were exposed to 10 ng/ml TGF-β (24 h) in the presence and absence of 20 μmol/L of LY2109761, 80 μg/ml of GTE, and 60 μmol/L of EGCG for 1 h. Then, the mRNA expression of (A) E-cadherin and (B) vimentin were detected with qRT-PCR, and the protein expression of (C) E-cadherin and (D) vimentin were detected with western blotting. GAPDH and β-actin were used as loading control in the protein and mRNA expression analyses, respectively. The results represented the mean ± SEM of three independent experiments. *P < 0.05 and **P < 0.01 compared to the untreated group, while #P < 0.05 and ##P < 0.01 compared with the TGF-β group. EGCG = Epigallocatechin-3-gallate;TGF-β = transforming growth factor-beta; GAPDH = glyceraldehyde 3-phosphate dehydrogenase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Green tea extract and EGCG repressed nuclear translocation, DNA binding, and transcriptional activity of Smad4 induced by TGF-β in cervical cancer cells

In whole-cell lysates, there was no significant difference in the Smad4 protein expression between experimental groups. However, TGF-β provoked the protein expression of nuclear Smad4 after 24 h of incubation. Meanwhile, the pre-incubation with 80 μg/ml of green tea and 60 μmol/L of EGCG effectively inhibited the expression of Smad4 protein stimulated by TGF-β1 (Fig. 5A).

To evaluate the binding of Smad4 to the promotor of EMT-related markers, we performed a ChIP assay in TGF-β-stimulated cervical cancer cells. The finding of the ChIP-PCR experiment showed that the Smad4 binding affinity to the EMT-related transcriptional factors Snail and ZEB2 in TGF-β-treated Hela and SiHa cells significantly increased relative to control conditions, and the treatment with EGCG could significantly decrease the binding of Smad4 to the target sequences (Fig. 5B). ChIP-PCR results were consistent with the real-time PCR results (Fig. 2B).

In the next step, the dual-luciferase reporter gene assay was utilized

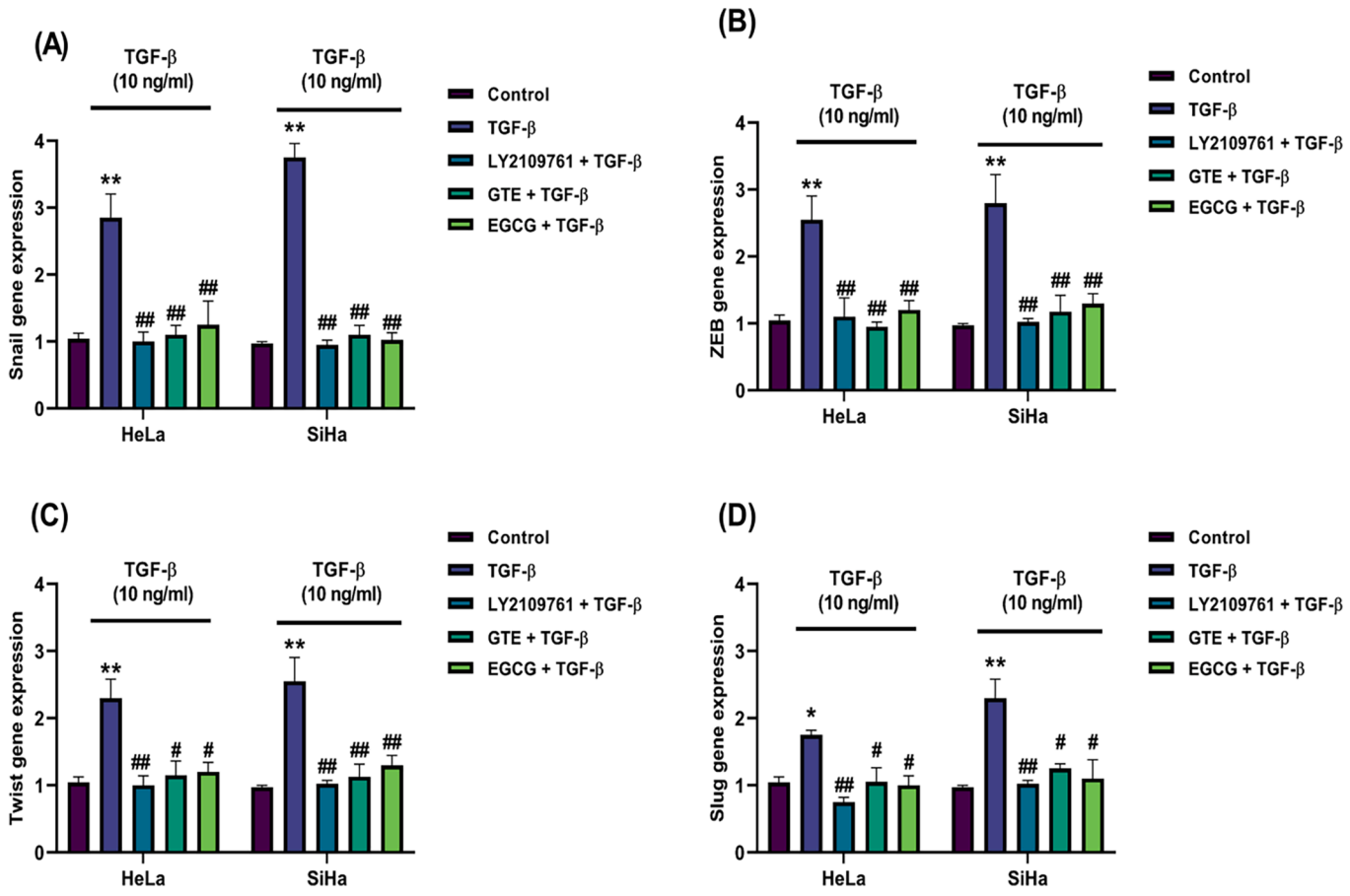


Fig. 3. Green tea extract (GTE) and EGCG suppress TGF-β-induced Snail, Twist, ZEB, and Slug in cervical cancer cells. HeLa and SiHa cells were exposed to 10 ng/ml TGF-β (24 h) in the presence and absence of 20 μmol/L of LY2109761, 80 μg/ml of GTE, and 60 μmol/L of EGCG for 1 h, and then the mRNA expression of (A) Snail, (B) ZEB, (C) Twist, and (D) Slug were detected with qRT-PCR. β-actin was used as an internal control in the mRNA expression analyses. The results represented the mean ± SEM of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the untreated group, while $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the TGF-β group. EGCG = Epigallocatechin-3-gallate; TGF-β = transforming growth factor-beta. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to evaluate the transcriptional activity of Smads complexes. After normalization by Renilla luciferase activity, a significant increase in the transcriptional activity of Smads was observed in HeLa and SiHa cells exposed to TGF-β1, which was significantly inhibited by EGCG and green tea pre-treatment ($p < 0.01$) (Fig. 5C).

3.7. Green tea extract and EGCG repressed TGF-β-induced ROS generation in cervical cancer cells

Preliminary comparisons explained that ROS was involved in TGF-β-induced EMT in various tumor cells (Muthuramalingam et al., 2020). Here, for investigating effects of green tea extract and EGCG on ROS generation, Hela and SiHa cells were pre-incubated with ROS scavenger, NAC (10 mmol/L), LY2109761 (20 μmol/L), EGCG (60 μmol/L), and green tea extract (80 μg/ml) for 1 h followed by stimulation with TGF-β1 (10 ng/ml) for 24 h and the ROS production were quantified by H2DCFDA assay. In response to TGF-β1 incubation (10 ng/ml; 24 h), the ROS level was significantly increased ($p < 0.05$, $p < 0.01$), and pre-incubation with TGFβRI/II inhibitor and NAC could reverse the ROS levels to previous levels ($p < 0.05$, $p < 0.01$). Also, pre-incubated with green tea extract and EGCG could significantly inhibit the ROS level in Hela cells treated with 10 ng/ml TGF-β1 ($p < 0.05$, $p < 0.01$) (Fig. 6).

3.8. Green tea extract and EGCG repressed H2O2-induced EMT markers in cervical cancer cells

To determine the inhibitory ability of green tea and EGCG on the ROS-mediated EMT, HeLa and SiHa cells were exposed to 250 μM H2O2 (24 h) in the presence and absence of 400 U/ml of CAT, 10 mmol/L of NAC, 80 μg/ml of GTE, and 60 μmol/L of EGCG for 1 h, and then the mRNA expression of E-cadherin and vimentin were detected q-RT-PCR assay. The findings showed that in response to H2O2 incubation, the mRNA of E-cadherin was significantly down-regulated ($p < 0.05$, $p < 0.01$) (Fig. 7A), while the mRNA of Vimentin was significantly up-regulated ($p < 0.05$) (Fig. 7B). When green tea, EGCG, CAT, or NAC were added, the mRNA levels of E-cadherin and vimentin return to the prior levels without any treatment ($p < 0.05$, $p < 0.01$).

4. Discussion

This manuscript explicated that Smad2 and Smad3 transcription factors are required for TGF-β1–induced EMT-associated markers in cervical cancer cells. We also hypothesized that intracellular ROS might play a role in TGF-β1–induced EMT through Smads activation. However, green tea extract and ECGC could attenuate EMT via suppressing ROS production and Smad signaling.

Numerous experimental and animal studies have indicated that green tea extract derived from fresh leaves and EGCG, as the most active polyphenols in green tea, possesses anti-tumor activity with few adverse

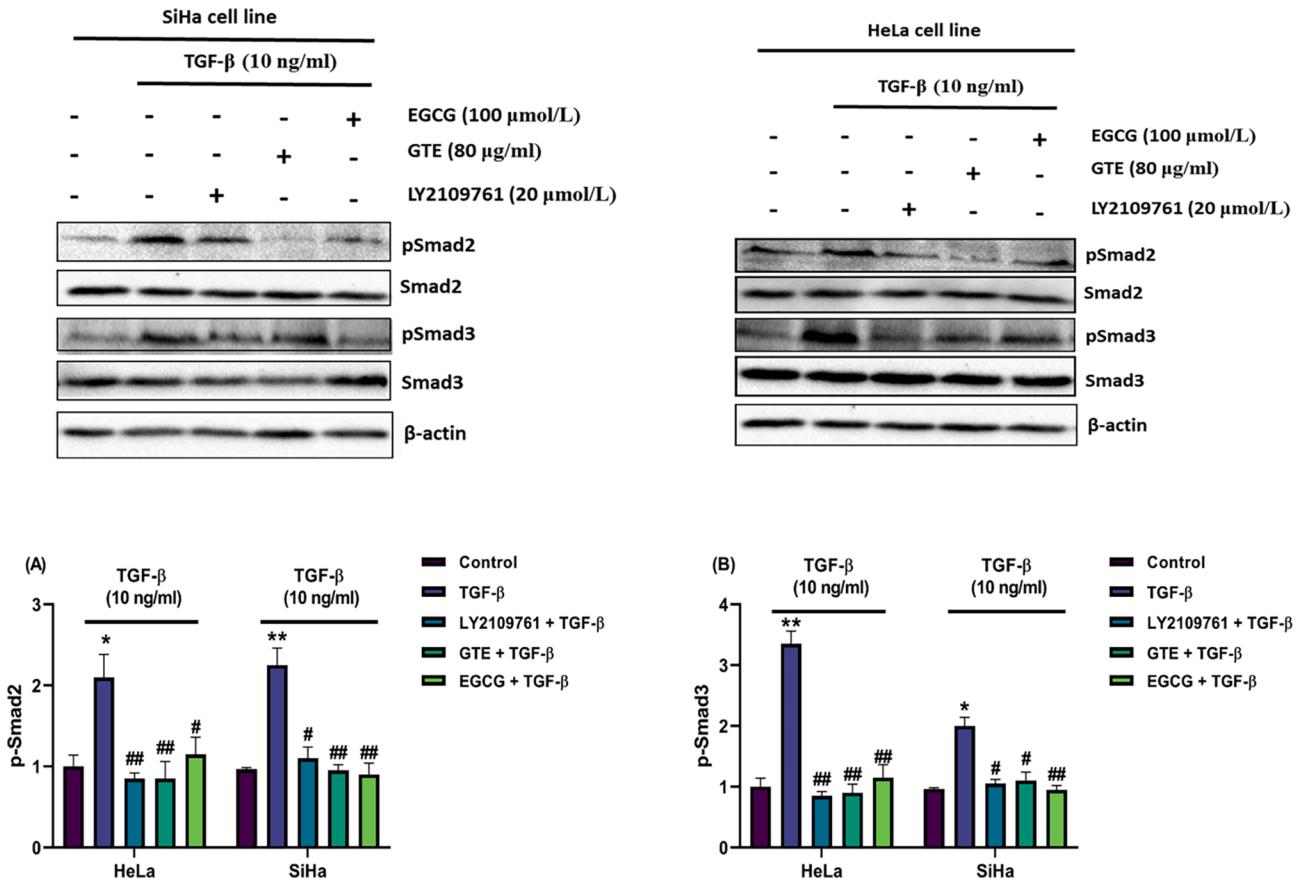


Fig. 4. Green tea extract (GTE) and EGCG suppress TGF-β-induced Smad2 and Smad3 phosphorylation in cervical cancer cells. HeLa and SiHa cells were exposed to 10 ng/ml TGF-β (24 h) in the presence and absence of 20 µmol/L of LY2109761, 80 µg/ml of GTE, and 60 µmol/L of EGCG for 1 h, and then the phosphorylation of (A) Smad2 and (B) Smad3 were detected with western blotting. β-actin was used as an internal control in the mRNA and protein expression analyses. The results represented the mean ± SEM of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the untreated group, while $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the TGF-β group. EGCG = Epigallocatechin-3-gallate; TGF-β = transforming growth factor-beta. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effects in several types of tumors. Furthermore, underlying molecular mechanisms appeared to be related to their actions on growth inhibition, angiogenesis, apoptosis, and EMT process (Schröder et al., 2019; Huang et al., 2016; Asadi et al., 2019). HUANG et al. revealed that the incubation with EGCG significantly inhibited the cell growth of MCF-7 compared to the untreated group in a dose-dependent manner (Huang et al., 2017). We initially explored the cytotoxicity of green tea extract and EGCG on Hela and SiHa cells in the present study. Then we prepared green tea extract from fresh green tea leaves and measured its polyphenols. Studies have confirmed that EGCG is the most critical polyphenol compound in green tea (Rady et al., 2018; Abazari et al., 2020). Our MTT data proved that the viability of cancer cells treated with EGCG or green tea extract was significantly attenuated in a dose-dependent manner. At 100 µg/ml EGCG and 80 µmol/L green tea extract, cell viability was inhibited by approximately 50%, and the higher concentrations of them had a high cytotoxic effect on the viability of cells. Previous studies have explicated that green tea extract and ECGC in human cancer cells could be suppressed in the EMT process (Ko et al., 2013).

The EMT process is a critical issue in tumor progression and metastasis, characterized by the impairment of epithelial cell–cell junctions and the acquisition of a mesenchymal phenotype (Fan et al., 2015; Lv et al., 2013). During EMT, a change in the expression of epithelial cell markers such as E-cadherin and mesenchymal markers such as Vimentin can alter epithelial cell phenotype into motile mesenchymal cells (Geng et al., 2014). The TGF-β signaling pathway participates in tumor invasive and metastasis by inducing the EMT (Musavi et al., 2020; Ji et al., 2015). Qing Ji et al. demonstrated TGF-β1-induced EMT in LoVo cells by down-regulation of E-cadherin and Vimentin up-regulation (Dongre and Weinberg, 2019). By western blotting and qRT-PCR assays, we confirmed that pretreatment with EGCG or green tea significantly increased E-cadherin expression and decreased Vimentin expression in TGF-β1-stimulated cervical cancer cell lines. EMT program is triggered by various transcription factors, including Twist, ZEB, Slug, and Snail, which down-regulate the expression of epithelial genes and upregulate the mesenchymal expression genes (Jia et al., 2016). The TGFβ pathway leads to the activation of EMT-associated transcription factors, which in turn down-regulate E-cadherin (Shafaei et al., 2017; Musavi et al., 2021; Zhang et al., 2016). H. Ko et al. reported that EGCG reverses TGF-β-induced Snail in human A549 lung cancer cells (Ko et al., 2013). Here, by qRT-PCR analysis, we discovered that TGF-β induced EMT-associated transcription factors, but green tea extract and EGCG could significantly inhibit this inducing effect in cervical cancer cells. TGF-β induces EMT-associated markers through ligand binding and activating cell-surface TGF-β receptors type 1 and type 2(TGF-βRI/TGF-βRII), leading to the phosphorylation and activation of Smad2 and Smsd3, which formed the complexes with Smad4. Then Smad complexes translocate into the nucleus, regulating the gene expression of EMT genes (Li et al., 2019). In human anaplastic thyroid carcinoma cells, Li et al. reported that EGCG attenuates TGF-β1-induced EMT by suppressing the nuclear translocation of Smad3/4 (Zhao et al., 2018). In the present study, we found that green tea extract and EGCG potently inhibited Smad complex nuclear translocation and TGF-β1-dependent transcription of the (CAGA) 12-Lux reporter in HeLa and SiHa cancer cells. The Smad3 and Smad4

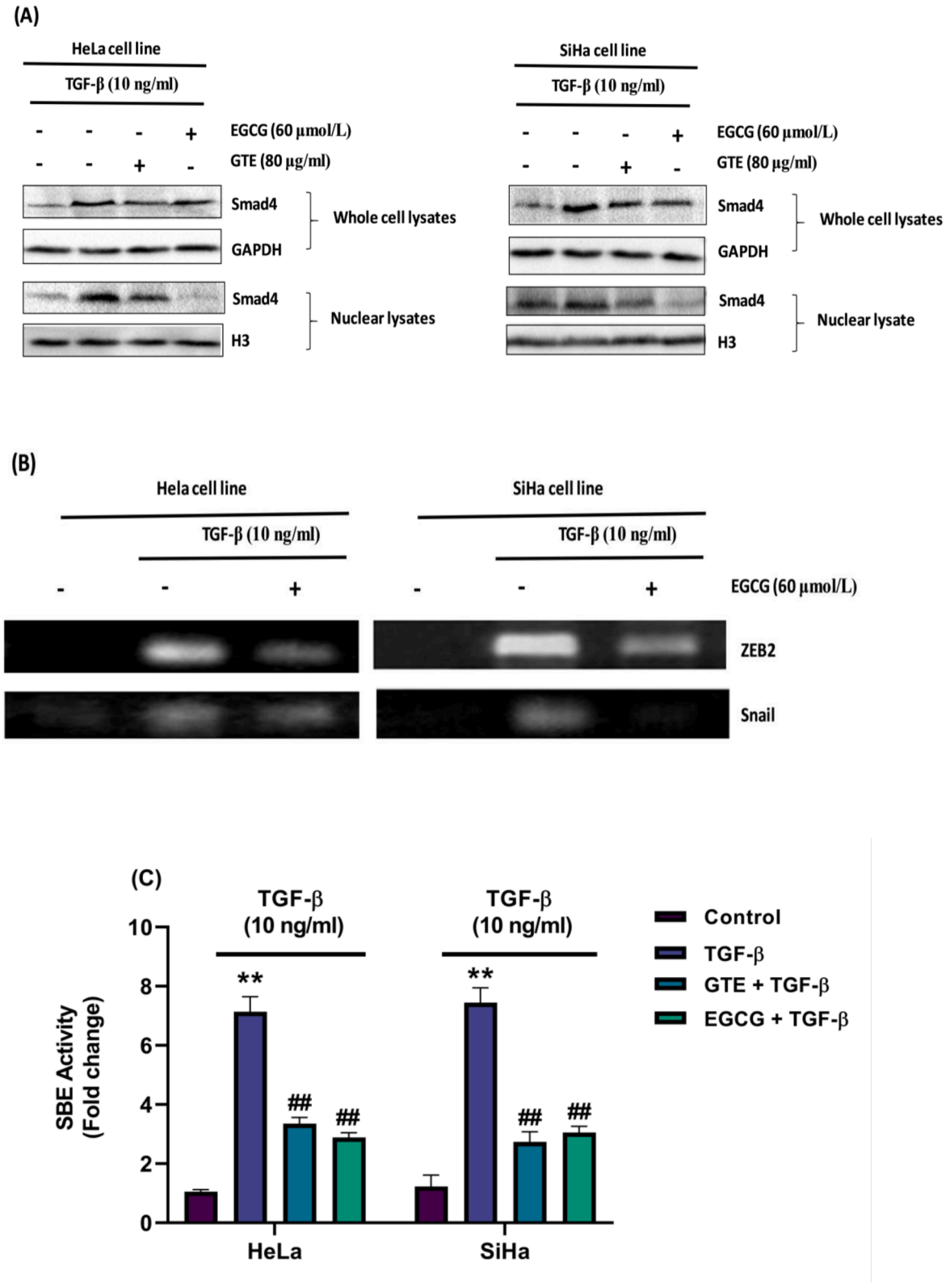


Fig. 5. Green tea extract (GTE) and EGCG reduces TGF-β- activated Smad4 pathway in cervical cancer cells. HeLa and SiHa cells were treated with TGF-β1 (5 ng/ml) in the absence or presence of 80 μg/ml of GTE or and 60 μmol/L of EGCG and (A) nuclear translocation of Smad4 was detected using western blotting, (B) The Smad4 binding to SBE was assessed by ChIP assay, and (C) was the Smads complex activity was determines using Dual-luciferase reporter gene assay. In western blot analysis, GAPDH and H3 were used as whole and nuclear internal control, respectively. In ChIP and Dual-luciferase reporter gene assay, IgG and Renilla luciferase were provided as the internal control. The results represented the mean ± SEM of three independent experiments. $^{**}P < 0.01$ compared to the untreated group, while $^{\#\#}P < 0.01$ compared with the TGF-β group. EGCG = Epigallocatechin-3-gallate; SBE = Smad binding element; ChIP = chromatin immunoprecipitation, TGF-β = transforming growth factor-beta. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

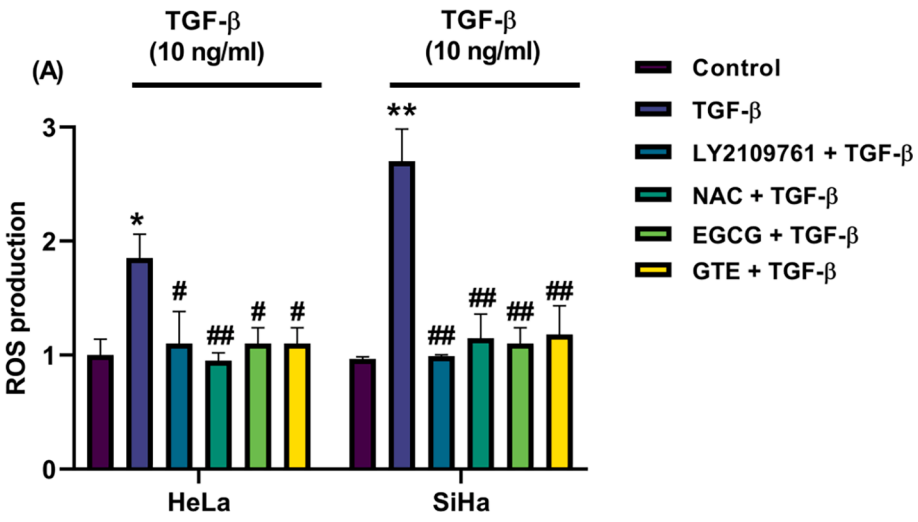


Fig. 6. Green tea extract (GTE) and EGCG suppress TGF-β-induced ROS production in cervical cancer cells. HeLa and SiHa cells were exposed to 10 ng/ml TGF-β (24 h) in the presence and absence of 20 μmol/L of LY2109761, 10 mmol/L of NAC, 80 μg/ml of GTE, and 60 μmol/L of EGCG for 1 h, and then the levels of intracellular ROS were detected with 2′,7′-dichlorofluorescein diacetate-based assay using a fluorimeter. The results represented the mean ± SEM of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the untreated group, while $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the TGF-β group. EGCG = Epigallocatechin-3-gallate; TGF-β = transforming growth factor-beta; NAC = N-acetylcysteine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

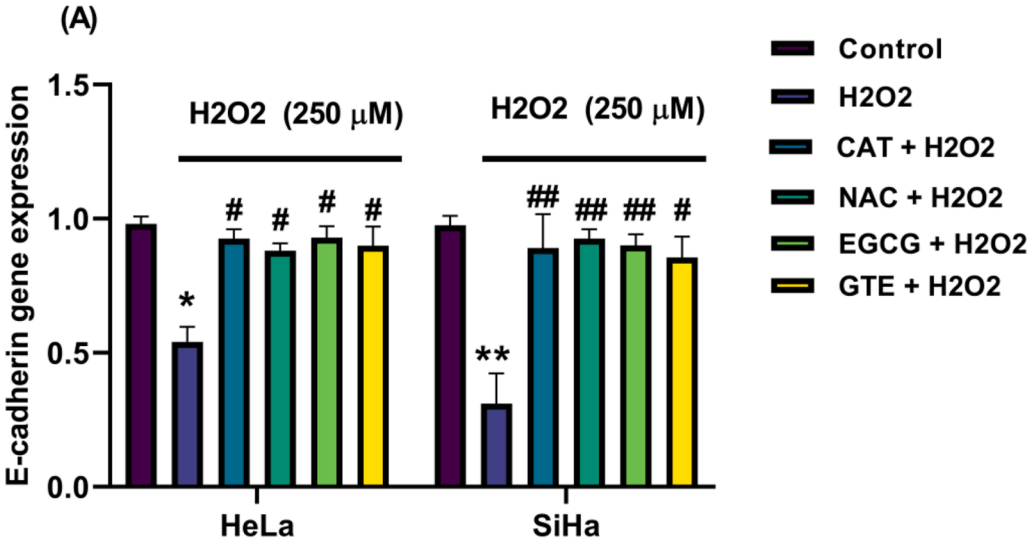
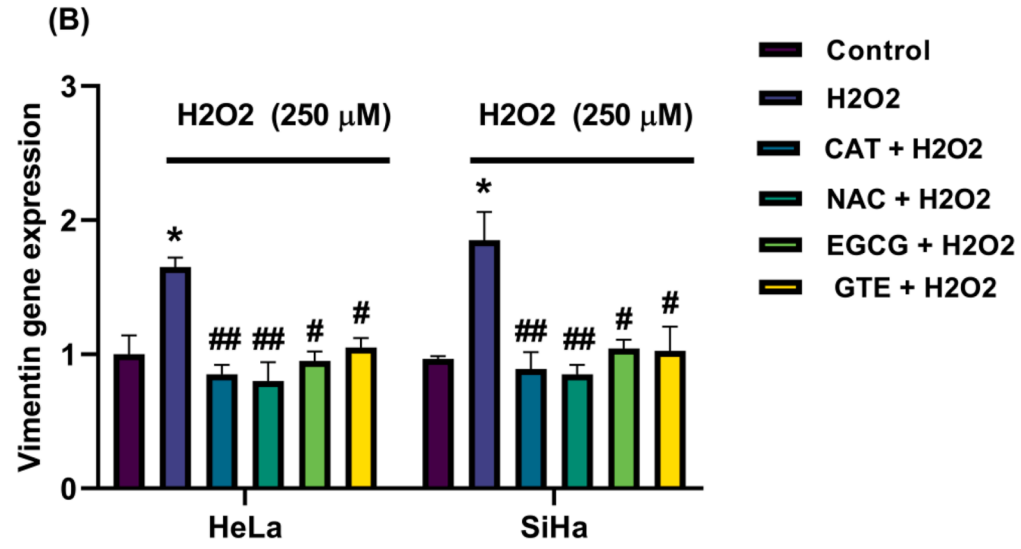


Fig. 7. Green tea extract (GTE) and EGCG suppress H2O2-induced EMT markers in cervical cancer cells. HeLa and SiHa cells were exposed to 250 μM H2O2 (24 h) in the presence and absence of 400 U/ml of CAT, 10 mmol/L of NAC, 80 μg/ml of GTE, and 60 μmol/L of EGCG for 1 h, and then the mRNA levels of E-cadherin and vimentin were detected q-RT-PCR assay. β-actin was used as an internal control. The results represented the mean ± SEM of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the untreated group, while $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the H_2O_2 group. EGCG = Epigallocatechin-3-gallate; TGF-β = transforming growth factor-beta; CAT = catalase; NAC = N-acetylcysteine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



transcription factors have a 'Mad-homology 1′ domain at their N-terminus, which is responsible for DNA binding by recognizing the SBE, while the MH2 domain can interact directly with the MH1 domain of other Smad proteins. The Smad activation domain (SAD) located at the C-terminal of linker region is important for the transcriptional activity of Smad4 protein (Ya et al., 2014). Previous studies indicated that Smad3 and Smad4 bind to the SBE of EMT target genes, including Snail, Slug, and N-cadherin, in a TGF-dependent pathway. Kang et al. illustrated that Smad4 binds to SBEs located within the N-cadherin promoter region, which is necessary for the expression of N-cadherin in the human pancreatic ductal epithelium (Ya et al., 2014). Our results of ChIP-PCR assay demonstrated that the Smad4 bound to the Snail and ZEB2 promoter and contributed to EMT-related markers expression. Many reports indicated that ROS is required for modulation of TGF-β1/Smad-induced EMT, and EGCG attenuates ROS generation (Muthuramalingam et al., 2020; Zhao et al., 2018). However, inadequate knowledge is known about the inhibitory impact of green tea extract on ROS generation-mediated EMT in cervical cancer cells. The present study determined that green tea extract and EGCG, like CAT and NAC, lowered vimentin and elevated E-cadherin levels in H_2O_2-treated Hela and SiHa cells. Our findings recommend that green tea may inhibit TGF-β1-induced EMT, possibly by suppressing ROS.

5. Conclusion

In this manuscript, our data imply that green tea extract and EGCG inhibit TGF-β/Smad signaling-mediated EMT in cervical cancer cells, and the underlying molecular mechanism may be related to ROS generation. The activation of TGF-β signaling leads to the ROS production and phosphorylation of Smad2 and Smad3, a response associated with the modulation of EMT markers genes, including E-cadherin, Vimentin, Snail, ZEB, Slug, and Twist. Green tea extract and EGCG block TGF-β–induced expression of EMT-related genes via inhibition of ROS generation, inhibition of Smad phosphorylation, and nuclear translocation. This might include one of the potential mechanisms of green tea extract and its polyphenolic compounds on inhibiting the invasion and metastases of cervical cancer cells. However, further studies are required to investigate other inhibitory effects of green tea extract and other herbs on the EMT process.

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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References

Abazari, O., Shafaei, Z., Divsalar, A., Eslami-Moghadam, M., Ghalandari, B., Saboury, A. A., 2016. Probing the biological evaluations of a new designed Pt (II) complex using spectroscopic and theoretical approaches: Human hemoglobin as a target. J. Biomol. Struct. Dyn. 34 (5), 1123–1131.

Abazari, O., Shafaei, Z., Divsalar, A., Eslami-Moghadam, M., Ghalandari, B., Saboury, A. A., et al., 2020. Interaction of the synthesized anticancer compound of the methylglycine 1, 10-phenanthroline platinum nitrate with human serum albumin and human hemoglobin proteins by spectroscopy methods and molecular docking. J. Iran. Chem. Soc. 1–14.

Abazari, O., Divsalar, A., Ghobadi, R., 2020. Inhibitory effects of oxali-Platin as a chemotherapeutic drug on the function and structure of bovine liver catalase. J. Biomol. Struct. Dyn. 38 (2), 609–615.

Abbasi, M., Abazari, O.O., 2018. Probing the Biological evaluations of a new designed Palladium (II) complex using spectroscopic and theoretical approaches: Human Hemoglobin as a Target. Archives of. Med. Laborat. Sci. 3 (3).

Asadi, A., Nezhad, D.Y., Javazm, A.R., Khanicheragh, P., Mashouri, L., Shakeri, F., et al., 2019. In vitro effects of curcumin on transforming growth Factor-β-mediated non-smad signaling pathway, oxidative stress, and pro-inflammatory cytokines production with human vascular smooth muscle cells. Iran. J. Allergy Asthma Immunol. 1–10.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254.

Chu, C., Deng, J., Man, Y., Qu, Y., 2017. Green tea extracts epigallocatechin-3-gallate for different treatments. Biomed Res. Int. 2017.

Costa A, Scholer-Dahirel A, Mechta-Grigoriou F, editors. The role of reactive oxygen species and metabolism on cancer cells and their microenvironment. Seminars in cancer biology; 2014: Elsevier.

Dongre, A., Weinberg, R.A., 2019. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. Nat. Rev. Mol. Cell Biol. 20 (2), 69–84.

Faghihloo E, Sadeghizadeh M, Shahmahmoodi S, MOKHTARI AT. Transcriptional suppression of E-cadherin by HPV-16 E6 and E7 oncogenes is independent of hypermethylation of E-cadherin promoter. 2017.

Fan, Q., Qiu, M.-T., Zhu, Z., Zhou, J.-H., Chen, L., Zhou, Y., et al., 2015. Twist induces epithelial-mesenchymal transition in cervical carcinogenesis by regulating the TGF-β/Smad3 signaling pathway. Oncol. Reports 34 (4), 1787–1794.

Fattah, A., Morovati, A., Niknam, Z., Mashouri, L., Asadi, A., Rizi, S.T., et al., 2021. The synergistic combination of cisplatin and piperine induces apoptosis in MCF-7 Cell Line. Iran. J. Public Health. 50 (5), 1037–1047.

Gasior, K., Wagner, N.J., Cores, J., Caspar, R., Wilson, A., Bhattacharya, S., et al., 2019. The role of cellular contact and TGF-beta signaling in the activation of the epithelial mesenchymal transition (EMT). Cell Sdhesion Migrat. 13 (1), 63–75.

Geng, J., Fan, J., Ouyang, Q., Zhang, X., Zhang, X., Yu, J., et al., 2014. Loss of PPM1A expression enhances invasion and the epithelial-to-mesenchymal transition in bladder cancer by activating the TGF-β/Smad signaling pathway. Oncotarget. 5 (14), 5700.

Huang, C.-Y., Han, Z., Li, X., Xie, H.-H., Zhu, S.-S., 2017. Mechanism of EGCG promoting apoptosis of MCF-7 cell line in human breast cancer. Oncol. Lett. 14 (3), 3623–3627.

Huang, S.-F., Horng, C.-T., Hsieh, Y.-S., Hsieh, Y.-H., Chu, S.-C., Chen, P.-N., 2016. Epicatechin-3-gallate reverses TGF-β1-induced epithelial-to-mesenchymal transition and inhibits cell invasion and protease activities in human lung cancer cells. Food Chem. Toxicol. 94, 1–10.

Ji, Q., Liu, X., Han, Z., Zhou, L., Sui, H., Yan, L., et al., 2015. Resveratrol suppresses epithelial-to-mesenchymal transition in colorectal cancer through TGF-β1/Smads signaling pathway mediated Snail/E-cadherin expression. BMC Dancer. 15 (1), 97.

Ji, Q., Liu, X., Han, Z., Zhou, L., Sui, H., Yan, L., et al., 2015. Resveratrol suppresses epithelial-to-mesenchymal transition in colorectal cancer through TGF-β1/Smads signaling pathway mediated Snail/E-cadherin expression. BMC Cancer. 15 (1), 1–12.

Jia, X., Wang, Z., Qiu, L., Yang, Y., Wang, Y., Chen, Z., et al., 2016. Upregulation of Lnc RNA-HIT promotes migration and invasion of non-small cell lung cancer cells by association with ZEB 1. Cancer Med. 5 (12), 3555–3563.

Ko, H., So, Y., Jeon, H., Jeong, M.-H., Choi, H.-K., Ryu, S.-H., et al., 2013. TGF-β1-induced epithelial–mesenchymal transition and acetylation of Smad2 and Smad3 are negatively regulated by EGCG in human A549 lung cancer cells. Cancer Lett. 335 (1), 205–213.

Krstić J, Trivanović D, Mojsilović S, Santibanez JF. Transforming growth factor-beta and oxidative stress interplay: implications in tumorigenesis and cancer progression. Oxidative medicine and cellular longevity. 2015;2015.

Lamouille, S., Xu, J., Derynck, R., 2014. Molecular mechanisms of epithelial–mesenchymal transition. Nat. Rev. Mol. Cell Biol. 15 (3), 178.

Li, T., Zhao, N., Lu, J., Zhu, Q., Liu, X., Hao, F., et al., 2019. Epigallocatechin gallate (EGCG) suppresses epithelial-Mesenchymal transition (EMT) and invasion in anaplastic thyroid carcinoma cells through blocking of TGF-β1/Smad signaling pathways. Bioengineered. 10 (1), 282–291.

Lv, Z.-D., Kong, B., Li, J.-G., Qu, H.-L., Wang, X.-G., Cao, W.-H., et al., 2013. Transforming growth factor-β 1 enhances the invasiveness of breast cancer cells by inducing a Smad2-dependent epithelial-to-mesenchymal transition. Oncol. Rep. 29 (1), 219–225.

Miyata, Y., Shida, Y., Hakariya, T., Sakai, H., 2019. Anti-cancer effects of green tea polyphenols against prostate cancer. Molecules 24 (1), 193.

Mohamed, R., Dayati, P., Mehr, R.N., Kamato, D., Seif, F., Babaahmadi-Rezaei, H., et al., 2019. Transforming growth factor–β1 mediated CHST11 and CHSY1 mRNA expression is ROS dependent in vascular smooth muscle cells. J. Cell Commun. Signal. 13 (2), 225–233.

Musavi, H., Abazari, O., Barartabar, Z., Kalaki-Jouybari, F., Hemmati-Dinarvand, M., Esmaeili, P., et al., 2020. The benefits of Vitamin D in the COVID-19 pandemic: biochemical and immunological mechanisms. Arch. Physiol. Biochem. 1–9.

Musavi, H., Abazari, O., Safaee, M.S., Variji, A., Koohshekan, B., Kalaki-Jouybari, F., et al., 2021. Mechanisms of COVID-19 Entry into the Cell: potential therapeutic approaches based on virus entry inhibition in COVID-19 Patients with Underlying Diseases. Iran. J. Allergy Asthma Immunol. 20 (1), 11–23.

Muthuramalingam, K., Cho, M., Kim, Y., 2020. Role of NAPDH oxidase and its therapeutic intervention in TGF-β-mediated EMT progression: an in vitro analysis on HeLa cervical cancer cells. Appl. Biol. Chem. 63 (1), 1–8.

Pai, H.-C., Chang, L.-H., Peng, C.-Y., Chang, Y.-L., Chen, C.-C., Shen, C.-C., et al., 2013. Moscatilin inhibits migration and metastasis of human breast cancer MDA-MB-231 cells through inhibition of Akt and Twist signaling pathway. J. Mol. Med. 91 (3), 347–356.

Panji, M., Behmard, V., Zare, Z., Malekpour, M., Nejadbiglari, H., Yavari, S., et al., 2021. Synergistic effects of green tea extract and paclitaxel in the induction of mitochondrial apoptosis in ovarian cancer cell lines. Gene 787, 145638.

Qureshi, R., Arora, H., Rizvi, M., 2015. EMT in cervical cancer: its role in tumour progression and response to therapy. Cancer Lett. 356 (2), 321–331.

Rady, I., Mohamed, H., Rady, M., Siddiqui, I.A., Mukhtar, H., 2018. Cancer preventive and therapeutic effects of EGCG, the major polyphenol in green tea. Egypt. J. Basic Appl. Sci. 5 (1), 1–23.

Sakamoto, T., Kobayashi, S., Yamada, D., Nagano, H., Tomokuni, A., Tomimaru, Y., et al., 2016. A histone deacetylase inhibitor suppresses epithelial-mesenchymal transition and attenuates chemoresistance in biliary tract cancer. PLoS One 11 (1), e0145985.

Schröder, L., Marahrens, P., Koch, J.G., Heidegger, H., Vilsmeier, T., Phan-Brehm, T., et al., 2019. Effects of green tea, matcha tea and their components epigallocatechin gallate and quercetin on MCF-7 and MDA-MB-231 breast carcinoma cells. Oncol. Rep. 41 (1), 387–396.

Sen, S., Chakraborty, R., 2017. Revival, modernization and integration of Indian traditional herbal medicine in clinical practice: Importance, challenges and future. J. Traditional Complementary Med. 7 (2), 234–244.

Shafaei, Z., Abazari, O., Divsalar, A., Ghalandari, B., Poursoleiman, A., Saboury, A.A., et al., 2017. Effect of a Synthesized Amyl-Glycine 1, 10-phenanthroline platinum nitrate on structure and stability of human blood carrier protein, Albumin: Spectroscopic and Modeling Approaches. J. Fluorescence. 27 (5), 1829–1838.

Sharifat, N., Jafari-Hafshejani, F., Dayati, P., Lorestanpoor, P., Paydar, A., BabaahmadiRezaei, H., 2017. Inhibitory effect of Curcumin on phosphorylation NFκB-p65 induced by hydrogen peroxide in Bovine Endothelial Cells. J. Fasa Univ. Med. Sci. 7 (2), 283–290.

Singleton VL, Orthofer R, Lamuela-Raventós RM. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods in enzymology. 299: Elsevier; 1999. p. 152-78.

Sun, S., Xie, F., Zhang, Q., Cui, Z., Cheng, X., Zhong, F., et al., 2017. Advanced oxidation protein products induce hepatocyte epithelial–mesenchymal transition via a ROS-dependent TGF-β/Smad signaling pathway. Cell Biol. Int. 41 (8), 842–853.

Wang, J., Man, G.C.W., Chan, T.H., Kwong, J., Wang, C.C., 2018. A prodrug of green tea polyphenol (–)-epigallocatechin-3-gallate (Pro-EGCG) serves as a novel angiogenesis inhibitor in endometrial cancer. Cancer Lett. 412, 10–20.

Xu, F., Zhang, J., Hu, G., Liu, L., Liang, W., 2017. Hypoxia and TGF-β1 induced PLOD2 expression improve the migration and invasion of cervical cancer cells by promoting epithelial-to-mesenchymal transition (EMT) and focal adhesion formation. Cancer Cell Int. 17 (1), 54.

Kang Ya, Ling J, Suzuki R, Roife D, Chopin-Laly X, Truty MJ, et al. SMAD4 regulates cell motility through transcription of N-cadherin in human pancreatic ductal epithelium. PloS one. 2014;9(9):e107948.

Zhang, J., Tian, X.-J., Xing, J., 2016. Signal transduction pathways of EMT induced by TGF-β, SHH, and WNT and their crosstalks. J. Clin. Med. 5 (4), 41.

Zhao, M., Mishra, L., Deng, C.-X., 2018. The role of TGF-β/SMAD4 signaling in cancer. Int. J. Biol. Sci. 14 (2), 111.