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Original Research Article

Thymol inhibits cell migration and invasion by downregulating the activation of PI3K/AKT and ERK pathways in human colon cancer cells

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Abstract

Purpose: *To assess the anti-metastasis effects of thymol on human colorectal cancer cells.*
Methods: *Human colorectal adenocarcinoma cell HT29 was incubated with varying concentrations of thymol. Cell viability, migration and invasion were determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-dipheny-tetrazoliumbromide (MTT) and Transwell assays, respectively. Matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) were analyzed by gel zymogram assay. Epithelial-mesenchymal transition (EMT)-associated gene expression and signaling pathway were analyzed using real-time quantitative polymerase chain reaction (PCR) and Western blotting, respectively.*
Results: *Thymol was significantly inhibited migration and invasion of HT29 cell ($p < 0.01$) and also markedly reduced the activity of matrix degrading enzymes MMP-2 and MMP-9 ($p < 0.01$). Moreover, the epithelial marker, E-cadherin, was elevated, while mesenchymal markers (vimentin and α-SMA), and associated transcription factors (snail and slug) decreased after thymol treatment ($p < 0.01$). In addition, thymol inhibited the phosphorylation of PI3K/AKT and ERK pathways ($p < 0.01$).*
Conclusion: *Thymol efficiently attenuates cell migration and invasion by decreasing EMT and downregulating the activation of PI3K/AKT and ERK signaling pathways in colorectal adenocarcinoma cells. It is, thus, a potential candidate drug for the management of colorectal cancer.*

Keywords: *Thymol, Colorectal cancer, Anti-metastasis, Epithelial-mesenchymal transition, Vimentin, PI3K/AKT and ERK pathway*

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INTRODUCTION

Colorectal cancer (CRC), one kind of the most digestive tract tumors, is the most common reason of cancer-related death, with between one and two million new cases diagnosed every year, and its incidence has been increasing year by year [1]. Modifiable risk factors for CRC related to lifestyle including smoking, physical activity habits, overweight, obesity and alcohol consumption. Chemotherapy and surgery are the most common treatment for CRC. CRC treatment has improved due to the application of a new generation of chemotherapy and

molecular-targeted drugs, but it remains unsatisfactory. Moreover, the toxic side effects of chemotherapy drugs and the failure of chemotherapeutic due to drug resistance are some of the drawbacks of clinical treatment [2]. The treatment of metastasis is still not satisfactory, mainly due to lack of effective drugs [3]. Therefore, it is necessary to find new effective drugs to fight against metastasis for CRC.

Thymol is an active monoterpene isolated from many medicinal herbs, such as *Thymus vulgaris, Monarda punctate* and *Origanum vulgare* spp [4]. It has been widely used for treatment inflammatory diseases, such as osteoarthritis [5] and asthma [6]. It reported that thymol has various bioactivities, such as anticancer [7], anti-bacterial [8] and antioxidant properties [9]. Despite thymol being known for its multifaceted activities, the anti-metastatic ability on colorectal carcinoma cells has not been studied.

The present study was designed to explore the effect of thymol on metastasis in human colorectal carcinoma cells. In view of its effects on the phosphorylation of PI3K/AKT and ERK pathways, the underlying mechanisms of how thymol inhibits cell migration and invasion, and EMT were explored.

EXPERIMENTAL

Chemicals and reagents

Thymol ($C_{10}H_{14}O$, MW: 150.22, purity ≥ 98 %) was purchased from Sigma (St. Louis, USA). It was dissolved in dimethylsulfoxide (DMSO) as stock solution of 10 M, stored at -20 °C, and freshly diluted with RPMI-1640 medium (Gibco, Carlsbad, CA) to the final concentration used in the study. [3-(4, 5-dimethylthiazol-2-yl)-2, 5-dipheny-tetrazoliumbromide] (MTT) was obtained from Sigma (St. Louis, USA). Antibodies against the following targets: AKT, phosphor-AKT, ERK, phosphor-ERK, and GAPDH were purchased from Bioworld Technology, Inc. (Louis Park, MN).

Cell culture

The human colorectal adenocarcinoma HT29 cell (American Type Culture Collection, Bethesda, MD, USA) were cultured in RPMI1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were cultured under a humidified 5 % CO_2 atmosphere at 37 °C.

Cell viability assay

Cell viability was measured using the colorimetric MTT assay as described previously [10,11]. The cells were cultured in 96-well plates overnight and treated with thymol (0.5, 1, 2, 4 mM) for 24 and 48 h. Thereafter, cell viability was determined by MTT.

Cell migration assay

A Transwell assay was employed to determine cell migration [12]. HT29 cells (1×10^4 cells/well) was added into the upper chamber of the Transwell plates, and treated with thymol (0.5, 1 and 2 mM), while the lower chamber contained 600 µL culture medium with 10 % FBS. After treatment for 24 h, the cells that migrated to the bottom face of the membranes were stained with crystal violet solution and further extracted with 10 % acetic acid. The absorbance at 540 nm represents the number of cells that migrated across the membrane.

Cell invasion assay

Cell invasive activity was performed by Matrigel assay [13]. Briefly, 5×10^4 cells were added to the chamber, and 100 µL Matrigel was added to the lower chamber. After incubation for 24 h, the cells were fixed with 4 % formaldehyde, stained with crystal violet and further extracted with 10 % acetic acid. The absorbance at 540 nm represents the number of cells that invaded across the Matrigel.

Gelatin zymography assay

The activity of MMP-2 and -9 were analyzed by using the gelatin zymography assay [14]. The HT29 cells were incubated with thymol (0.5, 1 and 2 mM) for 24 h. The supernatants were separated by 10 % SDS-PAGE containing 1 % (m/v) gelatin. The gels were visualized after staining with Coomassie blue and then photographed.

Western blotting

Cell lysates from HT29 cells were extracted in NP40 lysis buffer, separated by 10 % SDS-PAGE gel and further transferred to PVDF membranes. The membranes were blocked with 5 % nonfat milk for 2 h, and incubated with specific primary antibodies overnight at 4 °C, and then incubated with secondary antibody for 1 h at 37 °C. The protein bands were visualized with ECL reagent (Millipore, Billerica, MA, USA).

Quantitative polymerase chain reaction (qPCR) assay

Total RNA from HT29 cells was extracted with TRIzol according to the kit manufacturer's instructions. RNA was transcribed to CDNA, which were analyzed for the expression of E-cadherin, Vimentin, α-SMA, Snail and Slug by IQ SYBRGreen Supermix (Bio-Rad, Hercules, CA, USA). Primers were obtained from Sangon Biotech (Shanghai, China) and the details of the primers were listed in Table 1.

Statistical analysis

The data are given as mean ± SEM (n = 3). Differences between the groups were analyzed using SPSS software and one-way analysis of variance (ANOVA) followed by Dunnett's test. *P* < 0.05 was considered statistically significant.

RESULTS

Effect of thymol on viability in HT29 cells

Firstly, we investigated the effect of thymol on cell viability at different concentrations (0.5, 1, 2 and 4 mM). As shown in Figure 1, after treatment for 24 and 48 h, thymol markedly (4 mM) inhibited the cell viability in HT29 cells. Therefore, further studies were conducted using thymol (0.5, 1 and 2 mM) to avoid its cytotoxicity.

Effect of thymol on cell migration

To evaluate the anti-metastatic effect of thymol on HT29 cells, the migration of HT29 cells by Transwell assay was performed. As shown in Figure 2, thymol (1 and 2 mM) treatment led to an obvious decrease in HT29 cell migration across the membrane, compared to the control group.

Effect of thymol on cell invasion

HT29 cells were added into the upper chamber of Transwell insert pre-coated with Matrigel. After treatment with thymol (0.5, 1 and 2 mM) for 24 h, there was a concentration-dependent decrease on the invasion of HT29 cells, compared to the control (Figure 3).

Effect of thymol on MMP-2 and MMP-9 activities

We evaluated the effects of thymol on extracellular matrix degradation catalyzed by MMPs with gelatin zymography assay. Figure 4 showed that thymol (0.5, 1 and 2 mM) decreased the activity of MMP-2 and MMP-9 in a concentration-dependent manner.

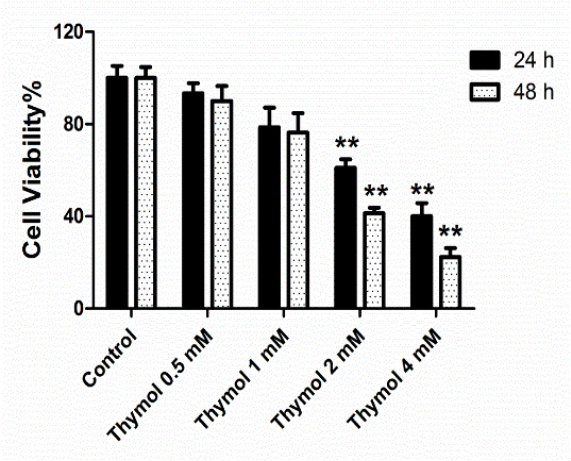

Figure 1: Effect of thymol on the cell viability in HT29 cells. HT29 cells were incubated with thymol (0.5, 1, 2, and 4 mM) for 24 and 48 h, and cell viability was examined by MTT assay. Data are expressed as mean ± SEM (n = 3); *$p < 0.05$, **: $p < 0.01$ *vs* control

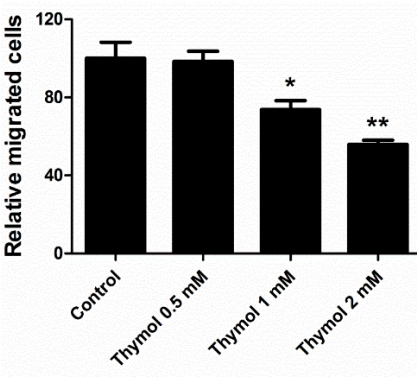

Figure 2: Effect of thymol on cell migration. HT29 cells were incubated with thymol (0.5, 1 and 2 mM) for 24 h, and cell migration was analyzed by Transwell assay. Migrated cells were stained with Crystal violet and the numbers of migrated cells were determined by absorbance at 540 nm. Data are expressed as mean ± SEM (n = 3); *$p < 0.05$, **$p < 0.01$ *vs* control

Table 1: Primer sequences of β-actin used in real-time PCR

arget gene	Forward primer 5'-3'	Reverse primer 5'-3'
-cadherin	GAGCCTGAGTCCTGCAGTCC	GTATTGCTGCTTGGCCTCA
mentin	AAAGTGTGGCTGCCAAGAAC	AGCCTCAGAGAGGTCAGCAA
-SMA	GGGTACCACCATGTACCCA	CACAGTTGTGTGCTAGAGGC
nail	CCCCAATCGGAAGCCTAACT	CGTAGGGCTGCTGGAAGGTA
ug	CCATTCCACGCCCAGCTA	CTCACTCGCCCCAAAGATGA
β-actin	CCAACCGCGAGAAGATGA	TCCATCACGATGCCAGTG

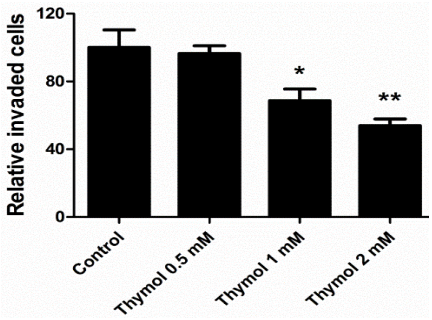

Figure 3: Effect of thymol on cell invasion. HT29 cells were treated with thymol (0.5, 1 and 2 mM) for 24 h, and cell invasion was detected by Transwell assay. Invaded cells were stained with Crystal violet and the numbers of invaded cells were obtained by absorbance at 540 nm. Data are expressed as mean ± SEM (n = 3); *$p < 0.05$, **: $p < 0.01$ *vs* control

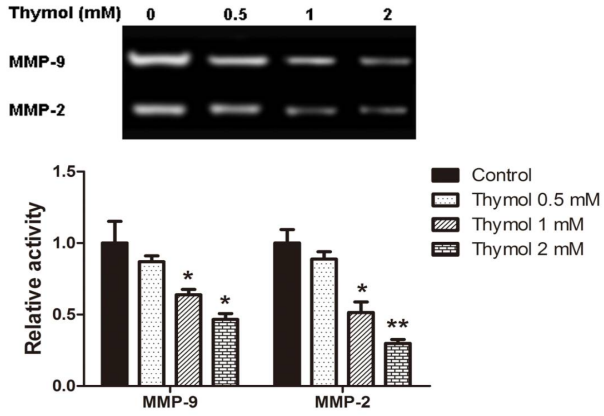

Figure 4: Effect of thymol on MMP-2 and MMP-9 activities. HT29 cells were treated with thymol (0.5, 1 and 2 mM) for 24 h. MMP-2 and MMP-9 on the degradation of gelatin were assessed. Data are expressed as mean ± SEM (n = 3); *$p < 0.05$, **: $p < 0.01$ *vs* control

Effect of thymol on epithelial-mesenchymal transition (EMT)

Whether thymol plays important roles in EMT of colon cancer cells HT29, we analyzed the mRNA expression of major EMT biomarkers, including E-cadherin, Vimentin, α-SMA, Snail and Slug by using real-time PCR. As shown in Figure 5, thymol (2 mM) markedly increased the mRNA expression of E-cadherin, while decreased the mRNA expression of α-SMA, Vimentin, Snail and Slug.

Effect of thymol on the PI3K/AKT and ERK pathway

To find out whether the effect of thymol on HT-29 cell invasion and migration involves PI3K/AKT or MAPK/ERK pathway, Western blot was performed to evaluate the protein levels of certain markers, including p-AKT and p-ERK. The results showed that thymol markedly decreased the phosphorylation levels of AKT and ERK, while it had no significant effect on the total levels of AKT and ERK in a concentration-dependent manner (Figure 6).

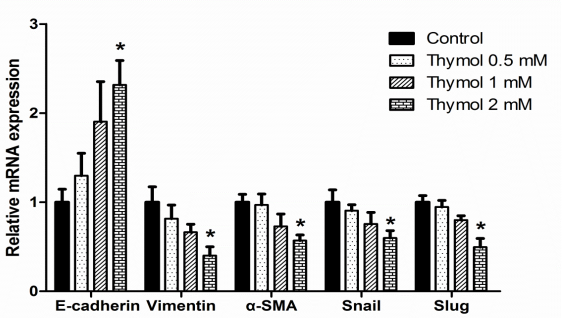

Figure 5: Effect of thymol on the mRNA expression of EMT-associated genes. HT29 cells were treated with thymol (0.5, 1 and 2 mM) for 24 h. Total RNA were extracted and the mRNA expression of E-cadherin, Vimentin, α-SMA, Snail and Slug were measured by real-time PCR assay. Gene expressions were normalized to β-actin. Data were expressed as means ± SEM of three independent experiments *: $P < 0.05$, **: $P < 0.01$ *vs* control

DISCUSSION

Metastasis is one of the major reasons of high mortality in CRC patients [15]. EMT, a process that tumor cell migrated and invaded from the surrounding tissue to the circulation, is characterized as the early step of the metastatic process [16]. Therefore, a compound that can effectively restrain cancer cell migration and invasion has the potential to be developed as a candidate drug for preventing or treating metastatic cancers. In this study, thymol significantly inhibited the migration and invasion in HT29 cells. It decreased the activity of MMP-2 and MMP-9. The mechanisms may involve the inhibition of EMT and downregulation of the activation of PI3K/AKT and ERK signaling pathways.

Invasion and migration have been acknowledged as the most lethal attributes of solid tumors and account for the majority of metastases [17]. Tumor cells have the ability to migrate from the original site to the blood and lymph, and invade surrounding or distant tissues, causing metastasis. Our results from the present study showed that thymol could suppress migration and invasion in HT29 cells.

MMPs, a group of zinc-dependent endopeptidases, are important mediators of invasion and degradation of basement membranes and extracellular matrix [18]. MMP-2

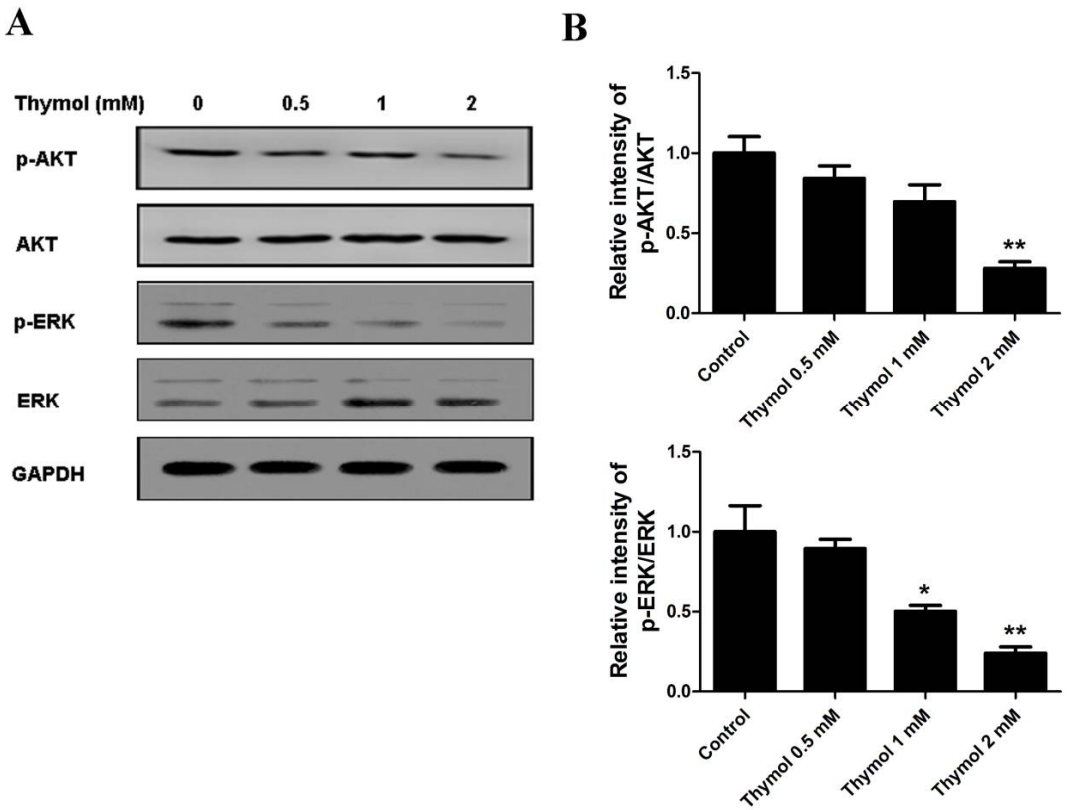

Figure 6: Effect of thymol on the activation of PI3K/AKT and ERK pathways. HT29 cells were treated with thymol (0.5, 1 and 2 mM) for 24 h. (A) Cells were harvested and lysed, and the levels of p-AKT, AKT, p-ERK, ERK and GAPDH were assessed by Western blot. (B) Densitometry analysis of immunoblotting was also shown. Data were presented as means ± SEM of three independent experiments *: $P < 0.05$, **: $P < 0.01$ *vs* control

and MMP-9 abundantly expressed in various cancers, are considered to play key roles in tumor invasion and metastasis [19]. In addition, mounting evidence suggests that inhibition of MMP-9 and MMP-2 by chemopreventive agents suppresses the invasiveness and metastases of many cancer cells [20,21]. In the present study, thymol (1 and 2 mM) markedly reduced the activity of MMP-2 and MMP-9 in HT29 cells.

Numerous studies have reported the correlation between EMT and cancer progression and metastasis [22]. Epithelial-derived tumor cells become malignant and obtain an invasive phenotype is mainly through an EMT process [23]. Several molecular markers, the down-regulation of epithelial cell surface marker E-cadherin, the up-regulation of mesenchymal markers vimentin and α-SMA, and the EMT-inducing transcription factors such as Snail and Slug, are the representative phenotypes of EMT [24]. Our results revealed that thymol inhibited EMT, evidenced by increasing expression of E-cadherin, decreasing the expression of vimentin and α-SMA, and associated transcription factors Snail and Slug.

PI3K/AKT pathway overactivation is frequently present in CRC and is associated with tumor progression processes, including cell proliferation, migration and invasion [25]. It has been shown to contribute to tumor metastasis by promoting the secretion of MMPs and the induction of EMT [26]. It reported that MAPKs, such as ERK seem to play a central role in regulating the expression of MMPs, inhibition of the MAPK pathway might also potentially prevent invasion and metastasis of a variety of tumors [27]. The Western blotting results suggested that thymol could significantly inhibit the activation of AKT and ERK in HT29 cells.

CONCLUSION

This study has demonstrated that thymol is able to inhibit the migration and invasion of HT29 human colon cancers, and reduce the activity of MMP-2 and MMP-9. The mechanisms may involve the inhibition of EMT and downregulation of the activation of PI3K/AKT and ERK signaling pathways. These results provided new insights into the anti-cancer mechanisms of thymol, which may be helpful in the development of thymol into a promising therapeutic agent against colorectal carcinoma.

DECLARATIONS

Conflicts of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

Ran Lv and Zhenzhou Chen conceived and designed the study; collected and analyzed the data; and wrote the manuscript. Both authors have read the manuscript and approved for publication.

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