



Anti-cancer activity and effect on oxidative status of caffeoylquinic acid derivatives in breast cancer cell lines

ROTSUKON POONBUD

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR MASTER DEGREE OF SCIENCE
IN BIOCHEMISTRY
FACULTY OF SCIENCE
BURAPHA UNIVERSITY

2023

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ฤทธิ์ต้านมะเร็งและผลที่มีต่อสภาวะออกซิเดชันของสารอนุพันธ์ Caffeoylquinic acids ใน
เซลล์มะเร็งเต้านม



รสสุคนธ์ พูลบุตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต

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ลิขสิทธิ์เป็นของมหาวิทยาลัยบูรพา

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The Thesis of Rotsukon Poonbud has been approved by the examining committee to be partial fulfillment of the requirements for the Master Degree of Science in Biochemistry of Burapha University

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KEYWORDS: CAFFEOYLQUINIC ACID/ MONO-CQAs/ DI-CQAs/ BREAST CANCER/ MDA-MB-231/ MCF-7/ ANTI-CANCER ACTIVITY/ REACTIVE OXYGEN SPECIES

ROTSUKON POONBUD : ANTI-CANCER ACTIVITY AND EFFECT ON OXIDATIVE STATUS OF CAFFEOYLQUINIC ACID DERIVATIVES IN BREAST CANCER CELL LINES. ADVISORY COMMITTEE: PANATA IAWSIPO, 2023.

Breast cancer is the most commonly diagnosed cancer in women worldwide. Although currently available chemotherapeutic drugs kill these cancer cells effectively, they often cause serious side effects. Previously, the crude extract from *Pluchea indica* tea leaves was found to be able to inhibit the proliferation of several cancer cells including those of breast, and caffeoylquinic acid (CQA) derivatives have been reported as the major phenolic compounds from *P. indica* tea leaves. Therefore, in the present study, the anti-cancer activity of selected CQAs was evaluated in breast cancer MCF-7 and MDA-MB-231 cells, in comparison to non-cancer Vero cells. Cytotoxicity of mono-CQAs (3-CQA, 4-CQA and 5-CQA) and di-CQAs (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) was assessed by the MTT assay. MTT result showed that mono- and di-CQAs could inhibit the growth of MDA-MB-231 than that of MCF-7 cells. Di-CQAs exerted the stronger effect than mono-CQAs and among them, 3,4-diCQA has the lowest IC₅₀ values (108 ± 31.06 μM). In addition, all CQA derivatives had low toxic effect on Vero cells (IC₅₀ values > 200 μM). The colony formation assay was further examined in MDA-MB-231 cells treated with di-CQAs at the IC₅₀ concentration. The result showed that 3,5-diCQA could inhibit cancer colony formation by 23.79 % at 100 μM and 38.79 % at 200 μM. Moreover, results from a transwell assay also showed that all di-CQAs tested could suppress the migration of the breast cancer MDA-MB-231 cells by 50% - 60% at 100 μM. Finally, the redox state of cells treated with di-CQAs was examined in order to determine the role of ROS in inducing cytotoxicity by the compounds. The results from DCFDA staining assay showed that an intracellular ROS level was increased in MDA-MB-231 exposed to di-CQAs, especially 3,5-diCQA, and was relative to a reduction of cell viability. This finding suggests that the cytotoxic effect of di-CQAs

was, at least, due to an induction of oxidative stress inside breast cancer cells. Overall results indicate the potential of CQAs in suppressing breast cancer cells with minimal side effect to those of non-cancer and can be developed as anticancer drugs for treatment of breast cancer.



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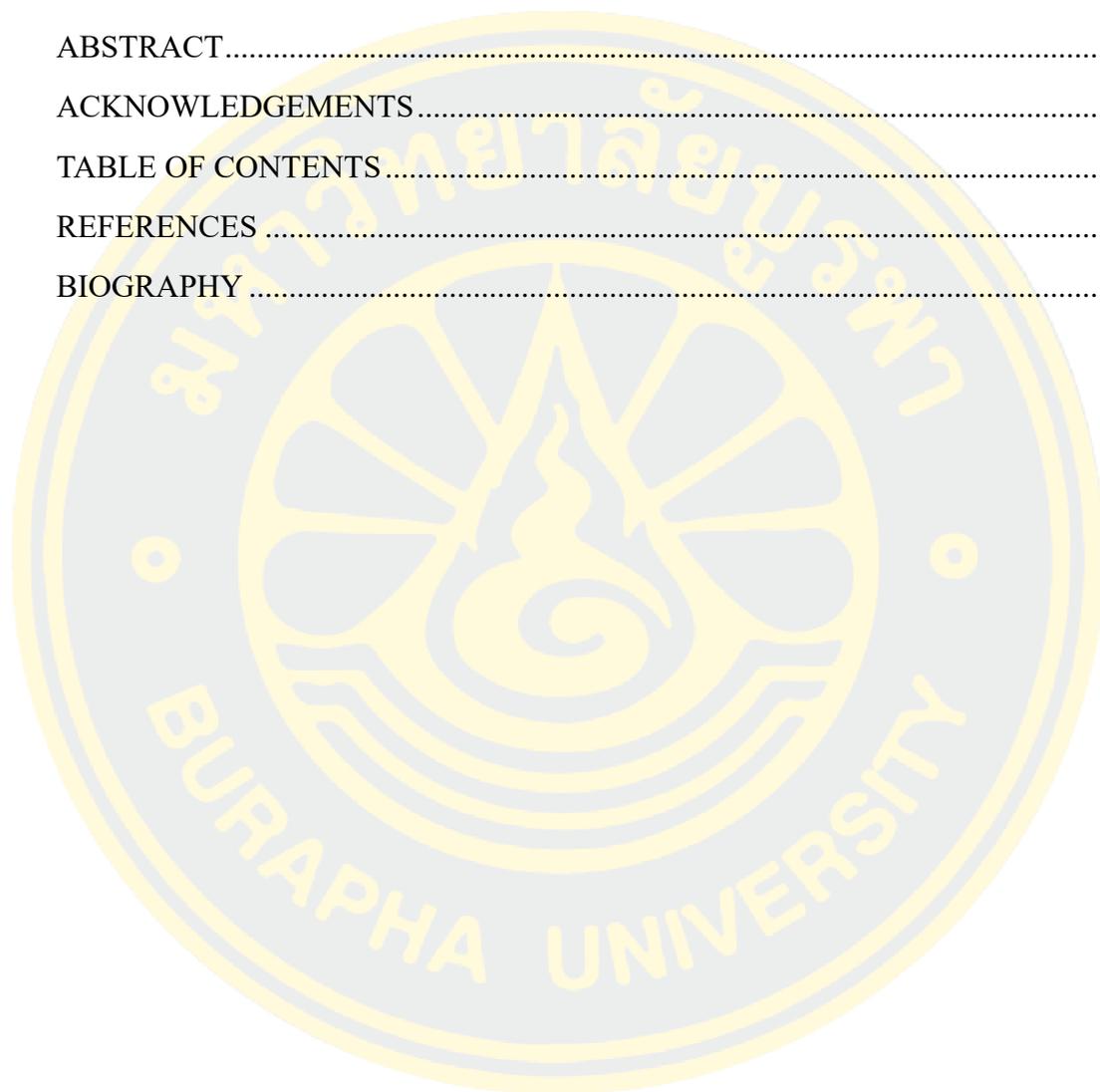


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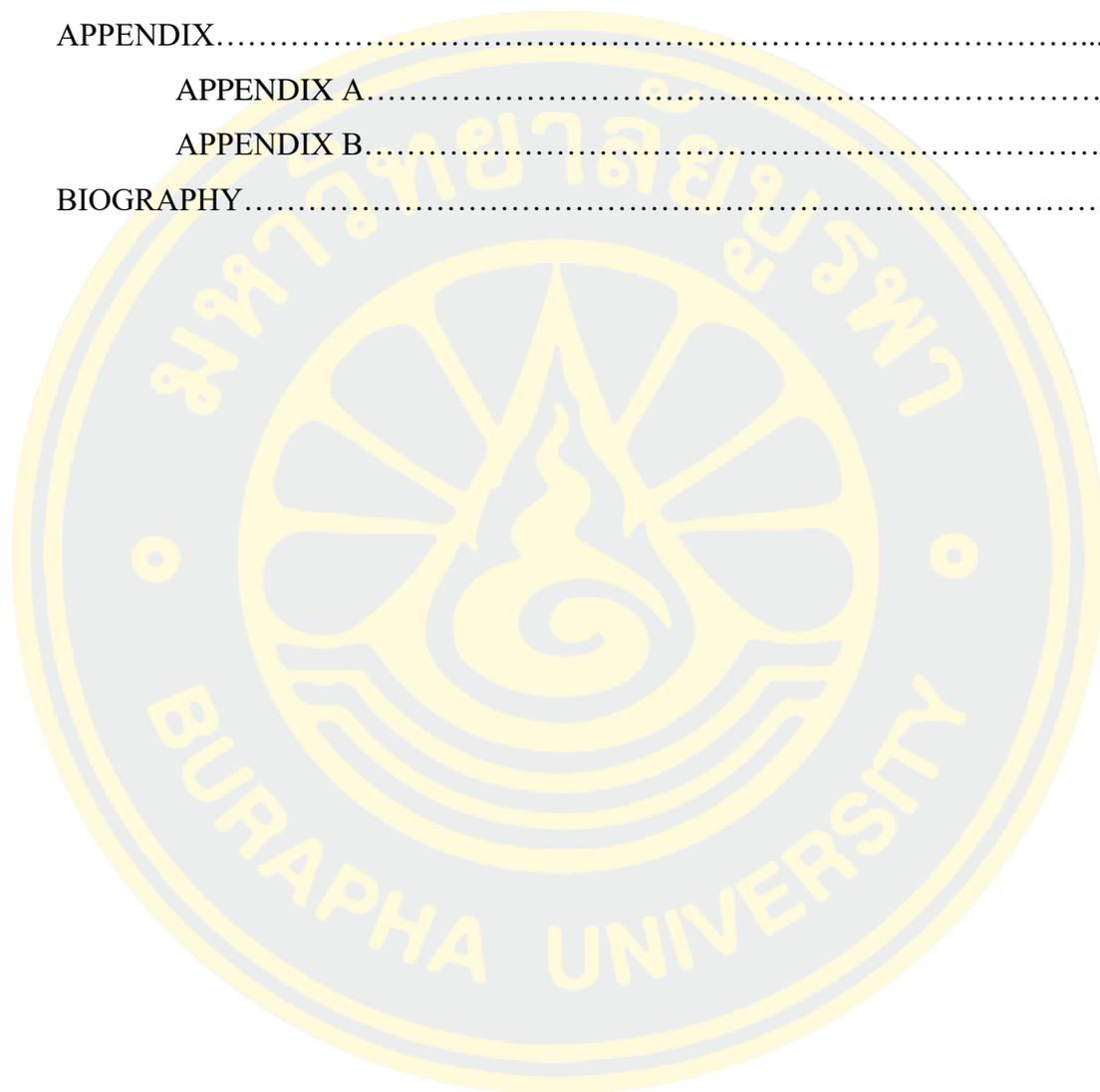
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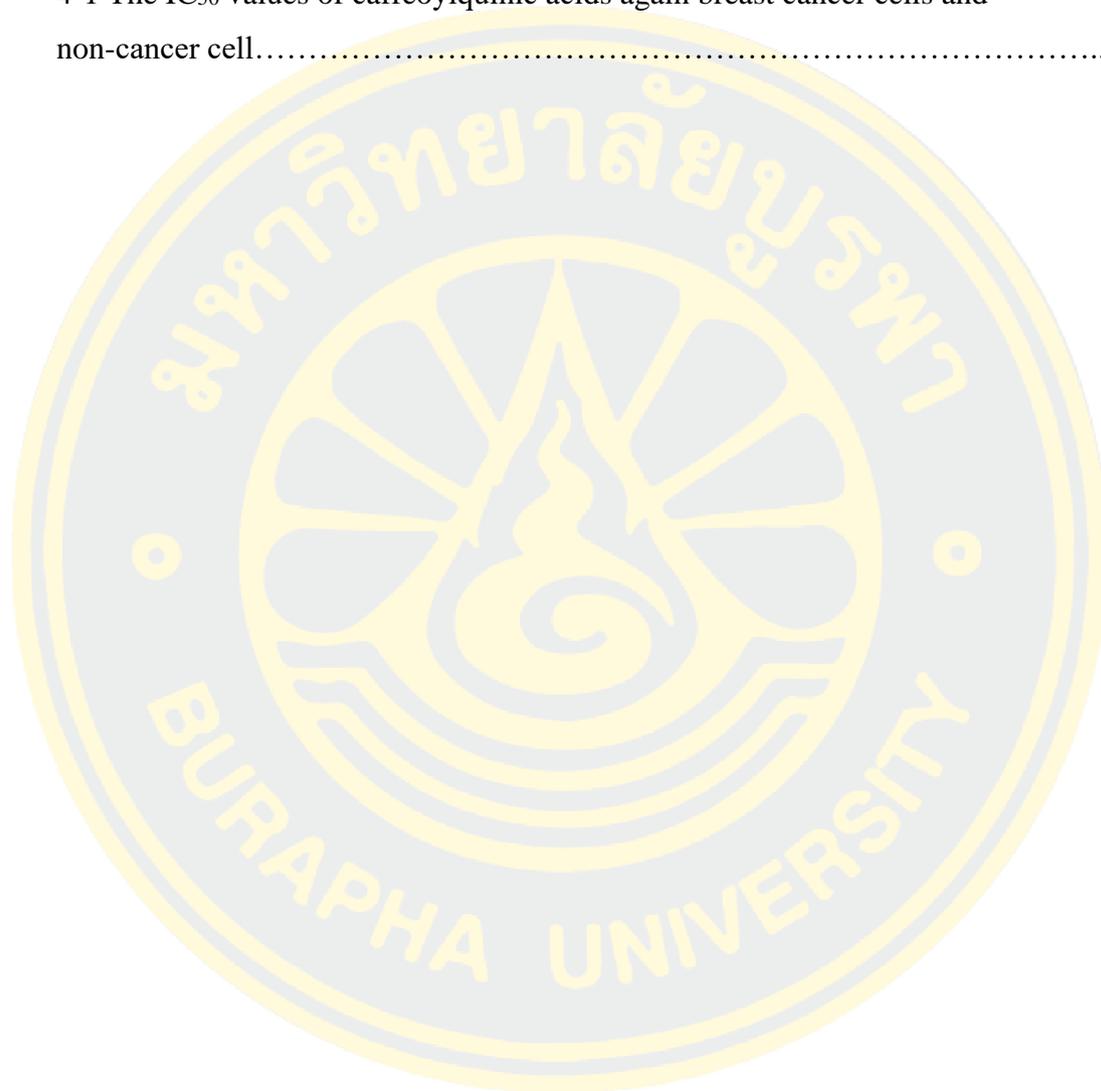
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CHAPTER 1

INTRODUCTION

1.1 Statements and significance of the problems

Cancer is one of the leading causes of death from non-communicable diseases (NCDs) worldwide. It is characterized by uncontrolled proliferation of abnormal cells, which arise from multiple gene mutations accumulating over time (Cooper, 2000). Moreover, cancer cells can migrate from where they originated to distant organs via the lymphatic or vascular circulations, and develop the secondary tumors. More than 5 years, breast cancer is the most commonly diagnosed cancers in females worldwide, contributing 11.7% of new cancer cases diagnosed in 2020 and an estimated 700,000 women died from this disease in the same year. (sung et al., 2021).

Currently, chemotherapy is one of the standard cancer treatments widely used in leukemia, lymphoma, and carcinoma because the chemotherapeutic drugs circulate in the blood and can be distributed throughout the body (Falzone et al., 2018). It can be used either alone or in a combination with other methods such as radiotherapy, and surgery. Basically, anti-cancer drugs are designed to kill rapidly-growing cells, mostly by inducing DNA damages or interfering DNA replicating process, lead to cell cycle arrest and ultimately cell death. In order to induce apoptotic cell death in cancer cells, anti-cancer drugs generate reactive oxygen species (ROS) to induce oxidative stress (Kenneth, 2004; Fulda et al., 2006). However, chemotherapeutic drug-induced ROS production have been implicated in drug resistance of cancer cells and partly cause adverse effects to patients. For example, doxorubicin, which is a common drug used for breast cancer, can cause

cardiomyopathy (Ilghami et al., 2020). In addition, 5-Fluorouracil, another drug for breast cancer treatment, also cause nephrotoxicity, and is hepatotoxic (Gelen et al., 2018). Therefore, natural antioxidants with anti-cancer properties may reduce ROS-induced side effects and, if used in combination with, may sensitize cancer cells to chemotherapeutic drugs (Danheir et al., 2014; Hseu et al., 2017; Yiang et al., 2014).

Caffeoylquinic acids (CQAs) are polyphenolic compounds. Due to the core of their chemical structure, which is compound of mono-to tri-esters of caffeic acids, class of quinic acids and derivatives (Islam et al., 2002). Naturally, they mostly found in various fruits, vegetables, and herbs, such as blueberry (Dawidowicz et al., 2014), propolis (Miyamae et al., 2011), sweet potato (Yoshimoto et al., 2002), coffee beans, and also tea leaves (Grujić-Letić et al., 2015). Leaves of *Pluchea indica* (L.) Less., which are used to produce tea leaves in Thailand, has been previously reported to contain various mono-CQAs, including 3-*O*-caffeoylquinic acid (Neo-chlorogenic acid), 4-*O*-caffeoylquinic acid (Cryptochlorogenic acid), 5-*O*-caffeoylquinic acid (Chlorogenic acid), as well as di-CQAs, that are 3,4-*O*-dicafeoylquinic acid (3,4-diCQA), 3,5-*O*-dicafeoylquinic acid (3,5-diCQA), and 4,5-*O*-dicafeoylquinic acid (4,5-diCQA), as main phenolic compounds (Chen et al., 2009, Kongkiatpaiboon et al., 2018). CQAs exhibit a variety of biological activities including antibacteria, anti-inflammation, anti-diabetes (inhibitory activity against α -glucosidase), anti-cancer, and anti-oxidation (Fiamegos et al., 2011; Wu et al., 2016; Islam et al., 2013; Jiao et al., 2019). Several CQA derivatives are recognized as natural antioxidants (Azuma et al., 1999; Xu et al., 2012; Teoh et al., 2018). Interestingly, they displayed protective effect on cardiomyocytes against H₂O₂ –induced damage (Yu et al., 2016). In addition, CQAs could inhibit cell growth of human colon cancer Caco-2 cells and also

inhibit proliferation and invasion of human lung carcinoma H1299 cells via PI3K/Akt signaling pathway (Carvalho et al., 2010; In et al., 2016). In contrast to function as antioxidants, CQAs were reported to be able to induce oxidative stress in order to anti-proliferate HCT116 human colon carcinoma cells (Teoh et al., 2018). However, the anti-cancer activity and effect on intracellular ROS of CQAs in breast cancer cells are still limited.

Therefore, in the current study, selected mono-and di-CQAs, which mainly presented in *P. indica* leaves, were assessed the potential in anti-proliferation and anti-migration on breast cancer cell lines, MCF-7 and MDA-MB-231 and compared to non-cancer cell line Vero. In addition, intracellular ROS level in CQAs-treated cancer cells were examined in order to propose underlying anti-cancer mechanism. The results from this study may provide useful information in developing CQAs as anti-breast cancer agents, or in combination treatment with doxorubicin for reducing side effects and resolving drug resistance in cancer cells.

1.2 Objectives

1. To investigate the effect on viability, proliferation and migration of selected CQAs in breast cancer cells (MDA-MB-231 and MCF-7) and compared to non-cancer cells (Vero)
2. To determine the modulatory effect of selected CQAs on intracellular ROS level in breast cancer cells

1.3 Hypotheses

1. Selected CQAs can inhibit viability, proliferation and migration of breast cancer cells (MDA-MB-231 and MCF-7) more than those of normal cells (Vero).

2. CQAs increase intracellular ROS level to induce anti- proliferation of breast cancer cells.

1.4 Contribution to knowledge

1. The knowledge of inhibitory effect of CQAs on breast cancer cells.
2. The knowledge of modulatory effect of CQAs on intracellular ROS level in breast cancer cells.
3. The knowledge of scientific method such as Transwell assay and DCFDA-staining method.

1.5 Scope of study

In this study, commercial CQAs, including 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA, were used to determine the anti-cancer activity in breast cancer cells (MDA-MB-231 and MCF-7) and compared to non-cancer Vero cells. First, the inhibitory effect on cancer or non-cancer cell growth was investigated using MTT assay with various concentrations of selected CQAs, as well as a referent drugs, doxorubicin. The IC_{50} values of each compound were determined. Second, selected CQAs were examined the effect on proliferation of cancer cells at IC_{50} concentration by colony formation assay, and the results were compared to that of Vero cells. Third, migration capacity of cancer cells treated with CQAs were examined by using transwell assay. Finally, breast cancer cells treated with CQAs, in combination with or without N-Acetyl-L-cysteine (NAC), were measured the intracellular reactive oxygen species (ROS) level by DCFDA-staining assay and compared to non-treated cells. All experiment were performed in triplicate. ANOVA

and Tukey's comparison test were applied to determine significant difference between treated and untreated cells, or cancer and noncancerous cells at $p < 0.05$ using Minitab 18 software.



CHAPTER 2

LITERATURE REVIEWS

2.1 Theory

2.1.1 Cancer (Sarkar et al., 2013)

Cancer is diseases characterized by the development of abnormal cells that uncontrolled cell division and tissue invasiveness (metastasis) caused by a series of mutations in the genes that regulate the cell cycle, typically involve either promotion of cell division (proto-oncogenes) or inactivation of cell cycle suppression (tumor suppressor genes).

2.1.2 Carcinogenesis (Sidiqui et al. 2015)

Carcinogenesis is a multi-stage process by which normal cells transform into malignant cells, which includes initiation, promotion, and progression.

1. Initiation involves the alteration, change, or mutation of genes arising spontaneously or induced by exposure to a carcinogenic agent. Genetic alterations can result in dysregulation of biochemical signaling pathways associated with cellular proliferation, survival, and differentiation, which can be influenced by a number of factors, including the rate and type of carcinogenic metabolism and the response of the DNA repair function.

2. Promotion stage is considered to be a relatively lengthy and reversible process in which actively proliferating preneoplastic cells accumulate. Within this period, the process can be altered by chemo preventive agents and affect growth rates.

3. Progression is the final stage of neoplastic transformation, where genetic and phenotypic changes and cell proliferation occur. This involves a fast increase in the tumor size, where the cells may undergo further mutations with invasive and metastatic potential.

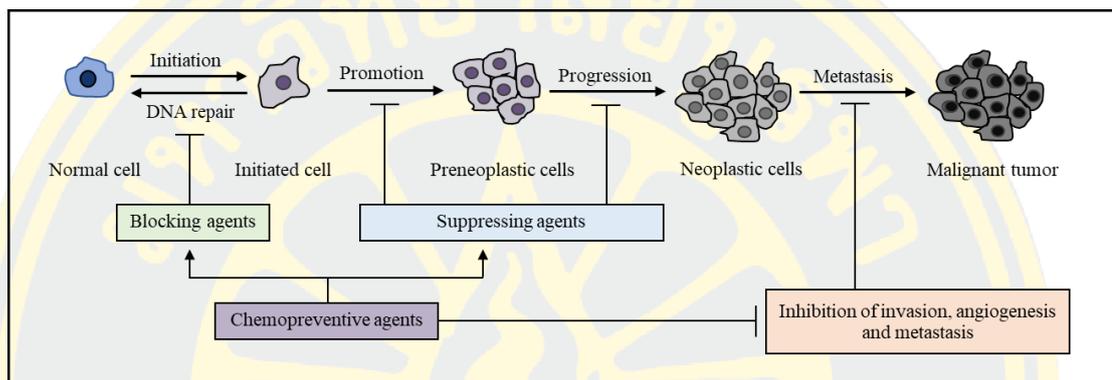


Figure 2-1 Carcinogenesis phases: initiation, promotion, progression, and metastasis
(Siddiqui et al., 2015)

2.1.3 Breast cancer

Breast cancer is the most common cancer in female (Bray et al., 2018). In 2020, the American Cancer Society on Cancer reported an estimated 700,000 women died from this disease and approximately 12.7% of total cancer cases in women were diagnosed as breast cancer in 2020 (sung et al., 2021).

Breast Lymph Nodes

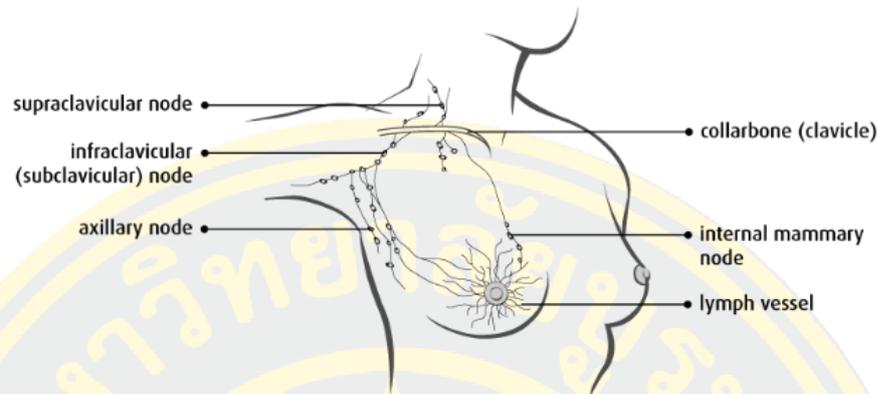


Figure 2-2 Breast structure (<https://www.cancer.ca>)

2.1.4 Stage of breast cancer (Akram et al., 2017)

Breast cancer stage is usually expressed as a scale of 0 through IV. Early-stage breast cancer – The tumor is smaller than 5 cm and the cancer has not spread to more than 3 lymph nodes. It includes stages 1A, 1B and 2A.

Locally advanced breast cancer – The tumor is larger than 5 cm. The cancer may have spread to the skin the muscles of the chest wall or more than 3 lymph nodes. It includes stages 2B, 3A, 3B and 3C. Inflammatory breast cancer is also considered locally advanced breast cancer.

Metastatic breast cancer – The cancer has spread to other parts of the body. It is stage IV

Stage 0: There are cancer cells only in the lining of a breast duct. This is called ductal carcinoma in situ (DCIS).

: There is a buildup of abnormal cells in the breast lobules. This is called lobular carcinoma in situ (LCIS).

: There is Paget disease of the breast without any invasive carcinoma, DCIS or LCIS.

Stage 1A: The tumor is 2 cm or smaller.

Stage 1B: The tumor is 2 cm or smaller, or no tumor can be seen in the breast. A small number of cancer cells are found in the lymph nodes (micrometastases). Each lymph node with cancer cells in it is no larger than 2 mm.

Stage 2A: The tumor is 2 cm or smaller, or no tumor can be seen in the breast. Cancer cells are found in 1 to 3 lymph nodes under the arm (axillary lymph nodes), in lymph nodes inside the chest around the breastbone (internal mammary lymph nodes) or in both areas. Or the tumor is larger than 2 cm but not more than 5 cm.

Stage 2B: The tumour is larger than 2 cm but not more than 5 cm. The cancer has also spread to 1 to 3 axillary lymph nodes, internal mammary lymph nodes or both areas. Or the tumour is larger than 5 cm.

Stage 3A: The tumour is 5 cm or smaller, or no tumour can be seen in the breast. Cancer cells are found in 4 to 9 axillary lymph nodes, or in internal mammary lymph nodes but not in axillary lymph nodes. Or the tumour is larger than 5 cm. The cancer has also spread to 1 to 9 axillary lymph nodes or to internal mammary lymph nodes. Or it may have spread to 1 to 3 axillary lymph nodes and internal mammary lymph nodes.

Stage 3B: The tumour has grown into the muscles of the chest wall or the skin or both. The cancer may have also spread to 1 to 9 axillary lymph nodes or to internal mammary lymph nodes. Or it may have spread to 1 to 3 axillary lymph nodes and internal mammary lymph nodes.

Stage 3C: The cancer has spread to 10 or more axillary lymph nodes or to lymph nodes below the collarbone (infraclavicular lymph nodes).

: The cancer has spread to more than 3 axillary lymph nodes and internal mammary lymph nodes.

: The cancer has spread to lymph nodes above the collarbone (supraclavicular lymph nodes).

Stage IV: The cancer has spread to other parts of the body (called distant metastasis), such as to the bone, liver, lungs or brain. This is also called metastatic breast cancer.

2.1.5 Treatment of breast cancer

The treatment of breast cancer depends on the stage and type of breast cancer. A multimodal approach is used in the treatment of breast cancer, including chemotherapy, surgery, radiation, and hormone therapy. Early breast tumor can be removed by surgery. For most cases, the chance of cancer recurrence is small if combined with radiation therapy. Radiation therapy is the use of radiation with high intensity to destroy or reduce tumors, cancer cells, and non-cancerous disorders (Moulder and Hortobagyi, 2008). Some cases may use chemotherapy to treat cancer. Anticancer medications are used to shrink or destroy malignant cells and stop cancer from spreading to other areas of the body. For example, doxorubicin can treat or alleviate a variety of malignant tumors effectively, such as breast cancer, bladder cancer, and cervical cancer. However, doxorubicin is highly toxic, and its long-term application may cause dose-dependent irreversible cardiomyopathy, severe cardiac toxicity, or liver damage, thereby limiting its application in clinical practice (Liquin

and Baolin, 2017; Ciarimboli, 2014). In addition, supplemental hormone therapy can also be used to prevent or halt tumor development or spread. For patients with late-stage breast cancer, which cancer cells have spread to several parts of the body, the goal of treatment is to control breast cancer for as long as possible (Wake & Winer, 2019).

2.1.6 Mechanisms of chemotherapeutic drugs

Chemotherapeutics like doxorubicin and cisplatin raise Reactive Oxygen Species (ROS) levels, which is a factor in their genotoxicity. ROS induces other forms of DNA damage through oxidizing nucleoside bases (e.g., the formation of 8-oxo guanine), which can lead to G-T or G-A transversions if left unrepaired. Additionally, ROS accumulation causes mitochondrial DNA strand breakage, lesions, and degradation (Srinivas et al., 2019). One of the processes by which several chemotherapeutic drugs promote tumor regression is by the amplification of ROS levels during chemotherapy. This promotes cell death by raising the proliferative rate of cancer cells above a certain threshold, such as with doxorubicin, daunorubicin, and epirubicin, which generate the highest levels of cellular ROS (Fig 2-3). The formation of cellular ROS is increased during chemotherapy by two main pathways: mitochondrial production of ROS and suppression of the cellular antioxidant defense. Chemotherapeutics induce a loss of mitochondrial membrane potential and inhibit complexes I and II, leading to disruption of the mitochondrial electron transport chain (ETC) and electronic leakage, and to elevated ROS production consequently. Another pathway to elevate cellular ROS production during chemotherapy is by the inhibition of the antioxidant system, such as GSH and ascorbic acid, enzymes regenerating the

reduced forms of antioxidants and ROS interacting enzymes such as superoxide dismutase (SOD), peroxidases and catalases. For example, Imexon is a small chemical drug that binds to thiols like GSH and cysteine, producing a reduction in cellular GSH and a buildup of ROS in individuals with metastatic cancer (Yang et al., 2018).

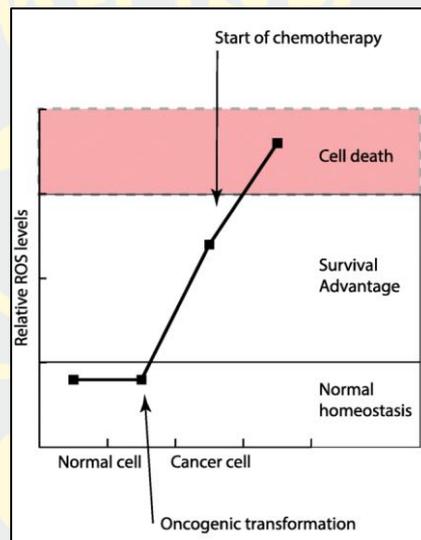


Figure 2-3 Chemotherapy raises ROS levels over a threshold that is thought to trigger biological processes that ultimately result in cell death, primarily via apoptosis (Yang et al., 2018).

2.1.7 Cell culture

1. Breast cancer MDA-MB-231 cell lines

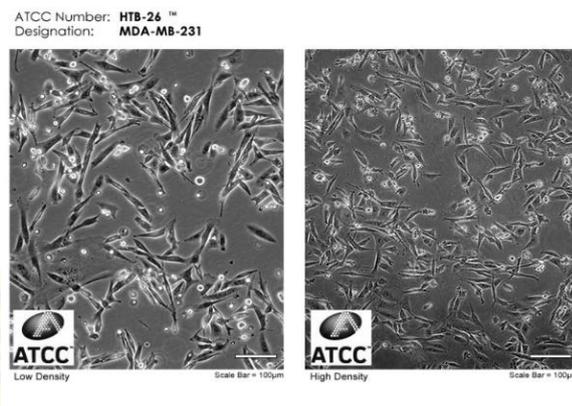


Figure 2-4 Morphology of MDA-MB-231 cells (<https://www.atcc.org>)

MDA-MB-231 cell line is an epithelial and highly aggressive, invasive, and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks estrogen receptor (ER), progesterone receptor (PR-), and Human Epidermal Growth Factor Receptor 2-negative; HER2-/neu (Zhaoming, Panpan and Jianhui, 2020).

2. Breast cancer MCF-7 cell lines

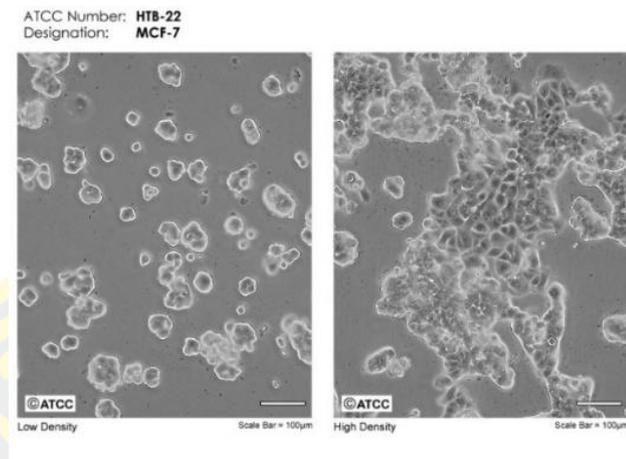


Figure 2-5 Morphology of MCF-7 cells (<https://www.atcc.org>)

MCF-7 cell line is an epithelial, and carcinoma. Routinely, it has the expression of estrogen receptor (ER+) and progesterone receptor (PR+) (Vincent, Roman and Philippe, 2010).

3. Non-cancer cell line (Vero)

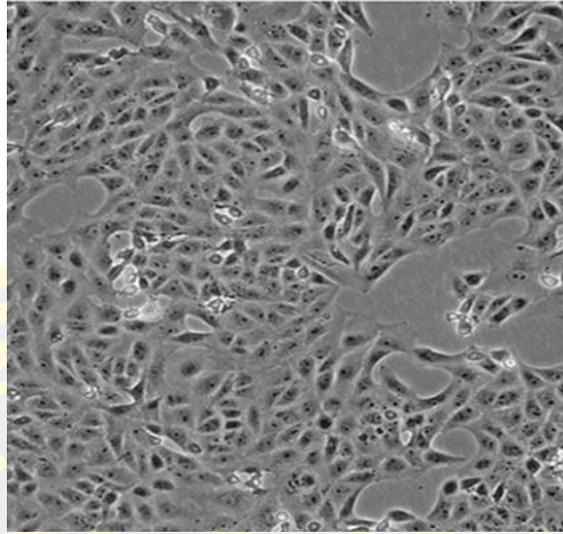


Figure 2-6 Morphology of Vero cells

Vero was isolated from kidney epithelial cells extracted from an African green monkey. Vero cells are extensively used for testing cytotoxicity.

(<https://www.atcc.org/products/ccl-81>)

2.1.8 MTT assay (Riss et al., 2016)

The MTT is a colorimetric assay for cell viability that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondria succinate dehydrogenase in metabolically active cells. The MTT solution enters the cells and passes into the mitochondria where MTT is converted into insoluble, purple colored formazan salts. The cells are then solubilized with DMSO and the resulting purple formazan solution is measured by a spectrophotometer at 540 nm. The intensity of purple color is proportional to the number of living cells.

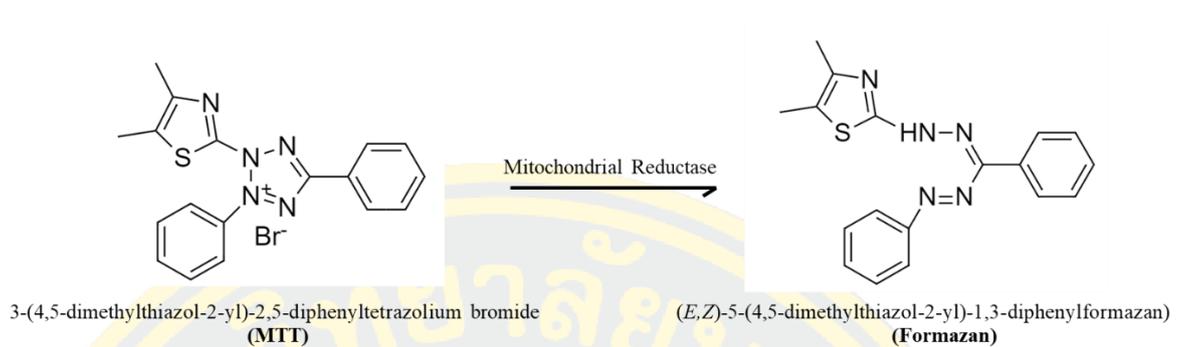


Figure 2-7 Reduction of the MTT, a yellow tetrazolium salt to purple formazan by succinate dehydrogenase in viable cells (<https://en.wikipedia.org>)

2.1.9 Colony formation assay (Franken et al., 2006)

The colony formation assay, also known as clonogenic assay, is an *in vitro* assay that measures the capability of a single cell to grow into a colony. A colony is considered to consist of at least 50 cells. This assay is commonly used to assess cell reproductive death after treatment with cytotoxic agents or radiation. The initiated cells are seeded at low densities and left for 1-3 weeks for colonies to form. Colonies are then fixed and stained to make them visible and easily counted under a stereomicroscope.

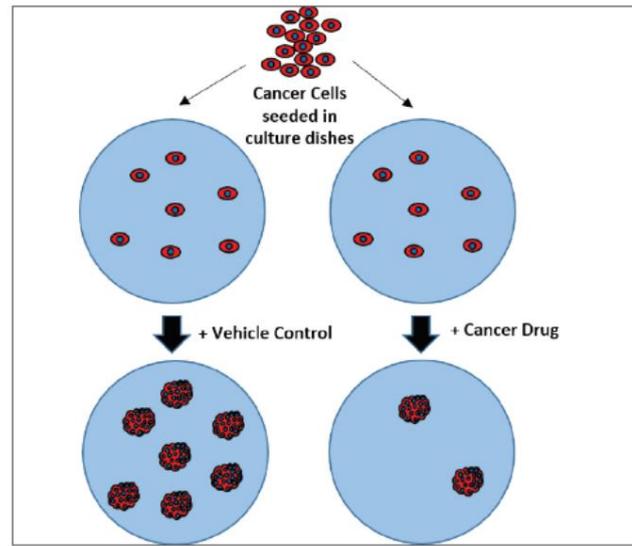


Figure 2-8 Schematic representation of the colony formation assay

(Ernest Heimsath, 2020).

2.1.10 Migration assay (Franken et al., 2006; Justus et al., 2014)

The transwell migration assay is a common technique for the migratory response of cells to chemical inducers or inhibitors. In order to determine the cell migration, cells are seeded on top of the filter permeable membrane in a transwell insert, and a solution containing the test agent is introduced below the permeable membrane, into the bottom of the lower chamber in a 24-well plate. If the invasive capability of cells is investigated, the membrane is usually additionally coated with some extracellular matrix component (such as collagen) which facilitates both adherence and migration. The cells that can migrate through the membrane are stained and counted after a 24-hour incubation period. In addition, cell migration is involved in the immune response and many pathological processes such as cancer metastasis and inflammation.

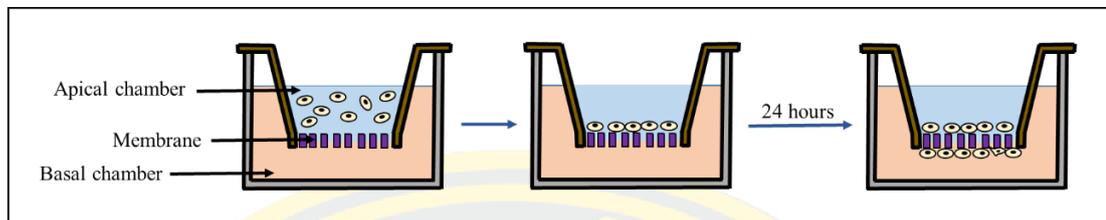


Figure 2-9 Schematic diagram of cell migration assay.

(<https://www.reactionbiology.com>)

2.1.11 Reactive Oxygen Species (ROS) generation in cells using DCFDA

(Banerjee et al., 2017)

Cellular ROS levels can be measured in live cells by 2',7' – dichlorofluorescein diacetate (DCFDA) dye which is oxidized to a fluorescence dye 2',7'-dichlorofluorescein (DCF). The fluorescence generated is directly proportional to the amount of oxidized DCFDA to DCF. Since the emission of the fluorescent dye is about 529nm, it can be measured in the FL-1 green channel by flow cytometry or fluorescent microscopy.

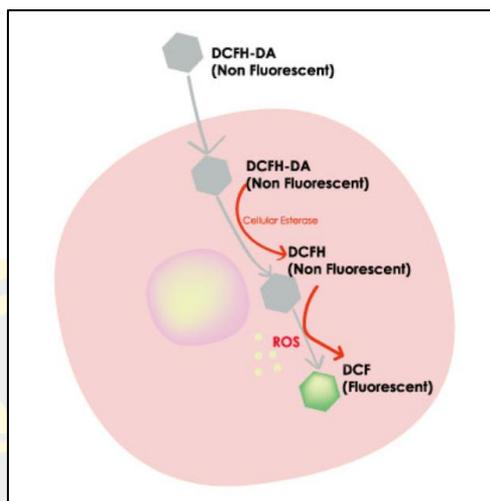


Figure 2-10 Action of ROS in DCFDA staining assay. (<https://dawinbio.com/product-information/?idx=8733746&bmode=view>)

2.2 Literature reviews

Literature survey revealed that caffeoylquinic acid (CQAs) is one of the phenylpropanoids found in coffee beans, sweet potatoes, propolis, and *Pluchea indica* (L.) Less. (*P. indica*) (Miyamea et al., 2011). The proliferation and viability of GBM8401 and HeLa cancer cells were both reduced by 75% and 70%, respectively, after being exposed to crude aqueous extracts of *P. indica* leaf and root for 48 hours. In GBM8401 and HeLa cells, it was discovered that p53 and p21 were activated (Cho et al., 2012). In addition, leaves of *P. indica* have been previously reported to contain a high amount of phenolic compounds such as CQAs derivatives, including 3-*O*-caffeoylquinic acid (17.34%), 4-*O*-caffeoylquinic acid (3.72%), 5-*O*-caffeoylquinic acid (2.52%), 3,4-*O*-dicaffeoylquinic acid (2.50%), 3,5-*O*-dicaffeoylquinic acid (10.50%), and 4,5-*O*-dicaffeoylquinic acid (24.24%) (Kongkiatpaiboon et al., 2018).

In addition, fresh potatoes contain CGAs that range from 0.10 to 0.19 mg of 5-CGA per 100 g of potato, which is equivalent to 90% of the total phenolic compounds present in potato tubers (Dao & Friedman, 1992). In addition, caffeoquinic acid derivatives were found effective as scavengers of the free radical, and caffeoquinic acid compounds could protect against oxidation stress such as Cu^{2+} -mediated low-density lipoprotein oxidation (Hung et al., 2006). Furthermore, the antioxidant effect of caffeoylquinic acid derivatives inhibits H_2O_2 -induced damage to the retinal ganglion cell line (RGC-5) as well as lipid peroxidation LPO in mouse forebrain homogenates (Nakajima et al., 2007).

In addition, In et al. (2016) that reported, CQAs derivatives showed anti-cancer activity. 5-caffeoylquinic acid (5-CQA) showed inhibitory effects on cell proliferation in human lung carcinoma H1299 cells via the PI3K/Akt signaling pathway. Meanwhile, 5-CQA treatment at 50 μM concentrations blocked mitogen-stimulated cell invasion in p53 wild-type A549 and p53-deficient H1299 NSCLC cells. Furthermore, caffeic acid and 5-caffeoylquinic acid inhibited the cell cycle in a human colon adenocarcinoma cell line (HT-29). In HT-29 cells, CA (10 mM) treatment promoted an increase of cells in the G₀/G₁ phase and a decrease in the G₂/M phase after 48 h. 5-CQA (5 mM and 10 mM) induced an increase in the percentage of cells in G₀/G₁ phase, followed by a decrease in cell number in G₂/M phase after 48 h and 96 h of treatment (Murad et al., 2015).

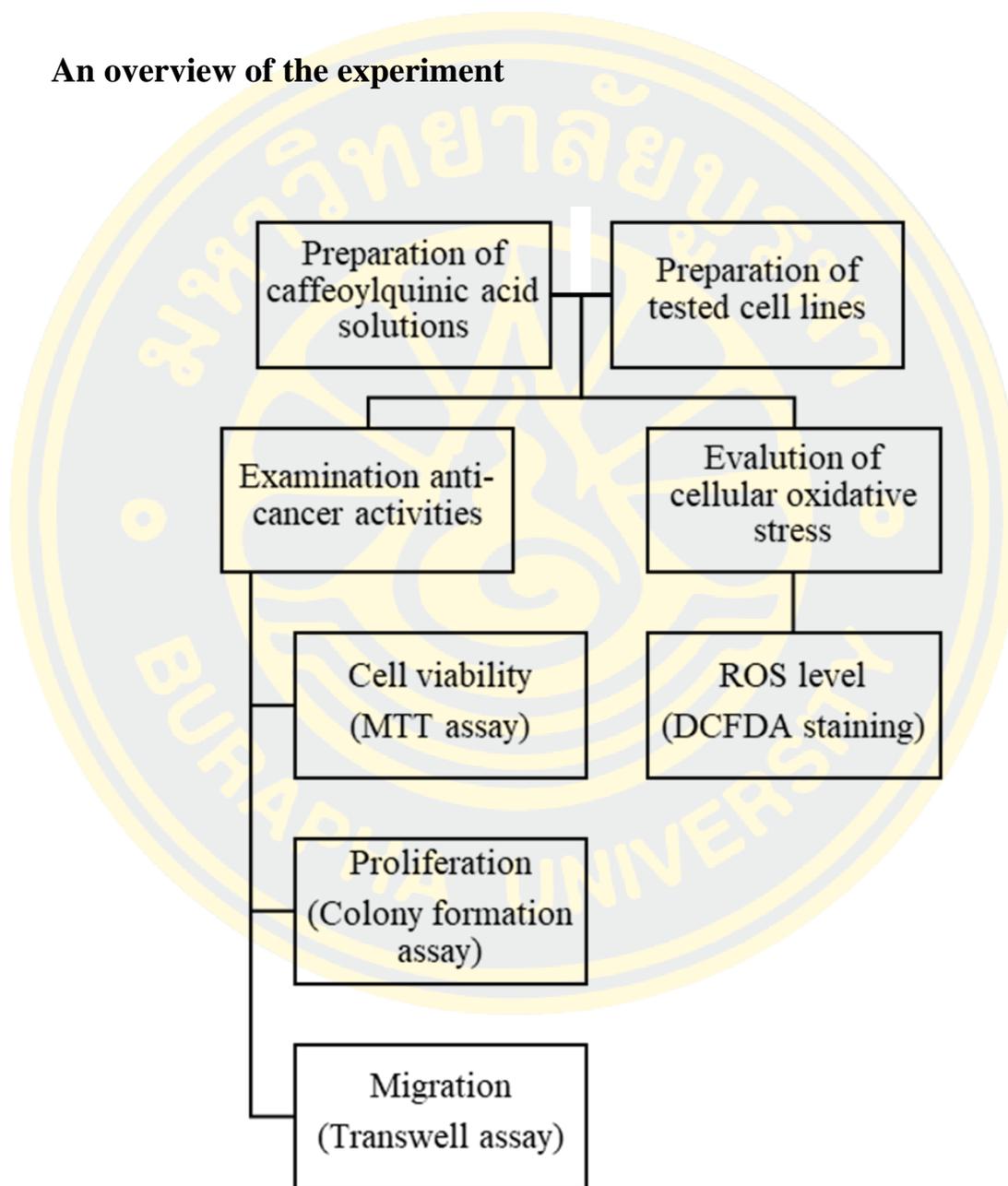
Moreover, the levels of MMP-2, MMP-9, or tissue inhibitor of metalloproteinase-2, an endogenous inhibitor of MMP, were not changed in 5-CQA-treated cells. Additionally, concentrations of 3,5-dicaffeoylquinic acid (DCQ) at 2 mg/ml for 48 h could induce a cell apoptosis increase of 76% in late apoptotic or

necrotic on HCT 116 cells human colon carcinoma cells. Moreover, 5-*O*-caffeoylquinic acid (CQ) and DCQ could induce HCT 116 cells arrested at S phase. At 1 and 2 mg/ml of CQ after 48 h treatments, the percentage of cells at S phase increased by 5.1 and 13.2 %, respectively. At 1 and 2 mg/ml of DCQ after 24 h treatments, the percentage of cells at S phase increased by 8.0 and 8.2 %, respectively (Teoh et al., 2018). Furthermore, 4,5-di-*O*-CQA has anti-migratory effects on lung cancer cells (A549) at concentrations of 30 μ M via the TRPV1 pathway in hypoxic microenvironments. (Kim et al., 2017). In addition, 1,3-DCQA and 1,5-DCQA inhibited the cells ability to form colonies in MCF-7 at concentrations of 24 μ M to 96 μ M. (Zhou et al., 2020). Moreover, 5-CQA induced hemeoxygenase-1 (HO-1) and glutamate cysteine ligase (GCL) expression, causing hydroperoxide-induced ROS production and GSH depletion in HepG2 cells. (Chen et al., 2020).

CHAPTER 3

RESEARCH METHODOLOGY

An overview of the experiment



3.1 Materials and equipments

3.1.1 Equipments

1. 24-well transwell inserts (SPL Life Sciences, Korea)
2. 6-cm culture plate (SPL Life Sciences, Korea)
3. 96-well black plate (SPL Life Sciences, Korea)
4. 96-well cell culture plate (Thermo Fisher Scientific, China)
5. Biological Safety Carbinet class II (NuAir, USA.)
6. Cell culture flask (Thermo Fisher Scientific, China)
7. Centrifuge tube 15 mL and 50 mL (Thermo Fisher Scientific, USA.)
8. CO₂ Incubator (NuAir, USA.)
9. Duran glass bottle (Schott AG, Germany)
10. Filter upper cup (Jet Biofil, China)
11. Fluorescence spectrophotometer (Agilent Technologies, USA.)
12. Hemocytometer slide (BOECO, Germany)
13. Inverted microscope eclipse TS100 (Nikon, Japan)
14. Measuring pipette 5 mL and 10 mL (Henneberg-Sander GmbH, Germany)
15. Microcentrifuge tube 1.5 mL (Kirgen, China)
16. Microplate Readers (Molecular Devieces, USA.)
17. pH Meter (Denver Instrument, Germany)
18. Pipette Aid (Thermo Fisher Scientific, USA.)
19. Pipette tips 10, 200 and 1000 µl
20. Spin down centrifuge (Hercuvan, Lab systems, Malaysia)
21. Stereo microscope (Olympus SZ51, Japan)
22. Vortex mixer (Isolab, Germany)

23. Water bath (Eyela, Japan)

3.1.2 Chemicals

1. 0.25% trypsin-EDTA (Gibco, USA.)
2. 0.4% trypan blue solution (Gibco, USA.)
3. 2',7'-Dichlorofluorescein diacetate (Sigma-Aldrich, USA.)
4. 3,4-dicaffeoylquinic acid (Isochlorogenic acid B) (MedChem Express, USA.)
5. 3,5-dicaffeoylquinic acid (Isochlorogenic acid A) (MedChem Express, USA.)
6. 3-caffeoylquinic acid (Neo-chlorogenic acid) (Sigma-Aldrich, USA.)
7. 4,5-dicaffeoylquinic acid (Isochlorogenic acid C) (MedChem Express, USA.)
8. 4-caffeoylquinic acid (Cryptochlorogenic acid) (MedChem Express, USA.)
9. 5-caffeoylquinic acid (Chlorogenic acid) (Sigma-Aldrich, USA.)
10. Crystal violet (CARLO ERBA Reagents, Italy)
11. Dimethyl sulfoxide (Amresco, USA.)
12. Dimethyl sulfoxide (Sigma-Aldrich, USA.)
13. Distilled water type I
14. Doxorubicin (MedChem Express, USA.)
15. Dulbecco's Modified Eagle Medium (Gibco, USA.)
16. Ethanol (Chemex, Thailand)
17. Fetal bovine serum (Gibco, USA.)

18. HEPES, free acid (Merck Millipore, China)
19. Methanol (Chemex, Thailand)
20. N-Acetyl-L-cysteine (TCI America, Japan)
21. Penicillin-Streptomycin solution (Gibco, USA.)
22. Phosphate-buffered saline
23. Potassium Chloride (Merck, USA.)
24. Potassium dihydrogen phosphate (Merck, USA.)
25. Sodium bicarbonate (Sigma-Aldrich, USA.)
26. Sodium Chloride (VetecTM Sigma-Aldrich, USA.)
27. Sodium hydrogen phosphate (Merck, USA.)
28. Thiazolyl Blue Tetrazolium Bromide (AppliChem GmbH, USA.)

3.2 Method

3.2.1 Compound preparation

3-CQA and 5-CQA were purchased from Sigma-Aldrich, USA. 4-CQA, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, and doxorubicin were purchased from MedChem Express, USA.

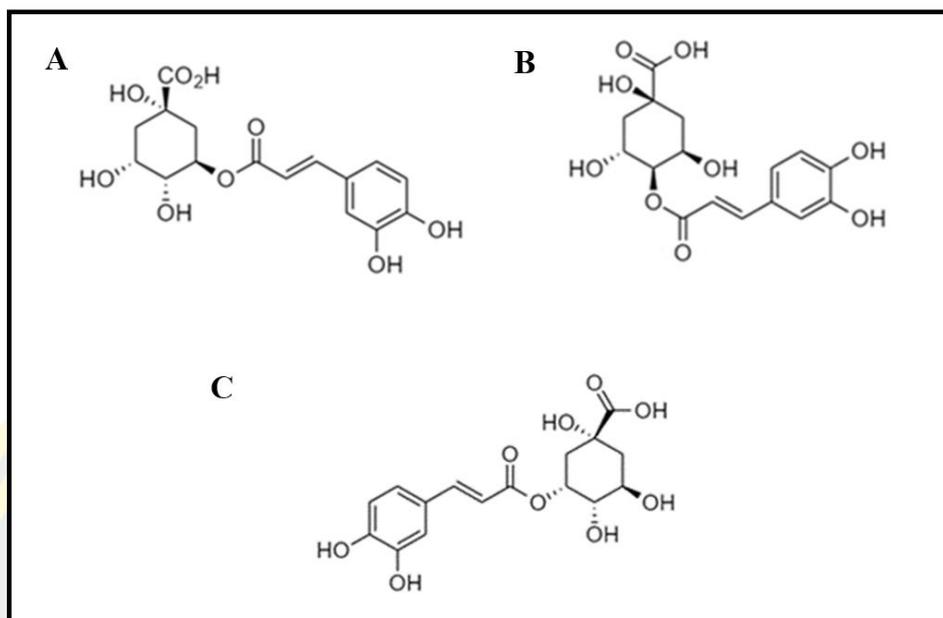


Figure 3-1 The chemical structure of selected mono-CQAs (A) 3-CQA, (B) 4-CQA and (C) 5-CQA (Liu et al., 2016)

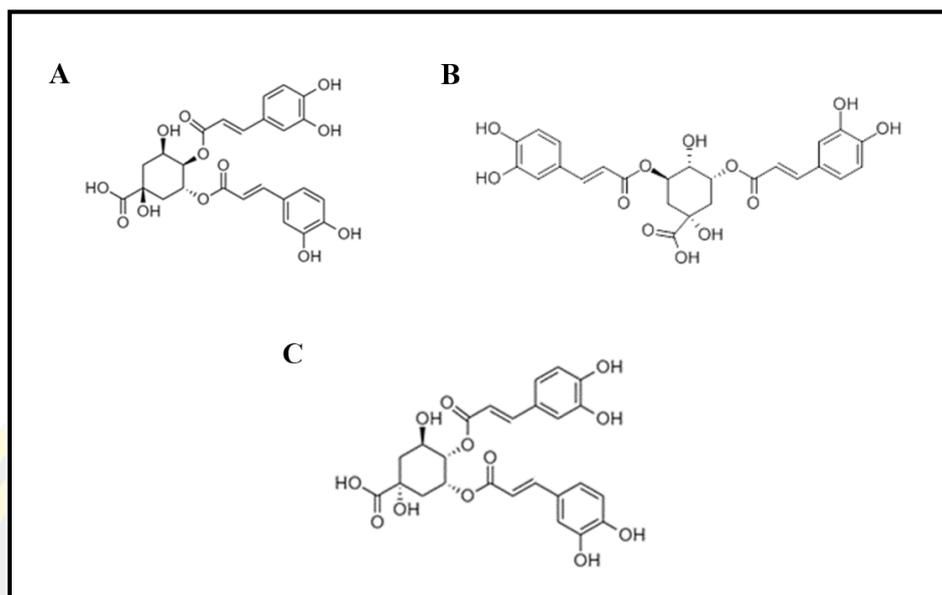


Figure 3-2 The chemical structure of selected di-CQAs (A) 3,4-dicaffeoylquinic acid, (B) 3,5-dicaffeoylquinic acid and (C) 4,5-dicaffeoylquinic acid

All CQAs solutions were prepared in DMSO. A stock solution (100 mM) was two-fold diluted to 50 mM and 25 mM, respectively. All working solutions were finally diluted in culture media to desired concentration as indicated. The final concentration of DMSO is limited to 0.2% for preventing its cytotoxicity.

3.2.2 Cell culture

Breast cancer cell lines (MDA-MB-231 and MCF-7), and non-cancerous cell line (Vero) were maintained in complete media which is Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and 1% antibiotics (Penicillin (10000 units/ml)/Streptomycin (10000 units/ml)). These cells

were incubated at 37 °C, in a humidified incubator with 5% CO₂. All cells used in this study were purchased from The American Type Culture Collection ATCC, USA.

3.2.3 Trypsinization and subculture

Cells were routinely subcultured every 3 - 4 days. When cells grown reach about 80-90% confluence, cells were subcultured by trypsinization. First, the culture media were removed. Second, cells were gently washed with 1x phosphate-buffered saline (PBS) twice and then the solution was completely removed. Then, appropriate quantity of 0.25% trypsin-EDTA solution was added to the side wall of the flask. The flask was gently swirled to cover the cell layer with trypsin solution, and then incubated in CO₂ incubator for 1-2 minutes or until most of cells were detached from culture surface. To inactivate trypsin, 9-fold volume of complete media was immediately added into the flask. After that, gently pipette up and down several times over the cell layer surface by using a P-1000 pipetman to dissociated the cells into single cells. Finally, 25% of cell suspension was transferred to the new flask, and the culture media was added up to complete 5 mL for 25-cm² flask or 10 ml for 75-cm² flask. The culture flask was then incubated in a CO₂ incubator, at 37 °C.

3.2.4 Counting cell with a Hemocytometer (Wilson & Walker, 2010)

In order to count cell using a hemocytometer, 10 µl of evenly-distributed cell suspension were mixed with 10 µl of 0.4% trypan blue. 10 µl of mixture was applied to the hemocytometer by using a P-20 pipetman and then covered the counting chamber with a cover glass. Only live, unstained cells were counted under

an inverted microscope (10x objective lens) using a tally counter and the concentration of viable cells was calculated using the following equation;

$$\text{Viable cell count (cell/ml)} = \frac{\text{Number of live cells counted}}{\text{number of squares counted}} \times \text{dilution factor} \times 10^4$$

3.2.5 Assessment of anti-cancer activity

3.2.5.1 Analysis of cell viability using MTT assay

Cells were trypsinized and counted using a hemocytometer. Cells were seeded onto 96-well plates at a density of 1.5×10^3 cells/well for MDA-MB-231, 3×10^3 cells/well for MCF-7 or 2×10^3 cells/well for Vero, and incubated at 37 °C, in a humidified incubator with 5% CO₂ for 24 h. After that, cells were treated with 50, 100, and 200 μM of CQAs or 0.25, 0.5, and 1 μM of doxorubicin. Each treatment was performed in triplicate. The inhibitory effect of compounds on cell growth were investigated after 72 h of incubation using MTT assay and normalized by untreated control cells (0.2% DMSO).

Once the treatments were complete, the compound containing solutions were replaced with 100 μl/well of DMEM media supplements with 10% MTT solution (Stock MTT solution is 5 mg in 1 ml of PBS buffer). After incubating for 3 h in CO₂ incubator, MTT solution was removed and 200 μl of DMSO was added into each well to dissolve the formazan product. The purple solution was brought to measure the absorbance at 540 nm using a microplate reader. The IC₅₀ value (concentration at which compound inhibits 50% of cell viability) of each CQA and doxorubicin were calculated from the plot between the concentration of compound

(X axis) and the percentage of cell viability (Y axis) (Fig 3-3). The obtained IC₅₀ values of treated cancer and non-cancer cells were compared.

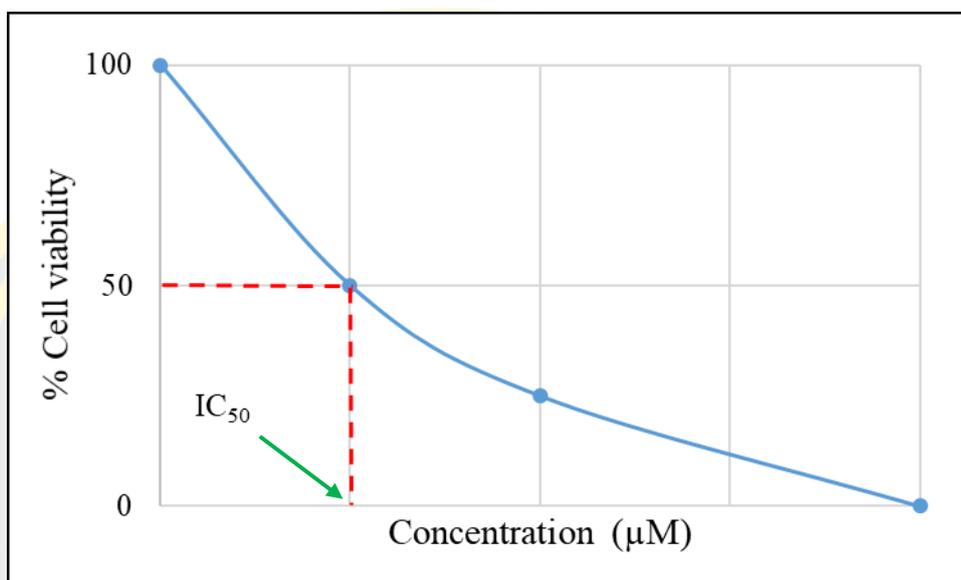


Figure 3-3 Determination of IC₅₀ value

3.2.5.2 Determination of cell proliferation using colony formation assay (Chen et al., 2015)

Single cells of MDA-MB-231, and Vero were seeded onto 60 mm dishes at a density of 1×10^3 cells/dish in triplicate, and incubated for 24 h. Then, cells were treated for 24 h with selected CQAs at IC₅₀ concentration or treated with 0.2% DMSO for negative control. Cells were washed twice with 1x PBS buffer and incubated further for 8-10 days in a CQAs-free complete medium to allow colony propagation. Finally, the medium was removed and cells were gently washed with cold 1x PBS buffer, twice. Cell was fixed with 95% cold methanol (3 ml/dish) for 10 min, and then stained with 0.5% crystal violet prepared in 95% ethanol (1 ml/dish) and let them

stand for 1 h at room temperature. Thereafter, the dishes were washed with the cold 1x PBS buffer for several times and were dried at room temperature. The visible colony which contains 50 cells or more were counted under a stereomicroscope.

3.2.5.3 Determination of cell migration using transwell assay (Tang et al., 2015; Deng et al., 2015)

Cell migration, a process associated with cancer metastasis, was measured using a transwell chamber model. First, MDA-MB-231 cells were suspended in media without FBS at a concentration of 5×10^5 cells/ml, and 200 μ l of the suspension was seeded into the upper chamber while 600 μ l of the medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the cells on the upper surface of the chamber were completely removed by cotton swabs. The lower surface of the membrane was gently washed with 1x PBS buffer and the migrated cells were fixed with cold methanol for 20 min at room temperature. The cells which have migrated through the membrane were stained in chamber with 0.1% crystal violet prepared in 95% ethanol for 30 min and counted under an inverted microscope (20x objective lens).

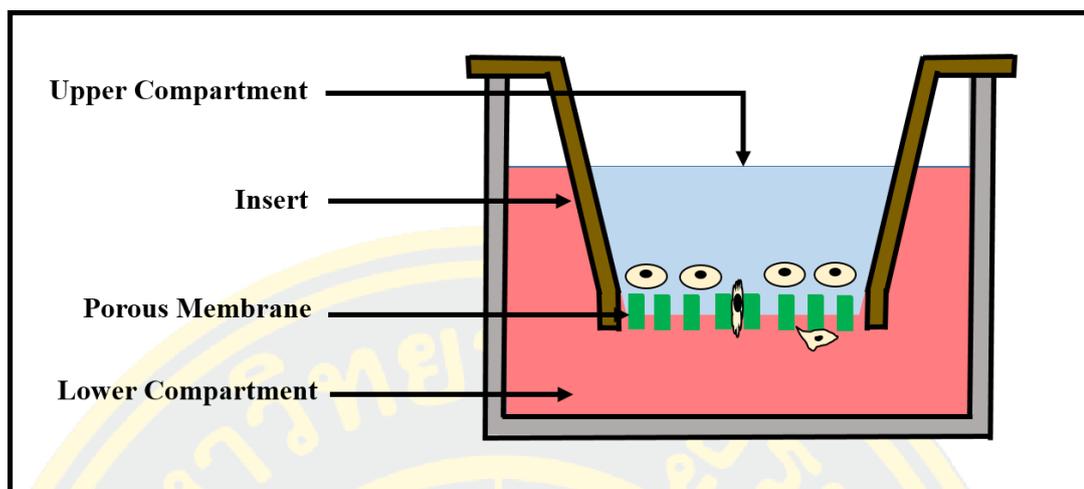


Figure 3-4 Transwell chamber model (Thomas and Robert., 2008)

3.2.6 Assessment of intracellular oxidative status in cancer cells by DCFDA staining method (Filippova et al., 2014)

Intracellular level of ROS was determined using the 2',7'-dichlorofluorescein diacetate (DCFDA) dye. Breast cancer MDA-MB-231 cells and non-cancer Vero cells at a concentration of 2.5×10^3 cells/well were cultured in 150 μ l of complete DMEM for 24 h at 37°C in a 96-well black plate. After that, media containing CQAs were added, and cells were incubated further for 72 h. Next, cells were washed twice with 1x PBS buffer and grown in media without FBS for 1 h. Then, cells were stained with 25 μ M DCFDA dissolved in culture media and incubated in CO₂ incubator for 30 min at 37°C, in the dark. After removing the DCFDA solution, cells were washed with 1x PBS buffer. Finally, 200 μ l of cold 1x PBS buffer were added to each well and the fluorescent intensity was measured using a fluorescence plate reader at excitation and emission wavelengths of 504 and 529 nm, respectively. It's counterpart with the same treatment condition was also performed in a clear 96-well plate for checking the cell

viability by MTT assay. The relative fluorescent intensity was calculated using the following equation;

$$\text{Relative fluorescence intensity (\%)} = \frac{\text{fluorescence intensity}}{\text{Cell viability}} \times 100$$

3.3 Statistical analysis

All experiments were performed in triplicate and repeated at least 3 times. Statistics of ANOVA and Tukey's comparison test were applied to determine significant difference between treated and untreated cells, or cancer and noncancerous cells at $p < 0.05$ using Minitab 18 software.

CHAPTER 4

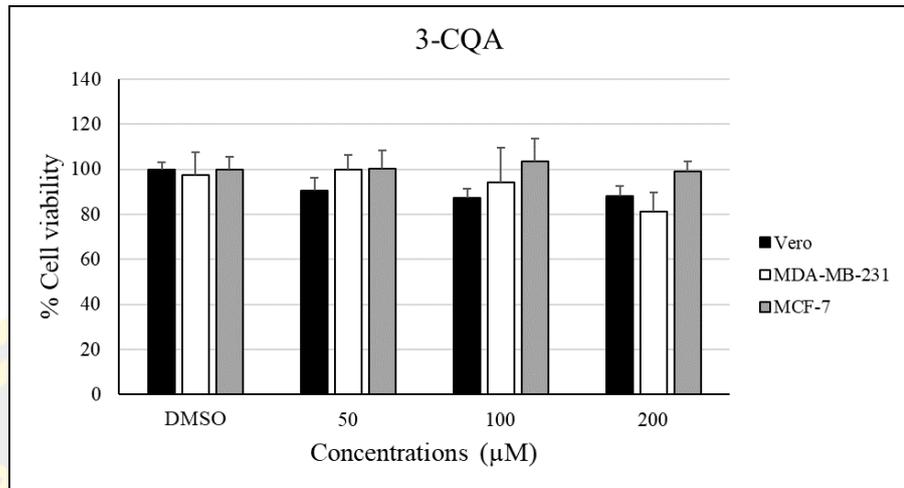
RESULTS

4.1 Cytotoxic effects of CQAs on breast cancer cells

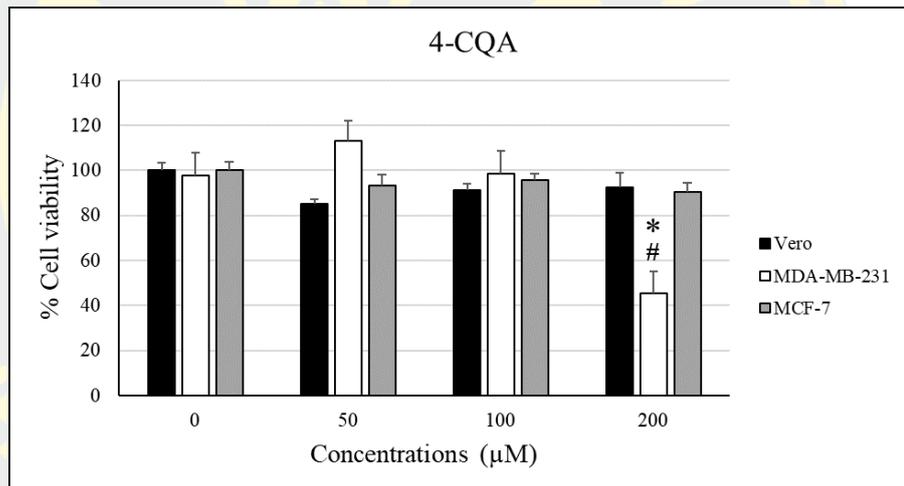
4.1.1 Effect of mono-CQAs on cell viability of breast cancer cells

The cytotoxicity of 3-CQA, 4-CQA, and 5-CQA was determined using the MTT assay. The breast cancer cells were plated in 96-well plates and treated with mono-CQAs at concentrations of 0 – 200 μM for 72 h. The results shown in Figure 4-1 demonstrated that two different breast cancer cell types responded differently to the mono-CQAs where MDA-MB-231 cells showed more sensitive to three mono-CQAs than MCF-7 cells. Mono-CQAs exhibited concentration-dependent cytotoxicity with a significant effect at 200 μM . At this concentration, more than half of MDA-MB-231 cells were growth inhibited by 4-CQA (54.51%) and 5-CQA (52.43%) while approximately 20% inhibition was detected from 3-CQA treatment. The IC_{50} values against all cell types are reported in Table 1. The IC_{50} values of 4-CQA and 5-CQA toward MDA-MB-231 cells were $191 \pm 27.64 \mu\text{M}$ and $194 \pm 27.01 \mu\text{M}$, respectively, whereas the rest were over 200 μM .

(A)



(B)



(C)

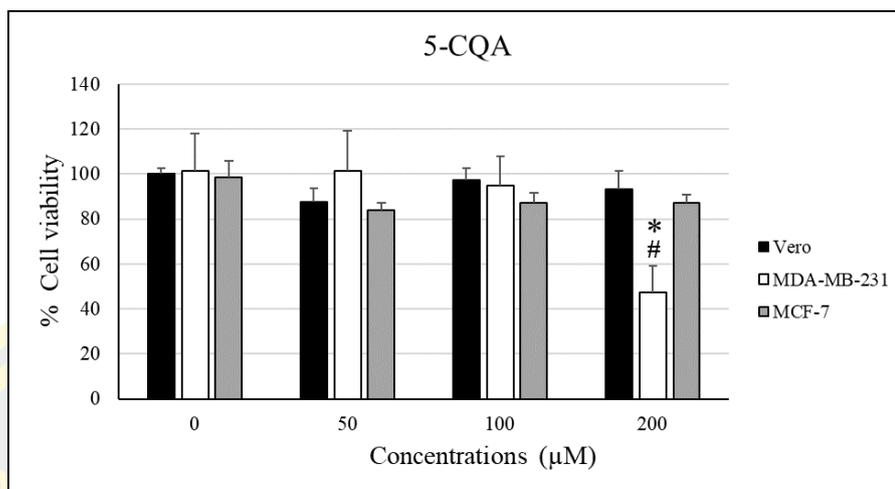


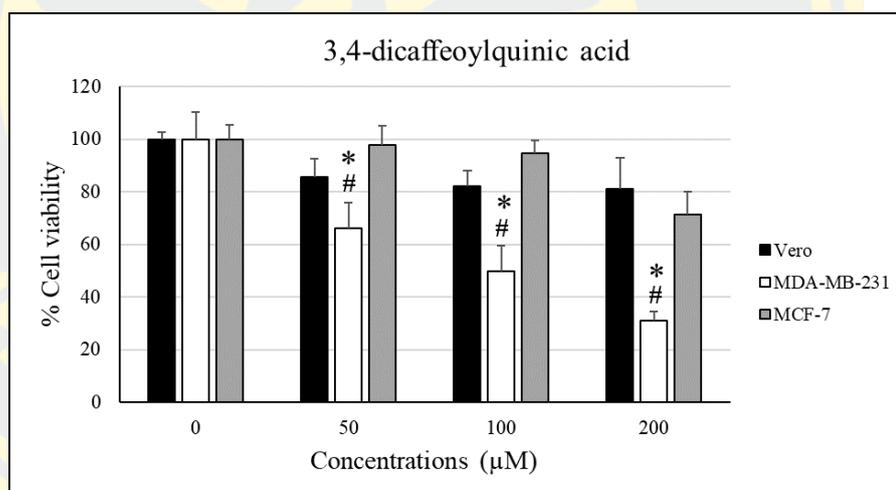
Figure 4-1 The effect of mono-CQAs on cell viability of breast cancer cells. Cells were treated with (A) 3-CQA, (B) 4-CQA, and (C) 5-CQA at the concentrations of 0 – 200 μM for 72 h and cell viability was examined by MTT assay. The data are presented as mean ± SD of at least three independent experiments with triplicate samples. # represents significant growth inhibition (>20%) of treated cells compared to untreated cells ($p < 0.05$), and * represents significant growth inhibition of cancer cells compared to non-cancerous cells at the same treatment condition ($p < 0.05$).

4.1.2 Effect of di-CQAs on viability of breast cancer cells

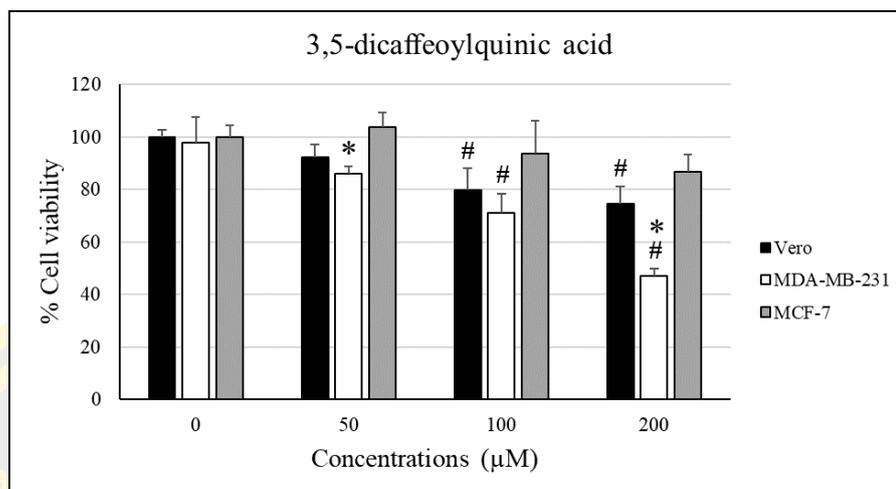
After 72-h treatment of di-CQAs on breast cancer MDA-MB-231 and MCF-7 cells, the cell viability was measured by MTT assay and the results were shown in Figure 4-2. The results showed that both breast cancer cells have a variable response to each di-CQA in a dose-dependent manner. As observed in mono-CQAs results, the viability of MDA-MB-231 cells were also more dramatically reduced by the three di-

CQAs than MCF-7. At 200 μM , 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA inhibited the growth of MDA-MB-231 cells by 31.01%, 46.96%, and 54.74%, respectively. Among di-CQA compounds, 3,4-diCQA exhibited the strongest inhibitory effect against MDA-MB-231 cells followed by 3,5-diCQA and 4,5-diCQA with the IC_{50} value of $108.25 \pm 31.06 \mu\text{M}$, $184 \pm 3.77 \mu\text{M}$, and $>200 \mu\text{M}$, respectively. Di-CQAs at a concentration of 200 μM were able to inhibit the proliferation of Vero cells slightly as 10 – 25%. The lower IC_{50} values indicates di-CQAs were more toxic to breast cancer cells than mono-CQAs (A).

(A)



(B)



(C)

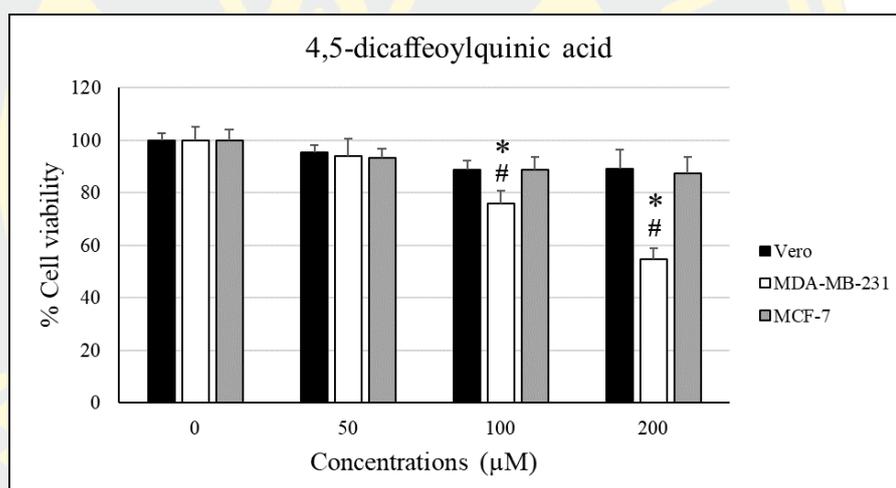


Figure 4-2 The effect of di-CQAs on viability of breast cancer cells. Cells were treated with (A) 3,4-diCQA, (B) 3,5-diCQA, and (C) 4,5-diCQA at the concentrations of 0 – 200 μM for 72 h and cell viability was examined by MTT assay. The data are presented as mean \pm SD of triplicate experiments and repeated at least three times. # represents significant growth inhibition (>20 %) of treated cells compared to untreated cells ($p < 0.05$), and * represents significant growth inhibition of cancer cells compared to non-cancerous cells at the same treatment condition ($p < 0.05$).

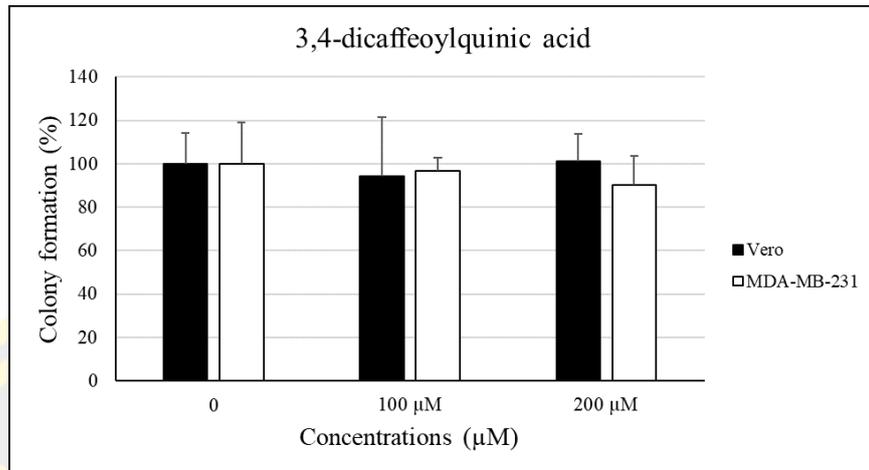
Table 1 The IC₅₀ values of caffeoylquinic acids again breast cancer cells and non-cancer cell

Compounds	Cells	IC ₅₀
3-CQA	Vero	>200 μM
	MDA-MB-231	>200 μM
	MCF-7	>200 μM
4-CQA	Vero	>200 μM
	MDA-MB-231	191 ± 27.64 μM
	MCF-7	>200 μM
5-CQA	Vero	>200 μM
	MDA-MB-231	194 ± 27.01 μM
	MCF-7	>200 μM
3,4- <i>O</i> -Caffeoylquinic acid (3,4-diCQA)	Vero	>200 μM
	MDA-MB-231	108.25 ± 31.06 μM
	MCF-7	>200 μM
3,5- <i>O</i> -Caffeoylquinic acid (3,5-diCQA)	Vero	>200 μM
	MDA-MB-231	184 ± 3.77 μM
	MCF-7	>200 μM
4,5- <i>O</i> -Caffeoylquinic acid (4,5-diCQA)	Vero	>200 μM
	MDA-MB-231	>200 μM
	MCF-7	>200 μM
Doxorubicin	Vero	0.43 ± 0.30 μM
	MDA-MB-231	0.17 ± 0.02 μM
	MCF-7	0.24 ± 0.07 μM

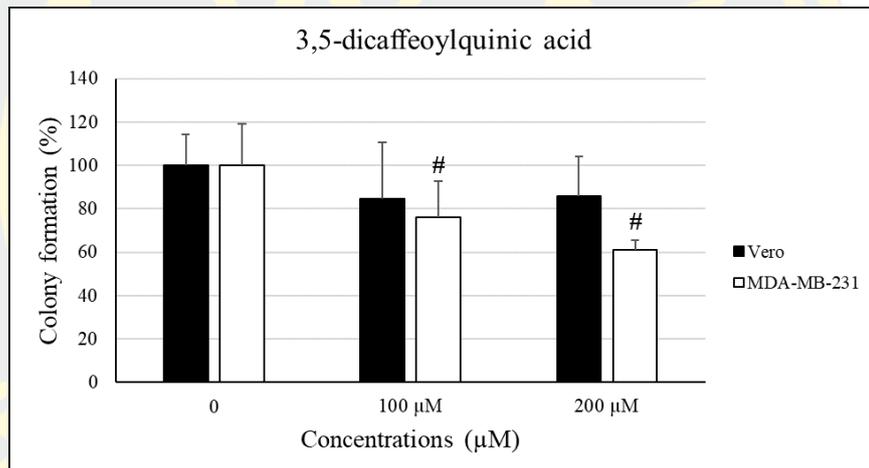
4.2 Effect of di-CQAs on cell proliferation of breast cancer cells

The colony formation assay was performed to determine the inhibitory effect of di-CQA on proliferation of breast cancer cells. Since the MTT results demonstrated the more sensitive response to CQAs of MDA-MB-231 cells than MCF-7 cells, and di-CQAs exhibited higher effect than mono-CQAs, therefore, MDA-MB-231 and Vero cells were treated with di-CQAs at the concentrations of 100 μ M and 200 μ M for 24 h. After 10 days of recovery from the treatment, number of colonies of MDA-MB-231 cells treated with 3,5-diCQA were reduced to 76.21% (at 100 μ M) and 61.21% (at 200 μ M) compared to the untreated control (Figure 4-3B). In contrast to cancer cells, Vero cells treated with 3,5-diCQA does not significantly change in colony formation capability when compared to the untreated control. Conversely to 3,5-diCQA, treatment of 3,4-diCQA and 4,5-diCQA at 100 μ M and 200 μ M could inhibit colony formation only 20% or less in both MDA-MB-231 and Vero cells. In conclusion, 3,5 diCQA showed the best suppression on MDA-MB-231 proliferation compared to other di-CQAs. Cancer has the ability to metastasize, so we investigated MDA-MB-231 cell migration to examine whether di-CQA has the effect of inhibiting cancer migration.

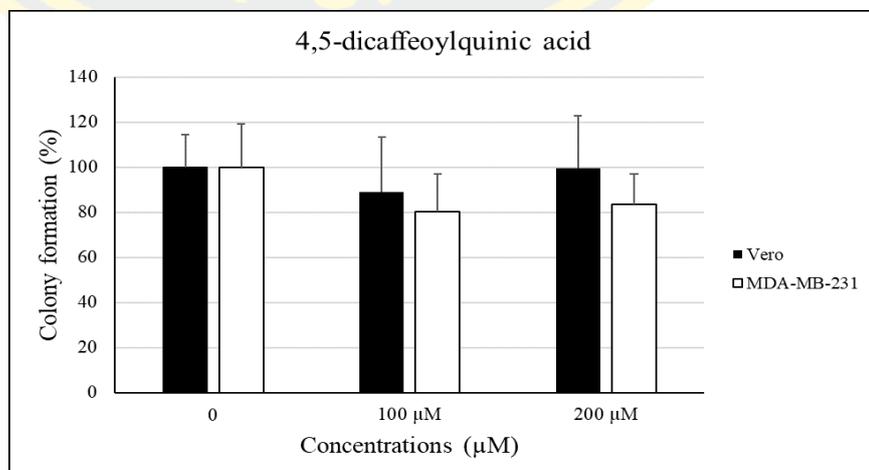
(A)



(B)



(C)



(D)

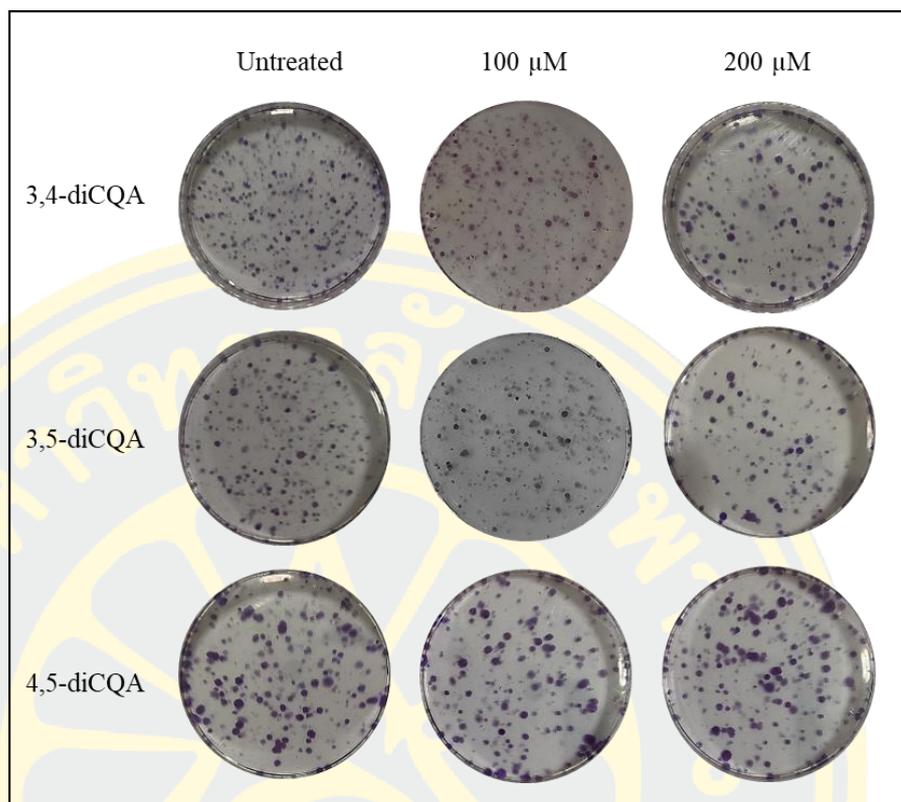
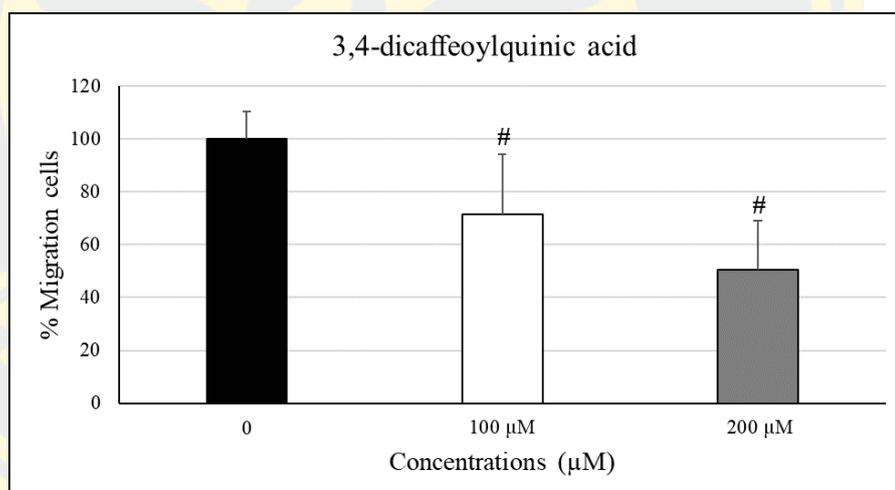


Figure 4-3 The effect of di-CQAs on colony formation of MDA-MB-231 and Vero cells. Cells were treated with (A) 3,4-diCQA, (B) 3,5-diCQA, and (C) 4,5-diCQA at the concentrations of 100 μ M and 200 μ M for 24 h and recovery in compound-free media for 10 days. After stained with crystal violet, the number of colonies was counted. The data are presented as mean \pm SD of at least three independent experiments with triplicate samples. # represents significant growth inhibition (>20%) of treated cells compared to untreated cells ($p < 0.05$). (D) The representative plates of MDA-MB-231 cells at individual condition were shown.

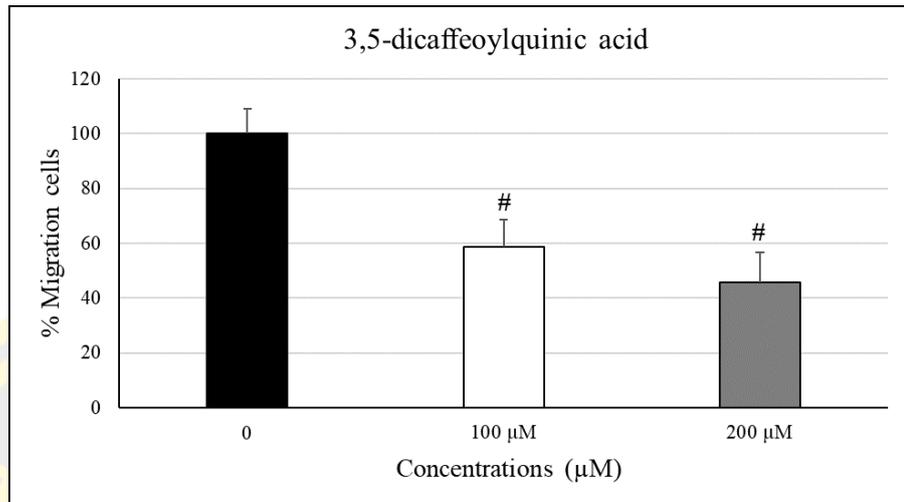
4.3 Effect of di-CQAs on cell migration of breast cancer cells

The ability to metastasis is one of characteristics of cancer cells. In this study, the transwell assay was used to determine the effect of di-CQAs on the migration of breast cancer cells. The results showed that di-CQAs could suppress the migration of breast cancer cells. The results showed that di-CQAs could suppress the migration of the breast cancer MDA-MB-231 cells in a dose-dependent manner. MDA-MB-231 cells treated with 200 μ M of 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA, were significantly decreased in cell migration up to 50%, 54%, and 60%, respectively, when compared to untreated cells (Figure 4-4). The results indicate that all di-CQAs tested had the anti-migration activity at the comparable level.

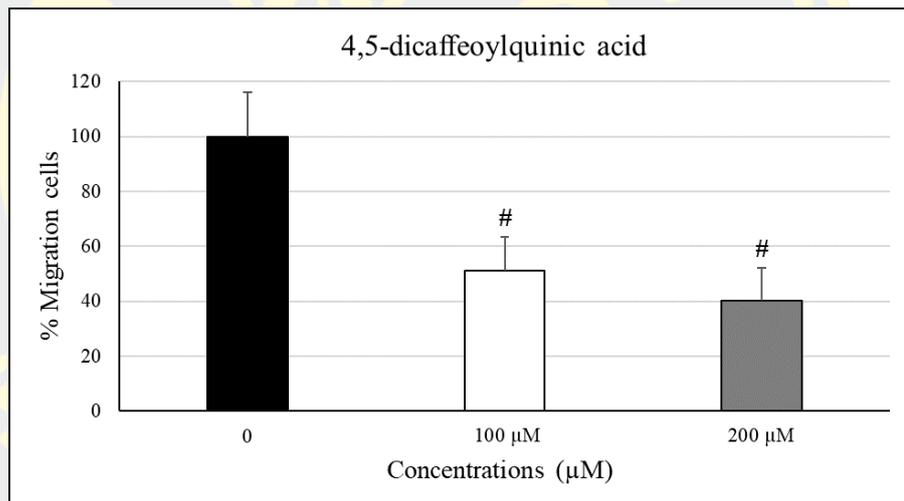
(A)



(B)



(C)



(D)

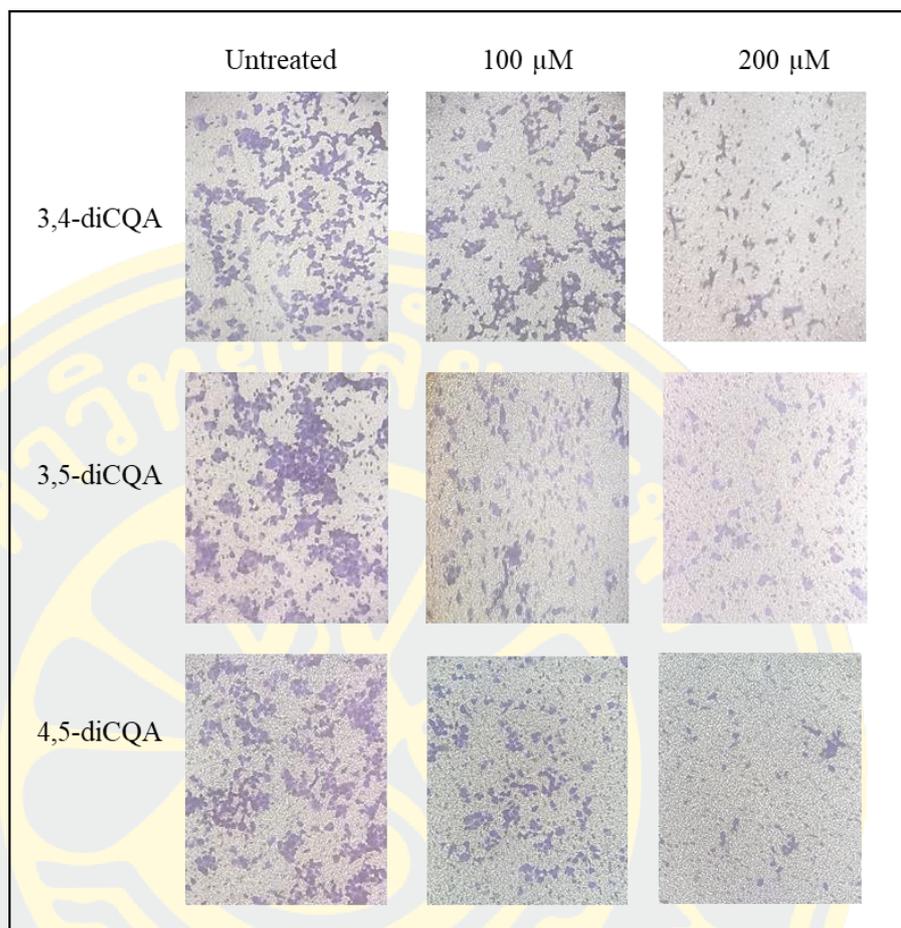
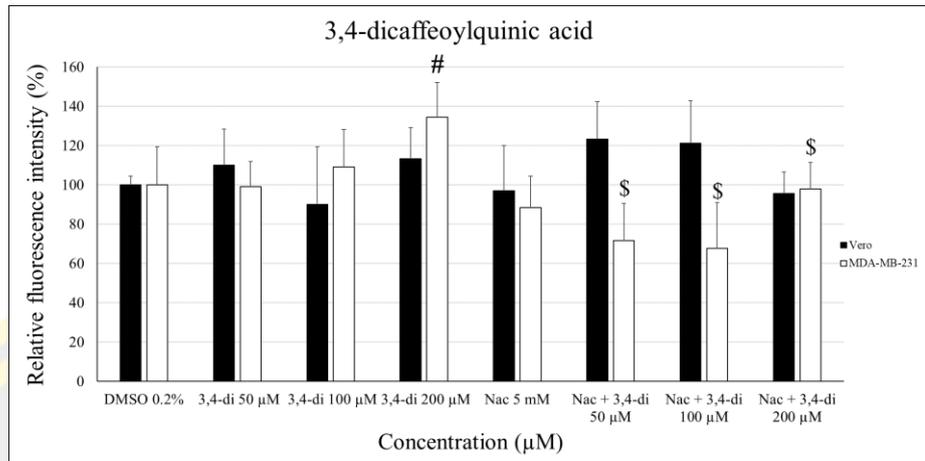


Figure 4-4 The effect of di-CQAs on cell migration of MDA-MB-231 determined by a transwell assay. Cells were treated with (A) 3,4-diCQA, (B) 3,5-diCQA, and (C) 4,5-diCQA at the concentrations of 100 μM and 200 μM for 24 h. Migrated cells were stained with crystal violet and counted under a stereomicroscope. The data are presented as mean \pm SD of three independent experiments with triplicate samples. # represents significant growth inhibition of treated cells compared to untreated cells ($p < 0.05$). (D) Representative images of the migrated cells from individual condition were taken under a stereomicroscope at 20x.

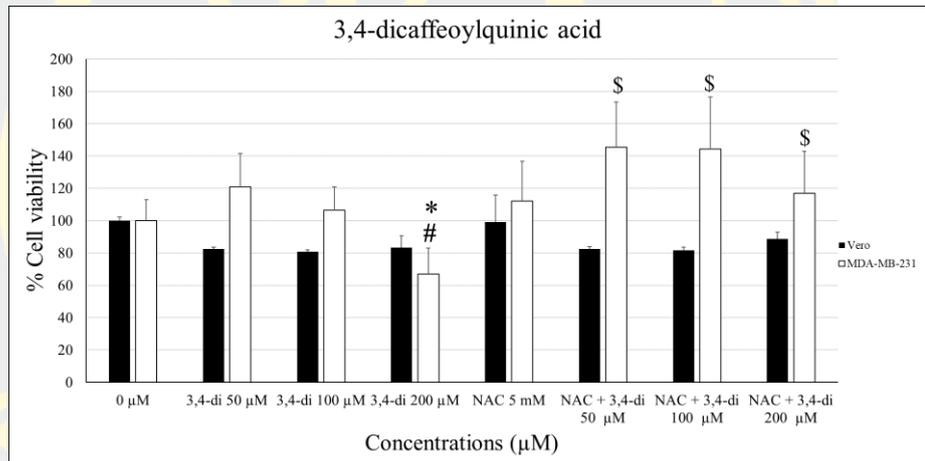
4.4 Investigation of intracellular ROS level in di-CQAs treated breast cancer cells

Since many chemotherapeutic drugs induce oxidative stress to kill cancer cells, therefore, the effect of di-CQAs on ROS generation in MDA-MB-231 and Vero cells was investigated by using DCFDA staining method. From Figure 4-5, the relative DCF fluorescent intensity of di-CQAs (A, C, E) were showed together with their effect on cell viability (B, D, F). For 3,4-diCQA treatment, the significant fluorescence induction was revealed when MDA-MB-231 cells were exposed to 200 μM of 3,4-diCQA where the cell viability was also significantly decreased (Figure 4-5 (A) and (B)). In cotreatment condition, NAC (a well-known antioxidant) could reduce the enhanced ROS production of 200 μM 3,4-diCQA and also rescued the viability of MDA-MB-231 cells. The stronger effect was detected when MDA-MB-231 cells were exposed to 3,5-diCQA (Figure 4-5 (C) and (D)). The augmentation of ROS production in concomitant with the increased cytotoxicity was observed at 50 μM up to 200 μM of 3,5-diCQA. In contrast to others, 4,5-diCQA treatment showed no significant change in neither ROS production nor cell viability in MDA-MB-231 and Vero cells (Figure 4-5 (E) and (F)). In conclusion, the increased in ROS production which negatively related to cell viability, and restored effect after NAC cotreatment indicate that the cytotoxicity of selected di-CQAs might be partly due to the induction of oxidative stress in breast cancer MDA-MB-231 cells.

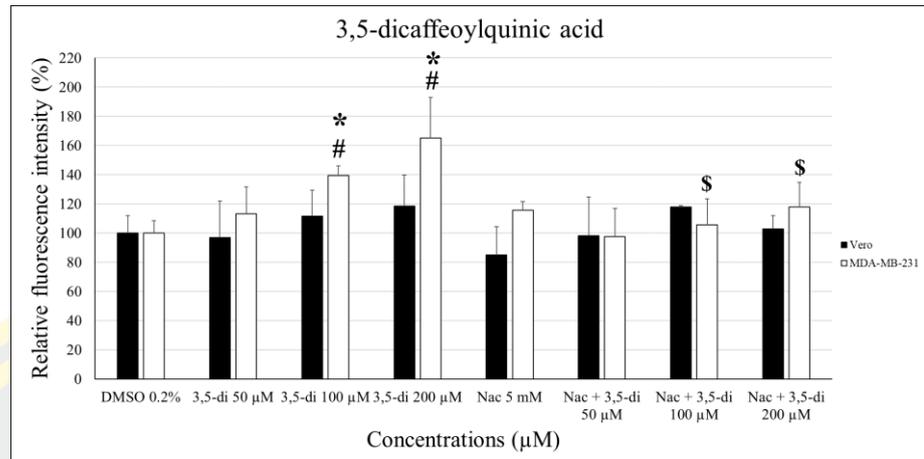
(A)



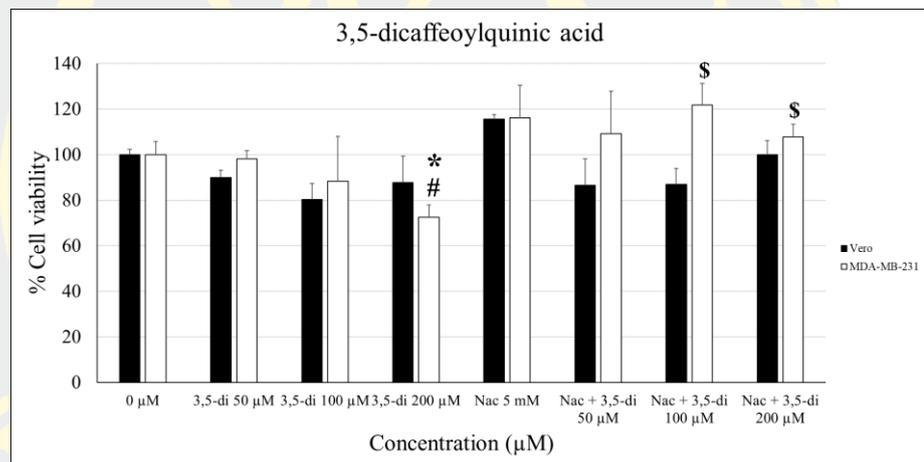
(B)



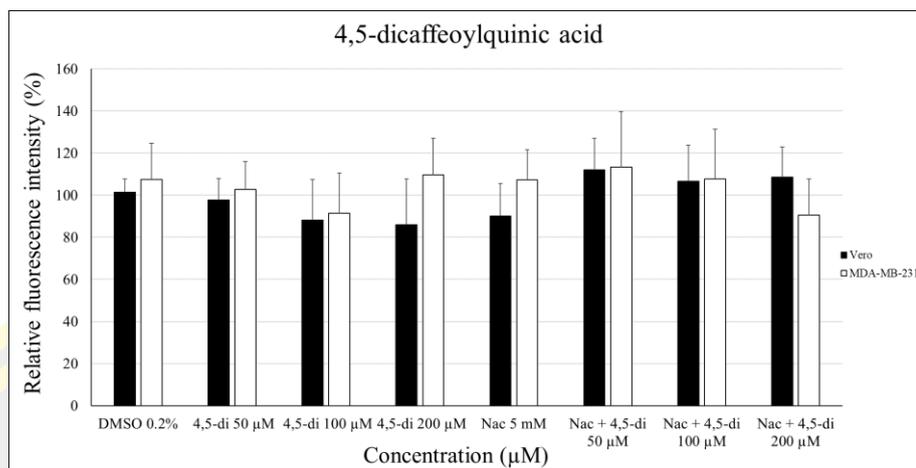
(C)



(D)



(E)



(F)

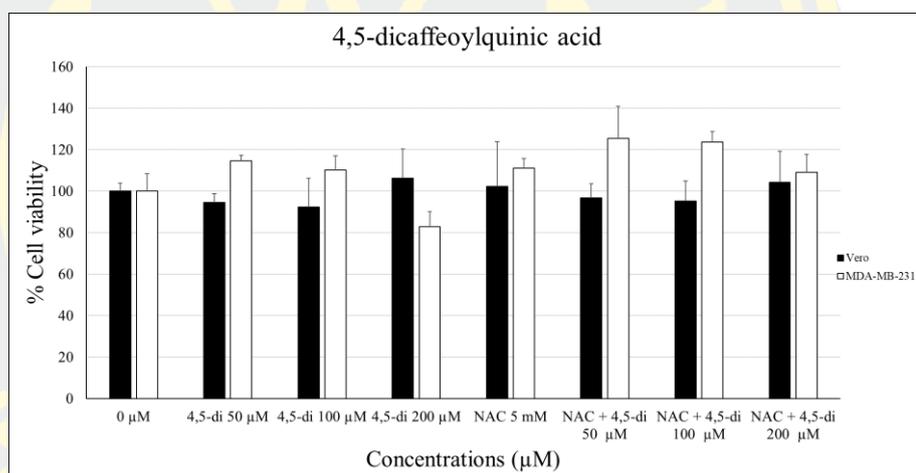


Figure 4-5 Relative Intracellular ROS level of MDA-MB-231 treated with di-CQAs.

The DCF fluorescent intensity from treatment of 3,4-diCQA (A), 3,5-diCQA (C), and 4,5-diCQA (E) were shown together with their effect on cell viability (B, D, F, respectively). The data are presented as the mean \pm SD of at least three independent experiments with triplicate samples. # represents significant growth inhibition of di-CQAs treated cells compared to untreated cells ($p < 0.05$). when * represents significant growth inhibition of cancer cells compared to non-cancerous cells at the same

condition ($p < 0.05$). The significant difference ($p < 0.05$) between di-CQAs treated with and without NAC was indicated as \$.



CHAPTER 5

DISCUSSION

In this study, the inhibitory effect of CQAs on two types of breast cancer cells, MCF-7 and MDA-MB-231 cells, were investigated. The cytotoxicity of mono-CQAs and di-CQAs was compared. The capability of di-CQAs on suppressing colony formation and migration of cancer cells was also demonstrated. Finally, the modulation of intracellular ROS level by di-CQAs was examined in relative to their cytotoxicity in order to hint the underlining mechanism.

CQAs is phenolic compound mostly available in *Erigeron breviscapus* (Zhang et al., 2007), *Ipomoea batatas* (Alcázar Magaña et al., 2021) and *Pluchea indica* (L.) Less. (*P. indica*) (Herman & Abdul., 2021). Previously, *P.indica* has been reported to contain a high amount of various kinds of CQAs derivatives, including 3-*O*-caffeoylquinic acid (17.34%), 4-*O*-caffeoylquinic acid (3.72%), 5-*O*-caffeoylquinic acid (2.52%), 3,4-*O*-dicaffeoylquinic acid (2.50%), 3,5-*O*-dicaffeoylquinic acid (10.50%), and 4,5-*O*-dicaffeoylquinic acid (24.24%) (Kongkiatpaiboon et al., 2018).

P.indica has been used as astringents, antipyretics, hepatoprotectives, diaphoretics in fevers, smooth muscle relaxants, nerve tonics, laxatives, and other traditional medicines since ancient times all over the world (Surendra and Naveen, 2011). Pharmacological studies have shown that *P. indica* exhibits many bioactives such as anti-inflammatory, anti-oxidant, anti-microbial, insecticidal, and anti-cancer activities (Ruan et al., 2018). Kao et al. (2015) reported that human nasopharyngeal cancer cells (NPC-TW 01 and NPC-TW 04) were suppressed by ethanolic extracts of

P. indica root in time- and dose-dependent manners, *P. indica* decreasing cellular survival to less than 50% of the control at 48 h. In addition, crude aqueous extracts of *P. indica* leaves and roots could inhibit human malignant glioma cancer cells (GBM8401) and human cervical cancer cells (HeLa) by 75% and 70%, respectively, at a concentration of 1 mg/ml for 48 h (Cho et al., 2012). Moreover, hexane fraction extract of *P. indica* root reduced cell viability in human glioblastoma cells (U87 cells and GBM cells) in a dose-dependent manner, with IC₅₀ values of 422.6 ± 38.3 µg/ml and 353.3 ± 18.7 µg/ml in U87 cells and 416.4 ± 35.5 µg/ml and 334.1 ± 29.6 µg/ml in GBM cells for 24 and 48 h, respectively (Cho et al., 2017). Previously, we demonstrated that crude ethanol extract of *P. indica* inhibited the growth of MDA-MB-231 and MCF-7 breast cancer cells with IC₅₀ values of 129 ± 8.19 and 282 ± 17.09 µg/mL, respectively (Iawsipo et al., 2022). However, the cytotoxic compounds in *P. indica* have not yet been identified.

Since CQAs are abundant in *P. indica* and they have been reported a wide range of biological activities, such as antioxidation, antibacterial, antiparasitic, neuroprotective, anti-inflammatory, antiviral, antidiabetic, and anticancer effects (Liu et al., 2020). Therefore, six CQA derivatives are subjected to investigate their anticancer activity in this study.

MTT assay was commonly used to evaluate cell viability after compound or drug treatment for assessing its cytotoxicity. From Figure 4-1, the MTT results revealed a dose-dependent growth inhibition by mono-CQAs against breast cancer cells of which MDA-MB-231 was prominent more than MCF-7 cells. Similar result of mono-CQAs against other cancer cell types were earlier demonstrated by other studies. The anticancer effect of 5-caffeoylquinic acids at concentrations of 2.5 mM -

80 mM at 96 h induced an average inhibition of 26% in human colon adenocarcinoma cells (HT-29). Additionally, the MTT assay showed that chlorogenic acid and caffeic acid could inhibit the promyelocytic leukemia cell line (HL-60) by 80% (at a concentration of 1,000 μ M) and 90% (at a concentration of 10 μ M), respectively (Murad et al., 2015).

When the cytotoxic effect of mono and di-CQA was compared, the results showed that di-CQAs showed the stronger effect than mono-CQA (Figure 4.1, 4.2 and Table 1). In our work, MTT results demonstrated 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA inhibited the growth of MDA-MB-231 cells by 31.01%, 46.96%, and 54.74%, respectively. Cytotoxic effect of di-CQAs was also reported by other researcher. 3,5-diCQA and 4,5-diCQA suppressed stomach cancer cells (Kato III) by 20 - 40% at concentrations of 100 - 500 μ M for 72 h by the WST-1 assay (Kurata et al., 2007). In addition, 1,5-diCQA and 4,5-diCQA inhibited melanogenesis in B16F1 murine melanoma cells by 61% and 84%, respectively (Ji Hoon & Soo Nam., 2018). The reason of this difference might be due to mono-CQA, which has one caffeoyl groups, having different chemical structures to di-CQAs, which have two caffeoyl groups. The caffeoyl group have been showed to be responsible for a number of biological activities. As the work of Taira et al., (2014) reported that, sweet potato leaf extracts such as mono (3, 4, and 5)-CQA and di-CQA (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) could inhibit human colorectal cancer HCT116 cells with IC_{50} values of mono (3, 4, and 5)-CQA $91.82 \pm 3.37 \mu$ M, $94.53 \pm 7.22 \mu$ M, and $97.17 \pm 2.51 \mu$ M, respectively and IC_{50} values of di-CQA (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) of $91.31 \pm 1.18 \mu$ M, $90.21 \pm 1.45 \mu$ M, and $93.34 \pm 3.20 \mu$ M, respectively. In addition, all CQAs could inhibit the β -catenin/Tcf-4 signaling in HCT116 cells. The inhibition of

the di-CQA was higher than that of the mono-CQA, suggesting that the caffeoyl structure in the presence of a catechol group plays a significant role in interfering with the -catenin/Tcf-4 signaling.

Regarding to the different response of breast cancer cells, MDA-MB-231 showed the more responsive to mono-CQAs and di-CQAs than MCF-7 cells. This might be because MDA-MB-231 cells are triple-negative breast cancer (TNBC), lacking of estrogen receptors negative (ER-) and progesterone receptors negative (PR-), as well as human epidermal growth factor receptor-2 (HER-2) (Lenci et al., 2016). In contrast, MCF-7 cells produce estrogen receptors (ER+) and progesterone receptors (PR+) but also are HER2-negative (Gaddy et al., 2004). For example, the differential response to having or not having ER and PR in both breast cancer cells is endocrine therapy, where a selective estrogen receptor modulator called tamoxifen is used against ER-positive breast cancer cells, resulting in the inhibition of their growth and apoptosis. On the other hand, the triple-negative breast cancer (TNBC) subtype has the highest likelihood of recurrence and the poorest survival prognosis (Li et al., 2017). Moreover, a difference in an intracellular ROS level between MDA-MB-231 and MCF-7 was reported (Sarmiento-Salinas et al., 2019). TNBC cells, like MDA-MB-231, produce higher ROS level than the luminal MCF-7 cells. Therefore, the exposure to prooxidant cytotoxic agents, which resulted in an increased ROS level, maybe lead MDA-MB-231 cells to reach a threshold level that induce cellular damage faster than MCF-7 cells. And this might be the reason why MDA-MB-231 cells appear to be more sensitive to prooxidant CQA derivatives than MCF-7. In addition, quercetin could inhibit the growth of MDA-MB-231 and MCF-7 cells, the IC₅₀ values of quercetin were 50 µm/mL for MDA-MB-231 cells and 80 µm/mL for MCF-7 cells,

respectively. This indicated that the quercetin could respond to the viability of MDA-MB-231 cells rather than MCF-7 cells (Tao et al., 2015).

Since the MTT assay only assesses enzyme metabolism in active cells and the reduced cell viability may be the result of proliferative inhibition or cell death induction by the cytotoxic compound. Therefore, the antiproliferative effect of di-CQAs was next examined by the colony formation assay.

The colony formation assay, or clonogenic assay, is a technique used to measure the ability of a single cell to grow into a large colony through clonal expansion to ensure its proliferation and differentiation patterns (Rajendran & Jain., 2018). The result of the proliferation shows that 3,5-diCQA could inhibit the cell viability to form colonies of MDA-MB-231 cells at concentrations of 100 μ M and 200 μ M by 76.21% and 61.21%, respectively. The results are consistent with reported of Zhou et al. (2020) that 1,3-diCQA and 1,5-diCQA inhibited cell proliferation to form colonies in MCF-7 cells at concentrations of 24 μ M to 96 μ M. Furthermore, 4,5-diCQA could also inhibit the proliferation of prostate cancer cells (DU-145) at 5 M for 72 h, resulting in an 88% decrease in colony formation (Lodise et al., 2019). The anticancer effect of di-CQAs may be mediated via a mechanism involving the inhibition of genes that control cell growth or cell division. Chiou et al. (2011) reported that 3,4-di-CQA was able to could act by down-regulate Akt, JNK, and part of the ERK1/2 pathways to inhibit angiotensin II-induced vascular smooth muscle cell (VSMC) proliferation and migration. In human colon cancer cells (HT-29 cells), 3,4-diCQA and 4,5-diCQA the increased the expression of genes controlling cell death through the Bax: Bcl-2 protein and activation of caspase-8 by cleavage of caspase-3, leading to the induction of apoptosis (Puangraphant et al., 2011). Additionally, 4,5-

diCQA could inhibit the proliferation of adenocarcinoma gastric cancer cells. 4,5-diCQA down-regulated cyclin D1, bcl2, VEGFA, c-myc, and survivin. Moreover, cell population increased at the G1 phase, and a number of cells at the G2/M phase of the cell cycle decreased after treatment (Jafari et al., 2018). Furthermore, polyphenolic extracts (AEs) from artichokes (*Cynara scolymus L.*) induce an increase in the upregulation of tumor suppressor genes p21^{Cip1/WaF1} and p16^{INK4a} in MDA-MB-231 cells. Both p21^{Cip1/WaF1} and p16^{INK4a} pathway inductions could lead to the inhibition of pRb phosphorylation by inhibiting CDK2/cyclinE and CDK4/cyclinD complexes, respectively. (Mileo et al. 2015).

Since cancer cells have the ability to metastasize which is the process by which cancer cells spread to another part of the body to form new tumors. Therefore, the anticancer effect of di-CQAs in inhibiting cancer migration was also investigated in this study. Cancer cells migrate in vivo by gradually degrading their surrounding extracellular matrix (ECM) through the secretion of matrix metalloproteinases (MMPs), to create their own migration tracks away from the primary tumor, intravasation into the bloodstream or lymphatic system, move along the circulation, extravasation to secondary tissues, and the formation of distant metastatic tumor colonies (Colin, Panagiotis and Konstantinos et al., 2017). From Figure 4-4, the result of transwell assay showed that of 200 μ M of 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA could suppress the migration of the breast cancer MDA-MB-231 cells by 50%, 54%, and 60%, respectively. Earlier, caffeoylquinic acid at a concentration of 1.8 mg/ml was reported to reduce the migration and invasion of pancreatic cancer cells (PANC-1) by 61.6% and 63.7%, respectively (Hagoel et al., 2019). Additionally, in the HCT116 cells, the expression of the gene caspase-3 could prevent the invasion and

metastasis of HCT116 cells by reducing the phenotypes associated with the epithelial-mesenchymal transition (EMT), increasing the expression of E-cadherin, and decreasing the expression of N-cadherin, Snail, Slug, and ZEB1 (Zhou et al., 2018).

The mostly cytotoxic agents inhibit cancer by inducing oxidative stress. (Tomris Ozben., 2007; Stepanic et al., 2015). Therefore, the intracellular ROS levels in MDA-MB-231 cells treated with the di-CQAs were measured and correlated with the cytotoxic activity. The results of intracellular ROS levels determined by the DCFDA staining method revealed that 3,4-diCQA and 3,5-diCQA could augment ROS production in MDA-MB-231 while also increasing cytotoxicity, which was observed at concentrations ranging from 50 to 200 μ M (Figure 4-5). Previous studies have demonstrated that *P. indica* ethanol crude extract exerts its anticancer effects through increased ROS levels in breast cancer cells (MDA-MB-231) and cervical cancer cells (C-33A), and cell viability is decreased at concentrations ranging from 100 to 200 μ M (Iawsipo et al., 2022).

In conclusion, our results in this study demonstrated that di-CQAs exert anti-cancer activity in MDA-MB-231 cells via inhibiting cell survival, cell proliferation, and cell migration. Cytotoxic effect of all CQAs tested was stronger in MDA-MB-231 than MCF-7 cells, contrasting to non-cancer Vero cells which were the least affected. The enhanced ROS production inside cancer cells was suggested to be one of action of di-CQAs in inducing cytotoxicity to MDA-MB-231 cells. Nevertheless, further study to identify the molecular mechanism underlining the anticancer activity of caffeoylquinic acid is needed. The obtained data suggest that caffeoylquinic acid might be useful in the development of functional foods or therapeutic agent for breast-cancer treatment.

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APPENDIX A

CULTURE MEDIA AND SOLUTIONS

1. Dulbecco's modified Eagle media (DMEM) with phenol red

DMEM powder (Gibco, USA; 13.5g), 3.7 g of NaHCO_3 , and 2.383 g of HEPES were dissolved in 900 mL of sterile water for injection. The pH of the solution was adjusted to 7.1-7.2 by adding concentrated of HCl. Then, 10 mL of the antibiotic Penicillin- Streptomycin was added to the solution. The solution was made to a volume of 1,000 mL with sterile water for injection. After that, the solution was sterilized by filter sterilization with membrane (pore size 0.22 μM) and stored in a refrigerator at 4 °C.

2. Phosphate-Buffer Saline (PBS, 10X)

80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4 and 2.4 g of KH_2PO_4 were dissolved in 800 mL of sterile water for injection. After that, the pH of the solution was adjusted to 7.4 and made up volumn to 1,000 mL with sterile water for injection. The solution was sterilized in an autoclave and stored in a refrigerator at 4 °C.

3. Thiazolyl Blue Tetrazolium Bromide (MTT) solution (5 mg/mL)

25 mg of MTT was dissolved in 5 mL of 1x PBS. Then, the MTT solution was aliquoted into a 1.5 mL tube and stored in a refrigerator at -20 °C.

4. Compound preparation of mono-CQAs

mono-CQAs: Preparation 100 mM (MW = 354.31 g/mol)

$$CV = g/MW$$

$$(100 \times 10^{-3} \text{ M}) \times (V)_L = (0.01 \text{ g}) / (354.31 \text{ g/mol})$$

$$V = 0.000282 \text{ L}$$

$$V = 282 \mu\text{l}$$

Therefore, weigh the compound: 0.01 g of mono-CQAs dissolved in DMSO 282 μl .

5. Compound preparation of di-CQAs

di-CQAs: Preparation 100 mM (MW = 516.4 g/mol)

$$CV = g/MW$$

$$(100 \times 10^{-3} \text{ M}) \times (V)_L = (0.01 \text{ g}) / (516.4 \text{ g/mol})$$

$$V = 0.000194 \text{ L}$$

$$V = 194 \mu\text{l}$$

Therefore, weigh the compound: 0.01 g of di-CQAs dissolved in DMSO 194 μl .

6. Preparation of doxorubicin

Preparation 0.5 mM (MW = 579.98 g/mol)

$$CV = g/MW$$

$$(0.5 \times 10^{-3} \text{ M}) \times (V)_L = (0.1 \text{ g}) / (579.98 \text{ g/mol})$$

$$V = 354 \mu\text{l}$$

Therefore, weigh the doxorubicin 0.1 g dissolved in 354 μl of DMSO.

7. Preparation of N-acetyl – L – cysteine (NAC)

Preparation 10 mM (MW = 163.19 g/mol)

$$CV = g/MW$$

$$(10 \times 10^{-3} \text{ M}) \times (V)_L = (2 \times 10^{-3} \text{ g}) / (163.19 \text{ g/mol})$$

$$V = 1.226 \times 10^{-3} \text{ L}$$

$$V = 1,226 \mu\text{l}$$

Therefore, weigh the NAC 2 mg dissolved in 1,226 μl of sterilized water.

8. 2',7' -dichlorofluorescein diacetate (DCFDA)

Preparation 12.5 mM (MW = 485.3 g/mol)

$$CV = g/MW$$

$$(12.5 \times 10^{-3} \text{ M}) \times (V)_L = (2 \times 10^{-3} \text{ g}) / (485.3 \text{ g/mol})$$

$$V = 0.000330 \text{ L}$$

$$V = 330 \mu\text{l}$$

Therefore, weigh the DCFDA 2 mg dissolved in 330 μl of DMSO.



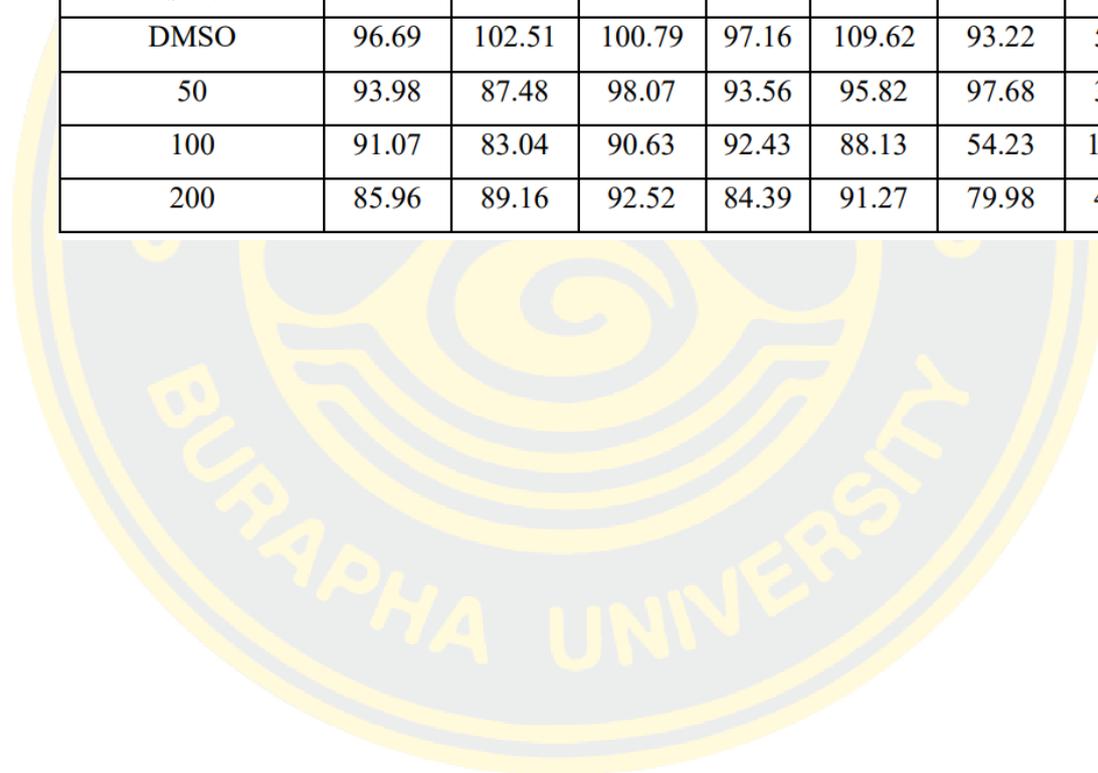
APPENDIX B

RAW DATA

The data obtained from the OD540 measurements were used to calculate the percentage growth of the cell tested with the 3-CQA at a concentration of 50–200 μM at 72 h.

Vero							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	101.88	99.10	99.02	104.21	97.76	98.02	2.527
50	95.87	92.70	96.55	97.67	93.87	97.02	1.931
100	88.18	89.31	88.95	87.21	92.56	89.02	1.810
200	83.64	89.90	89.60	84.27	91.70	96.20	4.713
Vero							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	101.75	104.12	94.13	100.72	103.61	95.67	4.164
50	82.45	84.80	87.97	85.28	88.45	83.07	2.465
100	84.97	83.60	79.75	86.31	85.59	94.47	4.849
200	84.43	90.21	93.66	85.08	85.99	84.29	3.817

MDA-MB-231							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	70.49	95.56	104.44	91.77	106.23	102.00	13.236
50	102.10	104.68	105.95	99.75	110.74	107.59	3.913
100	104.31	109.73	106.80	99.84	101.11	108.53	4.007
200	76.39	71.71	75.44	70.71	89.08	70.09	7.097
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	96.69	102.51	100.79	97.16	109.62	93.22	5.737
50	93.98	87.48	98.07	93.56	95.82	97.68	3.871
100	91.07	83.04	90.63	92.43	88.13	54.23	14.601
200	85.96	89.16	92.52	84.39	91.27	79.98	4.694



The data obtained from the OD540 measurements were used to calculate the percentage growth of the cell tested with the 4-CQA at a concentration of 50–200 μM at 72 h.

Vero							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	103.60	101.70	94.71	104.54	96.18	99.27	3.989
50	88.23	86.92	85.49	81.78	81.85	84.10	2.646
100	95.16	90.68	89.32	89.79	87.18	91.80	2.691
200	92.25	97.44	104.34	99.37	98.93	95.24	4.098
Vero							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	104.96	99.42	95.62	99.79	103.35	96.87	3.609
50	85.93	84.98	87.46	84.47	87.46	84.51	1.387
100	92.40	90.32	96.59	89.08	90.98	94.25	2.768
200	86.01	86.92	89.82	83.77	87.59	88.03	2.038

MDA-MB-231							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	71.82	91.99	108.01	90.33	104.15	105.52	13.649
50	118.80	127.95	126.74	115.09	102.89	106.53	10.274
100	115.94	99.60	115.66	89.04	87.59	84.34	14.200
200	58.12	57.03	39.66	55.69	56.18	52.50	6.893
MDA-MB-231							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	96.52	96.25	107.23	106.12	97.07	96.82	5.190
50	120.14	107.14	102.38	110.47	115.16	107.18	6.368
100	97.61	92.93	108.03	94.80	95.34	99.74	5.420
200	39.84	36.52	41.81	36.24	34.47	37.78	2.660

The data obtained from the OD540 measurements were used to calculate the percentage growth of the cell tested with the 5-CQA at a concentration of 50–200 μM at 72 h.

Vero							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	103.92	98.47	97.61	99.80	101.14	99.06	2.266
50	85.63	80.01	85.55	82.98	78.52	80.41	3.003
100	99.32	97.43	102.80	101.17	94.15	103.93	3.625
200	87.30	88.70	100.65	89.71	87.60	95.35	5.337
Vero							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	105.25	95.74	99.00	99.46	101.60	98.95	3.182
50	94.00	98.62	94.22	87.73	92.13	89.52	3.852
100	92.21	87.10	99.26	102.31	92.82	94.72	5.406
200	95.69	94.28	107.24	95.96	76.21	99.81	10.272

MDA-MB-231							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	100.07	98.06	101.87	101.79	94.54	103.68	3.279
50	96.75	100.69	96.34	85.80	90.77	85.83	6.197
100	89.63	92.80	91.74	91.69	80.82	88.77	4.385
200	58.08	60.73	59.83	53.68	59.16	57.28	2.498
MDA-MB-231							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	72.50	97.98	102.02	87.50	112.50	144.33	24.471
50	103.59	101.02	75.14	120.63	132.14	128.97	21.448
100	82.92	89.58	82.64	117.65	108.21	120.21	17.256
200	41.33	33.43	28.19	38.65	37.58	42.91	5.423

The data obtained from the OD540 measurements were used to calculate the percentage growth of the cell tested with the 3,4-diCQA at a concentration of 50–200 μM at 72 h.

Vero							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	102.07	101.60	96.33	104.20	99.51	96.29	3.226
50	93.58	93.84	90.01	91.81	94.52	89.06	2.225
100	92.33	92.52	85.43	78.56	84.25	86.68	5.280
200	73.55	77.95	66.53	70.05	67.39	65.62	4.770
Vero							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	101.40	101.09	97.51	99.20	103.16	97.63	2.262
50	79.37	77.95	79.15	79.24	79.51	79.43	0.584
100	75.72	76.52	80.20	77.61	75.72	79.66	1.960
200	87.64	96.31	87.63	91.66	96.18	90.55	3.893

MDA-MB-231							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	96.55	97.54	105.91	87.08	110.46	102.46	8.188
50	60.54	51.98	61.49	53.62	60.66	55.49	4.109
100	59.17	63.98	52.78	56.35	60.19	56.39	3.856
200	34.98	32.04	30.76	34.54	32.54	30.19	1.944
MDA-MB-231							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	78.52	111.79	109.69	92.92	98.36	108.72	12.831
50	72.32	71.84	76.63	73.78	73.19	81.25	3.564
100	45.85	47.50	40.32	35.95	38.82	39.68	4.415
200	27.23	33.71	23.70	34.15	30.63	28.35	4.018

The data obtained from the OD540 measurements were used to calculate the percentage growth of the cell tested with the 3,5-diCQA at a concentration of 50–200 μM at 72 h.

Vero							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	102.26	96.82	100.92	100.67	100.47	98.86	1.900
50	96.13	97.92	95.65	94.45	91.54	99.45	2.754
100	65.56	78.46	71.80	75.87	75.66	70.78	4.621
200	68.22	71.46	70.34	65.95	69.82	67.98	1.974
Vero							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	101.06	98.89	100.06	93.58	104.60	101.81	3.688
50	85.00	88.21	85.57	92.89	92.47	90.50	3.398
100	83.29	81.33	84.46	87.08	94.98	88.87	4.875
200	73.93	80.93	83.08	83.29	82.29	78.93	3.562

MDA-MB-231							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	74.56	94.73	105.27	93.04	98.32	108.64	12.005
50	85.14	89.03	84.18	80.21	86.90	87.32	3.084
100	83.63	79.82	80.74	66.85	66.39	67.95	7.964
200	46.24	43.28	45.09	48.20	46.82	47.07	1.724
MDA-MB-231							
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	86.64	106.20	107.16	103.36	96.89	99.74	7.604
50	81.45	86.99	89.80	87.85	86.23	87.89	2.836
100	64.09	61.37	68.77	73.41	75.24	66.08	5.383
200	52.87	46.59	42.24	49.97	47.25	47.87	3.553

The data obtained from the OD540 measurements were used to calculate the percentage growth of the cell tested with the 4,5-diCQA at a concentration of 50–200 μM at 72 h.

Vero							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	102.23	97.21	100.56	106.22	97.32	96.46	3.780
50	98.86	100.85	92.92	92.60	97.62	91.07	3.975
100	93.47	90.19	94.49	89.08	90.60	92.35	2.085
200	89.85	91.77	101.15	92.10	100.27	96.27	4.738
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	100.89	101.43	97.68	99.99	99.81	100.20	1.287
50	94.15	94.23	95.37	96.26	94.24	96.78	1.149
100	86.74	84.49	85.54	88.96	86.08	85.87	1.508
200	82.82	82.77	78.84	86.75	84.62	83.07	2.608

MDA-MB-231							
Concentrations (μM)	No 1			No 2			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	99.53	99.53	100.95	104.55	92.66	102.78	4.091
50	89.61	93.84	96.85	90.25	92.19	89.35	2.923
100	83.15	84.38	75.77	74.78	80.28	76.35	4.074
200	55.14	59.26	56.54	47.61	51.00	49.15	4.561
Concentrations (μM)	No 3			No 4			SD
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	92.79	96.15	111.06	100.21	101.44	98.34	6.228
50	92.67	88.88	89.13	108.71	93.32	105.73	8.618
100	77.41	69.39	75.46	70.97	70.23	73.76	3.186
200	54.79	51.79	60.32	58.36	53.73	59.19	3.404

The data obtained from the visible colony was counted under a stereomicroscope. measurements were used to calculate the percentage cell proliferation of the cell tested with the di-CQAs at a concentration of 100 and 200 μM at 8–10 days.

3,4-diCQA								
Concentrations (μM)	Vero			SD	MDA-MB-231			SD
	1 st	2 nd	3 rd		1 st	2 nd	3 rd	
DMSO	90.32	116.46	93.22	14.33	110.29	111.63	78.09	18.990
100	92.64	122.27	67.67	27.33	100.09	89.62	100.36	6.121
200	91.77	115.59	96.42	12.62	105.72	83.45	81.57	13.434

3,5-diCQA								
Concentrations (μM)	Vero			SD	MDA-MB-231			SD
	1 st	2 nd	3 rd		1 st	2 nd	3 rd	
DMSO	90.32	116.46	93.22	14.33	110.29	111.63	78.09	18.990
100	64.47	113.84	75.22	25.96	87.75	83.72	57.16	16.622
200	85.67	104.26	67.09	18.59	64.67	56.62	62.52	4.169

4,5-diCQA								
Concentrations (μM)	Vero			SD	MDA-MB-231			SD
	1 st	2 nd	3 rd		1 st	2 nd	3 rd	
DMSO	90.32	116.46	93.22	14.33	110.29	111.63	78.09	18.990
100	81.32	116.17	68.83	24.53	98.48	76.74	65.47	16.777
200	85.09	126.33	87.42	23.17	93.38	89.09	68.16	13.495

The data obtained from the transwell assay were counted under a stereomicroscope. measurements were used to calculate the percentage cell migration of the cell tested with the di-CQAs at a concentration of 100 and 200 μM at 72 h.

3,4-diCQA					
Concentrations (μM)	1 st		2 nd		SD
	Insert 1	Insert 2	Insert 1	Insert 2	
DMSO	112.31	87.69	102.75	97.25	10
100 μM	80.40	99.15	55.90	50.59	23
200 μM	51.83	75.74	32.74	41.18	19

3,5-diCQA					
Concentrations (μM)	1 st		2 nd		SD
	Insert 1	Insert 2	Insert 1	Insert 2	
DMSO	110.18	89.82	95.56	104.44	9
100 μM	60.59	69.18	60.06	45.27	10
200 μM	44.58	57.10	49.72	31.08	11

4,5-diCQA					
Concentrations (μM)	1 st		2 nd		SD
	Insert 1	Insert 2	Insert 1	Insert 2	
DMSO	118.81	81.19	93.49	106.51	16
100 μM	35.14	48.25	63.92	56.94	12
200 μM	40.37	23.81	46.20	51.11	12

The data obtained from the relative fluorescence intensity measurements were used to calculate the percentage ROS level and cell viability of the cell tested with the 3,4-diCQA

ROS	3,4-diCQA					
	Vero					
Concentrations (μM)	No 1			No 2		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd
DMSO	98.97	97.30	103.72	107.94	95.34	96.72
50 μM	99.55	85.84	100.15	136.16	119.67	86.12
100 μM	95.76	86.95	95.79	128.82	45.27	72.14
200 μM	128.16	101.99	130.30	96.83	102.93	119.35
Nac 5 mM	91.37	80.60	68.96	131.54	117.26	128.91
Nac + 50 μM	105.91	103.33	91.80	140.24	146.13	132.62
Nac + 100 μM	102.21	90.91	96.45	149.09	132.27	131.93
Nac + 200 μM	89.45	91.52	91.48	115.26	108.22	94.79

Concentrations (μM)	No 3			SD
	1 st	2 nd	3 rd	
DMSO	105.52	125.20	69.28	14.693
50 μM	113.96	99.58	103.88	16.102
100 μM	138.97	128.58	116.91	30.456
200 μM	76.66	159.08	85.56	25.547
Nac 5 mM	103.65	85.82	90.40	21.937
Nac + 50 μM	63.07	51.94	61.66	35.473
Nac + 100 μM	64.58	58.50	69.76	32.520
Nac + 200 μM	77.01	117.39	90.21	13.427

ROS	3,4-diCQA					
	MDA--MB-231					
Concentrations (μM)	No 1			No 2		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd
DMSO	104.99	84.48	110.53	122.66	99.07	78.28
50 μM	103.96	107.71	110.99	76.83	83.42	91.06
100 μM	102.47	110.02	109.00	111.71	85.53	77.59
200 μM	136.42	125.33	148.19	109.07	90.43	127.64
Nac 5 mM	60.66	80.60	79.86	89.04	88.83	120.63
Nac + 50 μM	97.58	91.92	96.28	60.49	71.51	49.14
Nac + 100 μM	112.06	102.29	70.41	40.60	45.10	64.06
Nac + 200 μM	97.66	89.59	93.02	85.60	119.80	108.68

Concentrations (μM)	No 3			SD
	1 st	2 nd	3 rd	
DMSO	105.52	125.20	69.28	19.275
50 μM	113.96	99.58	103.88	12.695
100 μM	138.97	128.58	116.91	19.145
200 μM	76.66	159.08	85.56	28.906
Nac 5 mM	103.65	85.82	90.40	16.522
Nac + 50 μM	63.07	51.94	61.66	18.980
Nac + 100 μM	64.58	58.50	69.76	23.697
Nac + 200 μM	77.01	117.39	90.21	14.641

MTT	3,4-diCQA						SD
	Vero						
Concentrations (μ M)	No 1			No 2			
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	98.10	101.23	100.67	103.26	100.01	96.73	2.32
50 μ M	81.00	82.41	82.04	82.80	82.97	84.00	1.00
100 μ M	79.68	79.28	81.95	80.37	82.12	81.44	1.20
200 μ M	89.28	91.37	87.45	72.42	79.75	80.19	7.19
Nac 5 mM	83.42	83.78	85.23	113.98	114.23	114.95	16.58
Nac + 50 μ M	80.93	81.81	84.00	81.43	84.15	82.16	1.35
Nac + 100 μ M	80.70	82.68	84.78	78.99	81.12	80.63	2.00
Nac + 200 μ M	83.14	89.38	87.65	87.37	89.35	95.57	4.05

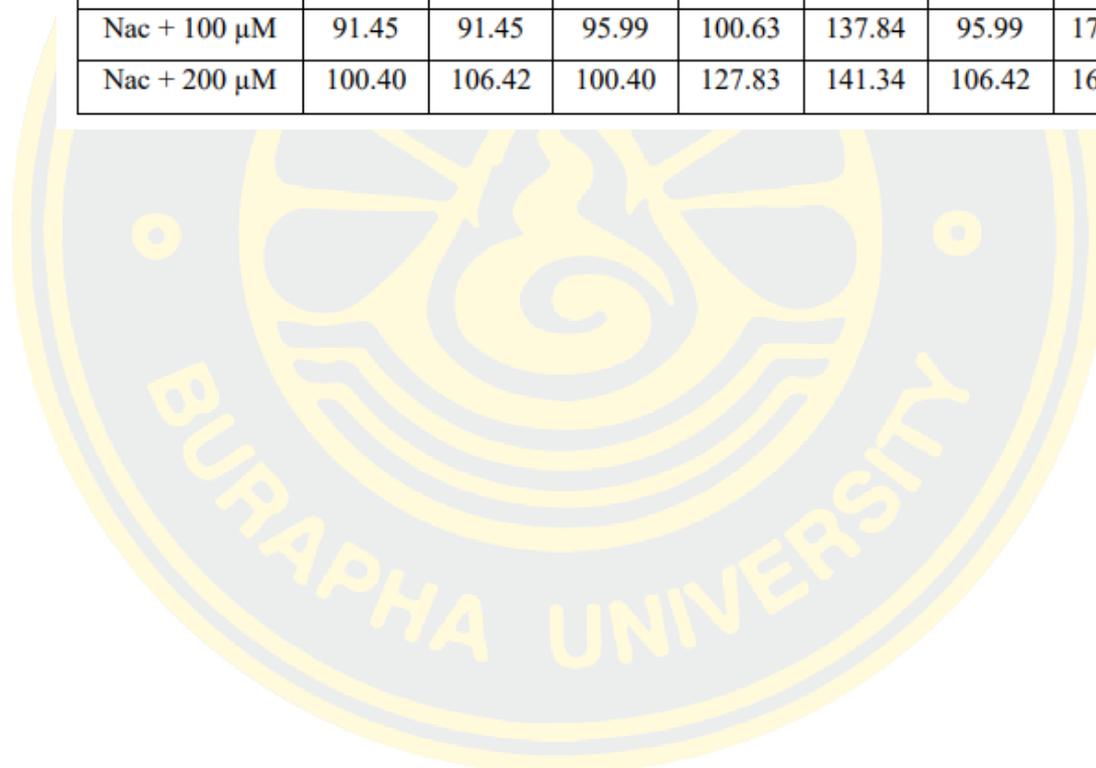
MTT	3,4-diCQA						SD
	MDA-MB-231						
Concentrations (μ M)	No 1			No 2			
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	78.47	106.17	115.35	91.48	103.47	105.06	13.02
50 μ M	103.21	99.49	104.55	131.86	143.80	141.91	20.61
100 μ M	92.18	94.58	94.27	114.85	124.08	119.18	14.40
200 μ M	43.84	52.76	62.36	81.59	78.68	81.51	16.25
Nac 5 mM	98.93	100.04	74.87	132.47	131.97	134.66	24.59
Nac + 50 μ M	112.59	129.48	122.40	154.92	180.68	172.00	27.93
Nac + 100 μ M	109.51	116.20	121.92	158.54	175.35	183.68	32.30
Nac + 200 μ M	99.38	94.88	86.39	145.60	136.00	138.48	25.99

The data obtained from the relative fluorescence intensity measurements were used to calculate the percentage ROS level and cell viability of the cell tested with the 3,5-diCQA

ROS	3,5-diCQA					
	Vero					
Concentrations (μM)	No 1			No 2		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd
DMSO	82.12	106.00	111.88	96.11	104.15	99.74
50 μM	127.58	119.15	112.32	73.47	67.67	63.03
100 μM	98.02	121.50	148.92	114.87	98.71	98.71
200 μM	124.22	135.11	118.41	133.64	144.32	87.27
Nac 5 mM	87.84	107.16	117.13	76.96	63.55	63.55
Nac + 50 μM	73.36	81.04	136.70	138.41	101.02	73.36
Nac + 100 μM	117.08	117.08	117.51	119.26	117.08	117.51
Nac + 200 μM	110.97	110.97	98.29	91.37	110.70	98.29

Concentrations (μM)	No 3		SD
	1 st	2 nd	
DMSO	115.18	84.82	11.89
50 μM	107.58	103.40	25.02
100 μM	98.02	114.87	17.78
200 μM	87.27	118.41	21.23
Nac 5 mM	76.96	87.84	19.23
Nac + 50 μM	81.04	101.02	26.56
Nac + 100 μM	119.26	117.51	0.93
Nac + 200 μM	91.37	110.70	8.95

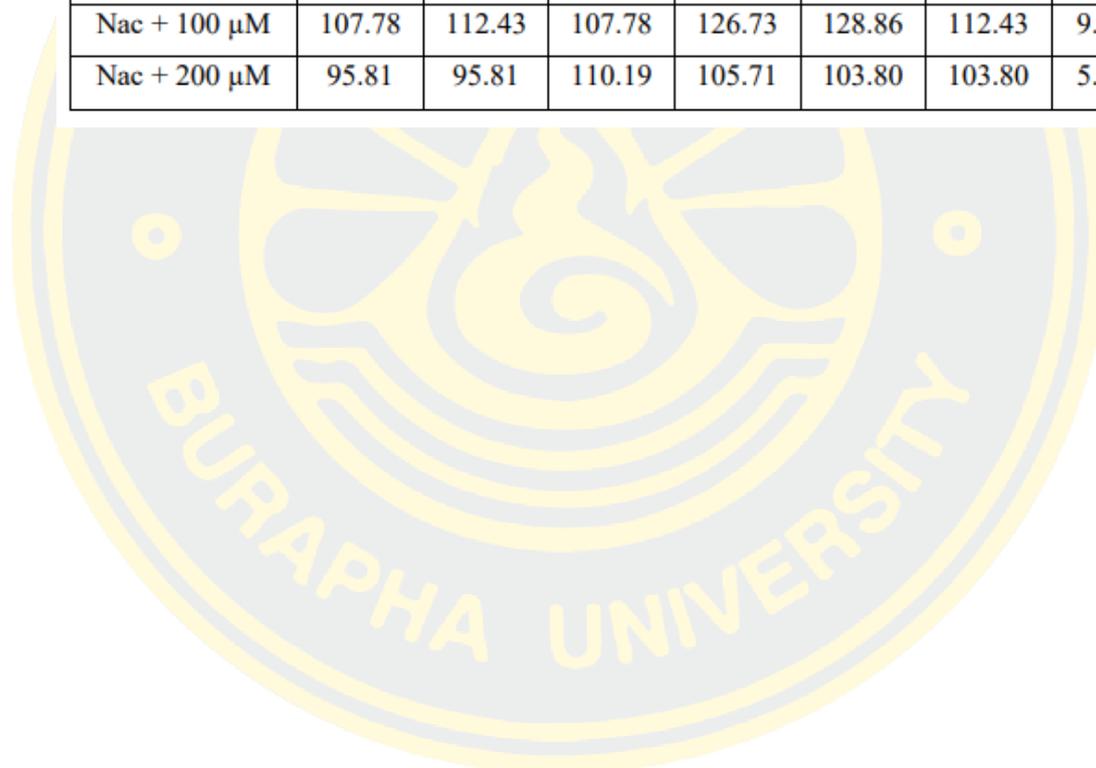
ROS	3,5-diCQA						
	MDA-MB-231						SD
	No 1			No 2			
Concentrations (μM)	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	93.20	99.65	107.15	104.65	87.61	87.61	8.47
50 μM	86.28	112.41	119.75	121.12	129.46	86.28	18.57
100 μM	146.56	137.08	133.99	132.60	129.46	129.46	6.42
200 μM	179.28	180.34	190.98	127.99	151.16	127.99	27.83
Nac 5 mM	114.85	117.67	119.00	106.94	107.22	105.52	5.99
Nac + 50 μM	73.28	111.11	116.88	85.85	105.90	73.28	19.40
Nac + 100 μM	91.45	91.45	95.99	100.63	137.84	95.99	17.78
Nac + 200 μM	100.40	106.42	100.40	127.83	141.34	106.42	16.88



MTT	3,5-diCQA					
	Vero					
Concentrations (μM)	No 1			No 2		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd
DMSO	104.29	97.95	97.76	102.67	99.59	97.74
50 μM	91.00	92.11	91.03	84.79	85.40	89.23
100 μM	86.86	83.76	83.84	83.20	69.15	69.15
200 μM	82.63	80.92	83.88	92.92	83.20	115.04
Nac 5 mM	119.35	115.22	114.14	114.54	117.97	114.14
Nac + 50 μM	79.09	105.10	78.97	82.87	79.09	105.44
Nac + 100 μM	80.16	80.16	84.83	85.16	97.89	84.83
Nac + 200 μM	90.35	90.35	105.23	104.14	99.93	105.23

Concentrations (μM)	No 3		SD
	1 st	2 nd	
DMSO	99.49	100.51	2.41
50 μM	92.87	93.32	3.26
100 μM	83.20	83.84	7.02
200 μM	80.92	82.63	11.67
Nac 5 mM	114.54	115.22	1.95
Nac + 50 μM	78.97	82.87	11.68
Nac + 100 μM	85.16	97.89	7.04
Nac + 200 μM	104.14	99.93	6.27

MTT	3,5-diCQA						
	MDA-MB-231						SD
	No 1			No 2			
Concentrations (μ M)	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	99.32	100.65	100.03	94.16	88.44	88.44	5.70
50 μ M	90.61	93.43	90.36	96.67	99.01	90.36	3.70
100 μ M	108.30	109.42	69.00	70.06	78.88	69.00	19.53
200 μ M	70.78	71.06	76.68	62.45	70.30	62.45	5.55
Nac 5 mM	125.30	126.16	118.61	98.10	98.81	96.87	14.18
Nac + 50 μ M	82.27	107.97	111.31	110.47	129.78	82.27	18.55
Nac + 100 μ M	107.78	112.43	107.78	126.73	128.86	112.43	9.39
Nac + 200 μ M	95.81	95.81	110.19	105.71	103.80	103.80	5.70



The data obtained from the relative fluorescence intensity measurements were used to calculate the percentage ROS level and cell viability of the cell tested with the 4,5-diCQA

ROS	4,5-diCQA					
	Vero					
Concentrations (μM)	No 1			No 2		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd
DMSO	91.46	110.63	97.91	97.60	104.86	97.55
50 μM	91.73	94.37	77.77	108.94	100.41	104.58
100 μM	78.38	69.68	90.02	112.86	56.84	119.38
200 μM	76.07	61.59	124.13	94.37	116.34	72.41
Nac 5 mM	92.81	86.25	79.47	119.89	102.06	97.88
Nac + 50 μM	113.09	120.59	108.32	103.14	122.04	77.37
Nac + 100 μM	101.84	112.99	82.56	128.27	109.48	120.31
Nac + 200 μM	80.79	94.37	115.69	118.63	113.68	110.16

Concentrations (μM)	No 3			SD
	1 st	2 nd	3 rd	
DMSO	110.42	100.40	100.79	6.326
50 μM	103.14	108.32	89.90	10.174
100 μM	90.15	88.56	87.06	19.400
200 μM	80.79	82.38	65.09	21.796
Nac 5 mM	73.74	69.56	88.81	15.482
Nac + 50 μM	125.74	123.52	115.01	14.989
Nac + 100 μM	91.20	86.07	126.13	17.128
Nac + 200 μM	98.94	116.67	127.29	14.415

ROS	4,5-diCQA					
	MDA-MB-231					
Concentrations (μM)	No 1			No 2		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd
DMSO	81.53	117.02	101.44	108.70	94.46	96.84
50 μM	110.20	112.58	118.20	88.30	79.14	107.10
100 μM	113.92	78.06	116.35	75.82	95.47	99.19
200 μM	123.38	103.29	134.21	119.47	111.48	91.78
Nac 5 mM	106.91	101.17	105.47	84.37	112.40	97.23
Nac + 50 μM	150.07	147.97	141.14	97.12	89.86	109.32
Nac + 100 μM	127.17	127.84	137.59	90.72	93.65	95.24
Nac + 200 μM	95.89	77.01	123.56	74.58	77.60	72.71

Concentrations (μM)	No 3			SD
	1 st	2 nd	3 rd	
DMSO	134.00	130.44	103.65	17.065
50 μM	97.80	95.66	115.61	13.302
100 μM	95.61	93.01	55.91	19.065
200 μM	126.28	88.72	88.78	17.324
Nac 5 mM	111.96	107.21	138.00	14.399
Nac + 50 μM	84.97	109.29	90.18	26.244
Nac + 100 μM	99.68	130.11	67.29	23.766
Nac + 200 μM	108.73	95.77	87.93	17.345

MTT	4,5-diCQA						SD
	Vero						
Concentrations (μ M)	No 1			No 2			
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	101.83	99.82	98.35	105.81	99.93	94.26	3.82
50 μ M	97.13	95.68	97.13	87.41	97.98	91.64	4.14
100 μ M	104.41	103.88	106.39	77.21	78.75	83.05	13.97
200 μ M	124.44	114.40	117.14	96.05	92.23	93.11	14.06
Nac 5 mM	121.38	121.92	122.24	81.36	83.15	83.57	21.46
Nac + 50 μ M	102.64	103.00	103.14	89.26	91.62	90.31	6.91
Nac + 100 μ M	103.13	104.92	104.04	88.06	85.04	85.22	9.89
Nac + 200 μ M	116.82	117.24	119.17	88.61	92.40	91.47	14.82

MTT	4,5-diCQA						SD
	MDA-MB-231						
Concentrations (μ M)	No 1			No 2			
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	
DMSO	92.14	101.54	106.32	87.26	107.21	105.53	8.36
50 μ M	106.86	106.84	106.33	104.92	108.36	112.29	2.55
100 μ M	102.95	104.62	98.55	114.36	102.40	115.03	6.79
200 μ M	73.78	86.94	93.74	76.53	84.22	87.39	7.41
Nac 5 mM	98.10	98.81	96.87	104.60	104.90	108.34	4.62
Nac + 50 μ M	117.35	115.95	117.32	145.99	148.52	139.67	15.53
Nac + 100 μ M	113.92	114.99	113.96	105.94	102.60	110.52	5.04
Nac + 200 μ M	100.45	100.66	96.85	93.53	91.53	77.50	8.61

BIOGRAPHY

NAME Miss Rotsukon Poonbud

DATE OF BIRTH 01 November 1995

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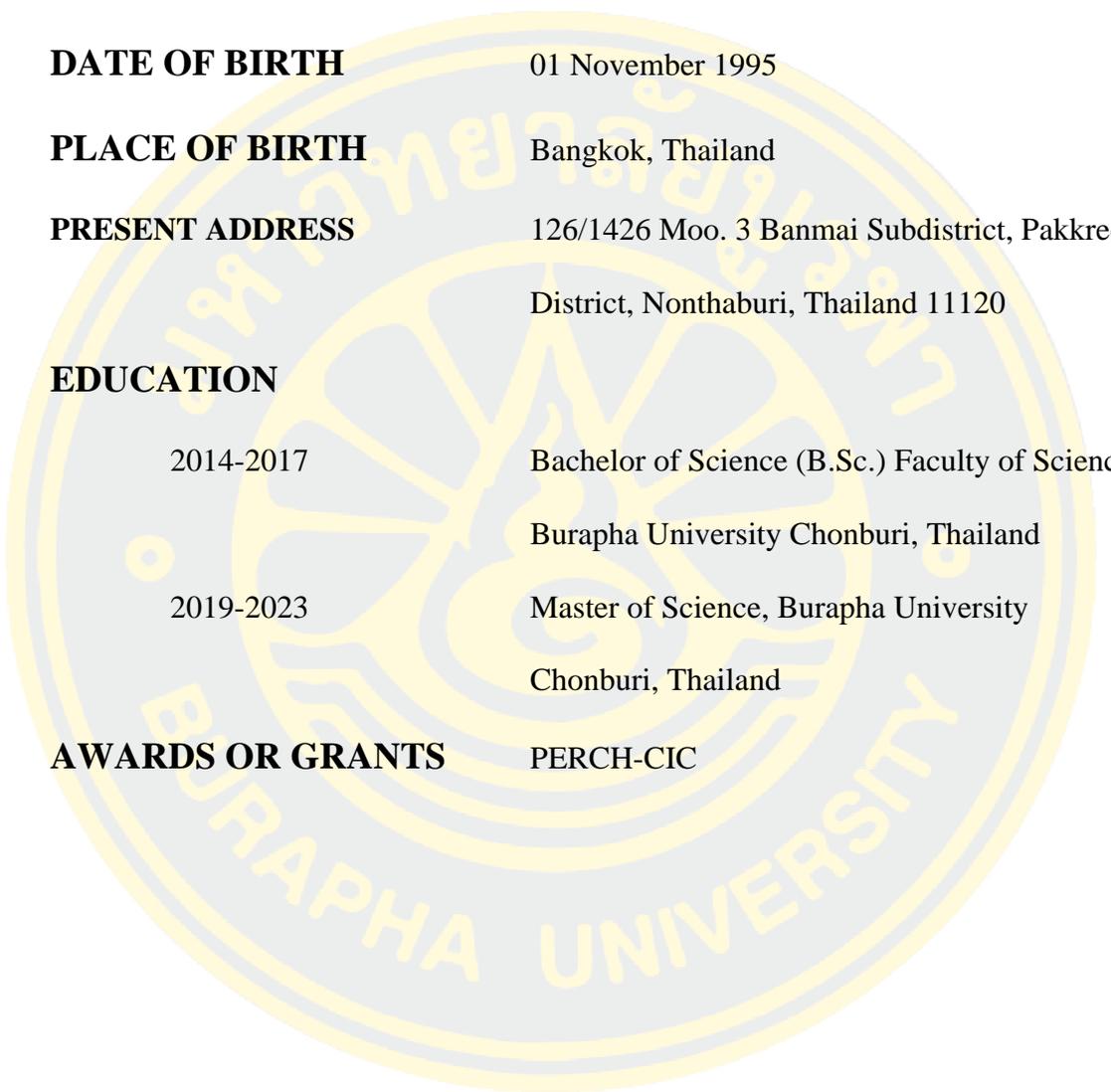
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The Thesis of Rotsukon Poonbud has been approved by the examining committee to be partial fulfillment of the requirements for the Master Degree of Science in Biochemistry of Burapha University

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Principal advisor (Assistant Professor Dr. Panata Iawsipo)	Principal examiner (Dr. Siriphatr Chamutpong)
 Member (Assistant Professor Dr. Panata Iawsipo)
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 Dean of the Faculty of Science (Associate Professor Dr. Usavadee Tuntiwaranuruk)

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