

Antiangiogenic Activity of 4-Chloro-Phenyl-Carbothioamide Derivatives In *Ex Vivo* and *In Vitro* Experimental Study

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Article Info

Submitted: 09-10-2024

Revised: 02-03-2025

Accepted: 25-03-2025

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ABSTRACT

Angiogenesis is a crucial and critical process in growth and development that has received study attention in recent decades. Angiogenesis inhibitors are one of the cornerstones of modern anticancer treatments, tumour develop resistance to drugs rapidly, thus providing new agents is crucial for patient's survival. The study investigated the effect of different concentrations of the novel 4-chloro-phenyl-carbothioamide derivative using different angiogenesis assays. The present study examined the potential antiangiogenic, antioxidant, and cytotoxic properties of a carbothioamide indole derivative and assessed VEGF gene expression. The tested indole derivative's antiangiogenic efficacy was assessed using the *ex vivo* rat aorta ring (RAR) assay. The DPPH test for scavenging activity was utilized to clarify the most likely cause of its antiangiogenic action. The MTT assay assessed the proliferation of the HUVEC cell line while the expression of the VEGF gene in the colon cancer (HCT116) cell line was analysed. The evaluated drug exhibited antiangiogenic efficacy with an IC₅₀ value of 17.99 µg/ml in the RAR assay. The drug successfully reduced the DPPH free radical in a concentration-dependent manner (IC₅₀ = 100.30 µg/ml). The evaluated drug exhibited negligible to non-toxic effects on the HUVEC cell line, with an IC₅₀ value of 733.60 µg/ml. It significantly downregulates the VEGF gene expression in HCT116 cells at 400 µg/ml. In conclusion, the 2-NHC compound exhibited significant antioxidant and anti-angiogenesis effects with minimum toxicity against normal human cells. 2-NHC appears to downregulate the VEGF gene expression in colon cancer cell lines.

Keywords: Anti-angiogenesis, VEGF gene, human umbilical ventricular endothelial cell (HUVEC), colon cancer (HCT116)

INTRODUCTION

The tumor is a biological tissue characterized by fast multiplication, intense metabolism, and resilient vitality, requiring significantly more oxygen and nutrients than normal tissue cells. The early phase of tumor growth is an avascular condition, wherein the tumor lacks aggressiveness and obtains oxygen and nutrients via diffusion from adjacent tissue

(Conway et al., 2001; Tashakori et al., 2024). Consequently, tumor angiogenesis is constrained to a dormant state due to the diminished quantities of pro-angiogenic factors and vascular inhibitory signals within the extracellular matrix, resulting in infrequent intratumoral vascularization (Ghnim et al., 2024; Kalluri, 2003). A microenvironment characterized by hypoxia, ischemia, acidosis, and elevated interstitial pressure progressively forms

within tumor tissue, resulting in the release of many growth factors and cytokines that promote angiogenesis and lymphangiogenesis to satisfy the demands of tumor growth and metabolism (Adams & Alitalo, 2007). The rapid proliferation of tumor cells has resulted in a microenvironment characterized by heightened hypoxia, acidosis, and elevated interstitial pressure in areas distant from blood arteries within tumor tissue, facilitating the growth and malignancy of the tumor. Numerous pro-angiogenic substances are continuously produced or upregulated by tumor cells to stimulate endothelial cells, pericytes, tumor-associated fibroblasts, endothelial progenitor cells, and immune cells (Ribatti & Crivellato, 2009; Shiga et al., 2015), therefore resulting in telangiectasia, degradation of the basement membrane, modification of the extracellular matrix, shedding of pericytes, and differentiation of endothelial cells to sustain an elevated state of angiogenesis, ultimately leading to tumor proliferation, dissemination, and metastasis (Parmar & Apte, 2021; Saadh et al., 2024). Moreover, metabolic stress in cancers can be induced by immunological stimulation, inflammatory responses, oncogene mutations, and pharmacological interventions, exacerbating tumor angiogenesis and facilitating tumor invasion and dissemination (Vakilzadehian et al., 2024; Viillard & Larrivee, 2017). To date, despite extensive research dedicated to anti-cancer therapies aimed at combating this incurable and fatal disease, none have demonstrated sustained clinical success (Bellou et al., 2013; Ebos & Kerbel, 2011).

Angiogenesis is a crucial and critical process in growth and development that received study attention in recent decades. It is a natural mechanism that forms new blood arteries from old ones through mechanisms of sprouting and division. Angiogenesis primarily entails cell proliferation, migration, and the release of vascular endothelial growth factor (VEGF); a disruption in this mechanism leads to clinical diseases (D'Alessio et al., 2015; Payne et al., 2024). Consequently, angiogenesis is seen as a possible treatment target for associated disorders. Nonetheless, the fundamental mechanisms remain inadequately comprehended (Ali et al., 2024; Hussein et al., 2018; Kadhim, 2016; Manna et al., 2019).

Aberrant angiogenesis is a critical characteristic and a significant mechanism in cancer progression. Angiogenesis provides the heightened demand for oxygen and nutrients characteristic of the rapidly proliferating

microenvironment of solid tumours (Fong, 2008; Raghif, 2016). Tumour angiogenesis facilitates the development of new blood vessels in an unregulated and disorganized manner, eventually leading to accelerated tumour growth and heightened metastatic potential. The angiogenic process is primarily governed by the interactions between tissue vascular endothelial growth factor receptors (VEGFRs) and their soluble ligands VEGFs, which are meticulously controlled under healthy conditions. VEGF molecules comprise many sub-variants, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (Zeena A. Hussein et al., 2018; Petrioli et al., 2022).

Reactive oxygen species (ROS) act as second messengers in response to hypoxia and serve an important role in stabilizing HIF-1 α protein and inducing the production of angiogenic factors. Furthermore, chemical antioxidants suppress HIF-1 α accumulation and inhibit the transcription of VEGF via the mechanism that involves ROS. ROS production results in an increase in viability and angiogenesis. As an important angiogenesis growth factor, VEGF can directly be promoted by various cell types and promotes cell proliferation, migration, cytoskeletal reorganization, and tubular morphogenesis in ECs (Cheng et al., 2019).

Studies focused on antiangiogenesis have propelled anti-angiogenic therapy as an attractive option in anti-tumor therapy. The predominant anti-angiogenic medicines comprise monoclonal antibodies and tyrosine kinase inhibitors (TKIs) that target the vascular endothelial growth factor (VEGF) pathway. Nonetheless, the clinical advantages of this modality remain constrained by various shortcomings, including adverse events, acquired drug resistance, tumor recurrence, and the absence of validated biomarkers. These issues necessitate further investigation into the mechanisms of tumor angiogenesis, the development of multiple pharmacological agents, and combination therapies to enhance therapeutic efficacy (Liu et al., 2023).

Indole is a naturally occurring heterocyclic molecule comprising a benzene ring fused to a pyrrole ring, characterized by its susceptibility to electrophilic substitution (Chadha & Silakari, 2018). Heterocyclic molecules containing nitrogen are gaining prominence in organic, medical, and pharmacological research. Indole derivatives, including carbothioamide, oxadiazole, and triazole, are esteemed as anticancer agents (Dhuguru & Skouta, 2020). Some of these indoles derivative

(thiadiazin) was shown to inhibit the process of angiogenesis (Mohamady et al., 2020).

Among natural and synthetic antioxidants, indole derivatives have effects against oxidative stress induced by hydroxyl radicals and peroxide anions formed in the Fenton reaction (Agircan et al., 2024). It has been proven that the presence of indole structure affects the effectiveness of antioxidants. Due to the free electron pair located on it, the heterocyclic nitrogen atom is an active indole redox center (Jasiewicz et al., 2021). Indolic compounds derivatives, inhibit VEGF-induced VEGFR-2 activation and subsequent angiogenesis, which suggests an interaction between indolic compounds and surface components of the endothelial cell membrane in a way that prevents VEGF from activating its receptor (Gallardo-Fernández et al., 2022)

Anti-angiogenic medication, as an innovative treatment, combats cancer by normalizing tumor vasculature, mitigating microenvironmental hypoxia, enhancing tissue drug concentration, and restricting distant invasion and metastasis of malignancies (Choi et al., 2022; Majidpoor & Mortezaee, 2021). In spite of the expanding array of FDA-sanctioned pharmaceuticals, the clinical advantages of anti-angiogenic monotherapies are ephemeral. Similar limitations in chemotherapy, such as developed drug resistance and tumor recurrence, have also been observed in anti-angiogenic therapy (Huinen et al., 2021; Ibraheem et al., 2024; Zhong et al., 2021). To date, despite extensive research dedicated to anti-cancer therapies aimed at combating this incurable and fatal disease, none have demonstrated sustained clinical success (Bellou et al., 2013; Ebos & Kerbel, 2011).

The current research sought to analyse the antiangiogenic properties of a carbothioamide indole derivative (2-NHC) and estimate the *VEGF* gene expression in cancer cell line.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were of pharmaceutical grade (supplementary Table S1).

Chemical synthesis of 2-NHC

All agents utilized were of pharmaceutical-grade purity ($\geq 98\%$). This study employed a test compound known as 2(5-bromo-1H-indole-2-carbonyl)-N-(4-chlorophenyl) hydrazine-1-carbothioamide [2-NHC]. The compound produced and thoroughly characterized by Hassan and

colleagues (O. M. Hassan et al., 2023); which is an indole derivative regarded as a new substance with potential antiproliferative properties (Al-Rubaye et al., 2024; Allawi et al., 2024; Heriz et al., 2024).

The chemical under investigation was utilized in powdered form, and a 1% concentrated solution was generated by dissolving it in DMSO at a final concentration of 10 mg/ml. Subsequently, this solution was employed to generate a series of different concentrations for the experiment. The initial chemical ingredient used for synthesizing novel indole derivatives was an indole derivative known as 5-bromoindole-2-carboxylic acid. Carbothioamides were chosen for the investigation among the synthesized compounds. The synthesis of carbothioamides derivatives, as described by Hassan et al., (2023) was carried out as previously reported (Figure 1).

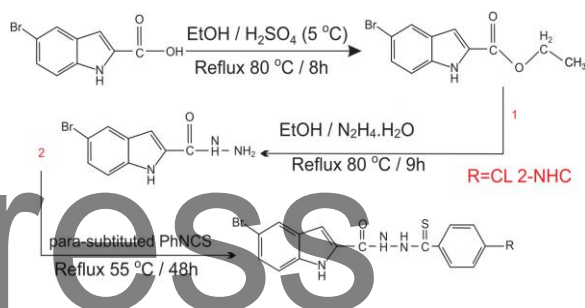


Figure 1: Chemical synthesis of 2-NHC (Hassan et al., 2023)

The derivatives were assessed for their chemical structure and the location of functional groups using ^1H NMR, ^{13}C NMR, and mass spectroscopy analysis (Al-Rubaye et al., 2024; Allawi et al., 2024; Hassan et al., 2023; Heriz et al., 2024; Yaseen et al., 2022).

Study design and settings

The antiangiogenic properties of 2-NHC were assessed through the ex-vivo rat aorta ring (RAR) assay to determine its likely mechanism of action; additionally, the DPPH assay was conducted to investigate the compound's capacity for free radical scavenging and its effect on the *VEGF* gene expression in HCT116.

The study was carried out in the animal house of Al-Nahrain University, College of Medicine, in the period between September 2023 and May 2024; the same institute approved the study (approval#: UNCOMIRB202405015, dated August 2023); all treatments involving animals, as well as tissue and cell culture, were executed in

accordance with the National Institutes of Health standards and the AVMA 2020 criteria (Underwood & Anthony, 2020).

Animal care

The animals were maintained in a sanitary environment at $23 \pm 2^\circ\text{C}$ with 40 – 50% humidity. They were subjected to a normal light and darkness cycle and given seven days to acclimatize; they were allowed ad libitum access to food and water.

Anti-angiogenic ex vivo activity

Sprague Dawley rats (n=8), 14 – 16 weeks, were euthanized using ketamine-xylazine (80 and 10 mg/kg) intraperitoneally (Obaid & Fawzi, 2024; Underwood & Anthony, 2020); postpartum, the aorta was dissected, washed with serum-free medium (M199), and sliced into fine rings of 1 mm thickness.

The procedure employed a 48-well tissue culture plate, each receiving 300 μl of fibrinogen-aptinin (3 and 5 mg/ml, respectively) in M199 media; the ring tissues were placed in the wells, and 10 μl of thrombin (50 U/ml) was placed in the wells. The tissues were maintained under a 5% CO₂ incubator for half an hour (37°C). The tested agent (2-NHC) was dispersed in 1.0% DMSO to provide a stock solution at a concentration of 0.1% (10 mg of the compound in 1 ml of DMSO). The stock solution was diluted in M199 medium to provide a range of concentrations (6.25, 12.5, 25, 50, and 100 $\mu\text{g}/\text{ml}$). Each concentration was performed in triplicate (Brown et al., 1996).

The cultured tissues were maintained for five days at 5% CO₂ (37°C), with the tissue median regularly replenished with media solution (for negative control, 1% of DMSO solution was given instead of the 2-NHC drug). On the 6th day, the number of blood vessels was calculated and divided by that of the negative control to obtain the percentage of angiogenesis (Nicosia, 2009). The measurement of blood vessels was undertaken using an inverted microscope at 40X power (Optika, Italy) and specialist software (SketchAndCalc™, Microsoft, USA).

The DPPH assay

100 μl of the examined material at serial concentrations of 2-NHC (15.625 – 500 μg), mixed with 200 μl DPPH (0.1 mM) and methanol (100 μl), after a half-hour incubation. The absorbance was assessed at 517nm by an ELISA reader (Human®, USA). The control comprised methanol and DPPH

(100 + 200 μl , respectively) (Pyrzynska & Pękal, 2013):

$$\text{DPPH scavenging activity (\%)} = \frac{Ab_{Cont} - Ab_{Samp}}{Ab_{Cont}} \times 100$$

Ab_{Cont} = absorbance of the control; Ab_{Samp} = absorbance of the sample

Cell lines and culture

The Human Umbilical Vein Endothelial Cells (HUVECs) and Colon Cancer Cells (HCT116) were procured from the American Type Culture Collection (ATCC, USA). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and a combination of antibiotics (penicillin, streptomycin, amphotericin B) under standard conditions (5% CO₂, 37°C, 95% humidity).

A penicillin-streptomycin-amphotericin B solution was purchased from ATCC®, Manassas, USA. The concentration of each antibiotic was 10,000 IU/ml, 10 mg/ml, and 25 $\mu\text{g}/\text{ml}$, respectively; 0.5 ml of the solution was added to 500 ml of the complete growth media to make the final concentration of 10 IU/ml, 10 $\mu\text{g}/\text{ml}$, and 0.025 mcg/ml of penicillin-streptomycin-amphotericin B, respectively.

The cells were allocated onto six-well plates at an approximate 6×10^4 cells/ml density. The cells were subsequently cultured for two to three days to promote proliferation and attain a confluency level of roughly 70% to 80%. (Unterleuthner et al., 2020).

Cell viability in vitro assay

The MTT test was utilized to assess the proliferative capacity. The cells were subjected to a serial concentration of 2-NHC (25, 50, 100, 200, and 400 $\mu\text{g}/\text{ml}$) for two days, then 20 μl of MTT solution (The MTT was made by adding a 5 mg/ml concentration in phosphate buffer saline) was introduced and allowed four hours of incubation; afterward, the supernatant was collected, and 200 μl of DMSO 1% was applied to the wells. The plates were vigorously agitated for one minute at ambient temperature to dissolve the blue crystals fully. An ELISA reader assessed the absorbance at 570 nm. The control comprised untreated cells, and cell viability was measured. Each concentration underwent four trials, and the experiment was replicated twice. The proportion of cell line inhibition was determined as the mean \pm standard deviation (Mosmann, 1983).

Thus, a graph was constructed to display the relationship between the percentage of cytotoxicity (y-axis) and the concentration (x-axis). Additionally, the IC₅₀ concentration was calculated for each test agent.

Gene expression analysis

Colon cancer cell line (HCT116) was exposed to 100, 200, and 400 µg/ml of the tested compound for 12 hours. Total RNA was extracted from 1 × 10⁴ treated cells using an extraction kit.

The technique principle involves utilizing RNA as a template to generate complementary DNA (cDNA) through Reverse Transcriptase. DNA polymerase will eventually amplify the resultant single-stranded cDNA to a double-stranded cDNA (Adams, 2020). Gene expression was assessed for the colon cancer cell line (HCT116). The targeted gene was (*VEGF*). The Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was the housekeeping gene (reference gene) to normalize the assessment results. Since the experiment involves measuring expressed genes, RNA samples are required. Cell samples were immediately kept in TRIzol® reagent to extract RNA templates.

Isolation of RNA templates from the compound was done according to the protocol of TRIzol® Reagent. For tissue samples, 0.5 ml of the reagent was added to about 30 – 50 mg of colon cancer tissue and shaken thoroughly using a vortex. In contrast, for cell culture, media was removed first, and 100 µl of the reagent was added directly to each well with pipetting up and down several times to ensure cell lysis. Incubate samples for 5 min to ensure complete dissociation of the nucleoprotein complex. 200 µl of chloroform for each 1 ml of TRIzol® reagent was added to each tube of the lysate and then shaken thoroughly. Incubate the samples for 2 – 3 min, then centrifuge at 12000 rpm for 10 – 15 min (Chomczynski & Sacchi, 1987).

The mixture divides into a lower red phenol/chloroform layer, an interphase, and a colorless top aqueous layer. The RNA-containing aqueous phase was moved to a fresh tube. 0.5 mL of isopropanol was incorporated to each 1 mL of TRIzol® reagent in the aqueous phase and incubated for 10 minutes at 4°C, followed by centrifugation for 10 minutes at 12,000 rpm. Total RNA was precipitated, resulting in the formation of a white gel-like pellet at the bottom of the tube, while the supernatant was discarded. For each tube, 0.5 mL of 70% ethanol was added, briefly

vortexed, and then centrifuged for 5 minutes at 10,000 rpm. The ethanol was subsequently aspirated, and the pellet was air-dried. The pellet was rehydrated in 50µl of Nuclease-Free Water and thereafter incubated in a water bath or heat block maintained at 55–60°C for 10 – 15 minutes (Chomczynski & Sacchi, 1987).

A Quantifluor RNA system was employed to ascertain the concentration of extracted RNA. For 1 µl of RNA, 200 µl of diluted Quantifluor Dye was combined. After a 5-minute incubation at room temperature in darkness, RNA concentration values were measured.

Reaction Step and Thermal Cycling (One-Step RT-PCR)

Primers were provided in a lyophilized state. Lyophilized primers were reconstituted in nuclease-free water to achieve a final 100 pmol/µl concentration as a stock solution. A functional solution of these primers was created by combining 10 µl of primer stock solution (stored at -20°C) with 90 µl of nuclease-free water, resulting in a working primer concentration of 10 pmol/µl (Table I).

Table I: Primers (forward and reverse) required for gene expression analysis by RT-qPCR.

Gene	Forward Primer	Reverse Primer
VEGF	GAGATGAGCTTCCTAC	TCACCGCCTCGGCTT
	AGCAC	GTCACAT
GAPDH	TGCCACCCAGAAGACT	TTCAGCTCAGGGAT
	GTGG	GACCTT

Table II: The master mix components and the general requirements for the one-step RT-qPCR technique

Component	Volume/sample	
qPCR master mix	5 µl	
RT mix	0.25 µl	
MgCl ₂	0.25 µl	
Forward primer	0.5 µl	
Reverse primer	0.5 µl	
Nuclease free water	2.5 µl	
RNA template	1 µl	
Step	Temp. & time	Cycle
RT enzyme activation	37°C for 15 min	1
Initial denaturation	95°C for 5 min	
Denaturation	95°C for 20 sec	40
Annealing	60°C for 20 sec	
Extension	72°C for 20 sec	

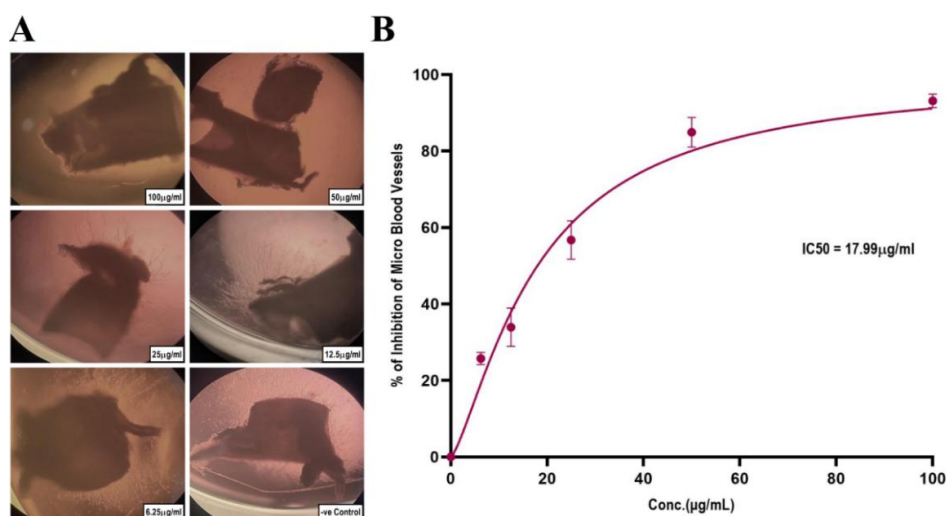


Figure 2: (A) Cultured ex-vivo RAR assay concentration-dependent inhibition of micro blood vessel growth for the test agent (2-NHC), (B) dose-response relationship of RAR assay for 2-NHC.

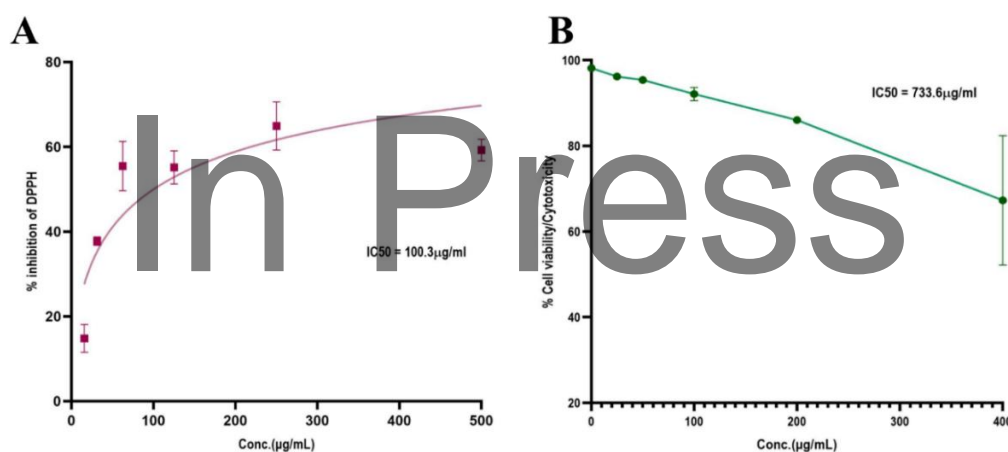


Figure 3: (A) concentration-dependent free radical scavenging activity by DPPH assay of 2-NHC, (B) MTT analysis of the cell viability of the HUVEC cell line after treatment with serial concentrations of 2-NHC.

Preparation of the master mix was the first and most important step in the analysis of gene expression, and the (one-step RT-PCR) technique with SYBR green was used. An appropriate volume of the reaction mixture was transferred to each well of a PCR tube/plate. The final volume of the sample to be analyzed was 10 µl. The reaction tube/plate was sealed and centrifuged briefly (Table II). The thermal cycler program was adjusted to the requirements of the qPCR protocol and then the device. The fluorescence was recorded, and gene expression was calculated relative to the reference

gene (GAPDH) using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Statistical Analysis

The normality test was undertaken using the Anderson Darling test; for a normally distributed variable, ordinary one-way ANOVA with post hoc Tukey was used, while for a non-normally distributed variable, the Kruskal Wallis test with post hoc Dunn test was used. The IC₅₀ was calculated using 4P log regression analysis. All analyses were performed using GraphPad Prism 10.2, with a significance level of ≤ 0.05 .

RESULTS AND DISCUSSIONS

Ex-Vivo RAR assay of 2-NHC

The assay results revealed that 2-NHC managed to inhibit the sprouting of micro-blood vessels in a concentration-dependent manner compared to the negative control that was treated with (DMSO 1%) only on day 6 ($IC_{50} = 17.99 \mu\text{g/ml}$) (Figure 2).

Free radical scavenging activity of 2-NHC using the DPPH assay

The 2-NHC effectively diminished the DPPH free radical in a concentration-dependent manner ($IC_{50} = 100.30 \mu\text{g/ml}$) (Figure 3).

Effect of 2-NHC on HUVEC viability

The findings demonstrated that 2-NHC exhibited minimal to negligible toxicity with an IC_{50} value of $733.60 \mu\text{g/ml}$ (Figure 3B).

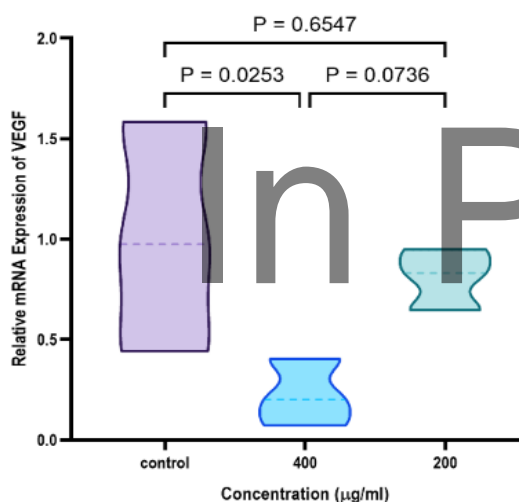


Figure 4: Violin plot of relative gene expression of *VEGF* in colon cancer cell line (HCT116) treated with 2-NHC

Effect of 2-NHC on the *VEGF* gene expression in HCT116 cell line

The 2-NHC at $400 \mu\text{g/ml}$ concentration showed significant downregulation of the *VEGF* gene expression (Figure 4).

The tasks of new blood vessel formation can be impaired in various diseases through mechanisms such as vasculogenesis, sprouting, and intussusceptive angiogenesis, which contribute to the creation of new vasculature in multiple pathological conditions, including cancer, atherosclerosis, arthritis, psoriasis, endometriosis, obesity, and SARS.SARS-CoV-2 (COVID-19) (Dudley

& Griffioen, 2023). Searching for and discovering novel agents that may show a promising effect as antiangiogenic activity is important. This study examined an emerging novel compound, 2-NHC, and evaluated its anti-angiogenesis leverage.

Nicosia's rat aortic ring model bridges the gap between *in vivo* and *in vitro* models since *in vivo* assays are time-consuming and expensive. The aortic ring assay is the most widely performed *ex vivo* model for studying angiogenesis to present. This study showed that heterocyclic indole derivatives significantly reduce micro-vessel sprouting in a dose-dependent manner compared to untreated rings. The study agent at a concentration of $100 \mu\text{g/ml}$ exhibited the most pronounced antiangiogenic action on rat aorta rings compared to other serial concentrations. Yao *et al.* examined the novel Indoles derivative compound in a wound healing assay (in vitro anti-angiogenesis assay) and confirmed that the compound could prevent HUVEC motility and migration (Yao *et al.*, 2022). A separate study investigated the migration of HUVECs induced by melatonin and other bioactive indolic compounds through migration wound-healing anti-angiogenesis assay. The findings reveal that indolic compounds originating from tryptophan metabolism, including melatonin, 3-indoleacetic acid, serotonin, and 5-hydroxytryptophan, inhibit VEGF-induced activation of VEGFR-2 and the ensuing angiogenesis, the authors propose a novel mechanism whereby Indolic compounds interact with the surface components of the endothelial cell membrane, thereby inhibiting VEGF from activating its receptor. This mechanism may serve as an additional explanation for the antiangiogenic effects of Indolic compounds (Cerezo *et al.*, 2017). These studies support the findings of this investigation, indicating that 2-NHC's inhibition of micro-vessel formation may be ascribed to its indole characteristics.

This study employed the DPPH radical to assess the radical scavenging activity of 2-NHC. The DPPH radical is a stable nitrogen-based organic free radical that can be reduced to a non-radical form (DPPH-H) by absorbing an electron or hydrogen from a hydrogen-donating antioxidant. Due to its rapid capacity to process numerous samples and its sensitivity in detecting active components at low concentrations, it has been widely employed to assess antiradical activity (Ai *et al.*, 2024). The free radical scavenging activity test for 2-NHC was crucial for elucidating the potential mechanism underlying their capacity to

inhibit angiogenesis; 2-NHC markedly decreased free radicals in a concentration-dependent manner. A study was conducted on various novel series of N-acyl substituted indoles to evaluate their antioxidant capacity against DPPH radicals; these compounds exhibited exceptional antioxidant activity (Jagadeesan & Karpagam, 2023). Another study supporting the current findings found that melatonin (an indole derivative) functions in a dual capacity to block angiogenesis, operating directly as a growth-associated inhibitor or indirectly as an antioxidant and free radical scavenger. Melatonin diminishes the survival and angiogenesis of hypoxia-damaged HUVECs by downregulating the hypoxia/HIF-1 α /ROS/VEGF pathway *in vitro* (Cheng et al., 2019). These studies may elucidate the anti-angiogenic action of 2-NHC and its antioxidant capabilities since potent antioxidants show recognizable anti-angiogenic effects (Iacopetta et al., 2020).

MTT assay is considered one of the important methods for determining cell viability; this assay measures mitochondrial function by measuring succinate dehydrogenase activity. The reduction of MTT by NADH results in the formation of purple formazan (Mngwengwe et al., 2024). In the present study, the *in vitro* screening revealed that 2-NHC possesses minimal to non-toxic effects on the HUVEC cell line with IC₅₀ equal to 733.60 μ g/ml. A closely similar study using novel Indole-thiazolidinone conjugates against human normal WI-38 cells by MTT assay was evaluated for their ability to induce cytotoxic effect against human normal lung fibroblast cell line (WI-38 cells) to investigate their safety. The results were expressed as IC₅₀ and showed non-significant cytotoxic action (Abo-Ashour et al., 2018). Another *in vitro* study showed the cytotoxic potential of Indoles hybrids towards human cancer cell lines in comparison to normal human lung epithelial cells (BEAS-2B); the safety profile of Indoles hybrids was disclosed by screening against normal human lung epithelial cell line by MTT assay (Tokala et al., 2020).

The VEGF family, comprising a group of related proteins, is considered a central mediator of angiogenesis (Das et al., 2023). Numerous studies support the role of VEGFs in increasing microvessel density and promoting metastasis; VEGFs are upregulated in multiple cancers, including breast, colorectal, and lung tumours (Saravanan et al., 2020). Results of HCT116 gene expression showed that the control cells expressed an elevated level of *VEGF*. In contrast, treatment with 400 μ g/ml of 2-NHC showed a significant reduction in the

expression of the *VEGF* gene compared to the control cells.

The gene expression results were consistent and supported the *ex vivo* (RAR) assay outcomes. The alteration in the gene expression of *VEGF* for 2-NHC could be attributed to its good antioxidant profile, as observed in the DPPH results, or by antiproliferative activity. These findings agree with a previous study in which the tested compounds inhibited the tyrosine kinase signalling of *EGFR* (Omeed M Hassan et al., 2023). Another 16 Indoles-based thalidomide analogues were designed and synthesized to obtain new effective antitumor immune modulatory agents. The synthesized compounds were evaluated for their cytotoxic activities against several cell lines, showing significant inhibition of VEGF (El-Zahabi et al., 2023). This study is consistent with a study of silico-designed cell-penetrating anticancer peptide that specifically inhibits VEGF-A to check the expression pattern of the molecules of VEGF-A signalling cascade expression with qPCR; outcomes of the two studies are promising, which are in agreement with the current research (Banerjee et al., 2024).

The study recommends further evaluation of the antiangiogenic potential using other *in vitro* assays, including endothelial cell migration, differentiation, and tube formation, followed by assessing important markers involved in each step. Since the agent is considered a novel indole derivative, an acute toxicity study is required to determine the LD₅₀. An animal tumour or angiogenesis induction model is recommended to understand the chemical's behaviour in a living *in vivo* setting.

CONCLUSION

The 2-NHC compound exhibited significant antioxidant and anti-angiogenesis effects with minimum toxicity against normal human cells. 2-NHC appears to downregulate the *VEGF* gene expression in colon cancer cell lines, indicating a potential molecular mechanism for its antiangiogenic and antiproliferative activity.

CONFLICT OF INTEREST

"The authors declare no conflict of interest".

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