Identification of anti-inflammatory agents from natural products by targeting NF-κB activity and their application to inflammation-associated diseases

Besse Hardianti

Graduate school of Medicine and Pharmaceutical Science for education, Pharmaceutical Science, Division of Bioscience

Institute of Natural Medicines,

University of Toyama

Japan

September 2022

Chapter 5. Anti-inflammatory activities of isopimara-8(9),15-diene diterpenoids and mode of action of kaempulchraols compound from *Kaempferia pulcra* rhizomes

Abbreviation

NF-κB	Nuclear factor-kappaB
COX-2	Cyclooxygenase-2
ROS	Reactive oxygen species
STAT3	Signal transducer and activator of transcription 3
AKT	Ak strain transforming
PKB	Protein Kinase B
TNF-α	Tumor Necrosis Factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
J	Coupling constant
$CDCl_3$	Deuterated chloroform
DMSO	Dimethyl sulfoxide
ESI	Electrospray ionization source
ELISA	Enzyme-linked immunosorbent assay
^{13}C NMR	Carbon Nuclear Magnetic Resonance
HPLC-MS	High performance liquid chromatography-mass spectrometry
δ	Ppm
^{1}H NMR	Proton Nuclear Magnetic Resonance

TLC	Thin Layer Chromatography
NMR	Nuclear Magnetic Resonance
MS	Mass
m/z	Mass-to-charge ratio
MHz	Megahertz
*μ*M	Micro molar
*μ*g/mL	Microgram per milliliter

Preface

This thesis has been submitted to the Affiliation of Graduate School of Medicine and Pharmaceutical Sciences for Education, University of Toyama, Japan, for the degree of Doctor of Philosophy Ph. D This research was conducted at the Institute of Natural Medicine's Laboratory of Cancer Biology and Immunology, Section of Host Defenses. The findings of this thesis were published in five papers, which are listed below:

1. Hardianti B, Umeyama L, Li F, Yokoyama S, Hayakawa Y. Anti-inflammatory compounds moracin O and P from (Sohakuhi) Morus alba Linn target the NF-κB pathway. Mol Med Rep. 2020 Dec;22(6):5385-5391. doi: 10.3892/mmr.2020.11615. Epub 2020 Oct 20. PMID: 33173971; PMCID: PMC7647032.

2. Win NN, Hardianti B, Ngwe H, Hayakawa Y, Morita H. Anti-inflammatory activities of isopimara-8(9),15-diene diterpenoids and mode of action of kaempulchraols B-D from Kaempferia pulchra rhizomes. J Nat Med. 2020 Mar;74(2):487-494. doi: 10.1007/s11418-020-01389-7. Epub 2020 Jan 31. PMID: 32006354.

3. Nwet Win N, Hardianti B, Kasahara S, Ngwe H, Hayakawa Y, Morita H. Anti-inflammatory activities of isopimara-8(14),-15-diene diterpenoids and mode of action of kaempulchraols P and Q from Kaempferia pulchra rhizomes. Bioorg

Med Chem Lett. 2020 Jan 15;30(2):126841. doi: 10.1016/j.bmcl.2019.126841. Epub 2019 Dec 3. PMID: 31836445.

4. Lallo, S., Hardianti, B., & Hayakawa, Y. Anti-Inflammatory and Cytoprotective Effect of *Kaempferia galanga* Extracts by Targeting NF-κB Activity. Asian Journal of Plant Sciences,2022, 21(2), 183-191.

5. Subehan Lallo, Hardianti B, Sartini S, Ismail I, Laela D, Hayakawa Y,. Ethyl P-Methoxycinnamate: An Active Anti-Metastasis Agent and Chemosensitizer Targeting NF-κB from Kaempferia galanga for Melanoma Cells. Life. 2022, Feb 24;12(3):337.

Abstract

Accumulating evidence indicates that inflammation is associated with multiple pathological processes and induces cellular and molecular damage by activating inflammatory signaling pathways, including the NF-κB pathway. The purpose of this study was to identify anti-inflammatory agents derived from natural products that target NF-κB activity and apply them to inflammation-associated diseases. Apparently, natural products play a significant role in regulating the inflammatory response pathways. NF-κB is a central mediator of pro-inflammatory gene induction and functions in both innate and adaptive immune cells; therefore, the anti-inflammatory regulation of NF-κB is required. In order to establish a novel therapeutic approach for inflammatory-induced pathogenic disease, the extensive pharmacological investigations of natural products were conducted to identify a potential anti-inflammatory agent by targeting NF-κB activity.

1. Firstly, the *Identification of natural products as a potential source for anti-inflammatory agents by* NF-κB *-targeted functional screening*

NF-κB -targeted functional screening of 112 natural products were conducted to determine their anti-inflammatory properties. Using a 4T1 breast cancer cell line that constitutively expresses the firefly luciferase gene under the control of the NF-κB reporter, the NF-κB -targeted functional screening were conducted and found that Sohakuhi (Morus alba Linn. bark) extract was found to significantly inhibit NF-κB activity without affecting cell viability. Secondary, 35 natural medicinal plants cultivated in Indonesia traditionally used to treat skin disease symptoms were also subjected to the NF-κB -targeted functional screening and two K. galanga extracts from different rhizome types were identified to exert strong suppression in NF-κB activity without affecting cell viability.

2. Anti-inflammatory compounds moracin O and P from Morus alba Linn (Sohakuhi) target the NF-κB *pathway*

To further determine the utility of Sohakuhi extract regarding its anti-inflammatory effect, we examined TRAIL-induced cellular damage of HaCaT human keratinocytes. While TRAIL triggered the phosphorylation of p65, a subunit of NF-κB, leading to cellular damage in HaCaT cells, treatment with Sohakuhi extract protected HaCaT cells against TRAIL-induced cellular damage and suppressed NF-κB activation, and up-regulation of anti-apoptotic BCL-XL and BCL-2 expressions. This mechanism could emphasis how interaction between the BCL-2 family address critical importance to further evaluate both the concentration and affinities of BCL-2 family protein to govern cell fate based on Justin kale at all review. Importantly, through chemical fractionation of Sohakuhi extract, we determined that Moracin O and P are responsible for the anti-inflammatory effect of Sohakuhi. Collectively, the present results indicate that Sohakuhi and Moracin compounds are promising targets for developing novel anti-inflammatory drugs.

3. Anti-inflammatory and cytoprotective effect of Kaempferia galanga extracts by targeting NF-κB *activity*

Based on the screening of 12 selected natural medicinal plants, we identified Two *K. galanga* extracts (29 and 35) derived from distinct rhizome types strongly inhibited NF-κB activity without affecting cell viability. The biological activity of *K. galanga* in extracts 29 and 35 was further evaluated by incubating HaCaT cells with recombinant TRAIL (rTRAIL) as a mediator of inflammatory stimuli that activate NF-κB activity and cytotoxicity. Pretreatment with extracts 29 and 35 did not show toxic effects on HaCaT cells, whereas rTRAIL induced a significant reduction in the viability of cells. Furthermore, pretreatment with extract 29 and 35 at concentrations of 16 and 32 μg/mL protected cell growth against rTRAIL-induced cellular damage. Cytoprotective effects of extracts 29 and 35 against tumour necrosis factor-related apoptosis-inducing ligand (rTRAIL)-induced cellular damage in human keratinocytes. These findings suggest that

the identified *K. galanga* extracts are promising candidates for the creation of novel anti-inflammatory natural medicines.

4. Ethyl P-methoxycinnamate: an active anti-metastasis agent and chemosensitizer targeting NF-κB *from Kaempferia galanga for melanoma cells*

The active compound in *K. galanga* was isolated and identified using chromatography and spectroscopy techniques, and given six compounds. Inhibitory activity on NF-κB activation and cell viability was determined using reporter assay methods. Among the isolated compounds, ethyl p-methoxycinnamate (EPMC) demonstrated potent NF-κB inhibitory activity against melanoma cell B16F10- NF-κB-Luc2 with an IC50 of 88.7 μM. Further investigation was conducted by evaluating the anti-metastasis effect of EPMC in vitro by using wound-healing assays, invasion tests, and molecular mechanism assays using Western blotting. NF-κB has been implicated in tumorigenesis through the PI3K/Akt/ NF-κB pathway. The results of this study indicated that EPMCs act as inhibitors of p38, this protein can directly or indirectly target proteins that could be a pre/post transcriptional response. The result could be speculated that p38 directly regulate Akt phosphorylation at serine 473 however,the present of EPMC downregulated Akt at serine 473 posporilation. As both protein could directrly target the downstream transcription factor(NF-κB), it can assume decrease level p38 and Akt affect NF-κB dependent transcription. Further analysis with paclitaxel demonstrated that the combinations could sensitize to apoptosis in response to well-known chemotherapy agents. Additional studies were conducted using the human melanoma cancer cell line SK-Mel 28. Along with the induction of apoptosis, we observed an increase in p-γH2AX expression (a molecular marker for double strand breaks in DNA damage) in response to treatment with paclitaxel and EPMC. The result showed EPMC to be a potential, viable adjuvant for improving the clinical efficacy of anti-metastatic and cancer chemotherapy.

5. Anti-inflammatory activities of isopimara-8(9),15-diene diterpenoids and mode of action of kaempulchraols compounds from Kaempferia pulchra rhizomes

Kaempulchraols, isopimara-8(9),15-diene diterpenoids isolated from *Kaempferia pulchra* rhizomes collected in Myanmar, were examined their anti-inflammatory activity. Among 21 isopimara-8,14(15)-diene diterpenoids, the most potent Kaempulchraols P and Q, with IC50 values of 39.88 and 36.05 μM, respectively, inhibited the NF-κB -mediated transactivation of a luciferase reporter gene, IL-6 production, and COX-2 expression, with an effective dose of 25 μM. Furthermore, other series of Kaempulchraols, Kaempulchraols B-D, isolated from *K.pulchra*, isopimara-8(9),15-diene diterpenoids were also effective as inhibitor NF-κB mediated transactivation of a luciferase reporter gene, IL-6 production, and COX-2 expression, with an effective dose of 25 μM. Thus, isopimarane diterpenoids are suggested to be potent inhibitors of NF-κB pathways and could be further explored as potential anti-inflammatory lead compounds.

In summary, these studies demonstrated the importance of natural products as a source of anti-inflammatory agents, particularly targeting NF-κB inflammatory signal pathway. The clinical application of those natural anti-inflammatory agents would be beneficial to treat inflammation-associated diseases.

Chapter 1

General Introduction

1.1 Background

1.1.1 Basic Principle of Inflammation

The ancients defined inflammation based on five cardinal signs: redness (rubor), swelling (tumour), heat (calor; only applicable to the body's extremities), pain (dolor), and loss of function (functio laesa). Celsus (30–38 B.C.) named the first four of these signs in ancient Rome, and Galen (A.D 130–200) named the last(1). As it has been traditionally described.

A greater blood flow through dilated vessels results in a more intense sensation of heat, as well as a more noticeable redness in the skin (because increase the number of erythrocytes passing through the area). oedema (swelling) occurs when fluid from dilated and permeable blood vessels enters the surrounding tissues, the cardinal sign of inflammation illustrated in (Figure 1). furthermore, cells invade the damaged area, and connective tissue accumulates as a result of an extended inflammatory response. Mediators, either from initial damage or caused by the inflammatory reaction itself and stretching of sensory nerves due to oedema, are responsible for pain. Oedema and pain can make a joint less mobile and cause scar tissue to replace the cells that make it work(2).

Once inflammation occurs, it serves as a defense mechanism for the body against infection and damage to tissue.The long-term effects of inflammation can be extremely detrimental to health status. Recent decades have seen an increase in the number of cases in which persistent inducers or genetic variations alter inflammatory responses. Different stimuli trigger different physiological and pathological inflammatory responses, the causes, and physiological and pathological outcomes of inflammation

illustrated in (Figure 2) (3). The elimination of infectious agents may be the result of a successful mediators-acute inflammatory response followed by a resolution and repair phase mediated by tissue-resident and recruited macrophages(4). During the acute phase of the inflammatory response, immune system cells move to the site of the injury in a properly orchestrated sequence that is helped along by acute-phase proteins, cytokines, and chemokines, this illustrated in (Figure 3). The state of injury became the starting point, this initial phase may be enough to fix the problem and start the healing process. However, Persistent inflammation can lead to the chronic phase, where tissue damage and fibrosis can occur. This can happen if the body is exposed to stimulation for a long time or if it reacts too strongly to its own molecules. Chronic inflammation has been linked to a lot of diseases and conditions that come with getting older, like arthritis, asthma, atherosclerosis, autoimmune diseases, diabetes, cancer, and even some forms of dementia(5).

1.1.2 NFκB regulate genes encoding inflammatory mediators

David Baltimore and Ranjan Sen discovered the transcription factor NF-κB 30 years ago, and since then, it has been proven to be a key regulator of immune system-inducible gene expression (6) NF-κB was initially described as a specific DNA-binding activity in B cells that identified an enhancer element in the gene encoding the immunoglobulin-light chain and was designated to reflect these characteristics. Afterward, it was discovered that NF-κB was expressed in nearly every type of cell, but the name NF-κB forced to stick (7). NF-κB regulates the expression of literally hundreds

of biologically significant genes (a growing list is available at (http://www.bu.edu/nnf-kb/generesources/target-genes/)

Nearly all multicellular organisms depend on the nuclear factor kappa B (NF-κB) family of dimeric transcription factors for the coordination of inflammatory responses as well as innate and adaptive immunity along with cell differentiation, proliferation, and longevity(8-11). Since its inception, NF-κB research has been at the leading edge of biochemistry, molecular biology, and genetics scientific breakthroughs. Researchers highlighted the complexity of NF-κB and its regulation due to its seemingly redundant and interconnected factors(12).

The NF-κB network in Mammalia is composed of five family member protein monomers (p65/RelA, RelB, cRel, p50, and p52) that form homodimers or heterodimers which thus connect DNA differentially(13-15) and are controlled by two different pathways: the canonical pathway, which is dependent on the NF-κB essential modulator (NEMO), and the noncanonical pathway, which is independent of the NEMO. These pathways exert a tight control over the rates and complexities of the transcriptionally active NF-κB dimer repertoire in both the constitutive state and in response to specific stimuli. As a result, they are able to regulate a wide variety of gene expression programs(16, 17) it is illustrated in (Figure 4). The canonical (classical) pathway core is IκB (NF-κB inhibitor) (18-20).

In unstimulated cells, NF-κB forms a complex with IκB and is therefore blocked in the cytoplasm. Multiple intracellular signaling pathways are activated if the cells are stimulated, and these pathways converge on the IκB kinase (IKK) complex. The most well-known form of this complex is composed of two functional non-redundant kinases

IKKα (IKK1) and IKKβ (IKK2). As well as and the regulatory subunit IKKγ, also known as NEMO ("basic regulator of NF-κB"). the IKK complex phosphorylates IκB at specific amino acid residues. IKKβ mainly mediates iκBα phosphorylation at Ser32 and Ser36. This site-specific phosphorylation is a prerequisite for subsequent post-translational modification, I.e., ubiquitination of IκBα, which marks the degradation of NF-κB inhibitors in the 26S proteasome. NF-κB can now be freely transferred to the nucleus, consequently regulating the expression of genes involved in essential physiological and pathophysiological cellular processes, such as controlling the immune system, especially the innate immune response, and the regulation of Inflammation and apoptosis.(21, 22)

The Central key of the non-canonical pathway regulates of NF-κB -inducing-kinase (NIK, also known as MAP3K14)(20, 23). This NIK-modified ubiquitin ligase complex generally consists of the TRAF3, TRAF2 proteins, and apoptosis inhibitor 1 or 2 (cIAP1 or 2). The non-classical NF-κB pathway activators include tumor necrosis factor superfamily receptor ligand (TNFSFR). It can be expressed by T cells, dendritic cells, B cells, macrophages, granulocytes, NK cells, and some non-immune cells. In dendritic cells, examples of TNFSFR include CD40, CD137 (41BB, TNFRSF9) and lymphotoxin receptor β (LTBR)(24). Finally, The translocation of NF-κB to the nucleus activates the transcription of several genes related to the innate and adaptive immune response, including inflammation and cell fates(25).

1.1.3 The role of NF-κB in inflammation-mediated cellular damage

NF-κB can play a vital role in cell fate by opposing or promoting cell death in multiple circumstances(26) It has been demonstrated that NF-κB has both proapoptotic and antiapoptotic functions(27) Necrotic cells have the potential to generate signals for master transcription factors such as NF-κB, AP1, and others. These transcription factors are responsible for the regulation of hundreds of genes that are involved in inflammation, innate immunity, damage maintenance and cell or tissue remodeling, In many tissue settings, the most prominent effect of NF-κB is to prevent apoptosis; however, in other settings, NF-κB can speed up cell death(26)

There are more than 150 stimuli that can trigger NF-κB activation therefore known as “central mediator of the human immune response” (28). The large majority of dissimilar ligands bind to similar cell surface and intracellular receptors, such as the cytokines (TNF-α, IL-1α/β, and TRAIL)(29). bacterial molecules (LPS, flagellin, and non-methylated dsDNA) thus, the binding between ligands and receptor could induce inflammatory responses(30).

Inflammatory and epithelial cells secrete reactive oxygen (ROS) and nitrogen species (RNS) during inflammatory process, which can damage DNA and cause mutations and genomic instability if not repaired. DNA damage response can also induce an inflammatory microenvironment characterized by hypoxia. Hypoxia increases intracellular RNS and ROS, causing DNA damage and a poor prognosis. DNA damage-producing inflammation can induce NFκB, resulting in iNOS-dependent DNA damage. Additionally, to date, all eukaryotic cells studied have used PI3-kinase-related protein kinases from the ATM/ATR family to initiate a variety of DNA damage responses. H2AX

is a variant histone that is specifically and rapidly phosphorylated within its C-terminal tail by both ATM and ATR in the nuclear microenvironment adjacent to DNA damage. (31-33)

1.1.4 The role of NF-κB in inflammation-associated carcinogenesis, metastasis and chemo resistant

NF-κB, a transcriptional factor, is involved in the regulation of inflammatory responses. which can be stimulated by a variety of stimuli such as proinflammatory cytokines, infectious agents, and cellular stresses, has been linked to carcinogenesis, metastasis and resistance to multiple drug therapy(34). Crosstalk between DNA damage and inflammation in the various stages of carcinogenesis (35) illustrated in (Figure 5). Because of the inflammatory microenvironment and various oncogenic mutations, a significant number of human cancers have constitutive NF-κB activity (35). Not only does NF-κB activity promote tumor cell proliferation, suppress apoptosis, and attract angiogenesis, but it also induces epithelial mesenchymal transition, which aids in distant metastasis.

Inflammation is a significant feature of cancer that can both initiate and sustain tumor development. In immuno-oncology, melanoma serves as a model tumor. Because of its rapid evolution and acquired drug resistance, metastatic melanoma remains one of the most aggressive and feared types of skin cancer. Because of the disease's ever-increasing prevalence, there is an urgent need to develop more effective follow-up and treatment options for melanoma patients(36-38).

Mechanisms of inflammation-associated melanoma figure out at (Figure 6). UVA and UVB rays both stimulate the production of high levels of ROS to induced such inflammation step in exposed skin, which can then activate three important pathways: MAPK cascade (a protein family that includes JNK and p38) with further activation of AP-1 transcription factor, AKT/PKB cascade with modulation of IKK via activation p50 and p65 proteins to enhance transcription factor NF-κB, via anti-apoptotic (the Bcl-2) pathway , JAK-2 and STAT-3 activation All of these pathways are linked to chronic inflammation and promote tumor progression by upregulating COX-2 and producing PGE2 at the tumor site(39). As a result, strategies to inhibit inflammation-induced disease via those pathways are still required.

1.1.5 NF-κB; target for Inflammation and inflammation-associated disease

The activation of NF-κB is triggered by a cocktail of proinflammatory agents. This inducible transcription factor controls the expression of a plethora of target genes. Because some target genes are also NF-κB activators, activating this transcription factor contributes to the establishment of a signal amplification loop. When the adaptive immune response against self-antigens builds up, some autoimmune diseases are often linked to dysregulation of the NF-κB mechanism and overexpression of inflammatory mediators(40). In order to anticipate several problems induced by NF-κB, it was important to searching new approach to inhibit NF-κB. Extensive research has revealed that several plants can inhibit NF-κB at the molecular level. Natural chemo preventive agents such as emodin, gingerol, resveratrol, lycopene, indole-3-carbinol, vitamin C, sulphoraphane, and ellagic acid have been found to be potent NF-κB inhibitors(41)

5.2 Aim and Purpose

As traditional medicine, hundreds of natural remedies have been widely used. Japan and Indonesia are well-known for their traditional medicines, Kampo and Jamu, respectively. Various anti-tumor, anti-inflammatory, and anti-aging activities derived from natural medicines have been identified, but the majority have not been investigated for their potential medical applications. Accumulating evidence suggests that inflammation is linked to multiple pathological processes and induces cellular and molecular damage through the activation of inflammatory signaling pathways, including the NF-κB pathway. The aim of the present study was to identify natural product as anti-inflammatory that can target NF-κB activity. Natural products reportedly play an important role in controlling the inflammatory response pathways. NF-κB is a central mediator of pro-inflammatory gene induction and functions in both innate and adaptive immune cells; therefore, the anti-inflammatory regulation of NF-κB is needed,

in order to establish a novel therapeutic approach for identify inflammatory agent induced pathogenic disease, I develop strategy to identify active anti-inflammatory agent from *Morus alba, Kaempferia galanga* and *Kaempferia pulchra* and their possible application on Inflammatory-associated disease. There is evidence to suggest that natural products play a significant part in the regulation of inflammatory response pathways, and the general introduction regarding inflammation and disease link to inflammation explained in this Chapter 1. I screened 112 natural products collected in institute of natural medicine library, Toyama university were tested for their anti-inflammatory properties, Sohakuhi (*Morus alba* Linn. bark) extract was observed to strongly suppress NF-κB activity without affecting cell viability. Furthermore, the aim in

this chapter is identify natural product by screening Indonesian medicinal plants that could target NF-κB activity as well, Yet, in chapter 2, I investigated the inhibition of NF-κB activation in 35 natural medicinal plants (1–35) cultivated in Indonesia traditionally used to treat skin disease symptoms. with particular focus given to *Kaempferia galanga* extracts as promising candidates. Based on the screening of 35 natural medicinal plants, we identified two *K. galanga* extracts (29 and 35) from different rhizome types that strongly suppressed NF-κB activity without affecting cell viability. To seeking their application on inflammation-associated cellular damage, I illustrated in chapter 3. Sohakuhi (*Morus alba* Linn. bark) extract was observed to strongly suppress NF-κB activity without affecting cell viability.

To further examine the anti-inflammatory effect of Sohakuhi, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cellular damage of human HaCaT keratinocytes was evaluated. While TRAIL triggered the phosphorylation of the p65 subunit of NF-κB, leading to cellular damage in HaCaT cells, treatment with Sohakuhi extract protected HaCaT cells against TRAIL-induced cellular damage. Moreover, Sohakuhi treatment also upregulated the anti-apoptotic proteins Bcl-xL and Bcl-2. Importantly, through chemical fractionation of Sohakuhi extract, moracin O and P were confirmed to mediate its anti-inflammatory effects.

Collectively, the present results indicated that Sohakuhi and moracin may represent potential candidates for the development of novel anti-inflammatory drugs. Future research to *Kaempferia galanga*, regarding to its potential anti-inflammatory effects, it exhibited the same profiles as Morus alba in terms of its cell-protective effect. The objective of purification is to identify compounds containing pharmacologically

active ingredient. I anticipated that unique active components would possess anti-inflammatory properties, and that their application in inflammation-associated disease efficacy.

Futhermore, Through a series of identification process we identified the active compound in *Kaempferia galanga*, Ethyl Para Methoxy cinnamate (EPMC) which could be used to treat melanoma as an anti-metastasis and chemosensitizer agent that could be trough NF-κB inflammatory signal. In this chapter 4 The purpose of this study was to identify the active compound in *Kaempferia galanga*, the active compound in *K. galanga* was isolated and identified using chromatography and spectroscopy techniques, and given six compounds. Inhibitory activity on NF-κB activation and cell viability was determined using reporter assay methods. Among the isolated compounds, ethyl p-methoxycinnamate (EPMC) demonstrated potent NF-κB inhibitory activity against melanoma cell B16F10- NF-κB Luc2.Further investigation was conducted by evaluating the anti-metastasis effect of EPMC in vitro by using wound-healing assays, invasion tests, and molecular mechanism assays using Western blotting. NF-κB has been implicated in tumorigenesis through the p38/Akt/NF-κB pathway. According to the findings of this study, EPMCs act as p38 and thus Akt phosphorylation inhibitors at serine 473, inhibiting NF-κB-dependent transcription. Future investigation with paclitaxel demonstrated that the combinations could sensitize to apoptosis in response to its well-known chemotherapy agents. Additional studies were conducted using the human melanoma cancer cell line SK-Mel 28. Along with the induction of apoptosis, we observed an increase in p-γH2AX expression (a molecular marker for double strand breaks in DNA damage) in response to treatment with paclitaxel and EPMC. The result

showed EPMC to be a potential, viable adjuvant for improving the clinical efficacy of anti-metastatic and cancer chemotherapy. However, Further confirmation of in vivo efficacy is still required.

In addition, Myanmar medicinal plants have pharmacological activity in inhibiting NF-κB activity without causing cell death was investigated. *Kaempferia pulchra* rhizomes compounds were collected in Myanmar further explore in this chapter 5. the anti-inflammatory activities of isopimara-8,14(15)-diene diterpenoids kaempulchraols P and Q, and isopimara-8(9),15-diene diterpenoids Kaempulchraols B-D have not yet been fully elucidated. Investigations of the mechanisms of action of those compounds revealed that they inhibit the NF-κB mediated transactivation of a luciferase reporter gene, IL-6 production, and COX-2 expression Thus, isopimarane diterpenoids are suggested to be potent inhibitors of NF-κB pathways and could be further explored as potential anti-inflammatory lead compounds.

Collectively, Anti-inflammatory agents isolated from natural products by targeting NF-κB activity and their application to inflammation-related diseases conducted to represent prospective candidates for novel anti-inflammatory drug development.

Chapter 2

Identification of natural products as a potential source for anti-inflammatory agents by NF-kB -targeted functional screening

2.1 Background

The World Health Organization defines traditional medicine as "the sum total of knowledge, skills, and practices based on theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as the prevention, diagnosis, improvement, or treatment of physical and mental illness." (42)

Natural remedies and traditional medicines are extremely valuable. Traditional Chinese medicine, Ayurveda, Kampo, traditional Korean medicine, Jamu and Unani have all been performed in some parts of the world and have flourished into well-organized medical systems(43). Over 80% of Japanese doctors practice Kampo, a traditional Japanese medicine. Kampo is used in western medicine because it is safe and effective. As of 2010, there were 345 randomized controlled trials in Japan that used Kampo(44) while Jamu is an Indonesian traditional medicinal herb that has been used for centuries to maintain health and treat the disease in the Indonesian society. Because jamu's biological activity is largely based on empirical data, more research is required to scientifically prove efficacy and ensure safety(45). Kampo and Jamu both primarily used herb as an ingredient that well known in Japan or in Indonesia. (46).

In traditional medicine, plants are used as natural resources to produce treatments that have traditionally been applied in human healthcare since ancient times. Generally, single plants or plant formulas are used to treat or prevent disease(47). This implies that natural products are a valuable source of new drugs, as well as good lead compoundsfor further modifications during drug development. Medicinal plants are the main source of drug discovery in traditional medicine. They have a secondary

metabolite, which has been reported for its pharmacological activity. In the past, drug discovery screening largely occurred by chance and drug development was based on clinical practice. In many plants, secondary metabolites possess structural diversity that makes them promising candidates in terms of their medicinal properties. Indeed, medicinal plants have played an important role in the development of the pharmaceutical industry(48, 49)

2.2 Materials and Methods.

2.2.1 Plant extracts

Total 112 Extracts from Japan collected in Library of institute of Natural medicines while extracts from Indonesia were collected from the South Sulawesi Province, Indonesia. The leaves or rhizomes of plants were extracted with 70% ethanol using the maceration method. Liquid extracts were then evaporated and lyophilized to obtain an EtOH extract. In total, extracts were collected from 35 natural medicinal plants, which were collected from a local traditional healer.

2.2.2 Cells and reagents

The murine B16F10 and 4T1 cell lines were obtained from American Type Culture Collection and maintained at 37˚C in Eagle's minimal essential medium (EMEM) or RPMI-1640 (Nissui Pharmaceutical Co., Ltd.) medium, respectively, supplemented with 10% FBS (Nichirei Biosciences, Inc.), 100 U/l penicillin G and 100 mg/L streptomycin at 37˚C with 5% CO_2.

2.2.3 In vitro NF-κB luciferase reporter assay.

B16F10 NF-κB, 4T1 NF-κB cells and of RAW264.7-NF-κB-luc2 cells expressing firefly luciferase under the control of an NF-κB response element were established as previously described (11,12) and maintained at 37˚C in RPMI-1640 medium containing 10% FBS. Briefly, B16F10 NF-κB and 4T1 NF-κB cells were generated by transfecting the B16F10 and 4T1 cell lines with pGL4.32-*luc2P*/NF-κB-RE/Hygro vector (Promega Corporation) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were selected on hygromycin B and cloned by limiting dilution. B16F10NF-κB cells and 4T1NF-κB cells in the exponential growth phase were seeded at a final concentration of $4x10^4$ cells/well in a 96-well plate. After 3-h incubation, the cells were co-cultured with 50 μg/mL extract from 112 natural products for 24 h. At the end of the assay, 900 μg/mL D-luciferin was added, and the plates were incubated for another 30 min. Luciferase activity was measured by the GloMax®-Multi Detection System (Promega Corporation).

Mouse RAW264.7-NF-κB-luc2 cells were cultured in DMEM containing 10% FBS, 100 U/L penicillin G, and 100 mg/L streptomycin in a 5% CO2 atmosphere at 37°C. In the experiments, cells were seeded in 35-mm dishes (Corning Inc., Corning, NY, USA), and confluent cells were incubated in medium DMEM for 24 h. To establish RAW264.7 cells expressing the NF-κB -mediated luciferase gene (RAW264.7-NF-κB-luc2), RAW 264.7 cells (RCB0535) (2×10^5/well) were seeded in 6-well plates and transfected with the pGL4.32 vector using Lipofectamine 2000. The cells were selected with hygromycin B (100 μg/mL) and cloned by limiting dilution. To evaluate the cellular response to NF-κB in vitro, RAW264.7-NF-κB-luc2 transfectants or RAW CMV control cells (2 ×

10^5/well) were cultured in 96-well plates and treated with LPS (100 ng/mL). After 6 h of incubation, the luciferase activity of cells was measured with a microplate reader.

2.2.4 Cell viability assay

Cell viability was quantified using the WST-1 cell proliferation reagent (Dojindo Molecular Technologies, Inc.). B16F10NF-κB , 4T1NF-κB or RAW 264.7 NF-κB cells were seeded on a 96-well plate and co-cultured with extracts from 112 natural products at 50 μg/mL for 24 h. on the other hand, 35 Indonesia extract were evaluated in the same technic in a separate experiment. After incubation, WST-1 solution was added and used according to the manufacturer's instructions, and absorbance was measured at 450 nm using a microplate reader. Cell viability was calculated as a percentage of the control.

2.3 Result

2.3.1 Sohakuhi suppresses NF-κB activity in murine cancer cell lines.

To identify novel anti-inflammatory agents in plant products, 112 medicinal plant extracts (Table 1) were screened for their effect on NF-κB activity using cell lines that express luciferase under the control of an NF-κB response element. Among the tested extracts, Sohakuhi markedly suppressed NF-κB activity without affecting the viability of 4T1 cells (Fig. 7A). Furthermore, NF-κB activity was suppressed by Sohakuhi extract in 4T1 cells and B16F10 cells in a dose-dependent manner (Fig. 7B). However, Sohakuhi extract did not display any cytotoxic effect, even at doses reaching 50 μg/mL (Fig. 7C).

2.3.2 *Kaempferia galanga* extract supress NF-κB activation versus cell viability in murine 4T1-NF-κB-Luc2 cells and RAW264.7-NF-κB-luc2 cells

We investigated the inhibition of NF-κB activation in 12 Indonesia natural medicinal plants from among collection extracts (table 2). Those extract reported traditionally used to treat skin disease symptoms: these were screened using a reporter assay method. Inhibitory activity against NF- κB was determined by co-culturing 4T1-NF-κB -Luc2 cells with extracts at 1 and 50 μg/mL for 24 hrs (Fig. 8A). Twelve extracts showed active inhibition of NF-κB in transfected cells. NF-κB inhibition results were compared with those from a viability assay in which WST-8 was used (Figure 8A)

The result of Fig. 8B illustrated the RAW264.7-NF-κB-luc2 cells were co-cultured with extracts 29, this extract effectively inhibited NF-κB activity without affecting viability cells.The result of Fig. 8C illustrated the RAW264.7-NF-κB-luc2 cells were co-cultured with extracts 35, this extract effectively inhibited NF-κB activity without affecting viability cells. The inhibitory effect (grey) of each plant extract on NF-κB activation relative to untreated controls is shown. Cell viability (black) was determined using a WST-8 assay and is shown as a percentage of untreated control cell viability

2.4 Discussion

The aim of the present study was to identify a novel anti-inflammatory drug candidate that can target NF-κB activation. A total of 112 Japan plant extracts were screened and among 35 extracts from Indonesia, identifying Sohakuhi and *Kaempferia galanga* as a promising anti-inflammatory extract. Sohakuhi in Japan is *Morus alba* species. The

white mulberry tree (*Morus alba* Linn.) is a deciduous tree originating from Asia, especially China, but currently cultivated in subtropical, tropical and mild environmental conditions(50, 51). *Morus alba* Linn. tree bark, fruits and leaves contain proteins, carbohydrates, calcium, iron, ascorbic acid, thiamine, folic acid and vitamin D (52). It has been used in conventional and natural medicine to treat diabetes, atherosclerosis, hyperlipidemia, hypertension, neurodegenerative disease and cancer (50).

Morus alba leaves have been investigated to contain constituents of its butanol extracts. Such as prenylflavanes and a glycoside, isoquercitrin, astragalin, scopolamine, skimming, roseoside II, and benzyl D-glucopyranoside (53). The fruit constituent existed; polyphenols, minerals, and vitamins beneficial to reduce hypertension, liver and kidney damage, and improving eyesight(54), Cyanidin-3-rutinoside and cyanidin-3-glucoside are the major anthocyanins isolated from mulberry fruits which reported have anti-cholesterol, anti-diabetic, antioxidative, and antiobesity effects (55, 56) stearic acids, myristic and fatty acid(57) ascorbic acid(58), many types of essential amino acids and non-essential where found in fruit with high antioxidant properties(59).

The root bark of Morus alba Linn contains 2-arylbenzofuran derivatives as good natural antioxidants and mulberrofuran H as a promising antioxidant and anti-browning agent that can be applied as a functional food additive(60). Phytochemical studies have reported that alkaloids, flavonoids, flavones, flavanones, stilbenes, benzophenones, coumarin derivatives, terpenoid stilbenes, oxyresveratrol and resveratrol were present in *Morus* species(61-63). Moreover, oxyresveratrol can suppress inflammation by inhibiting nitric oxide (NO) production, inducible NO synthase expression, prostaglandin

E2 production and NF-κB activation in macrophages (19). Moracin R, C, O, P and D belong to the 2-arylbenzofuran group, and display inhibitory activity against the differentiation of 3T3-L1 adipocytes and NO production in RAW264.7 cells(64). The 2-arylbenzofurans of *Morus* species, moracin O and P, markedly suppress hypoxia-inducible factor-1 (HIF-1) activity in the human Hep3B hepatocellular carcinoma cell line(65-67). Moracin-M-3'-O-β-D-glucopyranoside has been reported to suppress 12-O-tetradecanoylphorbol-13-acetate-induced tumor progression in mouse skin, and the underlying mechanism may involve the inhibition of leukocyte infiltration, epidermal hyper-proliferation, oxidative stress, and the endogenous tumor promoter TNF (68).

On the other hand, Screening resulted potent inhibitor extract from Indonesia were found on *Kaempferia galanga*. Almost all the plant family have been reported for their biological activity, including Zingiberaceae. An investigation of an isolated compound from K. galanga mainly reported it to contain essential oil(69); however, it is widely known that plants collected from distinct altitude habitats possess distinct essential oil characteristics(70). Kaempferia galanga, belonging to the Zingiberaceae family, is a plant that is widely used as a traditional medicine in Asia. According to the study by Khairullah at al., the plant is a stemless herb derived from tuberous rootstocks with a fibrous cylindrical root. It has dark reddish-brown skin, and the soft interior is nearly white(71). It is empirically used as a single plant or as a complementary ingredient in a medicinal formula. This plant grows easily and thrives in tropical climates such as Southeast Asia. The rhizome of the plant is mostly used as a traditional medicine, either in its fresh or in dry form. The empirical uses of the rhizome K. galanga were reported by Elshamy et al., as a cure for metabolic disorder, inflammation, urinary

tract infection, fever, coughs, hyper- tension, erectile dysfunction, abdominal and gastrointestinal ailment, asthma, wounds, rheumatism, epilepsy, and skin diseases(72). In Southeast Asian countries, this plant is used for the treatment of different kinds of diseases(73). Various research results have proven that this plant has anti-inflammatory(74), anti-angiogenic(75), anti-cancer and anti- proliferative activities, among others(76) in tests of murine RAW 264.7, stable cells transfected with NF-κB extracts 29 and 35 (*Kaempferia galanga*) were nontoxic to macrophage cells while NF-κB activity decreased significantly. Collectively, these data indicate that extracts 29 and 35 are promising candidates for targeting NF-κB in application inflammatory-associated disease particularly because they do not affect normal cell viability. Furthermore, these extracts might be useful candidates for advancing proinflammatory research.

Chapter 3

Anti-inflammatory compounds moracin O and P from Morus alba Linn (Sohakuhi) target the NF-κB pathway

3.1 Background

Mulberry (Morus L.) is an indigenous tree crop that provides long-term financial and environmental benefits to a great amount of people in rural areas across many Asian countries. Only a few of the 68 species globally recognized, such as *M. alba, M. bombycis, M. indica, M. latifolia*, and *M. multicaulis*, are cultivated for foliage, and *M. nigra* for fruit. The remaining species, as well as several landraces of the cultivated species, are considered wild, and thus have received little attention(77). Special focus on this chapter was given on *Morus alba* also known as mulberry or Sohakuhi. Sohakuhi was clinically prescribed for catharsis, diuresis, cough, edema, fever relief, and palsy prevention in two well-known Chinese medicine technical books, "Wakansho" and "Kampo Shoho Kaisetsu"(78). As found previously that *Morus alba* have effective inhibition to NF-κB.

Future research conducted to *Morus alba* determine their application on inflammation-induced cellular damage to normal human keratinocytes cells. The aim of the present study was to investigate anti-inflammatory properties of Sohakuhi, it was identified that Sohakuhi (*Morus alba* Linn. bark) extract markedly suppressed NF-κB-dependent luciferase reporter activity in murine 4T1 cells without affecting cell viability. The anti-inflammatory effect of Sohakuhi on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cellular damage was evaluated in human HaCaT keratinocytes. TRAIL triggered the phosphorylation of p65, a subunit of NF-κB, leading to cellular damage in HaCaT cells. However, treatment with Sohakuhi extract protected HaCaT cells against TRAIL-induced damage. Moreover, Sohakuhi also upregulated the expression of the anti-apoptotic proteins Bcl-xL and Bcl-2. Importantly, through chemical

fractionation of Sohakuhi extract, moracin O and P were determined to mediate its anti-inflammatory effect.

3.2 Materials and Methods

3.2.1 Cells and reagents

The murine B16F10 and 4T1 cell lines were obtained from American Type Culture Collection and maintained at 37˚C in Eagle's minimal essential medium (EMEM) or RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd.) containing 10% FBS (Nichirei Biosciences, Inc.), respectively. Human HaCaT keratinocytes (provided by Dr Takeda, Juntendo University) were maintained at 37˚C in culture medium consisting of DMEM (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% FBS, 100 U/l penicillin G and 100 mg/l streptomycin at 37˚C with 5% CO_2. Human recombinant TRAIL (rTRAIL) was purchased from PeproTech, Inc.

3.2.2 In vitro NF-κB luciferase reporter assay.

B16F10 NF-κB and 4T1-NF-κB cells expressing firefly luciferase under the control of an NF-κB response element were established as previously described (11,12) and maintained at 37˚C in RPMI-1640 medium containing 10% FBS. Briefly, B16F10 NF-κB and 4T1 NF-κB cells were generated by transfecting the B16F10 and 4T1 cell lines with pGL4.32-*luc2P*/NF-κB-RE/Hygro vector (Promega Corporation) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were selected on hygromycin B and cloned by limiting dilution. B16F10NFκB cells and 4T1NF-κB cells in the exponential growth phase were seeded at a final concentration of $4x10^4$ cells/well in a

96-well plate. After 3-h incubation, the cells were co-cultured with 50 μg/mL extract from 112 natural products (Table 1) for 24 h. At the end of the assay, 900 μg/ml D-luciferin was added, and the plates were incubated for another 30 min. Luciferase activity was measured by the GloMax®-Multi Detection System (Promega Corporation).

3.2.3 Cell viability assay

Cell viability was quantified using the WST-1 cell proliferation reagent (Dojindo Molecular Technologies, Inc.). B16F10NF-κB or 4T1NF-κB cells were seeded on a 96-well plate and co-cultured with extracts from 112 natural products (Table 1) at 50 μg/mL for 24 h. In a separate experiment, HaCaT cells were seeded on a 96-well plate at a density of 10^4 cells/well and pre-treated with 20 ng/ml rTRAIL for 1 h. The cells were then cultured with or without Sohakuhi extract (6. 25 to 50 μg/mL) for 24 h. After incubation, WST-1 solution was added and used according to the manufacturer's instructions, and absorbance was measured at 450 nm using a microplate reader. Cell viability was calculated as a percentage of the control.

3.2.4 Caspase-3 and -7 activity assay

For measurement of the activities of caspase-3 and -7, the Caspase-Glo® 3/7 assay system (Promega Corporation) was used according to the manufacturer's instructions. Briefly, HaCaT cells ($5x10^3$ cells/well in a 96-well plate) were pre-treated with 20 ng/mL rTRAIL for 1 h. The cells were then cultured with Sohakuhi extract for 24 h, and the Caspase-Glo® 3/7 reagent was then added. After 30-min incubation, caspase-3 and -7

activities were measured using the GloMax®-Multi Detection System (Promega Corporation).

3.2.5 Identification of the active components of Sohakuhi extract

To identify the bioactive components of Sohakuhi extract, 200 g root bark of *Morus alba* Linn. was decocted in 1 liter water for 50 min. The water extract was fractionated by water-methanol gradient reverse-phase medium-pressure liquid chromatography (RP-MPLC) fractionation. Mass spectrometry and ^{1}H-nuclear magnetic resonance (NMR) analysis were used to identify compounds in the isolated fractions. Extraction and isolation of Sohakuhi was conducted using a water-methanol gradient MPLC fractionation system and high- performance liquid chromatography (Accela™ HPLC system; Thermo Fisher Scientific, Inc.) profiles of water extracts of Sohakuhi were determined at 254-nm ultraviolet wavelength. A Capcell Pak C18 MG III S-5 (4.5x250 mm, 5 μm; Shiseido Co., Ltd.) column was used for HPLC analysis at a flow rate of 1 ml/min at 40˚C. A gradient elution system composed of 5% CH_3CN (v/v) (A) and H_2O was used as follows: 0-4 min, 5% A; 4-8 min, 5-10% B; 8-12 min, 10-15% B; 12-15 min, 15-20% B; 15-18 min, 20-25% B; 18-21 min, 25-30% B; 21-25 min, 30-35% B; 25 min, 40% B. The active fraction was further analyzed using a $CHCl_3$-MeOH gradient RP-MPLC fractionation system with mass spectrometry and ^{1}H-NMR analysis to identify the active compounds of Sohakuhi. The MS conditions were set as follows: Negative ESI mode, spray voltage 4.5 kV, capillary voltage 40.0 kV, tube lens 150 V, capillary temperature 270˚C, sheath gas flow rate 50 units, aux gas flow rate 10 units, and scan

range m/z 50-2,000. A polytyrosine solution was used for instrument calibration before each experiment.

3.2.6 Western blot analysis

HaCaT cells were seeded at a density of $5x10^5$ cells/well and cultured for 24 h in a 6-well plate. After treatment, the cells were washed in cold PBS, scraped and lysed in whole-cell lysis buffer (25 mmol/l HEPES, pH 7.7; 300 mmol/l NaCl; 1.5 mmol/l $MgCl_2$; 0.2 mmol/l EDTA; 0.1% Triton X-100; 20 mmol/l β-glycerophosphate; 1 mmol/l Na_3VO_4; 1 mmol/l phenylmethylsulfonylfluoride; 1 mmol/l dithiothreitol; 10 mg/ml aprotinin; 10 mg/ml leupeptin). Cell lysates (10 µL/lane) were resolved by SDS-PAGE on 7.5-15% gels, then transferred to an Immobilon-P nylon membrane (EMD Millipore). The membranes were treated with Block Ace (Dainippon Sumimoto Pharma Co., Ltd.) for at least 2 h at room temperature, then probed with primary antibodies at 4˚C for overnight, followed by horseradish peroxidase- conjugated secondary antibodies (P0448 or P0260, Dako; Agilent Technologies, Inc., used at 1:2,000 dilution). Bands were visualized using ECL reagents (Amersham; Cytiva). The primary antibodies used were specific for Bcl-2 (clone no. D55G8; cat. no. 4223), Bcl-xL (clone no. 54H6; cat. no. 2764), p65 (clone no. L8F6; cat. no. 6956), phosphorylated-p65, (clone no. 93H1; cat. no. 3033; all Cell Signaling Technology, Inc.) and β-actin (clone no. C4; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). All primary antibodies were used at 1:1,000 dilution.

3.2.7 Statistical analysis.

All data are presented as the mean ± SEM of three independent experiments. SPSS versions 23 and 25 software (IBM Corp.) were used to analyze data. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni correction. $P<0.05$ was considered to indicate a statistically significant difference.

3.3 Result

3.3.1 Cytoprotective effect of Sohakuhi extract on TRAIL-induced cellular damage in human keratinocytes.

To examine the biological utility of Sohakuhi extract, human HaCaT keratinocytes were incubated with rTRAIL as an inflammatory stimulus to induce NF-κB activation and cytotoxicity. Compared with the control, rTRAIL treatment induced significant cytotoxicity in HaCaT cells. However, pre-treatment with Sohakuhi extract significantly protected HaCaT cells from TRAIL-induced cytotoxicity in a dose-dependent manner (Fig. 9A). The cytoprotective effect of Sohakuhi extract was evident from microscopic observation of cell morphology (Fig. 9B). These results indicated the cytoprotective effect of Sohakuhi extract against TRAIL-induced cellular damage in human keratinocytes.

3.3.2 Anti-apoptotic effect of Sohakuhi extract on HaCaT cells.

To confirm whether Sohakuhi extract affects NF-κB activation in HaCaT cells, the expression of the p65 subunit of NF-κB and its phosphorylation status following rTRAIL stimulation was assessed in the presence or absence of Sohakuhi extract (Fig. 10A).

Phosphorylation of p65 was detectable in HaCaT cells following rTRAIL stimulation, but suppressed in the presence of Sohakuhi extract. While Sohakuhi extract treatment reduced total p65 expression in rTRAIL-stimulated HaCaT cells, there was almost no effect on the basal expression of p65 in unstimulated HaCaT cells, suggesting that the effect of Sohakuhi extract might be specific to TRAIL-induced NF-κB activity. Treatment with the Sohakuhi extract also showed an inhibitory effect on TRAIL-induced apoptosis, as seen in the suppression of caspase-3/7 activation (Fig. 10B). Importantly, the anti-apoptotic proteins Bcl-xL and Bcl-2 were upregulated in Sohakuhi extract-treated HaCaT cells following TRAIL stimulation (Fig. 10C). These results indicated that the cytoprotective effect of Sohakuhi extract might result to inhibit TRAIL-induced apoptosis via upregulation NF-κB as transcription factor which involve in regulation of pro-apoptotic proteins.

3.3.3 Identification of the active component of Sohakuhi extract to inhibit NF-κB activation.

As Sohakuhi extract significantly inhibited NF-κB activity and displayed a cytoprotective effect against inflammation-associated cellular damage of human keratinocytes, the bioactive components of Sohakuhi extract were then analyzed. A total of 8 yield fractions were isolated from a Sohakuhi water extract using RP-MPLC with methanol-water gradient (Fig. 11A). The preparative HPLC data for these 8 yield fractions are presented in Fig. 11B. Amongst those fractions, fraction 8 effectively and exclusively inhibited NF-κB activity (Fig. 11C).

Considering that fraction 8 was eluted with 100% methanol in the water-methanol gradient, the methanol extract of Sohakuhi was then fractionated into hexane, ethyl acetate, n-butanol and a residual water layer (Fig. 12A) to test the activity of each layer with respect to NF-κB inhibition. Amongst those layers, the ethyl acetate layer displayed very potent inhibition of NF-κB activity in 4T1 cells, even at the lowest dose tested, 5 μg/mL (Fig. 12B). These results indicated that the active component of Sohakuhi was fractionated into an ethyl acetate layer from the methanol extract.

3.3.4 Isolation of moracin O and P as active compounds of Sohakuhi

Using a CHCl3-MeOH gradient RP-MPLC fractionation system with mass spectrometry and ^{1}H-NMR analysis, two major compounds, moracin O and P, were identified in the ethyl acetate layer of Sohakuhi methanol extract (Fig. 13A). Importantly, both moracin O and P significantly inhibited NF-κB activity in 4T1 cells, starting at a dose of 3 nM (Fig. 13B). Additionally, both moracin O and P showed significant cytoprotective effects against TRAIL-induced cellular damage in HaCaT cells (Fig. 13C). Thus, moracin O and P were identified as active compounds of Sohakuhi that suppressed NF-κB activity and exerted a cytoprotective effect against TRAIL-induced damage in HaCaT cells.

3.4 Discussion

NF-κB plays a central role in inflammation, immunity and several other cellular responses (79). A variety of ligands and receptors can activate the NF-κB signaling pathway, including TNF and TNF receptor superfamily molecules that are key to inflammatory responses, Amongst these TNF superfamily members, TRAIL is known to

be a potent inducer of apoptosis through activation of inflammatory signaling pathways (80-82). In the present study, TRAIL induced cellular damage of HaCaT keratinocytes through NF-κB activation. Moreover, both moracin O and P significantly protected HaCaT cells against TRAIL-induced cytotoxicity, possibly through the inhibition of apoptosis. Considering that NF-κB -mediated inflammation has been implicated in the regulation of cellular damage by balancing anti- and pro-apoptotic protein expression (83-86).moracin O and P may protect against HaCaT cell damage through their anti-inflammatory activity by targeting the NF-κB pathway. In the present study, moracin O and P as novel anti-inflammatory compounds inhibiting cellular damage induced by NF-κB activation.

The aforementioned effects of Sohakuhi extract and its active components, moracin O and P, suggested that these compounds may prove beneficial for the treatment of inflammatory diseases. Although the exact mechanism of action remains to be determined, the present study demonstrated that both moracin O and P represent promising phytochemicals that may act as novel anti-inflammatory or cytoprotective agents through the suppression of the NF-κB pathway.

Chapter 4

Anti-inflammatory and cytoprotective effect of *Kaempferia galanga* extracts by targeting NF-κB activity

4.1 Background

Kaempferia galanga in Indonesia, it is commonly known as “kencur” and is used in local cuisines and traditional medicine. The rhizome of the plant has long been used in Indonesian traditional medicine as “jamu,” a plant material for “Jamu gendong” (a traditional medicine in liquid or other forms that is freshly prepared without preservation and sold without a label)(87). Many Southeast Asian countries use *K. galanga* as a traditional medicine or in other products. In Chinese traditional medicine, it used to treat cholera, contusion, constipation, and stomach ache; in Thailand, it is used as a treatment for menstrual disorder and dyspepsia; in Bangladesh, it is used for scenting vinegar, hair shampoo, cosmetics, flavorings, and beverages; and in Indian Ayurveda, it is used to treat muscular swelling and rheumatism (88-90).

Analysis of the macro- and micro-components of *K. galanga* has shown that it contains protein, fiber, and high amounts of essential minerals (e.g., potassium, phosphorous, and magnesium), along with considerable amounts of iron, manganese, zinc, cobalt, and nickel(73). In addition, compounds such as isopimarane, abietane, labdane, and clerodane diterpenoids, along with flavonoids, phenolic acids, phenyl-heptanoids, curcuminoids, tetrahydropyrano-phenolics, and steroids have been isolated from Kaempferia species(72). Previous studies have also reported that the biological activities of the plant include larvicidal, insect repellant, anticancer, antimicrobial, antioxidant, anti-obesity–induced dermatopathy, anticholinesterase, wound healing, antinociceptive, neuroprotective, antiallergenic, and anti-inflammatory activities(72, 91). The empirical uses of the rhizome *K. galanga* were reported by Elshamy et al., as a cure for metabolic disorder, inflammation, urinary tract infection, fever, coughs, hyper-

tension, erectile dysfunction, abdominal and gastrointestinal ailment, asthma, wounds, rheumatism, epilepsy, and skin diseases (72).

In Southeast Asian countries, this plant is used for the treatment of different kinds of diseases(73). Various research results have proven that this plant has anti-inflammatory(74), anti-angiogenic(75), anti-cancer and anti- proliferative activities, among others(76). Kumar et al. researched the chemical constituent of *K. galanga* rhizome, showing that this plant contains compounds such as esters, terpenoids, flavonoids, thiourea derivatives, polysaccharides, diarylheptanoids, phenolic acids, phenolic glycoside and cyclic lipodepsipeptide types(92). Propanoic acid, pentadecane, and ethyl p-methoxycinnamate (EPMC) compounds were reported as the most abundant compounds from the essential oil of this plant(91).

Various testing methods have been developed to explore the potential of materials that can be used as drugs, especially for cancer. Several drugs were found and used but were not able to completely overcome the problem of the disease. Efforts were made to find cancer drugs that can inhibit NF-κB activity and are not cytotoxic to normal cells. The NF-κB gene plays a role in the development of cancer in humans. There have been important concerns about NF-κB's role in the initiation, development, metastatic, and treatment resistance of human cancer. The suppression of NF-κB in myeloid cells or tumor cells usually leads to tumor regression, which makes the NF-κB pathway a promising therapeutic target(35). This study aimed to find medicinal ingredients sourced from traditional medicines, which have the ability to inhibit NF-κB with low toxicity.

As numerous reports have explained, inflammation is the primary control in the disease's development.

Consequently, the search for new anti-inflammatory medications is deemed essential. Inhibition of proinflammatory gene expression controlled by NF-κB is likely to reduce the production of inflammatory mediators and control excessive inflammation, thereby aiding in the prevention of inflammatory-associated diseases(93). So, the goal of this study was to find natural anti-inflammatory agents that could target NF-κB activity by looking at Indonesian medicinal plants, with a focus on *K. galanga* extracts and its compounds as promising candidates to anti-inflammatory-associated melanoma.

4.2 Materials and Methods

4.2.1 Extracts and Compounds from Plants

K. galanga was collected in the Indonesian province of South Sulawesi. Using the maceration technique, the rhizomes of plants were extracted with 70 percent ethanol. After liquid extracts were evaporated and lyophilized, an Et-OH extract was obtained (89). Using a combination of column chromatography and preparative thin layer chromatography (PTLC), six isolated compounds were obtained. To obtain six pure compounds, the isolated compounds were purified with PLTC silica gel and n-hexane:EtoAc (7:3) as the mobile phase. Using NMR and MS spectroscopy, the structure of the isolated compounds was determined and compared to the references. Paclitaxel was obtained from Fuji film Wako Pure Chemical Co., Osaka, Japan, under the SKN 5312 number.

4.2.2 Cells and Reagents

Human HaCaT keratinocytes (provided by Dr Takeda, Juntendo University) were maintained at 37˚C in culture medium consisting of DMEM (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% FBS, 100 U/l penicillin G and 100 mg/l streptomycin at 37˚C with 5% CO_2. Human recombinant TRAIL (rTRAIL) was purchased from PeproTech, Inc. Mouse RAW264.7-NF-κB-luc2 cells were cultured in DMEM containing 10% FBS, 100 U/L penicillin G, and 100 mg/L streptomycin in a 5% CO2 atmosphere at 37°C further transfection process as previous chapter.

B16F10 and SK-Mel 28 cell lin lines were obtained from the American Type Culture Collection and maintained at 37^{o}C in Eagle's minimal essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (FBS; Nichirei Biosciences, Inc., Tokyo, Japan). In order to create an NF-κB-mediated luciferase gene expressing B16F10 cells (B16F10 NF-κB), B16F10 cells (5×10^5/well) were seeded in a 6-well plate and the pGL4.32 vector was transfected with Lipofectamine 2000.

The cells were cloned using limiting dilution after being selected with Hygromycin B (200 g/mL). B16F10 NF-κB transfectants, or B16F10 CMV control cells (1×10^5/well), were cultured in a 96-well plate and treated with TNF (0.1–100 ng/mL) to assess the NF-κB response in vitro. After 6 h of incubation, luciferase activity was measured using a multiplate reader (2030 ARVO X; Perkin Elmer Life Sciences, Boston, MA, USA)(94).

4.2.3 Cell Viability

RAW264.7-NF-κB-Luc and B16F10-NF-κB-Luc2 cells were plated at a final concentration of 2×10^4 cells/well in a 96-well plate. After 24 h of incubation, the cells were pretreated with 50 μg/mL of extract or compounds and further incubated for 24 h. Subsequently, 10 μL of WST-8 (FUJIFILM Wako Pure Chemical Corporation) solution was added to the cells, which were incubated for an additional 1 h in a humidified atmosphere (37°C and 5% CO2) to allow for the formation of formazan dye and to increase sensitivity. Then, the absorbance was measured with a microplate reader (SunriseTM; Tecan Group Ltd., Männedorf, Switzerland) at wavelengths of 450/620 nm. Cell viability was determined based on the absorbance of the soluble formazan dye generated by the living cells. The same method was followed to determine the viability of B16F10-NF-κB-luc2 cells treated with selected extracts in a concentration-dependent manner. The WST-8 solution was added and used according to the manufacturer's instructions. The viability of the treated cells was calculated as a percentage(94, 95).

4.2.4 NF-κB Reporter Gene Assay

B16F10-NF-κB-luc2 cells were maintained in EMEM supplemented with 10% FBS, 1 mM L-glutamine, and antibiotics (100 U/L penicillin and 100 mg/L streptomycins) at 37 ◦C in a humidified atmosphere containing 95% air and 5% CO_2. The cells were seeded in black 96-well plates at a density of 2×10^4 cells per well and then incubated for 24 h. Subsequently, they were treated with the extracts, and an equal concentration of the solvent vehicle was included as a control after 24 h. At the end of the assay, 900 μg/mL D-luciferin was added, and the plates were incubated for another 30 min. Luciferase

activity was measured according to the luminescence of firefly luciferase, which was quantified using IVIS LUMINA II and Living Image 4.2 software (Caliper Life Science, Waltham, MA, USA) by determining the light emitted from cells. Data were expressed as photons/s and the fold NFκB activity was calculated as a total flux of extracts (photons/s) is divided by a total flux of vehicle as control (photons/s)(94, 95).

4.2.5 Wound Healing Assay

B16F10G5-Luc cells were seeded at a concentration of 1×10^5 cells/well in a 24-well plate and allowed to form a confluent mono-layer for 24 h. After scratching the mono-layer with a sterile pipette tip (1 mL), it was washed with medium to remove floating and detached cells and photographed (time 0 h). For 24 h, these cells were treated in medium containing 50 μM concentrations of EPMC. Scratched areas were photographed (magnification ×10 using a Biozero BZ-8000 microscope, Osaka, Japan) at 0 h as T-0 and again 24 h later as T-24 to assess wound healing. The wound closure percentage was calculated as follows: wound closure percent = 1 (wound area at T-24/wound area at T-0) 100 percent, where T-24 is the time after wounding and T-0 is the time immediately after wounding(96, 97).

4.2.6 Transwell Invasion Assay

A transwell chamber (Costar No. 3422, Cambridge, MA, USA) with an 8.0 μm pore size polycarbonate filter (Whatman, Clifton, NJ, USA) on the lower part and 1 g of Matrigel on the upper part of the filter was pre-coated with 10 g fibronectin (Fujifilm Wako Pure Chemical Corporation, Tokyo, Japan). B16F10 G5-Luc cells were pre-treated with 50

μM EPMC overnight before being suspended in serum-free media in the upper transwell chamber (3 × 10^5cells/100 μL/chamber) and placed in a 24-well plate (lower compartment of chamber) with media containing 0.1 percent (v/v) BSA, then incubated at 37°C for 7 h. Following incubation, the invading cells on the membrane's lower surface were fixed in methanol, stained with hematoxylin and eosin, and wiped with cotton swabs to remove non-invasive cells. The cells that invaded through the filter were photographed at magnification ×10 using a Biozero BZ-8000 microscope (Keyence, Osaka, Japan). Invading cells on the membrane were randomly selected in five visual fields, and the average value in the five fields was used to calculate the migration index. Image J software was used to count the cells that had infiltrated the filter(98).

4.2.7 Western Blotting

B16F10 G5-Luc Cells (10^6 cells/well) were treated for 12 h with the tested compounds EPMC 50 μM and PTX 20 μM. Trypsinized cells were harvested by adding PBS and centrifuged for 10 min at 14,000 rpm and 4°C. The supernatant was then discarded, and the cells were lysed in a whole-cell lysis buffer (25 mmol/L HEPES, pH 7.7, 300 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.1 percent Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenyl-methylsulfonyl fluoride, 1 mM dithiothreitol, 10 mg/mL of aprotinin, and 10 mg/mL of leupeptin). Cell lysates were electrophoretically separated using 10% SDS-PAGE and electrophoretically transferred to Immobilon-P nylon membranes (Millipore, Bedford, MA, USA). The membranes were blocked for at least 2 h with Block Ace (Dainippon Pharmaceutical, Co. Ltd., Osaka, Japan) before being probed with the indicated primary antibodies at 4°C overnight,

followed by incubation with horseradish-peroxidase-conjugated secondary antibodies (1:1000 dilutions) at room temperature. ECL reagents were used to visualize the bands (Amersham Bioscience, Piscataway, NJ, USA). Primary antibodies used were specific to p65 (LF6, #6956), p-p65 (93H1, #3033) (Cell Signaling Technology, Beverly, MA, USA), β-actin (C4, sc-47778) (Santa Cruz, CA, USA). Phospho-Akt (Ser473) Antibody #9271 Cell signaling (Beverly, MA, USA), p38α, sc-535 C20, (Santa Cruz, CA, USA), IKKβ #2684, Cell signaling (Beverly, MA, USA)(94).

4.2.8 Statistical analysis

All data are presented as the mean ± standard error of the mean of three independent experiments. SPSS version 25 software (IBM Corp., New York, NY, USA) was used to analyze data. Data were analyzed using a one-way analysis of variance, followed by a Bonferroni correction. p-values of <0.05 were considered to indicate a statistically significant difference(94, 95).

4.3 Result

4.3.1 Cytoprotective effects of K. galanga extract on TRAIL- induced cytotoxicity in HaCaT cells

The biological activity of *K. galanga* in extracts 29 and 35 was evaluated by incubating HaCaT cells with rTRAIL as a mediator of inflammatory stimuli that activate NF-κB activity and cytotoxicity. Pretreatment with extracts 29 and 35 did not show toxic effects on HaCaT cells, whereas rTRAIL induced a significant reduction in the viability of cells. Furthermore, pretreatment with extract 29 and 35 at concentrations of 16 and 32 μg/mL

protected cell growth against rTRAIL-induced cellular damage (Fig. 14a-b). Cytoprotective effects of extracts 29 (Fig. 14a) and 35 (Fig. 14b) against tumour necrosis factor-related apoptosis-inducing ligand (rTRAIL)-induced cellular damage in human keratinocytes.

4.3.2 Active Compounds of *Kaempferia galanga*

Dried rhizome of *K. galanga* was extracted with 70% EtOH to produce the ethanolic extract, and the compounds were isolated using a combination of column and preparative chromatography techniques, with silica gel as a stationary phase and various eluent systems. Six pure compounds were obtained. The compound's structure was identified using NMR and MS spectrometry and by comparing the existing reference data for the compound(99-102). The structure of the compound was obtained as shown in Figure 15. The compounds are diethyl-phthalate (1) with H-NMR (500 MHz, CDCl3) δ 7.70 (2H, m), 7.53 (2H, m), 4.21 (4H, m), 1.28 (6H, m), MS: m/z 222; ethyl-p-methoxycinnamate (2) H- NMR (500 MHz, CDCl3) 7.64 (1H, d,16 Hz), 7.46 (2H, d, 8 Hz), 6.88 (2H, d, 8 Hz), 6.30 (1H, d, 16 Hz), 4.25 (2H, q, 7.5 Hz), 3.81 (3H, s), 1.32 (3H, t, 8 Hz), MS: m/z 206; methyl-p- methoxycinnamate (3), H-NMR (500 MHz, CDCl3) 7.64 (1H, d, 16 Hz), 7.47 (2H, d, 8.5 Hz), 6.90 (2H, d, 8.5 Hz), 6.30 (1H, d, 16 Hz), 3.83 (3H, s), 3.79 (3H, s), MS: m/z 192; ethyl cinnamate (4), H-NMR (500 MHz, CDCl3) 7.69 (1H, d, 16 Hz), 7.51 (2H, m), 7.38 (3H, m), 6.43 (1H, d, 16 Hz), 4.27 (2H, m), 1.34 (3H, m), MS: m/z 176; p-hydroxycinnamic acid (103), H-NMR (500 MHz, CDCl3) 7.50 (1H, d, 16 Hz), 7.47 (2H, d, 16 Hz), 6.78 (2H, d, 16 Hz), 6.27 (1H, 8 Hz), MS: m/z 164; and p-methoxybenzoic acid

(6) H-NMR (500 MHz, CD3OD) 7.96 (2H, d, 8 Hz), 6.97 (2H, d, 8 Hz), 3.82 (3H, s), MS: m/z 152.

4.3.3 NF-κB Inhibition and Viability Cell against B16F10 Melanoma

As part of the process of exploring new bioactive components from *K. galanga*, the six isolates were then tested for their effects on NF-κB gene activity and cell viability for B16F10 melanoma skin cancer cells using the luciferin/luciferase reporter assay/WST-8 method. An investigation into the activity of nuclear factor-kappaB (NF-κB) and the viability of B16F10-NF-κB-luc cells was carried out, as shown in Figure 16A, B. BAY-11-7082 was applied as a positive control.

4.3.4 Evaluation Relative inhibitory versus viability effect of active compound EPMC on normal cell (RAW264.7-NF-κB-luc2)

Evaluation Relative inhibitory versus viability effect of active compound EPMC on normal cell (RAW264.7- NF-κB-luc2) As illustrated in Figure 17, RAW264.7-NF-κB-luc2 cells were co-cultured with EPMC at various concentrations and incubated for 24 hrs(10, 20, 40, 80 and 100 μM). The inhibitory effect of EPMC (bar graph) on NF-κB activation relative to untreated controls is shown. Cell viability (line) was determined using a WST-8 assay and is shown as percentage of viability cell compare to untreated control cell.

4.3.5 Molecular mechanism of EPMC

B16F10 G5-luc cells were cultured for 12 h and treated with EPMC before harvesting the protein. Furthermore, the molecular mechanism of EPMC 50 μM treatment's effect

on B16F10 G5-Luc cells was investigated. The Western blotting analysis result showed that EPMC 50 μM downregulated P38/ MAPK, and p-Akt could be interacted with Akt-at serine 473. We hypothesize that Akt kinase interaction is required for Akt-mediated NF-κB activation. Additionally, IKK- β and p-P65's serine536 level decreased (Figure 18).

4.3.6 Migration Assay

To better understand EPMC's possible effect on cancer activity, whether migratory or invasive, we performed a migration assay over 24 h. A concentration of 50 μM decreased melanoma cell migration from the clear area. The same result was found in invasion assay, while a 50 μM administration of EPMC was observed in B16F10G5-Luc cells (Figure 19A,B). Migration assay of B16F10G5-Luc in the presence of EPMC evaluated by the wound- healing method (19A) and invasion using transwell. The transwell cell migration assay measures the chemotactic ability of cells toward a chemo-attractant (19B). IC50 Paclitaxel against B16F10 G5-Luc Cells was shown inn Figure 20. Paclitaxel-treated effect on B16F10 G5-Luc. IC50 Paclitaxel on this cell is 23.8102 μM.

4.3.7 Sensivity test

When testing sensitized paclitaxel (PTX) 1–30 μM on B16F10 G5-Luc, PTX remained resistant, with IC50 = 23.81 μM. (Figure 20). Furthermore, both compounds were used for treatment at a nontoxic dose, EMPC 50 μM and PTX 20 μM, together with the co-treatment with both EPMC and PTX of B16F10 G5-Luc and human SK-Mel 28 melanoma cancer cells. The combination showed a toxic effect compared to single treatment (Figure 21A,B). The Western blotting results indicated that SK-Mel 28 has an

effect on EPMC and, combined with PTX, could activate the phosphorylation of γ-H2AX, causing a double-stranded DNA break in the cell and resulting in apoptosis induction (Figure 21C).

4.3.8 Dose response EPMC on both B16F10-NF-κB-luc cells and B16F10 G5-Luc

Figure 22 reveals B16F10-NF-κB-luc cells were cultured for 24 h and BAY-11-7082 was used as a positive control. The test was repeated at least three times, unless explicitly stated otherwise. Inhibitory activity of NF-κB (A) and cell viability for B16F10-NF-κB-luc (B) of 5, 10 and 20 μM and (C) dose response IC_{50} evaluated on B16F10 G5-Luc cells of EPMC was calculated using a calculator tool(103).

4.4 Discussion

Continuing to evaluate the application of *Kaempferia galanga*, which has shown promising candidates for targeting NF-κB in cancer cells, these extracts may be useful candidates for advancing proinflammatory research regarding inflammatory agents that can be applied in skin cancer-related inflammation. Evaluating the efficacy of extracts 29 and 35 in the HaCaT cell line as protector agent was further conducted, HaCaT cell has been widely used in the studies of skin biology and differentiation. At concentrations of 4, 8, 16 and 32 μg mL^{-1} extract 29 and 35 did not affect the viability of HaCaT cells, whereas, at concentrations of 16 and 32 μg mL^{-1}, the extracts showed protective effects in cells pretreated with TRAIL (Fig 14A,B).

Resistance of TRAIL can occur at different points in the TRAIL-induced apoptosis signalling pathway(104). Thus, these findings provide further evidence that the identified

Kaempferia species extracts may be beneficial for the treatment of inflammatory disease. Although their exact mechanism of action remains to be determined, the extracts may act as novel anti-inflammatory or cytoprotective agents through the suppression of the NF-κB pathway.

Futher research was conducted, the main compounds of *Kaempferia galanga* were analyzed in this study. The major compound was crystallized from the ethanolic extract at room temperature. Pure crystals were obtained after further purification via chromatography and recrystallization. The extract was further isolated, finding Six other compounds, identified in the results. In this research, activity of six compounds from K. galanga, of NF-κB inhibition and the viability of B16F10 NF-κB luc cells were identified, as shown in Figure 16. BAY-11-7082 was used as a positive control for NFκB inhibition, even though it continued to be cytotoxic to the cancer cell line that was used(105). The results of this study indicated that compound 2 (ethyl-p-methoxycinnamate, EPMC) inhibited cells that were transfected with the NF-κB luc gene in B16F10 cells (B16F10-NF-κB-Luc2). However, the other compounds demonstrated relatively similar results, with weak inhibition activity. Cytotoxicity testing on these cells revealed that none of the obtained compounds were toxic. This demonstrates that EPMC inhibits the activity of the NF-κB gene and is not toxic (Figure 16A, B).

To ensure the anti-inflammatory effect of the selected compound (EPMC), the ability to inhibit NF-κB and the cytotoxic effect on macrophages transfected with NF-κB (RAW246.7 NF-κB -Luc cells) were evaluated. This data indicated that EPMC could be a promising candidate for the treatment of inflammation-related skin diseases, specifically melanoma. Similarly, the objective of this research was to identify

compounds that, when used clinically would not harm normal human cells but would kill cancer cells that rely on the NF-κB gene for survival. As many reports has revealed that this gene is typically manipulated in abnormal cells, such as cancer cells, to allow cells to continue activating this primary regulatory gene, which in turn allows cells to survive, multiply, and stop producing the pro-inflammatory proteins that are used to inhibit cancer cell growth(106).

An additional investigation of the EPMC at concentrations of 5, 10, and 20 μM revealed a concentration-dependent activity and established that the compound is not cytotoxic at these concentrations (Figure 22A,B). These data indicate that EPMC is highly effective at inhibiting the NF-κB gene's activity, but it does not cause cell death at the tested concentrations (5, 10 and 20 μM). Additionally, the ensuing tests the were conducted to determine the compound's safety level using a parental cell transfected with luciferin (B16F10G5Luc cells). As can be seen, EPMC is cytotoxic at concentrations greater than 100 μM, with an IC50 of 88.7 μM (Figure 22C). As a result, further investigation with a nontoxic dose and a high level of NF-κB inhibition is necessary. This research was able to inhibit the NF-κB regulatory gene's activity in these melanoma cancer cells.

NF-κB has been shown to evoke invasion and metastasis and pro-inflammatory genes in cancerous cells, making them more resistant to therapy. Hence, inhibiting NF-κB has long been considered an effective strategy for delaying the onset of cancer(107). To assess NF-κB's role as a transcription factor, we examined the mechanism of action of this targeted therapy. Additionally, we discovered that the downregulation of NF-κB is

regulated by their upstream proteins p38 and AKT. The changes in NF-κB play a critical role on phosphorylation of serine536 and NF-κB's transcriptional activity.

EPMC treatment could dephosphorylate the p65 NF-κB sub-unit and inhibit transcriptional activity. The research showed that EPMC could interacts on phosphorylation Akt serine473 by dephosphorylation this protein. It also hypothesized that Akt interaction is required for the Akt-mediated regulation of NF-κB activity, it could depend on the level of inhibition of p38/MAPK. This is intimately connected to the signaling pathways p38MAPK/Akt/NFκB (Figure 18). However, further investigation of EPMC regulation in membrane-bound receptors is required.

Furthermore, while gaining a b

etter understanding of the possible effect of EPMC on cancer cells' ability to migrate or invade in relation to their ability to regulate the P38/AKT/ NF-κB pathway, it was found that EPMC administration also had an effect on B16F10G5-Luc cells' ability to migrate or invade (Figure 19A,B). The Boyden Chamber system was utilized in the invasion assay. The movement of a cell was tested in response to a chemical stimulus. Simple staining was used to investigate cell movement, with the morphology of the cells increasing as they move. Cells in melanoma usually acquire invasive abilities through the process known as epithelial-to-mesenchymal transition (EMT)(108). A similar migratory pattern was observed in B16F10G5-Luc cells in both wound healing and Boyden chamber migratory assays, suggesting that they may be related.

A new way to beat cancer chemoresistance with natural compounds could be by using the B16F10-NF-κB-Luc cell line(109). EPMC was identified to be an effective inhibitor of NF-κB activation in B16F10-NF-κB -Luc cells, while paclitaxel (PTX)

reportedly induced NF-κB activation, which affected resistance to cancer cell treatment(110).

To determine EPMC's ability to sensitize PTX, both compounds were co-treated in a non-toxic dose. The result indicated that PTX maintains resistance with IC50 23.81 μM on B16F10 G5-Luc melanoma cancer cells (Figure 20). Interestingly, the addition of EPMC (50 μM) to PTX (20 μM) activates the PLX's sensitivity to kill B16F10 cancer cells (Figure 21A). Thus, it will be necessary to determine whether the sensitivity of the two compounds is due to the reduction in these cells' ability to survive, because of the NF-κB gene being inhibited by the NF-κB inhibitor compound that was discovered in EPMC. The activity was continued for paclitaxel in combination with EPMC 50 μM using human malignant melanoma cancer cells, SK-Mel 28 a V600E type BRAF Mutation (Figure 21B). The molecular mechanism, as determined by protein expression, indicates that this is due to EPMC's ability to activate the phosphorylation of γ-H2AX, which means that the co-culture of EPMC and PTX resulted in a double-stranded DNA break in the cell, resulting in cell viability reduction (Figure 21C). EPMC significantly enhances the toxicity of PLX against the SK-Mel 28 cell line. Interestingly, our findings might show the phosphorylation of Akt residues, Ser473 in response to DNA damage. As this has been reported to be necessary for the full activation of Akt(110), we believe that Akt signaling may play an important role in the regulation of γH2A.X phosphorylation as well(111, 112).

As it was identified that the two compounds' sensitivity is due to an increase in phosporilation-γH2A.X expression, a molecular marker for DNA damage might be caused by NF-κB regulation. This demonstrates EPMC's potential for clinical trials with

human melanoma cancer cells. Some natural products have been reported for the inhibitory activity on melanoma cancer cell line: resveratrol, with an IC50 value range of 120–257 μM, and saikosaponin B, from *Bupleurum falcatnum*, which showed inhibitory activity with an IC50 value of 200 μM(109, 113). However, when comparing the inhibitory activity, EPMC performed more strongly than the previous reported natural products. EPMC was reported as the major compound from *K. galanga*. Other studies have reported the methanolic extract of this plant was 78% EPMC(114). High concentrations of EPMC from this plant provide natural resources for developing a new drug and its potential use.

Chapter 5

Anti-inflammatory activities of isopimara-8(9),15-diene diterpenoids and mode of action of kaempulchraols compounds from *Kaempferia pulchra* rhizome

5.1 Background

NF-κB stimulates the expression of enzymes, including the inducible cyclo-oxygenase (COX-2) that produces prostanoids (115-118). These metabolites contribute to the pathogenesis of the inflammatory process, which is activated by pro-inflammatory stimuli such as tumor necrosis factor (TNF-α) and lipopolysaccharide (LPS)(119).

Since NF-κB represents an important and very attractive therapeutic target for drugs to treat many inflammatory disorders, extensive attention has been paid to the identification of compounds that selectively interfere with this pathway. Natural products from herbal plants have contributed significantly to drug discovery in the past, and still provide effective leads for structure identification(120).

Numerous plant-derived substances have been evaluated as possible inhibitors of the NF-κB pathway. These plant-derived substances include several distinct classes of compounds, such as phenols and polyphenols (curcumin, resveratrol, caffeic acid phenethyl ester (CAPE), quercetin, apigenin, epigallocatechin-3-gallate), lignans (manassantins, (+)-saucernetin, (–)-saucerneol methyl ether), sesquiterpenes (costunolide, parthenolide), diterpenes (helenalin, excisanin, kamebakaurin), triterpenes (avicin, oleandrin), etc. [9]. The majority of these compounds act by blocking the protein kinase-mediated IκB degradation, thereby preventing NF-κB activation. The diterpenoids isolated from medicinal plants exert potent anti-NF-κB activities by acting on a different level of the NF-κB pathway(121-123). Among the different types of diterpenoids, isopimarane diterpenoids are structurally diverse and functionally noteworthy natural products. They are abundantly found in plants(124, 125) and fungi(126) and possess various biological activities, such as cytotoxicity(127), anti-

microbial (128), anti-malarial(129), anti-inflammatory(130), and anti-viral(131) properties, as well as inhibitory activities against acetylcholinesterase(132), α-glucosidase(126), and nitric oxide production(133). However, the NF-κB inhibition activities of the isopimarane diterpenoids have not been investigated widely. In our previous study, we isolated two types of isopimarane diterpenoids, isopimara-8(14),15-dienes and isopimara-8(9),15-dienes from *Kaempferia pulchra* rhizomes (Zingiberaceae). Some of the isopimara-8(14),15-dienes were potent NF-κB inhibitors as well as NO inhibitors(134). Herein, we report the anti-inflammatory effects of the second type of isopimara-8(9),15-dienes, including kaempulchraols A–D (**1**–**4**), G (103), N (**6**), O (**7**), and S (**8**) (Fig. 1), which were previously isolated from *Kaempferia pulchra* rhizomes as new compounds(124, 125, 135, 136).

5.2 Materials and methods

5.2.1 Chemicals and reagents

Lipopolysaccharide (LPS) from *E. coli*, sulfanilamide, *N*-1-naphthylethylene diamine dihydrochloride, α-MEM with L-glutamine and phenol red, the WST-8 cell counting kit, and the penicillin-streptomycin solution (×100) were purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich. Antibodies against COX-2 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The GmbH (Jena, pGL4.32 [luc2P/NF-κB-RE/Hygro] vector and D-luciferin were obtained from Promega (Sunnyvale, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Hygromycin B and phosphoric acid were obtained from Nacalai Tesque (Kyoto, Japan).

5.2.2 Cell culture

Mouse RAW264.7-NF-κB-luc cells were cultured in DMEM, containing 10% FBS, 100 unit/L penicillin G, and 100 mg/L streptomycin (DMEM culture medium), in a 5% CO_2 atmosphere at 37 °C. For experiments, the cells were seeded in 35 mm dishes (Corning Inc., Corning, NY, U.S.A.), and confluent cells were incubated in DMEM for 24 h. To establish RAW264.7 cells expressing the NF-κB-mediated luciferase gene (RAW264.7-NF-κB-luc), RAW 264.7 cells (RCB0535) (5×10^5/well) were seeded in 6-well plates and transfected with the pGL4.32 vector, using Lipofectamine 2000. The cells were selected with hygromycin B (100 μg/mL) and cloned by limiting dilution. To evaluate the cellular response to NF-κB *in vitro*, RAW264.7-NF-κB transfectants or RAW CMV control cells (1×10^5/well) were cultured in 96-well plates and treated with LPS (100 ng/mL). After 6 h incubation, the luciferase activity was measured with a microplate reader (2030 ARVO X, Perkin Elmer Life Science, Boston, MA).

3.2.3 Cell viability

RAW264.7-NF-κB-luc cells were plated at a final concentration of 2×10^4 cells/well in a 96-well plate. After 3 h incubation, the cells were pretreated with different doses of compound, followed by induction with or without 100 ng/mL of LPS for 1 h. After 24 h treatment, 10 μL of WST-8 solution was added. The cells were incubated for another 1 h in a humidified atmosphere (37 °C, 5% CO_2) to allow the formation of the formazan dye and to obtain higher sensitivity. The absorbance was measured with a microplate reader (Sunrise™; Tecan Group Ltd., Männedorf, Switzerland) at wavelengths of

450/620 nm. Cell viability was determined from the absorbance of the soluble formazan dye generated by the living cells.

3.2.4 NF-κB reporter gene assay

RAW264.7-NF-κB-luc cells (stable RAW cell line with NF-κB-driven luciferase reporter) were maintained in DMEM, supplemented with 10% FBS, 1 mM L-glutamine, and antibiotics (100 units/L penicillin and 100 mg/L streptomycin), in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were seeded in black 96-well plates at a density of 2×10^4 cells per well, and incubated for 3 h. The cells were then treated with the compounds, and an equal concentration of the solvent vehicle was always included as a control after 24 h. The luminescence of the firefly luciferase and the fluorescence of EGFP were quantified using the Living Image 4.2 software (Caliper Life Science) to determine the light emitted from the cells. All values were determined as photons per second (photon/sec).

5.2.5 ELISA

The supernatants from RAW264.7 cells (4×10^4/well) seeded in 96 well plates were collected to measure the IL-6 content, using a commercially available enzyme-linked immunosorbent assay (121) kit (mouse IL-6 ELISA MAX™ standard set, Biolegend), according to the manufacturer's instructions. The absorbance was measured with a microplate reader (Sunrise™; Tecan Group Ltd., Männedorf, Switzerland) at wavelengths of 450/570 nm.

5.2.6 Western blotting

RAW264.7 cells (5×10^5 cells/well) were seeded in 6-well plates for 24 h. The cells were treated with compounds for 1 h, and then induced with or without 100 ng/mL of LPS. After incubations for 12 h and 24 h, the treated cells were collected, washed with phosphate-buffered saline (PBS), and lysed in lysis buffer [25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 mg/mL aprotinin, and 10 mg/mL leupeptin].

The cell lysates were fractionated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes, using glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), and 20% (v/v) methanol]. After blocking with Block Ace for 4 h at room temperature, the membrane was incubated overnight with the primary antibodies (1:1,000), and then for 60 min with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, 1:2,000). Immunoreactive proteins of interest were visualized with an enhanced chemiluminescence system (Amersham Biosciences), and emitted signals were captured on Fuji X-ray film (Wako, Osaka, Japan). Equal protein loading was verified by β–actin.

5.2.7 Statistical analysis

Each experiment was performed three times, and all data are presented as mean ± standard deviation (38). All statistical analyses were done with JMP11.0 software. Comparisons between positive control and tested compounds were made by the student's *t*-test. *p* values less than 0.05 were assumed to indicate significance.

5.3 Result

5.3.1 Identification 21 isopimara-8,14(15)-diene diterpenoids

In a previous study, we identified 21 isopimara-8,14(15)-diene diterpenoids, including kaempulchraols E (1), F (2), H-M (3-8), P-R (9-11), T (12), V (13), W (14), 9α-hydroxyisopimara-8(14),15-dien-7-one (15), 7β,9α-dihydroxypimara-8(14),15-diene (16),(1S,5S,9S,10S,11R,13R)-1,11-dihydroxypimara-8(14),15-diene(17), sandaracopimaradien-1α,2α-diol (18), (2R)-ent-2-hydroxyisopimara-8(14),15-diene(19), (1R,2S,5S,9S,10S,11R,13R)-1,2,11-trihydroxypimara-8(14),15-diene (20), and 7α-hydroxyisopimara-8(14),15-diene (21), from a CHCl3-soluble extract of Kaempferia pulchra rhizomes (Fig. 23) and also Figure 24 illustrated Effects of 9 and 10 on NF-κB activation by a reporter gene assay (A), and on cell viability (B) in RAW 264.7- NF-κB -luc cells.

5.3.2 Identification isopimara-8(9),15-dienes 1–8

Structures of isopimara-8(9),15-dienes 1–8 isolated from K. pulchra rhizomes shown in the (Figure 25). Structures of isopimara-8(9),15-dienes 1–8 isolated from *K. pulchra* rhizomes and further NFκB activation as well as cytotoxicity of isopimara-8(9),15-dienes 1–8 isolated from K. pulchra rhizomes. Effects of compounds 1–8 on NF-κB activation by the reporter gene assay (25B) and on the viability (25C) of RAW264.7-NF-κB-luc cells.

5.3.3 IL-6 and COX-2 effect of compound 9 and 10

The effect of compounds 9 and 10 on IL-6 production indicated very significantly different from the LPS-treated control (Figure 26A) Effects of 9 and 10 on COX-2 expression in LPS-induced RAW 264.7 cells, analyzed by western blotting(Figure 26B). The data points and bars represent mean ± SD (n= 3). ***, $P < 0.0001$, Very significantly different from the LPS-treated control(26B)

5.3.4 IL-6 and COX-2 effect of compound 2,3 and 4

the isopimara-8(14),15-diene type kaempulchraols P and Q inhibited IL-6 production and COX-2 expression, with an effective dose of 25 μM (Figure 27 A, B) (134). As in the cases of kaempulchraols P and Q, the effects of 2–4 on the levels of IL-6 production and COX-2 expression in the LPS-induced macrophage RAW264.7 cells were analyzed by using enzyme-linked immunosorbent assay(ELISA) and western blot,respectively.

5-4 Discussion

Natural products reportedly play an important role in controlling the inflammatory response pathways. However, the anti-inflammatory activities of isopimara-8,14(15)-diene diterpenoids and isopimara-8(9),15-dienes have not yet been fully elucidated. Thus, some of the isolated 21 isopimara-8,14(15)-diene diterpenoids could be the anti-inflammatory phytoconstituents in this plant. To test this hypothesis, we investigated the anti-inflammatory activities of the 21 compounds and the more detailed inhibitory modes of action of two highly potent compounds. In particular, kaempulchraols P (9), Q (10). We selected compounds 9 and 10 and investigated their dose-dependent effects on the

activation of NF-κB, a central mediator of the human immune response in inflammation, by a reporter gene assay. As shown in Fig. 24A and 2B, both compounds inhibited the activation of NF-κB in RAW 264.7-NF-κB-luc cells at doses of 5, 10, and 25 μM. The preliminary NF-κB inhibition activities of **1**–**8** were evaluated against RAW 264.7-NF-κB-luc cells (Fig. 25B). The 50 μM concentrations of **2**–**4** and **5** inhibited the activation of NF-κB. The treatment with 50 μM of **2** and **4** reduced the cell viability, while **3** and **5** did not exhibit any cytotoxicity with RAW264.7-NF-κB-luc cells (Fig. 25A, B).

Altogether study, we reported that the isopimara-8(14),15-diene type kaempulchraols P and Q inhibited IL-6 production and COX-2 expression to prevent the NF-κB-mediated transactivation of a luciferase reporter gene, with an effective dose of 25 μM (Figure 26 A,B)(134). As in the cases of kaempulchraols P and Q, the effects of **2**–**4** on the levels of IL-6 production and COX-2 expression in the LPS-induced macrophage RAW264.7 cells were analyzed by using enzyme-linked immunosorbent assay (121) kits and western blotting, respectively. The immunosorbent assay revealed that 25 μM of **2**–**4** significantly inhibited the production of IL-6 (Fig. 27A). Furthermore, the western blotting analysis suggested that a 25 μM concentration of **2**–**4** inhibited the expression of COX-2 in the LPS-induced RAW264.7 macrophages (Fig. 27B).

In summary, kaempulchraols B (**2**), C (**3**), and D (**4**) were identified as potent anti-inflammatory agents, as in the case of the previously reported isopimara-8(14),15-diene type kaempulchraols P and Q isolated from *K. pulchra* rhizomes. These compounds functioned as potent inhibitors of the pro-inflammatory agent NO and the NF-κB signaling, which in turn reduces COX-2 gene expression and subsequent IL6 production. In addition to kaempulchraols P and Q, compounds **2**–**4** may also be

promising natural products to manage inflammation diseases through the inhibition of NF-κB.

Concluding remark

The topic of inflammation and the role of NFκB related to inflammation and inflammation-associated disease was covered in chapter 1. Furthermore, identification natural products from Morus alba Linn. Bark and Kaempferia galanga is covered in Chapter 2 of this dissertation. When 112 natural products collected in Institute of natural medicine, University of Toyama library were evaluated, the extract of Sohakuhi (Morus alba Linn. bark) was found to have a significant suppressive effect on NF-κB activity without having an adverse effect on cell viability. And also, we investigated the inhibition of NF-κB activation in 35 Indonesian medicinal plants (1–35) traditionally used to treat symptoms of skin condition and selected 12 active plants. especially from extracts of Kaempferia galanga, Two different *K. galanga* rhizome extracts strongly suppressed NF-κB activity without affecting cell viability.

In order to gain a deeper understanding of the anti-inflammatory properties of Sohakuhi and potential application regarding inflammatory-associated skin disease, the cellular damage that is caused by the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in human HaCaT keratinocytes was investigated. Treatment with Sohakuhi extract protected HaCaT cells against the cellular damage that was induced by TRAIL. TRAIL was found to trigger the phosphorylation of the p65 subunit of NF-κB, which led to cellular damage in HaCaT cells. In addition, treatment with Sohakuhi led to an increase in the levels of the anti-apoptotic proteins Bcl-xL and Bcl-2. Importantly, a chemical fractionation of the Sohakuhi extract confirmed that moracin O and P are the anti-inflammatory agents that mediate the effects of the Sohakuhi extract. Based on the

results shown here, Sohakuhi and moracin could be looked at as possible candidates for the development of new anti-inflammatory drugs.

TRAIL damages human HaCaT keratinocytes; however, *K. galanga* extracts 29 and 35 provide protection against TRAIL. On the basis of these results, it appears that *K. galanga* extracts could be used to produce natural inflammatory illustration all this things covered in chapter 3.

Furthermore, we identified the active compound in *Kaempferia galanga* as Ethyl Para Methoxy cinnamate (EPMC), which could be used to treat melanoma as an anti-metastasis and chemosensitizer agent that could be trough NF-κB inflammatory signal. This chapter's objective was to identify the active ingredient in Kaempferia galanga. Using chromatography and spectroscopy techniques, six compounds were assigned to the isolated and identified active component of *K. galanga*.. Among the isolated compounds, ethyl p-methoxycinnamate (EPMC) exhibited potent NF-κB inhibitory activity against melanoma cell B16F10- NF-κB-Luc2 with an IC50 value of 88.7 M. Evaluating the anti-metastasis effect of EPMC in vitro. Through the p38/Akt/NF-B pathway, NF-κB has been associated with tumorigenesis. According to the findings of this study, EPMCs inhibit p38 and Akt phosphorylation at serine 473, thereby inhibiting NF-κB-dependent transcription. Additional analysis with paclitaxel revealed that the combinations could increase sensitivity to apoptosis in response to well-known chemotherapy agents. The human melanoma cancer cell line SK-Mel 28 was utilized in additional research. In addition to inducing apoptosis, treatment with paclitaxel and EPMC led to an increase in p-H2AX expression (a molecular marker for double-strand

breaks in DNA damage). The results demonstrated that EPMC is a viable potential adjuvant for enhancing the clinical efficacy of anti-metastatic and cancer chemotherapy.

Additionally, *Kaempferia pulchra* was collected in Myanmar. More information about its compounds in chapters 5; The anti-inflammatory effects of isopimara-8(14),-15-diene diterpenoids and the mechanism of action of kaempulchraols P and Q, extracted from the rhizomes of Kaempferia pulchra, were investigated. Not yet fully understood are the anti-inflammatory effects of isopimara-8,14(15)-diene diterpenoids. To determine how isopimara-8,14(15)-diene diterpenoids reduce inflammation, we analyzed 21 isopimara-8,14(15)-diene diterpenoids isolated from Kaempferia pulchra rhizomes. At a concentration of 25 μM, the most potent kaempulchraols, P and Q, with respective IC50 values of 39.88 and 36.05 M, inhibited the NF-κB -mediated transactivation of a luciferase reporter gene, IL-6 production, and COX-2 expression.

Futhher research also evaluation the effects of isopimara-8(9),15-diene diterpenoids on inflammation and how kaempulchraols B-D from Kaempferia pulchra rhizomes were conducted. In this chapter, we found that Kaempulchraols B-D (2-4), which are isopimara-8(9), 15-diene diterpenoids, are strong NF-κB inhibitors. Inhibit the production of IL-6 and the expression of COX-2. So, it seems likely that isopimarane diterpenoids are strong NF-κB pathway inhibitors and could be looked into further as possible anti-inflammatory lead compounds.

Acknowledgments

I would like to express my appreciation to everyone who assisted me in writing this dissertation.

First, I'd like to express my sincere appreciation to my supervisor, Professor Dr. Yoshihiro Hayakawa, for his insightful advice, kind encouragement, and unwavering support throughout this study.

Second, I would like to express my deep appreciation to Dr. Satoru Yokoyama, associate professor, for his encouragement and suggestions throughout my studies. I would also like to thank professor emeritus Dr. Ikuo Saiki, for his encouragement and assistance. Assistant Professor Dr. Susukida Takeshi and Assistant Professor Sasaki So-ichiro, for the assistance and excellent guidance as well.

This study would not have been possible without the assistance of Professor Dr. Subehan, Professor Dr. Hirouki Morita, Dr. Nwe Nwe Win, Dr. Kodama, Sisca Ucchee, and drg. Muhammad Irfan. who helped me with important technical skill during this study.

I am extremely grateful to the ROMPAKU program for providing financial support for my visiting research.

Lastly, I am gratefully acknowledged to the staff and students of the Section Host defense Division of Bioscience at the University of Toyama, who have always been helpful and patient during my visiting research.

Reference

1. Hurley J. Acute inflammation: the effect of concurrent leucocytic emigration and increased permeability on particle retention by the vascular wall. British journal of experimental pathology. 1964;45(6):627.
2. Punchard NA, Whelan CJ, Adcock I. The Journal of Inflammation. Journal of Inflammation. 2004;1(1):1.
3. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454(7203):428-35.
4. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nature immunology. 2005;6(12):1191-7.
5. Germolec DR, Shipkowski KA, Frawley RP, Evans E. Markers of Inflammation. Methods Mol Biol. 2018;1803:57-79.
6. Sen R, Baltimore D. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell. 1986;47(6):921-8.
7. Baeuerle PA, Baltimore D. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell. 1988;53(2):211-7.
8. Gerondakis S, Grumont R, Gugasyan R, Wong L, Isomura I, Ho W, et al. Unravelling the complexities of the NF-κB signalling pathway using mouse knockout and transgenic models. Oncogene. 2006;25(51):6781-99.
9. Vallabhapurapu S, Karin M. Regulation and function of NF-κB transcription factors in the immune system. Annual review of immunology. 2009;27:693-733.

10. Hayden MS, Ghosh S. NF-κB, the first quarter-century: remarkable progress and outstanding questions. Genes & development. 2012;26(3):203-34.
11. Hoffmann A, Baltimore D. Circuitry of nuclear factor κB signaling. Immunological reviews. 2006;210(1):171-86.
12. Pasparakis M, Luedde T, Schmidt-Supprian M. Dissection of the NF-κB signalling cascade in transgenic and knockout mice. Cell Death & Differentiation. 2006;13(5):861-72.
13. Hoffmann A, Leung TH, Baltimore D. Genetic analysis of NF-κB/Rel transcription factors defines functional specificities. The EMBO journal. 2003;22(20):5530-9.
14. Leung TH, Hoffmann A, Baltimore D. One nucleotide in a κB site can determine cofactor specificity for NF-κB dimers. Cell. 2004;118(4):453-64.
15. Alkalay I, Yaron A, Hatzubai A, Orian A, Ciechanover A, Ben-Neriah Y. Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. Proceedings of the National Academy of Sciences. 1995;92(23):10599-603.
16. Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-κB signaling module. Oncogene. 2006;25(51):6706-16.
17. Shih VF-S, Tsui R, Caldwell A, Hoffmann A. A single NFκB system for both canonical and non-canonical signaling. Cell research. 2011;21(1):86-102.
18. Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev. 2004;18(18):2195-224.
19. Janssens S, Tschopp J. Signals from within: the DNA-damage-induced NF-kappaB response. Cell Death Differ. 2006;13(5):773-84.

20. Sun SC. The non-canonical NF-κB pathway in immunity and inflammation. Nat Rev Immunol. 2017;17(9):545-58.
21. Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat Cell Biol. 2006;8(4):398-406.
22. Chen F, Bhatia D, Chang Q, Castranova V. Finding NEMO by K63-linked polyubiquitin chain. Cell Death Differ. 2006;13(11):1835-8.
23. Xiao G, Harhaj EW, Sun SC. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. Mol Cell. 2001;7(2):401-9.
24. Moran AE, Kovacsovics-Bankowski M, Weinberg AD. The TNFRs OX40, 4-1BB, and CD40 as targets for cancer immunotherapy. Curr Opin Immunol. 2013;25(2):230-7.
25. Williams RA, Timmis J, Qwarnstrom EE. Computational Models of the NF-KB Signalling Pathway. Computation. 2014;2(4):131-58.
26. Seitz CS, Freiberg RA, Hinata K, Khavari PA. NF-κB determines localization and features of cell death in epidermis. The Journal of clinical investigation. 2000;105(3):253-60.
27. Lin B, Williams-Skipp C, Tao Y, Schleicher MS, Cano LL, Duke RC, et al. NF-κB functions as both a proapoptotic and antiapoptotic regulatory factor within a single cell type. Cell Death & Differentiation. 1999;6(6):570-82.
28. Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. Oncogene. 1999;18(49):6853-66.

29. Silverman N, Maniatis T. NF-κB signaling pathways in mammalian and insect innate immunity. Genes & development. 2001;15(18):2321-42.
30. Byrd-Leifer CA, Block EF, Takeda K, Akira S, Ding A. The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol. European journal of immunology. 2001;31(8):2448-57.
31. Norbury CJ, Zhivotovsky B. DNA damage-induced apoptosis. Oncogene. 2004;23(16):2797-808.
32. Shiloh Y. ATM and ATR: networking cellular responses to DNA damage. Current opinion in genetics & development. 2001;11(1):71-7.
33. Fernandez-Capetillo O, Chen H-T, Celeste A, Ward I, Romanienko PJ, Morales JC, et al. DNA damage-induced G2–M checkpoint activation by histone H2AX and 53BP1. Nature cell biology. 2002;4(12):993-7.
34. Wang H, Cho C. Effect of NF-κB signaling on apoptosis in chronic inflammation-associated carcinogenesis. Current cancer drug targets. 2010;10(6):593-9.
35. Xia Y, Shen S, Verma IM. NF-κB, an active player in human cancers. Cancer immunology research. 2014;2(9):823-30.
36. Gilmore S. Melanoma screening: informing public health policy with quantitative modelling. PloS one. 2017;12(9):e0182349.
37. Maio M. Melanoma as a model tumour for immuno-oncology. Annals of Oncology. 2012;23:viii10-viii4.
38. Gonzalez D, Fearfield L, Nathan P, Tanière P, Wallace A, Brown E, et al. BRAF mutation testing algorithm for vemurafenib treatment in melanoma:

recommendations from an expert panel. British Journal of Dermatology. 2013;168(4):700-7.

39. Tudor DV, Bâldea I, Lupu M, Kacso T, Kutasi E, Hopârtean A, et al. COX-2 as a potential biomarker and therapeutic target in melanoma. Cancer biology & medicine. 2020;17(1):20.
40. Bacher S, Schmitz ML. The NF-kB pathway as a potential target for autoimmune disease therapy. Current pharmaceutical design. 2004;10(23):2827-37.
41. Khuda-Bukhsh AR, Das S, Saha SK. Molecular approaches toward targeted cancer prevention with some food plants and their products: inflammatory and other signal pathways. Nutrition and cancer. 2014;66(2):194-205.
42. Che C-T, George V, Ijinu T, Pushpangadan P, Andrae-Marobela K. Traditional medicine. Pharmacognosy: Elsevier; 2017. p. 15-30.
43. Yuan H, Ma Q, Ye L, Piao G. The Traditional Medicine and Modern Medicine from Natural Products. Molecules. 2016;21(5):559.
44. Motoo Y, Seki T, Tsutani K. Traditional Japanese medicine, Kampo: Its history and current status. Chinese Journal of Integrative Medicine. 2011;17(2):85-7.
45. Elfahmi, Woerdenbag HJ, Kayser O. Jamu: Indonesian traditional herbal medicine towards rational phytopharmacological use. Journal of Herbal Medicine. 2014;4(2):51-73.
46. Chin Y-W, Balunas MJ, Chai HB, Kinghorn AD. Drug discovery from natural sources. The AAPS journal. 2006;8(2):E239-E53.

47. Wang M-W, Hao X, Chen K. Biological screening of natural products and drug innovation in China. Philosophical Transactions of the Royal Society B: Biological Sciences. 2007;362(1482):1093-105.
48. Sholikhah EN. Indonesian medicinal plants as sources of secondary metabolites for pharmaceutical industry. J Med Sci. 2016;48(4):226-39.
49. Dias DA, Urban S, Roessner U. A historical overview of natural products in drug discovery. Metabolites. 2012;2(2):303-36.
50. Flaczyk E, Kobus-Cisowska J, Przeor M, Korczak J, Remiszewski M, Korbas E, et al. Chemical characterization and antioxidative properties of Polish variety of Morus alba L. leaf aqueous extracts from the laboratory and pilot-scale processes. Agricultural Sciences. 2013;4(05):141.
51. Amarowicz R. Antibacterial activity of green tea polyphenols against. Escherichia coli K12 Nahrung. 2000;44:60-2.
52. Butt MS, Nazir A, Sultan MT, Schroën K. Morus alba L. nature's functional tonic. Trends in food science & technology. 2008;19(10):505-12.
53. Doi K, Kojima T, Makino M, Kimura Y, Fujimoto Y. Studies on the Constituents of the Leaves of Morus alba L. Chemical and Pharmaceutical Bulletin. 2001;49(2):151-3.
54. Pel P, Chae H-S, Nhoek P, Kim Y-M, Chin Y-W. Chemical Constituents with Proprotein Convertase Subtilisin/Kexin Type 9 mRNA Expression Inhibitory Activity from Dried Immature Morus alba Fruits. Journal of Agricultural and Food Chemistry. 2017;65(26):5316-21.

55. Suh HJ, Noh DO, Kang CS, Kim JM, Lee SW. Thermal kinetics of color degradation of mulberry fruit extract. Food/Nahrung. 2003;47(2):132-5.
56. Liu X, Xiao G, Chen W, Xu Y, Wu J. Quantification and purification of mulberry anthocyanins with macroporous resins. Journal of Biomedicine and Biotechnology. 2004;2004(5):326.
57. Ercisli S, Orhan E. Chemical composition of white (Morus alba), red (Morus rubra) and black (Morus nigra) mulberry fruits. Food Chemistry. 2007;103(4):1380-4.
58. Lale H, Ozcagiran R. A study on pomological, phenologic and fruit quality characteristics of Mulberry (Morus sp.) species. Derim. 1996;13(4):177-82.
59. Jiang Y, Nie WJ. Chemical properties in fruits of mulberry species from the Xinjiang province of China. Food Chem. 2015;174:460-6.
60. Paudel P, Seong SH, Wagle A, Min BS, Jung HA, Choi JS. Antioxidant and anti-browning property of 2-arylbenzofuran derivatives from Morus alba Linn root bark. Food Chemistry. 2020;309:125739.
61. Fukai T, Hano Y, HIRAKURA K, NOMURA T, Uzawa J, FUKUSHIMA K. Structures of two natural hypotensive diels-alder type adducts, mulberrofurans f and g, from the cultivated mulberry tree (morus lhou koidz.). Chemical and Pharmaceutical Bulletin. 1985;33(8):3195-204.
62. Ueda S, Matsumoto J, Nomura T. Four new natural Diels-Alder type adducts, mulberrofuran E, kuwanon Q, R, and V from callus culture of Morus alba L. Chemical and pharmaceutical bulletin. 1984;32(1):350-3.

63. Fukai T, Hano Y, Hirakura K, Nomura T, Uzawa J. Structure of mulberrofuran H, a novel 2-arylbenzofuran derivative from the cultivated mulberry tree (Morus lhou (ser.) Koidz.). Chemical and pharmaceutical bulletin. 1984;32(2):808-11.
64. Yang Z-G, Matsuzaki K, Takamatsu S, Kitanaka S. Inhibitory effects of constituents from Morus alba var. multicaulis on differentiation of 3T3-L1 cells and nitric oxide production in RAW264. 7 cells. Molecules. 2011;16(7):6010-22.
65. Kaur N, Xia Y, Jin Y, Dat NT, Gajulapati K, Choi Y, et al. The first total synthesis of moracin O and moracin P, and establishment of the absolute configuration of moracin O. Chemical communications. 2009(14):1879-81.
66. Dat NT, Jin X, Lee K, Hong Y-S, Kim YH, Lee JJ. Hypoxia-inducible factor-1 inhibitory benzofurans and chalcone-derived diels− alder adducts from Morus species. Journal of natural products. 2009;72(1):39-43.
67. Xia Y, Jin Y, Kaur N, Choi Y, Lee K. HIF-1α inhibitors: Synthesis and biological evaluation of novel moracin O and P analogues. European journal of medicinal chemistry. 2011;46(6):2386-96.
68. Khyade VB, Khyade VV, Khyade SV, Moser M-B. Influence of Moracin on DMBA-TPA induced skin tumerigenesis in the mouse. International Journal of Bioassays. 2014;3(11):3510-6.
69. Jirovetz L, Buchbauer G, Shafi PM, ABRAHAM GT. Analysis of the essential oil of the roots of the medicinal plant Kaempferia galanga L.(Zingiberaceae) from South-India. ACTA Pharmaceutica Sciencia. 2001;43(2).
70. Lallo S, Hasmiranti A, Hardianti B. Effect of the growth environment on Patchouli (Pogostemon cablin Benth.) oil character at Southeast Sulawesi, Indonesia.

Medicinal Plants-International Journal of Phytomedicines and Related Industries. 2020;12(1):77-81.

71. Khairullah AR, Solikhah TI, Ansori ANM, Hanisia RH, Puspitarani GA, Fadholly A, et al. Medicinal importance of Kaempferia galanga L.(Zingiberaceae): A comprehensive review. Journal of Herbmed Pharmacology. 2021;10(3):281-8.
72. Elshamy AI, Mohamed TA, Essa AF, Gawad A-E, Ahmed M, Alqahtani AS, et al. Recent advances in Kaempferia phytochemistry and biological activity: a comprehensive review. Nutrients. 2019;11(10):2396.
73. Srivastava N, Singh S, Gupta AC, Shanker K, Bawankule DU, Luqman S. Aromatic ginger (Kaempferia galanga L.) extracts with ameliorative and protective potential as a functional food, beyond its flavor and nutritional benefits. Toxicology reports. 2019;6:521-8.
74. Jagadish PC, Latha KP, Mudgal J, Nampurath GK. Extraction, characterization and evaluation of Kaempferia galanga L.(Zingiberaceae) rhizome extracts against acute and chronic inflammation in rats. Journal of Ethnopharmacology. 2016;194:434-9.
75. He Z-H, Yue GG-L, Lau CB-S, Ge W, But PP-H. Antiangiogenic Effects and Mechanisms of trans-Ethyl p-Methoxycinnamate from Kaempferia galanga L. Journal of agricultural and food chemistry. 2012;60(45):11309-17.
76. Amuamuta A, Plengsuriyakarn T, Na-Bangchang K. Anticholangiocarcinoma activity and toxicity of the Kaempferia galanga Linn. Rhizome ethanolic extract. BMC complementary and alternative medicine. 2017;17(1):1-11.

77. Vijayan K, Tikader A, Weiguo Z, Nair CV, Ercisli S, Tsou C-H. Morus. Wild crop relatives: genomic and breeding resources: Springer; 2011. p. 75-95.
78. Yamatake Y, Shibata M, Nagai M. Pharmacological studies on root bark of mulberry tree (Morus alba L.). The Japanese Journal of Pharmacology. 1976;26(4):461-9.
79. Baldwin Jr AS. The NF-κB and IκB proteins: new discoveries and insights. Annual review of immunology. 1996;14(1):649-81.
80. Harper N, Farrow SN, Kaptein A, Cohen GM, MacFarlane M. Modulation of tumor necrosis factor apoptosis-inducing ligand-induced NF-κB activation by inhibition of apical caspases. Journal of Biological Chemistry. 2001;276(37):34743-52.
81. Jeremias I, Debatin K-M. TRAIL induces apoptosis and activation of NFkB. European cytokine network. 1999;9(4):687-8.
82. Keane MM, Rubinstein Y, Cuello M, Ettenberg SA, Banerjee P, Nau MM, et al. Inhibition of NF-κB activity enhances TRAIL mediated apoptosis in breast cancer cell lines. Breast cancer research and treatment. 2000;64(2):211-9.
83. Kim YS, Schwabe RF, Qian T, Lemasters JJ, Brenner DA. TRAIL-mediated apoptosis requires NF-kB inhibition and the mitochondrial permeability transition in human hepatoma cells. Hepatology. 2002;36(6):1498-508.
84. Oya M, Ohtsubo M, Takayanagi A, Tachibana M, Shimizu N, Murai M. Constitutive activation of nuclear factor-κB prevents TRAIL-induced apoptosis in renal cancer cells. Oncogene. 2001;20(29):3888-96.
85. Southall MD, Isenberg JS, Nakshatri H, Yi Q, Pei Y, Spandau DF, et al. The platelet-activating factor receptor protects epidermal cells from tumor necrosis

factor (TNF) α and TNF-related apoptosis-inducing ligand-induced apoptosis through an NF-κB-dependent process. Journal of Biological Chemistry. 2001;276(49):45548-54.

86. Wang P, Du B, Yin W, Wang X, Zhu W. Resveratrol attenuates CoCl2-induced cochlear hair cell damage through upregulation of Sirtuin1 and NF-κB deacetylation. PLoS One. 2013;8(11):e80854.

87. Woerdenbag HJ, Kayser O. Jamu: Indonesian traditional herbal medicine towards rational phytopharmacological use. Journal of herbal medicine. 2014;4(2):51-73.

88. Tunsaringkarn T, Palanuvej C, Rungsiyothin A, Issaravanich S, Vipunngeun N, Chuthaputti A, et al. Pharmacognostic Specification of Kaempferia galanga Rhizome in Thailand= คุณสมบัติ ทาง เภสัชเวท ของ เหง้า เปราะหอม ใน ประเทศไทย. Journal of Health Research. 2007;21(3):207-14.

89. Kanjanapothi D, Panthong A, Lertprasertsuke N, Taesotikul T, Rujjanawate C, Kaewpinit D, et al. Toxicity of crude rhizome extract of Kaempferia galanga L.(Proh Hom). Journal of Ethnopharmacology. 2004;90(2-3):359-65.

90. Rahman M, Amin M, Ahamed T, Ali M, Habib A. Efficient plant regeneration through somatic embryogenesis from leaf base derived callus of Kaempferia galanga L. Asian journal of plant sciences. 2004;3(6):675-8.

91. Shetu HJ, Trisha KT, Sikta SA, Anwar R, Rashed SSB, Dash PR. Pharmacological importance of Kaempferia galanga (Zingiberaceae): A mini review. International Journal of Research in Pharmacy and Pharmaceutical Sciences. 2018;3(3):32-9.

92. Kumar A. Phytochemistry, pharmacological activities and uses of traditional medicinal plant Kaempferia galanga L.–An overview. Journal of ethnopharmacology. 2020;253:112667.
93. Sae-wong C, Tansakul P, Tewtrakul S. Anti-inflammatory mechanism of Kaempferia parviflora in murine macrophage cells (RAW 264.7) and in experimental animals. Journal of ethnopharmacology. 2009;124(3):576-80.
94. Takahashi K, Takeda K, Saiki I, Irimura T, Hayakawa Y. Functional roles of tumor necrosis factor-related apoptosis-inducing ligand-DR5 interaction in B16F10 cells by activating the nuclear factor-κB pathway to induce metastatic potential. Cancer Sci. 2013;104(5):558-62.
95. Lallo S, Hardianti B, Sartini S, Ismail I, Hayakawa Y. Anti-inflamatory and cytoprotective effect of Kaempferia galanga extracts by targeting NFκB activity. Asian J Plant Sci. 2022;21:183-91.
96. Yarrow JC, Perlman ZE, Westwood NJ, Mitchison TJ. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. BMC biotechnology. 2004;4(1):1-9.
97. Jonkman JE, Cathcart JA, Xu F, Bartolini ME, Amon JE, Stevens KM, et al. An introduction to the wound healing assay using live-cell microscopy. Cell adhesion & migration. 2014;8(5):440-51.
98. Shin M-K, Sasaki F, Ki D-W, Win NN, Morita H, Hayakawa Y. Anti-metastatic effects of ergosterol peroxide from the entomopathogenic fungus Ophiocordyceps gracilioides on 4T1 breast cancer cells. Journal of natural medicines. 2021;75(4):824-32.

99. Keire DA, Anton P, Faull KF, Ruth E, Walsh JH, Chew P, et al. Diethyl phthalate, a chemotactic factor secreted by Helicobacter pylori. Journal of Biological Chemistry. 2001;276(52):48847-53.

100. Hasali NHM, Omar MN, Zuberdi AM, Alfarra HY. Biotransformation of ethyl p-methoxycinnamate from Kaempferia galanga L. using Aspergillus niger. International Journal of Biosciences. 2013;3(7):148-55.

101. Świsłocka R, Kowczyk-Sadowy M, Kalinowska M, Lewandowski W. Spectroscopic (FT-IR, FT-Raman, 1H and 13C NMR) and theoretical studies of p-coumaric acid and alkali metal p-coumarates. Spectroscopy. 2012;27(1):35-48.

102. Hasegawa T, Hashimoto M, Fujihara T, Yamada H. Aroma profile of galangal composed of cinnamic acid derivatives and their structure-odor relationships. Natural product communications. 2016;11(10):1934578X1601101012.

103. Quest GraphTM I. Calculator, AAT Bioquest. Inc; 2021.

104. Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer gene therapy. 2005;12(3):228-37.

105. Rauert-Wunderlich H, Siegmund D, Maier E, Giner T, Bargou RC, Wajant H, et al. The IKK inhibitor Bay 11-7082 induces cell death independent from inhibition of activation of NFκB transcription factors. PloS one. 2013;8(3):e59292.

106. Li F, Zhang J, Arfuso F, Chinnathambi A, Zayed M, Alharbi SA, et al. NF-κB in cancer therapy. Archives of toxicology. 2015;89(5):711-31.

107. Dolcet X, Llobet D, Pallares J, Matias-Guiu X. NF-kB in development and progression of human cancer. Virchows archiv. 2005;446(5):475-82.

108. Guy J-B, Espenel S, Vallard A, Battiston-Montagne P, Wozny A-S, Ardail D, et al. Evaluation of the cell invasion and migration process: a comparison of the video microscope-based scratch wound assay and the boyden chamber assay. JoVE (Journal of Visualized Experiments). 2017(129):e56337.
109. Ma H, Yokoyama S, Saiki I, Hayakawa Y. Chemosensitizing effect of saikosaponin B on B16F10 melanoma cells. Nutrition and cancer. 2017;69(3):505-11.
110. An J, Huang Y-C, Xu Q-Z, Zhou L-J, Shang Z-F, Huang B, et al. DNA-PKcs plays a dominant role in the regulation of H2AX phosphorylation in response to DNA damage and cell cycle progression. BMC molecular biology. 2010;11(1):1-13.
111. Guo Z-F, Kong F-L. Akt regulates RSK2 to alter phosphorylation level of H2A. X in breast cancer. Oncology Letters. 2021;21(3):1-.
112. Brown Kristin K, Montaser-Kouhsari L, Beck Andrew H, Toker A. MERIT40 Is an Akt Substrate that Promotes Resolution of DNA Damage Induced by Chemotherapy. Cell Reports. 2015;11(9):1358-66.
113. Habibie H, Yokoyama S, Abdelhamed S, Awale S, Sakurai H, Hayakawa Y, et al. Survivin suppression through STAT3/β-catenin is essential for resveratroI-induced melanoma apoptosis. International journal of oncology. 2014;45(2):895-901.
114. Winingsih W, Husein SG, Ramdhani RPN. Analysis of Ethyl p-Methoxycinnamate from Kaempferia galanga L. Extract by High Performance Liquid Chromatography. Journal of Tropical Pharmacy and Chemistry. 2021;5(4):353-8.
115. Miller SC, Huang R, Sakamuru S, Shukla SJ, Attene-Ramos MS, Shinn P, et al. Identification of known drugs that act as inhibitors of NF-κB signaling and their mechanism of action. Biochemical pharmacology. 2010;79(9):1272-80.

116. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. The Journal of clinical investigation. 2001;107(2):135-42.
117. Brasier AR. The nuclear factor-κB–interleukin-6 signalling pathway mediating vascular inflammation. Cardiovascular research. 2010;86(2):211-8.
118. Zhang G, Ghosh S. Toll-like receptor–mediated NF-κB activation: a phylogenetically conserved paradigm in innate immunity. The Journal of clinical investigation. 2001;107(1):13-9.
119. van der Bruggen T, Nijenhuis S, van Raaij E, Verhoef J, Sweder van Asbeck B. Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the raf-1/MEK1-MEK2/ERK1-ERK2 pathway. Infection and Immunity. 1999;67(8):3824-9.
120. Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. Journal of natural products. 2012;75(3):311-35.
121. García-Piñeres AJ, Castro Vc, Mora G, Schmidt TJ, Strunck E, Pahl HL, et al. Cysteine 38 in p65/NF-κB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. Journal of Biological Chemistry. 2001;276(43):39713-20.
122. Hehner SP, Heinrich M, Bork PM, Vogt M, Ratter F, Lehmann V, et al. Sesquiterpene lactones specifically inhibit activation of NF-κB by preventing the degradation of IκB-α and IκB-β. Journal of Biological Chemistry. 1998;273(3):1288-97.

123. Leung C-H, Grill SP, Lam W, Han Q-B, Sun H-D, Cheng Y-C. Novel mechanism of inhibition of nuclear factor-κB DNA-binding activity by diterpenoids isolated from Isodon rubescens. Molecular pharmacology. 2005;68(2):286-97.
124. Win NN, Ito T, Aimaiti S, Imagawa H, Ngwe H, Abe I, et al. Kaempulchraols A–H, diterpenoids from the rhizomes of Kaempferia pulchra collected in Myanmar. Journal of natural products. 2015;78(5):1113-8.
125. Win NN, Ito T, Matsui T, Aimaiti S, Kodama T, Ngwe H, et al. Isopimarane diterpenoids from Kaempferia pulchra rhizomes collected in Myanmar and their Vpr inhibitory activity. Bioorganic & Medicinal Chemistry Letters. 2016;26(7):1789-93.
126. Xia X, Qi J, Liu Y, Jia A, Zhang Y, Liu C, et al. Bioactive isopimarane diterpenes from the fungus, Epicoccum sp. HS-1, associated with Apostichopus japonicus. Marine drugs. 2015;13(3):1124-32.
127. Wang R, Chen W-H, Shi Y-P. ent-kaurane and ent-pimarane diterpenoids from Siegesbeckia pubescens. Journal of natural products. 2010;73(1):17-21.
128. Dettrakul S, Kittakoop P, Isaka M, Nopichai S, Suyarnsestakorn C, Tanticharoen M, et al. Antimycobacterial pimarane diterpenes from the Fungus Diaporthe sp. Bioorganic & Medicinal Chemistry Letters. 2003;13(7):1253-5.
129. Thongnest S, Mahidol C, Sutthivaiyakit S, Ruchirawat S. Oxygenated pimarane diterpenes from Kaempferia marginata. Journal of natural products. 2005;68(11):1632-6.

130. Wu Z-Y, Zhang Y-B, Zhu K-K, Luo C, Zhang J-X, Cheng C-R, et al. Anti-inflammatory diterpenoids from the root bark of Acanthopanax gracilistylus. Journal of Natural Products. 2014;77(11):2342-51.
131. Zhang G-J, Li Y-H, Jiang J-D, Yu S-S, Wang X-J, Zhuang P-Y, et al. Diterpenes and sesquiterpenes with anti-Coxsackie virus B3 activity from the stems of Illicium jiadifengpi. Tetrahedron. 2014;70(30):4494-9.
132. Yılmaz A, Çağlar P, Dirmenci T, Gören N, Topçu G. A novel isopimarane diterpenoid with acetylcholinesterase inhibitory activity from Nepeta sorgerae, an endemic species to the Nemrut Mountain. Natural Product Communications. 2012;7(6):1934578X1200700602.
133. Jiang K, Chen L-L, Wang S-F, Wang Y, Li Y, Gao K. Anti-inflammatory terpenoids from the leaves and twigs of Dysoxylum gotadhora. Journal of Natural Products. 2015;78(5):1037-44.
134. Win NN, Hardianti B, Kasahara S, Ngwe H, Hayakawa Y, Morita H. Anti-inflammatory activities of isopimara-8 (14),-15-diene diterpenoids and mode of action of kaempulchraols P and Q from Kaempferia pulchra rhizomes. Bioorganic & Medicinal Chemistry Letters. 2020;30(2):126841.
135. Win NN, Ito T, Aimaiti S, Kodama T, Imagawa H, Ngwe H, et al. Kaempulchraols I–O: new isopimarane diterpenoids from Kaempferia pulchra rhizomes collected in Myanmar and their antiproliferative activity. Tetrahedron. 2015;71(29):4707-13.
136. Win NN, Ito T, Aimaiti S, Kodama T, Tanaka M, Ngwe H, et al. Kaempulchraols P–T, diterpenoids from Kaempferia pulchra rhizomes collected in Myanmar. Journal of Natural Products. 2015;78(9):2306-9.

137. Martincuks A, Andryka K, Küster A, Schmitz-Van de Leur H, Komorowski M, Müller-Newen G. Nuclear translocation of STAT3 and NF-κB are independent of each other but NF-κB supports expression and activation of STAT3. Cellular signalling. 2017;32:36-47.

9. Table and Figure

Table 1

No.	Extract Name	No.	Extract Name	No.	Extract Name	No.	Extract Name	No.	Extract Name	No	Extract Name
1	Inchinko	21	Kyonin	41	Sanshishi	61	Souzyutsu	81	Dokukatsu	101	Mao
2	Ukon	22	Kujin	42	Sanshichininnjin	**62**	**Sohakuhi**	82	Tochu	102	Mashinin
3	Uyaku	23	Kumazasa	43	Sanshuyu	63	Souyou	83	Nikujuyo	103	Mankeishi
4	Engosaku	24	Keigai	44	Sansyo	64	Zokudan	84	Ninjin	104	Mokko
5	Ougi	25	Keiketto	45	Sansonin	65	Soyou	85	Baimo	105	Motsuyaku
6	Ougon	26	Keihi	46	Sanryo	66	Daiyou	86	Hakusenpi	106	Yakuchi
7	**Oubaku**	27	Kouka	47	Jio	67	Taiso	87	Bakumondo	107	Yakumoso
8	Ouren	**28**	**Goukanpi**	48	Shigoka	68	Takusha	88	Hakka	108	Yakunin
9	Onji	29	Koujin	49	Jikoppi	69	Tanjin	89	Hange	109	Ryutan
10	Gaiyou	30	Koubushi	50	Shitsurishi	70	Chikusetsuninjin	90	Byakushi	110	Ryokyo
11	Kasyu	31	Kouboku	51	Syakuyaku	71	Chimo	91	Byakujutsu	111	Reishi
12	Gajutsu	32	Goshitsu	52	Syazenshi	72	Cyouji	92	Biwayo	112	Rengyo
13	Kakkon	33	Goshuyu	53	Zyukujio	73	Chotoko	93	Binroji		
14	Karokon	34	Goboushi	54	Syokyo	74	Chorei	94	Bukuryo		
15	Kankyo	35	Goma	55	Syoma	75	Chinpi	95	Bouji		
16	Kanzou	36	Gomishi	56	Shini	76	Tenma	96	Boukon		
17	Kikyo	37	Saiko	57	Jingyo	77	Tenmondo	97	Bofu		
18	Kikuka	38	Saishin	58	Sekisyaku	78	Touki	98	Hobushi		
19	Kijitsu	39	Saffron	59	Senkyu	79	Toujin	99	Hokotsushi		
20	Kyokatsu	40	Sankirai	60	Senburi	80	Tounin	100	Botanpi		

Table 1. Natural products from Japanese plant extracts were screening on mouse mammary carcinoma 4T1 and B16F10 cells that express luciferin reporter assay.

Table 2

No.	**Local Name**	**Plant Part**	**Latin Name**	**Family**
1	Klika Lelak	Bark	*Uvaria rufa* Blume	Annonaceae
2	Akar wangi	Root	*Chrysopogon zizanioides (L.) Roberty*	Poaceae

No.	Local Name	Plant Part	Latin Name	Family
3	Kersen	Leaf	*Muntingia calabura*	Muntingiaceae
4	Afrika	Leaf	*Vernonia amygdalina Delile*	Compositae
5	Rambai Laut	Leaf	*Sonneratia caseolaris (L.) Engl*	Lythraceae
6	Lakka-lakka	Rhizome	*Curculigo orchioides Gaertn*	Hypoxidaceae
7	Temu Kunci	Rhizome	*Boesenbergia rotunda (L.) Mansf*	Zingiberaceae
8	Kapuk Randu	Leaf	*Ceiba pentandra (L.) Gaertn*	Malvaceae
9	Faloak	Stem	*Sterculia abbreviata E.L.Taylor ex Mondragón*	Malvaceae
10	Degan	Fruit	*Dillenia serrata Thunb*	Dilleniaceae
11	Degan	Leaf	*Dillenia serrata Thunb*	Dilleniaceae
12	Kapuk Randu	Root	*Ceiba pentandra (L.) Gaertn*	Malvaceae
13	Kesambi	Leaf	*Schleichera oleosa (Lour.) Merr*	Sapindaceae
14	Faloak	Bark	*terculia abbreviata E.L.Taylor ex Mondragón*	Malvaceae
15	Leileum	Leaf	*terculia abbreviata E.L.Taylor ex Mondragón*	Malvaceae

No.	Local Name	Plant Part	Latin Name	Family
16	Kersen	Leaf	*Muntingia calabura L*	Muntingiaceae
17	Akar Laka	Leaf	*Dalbergia parviflora Roxb*	Leguminosae
18	Temu Ireng	Rhizome	*Curcuma aeruginosa Roxb*	Zingiberaceae
19	Temu Putih	Rhizome	*Curcuma zedoaria* (Christm.) Roscoe	Zingiberaceae
20	Curcuma Mangga	Rhizome	*Curcuma mangga Valeton & Zijp*	Zingiberaceae
21	Brotowali	Leaf	*Tinospora crispa (L.) Hook. f. & Thomson*	Menispermaceae
22	Bangle	Rhizome	*Zingiber montanum (J.Koenig) Link ex A.Dietr.*	Zingiberaceae
23	Lengkuas	Rhizome	*Alpinia galanga (L.) Willd*	Zingiberaceae
24	Jahe Merah	Rhizome	*Zingiber officinale Roscoe*	Zingiberaceae
25	Jahe Segar	Rhizome (fresh)	*Zingiber officinale Roscoe*	Zingiberaceae
26	Kunyit Putih	Rhizome	*Curcuma zedoaria (Christm.) Roscoe*	Zingiberaceae
27	Kunyit	Rhizome	*Curcuma longa L*	Zingiberaceae
28	Pangkal Kunyit Putih	Stem	*Curcuma zedoaria (Christm.) Roscoe*	Zingiberaceae
29	Kencur	Rhizome	*Kaempferia galanga L*	Zingiberaceae

No.	Local Name	Plant Part	Latin Name	Family
	Besar	(large)		
30	Temulawak	Rhizome	*Curcuma zanthorrhiza Roxb*	Zingiberaceae
31	Jahe kering	Rhizome (137)	*Zingiber officinale Roscoe*	Zingiberaceae
32	Bawang Putih Tunggal	Tuber (single)	*Allium sativum L*	Amaryllidaceae
33	Lempuyang	Rhizome	*Zingiber zerumbet (L.) Roscoe ex Sm*	Zingiberaceae
34	Temu Giring	Rhizome	*Curcuma heyneana Valeton & Zijp*	Zingiberaceae
35	Kencur Kecil	Rhizome (small)	*Kaempferia galanga L*	Zingiberaceae

Table 2. Natural products from Indonesian Plant extracts were screening on mouse mammary carcinoma 4T1 cells that express luciferin reporter assay.

Figure

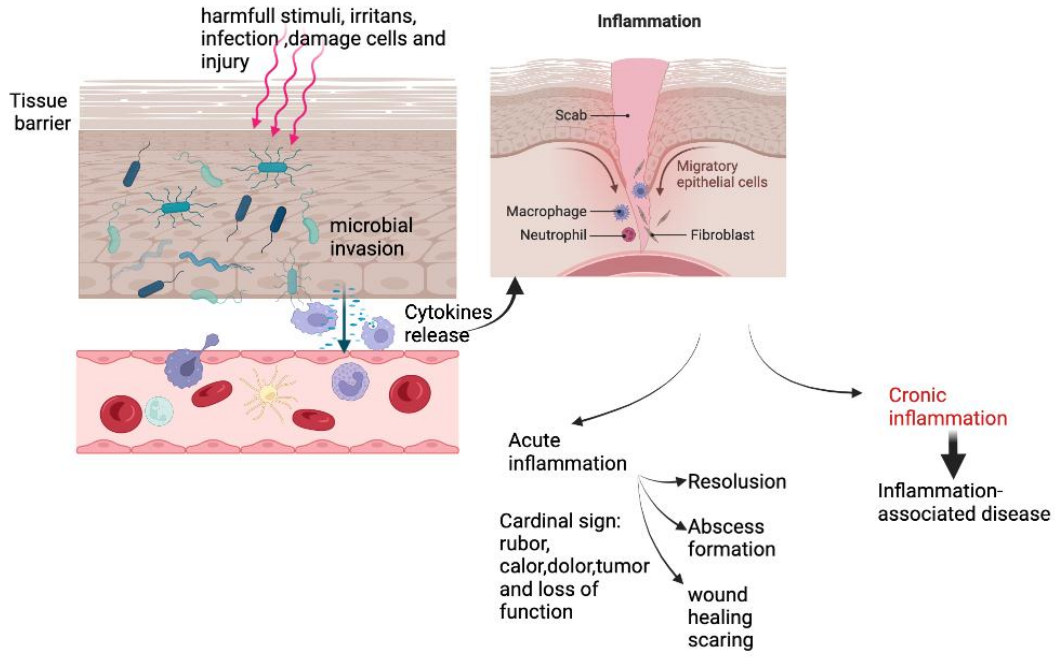


Figure 1. Basic cardinal sign of inflammation

- heat and redness (due to the additional number of erythrocytes passing through the area).
- Oedema is caused by increased fluid passage from dilated and permeable blood vessels into surrounding tissues, cell infiltration, and connective tissue deposition in prolonged inflammatory responses.
- Pain is caused by mediators from initial damage or the inflammatory response, and by oedema stretching sensory nerves.
- Loss of function refers to joint mobility due to oedema and pain or replacement of functional cells with scar tissue.

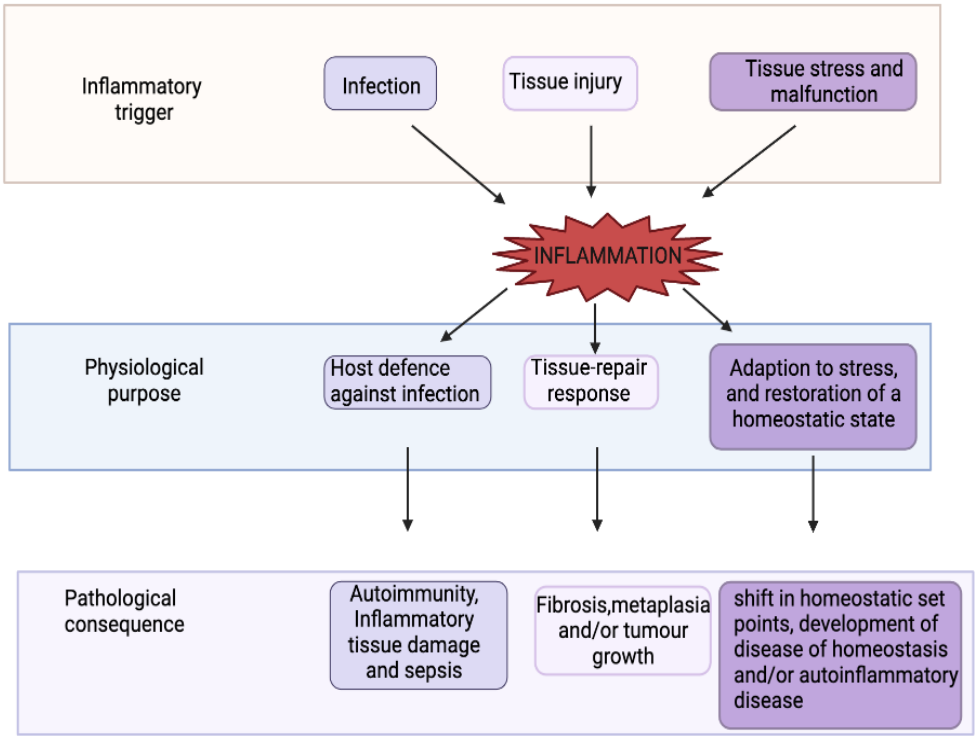


Figure 2. Causes, physiological and pathological outcomes of inflammation

Different stimuli elicit various physiological and pathological inflammatory responses, inflammatory causes, and physiological and pathological outcomes.

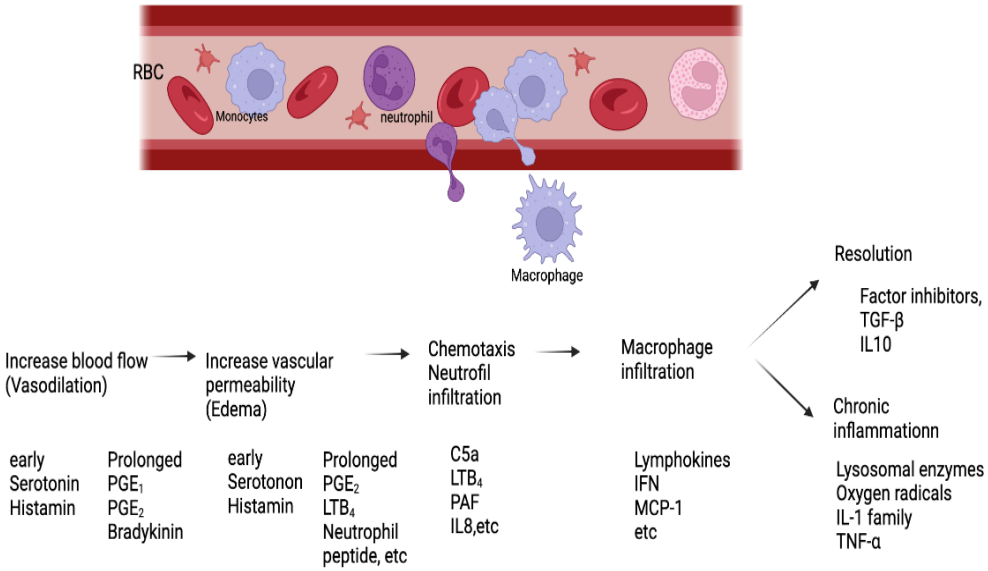


Figure 3. Mediators in the process inflammation from acute to chronic inflammation

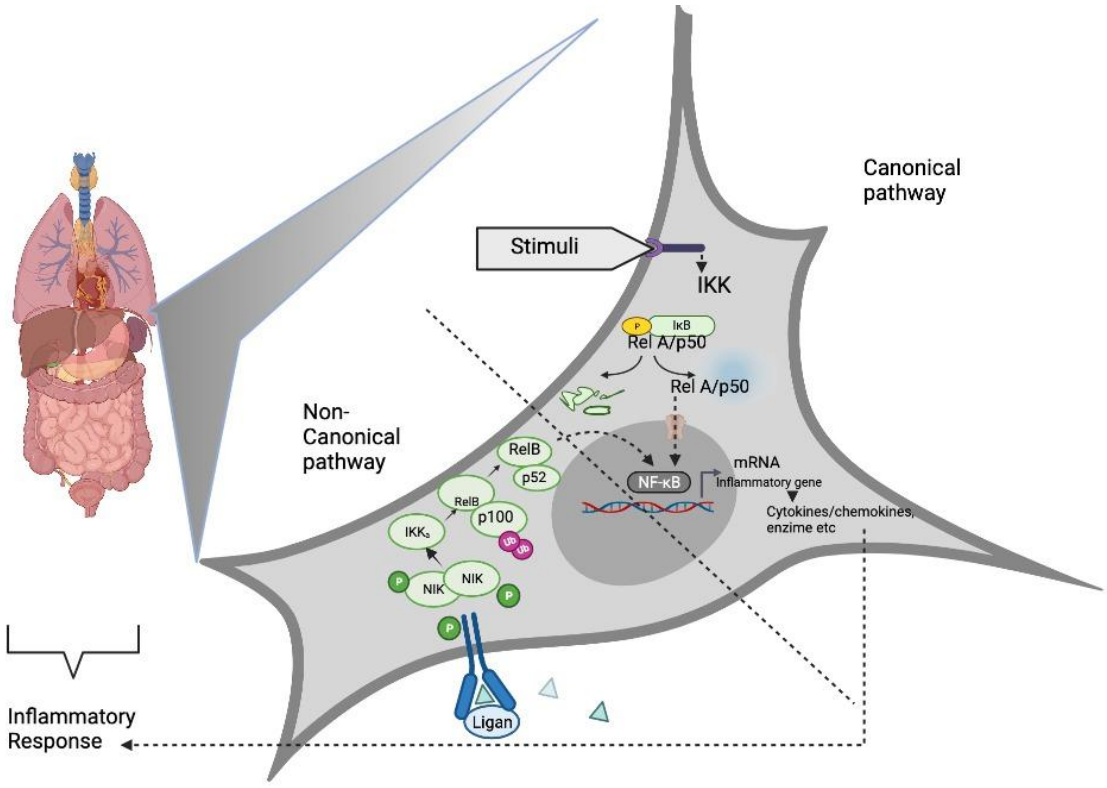


Figure 4. The canonical and non-canonical pathway on activation of NFκB

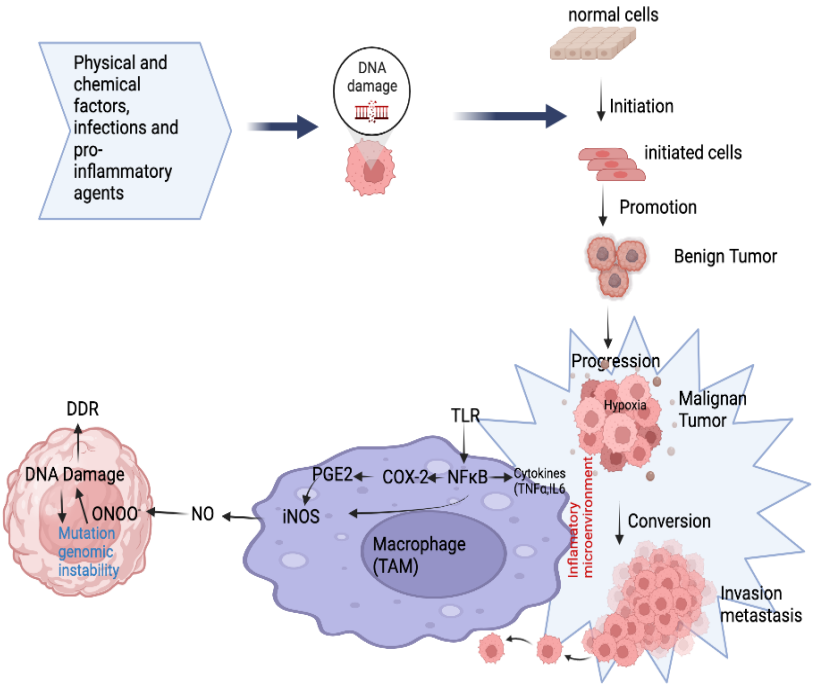


Figure 5. Crosstalk between DNA damage and inflammation in the various stages of carcinogenesis

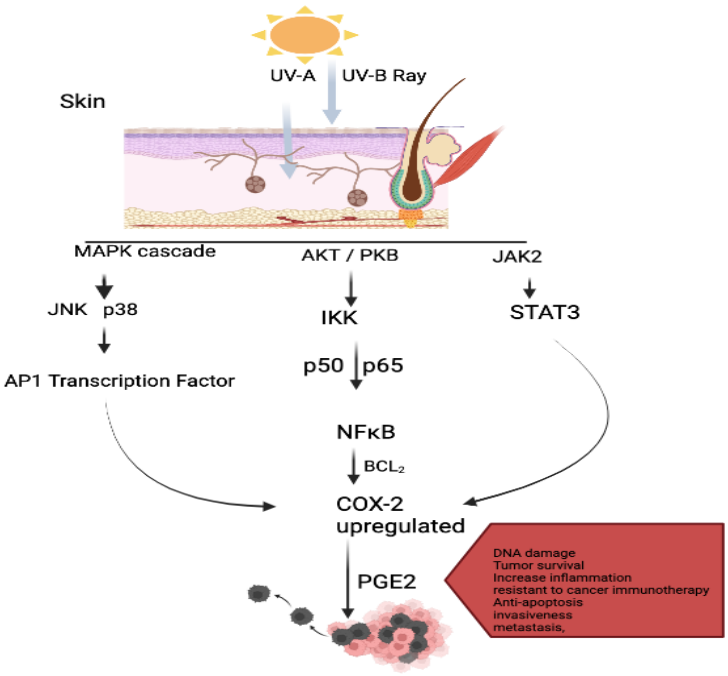


Figure 6. Mechanism of inflammation-associated melanoma

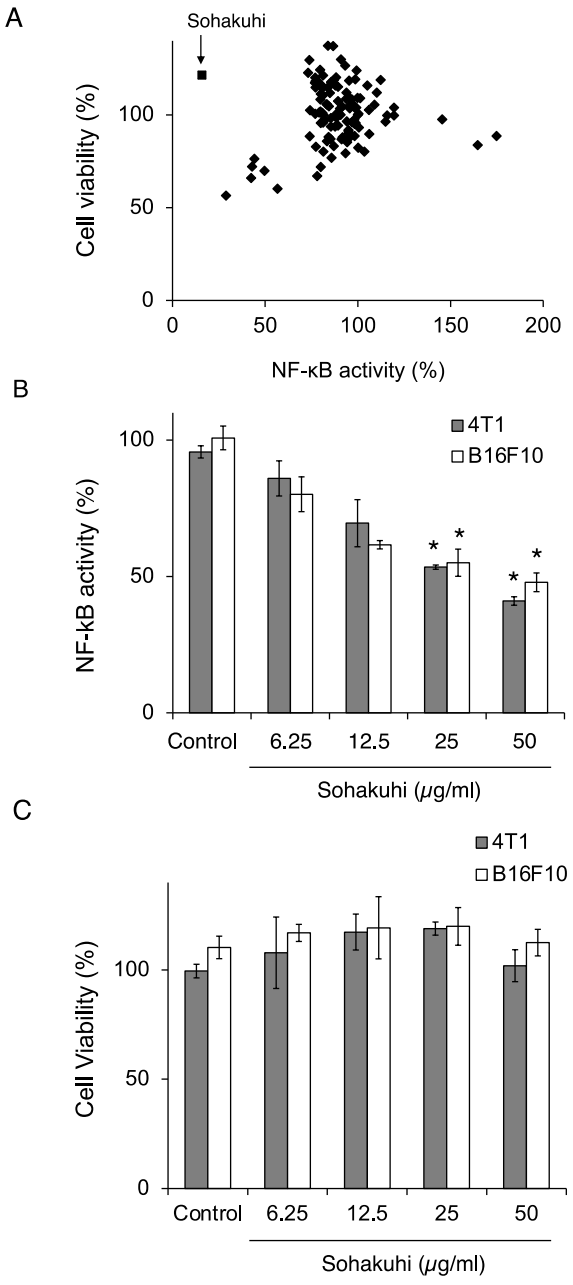


Figure 7. Sohakuhi water extract suppresses NF-κB activity in murine cancer cell lines. (A) 4T1NFκB cells were co-cultured with extracts from 112 natural products (50 μg/ml) for 24h. The inhibitory effect of each plant extract on NF-κB activation relative to untreated controls was determined. Cell viability was determined using a WST-1

assay and shown as a percentage of the untreated control. (B) NF-κB activation and (C) cell viability was determined in 4T1NFκB cells or B16F10NFκB cells treated with Sohakuhi extract at the indicated doses for 24 h. Data are normalized to the untreated controls and presented as the mean ± SEM; n=3. *P<0.05 vs. untreated control.

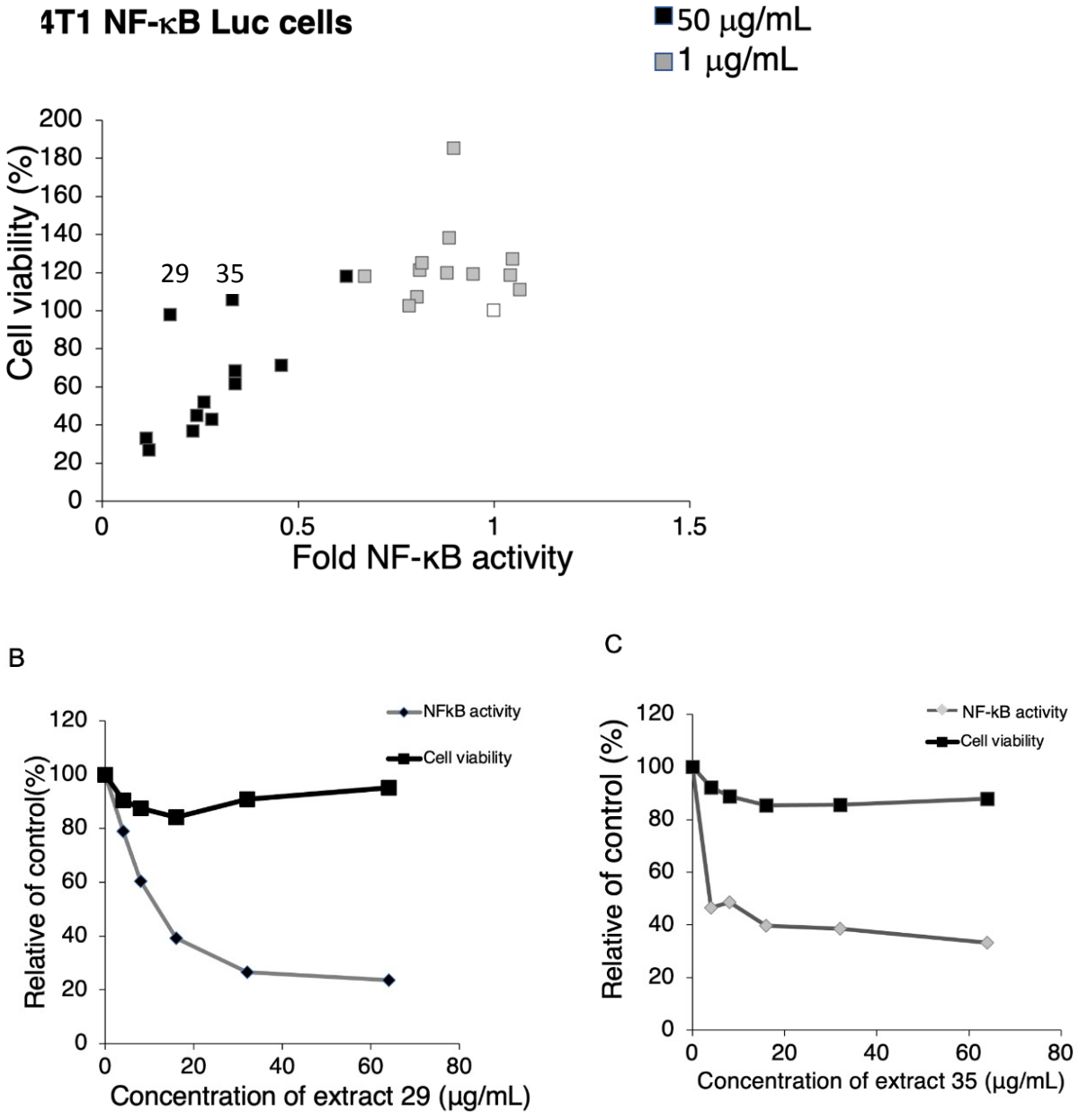


Figure. 8 Inhibitory activities against NF- κB was determined by co-culturing 4T1- NF-κB -Luc2 cells with extracts at 1 and 50 µg/ml for 24 hrs. Twelve extracts showed active inhibition of Figure 24. Effects of 9 and 10 on NF- κB activation by a reporter gene assay (A), and on cell viability (B) in RAW 264.7- NF- κB -luc cells. in transfected cells. NF- κB inhibition results were compared with those from a viability assay in which WST-8 was used (A) Futhermore, RAW264.7- NF– κB -luc2 cells were co-cultured with extracts 29, this extract effectively inhibited NF- κB activity without affecting viability cells(B). RAW264.7- NF- κB -luc2 cells were co-cultured with extracts 35, this extract effectively inhibited NF– κB activity without affecting viability cells(C). The inhibitory effect (grey) of each plant extract on NF- κB activation relative to untreated at various concentrations for 24 hrs. The inhibitory effect (grey) of each plant extract on NF- κB activation relative to untreated controls is shown. Cell viability (black) was determined using a WST-8 assay and is shown as a percentage of untreated control cell viability.

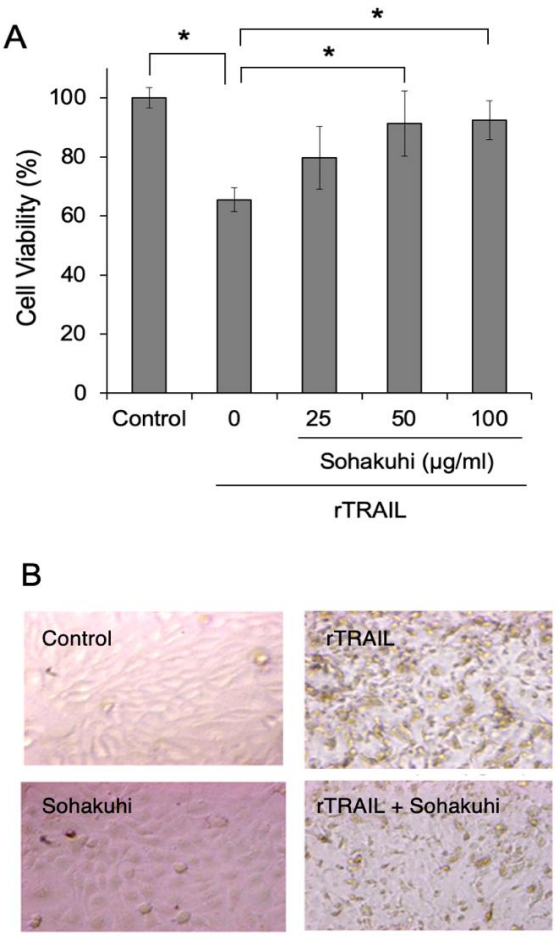


Figure 9. Cytoprotective effect of Sohakuhi extract against TRAIL-induced cellular damage in human keratinocytes. HaCaT cells were co-cultured with 20 ng/ml rTRAIL with or without Sohakuhi extract at the indicated doses. (A) Cell viability was determined using a WST-1 assay. Data are normalized to the untreated controls and presented as the mean ± SEM; n=3. *P<0.05 vs. 0 μg/ml Sohakuhi. (B) Representative images of the culture (magnification, x100). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; r, recombinant.

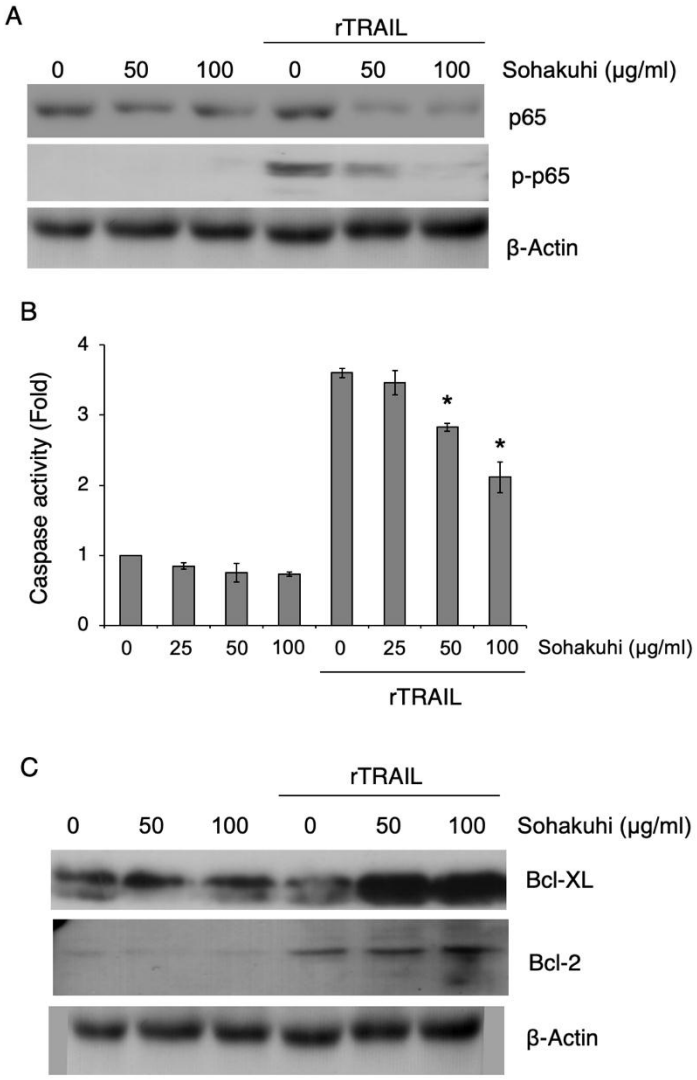


Figure 10. Anti-apoptotic effect of Sohakuhi extract on HaCaT cells. HaCaT cells were co-cultured with 20 ng/ml rTRAIL with or without Sohakuhi extract at the indicated doses. After 24-hr incubation, cell lysates were prepared for (A) western blot analysis of p65 and p-p65, and (B) measure- ment of caspase-3/7 activity using a commercial kit. (C) Protein levels of Bcl-xL, Bcl-2 are determined by western blot analysis. Data are presented as the mean ± SEM; n=3. *P<0.05 vs. untreated control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; r, recombinant; p-, phosphorylated.

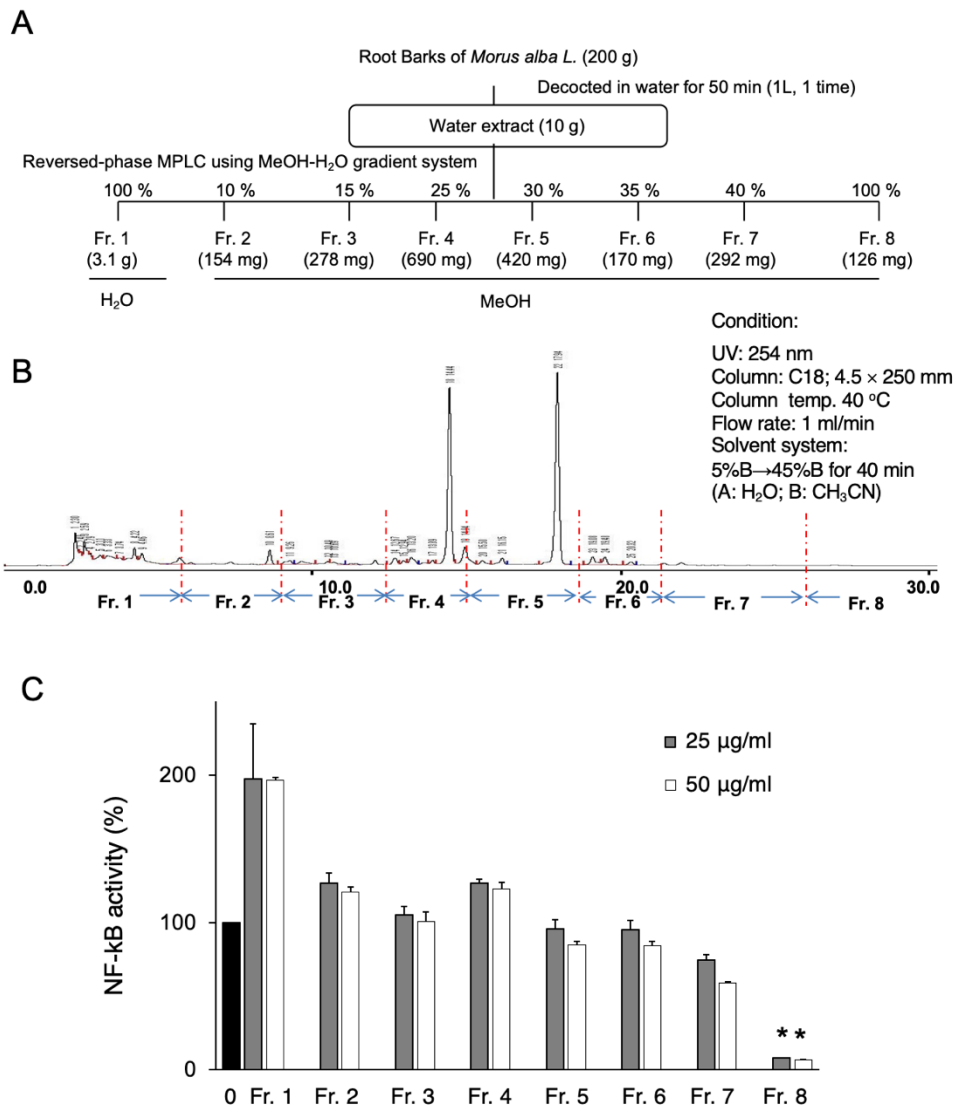


Figure 11. Identification of the active fraction of Sohakuhi extract that inhibit NF-□B activation. (A) Extraction and isolation flowchart for the identification of the active component of Sohakuhi extract using a water·methanol gradient MPLC fractionation. (B) High·performance liquid chromatography profile of water extracts of Sohakuhi at 254·nm UV wavelength (C) 4T1 NF-κB cells were co·cultured with the indicated fraction of Sohakuhi at 25 or 50 μg/ml for 24 h. Inhibition of NF-κB activation was determined relative to the untreated control. Data are presented as the mean ± SEM;

n=3. *P<0.05 vs. untreated control. UV, ultraviolet; MeOH, methanol; Fr, fraction; MPLC, medium-pressure liquid chromatography.

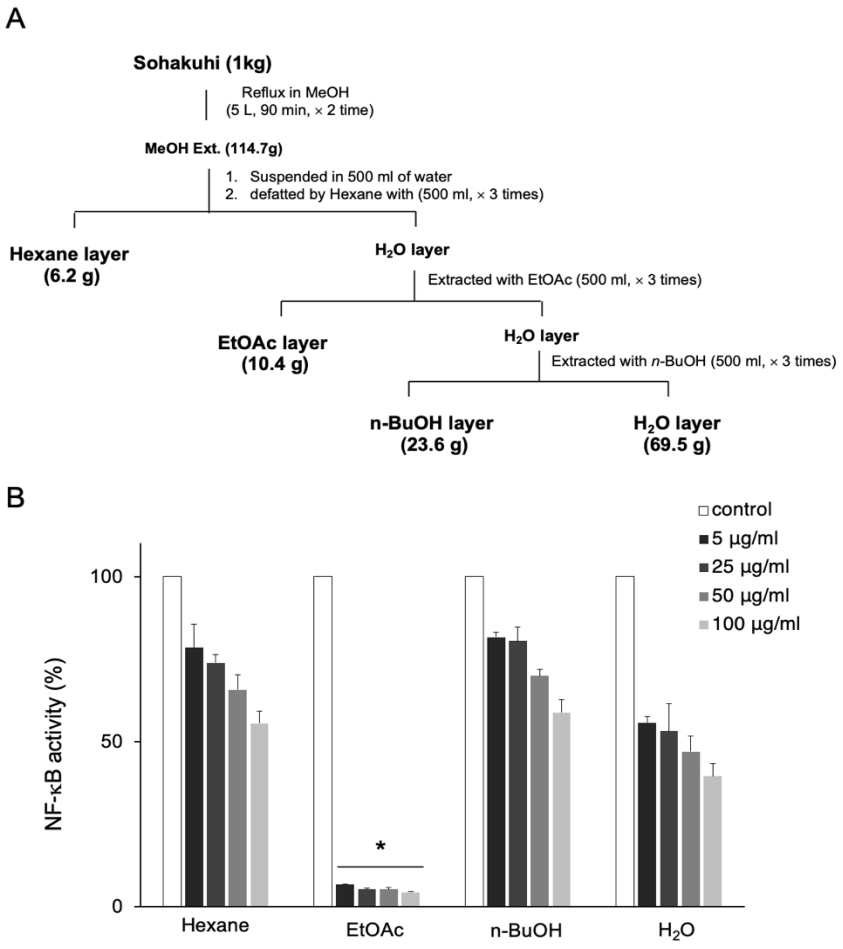


Figure 12. Identification of the active components of Sohakuhi that inhibit NF-κB activation.

(A) Extraction flowchart for the identification of the active frac- tion of Sohakuhi. (B) 4T1 NF-κB cells were co-cultured with the indicated solvent fraction at the indicated dose for 24 h. Inhibition of NF-κB activation was determined relative to the untreated control. Data are presented as the mean ± SEM; n=3. *P<0.05 vs. untreated control. MeOH, methanol; EtOAc, ethyl acetate; n-BuOH, n-butanol.

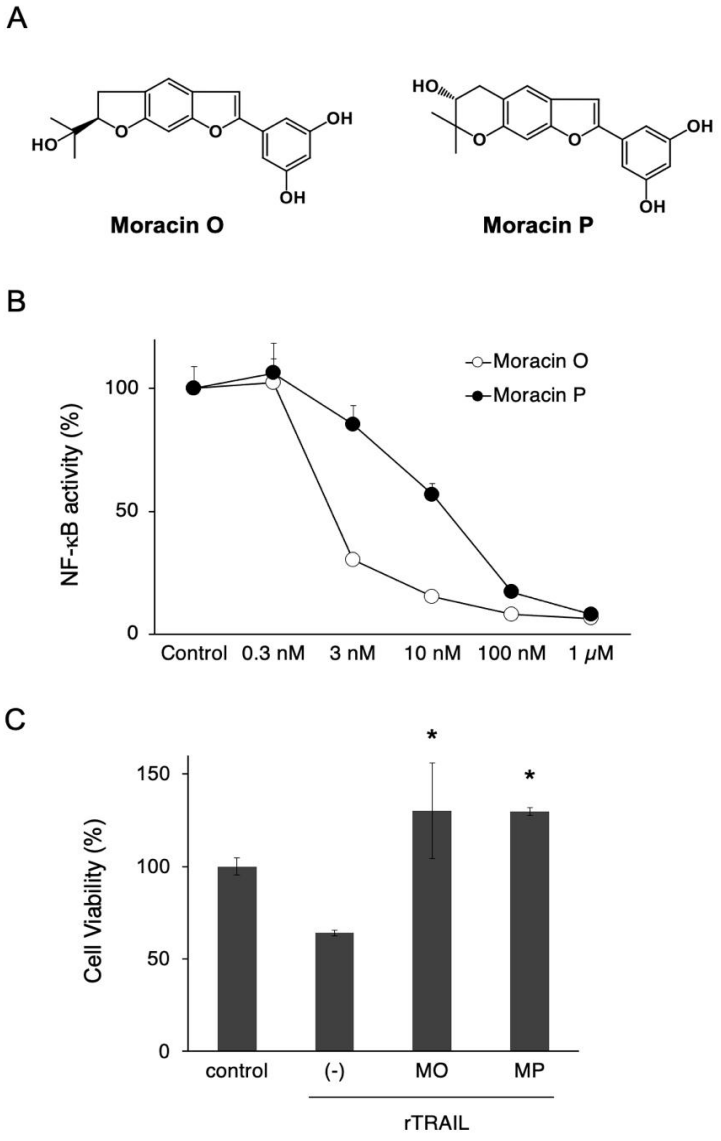


Figure 13. Isolation of moracin O and P as active compounds of Sohakuhi. (A) Chemicalstructures of moracin O and P. (B) 4T1NFκB cells were co-cultured with the indicated dose of moracin O or moracin P for 24 h. The inhibitory effect on NF-κB activation was determined relative to the untreated control. (C) HaCaT cells were pre-incubated with 10 μM of moracin O or moracin P for 1 h, then co-cultured with 20 ng/ml TRAIL for 24 h. Cell viability was determined using a WST-1 assay and normalized to the untreated control. Data are presented as the mean ± SEM; n=3. *P<0.05 vs.

untreated control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; r, recombinant.

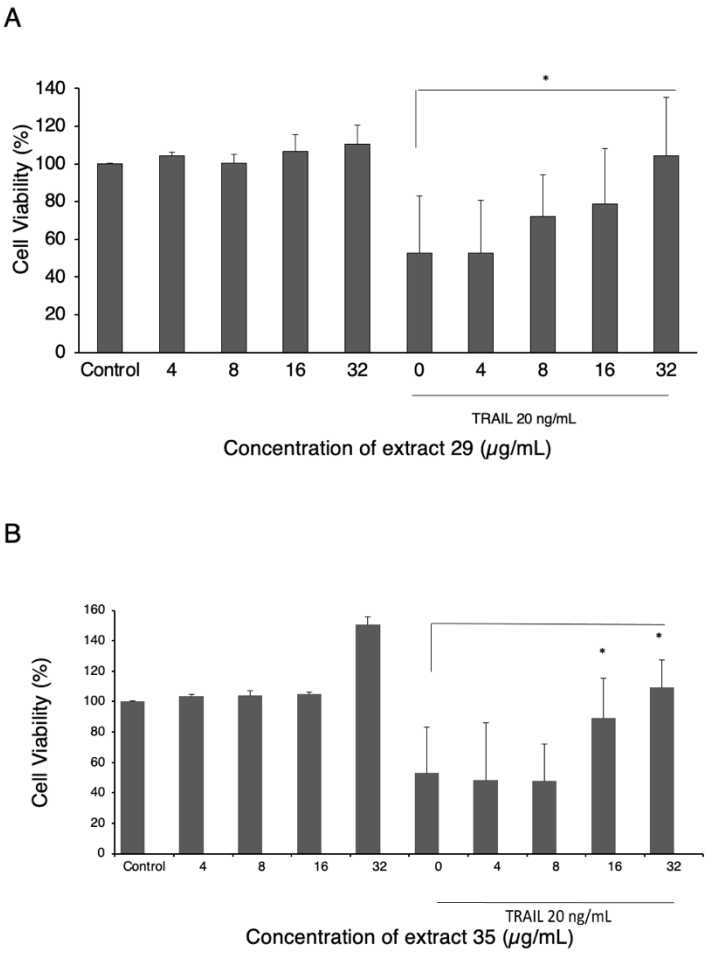


Figure 14. Cytoprotective effects of extracts 29 (A) and 35 (B) against rTRAIL-induced cellular damage in human keratinocytes Data are normalized to the untreated controls and presented as Mean± standard error of the mean (n = 3: *p<0.05 vs. cells without extract treatments)

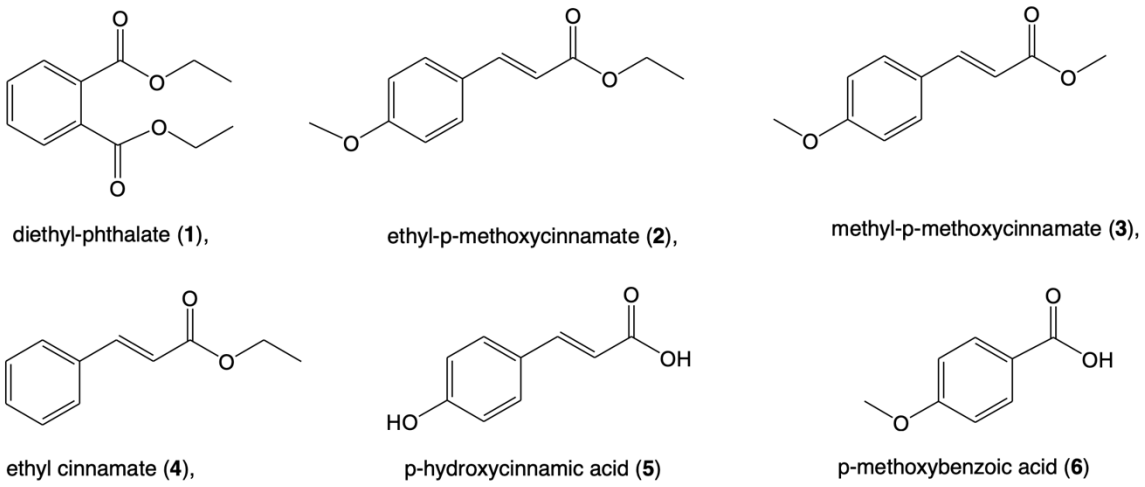


Figure 15. Chemical structure of the isolated compounds from *Kaempferia galanga*

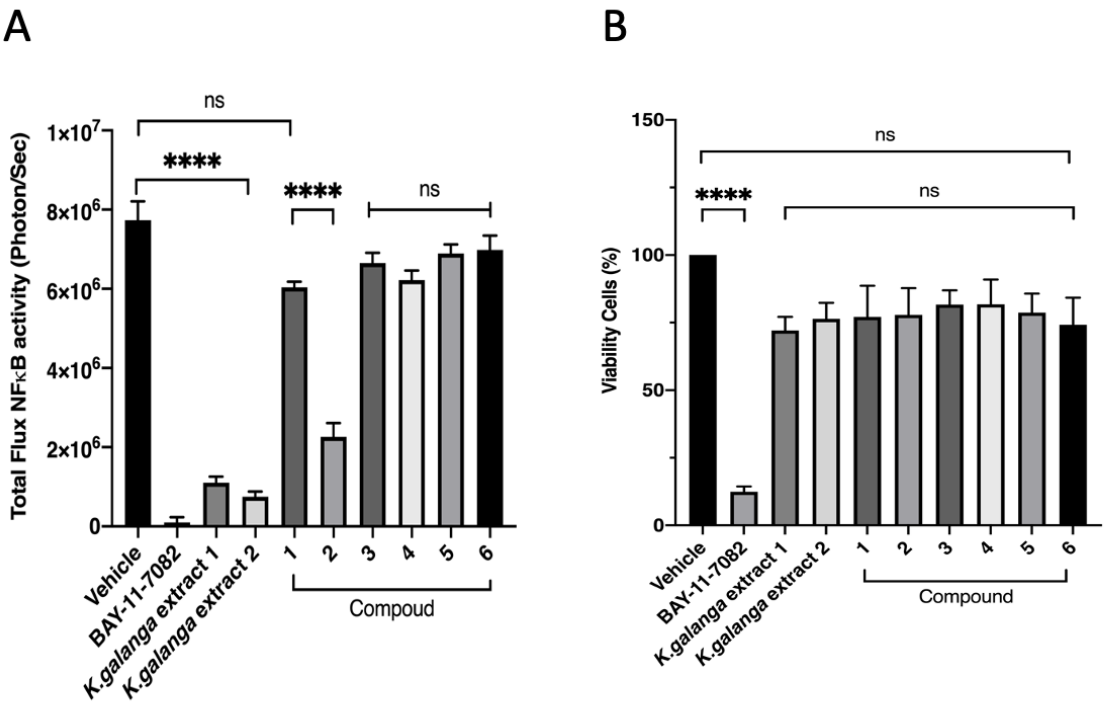


Figure 16. (A) Inhibitory activity regarding NF–κB and (B) cell viability of B16F10-NF-κB - luc for isolated compounds. B16F10- NF–κB-luc cells were cultured for 24 h, in 10 µg/mL and 10 µM extracts and compounds, respectively, with the positive control BAY-11-7082. The test was repeated at least three times, unless explicitly stated

otherwise. NF-κB activity and cell viability are expressed as the percentage of viability observed in untreated cells (Bonferroni post hoc test; **** $p < 0.0001$; ns, nonsignificant).

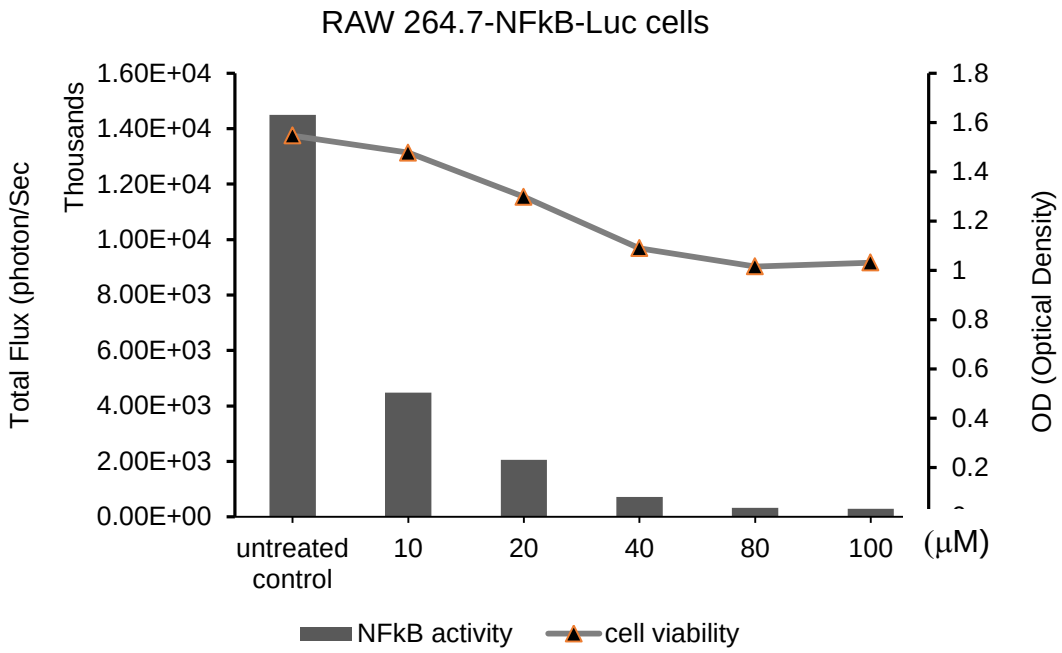


Figure 17. RAW264.7-NF–κB-luc2 cells were co-cultured with EPMC at various concentrations and incubated for 24 hrs (10, 20, 40, 80 and 100 μM). The inhibitory effect of EPMC (bar graph) on NF-κB activation relative to untreated controls is shown. Cell viability (line) was determined using a WST-8 assay and is shown as percentage of viability cell compare to untreated control cell.

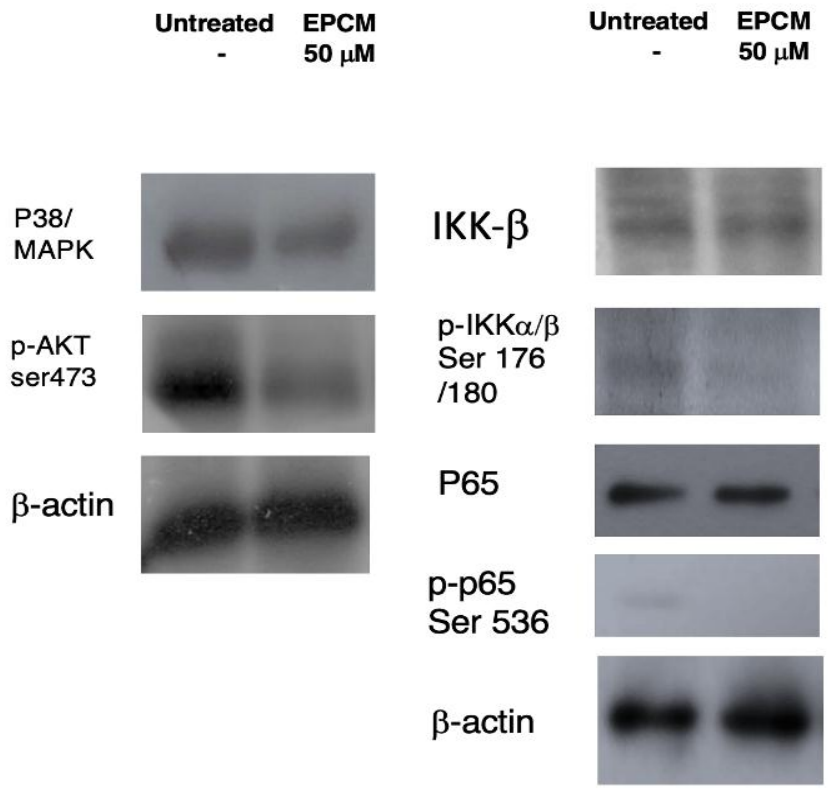


Figure 18. B16F10 G5-luc cells were cultured for 12 h and treated with EPMC before harvesting the protein. Furthermore, the molecular mechanism of EPMC 50 μM treatment's effect on B16F10 G5-Luc cells was investigated by Western blotting. In the protein level, pretreated B16F10 G5-Luc cells with EPMC detected the expression of p65 subunit of NFkB and its phosphorylation at serine 536, P38/MAPK and p-AKT ser-473 by western blotting.

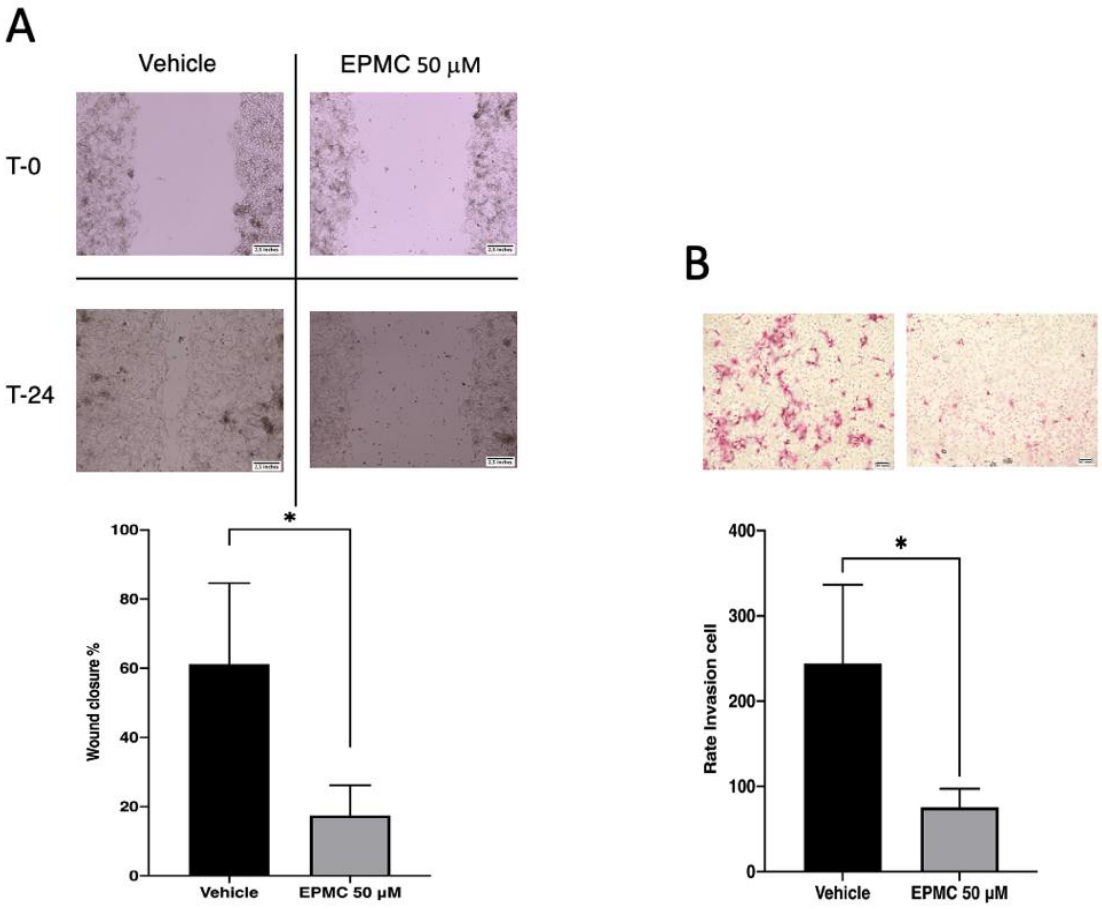


Figure 19. Migration assay of B16F10G5-Luc in the presence of EPMC evaluated by the wound-healing method (A) and invasion using transwell. The transwell cell migration assay measures the chemotactic ability of cells toward a chemo-attractant (B). Wound closure and invasion cells compared with untreated cells (Analyzed by one-way Anova followed by Bonferronni post hoc test; * $p < 0.05$).

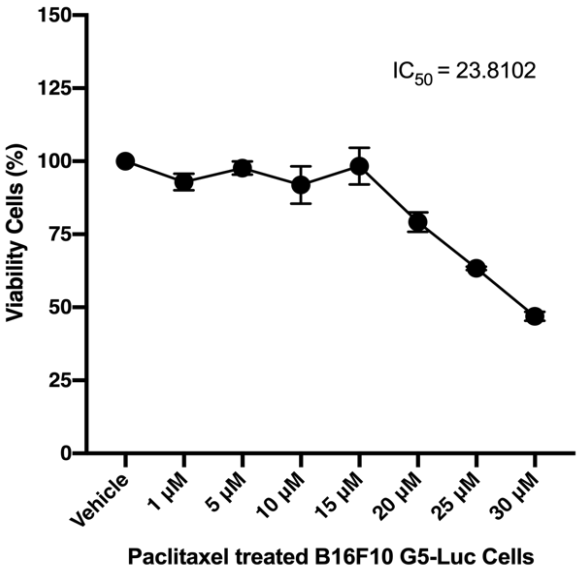


Figure 20. Paclitaxel-treated effect on B16F10 G5-Luc normalized by percentage of viability observed in untreated cells (Bonferroni post hoc test). IC_{50} Paclitaxel on this cell is 23.8102 μM.

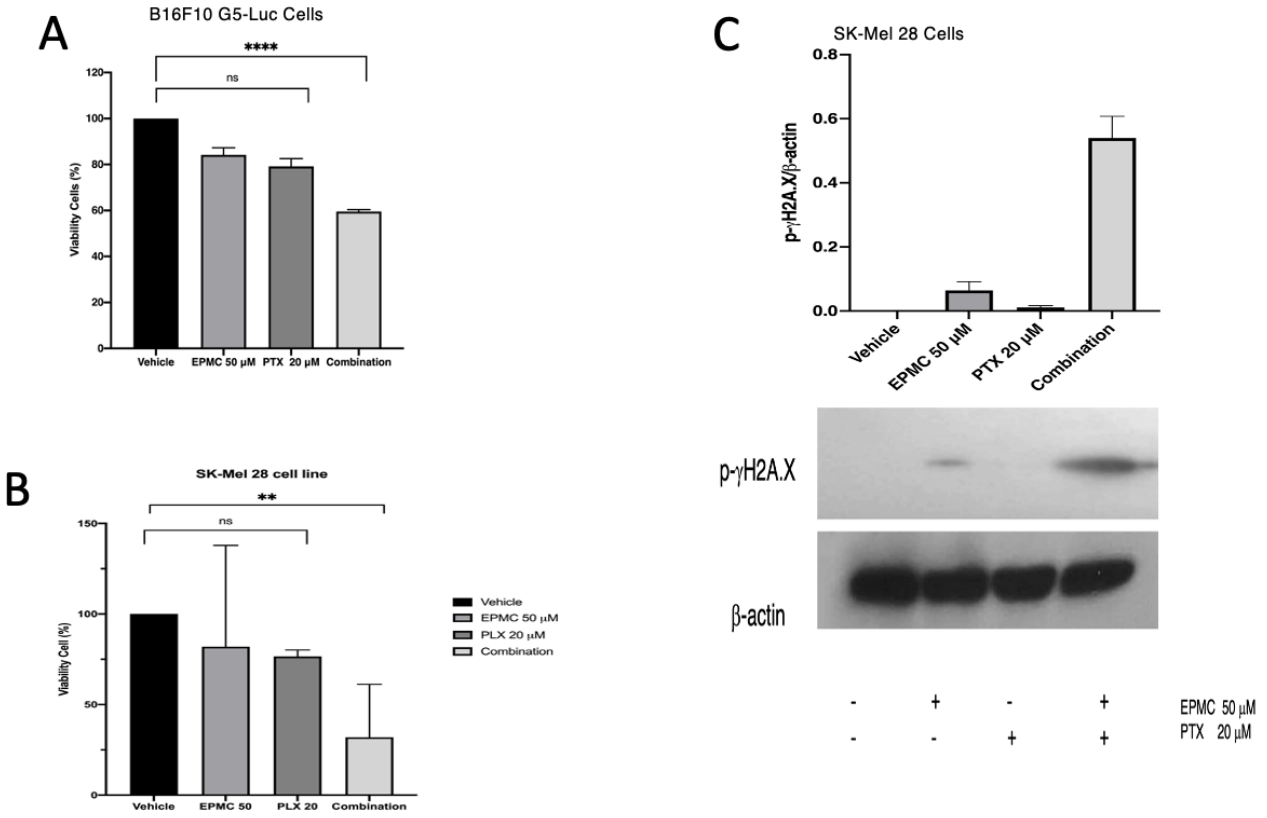


Figure 21. Paclitaxel and EPMC chemo-sensitizing effect on B16F10 G5-Luc (A) and human SK-Mel 28 melanoma cell line (B); cell viability normalized by percentage of viability observed in untreated cells, SK-Mel 28 cells were cultured for 12 h, then the protein was harvested. Additionally, the molecular mechanisms of EPMC 50 µM and PTX 20 µM co-treatment were analyzed using western blotting (C). Statistical analysis by one- way Anova followed by Bonferroni post hoc test; ** $p < 0.01$, **** $p < 0.0001$.

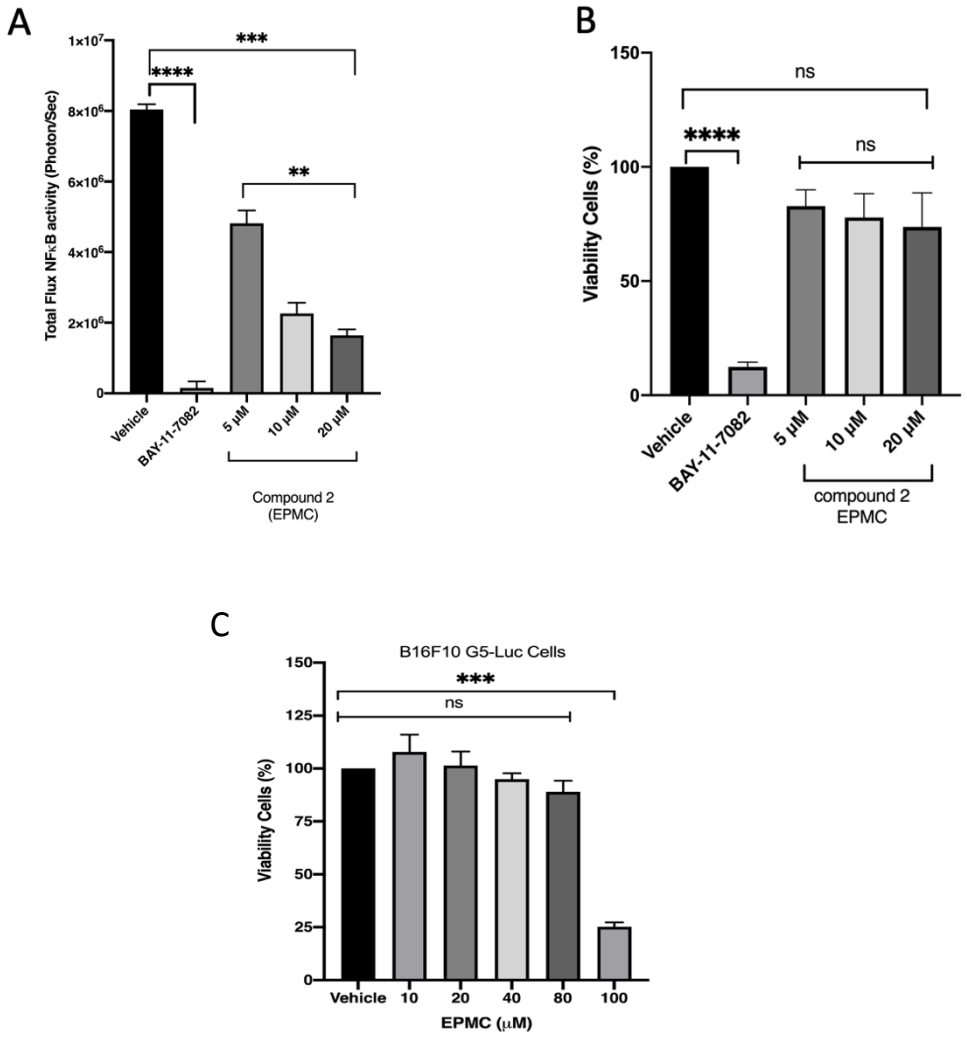
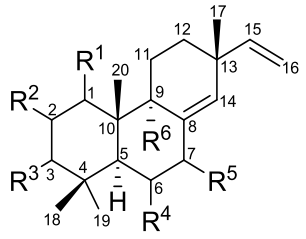
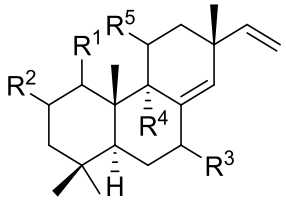


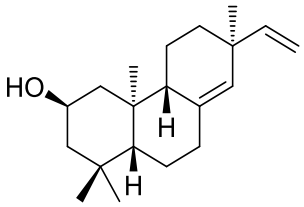
Figure 22. B16F10-NF-κB-luc cells were cultured for 24 h. BAY-11-7082 was used as a positive control. The test was repeated at least three times, unless explicitly stated otherwise. Inhibitory activity of NF−κB (A) and cell viability for B16F10-NF-κB-luc (B) of 5, 10 and 20 μM and (C) dose response IC_{50} evaluated on B16F10 G5-Luc cells of EPMC was calculated using a calculator tool (104). Cell viability is expressed as percentage of viability observed in untreated cells (Bonferroni post hoc test; ** $p < 0.01$, *** $p < 0.001$,**** $p < 0.0001$).





	R^1	R^2	R^3	R^4	R^5	R^6
1	α-OH	H	H	β-OH	H	H
2	α-OH	H	α-OH	H	H	H
3	α-OH	H	α-OH	β-OH	H	H
4	α-OH	H	H	H	H	H
5	α-OH	H	H	H	=O	H
6	H	H	H	β-OAc	H	OH
7	H	H	H	β-OH	H	OMe
8	α-OH	α-OH	H	H	H	OH
9	H	H	H	β-OH	H	H
10	α-OAc	H	H	β-OH	H	H
11	H	H	H	H	α-OAc	OH
12	H	H	H	β-OH	α-OAc	H
13	H	H	H	β-OH	β-OAc	OH
14	H	H	H	β-OH	β-OH	OH

	R^1	R^2	R^3	R^4	R^5
15	H	H	=O	OH	H
16	H	H	β-OH	OH	H
17	α-OH	H	H	H	α-OH
18	α-OH	α-OH	H	H	H
20	α-OH	α-OH	H	H	α-OH
21	H	H	α-OH	H	H



19

Figure 23. Identified 21 isopimara-8,14(15)-diene diterpenoids, including kaempulchraols E (1), F (2), H-M (3-8), P-R (9-11), T (12), V (13), W (14), 9α–hydroxyisopimara-8(14),15-dien-7-one(15),7β,9α-dihydroxypimara-8(14),15-diene(16),(1S,5S,9S,10S,11R,13R)-1,11-dihydroxypimara-8(14),15-diene(17), sandaracopimaradien-1α,2α-diol (18), (2R)-ent-2-hydroxyisopimara-8(14),15-diene(19), (1R,2S,5S,9S,10S,11R,13R)-1,2,11-trihydroxypimara-8(14),15-diene (20), and 7α-hydroxyisopimara-8(14),15-diene (21), from a CHCl3-soluble extract of *Kaempferia pulchra* rhizomes

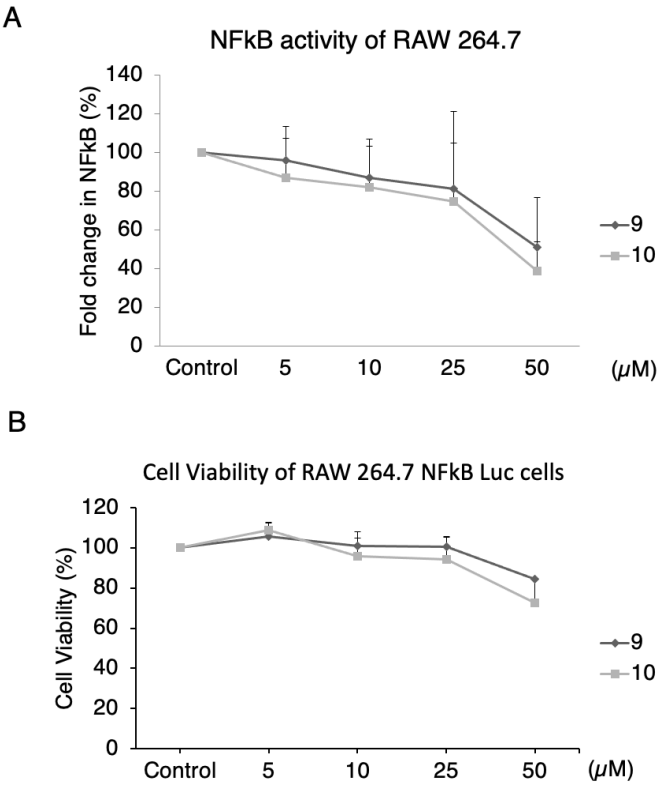
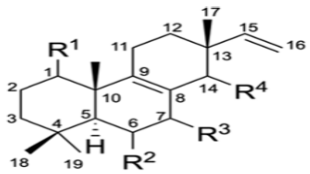


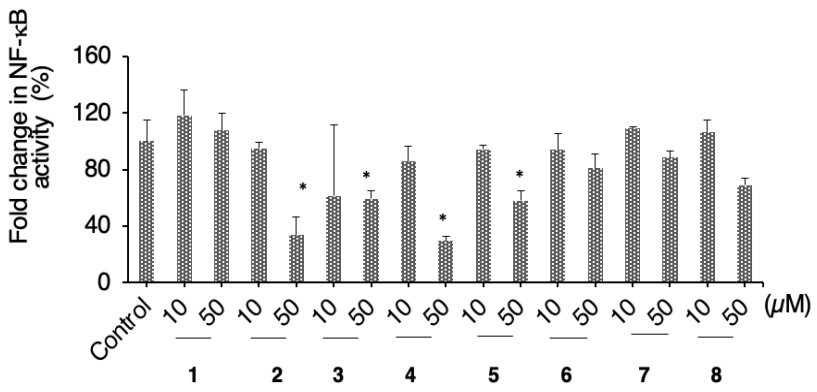
Figure 24. Effects of 9 and 10 on NF-κB activation by a reporter gene assay (A), and on cell viability (B) in RAW 264.7-NF-κB-luc cells. The data points and bars represent mean ± SD (n = 3).

A



	R^1	R^2	R^3	R^4
1	H	β-OH	H	α-OMe
2	H	β-OH	H	β-OMe
3	H	β-OH	H	α-OH
4	H	β-OH	H	β-OH
5	H	β-OH	H	=O
6	α-OH	β-OH	H	α-OH
7	α-OH	β-OH	H	β-OMe
8	H	H	=O	α-OH

B



C

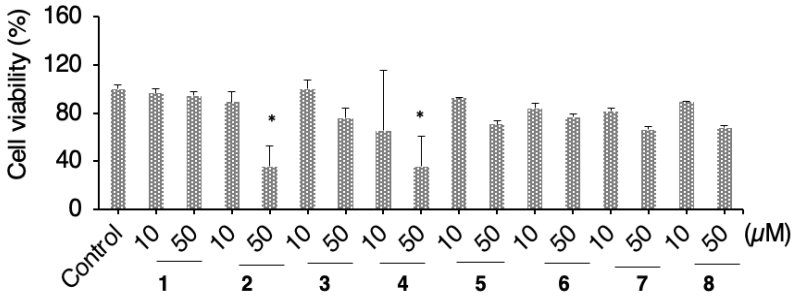


Figure 25. Structures of isopimara-8(9),15-dienes 1–8 isolated from K. pulchra rhizomes (A)and further effects of compounds 1–8 on NF-κB activation by the reporter gene assay (B) and on the viability (C) of RAW264.7- NF-κB-luc cells. The data points and bars represent mean ± SD (n=3). *P<0.05, significantly different from the control.

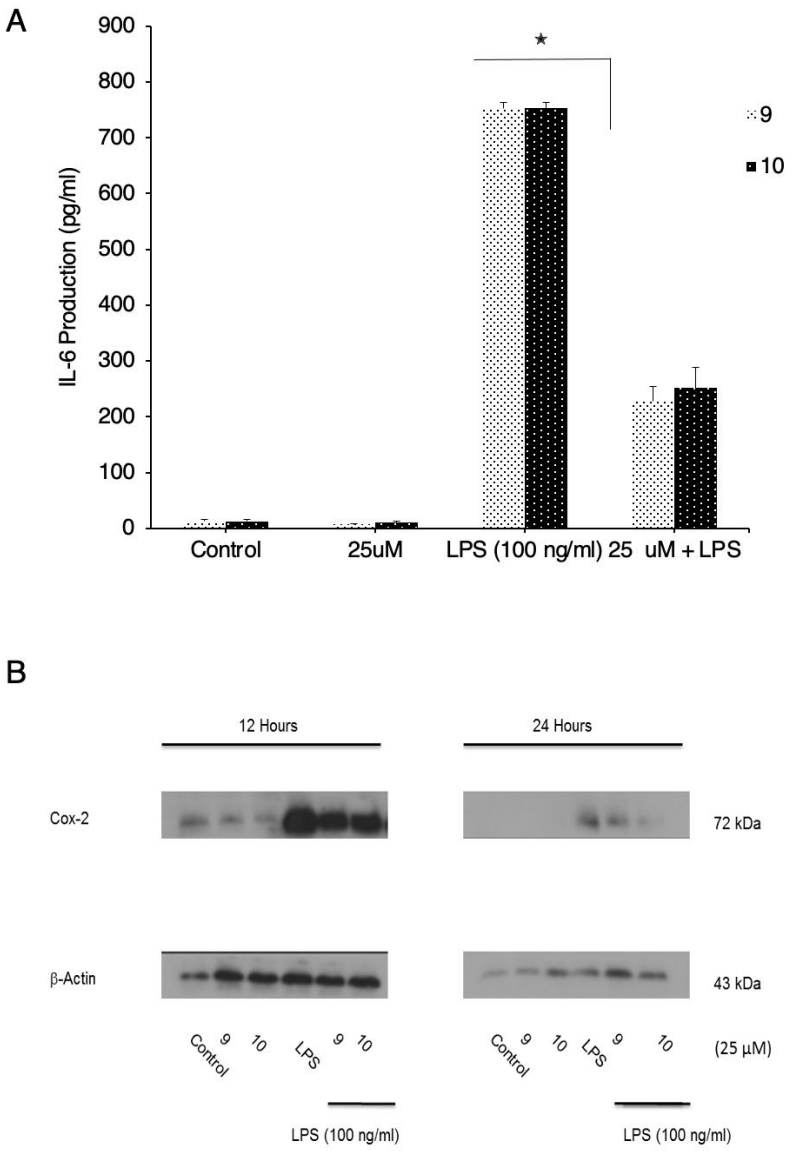


Figure 26. (A)Effects of compound **9** and **10** on IL-6 production. The data points and bars represent mean ± SD (n= 3). ***, $P < 0.0001$, very significantly different from the LPS-treated control (B) Effects of **9** and **10** on COX-2 expression in LPS-induced RAW 264.7 cells, analyzed by western blotting.

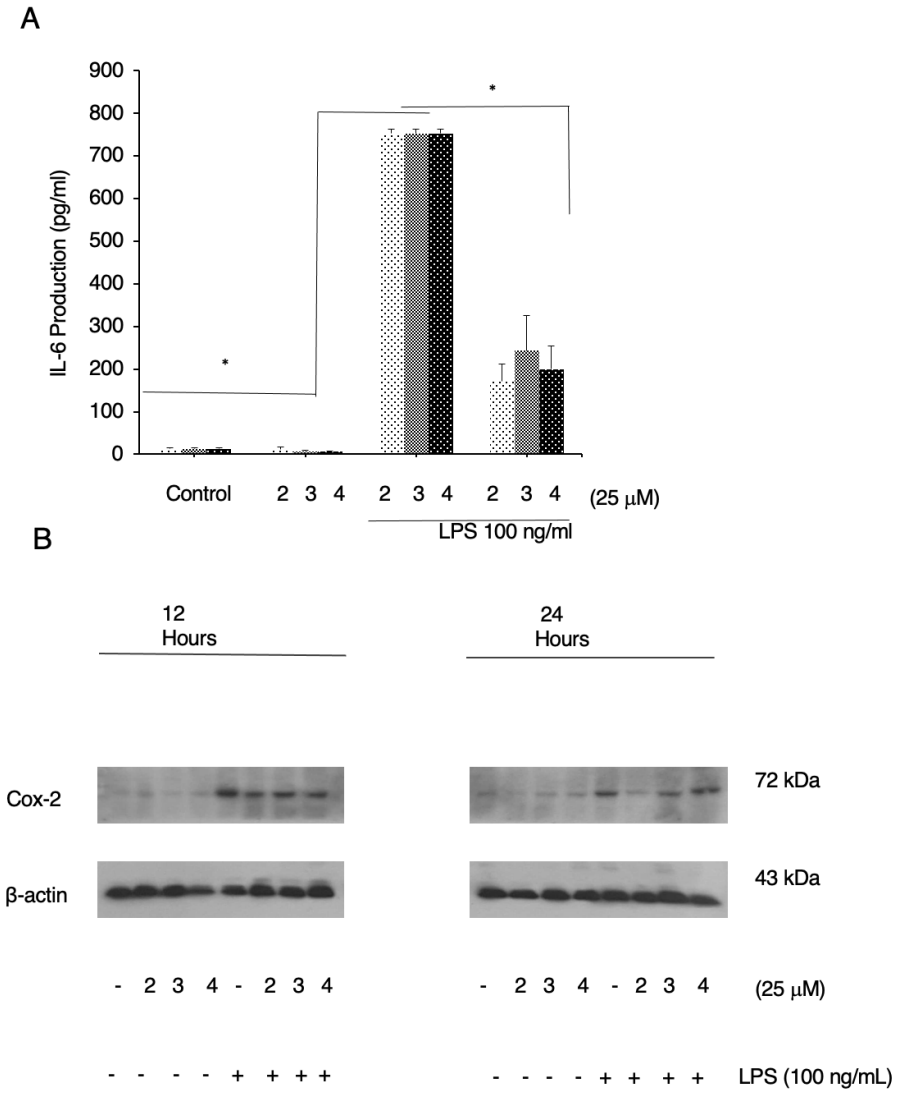


Figure 27. (A)Effects of **2,3** and **4** on IL-6 production. The data points and bars represent mean ± SD (n= 3). ***, $P < 0.0001$, Very significantly different from the LPS-treated control. (B) Effects of **2,3** and **4** on COX-2 expression in LPS-induced RAW 264.7 cells, analyzed by western blotting.