ANTI-ANGIOGENIC EFFECTS OF ASTAXANTHIN IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS INDUCED BY VASCULAR ENDOTHELIAL GROWTH FACTOR

By

ANIS ZUHAIDA MAMNUN BARIZI

Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Chairman : Yong Yoke Keong, PhD
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Angiogenesis is the development of new blood vessels from preexisting ones that is essential for tumour growth and metastasis. Anti-VEGF therapy such as Bevacizumab; Avastin has been used as anti-angiogenic agent in cancer treatment yet the treatment is frequently associated with severe adverse effects. Alternatives are investigated for complementary natural products with comparable anti-angiogenic effectiveness and minimal adverse effects. Despite research demonstrating that astaxanthin (ATX) possesses anti-oxidant and anti-inflammatory properties, its effect on the angiogenesis process is yet unknown. Therefore, the purpose of this study is to ascertain ATX's anti-angiogenic properties. By using in-vitro (cytotoxicity, proliferation, migration, and tube-formation assays) and in-silico (network pharmacology and molecular docking) studies, the effects of ATX on VEGF-induced human umbilical vein endothelial cells (HUVEC) angiogenesis were assessed. Concentrations used for cell cytotoxicity assay were ranging from 50 – 3.125

μM while for cell proliferation, cell migration and tube formation assays used concentrations ranging from 25 – 6.25 μM. The angiogenesis-inhibiting compound Suramin served as the positive control. The results revealed that ATX exhibited cytotoxic effect (IC50 = 75.09 μM) by statistically significant reduced cell viability (%) of HUVECs in concentration-dependent manner ($P<0.05$) and at 50μM ATX killed 33% of the HUVEC when treated for 24 h. Furthermore, ATX demonstrated an anti-proliferative effect, leading to a statistically significant reduction in cell viability (%) of VEGF-induced HUVECs in a concentration-dependent manner ($P < 0.05$). This effect was observed after a 72-hour treatment, using a 4-hour baseline for comparison. Next, ATX exhibited anti-migratory effect by statistically inhibited migration in concentration-dependent manner ($P<0.05$) through scratch wound healing assay and the migration rate (%) at 25 μM is 20% for 8 h. Moreover, ATX inhibited tube formation by statistically significant decreased the average of tube length (μm) in concentration-dependent manner ($P<0.05$) through tube formation assay for 6 h. To further elucidate the potential molecular mechanisms underlying these effects, we transitioned to in-silico studies. Four common hub genes: RXR, CCND1, CDK1, and HDAC1 were discovered through network pharmacology which were deemed to be key target genes of ATX against angiogenesis. Furthermore, ATX may exert its effects through serine/threonine/tyrosine kinase activity, modulating inflammatory responses in the cytoplasm, and interacting with pathways involved in cancer. Besides, the interactions of proteins and ligands of four hub genes (RXRA, CCND1, CDK1, and HDAC1) and VEGFR2, FGFR1, and Tie-2 were determined through molecular docking. The results revealed that ATX exhibits strong

binding affinity to all seven proteins, with a significantly higher affinity observed for VEGFR2 compared to the others. In conclusion, ATX demonstrated anti-angiogenic effects on VEGF-induced HUVECs through cytotoxicity, proliferation, migration, and tube-formation assays, with potential molecular mechanisms predicted through in silico studies. This study provides useful basis for further investigations on ATX against angiogenesis related diseases.

Keywords: Angiogenesis, Astaxanthin, VEGF, HUVECs, Suramin

SDG: GOAL 3: Good Health and Well-being

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

SIFAT ANTI-ANGIOGENIK ASTAXANTHIN DALAM SEL ENDOTELIUM VENA UMBILIKUS MANUSIA TERARUH OLEH FAKTOR PERTUMBUHAN ENDOTELIUM VASKULAR

Oleh

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Angiogenesis ialah pembentukan salur darah yang baru dari yang sedia ada yang penting untuk pertumbuhan tumor dan metastasis. Terapi anti-VEGF seperti Bevacizumab; Avastin telah digunakan sebagai agen anti-angiogenik dalam rawatan kanser tetapi malangnya menyebabkan kesan sampingan yang teruk. Rawatan alternatif telah disiasat demi produk pelengkap semula jadi yang memberi perbandingan serupa dengan agen anti-angiogenik yang lebih efektif dan kurang kesan sampingan. Walaupun terdapat kajian yang membuktikan astaxanthin (ATX) mempunyai sifat anti-oksidan and anti-inflamasi, namun sifat anti-angiogenik masih belum diselidik. Oleh itu, objektif kajian ini adalah untuk mengkaji sifat anti-angiogenik ATX. Dengan menggunakan kaedah-kaedah in-vitro (asai kesitotoksikan, pemproliferatan, migrasi dan pembentukan tiub) dan in-siliko (rangkaian farmakologi dan pengedokan molekul), sifat anti-angiogenik ATX dalam HUVEC terangsang oleh VEGF telah dikaji. Kepekatan yang digunakan untuk asai kesitotoksikan

sel berjulat dari 50 hingga 3.125 μM manakala kepekatan yang digunakan untuk asai-asai pemproliferatan, migrasi dan pembentukan tiub digunakan kepekatan yang berjulat dari 25 hingga 6.25 μM. Suramin, sebatian yang menghalang angiogenesis, berperanan sebagai kawalan positif. Keputusan kajian menunjukkan bahawa, ATX menunjukkan kesan sitotoksik (IC50 = 75.09 μM) dengan pengurangan yang bererti secara statistik dalam kebolehhidupan sel (%) HUVEC secara bergantung kepada kepekatan ($P<0.05$), dan pada 50μM ATX membunuh 33% HUVEC apabila dirawat selama 24 jam. Di samping itu, ATX menunjukkan kesan anti- pemproliferatan yang menyebabkan penurunan yang bererti secara statistik dalam kebolehhidupan sel (%) HUVEC yang dirangsang oleh VEGF dalam cara yang bergantung kepada kepekatan ($P < 0.05$). Kesan ini diperhatikan selepas rawatan selama 72 jam, dengan menggunakan 4 jam sebagai garis dasar untuk perbandingan. Seterusnya, ATX menunjukkan kesan anti-migrasi dengan menghalang migrasi yang bererti secara statistik dalam cara yang bergantung kepada kepekatan ($P<0.05$) melalui asai penyembuhan luka goresan dan kadar migrasi (%) pada 25 μM adalah 20% untuk 8 jam. Selanjutnya, ATX menghalang pembentukan tiub dengan mengurangkan purata panjang tiub yang bererti secara statistik dan bergantung kepada kepekatan ($P<0.05$) melalui asai pembentukan tiub selama 6 jam. Untuk menjelaskan lebih lanjut mekanisme molekul yang berpotensi mendasari kesan-kesan ini, kami beralih kepada kajian in-siliko. Empat gen pusat yang serupa: RXR, CCND1, CDK1, dan HDAC1 ditemui melalui rangkaian farmakologi yang dianggap sebagai gen sasaran utama ATX terhadap angiogenesis. Selain itu, interaksi protein dan ligand empat gen pusat (RXRA,

CCND1, CDK1, dan HDAC1) serta VEGFR2, FGFR1, dan Tie-2 ditentukan melalui pengedokan molekul. Keputusan menunjukkan bahawa ATX mempunyai keafinan pengikatan yang kuat terhadap kesemua tujuh protein, dengan keafinan yang jauh lebih tinggi diperhatikan untuk VEGFR2 berbanding yang lain. Kesimpulannya, ATX menunjukkan sifat anti-angiogenik pada HUVEC yang terangsang oleh VEGF melalui asai kesitotoksikan, pemproliferatan, migrasi dan pembentukan tiub dengan potensi mekanisme molekul yang diramalkan melalui kajian in siliko. Kajian ini menyediakan asas yang berguna untuk penyelidikan lanjut terhadap ATX dalam penyakit berkaitan angiogenesis.

Kata Kunci: Angiogenesis, Astaxanthin, VEGF, HUVECs, Suramin

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

(IL)-12	Interleukin 12
2D	Two-Dimensional
3D	Three-Dimensional
aFGF	Acidic Fibroblast Growth Factor
Akt	Protein Kinase B
Ang-1	Angopoietin-1
Ang-2	Angopoietin-2
AO-EB	Acridine Orange-Ethidium Bromide
ATX	Astaxanthin
AXL	Tyrosine-Protein Kinase Receptor Ufo
BALB/c	A Mouse Strain Of Albion Mice
BBB	Blood Brain Barrier
bFGF	Basic Fibroblast Growth Factor
BSC	Biosafety Cabinet
C57BL/6	C57 Black 6 (B6) Inbred Strain Of Laboratory Mouse
C57BL/6J	C57 Black 6 (B6j) Inbred Strain Of Laboratory Mouse
CAM	Chorioallantoic Membrane
CCND1	Cyclin D1
CDK1	Cyclin-Dependent Kinase 1
cKIT	Tyrosine-Protein Kinase Kit
cMET	Mesenchymal Epithelial Transition Factor (Met)
CO_2	Carbon Dioxide
CRC	Colorectal Cancer

DAVID	Database For Annotation, Visualizations and Integrated Discovery
DMSO	Dimethyl Sulfoxide
DU145	Prostate Cancer Cell Line
EC	Endothelial Cells
ECM	Extracellular Matrix
ECV304	Endothelium Cell Of Vessel 304
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
eNOS	Endothelial Nitric Oxide Synthase
FDA	The Food and Drug Administration
FGF	Fibroblast Growth Factors
FGF-1	Fibroblast Growth Factors-1
FGF-2	Fibroblast Growth Factors-2
FGF-3	Fibroblast Growth Factors-3
FGF-4	Fibroblast Growth Factors-4
FGFR	Fibroblast Growth Factor Receptors
FGFR-1	Fibroblast Growth Factor Receptors-1
FGFR-2	Fibroblast Growth Factor Receptors-2
FKHR/FOX01	Forkhead Box Protein O1
FLT3	Fms-Like Tyrosine Kinase 3
G1	Gap 1 Phase
GEJ	Gastroesophageal Junction
GIST	Gastrointestinal Stromal Tumour
GO	Gene Ontology
GRAS	Generally Recognized As Safe

HCC	Hepatocellular Carcinoma
HDAC1	Histone Deacetylase 1
HIF-1α	Hypoxia Inducible Factor 1 Subunit Alpha
HUVEC	Human Umbilical Vein Endothelial Cells
IC_{50}	Half-Maximal Inhibitory Concentration
IP	Intraperitoneal Injection
IV	Intravenous Injection
KEGG	Kyoto Encyclopaedia Of Genes And Genomes
KIT	Receptor Tyrosine Kinase Type III
LPA	Lysophosphatidic Acid
MCC	Maximal Clique Centrality
MET	Hepatocyte Growth Factor Receptor (Hgfr)
MKI	Multikinase Inhibitors
MMP	Matrix Metalloproteases
MMP-2	Matrix Metalloproteinase-2
MMP-9	Matrix Metalloproteinase-9
MNC	Maximum Neighborhood Component
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl-2h-Tetrazolium Bromide
NCBI	National Center For Biotechnology Information
NP-1	Neuropilin-1
NP-2	Neuropilin-2
OD	Optical Density
–OH	Hydroxyl Group
p53	Tumour Protein P53
PBS	Phosphate Buffered Saline

PC-12	Rat Pheochromocytoma Cells
PDB	Protein Data Bank
PDGFB	Platelet Derived Growth Factor Subunit B
PDGFRα	Platelet-Derived Growth Factor Receptor Alpha
PlGF	Placental Growth Factor
PNET	Primitive Neuro-Ectodermal Tumours
PPI	Protein-Protein Interaction
qRT-PCR	Real-Time Quantitative Reverse Transcription Pc
RCC	Renal Cell Carcinoma
RCSB	Research Collaboratory For Structural Bioinformatics
Ret	Rearranged During Transfection
ROS1	Ros Proto-Oncogene
RXRA	Retinoid X Receptor Alpha
S	Synthesis Phase
SHR	Spontaneously Hypertensive Rat
STRING	Search Tool For The Retrieval Of Interacting Genes/Proteins
TGF	Transforming Growth Factor
TGF-β	Transforming Growth Factor Beta
Tie-2	Angiopoietin-1 Receptor
TIMPs	Tissue Inhibitors Of Metalloproteinases
TKI	Tyrosine Kinase Inhibitors
TRKB	Tropomyosin Receptor Kinase B
TSP-1	Thrombospondin-1
TYRO3	Tyrosine-Protein Kinase Receptor Tyro3

UniProt	The Universal Protein Resource
uPAR	Urokinase-Type Plasminogen Activator Receptor
VEGF	Vascular Endothelial Growth Factor
VEGF-A	Vascular Endothelial Growth Factor-A
VEGF-B	Vascular Endothelial Growth Factor-B
VEGF-C	Vascular Endothelial Growth Factor-C
VEGF-D	Vascular Endothelial Growth Factor-D
VEGF-E	Vascular Endothelial Growth Factor-E
VEGFR-1	Vascular Endothelial Growth Factor Receptor-1
VEGFR-2	Vascular Endothelial Growth Factor Receptor-2
VEGFR-3	Vascular Endothelial Growth Factor Receptor-3
WS1	Human Dermal Fibroblasts Cell Line
^{o}C	Degree celsius

CHAPTER 1

INTRODUCTION

1.1 Background

Angiogenesis is the process by which new blood capillaries develop from preexisting ones that involves a series of intricate sequential phases. Through this intricate process the basement membrane degrades by proteases, endothelial cells migrate/invade and proliferate, tubes are formed, new capillary branches emerge and the newly created blood vessels survive (Folkman J, 1984; Petrioli, R et al., 2022). Under regular conditions, angiogenesis occurs during the generation of blood vessels in embryos, the healing of wounds, and the regeneration of organs (Yi et al., 2014; Liu, Z. L et al., 2023). In adults, angiogenesis is limited to certain areas, particularly the endometrium and ovarian follicle cells (Jang et al., 2007; Liu, Z. L et al., 2023). Vascular quiescence is predominantly maintained in healthy tissues through the action of endogenous angiogenesis inhibitors, as opposed to angiogenic stimuli (Ribatti et al., 2006; Stephen, N. M et al., 2023). As a result of strict regulation by stimulatory and inhibitory factors in physiological conditions leads to the formation of organised blood vessels (Rajasekar, J et al., 2019). However, in specific pathological conditions such as the development of solid tumours, autoimmune diseases, rheumatoid arthritis, atherosclerosis, cerebral ischemia and diabetic retinopathy, angiogenesis takes place in an unregulated manner (O'Reilly et al., 1997; La Mendola, D et al., 2022).

The initial framework for the development of angiogenesis in tumours, as put forth by Judah Folkman (1971) has garnered significant interest from researchers. This is due to the fact that tumour growth relies on angiogenesis, with each increase in tumour size necessitating a corresponding increase in blood vessel formation (Wang S et al., 2004; Aslan, C et al., 2019). Angiogenesis facilitates the progression of tumours by providing a constant supply of nutrients and oxygen to the tumour while eliminating metabolic waste generated by tumour cells (Papetti & Herman, 2002; Eelen, G et al., 2020). Throughout the years, the primary emphasis in cancer treatment has been on eradicating neoplastic cells (Mabeta et al., 2022). On the other hand, at this time, angiogenesis inhibition represents a potentially fruitful strategy for the development of novel drugs that able to impede the advancement of cancer (Cai W. Bin et al., 2012; Dudley, A. C., & Griffioen, A. W. 2023).

The therapeutic approach employs FDA-approved drugs that selectively inhibit the angiogenic factor or the signal cascade associated with it (Carmeliet & Jain, 2011; Ansari, M. J et al., 2022). VEGF, vascular endothelial growth factor is crucial in controlling tumour-related angiogenesis and is primarily found in adult tissues, making it an ideal focus for anti-tumour treatment. It is notable that a higher expression of VEGF is linked to increased tumour invasiveness and metastatic potential (Elice & Rodeghiero, 2012). Nevertheless, anti-VEGF drugs such as bevacizumab, sunitinib, sorafenib and pazopanib are associated with a range of adverse effects such as high blood pressure, high protein levels in urine, hemorrhage, gastrointestinal perforation, decreased wound recovery, and arterial and venous thromboembolism (Keefe et al., 2011). Thus, there is a pressing need for the discovery of a novel anti-

angiogenic therapy that demonstrates high efficacy and minimal adverse effects.

Complementary and alternative medicine, encompassing a wide range of treatments for cancer prevention and treatment, are gaining interest in oncology management (Khalid et al., 2016). Natural products have consistently been a valuable source of drug discovery due to their possibly low toxicological profile (Chen K et al., 2018). Naturally existing compounds known as carotenoids are present in bacteria, plants, fungi, and algae (Milani et al., 2017). These micro-components are found in fruits and vegetables and are accountable for the manifestation of their characteristic yellow, orange, and red hues (Gerster, 1997; Cassani, L et al., 2022). Over 600 carotenoids have been identified in nature thus far (Gerster, 1997; Britton, 2022). Carotenoids can be divided into two groups: oxygen-containing xanthophylls (like zeaxanthin, neoxanthin, and fucoxanthin) and oxygen-free carotenes (such as α-carotene, β-carotene, and lycopene) (Niranjana et al., 2015).

Recent years have witnessed a surge in interest surrounding the carotenoid astaxanthin (3,3′-dihydroxy-,′-carotene-4,4′-dione; $C_{40}H_{52}O_4$), an orange-red, lipophilic keto-carotenoid pigment (Patil et al., 2022) due to its effects of nutrition on human health, which numerous researchers have documented (Kidd, 2011; Guerin et al., 2003; Yang Y et al., 2014; Yuan J. P et al., 2010; Yamashita, 2013). Therefore, ATX may serve as a potential novel anti-angiogenic agent worthy of further investigation. Astaxanthin (ATX), a xanthophyll carotenoid derived primarily from marine sources (higher plants and microscopic organisms such as phytoplankton algae, yeasts and bacteria)

(Fassett & Coombes, 2011). Moreover, the Food and Drug Administration (FDA) has declared natural ATX to be generally recognised as safe (GRAS) (Capelli et al., 2014) which has boosted its consumption as a nutraceutical. In addition, ATX reportedly contains a number of attributes such as anti-oxidant (Liu et al., 2020), anti-cancer (Sowmya et al., 2017), anti-inflammation (Yaghooti et al., 2019) (Yaghooti et al., 2019), anti-diabetic (Penislusshiyan et al., 2020) (Penislusshiyan et al., 2020), osteoprotective (El-Baz et al., 2019), neuroprotection (Krestinina et al., 2020), hepatoprotection (Yang et al., 2015) and numerous additional properties.

Despite these revelations, the anti-angiogenic effects of ATX have not been systematically investigated. Therefore, in the present study, we demonstrated the anti-angiogenic properties of ATX on Human Umbilical Vein Endothelial Cells (HUVEC) induced by VEGF through a series of investigations, including in vitro studies (cytotoxic, proliferation, cell migration, and tube formation assays). In order to investigate the intricate interactions between components, diseases, and targets, network pharmacology combines systems biology, pharmacology, and computer analytic technologies (Yuan, C et al., 2021). Moreover, molecular docking is an in silico approach intended to predict the molecular interactions and binding patterns of small or macromolecules when they interact with a receptor (Santos, L. H. et al., 2019). Therefore, in silico studies (network pharmacology and molecular docking) were investigated to predict the underlying mechanisms of the anti-angiogenic effect of ATX, which may prove valuable for future research.

I.2 Problem Statement

A multitude of ailments, with cancer being a prime example, arise due to uncontrolled angiogenesis. Angiogenesis, the development of new blood vessels from existing ones, is critical for providing oxygen and nutrients while also facilitating the elimination of metabolic waste to promote the growth of solid tumors (Folkman, 1971). Inhibiting angiogenesis is regarded as a promising strategy for impeding the development of solid tumors since it impairs the essential supply, thereby incapacitating tumor growth. However, the existing anti-angiogenic agents are accompanied by side effects (Elice & Rodeghiero, 2012). Therefore, there is a pressing need of discovering a novel anti-angiogenic agent that possesses the potential for high efficacy and less side effects. Furthermore, the restricted range of available anti-angiogenic medicines in the pharmaceutical sector presents a limitation when it pertains to selecting appropriate alternative agents. Therefore, the discovery of new anti-angiogenic agents possesses the capacity to expand the range of available agents targeted against angiogenesis. ATX has been shown to provide numerous health benefits to humans, with well-documented major pharmacological actions including antioxidant (Chintong et al., 2019) and anti-inflammatory (Masoudi et al., 2021) properties. Regrettably, there is presently a lack of documentation about the anti-angiogenic properties of ATX. Therefore, it is imperative to do a study on the anti-angiogenic effects of ATX.

I.3 Objectives

I.3.1 General objectives

To investigate anti-angiogenic effect of astaxanthin (ATX) on vascular endothelial growth factor (VEGF)-stimulated human umbilical vein endothelial cells (HUVECs).

I.3.2 Specific objectives

I. To evaluate cell viability through cell cytotoxicity assay and cell proliferation assay utilizing MTT.
II. To examine anti-migratory effect of ATX on HUVECs utilising scratch wound healing assay.
III. To determine the inhibition effect of ATX on tube formation of HUVECs utilising tube formation assay.
IV. To investigate in silico study through network pharmacology and molecular docking approaches.

1.4 Hypothesis

It is hypothesised that ATX will demonstrate anti-angiogenic effect on VEGF-induced HUVECs through in vitro, characterised by exhibiting effects of cytotoxicity, anti-proliferative, anti-migratory and inhibition of tube formation. ATX also targets key angiogenic hub genes, specifically RXR, CCND1, CDK1, and HDAC1, and exhibits a strong binding affinity to VEGFR2, potentially inhibiting angiogenesis more effectively than other receptors

CHAPTER 2

LITERATURE REVIEW

2.1 Angiogenesis

The first organ system to develop into function in the mammalian embryo is the cardiovascular system (Dudley, A. C., & Griffioen, A. W., 2023). The cardiovascular system is made up of the heart, arteries, capillaries, and veins (Chaudhry, R et al., 2022). Beginning in the early stages of development, the circulatory system must enlarge to satisfy the demands of the developing tissues and organs as the embryo grows larger than the point at which vital substances can be delivered by simple diffusion.

Two fundamental mechanisms, vasculogenesis and angiogenesis, are responsible for the formation of new blood vessels. Vasculogenesis is characterised as the de novo development of early vascular networks by endothelial precursor differentiation, expansion, and merging. Once the vascular networks have established, new blood vessels are created by sprouting or splitting from pre-existing blood vessels in a process referred to as angiogenesis (Naito, H et al., 2020). A vast network of arteries, arterioles, veins, venules, and capillaries forms in all tissues and organs to transport oxygen and nutrients and eliminate waste products from metabolism during later stages of vascular development, which is caused by angiogenesis (Dudley, A. C., & Griffioen, A. W., 2023).

John Hunter, a British surgeon, coined the term “angiogenesis” for the first time in 1787 in his book “Treatise on the Blood, Inflammation and Growth Wounds” to describe blood vessel growth in reindeer antlers brought on by prolonged exposure to cold (Ribatti, 2014). In addition, according to Risau, (1997), angiogenesis is defined as the generation of primary vascular plexus which is a new differentiation of endothelial cells from unaltered precursor cells acquired from mesoderm through sprouting or non-sprouting mechanisms (Flournoy, J et al., 2022). Angiogenesis also comprises renewal, regeneration, and an elevation in the branching of blood vessels in addition to growth and development (Papetti & Herman, 2002; Ibragimov, A et al., 2023).

2.1.1 The stages of angiogenesis

As previously outlined, angiogenesis is a complex multi-process involving various activities of endothelial cells and can be divided into several stages; (1) Initiation (2) Endothelial cell activation, (3) Extracellular matrix degradation (4) Endothelial cell proliferation and migration, (5) Tube formation and (6) Maturation and stabilization. Each of these stages will be discussed at a greater length below.

In initiation stage, in order for the process of angiogenesis to start, the endothelial cells lining blood arteries must receive the proper signals. This may involve a variety of signalling molecules, hypoxia, and growth factors like VEGF (Carmeliet & Jain, 2000; Zimta, A. A et al., 2019). Relay proteins are activated when released signals attach to their receptor on the outermost layer of endothelial cells, sending signals into the cell nucleus (N. Nishida et al., 2006; Gluszko, A et al., 2019). Following the initiation, the second stage which

is the activation of endothelial cells will follow. The production of VEGF and other pro-angiogenic molecules are stimulated. It also triggers an inflammatory response, which draws in fibroblasts and macrophages, among other pro-angiogenic cells (Ferrara, 2002; Hsu, T et al., 2019).

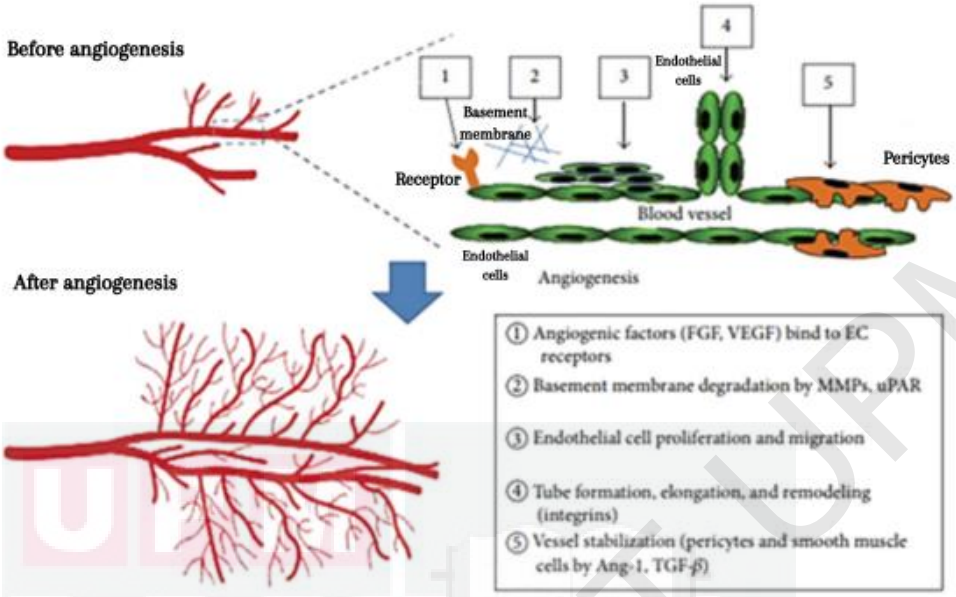
In the third stage, endothelial cells will then degrade Extracellular matrix (ECM) by the secretion of matrix metalloproteinases (MMPs) and cells such as macrophages and fibroblasts will be recruited in this extracellular matrix degradation stage (Risau, 1997; Bajbouj, K et al., 2021). The proteolytic activity of MMPs, such as MMP-2 and MMP-9 which is well acknowledged to be essential for angiogenesis, drives the degradation of basement membrane (Egeblad & Werb, 2002; Van Hinsbergh et al., 2006; Waszczykowska, A et al., 2020). When endothelial cells are in their quiescent state, MMPs will not be expressed. In contrast, under specific circumstances such as inflammation it will be expressed in high level (Hanemaaijer et al., 1993; Moracho, N et al., 2022). Tissue inhibitors of metalloproteinases control the endogenous activity of MMPs (Handsley & Edwards, 2005; Cerofolini, L et al., 2019).

Endothelial cells start to divide and move in the direction of the site of the angiogenesis. Additionally, they start to develop sprouts, a network of small blood arteries (Risau, 1997; van Hinsbergh, V. W. 2021). This stage is known as endothelial cell proliferation and migration which is the fourth stage. After proliferating and migrating, endothelial cells should be able to return to quiescence, create a new basal lamina, draw in perivascular cells, and create lumenised tubules (Muñoz-Chápuli et al., 2004; Ricard, N et al., 2021). In the fifth stage, through self-organisation into a tubular form, development of a

basement membrane, a functional lumen, and pericyte covering, the sprouts mature into functioning blood vessels. In order to create a functional network, the new vessels connect together with the existing ones (Risau, 1997; Al-Ostoot, F. H et al., 2021).

A vessel must mature and become stable in order to function. The recruitment of mural cells such as pericytes or smooth muscle cells and the formation of a basal lamina are necessary for the completion of new blood vessels (Carmeliet & Jain, 2011). The maturation stage is essential to guarantee the new vascular structures' persistence and functionality as well as the activated endothelium's return to quiescence and if vessels are not perfused, they will regress (Reginato et al., 2011). The detailed stages of angiogenesis are depicted in **figure 2.1.**

A.



B.

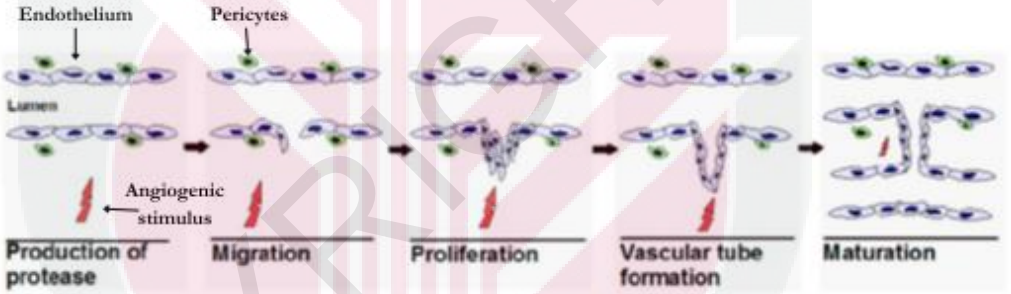


Figure 2.1: The stages of angiogenesis (Source: Ulus G, 2021) with some modifications — **A.** Starting at the top left with a blood vessel, proceeding to the subsequent stages and finally a new vascular network is formed at the bottom left. A small section of a blood vessels that is enlarged show the molecular mechanisms of angiogenesis (top right) which includes (1) Angiogenic factors (FGF, VEGF) triggered angiogenesis by binding to EC receptors; (2) The degradation of basement membrane by MMPs, uPAR; (3) Proliferation and migration of ECs; (4) Tube formations, elongation, and remodelling (integrins); (5) Vessel stabilization of pericytes and smooth muscle cells by Ang-1, TGF-β. **B.** This section depicts the cellular processes underlying angiogenesis which involve the production of proteases that degrade the basement membrane, endothelial cell migration and proliferation, the formation of vascular tubes, and the maturation of these new blood vessels, supported by pericytes and the diminishing angiogenic stimulus.

2.1.2 Physiological and pathophysiological conditions

Angiogenesis, the development of new blood vessels from pre-existing ones, can occur under both physiological and pathological circumstances (Folkman, 1971; Saman, H et al., 2020). During embryonic development and throughout life, angiogenesis is an essential process for organ creation. Numerous physiological processes, including pregnancy, placentation, and wound recovery, depend heavily on properly controlled angiogenesis (Alvarez-García et al., 2013). A multicellular organism's cells activate angiogenesis and vasculogenesis during growth in order to recruit in new blood supply. Therefore, cells must be situated between 100 and 200 μm from blood vessels, which serve as their supply of oxygen, in physiologic settings (Tonini et al., 2003; Aspriţoiu, V. M et al., 2021). Angiogenesis in normal adult tissues is a highly rare occurrence since physiological angiogenesis is mainly associated with tissue growth, tissue repair, and the reproductive cycle. As a result, the endothelium of most tissues is an incredibly stable population of cells with very little mitotic activity (Hall, 2005; Liu, Z. L et al., 2023). In physiological condition, once angiogenesis is intermittently switched on and it will then completely be inhibited (Folkman, 1984; van Hinsbergh, V. W., 2021).

Angiogenesis in physiological conditions believed to be tightly controlled and self-regulatory. Angiogenesis, in contrast, appears to be dysregulated in pathological conditions and is notably accelerated in many disorders (Ma. Q et al., 2019). Angiogenesis can also manifest pathologically in a number of illnesses, including rheumatoid arthritis, obesity, cancer (Risau, 1997; Shin, S. S., & Yoon, M. 2020). Cardiovascular diseases, diabetic retinopathy (Kuwano

et al., 2001; La Mendola, D et al., 2022). During pathological process, diseases that caused by persistent abnormal angiogenesis will progress even worse. A good illustration of this is the development of new capillaries in tumour tissue promotes the tumour's growth and metastasis. Another example is blindness is brought on by ocular neovascularization, a characteristic of many eye conditions. Therefore, in pathological settings, the dysregulation of angiogenesis is resulted from an imbalance between angiogenetic factors and inhibitory factors, contributing to significant diseases (Ma. Q et al., 2019). **Table 2.1** summarises the physiological and pathological conditions of angiogenesis.

Table 2.1: Physiological and pathological conditions of angiogenesis

Physiological	**Pathological**
Embryogenesis	Various malignant tumor types
Bone growth	Rheumatoid arthritis
Wound healing	Psoriasis
Corpus luteum	Atherosclerosis
Menstruation	Diabetic retinopathy
Uterus endothelium	Age-related macular degeneration
	Wound healing

[Source: Kuwano et al., (2001)]

2.1.2.1 Solid Tumor

The equilibrium between factors that promote angiogenesis and those that inhibit it is strictly regulated during the controlled process of angiogenesis. When this balance is disrupted, it can result in the emergence of unnatural blood vessels and the occurrence of disease such as cancer (Tonini et al., 2003; Ansari, M. J et al., 2022). When tumor cells take up an angiogenic phenotype, the proangiogenic mechanisms overpower the downregulating processes. As a result, endothelial cells go through a rapid growth phase that ultimately results in the formation of an oxygen and nutrient-rich microenvironment within the tumor that promotes the growth of the tumor and its dissemination to distant areas (Dalal et al., 2018).

A revolutionary notion put forth in 1971 by Drs. Folkman and Klagsbrun claimed that tumor angiogenesis is crucial for all phases of rapid tumour growth (Folkman & Klagsbrun, 1987; Brossa, A et al., 2019). Due to their requirement for oxygen and nutrients for survival and growth, malignant cells must be in close contact to blood vessels for it to enter the blood circulation system. This can be seen in the early observation by Judah Folkman that only rapidly growing tumours were heavily vascularized whereas dormant ones were not. This is when the theory the onset of tumour angiogenesis was necessary for tumour advancement came to light (Sherwood et al., 1971; Lugano, R et al., 2020). The requirement of a new vascular supply is one of the major hallmarks of cancer. Angiogenesis does not self-regulate in cancer. Once activated, tumor-induced angiogenesis lasts eternally until the host reaches death or the tumour is completely eliminated (Folkman, 1984).

The fact that cancer blood vessels never go dormant allows for the constant creation of new cancer blood vessels, which is one distinguishing trait that sets them apart. As a result, the cancer vasculature generates distinctive qualities and separates itself significantly from the regular blood circulatory system. The architecture of cancer blood vessels differs from that of regular blood vessels; their shapes are asymmetrical., convoluted, dilated, and can have dead ends. Rather than being organised into discrete arterioles, capillaries, and venules as what they usually are, they share the disordered features in every one of them. Overproduction of VEGF causes the vascular structure that forms in tumours to often be permeable and bleeding-related. (Bergers & Benjamin, 2003; Larionova, I et al., 2021).

Additionally, it has been observed that cancer cells have been incorporated into the vessel wall of tumour vasculature (Folberg et al., 2000; Silvestri, V. L et al., 2020). In tumour vessels, the flow of blood moves less steadily and occasionally even oscillates, leading to the dysfunctional of capillaries. Tumours can have a wide variety of vascular patterns and can over proliferate their capillary networks. Conversely, the metabolic requirements for nutrients and oxygen control the vessel density in healthy tissues is in a dynamic manner. Consequently, the pathogenic nature of their induction is evident in the anatomical and functional anomalies in the tumour vasculature (Bergers & Benjamin, 2003; Soliman, M. A et al., 2020).

2.1.3 Pro-angiogenic agents

Angiogenesis depends on the relatively dynamic homeostasis between pro-angiogenic and anti-angiogenic regulators secreted by the host and tumour cells. It includes several stages, such as the dissociation of endothelial cells from pericytes and the basement membrane, invasion and migration all over basement membranes, and finally expansion into the tumour body (Carmeliet, 2000; Larionova, I et al., 2021). The persistent secretion of pro-angiogenic substances by cancer cells and cancer-associated stromal cells such as macrophages, fibroblasts, neutrophils, and adipocytes during tumor growth triggers the angiogenic switch that causes endothelial cells (ECs) to proliferate and migrate to build new blood vessels (Chang et al., 2000; Lopes-Coelho, F et al., 2021).

An example of a well-performing classical model of the management of tumour angiogenesis is provided by a scale that has pro-angiogenic chemicals on one side and anti-angiogenic chemicals on the other. (**Figure 2.2**). The angiogenic switch is triggered by the extent to which that balance tips in the direction of pro-angiogenesis. (Bergers & Benjamin, 2003). Alternatively put, this process can be started by specific angiogenic molecules and stopped by specific inhibitory molecules.

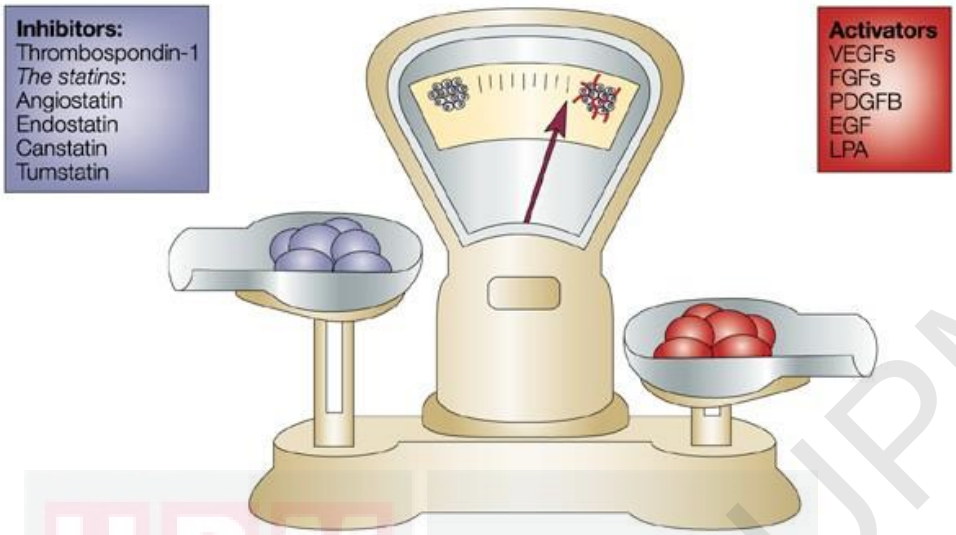


Figure 2.2: The angiogenic balance (Bergers & Benjamin, 2003). Activator and inhibitor levels determine whether an endothelial cell is in a quiescent or angiogenic state, and vice versa. The angiogenic switch is thought to be mediated by changes in the angiogenic balance. As indicated in the figure, when the angiogenic balance leans to the activators, angiogenesis occurs.

Pro-angiogenic gene expression is elevated by physiological stimuli, such as hypoxia, which occurs from increasing tissue mass (Dor et al., 2001; Singh, P. G et al., 2022), as well as by oncogene activation or tumour-suppressor mutation (Kerbel & Folkman, 2002; Deepa, K. 2021). Different places along the tumor-progression route may experience the angiogenic switch, in accordance with the type of tumour and the surrounding environment. (Bergers & Benjamin, 2003; Deepa, K. 2021). Typically, sprouting capillaries that has been induced will move toward the signal of pro-angiogenic stimulus (DiPietro, 2016).

Initial studies to test the theory that tumours create angiogenic factors was conducted by Greenblatt and Philippe (1968) in which melanoma or choriocarcinoma cell transplantation was discovered to increase blood vessel growth even in the presence of a filter, providing proof that the tumour's

angiogenesis was regulated by a diffusible factor (or factors) made by the tumour cells. Numerous factors have been discovered to stimulate angiogenesis, such as the members of the vascular endothelial growth factor (VEGF) family, angiopoietins, platelet-derived growth factor, transforming growth factors (TGF), tumour necrosis factor-α, interleukins and the members of the fibroblast growth factor (FGF) family (Ucuzian et al., 2010). Furthermore, a variety of variables regulate and affect angiogenesis such as soluble growth factors, membrane-bound proteins, cell–matrix and cell–cell interactions, and several interrelated systems (Papetti & Herman, 2002). Three pro-angiogenic agents namely vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and angiopoietins will be covered in more detail below.

2.3.1.1 Vascular Endothelial Growth Factor (VEGF)

The VEGF family has several members, but vascular endothelial growth factor-A (VEGF-A; commonly known as VEGF) is the best known and most extensively researched. It is a cytokine released by tumours that is crucial for both regular and tumour-associated angiogenesis (Rini & Small, 2005; Badodekar, N et al., 2021). VEGF-A interacts with cell surface receptors to produce its biological impact. These receptors are transmembrane tyrosine kinase receptors and they include the VEGF receptor-1 (VEGFR-1) and VEGFR-2, which are expressed specifically on vascular ECs, as well as the neuropilin receptors (NP-1 and NP-2), which are found on neurons and vascular endothelium (Dvorak, 2002; Yang, J., & Liu, Z. 2022).

Following the binding of VEGF-A to the extracellular domain of the receptor, the dimerization and autophosphorylation of the intracellular receptor tyrosine kinases will occur resulting the stimulation of a series of downstream proteins. The primary receptor that appears to be mediating VEGF-A-pro-angiogenic's actions is VEGFR-2. VEGF-A is known as the most potent pro-angiogenic agent which stimulates proliferation, sprouting and tube formation of endothelial cells (ECs) (Ferrara et al., 2003; Corrias, G et al., 2024). A significant range of angiogenic processes, such as wound healing, ovulation, blood pressure regulation, menstruation, and pregnancy are impacted by VEGF-A (Brown. L. F et al., 1992; Barbe, A et al., 2019). Practically every solid tumor that has been investigated in humans, as well as several haematological malignancies, express VEGF-A (Ferrara et al., 2003; Mancini, S. J et al., 2021). In contrast to VEGF-A which binds to VEGFR-2, VEGF-B binds specifically to

VEGFR-1 (Nash et al., 2006; Nascimento, C et al., 2021). Silvestre and colleagues were the first discovered that VEGF-B activated angiogenesis in in conjunction with the activation of Akt and eNOS-related pathways (Silvestre et al., 2003). Other VEGF-related molecules include VEGF-C and VEGF-D, which are structurally related to VEGF-A but differ in their degree of homology (Robinson & Stringer, 2001; Cho, W et al., 2022). Both VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3 (Achen et al., 1998; Mota, F et al., 2024). Finally, another member of the VEGF family is VEGF-E. Only 20 to 25% of the amino acids in the VEGF-E members are similar to those in VEGF-A (Lyttle et al., 1994; Bokhari, S. M. Z., & Hamar, P. 2023). VEGF-E is known as a potent angiogenic agent since the activation of VEGFR-2 by it alone can effectively increase angiogenesis (Meyer et al., 1999; Narayana, S et al., 2021).

2.3.1.2 Fibroblast Growth Factor (FGF)

Another growth factor that plays an important role in the regulation of angiogenesis is fibroblast growth factor (FGF). FGF-2 or basic FGF (bFGF) and FGF-1 or acidic FGF (aFGF) are heparin-binding protein mitogens (Thomas, 1987; Chen, M et al., 2020) and the most often researched forms. Both aFGF and bFGF stimulate numerous EC activities that are crucial to angiogenesis in vitro as well as promote EC migration (Gospodarowicz et al., 1989; Chen, M et al., 2020) and proliferation (Terranova et al., 1985; Chen, M et al., 2020). In the chick chorioallantoic membrane and cornea, FGFs cause blood vessels to sprout in vivo, demonstrating their role in angiogenesis (Folkman et al., 1983; Guo, Z et al., 2024).

FGFs bind to high affinity tyrosine kinase FGF receptors (FGFRs) on the surface of target cells to carry out their biological functions (Cross & Claesson-Welsh, 2001; Szybowska, P et al., 2021). Both forms frequently bind to the receptor tyrosine kinases FGFR-1 or FGFR-2 (Gerwins et al., 2000). As a result of receptor autophosphorylation, FGFR interaction entails the activation of numerous parallel signalling pathways. The stimulation of FGFR-1 or FGFR-2 and angiogenic FGFs (such as FGF-1, FGF-2, and FGF-4) results in the proliferation of EC (Cross & Claesson-Welsh, 2001; Farooq, M et al., 2021). The fact that VEGF is essential for the increases endogenous VEGF production and FGF-2-stimulated secretion of placental growth factor suggests that there is cross-talk and synergism between the FGF and other growth factor pathways (Landgren et al., 1996; Hosaka, K et al., 2020).

2.3.1.3 Angiopoietins

Next, another pro-angiogenic agent that plays a critical role in angiogenesis is Angiopoietin family, which consists extracellular ligands that mainly bind to Tie receptors located on ECs. Both Angiopoietins-1 (ANG-1) and Angiopoeietins-2 (ANG-2) bind to Tie-2 receptor which responsible in the maintenance of vessel, growth and stabilisation (Carmeliet, 2003; Akwii, R. G et al., 2019). Despite binding to the same receptor, ANG-1 and ANG-2 have entirely different actions. Similar to other growth factor receptors, tie receptors are a class of receptor tyrosine kinases that are primarily involved in angiogenesis and vascular remodelling (Maisonpierre et al., 1997; Akwii, R. G et al., 2019). ANG-1 is known as agonist where it accelerates the transphosphorylation of receptor which in turn activates the downstream protein kinase-B/Akt/FKHR

(FOX01) pathway, promoting EC survival and suppressing ANG-2 production (Daly et al., 2004; Akwii, R. G et al., 2019).

The inhibition of ANG-2 by ANG-1 is crucial as ANG-2 responsible for the activation of EC and initiation of angiogenesis. The substantial increase of ANG-2 in ECs following exposure to angiogenic stimuli including hypoxia and VEGF provides evidence for the early role of ANG-2 in angiogenesis (Yuan H. T et al., 2000; Akwii, R. G et al., 2019). ANG-2 phosphorylates the Tie-2 receptor by acting as an opposing antagonist of ANG-1 and signalling in the presence of continuous proangiogenic signals like VEGF since the release of ANG-2 triggers an autocrine response which involved in the disruption of the endothelial monolayer, and encourages EC survival, vessel sprouting, and angiogenesis (Maisonpierre et al., 1997; Akwii, R. G et al., 2019).

2.1.4 Current anti-angiogenic treatment

Folkman postulated in 1971 that anti-angiogenic therapy might be useful for the treatment of cancer because it could obstruct already-existing blood vessels and prevent the development of new ones, reducing the oxygen and nutrition supply to cancer cells and, as a result, slowing tumour growth (Folkman, 1989; Zetter, 2008). Antibodies like bevacizumab, the first VEGF-targeted drug approved by the Food and Drug Administration (FDA) for cancer treatment, became accessible for cancer therapy decades after Folkman made his statement (Vasudev & Reynolds, 2014). Following this, researchers have turned their attention to an anti-angiogenic method that could eventually lead to a unique therapeutic approach for the treatment of solid tumours. Angiostatin, endostatin, tumstatin, platelet factor-4, interleukin (IL)-12,

thrombospondin-1 (TSP-1), tissue inhibitors of metalloproteinases (TIMPs) and interferon-α, -β and -γ are specific, widely investigated anti-angiogenic regulators (Li. T et al., 2018).

There are four main approaches for creating anti-angiogenic drugs: blocking endogenous factors that encourage blood vessel growth, finding and using natural angiogenesis inhibitors, blocking molecules that encourage invasion of the surrounding tissue through tumour blood vessels, and impeding actively proliferating endothelial cells (Mousa & Davis, 2016). The most widely used anti-angiogenic medications at the moment are Bevacizumab, Ramucirumab, Aflibercept, Sunitinib, Sorafenib, Pazopanib, Axitinib, cabozantinib, and Lenvatinib for the treatment of different cancer types.

Although there is additional signalling pathways connected to angiogenesis, the interaction between VEGF and VEGFRs has been regarded as a crucial regulator and a prime candidate for the development of anti-angiogenic medicines (Yancopoulos et al., 2000; Ntellas, P et al., 2020). All bioactive forms of VEGF are recognised and neutralised by VEGF blockers, which stops VEGFR activation and, as a result, inhibits the growth of tumours (Kopetz et al., 2010). A small molecule multiple tyrosine kinase inhibitor that targets the VEGFR, PDGFR, stem cell factor receptor, colony-stimulating factor 1 receptor, fms-like tyrosine kinase receptor, and neurotrophic factor receptor in addition to bevacizumab is called sunitinib (Annese et al., 2019).

The simultaneous blocking of these receptors lowers tumour vascularization and causes cancer cells to go through apoptosis. This anti-angiogenic drug was approved by the FDA in 2006 and has been used to treat gastrointestinal

stromal tumours and renal cell carcinoma (Reddy, 2015). A second-generation small molecule multifunctional tyrosine kinase inhibitor called pazopanib targets the tyrosine kinase receptors on lymphocytes as well as VEGF, PDGF, FGFR, stem cell factor receptor, colony-stimulating factor 1 receptor, and PDGF (Schoon et al., 2018; Westin et al., 2018). Soft tissue sarcoma, epithelial ovarian carcinoma, and kidney cancer have all been treated with pazopanib (Bronte et al., 2017). Other anti-angiogenic drugs that approved b FDA are listed in (**Table 2.2**).

Table 2.2: The list of anti-angiogenic drugs approved by FDA

Drug name	Drug class	Targets	Indications
Bevacizumab	VEGF-A antibody	VEGF-A	Metastatic CRC, Metastatic RCC, Metastatic Ovarian cancer, recurrent glioblastoma
Ramucirumab	VEGFR2 antibody	VEGFR2	Metastatic Gastric or GEJ, Metastatic CRC
Aflibercept	VEGF-Trap	VEGF-A, VEGF-B	Metastatic CRC
Sunitinib	Tyrosine Kinase Inhibitor	All VEGFRs, PNET, FGFR1, cKIT, PDGFR	Metastatic GIST
Sorafenib	Tyrosine Kinase Inhibitor	ALL VEGFRs, FGFRs, PDGFRs FLT3	Metastatic RCC, Metastatic HCC, Metastatic thyroid carcinoma
Pazopanib	Tyrosine Kinase Inhibitor	All VEGFRs FGFR2, cKIT	Metastatic RCC, Metastatic soft tissue sarcoma
Axitinib	Tyrosine Kinase Inhibitor	All VEGFRs, PDGFRs, cKIT	Metastatic RCC

Table 2.2: Continued

Drug name	Drug class	Targets	Indications
Cabozantinib,	Tyrosine Kinase Inhibitor	All VEGFRs, cKIT, cMET, Ret	Metastatic medullary thyroid carcinoma, Metastatic HCC
Lenvatinib	Tyrosine Kinase Inhibitor	All VEGFRs, PDGFRs, FGFR1	Metastatic thyroid cancer, Metastatic HCC

[Source: Haibe et al., (2020)]

2.1.5 Side effects

There have been arguments in favour of the idea that anti-angiogenic drugs used in clinical settings could be less likely to produce unwanted side effects and drug resistance when treating cancer (Wu H. C et al., 2008). Theoretically, anti-angiogenic drugs are less likely to induce side effects because healthy individuals rarely experience neoangiogenesis, with the exception of the uterine endometrium during the monthly cycle (Kubota, 2012). Since endothelial cells are genetically stable, anti-angiogenic drugs are unlikely to result in resistance in these cells (Wu H. C et al., 2008). However, the development of new blood vessels is a very complex, multi-step biological process, and VEGF is crucial for the survival of endothelial cells as well as haematopoiesis and myelopoiesis (Kuenen et al., 2002). Therefore, the use of some anti-angiogenic drugs may develop unwanted side effects.

The adverse effects may differ depending on the anti-angiogenic therapy and the type of cancers. However, the common side effects reported on anti-VEGF agents are hypertension, proteinuria, bleeding, gastrointestinal perforation, impaired wound healing, and arterial and venous thromboembolism (Keefe et al., 2011). While the most common and significant adverse effects of bevacizumab have been listed as hypertension, tiredness, headache haemorrhage, proteinuria, arterial thromboembolic events, gastrointestinal perforations, and complications with wound healing (Sousa et al., 2019). Through its direct effect on extracellular VEGF, bevacizumab is utilised to reduce tumour vasculature, which in turn reduces angiogenesis and, subsequently, tumorigenesis. However, certain clinical findings indicate that

bevacizumab did not increase the overall patient survival rate. These failures are related to bevacizumab's poor blood-brain barrier (BBB) penetration efficiency, which led to an increase in administrations and off-target toxicity (Stegmayr et al., 2017). In comparison to other antiangiogenic drugs, ramucirumab may have a limited range of negative effects due to its unique action on VEGFR2. Hypertension and an elevated incidence of bleeding events are among the notable adverse effects linked to ramucirumab, as well as renal damage in all clinical trials (Khan U & Shah, 2019; Tiwari, 2016).

Additionally, aflibercept is a soluble recombinant protein that "decoy" receptor for VEGF that is given intravenously and eventually inhibits the binding of several VEGF family members' receptors, including VEGF-A, VEGF-B, and PLGF, to inactivate them. Aflibercept has similar adverse effects to most anticancer medications, such as fatigue and gastrointestinal issues like diarrhoea and abdominal pain. Leukopenia (neutropenia), thrombocytopenia, and poor wound healing are just a few of the major side effects that can result from mylosuppression, which is a typical side effect of most anticancer medications (Iatrakis, 2019).

Targeted therapies using small molecule tyrosine kinase inhibitors (TKIs) appear to have lower toxicity than traditional cytotoxic chemotherapeutic medicines, with a reasonably high therapeutic window, even if TKIs have significant and widespread side effects, such as fatigue, hypertension, hand-foot syndrome, rash, gastrointestinal toxicities with anorexia, nausea, and diarrhoea, and myelosuppression, which are reversible after treatment withdrawal (Kappers et al., 2010; Lai et al., 2020). However, there are worries

about the long-term negative effects of treatments, such as endocrine-related unfavourable effects, including thyroid function, bone metabolism, adrenal function, and glucose metabolism in addition to endothelial dysfunction, renal damage, and cardiovascular damage (Kappers et al., 2010; Lai et al., 2020).

An oral multikinase inhibitor known as sunitinib targets a number of tyrosine kinase inhibitors thought to be involved in tumour development, pathologic angiogenesis, and the spread of metastatic disease (O'Farrell et al., 2003; Šeparović et al., 2018). According to registration studies, common adverse effects observed in at least 20% of patients include hypertension, tiredness, skin disorders, hematologic disorders (anaemia, thrombocytopenia, granulocytopenia), hypothyroidism, and gastrointestinal disorders (Demetri et al., 2006; Raymond et al., 2011; Šeparović et al., 2018).

Next, a multikinase inhibitor called sorafenib inhibits c-kit, Raf signalling, PDGF, and VEGF in order to impede tumour angiogenesis and tumour cell proliferation (Llovet et al., 2008; Prejac et al., 2020). Rash, diarrhoea, and handfoot syndrome are sorafenib side effects that are frequently reported. Elevated blood pressure, leukopenia, nausea, vomiting, abnormal liver function tests, hypophosphatemia, and depression are among less frequent side effects (Berk et al., 2013; Khaja et al., 2019).

Furthermore, Pazopanib is an oral multi-targeted tyrosine kinase inhibitor (TKI) that targets c-Kit tyrosine kinases, platelet-derived growth factor receptors, and vascular endothelial growth factor receptors. Oftentimes, pazopanib causes adverse effects such as hepatotoxicity, hypertension, thrombocytopenia, anorexia, tiredness, and diarrhoea (Noda et al., 2019). Moreover, axitinib is an

oral second-generation tyrosine kinase inhibitor (TKI) (Chen L. T et al., 2017) that significantly and specifically inhibits VEGF receptors 1, and 2 (Bendell et al., 2017; Fu et al., 2022). More than 30% (Williams & Stein, 2019) of people who take axitinib experience its typical adverse effects, which include anaemia, diarrhoea, hypertension, lethargy, appetite loss (Henriksen & Chang, 2020), nausea, dysphonia (a hoarse, soft voice) (Li Z. Y et al., 2016), and fatigue (Fu et al., 2022).

Cabozantinib is a TKI that exhibits strong activity against several tyrosine kinases involved in cancer treatment resistance, tumour angiogenesis, aberrant bone remodelling, and tumour growth. VEGFR-1, VEGFR-2, VEGFR3, AXL and MET (hepatocyte growth factor receptor) and RET, the stem cell factor receptor KIT, FLT3, ROS1, MER, TYRO3, TRKB and TIE2 are important targets of cabozantinib (Katayama et al., 2015; Osanto & van der Hulle, 2018; Yakes et al., 2011). The most common adverse reactions are fatigue, rash, liver issues, muscle pains, and cough while immune-related lung, intestinal, liver, kidney, skin, or endocrine issues are examples of severe adverse effects (Satyadev, 2021).

Lenvatinib functions as a multityrosine kinase inhibitor and suppresses vascular endothelial growth factor receptor family (VEGFR1–3), fibroblast growth factor receptor family (FGFR1–4), platelet-derived growth factor receptor-alpha (PDGFRα), tyrosinekinase receptor (KIT) and reassembled during transfection receptor (RET), preventing the formation and maturation of new blood vessels and reducing the vascular permeability of the tumour microenvironment (Goel & Singla, 2021; Tohyama et al., 2014). Lenvatinib

therapy has been associated with a number of adverse effects that are comparable to those seen with other MKIs that target the VEGF signalling pathway (Takahashi S et al., 2017). The most frequent adverse effects were proteinuria, abdominal discomfort, exhaustion, diarrhoea, articular and muscular pain, weight loss, nausea, loss of appetite, vomiting, hypertension, and changes in voice while heart failure, liver and kidney damage, bowel perforation, and polycythemia were less frequent serious adverse events (Denaro et al., 2019; Lee H.J et al., 2015). Side effects caused by anti-angiogenic agents are listed in detail in **table 2.3.**

Table 2.3: The list of side effects

Drug name	Drug class	Side effects	Reference
Bevacizumab	VEGF-A antibody	Hypertension, tiredness, headache haemorrhage, proteinuria, arterial thromboembolic events, gastrointestinal perforations, and complications with wound healing.	(Sousa et al., 2019)
Ramucirumab	VEGFR2 antibody	Hypertension, an increased risk of bleeding events, and renal damage.	(Khan U & Shah, 2019; Tiwari, 2016)
Aflibercept	VEGF-Trap	Fatigue and gastrointestinal issues like diarrhoea and abdominal pain. Leukopenia (neutropenia), thrombocytopenia, and poor wound healing are just a few of the major side effects that can result from mylosuppression.	(Iatrakis, 2019)
Sunitinib	Tyrosine Kinase Inhibitor	Hypertension, tiredness, skin disorders, hematologic disorders (anaemia, thrombocytopenia, granulocytopenia), hypothyroidism, and gastrointestinal disorders	(Demetri et al., 2006; Raymond et al., 2011; Šeparović et al., 2018)

Table 2.3: Continued

Drug name	Drug class	Side effects	Reference
Sorafenib	Tyrosine Kinase Inhibitor	Rash, diarrhoea, and handfoot syndrome	(Berk et al., 2013; Khaja et al., 2019)
Pazopanib	Tyrosine Kinase Inhibitor	Hepatotoxicity, hypertension, thrombocytopenia, anorexia, tiredness, and diarrhoea	(Noda et al., 2019)
Axitinib	Tyrosine Kinase Inhibitor	Anaemia, diarrhoea, hypertension, lethargy, appetite loss, nausea, dysphonia (a hoarse, soft voice) and fatigue.	(Fu et al., 2022; Henriksen & Chang, 2020; Li Z. Y et al., 2016)
Cabozantinib,	Tyrosine Kinase Inhibitor	Fatigue, rash, liver issues, muscle pains, and cough while immune-related lung, intestinal, liver, kidney, skin, or endocrine issues are examples of severe adverse effects.	(Satyadev, 2021)

Table 2.3: Continued

Drug name	Drug class	Side effects	Reference
Lenvatinib	Tyrosine Kinase Inhibitor	Proteinuria, abdominal discomfort, exhaustion, diarrhoea, articular and muscular pain, weight loss, nausea, loss of appetite, vomiting, hypertension, and changes in voice	(Denaro et al., 2019; Lee H. J et al., 2015)

2.2 Astaxanthin (ATX)

In recent years, natural compounds have drawn significant attention and become excellent candidates for anti-angiogenic medications. One such product is carotenoids. Historically, Jons Jacob Berzelius, a Swedish chemist, identified xanthophylls (1837) as the designation given to the yellow pigments extracted from autumn leaves. Xanthos and phyllon are derived from the Greek, which means, yellow and leaf. Subsequently, carotenes and xanthophylls were separated and refined by the Russian-Italian botanist M.S. Tswett in 1911 using adsorption chromatography. He dubbed these substances carotenoids (Alcaino et al., 2014).

Carotenoids comprise a category of naturally occurring bioactive compounds that are produced by specific photosynthetic microorganisms and plants (Agarwal & Rao, 2000). Numerous carotenoids participate directly in photosynthesis, while others shield the host from the damaging effects of photooxidation (Hashimoto et al., 2016). A distinct subclass known as the xanthophylls is composed of oxygenated carotenoids containing hydroxy, epoxy, or oxo groups, whereas carotenes are composed of non-oxygenated carotenoids. Xanthophyll, like other bioactive compounds, has garnered significant interest from scientists due to its usefulness, bioavailability, and diversity. The xanthophyll variants comprise, lutein, zeaxanthin, β-cryptoxanthin, capsanthin, astaxanthin, and fucoxanthin (Yilmaz et al., 2018). The chemical structure of related carotenoids is displayed in **figure 2.3.**

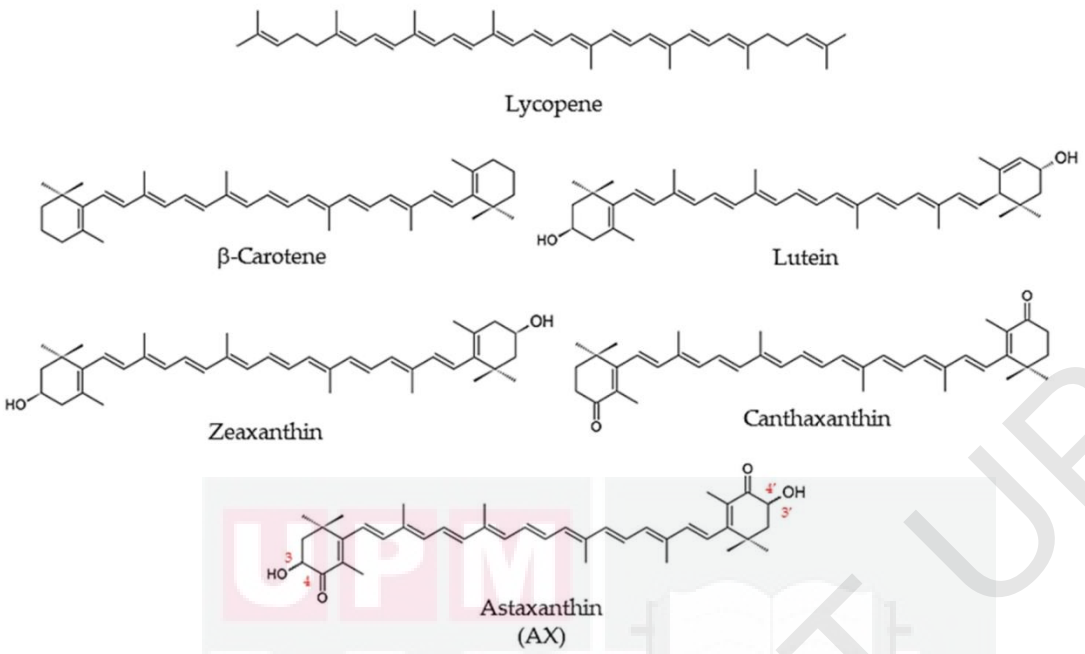


Figure 2.3: The chemical structure of related carotenoids (Nishida Y et al., 2022)

Prior studies have determined that astaxanthin exhibits a tenfold increase in antioxidant activity than other carotenoids including zeaxanthin, canthaxanthin, β-carotene, and lutein (Miki, 1991). Moreover, other carotenoids such as zeaxanthin, lutein, and fucoxanthin have been reported to exhibit anti-angiogenic effects (Pereira, A. G et al., 2021; Keegan, G et al., 2020). Therefore, since ATX exhibits stronger anti-oxidant effect compared to other carotenoids, ATX may as well exhibit anti-angiogenic if not stronger. Due to this rationale, in comparison to alternative carotenoids, ATX was chosen as a prospective tested compound in this specific investigation as a potential anti-angiogenic agent. Within the scope of this current investigation, we will concentrate on astaxanthin (ATX), also known as 3,3′-dihydroxy-β-carotene-4,4′-dione, which is a pigment that belongs to the xanthophyll family. It is water insoluble, fat-soluble pigment with a molecular formula of $C_{40}H_{52}O_4$, having molar mass of 596.84 g/mol, density of 1.081 g/L and a melting point of 224 ◦C

(Aneesh et al., 2022; Lorenz & Cysewski, 2000). ATX was originally first isolated from lobsters in 1938 by Kuhn and Sorensen (Kuhn & Sörensen, 1938; Stachowiak & Szulc, 2021).

2.2.1 Chemical structure of Astaxanthin (ATX)

Due to its molecular structure, astaxanthin possesses distinctive chemical properties in comparison to the molecular structure of other carotenoids. ATX possesses two carbonyl groups and two hydroxyl groups. Its backbone consists of 11 conjugated double bonds, which were formed from the sequential condensation of eight isoprene molecules (Ambati et al., 2014). The molecule has a β-ionone ring with hydroxy and keto groups at each end as depicted in **figure 2.4**. This structure allows ATX to either attract or give electrons for free radicals, which accounts for its antioxidant activity. The hydroxyl and keto groups present on each ionone ring account for some distinctive characteristics of the compound, including its esterification capability, enhanced antioxidant activity, and greater polarity compared to other carotenoids (Hussein et al., 2006).

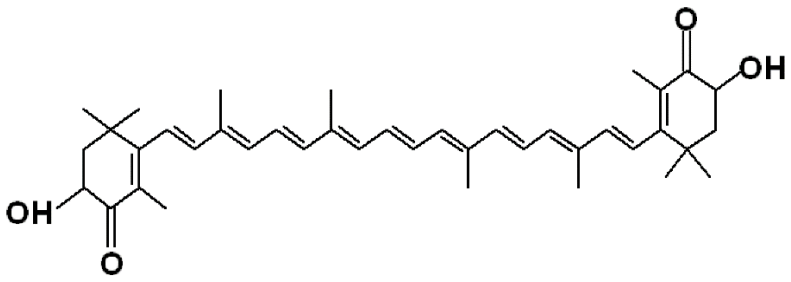


Figure 2.4 The structure astaxanthin (ATX) (Ambati et al., 2014).

ATX is susceptible to isomerization and degradation during the AST extraction process because of its structural properties that render it sensitive to light, heat, and oxygen (Fang et al., 2019). Astaxanthin is present in various form; optical isomers, geometric isomers, free and esterified forms. ATX's β-ionone rings have chiral carbon atoms at the 3 and 3′ positions that can have either R- or S- stereochemistry. Consequently, three configurational isomers as illustrated in **figure 2.5** are produced, including a meso form (3R, 3′ S) and two enantiomers (3S, 3′ R and 3R, 3′ S) (Brotosudarmo et al., 2020). Based on the available information, the distinct enantiomeric configurations of astaxanthin are determined by the manner in which the hydroxyl groups (–OH) are affixed to the carbon atoms located at these centres of asymmetry (Brotosudarmo et al., 2020).

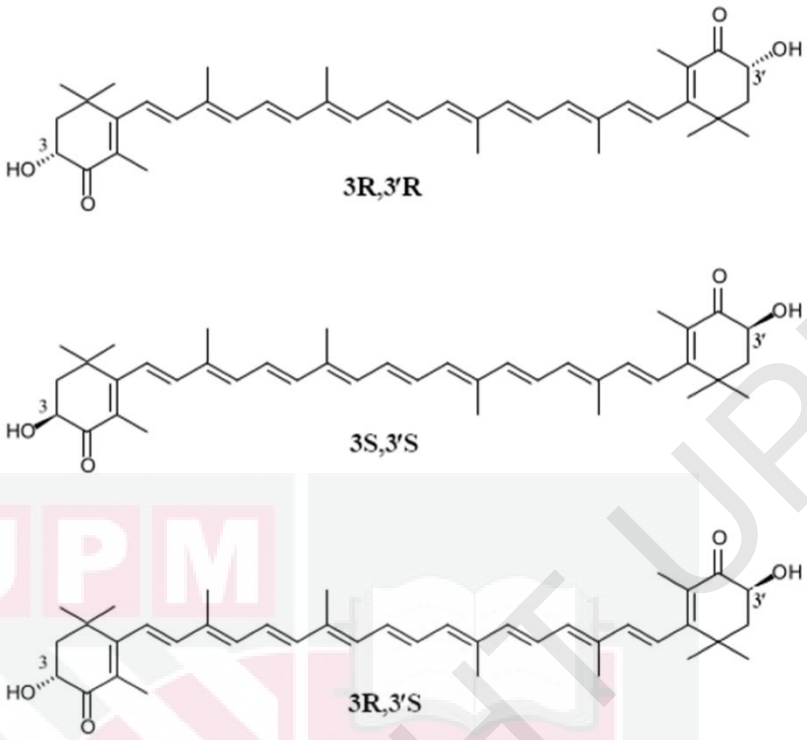


Figure 2.5: The structure of optical isomers of astaxanthin (ATX); two enantiomers (3S, 3′ R and 3R, 3′ S) and meso form (3R, 3′ S) (Stachowiak & Szulc, 2021).

Besides, by virtue of the arrangement of the double bonds in the polyene chain, ATX can also be found in cis and trans (E and Z) geometrical isomers mainly the all-trans, 9-cis, 13-cis and 15-cis isomers as illustrated in **figure 2.6** (Brotosudarmo et al., 2020). Under specific circumstances, including elevated temperature, light exposure, and the presence of acidic and organic solvents, the all-trans isomer and cis isomers are capable of exchanging positions (Yuan J. P & Chen, 1999). When compared to the trans isomers, cis-isomers exhibit lower thermodynamic stability (Higuera-Ciapara et al., 2006). While all-trans astaxanthin (also known as all-E-astaxanthin) is the predominant isomer, at least two cis-isomers (9-cis and 13-cis) are present in both natural and synthetic preparations, relying on the species of host and body area (Brotosudarmo et al., 2020).

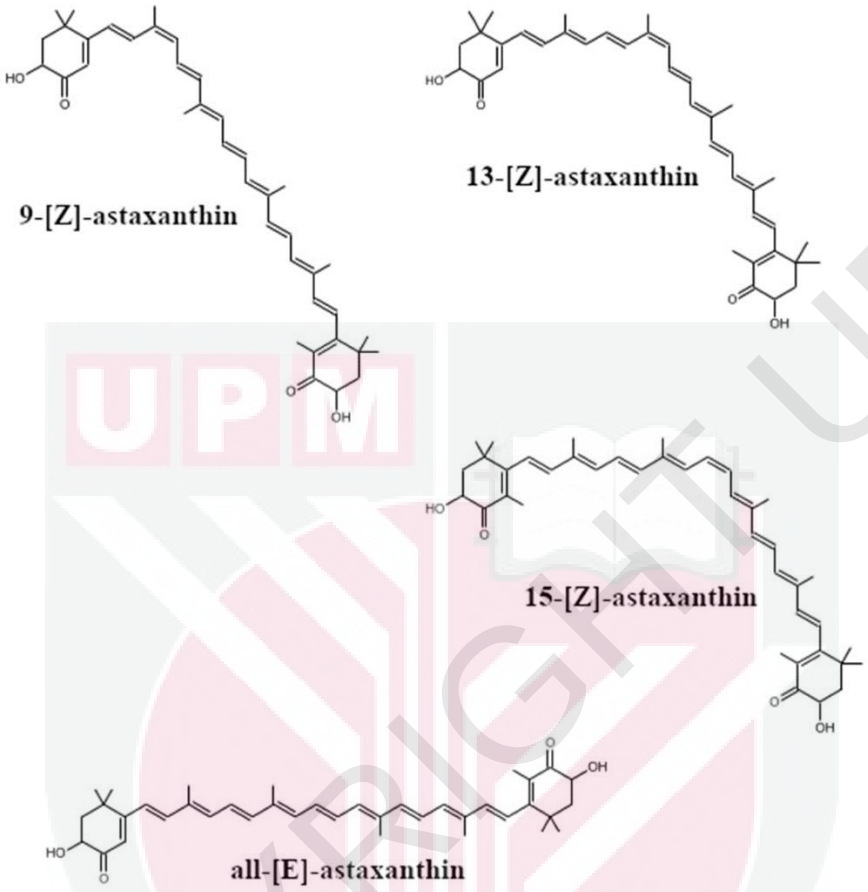


Figure 2.6: The structure of cis and trans (E and Z) geometric isomers of astaxanthin (ATX); all-trans (all-E), 9-cis, 13-cis and 15-cis isomers (Stachowiak & Szulc, 2021).

Moreover, ATX has the ability to undergo esterification, a process that enhances its solubility within the cell and improves its resistance to oxidation (Udayan et al., 2017). By binding to various fatty acids (e.g., palmitic, oleic, estearic, or linoleic acid), the hydroxy group located on one or both rings can generate monomers or diesters, respectively. In addition to its free form (hydroxyl group not esterified), astaxanthin can also be found in chemical complexes with proteins or lipoproteins (Brotosudarmo et al., 2020).

In addition, natural ATX has the structures (3S, 3′S) and (3R, 3′R) while synthetic ATX has a mixture of the three isomers in a ratio of roughly 1:2:1 (Fang et al., 2019). Every organism produces a different kind of ATx in its natural environment. For instance, whatever stereoisomer the fish consumed is what makes up the ATX in fish (Fang et al., 2019; Lorenz & Cysewski, 2000). Another example is the (3S, 3′S) stereoisomer of ATX produced by Haematococcus pluvialis is frequently utilised in animal feed that is then consumed by humans (Ambati et al., 2014). Interestingly, synthetic ATX's antioxidant capacity is 20–50 times lower than that of natural ATX (Fang et al., 2019; Lorenz & Cysewski, 2000).

2.2.2 Sources of Astaxanthin (ATX)

Higher plants as well as tiny creatures including bacteria, yeasts, and phytoplankton algae are the major sources of ATX (Stachowiak & Szulc, 2021). ATX is present in marine organisms in abundance. The renowned reddish-orange hue observed on the skin and flesh of salmon, crustaceans, and shrimp is attributed to this substance. Because they are unable to produce astaxanthin from scratch, crustaceans and fish must rely on eating algae and other microbes to provide them with astaxanthin precursors (Andersson et al., 2003). Similarly, humans lack the ability to produce carotenoids endogenously, necessitating their acquisition through dietary means. Seafood is the primary source of astaxanthin for human consumption (Lim K. C et al., 2018). Additionally, ATX is commonly found in, fungi, the feathers of several birds, including flamingos and quails, as well as in marine species such shrimp,

salmon, crab, lobsters, and crayfish (Cirino et al., 2017; Galasso et al., 2017; Routray et al., 2019). The natural sources of ATX are listed in **table 2**

Table 2.4: The list of natural sources of ATX

Source	Example of species	Astaxanthin concentration	Primary optical isomer	Reference
Microorganism				
Phytoplankton	*Haematococcus pluvialis* NIES-144	98,000 *d.w.*	3*S*,3*S*	(Domínguez-Bocanegra et al., 2004)
	Haemotococcus lacustris	43,100 *d.w.*	3*S*,3*S*	(Lee Y. -K & Soh, 1991)
	Haematococcus pluvialis CCAP-34/7	22,700 *d.w.*	3*S*,3*S*	(Orosa et al., 2001)
	Neochloris wimmeri CCAP-213/4	19,200 *d.w.*	—	
	Protosiphon botryoides SAG-731/1a	14,300 *d.w.*	—	

Table 2.4: Continued

Scotiellopsis oocystiformis SAG-277/1	10,900 *d.w.*	—	
Calanus finmarchicus	100–500 *d.w.*	3*S*,3*S*	(Hylander et al., 2015; Osanjo et al., 2009)
Calanus glacialis	100–500 *d.w.*	—	
Calanus hyperboreus	100–500 *d.w.*	—	
Calanus pacificus	—	—	
Diaptomus nevadensis	—	—	(Juhl et al., 1996)
Neocalanus tonsus	—	—	
Amphiascoides atopus	619 *d.w.*	—	(Caramujo et al., 2012)
Acartia bifilosa	293–487 *d.w.*	—	(Łotocka et al., 2004)
Pseudocalanus acuspes	239–305 *d.w.*	—	

Table 2.4: Continued

	Idotea metallica	—	—	(Herring, 1969)
Bacteria	*Agrobacterium aurantiacum*	140 *d.w.*	3*S*,3*S*	(Yokoyama et al., 1994)
	Brevundimonas sp. strain N-5	837 *d.w.*	3*S*,3*S*	(Asker, 2017)
	Sphingomicrobium astaxanthinifaciens	40 *d.w.*	—	(Shahina et al., 2013)
	Paracoccus bogoriensis	400 *w.w.*	—	(Osanjo et al., 2009)
	Brevundimonas spp.	27.6–365 *d.w.*	3*S*,3*S*	(Barredo, 2012)
	Brevundimonas sp. M7	1300 *d.w.*	3*S*,3*S*	

Table 2.4: Continued

	Sphingomonas astaxanthinifaciens	690 *d.w.*	—	
	Altererythrobacter ishigakiensis	—	—	(Matsumoto et al., 2011)
	Paracoccus haeundaensis	—	—	(Lee J. H et al., 2004)
	Paracoccus carotinifaciens	—	3*S*,3*S*	(Tsubokura et al., 1999)

Table 2.4: Continued

Yeast	*Phaffia rhodozyma* PR 190	970 *d.w.*	3*R*,3*R*	(Kusdiyantini et al., 1998)
	Phaffia rhodozyma UCD 67-210	387 *d.w.*	3*R*,3*R*	(E. A. Johnson & Lewis, 1979)
	Candida utilis	400 *d.w.*	—	(Miura et al., 1998)
Archaea	*Halobacterium salinarium* NRC-1	265 *d.w.*	—	(Calo et al., 1995)
	Haloarcula hispanica ATCC 33960	17 *d.w.*	—	
Chromista	*Thraustochytrium* sp. CHN-3	2800 *d.w.*	—	(Yamaoka et al., 2004)

Table 2.4: Continued

Crustaceans				
Shrimp	*Marsupenaeus japonicus*	418 *d.w.*	—	(Chien & Shiau, 2005)
	Litopenaeus setiferus	48.3 *w.w.*	—	(Pu et al., 2010)
	Penaeus sp.	0.96 *w.w.* (only head)	—	(Armenta-López et al., 2002)
	Litopenaeus vannamei	11.3–31.8 *w.w.*	3*R*,3*S*	(Parisenti et al., 2011)
	Penaeus monodon	24.9–67.5 *d.w.*	3*R*,3*S*	(Pan & Chien, 2003)
	Pandalus borealis	30.9–147.7 *w.w.*	3*R*,3*S*	(Visioli & Artaria, 2017)
Crawfish	*Procambarus clarkii*	78.5–197.9 *d.w.* (only shell)	—	(Balaban et al., 2001)
	Pleuroncodes planipes	—	—	(Coral-Hinostroza & Bjerkeng, 2002)

Table 2.4: Continued

Crabs	*Eriocheir sinensis*	3.5–4.7 *d.w.* (only carapace)	—	(Wang Z et al., 2018)
	Chionoecetes opilio	119.6 *d.w.*	—	(Higuera-Ciapara et al., 2006)
Lobster	*Jasus lalandii*	13 *w.w.*	—	(Auerswald & Gäde, 2005)
Antarctic krill	*Euphausia superba*	—	3*R*,3*R*	(Visioli & Artaria, 2017)
Fishes				
Salmonids	Atlantic salmon (*Salmo salar*)	6–8 *w.w.*	3*S*,3*S*	(Ambati et al., 2014)
	Sockeye salmon (*Oncorhynchus nerka*)	26–38 *w.w.*	3*S*,3*S*	

Table 2.4: Continued

Sockeye salmon (*Oncorhynchus nerka*)	26–38 *w.w.*	3*S*,3*S*
Chinook salmon (*Oncorhynchus tshawytscha*)	5.4 *w.w.*	3*S*,3*S*
Chum salmon (*Oncorhynchus keta*)	3–5 *w.w.*	3*S*,3*S*
Coho salmon (*Oncorhynchus kisutch*)	10–21 *w.w.*	3*S*,3*S*
Masu salmon (*Oncorhynchus masou*)	4.6 *w.w.*	3*S*,3*S*
Pink salmon (*Oncorhynchus gorbuscha*)	4–7 *w.w.*	3*S*,3*S*
Rainbow trout (*Oncorhynchus mykiss*)	24 *w.w.*	3*S*,3*S*

Table 2.4: Continued

Arctic char (*Salvelinus alpinus*)	8.6 *w.w.*	—

*d.w., dry weight; w.w., wet weight.

Source: Brotosudarmo et al., (2020)

Presently, chemical synthesis and biological extraction are the two methods used to manufacture ATX. The production of ATX from carotene is known as chemical synthesis. The FDA regulation prohibits the use of synthetic ATX in the human diet, despite the fact that it is less expensive and less stable, less absorbable, and less safe than natural ATX (Brown D. R et al., 2018; Fakhri et al., 2018; Parker, 1996). On the other hand, the second method for obtaining natural ATX is by extracting it from the waste of crustaceans (including krill, shrimp, and crab). Enzymatic hydrolysis and the acid-based approach can be used, and these processes not only boost economic benefits but also reduce environmental contamination and enable the low-carbon and effective utilisation of marine byproducts. However, because extraction is expensive, it is not ideal for mass production (Li J et al., 2020).

2.2.3 Uses of Astaxanthin (ATX)

ATX was originally identified in 1938 and was first commercialised as only for pigmentation in the aquaculture industry. To boost the amount of ATX in farmed salmonids and produce the flesh's distinctive orange-red colour, ATX has been permitted as a food colouring since 1991 which leads to improved quality and commercial acceptance (Zhang C et al., 2020). As of right now, ATX is a well-known substance for its commercial use in a variety of industries, including aquaculture, food, cosmetics, nutraceuticals, and pharmaceuticals (Begum et al., 2016; Davinelli et al., 2018; Dufossé et al., 2005; Dutta et al., 2005). Although plants, bacteria, and microalgae can also produce ATX, the chlorophyte alga Haematococcus pluvialis is thought to have the greatest capacity to accumulate ATX (Boussiba, 2000). Notably, more than 95% of

commercially available astaxanthin is derived synthetically from petrochemicals because it is more economical for large production; natural astaxanthin derived from Haematococcus pluvialis comprises less than 1% of commercial astaxanthin (Davinelli et al., 2018). In regard to the human consumption of synthetic astaxanthin, however, safety concerns have emerged (Brendler & Williamson, 2019). **Table 2.5** listed the products made with astaxanthin from different companies and their uses.

Table 2.5: Products made with natural ATX from different companies and their uses

Brand Name	Dosage form	Ingredients	Company Name	Purpose
Physician Formulas	Soft gel/Tablets	2 mg/4 mg-ATX	Physician formulas vitamin company	Antioxidant
Eyesight R_x	Tablet	ATX, vitamin-C, plant extracts	Physician formulas Vitamin company	Vision function
KriaXanthin	Soft gel	1.5 mg-ATX, EPA, DHA	Physician formulas vitamin company	Antioxidant
Astaxanthin Ultra	Soft gel	4 mg-ATX	AOR	Cardiovascular health/gastrointestinal
Astaxanthin Gold™	Soft gel	4 mg-ATX	Nutrigold	Eye/joint/skin/immune health
Best Astaxanthin	Soft gel	6 mg-ATX, CX	Bioastin	Cell membrane/blood flow

Table 2.5: Continued

Brand Name	Dosage form	Ingredients	Company Name	Purpose
Dr.Mercola	Capsules	4 mg ATX, 325 mg Omega-3 ALA	Dr. Mercola premium supplements	Aging/muscle
Solgar	Soft gel	5 mg-ATX	Solgar global manufacture	Healthy skin
Astaxanthin	Cream	ATX, herbal extracts	True botanica	Face moisturizing
astavita ex	Capsules	8 mg ATX, T3	Fuji Chemical Industry	Agingcare
astavita SPORT	Capsules	9 mg ATX, T3 and zinc	Fuji Chemical Industry	Sports nutrition
AstaREAL	Oil, powder, water soluble, biomass	ATX, ATX-esters	Fuji Chemical Industry	Soft gel, tablet, beverages, animal feed, capsules

Table 2.5: Continued

Brand Name	Dosage form	Ingredients	Company Name	Purpose
AstaTROL	Oil	ATX	Fuji Chemical Industry	Cosmetics
AstaFX	Capsules	ATX	Purity and products evidence based nutritional supplements	Skin/cardiovascular function
Pure Encapsulations	Capsules	ATX	Synergistic nutrition	Antioxidant
Zanthin Xp-3	Soft gel capsules	2 mg, 4 mg-ATX	Valensa	Human body
Micro Algae Super Food	Soft gel	4 mg ATX	Anumed intel biomed company	heart/eye/joint

[Source: Ambati et al., (2014)]

2.2.4 Pharmacological actions of Astaxanthin (ATX)

ATX has reportedly been associated with a variety of pharmacological action that are beneficial to human health which are anti-oxidant, anti-inflammatory, anti-apoptotic, anti-cancer, cardioprotective, anti-diabetic, hepatoprotective, neuroprotective, anti-dibesity, anti-hypertensive and gastroprotective. Summary of these activities are tabulated in **(Table 2.6).**

Table 2.6: The list of pharmacological actions of Astaxanthin

Pharmacological actions	Target disease	Study type	Cell lines	Animals	Doses	Reference
Anti-oxidant	Skin health	In vitro	Human dermal fibroblasts (WS1)	-	5–160 µg/mL of ATX	(Chintong et al., 2019)
Anti-inflammatory	Spinal cord injury	In vivo	-	Adult male Wistar rats (aged 2–3 months; 240–280 g in weight)	10 µl of 0.2 mM of ATX	(Masoudi et al., 2021)
Anti-apoptotic	Eye disease	In vivo	-	Newborn C57BL/6J-type mice	10 ng/µL IV- ATX, 100 ng/µL IV- ATX, 0.5 mg/kg IP- ATX, 5 mg/kg IP- ATX	(Küçüködük et al., 2019)

Table 2.6: Continued

Pharmacological actions	Target disease	Study type	Cell lines	Animals	Doses	Reference
Anti-cancer	Prostate cancer	In vivo	-	Nude mice inoculated with DU145 prostate cancer cell line	200 mg/kg of ATX	(Sun et al., 2020)
Cardioprotective	Myocardial infarction	In vivo	-	Male Wistar albino rats weighing between 150 and 200 g	10 mg/kg of ATX	(Zaafan & Abdelhamid, 2021)
Anti-diabetic	Type 2 diabetes mellitus	In vivo	-	Male Wistar rats (90–100 g)	15, 30, 50 mg/kg of ATX	(Zhuge et al., 2021)

Table 2.6: Continued

Pharmacological actions	Target disease	Study type	Cell lines	Animals	Doses	Reference
Hepatoprotective	Liver injury	In vivo	-	Male C57BL/6 mice	30 mg/kg/d or 60 mg/kg/d of ATX	(Zhang J et al., 2017; Aneesh et al., 2022)
Neuroprotective	Alzheimer's disease	In Vitro	Rat pheochromocytoma cells PC-12	-	0.1-50 μM of ATX	(Alghazwi et al., 2019)
Anti-obesity	Obesity	In vivo	-	Adult male mus musculus albino mice of Swiss strain weighing 25–35 g	6 and 30 mg/kg of ATX	(Bhuvaneswari et al., 2010; Xia et al., 2020)

Table 2.6: Continued

Pharmacological actions	Target disease	Study type	Cell lines	Animals	Doses	Reference
Anti-hypertensive	Blood pressure	In vivo	-	Sponta neously hypertensive rat (SHR) (7 weeks)	50 mg/kg of ATX	(Hussein et al., 2005; Raza et al., 2021)
Gastroprotective	Gastrointestinal diseases	In vivo	-	BALB/c female mice, 6–8-week-old	10 or 40 mg/kg of ATX	(Davinelli et al., 2019)

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

Complying with strict scientific protocol, **Table 3.1 - 3.4** presents an extensive inventory of the particular components used in all of the experiment processes. This inventory entails all of the chemicals, reagents, commercial kits, and lab apparatus that were used to execute the experiments.

3.1.1 Apparatus and Equipment

The apparatus and equipment required for the experimental procedures carried out in the current investigation are meticulously listed in **Table 3.1**, complemented by information about the model and manufacturer of each item.

Table 3.1: Apparatus/Equipment

Apparatus/Equipment	Model/Type	Brand/Manufacturer
Biological Safety Cabinet (BSC)	Safemate 1.2	BioAir/Esco, Singapore
CO_2 Incubator	CO170R-230-1000	New Brunswick Scientific, USA
Inverted Microscope	OLYMPUS CKX41	Olympus, Tokyo, Japan
Refrigerated centrifuge	UNIVERSAL 320R	Hettich Zentrifugen, Germany

Table 3.1: Continued

Apparatus/Equipment	Model/Type	Brand/Manufacturer
Water Bath	WNB-29	Memmert, Germany
Micropipette	Research® Plus	Eppendorf®, Germany
Serological pipette controller	SWIFTPET PRO	Corning HTL SA, Namur, Belgium
Freezer (-20°C)	FC186D4BWP	Hisense, Malaysia
Freezer (-80°C)		Thermo Scientific, USA
Analyyical balance	HR-250AZ	A&D Company, Limited, Tokyo, Japan
Microplate reader	Infinite M200	TECAN, Mannedorf, Switzerland
Haemacytometer	Neubauer-improved	Marienfeld Superior, Germany

3.1.2 Chemical/Reagents

A comprehensive list of all the compounds and reagents utilised in the current study is provided in **Table 3.2**, along with a list of the manufacturers of each.

Table 3.2: Chemical/Reagents

Chemical/Reagants	Brand/Manufacturer
EndoGRO Basal Medium supplemented with Kit	Merck Millipore, Darmstadt, Germany
Phosphate buffered saline (PBS)	Merck Millipore, Darmstadt, Germany
VEGF	Merck Millipore, Darmstadt, Germany
Astaxanthin	Sigma-Aldrich, Germany
Suramin	Sigma-Aldrich, Germany
2.5% Trypsin-EDTA solution 10x	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Dimethyl sulfoxide (DMSO)	ATCC, Virginia, USA
3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT)	Sigma Co. St Louis, MO, USA
Matrigel	BD Biosciences, San Jose, CA, USA
Trypan blue	Sigma-Aldrich, United Kingdom
Ethanol	SYSTERM, Malaysia
Distilled water	-

3.1.3 Glassware and disposable/consumable

Table 3.3 presents a succinct summary of the glassware and consumables used in this investigation, with information on the manufacturers of each item. It is of note that these things are intended for single use and will be properly disposed of following their initial use.

Table 3.3: Glassware and disposable/consumable

Glasswares/Plastics	Volume/Size	Brand/Manufacturer
Cell culture flask	25 cm^2	NEST, Wuxi, China
Serological pipette	5ml, 10ml	SPL Life Sciences, Gyeonggi-do, Korea
Micropipette tip	1000µl, 200µl, 10µl	Labcon, USA
Centrifuge tube	20ml, 50ml	NEST, Wuxi, China
Gloves	Small	Top Glove, Selangor, Malaysia
Cryovials	2ml	Kirgen, Wisconsin, USA
Well plate	96-well plate, 24-well plate	SPL Life Science, Gyeonggi-do, Korea

3.1.4 Software/databases

Table 3.4 offers a detailed summary of the databases and software programmes employed in this study, specifically with regard to in-silico experiments and assays that require further computational tool analysis.

Table 3.4: Software/databases

Software/databases
Database for Annotation, Visualizations and Integrated Discovery (DAVID)
Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database
Swiss Target Prediction Target
The Universal Protein Resource (UniProt) database
GeneCard Suite: Human Genes (GeneCards) database
DisGeNet database
National Center for Biotechnology Information (NCBI) Gene database
SuperPred database
InteractiVenn
Cytoscape 3.9.1 software
AutoDock Tools software version (v4.2.6)
Biovia Discovery Studio
RCSB Protein Data Bank
PubChem database
NIH's Image J software (National Institutes of Health, Bethesda, MD, USA)
GraphPad Prism Version 10

3.2 Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were procured from Merck Millipore (Darmstadt, Germany) and maintained in EndroGRO-LS Complete Culture Media Kit. The EndroGRO-LS Complete Culture Media Kit consist of basal medium, 0.2% EndroGRO-LS supplement, 5 ng/ml rh EGF, 50 µg/ml ascorbic acid, 10nM L-Glutamine, 1.0 µg/ml hydrocortisone hemisuccinate, 0.75 U/ml heparin sulfate and 2% FBS. All assays were executed utilising low passage cells (3–6 passages) to uphold cell morphology and preserve biological properties. HUVECs were cultivated in a humidified incubator with 5% CO^2 at 37°C, and the culture medium was refreshed every 2 days until the cells achieved 90% confluence. Subsequently, the cells were trypsinized using a 2.5% Trypsin-EDTA solution (10x), followed by centrifugation at 1200 rpm for 10 minutes, and subcultured into 25 cm^2 flasks.

3.3 Treatment protocol

For all experiments, unless otherwise specified in the protocol, HUVECs were seeded in either 96-well or 24-well plates. Astaxanthin (ATX) ≥97% (HPLC), from *Blakeslea trispora* was purchased form Sigma-Aldrich, Germany. A total of 3.8 mg of ATX was diluted in 1 ml of dimethyl sulfoxide (DMSO) to achieve a final DMSO concentration of 0.1%, ensuring non-toxicity to the cells. Additionally, 10 mg of VEGF was diluted in 1 ml of PBS, and 0.2 mg of suramin was diluted in 1 ml of PBS. These treatments (ATX, VEGF, Suramin) were then prepared at various concentrations as required for each specific assay. All experiments were conducted in triplicate and repeated in three independent tests for robustness and reliability.

Proliferation, migration and tube formation assay used suramin as positive control. Suramin was used because aside from its anti-trypanosomal properties, suramin was found to have strong anti-angiogenic and VEGF inhibitory properties (Vaskular, E et al., 2020). By preventing certain receptors from binding to PDGF, bFGF, VEGF, and neuropilin, suramin has been demonstrated to suppress angiogenesis (Ljoki, A., 2022).

3.4 Cell viability

The MTT assay, initially described by Mosmann (1983), serves as a widely employed preliminary screening method for determining cell viability. This colorimetric assay is a straightforward approach used to assess cell cytotoxicity, proliferation, and viability. This assay relies on the conversion of the yellow water-soluble substrate, 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT), into a dark blue formazan product. This transformation is catalyzed by mitochondrial dehydrogenase enzymes and results in an insoluble formazan product, providing a measurable indicator of cellular metabolic activity. Viable cells, characterized by an active metabolism, possess the capacity to enzymatically convert MTT into a purple-coloured formazan product. In contrast, non-viable cells lose the ability to undergo this conversion, resulting in the absence of formazan formation (Mosmann, 1983). Therefore, the resulting colour formation serves as a valuable and convenient marker exclusively for viable cells.

3.4.1 Cell Cytotoxicity Assay

The assessment of cell cytotoxicity was conducted using the MTT assay, following the methodology previously described by (Kepekçi et al., 2021) with slight modifications. In brief, HUVECs were seeded onto a 96-well plate at a density of 1 x 10^4 cells per well. To investigate the cytotoxic effects of ATX on HUVECs, the cells were exposed to varying concentrations of ATX, ranging from 3.125 to 50 µM. The experimental groups for the cell cytotoxicity assay, including the control, treatment groups with various concentrations of astaxanthin (ATX), and the blank, are outlined in **Appendix A (Table 1).** Each group consists of cells treated with media and different concentrations of ATX, ranging from 3.125 µM to 50 µM, alongside the respective MTT reagent addition. ATX solutions of varying concentrations were prepared in 100 µl of culture media. HUVECs were subjected to treatment with different ATX concentrations and subsequently incubated at 37°C for a duration of 24 hours. Post-treatment, 5 µl of MTT solution (2 mg/ml in PBS) was introduced into the wells containing 100 µl of the respective treatments, followed by an additional incubation period at 37°C for 4 hours. The MTT solution was aspirated, and 100 µl of dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystals. Subsequently, the optical density (OD) was quantified using a microplate reader at 570 nm wavelength (Infinite M200, TECAN, Mannedorf, Switzerland).

The obtained results were interpreted as cell viability, expressed as a percentage (%). The percentage of cell viability was derived from the mean absorbance readings using the following formula:

$$Cell\ viability\ (\%) = \frac{Abs\ (sample)}{Abs\ (control)} \times 100\%$$

Abs: absorbance reading at 570nm

This experimental procedure was repeated in three independent tests, each conducted in triplicate. Three optimal concentrations were selected for subsequent assays.

3.4.2 Cell Proliferation Assay

The assessment of cell proliferation was conducted using the MTT assay, following the methodology previously described by Lim C. P et al., (2019) with slight modifications. In brief, HUVECs were seeded onto a 96-well plate at a density of 1 x 10^4 cells per well. The three optimal concentrations were determined based on the results obtained from the preceding cytotoxic assay. To investigate the anti-proliferative effects of ATX on HUVECs, the cells were exposed to varying concentrations of ATX, ranging from 6.25 to 25 µM. HUVECs were subjected to co-treatment with various concentrations of ATX and 10 ng/ml of VEGF, followed by an incubation period at 37°C for 72 hours. The co-treatment involved the preparation of solutions containing varying concentrations of astaxanthin (ATX) and 10 ng/ml of vascular endothelial growth factor (VEGF) in 100 µl of culture media. A baseline group was incorporated and subjected to incubation for a duration of only 4 hours. The experimental groups for the cell proliferation assay, including control, baseline,

VEGF-only, and ATX treatment groups with varying concentrations (6.25 µM, 12.5 µM, and 25 µM of ATX combined with 10 µg/ml VEGF), alongside the respective MTT reagent addition, as well as a blank, are summarized in **Appendix A (Table 2).** Post-treatment, 5 µl of MTT solution (2 mg/ml in PBS) was introduced into the wells containing 100 µl of the respective treatments, followed by an additional incubation period at 37°C for 4 hours.

The MTT solution was aspirated, and 100 µl of dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystals. Subsequently, the optical density (OD) was quantified using a microplate reader at 570 nm wavelength (Infinite M200, TECAN, Mannedorf, Switzerland). This experimental procedure was repeated in three independent tests, each conducted in triplicate. The obtained results were interpreted as cell viability, expressed as a percentage (%). The percentage of cell viability was derived from the mean absorbance readings using the following formula:

$$Cell\ viability\ (\%) = \frac{Abs\ (sample)}{Abs\ (control)} \times 100\%$$

Abs: absorbance reading at 570nm

3.5 Scratch Wound Healing Assay

The evaluation of cell migration was conducted using the scratch wound healing assay, following the methodology described in a previous study Shi et al., (2022) with certain modifications. In brief, HUVECs were seeded onto a 24-well plate at a density of 1.5 x 10^5 cells per well. Upon seeding, cells were scratched using a 200 µl pipette tip (yellow tip) and subsequently washed with

PBS to eliminate sediment resulting from the scratching process. To investigate the anti-migratory effect of ATX on HUVECs, cells were co-treated with diverse concentrations of ATX ranging from 6.25 to 25 μM, along with 10 ng/ml of vascular endothelial growth factor (VEGF). Additionally, a control group was included with 20 ng/ml of Suramin serving as the positive control. The co-treatment involved the preparation of solutions containing varying concentrations of ATX and 10 ng/ml of VEGF in 500 μl of culture media. The experimental groups for the cell migration assay, including control, VEGF, Suramin, and astaxanthin (ATX) treatments with varying concentrations, are detailed in **Appendix A (Table 3).** The specific contents and concentrations are outlined in the table for easy reference. All groups were incubated at 37°C and underwent an 8-hour treatment duration. Images of each experimental group were captured at both the 0-hour and 8-hour time points, utilizing an inverted microscope fitted with a digital camera (OLYMPUS CKX41, Tokyo, Japan). The images were taken at the same location to ensure consistency in the observation area. The gap widths for each experimental group were measured using the NIH Image J software (National Institutes of Health, Bethesda, MD, USA http://imagej.nih.gov/ij/). This experimental procedure was repeated in three independent tests. Three readings were obtained for each group, and subsequently, the average gap width was calculated.

The result of scratch wound healing assay was interpreted as migration rate (%) using the formula of:

$$Migration\ rate\ (\%) = \frac{(A - B) \times 100}{A}$$

A: The width of initial scratch wound, B: The width of scratch wound at time 8h

3.6 Tube Formation Assay

The process of reassembling and establishing cell-to-cell contact during endothelial cell tube formation is a crucial angiogenic step that impacts the morphology of new capillaries (DeCicco-Skinner et al., 2014; Senger & Davis, 2011). In the presence of growth factors, HUVECs possess the ability of forming tubes by attaching to neighbouring cells on a matrix-coated surface. Since these tube vessels supply nutrients, compounds that can prevent tube formation may be helpful in treating a variety of illnesses, including cancer (Isaji et al., 1997).

The assessment of tube formation was carried out utilizing Matrigel, following the methodology outlined in a previous study (Lim H. J et al., 2019) with specific modifications. Prior to loading the plate, 24-well plate and 1ml pipette tips were refrigerated at 4°C for 30 minutes to prevent premature solidification of the Matrigel. 300 µl of Matrigel was carefully loaded into the wells to prevent the formation of bubbles. HUVECs were seeded onto the solidified Matrigel in each well at a density of 1.5 x 10^5 cells in 100 µl and distributed evenly. This step aimed to achieve a well-established network of tubes and prevent the formation of patches of confluent cells in the central region. Immediately after

seeding the cells, a co-treatment of ATX at concentrations ranging from 6.25 to 25 µl and 10 ng/ml VEGF was added, alongside other control groups. The experimental groups for the tube formation assay, including the control, VEGF, Suramin, and various concentrations of ATX, are detailed in **Appendix A (Table 4).**

The treatment incubation period lasted for 6 hours, and five random images of the tubular structures were captured at the conclusion of the 6-hour period using an inverted microscope equipped with a digital camera (OLYMPUS CKX41, Tokyo, Japan). The lengths of the tubular structures (µm) in five randomly selected images from each experimental group were quantified using the NIH Image J software (http://imagej.nih.gov/ij/) equipped with an angiogenesis plugin. Subsequently, the average tube lengths were computed. This experimental procedure was repeated in three independent tests. The obtained results were interpreted as tube lengths (µm).

3.7 Network Pharmacology

With the aim of identifying the connections between drugs, their targets, pathways, and related diseases, the emerging field of network pharmacology uses computational expertise to develop an understanding of drug action at various complexity levels, from a single drug target to multiple drug targets (Belenahalli Shekarappa et al., 2019). The next subsections utilised a series of network pharmacology approaches to investigate the putative targets of angiogenesis in relation to ATX.

3.7.1 Prediction of Target Genes of ATX

The Swiss Target Prediction database (http://www.swisstargetprediction.ch/, retrieved on 12 May 2023) (Lin et al., 2021) and the SuperPred database (https://prediction.charite.de/index.php, retrieved on 12 May 2023) (Yu et al., 2021) were used to determine the ATX target genes. To preserve the distinctive traits of human biology, the analysis was narrowed to only those present in homo sapiens, then afterwards the duplicated entries were removed to provide a proper list.

3.7.2 Prediction of Target Genes of Angiogenesis

Three prominent databases were utilised to identify target genes associated with the angiogenesis: Disease Gene Interaction (DisGeNet) https://www.disgenet.org/, retrieved on 12 May 2023) (Yun et al., 2021), GeneCards (http://www.genecards.org/, retrieved on 12 May 2023) (Fan & Sui, 2022), and Ncbi gene; (https://www.ncbi.nlm.nih.gov/gene/, retrieved on 12 May 2023) (G. Wang et al., 2022). To predict the potential relation between angiogenesis and ATX, the targets were queried through multiple databases with the keyword “Angiogenesis”. The duplicated entries were eliminated and standardised using UniProt database.

3.7.3 Construction of Venn Diagram

The potential target genes for both ATX and angiogenesis were analysed through InteractiVenn (http://www.interactivenn.net/, retrieved on 12 May 2023) (Zhao et al., 2019) in order to identify the association between ATX and angiogenesis. The end products of the approach were displayed in the manner of a Venn diagram, which revealed the intersection of potential target genes between ATX and angiogenesis.

3.7.4 Construction of Compound-Target Network

In order to more clearly demonstrate the relationships between ATX and angeogenesis, a compound-target network was built using the Cytoscape software version 3.9.1. (https://cytoscape.org/, retrieved on 12 May 2023) (Xiong et al., 2018). Following some organisation and categorization, the identified ATX and its target information were integrated into cytoscape. Then, the common target genes of ATX and angiogenesis are selected as network nodes, with lines connecting them demonstrating how these nodes are connected to one another. The above procedure eventually resulted in the establishment of a "Compound-Target-Disease" network.

3.7.5 Protein–Protein Interaction (PPI) Network Analysis

The overlapping target genes of ATX and angiogenesis were investigated through PPI network analysis by using STRING database version 11.5 (https://string-db.org/, retrieved on 12 May 2023) (Ma H et al., 2021). Only proteins from the *Homo sapiens* species were analysed, and only those with a confidence score of at least 0.900 were selected for network visualisation. The

end result of the network was visualised using the Cytoscape software version 3.9.1 (https://cytoscape.org/, retrieved on 12 May 2023) along with data from the STRING database. Through the application of the CytoHubba plugin (Ma H et al., 2021) and three algorithms—maximal clique centrality (MCC), maximum neighborhood component (MNC), and degree—the significance of the network's genes was evaluated and the top ten hub genes were determined according to the scores. The outcomes of the three algorithms were then merged to produce the ultimate set of hub genes, which were then presented as potential hub targets in the network.

3.7.6 Gene Ontology and Pathway Enrichment Analysis

To acquire a deeper knowledge of the fundamental biological processes of ATX and their potential implications against angiogenesis, a gene ontology (GO) and Kyoto Encyclopaedia of Genes and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed. The Database for Annotation, Visualisation, and Integrated Discovery (DAVID), version 2021 (https://david.ncifcrf.gov/home.jsp, retrieved on 12 May 2023) (Feng et al., 2019) was the tool utilised for the examination. The software programme serves as an extensive tool for gene functional analysis, comprising detailed information on biological processes, cellular components, molecular functions, and signalling pathways (Cai C et al., 2019; Ruan et al., 2020; Wu B & Xi, 2021). The top 10 from the GO analysis and the top 20 from the KEGG analysis were presented. The p-values were computed, and the results that were statistically significant had a p-value less than 0.05.

3.8 Molecular Docking

A computational approach known as molecular docking that is widely used in drug development since it predicts the binding modes and affinities of receptors and ligands was carried out to investigate the interactions between ATX and the hub genes discovered in the study. Four hub genes discovered from the network pharmacology study (*RXRA, CCND1, CDK1*, and *HDAC1*) and three additional main tyrosine kinase receptors VEGFR2, FGFR1 and Tie-2 which were considered as key targets in the process of angiogenesis were investigated through molecular docking. The crystal structure of the four hub genes—*RXRA* (PDB ID: 7B88), *CCND1* (PDB ID: 2W96), *CDK1* (PDB ID: 6GU6), and *HDAC1* (PDB ID: 4BKX) as well as the three additional main tyrosine kinase receptors—VEGFR2 (PDB ID: 3VHE), FGFR1 (PDB ID: 4V05), and TIE2 (PDB ID: 2WQB) were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/, retrieved on 19 May 2023) (Khan M. S et al., 2019). The 3D structure of ATX (Compound CID: 5281224) was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/, retrieved on 19 May 2023) (Taherkhani et al., 2020). The compound (ATX) acted as the ligands while the hub genes (*RXRA, CCND1, CDK1*, and *HDAC1*) and tyrosine kinase receptors (VEGFR2, FGFR1 and Tie-2) acted as the receptors. Water molecules and any preexisting ligand were taken out of the receptors in Biovia Discovery Studio 2021. AutoDockTools Version 4.2 (http://autodock.scripps.edu/, retrieved on 16 February 2023) (Sharma et al., 2021) was used to predict the ligand-receptor binding conformation. The binding energy (generally, ≤ 5 kcal/mol) obtained from molecular docking was used to estimate the potential of ligand-receptor interaction. Eventually, Biovia

Discovery Studio 2021 (https://discover.3ds.com/discovery-studio-visualizer, retrieved on 16 February 2023) (Patil et al., 2022) was used to visualise the molecular interactions between the receptors and ligands.

3.9 Statistical Analysis

The data were presented as means from three independent tests ± standard deviation (Mean ± SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Tukey's Post Hoc test, utilizing GraphPad Prism Version 10, San Diego, California, USA. Significance was established at $P < 0.05$.

CHAPTER 4

RESULTS

4.1 Cell Viability

4.1.1 Cytotoxicity assay of ATX in HUVECs utilising MTT

Certainly, in vitro toxicity testing has garnered considerable significance as a valuable tool for assessing the safety profile of various drugs, compounds, toxicants and chemicals. In this present study, the toxicity of ATX was assessed and the IC_{50} value was established.

4.1.1.1 Determination of IC_{50} in Cytotoxicity Assay of ATX in HUVECs

Inhibitory concentration (50%), also referred to as IC_{50}, is the concentration at which compound administration yields a 50% inhibition of net cell growth when compared to cells cultured in the absence of the compound. Put differently, the concentration at which half of the cell population is inhibited from the treatment of HUVECs by ATX is termed as the IC_{50}.

The IC_{50} of ATX was derived from a simple linear regression plotting cell viability (%) against concentration (μM), resulting in a linear equation: $Y = -0.5740 X + 93.10$ ($R^2 = 0.8385$) as shown in **Figure 4.2**. By setting Y (cell viability) to 50%, the corresponding x value was determined, yielding an IC_{50} of 75.09 μM. This value indicates the concentration at which, in the context of experiments, ATX suppressed cell viability by 50%. Inspecting the data, it is evident that the IC_{50} value (75.09 μM) exceeded the highest tested

concentration (50 μM). This suggests that the concentrations utilised are regarded as safe and not excessively toxic for HUVECs. Nevertheless, despite this safety margin, ATX remains to exhibit cytotoxic effects, demonstrated by the pattern that reveals a reduction in cell viability with increasing concentrations.

As examined in this research, there is a statistically significant reduction in the percentage of viable cells with an increase in the concentration of ATX ($P < 0.05$). Based on the graph depicted in **Figure 4.1,** in particular, the cell viability at the highest measured concentration was 67 ± 3.73%, indicating the reduction in cell population by 33% due to the cytotoxic effects of ATX**.** The corresponding cell viability percentages for other concentrations are as follows: 93 ± 1.87%, 86 ± 4.79%, 79 ± 3.56%, and 77 ± 4.86% for 3.125 μM, 6.25 μM, 12.5 μM, and 25 μM, respectively. It is notable that the concentrations of 50 μM, 25 μM, and 12.5 μM exhibited statistical significance when compared to the control group ($P < 0.05$).

Following the discovery of concentrations that demonstrate significance in comparison to the control group and the establishment of the IC_{50} value (75.09 μM), three intermediate concentrations were selected as they represented the most optimal range for observing the effects while minimizing potential extremes. This approach ensures a balanced analysis without the potential confounding effects of too high or too low concentrations. The concentrations are: 6.25 μM, 12.5 μM, and 25 μM.

In brief, ATX exhibits a cytotoxic effect on HUVECs through cell cytotoxicity assay utilising MTT within 24-hour period. In the context of anti-angiogenesis

research, this discovery is noteworthy as it indicates that ATX has a statistically significant impact on the cellular response. These results offer insightful information for future research on anti-angiogenic mechanisms as well as therapeutic approaches.

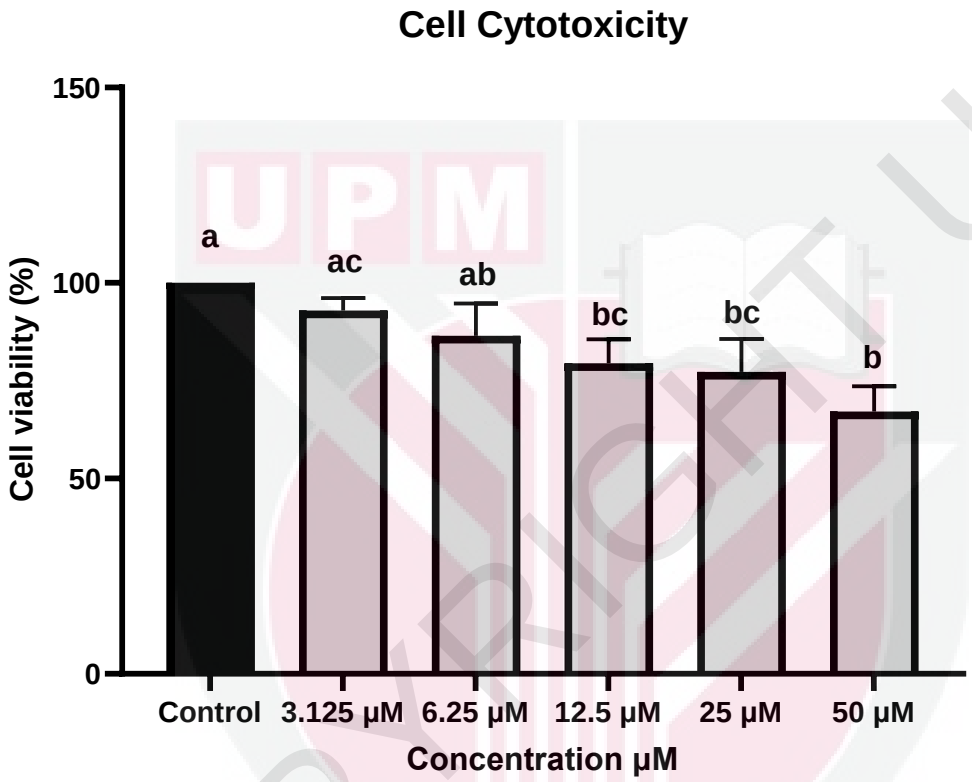


Figure 4.1: Cytotoxic effect of ATX in HUVECs after 24 hours of exposure using MTT at concentrations ranging from 3.125- 50 µM. The induction of HUVECs with ATX resulted in a significant decrease in cell viability (%) with increasing concentration ($P < 0.05$). The data represents as mean ± SD of three independent tests (n=3) in triplicates. Statistical analysis was performed using one-way ANOVA followed by post-hoc test Tukey and compared against each other. Different letters indicate statistically significant differences ($P<0.05$). Significant differences are found between the control group and the concentrations of 12.5 µM, 25 µM, and 50 µM, as well as between 3.125 µM and 50 µM.

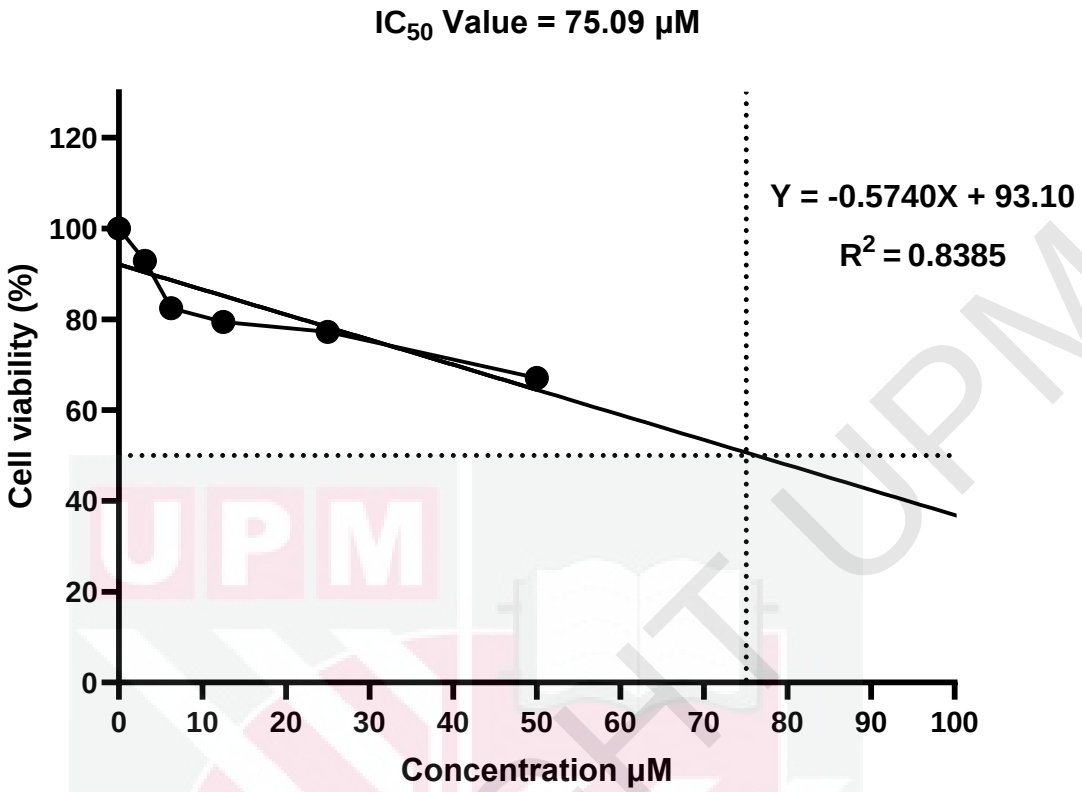


Figure 4.2: Dose-response graph by simple linear regression of MTT cytotoxic assay. The linear equation is Y = -0.5740X + 93.10 (R^2 = 0.8385). By setting Y (cell viability) to 50%, the corresponding X value was determined, yielding an IC_{50} of 75.09 µM.

4.1.2 Proliferation assay of ATX in HUVECs utilising MTT

In the context of the MTT cell proliferation assay, the assay captures the increase in the number of target cells during the exponential phase of proliferation (Hansen et al., 1989). By utilising the concentrations selected from previous cell cytotoxicity assay, cell proliferation assay was assessed.

In the current analysis, there is a statistically significant decline in cell viability percentage with an escalating concentration of ATX ($P < 0.05$). As shown in **figure 4.3,** all treated groups exhibited statistical significance compared to the control group, with the exception of the highest concentration of ATX (25 μM). This suggests that concentrations higher than 25 μM may be detrimental to the cells, as even at 25 μM, the treatment might be approaching a threshold where it begins to negatively affect cell viability.

Drawing from the results, it revealed the means of cell viability (%) are 141 ± 4.00%, 122 ± 3.41% and 114 ± 5.67% for the concentrations of 6.25, 12.5 and 25 μM respectively. There is a statistically significant difference between ATX treatment groups with varying concentrations in comparison to the VEGF-only group ($P < 0.05$), considering that both the VEGF-only group and the ATX treatment groups are induced by VEGF. This is attributed to vascular endothelial growth factors (VEGFs) a key regulator in vascular development during embryogenesis, encompassing vasculogenesis, and in the formation of blood vessels (angiogenesis) in the adult organism. The receptors of VEGF (VEGFRs) activate cellular processes that are shared with numerous growth-factor receptors, encompassing cell migration, survival, and proliferation (Olsson et al., 2006). As such, it is expected that when VEGF is induced, the

absorbance values would elevate, which will be correlated with an increase in the percentage of cell viability over the course of the experiment. This finding reinforces the preceding statement that ATX inhibits HUVEC growth despite the presence of VEGF stimulation proving its anti-proliferative effect.

In this proliferation assay, a baseline group comprising entirely of culture media was employed as the reference condition, with absorbance values taken at 4 hours. This is used as an indicator to quantify the level of absorbance in the 72-hour experimental groups relative to the 4-hour baseline. Given that adequate cellular proliferation is required under normal conditions, any observed proliferation that falls below the baseline is considered to be symptomatic of possible toxicity. The cell proliferation graph illustrates that all treatment groups that were exposed for 72 hours had significantly higher values than the baseline obtained at 4 hours ($P < 0.05$). Besides, the fact that every experimental group has exceeded the baseline implies the validity of the design.

Overall, ATX exhibits anti-proliferative effect on VEGF-induced HUVECs through cell proliferation assay utilising MTT within 72-hour period. This discovery bears particular relevance in light of the broader goal of the research on anti-angiogenic capabilities, since pathological angiogenesis is characterised by unrestricted proliferation of cells.

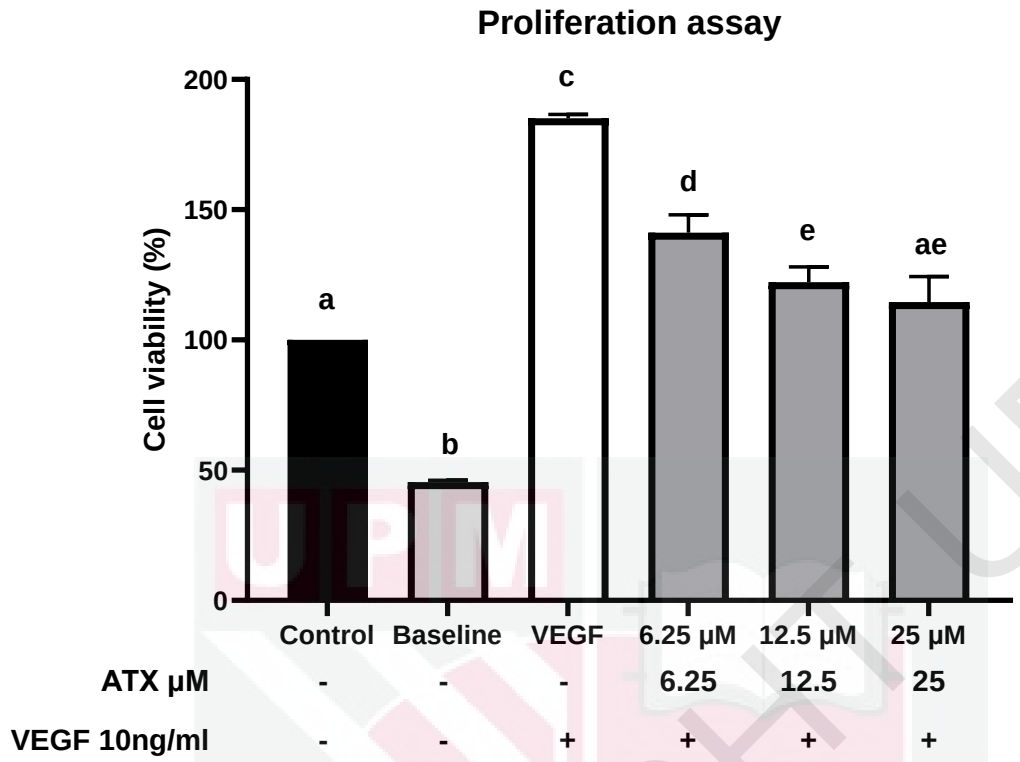


Figure 4.3: Anti-proliferative effect of ATX in HUVECs after 72 hours of exposure using MTT at concentrations ranging from 6.25- 25 µM. The treatment of HUVECs with ATX induced by VEGF resulted in a significant decrease in cell viability (%) with increasing concentration ($P < 0.05$). In terms of cell viability (%), all treatment groups consistently maintain values higher than both the control and baseline groups, yet lower than the VEGF group. The data represents as mean ± SD of three independent tests (n=3) in triplicates. Statistical analysis was performed using one-way ANOVA followed by post-hoc test Tukey and compared against each other. Different letters indicate statistically significant differences ($P<0.05$). The control group shows significant differences in cell viability when compared to the baseline, VEGF, 6.25 µM, and 12.5 µM group.

4.2 Scratch wound healing assay

Cell migration is essential for endothelial cells to form blood vessels during angiogenesis, and thus for cancer growth and metastasis. The anti-migratory effect of ATX on HUVECs cells was assessed utilising the scratch wound healing assay. Inhibiting migration is an excellent approach to govern the movement of cells during the angiogenesis process, which might impede the growth of tumours. In this experiment, further cell movement across opposing sides is associated with a higher percentage of migration rate.

Within the context of the study, there is a statistically significance decrease in migration rates as the concentration of ATX increases ($P<0.05$). Migration rates were quantified, resulting in means of 32 ± 2.45%, 36 ± 2.2%, and 17 ± 0.7% for the control group, VEGF group, and Suramin group, respectively. Moreover, the ATX treatment groups with 6.25 μM, 12.5 μM, and 25 μM exhibited migration rates of 26 ± 0.6%, 23 ± 1.9%, and 20 ± 1.9%, respectively.

The migration and invasion of endothelial cells are essential early event in angiogenesis initiation. VEGF, a potent angiogenic factor, induces a cascade of proteins and enzymes crucial to the breakdown process, establishing a pro-degradative environment that facilitates endothelial cell migration and sprouting (Takahashi H & Shibuya, 2005). Although there is a lack of significance difference between control group and VEGF-only group, **Figure 4.4** reveals that following VEGF stimulation, the VEGF-only group demonstrated the highest migration rate (36%), surpassing that of the control group of 32%, which was treated merely with media. To put it simply, VEGF

encourages HUVEC migration across the scratched regions. Also, the fact that ATX reduced migration rates when simultaneously induced by VEGF showed that ATX inhibits cell migration even in the presence of a growth factor, VEGF. This was further reinforced by the statistically significant difference between VEGF-only group and ATX of various concentration ($P<0.05$).

On the other hand, HUVECs that were simultaneously treated with Suramin and VEGF demonstrated the lowest migration rate, at 17%. As the positive control in this experiment, suramin is a well-known angiogenic inhibitor that was first identified for its anti-trypanosomal properties but has subsequently demonstrated strong anti-angiogenic and VEGF inhibitory capabilities. Thus, it is anticipated that the suramin group will migrate at the lowest rate compared to the control and VEGF groups. Furthermore, the results indicate a statistically significant reduction in the migration rate induced by suramin compared to both the control and VEGF groups, without the induction of suramin ($P < 0.05$). In summary, this present study underscores a concentration-dependent inhibitory effect of ATX on migration, evident over an 8-hour period.

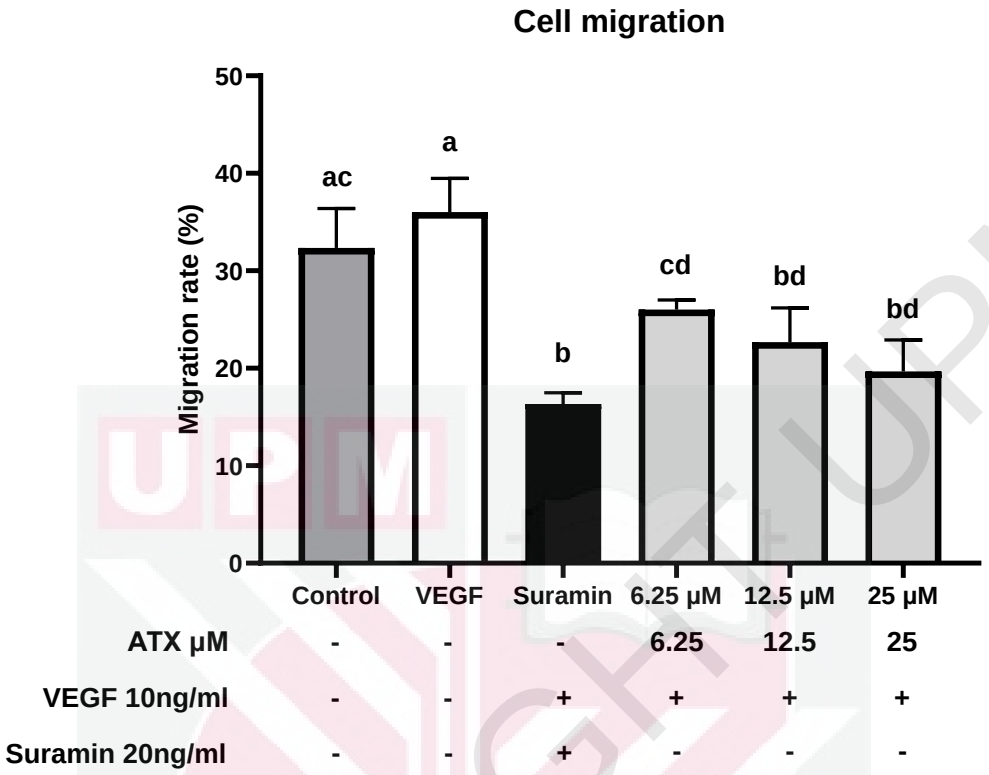


Figure 4.4: Anti-migratory effects of ATX in HUVECs induced by VEGF. HUVECs was co-cultured with 6.25 µM-25 µM of ATX and 10ng/ml of VEGF for 8 h. The migration inhibition is presented as migration rate (%). The value expressed are the mean ± SD of three independent experiments (n=3) and compared against each other. Different letters indicate statistically significant differences ($P<0.05$).

Visual evidence further supports the data pertaining to the rate of migration, based on the representative migration images presented in **figure 4.5.** The width of the gap at 0 hours represents as an indicator to analyse the initial extent of cell migration, gradually closing the gap by 8 hours. The migration rate was then computed by measuring the gap's width after 8-hours. The gap of VEGF group closed earlier than the control group, as the presence of VEGF promotes cell migration. In contrast, the gap of suramin group closed later than both the control and VEGF groups, given its potent anti-angiogenic properties that inhibit cell migration.

Based on the presented data, the gaps of all ATX treatment groups closed later than both the control and VEGF groups but earlier than the Suramin group. Furthermore, the closure of the gap aligned with the concentrations of ATX, with higher concentrations closing later than lower concentrations. Although the variations in gaps between each group may not be readily apparent, quantitative measurements were carried out to ensure a more precise evaluation of the outcomes. From the representative images, these findings verify that the quantitative data that is presented and the visual evidence are consistent. In summary, ATX demonstrates a notable anti-migration effect, thereby serving as a robust indicator of its anti-angiogenic efficacy in VEGF-induced HUVECs.

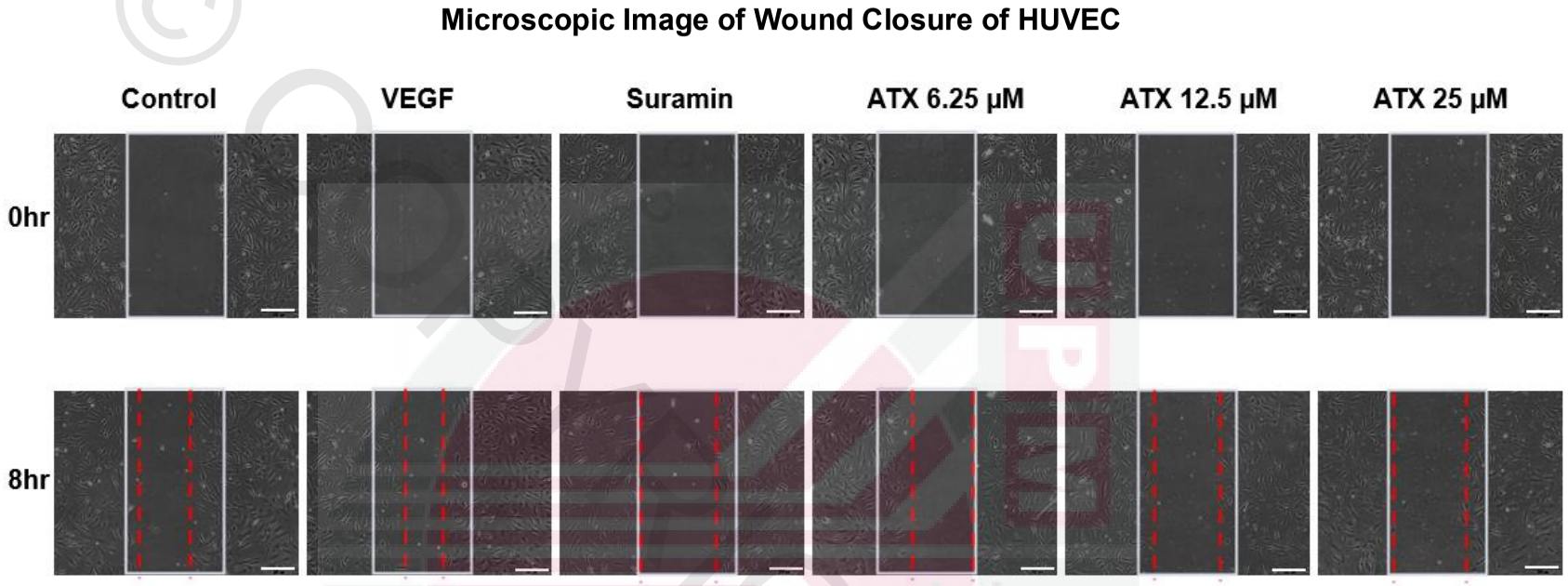


Figure 4.14: Microscopic images of Wound Closure of HUVECs induced by VEGF. Cells were scratched and treated in accordance with their concentrations. The white rectangle represents the initial scratch wound while the red lines represent the scratch wound at 8 h. Control: Media, VEGF (negative control): 10ng/ml of VEGF, Suramin (positive control): 20ng/ml of Suramin, 6.25µM of ATX, 12.5µM of ATX and 25µM of ATX. All group were induced by VEGF except for control group. Images were captured at 10x magnification at 0 h and 8 h. Scale bar: 200 µm

4.3 Tube formation assay

Given that the development of tubular structures by capillary endothelial cells implies vascular maturation during angiogenesis, the tube formation assay was conducted to examine the impact of a 6-hour exposure to ATX on VEGF-induced capillary-like structure formation.

The outcomes illustrated in **figure 4.6** are consistent with assertions, demonstrating that the tube length of the VEGF group, 8424 ± 735 µm, induced exclusively by VEGF, surpasses that of the control group, 6654 ± 715 µm, treated with medium alone. This is probable due to the stimulation of VEGFR2 by VEGF sets off mechanisms that lead to neovascularization, including endothelial cell migration, proliferation, survival, and tube formation (Manickam et al., 2011). Therefore, when compared to the control group lacking VEGF, the induction of VEGF encourages the growth of capillary-like tubular structures, which in turn explains an apparent increase in the tube lengths of cells produced by VEGF.

As previously mentioned in the migration assay, suramin was employed as an anti-angiogenic agent in this particular study owing to its proven ability to disrupt VEGF. Derived from the results, despite both groups were stimulated by VEGF, the tube lengths of the suramin group, which included suramin, exhibited a statistically significant difference ($P < 0.05$) when compared to the VEGF group, measuring 4049 ± 267 µm and 8424 ± 735 µm, respectively. This implies that suramin successfully suppresses VEGF activity, thereby affirming the reliability of the positive control.

Moreover, at the concentrations of, 6.25 μM, 12.5 μM, and 25 μM of ATX, the tube lengths were recorded to be 5266 ± 915 μm, 4832 ± 652 μm and 4704 ± 344 μm, respectively. In accordance with the results ATX significantly reduced the tube lengths in concentration-dependent manner compared to VEGF group ($P < 0.05$) in the presence of VEGF. This indicates that, despite being stimulated by VEGF, a definite pattern appeared, suggesting that lower tube lengths of HUVECs were linked to higher ATX concentrations. It is worth mentioning that the tube length observed at the highest concentration of ATX (25 μM) is comparatively lower than that of the positive control consisting of Suramin.

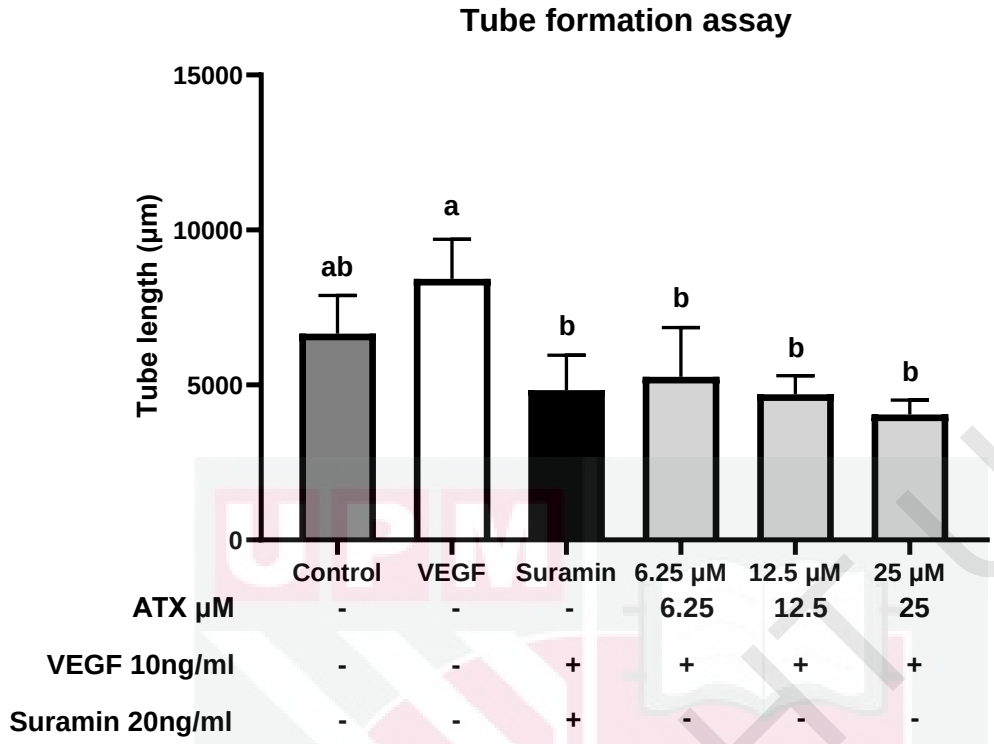


Figure 4.6: Anti-tube formation effects of ATX on HUVECs induced by VEGF. HUVECs was co-cultured with 6.25 µM-25 µM of ATX and 10ng/ml of VEGF for 6 h. The inhibition of tube formation is presented as tube length µm. The value expressed are the mean ± SD of three independent experiments and compared against each other. Different letters indicate statistically significant differences ($P<0.05$).

Furthermore, the morphology and structures of tube formation can be utilised for analysing its characteristics. Following seeding HUVECs on Matrigel, capillary-like tubular structures that connected to each other was formed, giving rise to the gradual development of a mesh-like structure on the Matrigel. Healthy tube formation appears as complete tube and organized web of the capillary-like structures. On the contrary, inhibited tube formation characterised as incomplete tubes, scattered cells, isolated clumps, and a failure of cells to establish the necessary connections with each other, resulting in a disrupted and fragmented pattern (Xie et al., 2016).

As displayed in the **figure 4.7**, both the control and VEGF groups exhibit a significant number of complete loops in tube formation, collectively with an organized web-like structure. This discovery suggests that the tube formation in these groups appears healthy and adopts to a normal pattern, with the main variable that distinguishes the two groups is a variance in tube length.

By contrast, the suramin group exhibits characteristics suggestive of inhibited tube development, as do groups treated with 6.25 µM, 12.5 µM, and 25 µM concentrations of ATX. Such characteristics include incomplete tubes, the presence of scattered cells, formation of isolated clumps, and an inability to establish cell-cell contacts. Based on the inhibited and disrupted nature of the observed tube structures, this observation suggests that the aforementioned groups fall into a category of abnormal tube formation, and the primary distinction between them is the variation in tube length.

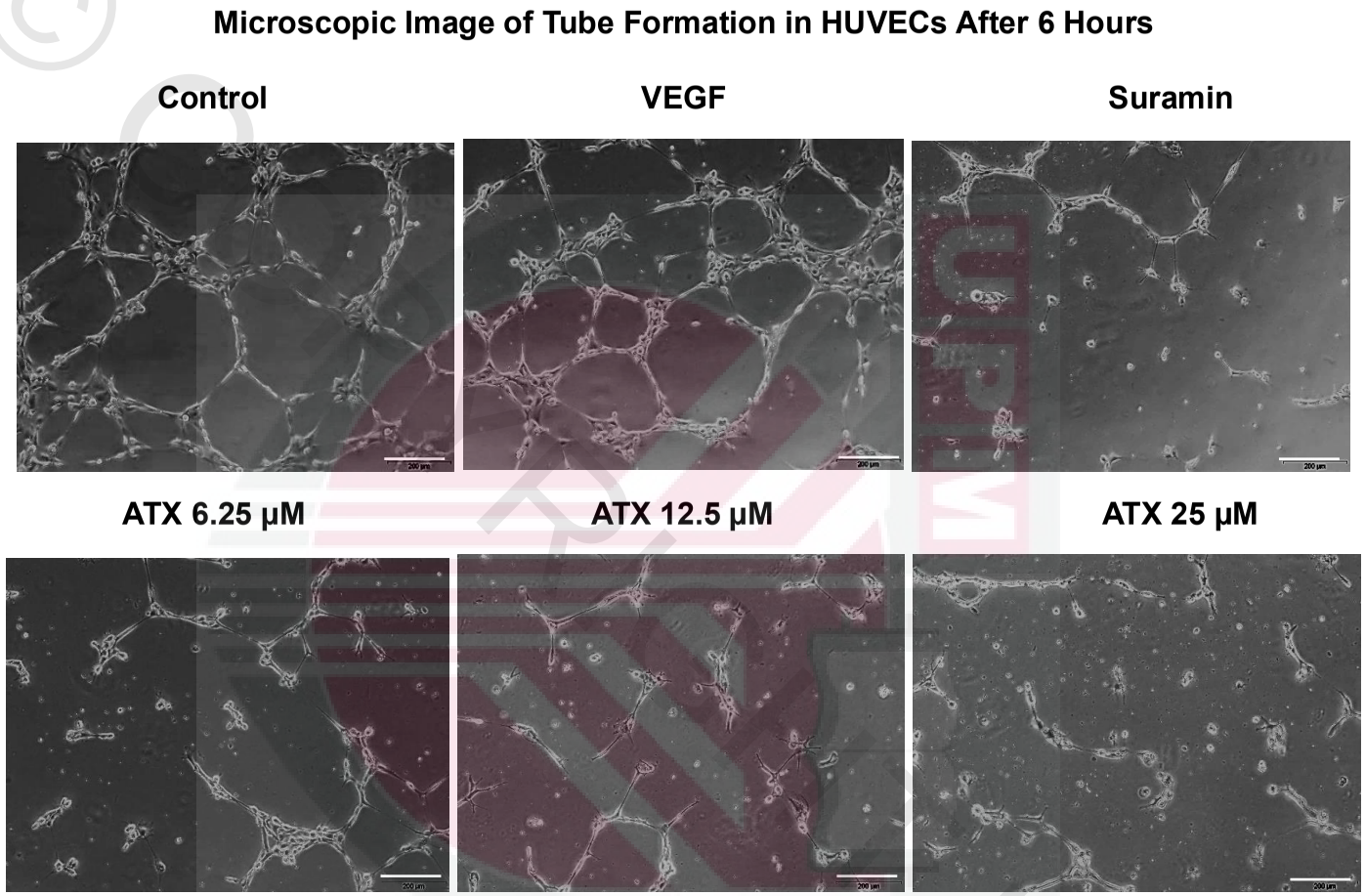


Figure 4.16: Microscopic Image of Tube Formation in HUVECs After 6 Hours induced by VEGF. Cells were seeded in Matrigel pre-coated wells and treated in accordance with their concentrations in 6 h. Control: Media, VEGF (negative control): 10ng/ml of VEGF, Suramin (positive control): 20ng/ml of Suramin, 6.25µM of ATX, 12.5µM of ATX and 25µM of ATX. All group were induced by VEGF except for control group. Images were captured at 10x magnification at 6 h. Scale bar: 200 µm

4.4 Network Pharmacology of ATX against angiogenesis

4.4.1 Target Acquisition of ATX and Angiogenesis Related Genes

To ascertain the potential correlation between angiogenesis and ATX, an extensive investigation was conducted by querying relevant targets across multiple databases using the keyword “Angiogenesis.” As of May 2023, the GeneCards database yielded 7242 targets, the DisGeNET database produced 822 targets, and the NCBI Gene database identified 2141 targets associated with angiogenesis. Concurrently, the SuperPred database and SwissTargetPrediction database identified 288 and 111 targets related to ATX, respectively, as of May 2023. Following the removal of duplicate values and standardization using the UniProt database, a consolidated dataset was obtained, comprising 7357 targets associated with angiogenesis and 385 targets linked to ATX.

4.4.2 Intersection Targets between Angiogenesis and ATX

To delineate the overlap among identified targets, the dataset encompassing 7357 angiogenesis-related targets and 385 ATX-related targets, obtained through prior analyses, was inputted into the online tool InteractiVenn. The resultant analysis revealed 171 intersecting targets, as depicted in **Figure 4.8**. These shared targets represent potential genes crucial to the involvement of ATX in the intricate processes of angiogenesis. The list of intersection target genes of ATX and angiogenesis is displayed in **Appendix B (Table 1)**.

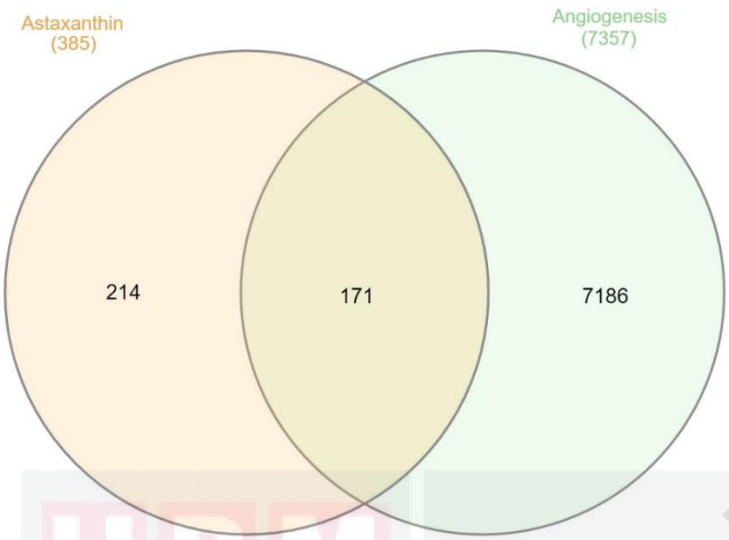


Figure 4.8: Intersection of 7357 angiogenesis-related targets and 385 ATX related targets illustrated as venn diagram.

4.4.3 Compound-Target Network Construction of ATX and Angiogenesis

To visualize the interactive relationships between ATX and its potential targets implicated in the regulation of angiogenesis, a network representation was constructed using the Cytoscape software, presented herein as the compound-target network **(Figure 4.9)**. This network serves to elucidate the interconnectedness between ATX and its associated targets within the regulatory framework of angiogenesis.

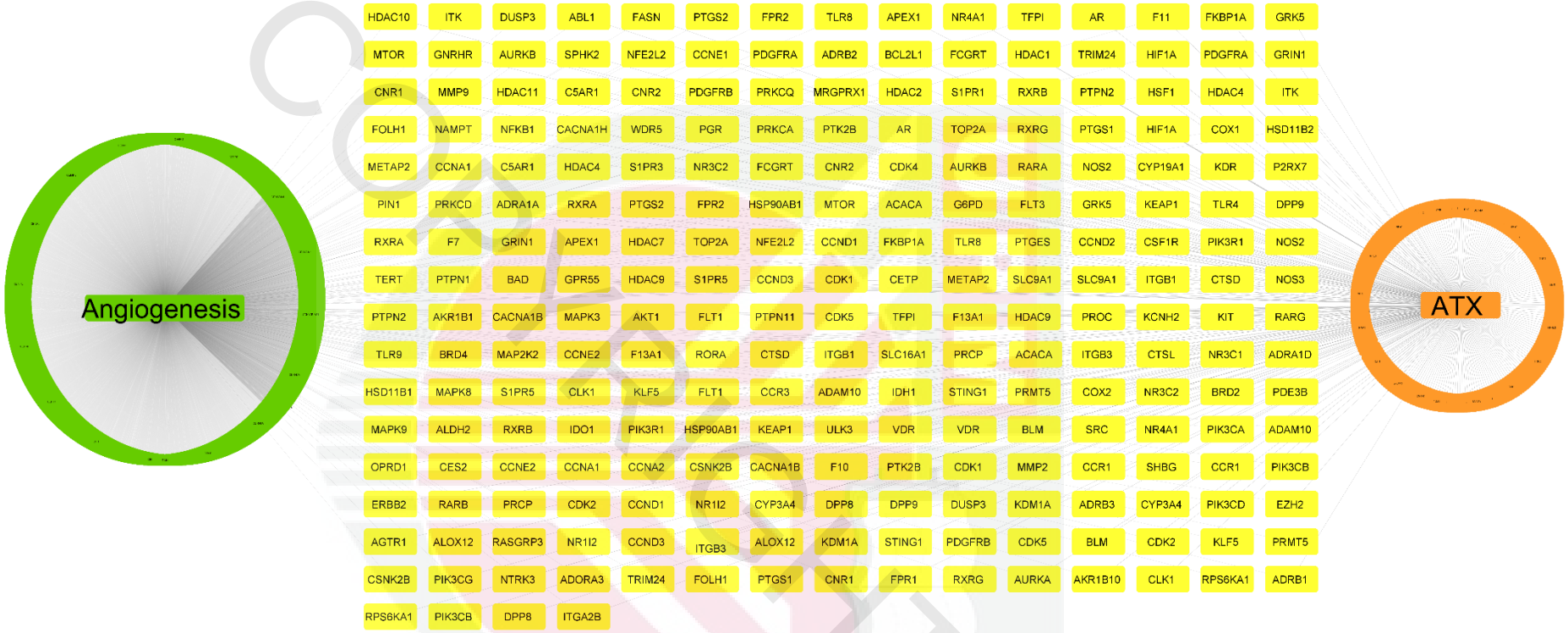


Figure 4.9: The compound-target network of 171 intersecting targets obtained from overlapping 7357 angiogenesis-related targets and 385 ATX-related targets. This network was built by using Cytoscape.

4.4.4 PPI Network Analysis of ATX and Angiogenesis

To systematically predict genes associated with disease based on interactions among genes with analogous functionalities, a Protein-Protein Interaction (PPI) network was constructed utilizing the STRING database. This network was established using the 171 intersection targets previously identified in the context of angiogenesis and ATX. Subsequent to the removal of isolated nodes and edges, the comprehensive PPI network analysis revealed 125 nodes and 440 edges. In this network, proteins are denoted as nodes, and their interactions are represented by edges. Employing the network analyzer plugin in Cytoscape software, each node was differentiated by its degree value, as depicted in **Figure 4.10**. The intensity of the node's coloration corresponds to its degree value, with darker shades indicating higher values. The average degree indicates the number of connections between nodes in a network (Khan, G. F et al., 2019). Notably, 49 nodes exhibited a degree value surpassing the average of 7.04. The average degree value of 7.04 indicates that each node in the network has approximately 7 connections and the fact that 49 nodes have a degree value surpassing 7.04 suggests that these nodes are more connected than the average node in the network. This observation suggests that targets associated with strongly linked nodes may play a pivotal role in angiogenesis regulation.

A further analysis utilizing the CytoHubba plugin in the Cytoscape software, incorporating algorithms such as maximal clique centrality (MCC), maximum neighborhood component (MNC), and degree, was conducted to pinpoint the top 10 hub genes within the established Protein-Protein Interaction (PPI)

network. The results, depicted in **Figure 4.11**, showcase the selected hub genes believed to be potential candidates for ATX in the regulation of angiogenesis. Notably, the intersection of the three algorithms identified four hub genes, namely RXR, CCND1, CDK1, and HDAC1, as illustrated in **Figure 4.12**. These identified hub genes represent key nodes within the network and are hypothesized to play pivotal roles in the regulatory control exerted by ATX on angiogenesis. This observation provides valuable insights into the importance of these pivotal targets in angiogenic processes and could establish a basis for future research inquiries. The significance ascribed to these key targets is strengthened by prior studies delving into their intricate association with angiogenesis (Daniel et al., 2014; Gao et al., 2019; Kim M. S et al., 2001; Salajegheh, 2016), thereby reinforcing the justification for their prominence as top hub genes in the network.

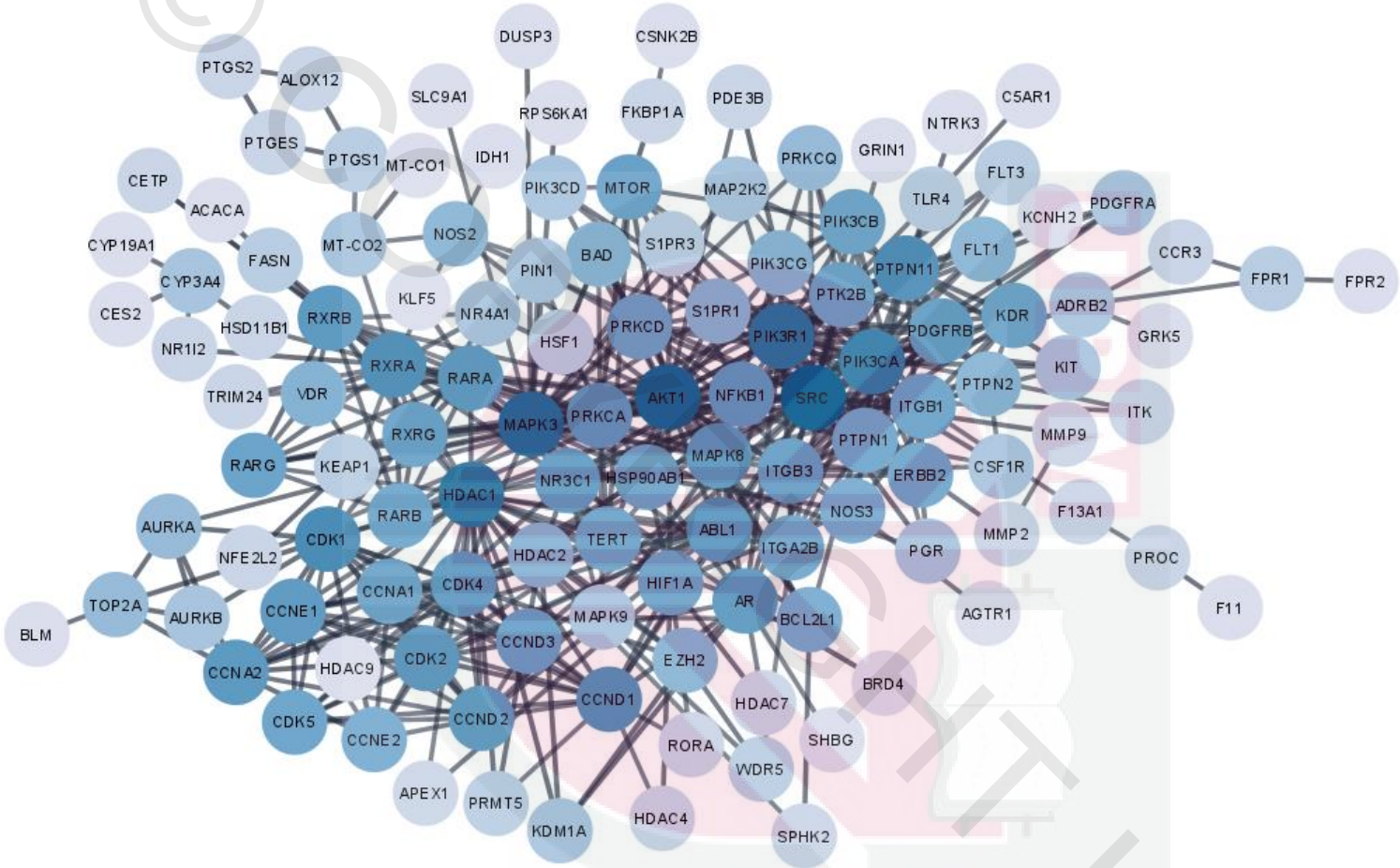


Figure 4.10: Protein-protein interaction networks are built using the STRING database to visualise how nodes were connected to one another by edges. According to this visualisation, one node may have only one or multiple connections to other nodes. The shade of the circle adjusted in proportion to the degree value given that a darker shade represents a greater degree

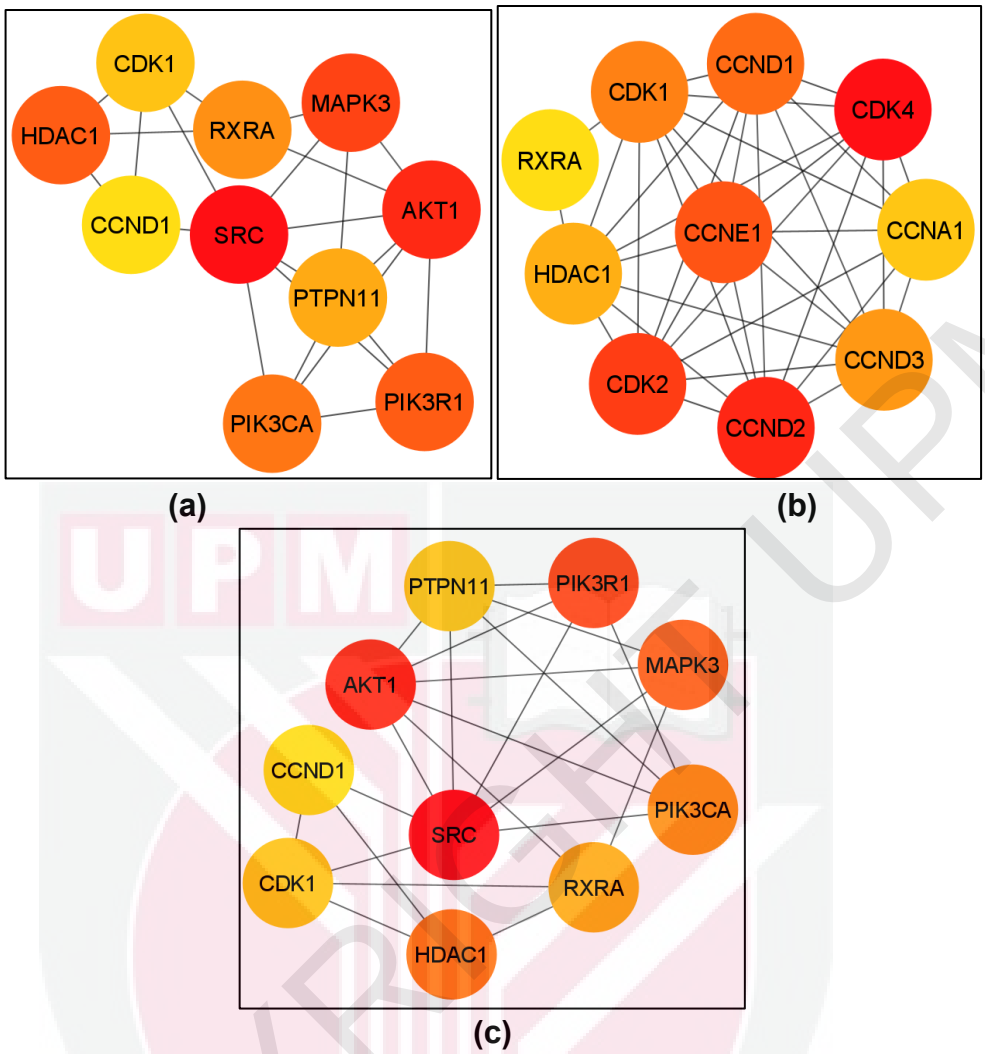


Figure 4.11: The top 10 hub gene networks of ATX against angiogenesis were identified by integrating three algorithms a) degree of nodes, **b)** maximal clique centrality (MCC), and **c)** maximum neighborhood component (MNC). The genes with the highest scores are deemed to be the most significant hub genes and are highlighted in dark red. Genes with lower values, on the other hand, were deemed to be less significant and are highlighted in a light-yellow colour

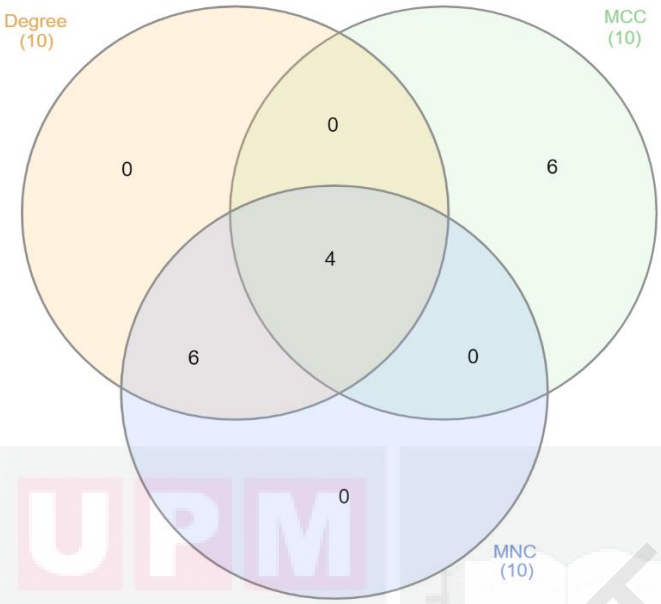


Figure 4.12: Intersection of the algorithms (Degree of nodes, MCC and MNC) illustrated as venn diagram in which revealed that there are 4 targets intersection.

4.1.5 Enrichment Analysis of GO and KEGG

To delve deeper into the diverse mechanisms underlying the anti-angiogenic properties of ATX, a comprehensive Gene Ontology (GO) enrichment analysis was conducted, encompassing biological processes, molecular functions, and cellular components. Additionally, a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed on the 171 predicted targets. These analytical procedures were executed utilizing the DAVID bioinformatics resources. The outcomes revealed the top 10 enriched terms for biological processes, molecular functions, and cellular components. **Figure 4.13** presents detailed insights into the results of the GO enrichment analysis

The predominant biological processes associated with the anti-angiogenic properties of ATX, as revealed by Gene Ontology (GO) enrichment analysis, included the following top five terms: (1) inflammatory response, (2) protein autophosphorylation, (3) protein phosphorylation, (4) positive regulation of cell proliferation, and (5) positive regulation of gene expression. In terms of molecular function, the top five enriched terms encompassed (1) protein serine/threonine/tyrosine kinase activity, (2) RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding, (3) protein binding, (4) enzyme binding, and (5) protein kinase activity. Regarding cellular components, the top five terms comprised (1) cytoplasm, (2) nucleoplasm, (3) cyclin-dependent protein kinase holoenzyme complex, (4) receptor complex, and (5) plasma membrane. Notably, the analysis underscores a significant association of these targets with processes regulating protein kinase activity.

Furthermore, KEGG pathway enrichment analysis was performed to categorize the principal effects associated with angiogenesis, and 171 common targets were scrutinized. The top 20 highly enriched pathways were selected based on P-values, as depicted in **Figure 4.14**. Noteworthy among these pathways are those implicated in cancer, such as pathways in cancer, the VEGF signaling pathway, chemical carcinogenesis-receptor activation, and viral carcinogenesis. Additionally, pathways contributing to neutrophil extracellular trap formation, thyroid hormone signaling, non-small cell lung cancer, and EGFR tyrosine kinase inhibition emerged prominently. These pathways are deemed to play a pivotal role in the intricate process of angiogenesis.

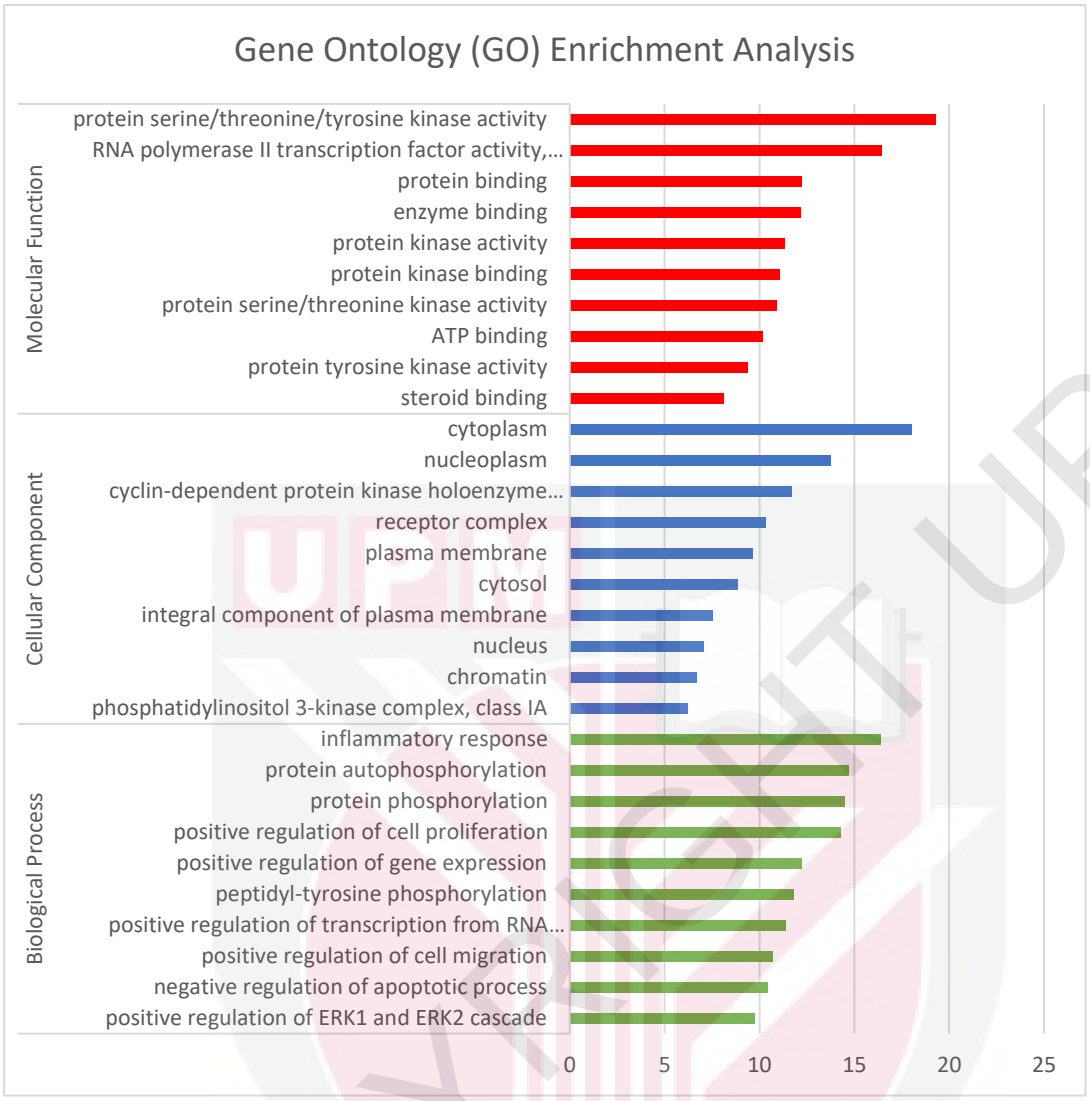


Figure 4.13: Gene ontology (GO) enrichment analysis. The top 10 terms that pertain to the target genes are represented in the bar chart as the most significantly enriched GO terms (log10 (p-value)) in biological processes, cellular elements, and molecular function.

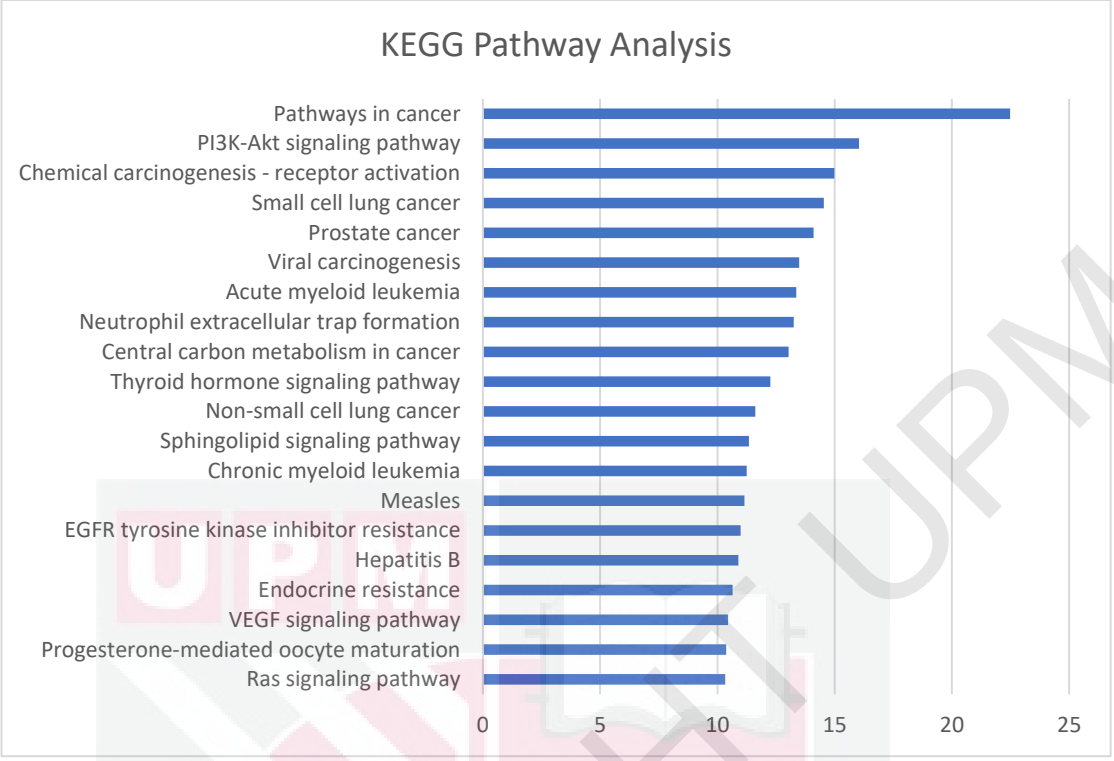


Figure 4.14: KEGG pathway enrichment analysis. The bar chart visualizes the top 20 enriched KEGG pathways of ATX against angiogenesis.

After conducting the KEGG enrichment pathway analysis, it was determined that the cancer pathway emerged as the top pathway. This signifies that the cancer pathway is a pivotal and potentially significant route through which ATX exerts its influence against angiogenesis. Armed with this insight, targeted intervention strategies aimed at disrupting angiogenesis through the cancer pathway can be devised, focusing on the manipulation of specific genes within the pathway cascade. To further elucidate the network of ATX-associated nodes within the cancer pathway, the KEGG Mapper was employed, and the identified cancer-associated nodes of ATX are illustrated in **Figure 4.15**.

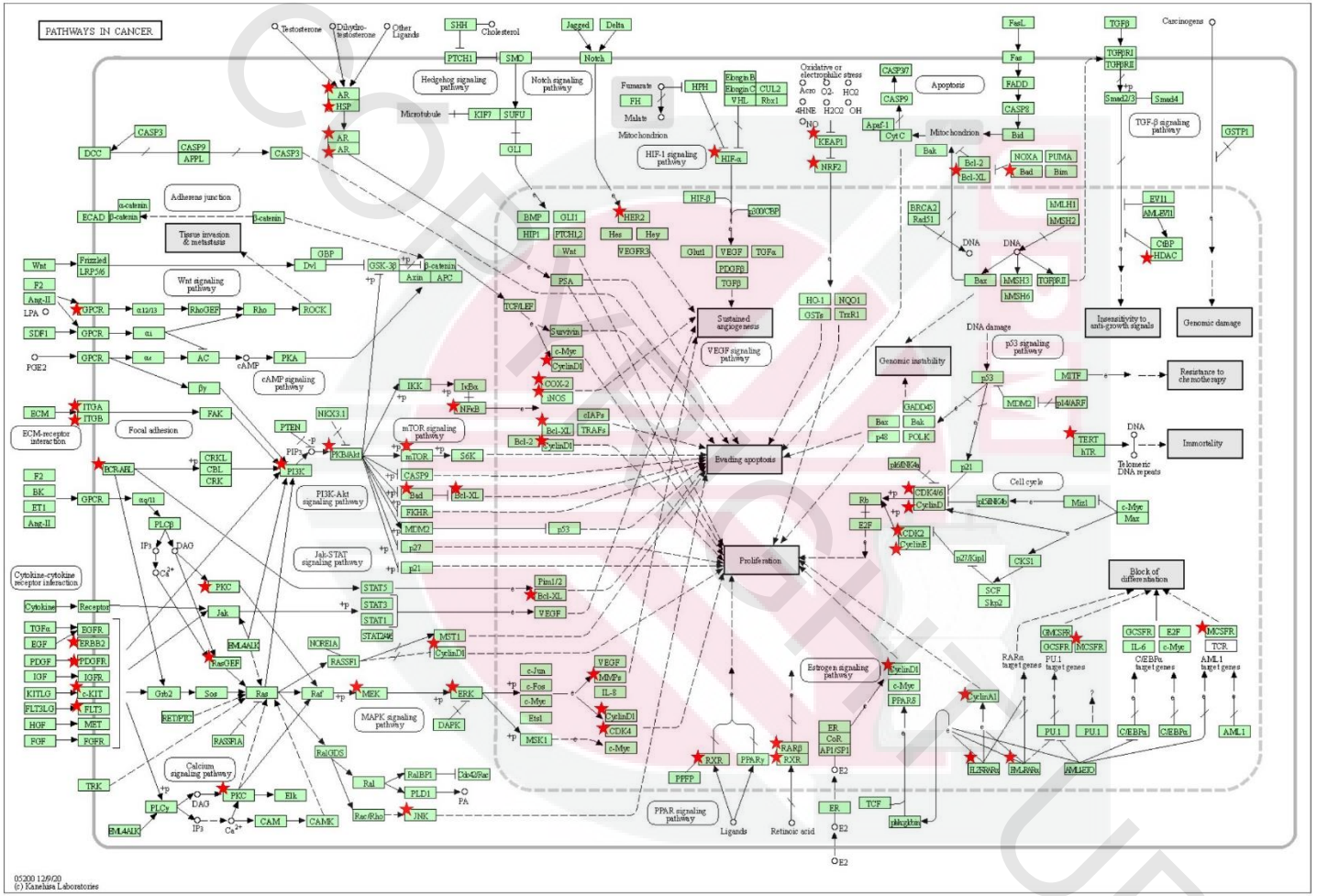


Figure 4.15: Potential targets and mechanism of ATX against angiogenesis. Red stars represent targeted genes involved in the cancer pathway.

4.5 Molecular Docking of ATX against Angiogenesis

To ascertain the potential significance of the previously identified targets (*RXRA, CCND1, CDK1, and HDAC1*) in the angiogenesis process, molecular docking was employed to predict the interaction models between ATX and these proteins at the atomic level. Additionally, considering the outcomes of GO and KEGG pathway enrichment analyses, which indicated a correlation between the anti-angiogenic effect of ATX and protein kinase activity, three additional key tyrosine kinase receptors—VEGFR2, FGFR1, and TIE2—were incorporated into the docking analysis.

The inclusion of the three other tyrosine kinase receptors in this molecular docking study is crucial for an in-depth evaluation, as they are recognised to be significant players in angiogenesis. This assertion is substantiated by, the main angiogenic growth factor that influences angiogenesis is vascular endothelial growth factor (VEGF), which is mediated by receptor tyrosine kinase VEGF receptors (VEGFRs). A variety of VEGFs (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) bind to VEGF receptors comprising VEGFR1, VEGFR2, and VEGFR3. Amidst these, the VEGFA/VEGFR-2 signalling pathway emerges to be preeminent in orchestrating angiogenesis-related cellular responses. In ECs, phosphorylated VEGFR2 triggers many cellular responses, such as a potent mitogenic signal and a survival signal, and activates downstream signalling pathways associated with angiogenesis (Abhinand et al., 2016). Therefore, it is nearly impractical to disregard the inclusion of VEGFR2 in the molecular docking analysis that is intended to clarify interaction between ATX and VEGFR2 in this research.

Moreover, fibroblast growth factor 2 (FGF2), commonly known as basic FGF, is a growth factor expressed in vascular endothelial cells and other tissues as well as cell types. Four receptor tyrosine kinases (FGFR1-4) are in interaction with FGF2 (Okada-Ban et al., 2000). FGFR1, on the other hand, is primarily expressed in endothelial cells (Esser et al., 2015), and aberrant angiogenesis is brought about by overexpressing dominant-negative FGFR1 (Lee S. H et al., 2000). Many tumour forms, including breast cancer, non-small cell lung cancer, ovarian cancer, urothelial carcinoma, and hepatocellular carcinoma, typically exhibit FGFR1 amplification or overexpression (Lee C et al., 2023). Thus, integrating FGFR1, a key aspect of angiogenesis, as part of our molecular docking study may offer insightful information regarding its interaction with angiogenesis.

Furthermore, the morphogenesis, homeostasis, and vascular remodelling during vascular inflammation, tumour angiogenesis, and metastasis are all critically impacted by the Angiopoietin (Angpt)-Tie receptor (TIE1 and TIE2) signalling (Augustin et al., 2009). The four members of the angiopoietin (Ang) family—Ang1, Ang2, Ang3, and Ang4—all bind to the receptor tyrosine kinase Tie2. In endothelial cells (ECs), these ligands behave in opposition to one another: whilst Ang2 and Ang3 act as context-dependent antagonists, Ang1 and Ang4 act as agonists and activate Tie2 (Ward & Dumont, 2002). Angiogenesis signals are regulated by Ang1–Tie2 after Ang1 binds to and stimulates Tie2 (Siavashi et al., 2016). Previous research revealing Tie2 expression and phosphorylation in adult tissues during angiogenesis implies a potential role for Tie2 signalling in pathological angiogenesis (Wong et al., 1997). Therefore, it is a prerequisite to include TIE2 in this molecular docking

study since efforts to study TIE2 signalling may yield significant insights into regulating angiogenesis.

Utilising the AutoDock Tools software, molecular docking analyses were conducted, yielding a total of seven docking results documented in **Table 4.1**. All seven outcomes demonstrated binding energy values below -6.0 kcal/mol, indicating a substantial interaction between the ligand and receptors. The results are as follow: VEGFR2; -9.34, FGFR1; -9.14, TIE2; -7.61, *CCND1*; -7.55, *CDK1*; -7.17, *HDAC1*; -6.60, *RXRA*; -6.42 kcal/mol. Notably, VEGFR2 exhibited the lowest binding energy, while *RXRA* displayed the highest among the seven receptors.

Consequently, these findings suggest that all seven receptors, including VEGFR2, represent potential anti-angiogenic targets, indicating the potential control of angiogenesis through the targeted modulation of these receptors. **Figure 4.16 - 4.22** depicts visualizations of the lowest binding energies between each receptor and ATX, generated using Biovia Discovery Studio.

Table 4.1: Compound-target molecular docking binding energy

Compound	Targets	PDB ID	Binding Energy (Kcal/Mol)
ATX	VEGFR2	3VHE	-9.34
	FGFR1	4V05	-9.14
	TIE2	2WQB	-7.61
	CCND1	2W96	-7.55
	CDK1	6GU6	-7.17
	HDAC1	4BKX	-6.60
	RXRA	7B88	-6.42

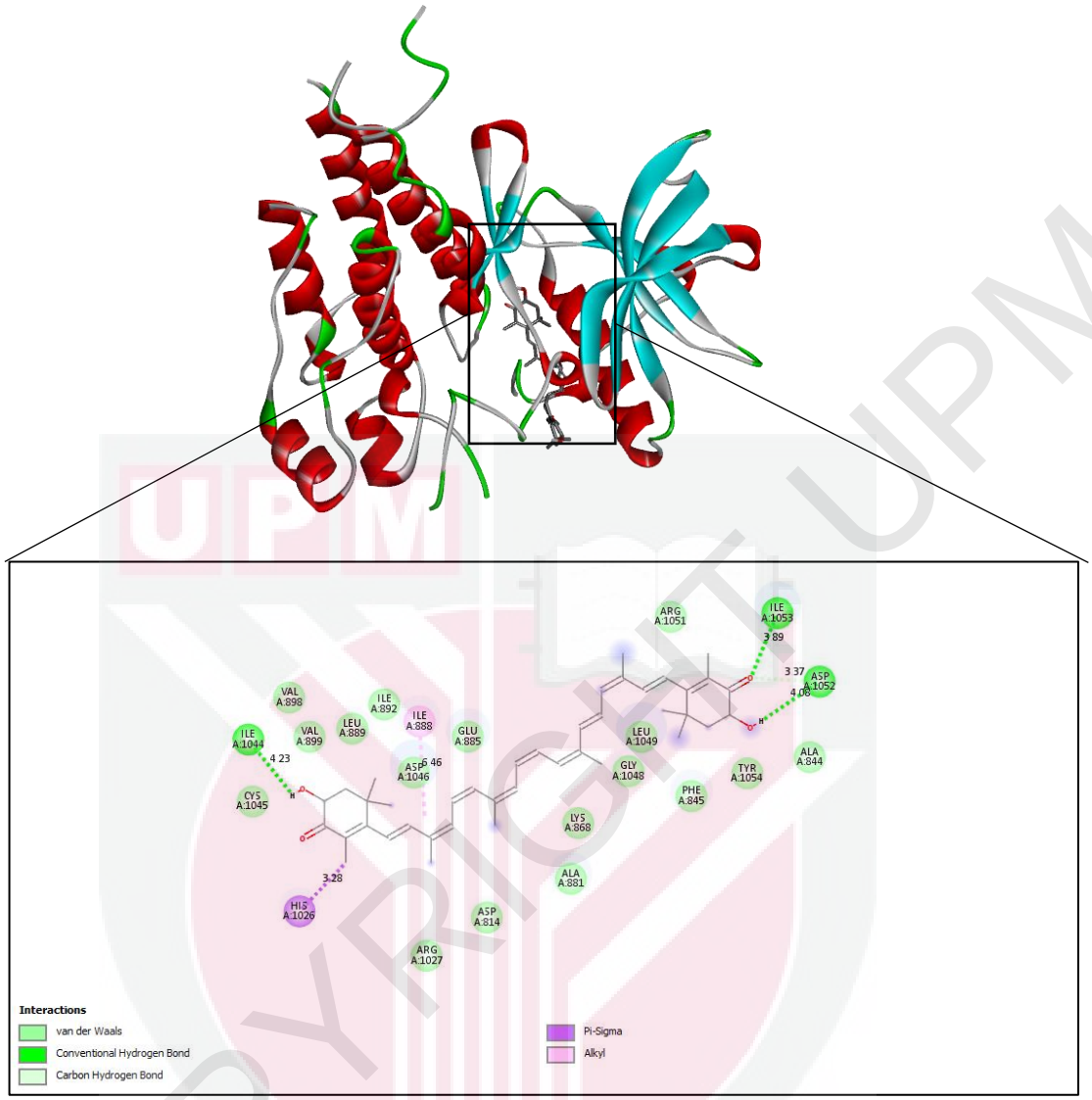


Figure 4.16: The diagram illustrates the prediction of 3D picture and 2D molecular interactions between ATX and the amino acid residues of VEGFR2 visualised by Biovia Discovery Studio. The amino acid residues are depicted by spheres of distinct colours based on interaction types surrounding the ATX structure represented by line model grey colour. Residues were shown as three-letter, type of chain and residue number. The interactions involved include van der Waals, Conventional Hydrogen Bond, Carbon Hydrogen Bond, Pi-Sigma and Alkyl. The distances of key interactions, represented by numbers along dashed lines, are measured in angstroms (Å): ILE A:888 (6.46 Å), ILE A:1044 (4.23 Å), ILE A:1053 (3.89 Å), HIS A:1026 (3.28 Å), and ASP A:1052 (3.37 Å and 4.08 Å).

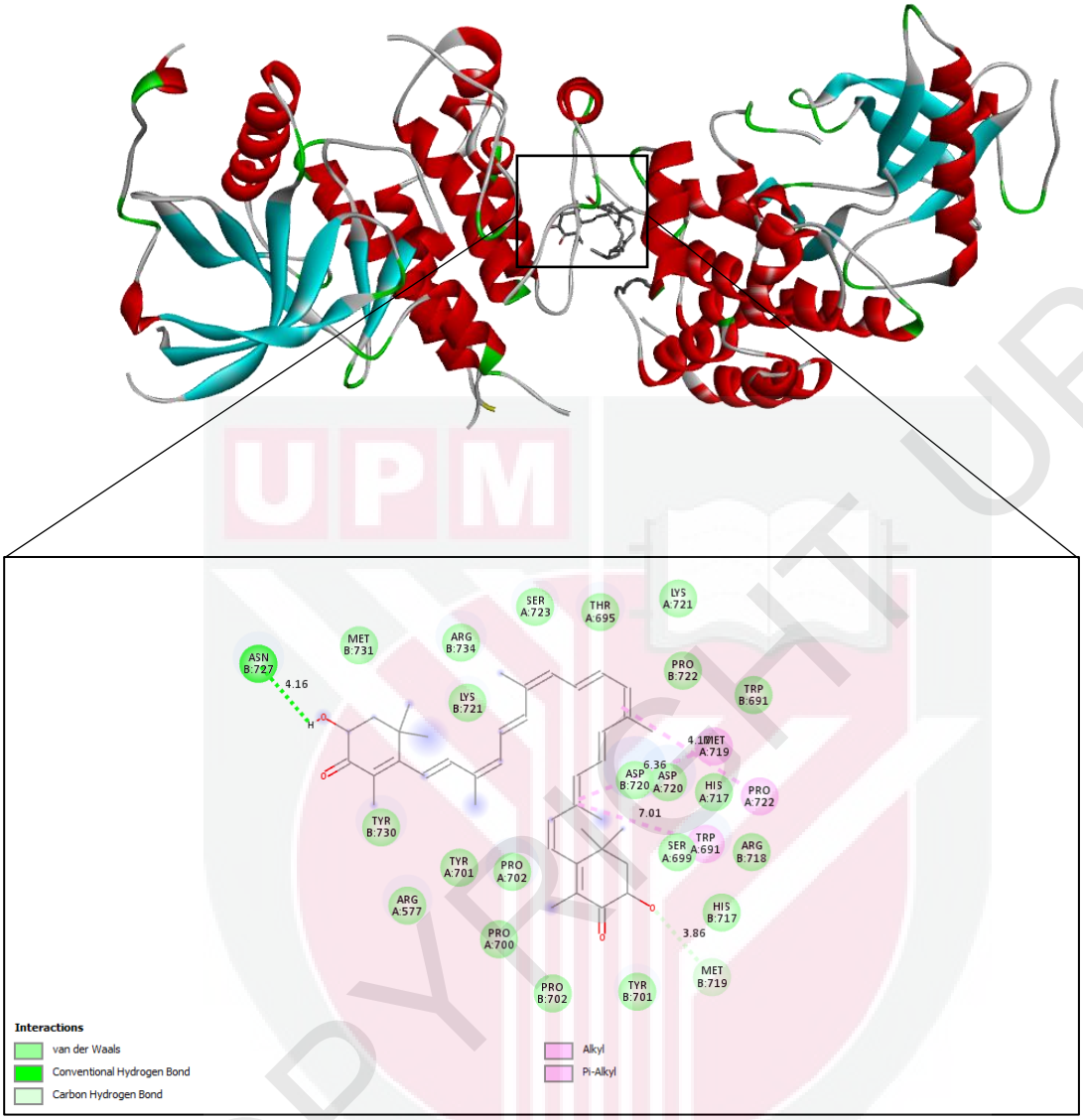


Figure 4.17: The diagram illustrates the prediction of 3D picture and 2D molecular interactions between ATX and the amino acid residues of FGFR1 visualised by Biovia Discovery Studio. The amino acid residues are depicted by spheres of distinct colours based on interaction types surrounding the ATX structure represented by line model grey colour. Residues were shown as three-letter, type of chain and residue number. The interactions involved include van der Waals, Conventional Hydrogen Bond, Carbon Hydrogen Bond, Alkyl and Pi-Alkyl. The distances of key interactions, represented by numbers along dashed lines, are measured in angstroms (Å): ASN B:727 (4.16 Å), MET A:719 (6.36 Å), MET B:719 (3.86 Å), PRO A:722 (4.17 Å), and TRP A:691 (7.01 Å).

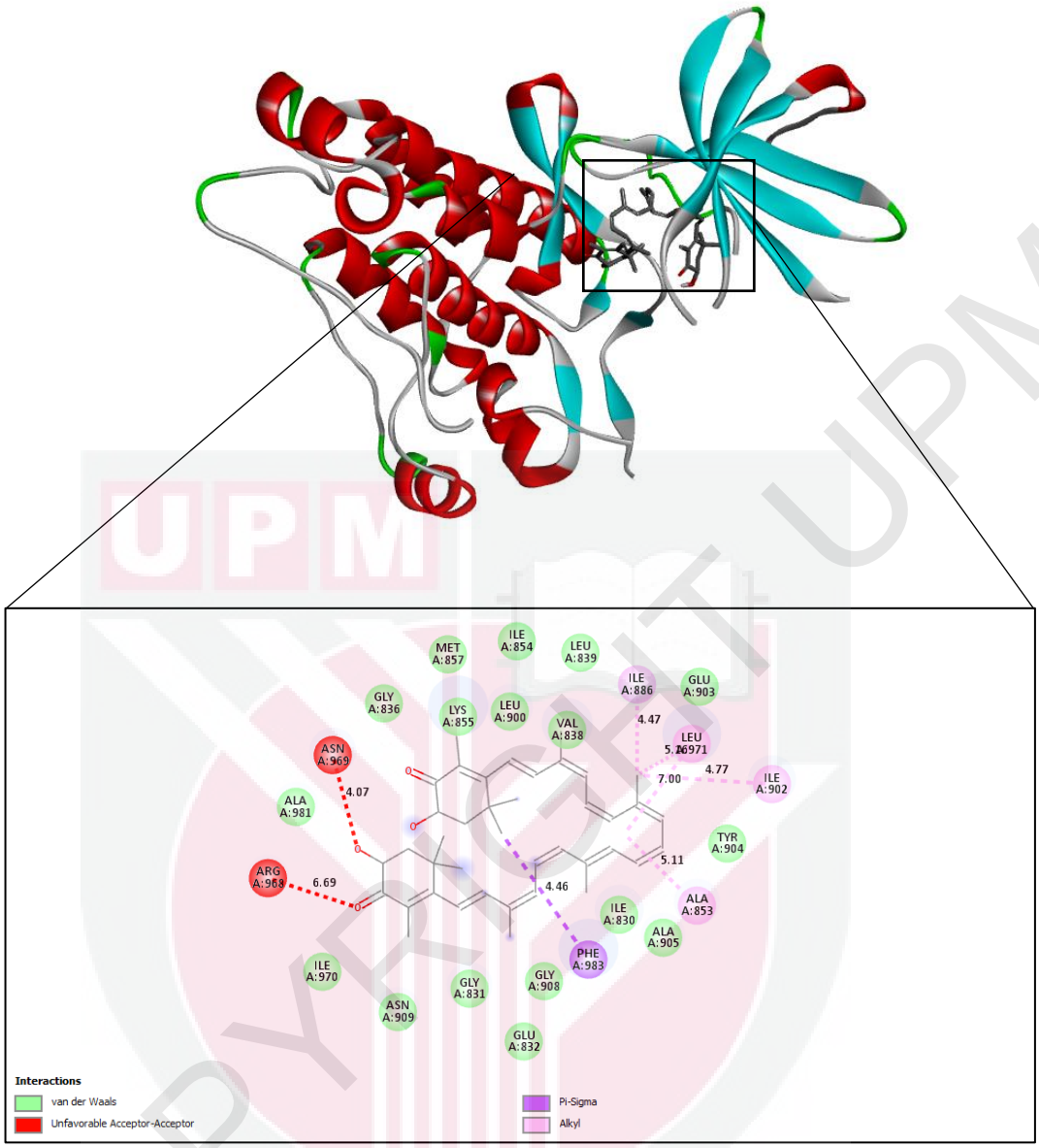


Figure 4.18: The diagram illustrates the prediction of 3D picture and 2D molecular interactions between ATX and the amino acid residues of TIE2 visualised by Biovia Discovery Studio. The amino acid residues are depicted by spheres of distinct colours based on interaction types surrounding the ATX structure represented by line model grey colour. Residues were shown as three-letter, type of chain and residue number. The interactions involved include van der Waals, Unfavorable Acceptor-Acceptor, Pi-Sigma and Alkyl. The distances of key interactions are represented by numbers along dashed lines and are measured in angstroms (Å): ASN A:969 (4.07 Å), ARG A:968 (6.69 Å), PHE A:983 (4.46 Å), ALA A:853 (5.11 Å), ILE A:902 (4.77 Å), ILE A:866 (4.47 Å), and LEU A:971 (5.26 Å and 7.00 Å).

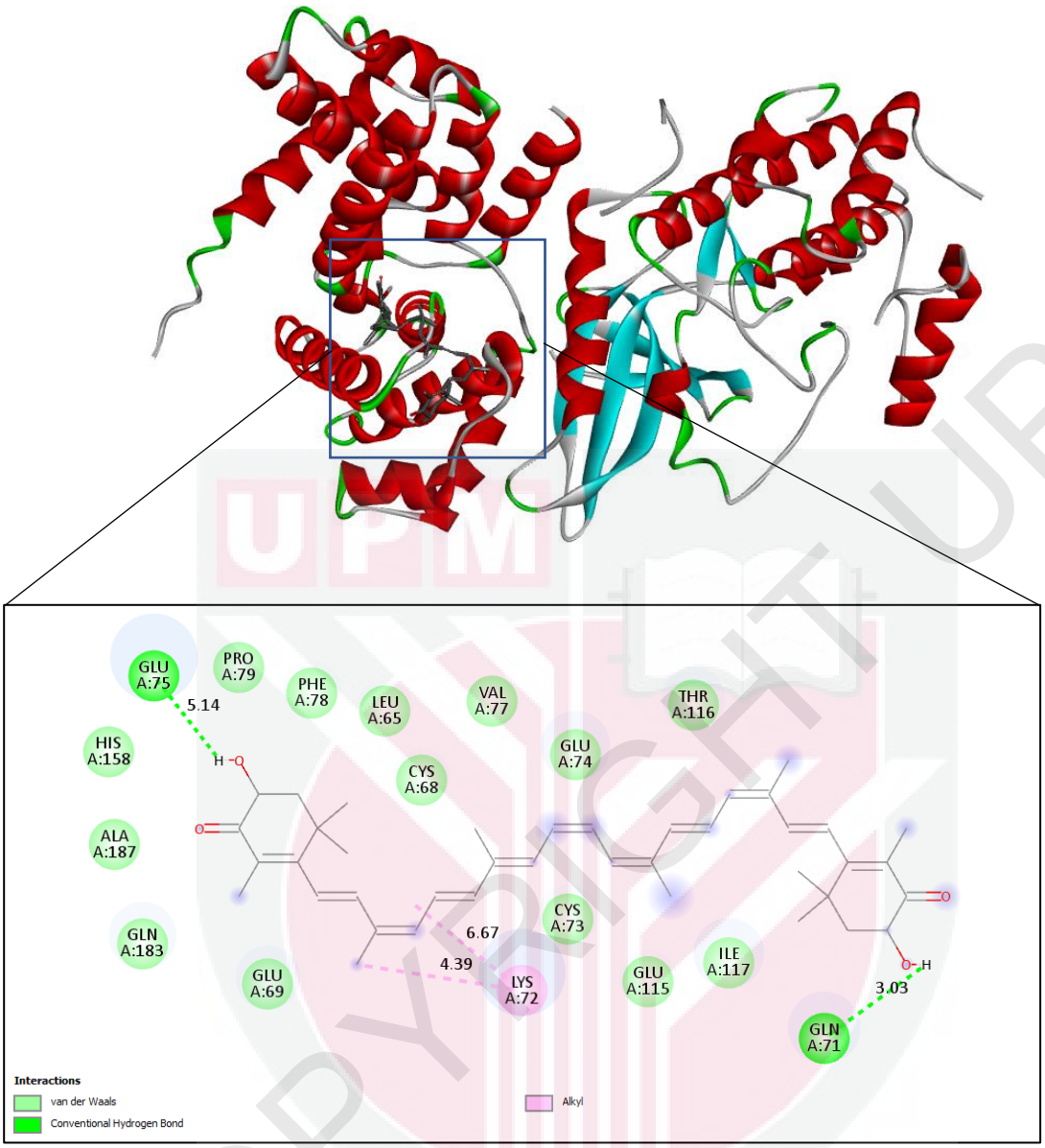


Figure 4.19: The diagram illustrates the prediction of 3D picture and 2D molecular interactions between ATX and the amino acid residues of *CCND1* visualised by Biovia Discovery Studio. The amino acid residues are depicted by spheres of distinct colours based on interaction types surrounding the ATX structure represented by line model grey colour. Residues were shown as three-letter, type of chain and residue number. The interactions involved include van der Waals, Conventional Hydrogen Bond and Alkyl. The distances of key interactions are represented by numbers along dashed lines and are measured in angstroms (Å): GLU A:75 (5.14 Å), GLN A:71 (3.03 Å) and LYS A:72 (4.39 Å and 6.67 Å).

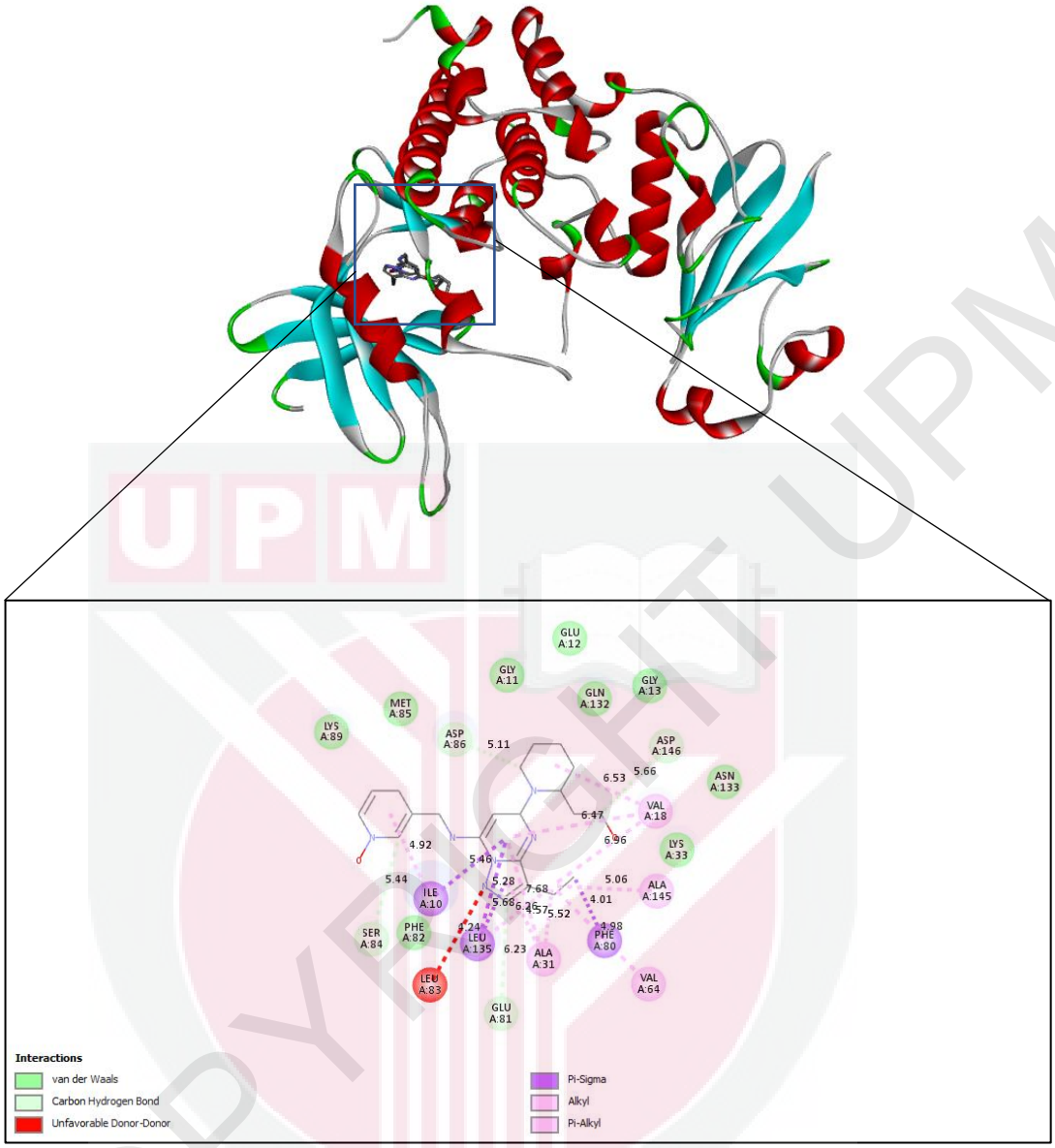


Figure 4.20: The diagram illustrates the prediction of 3D picture and 2D molecular interactions between ATX and the amino acid residues of *CDK1* visualised by Biovia Discovery Studio. The amino acid residues are depicted by spheres of distinct colours based on interaction types surrounding the ATX structure represented by line model grey colour. Residues were shown as three-letter, type of chain and residue number. The interactions involved include van der Waals, Carbon Hydrogen Bond, Unfavorable Donor-Donor, Pi-Sigma, Alkyl and Pi-Alkyl. The distances of key interactions are represented by numbers along dashed lines and are measured in angstroms (Å): ASP A:86 (5.11 Å), ASP A:146 (5.66 Å), VAL A:18 (6.53 Å, 6.47 Å and 6.96 Å), ALA A:145 (5.06 Å), PHE A:80 (4.01 Å), VAL A:64 (4.98 Å), ALA A:31 (5.52 Å, 7.68 Å and 6.26 Å), GLU A:81 (6.23 Å), LEU A:83 (4.24), ILE A:10 (5.46 Å and 4.92 Å), SER A:84 (5.44 Å) and LEU A:135 (5.28 Å, 5.68 Å and 4.57 Å).

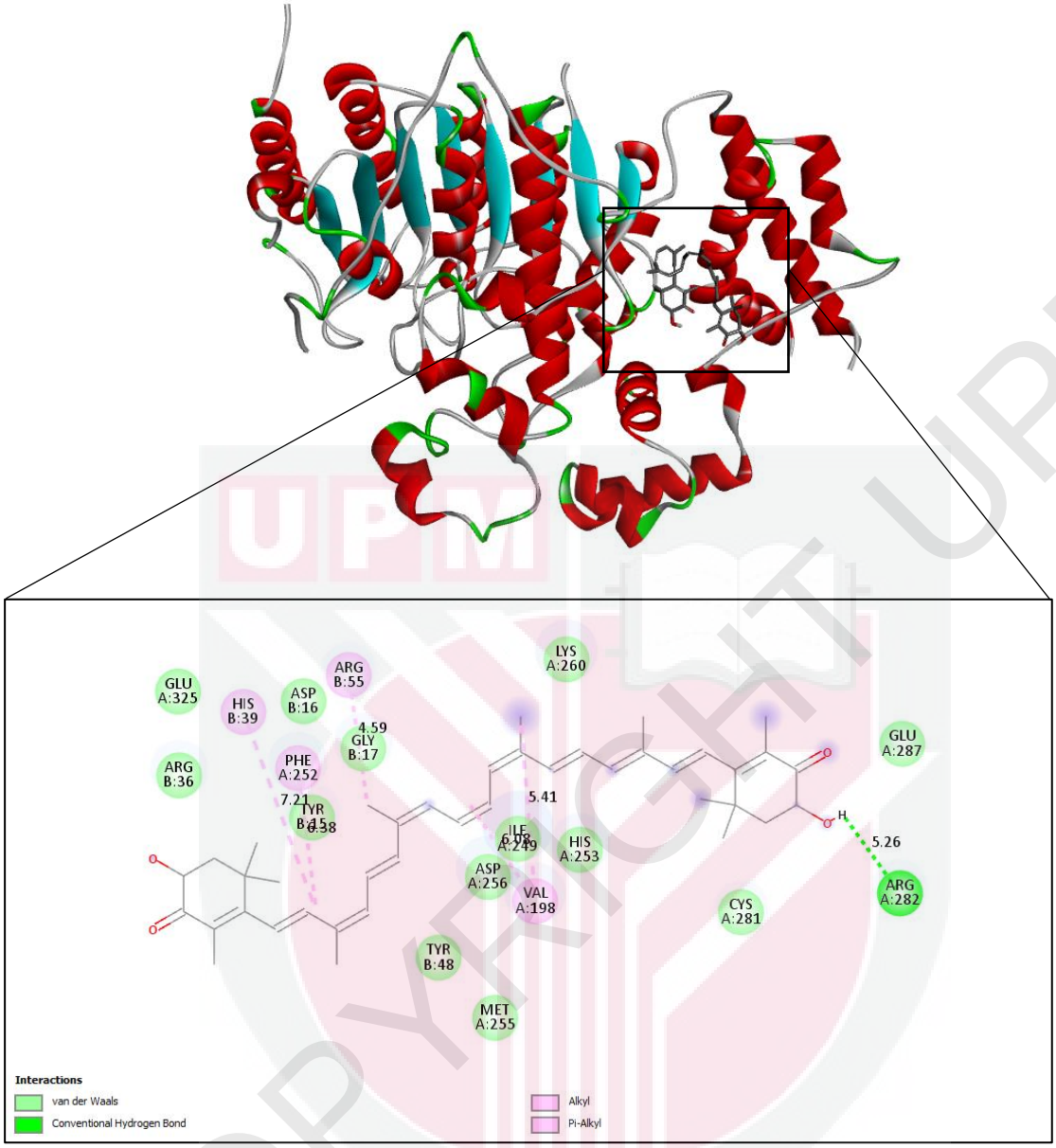


Figure 4.21: The diagram illustrates the prediction of 3D picture and 2D molecular interactions between ATX and the amino acid residues of *HDAC1* visualised by Biovia Discovery Studio. The amino acid residues are depicted by spheres of distinct colours based on interaction types surrounding the ATX structure represented by line model grey colour. Residues were shown as three-letter, type of chain and residue number. The interactions involved include van der Waals, Conventional Hydrogen Bond, Pi-Alkyl and Alkyl. The distances of key interactions are represented by numbers along dashed lines and are measured in angstroms (Å): ARG B:55 (4.59 Å), PHE A:252 (6.38 Å), HIS B:39 (7.21 Å), ARG A:282 (5.26 Å) and VAL A:198 (5.41 Å and 6.08 Å)

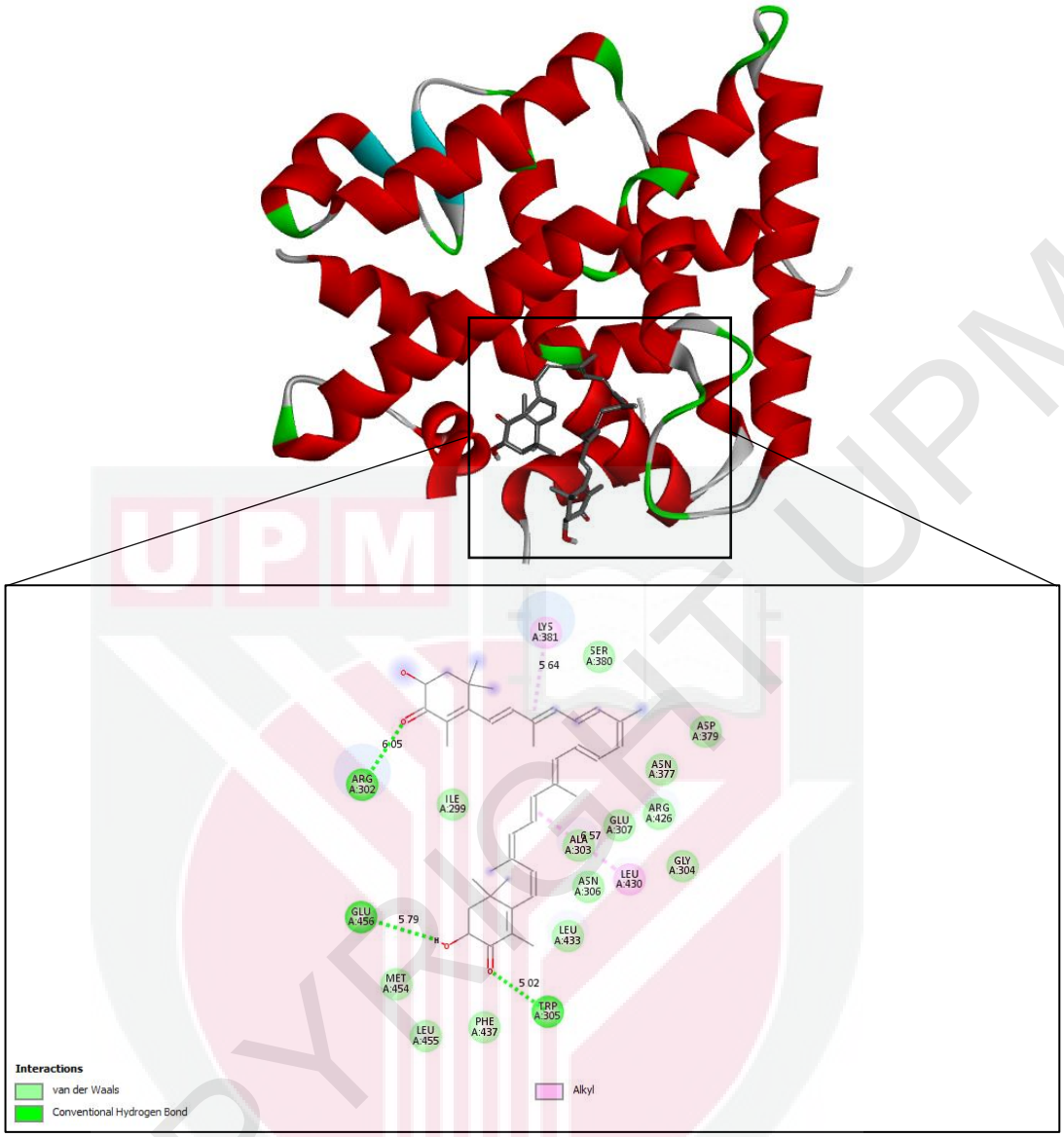


Figure 4.22: The diagram illustrates the prediction of 3D picture and 2D molecular interactions between ATX and the amino acid residues of *RXRA* visualised by Biovia Discovery Studio. The amino acid residues are depicted by spheres of distinct colours based on interaction types surrounding the ATX structure represented by line model grey colour. Residues were shown as three-letter, type of chain and residue number. The interactions involved include van der Waals, Conventional Hydrogen Bond and Alkyl. The distances of key interactions are represented by numbers along dashed lines and are measured in angstroms (Å): ARG A:302 (6.05 Å), LYS A:381 (5.64 Å), GLU A:456 (5.79 Å), TRP A:305 (5.02 Å) and LEU A:430 (6.57 Å)

CHAPTER 5

DISSCUSION

Angiogenesis plays a critical role in physiological processes involving wound healing, post-ischaemic tissue regeneration, embryonic growth, and endometrial alterations associated with the menstrual cycle. Inevitably, it is also linked to a number of pathophysiological disorders, such as cancer and non-cancerous conditions comprising atherosclerosis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus, and proliferative retinopathy (Walsh, 2007). Angiogenesis is defined as the formation of new blood vessels from existing ones, which serves as a precursor for tumour growth and metastasis (Folkman, 1984). This process effectively removes the metabolic wastes that the tumour cells produce while ensuring a constant supply of nutrients and oxygen to the tumour (Papetti & Herman, 2002). Hence, angiogenesis-focused cancer therapy approaches have drawn interest given that targeting the angiogenic mechanisms of tumours may yield beneficial outcomes.

The growing attention given to anti-angiogenic strategies has contributed in the invention of an array of anti-angiogenic agents. Bevacizumab (Avastin™; Genentech, San Francisco, CA), an anti-angiogenic medication, has exhibited a promising anti-tumour impact by targeting exclusively the VEGF pathway (Presta et al., 1997). This agent is administered in combination with chemotherapy to treat multiple kinds of malignancies, particularly lung, colorectal, prostate, and kidney cancers, demonstrating its promising therapeutic potential (Fox et al., 2002; Hurwitz et al., 2004; Johnson D. H et

al., 2004; Yang et al., 2003). In spite of this, pharmacologically inhibiting the VEGF pathway is anticipated to result in a multitude of adverse effects owing to its crucial function under normal physiological settings. These adverse effects comprise bleeding, proteinuria, and hypertension, and multiple others (Chen H. X & Cleck, 2009). Therefore, alternatives are investigated for complementary natural products with comparable anti-angiogenic effectiveness and minimal adverse effects.

Carotenoids are one of the most renowned natural pigments, having over 600 distinct compounds discovered thus far. Out of all these compounds, β-carotene appears the most notable (Olson & Krinsky, 1995). Increased intake of diets high in carotenoid-rich foods has been linked to a lower risk of a number of degenerative diseases, notably different types of cancer, heart disease, and eye diseases, as supported by numerous studies (Mayne, 1996).

On the opposite end of the spectrum, astaxanthin (ATX) is a naturally occurring reddish pigment that is produced by a variety of yeasts, microalgae, and bacteria. It is frequently present in aquatic species including salmon, trout, lobster, fish eggs, red seabream, and shrimp as well as in birds like quails and flamingos (Alcaino et al., 2014; Fakhri et al., 2018). Being a member of the xanthophylls carotenoid class, ATX participates in many of the same physiological and metabolic processes as carotenoids. However, ATX is more effective than β-carotene as an antioxidant (Strzałka et al., 2003). Scientists have therefore become increasingly interested in studying ATX and its possible capacities, which has resulted in the finding of ATX properties that include antioxidative, anticancer, anti-aging, anti-inflammatory, cardioprotective,

hepatoprotective, and a multitude other effect (Brotosudarmo et al., 2020). Nevertheless, no research has explicitly looked into the anti-angiogenic properties of ATX as of yet. In light of this, the primary purpose of this work is to investigate any possible anti-angiogenic effects of ATX in HUVECs that are stimulated by VEGF. In this current study, we have been demonstrated the analysis of cytotoxic, anti-proliferative, anti-migration and anti-tube formation effects, network pharmacology and molecular docking. Collectively, our investigations revealed a notable anti-angiogenic effect in HUVEC induced by VEGF.

5.1 Cell viability

5.1.1 Cytotoxic Effect of ATX in HUVECs by using MTT

The results revealed there is a statistically significant decreased of cell viability (%) in concentration-dependent manner of ATX in HUVECs ($p<0.05$), with an identified IC_{50} value of 75.09 µM. This suggests that ATX exhibits cytotoxic effects on HUVECs; moreover, it is expected to inhibit 50% of the HUVEC population at a specific concentration of 75.09 µM.

These results were coherent and in agreement with the published research, precisely the study carried out by Régnier et al., (2015). Throughout their study, they observed a concentration-dependent decline in the cell viability (%) of HUVECs after 48 hours of ATX treatment. In particular, cell viability was significantly reduced below the defined threshold of 70% starting at 15 µM. Furthermore, as per Nagaraj et al., (2012), ATX toxicity is exhibited via two discrete mechanisms: an increase in membrane permeability and an

interaction with microtubules, that eventually result in the hindrance of the mitotic cycle. This is especially noticeable at elevated concentrations in HepG2 cells, a cell line that represents liver cancer in humans.

Furthermore, taking into account the IC_{50} value and the statistically significant differences observed between the ATX treatment and the control groups, three particular concentrations were selected as the most optimal concentrations for further research. With this deliberate choice, the objective is to determine concentrations that will still exert a meaningful and pertinent impact in later experiments, without being unduly toxic to HUVECs.

The angiogenesis process involves basement membrane breakdown, cell migration, cell proliferation, and tube formation—all of which are accessible to in vitro testing, therefore methods to intervene can be directed towards altering each of these components (Auerbach et al., 2003). As that happened, a number of studies were conducted to precisely target the previously described steps of angiogenesis—MTT proliferation, scratch wound healing, and tube formation assays. These studies made it possible to examine the ability of ATX to impede angiogenesis at various phases.

5.1.2 Anti-Proliferative Effect of ATX in VEGF-Stimulated HUVECs utilising the MTT Assay

The initial phase of angiogenesis involves cell proliferation, as was previously explained. Thus, evaluation of cell proliferation offers important information on the angiogenic potential of tumours and their ability to initiate and promote the formation of blood vessels; as a result, targeted therapeutic interventions can be employed. The results revealed that ATX exhibited anti-proliferative effect in HUVECs induced by VEGF as there is a statistically significant decreased in cell viability (%) as concentration increased ($P < 0.05$).

In this experiment, VEGF stimulation was administered to HUVECs in ATX treatment groups, and the results were compared with control, baseline, and VEGF-only groups. It is anticipated that groups subjected to 72-hour ATX treatment will not display reduced cell proliferation compared to the four-hour baseline. It is sensible to presume that under standard conditions, cell proliferation will increase over time, leading in greater cell viability at 72-hours compared to the first 4-hours. It is also reasonable to anticipate that HUVECs treated for longer periods of time will proliferate more than those treated for shorter periods of time, confirming the baseline's validity as a point of reference. Any circumstance in which the ATX treatment group's cell proliferation declines less than the baseline suggests that ATX is excessively inhibiting cell proliferation. This insight is crucial since, in standard situations, cell proliferation is necessary and severe suppression of it may cause more harm than good.

Additionally, while all ATX-treated groups stimulated by VEGF showed a trend towards higher cell viability (%) compared to the control group, this difference was not statistically significant. This suggests that the experimental setting is appropriate, though there may be limitations that occurred during the experiments, which could have influenced the results. The limitations will be discussed further in future recommendation section (6.2). The observed trend aligns with the hypothesis that VEGF-induced HUVECs would proliferate more rapidly because endothelial cells stimulated by VEGF usually possess an increased proliferation, migration, cell cycle progression, and tube formation in endothelial cells in vitro (Guo et al., 2017).

As with the prior claim, it is reasonable to expect enhanced cell viability in ATX-treated groups triggered by VEGF compared to the control group, which simply contains the basal medium and no further inducers. Moreover, although VEGF stimulates cell proliferation in both the VEGF-only and ATX-treated groups, there are significant differences between these groups ($P < 0.05$). This demonstrates that even in the presence of an inducer that usually stimulates cell proliferation, ATX effectively restricted HUVECs' ability to proliferate.

The ATX proliferation experiment was previously carried out in a different cell line by another investigation. According to the study, ATX significantly reduced the proliferation of gastric cancer cells (KATO-III and SNU-1) in a dose-dependent manner. On the basis of this finding, the mechanism in which ATX exerts its anti-proliferative impact was characterised by its ability to cause cell cycle arrest in the G0/G1 phase and reduce cell proliferation at the S phase (Kim J. H et al., 2016). Therefore, it is feasible that anti-proliferative impact of

ATX in HUVECs might operate through a mechanism similar to that found in said investigation. To verify this, further investigations must be conducted.

5.2 Anti-Migratory Effect of ATX in HUVECs by utilising Scratch Wound Healing Assay

Moreover, cell migration is critical in adult organisms for maintaining tissue homeostasis, enabling wound repair, and maintaining an appropriate immune response. On the other hand, abnormal cell migration occurs in a number of pathological diseases (Trepat et al., 2012). Elevated migration of cells is observable during angiogenesis, as it is considered a crucial phase in the process. Gaining knowledge about the mechanisms that control cell migration enables to acquire greater insight into the dynamics of vessel development.

The outcomes revealed that ATX exhibits anti-migratory effect in HUVECs as there is a statistically significant decline in migration rate (%) as the ATX's concentrations increased ($P < 0.05$). In contrast to the VEGF-only group, ATX across various concentrations reveal a statistically significant reduction in migration rates observed ($P < 0.05$). This result demonstrates the concentration-dependent inhibitory effect of ATX on cell migration, which was demonstrated during the course of an 8-hour experiment.

Suramin group employed as the positive control in the study, offering an established benchmark for evaluating ATX as potential anti-angiogenic agent on cell migration. In comparison to suramin group, both the control and VEGF-only groups which did not receive Suramin treatment exhibit statistically significant reduction in the migration rate (%) ($P < 0.05$). The experiment is validated by the profound inhibitory effect of Suramin, which justifies its use as

a credible positive control for evaluating anti-angiogenic responses in the setting of VEGF-induced cell migration. It ought to be noted that none of the ATX-treated groups' migration rates fell below those of the suramin group. This indicates that ATX is not more potent than the well-known anti-angiogenic drug suramin. However, ATX is not significantly distant in terms of potency when compared to that of suramin. Consistent to a prior study, ATX has been reported to inhibit cell migration, specifically in the scratch wound healing assay in different cell line. In that investigation, it has been revealed that ATX, at a 50 μM concentration, effectively inhibited hypoxia-induced migration in the HUVEC derivative ECV304 cell line—a derivative of HUVECs originated through spontaneous transformation. To elucidate the manner in which ATX suppresses migration, immunoblot analysis revealed that ATX significantly decreased the expression levels of HIF-1α, VEGF, and MMP-2, hence impeding hypoxia-induced angiogenesis (Kowshik et al., 2014).

The mechanism in which ATX exerts anti-migratory effects may bear similarities to the previously described study, given the profound connection between angiogenesis and hypoxia. Reduced oxygen levels, or hypoxia, have been discovered to be the primary physiological factor that initiates the angiogenic switch (Giordano & Johnson, 2001). This correlation serves as a foundation for the consistency observed in the current study's findings. However, to further validate and identify the specific mechanism in which ATX exerts anti-migratory effect, an additional study must be conducted.

The suppression of VEGF-induced cell migration in anti-angiogenic agent is essential since increased migration is indicative of the angiogenic potential that

causes pathological illnesses. Therefore, this finding may lead to therapeutic approaches for angiogenesis-related disorders.

5.3 ATX Inhibited Tube Formation in VEGF-Induced HUVECs utilising Matrigel

Furthermore, tube formation is a specialised test intended to evaluate angiogenesis and is a crucial in vitro method for determining the capacity of a compound to induce angiogenesis (Madri et al., 1988). This suggests that the results of this assay may offer insights into the potential angiogenic or antiangiogenic properties of a particular compound. Before achieving a definitive finding, additional angiogenic assays must be performed for further validation.

The process of forming an in vitro tube mimics several phases of angiogenesis, including adhesion, basement membrane disruption, cell invasion/migration, differentiation, and basement membrane protein synthesis (MALINDA et al., 1999). After HUVECs were seeded on Matrigel, capillary-like tube structures begin to emerge gradually. The gel developed a structure resembling a mesh as a result of the interconnecting capillary-like tubes. Short and incomplete capillary-like tubes are expected to emerge in response to ATX, which is expected to reinforce its anti-angiogenic properties.

When compare to VEGF-only group, ATX significantly decreased the tube lengths as concentrations increased ($P < 0.05$). This observation implies that ATX has the ability to suppress tube formation in varying concentrations of ATX induce tube formation even while VEGF is present. To put it briefly, ATX has a

suppressive effect on tube development even under angiogenesis-promoting environments.

Besides, in contrast with the suramin group, no significant difference was detected between the ATX and suramin groups. This suggests that suramin and ATX possess comparable properties, suggesting a similar effect or efficacy as anti-angiogenic agents. Based on the information presented, the highest concentration (25 μM) implemented in this experiment revealed significantly shorter tube lengths (μm) as compared to the Suramin group, which is recognised for possessing potent anti-angiogenic effects. This discovery suggests that ATX is not only as potent as Suramin but may even be more effective in suppressing tube formation in angiogenesis. This discovery suggests that ATX is not only as potent as Suramin but may even be more effective in suppressing tube information in angiogenesis. This indicates that ATX possesses a potent anti-angiogenic effect and could be an attractive candidate for additional research or therapeutic development in the context of angiogenesis.

The VEGF-induced tube formation suppression of ATX in HUVECs is consistent with a prior study that reported ATX prevented hypoxia-induced tube formation of ECV 304 cells (Kowshik et al., 2014). Parallel to the study described in the section on the migration assay, the study showing the suppression of tube formation also showed the inhibition of cell migration. The mechanism by which ATX inhibits tube formation may be similar to the process that has been found to impede cell migration, which involves a decrease in the expression levels of MMP-2, VEGF, and HIF-1α. The manner in which ATX can

prevent the formation of tubes indicates that it may be able to restrict blood and nutritional supply, which would mitigate the angiogenesis process in pathological illnesses. This discovery holds significant importance in angiogenesis research, given that excessive angiogenesis is undesirable in numerous pathological conditions. Consequently, the treatment approach can be planned to specifically address this issue.

All things considered, ATX has proven to be an effective anti-angiogenic agent in the current study by a variety of assessments, such as those measuring cytotoxicity, proliferation, migration, and tube formation. Given the context of angiogenic disorders such as cancer and other related conditions, this discovery holds significance in the therapeutic strategy.

5.4 Network Pharmacology and Molecular Docking of ATX against Angiogenesis

To begin, both network pharmacology and molecular docking analyses were performed to filter for potential targets relevant to the objective of the study, particularly angiogenesis when analysed in the context of ATX. During the course of our network pharmacology investigation, RXR, CCND1, CDK1, and HDAC1 were identified as key nodes in the network. These nodes are believed to play crucial roles in the regulatory impacts that ATX has on angiogenesis. This identification clarifies the importance of these key targets in angiogenic processes and may provide a starting point for future research. Prior studies had addressed their intricate association with angiogenesis, reaffirming the significance of these critical targets and provided additional justification for their position as top hub genes in the network.

The first top hub gene identified is RXR, also referred to as retinoid X receptors. These nuclear receptors are transcription factors that are part of the steroid/thyroid hormone superfamily and are prevalent in cellular regulatory networks. Earlier research examined the role of RXRs in angiogenesis and discovered that macrophages exposed to RXR agonists stimulate the formation of new blood vessels (Daniel et al., 2014). Secondly, Cyclin D1, frequently referred to as CCND1, is an essential mediator of the G1 phase in cell cycle progression and plays a role in coordinating the cell's transition from the G1 phase to the S phase. The CCND1 gene is regarded as an oncogene that promotes angiogenesis, cell growth, proliferation, and resistance to radiation and chemotherapy (Malumbres & Barbacid, 2001; Chen et al., 2020).

Thirdly, Cyclin-Dependent Kinase 1 (CDK1) is essential for monitoring the complex regulatory mechanisms of the cell cycle. It modulates the centrosome cycle, promotes the G2 to M phase transition, and regulates the G1 phase. Research has demonstrated that CDK1 was highly expressed in pathological retinal angiogenesis, and CDK1 siRNAs decreased endothelial cell proliferation, tube formation and migration (Gao et al., 2019). Lastly, histone deacetyltransferases (HDACs) enhance histone acetylation, which is essential for transcriptional control. Previous studies have demonstrated that HDAC1, a member of the class I HDACs, significantly influences angiogenesis in HUVECs. In particular, it has been shown that over expression of HDAC1 promotes angiogenesis by downregulating the tumour suppressor von Hippel-Lindau and p53 (Kim M. S et al., 2001).

A molecular docking investigation incorporating the identified hub genes and ATX was carried out in order to strengthen and corroborate the results acquired by network pharmacology, particularly at the molecular level. However, the main proangiogenic factors, including VEGFR2, FGFR1, and TIE2, were noticeably lacking, despite the top hub genes showing a significant association with angiogenesis. In consequence, the four top hub genes and additional three genes were incorporated in the molecular docking assessment against ATX. The lowest binding energies for each of the targets were determined and thoroughly compared. A lower docking score suggests a higher binding strength between the ligand and the receptor; among the seven receptors, VEGFR2 demonstrated the lowest binding energy, while RXRA displayed the highest. However, the binding affinity of ATX for each of the seven targets remains potent displayed by binding energies that are consistently less than -6.0 kcal/mol. Drug design typically selects candidates with binding free energy values below -6.0 kcal/mol. However, to date, there is no established agreement on the specific range of binding energies that biologically active compounds should exhibit (Gligorić, E et al., 2023). This finding suggests that ATX has robust binding affinity for each of the seven receptors, with a particularly high affinity for VEGFR2.

The documented relationship between vascular endothelial growth factor A (VEGF-A) and its corresponding receptor, VEGF receptor 2 (VEGFR2), reinforces the recent discussion. Following this binding event, cellular components that control vital processes including angiogenesis, cell survival, and proliferation are activated (Eguchi et al., 2022). The binding of VEGF-A to VEGFR2 is well-documented for its angiogenesis-promoting effect, surpassing

the efficacy of other signalling pairings related to VEGF-VEGFR or VEGF-neuropilin (Ferrara, 2004).

While the in-silico study offers a convenient way to obtain insights in a research context pertaining to angiogenesis, relying only on in silico approaches is not sufficient to justify and validate the study in its entirety. As such, further research is necessary to validate and authenticate the insights obtained from the initial in-silico study.

CHAPTER 6

CONCLUSION AND FUTURE RECOMMENDATION

6.1 Conclusion

In summary, an in vitro cytotoxicity study using MTT determined that ATX exhibited cytotoxic activity, as evidenced by a concentration-dependent decrease in cell viability (%) in HUVECs ($P<0.05$); the IC50 value was 75.09 μM and at 50μM ATX killed 33% of the HUVEC when treated for 24 h. An additional indication of ATX's anti-proliferative effect was observed via a proliferation assay employing MTT: as the concentration of ATX increased, there was a statistically significant decrease in the percentage of viable cells ($P < 0.05$) in 72 hours with 4 h baseline as comparison. Furthermore, a scratch wound healing assay revealed that ATX exhibited an anti-migratory effect on HUVECs, as evidenced by the concentration-dependent decline in migration rates (%) as concentration increased ($P<0.05$). The migration rate observed at a concentration of 25 μM is 20% for a duration of 8 hours. Moreover, the inhibitory effect of ATX on tube formation was demonstrated by a Matrigel-based tube formation assay, which revealed a statistically significant decrease in the average tube length (μm) in a concentration-dependent manner as the concentration increased ($P<0.05$).

In this present study, network pharmacology identified potential targets of ATX for angiogenesis intervention, revealing 171 overlapping targets related to angiogenesis and ATX. A PPI network analysis using the STRING database showed 125 proteins and 440 interactions, with 49 nodes having high

interconnectivity (degree > 7.04). Using three algorithms (MNC, MCC, and degree) in Cytoscape, the top ten hub genes were identified, leading to four common hub genes: RXR, CCND1, CDK1, and HDAC1. These hub genes are crucial in regulating angiogenesis by ATX.

Molecular docking analysis identified interactions between ligands and seven key proteins: four hub gene proteins (RXRA, CCND1, CDK1, HDAC1) and three main tyrosine kinase proteins (VEGFR2, FGFR1, Tie-2). All interactions showed binding energies below -6.0 kcal/mol, with VEGFR2 exhibiting the strongest binding affinity (-9.34 kcal/mol) and RXRA the weakest (-6.42 kcal/mol). These results suggest ATX has a strong binding affinity to these proteins, particularly VEGFR2. However, in-silico analysis alone is insufficient, necessitating a series of in vitro experiments to validate the findings.

In conclusion, ATX exhibited anti-angiogenic effect on HUVEC induced by VEGF through cytotoxicity, proliferation, migration, and tube-formation assays. The present study provides an excellent basis for future studies concerning the therapeutic potential of ATX against diseases associated with angiogenesis.

6.2 Future recommendation

While this study has yielded promising results in understanding angiogenesis, there are some limitations that need to be acknowledged. Although VEGF is known to promote migration and tube formation, the VEGF-only group did not show a significant difference compared to the control group in both assays. This may be because we didn't allow enough time for VEGF to affect the cells, as a 72-hour proliferation assay showed a noticeable increase in the VEGF-only group. Initially, a 24-hour timeframe was chosen based on the study by Bishop et al., 2020. However, in this study, the wound gap between the control and VEGF-only groups had already closed by this time, making it difficult to determine which group closed first. Consequently, a shorter period of 8 hours was selected for more accurate observation.

For the tube formation assay, it is recommended to observe tube formation within 2-6 hours, as tubes typically form quickly (DeCicco-Skinner, K. L et al.,2014). In this study, we used a 6-hour timeframe, which may not have allowed sufficient time for VEGF to exert its full influence on the cells. To address this, using a higher concentration of VEGF might be necessary in future experiments to achieve a more pronounced effect. Another possible reason for this issue could be differences in the preparation of VEGF for the migration and tube formation assays compared to the proliferation assay. Variations in concentration, conditions, or temperature might have contributed to the discrepancy.

To validate the preliminary results obtained from each assay, it is crucial to undertake additional in-depth research within the framework of the present inquiry. Further comprehensive investigations are imperative in order to validate and establish the dependability of the results acquired in the present study. The cytotoxicity and proliferation assays utilising MTT alone do not offer enough information to understand the fundamental mechanisms by which ATX induces cytotoxic effects and suppresses proliferation. Therefore, additional methodologies, including flow cytometry, could be further investigated in order to analyse the dispersion of HUVECs throughout distinct phases of the cell cycle. Additionally, to differentiate between cells undergoing necrosis and those undergoing apoptosis, an apoptosis assay, such as the acridine orange and ethidium bromide (AO-EB) staining procedure, can be performed. In addition, alternative migration assays such as transwell migration assays or 3D invasion assays can be performed to verify and assess cell migration and invasion, respectively.

In order to validate the results obtained in vitro, further in vivo experiments are required. Thus, it is possible to conduct in vivo experiments employing the chorioallantoic membrane (CAM) assay or zebrafish angiogenesis assay in order to observe the anti-angiogenic effects of ATX in a more physiologically relevant and complex environment. Finally, assays such as Western Blot Analysis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) could potentially be used to detect alterations in the levels of gene and protein expression.

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APPENDIX A

Table 1: The content of experimental group cell cytotoxicity assay with the concentrations

Group	Content	MTT Reagent
Control	Media + (1 x 10^4) Cells	10 µL
ATX 1	Media + (1 x 10^4) Cells + 3.125 µM of ATX	
ATX 2	Media + (1 x 10^4) Cells + 6.25 µM of ATX	
ATX 3	Media + (1 x 10^4) Cells + 12.5 µM of ATX	
ATX 4	Media + (1 x 10^4) Cells + 25 µM of ATX	
ATX 5	Media + (1 x 10^4) Cells + 50 µM of ATX	
Blank	Media	

Table 2: The content of experimental group of cell proliferation assay with the concentrations

Group	Content	MTT Reagent
Control	Media + (1 x 10^4) Cells	10 µL
Baseline	Media + (1 x 10^4) Cells (only for 4 hours)	
VEGF	Media + (1 x 10^4) Cells + 10 µg/ml of VEGF	
ATX 1	Media + (1 x 10^4) Cells + 10 µg/ml of VEGF + 6.25 µM	
ATX 2	Media + (1 x 10^4) Cells + 10 µg/ml of VEGF + 12.5 µM	
ATX 3	Media + (1 x 10^4) Cells + 10 µg/ml of VEGF + 25 µM	
Blank	Media + (1 x 10^4) Cells	

Table 3: The content of experimental group of cell migration assay with the concentrations

Group	Content
Control	Media + (1.5 x 10^5) Cells
VEGF	Media + (1.5 x 10^5) Cells + 10 µg/ml of VEGF
Suramin	Media + (1.5 x 10^5) Cells + 10 µg/ml of VEGF + 20 µg/ml Suramin
ATX 1	Media + (1.5 x 10^5) Cells + 10 µg/ml of VEGF + 6.25 µM
ATX 2	Media + (1.5 x 10^5) Cells + 10 µg/ml of VEGF + 12.5 µM
ATX 3	Media + (1.5 x 10^5) Cells + 10 µg/ml of VEGF + 25 µM

Table 4: The content of experimental group of tube formation assay with the concentrations

Matrigel	Group	Content
300 µL	Control	Media + (1.5×10^5) Cells
	VEGF	Media + (1.5×10^5) Cells + 10 µg/ml of VEGF
	Suramin	Media + (1.5×10^5) Cells + 10 µg/ml of VEGF + 20 µg/ml Suramin
	ATX 1	Media + (1.5×10^5) Cells + 10 µg/ml of VEGF + 6.25 µM
	ATX 2	Media + (1.5×10^5) Cells + 10 µg/ml of VEGF + 12.5 µM
	ATX 3	Media + (1.5×10^5) Cells + 10 µg/ml of VEGF + 25 µM

APPENDIX B

Target genes and the database

Table 1: List of target genes of Astaxanthin

No	Target	Common name
1	Tyrosine-protein kinase ABL	ABL1
2	Acetyl-CoA carboxylase 1	ACACA
3	ADAM10	ADAM10
4	Adenosine A3 receptor	ADORA3
5	Alpha-1a adrenergic receptor	ADRA1A
6	Alpha-1d adrenergic receptor	ADRA1D
7	Beta-1 adrenergic receptor	ADRB1
8	Adrenergic receptor beta	ADRB2
9	Beta-3 adrenergic receptor	ADRB3
10	Type-1 angiotensin II receptor	AGTR1
11	Aldo-keto reductase family 1 member B10	AKR1B1
12	Aldo-keto reductase family 1 member B10	AKR1B10
13	Serine/threonine-protein kinase AKT	AKT1
14	Aldehyde dehydrogenase	ALDH2
15	Arachidonate 12-lipoxygenase	ALOX12
16	DNA-(apurinic or apyrimidinic site) lyase	APEX1
17	Androgen Receptor	AR
18	Serine/threonine-protein kinase Aurora-A	AURKA
19	Aurora kinase B/Inner centromere protein	AURKB

20	Bcl2-antagonist of cell death (BAD)	BAD
21	Apoptosis regulator Bcl-X	BCL2L1
22	Bloom syndrome protein	BLM
23	Bromodomain-containing protein 2	BRD2
24	Bromodomain-containing protein 4	BRD4
25	C5a anaphylatoxin chemotactic receptor	C5AR1
26	Voltage-gated N-type calcium channel alpha-1B subunit	CACNA1B
27	Voltage-gated T-type calcium channel alpha-1H subunit	CACNA1H
28	Cyclin-dependent kinase 2/cyclin A	CCNA1
29	Cyclin-dependent kinase 2/cyclin A	CCNA2
30	Cyclin-dependent kinase 4/cyclin D	CCND1
31	Cyclin-dependent kinase 4/cyclin D	CCND2
32	Cyclin-dependent kinase 4/cyclin D	CCND3
33	Cyclin-dependent kinase 2/cyclin E	CCNE1
34	Cyclin-dependent kinase 2/cyclin E	CCNE2
35	C-C chemokine receptor type 1	CCR1
36	C-C chemokine receptor type 3	CCR3
37	Cyclin-dependent kinase 1/cyclin B1	CDK1
38	Cyclin-dependent kinase 2/cyclin E	CDK2
39	Cyclin-dependent kinase 4/cyclin D	CDK4
40	Cyclin-dependent kinase 5	CDK5
41	Carboxylesterase 2	CES2
42	Cholesteryl ester transfer protein	CETP
43	Dual specificty protein kinase CLK1	CLK1

44	Cannabinoid CB1 receptor	CNR1
45	Cannabinoid CB2 receptor	CNR2
46	cytochrome c oxidase subunit I	COX1
47	cytochrome c oxidase subunit II	COX2
48	Macrophage colony stimulating factor receptor	CSF1R
49	Casein kinase II alpha/beta	CSNK2B
50	Cathepsin D	CTSD
51	Cathepsin L	CTSL
52	Cytochrome P450 19A1	CYP19A1
53	Cytochrome P450 3A4	CYP3A4
54	Dipeptidyl peptidase VIII	DPP8
55	Dipeptidyl peptidase IX	DPP9
56	Dual specificity protein phosphatase 3	DUSP3
57	Receptor protein-tyrosine kinase erbB-2	ERBB2
58	EZH2/SUZ12/EED/RBBP7/RBBP4	EZH2
59	Thrombin and coagulation factor X	F10
60	Coagulation factor XI	F11
61	Coagulation factor XIII	F13A1
62	Coagulation factor VII	F7
63	Fatty acid synthase	FASN
64	IgG receptor FcRn large subunit p51	FCGRT
65	FK506-binding protein 1A	FKBP1A
66	Vascular endothelial growth factor receptor 1	FLT1
67	Tyrosine-protein kinase receptor FLT3	FLT3

68	Glutamate carboxypeptidase II	FOLH1
69	Formyl peptide receptor 1	FPR1
70	Lipoxin A4 receptor	FPR2
71	Glucose-6-phosphate 1-dehydrogenase	G6PD
72	Gonadotropin-releasing hormone receptor	GNRHR
73	G-protein coupled receptor 55	GPR55
74	Glutamate NMDA receptor; GRIN1/GRIN2B	GRIN1
75	G protein-coupled receptor kinase 5	GRK5
76	Histone deacetylase 1	HDAC1
77	Histone deacetylase 10	HDAC10
78	Histone deacetylase 11	HDAC11
79	Histone deacetylase 2	HDAC2
80	Histone deacetylase 4	HDAC4
81	Histone deacetylase 7	HDAC7
82	Histone deacetylase 9	HDAC9
83	Hypoxia-inducible factor 1 alpha	HIF1A
84	11-beta-hydroxysteroid dehydrogenase 1	HSD11B1
85	11-beta-hydroxysteroid dehydrogenase 2	HSD11B2
86	Heat shock factor protein 1	HSF1
87	Heat shock protein HSP 90-beta	HSP90AB1
88	Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1
89	Indoleamine 2,3-dioxygenase	IDO1
90	Integrin alpha-IIb/beta-3	ITGA2B
91	Integrin alpha-5/beta-1	ITGB1

92	Integrin alpha-IIb/beta-3	ITGB3
93	Tyrosine-protein kinase ITK/TSK	ITK
94	HERG	KCNH2
95	LSD1/CoREST complex	KDM1A
96	Vascular endothelial growth factor receptor 2	KDR
97	Kelch-like ECH-associated protein 1	KEAP1
98	Stem cell growth factor receptor	KIT
99	Kruppel-like factor 5	KLF5
100	Dual specificity mitogen-activated protein kinase kinase 2	MAP2K2
101	MAP kinase ERK1	MAPK3
102	c-Jun N-terminal kinase 1	MAPK8
103	c-Jun N-terminal kinase 2	MAPK9
104	Methionine aminopeptidase 2	METAP2
105	Matrix metalloproteinase 2	MMP2
106	Matrix metalloproteinase 9	MMP9
107	Mas-related G-protein coupled receptor member X1	MRGPRX1
108	Serine/threonine-protein kinase mTOR	MTOR
109	Nicotinamide phosphoribosyltransferase	NAMPT
110	Nuclear factor erythroid 2-related factor 2	NFE2L2
111	Nuclear factor NF-kappa-B p105 subunit	NFKB1
112	Nitric oxide synthase, inducible	NOS2
113	Nitric-oxide synthase, endothelial	NOS3
114	Pregnane X receptor	NR1I2

115	nuclear receptor subfamily 3 group C member 1	NR3C1
116	Nuclear Receptor Subfamily 3 Group C Member 2	NR3C2
117	Nuclear receptor subfamily 4 group A member 1	NR4A1
118	NT-3 growth factor receptor	NTRK3
119	Delta opioid receptor	OPRD1
120	P2X purinoceptor 7	P2RX7
121	Phosphodiesterase 3B	PDE3B
122	Platelet-derived growth factor receptor alpha	PDGFRA
123	Platelet-derived growth factor receptor	PDGFRB
124	Progesterone receptor	PGR
125	PI3-kinase p110-alpha subunit	PIK3CA
126	PI3-kinase p110-beta subunit	PIK3CB
127	PI3-kinase p110-delta subunit	PIK3CD
128	PI3-kinase p110-gamma subunit	PIK3CG
129	PI3-kinase p110-alpha/p85-alpha	PIK3R1
130	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	PIN1
131	Lysosomal Pro-X carboxypeptidase	PRCP
132	Protein kinase C alpha	PRKCA
133	Protein kinase C delta (by homology)	PRKCD
134	Protein kinase C theta	PRKCQ
135	PRMT5/MEP50 complex	PRMT5
136	Vitamin K-dependent protein C	PROC
137	Prostaglandin E synthase	PTGES
138	Cyclooxygenase-1	PTGS1

139	Cyclooxygenase-2	PTGS2
140	Protein tyrosine kinase 2 beta	PTK2B
141	Protein-tyrosine phosphatase 1B	PTPN1
142	Protein-tyrosine phosphatase 2C	PTPN11
143	T-cell protein-tyrosine phosphatase	PTPN2
144	Retinoic acid receptor alpha (by homology)	RARA
145	Retinoic acid receptor beta (by homology)	RARB
146	Retinoic acid receptor gamma (by homology)	RARG
147	RAS guanyl releasing protein 3	RASGRP3
148	Nuclear receptor ROR-alpha	RORA
149	Ribosomal protein S6 kinase alpha 1	RPS6KA1
150	Retinoid X receptor alpha	RXRA
151	Retinoid X receptor beta	RXRB
152	Retinoid X receptor gamma	RXRG
153	Sphingosine 1-phosphate receptor Edg-1	S1PR1
154	Sphingosine 1-phosphate receptor Edg-3	S1PR3
155	Sphingosine 1-phosphate receptor Edg-8	S1PR5
156	Sex Hormone Binding Globulin	SHBG
157	Monocarboxylate transporter 1	SLC16A1
158	Sodium/hydrogen exchanger 1	SLC9A1
159	Sphingosine kinase 2	SPHK2
160	Tyrosine-protein kinase SRC	SRC
161	Stimulator of interferon genes protein	STING1
162	Telomerase reverse transcriptase	TERT

163	Tissue factor pathway inhibitor	TFPI
164	Toll-like receptor 4	TLR4
165	Toll-like receptor 8	TLR8
166	Toll-like receptor (TLR7/TLR9)	TLR9
167	DNA topoisomerase II alpha	TOP2A
168	Transcription intermediary factor 1-alpha	TRIM24
169	Serine/threonine-protein kinase ULK3	ULK3
170	Vitamin D receptor	VDR
171	WD repeat-containing protein 5	WDR5