ANTI-INFLAMMATORY PROPERTIES OF CITRUS LIMONOIDS AND THEIR ISOLATION AND CHARACTERIZATION

A Dissertation

by

JIN HEE KIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Horticulture

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Approved by:

Chair of Committee,	Bhimanagouda S. Patil
Committee Members,	G.K. Jayaprakasha
	Hisashi Koiwa
	Hongmin Qin
	Mariappan Muthuchamy
Head of Department,	Leland S. Pierson III

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ABSTRACT

Anti-inflammatory Properties of Citrus Limonoids and Their Isolation and Characterization. (December 2011)

Jin Hee Kim, B.S., Sahmyook University; M.S., Chung-Ang University

Chair of Advisory Committee: Dr. Bhimanagouda S. Patil

This dissertation investigates the role of limonoids in inflammation to reduce risk of breast cancer and cardiovascular disease. Radical scavenging activity and apoptotic effects of extracts from lemon seeds were investigated in human breast adenocarcinoma (MCF-7) cells and non-malignant breast (MCF-12F) cells. The MeOH:water (80:20) extract showed the highest (29.1%, $P < 0.01$) inhibition of MCF-7 cells without affecting the non-malignant breast cells. Further, the purified and modified limonoids were screened for their cytotoxicity on estrogen receptor (ER)-positive (MCF-7) or ER-negative (MDA-MB-231) human breast cancer cells. The MCF-7 cell was more susceptible to tested limonoids. Although most of limonoids induced anti-aromatase activity, the inhibition of proliferation was not related to the anti-aromatase activity. On the other hand, the anti-proliferative activity was significantly correlated with the level of caspase-7 activation by limonoids.

The next study investigated the mechanism of anti-breast cancer and anti-aromatase activities of obacunone through inhibition of MCF-7 cell proliferation without

affecting non-malignant breast cells. Treatment with obacunone resulted in an increased G1 cell cycle arrest and induction of apoptosis. Exposure of MCF-7 breast cancer cells to obacunone down-regulated expression of inflammatory molecules including nuclear factor-kappa B (NF-κB) and cyclooxygenase-2 (COX-2). Furthermore, potential of obacunone on inhibition of COX-2 and NF-κB by a mechanism involving activation of the p38 mitogen-activated protein (MAP) kinase was also investigated.

In the final study, nomilin was the most potent natural inhibitor for p38 MAP kinase activity in human aortic smooth muscle cells indicating that a seven-membered A ring with acetoxy group, present in nomilin, seems to be essential for its inhibitory activity on p38 MAP kinase. The possible mechanism of nomilin for prevention of cardiovascular disease was determined. Pre-treatment with nomilin resulted in significant inhibition of TNF-α induced HASMCs proliferation. The anti-proliferative activity of nomilin is due to apoptosis through mitochondrial dependent pathway.

DEDICATION

To my family, husband and my daughter

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Citrus is grown commercially in more than 140 countries around the world (1). Citrus production has shown increased demand for fresh juices and the processed market. Among citrus fruits, oranges, grapefruits, and lemons are considered to be the top three fruits for expanded consumption (1). Furthermore, lemons are widely used citrus crop for food additives, food products (essential oils, gum/resin, lipids), medicines folklore, and have shown to possess several potential health benefits. In the United States, lemon produced mainly in California and Arizona states (2). Per capita consumption of lemon is estimated to be 8.6 pounds in United States (3). Despite several uses of lemons, very little information is available about health benefits of lemon as compared to the other citrus fruits such as oranges and grapefruits.

Epidemiological studies have demonstrated the inverse correlation between increasing consumption of fruits and vegetables and incidences of human diseases such as cancer and heart disease (4, 5). Citrus fruits are rich in health-promoting phytochemicals. Limonoids constitute one of the major phytochemicals along with flavonoids, coumarins, and carotenoids in citrus (6). Citrus limonoids are a class of secondary metabolites known as triterpenoids, and act as a defense agents against insects. Until now, 62 limonoids, including 44 aglycones and 18 glucosides, have been

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This dissertation follows the style of Cancer Prevention Research.

identified from citrus fruits, and still the number is increasing (7-9). The early research focused on the identification of limonoids because of the delayed bitterness caused by certain limonoids such as limonin and nomilin. Limonin and other bitter limonoids were considered as an undesirable component to maintain the acceptable-quality level for the fresh or processed juice industry. However, recent research has discovered the various health benefits and pharmacological uses of limonoids such as anti-bacterial, anti-fungal, anti-viral, anti-oxidant, anti-inflammation, and anti-cancer (6, 8, 10). Additionally, consumption of about seven glasses of orange juice that contain 320 mg/L of limonoid glucosides is found to be nontoxic (11).

Limonoids were shown to possess anti-cancer properties. In the past quarter century, accumulated evidences advanced our understanding about the disease prevention mechanism of limonoids (12). It is speculated that limonoids suppress cell proliferation and induce apoptosis by inhibiting enzyme activities and signal transduction pathways. Several *in vitro* studies associated with citrus have demonstrated its antioxidant, anti-proliferative effects and its protective effects on cardiovascular disease and many other diseases (6, 13-16). In addition, limonoids were also shown to inhibit cell metastasis in cell culture and animal studies. However, further research is required to identify the cellular targets of active limonoids. Identification of the target mechanism at cellular level may result in the development of limonoids as anti-cancer agents.

1.2 Biosynthesis of Citrus Limonoids

Triterpenoids are the largest group of plant secondary metabolites, and over 4000 triterpenoids have been identified in the plant kingdom (17). The limonoids are biosynthesized in the cytosol and distributed in different parts of plant such as seed, stem bark, leaf, and fruit (18). The biosynthesis occurs based on the "biogenetic isoprene rule" (19). Isopentenyl diphosphate (IPP) and its allylic isomer dimethyl allyl diphosphate (DMAPP) are common precursors of isoprene biosynthesis. The limonoids are synthesized v*ia* a mevalonate pathway to produce triterpenoids (20). The triterpenoids consist of 30 carbons and are derived from squalene (21). Limonoids are degraded triterpenoids, also called tetranotriterpenoids, consist of a furan ring attached at C-17 and contain a functional group at C-3, C-4, C-7, C-16, C-17, and C-19 (8, 21). The limonoids are synthesized by squalene through oxidation and the rearrangement process and the basic skeleton come from euphol or tirucallol, which are considered precursors of limonoids (21).

By using radioactive tracer techniques, citrus metabolites were shown to possess two distinct precursors during limonoid biosynthesis. In principle, deacetylnomilinic acid is a precursor of limonoids in *Citrus* which gets converted to nomilin in the phloem region of the stem (22). In addition, stem tissue synthesizes nomilin directly from acetate (18). The synthesized nomilin is translocated from the stem to other parts of the plant, such as leaves, fruit, seed, and root tissues. Other limonoids are biosynthesized from nomilin in all the region of the plant except the cortex and inner core (18).

Citrus limonoids are present in the glucoside or aglycone. The aglycones are converted to glucosides by the UDP-D-glucoside: limonoid glucosyltransferase enzyme during the fruit maturation process (23). Higher concentration of total limonoids were found in the seeds as compared to fruits, suggesting that seeds act as sink for these compounds (24). However, the glucosides concentration is less than half of the aglycones in the seed (24).

1.3 Stability and Bioavailability of Citrus Limonoids

In addition to ascorbic acid, citrus contains flavonoids, carotenoids, coumarins, volatile oil, sitosterols, pectin, and limonoids (6, 25). Limonoids are present as glucosides as well as aglycone form. The solubility and bioavailability are often considered an important factor to improve the health effects of bioactive compounds. Accordingly, the identification of digestive metabolites, intracellular metabolism, plasma transport, and toxicity has been intensively studied for understanding the bioavailability properties (8, 15, 26). The limonin and limonin glucoside are the predominant limonoids in orange juice, and the limonoid glucosides concentration ranges between 350 and 400 ppm (27). Due to the bitterness of limonin, the levels of limonin are considered an important quality attribute of the citrus processing industries. The bitterness threshold of limonin in orange juice is 6.5 ppm, and the threshold is maximized at pH 3.8 because the optimum pH suppresses the limonin bitterness (28). The stability and degradation of limonin during juice processing need to be investigated. The limonin is a highly heat-stable and the chemical structure is stable up to 298°C (29). The stability and

decomposition rate of compounds could be affected by several factors such as humidity, temperature, pH, and oxygen level. Limonin is stable over a wide range of pH value (pH 2-9) and most stable at pH 5 at 45 ºC (*18*). However, the limonin is completely degraded at pH 10-12. On the other hand, the limonin produces an isomer named limonexic acid due to the effect of strong acids on the furan ring (29). Moreover, the limonin stability was affected by temperature in acidic and basic conditions. The limonin exhibited maximum stability at 45ºC compared to 70-80 ºC (29).

Several studies have been focused on understanding the bioavailability and toxicity of natural products due to the chemopreventive efficacy depending on its absorption, metabolism, and safety (30, 31). However, only one study was conducted on the bioavailability of limonoids till date. The limonin glucoside, upto 2 g/per day, was fed to the subjects and metabolites were measured in the plasma using LC-MS technique (10). The results of the study suggested that the limonin glucoside was converted into limonin as final metabolite with maximum plasma concentration at 6 h (10). During the experiment, epilimonin, which is an isomer of limonin, was also detected indicating presence of an additional metabolite. (32). Although there are limited human intervention studies on absorption of citrus limonoids, investigations using soy phytochemicals suggest that aglycones show much faster and higher absorption rates than glucosides in humans (33).

In addition, several toxicity studies were reported using animal models. Miller et al. (26) investigated the long term feeding of limonoids mixture on weight gain in pregnant rats. These results demonstrated that diet supplement with limonoids (0.25%

and 0.15%) caused a significant reduction in weight gain in female and male progeny, respectively (26). Even though the results suggest the exposure of high concentration of limonoids causes problem with weigh gain, such a correlation need to be determined in humans. For instance, daily consumption 130 glasses of orange juice for one week is required to reach a similar level for a 60 kg adult (26).

1.4 Cancer Chemoprevention by Citrus Limonoids

More than 290 bioactive compounds have been reported so far in citrus species, including 115 carotenoids (34), 60 flavonoids (34, 35), 62 limonoids (36), 31 volatile oils (37), 17 coumarins (38), and 6 amines (39). Due to the presence of these compounds, citrus is considered one of the promising healthy food. For instance, limonoids have shown strong activity to suppress tumor formation in a various cancers, reduce risks of heart disease, neurodegenerative disease, and autoimmune disease (6, 15, 40, 41). Among the chronic diseases, cancer is one of the common malignant, and more than 13% of human deaths (7.6 million) are associated with cancer in the world. In USA, approximately 560,000 Americans, more than a quarter-percent of the USA population die from cancer (42-44). However, the complexity of the disease, mortality, and morbidity of cancer, the mechanism of tumor formation and resistance to therapy is poorly understood. Interest in preventive therapies using a nutritional approach is now increasing as these alternative approaches may help in delaying the progression of carcinogenesis.

Limonoids have been identified for their anti-carcinogenic activity in cell cultures and animal model systems. The potential of an anti-carcinogenic property of limonoids from citrus species is being actively investigated in the colon cancer. Glutathione S-transferases (GST) are an important catalytic and binding protein family due to its ability to detoxify carcinogens (45). In 1989, Lam and coworkers reported the induction of GST enzyme in small intestinal mucosa by nomilin and limonin (46). In another study, while limonin showed minimal effect, nomilin increased the GST activity three times compared to the control group. Furthermore, recent study from Patil's lab demonstrated that a limonin analogue, limonin-7-methoxime was a potent inducer of GST in mice (47). Further, in 1994, it is demonstrated that limonin and nomilin suppress chemically induced tumor size in an animal model (48). Additionally, obacunone and limonin have shown the ability to inhibit formation of aberrant crypt foci (ACF) in azoxymethane (AOM) induced colon cancer model in rats (49). After 4 years, in 2005, limonin demonstrated the suppression of chemically induced ACF formation in similarly designed animal experiment (50). Based on the results, the authors speculated that the possible mechanism may be apoptosis mediated by down-regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are involved in the inflammatory process (50). Furthermore, limonoid aglycones such as limonin, nomilin, obacunone, and deacetyl nomilin showed anti-proliferative activity through induction of caspase 3/7 in colon carcinoma Caco-2 cells (51). In addition, ichanexic acid and isolimonexic acid inhibited the G2/M phase, inducing cell cycle arrest in human colon adenocarcinoma HT-29 cells (7). More recently, limonexic acid was shown to induce

cell cycle arrest in human colon adenocarcinoma HT-29 cells (52). Taken together, these results indicated that cell cycle arrest and apoptosis through inhibition of iNOS/COX-2 expression and induction of caspase 3/7 might be considered a mechanism by which limonoids may exhibit chemopreventive property in colon cancer.

Limonoids were reported to inhibit the cell proliferation in MDA-MB-435 estrogen receptor-negative (ER-) and MCF-7 estrogen receptor-positive (ER+) breast cancer cell lines (16, 53). However, the anti-proliferative activity by limonoids in breast cancer cells and the mechanism of cell growth inhibition by limonoids remains to be elucidated. Recent studies from Patil's lab and elsewhere reported that limonin and nomilin could inhibit cancer development in the neuroblastoma (51, 54), pancreatic *in vitro (36)*, stomach, lung, and skin *in vitro* and *in vivo (40)*. Furthermore, we also showed that obacunone induces apoptosis and inhibits inflammatory markers in pancreatic cancer Panc-28 cells (55).

Human cytochrome P450 oxidoreductase enzymes (CYPs) known to involve in drug metabolism, degradation of xenobiotics, and detoxification processes (56). Several studies have demonstrated that the overexpression of the CYPs has strongly associated with cancer susceptibility (56). The limonoids were evaluated as potent inhibitor of CYPs (57, 58). Fukuda *et al*. (57) found that the limonin and obacunone were potent inhibitor of cytochrome P450 monooxygenase 3A4 (CYP3A4) in rat and human liver microsomes. Furthermore, our recent study also indicated that nomilin, NAG, and LG is a potent inhibitor of CYP3A4 activity (58). However, the specific interaction of limonoids and CYPs is remained to be elucidated.

1.5 Structure-Activity Relationship of Limonoids

The structure-activity relationship of bioactive compounds is an important parameter for understanding the biological activity. Recent our results suggest that the presence of furan and an intact A ring structure are responsible for chemopreventive activity in cancer cells. For example, furan ring seems to play an important role in anti-proliferative activity of Panc-28 cells (36). In particular, the configuration of furan ring is critical for action. This is exemplified by the difference in activities of isolimonexic acid and limonexic acid, which differ in the configuration of furan ring. Other critical factors seem to be A-ring and glucose moiety. A-ring also seems to play important role in anti-proliferative activity of limonoids against MCF-7 cells (16). Specifically, presence of 7-membered A-ring (obacunone and nomilin) imparts higher activity compared to presence of A and A´ ring present in limonin.

1.6 Other Biological Activities of Citrus Limonoids

Limonoids exhibited anti-HIV, moderate radical scavenging, and anti-oxidant activity (54, 59). Recent studies have demonstrated the anti-microbial, anti-bacterial, and anti-fungal activity of limonoids in the khaya species, as well as in several other plants found in the *Rutaceae* family (8). For the first time, Patil's lab reported that certain citrus limonoids inhibit bacterial cell-cell signaling and biofilm formation (60, 61). Specially, obacunone was the most potent inhibitor of *Escherichia coli* O157:H7 biofilm formation (61). Michael *et al*. reported the structure-activity relationships of limonoids on anti-feedant activity (62). The results demonstrated that the furan ring and the epoxide group

have the highest activity against insects (62). Furthermore, studies have shown that the A ring of the limonoid nucleus may be a key regulator for anti-neoplastic activity and cancer chemopreventive activity (63, 64) whereas, the D ring may not be associated with biological activity (15, 65). Therefore, the structure-function activity of limonoids plays a vital role in understanding their biological action. More extensive research is required to understand the mechanism of proliferation inhibition by structurally related limonoids.

1.7 Role of Inflammation in Breast Cancer and Cardiovascular Disease

Inflammation is triggered in response to many genetic, familial, hormonal, and environmental factors and plays a role in many disease initiation and progression including breast cancer and cardiovascular disease (66). Pro-inflammatory factors, such as the transforming growth factor (TGF)-β, platelet derived growth factors, macrophage inhibitor factor (MIF), chemokines, cytokines, interferon gamma, and monocyte chemoattractant protein (MCP-1) play a role in the development of cell proliferation and cell migration (67-69). However, overproduction of these inflammatory factors have been implicated in inducing a variety of cancer and chronic cardiovascular diseases such as atherosclerosis, coronary artery disease, arrhythmia, heart failure, hypertension, and ischemia (68, 70-72). Therefore, chronic inflammation has been thought as a possible mechanism link between cancer and cardiovascular disease.

Breast cancer is one of the most common cancers among women. Unfortunately, reducing the risk of breast cancer is difficult due to the fact that most cases involve complex hormonal responses, increased obesity rates, and high blood estrogen levels for

postmenopausal women (73). Hormone sensitivity has been found to be one of the key signatures in breast cancer diagnosis (73). More than 60% of breast tumors exacerbated by excessive estrogen and the presence of estrogen receptors (ER) result in better prognosis than ER-negative (74). Although the ER positive breast tumors are more responsive to chemotherapy than negative breast tumors, the outcome of anti-cancer agents are not always followed by same mechanism due to the complex interaction of anti-cancer and human disease. After the discovery in 1973 of the biochemical interaction of the ERα with hormones (75), ERα antagonists, including tamoxifen and letrozole, were utilized for the treatment of breast cancer. While hormone therapy is currently the most prevalent breast cancer treatment, new models need to be explored (76).

The smooth muscle cells (SMCs) are closely related to the initiation of atherosclerosis and are involved in the disease's progression (77). The proliferation of SMCs is a key feature of development of the early atherosclerosis stage (77). Diverse signaling pathways such as mitogen activated protein kinase (MAPK), phosphoinositid (PI)-3 kinase, nuclear factor (NF)-KB, protein kinase C (PKC), and several growth factors indicate the progression of SMCs (78). Inflammatory responses continuously stimulate the migration and proliferation of SMCs, which results in accumulation of extracellular matrix and the thickening of the artery wall (78).

1.8 Alteration Cell Behavior and Apoptosis

Apoptosis is a regulated (or programmed) form of cell death that controls cellular organism through eliminating damaged or unwanted cells. These death signals lead to characteristic morphological changes such as membrane blebbing, chromosomal DNA fragmentation, and chromatin condensation (79). The cell death triggers through different signaling pathway in mammalian cells. Proper regulation of apoptosis allows counter balance of cell numbers and tissue size, and appropriate cellular homeostasis during the developmental and physiological process. A family of proteins known as caspases (cysteinyl aspartate-specific proteases) is key regulator that cleaves their substrates after an aspartate residue in early stage of apoptosis (80). There are two distinct classes of caspases: the initiator caspases, which include caspase-2, -8, -9 and -10 in mammals and the effector caspases, which include caspase-3, -6, and -7 in mammals (80).

Most caspases are synthesized as catalytically inactive zymogens and undergo a process of proteolytic activation during apoptosis (81). The initiator caspases are auto-activated under apoptotic conditions (81). The activation of an initiator caspase triggers a cascade of downstream caspase (called effector caspase) activation. As the activated caspases undergo proteolytic processing, a variety of cellular proteins are degraded during cell death (81). Dysregulation of apoptotic contributes to a number of human diseases including cancer, cardiovascular disease, autoimmune disorders and neurodegenerative diseases (82). Previous studies have shown that limonoids inhibit proliferation in different cancer cells. However, it is still unclear how limonoids are

regulating apoptosis mechanism. Therefore, further experiments are required to completely understand the function of limonoids in apoptotic cells.

1.9 Research Objectives

The main goal of this research is to determine the role of health promoting properties of limonoids from lemon in inflammatory disease such as breast cancer and cardiovascular disease, and to investigate the mechanism of the biological activities of limonoids relating to their potential in breast cancer and cardiovascular disease prevention. Additionally, structure-activity relationship of limonoids was demonstrated to determine the potential use of limonoids for human health. More specifically, the research objectives are as follows:

- Bioassay-derived isolation and purification of antioxidant potential and growth inhibition of human breast cancer and non-malignant cells using different polar extracts from lemon seed.
- Purification and characterization of aglycones and glucoside of limonoids using chromatographic techniques.
- Screen the potential biological activity in human breast cancer cells and delineate the possible mechanism underlying the inhibition of human breast cancer cell proliferation by limonoids.
- Investigate the possible downstream pathways to understand the mechanism of limonoids-induced inhibition of breast cancer cell growth.

- Elucidate the structure-function activity of limonoids on p38 mitogen activated protein kinase activity in human aortic smooth muscle cells.
- Explore the possible inhibition mechanism by nomilin on TNF-α induced HASMCs proliferation.

2. BIOASSAY-DERIVED PURIFICATION OF LEMON SEED EXTRACTS *

2.1 Introduction

Epidemiological studies have demonstrated the inverse correlation between increasing consumption of fruits and vegetables and incidences of breast cancer risk (4, 5). Recent cohort studies reported that fruit and vegetable consumption may not have a significant effect in reducing the risk for breast cancer (83). However, different bioactive compounds derived from fruits and vegetables, including flavonoids (84, 85), polyphenols (86), and vitamins (87, 88), have been evaluated for inhibition of breast cancer cell growth and metastasis inhibition using *in vitro* and *in vivo* model systems. Despite conflicting reports, fruits and vegetables are commonly recognized for their health benefits. While this is well understood, the mechanisms by which certain bioactive compounds in fruits and vegetables reduce the risk of cancer, as well as their absorption by the human body, have yet to be determined.

Citrus fruits, as a major contributor to human diet, have received attention by researchers due to their multitude of bioactive compounds. Recent *in vitro* studies suggested that several bioactive compounds from citrus contain many other health-promoting properties and have potential relevance for antioxidant, anti-proliferative, and anti-viral agents, as well as for the prevention of cardiovascular disease (8). In our previous studies, bioactive secondary metabolites from citrus, such as limonoids,

* Reprinted with permission from "Evaluation of chemopreventive and cytotoxic effect of lemon seed extracts on human breast cancer (MCF-7) cells" by Jinhee Kim, Guddadarangavvanahally K. Jayaprakasha, Ram M. Uckoo, and Bhimamagouda S. Patil, Food and Chemical Toxicology, In press.

flavonoids (naringin), and carotenoids (lycopene, lutein), were shown to suppress the growth rate of human breast cancer (16), colon cancer (7, 52, 89), neuroblastoma cells (51), and rat prostate carcinoma cells (90) using *in vitro* models, as well as azoxymethane-induced aberrant crypt foci in an *in vivo* study (50).

Apoptosis is an important regulatory mechanism in the development of tissues, involving biological events such as chromosome condensation, DNA laddering, membrane blebbing, and cytochrome C release, which leads to the removal of unnecessary cells (80). It is well known that cancer is due to mitochondria-generated reactive oxygen species (ROS) and DNA damage, apoptosis, or necrosis (91). Furthermore, studies have supported that ROS production, lipid peroxidation, and mitochondria function are related to many diseases, including cancer, diabetes, and neurodegenerative disorder (92). Recently, our studies have provided clear evidence that certain citrus bioactive compounds induce significant increases in the activity of detoxifying enzymes such as glutathione S-transferase and quinone reductase (47).

Citrus is grown commercially in more than 140 countries around the world. Among citrus, oranges, grapefruits, and lemons are considered to be the top three fruits for consumption throughout the world (1). Lemons are widely consumed as culinary fruit, and their economic importance has steadily increased due to the fresh fruit juice industry, medicinal folklore, and the ingredients market. During the processing of lemons, more than 50% of the fruit weight is discarded as waste (93). Byproducts such as molasses, seeds, and peels are being used for animal feed, health beneficial compounds (94), and fuel utilization (95). On the other hand, among the different parts

of the lemon fruit, seeds are one of the major byproducts which do not have significant use. The current research is an attempt towards making use of the seeds for understanding role of seeds. This will add economic benefits to citrus processing industry, citrus growers, and human society. Based on this information, we focused on evaluating the bioactivity of the lemon seeds. Despite several uses of lemons, very little information is available about the health-promoting properties and the mechanism of action of lemon bioactive components compared to other citrus fruits such as oranges and grapefruits.

To the best of my knowledge, no reports are available on lemon bioactive compounds and their effects on the growth of human breast cancer and non-malignant cells. Therefore, this study investigated the antioxidant potential and growth inhibition of human breast cancer and non-malignant cells using different polar extracts from lemon seed. The chemical composition was identified by LC-MS.

2.2 Materials and Methods

2.2.1 Chemicals

All solvents used in this study were analytical and HPLC grade (EDM Chemical Inc., Gibbstown, NJ). The following chemicals were purchased from Sigma (St. Louis, MO): 1, 1-diphenyl-2-picryl hydrazyl (DPPH); 2,2'-azino-di-(3-ethylbenzothiazoline)-6-sulfonic acid ($ABTS^{\cdot+}$) Folin-Ciocalteu; Dulbecco's Modified Eagle Medium (DMEM); trypsin-EDTA; penicillin; streptomycin; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); and dithiothreitol (DTT). Fetal bovine serum (FBS)

was purchased from Gibco (Grand Island, NY). Human breast cancer MCF-7 and MCF-12F non-malignant cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A cell fractionation kit was used to obtain the cytosolic cytochrome C extract (Bio-Vision incorporated, Mountain view, CA). Mouse monoclonal anti-Bax (sc-7480), anti-Bcl2 (sc-65392), anti-cytochrome C (sc-13156), and anti-β-actin (sc-81178) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit polyclonal anti-PARP (#9542) was purchased from Cell Signaling Biotechnology (Beverly, MA). Other chemicals and biochemical materials were purchased with the highest available purity.

2.2.2 Preparation of Lemon Seed Extracts

Dried lemon seeds (3 kg) were powdered and extracted with hexane in a Soxhlet extractor for 8 h to remove fatty material. The defatted powder (2.4 kg) was extracted sequentially with 6 L of ethyl acetate (EtOAc), acetone, methanol (MeOH), and MeOH:water (80:20) for 16 h each, successively. Each extract was concentrated by a rotary evaporator (Büchi, Switzerland) under a vacuum to obtain viscous liquid and stored at -20 °C until the analysis (Labconco Corp., Kansas, MO).

2.2.3 Identification by LC-MS

Lemon seed extracts were dissolved in either acetone or MeOH:water (1:1), filtered by a 0.45 μm membrane filter (Millipore Co., Bedford, MA) and analyzed by LC-MS. The LC system consisted of Finnigan Surveyor plus (West Palm174 Beach, FL)

coupled to a mass spectrometer-Ion Trap (LCQ-DECA, ThermoFinnigan). Separation of the compounds were conducted on an Aquasil, C-18 column (2.1 × 150 mm, 3 μm) (Keystone-Hypersil, Bellefonte, PA) using a gradient mobile phase of 0.1% formic acid (A) and acetonitrile (B), maintained at a flow rate of 0.2 mL/min. The gradient conditions consisted of (A) 80% to 75% in 7 min and maintained for 5 min, followed by linear change to 70% in 4 min and finally returned to 80% in 23 min. The mass spectrometer was operated using electron spray ionization in negative ion mode (ESI-) with the spray voltage set at 3.5 KV.

2.2.4 Quantification by HPLC

The chemical composition in lemon seed extracts were quantified by HPLC (Waters Corporation, Milford, MA) using a reversed phase C-18 Gemini (4.6 mm × 250 mm, 5 μm) series column (Phenomenex, Torrence, CA) at a flow rate of 1 mL/min, as described in our previous publications, with minor modifications (96). The column was eluted with a gradient mobile phase of (A) 3 mM phosphoric acid, and (B) acetonitrile. The elution of the column was as follows: 85% A - 50% A in 33 min, and 85% A / 15% B at the end of 35 min. Limonoids and flavonoids were detected at 210 and 280 nm, respectively.

2.2.5 Radical Scavenging Activity

DPPH Assay

Lemon seed extracts were dissolved in MeOH to make a stock solution (5 mg/ml), and pipetted different concentration (10, 20, 30, and 40 µl) into 96 well plate to provide linear concentration profiles. Next, 200 µl of methanolic DPPH (100 µM/L) solution was added and the total volume was adjusted to 240 µl by MeOH according to a published method (97). The final concentration of actual lemon seed extracts were reflected as 208, 417, 624, and 832 µg/ml. Ascorbic acid was used as a positive control for comparison. The degradation of the DPPH radical was measured by a KC4 microplate reader (BioTek Instruments, Winooski, VT) at 517 nm for 30 min.

$ABTS^{\cdot+}$ Assay

A solution of $ABTS^{\cdot+}$ was prepared by mixing ABTS (7 mM) and potassium persulfate (2.45 mM) in a 1:1 ratio and was then incubated in the dark for 16 h. The ABTS radical stock was diluted in MeOH up to 0.7 - 0.75 optical density. Different concentrations of lemon seed extracts were pipetted into a 96 well plate, and 200 µl of the ABTS solution was added. The degradation kinetics of radical cation (ABTS) was monitored by a KC4 microplate reader (BioTek Instruments, Winooski, VT) at 734 nm and was recorded every 3 min for 30 min.

2.2.6 Determination of Total Phenolic Content

The total phenolic concentration was measured using a Folin-Ciocalteu assay (98). A standard catechin solution (10, 20, 30, 40, 50, 72, and 100 µg/ml) was prepared

in water. Even though hesperidin (HEP), a flavonoid glucoside, is a predominant phenolic compound in lemon, it was not selected as a reference compound due to its low solubility in an aqueous solution (99). One hundred µL of each extract and different concentrations of catechin were pipetted into different tubes. Five hundred µL of Folin-Ciocalteu (1:1 diluted with water) was added to the tubes, and they were incubated for 10 min at 25 °C. One milliliter of sodium carbonate (7.5% w/v) was added and incubated for 30 min. The absorbance was recorded at 765 nm, and results were expressed as catechin equivalents (CE). All tests were performed in triplicate and averaged.

2.2.7 Cell Culture

The MCF-7 cells were cultured in DMEM medium containing 10% fetal bovine serum, 200 U/ml penicillin G, and 200 µg/ml streptomycin and incubated at 37 °C with 5% CO2. The MCF-12F cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium with 20 ng/ml of epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% chelex-treated horse serum. Dimethyl sulfoxide (DMSO) was used to dissolve the lyophilized extracts, and the same concentration of DMSO (< 0.2%) was used in the control cell group.

2.2.8 Cytotoxicity Assay

The cytotoxicity was determined by MTT assay (100). The cells (1×10^4 / well) were seeded in a 96 well microplate and cultured in the presence of 0, 12.5, 25, 50, 75, and 100 µg/ml of the EtOAc, acetone, MeOH, and MeOH:water (80:20) extracts, and

tamoxifen, and then were incubated for 24, 48, and 72 h. The cells were treated with 10 µL MTT reagents (5 mg/ml) and incubated for 2 h at 37 °C to obtain purple-colored formazan. The color was dissolved in 200 µL of DMSO and measured by an ELISA microplate reader (BioTek Instruments, Winooski, VT) at 570 nm. All values were calculated as a percent of the unviable cell number compared to the control from three independent experiments performed in triplicate.

2.2.9 DNA Fragmentation

MCF-7 cells (1×10^6) were seeded in a 100 mm petri dish and incubated, with four extracts of lemon seed and DMSO as controls, for 48 and 72 h at 37 °C. Cells were harvested and washed with cold phosphate buffered saline. For DNA extraction, a harvested cell pellet was resuspended with an extraction buffer (0.1 M NaCl, 0.01 M EDTA, 0.3 M Tris-Cl [pH 7.5], 0.2 M sucrose, 10% sodium dodecyl sulfate [SDS]) and incubated for 30 min at 65 °C. The DNA was extracted with a phenol, chloroform, and isoamyl alcohol (25:24:1) mixture and centrifuged for 10 min at 1200 rpm. The supernatant was transferred to a fresh tube and DNA was precipitated by 3M NaOAc (pH 5.7) and 100% ethanol and stored at -20 °C overnight. DNA was eluted in a Tris-EDTA buffer followed by ethanol precipitation. The extracted DNA was separated in 1.5% agarose gel and visualized by ethidium bromide staining under UV light (LAS 4000 imaging, Fuji Life Sciences, CT).

2.2.10 Western Blot

MCF-7 cells (1 x 10^6) were treated with the MeOH:water (80:20) extract (100 μg/ml) and incubated for 24, 48, and 72 h. Total protein was extracted in a lysis buffer (150 mM NaCl, 10 mM Tris-Cl [pH 7.2], 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA) with a protease inhibitor cocktail (GenDEPOT, TX). Cytosolic fractions for cytochrome c detection were prepared using a cell fractionation kit (Bio-Vision incorporated, Mountain view, CA) according to the manufacturer's instructions. Protein concentration was determined by the bicinchoninic acid method (Pierce, IL). Thirty μg of protein from each sample was separated by electrophoresis on 12% SDS-PAGE. The gel was blotted onto a PVDF membrane by semi-dry transfer (Bio-Rad, CA). The membranes were blocked with 5% non-fat dry milk in a TBS-T buffer (10 mM Tris-Cl [pH 8.0], 100 mM NaCl, and 0.1% Tween 20) for 1 h and then incubated overnight with a specific primary antibody such as anti-Bax (1:500), anti-Bcl2 (1:500), anti-cytochrome c (1:500), anti-β-actin (1:1000), and anti-PARP (1:1000)for each protein. Subsequently, the membrane was washed with a TBS-T buffer and was probed with a goat anti-mouse IgG-horseradish peroxidase conjugate (1:2000) (Santa Cruz Biotechnology, CA). The protein band signal was detected by a chemiluminescence (ECL) detection kit (Amersham Bioscience, NJ), and the image of the signal was visualized by the LAS 4000 mini-imaging system (Fuji Life Sciences, CT).

2.2.11 Statistical Analysis

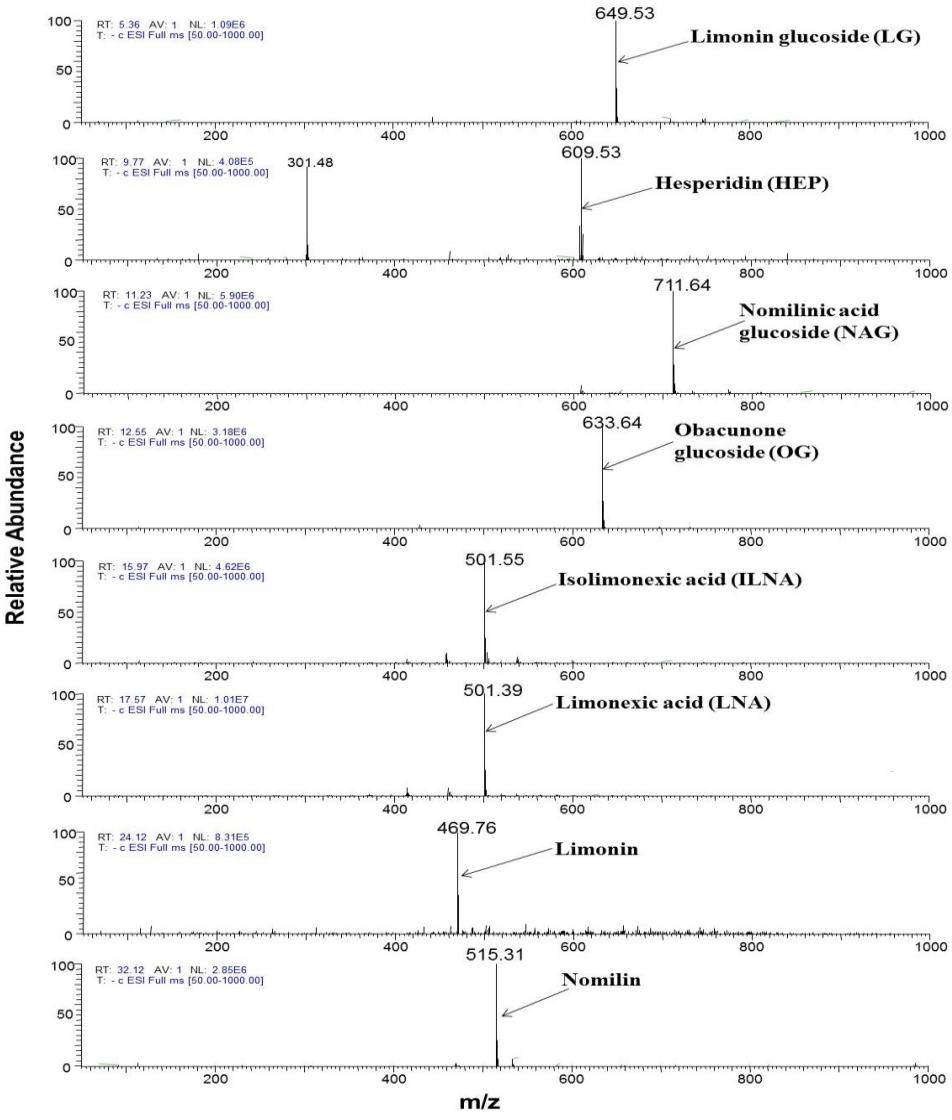
Statistical significance was analyzed by a Statistical Package for the Social Sciences (SPSS) 16.0 software (Chicago, IL) and expressed as means with either standard deviation or standard error with P-value. The statistical differences between the control and treatment groups were evaluated by one-way analysis of variance (ANOVA), followed by both Fisher's least significant difference and Turkey's multiple comparisons. $P < 0.05$ was considered significant.

2.3 Results and Discussion

2.3.1 Extraction and Identification of Bioactive Components in Lemon Seed Extracts

Successive solvent extraction of the lemon seed in a Soxhlet apparatus resulted in obtaining a yield of 78.62 g (3.27% w/w), 16.37 g (0.68% w/w), 221.50 g (9.23% w/w), and 402.60 g (16.78% w/w) for the EtOAc, acetone, MeOH, and MeOH:water (80:20) extracts, respectively. To investigate the effects of lemon seed components on human breast cancer (MCF-7) and non-malignant (MCF-12F) cells, all the extract were freeze-dried, and chemical constituents of lemon seed extracts were identified by LC-MS analysis. Our previous studies suggested that limonoids and flavonoids are the main bioactives present in citrus (7, 36, 47). Fig. 2.1 represents the molecular ion peaks of HEP (m/z 609.53), isolimonexic acid (ILNA) (m/z 501.55), limonexic acid (LNA) (m/z 501.39), limonin (m/z 469.76), and nomilin (m/z 515.31).

A



B

Extracts	LG	HEP	NAG	OG	ILNA	LNA	Limonin	Nomilin
EtOAc	-	+	-	+	+	+	+	+
Acetone	-	+	+	+	+	+	+	+
MeOH	+	+	+	+	-	-	+	+
MeOH:water	+	+	+	+	-	-	+	+

Fig. 2.1 Identification of bioactive compounds present in the lemon seed extracts by LC-MS using electron spray ionization (ESI) with negative ion mode. (A) Mass spectra of hesperidin and limonoids (B) Identified limonoids in different extracts.

Apart from the limonoid aglycones, three glucosides such as limonin glucoside (LG) (m/z 649.53), obacunone glucoside (OG) (m/z 633.64) and nomilinic acid glucoside (NAG) (m/z 711.64) were identified in the MeOH and MeOH:water (80:20) extracts. The molecular ion peaks are in agreement with previous reports (36, 101, 102).

2.3.2 Quantification of Bioactive Compounds in Lemon Seed Extracts

Limonoids and flavonoid based on LC-MS (Fig. 2.1), the compounds were quantified by reversed phased HPLC according to our previously established procedure (96, 103). Quantified limonoids and hespridin (HEP) from lemon seeds were presented in Table 2.1. The successive Soxhlet extraction of lemon seeds using different polar solvents enabled in obtaining selective ratio of polar and nonpolar compounds. Among the quantified compounds, the concentration of HEP was relatively higher in acetone extract (26%) in comparison to the lowest content (3%) in the EtOAc extract. The higher amount of limonoid aglycones, limonin (48%) and nomilin (37%) were present in the EtOAc extract whereas MeOH:water (80:20) extract which had the lowest levels, 5% and 1% respectively. In contrast, the MeOH:water (80:20) extract contained the highest level of glucosides (86%) as compared to the other extracts such as EtOAc extract (12%), acetone extract (46%), and MeOH extract (75%). Limonin glucoside was detected in MeOH and MeOH:water (80:20) extracts. ILNA and LNA were also detected in EtOAc and acetone extracts (Fig. 2.1). However, they were lower than the limit of quantification (Table 2.1). The failure of quantification for three compounds is due to lower detection sensitivity and selectivity of HPLC with UV absorbance than LC-MS (104).

Table 2.1 Levels of bioactive compounds (mg/g w/w) present in different lemon seed extracts*

Compounds	EtOAc	Acetone	MeOH	MeOH:water
Hesperidin	3.13 ± 0.40 (3%)	16.84 ± 0.00 (26%)	19.16 ± 0.19 (11%)	11.13 ± 0.16 (8%)
NAG	-	8.45 ± 0.19 (13%)	56.75 ± 0.53 (31%)	61.73 ± 0.34 (42%)
OG	11.87 ± 0.06 (12%)	21.02 ± 0.09 (33%)	79.92 ± 0.07 (44%)	65.19 ± 0.09 (44%)
Limonin	46.88 ± 0.34 (48%)	12.00 ± 0.14 (19%)	17.74 ± 0.10 (10%)	7.52 ± 0.42 (5%)
Nomilin	36.08 ± 0.33 (37%)	6.30 ± 0.15 (10%)	7.75 ± 0.35 (4%)	1.28 ± 0.06 (1%)

*Results are average of three independent experiments performed in triplicate.

2.3.3 Radical Scavenging Activity of Lemon Seed Extracts

ROS generation by up-regulating antioxidant systems is a bona fide method to develop anti-cancer agents (105). It is speculated that the inverse correlation between a diet rich in fruits and vegetables and cancer incidence is closely connected with the high amount of dietary antioxidants (106). Previously, we have demonstrated that citrus fruit (citron and blood orange varieties) extracts are a good source of free radical or reactive oxygen species scavengers (89). Indeed, the EtOAc and MeOH:water (80:20) extracts have demonstrated minimum and maximum antioxidant effects, respectively (Fig. 2.2). Lemon seed extracts were treated with DPPH and $ABTS^{\cdot+}$ to quench the free radicals, and the reaction was monitored for 30 min at different concentrations. In Fig. 2.2A and 2.2B, the kinetics graphs represent the degradation rate of free radicals. The capabilities of free radical quenching such as DPPH and $ABTS^{\cdot+}$ with lemon seed extracts is directly proportional to the radical scavenging activity. The MeOH:water (80:20) extract

exhibited the highest radical scavenging activity (62.2% and 91.3%), while the EtOAc extract showed the lowest radical scavenging activity (2.4% and 17.3%) at 833 µg/ml in the DPPH and $ABTS^{\cdot+}$ assay, respectively (Fig. 2.2C and 2.2D). The radical scavenging activity of lemon seed extracts showed in a concentration-dependent manner. The order of antioxidant activity was found in MeOH:water (80:20) > MeOH > acetone > EtOAc (Fig. 2.2C and 2.2D).

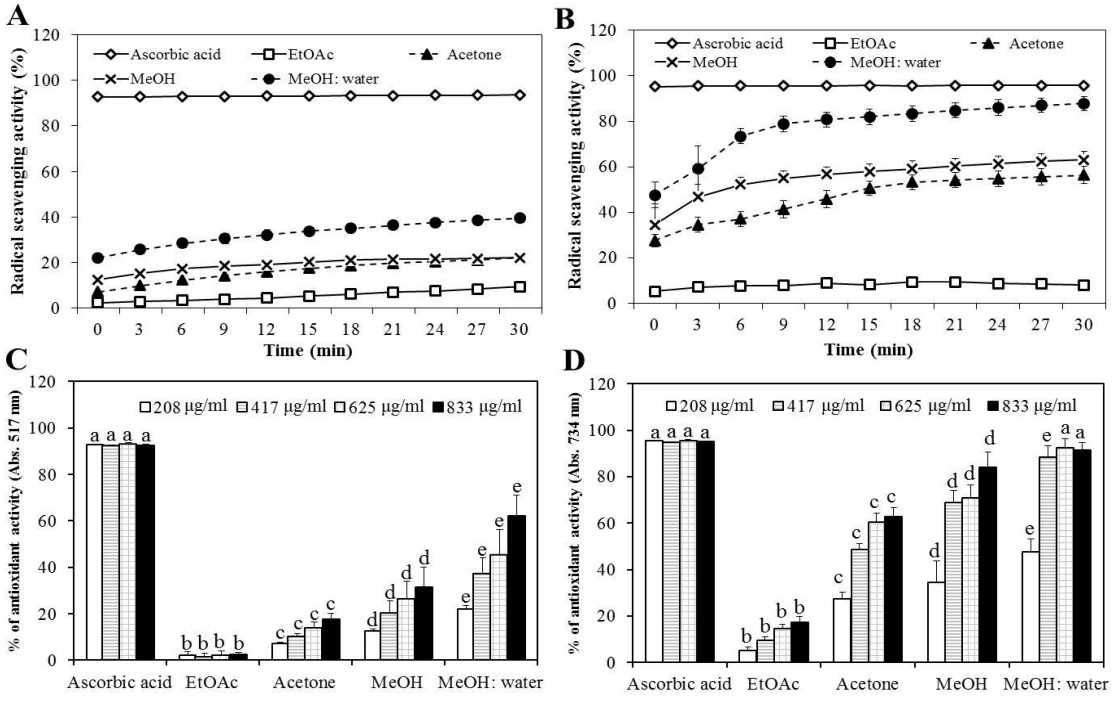


Fig. 2.2 Radical scavenging activity of various solvent extracts and ascorbic acid was measured as kinetic change of (A) DPPH radical and (B) $ABTS^{\cdot+}$. The value was monitored for every 3 min at a 208 µg/ml concentration. Values were expressed means of three independent experiments with standard deviation.

Generally, phenolic compounds are significant contributors of the antioxidant activities of fruit and vegetables. Thus, total phenolic content of the four extracts were determined by Folin-Ciocalteu method. The maximum (7.2 mg CE/g) and minimum (4.7 mg CE/g) phenolic content was observed in the MeOH:water (80:20) and the EtOAc extract, respectively (Fig. 2.3). According to Bocco et al. (107), the total content of six different glycosylated flavanones in the lemon seed MeOH extract was 2.15 mg/g by HPLC analysis. In the present study, MeOH extract has 5 mg CE/g total phenolics by Folin-Ciocalteu method. Higher levels of total phenolics determined in Folin-Ciocalteu method is not absolute measurements of the amounts of phenolic compounds, but are in fact based on their chemical reducing capacity relative to CE.

Comparison of present data and previous reports is difficult since there is no data on phenolic content of lemon seed. Sun et al. (108) found that the total phenolic content (0.81 ± 0.03 mg/g fresh fruit) and total antioxidant activity (42.8 ± 1.0 μM/g) of lemon extract were directly correlated with each other. Similar correlation results were observed in present investigation of lemon seed extracts (Fig. 2.2 and 2.3) (109). In the EtOAc extract, limonoid aglycones were determined as major constituents (Fig. 2.1 and Table 2.1). Since limonoids were inactive as a radical scavenger (110), it is possible that the lowest radical scavenger activity of the EtOAc extract is due to the presence of limonoid (Fig. 2.2 and 2.3). On the other hand, the MeOH and MeOH:water (80:20) mixture were used to elute polar compounds including limonoid glucosides and flavonoid glucoside. The higher antioxidant capacity of the methanolic extract may be due to the presence of flavonoid and polar compounds.

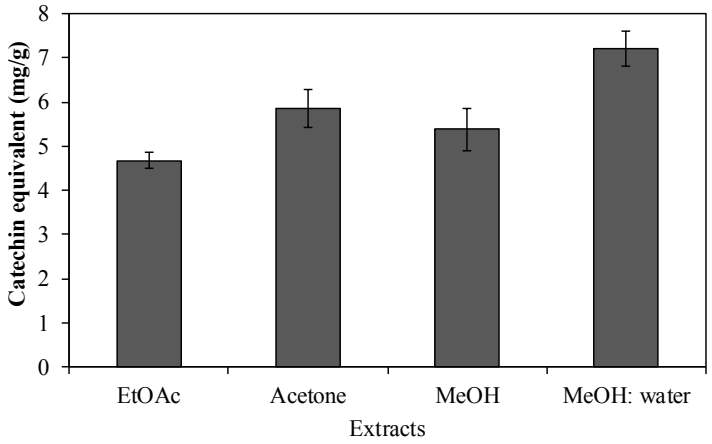


Fig. 2.3 Total phenolic content present in different lemon seed extracts. Values are expressed means of three independent experiments with standard deviation.

2.3.4 Effect of Different Extracts on Cytotoxicity

To evaluate the cytotoxicity of extracts, breast cancer (MCF-7) and non-malignant (MCF-12F) cells were treated with four lemon seed extracts and their proliferative ability was examined by MTT assay. Among the four extracts, EtOAc and MeOH:water (80:20) extracts exhibited 24% ($P < 0.01$) and 29.1% ($P < 0.01$) inhibition of MCF-7 cell growth respectively, at 100 µg/ml for 48 and 72 h. This inhibition was time- and concentration-dependent (Fig. 2.4) whereas acetone and MeOH extracts showed significant growth inhibition at 72 h for 100 µg/ml concentration. However, tamoxifen significantly inhibited breast cancer cells at all the tested concentrations and time (Fig. 2.4). Limonoids have been reported as potent inhibitor compared to hesperidin in MCF-7 cells (16, 111). In this study, the activity of EtOAc was directly proportional to the content of limonoids (Table 2.1). Tian et al. (16) reported that the mixture of limonoid glucosides (IC_{50} = 0.013 mM) was more potent than a single limonoid in the

anti-proliferative activity on MCF-7 cells. Indeed, these results suggest that higher cytotoxicity of the methanolic extracts may be due to the presence of limonoid aglycones, limonoid glucosides, and hesperidin (Fig. 2.1 and Table 2.1). Additionally, the presence of higher amount of phenolics could be relevant to the highest cytotoxicity of MeOH:water (80:20) extracts. Treatment with the same concentration of all extracts for 72 h did not show cytotoxicity in MCF-12F non-malignant cells. Tamoxifen was used as a positive control and significantly inhibited non-malignant breast cancer cells (MCF-12F) at all the tested concentrations and time (Fig. 2.4).

2.3.5 Apoptotic Phenotypes of MCF-7 Cells by Lemon Seed Extracts

One of the best-studied biochemical characteristics of apoptosis is internucleosomal DNA cleavage, which generates a characteristic DNA ladder (80). To investigate whether the inhibitory effect on MCF-7 cell proliferation was associated with the activation of apoptosis, a DNA fragmentation assay was conducted with the different extracts. As predicted from the antioxidant and anti-proliferative activity, the MeOH:water (80:20) extract induced DNA fragmentation at 75 and 100 μg/ml for 48 and 72 h (Fig. 2.5C).

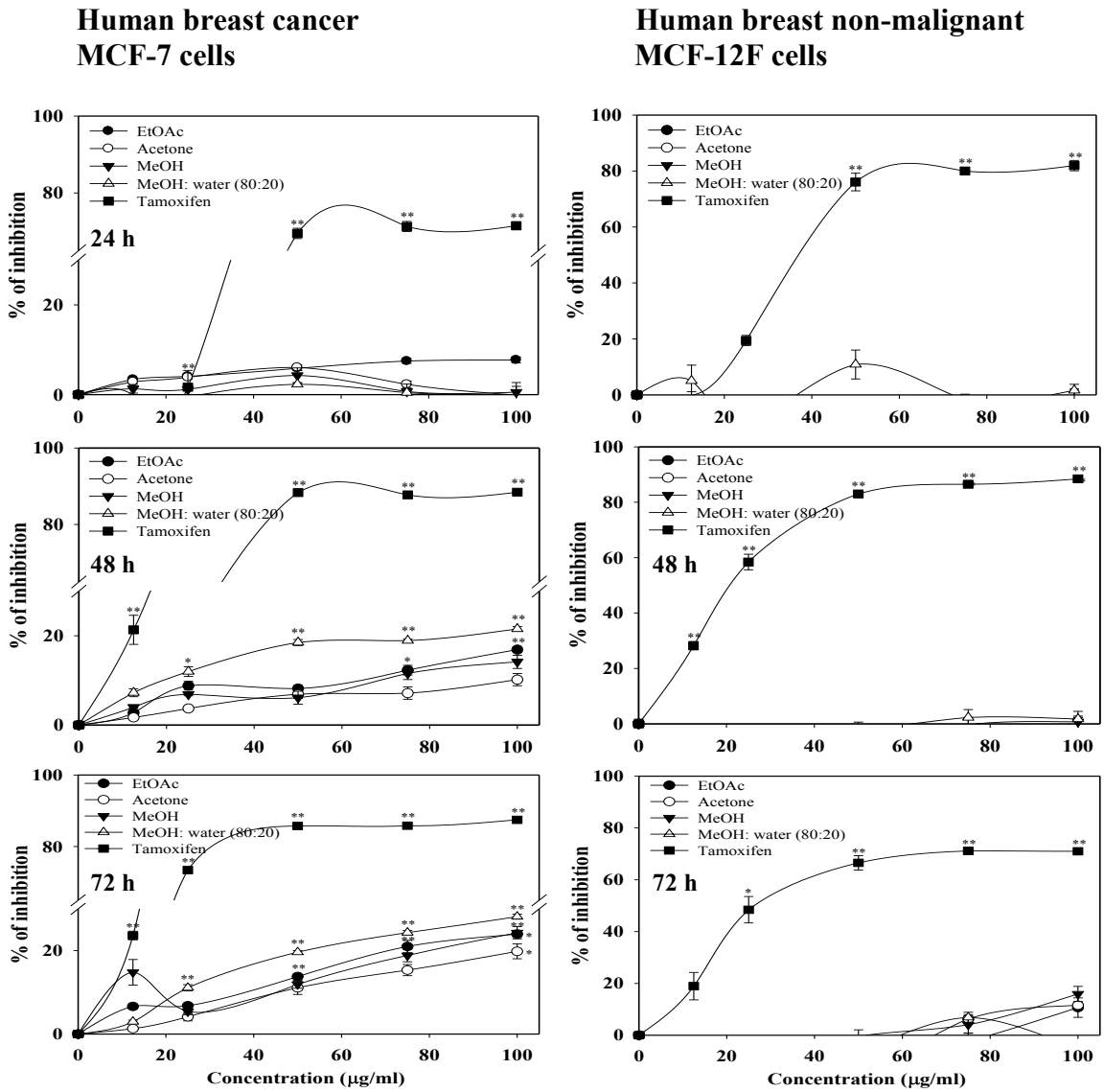


Fig. 2.4 Proliferation inhibition of human breast cancer cells (left panels) and non-malignant cells (right panels) by different lemon seed extracts was determined by the MTT assay. Cells (1 x 10^4 / well) were incubated with either each extract or tamoxifen at 0, 12.5, 25, 50, 75, and 100 μg/ml at different time points. The data are expressed as the percent of cell inhibition. The bars represent standard errors. (*P < 0.05, **P < 0.01)

In addition, the MeOH extract also induced the DNA fragmentation (Fig. 2.5B). However, the EtOAc and acetone extracts did not show the laddering patterns (Fig. 2.5A). This may be due to the high content of limonoid glucosides in the methanolic extracts. The order of total limonoid glucosides content was found in MeOH:water (80:20) extract (86%) > MeOH extract (75%) > acetone extract (46%) > EtOAc extract (12%) (Table 2.1).

Even though the EtOAc and acetone extracts did not show DNA fragmentation, both these extracts showed anti-proliferative activity at 100 µg/ml for 72 h. In our previous study, induction of G1 cell cycle arrest was demonstrated in human colon cancer cells by ILNA and sitosterol glucoside (52). Accordingly, the current study strongly supports that limonoid aglycones mainly present in EtOAc extract may induce cell cycle arrest.

Bcl-2 family proteins were demonstrated to cause apoptosis by up-regulation of pro-apoptotic protein (Bax) and down-regulation of anti-apoptotic protein (Bcl2, bcl-XL), leading to cytochrome C release from mitochondria and its complex with the Apaf-1 (apoptotic protease activating factor-1) activate downstream caspase cascade (caspase -8,-9,-3,-6,-7), as well as PARP cleavage (82, 112). Since MeOH:water (80:20) extracts showed the highest anti-proliferative activity in a time- and concentration-dependent manner, we used the MeOH:water (80:20) extract to understand the upstream mechanism of DNA fragmentation in MCF-7 cells.

As shown in Fig. 2.5D, Bax and cytosolic cytochrome C protein expression was up-regulated, and Bcl2 protein expression was down-regulated for 72 h using the

MeOH:water (80:20) extract. The increased cytochrome c in the cytosolic fraction (Fig. 5D) is enough to support inducing apoptosis. Similar results were published recently from our group (113) and others (114).

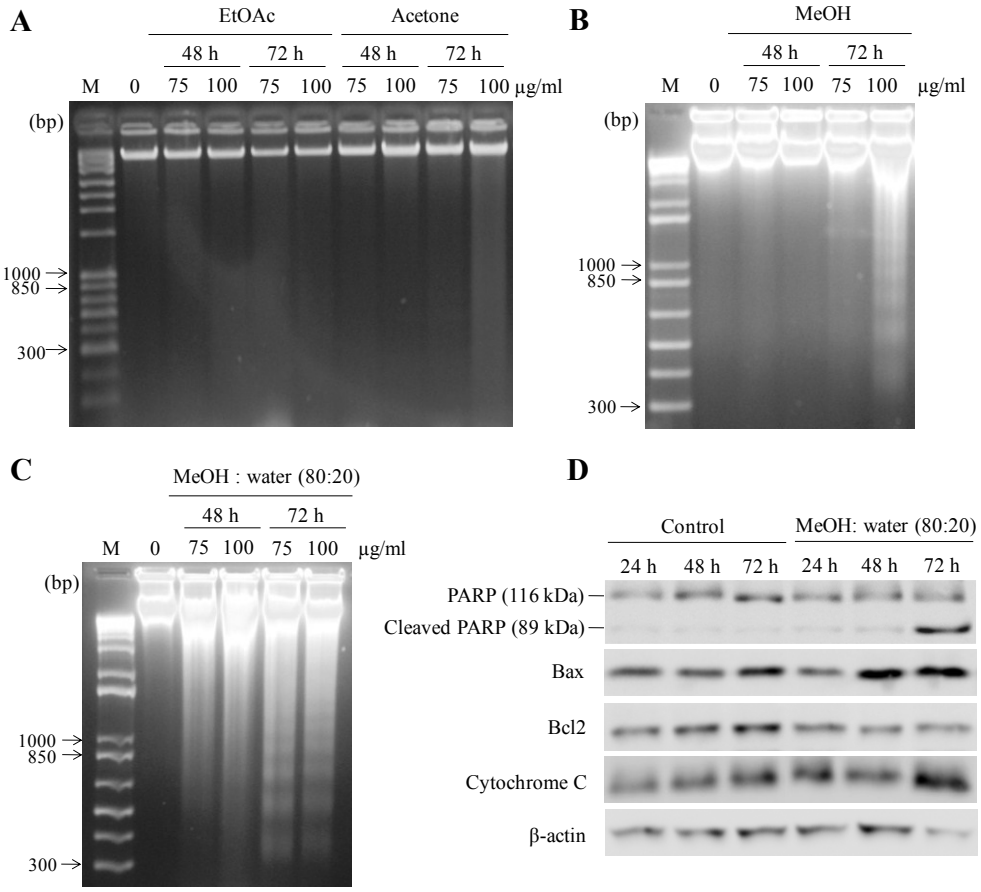


Fig. 2.5 The apoptotic features of lemon seed extracts. MCF-7 cells were treated with (**A**) EtOAc and acetone, (**B**) MeOH, and (**C**) MeOH:water (80:20) extracts and harvested after 48 and 72 h. The DNA fragmentation was assayed by 1.5% agarose gel electrophoresis in a TBE buffer. M (Marker). (**D**) The cells were treated with MeOH:water (80:20) extracts (100 µg/ml) for 24, 48, and 72 h and detected with a represented antibody by immunoblotting. β-actin levels served as a protein loading control. Protein was separated on 8-12% SDS-PAGE gel.

In addition, cleaved PARP was enhanced by the MeOH:water (80:20) extract, and these results are consistent with the DNA fragmentation in Fig. 2.5C. β-actin was used as an internal control. These results clearly demonstrated that the MeOH:water (80:20) extract induced apoptotic responses such as increased pro-apoptotic Bax, decreased anti-apoptotic Bcl2, released cytochrome C, and cleaved PARP in MCF-7 cells (Fig. 2.5C and 2.5D). Finally, it is possible that the solubilized bioactive compounds in the MeOH:water (80:20) extract may activate apoptosis in the MCF-7 cells at the cellular level.

Although the EtOAc extract has a significant amount of limonoid aglycones such as limonin, and nomilin, the anti-cancer activity is low as compared to MeOH:water (80:20) extract. It could be speculated that the concrete effect of the highest antioxidant and anti-cancer activity of the MeOH:water (80:20) extract from lemon seed may be due to the presence of higher amount of limonoid glucosides such as OG and NAG (Table 2.1).

2.4 Conclusion

Bioactive components in lemon seed extracts could be a good source of antioxidants and induce apoptosis in MCF-7 breast cancer cells through the mitochondrial apoptosis pathway attributed from the increased Bax, decreased Bcl2, released cytochrome C in cytosol, and cleaved PARP. Moreover, this research demonstrated for the first time that lemon bioactive compounds are non-toxic to non-malignant breast cells. Future studies should aim to purify and identify compounds

present in MeOH:water (80:20) extracts and understand their mechanism of apoptosis induction. These findings support the hypothesis that bioactive compounds from lemon may have cancer-preventive properties.

3. SCREENING OF LIMONOIDS FOR ANTI-PROLIFERATIVE ACTIVITY IN HUMAN BREAST CANCER CELLS

3.1 Introduction

Using different identification techniques, 62 limonoids have been identified and there is an ongoing effort to discover even more (9, 52). In 1949, a study was conducted to investigate the bitterness of citrus fruit (9). Later, the medicinal potential of limonoids on human health became newsworthy when their anti-tumor properties for leukemia cells were discovered in 1983 (115). It was later found that these cancer fighting properties could be extended to other type of human cancers such as colon (HT-29 and SW480) (50, 52, 116, 117), pancreatic (Panc-28) (36, 55, 118), leukemia (MOLT-4 and P388) (115, 119), breast (MCF-7 and MDA-MB-435) (16, 53), liver (HepG2) (41) and neuroblastoma (SH-SY5Y) (51, 54). Besides the anti-tumor properties, it may also be useful in prevention of coronary heart disease and inflammation (50, 120). Nevertheless, only two studies have been conducted to investigate the anti-proliferative effect of limonoids on estrogen receptor negative (ER-) and estrogen receptor positive (ER+) MCF-7 cell lines (16, 53). However, the cancer inhibition mechanism by limonoids in breast cancer remains to be investigated.

Patil's group has been investigating the potential health benefits of citrus bioactive compounds for improving human health (16, 50-52, 54, 55, 116-118, 120). Unfortunately, the limonoids are not commercially available except nomilin and limonin. It is imperative that limonoids be purified in order to test their efficacy *in vitro* as well as

in biological tests such as animal experimentation and clinical trials. Previously, Patil's group have reported efficient purification methods of diverse citrus species from sour orange to grapefruit using different analytical techniques such as liquid chromatography/electrospray ionization/ mass spectrometry (LC-ESI-MS), flash chromatography, column chromatography, and supercritical CO_2 extraction (89, 102, 121, 122). Even though lemon is in the top three most popular citrus fruit followed by orange and grapefruit, an importance of lemon as potential source of limonoids have not been yet evaluated.

Hormone sensitivity has been found to be one of the key signatures in breast cancer diagnosis (73). More than 60% of breast tumors exacerbated by excessive estrogen and the presence of estrogen receptors (ER) result in better prognosis than ER-negative (74). Although the ER positive breast tumors are more responsive to chemotherapy than negative breast tumors, the outcome of anti-cancer agents are not always followed by same mechanism due to the complex interaction of anti-cancer and human disease.

In this context, we aimed to purify different limonoids from lemon byproducts, particularly the seeds, and confirm their identity by using HPLC and mass spectra. Further, the purified limonoids were screened for potential biological activity in both ER-positive and ER-negative human breast cancer cells. Moreover, we further investigated the possible mechanism underlying the inhibition of human breast cancer cell proliferation by limonoids.

3.2 Materials and Methods

3.2.1 Materials

All solvents used for isolation and analysis were HPLC grade (EDM Chemical Inc., Gibbstown, NJ). TLC silica gel 60F-254 plate and silica gel (200-400 mesh) were obtained from Fisher Chemicals (Fair Lawn, NJ). Dowex-50 and Diaion HP-20SS resin were supplied from Supelco Incorporation (Bellefonte, PA). The ER-positive (MCF-7) and ER-negative (MDA-MB-231) human breast cancer cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Other chemicals and biochemical materials were purchased with highest available purity.

3.2.2 Extraction and Isolation

Dried lemon seeds (14 kg) were powdered and defatted with hexane using Soxhlet apparatus for 16 h. The defatted powder was sequentially extracted with 6 L of ethyl acetate (EtOAc), acetone, methanol (MeOH), and MeOH:water (80:20) (v/v) for 16 h each, successively. The EtOAc and MeOH concentrated extracts were obtained by a rotary evaporator (Büchi, Switzerland) under a vacuum. The dried EtOAc extract was placed onto a silica gel 60 (particle size 40-63 micron) column and mobile phases such as hexane, chloroform, acetone, and methanol with increasing polarity was injected into the column. Each fraction (1000 ml) was collected in a test tube and allowed to dry to achieve compound crystallization. Compound 1, 2, and 3 were eluted and crystalized with hexane: chloroform (50:50 (v/v), 25:75 (v/v), and 10:90 (v/v), respectively), while compounds 4 and 5 were crystallized in chloroform: acetone (80:20) (v/v) fractions.

Interestingly, after the elution of compound 4, compound 5 began to elute in the same mobile phase.

The MeOH and MeOH:water extracts were loaded to activated Dowex-50 (H^+) column connected with the SP-70 column. Then, the absorbed limonoids in the SP-70 column were eluted with linear gradient mobile phases consisting of acetonitrile (ACN) in water (v/v) according to previously published procedure (89). Compound 6 was eluted with 12.5% ACN in water. The 20- 25% ACN in water fractions were analyzed by HPLC and then pooled based on the retention time. The fractions with similar retention time were loaded onto the Diaion HP-20SS (Supelco, PA) cross-column and separated with mobile phases consisting of ACN in water (v/v). Compound 7 was eluted only with water. Compounds 8 and 9 were eluted with 5% (v/v) and 22.5% (v/v) ACN in water, respectively.

3.2.3 Preparation of Defuran Nomilin and Defuran Limonin

The modified compounds such as limonin methoxime (LM) and limonin oxime (LO) were prepared according to our previously established procedures (47, 120).
Defuran nomilin was prepared as follows, nomilin (0.514 g) acetonitrile (20 ml), carbon tetrachloride (10 ml), and water (15 ml) were mixed in a 100 ml round-bottom flask, and the reaction mixture was kept for stirring at room temperature for 10 min. Sodium periodate (4 g) and ruthenium trichloride (100 mg) were added, and stirring was continued until the completion of the reaction (～40 h). The reaction was monitored by thin layer chromatography. The reaction mixture was extracted with ethyl acetate in a

separating funnel, decolorized using activated charcoal, filtered, and concentrated under vacuum. The dried residue was extracted with chloroform and crystallized to obtain defuran nomilin (108 mg), while defuran limonin was prepared from limonin according to Perez et al (47).

3.2.4 Identification using HPLC/ LC-MS/NMR

All fractions were analyzed by thin layer chromatograph. The purified limonoids were identified with HPLC, LC-MS, and NMR spectra (58, 89, 120, 123). HPLC analysis was carried out using a Perkin Elmer instrument with diode array detector 235C using C18 reversed-phase Gemini column (4.6 mm × 250 mm, 5 µm, Phenomenex, CA). Compound 1-9 were detected at 210 nm. The mobile phase consisted of (a) 3 mM phosphoric acid, and (b) acetonitrile at a flow rate of 1 ml/min, as described previously (96).

Mass spectra of compounds were analyzed using an API QSTAR Pulsar Hybrid QTOF instrument (Applied Biosystems/MDS Sciex, Framingham, MA). The structure of purified compounds was identified by ^{1}H and ^{13}C NMR. The NMR spectra were recorded on a JEOL ECS-400 spectrometer using tetramethyl silane as the internal standard. The purified limonoids were dissolved in DMSO and chloroform, and analyses were conducted at 25 ºC. The spectra was recorded using a 5 mm broadband probe equipped with a shielded z-gradient and Delta software version 4.3.6. Tetramethylsilane was used as an internal reference.

3.2.5 Cell Culture

The MCF-7 cells were grown in a DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 200 units/ml penicillin G, and 200 µg/ml streptomycin. The MDA-MB-231 cells were cultured with the DMEM medium supplemented with 10% (v/v) FBS, 1% (v/v) sodium pyruvate, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 200 units/ml penicillin G, and 200 µg/ml streptomycin. Both cell lines were incubated at 37°C with 5% CO_2.

3.2.6 Cytotoxicity Assay

The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. In brief, both breast cancer cell lines were seeded (1×10^4 cells/well) in 96 well plates and incubated overnight to allow the cells to attach. To test the cytotoxic effect of limonoids, the limonoids were dissolved in DMSO and the dissolved limonoids (200 µM) were incubated with either MCF-7 or MDA-MB-231 cells for 72 h. At the end of the incubation period, 10 µl of MTT reagent (5mg/ml) was added to each well and exposed for another two hours. During this incubation period, viable cells convert tetrazolium salt to purple formazan due to mitochondrial dehydrogenase activity (124). After the two hour incubation period, the absorption of dissolved formazan using DMSO was read at 570 nm on a KC4 microplate reader (BioTek Instruments, Winooski, VT). All experiments were performed in triplicate three times. The MTT reduction was calculated as follows: (A of experimental group / A of control group) × 100.

3.2.7 Aromatase Activity Assay

In vitro aromatase inhibition assay was performed using CYP19/ Methoxy-4-trifluoromethyl-coumarin (MCF) high-throughput screening kit (BD Biosciences, Woburn, MA) following the manufacturer's protocol. Briefly, the tested limonoids were dissolved in acetonitrile. Various concentrations of limonoids were placed into 96-well black microtiter plates (BD Falcon^{TM}) and pre-incubated with NADPH-Cofactor mixture (8.1 μM $NADP^+$, 0.4 mM $MgCl_2$, 0.4 mM glucose-6-phosphate, 0.2 U/ ml glucose-6-phosphate dehydrogenase) for 10 min at 37 °C. After adding an enzyme and substrate (25 μM MFC and 1.5 pmol/well CYP19), the reactions were allowed for 30 min at 37 °C. The fluorescence was measured at 405 nm (excitation) and at 530 nm (emission). Ketoconazole was used as a positive control. Experiments were carried out on two separate occasions.

3.2.8 Western Blot

Whole cell lysates were prepared, as described previously (120). The protein concentration was measured using the BCA method. The same concentration (25 μg) of protein was subjected to 8% (PARP) or 12% (caspase-7) SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). The blots were proved with the polyclonal rabbit poly (ADP-ribosyl)polymerase (PARP) (1:1000), polyclonal rabbit caspase-7 (1:1000) antibody (Cell Signaling Technology, Beverly, MA) and monoclonal beta-actin (1:1000) antibody (Santa Cruz Biotechnology). Secondary horseradish peroxidase-labeled antibodies (1:2000) were evaluated under enhanced

chemiluminescence. The intensity of band signals was quantified by LAS 4000 mini (FUJI Film, Tokyo, Japan). The data were expressed as means ± standard error (S.E.) from three independent experiments.

3.2.9 Statistical Analysis

The statistical significance of the data was evaluated by one-way analysis of variance followed by Fisher's Least Significant Difference using the SPSS 16.0 program (Chicago, IL). Pearson's correlation was performed to evaluate the degree of correlation of the effects of limonoids between proliferations versus aromatase inhibition, aromatase inhibition versus caspase-7, and proliferation versus caspase-7. The P value (for two-tailed) was considered to be significant within 95% confidence intervals.

3.3 Results

3.3.1 Purification and Identification of Limonoids

Five aglycones (1-5) and four glucosides (6-9) were isolated from EtOAc and MeOH extracts, respectively. The chemical composition was analyzed by TLC and reversed phase HPLC. The compounds (1-5) were observed at retention time 43.7, 52.3, 41.4, 21.3, and 22.4 min, respectively. Compounds (6-9) were observed at retention time 19.49, 21.66, 34.61, and 36.12 min, respectively. Yield of compounds (1-9) were 4.0, 0.07, 16.0, 0.35, 0.98, 4.8, 0.12, 2.7, and 4.5 g, respectively. The mass spectra of compounds (1-9) were confirmed and matched to our previously reported value (58, 89, 123). Based on the HPLC and mass spectra data, compounds 1-9 were identified and

characterized as nomilin, obacunone, limonin, limonexic acid (LNA), isolimonexic acid (ILNA), limonin glucoside (LG), deacetyl nomilinic acid (DNAG), nomilinc acid (NAG), and obacunone glucoside (OG), respectively. The structures of purified limonoids were presented in Fig. 3.1.

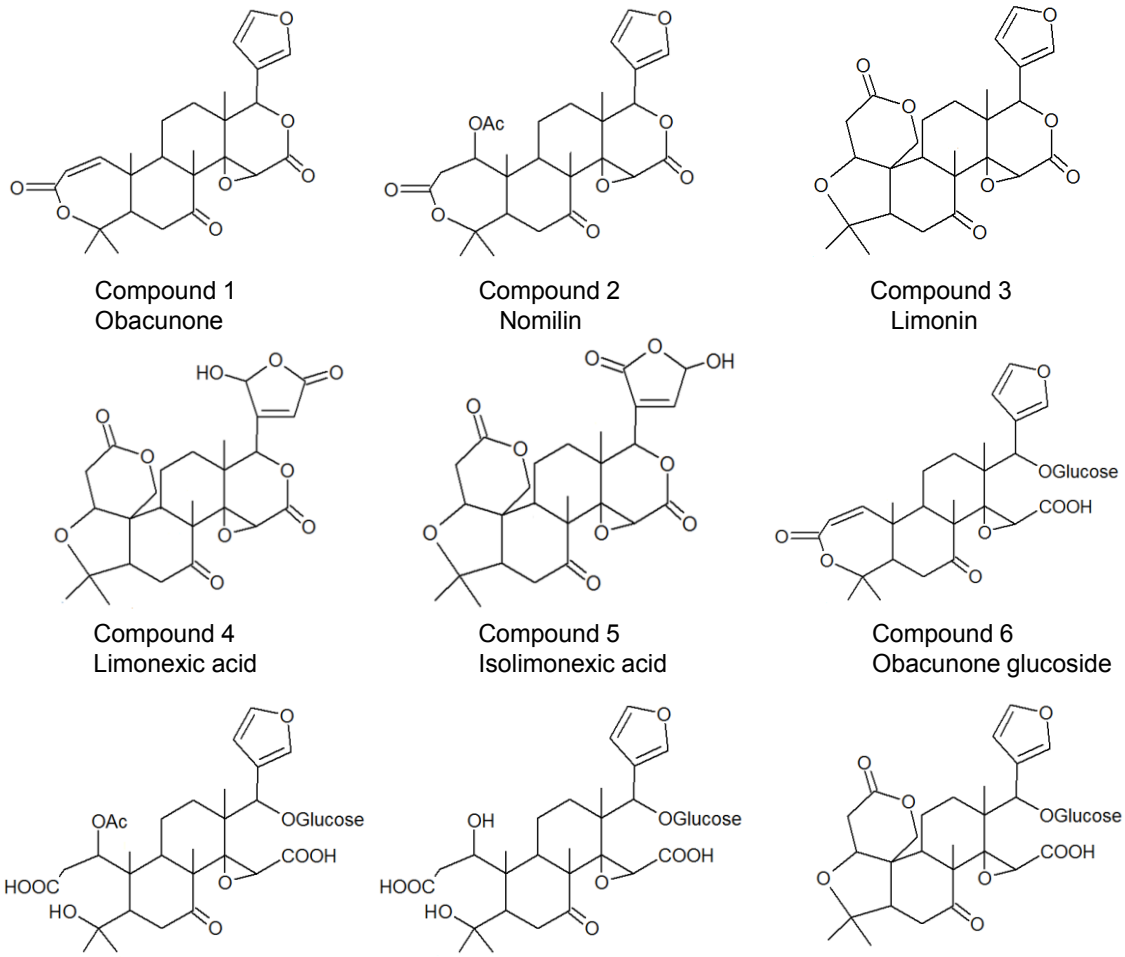


Fig. 3.1 The structure of purified limonoids from lemon seeds.

3.3.2 Modification of Limonoids

Defuran nomilin was prepared from nomilin by oxidative cleavage of furan ring at C20 position. The oxidation reaction was performed at 25 °C using ruthenium trichloride-sodium periodate in the presence of CCl4-CH3CN-H2O (2:2:3) solvent system (125) to obtain defuran nomilin. Fig. 3.2 depicts a complete assignment of ^{1}H and ^{13}C NMR signals of defuran nomilin, which was recorded in CD3OD. Proton spectra was quite complex specifically in the region between 1 and 3 ppm.

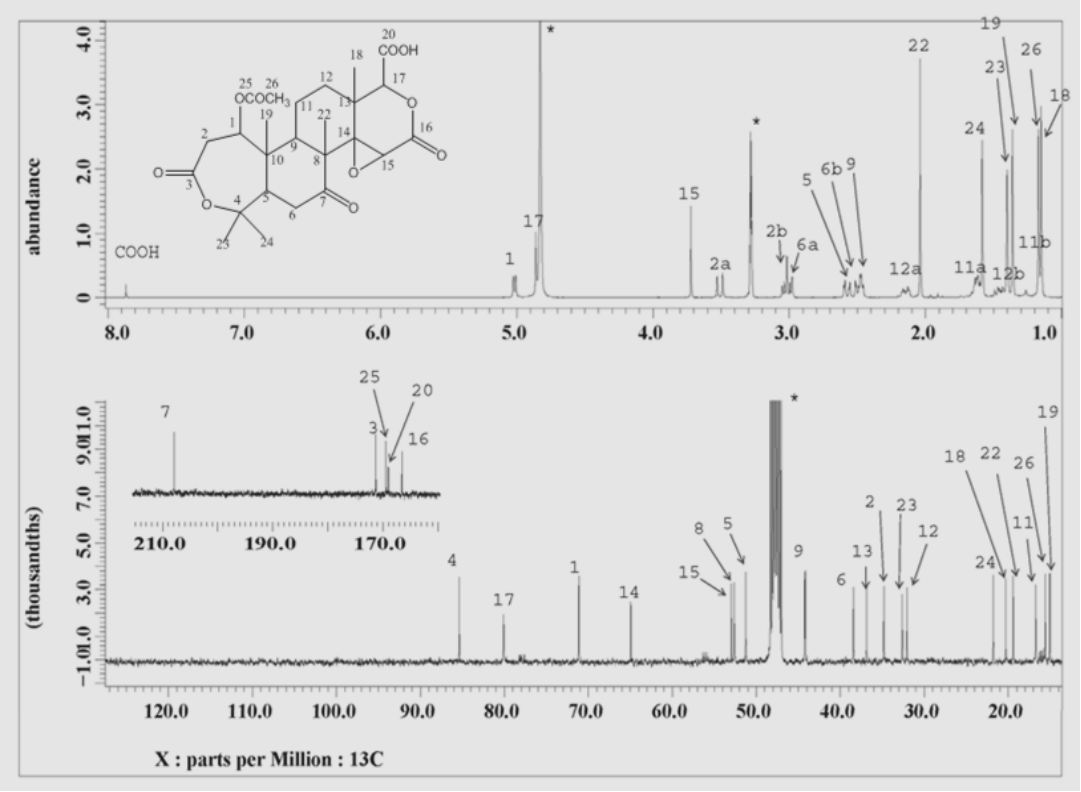


Fig. 3.2 ^{1}H and ^{13}C NMR spectra of defuran nomilin recorded using JEOL ECS spectrometer at 400 and 100 MHz respectively. Solvent used for NMR recording was CD_3OD and marked with asterisks. Assignments of all protons and carbons were made on the respective spectrum.

Six sharp single peaks for 3H with a characteristic chemical shifts and intensity between 1 and 2.1 ppm have been assigned to methyl groups. It is clear from the ^{1}H spectra that there are no aromatic signals at 6 to 8 ppm for the furan ring, which is further confirmed in ^{13}C spectra. An additional carbonyl signal at 168.5 ppm was assigned to carboxylic group at C20 position. The rest of the chemical shifts shown in Fig. 3.2 was matched to our previously reported values for nomilin (123).

3.3.3 Cytotoxicity of Limonoids in MCF-7 and MDA-MB-231

To evaluate the cytotoxic effect of limonoids, MTT reduction by tamoxifen (as a positive control), purified limonoids, and modified limonoids were measured as shown in Fig. 3.3. The MCF-7 cell lines were more susceptible than MDA-MB-231 to almost all the tested limonoids except the LG. Among 13 limonoids, six limonoids (limonin, DNAG, obacunone, OG, NAG, LO, and DN) showed significant cytotoxicity from both MCF-7 and the MDA-MB-231 cell. Notably, the obacunone exhibited the highest cytotoxicity of 44% ($P < 0.01$) and 18% ($P < 0.01$) in MCF-7 and MDA-MB-231 cells, respectively. However, no cytotoxicity was detected by LNA and DL in either cell lines.

The limonin (21%, $P < 0.01$ and 19%, $P < 0.01$) and its modified forms, such as LM (30%, $P < 0.01$ and null) and LO (23%, $P < 0.01$ and 17%, $P < 0.01$), exhibited significant cytotoxic effect in MFC-7 and MDA-MB-231, respectively, but the modification of limonoids did not show any significant effect on the cytotoxicity compared to the parent limonoid; whereas defuran limonin lost the cytotoxicity in both

cell lines. In contrast, defuran nomilin showed significant cytotoxicity 20% ($P < 0.05$) and 9% ($P < 0.01$) in MFC-7 and MDA-MB-231, respectively.

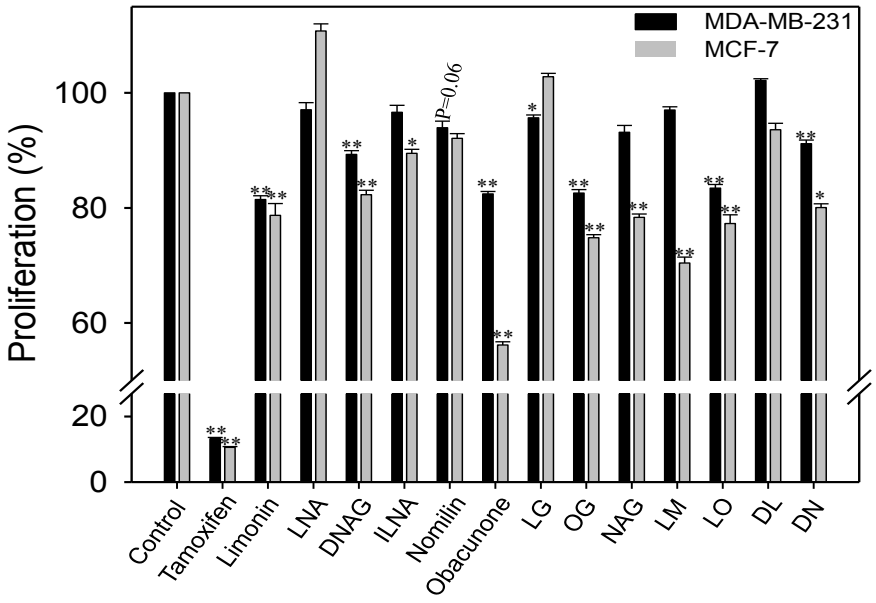


Fig. 3.3 Cytotoxic effect of purified or modified limonoids in human breast cancer cells. ER-positive breast cancer (MCF-7) or ER-negative breast cancer (MDA-MB-231) cells were treated with either DMSO (vehicle) or indicated limonoids (200 μM) for 72 h. After the 72 h incubation, the cytotoxic effect was determined by MTT assay. The bar represents means ± S.M.E. (n = 9-12). (* $P < 0.05$, ** $P < 0.01$)

3.3.4 Inhibition of Aromatase Activity by Limonoids

To determine if the susceptibility of MCF-7 for cytotoxicity by limonoids was due to estrogen receptor, aromatase activity was evaluated using CYP19 high throughput inhibition assay, shown in Table 3.1. The majority of limonoids were significantly inhibited the catalytic activity of aromatase enzyme except LNA (Table 3.1). Among 13

limonoids, LG and obacunone exhibited the most and least potent aromatase inhibition activity with an IC_{50} value of 1.27 μM ($P < 0.001$) and 28.04 ($P < 0.05$), respectively.

Table 3.1 IC_{50} for inhibition of aromatase by limonoids

Test compounds	**IC_{50} (μmol/L)**	***P*-value**
Ketoconazole (positive control)	0.85	0.000
Limonin	5.22	0.026
Limonexic acid (LNA)	20.02	0.344
Deacetyl nomilinic acid glucoside (DNAG)	4.41	0.008
Isolimonexic acid (ILNA)	25.60	0.034
Nomilin	18.86	0.000
Obacunone	28.04	0.024
Limonin glucoside (LG)	1.27	0.000
Obacunone glucoside (OG)	26.37	0.000
Nomilinic acid glucoside (NAG)	11.69	0.008
Limonin methoxime (LM)	1.65	0.000
Limonin oxime (LO)	2.00	0.000
Defuran limonin (DL)	1.65	0.000
Defuran nomilin (DN)	3.04	0.000

3.3.5 Effect of Limonoids on Activation of Caspase-7

Whether the cytotoxicity influences the caspase-7 activity in the presence of limonoids in MCF-7 was investigated using immunoblotting. As shown in Fig. 3.4, obacunone treated MCF-7 cells exhibited a 3.6-fold ($P < 0.01$) activation of caspase-7 followed by LG (2.8-fold, $P < 0.05$) while the effect of nomilin (2.4-fold, $P < 0.08$) and OG (2.4-fold, $P < 0.09$) on activation of caspase-7 was less significant.

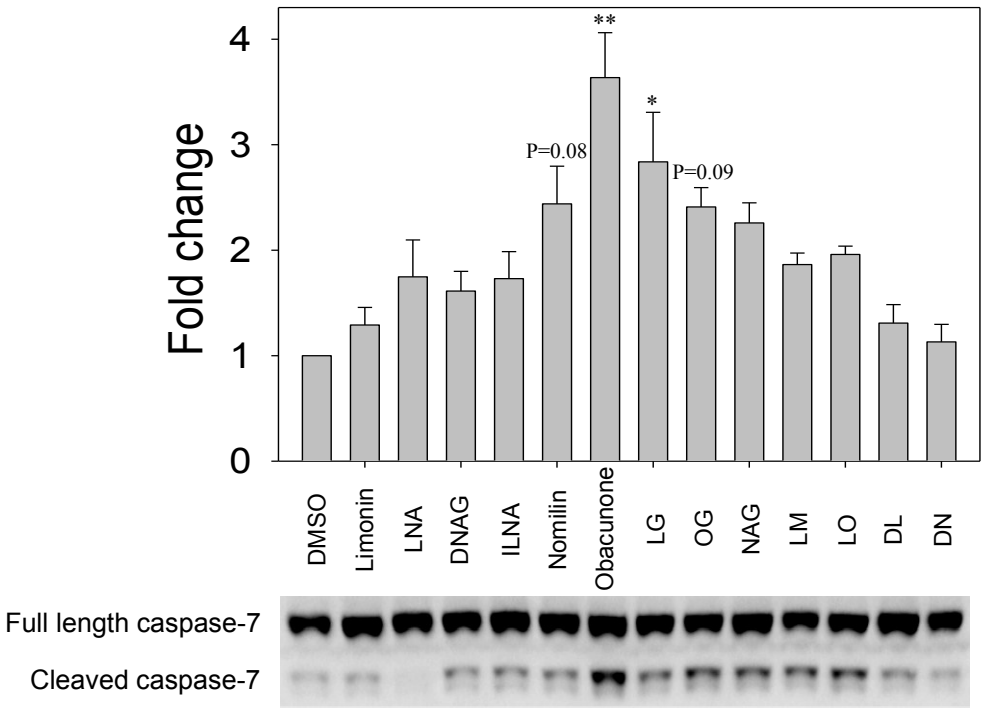


Fig. 3.4 Level of activated caspase-7 by different limonoids on ER-positive breast cancer cells. The MCF-7 cells were treated with limonoids for 72 h and the harvested cells were subjected to immunoblotting. Histograms shows the intensity of the chemiluminescent of activated caspase-7. The values were normalized by full length caspase-7. The bar indicates means ± S.E. from five independent experiments. The statistical significance of data was demonstrated with P value (* $P < 0.05$ ** $P < 0.01$). The representative immunoblot was probed with a specific caspase-7 antibody, as explained in Materials and methods.

3.3.6 Correlation Coefficients between the Biological Responses of Limonoids

To determine the correlation between the responses of limonoids on MCF-7 cells, the correlation between aromatase inhibition activity and proliferation inhibition activity by limonoids was measured by Pearson's correlation coefficient. As shown in

Fig. 3.5A. There were no significant correlations between aromatase inhibition and anti-proliferative activity by limonoids (r = 0.24, P = 0.43). Pearson's correlation coefficients indicated that the aromatase inhibition by limonoids tended to be associated with the activation of caspase-7 (r = 0.50, P = 0.08) (Fig. 3.5B). In contrast, the activation of caspase-7 showed significant positive association with anti-proliferative activity by limonoids in MCF-7 cells (r = 0.66, P = 0.02) (Fig. 3.5C).

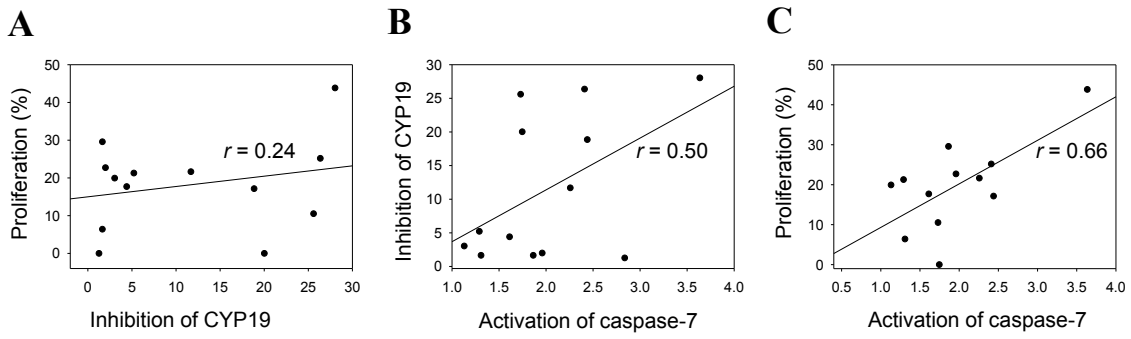


Fig. 3.5 Interactions between the tested biological reaction of limonoids in ER-positive breast cancer (MCF-7) cells. The correlation was determined by Pearson's coefficient of correlation method (two-tailed). (A) Correlation coefficient between aromatase inhibition and proliferation inhibition by limonoids (r = 0.24, P = 0.43), (B) Correlation coefficient between caspase-7 activation and aromatase inhibition by limonoids (r = 0.50, P = 0.08 (marginal significance)), and (C) Correlation coefficient between caspase-7 activation and proliferation inhibition by limonoids (r = 0.66, P = 0.02).

3.4 Discussion

The Food and Drug Administration has reported role of natural products (> 50%) as anti-cancer treatments (126). Among potential natural products, limonoids, abundantly present in citrus fruits, have been touted as one of the most promising anti-

cancer agent (6, 8, 9). Previous reports from Patil's lab and others using multiple experimental approaches demonstrated that limonoids have the potential risk reduction in many types of cancer such as colon (50, 52, 117), breast (16, 53), pancreatic (36, 55, 118), leukemia (115, 119), and neuroblastoma (51, 54).

This study demonstrated the importance of lemon seeds as a source of limonoids. Also, results demonstrated that the cytotoxic effect of limonoids from citrus is dependent on caspase-7 activation and is more susceptible to ER-positive MCF-7 than ER-negative MDA-MB-231 human breast cancer cells. However, even though limonoids exhibited anti-aromatase activity, sensitivity of MCF-7 to cytotoxicity by limonoids was independent of aromatase inhibition activity.

Due to the different biological profiles of MCF-7 (containing wild-type p53 gene and estrogen receptors) and MDA-MB-231 (containing mutant p53 gene and estrogen receptors) (127), the two cell lines are often selected for research as anti-cancer agents targeting breast cancer. Generally, the MCF-7 cell lines are more responsive to cancer therapy than the MDA-MB-231 cell lines, which have less sensitivity as a result of the p53 and ER mutation. In addition, recent papers have indicated that many anti-cancer agents were not occur apoptosis in MDA-MB-231 cell lines (127, 128). Similar results were obtained from present data that the cytotoxic effect of limonoids was stronger in MCF-7 cells than MDA-MB-231 cells. Moreover, these results are consistent with previous reports that the resveratrol and genistein, well known anti-cancer agents, are known to induce apoptosis in MCF-7 but not in MDA-MB-231 cells (128, 129).

Evidences demonstrated that breast tumors are ascribed to estrogen abundance (127) and limonoids have shown anti-proliferative activity in MCF-7 cells (Fig. 3.3). The aromatase enzyme is responsible for conversion from androgen to estrogen (130), so it was reasonable to investigate whether the limonoids have shown the anti-aromatase activity for the observed anti-proliferation activity. These results presented that limonoids have significant anti-aromatase properties (Table. 3.1). However, the anti-proliferation by limonoids was not associated with anti-aromatase activity (Fig. 3.5A). It should be noted that the aromatase activity is regulated by cyclooxygenase-2 (COX-2) suggesting the possible involvement of limonoids in regulation of inflammatory signaling pathways (131). Indeed, Patil's group previously demonstrated that certain limonoids (limonin and obacunone) have shown inhibition of COX-2, inducible nitric oxide synthase, and nuclear factor-kappa B translocation in *in vitro* and *in vivo* (50, 55).

Caspases, a member of the cysteine protease family, are the key mediators in cell death and the activation of effector caspases such as caspase-7 and caspase-3. These caspases are considered a last step of apoptosis (112). However, the caspase-3 is non-functional in MCF-7 cell lines due to deletion of exon 3 (132), therefore, it was important to determine whether caspase-7 is involved in the anti-proliferative activity of limonoids. Present data showed a significant positive correlation between the level of caspase-7 activation and cytotoxicity induction by limonoids (Fig. 3.5C). In addition, the correlation coefficient analysis between the caspase-7 activity and anti-aromatase activity exited marginal significant association indicating that the limonoids' role as an

aromatase inhibitor which cannot be disregarded for potential breast cancer prevention (Fig. 3.5B).

In conclusion, these results, for the first time, demonstrated that the anti-proliferative properties of limonoids are mediated by caspase-7 dependent pathways. In addition, the cytotoxic effect was more susceptible to estrogen responsive breast cancer cells. Also, we found for the first time that the caspase-7 activation showed a trend toward improvement of aromatase inhibitory activity of limonoids. Although there was no direct correlation between anti-aromatase activity and anti-proliferative activity, the potential association between limonoids and estrogen for breast cancer prevention needs to be investigated in the future.

4. OBACUNONE EXHIBITS ANTI-PROLIFERATIVE AND ANTI-AROMATASE ACTIVITY IN VITRO BY INHIBITING THE P38 MARK SIGNALING PATHWAY IN MCF-7 HUMAN BREAST CANCER CELLS

4.1 Introduction

Despite advances in novel therapeutic agents, breast cancer still remains the second leading cause of cancer-related death in women in the United States (133). Since about two-thirds of breast cancer patients are considered to have estrogen-receptor (ER)-dependent tumors (74), most patients are treated with hormone therapy, using drugs such as tamoxifen (an ER antagonist) and aromatase inhibitors, which block estrogen activity (76). However, concerns about the safety and effectiveness of hormone therapy still exist due to both the known and unknown side effects. Plant-based foods have been reported to have preventive and treatment-related potential in the clinical applications targeting cancer, heart disease, and other human disorders (134, 135). Therefore, numerous reports have focused on the identifying agents that naturally occur in plant products and can regulate the biological pathway to reduce risk from certain types of cancer.

Cancer prevention researchers have intensely studied the biological role of limonoids in cancer prevention. In animal models, limonoids (limonin, nomilin, and obacunone) have inhibited the formation of aberrant crypt foci in azoxymethane-induced colon cancer (49, 50). Although the suppression of cell growth has been observed in diverse types of cancer cells, little is known about the exact mechanism of inhibition in cancer disease. DNA damage or cell cycle regulation error results in apoptosis, which

has typical features of morphological changes and a highly conserved biological process including stress responses and inflammatory processes. The pro-inflammatory stimuli activate the numerous intracellular signaling pathways, such as mitogen-activated protein kinases (MAPKs) (67). The MAPKs are composed of extracellular signal-related kinases (ERKs), c-Jun N-terminal kinases (c-JNKs), and p38 kinases and play a convergent role as a signal transduction mediator, including nuclear transcription factor-kappa B (NF-κB), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS), in the regulation of cancer processes (67, 136). Consequently, the NF-κB is known to regulate the anti-apoptotic proteins such as Bcl-2, cycliin D1, and cyclin-dependent kinases (CDKs) as well as COX-2 expression (137, 138). Overexpression of COX-2 has been reported in malignant tumors, and the positive correlation between aromatase and COX-2 has been reported in breast tumors in several *in vitro* and *in vivo* studies (73, 131). Therefore, an approach to inhibit the aromatase enzyme by suppressing COX-2 is considered promising to reduce risk of breast cancer.

Our previous studies and elsewhere have proved that limonoids inhibit cell proliferation in human colon adenocarcinoma (SW-480 and HT-29) (7, 50, 139), pancreatic cancer (Panc-28) (36, 55, 140), neuroblastoma (SH-SY5Y) (51, 54), and ER-independent (MDA-MB-435) (53) and ER-dependent (MCF-7) breast cancer cells (16, 53). Additionally, obacunone was reported as a glutathione S-transferase (GST) enzyme inducer as well as a neuroprotective agent by induction of heme oxygenase-1 via the p38 MAPK pathway (49, 141). The anti-proliferation and anti-inflammatory activity of obacunone was also reported in pancreatic and colon cancer cell (55, 116). However, the

regulatory mechanisms of obacunone in anti-inflammation and pro-apoptosis in breast cancer are unknown. To evaluate the role of obacunone as potent anti-cancer agent, in the present study, we investigated the effect on anti-proliferation and anti-aromatase activity of obacunone in MCF-7 breast cancer cells. Additionally, the mechanism of obacunone-induced inhibition of MCF-7 cell growth were explored using possible downstream pathways such as cell cycle arrest, apoptosis, MAPK, COX-2, and NF-κB. Furthermore, the uptake level of obacunone into cells was measured by HPLC and confirmed by LC-MS analysis.

4.2 Materials and Methods

4.2.1 Cell Lines and Culture Conditions

Human breast cancer (MCF-7) and non-malignant immortalized breast epithelial (MCF-12F) cell lines were obtained from the American Type Culture Collection. The MCF-7 cells were cultured in DMEM medium containing 10% (v/v) fetal bovine serum, 200 U/ml penicillin G, and 200 µg/ml streptomycin. The MCF-12F cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium with 20 ng/ml of epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% (v/v) chelex-treated horse serum, and then the cells were incubated at 37 °C with 5% CO_2. Tested limonoids were dissolved in DMSO for all the *in vitro* studies (except the *in vitro* aromatase activity assay), and the same concentration of DMSO (< 0.2%) was used in the control cell group.

4.2.2 Cytotoxicity Assay

The MCF-7 or MCF-12F cells (1×10^4 / well) were seeded into a 96-well plate and allowed to adhere overnight. Cells were treated with different concentrations (10, 50, and 100 μM) of either obacunone or obacunone glucoside for 24, 48, and 72 h. Tamoxifen, a well-known anti-estrogen drug used for breast cancer therapy, was used as a positive control. After the various time points, 10 μl of the MTT (5 mg/ml) reagent were added to each well and incubated for 2 h at 37 °C. After 2 h incubation, the media was removed and 200 μl of DMSO were added to dissolve the purple formazan. The absorbance was measured by an ELISA microplate reader (BioTek Instruments, Winooski, VT) at 570 nm. The MTT reduction was calculated as follows: (A of experimental group / A of control group) × 100.

4.2.3 Flow-Cytometry Analysis

Exposure of anti-cancer agents may induce apoptosis following cell cycle arrest, in order to demonstrate the mechanism of anti-proliferation, obacunone was used to analyze the cellular DNA content using flow-cytometry. The MCF-7 (1×10^6) cells were plated into 100 mm culture dishes and allowed to attach overnight. Cells were incubated with a serum-free media for 24 h to synchronize and were treated with 10, 50, or 100 μM of obacunone for 72 h. After the 72 h incubation, cells were harvested and hypotonically lysed with a DNA staining solution (50 μg/ml propidium iodide, 200 μg/ml DNase-free RNase, 4 mM Sodium Citrate, 0.1% Triton X-100). The fluorescence was analyzed by

FACS Calibur (Becton Dickinson FACScan, San Jose, CA). The percentage of cells in each cell cycle was determined using ModFidLT V3.2.

4.2.4 Western Blot

Obacunone-treated MCF-7 cells were collected after 72 h of incubation, and total protein was extracted with a lysis buffer (150 mM NaCl, 10 mM Tris-Cl [pH 7.2], 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA) with a protease inhibitor cocktail (GenDEPOT, TX). Nuclear extracts were prepared using a nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. The total or nuclear protein concentration was determined by the bicinchoninic acid method (Pierce, IL). Equal amounts (25 μg per lane) of samples were subjected to 10% SDS-PAGE and transferred onto a PVDF membrane by semi-dry transfer (Bio-Rad, Hercules, CA). The membranes were blocked in 5% non-fat skim milk in a Tris-buffered saline containing 0.1% Tween 20 (TBS-T) buffer for 1 h and then incubated overnight at 4 ºC with the following primary antibodies: anti-cyclin D1, anti-CDK4, anti-p27, anti-p21, anti-p15, anti-caspase 7, anti-cleaved caspase 7, anti-COX-2 (Cell Signaling Technology, Beverly, MA), anti-Bcl2, anti-Bax, anti-p38, anti-phospho p38, anti-phospho ERK, anti-phospho c-JNK, anti-NF-κB (RelA, p52, and p50), anti-beta-actin, and anti-GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Subsequently, the membrane was washed with a TBS-T buffer and probed with a horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. The protein band signal was detected by a chemiluminescence (ECL) detection kit (Amersham Bioscience, NJ), and the image of

the signal was visualized using the LAS 4000 mini-imaging system (Fuji Life Sciences, CT).

4.2.5 In Vitro Aromatase Activity Assay

Aromatase inhibition was evaluated using a CYP19/MCF high-throughput screening kit (BD Biosciences, Woburn, MA). The fluorescent intensity of fluorescein, the hydrolysis product of 7-Methoxy-4-trifluoromethyl coumarin (MFC), by aromatase was measured according to the manufacturer's protocol. The obacunone was dissolved in acetonitrile since DMSO has shown to have an effect in CYP19 inhibition. Briefly, obacunone and ketoconazole (as a positive control inhibitor) were placed in 96-well black microtiter plates (BD FalconTM) by serial dilution. The NADPH-Cofactor mixture (8.1 μM NADP+, 0.4 mM MgCl2, 0.4 mM glucose-6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase) was added in each well and pre-incubated for 10 min at 37 °C. After the 10 min pre-incubation of the plate, the enzyme and substrate mixture (25 μM MFC and 1.5 pmol/well CYP19) was added to incubate for an additional 30 min at 37 °C. The fluorescence of metabolite was measured at excitation at 405 nm and emission at 530 nm. Experiments were carried out in duplicate on two separate occasions.

4.2.6 Extraction of Obacunone from MCF-7 Cell Pellet

The cells (1×10^6cells) were treated with obacunone (100 μM) and incubated for 0, 12, 24, 48, and 72 h. After the various time points, the pellet was collected and

washed with PBS. To remove fatty material from pellet, 1 ml of hexane was added into each tube containing pellet. After 30 min sonication, the supernatant was discarded. This process was repeated three times. The pellet was mixed with acetone (500 μl) and then, the mixture was loaded onto activated C18 column cartridge. The acetone eluate was collected into new tube and dried for HPLC analysis.

4.2.7 Quantification and Identification of Obacunone

Extracted obacunone was quantified and identified by HPLC and LC-MS analysis according to method described in Section 2.

4.2.8 Statistical Analysis

All data were presented as means ± S.E., and the statistical significance of the data was evaluated using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) by the SPSS 16.0 program (Chicago, IL). $P < 0.05$ was considered significant.

4.3 Results

4.3.1 Induction of Cytotoxicity in Human Breast Cancer Cells

To determine the cytotoxic effect of obacunone and OG on either MCF-7 or MCF-12F cells, mitochondrial dehydrogenase activity was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (100). Both obacunone and OG significantly inhibited the cell proliferation in a dose-dependent manner. As

shown in Fig. 4.1, treatment with obacunone and OG resulted in 44% ($P < 0.01$) and 25% ($P < 0.01$) at 200 μM for 72 h, respectively. However, treatment with the same concentration of obacunone and OG for 72 h did not show cytotoxicity in MCF-12F non-malignant cells. Tamoxifen was used as a positive control and significantly inhibited the MCF-7 and MCF-12F at 25μM (Fig. 4.1).

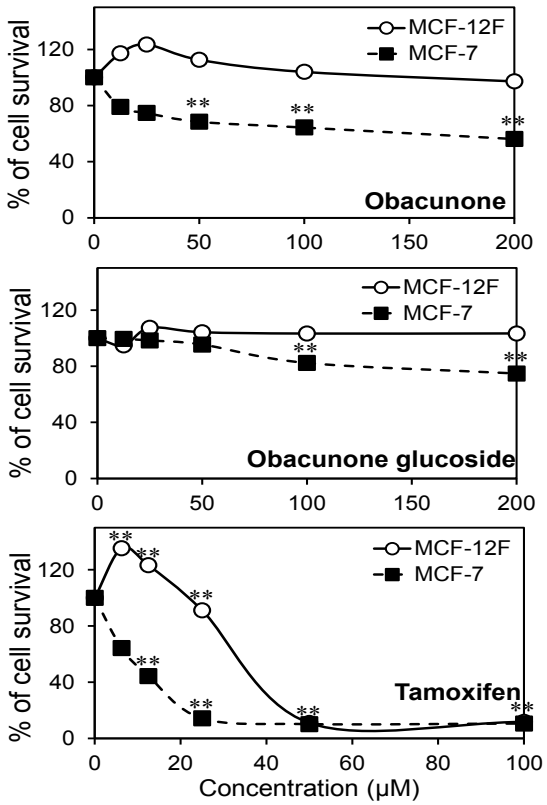


Fig. 4.1 Effect of obacunone and obacunone glucoside on cell proliferation. MCF-7 and MCF-12F cells were treated with 0 (DMSO, as vehicle control), 25, 50, or 100 μM for 72 h. Dose-dependent effects of (A) obacunone, (B) obacunone glucosides, or (C) tamoxifen (positive control) were examined by MTT assay. Values were expressed as mean ± S.E. of three independent experiments. The significant difference was compared with control by ANOVA (* $P < 0.05$, ** $P < 0.01$).

4.3.2 Cell Cycle Distribution and Expression of Cell Regulatory Protein

Cell cycle arrest is often observed before cells undergo apoptosis due to DNA damage (142). Since obacunone showed to be more effective in inhibition, reaching a maximal cytotoxic of 44%, followed by OG at 25%, in MCF-7 cell growth, we investigated the effect of obacunone on cell cycle distribution in MCF-7 cells by flow cytometry. In this experiment, we used limonoids until 100 μM (as the maximum concentration) since the underestimated growth inhibitory effect by MTT assay (143) is still enough to show significant inhibition. Treatment with obacunone caused significant growth arrest of 77% ($P < 0.05$) in the G0/G1 phase of the cell cycle and an increase of 18% ($P = 0.07$) in the Sub G1 phase (Fig. 4.2A). Therefore, we examined the levels of G0/G1 cell cycle regulatory proteins such as cyclin D1, CDKs, and CDK inhibitors. As shown in Fig. 4.2B, the expressions of cyclin D1 and CDK4 protein levels were 70% and 30% down-regulated, respectively, in a dose-dependent manner, whereas the cell cycle inhibitory proteins, such as $p27^{Kip1}$, $p21^{Waf/Cip1}$, and $p15^{INK4B}$, were up-regulated in obacunone (100 μM)-treated MCF-7 cells for 72 h (Fig. 4.2C). It is worth noting that trends to express the level of CDK inhibitors depended on the degree of cytotoxicity of obacunone. For instance, $p15^{INK4B}$ showed late response followed by expression $p27^{Kip1}$ and $p21^{Waf/Cip1}$ (Fig. 4.2C). Beta-actin level was confirmed for an equal amount of protein loading in each experiment.

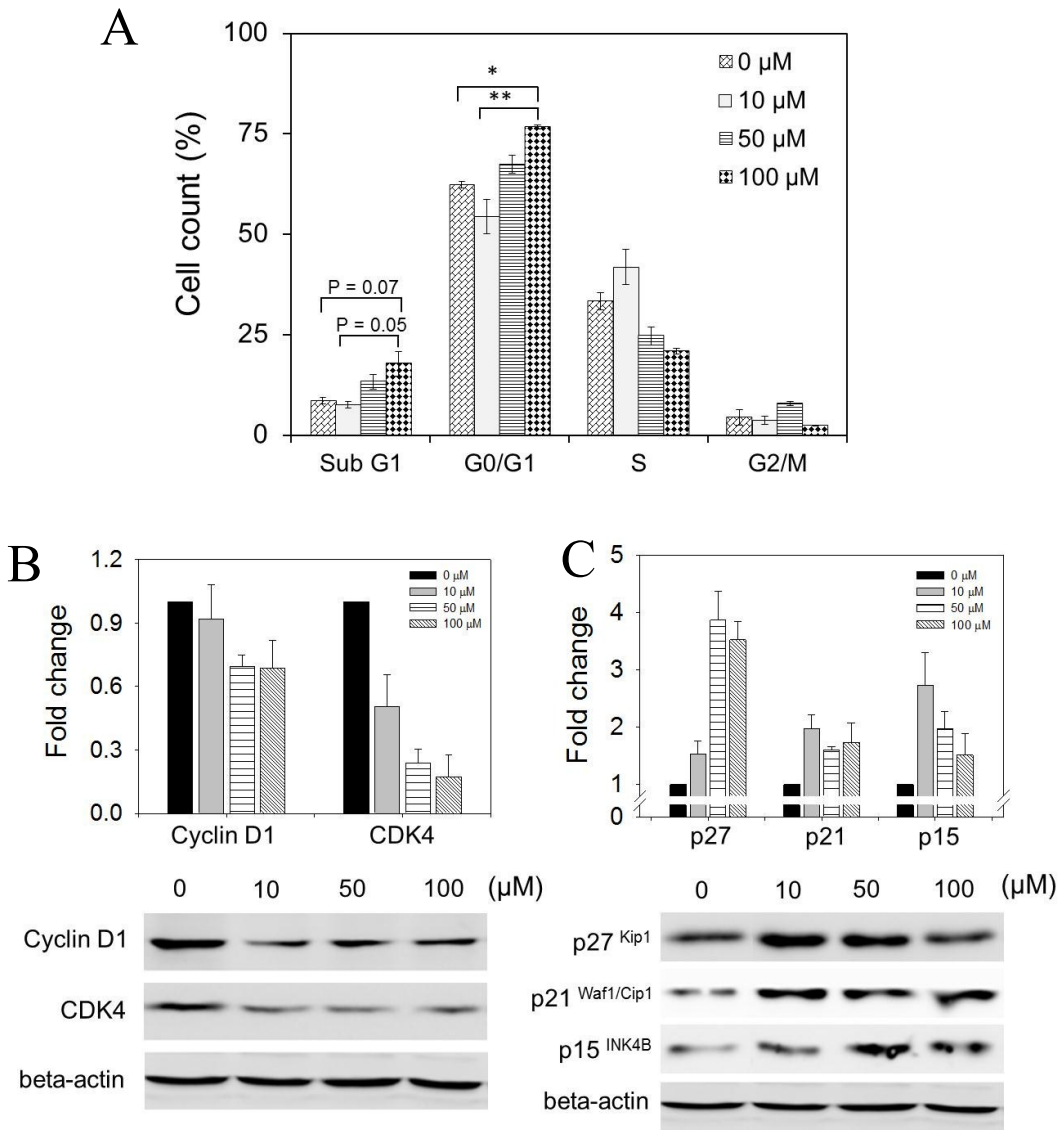


Fig. 4.2 Obacunone induces G1 cell cycle arrest in MCF-7 cells. The cells were treated with 0, 10, 50, and 100 μM of obacunone for 72 h. (A) The distribution of cells in each cell cycle phase was measured by flow cytometry. The data are indicated as mean ± S.M.E. from three independent experiments. Statistically significant differences between the groups are indicated as * $P < 0.05$ or ** $P < 0.01$. The expression level of G1 cell cycle related proteins was measured by western blot using (B) anti-cyclin D1, anti-CDK4, and (C) anti-CDK inhibitors. All bands were expressed as relative intensity using densitometry. The bars represent standard errors.

4.3.3 Effect on Apoptosis and Inflammation-Signaling Molecules

To further determine whether obacunone induces apoptosis as well as G0/G1 cell cycle arrest in MCF-7 cells, immunoblot analysis was performed with various apoptosis markers such as anti-apoptotic Bcl2, pro-apoptotic Bax, and pro- and active caspase-7 (Fig. 4.3A). Significant decreases in expression levels of the Bcl2/Bax ratio, which is associated with activation of apoptosis, were observed in obacunone-treated breast cancer cells. The Bcl2/Bax ratio was inhibited by 40, 57, and 60% using obacunone at 10, 50, and 100 μM, respectively. Since caspase-3 is nonfunctional in MCF-7 cells, the caspase-7 was evaluated as an apoptosis marker in downstream cascade by apoptosis signaling. When 100 μM of obacunone were used to treat MCF-7 cells, an 80% increase in expression level of active caspase-7 was detected.

The family of MAPKs is associated with various diseases, and inflammation has been reported in several *in vitro* and *in vivo* studies. Specifically, since the overexpression of p38 and JNK were considered as markers of breast tumorigenesis (136), we tested whether obacunone-induced cell cycle arrest/apoptosis is associated with the alteration of the MAPK pathway in MCF-7 cells. As shown in Fig. 4.3B, treatment with obacunone resulted in 58% ($P < 0.01$) inhibition of phospho-p38 levels without change in the total p38 protein level. Unlike phospho-p38, obacunone treatment did not affect the level of phospho-ERK and phospho-c-JNK at the overall range. These results suggest that attenuated phospho-p38 expression level correlates with cell cycle arrest and apoptosis using obacunone in MCF-7 human breast cancer cells.

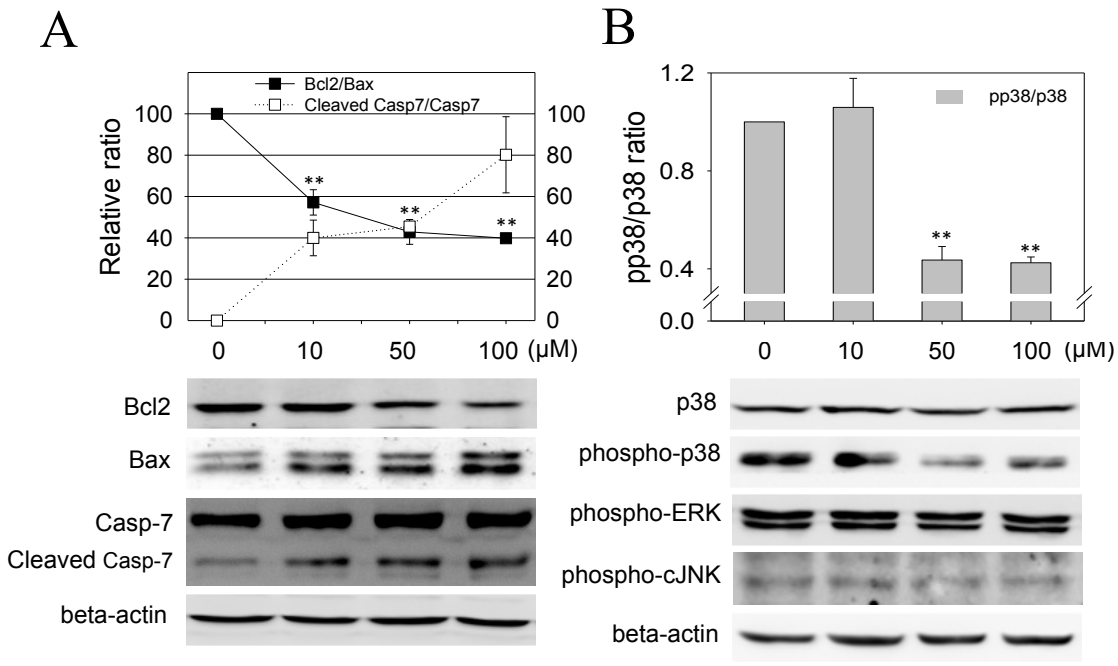


Fig. 4.3 Obacunone induces apoptosis through inhibition of p38 MAPK in MCF-7 cells. The cells were treated with 10, 50, and 100 μM obacunone or vehicle DMSO for 72 h. Cell lysates (25 μg) were analyzed by western blotting for (A) Bcl2, Bax, caspase-7 (casp-7), cleaved casp-7; and (B) p38, phospho-p38, phospho-ERK, phospho-cJUN, and beta-actin. Values were normalized to beta-actin. The relative ratios were determined as Bcl2 to Bax, cleaved casp-7 to casp-7, and phospho-p38 to p38. The data were represented as mean ± S.E. (n = 3) with statistically significant differences (** $P < 0.01$).

4.3.4 Inhibition of Aromatase Activity using Obacunone

Increased estrogen level and aromatase enzyme activity was detected in breast cancer tissue (73). Therefore, we determined whether obacunone has any aromatase inhibitor activity. *In vitro* MFC aromatase screening assay was used and determined the converted fluorescence substrate, 7-hydroxy-4-trifluoromethyl coumarin, by CYP19. As shown in Fig. 4.4, obacunone significantly inhibited the aromatase enzyme activity at

range 0.74 to 60 μM ($P < 0.01$). At the dose of 60 μM, the aromatase activity was inhibited 65.52% ($P < 0.01$) at maximum range. The required obacunone for 50% aromatase inhibition (IC50) was 28.04 μM. The ketoconazole, as a positive control, decreased aromatase activity with IC50 values of 0.85 μM. This value was consistent with the supplier.

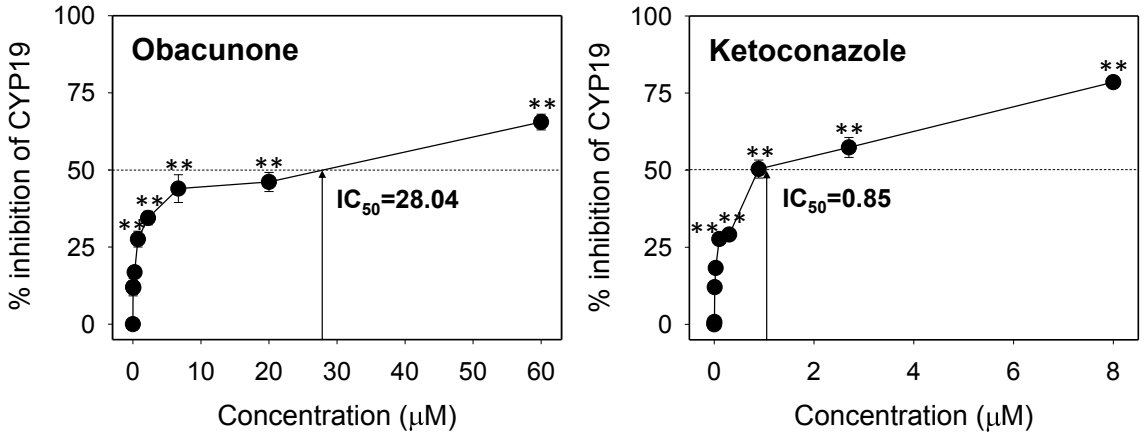


Fig. 4.4 Inhibition of aromatase (CYP19) activity by obacunone and ketoconazole (positive control inhibitor). Different concentrations of obacunone (0 to 60 μM by one-third serial dilution) and ketoconazole (0 to 8 μM by one-third serial dilution) were incubated with 7-methoxy-4-trifluoromethyl coumarin. The aromatase activity was measured as described in the Materials and Methods section. Values were expressed as means ± S.E. (n = 4 for obacunone or 3 for ketocozazole). The significant difference was compared with control by ANOVA (** $P < 0.01$).

4.3.5 Inhibition of NF-κB and COX-2

Evidence suggests that COX-2 regulates the aromatase activity through transcriptional regulation (131). Therefore, the suppression of aromatase activity by

obacunone led us to determine the COX-2 expression level. In addition, constitutive activation of NF-κB and COX-2 expression is known to be a critical factor for activating inflammation. Also, the down-regulated aromatase activity is known to inhibit the MAPK pathway and attenuate the activation of NF-κB. Therefore, the down-regulation of p38 MAPK by obacunone led us to investigate the NF-κB translocation activity from the cytosolic to the nucleus in MCF-7 cells. The measurement of nuclear fraction of MCF-7 cells was analyzed by western blot for NF-κB (Fig. 4.5A). In this experiment, all three forms of NF-κB were down-regulated, and the translocation inhibition levels were 37% ($P < 0.05$) of RelA, 63% ($P = 0.05$) of p52, and 45% ($P < 0.05$) at 100 μM for 72 h (Fig. 4.5A).

Since NF-κB has been reported as a transcriptional regulator of COX-2, experiment was conducted to determine whether obacunone treatment in MCF-7 involves an anti-inflammatory mechanism through COX-2 inhibition. As shown in Fig. 4.5B, obacunone treatment resulted in significant inhibition, and the inhibition rate was 60% ($P < 0.01$) at 100 μM followed by 30% ($P < 0.05$) at 50 μM.

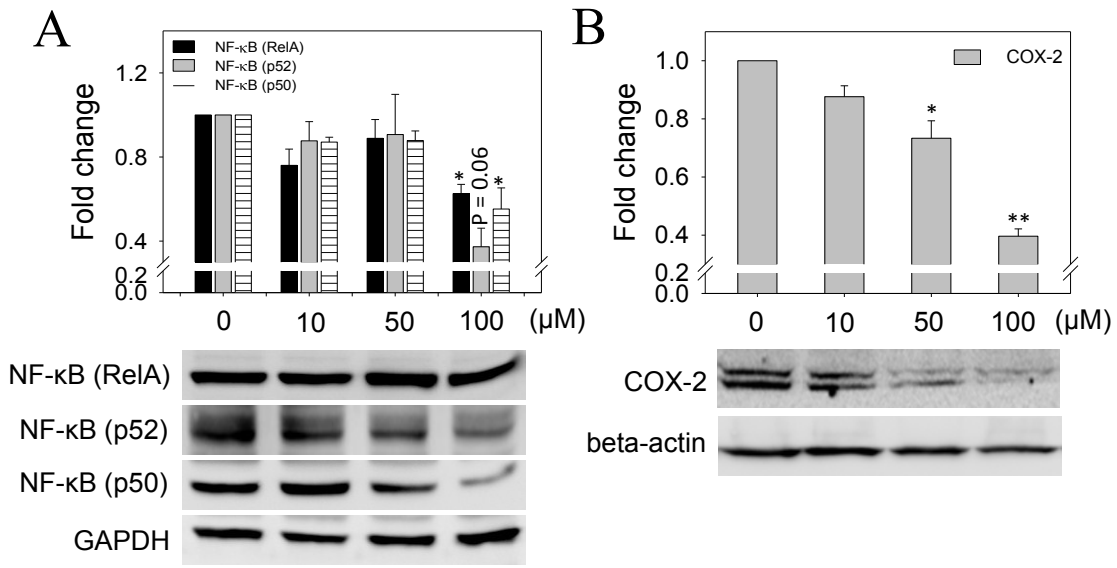


Fig. 4.5 Effect of obacunone on NF-κB activity and COX-2 expression. Cells were treated with indicated concentration of obacunone for 72 h, and the total protein lysates were analyzed by western blot with (A) COX-2, MMP-9, and beta-actin. (B) Nuclear extracts were analyzed by western blot with RelA, p52, p50, or GAPDH, which confirmed equal loading. The data were represented as mean ± S.E. (n = 3) with statistically significant differences (* $P < 0.05$, ** $P < 0.01$).

4.3.6 In Vitro Uptake Rate of Obacunone

To verify the absorption level and the stability of obacunone into MCF-7 cells, we determined the uptake level of obacunone in the cell pellet by HPLC analysis. As shown in Fig. 4.6A, about 13% obacunone was absorbed into MCF-7 cell as fast as 12 h incubation. However, the absorption level was constant during 72 h. Further, the stability of obacunone during the incubation was determined using LC-MS analysis. The LC-MS result indicated that obacunone is stable in the cell pellet for 72 h (Fig. 4.6B).

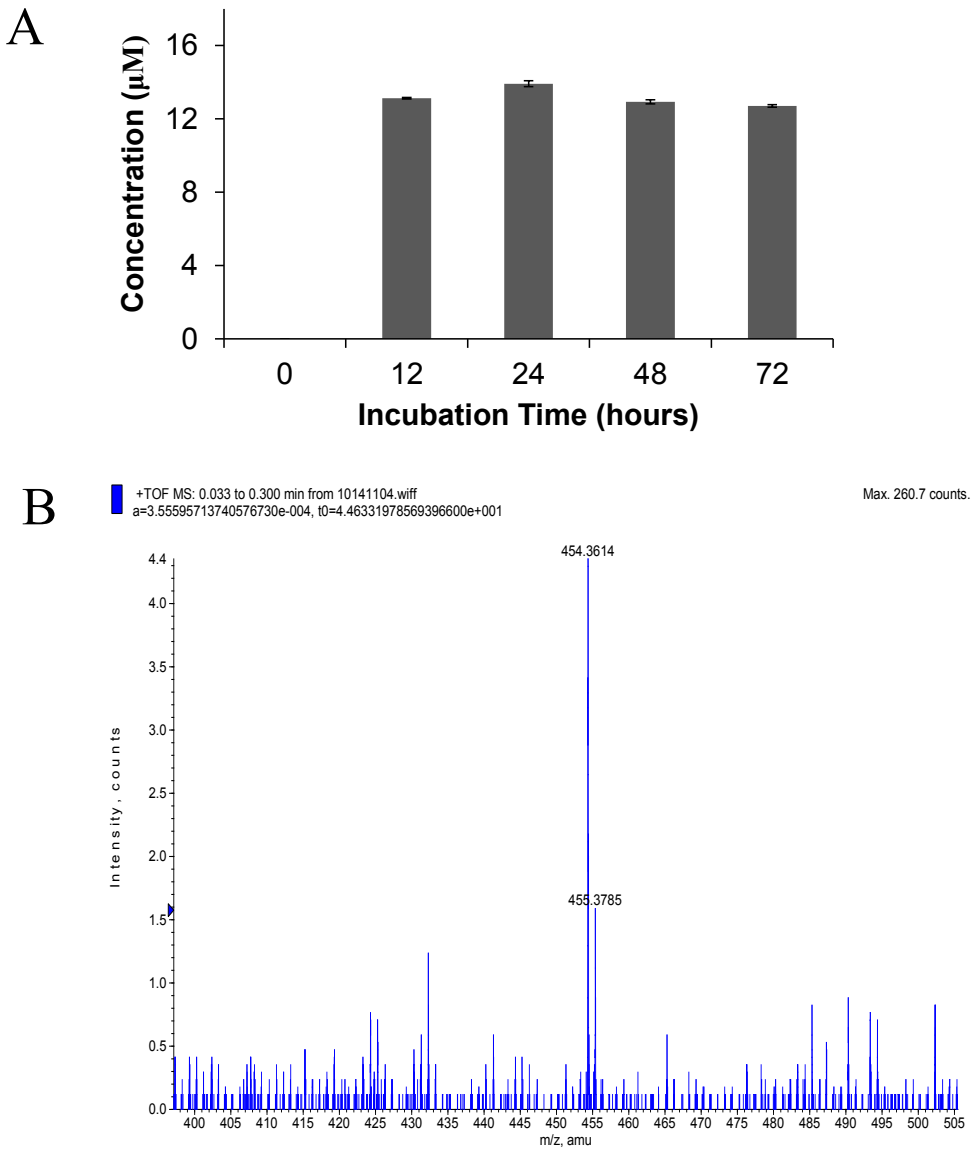


Fig. 4.6 Uptake rate of obacunone in MCF-7 cells. The Cells were treated with obacunone (100 µM) during indicated time period, and the cell pellet was extracted with chloroform as described in the Materials and Methods section. (A) The obacunone concentration present in pellet was quantified by HPLC. (B) The compound stability was measured using 72 h incubated-pellet fraction by LC-MS.

4.4 Discussion

Development of medicinal drugs has shown a trend toward the use of naturally occurring products as therapeutic agents or disease preventatives due to safety concerns. For instance, several clinical researchers have reported side effects, such as increased risk of cardiovascular disease, leukopenia, and liver toxicity (144), associated with well-known hormonal treatments, such as tamoxifen and aromatase inhibitor. Therefore, the use of natural compounds from plant product such as limonoids, abundantly present in citrus fruits, has been investigated due to their synergistic effect as disease-preventive agents (9, 12, 145).

In this current study, we demonstrated for the first time that obacunone, a natural compound from citrus, significantly inhibits estrogen-dependent breast cancer cell proliferation through inhibition of the p38 MAPK signaling pathway. The stimulated cytotoxicity of obacunone resulted in cell cycle arrest as well as apoptosis, or more specifically, a significant suppression of aromatase activity affected by suppression of NF-κB and COX-2 expression. The cytotoxic effect of obacunone did not affect non-malignant breast cell growth.

Lam et al. (146) demonstrated that obacunone has potential as a GST enzyme inducer in the forestomach, liver, and small intestine in animal models. Later, the effect was elaborated in other *in vivo* studies using rat models by demonstration enhancing detoxifying enzymes such as GST and QR by obacunone, thereby suppressing the formation of aberrant crypt foci (ACF) in azoxymethane (AOM)-induced colon cancer in rats (49, 147). In addition, previous studies from Patil's lab and others have shown the

cytotoxic effect in neuroblastoma, colon, pancreatic, and leukemia cancer cells (51, 55, 116, 148). The present study showed the significant inhibition of MCF-7 cell proliferation through the G0/G1 phase cell cycle arrest and apoptosis (Fig. 4.1-4.3).

The MAPK pathway is one of the important regulatory mechanisms to regulate cell growth and inflammation (67, 149). Accumulating data reported that the overexpression of MAPKs has been associated with malignancy of breast tissue (136, 150). This led us to consider the MAPK as a potential convergent target for breast cancer prevention using obacunone. Present result clearly showed the inhibition of p38 MAP kinase by obacunone at 50 μM and over (Fig. 4.3B). However, we did not see any significant change in other MAPK family members (Fig. 4.3B). This result was surprising because our recent study showed that obacunone was a significant inducer of p38 MAPK activity in a cardiovascular model (120). This contradictory role of obacunone in breast cancer and the cardiovascular system supports the notion that the major breast cancer treatment drugs, such as tamoxifen, letrozole (AI), and anastrozole (AI), increase the risk of cardiovascular diseases (144, 151). The increased estrogen hormone being linked with protective cardiovascular disorder obviously explains this contradiction. However, present data are not sufficient enough to explain the complex cross-talk between the several signaling pathways bridging breast cancer and cardiovascular disease. These results suggest that obacunone may have an estrogen-lowering effect in ER-dependent cancer cells and may lead to suppression of cell proliferation through attenuated p38 MAPK phosphorylation.

Since the imbalanced estrogen hormone causes breast cancer, a higher estrogen and estrogen-receptor level is considered the key biomarker of the risk of breast cancer (73, 151). Aromatase (CYP19) is one of the members of the cytochrome p450 superfamily, responsible for synthesizing estrogen from androgen (130). Therefore, blocking aromatase enzyme activity was considered a target mechanism to prevent breast cancer. Obacunone exhibited significant aromatase enzyme inhibition *in vitro* (Fig. 4.4). We also showed the significant down-regulation of COX-2 using treatment with obacunone in MCF-7 cells (Fig. 4.5B). This is consistent with previous reports of the positive correlation between COX-2 and aromatase enzyme activity (152). Moreover, the suppression of aromatase and COX-2 led to increased levels of cell cycle regulatory genes such as p21, p27, and p15 (Fig. 4.2B and 4.2C). Present data support studies that the decreased Bcl2 and cyclin D1 and increased cyclin inhibitors (p21 and p27) be induced by deprivation of estrogen level (153, 154).

The transcriptional activity of NF-κB has been implicated as the key mediator in regulation of cell proliferation and immune and inflammatory processes (137). The NF-κB/Rel family member following NF-κB1 (p105/p50), NF-κB2 (p100/p52), p65 (RelA), RelB, and c-Rel is one of the crucial transcription factors (155). The typical NF-κB complex is the p65/RelA heterodimer, which stimulates transcription, whereas the p50 and p52 subunits serve primarily to bind to DNA (156). The distinct role of evidences suggests that the expression of pro-inflammatory markers COX-2 and iNOS is responsible for the transcriptional activity of NF-κB because of the presence of the putative NF-κB binding site on the COX-2 promoter region (157). Present data

demonstrated the inhibition of different types of NF-κB translocation from the cytosol to the nucleus using obacunone (Fig. 4.5A). These data support the evidence of the direct entanglement of signaling regulation between NF-κB, COX-2, and MAPK pathways by extracellular stimuli, stress signals, or inflammatory processes. The present study demonstrate that the block in cell cycle progression and apoptosis may be due to the inhibition of p38 MAPK and continued suppression on its downstream pathways, such as NF-κB, COX-2, the aromatase enzyme, and CDK inhibitors.

In conclusion, the cancer preventive effect of obacunone was demonstrated. Furthermore, inhibition of p38 MAPK, an upstream target to regulate the downstream pathways such as NF-ΚB, COX-2, CYP19, cyclin D1, and CDK inhibitors in human breast cancer cells (Fig. 4.7), was demonstrated. Further *in vivo* or clinical studies may be needed, but the findings in this research may be useful for understanding the role of natural products as cancer prevention mechanisms in breast cancer.

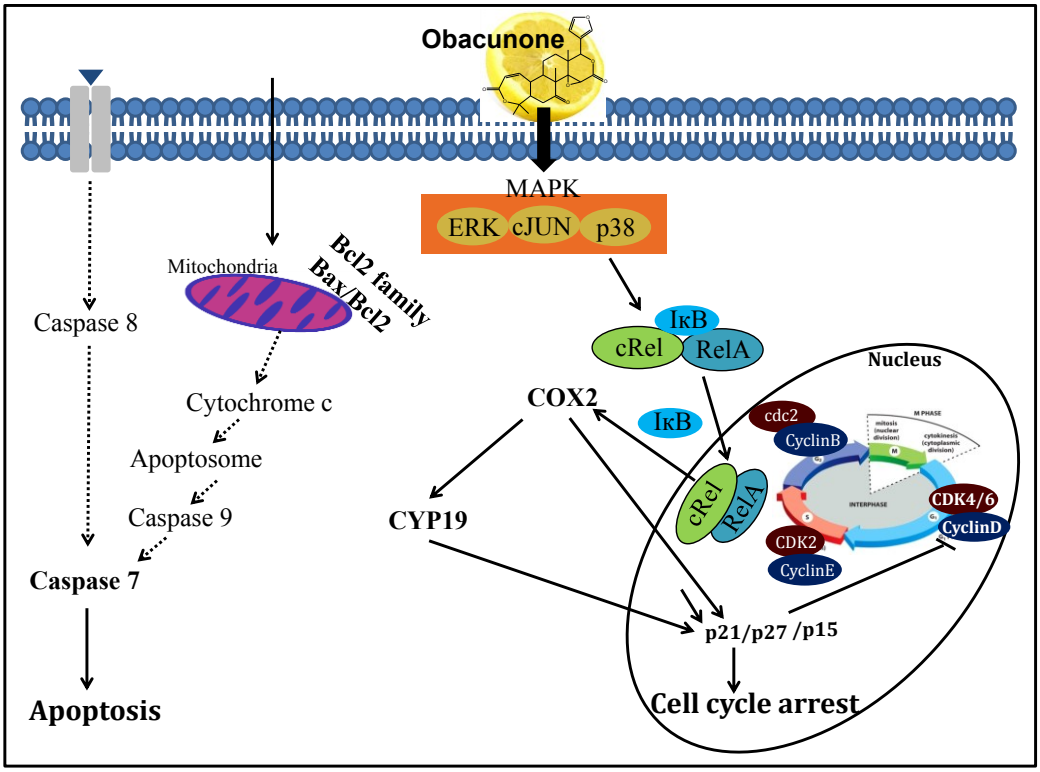


Fig. 4.7 Proposed model for signaling pathway leading to growth inhibition by obacunone in estrogen-responsive breast cancer (MCF-7) cell. The obacunone inhibits p38 phosphorylation, which activates NF-κB translocation from the cytosolic to the nucleus. The positive regulatory downstream targets COX-2 and CYP19 are attenuated due to the inhibition of NF-κB activation. The aromatase inhibition by sequential inhibitory effect decreases the cyclin D and CDK 4/6. Sequentially, the increased CDK inhibitors (p21, p27, and p15) inhibit the cell cycle progression. In addition, the decreased Bcl2/Bax ratio, which indicated mitochondrial dysfunction and activated caspase-7, indicates the apoptosis in MCF-7 cells.

5. STRUCTURE-FUNCTION RELATIONSHIPS OF LIMONOIDS ON P38 MAP KINASE ACTIVITY IN HUMAN AORTIC SMOOTH MUSCLE CELLS*

5.1 Introduction

Citrus fruits contain different groups of health promoting compounds such as flavonoids, ascorbic acid, vitamins, carotenoids, limonoids, and pectin (6). Among these health promoting compounds, tetranorterpenoid derivatives called limonoids are unique bitter compounds mostly present in citrus and neem (158). Accumulating evidences suggest that limonoids reduce the risk of diverse spectrum of diseases including cancer (15, 49, 52), cardiovascular (41, 159), and neurodegenerative disease (51). Previous studies suggest that the presence of furan and an intact A-ring of limonoids are associated with increasing chemopreventive activity in cancer cells (47, 63). While current evidences suggest that chemical structure of citrus limonoids play a critical role in reducing risk of certain cancers, very little information is available about the effects of limonoids on cardiovascular cells.

Pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and IL-12, and tumor necrosis factor-α (TNF-α) play an important role in the regulation of inflammatory mechanisms in mammalian cells (67, 68). However, overproduction of these cytokines have been implicated in inducing a variety of chronic cardiovascular

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diseases such as atherosclerosis, coronary artery disease, arrhythmia, heart failure, hypertension, and ischemia (68, 70). Since the TNF-α is regulated by the p38 mitogen-activated protein (MAP) kinase signalling cascade (160), the regulation of the p38 MAP kinase signal transduction pathway is a significant target for anti-inflammatory therapy. Indeed, accumulating evidences suggest that up-regulation of p38 is associated with the development of human cardiovascular disease (161, 162). Therefore, p38 MAP kinase is one of the important central targets for therapeutic approaches in controlling human cardiovascular disease. Although synthetic p38 inhibitors are commercially used in cardiovascular disease treatments, clinical trials are still ongoing due to side effect (mainly hepatotoxicity) and the interruption of other signalling pathways (163). Therefore, there is a critical need for potential natural bioactive compound, such as limonoids, which distinctly suppress p38 MAP kinases without any side effects.

We have previously shown that limonoids decrease proliferation of cancer cells (16, 51, 52, 118). Several studies have demonstrated that p38 MAP kinase pathway is one of the primary determinants in modulating cell cycle signalling and inflammation (67, 70). Hence, we have hypothesized that limonoids will have an effect on p38 MAP kinase activity in cardiovascular cells. Therefore, the present study was aimed to determine the effect of citrus limonoids on p38 MAP kinase activity in human aortic smooth muscle cells (HASMCs). In addition, structure-function relationships were investigated using seven limonoids to determine which functional groups of limonoids and/or how many numbers of ring structures in limonoids are essential in modulating p38 MAP kinase activity.

5.2 Materials and Methods

5.2.1 Materials

HASMCs, smooth muscle cell medium (SMCM), and SMC growth supplement were purchased from ScienCell (Carlsbad, CA, USA). Super Signal West Femto chemiluminescence substrate and BCA protein assay kit were purchased from Pierce (Pierce, IL, USA). Anti-p38 MAPK (sc-7972), anti-phospho p38 MAPK (sc-7973), and anti-β-actin (sc-81178) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human TNF-α was obtained from R&D systems (Minneapolis, MN, USA). A chemiluminescence (ECL) detection kit was obtained from Amersham Pharmacia (Piscatawa, NJ, USA), and PVDF membrane was obtained from Bio-Rad (Hercules, CA, USA). All other reagents were analytical grades and obtained from Fisher Scientific (Atlanta, GA, USA).

5.2.2 Cell Culture

The HASMCs were cultured in SMCM containing a growth supplement at 37 ºC in a humidified atmosphere of 5% CO2 in an incubator. HASMCs were grown to 80-90% confluence, and cell passage 4-6 was used for all the experiments. For treatments, cells were serum starved for 24 h. After the 24 h incubation, the cells were provided with fresh media that contained limonoids at different concentrations (12.5 to 50 mM) and the incubation was continued for 72 h without changing the media. Control cell group was treated with appropriate concentration of DMSO, which was used for solubilizing the limonoids.

5.2.3 Western Blot

HASMCs were collected at the end of appropriate incubation period and the cell pellet was washed with PBS containing phosphatase inhibitors and lysed in a protein lysis buffer containing 150 mM NaCl, 10 mM Tris-Cl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA and with a protease inhibitor cocktail (GenDEPOT, TX, USA)). Protein concentration was determined by the BCA method. Equal amount of protein (30 μg) was separated by 10% SDS-PAGE, and the gel was blotted onto a PVDF membrane by semi-dry transfer (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat dry milk in a Tris-buffered saline containing 0.1% Tween 20 (TBST) buffer for 1 h and then incubated overnight at 4 ºC with primary mouse IgG anti-p38 (1:800) and primary mouse IgM anti-pp38 (1:500). After being washed four times for 5 min with the TBST buffer, the membrane was continuously incubated with a horseradish peroxidase conjugated secondary antibody (1:2500). The protein band signal was detected by either ECL (β-actin and p38) or Super Signal West Femto chemiluminescence (pp38). To reprobe the membranes with an anti-β-actin antibody, membranes were incubated with a stripping buffer (100 mM 2-Mercaptoehtanol, 62.5 mM Tris-HCl (pH 6.7), 2% SDS) for 30 min at 50 ºC. Sequentially, blocking the membrane with 5% non-fat dry milk in TBST buffer for 1 h was done, and then the immunodetection protocol was repeated. The protein levels and densitometry were analyzed by LAS 4000 mini (FUJI Film, Tokyo, Japan). Western blot analyses of HASMCs proteins followed by quantification were performed three times for each sample and the resulting mean ± standard error of mean (S.E.) was calculated.

5.2.4 Statistical Analysis

All data were presented as means ± S.E. of at least three independent experiments. The data was determined using one-way analysis of variance (ANOVA) with Student's t-test by the SPSS 16.0 program (Chicago, IL). $P < 0.05$ was considered significant.

5.3 Results

5.3.1 Differential Inhibition of p38 MAP Kinase Activity by Parent and Defuran Limonoids

The typical structures of citrus limonoids consist of five rings termed as A, A', B, C and D (Fig. 5.1 and 5.2). Nomilin differs from limonin in the absence of the A' ring and furan ring has been removed from nomilin and limonin to generate defuran nomilin and defuran limonin, respectively (Fig. 5.1 top panel). Nomilin and deacetyl nomilin consists of saturated seven- membered ring with one asymmetric center at C-1 position (Fig. 5.2A). Moreover, C-1 position was attached with acetoxy group in nomilin whereas hydroxyl group present in deacetyl nomilin. As seen in Fig. 5.2A, methyl nomilinate is similar to nomilin except 'A' ring is opened and C-3 position is methylated. Obacunone has unsaturated double bond between C-1 and C-2 position.

To investigate the role of furan ring on p38 MAP kinase activity, HASMCs were treated with limonin, nomilin, defuran limonin and defuran nomlin at various concentrations. At 50 mM concentration, limonin, nomilin, and defuran nomilin exhibited significant decrease in p38 MAP kinase activity by 19%, 38% and 17%,

respectively; whereas defuran limonin did not show any significant effect on p38 MAP kinase (Fig. 5.1). Nomilin that possesses seven-membered A ring with acetoxy group showed significant suppression of p38 MAP kinase activity when compared with the defuran nomilin (38% versus 17%, respectively) (Fig. 5.1B).

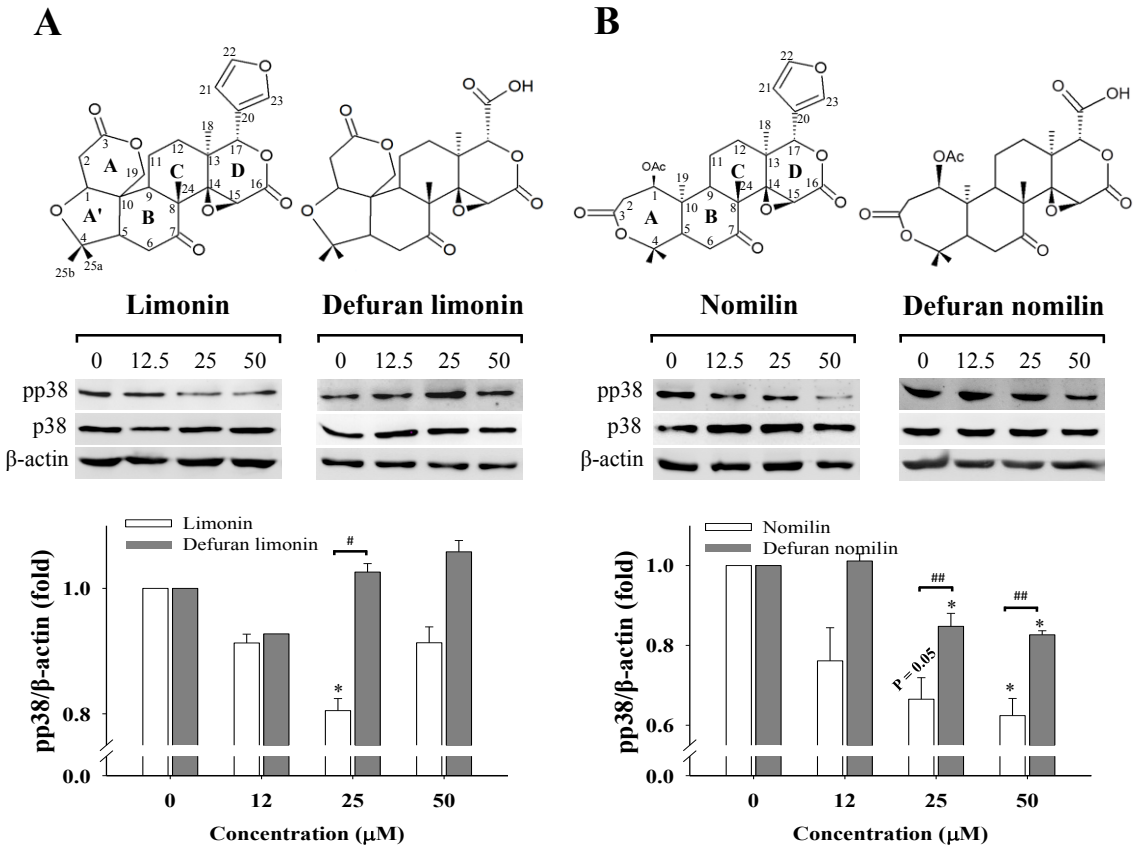

Fig. 5.1 Effect of furan moiety on p38 MAP kinase activation in HASMCs. (Top panel) Structure of purified and modified at the side chains of limonoids. The cells were incubated with various concentrations of (A) limonin and defuran limonin, and (B) nomilin and defuran nomilin, for 72 h. p38 MAP kinase activity was evaluated by western blots as described in the Methods section. β-actin was used as the loading control. The bar graphs represent the mean ± S.E. of three independent experiments. * $P < 0.05$, # $P < 0.05$, ## $P < 0.01$ by ANOVA.

5.3.2 Differential Effects on the p38 MAP Kinase Activity by Seven-Membered A Ring Limonoids

To further determine the effects of substitution of seven-membered with different functional groups on p38 MAP kinase activity, four different A ring compounds at various concentrations were tested on HASMCs.

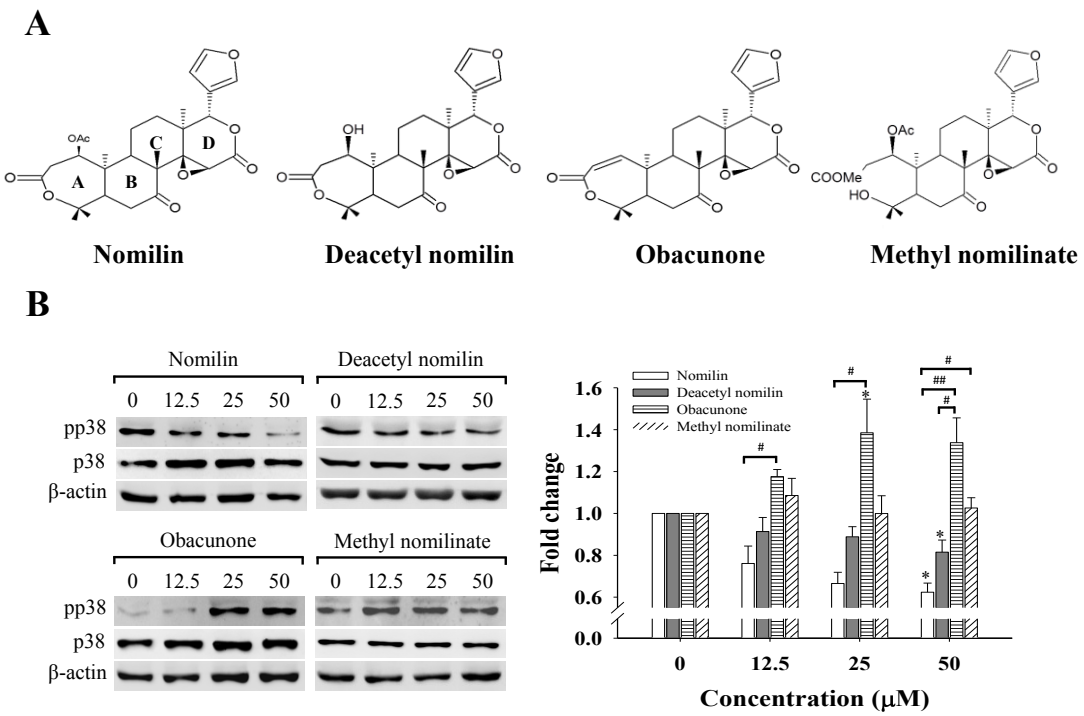

Fig. 5.2 Effect of A-ring modifications on p38 MAP kinase activation in HASMCs. (A) Structure of purified limonoids containing slight A-ring modifications. The cells were incubated with (B) nomilin and A-ring modified compounds under the indicated concentrations for 72 h. The expression of total and phospho p38 MAP kinase was examined by western blots. β-actin was used as the loading control. Data represent the mean ± S.E. of three independent experiments. * $P < 0.05$, # $P < 0.05$, ## $P < 0.01$ by ANOVA.

At 50 μM concentrations, while the parent molecule nomilin decreased p38 MAP kinase activity by 38%, deacetyl nomilin showed a 19% inhibition of p38 MAP kinase activity (Fig. 5.2). In contrast, obacunone increased p38 MAP kinase activity by 38% and methyl nomilinate, which possesses an open A ring structure, did not show any effect on p38 MAP kinase.

5.3.3 Nomilin Inhibits TNF-α Induced p38 MAP Kinase Activity in HASMCs

The phosphorylation of the p38 MAP kinase is mediated through pro-inflammatory molecule, TNF-α. Therefore, the most active limonoid, nomilin, was examined to elucidate its effect on TNF-α induced p38 activity in HASMCs.

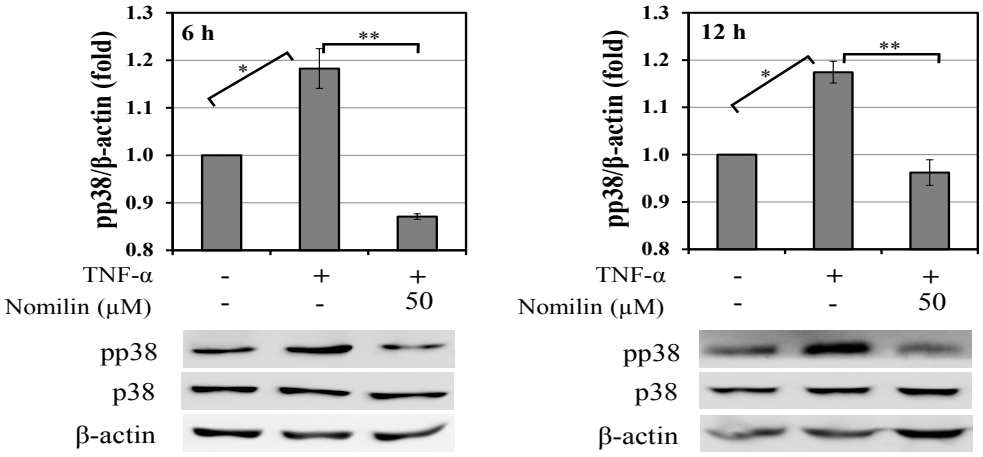

Fig. 5.3 Nomilin inhibits TNF-α induced p38 MAP kinase activity in HASMCs. The cells were pretreated with 0.1% DMSO (vehicle) or nomilin (50 μM) for 1 h and then stimulated by TNF-α (100 ng/ml) for 6 and 12 h. Cell extracts were analyzed using western blot with anti-p38, anti-phospho-p38, and anti-β-actin. β-actin was used as the loading control. Data represent the mean ± S.E. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ by ANOVA.

The cells were pretreated with 50 mM of nomilin for 1 h and then exposed to TNF-α for 6 and 12 h (Fig. 5.3). Pretreatment of nomilin completely suppressed the TNF-α induced phosphorylation of p38 MAP kinase activity as early as 6 h. The maximum p38 MAP kinase inhibition activity (31%) could be seen at 6 h, and the inhibitory activity (21%) could be sustained at 12 h (Fig. 5.3).

5.4 Discussion

The data clearly demonstrated for the first time that citrus limonoids are potent inhibitors for p38 MAP kinase activity in HASMCs. Structure-function studies of seven limonoids revealed that nomilin is the most potent inhibitor of p38 MAP kinase. In addition, nomilin completely inhibits the TNF-a induced p38 MAP kinase activity in HASMCs.

In recent years, several studies from Patil's laboratory and other groups indicate that limonoids have strong positive effects on different types of cancer cells by inhibiting cell proliferation (16, 53, 164), inducing apoptosis (36, 140), arresting cell cycles (7), or increasing detoxifying enzymes (64). In addition, using in vivo model systems it has been shown that limonoids significantly reduce the tumor size (49, 50). Recently, in vivo studies demonstrated no risk of toxicity in using limonin and nomilin (165). Although numerous epidemiological studies demonstrate that the risk of cardiovascular disease and fruit consumption have inverse relationship (166-168), the specific mechanism of actins using citrus bioactives remains to be elucidated.

Accumulating evidences demonstrate that several kinases such as MAP kinases, phosphoinositide 3-kinase (PI3K)/Akt, and the small GTPase Rho/Rho kinases (ROCKs) play significant roles in modulating human cardiovascular diseases (169, 170). These kinases are central hierarchies in various signalling pathways that have been demonstrated in various physiological functions including protein phosphorylation, cell adhesion, cell survival, and cell contraction (170). Although these pathways are regulated by complex interactions and cross-talking with other signal transduction pathways, the regulatory mechanisms in a specific cell, downstream substrates, and outcomes of responses are highly specialized to each cell type. As possible downstream targets of PI3K/Akt and ROCK pathway, MAP kinases play a convergent role in the modulation of the heart disease processes (169). The three major MAP kinase subfamilies in mammalian cells are the extracellular signal regulated kinases (ERK1/2), the c-Jun N-terminal kinases (JNKs), and the p38 MAP kinase (67). In particular, it has been demonstrated that p38 MAP kinase plays a central role in the regulation of human cardiovascular diseases (171). Elevated p38 MAP kinase activity was shown in failing human heart secondary to ischemic cardiomyopathy but the activity of ERK1/2 was unchanged (172). Furthermore, an increase in p38 kinase activity was reported in collagen- or thrombin-induced activation of human platelet (173, 174). In addition, p38 inhibitors have been shown to inhibit collagen- or thrombin-induced platelet aggregation (175) and cardiac hypertrophy in rat model (163). In the present study, we have shown that citrus limonoids are potent p38 MAP kinase inhibitors in HASMCs. Since p38 MAP kinase pathway plays a central role in inflammation, one of the key mediators in

cardiovascular diseases, citrus limonoids can modulate such pathway to reduce the risk of cardiovascular diseases. However, further experiments are warranted to test the effect of limonoids on various down-stream signalling cascades of p38 MAP kinase activity.

Most of the citrus limonoids contain a furan ring at C-17 position. The existence of furan ring has been thought to play an important role in biological activity. Limonoids are thermally stable and the melting point is 298-300 °C (101). Limonin is stable over a wide range of pH at 2-9, but completely degraded at pH 10-12 (29). Patil's group have isolated and identified several limonoids at different temperatures and purified by various methods (58, 117, 123) and the change in furan ring was not observed. Therefore, the functional groups including furan ring will be stable during isolation process.

A study about structure-activity relationships of limonin on anti-feedant activity demonstrated that the furan ring and epoxy group have the highest activity against insect (62). Lam et al. have shown that the furan moiety is critical to enhance glutathione S-transferase (GST) enzyme activity in various organs using animal models (63, 146, 176). Additionally, our recent in vivo study demonstrated that defuran limonin lost its ability to induce GST enzyme activity (47). Present study also demonstrated that defuran nomilin inhibited the p38 MAP kinase activity by 17%, while the parent molecule (nomilin) showed 38% inhibition. Interestingly, defuran limonin did not show inhibition but parent molecule (limonin) showed 19% inhibition of p38 MAP kinase activity. Therefore, this data indicate that the existence of the furan ring has a potential impact to increase the inhibition of p38 MAP kinase activity. In addition, nomilin exhibited a

greater inhibition of p38 MAP kinase compared to limonin (38% versus 19%). It is possible that seven-membered A ring in nomilin plays a key role in inhibition of p38 MAP kinase activity. The p38 MAP kinase inhibition activity of the different limonoids (Fig. 5.1 and 5.2) are ranked in the following order: nomilin (38%) > limonin (19%) = deacetyl nomilin (19%) > defuran nomilin (17%). Defuran limonin and methyl nomilinate have no activity on p38 MAP kinase. These data suggest that the p38 MAP kinase suppression by specific limonoids is more affected by the seven-membered A ring with acetoxy group and the furan moiety. In contrast, obacunone, which has a double bond in the A-ring, promotes p38 MAP kinase activity in HASMCs. These results seem to suggest that, saturation of ring A by an acetyl group at the C-1 position of nomilin will change in the conformation of large part of the molecule and it leads to significant p38 MAP kinase inhibition. Obacunone has also been reported as one of the most potent limonoids in reducing the risk of carcinogen induced cancer in animal model (49). Thus, it is critical to understand the role of double bond in the seven-membered A ring compound on the modulation of p38 MAP kinase activity in cancer and cardiovascular cells.

TNF-α production is mediated by macrophages and the neointimal vascular region as an inflammatory response after damage in the cardiovascular region (177, 178). Furthermore, TNF-α is present in the human arterial wall and its activation initiates the progression of atherosclerosis (178). Currently, the TNF-α antibody, infliximab, and anti-inflammatory drugs such as aspirin, indomethacin, and ibuprofen are prescribed for the treatment of inflammatory disorders, including cardiovascular disease (179, 180). In

the present study, phosphorylation of p38 MAP kinase induced by TNF-α in HASMCs was completely blocked by nomilin, suggesting that nomilin may potentially be involved in the anti-inflammatory response of citrus fruits by preventing phosphorylation of p38 MAP kinase cascade in HASMCs.

In conclusion, these data demonstrated that certain citrus limonoids inhibited p38 MAP kinase activity and the activity was affected by the existence of a furan ring and A ring modification. Specifically, nomilin, which has seven-membered A ring with acetylation at the C-1 position as well as a furan ring, showed a greater inhibition of p38 MAP kinase activity in both HASMCs and TNF-α induced HAMSCs. Future studies addressing the effect of limonoids on the down-stream signalling cascades of p38 MAP kinase in cardiovascular cells will provide additional insights in understanding the biological and physiological roles of limonoids for cardiovascular disease prevention.

6. NOMILIN INHIBITS THE TNF-α-INDUCED HUMAN AORTIC SMOOTH MUSCLE CELL PROLIFERATION THROUGH MITOCHONDIRAL-DEPENDENT PATHWAY

6.1 Introduction

The limonoids demonstrate biochemical and biological effects in reducing risks against cancer(9, 181), cardiovascular disease (182), and degenerative disease (181, 183). In addition citrus limonoids also exhibit anti-malarial, anti-fungal, and anti-viral activities (181, 184). Additionally, our previous in vivo and in vitro research showed that limonoids induce phase II enzymes (glutathione S-transferase and quinone reductase) (185-188) and inhibited cell proliferation of MCF-7 breast cancer (16, 189), HT-29 colon cancer (190), panc-28 pancreatic cancer (191, 192), and SH-SY5Y neuroblastoma cells (183). Furthermore, certain limonoids induced apoptosis in rats through the suppression of anti-inflammatory proteins such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (49, 50).

Atherosclerosis is initiated by chronic inflammatory processes such as endothelial injury, modification of lipoprotein by a long-term oxidation process, adhesion of blood monocytes, lymphocytes, and platelets to the artery walls, and proliferation of smooth muscle cells (SMCs) (77). Previous research has suggested that SMCs are closely related to the initiation of atherosclerosis and are involved in every type of lesion during the disease's progression (193). In addition, it has been suggested that the SMCs are considered the origin for secretion of important inflammatory molecules such as the transforming growth factor (TGF)-β, platelet derived growth

factors, macrophage inhibitor factor (MIF), chemokines, interferon gamma, and monocyte chemoattractant protein (MCP-1), which play a role in the development of cell proliferation and cell migration as well as extracellular matrix (ECM) production in atherosclerosis (193). In addition, SMCs have an anti-apoptotic effect on monocytes through increased Bcl2, AKT, and MAPK. These evidences suggest the possibility that induction of SMC apoptosis in the early stages may result in the prevention of atherosclerosis (194). The SMCs are involved throughout the development of atherosclerosis, and the proliferation of SMCs is a key feature of development of the early atherosclerosis stage (77). Inflammatory responses continuously stimulate the migration and proliferation of SMCs, which results in accumulation of ECM and the thickening of the artery wall (78).

To the best of my knowledge, very little information is available related to limonoids and anti-inflammatory properties. To explore the possible role of limonoids, nomilin was evaluated for the anti-proliferative activity against TNF-α induced human aortic smooth muscle cells (HASMCs), and the possible mechanism of growth-inhibition was elucidated.

6.2 Materials and Methods

6.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM); trypsin-EDTA; penicillin; streptomycin; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); and dithiothreitol (DTT). HASMCs, smooth muscle cell medium (SMCM), and SMC growth

supplement were purchased from ScienCell (Carlsbad, CA, USA). Mouse monoclonal anti-Bax (sc-7480), anti-Bcl2 (sc-65392) and anti-β-actin (sc-81178) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nomilin was purified and identified as described in Section 2.

6.2.2 Cell Culture and Cytotoxicity Assay

The HASMCs were cultured in SMCM containing a growth supplement followed by previously described in Section 5. For MTT assay, cells (0.5 × 104/well) were seeded into a 96-well plate and allowed to grow overnight. Next day, the cells were serum starved for 24 h. After the 24 h incubation, the cells were pretreated with 0.1% DMSO (vehicle) or nomilin (50 μM) for 1 h and then stimulated by TNF-α (20 ng/ml) for 12 and 24 h. At the end of the treatment period, 10 μl of MTT (5 mg/ml in PBS) solution was added into each well, and then the plate was incubated at 37 °C for 2 h. After the incubation, the media was removed, and 200 μl DMSO was added to dissolve the formazon; the absorbance values read 570 nm on the KC4 microplate reader (BioTek Instruments, Winooski, VT).

6.2.3 Western Blot

For western blot, HASMCs (0.5×10^6) cells were seeded into 6 well plates and serum starved for 24 h. After the serum starvation, either 0.1% DMSO or nomilin (50 μM) was pretreated into HASMCs cells and then stimulated by TNF-α (20 ng/ml) for 12

and 24 h. After the incubation time, cells were harvested and lysed. Protein expression level was measured by western blot according to described in Section 2.

6.3 Results and Discussion

Since the proliferation of SMCs is a key feature of development of cardiovascular disease (78), the purified nomilin was evaluated for the effect on TNF-α induced HASMCs proliferation. As shown in Fig. 6.1, HASMCs proliferation was significantly induces by treatment of TNF-α (20 ng/ml) for 24 h. Nomilin was significantly inhibited the TNF-α induced proliferation. The activated proliferation by TNF- α was returning back to normal stage by pre-treatment of nomilin.

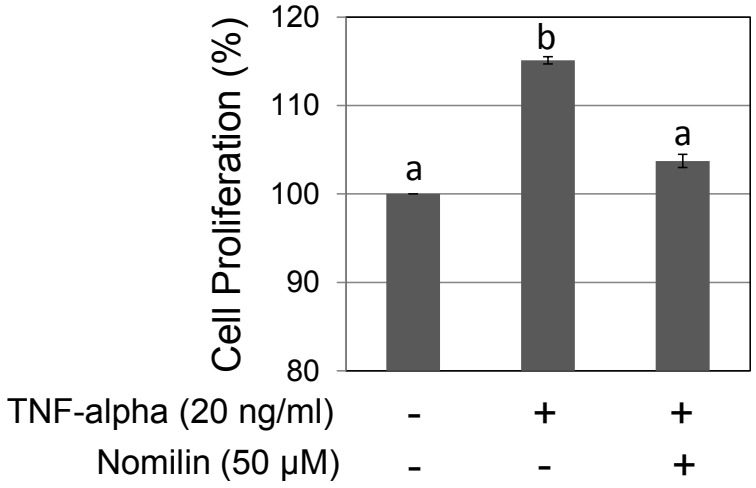

Fig. 6.1 Nomilin inhibits the TNF-α induced HASMCs proliferation. The cells were incubated with DMSO or nomilin (50 μM) for 1 h and then activated with TNF-α (20 ng/ml) for 23 h. The HASMCs proliferation was determined using MTT assay. The bar graphs represent the mean ± S.E. of three independent experiments. The bar graph with different alphabet is significantly different at $P < 0.05$ by ANOVA.

To determine the induction of apoptosis, the Bcl2 and Bax protein level were evaluated by western blot. The decreased ratio of Bcl2/Bax is suggested to be involved in the apoptosis process and is representative of the mitochondrial dependent apoptosis marker. The TNF-α induced HASMCs exhibited 70% increased Bcl2/Bax ratio, while nomilin treatment depicts 50% decreased Bcl2/Bax than DMSO (vehicle control) treatment (Fig. 6.2).

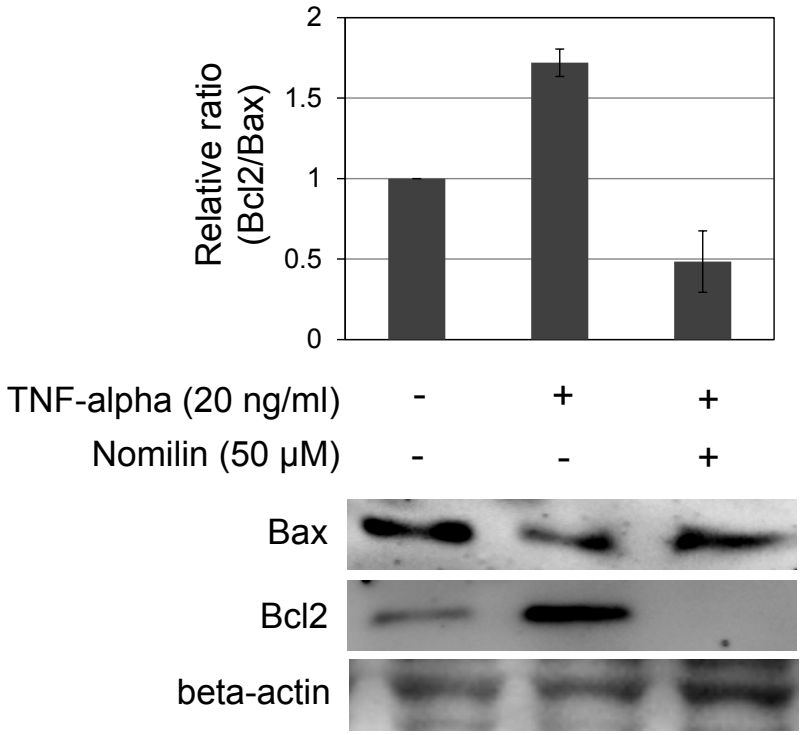

Fig. 6.2 Nomilin inhibits TNF-α induced HASMCs proliferation by mitochondrial-dependent pathway. The cells were incubated with DMSO or nomilin (50 μM) for 1 h and then activated with TNF-α (20 ng/ml) for 23 h. The protein expression level was detected by western blot analysis. Beta-actin was used for loading control.

Several *in vitro* and *in vivo* studies proved that citrus bioactive components have antioxidant, prevention of cancer, degenerative disease and cardiovascular disease (12). However, the mechanism by which limonoids inhibit cardiovascular disease is warranted. studied. In the Section 5, results demonstrated that nomilin inhibit the TNF-α induced p38 MAPK activity in HAMSCs. Since the SMCs are thought to be a principle cell involved in development of cardiovascular disease, this present study was conducted to elucidate the further mechanism of nomilin in TNF-α induced HAMSCs. Nomilin showed significant inhibition of TNF-α induced HASMCs proliferation (Fig. 6.1).

Dysregulation of cell growth contributes to a number of human diseases including cancer, cardiovascular disease, autoimmune disorders and neurodegenerative diseases (195). Thus, we postulated that the proliferation inhibition by nomilin might be due to induction of apoptosis. To test this hypothesis, the expression levels of Bcl2 and Bax, which are responsible for apoptosis, were measured (Fig.6.2). Nomilin decreased the ratio of Bcl2/Bax ratio in TNF-α induced HASMCs for 24 h. Since the decreased Bcl2/Bax ratio is considered to induce apoptosis due to mitochondrial dysfunction (196), these results demonstrated that the anti-proliferative activity of nomilin is due to apoptosis through mitochondrial dependent pathway. However, in order to make any conclusions with regard to health claim, further studies are still needed.

7. SUMMARY AND CONCLUSIONS

The major objective of this research was to determine the health beneficial effects of limonoids from lemon seed and to investigate the mechanism and effectiveness of anti-breast cancer and anti-cardiovascular disease properties. The work done in this dissertation supports the following conclusions.

Bioactive components in lemon seed extracts could be an excellent source of antioxidants and induce apoptosis in MCF-7 breast cancer cells through the mitochondrial apoptosis pathway attributed from the phosphorylation of Bax, Bcl2, released cytochrome C in cytosol, and cleaved PARP. Moreover, this research demonstrated for the first time that lemon bioactive compounds are non-toxic to non-malignant breast cells. These findings support the hypothesis that bioactive compounds from lemon may have cancer-preventive properties. Therefore, purification and identification of compounds present in each extracts was carried out to understand their mechanism of apoptosis induction.

Nine limonoids in lemon seed were purified and identified using ethyl EtOAc) and MeOH extracts with different types of column chromatography such as silica gel and ion-exchange column. The compounds were identified by employing TLC, HPLC, and LC-MS analytical techniques. Purified and modified limonoids, including limonin, nomilin, obacunone, limonexic acid (LNA), isolimonexic acid (ILNA), nomilinic acid glucoside (NAG), deacetyl nomilinic acid glucoside (DNAG), limonin glucoside (LG), obacunone glucoside (OG), limonin methoxime (LM), limonin oxime (LO), defuran limonin (DL), and defuran nomilin (DN), were screened for their cytotoxicity on

estrogen receptor (ER)-positive (MCF-7) or ER-negative (MDA-MB-231) human breast cancer cells. We further tested the mechanism of anti-proliferative activity of limonoids using *in vitro* aromatase enzyme assay and western blot with anti-caspase-7. Although most of limonoids showed anti-aromatase activity, the inhibition of proliferation was not related to the anti-aromatase activity. On the other hand, the anti-proliferative activity was significantly correlated with caspase-7 activation by limonoids. These results indicated that the citrus limonoid may have potential for the prevention of estrogen-responsive breast cancer (MCF-7) via caspase-7 dependent pathways.

Obacunone was the most potent to inhibit MCF-7 cell proliferation without affecting non-malignant breast cells. Treatment with obacunone also resulted in an increased apoptotic effect through up-regulated expression of the tumor suppressor p53, pro-apoptotic protein Bax, and anti-apoptotic protein Bcl2, as well as G1 cell cycle arrest molecules. In addition, obacunone most effectively inhibited aromatase activity in an *in vitro* enzyme assay. Exposure of MCF-7 breast cancer cells to obacunone down-regulated expression of inflammatory molecules including nuclear factor-kappa B (NF-κB) and cyclooxygenase-2 (COX-2). Furthermore, we also tested the possibility that obacunone inhibits COX-2 and NF-κB by a mechanism involving activation of the p38 mitogen-activated protein kinase (MAPK). This study demonstrated for the first time that obacunone, a citrus limonoid, may have potential in the prevention of estrogen-responsive breast cancer through inhibition of the aromatase enzyme, inflammatory pathways, and apoptotic markers.

Since up-regulation of p38 is associated with the development of human cardiovascular disease, we tested that eight structurally different limonoids would involve in inflammatory pathway via modulating p38 MAP kinase activity at various extent in vascular smooth muscle cells. Results demonstrated that certain citrus limonoids inhibited p38 MAP kinase activity and the activity was affected by the existence of a furan ring and A ring modification. Specifically, nomilin, which has seven-membered A ring with acetylation at the C-1 position as well as a furan ring, showed a greater inhibition of p38 MAP kinase activity in both HASMCs and TNF-α induced HAMSCs.

Nomilin was significantly inhibited the TNF-α induced proliferation in HASMCs. Furthermore, the anti-proliferative activity of nomilin is due to apoptosis through mitochondrial dependent pathway. Future studies addressing the effect of limonoids on the down-stream signalling cascades of p38 MAP kinase in cardiovascular cells will provide additional insights in understanding the biological and physiological roles of limonoids for cardiovascular disease prevention.

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VITA

Name: Jin Hee Kim

Address: Department of Horticultural Sciences
c/o Dr. Bhimanagouda S. Patil
Texas A&M University
College Station, TX 77843-2119

Email Address: jhkimtamu@gmail.com

Education: B.S., Horticulture, Sahmyook University, 2004
M.S., Biochemistry, Chung-Ang University, 2006
Ph.D., Horticulture, Texas A&M University, 2011