

Evaluation of the Antiproliferative, Anti-angiogenic, and Antioxidant Activity of a Newly Synthesized Indole Derivative

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Abstract:

Background: Angiogenesis is a physiological process whereby preexisting blood vessels divide to generate new ones. Abnormal angiogenesis is the major cause of various diseases.

Objective: In this work, a newly synthesized indole derivative, 2-(5-bromo-1H-indole-2-carbonyl)-N-(p-tolyl) hydrazine-1-carbothioamide (BIHC), was evaluated for antiproliferative and antiangiogenic activities using experimental models of neovascularization, while its antioxidant activity was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay. **Methods:** The antiproliferative effect on the human umbilical vein endothelial cell line (HUVECs) and MCF-7 cell line was assessed using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay. *An ex vivo* rat aorta ring assay and an *in vivo* chorioallantoic membrane experimental model were used to investigate its antiangiogenic activity; these assays were also used to assess the dose-response effects of the tested compounds by preparing serial concentrations. Antioxidant activity was analyzed using the DPPH free radical scavenging assay.

Results: The compound inhibits the proliferation of HUVECs with an IC₅₀ of 80.24 µg/mL and MCF-7 with an IC₅₀ of 96.32 µg/mL. The tested compound at serial concentrations showed significant dose-dependent inhibitory activity (P < 0.0001) in the rat aorta ring (RAR) assay, with an IC₅₀ of 30.47 µg/mL. The study proceeded by applying the chorioallantoic membrane (CAM) assay, in which serial dilutions of the derivative were added and tested separately. Furthermore, the tested compounds exhibited significant antioxidant activity in the DPPH radical scavenging assay (P < 0.0001), with an IC₅₀ of 5.48 µg/mL.

Conclusion: The findings suggest that the new indole derivative has an antiproliferative effect and significant anti-angiogenic and antioxidant properties.

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Keywords: indole derivative, antiproliferative, anti-angiogenic, antioxidant activity.

Introduction

Angiogenesis denotes the formation of new blood vessels from existing ones [1]. Angiogenesis plays a key role in the repair of injured tissues [2]. Tissue hypoxia is considered a potent trigger for this angiogenic process [3]. Hypoxia is resulting from decreased supply and/or increased oxygen demand. Angiogenesis is an adaptive response to hypoxia and is typically regulated by hypoxia-inducible factors (HIFs) [4]. The central role of HIF in the activation of angiogenic-related genes [5]. It activates the genes encoding pro-angiogenic proteins and their receptors, as well as the main initiator and stimulator of angiogenesis, namely vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) [6]. VEGF binds to its receptor (VEGFR) to activate its downstream intracellular signaling. Blocking VEGF prevents the activation of this signaling, leading to the anti-angiogenic effects [7]. Sprouting angiogenesis and splitting angiogenesis are the two types of angiogenesis in normal cells. Sprouting angiogenesis, the most common mechanism of vascular growth, is regulated by angiogenic growth factors produced by hypoxic tissue. Splitting angiogenesis, also known as intussusception, occurs when a groove forms within an existing vessel, inevitably splitting it into two vessels. Chaining angiogenesis occurs when vessel loops are instinctively brought into the tissue. Both types of angiogenesis play vital roles in the advancement and regeneration of organ systems [8]. As abnormal angiogenesis is a main cause of various conditions, angiogenesis itself can be useful for prognostic and diagnostic applications and can be modified and optimized for future clinical applications. In ischemic disorders that result from insufficient angiogenesis, a stimulator of angiogenesis may be used for therapeutic

purposes. In the case of tumors with excessive angiogenesis, anti-angiogenic agents can be used to attenuate it [9].

Indole-containing compounds have applications spanning antioxidant, anti-inflammatory, antimicrobial, antiviral, anticancer, antihypertensive, and antidiabetic activities [10].

In the present investigation, the anti-proliferative effect of the test compound on HUVEC and MCF-7 cell lines was determined using the MTT assay; the IC_{50} of the test compound and dose-response curve in HUVECs and the MCF-7 cell line were estimated using the MTT assay. Ex-vivo activity of the compound was determined using a rat aorta ring (RAR) assay, and In-vivo activity was determined using the chorioallantoic membrane (CAM) assay. Furthermore, to determine the free radical scavenging activity of the indole derivative, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was used.

Materials and Methods

Preparation of the compound: bromo-5)-2-1H-indole-2-carbonyl)-N-(ptolyl) hydrazine,carbothioamide-1- BIHC

A newly synthesized compound, 2-(5-bromo-1H-indole-2-carbonyl)-N-(p-tolyl) hydrazine-1-carbothioamide (BIHC), has been synthesized in the University of Baghdad's Department of Pharmaceutical Chemistry. The molecular structure of the compound was confirmed by a combination of Fourier-transform infrared spectroscopy (FTIR), 1H – NMR (Nuclear magnetic resonance spectroscopy), ^{13}C - NMR, and mass spectrometry. The FTIR spectrum displayed a characteristic sharp band at 1647 cm^{-1} assigned to the amide (C=O) stretching alongside a prominent peak at 1168 cm^{-1} ,



corresponding to the C=S function. In the ^1H -NMR spectrum, the presence of four distinct downfield singlets integrated for the NH proton, combined with the characteristic A_2B_2 spin system of the p-tolyl ring ($\delta=7.80$ and 7.13 ppm), confirmed the successful condensation. This was further substantiated by ^{13}C -NMR signals at $\delta=181.20$ ppm (C=S) and 160.70 ppm (C=O). Finally, the MS spectrum revealed an $[\text{M}+1]^+$ peak at $m/z = 403.00$, which is in excellent agreement with the calculated mass (403.01), thereby providing absolute evidence for the proposed structure. The purity of the compound was initially evaluated by thin-layer chromatography (TLC) using a hexane/ethyl acetate (60:40 v/v) solvent system, which revealed a single spot with an R_f value of 0.62 ; the sharp melting point range (211 - 215°C) further confirmed the crystalline purity and homogeneity of the synthesized compound. [11]. The stock solution of the tested compound was dissolved in DMSO and then diluted in the growth medium to make the final DMSO concentration of 1% .

Animals

Ten male albino rats aged 12-14 weeks were used in the RAR assay. Food and water were provided ad libitum to the rats, which were kept at 28 - 30°C . The animals were obtained from the animal house facility at the College of Pharmacy, Mustansiriyah University, Baghdad, Iraq. The experiments were approved by the College of Pharmacy at Mustansiriyah University before the study's initiation.

MTT assay

Cell culture

Human umbilical vein endothelial cell line HUVECs were propagated at the University of Jordan, Faculty of Science [12]. The MCF-7 breast cancer cell line was purchased from the

American Type Culture Collection (ATCC, USA). Cells were cultured in M-199 medium and RPMI-1640 medium, respectively. To create the full growth medium, 1% L-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin were added to both medium types. Additionally, 0.1 mg/ml heparin and 10 $\mu\text{g}/\text{ml}$ endothelial cell growth factor were added to M-199 medium. Cells were incubated at 37°C in 5% carbon dioxide (CO_2).

Anti-proliferation assay (MTT)

96-well plates were seeded with 1×10^4 cells/well in 100 μl of drug-free medium and incubated overnight to allow cell attachment. The 96-well plate, used only for the HUVEC cell line, was first coated with 50 μl of 0.5% gelatin before the trials and incubated for 30 minutes. Then, this solution was aspirated before seeding, whereas the plate used for MCF-7 is not coated. 100 μL of new medium with varying drug doses was added to each well. The applied concentrations were serial dilutions of the synthetic compound at 1000 , 500 , 250 , 125 , 62.5 , 31.25 , 16.625 , 7.812 , 3.906 , and 1.953 $\mu\text{g}/\text{mL}$. Moreover, the untreated cells received only DMSO as a negative control, and a blank well contained media without cells. Cells were incubated for 48 hours under standard culture conditions. Each concentration was examined in triplicate. After incubation, the medium was removed, 110 μl of MTT dye (5 mg/ml in PBS) was added, and the cells were incubated at 37°C with 5% CO_2 for 4 hours. The plates were subsequently extracted from the incubator, the supernatant was aspirated, and 130 μl of DMSO was introduced into each well. All plates were agitated vigorously for roughly 10 minutes (12, 13). Optical density reading (OD) was recorded using a microplate reader at 590 nm. Cell growth viability and inhibition were calculated using the following formula [15].



$$\% \text{ Viable cells} = \frac{\text{abs sample} - \text{abs blank}}{\text{abs control} - \text{abs blank}} \times 100$$

* “abs” stands for absorbance

$$\text{Inhibition \%} = 100 - \text{cell viability \%}$$

RAR anti-angiogenesis assay

The rat aortic ring assay experiment was conducted after revision and approval of the experimental procedures by the Ethics Committee of Mustansiriya University/College of Pharmacy/Approval No. on 75 1/2/2026.

The method developed by Brown and coworkers was used for the angiogenesis assay, with slight modifications [16]. Fresh thoracic aortas were collected, rinsed with serum-free medium, cleaned of the fibro-adipose tissue and residual blood clots, and then the aorta was cut into rings of 1 mm in thickness. To each well of a 48-well plate, 300 μ L of fibrinogen (3 mg/mL) in medium M199 with aprotinin (5 μ g/mL) was loaded and used as the lower layer.

Subsequently, one aortic ring was added to each well, and 10 μ L of thrombin in 0.15 M NaCl was added. Then, the section was incubated in 5% CO₂ and allowed to solidify for 1 radhour. The top-layer medium was prepared by adding the following to 300 μ L of medium supplemented with 20% HIFBS, 0.6% gentamicin, 1% amphotericin B, 0.1% aminocaproic acid, and 1% L-glutamine, and then adding it to each well. A stock solution was prepared by dissolving it in DMSO and diluting it with M199 growth medium to a final concentration of 1%. Serial dilutions of the tested compounds were prepared at concentrations of 1.953, 3.906, 7.812, 16.625, 31.25, 62.5, 125, 250, 500, and 1000 μ g/mL and added to the top. Wells without test samples received 100 μ g/mL aspirin as a positive control, and medium with 1% DMSO as a negative control. Each procedure was done in six replicates. Cultures were maintained in an incubator for 5 days. Fresh medium was

added on the fourth day of the procedure. Vessel inhibition was quantified with ImageJ as the percentage reduction in sprouting density relative to the control [17].

CAM in vivo assay

The chorioallantoic membrane assay was conducted after revision and approval of the experimental procedures by the Ethics Committee of Mustansiriya University/College of Pharmacy/Approval No. on 75 1/2/2026.

A local hatchery was the supplier of the fertilized chicken eggs. Six eggs per concentration. They were cleaned with ethanol 70%, then incubated in a CO₂ incubator for 72 hours at 37°C with approximately 60% humidity. The eggs were placed in the incubator horizontally and rotated frequently. Following 72 hours of incubation, 2 ml of albumin was aspirated through a pinpoint hole created on the side of the shell; the hole was subsequently sealed to facilitate CAM separation from the shell, and the eggs were placed again in the incubator. On the tenth embryonic day, a 3-4 cm window was opened over the air sac under sterile conditions, and sterile filter paper discs were loaded with 20 μ L of serial dilutions of the tested compounds, prepared at concentrations of 1.953, 3.906, 7.812, 16.625, 31.25, 62.5, 125, 250, 500, and 1000 μ g/mL. The sample's stock solution was produced by dissolving it in DMSO and diluting it with phosphate buffer solution (PBS). The negative control discs that had no samples were treated with PBS containing 1% DMSO. A working aspirin solution was freshly prepared at a concentration of 100 μ g/mL, 20 μ L of the prepared aspirin solution was loaded onto a sterile filter-paper disc. The



aspirin was used as the positive control. One disc was placed in an avascular region adjacent to small branching vessels; the shell window was sealed with sterile adhesive tape, and eggs were placed in the incubator for a further 72 hours at 37 °C and 60% relative humidity. Images were acquired around the disc and quantified using ImageJ. Anti-angiogenic activity was expressed as the percentage inhibition of vessel growth relative to the vehicle-treated control (17, 18).

DPPH scavenging activity

The DPPH was used to measure the antioxidant activity of the tested compounds.

The following serial concentrations of 100 µl of the tested compound were used: 1.953, 3.906, 7.812, 16.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL. These concentrations were mixed with 200 µl of 0.1 mM DPPH dissolved in methanol. Ascorbic acid was used as a positive control. In contrast, a negative control consisting of 200 µL of DPPH and 100 µL of methanol and methanol alone served as a blank. Absorbance was measured at 517 nm using a plate reader after 30 minutes of incubation in the dark. The tests were performed in triplicate. The free radical scavenging activity (F) was calculated by the following formula (19):

$$F \% = (A_0 - AC)/A_0 \times 100$$

A_0 = Absorbance of control

AC = Absorbance after 30 minutes of incubation

IC_{50} is the concentration that inhibits 50% of the sample required to scavenge 50% of DPPH free radicals (FRs). IC_{50} values were obtained by GraphPad Prism from concentration-response curves [21,22].

Statistical analysis

The results were expressed as mean $\pm$ SEM and plotted using GraphPad Prism 8.0.2. The obtained data were analyzed and compared using a one-way ANOVA, followed by Tukey's post hoc test, with a significance level of $p < 0.05$. Concentration-response curves and IC_{50} values were obtained by nonlinear regression.

Results

MTT assay

This indole derivative exhibited a dose-dependent anti-proliferative effect. For HUVECs, concentrations of 1000, 500, 250, 125, 62.5, 31.25, 16.625, 7.812, 3.906, and 1.953 µg/mL inhibited cell proliferation by 95.93 ± 1.47 , 78.31 ± 1.48 , 75.61 ± 1.72 , 63.67 ± 1.84 , 47.42 ± 1.62 , 32.55 ± 1.50 , 20.18 ± 1.55 , 12.91 ± 1.71 , and 9.61 ± 1.16 , respectively (Figure 1.A). The IC_{50} value was

80.24 µg/mL (48 h). The regression fits were quite good, $R^2 = 0.9918$. MCF-7 inhibited cell proliferation by 89.92 ± 1.81 , 76.13 ± 1.62 , 70.14 ± 1.72 , 59.91 ± 1.65 , 39.71 ± 1.50 , 25.23 ± 1.39 , 20.16 ± 1.52 , 12.69 ± 1.70 , 9.46 ± 1.63 , and 7.93 ± 1.53 , respectively. (Figure 1. B). The IC_{50} value was 96.32 µg/mL (48 h); the $R^2 = 0.9940$.

RAR assay

The rat aortic ring (RAR) assay provided clear evidence that the newly synthesized indole derivative, BIHC, exerts a potent anti-angiogenic effect in a dose-dependent manner. The rat aortic ring (RAR) assay demonstrated that the synthetic indole derivative, BIHC, exerted a clear dose-dependent suppression of angiogenesis. The dose-response curve (Figure 2) revealed a steep increase in inhibition at lower concentrations, followed by a plateau at higher concentrations, indicating



saturation of the inhibitory effect. Quantitative analysis (Table 1) confirmed this trend, with inhibition values ranging from $46.93 \pm 2.12\%$ at $1.953 \mu\text{g/mL}$ to a maximum of $81.99 \pm 2.54\%$ at $1000 \mu\text{g/mL}$. Intermediate concentrations ($62.5\text{--}250 \mu\text{g/mL}$) consistently maintained inhibition levels above 70%, highlighting the compound's sustained efficacy across a broad range. Nonlinear regression analysis determined the IC_{50} value to be $30.47 \mu\text{g/mL}$, underscoring the compound's potency at relatively low concentrations.

Figure 3 further supports these observations by presenting the comparative inhibition percentages across treatment groups. The control (DMSO) exhibited negligible inhibition, whereas aspirin at $100 \mu\text{g/mL}$ produced a statistically significant reduction in vessel growth ($p < 0.0001$). Importantly, the indole derivative demonstrated consistently higher inhibition across all tested concentrations, with significance maintained relative to the DMSO group ($p < 0.0001$). This comparative analysis underscores the superior anti-angiogenic activity of the compound when benchmarked against aspirin, a known anti-angiogenic agent.

Figure 4 provides visual confirmation of these quantitative findings, showing representative micrographs of rat aortic rings treated with increasing concentrations of the compound. At lower doses ($3.906\text{--}16.625 \mu\text{g/mL}$), microvessel sprouting was still evident, though reduced compared to the control. As concentrations increased ($31.25\text{--}62.5 \mu\text{g/mL}$), a progressive suppression of angiogenic outgrowth was observed, culminating in near-complete inhibition at higher doses. These images vividly demonstrate the concentration-dependent suppression of neovascularization, aligning with the quantitative data presented in Figures 2 and 3.

CAM assay

The compound again demonstrated a dose-dependent suppression of angiogenesis, with inhibition values ranging from $37.72 \pm 0.80\%$ at $1.953 \mu\text{g/mL}$ to $69.53 \pm 0.49\%$ at $1000 \mu\text{g/mL}$ (Table 2). The dose–response curve (Figure 5) revealed a plateau around 60–70% inhibition, consistent with saturation kinetics observed in the RAR assay. Statistical analysis (Figure 6) confirmed that both the compound and aspirin significantly inhibited angiogenesis compared with the DMSO control ($p < 0.0001$). Representative CAM images (Figure 7) visually corroborated these findings, showing progressive suppression of vascular branching with increasing concentrations of the compound. The consistency between ex vivo and in vivo models strengthens the evidence that the compound acts as a reliable angiogenesis inhibitor. Importantly, the compound's inhibitory effect was comparable to, and in some cases exceeded, that of aspirin, a well-established anti-angiogenic agent.

DPPH Scavenging Activity Assay

The antioxidant potential of BIHC was evaluated using the DPPH radical scavenging assay. Ten serial dilutions ranging from 1000 to $1.953 \mu\text{g/mL}$ were tested, with ascorbic acid serving as the standard reference compound. The compound demonstrated a concentration-dependent FRSA, as shown in Figure 8. At the highest concentration ($1000 \mu\text{g/mL}$), the compound achieved $84.21 \pm 0.13\%$ scavenging activity, while at the lowest concentration ($1.953 \mu\text{g/mL}$), activity decreased to $42.06 \pm 1.99\%$ (Table 3). Nonlinear regression analysis performed in GraphPad Prism yielded an IC_{50} value of $5.48 \mu\text{g/mL}$, indicating strong antioxidant potency. A lower IC_{50} value reflects higher antioxidant activity, and this result highlights the



compound's ability to neutralize free radicals effectively at relatively low concentrations. Ascorbic acid exhibited robust FRSA across all tested concentrations, with activity values ranging from $97.51 \pm 0.13\%$ at $1000 \mu\text{g/mL}$ to $48.95 \pm 1.38\%$ at $1.953 \mu\text{g/mL}$ (Table 4). The dose-response curve (Figure 9) showed near-complete scavenging efficiency at higher concentrations, consistent with its established role as a potent antioxidant. Nonlinear regression analysis determined the IC_{50} value for ascorbic acid to be $1.12 \mu\text{g/mL}$, confirming its superior radical scavenging capacity compared with the synthetic compound.

Figure 10 presents the comparative bar graphs for both the synthetic compound (Panel A) and ascorbic acid (Panel B). One-way ANOVA revealed that both agents significantly reduced DPPH radical concentrations in a dose-dependent manner compared with the control ($p < 0.0001$). While ascorbic acid demonstrated stronger activity overall, the synthetic compound consistently exhibited substantial scavenging activity across all concentrations, reinforcing its antioxidant potential.



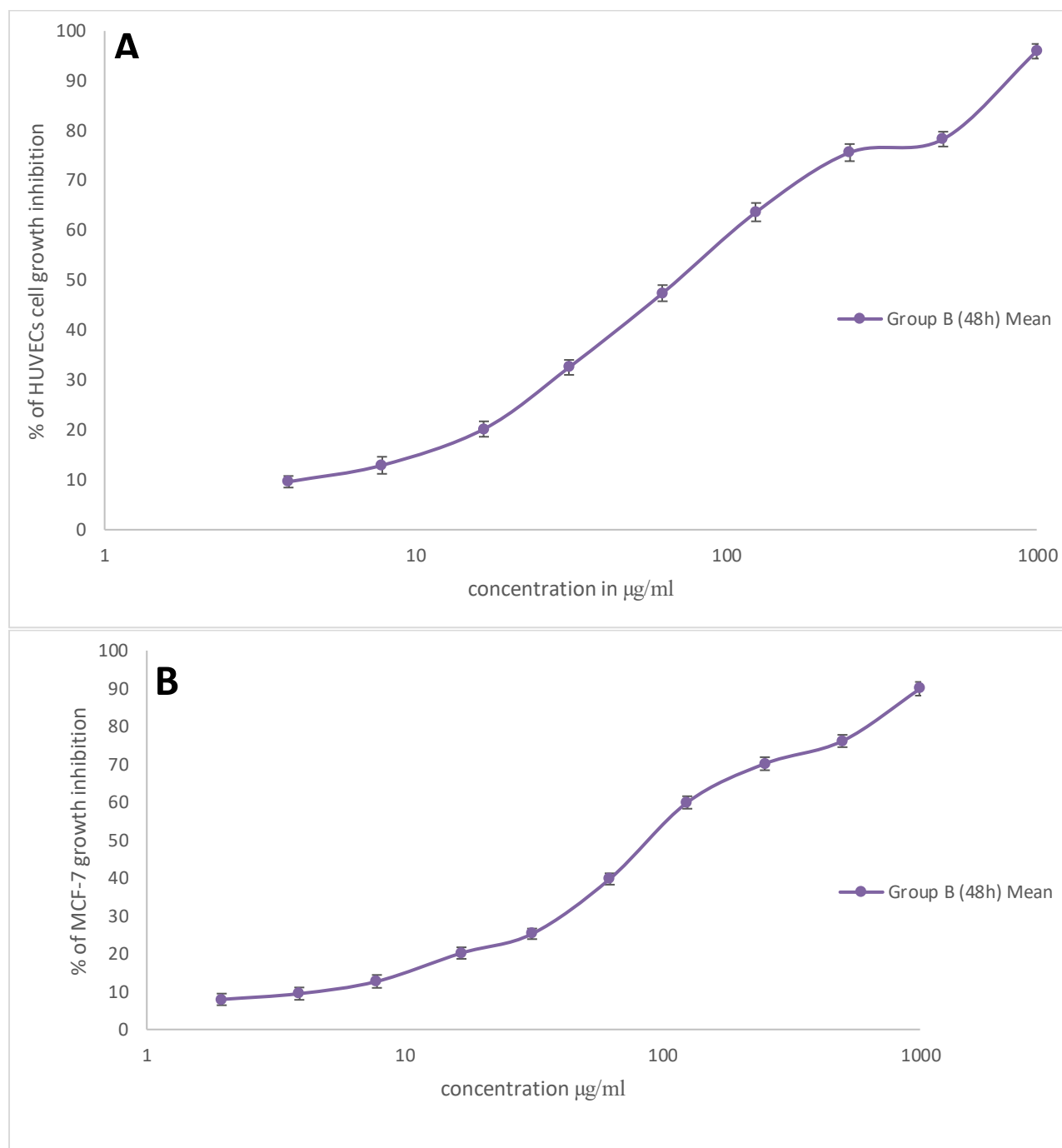


Figure 1: The anti-proliferative activity of the 2-(5-Bromo-1H-indole-2-carbonyl)-N-(p-tolyl) hydrazine-1-carbothioamide against HUVECs and MCF-7 cell lines:

A: The synthetic compound's dose-response curve against the HUVECs cell line.

B: The synthetic compound's dose-response curve against the MCF-7 cell line.

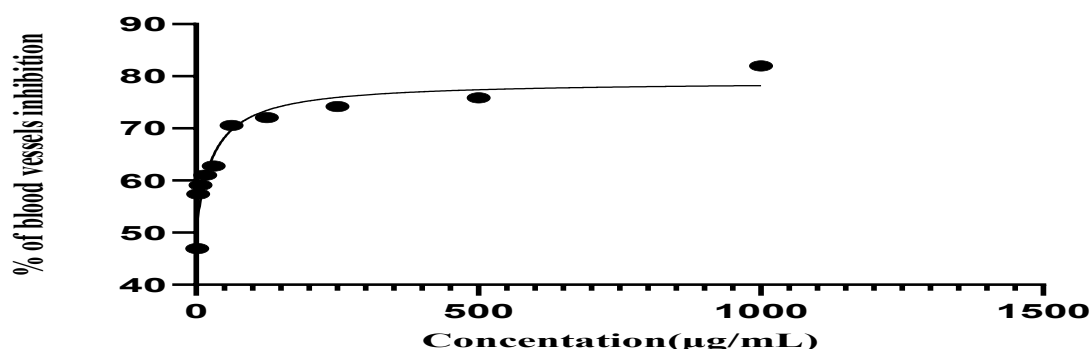


Figure 2: Dose response curve of BIHC against blood vessel growth in rat aortic ring assay.

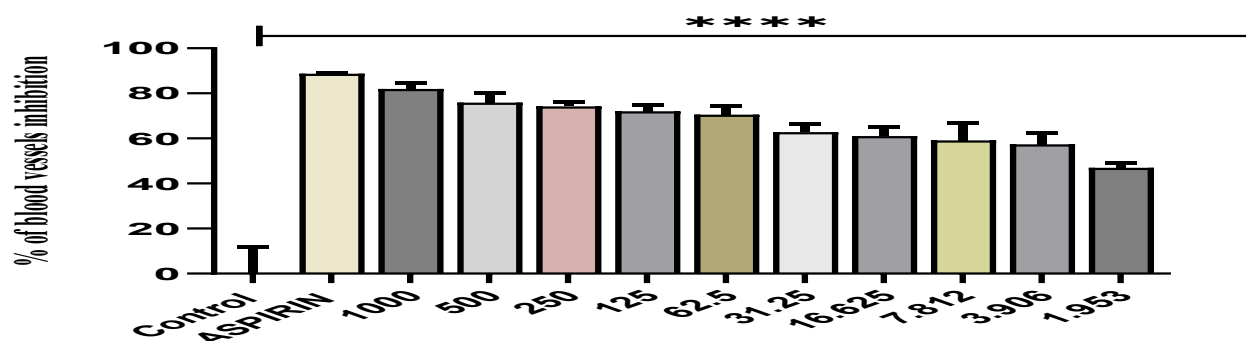


Figure 3: Graphical presentation of the effect of BIHC on blood vessel growth inhibition, assessed by the rat aortic ring assay (ex vivo assay), at ten serial concentrations spanning 1000-1.953 µg/ml, with aspirin at 100 µg/ml and DMSO. There was a significant difference in blood vessel inhibition between the tested compound and aspirin compared with the DMSO group (**** = $P < 0.0001$).

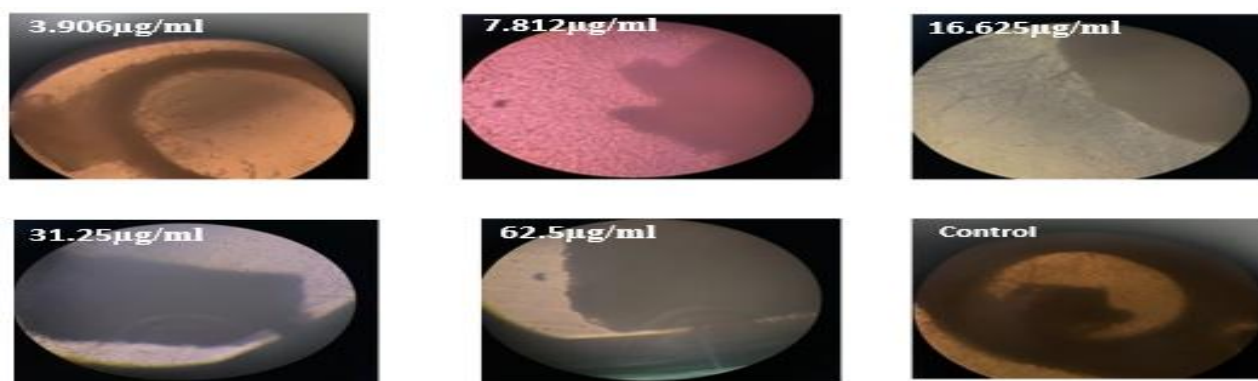


Figure 4: Effect of different concentrations of BIHC on blood vessel outgrowth in the rat aortic ring assay (ex vivo). After 5 days, microvessel sprouting was examined under an inverted microscope (10× magnification), and images were captured with a specialized microscope camera.

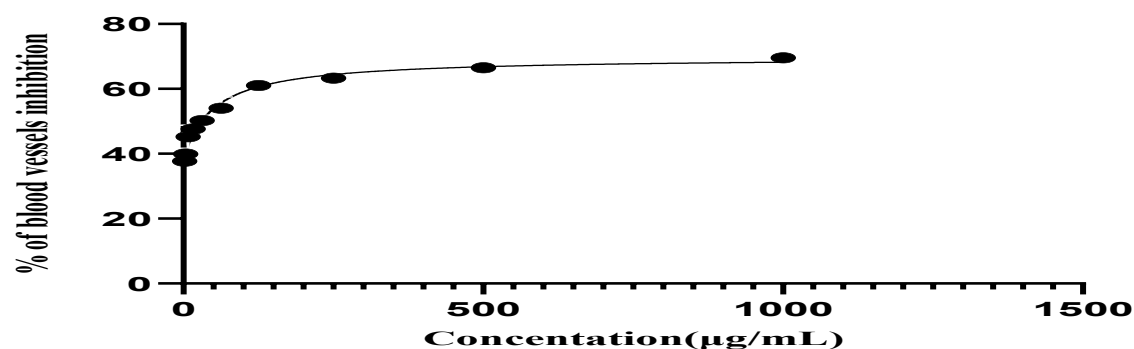


Figure 5: Antiangiogenic effects in CAM assay by BIHC.

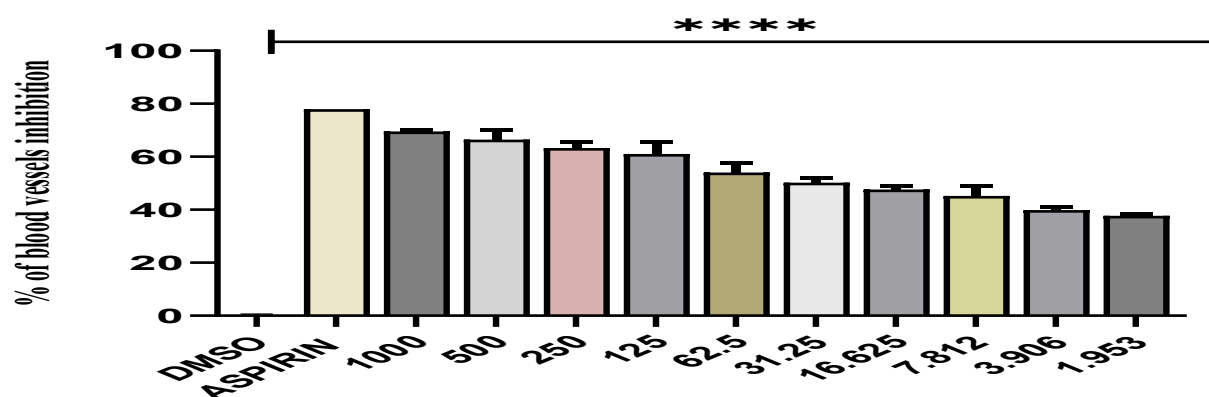


Figure 6: Graphical presentation of the effect of BIHC on blood vessel growth inhibition, assessed in *chick chorioallantoic membrane* assay (in vivo assay), at ten serial concentrations spanning 1000-1.953 µg/ml, with aspirin at 100 µg/ml and DMSO. There was a significant difference in the blood vessel inhibition between the tested compound and aspirin compared with the DMSO group (**** = $P < 0.0001$).

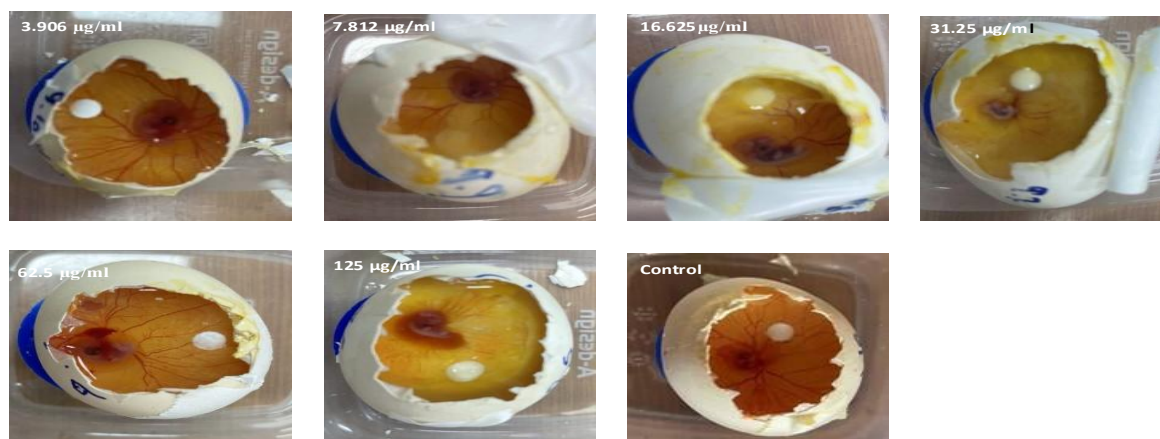


Figure 7: Images of the CAM assay illustrating the effect of different concentrations of BIHC on blood vessel growth

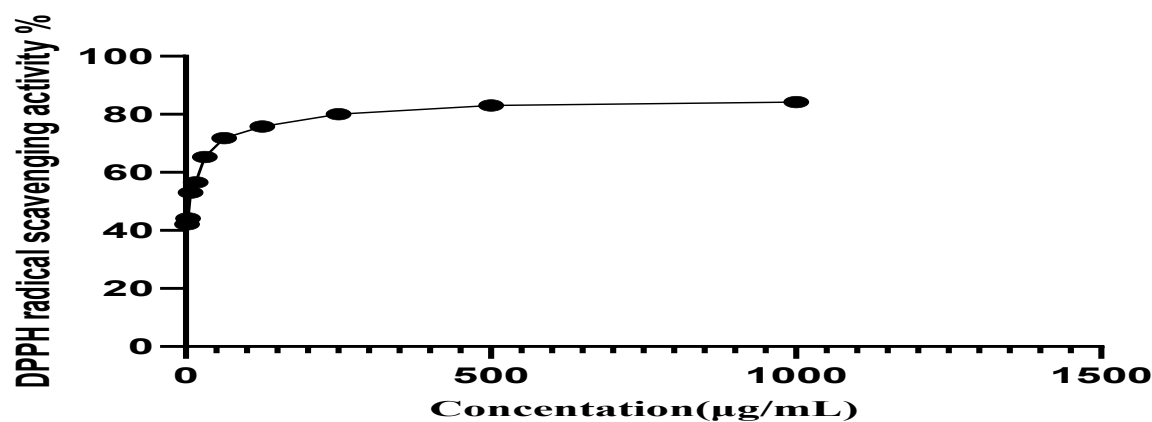


Figure 8: Free radical scavenging activity (FRSA) of BIHC.

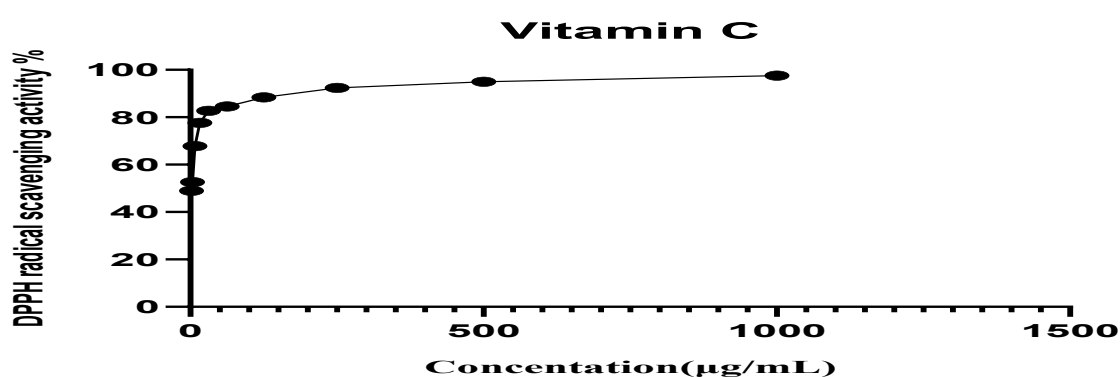


Figure 9: Free radical scavenging activity (FRSA) of ascorbic acid

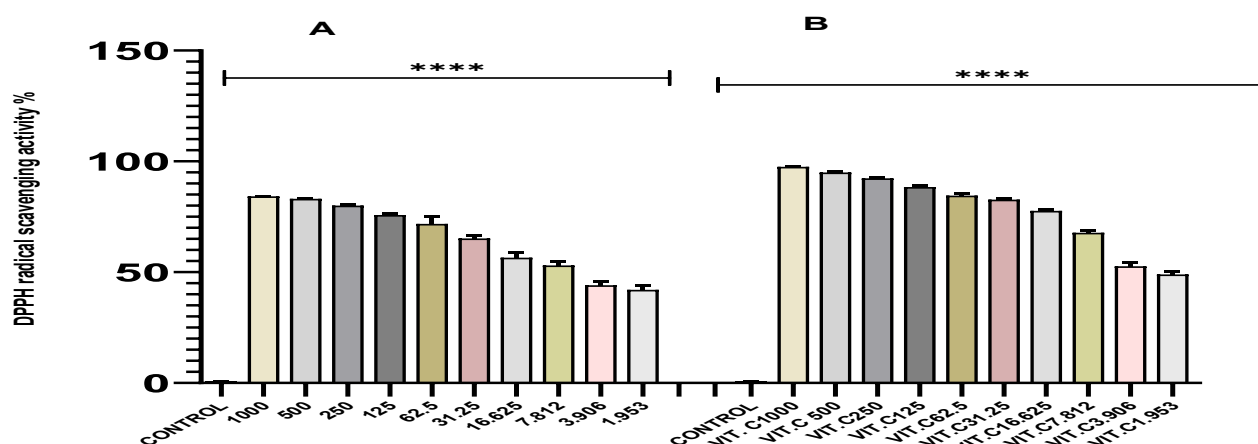


Figure 10: DPPH radical scavenging activity of: (A) BIHC, at ten serial concentrations spanning 1000-1.953 µg/ml with a negative control. (B) ascorbic acid, at ten serial concentrations spanning 1000-1.953 µg/ml with a negative control. One-way ANOVA indicates that the synthetic compound and ascorbic acid demonstrated significant concentration-dependent DPPH radical-scavenging activity compared with the control (****, $P < 0.0001$).

Table 1: Serial concentrations of BIHC compound and their respective blood vessel inhibition percentage in rat aortic ring assay

Concentration	Blood vessel inhibition % (mean $\pm$ SD)
1000	81.99 $\pm$ 2.54
500	75.85 $\pm$ 4.21
250	74.21 $\pm$ 1.77
125	72.05 $\pm$ 2.63
62.5	70.56 $\pm$ 3.61
31.25	62.78 $\pm$ 3.67
16.625	61.03 $\pm$ 3.85
7.812	59.12 $\pm$ 7.64
3.906	57.38 $\pm$ 5.17
1.953	46.93 $\pm$ 2.12

Table 2: Effect of BIHC on blood vessel inhibition in CAM assay.

Concentration	Blood vessel inhibition % (mean $\pm$ SD)
1000	69.53 $\pm$ 0.49
500	66.4 $\pm$ 3.59
250	63.31 $\pm$ 2.05
125	61.01 $\pm$ 4.72
62.5	54.01 $\pm$ 3.47
31.25	50.22 $\pm$ 1.67
16.625	47.6 $\pm$ 1.20
7.812	45.2 $\pm$ 3.56
3.906	39.89 $\pm$ 0.99
1.953	37.72 $\pm$ 0.80

Table 3: DPPH Radical Scavenging Activity (%) of BIHC compound at Different Concentrations.

Concentration	DPPH scavenging activity % (mean $\pm$ SD)
1000	84.21 $\pm$ 0.13
500	83.07 $\pm$ 0.20
250	80.04 $\pm$ 0.33
125	75.79 $\pm$ 0.66
62.5	71.8 $\pm$ 3.26
31.25	65.25 $\pm$ 1.45
16.625	56.54 $\pm$ 2.14
7.812	53.03 $\pm$ 1.60
3.906	44.08 $\pm$ 1.84
1.953	42.06 $\pm$ 1.99



Table 4: DPPH FRSA (%) of ascorbic acid at Different Concentrations.

Concentration	DPPH scavenging activity % (mean $\pm$ SD)
1000	97.51 $\pm$ 0.13
500	94.95 $\pm$ 0.45
250	92.36 $\pm$ 0.24
125	88.4 $\pm$ 0.72
62.5	84.52 $\pm$ 0.88
31.25	82.71 $\pm$ 0.32
16.625	77.63 $\pm$ 0.82
7.812	67.76 $\pm$ 1.13
3.906	52.63 $\pm$ 1.52
1.953	48.95 $\pm$ 1.38

Discussion

Anti-angiogenesis has been investigated as a modality for preventing tumor growth and other angiogenesis-dependent diseases [23]. Biological and clinical applications have been demonstrated for many bioactive aromatic compounds containing the indole nucleus. The indole scaffold is found in many synthetic drug molecules, providing a valuable basis for treatment, and it binds with high affinity to multiple receptors, making it a promising pharmacophore for the development of novel therapeutic molecules [24].

This study showed that the synthesized indole derivative inhibited the proliferation of both HUVEC and MCF-7 cells in a concentration-dependent manner. This concentration-dependent cytotoxicity is considered a fundamental pharmacological property of compounds with true antiproliferative activity and suggests that greater intracellular exposure leads to greater interference with signaling pathways that control cell growth and survival [25,26]. The lower IC₅₀ value in HUVECs in comparison with MCF-7 cells indicates that endothelial cells are more sensitive to the compound used. This is desirable as endothelial cells depend on the VEGF/VEGFR system for proliferation and migration. Blocking this pathway has immediate effects on endothelial cell activity and blood vessel

formation. VEGFR-2 is now identified as the major receptor regulating endothelial proliferation and is the main molecular target for many newly developed indole-based anti-angiogenic agents [27,28]. The MCF-7 cells were somewhat more resistant than HUVECs, but the compound did induce a distinct dose-dependent decrease in cell viability. Multiple compensatory signaling pathways including PI3K/Akt, MAPK/ERK, EGFR, and estrogen receptor signaling have been identified to be activated in breast cancer cells, leading to decreased sensitivity of tumor cells to single-target drugs. Therefore, in most cases, a higher drug concentration is required to achieve similar anti-proliferative effects. Recent studies of the anticancer activity of indole-derived compounds have shown similar differences in sensitivity between some breast cancer cell lines [29,30].

A newly synthesized indole derivative was evaluated for its anti-angiogenic activity using both ex vivo and in vivo assays and for its antioxidant activity using the DPPH radical-scavenging assay.

The RAR assay is an angiogenic assay that has shown particular benefit in the study of microvascularization [19]. Ex vivo models bridge the gap between in vitro and in vivo models, combining the advantages of both systems and allowing the angiogenic and anti-angiogenic effects of various soluble or matrix



factors to be easily assessed [31]. Based on the current research data for the RAR assay, the present study demonstrates that the newly synthesized indole derivative inhibits angiogenesis. The IC_{50} of the tested compound was 30.47 $\mu\text{g/mL}$. Both the test compound and aspirin significantly inhibit angiogenesis compared with the control group ($P < 0.0001$). The CAM assay is widely used to study angiogenesis [32]. The current study showed that the newly synthesized indole derivative inhibits angiogenesis in vivo in a concentration-dependent manner. It produces a maximum inhibitory effect of 69.53% at the highest tested concentration (1000 $\mu\text{g/mL}$). Statistical analysis using one-way ANOVA demonstrated that the newly synthesized compounds and aspirin significantly inhibited angiogenesis

($p < 0.0001$). Based on current findings from RAR and CAM experimental models, the newly synthesized compound significantly inhibits blood vessel formation in both. Therefore, inhibiting blood vessel formation may occur via more than one pathway; in fact, studies have also implicated an antiangiogenic compound in reducing blood vessel sprouting [14] decreasing blood vessel creation [33], and reducing the expression of bFGF, VEGF, VEGFR2, and HIF-1 α [34].

The DPPH assay is used to test radical-scavenging activity [35]. There is substantial evidence that an excess of FRs is linked to the development of various diseases, including inflammatory and cardiovascular diseases, tumors, aging, and other disorders [36]. The DPPH assay showed that the tested compounds exhibited antioxidant activity, with an IC_{50} of 5.48 $\mu\text{g/mL}$. A lower IC_{50} value implies higher antioxidant activity. Both the newly synthesized compounds and ascorbic acid, as a positive control, demonstrated significant concentration-dependent DPPH radical-scavenging activity ($P < 0.0001$). The tested compound could therefore inhibit

angiogenic signaling by reducing oxidative stress-induced signals that promote it [37].

Conclusion

In conclusion, the synthesized indole derivative demonstrated a concentration-dependent antiproliferative effect against both HUVEC and MCF-7 cells, with greater potency toward HUVECs, suggesting preferential antiangiogenic activity. The new indole derivative possesses significant antiangiogenic and antioxidant properties. The compound effectively inhibited neovascularization when applied at varying concentrations in both ex vivo and in vivo experiments (i.e., RAR and CAM models) in a concentration-dependent manner. In addition, the tested compound exhibited a marked and significant antioxidant activity. Collectively, the synthesized indole derivatives represent promising bioactive compounds. However, the current results should be regarded as preliminary proof-of-concept evidence rather than definitive mechanistic validation. Future research should serve to clarify the molecular mechanism of action via apoptosis and cell cycle studies; verify the involvement of VEGFR-2 and signaling pathways; study pharmacokinetics and toxicity; and carry out efficacy studies in relevant mammalian cancer models.

CONFLICT OF INTERESTS

The authors declare that there was no conflict of interest.

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Author Contributions

Raghad Shakir Mahmood conducted the experiments, collected and analyzed the data, performed the statistical analysis, interpreted the results, and prepared the initial manuscript.



Gaith Ali Jasim conceived the study, supervised the research, followed up on the preparation and development of the manuscript, and critically reviewed the manuscript.

Basma Talib Al-Sudani co-supervised the research, followed up on the experimental work performed the statistical analysis and contributed to the preparation and critical revision of the manuscript.

Asmaa Q. Ibrahim isolated and provided the HUVECs and trained Raghad Shakir Mahmood in HUVEC culture and handling.

All authors reviewed and approved the final version of the manuscript.

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