APOPTOSIS INDUCTION ON COLON CANCER CELLS FROM THE *CALOTROPIS GIGANTEA* STEM BARK EXTRACT

THANWARAT WINITCHAIKUL

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Physiology
2021

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Thesis entitled "Apoptosis induction on colon cancer cells from the *Calotropis gigantea* stem bark extract"

By THANWARAT WINITCHAIKUL

has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science in Physiology of Naresuan University

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ABSTRACT

The drawbacks of chemotherapeutic agents in colorectal cancer cells (CRC) are systemic side effects, and drug resistance which generates limitations during the therapy. Natural products are being greatly focused on in the hope that they will be able to replace the medications. The anticancer effects of different parts of the *Calotropis gigantea* (*C. gigantea*) have been reported, however, the inhibitory effects on cancer cell proliferation from its stem bark extracts in CRC have not yet been examined. This study investigated the anticancer effects of the four fractions from the stem bark extracts of *C. gigantea*, both singularly and combined with 5-fluorouracil (5-FU). Dry powder from the *C. gigantea* bark was extracted using 95% ethanol to obtain the ethanolic crude extract (CGEtOH), then dichloromethane (CGDCM) was added to the extract, which created ethyl acetate (CGEtOAC), and water (CGW) layers. The cytotoxic effect from one CGDCM fraction showed the highest potency in both HCT116 and HT-29 cells, while the HCT116 cells combined with 5-FU demonstrated a more significant effect. The resistance of normal fibroblast HFF-1 cells to the treatment demonstrated a safety usage for normal cells, while the combination significantly enhanced apoptosis via the mitochondrial-dependent pathway. Reducing the ATP production and increasing ROS generation after treatment with the combination caused apoptotic induction. Our results suggest that DCM extracts from the stem bark of the *C. gigantea* demonstrated promising anti-cancer activities, and potential effects to facilitate apoptotic induction with 5-

fluorouracil in the treatment of CRC with reduced toxicity to normal human cells.

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CHAPTER I
INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide, with the third and second highest rate in males and females, respectively (Organization, 2020). The most common cancer-related cause of death is colorectal metastases, which has a 5-year survival rate of less than 15%. The spread of CRC burden varies widely, with more than two-thirds of all cases and approximately 60% of all deaths occur in countries with a high human development index (HDI) (Sung et al., 2021). Nowadays, chemotherapy treatment for CRC patients has been found to be less effective than before (Van der Jeught et al., 2018). Chemotherapeutic agents for treatment CRC patients such as irinotecan, oxaliplatin, capecitabine, and 5-fluorouracil (5-FU) that are thought to be effective, but at the same time have a high incidence of drug resistance, recurrence, and side effects (Braun et al., 2011). This research concentrated on the combination of chemotherapeutic drugs, along with compounds derived from natural products that can be used as a specific treatment for cancer.

Calotropis gigantea (*C. gigantea*) is part of the family of Apocynaceae and sub-family of Asclepiadaceae, which is a native plant found in Africa, Eastern and Southeast Asia, and Thailand. There have been many interesting pharmacological studies of the extracts from several parts of the *C. gigantea*, with the most prolific being cardenolides (Parhira et al., 2016; Parhira et al., 2014; You et al., 2013), followed by alkaloid, pregnanones (Z.-N. Wang et al., 2008), triterpenes (Thakur et al., 1984), and triterpenoids and steroids (Hasballah et al., 2021; Omer et al., 2017). This plant has been reported to exhibit other pharmacological properties, which include antibacterial (Sharma et al., 2015) ,anti-inflammatory (H. Wang et al., 2021) and antitumor activities (Parhira et al., 2016; You et al., 2013). In addition, several compounds were isolated from this plant such as 12,16-dihydroxycalotropin, 19-dihydrocalactin, calactin, calotropin, corotoxigenin 3-O-glycoside, desglucouzarin, uscharin, and 2´´-oxovoruscharin have shown–potential anticancer agents, which induced apoptosis cell death and other pathways (Khan et al., 2015; Rascón-Valenzuela et al., 2016; Van Quaquebeke et al., 2005).In earlier studies, the aerial

parts of the ethanolic *C. gigantea* extracts were found to induce apoptotic cell death the activation of extrinsic, intrinsic pathways, and cell cycle arrest via the enhancing of reactive oxygen species (ROS) in non-small cell lung carcinoma cells (Lee et al., 2019). In *in vitro* and *in vivo* models, the flowers, roots, root bark, and leaf extracts from the *C. gigantea* exhibited cytotoxic effects (M Rowshahul Habib, 2011; Muhammad Rowshanul Habib et al., 2010; Mutiah et al., 2016). In addition, the cardenolides isolated from the ethanolic bark extracts of the *C. gigantea,* showed inhibitory effects on both Hela and the A549 cells (Van Khang et al., 2014). it has been reported that combined treatment with one of the cardenolides from the *C. gigantea* and irradiation did not affect normal epithelial cells (Sun et al., 2017). *C. gigantea* has also been shown to augment the responses of cancer cells to other forms of cancer therapy. Coroglucigenin is one of the cardenolides isolated from the stem and leaves of the *C. gigantea,* which increased the sensitivity to radiation therapy on human lung cancer cells (Sun et al., 2017). However, the mechanism underlying the induction of apoptosis on cancer cells by the stem bark extract of *C. gigantea* has not yet been identified.

5-fluorouracil (5-FU) is the chemotherapeutic agent for treatment CRC as an adjuvant and neoadjuvant, to achieve positive clinical outcomes (Longley et al., 2003; Miura et al., 2010; Öven Ustaalioğlu et al., 2018). Several studies have suggested that the combination of 5-FU and other natural products are able to decrease proliferation in many cancers (Y. Wei et al., 2018; Yang et al., 2017). In addition, there is a need to enhance the anticancer activities of 5-FU which will allow it to reduce toxicity in patients. Plant extracts have been reported to improve the cytotoxic effect of 5-FU in cancer cells. The anti-cancer effect of *Piper betle* enhanced 5-FU activity in HT-29 and HCT116 colon cancer cells (Ng et al., 2014). The synergistic anti-cancer effect mediated through mitochondrial-dependent apoptosis of a combined longan flower extract and 5-FU was found in colorectal cancer cells (Chen et al., 2020). Rutin, a glycoside component in green tea and apple trees combined with 5-FU, exhibited synergistic drug-herb interactions that induced apoptosis in prostate cancer cells (Satari et al., 2019). In addition, Verbascoside is a phynylethanoid glycoside isolated from *Plantago* seeds, had a sensitization effect on HCT116 and Caco2

adenocarcinoma cells to 5-FU by downregulating the phosphatidylinositol 3-kinases (PI3K)/ protein kinase B (Akt) pathway (Attia et al., 2018).

Therefore, this study aimed to determine the extracts from stem bark of *C. gigantea* on inhibition of growth and inducing apoptosis in colon cancer cells. Moreover, Moreover, in combination of *C. gigantea* with the least concentration of standard cancer chemotherapeutic drug 5-FU having cellular cytotoxicity was hypothesized to provide higher suppressing effect on cancer proliferation than single treatment. These results may provide novel important information for further investigation on discovery of new acceptable anti-cancer strategies of *C. gigantea* in combination therapy to accelerate the treatment outcome in cancer cells.

Objectives

1. To evaluate the effects of *C. gigantea* stem bark extracts on the inhibition of growth and induction of apoptosis in colon cancer cells.

2. To study the effect of 5-FU combination and *C. gigantea* increased ROS production and decreased ATP mediated apoptosis in HCT116 cells.

Research Scope

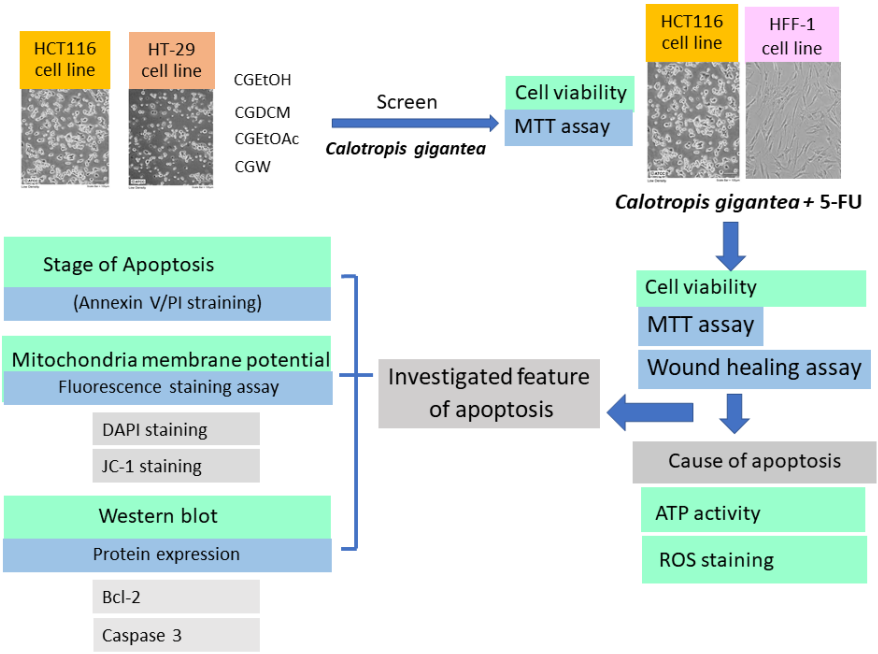


Figure 1 Scope of this study

Keywords

Colon cancer cells, *Calotropis gigantea* stem bark extract, Cardenolides, Reactive oxygen species (ROS), apoptosis

Research Hypothesis

The *Calotropis gigantea* stem bark extract exhibited cytotoxic effects against colon cancer cells both single or combined with the low dose 5-FU by apoptotic induction through decreasing of ATP and enhance ROS production.

CHAPTER II
REVIEW OF RELATED LITERATURE AND RESEARCH

Colorectal cancer

Colorectal cancer (CRC) is one of the most common malignancies worldwide with the third and second highest in males and females, respectively. Figure 2 shows that CRC is one of ten highest incidence rate amongst cancers in the GLOBOCAN 2020 database (Sung et al., 2021). Approximately 60% of cancer burden in Thailand is due to five types of cancers which are breast, cervix, colorectal, liver, and lung. Excluding melanoma, these five cancers account for 59.2% of incidence, 63.1% of mortality and 54.3% of 5-year prevalence in 2012. However, the number of cases expected for the year 2025 using cancer registry data in Thailand found that breast, colorectal and liver cancer are increasing nationwide (Virani et al., 2017).

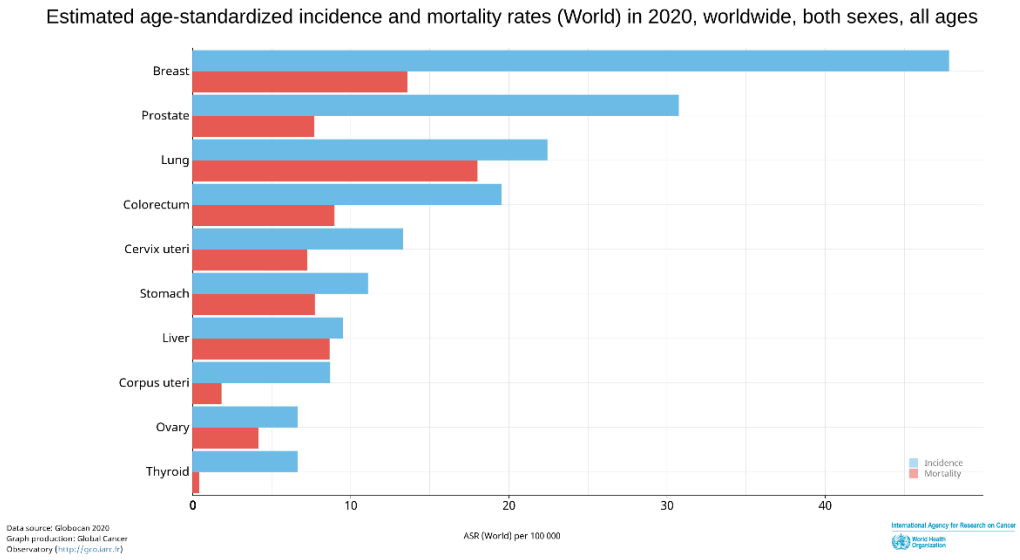


Figure 2 Incidence and mortality rates for the Top 10 Most Common Cancers in GLOBOCAN 2020

Source:(Sung et al., 2021)

Colorectal cancer staging

Colorectal cancer is a bowel decease caused by aberrant growth and development cells in colon or rectum. Progression of cancer can be separated into 5 stages

Stage 0: It is considered a non-invasive stage. Aberrant cells only locate in the mucosa. Cells are not grown beyond the inner lining of the colon or rectum wall. Tumor mass is defined as “polyp”. This stage is very treatable and often curable by surgery.

Stage I: Cells increasingly grow and proliferate contributing to progressing tumor mass. As a consequence, cancers invade into the muscular layer of the colon wall. However, there is no spreading to outside or lymph nodes. Treatments are performed by surgery.

Stage II: Cancer cells in the muscular layer grow and possibly invade the nearby tissue, but they have not penetrated into lymph nodes. Removing of malignant tumor provides the efficiency of therapeutic results. Medicinal management is carried out by surgery and chemotherapy.

Stage III: Cancer cells spread through nearby lymph nodes, but they do not yet move to other organs.

Stage IV: Spreading to distant organs occurs in this stage that is defined as the "metastasis". Target organs are often threatened such as the liver, lung, bone, and brain. Mostly, palliative treatments are the main remedy.

Signs and symptoms of colon cancer

The common signs and symptoms are different in patients with bowel cancer. They can be classified into local and systemic symptoms.

Local symptoms of colorectal cancer

Local symptoms are those that affect only the colon and/or rectum and have no spreading to distant organs. Common local symptoms include constipation, diarrhea, alternating diarrhea, and constipation, or other changes in bowel habits, rectal bleeding or blood in the stool, abdominal bloating, cramps or discomfort, feeling that the bowel doesn't empty completely, and stools that are thinner than normal.

Systemic symptoms of colorectal cancer

Systemic colorectal cancer symptoms may impact more than the digestive tract and affect the entire body. Common systemic symptoms of colorectal cancer include unexplained loss of appetite, unexplained weight loss, nausea, vomiting, jaundice, anemia, weakness, and fatigue (Rasmussen et al., 2015)

Treatment of colorectal cancer

In present, medical performances aim to conduct prevention, cure, and palliation of tumor recurrence. To provide the efficiency for treatment and discard tumor as much as possible, stages of colorectal cancer must be concerned.

1. Surgery is the major carrying out and often performs in the early stage of colorectal cancer to remove the cluster of aberrant cells (polypectomy). It gives the efficiency therapeutic results in patients with a stage of 0-II of colorectal cancer. The surgery also refers to colectomy (remove all or part of the colon). Generally, it usually combines with other medical management, especially radiation therapy and chemotherapy (Rentsch et al., 2016).

2. Radiation therapy can be divided into external beam radiotherapy, internal radiation therapy, and radioembolization. Radiation therapy uses high-energy rays (such as x-rays) or particles to destroy cancer cells. It is more often used to treat people with rectal cancer than for people with colon cancer. At the same time, to make radiation therapy works better, it must combine with chemotherapy (C.-M. Huang et al., 2017; Kye et al., 2014).

3. Cell immunity therapy is the new technology that extracts immunologic cell (DC-CIK) from the body and then cultures it outside the body. Consequently, reinfusion is performed in order to regulate immunity functions, enhance the antitumor capability, and completely kill tumor (Boland et al., 2017).

4. Chemotherapy is a classically treatment for colorectal cancer for every stage. There are various anti-neoplastic drugs that predominantly act to inhibit cell growth and proliferation. Chemotherapeutic drugs are applied to surgery and radiotherapy. Unfortunately, they exhibit the deleterious effects on normal cells. Side effects include nausea, vomiting, fatigue, hair loss, and weight loss, etc. Common chemotherapeutic drugs for colorectal cancer treatment are listed below.

• Capecitabine (Xeloda)

• 5-Fluorouracil (5-FU, Adrucil)

• Irinotecan (Camptosar)

• Oxaliplatin (Eloxatin)

• Trifluridine / tipiracil (TAS-102, Lonsurf) (Akhtar et al., 2014; Saltz et al., 2000)

5-Fluorouracil (5-FU)

5-Fluorouracil (5-FU) is a fluoropyrimidine analog which is widely used for patients in the treatment of a range of cancers since 1957, including colorectal cancer, breast cancers, and cancers of the aerodigestive tract. It is uracil analogue with a fluorine atom at the C-5 position in place of hydrogen which a heterocyclic aromatic organic compound of 5-FU has a structure similar to that of the pyrimidine molecules of DNA and RNA (Grem, 2000) Meanwhile, it is an inhibitor for thymidylate synthase (TS) affecting the synthesis of the pyrimidine thymidine, the precursor for DNA replication. Interference of DNA synthesis influences cell proliferation. (Figure 3)

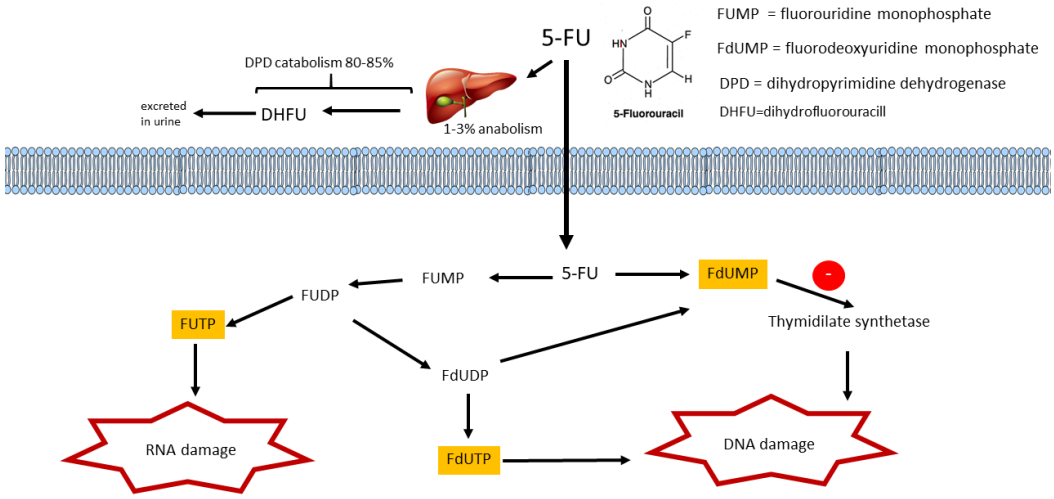


Figure 3 The mechanism of 5-Fluorouracil

Source: Adaptation from https://www.nature.com/articles/nrc1074

Metabolism of 5-FU, it has been demonstrated that 80% to 85% of 5-FU is broken down primarily in the liver to inactive metabolites by enzyme dihydropyrimidine dehydrogenase (DPD), which converts 5-FU to dihydrofluorouracil (DHFU), finally leading to the formation of α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine (FBAL) was excreted in urine as FBAL within 24 hours. Only 1 to 3% anabolism of 5-FU exhibited the cytotoxic effects on tumor cells and normal tissues (Longley et al., 2003; Miura et al., 2010). Mechanism of action 5-FU in mammalian cells, 5-FU is entered cell using the same transport mechanism as uracil. It converted to fluorodeoxyuridine monophosphate (FdUMP), which forms a ternary complex with thymidylate synthase (TS) to inhibited synthesis of DNA and then 5-FU is converted to Fluorouridine triphosphate (FUTP), incorporated into RNA instead of UTP. The metabolite of 5-FU converted to Fluorodeoxyuridine triphosphate (FdUTP) cause DNA damage (Miura et al., 2010).

Although 5-fluorouracil (5-FU) is one of the most generally used chemotherapy drugs but its use is limited due to chemotherapeutic resistance and side effects upon prolonged chemotherapy. Effects of 5-FU including GI toxicity, myelotoxicity, Cardiac toxicity (Shirasaka et al., 1993; Sorrentino et al., 2012) Several studies have suggested that the combination of 5-FU can decrease proliferation in many cancers including colorectal cancer, esophageal squamous cell carcinoma, liver cancer, nasopharyngeal cancer, pancreatic cancer, and gastric cancer (Y. Wei et al., 2018; Yang et al., 2017). In addition, there is a need to enhance anticancer activity of 5-FU and to reduce toxicity in patients. Thus, new medications from potential herbal sources should be explored. It has been demonstrated that combination 5-FU with other compound or drug show sensitivity on several antitumor such as the combination of casticin (a polymethexyflavone isolate from Vitex rotundifolia) and 5-FU induce apoptotic WEHI-3 cell death through the expression of antioxidant molecule (SOD and catalase) to enhanced ROS production and ER stress (Cheng et al., 2020).The combination of 5-FU and Rutin, is a glycoside from quercetin flavonoid that show a synergistic effect on PC3 prostate cancer cells and anti-proliferation after treatment for 48 h. and induced apoptosis by downregulated Bcl-2 expression through p53 gene expression (Satari et al., 2019). Meanwhile, the

treatment PI3K/AKT inhibitor can increase sensitivity 5-FU chemotherapeutic drug on esophageal cancer cell (B. Li et al., 2014)

Apoptosis cell death

Apoptosis is one type of program cell death pathway controlled by genes. It plays an important role in the development and balance of the organism. Risk factors for promoted apoptosis have various stimuli including radiation, toxic chemical, oxidative stress and hypoxia which leads to abnormality of cells such as DNA damage. Cell changes will occur after the cascade of cell signaling and caspase-mediated events which exert the control of proapoptotic proteins involving cell death induction and antiapoptotic proteins involving cell death inhibition. The characteristic cell change (morphology) of apoptotic cells shows blebbing of plasma membrane, nuclear condensation, chromatin aggregation, and DNA fragments. Induction of apoptosis occurs in two ways including extrinsic and intrinsic pathway. Both pathway leading to apoptosis are involved by activating the effector caspases enzyme, such as actin, lamin A, ICAD / CAD, PARP, Rb, p27, p21 that digest proteins in various cells (Elmore, 2007).

Death-receptor induced extrinsic pathway: The extrinsic signaling pathway that initiates apoptosis involves transmembrane receptor-mediated interactions. The death domain of death receptors has various type in cell such as death receptor 3 (DR3), death receptor 4 (DR4), death receptor 5 (DR5), and tumor necrosis factor (TNF) receptor by binding to their ligands. It plays a critical role in transmitting the death signal by activating procaspase to be an caspase leading to apoptosis from activating at the cell surface to induce effector caspases in the intracellular signaling pathways (Galluzzi et al., 2018; Xu et al., 2007).

Mitochondrial-mediated apoptosis intrinsic pathway: The intrinsic signaling pathway initiated by, for example, chemotherapy and/or radiotherapy mediated stimuli intracellular stress that cause mitochondrial-initiated apoptotic (Hengartner, 2000; M. C. Wei et al., 2000). In figure 4, apoptosis has many other regulators which can be divided into 2 group of control including pro-apoptosis members (e.g. PUMA, BAX, BAK) and anti-apoptotic members (BCL-2, BCL-XL) when pro-apoptosis promoted mitochondrial outer membrane permeabilization (MOMP) and release cytochrome C into cytosol. Cytochrome C associated with

APAF-1and Caspase-9 to from the apoptosome, which activated caspase-3 leading to apoptosis (C. Wang et al., 2009).

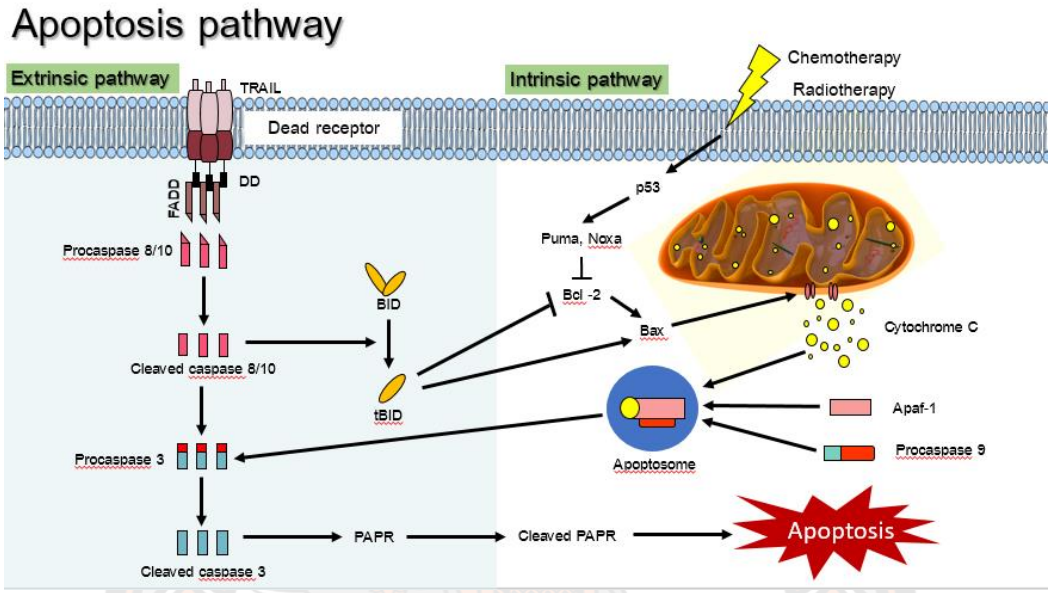


Figure 4 Apoptosis pathway

Source: Adaptation from https://biologydictionary.net/apoptosis/

Reactive oxygen species (ROS)

ROS are radical, ions that have a single unpaired electron. It can be categorized into two groups: free oxygen radicals and non-radical ROS. Free oxygen radicals include superoxide ($O2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$), organic radicals ($R^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$), thiyl radicals ($RS^{\bullet}$), sulfonyl radicals ($ROS^{\bullet}$), thiyl peroxyl radicals ($RSOO^{\bullet}$), and disulfides (RSSR). Non-radical ROS include hydrogen peroxide (H_2O_2), singlet oxygen (1O2), ozone/trioxygen (O_3), organic hydroperoxides (ROOH), hypochloride (HOCl), peroxynitrite (ONO^-), nitrosoperoxycarbonate anion ($O{=}NOOCO_2^-$), nitrocarbonate anion ($O_2NOCO_2^-$), dinitrogen dioxide (N_2O_2), nitronium (NO_2^+), and highly reactive lipid-or carbohydrate-derived carbonyl compounds. In mitochondria, ROS produced from the process of oxidative phosphorylation or generate from interactions with exogenous sources. In general, the cellular redox balance is regulated homeostasis by

antioxidant enzymes such as superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) in figure 5.

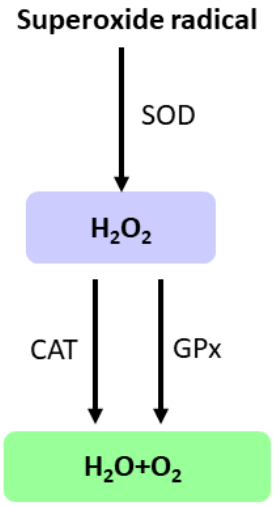


Figure 5 Reactive oxygen species (ROS) scavenging mechanism

SOD is considered as the first detoxification enzyme which defense against formed ROS. SOD converts the superoxide anion into O_2 and H_2O_2. On the other hand, CAT and Glutathione peroxidase (GPx) enzyme hydrolyses hydrogen peroxide (H_2O_2) into water and molecular oxygen. Antioxidant enzymes plays an important role to protect the cell from the superoxide injury via inhibiting the reaction of superoxide from the imbalance between the antioxidants and increased oxidative stress on cellular (Verma et al., 2018).

GST is considered as the cytoprotective phase II enzyme, which is involved in the detoxification of H_2O_2. The prime role of GST is protecting the cell from the free radical induced damage via catalyzing the production of lipid hydro-peroxides, which are regularly generated during the oxidative damage of cellular lipid molecules.

GPx is a selenoprotein endogenous anti-oxidant enzyme in the cytosol and mitochondrial matrix, which catalyzes the reduction of hydroperoxides to H_2O via oxidation of reduced GSH into its oxidized disulfide form GSSH to protects the cell from the oxidative damage and inhibit the lipid hydro-peroxides (Shaban et al., 2013; Weydert et al., 2010).

Although, oxidative stress occur in cell causes by either overproduction of reactive oxygen species, decreased of antioxidant enzymes or both. It has been reported oxidative stress caused by ROS, which the results of ROS are mediated to damage of nucleic acids, proteins, and lipids, and accumulation of ROS can induce cell death (Ray et al., 2012; Velu et al., 2016). Previous study, it has interesting in ROS-mediated apoptosis cell death in several cancer. Example, Inhibition of antioxidant molecules (NQO-1,HO-1) through Nrf2 affect to ROS enhancing on human lung cancer cells from *C. gigantea* (Sun et al., 2017). On A549 and NCI-H1299 non-small cell lung cancer cells, the ethanol extracts from the whole plant of *C. gigantea* at dose 3.75, 7.5, 15 µg/ml for 48 hours induced apoptotic cell death through the expression of ROS scavenger genes (SOD2 and catalase) to enhanced ROS production (Lee et al., 2018). Galangin a flavonol extracted from the Alpinia galangal root at dose 20, 40 µM for 24 h. induces apoptosis by upregulated NADPH activity which was enhanced ROS production in MCF-7 and T47D human breast cancer cell (Song et al., 2017). Compound name oxypregnane-oligoglycosides (Calotroposides) from the ethanolic extract of root bark *Calotropis gigantea* induces apoptosis by enhance production ROS in MDA-MB-231 breast cancer cells (Mahar et al., 2016). In addition, Isorhamnetin (3′-methoxy-3,4′,5,7-tetrahydroxyflavone) is a flavonol aglycone at dose 100 µM for 48 h. showed cytotoxic effect through enhancing of ROS to induced apoptosis pathway in human bladder cancer cells (C. Park et al., 2019).

Calotropis gigantea

Figure 6 *Calotropis gigantea* in Thailand

Source: Photo by Miss. Thanwarat Winitchaikul on February 28th, 2021

Calotropis species, belonging to the family of Asclepiadaceae in the plant kingdom, are the well-known native plants in wastelands of Asia and Africa. It has two species include *Calotropis procera* and *Calotropis gigantea*. *Calotropis gigantea* (*C. gigantea*) is a commonly called giant milk weed or crown flower. It is commonly known as "รัก Rak" in Thailand shown in Figure 6. The flowers are generally purple and white. In general, this plant has been tradition medicines for using in Ayurveda both of the people in Indonesia and China to cure many diseases and symptoms including latex is used for stings, toothache, caries, leprosy, ringworm, syphilis, tumours, rheumatism, antiseptic, vermifuge and purgative. The flowers are also used for treating jaundice, inflammation, ulcer and asthma. The whole plant is used for skin diseases such as boils, sores, as a tonic and purgative. In particular, the stem bark is used as diaphoretic, expectorant and useful in complaints such as dysentery, spleen enlargement, convulsions, scabies, ringworm, pneumonia and to induce labour in pregnant women (Kadiyala et al., 2013).The leaves for rashes, scabies, boils, cough, trachoma, and constipation, flowers for asthma, nausea, and stomach pain, roots for syphilis, and viper bites, root bark for cutaneous infections, intestinal worms,

helmintic infections, cough and ascites (Muhammad R Habib et al., 2013; Kadiyala et al., 2013; Kumar et al., 2011; Rathod et al., 2009).

In last few decades, *C. gigantea* is extensively studied for its medicinal properties and variety of bioactive compounds have been isolated from the different parts of the plant such as leaves, latex, flowers, roots and root bark (M Rowshanul Habib et al., 2007; Lhinhatrakool et al., 2006; Lodhi et al., 2009; Murti et al., 1943; Pari et al., 1998; Seeka et al., 2010; Sen et al., 1992; Van Khang et al., 2014; You et al., 2013). The *C. gigantea* extracts present many chemical constituents (Kiuchi et al., 1998; Lhinhatrakool et al., 2006), flavonoids (Sen et al., 1992), terpenes (Parhira et al., 2014), steroid, terpenoid, saponin (Seniya et al., 2011), alkaloid, tannins ((Rathod et al., 2009)), pregnanes (Kitagawa et al., 1992), and a nonprotein amino acid (Pari et al., 1998) as shown in figure7. However, the bioactive molecules reported from different parts of *C. gigantea* are rich in metabolites of triterpenoids, flavonoid, alkaloids, and cardenolides. These classes of compounds are known to have a wide range of biological and pharmacological activities. Ethyl acetate leaves extract show antibacterial activity of *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Salmonella typhi*, and *Micrococcus luteus strains* (Seniya et al., 2011), anti-diarrheal activity from aerial part of the plant are reported on castor oil-induced-diarrhea model in rats (Chitme et al., 2004), The ethanolic extract of root bark extract treatment accelerates wound healing in Wistar albino rats (Deshmukh et al., 2009). The ethanolic extract of stem decreases liver damage by lower level of AST, ALT, and antioxidant levels (Lodhi et al., 2009).

Chemical constituent	Plant part	Chemical nature
19-Nor- and 18,20-Epoxy-cardenolides	Leaves	Cardenolides
15beta-hydroxycardenolides	Leaves	Cardenolides
16alpha-hydroxycalactinic acid methyl ester	Leaves	Cardenolides
Isorhamnetin-3-*O*-rutinoside	Arial parts	Flavonol
Isorhamnetin-3-*O*-Glucopyranoside	Arial parts	Flavonol
Taraxasteryl acetate	Arial parts	Flavonol
Calotropain-F1 and	Latex	Proteinases
Calotropain-FII	Latex	Proteinases
3′-methylbutanoates of α-amyrin	Latex	triterpene esters
ψ-taraxasterol	Latex	triterpene esters
Calotropins DI	Latex	Proteinases
Calotropins DII	Latex	Proteinases
Di-(2-ethylhexyl) Phthalate	Flowers	Triterpenoids
Anhydrosophoradiol-3-acetate	Flowers	Triterpenoids
Calotropone	Roots	Cardiac glycoside
Calotropises juiterpenol	Roots	Terpene
Calotropisesterterpenol	Roots	Terpene
Calotropbenzofuranone	Roots	Aromatic product
Coroglaucigenin	Roots	Cardenolides
Frugoside	Roots	Cardenolides
Stigmasterol	Root bark	Sterols
β-sitosterol	Root bark	Sterols
Giganticine	Root bark	Nonprotein amino acid

Figure 7 Some bioactive molecules reported from different parts of *C. gigantea* Linn.

Source: A Review on Pharmacological and Phytochemical Profile of *Calotropis Gigantea* Linn.

In addition, the major components of *C. gigantea*, cardiac glycosides or cardiotonic steroids consisting of a steroid ring, a lactone ring with five or six carbons, and a sugar moiety. It can inhibit the membrane sodium-potassium (Na+-K+) pump that increase cardiac contractile force of the heart (Fozzard et al., 1985). The member in the class of cardenolides shown in figure 8, exhibit potential anticancer activity. A well-known cardenolide, digitalis, and its congeners such as digoxin and digitoxin which have been widely use for treatment of heart disease, is approved by the Food and Drug Administration of the United States to be used for treatment of prostate cancer (PCa) growth in human (Cerella et al., 2015; Lin et al., 2014). In addition, a substance called UNBS1450, a hemi-synthetic cardenolide is modified from cardiac glycosides (2″-oxovorusharin,) deried from *C. procera* extracted, which is in the same genus as the *C. gigantea* found in Thailand. It has been approved for using in human leukemia cells (Cerella et al., 2015; Juncker et al., 2011; Mijatovic et

al., 2007). Therefore, cardenolides composing in the extract of C. gigantea are interesting to further study a therapy for malignancies.

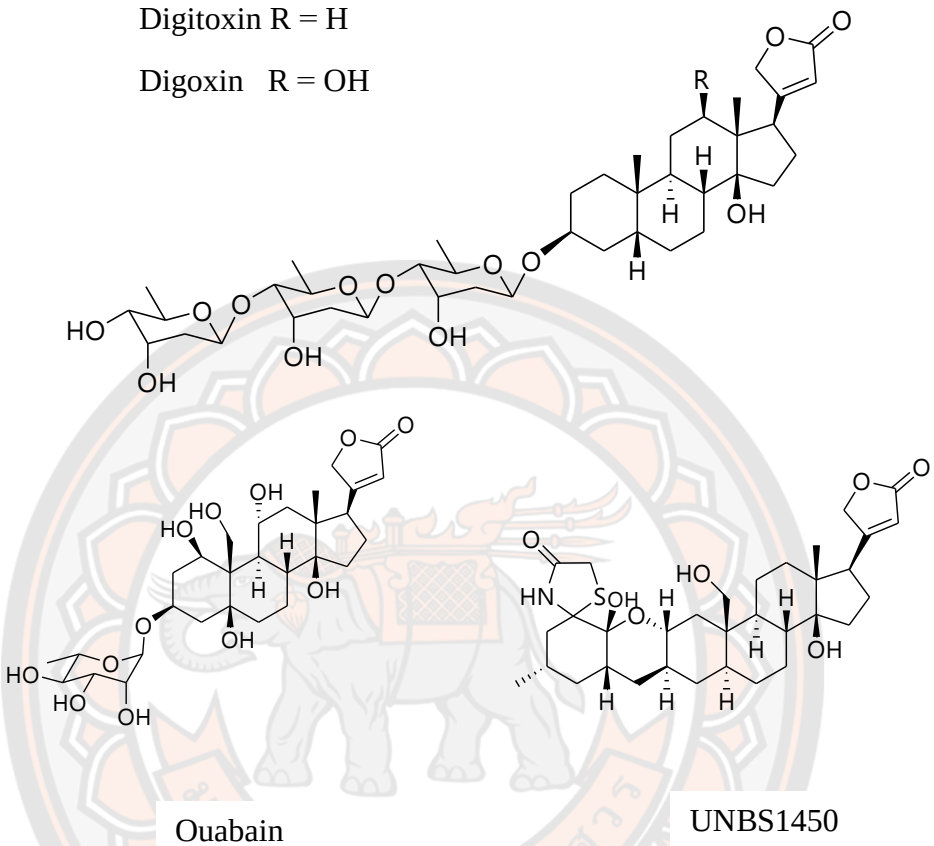


Figure 8 Chemical structures of cardenolides

Source: (Kataoka, 2009; Mijatovic et al., 2007; Prassas et al., 2008)

Moreover, previous study results suggest that the ethyl acetate extract from the flowers *C. gigantea* can induce antitumor activity in Ehrlich's ascites carcinoma (EAC) tumor baring mice (Muhammad Rowshanul Habib et al., 2010). The methanol extract and chloroform fractions from roots of *C. gigantea* deceased viable tumor cell growth of Ehrlich ascites carcinoma (EAC) tumor bearing mice in Swiss albino mice (M Rowshahul Habib, 2011). It has been reported that the cytotoxic potential of the fraction CH_2C_{l2} extract, and n-BuOH extract of the leaves cardenolide compound antiproliferation of breast cancer cell MCF7, skin cancer cells KB, and lung cancer cells NCL-H18 (Seeka et al., 2010). The dichloromethane cytotoxic extracted from

the leaves of *C. gigantea* is potential to inhibit growth of breast cancer cells MCF-7 and MDA-MB-231, Hela cells, colon cancer cells HT-29, ovarian cancer cells Skov3, and liver cancer cells Hep-G2 (Wong et al., 2011). The cardenolide named coroglaucigenin (CGN) isolated from ethanol extract of the stem of *C. gigantea* has been shown inhibition effect of proliferation in AS49 human lung cancer cells with X-Ray at dose IC50< 6 µM (Sun et al., 2017). The subfraction calotroposid A, a triterpenoid glycoside compound, isolated by ethyl acetate of root *C. gigantea* at IC50 dose 17.23 µg/ml has been reported to induce apoptosis in colon WiDr cells through cell cycle arrest at G2/M phase and increase caspase 8 protein expression (Mutiah et al., 2018). Thus, these reports provide a strong proposal idea for studying mechanism of anticancer activity of C. gigantea extract which will be benefit for further promising anticancer therapeutic clinical study.

CHAPTER III
METHODOLOGY

Research instrument

CO_2 incubator (Sanyo, MCO-20AIC)

Inverted optical microscope (IX71, Olympus, Japan)

Fluorescence microscopy (BX53F2, Olympus Corporation, Japan)

Microplate reader (Biotek, USA)

Muse Cell Analyzer (0500-3115, Merck, Germany).

Chemiluminescence western blot detection Image Quant LAS 4000 (GE Healthcare Life Sciences, USA)

Plant extract

The four fractions extract, CGEtOH, CGDCM, CGEtOAc and CGW from the fresh stem barks of *Calotropis gigantea* (L.) Dry and. were collected from Thoen District, Lampang Province, Thailand, during June, 2015 to April, 2018 (latitude/longitude: 17º36/´9´´N/ 99º12´50´´E) and purchased from Dr. Supawadee Parhira, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Naresuan University.

Cell culture

Human colorectal carcinoma HCT116 (CCL-247, ATCC, USA) and colorectal adenocarcinoma HT-29 (HTB-38, ATCC, USA) were purchased from American Type Culture Collection. The culture of both cell line was cultured with McCoy's media (Corning, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% Antibiotic-Antimycotic (Gibco, USA). Cells were incubated at 37 ºC in a 5% CO_2 incubator. The culture medium was replaced every 2 days. Human foreskin fibroblast HFF-1 (SCRC-1041, ATCC, USA) cells were provided from Assistant Professor Metawee Srikummool, Ph.D., Department of Biochemistry, Faculty of Medical Science, Naresuan University. Cell were cultured in Dulbecco's Modified Eagle's media (Gibco, USA) supplemented with 15 % FBS (Gibco, USA). The cells were maintained in a humidified incubator at 37 ºC under a 5% CO_2 atmosphere. The

medium was replaced every 2 days. When cell confluence reached 80–90%, the cells were sub-cultured.

MTT assay

Cells were seeded at a density of 20,000 cells/well in 96-well plates (SPL, Korea), and incubated at 37 ºC for 24 h. Cells were incubated with fraction of *C. gigantea* extract, 5-FU and combination of both. After treatment, the cells were incubated with 2 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Merck, Germany) at 37 ºC for 4 h in a 5% CO_2 incubator. The mitochondrial reductase enzyme converts MTT (yellow color) into formazan crystals (purple). Then, the formazan crystals were dissolved by 100%DMSO and the optical density (OD) of the samples was measured at 595 nm using a microplate reader (Biotek, USA).

Wound healing assay

Cells were seeded in a 12-well plate at a density of 2.5×10^5 cells/well and incubate 48 h. Then, migration ability of HCT116 cells treated with CGDCM, 5-FU, or a combination of both. Cell migration from the wound edges to the wound gap was visualized by an inverted optical microscope (IX71, Olympus, Japan), and quantified by measuring the wound distance using the program cellSens standard [Ver.2.3] Images were taken at a 10× magnification.

Nuclear staining

Cells were seeded in a 35-mm cell culture dish at a density of 3×10^5 cells/dish. Cells were treated with CGDCM, 5-FU, or a combination of both for 24 h. The cells were harvested and fixation onto a glass slide (Thermo Fisher Scientific, USA) with 10% formalin at room temperature. After, the cells were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (Invitrogen, USA) at room temperature for nuclear staining, and visualized by fluorescence microscopy (BX53F2, Olympus Corporation, Japan).

JC-1 staining assay

Cells were seeded in a 35-mm cell culture dish at a density of 3×10^5 cells/dish. Cells were treated with CGDCM, 5-FU, or a combination of both for 24 h. The cells were harvested and fixation onto a glass slide (Thermo Fisher Scientific, USA) with 10% formalin at room temperature. The cells were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanine iodide (JC-1) dye, a mitochondrial membrane potential probe (Invitrogen, USA), at room temperature. The normal ΔΨm, with JC-1 aggregation in mitochondria represent red fluorescence but in depolarization of ΔΨm was presenced of monomeric JC-1 in the cytosol that expressed as green fluorescence. The stained cells were analyzed by fluorescence microscopy (BX53F2, Olympus Corporation, Japan).

Apoptosis by flow cytometry

Cells were seeded in a 24-well plate at a density of 0.6×10^5 cells/dish. Cells were treated with CGDCM, 5-FU, or a combination of both for 24 h. Cells were harvested and stained with Annexin V and Dead Cell Assay Kit (MCH100105, Merck, Germany), according to the manufacturer's instructions. In the early stages of apoptosis, Annexin-V were stained phosphatidylserine. In the late apoptotic stages, both of annexin V and 7-amino-actinomycin D (7-AAD) were stained phosphatidylserine and DNA in cells. Briefly, cells in 1% FBS phosphate buffered saline were stained with annexin V and 7-AAD reagent and incubated for 20 min in the dark. Apoptotic cells were analyzed using Muse Cell Analyzer (0500-3115, Merck, Germany).

ROS quantification

Cells were seeded in a 35-mm cell culture dish at a density of 3×10^5 cells/dish. Cells were treated with CGDCM, 5-FU, or a combination of both for 24 h by using CM-H2DCFDA, a chloromethyl derivative of H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) (Thermo Fisher Scientific, USA) was added to each sample and incubated at 37 ºC for 30 min under a light protection. Cell staining

indicated the accumulation of intracellular ROS. The cells were visualized using a fluorescence microscopy (BX53F2, Olympus Corporation, Japan).

Intracellular adenosine triphosphate (ATP) assay

Cells were seeded in a 35-mm cell culture dish at a density of 1×10^6 cells/dish. Following treatment with CGDCM, 5-FU, or a combination of both for 24 h, the intracellular ATP levels were determined using the ATP assay kit (Elabscience, USA), as per the manufacturer's instructions. The cellular ATP content was evaluated by measuring the OD at 636 nm using a microplate reader (Biotek, USA).

Western blot analysis

Cells were seeded in a 35-mm cell culture dish at a density of 3×10^5 cells/dish and Following treatment with CGDCM, 5-FU, or a combination of both for 24 h. The cells were harvested and extracted total protein by using Mammalian Protein Extraction Reagent (M-PER; Thermo Fisher Scientific, USA), containing a proteinase inhibitor cocktail (HIMEDIA, India). Proteins from the cell lysate were collected and quantified concentration by addition of bicinchoninic acid assay reagent (Thermo Fisher Scientific, USA), and the OD was measured at 590 nm using a microplate reader. Equal amounts of proteins were separated by 12 % sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. After incubation with blocking solution (GeneDireX, USA) for 1 h, the membranes were incubated with anti−B-cell lymphoma 2 (Bcl-2) (Thermo Fisher Scientific, USA) and anti-cleave caspase-3 (Cell signaling Technology, USA) primary antibodies, and then exposed to horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Life Technologies, Invitrogen). β-actin (Cell signaling Technology, USA) was used as an internal standard. Protein bands were visualized using LuminataTM Forte Western HRP Substrate (Merck Millipore, USA) and detected by Chemiluminescence western blot detection (Image Quant LAS 4000; GE Healthcare Life Sciences, USA). Relative expression levels (%) of protein/β-actin were calculated using Image J software version 1.46.

Statistical analysis

Data from three independent experiments is shown as mean ± SD. One-way analysis of variance (ANOVA) or Student's t-test with Tukey's post hoc analysis were used to determine the statistical significance of differences between the experimental and control groups. Differences were considered statistically significant when $p < 0.05$. Data analysis was done using Graph Prism Software version 9.

CHAPTER IV
RESULTS

C. gigantea stem bark extracts induced cytotoxic effect on colon cancer cells

The ability of cytotoxic effects of CGEtOH, CGDCM, CGEtOAC, and CGW fraction from *C. gigantea* extract on HCT116 and HT-29 cells were determined by MTT assay with various concentrations for 24 h. The cytotoxic effect of HCT116 and HT-29 cells increased in a dose-dependent manner after treated with CG extract 4 fractions. IC50 values ranging were CGDCM 5.9 µg/ml, CGEtOAC 7.7 µg/ml, CGEtOH 32.8 µg/ml, and CGW 39.0 µg/ml on HCT116 cells and on HT-29 cells were CGEtOAC 43.6 µg/ml, CGDCM 44.0 µg/ml, CGEtOH 60.6 µg/ml, and CGW 86.7 µg/ml as shown in Figure 9A- 9D. It is noted that the most potency effect was seen by the CGDCM and CGEtOAC extracts treated in both cells. 5-FU showed cytotoxicity in a dose dependent manner with IC50 in HCT116 at 248.1 µM and in HT-29 at 3,598 µM (Figure 10A, B). In accordance with our data, the inhibitory effect of 5-FU in HCT116 were approximately 60% at dose of 100 µM for 24 h (Focaccetti et al., 2015). Based on results obtained here, the concentrations of CGDCM and CGEtOAC at dose 1, 2, 4, 8, and 10 µg/ml which sub-IC50 and supra-IC50 were selected to combine with 5-FU at dose 5 µM which showed a minimum significant response in HCT116 cells for the further combination experiments.

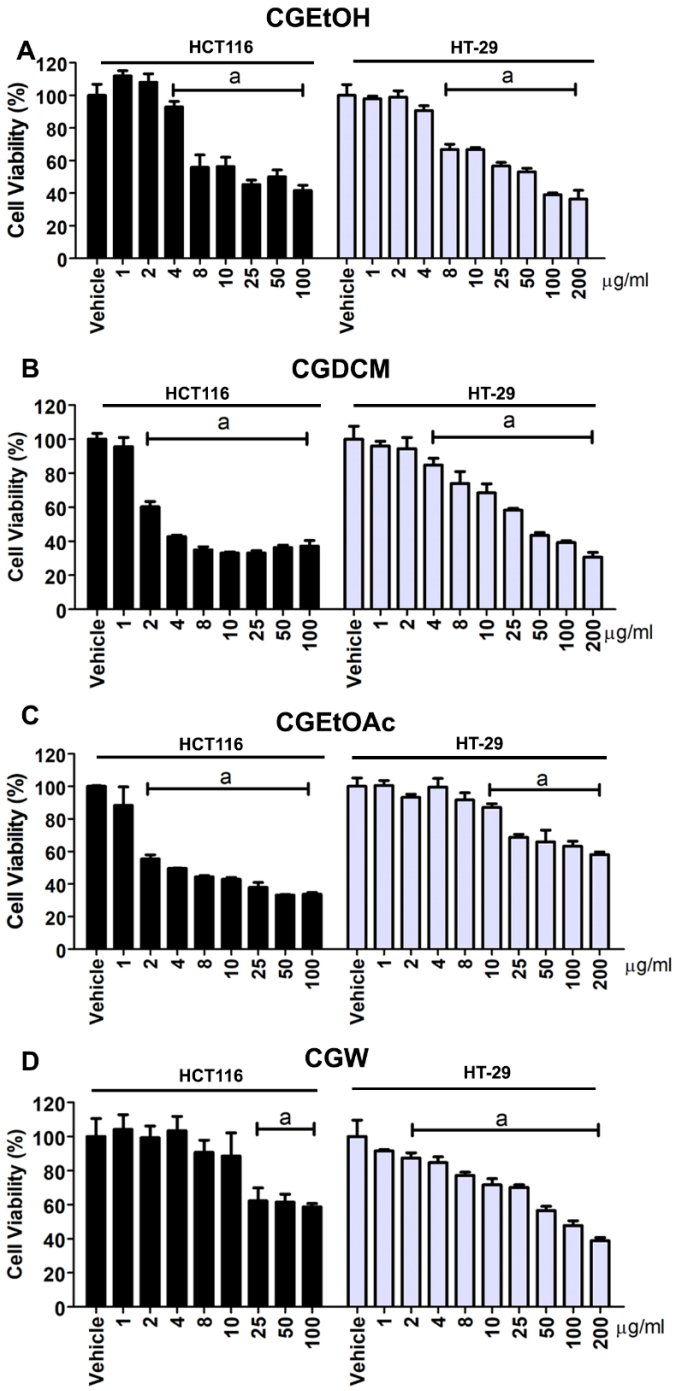


Figure 9 *C. gigantea* stem bark extracts induced cytotoxic effect on colon cancer HCT116 and HT-29 cells.

CGEtOH fraction (A), CGDCM fraction (B), CGEtOAc (C), and CGW fraction (D) of *C. gigantea* stem bark extracts were treated in HCT116 and HT-29 cells measured by MTT assay at 24 h. The vehicle control group was 0.8% DMSO.

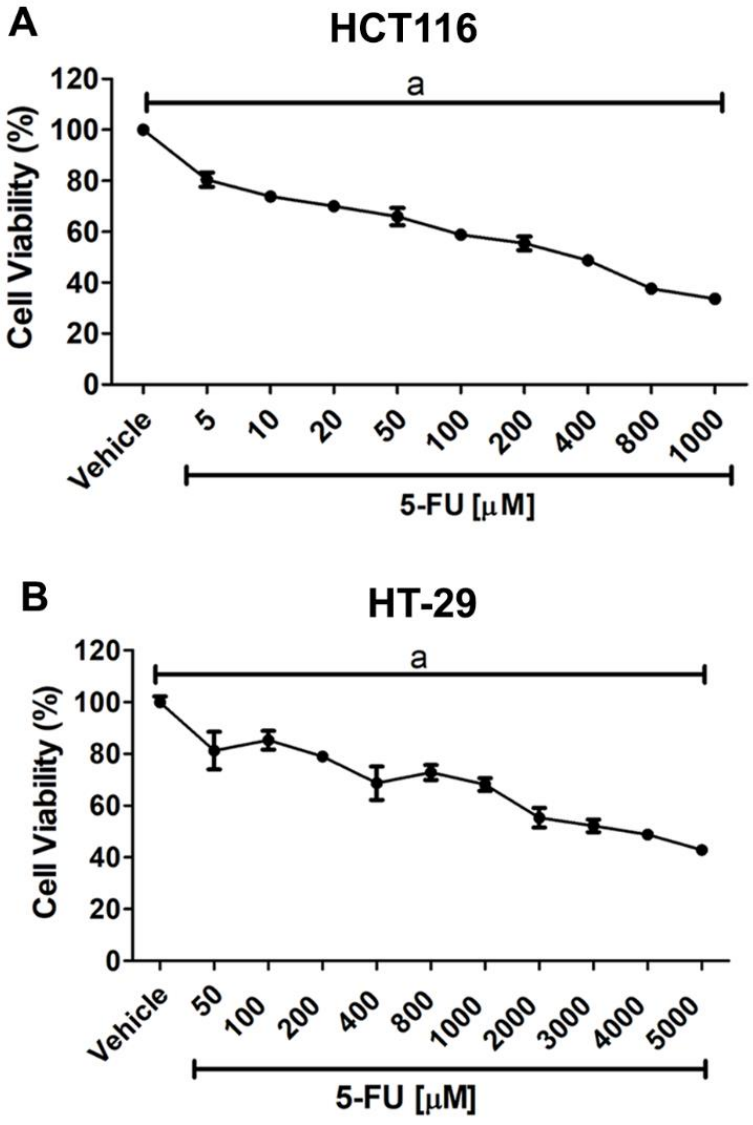


Figure 10 The cytotoxic effect of 5-FU on colon cancer HCT116 and HT-29 cells.

HCT116 (A) and HT-29 (B) cells were treated with 5-FU in HCT116 and HT-29 cells measured by MTT assay at 24 h. The vehicle control group was 0.8% DMSO. a; $p < 0.05$ compared with the vehicle.

The cytotoxicity in HCT116 cells treated by combination of CGDCM and CGEtOAc with 5-FU

To evaluated combination of cytotoxicity of HCT116 cells after received CGDCM and 5-FU (5 µM). The results showed that at 24 h incubation period, Treatment of CGDCM (1, 2 µg/mL) with 5-FU (5 µM) did not exhibit combination of cytotoxicity in HCT116 cells (Figure 11A). At concentrations of CGDCM (4, 8, and 10 µg/ml) with 5-FU (5 µM) significantly decreased cell viability compared with 5-FU alone, while the co-treatment of CGDCM at 8 and 10 µg/mL and 5-FU (5 µM) exhibited combination effects, showing greater inhibition of cell viability, compared with either drug alone. However, the cytotoxic effect of the combination of CGDCM at 8 µg/ml with 5-FU was not significantly different from that of CGDCM dose 10 µg/mL and 5-FU. The treatment with CGDCM and 5-FU (5 µM), alone and in combination at 48 h; potent cytotoxic effects were observed, but no combination effects (Figure 11A). In addition, in Figure 11B, the effect on reducing cell viability by a combination of CGEtOAC (1, 2, 4, 8, and 10 µg/ml) and 5 µM 5-FU for 24 h showed less potency than that of CGDCM. A 48-h incubation treatment of CGEtOAC, 5-FU, and their combination showed similar efficiency as of CGDCM at 48 h (Figure 11B). Moreover, studied in normal HFF-1 cells showed their resistance to 4, 8, and 10 µg/ml CGDCM, 5-FU, and their combinations treatment in 24 h as shown in Figure 11C.

Therefore, incubation period of 24 h of the combinations CGDCM (4, 8, and 10 µg/mL) with 5-FU (5 µM) were selected for the apoptotic induction experiments in HCT116 cells.

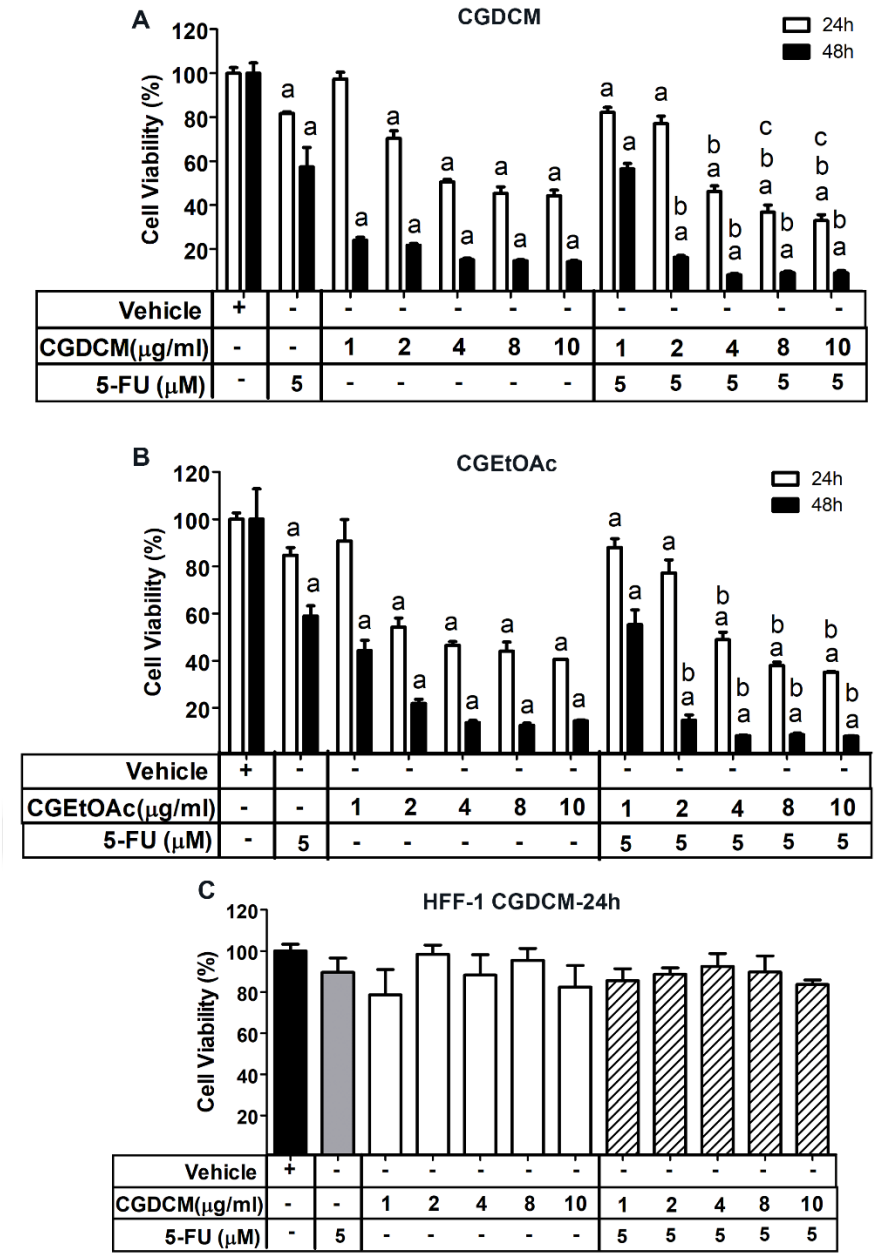
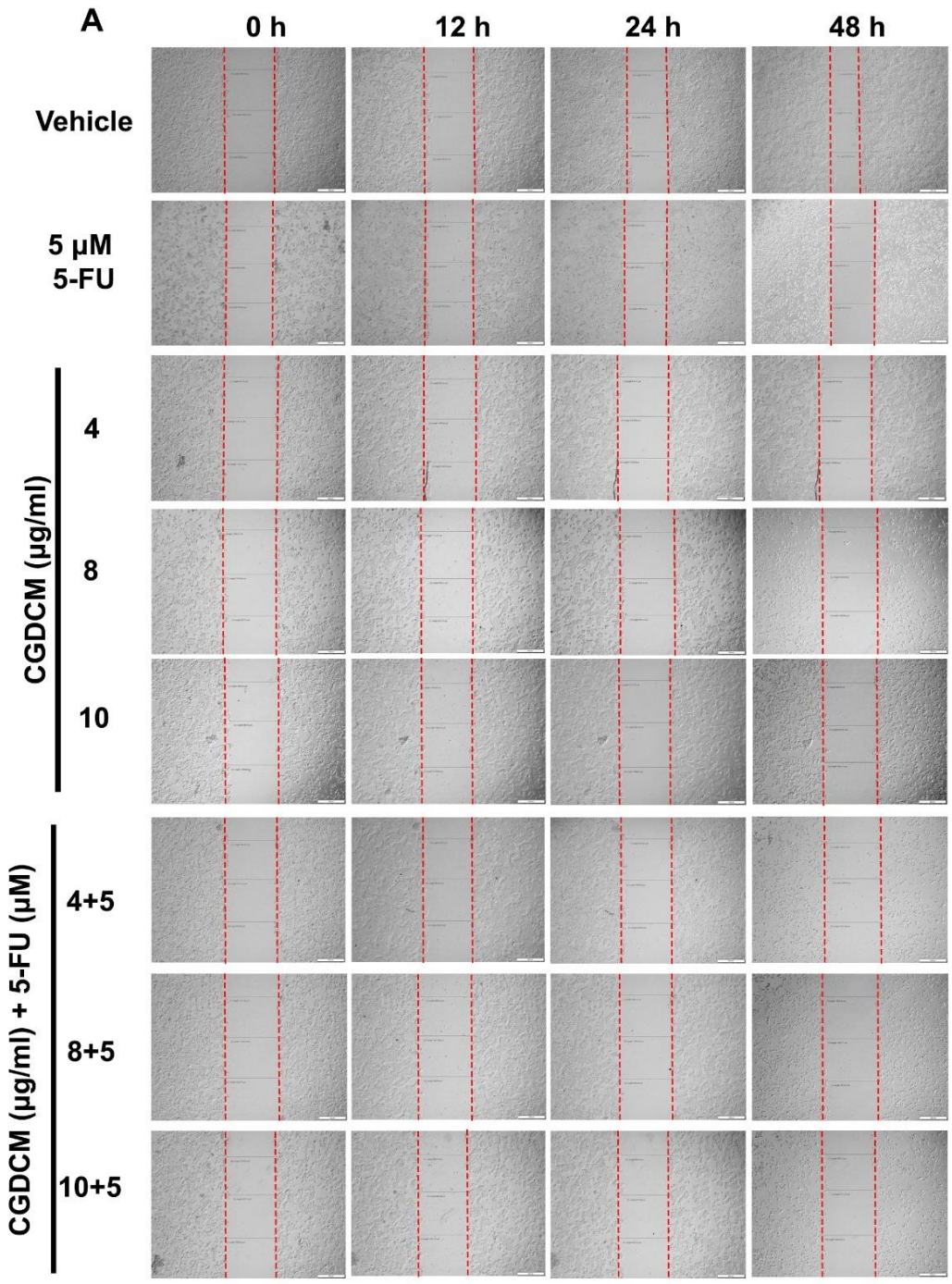


Figure 11 The cytotoxicity in HCT116 cells treated by a combination of CGDCM and CGEtOAc with 5-FU.

The viability was measured by MTT assay in HCT116 cells treated CGDCM (with/ without 5-FU) at 24 h and 48 h (A) and CGEtOAc (with/ without 5-FU) at 24 h and 48 h (B). The cytotoxicity was evaluated in normal HFF-1 cells for 24 h (C). a; $p < 0.05$ compared with the vehicle, b; $p < 0.05$ vs. 5-FU group, and c; $p < 0.05$ vs. treatment with CGDCM alone.

Anti-migration activity of combination effects the *C. gigantea* stem bark extracts and 5-FU in HCT116 cells

To evaluated reducing of cell proliferation and migration in the HCT116 cells after received CGDCM and a combination of CGDCM with 5-FU (Figure 12A and 12B). Cell migration can also be measured using the wound gap. After treatment with CGDCM (4, 8, 10 µg/mL) in combination with 5-FU (5 µM) for 12, 24, and 48 h, the gap distance exhibited unchanged that suggesting inhibitory effect on proliferation and migration of HCT116 cells line. Moreover, studied in normal HFF-1 cells showed their resistance to 4, 8, and 10 µg/ml CGDCM, 5-FU, and their combinations treatment in 24 h as shown in Figure 12C. This result suggests a selective cytotoxic efficacy of CGDCM to cancer cells.


A
0 h
12 h
24 h
48 h
Vehicle
5 μM
5-FU
CGDCM (μg/ml)
4
8
10
CGDCM (μg/ml) + 5-FU (μM)
4+5
8+5
10+5

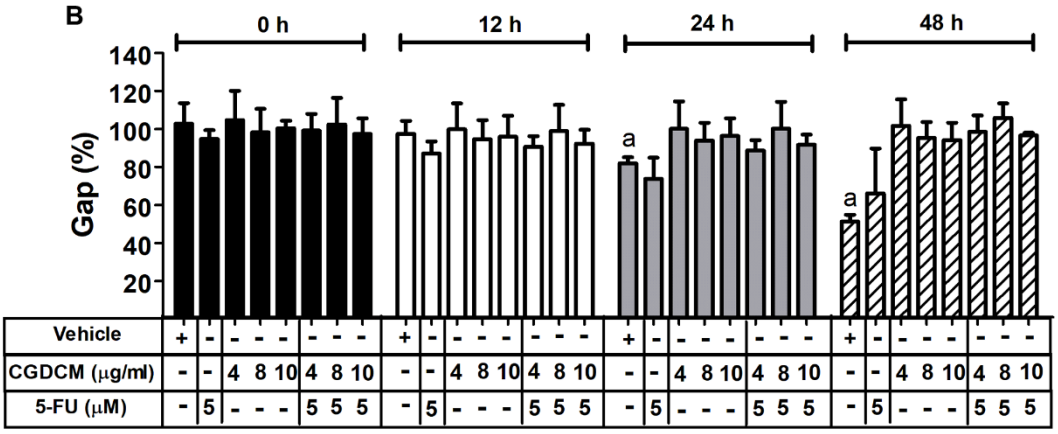


Figure 12 Anti-migration activity of combination effects the *C. gigantea* stem bark extracts and 5-FU in HCT116 cells.

Anti-migration activity of CGDCM and 5-FU on HCT116 cells were evaluated for 48 h, bars = 500 μm (A and B). a; $p < 0.05$ vs. vehicle group.

Apoptotic effect of CGDCM and 5-FU combination in HCT116 cells

To determine effect CGDCM, 5-FU, and a combination of both induced apoptosis in HCT116 cells for 24 h. In Figure 13A, B, cells were treated with 5-FU (5 µM) for 24 h did not significantly increase apoptosis at 15.49% compared with vehicle. The apoptotic cell population significantly increased from 27.18%, 30.23%, and 33.63% treated with 4, 8, and 10 µg/mL CGDCM, respectively than those treated with the vehicle. Likewise, compared with either of the drugs alone-treated cells, CGDCM (4, 8, and 10 µg/mL) together with 5-FU (5 µM) significantly enhances apoptosis induction at 30.06%, 40.35%, and 41.37%, respectively. In combination of CGDCM at dose 8 µg/mL and 10 µg/mL with 5 µM of 5-FU showed the same result of apoptotic effect. These results suggest that effects of co-treatment with CGDCM and 5-FU increased apoptotic cell death in HCT116 cells.

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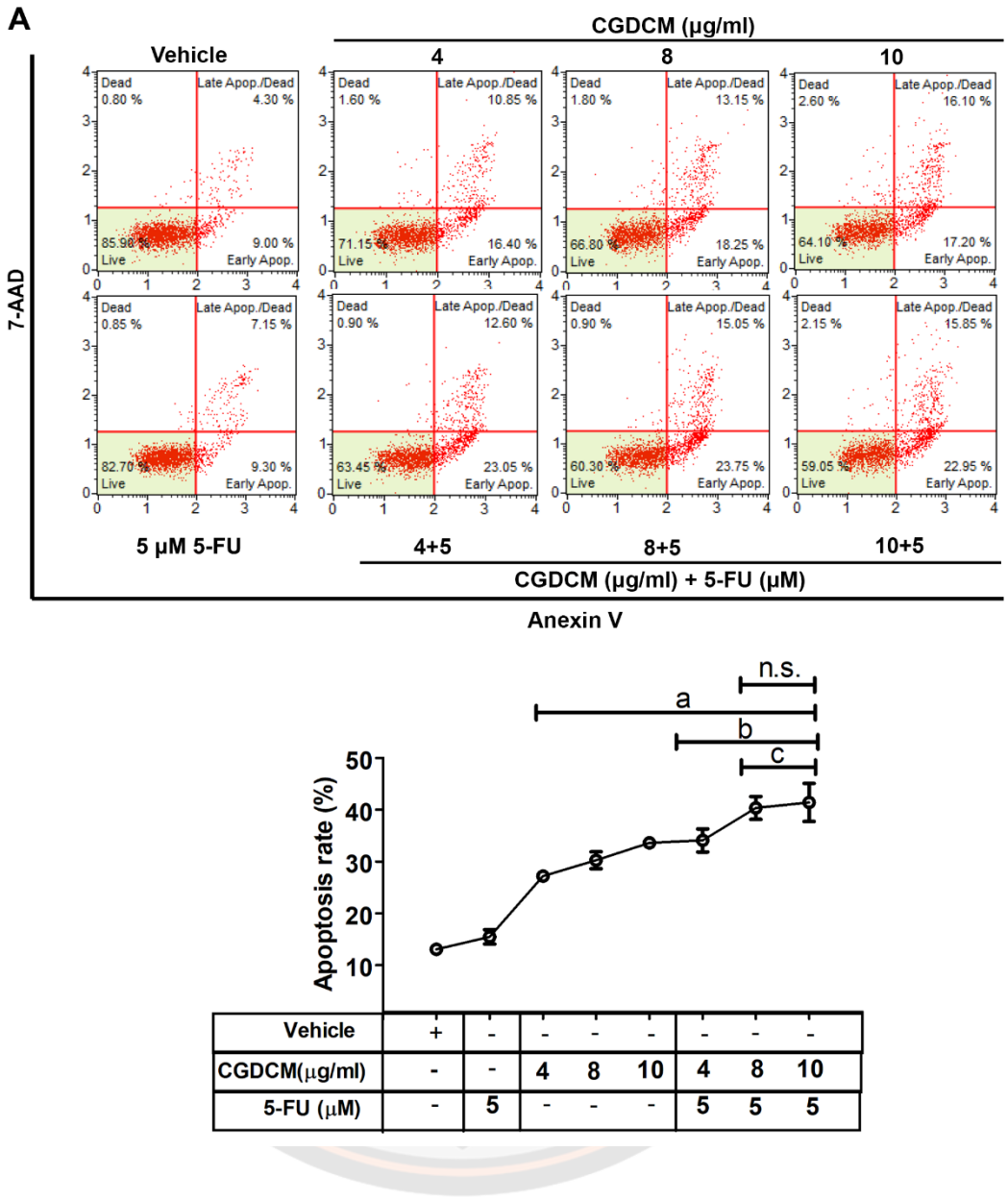


Figure 13 Apoptotic effect of CGDCM and 5-FU combination in HCT116 cells.

At 24 h, the rate of apoptosis in HCT116 cells treated with CGDCM (with/ without 5 μM 5-FU). Total apoptotic (early and late stage) cells were measured by annexin-V and 7-AAD staining (A) and calculated into percentage of the total cells (B). 0.8% DMSO is the vehicle control group. a; $p < 0.05$ vs. vehicle group. b; $p < 0.05$ vs. 5-FU group, and c; $p < 0.05$ vs. treatment with CGDCM alone.

Inhibitory effect of CGDCM and 5-FU combination therapy on mitochondrial membrane potential (ΔΨm) in HCT116 cells

Mitochondrial damage is an initiator of apoptosis in various studies report (Giampazolias et al., 2016). Induction of apoptosis through loss of ΔΨm was investigated by fluorescence JC-1 dye staining. In figure 14. After treatment with CGDCM (4, 8, and 10 µg/mL), 5-FU, and combinations of both on HCT116 cells, results show that the formation of JC-1 higher green and lower red fluorescence intensity than the vehicle cell, indicating depletion of mitochondrial function. Thus, these results demonstrated that effect of CGDCM, 5-FU, and combination of both promoted loss of ΔΨm mediated apoptosis in HCT116 cells.

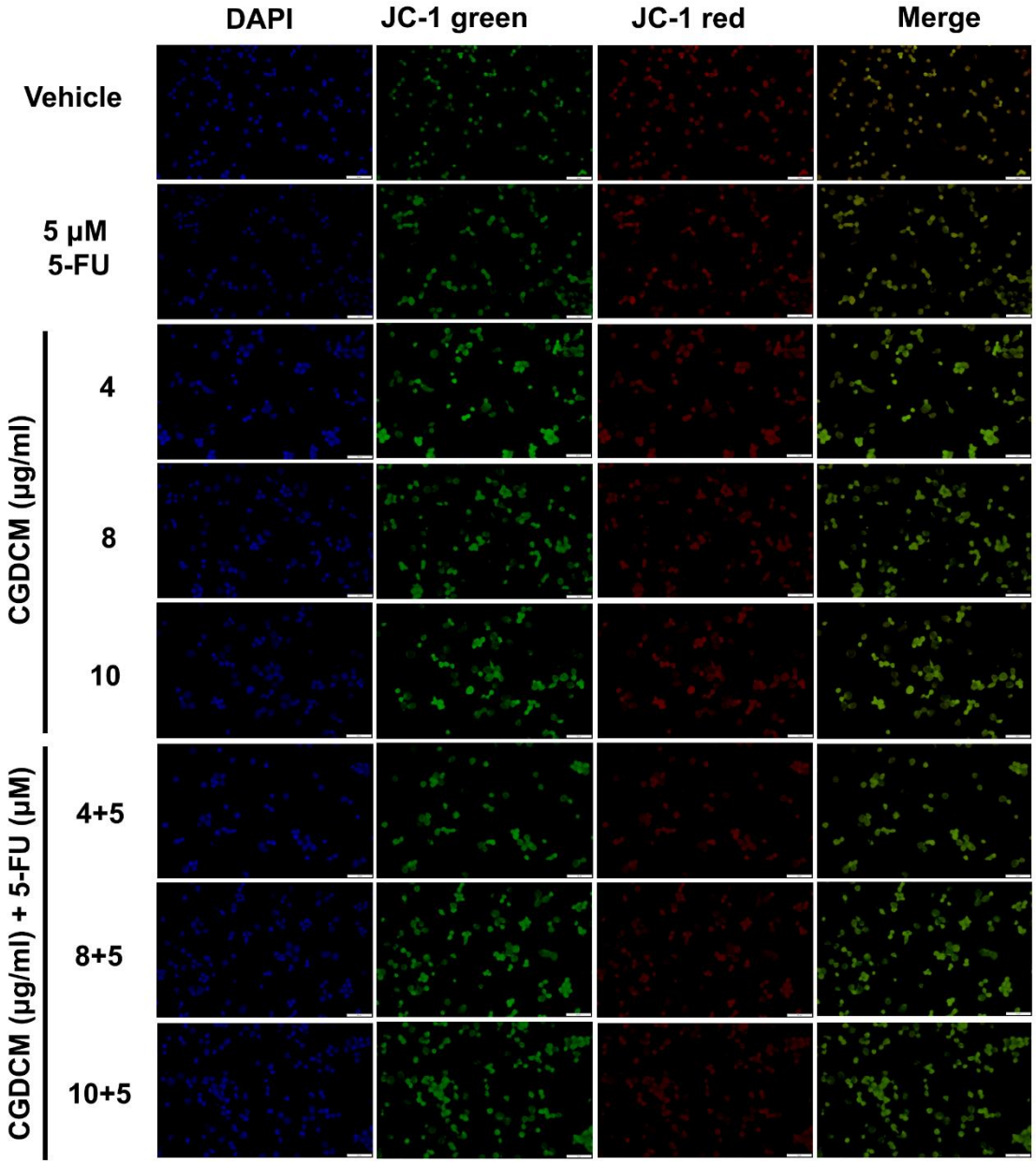


Figure 14 Inhibitory effect of CGDCM and 5-FU combination therapy on mitochondrial membrane potential (ΔΨm) in HCT116 cells.

Cells were treated with CGDCM (with/ without 5 μM 5-FU) for 24 h. JC-1 staining was visualized by fluorescence microscopy, bars = 100 μm.

The combination CGDCM and 5-FU was affected on apoptosis protein expressions in HCT116 cells

We next determined the expression of Bcl-2 and cleaved caspase 3 following CGDCM, 5-FU, and their combination treatments. The result was expressed in Figure 15A-D and showed that the expression of cleaved-caspase-3 following combination treatments was significantly increased when compared to single treatment with CGDCM and 5-FU. Bcl-2 level was significantly decreased after cells were exposed to combination treatments when compared to single treatment with 5-FU. Thus, the effect of CGDCM combined to 5-FU affirmed a combination effect on apoptosis induction that involved the upregulation of cleaved caspase 3 and downregulation of Bcl-2 protein expression.

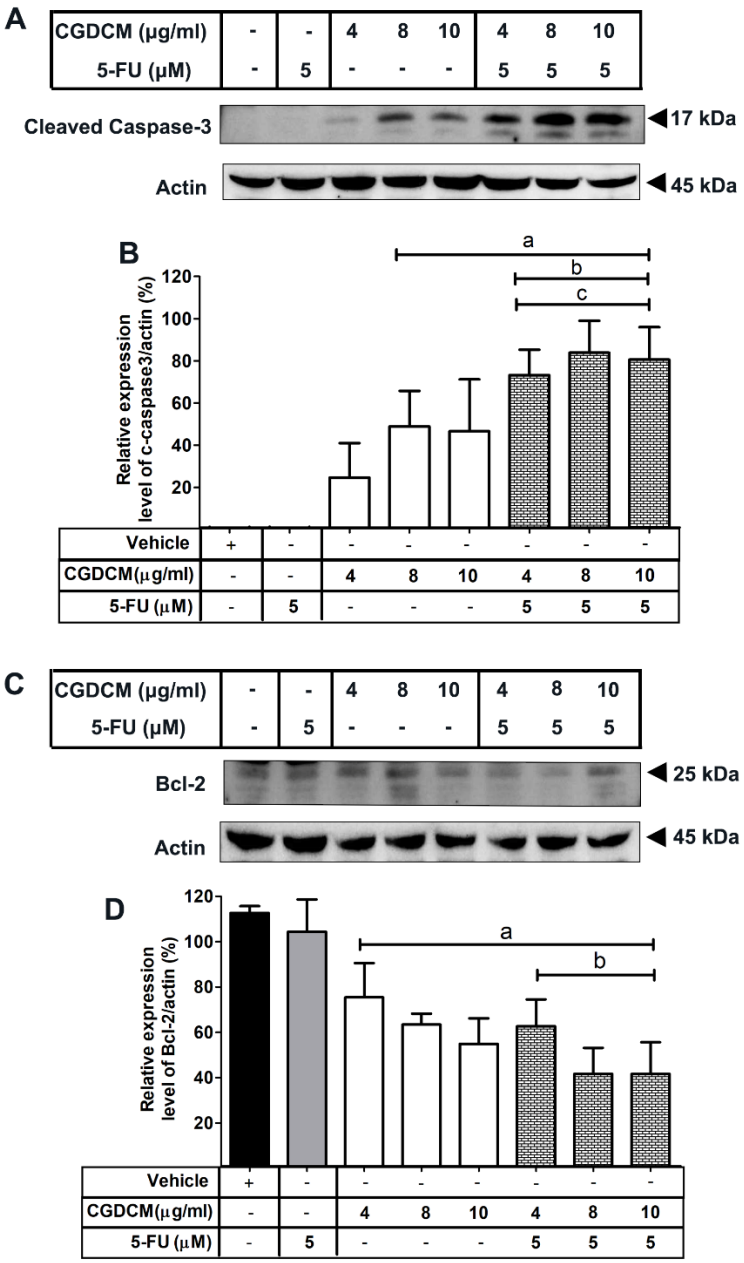


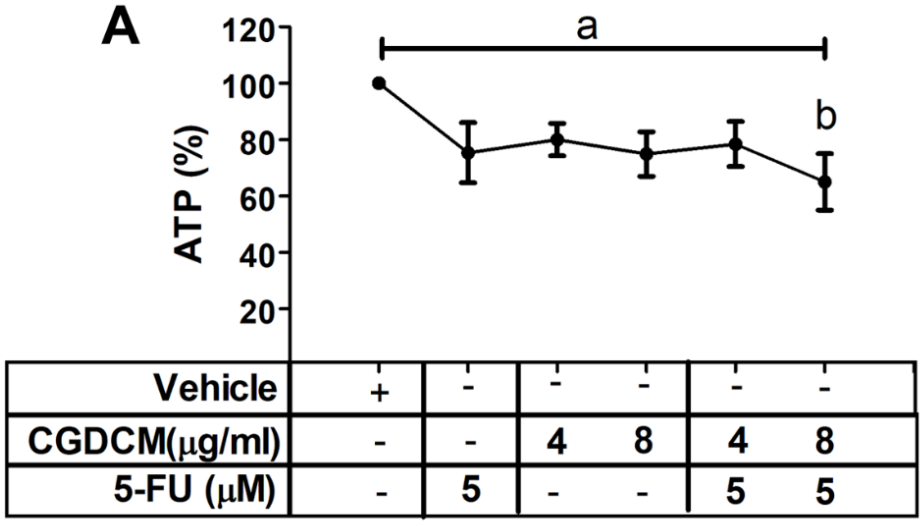
Figure 15 The combination CGDCM and 5-FU was affected on apoptosis protein expressions in HCT116 cells.

The treatment of CGDCM (with/ without 5 μM 5-FU) in HCT116 cells for 24 h analyze by protein expression levels of cleaved caspase-3(A and B), Bcl-2 (C and D) with western blotting. a; $p < 0.05$ vs. vehicle group, b; $p < 0.05$ vs. 5-FU group, and c; $p < 0.05$ vs. treatment with CGDCM alone.

The combination CGDCM and 5-FU was caused apoptosis via the regulation of ATP and enhancing of ROS in HCT116 cells

To recognize the mechanism of apoptosis cell death associated intracellular ATP production in HCT116 cell. ATP has been reported to play important role in regulating apoptosis in many cancer cells (Soebagjo et al., 2019; Tatsumi et al., 2003).Based on results of apoptotic effect, it is noted that the effect of CGDCM (4 and 8 µg/ml) and 5-FU was more potent when their combination was performed. Next, HCT116 cells exposed to CGDCM (4 and 8 µg/ml) in combination with 5-FU were further evaluated the underlying mechanism of apoptosis induction. Our data in Figure 16A indicated that the combination of CGDCM 8 µg/ml and 5-FU 5 µM after treatment for 24 h significantly diminished the cellular ATP levels in HCT116 cells.

The DNA damage can cause by an increase in the generation of ROS that leads to apoptosis in cancer cells has been reported (Srinivas et al., 2019). The present study focused the apoptotic effect of CGDCM (4 and 8 µg/ml) in combination with 5-FU was correlated to activation of ROS production. The green fluorescence intensity of ROS production in HCT116 cells increased in 24 h after treated with CGDCM at 4 and 8 µg/ml, 5 µM 5-FU, and their combinations (Figure 16B), suggesting an increase of ROS mediating apoptosis.



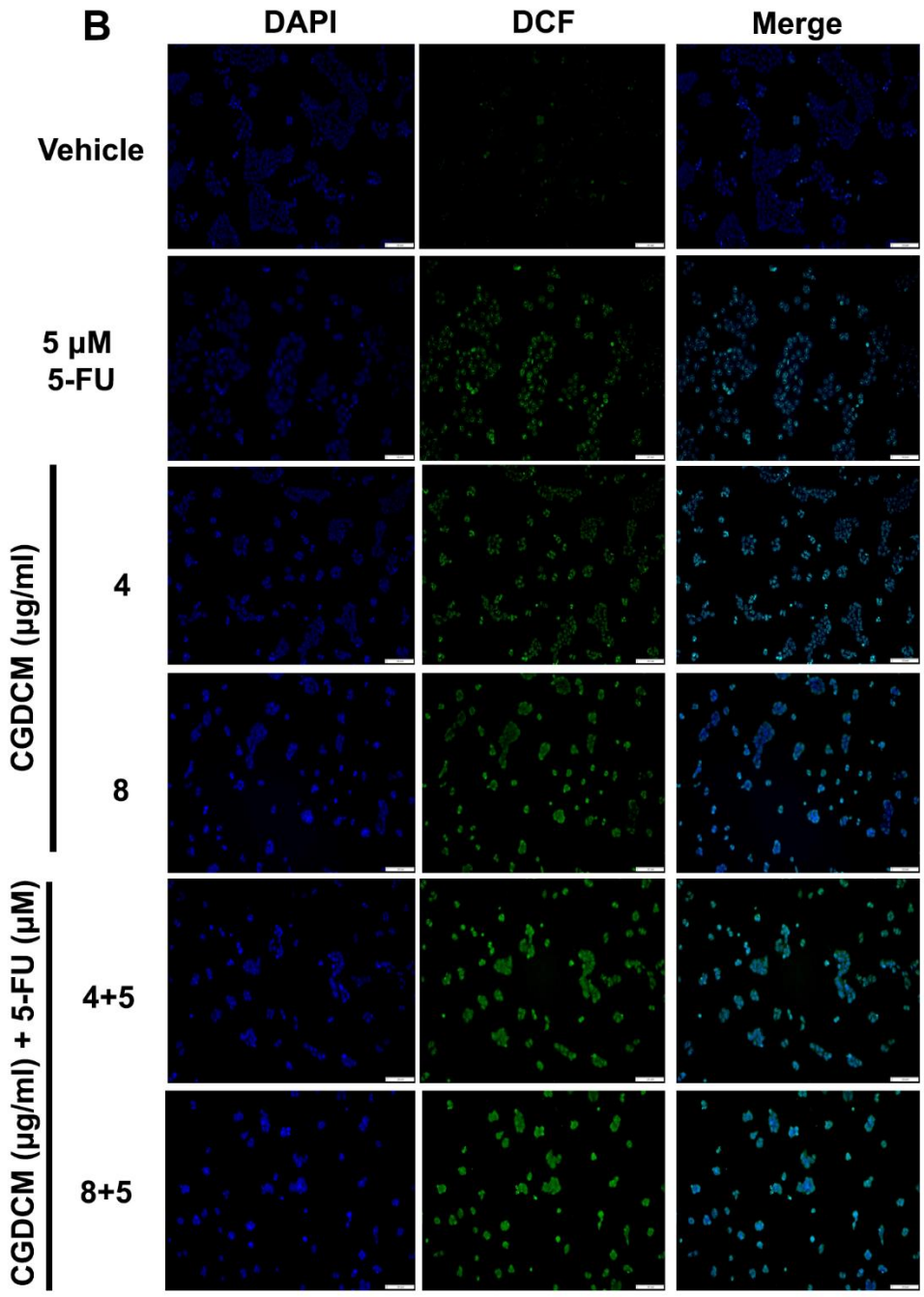


Figure 16 The combination CGDCM and 5-FU was caused apoptosis via the regulation of ATP and enhancing of ROS in HCT116 cells.

Cells were treated with 4, 8, and 10 μg/mL CGDCM (with/ without 5 μM 5-FU) for 24 h. The ATP levels (A). The ROS levels by fluorescence microscopy, bars = 50 μm (B). a; $p < 0.05$ vs. vehicle group. b; $p < 0.05$ vs. 5-FU group.

CHAPTER V
DISCUSSION AND CONCLUSION

In the present study, fractions possessing more hydrophobia (CGDCM and CGEtOAc) had more cytotoxic to HCT116 cells with IC50 values ranging from 5.91 µg/ml and 7.71 µg/ml than the hydrophilic fractions. Based on these results, CGDCM and CGEtOAc may contain active ingredients that operate high potential biological activity, especially anticancer activity. The extracts from several parts of *C. gigantea* have long been reported to treat many cancer cells. The ethanolic extracts from the whole plant of *C. gigantea* exhibited cytotoxic effect at 48 h in non-small cell lung cancer cells (Lee et al., 2019). The *C. gigantea* isolated by ethanolic from roots and leaves extracts has more cytotoxic effect on breast cancer cells than the extracts from flowers in 24 h (Mutiah et al., 2016). The leaves of *C. gigantea* extracted by ethanol has been inhibit fibrosarcoma growth via induced caspase-3 expression in mice-induce by DMBA (7,12 dimethylbenz(α) anthracene) acetone (Muti'ah et al., 2016). The cytotoxic effect from leave extraction of *C. gigantea* against colon cancer WIDR cell was observed in fractions of DCM (IC50=40.57 µg/ml), EtOAc (IC50=41.79 µg/ml), and EtOH (IC50=48.5 µg/ml) (Muhammad Rowshanul Habib et al., 2010; Mutiah et al., 2017).

It has been reported that secondary metabolites in the extracts are considered to exhibit therapeutic efficacies against cancer cells. The flavonoid and terpenoid compounds found in 70% ethanolic extract, dichloromethane, and ethyl acetate fractions from leaves of *C. gigantea* cause cytotoxic in human colon cancer WiDr cell lines (Mutiah et al., 2017). The tumor cells in Ehrlich ascites carcinoma (EAC) tumor bearing mice was decreased by the methanolic and chloroform extracts from root bark and ethyl acetate extracts from the flower of *C. gigantea* (M Rowshahul Habib, 2011). The evidence of several cell lines established that efficient cytotoxic effect against cancer cells of the extracts from *C. gigantea* is based on the presence of cardiac glycosides, triterpenoids, and phenolic compounds.

Cardenolides extract from *C. gigantea* possessed the anti-cancer activity on triple-negative breast cancers (TNBC) (Pederson et al., 2020), and from the root bark has been reported in A549 and Hela cancer cells (You et al., 2013). The potential

cytotoxicity against human breast cancer cells of cardenolides from *C. gigantea* was considered through inhibiting hypoxia-inducible factor-1 transcriptional activity (Parhira et al., 2016). The isolated from latex and fruit contain with uscharin, 15β-hydroxyuscharin, 19deoxy-15β-hydroxyuscharin, 2″-oxovoruscharin, calactin, calotropin, gomphoside, and asclepin exhibited cytotoxic effect through inhibiting HIF-1 on human breast cancer MCF-7 cells (Parhira et al., 2016). The methanolic extract (CGME) from leaves and stems of *C. gigantea* has been reported that have cardenolide including calactin, calotropagenin, usharin, afroside, calatoxin, gamphoside and two unknown cardenolide that inhibited cell growth in breast cancer MCF-7 cells (Kharat et al., 2019). Calotropin (CTP) one of cardenolide isolated of *C. gigantea* induced apoptotic cell death by inhibiting Wnt signaling pathway in SW480 cells (H. Y. Park et al., 2014). Coroglaucigenin extracted from root of *C. gigantea* induced senescence by downregulation of CDK4 and dephosphorylation of Akt to induce autophagy in colorectal cancer cells (Y. H. Huang et al., 2018). The calotropin, calactin, and coroglaucigenin (CGN) that is cardenolides were isolated from ethyl acetate fraction of the stems and leave of *C. gigantea*, effectively exhibited cytotoxic effect in human lung cancer cells (Sun et al., 2017). Meanwhile, Calotropin (CTP), a cardenolide isolated from *C. gigantea* at dose 0.1, 0.2 μM for 24 h inhibited cell proliferation in HCT116 and HT-29 cells (Zhou et al., 2019). The isolated cardenolides from the root bark of C. gigantea from Vietnam consist of (1–3), 3'-*O*-methylcalotropin, 3'-*O*-acetylfrugoside, and 3'-*O* benzoylfrugoside have cytotoxic activity against HeLa and A549 cell (Van Khang et al., 2014). Isolated cardenolide from root bark extracts of *C. gigantea* have the C-10 formyl and hydroxymeyhyl group including the double linked six-membered ring sugar unit that enhances the anti-proliferative activity of this compounds in A549 human lung carcinoma and Hela cervical cancer cells (You et al., 2013).

Besides, triterpenoid has been reported as highly potential cytotoxicity on cancer cells. The triterpenoid glycoside isolated from ethyl acetate of the root of *C. gigantea* is Calotroposid A that inhibited WiDr colon cancer cells growth (Mutiah et al., 2018). It has been found that anhydrosophoradial-3-acetate (A3A), one of triterpenoids isolated from ethyl acetate of the flower of *C. gigantea* at dose 10, 20 mg/kg BW decreased viability of Ehrlich ascites carcinoma bearing mice

(Muhammad R Habib et al., 2013). Thus, accumulation of these given backgrounds, it is speculated that cardenolides and triterpenoids might be responsible for the cytotoxicity of the *C. gigantea* stem bark extracts in our study.

Although the present study determined that a single treatment with CGDCM from *C. gigantea* stem bark extract showed potent cytotoxicity on HCT116 cells, a combination of low concentration of both CGDCM and 5-FU intriguingly caused a stronger apoptotic effect than single treatment. More importantly, this treatment also showed the selectivity of the potential effect to cancer cells that are sensitive at doses that have less toxic to normal fibroblast cells. Administration of CGDCM caused anti-cancer effect of 5-FU at a low concentration being more pronounce. 5-FU concentration that less than 10 µM. has been found to exhibit synergistic effect and decreased resistance of CRC (Attia et al., 2018). However, a better the treatment gains successfulness, a higher dose of drugs must be used for cancer cells. Cancer treatment with 5-FU at high dose and prolong duration of application face resistance to the treatment and systemic toxic side effects (Tecza et al., 2018; N. Zhang et al., 2008). Treatment of reducing dose of 5-FU combined with compounds extracted from plants that possess anti-cancer properties has been showed to produce a higher cell viability suppression effect than single treatment or high dose of 5-FU usage (López et al., 2019), and the combination with plant extract abolished drug resistance to 5-FU (Blondy et al., 2020). Thus, the efficient therapeutic approach to overcome drug resistance and minimize undesired side effects by using the combination therapy with a lower dose of drugs to a single treatment to enhanced superior anti-cancer effect.

We also reported that the mechanism of the selective cytotoxic effect of CGDCM and the combination with 5-FU in HCT116 cells targeted downregulation of ATP and upregulation of ROS. The accumulation of ROS is one of cellular stress markers that initiates cell death apoptosis in cancer cells (M. Zhang et al., 2015). It has been reported that 95% ethanolic extract from the whole plant of *C. gigantea* showed apoptotic induction through enhancing of ROS production following suppression of ROS scavenger SOD2 and catalase in human non-small cell lung cancer A549 and NCI-H1299 cells (Lee et al., 2019). The Coroglaucigenin (CGN) isolated from ethyl acetate fraction of the stems and leaves from *C. gigantea* induced apoptosis in human lung cancer cells via inhibiting antioxidant molecules (NQO-1,

HO-1) that led to inducing generation of ROS (Sun et al., 2017). In addition to *C. gigantea*, bioactive compounds, flavonoid, triterpenoid, and phenolic found in whole fruit without seeds of bitter melon extract (BME) suppressed the *de novo* lipogenesis pathway and accumulation of ROS leading to apoptosis in Cal27 and JHU022 oral cancer cells (Sur et al., 2019). Galangin, a flavanol extracted from the *Alpinia galangal* root induced apoptosis by upregulating NADPH activity which led to enhanced ROS production in MCF-7 and T47D human breast cancer cell (Song et al., 2017). Curcumin (diferuloylmethane), a polyphenol isolated from the zingiberaceae *Curcuma longa* enhanced ROS production and malondialdehyde (MDA) level, a marker of lipid peroxidation, which resulted in inducing apoptosis in tumor cell *in vitro* and *in vivo* model (Bianchi et al., 2018).

The mechanisms of ROS mediating apoptosis in cancer cells have been reported to involve mitochondrial ATP production following the treatment of flavanol aglycone is isorhamnetin (3′-methoxy-3,4′,5,7-tetrahydroxyflavone) isolated from fruit and leaves of various plants such as *Hippophae rhamnoides* L., *Oenanthe javanica*, and *Ginkgo biloba* L.in human bladder cancer cells (C. Park et al., 2019). As the main source of energy for cellular processes, a reduction of ATP generation induces apoptosis in several cancer cells. However, the mechanism of ATP activating apoptosis by *C. gigantea* extracts in cancer cells has not yet been identified. A bioactive flavonoid is Wogonoside (wogonin-7-glucuronide), isolated from the root of *Scutellaria baicalensis Georgi* treated on A549 human non-small cell lung cancer cells for 48 h induced apoptosis via promoting mitochondrial dysfunction related to decreasing ATP level that consequently activated AMPK/mTOR signaling (Luo et al., 2018). Decreasing ATP levels by isorhamnetin (3′-methoxy-3,4′,5,7-tetrahydroxyflavone), a flavonol aglycone showed cytotoxic apoptotic induction effect through mTOR/p70S6K/ACC1 signaling pathway in human bladder cancer cells (C. Park et al., 2019).

The number of reports has determined that activating endoplasmic reticulum (ER) stress is responsible for the potential cytotoxicity of secondary metabolites from *C. gigantea* and other herbal plants, which include cardiac glycosides, triterpenoids, and phenolic compounds. The cardiac glycoside is Oleandrin isolated from the leaves of *Nerium oleander* enhanced apoptosis via activating ER stress by enhancing

phospho-PERK phosphorylating sequential target proteins including eIF2α and ATF4, finally upregulating CHOP protein in breast cancer MCF7 and MDA-MB-231 cells (X.-x. Li et al., 2020). Likewise, *C. gigantea* extract showed riches of bioactive compounds similarly found in bitter melon extract (BME) from whole fruit without seeds that induced apoptosis by activated ER stress in Cal27 and JHU022 oral cancer cells (Sur et al., 2019). In addition to ER stress, apoptotic induction effect of active biological compounds in *C. gigantea* also correlates downregulation of fatty acid pathway in cancer cells. Kahweol, a coffee-specific diterpene exhibited apoptotic induction in HER2 overexpressing cancer cells through expression of PI3K/Akt/mTOR/SREBP1 signaling pathway that regulates fatty acid synthesis (Oh et al., 2018). Downregulation of fatty acid synthesis triggered apoptosis by the regulation of AKT/mTOR/SREBP-1c signaling pathway in many cancer cells (H. Li et al., 2018; Oh et al., 2018; Shi et al., 2020; Tao et al., 2019).

5-FU has been used for the chemotherapy drug for a long time worldwide including colorectal, breast, and head and neck cancer (Longley et al., 2003). The active metabolites of 5-FU included fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP) function to inhibit thymidylate synthase (TS) that results in disrupting the synthesis of DNA/RNA for the antitumor activity (Miura et al., 2010). However, the limitation of the therapeutic outcome is the systemic side effects and drug resistance (Tecza et al., 2018). In a previous study, the resistance of esophageal cancer cells to 5-FU treatment corresponded with an upregulation of PI3K/AKT pathway (B. Li et al., 2014). The evidence of several cell lines demonstrated the potential effect of plants compounds that can augment the sensitivity of cancer cells to 5-FU. A previous study showed that in Caco2 colon cancer cells treated with 5-FU singly was caused apoptosis and cell cycle arrest. A combination with verbascoside, a phynylethanoid glycoside improved the sensitization of cancer cells to 5-FU treatment (Attia et al., 2018). In addition, the combination of 5-FU with rutin, a glycoside from quercetin flavonoid showed a synergistic apoptotic induction effect on PC3 prostate cancer cells (Satari et al., 2019). The combination of Casticin which a member of polymethexyflavone from Vitex rotundifolia L. and Vitex species of the family Verbenaceae with 5-FU on WEHI-3 mouse leukemia cells induced apoptosis (Cheng et al., 2020).

In conclusion, the present study represented that a co-treatment of 5-FU and the dichloromethane fraction of the *C. gigantea* stem bark extract showed enhanced potency to induced apoptotic activity in HCT116 cells and non-toxicity in normal human fibroblast cells. Therefore, this extract is an encouraging alternative anti-cancer drug that may be used in combination with base chemotherapeutics in the treatment of colon cancer.

REFERENCES

Akhtar, R., Chandel, S., Sarotra, P., & Medhi, B. (2014). Current status of pharmacological treatment of colorectal cancer. *World journal of gastrointestinal oncology*, *6*(6), 177.

Attia, Y. M., El-Kersh, D. M., Wagdy, H. A., & Elmazar, M. M. (2018). Verbascoside: Identification, quantification, and potential sensitization of colorectal cancer cells to 5-FU by targeting PI3K/AKT pathway. *Scientific reports*, *8*(1), 1-12.

Bianchi, G., Ravera, S., Traverso, C., Amaro, A., Piaggio, F., Emionite, L., . . . Raffaghello, L. (2018). Curcumin induces a fatal energetic impairment in tumor cells in vitro and in vivo by inhibiting ATP-synthase activity. *Carcinogenesis*, *39*(9), 1141-1150.

Blondy, S., David, V., Verdier, M., Mathonnet, M., Perraud, A., & Christou, N. (2020). 5-Fluorouracil resistance mechanisms in colorectal cancer: From classical pathways to promising processes. *Cancer Science*, *111*(9), 3142.

Boland, P. M., & Ma, W. W. (2017). Immunotherapy for colorectal cancer. *Cancers*, *9*(5), 50.

Braun, M. S., & Seymour, M. T. (2011). Balancing the efficacy and toxicity of chemotherapy in colorectal cancer. *Therapeutic advances in medical oncology*, *3*(1), 43-52.

Cerella, C., Muller, F., Gaigneaux, A., Radogna, F., Viry, E., Chateauvieux, S., . . . Diederich, M. (2015). Early downregulation of Mcl-1 regulates apoptosis triggered by cardiac glycoside UNBS1450. *Cell death & disease*, *6*(6), e1782-e1782.

Chen, S.-J., Chung, Y.-C., Chang, H.-L., Chang, H.-P., Chou, J.-L., Lin, C.-C., . . . Hsu, C.-P. (2020). Synergistic effect of combined treatment with Longan flower extract and 5-fluorouracil on colorectal Cancer cells. *Nutrition and cancer*, *72*(2), 209-217.

Cheng, Z. Y., Chueh, F. S., Peng, S. F., Lin, C. H., Kuo, C. L., Huang, W. W., . . . Chung, J. G. (2020). Combinational treatment of 5-fluorouracil and casticin induces apoptosis in mouse leukemia WEHI-3 cells in vitro. *Environmental toxicology*, *35*(9), 911-921.

Chitme, H. R., Chandra, R., & Kaushik, S. (2004). Studies on anti-diarrhoeal activity of *Calotropis gigantea* R. Br. in experimental animals. *J Pharm Pharm Sci*, *7*(1), 70-75.

Deshmukh, P. T., Fernandes, J., Atul, A., & Toppo, E. (2009). Wound healing activity of *Calotropis gigantea* root bark in rats. *Journal of ethnopharmacology*, *125*(1), 178-181.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology*, *35*(4), 495-516.

Focaccetti, C., Bruno, A., Magnani, E., Bartolini, D., Principi, E., Dallaglio, K., . . . Noonan, D. M. (2015). Effects of 5-fluorouracil on morphology, cell cycle, proliferation, apoptosis, autophagy and ROS production in endothelial cells and cardiomyocytes. *PloS one*, *10*(2), e0115686.

Fozzard, H. A., & Sheets, M. F. (1985). Cellular mechanism of action of cardiac glycosides. *Journal of the American College of Cardiology*, *5*(5S1), 10A-15A.

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., . . . Andrews, D. W. (2018). Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death & Differentiation, 25*(3), 486-541.

Giampazolias, E., & Tait, S. W. (2016). Mitochondria and the hallmarks of cancer. *The FEBS journal, 283*(5), 803-814.

Grem, J. L. (2000). 5-Fluorouracil: forty-plus and still ticking. A review of its preclinical and clinical development. *Investigational new drugs, 18*(4), 299-313.

Habib, M. R. (2011). Evaluation of antitumour activity of *Calotropis gigantea* L. root bark against Ehrlich ascites carcinoma in Swiss albino mice. *Asian Pacific journal of tropical medicine, 4*(10), 786-790.

Habib, M. R., Aziz, M. A., & Karim, M. R. (2010). Inhibition of Ehrlich's ascites carcinoma by ethyl acetate extract from the flower of *Calotropis gigantea* L. in mice. *Journal of Applied Biomedicine, 8*(1), 47-54.

Habib, M. R., & Karim, M. R. (2013). Effect of anhydrosophoradiol-3-acetate of *Calotropis gigantea* (Linn.) flower as antitumoric agent against Ehrlich's ascites carcinoma in mice. *Pharmacological Reports, 65*(3), 761-767.

Habib, M. R., Nikkon, F., Rahman, M., Haque, M. E., & Karim, M. R. (2007). Isolation of stigmasterol and beta-sitosterol from methanolic extract of root bark of *Calotropis gigantea* (Linn). *Pakistan journal of biological sciences: PJBS, 10*(22), 4174-4176.

Hasballah, K., Sarong, M., Rusly, R., Fitria, H., Maida, D. R., & Iqhrammullah, M. (2021). Antiproliferative Activity of Triterpenoid and Steroid Compounds from Ethyl Acetate Extract of *Calotropis gigantea* Root Bark against P388 Murine Leukemia Cell Lines. *Scientia Pharmaceutica, 89*(2), 21.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Huang, C.-M., Huang, M.-Y., Ma, C.-J., Yeh, Y. S., Tsai, H.-L., Huang, C.-W., . . . Wang, J.-Y. (2017). Neoadjuvant FOLFOX chemotherapy combined with radiotherapy followed by radical resection in patients with locally advanced colon cancer. *Radiation Oncology, 12*(1), 1-10.

Huang, Y. H., Lei, J., Yi, G. H., Huang, F. Y., Li, Y. N., Wang, C. C., . . . Tan, G. H. (2018). Coroglaucigenin induces senescence and autophagy in colorectal cancer cells. *Cell proliferation, 51*(4), e12451.

Juncker, T., Cerella, C., Teiten, M.-H., Morceau, F., Schumacher, M., Ghelfi, J., . . . Dicato, M. (2011). UNBS1450, a steroid cardiac glycoside inducing apoptotic cell death in human leukemia cells. *Biochemical pharmacology, 81*(1), 13-23.

Kadiyala, M., Ponnusankar, S., & Elango, K. (2013). *Calotropis gigantiea* (L.) R. Br (Apocynaceae): a phytochemical and pharmacological review. *Journal of ethnopharmacology, 150*(1), 32-50.

Kataoka, T. (2009). Chemical biology of inflammatory cytokine signaling. *The Journal of antibiotics, 62*(12), 655-667.

Khan, M., Maryam, A., Qazi, J. I., & Ma, T. (2015). Targeting apoptosis and multiple signaling pathways with icariside II in cancer cells. *International journal of biological sciences, 11*(9), 1100.

Kharat, K. R., & Kharat, A. S. (2019). The *Calotropis Gigantea* Methanolic Extract Induces Apoptosis in Human Breast Carcinoma Cells. *Iranian journal of medical sciences*, *44*(6), 483.

Kitagawa, I., ZHANG, R.-s., Park, J. D., Baek, N. I., Takeda, Y., YOSHIKAWA, M., & SHIBUYA, H. (1992). Indonesian medicinal plants. I. Chemical structures of calotroposides A and B, two new oxypregnane-oligoglycosides from the root of *Calotropis gigantea* (Asclepiadaceae). *Chemical and Pharmaceutical Bulletin*, *40*(8), 2007-2013.

Kiuchi, F., Fukao, Y., Maruyama, T., Obata, T., Tanaka, M., Sasaki, T., . . . Tsuda, Y. (1998). Cytotoxic principles of a Bangladeshi crude drug, akond mul (roots of *Calotropis gigantea* L.). *Chemical and Pharmaceutical Bulletin*, *46*(3), 528-530.

Kumar, G., Karthik, L., & Rao, K. V. B. (2011). A review on pharmacological and phytochemical profile of *Calotropis gigantea* Linn. *Pharmacologyonline*, *1*, 1-8.

Kye, B.-H., & Cho, H.-M. (2014). Overview of radiation therapy for treating rectal cancer. *Annals of coloproctology*, *30*(4), 165.

Lee, J., Jang, H.-J., Chun, H., Pham, T.-H., Bak, Y., Shin, J.-W., . . . Oh, S. R. (2019). *Calotropis gigantea* extract induces apoptosis through extrinsic/intrinsic pathways and reactive oxygen species generation in A549 and NCI-H1299 non-small cell lung cancer cells. *BMC complementary and alternative medicine*, *19*(1), 1-14.

Lee, J., Jang, H., Bak, Y., Shin, J.-W., Jin, H., Kim, Y.-I., . . . Yoon, D.-Y. (2018). *Calotropis gigantea* extract induces apoptosis through extrinsic/intrinsic pathways and upregulation of reactive oxygen species in non-small-cell lung cancer cells. *bioRxiv*, 441162.

Lhinhatrakool, T., & Sutthivaiyakit, S. (2006). 19-Nor-and 18, 20-epoxy-cardenolides from the leaves of *Calotropis gigantea*. *Journal of natural products*, *69*(8), 1249-1251.

Li, B., Li, J., Xu, W. W., Guan, X. Y., Qin, Y. R., Zhang, L. Y., . . . Cheung, A. L. (2014). Suppression of esophageal tumor growth and chemoresistance by directly targeting the PI3K/AKT pathway. *Oncotarget*, *5*(22), 11576.

Li, H., Xiang, L., Yang, N., Cao, F., Li, C., Chen, P., . . . Wang, X. (2018). Zhiheshouwu ethanol extract induces intrinsic apoptosis and reduces unsaturated fatty acids via SREBP1 pathway in hepatocellular carcinoma cells. *Food and Chemical Toxicology*, *119*, 169-175.

Li, X.-x., Wang, D.-q., Sui, C.-g., Meng, F.-d., Sun, S.-l., Zheng, J., & Jiang, Y.-h. (2020). Oleandrin induces apoptosis via activating endoplasmic reticulum stress in breast cancer cells. *Biomedicine & Pharmacotherapy*, *124*, 109852.

Lin, J., Zhan, T., Duffy, D., Hoffman-Censits, J., Kilpatrick, D., Trabulsi, E. J., . . . Kennedy, B. (2014). A pilot phase II Study of digoxin in patients with recurrent prostate cancer as evident by a rising PSA. *American journal of cancer therapy and pharmacology*, *2*(1), 21.

Lodhi, G., KUMAR SINGH, H., Pant, K. K., & Hussain, Z. (2009). Hepatoprotective effects of *Calotropis gigantea* extract against carbon tetrachloride induced liver injury in rats. *Acta Pharmaceutica*, *59*(1), 89-96.

Longley, D. B., Harkin, D. P., & Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. *Nature reviews cancer, 3*(5), 330-338.

López, E. P.-F., García, F. G., & Jornet, P. L. (2019). Combination of 5-Florouracil and polyphenol EGCG exerts suppressive effects on oral cancer cells exposed to radiation. *Archives of oral biology, 101*, 8-12.

Luo, M., Mo, J., Yu, Q., Zhou, S., Ning, R., Zhang, Y., . . . Cui, J. (2018). Wogonoside induces apoptosis in human non-small cell lung cancer A549 cells by promoting mitochondria dysfunction. *Biomedicine & Pharmacotherapy, 106*, 593-598.

Mahar, R., Dixit, S., Joshi, T., Kanojiya, S., Mishra, D. K., Konwar, R., & Shukla, S. K. (2016). Bioactivity guided isolation of oxypregnane-oligoglycosides (calotroposides) from the root bark of *Calotropis gigantea* as potent anticancer agents. *RSC advances, 6*(106), 104215-104226.

Mijatovic, T., Lefranc, F., Van Quaquebeke, E., Van Vynckt, F., Darro, F., & Kiss, R. (2007). UNBS1450: A new hemi-synthetic cardenolide with promising anti-cancer activity. *Drug Development Research, 68*(4), 164-173.

Miura, K., Kinouchi, M., Ishida, K., Fujibuchi, W., Naitoh, T., Ogawa, H., . . . Haneda, S. (2010). 5-fu metabolism in cancer and orally-administrable 5-fu drugs. *Cancers, 2*(3), 1717-1730.

Murti, P. B. R., & Seshadri, T. (1943). *Chemical composition of Calotropis gigantea.* Paper presented at the Proceedings of the Indian Academy of Sciences-Section A.

Muti'ah, R., Griana, T. P., Ula, Q. N., & Andhyarto, Y. (2016). The Effect of *Calotropis gigantea* Leaves Extract on Fibrosarcoma Growth and Caspase 3 Expression. *International Journal of Pharmaceutical and Clinical Research, 8*(03), 167-171.

Mutiah, R., Sukardiman, S., & Widyawaruyanti, A. (2017). Cytotoxic effect of crude extract and fraction from *Calotropis gigantea* leaves on human colon cancer widr cell lines. *International Journal of Pharmacy and Pharmaceutical Sciences, 9*(1), 83-86.

Mutiah, R., Sukardiman, S., Widyawaruyanti, A., & Zulaikah, S. (2016). Comparison of Ethanol Extract from Roots, Leaves, and Flowers of *Calotropis gigantea* as Anticancer on T47D Breast Cancer Cell Lines. *Alchemy, 5*(1), 1-4.

Mutiah, R., Widyawaruyanti, A., & Sukardiman, S. (2018). Calotroposid a: a glycosides Terpenoids from *Calotropis gigantea* induces apoptosis of Colon Cancer WiDr cells through cell cycle arrest G2/M and caspase 8 expression. *Asian Pacific journal of cancer prevention: APJCP, 19*(6), 1457.

Ng, P. L., Rajab, N. F., Then, S. M., Yusof, Y. A. M., Ngah, W. Z. W., Pin, K. Y., & Looi, M. L. (2014). Piper betle leaf extract enhances the cytotoxicity effect of 5-fluorouracil in inhibiting the growth of HT29 and HCT116 colon cancer cells. *Journal of Zhejiang University-SCIENCE B, 15*(8), 692-700.

Oh, S. H., Hwang, Y. P., Choi, J. H., Jin, S. W., Lee, G. H., Han, E. H., . . . Jeong, H. G. (2018). Kahweol inhibits proliferation and induces apoptosis by suppressing fatty acid synthase in HER2-overexpressing cancer cells. *Food and Chemical Toxicology, 121*, 326-335.

Omer, I., Abdurrahman, I., & Cai-Xia, Y. (2017). New triterpenoid from the roots of *Calotropis gigantea* (L) Dryand (Asclepiadaceae). *Am. J. Org. Chem, 7*, 13-18.

Organization, W. H. (2020). WHO report on cancer: setting priorities, investing wisely and providing care for all.

Öven Ustaalioğlu, B. B., Bilici, A., Tilki, M., Sürmelioğlu, A., Erkol, B., Figen, M., & Uyar, S. (2018). Capecitabine-cisplatin versus 5-fluorouracil/leucovorin in combination with radiotherapy for adjuvant therapy of lymph node positive locally advanced gastric cancer.

Parhira, S., Zhu, G.-Y., Chen, M., Bai, L.-P., & Jiang, Z.-H. (2016). Cardenolides from *Calotropis gigantea* as potent inhibitors of hypoxia-inducible factor-1 transcriptional activity. *Journal of ethnopharmacology, 194*, 930-936.

Parhira, S., Zhu, G.-Y., Jiang, R.-W., Liu, L., Bai, L.-P., & Jiang, Z.-H. (2014). 2′-Epi-uscharin from the latex of *Calotropis gigantea* with HIF-1 inhibitory activity. *Scientific reports, 4*(1), 1-7.

Pari, K., Rao, P., Devakumar, C., & Rastogi, J. (1998). A novel insect antifeedant nonprotein amino acid from *Calotropis gigantea*. *Journal of natural products, 61*(1), 102-104.

Park, C., Cha, H.-J., Choi, E. O., Lee, H., Hwang-Bo, H., Ji, S. Y., . . . Cheong, J. (2019). Isorhamnetin induces cell cycle arrest and apoptosis via reactive oxygen species-mediated AMP-activated protein kinase signaling pathway activation in human bladder cancer cells. *Cancers, 11*(10), 1494.

Park, H. Y., Toume, K., Arai, M. A., Sadhu, S. K., Ahmed, F., & Ishibashi, M. (2014). Calotropin: a cardenolide from *Calotropis gigantea* that inhibits Wnt signaling by increasing casein kinase 1α in colon cancer cells. *Chembiochem, 15*(6), 872-878.

Pederson, P. J., Cai, S., Carver, C., Powell, D. R., Risinger, A. L., Grkovic, T., . . . Cichewicz, R. H. (2020). Triple-Negative Breast Cancer Cells Exhibit Differential Sensitivity to Cardenolides from *Calotropis gigantea*. *Journal of natural products, 83*(7), 2269-2280.

Prassas, I., & Diamandis, E. P. (2008). Novel therapeutic applications of cardiac glycosides. *Nature reviews Drug discovery, 7*(11), 926-935.

Rascón-Valenzuela, L., Velázquez, C., Garibay-Escobar, A., Vilegas, W., Medina-Juárez, L., Gámez-Meza, N., & Robles-Zepeda, R. (2016). Apoptotic activities of cardenolide glycosides from *Asclepias subulata*. *Journal of ethnopharmacology, 193*, 303-311.

Rasmussen, S., Larsen, P. V., Søndergaard, J., Elnegaard, S., Svendsen, R. P., & Jarbøl, D. E. (2015). Specific and non-specific symptoms of colorectal cancer and contact to general practice. *Family practice, 32*(4), 387-394.

Rathod, N., Raghuveer, I., Chitme, H., & Chandra, R. (2009). Free radical scavenging activity of *Calotropis gigantea* on streptozotocin-induced diabetic rats. *Indian journal of pharmaceutical sciences, 71*(6), 615.

Ray, P. D., Huang, B.-W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular signalling, 24*(5), 981-990.

Rentsch, M., Schiergens, T., Khandoga, A., & Werner, J. (2016). Surgery for colorectal cancer-trends, developments, and future perspectives. *Visceral medicine, 32*(3), 184-191.

Saltz, L. B., Cox, J. V., Blanke, C., Rosen, L. S., Fehrenbacher, L., Moore, M. J., . . . Pirotta, N. (2000). Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. *New England Journal of Medicine, 343*(13), 905-914.

Satari, A., Amini, S. A., Raeisi, E., Lemoigne, Y., & Heidarian, E. (2019). Synergetic impact of combined 5-fluorouracil and Rutin on apoptosis in PC3 cancer cells through the modulation of P53 gene expression. *Advanced pharmaceutical bulletin, 9*(3), 462.

Seeka, C., & Sutthivaiyakit, S. (2010). Cytotoxic cardenolides from the leaves of *Calotropis gigantea*. *Chemical and Pharmaceutical Bulletin, 58*(5), 725-728.

Sen, S., Sahu, N. P., & Mahato, S. B. (1992). Flavonol glycosides from *Calotropis gigantea*. *Phytochemistry, 31*(8), 2919-2921.

Seniya, C., Trivedia, S. S., & Verma, S. K. (2011). Antibacterial efficacy and phytochemical analysis of organic solvent extracts of *Calotropis gigantea*. *J. Chem. Pharm. Res, 3*(6), 330-336.

Shaban, N. Z., El-Kersh, M. A., El-Rashidy, F. H., & Habashy, N. H. (2013). Protective role of *Punica granatum* (pomegranate) peel and seed oil extracts on diethylnitrosamine and phenobarbital-induced hepatic injury in male rats. *Food chemistry, 141*(3), 1587-1596.

Sharma, M., Tandon, S., Aggarwal, V., Bhat, K. G., Kappadi, D., Chandrashekhar, P., & Dorwal, R. (2015). Evaluation of antibacterial activity of *Calotropis gigentica* against *Streptococcus mutans* and *Lactobacillus acidophilus*: An in vitro comparative study. *Journal of conservative dentistry: JCD, 18*(6), 457.

Shi, Y., Fan, Y., Hu, Y., Jing, J., Wang, C., Wu, Y., . . . Dong, D. (2020). α-Mangostin suppresses the de novo lipogenesis and enhances the chemotherapeutic response to gemcitabine in gallbladder carcinoma cells via targeting the AMPK/SREBP1 cascades. *Journal of cellular and molecular medicine, 24*(1), 760-771.

Shirasaka, T., Shimamoto, Y., & Fukushima, M. (1993). Inhibition by oxonic acid of gastrointestinal toxicity of 5-fluorouracil without loss of its antitumor activity in rats. *Cancer research, 53*(17), 4004-4009.

Soebagjo, H. D., Fatmariyanti, S., Paulus Sugianto, N., Purwanto, B., Bintoro, S. U. Y., & Widagdo, E. R. K. (2019). Detection of the Calcium and ATP Role in Apoptosis of Retinoblastoma Culture Cells through Caspase-3 Expression. *Research Journal of Pharmacy and Technology, 12*(03), 1307-1314.

Song, W., Yan, C.-y., Zhou, Q.-q., & Zhen, L.-l. (2017). Galangin potentiates human breast cancer to apoptosis induced by TRAIL through activating AMPK. *Biomedicine & Pharmacotherapy, 89*, 845-856.

Sorrentino, M. F., Kim, J., Foderaro, A. E., & Truesdell, A. G. (2012). 5-fluorouracil induced cardiotoxicity: review of the literature. *Cardiology journal, 19*(5), 453-457.

Srinivas, U. S., Tan, B. W., Vellayappan, B. A., & Jeyasekharan, A. D. (2019). ROS and the DNA damage response in cancer. *Redox biology, 25*, 101084.

Sun, M., Pan, D., Chen, Y., Li, Y., Gao, K., & Hu, B. (2017). Coroglaucigenin enhances the radiosensitivity of human lung cancer cells through Nrf2/ROS pathway. *Oncotarget, 8*(20), 32807.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians, 71*(3), 209-249.

Sur, S., Nakanishi, H., Flaveny, C., Ippolito, J. E., McHowat, J., Ford, D. A., & Ray, R. B. (2019). Inhibition of the key metabolic pathways, glycolysis and lipogenesis, of oral cancer by bitter melon extract. *Cell Communication and Signaling, 17*(1), 1-13.

Tao, T., Su, Q., Xu, S., Deng, J., Zhou, S., Zhuang, Y., . . . Peng, M. (2019). Down-regulation of PKM2 decreases FASN expression in bladder cancer cells through AKT/mTOR/SREBP-1c axis. *Journal of cellular physiology, 234*(3), 3088-3104.

Tatsumi, T., Shiraishi, J., Keira, N., Akashi, K., Mano, A., Yamanaka, S., . . . Nakagawa, M. (2003). Intracellular ATP is required for mitochondrial apoptotic pathways in isolated hypoxic rat cardiac myocytes. *Cardiovascular research, 59*(2), 428-440.

Tecza, K., Pamula-Pilat, J., Lanuszewska, J., Butkiewicz, D., & Grzybowska, E. (2018). Pharmacogenetics of toxicity of 5-fluorouracil, doxorubicin and cyclophosphamide chemotherapy in breast cancer patients. *Oncotarget, 9*(10), 9114.

Thakur, S., Das, P., Itoh, T., Imai, K., & Matsumoto, T. (1984). Latex extractables of *Calotropis gigantea*. *Phytochemistry, 23*(9), 2085-2087.

Van der Jeught, K., Xu, H.-C., Li, Y.-J., Lu, X.-B., & Ji, G. (2018). Drug resistance and new therapies in colorectal cancer. *World journal of gastroenterology, 24*(34), 3834.

Van Khang, P., Zhang, Z.-G., Meng, Y.-H., Guo, D.-A., Liu, X., Hu, L.-H., & Ma, L. (2014). Cardenolides from the bark of *Calotropis gigantea*. *Natural product research, 28*(15), 1191-1196.

Van Quaquebeke, E., Simon, G., André, A., Dewelle, J., Yazidi, M. E., Bruyneel, F., . . . Decaestecker, C. (2005). Identification of a Novel Cardenolide (2 ‘‘-Oxovoruscharin) from *Calotropis procera* and the Hemisynthesis of Novel Derivatives Displaying Potent in Vitro Antitumor Activities and High in Vivo Tolerance: Structure− Activity Relationship Analyses. *Journal of medicinal chemistry, 48*(3), 849-856.

Velu, P., Vijayalakshmi, A., Iyappan, P., & Indumathi, D. (2016). Evaluation of antioxidant and stabilizing lipid peroxidation nature of *Solanum xanthocarpum* leaves in experimentally diethylnitrosamine induced hepatocellular carcinogenesis. *Biomedicine & Pharmacotherapy, 84*, 430-437.

Verma, A., Singh, D., Anwar, F., Bhatt, P. C., Al-Abbasi, F., & Kumar, V. (2018). Triterpenoids principle of *Wedelia calendulacea* attenuated diethynitrosamine-induced hepatocellular carcinoma via down-regulating oxidative stress, inflammation and pathology via NF-kB pathway. *Inflammopharmacology, 26*(1), 133-146.

Virani, S., Bilheem, S., Chansaard, W., Chitapanarux, I., Daoprasert, K., Khuanchana, S., . . . Siriarechakul, S. (2017). National and subnational population-based incidence of cancer in Thailand: assessing cancers with the highest burdens. *Cancers, 9*(8), 108.

Wang, C., & Youle, R. J. (2009). The role of mitochondria in apoptosis. *Annual review of genetics, 43*, 95-118.

Wang, H., Zhang, H., Fan, K., Zhang, D., Hu, A., Zeng, X., . . . Wang, H. (2021). Frugoside delays osteoarthritis progression via inhibiting miR-155-modulated synovial macrophage M1 polarization. *Rheumatology*.

Wang, Z.-N., Wang, M.-Y., Mei, W.-L., Han, Z., & Dai, H.-F. (2008). A new cytotoxic pregnanone from *Calotropis gigantea*. *Molecules, 13*(12), 3033-3039.

Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., . . . Korsmeyer, S. J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes & development, 14*(16), 2060-2071.

Wei, Y., Yang, P., Cao, S., & Zhao, L. (2018). The combination of curcumin and 5-fluorouracil in cancer therapy. *Archives of pharmacal research, 41*(1), 1-13.

Weydert, C. J., & Cullen, J. J. (2010). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nature protocols, 5*(1), 51-66.

Wong, S. K., Lim, Y. Y., Abdullah, N. R., & Nordin, F. J. (2011). Assessment of antiproliferative and antiplasmodial activities of five selected Apocynaceae species. *BMC complementary and alternative medicine, 11*(1), 1-8.

Xu, G., & Shi, Y. (2007). Apoptosis signaling pathways and lymphocyte homeostasis. *Cell research, 17*(9), 759-771.

Yang, H., Huang, S., Wei, Y., Cao, S., Pi, C., Feng, T., . . . Ren, G. (2017). Curcumin enhances the anticancer effect of 5-fluorouracil against gastric cancer through down-regulation of COX-2 and NF-κB signaling pathways. *Journal of Cancer, 8*(18), 3697.

You, H., Lei, M., Song, W., Chen, H., Meng, Y., Guo, D., . . . Hu, L. (2013). Cytotoxic cardenolides from the root bark of *Calotropis gigantea*. *Steroids, 78*(10), 1029-1034.

Zhang, M., Harashima, N., Moritani, T., Huang, W., & Harada, M. (2015). The roles of ROS and caspases in TRAIL-induced apoptosis and necroptosis in human pancreatic cancer cells. *PloS one, 10*(5), e0127386.

Zhang, N., Yin, Y., Xu, S.-J., & Chen, W.-S. (2008). 5-Fluorouracil: mechanisms of resistance and reversal strategies. *Molecules, 13*(8), 1551-1569.

Zhou, L., Cai, L., Guo, Y., Zhang, H., Wang, P., Yi, G., & Huang, Y. (2019). Calotropin activates YAP through downregulation of LATS1 in colorectal cancer cells. *OncoTargets and therapy, 12*, 4047.