ANTI-PROLIFERATIVE ANTI-ANGIOGENIC AND ANTI-MIGRASTATIC EFFECTS OF *OROXYLUM INDICUM* (L.) KURZ EXTRACT ON BREAST CANCER CELL

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ผลการยับยั้งการเจริญเติบโตของเซลล์การสร้างหลอดเลือดใหม่และการเคลื่อนที่ของเซลล์ โดยสารสกัดเพกา (*OROXYLUM INDICUM* (L.) KURZ) ต่อเซลล์มะเร็งเต้านม

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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การกลับมาเป็นซ้ำของมะเร็งเต้านมยังคงเป็นปัญหาทางคลินิกที่สำคัญ แม้ว่าการวินิจฉัยและการรักษาเชิงรุกที่ก้าวหน้าอย่างมีประสิทธิภาพก็ตาม ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์การต้านมะเร็งของสารสกัดเพกา โดยการตรวจสอบการเพิ่มจำนวนของเซลล์ การเคลื่อนที่ของเซลล์และการสร้างหลอดเลือดใหม่โดยการชักนำของเซลล์มะเร็งเต้านม (MDA-MB-231) โดยความสามารถในการเพิ่มจำนวนของเซลล์ ได้ทำการตรวจสอบโดยใช้ชุดตรวจ CCK-8 assay และตรวจสอบการสร้างโคโลนีของเซลล์ ฤทธิ์ของสารสกัดเพกาต่อการเคลื่อนที่ของเซลล์ถูกตรวจสอบด้วยเทคนิค wound healing assay และ transwell assay ในขณะเดียวกัน การสร้างหลอดเลือดใหม่โดยเซลล์มะเร็งเต้านม MDA-MB-231 ในโมเดล CAM (Chick Chorioallantoic Membrane) ถูกนำมาประยุกต์ใช้สำหรับการศึกษาฤทธิ์การต้านการสร้างหลอดเลือดใหม่ของสารสกัดเพกา พบว่าสารสกัดเพกาที่ความเข้มข้นต่ำกว่า 600 ไมโครกรัม/มิลลิลิตร ไม่มีผลในความเป็นพิษต่อเซลล์มะเร็งเต้านม และยังพบว่าฤทธิ์การยับยั้งการสร้างโคโลนีของเซลล์มะเร็งเต้านมในระยะยาวของสารสกัดเพกาเพิ่มมากขึ้นตามความเข้มข้นที่สูงขึ้นของสารสกัด การต้านการเคลื่อนที่ของเซลล์และการสร้างหลอดเลือดใหม่ถูกตรวจสอบโดยใช้ความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ของสารสกัดเพกาซึ่งอยู่ในช่วง 25 -150 ไมโครกรัม/มิลลิลิตร สารสกัดเพกามีฤทธิ์ในการยับยั้งการเคลื่อนที่ของเซลล์มะเร็งเต้านมเพิ่มมากขึ้นตามความเข้มข้นของสารที่เพิ่มขึ้น สารสกัดเพกาสามารถยับยั้งการสร้างหลอดเลือดใหม่ที่เกิดจากการชักนำของเซลล์มะเร็งเต้านม อย่างมีนัยสำคัญและพบว่าที่ความเข้มข้นดังกล่าวไม่เป็นพิษต่อการสร้างหลอดเลือดใหม่ที่เกิดตามโดยธรรมชาติ ที่น่าสนใจคือ สารสกัดเพกาที่ความเข้มข้นสูงสุดที่ใช้ในการทดสอบที่ 150 ไมโครกรัม/มิลลิลิตร มีประสิทธิภาพใกล้เคียงกับยาต้านมะเร็งพาโซพานิบที่ความเข้มข้น 4.37 ไมโครกรัม/มิลลิลิตร ซึ่งมีผลในการยับยั้งการเคลื่อนที่ของเซลล์และการสร้างหลอดเลือดใหม่โดยการชักนำของเซลล์มะเร็งเต้านม ดังนั้น ผลของสารสกัดเพกาในการยับยั้งการเพิ่มจำนวนและการเคลื่อนที่ของเซลล์ ร่วมกับการต้านการสร้างเส้นเลือดใหม่ในเซลล์มะเร็งเต้านม ซึ่งบ่งชี้ว่าสารสกัด

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BENJAMAS CHIRAATTHAKIT : ANTI-PROLIFERATIVE ANTI-ANGIOGENIC AND ANTI-MIGRASTATIC EFFECTS OF *OROXYLUM INDICUM* (L.) KURZ EXTRACT ON BREAST CANCER CELL. THESIS. THESIS ADVISOR : PROF. GRIANGSAK EUMKEB, Ph.D. 60 PP.

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Breast cancer recurrence continues to pose a major clinical problem, despite significant advancements in early diagnosis and an aggressive mode of treatment. This study aimed to investigate the anti-cancer activity of *O. indicum* extract (OIE) by assessing cell proliferation, cell migration, and angiogenesis in metastatic breast cancer MDA-MB-231 cell lines. Cell proliferation capacity was performed by cell counting kit-8 (CCK-8) and colony formation assays. The effect of OIE on cell migration was determined using wound healing and transwell assays. Meanwhile, MDA-MB-231-induced angiogenesis on Chick Chorioallantoic Membrane (CAM) was applied to investigate the *ex vivo* anti-angiogenesis activity of the extracts. OIE at concentrations lower than 600 µg/mL had no cytotoxic effects against MDA-MB-231 cells. OIE was found to inhibit the long-term colony formation ability of MDA-MB-231 cells in a concentration-dependent manner. Anti-migration and anti-angiogenesis activities were further investigated using non-cytotoxic concentrations of OIE ranging from 25 -150 µg/mL. OIE significantly reduced the migration of MDA-MB-231 breast cancer cells in a dose-dependent manner. OIE significantly suppressed the MDA-MB-231-induced angiogenesis, and there was no substantial toxic effect on natural angiogenesis. Interestingly, the highest concentration of OIE (150 µg/mL) was as potent as the positive anticancer drug, pazopanib, at 4.37 µg/mL, in inhibiting MDA-MB-231 cell migration and angiogenesis induced by these cells. Therefore, the inhibitory effects of OIE in cell proliferation and cell migration with anti-angiogenesis in MDA-MB-231 breast

cancer cells suggest that OIE may be developed as a new candidate for breast cancer chemotherapeutic agent in the future.

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LIST OF ABBREVIATIONS

µg/mL	=	Microgram per milliliter
µL	=	Microliter
µM	=	Micromolar
g	=	Gravitational acceleration
ANOVA	=	Analysis of variance
BSA	=	Bovine serum albumin
DI water	=	Distilled water
DMSO	=	Dimethylsulfoxide
EDTA	=	Ethylenediaminetetraacetic acid
FBS	=	Fetal bovine serum
g	=	Gram
h	=	Hour
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	=	Median inhibition concentration
kg	=	Kilogram
L	=	Liter
M	=	Molar
min	=	Minute
mg	=	Milligram
mg/mL	=	Milligram per milliliter
mL	=	Milliliter
mM	=	Millimolar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	=	Optical density
OIE	=	*O. indicum* extract
PBS	=	Phosphate buffered saline

LIST OF ABBREVIATIONS (Continued)

rpm	=	Revolution per minute
RT	=	Room temperature
SD	=	Standard deviation
VH	=	Vehicle
v/v	=	volume by volume
w/v	=	weight by volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Cancer is one of the leading global causes of death and disability. The global burden of cancer worldwide uses the GLOBOCAN 2021 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer, focusing on geographic variability across 20 world regions. There will be an estimated 19.3 million new cancer cases and 10.0 million cancer deaths in 2020 (H. Sung et al., 2021).

Breast cancer is the leading cause of cancer-associated mortality among women. Breast cancer recurrence continues to pose a major clinical problem, despite significant advancements in early diagnosis and an aggressive mode of treatment. Breast cancer treatment can become complicated if the tumor cells metastasize into distant body organs. Also, an essential biological phenomenon supports the growth of cancer: angiogenesis. Initiation of angiogenesis plays a critical role in breast cancer progression, invasion, and metastasis (Soung et al., 2016).

Angiogenesis, the development of new microvascular networks, is required for invasive tumor growth and metastasis and constitutes a key point in controlling the development and progression of various cancers. Accumulating evidence has demonstrated that vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (HIF-1α) play a critical role in tumor angiogenesis (Li et al., 2012; Ramakrishnan et al., 2014). Angiogenesis is the complex process of generating new capillaries from existing blood vessels. Usually, it includes the following steps: vascular endothelial growth factor (VEGF), enzymatic degradation of the vascular basement membrane, proliferation, migration, and germination of vascular endothelial cells, branching, and tube formation. Under physiological conditions in normal adults, the net balance between proangiogenic factors and antiangiogenic factors controls the process of angiogenesis, and blood vessels remain static and rarely form new branches. Tumors secrete various substances to break this balance and induce pathological

angiogenesis, which eventually develops into a complex, mature vascular system. This system provides the tumor with the necessary nutrients and oxygen to promote tumor growth and causes the tumor cells to leave the tumor's original site and spread to distant organs through the blood. Hypoxia is the main force driving tumor angiogenesis, and VEGF plays a key role in tumor angiogenesis by enhancing all the steps of angiogenesis (Muz et al., 2015). The ability of tumors to recruit endothelial cells and stimulate their proliferation, migration, or survival is thought to be central in tumor-induced angiogenesis. Much attention has been focused on the vascular endothelial growth factor (VEGF) family of growth factors and the receptor tyrosine kinases that mediate their angiogenic effects (Holmes and Zachary, 2005). In growing cancers, endothelial cells are vigorously active because of the release of many proteins, such as EGF, estrogen, basic and acidic FGF, IL-8, prostaglandin E1, and E2, TNF-α, and VEGF, that can activate endothelial cell growth and motility when the anti-angiogenic factors' production is reduced. VEGF and bFGF are essential to tumor angiogenesis, but the redundancy of (other) pro-angiogenic factors helps explain the current suboptimal effectiveness in the oncology of the pharmacological inhibitors of single endogenous angiogenic agents (Rajabi and Mousa, 2017). VEGF is a hypoxia-inducible secreted glycoprotein that predominantly regulates angiogenesis. It belongs to a family that includes platelet-derived growth factors and a group of proteins closely related to the primary structure of VEGF: placental growth factor and VEGF homologous VEGF B, C, D, and E. VEGF acts via high-affinity binding to three tyrosine kinase receptors, VEGFR-1, VEGFR-2, and VEGFR-3. Studies involving gene activation or inhibition have demonstrated that VEGFR-2 transmits essential angiogenic signals in response to VEGF (Sentilhes et al., 2011). Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits and is a major regulator of VEGF expression in response to hypoxia (Krenn and Paper, 2009). HIF-1 is a key factor in carcinogenesis, tumor angiogenesis, tumor growth, invasion, and metastasis, which can be induced by hypoxia and growth factors (Pennacchietti et al., 2003). HIF-1α is often up-regulated in human cancers to regulate VEGF expression by binding to the hypoxia response element of the VEGF promoter. It has been well demonstrated that AKT is one of the important upstream regulators of HIF-1α. This signaling pathway is

one of the major pathways in regulating tumor angiogenesis and tumor growth in ovarian, prostate, and lung cancer cells (Masoud and Li, 2015). The growth of new blood vessels is intrinsic to inflammation. It is associated with structural changes, including activation and proliferation of endothelial cells and capillary and venule remodeling, all of which will expand the tissue microvascular bed (Danese et al., 2007). A potential functional consequence of this expansion is the promotion of inflammation through various correlated mechanisms. First, the influx of inflammatory cells may increase; second, there is an increased nutrient supply that keeps feeding a metabolically active immune process; and third, activate endothelial cells due to the local production of cytokines, chemokines, and matrix metalloproteinases. Therefore, the anatomic expansion of the microvascular bed combined with its increased functional activation can foster further recruitment of inflammatory cells, and angiogenesis and inflammation may become chronically codependent processes (Danese et al., 2006). Inflammation may promote angiogenesis in a number of ways. Inflammatory tissue is often hypoxic, and hypoxia can induce angiogenesis through upregulation of factors such as VEGF. The extravasated plasma proteins, particularly fibrinogen products, may stimulate neovascularization. Inflammatory cells, such as macrophages, lymphocytes, mast cells, and fibroblasts, and the angiogenic factors produced by these cells can stimulate vessel growth. Many pro-inflammatory cytokines like tumor necrosis factor (TNF)-α, may exhibit angiogenic activity in addition to pro-inflammatory activity. Increasing blood flow itself may also stimulate angiogenesis through shear stresses directly on the endothelium. The inflammation can then upregulate the expression of angiogenic growth factors such as VEGF and fibroblast growth factor-1 (FGF-1) by resident cells, local fibroblasts (Wang et al., 2001). IL-8 expression has profound biological consequences. It is also a potent angiogenic, mitogenic and chemotactic factor in several malignancies, including breast and prostate cancer. Still, many studies have suggested that FGF-2, acting in an autocrine and paracrine fashion, can induce IL-6 expression through p38. In addition, it has been reported that IL-1α promotes angiogenesis by the activation of the VEGF-2 signaling pathway between inflammatory cells and blood vessel endothelial cells (Salven et al., 2002)

Medicinal plants are well known to be a rich source of bioactive constituents with a wide range of therapeutic properties acting against various diseases. The popularity of medicinal plants is popularly increasing to reduce the risk of side effects caused by modern medicine. Hence, Phytotherapy is still being used by most of the world's population. Natural products play a crucial role in the healthcare systems of Ayurveda, tribal, folk, and herbal medicine. Hence, it will be necessary to identify the chemical components responsible for plants' potential activities against disease-causing entities. Thus, they may be considered potential nutraceuticals, which may modulate angiogenic processes (Bharali et al., 2017).

Oroxylum indicum (L.) Kurz (*O. indicum*) has been used for centuries as a traditional medicine in Asia in ethnomedicinal systems for the prevention and treatment of several diseases, such as jaundice, arthritic and rheumatic problems gastric ulcers, tumors, respiratory diseases, diabetes, diarrhea, and dysentery, among others. The major flavonoids of the stem bark, such as baicalein, chrysin, and oroxylin A, were reported for the first time as natural flavonoids with potent inhibitory activity against endoprotease enzymes and proprotein convertases, which play a key role in the growth of cancer, viral and bacterial infections. Flavonoids are the active components purified from bioactive extracts (Dinda et al., 2015).

As angiogenesis is essential in almost all tumor growth, progression, and metastasis, it is related to and promoted by inflammation. *O. indicum*, which possesses anti-inflammatory properties, may exert its activity against various cancers by targeting the angiogenesis pathway (Hengpratom et al., 2018).

1.2 Research objectives

1. To investigate the anti-proliferative effect of *O. indicum* extract (OIE) against metastasis human breast cancer MDA-MB-231 cells.
2. To investigate the effects of OIE on the migration potential of metastasis human breast cancer MDA-MB-231 cells.
3. To investigate the anti-angiogenic activity of OIE on MDA-MB-231-induced angiogenesis by using the chick chorioallantoic membrane (CAM) model.

1.3 Scope and limitation of the study

This thesis was only focused on the inhibitory effect of OIE on cell proliferation, cell migration, and angiogenesis in MDA-MB-231 cells. Cell proliferation was investigated on metastatic human breast cancer MDA-MB-231 cells by using CKK-8 assay and colony formation assays. The effect of OIE on cell migration was also determined by using wound healing and transwell assays. The anti-angiogenic activity of OIE was further investigated by using the *ex vivo* on the chick CAM model.

1.4 Expected results

The *O. indicum* may possess anticancer activity mediated through anti-angiogenesis mechanisms. This information can be used as primary pharmacological data for a consideration of its therapeutic potential in the future.

CHAPTER II

LITERATURE REVIEW

2.1 Cancer

Cancer is the first or second leading cause of death before the age of 70 in 112 of 183 countries and ranks third or fourth in a further 23 countries. The global cancer burden using the GLOBOCAN 2020 estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer. Worldwide, an estimated 19.3 million new cancer cases (18.1 million excluding nonmelanoma skin cancer) and almost 10.0 million cancer deaths (9.9 million excluding nonmelanoma skin cancer) occurred in 2020. The global cancer burden is expected to be 28.4 million cases in 2040, a 47% rise from 2020, with a more significant increase in transitioning (64% to 95%) versus transitioned (32% to 56%) countries due to demographic changes. However, this may be further exacerbated by increasing risk factors associated with globalization and a growing economy (Hyuna Sung et al., 2021). Cancer results from a series of molecular events that fundamentally alter the typical properties of cells. In a cancer cell, the standard control systems that prevent cell overgrowth and the invasion of other tissues are disabled. These altered cells divide and grow in the presence of signals that generally do not activate or inhibit cell growth; therefore, they no longer require special signals to induce cell growth and division. Cancer refers to any one of many diseases characterized by the development of abnormal cells that divide uncontrollably and can infiltrate and destroy normal body tissue. Cancer can often spread throughout our body. The abnormalities in cancer cells usually result from mutations in protein-encoding genes that regulate cell division. During tumorigenesis, cancer cells acquire, through a multistep process, a new set of properties that allows them to overcome physiological homeostasis. These properties include unlimited proliferation potential, self-sufficiency in growth signals, resistance to antiproliferative and apoptotic signals, and escape immune system. These alterations, on the other hand, contribute to a process known as the stress phenotype

of cancer, which includes DNA damage/replication stress, proteotoxic stress, mitotic stress, metabolic stress, and oxidative stress. To survive the tumorigenic process, a cancer cell undergoes several modifications to its genomic circuitry, such as activating mutations in oncogenes and aberrant activation of non- oncogenic pathways (De Carvalho et al., 2012)

Breast cancer is the second most common life-threatening disease seen in women worldwide, with approximately 70–80% of patients with early-stage, non-metastatic disease (Harbeck et al., 2019). The two main reasons for the high mortality rates associated with breast cancer and the leading causes of poor clinical outcomes are invasion and metastasis (Akram et al., 2017). Metastasis of breast cancer is a complex event involving several coordinated sequential steps such as intravasation, survival in circulation, extravasation into distant organs, and angiogenesis. Recent studies have identified transforming growth factor-beta (TGFβ) as one of the factors produced abundantly by stromal cells essential for tumor cell metastasis. Activation of TGFβ signaling promotes breast cancer metastasis by increasing cell proliferation, epithelial-mesenchymal transition (EMT), migration, invasion colonization at distant organs, and inhibiting the immune cell function (Sung et al., 2016). Treatment with TGFβ has been shown to cause the delocalization or downregulation of cell junction proteins such as ZO-1, E-cadherin, and β-catenin. It promotes cytoskeleton reorganization by forming stress fiber assembly and by myosin light chain phosphorylation. The activation of TGFβ/Smad pathway induces breast cancer cell invasion by increasing the expression of matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9). Therefore, a drug that can suppress the TGFβ signaling can be a promising agent for blocking breast cancer metastasis (Tripathy et al., 2018). It is clear that there are thousands of point mutations, translocations, amplifications and deletions that may contribute to cancer development. The mutational range can differ even among histopathologically identical tumors. Detailed bioinformatic analyses have suggested that cancer-related driver mutations affect a dozen or more cores signaling pathways and processes responsible for tumorigenesis (Cairns et al., 2011). In the case of cancer cells, these adaptations must be implemented in the stressful and dynamic microenvironment of the solid tumor, where concentrations of crucial nutrients such

as glucose, glutamine, and oxygen are spatially and temporally heterogeneous. The nature and importance of metabolic restriction in cancer have often been masked owing to tissue culture conditions in which oxygen and nutrients are always in excess. Breast cancer has become one of the most common malignant tumors, and the latest dates from CA-cancer magazine show that the incidence rate is increasing yearly. In 2019, approximately 316 700 new breast cancer cases were confirmed in US women, and the growth rate is nearly 0.3% per year (He et al., 2020). Metastasis of breast cancer is a complex event involving several coordinated sequential steps such as intravasation, survival in circulation, extravasation into distant organ and angiogenesis. Recent studies have identified transforming growth factor beta (TGFβ) as one of the factors produced abundantly by stromal cells that is essential for tumor cell metastasis. Activation of TGFβ signaling promotes breast cancer metastasis by increasing cell proliferation, epithelial–mesenchymal transition, migration, invasion, colonization at distant organs and by inhibiting the immune cell function (Xie et al., 2018). The cells of the blood vessels that divide to make new capillary vessels are inactive in normal tissue; however, tumors make angiogenic factors, which activate these blood vessel cells to divide. Without the additional blood supplied by angiogenesis, tumors can grow no more significant than half a millimeter. Without a blood supply, tumor cells cannot spread or metastasize to new tissues (Chikezie O. Madu et al., 2020). Tumor cells can cross through the walls of the capillary blood vessel at a rate of about one million cells per day. However, not all cells in a tumor are angiogenic (Jain et al., 2020). Both angiogenic and non-angiogenic cells in a tumor cross into blood vessels and spread; however, non-angiogenic cells give rise to dormant tumors when they grow in other locations. In contrast, the angiogenic cells quickly establish themselves in new locations by growing and producing new blood vessels, resulting in the rapid growth of the tumor (Robertson-Tessi et al., 2015). Cells can respond to various environmental cues, and in many cases, these cues induce directed cell migration toward or away from these signals. Cell migration is critical in many physiological, developmental, and disease-related processes, and basic tenets governing this process have been uncovered over the years. *In vivo* cells must be able to perceive various cues in their environment and migrate toward or away from these

cues to execute morphogenetic programs during development, mount an immune response, and repair damaged tissues (SenGupta et al., 2021).

2.2 Angiogenesis

All cells need a constant source of oxygen and nutrients such as glucose. Our cells get their nutrients delivered to them via the blood. Nutrients and oxygen are pumped through the body via the circulatory system. Once in the tissues, the nutrients cross the blood vessel walls and enter the spaces around the cells. Cells need nutrients constantly, and the process by which nutrients float over to cells takes time. Angiogenesis is the formation of new blood vessels (Tahergorabi and Khazaei, 2012). This process involves the migration, growth, and differentiation of endothelial cells, which line the inside wall of blood vessels. Angiogenesis refers to the development of new capillaries from pre-existing capillaries and post-capillaries. This has been stimulated by the therapeutic opportunities offered by the ability to manipulate the vasculature in pathologies such as cancer (Bikfalvi and Bicknell, 2002).

2.2.1 Angiogenesis in cancer

Tumor growth and metastasis depend on angiogenesis, and lymphangiogenic is triggered by chemical signals from tumor cells in a phase of rapid growth (Folkman, 1971). Angiogenesis is a significant event in a wide range of healthy and diseased conditions. It is a normal and complex process controlled by specific biomolecules produced in the body. Endogenous local or systemic chemical signals coordinate functions of endothelial cells and smooth muscle cells to repair damaged blood vessels. The generation of new blood vessels is from pre-existing blood cells via the “sprouting” of endothelial cells, thus expanding the vascular tree (Figure 2.1). Steps toward angiogenesis include protease production, endothelial cell migration and proliferation, vascular tube formation, anastomosis of newly formed tubes, synthesis of a new basement membrane, and incorporation of pericytes and smooth muscle cells (Rajabi and Mousa, 2017). The cancer cells without blood circulation grew to 1–2 mm^3 in diameter and then stopped but grew beyond 2 mm^3 when placed in an area where angiogenesis was possible. In the absence of vascular support, tumors may become necrotic or even apoptotic. Therefore, angiogenesis is an important factor in

the progression of cancer. Neovascularization, including tumor angiogenesis, is basically a four-step process. First, the basement membrane in tissues is injured locally. There is immediate destruction and hypoxia. Second, endothelial cells activated by angiogenic factors migrate. Third, endothelial cells proliferate and stabilize. Fourth, angiogenic factors continue to influence the angiogenic process (Nishida et al., 2006).

The growth and metastasis of tumors largely depend on angiogenesis. When blood supply is deficient, tumors are incapable of growing, and necrosis occurrs. After a while, a subsequent metastatic spread to the systemic circulation is prevented. Research of the mechanism and the various factors surrounding angiogenesis have helped scientists understanding its impact on breast cancer and mount a countermeasure against tumor progression. Due to the dual nature of this process, however, it is crucial to carefully analyze and distinguish between the mechanism that leads to normal angiogenesis, such as wound repair, normal growth, and embryo nourishment, and that of tumor-related angiogenesis (Chen et al., 2005).

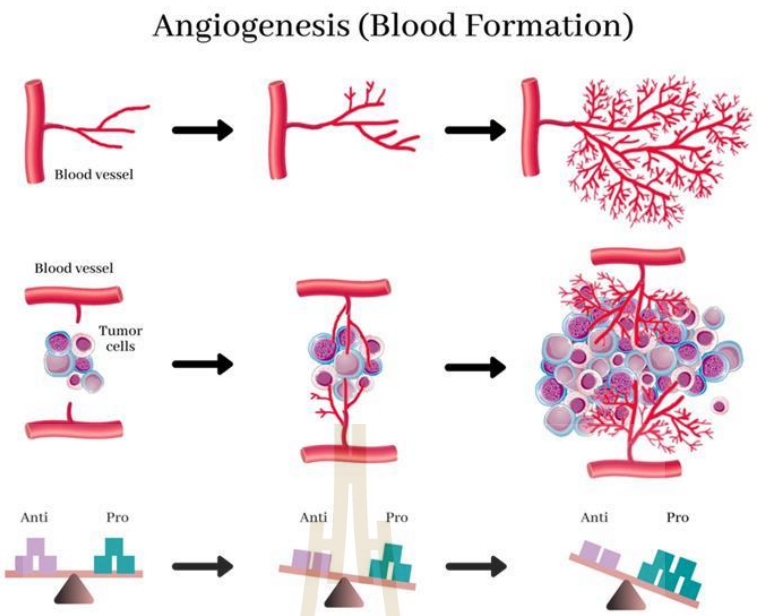

Figure 2.1 Angiogenesis is the physiological process of developing new blood vessels from preexisting vessels (C. O. Madu et al., 2020).

The importance of angiogenesis for the growth of solid tumors is now well recognized. The differential activation of angiogenesis between normal and tumor cells makes this process an attractive strategic target for anti-tumor drug discovery. Angiogenesis is a critical factor in developing tumors and metastases in numerous cancers. An increased level of angiogenesis is associated with decreased survival in breast cancer patients. Therefore, a good understanding of the angiogenic mechanism promises to provide effective treatments for breast cancer, which enhancing patients' survival. Disrupting the initiation and progression of this process by targeting angiogenic factors such as vascular endothelial growth factor (VEGF)-one of the most potent member of the VEGF family- or by targeting transcription factors, such as hypoxia-Inducible factors (HIFs) that act as angiogenic regulators, have been considered as potential treatment options for several types of cancers (Chikezie O. Madu et al., 2020). The concept of antiangiogenic therapy in cancer patients started after observations performed by Judah Folkman approximately 45 years ago. He noticed that to grow beyond 1–2 mm^3, tumors require blood supply and, for that reason, induce the generation of new vessels in the process of angiogenesis. Based on such observations, it was proposed that inhibition of tumor vessel formation could

suppress tumor growth, and that concept was called antiangiogenic therapy. Angiogenesis, the rapid increase in the formation of blood vessels, is required for the supply of sufficient oxygen and nutrition for breast tumor growth. Breast cancer cells, like all-body tissues, need constant nourishment and oxygen supply through the vascular network of capillaries in the system. These capillaries usually do not increase because the cells that line the interior surface of blood vessels, endothelial cells (ECs), do not multiply. However, low levels of O2 (hypoxia) can trigger numerous transcriptional responses mediated by transcription factors, referred to as hypoxia-inducible factors (HIFs). HIFs are highly conserved transcription factors that regulate the expression of multiple genes responsible for stimulating specific physiological responses, such as metabolism, cell division, and angiogenesis. Local angiogenesis is one of the tumor's microenvironment long-term primary adaptations to low O2 levels (Muz et al., 2015). It involves the convergence of EC precursors that give rise to the capillary plexus, subsequently developing into blood vessels. Angiogenesis is really crucial in normal processes, such as embryonic development, growth, and wound healing. More importantly, these angiogenic activators had been confirmed to play an essential role in the development of tumors (Nishida et al., 2006). Angiogenesis is vital in solid tumor cells' development, progression, and metastasis. During its onset, the tumor does not stimulate angiogenesis and, with low nutrient and oxygen supply, will remain limited in its growth to about 1-2 mm in diameter. The balance between this switch determines whether the tumor will turn on angiogenesis or not. It will be stimulated whenever tumor tissues require nutrients and oxygen. Angiogenesis is regulated by both activator and inhibitor molecules. However, up-regulation of the activity of angiogenic factors is insufficient for angiogenesis of the neoplasm. Negative regulators or inhibitors of vessel growth need to also be down-regulated (Figure 2.2). Further experiments indicated a decrease in the production of the anti-angiogenic proteins turns on the tumor angiogenic switch and consequently promotes tumor growth and metastases. Stimulating angiogenesis in a tumor and creating the subsequent endothelial tubes involves a multistep process regulated by hypoxia in every step. This process relies extensively on ECs expressing the heterodimeric transcription factor, HIF-1α. This protein is stabilized and able to form a heterodimer

with HIF-1β under hypoxic conditions (C. O. Madu et al., 2020), and this duo can, later, activate the transcription of several target genes to survive in the hypoxic environment in human cancer cells (Chikezie O. Madu et al., 2020).

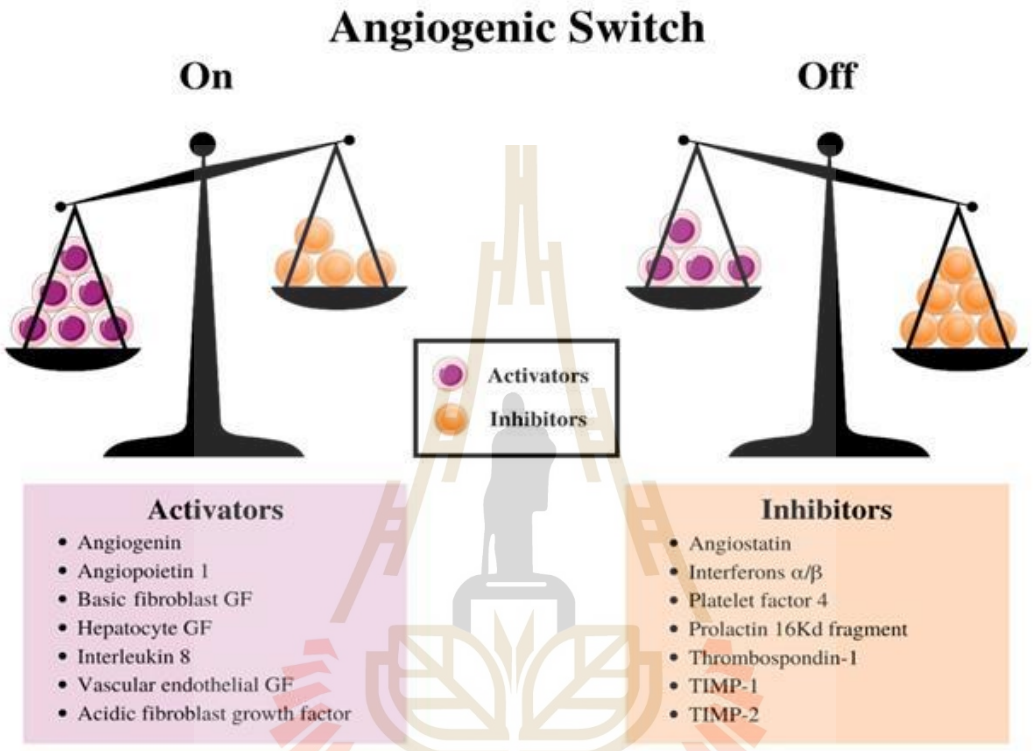

Figure 2.2 Schematic diagram illustrating the balance hypothesis of the angiogenic switch (C. O. Madu et al., 2020).

In addition, the studies on platelet-derived growth factor (PDGF) and PDGF receptor (PDGFR) and fibroblast growth factor (FGF) and FGF receptor (FGFR) pathways, for example, also provide potential escape mechanisms from anti-VEGF/VEGFR therapy that could facilitate the resumption of tumor growth. Accordingly, more recent treatments have focused on inhibiting multiple signaling pathways simultaneously. Many cancers exploit angiogenic mechanisms to stimulate tumor growth and disease progression (Y. Zhao and A. A. Adjei, 2015) (Figure 2.3). Several antiangiogenic treatments that have been approved for clinical use targeting these pro-angiogenic growth factors and/or their associated receptors, cytokines, and proteases. Some of

the examples of compounds/drugs that have gone through clinical research and approval will be covered in the next section.

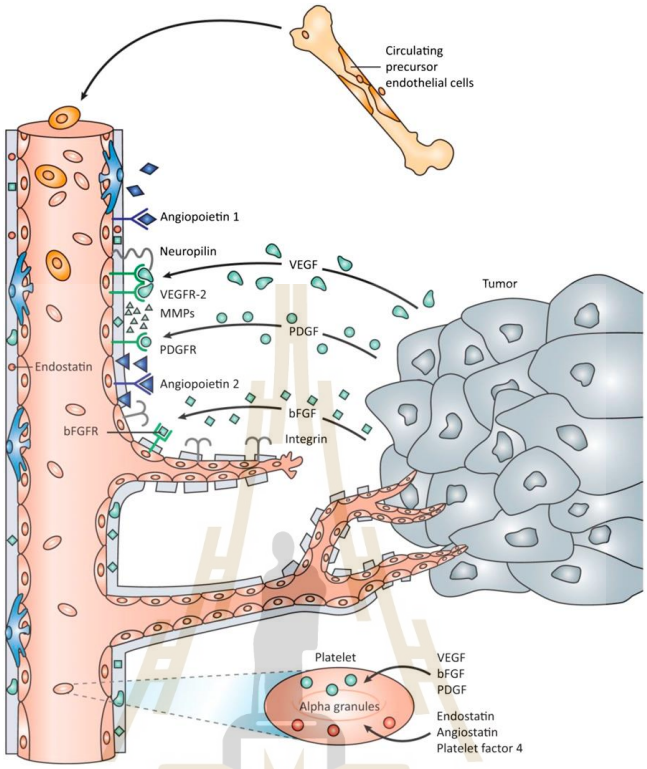

Figure 2.3 Tumor angiogenesis mechanisms. Soluble angiogenic factors (e.g., VEGF, PDGF, FGF) are secreted from the tumor and surrounding cells to induce and regulate key steps in angiogenesis and reproduced with permission from (Y. Zhao and A. Adjei, 2015).

2.3 Cancer cell migration

The migration of cancer cells into surrounding tissue and the vasculature is an initial step in tumor metastasis. Cancer cell migration and invasion are integral components of metastatic disease, the major cause of death in cancer patients. Cancer cells can move out of the primary tumor and invade proximal and distant tissues, where they can form metastases, ultimately responsible for 90% of cancer-associated deaths (Irimia and Toner, 2009). Cancer cells can disseminate and migrate via several alternative mechanisms, including amoeboid cell migration, mesenchymal cell migration, and collective cell migration. These diverse movement strategies display

certain specific and distinct hallmarks in cell-cell junctions, actin cytoskeleton, matrix adhesion, and protease activity involvement. During tumor progression, cells pass through complex microenvironments and adapt their migration strategies by reversible mesenchymal-amoeboid and individual-collective transitions (Wu et al., 2021). The first step of the metastatic cascade is invasion, in which tumor cells penetrate their surrounding basement membrane and migrate through the extracellular matrix (ECM) into the surrounding tissue (Figure. 2.4). The movement of cancer cells into tissue surrounding the tumor and the vasculature is the first two concurrent steps needed for spreading metastatic cancers (Yamaguchi et al., 2005).

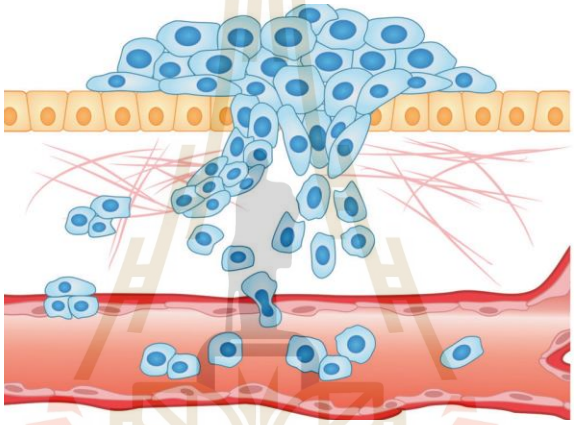

Figure 2.4 The model of cancer cell invasion (Novikov et al., 2021).

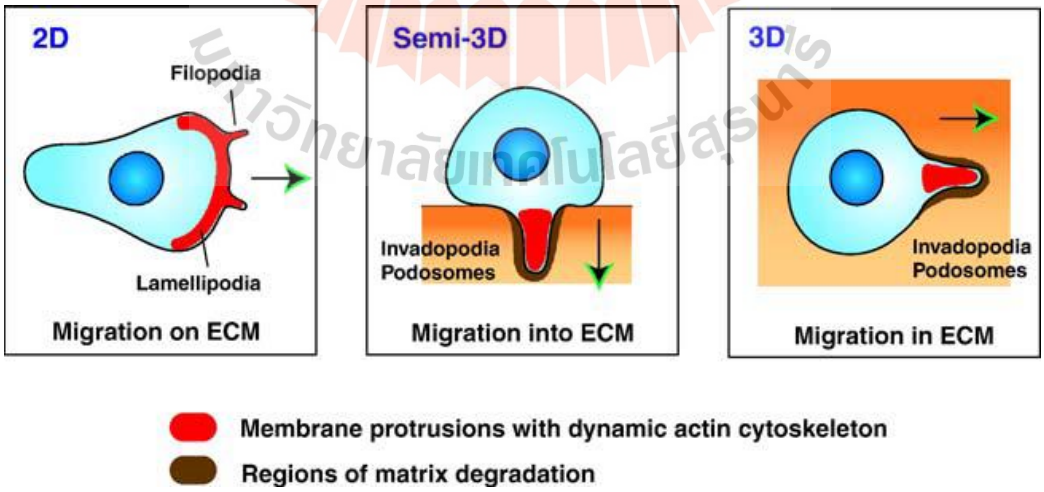

Figure 2.5 Cell migration and membrane protrusions in different environments (Yamaguchi et al., 2005)

Cell migration is required for many biological processes, such as embryonic morphogenesis, immune surveillance, and tissue repair and regeneration. Aberrant regulation of cell migration drives the progression of many diseases, including cancer invasion and metastasis. Therefore, understanding the fundamental mechanisms of cell migration is critical for our understanding of both basic biology and the pathology of the disease. Cell migration is a highly integrated multistep process initiated by the cell membrane's protrusion (Yamaguchi and Condeelis, 2007) (Figure 2.5). In contrast, the migratory capacity of leukocytes to move through the body to keep the organism under immunological surveillance and respond to pathogenic invaders is well described. The most active migrating cells among leukocytes are natural killer cells, T lymphocytes, macrophages, dendritic cells, and neutrophil granulocytes. Neutrophils, with a maximum speed of 15 to 20 Am/min, are the fastest cells. In comparison to these cells, fibroblasts are slow-moving cells. Their activity is necessary during tissue regeneration after injury. The morphology of these fibroblasts and their manner of migration resembles the migration of tumor cells. They share some structural elements with these cells, e.g., the focal adhesion contacts, which coordinate the regulated

adhesion between the extracellular matrix (ECM) and the intracellular cytoskeleton via integrin receptors. In contrast to the aforementioned physiological migration, the pathological migration of tumor cells is a component of the tumor progression with resulting invasion in surrounding tissues and metastasis development in distant organs (Entschladen et al., 2005). Cell migration is a central process in developing and maintaining multicellular organisms. Tissue formation during embryonic development, wound healing and immune responses require the orchestrated movement of cells in particular directions to specific locations.

Cells commonly move in response to specific external signals, including chemical signals and mechanical signals (Mak et al., 2016). Errors during this process have serious consequences, including intellectual disability, vascular disease, tumor formation, and metastasis (Franz et al., 2002).

2.4 *Oroxylum indicum* (L.) Kurz

Oroxylum indicum (L.) Kurz (earlier name: *Oroxylum flavum* Rehder; Bignoniaceae) is a medium-sized tree that grows in Asian tropical and subtropical low-altitude open forests. *Oroxylum indicum* is a deciduous tree found in many parts of the world, particularly in South Asia, Southeast Asia, and China. The plant has been used in Indian Ayurvedic and Chinese folk medicine to cure stomach problems, diarrhea, dysentery, and rheumatism (Dinda et al., 2015). The fruits of this tree are consumed as a part of the diet in the north and northeastern parts of Thailand. Its local names in other Southeast Asian countries are as follows; Indonesia: pongporang (Sundanese), Kayu lanang, mungli (Javanese); Malaysia: beko, bonglai, Kulai; the Philippines: pingka-pingkahan (Tagalog), abong-abong (Bisaya), kamkampilan (Iloko); Cambodia: pika; Laos: linmay, ungka; Thailand: Phe-ka (central). Recently, phytochemical studies of the tree have revealed the presence of flavonoids, such as chrysin, oroxylin, baicalein, baicalein glycosides, and benzoic acid and fatty acids (Usman and Osuji, 2007).

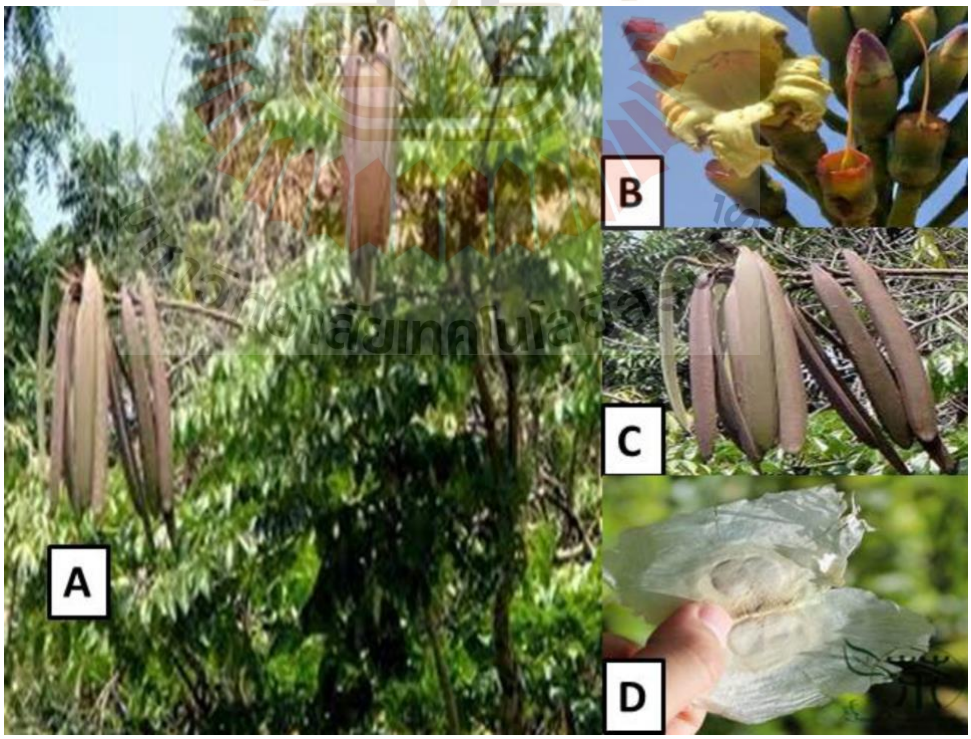

Figure 2.6 *O. indicum* (A) whole plant, (B) flower, (C) fruit, (D) seed (Kalra and Kaushik, 2017)

Flavonoids have been reported that baicalein, chrysin, and oroxylin A are the major flavones present in stem bark. In contrast, baicalein, chrysin, baicalein7-O-glucoside, baicalein 7-O-diglucoside, and chrysin7-O-diglucoside are the major chemical constituents of the seeds. In fruits, baicalein was present as the major constituent at approximately 4% of freeze-dried fruits. In root bark, oroxylin A and baicalein are the major constituents (Dinda et al., 2015). Aqueous and methanolic extracts of stem bark of *O. indicum* were evaluated for their anticancer potential against Hep3B (human hepatic carcinoma), MDA-MB-435S (human breast carcinoma) and PC-3 (human prostate cancer) cell lines and it was concluded that the phytoconstituents of the extract were competent to bring apoptosis in cancerous cells. Kumar et al. showed that petroleum ether hot-extract of the stem bark of *O. indicum* could efficiently target both ER-negative viz (Naveen Kumar et al., 2012). MDA-MB-231 and ER-positive viz. MCF-7, breast cancer cell lines, help induce apoptosis without any side effects on normal cells due to cancer-specific cytotoxicity. *O. indicum* has been found to have an IC_{50} value of 19.6 µg/mL for CEM (leukemia), 17.2 µg/mL for B-16 (murine melanoma), 14.2 µg/mL for HL-60 (leukemia) and 32.5 µg/mL for HCT-8 (human colon carcinoma). *O. indicum* was also examined on the sea urchin eggs and found to inhibit the development of the cell cycle by inhibiting the first cleavage (IC_{50} = 13.5 µg/mL) (Ong et al., 2009). The ethyl acetate extract of *O. indicum* was also found to be the potent NF-kB inhibitor with IC_{50} values of 47.45 µg/mL. Furthermore, ethyl acetate extract of *O. indicum* was also found to inhibit PGE2 as well as *in vitro* lipid-peroxidation. Baicalein suppresses the growth of primary myeloma cells by downregulating NF-kB (Liu et al., 2018) and inhibits IL-6 and IL-8 production in human retinal pigment epithelial cells at the transcriptional level (Nakamura et al., 2003). Antiproliferative and antimitotic activities of stem bark of *O. indicum* were studied and found that hydroalcoholic extract at concentrations of 4, 5, and 6 mg/ml exhibited a significant inhibitory effect against the dividing cells of Allium roots and thus decreased root growth and mitotic index (Sijoria et al., 2016). The anti-angiogenic potential of *O. indicum* has been reported by a chick chorioallantoic membrane (CAM), a significant inhibition of vascular endothelium growth factor (VEGF), and induced neovascularization (Maheswari and Revathi, 2017). Several reports are availably

attributed to bioactive molecules from *Oroxylum indicum*. Phytochemical screenings done on various plant parts indicate that this particular plant is rich in flavonoids, tannins, terpenoids, carotenoids, stilbenoids, and anthocyanin. However, considering chemotherapeutic aspects of *O. indicum* extract, additional prominence has been given to baicalein (flavonoid), the most potent antitumor compound (Islam et al., 2010).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell lines

MDA-MB 231 cells were grown in L-15 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

3.1.2 Chemicals and instruments

The chemicals and instruments employed in the present studies are summarized in Tables 3.1 and 3.2.

Table 3.1 List of chemicals.

Name	Source
Absolute ethanol	Carlo Erba
Ammonium sodium phosphate tetrahydrate	Acros organics
Cell Counting kit-8 (CCK-8)	Sigma
Dimethyl sulfoxide (DMSO)	Amresco
EDTA (Ethylenediaminetetraacetic acid)	Sigma
Magnesium sulfate	Carlo Erba
Potassium chloride	Carlo Erba
Potassium phosphate, dibasic (anhydrous)	Univar
Sodium acetate	Aldrich
Sodium monohydrogen phosphate heptahydrate	Merck
Streptomycin solution sulfate	Sigma-Aldrich
Trypan blue	Fluka

Table 3.2 List of instruments.

Name	Source
Bruker Vertex 70 spectrometer coupled with a Bruker-Hyperion 2000 microscope	Bruker Optics
Centrifuge (model CT15RT)	Techcomp
Inverted microscope (model CKX41)	Olympus
Class II Biohazard safety cabinet	ESCO
Light microscope (model CX21)	Olympus
Lyophilize (model Freeze-zone 12 plus)	Labconco Corporation
Microcentrifuge	Sorvall
Microplate spectrophotometer	Bio-Rad
pH meter	Selecta
Rotary evaporator with a vacuum (model R205)	Buchi
Microplate reader	Benchmark
Stereomicroscope (model SZX7)	Olympus

3.2 Methods

3.2.1 Preparation of *O. indicum* extract (OIE)

O. indicum (fruit pods) fresh samples were purchased from the local market at Wang Nam Khiao district, Nakhon Ratchasima province, Thailand. Fresh pods were washed thoroughly with tap water, cut into small pieces, and then dried in the oven at 40 °C for 2 days. The dried pieces were pulverized using a mechanical grinder, and the resulting coarse powder was preserved from moisture. The *O. indicum* dry powder (500 g) was extracted with 95% ethanol by a soxhlation for 8 hrs. The extract was filtered through Whatman filter paper and concentrated using a rotary evaporator at 50 °C under a vacuum to remove remnant of ethanol. Subsequently, the sample was lyophilized in a freeze dryer (LABCONCO) into a crude powder. The extracted powder was stored at -20 °C until required. The lyophilized OIE was used within 3 months of preparation. The extract was resuspended in the differentiation medium containing 0.1 v/v DMSO (vehicle) and added to the cells at concentrations ranging from 0 to 1500 µg/mL (Hengpratom et al., 2018).

3.2.2 Cytotoxic test (CKK-8 assay)

Cell Counting Kit-8 (CCK-8) allows sensitive colorimetric assays to determine cell viability in cell proliferation and cytotoxicity assays. Highly water-soluble tetrazolium salt, WST-8, is reduced by cell dehydrogenase activities to give a yellow-color formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye generated by the activities of dehydrogenases in cells is directly proportional to the number of living cells. The detection sensitivity of CCK-8 is higher than the other tetrazolium salts such as MTT, XTT, MTS, or WST (Jin et al., 2015).

MDA-MB-231 (1.5×10^4 cells/well) were seeded in a 96-well plate for 24 hrs and treated with various concentrations of OIE (25, 50, 100, 250, 500, or 1,000 µg/mL). After 24 hrs, 10 µL of CKK-8 solution was added to the cells and further incubated for 2 hrs at 37 °C. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, USA) (Paquette et al., 2011). The percentage of cell viability was calculated according to the following equation:

$$\text{Percent cell viability} = \frac{\text{Average OD for the test group}}{\text{Average OD for the control group}} \times 100$$

3.2.3 Colony formation assay

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The effect of OIE on the ability of MDA-MB-231 cells to form colonies was investigated using colony formation assay as described previously (Brix et al., 2020). MDA-MB-231 cells (500 cells/well) were seeded for 24 hrs. Then, the cells were treated with the indicated concentration of OIE (50, 150, 300, and 600 µg/mL), baicalein 3.75 µg/mL, or pazopanib 4.37 µg/mL for 24 hrs. After incubation, the culture medium was removed and washed twice with PBS. The culture medium was replaced every 2 days for two weeks. Then, the cells were washed twice with PBS, fixed with methanol: acetone (3: 1), and stained with 0.5% crystal violet solution for 15 min at room temperature. The number of colonies was photographed and counted. The percentage of colony formation was calculated according to the following equation:

$$\% \text{ Colony formation} = \frac{N_{sample}}{N_{control}} \times 100$$

Where N_{sample} = The colony number of treated group.

$N_{control}$ = The colony number of the control group.

3.2.4 Cell migration and invasion assay

3.2.4.1 wound-healing assay

The wound-healing assay is simple, inexpensive, and one of the earliest developed methods to study directional cell migration *in vitro*. This method mimics cell migration during wound healing *in vivo*. It is particularly suitable for studies on the effects of cell-matrix and cell-cell interactions on cell migration (Rodriguez et al., 2005). The basic steps involve creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells. MDA-MB-231 cells were seeded at a density of 1×10^5 cells/well in 6-well plates and grown for 24 hrs. Next, a linear scratch wound was created across the middle of the well's surface using a 200 µL pipette tip. After that, the culture medium was replaced with a fresh culture medium containing OIE (50, 150, 300, and 600 µg/mL), baicalein 3.75 µg/mL, or pazopanib 4.37 µg/mL and further incubated for 24 hrs. At predetermined time points (18 and 24 hrs), the images of cells that migrated into the denuded areas were taken using an inverted microscope with 40× magnification equipped with a DinoEye digital eyepiece. The widths of the wound were measured using ImageJ software. The percentage of wound closure was calculated according to the following equation:

$$\% \text{ wound closure} = \frac{A_0 - A_{18 \text{ or } 24}}{A_0} \times 100$$

Where $A_{18 \text{ or } 24}$ = The wound area post-treatment at 18 or 24 hrs.

A_0 = The wound area pre-treatment at 0 hrs.

3.2.4.2 Transwell migration assay

To examine the effect of OIE on the migration ability of MDA-MB-231 cells, the *in vitro* migration assay was performed using the Transwell chamber system (6.5 mm diameter, 0.4 µm pore size). Briefly, MDA-MB-231 cells (1×10^4 cells/well) were seeded on the upper well of chambers placed in a 24-well plate in 450 µL of serum-free medium containing indicated concentrations of OIE (25, 50, 100, and 150 µg/mL), baicalein 3.75 µg/mL or pazopanib 4.37 µg/mL. The lower chamber was filled with 750 µL of culture medium supplemented with 10% FBS. Cells were incubated undisturbed in the CO_2 incubator for 18 hrs. The cells on the upper side of the inserts were removed with a cotton swab. The migrated cells on the lower surface were fixed and stained with crystal violet. The images were captured, and the numbers of migrated cells were counted in four different microscopic fields. Results are expressed as the percentage of migrated cells as compared to the respective control. The percentage of migration was calculated according to the following equation:

$$\%\text{transwell migration} = \frac{N_{\text{test group}}}{N_{\text{control}}} \times 100$$

Where N_{sample} = the cell numbers of the test group in the lower chamber.

$N_{control}$ = the cell numbers of the control group in the lower chamber.

3.2.5 Cell migration assay

3.2.5.1 The toxicity of OIE on natural angiogenesis in chick chorioallantoic membrane (CAM) model

To evaluate the effect of OIE on natural angiogenesis in the CAM was evaluated at 24 and 48 hrs post-exposure, and the CAM assay was performed following the method of Sala-ngam (Sala-ngam et al., 2016). Briefly, the fertilized chicken eggs were used after incubating them for 4 days. A 2 cm^2 window was made in the shell to create a pocket to expose the CAM. A filter disc in the presence or absence of OIE with indicated concentrations (25, 50, 100 and 150 µg/mL), baicalein 3.75 µg/mL or pazopanib 4.37 µg/mL was placed upon the CAM, and 20 µL of 100 U/mL penicillin was immediately added. The exposed hole in each egg was closed with tape and

further incubated for 24 and 48 hrs. After incubation, the CAM images were observed using a stereomicroscope with 40×magnification equipped with a DinoEye digital eyepiece and photographed. From the take, a photograph, the numbers of blood vessels in direct contact with the filter disk placed on the CAM were counted, as shown in Figure 3.1. The neovascularization was calculated according to the following equation:

$$\text{Neovascularization} = V_{24 \text{ or } 48 \text{ hrs}} - V_0$$

Where $V_{24 \text{ or } 48}$ = The vessel numbers around the disk at 24 or 48 hrs

V_0 = The vessel Numbers around the disk at 0 hrs

Figure 3.1 Blood vessels on CAM. Arrows indicate total blood vessels around the CAM (magnified×40).

3.2.5.2 The anti-angiogenic effect of OIE on MDA-MB-231-induced angiogenesis in the CAM model

The inhibitory effect of OIE on MDA-MB-231-induced angiogenesis was evaluated in the CAM model. Briefly, the fertilized chicken eggs were used after incubating them for 4 days. A 2 cm^2 window was made in the shell to create a pocket to expose the CAM. MDA-MB-231 cells at a density of 1×10^6 cells were placed directly onto the CAM, then a filter disc in the presence or absence of OIE (25, 50, 100, and 150 µg/mL), baicalein 3.75 µg/mL, or pazopanib 4.37 µg/mL, was then placed upon the CAM. After that, 20 uL of penicillin (100 U/mL) was immediately added to the CAM. The exposed hole in each egg was closed with tape and further incubated for 24 and

48 hrs. After incubation, the CAM images were observed using a stereomicroscope with 40×magnification equipped with a DinoEye digital eyepiece and photographed. Angiogenesis was quantitated by counting the number of blood vessels in direct contact with the filter disk.

3.2.6 Statistical analysis

All statistical significances (Statistical Package for the Social Sciences, version 19) were determined by performing a one-way analysis of variance (ANOVA) with a Tukey's post hoc analysis to determine differences among each treated and control groups. Values were considered statistically significant when $p < 0.05$, and data was representative of at least three independent experiments.

CHAPTER IV
RESULT AND DISCUSSION

4.1 Extraction yield

A 1.0 kg weight of fruit pods from *O. indicum* was extracted with 95% ethanol by a soxhlation for 8 h. The ethanol extract was evaporated and lyophilized to obtain a final yield of 18.41% (w/w) *O. indicum* extract (OIE).

4.2 Cytotoxic effect of OIE against MDA-MB-231 cells

The effects of OIE on the proliferation of human breast cancer cells were investigated. Briefly, MDA-MB-231 cell lines were treated with various concentrations of OIE, and then the viable cell was determined by CKK-8 assay. The CKK-8 assay has been widely used for measuring cell survival and proliferation. Dojindo's highly water-soluble tetrazolium salt, WST-8, is reduced by dehydrogenase activities in cells to give a yellow-color formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye generated by the activities of dehydrogenases in cells is directly proportional to the number of living cells. The cells were incubated for 24 hrs with various concentrations of OIE ranging from 25-1,000 µg/mL. Figure 4.1 showed that MDA-MB-231 cells exhibited different susceptibility to OIE in a dose-dependent manner. OIE up to concentration of 600 µg/mL had no toxicity towards MDA-MB-231 cells ($p > 0.05$). However, 600 µg/mL of OIE displays slightly decreased the viability of MDA-MB-231 by 9.5% ($p > 0.05$) but not significant. At high concentrations (800 and 1,000 µg/mL), OIE showed the cytotoxicity on MDA-MB-231 cells by approximately 50-60%. At 24 hrs, OIE showed cytotoxicity on MDA-MB-231 cells with IC_{50} values of 800 µg/mL. Therefore, OIE at concentrations of 25, 50, 100, and 150 µg/mL should be considered relatively safe for further investigation to exclude the OIE's cytotoxicity affecting the cell's migrastatic and angiogenic abilities in subsequent experiments.

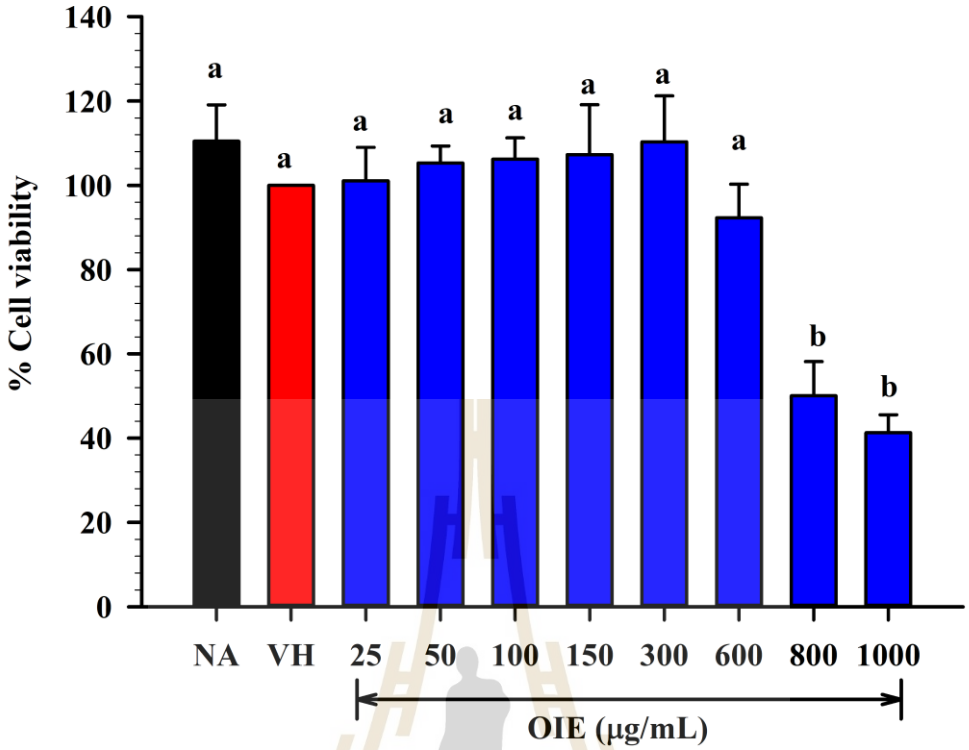

Figure 4.1 MDA-MB 231 were treated with indicated concentrations of OIE for 24 hrs, and then cell viability was measured using CCK-8 assay. Values are expressed as a percentage of the control. The data represent the mean ± SD of three independent experiments. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA with the Tukey post hoc test. NA is Naïve, cells alone in media, VH is vehicle control, cells in 0.1% v/v of DMSO diluted in media.

4.3 OIE inhibited the colony-forming ability of MDA-MB-231 cells

A colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony formation assays were performed to examine the long-term anti-carcinogenic effect of OIE on cell proliferation in MDA-MB-231 human breast cancer cells. The number of colonies was dramatically decreased in MDA-MB-231 cells treated with OIE in a dose-dependent manner compared to the control group (Figure 4.2A and 4.2B). The results showed that OIE treatment significantly inhibited the colony-forming ability of MDA-MB-231 cells starting from 150 to 600 µg/mL compared to control ($p < 0.05$). OIE at a concentration of 150, 300, and 600 µg/mL inhibited the colony formation by approximately 40%, 69%, and 100%,

respectively. Interestingly, OIE at a concentration of 600 µg/mL was as potent as the anticancer drug, pazopanib 4.37 µg/mL, in inhibiting MDA-MB-231 colony formation. According to the study of Benjawan and teams (2020), they reported that the amount of baicalein was detected in OIE with a concentration of 25,498.16 µg/g. Based on these findings, OIE at a concentration of 150 µg/mL may contain approximately 3.75 µg/mL baicalein. In order to clarify whether baicalein could be employed as the main active ingredient to inhibit the colony formation or not, baicalein at a concentration of 3.75 µg/mL was used as a positive control. The result showed that baicalein decreased the MDA-MB-231 colony formation by approximately 10% (Figure 4.2B). This result indicates that baicalein in OIE could inhibit the colony formation of MDA-MB231 cells. However, OIE at 150 µg/mL, which contained baicalein around 3.75 µg/mL, showed a significantly stronger inhibitory effect on MDA-MB231 colony formation than baicalein alone ($p < 0.05$).

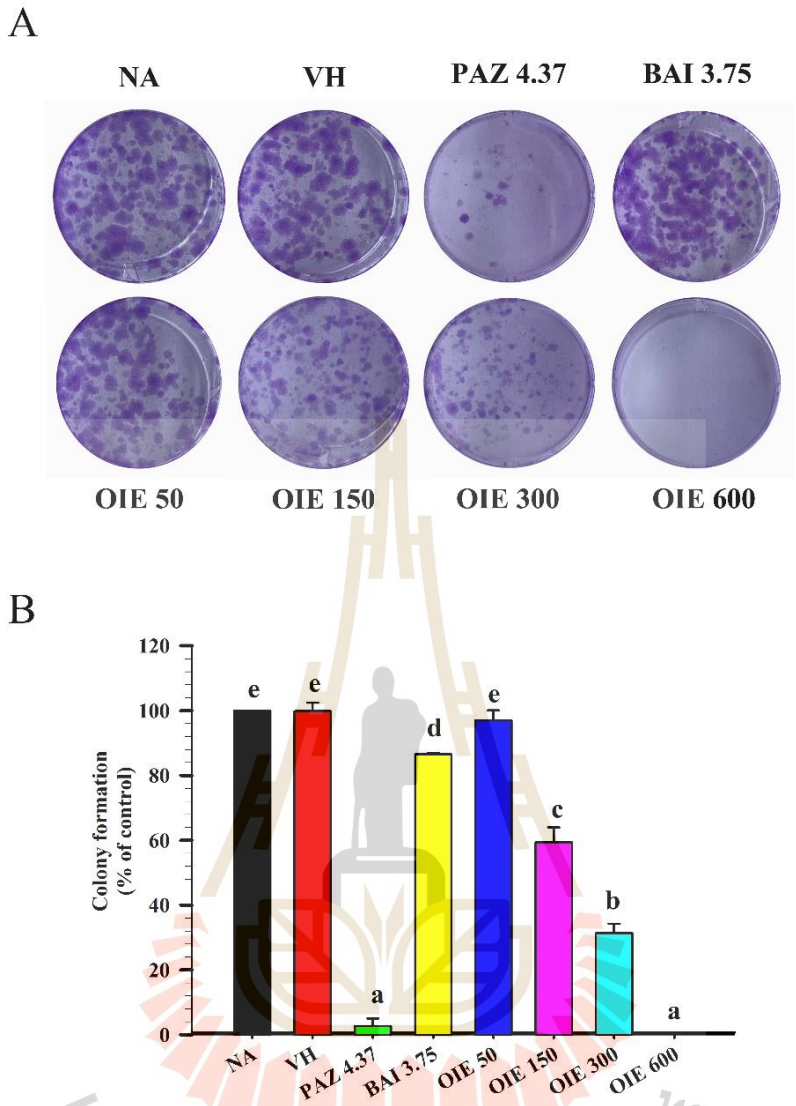

Figure 4.2 Effect of OIE on colony formation. MDA-MB-231 cells were treated with the indicated concentration of OIE and further incubated for 14 days. After the incubation, the colony formation was staining with crystal violet. A) The representative image of the colony formation after stained with crystal violet. B) The number of colonies was counted and expressed as the percentage of control. The data represent the mean ± SD of three independent experiments. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA with the Tukey post hoc test. NA = Naïve, cells alone in media; VH = vehicle control, cells in 0.1% v/v of DMSO diluted in media; PAZ 4.37, BAI 3.75, and OIE 50-OIE 600 = cells were treated with pazopanib 4.37 µg/mL, baicalein 3.75 µg/mL, and *O. indicum* extract at ranges of 50-600 µg/mL, respectively.

4.4 OIE suppressed the migration of MDA-MB-231 cells

Cell migration is an essential step in cancer metastasis (Lou et al., 2021). The effect of OIE on cell migration was examined by performing a wound-healing assay with non-lethal concentrations and incubation for 18 and 24 hrs. Treatment with OIE significantly suppressed the closing of wounds in a concentration-dependent manner (Figures 4.3A and 4.3B). The highest concentration of OIE strongly inhibited the migration of MDA-MBA-231 cells, as seen by the decrease in the percentages of wound closure by approximately 25% and 32% after OIE treatment for 18 and 24 hrs, respectively. These results suggest that OIE possessed the inhibitory effect on MDA-MB-231 cell migration. As expected, pazopanib (4.37 µg/mL) and baicalein (3.75 µg/mL) significantly decreased the wound closure compared to VH control cells at both 18 and 24 hrs ($p < 0.05$). Interestingly, OIE at 150 µg/mL showed a significantly stronger inhibitory effect on MDA-MB-231 cell migration than baicalein alone ($p < 0.05$) at 18 and 24 hrs of incubation. These additional inhibitory effects may act from other flavonoids or compounds in OIE. The results indicate that OIE can effectively inhibit the migration of MDA-MB-231 cells while had no cytotoxic effect on cell viability at these concentrations.

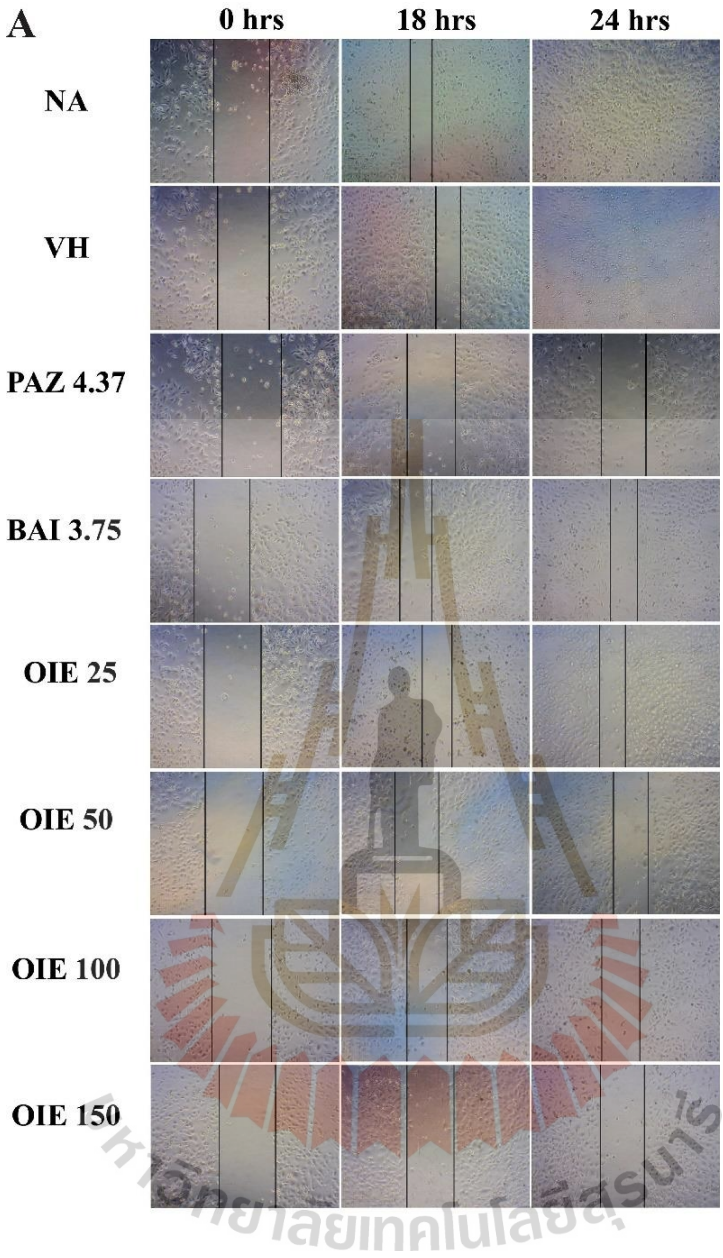

Figure 4.3 Effects of OIE on the migration of MDA-MB-231 cells. Cells were treated with indicated concentrations of OIE for 18 and 24 hrs. Then, the inhibitory effect of OIE on MDA-MB-231 cell migration was evaluated by wound-healing assay. (A) Representative images of wound scratches were taken at 0, 18, and 24 hrs under the inverted microscope (40×magnification). The scale bar in the figure is 100 μm.

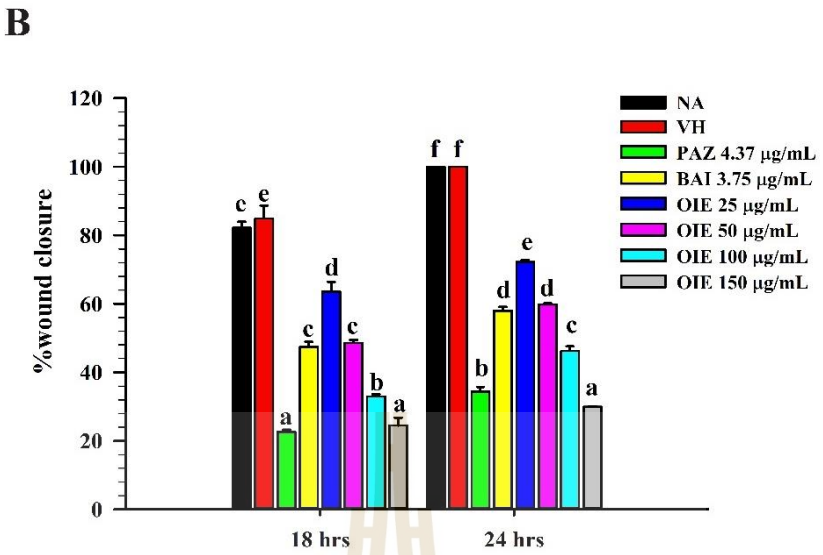

Figure 4.3 (Continued) (B) The migration rate of the cells toward the wounds was expressed as the percentage of wound closure at 18 and 24 hrs. The percentage of wound closure was normalized to the wound area at 0 h. The data represent the mean ± SD of three independent experiments. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA with the Tukey post hoc test. NA = Naïve, cells alone in media; VH = vehicle control, cells in 0.1% v/v of DMSO diluted in media; PAZ 4.37, BAI 3.75, and OIE 25-OIE 150 = cells were treated with pazopanib 4.37 µg/mL, baicalein 3.75 µg/mL, and *O. indicum* extract at ranges of 25-150 µg/mL, respectively.

4.5 OIE inhibited the transwell migration of MDA-MB-231 cells

The cell migration was further confirmed by Transwell assay. Similar to the wound healing assay results, OIE significantly decreased MDA-MB-231 cell migration toward a serum chemoattractant in a concentration-dependent manner ($p < 0.05$; Figure 4.4A and 4.4B). Quantification analysis indicated that migration reduced by 31%, 46%, 57%, and 77% after being treated with OIE at 25, 50, 100, and 150 µg/mL, respectively. The results indicate that OIE can effectively inhibit the motility of MDA-MB-231 cells since OIE had no cytotoxic effect on cell viability at these concentrations. This result confirmed the inhibitory effect of OIE in MDA-MB-231 cell migration. As expected, Pazopanib showed a highly anti-migration effect on MDA-MB-231 cells, while baicalein did not exhibit such an effect.

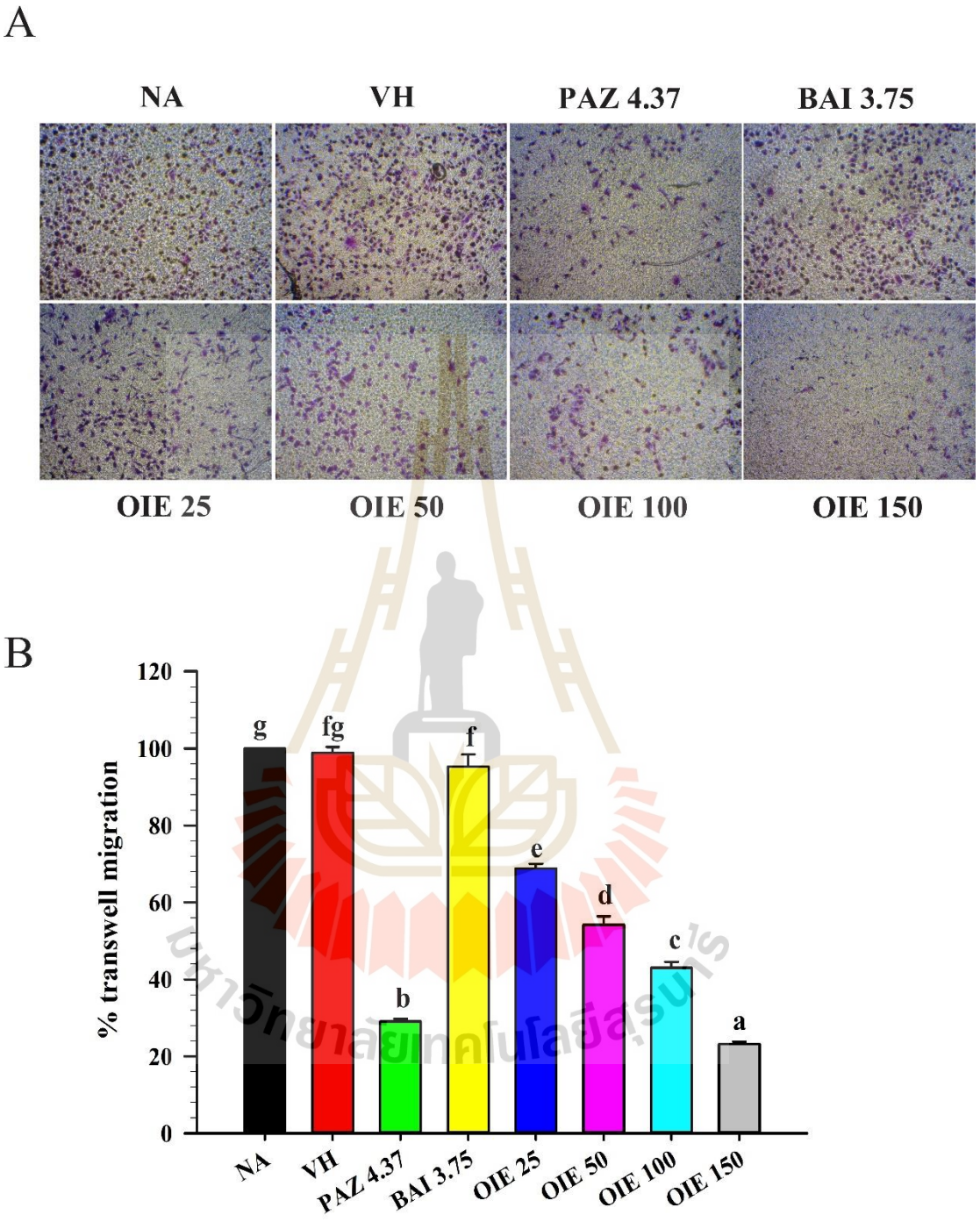

Figure 4.4 Effect of OIE on MDA-MB-231 cell migration. MDA-MB-231 cells were treated with indicated concentrations of OIE for 18 hrs. After incubation, the cells on the upper side of the membrane were removed with a cotton swab. The migrated cells on the lower surface were fixed and stained with crystal violet. (A) The images were captured under the inverted microscope (40× magnification). (B) The numbers of invading cells

were counted in four different microscopic fields and the results were expressed as the percentage of migrated cells. The data represent the mean ± SD of three independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA with the Tukey post hoc test. NA = Naïve, cells alone in media; VH = vehicle control, cells in 0.1% v/v of DMSO diluted in media; PAZ 4.37, BAI 3.75, and OIE25-OIE150 = cells were treated with pazopanib 4.37 µg/mL, baicalein 3.75 µg/mL, and *O. indicum* extract at ranges of 25-150 µg/mL, respectively.

4.6 Toxicity of OIE on the Chick Chorioallantoic Membrane (CAM) model

The optimum concentration of OIE used in the Chick Chorioallantoic Membrane (CAM) model was performed. The effects of OIE on natural angiogenesis in the CAM was evaluated at 24 and 48 hrs post-exposure, as shown in Figure 4.5A and 4.5B. The results demonstrate that VH control (0.1% DMSO) did not affect the number of neovascular formation at both 24 and 48 hrs, compared to NA (media alone) ($p > 0.05$). These results indicate that 0.1% DMSO solution should be considered safe for CAM. Likewise, the exposure to OIE ranging from 25-150 µg/mL for 24 and 48 hrs had no significant alteration in the number of neovascular formations compared to VH ($p > 0.05$). Consequently, the concentration up to 150 µg/mL of OIE, which had no suppression on natural angiogenesis generation, was chosen for subsequent anti-angiogenesis study.

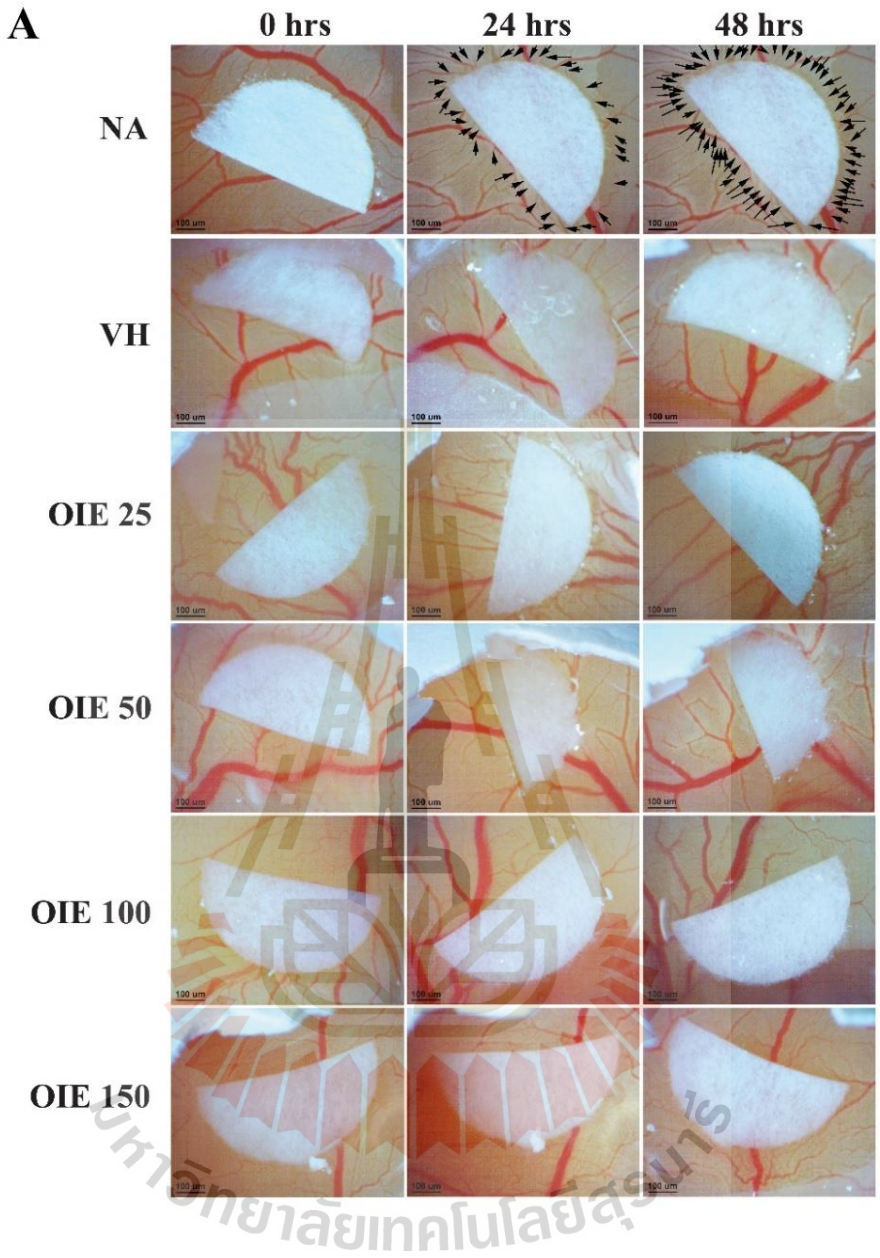

Figure 4.5 Effect of OIE on normal angiogenesis in the CAM model at 24 and 48 hrs. (A) Representative images of OIE on natural angiogenesis in the Chick Chorioallantoic Membrane (CAM) model under the inverted microscope (40× magnification). (B) The numbers of the number of neovascular formations at 24 and 48 hrs post-exposure. Values are expressed as means ± SD (n = 4) and represent two independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA with the Tukey post hoc test.

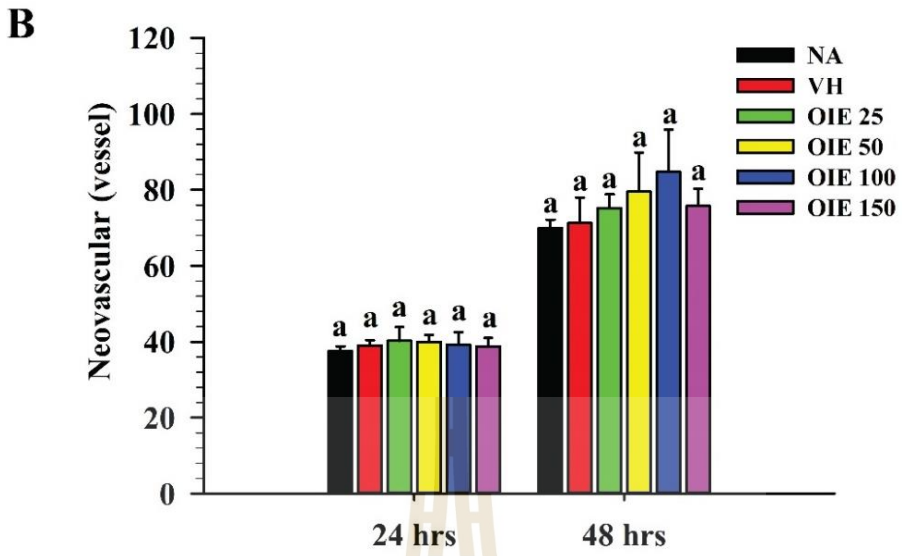

Figure 4.5 (Continued) NA = Naïve, culture medium; VH = vehicle control, 0.1% v/v of DMSO diluted in media; OIE25-OIE150 = cells were treated with *O. indicum* extract at ranges of 25-150 µg/mL, respectively.

4.7 Anti-angiogenic activity of OIE on MDA-MB-231-induced angiogenesis in the CAM model

A CAM assay was performed to evaluate the anti-angiogenic effect of OIE on MDA-MB-231-induced angiogenesis. Figures 4.6A and 4.6B show that OIE clearly produced a dose-dependent suppression of MDA-MB-231-induced neovascularization in the CAM model ($p < 0.05$) at both 24 and 48 hrs. The highest concentration of OIE at 150 µg/mL caused the reduction of MDA-MB-231-induced neovascularization by 89% and 86% after OIE treatment at 24 and 48 hrs, respectively, compared to VH control (MDA-MB-231-induced neovascularization in CAM). Likewise, the number of neovascularization induced by MDA-MB-231 was also reduced by approximately 49% and 20% after exposure to baicalein at 24 and 48 hrs, respectively. These findings support the claim that baicalein is the main active ingredient in OIE and that it is responsible for its anti-angiogenic activities. As expected, pazopanib exhibited a strongly anti-angiogenic potential by reducing MDA-MB-231-induced neovascularization by approximately 88-90% compared to VH control at both 24 and 48 hrs.

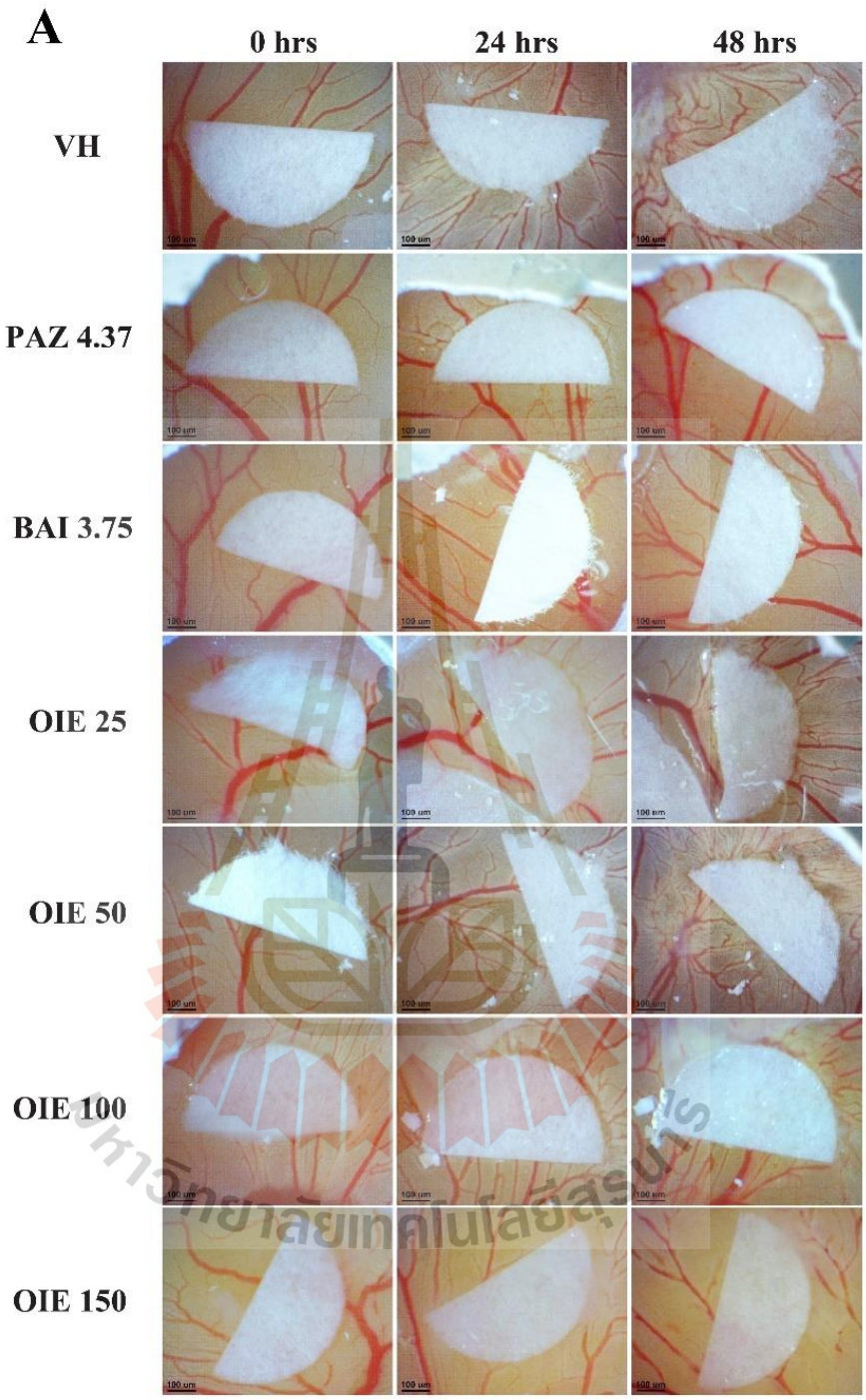

Figure 4.6 Anti-angiogenic activity of OIE on MDA-MB-231-induced angiogenesis in the CAM model at 24 and 48 hrs. Values are expressed as means ± SD (n = 4) and are representative of three independent experiments with similar results. Bars marked with different letters are significantly different at $p < 0.05$ as determined by one-way ANOVA.

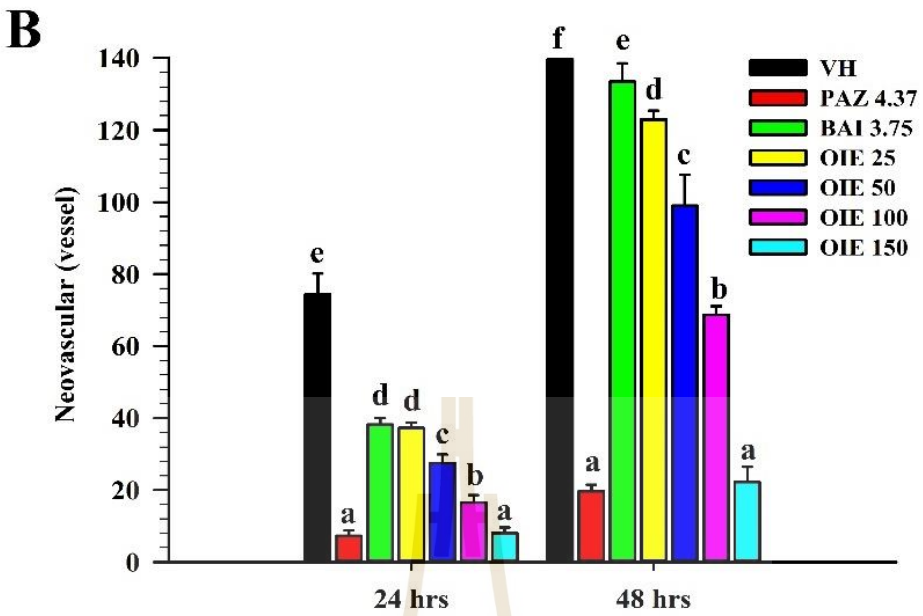

Figure 4.6 (Continued) NA = Naïve, cells alone in media; VH = vehicle control, cells in 0.1% v/v of DMSO diluted in media; PAZ 4.37, BAI 3.75, and OIE25-OIE150 = cells were treated with pazopanib 4.37 µg/mL, baicalein 3.75 µg/mL, and *O. indicum* extract at ranges of 25-150 µg/mL, respectively.

CHAPTER V
DISCUSSION

Breast cancer is the leading cause of cancer-associated mortality among women. Treatment of breast cancer becomes difficult after the tumor cells metastasize into distant body organs. Initiation of angiogenesis plays a critical role in breast cancer progression, invasion, and metastasis (Sinha et al., 2016). Therefore, it is of great need to develop novel diagnostic and therapeutic agents to improve breast cancer treatment. Although the chemical drugs significantly destroy tumor cells but also have a strong toxic side effect. This study was particularly interested in using a highly metastatic aggressive breast cancer, MDA-MB-231, cell lines as a model for investigating the anticancer activity of *Oroxylum indicum* (*O. indicum*) in breast cancer.

Since most medicinal plants occur naturally in many countries, a plant of potential importance in one country may well have been studied by scientists elsewhere. Natural products from plants, animals, and minerals are the basis for treating human diseases. *O. indicum* belongs to the family Bignoniaceae and is a well-known food and herbal medicine in many Asian countries, including Thailand. *O. indicum* has been used as a single drug or as a component of specific compound drug preparations in the Indian Ayurvedic medicine system for various disorders, including cancer (Dev et al., 2010). *O. indicum* also possesses strong hepatoprotective, antiprostatic hyperplasia, antioxidant, anti-inflammatory, antiproliferative, anti-adipogenesis, gastroprotective, and antitumor activities (Chetry and Bharali, 2018; Dunkhunthod et al., 2020; Hengpratom et al., 2020). Notably, the crude extracts and compounds from *O. indicum* have been shown to inhibit several cancer cell types, such as liver cancer, leukemia, and cervical cancer, including breast cancer cells (Dinda et al., 2015; Liu et al., 2018; Naveen Kumar et al., 2012). In addition, *O. indicum* has been reported to possess anti-cancer activity against both estrogen receptor (ER)-negative breast cancer cells (MDA-MB-231) and the ER-positive breast cancer cells (MCF-7) (Buranrat et al., 2020; Buranrat et al., 2018; Naveen Kumar et al., 2012). The

study by Naveen Kumar et al. (2012) indicated that the stem bark extract from *O. Indicum* more specifically reacts to ER- negative breast cancer cells (MDA-MB-231) than ER-positive breast cancer cells (MCF-7) cell lines. Some previous reports have demonstrated the anti-angiogenic effect of *O. indicum* on vascular endothelium growth factor (VEGF)-induced neovascularization in the CAM model (Talari et al., 2017). However, to our knowledge, no previous study has verified the anti-angiogenic effect of *O. indicum* using breast cancer cells-induced angiogenesis. This study aimed to investigate the anticancer activity of OIE in breast cancer MDA-MB-231 cell lines, assessing proliferation, colony formation, and migration of these cells, as well as the anti-angiogenic activity of OIE.

The cytotoxic effect of OIE was first investigated using the CCK-8 assay. The results of the current study demonstrate that OIE up to a concentration of 600 µg/mL had no directed cytotoxicity against MDA-MB-231. In contrast, the high dose (800-1000 µg/mL) induced approximately 50-60% cell death. The 600 µg/mL OIE concentration could be considered a maximum tolerated concentration as this level caused less than 10% cell death in MDA-MB-231 in our experiments.

The ability to self-renew and differentiate gives rise to the heterogeneous phenotype of the tumor cells. These cells are believed to be involved in metastasis, recurrence, and therapy resistance in various cancers (Chen et al., 2014). The colony formation assay was applied to detect the ability of single cells to survive and reproduce to form colonies after OIE treatment. According to cell viability results, the non-cytotoxic concentration ranges of OIE (25-600 µg/mL) were chosen to treat MDA-MB-231 for 24 hrs and then incubated in a culture medium for 14 days. Indeed, the colony formation of MDA-MB-231 cells was significantly suppressed with an increasing concentration of OIE up to more than 95% at 600 µg/mL compared to the control. These results suggest that OIE demonstrates a long-term suppressive effect on cell proliferation of MDA-MB-231 cells in a concentration-dependent manner.

Cell migration and invasion play an important role in the development of cancer. Regardless of advancements in local cancer treatments, there is a higher chance of clinical challenges combating systemic metastatic disease (Shevde-Samant and Welch, 2003). The wound-healing assay is straightforward and gives valuable initial

information about cell front migration but does not provide dynamic information. The transwell migration was further used to analyze the ability of single cells to respond to chemoattractants directionally. The wound healing and transwell migration experiments were performed on MDA-MB-231 breast cancer cells to investigate the anti-migration activity of OIE. To more compromise for exclusion of the concentration that was cytotoxic MDA-MB-231, the concentration up to 150 µg/mL of OIE was selected for investigation in the experiment. The wound healing assays showed that OIE significantly suppressed cell migration of MDA-MB-231 breast cancer cells in a dose-dependent manner (Figure 3A and 3B). Furthermore, the migratory function of cells was confirmed by transwell migration showing that OIE drastically inhibited the migration of MDA-MB-231 breast cancer cells. Together, these results strongly suggest that OIE could contribute to hindering metastasis progression in breast cancer. Results similar to this have also been reported by Naveen Kumar et al. (2012), who found that the petroleum ether hot extract of *O. indicum* has demonstrated an obvious inhibition of cell migration in MDA-MB-231 cells along with few morphological changes to indicate arrest in the cell proliferation and migration. A similar observation was also reported in MCF-7 breast cancer cells, where *O. indicum* leaf and fruit extracts exhibited an anti-cancer effect by inhibiting colony formation and cell migration, reducing both MMP9 and ICAMP1 gene expression and MMP9 protein expression (Buranrat et al., 2020; Buranrat et al., 2018). Expressions of MMP-9 and ICAMP1 are closely linked to the growth, invasion, metastasis, and angiogenesis of cancer cells (Ahn and Brown, 2008; Bergers et al., 2000; Deng et al., 2007). This literature triggers us believe that *O. indicum* may also be able to reduce angiogenesis. Therefore, the anti-angiogenic effects of OIE were further investigated using the CAM model.

Angiogenesis is a term that describes the formation of new blood and lymphatic vessels from pre-existing vasculature. Angiogenesis plays a key role in tumor growth, invasion, and metastasis of cancer diseases; therefore, the inhibition of angiogenesis could be a strategy to arrest tumor growth and metastasis (Carmeliet and Jain, 2000; Saman et al., 2020). In this study, the toxicity of OIE on chick embryos was first evaluated to establish the safe dosing of the extract in the CAM model. The results revealed that a wide range of OIE concentrations (25-150 µg/mL) had no toxicity on

chick embryos. The tested concentration did not suppress the natural angiogenesis compared to control at 24 and 48 hrs. Therefore, OIE concentration up to 150 µg/mL was selected to investigate the anti-angiogenesis effect of OIE on the CAM model. The result exhibited that OIE could show anti-angiogenic effects on the CAM model in a concentration-dependent manner. OIE at the concentration range of 25-150 µg/mL suppressed MDA-MB-231-induced angiogenesis by approximately 49-98% and 23-87% after OIE exposure at 24 and 48 hrs, respectively, compared to the control group. In the safety assay, OIE at concentration ranges of 25-150 µg/mL did not show any toxicity on the MDA-MB-231 cells, and there was no substantial toxic effect on natural angiogenesis. The data confirm that OIE shows a full anti-angiogenesis effect without significantly harming the cells and natural angiogenesis. This study's data agree with the results of Talari et al. (2017), showing that *O. indicum* exhibited the anti-angiogenic effect on VEGF-induced neovascularization in the CAM model. In addition, the present work elucidates for the first time the anti-angiogenesis properties of *O. indicum* toward breast cancer cells-induced angiogenesis in the CAM model.

Our team's previous study found that five flavonoids and 27 volatile compounds were found in OIE (Benjawan et al., 2020; Hengpratom et al., 2020). The major flavonoid compounds in OIE were baicalein, oroxylin A, luteolin, quercetin, and apigenin. They have also reported that the amount of baicalein was detected in OIE with a concentration of 25,498.16 µg/g (Dunkhunthod et al., 2020). Therefore, the amount of baicalein present in OIE at a 150 ug/mL concentration was approximately 3.75 ug/mL. To clarify whether baicalein contributed to the anti-cancer activity of OIE or not? So, baicalein 3.75 µg/mL was used as a positive control. Many studies have shown that baicalein exhibits anticancer properties, which are attributed at least partially through the inhibition of cell proliferation, cell migration and invasion in many cell types, including AGS human gastric cancer cells, DLD1 colorectal cancer cells, B16F10 melanoma cells, vein endothelial cells (HUVECs), and MDA-MB-231 breast cancer cells (Chen et al., 2014; Choi et al., 2017; Gao et al., 2015; Rui et al., 2016). Likewise, many researchers have also reported the anti-angiogenic activity of baicalein (Huang et al., 2017; Liu et al., 2003; Park et al., 2017). Similar to other investigators, this study further confirmed the anticancer activity of baicalein since baicalein showed the

inhibition of colony formation, cell migration, and angiogenesis in breast cancer MDA-MB-231 cells. Compared to OIE at a 150 ug/mL concentration, baicalein alone had a lower anticancer potency than OIE (150 ug/mL) throughout each investigation. The higher potency of OIE than baicalein may be due to OIE's other bioactive compounds, such as luteolin, apigenin, quercetin, and other volatile oils compounds (Dunkhunthod et al., 2020). These compounds have been demonstrated to possess anti-cancer properties and suppress metastasis and angiogenesis progression in several cancers (Kim et al., 2011; Li et al., 2017; Qian et al., 2022; Song et al., 2017). These reports lead us to believe synergistic activity could occur between baicalein and other flavonoids or volatile compounds in enhancing the anticancer activity of OIE.

In the present study, the anticancer mechanism of OIE was confirmed by using pazopanib as a positive anticancer drug. Pazopanib is a pan-vascular endothelial growth factor receptor inhibitor, and preclinical work indicates that pazopanib exerts an anti-cancer effect by inhibiting both angiogenic and oncogenic signaling pathways (Lee et al., 2019; Zhu et al., 2011). As expected, pazopanib exhibited a strongly anti-cancer potency by suppressing colony formation, cell migration, and angiogenesis in breast cancer MDA-MB-231 cells. Interestingly, the highest concentration of OIE (150 µg/mL) was as potent as the positive anticancer drug, pazopanib, at 4.37 µg/mL, in inhibiting MDA-MB-231 cell migration and angiogenesis induced by these cells.

The findings in the current study suggest that OIE possesses anticancer activity in suppressing colony formation and cell migration and also inhibits MDA-MB-231-induced angiogenesis. The underlying mechanism behind the anti-migratory and anti-angiogenic effects of OIE in MDA-MB-231 breast cancer cells should be further investigated. Nevertheless, this inhibition of cell proliferation, cell migration, and tumor-induced angiogenic activities in OIE could explain, at least in part, the claimed property of anti-cancer activity of *O. indicum*.

CHAPTER VI
CONCLUSION

O. indicum has been used as a traditional medicine in Asian countries to prevent and treat several diseases, including cancer. This plant has been reported to possess anti-cancer activity against several cancer cell types, including breast cancer cells. The results of the current study demonstrated that OIE up to a concentration of 600 µg/mL had no directed cytotoxicity against MDA-MB-231 cells. So, the non-cytotoxic concentration of OIE was chosen to treat MDA-MB-231 in subsequence experiments. The results showed that the colony formation of MDA-MB-231 cells was significantly suppressed with increasing concentration of OIE up to more than 95% at 600 µg/mL, compared to the control ($p < 0.05$). To further investigate the anti-migration activity of OIE, the results from wound healing and transwell assays indicated that OIE significantly suppressed cell migration of MDA-MB-231 breast cancer cells in a dose-dependent manner ($p < 0.05$). The present study elucidates for the first time the anti-angiogenesis properties of *O. indicum* toward breast cancer cells-induced angiogenesis. The anti-angiogenesis study revealed that OIE showed a full anti-angiogenesis effect without any substantial toxic impact on the cells and natural angiogenesis. To clarify whether baicalein contributed to the anti-cancer activity of OIE or not? This study also revealed that the anticancer capacity of the OIE was more potent than that of baicalein alone. This finding suggests that synergistic activity between baicalein, and other flavonoids or volatile compounds could enhance the anticancer activity of OIE. The anticancer capacity of OIE and pazopanib was compared. The highest concentration of OIE (150 µg/mL) was practically potent, the same as the positive anticancer drug, pazopanib at 4.37 µg/mL, in inhibiting MDA-MB-231 cell migration and angiogenesis induced by these cells. In summary, these findings provide evidence that OIE possesses anticancer activity against human breast cancer MDA-MB-231 cells by inhibiting cell proliferation, cell migration and suppressing angiogenesis induced by these cells. OIE

may be developed as a promising agent for treating breast cancer metastasis in the future.

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APPENDICES

APPENDIX A

PREPARATION OF REAGENTS FOR CELL CULTURE

A.1 Phosphate buffer saline (PBS), 1X, pH 7.4

- KH2PO4 0.144 g
- $Na_2HPO_4.7H_2O$ 0.795 g
- NaCl 9.0 g
- DI water 1 L

Adjust pH to 7.2 ± 0.1 and filter sterile (store at 4 °C).

A.2 Culture media preparation

- FBS (heat-inactivated)
  - Slowly thaw the frozen FBS in a beaker filled with water.
  - Put in a water bath at 37 °C till it completely thaws.
  - Heat inactivate (56 °C, 30 min), gentle mix every 15 min.

  (Store at -20 °C).
- Leibovitz's L-15, 1X (incomplete medium)
  - Leibovitz's L-15, 1X with L-glutamine and phenol red 1 pack
  - $NaHCO_3$ 9.0 g
  - DI water 1 L

    Adjust pH to 7.2-7.4 and filter sterile (Store at 4 °C).
- Leibovitz's L-15, 1X (complete medium)
  - Inactivated FBS 20 mL
  - Penicillin/Streptomycin 2 mL
  - HEPES buffer, 1M 3 mL

    Adjust volume to 200 ml with RPMI 1640, 1X (incomplete medium, store at 4 °C).

APPENDIX B

PREPARATION OF REAGENTS FOR CELL CULTURE

B.1 Phosphate buffer saline (PBS), 1X, pH 7.4

- KH2PO4 0.144 g
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  - RPMI 1640, 1X with L-glutamine and phenol red 1 pack
  - $NaHCO_3$ 9.0 g
  - DI water 1 L

    Adjust pH to 7.2-7.4 and filter sterile (Store at 4 °C).
- RPMI 1640, 1X (complete medium)
  - Inactivated FBS 20 mL
  - Penicillin/Streptomycin 2 mL
  - HEPES buffer, 1M 3 mL

    Adjust volume to 200 ml with RPMI 1640, 1X (incomplete medium, store at 4 °C).

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INTERNATIONAL PUBLICATIONS (PEER-REVIEWED PAPERS)

1. Dunkhunthod, B., **Chiraatthakit, B.**, Chitsomboon, B., Kiatsongchai, R., Thumanu, K., Musika, S., and Sittisart, P. (2021). Apoptotic induction of the water fraction of *Pseuderanthemum palatiferum* ethanol extract powder in Jurkat cells monitored by FTIR microspectroscopy. *ScienceAsia. 47*(5): 556-566.

ORAL PRESENTATION AND PROCEEDING

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