รายงานวิจัยฉบับสมบูรณ์

กลไกการออกฤทธิ์ยับยั้งของสารสกัดจากว่านชักมดลูก (*Curcuma comosa*) และสารออกฤทธิ์ต่อการส่งสัญญาณของโปรตีนนิวคลีโอฟอสมินที่เกี่ยวข้องกับการดื้อยาและการแบ่งตัวของเซลล์ในเซลล์สายพันธุ์มะเร็งเม็ดเลือดขาวดื้อยาชนิด K562/ADR

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กลไกการออกฤทธิ์ยับยั้งของสารสกัดจากว่านชักมดลูก (*Curcuma comosa*) และสารออกฤทธิ์ต่อการส่งสัญญาณของโปรตีนนิวคลีโอฟอสมินที่เกี่ยวข้องกับการดื้อยาและการแบ่งตัวของเซลล์ในเซลล์สายพันธุ์มะเร็งเม็ดเลือดขาวดื้อยาชนิด K562/ADR

บทคัดย่อ

ว่านชักมดลูก เป็นพืชที่อยู่ในวงศ์ขิง (Zingiberaceae) เหง้าของว่านชักมดลูกถูกนำมาใช้ประโยชน์ทางการแพทย์แผนไทย โดยใช้รักษาอาการของสตรี เช่น ประจำเดือนมาไม่ปกติ ปวดท้องระหว่างมีประจำเดือน การศึกษาในครั้งนี้ได้ทำการสกัดสารออกฤทธิ์ทางชีวภาพและวิเคราะห์องค์ประกอบทางเคมีที่สำคัญ รวมทั้งทดสอบกลไกการออกฤทธิ์ยับยั้งของสารสกัดจากว่านชักมดลูก ต่อการส่งสัญญาณของโปรตีนนิวคลีโอฟอสมินที่เกี่ยวข้องกับการดื้อยาและการแบ่งตัวของเซลล์ในเซลล์สายพันธุ์มะเร็งเม็ดเลือดขาวดื้อยาชนิด K562/ADR จากการศึกษาพบว่าสามารถแยกสารออกฤทธิ์ที่สำคัญคือ diarylheptanoids ประกอบด้วย (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol โดยสกัดแยกจากการหมักด้วยตัวทำละลายเอทิลอะซิเตท และสาร (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol จากการหมักด้วยตัวทำละลายเฮกเซน เมื่อนำสารสกัดทั้งสองชนิดมาทดสอบฤทธิ์ในการต้านมะเร็งเม็ดเลือดขาวชนิดต่าง ๆ พบว่าสารสกัด (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol สามารถต้านเซลล์มะเร็งเม็ดเลือดขาวและสามารถยับยั้งการสร้างโปรตีน WT1 ที่ทำหน้าที่ในการแบ่งตัวของเซลล์มะเร็งได้ดีกว่าสารสกัด (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol สารสกัด (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol ยังสามารถต้านอนุมูลอิสระและต้านการอักเสบได้ ยิ่งไปกว่านั้นสารสกัดทั้งสองไม่มีฤทธิ์ในการทำลายเม็ดเลือดแดง จากผลของสารสกัด diarylheptanoids ต่อการสร้างโปรตีน P-gp และ nucleophosmin ที่มีความสำคัญต่อการดื้อยาของเซลล์มะเร็งเม็ดเลือดขาว พบว่าสามารถยับยั้งการแสดงออกของโปรตีนทั้งสองได้ รวมทั้งเหนี่ยวนำให้เกิดการตายแบบอะพอพโทซิส (apoptosis) ในเซลล์มะเร็งเม็ดเลือดขาวชนิดดื้อยา (K562/ADR) สารสกัด diarylheptanoids ยังสามารถต้านเซลล์มะเร็งเม็ดเลือดขาวชนิด HL-60 ได้ โดยยับยั้งการทำงานของเอนไซม์ทีโลเมอเรส (telomerase) ซึ่งเป็นเอนไซม์ที่มีการแสดงออกมากในเซลล์มะเร็งเม็ดเลือดชนิดปกติและดื้อยา ผ่านการยับยั้งการแสดงออกของยีน *c-Myc* ส่งผลให้การแสดงออกของยีน *hTERT* ลดลงไปด้วย ดังนั้นฤทธิ์ต้านมะเร็งเม็ดเลือดขาวชนิด HL-60 ของสารสกัด diarylheptanoids เกิดจากการลดการทำงานเอนไซม์ทีโลเมอเรส โดยลดการแสดงออกของโปรตีน c-Myc ทำให้ลดการแสดงออกของโปรตีน hTERT ที่เป็นส่วนหนึ่งของเอนไซม์ทีโลเมอ

เรส ดังนั้นสารสกัด diarylheptanoids จากว่านชักมดลูก จึงเป็นสารที่น่าสนใจ ที่จะนำไปศึกษาต่อถึงกลไกในเชิงลึก เพื่อนำไปพัฒนาเป็นยารักษาโรคมะเร็งเม็ดเลือดขาวต่อไปในอนาคต โดยเฉพาะมะเร็งเม็ดเลือดขาวเฉียบพลันสายมัยอิลอยด์ รวมทั้งมะเร็งเม็ดเลือดขาวที่ดื้อต่อยาเคมีบำบัด

Inhibitory mechanism of Wan Chak Motluk (*Curcuma comosa*) extracts and its active compounds on nucleophosmin protein signaling related to drug resistance and cell proliferation in multidrug-resistant leukemic K562/ADR cell line

Abstract

Curcuma comosa belongs to the Zingiberaceae family. The rhizome of the plant has been used for medicinal purposes, in particular, to manage of the unpleasant symptoms in urogenital organ system in women in Thai traditional medicine. In this study, natural compounds were isolated from *C. comosa* and structurally determined by spectroscopic methods and nuclear magnetic resonance. The two isolated compounds were identified as diarylheptanoids (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol (1) and diarylheptanoids (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol (2). These diarylheptanoids were investigated for anticancer activities. Compound 1 showed the strongest cytotoxicity against HL-60 cells. The antioxidant and anti-inflammatory properties of compound 1 were higher than that of compound 2, additionally, compound 1 were also a potent antioxidant as compared to ascorbic acid. Moreover, both compounds had no effect on red blood cell hemolysis. Moreover, compound 1 had significantly affected on WT1 protein suppression and cell proliferation inhibition. Compound 1 with cancer cytotoxicity against the cancer cell lines, high antioxidant activity, and high anti-inflammatory were identified in pure compound from *C. comosa*. Moreover, our finding that diarylheptanoids could reduce P-gp and Nucleophosmin expression suggests that diarylheptanoids can induce apoptosis in drug resistant leukemia, K562/ADR. Furthermore, anticancer role of diarylheptanoids from *C. comosa* in human leukemic cells is achieved through down regulation of enzyme telomerase by suppression of c-Myc and hence decreasing expression of its target hTERT. Therefore, diarylheptanoids from *C. comosa* has strong potential for further study on the molecular mechanism of telomerase inhibition. Medicinal plants seem to offer a wealth of potential candidate compound. Our results suggest that active compound from *C. comosa* can be used as a chemotherapeutic agent for the treatment of human leukemia, particularly AML.

Contents

List of abbreviations

%	=	Percent
°C	=	Degree Celsius
µl	=	Microliter
µM	=	Micromolar
ml	=	Milliliter
U	=	Unit
ADR	=	Adriamycin
mM	=	MilliMolar
ng	=	Nanogram
rpm	=	Revolution per minute
ddH_2O	=	Distilled water
NPM	=	Nucleophosmin
P-gp	=	P-glycoprotein
PBMCs	=	Peripheral blood mononuclear cells
DNA	=	Deoxyribonucleic acid
DEX	=	Dexamethasone
EtOAc	=	Ethyl acetate
nm	=	Nanometer
Hex	=	Hexane
MDR	=	Multi-drug resistance
hTERT	=	Human telomerase reverse transcriptase
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
IC50	=	50% inhibitory concentration
DPPH	=	Diphenyl-1-picrylhydrazyl-hydrate

List of Figures

List of Tables

Introduction

The leukemias are common hematologic malignancies worldwide, with approximately 200,700 new cases (1.4% of all cancer cases) and 265,500 deaths (3.2% of all cancer death) annually (1). Despite ongoing improvements in the outcomes of patients with leukemia, only 30%-40% of patients achieve long-term, disease-free survival due to drug resistance and disease relapse (2). Drug-resistance in leukemic cells is one of the principal causes for chemotherapy failure in clinical practice, including the surface high expressed drug resistant protein mediated multi-drug resistance (MDR) and the over-expressed anti-apoptosis factor induced apoptosis resistance. The drug-resistant proteins currently reported are mainly: P-glycoprotein (P-gp), breast cancer resistance protein 1 (BCRP1), MDR-associated protein 1 (MRP1), etc. Most of the chemotherapeutic agents used in clinical practice are substrates of these drug-pump proteins or the substances that may induce the expressions of them in tumor cells, leading to MDR of the tumor (3, 4). Nowadays, other molecules that are involved in drug resistant mechanism are being studied to determine their potential role in resistant of leukemia.

Nucleophosmin (NPM) is a major nucleolarphosphoprotein that displays a number of activities. These include a potential role as a positive regulator of cell proliferation, cell differentiation and apoptosis. Nucleophosmin is significantly more abundant in tumor than normal cells (5-6). Nucleophosmin is obviously over-expressed in many leukemic cells, especially in the drug resistant leukemic cell lines. It is revealed that an expression of NPM has relationship to drug resistance. Previous study indicated that down-regulation of NPM expression inhibits leukemic cells proliferation, blocks cell cycle progression and induces cellular apoptosis in K562 leukemic cells (7). In 2013, Lin and his colleague (8) reported that knockdown of NPM by RNA interference inhibits cells proliferation, arrested cell cycle progression, induced apoptosis, promoted differentiation and also reverse multidrug resistance in drug resistant leukemic HL-60 cells. Moreover, decreased expression of NPM by RNA interference reverse the drug resistance of adriamycin-resistant Molt-4 leukemia cells through mdr-1 (P-gp) down-regulation and Akt/mTOR signaling was also reported by Wang et al (9). Recently, NPM was found as a

novel BAX binding protein with this interaction involved in activation and translocation of BAX in mitochondrial dysfunction and apoptotic cell death. NPM translocates from nucleolus to cytosol, binds to BAX, and blocks mitochondrial translocation, oligomerization, and activation of BAX, thereby rendering cells resistant to death induction (10). NPM exhibits both the oncogenic potential role in leukemia and has an important role in drug resistant of leukemia. However, further investigation is needed to explore more precise mechanisms of NPM gene in drug-resistance in leukemia.

Until now, there is still no effective drug that removes drug resistance from tumors. Thus, finding new effective substances with minimal side effects and substances that removes drug resistance from tumors for leukemia treatment has become a hot-spot of current tumor research. Discovery of local medicinal plants provide an important source of the new drug development. Herb extracts have been used against several cancer types such as breast, colon, cervical, liver and lung cancers. For example, Taxol and related compounds are good examples of the development of the anticancer medicines. Development of naturally derived anticancer drugs is, therefore, a crucial process. Isolations of novel compounds as well as the studying the effect of those compounds on cancer cells have become an important part of anticancer research. Most anticancer drugs have been discovered through random screening of potential plants and organisms. Results from various studies support the ability of medicinal plants to treat a number of cancer cases, allowing that medicinal plants have a great capability as the agents of choice for leukemia treatment. Curcuma spp. is one of the potential plants for long term anticancer chemoprevention.

Since NPM is highly express in leukemic cells and multidrug-resistant leukemic cells and also serve as a potential moderator in drug resistant process of MDR-resistant leukemic cells, using Thai natural products to target NPM may suppress the growth of leukemic cell lines and their corresponding MDR-resistant cell lines.

Telomerase, the enzyme responsible for maintaining telomeres, is highly expressed in acute myeloid leukemias, but the expression is low in normal cells. Telomeric TTAGGG tandem repeats at the ends of chromosomes protect the chromosomes from end-to-end fusion, rearrangement and translocation (11, 12). In normal somatic cells with low telomerase levels, DNA polymerase cannot completely replicate the 5′ end of the lagging strand, leading to telomere shortening with each cell division. This telomere shortening

triggers the cell to undergo replicative senescence, cell cycle arrest and DNA damage signaling (13, 14). Telomerase consists of two core subunits required for its activity: the human telomerase RNA template (hTERC) and the catalytic subunit of human telomerase reverse transcriptase (hTERT) (15, 16). Telomerase plays a role in cell proliferation as a protective mechanism against end-replication problems by adding TTAGGG repeats to the telomeres (17). Telomerase is highly active only in germ cells and in tissue stem cells. Increased expression of telomerase is strongly associated with neoplastic growth and the expression of telomerase in cancer cells may be a necessary step for tumor progression. Therefore, the development of agents having activity against telomerase may be a productive approach to develop novel breast cancer therapies. Chemotherapeutic drugs are quite toxic for patients and often result in drug resistance after a certain period. The use of alternative therapies is widespread in all regions of the developing world, and is rapidly growing in industrialized countries (18).

In this study, we determined the biological activity of active compounds of Curcuma comosa in human leukemic cells, the antiproliferative effect and the molecular mechanism underlining the anticancer activity of active compounds of *Curcuma comosa* on multidrug-resistant leukemic cell line as compared to the parental cell line and also examined the effect of *Curcuma comosa* on telomerase activity and molecular pathway in human leukemic cell line

Literature review

1. Cancer

Cancer is the leading cause of death in economically developing countries and the second leading cause of death in developing countries. The global burden of cancer continues to increase largely because of the aging and growth of the world population, along with an increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries (1). Based on the GLOBOCAN, IARC estimated that approximately 14 million cancer case and 8.2 million cancer deaths occurred in 2012 worldwide. Of these, 57% of the cases and 65% of the deaths occurred in the developing world (1).

Cancers are usually recognized by the fact that the cells have shown abnormal immortal growth (19). Cancer cells differ from normal cells in that they are no longer responsive to normal growth controlling

mechanisms. There are many different kinds of cancers which are developed in almost any organ or tissue, such as the lung, colon, breast, skin, bone, or nerve tissue (20). Breast cancer in females and lung cancer in males are the most frequent cancers and are the leading causes of cancer deaths (1). Treatment for cancer varies, based on the type and stage of cancer. Cancer is usually treated with surgery and then possibly with chemotherapy or/and radiation. However, radiation and chemotherapy, though they are essential part of the multidisciplinary treatment of cancers, can induce unwanted complications including skin wound and a certain degree of destroying effects to the bone, nervous system, gastrointestinal, and other complications (21, 22). Researchers have been increasingly interested in the agents by which their actions can change the biological properties of cancer cells, so that one of their major malignant characteristics is lose.

2. Leukemias (23, 24)

Leukemia is a form of cancer that targets the blood. Blood contains different types of cells such as red blood cells, white blood cells (WBCs), and platelets. The normal life cycle of these cells (formation, growth, function and death) is controlled in part by the bone marrow. In many instances, if the control over WBCs life cycle is disturbed, leukemia is the result. The number of WBCs is higher than other blood cells, they stop dying normally and they not carry out their function in the body, such as fighting infections and healing wounds.

In general, leukemia is the most common cancer in children. After disrupting the functions of the immune system, causing fevers and infections, the cancer interferes with the production of other blood cells, resulting in anemia and bleeding problems. An affected child may look pale, be often breathless, bruise and bleed easily for a prolonged period of time. Leukemia cells can also accumulate in different organs in the body triggering a set of symptoms including headaches, confusion, joint or bone pains, and painful swellings. The different types of leukemia are classified according to the length of the disease, the number of blood cells in the blood, and the specific type of blood cell involved. The four main types of leukemia are acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), and chronic lymphocytic leukemia (CLL)

Risk factors of leukemia including exposure to ionizing radiation (as might be given to treat another cancer), exposure to benzene or certain other organic chemicals, previous treatment with chemotherapy drugs, especially alkylating agents, for other cancers, inflammatory conditions, or certain genetic conditions, such as Down syndrome.

3. Drug resistant in leukemia

Drug resistance remains a major obstacle to cure of patients with acute leukemia. At the present, most centers are reporting 90% complete remission rates in acute lymphatic leukemia (ALL) and 70%-80% complete remission rates in patients with acute non-lymphocytic leukemia (ANLL). However, 5-year disease-free survival rates are only 50% and 10%-15%, respectively, in these diseases. This is almost certainly due to the development of drug resistance even to the combination chemotherapy programs utilized to treat these diseases (25). Resistance of leukemia cells to chemotherapeutic drugs occurs through several different mechanisms, including expression of one or more energy-dependent transporters that defect and eject drugs, induction of drug detoxifying mechanisms, increased or altered drug targets, decreased membrane permeability and/or insensitivity to drug-induced apoptosis (26). These mechanisms are summarized in Figure 1.

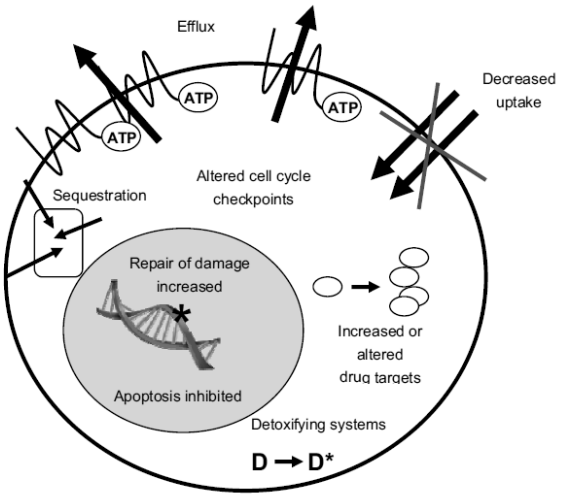


Figure 1. Schematic representation of several of the numerous different mechanisms by which cells become resistant to chemotherapeutic drugs (26).

After chemotherapeutic drugs treatments, it is a common clinical finding that patients develop cross-resistance towards a broad spectrum of structurally unrelated drugs to which the cells have never exposed (27). The cell's ability to protect itself from toxins is an essential biological function. Protection against, or resistance towards, a single class of drugs can be achieved in several ways, but for multidrug resistance (MDR) the main mechanism is active drug transport out of the cell (28). Multidrug transporters belong to a number of distinct transporter superfamilies of which the ABC (adenosine triphosphate (ATP) binding cassette) superfamily is the most important in the context of leukemia. In leukemia the MDR phenotype has been associated with clinical resistance and poor treatment outcome (29, 30)

3.1 ATP-binding cassette (ABC) protein

The ABC superfamily is a large family of transmembrane proteins that was first defined in the 1980s. In human, there are 49 known members of this family that efflux compounds in an active ATP-dependent manner (31). However, these proteins are present in normal tissues and involved not only in drug efflux but also in moving nutrients, hormones and other biologically important molecules into and out of cells, and across plasma membranes (32). To translocate substrates, these proteins require a minimum of four domains, i.e. two transmembrane domains and two nucleotide-binding domains, plus energy which is derived from ATP (33, 34). In this context transporter protein substrates are defined as compounds that are transported and inhibitors are compounds that restrict the function of the transporters. The ABC-proteins are organized in seven subfamilies, i.e. ABCA – ABCG, as described below (35-37)

1. The ABCA subfamily contains some of the largest ABC genes (>2100 amino acids). Two members of this family have been extensively studied; ABCA1 which is involved in cholesterol transport and high density lipoprotein synthesis, and ABCA4 which transports vitamin A derivatives.

2. The ABCB subfamily is unique in that it contains both full and half transporters. The ABCB1 protein is the most extensively studied and in AML and multidrug resistance the most important member of this family. ABCB1 or permeability glycoprotein (P-gp) is further described below.

3. The ABCC subfamily contains proteins with a diverse functional spectrum. Of these, the ABCC1, ABCC2 and ABCC3 (or multidrug resistance related proteins (MRP1-3)) transport drug conjugates to glutathione and other organic anions, which is further described below.

4. The ABCD subfamily function in the regulation of very long fatty acid transport.

5. The ABCE and ABCF subfamilies have ATP-binding domains but no transmembrane domains and are not known to be involved in any cross-membrane transport.

6. The ABCG subfamily contains half transporters that function as homodimers. The most relevant of the ABCG proteins, in the context of AML, is the ABCG2 or breast cancer resistance protein (BCRP), which is further described below.

3.2 Permeability glycoprotein (P-gp)

The permeability glycoprotein (P-gp) is one of the most well characterized proteins that have been linked to multidrug resistance. It was first found to be over-expressed in cell lines selected for resistance to colchicin and vinblastin and was believed to alter the permeability of the cell membrane (38). P-gp is the protein product of the MDR1 gene on chromosome 7q21. It has a molecular mass of 170 kDa and contains 1280 amino acids (39). P-gp is a transmembrane glycoprotein found in several normal human tissues such as liver, kidney, pancreas, colon, jejunum and placenta, as well as in numerous cancers. P-gp consists of two similar halves, joined by a linker region. Each half forms a total of six transmembrane domains and one cytoplasmic domain with ATPase activity (the ATP binding cassette) that hydrolyses ATP during molecular efflux. Both halves interact to form a single transporter. This interaction is necessary for functional drug transport (40). Binding of a drug results in activation of one of the ATP-binding domains, and the subsequent hydrolysis of ATP causes a major change in the configuration of P-gp, which results in release of the drug into the extracellular space. The substrates are transported against a concentration gradient across the membrane. To restore the shape of P-gp, hydrolysis of a second molecule of ATP is needed (41, 42). P-gp expression correlates with a reduced rate of complete remission and poor prognosis in AML. About one third of AML patients express P-gp at diagnosis and at relapse AML patients often display increased drug resistance. However, P-gp has not been proven to be up-

regulated at relapse indicating that this increase might be due to selection of resistant subpopulations and/or clonal expansion during chemotherapy (43). In vitro, long-term exposure of leukemia cell lines to cytostatic drugs, in increasing concentrations, results in a drug resistant phenotype with increased P-gp expression. It has also been shown that both P-gp substrates and non-P-gp substrates can induce P-gp mRNA and protein expression within 4 hours of exposure in leukemia cell lines and within 16 hours of exposure in AML patient samples (44, 45).

3.3 Multidrug resistance related proteins (MRP)

MRP is also a member of the ABC family of transporters which has been shown to confer resistance. MRP is 1531 amino acid glycosylated integral membrane protein that is overexpressed in several MDR cell lines (46). Like P-glycoprotein MRP1 transports drugs out of the cell, reducing the concentration at the intracellular target. MRP1 has been detected in several normal tissues with high levels of the protein being detected in adrenal gland, lung, heart and skeletal muscle and lower expressions detected in liver, spleen, kidney and erythrocyte membranes (47). MRP1 protein expression has also been detected in several human cancers and has been implicated in the clinical resistance in a number of solid malignancies and in certain hematological malignancies. Since the initial discovery of MRP1 and the recognition of the importance of MRP in MDR, seven additional members of the MRP family have been described, MRP2-8 (48, 49). In some studies, the expression of MRP 1 and not P-gp has been found to be higher at relapse than at diagnosis in AML patients. However, in general MRP1 expression at diagnosis is not associated with clinical response to induction chemotherapy and survival in most studies. It has been reported that intermediate or high MRP1 expression may be associated with shorter overall survival compared to patients with low MRP1 expression (50). AML (subtype M4E0) patients with an inversion of chromosome 16 (inv[16]) leading to the deletion of the MRP1 gene, have a relatively favorable outcome which suggests that MRP1 may play a role in determining clinical outcome in these patients. In CLL MRP1 mRNA expression has been detected in both chemotherapies treated and untreated CLL. Several studies investigating such roles for these proteins are currently underway. MRP1, MRP2 and MRP3 have

all been shown to cause resistance to neutral organic drugs and MRP4 overexpression has been associated with resistance to nucleoside analogues used in immunodeficiency virus drugs (51, 52).

3.4 Breast cancer resistance protein (BCRP)

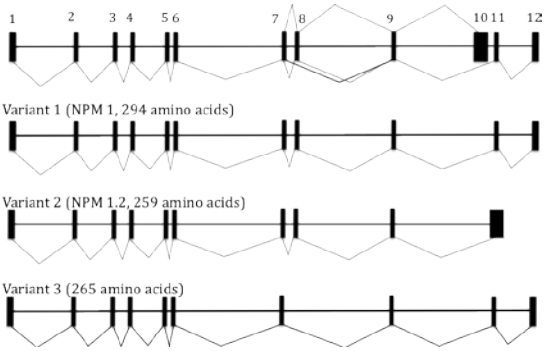
Studies on a breast cancer cell line resistant to mitoxantrone, but lacking over-expression of P-gp or MRP1, led to the identification of the breast cancer resistance protein (BCRP). The expression of BCRP is not specific for breast cancer cells, which is reflected in the names given by Miyake and co-workers and Allikmets and co-workers who simultaneously cloned the gene and called it mitoxantrone resistance (MXR) and placental ABC protein (ABCP), respectively (53, 54). BCRP is the protein product of the ABC (adenosine triphosphate (ATP) binding cassette) superfamily gene that maps to chromosome 4q22 (55). It has a molecular mass of 95 kDa and contains 655 amino acids (56). BCRP has only one transmembrane domain and one nucleotide binding domain and is therefore known as a half-transporter, which is likely to form homodimers or homotetramers to function (57). The physiological role of BCRP is not fully understood but it is highly expressed in placenta, the intestine, and in a subpopulation of hematopoietic stem cells (side population). During differentiation of hematopoietic cells, the expression of BCRP decreases (58). In AML, expression of BCRP at diagnosis has been correlated to a drug resistant phenotype and poor prognosis. However, conflicting data has been published on whether BCRP is up-regulated at relapse compared to diagnosis (44, 59).

4. Nucleophosmin

Nucleophosmin (NPM, also known as B23, numatrin or NO38 (60-62)), was first identified as a no-ribosomal nucleolarphosphoprotein with pleiotropic functions in various cellular processes, such as ribosome biogenesis, centrosome duplication, cell cycle progression, cell differentiation and apoptosis. NPM expresses at high level in the granular region of the nucleolus (63, 64). It is a highly conserved protein in vertebrates, which widely distributed among different species with molecular weight ranged from 35 to 40 kDa. In human and rat, NPM exists at least in two isoforms, NPM1 and NPM1.2 (B23.1 and B23.2, respectively), which are generated from a single gene via alternative splicing (65). Expression of

NPM rapidly increases in response to mitogenic stimuli, and that increased amounts of the protein are detected in highly proliferating cells and malignant cells (66).

A.



B.

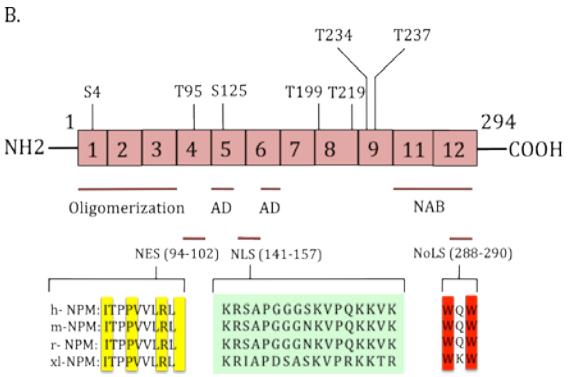


Figure 2. A schematic representation of the human nucleophosmin gene. (A), nucleophosmin exists as at least three isoforms, variants 1, 2 and 3. The relative sizes and positions of exons are represented by the numbers 1-12. The line (-) represents multiple alternative splicing events. (B), Structure of the wild-type human NPM gene. The regular-spliced NPM gene has 11 exons encoding 294 amino acids. The N-terminal and C-terminal portions of NPM are essential for oligomer formation and nucleic acid binding activity, respectively. The central region of NPM is highly acidic. AD denotes the acidic domain and NAB, the nucleic acid domain. (67).

4.1 Nucleophosmin structure

In 1989, human cDNA encoding a 294 amino acid called nucleophosmin was firstly cloned by Chan WY et al (66). The human nucleophosmin gene was mapped to chromosome 5q35, spanning 25 kb. The gene contains 12 exons with sizes ranging from 58 to 358 bp (Figure 2) (67). The human NPM gene can be transcribed as three isoforms, variant 1, variant 2, and variant 3. Among these, variant 1 is the predominant type and results in the longest transcript. The splice variant 2, differs at 3'-terminal exon compared with variant 1, resulting in a shorter protein with a distinct C-terminus. Variant 1 is mostly nucleolar, while variant 2 is detected at a low level in cells. Variant 2 is localized in both the cytoplasm and nucleoplasm, suggesting that the C-terminal end of nucleophosmin is necessary for subcellular localization of the protein. Variant 3, a new transcript variant in human, lacks an alternate inframe exon 8, resulting in a shorter protein. Functional significance of this variant is unknown.

4.2 Nucleophosmin protein

Nucleophosmin (NPM) is a 294 amino acid protein of 37 kDa and has multiple functional domains, i.e. oligomerization domain, histone binding domain, ribonuclease activity domain, and DNA/RNA binding domain as shown in Figure 3 (68). NPM exists as an oligomer under resting and proliferating condition (69). The N-terminus portion contains one hydrophobic segment that regulates self-oligomerization as well as chaperon activity towards proteins, nucleic acid, and histones (70). The middle part of nucleophosmin contains two acidic stretches that are important for histone binding. The central portion between the two acidic portions together with the C-terminal domain functions in ribonuclease activity. The C-terminus contains basic region that also involved in nucleic acid binding (71). The basic cluster is followed by an aromatic short stretch encompassing two tryptophans 288 and 290 residues which are critical for nucleolar localization of the protein as both tryptophan residues act as nucleolar localization signal (NuLs) (72). In addition, nucleophosmin also contains a bipartite nuclear localization signal (NLS), Leucine-rich nuclear export signal (NES), and several phosphorylation sites. The latter participates in association with centrosomes (73) and sub-nuclear compartments (74).

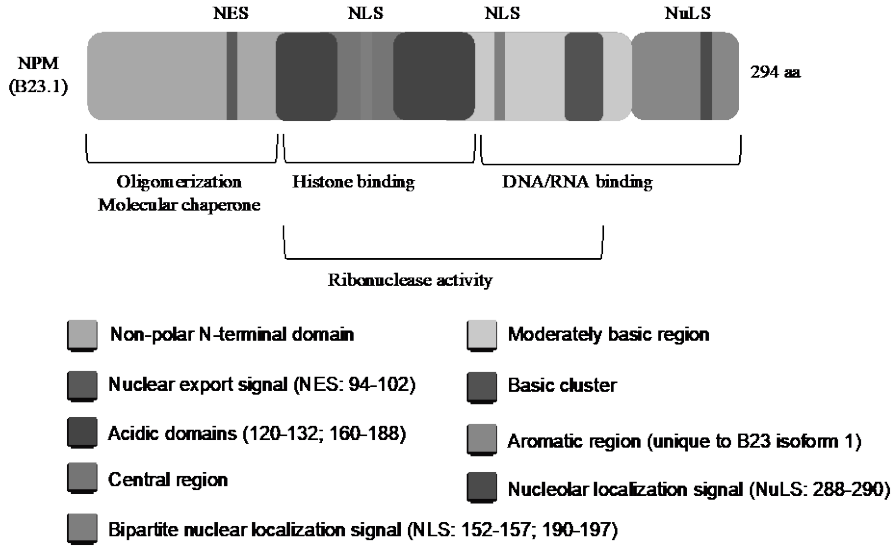


Figure 3. Structural domains of the nucleophosmin protein (68)

3.4 Nucleophosmin functions

NPM is a nucleolar multifunctional phosphoprotein that constantly shuttles between nucleolus and cytoplasm. NPM contains distinct functional domains through which it contributes many functions in the cell. This protein plays an important role in cellular cycling activities related with both proliferation and suppression pathways. The diverse cellular activities make this protein believed to be both potential oncoprotein and potential tumor suppressor protein, depending on its functional circumstances (68).

3.5 Ribosome biogenesis

NPM is a key molecule participating in ribosome biogenesis. It mediates 5S rRNA nuclear export and interacts with ribosomal protein L5 (rpL5), a known chaperone for the 5S rRNA (75). Direct interaction of NPM with rpL5 mediates the colocalization of NPM with the maturing nuclear 60S ribosomal subunits. The intervention also mediates the export and assembly of 80S ribosomes and polysomes. NPM intervenes in processing and/or assembly of ribosome through its nucleo-cytoplasmic shuttling properties, intrinsic RNAse activity, and its ability to bind nucleic acid. It can also act as a chaperone to prevent protein aggregation in the nucleolus during the ribosome assembly (76).

3.6 Embryonic development and genomic instability

NPM is essential for embryonic development and maintenance of genomic stability. Inactivation of the gene encoding for nucleophosmin in the mouse germ line leads to the developmental defects that cause embryonic death in mid-gestation. NPM-/- mice have aberrant organogenesis and die between embryonic day E11.5 and E16.5 owing to severe anemia and defects in primitive hematopoiesis. NPM function is required to maintain genomic stability through the regulation of centrosome duplication. Haploid-insufficiency of nucleophosmin leads to unrestricted centrosome duplication and genomic instability. The mice eventually developed myelodysplasia with an acceleration of oncogenesis (77).

3.7 Chaperone activity

NPM is nuclear chaperone protein that functions as a histone assembly and chromatin decondensation factor. The N-terminal hydrophobic portion of NPM contains the regions that are responsible for the self-oligomerization and chaperone activity (Figure 4). The chaperone activity of NPM prevents protein aggregation in the nucleolus during ribosome assembly and favors histone and nucleosome assembly as well as increases acetylation-dependent transcriptional activity. The chaperone activity of NPM depends upon the first of the two acidic domains (stretches of aspartic and glutamic acids) that are located in the middle portion of the protein (70).

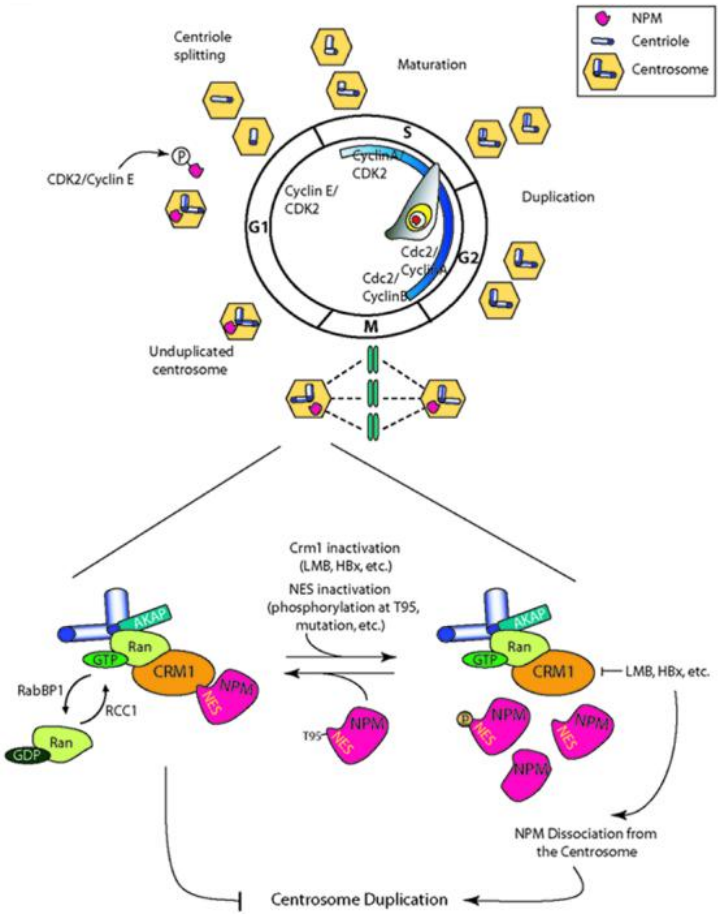


Figure 4. Nucleophosmin acts as a licensing factor for centrosome duplication and its transportation to the centrosome is regulated by the Ran/CRM1 complex. Nucleophosmin associates specifically with unduplicated centrosomes, and dissociates from centrosomes following CDK2 kinase phosphorylation during late G1 phase. This event allows centrosome duplication to take place. During cell cycle progression from the S to the G2 phase, nucleophosmin is mainly located in the nucleolus where it acts as a regulator of ribosome biogenesis. When the nuclear membrane breaks down at the beginning of mitosis, nucleophosmin is relocated to the centrosome, resulting in the prevention of centrosome reduplication. Newly divided cells contain a NPM-bound centrosome. Nucleophosmin movement to the centrosomes during mitosis occurs by interacting with the Ran/CRM1 complex through the NES motif. Inhibition of CRM1 by LMB or HBx, and NES inactivation, leads to dissociation from the centrosome and eventually allows centrosome amplication (67).

3.8 Centrosome duplication (67, 78)

NPM functions as a licensing factor for centrosome duplication. Its transportation to the centrosome is regulated by the Ran/CRM1 complex. NPM associated specifically with unduplicated centrosomes and dissociates from centrosomes upon Thr199 phosphorylation by Cdk2/Cyclin E at late G1 phase which allows centrosome duplication to take place.

During cell cycle progression from the S to the G2 phase, NPM is mainly located in nucleolus where it acts as a regulator of ribosome biogenesis. When the nuclear membrane breaks down at the beginning of the mitosis, nucleophosmin is relocalized to the centrosome, resulting in the prevention of centrosome reduplication. In the mitotic phase, NPM relocalization to the centrosomes occurs by interacting with the Ran/CRM1 complex through its NES motif. The Ran protein is a small GTPase which can cycle between an active GTP and an inactive GDP-bound form by the aid of RCC1 and RanBP, respectively. The active GTP-bound Ran localizes at the centrosome with AKAP and CRM1. Inhibition of CRM1 can be occurred by leptomycin B (LMB), hepatitis B virus protein (HBx). CRM1 inhibition can also induced by NES inactivation of NPM by phosphorylation at T95, or by mutation to NES motif of nucleophosmin. The CRM1 inhibition leads to dissociation of this complex from the centrosome and eventually allows centrosome amplification. All these data suggest that NPM could involve significantly in the duplication of centrosome during mitosis (Figure 5).

The nucleophosmin gene appears to be involved in control of cell growth, differentiation and programmed cell death. NPM over-expression in RNA and protein levels may contribute to the onset of cancer. It can thus be speculated that an excess amount of nucleophosmin may be an important cause of cancer, not just a consequence (Figure 5). Therefore, nucleophosmin, represent a potentially useful approach to anticancer biotherapeutics

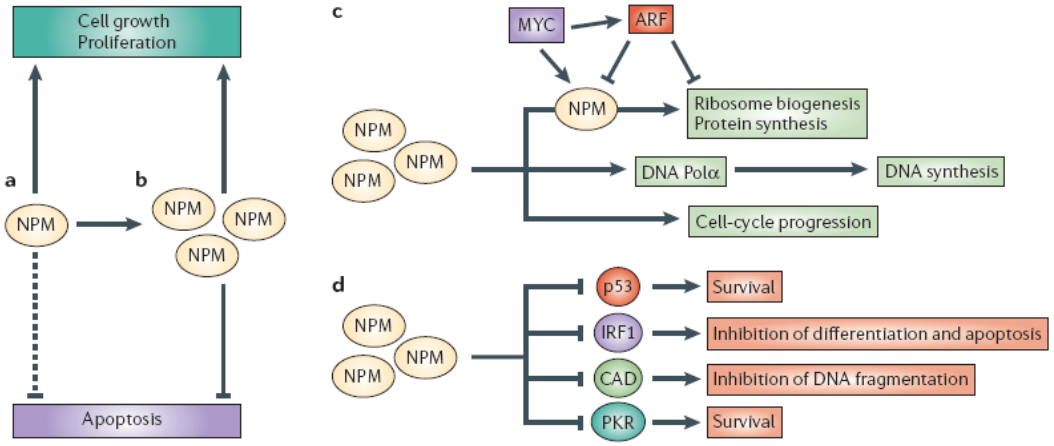


Figure 5. Nucleophosmin overexpression in tumour cells leads to increased proliferation and inhibition of apoptosis. (A), In normally proliferating tissues, nucleophosmin (NPM) is expressed at physiological levels, and the balance between cell proliferation and apoptosis is preserved. (B), In tumour cells, nucleophosmin is often overexpressed, and can exert its oncogenic potential both by stimulating cell proliferation and preventing apoptosis. (C), Nucleophosmin overexpression can lead to increased cell growth and proliferation through hyperactivation of the ribosome machinery, in view of its role as a ribosome biogenesis factor. NPM, together with a number of ribosomal proteins, is a transcriptional target of MYC. The altered activity of MYC in tumour cells correlates with increased protein synthesis. In addition, NPM is a putative stimulatory factor for DNA polymerase-α (DNA Polα), and contributes to cell-cycle progression. (D), Overexpressed NPM shows oncogenic potential by opposing apoptosis through different mechanisms: it prevents the DNA-binding activity of the transcription factor interferon regulatory factor 1 (IRF1); it binds to and inhibits the catalytic function of eukaryotic initiation factor 2 kinase PKR; it opposes the DNA-fragmentation activity of caspase-activated DNAse (CAD) in neural cells, thereby suppressing its pro-apoptotic activity; and finally, it interacts with and inhibits p53 in response to apoptotic stimuli (68)

4. Nucleophosmin and cancer

NPM is overexpressed in many types of major human solid tumors including tumors of colon, liver, stomach, ovary and prostate (79). In addition, disruption of the NPM gene by translocation is frequently

found in human hematopoietic malignancies (80-82). Recent studies indicate that about one-third of adult acute myeloid leukemia (AML) contain aberrant cytoplasmic expression of NPM with mutations occurring at the exon-12 of the NPM gene (83). The recent article by Falini et al (83) and the letter by Nakagawa et al (84) put forward a working model on the mechanism underlying the abnormal cytoplasmic localization of NPM in AML. They suggested that mutated NPM may utilize a Crm1-mediated nuclear export mechanism to lend its cytoplasmic accumulation through a frameshift mutation resulting in the creation of a functional NES motif or by amino acid substitutions at residues 288 and 299 to alter nucleolar localization. Further analyses indicate that the new NES motif created by the mutational event is not in itself sufficient to cause nuclear export of NPM but needs to act in concert with the mutated tryptophan(s) 288 and 290 at the mutant C-terminus (85).

Furthermore, they found that two NESs, i.e., a physiological NES at position 92-104 and a 'pathological' NES at the C-terminus created by mutations, are better than one to promote NPM cytoplasmic expression (85). It remains intriguing whether NPM cytoplasmic accumulation is associated with carcinogenesis, as we and others demonstrated its key role in the maintenance of centrosome duplication contributed by the presence of a physiological NES in NPM.

5. Nucleophosmin and multidrug resistant leukemic cell

Durable leukemic cell eradication by chemotherapy is challenged by the development of multidrug-resistance (MDR). As experimental models, chemotherapy-resistant cell line can be generated by in vitro selection with cytotoxic agents, which can become resistant to a single drug or a class of drugs. Many studies were concentrated on the mechanisms of drug resistance. General mechanisms of drug resistance can be classified into two classes (86): the genetic and epigenetic alternations of tumor cells that affect drug sensitivity, and the impairment of drug delivery system of tumor cells. An abundant number of reports persuasively confirmed that NPM was highly expressed in several malignant tumors and correlated with poor prognosis (87, 88). As previous research revealed, NPM was highly expressed in refractory or relapsed patients as well as drug-resistant myeloid cell lines (89). NPM regulate the expression of P-gp

through Akt/mTOR pathway mediated drug efflux to maintain drug resistance in Molt-4/ADR leukemia cells (9).

Most chemotherapeutic drugs are cytotoxic by inducing tumor cell apoptosis. In contrast, tumor cells could also survive and produce MDR through anti-apoptosis mechanism. Apoptosis is a balance of pro-apoptotic genes and anti-apoptotic genes, in which multiple genes and signaling pathways are involved. Abnormal expression and functional changes of these genes are closely related to tumorigenesis. BCL-2 gene is the first discovered as anti-apoptotic gene. BAX is one of the important pro-apoptosis genes. After NPM gene silencing, BCL-2 gene expression level was down-regulated, but BAX expression was up-regulated. Therefore, NPM was a BAX binding protein with this interaction involved in activation and translocation of BAX in mitochondrial dysfunction and apoptotic cell death. NPM translocates from nucleolus to cytosol, binds to BAX, and blocks mitochondrial translocation, oligomerization, and activation of BAX, thereby rendering cells resistant to death induction (10).

6. Telomerase

Telomerase, the enzyme responsible for maintaining telomeres, is highly expressed in over 90% of breast cancer tumors, but the expression is low in normal breast tissue (90, 91). Telomeric TTAGGG tandem repeats at the ends of chromosomes protect the chromosomes from end-to-end fusion, rearrangement and translocation (11, 12). In normal somatic cells with low telomerase levels, DNA polymerase cannot completely replicate the $5'$ end of the lagging strand, leading to telomere shortening with each cell division. This telomere shortening triggers the cell to undergo replicative senescence, cell cycle arrest and DNA damage signaling (13, 14). Telomerase consists of two core subunits required for its activity: the human telomerase RNA template (hTERC) and the catalytic subunit of human telomerase reverse transcriptase (hTERT) (15, 16). Telomerase plays a role in cell proliferation as a protective mechanism against end-replication problems by adding TTAGGG repeats to the telomeres (17). Telomerase is highly active only in germ cells and in tissue stem cells. Increased expression of telomerase is strongly associated with neoplastic growth and the expression of telomerase in cancer cells may be a necessary step for tumor progression. Therefore, the development of agents having activity against

telomerase may be a productive approach to develop novel breast cancer therapies (92-94). Chemotherapeutic drugs are quite toxic for patients and often result in drug resistance after a certain period. The use of alternative therapies is widespread in all regions of the developing world, and is rapidly growing in industrialized countries

7. *Curcuma comosa*

The use of herbs as complementary or alternative medicine (CAM) among patient with cancer, especially advanced cancer, has increased recently (95). The use of herbal intervention is widespread in all regions of the developing world, and is rapidly growing in industrialized countries (96, 97). Despite broad use, there are insufficient scientific data on the safety and efficacy of herbal health promotion and therapy. Some herbs have been shown to possess anti-cancer activities, but how they work is poorly understood. Lack of scientific evidence showing the molecular pathways of their action diminishes their clinical utility; therefore, basic research aimed at elucidating the mechanisms of action underlying the herbal effects should be a first priority.

Curcuma comosa is one of the Curcuma genus. In Thailand, *C. comosa* is commonly known as Wan Chak Motluk (Thai common name) and is found in Northern and Southeast. Rhizomes are round without horizontal branch, brown inside, and aromatic smell. It has been used as folk medicine in women for management of the unpleasant symptoms in urogenital organ system such as vaginal dryness, dysmenorrhea (painful menstruation), amenorrhea (absence of menstruation), menorrhagia (abnormal menstruation, too much menstruation) (98). Moreover, Curcuma genus shows anti-cancer (99), antioxidant activities (100), and anti-inflammatory effects (101), it also can suppress abdominal pain and chronic pelvic disorders by enables the contractions of the organ in urogenital system (102). It has been expected to contain bioactive compounds which have anti-cancer, antioxidant, or anti-inflammatory properties. Compound-092, (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol from *C. comosa* provide evidence for the pro-oxidant activity (GSH and ROS determination) of the diarylheptanoid bearing a catechol moiety in the induction of apoptosis in murine P388 leukemia (103).

Figure 6. *Curcuma comosa*

Objectives

The aims of this study are:

1. To study the biological activity of isolated diarylheptanoids from *Curcuma comosa* rhizomes
2. To study the antiproliferative effect and the molecular mechanism underlining the anticancer activity of active compounds of *Curcuma comosa* on multidrug-resistant leukemic cell line as compared to the parental cell line
3. To investigate the effect of *Curcuma comosa* on telomerase activity and molecular pathway in human leukemic cell line

Materials and Methods

1. Plant maceration

Curcuma comosa were harvested form Chiang Dao District, Chiang Mai province, Thailand in August 2018. A voucher specimen No. 023237 was deposited at the herbarium, Northern Research Center for medicinal plants, Faculty of Pharmacy, Chiang Mai University, Thailand. Herbarium specimen has been studied and annotated by traditional methods of herbarium taxonomy. Fresh rhizomes of *C. comosa* (5 kg) were peeled and dried at 50 °C. The dried rhizomes were grounded into powder and macerated in hexane for 3 days. Liquid portion was collected and repeated 3 times. The liquid portions of extraction were pooled together and filtrated. The filtrated was evaporated by using a rotary evaporator (EYELA N-1000) and subsequently dried to obtain hexane fraction (F-Hex). The residual powder will be dried in hot air oven (45 °C) and subjected to next maceration with ethyl acetate to obtain ethyl acetate fraction (F-EtOAc).

2. Column Chromatography

Silica gel grade 60 (Merck, Germany) was used as a solid phase in a column. Hexane and ethyl acetate were used as liquid phases with different ratios by increasing polarity to separate different compounds. Fractions were collected at least 8 mL in a test tube every 6-8 min. A thin layer chromatography was used to determine fractions that contain compounds. Fractions containing purified main compounds were pooled and characterized at the Faculty of Science, Chiang Mai University to determine chemical structures by Nuclear Magnetic Resonance (NMR). C. comosa fractional extracts and main compounds were kept at -20 °C. The fractional extracts or main compounds were dissolved in DMSO to obtain the working concentration (25 mg/mL) and kept at -20 °C before use.

Silica gel 60 (Brand) were packed in column and PPF-EtOAc or PPF-Hex were added to the top of the silica gel. PPF-EtOAc 1.577 g was first separated. Column were eluted with Hex:EtOAc by the ratio of 1:1. Fractions (8 mL/tube) were collected and observed by thin-layer chromatography (TLC). Similar TLC pattern of each fractions were selected and pooled together. Pooled fractions were observed by TLC again. The most purified pooled fractions (% yield = 32.40) were selected to do the second separated with the same procedure. Column were eluted with hexane/diethyl ether by the increasing ratios of 1:1. 1:2, and 1:3. First PPF- hex (3.025 g) purifications was done by the same procedure but different gradient elution of hexane/ethyl acetate of 1:1, 97.5:2.5, 96.5:3.5, 95:5, 90:10, 85:15, and 80:20.

3. Structure Identification

The purity of each collected fraction was determined by TLC and 1H NMR spectrum. The structure of two pure compounds was characterized by spectroscopic analyses. Optical rotations were measured on an Autopol I automatic polarimeter at the sodium lamp (λ = 589 nm) D-line and are reported as follows: [$\propto$]TD (c g/100 mL, solvent). 1H and 13C NMR spectra on a on a Bruker AVANCE 400 (400MHz) in deuterated chloroform (CDCl3; Sigma-Aldrich) and deuterated methanol (CD3OD; Sigma-Aldrich). 1H NMR spectra are reported as follows: chemical shift (δ, ppm), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants (J) in Hz, integration, and assignments. 13C NMR spectra are reported in terms of chemical shift (δ, ppm).

4. Cell cultures

Leukemic cell lines including K562 (human erythroid leukemic cell line), K562/ADR, and HL-60 (human promyelocytic leukemic cell line) was maintained in RPMI 1640 medium containing 1mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% fetal bovine serum (FBS). KG-1a (leukemic stem cell-like cell line with

stem cell population) was cultured in IMDM medium containing 1mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and supplemented with 20% FBS. A549 (human lung cancer cell line) and MCF-7 (breast cancer cell line) was cultured in DMEM medium containing 1mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% FBS. All cancer cell lines were incubated at 37 °C under 95% humidified and 5% CO_2.

5. Cytotoxicity determinations by MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for detecting the cytotoxicity of *C. comosa* extracts and purified compounds on cancer cells. The cytotoxicity of F-EtOAc, F-Hex, and main compounds was investigated by MTT assay in K562 KG-1a, HL-60, A549, and MCF-7 cell lines. K562 (1.0×10^5 cells/mL), HL-60 (1.0×10^5 cells/mL), KG-1a (1.5×10^5 cells/mL), and A549 (5.0×10^4 cells/mL), MCF-7 (5.0×10^4 cells/mL) were added then incubated for 48 h. After that, 100 µL of medium was removed and 15 µL of MTT dye solution was added, and cells were further incubated for 4 h. After supernatant was removed, 200 µL of DMSO were added to each well and mixed thoroughly to dissolve the purple formazan crystals. The optical density was measured by using ELISA plate reader at 578 nm with reference wavelength at 630 nm. The percentage of surviving cells was calculated from the absorbance values of the test and control wells using the following equation:

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test well}}{\text{Mean absorbance in vehicle control well}} \square \ 100$$

The average percentage of cells surviving at each concentration obtained from triplicate experiments was plotted as a dose-response curve. The 50% inhibitory concentration (IC_{50}) was defined as the lowest concentration that inhibited cell growth by 50% compared to the untreated control.

6. Trypan blue exclusion

Total cell numbers were counted by trypan blue exclusion method. Live cells have intact membrane and able to exclude trypan blue dye. In contrast, death cells with compromised membrane were stained by trypan blue dye solution. Cell suspension and 0.2% trypan blue was mixed and count viable (unstained) and death cell (stained) in the hemacytometer. The percentage of viable cells was calculated.

7. Cytotoxicity of PBMCs.

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque density-gradient centrifugation using Lymphoprep^{TM} solution (Axis-Shield, Oslo, Norway). The PBMCs (1×10^6 cells/mL) were plated in flat-bottom 96-well plates overnight in a 5% CO_2 incubator at 37 °C .Then, Various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) of *C. comosa* extracts and purified compounds were added, and the cells were then incubated for 48 h .The cell survival rate was assessed by means of the MTT colorimetric assay as previously described.

8. Antioxidant activities determination

8.1 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulphonate (ABTS) assay

The ABTS $^{\bullet+}$ scavenging activity of *C. comosa* extracts and purified compounds were investigated using ABTS assay. Briefly, ABTS$^{\bullet+}$ solution was prepared by mixing 2 mL of 7 mM ABTS solution with 3 mL of 2.45 mM potassium persulfate solution and incubated in the dark. After 24 h, the resulting ABTS$^{\bullet+}$ solution was diluted 1:20 in absolute ethanol. Then 20 μL of the sample was mixed with 180 μL of ABTS$^{\bullet+}$ solution, incubated at room temperature for 5 min, and measured for UV absorbance at 750 nm using a microplate reader (Spectrostar Nano, BMG Labtech GmbH, Ortenberg, Germany). The results were reported as Trolox equivalent antioxidant capacity (TEAC). All experiments were done in triplicate.

8.2 2,2'-Diphenyl-1-picrylhydrazyl-hydrate (DPPH) assay

The DPPH$^{\bullet}$ scavenging activity of *C. comosa* extracts and purified compounds were investigated using DPPH assay. Briefly, 20 μL of the sample was mixed with 180 μL of 167 μM DPPH solution, incubated at room temperature in the dark for 30 min, and measured for UV absorbance at 520 nm using a microplate reader (Beckman CoulterDTX880, Fullerton, CA, USA). The scavenging activity was calculated using the following equation:

$$\%\ \text{DPPH}\bullet\ \text{inhibition} = \frac{A\text{-}B}{A} \times 100$$

where *A* is a UV absorbance of a mixture without sample solution and *B* is a UV absorbance of a mixture with sample solution. L-ascorbic acid was used as a positive control. All experiments were done in triplicate.

8.3 Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power of *C. comosa* extracts and purified compounds were investigated by FRAP assay. Briefly, FRAP solution was freshly prepared by mixing 10 mL of 0.3 M acetate buffer pH 3.6, 1 mL of 10 mM 2,4,6 tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 1 mL of 20 mM ferric chloride. Then 20 μL of the sample was mixed with 180 μL of FRAP solution, incubated at room temperature in the dark for 5 min, and measured for UV absorbance at 595 nm using a microplate reader (Beckman CoulterDTX880, Fullerton, CA, USA). The results were expressed as equivalent capacity (EC_1). L-ascorbic acid was used as a positive control. All experiments were done in triplicate.

9. Anti-inflammatory activities determination

Anti-inflammatory activity of *C. comosa* extracts and purified compounds were investigated by means of inhibitory activities against IL-6 and TNF-α secretion. The mouse monocyte macrophage RAW 264.7 cells (American Type Culture Collection, ATCCTIB-71) were stimulated with lipopolysaccharide (LPS) to induce an inflammatory process. The cell incubated with LPS served as a vehicle control, of which the secreted cytokines was defined as 100% , whereas, non-treated RAW 264.7 cells served as a negative control. Dexamethasone, a well-known anti-inflammatory drug, was used as a positive control.

Briefly, 1×10^5 cells per well in DMEM were seeded and incubated for 24 h in a CO_2 incubator set at 37°C and 5% CO_2 -95% air. Then 1 μL of *C. comosa* extracts or purified compounds were added. After 2 h incubation in a CO_2 incubator, LPS was added to make a final concentration of 1 μg/mL. After 24 h incubation in a CO_2 incubator, the media was removed and centrifuged at 13,500×g for 10 min and 100 μL of the supernatant was analyzed by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). The optical density was measured at 450 nm, corrected with the reference wavelength of 570 nm using multimode detector (Beckman CoulterDTX880, Fullerton, CA, USA).

RAW 264.7 cell viability was also determined simultaneously with the ELISA by MTT assay. The supernatant was totally removed and MTT was added to the cells. After 2 h incubation in a CO_2 incubator, the supernatant was totally removed, and the cells were lysed with DMSO. The optical density was measured at 570 nm, corrected with the reference wavelength of 690 nm using multimode detector (Beckman CoulterDTX880, Fullerton, CA, USA). IL-6 and TNF-α secretion inhibitions were calculated using the following equation:

$$\%\ \text{Cytokine inhibition} = \frac{A\text{-}B}{A} \times 100$$

where A is an optical density of the mixture without sample and B is an optical density of the mixture with sample. Dexamethasone was used as a positive control. All experiments were done in triplicate.

10. RBC hemolysis induction

EDTA blood samples were collected from normal subjects. After centrifugation, RBCs were collected and washed twice with 0.9 % NaCl .For RBC hemolysis induction, 1 mL of 5 % RBC suspension was then incubated with F-EtOAc, Compound **1**, F-Hex, and Compound **2** at 37°C water bath for 3 h .Triton-X 100) 0.05 (% NaCl) 0.9 (% were used as positive and negative controls, respectively .After incubation, the supernatant was collected by centrifugation at 4400 rpm for 5 min at room temperature and measured hemoglobin concentration by spectrophotometry at 540 nm.

11. Western Blot analysis for WT1 expression

KG-1a cells were adjusted to concentration of 1.0 x 10^5 cells/mL and cultured with F-EtOAc, Compound **1**, F-Hex, and Compound **2** at concentration at 20% growth (IC_{20}) of each extract. After incubation for 48 h, cells were washed, and the whole protein were extracted using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 0.1% SDS, and a protease inhibitor cocktail). The protein concentration was determined by the Folin-Lowry method. Twenty micrograms of each sample were loaded to 7.5% SDS-PAGE and then transferred to PVDF membranes. The membranes were shaken in PBS for 5 min then incubated membranes in 5% skim milk in PBS to block nonspecific binding for 2 h. Each membrane was incubated with rabbit polyclonal anti-WT1 IgG (Santa Cruz Biotechnology, CA, USA) and rabbit polyclonal anti-human GAPDH IgG (Santa Cruz Biotechnology, CA, USA) at dilution 1:1000 with shaking for 2 h. After that, membranes were washed then incubated with HRP-conjugated goat anti-rabbit IgG (Invitrogen™, CA, USA) at 1:20,000 dilution with shaking for 2 h. To detect

protein band, Luminata TM Forte Western HRP substrate (Merck, Darmstadt, Germany) was added to membranes. After that, the membranes were placed onto a film cassette and exposed on X-ray film (Sakura, Japan). Densitometry was quantitated using Quantity One 1-D Analysis software (Bio-Rad, USA). The density values of WT1 bands were normalized to GAPDH bands.

12. Cell cycle analysis

Cell cycle analysis of main active compounds of C. comosa treated cells. After treated with Curcuma comosa extracts, leukemic cell lines (K562) and their corresponding MDR cell lines (K562/ADR) were subjected for cell cycle analysis by using CycleTESTTM PLUS DNA Reagent Kit. The method involves dissolving the cell membrane lipids with a nonionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting the cellular RNA with a ribonuclease A, and stabilizing the nuclear chromatin with spermine. Propidium iodide (PI) is stochiometrically bound to the clean, isolated nuclei which are then run on a flow cytometer to analyze light emitted between 580 and 650 nm from the stained cells.

13. Apoptosis analysis

Assay of cell apoptosis induced by main active compounds of C. comosa. Apoptotic cells were detected using an Annexin-V-FITC Apoptosis Detection Kit (ACT Gene) following the manufacturer's instructions. Briefly, After 48 h of treatment with herb extracts, leukemic cell line (K562) and their corresponding MDR cell line (K562/ADR) were incubated with FITC Annexin-V and propidium iodide (PI). Dead cells were identified by flow cytometer. The results are compared between control cells and herb-treated cells.

14. Cell cytotoxicity assay of various chemotherapeutic agents using MTT assay

Leukemic cell lines (K562) and their corresponding MDR cell lines (K562/ADR) were passaged in log phase growth, counted, and added to 96-well tissue culture plates at a

concentration of $1x10^4$ cells per well. The cell was incubated with various concentration of active compounds of *C. comosa* for 48 hr at 37 °C. ADR (Adriamycin) was added. Fifteen µl of a 5 mg/ml MTT solution was added to each well and the plate was incubated in dark for 4 hr. The resultant MTT-formazan crystals were dissolved in 200 µl of DMSO as the solubilizing agent. The absorbance at 570 nm was read on a spectrophotometric plate reader. The experiment was repeated for three times. IC50 values were calculated by SPSS 11.0 software. Relative reversal rate = (IC50 A − IC50 B)/(IC50 A − IC50 C), where IC50 A was IC50 values of resistant cells before treated, IC50 B was IC50 values of resistant cells after treated, IC50 C was IC50 values of parental cells.

15. Western blot analysis for P-gp and NPM expression

Study the expression level of proteins that involved in the inhibitory mechanism of active compounds of C. comosa on nucleophosmin protein signaling related to drug resistance and cell proliferation by Western blot analysis. Treated and untreated cells were rinsed twice with ice-cold phosphate buffer saline (PBS) and lysed with the lysis buffer at 4 °C with 50 mM HEPES-KOH, pH 7.5, 150 mM NaCI, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, and 10% glycerol supplemented with the following proteinase inhibitors: 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin. Cell lysates (50 mg) were loaded onto 10 % SDS-polyacrylamide gel electrophoresis and transferred to Immobilion-P membranes. The equal loading of proteins was confirmed by amido black staining. The membranes were probed with monoclonal antibodies (NPM, P-gp). The primary antibodies were detected with sheep anti-mouse-horseradish peroxide secondary antibody and subjected to enhanced chemiluminescence reagents. The levels of expression were quantified using a densitometric scanning system. Mouse monoclonal antibody specific to GAPDH is used as a control antibody to ensure equal protein loading.

16. Real-time RT-PCR

Study the effect of active compounds of *C. comosa* on mRNA expression of genes that involved cell proliferation inhibition. After treat cells with active compounds of *C. comosa*, RNAs were extracted from each cells then check integrity of mRNA of each gene (including c-Myc, NPM, P-gp, and hTERT) by real-time RT-PCR. In brief, RNA was isolated using available RNA purification kit. Expression of genes in each cell was studied by Real-time RT-PCR using optimum condition and specific primers for each fragment. GAPDH primers were used for Real-time RT-PCR internal control.

Table 1. Oligonucleotide primer sequences for Real-time RT-PCR

Genes	Primers		Fragment size (bp)
	Forward	Reverse	
c-Myc	ACCACCAGCAGCGACTCTGA	TCCAGCAGAAGGTGATCCAGACT	117
NPM	CCAGTGGTCTTAAGGTTGAAGTGTGG	TCCAGATATACTTAAGAGTTTCACATCCTCCTC	132
P-gp	CCCATCATTGCAATAGCAGG	GTTCAAACTTCTGCTCCTGA	150
hTERT	TCCACTCCCCACATAGGAATAGTC	TCCTTCTCAGGGTCTCCACCT	110
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	225

17. Telomerase activity

Telomerase activity was measured by the PCR-based Telomeric Repeat Amplification Protocol (TRAP) assay, using the TRAPEZE ELISA Telomerase Detection Kit (Millipore, Billerica, MA, USA). The assay was performed following the manufacturer's instructions. Briefly, 5×10^5 cells per well were seeded into 6-well plates and incubated with curcuma isolates at the specified concentration for 48 h. The cell pellets were then cooled on ice, lysed with CHAPS lysis buffer for 30 min and centrifuged at 12,000 × g at 4 °C for 20 min. The TRAP PCR reaction mixture contained 1 μg of protein from each cell extraction, 1 μL of biotinylated TS primer, 1 μL of RP

primer, 1 μL of internal control (K1 primer and TSK1 template), 2.5 mM dNTPs and 2 units Taq DNA polymerase. The PCR was performed for 33 cycles of 94 °C for 30 sec, 55 °C for 30 s, 72 °C for 30 s and followed by a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis through 10% non-denaturing polyacrylamide gels to determine the degree of telomeric repeats and quantitated by ELISA to quantitatively determine telomerase activity.

For ELISA, 5 μL of the PCR product was mixed with 100 μL of the blocking buffer into a well of a streptavidin-coaded microtiter plate and the samples were incubated at 37 °C for 1 h. The blocking solution was completely removed and the well was rinsed 5 times with 250 μL of washing buffer. Afterthen, 100 μL of a horseradish peroxidase conjugated anti-dinitrophenyl (DNP) antibody was added to each well. The plate was incubated at room temperature for 30 min in a dark chamber. Antibody solution was removed and the wells were rinsed 5 times with 250 μL of washing buffer before the addition of 100 μL Tetramethylbenzidine (TMB). Samples were incubated for 10 min at room temperature before the addition of 100 μL of stop solution. Using a microplate reader, the amount of TRAP product was determined by measuring the absorbance ratio at 450 and 690 nm (reference wavelength) within 30 min of stopping the reaction.

18. Statistical analysis

All data are expressed as the mean ± standard deviation (SD) or the mean ± standard error of mean (SEM) from triplicate samples of three independent experiments. The statistical differences between the means were determined using one-way ANOVA. The differences were considered significant when the probability value obtained was found to be less than 0.05 (p<0.05) or and 0.001 (p<0.001).

Results

<u>Part 1</u> To study the biological activity of isolated diarylheptanoids from *Curcuma comosa* rhizomes

1. Plant extraction

5 kilograms of dried rhizome were extracted using ethyl acetate and hexane having relative polarities of 0.228 and 0.009, respectively, compared to water (1.000). Ethyl acetate fraction (F-EtOAc) and hexane fraction (F-Hex) were obtained by maceration and evaporation (Figure 7). The yields of the extracts were 2.09 and 2.80%, respectively.

Figure 7. Extraction *C. comosa* was extracted with solvent (ethanol, hexane, ethyl acetate and methanol) by maceration method.

Both fractions were separated by silica gel column chromatography which led to the isolation of two known compounds **1** and **2**. The structures of these purified compounds were identified using 1D and 2D NMR spectroscopy and confirmed by comparison of their ^{1}H and/or ^{13}C NMR data with those previously published data.

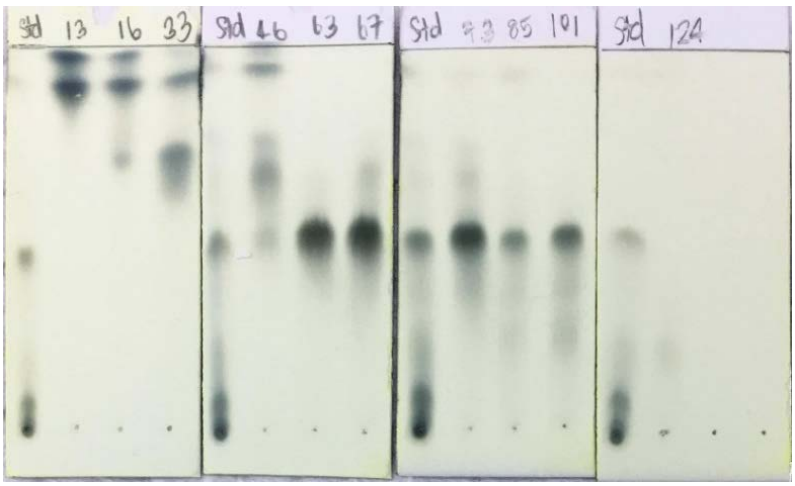


Figure 8. Thin layer chromatography

Compound **1** was obtained as brown gum. The ^{1}H NMR spectrum showed two sets of aromatic protons. The signals at δ 7.33—7.14 (m, 5H) could be the five protons of phenyl ring. Another set of aromatic protons displayed at δ 6.64 (m, 2H) for two protons and δ 6.50 for one proton (dd, J = 8.0, 2.0 Hz) which could be 1,3,4-trisubstituted aromatic rings. The two signals at δ 6.37 (d, J = 15.8 Hz) and 6.22 (dt, J = 15.8, 6.8 Hz) were the signals of trans-olefinic protons which attached to the phenyl ring. The signal at δ 3. 57 (m, 1H) suggested the presence of methine proton attached to hydroxy group. The ^{13}C NMR spectrum of compound **1** displayed 19 signals for twelve aromatic carbons (δ 146.1, 144.2, 139.2, 135.3, 131.4, 131.3, 129.4 (2C), 127.8, 126.9 (2C), 120.7), two olefinic carbons (δ 116.6, 116.3), one methine carbon bearing hydroxy group (δ 71.2) and four methylene carbons (δ 40.6, 38.1, 32.4, 30.3), respectively. The absolute configuration at C-5 was assigned to be *S* by comparing optical rotation with the literature ($[\alpha]_D^{30}$ -108 in EtOH, lit $[\alpha]_D^{28}$ -5.2). By comparison spectroscopic data with those reported in the literature, compound **1** were suggested to be diarylheptanoids (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol as shown in Figure.9.

Compound **2** was obtained as colorless oil. The ^{1}H NMR of compound **2** was similar to that of compound **1** except the signal of aromatic protons which showed ten protons. This signal

indicated that compound **2** had two phenyl rings in the structure. It was also confirmed by the ^{13}C NMR which displayed the presence of only two quaternary carbons (δ 142.2 and 137.7). The absolute configuration at C-5 was also assigned to be S by comparing optical rotation with the literature ($[\alpha]_D^{30}$ -1 in EtOH, lit $[\alpha]_D^{27}$ -2.6). By comparison spectroscopic data with those reported in the literature, compound **2** were suggested to be (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol as shown in Figure 10. Diarylheptanoids were isolated from the rhizomes of *Curcuma comosa* Roxb. Some of the isolated diarylheptanoids exhibited estrogenic activity comparable to or higher than that of the phytoestrogen genistein. Phenolic diarylheptanoids, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl(1*E*)-1-heptene and 7-(3,4- dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene, were isolated from the rhizomes of *Curcuma xanthorrhiza*. These two compounds exhibited hypolipidemic action by inhibiting hepatic triglyceride secretion.

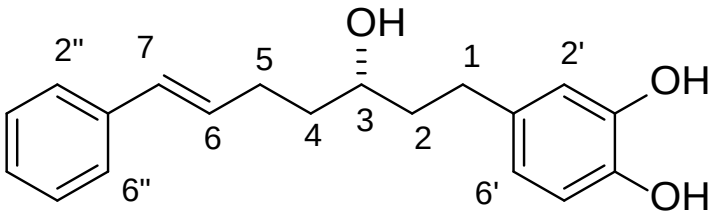


Figure 9. The chemical structure of compound **1** (diarylheptanoids (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol)

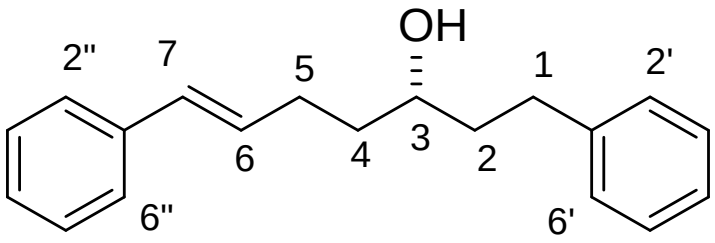


Figure 10. The chemical structure of compound **2** (diarylheptanoids (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol)

2. Cytotoxicity determinations by MTT assay

The cytotoxicity of F-Hex, F-EtOAc, and two purified active compounds were investigated by MTT assay in K562, KG-1a, HL-60, A549, and MCF-7 cells. F-EtOAc, compound 1 (purified active compound from F-EtOAc), PPF-Hex, and compound 2 (purified active compound from F-Hex) were treated in K562, KG-1a, HL-60, A549, and MCF-7 cells. All the extracts and purified compounds show cytotoxic effects on K562, KG-1a, HL-60, A549, and MCF-7 cells by MTT assay. The IC50 values (inhibitory concentration at 50% growth) of F-EtOAc, compound 1, F-Hex, and compound 2 on K562 cells were 37.35 ± 1.34, 28.92 ± 3.04, 40.52 ± 1.10, and 26.67 ± 2.81 µg/mL, respectively. The IC50 values of F-EtOAc, compound 1, F-Hex, and compound 2 on KG-1a cells were 18.52 ± 7.21, 43.62 ± 6.28, 53.56 ± 6.41, and 35.21 ± 2.19 µg/mL, respectively. The IC50 values of F-EtOAc, compound 1, F-Hex, and compound 2 on HL-60 cells were 19.93±1.28, 10.79±0.91, 27.19±1.66 and 19.47±0.49 µg/mL, respectively. The IC50 values of F-EtOAc, compound 1, F-Hex, and compound 2 on A549 cells were 38.87±1.74, 63.49±8.71, 31.81±5.25, and 35.30±1.60 µg/mL, respectively. The IC50 values of F-EtOAc, compound 1, F-Hex, and compound 2 on MCF-7 cells were 15.26±6.87, 34.28±1.34, 28.58±3.28, and 35.70±0.98 µg/mL, respectively (Figure 11).

The cytotoxicity of (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol (compound-092, compound 1 in this study) and (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol (compound-001; compound 2 in this study) were reported on P388 leukemic cells by MTT assay with the IC50 values of 3.8±0.1 and 39.1±1.5 µM, respectively and therefore, induced apoptosis.6 This result related to this study, compound 1 also showed a potent cytotoxicity against leukemic cells (HL-60) more than compound 2. However, cytotoxicity of compound 2 was better than compound 1 in K562 and KG-1a cells. The reason of these difference responses might be from the different compounds and type of cancer cell lines.

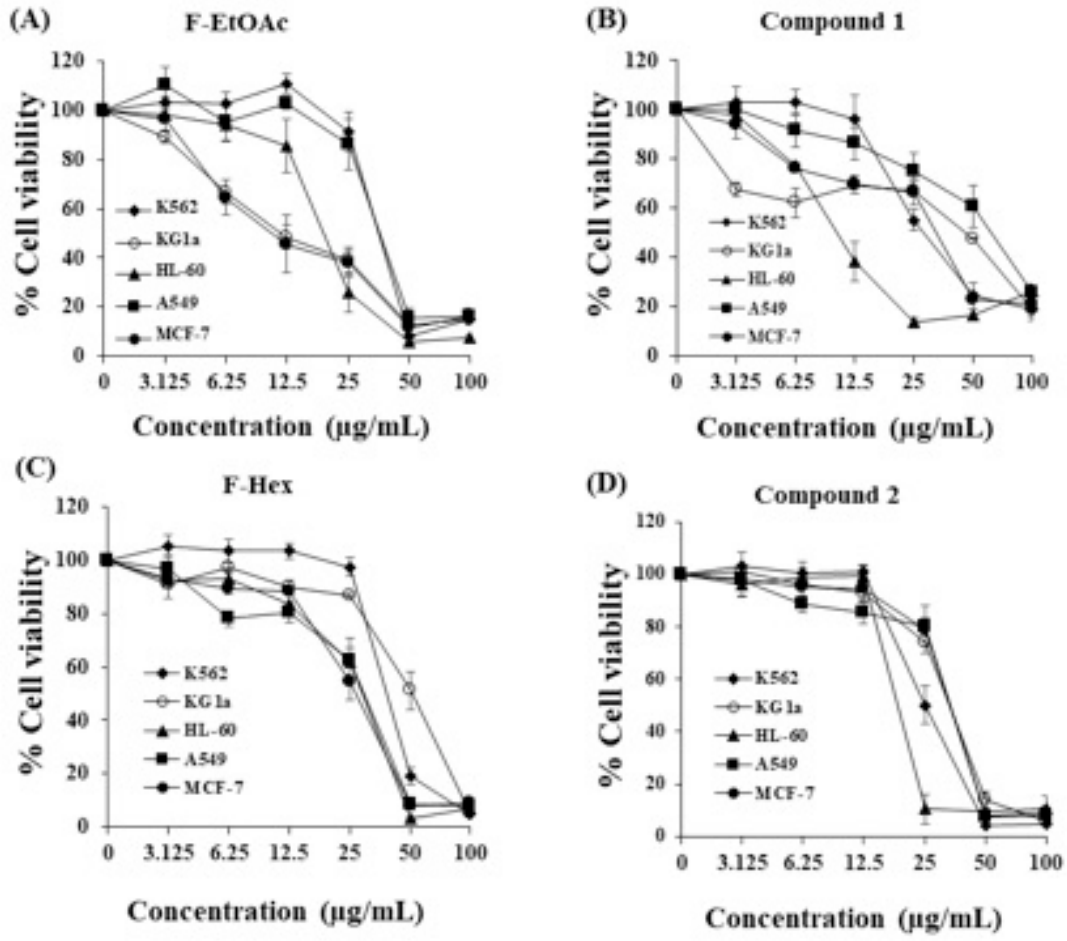


Figure 11. Cytotoxicity of crude fractional extracts and pure compounds from *C. comosa* to cancer cell lines. K562, KG-1a, HL-60, A549, and MCF-7 cells were treated with (A) ethyl acetate fraction (F-EtOAc), (B) compound **1**, (C) hexane fraction (F-Hex), and (D) compound **2** for 48 hr. The cell viability was determined by the MTT assay. Each point represents the mean ± standard deviation (SD) of three independent experiments, each performed in triplicate.

3. Cytotoxicity of PBMCs.

The cytotoxicity of F-Hex, F-EtOAc, and two purified active compounds were evaluated in peripheral blood mononuclear cells (PBMCs). PBMCs are collected from the volunteers

including 3 males and 2 females. The IC50 values of F-EtOAc, compound 1, F-Hex, and compound 2 on PBMCs were 30.91 ± 8.47, 22.40 ± 0.85, 44.38 ± 6.08, and 38.40 ± 1.50 µg/mL, respectively. The IC50 values of PBMCs indicated that extracts from *C. comosa* had cytotoxicity on PBMCs according to National Cancer Institute reference value. However, when observed the concentration at IC20 values of KG-1a cell line for Western Blot analysis, the concentrations had no cytotoxicity to PBMCs. Cytotoxicity of fractional extracts and compounds on PBMCs are shown in Figure 12.

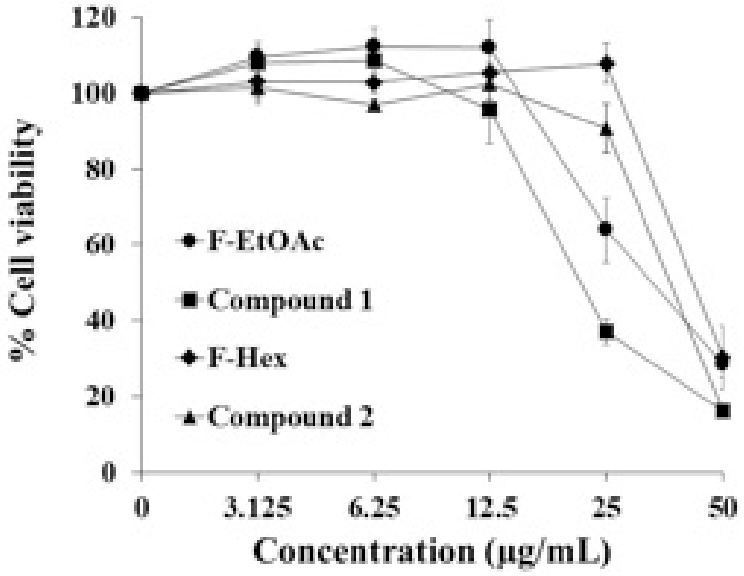


Figure 12. Cytotoxicity of crude fractional extracts and pure compounds from C. comosa to peripheral blood mononuclear cells (PBMCs). PBMCs were treated with ethyl acetate fraction (F-EtOAc), compound 1, hexane fraction (F-Hex), and compound 2 for 48 h. The cell viability was determined by the MTT assay. Each point represents mean ± SE of five independent experiments performed in triplicate.

4. Antioxidant activities determination

The antioxidant activities of purified compounds from *C. comosa* extract are shown in Table 2. Compound **1** possessed significantly higher antioxidant activities than compound **2** ($p<0.05$). Interestingly, compound **1** showed comparable TEAC and EC_1 value to that of ascorbic acid, a widely known potent antioxidant both directly via radical scavenging and indirectly through regeneration of other antioxidant systems. Therefore, compound **1** was suggested as an antioxidant with potent radical scavenging property and ferric reducing antioxidant power. Furthermore, *C. comasa* was hence a natural source of a potent antioxidant. Since antioxidants had an ability to reduce the oxidative stress in cells, they were hence useful for the treatment of various condition, e.g. cancer, cardiovascular diseases, gastrointestinal diseases, inflammation, neurodegenerative diseases. Compound-092 or compound **1** in this study, (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol) decreased GSH level but did not significantly increase the intracellular ROS. Compound **1**, which was a potent antioxidant, would have a beneficial effect on human health.

Table 2. Antioxidant activity of compound **1** and compound **2**

Samples	**TEAC (µM Tolox/g extract)**	**EC_1 (mM FeSO4/g extract)**	**IC_{50} DPPH (µg/mL)**
Ascorbic acid	1.7 ± 0.2^{a}	23.7 ± 0.7^{a}	13.9 ± 0.5^{a}
Compound **1**	0.9 ± 0.2^{a}	23.2 ± 1.0^{a}	13.0 ± 0.3^{b}
Compound **2**	0.1 ± 0.0^{b}	1.5 ± 0.0^{b}	$> 100^{c}$

Results expressed as mean ± SD of triplicate samples. Superscript letters (a, b, and c) within the same column denote significant differences in means between different samples determined by one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

The dose response curve on RAW 264.7 cell viability of purified compounds from *C. comosa* extract is shown in Figure 13. The IC_{20} values, which were the concentration that 80% of RAW 264.7 cells were viability, of compound **1** and **2** were 18.47 ± 0.36 and 26.61 ± 0.40 μg/ mL, respectively. Therefore, compound **2** tend to be safer on RAW 264.7 cells than compound **1**. The concentration at IC_{20} value of each sample was used in further anti-inflammatory activity determination.

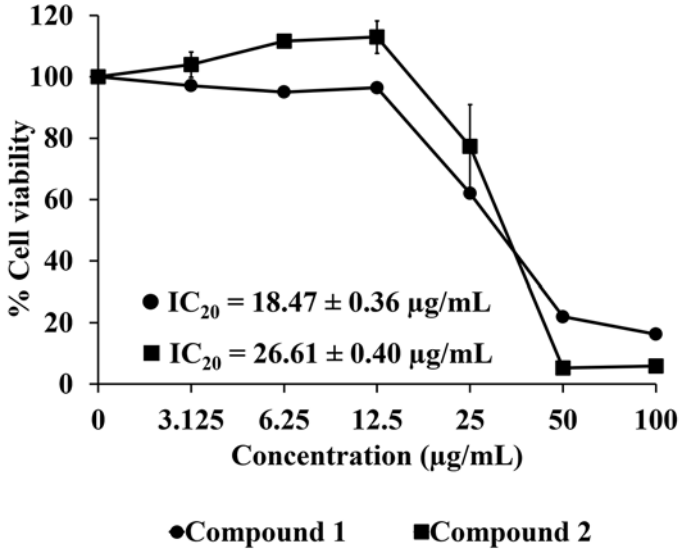


Figure 13. The effect of compound **1** and compound **2** on viability of RAW 264.7 cell lines by MTT assay. Each point represents mean ± SD of three independent experiments performed in triplicate.

5. Anti-inflammatory activities determination

Compound **1** possessed the potent inhibitory activities against both IL-6 and TNF-α. Interestingly, the IL-6 inhibition of compound **1** were significantly more potent than that of dexamethasone, a well-known anti-inflammatory drug (p<0.05). In addition, compound **1** exhibited a comparable TNF-α inhibition to that of dexamethasone (p>0.05). On the other hand, compound **2** had no effect on IL-6 and only slight inhibitory effect on TNF-α secretion. Therefore, compound **1** was suggested as a potent anti-inflammatory agent against both IL-6 and TNF-α mediated inflammation. Since IL-6 and TNF-α plays an important role on various diseases related to the inflammatory process and autoimmune diseases, such as rheumatoid arthritis, asthma, diabetic, nephropathy, etc., the biological active compounds that possessed inhibitory effect on IL-6 are increasingly considered as therapies for chronic diseases, as well as cancer. Diarylheptanoids from *C. comosa* was reported to inhibit lipopolysaccharide-induced nitric oxide production in macrophage RAW 264.7 cells. Moreover, pretreatment with hexane or ethanol extract or two diarylhepatanoids (5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene) of *C. comosa* was reported to significantly decrease the release of TNF-α and IL-1β from phorbol-12-myristate-13-acetate (PMA)-stimulated PBMC and U937 cells. The present study suggested that compound **1** could be used as an alternative natural compound for treating these conditions.

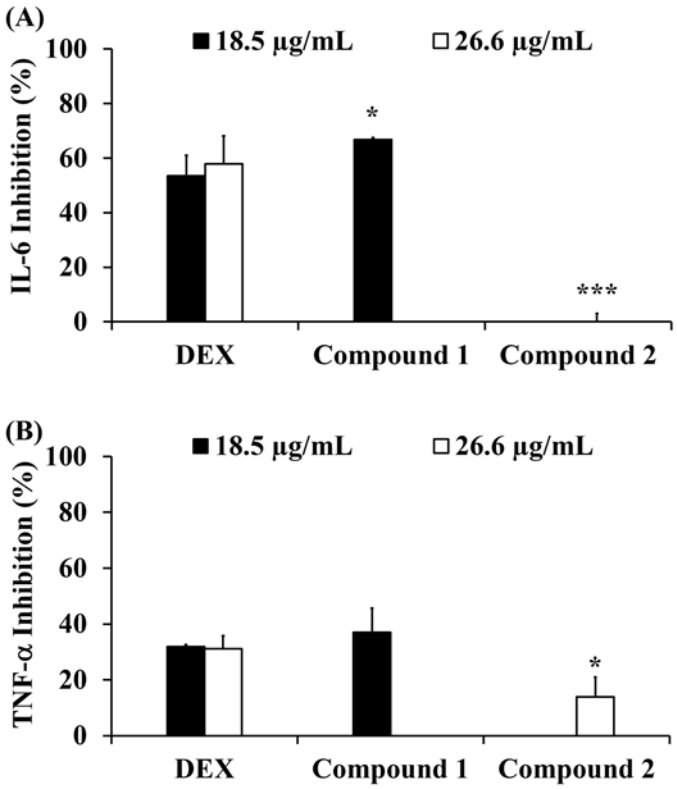


Figure 14. (A) IL-6 and (B) TNF-α inhibition of dexamethasone (DEX), compound **1**, and compound **2**. Each point represents mean ± SD of three independent experiments performed in triplicate. Asterisks (*) designate significant differences between the purified compounds from *C. comosa* extract and dexamethasone. (*p<0.05 and *** p<0.001).

6. RBC hemolysis induction

Hemolysis is the destruction of red blood cells which cause the release of hemoglobin and can cause hemolytic anemia. Hemolysis is caused by drugs or toxins in the body, blood disorders, or infection, and thus, the effects of crude extracts, fractions, and purified compound extracts on red blood cell hemolysis should be determined before prior use. A red blood cell hemolysis assay was performed to determine the effects of F-EtOAc, Compound **1**, F-Hex, and Compound **2** on red blood cells. A 0.05% Triton X-100 and 0.9% normal saline solution were used as positive (100% hemolysis) and negative controls (0% hemolysis). Figure 15 showed the

effect of F-EtOAc, Compound **1**, F-Hex, and Compound **2** at indicated doses on red blood cell hemolysis. Interestingly, at all concentration tested of F-EtOAc, Compound **1**, F-Hex, and Compound **2** showed less than 5% hemolysis, suggesting that they are not hemolysis inducing agents.

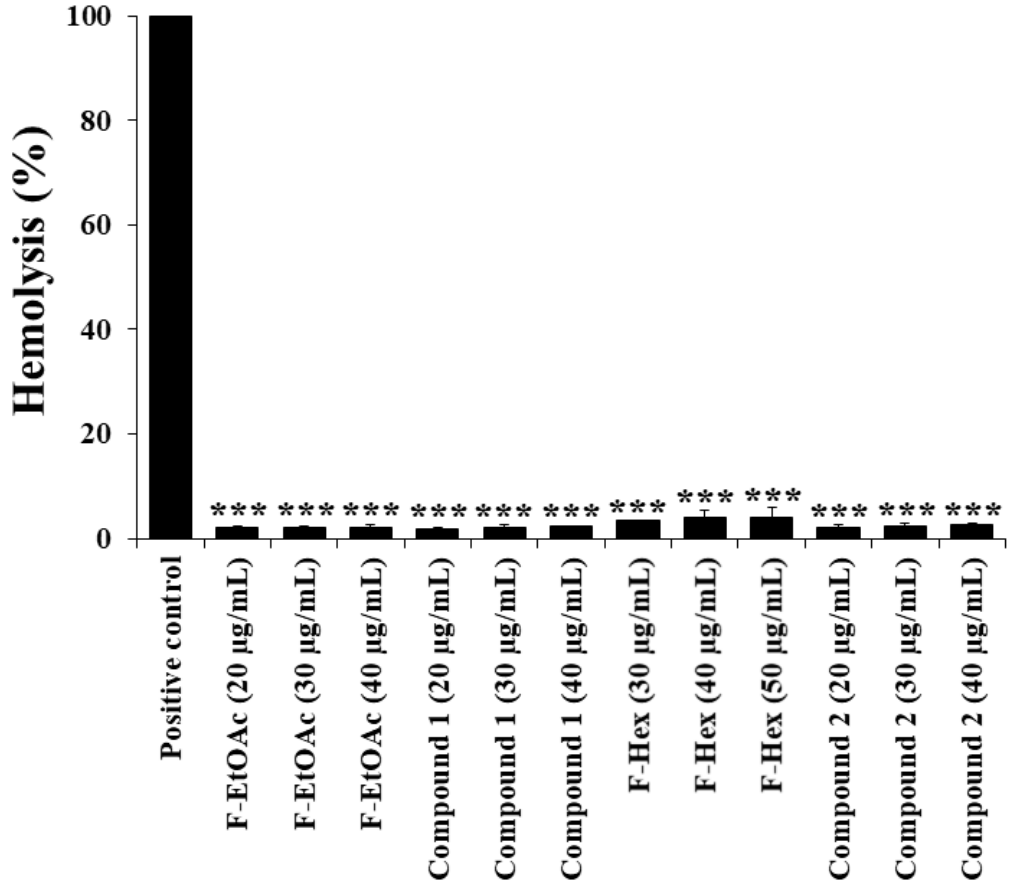


Figure 15. RBC hemolysis after incubated with F-EtOAc, Compound **1**, F-Hex, and Compound **2**. Each bar represents mean ± SD of three independent experiments performed in triplicate. Asterisks (*) designate significant differences between *C. comosa* extracts and positive control. (*** p<0.001).

7. The effect of active compound of Curcuma comosa on WT1 protein

In this study, WT1 protein was used as a biomarker of leukemic cell proliferation and determined by Western blotting. KG-1a cells were used as the leukemic cell model because this cell line has high level of WT1 protein and has capacity of self-renewal and can give rise to heterogeneous lineage of cell with abnormal proliferation and differentiation to malignant blasts, called leukemic cells. The IC_{20} values of F-EtOAc, compound **1**, F-Hex, and compound **2** (5.11, 2.50, 31.08, and 23.21 µg/mL, respectively) were used to treat and evaluate WT1 protein expressions. WT1 levels were normalized by using GAPDH protein levels. WT1 expressions after treatments were 35.93 ± 5.04, 39.48 ± 7.82, 47.60 ± 7.99, and 85.99 ± 11.27 µg/mL, respectively when compared to vehicle control (Figure 16A). The levels of WT1 protein after treatment with F-EtOAc, compound **1**, and F-Hex were significantly decreased and correlated to their effect on total cell numbers as shown in Fig. 8B. Compound **2** was observed to reduce total cell number in KG-1a cells but did not significantly decrease WT1 protein expression in this study. Thus, this result was suggested that compound **2** may involve other target proteins that related to cell proliferation. Moreover, F-Hex may contain other compounds beside of compound 2 to suppress WT1 protein expression.

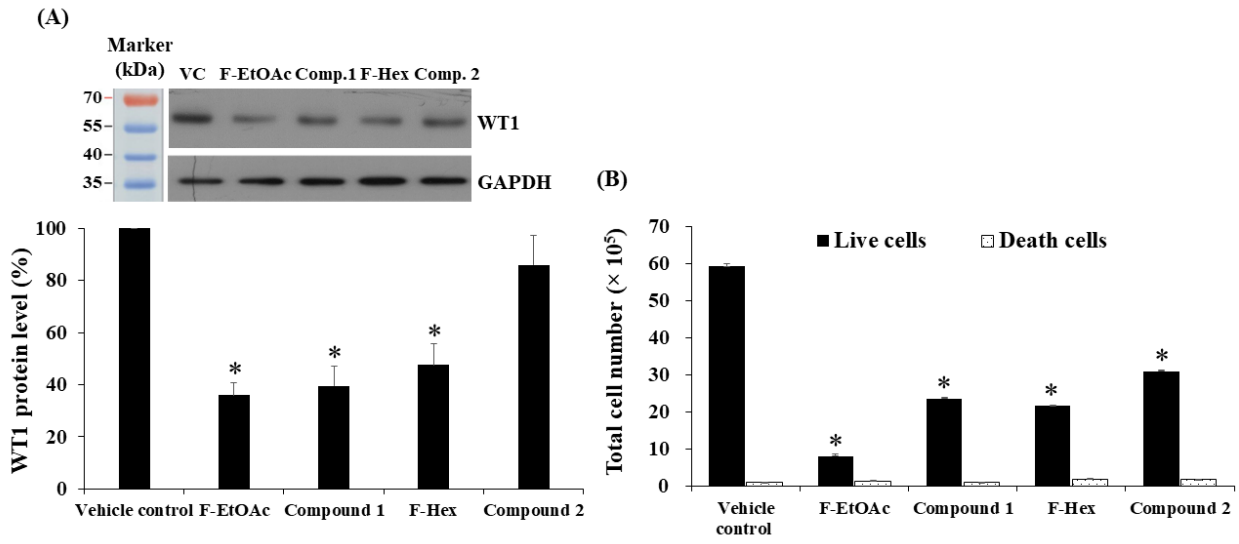


Figure 16. Effect of F-EtOAc, compound **1**, F-Hex, and compound **2** on KG-1a cells. (A) The level of WT1 protein after treatments with F-EtOAc, compound **1** (comp. **1**), F-Hex, and compound **2** (comp. **2**) for 48 h. Protein level were evaluated by Western Blot and analyzed by scan densitometer. The levels of WT1 were normalized by using GAPDH protein levels. (B) Total cell number after treatments with F-EtOAc, compound 1, F-Hex, and compound 2 for 48 h, determined by Trypan blue exclusion method. Each bar represented mean ± SD of three independent experiments performed in triplicate. Asterisks (*) designate significant differences between *C. comosa* extracts and vehicle control. (*p<0.05).

<u>Part 2</u> To study the antiproliferative effect and the molecular mechanism underlining the anticancer activity of active compounds of *Curcuma comosa* on multidrug-resistant leukemic cell line as compared to the parental cell line

1. Cytotoxic effect of *Curcuma comosa* (Wan Chak Motluk) fraction extracts on K562 and K562/ADR leukemic cell lines

To study the cytotoxic effect of *Curcuma comosa*. (Wan Chak Motluk) fraction extracts, K562 and K562/ADR cells at constant initial number (1x 10^4 cells/ml) were exposed to various concentrations prepared from *C. comosa* fraction extracts for 48 hours in the CO_2 incubator and then were assayed by MTT assay to determine cell viability after treating with each extract concentration compare to non-treated cells (Control). The results were expressed as % of control.

Various extracts concentrations (Fraction 4, 5, 6, Ethyl acetate extract, Hexane extract, Ethanol extract) were prepared from stock extracts solutions in RPMI 1640 cell culture medium supplemented with 10% FBS. The concentrations range for *C. comosa* fraction extracts was from 100 to 3.125 µg/mL. DMSO concentrations were also prepared to eliminate any DMSO toxicity in *C. comosa* extract solutions because their stock solutions were prepared in DMSO. Experimented DMSO concentrations were in the range from 1 to 0.01325% (v/v). Percentage viability at each extract concentration was calculated and plotted against extract concentrations (Figure 17, 18).

The K562 and K562/ADR cells were incubated with various concentrations of the *C. comosa* extracts as well as DMSO respective concentrations for 48 hours and then were assayed by MTT assay to determine the extracts and DMSO toxicity to the cells. The results were expressed as a mean percentage ± standard deviation (SD) of the mean negative control (untreated cells) absorbance. These data were then analyzed using GraphPad Prism software which used to construct dose response curves of the various extracts on the cells.

The *C. comosa* extracts have been dissolved in DMSO solvent and by comparison to the DMSO effect, it is clear that in the range of the inhibitory concentrations of *C. comosa* extract, the DMSO had little activity on the cells, so the dose dependent inhibition seen was attributed to the *C. comosa* extracts alone. We also tested the Cell cytotoxicity effect of chemotherapeutic agents; Doxorubicin by using MTT assay as shown in Figure 19.

Therefore, *C. comosa* fraction had cytotoxic effect on K562 and K562/ADR leukemic cell lines. Interestingly, its activity of extracts correlated to the purified compounds No.5 and 6. We found that pooled fraction No.5 from ethyl acetate fraction had the strongest activity which correlated to the previous study of Autdani *et al* in 2015.

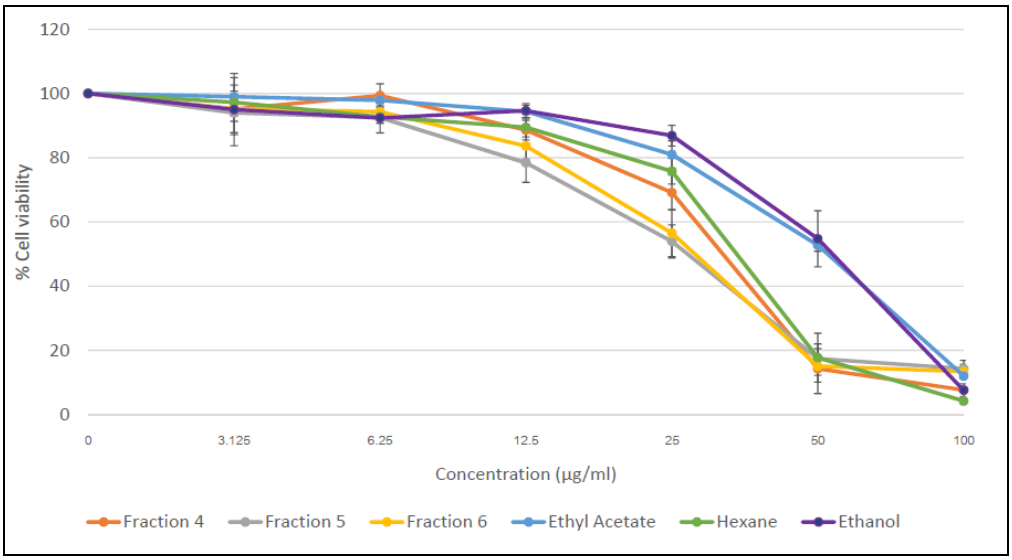


Figure 17. Effect of *C. comosa* fraction on K562/ADR leukemic cell line. Cytotoxic effect was measured using MTT assay after treatment with *C. comosa* fraction. (Fraction 4, 5, 6, Ethyl acetate, Hexane, Ethanol)

Table 3. Cytotoxic effect of *C. comosa* fraction on K562/ADR leukemic cell line

K562/ADR cell line		
Agents	**IC_{50} (µg/mL)**	**IC_{20} (µg/mL)**
Fraction 4	33.77±2.54	18.24±2.79
Fraction 5	27.73±3.14	12.06±2.44
Fraction 6	28.79±3.88	13.99±1.76
Crude ethanolic extract	67.44±12.18	30.56±2.76
Hexane fractional extract	36.20±2.47	21.42±2.90
Ethyl acetate fraction	52.29±1.72	29.09±1.02

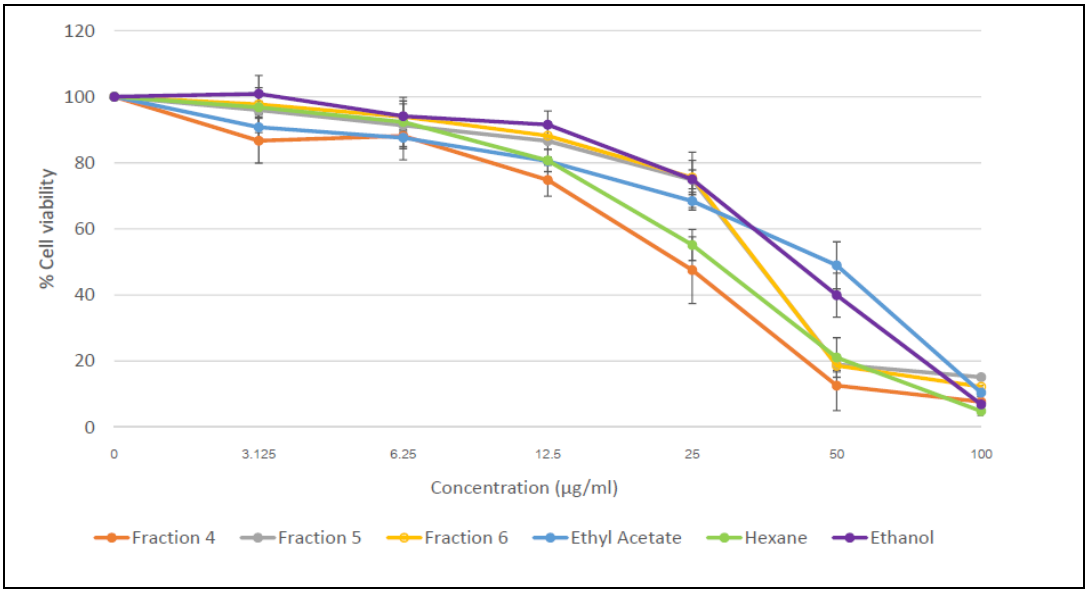


Figure 18. Effect of *C. comosa* fraction on K562 leukemic cell line. Cytotoxic effect was measured using MTT assay after treatment with *C. comosa* fraction**.**

Table 4. Cytotoxic effect of *C. comosa* fraction on K562 leukemic cell line

K562 cell line		
Agents	**IC_{50} (µg/mL)**	**IC_{20} (µg/mL)**
Fraction 4	32.88±9.66	11.30±1.70
Fraction 5	38.14±3.99	23.77±9.19
Fraction 6	36.15±1.53	23.14±7.85
Crude ethanolic extract	43.09±3.78	28.17±7.24
Hexane fractional extract	32.18±6.71	19.45±3.42
Ethyl acetate fraction	40.43±6.71	11.64±3.47

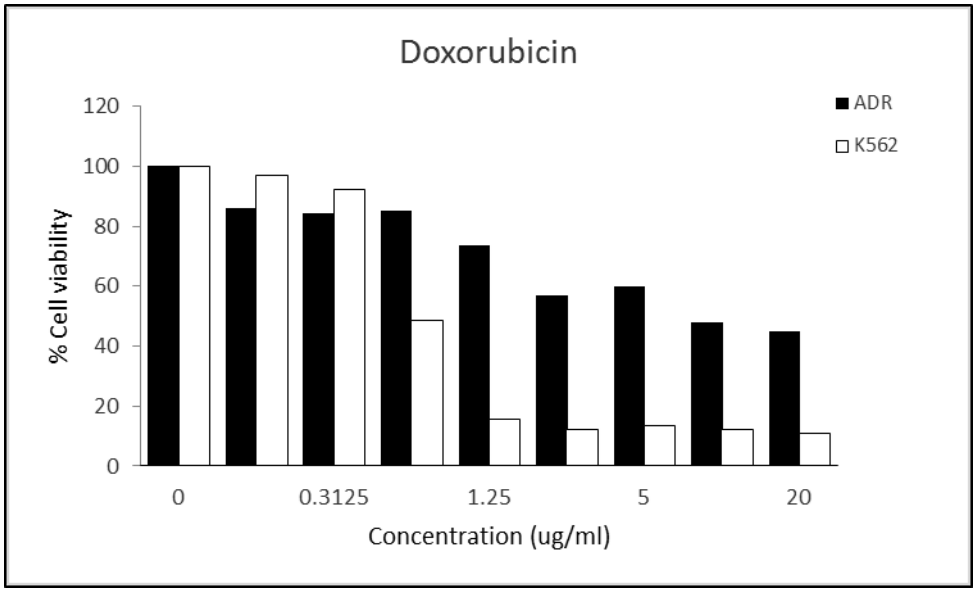


Figure 19. The cytotoxic effect of doxorubicin in K562 and K562/ADR cells.

2. Assessment of *C. comosa* ethyl acetate extract effects on peripheral blood mononuclear cells (PBMCs) by MTT assay.

The degree of toxicity expressed by extracts of *C. comosa* on K562, K562/ADR cells by means of MTT assay have been investigated as seen in the previous section. Investigation of the possible expression of toxicity of the extracts in normal, healthy human cells was judged as the next logical step. Extracts concentrations ranges tested on PBMCs was from 100 to 3.125 µg/mL. DMSO concentrations were from 1 to 0.01325% (v/v). Percentage viability at each extract concentration was calculated and plotted against extract concentrations. PBMCs were separated by Ficoll-hyplaqe and the effect of the *C. comosa* extracts on them was assessed by MTT assay after exposing the cells to various extract concentrations as with K562 and K562/ADR cell. Dose response curves of the extracts effects on PBMCs are shown in Figure 20. We also tested the DMSO effect on peripheral blood mononuclear cells (PBMCs), since we used DMSO as a diluent for preparation of *C. comosa* extract solutions as shown in Figure 21.

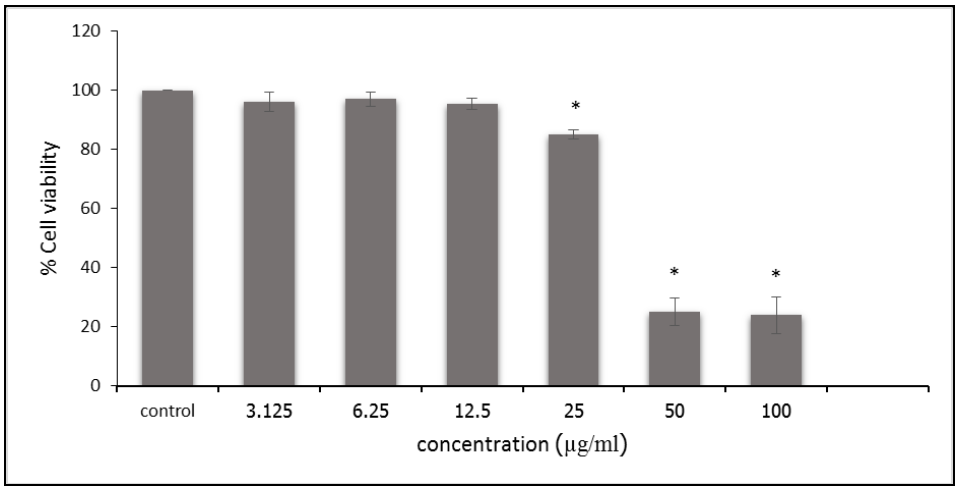


Figure 20. Assessment of *C. comosa* ethyl acetate extract effects on peripheral blood mononuclear cells (PBMCs) at final concentration 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL by MTT assay

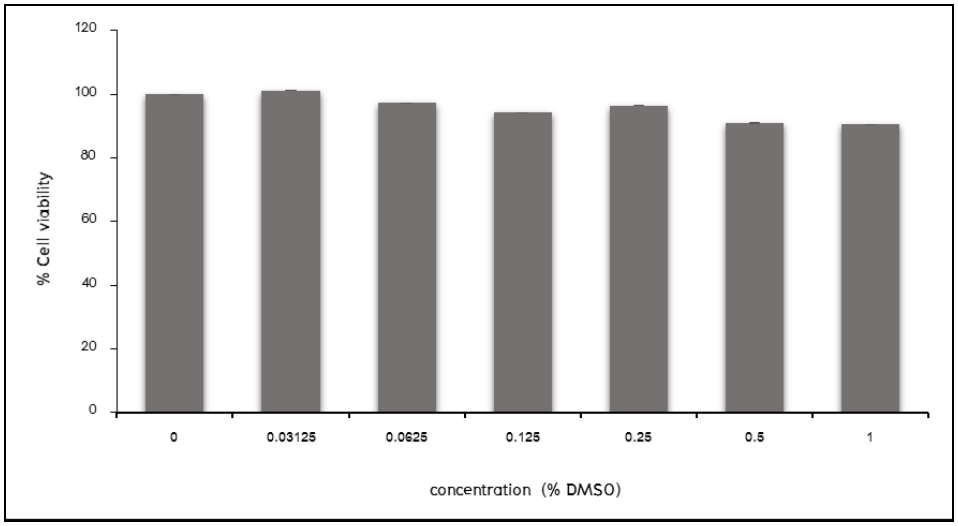


Figure 21. Assessment of DMSO effects on peripheral blood mononuclear cells (PBMCs) at final concentration 0.03125, 0.0625, 0.125, 0.25, 0.50 and 1 % by MTT assay

3. Effects of crude fractional extracts and purified compound from *C. comosa* on P-gp and NPM expression in K562/ADR leukemic cell line

P-gp or MDR1 protein is the crucial protein that involved in drug resistant mechanism of cancer cell. Therefore, P-gp protein is the drug target for cancer therapy especially in multidrug-resistant leukemic K562/ADR. Since NPM is highly express in leukemic cells and multidrug-resistant leukemic cells and also serve as a potential moderator in drug resistant process of MDR-resistant leukemic cells, using Thai natural products to target NPM may suppress the growth of leukemic cell lines and their corresponding MDR-resistant cell lines.

To study the ability of crude fractional extracts and purified compound of *C. comosa* on P-gp and NPM expression. K562/ADR were incubated with IC20 concentration of the compounds of *C. comosa*. Treated cells were measured the expression of P-gp and NPM protein by western blot analysis. The results are compared between control cells and treated cells.

From our result as shown in figure 22, IC_{20} value of each extract was further treated K562/ADR leukemic cell line and the result showed that pooled fraction No. 6 is the most effective extract for p-gp expression suppression. Moreover, The IC20 values (non-toxic concentration of extract) were used as concentrations for treatment and evaluation of NPM expression in K562/ADR cells. Crude ethanolic extract, hexane fractional extract, ethyl acetate fraction, pooled fraction No. 4, 5, and 6 were used at the concentration of 34, 15, 26, 17, 18 และ 18 µg/ml, respectively. The levels of NPM proteins was normalized by using GAPDH protein. The percentages of NPM suppression were 28.0±18.3, 29.53±10.50, 38.2±5.0, 38.5±4.7, 39.2±2.5, and 10.4±9.0% when compared to vehicle control, in response to crude ethanolic extract, hexane fractional extract, ethyl acetate fraction, pooled fraction No. 4, 5, and 6 respectively. Pooled fraction No. 5 was the most effective compound for NPM expression suppressions in K562/Adr cells. As shown in figure 23.

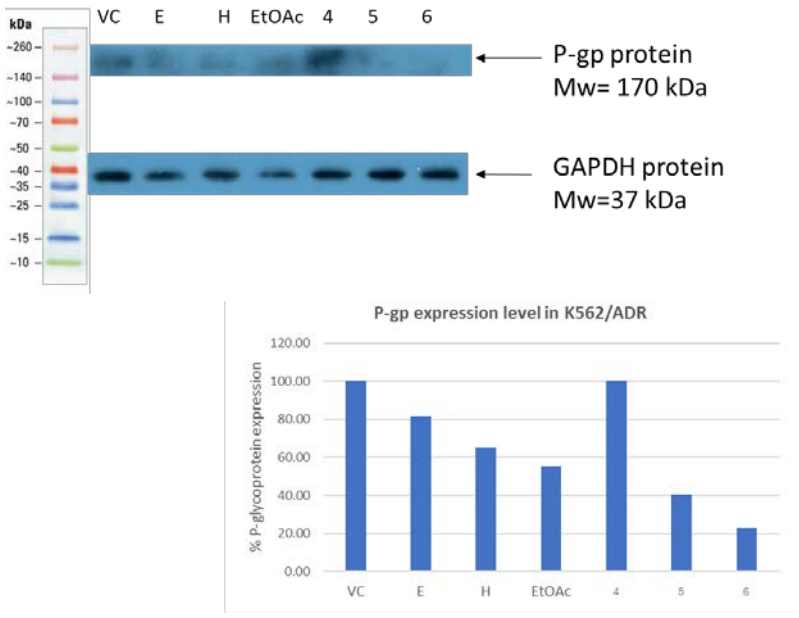


Figure 22. The effect of extracts from *C. comosa* on the expression of p-gp protein in K562/ADR cell line **(**VC: vehicle control, E: ethanol fraction, H: hexane fraction, EtOAc: ethyl acetate fraction, 4: fraction 4, 5: fraction 5, 6: fraction 6)

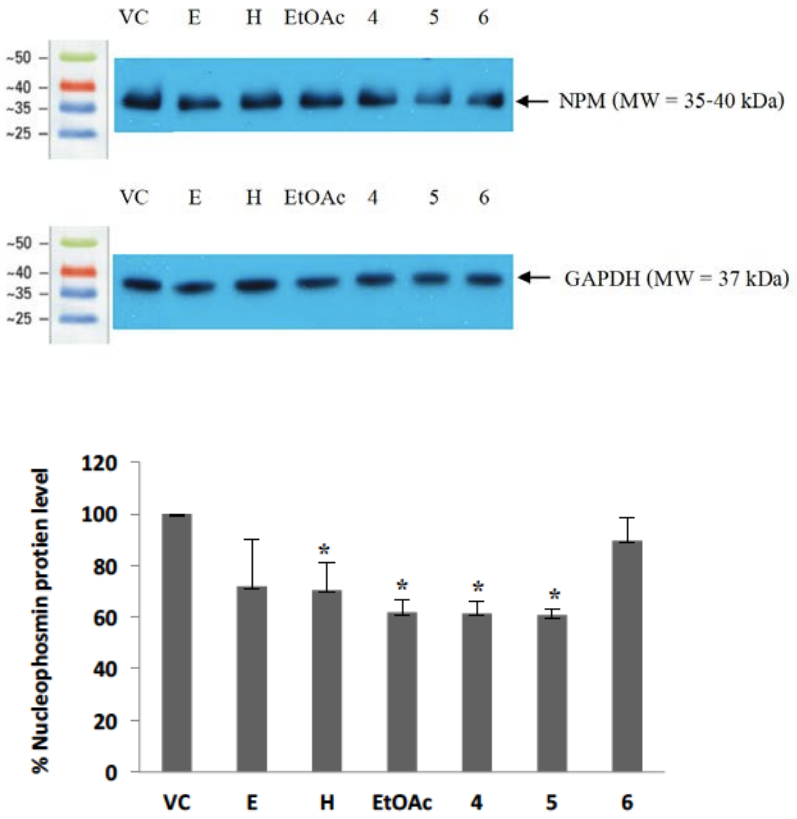


Figure 23. The effect of extracts from *C. comosa* on the expression of NPM protein in K562/ADR cell line (VC: vehicle control, E: ethanol fraction, H: hexane fraction, EtOAc: ethyl acetate fraction, 4: fraction 4, 5: fraction 5, 6: fraction 6)

4. Effect of *C. comosa* extracts on cell cycle distribution in K562 and K562/ADR leukemic cell lines.

Cell cycle analysis of the compounds of *C. comosa* treated cells. After treated with *C. comosa* extracts, K562/ADR was subjected for cell cycle analysis by using CycleTESTTM PLUS DNA Reagent Kit following the manufacturer's instructions. The results are compared between control cells and treated cells. The cell cycle/DNA content histograms represent the amount of cell population at each level of cellular DNA content in each treated group.

The pooled fraction No. 5 was examined for cell cycle by using IC_{20} value and IC_{50} value, respectively. We found that the blockage at G2/M was observed in fraction 5 treated groups. Therefore, fraction 5 could inhibit the cell cycle at G2/M phase as shown in figure 24.

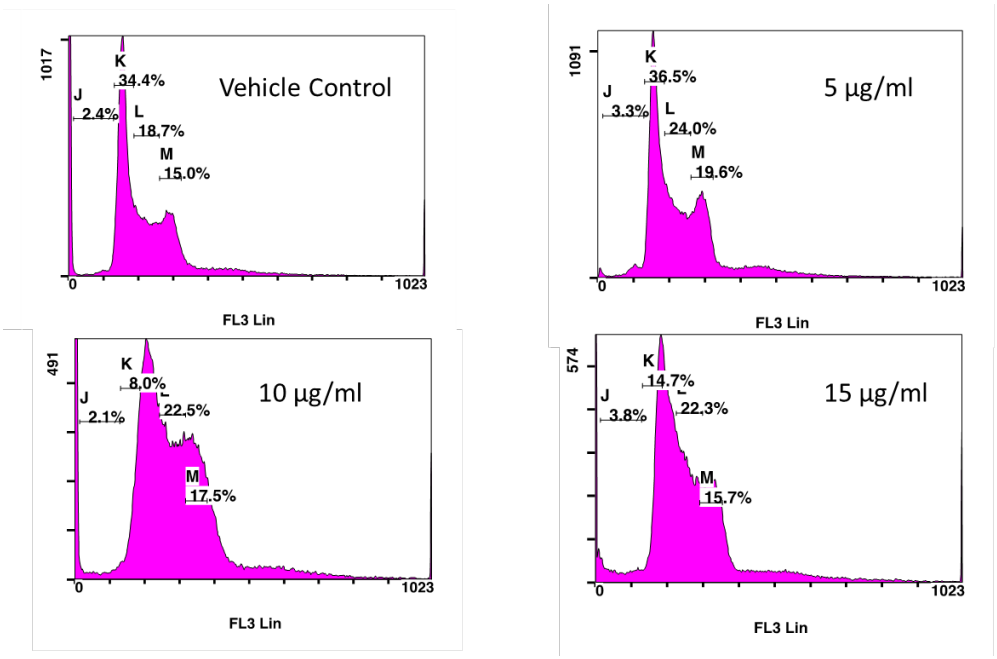


Figure 24. Effect of fraction 5 on cell cycle distribution in K562/ADR leukemic cell lines. K562/ADR were treated with 5, 10, and 15 µg/mL of fraction 5 for 48 hr, and then stained with propidium iodide (PI) before subjected to flow cytometric analysis.

5. Effect of *C. comosa* extracts on cell apoptosis in K562 and K562/ADR leukemic cell lines.

Assay of cell apoptosis induced by active compounds of C. comosa. Apoptotic cells were detected using an Annexin-V-FITC Apoptosis Detection Kit (ACT Gene) following the manufacturer's instructions. This assay revealed that the negatively charged phosphatidylserine found on the interior surface of the plasma membrane of the cells is translocated to the cell surface during apoptosis. After 48 hr incubation with 10, 20, and 30 µg/mL of fraction 5, cells were stained and subjected to bivariate flow cytometric analysis. As shown in figure 25, cell

population in all condition were already death including vehicle control so that we cannot interpreted the result from apoptosis assay. We discuss that the cause of cell death in this experiment due to the reagent that use in this study such as PBS. Therefore, the apoptosis assay should be repeat again for the final conclusion.

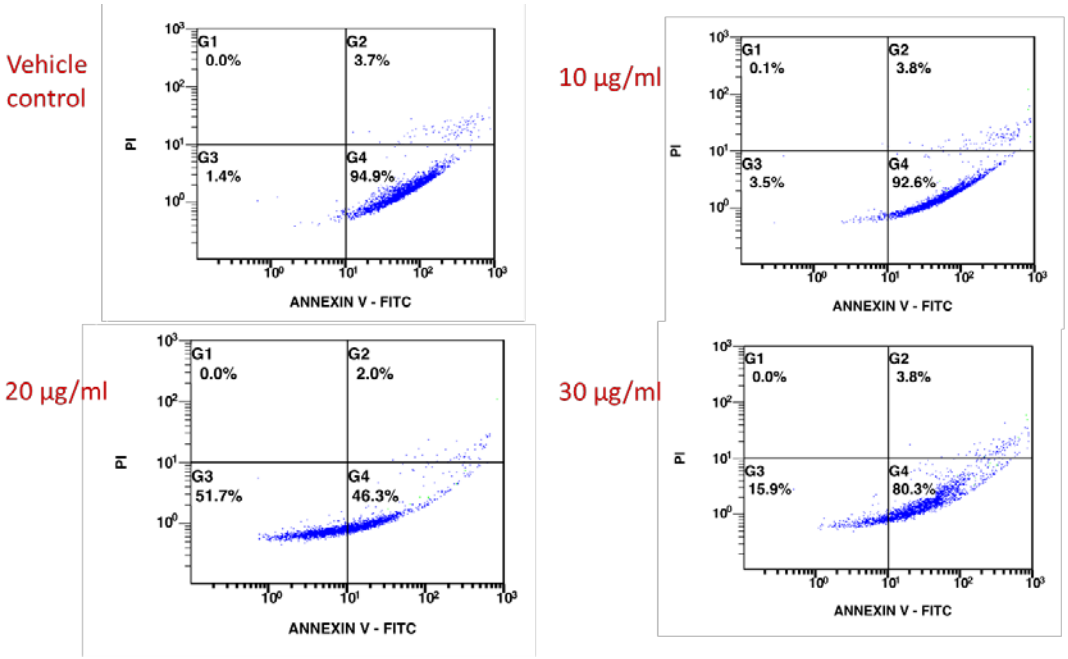


Figure 25. Effect of *C. comosa* extracts on cell apoptosis in K562/ADR leukemic cell line. K562/ADR was incubated with fraction 5 for 48 hr and apoptosis was analyzed by staining phosphatidylserine translocation with FITC-Annexin

Among all fractions, pooled fraction No.5 showed the highest cytotoxicity effect on K562/ADR with IC50 value of 38.14±3.99 µg/mL. IC20 value of K562/ADR is 38.14±3.99 µg/mL. Therefore, chromatography was done to purify active compound from ethyl acetate fraction and the structure was analyzed by Nuclear Magnetic Resonance (NMR). The result showed that the most purified compound is diarylheptanoids. It was found that diarylheptanoids the strongest cytotoxic activity on K562/ADR cells. The protein expression level of P-glycoprotein (P-gp) and Nucleophosmin, cell cycle and apoptosis assay were determined in

K562/ADR after treatment with diarylheptanoids. Our results show that the expression of P-glycoprotein and Nucleophosmin were inhibited by diarylheptanoids. Moreover, cell cycle analysis and annexin V assay indicated that diarylheptanoids also interferes cell cycling at G2/M period within 24 hours and also increase apoptosis cell fraction in K562/ADR cell line. Our finding lead to the new knowledge of anticancer mechanism of active compounds of *C.* comosa leukemic cell and multidrug resistant leukemic cell lines.

<u>Part 3</u> To investigate the effect of *Curcuma comosa* crude extract on telomerase activity and molecular pathway in human leukemic cell line

We also tested the effectiveness of ethyl acetate extract from *Curcuma comosa* on inhibition of telomerase activity in HL-60 cell lines. Telomerase, the enzyme responsible for maintaining telomeres, is highly expressed in over 75% of leukemia and provide the leukemic cell's ability to survive and proliferate. Therefore, the development of agents having activity against telomerase may be a productive approach to develop novel leukemia therapies. In this study, we determined the anticancer activity of Thai medicinal plant (*Curcuma comosa*) on telomerase activity and the expression of genes associated with telomerase in human leukemic cell lines (HL-60). HL-60 cells were treated with ethyl acetate extract from *C. comosa* at various concentration. Telomeric Repeat Amplification Protocol (TRAP) assay and Real Time RT-PCR were performed to investigate telomerase activity and the expression of human telomerase reverse transcriptase gene (*hTERT*), respectively. Since c-Myc is khown to regulate *hTERT*, expression of *c-Myc* was also determined.

1. To study the antiproliferative effect of *Curcuma comosa* crude extracts on human promyelocytic leukemic cell line

MTT assay cell survival was determined by using the MTT assay. HL-60 cell were treated with crude extracts at various concentrations. HL-60 cells were treated with ethyl acetate extract from *C. comosa* at various concentration. The results shown that 4 fraction exhibit a cytotoxic effect on HL-60 cell. Inhibitory concentration at 50 % (IC50) of ethanol, hexane, ethyl acetate and methanol fraction was 23.81 ± 2.10 µg/ml, 27.19 ± 1.66 µg/ml, 19.93 ± 1.28 µg/ml and 47.16 ± 1.81 µg/ml respectively. The results were shown in figure 26-29.

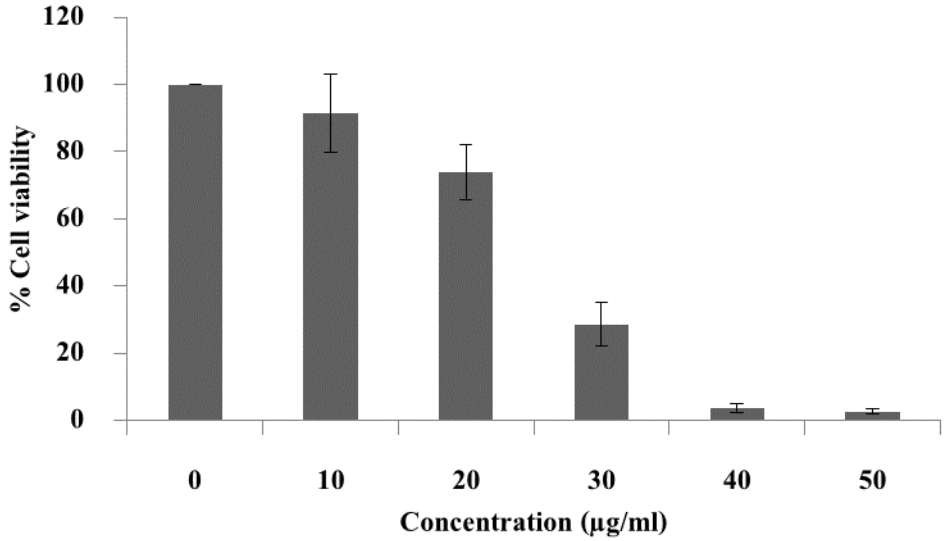


Figure 26. Antiproliferative effects of ethanol extract on the HL-60 cell.

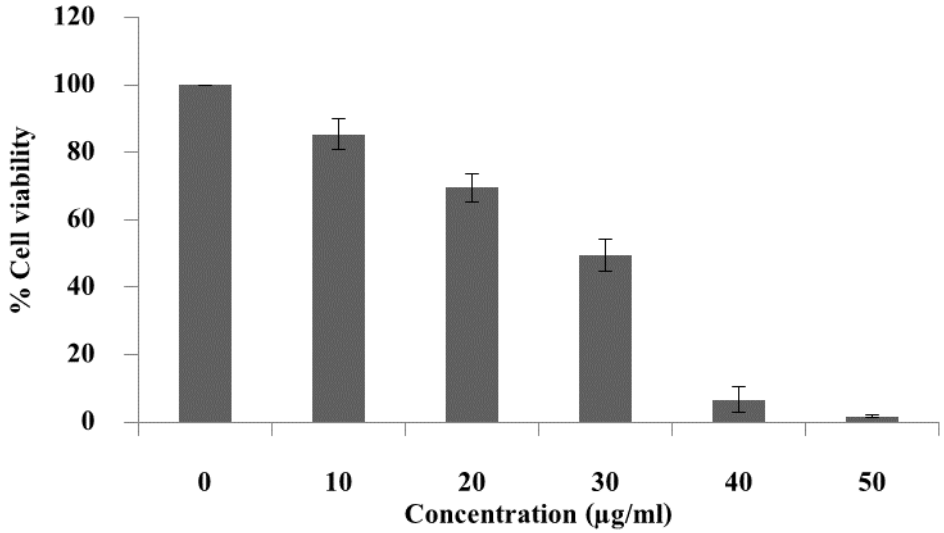


Figure 27. Antiproliferative effects of hexane extract on the HL-60 cell.

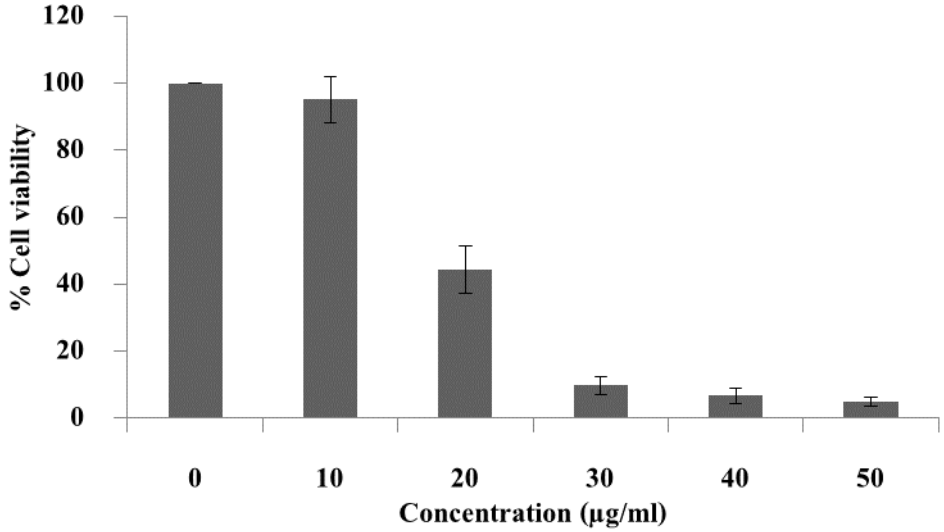


Figure 28. Antiproliferative effects of ethyl acetate extract on the HL-60 cell.

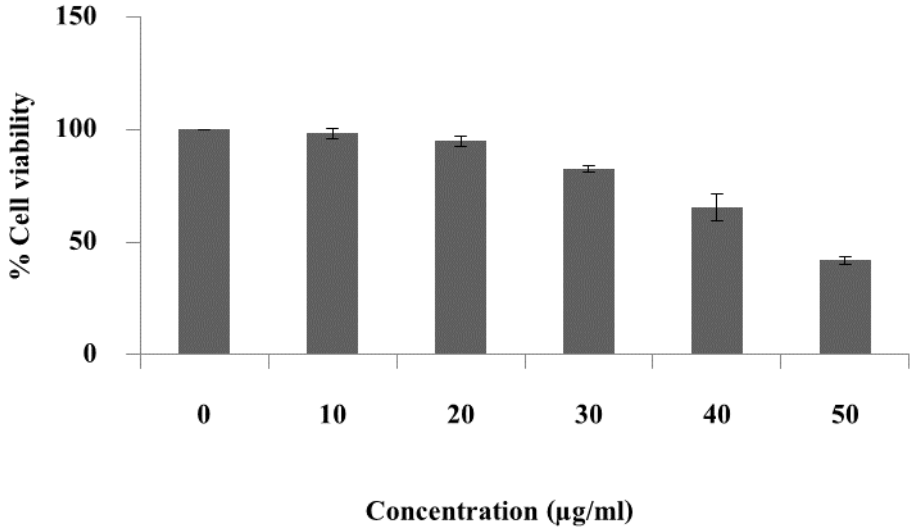


Figure 29. Antiproliferative effects of methanol extract on the HL-60 cell.

2. Apoptosis analysis

In order to investigate apoptosis induction, HL-60 cell were treated with crude extracts, otherwise cells treated with Cyclophosphamide (chemotherapeutic drug) which used as positive control then detected by DAPI staining techniques. Apoptosis was observed by morphological changes of the cells after DAPI staining under fluorescence microscope. Flow cytometry was used to study the effect of crude extracts on cell cycle. HL-60 cell were treated with ethyl acetate extract and stained with propidium iodide and analyzed using flow cytometry. The nuclear staining showed that cells treated with crude extracts had a little effect on apoptosis. Interestingly, number of cells decreased that mean treated cells stop dividing. Ethyl acetate extract at a 19.93 ± 1.28 µg/ml can inhibit the cell cycle at G0 /G1 phase, resulting in an accumulation of cells at G0 / G1. Crude extracts of *Curcuma comosa* can effectively inhibit the proliferation of HL-60 cell line which cloud be attributed to the cell cycle inhibition. Among the 4 extracts, ethyl acetate extract has the highest effect on cell proliferation as well as can inhibit cell cycle at G0/G1 phase. Hence, Crude extracts of Curcuma comosa might be a potential way in leukemia treatment.

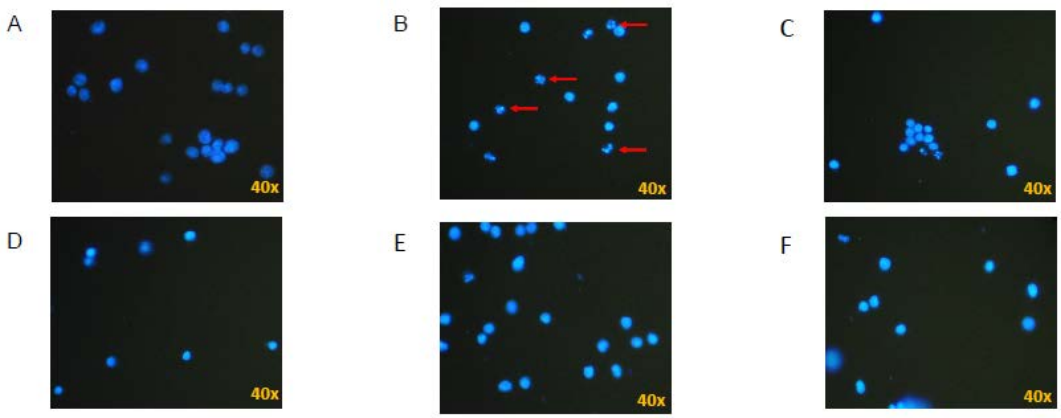


Figure 30. DAPI staining (A) untreated control cell (B) positive control cell (Cyclophosphamide) (C) ethanol 23.81 µg/ml (D) hexane 27.19 µg/ml (E) methanol 47.16 µg/ml (F) ethyl acetate 19.93 µg/ml

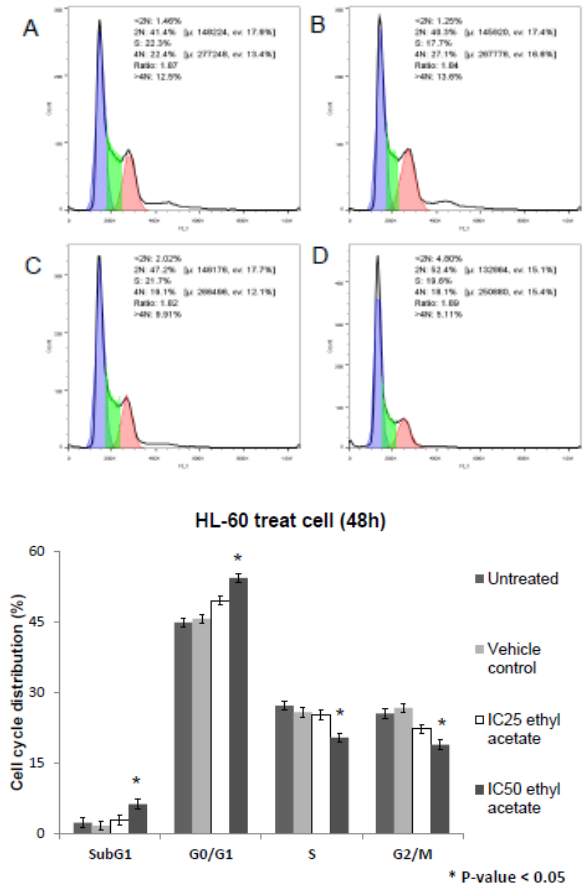


Figure 31. Cell cycle of HL-60 at 48 h treated with (A). untreated (B) DMSO0.4% (C) 14.47 µg/ml ethyl acetate (D) 19.93 µg/ml ethyl acetate

3. The effect of *Curcuma comosa* crude extract on telomerase activity

Telomeric Repeat Amplification Protocol (TRAP) assay and Real Time RT-PCR were performed to investigate telomerase activity and the expression of human telomerase reverse transcriptase gene (*hTERT*), respectively. Since c-Myc is khown to regulate *hTERT*, expression of *c-Myc* was also determined. We found that telomerase activity was inhibited by ethyl acetate extract from *C. comosa* in dose dependent manner. Realtime RT-PCR was undertaken to quantitate levels of the transcripts. Figure 32 show that only two concentrations of ethanolic extract were tested in parallel with untreated cells: 10 and 20 µg/mL and incubations were

performed for 48 hr as mentioned in method part. Results from the RT-PCR confirm that ethanolic extract from *C. comosa* inhibits gene expression of hTERT and c-Myc. Expression of hTERT was significantly reduced. Moreover, not only hTERT mRNA but also c-Myc mRNA were decreased after treated with ethyl acetate extract from *C. comosa*. This result implies that the pathway of c-Myc and its downstream target, hTERT, could involve in reduction of telomerase activity in treated cells. We believe that anticancer role of ethyl acetate extract from *C. comosa* in human leukemic cells is achieved through down regulation of enzyme telomerase by suppression of c-Myc and hence decreasing expression of its target hTERT. Therefore, ethyl acetate extract from *C. comosa* has strong potential for further study on the molecular mechanism of telomerase inhibition. This important finding gives a hope that *C. comosa* active materials can be useful in leukemia treatment development.

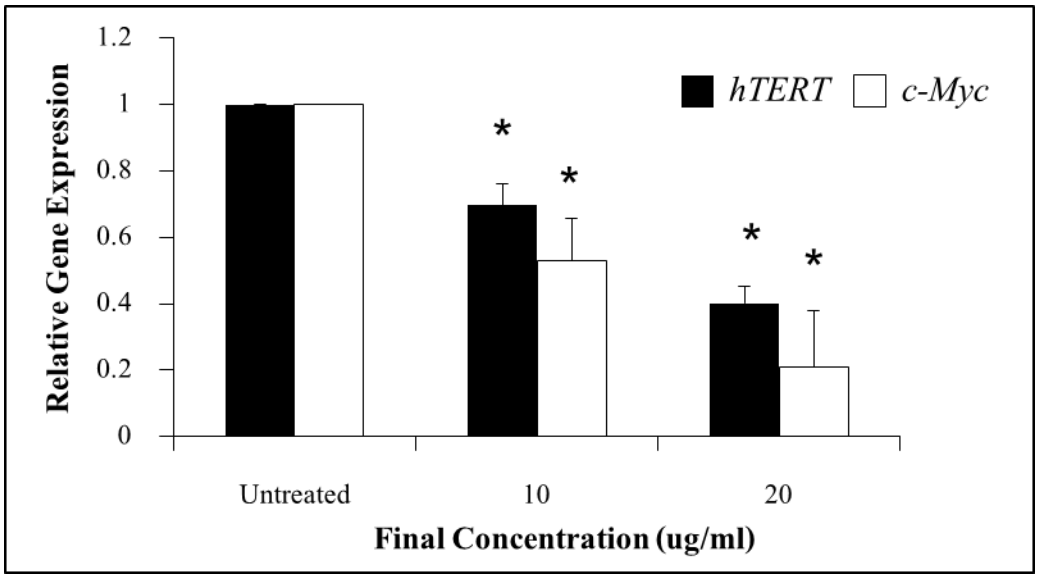


Figure 32. *hTERT* and *c-Myc* expression after treated with ethyl acetate extract.

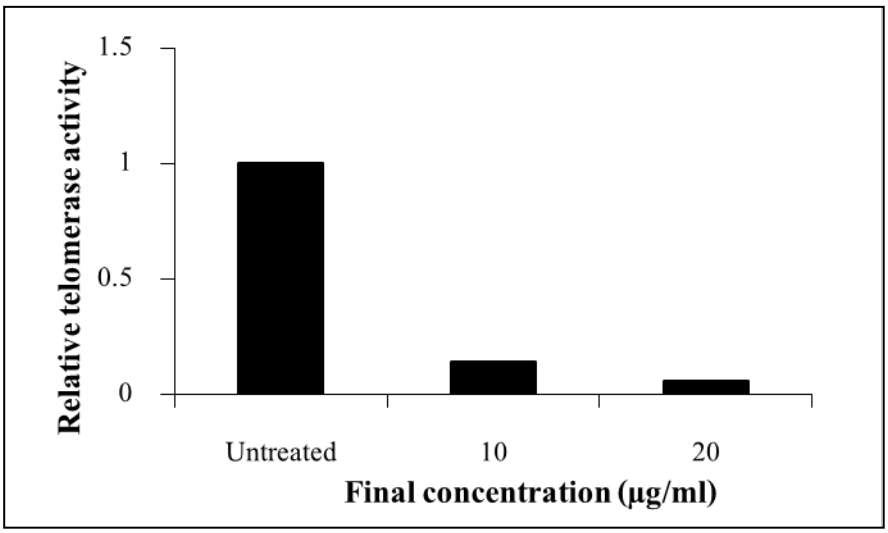


Figure 33. Effect of ethyl acetate extract on telomerase activity in HL-60 cell lines.

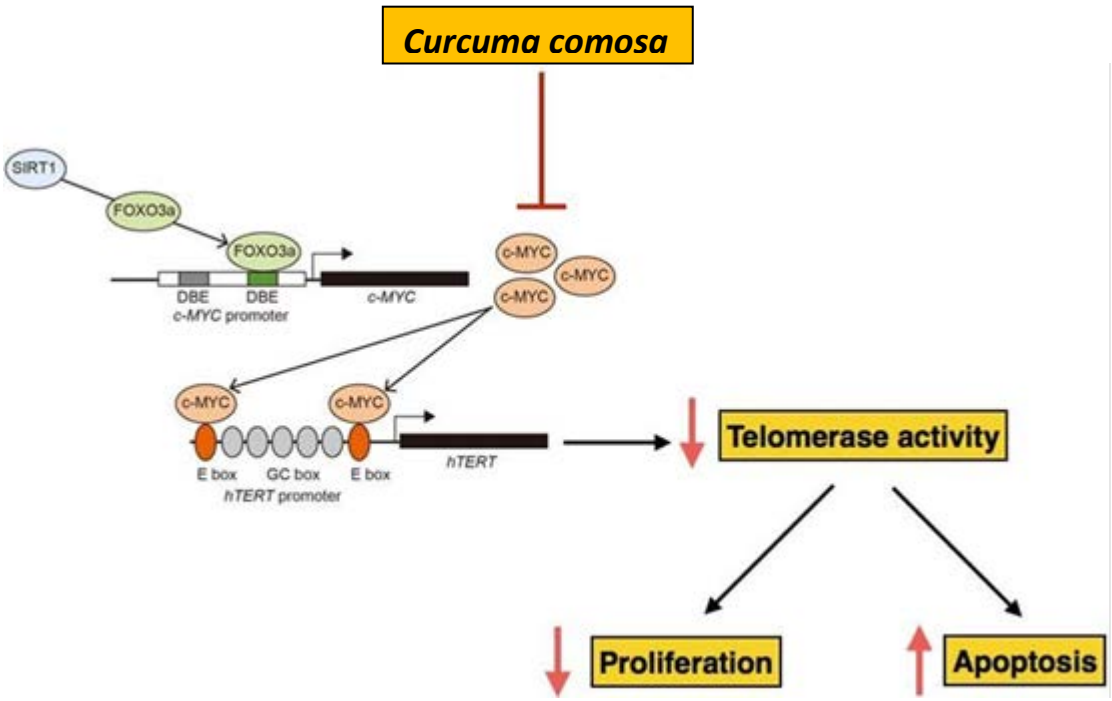


Figure 34. Hypothesized model

Discussion

Curcuma comosa belongs to the Zingiberaceae family. The rhizome of the plant has been used for medicinal purposes, in particular, to manage of the unpleasant symptoms in urogenital organ system in women in Thai traditional medicine. In this study, natural compounds were isolated from Curcuma comosa and structurally determined by spectroscopic methods and nuclear magnetic resonance. The two isolated compounds were identified as diarylheptanoids (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol (1) and diarylheptanoids (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol (2). Diarylheptanoids were isolated from the rhizomes of Curcuma comosa Roxb. Some of the isolated diarylheptanoids exhibited estrogenic activity comparable to or higher than that of the phytoestrogen genistein.8 Phenolic diarylheptanoids, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl(1E)-1-heptene and 7-(3,4- dihydroxyphenyl)-5-hydroxy-1-phenyl-(1E)-1-heptene, were isolated from the rhizomes of Curcuma xanthorrhiza. These two compounds exhibited hypolipidemic action by inhibiting hepatic triglyceride secretion (104). We show herein these diarylheptanoids were investigated for anticancer activities. Compound 1 showed the strongest cytotoxicity against HL-60 cells. The antioxidant and anti-inflammatory properties of compound 1 were higher than that of compound 2, additionally, compound 1 were also a potent antioxidant as compared to ascorbic acid. Moreover, both compounds had no effect on red blood cell hemolysis. Moreover, compound 1 had significantly affected on WT1 protein suppression and cell proliferation inhibition.

Nucleophosmin (NPM) is a major nucleolarphosphoprotein that displays a number of activities. These include a potential role as a positive regulator of cell proliferation, cell differentiation and apoptosis. Nucleophosmin is obviously over-expressed in many leukemic cells, especially in the drug resistant leukemic cell lines. It is revealed that an expression of NPM has relationship to drug resistance. Previous study indicated that down-regulation of NPM expression inhibits leukemic cells proliferation, blocks cell cycle progression and induces cellular

apoptosis in K562 leukemic cells (7). In 2013, Lin and his colleague (8) reported that knockdown of NPM by RNA interference inhibits cells proliferation, arrested cell cycle progression, induced apoptosis, promoted differentiation and also reverse multidrug resistance in drug resistant leukemic HL-60 cells. Moreover, decreased expression of NPM by RNA interference reverse the drug resistance of adriamycin-resistant Molt-4 leukemia cells through mdr-1 (P-gp) down-regulation and Akt/mTOR signaling was also reported by Wang et al (9). Recently, NPM was found as a novel BAX binding protein with this interaction involved in activation and translocation of BAX in mitochondrial dysfunction and apoptotic cell death. NPM translocates from nucleolus to cytosol, binds to BAX, and blocks mitochondrial translocation, oligomerization, and activation of BAX, thereby rendering cells resistant to death induction (10).

In this study, we found that C. comosa extracts, diarylheptanoids exhibit the antiproliferative on both K562/ADR and its parental cells line. Cell cycle analysis and annexin V assay indicated that diarylheptanoids also interferes cell cycling at G2/M period within 24 hours and also increase apoptosis cell fraction in K562/ADR cell line. To identify the target molecules of diarylheptanoids in K562/ADR cells, real-time RT-PCR, and western blot analysis were used to explore. The results revealed that the expression of nucleophosmin and P-gp decreased markedly after diarylheptanoids treatment. Nucleophosmin distributes mostly in the nucleolus of the untreated cells. It is a multifunctional phosphoprotein shuttling continuously between nucleolus and cytoplasm. This protein has an important role in controlling cellular cycling activities related to both cell proliferation and apoptosis (105). Previous studies showed that nucleophosmin is down-regulated in cancer cells during drug-induced apoptosis (106, 107). There are several anticancer drugs (i.e., actinomycin D, daunomycin, camptothecin) known to induce translocation of nucleophosmin from nucleoli to the nucleoplasm and triggers apoptosis (108-110). Thus, the changes of expression of nucleophosmin could play crucial role in apoptotic process. Our finding that diarylheptanoids could induce nucleophosmin downregulation in the

same way as the other anticancer drugs mentioned above suggests that diarylheptanoids can induce apoptosis in K562/ADR

The c-Myc has been proposed to be involved in multiple cellular functions including cell cycle regulation, differentiation and apoptosis (111, 112). c-Myc is able to bind promoter of nucleophosmin at Myc-binding site and regulates the expression of nucleophosmin (113). Recently, retinoic acid (RA) has been reported to reduce the expression of c-Myc and also affect the binding of c-Myc to nucleophosmin expression, leading to down-regulated expression of nucleophosmin (114). Thus, c-Myc is one of the factors that regulate nucleophosmin promoter. In this study, we found that the expressions of both nucleophosmin and c-Myc were decreased after diarylheptanoids treatment. Therefore, down-regulation of nucleophosmin during diarylheptanoids treatment may be a consequence of the decreased expression of c-Myc and affected binding of c-Myc to nucleophosmin promoter.

Over the last few years, telomerase has increasingly been seen as a viable target for cancer therapy, and as such there has been an increase in investigations seeking telomerase inhibitors as well as studies investigating the mode of action of these inhibitors. Inhibition of telomerase leads to progressive telomeric shortening during successive cell cycles and subsequently results in growth arrest and cell death (115). Several reports have shown that telomerase activity can be inhibited by certain medicinal plants and extracts thereof. Medicinal plants are those in which some part of the plant can be used for a therapeutic purpose, and many of the drugs in use today are derived from medicinal plants (116). Moreover, many studies seek to find new drug compounds from plants used in traditional medicine (117). In 2005, Choi and colleagues reported that costunolide, a natural sesquiterpene compound derived from the bark of Magnolia sieboldii (a member of the Magnoliaceae family, which are commonly used in oriental traditional medicine (118), inhibited the growth of a human breast cancer cell line by reducing telomerase activity (119). Similarly, Ramachandran and colleagues reported that curcumin, derived from the rhizomes of turmeric (Curcuma longa L.), a plant with a long history of natural

medicinal usage, can inhibit activity of telomerase by down-regulating hTERT expression in MCF-7 breast cancer cells (120). More recently, Mittal et al. reported the down-regulation of telomerase by (-)-epigallocatechin-3-gallate (EGCG), a constituent of green tea, in MCF-7 cells, leading to suppressed viability of the cell and the induction of apoptosis (121). In this work, we report the antiproliferative effect of the extracts derived from Curcuma comosa. This plant is widely cultivated in Asia and the Indian sub-continent, where it has a long history of medicinal use for a variety of disorders, including malignant growths (122, 123). The extract, consisting of diarylheptanoids (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol and diarylheptanoids (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol, was shown to have significant antiproliferative effect on the human human leukemic cell lines, HL-60. Cell cycle blockages at G0/G1 phase as well as a certain degree of apoptosis were seen in HL-60 cell lines. The extract of Curcuma comosa reported here affected telomerase activity as well as the expression of genes associated with regulation of this enzyme in HL-60 cell line. Expression of the hTERT gene is directly correlated with telomerase activity as the hTERT protein is the catalytic rate-limiting determinant subunit of telomerase (124). hTERT is a downstream target of c-Myc factor which binds to E-box (CACGTG) and promotes expression of the hTERT gene (125). Hence, c-Myc upregulates telomerase by enhancing hTERT expression (126, 127). Hence, it is possible that Curcuma comosa directly modulates subsequent signaling pathway as is suggested by studies that shown the down regulation of the JAK/STAT pathway (128). In both cases, C. comosa extracts could be acting through c-Myc, directly or indirectly, leading to the reduction of telomerase activity and subsequent loss of cellular proliferation, as confirmed by the finding of cell cycle G0/G1 blockage after C. comosa treatment. Inhibition of telomerase is therefore an attractive approach for leukemia therapeutic goal, and medicinal plants seem to offer a wealth of potential candidate compounds.

Conclusion

The present study identified the two isolated compounds as diarylheptanoids (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol and diarylheptanoids (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol. Both are the active components in the ethyl acetate and hexane fractional extracts of C. comosa. This is the report of diarylheptanoids isolated from the C. comosa plant which related to previous reports. Bioassays of diarylheptanoids against cancer cells confirmed its anti-leukemic, antioxidant, and anti-inflammatory activities. (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol is a potent antioxidant and a potent anti-inflammatory agent against both IL-6 and TNF-α mediated inflammation. (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-hepten-3-ol also significantly showed suppression of WT1 protein expression and cell proliferation in leukemic stem cell line. Moreover, our finding that diarylheptanoids could induce P-gp and nucleophosmin downregulation suggests that diarylheptanoids can induce apoptosis in drug resistant leukemia. Anticancer role of diarylheptanoids from C. comosa in human leukemic cells is achieved through down regulation of enzyme telomerase by suppression of c-Myc and hence decreasing expression of its target hTERT. Therefore, diarylheptanoids from C. comosa has strong potential for further study on the molecular mechanism of telomerase inhibition. Medicinal plants seem to offer a wealth of potential candidate compound. Our results suggest that active compound from C. comosa can be used as a chemotherapeutic agent for the treatment of human leukemia, particularly AML.

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the JAK/STAT pathway and potentiates antiproliferative effects of gemcitabine on pancreatic cancer cells. Cancer Res. 2009;69:5876–5884.

Appendix

Biological activity of isolated diarylheptanoids from *Curcuma comosa* rhizomes

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ABSTRACT

Curcuma comosa belongs to the Zingiberaceae family. The rhizome of the plant has been used for medicinal purposes, in particular, to manage of the unpleasant symptoms in urogenital organ system in women in Thai traditional medicine. In this study, natural compounds were isolated from *Curcuma comosa* and structurally determined by spectroscopic methods and nuclear magnetic resonance. The two isolated compounds were identified as diarylheptanoids (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol (**1**) and diarylheptanoids (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol (**2**). These diarylheptanoids were investigated for anticancer activities. Compound **1** showed the strongest cytotoxicity against HL-60 cells. The antioxidant and anti-inflammatory properties of compound **1** were higher than that of compound **2**, additionally, compound **1** were also a potent antioxidant as compared to ascorbic acid. Moreover, both compounds had no effect on red blood cell hemolysis. Moreover, compound **1** had significantly affected on WT1 protein suppression and cell proliferation inhibition. In summary, compound **1** with cancer cytotoxicity against the cancer cell lines, high antioxidant activity, and high anti-inflammatory were identified in pure compound from *Curcuma comosa*.

Keywords: Zingiberaceae; *Curcuma comosa*; diarylheptanoids; cytotoxicity; antioxidant; anti-inflammatory; hemolysis

Introduction

Curcuma comosa is one of the Curcuma genus. In Thailand, *C. comosa* is commonly known as Wan Chak Motluk (Thai common name) and is found in Northern and Southeast. Rhizomes are round without horizontal branch, brown inside, and aromatic smell. It has been used as folk medicine in women for management of the unpleasant symptoms in urogenital organ system such as vaginal dryness, dysmenorrhea (painful menstruation), amenorrhea (absence of menstruation), menorrhagia (abnormal menstruation, too much menstruation).[1] Moreover, Curcuma genus shows anti-cancer,[2] antioxidant activities,[3] and anti-inflammatory effects.[4] It also can suppress abdominal

pain and chronic pelvic disorders by enables the contractions of the organ in urogenital system.[5] It has been expected to contain bioactive compounds which have anti-cancer, antioxidant, or anti-inflammatory properties. Compound-092, (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol from *C. comosa* provide evidence for the pro-oxidant activity (GSH and ROS determination) of the diarylheptanoid bearing a catechol moiety in the induction of apoptosis in murine P388 leukemia.[6] This study will reveal the role of *C. comosa* on cancer cytotoxicity, red blood cell hemolysis, antioxidant, and anti-inflammatory activities. Furthermore, antiproliferation is also observed via WT1 protein suppression in KG-1a leukemic cell model. WT1 protein is a leukemic cell biological marker. It is involved in leukemic cell proliferation.[7]

Thus, the goal of this study is to purify, characterize the main chemical structures, and study the activities of main compounds from *C. comosa*.

Results and discussion

In the present study, 5 kilograms of dried rhizome were extracted using ethyl acetate and hexane having relative polarities of 0.228 and 0.009, respectively, compared to water (1.000). Ethyl acetate fraction (F-EtOAc) and hexane fraction (F-Hex) were obtained by maceration and evaporation. The yields of the extracts were 2.09 and 2.80%, respectively. Both fractions were separated by silica gel column chromatography which led to the isolation of two known compounds **1** and **2**. The structures of these purified compounds were identified using 1D and 2D NMR spectroscopy and confirmed by comparison of their ^{1}H and/or ^{13}C NMR data with those previously published data.

Compound **1** was obtained as brown gum. The ^{1}H NMR spectrum showed two sets of aromatic protons. The signals at δ 7.33—7.14 (m, 5H) could be the five protons of phenyl ring. Another set of aromatic protons displayed at δ 6.64 (m, 2H) for two protons and δ 6.50 for one proton (dd, J = 8.0, 2.0 Hz) which could be 1,3,4-trisubstituted aromatic rings. The two signals at δ 6.37 (d, J = 15.8 Hz) and 6.22 (dt, J = 15.8, 6.8 Hz) were the signals of trans-olefinic protons which attached to the phenyl ring. The signal at δ 3.57 (m, 1H) suggested the presence of methine proton attached to hydroxy group. The ^{13}C NMR spectrum of compound **1** displayed 19

signals for twelve aromatic carbons (δ 146.1, 144.2, 139.2, 135.3, 131.4, 131.3, 129.4 (2C), 127.8, 126.9 (2C), 120.7), two olefinic carbons (δ 116.6, 116.3), one methine carbon bearing hydroxy group (δ 71.2) and four methylene carbons (δ 40.6, 38.1, 32.4, 30.3), respectively. The absolute configuration at C-5 was assigned to be *S* by comparing optical rotation with the literature ($[\alpha]_D^{30}$ -108 in EtOH, lit $[\alpha]_D^{28}$ -5.2).[8] By comparison spectroscopic data with those reported in the literature, compound **1** were suggested to be diarylheptanoids (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol as shown in Fig.1.[8, 9]

Compound **2** was obtained as colorless oil. The ^{1}H NMR of compound **2** was similar to that of compound **1** except the signal of aromatic protons which showed ten protons. This signal indicated that compound **2** had two phenyl rings in the structure. It was also confirmed by the ^{13}C NMR which displayed the presence of only two quaternary carbons (δ 142.2 and 137.7). The absolute configuration at C-5 was also assigned to be S by comparing optical rotation with the literature ($[\alpha]_D^{30}$ -1 in EtOH, lit $[\alpha]_D^{27}$ -2.6).[8] By comparison spectroscopic data with those reported in the literature, compound **2** were suggested to be (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol as shown in Fig. 2.[8, 10] Diarylheptanoids were isolated from the rhizomes of *Curcuma comosa* Roxb. Some of the isolated diarylheptanoids exhibited estrogenic activity comparable to or higher than that of the phytoestrogen genistein.[8] Phenolic diarylheptanoids, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl(1*E*)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene, were isolated from the rhizomes of *Curcuma xanthorrhiza*. These two compounds exhibited hypolipidemic action by inhibiting hepatic triglyceride secretion.[9]

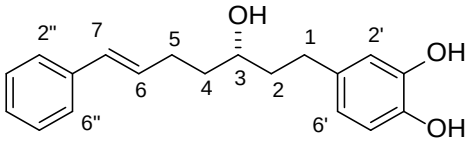


Fig. 1. The chemical structure of compound **1** (diarylheptanoids (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol)

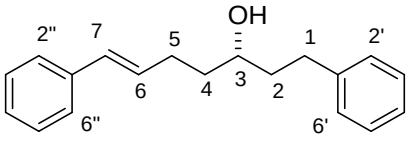


Fig. 2. The chemical structure of compound **2** (diarylheptanoids (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol)

The cytotoxicity of F-Hex, F-EtOAc, and two purified active compounds were investigated by MTT assay in K562, KG-1a, HL-60, A549, and MCF-7 cells. F-EtOAc, compound **1** (purified active compound from F-EtOAc), PPF-Hex, and compound **2** (purified active compound from F-Hex) were treated in K562, KG-1a, HL-60, A549, and MCF-7 cells. All the extracts and purified compounds show cytotoxic effects on K562, KG-1a, HL-60, A549, and MCF-7 cells by MTT assay. The IC_{50} values (inhibitory concentration at 50% growth) of F-EtOAc, compound **1**, F-Hex, and compound **2** on K562 cells were 37.35 ± 1.34, 28.92 ± 3.04, 40.52 ± 1.10, and 26.67 ± 2.81 µg/mL, respectively. The IC_{50} values of F-EtOAc, compound **1**, F-Hex, and compound **2** on KG-1a cells were 18.52 ± 7.21, 43.62 ± 6.28, 53.56 ± 6.41, and 35.21 ± 2.19 µg/mL, respectively. The IC_{50} values of F-EtOAc, compound **1**, F-Hex, and compound **2** on HL-60 cells were 19.93±1.28, 10.79±0.91, 27.19±1.66 and 19.47±0.49 µg/mL, respectively. The IC_{50} values of F-EtOAc, compound **1**, F-Hex, and compound **2** on A549 cells were 38.87±1.74, 63.49±8.71, 31.81±5.25, and 35.30±1.60 µg/mL, respectively. The IC_{50} values of F-EtOAc, compound **1**, F-Hex, and compound **2** on MCF-7 cells were 15.26±6.87, 34.28±1.34, 28.58±3.28, and 35.70±0.98 µg/mL, respectively (Fig. 3).

The cytotoxicity of (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol (compound-092, compound **1** in this study) and (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol (compound-001; compound **2** in this study) were reported on P388 leukemic cells by MTT assay with the IC_{50} values of 3.8±0.1 and 39.1±1.5 µM, respectively and therefore, induced apoptosis.[6] This result related to this study, compound **1** also showed a potent cytotoxicity against leukemic cells (HL-60) more than compound **2**. However, cytotoxicity of compound **2** was better than compound **1** in K562 and KG-1a cells. The reason of these difference responses might be from the different compounds and type of cancer cell lines.

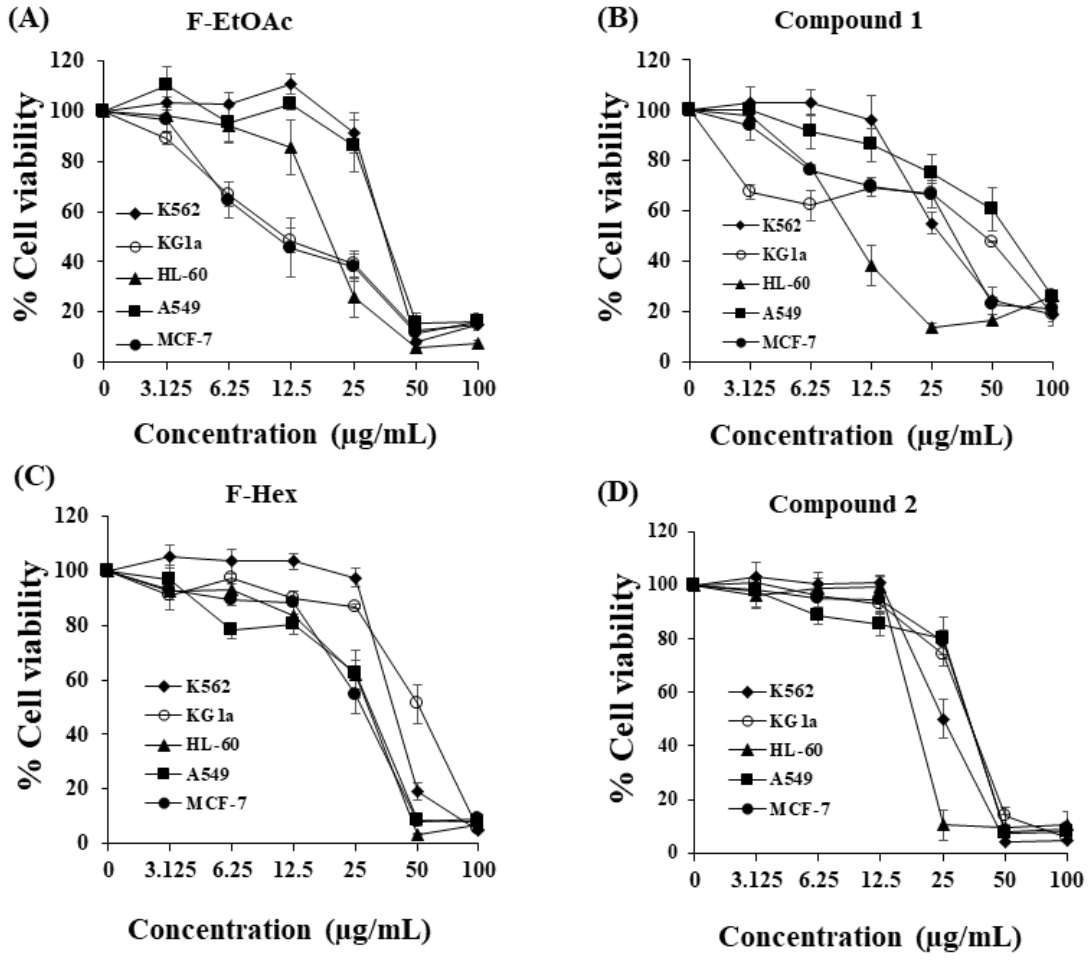


Fig. 3. Cytotoxicity of crude fractional extracts and pure compounds from *C. comosa* to cancer cell lines. K562, KG-1a, HL-60, A549, and MCF-7 cells were treated with (A) ethyl acetate fraction (F-EtOAc), (B) compound **1**, (C) hexane fraction (F-Hex), and (D) compound **2** for 48 h. The cell viability was determined by the MTT assay. Each point represents the mean ± standard deviation (SD) of three independent experiments, each performed in triplicate.

The cytotoxicity of F-Hex, F-EtOAc, and two purified active compounds were evaluated in peripheral blood mononuclear cells (PBMCs). PBMCs are collected from the volunteers including 3 males and 2 females. The IC_{50} values of F-EtOAc, compound **1**, F-Hex, and compound **2** on PBMCs were 30.91 ± 8.47, 22.40 ± 0.85, 44.38 ± 6.08, and 38.40 ± 1.50 µg/mL, respectively. The IC_{50} values of PBMCs indicated that extracts from *C.*

comosa had cytotoxicity on PBMCs according to National Cancer Institute reference value.[11] However, when observed the concentration at IC_{20} values of KG-1a cell line for Western Blot analysis, the concentrations had no cytotoxicity to PBMCs. Cytotoxicity of fractional extracts and compounds on PBMCs are shown in Fig. 4.

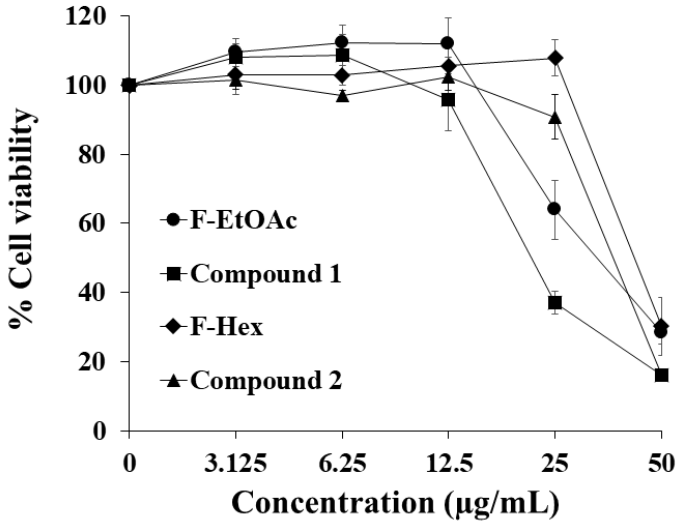


Fig. 4. Cytotoxicity of crude fractional extracts and pure compounds from *C. comosa* to peripheral blood mononuclear cells (PBMCs). PBMCs were treated with ethyl acetate fraction (F-EtOAc), compound **1**, hexane fraction (F-Hex), and compound **2** for 4 8 h. The cell viability was determined by the MTT assay. Each point represents mean ± SE of five independent experiments performed in triplicate.

The antioxidant activities of purified compounds from *C. comosa* extract are shown in Table 1. Compound **1** possessed significantly higher antioxidant activities than compound **2** (p<0.05). Interestingly, compound **1** showed comparable TEAC and EC_1 value to that of ascorbic acid, a widely known potent antioxidant both directly via radical scavenging and indirectly through regeneration of other antioxidant systems.[12] Therefore, compound **1** was suggested as an antioxidant with potent radical scavenging property and ferric reducing antioxidant power. Furthermore, *C. comasa* was hence a natural source of a potent antioxidant. Since antioxidants had an ability to reduce the oxidative stress in cells, they were hence useful for the treatment of various condition, e.g. cancer, cardiovascular diseases, gastrointestinal diseases, inflammation, neurodegenerative diseases.[13-15] Compound-092 or

compound **1** in this study, (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol) decreased GSH level but did not significantly increase the intracellular ROS.[6] Compound **1**, which was a potent antioxidant, would have a beneficial effect on human health.

Table 1. Antioxidant activity of compound **1** and compound **2**

Samples	**TEAC (μM Tolox/g extract)**	**EC_1 (mM FeSO4/g extract)**	**IC_{50} DPPH (μg/mL)**
Ascorbic acid	1.7 ± 0.2^{a}	23.7 ± 0.7^{a}	13.9 ± 0.5^{a}
Compound **1**	0.9 ± 0.2^{a}	23.2 ± 1.0^{a}	13.0 ± 0.3^{b}
Compound **2**	0.1 ± 0.0^{b}	1.5 ± 0.0^{b}	$> 100^{c}$

Results expressed as mean ± SD of triplicate samples. Superscript letters (a, b, and c) within the same column denote significant differences in means between different samples determined by one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

The dose response curve on RAW 264.7 cell viability of purified compounds from *C. comosa* extract is shown in Fig. 5. The IC_{20} values, which were the concentration that 80% of RAW 264.7 cells were viability, of compound **1** and **2** were 18.47 ± 0.36 and 26.61 ± 0.40 μg/mL, respectively. Therefore, compound **2** tend to be safer on RAW 264.7 cells than compound **1**. The concentration at IC_{20} value of each sample was used in further anti-inflammatory activity determination.

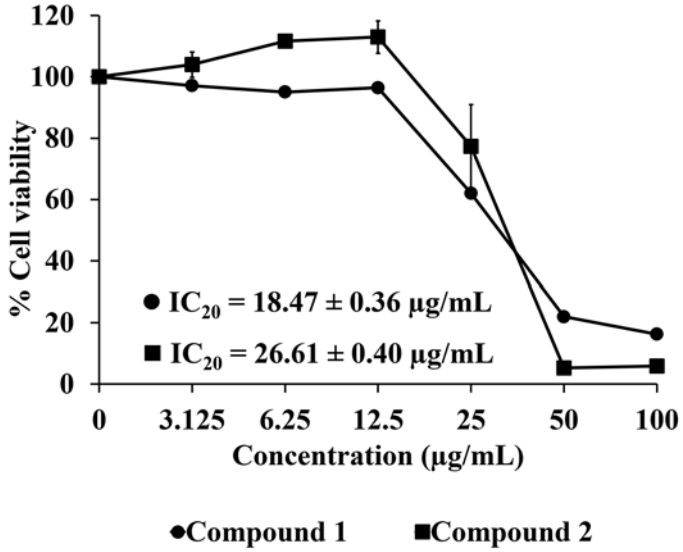


Fig. 5. The effect of compound **1** and compound **2** on viability of RAW 264.7 cell lines by MTT assay. Each point represents mean ± SD of three independent experiments performed in triplicate.

Fig. 6 illustrates the anti-inflammatory activity of purified compounds from *C. comosa* extract. Compound **1** possessed the potent inhibitory activities against both IL-6 and TNF-α. Interestingly, the IL-6 inhibition of compound **1** were significantly more potent than that of dexamethasone, a well-known anti-inflammatory drug (p<0.05). In addition, compound **1** exhibited a comparable TNF-α inhibition to that of dexamethasone (p>0.05). On the other hand, compound **2** had no effect on IL-6 and only slight inhibitory effect on TNF-α secretion. Therefore, compound **1** was suggested as a potent anti-inflammatory agent against both IL-6 and TNF-α mediated inflammation. Since IL-6 and TNF-α plays an important role on various diseases related to the inflammatory process and autoimmune diseases, such as rheumatoid arthritis, asthma, diabetic, nephropathy, etc., the biological active compounds that possessed inhibitory effect on IL-6 are increasingly considered as therapies for chronic diseases, as well as cancer.[16-20] Diarylheptanoids from *C. comosa* was reported to inhibit lipopolysaccharide-induced nitric oxide production in macrophage RAW 264.7 cells.[21] Moreover, pretreatment with hexane or ethanol extract or two diarylhepatanoids (5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene) of *C. comosa* was reported to significantly decrease the release of TNF-α and IL-1β from phorbol-12-myristate-13-acetate (PMA)-stimulated PBMC and U937 cells.[22]

The present study suggested that compound **1** could be used as an alternative natural compound for treating these conditions.

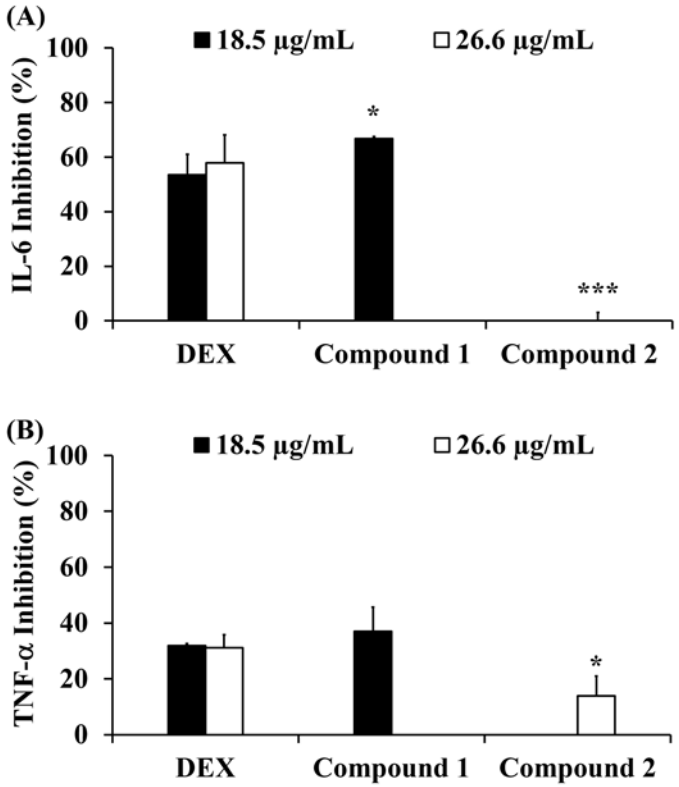


Fig. 6. (A) IL-6 and (B) TNF-α inhibition of dexamethasone (DEX), compound **1**, and compound **2**. Each point represents mean ± SD of three independent experiments performed in triplicate. Asterisks (*) designate significant differences between the purified compounds from *C. comosa* extract and dexamethasone. (*p<0.05 and *** p<0.001).

Hemolysis is the destruction of red blood cells which cause the release of hemoglobin. and can cause hemolytic anemia. Hemolysis is caused by drugs or toxins in the body, blood disorders, or infection, and thus, the effects of crude extracts, fractions, and purified compound extracts on red blood cell hemolysis should be determined before prior use. A red blood cell hemolysis assay was performed to determine the effects of F-EtOAc, Compound **1**, F-Hex, and Compound **2** on red blood cells. A 0.05% Triton X-100 and 0.9% normal saline solution were used as positive (100% hemolysis) and negative controls (0% hemolysis). Fig. 7 showed the effect of F-EtOAc, Compound **1**, F-Hex, and Compound **2** at indicated doses on red blood cell hemolysis. Interestingly, at all

concentration tested of F-EtOAc, Compound **1**, F-Hex, and Compound **2** showed less than 5% hemolysis, suggesting that they are not hemolysis inducing agents.[23]

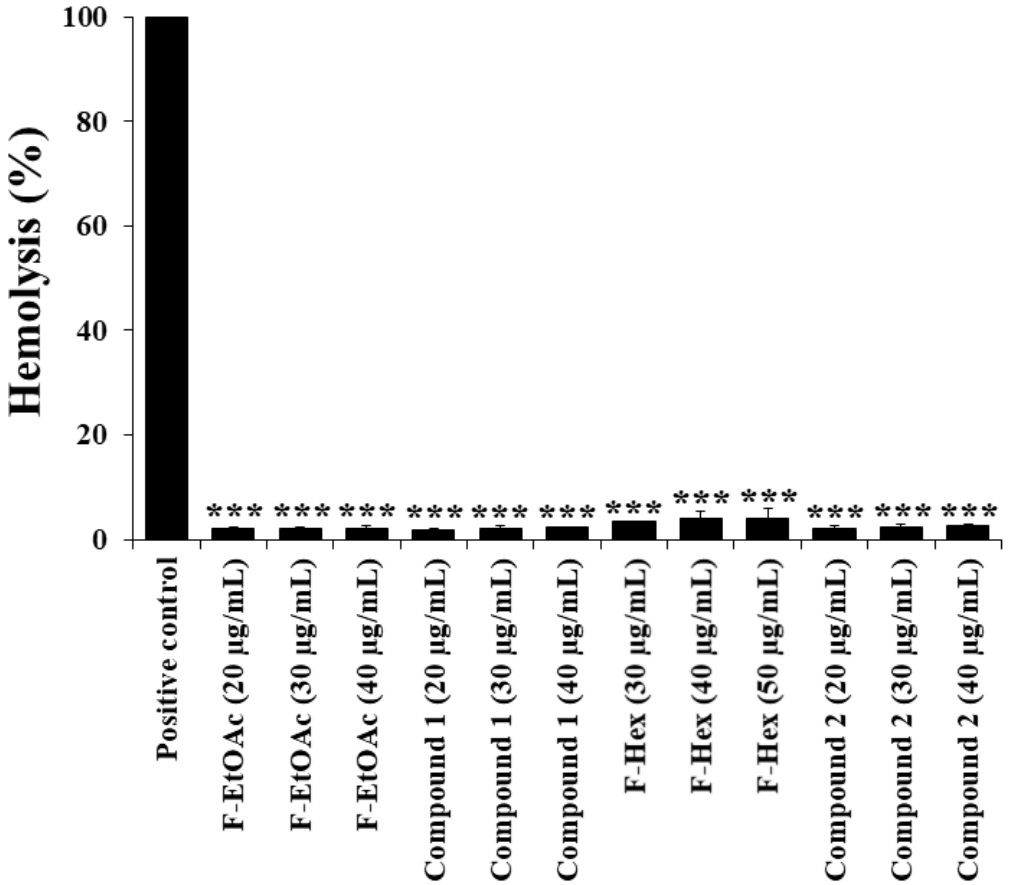


Fig. 7. RBC hemolysis after incubated with F-EtOAc, Compound **1**, F-Hex, and Compound **2**. Each bar represents mean ± SD of three independent experiments performed in triplicate. Asterisks (*) designate significant differences between *C. comosa* extracts and positive control. (*** p<0.001).

In this study, WT1 protein was used as a biomarker of leukemic cell proliferation and determined by Western blotting. KG-1a cells were used as the leukemic cell model because this cell line has high level of WT1 protein[24, 25] and has capacity of self-renewal and can give rise to heterogeneous lineage of cell with abnormal proliferation and differentiation to malignant blasts, called leukemic cells.[26] The IC_{20} values of F-EtOAc, compound **1**, F-Hex, and compound **2** (5.11, 2.50, 31.08, and 23.21 µg/mL, respectively) were used to treat and evaluate WT1 protein expressions. WT1 levels were normalized by using GAPDH protein levels. WT1

expressions after treatments were 35.93 ± 5.04, 39.48 ± 7.82, 47.60 ± 7.99, and 85.99 ± 11.27 µg/mL, respectively when compared to vehicle control (Fig. 8A). The levels of WT1 protein after treatment with F-EtOAc, compound **1**, and F-Hex were significantly decreased and correlated to their effect on total cell numbers as shown in Fig. 8B. Compound **2** was observed to reduce total cell number in KG-1a cells but did not significantly decrease WT1 protein expression in this study. Thus, this result was suggested that compound **2** may involve other target proteins that related to cell proliferation. Moreover, F-Hex may contain other compounds beside of compound 2 to suppress WT1 protein expression.

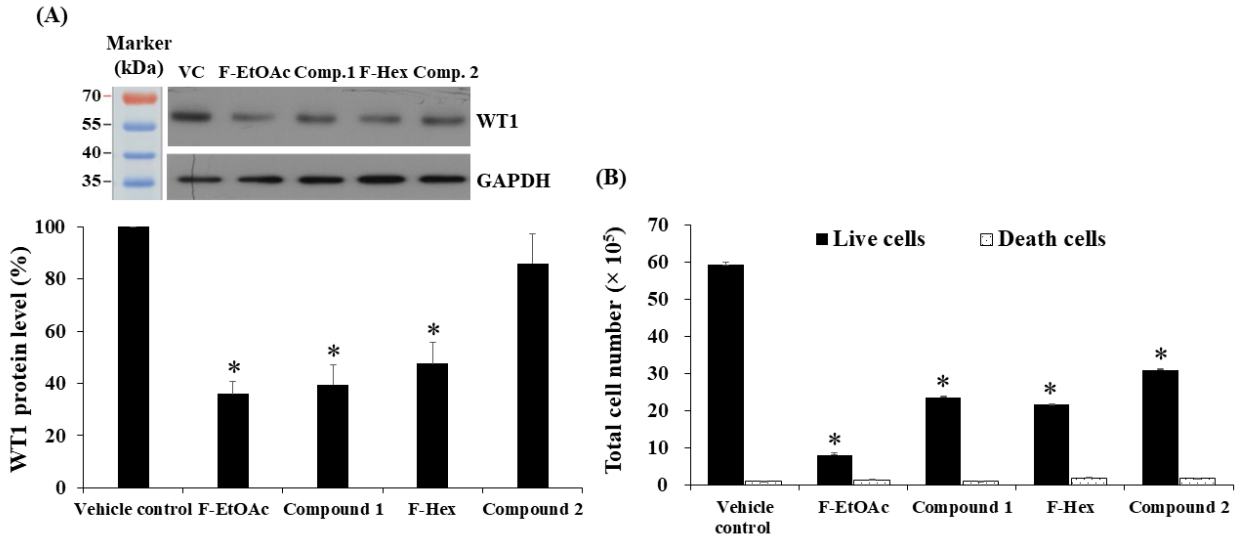


Fig. 8. Effect of F-EtOAc, compound **1**, F-Hex, and compound **2** on KG-1a cells. (A) The level of WT1 protein after treatments with F-EtOAc, compound **1** (comp. **1**), F-Hex, and compound **2** (comp. **2**) for 48 h. Protein level were evaluated by Western Blot and analyzed by scan densitometer. The levels of WT1 were normalized by using GAPDH protein levels. (B) Total cell number after treatments with F-EtOAc, compound 1, F-Hex, and compound 2 for 48 h, determined by Trypan blue exclusion method. Each bar represented mean ± SD of three independent experiments performed in triplicate. Asterisks (*) designate significant differences between *C. comosa* extracts and vehicle control. (*p<0.05).

Conclusion

The present study identified the two isolated compounds as diarylheptanoids (3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol (**1**) and diarylheptanoids (3S)-1,7-diphenyl-(6E)-6-hepten-3-ol (**2**). Both are the active components in the ethyl acetate and hexane fractional extracts of *C. comosa*. This is the report of diarylheptanoids isolated from the *C. comosa* plant which related to previous reports.[6, 8, 9] Bioassays of diarylheptanoids against cancer cells confirmed its anti-leukemic, antioxidant, and anti-inflammatory activities. Compound **1** is a potent antioxidant and a potent anti-inflammatory agent against both IL-6 and TNF-α mediated inflammation. Compound **1** also significantly showed suppression of WT1 protein expression and cell proliferation in leukemic stem cell line. These results suggest that Compound **1** can be used as a chemotherapeutic agent for the treatment of human leukemia, particularly AML.

Materials and methods

Plant maceration

Curcuma comosa were harvested form Chiang Dao District, Chiang Mai province, Thailand in August 2018. A voucher specimen No. 023237 was deposited at the herbarium, Northern Research Center for medicinal plants, Faculty of Pharmacy, Chiang Mai University, Thailand. Herbarium specimen has been studied and annotated by traditional methods of herbarium taxonomy. Fresh rhizomes of *C. comosa* (5 kg) were peeled and dried at 50°C. The dried rhizomes were grounded into powder and macerated in hexane for 3 days. Liquid portion was collected and repeated 3 times. The liquid portions of extraction were pooled together and filtrated. The filtrated was evaporated by using a rotary evaporator (EYELA N-1000) and subsequently dried to obtain hexane fraction (F-Hex). The residual powder will be dried in hot air oven (45°C) and subjected to next maceration with ethyl acetate to obtain ethyl acetate fraction (F-EtOAc).

Column Chromatography

Silica gel grade 60 (Merck, Germany) was used as a solid phase in a column. Hexane and ethyl acetate were used as liquid phases with different ratios by increasing polarity to separate different compounds. Fractions were collected at least 8 mL in a test tube every 6—8 min. A thin layer chromatography was used to determine fractions that contain compounds. Fractions containing purified main compounds were pooled and characterized at the Faculty of Science, Chiang Mai University to determine chemical structures by Nuclear Magnetic Resonance (NMR). *C. comosa* fractional extracts and main compounds were kept at -20°C. The fractional extracts or main compounds were dissolved in DMSO to obtain the working concentration (25 mg/mL) and kept at -20°C before use.

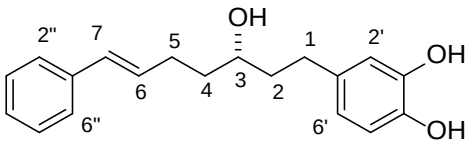
Silica gel 60 (Brand) were packed in column and PPF-EtOAc or PPF-Hex were added to the top of the silica gel. PPF-EtOAc 1.577 g was first separated. Column were eluted with Hex:EtOAc by the ratio of 1:1. Fractions (8 mL/tube) were collected and observed by thin-layer chromatography (TLC). Similar TLC pattern of each fractions were selected and pooled together. Pooled fractions were observed by TLC again. The most purified pooled fractions (% yield = 32.40) were selected to do the second separated with the same procedure. Column were eluted with hexane/diethyl ether by the increasing ratios of 1:1. 1:2, and 1:3. First PPF- hex (3.025 g) purifications was done by the same procedure but different gradient elution of hexane/ethyl acetate of 1:1, 97.5:2.5, 96.5:3.5, 95:5, 90:10, 85:15, and 80:20.

Structure Identification

The purity of each collected fraction was determined by TLC and ^{1}H NMR spectrum. The structure of two pure compounds was characterized by spectroscopic analyses. Optical rotations were measured on an Autopol I automatic polarimeter at the sodium lamp (λ = 589 nm) D-line and are reported as follows: $[\alpha]^{T}_{D}$ (*c* g/100 mL, solvent). ^{1}H and ^{13}C NMR spectra on a on a Bruker AVANCE 400 (400MHz) in deuterated chloroform ($CDCl_3$; Sigma-Aldrich) and deuterated methanol (CD_3OD; Sigma-Aldrich). ^{1}H NMR spectra are reported as follows: chemical shift (δ, ppm), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling

constants (*J*) in Hz, integration, and assignments. ^{13}C NMR spectra are reported in terms of chemical shift (δ, ppm).

(3*S*)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol (**1**)

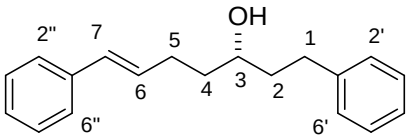


^{1}H NMR (400 MHz, MeOD) δ 7.33 – 7.14 (m, 5H, H-2”-6”), 6.64 (m, 2H, H-2’,5’), 6.50 (dd, *J* = 8.0, 2.0 Hz, 1H, H-6’), 6.37 (d, *J* = 15.8 Hz, 1H, H-7), 6.22 (dt, *J* = 15.8, 6.8 Hz, 1H, H-6), 3.57 (m, 1H, H-3), 3.30 (br s, 1H, 3-OH), 2.62 (m, 1H, 1a), 2.49 (m, 1H, 1b), 2.27 (m, 2H, H-5), 1.70 (m, 2H, H-2), 1.60 (m, 2H, H-4).

^{13}C NMR (100 MHz, MeOD) δ 146.1 (C), 144.2 (C), 139.2 (C), 135.3 (C), 131.4 (CH), 131.3 (CH), 129.4 (2xCH), 127.8 (CH), 126.9 (2xCH), 120.7 (CH), 116.6 (CH), 116.3 (CH), 71.2 (CH), 40.6 (CH_2), 38.1 (CH_2), 32.4 (CH_2), 30.3 (CH_2)

$[\alpha]_D^{30}$ -108 (c 0.37 in EtOH)

(3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol (**2**)



^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 – 7.22 (m, 10H, Ar-H), 6.47 (d, *J* = 15.8 Hz, 1H, H-7), 6.29 (dt, *J* = 15.8, 6.9 Hz, 1H, H-6), 3.75 (m, 1H, H-3), 2.85 (m, 1H, 5a), 2.73 (m, 1H, 5b), 2.39 (m, 2H, H-1), 1.86 (m, 2H, H-4), 1.73 (m, 2H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$) δ 142.2 (C), 137.7 (C), 130.4 (2xCH), 128.6 (2xCH), 128.52 (2xCH), 128.50 (2xCH), 127.1 (CH), 126.1 (2xCH), 125.9 (CH), 70.9 (CH), 39.2 (CH_2), 37.1 (CH_2), 32.1 (CH_2), 29.4 (CH_2)

$[\alpha]_D^{30}$ -1 (c 1.0 in EtOH).

Cell cultures

Leukemic cell lines including K562 (human erythroid leukemic cell line) and HL-60 (human promyelocytic leukemic cell line) was maintained in RPMI 1640 medium containing 1mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% fetal bovine serum (FBS). KG-1a (leukemic stem cell-like cell line with stem cell population) was cultured in IMDM medium containing 1mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and supplemented with 20% FBS. A549 (human lung cancer cell line) and MCF-7 (breast cancer cell line) was cultured in DMEM medium containing 1mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% FBS. All cancer cell lines were incubated at 37°C under 95% humidified and 5% CO_2.

Cytotoxicity determinations by MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for detecting the cytotoxicity of *C. comosa* extracts and purified compounds on cancer cells. The cytotoxicity of F-EtOAc, F-Hex, and main compounds was investigated by MTT assay in K562 KG-1a, HL-60, A549, and MCF-7 cell lines. K562 (1.0×10^5 cells/mL), HL-60 (1.0×10^5 cells/mL), KG-1a (1.5×10^5 cells/mL), and A549 (5.0×10^4 cells/mL), MCF-7 (5.0×10^4 cells/mL) were added then incubated for 48 h. After that, 100 µL of medium was removed and 15 µL of MTT dye solution was added, and cells were further incubated for 4 h. After supernatant was removed, 200 µL of DMSO were added to each well and mixed thoroughly to dissolve the purple formazan crystals. The optical density was measured by using ELISA plate reader at 578 nm with reference wavelength at 630 nm. The percentage of surviving cells was calculated from the absorbance values of the test and control wells using the following equation:

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test well}}{\text{Mean absorbance in vehicle control well}} \times 100$$

The average percentage of cells surviving at each concentration obtained from triplicate experiments was plotted as a dose-response curve. The 50% inhibitory concentration (IC_{50}) was defined as the lowest concentration that inhibited cell growth by 50% compared to the untreated control.

Trypan blue exclusion

Total cell numbers were counted by trypan blue exclusion method. Live cells have intact membrane and able to exclude trypan blue dye. In contrast, death cells with compromised membrane were stained by trypan blue dye solution. Cell suspension and 0.2% trypan blue was mixed and count viable (unstained) and death cell (stained) in the hemacytometer. The percentage of viable cells was calculated.

Cytotoxicity of PBMCs.

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque density-gradient centrifugation using Lymphoprep^{TM} solution (Axis-Shield, Oslo, Norway). The PBMCs (1×10^6 cells/mL) were plated in flat-bottom 96-well plates overnight in a 5% CO_2 incubator at 37°C . Then, Various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) of *C. comosa* extracts and purified compounds were added, and the cells were then incubated for 48 h . The cell survival rate was assessed by means of the MTT colorimetric assay as previously described.

Antioxidant activities determination

1) 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulphonate (ABTS) assay

The $ABTS^{\bullet+}$ scavenging activity of *C. comosa* extracts and purified compounds were investigated using ABTS assay.[27] Briefly, $ABTS^{\bullet+}$ solution was prepared by mixing 2 mL of 7 mM ABTS solution with 3 mL of 2.45 mM potassium persulfate solution and incubated in the dark. After 24 h, the resulting $ABTS^{\bullet+}$ solution was diluted 1:20 in absolute ethanol. Then 20 μL of the sample was mixed with 180 μL of $ABTS^{\bullet+}$ solution, incubated at room temperature for 5 min, and measured for UV absorbance at 750 nm using a microplate reader (Spectrostar

Nano, BMG Labtech GmbH, Ortenberg, Germany). The results were reported as Trolox equivalent antioxidant capacity (TEAC). All experiments were done in triplicate.

2) 2,2'-Diphenyl-1-picrylhydrazyl-hydrate (DPPH) assay

The DPPH$^{\bullet}$ scavenging activity of *C. comosa* extracts and purified compounds were investigated using DPPH assay[27]. Briefly, 20 μL of the sample was mixed with 180 μL of 167 μM DPPH solution, incubated at room temperature in the dark for 30 min, and measured for UV absorbance at 520 nm using a microplate reader (Beckman CoulterDTX880, Fullerton, CA, USA). The scavenging activity was calculated using the following equation:

$$\%\ \text{DPPH}^{\bullet}\ \text{inhibition} = \frac{A-B}{A} \times 100$$

where *A* is a UV absorbance of a mixture without sample solution and *B* is a UV absorbance of a mixture with sample solution. L-ascorbic acid was used as a positive control. All experiments were done in triplicate.

3) Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power of *C. comosa* extracts and purified compounds were investigated by FRAP assay.[27] Briefly, FRAP solution was freshly prepared by mixing 10 mL of 0.3 M acetate buffer pH 3.6, 1 mL of 10 mM 2,4,6 tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 1 mL of 20 mM ferric chloride. Then 20 μL of the sample was mixed with 180 μL of FRAP solution, incubated at room temperature in the dark for 5 min, and measured for UV absorbance at 595 nm using a microplate reader (Beckman CoulterDTX880, Fullerton, CA, USA). The results were expressed as equivalent capacity (EC_1). L-ascorbic acid was used as a positive control. All experiments were done in triplicate.

Anti-inflammatory activities determination

Anti-inflammatory activity of *C. comosa* extracts and purified compounds were investigated by means of inhibitory activities against IL-6 and TNF-α secretion.[28] The mouse monocyte macrophage RAW 264.7 cells (American Type Culture Collection, ATCCTIB-71) were stimulated with lipopolysaccharide (LPS) to induce an

inflammatory process. The cell incubated with LPS served as a vehicle control, of which the secreted cytokines was defined as 100%, whereas, non-treated RAW 264.7 cells served as a negative control. Dexamethasone, a well-known anti-inflammatory drug, was used as a positive control.

Briefly, 1×10^5 cells per well in DMEM were seeded and incubated for 24 h in a CO_2 incubator set at 37°C and 5% CO_2 -95% air. Then 1 μL of *C. comosa* extracts or purified compounds were added. After 2 h incubation in a CO_2 incubator, LPS was added to make a final concentration of 1 μg/mL. After 24 h incubation in a CO_2 incubator, the media was removed and centrifuged at 13,500×g for 10 min and 100 μL of the supernatant was analyzed by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). The optical density was measured at 450 nm, corrected with the reference wavelength of 570 nm using multimode detector (Beckman CoulterDTX880, Fullerton, CA, USA).

RAW 264.7 cell viability was also determined simultaneously with the ELISA by MTT assay. The supernatant was totally removed and MTT was added to the cells. After 2 h incubation in a CO_2 incubator, the supernatant was totally removed, and the cells were lysed with DMSO. The optical density was measured at 570 nm, corrected with the reference wavelength of 690 nm using multimode detector (Beckman CoulterDTX880, Fullerton, CA, USA). IL-6 and TNF-α secretion inhibitions were calculated using the following equation:

$$\%\ \text{Cytokine inhibition} = \frac{A\text{-}B}{A} \times 100$$

where A is an optical density of the mixture without sample and B is an optical density of the mixture with sample. Dexamethasone was used as a positive control. All experiments were done in triplicate.

RBC hemolysis induction

EDTA blood samples were collected from normal subjects. After centrifugation, RBCs were collected and washed twice with 0.9 % NaCl .For RBC hemolysis induction, 1 mL of 5 % RBC suspension was then incubated with F-EtOAc, Compound **1**, F-Hex, and Compound **2** at 37°C water bath for 3 h .Triton-X 100)0.05 (%and NaCl) 0.9 (% were used as positive and negative controls, respectively .After incubation, the supernatant was collected by centrifugation at 4400 rpm for 5 min at room temperature and measured hemoglobin concentration by spectrophotometry at 540 nm.

Western Blot analysis

KG-1a cells were adjusted to concentration of 1.0 x 10^5 cells/mL and cultured with F-EtOAc, Compound **1**, F-Hex, and Compound **2** at concentration at 20% growth (IC_{20}) of each extract. After incubation for 48 h, cells were washed, and the whole protein were extracted using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 0.1% SDS, and a protease inhibitor cocktail). The protein concentration was determined by the Folin-Lowry method. Twenty micrograms of each sample were loaded to 7.5% SDS-PAGE and then transferred to PVDF membranes. The membranes were shaken in PBS for 5 min then incubated membranes in 5% skim milk in PBS to block nonspecific binding for 2 h. Each membrane was incubated with rabbit polyclonal anti-WT1 IgG (Santa Cruz Biotechnology, CA, USA) and rabbit polyclonal anti-human GAPDH IgG (Santa Cruz Biotechnology, CA, USA) at dilution 1:1000 with shaking for 2 h. After that, membranes were washed then incubated with HRP-conjugated goat anti-rabbit IgG (Invitrogen™, CA, USA) at 1:20,000 dilution with shaking for 2 h. To detect protein band, Luminata TM Forte Western HRP substrate (Merck, Darmstadt, Germany) was added to membranes. After that, the membranes were placed onto a film cassette and exposed on X-ray film (Sakura, Japan). Densitometry was quantitated using Quantity One 1-D Analysis software (Bio-Rad, USA). The density values of WT1 bands were normalized to GAPDH bands.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) or the mean ± standard error of mean (SEM) from triplicate samples of three independent experiments. The statistical differences between the means were determined using one-way ANOVA. The differences were considered significant when the probability value obtained was found to be less than 0.05 ($p<0.05$) or and 0.001 ($p<0.001$).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/xxxx/xxxxxxx.

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