**Abstract**

**Background:** Breast cancer is known to be one of the most prevalent malignancies in women worldwide. Umbelliprenin is a naturally-occurring component derived from plant species, which has shown anti-cancer properties. The present study aimed to evaluate the effect of umbelliprenin on the PI3K / Akt / ERK signaling pathway and their products HIF1α / VEGF in the MDA-MB-231 cell line.

**Method:** In this experimental study, the cytotoxic effect of umbelliprenin on MDA-MB-231 cells was evaluated using the MTT assay and the umbelliprenin concentrations of IC5 and IC10 were selected for the signaling pathway study. MDA-MB-231 cells were stimulated with EGF and CoCl2 and umbelliprenin IC5 and IC10 effects on gene expression and translation was studied. PI3K / Akt / mTOR / S6K / Erk1 and 2 / 4E-BP1 / HIF-1α / HIF-1β / EGFR / VEGFR and VEGF mRNA expression, and VEGF / HIF-1α proteins were evaluated employing real time PCR and western blot analysis, respectively.

**Results:** The concentrations of umbelliprenin in IC10 and IC5 were 20 and 10 μM, respectively. Umbelliprenin, specifically IC10, significantly inhibited PI3K, ERK1, ERK2, Akt, mTOR, HIF1-β, HIF1-α mRNA, as well as HIF-1α and VEGF protein expression.

**Conclusion:** Our results suggested that UMB, a cytotoxic agent, inhibits PI3K / Akt / ERK signal pathway in the CoCl2 or EGF-stimulated MDA-MB-231 cells.

**Keywords:** Umbelliprenin, MDA-MB-231, Cobalt Chloride, EGF, HIF1α, VEGF, Angiogenesis, Cancer
Introduction
Breast cancer is one of the most prevalent malignancies in women worldwide. To date, a lot of research has been done in order to find new strategies for breast cancer treatment. Anti-angiogenic drugs have opened a promising window in this regard. Angiogenesis is defined as the formation of new blood vessels from pre-existing vessels that occur under both physiological and pathological conditions and ensure the supply of oxygen and nutrients to tissues. In normal conditions, such as wound healing, angiogenesis is a critical stage for tissue repair. However, in pathological circumstances, such as solid tumors, an increase in blood vessels in the tumor environment will significantly increase the spread of cancer cells. Angiogenesis may therefore be the ideal target for the treatment of tumors.

Angiogenesis is a multistep process regulated by a large number of pro-angiogenic and anti-angiogenic factors. A delicate balance between the secretion of numerous angiogenic factors, such as VEGF and EGF, in addition to activation of various transcriptional factors, such as HIF-1, play an essential role in promotion of angiogenesis. In certain physiological conditions, the changes in the microenvironment of the cells directly or indirectly affect the secretion of angiogenic factors. Hypoxia is a physiological situation defined through a decrease in oxygen levels in microenvironmental cells, leading to the activation of pro-angiogenic signaling pathways. HIF-1 is one of the pro-angiogenic transcription factor members of HIF family, which is activated by hypoxia. This heterodimer transcription factor contains two different subunits, namely HIF-1α and HIF-1β. In the presence of oxygen (normal condition), the two proline residues of HIF-1α (P402/P564) are hydroxylated by prolyl hydroxylase domain (PHD) enzyme, leading to binding to the von Hippel-Lindau tumor suppressor (pVHL) and eventually ubiquitination and degradation of HIF-1α. Once hypoxia occurs, the two subunits of HIF-1 form an activated heterodimer that binds to the hypoxia-responsive elements of various genes whose products are involved in angiogenesis, including VEGF and its receptors. The HIF-1α up-regulation also occurs in response to other growth factors and cell signaling pathways. It has been reported that EGF elevates the expression of HIF-1α. Ligand binding to extracellular domains of EGF receptor and other tyrosine kinase receptors trigger various intracellular signaling pathways, including the PI3K / AKT / mTOR, RAS / RAF / MEK / MAPK, and STAT pathways.

HIF-1α and VEGF are common in the angiogenesis and metastasis. A number of recent studies have provided compelling evidence of the close association between elevated HIF-1α levels and tumor metastases. HIF-1α promotes tumor metastases to distant and more oxygenated tissues by transcriptional activation of oncogenic growth factors, for instance, transforming growth factor beta 3 (TGF-β3) and the epidermal growth factor (EGF). Overall, HIF-1α activation in tumor cells is one of the key masters of their adaptation mechanisms to the hypoxia environment. In brief, on account of the vital role of HIF-1α transcription factor in angiogenesis, it has been a favorable target in new cancer therapeutic approaches. Today, a great deal of scientific attention has been drawn to development of the effective drugs against HIF-1α transcription factor. Over the recent years, several attempts have been made to find the effective herbal components with a desirable anti-angiogenic effect. Umbelliprenin (UMB), a prenylated coumarin, is a naturally-occurring component in various plant species, such as coriander, celery, lemon, angelica, and
especially ferula species with the molecular formula of C24H30O3.20 UMB possess different characteristics, including anti-tumor, anti-oxidant, and anti-inflammatory properties, as well as lipoxygenase and acetylcholinesterase inhibitory effects.18, 21 So far, various studies have proven the anti-tumor effects of UMB.22, 23 It has been found that in mouse models of breast and colorectal cancer, UMB exerts its anti-tumor activity through inhibition of various proteins involved in angiogenesis and metastasis, including VEGF, MMP9, and MMP2.24, 25 However, the exact mechanism for the role of UMB has not yet been explained. The MDA-MB-231 is a triple-negative cell line of breast cancer. Triple-negative breast cancer (TNBC) is clinically defined by the absence of estrogen receptor (ER) and progesterone receptor (PR) positivity and the lack of HER2 overexpression by immunohistochemistry.2 To date, no research has been conducted on the effect of UMB on this cell line. Our previous study on UMB effects on angiogenesis markers was conducted in vivo and this is a confirmatory in vitro study of UMB effects on angiogenesis signaling pathways. Throughout this in vitro research, non-toxic concentrations of UMB (IC5 and IC10) were used to investigate the impact of umbelliprenin on the mechanism of angiogenesis by examining genes and proteins involved in the formation of VEGF in the human TNBC cell line MDA-MB-231 (Scheme I).

Materials and Methods

Materials
This experimental in vitro study was performed on the MDA-MB-231 human breast cancer cell line purchased from the Iranian Biological Resources Center (IBRC). This triple-negative breast cancer cell line was derived from human breast adenocarcinoma. MDA-MB-231 cells were routinely cultured in RPMI medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and were incubated at 37 °C, 95% humidified air, and 5% CO2. RPMI medium, FBS, antibiotics, and trypsin-EDTA were provided from Gibco BRL (Grand Island, NY, USA). UMB (C24H30O3, MW: 366) was purchased from Department of Pharmacognosy and Biotechnology of Mashhad University of Medical Sciences, Iran. Umbelliprenin was dissolved in DMSO at different concentrations (μM) and stored at -20°C. Immediately before use, they were diluted in the culture medium to obtain a final DMSO concentration of 0.25% (v/v). Recombinant human EGF proteins and Cobalt Chloride (CoCl2) were purchased from Sigma-Aldrich Merck (Dublin, Ireland) and Sigma-Aldrich Merck KGaA (Darmstadt, Germany), respectively. Preparations were done according to the manufacturer’s instructions.

Cell viability assay (MTT Assay)
Cell viability was determined using 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent (BioBasic, Canada). Briefly, approximately 5 x 103 breast cancer MDA-MB-231 cells were seeded in 96-well plates and incubated for 24 h at 37 °C under 5% CO2. The cultured cells were treated with different concentrations of UMB, including 200, 100, 50, 25, 12.5, 6.2, and 3.1 μM for 24, 48, and 72 h. The cells treated with the medium only (RPMI medium containing 10% FBS, 1% penicillin-streptomycin) served as a negative control group. At the end of the incubation time, the supernatant of each well was removed and the cells were washed with PBS. Afterwards, 20 μl of MTT solution (5 mg/ml MTT) was added to each well and incubated for 4 h at 37 °C (final MTT concentration was 0.5 mg/mL). The resultant formazan crystals were dissolved
in 100 μl of dimethyl sulfoxide (DMSO) Merck (Germany) and absorbance intensities were measured in a microplate reader (biotek, USA) at 550 nm with a reference wavelength at 630 nm. We performed all the experiments in triplicate. The effects of UMB on cell viability were assessed as the percentage of viable cells compared with the control cells. IC<sub>50</sub> was calculated and IC<sub>5</sub> and IC<sub>10</sub> of UMB were utilized for angiogenesis study (10 and 20 μM). According to previous studies, the number of cells in these concentrations does not decrease. In fact, the reason behind selecting these concentrations from UMB is that 90-95% of the cells are still alive.

**Treatment groups**

When MDA-MB-231 breast cancer cells reached 80% of confluence, the cells were serum starved overnight. They were then stimulated with EGF (20 ng/ml) or CoCl<sub>2</sub> (50 μM) for 30 min, followed by post-treatment with different concentrations of UMB (10 and 20 μM) for 24 h. The groups that were not treated with EGF or CoCl<sub>2</sub> were considered as the control groups.

**Real time PCR**

Total cellular RNA was extracted from the cultured cells using the RNeasy kit (BioBasic, Canada), according to the manufacturer’s protocol. The extracted RNAs were quantified with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and then stored at -70 °C. cDNA was synthesized using a PrimeScript ® RT reagent kit (Takara Bio, Inc., Otsu, Japan) based on the random hexamer priming according to the manufacture instructions. The cDNA synthesis was carried out at 37 °C for 15 minutes, 85 °C for 5 second, and 4 °C for 5 minutes. The cDNA was stored at -20 °C.

To determine the expression levels of EGF, HIF-1α, 4E-BP1, EGFR, PI3K, Akt, mTOR, S6K, Erk1/2, HIF-1β, and VEGFR genes (Table 1), real time PCR was performed with ABI Step One plus detection system using RealQ Plus 2x Master Mix Green kit (Ampliqon, Denmark) according to the manufacture instructions. β-actin, as a house keeping gene, was utilized to normalize the relative expression level of the target genes based on the 2−ΔΔCT method. Real-time PCR cycles started with 95 °C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60 °C for 60 sec (annealing and extension, respectively). Each measurement was repeated in duplicate. Table 1 lists the specific primers used in the present study.

**Western blot analysis**

When MDA-MB-231 breast cancer cells reached 80% of confluence, we did various treatments as described. Whole cell lysates were prepared using RIPA-lysis buffer Santa Cruz Biotechnology (Santa Cruz, CA, USA) based on the manufacturer’s instructions. Protein concentration was measured with the Bradford protein assay. 30 μg of total protein was resolved in 10 % SDS-PAGE in the Bio-Rad Criterion System. Following the transfer of resolved protein onto PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA), the non-specific sites were blocked with 2% non-fat dried milk in Tris-buffered saline containing 20% Tween-20 (TBST) for 75 min at room temperature. Subsequently, the membranes were incubated overnight at 4 °C with primary specific antibodies against VEGF, HIF-1α, and β-actin (molecular weight of VEGF, HIF-1α, and β-actin were 25, 120, and 45 kDa, respectively). After being washed with TBST, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (monoclonal anti-HIF-1a, VEGF, and β-actin from R and D Systems, Minneapolis, MN, USA) for 1 h. Protein bands were visualized employing the chemiluminescence detection substrate ECL Plus Western Blotting Detection System. The protein bands were quantified via
ImageJ software version (NIH) and normalized with respective β–actin level.

**Statistical analysis**

MTT assay presentation and their IC$_{50}$, IC$_{10}$, and IC$_{5}$ calculations were rendered with Graph Pad Prism version 6.0.1. The changes in mRNA expression and their statistical comparisons were conducted utilizing the Relative Expression Software Tool V2.0.13 (REST 2009). All the experiments were performed in triplicates and the results were expressed as mean ± SD from three independent experiments. The comparisons between protein expression groups were analyzed with T-test or Mann-Whitney U test. In this study, P ≤ 0.05 was considered to be statistically significant.

**Results**

**Cytotoxic effects of UMB and CoCl$_2$ on the MDA-MB-231 breast cancer cell line**

Before evaluating the pharmacological potentiality of UMB, its cytotoxic effects on the MDA-MB-231 was primarily examined via MTT assay. MDA-MB-231 cells were treated with different concentrations of UMB ranging from 3.1 to 200 µM for 24h, after which the MTT assay was performed to assess the survived cells and presented as percentage of control. The results of the MTT assay showed that UMB exerted its cytotoxic effect in a concentration-dependent manner (Figure 1). The IC$_{50}$ for UMB at 24h was 75 (CI of 95%: 65 to 86) µM. Subsequent experiments were performed using non-toxic IC$_{10}$ and IC$_{5}$ of UMB concentrations (20 and 10 µM, respectively).

The MTT assay was utilized to determine the viability of MDA-MB-231 cells in the presence of CoCl$_2$. MDA-MB-231 cells were treated with different concentrations of CoCl$_2$ ranging from 50 to 600 µM and incubated at the aforementioned conditions for 24h (Figure 2). IC$_{50}$ for CoCl$_2$ was 158 (CI of 95%: 133 to 189) µM and the non-toxic CoCl$_2$ concentration of 50 µM was used for the rest of experiments in this investigation.

**UMB treatment effect on the expression of mRNAs and proteins involved in angiogenesis**

We evaluated the effects of EGF and CoCl$_2$ on the expression levels of EGFR, PI3K, Akt, mTOR, S6K, 4EBP1, ERK1, ERK2, HIF-1α, HIF-1β, VEGF, VEGFR mRNAs, HIF-1α, and VEGF proteins (Figure 2). Our results revealed that the EGF-treated MDA-MB-231 cells significantly increased the expression levels of all the mRNAs described, except for mTOR and VEGFR. The EGF treatment of MDA-MB-231 cells dramatically increased the expression levels of VEGF mRNA by approximately 7000 folds compared to those in the control.

The EGF-treated MDA-MB-231 cells were then treated with IC$_{5}$ (10 µM) and IC$_{10}$ (20 µM) UMB. In EGF-induced cells, the IC$_{5}$ concentration of UMB did not significantly reduce the expression of all the listed mRNAs compared to EGF-treated cells, except for ERK1/2 and 4EBP1. UMB IC$_{10}$ displayed a significant decrease in expression levels of ERK1/2, HIF-1β, and HIF-1α mRNAs (Figure 2).

In this analysis, CoCl$_2$ was used to induce hypoxic conditions in MDA-MB-231 cells. After the treatment of MDA-MB-231 cells with CoCl$_2$, expression of all the described mRNAs increased, except for that of ERK2 and VEGFR. The CoCl$_2$ effect of the increase in ERK1 and VEGF mRNAs was dramatic. According to our results, CoCl$_2$ increased the expression levels of ERK1 (~170 folds) and VEGF (~11000 folds) in the MDA-MB-231 cells compared to those of the control group (Figure 2). Following the treatment of the CoCl$_2$-induced hypoxic cells with UMB IC$_{5}$, the mRNA expression of Akt, ERK1, and mTOR decreased significantly. The IC$_{10}$ concentration of UMB showed significantly lower
expressions of PI3 K, ERK1/2, Akt, and mTOR mRNAs compared to the CoCl2 treated cells (Figure 2).

In accordance with the real-time PCR findings, the MDA-MB-231 cells in the presence of EGF and CoCl2 displayed elevated levels of expression of HIF-1α and VEGF proteins. Afterwards, the effects of UMB at IC5 and IC10 concentrations (10 and 20 μM) on EGF and CoCl2 -induced cells, where β-actin was used as an internal control, were investigated. For note, only UMB IC10 significantly decreased the expression of HIF-1α and VEGF protein (Figure 2).

Discussion

In this in vitro study of MDA-MB-231 cells, UMB was found to have concentration-dependent cytotoxicity for MDA-MB-231 cells with IC50, IC10, and IC5 at 75 μM, 20 μM, and 10 μM, respectively. We found that EGF and CoCl2 could induce cells and increase the transcription of PI3K / Akt / ERK pathway genes (Scheme I) and the translation of VEGF and HIF-1α. These stimulating effects were inhibited by sub-toxic IC5 and IC10 UMB.

Today, different strategies are being used to treat different types of cancer, each targeting vital biological mediators in the cancer cell. The use of chemical drugs and antibodies covers a large part of the drugs used in cancer therapy. One of the successful strategies for cancer treatment is to target pro-angiogenic factors. Angiogenesis mediators within the cancer microenvironment accelerate the formation of new vessels at the tumor site, which facilitates the supply of nutrients and the access of tumor cells to new niches. Various investigations have therefore been conducted to find effective anti-angiogenic drugs. The cytotoxic effects of UMB as a natural compound derived from the Ferula species on various cancer cell lines have been well documented; for example, Rashidi et al. investigated the cytotoxic effects of UMB on the MCF-7 line of breast cancer cells. In accordance with the results of our study, they showed that UMB had a concentration-dependent cytotoxic effect. They also obtained the same results when assessing the cytotoxic effect of UMB on different cell lines (HT29, A172, and CT26). This evidence, consistent with our findings, confirms that the concentration-dependent cytotoxic effect of UMB is not specific to a particular cell line.

A number of studies have been conducted to clarify the role of the EGF in the development of cancer. EGF binds to its EGFR receptor, which activates a signal cascade leading to angiogenesis (Scheme I). The first cross-talk report between EGF signaling and VEGF secretion was obtained from the Goldman et al. study, which found that the EGF significantly increased the expression of VEGF in glioma cells and hyperproliferative keratinocytes. In our study, almost all the genes involved in the angiogenesis pathway and protein expressions of VEGF and HIF-1α significantly increased in the presence of EGF.

High metabolism of tumor cells induces hypoxic condition and promotes angiogenesis in their microenvironment. It is well documented that the increased hypoxic condition in the microenvironment of cancer cells is associated with an increase in tumor size and metastases. Various papers are conducted every year to identify the biological events in hypoxic-induced angiogenesis in tumor cells. Cobalt ions are used to mimic the hypoxic condition in experimental studies. We found that the treatment of MDA-MB-231 cells with CoCl2 significantly increased the expression rates of VEGF signaling pathway mRNAs and HIF-1α and VEGF proteins. Our results were consistent with those of Rana et al.

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who reported that the treatment of CoCl$_2$ increased the expression of the VEGF and HIF-1α genes. However, there are certain contradictory data on the effect of CoCl$_2$ on the expression of the HIF-1α gene; for instance, Li et al. reported that the treatment of MCF-7 cells with CoCl$_2$ increased the expression of HIF-1α only at protein level. Our work showed that CoCl$_2$ had an extremely large effect on the production of VEGF mRNA approximately 11000 times more than the non-stimulated control MDA-MB-231 cells. This huge production of VEGF mRNA is also seen in the cells stimulated by the EGF. In contrast to ERK2, ERK1 was another gene whose expression greatly increased by CoCl$_2$. Our findings implied that the non-toxic concentrations of UMB (IC$_5$ and IC$_{10}$) reduced the gene expressions of VEGF and HIF-1α in the EGF and CoCl$_2$-stimulated cells in a concentration-dependent manner. However, only IC$_{10}$ concentration of UMB could reduce the production of HIF-1α and VEGF proteins in these stimulated cells. These data were consistent with the results obtained by Alizadeh et al., where the injection of UMB in the mouse colorectal cancer model showed a significant reduction in the proteins involved in angiogenesis and metastasis, including VEGF, MMP2, and MMP9. In another study, the anti-angiogenic effect of auraptene was attributed to its ability to block HIF-1α. Auraptene and UMB are structurally closely similar and the only difference between these two compounds is that there is a longer 7-prenyloxy chain in the UMB. It is worth noting that UMB inhibited the production of ERK1/2, PI3K, Akt, mTOR, and HIF-1α mRNA in stimulated MDA-MB-231 cells in our research. It shows that UMB could affect angiogenesis genes and should be considered as a cytotoxic agent that kills cancer cells and also has anti-angiogenic effects. Akt serves a key center for the signaling pathways Wnt / β-catenin and PI3K. Pharmacological inhibition of Akt slows tumor growth and control malignant stem cells. The PI3K / Akt / mTOR pathway and its crosstalk along the RAS / RAF / MEK / MAPK pathway play an essential role in the growth, survival, differentiation, and proliferation of cancer cells. In addition, the PI3K / AKT / mTOR pathway is involved in the complex control of cell energy, glucose metabolism, senescence and angiogenesis. Chang et al. reported that angiogenesis and metastasis of MDA-MB-231 cells were inhibited by Nelumbo nucifera Gaertn leaves extract through down-regulation of connective tissue growth factor (CTGF) mediated PI3K/AKT/ERK signaling. Further research in this context should improve our understanding of how UMB influences the signaling pathways in cancer cells.

**Conclusion**

The obtained results herein provided evidence of the effects of UMB on MDA-MB-231 breast cancer cells treated with CoCl$_2$ / EGF. By lowering the expression of many genes in the PI3K / Akt/ MAPK, it is suggested that UMB at a concentration of 20 μM (or higher) be used as an appropriate anti-angiogenic, anti-metastatic, and cytotoxic agent for cancer therapy.

**Acknowledgment**

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**Conflict of Interest**

None declared.
References


Umbelliprenin is potentially toxic against the HT29, CT26, MCF-7, 4T1, A172, and GL26 cell lines, potentially harmful against bone marrow-derived stem cells, and non-toxic against peripheral blood mononuclear cells. *Iran Red Crescent Med J.* 2016;18(7)doi: 10.5812/ircmj.35167.


Table 1. The specific primes for amplification of genes involved in angiogenesis

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Scheme I. This scheme shows EGFR signaling pathway in tumor angiogenesis and metastasis.
Figure 1. Cytotoxicity of 24 hr MDA-MB-231 exposure to different concentrations of UMB (left) CoCl₂ (right) measured via MTT assay. Each point represents the mean of independent assays performed in triplicate.
Figure 2. EGF and CoCl₂-stimulated MDA-MB-231 cells treated with IC₅ (10 μM) and IC₁₀ (20 μM) UMB concentrations. The mRNA levels (white bars) were determined with real-time PCR. Each white bar represents the mean ± SE of the mean of mRNA levels calculated via the REST 2009 software for three independent experiments. The levels of HIF-1α and VEGF proteins (black bars) were determined with western blot analysis. Each black bar represents the mean ± SD of three separate experiments quantified employing the ImageJ software. There is a photograph of the blots in the bottom.

* P ≤ 0.05, ** P ≤ 0.01