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Dihydromyricetin Inhibits Cell Invasion and Down-regulates MMP-2/-9 Protein Expression Levels in Human Breast Cancer Cells*

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Abstract Dihydromyricetin (3, 5, 7, 3′, 4′, 5′-hexahydroxy-2, 3-dihydroflavonol, DMY) isolated from *Ampelopsis grossedentata* is known to have an anti-proliferative effect *in vitro* on a few cancer cells. However, the exact mechanisms retain unclear. The aim of this article was to study the effect of DMY on cell invasion of highly metastatic human breast cancer MDA-MB-231 cells, and investigate the possible mechanisms. The anti-proliferation effect of DMY on MDA-MB-231 cells was determined by the MTT assay. The gelatinolytic activity was assessed by gelatin zymography. The mRNA and the protein expression levels of MMP-2/-9 were evaluated by Real-time PCR and Western blot assay, respectively. The effect of DMY on cell invasion was observed by a transwell model. Results indicated that DMY inhibited the proliferation of MDA-MB-231 cells in a dose-dependent manner, and the IC_{50} value was about 73.6 mg/L after treated by DMY for 48 h. DMY suppressesed gelatinase activity and MMP-2/-9 protein expression levels in a dose-dependent manner, and restrained MMP-2/-9 mRNA expression levels. In addition, DMY inhibited the invasion of MDA-MB-231 cells in a dose-dependent manner without cytotoxicity against the cancer cells. DMY can inhibit proliferation and invasion of MDA-MB-231 cells, and invasion inhibition may be related to the down-regulation of the expression levels of MMP-2/-9 proteins.

Key words dihydromyricetin, breast cancer, cell invasion, matrix metalloproteinases
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Cancer metastasis is the major cause of death for cancer patients. Many clinical evidences indicated that the cure rate has a great decline once breast cancer cells leave the primary site, and the 5-year survival rate has decreased to 25%～30% of lymph node-positive patients from 70%～75% of lymph node-negative patients [1]. Therefore, the suppression of tumor cells invasion and metastasis would be the critical factor for breast cancer and other malignant tumor therapy systems. Matrix metalloproteinases (MMPs), which are a family of secreted or membrane-associated proteins capable of digesting extracellular matrix components, are thought to play an essential role in the facilitation of cancer metastasis [2]. Among MMPs, it has been suggested that gelatinases/type Ⅳ collagenases (MMP-2/-9), which are secreted by invasive cancer cells, are important in cancer cell invasion and metastasis because tumor cells must cross the type Ⅳ collagen-rich basement membrane of the vessel wall to spread to sites distant from the primary tumor[3-4].

Dihydromyricetin (3, 5, 7, 3′, 4′, 5′-hexahydroxy-2, 3-dihydroflavonol, DMY), abundant in the tender stem and leaves of *Ampelopsis grossedentata*

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(Hand-Mazz)[5], is an important dihydroflavonol. It has recently been reported that DMY has various physiological activities, such as anti-oxidation, anti-inflammatory, anti-mutation, relieving cough, removing sputum, anti-bacterial action [6], and anti-proliferative and anti-cancer effects in several cancer cell lines[7–10]. Several mechanisms have been suggested to explain these effects, including cell cycle arrest [11], cell apoptosis[8], as well as interaction with enzymes or proteins involved in cancer progression (*e.g.*, telomerase) [12–13]. Furthermore, it has been found that DMY inhibits the adhesion of B16 mouse melanoma cells to fibronectin, laminin, or matrigel, and inhibits the invasion into the reconstituted basement membrane [14–15].

However, it is still not fully understood about the precise mechanism by which DMY exerts its anti-cancer effect, and also there is no information about the effect of DMY on the growth of human breast cancer MDA-MB-231 cell with highly metastasis. The purpose of this study was to examine the anti-proliferative effects and invasion- inhibiting properties of DMY in human breast cancer (MDA-MB-231) cells. We found that DMY inhibited cell proliferation and cell invasion, which may be related to the down-regulation of MMP-2/-9 protein expression levels both in the extracellular matrix and in the intracellular space.Those results will enlarge the clinical application of DMY.

1 Materials and methods

1.1 Materials and reagents

Dihydromyricetin (>95% of purity by HPLC) was isolated from the tender stem leaves of Ampelopsis grossedentata, purified by recrystallization, and detected and identified by high performance liquid chromatography, ultraviolet spectrometry, infrared spectrometry and mass spectrum [16]. MTT and DMSO were purchased from Sigma Chemical Company. Highly metastatic human breast cancer cell lines (MDA-MB-231) were obtained from the American Type Culture Collection, ATCC. The BCA Protein assay kit was purchased from Beyotime Institute of Biotech (Shanghai, China). PVDF membrane and Super Signal West Dura were purchased from Pierce (USA) and polyclonal primary antibodies (ab38898, human anti-MMP-9 and ab37150, anti-MMP-2) were obtained from Abcam Biotechnology (Cambridge, MA, USA). Anti-GAPDH monoclonal primary antibody and the horseradish peroxidase-conjugated rabbit anti-human antibody were purchased from Boster Biological Technology, LTD (Wuhan, China).

1.2 Cell culture

Stock culture of MDA-MB-231 cells were cultured in 75-cm^2 flasks and were incubated in DMEM/F12 (Gibco, USA) supplementing with 10% fetal bovine serum (Gibco, U.S.A.), 2 mmol/L L-glutamine and antibiotics in a humidified incubator with 5% CO_2 at 37℃.

1.3 Cell viability assay

Effect of DMY on the viability of MDA-MB-231 cells was determined by MTT assay following the method of Mosmaan[17]. 5000 cells per well were plated in 96-well plates and incubated for 12 h. Thereafter, DMY was added with the indicated concentrations of DMY. After 24 h or 48 h exposure, media were removed, 20 μl of MTT (5 g/L) was added and incubated for 4 h at 37℃. Then MTT was removed and the formazan crystals were solubilized with 200 μl DMSO for 5 min. Absorbance was measured at 570 nm with a Bio-Rad microplate reader. The percent viability was calculated by comparing the absorbance of the treated cells to the control. Then, IC_{50} value of DMY was calculated[18].

1.4 Gelatin zymography

The gelatinase secretion was examined by gelatin zymography according to the protocol described previously [19]. Briefly, 2 ml MDA-MB-231 cells suspension (1×10^5 viable cells/ml) were plated in 6-well plates. Subconfluent monolayers were cultured for 48 h in the presence of different concentrations of DMY. Supernatant samples were quantitated by BCA protein assay kit, and equal amounts of protein (23 μg/sample) were loaded with a nonreducing sample buffer onto a 12% SDS-PAGE containing 1 g/L gelatin and electrophoresed. After removal of SDS from the gels by washing with 2.5% Triton X-100 solution for 30 min twice and 50 mmol/L Tris-HCl buffer (pH 7.6) for 10 min with shaking, the gels were incubated in 50 mmol/L Tris-HCl (pH 7.5) containing 0.5 mmol/L $CaCl_2$ and 0.1 μmol/L $ZnCl_2$ at 37℃ for 48 h to activate MMPs. The activated gels were stained with 0.1% coomassie Blue R-250 (in 10% methaanol and 5% acetate) and subsequently destained with 10% methanol and 5% acetate solution. The gelatinolytic activity of each gelatinase was detected as a clear band

against a blue background.

1.5 Real-time RT-PCR analysis

The cell were grown to about 50% confluence in 6-well plates and treated with DMY(20, 40 and 80 mg/L) for 48 h. One micrograms of total RNA from each sample were subjected to reverse transcription by using the SYBR PrimeScript RT-PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. Each real-time PCR was carried out in triplicate in a total of 20 μl reaction mixture (1 μl of cDNA, 10 μl of SYBR Premix Ex Taq, 0.4 μl of each 10 μmol/L forward and reverse primers, and 8.2 μl of ddH_2O) on BIORAD Chrom 4 Real-time PCR System. Primers used for real-time PCR analysis were presented in Table 1. The PCR program was initiated by 30 s at 95℃ before 40 thermal cycles, each of 5 s at 95℃, 15 s at 61℃ and 15 s at 72℃. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method [20] and were normalized to 18 S rRNA expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

Table 1 Primers used for real-time PCR analysis

Gene	Primer sequence	PCR product
MMP-9	5′ GAGGTTCGACGTGAAGGCGCAGATG 3′	200 bp
	5′ CATAGGTCACGTAGCCCACTTGGTC 3′	
MMP-2	5′ TGACATCAAGGGCATTTCAGGAGC 3′	180 bp
	5′ GTCCGCCAAATGAACGGTCCTTG 3′	
18 S rRNA	5′ AATTGACGGAAGGGCACCAC 3′	153 bp
	5′ CACCAACTAAGAACGGCCATG 3′	

1.6 Western blot analysis

Western blot analysis was done to determine the expression of MMP-2/-9 proteins. MDA-MB-231 cells were treated with different concentrations of DMY (20, 40 and 80 mg/L) for 48 h. Cells were harvested, washed with cold PBS (pH 7.4) and lysed with ice-cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mmol/L PMSF, pH 7.4) for 30 min. The cell lysates were transferred to microfuge tube and centrifuged at 20 000 *g* for 30 min at 4℃. The supernatant (total cell lysate) was used immediately or stored at –80℃. Sample protein concentration was measured *via* the BCA protein assay strictly following the manufacturer's instructions. Equal amounts of protein (25 μg) were loaded onto a 12% SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in blocking buffer (5% nonfat dry milk in 20 mmol/L TBS, pH 7.6) for 1 h at room temperature, and subsequently incubated overnight at 4℃ with primary antibody. Then the membranes were incubated with secondary antibody conjugated to horse radish peroxidase for 1 h at room temperature. Chemiluminescence was detected using the ECL reagent according to the manufacturer's protocol. Different exposure times were used to ensure that bands were not saturated. For detection of GAPDH, the same membranes were incubated with rabbit polyclonal anti-GAPDH antibody overnight at 4℃ and processed as described. Densitometric measurements were performed using Image J 1.42q (Wayne Rasband National Institutes of Health, USA) to quantify the intensity of the bands from there independent Western blots.

1.7 Cell invasion assay

Cell invasion assay was performed in 12-well plates receiving 8 μm pore size polyethylene terephtalate membrane cell culture insert (Millipore), coated with 25 μg/ filter Matrigel (Beckton Dickinson). Cells growing in 100-mm Petri dishes were exposed to the indicated concentrations of DMY. After 24 h exposure, the cells were harvested by a mild trypsinization and were washed twice with FBS-free medium (1% BSA) and counted. Cells (8×10^4 viable cells/sample) were allowed to invade matrigel toward 10% FBS DMEM/F12 medium at 37℃, 5% CO_2. After 24 h, the remaining cells were removed from the upper side of

the membrane, and cells on the lower surface of the filter were fixed in methanol for 20 min, stained with crystal violet for 5 min, and 5 fields/ filter were counted at 100 × magnification. Data represent the average, and all performed in triplicate in 3 separate experiments (control group, 10, 20 and 40 mg/L DMY-treated groups). For easier comparison between groups, results obtained were normalized to the control condition.

1.8 Statistical analysis

All data were expressed as the $\bar{x} \pm s$ from three independent experiments. Statistical significance was determined with One-way ANOVA and Student's t test. Results were considered significant at $P < 0.05$.

2 Results

2.1 Effect of DMY on proliferation of MDA-MB-231 cells

After 48 h exposure, DMY decreased the proliferation of MDA-MB-231 cells significantly in a dose-dependent manner, however, the cell viability was not affected when exposed to 20 or 40 mg/L DMY for 24 h (Figure 1). The IC_{50} value is about 73.6 mg/L after treated by DMY for 48 h.

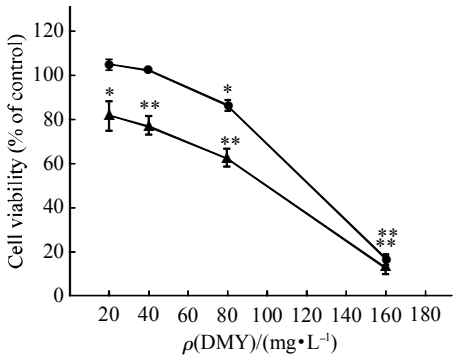

Fig. 1 Effect of DMY on the viability of MDA-MB-231 cells

*$P < 0.05$ and **$P < 0.01$ versus control groups, n=3.●—●: 24 h; ▲—▲: 48 h.

2.2 Effect of DMY on the gelatinolytic activity

To clarify whether DMY affect the gelatinolytic activity of MDA-MB-231 cells, we performed gelatin zymography. Subconfluent monolayers of MDA-MB-231 cells were cultured for 48 h in the presence of different concentrations (20, 40 and 80 mg/L) of DMY. The samples of the conditioned media were analyzed by gelatin zymography. DMY reduced the gelatinolytic activity of the 92 ku MMP-9 protein (gelatinase B) and the 72 ku MMP-2 protein (gelatinase A) in a dose-dependent manner (Figure 2).

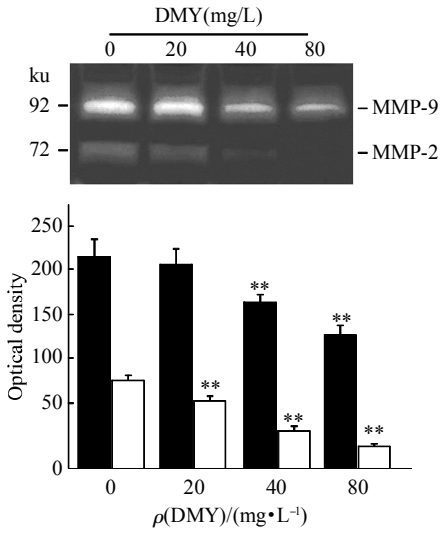

Fig. 2 Gelatin zymogram showing the effects of DMY on the gelatinolytic activities

**$P < 0.01$ versus control groups, n=3. ■: MMP-9; □: MMP-2.

2.3 Real-time RT-PCR analysis

To determine whether DMY affect the alteration of MMP-2/-9 mRNA levels in MDA-MB-231 cells, we conducted real-time RT-PCR analysis. After treated with 20 and 40 mg/L of DMY for 48 h, the MMP-9 mRNA expression levels were decreased 2.1 and 1.8 folds, and the MMP-2 mRNA levels were decreased 1.7 and 1.2 folds, respectively.However, treated with 80 mg/L of DMY for 48 h, MMP-9 and MMP-2 mRNA expression levels were elevated 2.7 and 3.5 folds, respectively (Figure 3).

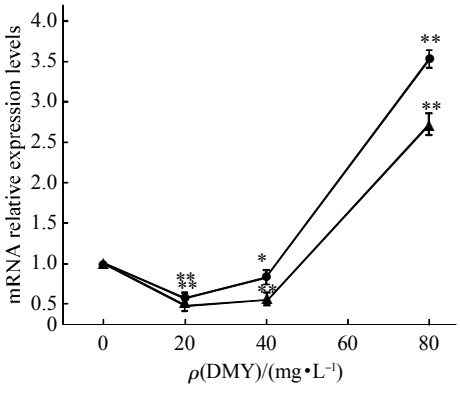

Fig. 3 MMP-2/-9 mRNA relative expression levels after treated with different concentration of DMY

*$P < 0.05$ and **$P < 0.01$ versus control groups, n = 3. ●—●: MMP-2; ▲—▲: MMP-9.

2.4 Western blot analysis

In order to verify whether the alterations of MMP-2/-9 mRNA expression levels accompanied with the change of protein expression levels, we conducted Western blot analysis. As shown in Figure 4, DMY down-regulated MMP-2/-9 protein expression levels in a dose-dependent manners, both in media and in intracellular.

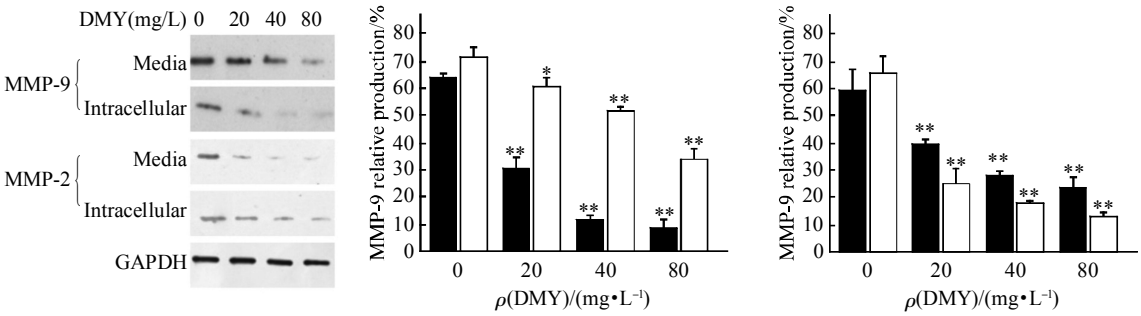

Fig. 4　Effect of DMY on the expression of MMP-2/-9 detected by Western blot

*P < 0.05 and **P < 0.01 versus vihicle-treated groups, n = 3. ■ : Intracellula; □ : Media.

2.5 Effect of DMY on invasion of MDA-MB-231 cells

Next we evaluated the effect of DMY on the invasion of MDA-MB-231 cells by a transwell model. 10, 20 and 40 mg/L DMY were employed because the cells viability was not affected when exposed to it for 24 h. Subconfluent monolayers of MDA-MB-231 cells were cultured for 24 h in the presence of DMY. Cells were harvested, counted and allowed to invade Matrigel for 24 h. The results shown that DMY dramatically suppressed the invasion of MDA-MB-231 cells (Figure 5). After 24 h incubation with the indicated concentrations of DMY, the number of migrating cells were decreased to (85 ± 9), (56 ± 4) and (23 ± 3) from (130 ± 11) of control group. The relative invasion of MDA-MB-231 cells was 65.4%, 53.1%, 17.7% respectively, compared with control group (Figure 5e).

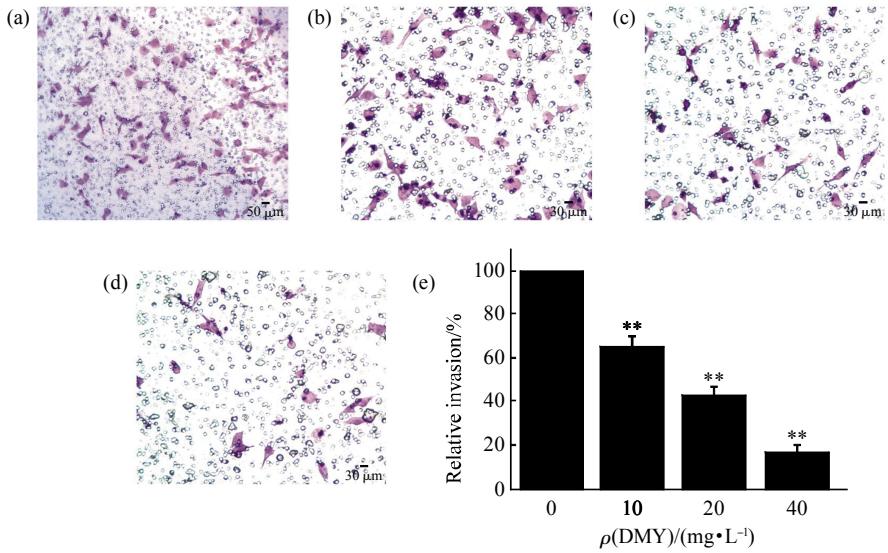

Fig. 5　Effect of DMY on invasion of MDA-MB-231 cells

After 24 h incubation, the invated cells (bottom) under light microscope: (a) Control group; (b) 10 mg/L; (c) 20 mg/L; (d) 40 mg/L. The cells were counted at 100× magnification. (e) Relative invasion rate (normalized to vehicle-treated cell as 100) from three duplicate experiments are shown. **P < 0.01, compared with the control.

3 Discussion

DMY, occurring abundantly in the tender stem and leaves of *Ampelopsis grossedentata*, is the principal biochemical components of Tengcha, which is an ancient plant used as traditional Chinese medicine and tea among the populace of south China. Then content of DMY was more than 27% in the tender stem leaves of this species, especially, more than 40% in the cataphyll [6]. Very recently, the anti-cancer action of DMY is drawing increasing attention for its natural and low toxicity. Previous researches mainly focused on the apoptosis induction and anti-proliferation action. However, the precise mechanism of anti-tumor is still unclear.

It has been shown DMY inihibits the adhesion of B16 mouse melanoma cells to fibronectin, laminin, or matrigel, and inhibits the invasion into the reconstituted basement membrane [14-15]. In this paper, we demonstrated that DMY could inhibit the proliferation and invasion of human breast cancer (MDA-MB-231) cells with highly metastasis in a dose-dependent manner. Our other research results showed that DMY suppressed the proliferation and invasion of 4T1 mice breast carcinoma cells *in vitro*, and distant pulmonary metastasis *in vivo* can be inhibited by DMY administration (data not shown). Those results confirmed further the anti-proliferation and the anti-invasion potential of DMY.

Many factors could affect invasion and metastasis. However, the alteration of MMPs expression, especially the up-regulation of MMP-2/-9 expression, and the enhencement of degradation ability of Ⅳ collagen in basement membrane are the key steps of cancer cell invasion and metastasis. MMP-2/-9 appear to have clinical value as diagnostic factors for breast cancer or predictive factors of metastasis. Previous research found that the expression of both MMP-2 and MMP-9 protein were enhanced in breast cancer tissue, which led to the enhancement of ECM degradation and tumor metastasis[21-22]. The correlation between tumor cell activation efficiency of MMPs and invasion through basement membrane-like matrigel barriers had been proved [23]. In this paper MDA-MB-231 cells were treated with DMY (0, 20, 40 and 80 mg/L), then the expression of MMP-2/-9 genes were detected. Results shown that MMP-2/-9 proteins expression both in extracellular matrix and in intracellular space were decreased significantly by DMY treatment with a dose-depended manner, however the MMP-2/-9 mRNA levels firstly decreased then increased sharply with the elevation of DMY concentration. These results implied that MMP-2/-9 proteins expression and mRNA expression could be associated *via* a negative- feedback regulatory mechanism.

As far as the concentration of DMY is concerned, MDA-MB-231 cells viability was not affected, while the cell invasion was inhibited when exposed to DMY of 10, 20 or 40 mg/L for 24 h. These results suggest that the cancer cell invasive inhibitory effect of DMY is not caused by cytotoxicity.In conclusion, DMY can reduce the secreted gelatinase activities and inhibit invasion of MDA-MB-231 cells *in vitro*, which may be related to the down-regulation of MMP-2/-9 protein expression levels instead of mRNA expression levels. And it implies DMY could be the agent with potential antimetastasis activity because it inhibited invasion in the absence of cytotoxicity.

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二氢杨梅素抑制人乳腺癌细胞侵袭和下调 MMP-2/-9 蛋白表达研究 *

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摘要 藤茶活性成分二氢杨梅素(3, 5, 7, 3′, 4′, 5′- 六羟基 -2, 3- 二氢黄酮醇，DMY)体外对几种癌细胞具有抗增殖作用，但机制尚未完全清楚．本文研究 DMY 对人高转移型乳腺癌 MDA-MB-231 细胞侵袭的影响，并探讨可能的机制．用 MTT 法检测 DMY 对 MDA-MB-231 细胞的增殖抑制率；明胶酶谱分析明胶酶活力；基质金属蛋白酶(MMP-2/-9)的基因表达水平和蛋白质表达水平分别利用实时定量 PCR 和 Western blot 分析进行检测．Transwell 模型检测 DMY 对肿瘤细胞侵袭的影响．结果显示，DMY 以剂量依赖方式抑制 MDA-MB-231 细胞的增殖，作用 48 h 的 IC_{50} 为 73.6 mg/L．DMY 显著抑制明胶酶活性和 MMP-2/-9 蛋白表达，并抑制 MMP-2/-9 的 mRNA 表达水平．此外，DMY 不依赖细胞毒作用和以剂量依赖方式抑制 MDA-MB-231 细胞的侵袭．这些结果提示：DMY 能显著抑制人乳腺癌 MDA-MB-231 细胞的侵袭和增殖, 其侵袭抑制的机制可能与其下调 MMP-2/-9 蛋白表达水平相关.

关键词 二氢杨梅素，乳腺癌，细胞侵袭，基质金属蛋白酶
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