UNIVERSITI PUTRA MALAYSIA

THE EFFECTS OF NOVEL PYRAZOLE DERIVATIVES (HEXUR-2) ON THE CYTOTOXICITY AND MIGRATION OF MDA-MB-231 HUMAN BRE AST CANCER CELLS

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SITI SARAH BINTI HASRAN

A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE (BIOMEDICAL SCIENCES)

DEPARTMENT OF BIOMEDICAL SCIENCES
FACULTY OF MEDICINE AND HEALTH SCIENCES
UNIVERSITI PUTRA MALAYSIA

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ABSTRACT

The Effects of Novel Pyrazole Derivatives (HEXUR-2) on the Cytotoxicity and Migration of MDA-MB-231 Human Breast Cancer Cells.

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Introduction: Breast cancer is the most frequent malignant cancer in women and the leading cause of cancer death worldwide. According to GLOBOCAN (2020), the percentage of new cases for women of all ages in Malaysia is 32.9%. Like any other cancer, it possesses the ability to metastasize and invade the surrounding tissues. This ability is often used to distinguish between aggressive and non-aggressive cancers. The treatments for breast cancers are already available, either chemotherapy treatment or removal of the tumor via surgery. In certain cases, both treatments may be required. However, there is still no effective therapy that is capable of killing cancer cells without destroying normal cells. Therefore, in this study, novel pyrazole derivatives (HEXUR-2) will be used to investigate the cytotoxic and migration effects on the MDA-MB-231 Human Breast Cancer Cells. **Objectives:** The objective of this study is to determine the effects of novel pyrazole derivatives (HEXUR-2) of the cytotoxicity and migration effects on MDA-MB-231 cells. **Methodology:** This study used MDA-MB-231 cells and were cultured in complete growth medium DMEM under humidified conditions. The compounds used are novel pyrazole derivatives (HEXUR-2). Cell viability and cytotoxic effects of HEXUR-2 on MDA-MB-231 cells were measured using MTT assay. The migration of MDA-MB-231 cells was observed using scratch migration assay. **Results:** MTT assay results showed that the IC_{50} values for MDA-MB-231 cells treated with HEXUR-2 were 75 ± 9.7, 33 ± 9.3 and 9 ± 9.5 µM at 24, 48 and 72 hours of incubation, respectively. The morphological changes such as apoptotic bodies, nuclear fragmentation and cell shrinkage were observed in MDA-MB-231 cells treated with HEXUR-2. Meanwhile, the treatment of HEXUR-2 on the MDA-MB-231 cells migration was observed at 0 and 24 hours. **Discussion:** HEXUR-2 was shown to significantly reduced the cytotoxicity effects in a dose-dependent manner with the IC_{50} observed at three different incubation periods. However, HEXUR-2 did not significantly reduce the cytotoxicity effects of MDA-MB-231 cells in a time-dependent manner. MDA-MB-231 cells treated with HEXUR-2 also showed the apoptotic characteristics such as cell shrinkage, nuclear fragmentation, and cells detachment, suggesting that the mode of the cell death might be due to the apoptosis. The treatment of HEXUR-2 also shown not to inhibit the migration of MDA-MB-231 cells compared to the untreated cells. **Conclusion:** Therefore, it is concluded that HEXUR-2 exhibits cytotoxic effects however the migration effects were not shown in the MDA-MB-231 cells.

Keywords: Novel pyrazole derivatives, cytotoxicity, metastasis, MDA-MB-231

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ABSTRAK

KESAN TERBITAN PYRAZOLE BARU (HEXUR-2) PADA SITOTOKSISITI DAN MIGRASI SEL KANSER PAYUDARA MANUSIA (MDA-MB-231)

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Pengenalan: Kanser payudara merupakan kanser malignan yang paling kerap dan penyebab utama kematian kanser dalam kalangan wanita di seluruh dunia. Menurut GLOBOCAN (2020), peratusan kes baharu dalam kalangan wanita dari semua peringkat umur di Malaysia adalah 32.9%. Seperti kanser yang lain, kanser payudara mempunyai kemampuan untuk bermigrasi dan menyerang tisu-tisu di sekitarnya. Keupayaan ini sering digunakan untuk membezakan kanser yang agresif dan tidak agresif. Rawatan untuk kanser payudara sedia ada termasuklah rawatan kemoterapi atau rawatan pembuangan barah melalui pembedahan. Dalam kes tertentu, kedua-dua rawatan mungkin diperlukan. Namun begitu, masih belum ada terapi yang berkesan mampu membunuh sel kanser tanpa memusnahkan sel normal. Oleh itu, dalam kajian ini, terbitan pyrazole (HEXUR-2) akan digunakan untuk menyiasat kesan sitotosik dan migrasi pada sel kanser payudara manusia (MDA-MB-231). **Objektif:** Secara amnya, kajian ini bertujuan untuk menentukan kesan terbitan pyrazole (HEXUR-2) pada sitotoksik dan migrasi sel kanser payudara manusia (MDA-MB-231). **Metodologi:** Sel MDA-MB-231 digunakan dalam kajian ini dan dikultur dalam media lengkap Dulbecco's modified eagle medium (DMEM) di bawah keadaan lembab. Sebatian yang digunakan adalah terbitan pyrazole baru (HEXUR-2). Keupayaan sel dan kesan sitotoksik HEXUR-2 pada sel MDA-MB-231 diuji mengunakan ujian MTT. Migrasi sel MDA-MB-231 diperhatikan menggunakan ujian "scratch migration". **Keputusan:** Keputusan ujian MTT bagi nilai IC_{50} untuk sel MDA-MB-231 yang dirawat dengan HEXUR-2 adalah 75 ± 9.7, 33 ± 9.3 and 9 ± 9.5 µM pada tempoh pengeraman yang berbeza (24, 48 dan 72 jam). Perubahan morfologi seperti pembentukan jasad apoptosis, pemecahan nuklear dan pengecutan sel diperhatikan pada sel MDA-MB-231 yang dirawat dengan HEXUR-2. Sementara itu, rawatan HEXUR-2 pada migrasi sel MDA-MB-231 diperhatikan pada 0 dan 24 jam. **Perbincangan:** HEXUR-2 terbukti dapat mengurangkan kesan sitotoksiti dengan bergantung kepada dos dan IC50 juga diperhatikan pada tiga tempoh pengeraman yang berbeza. Walau bagaimanapun, HEXUR-2 tidak mengurangkan kesan sitotoksisiti sel MDA-MB-231 secara signifikan bergantung kepada masa. Sel MDA-MB-231 yang dirawat dengan HEXUR-2 juga menunjukkan ciri-ciri apoptosis seperti penyusutan sel, pemecahan nuklear dan sel tercabut, menunjukkan bahawa cara kematian sel mungkin disebabkan oleh apoptosis. Rawatan HEXUR-2 juga terbukti tidak menghalang penghijarahan sel MDA-MB-231 berbanding dengan sel yang tidak dirawat. **Kesimpulan:** Oleh itu, keputusan menunjukkan HEXUR-2 menunjukkan kesan sitotoksik namun tidak menunjukkan kesan migrasi pada sel MDA-MB-231.

Kata kunci: terbitan pyrazole, sitotosik, metastatik, MDA-MB-231

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LIST OF ABBREVIATIONS

µL	Microliter
5-FU	5-fluorouracil
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BPA	Bisphenol A
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
BSC	Biosafety cabinet
CTC	Circulating tumour cell
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDC	Endocrine disrupting compounds
ER	Estrogen receptor
FBS	Fetal Bovine Serum
FHS	Family history score
GLOBOCAN	Global Cancer Data
HER2	Human epidermal Growth Factor Receptor 2
IC_{50}	Half maximal inhibitory concentration
MAPK	Mitogen-activated protein kinase
P13K	Phosphoinositide 3-kinase
P53	Tumor protein P53
PBS	Phosphate-buffered saline
PR	Progesterone receptor
SDS	Sodium dodecyl sulfate
SEER	Surveillance, Epidemiology and End Results Program
SEM	Standard Error Mean
TNBC	Triple negative breast cancer
VEGF	Vascular endothelial growth factor

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CHAPTER 1

INTRODUCTION

1.1 Study background

Breast cancer is the most prevalent type of cancer in women and the major cause of cancer death. According to GLOBOCAN statistics, 2.3 million new cases (11.7%) are estimated to be recorded worldwide in 2020 (Sung et al., 2020). It was shown to be the highest rank among new cases in Malaysian women of all ages in females in 2020 (GLOBOCAN, 2020). The rising number of new cases contributes significantly to Malaysia's and the world's high mortality rates of breast cancer.

Breast cancer is a type of cancer that develops in the breast cells. It affects more single women than married women and it caused by the interaction of genetic and environmental factors. Breast cancer is categorized into two types which are non-invasive and invasive breast cancer. The example for non-invasive breast cancer is ductal carcinoma in situ (DCIS) whereas for invasive breast cancer is invasive lobular carcinoma. Invasive ductal carcinoma is the most prevalent type of invasive breast cancer, followed by invasive lobular carcinoma (Thomas et al., 2019).

Breast cancer stages range from 0 to 4. Breast cancer in stage 0 is non-invasive, whereas the cancer in stages 2, 3, and 4 has begun to beome invasive. Stage 4 is also described as metastatic and advanced invasive stage. Tumor metastasis, which involves cellular migration and dissemination to the other organs in the body, is one of the hallmarks of breast cancer. Breast cancer metastasis usually spreads to the lung, brain, liver, and bone, where it segregates to form a new cancer. However, it is still referred to as breast cancer (Akram et al., 2017).

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Triple Negative Breast Cancer (TNBC) is another type of breast cancer. TNBC is distinguished by the lack of progesterone receptor, estrogen receptor expression and human epidermal growth factor receptor 2. It is a destructive, aggressive that is common in premenopausal females. It also has demonstrated that women with TNBC have are more susceptible to develop brain metastases. Apart from that, the median survival time following brain metastasis is shorter than in other breast cancer phenotypes (Kumar et al., 2016).

Breast cancer can be diagnosed based on the patient and family medical history, as well as a physical examination that includes self-examination, ultrasound breast imaging, magnetic resonance imaging, breast biopsy and digital mammography as the standard method for breast cancer diagnosis. Breast cancer treatment and management include surgery, chemotherapeutic agents, and a variety of therapies such as targeted, radiation and hormonal therapy. Multi-targeted therapy has been suggested as a treatment option for metastatic breast cancer. Apart from that, chemotherapy has also been shown to slow the progression of metastatic breast cancer. However, there are still side effects from the treatment such as fatigue, nausea and diarrhea which serve as motivating factors to seek out other options for breast cancer treatment (Akram et al., 2017).

Pyrazole, an aromatic heterocyclic with three carbon atoms and two nitrogen atoms comes from azole family. According to Karrouchi et al. (2018), pyrazole has the antibacterial, anti-inflammatory, and antitumor properties. Pyrazolyl-urea, [ethyl 1-(2-hydroxypentyl)-5-(3-(3-(trifluoromethyl)phenyl)ureido)-1H-pyrazole-4-carboxylate] also known as GeGe3, is a pyrazole derivatives that has been shown to inhibit

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endothelial cells proliferation and migration. Thus, it was stated that it had the potential to be an antitumor compound (Meta et al., 2017).

1.2 Problem statement

The ability to migrate and infiltrate to the other tissues is one of the characteristics of malignant cancer (Tieng et al., 2019). Breast cancer is a type of malignant cancers that can cause death. Most breast cancer deaths are caused by metastasis to other organs in the body. Chemotherapy, which uses cytotoxic drugs such as 5-fluorouracil, anthracyclines and taxanes, is one of the treatments given for metastatic breast cancer. Resistance against chemotherapy and chemical drug toxicity continues to be a significant barrier to successful breast cancer treatment, resulting in recurrence, metastases, and poor outcomes (Fei, Huimei, & Dongmin, 2020). As a result, finding an alternative drug with lower side effects, toxicity and can inhibit the cancer metastatic ability has received a lot of attention around the world.

1.3 Justification

There is a need to investigate the cytotoxic and migratory effects of HEXUR-2 on MDA-MB-231 cells by studying the ability of the compounds to kill half of the cell's population and low dose drug concentration in the search as potential anticancer drugs.

1.4 Objectives

1.4.1 General objectives

The general objective of this study is to determine the cytotoxic and migratory effects of novel pyrazole derivatives (HEXUR-2) on MDA-MB-231 cells.

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1.4.2 Specific objectives

The specific objective of this study is to evaluate the cytotoxic effects of novel pyrazole derivatives (HEXUR-2) on MDA-MB-231 cells by using MTT assay and to study the migratory effects of novel pyrazole derivatives (HEXUR-2) on migration of MDA-MB-231 cells by using Scratch Migration assay.

1.5 Hypothesis

It is hypothesized that Novel Pyrazole derivatives (HEXUR-2) is capable to elicit cytotoxic and migratory effects on MDA-MB-231 human breast cancer cells.

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CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer

2.1.1 Introduction of breast cancer

Breast cancer is a type of cancer that develops in the breast cells. Breasts consist of three main parts which are connective tissue, lobules, and ducts. Majority of the breast cancers develop in the lobules or ducts. Breast cancer is classified into two types which are non-invasive and invasive breast cancer. Invasive breast cancer is a type of breast cancer that already metastasize surrounding the nearby organ (Akram et al., 2017). Ductal carcinoma in situ (DCIS) and invasive carcinoma are among the most frequent types of the breast cancer.

Breast cancer has several types which including Triple Negative Breast cancer (TNBC). TNBC is known for its absence of estrogen receptor expression (ER), Human epidermal Growth Factor Receptor 2 (HER2) and progesterone receptor (PR). TNBC is more frequent in certain races such as Latin, African American, and African contributing for approximately 10-15% of all breast cancer cases. TNBC is also known by its early relapse, aggresiveness and metastatic dissemination to the surrounding organs and tissues such as nervous system, lung, and liver, as well as having a poorer prognosis (Silva et al., 2020).

2.1.2 Epidemiology of breast cancer

Breast cancer is the most diagnosed cancer and the most mortarlity cases among females in the world. According to GLOBOCAN (2020), there were around 2.3 million new breast cancer cases among females of all ages worldwide, with the

estimated incidence rate at 24.5%. The overall mortality rate for female breast cancer is 15.5%, with 684 996 deaths (GLOBOCAN, 2020).

There are 21,634 cases of female breast cancer in Malaysia in 2012-2016, compared to 18,206 cases in 2007-2011. The Chinese had the most breast cancer cases, followed Indians and Malays (Malaysia National Cancer Registry, 2019). In 2020, breast cancer in Malaysia ranked as the highest incidence case with 8418 cases (32.9%), followed by colon rectum and ovary. The mortality rate among Malaysian females is 25.1%, with estimation of 3503 deaths.

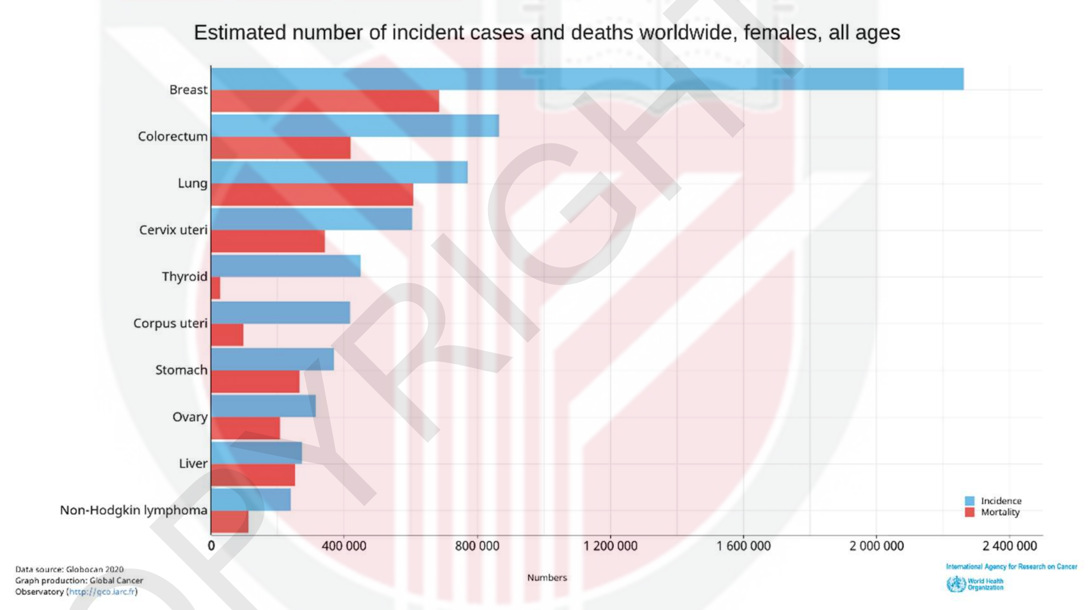

Figure 2.1.1. Multi-bar graph of incident and mortality of all cancers in females of all ages in 2020 (GLOBOCAN, 2020).

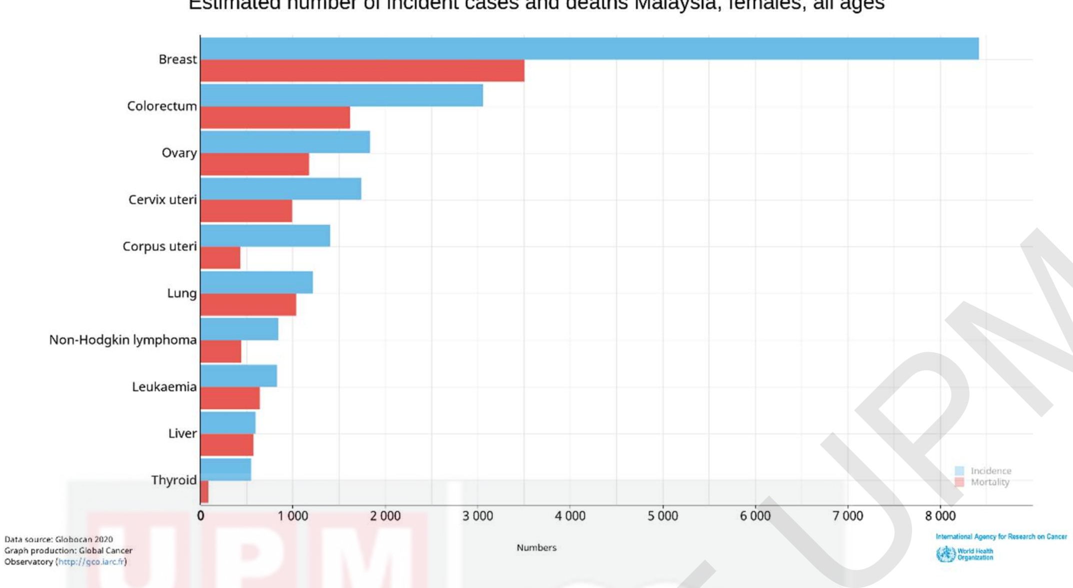

Figure 2.1.2. Multi-bar graph of incident and mortality of all cancers in Malaysian females of all ages in 2020 (GLOBOCAN, 2020).

2.1.3 Pathogenesis of breast cancer

Breast cancers are frequently caused by the association between genetic and environmental factors. Deoxyribonucleic acid (DNA) is the genetic material that is passed down from generation to generation. When there is an alteration in DNA, the alternate gene can trigger an imbalance in the body's system, leading to breast cancer. P53, BRCA1 and BRAC2 genes are among those linked to breast cancer. A study done by Brewer et al. (2017) discovered that the families with a history of breast cancer were more likely to inherit this gene and develop breast cancer. Apart from that, a mutation in genes such as P13K/AKT pathway can increase the probability of getting breast cancer. The P13K/AKT pathway is a pathway that is important in cellular growth and apoptosis. When a mutation occurs, the cells continue to proliferate, eventually leading to cancer development. These mutations were studied to see whether there is any association with estrogen exposure (Khatpe et al., 2021).

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In addition, exposure to environmental such as chemicals or toxicants can increase the risk of getting breast cancer. The environmental exposure includes endocrine disrupting compounds (EDCs), tobacco smoking and radiation. An *in vitro* study on Bispheno*l* A (BPA), which is also part of EDC showed that BPA can reduces the chemotherapy efficiency by inhibiting the growth of the breast cancer cells (Gray et al., 2017). The same situation can be seen on tobacco smoking and radiation as more exposure from it can increase the development of the breast cancer.

The development of breast cancer is also related to the function of the estrogen receptor (ER). ER is a transcriptional factor that either activates the ligand or represses the gene expression upon ligand binding. Erα, a type of ER has been found to be highly expressed in approximately 75% of breast cancers by stimulating the growth of breast tumor cells as part of the pathogenesis of breast cancer (Feng et al., 2017).

2.1.4 Stages of breast cancer

Staging system for breast cancer was organized by the American Joint Committee on Cancer (AJCC). Previous staging systems focused on the anatomic extents disease, which later classified the anatomic breast cancer into several stages, starting with stage 0 and progessing to stage IV as shown in figure 2.3. However, AJCC published a revised staging system that addressed the biological factors such as hormone receptor expression and tumor grade, known as the prognostic stage. This is as important as the anatomic extent of disease to determine for better treatment decision and prognosis.

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Table 2 Anatomic stage groups of breast cancer.

Stages		Definition
Stage 0		Ductal Carcinoma In Situ
Stage I	IA	Primary invasive tumor with a size of ≤20 mm No nodal involvement
	IB	Nodal micrometastases (>0.2 mm, <2.0 mm) with or without ≤ 20 mm primary tumor
Stage II	IIA	Movable ipsilateral Level I, II lymph node metastases with ≤20 mm primary tumor; Or > 20 mm, ≤ 50 mm tumor with no nodal involvement
	IIB	Movable ipsilateral Level I, II lymph node metastases with >20 mm, ≤ 50 mm tumor; Or > 50 mm tumor with no nodal involvement
Stage III	IIIA	Movable ipsilateral Level I, II lymph node metastases with >50 mm tumor; Or any size primary tumor with fixed ipsilateral Level I, II or internal lymph node metastases
	IIIB	Primary tumor with chest wall and/or skin invasion
	IIIC	Any size primary tumor with supraclavicular or ipsilateral Level III lymph node metastases; Or with ipsilateral Level I, II and internal lymph node metastases
Stage IV		Any case with distant organ metastasis

Notes: 1). Lobular carcinoma in situ is now considered benign thus removed from the breast cancer staging system.
2). The Anatomic Stage Group is to be used when biomarker tests are not available.
Source: AJCC Cancer Staging Manual, Eighth Edition, The American College of Surgeons (ACS), Chicago, IL, USA. With reprint permission of ACS.

Figure 2.1.3. Anatomic stage groups of breast cancer
(Feng et al., 2017).

2.1.5 Metastasis of breast cancer

Metastasis is defined as the spread of cancer cells from its primary tumor to the surrounding tissue and organs and as the causes for the cancer mortality and morbidity (Seyfried & Huysentruyt, 2013). One of the hallmarks of breast cancer is tumor metastasis which involves cellular migration and invasion. Breast cancer progression with metastasis were seen more in advanced stages with poorer prognosis (Tieng et al., 2019). Breast cancer metastasis most frequently occurs in the bone, brain, lung, and liver. The development of metastasis begins with invasion, intravasation, circulation, extravasation, and colonization.

Invasion means the cancer cells are required to leave their primary site and invade the extracellular matrix (ECM) and basement membrane. Then, the cancer cells

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will penetrate the lymphatic or vascular circulation via intravasation (Martin et al., 2013). The cancer cells will circulate in the lymph or bloodstream as circulating tumor cells (CTC) either as single cells or clusters. The circulating clusters are much likely to contribute to the metastasis process. Most CTCs have the ability to avoid the immune system by suppressing the leukocyte activation for CTC survival. The CTC also has the ability to attach and extravasate through endothelial cells, causing microvascular rupture or forcing the cell to extravasate. Then, CTC will colonize the new area and grow at the secondary site (Fares et al., 2020).

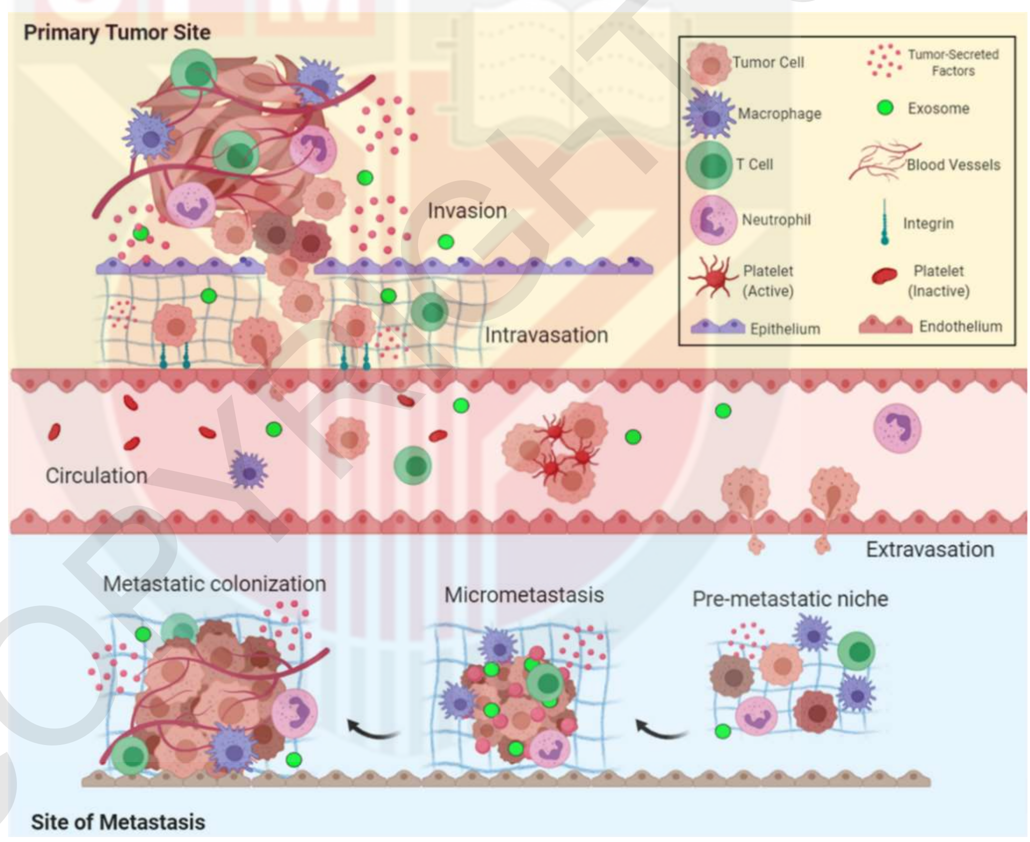

Figure 2.1.4. Overview of metastatic cascade that involve invasion, intravasation, circulation, extravasation, and colonization (Fares et al., 2020).

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2.1.6 Risk factor of breast cancer

Breast cancer risk can be influenced by various factors which are family history, age, reproductive factors, estrogen, and lifestyle (Sun et al., 2017).

Family history. A cohort study reported a 3.5-fold difference in risk between the lowest and highest FHS groups, and a 2.5-fold difference in risk among women who had relatives with breast cancer (Brewer et al., 2017).

Age. Breast cancer has become more frequent as people get older. According to Surveillance, Epidemiology and End Results Program (SEER), around 23.8% of female breast cancer deaths in the United States were reported among women aged 65 to 74, with the median age of death at 69 years old.

Reproductive factors. Apart from that, reproductive factors such as having a large number of children reduce the risk of developing breasr cancer when compared to women who have fewer children (Sun et al., 2017).

Estrogen. Furthermore, estrogen which is a hormone that play a role in female reproductive system are also linked with high risk of breast cancer. Tian et al. (2018) found that the administration of estrogen alone promoting the MCF-7 cell proliferation and clonogenic abilities.

Lifestyle. Lifestyle also can be one of the contributor factors for the breast cancer. This includes too much dietary fat intake and excessive alcohol intake. Apart from that, the risk is also higher in the obese women. The probability of distant breast cancer recurrence is higher in obese women than non-obese women, which later acts as a great substitute of breast cancer death (Ecker et al., 2019). This is due to the

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adverse effect of obesity that cause higher levels of estrogen via increasing in aromatase activity within the excess adipose tissue.

2.1.7 Current treatment of breast cancer

Breast cancers are treated depending on its type and how far it has spread. This is to ensure the quality of life with prolonged life expectancy (Akram et al.,2017). Current breast cancer treatments include surgery, chemo-, radio- and hormonal therapy.

Surgery. Surgery is the standard treatment options for breast cancer patients. A breast cancer surgeon will conduct an operation to cut out the cancer tissue. Among the two main types of breast cancer surgery include lumpectomy (breast-conserving surgery) and mastectomy. Breast-conserving surgery usually involves the removal of both malignant tumor and some normal breast tissue. The side effects of this type of surgery are temporary inflammation, sclerosis, and tenderness (Akram et al., 2017). Meanwhile, mastectomy refers to the surgical removal of all the breast tissue to reduce the chance of developing to breast cancer. It is considered as the most effective method compared to lumpectomy in dealing with diffused case of breast cancer. However, this could lead to most women having a depression, loss of self-image and feeling of asexuality after having a mastectomy (Akram et al., 2017).

Chemotherapy. Chemotherapy is the special drugs that use to shrink or kill the tumor cells. It targets the cancer cells and may be used before and after surgery, depending on the patient's condition. It may be used as neoadjuvant chemotherapy, adjuvant chemotherapy, as support to other therapies to make it more effective and prevent cancer cells from spreading to the other parts of the body. According to the American Cancer Society, the chemotherapy drugs used in treating breast cancer

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includes Taxanes, Anthracyclines, 5-fluorouracil (5-FU) and platinum agents such as cisplatin. However, the adverse effects of chemotherapy often worrying in breast cancer patients as it can cause nausea, vomiting, diarrhea and some affects the blood cells which lead to increased chances of infections, bleeding, and fatigue.

Radiation therapy. To effectively eliminate cancer cells, high energy of radiation was used thus lowering the likelihood of breast cancer recurrence following the surgery. It is commonly combined with other treatments such as surgery and chemotherapy. A study done by Speers and Pierce (2016) shows that the combination of postoperative surgery and radiotherapy were significantly reduces the breast cancer recurrence and increases overall survival.

2.2 Apoptosis

Apoptosis is described as programmed cell death, which occurs when a cell initiates a process that results in controlled death of the cells without leaking its contents into the surrounding environment (D'Arcy. 2019). Apoptosis also occurs as a defensive mechanism during the cells damaged due to disease or toxic substances (Barber, 2001). Apoptosis can be characterized by observing the morphological changes such as cell shrinkage (CS), nuclear fragmentation (NF), apoptotic bodies (AB) and membrane blebbing (MB) (Hosseinpour et al., 2014). Apoptosis also does not result in an inflammatory reaction because apoptotic bodies containing dead cell contents can be phagocytosed by the surrounding cells and reduce inflammation (D'Arcy. 2019). Apoptosis failure can result in many pathological conditions including cancer, autoimmune diseases, and neurological disorders (Favaloro et al., 2012).

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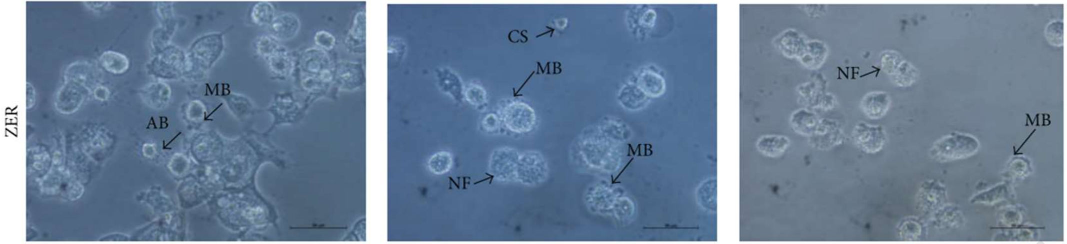

Figure 2.2.1. Morphological changes of MDA-MB-231 cells when treated with Zerumbone treatment (Hosseinpour et al., 2014).

2.3 Pyrazole and its derivatives

Pyrazole, an aromatic heterocyclic with three carbon atoms and two nitrogen atoms comes from azole family. The first discovered pyrazole known as 3-n-nonyl-1H-pyrazole was isolated from *Houttuynia cordata*, a common plant in Tropical Asia. The biological active of pyrazole compounds has been synthesized for use in the medical field for its antibacterial, anti-inflammatory, antifungal and antitumor activity (Faria et al., 2017).

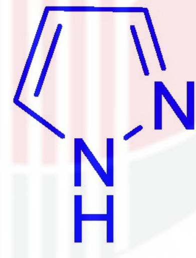

Figure 2.3.1. Structure of Pyrazole
(Karrouchi et al, 2018).

Pyrazole derivatives are among the most active classes of compounds, with a broad range of biological activities. Example of drugs that been developed from pyrazole derivatives includes celecoxib, fomepizole and rimonabant (Ansari, 2017). Another type of pyrazole derivative is serine/threonine kinase-inhibiting pyrazole

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derivatives, which have anti-cancer and anti-angiogenic properties has been extensively researched around the world. A study done by Meta et al. (2017) discovered that a pyrazolyl-urea derivatives, (ethyl 1-(2-hydroxypentyl)-5-(3-(3-(trifluoromethyl) phenyl)ureido)-1*H*-pyrazole-4-carboxylate) or GEGE3 has the anti-angiogenic properties. GeGe3 was also reported to interfere the cell proliferation and tumor angiogenesis *in vivo* via phosphoinositide 3-kinase (P13K) and mitogen-activated protein kinases (MAPK) pathways.

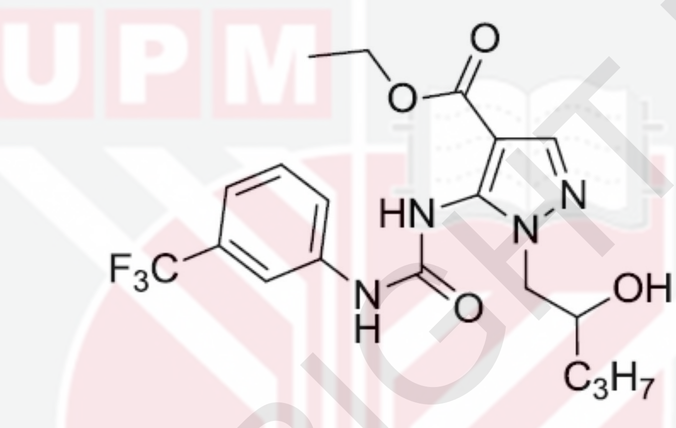

Figure 2.3.2. Chemical structure of Pyrazolyl-urea (GEGE3)
(Meta et al., 2017).

2.4 Methods for determination of cytotoxic and migration process

2.4.1 MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay is a colorimetric assay that widely used to study the cytotoxicity effects of any compounds and is ideal for high throughput screening (HTS) (Riss et al., 2016). It is commonly used in the laboratory as evidenced by thousands of published articles. The principle of MTT is to measure the enzymatic activity of mitochondrial succinate dehydrogenase, which in the living cells that converts yellow, MTT to an insoluble dark, blue-colored formazan product. The formazan product accumulates as an

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insoluble precipitate inside cells, which later solubilize by using Dimethyl Sulphur Oxide (DMSO), dimethylformamide, SDS, acidified isopropanol and other detergent and organic solvent combinations (Riss et al., 2016). The solubilization causes the formazan to change from dark blue into yellow color.

2.4.2 Wound healing assay

Scratch migration assay is the conventional method in the research laboratory to study the cell migratory behavior. It is also one of the simplest, cheaper and faster methods to observe the morphological features of the cell during migration (Pijuan, 2019). The principle of the assay is to create a vertical wound on the cell using pipette tips and the closure of wound distance was observed over time.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell lines

The MDA-MB-231 human breast carcinoma cells were purchased from American Type Culture Collection (ATCC®) (ATCC accession no. MDA-MB-231 (HTB-26^{TM}). The cells were cultured in T75 and T25 tissue culture flasks in DMEM with 1% Penicillin-Streptomycin solution (10x) and 10% FBS. The cells were then incubated in a 5% CO_2 incubator at 37°C.

3.1.2 Pyrazole derivatives

Pyrazole derivates, HEXUR-2 were kindly given by our collaborator, Professor Chiara Brullo from Italy. It arrived in powder form and was diluted with DMSO in the laboratory.

3.1.3 Reagents

Phosphate-buffered saline (PBS) tablet was purchased from VWR® Life Science AMRESCO (Ohio, USA). Dulbecco's Modified Eagle Medium (DMEM), 0.25% (1x) Trypsin-EDTA and Fetal Bovine Serum (FBS) were purchased from GE Healthcare Life Sciences HyClone^{TM} (Utah, USA). Dimethyl Sulfoxide (DMSO) was purchased from purchased from Fisher BioReagents^{TM} (Massachusetts, USA). Tryphan blue solution (0.4%) was purchased from Sigma Aldrich (St. Louise, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma Aldrich® (St. Louise, USA).

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3.2 Methods

3.2.1 Cell culture

Biosafety cabinet (BSC) class II was used to perform all the cell culture procedures to ensure a sterile environment for culturing cells. The MDA-MB-231 cells were cultured in T75 and T25 tissue culture flasks in complete growth medium (DMEM, 1% Penicillin-Streptomycin solution (10x) and 10% FBS) and incubated in a 5% CO_2 incubator. The cells were monitored daily under an inverted light microscope (Olympus Research Microscope, CH20i (Binocular Version), Tokyo, Japan) to ensure that they were growing healthy and were not contaminated. Cell passaging was performed when the cells reached 70-80% confluency.

3.2.2 Stock cell thawing

The materials and medium for cell thawing were prepared. The cell line name, date and passage number were labelled on the culture flask. The cryovial was retrieved from the -80°C freezer and placed in a water bath to melt the contents. The ampoule was swabbed with 70% alcohol before being placed in the BSC. The ampoule contents were transferred to a T25 culture flask, and media was added. The culture was observed under an inverted light microscope and incubated in a 5% CO_2 incubator.

3.2.3 Cell passaging

The complete growth media of DMEM, trypsin, and PBS were prewarmed in a water bath at 37°C. After preparing the BSC, the reagents and materials were sprayed with alcohol. The culture was thoroughly examined for signs of contamination. The medium was discarded as the culture was brought into the BSC. The cells were prewashed with PBS to remove any serum interfering with trypsin activity, and the rinse was discarded. The cells were trypsinized and incubated for 3 minutes. To ensure

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that the cells were detached, the culture flask was examined under an inverted light microscope. After that, the medium was added to inhibit the trypsin activity. The media was then disseminated by pipetting over the surface of the culture flask several times. The cell suspension was transferred into a new falcon tube and a small amount of the cell suspension was counted using a hemocytometer. The cell suspension was diluted to the appropriate seeding concentration before being distributed among the flasks. After that, the flask was then incubated.

3.2.4 Cell counting

Cell confluency was observed under the microscope. The medium was discarded and washed with PBS. Trypsin-EDTA was used to trypsinize the cells, which were then incubated for 3 minutes. Then, the cells were examined under the microscope to ensure that they were detached. The media was then added, and the cells were dispersed by pipetting all over the surface of the culture flask several times. A small amount of cell suspension was transferred in an Eppendorf tube and mixed in a 1:1 ratio with tryphan blue. The hemocytometer's surface and coverslip were cleaned with 70% alcohol. The counting chamber was covered with the coverslip, and 10 µl of mixed cell suspension was loaded on the hemocytometer. The cell suspension was observed under the microscope and the total number of cells in each quadrant of the grid were counted. The following formula was used to calculate the number of cells per ml:

Cell concentration (number of cells/ml)

$$= \frac{\text{Total number of cells in 4 quadrants}}{4} \times \text{dilution factor} \times 10^4$$

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3.2.5 Cryopreservation of cells

Freezing medium was prepared with addition of 10% DMSO and 90% FBS and placed in the ice bucket filled with ice. Cells were harvested, counted, and centrifuged. The freezing medium was mixed with the cell suspension in a falcon tube. Then, the suspension was dispensed into a prelabeled cryovial and tightly sealed with parafilm. The cells were then placed in a -80°C freezer for storage.

3.2.6 MTT assay for cytotoxicity

MTT assay or cytotoxicity assay is a commonly used test to determine the cytotoxicity effect on cell proliferation. It is a colorimetric assay that measures the enzymatic activity of mitochondrial succinate dehydrogenase which changes the colour of yellow, MTT to an insoluble, dark-blue colored formazan product in living cells. MTT assay were used in this experiment to evaluate the cell viability of MDA-MB-231 cells treated with novel pyrazole derivatives (HEXUR-2).

The MDA-MB-231 cells were seeded at a density of $2x10^4$ cells/well in a 96-well plate. Next, the plates were incubated for overnight at 37 °C in a 5% CO_2 incubator for the cell attachment on the plates. After 24 hours, the media was removed and the cells were subsequently treated with eight different concentrations of HEXUR-2 (200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µM). The stock solution of HEXUR-2 (100 mM in DMSO) was used to prepare the highest concentration of HEXUR-2 (200 µM) with complete growth medium. Then, a serial dilution was performed from the highest concentration for the other respective concentrations. Fluorouracil (5-FU) was used as positive control and were prepared with three different concentrations (94, 23 and 10 µM) following the incubation period (24, 48 and 72 hours), respectively. To prepare the concentrations of 5-FU with complete growth medium, a stock solution of

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5-FU (10 mM in DMSO) was used. The treated cells with HEXUR-2, positive control, and untreated cells (negative control) were incubated at according to its incubation period. After each incubation periods, 10 µL of MTT solution was added into each well and incubated for 3 to 4 hours.

After 3 to 4 hours, the formazan precipitates were formed in each well which were then dissolved with 100 µL of DMSO. A microplate reader (Tecan, infinite® M200, Männedorf, Switzerland) was used to measure the absorbance at 570 nm for each well with reference wavelength of 630 nm. The data were obtained, and the viability of cell precentage was calculated by using the formula:

Percentage of cell viability (%):

$$= \frac{\text{The average of sample absorbance}}{\text{The average of control absorbance}} \times 100\%$$

The values of half-maximal inhibitory concentration (IC_{50}) were determined and the graph of the percentages of cell viability against the concentrations were plotted.

3.2.7 Cell seeding optimization

Cell seeding optimization was performed to find the appropriate cell density in the 12-well plate for the scratch migration assay. In a 12-well plate, the cells were seeded at densities ranging from 3×10^5 to 1×10^6. The cells' confluency was examined under inverted light microscope and images were captured. The cell seeding optimization was determined based on cell confluency, which is at 70-80% confluency.

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3.2.8 Scratch migration assay

The MDA-MB-231 cells ($5x10^5$ cells/well) were seeded in a 12-well plate and left incubated for 24 hours at 37 °C in a 5% CO_2 incubator. After the cells had reached 70-80% of confluency, a scratch was created with a sterile yellow pipette tip. The medium was then discarded, and the cells were subsequently treated with four different concentrations of HEXUR-2 (2, 4, 6, 9 and 15 µM) based on cytotoxicity data with concentration of 1% FBS. The wound gap images were taken at 0, 24 and 48 hours under the inverted light microscope. The gap difference for each concentration was observed and compared to the control group.

3.2.9 Statistical analysis

Statistical analysis was performed by using GraphPad Prism 9. The data were analyzed to determine the significance of mean differences through One and Two-Way Analysis of Variance (ANOVA), followed by Tukey HSD or Dunnett's multiple comparison. Results of each independent experiments were expressed as mean ± standard error of mean (mean ± SEM). A p-value less than 0.05 ($p \leq 0.05$) was considered as statistically significant.

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CHAPTER 4

RESULTS

The cytotoxicity effects of novel pyrazole derivatives (HEXUR-2) on MDA-MB-231 cells were evaluated by using MTT assay in three independent experiments. The cells were treated with eight different concentrations of HEXUR-2 ranging from 1.56 to 200 μM at three different incubation periods which were 24, 48 and 72- hours, respectively. Following the MTT assay, scratch migration assay was performed to study the migratory effects of HEXUR-2 on the MDA-MB-231 cells.

4.1 Cell viability analysis of MDA-MB-231 Human Breast Cancer Cells treated with HEXUR-2

Figure 4.1.1 showed the cell viability analysis of MDA-MB-231 cells treated with different concentrations of HEXUR-2. The MDA-MB-231 cell viability was significantly reduced after 48 and 72 hours of treatment between 12.5 to 200 μM of HEXUR-2. The MDA-MB-231 cell viability was also significantly reduced after 24 hours of HEXUR-2 treatment, starting from 100 to 200 μM at 24 hours. Table 4.1.1 summarizes the half-maximal concentration (IC_{50}) values obtained after 24 (75 μM ± 9.717), 48 (33 μM ± 9.346) and 72 hours (9 μM ± 9.523) incubation. The IC50 values of MDA-MB-231 cells that have been treated with HEXUR-2 were shown to reduce with increasing timepoint. However, there are no significant differences observed between each timepoint, as shown in figure 4.1.2.

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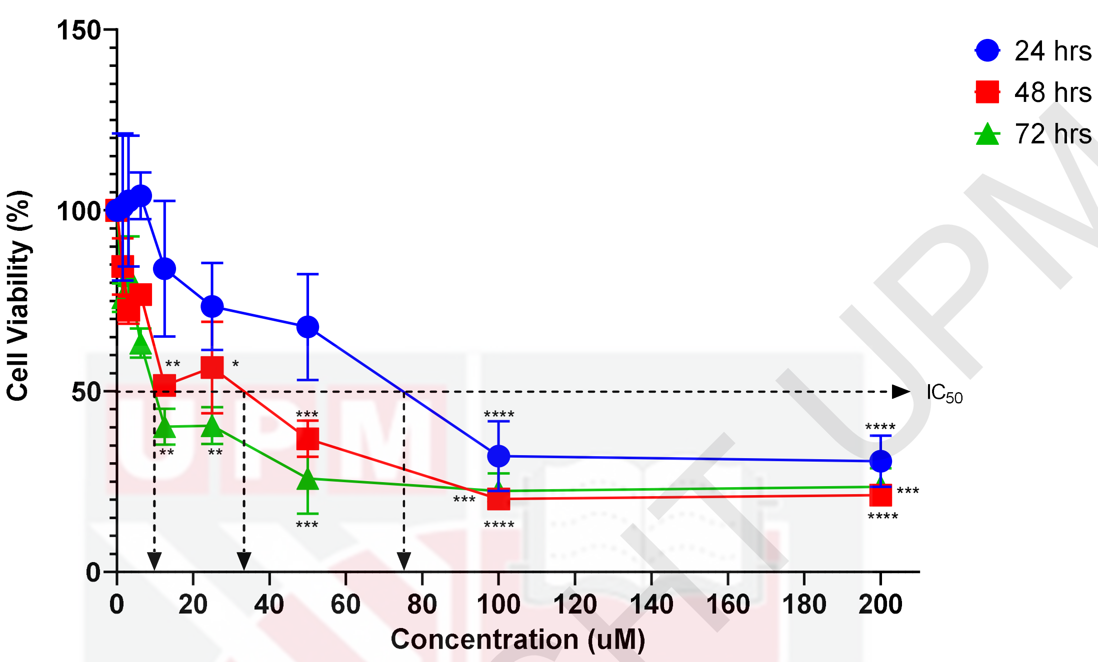

Figure 4.1.1. Cytotoxic effects of HEXUR-2 on MDA-MB-231 cells via MTT assay. Mean data were expressed as ± SEM based on three replicated experiments. Results were analyzed by using Two-Way ANOVA followed by Dunnett's test. The means marked with *, **, ***, **** were significantly different with p-value less than 0.05 as compared to control group (untreated cells).

Table 4.1. IC_{50} values of HEXUR-2 on MDA-MB-231 cells. IC_{50} values was determined at 24, 48 and 72 hours, respectively.

Drugs	Proliferation IC_{50} MDA-MB-231 cells		
	24 hours	48 hours	72 hours
HEXUR-2	75 µM ± 9.717	33 µM ± 9.346	9 µM ± 9.523

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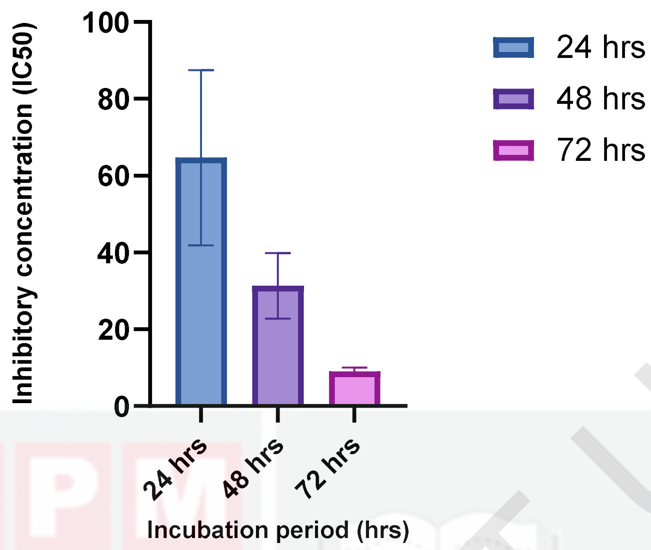

Figure 4.1.2. IC_{50} graph of HEXUR-2 on MDA-MB-231 cells at 24, 28 and 72 hours via MTT assay. Mean data expressed as ± SEM based on three independent experiments. Results were analyzed by one-way ANOVA and followed by Tukey's test.

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4.2 The effects of HEXUR-2 on the MDA-MB-231 cell morphology

A morphological study was conducted to observe the effects of HEXUR-2 on MDA-MB-231 cells. Table 4.2.1 showed the morphology pre- and post- treatment of MDA-MB-231 cells with the positive control (5-FU), and with different concentrations of HEXUR-2 ranging from 1.56 to 200 µM after 24-, 48- and 72-hours incubation. Figure 4.2.1 showed the morphology of MDA-MB-231 cells at the concentrations of 6.25, 12.5 and 25 µM following the IC50 values after 72 hours (9 µM ± 9.523). At 6.25 µM of HEXUR-2, the cells appeared to have an apoptotic characteristic such as nuclear fragmentation whereas at 12.5 µM, the cells exhibited apoptotic characteristics such as cell shrinkage.

Table 4.2. The effects of HEXUR-2 on the MDA-MB-231 cell morphology. The morphology of untreated cells, cells with the positive control (5-FU) and different concentrations of HEXUR-2 were observed after 24, 48 and 72 hours, respectively (x40 magnification).

Concentration of HEXUR-2 (µM)	0 hr	24 hrs	48 hrs	72 hrs
Negative control				

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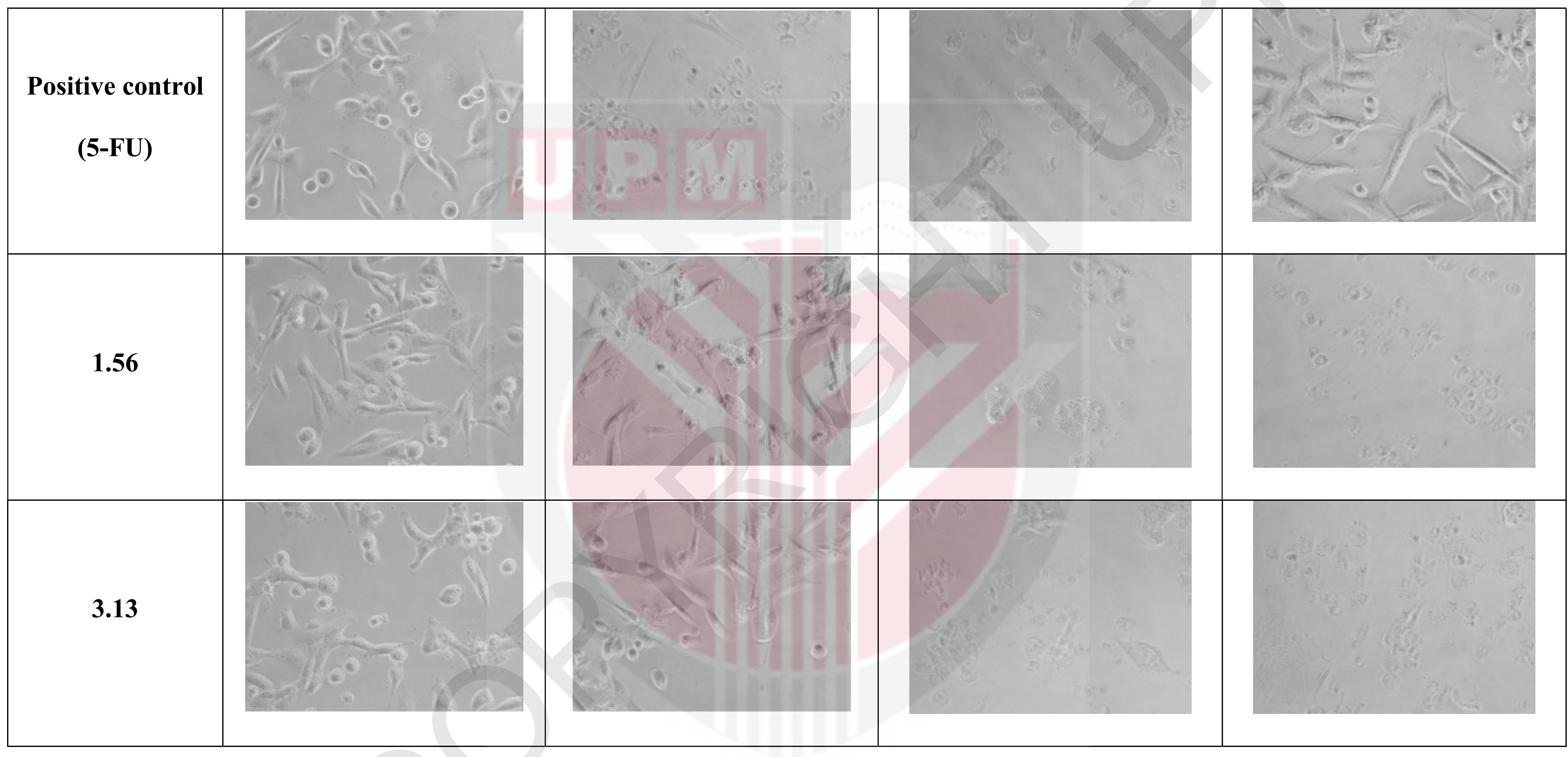

Positive control (5-FU)				
1.56				
3.13				

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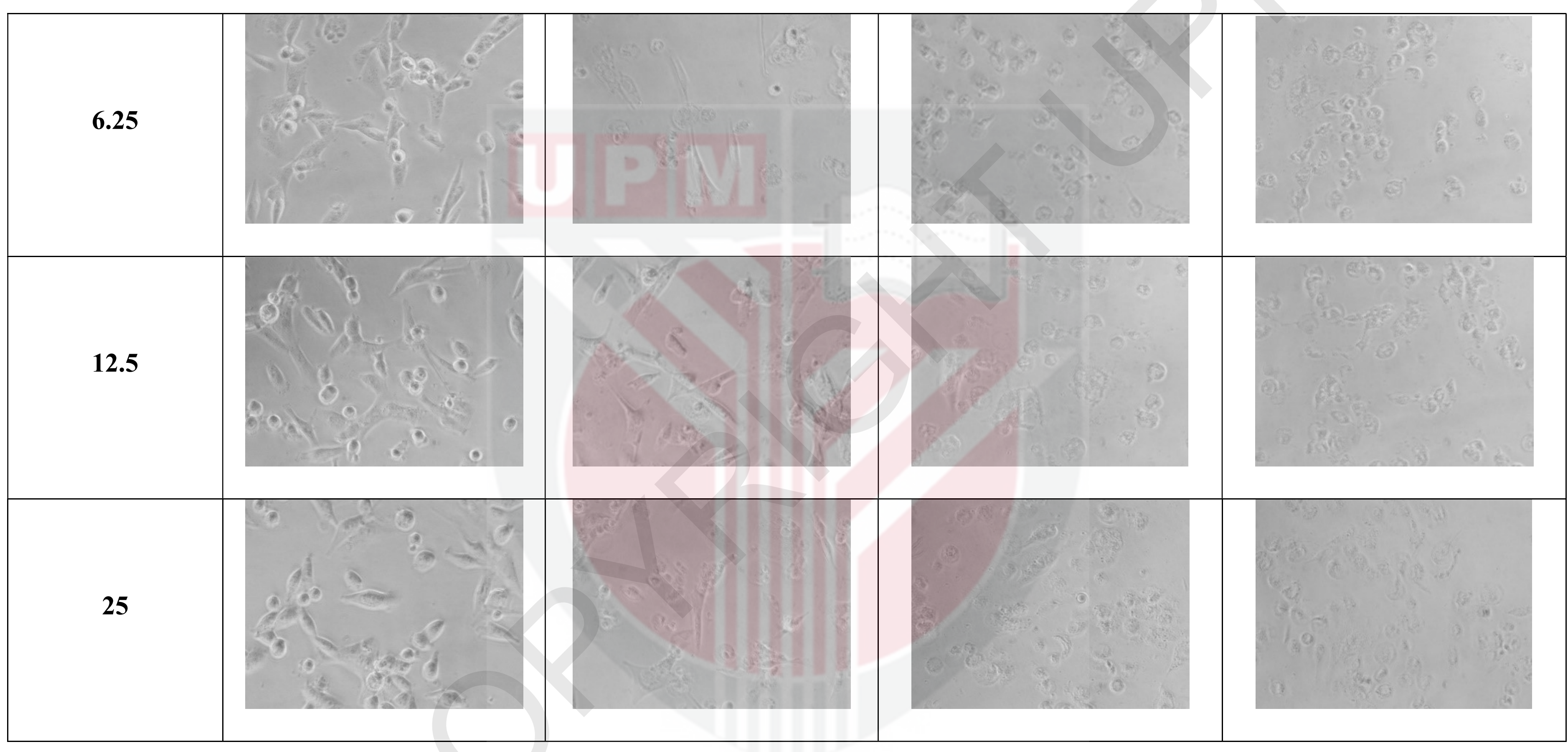

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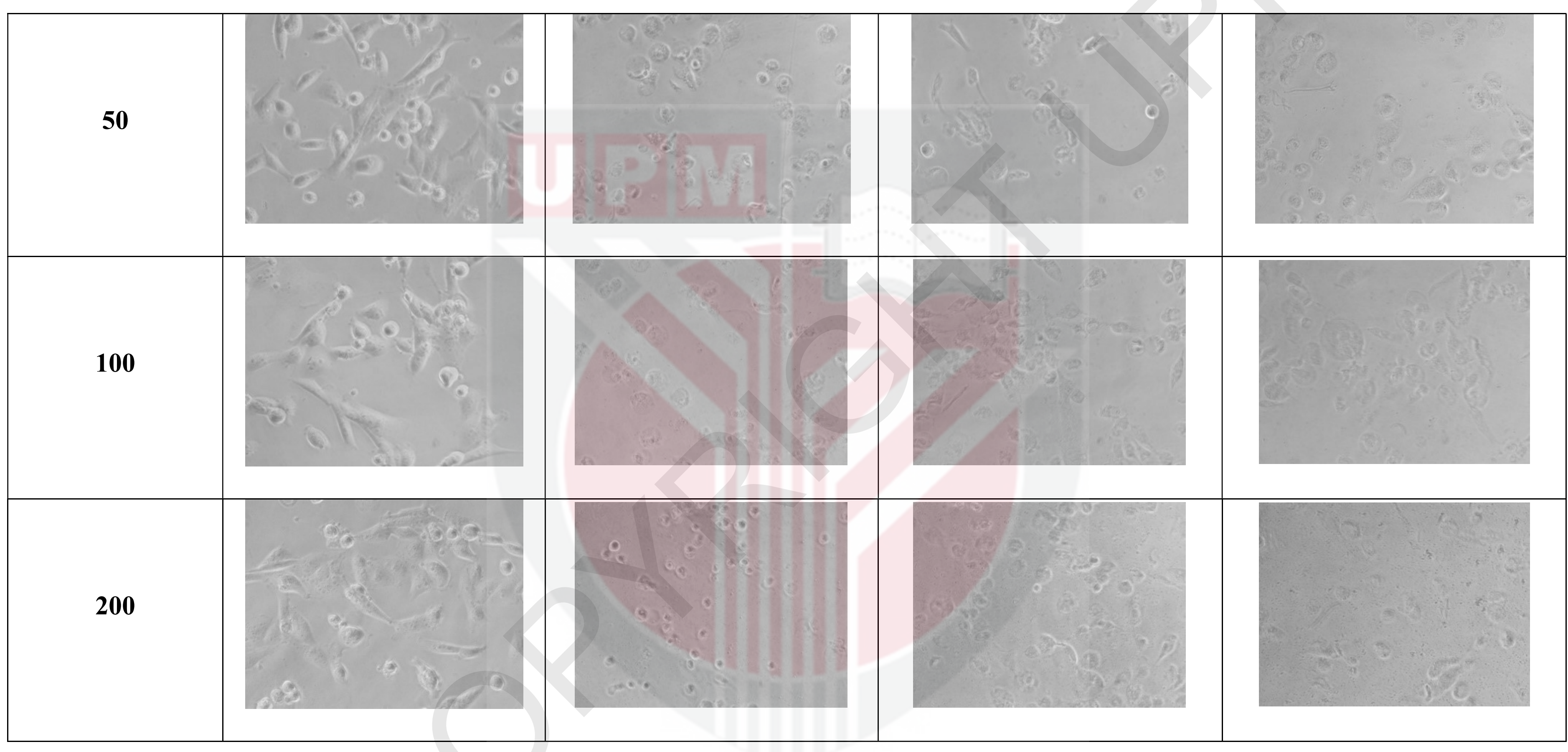

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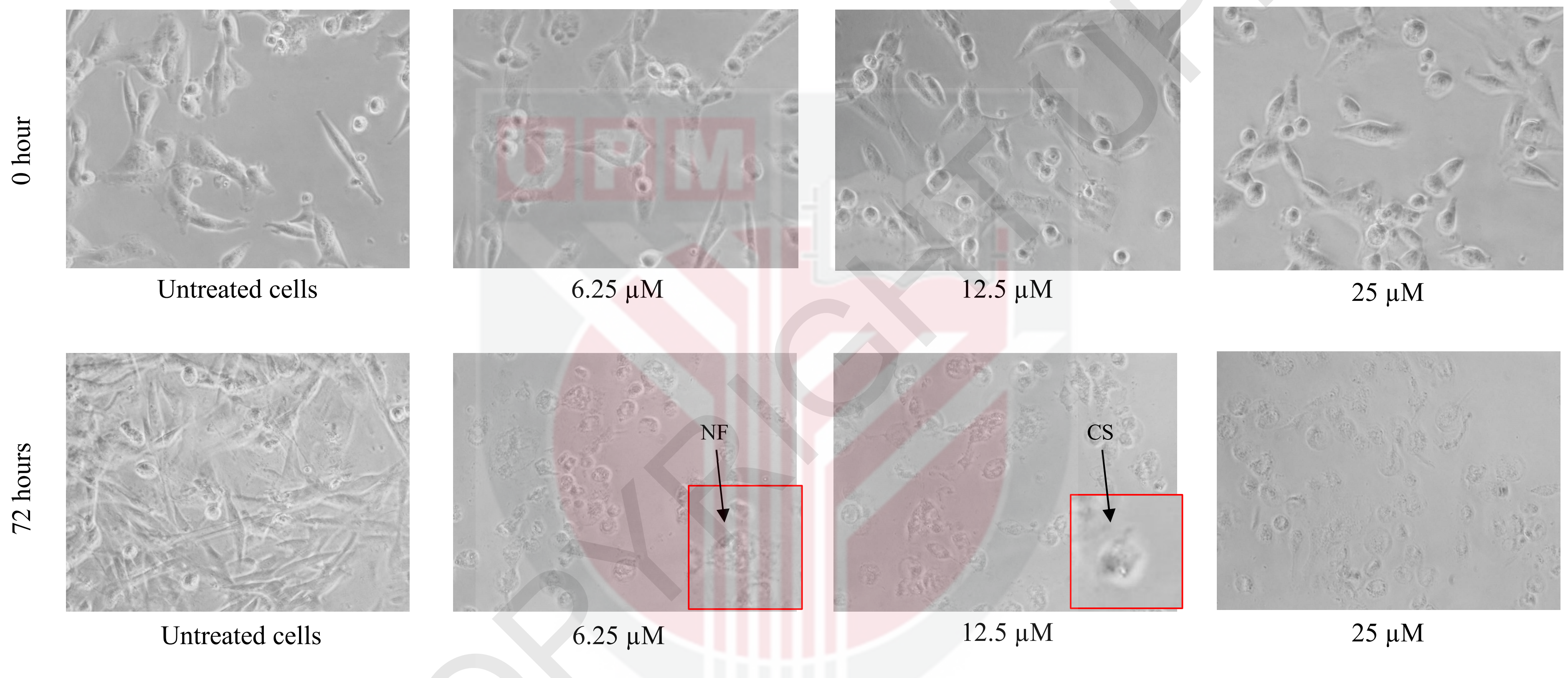

Figure 4.2.1. Morphological changes of MDA-MB-231 cells treated with HEXUR-2. Cells (2x104) were treated with 6.25µM and 12.5µM of HEXUR-2 at 72 hours showed the characteristics of apoptosis such as nuclear fragmentation (NF) and cell shrinkage (CS) compared to control (40x magnification).

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4.3 The effects of HEXUR-2 on the MDA-MB-231 cell migration

The scratch assay was performed to determine the migratory effects of HEXUR-2 on MDA-MB-231 cells. Figure 4.3 showed the scratch migration assay images of MDA-MB-231 cells on different concentrations of HEXUR-2 (2, 4, 6, 9 and 15 µM) at 0 and 24 hours.

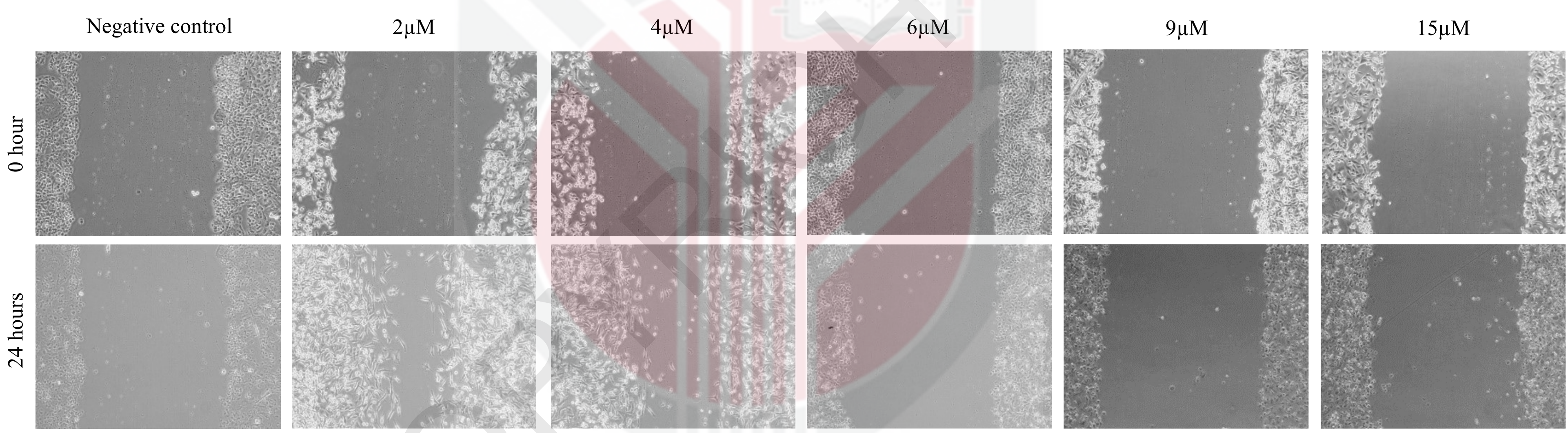

Figure 4.3. Scratch migration assay of MDA-MB-231 cells. Images from scratch migration assay of MDA-MB-231 cells treated HEXUR-2 at 0- and 24- hours incubation time under 20x magnification.

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CHAPTER 5

DISCUSSION

The ability to invade and metastasize is one of the characteristics of cancer (Hanahan & Weinberg, 2011) in which the cancer cells undergo phenotypic changes and lose their cell adhesion, allowing them to detach and invade into the extracellular matrix (ECM). The outcome of the migratory process could result in cancer recurrence by forrming new tumors in the surrounding tissues. In addition, the migration of cancer cells supported by forming new blood vessels known as angiogenesis by providing the necessary resources such as vascular endothelial growth factor (VEGF) for tumor development, facilitating metastatic invasion, and metastasize thorughout the body (Martin et al., 2013).

Pyrazole and its derivatives were known to have antibacterial, antifungal, and antitumor properties (Karrouchi, 2018). In this study, the compound used was a novel pyrazole derivatives compound (HEXUR-2) to study its cytotoxicity and migratory effects on the MDA-MB-231 cells. HEXUR-2 was derived from a parental compound of GEGE3, that has been shown to interfere with cell growth and migration by blocking MAPK and P13K signaling in the human umbilical vein endothelial cells (HUVEC). In addition, GEGE3 also has proven to be a potential antitumor and antiangiogenic compound. Therefore, Meta et al. (2017) suggests that further study on GEGE3 is required to determine its cytotoxicity and inhibitory activity on different tumor cell lines.

The cytotoxic effects of HEXUR-2 on MDA-MB-231 cells were determined by using MTT assay. This assay evaluates the cytotoxicity by measuring the cell viability with active metabolism that converts MTT into a purple formazan product

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with an absorbance reading of 570 nm (Riss et al., 2016). From the absorbance reading, the cell viability against HEXUR-2 concentrations and half-maximal inhibitory concentration (IC_{50}) of HEXUR-2 towards MDA-MB-231 cells were extrapolated. MDA-MB-231 was chosen for this study because it is a triple-negative breast cancer, which is extremely invasive and metastatic form of breast cancer (Holliday & Speirs, 2011).

Based on Figure 4.1.1, the MDA-MB-231 cell viability was reduced with the increasing HEXUR-2 concentrations throughout three different incubation periods. HEXUR-2 was proven to significantly reduce the MDA-MB-231 cell viability at 24 hours between 12.5 μM and 200 μM. The cell viability at 100 and 200 M decreases significantly after 48 and 72 hours. This is consistent with another pyrazole derivative, Ferrocenyl pyrazole, which is shown to be cytotoxic in MDA-MB-231 cells via inducing apoptotic cell death (Atmaca et al., 2017). As a result, it was discovered that the cytotoxic effects of HEXUR-2 are in a dose-dependent manner.

The IC_{50} of HEXUR-2 in MDA-MB-231 cells was determined at each incubation period. Based on table 4.1.1, 75 μM, 33 μM and 9 μM killed half of the MDA-MB-231 cells population (50%) after 24, 48 and 72 hours, respectively. This showed that HEXUR-2 effectively inhibited cell proliferation after 24 hours of incubation, suggesting that HEXUR-2 could be a potent drug for the MDA-MB-231 cells. Based on figure 4.1.2, the decreasing trend of the IC_{50} values of MDA-MB-231 cells treated with HEXUR-2 were shown to be in a time-independent manner. This is contrast to an unpublished data which reported that the IC_{50} values of novel pyrazole derivatives (BK-31 and BK-33) was significantly reduced in a time dependent manner (48 and 72 hours) (Nadia et al., 2020).

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For the morphological part, cell death was observed in MDA-MB-231 cells at lower concentrations ranging from 1.56to 200 μM of HEXUR-2 after 48 and 72 hours. Meanwhile, at 50 μM of HEXUR-2, cell death of MDA-MB-231 cells began to be observed after 24 hours. The morphology of the cells for 72 hours, with an IC_{50} of 9 μM was compared with 0 hour and the cells were observed at 6.25 μM and 12.5 μM. It is well established that apoptotic features such as cell shrinkage and nuclear fragmentation are usually seen in the treated cells after the exposure to lower doses of cytotoxic chemical (Williams et al., 1998). Thus, the cell death of MDA-MB-231 cells treated with HEXUR-2 may be due to apoptosis as it exhibits apoptotic characteristics such as cell shrinkage, nuclear fragmentation, and cell detachment (Hosseinpour et al., 2014).

In the preliminary study on scratch migration assay, the IC_{20} values from the MTT assay were used for the concentration. The cells were observed for their migratory phenotype in response to the treatment. Based on figure 4.3, the cells treated with 2 and 4 μM were growing and nearly closing the wound gap compared to the negative control and other concentrations. This suggests that HEXUR-2 induces migration however, the results are inconclusive because the study was only conducted once. The inhibition of cell migration can also be observed in negative control which may be related to the longer assay time (24 hours); cell proliferation becomes a confounding variable towards the experiment. Optimization is essential for scratch assay to minimize assay time and prevents unwanted cell death throughout the experiment (Cormier et al., 2015). Furthermore, Meta et al. (2017) also found that another series of pyrazole derivatives exhibited the trend of promoting endothelial cell migration rather than inhibiting it. As a result, the cells were primarily observed for

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their migratory effects, and no statistical analysis was performed to determine the migration gap across timepoints.

In both MTT and scratch migration studies, various factors can affect the results, including pipetting error. The pipetting error occurs when the number of cells required for seeding is inconsistent for each well, affecting the consistency of absorbance readings on the experimental results. The longer incubation time. the more color accumulation occurs, which increases sensitivity during the reading (Riss et al., 2013). In addition, all reagents must be kept free of contaminants that may contribute to cell contamination. The cell images must also be captured at different quadrants of each well of the plate to represent the condition of cells treated with HEXUR-2 in each well. For the scratch migration assay, one of the factors that may need to be considered for the inhibition of the cells that contributed to negative results at higher concentrations is the cell confluency which may affect the morphology of the cells, for instance if it is too confluent it may induce the cell death.

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CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, this study has found that novel pyrazole derivatives (HEXUR-2) do have cytotoxicity effects on MDA-MB-231 cells after 24, 48 and 72 hours. From the morphological analysis, apoptotic features such as cell shrinkage, nuclear fragmentation and cell detachment were observed in MDA-MB-231 cells treated with HEXUR-2. Interestingly, the preliminary results of scratch migration assay showed that HEXUR-2 did not inhibit the migration of MDA-MB-231 cells however, further experiments and validation work need to be done to confirm this.

6.2 Future recommendations

The current MTT study showed that HEXUR-2 is cytotoxic to MDA-MB-231 cells. Yet, the results for normal cells are uncertain. Therefore, the recent MTT results of HEXUR-2 need to be validated using normal cells following the previous study of novel pyrazole derivatives (BK-31 and BK-33) that used NIH/3T3 cells (unpublished data). Furthermore, to get a better understanding about the data, moreexperiment needs to be repeated and further validated in a three-dimensional (3D) invasion assay as the current method used is in a 2-dimensional (2D) setting. Among the advantages of using a 3D invasion assay is that it provides a clearer picture of both structure and barrier to the cells as they migrate through the extracellular matrix (ECM) thus provides more information about the cell migration and invasion process (Liu et al., 2020).

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