Celastrus Orbiculatus Extract could inhibit human colorectal carcinoma HT-29 cells metastasis via suppression of the mTOR signaling pathway

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ABSTRACT: Objective The ethanol extract from the stem of *Celastrus Orbiculatus* was found to exhibit significant anti-cancer activity. This study aims to investigate whether *Celastrus Orbiculatus* extract (COE) could inhibit human colorectal carcinoma HT-29 cells metastasis and the mTOR signaling pathway with special reference to the process of metastasis. **Methods** The human colorectal carcinoma HT-29 cells were divided into negative and positive control group and COE groups (20, 40, 80, 160, 320 μg/mL, respectively). The cytotoxicity effect was measured by using the MTT assay. The invasion and migration ability of HT-29 cells was assayed by Transwell invasion assay and wound-healing repair assay. Western blot was carried out to examine protein expression of each group. The protein we detected consisted of mTOR, its upstream regulators and downstream effectors such as Akt, p70S6K, 4EBP1 and MMP-9. **Results** COE suppressed the migration and invasion ability of HT-29 cells in a dose-dependant manner. COE treatment dose-dependently inhibited the expression of mTOR and p-mTOR, phospho-4E-BP1, phospho-p70S6K, p70S6K and MMP-9 compared to the control group, whereas there was no significant effect on Akt. **Conclusions** In summary, COE exerts an anti-metastasis effect on HT-29 cells. The mechanisms of the anti-metastasis activity of COE might be due to the down-regulation of mTOR signaling pathway.

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1. Introduction

Colorectal cancer is one of the most frequent cancers and one of the leading causes of cancer death worldwide[1].About 50–60% of patients with colorectal cancer will experience metastasis[2]. Metastasis is one of the remarkable characteristics of colorectal cancer and also the primary cause of colorectal cancer mortality. The process of metastasis occurs by a series of steps including vessel formation, cell attachment, migration, invasion, and cell proliferation, which is regulated by extremely complicated mechanisms. Among them, mTOR pathway has drawn a great attention. The mammalian target of rapamycin (mTOR), a highly conserved and ubiquitously expressed serine/threonine (Ser/Thr) kinase, plays a critical role in the regulation of cancer metastasis [3-4].

mTOR is considered as a downstream target of Akt and involved in the translational initiation of many survival proteins through its activation of p70S6 kinase (p70S6K) and 4EBP1. Recent study also reveals that mTOR signaling promotes cell migration and invasion through increased expression and proteolytic activities of MMP-9[5]. MMP-9 is an important member of matrix metalloproteinases which act an important role in the proteolytic destruction of extracellular matrix and basement membranes, which is essential for tumor invasion and metastasis[6]. Therefore, the inhibition of mTOR pathway could be a potential treatment for inhibiting metastasis of colorectal cancer.

The Celastraceae plant *Celastrus orbiculatus* (*Celastrus orbiculatus* Thunb., Nansheteng, *celastrus*), which is widely distributed in China, has been used as a folk medicine in China for the treatment of many diseases, including arthritis and other inflammatory diseases[7]. Our previous study found that *C. orbiculatus* ethyl acetate extract (COE) displays anti-cancer effects in vitro and in vivo through the inhibition of proliferation, angiogenesis, invasion and adhesion ability[8-11].

The effects were observed in human gastric cancer cells, Hepatocarcinoma cells, cervical-cancer cells, etc, but not in colorectal cancer cells. As we all know that 5-Fu is one of

most clinically-used chemical drugs for colorectal cancer. In the present study, we investigated for the first time that the possible involvement of the mTOR signaling pathway in anti-metastatic activities of COE against human colorectal carcinoma HT-29 cells. Therefore, in the present study, we examined the anti-metastatic activities of COE and 5-Fu (as the positive control) against HT-29 cells in vitro, and further investigated that the effects of COE on mTOR signalling with special reference to the process of cell proliferation, invasion and migration.

2. Materials and methods

2.1 Plant Material

The stems of *C. orbiculatus* plants (batch No.070510) were purchased from Guangzhou Zhixin Pharmaceutical Co., Ltd. (Guangzhou, China) in 2007. The extraction and characterization of COE were kindly provided by Prof. Wang Qiang, Department of Chinese Material Medical Analysis, China Pharmaceutical University, Nanjing, China. The extraction process is specified as follows: First, we grinded the dried *C. orbiculatus* stems and extracted the powder with 95% ethanol for 3 h; this procedure was repeated 3 times. Then we filtered and evaporated the combined extract to dryness. The ethanol extract was subsequently partitioned between ligarine and water and the aqueous layer was further partitioned by ethyl acetate. Finally we condense the final ethyl acetate extract and lyophilize it into powder[12]. The resultant micropowder was dissolved in DMSO (Sigma, St. Louis, MO, USA) at 1600 μg/ml concentration as a stock solution which was stored at −20°C before evaluation of its anti-metastatic properties.

2.2 Regents

RPMI medium 1640 and trypsin-EDTA solution were purchased from Gibco Co. (Carlsbad, CA). Fetal bovine serum(FBS) was from Hyclone Biotechnology Inc. 3- (4,5- dimethylthiazol-2-yl) -2,5 –diphenyltetrazo lium bromide (MTT) and matrigel was purchased from Sigma Chemical Co.(St. Louis, MO).Transwell chambers were obtained from Corning Co. Rabbit monoclonal Anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-p70S6 kinase, anti-p70S6 kinase, anti-phospho-4E-BP1 (Tyr37/46), anti-4E-BP1, anti-actin were all purchased from Cell Signaling Biotechnology (Beverly, MA). Rabbit monoclonal anti-MMP-9 was from Millipore Co.(Bedford, MA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was from Bioworld Biotechnology. 5-Fu was purchased from Shanghai Xudong Pharmaceutical Co. Ltd. (batch No.090724).

2.3 Cell culture

Human colon cancer HT-29 cells was obtained from the Cell Bank of Chinese Academy of Sciences Shanghai Institute of Cell Biology (Shanghai, China). HT-29 cells were maintained in RPMI 1640 supplemented with 10% FBS at 37°C in a humidified atmosphere with 5% CO2.

2.4 Assessment of cell viability

Cell viability was monitored by the MTT colorimetric assay. Briefly, exponentially growing HT-29 cells (1×10^4 cells in 96 well plates) were treated with COE at various concentrations (20, 40, 80,160 and 320 μg/ml) for 24h before MTT (5 mg/ml in PBS) was added to each well. After incubation for 4 h, media was discarded and 150 μl of DMSO was added to each well to dissolve formazan. The absorbance was measured at 490 nm. Cell viability (%) was measured as follows: the OD value of experimental group/ the OD value of control group×100.

2.5 Cell invasion assay

HT-29 cells incubated with various concentrations (0, 20, 40 and 80 μg/ml) of COE and 5-Fu (25 μg/ml) for 24 h were removed by trypsin, and their invasiveness was tested by the Transwell chamber invasion assay. The bottom of Transwell chamber is 6.5 mm in diameter with 8.0 μm prore polycarbonate membrane insert. Matrigel was diluted to 25 mg/50 ml with cold filtered distilled water and applied to 8-μm-pore size polycarbonate membrane filters for 1h to form a genuinely reconstituted basement membrane. The prepared chamber was then sterilized by ultraviolet rays for 2h and hydrated with serum-free medium before using. Briefly, the treated cells were seeded to the upper part of the Transwell chamber at a density of 5×10^5 cells/ml in 100 μl of serum-free medium. The bottom chambers were filled with 500 μl RMPI 1640 media supplemented with 20% FBS. Cells were allowed to migrate for 8 h at 37 °C. After the chambers were incubated at 37℃for 12 h, non-invasive cells in the upper chamber were removed by wiping with a cotton swab. The invasive cells on the bottom side of the membrane were fixed in methanol for 15 min and washed twice with PBS. The cells were stained with crystal violet and then washed twice with PBS. Images

were observed and photographed under fluorescent microscopy (400×magnification), and invasive cells were quantified by manual counting. Percentage inhibition of invasive cells was quantified, with untreated cells representing 100%.

2.6 Cell migration assay

For wound healing assay, HT-29 cells were seeded into a 24-well plate and grown in RMPI 1640 containing 10% FBS to a nearly confluent cell monolayer. A plastic 100 µl pipette tip was used to scratch the center of the plate to produce a clean 1-mm-wide wound area. Cellular debris was removed by washing with PBS twice, and then the cells were incubated with various concentrations of COE (20 to 80 µg/ml) and 5-Fu (25 µg/ml) for 24 h. Images were photographed (200×magnification) at 0 and 24 h to monitor the migration of cells into the wounded area, and the migrated cells in the closure of wounded area was counted. Cell migration rate (%) was measured as follows: the migrated cell numbers of experimental group/the migrated cell numbers of control group×100.

2.7 Western blot analysis

The cells were incubated with various concentrations (0, 20, 40, 80 and 160 µg/ml) of COE and 5-Fu (25 µg/ml) for 24 h and washed twice in cold PBS, and then resuspended in lysis buffer (50 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 µg/ml phenylmenthanesulfonyl fluoride (PMSF), 10 µg/ml leupeptin, and 5 mmol/L Na_3VO_4). The suspension was put on ice for 20 min and then centrifuged at 12 000 r/min for 15 min. Total protein content was determined using the Bio-Rad protein assay kit. Equal amount of protein was electrophoresed on 6% to 15% SDS polyacrylamide gels and then transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk, the membranes were incubated overnight at 4 °C. On the next day, the membranes were incubated with corresponding HRP-conjugatedsecondary antibody. The blots were developed using ECL Western blotting detection reagents.

3. Results

3.1 Effect of COE on cells growth

The anti-proliferative potential of COE on HT-29 human colon cancer cells was measured by MTT assay. As shown in **Figure 1**, cells treated with various concentrations of COE (20 to 320 µg/ml) exhibited a dose-dependent growth inhibition with an IC50 value of 166.6 µg/ml. The high inhibition of cells growth would cause interference in cancer migration and invasion assay, so we used the treatment with 20 to 80 µg/ml COE on HT-29 cells for 24 h in subsequent experiment. According to our study and related literature research, we realized that the IC_{50} value of 5-Fu on HT-29 cells is approximately 25 µg/ml. Thus, we used the treatment with 25 µg/ml 5-Fu as positive control in subsequent experiment.

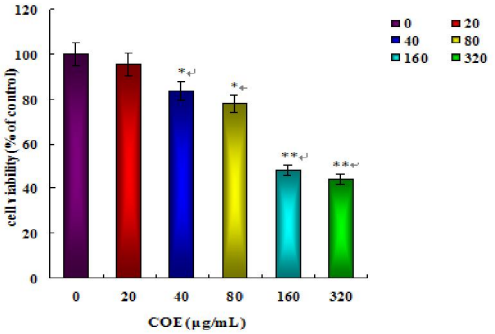

Figure 1 Concentration-dependent effects of COE on the viability of HT-29 cells.1.Cells were either untreated or treated with 20, 40, 80, 160 and 320µg/mL of COE for 24 h. Results are presented as mean ± SD of three assays. *, $P<0.05$; **, $P<0.01$, compared with the control group.

3.2 Effect of COE on HT-29 cells invasion and migration

To evaluate the anti-metastatic activity of COE, we first assessed the inhibitory effect of COE on the migration of HT-29 cells by the Transwell invasion assays. As shown in Figure 3A, the invasion inhibition rate of 20, 40 and 80 µg/ml of COE-treated cells were 9.18±4.45%, 33.87±4.27% and 55.86±7.39%, respectively. Compared with the control group, the difference was statistically significant ($P<0.05$ or $P<0.01$) in a dose-dependent manner by COE. Furthermore, to determine the effects of COE in vitro on HT-29 cells migration, wound healing assay were performed. The results indicated that COE suppressed the migration of HT-29 cells across the wounded space in a dose-dependent manner (**Figure 2**).

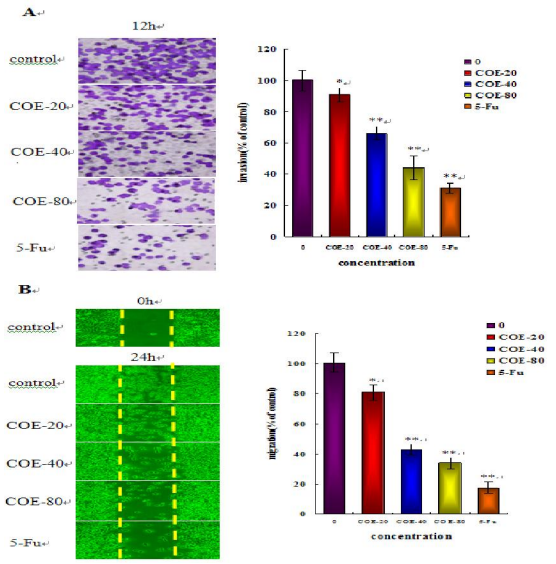

Figure 2 COE inhibits the invasion and migration ability of HT-29 cells in transwell invasion assay and wound healing assay.(A)HT-29 cells were pretreated with various concentrations (0, 20, 40 and 80μg/mL) of COE and 5-Fu(25 μg/ml). After 12 h, cells invading under the membrane were photographed (400×magnification). Percentage inhibition of invasive cells was quantified, with untreated cells representing 100%. (B) Cells were scratched and treated with various concentrations and 5-Fu, and migration was observed and photographed under fluorescent microscopy (200×magnification) at 0 and 24 h, and the closure of area was calculated. Results are presented as mean ± SD of three assays. *, $P<0.05$; **, $P<0.01$, compared with the control.

3.3 Effect of COE on p-mTOR and mTOR expression

To determine the effect of COE on the expression of p-mTOR and mTOR proteins in HT-29 cells, Western blot analysis was carried out. Compared to the control group, both p-mTOR and mTOR proteins level of the cells treated with different concentrations of COE was decreased in a dose-dependent manner (**Figure 3**).

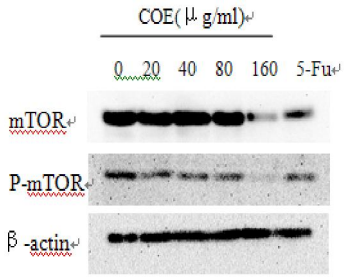

Figure 3. Inhibitory effects of COE on p-mTOR and mTOR expression. Cells were treated with various concentrations (0, 20, 40, 80 and 160μg/mL) of COE and 5-Fu (25 μg/ml) for 24 h .Proteins from each sample were resolved on 6–15% SDS–PAGE, and Western blot was performed. β-actin was used as a control.

3.4 Effect of COE on the expression of upstream regulators and downstream effectors of mTOR signaling

To further elucidate the mechanisms of COE-mediated anti-metastasis activity on HT-29 cells, the proteins related to mTOR-mediated signaling pathway in the cancer cells were examined. High concentration of COE repressed the expression of phospho-Akt, whereas there was no effect on total Akt expression. Next we examined the effect of COE on expression of downstream effectors of mTOR signalling. COE treatment dose-dependently inhibited the expression of phospho-4EBP1, phospho-p70S6K, and p70S6K compared to the control group. Moreover, the inhibitory effect of high concentration of COE on these three proteins was more significant than that of 5-Fu. COE at 160 μg/ml markedly inhibited 4E-BP1 expression, while other concentrations of COE and 5-Fu had a nearly negligible impact on it. Compared to the control, the MMP-9 protein level in the cells treated with various concentrations of COE was decreased in a concentration -dependent manner (**Figure 4**).

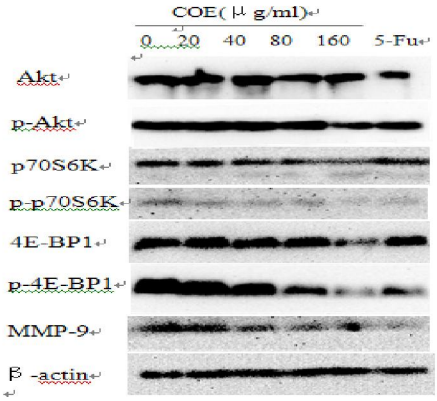

Figure 4 Effect of COE on the expression of upstream regulators and downstream effecter of mTOR signalling .Cells were treated with various concentrations (0, 20, 40, 80 and 160μg/mL) of COE and 5-Fu (25 μg/ml) for 24 h .Proteins from each sample were resolved on 6–15% SDS–PAGE, and Western blot was performed. β-actin was used as a control.

4. Discussion

Approximately half of patients with colorectal cancer develop metastasis and ultimately die as a result of the effects of the disease. Thus, metastasis has been a major challenge for the successful treatment of this cancer. Many anti-tumor agents are believed to block or delay the promotion or progression of tumor cells by modulating cell proliferation or differentiation [13]. These agents also have severe side effects, which limit their clinical applications. In recent years, attention has been focused on the anti-cancer properties of natural agents. Our previous study shows that COE exhibited potent anti-tumor activity in vitro and in vivo, including anti-proliferation, anti-angiogenesis, etc. However, the molecular mechanism underlying the COE-mediated inhibition of cancer cells migration and invasion has not been elucidated until the present study shows that the down-regulation of mTOR pathway might be responsible for the inhibitory effect of COE.

To demonstrate COE as a regulator of cell migration and invasion, we excluded the anti-proliferative effect of COE on tumor cell growth by MTT assay showing that the cell viability was not significantly altered by the treatment of COE at concentrations between 20 and 80 μg/ml (Figure 1). This dose range was applied in all subsequent experiments to avoid the influence of cell cytotoxicity on the observed parameters. We also used the treatment with 25 μg/ml 5-Fu (IC_{50} value) as positive control. COE treatment was shown to inhibit both the migration and invasion potential of HT-29 cells by wound-healing analysis and Transwell assay in a dose-dependent manner (Figures 2 and 3).

mTOR is known as a central regulator of cell growth, proliferation, differentiation and survival[14-16].Recent studies have shown that mTOR pathway also plays an essential role in the regulation of tumor cell invasion, mobility and cancer metastasis[17-18].The data demonstrated that COE suppressed the expression of mTOR and p-mTOR proteins in HT-29 cells. COE at 160 μg/ml almost completely blocked the expression of mTOR. It suggested that the anti-metastasis effect of COE may be associated in part with the regulation of mTOR-mediated signaling.

Based on the effect of COE on mTOR expression, mTOR-mediated upstream and downstream signaling was further investigated in HT-29 cells. mTOR is mainly regulated by the phosphatidylinositol 3'-kinase/Akt pathway, and p70S6K and 4EBP1 are two best characterized downstream targets of mTOR. 4EBP1 is a translation inhibitory factor which binds tightly to the cap-binding protein eIF4E and represses cap-dependent mRNA translation by blocking the interaction of eIF4E with the eIF4G protein [19]. The phosphorylation of 4EBP1 through activation of mTOR at multiple site releases eIF4E to promote protein translation. On the other hand, p70S6K is ubiquitously expressed and plays a critical role in the control of cell growth and cell motility. Interestingly, various concentrations of COE had a nearly negligible impact on Akt expression and COE at 160 μg/ml inhibited p-Akt expression. It suggested that other proteins may involve in regulation of mTOR pathway. As illustrated in Figure 4, COE concentration-dependently suppressed p70S6K and the activation of 4E-BP1 and p70S6K. These data further confirm the involvement of down-regulation of mTOR-mediated signaling in the inhibitory effect of COE against HT-29 cancer cells.

MMP-9 is an extremely important member of MMPs which play an important role in the proteolytic destruction of extracellular matrix and basement membranes for tumor growth, invasion and tumor-induced angiogenesis[6]. The data revealed that activated PI3K-Akt-mTOR signaling pathway promotes invasion and metastasis in hepatocellular carcinoma though up-regulation of MMP-9[5]. Meanwhile, knockdown of mTOR expression resulted in the abrogation of MMP-2, MMP-9 activity[20]. In our

study, we found that COE inhibited the expression of MMP-9 in a dose-dependent manner. Thus, it seems quite likely that COE could inhibit or delay cancer invasion and migration in HT-29 cells via modulation of MMPs.

Taken together, our observations indicate that COE exerts an inhibitory effect on several essential steps of metastasis, including proliferation, migration and invasion of HT-29 cells. The inhibitory effect of COE might be in part related with the down-regulation of mTOR pathway. Based on these results, we proposed a schematic presentation of the possible molecular mechanisms for the inhibitory effect of COE on migration and invasion of HT-29 cells (**Figure 5**). Together with those of our previous report [12, 21, 22, 23, 24], these findings suggest that COE could be recognized to be a potential candidate for the development of a preventive agent for cancer.

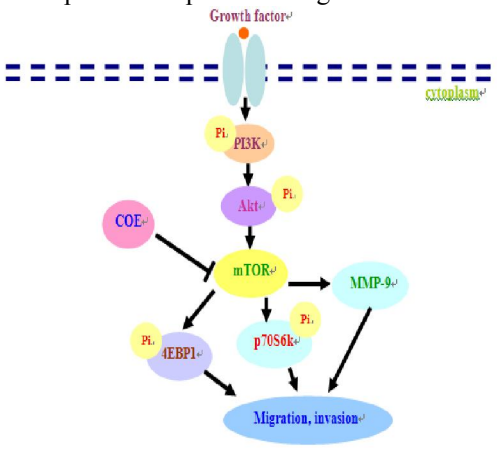

Figure 5. A proposed model for the inhibitory effect of COE on the migration and invasion of human colorectal carcinoma HT-29 cells. See the text for discussion.

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