Demethylzelasteral Contributes to Epithelial-Mesenchymal Transition in esophageal squamous cell carcinoma through the Wnt/β-catenin Signaling Pathway

Jiarui Yu¹², Wei Wang², Baolin Liu¹, Jinling Gu², Meiyue Liu¹, Siyuan Chen², Guogui Sun¹² *

1. School of Public Health, Affiliated Hospital, North China University of Science and Technology, Tangshan, Hebei 063000, China
2. Department of Radiation Oncology, North China University of Science and Technology, Affiliated People's Hospital, Tangshan, Hebei 063000, China

Jiarui Yu and Wei Wang contributed equally to this article.

Abstract
Esophageal squamous cell carcinoma (ESCC) is a common malignant tumor in China. Nowadays, no quite effective treatment is available. Therefore, seeking a new treatment is urgent. Demethylzeylasteral(T-96) isolated from Tripterygium wilfordii root bark embraces outstanding good antitumor activity. However, as for the mechanism of Demethylzeylasteral's work on ESCC cells, it is rarely reported. In this study, it is found out that Demethylzeylasteral can inhibit the proliferation, migration and clonogenesis of ESCC cells in a dose and time dependent manner. And Demethylzeylasteral can result in the stop of G2 / M phase and induce apoptosis of ESCC cells. Besides, when observing cells processed by Demethylzeylasteral, the expression of Cyclin B1, Cyclin D1, Bcl-2, PARP1 and Survivin decreased, while the expression of BAX, Cleaved PARP1 increased. In addition, the expression of E-cadherin increased obviously, while that of N-cadherin, Vimentin and MMP9 decreased after Demethylzeylasteral treatment. Moreover, the expression of Wnt / β-Catenin pathway related proteins β-Catenin, c-Myc and Wnt3a decreased. Based on our research, it is demonstrated that Demethylzeylasteral inhibits the proliferation and migration of esophageal cancer cells through the Wnt / β-catenin pathway and induces its cell cycle arrest and apoptosis. And the result indicates that Demethylzeylasteral is likely to be applied when treating ESCC patients, which lays the experimental foundation for clinical research.

Keywords: Demethylzeylasteral; ESCC; Proliferation; Cell cycle; Apoptosis; EMT; Wnt/β-catenin pathway

Introduction
Esophageal squamous cell carcinoma (ESCC) is one of the most common cancers with a lower five-year survival rate, ranking eighth worldwide and third in China[1].Surgical resection is only applicable to patients with early esophageal cancer. Unfortunately, esophageal lesions are usually neglected, causing a delay in the treatment of esophageal cancer[2, 3]. Advanced esophageal cancer is prone to metastasis, and difficult to be cured by surgery. Chemotherapy plays an important role in cancer treatment, but the
present chemotherapeutics failed to achieve satisfactory results[4]. Given that esophageal cancer cells exhibit drug resistance, developing effective chemotherapy drugs is urgently needed and will be beneficial for the cancer treatment. Demethylzeylasteral (T-96), an active ingredient was isolated from the root and bark of Tripterygium wilfordii in the 1990s. This compound has been shown to have anti-inflammatory and anti-tumor effects[5]. T-96 displays significant anticancer activities in various human malignancies, including glioma, melanoma, breast, and pancreatic cancer[6-9]. Multiple studies have shown that T-96 can attenuate the cancer cell growth, affect the tumor cell cycle, promote apoptosis, and inhibit metastasis. However, it remains to be investigated whether T-96 has antitumor effects in ESCC. Here, we aimed to examine the effects of T-96 on ESCC cell proliferation and migration, as well as explore the underlying mechanisms. This study could contribute to a better understanding of the biological relevance and therapeutic potential of T-96 in ESCC.

It is known that the Wnt/β-catenin signaling is critically involved in cell growth, metastasis, and apoptosis, and has been closely related to epithelial-mesenchymal transition (EMT)[10, 11]. As a transmembrane protein, N-cadherin (N-cad) functions in cell-to-cell adhesion, while E-cadherin (E-cad) is required for forming intercellular connections. Loss of E-cadherin may lead to enhanced tumor infiltration and metastasis[12, 13]. β-catenin is a key effector of Wnt signaling pathway, and inactivation of the Wnt signaling may lead to the development of cancer[14]. In this study, we showed that T-96 can effectively inhibit cell proliferation, migration and EMT, and enhance apoptosis through regulating Wnt/β-catenin pathway. This study revealed an anti-tumor potential of T-96 in ESCC.

Materials and Methods

Reagents, antibodies and kits
Reagents, antibodies and kits in the study were purchased as follows: T-96 from Selleck (Selleckchem, Houston, USA), antibodies against N-cadherin, E-cadherin, Vimentin, MMP9, Bcl-2, GAPDH, Wnt3a and β-actin from Proteintech (Wuhan, China), antibodies against Survivin, BAX, cyclinD1, β-catenin and c-Myc from Cell Signaling (Danvers, MA, USA), CCK8 kit from Beyotime Biotechnology (Shanghai, China). The kit for cell cycle detection and FITC-conjugated Annexin V kit for apoptosis detection from NEOBIOISCINECE (Shenzhen, China).

Cell culture and treatments
KYSE150 and KYSE410 cell lines were obtained from the laboratory of Prof. Yutaka Shimada at Kyoto University in Japan. The ESCC cells were grown in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin in a 37°C incubator with humidified 5% CO₂.

Cell viability assay
Cell Counting Kit-8 (CCK8) assay was carried out for assessing the cell survival rate. The ESCC cells at logarithmic growth phase were collected and uniformly seeded into three 96-well plates (3×10³ cells/well). After 24 hours culture, the cells were incubated with 0, 5, 10 or 20 μM T-96 (6 sub-holes per concentration) for 24, 48, and 72 hours, respectively. Subsequently, 10 μL of CCK8 was applied to each well with 90 μL of culture medium, and the cells were grown for 2 hours in the dark. Finally, a microplate
reader was used to determine the absorbance at a wavelength of 450 nm, and the cell inhibition rate was measured according to the following formula: the inhibition rate (%) = \((1 - \text{absorbance value in the treated group} / \text{that in the control one}) \times 100\%\). Each experiment was conducted in triplicate, and the average value was taken as the experimental results.

**Colony formation assay**
For this assay, logarithmically growing ESCC cells were uniformly inoculated into three 6-well plates. After 24 hours incubation, 0, 5 or 10 μM T-96 were applied to the plates, respectively, and the cells were continuously cultured for 12 hours. Then, the cells were harvested by centrifugation, and 2000 cells in the normal medium were subcultured in a six-well plate. After 14 days, the surviving colonies were fixed in 100% methanol, followed by staining using 1% crystal violet for 10 minutes. Finally, the plate was washed with running tap water three times and air dried at RT. For each group, the experiments were performed in triplicate.

**Transwell assay for cell migration**
Logarithmic growth of the ESCC cells was carried out in a 6-well plate. After 24 hours culture, 0, 5 or 10 μM T-96 was applied to the growing cells, respectively. The cells continued to grow for 12 hours and were then subjected to digestion and counting. 100,000 viable cells were resuspended in serum-free medium and uniformly seeded into the upper chamber of a Transwell plate. Subsequently, 700 μL of culture medium with 30% FBS was administered to each lower chamber. After incubation for 24 hours, the remaining cells were immersed with pre-cold methanol for 10 min, followed by staining with 0.5% crystal violet for 10 minutes. The migrated cells were photographed and quantified by using a microscope. The experiment was performed thrice.

**Cell cycle analysis**
The ESCC cells were treated with different concentrations of T-96 (0, 5 or 10 μM). After T-96 treatment for 48 hours, cells were harvested, washed with PBS, and fixed with ice 70% ethanol overnight at 4°C. Then, the fixed cells were washed with PBS again and treated with RNase A at 37°C for 20 minutes and stained with PI in the dark for 30 minutes. The DNA content was detected using a flow cytometer (BD Biosciences, NJ, USA). For each group, the experiments were performed in triplicate.

**Cell apoptosis analysis**
Flow cytometry was carried out for detecting apoptosis using the Annexin V-FITC kit (NEOBIOSCINECE, Shenzhen, China). Briefly, the ESCC cells were incubated with 0, 5 or 10 μM T-96 for 48 hours and then subjected to a Leica DMI4000B microscope (Leica Microsystems GmbH, Wetzlar, Germany) for a morphological examination. The attached and floating cells were treated with trypsin, followed by incubation with 5 μL of FITC-conjugated annexin V (0.5 mg / mL) for 15 minutes, and then 5 μL of PI (0.5 mg/mL) for another 15 minutes at RT in the dark. A BDTM LSR-II flow cytometer (BD Biosciences, NJ, USA) was employed to detect apoptotic cells that were identified as
annexin V-positive cells in this experiment. For each group, the experiments were performed in triplicate.

**RNA extraction and qRT-PCR**
Total RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions [15]. cDNA was synthesized with PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Kusatsu, Japan). Real-time PCR was performed with CFX Connect Real-time PCR system (BioRad) using SYBR Premix Ex Taq kit (TaKaRa). The above listed primers are listed in Table 1.

Table 1 Primer Sequences Used in This Article

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward(5’-3’)</th>
<th>Reverse(5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP9</td>
<td>ATCCCCCAACCTTTACCA</td>
<td>TCAGAACCGACCTACAA</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>CGAGAGCTACAGTTCACGG</td>
<td>GGGTGTCGAGGGAAAAATAGG</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>TTTGATGGAGGTCTCCTAACA</td>
<td>ACGTTTAAACAGTTGGAAATGTG</td>
</tr>
<tr>
<td>Vimentin</td>
<td>GACAATGCGTCTCTGGCACGTCTT</td>
<td>TCCCTCGCCCTCCTGCAGGTCTTT</td>
</tr>
<tr>
<td>β-actin</td>
<td>CTCCATCCTGGCCTCGCTGT</td>
<td>GCTGTCACCTTCAACCTGTTCC</td>
</tr>
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**Western blotting**
The ESCC cells were incubated with various concentrations of T-96 (0, 5 or 10 μM) for 24 hours, respectively. After washing with PBS twice, the cells were harvested with trypsin digestion and centrifugation, and then subjected to a total protein extraction. Protein concentrations were determined by using Plyle BCA protein quantitation kit. The equal amount of protein samples was separated by SDS-PAGE gel electrophoresis and transferred onto a PVDF membrane (Millipore, USA). The membrane was blocked in 5% skim milk or 2% BSA and then incubated with primary antibodies, followed by the corresponding secondary antibodies (Abbkine, Wuhan, China). The target proteins in the membrane were detected and visualized by using the Chemiluminescence Luminol Kit. The following primary antibodies were used in the experiments: E-cadherin, N-cadherin, Vimentin, MMP9, Bcl-2, β-actin, Survivin, BAX, cyclinD1, Cyclin B1, β-catenin, Wnt3a and c-Myc. The experiment was performed thrice.

**Statistical analysis**
GraphPad Prism 8.0 was used to test the statistical differences between the two groups by Student's t test. All experiments in the bar chart were carried out at least three times, and all samples were analyzed in triplicate. (SD). In all cases, differences were considered significant at \( p < 0.05 \).

**Results**
**An inhibitory effect of T-96 on ESCC cell proliferation**
The chemical structure and 3D structure diagram of Demethylzeylasteral(T-96) are shown in Figure 1A-C. CCK8 assay was performed to study the effects of T-96 on the viability of esophageal cancer cells. For this purpose, KYSE150 and KYSE410 cells were incubated with various concentrations of T-96 (0, 5, 10 or 20 μmol/L) for 24 hours, 48 hours and 72 hours, respectively. The chemical structural formula of T-96 was shown in Figure 1D. The cell viability test revealed that treatments with T-96 led to a
significant dose- and time-dependent inhibition in the ESCC cell proliferation (Figure 1E).

**T-96 inhibits the cancer cell migration and colony formation in ESCC**
To study the effect of T-96 on the biological function of KYSE150 and KYSE410 cell migration, Transwell analysis was performed. The results showed that when KYSE150 and KYSE410 cells were treated with different concentrations of T-96 (0, 5 or 10 μmol / L) for 24 hours, the number of migrating cells was significantly less than that of the control group (Figure 2A), indicating that T-96 was dose dependent Mode inhibits the migration capacity of ESCC cells. To study the effect of T-96 on colony forming ability As the concentration of T-96 increased, the colony forming ability decreased significantly (Figure 2B).

**T-96 induces Cell Cycle Arrest in ESCC Cells**
To study the effect of T-96 on ESCC cells’ cell cycle, flow cytometry analysis was carried out after treating KYSE150 and KYSE410 cells with (0,5 or 10 μmol / L) T-96 for 48 hours. Compared with the control group, the cells exposed to Demethylzeylasteril at 5 and 10 μmol / L concentration will result in the aggregation of a large amount of G2 / M, and the population of G0 / G1 reduced accordingly(Figure 3A). In order to study whether the potential mechanism of G2 / M phase arrest is related to T-96 treatment, the effect of T-96 on the expression of CyclinB1 was tested, which is a key regulator of cell cycle. As expected, the expression of CyclinB1 protein and mRNA in KYSE150 and KYSE410 cells treated with T-96 decreased(Figure 3B-C).These findings indicated that T-96 arrested the progression of the cell cycle thereby hindering the proliferation of ESCC cells.

**T-96 induces apoptosis in ESCC Cells**
To assess whether T-96 induces apoptosis in ESCC, the ESCC cells administered with T-96were stained using Annexin V/PI and then subjected to a flow cytometry analysis. As depicted in Figure 4A and 4B, increased concentrations of T-96 led to a significant increase in the proportion of apoptotic cells in the cancer cell lines compared with the control group. The apoptotic rates of KYSE150 and KYSE410 cells were 1.47 ± 0.25% and 1.6 ± 0.1% respectively in untreated groups, while they were 8 ± 0.62% and 7 ± 0.26% in cells treated with 5μM T-96, 9 ± 0.2% and 9.87 ± 0.21% in cells treated with 10μM T-96(Figure 4A-B). Meanwhile, we observed that treatments with T-96 for 24 hours caused a significant change in the morphology of the cancer cells reminiscent of apoptosis. Together, these findings demonstrated that T-96 can elicit apoptosis in ESCC.

**Altered expression of apoptotic proteins in ESCC cells treated with T-96**
We further characterized T-96-induced apoptosis in the ESCC cells by analyzing the expression of apoptotic proteins. As indicated by western blot analysis, treatments with T-96 resulted in increased levels of Cleaved-PARP1 and BAX proteins, but decreased expression of Bcl2, Survivin and PARP1 in the cancer cells in comparison with the control group (Figure 5). Furthermore, an increased ratio of BAX/Bcl-2 was observed
in the experimental group. All these data provided more evidence that T-96 can induce apoptosis in esophageal cancer cells.

**T-96 inhibits EMT by regulating Wnt/β-catenin signaling pathway**
It is known that EMT is essential for tumor cells to migrate, and Wnt/β-catenin pathway is involved in the regulation of this process. Given the inhibitory effect of T-96 on ESCC cell migration, we set out to investigate whether EMT was altered in demethylzeylasteral-treated ESCC cells by examining the expression of EMT markers. As shown in Figure 6A-B, increased concentrations of T-96 led to an up-regulation of E-cadherin as well as a significant reduction in the expression of Vimentin, N-cadherin and MMP-9 in the cancer cells treated with T-96 for 24 hours. Moreover, western blot analysis revealed a reduced level of β-catenin protein as well as a significant dose-dependent decrease in the expression of Wnt/β-catenin signaling downstream target genes cyclin D1,c-Myc and Wnt3a (Figure 6C-D, Supplementary Figure1). Collectively, these data indicated that T-96 inhibits EMT by regulating Wnt/β-catenin pathway.

**Discussion**
Esophageal cancer ranks fourth in incidence in China, and more than 90% of esophageal cancers are squamous cell carcinoma[16]. Patients with ESCC have a poor prognosis and do not respond to most conventional chemotherapy drugs[17]. Increasing evidence has shown that natural small molecule drugs have the potential to become new compounds for cancer treatment[18, 19]. T-96 is an active ingredient extracted from the bark and roots of Tripterygium wilfordii that exhibits various pharmacological effects, including metabolic modulation of hormones, immunosuppressive and antitumor effects[20-23]. In this study, we found that treatments with T-96 attenuated the ESCC cell proliferation and migration and induced cell cycle arrest and apoptosis in ESCC. Furthermore, T-96 inhibits EMT in the cancer cells by regulating Wnt/β-catenin pathway.

This study showed that T-96 extracted from traditional Chinese medicine Tripterygium wilfordii with recognized anti-tumor activity effectively attenuated ESCC's proliferative capacity in vitro, and increased concentrations of the compound and prolonged time for treatment led to an inhibition in the cancer cell proliferation. T-96 can not only suppress the cancer cell growth but also the colony forming ability of ESCC. Notably, the colony forming ability of esophageal cancer cells was inversely proportional to the concentration of T-96 used in the experiments. Meanwhile, we observed that T-96 displays an inhibitory effect on the cancer cell migration. It is known that as an important biological feature of many cancers, metastasis is associated with cancer progression[24]. Cancer metastasis, rather than the primary tumors, accounts for 90% of cancer mortality[25]. T-96 has been shown to exert an inhibitory effect on proliferation and migration of breast, pancreatic, and glioma cells[6-8].

Generally, tumor cells are characterized by abnormal cell cycle regulation, which should be attributed to the structure and function that change cyclin and cyclin
dependent kinases[26]. According to relevant studies, lots of small molecular products extracted from natural drugs inhibit the proliferation of cancer cells by inducing cell cycle arrest and apoptosis[27]. Similarly, in lots of cancers, such as pancreatic cancer, it is proved that Demethylzeylasteral is able to induce cell cycle arrest and apoptosis[7]. It lives up to our expectation that 48 hours after Demethylzeylasteral treatment, significant G2 / M phase arrest happened in KYSE150 and KYSE410 cells. It is known to all that the cell transformation from G2 to M phase is dominated by CyclinB1. G2 / M serves as a DNA damage checkpoint that prevents cells with genomic DNA damage from entering M phase[28]. As expected, the expression of Cyclin B1 protein and mRNA in KYSE150 and KYSE410 cells treated with Demethylzeylasteral decreased. We finding indicated that Demethylzeylasteral arrested the progression of the cell cycle thereby hindering the proliferation of ESCC cells. Numerous studies have found that apoptosis is a promising target for anti-cancer therapy and has been used in screening and evaluating chemotherapy drugs[29, 30]. Further research findings that apoptosis is a highly regulated and genetically controlled process in which dead cells are cleared without causing inflammation and damage to the surrounding cells. Here, we found that T-96 treatment significantly increased the percentage of apoptotic cells in the ESCC cell lines, while down-regulated Bcl2, an anti-apoptotic factor. This finding was consistent with a recent report that T-96 can attenuate the pancreatic cancer cell proliferation and accelerate the cancer cell apoptosis.

Despite the observations that T-96 negatively regulates tumor cell proliferation and migration in most cancers, it remains to be investigated whether it affects EMT, thereby inhibiting tumor cell migration. Metastasis is the most important biological behavior of malignant cells, and EMT is a key process involved in the cancer invasion and metastasis[31, 32]. It has been reported that many signaling pathways, including Wnt/β-catenin pathway, are implicated into EMT regulation. EMT is a complex multi-step process in which epithelial markers such as E-cadherin are down-regulated, while mesenchymal markers, including N-cadherin and vimentin are up-regulated. It has been demonstrated that Wnt/β-catenin signaling is activated during EMT, and inactivation of Wnt/β-catenin pathway inhibits and reverses EMT[33, 34]. In the present study, we analyzed the mRNA and protein levels of E-cadherin, N-cadherin, Vimentin, and MMP9 in the ESCC cells incubated with Demethylzeylasteral. As expected, T-96 inhibited EMT in the cancer cells. The Wnt/β-catenin signaling is involved in various biological processes as well as the regulation of EMT. Blocking Wnt/β-catenin signaling activities can inhibit EMT by inducing epithelial differentiation. Therefore, developing new drugs and effective approaches for inhibiting Wnt/β-catenin pathway and EMT is crucial for controlling cancer metastasis.

Wnt signaling pathway is important for the growth control of epithelial cells, and has been used as a target for developing anti-cancer drugs[35]. Increasing evidence suggests that Wnt/β-catenin signaling plays a key role in regulating stem cell-like characteristics and EMT in metastasis. Dysregulation of Wnt/β-catenin pathway is associated with various types of cancer, including esophageal cancer, suggesting the
importance of the canonical Wnt pathway in tumor development[36, 37]. T-96 can inhibit the growth of clones and function in the regulation of cell proliferation, metastasis and metastasis pathways through regulating the expression of Wnt/β-catenin signaling responsive genes. β-catenin acts as a key indicator for cell fatalization and EMT in metastatic cancers, and was found to be related to poor prognosis, drug resistance and tumor recurrence in esophageal cancer patients[38-40]. T-96 inhibits the expression of Wnt signaling downstream target genes such as cyclinD1 and c-Myc in the ESCC cells, showing an inhibitory effect on Wnt/β-catenin signaling activities.

Here, we provided the first demonstration that T-96 has a significant inhibitory effect on ESCC proliferation, and treatment with this compound reversed the EMT of malignant ESCC. However, while the targets of T-96 have not yet been identified, the in vivo role of this compound needs to be further investigated. T-96 displays far fewer side effects than Tripterygium wilfordii and has little effect on the weight of nude mice. In addition, T-96 exhibits the same immunosuppressive and antitumor activities as other Tripterygium wilfordii monomers. While T-96 is less toxic than other monomers in Tripterygium wilfordii, this compound is 1000 times less cytotoxic than triptolide. Thus, the relative safety of T-96 is higher. Given that in comparison with triptolide, T-96 has different targets, further studies should be focused on the mechanism of action for identifying their targets. Besides, T-96 has low oral bioavailability with 4.2% of the absolute bioavailability[22]. Therefore, development of new formulations or semi-synthetic derivatives for improving the bioavailability of T-96 will be beneficial for its possibly clinical application.

In summary, we showed that T-96 inhibits the ESCC cell proliferation and migration, and induces the cancer cell cycle arrest and apoptosis through regulating Wnt/β-catenin signaling, suggesting a clinical potential for ESCC treatment.

Acknowledgements
This work was supported by the Young Top-Notch talent Project of Hebei province [No.JI2016(10),http://www.hebgcdy.com/], Talent Project of Hebei province (A201801005,http://rst.hebei.gov.cn/index.html), Academician Workstation Construction Special Project Of Tangshan People's Hospital (199A77119H,https://kjt.hebei.gov.cn/www/index_ssl/index.html), Natural Science Foundation of Outstanding Youth of Hebei Province (H2019105026,https://kjt.hebei.gov.cn/www/index_ssl/index.html), and Basic Research Cooperation Project of Beijing-Tianjin-Hebei [H2019105143,19JCZDJC64500(Z),https://kjt.hebei.gov.cn/www/index_ssl/index.html]. No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this study.

Conflicts of interest
The authors declare that they have no conflicts of interest.
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19. Feng YL, Chen DQ, Vaziri ND, Guo Y, Zhao YY. Small molecule inhibitors of epithelial-mesenchymal transition for the treatment of cancer and fibrosis. Medicinal research reviews. 2020; 40: 54-78.


**Figure Legend**

Figure 1. Demethylzeylasteral(T-96) inhibits the proliferation of ESCC cells. A. Leaves and roots of Chinese herbal medicine Tripterygium wilfordii. B. Chemical structure of Demethylzeylasteral. C. 3D Conformerstructure of Demethylzeylasteral. D-E. After the KYSE150 and KYSE410 cells were treated with 24, 48 and 72 hours with Demethylzeylasteral of different concentration gradients (0, 5,10 or 20 µM), CCK8 assay was used to detect the viability of the ESCC cells. Data shown are means ± SEM from 3 independent experiments in duplicate. *P < 0.05, **P < 0.01, * P < 0.001 as compared to the control group.
Figure 2. Demethylzeylasteral (T-96) inhibits the migration and colony formation of ESCC cells. The effects of different concentrations of Demethylzeylasteral on ESCC migration were determined by Transwell assay. A. Demethylzeylasteral treatment reduced the migration capacity of KYSE150 and KYSE410 cells in a dose-dependent manner. B. Bar chart of number of migrating cells. C. ESCC cells clone formation ability. Demethylzeylasteral reduced colony formation in KYSE150 and KYSE410 cells in a dose-dependent manner. D. Bar chart of number of colony formation cells. Data shown are means ± SEM from 3 independent experiments in duplicate. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as compared with the control group.
Figure 3. Demethylzeylasteral(T-96) causes cell cycle arrest at G2/M phase in ESCC cells. A. Representative results of cell cycle distribution. After a 48 hours exposure to osthole at concentrations of 0, 5 and 10μM, the cell cycle distributions of KYSE150 and KYSE410 cells were measured by flow cytometry with PI staining. B. The CyclinB1 was detected by Western blot. C. The protein levels of cyclinB1 was measured by western blotting. β-actin was served as loading control. *P < 0.05, **P < 0.01 as compared to the control group.
Figure 4. Demethylzeylasteral(T-96) promotes apoptosis in ESCC cells. A. Apoptosis was assessed based on Annexin V-FITC/PI dual staining and flow cytometry. B. Representative results are shown and the percentage of apoptotic cells is plotted.* $P < 0.05$, ** $P < 0.001$ as compared to the control group.
Figure 5. The effects of Demethylzeylasteral(T-96) on apoptosis related proteins. A-B. Western blot was used to detect the expression of PARP1, cleaved PARP1 (C-PARP1), Bcl-2, Bax and Survivin. Bar graphs represent quantitative differences in the expression of integrin PARP1, cleaved PARP1 (C-PARP1), Bcl-2, Bax and Survivin. Data represent the means ± SD of four independent experiments. β-actin was used as a load control. β-actin was served as loading control.* $P <0.05$ ,** $P <0.01$ and *** $P <0.001$ as compared with the control group.
Figure 6. Demethylzeylasteral(T-96) inhibits EMT by regulating Wnt/β-catenin signaling pathway. A-B. Western blot was used to detect the expression of E-cadherin, N-cadherin, Vimentin and MMP-9. Bar graphs represent quantitative differences in the expression of integrin E-cadherin, N-cadherin, Vimentin and MMP-9. Data represent the means ± SD of four independent experiments.C-D. Western blot was used to detect the expression of β-catenin, cyclinD1 and c-Myc. Bar graphs represent quantitative differences in the expression of integrin β-catenin, cyclinD1 and c-Myc. β-actin was used as a load control. Data represent the means ± SD of four independent experiments.* $P<0.05$, ** $P<0.01$ and *** $P<0.001$ as compared with the control group.

Supplementary Figure 1. The Wnt3a was detected by Western blot. A. The protein levels of Wnt3a was measured by western blotting. Bar graphs represent quantitative differences in the expression of integrin Wnt3a was used as a load control. GAPDH was served as loading control. **$P<0.01$ and ***$P<0.001$ as compared to the control group.
Supplementary Figure 2. The mRNA levels of E-cadherin, N-cadherin, Vimentin and MMP9 were detected by qRT-PCR. A-D. Relative mRNA expression of E-cadherin, N-cadherin, Vimentin and MMP9 in KYSE150 cells was analysed by quantitative real-time PCR and normalized to β-actin mRNA expression. The data represent three independent experiments. ns, not significant, *P < 0.05 and **P < 0.01 as compared to the control group.