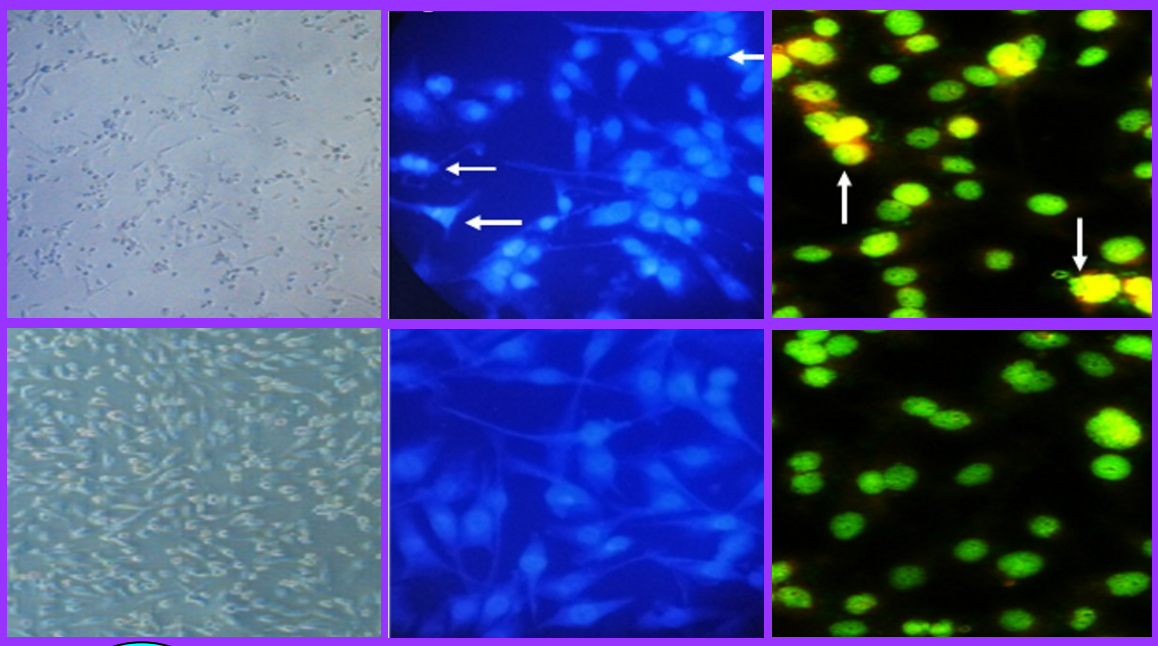

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Research Article

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# Berberine chloride inhibits proliferation and angiogenesis in triple-negative breast cancer by down-regulating HIF-1α/VEGF/Ang pathway

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## Abstract

In triple-negative breast cancer, intratumoral hypoxia drives tumor progression via HIF-1α-mediated angiogenesis. The study aims to analyze the efficacy of berberine chloride on tumor proliferation and angiogenesis in triple-negative breast cancer mediated by HIF-1α. The triple-negative breast cancer cells were subjected to berberine chloride, and the effects on cell viability, apoptosis, and angiogenic gene expression were assessed. MTT assay determined the $IC_{50}$ as 9.5 µM. Fluorescent imaging confirmed apoptosis through membrane disintegration, fragmented DNA, and the presence of apoptotic bodies. Real-time PCR analysis showed significant down-regulation of HIF-1α ($p<0.01$), VEGF ($p<0.05$), Ang1 ($p<0.05$), and Ang2 ($p<0.01$) after berberine chloride treatment. These findings indicate the efficacy of berberine chloride in inhibiting triple-negative breast cancer cell proliferation and angiogenesis, highlighting its potential as a therapeutic candidate.

## Introduction

Triple-negative breast cancer is devoid of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor, which are key markers used to classify its clinical characteristics. Based on gene expression patterns associated with cell division, DNA repair, and cellular proliferation, triple-negative breast cancer is further divided into six distinct subtypes, each requiring different therapeutic approaches (Bianchini et al., 2016). Epidemiological studies reveal triple-negative breast cancer is most common among premenopausal women under 40, contributing to 15–20% of breast cancer cases (Yam et al., 2017). The mean survival rate within five years of diagnosis is poor, with a 40% mortality rate (Den et al., 2007). Triple-negative breast cancer is highly invasive (46%), with an average post-metastasis survival of ~13.3 months and a relapse rate of 25%.

Angiogenesis is a regulated physiological process involved in embryogenesis, organogenesis, wound healing, growth and reproduction (Yadav et al., 2015). Deregulation of angiogenesis is observed in cardiovascular diseases and cancer. Tumor cells undertake angiogenesis to fulfil their increased demand to supply oxygen, nutrients thereby to support their uncontrolled proliferation, increased metabolism and establishment in new areas (Hoff and Machado, 2012).

Intra-tumoral hypoxia negatively impacts triple-negative breast cancer by promoting tumor aggression, invasion, drug resistance, and inflammation (Liu et al.,

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2015). HIF-1α (Hypoxia-inducible factor-1α) is a transcription factor activated under hypoxic conditions within tumor cells. It is central in driving the complexity of triple-negative breast cancer by modulating various biological pathways (Slemc and Kunej, 2016). Its overexpression promotes angiogenesis in triple-negative breast cancer by up-regulating key factors such as vascular endothelial growth factor (VEGF) and angiopoietins (Ang1 and Ang2). While VEGF primarily facilitates angiogenesis, Ang1 and Ang2 - crucial angiogenic mediators regulated by HIF-1α - contribute to cell survival, vascular permeability, and cell migration (Ciu et al., 2015; Briggs et al., 2016; Jia et al., 2016). Thus, targeting HIF-1α may offer a promising strategy to control angiogenesis in triple-negative breast cancer.

Current triple-negative breast cancer treatments are associated with significant toxicity, with 43% of patients experiencing severe adverse effects despite alternative therapies (Jia et al., 2016). The need for novel therapeutic approaches is growing due to the high toxicity and mortality of existing treatments. Natural bioactive compounds derived from plants, microbes, and marine organisms are being explored as alternatives.

Berberine, an isoquinoline alkaloid found in Berberidaceae, Papaveraceae, and Ranunculaceae plant families, is primarily extracted from Berberis, Coptidis, and Mahonia species. Traditionally used in Ayurvedic and Chinese medicine (Filli et al., 2020), berberine has therapeutic effects, including antineoplastic, antipyretic, antibacterial, anti-inflammatory, detoxifying, anti-lipidemic, and anti-diabetic properties (El Khalki et al., 2020). It has demonstrated anti-cancer activity in lung, liver, breast, and cervical cancers by inducing apoptosis, inhibiting proliferation, arresting the cell cycle, and triggering DNA damage (El Khalki et al., 2020; Letasiova et al., 2006). Berberine also suppresses metastasis in triple-negative breast cancer by inhibiting matrix metalloproteinases (MMP-1, 3, 7, 9, 11) (Rajoriya et al., 2021). However, natural berberine has poor solubility, bioavailability, and inherent toxicity. To overcome these limitations, its hydrochloride salt form, berberine chloride, is recommended due to enhanced bioavailability, safety, and potency (Filli et al., 2020).

This study evaluates berberine chloride's efficacy in inhibiting HIF-1α-mediated triple-negative breast cancer cell proliferation and angiogenesis *in vitro*. The findings may support the development of herbal bioactives as targeted anti-cancer agents or lead molecules for triple-negative breast cancer treatment. This could lower treatment cost and improve progression-free survival for triple-negative breast cancer patients.

## Materials and Methods

### Materials

Dulbecco's Modified Eagles Medium (DMEM)-high glucose (HiMedia, USA), trypsin (Thermo Scientific, USA), FBS (fetal bovine serum) (HiMedia, USA), berberine chloride (Otto, Germany), antibiotic antimycotic cocktail (HiMedia, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (HiMedia, USA), TRIzol (Invitrogen, USA), RevertAid cDNA synthesis kit (Fermentas, USA), PowerUp SYBR green master mix (Applied Biosystems, USA)

### Cell culture

The study utilized triple-negative breast cancer cell line (MDA-MB-231) gifted by Dr. Ashok Kumar. The cells were grown in complete DMEM (high glucose) with

### Box 1: MTT assay

**Principle**

The MTT assay is a color-based technique commonly used to evaluate cell viability by measuring mitochondrial function. Live cells possess oxidoreductase enzymes that convert the yellow MTT compound into insoluble formazan crystals. The strength of the resulting color, typically read at a wavelength of 570 nm, reflects the number of cells that are metabolically active.

**Requirements**

96-well plate, Antibiotic antimycotic cocktail, Berberine chloride, Dulbecco's Modified Eagles Medium (DMEM)-high glucose, FBS, Isopropanol, MDA-MB-231 cell line, MTT, Multimode plate reader (Alpha Tech Perkin Elmer, USA), Trypsin

**Procedure**

*Step 1:* The MDA-MB-231 cells were seeded in a 96-well plate at $1x10^5$ cells/well. The cells were allowed to adhere and proliferate for 24 hours.

*Step 2:* Actively dividing cells were exposed to varying concentrations of berberine chloride (0-30 µM) dissolved in DMSO (0.1%) for 24 hours.

*Step 3:* The media was removed and MTT (20 µL/well) reagent was added to each well and incubated for 3 hours.

*Step 4:* Cells were observed for formation of formazan crystals. MTT reagent was removed.

*Step 5:* Isopropanol (100 µL) was added to each well and incubated for 20 min.

*Step 6:* The color developed was read at 570 nm in a multimode plate reader and the cell viability was calculated. Cells exposed to DMSO alone served as control. Similarly, cells exposed to doxorubicin (0-5 µM) served as standard. Cell viability was determined using the following formula:

$$\%\text{Inhibition} = \frac{\text{Mean OD (control)} - \text{Mean OD (treated)}}{\text{Mean OD (control)}} \times 100$$

**Reference**

Fazeela et al., 2024

10%FBS and 1%antibiotic antimycotic solution in cell culture incubator (Galaxy Eppendorf, USA) (5%$CO_2$, 37°C) and utilized for further analysis.

### *Determination of cell death by fluorescent imaging*

To evaluate the effect of berberine chloride in inducing cell death, MDA-MB-231 cells subjected to $IC_{50}$ concentration of berberine chloride for 24 hours were examined under a phase-contrast microscope to observe morphological alterations resulting from berberine chloride exposure. Further, the treated cells were analyzed by fluorescent microscope using fluorescent stains like DAPI, acridine orange and ethidium bromide for the hall marks of apoptosis. The fluorescent stain acridine orange can stain DNA in both living and dead cells. On the other hand, ethidium bromide can penetrate the cells with altered membrane integrity.

### *Analysis of antiangiogenic activity*

To evaluate the impact of berberine chloride on HIF1α-mediated angiogenesis, the mRNA expression levels of HIF1α, VEGF, Ang1, and Ang2 were analyzed using RT -PCR in berberine chloride-treated cells. The MDA-MB-231 cells, subjected to $IC_{50}$ concentration of berberine chloride for 24 hours were used for analysis. The mRNA was isolated by TRIzol method as prescribed by the manufacturer. The extracted RNA was utilized for synthesis of complementary DNA using Revert Aid cDNA synthesis kit as per the manufacturers protocol. Following this, the synthesized cDNA was used for detecting the expression levels of HIF-1α, VEGF, Ang1 and Ang2 mRNAs using appropriate primers in BioRad RT-PCR. The expression levels were normalized with β -actin expression levels. The RT-PCR cycle used the following steps: Initial denaturation (95°C - 5 min), denaturation (94°C - 30 sec), annealing (56-60°C - 30 sec), extension (72°C - 40 sec). Steps were repeated for 35 cycles.

### *Statistical analysis*

All experiments were done in triplicate and repeated to a minimum of three times. Data generated were analyzed as mean ± standard deviation (SD). The values were analyzed using IBM SPSS 23 software, employing the student's t-test to assess the level of statistical significance with $p<0.05$.

## Results

### *Viability of MDA-MB-231 cells*

Berberine chloride displayed cytotoxicity in the cell line in a dosage-dependent manner. The $IC_{50}$ was determined as 9.5 µM (Figure 1). The outcomes of the study showed that berberine chloride suppressed the viability of triple-negative breast cancer cells under *in vitro* conditions.

### *Apoptosis in MDA-MB-231 cells*

The results of phase contrast microscopy showed that treatment with berberine chloride resulted in decreased cell confluence and loss of membrane integrity which were indications of apoptosis. Similarly, the observation of floating cells indicated that berberine chloride treatment inhibited cell adherence whereas, cells unexposed to berberine chloride were attached and exhibited more than 90% confluence (Figure 2). Following this, fluorescent microscopy analysis with DAPI was carried out to analyze the hallmarks of apoptosis including changes in nucleus morphology and apoptotic body formation. Cells treated with berberine chloride exhibited shrunk nuclei and formation of apoptotic bodies whereas, the

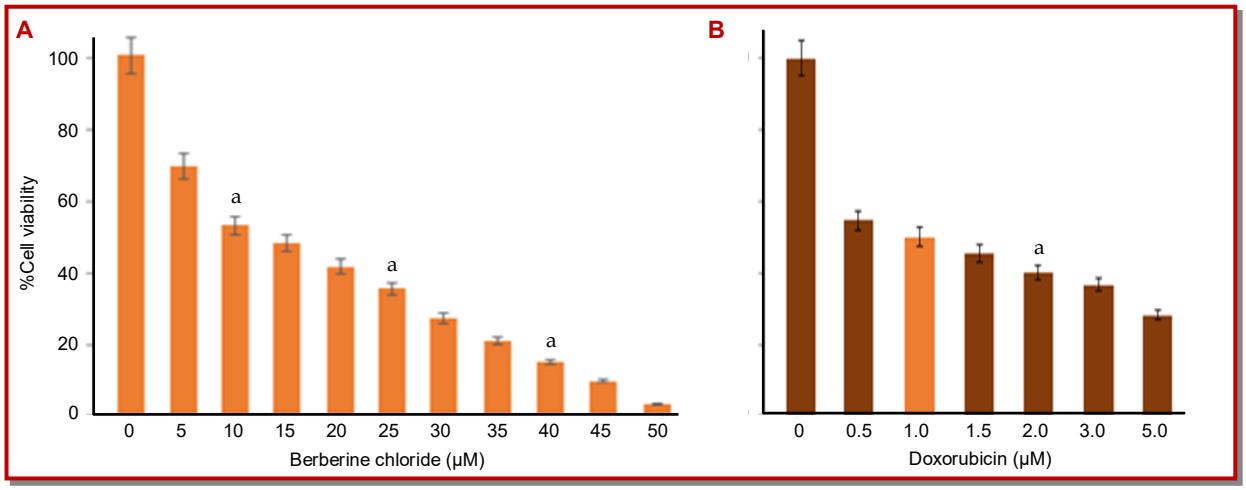


Figure 1. Cell viability assay of berberine chloride (A)- and doxorubicin (B)-induced cell death in MDA-MB-231 cell line in dose-dependent manner. The inhibitory concentration of berberine chloride and doxorubicin (reference drug) was determined at 24 hours. The experiments were carried out in triplicate and repeated three times. The values expressed are mean ± S.D. [a]$p<0.05$ indicates statistically significant

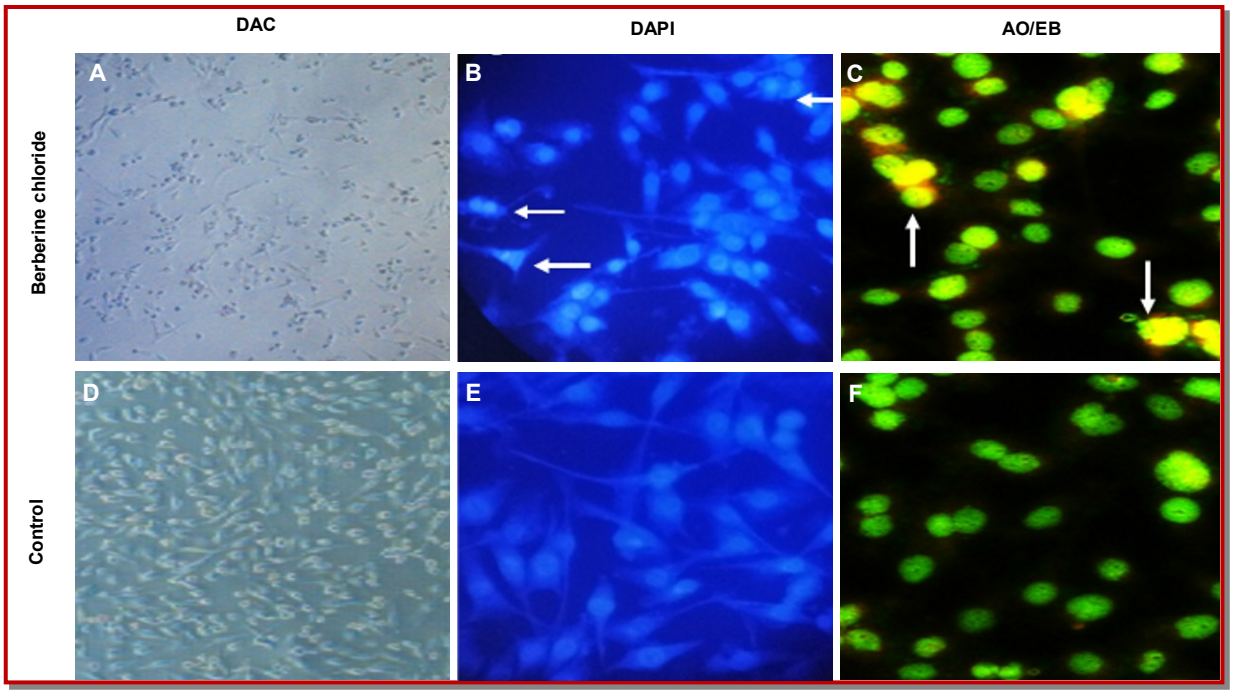


Figure 2: Determination of apoptosis. MDA-MB-231 cells were treated with $IC_{50}$ concentration of berberine chloride for 24 hours and analyzed for apoptotic features by fluorescent imaging using stains including DAPI, acridine orange/ethidium bromide

untreated cells exhibited round nuclei with even staining of DAPI. The observation of hallmarks of apoptosis by DAPI stain indicated that berberine chloride treatment caused cell death through induction of apoptosis in triple-negative breast cancer cells.

When cells were stained with acridine orange and ethidium bromide, untreated cells appeared with green fluorescence, whereas cells treated with berberine chloride exhibited greenish-yellow fluorescence, indicating that the cells were undergoing early apoptosis (Figure 2). Taken together, the results of microscopic studies revealed that berberine chloride induces apoptosis-mediated cell death in MDA-MB-231 cells.

***Angiogenesis in MDA-MB-231 cells***

The results showed a noticeable reduction in HIF1α mRNA expression in cells treated with berberine chloride compared to untreated controls (Table I). Likewise, the mRNA levels of VEGF, Ang1, and Ang2—key down-stream targets in the HIF1α signaling pathway—were also down-regulated following berberine chloride treatment. These findings suggest that berberine chloride inhibits angiogenesis by suppressing the expression of genes involved in the HIF1α-mediated angiogenic pathway.

**Table I**

**RT-PCR analysis of gene expression in angiogenic pathway**

| Genes | Expression of genes | p value |
|---|---|---|
| HIF1α $2^{\Delta\Delta CT}$ | 37.5 ± 1.6 | 0.01 |
| VEGF $2^{\Delta\Delta CT}$ | 31.78±1.98 | 0.05 |
| ANG 1 $2^{\Delta\Delta CT}$ | 37.5±1.15 | 0.05 |
| ANG 2 $2^{\Delta\Delta CT}$ | 24.7±0.017 | 0.01 |

Data are mean ± SD; n=3; repeated 3 times

## Discussion

Findings of this study demonstrated that berberine chloride exhibited cytotoxicity in MDA-MB-231 cells by inducing apoptosis. Similarly, treatment with berberine chloride resulted in a decrease of HIF-1α expression, and its downstream targets—VEGF, Ang1, and Ang2 in MDA-MB-231 cells. These findings suggest that suppessing HIF-1α mRNA expression may serve as an effective strategy to inhibit angiogenesis during cancer progression.

The findings of the current study align with previous studies where berberine chloride was shown to reduce the viability of A549 cells with an $IC_{50}$ of 200 µM. Additionally, prior studies have noted the ability of berberine to impact the viability of various cancer lines, including MDA-MB-231 ($IC_{50}$ 16.7 µM) and MDA-MB-468 ($IC_{50}$ 0.48 µM), as well as HT29 ($IC_{50}$ 52.37 µM), HCC ($IC_{50}$ 0.19 µM), HCC70 ($IC_{50}$ 1.67 µM), HCC38 ($IC_{50}$ 3.24 µM), and BT 20 ($IC_{50}$ 0.23 µM). These results indicate that both berberine and its derivative, berberine chloride, are effective in suppressing cancer cell proliferation.

The current observations on angiogenesis coincide with earlier observations which showed that berberine

chloride exhibited antiangiogenic activity by inhibiting tumor directed capillary formation and expression of proangiogenic factors including VEGF, GM-CSF, NF-κB, COX-2 and HIF1 (Hamsa et al., 2011). In a previous study, berberine iodide was shown to be more effective in inhibiting angiogenesis in HER2 overexpressed breast cancer cells compared to berberine (Elisa et al., 2015). Similarly, berberine hydrochloride loads small extracellular vesicles were more effective in reducing capillary formation in HUVECS (Abir et al., 2022). Further, the current findings are in line with small molecules and natural products like sanguinarine (Su et al., 2021), cardamon (Jin et al., 2019), nanoliposomal echinomycin (Bailey et al., 2020), melittin (Mir Hassani et al., 2021), isoliquiritigenin (Wang et al., 2017, were shown to inhibit HIF-1α mRNA expression and also the expression of its down-stream components. These compounds are employed in antiangiogenic therapy for the management of breast cancer.

In triple-negative breast cancer, hypoxia-driven angiogenesis is crucial for tumor growth, metastasis, and colonization at distant sites. HIF-1α plays a central role in promoting angiogenesis in triple-negative breast cancer by regulating the expression of key proangiogenic factors such as VEGF, Ang1, and Ang2. Under hypoxic conditions, HIF-1α binds to the VEGF gene promoter, enhancing its transcription and thereby promoting new blood vessel formation. In triple-negative breast cancer, studies have reported significantly elevated levels of HIF-1α-induced VEGF mRNA compared to other breast cancer subtypes. Similarly, the accumulation of HIF-1α leads to the up-regulation of several proangiogenic genes, including Ang1 and Ang2, which play crucial roles in the formation of new blood vessels. VEGF, Ang1, and Ang2 are considered key drivers of tumor-associated angiogenesis. In addition to promoting vessel formation, VEGF can stimulate the expression of other proangiogenic molecules and their receptors, contributing to a process known as angiogenic switching. This mechanism allows tumor cells to initiate and sustain angiogenesis, thereby supporting tumor growth by supplying essential nutrients and oxygen through newly developed capillaries. In the current study, treatment with berberine chloride down-regulated the expression of HIF 1α and its down-stream angiogenic components including VEGF, Ang1, and Ang2. These outcomes prove the antiangiogenic activity of berberine chloride in triple negative breast cancer.

## Conclusion

Berberine chloride effectively reduced the mRNA expre-ssion of HIF-1α, VEGF, Ang1, and Ang2 indicating its potential to suppress angiogenesis.

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## Ethical Issue

The guidelines about the development, acquisition, authentication, cryopreservation, and transfer of cell lines between laboratories were strictly followed. Besides, microbial contamination (commonly mycoplasma), characterization, instability, and misidentification was considered seriously.

## Conflict of Interest

Authors declare no conflict of interest

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