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투실라고논, 이갈란 및 치쿠셋수사포닌 포에이 메틸 에스테르에 의한 화학적 암 예방 효과 및 분자 기전 연구

Cancer chemopreventive effects of tussilagonone, igalan and chikusetsusaponin IVa methyl ester and their molecular mechanisms

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ABSTRACT

Cancer chemopreventive effects of tussilagonone, igalan and chikusetsusaponin IVa methyl ester and their molecular mechanisms

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Cancer is widespread and growing worldwide. To combat it, people must avoid smoking and drinking alcohol, two major causes of cancer, and instead cultivate healthy lifestyle habits. In addition, the study of assistive drugs and foods that can supplement with cancer prevention and active cancer treatment must continue apace.

Cancer is not onset suddenly. Its prognosis and symptoms appear at the cellular level, step by

step, divided into four stages. An initiation step of changing from normal cells to initiated cells; a promotion step to preneoplastic cells; and a progression step of forming into neoplastic cells. These processes are referred to as carcinogenesis. Cancer chemopreventive activity, then, inhibits the development of cancer with suppressing and delaying the steps of carcinogenesis, and represses the progress of cancer. In this study, new natural substances that have the potential for chemopreventive activities and related mechanisms have been identified.

In order to prevent the formation of cancer, tussilagonone, which is a compound isolated from *Tussilago farfara* L., and igalan, isolated from *Inula helenium* L., were identified by screening for natural products that can act in the initiation stage. The quinone reductase (QR) assay which measures chemopreventive activity through detoxification activity was used for screening. Tussilagonone showed greater than two-fold activity at a concentration of 2.5 μM, and igalan also started to show a two-fold activity at a concentration of 5 μM. Based on these assays, relevant molecular experiments were performed. And based on the results of the experiments, the Nrf2 activity was induced in HepG2 cells using two compounds, and an increase of the expression level of NQO1 and HO-1, target genes of Nrf2, was observed at the protein level. In order to investigate molecular mechanisms, a reporter assay and an oligo pull down assay were performed. The increase of binding to the ARE (Antioxidant Response Elements), on the promoter of the target genes, of Nrf2 was confirmed to induce the expression of target genes. To investigate which kinases are involved in the Nrf2 activation under each compound, various kinase inhibitors were used. Tussilagonone activated MEK and ERK1/2, and igalan inactivates GSK3β while also activating AKT by phosphorylation.

In addition to detoxification, when strong reactive oxygen species was induced by *t*-BHP, ROS production was suppressed and cell death was reduced by tussilagonone. These results suggest

that tussilagonone induces the activity of Nrf2 to detoxifying and antioxidative effects. Igalan has been shown to decrease the activity of NF-κB, a major marker of immune function, in addition to detoxify. Under exposure to TNFα, which induced an immune response, the inflammatory proteins of cells exposed to igalan was significantly lower than that of the control. ROS, xenobiotics, and immune responses are known to play a role in the development of cancer in the initial stage of normal cell transformation into initiated cells. Tussilagonone and igalan inhibit cancer development by suppressing carcinogenic activities.

Next, the inhibitory effects of chikusetsusaponin IVa methyl ester (CME) on cell growth was evaluated by MTT assay. G0/G1 arrest was induced by this compound in colorectal cancer cell lines as a result of FACS analysis. In order to investigate molecular mechanisms to induce cell cycle arrest by CME, western blot analysis and other molecular approaches were performed. It is showed that Cyclin D1 expression, which is a representative target genes of Wnt/β-catenin signaling, was markedly decreased and reporter assays were performed using TOP flash. As a result, it was confirmed that the activity of β-catenin is inhibited. The inhibition of β-catenin nuclear translocation was confirmed by oligo pull down assay and protein expression in the nucleus. Cyclin D1, c-Myc and other CDK proteins, which promote cell division, are also decreased. It was confirmed that CME could inhibit the progression of cancer by inhibiting the nuclear translocation of β-catenin to promote cell proliferation.

Taken together, it has been identified that novel functions of tussilagonone, igalan, and chikusetsusaponin IVa methyl ester, all of which are from natural products and have been shown to inhibit cancer development and progression. It means that those natural occurring compounds have chemopreventive activities and can be used to inhibit cancer development and progression.

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keywords : Tussilagonone, *Tussilago farfara* L., Igalan, *Inula helenium* L., Chikusetsusaponin IVa methyl ester, *Achyranthes japonica*, Nrf2, NF-κB, β-catenin, anti-cancer, ERK1/2, GSK3β, Cyclin D1

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I. INTRODUCTION

Cancer is a major public health problem. In fact, it is the second leading cause of mortality worldwide. In 2014, cancer was the leading cause of death in South Korea [1]. According to studies, more than 200,000 new cancer patients were diagnosed that year, and more than 70,000 people died from cancer [2]. Increased incidence of cancer is seen as a result of an increasingly aging population and the adoption of Westernized lifestyles [3]. And so, cancer chemoprevention that can inhibit the development and progression of cancer may play an important role in solving this problem.

1. Cancer chemoprevention

This term “cancer chemoprevention” was first used by Dr. Michael Sporn in the 1970s to explain the effects of retinoic acid, a vitamin A derivative, in inhibiting experimentally induced tumorigenesis [4]. Cancer chemoprevention is intended to inhibit, reverse, or delay the carcinogenic process from the earliest stages to the formation of preneoplastic cells using agents.

1.1. Carcinogenesis

Carcinogenesis consists of multiple steps with cellular alterations. The accumulation of genetic changes are involved in the carcinogenesis that triggers malignancy. There are three main steps in multistage of carcinogenesis (Fig. 1) [5, 6].

1) Initiation: The transformative step from normal cells to cancer cells and a rapid

and irreversible step. The exposure to carcinogenic factors including ROS, inflammation, and distribution can translocate the factors to organs and tissues or damage DNA, and this is one of the causes that initiate carcinogenesis.

2) Promotion: The slower stage of carcinogenesis. The initiated cells undergo cancer promotion steps to transform to preneoplastic cells with the ability to proliferate actively thereafter. This step is reversible.

3) Progression: The final stage of carcinogenesis. This step is characterized by genetic instability along with invasive and metastatic abilities.

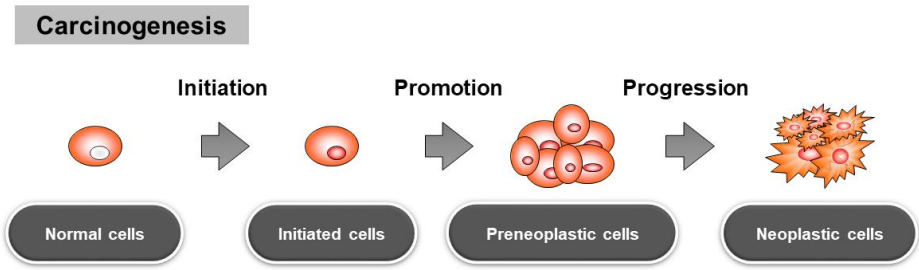


Figure 1. Overview of Carcinogenesis.

The initiation is transformation of normal cells into initiated cells. The initiated cells go through promotion to develop preneoplastic cells (precancerous cells). The preneoplastic cells develop to neoplastic cells with cancer activities including invasive or metastatic ability through progression stage [5, 6].

1.2. Cancer chemopreventive agents

Cancer chemopreventive agents can inhibit or reverse each step in carcinogenesis. Chemopreventive agents are separated into two broad categories: blocking agents and suppressing agents [7].

Blocking agents are used to inhibit cancer initiation. The agents may function by preventing the interactions between endogenous free radicals or chemical carcinogens and DNA. Therefore, the blocking agents reduce damage or mutations that induce cancer initiation and genomic instability leading to neoplastic transformation. Blocking agents may achieve the regulation of carcinogen metabolism through phase I enzyme and/or increasing detoxification and conjugation of activated metabolites mediated by phase II enzymes. Phase I and II enzymes can change rates of DNA repair, remove reactive oxygen, and decrease production of other free radical species involved in the blocking mechanisms [8, 9].

Suppressing agents inhibit the transformation of initiated cells into malignant ones in the promotion and progression stages. They can suppress or delay the progression or development of precancerous cells into cancer cells. Suppressing agents can also reduce the proliferation of initiated cells or recover apoptosis to a normal state in order to repress the abnormal gene expression and by doing so prevent an increase in abnormal cells caused by damage [10]. It is important to inhibit signal transduction pathways, including the NF-κB pathway, to disrupt the promotion and progression stages [11]. Moreover, growth arrest and apoptosis can be an effective chemopreventive activities because cancer comes from an imbalance between

apoptosis and proliferation. It might be a useful way for chemoprevention to induce the apoptosis of damaged cells with inappropriate cell division [12-16]. It has also been reported that interference between energy homeostasis and cancer cell metabolism by effects on signaling pathways including AMPK and mTOR can be a target for chemopreventive agents [17].

1.3. Natural compounds in cancer chemoprevention

Extensive studies have been searched for natural-occurring compounds with chemopreventive activities. The compounds are found in fruits, vegetables, nuts, herbs, and tea. The promising natural compounds with chemopreventive activities are in Fig. 2 and the activities are in Table.1.

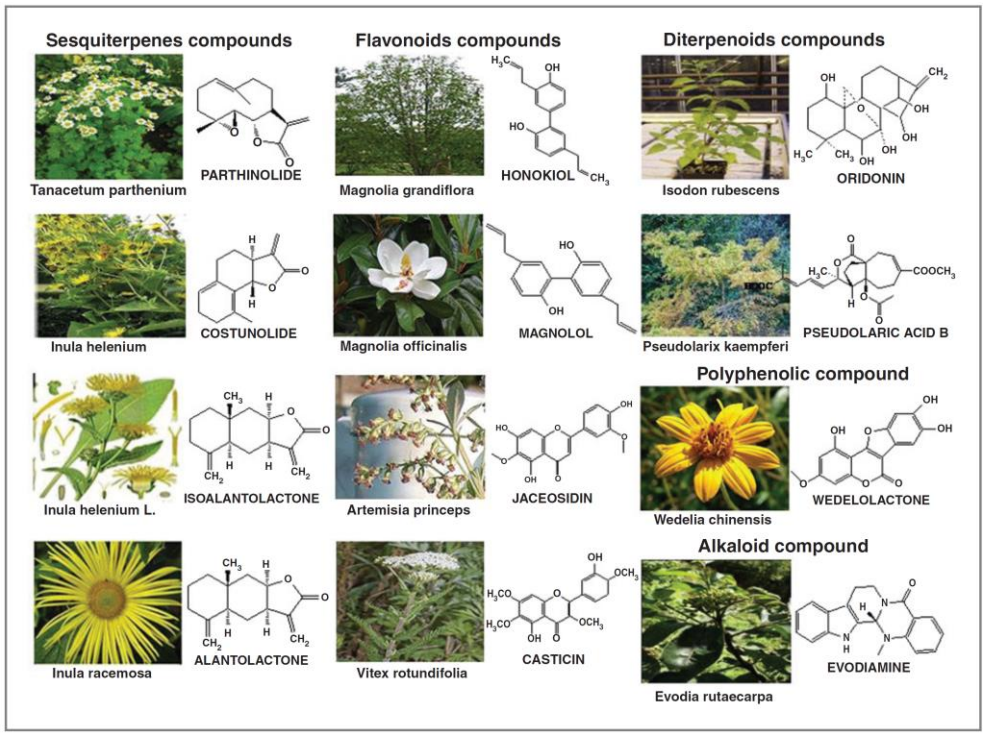


Figure 2. The chemical structure of natural compounds with chemopreventive activities (Adapted from ref. [18]).

Table 1. Natural-occurring compounds with cancer chemopreventive activities (Adapted from ref. [18] with rearrangement).

	Compound	Activity
Flavonoids	Honokiol	Antioxidant, antiproliferation (cell-cycle arrest and apoptosis), anti-inflammation, antiangiogenesis, antiautophagy [19-35]
	Magnolol	Antiproliferation (cell-cycle arrest and apoptosis), anticancer, antioxidant, anti-inflammation, hepatoprotective effects [36-43]
	Jaceosidin	Antioxidant, antiproliferation (cell-cycle arrest and apoptosis), anti-inflammation [44-46]
	Casticin	Antioxidant, antiproliferation (cell-cycle arrest and apoptosis), anti-inflammation [47-51]
Sesquiterpenes	Costunolide	Antioxidant, antiproliferation (cell-cycle arrest and apoptosis), Anti-inflammation, anticancer [52-58]
	Parthenolide	Antioxidant, antiproliferation (cell-cycle arrest and apoptosis), Anti-inflammation, antiangiogenesis [59-74]

	Alantolactone	Anti-inflammation, antiproliferation anticancer, oxidoreductase [75-81]
	Isoalantolactone	Anti-inflammation, anticancer, oxidoreductase, antiproliferation [82, 83]
Diterpenoids	Oridonin	Anti-oxidant, antiproliferation (cell-cycle arrest and apoptosis), Anti-inflammation [84-94]
	Pseudolaric Acid B	Antioxidant, antiproliferation (cell-cycle arrest and apoptosis), anticancer, anti-inflammation, antiangiogenesis [95-98]
Polyphenolic	Wedelolactone	Antioxidant, antiproliferation (cell-cycle arrest and apoptosis), anti-inflammation, hepatoprotective effects [99-101]
Alkaloids	Evodiamine	Antioxidant, antiproliferation (cell-cycle arrest and apoptosis), Anti-inflammation, anticancer, anticarcinogenesis [102-109]

2. Targets for chemopreventive agents

The well-known signaling for chemopreventive agents are regulation of nuclear factor-kappa B (NF-κB) activity [26, 34, 48, 52, 64, 66, 75, 95, 96, 110-118], nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [86, 119-128] and β-catenin signaling[129-131]. The activities or regulations by these chemopreventive phytochemicals influence on carcinogen detoxification, DNA repair, cell proliferation, cell-cycle regulation, apoptosis and metastasis. Moreover, many epidemiological studies suggest that phytochemicals may attenuate the incidence of variety of cancers [132-134].

2.1. Nuclear factor erythroid 2-related factor 2 (Nrf2)

It is an important approach to reduce DNA damage by carcinogens in the initiation stage of carcinogenesis. The phase II enzyme has an effect on cellular stresses including electrophilic and oxidative stress.

2.1.1. Nrf2 pathway

The phase II enzymes play roles in detoxification of toxic xenobiotic chemicals and anti-oxidative effects by removal of electrophilic and oxidative toxicants [135, 136]. Nrf2 is ubiquitously expressed and particularly active in metabolic and detoxifying organs, including the liver [137]. As such, Nrf2 activation has been extensively studied as a therapeutic target for the prevention and treatment of liver diseases [138]. In fact, previous research supports that Nrf2 pathway activation can mitigate the

development of hepatitis, alcoholic or non-alcoholic liver diseases, fibrosis, and liver cancer [138, 139]. Thus, both natural and synthetic molecules regulating Nrf2 activity have been evaluated as drug candidates for such liver diseases.

Cells have developed various antioxidant defense mechanisms to detoxify reactive products and limit oxidative stress [140]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is known to be a basic leucine zipper family and redox-sensitive transcription factor regulating antioxidant response element (ARE)-induced phase II detoxifying and antioxidant enzymes [141, 142]. Under exposed to oxidants or electrophiles, Nrf2 increases the transcription of antioxidants and detoxification genes including glutamyl cysteine ligase-modulator (GCLM), glutathione-S-reductase (GSR), glutamyl cysteine ligase-catalytic (GCLC), NAD(P)H:quinone oxidoreductase 1 (NQO1), and heme oxygenase-1 (HO-1) [143-145]. Under basal level, Nrf2 is in the cytoplasm by binding with Kelch-like ECH-associated protein 1 (Keap1) [146]. Keap1 as a negative regulator leads Cul3/Rbx1 E3 ubiquitin ligase complex mediated ubiquitination and degradation of Nrf2 [147]. Under exposed to oxidative or electrophilic stress, Nrf2 is disassociated with Keap1 [147]. The division between Nrf2 and Keap1 has been explained by some mechanisms including the modification of cysteine residue in Keap1 and phosphorylation of Nrf2 [147, 148].

Various protein kinases have been involved in release of Nrf2 from Keap1. Thus Nrf2 moves into nucleus to bind to the ARE and controls gene expression of a variety of antioxidant and detoxifying enzymes. Previous studies have reported that extracellular signal-regulated kinase (ERK1/2) [149], protein kinase C (PKC) [150], phosphoinositide 3-kinase (PI3K) and 5'AMP-activated protein kinase (AMPK)

[151] are involved in the activation of Nrf2. While, another kinase can play a role in the nuclear export of Nrf2. Activation of GSK3β by phosphorylation of tyrosine 216 residue or de-phosphorylation of serine 9 leads to phosphorylate Fyn at threonine residue causing nuclear localization of Fyn [152, 153]. Fyn phosphorylates Nrf2 at tyrosine 568 residue leading to export of Nrf2 from nucleus, ubiquitination and degradation of it [154]. The mechanism of activation of Nrf2 pathway is shown in Fig. 3.

2.1.2. Nrf2 and Cancer

Although moderate ROS levels are needed for promoting cell proliferation and differentiation, a high level of ROS induces irreversible oxidative damages [155, 156]. However, Phase II antioxidant and detoxification enzymes play an important role as defense mechanisms against oxidative stress, and these genes are regulated by Nrf2 binding to ARE in their promoter [157]. Nrf2 has been known as its cytoprotective function and a main regulator of cell survival [158]. Consumption of dietary phytochemicals promote antioxidant effects by activation of the expression of antioxidants and detoxification enzymes through the Nrf2 signaling pathway [125, 126]. The protective role of Nrf2 in the prevention of cancer development has been widely studied in multiple organs [121, 122, 159]. There is abundant evidence describing the effects of Nrf2 in cancer chemoprevention [121]. It is more sensitive to carcinogenesis in Nrf2-deficient mice, and the activation of Nrf2 suppresses carcinogenesis [160-162].

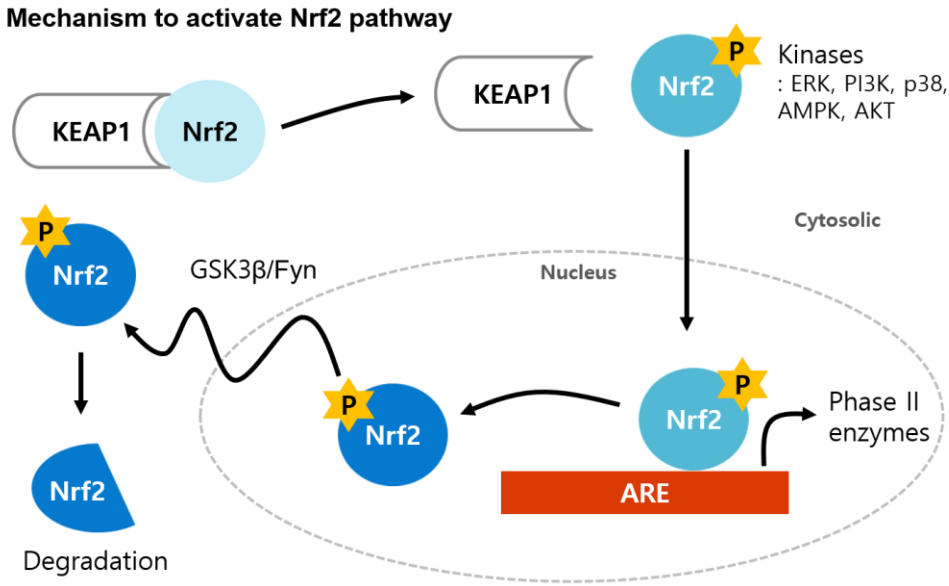


Figure 3. Mechanism to activate Nrf2 pathway.

Under oxidative stress, Nrf2 is released from KEAP1 through phosphorylation of it by various kinases. Nrf2 translocates into the nucleus and binds to ARE on promoter of target genes. The binding between Nrf2 and ARE induces transcription of target genes including NQO1 and HO-1. In other wise, GSK3β and Fyn kinase are involved in the degradation of Nrf2 by driving it out of nucleus [136].

2.2. Nuclear factor-kappa B (NF-κB)

2.2.1. NF-κB pathway

As a transcription factor, the NF-κB plays an important role in the regulation of biological processes including immunological functions [115, 163]. NF-κB controls a variety of cellular functions such as survival, stress-mediated responses, apoptosis, and the progression of chronic diseases [164-166]. It has been argued that chronic inflammation is related to the initiation and promotion of cancer. The increase of local expression of cytokines and growth factors lead the survival pathways in cancer cells. Thus, aberrant activation of NF-kB is involved in cancer initiation and progression [113].

NF-κB is stimulated by various inflammatory signals including lipopolysaccharide (LPS), interleukin-1 (IL-1) and tumor necrosis factor α (TNFα). TNFα induces two different signaling pathways leading to apoptosis or proliferation by binding to its receptors (TNFR1/TNFR2) [167]. Binding between ligand such as TNFα and its receptors leads to activate IKK, resulting in IκBα phosphorylation, and then following IκB degradation. NF-κB moves into the nucleus to induce downstream genes. Cytokines, chemokines, and cell adhesion molecules are among the key factors of the immune response and main targets for NF-κB [116]. Regulators of apoptosis, such as the anti-apoptotic family and cell-cycle related proteins, are also target genes having effects on cell proliferation and survival [168-170]. The mechanism of activation of the NF-κB pathway and its target gene are shown in Fig.

4 and 5.

2.2.2. NF-κB pathway and cancer

Genomic instability and genetic mutations leading to cancer initiation and development can be caused by sustained chronic inflammation [171]. Inflammatory factors generated by immune cells are strongly related to inflammation and cancer. Among those factors, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 are well-known cytokines correlated with inflammation and cancer development, and the factors are involved in the progression of cancer [172, 173]. These factors are mainly regulated by the NF-κB pathway leading to induction of COX2 expression and ROS production [174, 175]. In the initiation stage, an increase in the rates of DNA mutation and mutated cells and an inactivation of DNA repair and induction of the growth factors and cytokines activated by NF-κB are caused by the inflammatory microenvironment [176-178]. Chronic inflammation also induces the progression of cancer. Tumor-promoting cytokines such as IL-1β, TNF-α, and IL-6 secreted by inflammatory cells are needed for carcinogenesis [172, 173]. A high level of inflammatory cytokines and macrophage infiltration stimulate angiogenesis [179]. Likewise, chronic inflammation is involved in cancer development and metastasis [180].

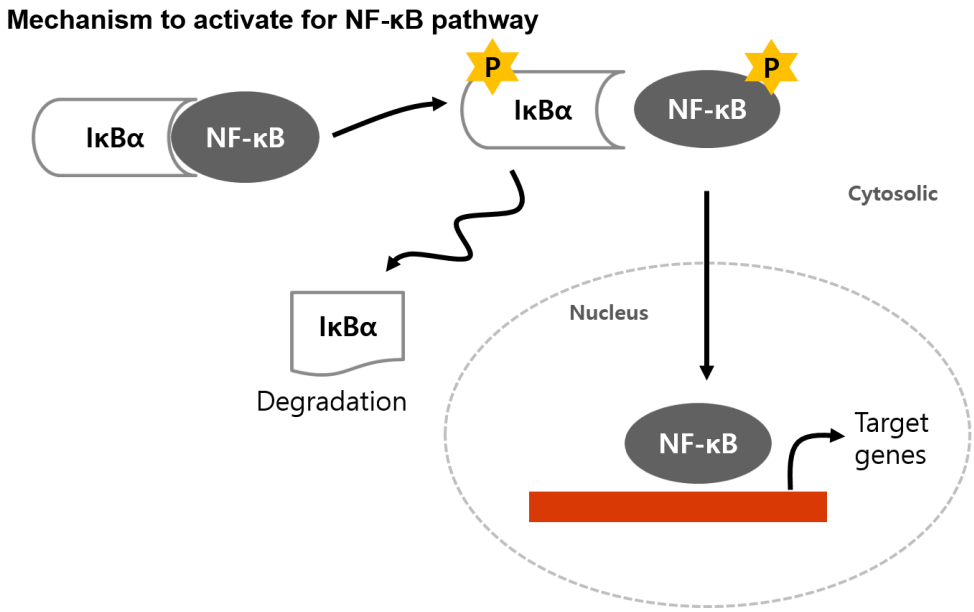


Figure 4. Mechanism to activate NF-κB pathway.

Under inflammatory signal, IκBα is phosphorylated by upstream kinase, thereby disassociates with NF-κB to degradation. Released NF-κB moves into nucleus to bind NF-κB binding site. The target genes mediated by NF-κB is transcriptionally activated [116].

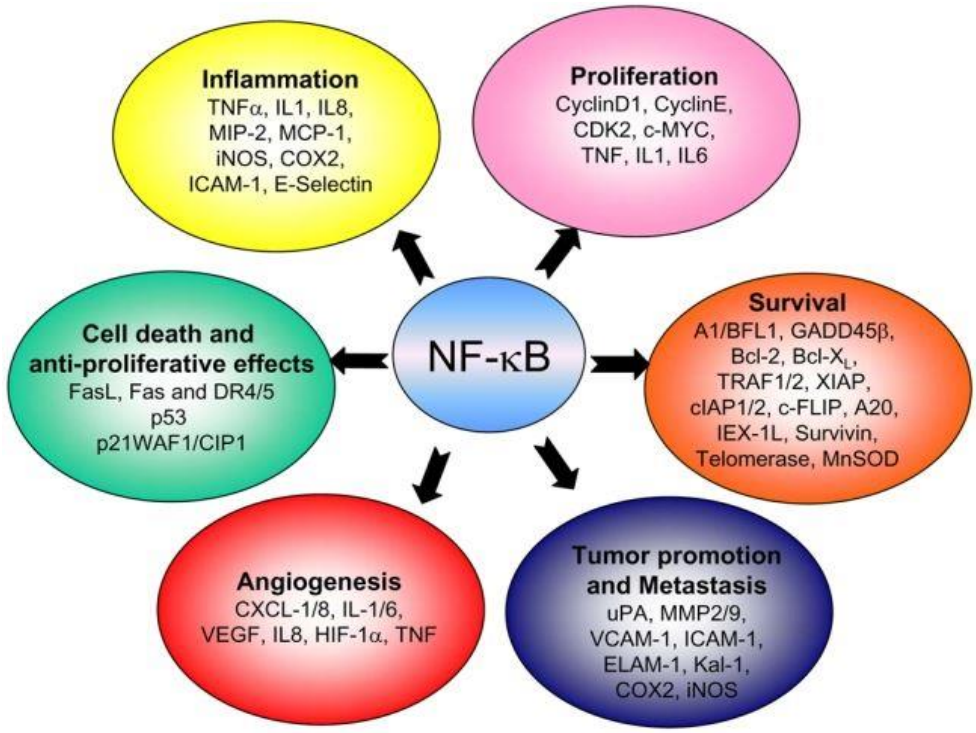


Figure 5. Target genes of NF-κB pathway.

NF-κB target genes have effects on the cancer development and progression by increasing expression of genes involved in cell proliferation, tumour promotion, and survival (Adapted from ref. [112]).

2.3. WNT/ β-catenin

2.3.1. WNT/ β-catenin pathway

Wnt signalling is an important molecular cascade. The signalling is known to control cell fate. During embryonic development, Wnt/β-catenin signalling plays a role in the development of the body axis, tissues, and organs. In adult organs, the signalling is involved in tissue homeostasis, cell renewal, and regeneration. When Wnt ligands interact with receptors, intracellular signalling cascades are triggered by receptor complexes. β-catenin plays a crucial role in signal transduction, with nuclear translocation of it in canonical pathways. β-catenin induces the expression of genes which regulate cell fate in cells and tissues [181]. As a result of the mutation of the Wnt/β-catenin signalling, β-catenin has been known to be associated with human cancers including colon cancer [182, 183].

A destruction complex including adenomatous polyposis coli (APC), axis inhibitor (AXIN), and glycogen synthase kinase-3-β (GSK3β) phosphorylates cytosiloc β-catenin leading to degradation by the ubiquitin-proteasome system [184, 185]. When the Wnt ligand binds to receptors, GSK3β becomes inactivated by phosphorylation of ser 9, and β-catenin can be stabilized in the cytoplasm [152]. The stabilization of β-catenin is also caused in relation to APC and axin [186, 187]. The mechanism for the Wnt/β-catenin pathway is shown in Fig. 6.

This stabilized β-catenin moves into the nucleus and interacts with T-cell factor/lymphoid enhancer factor (LEF/TCF) in the promoter of target genes, then the

expression of target genes is activated [183, 188]. The target genes, including c-Myc, CyclinD1, MMP7, and Keratin1 are involved in the regulation of cell-cycle, cell-adhesion, and cellular development [189, 190]. According to recent reports, it is characterized that the TCF/LEF-binding elements (TBE) is in the COX-2 promoter and β-catenin upregulates COX2 gene expression [191].

2.3.2. WNT/ β-catenin pathway and Cancer

Wnt/β-catenin signaling regulates development and stemness. However, the abnormal activity of the Wnt/β-catenin pathway is involved in cancer development. Among cancers, colorectal cancer is well-studied in regard to the Wnt/β-catenin pathway in carcinogenesis. Aberrant activity of the Wnt/β-catenin pathway is mainly caused by a loss of APC in colorectal cancer [192]. In addition, the canonical pathway is linked to triple negative breast cancer development [193-195]. Somatic mutations of pathway regulators including β-catenin are only in a small fraction of tumors [193], whereas Wnt ligands and receptors of the canonical pathway are often overexpressed in tumors [196, 197]. Recent studies also investigated the links between the Wnt/β-catenin pathway and cancer stem cells with the determination that the level of the Wnt/β-catenin pathway controls normal hematopoietic stem cells for long-term maintenance. However, it has been shown that Wnt activity is constitutively enhanced in most leukemia [198]. Although the Wnt/β-catenin pathway plays an important role in development, it can also be one of causes of cancer development.

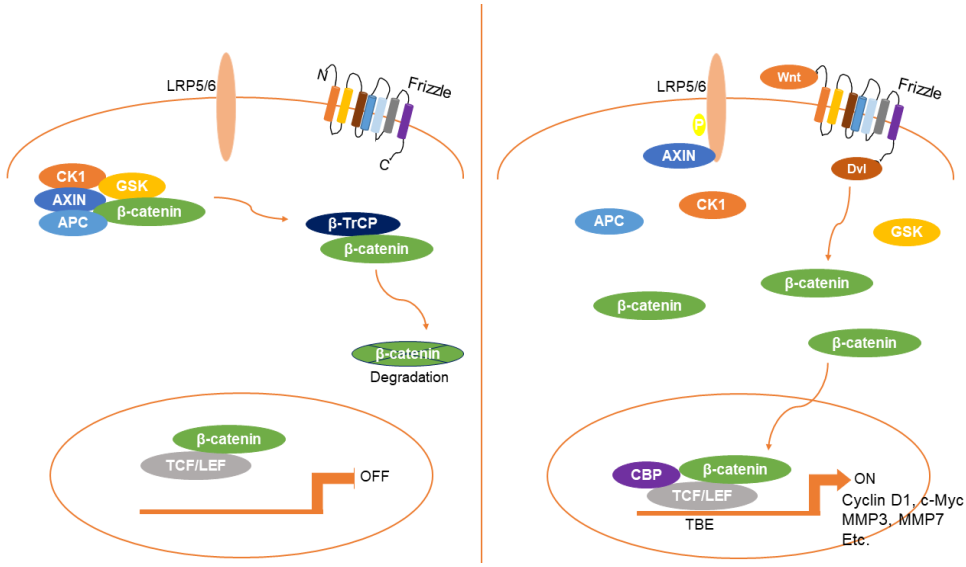


Figure 6. Wnt/β-catenin signaling pathway.

In the absence of Wnt ligand, APC-Axin1-GSK3β phosphorylates β-catenin in cytoplasm. The phosphorylated form of β-catenin leads to degradation of it. In the presence of Wnt ligand, it causes the phosphorylation of GSK3β at ser 9 to inactivate it. The inactivation of GSK3β leads to release β-catenin from APC-Axin1-GSK3β complex. The β-catenin translocates to nucleus to activate the expression of target genes [199].

3. *Tussilago farfara* L.

Tussilago farfara L.belonging to the asteraceae family—known as coltsfoot—is a plant found largely throughout Korea, China, North Africa, Siberia, and Europe. It has been used for traditional medicine. The flower buds of *T. farfara* are used to treat cough, bronchitis, and asthma [200, 201]. Moreover, pharmacological studies demonstrate that compounds isolated from *T. farfara* exhibit anti-inflammatory, anti-oxidative, and neuroprotective effects [202-204]. In particular, tussilagonone as one of sesquiterpenoies exerts its anti-inflammatory activity via the Nrf2/HO-1 pathway activation in RAW264.7 cells [205]. The chemical structure of tussilagonone is shown in Fig. 7.

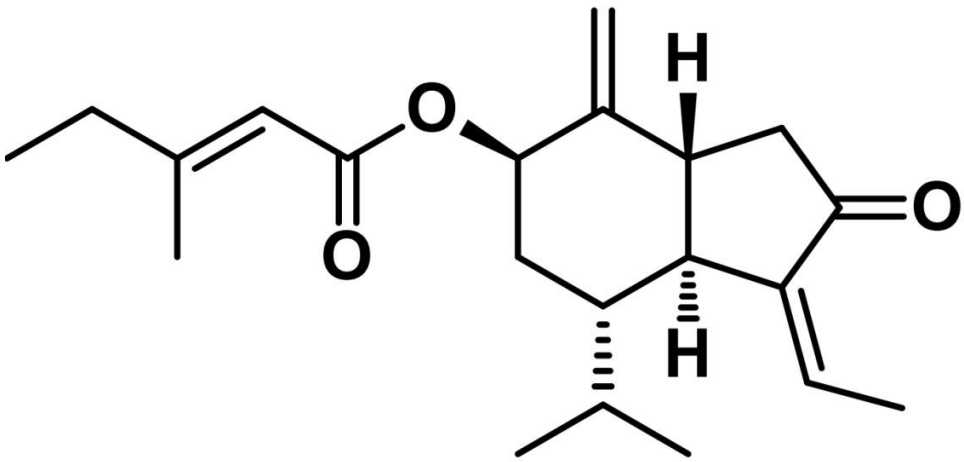


Figure 7. The chemical structure of tussilagonone.

4. *Inula helenium. L.*

Inula helenium. L as a perennial plant is widely spread in Europe and East Asia. Its roots have been used as traditional medicine for a diaphoresis and a diuretic expectorant agent in Europe and a fragrance agent for home medicines in Japan [206]. In china, it is commonly used as traditional medicines to treat inflammatory diseases including enterogastritis, tuberculotic enterorrhea and bronchitis in China [207-209].

Recently, it is identified that main ingredients of roots are sesquiterpene lactones including alantolactone and isoalantolactone [209]. These factors have various biological activities such as anti-inflammatory, anti-bacterial, and anti-tumor activities [82, 210]. Igalan, one of the sesquiterpene lactones, are also isolated from *Inula helenium*. L and the chemical structure of it is shown in Fig. 8.

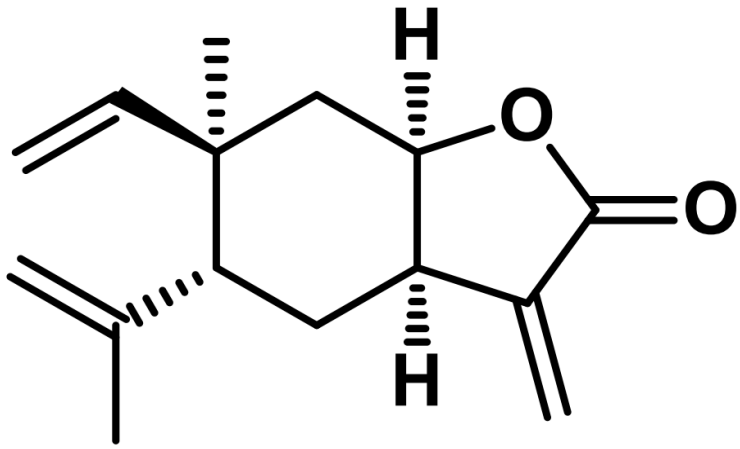


Figure 8. The chemical structure of igalan.

5. Achyranthes japonica.

Achyranthes japonica Nakai is a perennial herb and widely spread throughout in East Asia including Korea and Japan. The plant is mainly used as a tranditional medicine [211]. The roots of *Achyranthes japonica* N. is commonly called “Soe-moo-reup” in Korean and the name comes from their shape. *Achyranthes japonica* N. has been known to effect on edema, arthritis, and delayed menses [212]. According to previous studies, it is known that the roots of *Achyranthes japonica* N. have various pharmacological effects, including anti-inflammatory and antioxidative effects [111, 213]. Moreover, it has been reported that *Achyranthes japonica* N. inhibits platelet aggregation [214] and has anti-fungal activity [215].

Chikusetsusaponin IVa methyl ester (CME), a triterpenoid saponin, is from the root of *Achyranthes japonica* N. and the chemical structure of CME is shown in Fig. 9. Besides, the compounds such as Chikusetsusaponin IVa, ecdysterone, inokosterone, and oleanolic acid have also been isolated from this plant [111].

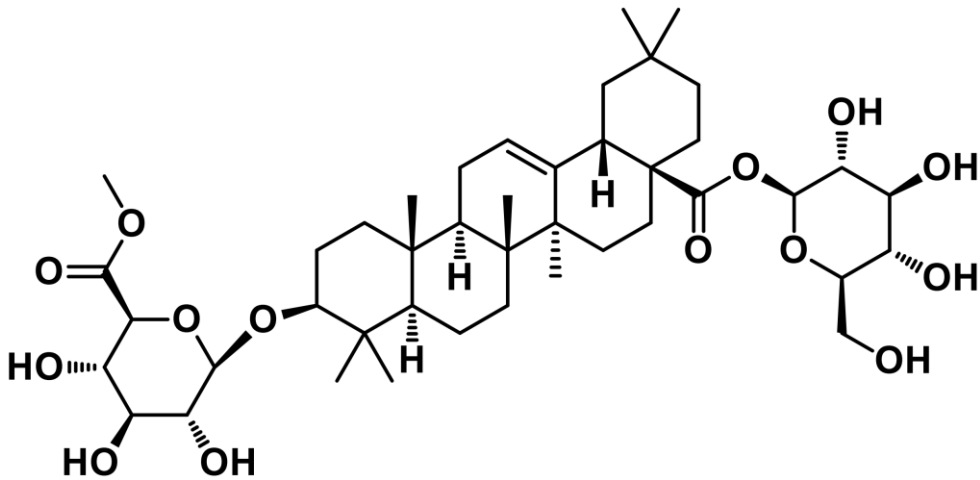


Figure 9. The chemical structure of Chikusetsusaponin IVa methyl ester.

II. STATE OF THE PROBLEM

In this study, the molecular mechanisms underlying detoxification, anti-oxidants, and anti-proliferation of tussilagonone, a sesquiterpenoid from *Tussilago farfara* L., igalan, a sesquiterpene lactone from *Inula helenium* and chikusetsusaponin IVa methyl ester isolated from *Achyranthes japonica* were investigated in cells with molecular approaches.

Cancer is one of the most life-threatening issues worldwide [136]. Thus, sustaining healthy lifestyles is in the spotlight. To that end, cancer chemoprevention can be a valuable research project. Chemopreventive agents are categorized by blocking and suppressing agents. Blocking agents can block DNA damage mediated by carcinogens in normal cells, and suppressing agents can inhibit, reverse, or retard the promotion and progression stage of carcinogenesis.

As a carcinogenic factor, it is known to produce reactive oxygen species (ROS). The cellular redox system is necessary for reactive oxygen species (ROS) homeostasis under normal conditions; however, certain pathological conditions such as chronic inflammatory disorders result in excessive ROS accumulation and abnormal cellular function [216, 217]. This imbalance between the formation and removal of ROS disrupts cellular homeostasis [218] and is responsible for the pathophysiology of various diseases including cancer. To regulate this ROS which induce disorders, Nrf2 does function as a master regulator of anti-oxidation and detoxification. The regulation of the Nrf2 pathway is a well-known method to induce chemopreventive effects by decreasing carcinogenic factors.

Another factor causing the formation of cancer is NF-κB which regulates

inflammatory signaling. Abnormal activity of NF-κB leads to cell proliferation in malignant cells because the target genes of NF-κB are associated with cell proliferation and the inhibition of apoptosis [219-221]. Chemopreventive agents have been targeted for NF-κB to inhibit constitutive activation in malignant cells [136].

In addition, colorectal cancer is caused by multiple transition steps, including the accumulation of genetic errors [222, 223]. Previous studies discovered several critical genes and pathways that regulate the progression of colorectal cancers [224]. Among those mutated genes, genetic mutations in the Wnt signaling pathway increase the progression of colorectal cancer through the up-regulation of β-catenin transcriptional activity [225-227]. The Wnt/ β-catenin signaling pathway affects cellular developmental processes and human carcinogenesis through the activation of genes associated with cell proliferation [186, 228-231]. Thus, inhibition of the Wnt/ β-catenin signaling pathway can be an important candidate for drug targeting for the colorectal cancers. Inhibition of the Wnt/β-catenin signaling pathway may be a useful way for chemoprevention particularly in colorectal cancer. And among natural compounds, saponins are well known to have an anticancer activity [17-19]. However, it is not well known that saponins may serve as an inhibitor for Wnt/β-catenin signaling.

Thus, three compounds that can be candidates for chemoprevention were selected by screening. As blocking agents that regulate Nrf2 pathway, tussilagonone and igalan were selected. Using HepG2 cells, it is characterized by having

chemopreventive potential in the initiation stage of carcinogenesis. Igalan was identified by not only a blocking agent but also a suppressing agent with an inhibitory effect on NF-κB activity. As a suppressing agent, chikusetsusaponin IVa methyl ester is also characterized by the inhibition of β-catenin activity in reducing cell proliferation and the induction of apoptosis in the promotion and progression stage of carcinogenesis.

III. RESULTS

1. Screening for cancer chemopreventive effects

1.1. Screening for blocking agents

Initially, over 400 compounds were used for screening. Before performing experiments for screening, common compounds with anti-oxidative, detoxifying, or activating effects on Nrf2 pathways was excluded. In the screening system, *t*-BHP was used as a ROS inducer. The cells were treated with various compounds before treatment with *t*-BHP. And after treatment with *t*-BHP, the cells were analyzed by an MTT assay. The results are shown in Table 2. Primarily, compounds with the recovery activity of cell viability reduced by *t*-BHP were selected for the next screening. Through this assay, the protective effects against oxidative stress were examined.

Next, a QR assay was used for quinone reductase activity. The chemopreventive index was also calculated using the value of CD and IC_{50}. Compounds with a CD value of 2 or more were selected. The results are shown in Table 3.

A detailed results of the screening is in the appendix.

Table 2. The evaluation of protective effects.

Before treatment with *t*-BHP (500μM), HepG2 cells were treated with various compounds for 24hr. Cell viability was analyzed by MTT assay.

	Cell Viability (%)		
Compound	Control	*t*-BHP	10 or 12.5μM
Ginsenoside Rg1	100 (±3.18)	30.39 (±0.44)	30.95 (±0.50)
Ginsenoside Rd	100 (±3.18)	30.39 (±0.44)	28.16 (±1.67)
Ginsenoside Rb1	100 (±3.18)	30.39 (±0.44)	36.33 (±2.97)
Ginsenoside Rb2	100 (±3.18)	30.39 (±0.44)	56.32 (±0.95)
Ginsenoside F4	100 (±3.18)	30.39 (±0.44)	52.23 (±4.41)
Ginsenoside Rg5	100 (±3.18)	30.39 (±0.44)	35.78 (±0.91)
Ginsenoside Rh4	100 (±3.18)	30.39 (±0.44)	45.17 (±2.14)
Ginsenoside Rg3	100 (±3.18)	30.39 (±0.44)	73.79 (±1.53)
α-Cyperon	100 (±3.18)	30.39 (±0.44)	85.04 (±4.94)
Nodakenin	100 (±3.18)	30.39 (±0.44)	39.77 (±1.35)
Honokiol	100 (±3.18)	30.39 (±0.44)	76.77 (±0.91)
Spicatoside A	100 (±3.18)	30.39 (±0.44)	72.58 (±1.85)
Igalan	100 (±1.24)	22.70 (±3.13)	45.81 (±1.48)
Compound #7	100 (±3.18)	30.39 (±0.44)	64.87 (±2.31)
Lirioresinol B dimethyl ether	100 (±3.18)	30.39 (±0.44)	45.82 (±2.16)
Puerarin	100 (±3.18)	30.39 (±0.44)	46.10 (±2.95)
Tussilagonone	100 (±3.88)	46.72 (±0.47)	70.86 (±0.97)
Vanillin	100 (±3.18)	30.39 (±0.44)	53.25 (±1.39)
(-)-Maackianin	100 (±3.18)	30.39 (±0.44)	75.10 (±1.84)
Byakangelicol	100 (±3.18)	30.39 (±0.44)	39.68 (±0.85)
Coptisine	100 (±3.18)	30.39 (±0.44)	26.77 (±3.75)
Chrysin	100 (±3.18)	30.39 (±0.44)	28.72 (±0.46)
Sulforaphane	100 (±3.18)	30.39 (±0.44)	109.48 (±0.46)
(Positive Control)			

Table 3. The evaluation of QR activity.

The calculation of chemopreventive index by analyzing the result of QR assay.

Compound	CD	IC_{50}	CI
Chikusetsaponin Iva methyl ester	>200	>200	<0.1
Ginsenoside F4	>200	>200	<0.1
Ginsenoside Rb1	>200	>200	<0.1
Ginsenoside Rb2	>200	>200	<0.1
Ginsenoside Rd	>200	>200	<0.1
Ginsenoside Rg1	>200	168.364	<0.1
Ginsenoside Rg3	>200	>200	<0.1
Ginsenoside Rg5	>200	>200	0.836
Icariin	>200	>200	<0.1
Puerarin	>200	65.398	<0.1
Coumarin	>200	>200	<0.1
Bisdemethoxycurcumin	73.206	171.345	2.341
Gallic acid methyl ester	>200	>200	0.653
Kaurenoic acid	98.842	>200	2.023
Nootkatone	>200	>200	0.686
α-Cyperone	130.802	>200	1.529
Honokiol	117.493	>200	1.702
Igalan	2.597	10.542	4.059
Tussilagonone	1.841	25	13.58
Sulforaphane (Positive Control)	0.423	10.715	25.348

The value of IC_{50} means the concentration of 50 % cell viability; CD means the concentration for 2 fold compared to control; CI means the chemopreventive index by calculated with IC_{50} and CD values.

1.2. Screening for suppressing agents

In this screening, over 400 compounds were used. Before the screening began, the most common compounds with cell cycle arrest and apoptosis was excluded. As a screening system, the MTT assay was used. The cells were treated with various compounds, and the value of IC_{50} was evaluated. The results are shown in Table. 4. Compounds with the value of IC_{50} below 50μM were selected for the second screening experiment, then selected compounds were used for an FACS analysis to measure cell cycle arrest or apoptosis and observation of morphology (Table. 5). Detailed results of the screening are in the appendix. Through those assays, compounds with the inhibitory effects of proliferation were selected.

Table 4. The measurement of cell viability.

Compound	IC_{50} (μM)
2-Methoxycinnamaldehyde	34.4
α-Hederin	14.7
Chikusetsusaponin Iva methyl ester	24.19
Spicatoside A	21.9
Ginsenoside Rg3	92.09
Ginsenoside Rh4	101.71
Chrysin	50.39
Licoricidin	31.82
Luteolin	23.21
Honokiol	29.03
Berberine	31.78
Coptisine	91.57
Palmatine	108.42
Aurapten	47.79
Byakangelicol	108.26
Demethylsuberosin	73.60
Oxypeucedanin	149.7
Demethoxycurcumin	22.31
Gallic acid methyl ester	183.04
Kaurenoic acid	95.62
α-cyperone	125.88
Schisandrin	29.84
Schisandrin C	46.04
Schisandrol A	40.38
Schisandrol B	>200

Ent-Pimara-8(14),15-diene-19-oic acid	96.46
Anomalin	19.18
Lirioresinol B dimethyl ether	10.63
(-)-Maackianin	51.67

The value of IC_{50} means the concentration of 50 % cell viability.

Table 5. The results of FACS analysis and observation of cell morphology.

Compound	IC_{50} (μM)	Morphology (blebbing & apoptotic body)	subG1 (%)	G0/G1 (%)
2-Methoxycinnamaldehyde	34.4	blebbing, low cell population, No noticeable difference from control	40μM : 2.18	40μM : 63.92
α-Hederin	14.7	blebbing , cell death	20μM : 30.7	20μM : 51.20
Chikusetsusaponin Iva methyl ester	24.2	low cell population, cell death with dose dependency	20μM : 9.32 30μM : 31.80 40μM : 53.41	20μM : 64.53 30μM : 38.80 40μM : 29.54
Spicatoside A	21.9	low cell population, cell death with dose dependency	25μM : 1.47 50μM : 52.24	25μM : 82.24 50μM : 35.38
Licoricidin	31.8	cell death	40μM : 10.88	40μM : 63.28
Schisandrin	29.8	low cell population	50μM : 1.46	50μM : 60.86
Schisandrol A	40.4	low cell population	50μM : 4.23	50μM : 78.18
Anomalin	19.2	low cell population	15μM : 2.97 30μM : 4.71	15μM : 67.98 30μM : 77.60
Lirioresinol B dimethyl ether	10.6	no difference from control	10μM : 2.50 20μM : 9.34	10μM : 56.33 20μM : 59.95

The value of IC_{50} means the concentration of 50 % cell viability; The percentage of Sub G1 means the apoptosis; The value of G0/G1 means the percentage of cells in cell-cycle arrest.

2. Activity of tussilagonone from *Tussilago farfara* L. and igalan from *Inula helenium. L.* in initiation stage of carcinogenesis

2.1. Tussilagonone-induced Nrf2 pathway activation protects HepG2 cells from oxidative injury.

Previously, it was shown that tussilagonone induces the Nrf2 pathway to protect cells from oxidative stress [233]. In this study, the underlying mechanism and the function of tussilagonone-mediated Nrf2 pathway were investigated in HepG2 cells.

2.1.1. Tussilagonone induces specific QR activity.

To examine the activity and Nrf2 pathway effects of tussilagonone in HepG2 cells, MTT assays was used to choose concentration of the compound. As expected, no significant effects in cell viability were observed after 24 h treatment with concentrations up to 5 μM in the previous study [233].

NQO1 is a phase II detoxification enzyme that exhibits QR activity to protect cells from semiquinone radical-induced cytotoxicity [232]. Notably, tussilagonone induced specific QR activity in a dose-dependent manner (Fig. 10) and the value of chemopreventive index (CI) also is significantly increased (Table. 6) suggesting that the compound elicits induction of detoxification enzymes and cellular antioxidant defenses similar to the function of Nrf2.

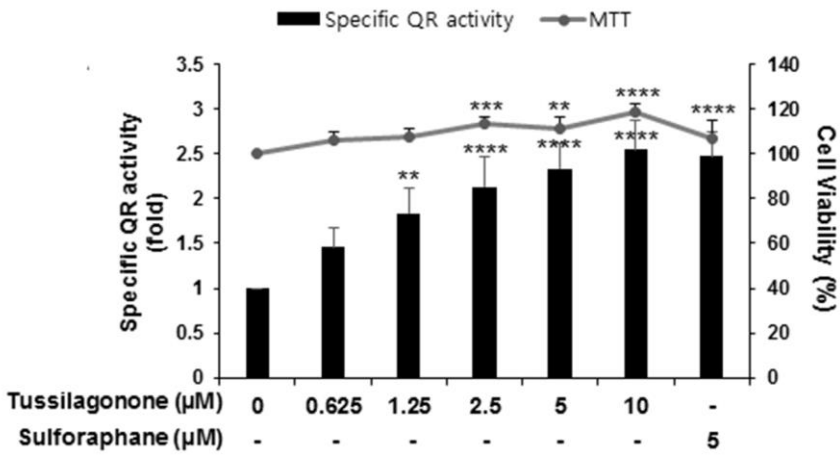


Figure 10. Tussilagonone induces quinone reductase (QR) activity.

Effect of tussilagonone on QR activity in Hepa1c1c7 cells. Cells are treated with the indicated concentrations (0–10 or 20 μM) for 24 h.

Table 6. Tussilagonone increases chemopreventive index.

The calculation of chemopreventive index by analyzing the result of QR assay.

	IC_{50}	CD	CI
Tussilagonone	>20	2.3	8.9
Sulforaphane	>20	1.5	13.4

The value of IC_{50} means the concentration of 50 % cell viability; CD means the concentration for 2 fold compared to control; CI means the chemopreventive index by calculated with IC_{50} and CD values.

2.1.2. Tussilagonone attenuates *t*-BHP-induced ROS production and cell death.

Intracellular ROS accumulation increases susceptibility of cells to oxidative stress-induced cell death. It was shown that the increase in intracellular ROS induced by *t*-BHP (500 μM) was hampered by tussilagonone in a dose-dependent manner and tussilagonone protected cells from *t*-BHP-induced oxidative damage and cell death in a dose-dependent manner to a similar magnitude as that obtained with the known antioxidant sulforaphane in the previous study [233]. To further investigate whether tussilagonone protect cells from *t*-BHP-induced oxidative stress, LDH assay was performed (Fig. 11 A). It was found that tussilagonone attenuated *t*-BHP-induced LDH release and recovered expression of the cell death-associated proteins PARP and caspase 3 (Fig. 11 A, B). Collectively, these data suggested that tussilagonone protects HepG2 cells from ROS-induced oxidative damage and cell death.

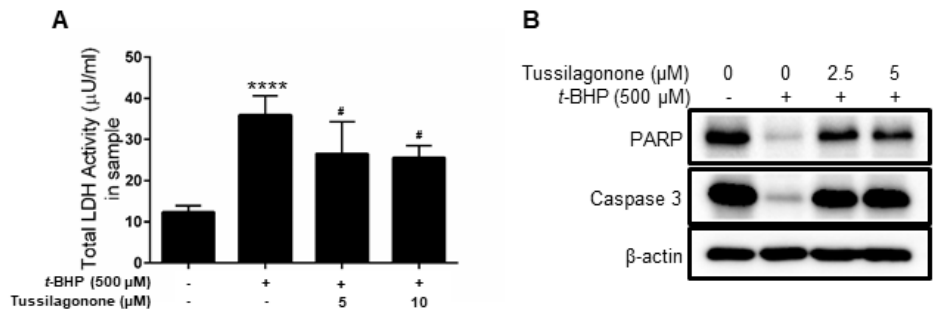


Figure 11. Tussilagonone protects cells from ROS-induced oxidative stress.

(A) Cell membrane permeability was monitored by measuring LDH release from the cytosol of *t*-BHP-damaged cells. (B) Immunoblots for apoptotic proteins, precursor PARP, and pro-caspase 3 in lysates from cells treated with tussilagonone and/or *t*-BHP.

2.1.3. Tussilagonone induces Nrf2 expression and nuclear accumulation.

It was recently reported that tussilagonone activates the Nrf2/HO-1 pathway to exert anti-inflammatory effects [205]. Moreover, it was shown that tussilagonone increases the expression and the nuclear accumulation of Nrf2 in HepG2 cells [233]. To confirm this finding, tussilagonone were treated in HepG2 cells for 6 h. As expected, tussilagonone increased the nuclear Nrf2 expression leading to subsequent target gene transcription (Fig. 12).

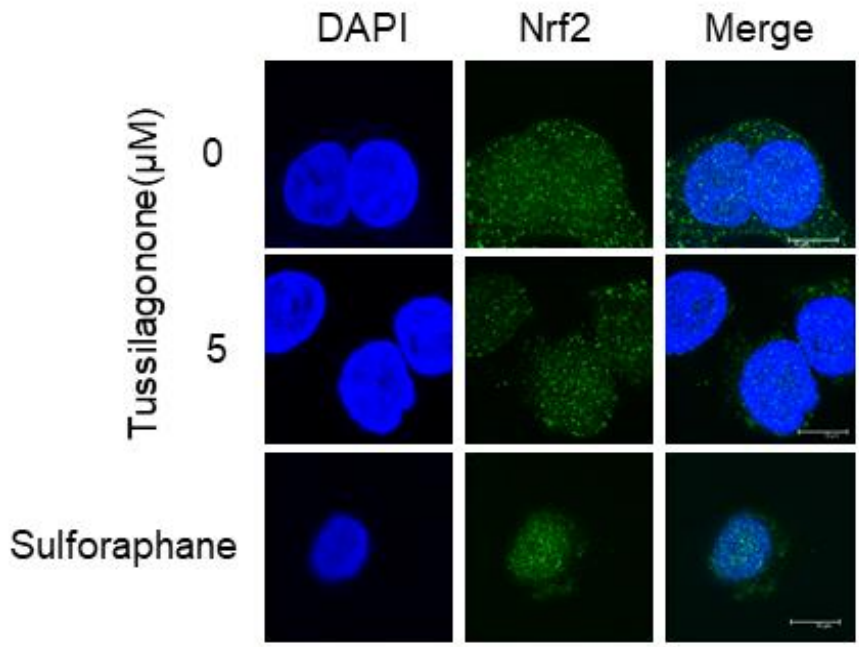


Figure 12. Tussilagonone increases Nrf2 nuclear expression and activation.

Nrf2 nuclear translocation was examined by immunocytochemistry after 6 h tussilagonone treatment. Sulforaphane (10 μM)-treated cells served as a positive control (scale bar, 10 μm).

2.1.4. Tussilagonone upregulates cellular antioxidants via Nrf2 in HepG2 cells.

To confirm that the tussilagonone-induced nuclear translocation of Nrf2 effectively upregulated target gene expression, HO-1 and NQO1 protein expressions were examined. As expected, treated cells showed a marked increase in HO-1 and NQO1 protein levels (Fig. 13A). This result was further validated in cells transfected with Nrf2 siRNA, which demonstrated that tussilagonone-induced HO-1 expression was repressed in Nrf2-knockdown cells (Fig. 13B). Thus, these findings suggest that tussilagonone induces HO-1 expression in an Nrf2-dependent manner to prevent oxidative damage.

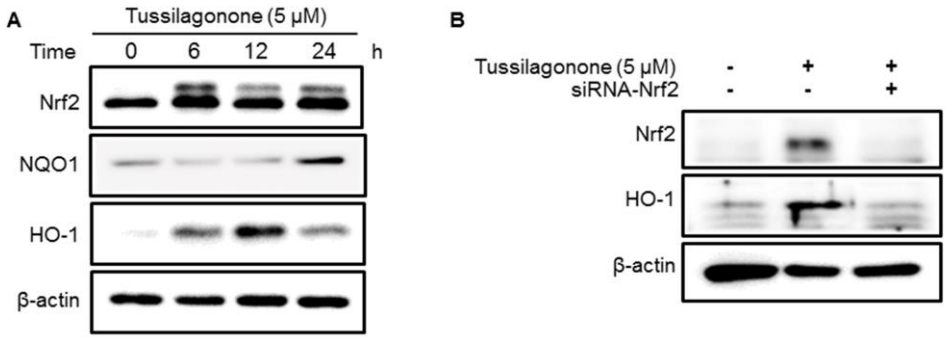


Figure 13. Tussilagonone increases Nrf2 target gene expression.

(A) Time-course expression analysis of Nrf2 and the indicated target genes in HepG2 cells treated with tussilagonone (5 μM). (B) Tussilagonone-induced HO-1 expression analysis in cells transiently transfected with Nrf2-specific siRNA for 24 h.

2.1.5. Tussilagonone increases Nrf2 binding to AREs to promote target gene expression.

It was elucidated the effects of tussilagonone-induced Nrf2 activation on target gene expression. Nrf2 binds AREs in the promoter region of target genes to induce their transcription [234]. Importantly, exposure to 5 μM tussilagonone caused a significant increase in ARE luciferase reporter activity (Fig. 14A). It was subsequently confirmed that tussilagonone increases Nrf2 binding to ARE sequences with oligonucleotide pull down assays (Fig. 14B). Consistently, qRT-PCR analysis showed that tussilagonone increased NQO1 and HO-1 mRNA expression, both of which harbor AREs in their promoter region (Fig. 14C, D).

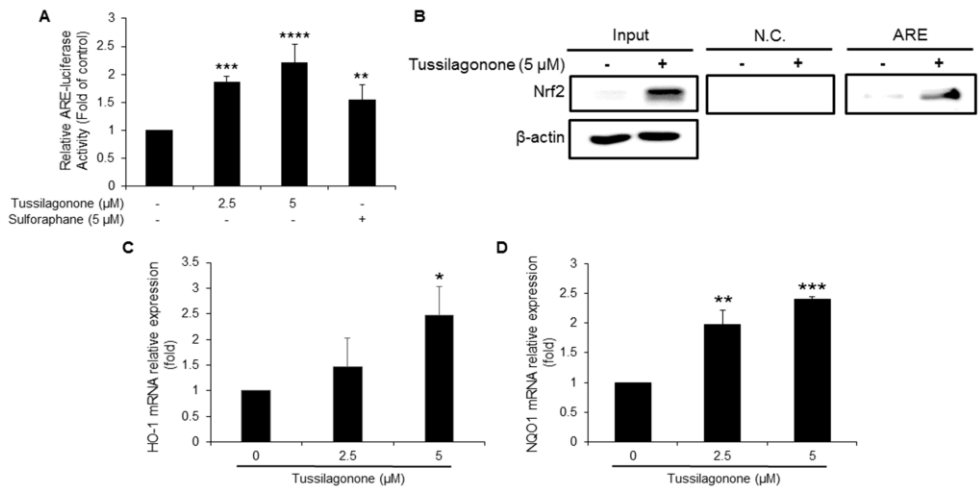


Figure 14. Tussilagonone elicits Nrf2 pathway and antioxidant response element (ARE) binding.

(A) Tussilagonone-induced ARE luciferase reporter activity in HepG2 cells. (B) Nrf2-ARE binding was examined after tussilagonone treatment (5 μM) with oligonucleotide pulldown assays. (C) Expression of the Nrf2 target genes NQO1 and HO-1 following tussilagonone treatment was examined by qRT-PCR.

2.1.6. Nrf2 activation by tussilagonone is mediated by ERK1/2.

Multiple kinases are involved in Nrf2 activation [149, 150, 235-237]. To investigate whether tussilagonone-mediated Nrf2 activation and translocation are regulated by kinase activity, HO-1 expression in the presence or absence of various kinase inhibitors was measured. Notably, western blot analysis revealed that treatment with U0126—an inhibitor of MEK1/2 upstream of ERK1/2—attenuated tussilagonone-induced HO1 expression in HepG2 cells (Fig. 15A), supporting that ERK1/2 is involved in tussilagonone-induced Nrf2 activation. In addition, Nrf2 expression and nuclear accumulation was markedly lower in HepG2 cells co-treated with tussilagonone and U0126 as compared to those treated with tussilagonone alone (Fig. 15B, C). Further, a time-course analysis revealed that tussilagonone also increased Nrf2 protein expression and phosphorylated forms of MEK1/2 and ERK1/2 (Fig. 15D). Collectively, these results suggested that tussilagonone activates MEK1/2 and ERK1/2 to induce Nrf2 nuclear translocation and target gene expression.

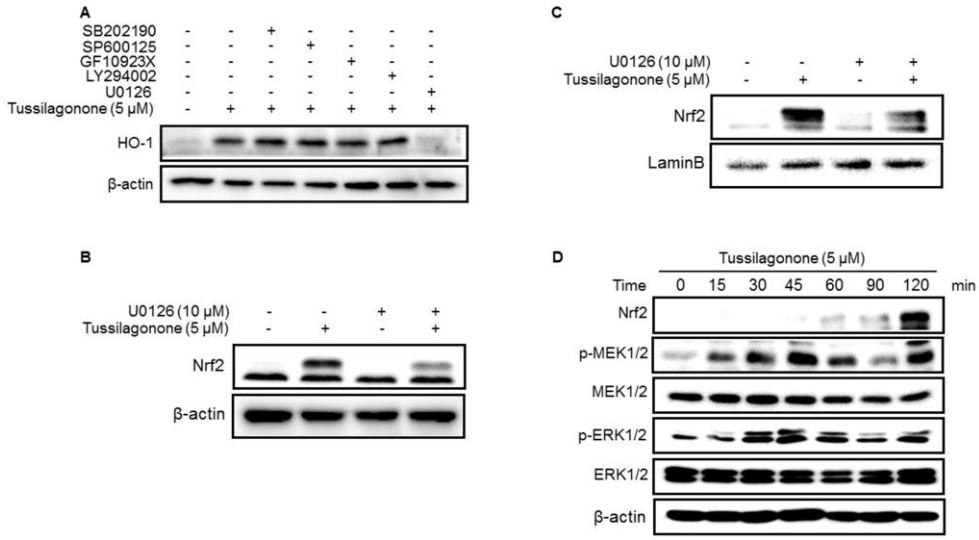


Figure 15. Tussilagonone elicits Nrf2 pathway activity via ERK phosphorylation.

(A) HO-1 expression analysis in HepG2 cells pretreated U0126 (MEK inhibitor), SB202190 (p38 inhibitor), SP600125 (JNK inhibitor), GF10923x (PKC inhibitor), or LY294002 (PI3K inhibitor) for 1 h and then with tussilagonone (5 μM). (B) Nrf2 protein expression and (C) nuclear accumulation following treatment with tussilagonone (5 μM) and/or U0126 for 12h and 6 h. (D) Immunoblot analysis of tussilagonone-induced MEK and ERK phosphorylation and Nrf2 expression.

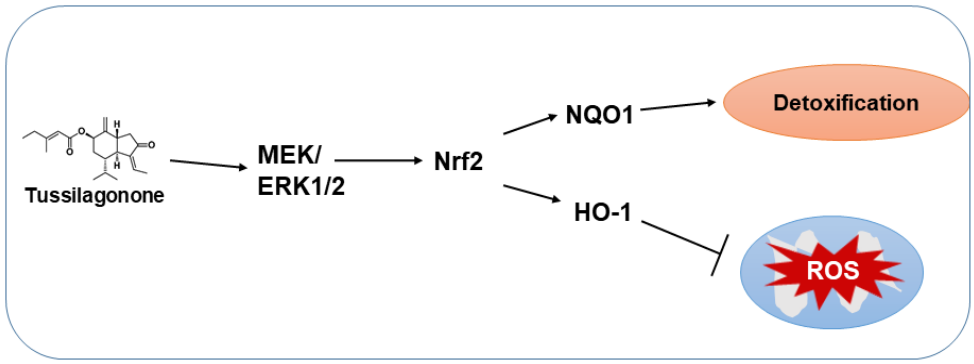


Figure 16. Proposed model for Tussilagonone on Nrf2 pathway.

Tussilagonone induces the Nrf2 pathway through phosphorylation and activation of MEK/ERK1/2. The activation of Nrf2 pathway leads the transcriptional activation of NQO1 and HO-1. As representative enzymes of detoxification and antioxidation, target genes of Nrf2 pathway have effect on the cellular defense system. Through the protective effect from the ROS which one of carcinogenic factors, tussilagonone could be functioned as one of the chemopreventive agents.

2.2. Activation of Nrf2 pathway and inhibition of NF-kB pathway mediated by igalan in HepG2 cells.

2.2.1. Igalan increases the QR activity.

In a preliminary study, it was found that igalan induces specific quinone reductase QR activity, which can be a marker for cancer prevention (Fig. 17A and Table 7). It was hypothesized that igalan pays a role in defense mechanisms in cells. To study the mechanism of the activity of igalan, it was measured cytotoxicity using the MTT assay in HepG2 cells. As shown in Fig. 17 B, concentrations of 5-10 μM did not induce cytotoxicity in HepG2 cells.

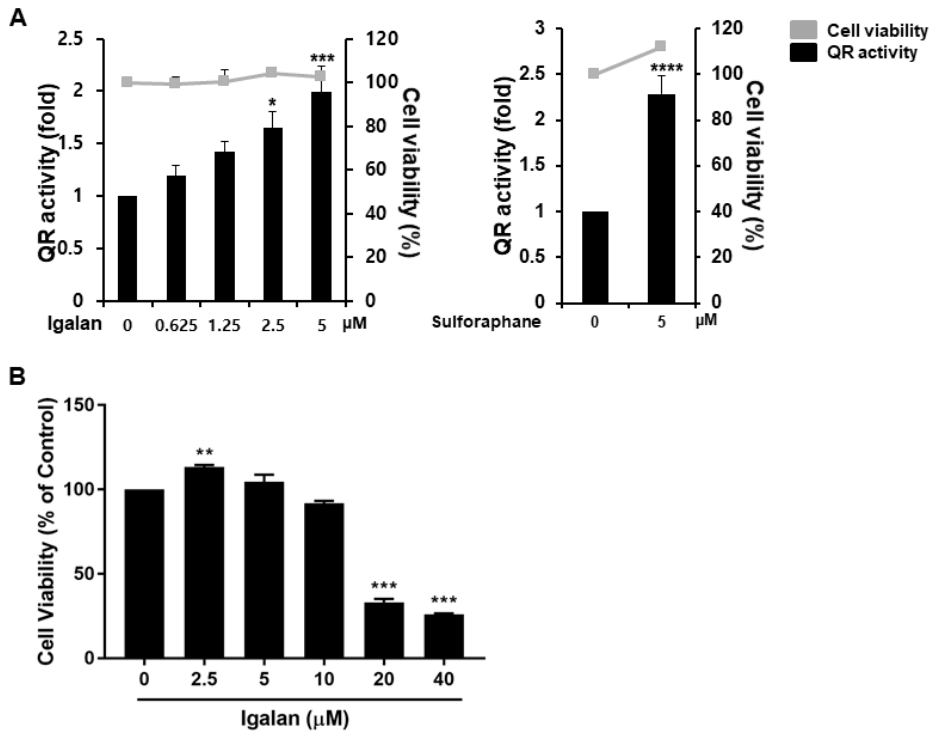


Figure 17. Igalan induces quinone reductase (QR) activity.

(A) The measurement of cell viability in HepG2 cells. (B) Effect of tussilagonone on QR activity in Hepa1c1c7 cells. Cells are treated with the indicated concentrations (0–10 or 20 μM) for 24 h.

Table 7. Igalan increases chemopreventive index.

The calculation of chemopreventive index by analyzing the result of QR assay.

	IC_{50}	CD	CI
Igalan	12.7	6.3	2.0
Sulforaphane	>20	1.6	12.6

The value of IC_{50} means the concentration of 50 % cell viability; CD means the concentration for 2 fold compared to control; CI means the chemopreventive index by calculated with IC_{50} and CD values.

2.2.2. Igalan activates Nrf2 pathway.

Because Nrf2 increases genes, including antioxidant proteins and phase II detoxifying enzymes, that function in defense mechanisms in cells, it was analyzed the expression of Nrf2 and its target genes in HepG2 cells treated with igalan. Treatment with igalan gradually increased the expression of Nrf2 in a dose-dependent manner (Fig. 18A). Increases in NQO1 and HO-1, which are target genes of Nrf2, was observed over time in response to igalan (Fig. 18B). Igalan induced the expression of both Nrf2 and its target genes. To confirm that the increase in the target genes was mediated through the Nrf2 pathway, an Nrf2 siRNA system was used (Fig. 18C). The increase in HO-1 expression induced by igalan was reduced in Nrf2-deficient cells following treatment with siRNA.

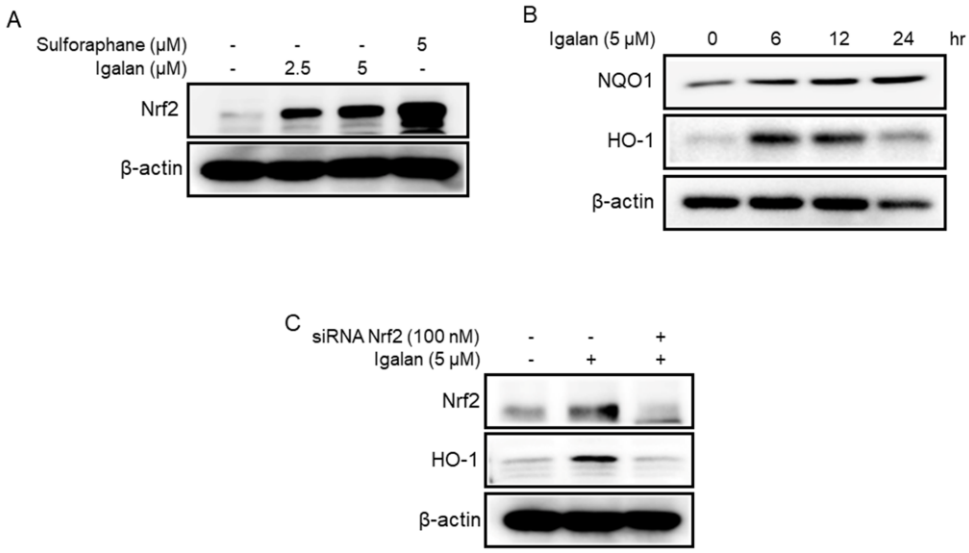


Figure 18. Upregulation of the expression of Nrf2 and target genes by igalan.

(A) Nrf2 was increased by igalan in HepG2 cells. Sulforaphane was used as a positive control. (B) Igalan induced NQO1 and HO1 in a time-dependent manner in HepG2 cells. (C) No increase in HO-1 expression occurred in Nrf2 knocked-down cells following transfection with Nrf2 siRNA. The immunoblot assay was used to measure the expression.

2.2.3. Nuclear accumulation of Nrf2 is increased by igalan to increase its target genes.

Nrf2 translocates to the nucleus in order to activate its target genes [140]. The nuclear Nrf2 expression was measured in HepG2 cells. Igalan gradually increased the nuclear accumulation of Nrf2 (Fig. 19A). To investigate the role of Nrf2 in the activation of target gene expression by igalan, the luciferase activity of the ARE was measured. When cells were exposed to igalan, the binding of Nrf2 increased in a dose-dependent manner (Fig. 19B). Because nuclear Nrf2 binds to ARE sites and increases its target genes, the HO-1 and NQO1 mRNA levels were evaluated. It was found that the HO-1 and NQO1 mRNA levels were induced by igalan in HepG2 cells (Fig. 19C, D).

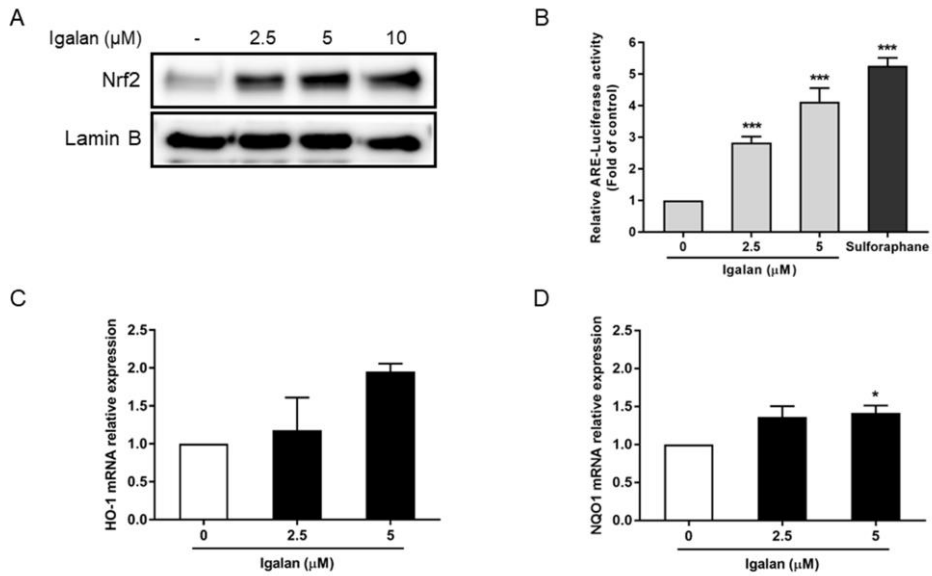


Figure 19. Igalan induces target genes by increasing nuclear accumulation of Nrf2.

(A) The nuclear expression of Nrf2 increased in a dose-dependent manner in HepG2 cells. (B) Igalan induced ARE luciferase activity in a reporter assay. (C-D) HO-1 and NQO1 mRNA expression was increased by igalan in HepG2 cells.

2.2.4. Igalan inactivates GSK3β and activates AKT to induce Nrf2 activation.

A variety of kinases are involved in the activation of Nrf2 [149, 150, 235-237]. To determine which kinases play a role in Nrf2 activation, the expression of kinases following exposure to igalan was measured over time (Fig. 20A). Our results showed that igalan regulated Nrf2 activation through the phosphorylation of GSK3β at its serine 9 residue and AKT (Fig. 20A). To further confirm these findings, inhibitors for each kinase were used. The results indicated that the expression of HO-1 increased in cells exposed to both igalan and LiCl, synergistically and decreased in cells exposed to igalan and LY294002, or igalan and wortmannin (Fig. 20B-D).

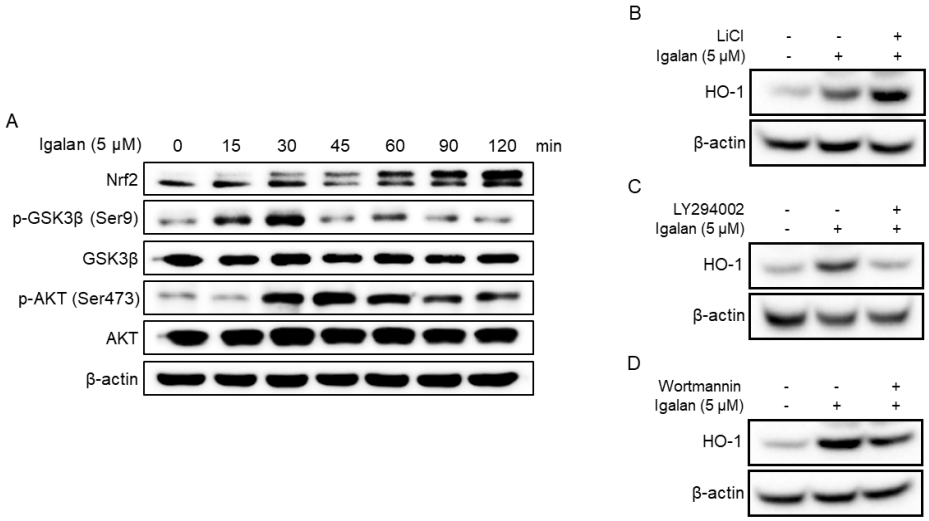


Figure 20. Igalan activates Nrf2 through GSK3β (ser 9) and AKT pathway.

(A) Igalan induced the phosphorylation of AKT and GSK3β (serine 9) in a time-dependent manner. (B) HO-1 expression increased following treatment with igalan and LiCl (GSK3β inhibitor) and decreased following treatment with igalan and LY294002 and wortmannin. The immunoblot assay was used to measure the expression.

2.2.5. NF-κB pathway is attenuated by igalan.

To determine whether igalan suppresses NF-κB signaling, an NF-κB reporter gene assay system was used in HepG2 cells (Fig. 21A). TNFα treatment increased the relative NF-κB luciferase activity, and the activity decreased in HepG2 cells exposed to igalan with TNFα (Fig. 21A). To determine whether igalan regulates NF-κB nuclear translocation resulting in an inhibitory effect on TNFα-induced NF-κB activation, the expression of phosphorylated NF-κB and IκBα was examined by western blotting (Fig. 21B). Phosphorylated NF-κB was decreased by igalan treatment, and the phosphorylated form of IκBα was also decreased by igalan in HepG2 cells treated with TNFα. To further confirm these findings, it was assessed the translocation and expression of IκBα and NF-κB. The decreases in IκBα and NF-κB in the cytosol induced by TNFα were reversed by igalan, whereas the accumulation of NF-κB in the nucleus was attenuated by igalan (Fig. 21C). NF-κB plays an important role in the regulation of pro-inflammatory gene expression, and NF-κB is involved in the synthesis of cytokines, including IL-8, IL-6, and TNFα [238]. Consistent with these results, quantitative PCR analysis also revealed that igalan inhibited the expression of pro-inflammatory cytokines such as TNFα, IL-8, and IL-6 in HepG2 cells treated with igalan in the presence of TNFα (Fig. 22D-F).

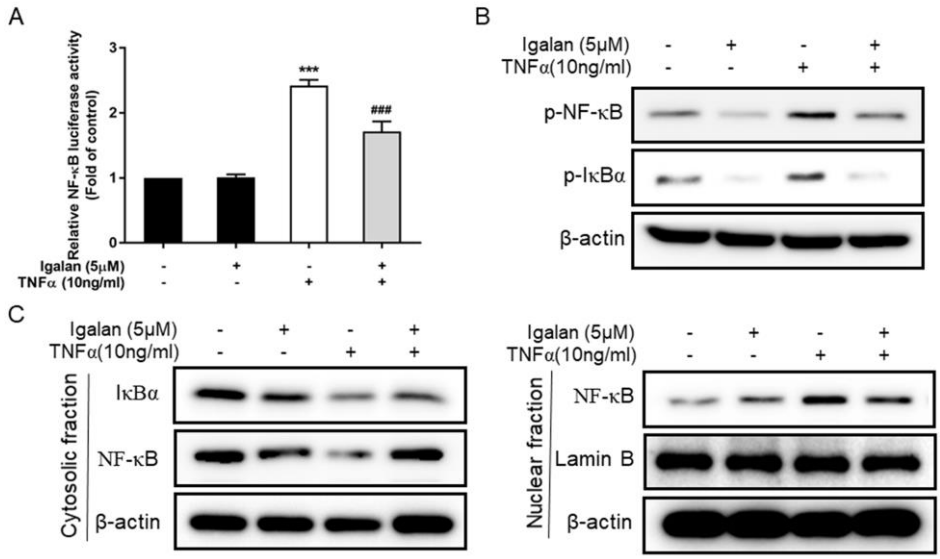


Figure 21. Igalan suppresses inflammatory pathway via inhibition of NF-κB activity.

(A) NF-κB luciferase activity was measured with a reporter assay. Igalan reduced TNFα-induced NF-κB luciferase activity. The expression of NF-κB and IκBα was measured with an immunoblot assay. (B) Igalan decreased TNFα-mediated NF-κB activation. (C) Igalan reduced the TNFα-induced NF-κB accumulation in the nucleus.

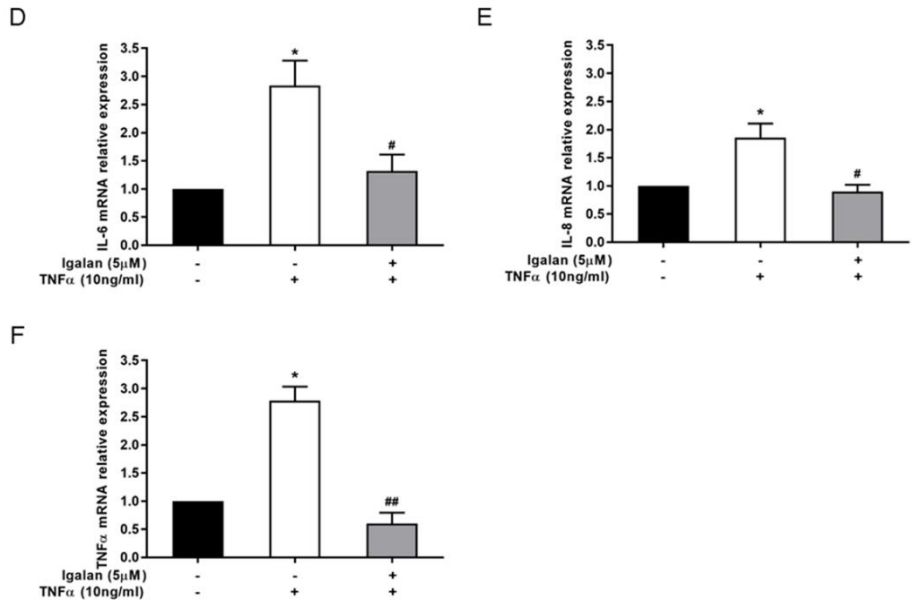


Figure 22. Igalan suppresses inflammatory pathway via inhibition of NF- κB activity.

(D-F) The expression of proinflammatory genes was determined by real-time PCR. The increases in proinflammatory genes, including IL-6, IL-8, and TNFα, induced by TNFα treatment were attenuated by igalan.

3. Activity of chikusetsusaponin IVa methyl ester isolated from *Achyranthes japonica* in promotion and progression stages of carcinogenesis.

3.1. Chikusetsusaponin IVa methyl ester inhibits Wnt signaling through β-catenin for induction of cell cycle arrest and induces apoptosis.

3.1.1. Chikusetsusaponin IVa methyl ester (CME) inhibits cell proliferation in colorectal cancer cells.

To investigate whether CME reduces cell viability in colorectal cancer cells, a cell viability assay that measures cellular mitochondrial dehydrogenase activity was performed. The cells were treated with different concentrations of CME as shown in Fig. 23A. CME decreased the cell proliferation of colorectal cancer cells in the range of 25 μM and 50 μM and caused severe cell death at concentrations above 50 μM (Fig. 23A).

Next, it was evaluated whether CME induces cell cycle arrest to inhibit cell proliferation in colorectal cancer by DNA content analysis by flow cytometry. As shown in Fig. 23B, C, the proportion of cells in G0/G1 phase changed from 40.9 % to 78.5 % at 20 μM CME and 63.0 % at 30 μM CME in HCT116 cells and also increased the population of cells in G0/G1 phase in SW480 cells. These results

suggested that CME inhibits cell proliferation by induction of cell cycle arrest in G0/G1 in both HCT116 and SW480 cells.

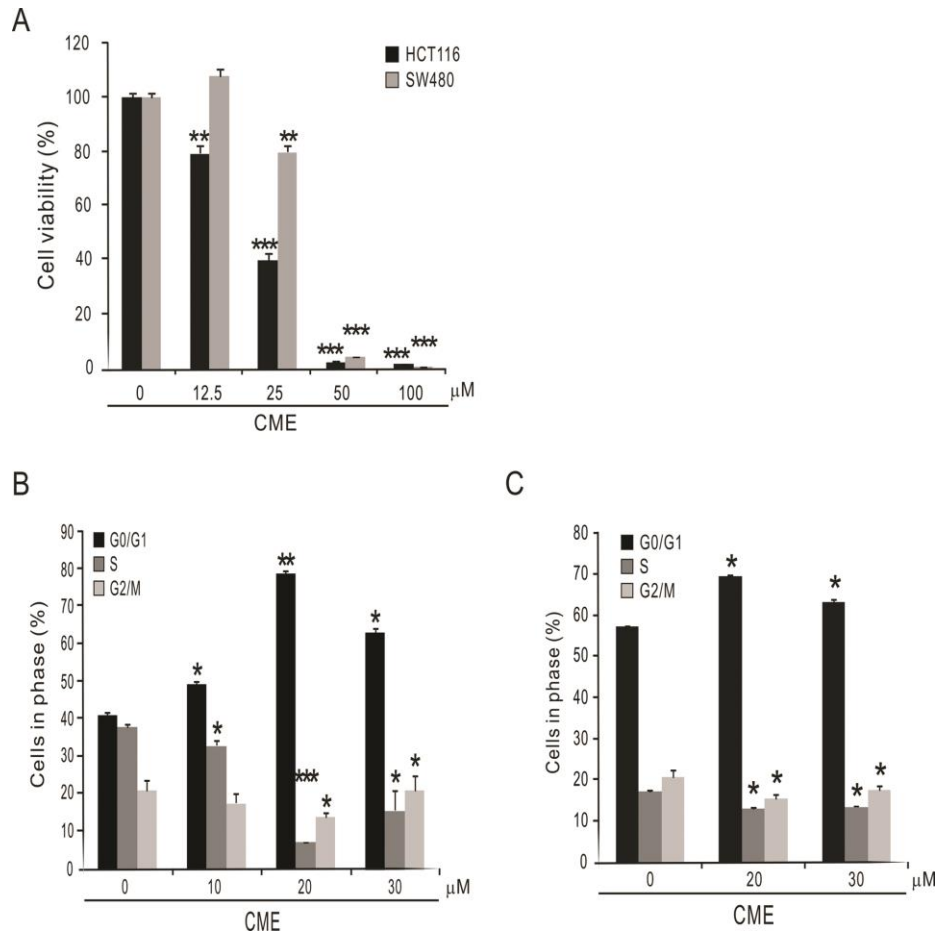


Figure 23. Inhibition of cell proliferation by Chikusetsusaponin IVa methyl ester (CME) in colorectal cancer cells.

Cell survival was determined using a cell viability assay. (A) HCT116 cells were treated with CME at the indicated concentrations for 24 hr. Cell cycle distribution was measured by flow cytometry DNA content analysis (FACS) in HCT116 (B) and SW480 (C) cells.

3.1.2. CME reduces cyclin D1, a representative target of Wnt/β-catenin signaling, and regulates cell cycle regulatory proteins to induce G0/G1 cell cycle arrest.

As shown in Fig. 23, it was shown that CME induced cell cycle arrest in colorectal cancer cells through FACS analysis. To determine whether CME regulates cell cycle regulatory proteins to induce cell cycle arrest, the expression of cell cycle regulatory proteins was measured by Western blot. The expression of cyclin D1, which is a target of the Wnt signaling pathway and is known to regulate cell cycle arrest at G0/G1 was observed [239, 240]. HCT116 and SW480 colorectal cancer cells treated with CME showed a marked decrease in cyclin D1 expression in time and dose-dependent manner (Fig. 24). The expression level of other cell cycle regulatory proteins including CDK2, CDK4, and p21 was also measured to determine whether CME affects them. It was observed that the expression of cell cycle regulatory proteins involved the regulation of G1 progression and G1/S transition [228, 229] were also affected by CME. These results showed that CME plays a crucial role in the co-regulation of cyclin D1and cell cycle regulatory proteins such as CDK 2, CDK 4 and p21.

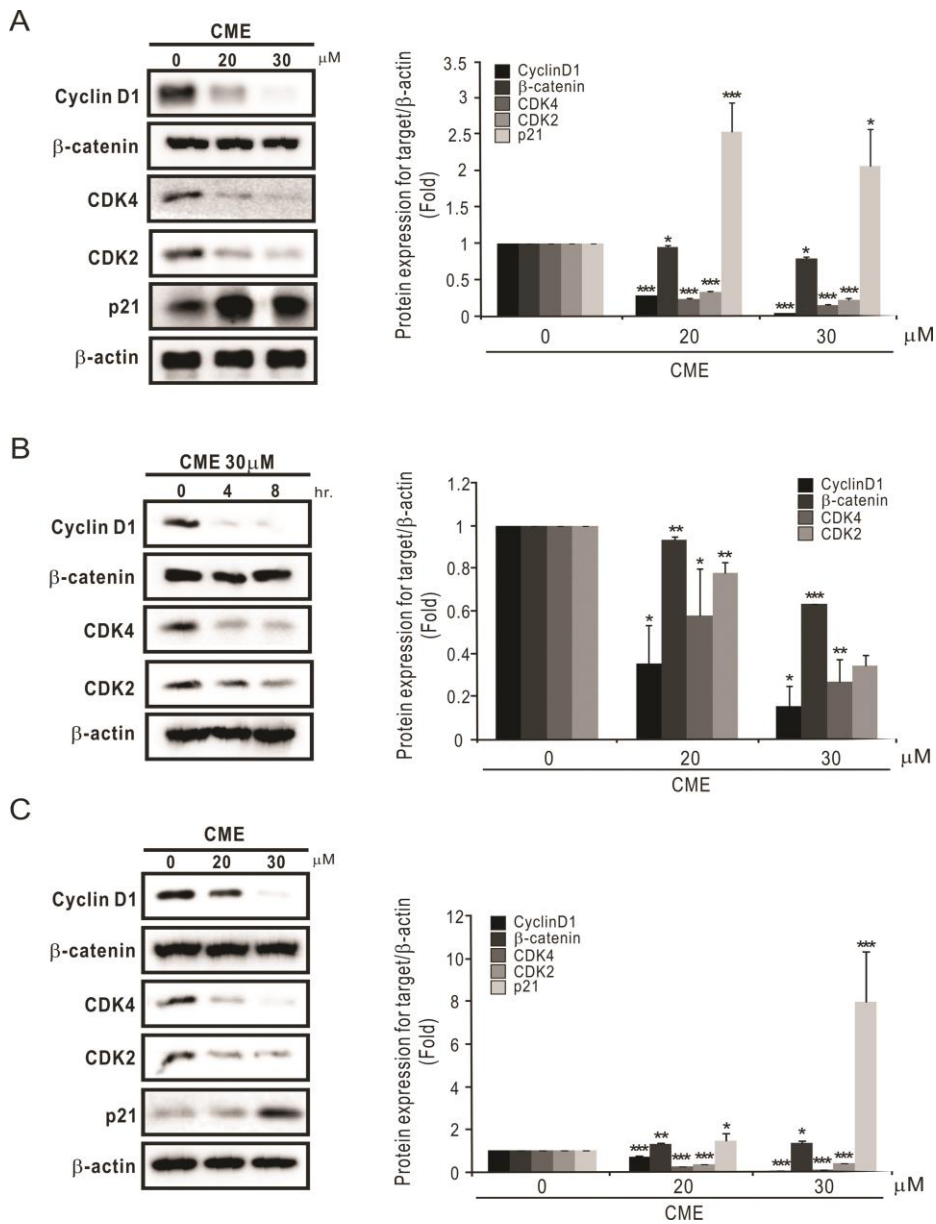


Figure 24. Regulation of cyclin D1, a representative target of Wnt/β-catenin signaling, and cell cycle regulatory proteins by Chikusetsusaponin IVa methyl ester (CME).

HCT116 Cells were treated with 20 μM and 30 μM CME for 4 hr, 8 hr (A) and 24 hr (B). SW480 Cells were treated with 20 μM and 30 μM CME for 24 hr. The cell

lysates were used for Western blot analysis (B).

3.1.3. CME inhibits TCF/β-catenin-dependent transcriptional activity by decreasing β-catenin binding to TCF binding element (TBE) and translocation to nucleus.

HCT116 cells have a mutated β-catenin that is not degraded by the proteasome system, resulting in abnormal cell proliferation [227]. SW480 cells contain a mutated APC that maintains activation of the wnt signaling pathway, causing cancer cells to proliferate [227]. It was observed that CME reduces cyclin D1 protein, one of main target of wnt signaling pathway, significantly, at early and late time (Fig. 24A, B). To investigate whether cell cycle arrest by CME involves the regulation of the wnt signaling pathway, cells were transiently co-transfected with reporter plasmids including TOP flash and FOP flash as a negative control and pRL-CMV for normalization. β-catenin transcriptional activity was decreased by CME in time and dose-dependent manner (Fig. 25A).

The β-catenin usually translocates from the cytoplasm to the nucleus to increase the transcriptional activity of target genes such as cyclin D1 and c-myc [229, 231]. To examine the accumulation of β-catenin in the nucleus, the expression of β-catenin in the nucleus was measured using immunofluorescence and western blot analysis for nuclear fractions (Fig. 25B and C). As shown in Fig. 25B and C, the level of β-catenin was decreased in nucleus in the dose and time-dependent manner. To confirm the decrease of β-catenin activity in the nucleus, the binding of β-catenin to specific DNA sequences, called TCF-binding elements (TBE), was measured in HCT116

cells treated with CME (Fig. 25C). The binding activity of β-catenin was also decreased by CME in a dose-dependent manner. These results suggested that CME inhibits the wnt signaling pathway by disrupting β-catenin binding to TBE in the promoters of target genes. As the amount of β-catenin in nucleus decreased, mRNA expressions of cyclin D1 and c-myc, targets of the wnt signaling pathway, were decreased in HCT116 cells after CME treatment (Fig. 25D).

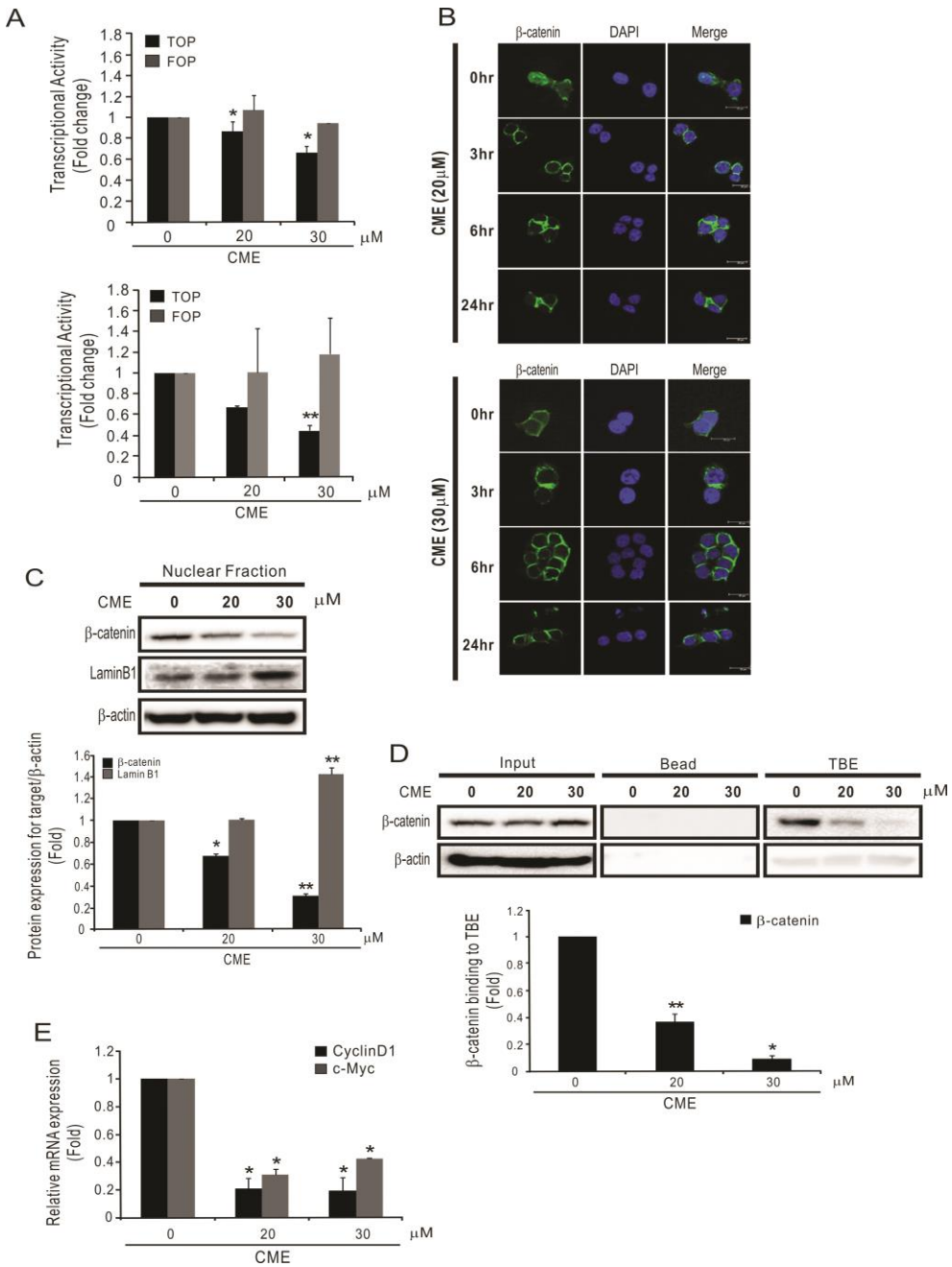


Figure 25. Inhibition of TCF/β-catenin-dependent transcriptional activity by decreasing β- catenin binding to TCF binding element (TBE) and translocation to the nucleus.

(A) Luciferase reporter assay. HCT116 cells were treated with luciferase reporter plasmids and then treated with 20 μM or 30 μM CME for 6 hr (left) and 24 hr (right). (B) Immunofluorescence staining of β-catenin nuclear translocation under 20 μM and 30 μM CME for 0 hr, 3 hr, 6 hr and 24 hr in HCT116 cells (Bar=20μm). (C) Western blot analysis of nuclear fraction in HCT116 cells with 20 μM and 30 μM CME for 24 hr. (D) Oligo pull-down assay in HCT116 cells with TBE elements. After cells were treated with 20 μM and 30 μM CME for 24 hr, cell lysates were used for the oligo pull-down assay. (E) Real-time PCR analysis of cyclin D1and c-myc expression in HCT116 cells. The cells were treated with 20 μM and 30 μM CME for 24 hr.

3.1.4. CME also induces apoptosis to inhibit proliferation in colorectal cancer cells.

Because apoptosis is a major mechanism of cancer cell death, it was investigated whether CME causes apoptosis by measuring DNA contents with flow cytometry in HCT116 cells treated with CME. As shown in Fig. 26A, CME markedly increased the sub G1 population of cells from 2.57 % at 0 μM to 10.73 % at 40 μM. To further confirm that apoptosis was induced by CME in HCT116 cells, the externalisation of phosphatidylserine, a more precise indicator of apoptosis was measured. In line with the previous results, as the dose of CME increased, apoptosis increased. As shown in Fig. 26B, the cell population undergoing apoptosis increased from 17.78 % to 35.73% after 24 hr in HCT116 cells treated with 40 μM CME. These results suggested that a high dose (e.g., 40 μM) of CME induced apoptosis in colorectal cancer cells.

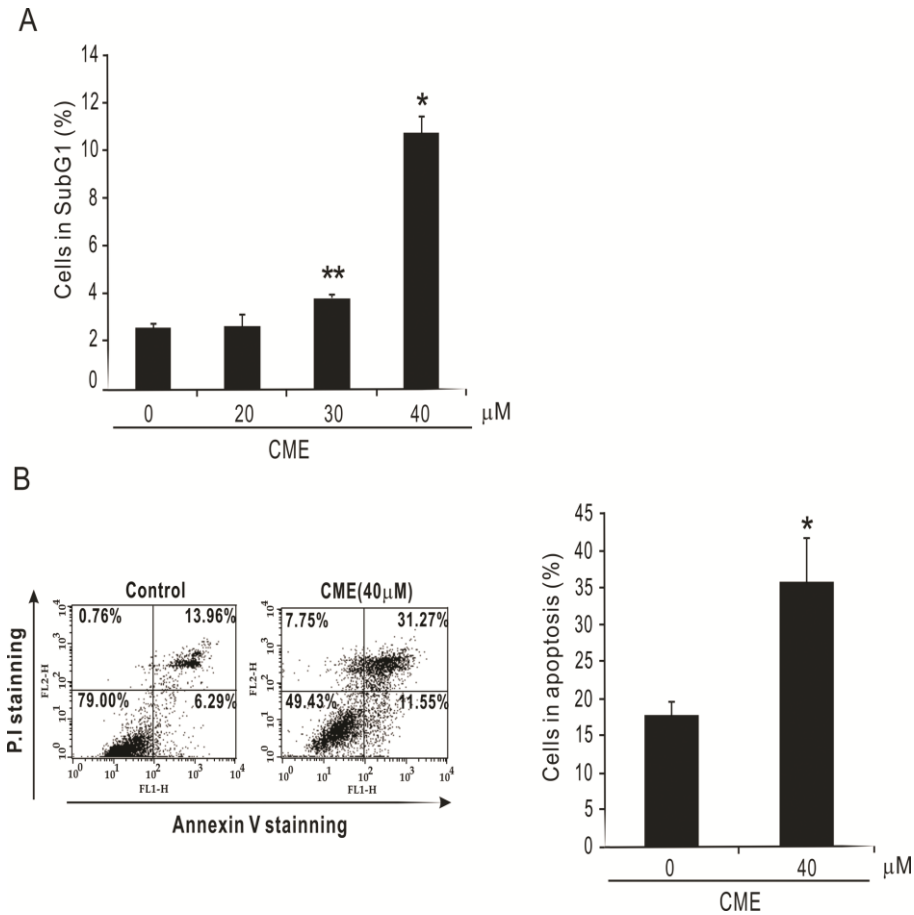


Figure 26. Apoptosis by Chikusetsusaponin IVa methyl ester (CME) in colorectal cancer cells.

(A) The sub G1 population was analysed by FACS in HCT116 cells (B) Cells were treated with 40 μM CME for 24 hr, and the externalisation of phosphatidylserine was evaluated by FACS.

3.1.5. CME induces death receptor-mediated apoptosis by activating JNK.

As shown in Fig. 26, colorectal cancer cells treated with more than 30 μM CME underwent apoptosis. To determine the mechanism of CME-induced apoptosis, the protein expression of apoptotic regulatory genes was measured (Fig. 27A and Fig. 28A). It was found that CME activated caspase 8 caused the cleavage of caspase 3 and PARP (Fig. 27A and Fig. 28A). To identify which kinase pathway is involved in caspase 8-mediated apoptosis, the protein expression of various kinases was measured. As shown in Fig. 27B and Fig. 28B, CME increased the phosphorylation of JNK in a time-dependent manner. To further confirm that CME induces apoptosis via the JNK pathway, a JNK inhibitor (SP600125) was used in the presence of CME in HCT116 cells. Activation of Fas ligand and cleaved caspase 8, clearly, was decreased in cells co-treated with SP600125 and 40 μM CME (Fig. 27C and 28C). Moreover, the expression of cleaved caspase 3 also was reduced in cells co-treated with SP600125 and 40 μM CME as a result of the decreased caspase 8 activation. These results suggest that CME induces JNK-mediated extrinsic apoptotic pathway.

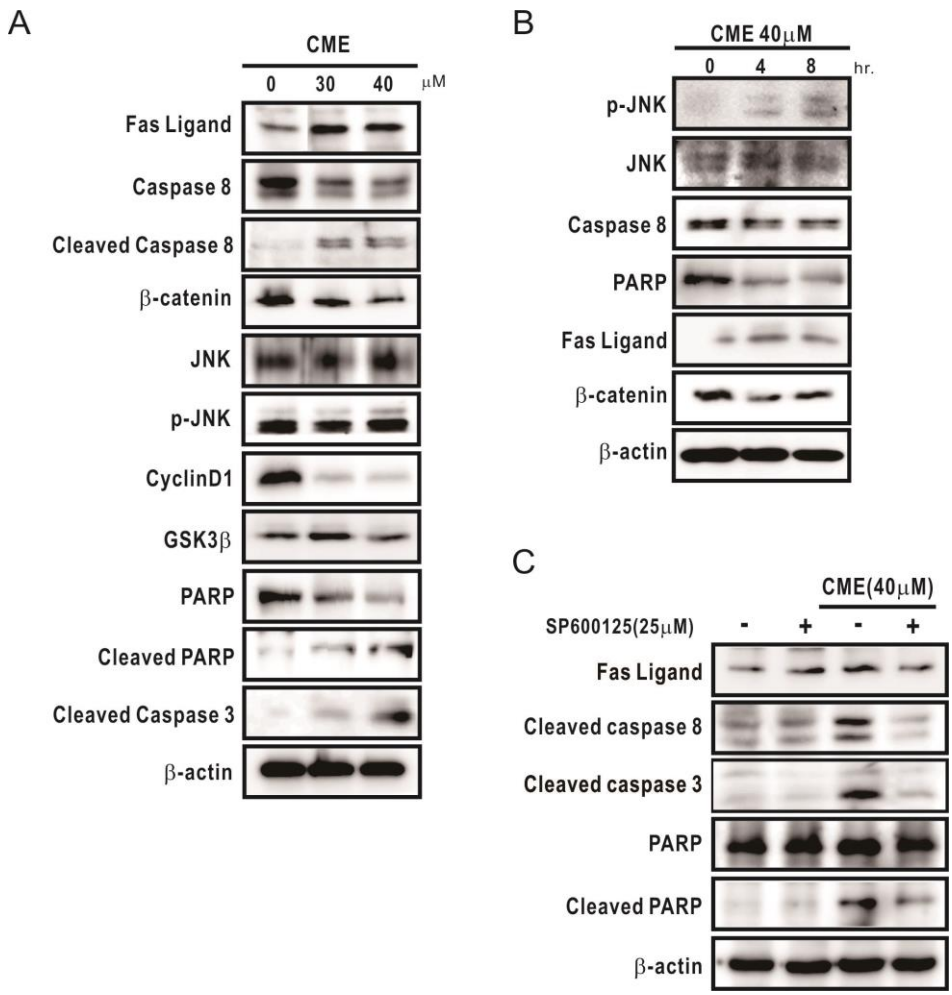


Figure 27. Increase in the expression level of receptor-mediated apoptotic genes by JNK.

Western blot analysis in HCT116 cells. (A) Cells were treated with 30 μM or 40 μM CME for 24 hr. HCT116 cell lysates were used for Western blot analysis. (B) Cells were treated with 40 μM CME for 6 hr. (C) The cells were pre-treated with 25 μM SP600125 (JNK inhibitor) for 2 hr and then treated with 40 μM CME for 24 hr.

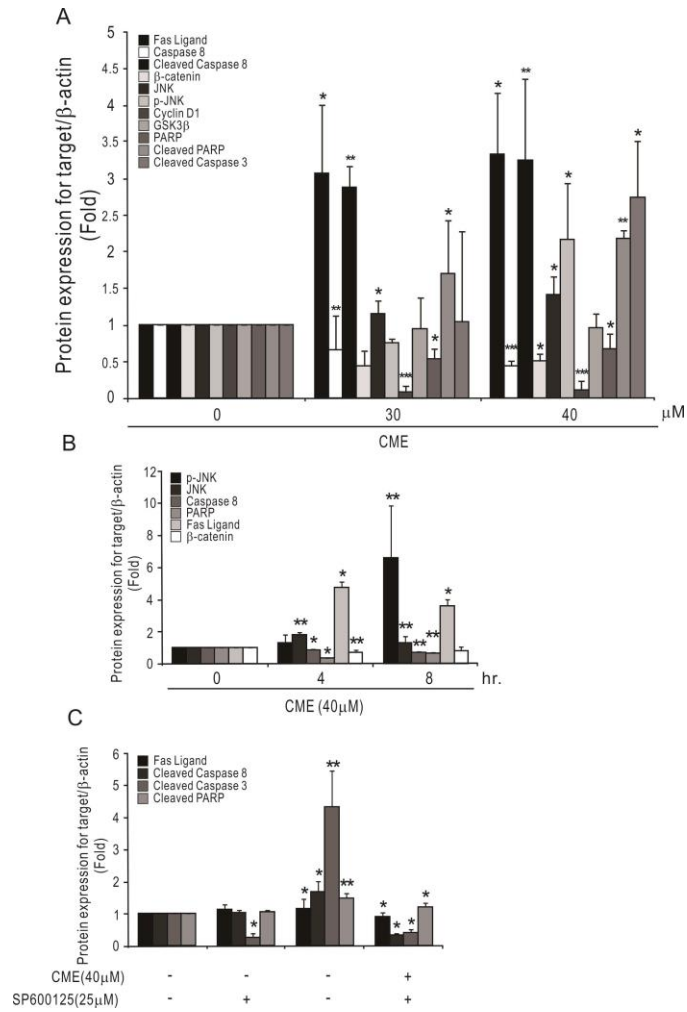


Figure 28. The quantification data for the expression level of receptor-mediated apoptotic genes by Chikusetsusaponin IVa methyl ester (CME).

Western blot analysis in HCT116 cells. (A) Cells were treated with 30 μM or 40 μM CME for 24 hr. HCT116 cell lysates were used for Western blot analysis. (B) Cells were treated with 40 μM CME for 6 hr. (C) The cells were pre-treated with 25 μM SP600125 (JNK inhibitor) for 2 hr and then treated with 40 μM CME for 24 hr.

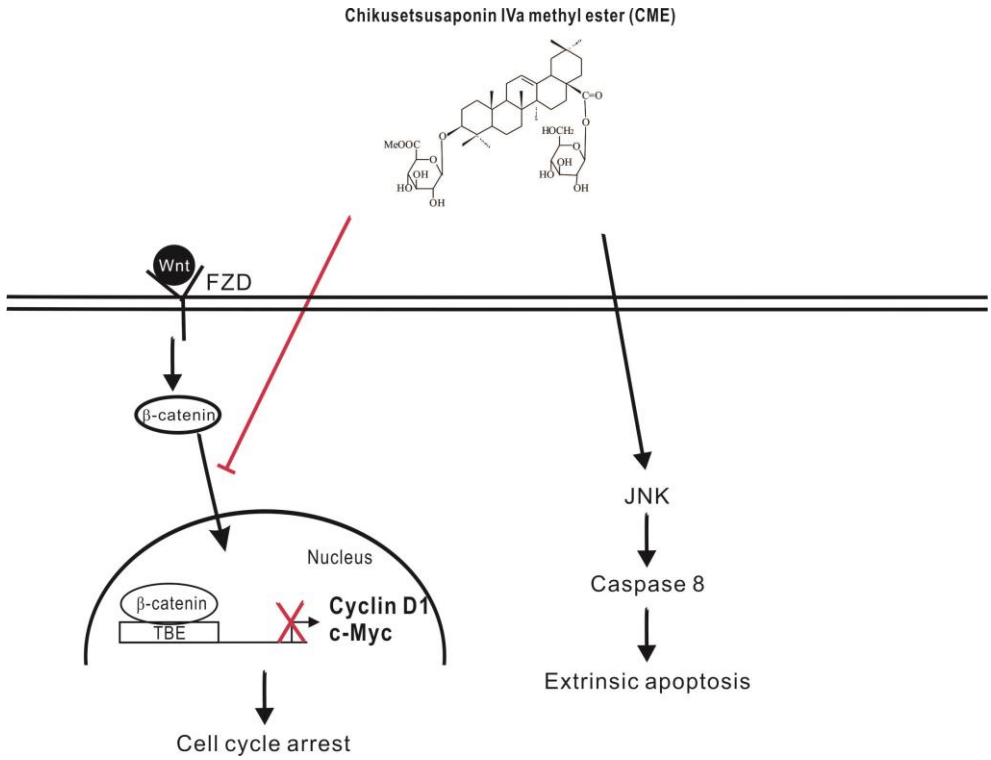


Figure 29. Hypothetical model of Chikusetsusaponin IVa methyl ester (CME)-induced cell cycle arrest and apoptosis through regulation of Wnt signaling.

CME inhibits translocation of β-catenin to induce cell proliferation. The expression of cell cycle regulatory proteins including cyclin D1 is altered in cells treated with CME. Thus, CME induces cell cycle arrest at G0/G1. A high dose of CME increases apoptosis via the JNK pro-apoptotic pathway.

IV. DISCUSSION

1. Cancer chemopreventive activity of tussilagonone from *Tussilago farfara* L. in the initiation stage of carcinogenesis.

T. farfara L. is a medicinal plant with various pharmacological effects. For instance, *T. farfara* flower bud extract exhibits anti-inflammatory effects, including inhibition of iNOS and COX-2 expression in microglial cells [204]. Tussilagonone, one of the sesquiterpenoids, is derived from *T. farfara* flower buds that inhibits the production of pro-inflammatory mediators such as NO and PGE_2 and the expression of iNOS, COX2, TNFα, and IL-6 in LPS-activated RAW264.7 cells by increasing Nrf2/HO1 expression [205]. However, the activity of this compound in HepG2 cells has not yet been characterized.

The present study demonstrated that tussilagonone protects HepG2 cells from ROS-induced oxidative stress and cell death by inducing the expression of defense-associated genes. Antioxidant and phase II detoxification enzymes mitigate the harmful effects of oxidative stress and detoxify carcinogens. The antioxidant NQO1 has shown hepatoprotective and chemopreventive effects in previous studies [241-243]. Consistently, it was found that tussilagonone activated phase II enzymes in Hepa1c1c7 murine hepatoma cells; thus, it was hypothesized that tussilagonone may be involved in these defense mechanisms.

ROS function as subcellular messengers in complex cellular processes, including

mitogenic signal transduction, the expression of gene, and the regulation of cell proliferation [244]. However, excessive ROS accumulation causes oxidative stress, loss of cell function, and ultimately apoptosis or necrosis. Oxidative stress is implicated in the etiology of several disorders, including liver diseases [245] and can be triggered by inflammation-induced free radical production. Since tussilagonone represses inflammatory signaling, it was sought to determine whether it also displayed anti-oxidative effects. As expected, tussilagonone effectively suppressed ROS production and consequent cell death following treatment with the *t*-BHP.

While Nrf2 is upregulated in response to oxidative stress in a variety of tissues and cells, it is particularly abundant in metabolically active organs such as the liver [137]. Moreover, oxidative stress is highly associated with the development and progression of liver diseases [246]. Nrf2 induces the expression of factors that detoxify carcinogens and repress oxidative stress, and are thus an important defense mechanism. Notably, these enzymes are often used as biomarkers and therapeutic targets for cancer prevention [242]. In this study, it was observed that tussilagonone increases Nrf2 expression and nuclear Nrf2 accumulation in HepG2 cells. In turn, the increase in nuclear Nrf2 induced the transcription of NQO1 and HO-1. Consequently, Nrf2 activation by tussilagonone represses ROS production and cell death.

A previous study showed that tussilagonone elicits Nrf2 pathway activity, but did not investigate the underlying mechanism of action [205]. Therefore, it was investigated the kinases responsible for tussilagonone-induced Nrf2 activation in the

present study. ERK1/2, PKC, PI3K, and AMPK have been reported to affect Nrf2 activation by phosphorylating Nrf2 for stabilization and activation [149-151]. Indeed, ERK1/2 kinase activity is required for Nrf2 nuclear localization and may disrupt the interaction between Nrf2 and Keap1 [149]. PKC also phosphorylates Nrf2 and inhibits its association with Keap1 [237]. It has been reported that the kinases GSK3β and Fyn also phosphorylate Nrf2 and regulate its activity [247].

In the current study, it was found that tussilagonone enhances MEK1/2 and ERK1/2 phosphorylation and ARE-mediated gene expression, whereas the MEK1/2 inhibitor U0126 blocked these effects; however, it should be noted that U0126 did not fully suppress Nrf2 nuclear accumulation. Based on these findings, it was speculated that other kinases are likely involved in this process and should be a subject of focus in future studies. In addition, the efficacy and effectiveness of tussilagonone remain to be assessed in animal models of liver diseases.

It is strongly indicated that tussilagonone is a potential therapeutic to prevent and/or treat liver disease by increasing the expression of cellular antioxidants.

2. Cancer chemopreventive activity of igalan from *Inula helenium.* L. in the initiation stage of carcinogenesis.

Sesquiterpene lactones represent one class of active compounds isolated from many herbs used in traditional medicines for anti-inflammatory remedies. Sesquiterpene-enriched plant extracts are commonly used in traditional medicines for infections and inflammation [248]. *Inula helenium* L. is a member of the Asteraceae family, and its dried roots are used in traditional Chinese medicine to treat inflammatory diseases [207, 208]. Alantolactone and isoalantolactone, which are sesquiterpene lactones, are the main compounds isolated from *Inula helenium* L. Alantolactone induces detoxifying enzymes such as QR and GST through the activation of the PI3K and JNK pathways, which leads to the accumulation of Nrf2, and isoalantolactone also induces QR and the nuclear translocation of Nrf2. Isoalantolactone is also a potent phase II enzyme inducer [83, 249]. Thus, in this study, it was determined whether igalan, a sesquiterpene isolated from *Inula helenium* L., induces detoxifying enzymes.

Igalan induced specific QR activity by activating enzymes mediated by Nrf2. To activate downstream genes, Nrf2 has to translocate to the nucleus. During this process, various kinases are associated with the accumulation of Nrf2 [149-151]. In contrast, GSK3β functions to export of Nrf2 from the nucleus by inducing phosphorylation at the tyrosine 216 residue [152, 153]. In the present study, it was

also determined which kinases are involved in the activation of Nrf2 by igalan (Fig. 20). In the present study, it was also determined which kinases are involved in the activation of Nrf2 by igalan (Fig. 20). It was found that igalan increases the phosphorylation of GSK3β at its serine 9 residue. Igalan also induced the phosphorylation of AKT (Fig. 20A). Interestingly, following treatment with both igalan and LiCl, an inhibitor of GSK3β, HO-1 expression increased more than that in cells treated with only igalan (Fig. 20B), indicating that the activation of the Nrf2 pathway mediated by igalan occurs through the inactivation of GSK3β. It was thought that the phosphorylation AKT may be an upstream regulator of the phosphorylation of GSK3β (serine 9). However, the phosphorylation of the kinases occurred discontinuously; thus, it was concluded that the activity of each kinase might act independently. Interestingly, it was found that GSK3β (serine 9) phosphorylation occurred first and that the phosphorylation of AKT then occurred later. It was proposed that the timing for the activation and phosphorylation of GSK3β (serine 9) and AKT by igalan is regulated differentially; however, the relationships between the regulation of these kinases by igalan need to be clarified in future studies.

GSK3β plays an important role in the regulation of neuronal survival or death in normal and pathological physiology [250]. The abnormal activation of GSK3β has also been shown to be involved in neurodegenerative disorders [250]. Following exposure to human immunodeficiency virus type-1 Tat protein [251], amyloid β-peptide (Aβ) [252], or mitochondrial toxins [152], GSK3β is associated with the

dephosphorylation of the serine 9 residue. That is, the phosphorylation of the serine 9 residue by various kinases leads to the inhibition of the activity of GSK3β [250, 253]. It was hypothesized that igalan plays a role in neurodegenerative disorders by changing GSK3β to its inactive form.

Recent studies have suggested there is a relationship between Nrf2 and NF-κB under oxidative stress. Particularly, it was reported that p65 suppresses the Nrf2-ARE pathway [254]. Moreover, a naturally occurring compound was shown to inhibit NF-κB activity through the Nrf2/HO-1 pathway [205]. It was also hypothesized that igalan attenuated the NF-κB pathway by activating the Nrf2/HO-1 pathway. It was found that igalan repressed the TNFα-induced inflammatory signaling pathway by decreasing the translocation of NF-κB to the nucleus (Fig. 21). Thus, SnPP and an Nrf2 siRNA system were used to test our hypothesis (data not shown). However, it was not able to show that SnPP and Nrf2 siRNA restored the inhibition of the TNFα-induced NF-κB pathway by igalan (data not shown), leading us to conclude that igalan inhibited NF-κB activity through another pathway.

Interestingly, it was found that NF-κB activity can be regulated by GSK3β [152]. Previous studies [152, 255] reported that the inhibition of GSK3β by lithium decreased TNFα-mediated NF-κB activity in HEK293 cells. These results indicate that GSK3β activity is required for TNFα-induced NF-κB activation [152]. In the present study, NF-κB activation was induced by stimulation with TNFα. It was hypothesize that igalan may repress NF-κB activity by regulating GSK3β activation. However, additional studies are needed to fully determine the relationship between NF-κB and the regulation of GSK3β by igalan.

Additionally, it was found that igalan inhibits STAT3 activation (phosphoform of tyrosine 705) (data not shown). STAT3 is activated in about 60% of hepatocellular carcinoma (HCC) cases [256]. IL-6, a cytokine that is a major activator of STAT3, is increased in human liver diseases and HCC [257, 258]. Moreover, IL-6 is an important NF-κB-dependent cytokine [259]. However, STAT3-positive HCC is not associated with NF-κB activation, and NF-κB-positive HCC does not involve activated STAT3 [256]. Thus, the main factor that activates STAT3 in human HCC could simply be the increased expression of IL-6 and other cytokines, including IL-11 and IL-22 [259]. The activation of STAT3 may contribute to inducing the activation of NF-κB constitutively in some cancers [259]. It was proposed that igalan has a potential anti-cancer activity through its regulation of STAT3 activation; however, further studies are needed to determine its anti-cancer activity in various types of cancer cells.

These activities of igalan indicate its potential for use as a chemoprevention agent and in the treatment of inflammatory-related diseases.

3. Cancer chemopreventive activity of igalan and tussilagonone in the initiation stage of carcinogenesis.

Tussilagonone and igalan are involved in the sesquiterpenoid family. However, they have different mechanisms for the activation of the Nrf2 pathway and functions in HepG2 cells. Although tussilagonone has an anti-inflammatory activity [205], it has shown mainly anti-oxidative activity in HepG2 cells, whereas igalan has shown anti-inflammatory and detoxifying activities in HepG2 cells. Moreover, tussilagonone induces the Nrf2 pathway through ERK, but igalan does so through the regulation of GSK3β and AKT.

These differences may be caused by the structure of compounds. The effects of sesquiterpene lactones mostly focus on the regulation of NF-κB pathway. The inhibitory effect of NF-κB is manifests as the prevention of NIK and MEKK1 signaling pathways from functioning [260, 261]. Since igalan is one of the sesquiterpene lactones and ERK is known to be related with MEKK1 pathways, the activation of the Nrf2 pathway induced by igalan may be through GSK3β and AKT, not ERK. Additionally, in the activation of the Nrf2 pathway mediated by tussilagonone, ERK was involved in the Nrf2 activation effects on the inhibition of NF-κB activity [205]. This may be due to tussilagonone's involvement in the sesquiterpenoids, not with a lactone ring. However, this hypothesis requires the further investigation regarding the differences between mechanisms for the activation of the Nrf2 pathway depending on the different structures.

4. Cancer chemopreventive activity of chikusetsusaponin IVa methyl ester isolated from *Achyranthes japonica* in the promotion and progression stages of carcinogenesis.

Here, it was reported the anticancer activity of chikusetsusaponin IVa methyl ester (CME) and elucidate its molecular mechanism. CME induced cell cycle arrest and apoptosis by regulation of the Wnt signaling pathway. Inhibitors of the Wnt signaling pathway have been thought to be potential chemotherapeutic agents for colorectal cancer [240, 262-265]. CME inhibited the transcriptional activity of the Wnt signaling pathway in HCT116 cells by disrupting β-catenin binding to TCF binding element (TBE) (Fig. 4). Thus, CME caused G0/G1 arrest by inhibiting the translocation of β-catenin to the nucleus to induce target genes, such as cyclin D1 or c-myc, involved in cell cycle regulation to induce proliferation [186, 228, 229]. Like CME, quercetin [263] and lignans [262] are also known to inhibit the translocation of β-catenin to the nucleus. However, the mechanism by which they disrupt the translocation of β-catenin is still unclear. Investigating the detailed mechanism of inhibition of β-catenin binding or β-catenin translocation by natural products may be an important approach for searching potential anti-cancer agents such as CME.

It was measured the expression level of other crucial G1 phase cell cycle regulatory proteins in cells treated with CME [239, 266]. CME decreased the expression of not

only cyclin D1, a target of the Wnt signaling pathway, but also CDK4 and CDK2 (Fig. 3), which play a crucial role as cyclin-dependent kinases that control the G1/S transition and G1 progression [239, 266]. Interestingly, CME increased the expression of p21 involved in regulation of cell cycle arrest [239, 266] in a p53-independent manner and the p21 repressed CDK4 and CDK2 [267] (Fig. 3). It was indicated that CME regulates multiple genes associated with the cell cycle and inhibits the Wnt signaling pathway.

It was shown that CME induced apoptosis at concentrations above 30 μM CME and cell cycle arrest at concentrations under 30 μM CME. It was hypothesized that there may be a factor that regulates the switch between cell cycle arrest and apoptosis. According to a recent study, dysregulation of Wnt signaling induces a variety of cancers because secreted Wnt antagonists are silenced by promoter hypermethylation [268]. Restoration of SFRP1 induces apoptosis in HCT116 and SW480 cells [269]. Moreover, Epigallocatechin-3-gallate (EGCG) induces reactivation of SFRP1 and increases apoptosis in hepatoblastoma cells harbouring β-catenin mutations [270]. However, the mechanism for the relationship between SFRP1 and apoptosis still needs to be studied in terms of the regulation of Wnt signaling [270]. It is likely to the function of EGCG which CME increased transcription of SFRP1 in HCT116 cells treated with 40 μM of CME (data unknown). However, the detailed molecular mechanism of SFRP1 induction of apoptosis needs to be confirmed and investigated in depth.

It was also found that CME activates ERK and JNK activation. Each kinase inhibitor decreased cleavage of caspase 3, but the ERK inhibitor (PD98059) did not decrease caspase 8 cleavage but reduced p21 expression significantly in CME-treated HCT116 cells. ERK1/2 kinase appears to increase p21 expression to induce cell cycle arrest in CME-treated HCT116 cells. However, it may require further study for ERK. An inhibitor for JNK decreased Fas ligand expression and caspase 8 cleavage in CME-treated HCT116 cells as shown in Fig. 6.

Finally, CME strongly inhibited the cell proliferation in HCT116 cells. It is important to note that CME, a naturally occurring compound, might act as a putative anticancer drug in colon cancer even in the cases of mutations in Wnt signaling components.

V. CONCLUSION

The molecular mechanisms for cancer chemopreventive effects including anti-oxidation, detoxification, anti-inflammation, anti-proliferation, apoptosis of tussilagonone, a sesquiterpenoid from *Tussilago farfara* L., igalan, a sesquiterpene lactone from *I. helenium*, and Chikusetsusaponin IVa methyl ester, a triterpenoid saponin from the root of *Achyranthes japonica* were investigated in this study.

First of all, through the screening for identifying the chemopreventive index with QR activity, the QR assay system was used, while the MTT assay was used to measure the capability to regulate cell proliferation. As a result of the QR assay, tussilagonone and igalan were characterized for cytoprotective effects including anti-oxidant, detoxification, and anti-inflammation. As well, chikusetsusaponin IVa methyl ester was used for studying the inhibitory effect of proliferation by the MTT assay.

First, present study reports on the cytoprotective effects of tussilagonone-induced antioxidant and detoxification enzymes in HepG2 cells. Most importantly, it is shown that tussilagonone activates Nrf2 and promotes expression of the target genes NQO1 and HO-1 in HepG2 cells. Furthermore, tussilagonone significantly attenuates ROS production and oxidative damage following *t*-BHP exposure.

Second, igalan isolated from *I. Helenium* L. was used to study the regulation of the Nrf2 pathway as a defense mechanism in HepG2 cells. It was found that igalan activated the Nrf2 pathway to induce detoxifying and anti-oxidative effects and that the activation of Nrf2 is regulated by the inactivation of GSKβ and the activation of AKT. Moreover, it was found that igalan inhibits TNFα-induced NF-kB activity and

cytokines such as IL-6 and IL-8.

Last, it was demonstrated that CME, isolated from the root of *Achyranthes japonica*, inhibited the Wnt/β-catenin signaling pathway to induce cell cycle arrest (Fig. 4). CME disrupted β-catenin nuclear translocation and the transcriptional activity of β-catenin also repressed in HCT116 cells. Thus, cyclin D1, a target gene for β-catenin, also decreased and was accompanied by a decrease in the expression of CDK2 and CDK4 in cell cycle arrest at the G0/G1 phase. And CME also induces apoptosis by JNK mediated extrinsic apoptotic pathway.

Overall, tussilagonone and igalan have effects on the initiation stage of carcinogenesis by decreasing carcinogenic factors including ROS, whereas chikusetsusaponin IVa methyl ester can act as a suppressing agent by inhibiting cell proliferation and inducing apoptosis.

VI. EXPERIMENTAL

SECTION

1. Materials

1.1. Tussilagonone

Tussilagonone, isolated as described by Lee *et al*. (2016), was provided by Prof. Eun Kyoung Seo in College of Pharmacy, Graduate School of Pharmaceutical Sciences, Ehwa Womans University.

1.2. The isolation of igalan from *Inula helenium*

The root of *Inula helenium* was purchased from the herb market in Jechun, Chungbuk in South Korea. The roots were ground and extracted with methanol (sonication, 2h, and room temperature). The extract was filtered and evaporated with a rotary evaporator under reduced pressure. The dried extract was dissolved in distilled water and solvent-partitioned with *n*-hexane. Then, the *n*-hexane fraction was further separated by counter-current chromatograpy with optimum solvent system composed of *n*-hexane-acetonitrile-methanol-water (5:3:1:2, v/v/v/v). The *n*-hexane fraction and isolated igalan were analyzed by high-performance liquid chromatography (HPLC). HPLC conditions were as follows: INNO C_{18} column (50 mm x 2.0 mm, 3.0 μm); eluent A, water with 0.1% formic acid; eluent B, acetonitrile with 0.1% formic acid; gradient, 0-8 min (45-65% B), 8-13 min (65-100% B), and then washed with 100% B for 5 min at a flow rate of 0.3 mL/min. The purity of separated igalan was greater than 98% as assessed by HPLC-PDA (photodiode array

detector). The compound was dissolved in dimethyl sulfoxide (DMSO) for pharmaceutical test.

1.3. Chikusetsusaponin IVa methyl ester

Chikusetsusaponin IVa methyl ester was provided by Prof. Kun Ho Son in department of food science and nutrition, andong national university. The roots of *Achyranthes japonica* were purchased and identified by Professor Je hyun Lee (College of Oriental Medicine, Dongguk University, Gyeongju, Korea). The dried roots of *Achyranthes japonica* (12.15 kg) were extracted with MeOH (60 ℓ) under reflux 3 times, and the filtrate was concentrated under reduced pressure to give the MeOH extract (962.16 g). The MeOH extract was suspended in H_2O and partitioned with hexane (20 ℓ), EtOAc (20 ℓ) and BuOH (20 ℓ) to yield the hexane (45.04 g), EtOAc (28.81 g), BuOH (79.90 g) and H_2O (770.62 g) extracts, respectively. A part of the BuOH extract (76.37 g) was separated by RP-18 column chromatography with a CH_3CN:H_2O (gradient) to give 11 fractions. Fraction 4 was chromatographed again by silica gel column chromatography with $CHCl_3$: MeOH (gradient) and an RP-18 column eluted with a gradient mixture of MeOH and H_2O to give chickusetsusaponin IVa methyl ester.

1.4. Reagents and Chemicals

The JNK inhibitor SP600125, the p38 inhibitor SB203580, the ERK1/2 inhibitor U0126, the PI3K/AKT inhibitor LY294002 and Wortmannin, the PKC inhibitor GF10923x, and the GSK3β inhibitor LiCl (Lithium Chloride) were purchased from Sigma Aldrich (St. Louis, MO). Annexin V-FITC and a propidium iodide (PI) kit were purchased from BD Biosciences (Bedford, MA). Antibodies for p-MEK1/2, MEK, p-ERK1/2, ERK1/2, p-GSK3β (ser9), GSK3β, p-NF-κB, NF-κB, p-IκBα cyclin D1, β-catenin, caspase 8, cleaved caspase 8, JNK, phospho-SAPK/JNK (Thr183/Tyr185), GSK3 β, PARP and cleaved caspase 3 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for HO-1, NQO1, β-actin, Lamin B, CDK4, CDK2, p21, and Fas-L as well as the corresponding secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Nrf2 antibody was from Abcam (Cambridge, MA). Penicillin, streptomycin, DMEM (high glucose), RPMI 1640 medium, and α-MEM were purchased from hyclone (Logan, UT). Fetal bovine serum (FBS) were obtained from Gibco (Thermo scientific, Waltham, MA). All other chemicals were purchased from Sigma Aldrich unless otherwise specified.

1.5. Cell culture

HepG2 (human liver hepatocellular carcinoma cells) and Hepa1c1c7c (murine hepatoma cells) cells were obtained from the American Type Culture collection (Manassas, VA). DMEM and α-MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin were used for routine subculture. HCT116 cells and SW480 cells also were obtained from the American Type Culture

Collection (ATCC, Rockville, MD, USA). RPMI1640 was used for cell cultivation. They were supplemented with 10% FBS (Hyclone, Logan, UT, USA), penicillin (100 units/mL) and streptomycin (100 μg/mL). They were maintained at sub-confluence in a 37ºC humidified atmosphere with 5% CO2.

2. Methods

2.1. Cell viability assay

Cell viability was measured by EZ cytox (Suwon, Korea). Briefly, cells were seeded in 96-well plates at 1×10^4 cells/well, incubated at 37°C for 24 h, and then treated with various concentrations of compounds. After a 24-h incubation, MTT assay was performed according to manufacturer's instructions.

2.2. Measurement of cell viability for cytoprotective effects

To assess cytoprotective effects, HepG2 cells were seeded in 96-well plates at 5×10^4 cells/well, incubated at 37°C for 24 h, and then exposed to various concentrations of tussilagonone (0, 0.625, 1.25, 2.5, or 5 μM). After a 24-h incubation, some wells were treated with *t*-BHP (500 μM) for 4 h to induce oxidative stress before MTT analysis. After 4 h, Ez cytox solution was added to each well and the plate was incubated for an additional 2 h under the same conditions. After addition of the

solution, the method was performed identically to the cell viability assay described above [205, 271].

2.3. Intracellular ROS quantification

Intracellular ROS-scavenging was measured with the fluorescent marker DCFH-DA. Briefly, HepG2 cells were seeded in 96-well plates at 5×10^4 cells/well, cultured for 24 h, and then treated with or without the indicated concentrations of tussilagonone for 2 h. Oxidative stress was induced by adding *t*-BHP (500 μM) into the culture medium for 1 h. After induction of ROS production, the cells were labeled with DCFH-DA (20 μM) for 30 min, washed twice with PBS, and then fluorescence intensity was measured with a 96-well microplate fluorometer (Molecular Devices Gemini XS, Sunnyvale, CA, UA) at excitation and emission wavelengths of 485 nm and 528 nm, respectively.

2.4. Preparation of nuclear and cytosolic fractions

Cells (6×10^5 cells/well) were seeded in a 6-well plate and treated with tussilagonone as indicated. Nuclear and cytoplasmic fractions were then prepared using a nuclear extraction kit (Cayman Chemical, Ann Arbor, MI).

2.5. Western blot analysis

Cells (6×10^5 cells/well) were cultured in 6-well plate and treated with compounds as indicated. Cell lysate was extracted with ice-cold RIPA buffer (Sigma-Aldrich, St Louis, MO) containing phenylmethanesulfonyl fluoride and a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) for 30 min. Protein concentrations were determined using protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein was separated with 10% SDS-PAGE gels and then transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 3% BSA in 1% Tween-20 in PBS for 1 h and followed by primary and secondary antibodies. Immunoreactive bands were then visualized with enhanced chemiluminescence western blotting reagent (Thermo scientific, Waltham, MA).

2.6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from HepG2 cells with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (1 μg) was used to synthesize cDNA with an Prime Script 1st strand cDNA synthesis Kit (Takara Clontech, Kyoto, Japan) and a Genius FGEN05TD thermocycler (Teche, England) using the following cycles: 30°C for 10 min, 42°C for 60 min, and 95°C for 5 min. qRT-PCR analysis was conducted with an Applied Biosystems 7300 real-time PCR system and software (Life Technologies, Carlsbad, CA) in PCR tubes using a custom PCR master mix containing forward and reverse primers and SYBR green working solution (iTaq™ Universal SYBR Green Supermix; Bio-Rad, Hercules, CA).

2.7. Reporter assays

Cells were seeded in 24-well culture plates for 24 h before transfection at 50–60% confluency. Cells were transiently co-transfected with 0.2 μg each of ARE-luc reporter construct harboring the ARE derived from human NQO1, NF-kB-luc reporter construct harboring the NF-kB binding site, or TOP/FOP flash. And pRL-CMV was used as a transfection control vector (Promega, Madison, WI) using the TransIT-2020 transfection reagent (Mirrus Bio LLC, Madison, WI) according to the manufacturer's instructions. Luciferase assays were performed 24 h post-transfection with a dual luciferase assay kit (Promega, Madison, WI) and a Synergy HT Multi-microplate reader (Bio-Tek Instruments, Winooski, VT) according to the manufacturer's instructions.

2.8. Quinone reductase (QR) assay

Specific QR activity was measured using QR assays as described previously [241, 272] with minor modifications. Hepa1c1c7 cells (7×10^3 cells/well) were seeded into 96-well plates and cultured for 24 h prior to treatment with compounds for an additional 24 h. Sulforaphane (Sigma-Aldrich, St Louis, MO) was used as a positive control. Absorbance at 610 nm was measured five times at 50-s intervals using a Synergy HT Multi-microplate reader. The specific QR activity was analyzed by the calculation of comparing the specific QR activity of the cells treated with compounds of vehicle only treated cells. As a result of enzymatic activity, the value of CD meant the concentration required to double QR activity. The half concentration of cell viability compared to control is IC_{50}. Chemoprevention index (CI) was calculated by

dividing the half-maximal cell growth inhibitory concentration (IC_{50}) values by the CD values.

2.9. Oligonucleotide pulldown assay

Cells were lysed in HKMG buffer (10 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 0.5% NP-40). Cellular extracts were incubated for 16 h with biotinylated, double-stranded oligonucleotides described elsewhere [273]. To collect DNA-bound proteins, the mixtures were incubated with streptavidin agarose beads for 6 h, washed with HKMG buffer, and precipitated by centrifugation. The precipitates were then analyzed by western blotting as described above.

2.10. Immunocytochemistry

Immunocytochemistry was performed as previously described with minor modifications. Briefly, the cells were seeded onto glass coverslips in 24-well plates (2×10^3 cells/well) and treated with tussilagonone (0–5 μM) or sulforaphane (5 μM) for 6 h. Cells were subsequently incubated with anti-Nrf2 (1:100, Abcam, Cambridge, MA) or anti-β-catenin (1:150, Cell Signaling Technology,Beverly, MA) overnight at 4ºC, followed by Alexa Flour 488-conjugated anti-rabbit secondary antibody (1:200, Invitrogen, Carlsbad, CA) for 1 h at room temperature. Images were obtained using a Leica TCS SP5 confocal system (Leica, Wetzlar, Germany) [243].

2.11. Lactate dehydrogenase (LDH) quantification

LDH was measured using an LDH kit (Cayman, Ann. Arbor, MI) according to the manufacturer's protocol and absorbance at 490 nm was measured with a microplate reader (HT Multi-microplate reader; Bio-Tek Instruments, Winooski, VT)

2.12. siRNA treatment

To knockdown the expression of human Nrf2 expression, RNA interference methods were performed using small interfering RNAs (siRNAs). AccuTarget Negative control siRNA (Bioneer, Daejeon, Korea) was used as a control scrambled siRNA. The siRNAs specific for Nrf2 was purchased from Bioneer. The cells were transfected with control scrambled siRNA or Nrf2 siRNA at the 100nM concentrations using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as recommended by the manufacturer.

2.13. Annexin V/Propidium Iodide (PI) Staining

The cells treated with various concentrations of CME were collected and washed with PBS, resuspended in binding buffer, and stained with annexin V-FITC and PI for 15 min at room temperature in the dark. Then, the samples were analysed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.14. Flow Cytometry Analysis

After treatment with various concentrations of CME, cells were collected and washed with PBS followed by fixation with 70% ethanol and were incubated at −20 °C overnight. Cells were then collected by centrifugation and washed. The pellet was resuspended in PBS and incubated with RNase A (50 μg/mL) for 30 min at room temperature, then stained with PI for 10 min. The samples were analysed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.15. Statistical analysis

Statistical significance was assessed with a one-way analysis of variance (ANOVA) followed by Dunnett's and/or Bonferroni's multiple comparisons test. Data represent the mean ± standard error of the mean (SEM) of three replicates. $P < 0.05$ was considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ indicate a significant difference from the control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ indicate a significant difference from the group treated with *t*-BHP or TNFα) using GraphPad Prism version 7.00.

REFERENCES

[1] Statistics Korea; 2016, in: S. Korea (Ed.)Daejeon, 2016.

[2] Jung, K.W., Won, Y.J., Oh, C.M., Kong, H.J., Lee, D.H., Lee, K.H., Community of Population-Based Regional Cancer, R., Cancer Statistics in Korea: Incidence, Mortality, Survival, and Prevalence in 2014, Cancer Research and Treatment, 2017; 49:292-305.

[3] Jung, K.W., Won, Y.J., Oh, C.M., Kong, H.J., Cho, H., Lee, D.H., Lee, K.H., Prediction of cancer incidence and mortality in Korea, 2015, Cancer Research and Treatment, 2015; 47:142-8.

[4] Sporn, M.B., Dunlop, N.M., Newton, D.L., Smith, J.M., Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids), Federation Proceedings, 1976; 35:1332-8.

[5] Molecular Cancer Society, K.G.T.R.F., Cancer chemoprevention, 2000.

[6] PK, G., Cancer Chemoprevention by Natural Products: Current&Future prospects., Journal of integrative oncology, 2012; 1.

[7] Wattenberg, L.W., Chemoprevention of cancer, Cancer Research, 1985; 45:1-8.

[8] Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., Free radicals and antioxidants in normal physiological functions and human disease, The International Journal of Biochemistry & Cell Biology, 2007; 39:44-84.

[9] Yu, S., Kong, A.N., Targeting carcinogen metabolism by dietary cancer preventive compounds, Current Cancer Drug Targets, 2007; 7:416-24.

[10] Manson, M.M., Gescher, A., Hudson, E.A., Plummer, S.M., Squires, M.S.,

Prigent, S.A., Blocking and suppressing mechanisms of chemoprevention by dietary constituents, Toxicology Letters, 2000; 112:499-505.

[11] Karin, M., Nuclear factor-kappaB in cancer development and progression, Nature, 2006; 441:431-6.

[12] Bange, J., Zwick, E., Ullrich, A., Molecular targets for breast cancer therapy and prevention, Nature Medicine, 2001; 7:548-52.

[13] Campisi, J., Cellular senescence as a tumor-suppressor mechanism, Trends in Cell Biology, 2001; 11:S27-S31.

[14] Huang, P., Oliff, A., Signaling pathways in apoptosis as potential targets-for cancer therapy, Trends in Cell Biology, 2001; 11:343-+.

[15] Malumbres, M., Barbacid, M., To cycle or not to cycle: A critical decision in cancer, Nature Reviews Cancer, 2001; 1:222-31.

[16] Waddick, K.G., Uckun, F.M., Innovative treatment programs against cancer - II. Nuclear factor-kappa B (NF-kappa B) as a molecular target, Biochemical Pharmacology, 1999; 57:9-17.

[17] Din, F.V.N., Valanciute, A., Houde, V.P., Zibrova, D., Green, K.A., Sakamoto, K., Alessi, D.R., Dunlop, M.G., Aspirin Inhibits mTOR Signaling, Activates AMP-Activated Protein Kinase, and Induces Autophagy in Colorectal Cancer Cells, Gastroenterology, 2012; 142:1504-15.

[18] Millimouno, F.M., Dong, J., Yang, L., Li, J., Li, X.M., Targeting Apoptosis Pathways in Cancer and Perspectives with Natural Compounds from Mother Nature, Cancer Prevention Research, 2014; 7:1081-107.

[19] Arora, S., Bhardwaj, A., Srivastava, S.K., Singh, S., McClellan, S., Wang, B.,

Singh, A.P., Honokiol Arrests Cell Cycle, Induces Apoptosis, and Potentiates the Cytotoxic Effect of Gemcitabine in Human Pancreatic Cancer Cells, Plos One, 2011; 6.

[20] Arora, S., Singh, S., Piazza, G.A., Contreras, C.M., Panyam, J., Singh, A.P., Honokiol: A Novel Natural Agent for Cancer Prevention and Therapy, Current Molecular Medicine, 2012; 12:1244-52.

[21] Chen, X.R., Lu, R., Dan, H.X., Liao, G., Zhou, M., Li, X.Y., Ji, N., Honokiol: a promising small molecular weight natural agent for the growth inhibition of oral squamous cell carcinoma cells, International Journal of Oral Science, 2011; 3:34-42.

[22] Dikalov, S., Losik, T., Arbiser, J.L., Honokiol is a potent scavenger of superoxide and peroxyl radicals, Biochemical Pharmacology, 2008; 76:589-96.

[23] Fried, L.E., Arbiser, J.L., Honokiol, a Multifunctional Antiangiogenic and Antitumor Agent, Antioxidants & Redox Signaling, 2009; 11:1139-48.

[24] Hahm, E.R., Arlotti, J.A., Marynowski, S.W., Singh, S.V., Honokiol, a constituent of oriental medicinal herb Magnolia officinalis, inhibits growth of PC-3 xenografts in vivo in association with apoptosis induction, Clinical Cancer Research, 2008; 14:1248-57.

[25] Jeong, J.J., Lee, J.H., Chang, K.C., Kim, H.J., Honokiol exerts an anticancer effect in T98G human glioblastoma cells through the induction of apoptosis and the regulation of adhesion molecules, International Journal of Oncology, 2012; 41:1358-64.

[26] Kim, B.H., Cho, J.Y., Anti-inflammatory effect of honokiol is mediated by PI3K/Akt pathway suppression, Acta Pharmacologica Sinica, 2008; 29:113-22.

[27] Li, Z.Y., Liu, Y., Zhao, X., Pan, X.L., Yin, R.T., Huang, C.H., Chen, L.,Wei, Y.Q., Honokiol, a natural therapeutic candidate, induces apoptosis and inhibits angiogenesis of ovarian tumor cells, European Journal of Obstetrics Gynecology and Reproductive Biology, 2008; 140:95-102.

[28] Liu, H., Zang, C., Emde, A., Planas-Silva, M.D., Rosche, M., Kuhnl, A., Schulz, C.O., Elstner, E., Possinger, K., Eucker, J., Anti-tumor effect of honokiol alone and in combination with other anti-cancer agents in breast cancer, European Journal of Pharmacology, 2008; 591:43-51.

[29] Liu, S.H., Shen, C.C., Yi, Y.C., Tsai, J.J., Wang, C.C., Chueh, J.T., Lin, K.L., Lee, T.C., Pan, H.C., Sheu, M.L., Honokiol inhibits gastric tumourigenesis by activation of 15-lipoxygenase-1 and consequent inhibition of peroxisome proliferator-activated receptor-gamma and COX-2-dependent signals, British Journal of Pharmacology, 2010; 160:1963-72.

[30] Rajendran, P., Li, F., Shanmugam, M.K., Vali, S., Abbasi, T., Kapoor, S., Ahn, K.S., Kumar, A.P., Sethi, G., Honokiol inhibits signal transducer and activator of transcription-3 signaling, proliferation, and survival of hepatocellular carcinoma cells via the protein tyrosine phosphatase SHP-1, Journal of Cellular Physiology, 2012; 227:2184-95.

[31] Singh, T., Katiyar, S.K., Honokiol, a phytochemical from Magnolia spp., inhibits breast cancer cell migration by targeting nitric oxide and cyclooxygenase-2, International Journal of Oncology, 2011; 38:769-76.

[32] Tian, W., Deng, Y.C., Li, L., He, H.F., Sun, J., Xu, D., Honokiol synergizes chemotherapy drugs in multidrug resistant breast cancer cells via enhanced apoptosis

and additional programmed necrotic death, International Journal of Oncology, 2013; 42:721-32.

[33] Tian, W., Xu, D., Deng, Y.C., Honokiol, a multifunctional tumor cell death inducer, Pharmazie, 2012; 67:811-16.

[34] Vaid, M., Sharma, S.D., Katiyar, S.K., Honokiol, a phytochemical from the Magnolia plant, inhibits photocarcinogenesis by targeting UVB-induced inflammatory mediators and cell cycle regulators: development of topical formulation, Carcinogenesis, 2010; 31:2004-11.

[35] Wu, J.P., Zhang, W., Wu, F., Zhao, Y., Cheng, L.F., Xie, J.J., Yao, H.P., Honokiol: an effective inhibitor of high-glucose-induced upregulation of inflammatory cytokine production in human renal mesangial cells, Inflammation Research, 2010; 59:1073-79.

[36] Chen, L.C., Lee, W.S., P27/Kip1 Is Responsible for Magnolol-Induced U373 Apoptosis in Vitro and in Vivo, Journal of Agricultural and Food Chemistry, 2013; 61:2811-19.

[37] Chen, M.C., Lee, C.F., Huang, W.H., Chou, T.C., Magnolol suppresses hypoxia-induced angiogenesis via inhibition of HIF-1 alpha/VEGF signaling pathway in human bladder cancer cells, Biochemical Pharmacology, 2013; 85:1278-87.

[38] Kang, Y.J., Park, H.J., Chung, H.J., Min, H.Y., Park, E.J., Lee, M.A., Shin, Y., Lee, S.K., Wnt/beta-Catenin Signaling Mediates the Antitumor Activity of Magnolol in Colorectal Cancer Cells, Molecular Pharmacology, 2012; 82:168-77.

[39] Park, H.J., Kang, Y.J., Chung, H.J., Kim, K.D., Lee, S.K., WNT/beta-CATENIN SIGNALING MEDIATES THE ANTITUMOR ACTIVITY OF MAGNOLOL IN

COLORECTAL CANCER CELLS, Pharmaceutical Biology, 2012; 50:572-72.

[40] Park, J.B., Lee, M.S., Cha, B.Y., Lee, J.S., Sul, J.Y., Song, I.S., Kim, J.Y., Magnolol-Induced Apoptosis in HCT-116 Colon Cancer Cells Is Associated with the AMP-Activated Protein Kinase Signaling Pathway, Biological & Pharmaceutical Bulletin, 2012; 35:1614-20.

[41] Rasul, A., Yu, B., Khan, M., Zhang, K., Iqbal, F., Ma, T.H., Yang, H., Magnolol, a natural compound, induces apoptosis of SGC-7901 human gastric adenocarcinoma cells via the mitochondrial and PI3K/Akt signaling pathways, International Journal of Oncology, 2012; 40:1153-61.

[42] Dong, L.Q., Zhou, S., Yang, X.H., Chen, Q.M., He, Y., Huang, W., Magnolol Protects Against Oxidative Stress-Mediated Neural Cell Damage by Modulating Mitochondrial Dysfunction and PI3K/Akt Signaling, Journal of Molecular Neuroscience, 2013; 50:469-81.

[43] Li, M.H., Kothandan, G., Cho, S.J., Huong, P.T., Nan, Y.H., Lee, K.Y., Shin, S.Y., Yea, S.S., Jeon, Y.J., Magnolol Inhibits LPS-induced NF-kappa B/Rel Activation by Blocking p38 Kinase in Murine Macrophages, Korean Journal of Physiology & Pharmacology, 2010; 14:353-8.

[44] Ji, H.Y., Kim, S.Y., Kim, D.K., Jeong, J.H., Lee, H.S., Effects of Eupatilin and Jaceosidin on Cytochrome P450 Enzyme Activities in Human Liver Microsomes, Molecules, 2010; 15:6466-75.

[45] Lee, J.G., Kim, J.H., Ahn, J.H., Lee, K.T., Baek, N.I., Choi, J.H., Jaceosidin, isolated from dietary mugwort (Artemisia princeps), induces G2/M cell cycle arrest by inactivating cdc25C-cdc2 via ATM-Chk1/2 activation, Food and Chemical

Toxicology, 2013; 55:214-21.

[46] Lv, W., Sheng, X., Chen, T., Xu, Q., Xie, X., Jaceosidin induces apoptosis in human ovary cancer cells through mitochondrial pathway, Journal of Biomedicine and Biotechnology, 2008.

[47] Chen, D., Cao, J.G., Tian, L., Liu, F., Sheng, X.F., Induction of apoptosis by casticin in cervical cancer cells through reactive oxygen species-mediated mitochondrial signaling pathways, Oncology Reports, 2011; 26:1287-94.

[48] Koh, D.J., Ahn, H.S., Chung, H.S., Lee, H., Kim, Y., Lee, J.Y., Kim, D.G., Hong, M., Shin, M., Bae, H., Inhibitory effects of casticin on migration of eosinophil and expression of chemokines and adhesion molecules in A549 lung epithelial cells via NF-kappa B inactivation, Journal of Ethnopharmacology, 2011; 136:399-405.

[49] Shen, J.K., Du, H.P., Yang, M., Wang, Y.G., Jin, J., Casticin induces leukemic cell death through apoptosis and mitotic catastrophe, Annals of Hematology, 2009; 88:743-52.

[50] Yang, J., Yang, Y., Tian, L., Sheng, X.F., Liu, F., Cao, J.G., Casticin-induced apoptosis involves death receptor 5 upregulation in hepatocellular carcinoma cells, World Journal of Gastroenterology, 2011; 17:4298-307.

[51] Zeng, F.X., Tian, L., Liu, F., Cao, J.G., Quan, M.F., Sheng, X.F., Induction of apoptosis by casticin in cervical cancer cells: reactive oxygen species-dependent sustained activation of Jun N-terminal kinase, Acta Biochimica Et Biophysica Sinica, 2012; 44:442-49.

[52] Butturini, E., Di Paola, R., Suzuki, H., Paterniti, I., Ahmad, A., Mariotto, S., Cuzzocrea, S., Costunolide and Dehydrocostuslactone, two natural sesquiterpene

lactones, ameliorate the inflammatory process associated to experimental pleurisy in mice, European Journal of Pharmacology, 2014; 730:107-15.

[53] Choi, J.H., Lee, K.T., Costunolide-Induced Apoptosis in Human Leukemia Cells: Involvement of c-Jun N-Terminal Kinase Activation, Biological & Pharmaceutical Bulletin, 2009; 32:1803-08.

[54] Hsu, J.L., Pan, S.L., Ho, Y.F., Hwang, T.L., Kung, F.L., Guh, J.H., Costunolide Induces Apoptosis Through Nuclear Calcium(2+) Overload and DNA Damage Response in Human Prostate Cancer, Journal of Urology, 2011; 185:1967-74.

[55] Liu, C.Y., Chang, H.S., Chen, I.S., Chen, C.J., Hsu, M.L., Fu, S.L., Chen, Y.J., Costunolide causes mitotic arrest and enhances radiosensitivity in human hepatocellular carcinoma cells, Radiation Oncology, 2011; 6.

[56] Pae, H.O., Jeong, G.S., Kim, H.S., Woo, W.H., Rhew, H.Y., Kim, H.S., Sohn, D.H., Kim, Y.C., Chung, H.T., Costunolide inhibits production of tumor necrosis factor-Cc and interleukin-6 by inducing heme oxygenase-1 in RAW264.7 macrophages, Inflammation Research, 2007; 56:520-26.

[57] Rasul, A., Bao, R., Malhi, M., Zhao, B., Tsuji, I., Li, J., Li, X.M., Induction of Apoptosis by Costunolide in Bladder Cancer Cells is Mediated through ROS Generation and Mitochondrial Dysfunction, Molecules, 2013; 18:1418-33.

[58] Yang, Y.I., Kim, J.H., Lee, K.T., Choi, J.H., Costunolide induces apoptosis in platinum-resistant human ovarian cancer cells by generating reactive oxygen species, Gynecologic Oncology, 2011; 123:588-96.

[59] Cheng, G., Xie, L.P., Parthenolide Induces Apoptosis and Cell Cycle Arrest of Human 5637 Bladder Cancer Cells In Vitro, Molecules, 2011; 16:6758-68.

[60] Czyz, M., Lesiak-Mieczkowska, K., Koprowska, K., Szulawska-Mroczek, A., Wozniak, M., Cell context-dependent activities of parthenolide in primary and metastatic melanoma cells, British Journal of Pharmacology, 2010; 160:1144-57.

[61] Lee, C.S., Kim, Y.J., Lee, S.A., Myung, S.C., Kim, W., Combined Effect of Hsp90 Inhibitor Geldanamycin and Parthenolide via Reactive Oxygen Species-Mediated Apoptotic Process on Epithelial Ovarian Cancer Cells, Basic & Clinical Pharmacology & Toxicology, 2012; 111:173-81.

[62] Li, Y., Zhang, Y.L., Fu, M.M., Yao, Q., Zhuo, H.Q., Lu, Q.Y., Niu, X., Zhang, P., Pei, Y., Zhang, K.J., Parthenolide induces apoptosis and lytic cytotoxicity in Epstein-Barr virus-positive Burkitt lymphoma, Molecular Medicine Reports, 2012; 6:477-82.

[63] Liu, J.W., Cai, M.X., Xin, Y., Wu, Q.S., Ma, J., Yang, P., Xie, H.Y., Huang, D.S., Parthenolide induces proliferation inhibition and apoptosis of pancreatic cancer cells in vitro, Journal of Experimental & Clinical Cancer Research, 2010; 29:1-7.

[64] Nakabayashi, H., Shimizu, K., Involvement of Akt/NF-kappa B pathway in antitumor effects of parthenolide on glioblastoma cells in vitro and in vivo, BMC Cancer, 2012; 12.

[65] Shanmugam, R., Kusumanchi, P., Appaiah, H., Cheng, L.A., Crooks, P., Neelakantan, S., Peat, T., Klaunig, J., Matthews, W., Nakshatri, H., Sweeney, C.J., A water soluble parthenolide analog suppresses in vivo tumor growth of two tobacco-associated cancers, lung and bladder cancer, by targeting NF-kappa B and generating reactive oxygen species, International Journal of Cancer, 2011; 128:2481-94.

[66] Sohma, I., Fujiwara, Y., Sugita, Y., Yoshioka, A., Shirakawa, M., Moon, J.H.,

Takiguchi, S., Miyata, H., Yamasaki, M., Mori, M., Doki, Y., Parthenolide, An NF-kappa B Inhibitor, Suppresses Tumor Growth and Enhances Response to Chemotherapy in Gastric Cancer, Cancer Genomics & Proteomics, 2011; 8:39-47.

[67] Sun, Y.L., Clair, D.K.S., Xu, Y., Crooks, P.A., Clair, W.H.S., A NADPH Oxidase-Dependent Redox Signaling Pathway Mediates the Selective Radiosensitization Effect of Parthenolide in Prostate Cancer Cells, Cancer Research, 2010; 70:2880-90.

[68] Watson, C., Miller, D.A., Chin-Sinex, H., Losch, A., Hughes, W., Sweeney, C., Mendonca, M.S., Suppression of NF-kappa B Activity by Parthenolide Induces X-Ray Sensitivity through Inhibition of Split-Dose Repair in TP53 Null Prostate Cancer Cells, Radiation Research, 2009; 171:389-96.

[69] Wyrebska, A., Gach, K., Szemraj, J., Szewczyk, K., Hrabec, E., Koszuk, J., Janecki, T., Janecka, A., Comparison of Anti-Invasive Activity of Parthenolide and 3-Isopropyl-2-Methyl-4-Methyleneisoxazolidin-5-One (MZ-6) - A New Compound with alpha-Methylene-gamma-Lactone Motif - on Two Breast Cancer Cell Lines, Chemical Biology & Drug Design, 2012; 79:112-20.

[70] Wyrebska, A., Szymanski, J., Gach, K., Piekielna, J., Koszuk, J., Janecki, T., Janecka, A., Apoptosis-mediated cytotoxic effects of parthenolide and the new synthetic analog MZ-6 on two breast cancer cell lines, Molecular Biology Reports, 2013; 40:1655-63.

[71] Xu, Y., Fang, F., Miriyala, S., Crooks, P.A., Oberley, T.D., Chaiswing, L., Noel, T., Holley, A.K., Zhao, Y., Kiningham, K.K., Clair, D.K., Clair, W.H., KEAP1 Is a Redox Sensitive Target That Arbitrates the Opposing Radiosensitive Effects of

Parthenolide in Normal and Cancer Cells, Cancer Research, 2013; 73:4406-17.

[72] Yun, B.R., Lee, M.J., Kim, J.H., Kim, I.H., Yu, G.R., Kim, D.G., Enhancement of parthenolide-induced apoptosis by a PKC-alpha inhibition through heme oxygenase-1 blockage in cholangiocarcinoma cells, Experimental and Molecular Medicine, 2010; 42:787-97.

[73] Zhang, D.L., Qiu, L., Jin, X.Q., Guo, Z.H., Guo, C.B., Nuclear Factor-kappa B Inhibition by Parthenolide Potentiates the Efficacy of Taxol in Non-Small Cell Lung Cancer In vitro and In vivo, Molecular Cancer Research, 2009; 7:1139-49.

[74] Zhao, L.J., Xu, Y.H., Li, Y., Effect of parthenolide on proliferation and apoptosis in gastric cancer cell line SGC7901, Journal of Digestive Diseases, 2009; 10:172-80.

[75] Chun, J., Choi, R.J., Khan, S., Lee, D.S., Kim, Y.C., Nam, Y.J., Lee, D.U., Kim, Y.S., Alantolactone suppresses inducible nitric oxide synthase and cyclooxygenase-2 expression by down-regulating NF-kappa B, MAPK and AP-1 via the MyD88 signaling pathway in LPS-activated RAW 264.7 cells, International Immunopharmacology, 2012; 14:375-83.

[76] Khan, M., Li, T., Khan, M.K.A., Rasul, A., Nawaz, F., Sun, M.Y., Zheng, Y., Ma, T.H., Alantolactone Induces Apoptosis in HepG2 Cells through GSH Depletion, Inhibition of STAT3 Activation, and Mitochondrial Dysfunction, Biomed Research International, 2013.

[77] Khan, M., Yi, F., Rasul, A., Li, T., Wang, N., Gao, H.W., Gao, R., Ma, T.H., Alantolactone induces apoptosis in glioblastoma cells via GSH depletion, ROS generation, and mitochondrial dysfunction, Iubmb Life, 2012; 64:783-94.

[78] Lei, J.C., Yu, J.Q., Yin, Y., Liu, Y.W., Zou, G.L., Alantolactone induces

activation of apoptosis in human hepatoma cells, Food and Chemical Toxicology, 2012; 50:3313-19.

[79] Seo, J.Y., Lim, S.S., Kim, J.R., Lim, J.S., Ha, Y.R., Lee, I.A., Kim, E.J., Park, J.H., Kim, J.S., Nrf2-mediated Induction of Detoxifying Enzymes by Alantolactone Present in Inula helenium, Phytotherapy Research, 2008; 22:1500-05.

[80] Shi, Y., Bao, Y.L., Wu, Y., Yu, C.L., Huang, Y.X., Sun, Y., Zheng, L.H., Li, Y.X., Alantolactone Inhibits Cell Proliferation by Interrupting the Interaction between Cripto-1 and Activin Receptor Type II A in Activin Signaling Pathway, Journal of Biomolecular Screening, 2011; 16:525-35.

[81] Wei, W., Huang, H., Zhao, S., Liu, W., Liu, C.X., Chen, L., Li, J.M., Wu, Y.L., Yan, H., Alantolactone induces apoptosis in chronic myelogenous leukemia sensitive or resistant to imatinib through NF-kappa B inhibition and Bcr/Abl protein deletion, Apoptosis, 2013; 18:1060-70.

[82] Rasul, A., Khan, M., Ali, M., Li, J., Li, X., Targeting apoptosis pathways in cancer with alantolactone and isoalantolactone, Scientific World Journal, 2013; 2013:248532.

[83] Seo, J.Y., Park, J., Kim, H.J., Lee, I.A., Lim, J.S., Lim, S.S., Choi, S.J., Park, J.H., Kang, H.J., Kim, J.S., Isoalantolactone from Inula helenium caused Nrf2-mediated induction of detoxifying enzymes, Journal of Medicinal Food, 2009; 12:1038-45.

[84] Bu, H.Q., Luo, J., Chen, H., Zhang, J.H., Li, H.H., Guo, H.C., Wang, Z.H., Lin, S.Z., Oridonin enhances antitumor activity of gemcitabine in pancreatic cancer through MAPK-p38 signaling pathway, International Journal of Oncology, 2012;

41:949-58.

[85] Cheng, Y., Qiu, F., Ikejima, T., Molecular mechanisms of oridonin-induced apoptosis and autophagy in murine fibrosarcoma L929 cells, Autophagy, 2009; 5:430-31.

[86] Du, Y., Villeneuve, N.F., Wang, X.J., Sun, Z., Chen, W.M., Li, J., Lou, H., Wong, P.K., Zhang, D.D., Oridonin confers protection against arsenic-induced toxicity through activation of the Nrf2-mediated defensive response, Environmental Health Perspectives, 2008; 116:1154-61.

[87] Gao, F.H., Hu, X.H., Li, W., Liu, H., Zhang, Y.J., Guo, Z.Y., Xu, M.H., Wang, S.T., Jiang, B., Liu, F., Zhao, Y.Z., Fang, Y., Chen, F.Y., Wu, Y.L., Oridonin induces apoptosis and senescence in colorectal cancer cells by increasing histone hyperacetylation and regulation of p16, p21, p27 and c-myc, BMC Cancer, 2010; 10.

[88] Hu, H.Z., Yang, Y.B., Xu, X.D., Shen, H.W., Shu, Y.M., Ren, Z., Li, X.M., Shen, H.M., Zeng, H.T., Oridonin induces apoptosis via PI3K/Akt pathway in cervical carcinoma HeLa cell line, Acta Pharmacologica Sinica, 2007; 28:1819-26.

[89] Huang, J., Wu, L.J., Tashiro, S., Onodera, S., Ikejima, T., Reactive oxygen species mediate oridonin-induced HepG2 apoptosis through p53, MAPK, and mitochondrial signaling pathways, Journal of Pharmacological Sciences, 2008; 107:370-79.

[90] Li, C.Y., Wang, E.Q., Cheng, Y., Bao, J.K., Oridonin: An active diterpenoid targeting cell cycle arrest, apoptotic and autophagic pathways for cancer therapeutics, International Journal of Biochemistry & Cell Biology, 2011; 43:701-04.

[91] Li, X., Li, X., Wang, J.X., Ye, Z.Y., Li, J.C., Oridonin Up-regulates Expression

of P21 and Induces Autophagy and Apoptosis in Human Prostate Cancer Cells, International Journal of Biological Sciences, 2012; 8:901-12.

[92] Wang, S.P., Zhong, Z.F., Wan, J.B., Tan, W., Wu, G.S., Chen, M.W., Wang, Y.T., Oridonin Induces Apoptosis, Inhibits Migration and Invasion on Highly-Metastatic Human Breast Cancer Cells, American Journal of Chinese Medicine, 2013; 41:177-96.

[93] Zang, L., He, H., Ye, Y., Liu, W., Fan, S., Tashiro, S., Onodera, S., Ikejima, T., Nitric oxide augments oridonin-induced efferocytosis by human histocytic lymphoma U937 cells via autophagy and the NF-kappaB-COX-2-IL-1beta pathway, Free Radical Research, 2012; 46:1207-19.

[94] Zhang, Y.H., Wu, Y.L., Tashiro, S., Onodera, S., Ikejima, T., Reactive oxygen species contribute to oridonin-induced apoptosis and autophagy in human cervical carcinoma HeLa cells, Acta Pharmacologica Sinica, 2011; 32:1266-75.

[95] Hou, L., Xu, B., Guo, W., Ran, F.X., Liu, J.T., Yuan, X., Fu, H.Z., Cui, J.R., Pseudolaric acid B inhibits inducible cyclooxygenase-2 expression via downregulation of the NF-kappa B pathway in HT-29 cells, Journal of Cancer Research and Clinical Oncology, 2012; 138:885-96.

[96] Li, T., Wong, V.K.W., Yi, X.Q., Wong, Y.F., Zhou, H., Liu, L., Pseudolaric Acid B Suppresses T Lymphocyte Activation Through Inhibition of NF-kappa B Signaling Pathway and p38 Phosphorylation, Journal of Cellular Biochemistry, 2009; 108:87-95.

[97] Tong, J., Yin, S.T., Dong, Y.H., Guo, X., Fan, L.H., Ye, M., Hu, H.B., Pseudolaric Acid B Induces Caspase-Dependent Apoptosis and Autophagic Cell

Death in Prostate Cancer Cells, Phytotherapy Research, 2013; 27:885-91.

[98] Zhao, D.D., Lin, F., Wu, X.D., Zhao, Q.S., Zhao, B.J.H., Lin, P., Zhang, Y., Yu, X.G., Pseudolaric acid B induces apoptosis via proteasome-mediated Bcl-2 degradation in hormone-refractory prostate cancer DU145 cells, Toxicology in Vitro, 2012; 26:595-602.

[99] Benes, P., Alexova, P., Knopfova, L., Spanova, A., Smarda, J., Redox state alters anti-cancer effects of wedelolactone, Environmental and Molecular Mutagenesis, 2012; 53:515-24.

[100] Chen, Z.M., Sun, X.X., Shen, S.S., Zhang, H.H., Ma, X.Q., Liu, J.L., Kuang, S., Yu, Q., Wedelolactone, a Naturally Occurring Coumestan, Enhances Interferon-gamma Signaling through Inhibiting STAT1 Protein Dephosphorylation, Journal of Biological Chemistry, 2013; 288:14417-27.

[101] Kobori, M., Yang, Z., Gong, D., Heissmeyer, V., Zhu, H., Jung, Y.K., Gakidis, M.A., Rao, A., Sekine, T., Ikegami, F., Yuan, C., Yuan, J., Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK Complex, Cell Death and Differentiation, 2004; 11:123-30.

[102] Chao, D.C., Lin, L.J., Hsiang, C.Y., Li, C.C., Lo, H.Y., Liang, J.A., Kao, S.T., Wu, S.L., Ho, T.Y., Evodiamine Inhibits 12-O-Tetradecanoylphorbol-13-acetate-induced Activator Protein 1 Transactivation and Cell Transformation in Human Hepatocytes, Phytotherapy Research, 2011; 25:1018-23.

[103] Chen, M.C., Yu, C.H., Wang, S.W., Pu, H.F., Kan, S.F., Lin, L.C., Chi, C.W., Ho, L.L., Lee, C.H., Wang, P.S., Anti-Proliferative Effects of Evodiamine on Human Thyroid Cancer Cell Line ARO, Journal of Cellular Biochemistry, 2010; 110:1495-

503.

[104] Jiang, J.L., Hu, C.P., Evodiamine: A Novel Anti-Cancer Alkaloid from Evodia rutaecarpa, Molecules, 2009; 14:1852-59.

[105] Pan, X.B., Hartley, J.M., Hartley, J.A., White, K.N., Wang, Z.T., Bligh, S.W.A., Evodiamine, a dual catalytic inhibitor of type I and II topoisomerases, exhibits enhanced inhibition against camptothecin resistant cells, Phytomedicine, 2012; 19:618-24.

[106] Wei, W.T., Chen, H., Wang, Z.H., Ni, Z.L., Liu, H.B., Tong, H.F., Guo, H.C., Liu, D.L., Lin, S.Z., Enhanced Antitumor Efficacy of Gemcitabine by Evodiamine on Pancreatic Cancer via Regulating PI3K/Akt Pathway, International Journal of Biological Sciences, 2012; 8:1-14.

[107] Yang, J., Wu, L.J., Tashino, S.I., Onodera, S., Ikejima, T., Critical roles of reactive oxygen species in mitochondrial permeability transition in mediating evodiamine-induced human melanoma A375-S2 cell apoptosis, Free Radical Research, 2007; 41:1099-108.

[108] Yang, J., Wu, L.J., Tashino, S.I., Onodera, S., Ikejima, T., Reactive oxygen species and nitric oxide regulate mitochondria-dependent apoptosis and autophagy in evodiamine-treated human cervix carcinoma HeLa cells, Free Radical Research, 2008; 42:492-504.

[109] Yang, J., Wu, L.J., Tashiro, S.I., Onodera, S., Ikejima, T., Nitric oxide activated by p38 and NF-kappa B facilitates apoptosis and cell cycle arrest under oxidative stress in evodiamine-treated human melanoma A375-S2 cells, Free Radical Research, 2008; 42:1-11.

[110] Anand, P., Sung, B., Kunnumakkara, A.B., Rajasekharan, K.N., Aggarwal, B.B., Suppression of pro-inflammatory and proliferative pathways by diferuloylmethane (curcumin) and its analogues dibenzoylmethane, dibenzoylpropane, and dibenzylideneacetone: role of Michael acceptors and Michael donors, Biochemical Pharmacology, 2011; 82:1901-9.

[111] Bang, S.Y., Kim, J.H., Kim, H.Y., Lee, Y.J., Park, S.Y., Lee, S.J., Kim, Y., Achyranthes japonica exhibits anti-inflammatory effect via NF-kappa B suppression and HO-1 induction in macrophages, Journal of Ethnopharmacology, 2012; 144:109-17.

[112] Baud, V., Karin, M., Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls, Nature Reviews Drug Discovery, 2009; 8:33-40.

[113] DiDonato, J.A., Mercurio, F., Karin, M., NF-kappaB and the link between inflammation and cancer, Immunological Reviews, 2012; 246:379-400.

[114] Harikumar, K.B., Sung, B.Y., Tharakan, S.T., Pandey, M.K., Joy, B., Guha, S., Krishnan, S., Aggarwal, B.B., Sesamin Manifests Chemopreventive Effects through the Suppression of NF-kappa B-Regulated Cell Survival, Proliferation, Invasion, and Angiogenic Gene Products, Molecular Cancer Research, 2010; 8:751-61.

[115] Hayden, M.S., Ghosh, S., NF-kappaB in immunobiology, Cell Research, 2011; 21:223-44.

[116] Oeckinghaus, A., Ghosh, S., The NF-kappaB family of transcription factors and its regulation, Cold Spring Harbor Perspectives in Biology, 2009; 1:a000034.

[117] Park, K.K., Chun, K.S., Lee, J.M., Lee, S.S., Surh, Y.J., Inhibitory effects of [6]-gingerol, a major pungent principle of ginger, on phorbol ester-induced

inflammation, epidermal ornithine decarboxylase activity and skin tumor promotion in ICR mice, Cancer Letters, 1998; 129:139-44.

[118] Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B.B., Oxidative stress, inflammation, and cancer: how are they linked?, Free Radical Biology & Medicine, 2010; 49:1603-16.

[119] Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C.J., Foresti, R., Alam, J., Motterlini, R., Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element, Biochemical Journal, 2003; 371:887-95.

[120] Halliwell, B., and Gutteridge, J. M. C., Free Radicals in Biology and Medicine, Oxford University Press, Oxford, 2015.

[121] Hayes, J.D., McMahon, M., Chowdhry, S., Dinkova-Kostova, A.T., Cancer Chemoprevention Mechanisms Mediated Through the Keap1-Nrf2 Pathway, Antioxidants & Redox Signaling, 2010; 13:1713-48.

[122] Kensler, T.W., Wakabayashi, N., Nrf2: friend or foe for chemoprevention?, Carcinogenesis, 2010; 31:90-99.

[123] Li, W.J., Guo, Y., Zhang, C.Y., Wu, R.Y., Yang, A.Y., Gaspar, J., Kong, A.N.T., Dietary Phytochemicals and Cancer Chemoprevention: A Perspective on Oxidative Stress, Inflammation, and Epigenetics, Chemical Research in Toxicology, 2016; 29:2071-95.

[124] Ramos-Gomez, M., Kwak, M.K., Dolan, P.M., Itoh, K., Yamamoto, M., Talalay, P., Kensler, T.W., Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-

deficient mice, Proceedings of the National Academy of Sciences of the United States of America, 2001; 98:3410-15.

[125] Reuland, D.J., Khademi, S., Castle, C.J., Irwin, D.C., McCord, J.M., Miller, B.F., Hamilton, K.L., Upregulation of phase II enzymes through phytochemical activation of Nrf2 protects cardiomyocytes against oxidant stress, Free Radical Biology and Medicine, 2013; 56:102-11.

[126] Saw, C.L., Wu, Q., Su, Z.Y., Wang, H., Yang, Y., Xu, X., Huang, Y., Khor, T.O., Kong, A.N., Effects of natural phytochemicals in Angelica sinensis (Danggui) on Nrf2-mediated gene expression of phase II drug metabolizing enzymes and anti-inflammation, Biopharmaceutics & Drug Disposition, 2013; 34:303-11.

[127] Sporn, M.B., Liby, K.T., NRF2 and cancer: the good, the bad and the importance of context, Nature Reviews Cancer, 2012; 12:564-71.

[128] Suzuki, T., Wakai, K., Matsuo, K., Hirose, K., Ito, H., Kuriki, K., Sato, S., Ueda, R., Hasegawa, Y., Tajima, K., Effect of dietary antioxidants and risk of oral, pharyngeal and laryngeal squamous cell carcinoma according to smoking and drinking habits, Cancer Science, 2006; 97:760-67.

[129] Saud, S.M., Young, M.R., Jones-Hall, Y.L., Ileva, L., Evbuomwan, M.O., Wise, J., Colburn, N.H., Kim, Y.S., Bobe, G., Chemopreventive activity of plant flavonoid isorhamnetin in colorectal cancer is mediated by oncogenic Src and beta-catenin, Cancer Research, 2013; 73:5473-84.

[130] Verma, S., Das, P., Kumar, V.L., Chemoprevention by artesunate in a preclinical model of colorectal cancer involves down regulation of beta-catenin, suppression of angiogenesis, cellular proliferation and induction of apoptosis,

Chemico-Biological Interactions, 2017; 278:84-91.

[131] Lin, L.C., Yeh, C.T., Kuo, C.C., Lee, C.M., Yen, G.C., Wang, L.S., Wu, C.H., Yang, W.C., Wu, A.T.H., Sulforaphane Potentiates the Efficacy of Imatinib against Chronic Leukemia Cancer Stem Cells through Enhanced Abrogation of Wnt/beta-Catenin Function, Journal of Agricultural and Food Chemistry, 2012; 60:7031-39.

[132] Boyanapalli, S.S., Tony Kong, A.N., "Curcumin, the King of Spices": Epigenetic Regulatory Mechanisms in the Prevention of Cancer, Neurological, and Inflammatory Diseases, Current Pharmacology Reports, 2015; 1:129-39.

[133] Shukla, S., Gupta, S., Apigenin: a promising molecule for cancer prevention, Pharmaceutical Research, 2010; 27:962-78.

[134] Wong, C.P., Hsu, A., Buchanan, A., Palomera-Sanchez, Z., Beaver, L.M., Houseman, E.A., Williams, D.E., Dashwood, R.H., Ho, E., Effects of sulforaphane and 3,3'-diindolylmethane on genome-wide promoter methylation in normal prostate epithelial cells and prostate cancer cells, Plos One, 2014; 9:e86787.

[135] Sporn, M.B., Liby, K.T., Cancer chemoprevention: scientific promise, clinical uncertainty, Nature Clinical Practice Oncology, 2005; 2:518-25.

[136] Surh, Y.J., Cancer chemoprevention with dietary phytochemicals, Nature Reviews Cancer, 2003; 3:768-80.

[137] Lee, J.M., Li, J., Johnson, D.A., Stein, T.D., Kraft, A.D., Calkins, M.J., Jakel, R.J., Johnson, J.A., Nrf2, a multi-organ protector?, Faseb Journal, 2005; 19:1061-66.

[138] Shin, S.M., Yang, J.H., Ki, S.H., Role of the Nrf2-ARE Pathway in Liver Diseases, Oxidative Medicine and Cellular Longevity, 2013.

[139] Bataille, A.M., Manautou, J.E., Nrf2: A Potential Target for New Therapeutics

in Liver Disease, Clinical Pharmacology & Therapeutics, 2012; 92:340-48.

[140] Ma, Q., Role of nrf2 in oxidative stress and toxicity, Annual Review of Pharmacology and Toxicology, 2013; 53:401-26.

[141] Moi, P., Chan, K., Asunis, I., Cao, A., Kan, Y.W., Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region, Proceedings of the National Academy of Sciences of the United States of America, 1994; 91:9926-30.

[142] Motohashi, H., Yamamoto, M., Nrf2-Keap1 defines a physiologically important stress response mechanism, Trends in Molecular Medicine, 2004; 10:549-57.

[143] Dhakshinamoorthy, S., Jaiswal, A.K., Small maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:Quinone oxidoreductase1 gene, Journal of Biological Chemistry, 2000; 275:40134-41.

[144] Nioi, P., McMahon, M., Itoh, K., Yamamoto, M., Hayes, J.D., Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence, Biochemical Journal, 2003; 374:337-48.

[145] Prestera, T., Talalay, P., Electrophile and antioxidant regulation of enzymes that detoxify carcinogens, Proceedings of the National Academy of Sciences of the United States of America, 1995; 92:8965-9.

[146] Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D.,

Yamamoto, M., Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, Genes & Development, 1999; 13:76-86.

[147] Niture, S.K., Khatri, R., Jaiswal, A.K., Regulation of Nrf2-an update, Free Radical Biology & Medicine, 2014; 66:36-44.

[148] Bryan, H.K., Olayanju, A., Goldring, C.E., Park, B.K., The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation, Biochemical Pharmacology, 2013; 85:705-17.

[149] Zipper, L.M., Mulcahy, R.T., Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells, Toxicological Sciences, 2003; 73:124-34.

[150] Huang, H.C., Nguyen, T., Pickett, C.B., Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription, Journal of Biological Chemistry, 2002; 277:42769-74.

[151] Liu, X.M., Peyton, K.J., Shebib, A.R., Wang, H., Korthuis, R.J., Durante, W., Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival, American Journal of Physiology-Heart and Circulatory Physiology, 2011; 300:H84-H93.

[152] Grimes, C.A., Jope, R.S., The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling, Progress in Neurobiology, 2001; 65:391-426.

[153] Jain, A.K., Jaiswal, A.K., GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2, Journal of Biological

Chemistry, 2007; 282:16502-10.

[154] Jain, A.K., Jaiswal, A.K., Phosphorylation of tyrosine 568 controls nuclear export of Nrf2, Journal of Biological Chemistry, 2006; 281:12132-42.

[155] Trachootham, D., Lu, W.Q., Ogasawara, M.A., Valle, N.R.D., Huang, P., Redox regulation of cell survival, Antioxidants & Redox Signaling, 2008; 10:1343-74.

[156] Trachootham, D., Alexandre, J., Huang, P., Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?, Nature Reviews Drug Discovery, 2009; 8:579-91.

[157] Wasserman, W.W., Fahl, W.E., Functional antioxidant responsive elements, Proceedings of the National Academy of Sciences of the United States of America, 1997; 94:5361-66.

[158] Menegon, S., Columbano, A., Giordano, S., The Dual Roles of NRF2 in Cancer, Trends in Molecular Medicine, 2016; 22:578-93.

[159] Hu, R., Saw, C.L.L., Yu, R., Kong, A.N.T., Regulation of NF-E2-Related Factor 2 Signaling for Cancer Chemoprevention: Antioxidant Coupled with Antiinflammatory, Antioxidants & Redox Signaling, 2010; 13:1679-98.

[160] Iida, K., Itoh, K., Kumagai, Y., Oyasu, R., Hattori, K., Kawai, K., Shimazui, T., Akaza, H., Yamamoto, M., Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis, Cancer Research, 2004; 64:6424-31.

[161] Ramos-Gomez, M., Dolan, P.M., Itoh, K., Yamamoto, M., Kensler, T.W., Interactive effects of nrf2 genotype and oltipraz on benzo[a]pyrene-DNA adducts and tumor yield in mice, Carcinogenesis, 2003; 24:461-67.

[162] Fahey, J.W., Haristoy, X., Dolan, P.M., Kensler, T.W., Scholtus, I., Stephenson, K.K., Talalay, P., Lozniewski, A., Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors, Proceedings of the National Academy of Sciences of the United States of America, 2002; 99:7610-15.

[163] Vallabhapurapu, S., Karin, M., Regulation and function of NF-kappaB transcription factors in the immune system, Annual Review of Immunology, 2009; 27:693-733.

[164] Baichwal, V.R., Baeuerle, P.A., Activate NF-kappa B or die?, Current Biology, 1997; 7:R94-6.

[165] Pahl, H.L., Activators and target genes of Rel/NF-kappaB transcription factors, Oncogene, 1999; 18:6853-66.

[166] Wang, T., Zhang, X., Li, J.J., The role of NF-kappaB in the regulation of cell stress responses, International Immunopharmacology, 2002; 2:1509-20.

[167] Beyaert, R., Van Loo, G., Heyninck, K., Vandenabeele, P., Signaling to gene activation and cell death by tumor necrosis factor receptors and Fas, International Review of Cytology, 2002; 214:225-72.

[168] Chen, C.C., Manning, A.M., Transcriptional regulation of endothelial cell adhesion molecules: a dominant role for NF-kappa B, Agents and Actions Supplements, 1995; 47:135-41.

[169] Kopp, E.B., Ghosh, S., NF-kappa B and rel proteins in innate immunity, Advances in Immunology, 1995; 58:1-27.

[170] Wissink, S., van de Stolpe, A., Caldenhoven, E., Koenderman, L., van der Saag,

P.T., NF-kappa B/Rel family members regulating the ICAM-1 promoter in monocytic THP-1 cells, Immunobiology, 1997; 198:50-64.

[171] Elinav, E., Nowarski, R., Thaiss, C.A., Hu, B., Jin, C.C., Flavell, R.A., Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms, Nature Reviews Cancer, 2013; 13:759-71.

[172] Jedinak, A., Dudhgaonkar, S., Sliva, D., Activated macrophages induce metastatic behavior of colon cancer cells, Immunobiology, 2010; 215:242-9.

[173] Thejass, P., Kuttan, G., Inhibition of endothelial cell differentiation and proinflammatory cytokine production during angiogenesis by allyl isothiocyanate and phenyl isothiocyanate, Integrative Cancer Therapies, 2007; 6:389-99.

[174] Ben-Neriah, Y., Karin, M., Inflammation meets cancer, with NF-kappa B as the matchmaker, Nature Immunology, 2011; 12:715-23.

[175] Kuraishy, A., Karin, M., Grivennikov, S.I., Tumor Promotion via Injury- and Death-Induced Inflammation, Immunity, 2011; 35:467-77.

[176] Colotta, F., Allavena, P., Sica, A., Garlanda, C., Mantovani, A., Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability, Carcinogenesis, 2009; 30:1073-81.

[177] Blaylock, R.L., Cancer microenvironment, inflammation and cancer stem cells: A hypothesis for a paradigm change and new targets in cancer control, Surgical Neurology International, 2015; 6:92.

[178] Federico, A., Morgillo, F., Tuccillo, C., Ciardiello, F., Loguercio, C., Chronic inflammation and oxidative stress in human carcinogenesis, International Journal of Cancer, 2007; 121:2381-6.

[179] Ono, M., Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy, Cancer Science, 2008; 99:1501-06.

[180] Chiodoni, C., Colombo, M.P., Sangaletti, S., Matricellular proteins: from homeostasis to inflammation, cancer, and metastasis, Cancer and Metastasis Reviews, 2010; 29:295-307.

[181] Valenta, T., Hausmann, G., Basler, K., The many faces and functions of beta-catenin, The EMBO Journal, 2012; 31:2714-36.

[182] Clevers, H., Wnt/beta-catenin signaling in development and disease, Cell, 2006; 127:469-80.

[183] Morin, P.J., beta-catenin signaling and cancer, Bioessays, 1999; 21:1021-30.

[184] Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., Polakis, P., Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein, Proceedings of the National Academy of Sciences of the United States of America, 1995; 92:3046-50.

[185] Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., Polakis, P., Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly, Science, 1996; 272:1023-6.

[186] Polakis, P., Wnt signaling and cancer, Genes & Development, 2000; 14:1837-51.

[187] Satoh, S., Daigo, Y., Furukawa, Y., Kato, T., Miwa, N., Nishiwaki, T., Kawasoe, T., Ishiguro, H., Fujita, M., Tokino, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., Yamaoka, Y., Nakamura, Y., AXIN1 mutations in hepatocellular carcinomas, and

growth suppression in cancer cells by virus-mediated transfer of AXIN1, Nature Genetics, 2000; 24:245-50.

[188] Novak, A., Dedhar, S., Signaling through beta-catenin and Lef/Tcf, Cellular and Molecular Life Sciences, 1999; 56:523-37.

[189] Kolligs, F.T., Nieman, M.T., Winer, I., Hu, G., Van Mater, D., Feng, Y., Smith, I.M., Wu, R., Zhai, Y., Cho, K.R., Fearon, E.R., ITF-2, a downstream target of the Wnt/TCF pathway, is activated in human cancers with beta-catenin defects and promotes neoplastic transformation, Cancer Cell, 2002; 1:145-55.

[190] Wong, N.A., Pignatelli, M., Beta-catenin--a linchpin in colorectal carcinogenesis?, American Journal of Pathology, 2002; 160:389-401.

[191] Araki, Y., Okamura, S., Hussain, S.P., Nagashima, M., He, P., Shiseki, M., Miura, K., Harris, C.C., Regulation of cyclooxygenase-2 expression by the Wnt and ras pathways, Cancer Research, 2003; 63:728-34.

[192] Polakis, P., Wnt Signaling in Cancer, Cold Spring Harbor Perspectives in Biology, 2012; 4.

[193] Geyer, F.C., Lacroix-Triki, M., Savage, K., Arnedos, M., Lambros, M.B., MacKay, A., Natrajan, R., Reis-Filho, J.S., beta-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation, Modern Pathology, 2011; 24:209-31.

[194] Khramtsov, A.I., Khramtsova, G.F., Tretiakova, M., Huo, D.Z., Olopade, O.I., Goss, K.H., Wnt/beta-Catenin Pathway Activation Is Enriched in Basal-Like Breast Cancers and Predicts Poor Outcome, American Journal of Pathology, 2010; 176:2911-20.

[195] Xu, J.H., Prosperi, J.R., Choudhury, N., Olopade, O.I., Goss, K.H., beta-Catenin Is Required for the Tumorigenic Behavior of Triple-Negative Breast Cancer Cells, Plos One, 2015; 10.

[196] Yang, L., Wu, X., Wang, Y., Zhang, K., Wu, J., Yuan, Y.C., Deng, X., Chen, L., Kim, C.C., Lau, S., Somlo, G., Yen, Y., FZD7 has a critical role in cell proliferation in triple negative breast cancer, Oncogene, 2011; 30:4437-46.

[197] Liu, C.C., Prior, J., Piwnica-Worms, D., Bu, G.J., LRP6 overexpression defines a class of breast cancer subtype and is a target for therapy, Proceedings of the National Academy of Sciences of the United States of America, 2010; 107:5136-41.

[198] Lento, W., Congdon, K., Voermans, C., Kritzik, M., Reya, T., Wnt Signaling in Normal and Malignant Hematopoiesis, Cold Spring Harbor Perspectives in Biology, 2013; 5.

[199] Gattinoni, L., Ji, Y., Restifo, N.P., Wnt/beta-catenin signaling in T-cell immunity and cancer immunotherapy, Clinical Cancer Research, 2010; 16:4695-701.

[200] Judzentiene, A., Budiene, J., Volatile oils of flowers and stems of Tussilago farfara L. from Lithuania, Journal of Essential Oil Bearing Plants, 2011; 14:413-16.

[201] Barceloux, D.G., Coltsfoot (Tussilago farfara L.), Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants, and Venomous Animals, 2008:446-48.

[202] Hwangbo, C., Lee, H.S., Park, J., Choe, J., Lee, J.H., The anti-inflammatory effect of tussilagone, from Tussilago farfara, is mediated by the induction of heme oxygenase-1 in murine macrophages, International Immunopharmacology, 2009; 9:1578-84.

[203] Kim, M.R., Lee, J.Y., Lee, H.H., Aryal, D.K., Kim, Y.G., Kim, S.K., Woo, E.R., Kang, K.W., Antioxidative effects of quercetin-glycosides isolated from the flower buds of Tussilago farfara L, Food and Chemical Toxicology, 2006; 44:1299-307.

[204] Lim, H.J., Dong, G.Z., Lee, H.J., Ryu, J.H., In vitro neuroprotective activity of sesquiterpenoids from the flower buds of Tussilago farfara, Journal of Enzyme Inhibition and Medicinal Chemistry, 2015; 30:852-6.

[205] Lee, J., Kang, U., Seo, E.K., Kim, Y.S., Heme oxygenase-1-mediated anti-inflammatory effects of tussilagonone on macrophages and 12-O-tetradecanoylphorbol-13-acetate-induced skin inflammation in mice, International Immunopharmacology, 2016; 34:155-64.

[206] Okuda T. (ed.), Encyclopedia of Natural Medicine, Hirokawa, Tokyo, Japan, 1986.

[207] China, E.b.o.F.o., Flora of China 75, Science Press, PR China, Beijing, 1979.

[208] Commission, C.P., Chinese Pharmacopoeia 1., China Medical Science Press, PR China, Beijing, 2015.

[209] Gao, S., Wang, Q., Tian, X.H., Li, H.L., Shen, Y.H., Xu, X.K., Wu, G.Z., Hu, Z.L., Zhang, W.D., Total sesquiterpene lactones prepared from Inula helenium L. has potentials in prevention and therapy of rheumatoid arthritis, Journal of Ethnopharmacology, 2017; 196:39-46.

[210] Chun, J., Choi, R.J., Khan, S., Lee, D.S., Kim, Y.C., Nam, Y.J., Lee, D.U., Kim, Y.S., Alantolactone suppresses inducible nitric oxide synthase and cyclooxygenase-2 expression by down-regulating NF-kappaB, MAPK and AP-1 via the MyD88 signaling pathway in LPS-activated RAW 264.7 cells, International

Immunopharmacology, 2012; 14:375-83.

[211] Jung, S.M., Choi, S.I., Park, S.M., Heo, T.R., Synergistic antimicrobial effect of Achyranthes japonica Nakai extracts and Bifidobacterium supernatants against Clostridium difficile, Food Science and Biotechnology, 2008; 17:402-07.

[212] Ahn, D.K., Illustrated Book of Korean Medicinal Herbs., Kyohak Publishing Co.2003.

[213] Park, J.H., Kang, S.N., Shin, D., Hur, I.C., Kim, I.S., Jin, S.K., Antioxidant Activities of Achyranthes japonica Nakai Extract and Its Application to the Pork Sausages, Asian-Australasian Journal of Animal Sciences, 2013; 26:287-94.

[214] Yun-Choi, H.S., Kim, S.O., Kim, J.H., Lee, J.R., Cho, H.I., Modified smear method for screening potential inhibitors of platelet aggregation from plant sources, Journal of Natural Products, 1985; 48:363-70.

[215] Kim, J.C., Choi, G.J., Lee, S.W., Kim, J.S., Chung, K.Y., Cho, K.Y., Screening extracts of Achyranthes japonica and Rumex crispus for activity against various plant pathogenic fungi and control of powdery mildew, Pest Management Science, 2004; 60:803-08.

[216] Finkel, T., Signal transduction by reactive oxygen species, Journal of Cell Biology, 2011; 194:7-15.

[217] Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., Telser, J., Free radicals and antioxidants in normal physiological functions and human disease, International Journal of Biochemistry & Cell Biology, 2007; 39:44-84.

[218] Rodriguez, R., Redman, R., Balancing the generation and elimination of reactive oxygen species, Proceedings of the National Academy of Sciences of the

United States of America, 2005; 102:3175-76.

[219] Beg, A.A., Baltimore, D., An essential role for NF-kappa B in preventing TNF-alpha-induced cell death, Science, 1996; 274:782-84.

[220] Manson, M.M., Cancer prevention -- the potential for diet to modulate molecular signalling, Trends in Molecular Medicine, 2003; 9:11-8.

[221] Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., Baldwin, A.S., NF-kappa B antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation, Science, 1998; 281:1680-83.

[222] Davies, R.J., Miller, R., Coleman, N., Colorectal cancer screening: prospects for molecular stool analysis, Nature Reviews Cancer, 2005; 5:199-209.

[223] Watson, A.J., An overview of apoptosis and the prevention of colorectal cancer, Critical Reviews in Oncology/Hematology, 2006; 57:107-21.

[224] Fearon, E.R., Molecular genetics of colorectal cancer, Annual Review of Pathology, 2011; 6:479-507.

[225] Barker, N., Clevers, H., Mining the Wnt pathway for cancer therapeutics, Nature Reviews Drug Discovery, 2006; 5:997-1014.

[226] Klaus, A., Birchmeier, W., Wnt signalling and its impact on development and cancer, Nature Reviews Cancer, 2008; 8:387-98.

[227] Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., Kinzler, K.W., Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC, Science, 1997; 275:1787-90.

[228] Cadigan, K.M., Nusse, R., Wnt signaling: a common theme in animal development, Genes & Development, 1997; 11:3286-305.

[229] He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., Kinzler, K.W., Identification of c-MYC as a target of the APC pathway, Science, 1998; 281:1509-12.

[230] Mann, B., Gelos, M., Siedow, A., Hanski, M.L., Gratchev, A., Ilyas, M., Bodmer, W.F., Moyer, M.P., Riecken, E.O., Buhr, H.J., Hanski, C., Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas, Proceedings of the National Academy of Sciences of the United States of America, 1999; 96:1603-8.

[231] Tetsu, O., McCormick, F., Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells, Nature, 1999; 398:422-6.

[232] Prochaska, H.J., Talalay, P., Purification and characterization of two isofunctional forms of NAD(P)H: quinone reductase from mouse liver, Journal of Biological Chemistry, 1986; 261:1372-8.

[233] Kwon, T.Y., Tussilagonone from *Tussilago farfara* attenuates t-BHP-induced oxidative stress through Nrf2-dependent pathway in HepG2 cells, pharmacology, Seoul National University, Seoul, 2015.

[234] Lee, S.B., Kim, C.Y., Lee, H.J., Yun, J.H., Nho, C.W., Induction of the Phase II Detoxification Enzyme NQO1 in Hepatocarcinoma Cells by Lignans from the Fruit of Schisandra chinensis through Nuclear Accumulation of Nrf2, Planta Medica, 2009; 75:1314-18.

[235] Sun, Z., Huang, Z.P., Zhang, D.D., Phosphorylation of Nrf2 at Multiple Sites by MAP Kinases Has a Limited Contribution in Modulating the Nrf2-Dependent Antioxidant Response, Plos One, 2009; 4.

[236] Surh, Y.J., Kundu, J.K., Na, H.K., Nrf2 as a Master Redox Switch in Turning on the Cellular Signaling Involved in the Induction of Cytoprotective Genes by Some Chemopreventive Phytochemicals, Planta Medica, 2008; 74:1526-39.

[237] Bloom, D.A., Jaiswal, A.K., Phosphorylation of Nrf2 at Ser(40) by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H : quinone oxidoreductase-1 gene expression, Journal of Biological Chemistry, 2003; 278:44675-82.

[238] Tak, P.P., Firestein, G.S., NF-kappaB: a key role in inflammatory diseases, Journal of Clinical Investigation, 2001; 107:7-11.

[239] Diehl, J.A., Cycling to cancer with cyclin D1, Cancer Biology & Therapy, 2002; 1:226-31.

[240] Tarapore, R.S., Siddiqui, I.A., Mukhtar, H., Modulation of Wnt/beta-catenin signaling pathway by bioactive food components, Carcinogenesis, 2012; 33:483-91.

[241] Lee, S.B., Cha, K.H., Selenge, D., Solongo, A., Nho, C.W., The chemopreventive effect of taxifolin is exerted through ARE-dependent gene regulation, Biological & Pharmaceutical Bulletin, 2007; 30:1074-79.

[242] Lee, S.B., Kang, K., Oidovsambuu, S., Jho, E.H., Yun, J.H., Yoo, J.H., Lee, E.H., Pan, C.H., Lee, J.K., Jung, S.H., Nho, C.W., A polyacetylene from Gymnaster koraiensis exerts hepatoprotective effects in vivo and in vitro, Food and Chemical Toxicology, 2010; 48:3035-41.

[243] Yun, J.H., Lee, S.B., Lee, H.J., Kim, C.Y., Kim, M.A., Sohn, Y.C., Nho, C.W.,

Bi-functional induction of the quinone reductase and cytochrome P450 1A1 by youngiasides via Nrf2-ARE and AhR-XRE pathways, Biological and Pharmaceutical Bulletin, 2010; 33:1650-7.

[244] Esposito, F., Ammendola, R., Faraonio, R., Russo, T., Cimino, F., Redox control of signal transduction, gene expression and cellular senescence, Neurochemical Research, 2004; 29:617-28.

[245] Lima, C.F., Valentao, P.C., Andrade, P.B., Seabra, R.M., Fernandes-Ferreira, M., Pereira-Wilson, C., Water and methanolic extracts of Salvia officinalis protect HepG2 cells from t-BHP induced oxidative damage, Chemico-Biological Interactions, 2007; 167:107-15.

[246] Zhu, R.Z., Wang, Y.J., Zhang, L.Q., Guo, Q.L., Oxidative stress and liver disease, Hepatology Research, 2012; 42:741-49.

[247] Jain, A.K., Jaiswal, A.K., GSK-3 beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2, Journal of Biological Chemistry, 2007; 282:16502-10.

[248] Heinrich, M., Robles, M., West, J.E., Ortiz de Montellano, B.R., Rodriguez, E., Ethnopharmacology of Mexican asteraceae (Compositae), Annual Review of Pharmacology and Toxicology, 1998; 38:539-65.

[249] Seo, J.Y., Lim, S.S., Kim, J.R., Lim, J.S., Ha, Y.R., Lee, I.A., Kim, E.J., Park, J.H., Kim, J.S., Nrf2-mediated induction of detoxifying enzymes by alantolactone present in Inula helenium, Phytotherapy Research, 2008; 22:1500-5.

[250] Woodgett, J.R., Recent advances in the protein kinase B signaling pathway, Current Opinion in Cell Biology, 2005; 17:150-7.

[251] Maggirwar, S.B., Tong, N., Ramirez, S., Gelbard, H.A., Dewhurst, S., HIV-1 Tat-mediated activation of glycogen synthase kinase-3beta contributes to Tat-mediated neurotoxicity, Journal of Neurochemistry, 1999; 73:578-86.

[252] Takashima, A., Noguchi, K., Michel, G., Mercken, M., Hoshi, M., Ishiguro, K., Imahori, K., Exposure of rat hippocampal neurons to amyloid beta peptide (25-35) induces the inactivation of phosphatidyl inositol-3 kinase and the activation of tau protein kinase I/glycogen synthase kinase-3 beta, Neuroscience Letters, 1996; 203:33-6.

[253] Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B, Nature, 1995; 378:785-9.

[254] Liu, G.H., Qu, J., Shen, X., NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK, Biochimica et Biophysica Acta, 2008; 1783:713-27.

[255] Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., Woodgett, J.R., Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation, Nature, 2000; 406:86-90.

[256] He, G., Yu, G.Y., Temkin, V., Ogata, H., Kuntzen, C., Sakurai, T., Sieghart, W., Peck-Radosavljevic, M., Leffert, H.L., Karin, M., Hepatocyte IKKbeta/NF-kappaB inhibits tumor promotion and progression by preventing oxidative stress-driven STAT3 activation, Cancer Cell, 2010; 17:286-97.

[257] Tilg, H., Wilmer, A., Vogel, W., Herold, M., Nolchen, B., Judmaier, G., Huber, C., Serum levels of cytokines in chronic liver diseases, Gastroenterology, 1992;

103:264-74.

[258] Trikha, M., Corringham, R., Klein, B., Rossi, J.F., Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence, Clinical Cancer Research, 2003; 9:4653-65.

[259] He, G., Karin, M., NF-kappaB and STAT3 - key players in liver inflammation and cancer, Cell Research, 2011; 21:159-68.

[260] Siedle, B., Garcia-Pineres, A.J., Murillo, R., Schulte-Monting, J., Castro, V., Rungeler, P., Klaas, C.A., Da Costa, F.B., Kisiel, W., Merfort, I., Quantitative structure - Activity relationship of sesquiterpene lactones as inhibitors of the transcription factor NF-kappa B, Journal of Medicinal Chemistry, 2004; 47:6042-54.

[261] Chadwick, M., Trewin, H., Gawthrop, F., Wagstaff, C., Sesquiterpenoids lactones: benefits to plants and people, International Journal of Molecular Sciences, 2013; 14:12780-805.

[262] Kang, K., Lee, K.M., Yoo, J.H., Lee, H.J., Kim, C.Y., Nho, C.W., Dibenzocyclooctadiene lignans, gomisins J and N inhibit the Wnt/beta-catenin signaling pathway in HCT116 cells, Biochemical and Biophysical Research Communications, 2012; 428:285-91.

[263] Park, C.H., Chang, J.Y., Hahm, E.R., Park, S., Kim, H.K., Yang, C.H., Quercetin, a potent inhibitor against beta-catenin/Tcf signaling in SW480 colon cancer cells, Biochemical and Biophysical Research Communications, 2005; 328:227-34.

[264] Teiten, M.H., Gaascht, F., Dicato, M., Diederich, M., Targeting the wingless signaling pathway with natural compounds as chemopreventive or chemotherapeutic

agents, Current Pharmaceutical Biotechnology, 2012; 13:245-54.

[265] Yoo, J.H., Lee, H.J., Kang, K., Jho, E.H., Kim, C.Y., Baturen, D., Tunsag, J., Nho, C.W., Lignans inhibit cell growth via regulation of Wnt/beta-catenin signaling, Food and Chemical Toxicology, 2010; 48:2247-52.

[266] Eastman, A., Cell cycle checkpoints and their impact on anticancer therapeutic strategies, Journal of Cellular Biochemistry, 2004; 91:223-31.

[267] Niehrs, C., Acebron, S.P., Mitotic and mitogenic Wnt signalling, The EMBO Journal, 2012; 31:2705-13.

[268] Rubin, J.S., Barshishat-Kupper, M., Feroze-Merzoug, F., Xi, Z.F., Secreted WNT antagonists as tumor suppressors: pro and con, Frontiers in Bioscience, 2006; 11:2093-105.

[269] Suzuki, H., Watkins, D.N., Jair, K.W., Schuebel, K.E., Markowitz, S.D., Chen, W.D., Pretlow, T.P., Yang, B., Akiyama, Y., Van Engeland, M., Toyota, M., Tokino, T., Hinoda, Y., Imai, K., Herman, J.G., Baylin, S.B., Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer, Nature Genetics, 2004; 36:417-22.

[270] Godeke, J., Maier, S., Eichenmuller, M., Muller-Hocker, J., von Schweinitz, D., Kappler, R., Epigallocatechin-3-gallate inhibits hepatoblastoma growth by reactivating the Wnt inhibitor SFRP1, Nutrition and Cancer, 2013; 65:1200-7.

[271] Yang, J.H., Shin, B.Y., Han, J.Y., Kim, M.G., Wi, J.E., Kim, Y.W., Cho, I.J., Kim, S.C., Shin, S.M., Ki, S.H., Isorhamnetin protects against oxidative stress by activating Nrf2 and inducing the expression of its target genes, Toxicology and Applied Pharmacology, 2014; 274:293-301.

[272] Fahey, J.W., Dinkova-Kostova, A.T., Stephenson, K.K., Talalay, P., The "Prochaska" microtiter plate bioassay for inducers of NQO1, Quinones and Quinone Enzymes, Pt B, 2004; 382:243-58.

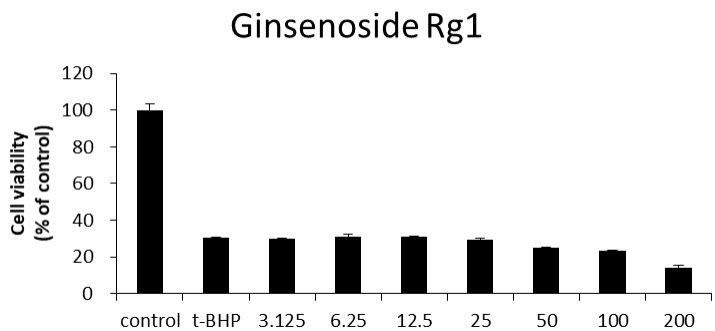
[273] Lee, K.M., Kang, K., Lee, S.B., Nho, C.W., Nuclear factor-E2 (Nrf2) is regulated through the differential activation of ERK1/2 and PKC alpha/beta II by Gymnasterkoreayne B, Cancer Letters, 2013; 330:225-32.

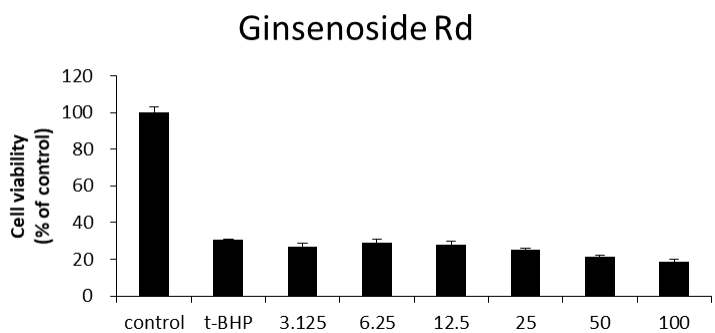
VIII. APPENDIX

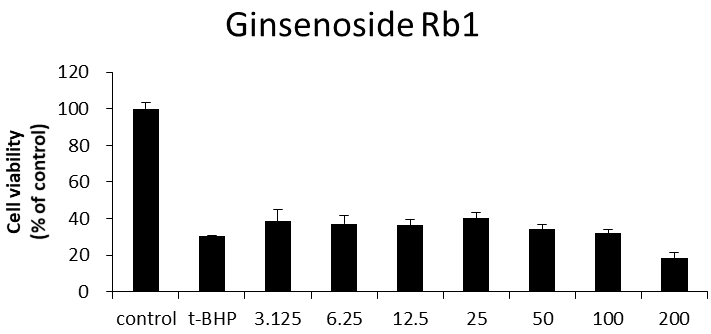
1. Screening for cancer chemopreventive effects

1.1. Screening for blocking agents

1.1.1. The measurement of anti-oxidative activity against cell death induced by *t*-BHP as excessive ROS production.






Ginsenoside Rb1
Cell viability (% of control)
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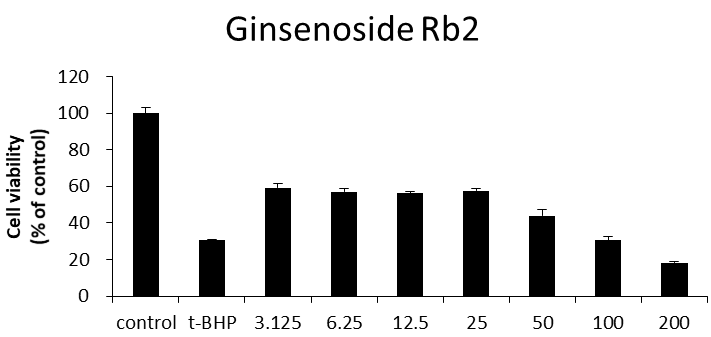
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25
50
100
200


Ginsenoside Rb2
Cell viability (% of control)
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60
40
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control
t-BHP
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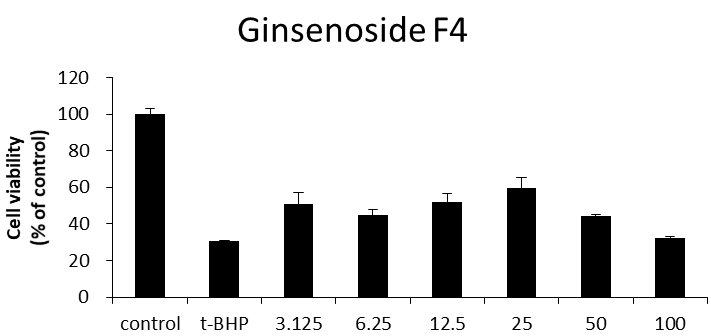
6.25
12.5
25
50
100
200


Ginsenoside F4
Cell viability (% of control)
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control
t-BHP
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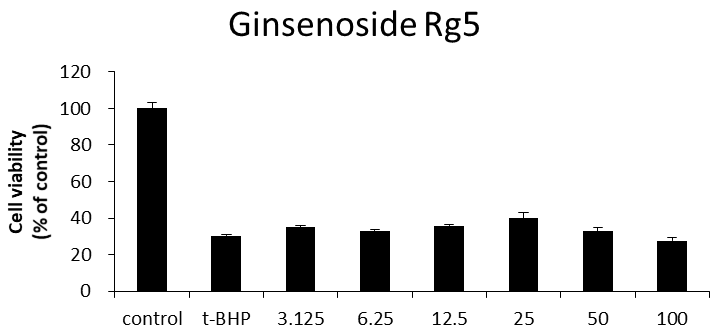
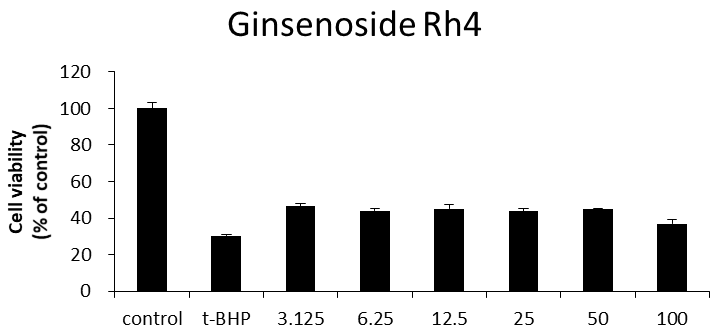
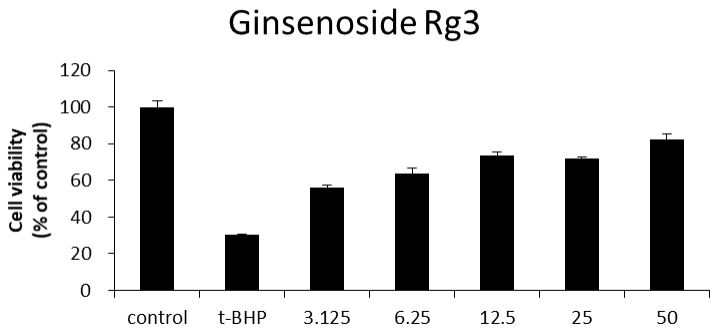
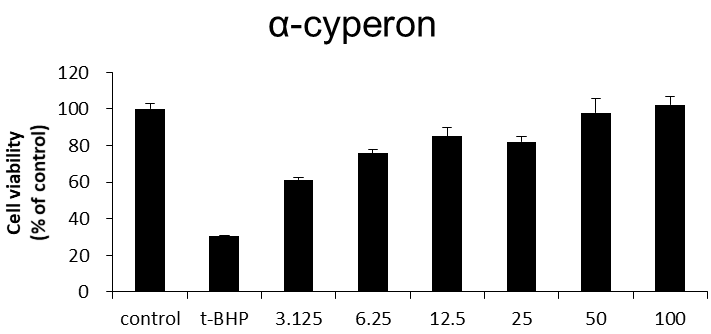
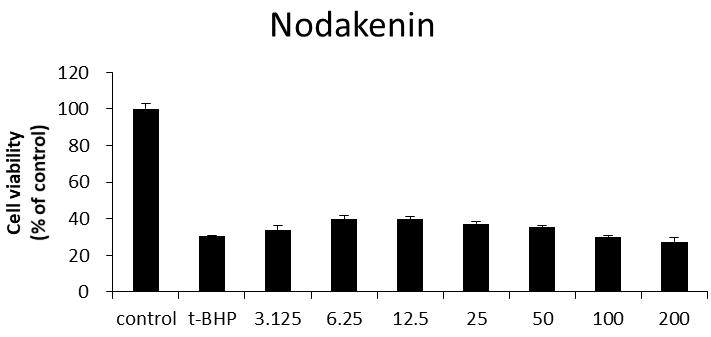
6.25
12.5
25
50
100

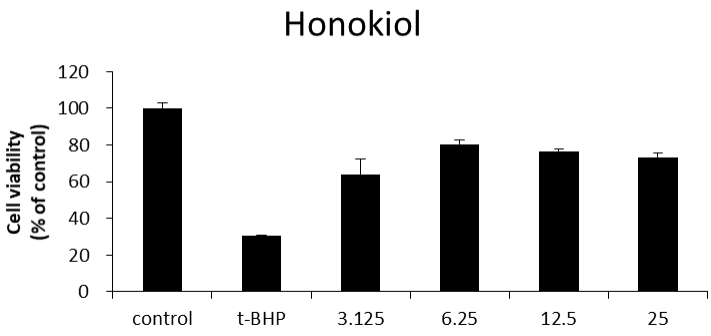

Ginsenoside Rg5
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25
50
100

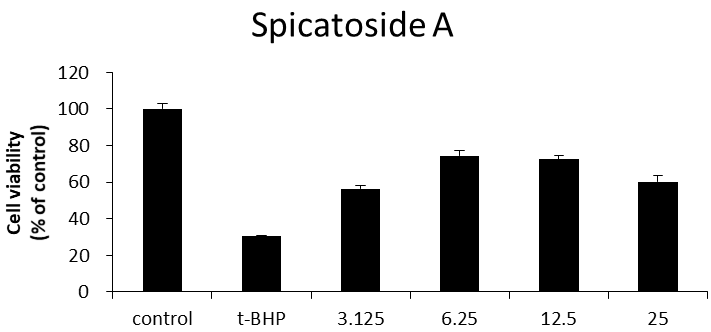

Ginsenoside Rh4
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25
50
100

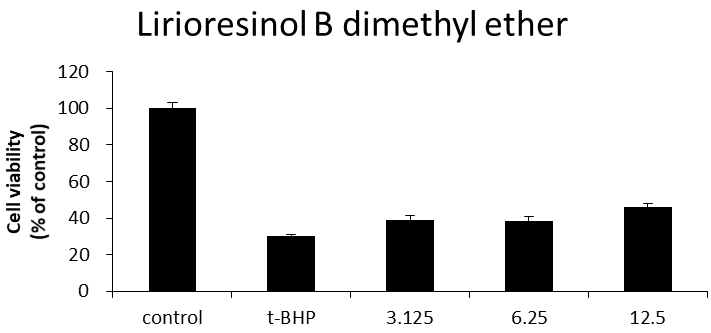

Ginsenoside Rg3
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25
50

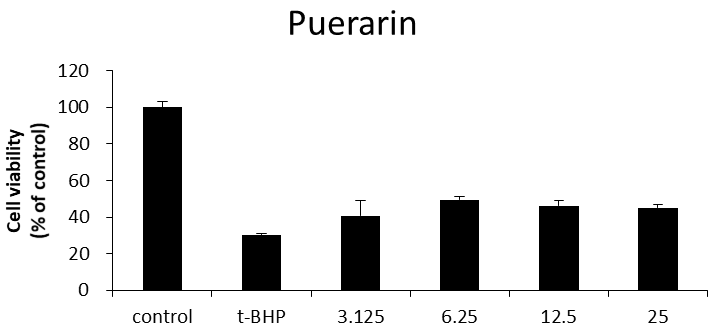

α-cyperon
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25
50
100

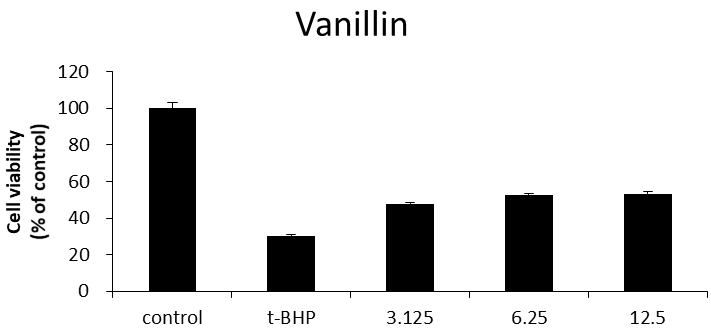

Nodakenin
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25
50
100
200

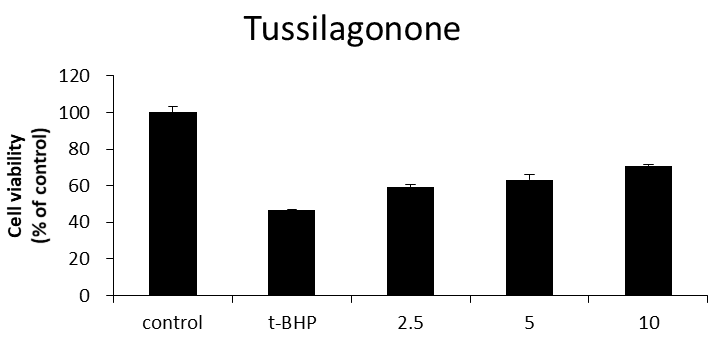

Honokiol
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25

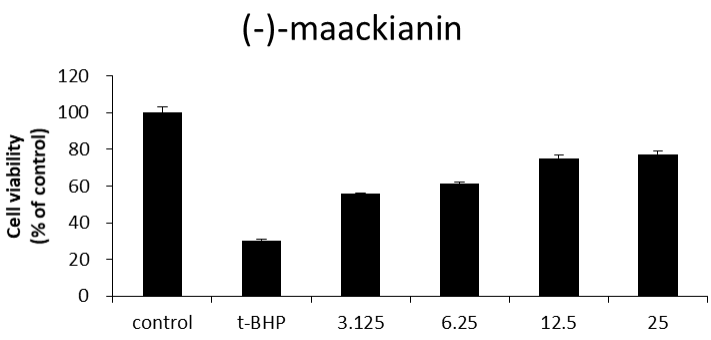

Spicatoside A
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25

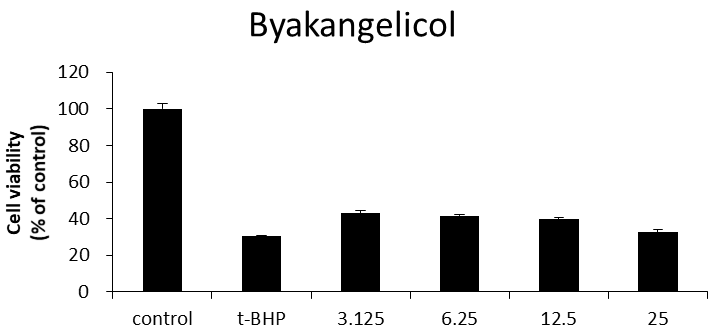

Lirioresinol B dimethyl ether
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5

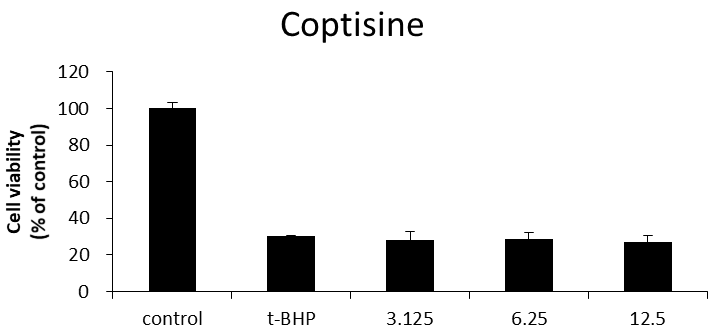

Puerarin
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25

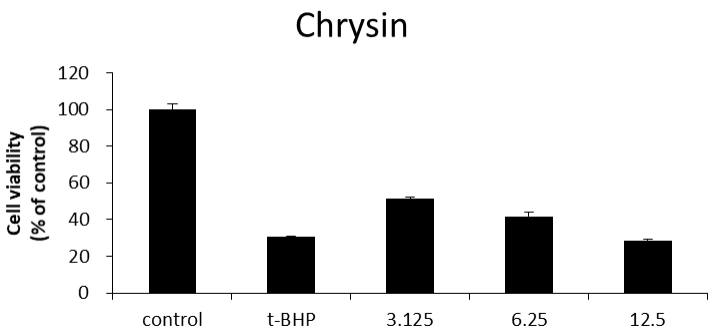

Vanillin
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5


Tussilagonone
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
2.5
5
10


(-)-maackianin
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25


Byakangelicol
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5
25


Coptisine
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5


Chrysin
Cell viability (% of control)
120
100
80
60
40
20
0
control
t-BHP
3.125
6.25
12.5

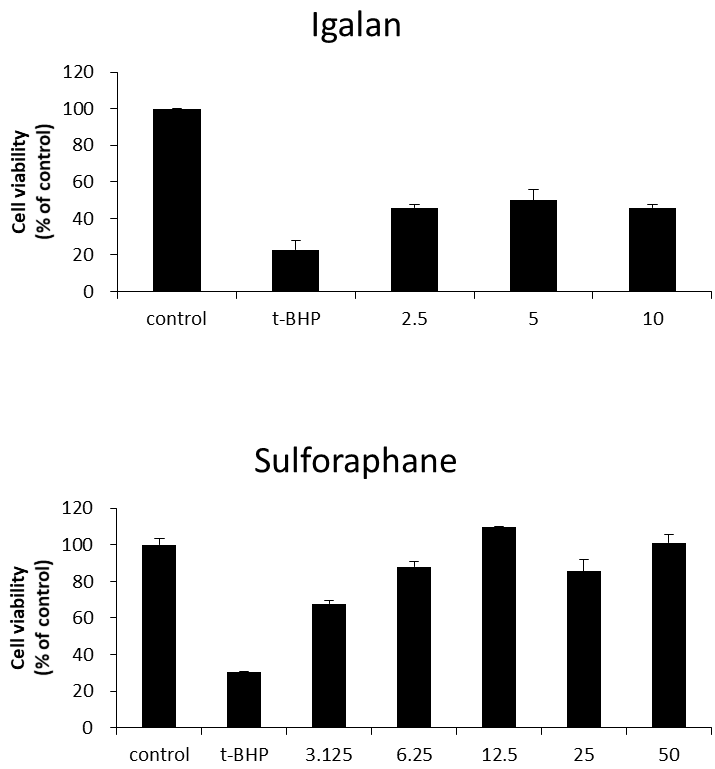
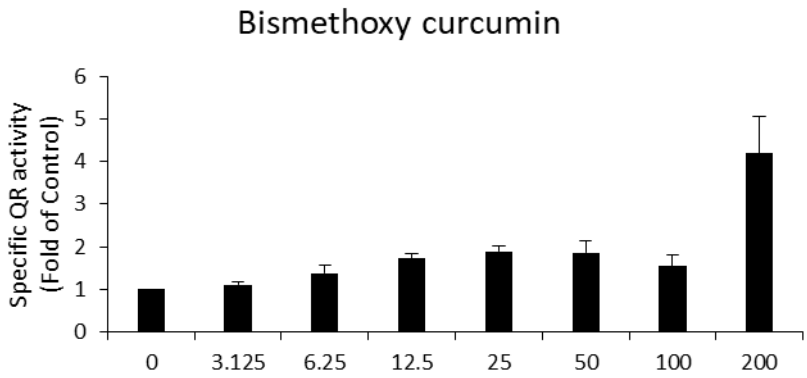
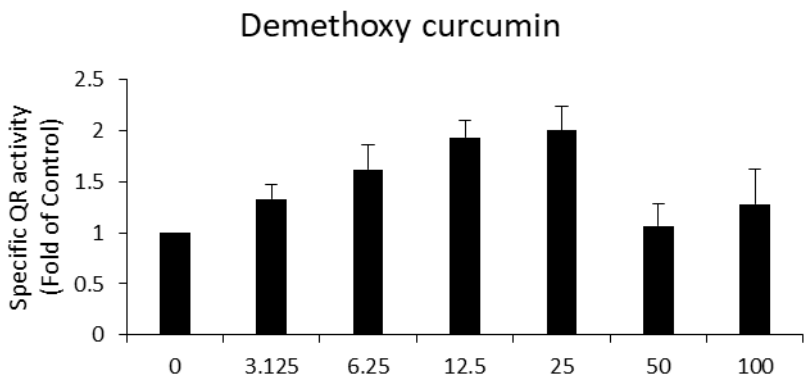


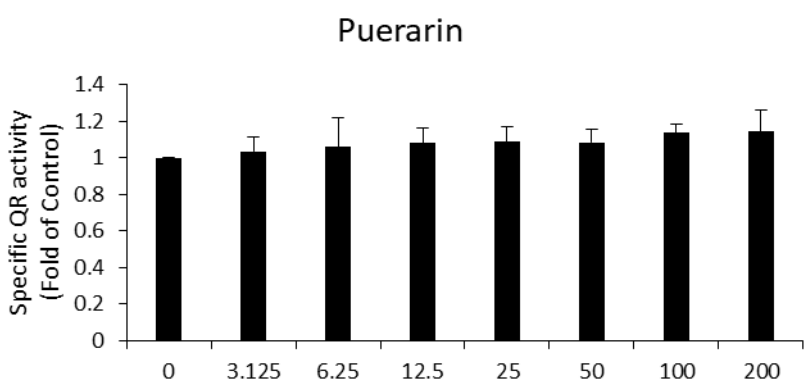
Figure 30. The measurement of cytoprotective effect.

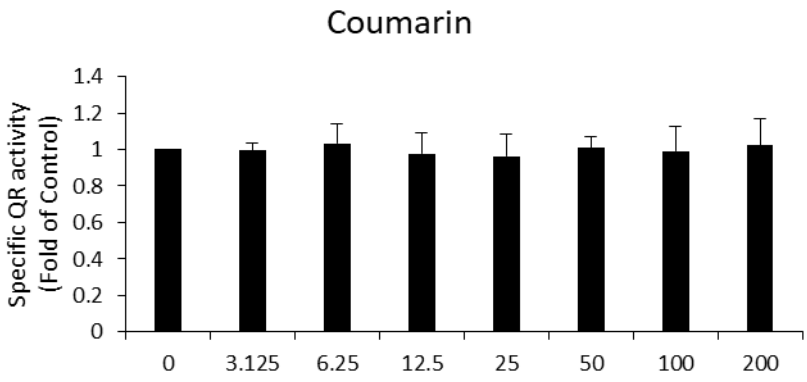
Effects of compounds on protective activity from *t*-BHP induced ROS in HepG2 cells with indicated concentrations (μM). Sulforaphane was used as a positive control.

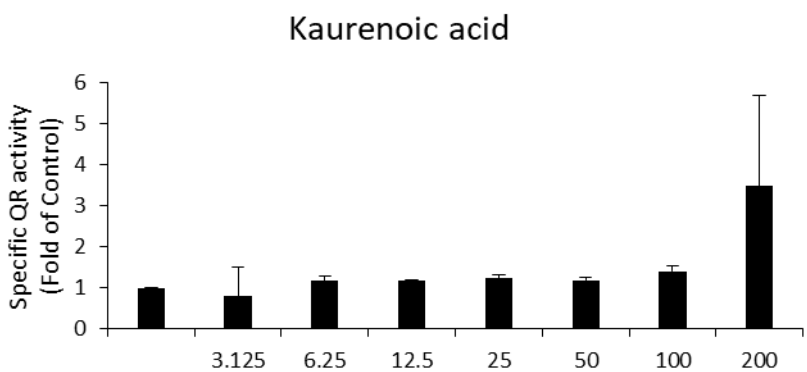
1.1.2. The measurement of QR activity

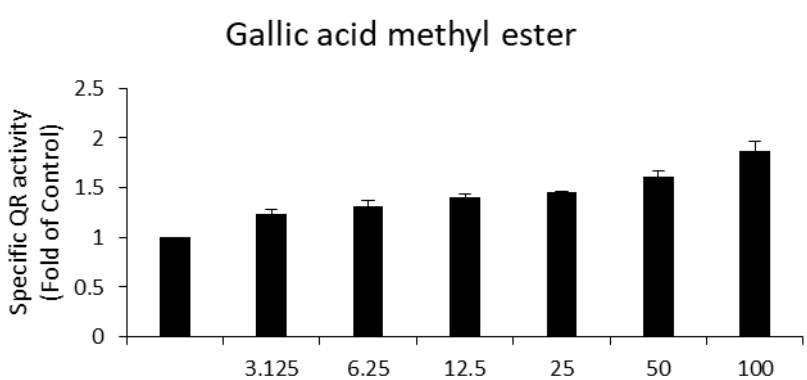


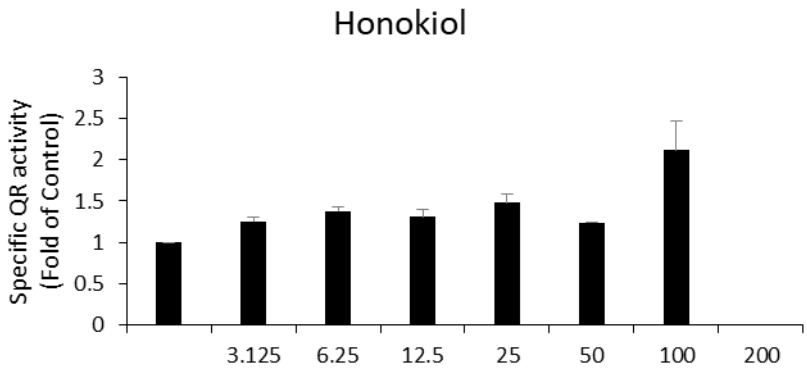


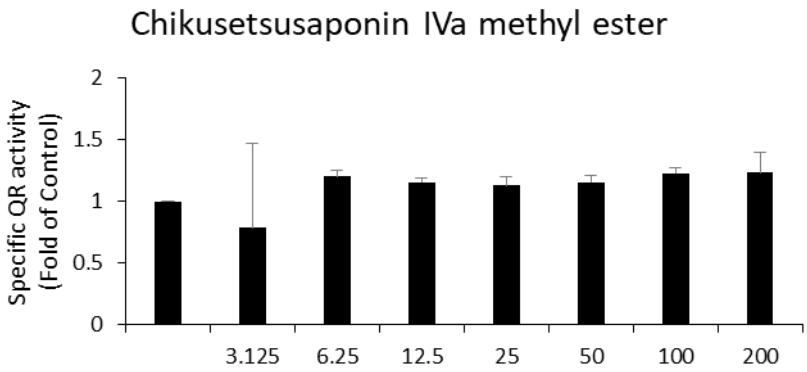


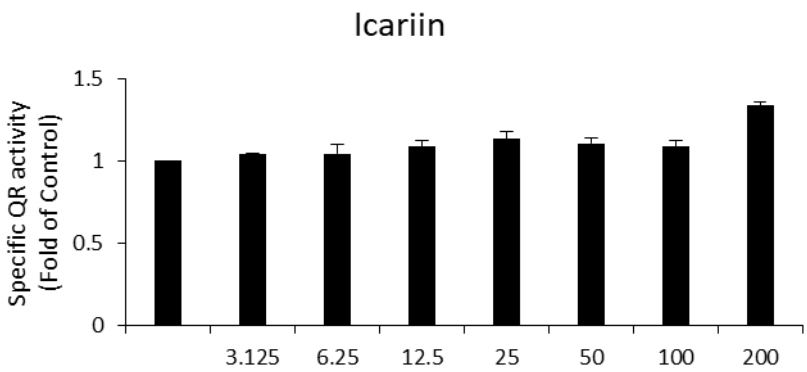

Coumarin
Specific QR activity (Fold of Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
3.125
6.25
12.5
25
50
100
200

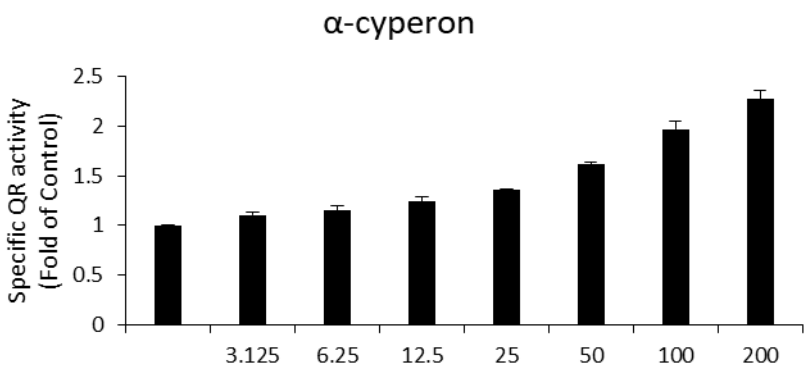

Kaurenoic acid
Specific QR activity (Fold of Control)
6
5
4
3
2
1
0
3.125
6.25
12.5
25
50
100
200

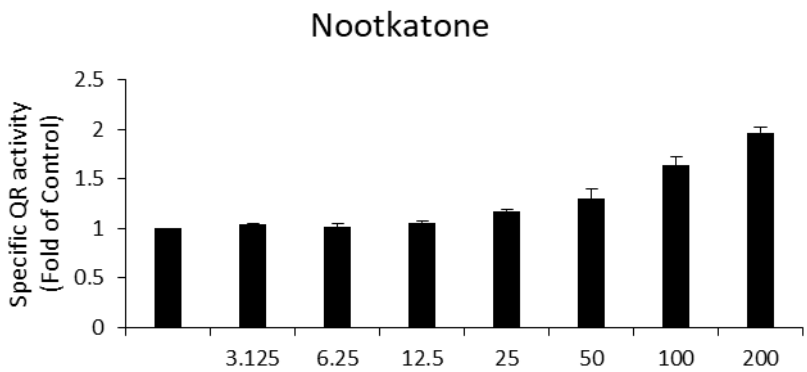

Gallic acid methyl ester
Specific QR activity (Fold of Control)
2.5
2
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100

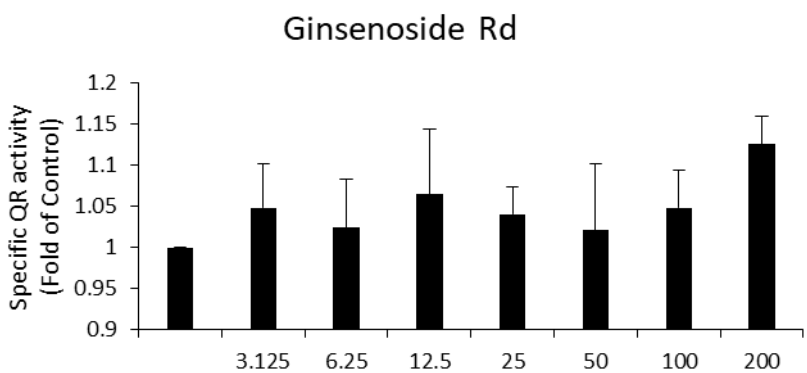

Honokiol
Specific QR activity (Fold of Control)
3
2.5
2
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100
200

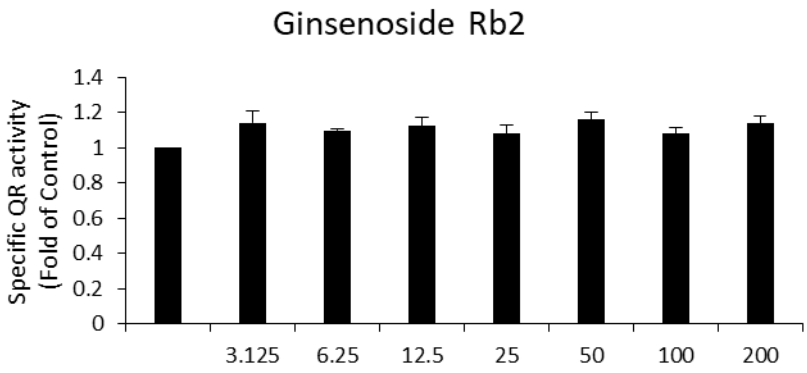

Chikusetsusaponin IVa methyl ester
Specific QR activity (Fold of Control)
2
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100
200

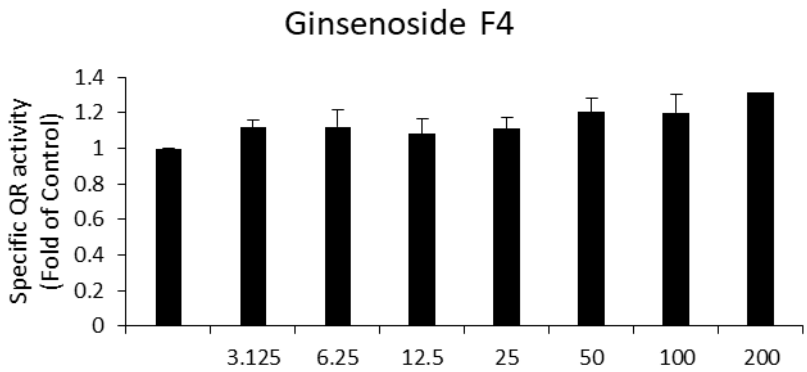

Icariin
Specific QR activity (Fold of Control)
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100
200

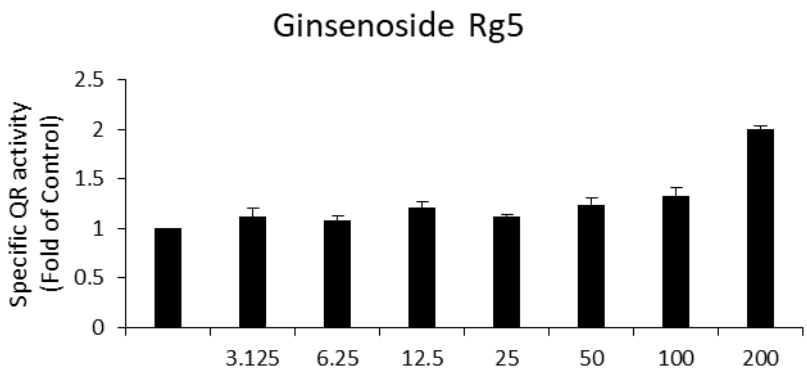

α-cyperon
Specific QR activity (Fold of Control)
2.5
2
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100
200

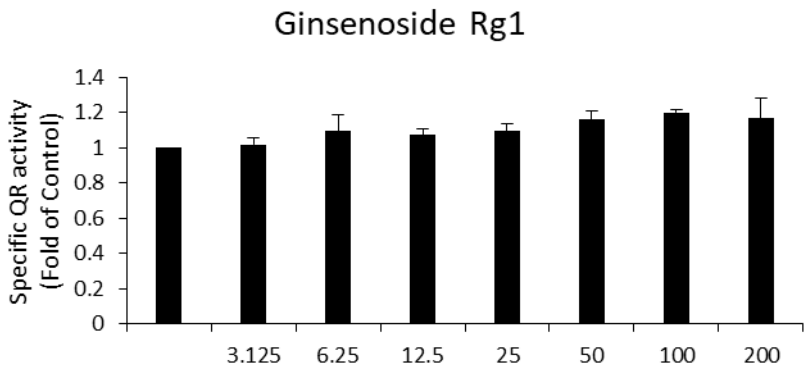

Nootkatone
Specific QR activity (Fold of Control)
2.5
2
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100
200

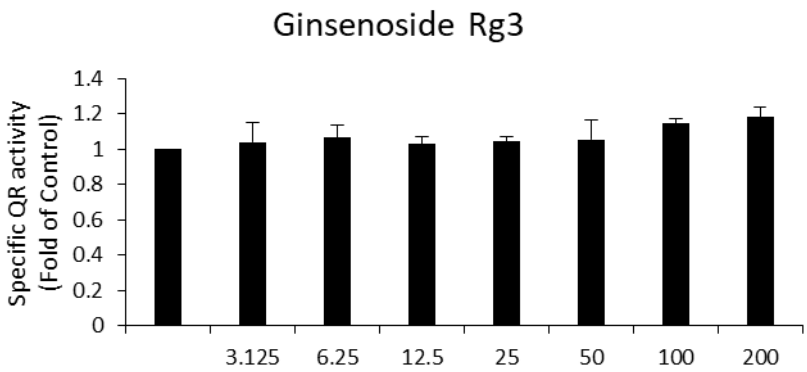

Ginsenoside Rd
Specific QR activity (Fold of Control)
1.2
1.15
1.1
1.05
1
0.95
0.9
3.125
6.25
12.5
25
50
100
200

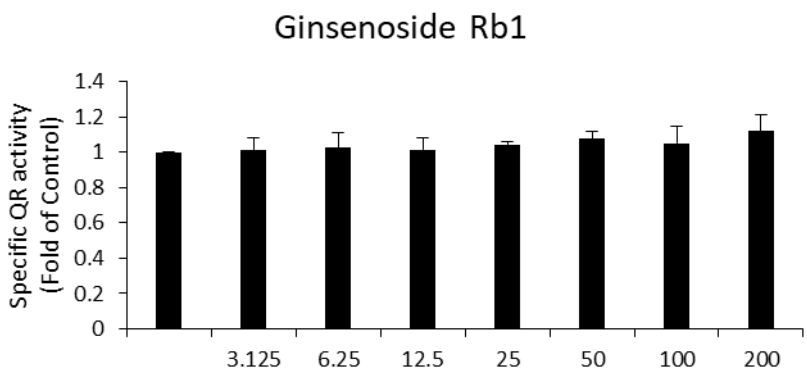

Ginsenoside Rb2
Specific QR activity (Fold of Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3.125
6.25
12.5
25
50
100
200

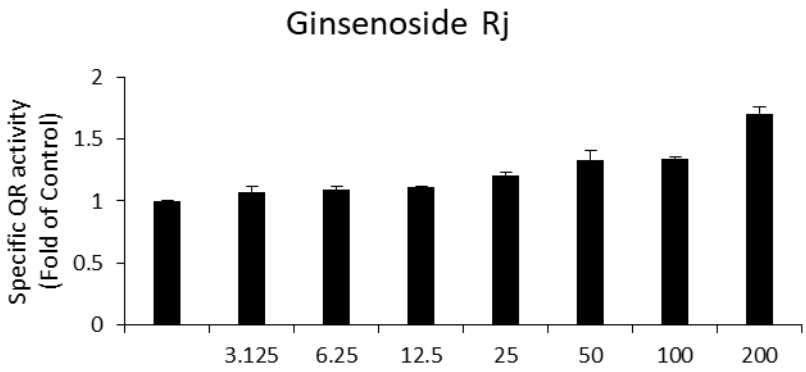

Ginsenoside F4
Specific QR activity (Fold of Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3.125
6.25
12.5
25
50
100
200

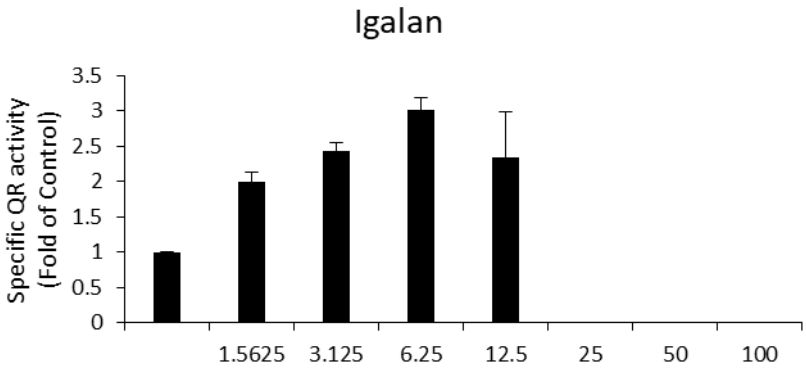

Ginsenoside Rg5
Specific QR activity (Fold of Control)
2.5
2
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100
200

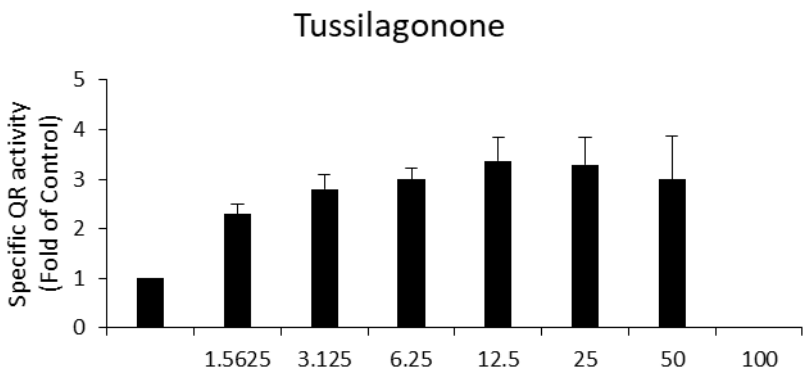

Ginsenoside Rg1
Specific QR activity (Fold of Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3.125
6.25
12.5
25
50
100
200


Ginsenoside Rg3
Specific QR activity (Fold of Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3.125
6.25
12.5
25
50
100
200


Ginsenoside Rb1
Specific QR activity (Fold of Control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3.125
6.25
12.5
25
50
100
200


Ginsenoside Rj
Specific QR activity (Fold of Control)
2
1.5
1
0.5
0
3.125
6.25
12.5
25
50
100
200


Igalan
Specific QR activity (Fold of Control)
3.5
3
2.5
2
1.5
1
0.5
0
1.5625
3.125
6.25
12.5
25
50
100


Tussilagonone
Specific QR activity (Fold of Control)
5
4
3
2
1
0
1.5625
3.125
6.25
12.5
25
50
100

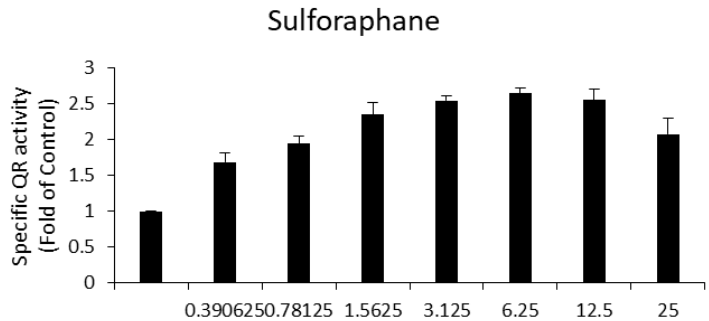


Figure 31. The results of QR assay.

Effect of compounds on QR activity in Hepa1c1c7 cells. Cells are treated with indicated concentrations (μM) for 24 h. Sulforaphane was used as a positive control.

1.2. Screening for suppressing agents

1.2.1. FACS screening

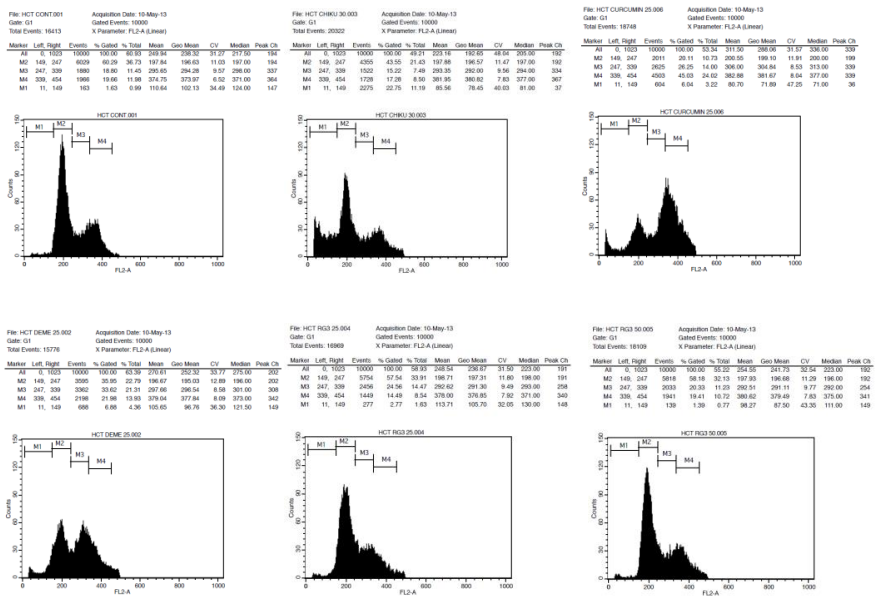
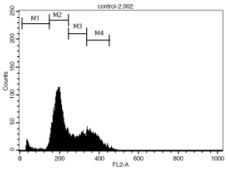


Figure 32. The first screening for measurement of cell viability using MTT assay in HCT 116 cells .

The results of FACS analysis in cells exposed to vehicle, Chikusetsusaponin IVa methyl ester (30 μM), Curcumin (25 μM), Demethoxy curcumin (25 μM), and Ginsenoside Rg3 (25, 50 μM).

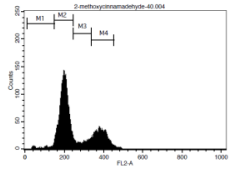
File: control-2.002 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 17294 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	57.82	249.19	233.88	33.61	219.00	201
M2	149, 247	5677	56.77	32.83	196.87	195.59	11.39	196.00	201
M3	247, 339	2061	20.61	11.92	294.45	293.13	9.41	296.00	339
M4	339, 454	1959	19.59	11.33	378.41	377.47	7.13	375.00	339
M1	11, 149	339	3.39	1.96	81.88	70.15	55.24	57.00	38



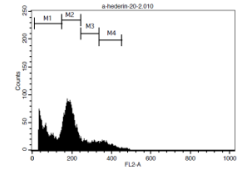
File: 2-methoxycinnamaldehyde-40.004 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 15363 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	65.09	253.79	239.57	34.65	213.00	196
M2	149, 247	6382	63.82	41.61	199.43	198.40	10.11	199.00	196
M3	247, 339	935	9.35	6.09	296.89	295.46	9.72	301.00	339
M4	339, 454	2427	24.27	15.80	387.01	386.08	6.96	385.00	377
M1	11, 149	218	2.18	1.42	105.27	96.34	36.66	114.50	149



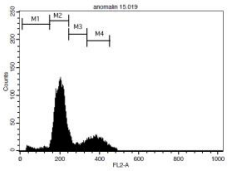
File: a-hederin-20-2.010 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 9793
Total Events: 34140 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	9793	100.00	28.68	183.79	157.31	50.55	182.00	177
M2	149, 247	5014	51.20	14.69	191.13	189.68	12.41	189.00	177
M3	247, 339	1023	10.45	3.00	292.35	291.04	9.43	293.00	282
M4	339, 454	771	7.87	2.26	379.69	378.64	7.55	374.00	354
M1	11, 149	3004	30.67	8.80	80.90	73.74	42.68	73.00	36



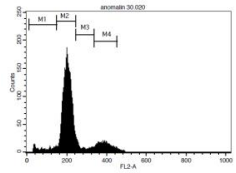
File: anomalin 15.019 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 24236 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	41.26	243.64	229.99	34.30	214.00	205
M2	149, 247	6798	67.98	28.05	202.15	201.64	10.40	202.00	205
M3	247, 339	1072	10.72	4.42	287.40	285.82	10.56	284.00	249
M4	339, 454	1780	17.80	7.34	389.39	388.26	7.62	387.00	384
M1	11, 149	297	2.97	1.23	87.41	78.75	42.89	88.00	36



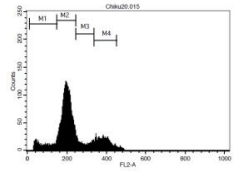
File: anomalin 30.020 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 21812 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	45.85	225.96	214.59	32.19	208.00	201
M2	149, 247	7760	77.60	35.58	204.06	203.17	9.29	204.00	201
M3	247, 339	613	6.13	2.81	282.01	280.42	10.77	273.00	247
M4	339, 454	1135	11.35	5.20	389.31	388.26	7.39	388.00	380
M1	11, 149	471	4.71	2.16	90.97	81.63	43.17	90.00	37



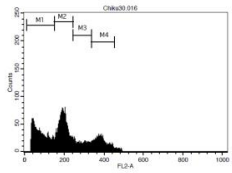
File: Chiku20.015 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 21037 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	47.54	230.36	212.16	38.56	208.00	193
M2	149, 247	6453	64.53	30.67	199.77	198.53	11.02	199.00	193
M3	247, 339	1003	10.03	4.77	290.98	289.44	10.29	289.00	252
M4	339, 454	1568	15.68	7.45	388.16	387.06	7.56	386.50	346
M1	11, 149	932	9.32	4.43	88.82	81.25	39.94	88.00	45



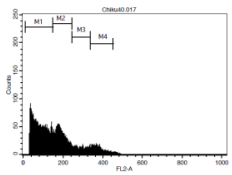
File: Chiku30.016 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 20671 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	47.91	206.29	171.47	54.70	193.00	205
M2	149, 247	3880	38.80	18.59	195.55	194.20	11.74	194.00	205
M3	247, 339	1180	11.80	5.65	292.07	290.72	9.58	293.00	250
M4	339, 454	1712	17.12	8.20	386.41	385.35	7.47	383.00	372
M1	11, 149	3180	31.80	15.24	83.02	76.25	49.24	78.00	37



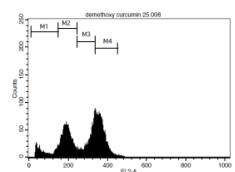
File: Chiku40.017 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 17552 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	56.97	159.96	130.80	62.39	140.00	38
M2	149, 247	2954	29.54	16.83	190.40	188.76	13.26	187.00	174
M3	247, 339	923	9.23	5.26	292.39	291.04	9.59	291.00	299
M4	339, 454	769	7.69	4.38	379.62	378.56	7.59	375.00	367
M1	11, 149	5341	53.41	30.43	65.70	78.97	38.78	83.00	38



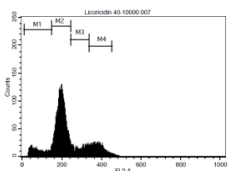
File: demethoxy curcumin 25.008 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 16817 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	59.46	276.79	252.76	34.37	314.00	338
M2	149, 247	3107	31.07	18.48	193.80	192.44	11.88	192.00	191
M3	247, 339	2436	24.36	14.49	310.41	309.34	8.08	318.00	338
M4	339, 454	3660	36.60	21.76	368.93	368.27	6.09	365.00	340
M1	11, 149	867	8.67	5.16	86.17	77.19	45.40	76.00	39



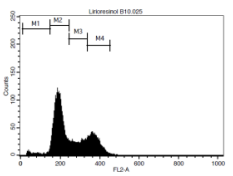
File: Licoricidin 40-10000.007 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 19851 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	50.38	227.63	209.14	38.38	205.00	200
M2	149, 247	6299	62.99	31.73	197.71	196.54	10.83	197.00	200
M3	247, 339	1151	11.51	5.80	295.73	294.34	9.59	299.00	334
M4	339, 454	1562	15.62	7.87	381.69	380.72	7.17	379.00	368
M1	11, 149	986	9.86	4.97	85.76	78.41	41.11	79.00	46



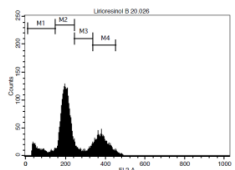
File: Liriresinol B10.025 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 19388 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	51.56	252.04	238.05	33.03	217.00	186
M2	149, 247	5633	56.33	29.05	194.62	193.49	10.84	193.00	186
M3	247, 339	1916	19.16	9.88	295.40	294.09	9.35	296.00	339
M4	339, 454	2244	22.44	11.57	375.85	375.07	6.52	373.00	359
M1	11, 149	250	2.50	1.29	91.96	82.93	42.59	88.00	43



File: Liriresinol B 20.026 Acquisition Date: 18-May-13
Gate: G1 Gated Events: 10000
Total Events: 22895 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	43.68	239.77	218.15	39.63	210.00	197
M2	149, 247	5995	59.95	26.18	201.05	200.08	9.82	201.00	197
M3	247, 339	832	8.32	3.63	295.40	293.67	10.74	298.00	336
M4	339, 454	2212	22.12	9.66	384.62	383.65	7.15	381.00	380
M1	11, 149	934	9.34	4.08	79.81	72.96	42.57	73.00	37



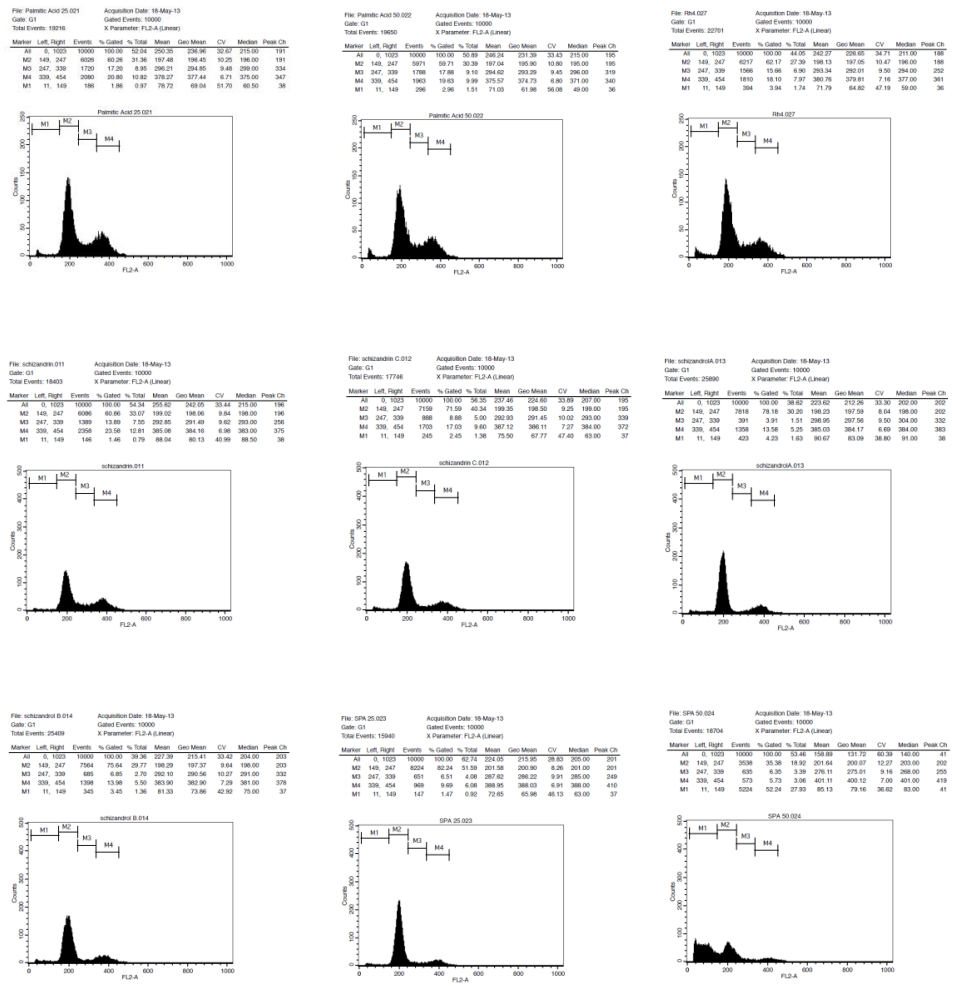


Figure 33. The second screening for measurement of cell viability using MTT assay in HCT 116 cells.

The results of FACS analysis in cells exposed to vehicle, 2-Methoxycinnamaldehyde (40 μM), α-Hederin (20 μM), Anomalin (15, 30 μM), Chikusetsusaponin IVa methyl ester (20, 30, 40 μM, Demethoxy curcumin (25 μM), Licoricidin (40 μM), Lirioresinol B dimethyl ether 10, 20 μM), Palmitic acid (25, 50 μM), Ginsenoside Rh4 , Schizandrin, Schizandrin C, Schizandrol A, Schizandrol B, and Spicatoside A

(25, 50 μM) .

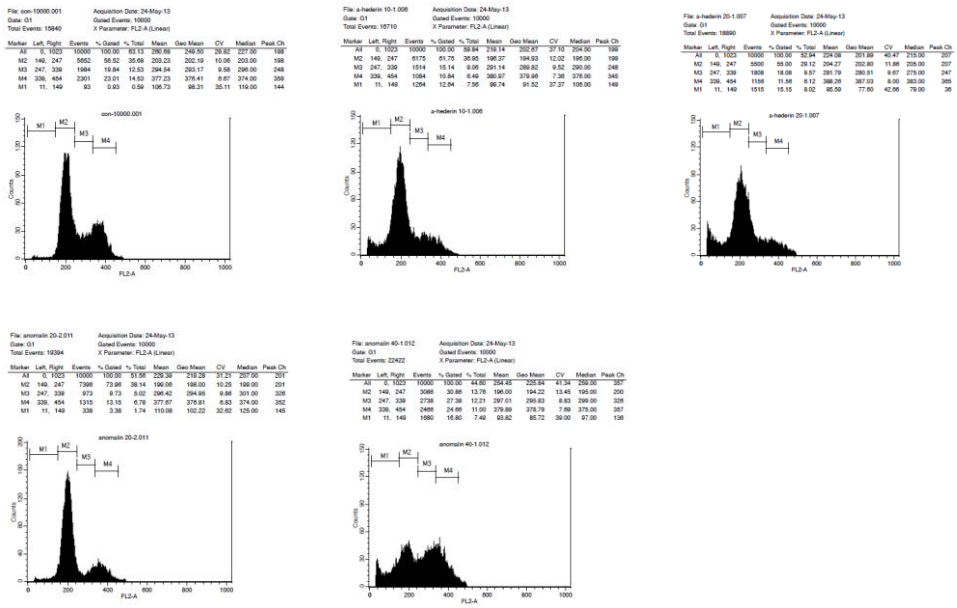


Figure 34. The third screening for measurement of cell viability using MTT assay in HCT 116 cells.

The results of FACS analysis in cells exposed to vehicle, α-Hederin (10, 20 μM), and Anomalin (20, 40 μM).

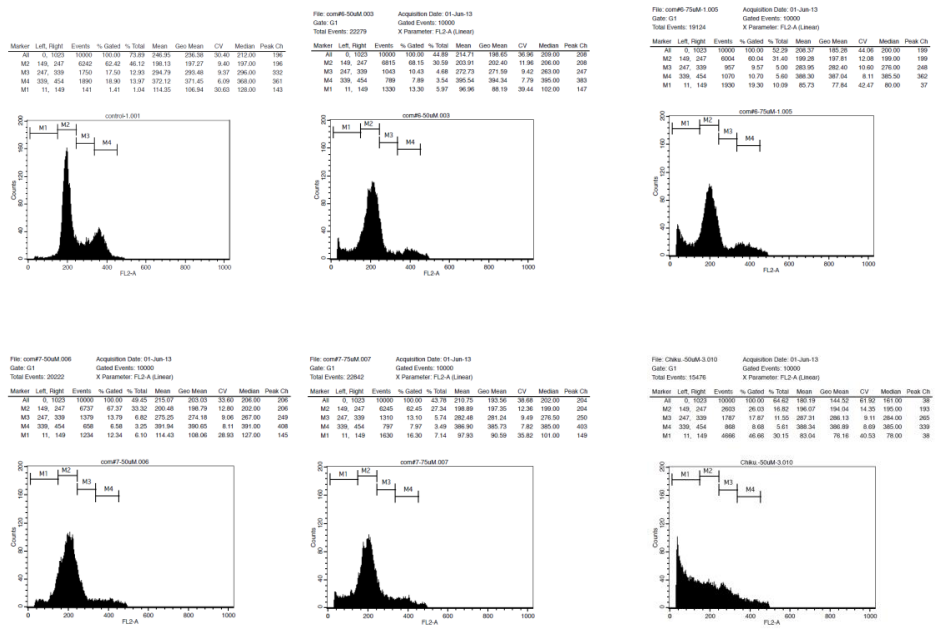
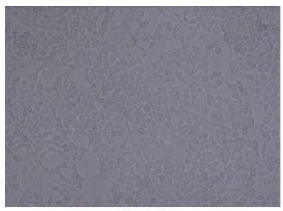


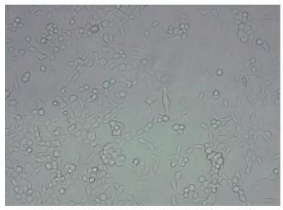
Figure 35. The fourth screening for measurement of cell viability using MTT assay in HCT 116 cells.

The results of FACS analysis in cells exposed to vehicle, Compound #6 (50, 75 μM), and Compound #7 (50, 75 μM).

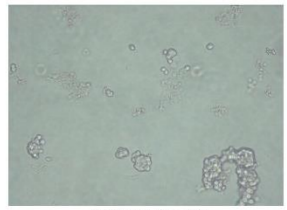
1.2.2. The observation of morphology



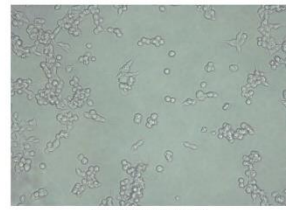
Control



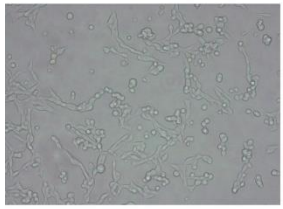
2-methoxycinnamaldehyde (40μM)



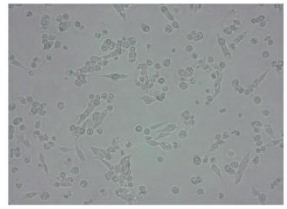
α-Hederin (20μM)



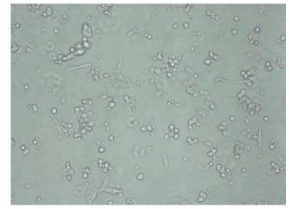
Licoricidin (40μM)



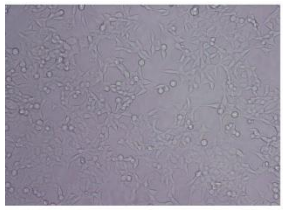
Chikusetsaponin Iva methyl ester (20μM)



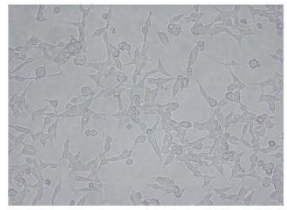
Chikusetsaponin Iva methyl ester (30μM)



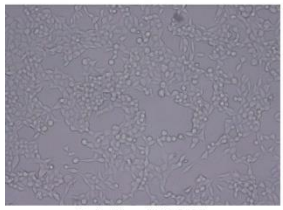
Chikusetsaponin Iva methyl ester (40μM)



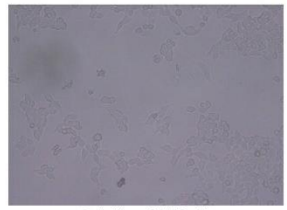
Lirioresinol B dimethyl ether (10μM)



Lirioresinol B dimethyl ether (20μM)



Palmitic acid (25μM)



Palmitic acid (50μM)

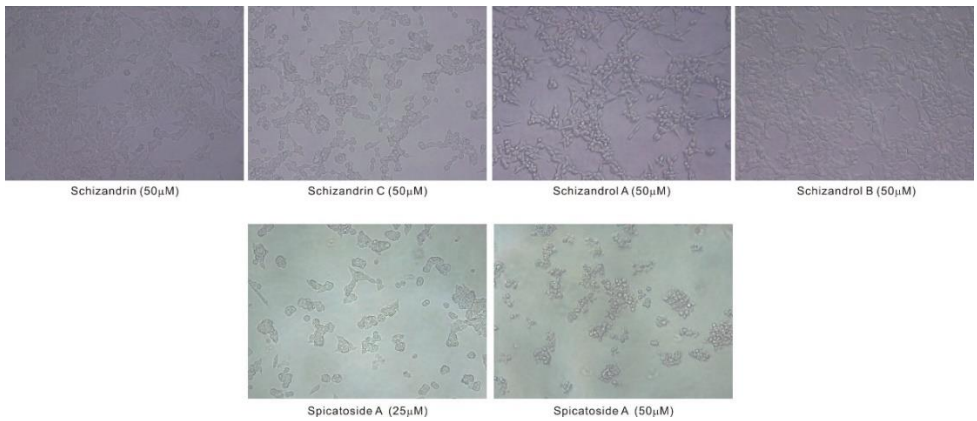


Figure 36. The morphology of cell exposed to various compounds with indicated concentration.

ABSTRACT IN KOREAN

세계적으로 암의 발생은 끊기지 않는 이슈이자 사회적 문제로 자리매김하고 있다. 이를 해결하기 위해, 주요 암들의 원인으로 분리되고 있는 흡연과 음주를 지양하고 건강한 생활 습관을 구축하도록 독려 하고 있다. 또한, 암환자 들에게는 적극적인 치료와 함께 보조적으로 도움이 될 수 있는 보조 약품이나 식품들의 개발이 지속적으로 진행되고 있는 추세이다.

암의 발생은 급작스럽게 이루어 지는 것이 아니라, 단계별로 그 예후와 증상들이 세포수준에서 나타나며 단계적으로 진행된다. 암 화 과정은 4단계로 나뉘어지며, 정상 세포에서 비정상 세포로 변하는 '개시' 단계, 전 암 세포로 가는 '촉진' 단계, 그리고 암의 특징을 가지며 암으로 발전되어 진행중인 '진행' 단계로 나뉜다. 암 화 과정 의 각 단계에서 작용하여, 암의 발생과 암의 진행을 억제 또는 지연시키는 과정을 화학적 암 예방이라 한다. 본 연구에서는 이러한 암 화 과정을 억제하는 진행을 지연시킬 수 있는 새로운 천연물 유래 물질들을 발굴하고, 관련된 기전을 규명 하여 화학적 암 예방의 활성을 가진 물질로서 활용 가능함을 확인 하였다.

먼저 정상세포에서 발암 개시 세포로 변하는 개시 단계에서 작용할 수 있는 천연물로 관동화 유래 화합물인 투실라고논 (tussilagonone) 과 토목향 유래 화합물인 이갈란 (igalan) 을 스크리닝을 통해 확인 하였다. 스크리닝에는 암 예방 수치를 측정해 볼 수 있는 퀴논 리덕테이즈 어세이를 사용하였다. 투실라고논과 이갈란 모두 5μM 이하의 농도에서 2배 이상의 활성을 나타내었고 해독작용의 활성이 증가함에 따라 이를 바탕으로 분자적 수준의 실험들을 수행하였다. 기존에 실험 결과들을 바탕으로, 두 가지 화합물을 사용하여 간암 세포주 에서 Nrf2의 활성을 유도함을 확인 하였고, Nrf2의 하위 단계 유전자들인 NQO1과 HO-1의 발현이 증가함을 단백질 수준에서 관찰 하였다. 분자적 기전 연구를 위하여 두 가지 화합물을 세포에 처리 하였을 때, Nrf2의 ARE (Antioxidant Response Elements) 에 결합 함을 리포터 어세이와 올리고 풀다운 어세이를 통해 확인하여, 하위 단계의 유전자의 프로모터 상에 존재하는 ARE 부위에 Nrf2를 결합시켜 항 산화와 해독효소들의 전사를 증가 시켜

활성을 유도함을 확인하였다. 투실라고논은 해독작용과 더불어 *t*BHP를 사용하여 강한 ROS를 유도하였을 때, ROS 생성을 억제하여 세포사멸이 감소함을 확인하였다. 이로써 투실라고논이 Nrf2의 활성을 유도하여 해독작용과 항 산화 작용을 함을 보았다. 이갈란은 해독작용과 더불어 면역작용에서 주요 역할을 하는 NF-κB의 활성이 감소함을 확인하였다. 이갈란을 세포를 노출 시킨 후, TNFα 를 사용하여 면역 작용을 유도한 결과 이갈란에 노출 된 세포에서는 면역반응이 대조군과 비교했을 때 현저히 낮아진 것을 확인 하였다. ROS, xenobiotics, 그리고 면역 반응은 정상세포가 발암 개시 세포로 변하는 시작 단계에서 암 발생의 원인으로 작용하는 것으로 투실라고논과 이갈란이 이를 억제함으로써 암 발생을 억제해 줄 수 있음을 확인 하였다.

다음으로 엠티티로 세포 생존률의 스크리닝 결과를 통하여 치쿠셋수사포닌 포에이 메틸 에스테르 (chikusetsusaponin IVa methyl ester (CME))의 세포 성장 억제 효과가 가장 좋았기에 이를 사용하여 실험을 수행 하였다. 대장암 세포주 에서 이 화합물을 사용하여 팩스 분석을 통하여 G0/G1기에서 세포주기 정지 (cell cycle arrest)가 유도되는 것을 확인하였다. 이를 조절하는 세포주기 조절 단백질 중 Cyclin D1의 발현 감소가 두드러졌고 이와 관련된 분자적 기전을 연구하기 위하여 TOP flash를 사용하여 리포터 어세이를 수행한 결과, 대장암 세포를 포함하는 암세포가 되는 원인인 베타 카테닌 (β-catenin)의 활성이 억제 됨을 확인하였다. 치쿠셋수사포닌 포에이 메틸 에스테르를 처리함에 따라 베타 카테닌의 핵 내 이동이 억제 되는 것을 올리고 풀 다운 어세이와 핵 내의 단백질 발현을 통해 확인하였고, 그 하위 단계 유전자인 세포 분열을 촉진하는 cyclin D1과 c-Myc도 감소함을 보았다. 그뿐 아니라 세포분열 관련 유전자인 CDK 단백질들도 감소함을 확인하였다. 이로써 치쿠셋수사포닌 포에이 메틸 에스테르가 종양 세포로의 발전단계에서 베타 카테닌의 발현을 억제함으로써 세포 분열을 억제하여 암의 진행 억제 시켜 줄 수 있음을 확인하였다.

위의 실험들을 볼 때, 관동화 유래의 투실라고논 과 토목향 유래의 이갈란은 기존에 밝혀지지 않았던 기능인 항 산화 기능, 해독작용, 그리고 항 염증의 효

능을 가지며, 암의 발생을 예방하는 물질의 후보군으로 가치가 있을 것으로 기대된다. 또한 우슬 에서 분리된 치쿠셋수사포닌 포에이 메틸 에스테르의 경우, 세포 성장과 분열을 억제하며 세포 자멸을 유도함으로써, 암의 진행을 억제하는 효과의 새로운 기능을 확인하였다. 이러한 물질들은 암의 형성과 진행을 억제하는 화학적 암 예방 물질로서 향후, 천연물을 통한 암의 발생과 진행 억제를 위한 연구에 중요한 자료가 될 것으로 사료된다.

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주요어 : Tussilagonone, *Tussilago farfara* L., Igalan, *Inula helenium* L., Chikusetsusaponin IVa methyl ester, *Achyranthes japonica*, Nrf2, NF-κB, β-catenin, anti-cancer, ERK1/2, GSK3β, Cyclin D1

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