nutrición clínica y Dietética Hospitalaria

Artículo Original

Nutr Clín Diet Hosp. 2025; 45(3):129-137
DOI: 10.12873/453aldeeb

Anti-Angiogenic effects of *Ocimum basilicum* ethanolic extract: In vitro and ex vivo evidence of endothelial inhibition

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Recibido: 21/mayo/2025. Aceptado: 13/agosto/2025.

ABSTRACT

Background: Angiogenesis and inflammation are two linked processes that exacerbate solid malignancies such as colorectal cancer. Bearing this in mind, it was important to study the anti-angiogenic properties of a well-known anti-inflammatory herb, *Ocimum basilicum* Lamiaceae (OB). Although many studies have explored the OB cytotoxic properties against a panel of cell lines, to the best of our knowledge, this is the first study that aims to investigate the *in vitro* and *ex vivo* anti-angiogenic properties of OB.

Methods: Ethanolic extract of *Ocimum basilicum* leaves (OBL70) was prepared in 70% ethanol. Its half-maximal (IC_{50}) inhibitory concentration on HT29 and EA.hy926 cells were evaluated using viability assay. Its antiangiogenic activities were investigated through ex vivo rat aorta ring assay, migration assay, invasion assay, and tube formation assay.

Results and Conclusion: OBL70 demonstrated selective cytotoxicity against EA.hy926 endothelial cells and HT29 cells with IC_{50} value of 79.78± 2.35 and >100 µg/ml respectively. At 100 µg/ml, 7 days exposure to OBL70 significantly inhibited rat aortic formation of new blood vessels by 77.46 ± 9.3% while the percentage of EA.hy926 survival after 96h exposure was reduced to 71.6± 4.1 %. In addition, OBL70 inhibited EA.hy926 migration by 59.1 % ± 1.17 and 49.5 % ± 3.79 at 12 and 24 h. OBL70 also decreased the number of endothelial cell invasion through the Matrigel by 31.51 ± 2.37%. Notably, at both 200 and 100 µg/ml, OBL70 was able to stop tube formation totally. These significant findings suggest promising potential for translation into anti-angiogenic therapeutic agent.

KEYWORDS

Angiogenesis, anti-inflammatory, cytotoxicity, *Ocimum basilicum*.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, plays a crucial role in tumor progression. This complex process involved several steps, including extracellular remodeling, endothelial cells (EC) proliferation, migration, invasion, capillary formation and vascular connections. While angiogenesis is essential in physiological processes like wound healing and fetal development, pathological angiogenesis supports tumor growth by supplying oxygen and nutrients to tumors enabling the dissemination of tumor cells, leading to secondary tumor formation[1].

The angiogenesis process is driven by sustained release of angiogenic factors, which can originate from tumor cells or stromal cells. Key factors include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), primarily released by tumor cells. Additionally, chronic inflammation can initiate angiogenesis through the release of pro-inflammatory cytokines such as VEGF, IL-1α, IL-1β, and TNF-α, mainly by leukocytes[2]. Targeting these factors has emerged as a promising strategy for cancer treatment.

Recent studies have focused on targeting VEGF, the primary soluble factor regulating EC behavior and barrier functions[3].

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In mammals, the VEGF family consists of five members, glycoproteins released by various tumor malignancies as well as by ECs, neutrophils, platelets, macrophages, activated T cells, dendritic cells, and pericytes[4]. VEGF-A, the most potent stimulator of angiogenesis, binds with high affinity to tyrosine kinase receptors (VEGFR-1 and VEGFR-2) predominantly expressed on ECs. Although targeting VEGF has demonstrated proven efficiency and safety for colorectal cancer treatment when combined with chemotherapy, such as bevacizumab (a humanized anti-VEGF antibody) with 5-fluorouracil-leucovorin for colorectal cancer treatment[5], tumors can develop resistance by producing alternative angiogenic factors[6].

To overcome this challenge, recent research has shifted towards vascular targeting agents that directly target ECs, preventing them from responding to angiogenic stimuli[7]. This approach is based on the premise that ECs, being genetically stable, are less likely to develop drug resistance and may offer a more direct anti-angiogenic effect.

In this study, we investigated the in vitro and ex vivo anti-angiogenic effects of *Ocimum basilicum* Lamiaceae (OB), a common herb known for its ornamental and therapeutic uses, which has reported anti-inflammatory and antioxidant properties. Previous studies demonstrated the in vivo anti-angiogenic effect of OB's ethanolic extract using a duck embryo chorioallantoic membrane (CAM) assay, showing a significant decrease in new blood vessel formation compared to controls[8]. Our study aims to explore the anti-angiogenic potential of OB extract with minimal cytotoxicity. While many cytotoxic compounds or extracts exhibit anti-angiogenic effects by directly targeting cancer and endothelial cells, this study investigates the non-cytotoxic concentration of OB extract to elucidate the molecular mechanism behind its anti-angiogenic properties. To our knowledge, this is the first study to explore the anti-angiogenic effects of OB leaves extract.

MATERIAL AND METHODS

Plant Sample Collection And Extraction

The OB plant used in this study was collected from Taif city, Saudi Arabia, during August of 2017. Its taxonomic identification was conducted in the College of Pharmacy, Umm Al-Qura University, Makkah. Cleansed leaves were dried and grinded to make a fine powder. Then, 2g of OB leaf powder was sonicated in 70% ethanol (Merck, USA) for 8 minutes, followed with 48 h of continuous shaking at 55°C using KS 4000 i control incubator shaker (IKA®-Werke GmbH & Co. KG, Germany), and then filtered. The filtrate was then left to dry in a petri dish for 24 h with gentle shaking at 50°C. Dried extract was dissolved in Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, USA) to make a 200 mg/ml stock solution. This extract, hereafter referred to as OBL70, is stored at -20°C until further use.

Cell Culture And Incubation Conditions

HT29 epithelial colorectal cancer cell line and EA.hy926 endothelial cell line were provided by Dr. Amin Malek Shah (School of Pharmaceutical sciences, USM, Malaysia). HT29 cells were grown in RPMI-1640 (Gibco/Life technology, USA) while EA.hy926 cells were grown DMEM medium (Gibco/Life technology, USA). All culture media were supplemented with 1% Penicillin/streptomycin (Gibco/Life technology, USA) and 10% Fetal bovine serum (FBS) (Gibco/Life technology, USA). Incubation conditions were set at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was utilized to assess the growth inhibitory properties of OBL70. Briefly, HT29 and EA.hy926 cells were suspended in 96-well plate at density of 2000 and 3000 cells/well, respectively. After overnight incubation, adhered cells were exposed to OBL70 at various concentrations, ranging from 1.56 µg/ml to 200 µg/ml. Vehicle control group was exposed to 0.05% DMSO, while blank wells contained cell-free medium. The treatment was carried out for 96 h, after which the supernatant was aspirated and replaced with fresh medium containing 0.5 mg/ml MTT (Sigma-Aldrich). After 4 h incubation with MTT solution, all media was removed from wells and formazan was dissolved in 150 µl DMSO with gentle mixing. Optical density was measured at 570 nm using a spectrophotometer (Biotek ELX808, USA), and the IC_{50} and percentage of cell viability at 100 µg/ml was calculated for three independent experiments and expressed as mean ± SD.

Neovascularization Inhibition Of OBL70 Using Rat Aorta Ring Assay

This tissue-isolated method was done as described by (Nicosia and Ottinetti, 1990) with minor modification[9]. Female Wistar rats (12- 14 weeks old) were obtained from the animal house facility of the college of pharmacy of Taif University, KSA. The rats were kept under standards of animal facility conditions and were provided with free access to food and water. The rats were sustained in a 12 h day/night cycle at 25±2 °C with relative humidity of 50-60%. The in vivo protocol was approved by the Medical Ethics Committee of the department of pharmacology and Toxicology of Taif University college of pharmacy. Briefly, rats were anesthetized with ketamine/xylazine IP injection (70 µg/kg ketamine and 10 µg/ml xylazine) (Sigma-Aldrich, USA) and humanely euthanized by neck dislocation. Then, the thoracic aorta was picked, cleaned from adipose-tissue, and placed into M199 medium (Gibco/Life technology, USA). Next, the aorta was cut into similar lengths of 1 mm, and each ring was placed into a single well in 48-well plates preloaded with freshly prepared 300 µl/well M199 medium containing fib-

rinogen (3 µg/ml) (Merck, Germany) and aprotinin (5 µg/ml) (Sigma-Aldrich, USA). After explanting the rings, 10 µl of thrombin (50 U/ml in normal saline: bovine serum albumin) (Sigma-Aldrich, USA) was added to harden the layer. The plate was gently incubated for 1.5 h in the incubator. During that time, the upper layer (300 µl/ well) was prepared by mixing M199 medium with 20% FBS, 2 mM L-glutamine (Gibco/Life technology, USA), 0.1% (w/v) aminocaproic acid (Sigma-Aldrich, USA) and 1% penicillin streptomycin (Lonza Group, Ltd., Basel, Switzerland) and amphotericin B antibiotics (Lonza Group, Ltd., Basel, Switzerland). The samples were treated with 100 µg/ml OBL70. Suramine (Sigma-Aldrich, USA) at 100 µg/ml was used as positive control whereas the negative control was 0.05 % DMSO. The treatments were carried out in incubation for seven days. However, on day four, the upper layer was pipetted and replaced by fresh layer containing similar treatments and concentrations as previously mentioned. Finally, on day seven, the neovascularization formation was investigated under inverted microscope supported with digital camera (EVOS fl) (Advanced Microscopy Group, USA) at ×4 magnification power. The pictures were analyzed by imageJ 1.51n® software (National Institute of Health, Bethesda, MA, USA) by measuring the average length of newly formed vessels exposed to a treatment (4 rings/treatment) and calculating percentage of OBL70 inhibition compared to the negative control. Results were represented as mean ± SD.

Anti-Migration Properties Of OBL70 Against EA.hy926 (Scratch Assay):

Scratch assay was done as described by (10) with minor changes. Briefly, 5×10^5 cells/well were seeded into 6 well plates and left in the incubator to be almost 90% confluent. After that, a longitudinal scratch was made in the middle of cell layer using sterile 200 µl tips. The medium was removed, and wells were washed twice with PBS. Proceeding, 2 ml of low FBS containing-medium (2%) as well as OBL70 concentrations (100, 50, 25 and 12.5 µg/ml) were added to the wells. 0.05% DMSO was used as negative control. Pictures were taken for the scratches at 0, 12 and 24 h of treatment under the inverted microscope. Five pictures for each scratch were taken to measure the average width of scratch using ImageJ 1.51n® software (National Institute of Health, Bethesda, MA, USA). The rate of ECs migration was calculated by dividing the scratch width over the time, then the percentage of inhibition for each treatment concentration at specific period was calculated as the following:

$$\%\ \textit{inhibition of EA.hy926 migration} = (1 - (\textit{Rtr/Rc})) \times 100$$

Where Rtr: The rate of EA.hy926 migration for treated group.

Rc: The rate of EA.hy926 migration for untreated group.

Invasion Of EA.Hy926 Through Matrigel After OBL70 Treatment

This assay measures the ability of OBL70 to inhibit degradation of extracellular matrix by EA.hy926, which is considered a very important step in the angiogenesis. Matrigel (Corning, USA) was used in this assay by adding it into 96 well plates (50 µl/well 1:1; Matrigel: DMEM medium) and was left for one hour in the incubator to solidify. Next, 5000 cell/well suspended in DMEM medium containing OBL70 (200 and 100 µg/ml), or 10 µM vinblastine as positive control or 0.1% DMSO as negative control, were added carefully to each well. The plates were re-incubated for 24 h. Next day, the upper layer was removed, and the lower layer was rinsed with PBS carefully to remove uninvaded cells. Finally, pictures for each well were taken under inverted microscope at ×10 magnification. The average number of invaded cells/mm^2 were calculated using ImageJ 1.51n® software. Then, the percentage of cell invasion inhibition was calculated according to this formula:

$$\%\ \textit{cell invasion inhibition} = (1 - (\textit{Nt/Nc})) \times 100$$

Where Nt: Mean cell number invading Matrigel for treated group.

Nc: Mean cell number invading Matrigel for untreated group.

Effect Of OBL70 Towards EA.hy926 Capability To Form Capillary-Like Structures (Tube Formation Assay)

The tube formation capability of EA.hy926 endothelial cells is the last step in the angiogenesis process before remodelling. It is the most important in vitro assay which reflects ECs proliferation, migration and invasion across Matrigel to form capillary-like structures[11]. Here, 2×10^5 cells/ml was seeded into each culture flask and left for 48 h in the incubator. After that, old medium was discarded and replaced by fresh medium containing OBL70 (200 and 100 µg/ml), 10 µM vinblastine as positive control, or 0.1% DMSO as negative control. The cultured flasks were kept in the incubator for another 24 h. Then, treated cells were detached using trypsin and 500 µl/well containing 1×10^5 cells were seeded into 48-well plates preloaded with Matrigel (150 µl/well 1:1; Matrigel: DMEM medium). Plates were re-incubated with continuous check under the inverted microscope until forming tube structures (seen after 2 h). Pictures were taken under an inverted microscope at 4× magnification for each well and the enclosed area surrounded by tubes were measured using ImageJ 1.51n® software. Finally, the percentage of tube formation inhibition by OBL70 was calculated as the following:

$$\%\ \textit{tube formation inhibition} = (1 - (\textit{Aeat/Aeac})) \times 100$$

Where Aeac: Average enclosed area for the untreated group.

Aeat: Average enclosed area for the treated group.

RESULTS

OBL70 Showed Selectivity Toward EA.hy926 Cells

OBL70 showed varying effects on proliferation of HT29 and EA.hy926 cells. As summarised in Table 1, OBL70 exhibited greater inhibitory effect on EA.hy926 cells, with a lower IC_{50} value achieved at after the 96h treatment. The significant inhibition of EA.Hy926 cell proliferation by OBL70, with no notable effect on HT29 cells even at 100 µg/ml, suggests a targeted antiangiogenic effect rather than broad cytotoxicity. Based on the IC_{50} results obtained, selected concentrations was used in the subsequent assays.

Table 1. OBL70 IC50 and viability of HT29 and EA.hy926 cells after treatment with OBL70 in MTT assay

Cell line	Half-maximal inhibitory concentration (IC_{50}) (µg/ml)
HT29	>100
EA.hy926	79.78 ± 2.35

Neovascularization formation inhibition by OBL70 using rat aorta ring assay:

Given that ECs are the principal cell type in angiogenesis, we investigated whether the inhibitory activity of OBL70 exhibited in in-vitro viability assay is sustained in ex-vivo settings. In other words, this method allowed us to prove whether OBL70 had potent anti-angiogenic effect in an ex vivo tissue-isolated setting. Our results revealed that 100 µg/ml OBL70 significantly inhibited the formation of new blood vessels comparable to the positive control, suramine. Specifically, OBL70 inhibited neovascularization by 77.46 ± 9.3 % after 7 days exposure, compared to the positive control (100 µg/ml suramine) which achieved 66.39 ± 2.5% inhibition (Figure 1). This finding highlights the antiangiogenic potential of OBL70 in a more complex biological settings.

OBL70 Inhibited Migration Of EA.hy926

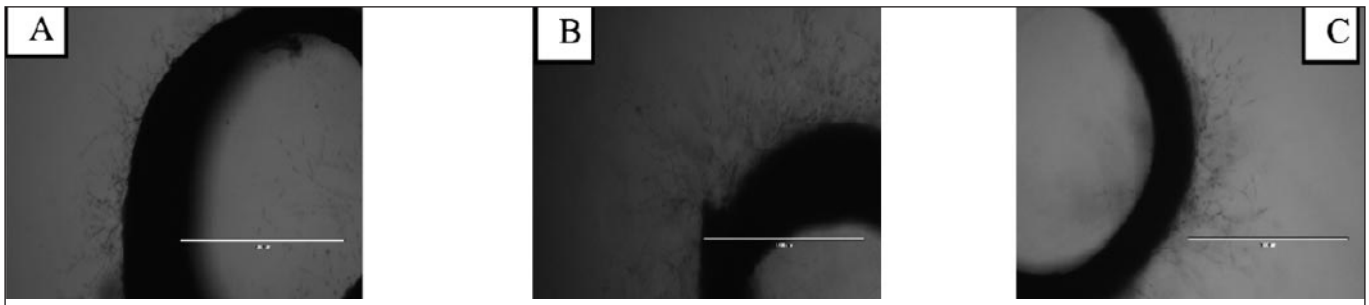
Migration is a key step in angiogenesis process. Four different concentrations of OBL70 (100, 50, 25 and 12.5 µg/ml) were used to clarify its anti-migratory properties towards EA.hy926 after 24 h exposure. The results were represented as % inhibition for scratch closure after 12 and 24 h exposure in respect to negative control (0.05% DMSO). After 12 h, OBL70 at concentrations 100, 50, 25 and 12.5 µg/ml decreased scratch closure by 59.1 % ± 1.17, 28.6 % ± 1.67, 22.4 % ± 0.28 and -3.0 % ± 0.14, respectively. After 24 h, these values changed to 49.5 % ± 3.79, 15.0 % ± 3.75, 12.1 % ± 2.56 and -3.7 % ± 0.49 (Figure 2. A & B). All results are statistically significant compared to untreated cells, with exception for the 12.5 µg/ml treatment at 12 and 24 hours ($P \leq 0.05$). Interestingly, no significant difference in result for 100 µg/ml was noted between the two periods, while a significant reduction in the inhibitory effect of 50 and 25 µg/ml was noted between 12 and 24 h ($P \leq 0.05$).

Inhibition Of EA.Hy926 Invasion Capability Through Matrigel Barrier

OBL70 treatment showed suppressive activity on EA.hy926 invasion. The 24 h exposure to OBL70 (200 and 100 µg/ml), vinblastine (10 µM) and 0.1% DMSO produced varying number of invaded cells/mm^2 – 32.09 ± 1.8, 59.10 ± 2.05, 36.41 ± 2.35 and 86.29 ± 2.17, respectively. These values, when compared to untreated cells, correspond to cell invasion inhibition rates of 62.81 ± 2.09%, 31.51 ± 2.37%, and 57.81 ± 2.73%, respectively (Figure 3 A, B and C), all of which are significant ($P \leq 0.05$).

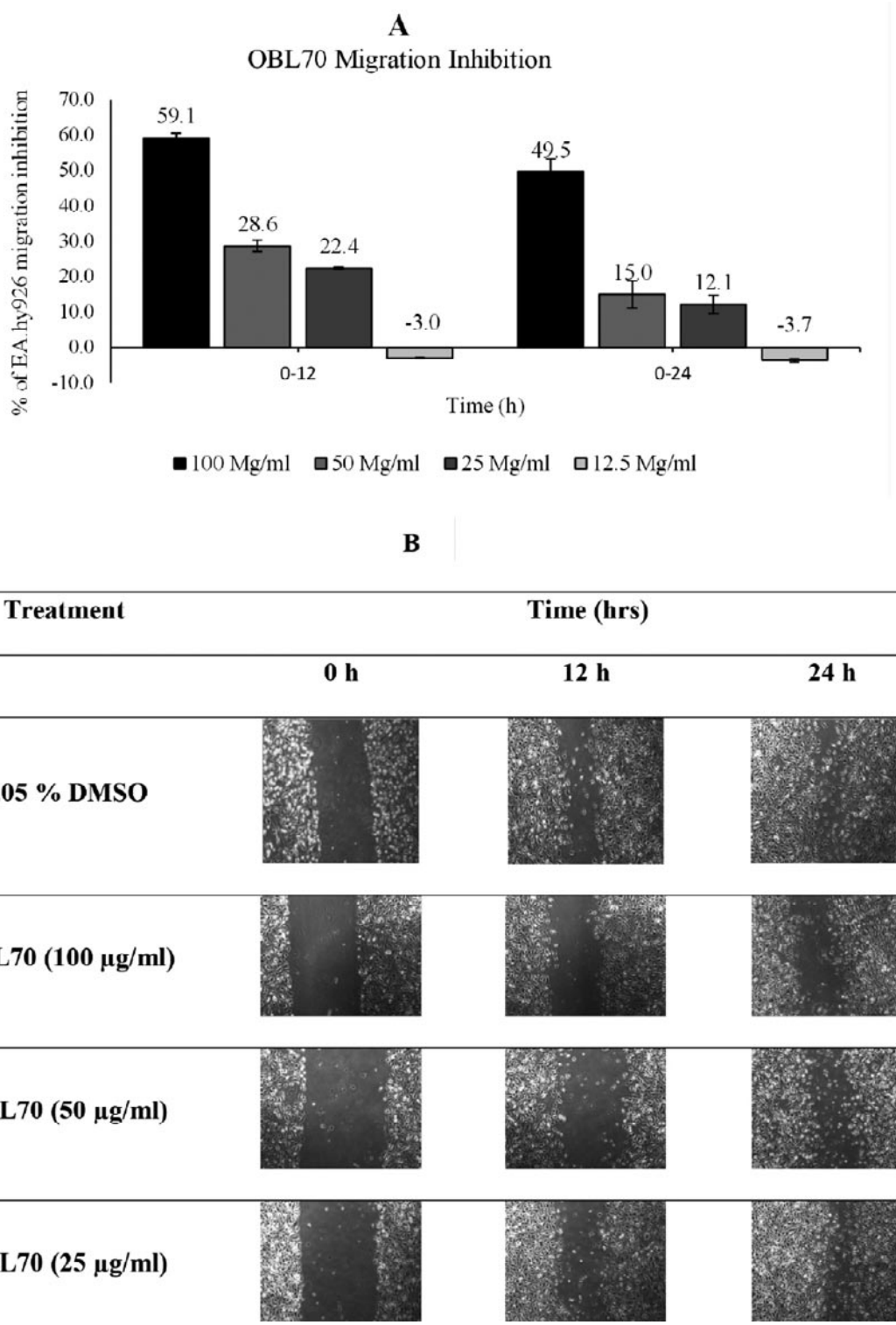
EA.hy926 Tube-Like Structures Inhibition By OBL70:

Assessment on the effects of OBL70 on tube formation revealed remarkable inhibitory activity. As depicted in Figure 4,



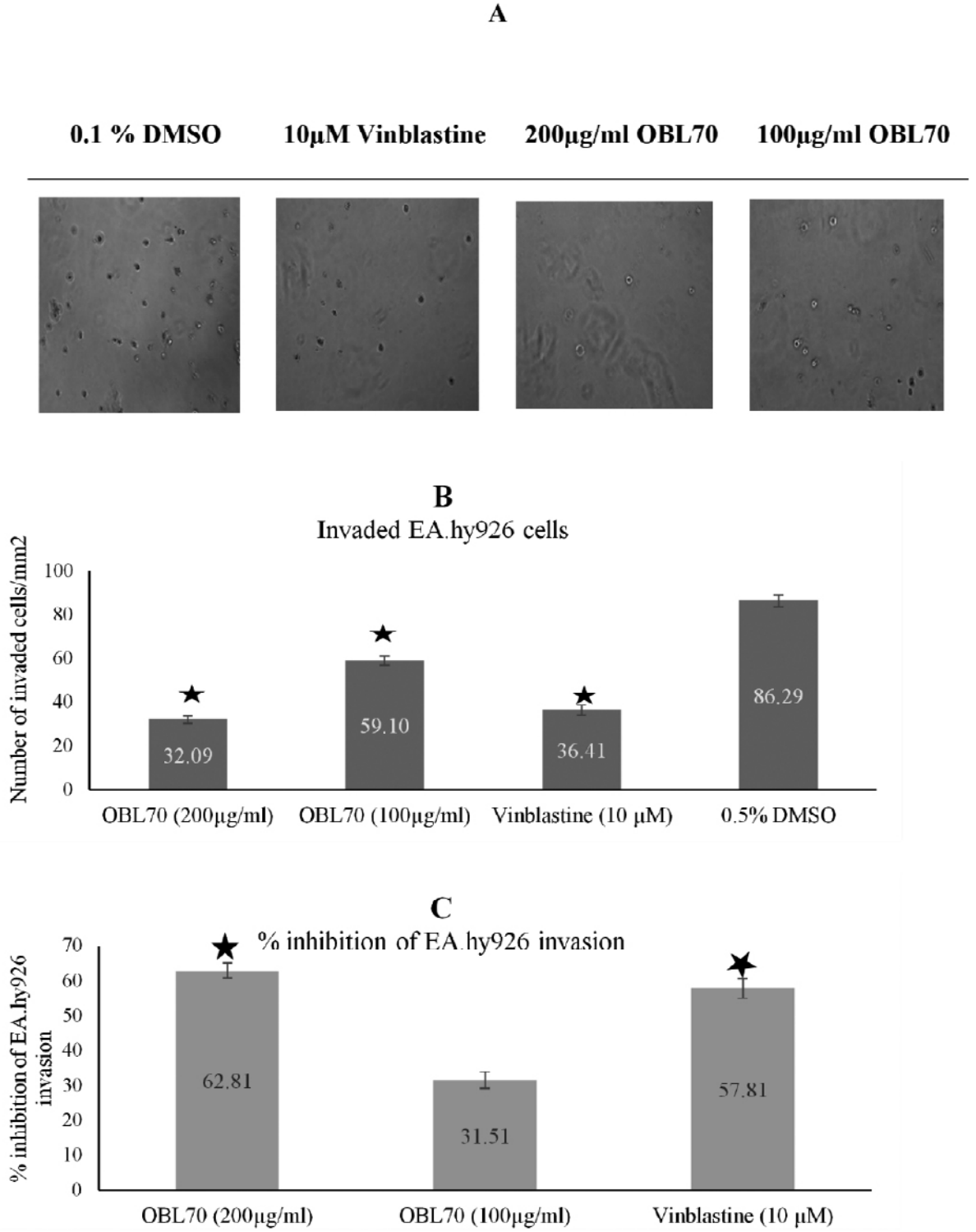
OBL70 exhibited inhibited neuvascularization formation by 77.46 ± 9.3 % ($P \leq 0.05$).
Pictures were taken under inverted microscope at 5×magnification.

Figure 1. Neovascularization formation inhibition after 7 days treatment with 100 µg/ml of (A) OBL70; (B) Negative control (0.05% DMSO) and (C) 100 µg/ml Suramine



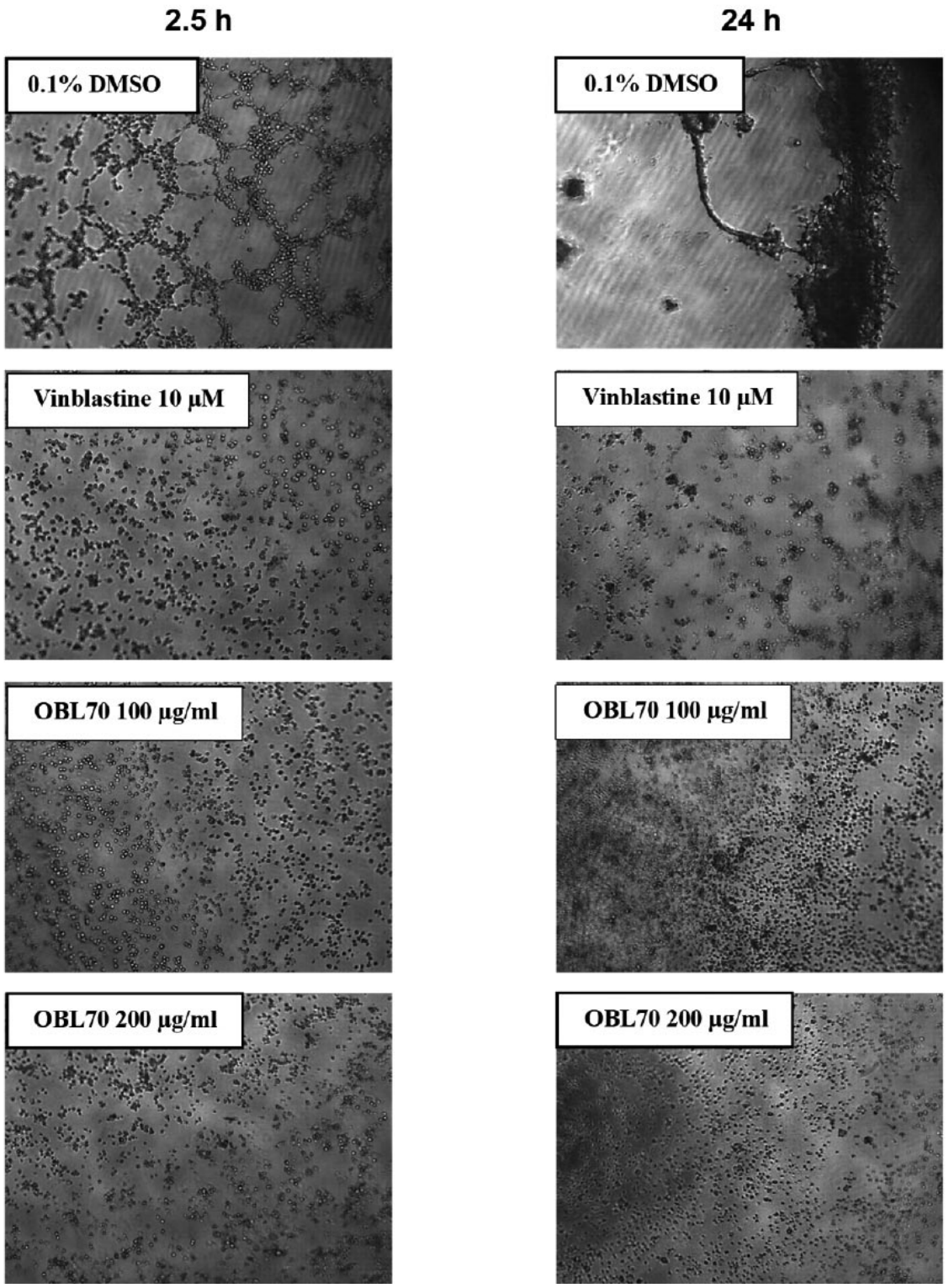
OBL70 extract inhibited migration of EA.hy926 cells after exposure 24 h. Pictures were taken at 12 and 24 h. (A) % of migration inhibition after 12 h and 24 h for 100 µg/ml was 59.1 % ± 1.17 and 49.5 % ± 3.79, for 50 µg/ml was 28.6 % ± 1.67 and 15.0 % ± 3.75, for 25 µg/ml was 22.4 % ± 0.28 and 12.1 % ± 2.56, and for 12.5 µg/ml was -3.0 % ± 0.14 and -3.7 % ± 0.49, respectively. (B) Pictures taken at 0, 12 and 24 h under inverted microscope at ×4 magnification. 0.05 % DMSO was used as negative control.

Figure 2. Percentage of EA.hy926 migration inhibition



(A) Pictures taken under the inverted microscope at ×10 magnification. (B) number of EA.hy926 invaded Matrigel®/mm2 after 24 h treatment with either 200 µg/ml OBL70, 100 µg/ml OBL70, 10 µM vinblastine or 0.1% DMSO as negative control. (C) Percentage of inhibiting EA,hy926 invasion by OBL70 or vinblastine when normalized in respect to negative control. ★ Indicate significant results (P≤0.05).

Figure 3. OBL70 inhibits invasion of EA.hy926 Human endothelial cell lines



OBL70 at both concentrations was able to inhibit the tube formation process almost 100% when results are normalized in respect to untreated cells (0.1% DMSO). These results were clear at 2.5 h and 24 h. Pictures were taken under inverted microscope at 20x total magnification power.

Figure 4. Inhibition of EA.hy926 tube formation by OBL70

OBL70 had the capacity to completely prevent tube formation at both tested concentrations (200 and 100 µg/ml) in respect to untreated EA.hy926.

DISCUSSION

This study presents compelling evidence for the antiangiogenic activity of *Ocimum basilicum* ethanolic extract (OBL70) demonstrating its efficacy across multiple in vitro and ex vivo models of angiogenesis. Collectively, the data suggest that OBL70 exerts its effects by selectively targeting endothelial cells (ECs) impeding critical angiogenic processes including proliferation, migration, invasion, neovascularization and tube formation.

The selective cytotoxicity of OBL70 towards EA.hy926 endothelial cells while sparing HT29 colorectal cancer cells indicates a level of cellular specificity. This selectivity may reflect OBL70's interference with endothelial-specific signaling pathways rather than general cytotoxicity highlighting its therapeutic potential as an antiangiogenic agent with minimal off-target toxicity. Given the known anti-inflammatory properties of *O. basilicum*, the extract likely mediates these effects through modulation of the VEGF/VEGFR signaling axis and other inflammation-associated pathways such as NFκB and TNFα, which are central regulators of angiogenesis and tumor progression[2,12,13].

The rat aortic ring assay, an ex vivo model that retains the complexity of the vascular microenvironment including pericytes, fibroblasts and extracellular matrix components provided strong confirmation of the in vitro findings. OBL70 inhibited neovascular sprouting by over 77 percent, outperforming the known angiogenesis inhibitor suramin. These results validate the extract's potential for antiangiogenic intervention in a physiologically relevant context aligning with previous findings that emphasized the role of OB extract in modulating vascular responses[8,14].

The observed inhibition of EC migration and invasion two fundamental processes required for capillary sprouting and remodeling, further underscores OBL70's impact on angiogenesis. At sub-cytotoxic concentrations OBL70 effectively impaired EC motility suggesting modulation of molecular pathways beyond cell viability. Such effects are often mediated by downregulation of integrins, MMPs and inflammatory mediators like COX2, VEGF and IL1β [2,15,16]. The sustained inhibitory effect at 100 µg/ml, compared to partial recovery at lower concentrations, could indicate dose-dependent engagement of multiple regulatory pathways or a threshold effect needed to overcome compensatory angiogenic signals.

Moreover, the inhibition of EC invasion through Matrigel a model mimicking extracellular matrix degradation, supports a mechanistic role for OBL70 in interfering with proteolytic enzymes such as MMP2 and MMP9 which are typically regulated by VEGFA/VEGFR2 signaling[17,18]. The similar inhibitory profile to vinblastine a known VEGFR2 inhibitor further reinforces this hypothesis[19].

The most striking finding was OBL70's complete inhibition of tube formation in EA.hy926 cells, a hallmark of in vitro angiogenesis. The tube formation assay integrates several steps of the angiogenic cascade including adhesion, migration, alignment, proteolysis, and tube assembly and is highly specific to EC behavior[16,20]. The absolute inhibition observed mirrors the effect of potent antiangiogenic compounds like catechins, which block VEGFR signaling and associated downstream pathways including PI3K/Akt and IL8 production[21,22]. OBL70's capacity to suppress these processes suggests the presence of active phytochemical constituents capable of targeting multiple angiogenic regulators concurrently.

Study Limitations

A key limitation of this study is the absence of phytochemical characterization of OBL70. The extract was used in crude form and therefore the specific bioactive constituents responsible for the antiangiogenic effects remain unidentified. Future investigations employing chromatographic and spectrometric techniques are necessary to isolate, identify and quantify the active compounds within the extract.

Furthermore, while the results suggest modulation of angiogenic pathways such as VEGFA/VEGFR2 signaling and matrix metalloproteinase activity these mechanisms were inferred based on phenotypic assays rather than directly validated. Elucidating the underlying molecular targets and signaling cascades through transcriptomic, proteomic or reporter-based analyses would strengthen the mechanistic basis of OBL70's activity and support its development as a targeted antiangiogenic agent.

CONCLUSION

This study presents the first comprehensive evaluation of the anti-angiogenic potential of *Ocimum basilicum* ethanolic leaf extract (OBL70) using both in vitro and ex vivo models. OBL70 demonstrated selective cytotoxicity toward endothelial cells, significantly inhibiting key steps of angiogenesis, including endothelial migration, invasion, and tube formation, as well as sprouting in the rat aortic ring assay. These findings underscore the therapeutic promise of OBL70 as a natural anti-angiogenic agent and warrant further investigation into its molecular mechanisms and in vivo efficacy in the context of solid tumor progression.

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