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Anticancer activities of the bioactive compounds from *Citrus unshiu* leaf: molecular targets and mechanisms

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ABSTRACT

Citrus leaves are reputedly general tonics for gastrointestinal disorders and fevers. The leaf extract is consumed as an herbal tea and the leaf hydrosol from numerous *Citrus* species is used for some traditional dishes in Tunisian cuisine. The appropriate tree canopy for *Citrus unshiu* should be maintained by skirting and pruning. Therefore, *Citrus unshiu* leaves are readily available agricultural byproducts. Although there are reports on the biological effects and chemical compositions of certain species of citrus leaves, very limited information about the anticancer properties of *Citrus unshiu* leaves has been reported. Therefore, *Citrus unshiu* leaves usages as anticancer reagents in food and pharmaceutical industries has been neglected.

In **CHAPTER II**, we assessed antiproliferative activities of the methanolic extract of *Citrus unshiu* leaves (MECL) using several cancer cell lines and marked cytotoxicity was observed in AGS human gastric cancer cells. Treatment with MECL induced non-apoptotic cell death and increased formation of the acidic vesicular organelles and LC3 puncta. MECL-induced cell death was significantly reduced by pretreatment with an autophagy inhibitor, 3-methyladeinine. Gas chromatography-mass spectrometry identified the major compound of MECL to be phytol (47.35%).

In **CHAPTER III**, we investigated cytotoxic effects of phytol, the major component of MECL, on cancer cells. Phytol induces apoptosis in AGS cells, as evidenced by an increase of the sub-G1 population, activation of caspases, and depolarization of the mitochondrial membrane. Co-treatment with chloroquine (CQ), a lysosomal inhibitor, strongly enhanced phytol-induced apoptosis in AGS cells,

suggesting that phytol could induce protective autophagy. Furthermore, N-acetyl-L-cysteine, a reactive oxygen species (ROS) scavenger, increased the phytol-induced cytotoxicity, but decreased the levels of p62 and the formation of the acidic vesicular organelles, suggesting that the Nrf2 pathway was induced by phytol.

In **CHAPTER IV**, we fractionated methanolic extract of *Citrus unshiu* leaves by using *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water; we found 2,4-di-tert-butylphenol (DTBP) as the most potent candidate for anticancer agent in *Citrus unshiu* leaves. DTBP increased acetylation of α-tubulin, p21 and Rb expression, but reduced β-catenin expression and cell proliferation. Increase of α-tubulin acetylation by DTBP resulted in tubulin polymerization, consequently, inducing aberrant mitosis.

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CHAPTER I. Introduction

1. Anticancer properties of phytochemicals

Dietary phytochemicals are plant based chemical compounds (‘phyto’ is from the Greek word meaning plant). Plants produce phytochemicals to protect them from external threats such as ultraviolet and generators of damaging free radicals. Plant derived phytochemicals have attracted significant attention to prevent the onset of many diseases including cardiovascular disease, inflammation, and cancer. These phytochemicals exert beneficial effects by degenerating reactive oxygen species (ROS) and reducing inflammatory signaling molecules. Phytochemicals also influence the proliferation, growth, and metastasis of tumors (Vauzour et al., 2010). Thus, these plant-derived substances may represent natural anti-cancer drugs.

Many natural products and their analogs have been identified as potent anticancer agents, and anticancer properties of various phytochemicals are being identified. Several plant-based anticancer agents including taxol, vincristine, camptothecin derivatives, topotecan, and irinotecan are in clinical use all over the world (Shukla and Metha, 2015).

Phytochemicals can interact with the metabolic pathways and signaling cascades that control growth, proliferation, and cell survival or death. Upon entering cells, these phytochemicals can scavenge free radicals and generate chemical or electrophilic stress signals that cause proteins to trigger various cellular signaling pathways (Finley et al., 2011; Kong et al., 2001). Phytochemicals can alter target proteins and signaling mechanisms, such as STAT3, PI3K, NF-κB, and Nrf2, as well as histone modification. Although natural phytochemicals are generally considered non-toxic, they can exhibit toxicities on certain situation and concentration.

Therefore, understanding their pharmacological effects and molecular targets are essential for phytochemicals' druggability.

Table 1-1. Association among phytochemicals, cancer type and scientific evidence. Reproduced from Baena Ruiz and Salinas Hernández. Maturitas 2016; 94: 13-19, with permission of Elsevier.

Phytochemicals	**Cancer type (risk reduction)**	**Scientific evidence**
Carotenoids: Lycopene, alpha- and beta-carotene.	Prostate, Breast, Lung, Pancreatic	Strong +++, ++, +
Soybean isoflavones: Daidzein, genistein, glycitein.	Breast, Colon, Prostate, Lung	Strong +++, ++,+
Isothiocyanates: Sulforaphane, phenethyl and benzyl isothiocyanate.	Breast, Colorectal, Prostate, Renal, Gastric, Bladder	Strong +++, ++, +
Indoels: Indole-3-carbinol.	Hormone-dependent tumors Breast, Prostate, Cervical	Weak *
Epigallocatechin gallate (EGCG)	Colon, Breast, Prostate, Lung, Oesophagus, Head and Neck	Limited *, +
Allyl sulfur compounds: Diallyl trisulfide (DTS)	Upper aerodigestive tract, Gastric, Prostate, Colorectal, Kidney	Strong +++, ++, +
Reveratrol	Breast, Colon, Prostate, Liver, Pancreatic	Limited *, +
Curcumin	Breast, Colorectal, Prostate, Lung, Pancreatic	Limited *, +

+++ meta-analysis studies, ++ cohort studies, + case-control studies, *animals and/or in vivo studies.

1-1. Nuclear factor erythroid-2-related factor 2 (Nrf2)

Sulfur-containing phytochemicals, phenethyl isothiocyanate (PEITC) and sulforaphane for instance, are known as potent phase II gene inducers, and these inductions are Nrf2 dependent (Cheung et al., 2009; Cheung and Kong 2010). PEITC can induce the ERK and JNK phosphorylation and subsequently phosphorylate Nrf2 to induce its translocation to nucleus. Sulforaphane modifies the Keap1-Nrf2 interaction and attenuates Nrf2 degradation, which results in the translocation of Nrf2. Sulforaphane can react with thiols of Keap1 by forming thionoacyl adducts, thereby releasing Nrf2 from Keap1 binding (Hong et al., 2005). Epigallocatechin gallate (EGCG) and curcumin have been reported to be capable of regulating Nrf2 activity via a similar pathway. Treatment with EGCG increased the nuclear accumulation of Nrf2, ARE binding, and transcriptional activity in human breast epithelial MCF10A cells (Na et al., 2008). *In vivo* study showed that curcumin administration enhanced nuclear translocation and ARE-binding of Nrf2 in dimethylnitrosamine (DMN)-induced rats, suggesting that curcumin has hepatoprotection potential in DMS-induced hepatotoxicity through Nrf2 activation (Farombi et al., 2008).

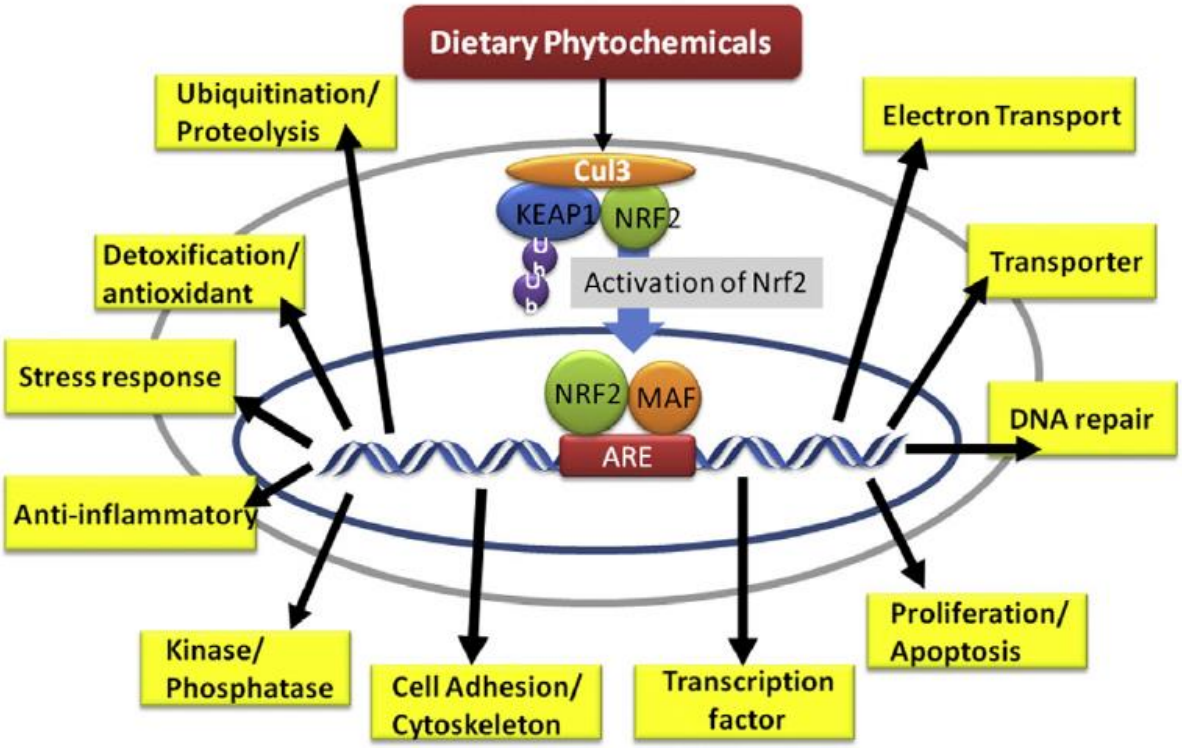

Figure 1-1. Phytochemicals on the regulation of Nrf2 signaling. Adapted from Lee et al. Pharmacol Ther 2013; 137: 153-171, with permission of Elsvier.

1-2. Histone modifications

Histone modification such as lysine acetylation, causes the chromatin to open up by the negative charge conferred by the acetyl moieties, which reduces the histone-DNA interactions. The lysine acetylation reactions are catalyzed by histone acetyltrasferases (HATs), which transfer the acetyl groups from acetyl coenzyme A to the lysine moieties in the nucleosomes. HATs also play important roles in regulating cell cycle related proteins expression (Wang et al., 2001).

Histone deacetylases (HDACs) remove acetyl groups from lysine residues to reduce the negative charge, leading to chromatin compaction. Overexpression and altered activities of HDACs are associated generally with the silencing of tumor suppressor genes, epithelial to mesenchymal transitions and metastasis (Chen et al., 2013; Park et al., 2011).

Accumulated evidences show that phytochemicals which were earlier known only for their antioxidant or chemopreventive effects are also potent epigenetic regulators. These compounds can target or revert abnormal epigenetic modifications in various human pathologies including cancer (Gerhauser, 2013; Hauser and Jung, 2008; Huang et a., 2011; Tharkur et al., 2014).

Table 1-2. Phytochemicals and histone modification. Reproduced from Shankar et al. Semin Cancer Biol 2016; 40-41: 82-99, with permission of Elsevier

Phytochemicals	**Molecular mechanisms**	**Target genes**
Apigenin	HDAC inhibitor	P21
Epigalocatechin-3-gallate	HAT inhibitor HMT inhibitor	H3/H4 acetylation NF-kB, IL-6, BMI-1, EZH2, SUZ12
Genistein	HAT inhibitor	Acetylation H2A/H2B/H3/H4
Daidzein	HDAC inhibitor	Histone acetylation
Allyl mercaptan	SIRT1 inhibitor	H3/H4 acetylation P21/WAF1
Diallyl disulphide	HDAC inhibitor	H3/H4 acetylation P21/WAF1
Reveratrol	SIRT1 activator	TNF-α
Sulforaphane	HDAC inhibitor	H3/H4 acetylation RARβ, HBD-2, p21, Bax

1-3. Histone deacetylases (HDACs)

Histone acetylation and deacetylation are the greatest interest in the field of post-translational modifications. These processes are regulated by two groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyze the transfer of an acetyl group from acetyl coenzyme A to the ε-amino site of lysine, neutralizing the positive charge on histones and results in an open chromatin that frequently leads to increased DNA transcription (Glozak et al., 2005). HDACs deacetylate lysine residues resulting in a closed chromatin conformation and repressed transcription.

There are 18 HDACs, classified into Zn^{2+}-dependent (class I, II and IV) and Zn^{2+}-independent, NAD-dependent (class III) enzymes. Class I HDACs comprise HDACs 1-3 and 8 and these are ubiquitously expressed in all cells and have histones as substrates. HDACs 1, 2 and 3 are located in the nucleus and target multiple substrates, such as p53, signal transducer and activator of transcription 3 (STAT3), E2F1, myo-D, Rel-A, pRb, and YY1 (Witt et al., 2009).

Class II HDACs comprise HDACs 4, 5, 6, 7, 9 and 10. They can be present in either the cytoplasm or nucleus. They are sub-divided into two classes: class IIa HDACs (4, 5, 7 and 9) and class IIb HDACs (6 and 10) (de Ruijter et al., 2003). Their functions seem to be more tissue specific, with roles in the vascular and nervous systems, bone, heart and skeletal muscle. HDAC9 is involved in cardiomyocyte differentiation (Zhang et al., 2002). HDAC4 functions as a repressor of chondrocyte hypertrophy (Vega et al., 2004), and HDAC5 is a negative regulator of angiogenesis in endothelial cells (Urbich et al., 2009). HDAC7 functions in the

negative regulation and apoptosis of T cells (Dequiedt et al., 2003).

Class IIb HDACs (6 and 10) are located in the cytoplasm. HDAC6 is the only HDAC to have two catalytic sites and a ubiquitin-binding site. It can bind directly to ubiquitinated proteins through a ubiquitin-binding domain, targeting proteins for subsequent processing (Boyault et al., 2006). HDAC6 to have a vital role in aggresome formation and may participate in regulating cell viability in response to misfolded proteins (Kawaguchi et al., 2003).

HDAC6 has several specific non-histone substrates, including α-tubulin, cortactin and heat-shock protein 90 (HSP90) (Marks, 2010).

2. Cell death mechanisms

2-1. Apoptosis

The most common and well-defined form of programmed cell death (PCD) is apoptosis, a cell-suicide program that is essential for embryonic development, immune-system function and the maintenance of tissue homeostasis (Jacobson et al., 1997). Apoptosis in mammalian cells is mediated by a family of cysteine proteases known as the caspases. To keep the apoptotic program under control, caspases are initially expressed in cells as inactive procaspase precursors. When initiator caspases are activated, they cleave the precursor forms of executioner caspases (Okada and Mak, 2004; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998).

There are two types of apoptotic pathways – the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is activated by the engagement of death receptors on the cell surface. The intrinsic pathway is triggered by various extracellular and intracellular stresses. Signals that are transduced in response to these stresses converge mainly on the mitochondria.

Cell death is also modified by some other mitochondrial proteins. Endonuclease G and apoptosis-inducing factor (AIF) might induce cell death independently of caspase activation. DIABLO (also known as SMAC) (Verhagen et al., 2000) and Omi (also known as HtrA2) (Hegde et al., 2002; Martins et al., 2002) can promote caspase activation by counteracting inhibitor of apoptosis (IAP)-mediated caspase inhibition.

2-2. Autophagy

Macroautophagy (hereinafter referred to as autophagy) is a catabolic process that involves the degradation of cytoplasmic components, protein aggregates and organelles through the formation of autophagosomes and degradation by fusion with lysosomes (Mizushima and Komatsu, 2011). Autophagy depends on an evolutionarily conserved autophagy-related (ATG) genes. In accordance with the protective function of autophagy, silencing or deletion of ATG genes results in accelerated cell death (Maiuri et al., 2007). However, in certain conditions (and depending on the organism), it has been suggested that autophagy can lead to or contribute to cell death.

At the molecular level, the signaling pathway that leads to autophagy involves the activities of phosphatidylinositol 3-kinase (PI3K) and the target of rapamycin (TOR). Class-III PI3K activity is particularly important for the early stages of autophagosomes formation. In contrast, mTOR negatively regulates autophagosome formation and expansion. Therefore, inhibition of mTOR by rapamycin blocks cell-cycle progression and eventually results in autophagy (Noda and Oshumi, 1998; Rohde et al., 2001). Deprivation of amino acids, nutrients or growth factors can also downregulate mTOR signaling pathway. The mTOR pathway therefore coordinates signaling pathways that are initiated by nutritional and mitogenic factors, and also controls both protein synthesis and degradation.

2-3. Necroptosis

Necrosis was regarded as an unregulated mode of cell death caused by overwhelming trauma. However, many recent studies revealed the existence of regulated necrosis. Necrosis is characterized by swelling of organelles and cells, rupture of the plasma membrane and release of the intracellular contents (Vanden Berghe et al., 2014).

The TNFR signaling pathway best exemplifies how the necroptotic program can be induced. Following TNF binding to TNRRF1 induces the formation of complex I, comprising RIPK1, TNFR1-associated death domain (TRADD), cIAP1 or cIAP2, and TNF-receptor-associated factor 2 (TRAF2) (Micheau and Tschopp, 2003). cIAPs mediate Lys63-linked ubiquitination of RIPK1, which enables the docking of TGFβ-activated kinase 1 (TAK1), TAK1-bidng protein2 (TAB2), and TAB3; this results in the activation of the inhibitor of nuclear factor-κB (NF-κB) kinase IKK complex. This complex leads to NF-κB and mitogen-activated protein kinase (MAPK) activation (Silke and Brink, 2010; Wertz et al., 2004). Such pro-survival NF-κB target genes encode zinc-finger protein A20 and FLICE-like inhibitory protein (FLIP). A20 polyubiquitinates RIPK1, thereby marking it for proteasomal degradations. The deubiquitinating enzyme cylindromatosis (CYLD) eliminates Lys63-linked ubiquitin chains from RIPK1, leading to the dissociation of RIPK1 from complex I (Hitomi et al., 2008). This deubiquitination changes the function of RIPK1 from promoting survival to death through the formation of DISC (also known as complex IIa), which comprises of RIPK1, RIPK3, TRADD, FADD, caspase 8, and FLIP (Micheau and Tchopp, 2003; Vanden Berghe et al., 2014).

In the absence of caspase-8 and FADD, RIPK1 associates with RIPK3, resulting in phosphorylation and formation of the necrosome (Cho et al., 2009). Accordingly, targeting the kinase domain of RIPK1 with necrostatin 1 inhibits the interaction between RIPK1 and RIPK3 but does not affect the pro-survival NF-κB pathway downstream of RIPK1 (Christofferson and Yuan, 2010; Degterev et al., 2005). Phosphorylation of RIPK3 results in the recruitment of mixed-lineage kinase domain-like protein (MLKL), which initiates necroptosis (Sun et al., 2012). MLKL is phosphorylated by RIPK3, causing the formation of MLKL oligomers that translocate to the plasma membrane, bind to phosphatidylinositol phosphates and form membrane-disrupting pores (Dondelinger et al., 2014).

2-4. Caspase-independent cell death

Pro-apoptotic stimuli that cause mitochondrial outer membrane permeabilization (MOMP) can engage cell death even in the absence of caspase activity – so called caspase-independent cell death (CICD) (Tait and Green, 2008). CICD clearly shares similarities to apoptosis but is distinct morphologically, biochemically and kinetically.

Following the mitochondrial outer membrane permeabilization, various intermembrane-space proteins such as SMAC, HtrA2/Omi and cytochrome c are released into the cytoplasm. Some of these, such as apoptosis-inducing factor (AIF) and endonuclease G (Endo G), may actively kill the cell in a caspase-independent manner. Over time, mitochondrial functions also decline following outer membrane permeabilization. This mitochondrial dysfunction includes a loss of mitochondrial protein import due to cleavage of TIM23 and degradation of cytochrome c. Collectively, these events lead to progressive loss of respiratory chain function and reduced mitochondrial membrane potential ($\Delta\Psi_m$), ultimately to bioenergetic crisis and cell death.

2-5. Senescence

Senescence can be considered as a type of ‘living cell death’ because, although senescent cells maintain the integrity of their plasma membranes, they undergo permanent growth arrest and lose their clonogenicity.

Primary cells in culture initially undergo a period of rapid proliferation, during which the telomeres of their chromosomes become shorter. Eventually, cell growth decelerates and the cells enter a form of permanent cell-cycle arrest known as replicative senescence. Senescent cells show morphological changes, such as flattened cytoplasm and increased granularity, and at the biochemical level, senescent cells have increased β-galactosidase activity (Dimri et al., 1995).

Tumorigenic stresses such as DNA damage and oncogene activation, as well as anticancer agents can also trigger cells to enter senescence. In this case, a senescence program is initiated that induces the activation of various cell-cycle inhibitors and requires the functions of p53, p21/WAF1, p16/INK4A, and the retinoblastoma protein (Rb) (Sage et al., 2003; Stein et al., 1999).

3. Research rationale

Potential anticancer phytochemicals are often extracted from medicinal plants, vegetables, and fruits. The abundance and easy-availability of plants make phytochemicals diverse and economical to study. Moreover, phytochemicals derived from these edible sources are usually safe for human consumption, and even beneficial for normal physiological functions. This means that they exhibit low or no toxicity on normal cells when used at a physiologically relevant concentration (Xie et al., 2016). Further studies on pharmacological mechanisms of natural anticancer compounds showed that they not only show bioactivities such as anti-inflammation and antioxidation, but also target multiple cancer-related signaling pathways (Wang et al., 2012). Their anticancer properties, including apoptosis promoting, anti-proliferative and anti-metastatic activities, work alone or in combination to kill cancer cells.

Once bioactive phytochemicals are identified, they are usually used as a foundation for structural modification or as lead compounds for synthesizing new compounds based on structure-function relationships in order to improve their bioavailability and minimize their toxicity for further development (Tan et al., 2014).

In this study, we firstly extracted *Citrus unshiu* leaves with aqueous methanol to evaluate its anticancer activity. To assess anticancer potential of *Citrus unshiu* leaves extracts, we performed cell viability assay, cell cycle analysis, Hoechst 33342 and acridine orange staining, Western blotting and gas chromatography mass spectrometry (GC-MS) analysis. Secondly, we investigated cytotoxicity and cell death mechanism of phytol, which was identified as a major component of *Citrus*

unshiu leaves extract, through GC-MS. Lastly, we fractionated the crude methanolic extract and we found 2,4-di-tert-butylphenol as the most potent candidate for anticancer agent in *Citrus unshiu* leaves. Furthermore, we elucidated the mechanism of antiproliferative property of 2,4-di-tert-butylphenol to develop this compound as a promising therapeutic agent.

CHAPTER II. *Citrus unshiu* leaf extract induces autophagic cell death in human gastric adenocarcinoma AGS cells

1. ABSTRACT

The pharmaceutical potential of the methanolic extract of *Citrus unshiu* leaves (MECL) was assessed through analysis of its inhibitory effect on cancer cells. The antiproliferative activities of the leaves were evaluated using several cancer cell lines and considerable cytotoxicity was observed in human gastric adenocarcinoma AGS cells. Inhibition of AGS cell viability was both time- and dose-dependent and MECL induced non-apoptotic cell death. AGS cells treated with MECL increased the formation of acidic vesicular organelles and GFP-LC3 puncta. Pretreatment with an autophagy inhibitor, 3-methyladenine, inhibited MECL-induced cell death. These results indicated that the mechanism underlying the anticancer effects of MECL in AGS cells could be via the induction of autophagic cell death. The major compounds of MECL were identified as phytol, 4-ethenyl-2-methoxyphenol, hexadecanoic acid, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, and vitamin E using gas chromatography-mass spectrometry. These results indicate that *Citrus unshiu* leaves can be exploited for numerous pharmaceutical applications as a source of anticancer ingredients.

2. INTRODUCTION

An analysis of chemotherapeutic agents and their sources indicated that over 74.8% of approved drugs are derived from natural compounds (da Rocha et al., 2001; Newman and Cragg, 2012). Some pharmaceuticals derived from natural sources have received a great deal of attention from both the scientific community and the public owing to their demonstrated ability to suppress cancers (da Rocha et al., 2001). In the past decade, various fruit extracts have been screened for potential use as alternative remedies for the treatment of infectious diseases and as cancer chemopreventive agents. Several studies have reported that the lower incidence of gastric cancer could be associated with citrus fruit intake (Bae et al., 2008) and have found that citrus fruits exhibit anticancer effects by inducing apoptosis in cancer cells (Patil et al., 2009; Ko et al., 2010; Lim et al., 2009). *Citrus unshiu,* locally known as “unshiu” or “onjumilgam”, is one of the most popular citrus fruits in Korea and has been reported to possess diverse therapeutic activities including anticancer effects (Lee et al., 2011; Kim et al., 2011). Its peel has been used for traditional medicines against cancer in East Asia (Kamei et al., 2000; Morley et al., 2007; Park et al., 2008).

Apoptosis is programmed cell death and cancer cells elude apoptosis through a variety of pathways. The induction of apoptosis and the inhibition of cancer cell proliferation have been used to evaluate phytochemical anticancer activities and develop new anticancer drugs (Sun et al., 2004; Beevi et al., 2010). On the other hand, autophagy is an evolutionarily conserved and genetically programmed process that degrades long-lived cellular proteins and organelles (Park et al., 2011). In response to stress conditions, cytoplasmic organelles are engulfed by

autophagosomes and subsequently fused with lysosomes, resulting in their degradation and recycling to promote cell survival (Yorimitsu and Klionsky, 2005; Meijer and Codogno, 2004; Mariño and López-Otín, 2004). During the initial stages of autophagy, cellular proteins, organelles, and cytoplasm are sequestered and engulfed by intracellular double-membrane-bound structures called autophagosomes. Autophagosomes mature by fusing with lysosomes to form autolysosomes, in which the sequestered proteins and organelles are digested by lysosomal hydrolases and recycled to sustain cellular metabolism (Mizushima, 2004). The role of autophagy in cancer is quite complicated and controversial; autophagy is assumed to be tumor suppressive during cancer development but to contribute to tumor cell survival during cancer progression (Yang and Klinosky, 2010). Regardless of whether they promote cell survival or cell death, the two processes engage in complex and poorly understood molecular cross talk (Maiuri et al., 2007).

With the aim of exploring the anticancer activity of *Citrus unshiu* leaves, we analyzed the chemical composition of methanolic extracts of *Citrus unshiu* leaves (MECL) using GC-MS and sought to elucidate the ability of MECL to induce apoptosis and/or autophagy in human gastric adenocarcinoma AGS cells. Our results demonstrated for the first time that MECL contain bioactive compounds capable of suppressing the proliferation of AGS cells by inducing autophagy. Thus, autophagic cell death must be considered a therapeutic approach to reduce AGS proliferation and a promising strategy to develop an anticancer reagent from MECL.

3. MATERIALS AND METHODS

3-1. Plant samples

C. unshiu Marc. cv. Miyagawa leaves were collected from National Institute of Subtropical Agriculture, Jeju Province, Korea. Botanical samples were taxonomically identified and a voucher specimen (number SKC.111022) was deposited in the herbarium of the Subtropical Research Institute of Jeju National University. The lyophilized leaves were pulverized using a milling machine and extracted with 80% methanol by sonication for 45 min. The extract was filtered, concentrated with a vacuum rotary evaporator, and lyophilized. The extract was refrigerated (4°C) until use. The extract was dissolved in DMSO to a concentration of 200 mg/mL during the experiments.

3-2. Cell culture

AGS, HeLa, MCF-7, and HepG2 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). AGS and MCF-7 cells were cultured in RPMI-1640 and HeLa, HepG2, and fibroblast cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO_2.

3-3. Reagents

RPMI 1640 medium, DMEM medium, trypsin/EDTA, and fetal bovine serum (FBS) were purchased from Invitrogen. Hoechst 33342 dye, acridine orange dye, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI), and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO). The Annexin V-FITC Apoptosis Detection Kit I was purchased from BD™ Biosciences

(Franklin Lakes, NJ).

3-4. Cell viability

The effect of MECL on cell viability was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, which involves the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Briefly, cells were plated in 96-well plates at an initial density of 5×10^4 cells/mL per well. After 24 h incubation, the cells were pre-treated with or without 1 mM 3-methyladenine for 2 hand then various concentrations (from 0 to 200 μg/mL) of MECL were added to the cells. At the indicated time points, 20 μL of MTT solution (5 mg/mL) was added to each well and the cells were kept in a humidified environment for 3–4 h. The supernatant was removed and dissolved in 150 μL DMSO. Absorbance was detected in a microplate reader at 570 nm (Tecan, Salzburg, Austria).

3-5. Cell morphology analysis

AGS cells were placed in 6-well plates at 5×10^4 cells/mL and treated with the *Citrus unshiu* leaf extract after 24 h of seeding. After 24 h, 10 μM of Hoechst or AO were added to the solution in each well, and the plates were incubated for 10 min at 37°C. The stained cells were then observed under a fluorescence microscope (Olympus, UK).

3-6. Flow cytometry

For sub-diploid population detection, cells were detached from plates by the addition of trypsin-EDTA, washed in phosphate-buffered saline (PBS), fixed in 70% ethanol, treated with RNase A (25 ng/mL), and stained with propidium iodide (PI) (40 μg/mL). The annexin V-FITC apoptosis detection kit I was used according to the

manufacturer's protocol to detect phosphatidylserine translocation from the inner to the outer plasma membrane. For each assay, cells were washed with PBS, diluted in annexin V binding buffer containing annexin V and PI, and incubated for 15 min at room temperature (RT). For autophagy detection, we stained the cells with 10 μM acridine orange, harvested the cells, and kept them in 2 mM EDTA-PBS containing 10% FBS. Data from 10,000 cells/sample were analyzed with Cell Quest software (Becton Dickson, USA). Each experiment was repeated at least 3 times.

3-7. LC3-GFP transfection

Vector pEGFP-LC3B was a kind gift from Professor Tamotsu Yoshimori (Hayama, Japan) and Junsoo Park (Yonsei University, Wonju, Republic of Korea). The transfection was carried out with Lipofectamine (Invitrogen) according to the manufacturer's protocol. After 24 h of incubation with the plasmid-Lipofectamine complex, the indicated doses of extract were applied, followed by further incubation for 24 h before visualization of the LC3-II in autophagosomes under a fluorescent microscope (Olympus, UK).

3-8. GC-MS analysis

Chromatographic analysis was carried out using a Shimadzu GC–MS (Model QP-2010, Shimadzu Co., Kyoto, Japan) in EI (Electron Impact) mode. The ionization voltage was 70 eV and the temperatures of the injector and detector were 250°C and 290°C, respectively. The capillary column used was an RTX-5MS (30 m long x 0.25 mm internal diameter and 0.25 μm, film thickness). The oven temperature programmed at 60°C (isothermal for 2 min) was ramped to 250°C at 5°C/min and then to 310°C at 5°C/min (isothermal for 12 min). Helium was used as the carrier gas

at a flow rate of 1 ml/min, with an injector volume of 1 μL using 1:10 split ratio. The dried methanolic extract powder was dissolved in methanol, filtered through a 0.20 μm syringe filter (Advantec, Tokyo, Japan), and aliquots were injected onto the GC–MS. The mass spectra of each compound obtained from GC-MS were tentatively identified using the spectral data of compounds contained within the WILEY7 and NIST libraries. Furthermore, tentative identification was completed by comparison of their mass spectra with authentic published data (Adams, 1995).

3-9. Statistical analysis

Data are expressed as mean ± standard deviation of three independent determinations. The significance of differences between groups was determined through one-way analysis of variance (ANOVA).

4. RESULTS

4-1. *Citrus unshiu* leaves extract reduced viability of AGS cells

To investigate the cytotoxic effects of unshiu leaves on human cancer cell lines, MTT assay was conducted. The cells incubated with various concentrations of the methanolic extract of *Citrus unshiu* leaves (MECL) showed a dose-dependent reduction in cell viability. The AGS cells appeared to be the most sensitive among the cancer cell lines tested with treatment of MCEL for 72 h (Fig. 2-1A). MECL reduced cell viability both time- and dose-dependently in AGS cells (Fig. 2-1B) and no obvious cytotoxicity in normal human dermal fibroblasts was observed even after treatment for 72 h (Fig. 2-1C).

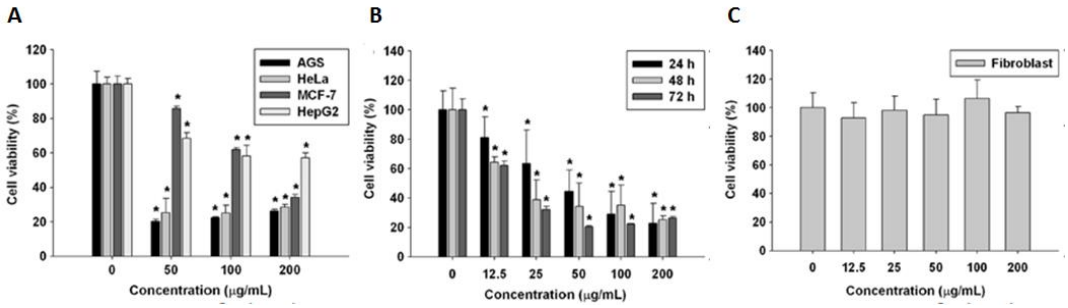

Figure 2-1. Antiproliferative activities of MECL. Cell viability was assessed based on MTT reduction. (A) Several cancer cell lines were treated with the indicated concentrations of MECL for 72 h. The statistically significant differences are presented as *p< 0.05. (B) AGS cells were treated with the indicated concentrations of MECL for 24, 48 and 72 h. *p< 0.05. (C) Normal human dermal fibroblast cells were treated with the indicated concentrations of MECL for 72 h.

4-2. Analysis of cell cycle and the effects of 3-MA on MECL-induced cell death

To evaluate whether the MECL-induced decrease in viability was attributable to apoptosis, we first investigated cell cycle distribution. There were no significant changes in the cell cycles of MECL-treated cells (Fig. 2-2A). Thus, the inhibition of growth observed in response to MECL is not associated with sub-G1 arrest of the cell cycle. Next, we investigated whether MECL induces autophagy in AGS cells since autophagy has been reported to increase as a result of chemotherapy (Liu et al., 2011). Initiation of the autophagy process requires class III PI3K (PI3KC3) and the initiation process can be suppressed by 3-MA, a specific inhibitor of endogenous lysosomal protein degradation that targets PI3KC3 but not the other PI3Ks (Seglen and Gordon, 1982; Araki et al., 2006). For this reason, we measured and compared the viability of cells pre-treated with an autophagy inhibitor, 3-methyladenine (3-MA) to the viability of cells without pre-treatment of 3-MA. The cell viability of AGS cells pre-treated with 3-MA was reversed, indicating that the MECL induced autophagic cell death in AGS cells (Fig. 2-2B).

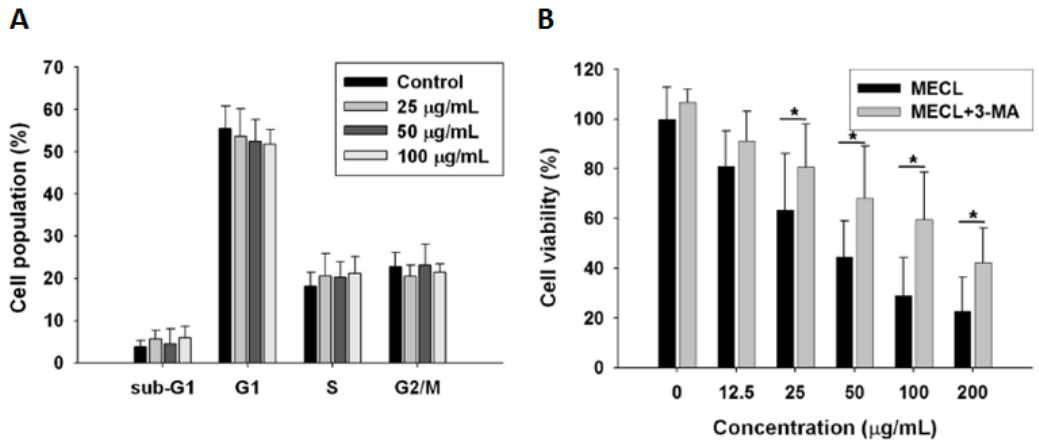

Figure 2-2. Cell cycle analysis and the effect of 3-MA on MECL-induced cell death. (A) AGS cells were treated for 24 h and analyzed for DNA content using flow cytometry. (B) AGS cells were pre-treated with or without 1 mM 3-MA for 2 h and then incubated with the various concentrations (from 0 to 200 μg/mL) of MECL for 24 h. Cell viability was detected using MTT assay. *p< 0.05.

4-3. MECL induced autophagy in AGS cells

To confirm that the MECL-induced decrease in viability was attributable to autophagy, we performed flow cytometric analysis and staining of the cells. Flow cytometric analysis using annexin V/PI double-staining revealed that the percentage of annexin V-positive/PI-negative AGS cells under apoptosis only slightly exceeded the control level (Fig. 2-3A), whereas acidic vesicular organelles (AVO) increased by about 13.49% in the AGS cells treated with 100 μg/mL of MECL (Fig. 2-3B), which is characteristic of autophagy (Herman-Antosiewicz et al., 2006; Yo et al., 2009). Nuclear staining with Hoechst 33342 revealed fragmented and condensed nuclei in the quercetin-treated cells whereas no morphological changes of cells or condensed nuclei were found when the cells were treated with the high concentration of MECL (Fig. 2-3C). As shown in Figure 2-3D, higher numbers of AVOs were detected in the AGS cells treated with MECL. The LC3 has been proposed to serve as a marker for autophagic vesicles and the lipidated form of LC3 has been considered an autophagosomal marker because of its localization and aggregation on autophagosomes (Kabeya et al., 2000; Li et al., 2010; Mizushima, 2004). Therefore, we transiently transfected the AGS cells with a pEGFP-LC3 plasmid and observed that the control cells revealed diffused and weak GFP-LC3 punctuate dots, whereas MECL-treated cells exhibited green LC3 punctuate dots in the cytoplasm (Fig. 2-3E). Collectively, these results indicate that MECL induced not apoptotic cell death but autophagic cell death in the AGS cells.

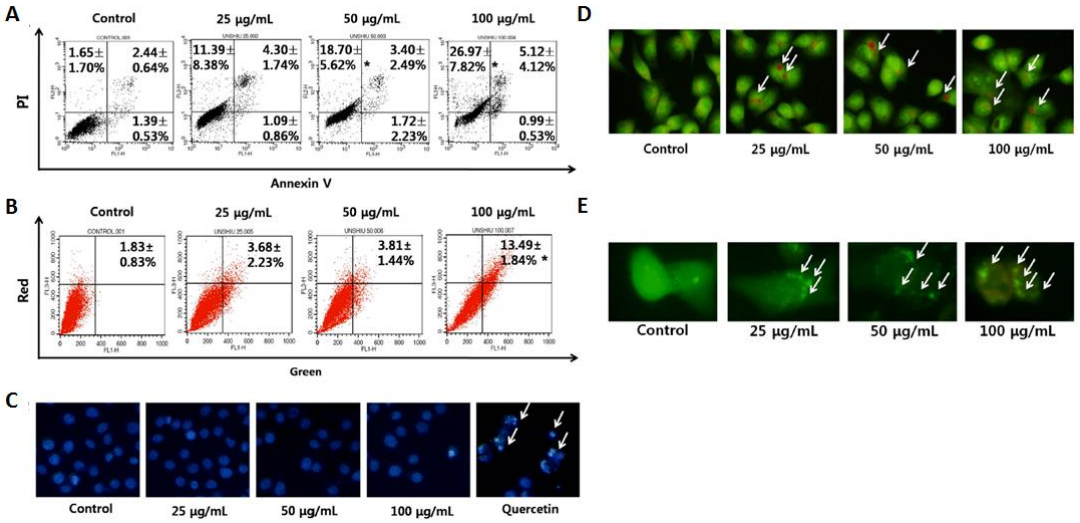

Figure 2-3. MECL induces non-apoptotic but autophagic cell death. (A) Cells were treated with 0 to 100 μg/mL MECL for 24 h. After treatment, the cells were collected and stained with annexin V and PI and then analyzed by flow cytometry. *p< 0.05. (B) Quantification of AVO-positive cells by flow cytometry. Cells were seeded in 60 mm dishes and treated with the indicated concentration of MECL. After 24 h, the cells were stained with 10 μM of AO, trypsinized, and analyzed. *p< 0.05. (C) Nuclear Hoechst staining. Cells were treated with the indicated concentrations of MECL, DMSO (control), or Quercetin 50 μM (positive control) for 24 h, then stained with Hoechst 33342 and observed under a fluorescent microscope. Arrows indicate the formation of apoptotic bodies. (D) Representative images for increased AVO-positive cells. Cells were seeded in 60 mm plates for 24 h and treated with the indicated concentrations of MECL for an additional 24 h. Arrows indicate the formation of AVO. (E) AGS cells were transfected with an expression construct for LC3 fused to green fluorescent protein (GFP-LC3) for 24 h. Cells were treated with or without MECL for 24 h and visualized under a fluorescent microscope. Arrows indicate the puncta of LC3.

4-4. Compositional analysis by GC–MS

These data suggest that *Citrus unshiu* leaves-derived phytochemicals play a vital role in preventing human gastric cancer cell proliferation. Thus, we analyzed the composition of MECL by GC–MS and tentatively identified 30 compounds that account for nearly 90% of the constituents (Fig. 2-4 and Table 2-1). MECL contains different groups of phytochemicals including hydrocarbons, alcohols, lactones, pyrans, flavones and sterols. Phytol was the major compound and the sequence of compounds in relative concentration was: 4-ethenyl-2-methoxyphenol, hexadecanoic acid, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, and vitamin E. Stigmasterol, γ-sitosterol, and δ5-ergostenol were identified as minor steroidal compounds. It is possible that therapeutic properties are expressed by the constituents of MECL, in which case the above compounds, either individually or synergistically, would be responsible for the anticancer activity. We are currently exploring the effects of the five major compounds and the potential of these compounds as therapeutic agents for the prevention of gastric cancer.

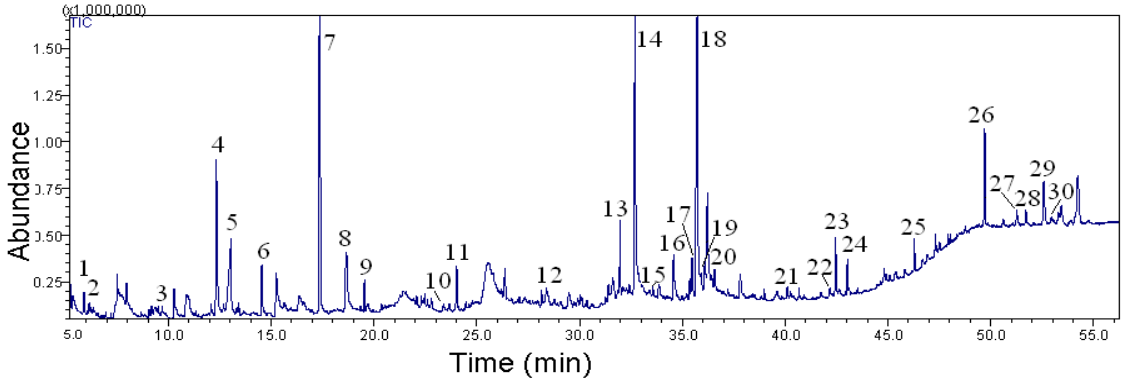

Figure 2-4. GC-MS chromatogram of MECL.

Table 2-1. Compounds identified from the methanolic extract of *Citrus unshiu* leaf by GC-MS

No.	RI[a)]	Name [b)]	Area % [c)]
1	923	Butyrolactone	0.47
2	933	1,2-Cyclopentadione	0.22
3	1060	Furaneol	0.38
4	1147	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	3.28
5	1170	Benzoic acid	1.53
6	1219	4-Vinylphenol	1.06
7	1316	4-Ethenyl-2-methoxyphenol	13.81
8	1363	p-Hydroxybenzaldehyde	1.17
9	1394	β-Elemene	0.69
10	1554	Elemol	1.25
11	1568	3,4-dimethoxyacetophenone	0.90
12	1743	Coniferyl alcohol	0.34
13	1919	Hexadecanoic acid, methyl ester	1.57
14	1955	Hexadecanoic acid	6.58
15	1964	Bis(2-methoxyethyl)-phthalate	0.18
16	2054	Heptadecanoic acid	0.31
17	2090	(Z,Z)-9,12-Octadecadienoic acid methyl ester	0.42
18	2109	(E)-Phytol	47.35
19	2128	(Z,Z)-9,12-Octadecadienoic acid	0.54
20	2155	(Z)-9-Octadecenoic acid	0.47
21	2398	Tetracosane	0.31
22	2499	Pentacosane	0.31
23	2507	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	1.23
24	2551	Bis(2-ethylhexyl) phthalate	0.72
25	2826	Squalene	0.70
26	3000<	Vitamin E	2.18
27	3000<	delta.5-Ergostenol	0.28
28	3000<	Stigmasterol	0.29
29	3000<	gamma-Sitosterol	0.91
30	3000<	Nobiletin	0.15
		Total	89.60

[a)] Retention index

[b)] Compounds tentatively identified based on parent molecular ions, retention times, retention indices and elution order, and fragmented spectra compared with the literature

[c)] Peak area percentage (peak area relative to the total peak area %)

5. DISCUSSION

This is the first report on the antiproliferative activity of *Citrus unshiu* leaves extract against AGS human gastric adenocarcinoma cells. Leaves of *Citrus unshiu* are rich in phytol, 4-ethenyl-2-methoxyphenol, hexadecanoic acid, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, and vitamin E, and possess potent anticancer activity. Several ingredients maybe responsible for the induction of cell death, a process in which squalene, for example, is therapeutically active (Reddy and Couvreur, 2009). In particular, phytol, a major compound of MECL dramatically induced AVO formation in AGS cells (data not shown). This result implies that the effect of MECL might come from a phytol. 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one has also been reported to have an anti-proliferative and anti-apoptotic effect in human colon cancer (Ban et al., 2007). Vitamin E may protect against ovarian cancer growth and progression by reducing cancer cell growth and/or promoting apoptosis (Kline et al., 2007). Furthermore, vitamin E was reported as a novel enhancer of autophagy (Karim et al., 2010). In addition, nobiletin, the only flavone identified, has been reported to inhibit the proliferation of several kinds of human cancer cell lines (Kandaswami et al., 1991; Kandaswami et al., 1992; Yanez et al., 2004). It has also been reported to decrease the incidence of chemically induced tumors in mice and rats (Murakami et al., 2000) and to control many human cancer cell lines (Manthey and Guthrie, 2002).

MECL induced autophagy was evidenced by the increase in acidic vacuole organelle formation and GFP-LC3 puncta as well as the inhibition of MECL-induced cell death by pre-treatment with the autophagy inhibitor 3-MA. Our results suggest

that *Citrus unshiu* leaves extract could be effective as a preventive agent against cancer cells. Further studies must be carried out to isolate and purify the compounds that are responsible for the autophagy.

CHAPTER III. Phytol, a major compound of methanolic extract of *Citrus unshiu* leaf, induces apoptosis and ROS-mediated autophagy in AGS cells

1. ABSTRACT

Phytol, a diterpene alcohol produced from chlorophyll, is used widely as a food additive and an aromatic ingredient. However, the molecular mechanisms behind the cytotoxic effects of phytol in cancer cells are not understood. The current study demonstrated that phytol induces apoptosis in human gastric adenocarcinoma AGS cells, as evidenced by an increased cell population in the sub-G1 phase, downregulation of Bcl-2, upregulation of Bax, the activation of caspase-9 and -3, PARP cleavage, and depolarization of the mitochondrial membrane. In addition, phytol induced autophagy, as evidenced by the induction of acidic vesicle accumulation, the conversion of microtubule-associated protein LC3-I to LC3-II, and the suppression of Akt, mTOR, and p70S6K phosphorylation. Most importantly, pretreatment with chloroquine, a lysosomal inhibitor, strongly augmented phytol-induced apoptosis in AGS cells, suggesting that phytol could induce protective autophagy. Furthermore, co-treatment with a reactive oxygen species (ROS) scavenger enhanced the phytol-induced cytotoxicity, which is accompanied by the decrease in the levels of p62 as well as the percentage of acidic vesicular organelles (AVOs) positive cells, suggesting that the cytoprotective Nrf2 pathway was induced by phytol. Taken together, these findings provide valuable insights into the mechanisms by which phytol induces cell death in AGS cells.

2. INTRODUCTION

Phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol) is an acyclic monounsaturated diterpene alcohol from chlorophyll. It is an aromatic ingredient used in many fragrant compounds, and is also found in some cosmetic and non-cosmetic products (McGinty et al., 2010). Known for its medicinal value, phytol has antinociceptive and antioxidant activities (Santos et al., 2013), as well as anti-inflammatory (Ryu et al., 2011) and immunostimulatory effects (Lim et al., 2006). Phytol also elicits antimicrobial activity against *Mycobacterium tuberculosis* (Rajab et al., 1998; Saikia et al., 2010) and *Staphylococcus aureus* (Inoue et al.,2005). Several reports have assessed the anticancer activities of plant extracts that contain phytol as a major component (Hsu et al., 2011; Hsu et al., 2011; Jiang et al., 2014). Similar to these findings, recent reports assessed the apoptosis induction in hepatocellular carcinoma cells (Kim et al., 2015) and the cytotoxic effects of phytol using MTT assays in several tumor cell lines *in vitro* (Pejin et al., 2014). However, the molecular mechanism by which phytol induced cell death remains unclear. In this study, we explored the molecular mechanisms by which phytol induces cell death in AGS gastric cancer cells.

Cell death plays an important role in cancer growth and progression, as well as the efficacy of chemotherapy. Apoptosis is thought to be the principal mechanism by which anti-cancer drugs kill cells. Apoptosis is a highly conserved form of cell suicide that is regulated by programmed cellular signaling pathways that control tissue homeostasis and/or eliminate damaged or infected cells (Elmore, 2007). There are two major representative apoptotic pathways: the extrinsic pathway, which is

mediated by death receptors (DRs), and the intrinsic pathway, which is mediated by mitochondria. The death-receptor-mediated pathway activates caspase-8 by forming the death-induced signaling complex (DISC) (Debatin and Krammer, 2004). Activated caspase-8 then cleaves Bid into truncated Bid (tBid), which connects the death receptor pathway to the mitochondrial pathway by translocating from the cytosol to the mitochondria. The mitochondrial pathway is initiated by the loss of mitochondrial membrane potential (MMP, $\Delta\Psi m$) and release of cytochrome c, which activates caspase-9 and its downstream effector caspases that cleave different substrates to eventually lead to dismantling of the cell (Cory and Adams, 2002; Wolf and Green, 1999).

Autophagy is an evolutionarily conserved cellular degradation pathway for long-lived proteins and organelles (Park et al., 2011). Induction of autophagy might maintain cancer survival under metabolic stress and mediate resistance to anticancer therapies (Yuan et al., 2014). Thus inhibition of autophagy enhances cancer cell death and potentiates various anticancer therapies (Apel et al., 2008; Kim et al., 2006). High levels of autophagy can induce a form of cell death known as type II cell death or autophagic cell death (Baehrecke, 2005). From a molecular point of view, several cellular signaling pathways play a role in regulating autophagy, including phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR). Previous studies have shown that inhibiting Akt and its downstream target mTOR contribute to the initiation of autophagy (Guertin and Sabatini, 2007). During the initial stages of autophagy cellular proteins, organelles, and the cytoplasm are sequestered and engulfed by intracellular double membrane-bound structures called

autophagosomes. The microtubule-associated protein 1 light chain 3 (LC3), a mammalian homolog of the yeast Atg8, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II, which is recruited to autophagosomal membranes (Tanida and Waguri, 2010). These autophagosomes mature by fusing with lysosomes to form the autolysosomes, in which the sequestered proteins and organelles are digested by lysosomal hydrolases and recycled to sustain cellular metabolism (Mizushima, 2004). Through a comprehensive analysis of LC3-binding, p62 was identified as an LC3-interacting protein (Bjørkøy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007). Because LC3-II present in the inner membrane of the autophagosome is degraded together with other cellular constituents by lysosomal proteases, p62 trapped by LC3 is transported selectively into the autophagosome. The p62 protein localizes to sites of autophagosome formation and can associate with both the LC3 and ubiquitinated proteins (Bjørkøy et al., 2005). Therefore, p62 is considered to act as a receptor for ubiquitinated proteins, which it sequesters into the autophagosome (Johansen and Lamark, 2011) and the impaired autophagy is accompanied by accumulation of p62.

Reactive oxygen species (ROS) is a term that describes the collective species formed by the incomplete reduction of oxygen, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO·) (Chandel and Budinger, 2007). Intracellular redox homeostasis is a key determinant of cell fate: excessive ROS production usually results in cytotoxic effects and might lead to apoptotic cell death, whereas moderate levels of ROS function as second messengers to regulate diverse cellular processes such as cell survival, proliferation, and metastasis

(Inokuma et al., 2009; Wu, 2006). The accumulation of ROS can induce autophagy, which in turn facilitates the clearance of excessive ROS to protect cells from oxidative damage; this might reflect the balance of either cell survival or death (Scherz-Shouval and Elazar, 2011; Shi et al., 2012). During oxidative stress, ROS-mediated KEAP1 inhibition results in the activation of Nuclear factor-erythroid 2-related factor 2 (Nrf2) and the expression of its target genes p62, heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase, quinine 1 (NQO1) (Jain et al., 2010). Moreover, p62 can interact directly with KEAP1 to promote the non-canonical activation of Nrf2 and creating a positive feedback loop that further amplifies p62 expression (Komatsu et al., 2010). Therefore, p62 stimulates autophagy and the Nrf2-mediated antioxidant response during oxidative stress.

The main objective of this study was to examine the cytotoxic mechanism of action of phytol, an aromatic ingredient used in many fragrant compounds. Here, we demonstrate that phytol induces apoptosis and autophagy in human gastric adenocarcinoma AGS cells. Phytol-induced apoptosis was enhanced by inhibiting autophagy or suppressing ROS accumulation. These findings strongly suggest that phytol-induced autophagy might function as a survival pathway against apoptosis, and that the combination treatment with phytol and an autophagy inhibitor or ROS scavenger might exert promising therapeutic effects in stomach cancer.

3. MATERIALS AND METHODS

3-1. Cell culture

AGS human gastric adenocarcinoma cells and human dermal fibroblast cells were obtained from the ATCC (Manassas, VA, USA). AGS cells were cultured in F-12K medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO_2.

3-2. Reagents

F-12K medium, trypsin/EDTA, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Phytol, Hoechst 33342 dye, acridine orange dye (AO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), chloroquine (CQ), RNase A, 2',7'-dichlorofluorescin diacetate (H_2DCF-DA), and anti-LC3 and -actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The BD^{TM} Mitoscreen (JC-1) kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-caspase-9, -caspase-3, -PARP, -Bcl-2, -Akt, -p-Akt, -mTOR, -p-mTOR, - p70S6K, -p-p70S6K, and -Bax antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Western blotting polyvinylidene fluoride (PVDF) membranes were purchased from Merck Millipore (Darmstadt, Germany).

3-3. Cell viability assay

The effects of the treatments on cell viability were determined using MTT assays, which are based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Briefly, cells were plated in 96-well plates at an initial density of 6 × 10^3 cells/well. After a 24-h incubation, the cells were treated with phytol alone or

combination of 60 μM chloriquine or 2 mM NAC and then incubated for an additional 24 h. At the indicated time points 20 μL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated in a humidified environment for 3–4 h. The supernatant was then removed, and the crystals were dissolved in 150 μL DMSO. The absorbance at a wavelength of 570 nm was measured in the microplate reader (Tecan, Grodig, Austria).

3-4. Cell morphology

AGS cells were placed in 60 mm plates at a density of 3×10^4 cells/mL, and then treated with phytol after 24 h. After an additional 24 h, 10 μM Hoechst or AO was added to each well, and the plates were incubated for 10 min at 37°C. The stained cells were then observed under a fluorescence microscope (Olympus, Tokyo, Japan).

3-5. Flow cytometry

To detect the sub-diploid population cells were detached from plates by the addition of trypsin-EDTA, washed in phosphate-buffered saline (PBS), fixed in 70% ethanol, treated with RNase A (25 ng/mL), and finally stained with 40 μg/mL PI. The generation of ROS was measured using H_2DCF-DA as a substrate. Cells were incubated with 10 μM H_2DCF-DA for 30 min, harvested, resuspended in PBS, and analyzed. To detect autophagy cells were stained with 10 μM AO, harvested, and suspended in PBS. The data from 10,000 cells/sample were analyzed using CellQuest software (Becton Dickson, Franklin Lakes, NJ, USA). Each experiment was repeated at least three times.

3-6. Western blotting analysis

Cell lysates were prepared by incubating cells in 150 μL of lysis buffer (100 mM

Tris-HCl, pH 8, 250 mM NaCl, 0.5% Nonidet P-40, 1× protease inhibitor cocktail), disrupted by sonication, and extracted at 4°C for 30 min. The protein concentration was determined using BCA™ protein assays (Pierce, Rockford, IL, USA). Aliquots of the lysates were separated using 12–15% SDS-PAGE and transferred to PVDF membranes using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl pH 8.8, and 20% methanol (v/v). After blocking with 5% non-fat dried milk, the membrane was incubated for 2 h with primary antibodies followed by 30 min with secondary antibodies in Tris-buffered saline (TBS) containing 0.5% Tween-20. Most primary antibodies were used in 1:1,000 dilutions except for β-actin (1:10,000 dilution), and the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were used in 1:5,000 dilutions. Protein bands were detected using the BS ECL-Plus Kit (Biosesang, Gyeonggi-do, Korea).

3-7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Reverse transcription was carried out using the reverse transcription system (Promega, Madison, WI, USA). Polymerase chain reaction (PCR) was performed using Taq polymerase (Enzynomics, Daejeon, Korea) with the following cycling conditions: initiation at 95°C for 1 min, followed by 35 cycles of 40 sec denaturation at 95°C, 40 sec annealing at 57°C, and 40secelongation at 72°C. PCR products (5 μL) were separated on 1.2% agarose gels and then stained with ethidium bromide. PCR primers for amplification were as follows: p62, forward 5’-AAGCCGGGTGGGAATGTTG-3’, reverse 5’-GCTTGGCCCTTCGGATTCT-3’;

HO-1, forward 5’-ACTTCCCAGAAGAGCTGCAC-3’, reverse 5’-GCTTGAACTTGGTGGCACTG-3’; NQO-1, forward 5’-GGGCAAGTCCATCCCAACTG-3’, reverse 5’-GCAAGTCAGGGAAGCCTGGA-3’; GAPDH, forward 5’-GAGAAGGCTGGGGCTCATTT-3’, reverse 5’-AGTGATGGCATGGACTGTGG-3’.

3-8. Statistical analysis

The significance of differences between groups was determined using one-way analysis of variance (ANOVA). The Western blots shown are representative of a group of experiments, and graphs represent the means ± standard deviations.

4. RESULTS

4-1. Phytol induces apoptosis in AGS cells

The antiproliferative effects of phytol on AGS cells are shown in Fig. 3-1. Phytol significantly inhibited the growth of AGS cells compared to untreated control. To determine whether the reduced cell viability of cells was attributable to apoptosis, we assessed morphological changes by staining cell nuclei with Hoechst 33342. Phytol induced the fragmentation and condensation of AGS cell nuclei in a concentration-dependent manner (Fig. 3-2A). Next, the effects of phytol on the cell cycle were analyzed using flow cytometry. The percentage of cells in the sub-G1 peak is shown in Fig. 3-2B. There was a significant and dose-dependent increase in the sub-G1 fraction from 5.02% in control cells to 22.03% in the cells treated with 200 μM phytol. The results of Western blotting revealed that phytol decreased the levels of the anti-apoptotic protein Bcl-2 and increased the cleaved form of caspase-9, -3, PARP, and Bax (Fig. 3-2C). It was suggested that chemical-induced apoptosis is often, but not always, associated with a loss of the mitochondrial membrane potential (ΔΨm) that is caused by increased leakiness of the inner mitochondrial membrane (Salvioli et al., 1997; Sun et al., 1999). The non-toxic fluorescent probe JC-1 becomes concentrated in mitochondria as red fluorescent aggregates when the ΔΨm is high, and is converted to green monomers when the ΔΨm is lost. As shown in Fig. 3-2D, phytol increased the number of mitochondria with disrupted membrane potential in a dose-dependent manner. These results suggest that phytol induced apoptotic cell death by causing DNA damage and mitochondrial membrane dysfunction.

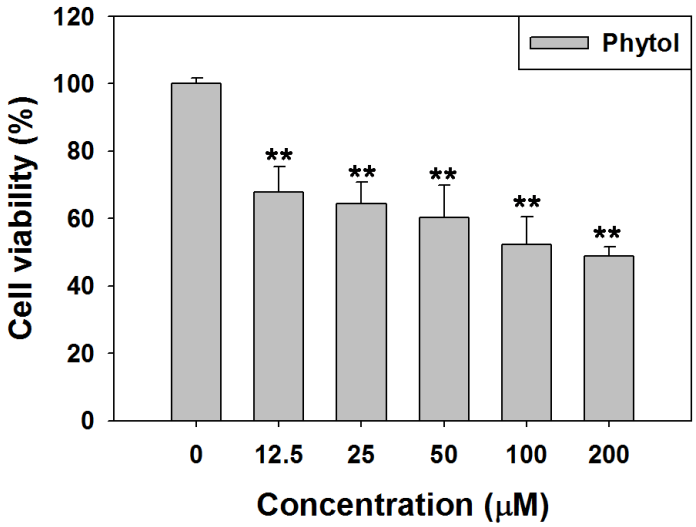

Figure 3-1. The viability of cells treated with various concentrations of phytol for 24 h. The results shown are the means ± S.Ds. of three independent experiments (**p<0.01 vs. control).

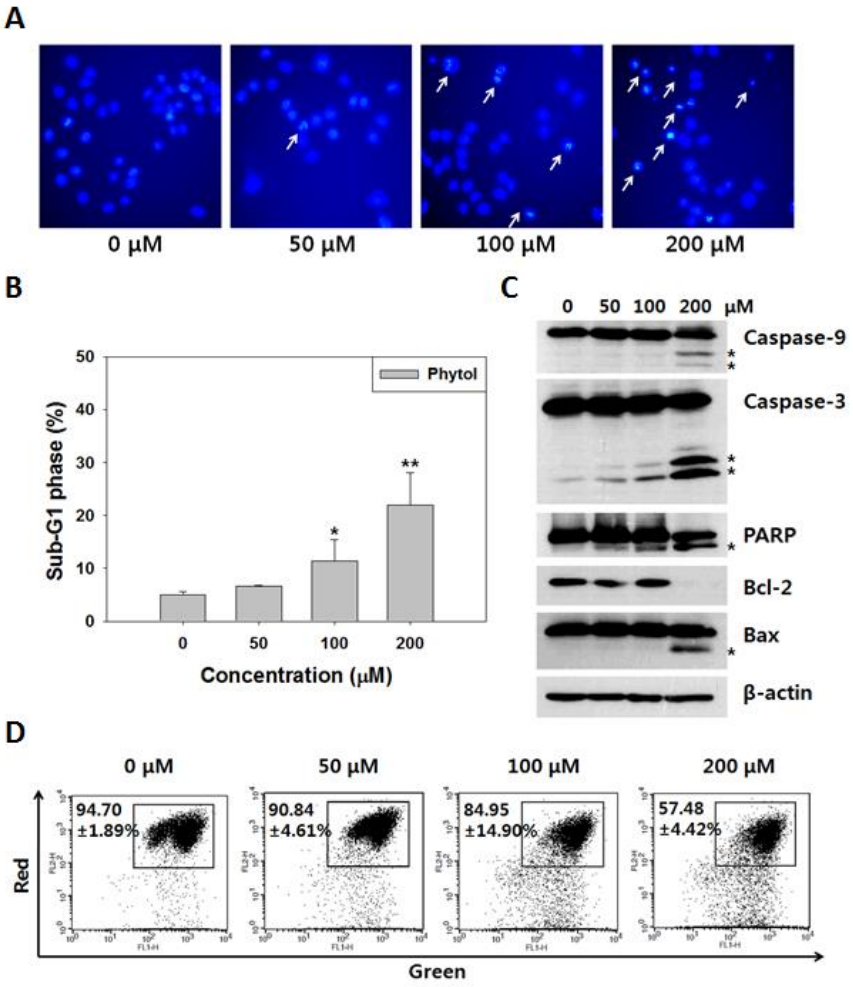

Figure 3-2. Phytol induces apoptosis in AGS cells. (A) Cells were treated with the indicated concentrations of phytol for 24 h, stained with Hoechst 33342, and observed under a fluorescent microscope. (B) AGS cells were treated for 24 h and the DNA content was analyzed using flow cytometry. Statistically significant differences are presented as *p< 0.05 and **p< 0.01. (C) Lysates were prepared from cells treated with DMSO or phytol for 24 h, and Western blotting was performed using caspase-9, caspase-3, PARP, Bcl-2, and Bax antibodies; (*) indicates the cleaved form of the protein. (D) Mitochondrial membrane potential, as assessed using JC-1 staining. **p< 0.01.

4-2. Phytol induces autophagy in AGS cells

Acridine orange (AO) staining was used to analyze the formation of acidic vesicular organelles (AVOs) or autophagolysosome vacuoles, which occurs as the result of fusion between autophagosomes and lysosomes and is a key feature of autophagy (Kanzawa et al., 2004). Large numbers of AVOs (red aggregates) were detected in phytol-treated AGS cells, indicating an induction of autophagy (Fig. 3-3A). The conversion of LC3-I into LC3-II followed by the localization and aggregation of LC3-II in autophagosomes is an autophagosomal marker (Kabeya et al., 2000). Previous studies confirmed that inhibition of the Akt/mTOR pathway triggers autophagy (Kim et al., 2006). Activated Akt, which provides key signals to downstream molecules such as mTOR, can inhibit autophagy and promote cell survival (Yap et al., 2008). As shown in Fig. 3-3B, phytol suppressed the phosphorylation of Akt, mTOR, and p70S6K, and increased the conversion of LC3-I to LC3-II. Because p62 is localized to autophagosomes via an interaction with LC3 and is subsequently degraded by the autophagy-lysosome system, treatment of a lysosomotropic agent can lead to a marked accumulation of p62 (Pankiv et al., 2007). The levels of p62 were greater in the cells treated with both phytol and chloroquine (CQ), a lysosomotropic agent, than those in the cells treated with phytol alone, indicating that phytol activated the autophagic response (Fig. 3-3C and D).

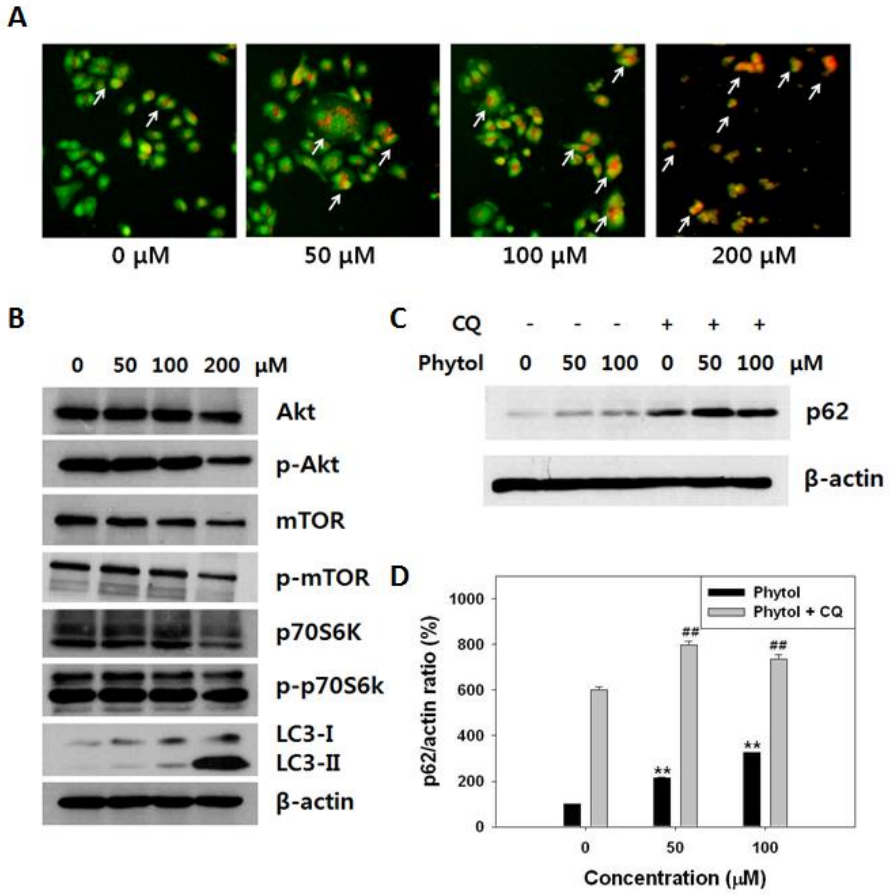

Figure 3-3. Detection of autophagy in AGS cells treated with phytol for 24 h. (A) Acidic vesicular organelles (AVOs) were examined by incubating the cells with 10 μM acridine orange (AO), and the images were analyzed using a fluorescence microscope. (B) Western blotting for Akt, p-Akt, mTOR, p-mTOR, p70S6K, p-p70S6K, LC3, and β-actin after the treatment of cells with the indicated concentrations of phytol for 24 h. (C) AGS cells were treated with phytol in the absence or presence of 60 μM chloroquine (CQ). Cells were then harvested and analyzed by Western blotting using the indicated antibodies. (D) The band intensity of the blots shown in (C) was quantified using the ImageJ software. Data were normalized to the amount of β-actin in each sample. (**p< 0.01 vs. control, ##p< 0.01 vs. CQ treated cells).

4-3. Phytol induces protective autophagy in AGS cells

Because the role of autophagy in the fate of cancer cells remains controversial, we next investigated whether phytol-induced autophagy functioned as a pro-survival or pro-death mechanism. To determine the biological role of autophagy in phytol-mediated apoptotic cell death, CQ was used to disrupt lysosomal function and prevent completion of the autophagic flux. As shown in Fig. 3-4A, the number of apoptotic bodies was increased when cells were co-treated with phytol and CQ compared with phytol alone. In addition, the sub-G1 population was increased two-fold by co-treatment with phytol and CQ compared with those treated with phytol alone (Fig. 3-4B). Furthermore, the amount of the cleaved forms of caspase-3 and PARP proteins was also increased after co-treatment with phytol and CQ (Fig. 3-4C). The results of MTT assays showed that cell viability was decreased further when autophagy was blocked using CQ (Fig. 3-4D). Taken together, these data strongly suggest that inhibiting autophagy enhanced apoptotic cell death in AGS cells treated with phytol.

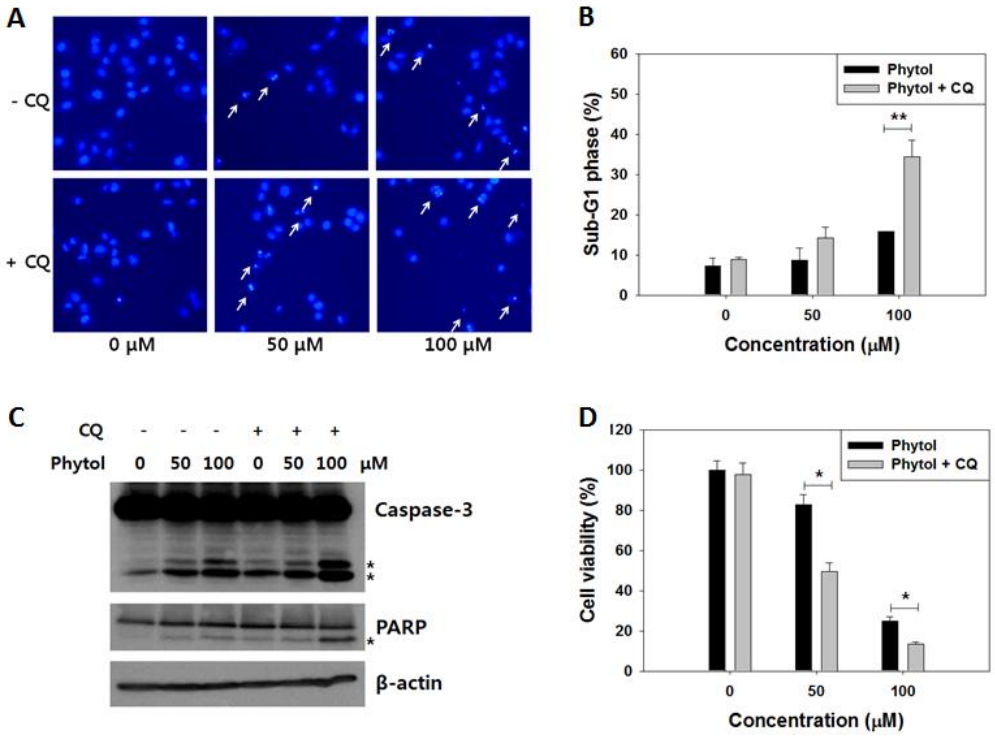

Figure 3-4. Phytol induces protective autophagy in AGS cells. Cells were treated with phytol alone or together with 60 μM chloroquine (CQ) for 24 h. (A) Apoptotic cells were analyzed using Hoechst staining. (B) AGS cells were treated as indicated for 24 h and DNA content was analyzed using flow cytometry. Results are presented as the means ± S.Ds. of three independent experiments. **p< 0.01. (C) Western blotting was performed using caspase-3 and PARP antibodies; (*) indicates the cleaved form of the protein. (D) Cell viability was determined using MTT assays. *p< 0.05.

4-4. Phytol induces ROS mediated Nrf2 cytoprotective pathways

Because the results described above showed that phytol increased the levels of p62 (Fig. 3-3C) and it has been reported p62 promotes ROS production (Mathew et al., 2009), we speculated that phytol might activate the Nrf2 cytoprotective pathway. To investigate this possibility, we first examined ROS levels in AGS cells by measuring the percentage of H_2DCF-DA-positive cells, which is proportional to ROS production. As shown in Fig. 3-5A, treatment with 50 and 100 μM phytol increased the percentage of H_2DCF-DA-positive cells by 17.65% and 18.58%, respectively, compared with control (14.81%). Interestingly, the expressions of the Nrf2 target genes, *p62*, *HO-1*, and *NQO1* were increased dramatically, in contrast to the slight increase in ROS production (Fig. 3-5B and C). In order to confirm that all these responses are due to the increase in ROS production, the cells were treated with the ROS scavenger N-acetyl-L-cysteine (NAC). The percentage of phytol-induced H_2DCF-DA-positive cells was reduced in the presence of NAC (Fig. 3-5A) and correspondingly p62 protein levels as well as the percentage of AVOs positive cells were reduced (Fig. 3-5D-F). In particular, co-treatment with NAC enhanced the cytotoxic effects of phytol (Fig. 3-5G). Together, these results suggest that phytol-induced ROS can facilitate protective autophagy via Nrf2 antioxidant pathway (Fig. 3-6).

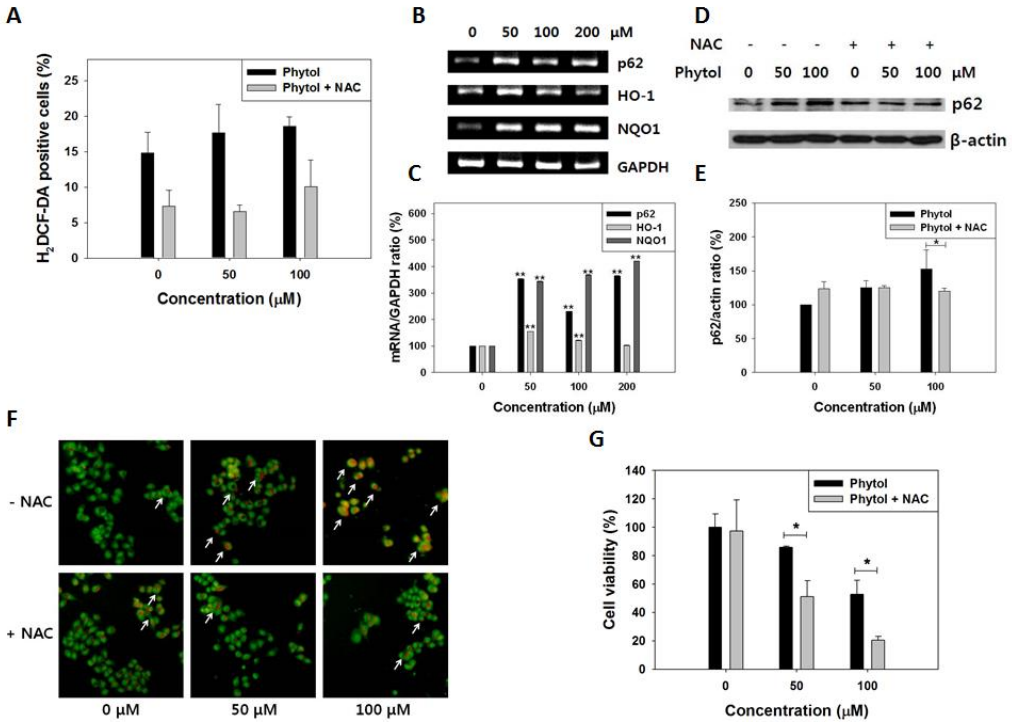

Figure 3-5. Phytol induces ROS-dependent cytoprotective pathways in AGS cells. (A) AGS cells were treated with phytol in the absence or presence of 2 mM NAC for 24 h, and then stained with H_2DCF-DA. The H_2DCF-DA fluorescence intensity was measured using flow cytometry. (B) The mRNA levels of Nrf2 target genes were assessed after treatment with phytol for 24 h. Total RNA was harvested from AGS cells, reverse transcribed, and amplified using gene-specific primers. (C) The values were normalized to the amount of each mRNA in untreated AGS cells. **p< 0.01. (D) AGS cells were treated with phytol in the absence or presence of 2 mM NAC for 24 h, and p62 protein levels were measured using Western blotting. (E) The intensities of the bands shown in (D) were quantified using the ImageJ software. *p< 0.05. (F) Acidic vesicular organelles (AVOs) were analyzed using fluorescent microscopy in AGS cells treated with phytol and/or NAC for 24 h. (G) The viability of AGS cells was determined using MTT assay. *p< 0.05.

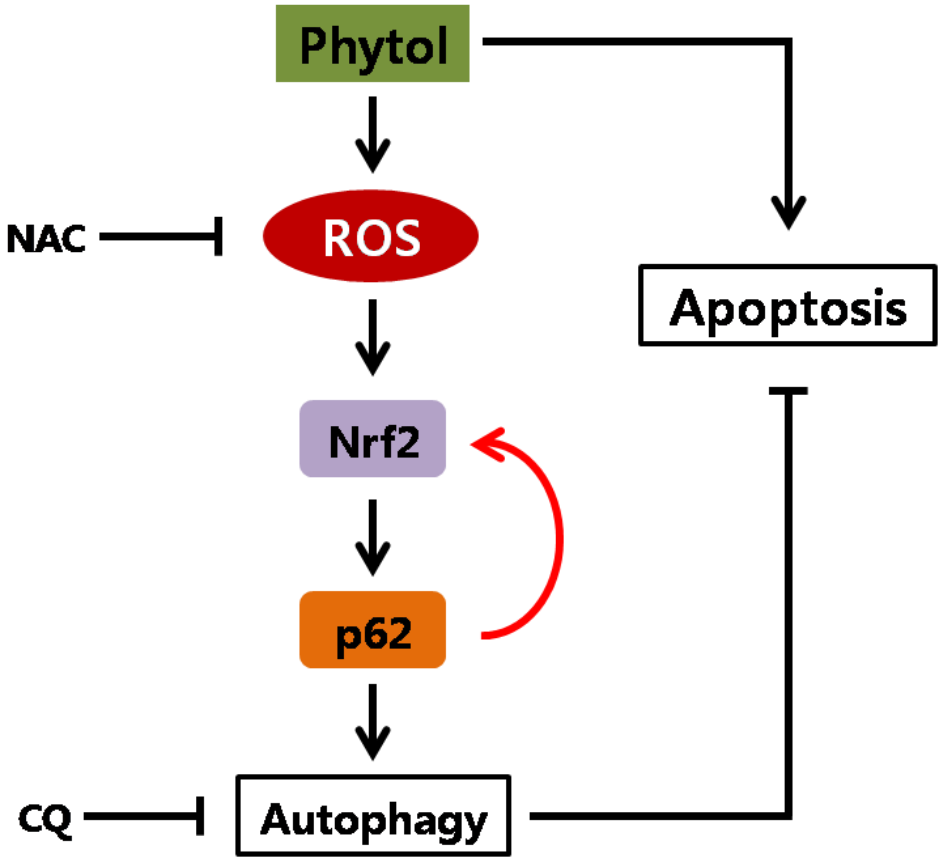

Figure 3-6. A schematic diagram summarizing the cytotoxic effects of phytol in human gastric adenocarcinoma AGS cells. Phytol increases ROS levels, which results in the induction of autophagy. ROS-induced Nrf2 enhances the expression of the antioxidant genes and *p62*. In turn, p62 protein associates with Keap1 (Jain et al., 2010; Komatsu et al., 2010) and activates Nrf2, completing a positive feedback loop (red arrow). Increased p62 levels can induce autophagy in AGS cells, and also have a protective role against phytol-induced apoptosis. Therefore, the induction of apoptosis by phytol treatment can be enhanced by targeting ROS generation or inhibiting the protective autophagy. ROS, Reactive oxygen species; Nrf2, Nuclear factor-erythroid 2-related factor 2; NAC, N-acetyl-L-cysteine; CQ, Chloroquine.

5. DISCUSSION

Phytol is widespread in nature because it is a constituent of chlorophyll (Baxter, 1968). It is a common food additive, and information regarding the oral bioavailability of phytol in mice revealed that it is well absorbed (Mize et al., 1966). Moreover, comprehensive toxicological data are available. For example, the acute oral LD50 of phytol in rats was reported to be >10,000 mg/kg, and it was not mutagenic (McGinty et al., 2010). Some reports suggested that phytol derivatives might activate nuclear hormone receptors and modulate gene expression and cell differentiation (Hansen, 1966). Phytol can activate peroxisome proliferator-activated receptor α (PPARα) directly and thereby regulate the expression of genes related to lipid metabolism (Goto et al., 2005). It also exerts therapeutic effects in models of arthritis and seizures in mice (Hultqvist et al., 2006; Costa et al., 2012). In addition, several reports have revealed that plant extracts containing phytol as a major component have anticancer activities (Hsu et al., 2011a; Hsu et al., 2011b; Jiang et al., 2014).

Our laboratory has been interested in phytol since we identified that *Citrus unshiu* leaf extract, which contains phytol as a major component, induces autophagic cell death in human gastric adenocarcinoma AGS cells (Song et al., 2015). While we were investigating the cytotoxic mechanism of action of phytol, one research group reported that phytol induced concentration-dependent cytotoxic responses in seven cell lines (MCF-7, HeLa, HT-29, A-549, Hs294T, MDA-MB-231, and PC-3 cells) as evaluated using the MTT assays that revealed IC_{50} values ranging from 8.79 to 77.85 μM (Pejin et al., 2014). However, in the current studies milder toxicity was detected

against human gastric adenocarcinoma AGS cells at the concentrations used (IC_{50} 147.67 ± 5.63 μM). This suggests that phytol might induce protective pathways in AGS to render cells resistant to cell death. Therefore, these results emphasize the importance of understanding the molecular mechanisms behind the effects of phytol to enhance its therapeutic efficacy.

The current data demonstrated that phytol inhibited proliferation and induced both apoptosis and autophagy in AGS cells. Apoptosis and autophagy have many common regulators, and crosstalk between these pathways regulates cell fate in response to cellular stress. The complex interaction between apoptotic and autophagic pathways necessitates the careful consideration of both pathways to understand cell death phenomena fully (Filippi-Chiela et al., 2011). Previous studies revealed that several anticancer drugs induced both apoptosis and autophagy in cancer cells (Song et al., 2008). The relationship between autophagy and apoptosis is complex, and varies according to the individual cell types and the specific stress placed upon the cell (Zhang et al., 2009). Although it remains controversial whether autophagy kills cancer cells or sustains their survival under conditions of stress, an increasing number of reports have provided data to support the hypothesis that autophagy promotes the survival of cancer after chemotherapy or radiation therapy, thereby allowing cancer cells to escape drug-induced apoptosis (Carew et al., 2007; Fritsche et al., 2009). CQ is a lysomotropic drug that alters lysosomal pH, prevent autophagosome-lysosome fusion. By blocking the last step of the autophagy pathway, leads to the accumulation of ineffective autophagosomes and cell death (Amaravadi et al., 2007). The current results revealed that the combination of CQ

and phytol inhibited proliferation and increased apoptosis significantly in AGS cells, as evidenced by the cleavage of caspase-3 and PARP. These results suggest that phytol-induced autophagy protects against apoptosis.

The KEAP1-Nrf2 system is a major cellular defense mechanism against oxidative stress (Hayes and McMahon, 2009). Several reports have revealed that during oxidative stress, ROS-mediated KEAP1 inhibition results in the activation of Nrf2 and the expression of its target gene *p62* (Jain et al., 2010). In addition, p62 can interact with KEAP1 via its KIR domain to promote the non-canonical activation of Nrf2, thereby creating a positive feedback loop that further amplifies p62 expression (Komatsu et al., 2010). In particular, p62 has a positive effect on autophagy and the Nrf2-mediated antioxidant response during conditions of oxidative stress. Furthermore, p62 is the highest specific adaptor protein for autophagy and plays a central role in clearance of protein aggregates. (Johansen and Lamark, 2011; Komatsu et al., 2010). Considering that p62 is a receptor for ubiquitinated proteins and organelle to degrade them into the lysosomes, the current results showed that phytol induces ROS production and increases p62 expression and then the Nrf2-p62 loop may function to keep the level of p62 protein. Consistent with this, scavenging ROS by co-treatment with NAC reduced the levels of p62 and acidic vesicular organelles (AVOs) as well as overall cell viability.

Several stimuli that induce ROS production can also induce autophagy, including nutrient starvation, hypoxia, oxidative stress, and some chemotherapeutic agents (Lee et al, 2012). Alterations in ROS levels and autophagy play a crucial role in the initiation and progression of cancer, and both are recognized as potential

targets for cancer treatment (Hale et al., 2013; Mah and Ryan, 2012). Major sources of cellular ROS are the mitochondrial electron transport chain, the NADPH oxidase (NOX) complex, and the endoplasmic reticulum (Chandel and Budinger, 2007; Poli et al., 2004). A recent report revealed that gambogic acid, a polyprenylated xanthone, could promote apoptosis and autophagy in colorectal cancer cells, and that inhibiting autophagy enhanced the sensitivity to gambogic acid treatment. In addition, the accumulation of intracellular ROS via 5-lipoxygenase (5-LOX) was required for gambogic acid-induced autophagy (Zhang et al., 2014). Similarly, the current results showed that phytol-induced autophagy was prevented when ROS generation was blocked by treatment with NAC. Further studies to identify the markers responsible for ROS generation after treatment with phytol are required to elucidate the precise connection between ROS and autophagy.

In summary, the current study revealed that phytol could induce both apoptosis and protective autophagy in AGS cells. The autophagy inhibitor CQ enhanced the inhibitory effects of phytol on cell proliferation and promoted phytol-induced apoptosis in gastric cancer cells. These effects were regulated by ROS generation, which could lead to protective autophagy via a Nrf2-p62 feedback loop. These data suggest that there is crosstalk between apoptosis and ROS-induced autophagy in phytol-treated AGS cells, and provide novel insights into the treatment of cancer using phytol. The effects of phytol could be enhanced by combination treatment with either autophagy inhibitors or ROS scavengers.

CHAPTER IV. 2,4-Di-tert-butylphenol, a component of chloroform fraction of *Citrus unshiu* leaf, induces senescence and mitotic catastrophe in AGS cells

1. ABSTRACT

Histone deacetylase 6 (HDAC6) is a unique cytoplasmic enzyme which contributes to malignant progression in various cancer. Such effect on cancer brings more interest on developing HDAC6 inhibitors. Here, we found that 2,4-di-tert-butylphenol inhibits HDAC6 activity, increases acetylated α-tubulin and reduces β-catenin expression and cell proliferation. Increase of α-tubulin acetylation by DTBP resulted in tubulin polymerization, and consequently, induced aberrant mitosis. Moreover, DTBP elicits different effects depending on different concentrations. Treatment with high concentrations of DTBP induces cell death by mitotic catastrophe, whereas low concentration of DTBP induces senescence with upregulation of p21 and Rb, and increase in the phosphorylation of mTOR and the β-galactosidase activity. We report for the first time that DTBP, which is known as antioxidant and antifungal compound, can have anti-proliferative activity due to the inhibition of HDAC6 enzyme activity. Therefore, DTBP may be considered as a promising new candidate for anti-cancer drug development.

2. INTRODUCTION

Histone deacetylase (HDACs) are group of enzymes that remove the acetyl groups of lysine residues in histone and non-histone proteins (Minucci and Pelicci, 2006). Eighteen distinct HDACs have been identified and are classified into four groups; namely, Class I (HDAC1, 2, 3 and 8), Class II (HDAC4, 5, 6, 7, 9 and 10), Class III (SIRT1-7), and Class IV (HDAC11) (Ecker et al., 2013). Among the 18 HDAC isoenzymes, HDAC6 is mainly localized in the cytoplasm and it deacetylates many non-histone proteins such as α-tubulin, heat shock protein HSP90, and cortactin (Aldana-Masangkay and Sakamoto, 2011). Deacetylation of α-tubulin by HDAC6 is associated with microtubular depolymerization (Hubber et al., 2002; Zhang et al., 2007).

α-Tubulin is known to be acetylated on the lysine residue at position 40 in the amino terminus (LeDizet and Piperno, 1987). The acetylation of α-tubulin plays an important role in regulating microtubule properties and microtubule-based functions such as cell division and intracellular trafficking (Hammond et al., 2008; Sasse and Gull 1988). HDAC6 also has the capacity to bind both polyubiquitinated misfolded protein cargo and dynein motors for facilitating the transport of ubiquitinated proteins to aggresomes. Therefore, HDAC6 is required for autophagic degradation (Kawaguchi et al., 2003; Iwata et al., 2005).

Mitotic catastrophe is a form of aberrant mitosis. Mis-segregation chromosomes leads to formation of cells with multinuclei, often followed by impaired viability (Roninson et al., 2001). Mitotic catastrophe can be induced by agents that disturb the mitotic spindle such as taxol (paclitaxel) (Vakifahmetoglu et al., 2008). In addition to

induction of apoptosis, drug-induced senescence, and mitotic catastrophe can provide another target for cancer therapy (Morse et al., 2005). Cellular senescence is a permanent growth arrest (Muñoz-Espín and Serrano, 2014) which can be induced upon treatment with DNA damaging agents that lead to unrepairable double strand DNA breaks (Saretzki, 2010).

2,4-Di-tert-butylphenol (DTBP), a compound that can be isolated from various bacterial, fungal, and plant sources, has been reported to contain antibacterial (Dehpour et al., 2012), antifungal (Dharni et al., 2014; Rangel-Sánchez et al., 2013; Sang et al., 2011), antibiofilm (Padmavathi et al., 2014; Viszwapriya et al., 2016), antioxidant (Choi et al., 2013) and anticancer (Sathuvan et al., 2012; Varsha et a., 2015) activities. Despite recent findings, the mechanisms underlying the anticancer activity of DTBP are still poorly understood. In the present study, we found that DTBP can inhibit proliferation, induce senescence, and cause mitotic catastrophe in AGS human gastric cancer cells. DTBP inhibits HDAC6 activity and this effect results in accumulation of acetylated α-tubulin and increases of p21 expression. Furthermore, DTBP also exerts its antimitotic activity on A549 human lung cancer and MDA-MB-231 breast cancer cells, which proves that its mode of action is not restricted to a particular type of cancer. Our results suggest that DTBP is a potential lead compound for the development of cancer therapeutic agents.

3. MATERIALS AND METHODS

3-1. Cell culture

The human gastric adenocarcinoma cell line AGS was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The A549 human lung adenocarcinoma cell line and MDA-MB-231 human breast carcinoma were kindly provided by Professor Min Young Kim of the Department of Biotechnology, and Professor Moonjae Cho, Department of Medicine, Jeju National University, Korea, respectively. The cells were cultured in DMEM or F-12K containing 10% (v/v) heat inactivated FBS. The cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2.

3-2. Cell viability assay

The effects of the treatments on cell viability were determined using MTT assays as previously described.

3-3. Colony formation assay

AGS cells were seeded in 24-well plates at 5,000 cells/well and exposed to DTBP for 5 days. After treatment, cells were fixed with 4% paraformaldehyde and stained with crystal violet for 15 min.

3-4. Flow cytometry

Cells were seeded in 60-mm plates and treated with DTBP for 24 h. For the Annexin V/PI assay, the cells were stained with Annexin V-FITC and PI and analyzed using flow cytometry. For the cell cycle and sub-G1 analysis, the cells were harvested, washed with phosphate-buffered saline (PBS), fixed in 70% ethanol, rehydrated in 2 mM EDTA–PBS supplemented with RNase A (25 ng/mL) and stained with PI (40

μg/mL). The PI fluorescence intensities of the cells were measured in each sample using flow cytometry.

3-5. Western blotting analysis

Cell lysates were prepared by RIPA lysis buffer. The protein concentration was determined using BCA protein assays kits. Aliquots of the lysates were separated using 10–15% SDS-PAGE and transferred to PVDF membranes using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl pH 8.8, and 20% methanol (v/v). After blocking with 5% non-fat dried milk, the membrane was incubated with primary antibodies followed by 30 min with secondary antibodies in Tris-buffered saline (TBS) containing 0.5% Tween-20. Most primary antibodies were used in 1:1,000 dilutions except for β-actin (1:10,000 dilution), and the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were used in 1:5,000 dilutions. Protein bands were detected using the BS ECL-Plus Kit (Biosesang, Gyeonggi-do, Korea).

3-6. Immunofluorescence staining

AGS cells were treated with DTBP or paclitaxel for 24 h and then fixed in 4% paraformaldehyde for 30 min at room temperature followed by blocking and permeabilization in PBS containing 10% serum and 0.1% Triton X-100 for 1 h at room temperature. After blocking, cells were incubated with the primary antibody for overnight at 4°C. Following incubation, cells were washed with three times with PBS and incubated with secondary antibody for 1 h. After rinse with PBS, Hoechst 33342 were added, cells were mounted and examined under microscope.

3-7. Tubulin polymerization assay

Cells were treated with DTBP or paclitaxel for 24 h and then lysed with tubulin extraction buffer. After a short and vigorous vortex, the cell lysates were incubated at room temperature for 5 min and then centrifuged at 21,000×g for 10 min. Each supernatant and pellet fraction was resolved on 10% SDS-polyacrylamide gels. The resolved proteins were then subjected to Western blotting with a specific α-tubulin antibody.

3-8. Senescence associated-β-galactosidase staining

AGS cells were cultured in 12-well plate and then treated with DTBP for 3, 4 and 5 days. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Cell were incubated overnight at 37°C in freshly made staining solution and photographed under a microscope.

3-9. In silico docking

The process for the docking experiments followed to the methods reported previously (Shin et al., 2013).

3-10. Statistical analysis

The significance of differences between groups was determined using one-way analysis of variance (ANOVA). The Western blots shown are representative of a group of experiments, and graphs represent the means ± standard deviations of at least two independent experiments.

4. RESULTS

4-1. DTBP as a potential therapeutic compound detected in *Citrus unshiu* leaves extract

The n-hexane, chloroform, ethyl acetate, n-butanol, and H2O fractions, obtained in a stepwise manner to investigate the anti-cancer compounds from the *Citrus unshiu* leaves methanol extracts. The effects of various concentrations of extracts on the growth of human gastric cancer AGS cells were examined using a MTT-based assay. Among tested extracts, the chloroform fraction appeared to exert the most potent effects on AGS cells (Fig. 4-1A). The GC-MS analysis of chloroform fraction identified 14 compounds in the fraction (Table 4-1). In terms of % peak area, cholest-5-en-3.beta-yl-methyl ether (32.14%), alpha-tocopherol (13.42%), campesterol (6.22%), stigmasterol (6.00%), lupeyl acetate (5.05%), beta-tocopherol (3.69%), and 2,4-di-tert-butylphenol (1.31%) were found to be seven major compounds. MTT assay showed that 2,4-di-tert-butylphenol (DTBP) has the highest toxicity towards the AGS cells (Fig. 4-1B). Thus, we presume that DTBP is likely to be the most potent anticancer candidate present in *Citrus unshiu* leaves.

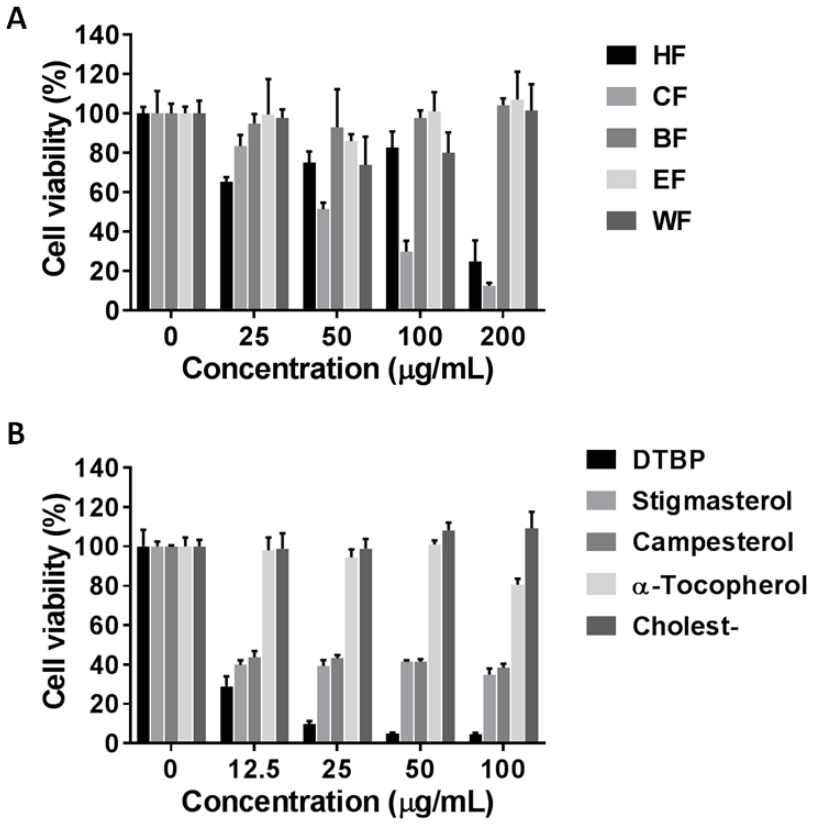

Figure 4-1. Cell viability of AGS cells. Effects of solvent fractions (A) and compounds (B) on the viability were determined by MTT assay.

Table 4-1. Compounds identified in chloroform fraction of *Citrus unshiu* leaf.

No.	RT[a)]	Constituent [b)]	Area % [c)]
1	19.60	2,4-Di-tert-butylphenol	1.31
2	21.98	4-(1,1,3,3-Tetramethylbutyl)phenol	0.45
3	25.13	Coniferol	0.44
4	30.19	Pentadecanoic acid	0.18
5	31.00	6,7-Dimethoxycoumarin	0.14
6	35.01	Phytol	0.90
7	41.10	(Z)-9-Octadecenamide	0.19
8	43.03	Tetracosane	0.38
9	45.60	beta-Tocopherol	3.69
10	47.83	Cholest-5-en-3.beta-yl-methyl ether	32.14
11	48.68	alpha-Tocopherol	13.42
12	51.31	Lupeyl acetate	5.05
13	51.72	Campesterol	6.22
14	53.09	Stigmasterol	6.00

[a] Retention time (min)

[b] Compounds tentatively identified based on parent molecular ions, retention times, and elution order, as well as the fragmentation pattern described in the literature

[c] Relative peak area percentage (peak area relative to the total peak area %).

4-2. Growth inhibitory effect of DTBP

To examine the anticancer activity of DTBP, we first measured the effect of various concentrations of DTBP on the proliferation by MTT assay and crystal violet staining. As indicated in Fig. 4-2A and B, treatment of AGS cells with DTBP resulted in a dose-dependent reduction of cell viability. A similar dose-dependent reduction of cell viability was also observed in A549 and MDA-MB-231 cells (data not shown). The colony formation assay also demonstrated decrease in survival fraction after treatment with DTBP for 5 days (Fig. 4-2C). However, Annexin V/PI staining revealed that the cells after treatment with 5, 10, and 20 μM did not induce any cell death, suggesting that proliferation of the cells at these concentrations is inhibited and cell death is minimal (Fig. 4-2D and E).

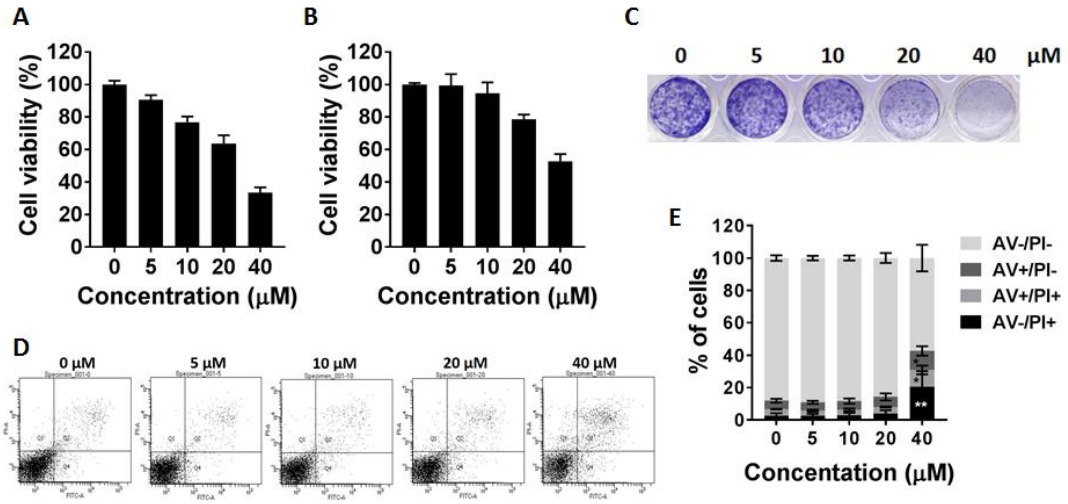

Figure 4-2. DTBP inhibits the proliferation of AGS cells. AGS cells were treated with various concentrations of DTBP for 24 h, and the cell viability was determined by MTT assay (A) and crystal violet staining (B). (C) The growth inhibition effects were measured by colony formation assay. (D and E) Cells were treated with DMSO or various concentrations of DTBP for 24 h. Then they were stained with annexin V and PI and analyzed by flow cytometry.

4-3. Effects of DTBP on the cell cycle

To examine the mechanism for DTBP-induced growth inhibition, cell cycle distribution was analyzed by flow cytometry. As shown in Fig. 4-3A, concentration of DTBP 5, 10, and 20 μM did not significantly change cell cycle distribution while 40 μM dramatically induced apoptotic cells (sub-G1). However, doublet or multinucleated populations were dose-dependently increased (Fig. 4-3B), and phosphorylated histone H3, a marker of mitosis, was decreased in response to DTBP treatment (Fig. 4-3C). Moreover, cleavage of caspase-3 and PARP were detected only at 40 μM (Fig. 4-3D). These data suggest that low concentrations of DTBP could induce mitotic slippage in AGS cells.

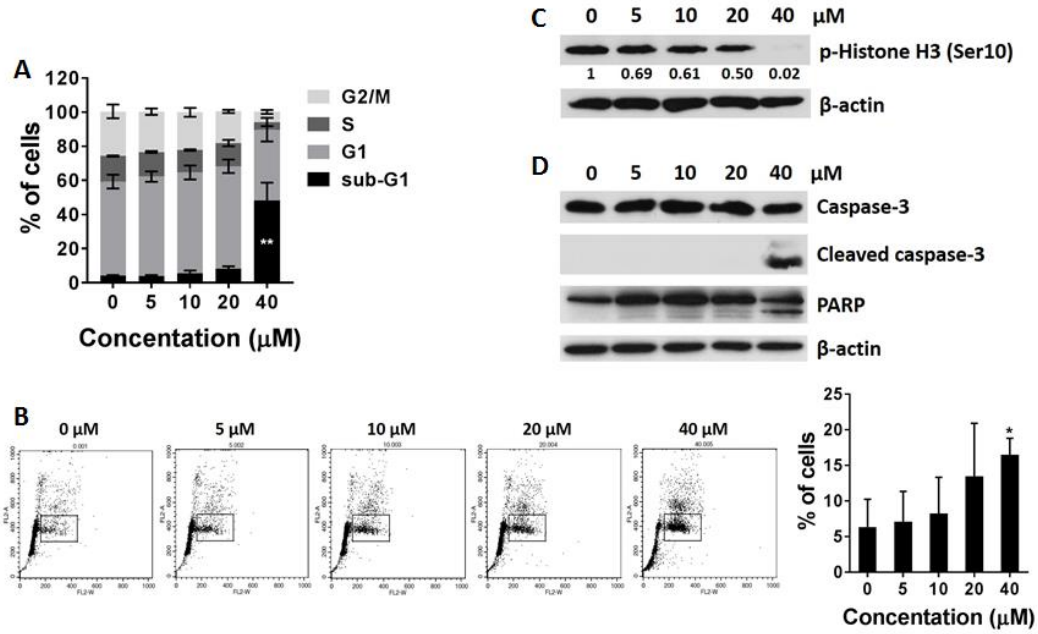

Figure 4-3. DTBP induces mitotic slippage in AGS cells. (A and B) The cell cycle distribution of AGS cells treated with DTBP for 24 h was measured by flow cytometry with PI staining. (C and D) Cells were treated with DTBP for 24 h, then whole cell extracts were extracted and subjected to Western blot analysis.

4-4. DTBP induces senescence in AGS cells

AGS cells treated with low concentration (5, 10, and 20 μM) of DTBP exhibited characteristics of senescence such as cell viability, metabolic activeness, and permanent growth arrest. Therefore, we determined whether growth arrested AGS cells do undergo senescence. It is well known that senescent cells express a senescence-associated β-galactosidase (SA-β-Gal), which is detectable at pH 6.0 using X-gal. Our results showed that DTBP-treated cells expressed SA-β-galactosidase (Fig. 4-4A). Moreover, expression of p21 and Rb proteins, which are believed to have important roles in regulating cellular senescence (Borlon et al., 2008; Schmitt et al., 2002), were up-regulated by DTBP treatment (Fig. 4-4B). mTOR activity is known to be related with senescence, and inhibition of mTOR by rapamycin delays senescence (Leontieva and Blagosklonny, 2010; Xu et al., 2014). As shown in Fig. 4-4C, DTBP treatment increased phosphorylation of mTOR but not Akt. Altogether, these results indicate that induction of senescence contributes to the inhibitory effect of DTBP on the proliferation of AGS cells.

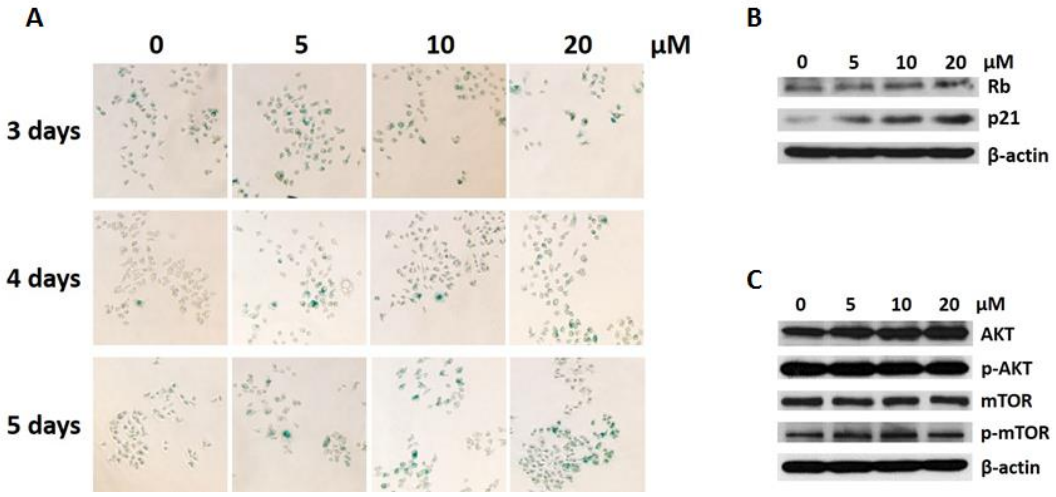

Figure 4-4. DTBP induces senescence via mitotic slippage. (A) AGS cells were incubated with DTBP for 3-5 days and stained for SA-β-galactosidase activity to detect senescence. (B and C) The expression of proteins in AGS cells treated with DTBP for 24 h was analyzed by Western blot.

4-5. DTBP induces aberrant mitosis and multinucleated cells

The microscopic inspection of AGS cells revealed that DTBP increased the level of multinucleated cells and scattered microtubule fragments (Fig. 4-5A). To evaluate the effect of DTBP on microtubule assembly dynamics, we determined cellular tubulin levels. As shown in Fig. 4-5B, DTBP exposure in AGS cells caused an increase in the proportion of polymerized (pellet) tubulin. Moreover, we also observed tubulin hyperpolymerization by DTBP treatment in A549 and MDA-MB-231 cells (Fig. 4-5C). Since dysregulated mitosis can lead to DNA damage, we evaluated the levels of phosphorylated H2AX. Only at high concentrations of DTBP (40 or 60 μM) significantly increased H2AX phosphorylation and PARP cleavage (Fig. 4-5D). Thus, we concluded that DTBP induce cell death by mitotic catastrophe at high doses.

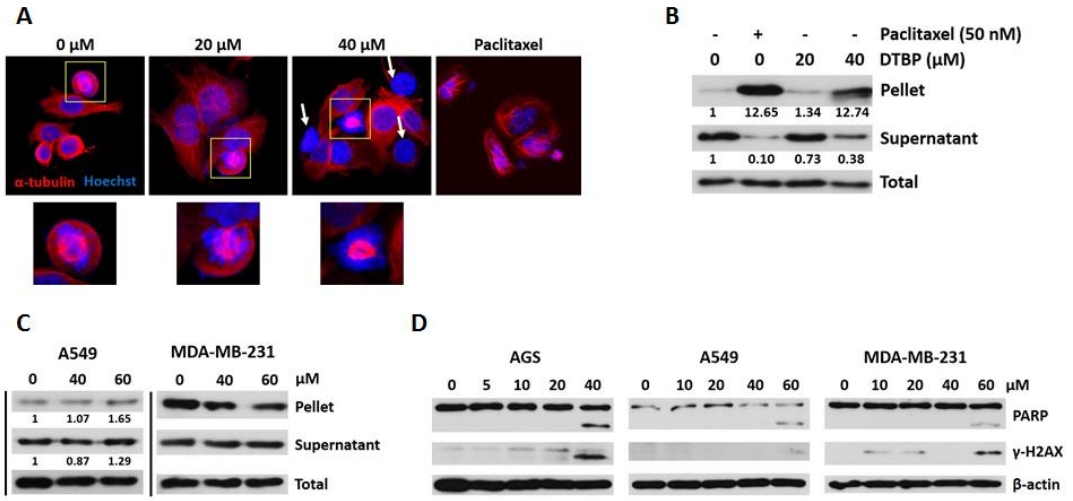

Figure 4-5. DTBP induces microtubule polymerization and mitotic catastrophe. (A) Microtubular polymerization was analyzed by immunostaining of α-tubulin (red) and nuclei staining by Hoechst 33342 (blue). (B and C) The levels of polymerized and depolymerized tubulin in AGS, A549, and MDA-MB-231 cells treated with DTBP for 24 h were analyzed by Western blot. (D) The expression of PARP and γ-H2AX proteins in cells treated with DTBP for 24 h was analyzed by Western blot.

4-6. DTBP inhibits HDAC6 activity

It is widely accepted that acetylation of α-tubulin at lysine 40 is a posttranslational modification associated with more stable, long-lived, and less dynamic microtubules (Cambray-Deakin and Burgoyne, 1987; Houliston and Maro, 1989; Maruta et al., 1986; Matov et al., 2010; Matsutama et al., 2002; Piperno et al., 1987; Tran et al., 2007; Webster and Borisy, 1989). HDAC6, the tubulin deacetylase, plays a key role in maintaining typical distribution of acetylated microtubules in cells (Hubbert et al., 2002; Matsuyama et al., 2002). DTBP treatment increased acetylation of α-tubulin and decreased β-catenin expression (Fig. 4-6). β-catenin is phosphorylated by GSK3β and becomes a target for β-TrCP-mediated degradation (Zhou et al., 2004). However, DTBP treatment did not change the level of GSK3β phosphorylation (Fig. 4-6A) and Ser473 phosphorylation of Akt, an active form of upstream kinase hat phosphorylates Ser9 of GSK3β (Fig. 4-4C). Thus, Akt/GSK3β pathway is not responsible for the β-catenin degradation. HDAC6 deacetylates β-catenin and enhances its nuclear translocation and promoter binding. Inhibition of HDAC6 activity leads to increased β-catenin acetylation and phosphorylation, suggesting that acetylation of β-catenin regulates its stability (Schofield et al., 2013; Wang et al., 2014; Zhu et al., 2011). We next investigated the molecular binding mode between DTBP and HDAC6 was elucidated using in silico docking. Since the three-dimensional (3D) structure of human HDAC6 deposited in the protein data bank only contains zinc-finger domain, the 3D structure of zebrafish including zinc-finger domain as well as catalytic domain was selected. Among 18 3D structures deposited in the protein data bank, 5g01.pdb is consisted of the most residues, 25 – 831

(Miyake et al., 2016). It is a X-ray crystallographic structure with 1.99Å resolution and contains 1-(4-(hydroxycarbamoyl)benzyl)-1-butyl-3-phenylurea (named as Nexturastat A) as its ligand (Fig. 4-7). The HDAC6 structure (5g01.pdb) includes its ligand as well as zinc, potassium and chloride ions. It consists of a homodimer, but chain A was selected for use. To obtain the apo-protein, only its ligand was deleted using the Sybyl 7.3 program (Tripos, St. Louis, MO, USA) (Shin et al., 2013). HDAC6 is composed of two catalytic domains, CD1 and CD2. Since they participate in the activity of the enzyme, both CD1 and CD2 have been used for in silico docking. The binding site of the ligand was analyzed using the LigPlot program [Proteins;37:228] as follows: (CD1) His82, Pro83, Ser150, His193, Gly201, Phe202, Asp230, His232, Trp261, Asp323, and Tyr363, (CD2) His463, Pro464, Ser531, Pro571, His574, Gly582, Phe583, Asp612, His614, Phe643, Asp705, Leu712, and Tyr745. The 3D structure of DTBP was obtained from PubChem (CID: 8372). We followed the methods reported previously (Shin et al., 2013) for detailed docking experiment process. Since 30 iteration of the docking process was performed, 30 complexes were generated. Of those complexes, one showing the best binding energy and docking pose was chosen. While the binding energy between HDAC6 and its original ligand, Nexturastat A in CD1 ranged from -29.04 kcal/mol·Å to -27.48 kcal/mol·Å, that between HDAC6 and DTBP in CD1 ranged from -6.54 kcal/mol·Å to -2.59 kcal/mol·Å. Likewise, the binding energy in CD2 of the HDAC6 - Nexturastat A complex ranged between -27.10 kcal/mol·Å and -23.32 kcal/mol·Å, and that of the HDAC6 - DTBP complex ranged between 8.12 kcal/mol·Å and 5.28 kcal/mol·Å. As shown in Fig. 4-8, while the ligands were docked inside the binding

pocket in the HDAC6 - Nexturastat A complex (A), the ligands were bound at the entrance of the binding pocket in the HDAC6 - DTBP complex (B). As shown in Fig. 4-9, the entrance of the binding pocket consists of hydrophobic residues. However, the deep site of the binding pocket is composed of hydrophilic residues, and zinc ion is placed inside the pocket. As a result, DTBP can be docked at the entrance of the binding pocket, but cannot enter inside the pocket because of its high logP value, which was calculated to be 5.05 by the ChemDraw program (PerkinElmer, Shelton, CT, USA). This is the reason why the binding energy of the HDAC6 - DTBP complex is less than that of the HDAC6 - Nexturastat A complex.

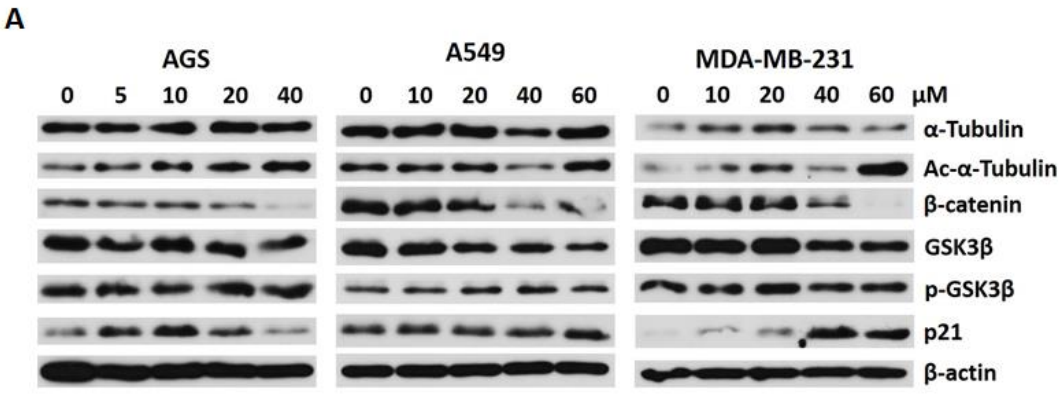

Figure 4-6. DTBP inhibits HDAC6 enzyme activity. Cells treated with various concentrations of DTBP for 24 h were analyzed by Western blotting and probed for α-tubulin, acetyl-α-tubulin, β-catenin, GSK3β, p-GSK3β, p21 and β-actin (loading control).

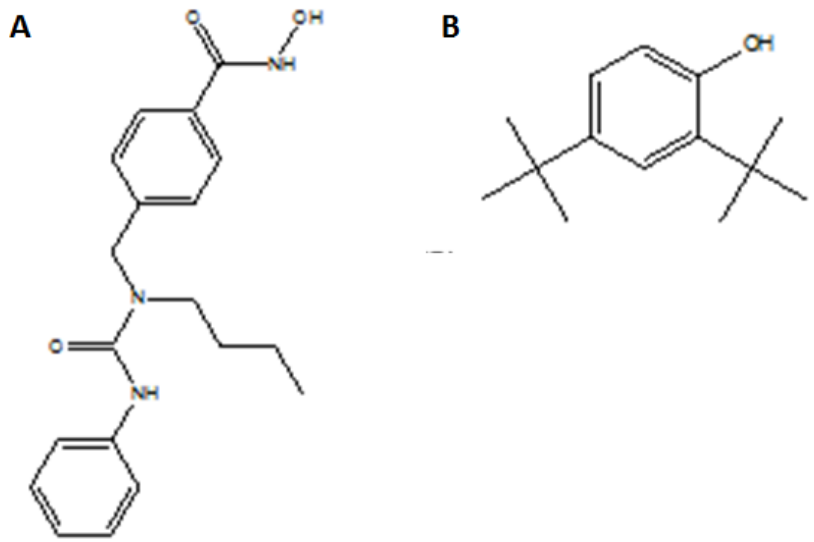

Figure 4-7. The structures of (A) 1-(4-(hydroxycarbamoyl)benzyl)-1-butyl-3-phenylurea (named as Nexturastat A) and (B) 2,4-di-tert-butylphenol (DTBP).

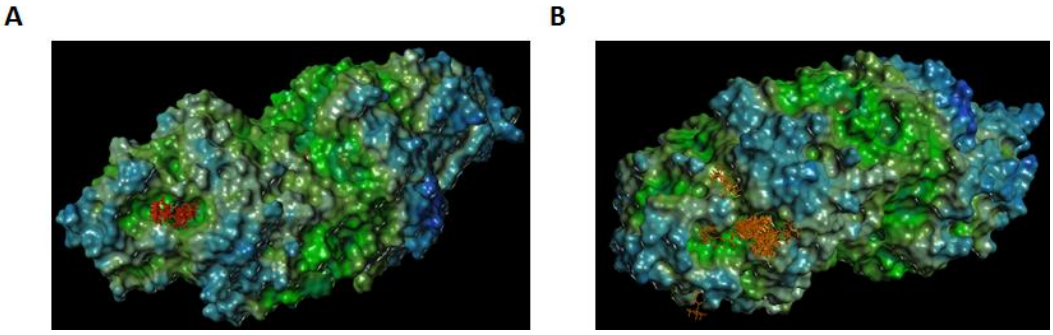

Figure 4-8. (A) A total of 30 HDAC6(CD1) - Nexturastat Acomplexes obtained from in silico docking experiments and (B) 30 HDAC6(CD1) - DTBP complexes.

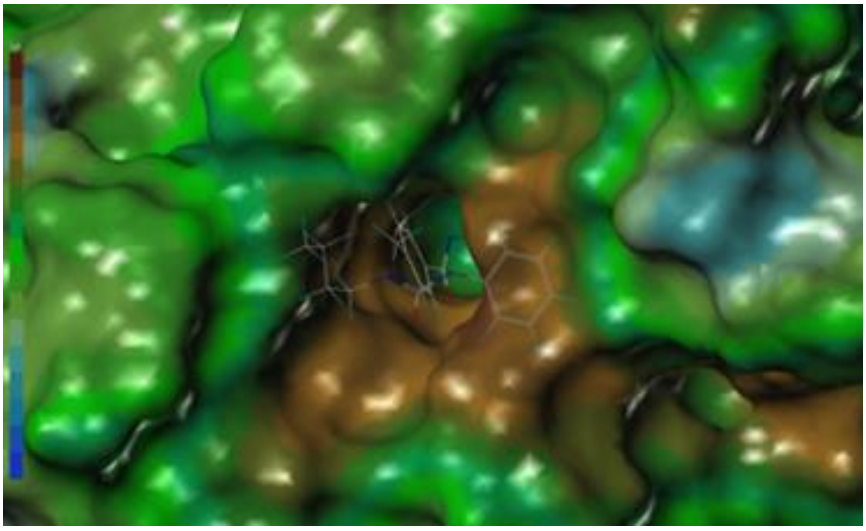

Figure 4-9. The image of the binding pocket of HDAC6 obtained from the X-ray crystallographic structure (5g01.pdb) where brown color and green color indicate hydrophobicity and hydrophilicity, respectively.

5. Discussion

The experiments presented here show that DTBP induces cell death by mitotic catastrophe in cancer cells. Specifically, treatment with DTBP inhibits HDAC6 activity, and this inhibitory effect increases acetylated α-tubulin and disrupts microtubule dynamics.

It is well known that HDACs are promising therapeutic intervention targets in cancer and cancer-related diseases (Tang et al., 2013). HDAC6 is a member of the classIIb family of HDACs, and it acts as a cytoplasmic deacetylase associated with microtubules and deacetylates α-tubulin (Hubbert et al., 2002). In addition, HDAC6 has been reported to be overexpressed in a number of human cancer cell lines. Many studies have suggested that HDAC-mediated gene suppression can cause uncontrolled cell growth because HDACs suppress the transcription of cyclin-dependent kinase inhibitors (CDKIs), allowing continued cellular proliferation (Noh et al., 2011; Xie et al., 2012).

Common cancer treatment drugs aim to induce cell death and apoptosis, which are considered as prerequisites for preventing malignant cell growth. Several studies demonstrated that cellular senescence can occur in vivo and may provide a critical barrier for cancer development and cancer progression (Ewald et al., 2010). In fact, treatment of cancer cell lines with different chemotherapeutic drugs induces irreversible growth arrest associated with senescence like-phenotype (Chang et al., 1999; Vergel et al., 2010). Indeed, induced-senescence appears to be a promising alternative strategy for cancer treatment. Our results show that low concentrations of DTBP induce senescence in AGS cells. We found that DTBP-treated AGS cells

exhibited markers of senescence including SA-β-galactosidase activity, cell cycle arrest, and elevated expression of the cyclin-dependent kinase inhibitor, p21.

Senescence is triggered by DNA damage to cells which respond via the induction of a group of genes responsible for the development of the senescence phenotype (Larsson et al., 2004). The decision of the cell to undergo either apoptosis or senescence is dependent, at least in part, upon the magnitude of DNA damage of cancer cells. Low levels of DNA damage may induce senescence without activating the apoptotic pathway. Several studies reported that many senescence inducing drugs generate DNA damage. In fact, treatment of prostate cancer cells with high doses of doxorubicin, a DNA-damage inducing chemotherapeutic drug, lead to apoptosis, while a lower dose of doxorubicin induces senescence (Rebbaa et al., 2003).

The expression of the cyclin-dependent kinase inhibitor p21 has been implicated in chemotherapy induced cell cycle arrest in various human cancers (Gao et al., 2010; Han et al., 2002; Schwarze et al., 2001). The contribution of p21 to cellular senescence has been demonstrated by numerous studies (Han et al., 2002; Schwarze et al., 2001; Romanov et al., 2010). In fact, p21 has been found to be upregulated in senescent cells. Moreover, forced overexpression of p21 was shown to be sufficient to induce cell cycle arrest, and premature senescence in wild-type and mutant p53 cells (Larsson et al., 2004). In line with these data, we report that DTBP at low concentrations is able to induce maintained upregulation of p21 expression in the cancer cells. In particular, DTBP upregulated p21 expression in p53 mutated MDA-MB-231 cells. It has been reported that histone hyperacetylation is directly linked to the upregulation of p21, and this activation can also occur independent of p53 (Wang

et al., 1998).

In summary, DTBP treatment with low concentration induces a limited DNA damage and p21 upregulation which subsequently results in the induction of senescence. In contrast, high concentrations of DTBP triggers cell death by disrupting microtubules. These results reveal a novel mechanism of DTBP against cancer cells and provides experimental evidence that this compound represent promising lead structures for the development of more potent HDAC6 inhibitors.

REFERENCES

Aldana-Masangkay GI, Sakamoto KM, 2011. The role of HDAC6 in cancer. J Biomed Biotechnol 2011: 875824.

Amaravadi RK, Yu D, Lum JJ, Bui T, Chrostophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB, 2007. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 117: 326-336.

Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A, 2008. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res 68: 1485-1494.

Araki N, Hamasaki M, Egami Y, Hatae T, 2006. Effect of 3-methyladenine on the fusion process of macropinosome in EGF-stimulated A431 cells. Cell Struct Funct 31: 145-157.

Arias BA, Ramon-Laca L, 2005. Pharmacological properties of citrus and their ancient and medieval uses in the Mediterranean region. J Enthnopharmacol 97: 89-95.

Bae JM, Lee EJ, Guyatt G, 2008. Citrus fruit intake and stomack cancer risk: a quantitative systematic review. Gastric Cancer11: 23-32.

Baehrecke EH, 2005. Autophagy: dual roles in life and death? Nat Rev Mol Cell Biol 6: 505-510.

Baena Ruiz R, Salinas Hernández P, 2016. Cancer chemoprevention by dietary phytochemicals: Epidemiological evidence. Maturitas 94: 13-19.

Ban JO, Hwang IG, Kim TM, Hwang BY, Lee US, Jeong HS, Yoon YW, Kim DJ,

Hong JT, 2007. Anti-proliferate and pro-apoptotic effects of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyranone through inactivation of NF-kappaB in human colon cancer cells. Arch Pharm Res 30: 1455-1463.

Baxter JH, 1968. Absorption of chlorophyll phytol in normal man and in patients with Refsum's disease. J Lipid Res 9: 636-641.

Beevi SS, Mangamoori LN, Subathra M, Edula JR, 2010. Hexane extract of Raphanussativus L. roots inhibits cell proliferation and induces apoptosis in human cancer cells by modulating genes related to apoptotic pathway. Plant Foods Hum Nutr 65: 200-209.

Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T, 2005. P62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 171: 603-614.

Borlon C, Vankoningsloo S, Godard P, Debacq-Chainiaux F, Toussaint O, 2008. Identification of p53-dependent genes potentially involved in UVB-mediated premature senescence of human skin fibroblasts using siRNA technology. Mech Ageing Dev 129: 109-119.

Boyault C, Gilquin B, Zhang Y, Rybin V, Garman E, Meyer-Klaucke W, Matthias P, Müller CW, Khochbin S, 2006. HDAC6-p97/VCP controlled polyubiquitin chain turnover. EMBO J 25: 3357-3366.

Cambray-Deakin MA, Burgoyne RD, 1987. Acetylated and detyrosinated alpha-tubulin are co-localized in stable microtubules in rat meningeal fibroblasts. Cell Motil Cytoskeleton 8: 284-291.

Carew JS, Nawrocki ST, Cleveland JL, 2007. Modulating autophagy for therapeutic benefit. Autophagy 3: 464-467.

Chandel NS, Budinger GR, 2007.The cellular basis for diverse responses to oxygen. Free Radic Biol Med 42: 165-174.

Chang BD, Broude EV, Dokmanovic M, Zhu H, Ruth A, Xuan Y, Kandel ES, Lausch E, Christov K, Roninson IB, 1999. A senescence-like phenotype distinguishes tumor cells that undergo terminal proliferation arrest after exposure to anticancer agents. Cancer Res 59: 3761-3767.

Chen X, Xiao W, Chen W, Luo L, Ye S, Liu Y, 2013. The epigenetic modifier trichostatin A, a histone deacetylase inhibitor, suppressed proliferation and epithelial-mesenchymal transition of lens epithelial cells. Cell Death Dis 4: e884.

Cheung KL, Khor TO, Kong AN, 2009. Synergistic effect of combination of phenethyl isothiocyanate and sulforaphane or curcumin and sulforaphane in the inhibition of inflammation. Pharm Res 26: 224-231.

Cheung KL, Kong AN, 2010. Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. AAPS J 12: 87-97.

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK, 2009. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137: 1112-1123.

Choi SJ, Kim JK, Kim HK, Harris K, Kim CJ, Park GG, Park CS, Shin DH, 2013. 2,4-Di-tert-butylphenol from sweet potato protects against oxidative stress in PC12 cells and mice. J Med Food 16: 977-983.

Christofferson DE, Yuan J, 2010. Necroptosis as an alternative form of programmed

cell death. Curr Opin Cell Biol 22: 263-268.

Cory S, Adams JM, 2002. The Bcl2 family: regulators of the cellular life-of-death switch. NatRev Cancer 2: 647–656.

Costa JP, Ferreira PB, De Sousa DP, Jordan J, Freitas RM, 2012. Anticonvulsant effect of phytol in a pilocarpine model in mice. Neurosci Lett 523: 115-118.

da Rocha AB, Lopes RM, Schwartsmann G, 2001. Natural products in anticancer therapy. Curr Opinion Pharmacol 1: 364-369.

Dharni S, Sanchita, Maurya A, Samad A, Srivastava SK, Sharma A, Patra DD, 2014. Purification, characterization, and in vitro activity of 2,4-Di-tert-butylphenol from Pseudomonas monteilii PsF84: conformational and molecular docking studies. J Agric Food Chem 62: 6138-6146.

Debatin KM, Krammer PH, 2004. Death receptors in chemotherapy and cancer. Oncogene 23: 2950-2966.

Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J, 2005. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 1: 112-119.

Dehpour AA, Yousefian M, Jafary Kelarijani SA, Koshmoo M, Mirzanegad S, Mahdavi V, Javad Bayani MJ, 2012. Antibacterial activity and composition ofessential oils of flower Allium rotundum. Adv Environ Biol 6: 1020–1025.

Dequiedt F, Kasler H, Fischle W, Kiermer V, Weinstein M, Herndier BG, Verdin E, 2003. HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity 18: 687-698.

de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB, 2003.

Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J 370(Pt 3): 737-749.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubeli I, Pereina-Smith O, Peacocke M, Campisi J, 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A 92: 9363-9367.

Dondelinger Y, Declercq W, Montessuit S, Roelandt R, Goncalves A, Bruggeman I, Hulpiau P, Weber K, Sehon CA, Marquis RW, Bertin J, Gough PJ, Savvides S, Martinou JC, Bertrand MJ, Vandenabeele P, 2014. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. Cell Rep 7: 971-981.

Ecker J, Witt O, Milde T, 2013. Targeting of histone deacetylases in brain tumors. CNS Oncol 2: 359-376.

Elmore S, 2007. Apoptosis: a review of programmed cell death. Toxicol Pathol 35: 495-516.

Ewald JA, Desotelle JA, Wilding G, Jarrard DF, 2010. Therapy-induced senescence in cancer. J Natl Cancer Inst 102: 1536-1546.

Farombi EO, Shrotriya S, Na HK, Kim SH, Surh YJ, 2008. Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of heme oxygenase-1. Food Chem Toxicol 46: 1279-1287.

Filippi-Chiela EC, Villodre ES, Zamin LL, Lenz G, 2011.Autophagy interplay with apoptosis and cell cycle regulation in the growth inhibiting effect of resveratrol in gliomacells. PLoS One 6: e20849.

Finley JW, Kong AN, Hintze KJ, Jeffery EH, Ji LL, Lei XG, 2011. Antioxidant in foods: state of the science important to the food industry. J Agric Food Chem 59: 6837-6846.

Fritsche P, Seidler B, Schüler S, Schnieke A, Göttlicher M, Schmid RM, Saur D, Schneider G, 2009. HDAC2 mediates therapeutic resistance of pancreatic cancer cells via the BH3-only protein NOXA. Gut 58: 1399-1409.

Gao FH, Hu XH, Li W, Liu H, Zhang YJ, Guo ZY, Xu MH, Wang ST, Jiang B, Liu F, Zhao YZ, Fang Y, Chen FY, Wu YL, 2010. Oridonin induces apoptosis and senescence in colorectal cancer cells by increasing histone hyperacetylation and regulation of p16, p21, p27 and c-myc. BMC Cancer 10: 610.

Gawlik-Dziki U, Świeca M, Sulkowski M, Dziki D, Baraniak B, Czyż J, 2013. Antioxidant and anticancer activities of Chenopodium quinoa leaves extracts – in vitro study. Food Chem Toxicol 57: 154-160.

Gerhauser C, Cancer chemoprevention and nutriepigenetics: state of the art and future challenges. Top Curr Chem 329: 73-132.

Glozak MA, Sengupta N, Zhang X, Seto E, 2005. Acetylation and deacetylation of non-histone proteins. Gene 363: 15-23.

Gorrini C, Harris IS, Mak TW, 2013. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov. 12: 931-947.

Goto T, Takahashi N, Kato S, Egawa K, Ebisu S, Moriyama T, Fushiki T, Kawada T, 2005. Phytol directly activates peroxisome proliferator-activated receptor alpha (PPARalpha) and regulates gene expression involved in lipid metabolism in PPARalpha-expressing HepG2 hepatocytes. Biochem Biophys Res Commun

337: 440-445.

Guertin DA, Sabatini DM, 2007. Defining the role of mTOR in cancer. Cancer Cell 12: 9-22.

Hale AN, Ledbetter DJ, Gawriluk TR, Rucker EB 3rd, 2013. Autophagy: regulation and role in development. Autophagy 9: 951-972.

Hammond JW, Cai D, Verhey KJ, 2008. Tubulin modifications and their cellular functions. Curr Opin Cell Biol 20: 71-76.

Han Z, Wei W, Dunaway S, Darnowski JW, Calabresi P, Sedivy J, Hendrickson EA, Balan KV, Pantazis P, Wyche JH, 2002. Role of p21 in apoptosis and senescence of human colon cancer cells treated with camptothecin. J Biol Chem 277: 17154-17160.

Hansen RP, 1966. Occurrence of phytanic acid in rumen bacteria. Nature 210: 841.

Hauser AT, Jung M, 2008. Targeting epigenetic mechanisms: potential of natural products in cancer chemoprevention. Planta Med 74: 1593-1601.

Hayes JD, McMahon M, 2009. NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. Trends Biochem Sci 34: 176-188.

Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, DuBois G, Lazebnik Y, Zervos AS, Fernandes-Alnemri T, Alnemri ES, 2002. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem 277:432-438.

Herman-Antosiewicz A, Johnson DE, Singh SV, 2006. Sulforaphane causes autophagy to inhibit release of cytochrome C and apoptosis in human prostate cancer cells. Cancer Res 66: 5828-5835.

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J, 2008. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135: 1311-1323.

Hong F, Freeman ML, Liebler DC, 2005. Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. Chem Res Toxicol 18: 1917-1926.

Hosni K, Hassen I, M'Rabet Y, Sebei H, Casabianca H, 2013. Genetic relationship between some Tunisian Citrus species based on their leaf volatile oil constituents. Biochem Syst Ecol 50: 65-71.

Houliston E, Maro B, 1989. Posttranslational modification of distinct microtubule subpopulations during cell polarization and differentiation in the mouse preimplantation embryo. J Cell Biol 108: 543-551.

Hsu HF, Huang KH, Lu KJ, Chiou SJ, Yen JH, Chang CC, Houng JY, 2011a. Typhoniumblumei extract inhibits proliferation of human lung adenocarcinoma A549 cells via induction of cell cycle arrest and apoptosis. J Ethnopharmacol 135: 492-500.

Hsu HF, Wu YC, Chang CC, Houng JY, 2011b. Apoptotic effects of bioactive fraction isolated from Glossogynetenufolia on A549 human lung cancer cells. J Taiwan Inst Chem E 42: 556-562.

Huang J, Plass C, Gerhauser C, 2011. Cancer chemoprevention by targeting the epigenome. Curr Drug Targets 12: 1925-1956.

Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang XF, Yao TP, 2002. HDAC6 is a microtubule-associated deacetylase. Nature

417:455-458.

Hultqvist M, Olofsson P, Gelderman KA, Holmberg J, Holmdahl R, 2006.A new arthritis therapy with oxidative burst inducers. PLoS Med 3: e348.

IFRA (International Fragrance Association), 2004. Use Level Survey, August 2004.

Inokuma T, Haraguchi M, Fujita F, Tajima Y, Kanematsu T, 2009.bOxidative stress and tumor progression in colorectal cancer. Hepatogastroenterology 56: 343-347.

Inoue Y, Hada T, Shiraishi A, Hirose K, Hamachima H, Kobayashi S, 2005. Biphasis effects of geranylgeraniol, teprenone, and phytol on the growth of Staphylococcus aureus. Antimicrob Agents Chemother 49: 1770-1774.

Iwata A, Riley BE, Johnston JA, Kopito RR, 2005. HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. J Biol Chem 280: 40282-40292.

Jacobson MD, Weil M, Raff MC, 1997. Programmed cell death in animal development. Cell 88: 347-354.

Jain A, Lamark T, Sjøttem E, Larsen KB, Awuh JA, Ø vervatn A, McMahon M, Hayes JD, Johansen T, 2010.P62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. J Biol Chem28:, 22576-22591.

Jaramillo MC, Zhang DD, 2013. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. Genes Dev 27: 2179-2191.

Jiang R, Sun L, Wang Y, Liu J, Liu X, Feng H, Zhao D, 2014.Chemical composition, and cytotoxic, antioxidant and antibacterial activities of the essential oil from ginseng leaves. Nat Prod Commun 9: 865-868.

Johansen T, Lamark T, 2011. Selective autophagy mediated by autophagic adapter proteins. Autophagy 7: 279-296.

Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Oshumi Y, Yoshimori T, 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membrane after processing. EMBO J 19: 5720-5728.

Kamei T, Kumano H, Iwata K, Nariai Y, Matsumoto T, 2000. The effect of a traditional Chinese prescription for a case of lung carcinoma. J Altern Complement Med 6: 557-559.

Kandaswami C, Perkins E, Drzewiecki G, Soloniuk DS, Middleton Jr E, 1992. Differential inhibition of proliferation of human squamous cell carcinoma, gliosarcoma and embryonic fibroblast-like lung cells in culture by plant flavonoids.Anticancer Drugs 3: 525–530.

Kandaswami C, Perkins E, Soloniuk DS, Drzewiecki G, Middleton Jr E, 1991. Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma in vitro. Cancer Lett 56: 147–152.

Kanzawa T, Germano IM, Komato T, Ito H, Kondo Y, Kondo S, 2004.Role of autophagy in temozolomide-induced cytotoxicity for malignant gliomacells. Cell Death Differ 11: 448-457.

Karim MR, Fujimura S, Kadowak M, 2010. Vitamin E as a novel enhancer of macroautophagy in rat hepatocytes and H4-II-E cells. Biochem Biophys Res Commun 394: 981-987.

Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP, 2003. The

deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell 115: 727-738.

Kensler TW, Wakabayashi N, Biswal S, 2007. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Phamacol Toxicol 47: 89-116.

Kim CW, Lee HJ, Jung JH, Kim YH, Jung DB, Sohn EJ, Lee JH, Woo HJ, Baek NI, Kim YC, Kim SH, 2015. Activation of Caspase-9/3 and Inhibition of Epithelial Mesenchymal Transition are Critically Involved in Antitumor Effect of Phytol in Hepatocellular Carcinoma Cells Phytother Res 29: 1026-1031.

Kim H, Moon JY, Mosaddik A, Cho SK, 2010. Induction of apoptosis in human cervical carcinoma HeLa cells by polymethoxylated flavone-rich Citrus grandis Osbeck (Dangyuja) leaf extract. Food Chem Toxicol 48: 2435-2442.

Kim HG, Kim GS, Lee JH, Park SM, Jeong WY, Kim YH, Kim JH, Kim ST, Cho YA, Kline K, Lawson AK, Yu W, Sanders GB, 2007 Vitamine E and Cancer. Vitam Horm 76: 435-461.

Ko HC, Jang MG, Kang CH, Lee NH, Kang SI, Lee SR, Park DB, Kim SJ 2010. Preparation of a polymethoxylflavone-rich fraction (PRF) of Citrus sunki Hort. Ex Tanaka and its antiproliferative effects. Food Chem 123: 484-488.

Kim KW, Mutter RW, Cao C, Albert JM, Freeman M, Hallahan DE, Lu B, 2006. Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. J Biol Chem 281: 36883-36890.

Kim SK, Yang JW, Kim MR, Roh SH, Kim HG, Lee KY, Jeong HG, Kang KW, 2008. Increased expression of Nrf2/ARE-dependent anti-oxidant proteins in

tamoxifen-resistant breast cancer cells. Free Radic Biol Med 45: 537-546.

Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, Sou YS, Ueno I, Sakamoto A, Tong KI, Kim M, Nishito Y, Iemura S, Natume T, Ueno T, Kominami E, Motohashi H, Tanaka K, Yamamoto M, 2010. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat Cell Biol 12: 213-223.

Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, Mizushima N, Iwata J, Ezaki J, Murata S, Hamazaki J, Nishito Y, Iemura S, Natume T, Yanagawa T, Uwayama J, Warabi E, Yoshida H, Ishii T, Kobayashi A., Yamamoto, M, Yue Z, Uchiyama Y, Kominami E, Tanaka K, 2007. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131: 1149-1163.

Kong AN, Owuor E, Yu R, Hebbar V, Chen C, Hu R, Mandlekar S, 2001. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). Drug Metab Rev 33: 255-271.

Larsson O, Scheele C, Liang Z, Moll J, Karlsson C, Wahlestedt C, 2004. Kinetics of senescence-associated changes of gene expression in and epithelial, temperature-sensitive SV40 large T antigen model. Cancer Res 64: 482-489.

LeDizet M, Piperno G, 1987. Identification of an acetylation site of Chlamydomonas alpha-tubulin. Proc Natl Acad Sci U S A 84: 5720-5724.

Lee J, Giordano S, Zhang J, 2012. Autophagy, mitochondria and oxidative stress: cross-talk and redox signaling. Biochem J 441: 523-540.

Lee JH, Khor TO, Shu L, Su ZY, Fuentes F, Kong AN, 2013. Dietary phytochemicals

and cancer prevention: Nrf2 signaling, epigenetics, and cell death mechanisms in blocking cancer initiation and progression. Pharmacol Ther 137: 153-171.

Lee S, Ra J, Song JY, Gwak C, Kwon HJ, Yim SV, Hong SP, Kim J, Lee KH, Cho JJ, Park YS, Park CS, Ahn HJ, 2011. Extracts from Citrus unshiu promote immune-mediated inhibition of tumor growth in a murine renal cell carcinoma model. J Ethnopharmacol 133: 973-979.

Lee WS, Lee SJ, Jin JS, Shin SC, 2011, Determination of the change of flavonoid components as the defense materials of Citrus unshiu Marc. Fruit peel against Penicilliumdigitatum by liquid chromatography coupled with tandem mass spectrometry. Food Chem 128: 49-54.

Leontieva OV, Blagosklonny MV, 2010. DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence. Aging (Albany NY) 2: 924-935.

Li DD, Gua JF, Huang JJ, Wang LL, Deng R, Liu JN, Feng GK, Xiao DJ, Deng SZ, Zhang XS, Zhu XF, 2010. Rhabdastrellic acid-A induced autophagy-associated cell death through blocking Akt pathway in human cancer cells. PLos One 5: e12176.

Lim HK, Moon JY, Kim H, Cho M, Cho SK, 2009. Induction of apoptosis in U937 human leukaemia cells by the hexane fraction of an extract of immature Citrus grandisOsbeck fruits. Food Chem 114: 1245-1250.

Lim SY, Meyer M, Kjonaas RA, Ghosh SK, 2006. Phytol-based novel adjuvants in vaccine formulation: 1. assessment of safety and efficacy during stimulation of humoral and cell-mediated immune responses. J Immune Based Ther Vaccines 4:

6. doi:10.1186/1476-8518-4-6.

Liu D, Yang Y, Liu Q, Wang J, 2011. Inhibition of autophagy by 3-MA potentiates cisplatin-induced apoptosis in esophageal squamous cell carcinoma cells. Med Oncol 28: 105-111.

Lota M, de Rocca Serra D, Tomi F, Casanova J, 2001. Chemical variability of peel and leaf essential oils of 15 species of mandarins. Biochem Syst Ecol 29: 77-104.

Mah LY, Ryan KM, 2012. Autophagy and cancer. Cold Spring Harb Perspect Biol 4: a008821.

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G, 2007. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 8: 741-752.

Manthey JA, Guthrie N, 2002. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. J Agric Food Chem 50: 5837–5843.

Mariño G, López-Otín C, 2004. Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. Cell Mol Life Sci 61: 1439-1454.

Marks PA, 2010. The clinical development of histone deacetylase inhibitors as targeted anticancer drugs. Expert Opin Investig Drugs 19: 1049-1066.

Maruta H, Greer K, Rosenbaum JL, 1986. The acetylation of alpha-tubulin and its relationship to the assembly and disassembly of microtubules. J Cell Biol 103: 571-579.

Martins LM, Iaccarino I, Tenev T, Gschmeisser S, Totty NF, Lemoine NR, Savopoulos J, Gray CW, Creasy CL, Dingwall C, Downward J, 2002. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a

reaper-like motif. J Biol Chem 277: 439-444.

Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G, Gelinas C, Dipaola RS, Karantza-Wadsworth V, White E, 2009. Autophagy suppresses tumorigenesis through elimination of p62. Cell 137: 1062-1075.

Matov A, Applegate K, Kumar P, Thoma C, Krek W, Danuser G, Wittmann T, 2010. Analysis of microtubule dynamic instability using a plus-end growth marker.

Matsuyama A, Shimazu T, Sumida Y, Saito A, Yoshimatsu Y, Seigneurin-Berny D, Osada H, Komatsu Y, Nishino N, Khochbin S, Horinouchi S, Yoshida M, 2002. In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. EMBO J 21: 6820-6831.

McGinty D, Letizia CS, Api AM, 2010. Fragrance material review on phytol. Food Chem Toxicol 48: Suppl 3:S59-S63.

Meijer AJ, Codogno P, 2004, Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol 36: 2445-2462.

Micheau O, Tschopp J, 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114: 181-190.

Minucci S, Pelicci PG, 2006. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatment for cancer. Nat Rev Cancer 6: 38-51.

Miyake Y, Keusch JJ, Wang L, Satio M, Hess D, Wang X, Melancon BJ, Helquits P, Gut H, Matthias P, 2016. Structural insights into HDAC6 tubulin deacetylation and its selective inhibition. Nat Chem Biol 12: 748-754.

Mize CE, Avigan J, Baxter JH, Fales HM, Steinberg D, 1966. Metabolism of phytol-

U-14C and phytanic acid-U-14C in the rat. J Lipid Res 7: 692-697.

Mizushima N, 2004. Methods for monitoring autophagy. Int J Biochem Cell Biol 36: 2491-2502.

Mizushima N, Komatsu M, 2011. Autophagy: renovation of cells and tissues. Cell 147: 728-741.

Moon JY, Kim H, Cho M, Chang WY, Kim CT, Cho SK, 2009. Induction of apoptosis in SNU-16 human gastric cancer cells by the chloroform fraction of an extract of Dangyuja (Citrus grandis) leaves. J Korean Soc Appl Biol Chem 52: 168-175.

Morley KL, Ferguson PJ, Koropatnick J, 2007. Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. Cancer Lett 251: 168-178.

Morse DL, Gray H, Payne CM, Gillies RJ, 2005. Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. Mol Cancer Ther 4: 1495-1504.

Muñoz-Espín D, Serrano M, 2014. Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol 15: 482-496.

Murakami A, Nakamura Y, Torikai K, Tanaka T, Koshiba T, Koshimizu K, Kuwahara S, Takahashi Y, OgawaK, Yano M, Tokuda H, Nishino H, Mimaki Y, Sashida Y, Kitanaka S, Ohigashi H, 2000. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. Cancer Res 60: 5059–5066.

Na HK, Kim EH, Jung JH, Lee HH, Hyun JW, Surh YJ, 2008. (-)-Epigallocatechin

gallate induces Nrf2-mediated antioxidant enzyme expression via activation of PI3K and ERK in human mammary epithelial cells. Arch Biochem Biophys 476: 171-177.

Nakamura T, Cho DH, Lipton SA, 2012.Redox regulation of protein misfolding, mitochondrial dysfunction, synaptic damage, and cell death in neurodegenerative diseases. Exp Neurol 238: 12-21.

Newman DJ, Cragg GM, 2012. Natural products as sources of new drugs over 30 years from 1981 to 2012. J Nat Prod 75: 311-335.

Noda T, Oshumi Y, 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem 273: 3963-3966.

Noh JH, Jung KH, Kim JK, Eun JW, Bae HJ, Xie HJ, Chang YG, Kim MG, Park WS, Lee JY, Nam SW, 2011. Aberrant regulation of HDAC2 mediates proliferation of hepatocellular carcinoma cells by deregulating expression of G1/S cell cycle proteins. PLos One 6: e28103.

Okada H, Mak TW, 2004. Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer 4: 592-603.

Padmavathi AR, Abinaya B, Pandian SK, 2014. Phenol, 2,4-bis(1,1-dimethoxylethyl) of marine bacterial origin inhibits quorum sensing mediated biofilm formation in the uropathogen Serratia marcescens. Biofouling 30: 1111-1122.

Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Ø vervatn A, Bjørkøy G, Johansen T, 2007. P62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282: 24131-24145.

Park EJ, Choi KS, Kwon TK, 2011. β-Lapachone-induced reactive oxygen species (ROS) generation mediates autophagic cell death in glioma U87 MG cells. Chem Biol Interact 189: 37-44.

Park HJ, Kim MJ, Ha E, Chung JH, 2008, Apoptotic effect of hesperidin through caspase3 activation in human colon cancer cells, SNU-C4. Phytomedicine 15: 147-151.

Park SY, Jun JA, Jeong KJ, Heo HJ, Sohn JS, Lee HY, Park CG, Kang J, 2011. Histone deacetylases 1, 6 and 8 are critical for invasion in breast cancer. Oncol Rep 25: 1677-1681.

Patil JR, Jayaprakasha GK, Chidambara Murthy KN, Tichy SE, Chetti MB, Patil BS, 2009. Apoptosis-mediated proliferation inhibition of human colon cancer cells by volatile principle of Citrus aurantifolia. Food Chem 114: 1351-1358.

Pejin B, Kojic V, Bogdanovic G, 2014. An insight into the cytotoxic activity of phytol at in vitro conditions. Nat Prod Res 28: 2053-2056.

Piperno G, LeDizet M, Chang XJ, 1987. Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. J Cell Biol 104: 289-302.

Poli G, Leonarduzzi G, Biasi F, Chiarpotto E, 2004. Oxidative stress and cell signaling. Curr Med Chem 11: 1163-1182.

Rajab MS, Cantrell CL, Franzblau SG, Fischer NH, 1998. Antimycobacterial activity of (E)-phytol and derivatives: a preliminary structure-activity study. Planta Med 64: 2-4.

Rangel-Sánchez G, Castro-Mercado E, García-Pineda, 2014. Avocado roots treated with salicylic acid produce phenol-2,4-bis (1,1-dimethylethyl), a compound

with antifungal activity. J Plant Physiol 171: 189-198.

Rebbaa A, Zheng X, Chou PM, Mirkin BL, 2003. Caspase inhibition switches doxorubicin-induced apoptosis to senescence. Oncogene 22: 2805-2811.

Reddy LH, Couvreur P, 2009. Squalene: A natural triterpene for use in disease management and therapy. Adv Drug Deliv Rev 61: 1412-1426.

Rohde J, Heitman J, Cardenas ME, 2001. The TOR kinase link nutrient sensing to cell growth. J Biol Chem 276: 9583-9586.

Romanov VS, Abramova MV, Svetlikova SB, Bykova TV, Zubova SG, Aksenov ND, Fornace AJ Jr, Pospelova TV, Pospelov VA, 2010. P21(Waf1) is required for cellular senescence but not for cell cycle arrest induced by the HDAC inhibitor sodium butyrate. Cell Cycle 9: 3945-3955.

Roninson IB, Broude EV, Chang BD, 2001. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Updat 4: 303-313.

Rotblat B, Melino G, Knight RA, 2012. NRF2 and p53: Januses in cancer? Oncotarget 3: 1272-1283.

Ryu KR, Choi JY, Chung S, Kim DH, 2011. Anti-scratching behavioral effect of the essential oil and phytol isolated from Artemisia principles Pamp. in mice. 2011. Planta Med 77: 22-26.

Sage J, Miller AL, Pérez-Mancera PA, Wysocki JM, Jacks T, 2003. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. Nature 424: 223-228.

Saikia D, Parihar S, Chanda D, Ojha S, Kumar JK, Chanotiya CS, Schanker K, Negi

AS, 2010. Antitubercular potential of some semisynthetic analogues of phytol. Bioorg Med Chem Lett 20: 508-512.

Salvesen GS, Dixit VM, 1997. Caspases: intracellular signaling by proteolysis. Cell 91: 443-446.

Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A, 1997. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implication for studies on mitochondrial functionality during apoptosis. FEBS Lett 411: 77-82.

Sang MK, Kim JD, Kim BS, Kim KD, 2011. Root treatment with rhizobacteria antagonistic to Phytophthora blight affects anthracnose occurrence, ripening, and yield of pepper fruit in the plastic house and field. Phytopathology 101: 666-678.

Santos CCMP, Salvadori MS, Mota VG, Costa LM, Almeida AACO, Oliveira GAL, Costa JP, Sousa DP, Freitas RM, Almeida RN, 2013. Antinociceptive and antioxidant activities of phytol in vivo and in vitro models. Neurosci J Article ID 949452. doi:10.1155/2013/949452.

Saretzki G, 2010. Cellular senescence in the development and treatment of cancer. Curr Pharm Des 16: 79-100.

Sasse R, Gull K, 1988. Tubulin post-translational modifications and the construction of microtubular organelles in Trypanosoma brucei. J Cell Sic 90(Pt 4): 577-589.

Sathuvan M, Vignesh A, Thangam R, Palani P, Rengasamy R, Murugesan K, 2012. In vitro antioxidant and anticancer potential of bark of Costus pictus D.DON. Asian Pac J Trop Biomed 2: S741-S749

Scherz-Shouval R, Elazar Z, 2011. Regulation of autophagy by ROS: physiology and pathology. Trends Biochem Sci 36: 30-38.

Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM, Lowe SW, 2002. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell 109: 335-46.

Schofield AV, Gamell C, Bernard O, 2013. Tubulin polymerization promoting protein 1 (TPPP1) increases β-catenin expression through inhibition of HDAC6 activity in U2OS osteosarcoma cells. Biochem Biophys Res Commun 436: 571-577.

Schwarze SR, Shi Y, Fu VX, Watson PA, Jarrard DF, 2001. Role of cyclin-dependent kinase inhibitors in the growth arrest at senescence in human prostate epithelial and uroepithelial cells. Oncogene 20: 8184-8192.

Seglen PO, Gordon PB, 1982. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci USA 79: 1889-1992.

Shankar E, Kanwal R, Candamo M, Gupta S, 2016. Dietary phytochemicals as epigenetic modifiers in cancer: Promise and challenges. Semin Cancer Biol 40-41: 82-99.

Shi Y, Tang B, Yu PW, Tang B, Hao YX, Lei X, Luo HX, Zeng DZ, 2012. Autophagy protects against oxaliplatin-induced cell death via ER stress and ROS in Caco-2 cells. PLoS One 7: e51076.

Shim GS, Manandhar S, Shin DH, Kim TH, Kwak MK, 2009. Acquisition of doxorubicin resistance in ovarian carcinoma cells accompanies activation of the NRF2 pathway. Free Radic Biol Med 47: 1619-1631.

Shin SY, Yoon H, Hwang D, Ahs S, Kim DW, Koh D, Lee YH, Lim Y, 2013. Benzochalcones bearing pyrazoline moieties show anti-colorectal cancer activities and selective inhibitory effects on aurora kinases. Bioorg Med Chem 21: 7018-7024.

Shukla S, Metha A, 2015. Anticancer potential of medicinal plants and their phytochemiclas: a review. Braz J Bot 38: 199-210.

Silke J, Brink R, 2010. Regulation of TNFRSF and innate immune signaling complexes by TRAFs and cIAPs. Cell Death Differ 17: 35-45.

Song KS, Kim JS, Yun EJ, Kim YR, Seo KS, Park JH, Jung YJ, Park JI, Kweon GR, Yoon WH, Lim K, Hwang BD, 2008. Rottlerin induces autophagy and apoptotic cell death through a PKC-delta-independent pathway in HT1080 human fibrosarcoma cells: the protective role of autophagy in apoptosis. Autophagy 4: 650-658.

Song YW, Shrestha S, Gyawali R, Lee DS, Cho SK, 2015. Citrus unshiu leaf extract containing phytol as a major compound induces autophagic cell death in human gastric adenocarcinoma AGS cells. J Korean Soc Appl Biol Chem 58: 257-265.

Stein GH, Drullinger LF, Soulard A, Dulić V, 1999. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. Mol Cell Biol 19: 2109-2117.

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X, 2012. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148: 213-227.

Sun SY, Hail N, Lotan R, 2004. Apoptosis as a novel target for cancer

chemoprevention. J Natl Cancer Inst 96: 662-672.

Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM, 1999. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J Biol Chem 274: 5053-5060.

Tait SW, Green DR, 2008. Caspase-independent cell death: leaving the set without the final cut. Oncogene 27: 6452-6461.

Tan HK, Moad AI, Tan ML, 2014. The mTOR signalling pathway in cancer and the potential mTOR inhibitory activities of natural phytochemicals. Asian Pac J Cancer Prev 15: 6463-6475.

Tang J, Yan H, Zhuang S, 2013. Histone deacetylases as targets for treatment of multiple diseases. Clin Sci (Lond) 124: 651-662.

Tanida I, Waguri S, 2010 Measurement of autophagy in cells and tissues. Methods Mol Biol 648: 193-214.

Thakur VS, Deb G, Babcook MA, Gupta S, 2014. Plant phytochemicals as epigenetic modulators: role in cancer chemoprevention. AAPS J 16: 151-163.

Thornberry NA, Lazebnik Y, 1998. Caspases: enemies within. Science 281: 1312-1316.

Tran AD, Marmo TP, Salam AA, Che S, Finkelstein E, Kabarriti R, Xenias HS, Mazitschek R, Hubbert C, Kawaguchi Y, Sheetz MP, Yao TP, Bulinski JC, 2007. HDAC6 deacetylation of tubulin modulates dynamics of cellular adhesions. J Cell Sci 120(Pt 8): 1469-1479.

Urbich C, Rössig L, Kaluza D, Potente M, Boeckel JN, Knau A, Diehl F, Geng JG, Hofmann WK, Zeiher AM, Dimmeler S, 2009. HDAC5 is a repressor of

angiogenesis and determines the angiogenic gene expression pattern of endothelial cells. Blood 113: 5669-5679.

Vakifahmetoglu H, Olsson M, Zhivotovsky B, 2008. Death through a tragedy: mitotic catastrophe. Cell Death Differ 15: 1153-1162.

Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P, 2014. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. Nat Rev Mol Cell Biol 15: 135-147.

Varsha KK, Devendra L, Shilpa G, Priya S, Pandey A, Nampoothiri KM, 2015. 2,4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated Lactococcus sp. Int J Food Microbiol 211: 44-50.

Vauzour D, Rodiriguez-Mateos A, Corona G, Oruna-Concha MJ, Spencer JP, 2010. Polyphenols and human health: prevention of disease and mechanisms of action. Nutrients 2: 1106-1131.

Vega RB, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, McAnally J, Pomajzl C, Shelton JM, Richardson JA, Karsenty G, Olson EN, 2004. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell 119: 555-566.

Vergel M, Marin JJ, Esteves P, Carnero A, 2010. Cellular senescence as a target in cancer control. J Aging Res 2011: 725365.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL, 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102: 43-53.

Viszwapriya D, Prithika U, Deebika S, Balamurugan K, Pandian SK, 2016. In vitro and in vivo antibiofilm potential of 2,4-Di-tert-butylphenol from seaweed surface associated bacterium Bacillus subtilis against group A streptococcus. Microbiol Res 191: 19-31.

Wang C, Fu M, Mani S, Wadler S, Senderowicz AM, Pestell RG, 2001. Histone acetylation and the cell-cycle in cancer. Front Biosci 6: D610-D629.

Wang H, Khor TO, Shu L, Su ZY, Fuentes F, Lee JH, Kong AN, 2012. Plants vs. cancer: a review on natural phytochemicals in preventing and targeting cancers and their druggability. Anticancer Agents Med Chem 12: 1281-1305.

Wang SH, Li N, Wei Y, Li QR, Yu ZP, 2014. β-catenin deacetylation is essential for WNT-induced proliferation of breast cancer cells. Mol Med Rep 9: 973-978.

Wang X, Wong SC, Pan J, Tsao SW, Fung KH, Kwong DL, Sham JS, Nicholls JM, 1998. Evidence of cisplatin-induced senescent-like growth arrest in nasopharyngeal carcinoma cells.

Webster DR, Borisy GG, 1989. Microtubules are acetylated in domains that turn over slowly. J Cell Sci 92(Pt 1): 57-65.

Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM, 2004. De-ubiquitination and ubiquitin ligase domains of A20 downregulates NF-kappaB signaling. Nature 430: 694-699.

Witt O, Deubzer HE, Milde T, Oehme I, 2009. HDAC family: What are the cancer relevant targets? Cancer Lett 277: 8-21.

Wolf BB, Green DR, 1999. Suicidal tendencies: apoptotic cell death by caspase

family proteinases. J Biol Chem 274: 20049–20052.

Wu WS, 2006. The signaling mechanism of ROS in tumor progression. Cancer Metastasis Rev 25: 695-705.

Xie HJ, Noh JH, Kim JK, Jung KH, Eun JW, Bae HJ, Kim MG, Chang YG, Lee JY, Park H, Nam SW, 2012. HDAC1 inactivation induces mitotic defect and caspase-independent autophagic cell death in liver cancer. PLoS One 7: e34265.

Xie J, Yang Z, Zhou C, Zhu J, Lee RJ, Teng L, 2016. Nanotechnology for the delivery of phytochemicals in cancer therapy. Biotechnol Adv 34: 343-353.

Xu Y, Li N, Xiang R, Sun P, 2014. Emerging roles of the p38 MAPK and PI3K/AKT/mTOR pathways in oncogene-induced senescence. Trends Biochem Sci 39: 268-276.

Yanez J, Vicente V, Alcaraz M, Castillo J, Benavente-Garcia O, Canteras M, Teruel JA, 2004. Cytotoxicity and antiproliferative activities of several phenolic compounds against three melanocytes cell lines: relationship between structure and activity. Nutr Cancer 49: 191–199.

Yang EJ, Lee HJ, Kang GJ, Park SS, Yoon WJ, Kang HK, Cho SK, Yoo ES, 2009. Anti-inflammatory effect of Dangyuja (Citrus grandis Osbeck) leaves in LPS-stimulated RAW 264.7 cells. Food Sci Biotechnol 18: 1063-1070.

Yang Z, Klinosky DJ, 2010. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 22: 124-131.

Yap TA, Garrett MD, Walton MI, Raynaud F, de Bono JS, Workman P, 2008.Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. Curr Opin Pharmacol 8: 393-412.

Yo YT, Shieh GS, Hsu KF, Wu CL, Shiau AL, 2009. Licorice and licochalcone-A induce autophagy in LNCaP prostate cancer cells by suppression of Bcl-2 expression and the mTOR pathway. J Agric Food Chem 57: 8266-8273.

Yorimitsu T, Klionsky DJ, 2005. Autophagy: molecular machinery for self-eating. Cell Death Differ 12: 1542-1552.

Yuan G, Yan SF, Xue H, Zhang P, Sun JT, Li G, 2014. Cucurbitacin I induces protective autophagy in glioblastoma in vitro and in vivo. J Biol Chem 289: 10607-10619.

Zhang C, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN, 2002. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 110: 479-488.

Zhang H, Kong X, Kang J, Su J, Li Y, Zhong J, Sun L, 2009. Oxidative stress induces parallel autophagy and mitochondria dysfunction in human glioma U251 cells. Toxicol Sci 110: 376-388.

Zhang H, Lei Y, Yuan P, Li L, Luo C, Gao R, Tian J, Feng Z, Nice EC, Sun J, 2014. ROS-mediated autophagy induced by dysregulation of lipid metabolism plays a protective role in colorectal cancer cells treated with gambogic acid. PLoS One 9: e96418.

Zhang X, Yuan Z, Zhang Y, Yong S, Salas-Burgos A, Kooemn J, Olashaw N, Parsons JT, Yang XJ, Dent SR, Yao TP, Lane WS, Seto E, 2007. HDAC6 modulates cell motility by altering the acetylation level of cortactin. 27: 197-213.

Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC, 2004. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-

mesenchymal transition. Nat Cell Biol 6: 931-940.

Zhu J, Coyne CB, Sarkar SN, 2011. PKC alpha regulates Sendai virus-mediated interferon induction through HDAC6 and β-catenin. EMBO J 30: 4838-4849.