*Research Article*

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# Effects of *Celastrus Orbiculatus* Extracts On the Invasion and Metastasis Via Targeting Integrin B4 in Gastric Cancer Cells

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## ABSTRACT

**Background:** Gastric cancer is a malignant tumor that originates from the gastric mucosal epithelium and is formed by continuous cloning and proliferation of gastric epithelial cells and progenitor cells. *Celastrus orbiculatus* extract (COE) has been shown to inhibit the activity of many types of tumors.

**Objective:** The study aims to investigate the effects and the mechanisms of COE inhibiting the invasion and migration by targeting integrin β4 (ITGB4) in AGS and MKN45 cells.

**Methods:** COE was diluted to different concentrations (100, 200 and 400 μg/mL), and the cell proliferation was detected using the MTT assay. The effects of COE on the invasion and migration in the gastric cancer cells were evaluated by Transwell and wound healing assays. The invasion and migration of cells were further observed with a living cell motion tracking system. The expression of matrix metalloproteinases (MMPs) and epithelial-mesenchymal transformation (EMT)-related proteins was detected by Western Blotting after COE treament for 24 h in the gastric cancer cells.

**Results:** COE obviously inhibited the proliferation of AGS and MKN45 cells. After COE treatment, the expression levels of MMP-2 and MMP-9 protein decreased, while the expression level of TIMP-1 increased. The expression level of N-cadherin and Vimentin decreased, while the expression level of E-cadherin increased. The protein expression levels of ITGB4, Akt, PI3K and Palladin were decreased.

**Conclusion:** COE inhibited the invasion and migration of AGS and MKN45 cells. which may be associated with the inhibition of ITGB4/PI3K/Akt/Palladin signaling pathway.

**Keywords**: *Celastrus orbiculatus* extract; invasion; migration; human gastric cancer; ITGB4

## INTRODUCTION

Gastric cancer is one of the most common malignancies, which caused by a variety of factors, and represents the third leading cause of cancer death. In China, the incidence of gastric cancer shows a significant upward trend[1]. The prognosis of advanced gastric cancer is poor with a median survival of less than 12 months. Early diagnosis and prompt treatment are very important[2, 3]. In recent years, the early cancer screening has effectively inhibited cancer progression in China[4-6]. Although surgery and other treatment methods are widely used, the patient's postoperative quality of life remains unsatisfactory[7]. Tumor invasion and migration refers to the process of tumor cells infiltrating into surrounding tissues from the origin site or leaving the primary lesion, entering the blood circulation and lymphatic circulation and reaching other body parts. Tumor invasion and migration are important indicators of malignant tumor development, and inhibiting these processes can delay tumor progression[8, 9]. Recently, many kinds of traditional Chinese medicines have shown obvious antitumor effects[10].

*Celastrus orbiculatus* is a traditional Chinese medicine belonging to the *Celastraceae* family, and it is widely distributed in China and has many pharmacological effects[11]. This medicine is

used for the treatment of rheumatoid arthritis and shows anti-inflammatory and other effects[12]. Many experiments have shown that COE has a significant effect on tumors. Moreover, it can enhance the sensitivity of gastric cancer to cisplatin through caspase-dependent apoptosis[13] and promote human squamous carcinoma apoptosis through the PI3K/Akt/mTOR signaling pathway[14]. COE also has a significant effect on promoting the apoptosis of AGS and BGC-823 cells[15], and it can also inhibit the epithelial-mesenchymal transformation of gastric cancer through the NF-kB/Snail signaling pathway[16].

Based on the results of previous studies, our study explored the effects of COE on the inhibition of invasion and migration of AGS and MKN45 cells, as well as the related targets and possible mechanisms involved.

## MATERIALS AND METHODS

### Drugs

Guangzhou Zhixin Pharmaceutical Co., Ltd provided *Celastrus orbiculatus* Thunb. The purchased Chinese medicine was identified by Professor Qin Minjian of the Chinese Medicine Resource Laboratory of China Pharmaceutical University. Professor Wang Qiang from China Pharmaceutical University was responsible for the extraction and identification of the compounds.

### Cells and Reagents

Human gastric cancer cell lines AGS and MKN45 were derived from the Cell bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences and cultured in medium containing 10% fetal bovine serum RPMI 1640. The cells were incubated in an incubator at 37 °C with 5% $CO_2$. RPMI 1640 cell culture medium and trypsin were obtained from HyClone (United States). Fetal bovine serum (FBS) was obtained from Gibco (United States). Artificially reconstituted basement membrane and Transwell chambers were obtained from Corning (United States). Thiazole blue (MTT) powder was obtained from Sigma (United States). Trypsin was purchased from Beyotime Shanghai, and MTT powder was purchased from Sigma-Aldrich (United States). ITGB4, AKT, p-AKT, PI3K and p-PI3K antibodies were purchased from Cell Signaling Technology (United States). MMP-2, MMP-9, TIMP-1, E-cadherin, N-cadherin, vimentin, β-actin, HRP-labeled goat anti-rabbit IgG, goat anti-rat IgG, and goat anti-mouse IgG antibodies were purchased from Thermo (United States). Palladin antibodies were purchased from AbCAM (Britain).

### MTT Assay

AGS and MKN45 cells were digested, resuspended and evenly inoculated into 96-well culture plates with 180 µl RPMI 1640 to ensure that the number of cells was approximately 3-4×$10^4$/well. Replace the medium when the cells grow logarithmically. The medium contained COE at concentrations of 100, 200, and 400µg/ml. After the medium was changed, the cells were cultured again for 48 hours. 20µl MTT (5% MTT) was added to each well, which were incubated at 37 °C for 4 h, and then 150 µl DMSO was added to each well and the supernatant was discarded. The fully automatic enzyme plate apparatus vibrated at medium speed for 10 min to fully dissolve the crystals. The absorbance (A) value of each well was determined at 490 nm wavelength. To evaluate the effect of COE on cell growth, the 50% concentration of inhibition (IC50) was calculated.

### Cell Invasion and Migration Assay

Cell migration assays were performed in a 24-well Transwell chamber with an 8.0 µm pore size polycarbonate membrane (Corning). The cell invasion assay was performed using the above procedure with precoating with matrix gel. The medium contained COE at concentrations of 100, 200, and 400 µg/ml was added to the lower chamber. Cell suspension was added to the upper chamber. After incubation for 48 h, the cancer cells in the upper compartment were removed with cotton swabs. The cells on the inferior membrane were fixed and stained with 5% crystal violet solution. Matrix gel precoating was not used in the cell migration tests. Images were obtained under a 100× magnification microscope (Japan). Three images of 10 random fields in each layer were collected, and migratory cells were counted.

### Wound Healing Assay

AGS and MKN45 cells were cultured to 90% confluence in RPMI 1640 medium. Linear scratches were made with micropipette tips, and then the cells were washed three times with PBS to remove detached cells. Cells in the experimental group were cultured in medium containing COE in concentrations of 100, 200 and 400 µg/ml for 24 h. The experiment was repeated three times. The wound healing degree (%) was calculated as [(scratch width of control - experimental group) / scratch width of control group] × 100%, and the cell migration ability was measured.

### Western Blot Assay

AGS cells and MKN45 cells at the logarithmic growth stage were evenly plated in a 6-well dish. Cells were cultured with RPMI 1640 medium containing COE in concentrations of 100, 200 and 400 µg/mL for 48 h. Cells were removed from the incubator and washed with PBS, and total cell proteins were extracted with protein lysates containing protease inhibitors and phosphatase inhibitors. A BCA kit was used to determine the total protein concentration, and then an SDS-PAGE gel was concentrated at 80 V constant voltage for 20 min,

changed to 120 V constant voltage for 90 min for separation, and transferred to a PVDF membrane. The PVDF membrane was placed in 5% skim milk and left at room temperature for 2-3 hours. The corresponding antibody was added and incubated overnight at 4 °C. After rinsing in TBST solution 3 times, the corresponding secondary antibody was added for 2 h.Subsequent to rinsing in TBST 3 times, imaging was performed under the gel imaging system.

### Cell Migration Ratio Analysis

Cells were plated in 96-well plates and cultured for 24 h. When the cells showed logarithmic growth, the cells were cultured with dosing for 12 h. The 96-well plate was placed into a PerkinElmer Operetta CLS high-content imaging analysis system (PerkinElmer, Waltham, USA). After 12 h, data were collected and analyzed with Harmony 4.1 software.

### Data Analysis

Data were analyzed using IBM SPSS 25.0. Scientific graphics were generated using GraphPad Prism 8.0. Values of $p < 0.05$ were considered as statistically significant. All experimental data are expressed as x ± s. Analysis of variance (ANOVA) was performed to compare the different groups among multiple factors.

## RESULTS

### COE inhibited the proliferation of AGS and MKN45 cells.

AGS and MKN45 human gastric cancer cells were treated with different concentrations of COE (100, 200 and 400 μg/ mL) for 48 hours, respectively. The effects of COE on the proliferation of the cells were analyzed by using MTT assay. As shown in Fig. 1, compared with the control group, COE significantly inhibited the proliferation of gastric cancer cells ($p < 0.05$, Figure 1). Subsequent experiments used the same concentration gradient to investigate the inhibitory effect of COE on the invasion and migration of AGS and MKN45 cells.

### COE inhibited the invasion and migration of AGS and MKN45 cells.

Transwell migration and invasion assays were performed to analyze the effects of COE on the migration and invasion of AGS and MKN45 cells. On the membrane of the transwell compartment, the cells were stained blue-purple with crystalline violet. Compared with the control group, the COE treatment reduced the number of transmembrane cells. The results showed that COE could significantly inhibit the invasion and migration of gastric cancer cells ($p < 0.01$, Figure 2).

The effects of COE on the invasion and migration ability of AGS and MKN45 cells were analyzed by using wound healing assay. After COE treatment, the scratch width was larger than that of the control group. Thus, the drug treatment group had a lower scratch healing ability than the control group. The results showed that COE significantly inhibited the migration of AGS and MKN45 cells ($p < 0.05$, Figure 3).

To more intuitively and specifically detect the degree of cell migration, we used high-content imaging technology to track the migration of gastric cancer cells. Harmony 4.1 software was used to analyze the mean squared displacement of cells. The results showed that the degree of cell migration after the COE treatment was lower than that of the control group (Figure 4A and B). The results showed that the migration ability of cells was inhibited after the COE treatment.

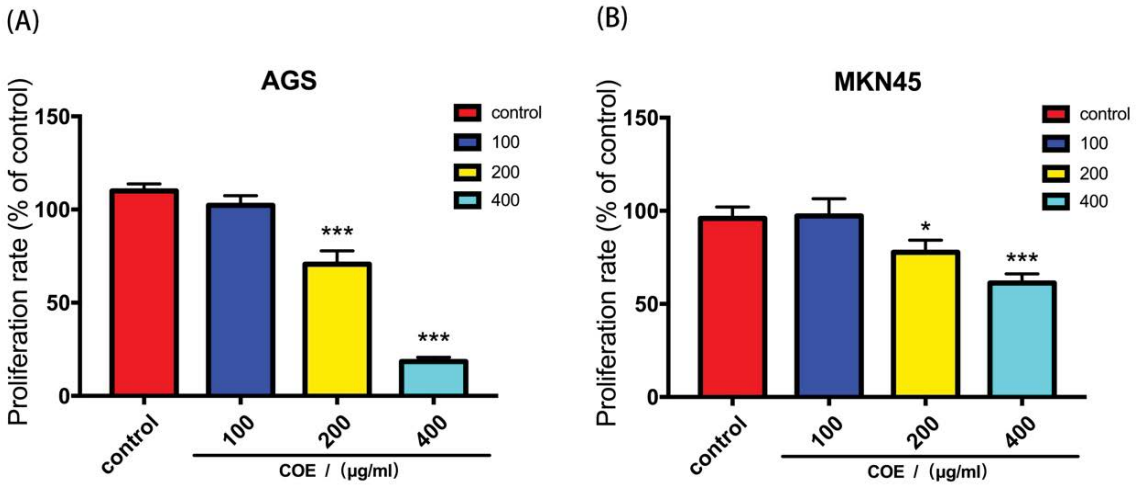


**Figure 1**. Effects of COE on the proliferation of AGS and MKN45 cells. (A) MTT assay was used to detect the growth inhibition of COE (100, 200 and 400 μg/ mL) on AGS cells. (B) MTT assay was used to detect the growth inhibition of COE (100, 200 and 400 μg/ mL) on MKN45 cells.

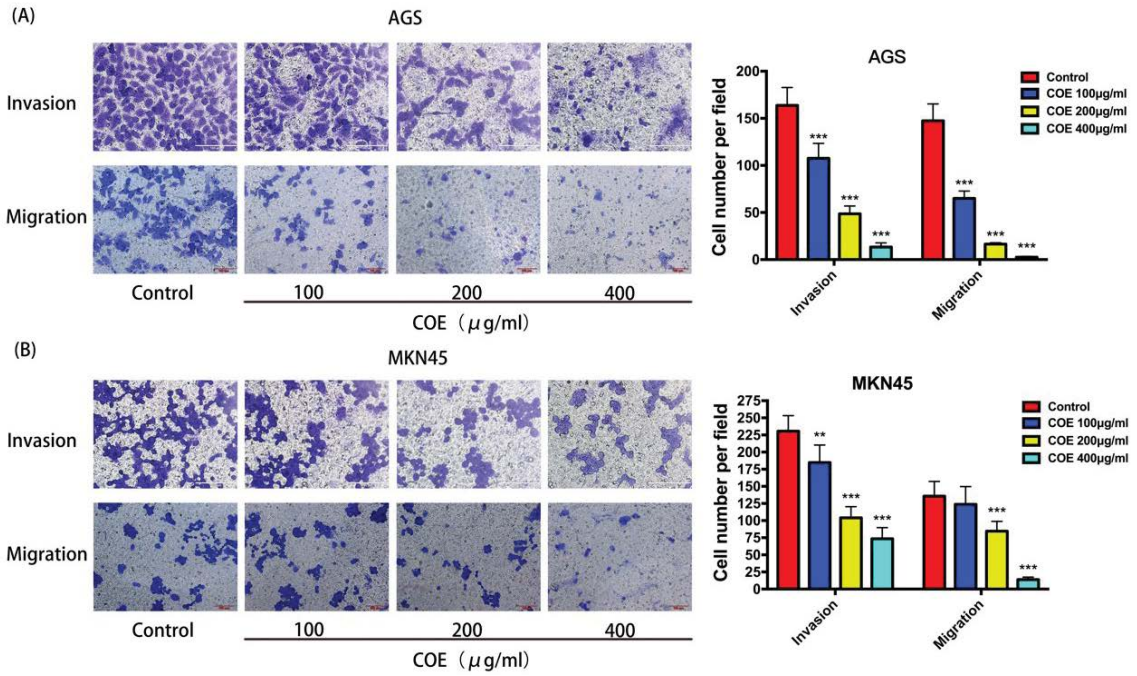


**Figure 2**. Effects of COE on the invasion and migration of AGS and MKN45 cells. (A) Transwell assays performed to investigate the effect of COE (100, 200 and 400 μg/ mL) on the invasion and migration of AGS cells. AGS cells. Image obtained under 100 x magnification microscope (Japan). (B) Transwell assays performed to investigate the effect of COE (100, 200 and 400 μg/ mL) on the invasion and migration of MKN45 cells. Image obtained under 100 x magnification microscope (Japan).

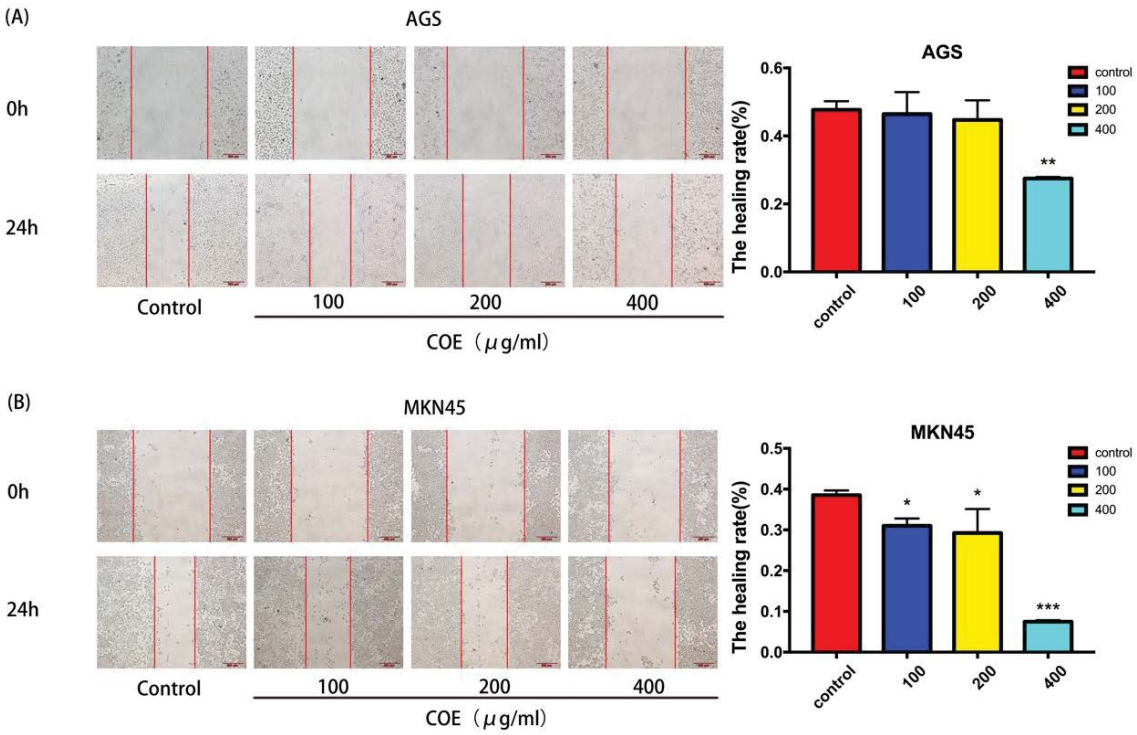


**Figure 3**. The effect of COE on migration of AGS and MKN45 cells was examined by wound healing assay. (A) After 24 hours of COE (100, 200 and 400 μg/ mL) treatment, AGS cells showed reduced scratch healing ability. Image obtained under 200 × magnification microscope (Japan). (B) After 24 hours of COE (100, 200 and 400 μg/ mL) treatment, MKN45 cells showed reduced scratch healing ability. Image obtained under 200 × magnification microscope (Japan).

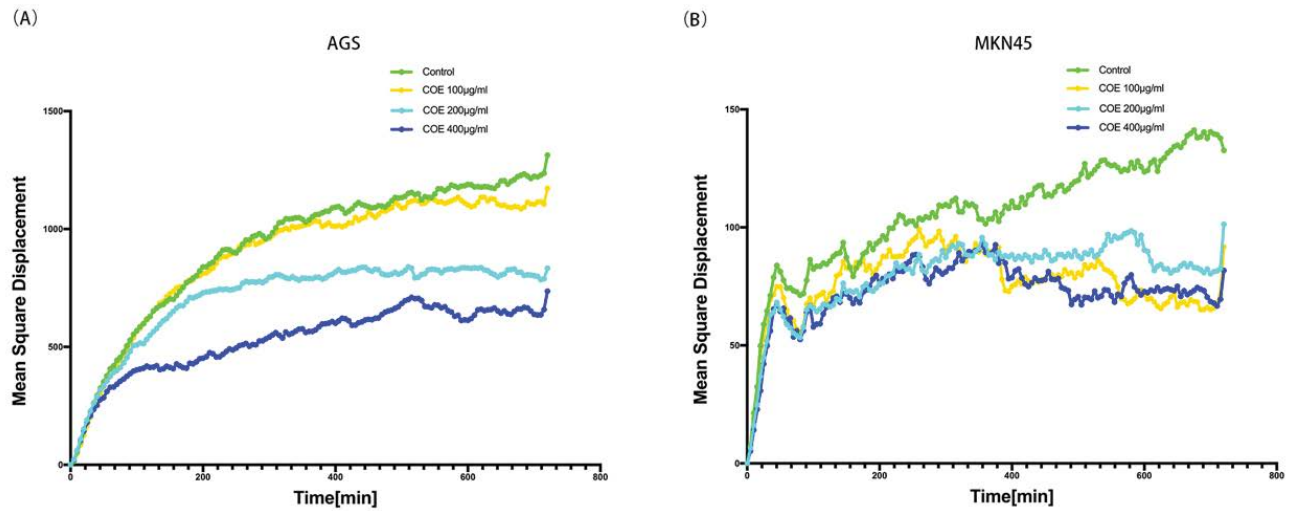


**Figure 4.** Cell mobility was detected by PerkinElmer Operetta CLS high-content Imaging System. The cells were treated with different concentrations of COE (100, 200 and 400 μg/ mL) for 24h. (A, B) Plot of mean squared displacement vs. the observation time from well-level data.

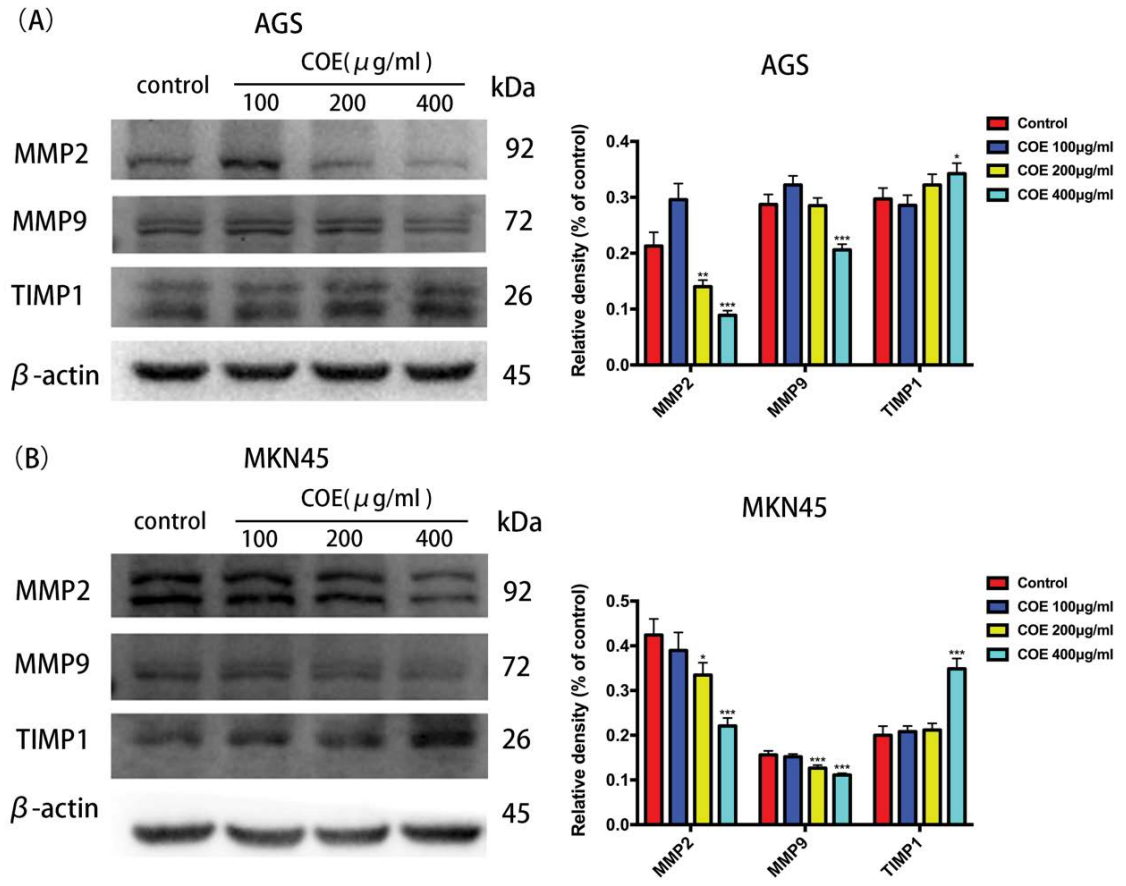


**Figure 5.** Western Blot was used to detect the effects of COE on the expression of MMP-2, MMP-9 and TIMP-1 in AGS and MKN45 cells. (A) Changes in expression of matrix metalloproteinase (MMP) related proteins MMP-2, MMP9 and TIMP-1 in AGS cells after 24 h of COE (100, 200 and 400 μg/ mL) treatment. (B) Changes in expression of matrix metalloproteinase (MMP) related proteins MMP-2, MMP9 and TIMP-1 in MKN45 cells after COE (100, 200 and 400 μg/ mL) treatment for 24 h.

### COE regulated the expression of matrix metalloproteinases (MMPs) in AGS and MKN45 cells.

Western blotting was performed to observe the expression levels of intracellular-related proteins. ($p < 0.05$, Figure 5). We explored the matrix metalloproteinase-related proteins. Compared with the control group, the expression levels of intracellular MMP-2 and MMP-9 proteins were decreased after COE treatment while the expression level of TIMP-1 was increased. The results suggested that COE may inhibit the invasion and migration of gastric cancer cells by acting on MMPs.

### COE regulated the expression of EMT-related proteins.

EMT is an important biological process for tumor cell invasion and migration. To investigate whether COE inhibited the invasion and migration of gastric cancer cells by inhibiting the EMT process, we verified the expression of EMT-related proteins. The results showed that the expression levels of N-cadherin and Vimentin were decreased, and the expression level of E-cadherin was increased simultaneously. The results confirmed that COE could inhibit the EMT process and thus inhibit the invasion and migration of AGS and MKN45 cells ($p < 0.05$, Figure 6).

### Effects of COE on the expression of ITGB4 signaling pathway.

As shown in Figure 7, Compared with the control group, the protein expression levels of ITGB4, Akt, PI3K and palladin were decreased after the COE treatment. The results showed that COE inhibited cell invasion and migration by acting on the ITGB4 and its downstream proteins.

## DISCUSSION

Metastasis of tumor will lead to poor prognosis of patients. There are many ways of metastasis of gastric cancer, which can be roughly divided into implantation metastasis, lymphatic metastasis and hematogenous metastasis. Studies have found that patients with ovarian metastasis of gastric cancer die

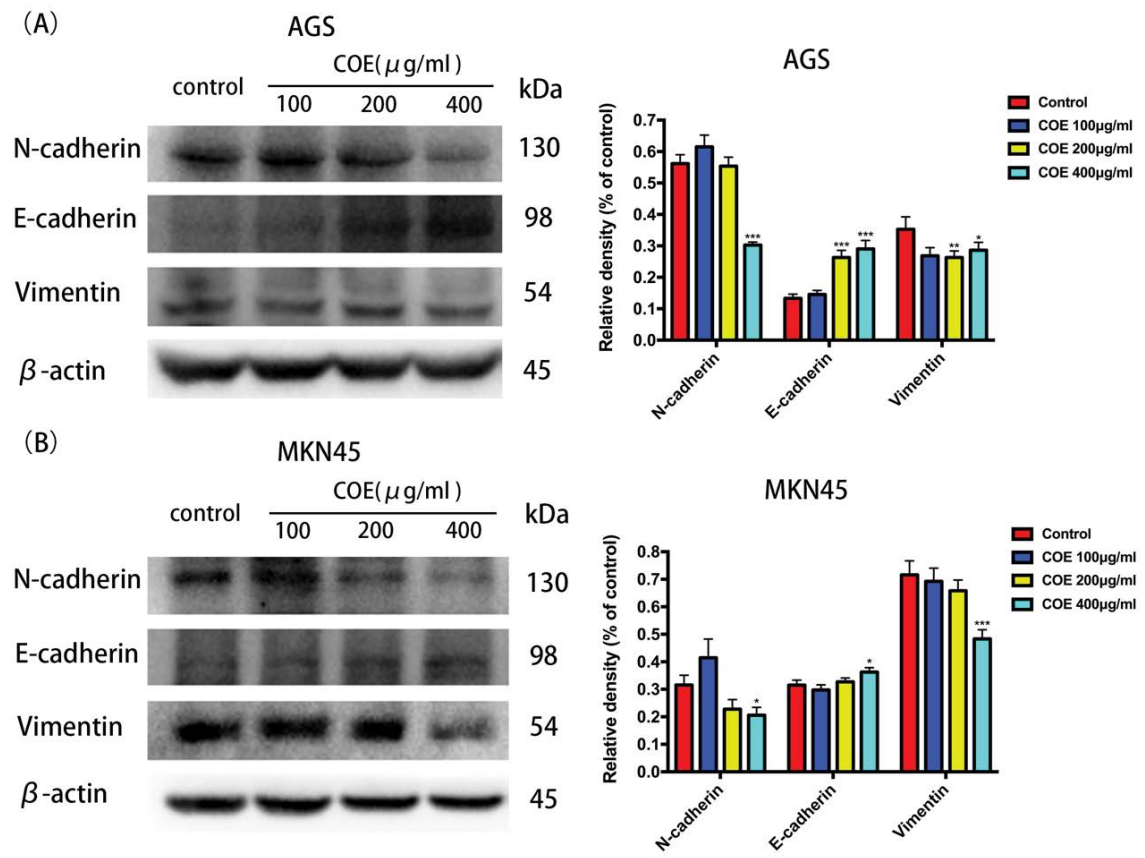


**Figure 6.** Western Blot was used to detect the effects of COE on the expression of N-cadherin, E-cadherin and Vimentin in AGS and MKN45 cells. (A) After 24 h of COE (100, 200 and 400 μg/ mL) treatment, the expression of EMT-related proteins N-cadherin, E-cadherin and Vimentin in AGS cells was detected. (B) After 24 h of COE (100, 200 and 400 μg/ mL) treatment, the expression of EMT-related proteins N-cadherin, E-cadherin and Vimentin in MKN45 cells was detected.

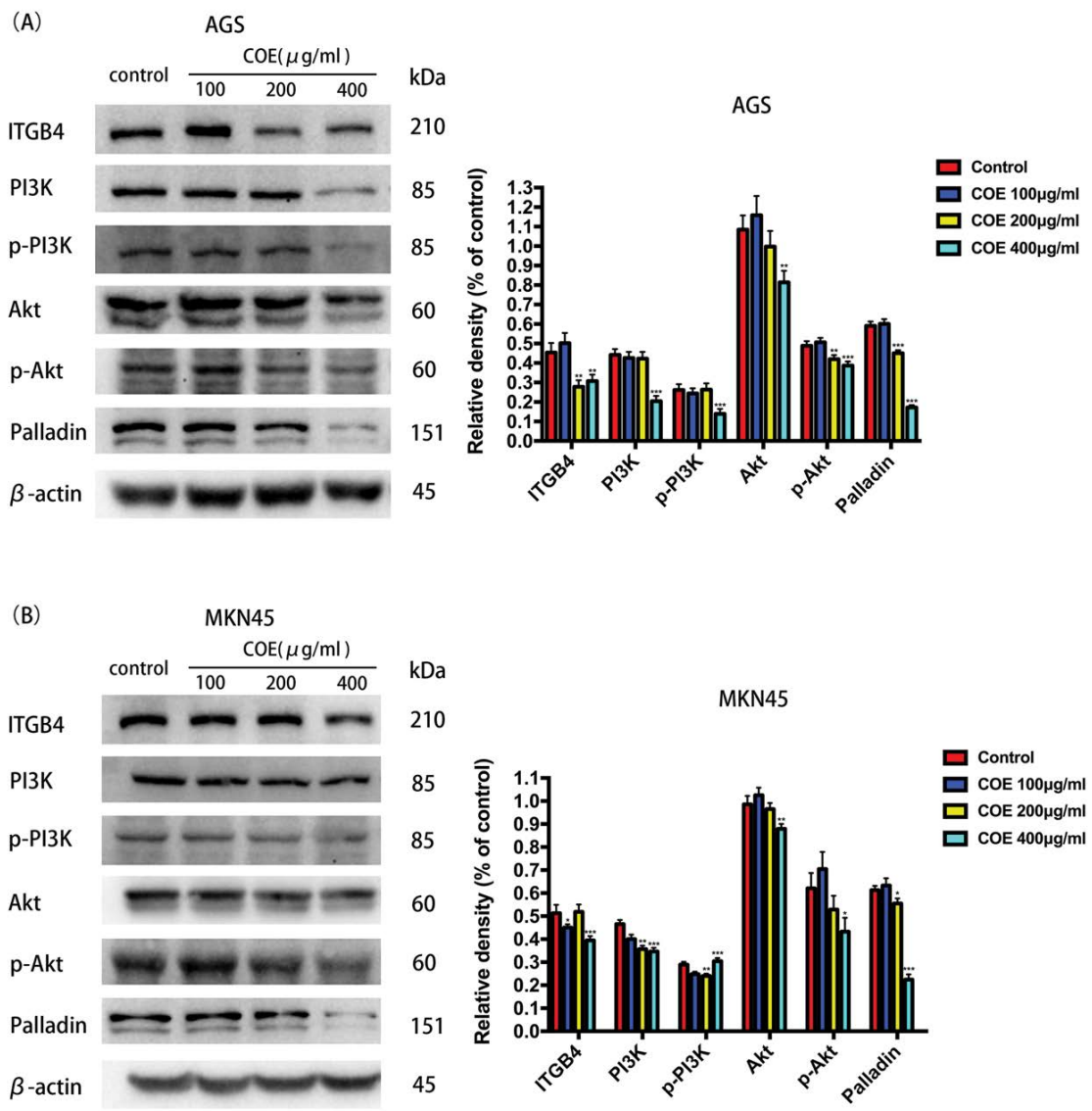


**Figure 7.** Effects of COE on the expression of ITGB4, PI3K, p-PI3K, Akt, p-Akt and Palladin in AGS and MKN45 cellsn were detected by Western Blot. (A) The cells were treated with different concentrations of COE (100, 200 and 400 μg/ mL) for 24h. Changes in protein expression levels related to the pathway in AGS cells after COE treatment were determined. (B) The cells were treated with different concentrations of COE (100, 200 and 400 μg/ mL) for 24h. Changes in protein expression levels related to the pathway in MKN45 cells after COE treatment were determined.

within 1-2 years[17]. The relevant study reviewed 808 cases of gastric cancer and found that 30% of the patients had metastasis, among which abdominal metastasis was the most common, and other cases included lung metastasis and liver metastasis [18]. Thus, the invasion and migration of gastric cancer is closely related to the prognosis of patients. Therefore, it's imperative to discover new anti-cancer drugs targeting the invasion and metastasis of gastric cancer.

Traditional Chinese herbal medicine *Celastrus orbiculatus* has the effects of anti-inflammation. By inhibiting the binding activity of TLR4/NF-κB and thus inhibiting the migration and invasion of lipopolysaccharide (LPS) -stimulated fibroblast-like synovial cells (FLS), this natural medicine can be used for the treatment of rheumatoid arthritis(RA)[19, 20]. Studies have shown that Celastrus orbiculatus extract can reverse the resistance of many cells to anti-tumor drugs such as paclitaxel[21]. In addition, previous studies of our group found that COE could inhibit the invasion and migration of gastric cancer. Recent studies have shown that COE has significant antitumor activity *in vitro* and *in vivo*.

Integrin β4 (ITGB4), as a member of the integrin family, usually forms a heterodimer with integrin α6 (ITGA6) to play its role[22]. ITGB4 expression in papillary thyroid carcinoma (PTC) is up-regulated compared with normal thyroid tissue, and its high expression is related to high TNM stage of PTC and reduced overall survival rate, and ITGB4 can help tumor cells overcome the influence of Anoikis to achieve lymph node metastasis [23]. Exosome 231-EXO derived from breast cancer MDA-MB-231 cells can induce lung metastasis of breast cancer through the high affinity between ITGB4 highly expressed on exosomes and lung surfactant protein C (SPC) [24]. Extracellular matrix 1 (ECM1) is associated with the invasion and poor prognosis of disgusting tumors. Studies have shown that ECM1 directly interacts with ITGB4 to activate the ITGB4/FAK signaling pathway and induce the expression of transcription factor SOX2, which alters the expression of epithelial-mesenchymal transformation (EMT) related genes. Moreover, HIF-1α promoter activity was enhanced to regulate glucose metabolism. ECM1 regulates gastric cancer cell metastasis and glucose metabolism by inducing ITGB4/FAK/SOX2/HIF-1α signaling pathway [25].

We aimed to investigate the role of ITGB4 in suppressing the invasion and migration of gastric cancer after COE treatment. MTT assay showed that COE could inhibit the proliferation of AGS and MKN45 cells. Transwell results showed that the number of transmembrane cells decreased with increasing drug concentration. Wound healing experiment results showed that the healing ability of the experimental group was inferior to that of the control group. We also conducted cell migration ratio analysis using high-content imaging system. It can be seen from these results that COE has an inhibitory effect on the invasion and migration of cells. In order to explore the mechanism of COE inhibiting the invasion and migration of AGS and MKN45 cells, we conducted a series of Western blotting experiments.

EMT is a cellular procedure that is critical for the malignant progression of tumors and increases the likelihood that tumor cells will metastasize. Normally, epithelial cells are of apical to basal polarity and are connected to each other by e-cadherin. After EMT, the expression of E-cadherin was inhibited, and the polygonal and elliptic morphology of epithelial characteristic cells disappeared. On the other hand, cells present spindle mesenchymal morphology, and n-cadherin and Vimentin expressions are reduced [26]. To explore the mechanism of COE, we examined the effect of the extract on the expression of EMT-related proteins. In this study, we found that e-cadherin expression increased in gastric cancer cells after COE treatment, while N-cadherin and Vimentin expression decreased. Our results suggest that COE may inhibit the invasion and migration of human gastric cancer cells by blocking EMT progression. Moreover, our experimental results showed that COE could inhibit the invasion and migration of AGS and MKN45 cells by acting on MMPs. Invasion and migration of tumor cells require the degradation of extracellular matrix (ECM) and destruction of basal membrane (BM). MMPs are the most important proteases for the degradation of extracellular matrix. Extracellular vesicles containing metalloproteinases can directly participate in the remodeling of ECM, which generates specialized cellular processes capable of degradation, namelyinvadopodia. ECM remodeling helps tumor cells migrate between tissues[27, 28]. MMP activity is inhibited in a specific and reversible way by endogenous MMP inhibitors, namely TIMPs[29]. TIMPs act in conjunction with MMPs, thereby inhibiting the function of MMPs. In addition, TIMPs inhibit the invasion and migration of gastric cancer cells. MMPs can promote the EMT process by processing a variety of cell surface adhesion molecules. The results showed that the expression of MMP-2 and MMP-9 was decreased and the expression of TIMP-1 was increased.

Integrin-mediated cell adhesion is involved in many physiological and pathological functions of cells, not only in ECM remodeling and immune escape of tumor cells, but also in almost every process of tumor genesis, development, invasion and migration[26, 30]. After COE acting on the relevant signaling pathway, the expression of ITGB4 is downregulated, which can effectively inhibit the metastasis and invasion of tumor cells. To further explore the mechanism of COE, we detected the downstream protein PI3K of ITGB4. PI3Ks are a family of heterodimeric lipid kinases. As a serine/threonine kinase, Akt is the main downstream effector molecule of PI3K. The PI3K/Akt pathway is closely related to the genesis, proliferation, invasion, migration and EMT of tumor cells[31]. The experimental results suggested that the protein levels of Akt, p-Akt, PI3K and p-PI3K decreased after COE was applied to human gastric cancer cells, which suggests that COE may act on the PI3K/Akt signaling pathway. Next, we examined the downstream protein Palladin of Akt, a protein that directly regulates cell migration. Palladin, as a cytoskeleton protein, plays a role in tumor cell migration by controlling the formation of pseudopodia, degradation of matrix and cadherin binding[32]. Studies have found that Palladin can be used as a prognostic indicator of a variety of tumors, and its high expression is related to the metastasis of a variety of tumors. For example, miR-96 / miR-182 inhibits invasion and migration of breast cancer by down-regulating the level of Palladin protein[33]. The reduced expression of Palladin inhibited cell migration, and this finding was consistent with the Western blot results in this study, which suggested that the inhibition of COE on the invasion and migration of human gastric cancer cells was related to the ITGB4/PI3K/Akt/Palladin

signaling pathway. Through a series of in vitro experiments, we verified and explored the effect of COE on inhibiting the invasion and migration of AGS and MKN45 cells in gastric cancer. These preliminary results will provide a basis for our further exploration.

## CONCLUSION

COE significantly inhibited the invasion and migration of AGS and MKN45 human gastric cancer cell by regulating ITGB4/ PI3K/Akt/ Palladin signaling pathway. This study provides a new basis for the development of COE as an antitumor agent and contributes to the further study of the effect of COE targeting ITGB4.

## ABBREVIATIONS

COE: *Celastrus orbiculatus* extracts; ITGB4: Integrin β4

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## FUNDING

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## AUTHOR'S CONTRIBUTIONS

Yin developed the ideas and methods; Yin performed the relevant experiments; Yin and Qin analyzed the experimental data; Yin prepared the manuscript; Qin and Chu reviewed and edited the manuscript; Liu provided project management; Qian provided experimental equipment and financial support.

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