The chemopreventive activities and molecular mechanisms of loquat (*Eriobotrya japonica*) tea

（ビワ茶の生体調節機能および分子機構に関する研究）

A DISSERTATION PRESENTED TO THE UNITED GRADUATED SCHOOL OF KAGOSHIMA UNIVERSITY IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Abbreviations

3-CQA, 3-caffeonylquinic acid

5-CQA, 5-caffeonylquinic acid

AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride

ANOVA, Analysis of variance

ATR, aircraft manufacturer

AUC, area under curve

Bax, Bcl-2-associated X protein

BW, body weight

CH_3CN, acetonitrile

CO_2, carbondioxide

COX-2, cyclooxygenase-2

d, day

DCFH-DA, dichlorofluorescein-diacetate

DHB, 2,5-dihydroxybenzoic acid

$DiOC_6(3)$, 3,3'-Dihexyloxacarbocyanine iodide

DMEM, Dulbecco's Modified Eagle Medium

DMSO, Dimethyl sulfoxide

DMSO-d_6, dimethyl sulfoxide-d6

DPPH, 1-diphenyl-2-picrylhydrazyl

EC, (-)-epicatechin

ECL, enhanced chemiluminescence

ELISA, enzyme linked immunosorbent assay

eNOS, endothelial nitric oxide synthase

ERK, extracellular signal-regulated kinase

EtOH, Ethanol

FBS, Fetal bovine serum

FTIR, fourier transform infrared spectroscopy

GAE, gallic acid equivalent

G-CSF, granulocyte colony-stimulating factor

GM-CSF, granulocyte-macrophage colony-stimulating factor

h, hour

H_3PO_4, phosphoric acid

HL-60, human promyelocytic leukemia cells

H-NMR, hydrogen nuclear magnetic resonance

HPLC, High performance liquid chromatography

HRP, horseradish peroxidase

IFN- γ, interferon-gamma

IKK, IκB kinase

IL-10, interleulin-10

IL-12 p40, interleulin-12 p40

IL-12 p70, interleulin-12 p70

IL-13, interleulin-13

IL-17, interleulin-17

IL-1a, interleulin-1alpha

IL-1b, interleulin-1beta

IL-2, interleulin-2

IL-3, interleulin-3

IL-4, interleulin-4

IL-5, interleulin-5

IL-6, interleulin-6

IL-6, interleulin-6

IL-9, interleulin-9

iNOS, inducible nitric oxide synthase

IRF3, interferon regulating factor 3

IκB, I kappa B

JNK, c-Jun NH_2-terminal kinase

KC, keratinocyte chemoattractant

LPS, lipopolysaccharide

LTE, loquat tea extract

MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time to flight mass spectrometer

MAPK, mitogen-activated protein kinase

Mcl-1, anti-apoptotic myeloid cell leukemia 1

MCP-1, monocyte chemotactic protein-1

MeOH, Methanol

mg/ml, milligram per milliliter

MIP-1a, macrophage inflammatory protein-1alpha

MIP-1b, macrophage inflammatory protein-1beta

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliun bromide

Na_2CO_3, Sodium carbonate

NaCl, sodium chloride

NF-κB, nuclear factor kappa B

NMR, nuclear magnetic resonance

nNOS, neuronal NOS

NO, nitric oxide

Nrf2, Nuclear factor-erythroid 2 p45-related factor 2

ORAC, oxygen radical absorbance capacity

pg/ml, picograms per milliliter

PGE_2, prostaglandin E_2

ProB2, procyanidin B2

PVDF, polyvinylidene difluoride

RANTES, regulated on activation, normal T cell expressed and secreted

RAW 264.7, Mouse macrophage-like cells

RLL, Run length limited

ROS, reactive oxygen species

S.D, standard deviation

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TBK1, TANK-binding kinase 1

TGF-β, transforming growth factor beta

TNF-α, transforming growth factor alpha

TRIF, Toll like/IL1 receptor domain containing adaptor inducing IFN-β

Trolox, 6-Hydroxy, 2.5.7.8-tetramethyl chroman-2-carboxylic acid

µg/ml, microgram per millimeter

Abstract

Loquat tea is called “Biwa Cha” in Japanese which is made from loquat leaves roasted to 350 ºC and it is now using for health beverage in Japan. Although accumulated data revealed that fresh loquat leaves have benefits to human health such as chemoprevention and treatment for chronic inflammatory diseases, the bioactivity of roasted loquat leaves (loquat tea) is unknown yet. Therefore, I chose loquat tea as my research objective to clarify the antioxidant, anti-inflammatory and anticancer activities and underlying molecular mechanisms in the present study.

First of all, several types of column chromatography were used to separate the bioactive fractions of loquat tea by antioxidant activity-guided fractionation. The antioxidant potency of the ethanol fractions of loquat tea extract (LTE) and fresh loquat leaves (FLL) was determined by oxygen radical absorbance capacity (ORAC) and DPPH radical scavenging activity assays. Moreover, LTE increased total antioxidant activities *in vitro* by suppressing cellular reactive oxygen species (ROS) in murine RAW 264.7 cells and increasing nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)-mediated expression of heme oxygenase-1 (HO-1).

Since antioxidant activities are linked to anti-inflammatory activities by modulating signal transduction pathways, I next clarified the anti-inflammatory effects and molecular mechanisms of LTE in both cell and animal models. LTE inhibited the production of pro-inflammatory factors including cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2), inducible nitric oxide synthase (iNOS), nitric oxide (NO), interleukin 6 (IL-6), RANTES and tumor necrosis factor (TNF-α). Cellular signaling data revealed that the downregulation of TGF-β-activated kinase (TAK1)-mediated mitogen-activated protein kinase (MAPK) and

nuclear factor-κB (NF-κB) pathways as well as interferon regulatory transcription factor 3 (IRF3) pathway were involved in the inhibition of pro-inflammatory factors by LTE. Animal experiment data confirmed the *in vivo* anti-inflammatory effects of LTE by attenuating LPS-induced mouse paws edema and serum cytokines level.

Because of the bioactive compounds possessing the antioxidant and anti-inflammatory activities will also have anticancer activity, the antiproliferation and apoptosis induction activities of LTE were investigated using human promyelocytic leukemia cells (HL-60). LTE suppressed the proliferation of HL-60 cell and inducing apoptosis by releasing proapoptic proteins from mitochondria membrane loss ($\Delta\Psi m$).

Finally, the bioactive compounds contributing to the antioxidant and anti-inflammatory and chemopreventive activities in LTE were analyzed with the methodologies of high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FT-IR), mass spectroscopy (MS) and nuclear magnetic resonance (NMR). The results revealed that the original flavonoid in fresh loquat leaves such as 3-caffeonylquinic acid, 5-caffeonylquinic acid, epicatechin and procyanidin B2 were reduced in LTE. In place of them, several kinds of new phenolic compounds were detected in LTE, that are produced from the release and/or degradation of bound phenolic compounds from fresh loquat leaves during roasting process.

In summary, LTE possessed stronger antioxidant activity, and could suppress the production of inflammatory mediators by downregulating inflammatory signaling pathways *in vitro* and *in vivo*. LTE also suppressed the proliferation of HL-60 cell by inducing mitochondrial dysfunction pathway. The results provide insight for understanding chemopreventive effects and molecular mechanisms of loquat tea.

要旨

ビワの葉は、古くから漢方薬や民間薬などとして利用され、抗酸化、抗菌や抗癌活性などを有することが報告されている。近年、ビワ茶はビワの葉を乾燥し、焙煎により作られた飲料茶として親しまれている。一方、ビワ茶の機能性成分や生体調節機能についてはまだ十分に解明されていない。本研究では、ビワ茶の抗酸化、抗炎症と抗がん活性などの生体調節機能およびそれらの作用分子機構について解析を行った。

はじめに種々のカラムクロマトグラフィーを用い、ビワ茶の成分分画を行った。抗酸化活性を示す成分を機能性画分として更なる分析を行った。ビワ茶のエタノール画分の抗酸化活性（ORAC 活性および DPPH ラジカル消去活性）がビワの生葉より高い値を示した。また、培養細胞レベルにおいてもビワ茶のエタノール画分は、抗酸化に関する転写因子 Nrf2 を介した抗酸化タンパク質（HO-1 など）の発現を促進し、細胞内活性酸素の産生を抑制した。

つぎに、抗酸化活性は細胞内信号伝達経路を介し抗炎症活性に関連することから、ビワ茶の抗炎症効果およびその分子機構について細胞ならびに動物実験により明らかにした。その結果、ビワ茶のエタノール画分は細菌リポ多糖により誘発された炎症性因子（COX-2、PGE_2、iNOS、NO、IL-6、RANTES、TNF-α）の産生を著しく抑制した。また、ビワ茶のエタノール画分が 炎症性細胞内信号伝達系（MAPK、NF-κB および IRF3 経路）を抑制することで上記の炎症性因子を阻害することも明らかにした。マウスの炎症性足浮腫モデル実験ではビワ茶のエタノール画分が細菌リポ多糖により誘発されたマウスの血清中の炎症性因子の水準を低下させ、足浮腫を緩和する効果が顕著に認められた。よって、実験動物においてもビワ茶が抗炎症作用を有することを実証した。

さらに、ヒト骨髄性白血病細胞（HL-60）に対するビワ茶の細胞増殖抑制能およびアポトーシス誘導効果を調べた。その結果、ビワ茶のエタノール画分が HL−60 細胞の増殖を顕著に抑制した。また、ビワ茶のエタノール画分が HL-60 細胞の DNA 断片化、カスパーゼ活性化、ミトコンドリア膜電位の減衰およびシトクロム *c* の細胞質への放出を促進し、ミトコンドリア機能喪失によるがん細胞アポトーシスを引き起こすことを明らかにした。

最後に、種々の化学分析法（HPLC, FT-IR, NMR）を用い、抗酸化、抗炎症、抗がん活性に寄与するビワ茶の成分についての解析を行った。これらの解析データから、ビワの生葉に含有されているカフェオイルキナ酸、エピカテキン、プロシアニジン B2 等のフラボノイドがビワ茶では減少し、その代わりに複数のフェノール性化合物が新規に生成していることを確認した。これらの生体調節機能を有する化合物はビワの生葉を焙煎する過程で産生された成分だと考えられた。

以上のように、焙煎したビワ茶はビワの生葉より強い抗酸化活性を有し、細胞内酸化系や炎症系の信号伝達経路や炎症性因子の産生を阻害することで抗炎症作用を示した。さらに、ビワ茶ががん細胞のミトコンドリアの機能障害によるアポトーシスを引き起こし、ヒト骨髄性白血病細胞の増殖を抑制した。これらの結果は、ビワ茶の生体調節機能に関する貴重な分子エビデンスと科学的知見を提供するものである。

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Chapter 1

Introduction

1.1 Loquat (*Eriobotrya japonica*)

Loquat (*Eriobotrya japonica*) belongs to the Rosaceae family which includes apples, pears and quinces. Loquat grows in subtropical and mild-temperate climates, but may be found from 20° to 35° North [1]. Its origin is in south-eastern China and it has been cultivated in China and Japan since ancient times [2]. In Japan, this tree is called “Biwa”, and is also known as the Japanese medlar or Japanese plum. It is also cultivated in other regions, namely in the Mediterranean area, Australia, South Africa, South America, California and India [3]. The plant is an evergreen shrub with narrow leaves, dark green on the upper surface, and a lighter woolly under surface. All parts of loquat such as fruits, leaves and peels have many benefits to health. Its leaves have astringent flavor and have been used as folk medicine for a long time to treat chronic bronchitis, coughs, phlegm, high fever and gastroenteritic disorders [4]. A decoction of the leaves has been known to be a cooling beverage preventing sunstoke and thirst, and has also been applied locally to wounds, ulcers and cancers [5]. Fresh loquat leave had antitumor effect [6,7], anti-inflammatory effect [8] and antinociceptive effect [9], antioxidant effect [2,10]. Accumulated data showed that fresh loquat leave contained hydroxycinamoyl chlorogenic acids [2,11,12], various triterpenes [13], tannins and megastigmane glycosides [6], flavonoids, procyanidins and related flavan 3-ols [4,14].

1.2 Loquat tea (*Eriobotrya japonica* tea)

Loquat tea is made from loquat leaves in Totsukawa Noujyo Ltd. In brief, loquat leaves were roasted to 350 ºC in a ceramics vessel after washing and drying. Loquat tea is now used for health beverage in Japan and called as “Biwa Cha” in Japanese.

1.3 Antioxidant

Antioxidants are defined as compounds that can delay, inhibit, or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Epidemiological evidence observed that antioxidants involved in fruit and vegetable can help to prevent inflammatory diseases. Therefore, the consumption of antioxidant rich food might have beneficial effects in treatment and prevention of human disease.

The most widely used assays for quick quantification of antioxidant are DPPH radical scavenging assay [15] and oxygen radical absorbance capacity (ORAC) assay [16]. The DPPH method is a radical generating substance that is rapid, accurate assay to monitor the free radical scavenging abilities. ORAC assay employs a fluorescin to compete with sample antioxidant for peroxyl radicals generated by decomposition of 2,2’-azobis dihydrochloride (AAPH). The fluorescence intensity is measured every minute at physiological conditions (pH 7.4, 37 ºC) to obtain a kinetic curve of fluorescence decay. Thus, ORAC method is better than other methods to estimate the antioxidant activity of samples [17].

The imbalance state of reactive oxygen species (ROS)/reactive nitrogen species (RNS) lead to oxidative stress *in vivo*. Oxidative stress caused tissue damage and cytotoxicity through the formation of ROS and/or RNS that associated with chronic inflammatory disease. Antioxidants can diminish oxidative stress by both directly scavenging radical species such as ROS/RNS and indirectly reducing ROS/RNS formation through the upregulation of antioxidant enzymes expressions. ROS activates redox-sensitive I kappa B

kinase (IKK) [18,19] and mitogen-activated protein kinases (MAPK) [20] and interferon regulatory transcription factor 3 (IRF3) [21]. The production of ROS can be reduced by overexpression of heme oxygenase (HO-1) [22]. HO-1 not only provides antioxidant functions [23] but also potent anti-inflammatory properties [24,25]. Upregulation of HO-1 is mediated through activation of Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), activator protein (AP-1) and nuclear factor kappa B (NF-κB) [26,27]. In normal condition, Nrf2 kept inactive in association with Keap1. Upon oxidative stimulation, Nrf2 dissociates from kelch-like ECH-associated protein 1 (Keap1), translocates into the nucleus and stimulates to antioxidant enzymes such as HO-1 [26,27] that leads to reduce oxidation.

The formation of ROS in cancer is found to be higher concentration compared with normal cell and they act as initiator and/or promotor in carcinogenesis (Ito et al., 1986).

1.4 Inflammation

Inflammation is the first physiological defense system in the human body and it can protect against invaded microbes and injuries caused by physical wounds, poisons, etc. The defense system also includes short-term inflammation or acute inflammation and long-term inflammation or chronic inflammation. Acute inflammation can destroy infectious microorganisms and eliminate irritants, and maintain normal physiological functions within a few days. Chronic inflammation results from prolonged chemical exposures, persistent foreign bodies, recurrent acute inflammation or certain pathogens and might cause dysfunctions of the regular physiology. Most common chronic diseases such as Alzheimer's disease, asthma, cardiovascular disease, cancer and obesity can occur in chronic inflammation [28-32]. During inflammatory disease, the macrophages is activated by many inflammatory factors such as bacterial lipopolysaccharide (LPS) which stimulates Toll-like receptor 4 (TLR4) triggers several inflammatory reactions. TLR4 leads to signal activation

through the downstream signaling factors which include myeloid differentiation primary response gene 88 (MyD88) and Toll-like/IL1 receptor domain containing adaptor inducing IFN-β (TRIF). MyD88 is an adaptor which recruit to interleukin-1 receptor-associated kinase (IRAKs), TRAF6 and transforming growth factor-β-activated kinase-1 (TAK1), and thereafter activates MAPK and NF-κB [33,34] which release excess amounts of mediators such as prostanoids, nitric oxide (NO) and pro-inflammatory cytokines [27,35,36]. Both MyD88- and TRIF-dependent signals downregulate MAPK and NF-κB pathways and control inflammatory responses [34].

1.4.1 Pro-inflammatory mediators

1.4.1.1 Cyclooxygenase and Prostagladin E_2

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins E_2 (PGE_2), and exists in two isoforms, COX-1 and COX-2. COX-1 is constantly expressed in most tissue and is responsible for the production of prostagladins under physiological condition [37]. However, COX-2 is expressed only during inflammation which is induced by proinflammatory stimuli including bacterial LPS, growth factor and cytokines [38]. Excess amount of COX-2 produces pro-inflammatory prostagladins at the inflammatory site [39]. COX-2 and prostaglandins are involved in various carcinomas and play a key role in inflammation and tumorigenesis [40,41].

1.4.1.2 Inducible nitric oxide synthase and nitric oxide

Nitric oxide synthase (NOS) exits three isoforms, endothelial NOS (eNOS), Neural NOS (nNOS) and inducible (iNOS). They are enzymes and responsible for the catalysis of nitric oxide (NO). eNOS is nitric oxide synthase 3 (NOS3) and generates NO in blood vessels and

is involved in regulating vascular function. nNOS produces NO in both central and peripheral nervous system [42]. iNOS is only induced by various inflammatory cytokines in macrophages, hepatocytes and endothelial cells and it catalyses large amount of NO. iNOS and NO play a key role in the various forms of inflammation and carcinogenesis [43,44]. NO overproduction leads to various harmful responses including apoptosis and necrosis [45], and organ destruction in autoimmune diseases [46].

1.4.1.3 Cytokines

Cytokines comprise interleukins, lymphokines, monokines, interferons, colony-stimulating factors, chemokines and a variety of proteins [47]. Cytokines are soluble mediators of inter and intra cellular communication that regulates development, tissue repair, hemopoiesis, inflammation, and the specific and non-specific immune responses. In addition, they act essentially between immune cells that coordinate the inflammatory response across time and space, temporally and spatially [48]. The production of cytokines in macrophages is stimulated by LPS which recruit or alert neighboring cells for mounting an effective immune response [49,50] and one cytokine influences the productions of other cytokines and the production of one cytokine is responsibility on many other cytokines [51,52].

1.4.2 Major signaling pathways

1.4.2.1 Mitogen-activated protein kinases pathway

MAPKs are a group of signaling molecules that include extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK) and C-Jun NH_2-terminal kinase (JNK) [53-55]. MAPK signaling molecules activated by LPS [33] are key regulators

in the expression of inflammatory mediators including COX-2 and iNOS [56,57] and cytokines [58].

1.4.2.2 Nuclear factor-κB pathway

COX-2, iNOS and cytokines expressions are regulated by nuclear factor-κB (NF-κB) [59,60] which acts as a key signaling pathway [61]. In unstimulated cells, the dimeric form of NF-κB is trapped in the cytoplasm by physical association with an inhibitory protein, I-κB. Many stimuli including LPS, cytokines and virus activate NF-κB via several signal transduction pathways, leading to the phosphorylation of I-κB by a multisubunit I-κB kinase (IKK) [62] following I-κB degradation [63]. IKK is activated by phosphorylation which is stimulated by TAK-1[64]. Once activated, NF-κB is translocated to the nucleus and causes transcriptional expression of many pro-inflammatory mediators such as COX-2, iNOS and cytokines [58].

1.4.2.3 Activation of interferon regulatory transcription factor 3

In unstimulated cells, interferon regulatory transcription factor 3 (IRF3) is primary present in the cytoplasm in an inactive form. However, stimulation with TLR4 ligands or viral infection causes activation of IRF3 via phosphorylation and then mediated the expression of type 1 IFN [65]. Activation of IRF3 is controlled by TRIF-dependent signaling pathway [66,67]. Furthermore, activated IRF3 participates in the late phase of NF-κB activation [34].

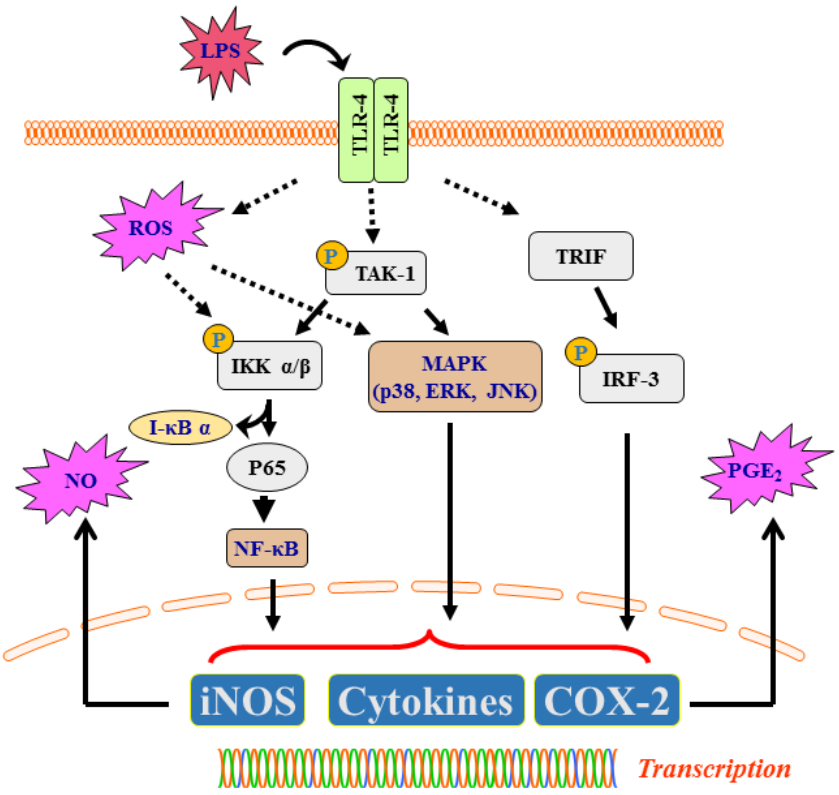

Fig.1.1. Molecular mechanism of LPS mediated inflammatory pathway

1.5 Apoptosis

Apoptosis is programmed cell death and it plays an important role in anti-carcinogenesis [68]. It is well characterized by cell shrinkage, cytoplasm vacuolization, chromatin condensation, DNA fragmentation, cellular breakdown into apoptotic bodies [69]. There are two signal pathways to trigger apoptosis: extrinsic and intrinsic pathways. The extrinsic pathway is signaling pathway of transmitting the death signal from the surface to the inside of the cell and it is activated by external death signals such as the Fas ligand (FasL) and tumor necrosis factor α (TNFα) which are the best characterized signals for caspase activation pathway, especially for procapase-8 [70-71]. Activation of caspase-8 activates caspase-3 and mitochondrial apoptotic pathway [72-74]. The mitochondria is an essential component of the intrinsic apoptotic pathway and it initiates death signal inside the cells.

Active apoptotic mitochondrial pathway is especially controlled by changes of upstream Bcl-2 family members: pro-apoptotic proteins (eg: Bax, Bak) and anti-apoptotic proteins (eg: Mcl-1, Bcl-2 and Bcl-xl). Anti-apoptotic proteins are initially integrated in mitochondria, endoplasmid reticulum or nuclear membrane; pro-apoptotic proteins are localized to cytosol or cytoskeleton prior to active mitochondrial events [73]. Antiaopoptotic proteins can inhibit activation of proapoptotic protein following active events [75]. Increased amount of pro-apoptotic proteins causes activation of caspase pathway and mitochondrial dysfunction. On the other hand, one of mitochondrial dysfunction, a decreasing mitochondrial membrane potential is associated with cytotoxic agents that cause overexpression of ROS amount. Loss of mitochondrial membrane potential leads to release of cytochrome *c* to the cytosol from the mitochondrial intermembrane that is the most important factor in the caspase-dependent mitochondrial pathway of apoptosis [76]. Both extrinsic and intrinsic pathways induce caspase-3 activation that is primary activator of apoptotic DNA fragmentation [70, 76-77].

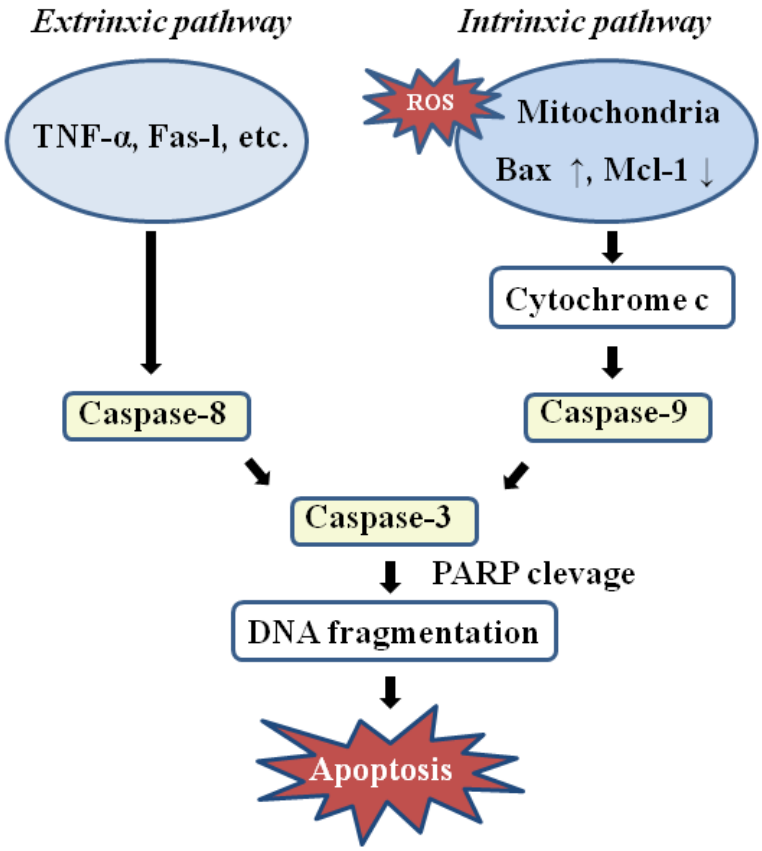

Fig.1.2. Molecular mechanism of apoptosis pathway

1.6 Polyphenols

Polyphenols are a group of natural compounds with phenolic structural features (ie, several hydroxyl groups on aromatic rings). They give color and flavour in fruit and vegetables, and are also the components showing astringent and bitter taste [78]. Polyphenols are widely distributed in the plant kingdom. Fruit, vegetables, the whole grains and other types of foods and beverages such as tea, chocolate and wine are rich sources of polyphenols. More than 8000 phenolic compounds are currently known, ranging from simple molecules such as phenolic acid to highly polymerized substances such as tannins. Among them over 4000 flavonoids have been identified [79-80] as phenolic acid, flavonoid, tannin, stilbene and lignins.

Phenolic acid are non-flavonoid polyphenolic compounds which can be divided into two classes: benzoic acid such as gallic acid and cinnamic acid derivatives as coumaric, caffeic and ferulic acid. The most abundant phenolic acid in fruit and vegetables is caffeic acid that can be found as free form, and in grains and seeds, particularly in the bran or hull in which phenolic acids are often bound form [81-82]. Flavonoids are the most abundant polyphenols in our diets and divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central ring. Tannins are another major group of polyphenols in our diets and usually divided into hydrolysable tannins and condensed tannins. Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterfied with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called eallgitannins. They are referred as oligomeric compounds after intermolecular oxidation reactions. Condensed tannins are oligomers or polymers and referred as proanthocyanidins which decomposed to anthocyanidins through acid-catalysed oxidation upon heating in acidic alcohol solutions. Other less common polyphenols are stilbene and lignin. Stilbene such as resveratrol is found in grapes and red wine. Lignans exist in the bound forms in flax, sesame and many grains.

Polyphenols present in plant foods as bioactive antioxidant molecules. Moreover, phenolic compounds are able to inhibit the expression of pro-inflammatory proteins by modulating the signaling pathways of inflammation, and are considered as potential therapeutic agents against inflammatory processes [83]. Several lines of data have suggested that the bioactive compounds such as phenolic compounds possessing the antioxidant and anti-inflammatory activities will have also anticancer activity [84-85].

1.6.1 Extractions, fractionation of polyphenols

There is no universal extraction procedure suitable for extraction of all plant phenolics because plant materials may contain phenolic compounds varying from simple (e.g., phenolic acids, anthocyanins) to highly polymerized subsatances (e.g., tannin) in different quantities. However, the phenolic nature of polyphenols makes them relatively hydrophilic, thus free polyphenols, including aglycones, glycosides, and oligomers, are extracted using water. Methanol has generally found to be more efficient in easier extraction of lower molecular weight polyphenols while the higher molecular weight flavanols are better extracted with aqueous acetone [86-87]. Ethanol is another good solvent for polyphenol extraction and is safe for human consumption.

In order to accurately quantify and identify the individual polyphenols, firstly these compounds must be separated by fractionation. Fractionations of phenolic extracts were achieved by column chromatography over sepahdex LH-20, MCI gel CHP20P, and ODS gel [12, 88]. Among them, MCI gel CHP20P designed for separation of aromatic compounds, peptides, steroids, desalting, etc. Moreover, ODS gel is suitable for hydrophilic compound separation. Ethanol, methanol, acetone, and water and their combinations are commonly used as eluents in column chromatography.

1.6.2 Identification of polyphenols

High performance liquid chromatography (HPLC) coupled with diode array detector (DAD) is the most widely analytical tool for quantification of polyphenols. Moreover, HPLC techniques offer a unique chance to analayse simultaneously all components of interest together with their possible derivatives or degradation products [89-90]. In HPLC analysis, column temperature may affect the separation of phenolics, the constant temperature should be recommended depend on manufacture’s instruction. RP C18 column

ranging from 100 to 250 mm in length and usually with an internal diameter of 3.9 to 4.6 mm is enhanced HPLC separation of different classes of phenolic compound [91]. Sample particle size is also important in HPLC separation. Acetonitrile and methanol are the most commonly used as mobile phase in HPLC. Though CH_3CN is expensive than MeOH, it is the best choice in HPLC because it reduces noise production, makes less pressure inside column, decreases air bubbles and gives good elution strength than MeOH and finally it causes beautiful peak separation in HPLC method. Due to that abilities, acetonitrile gives sharper and beautiful peak shapes than methanol. The pH value has been influenced on HPLC assay. The recommended pH range for HPLC assay is pH=2-4. The pH value is controlled by adding small amounts of acids such as acetic, formic, phosphoric and most rarely perchloric acid to the mobile phase. The acid concentration can vary from 5 to 50 mM. Detection for the simultaneous separation of mixtures of phenolic acids at 280 nm is most generally used although for dual monitoring 254 and 280 nm, or 280 and 320 nm [17,91].

1.6.3 Determination of chemical structure

Fourier transform infrared spectroscopy (FTIR), Mass Spectroscopy (MS) [92-94] and Carobons/Hydrogens nuclear magnetic resonance (NMR) [95-96] are primary methods for determining the structure of phenolic compound. FTIR spectroscopy identifies specific functional groups within a molecule on the basic of absorption bands at characteristic frequencies [97]. This method with very small amounts of sample (i.e. 10-25 mg) is nondestructive and can be applied to study both dry and wet samples [98-99]. FTIR data is helpful to have other data from other instrumentation such as mass spectra or number of carbons/hydrogens (NMR), to confirm a proposed molecular model. Mass spectroscopy (MS) measures the mass to charge ratio of ions that containe in sample by electrostatic

acceleration and magnetic field perturbation and it provides a precise molecular weight and it is also helpful for nuclear magnetic resonance (NMR).

1.6.4 Dietary consumption and metabolism of polyphenols

Polyphenol intake is high variable in individual countries. Intake of phenolic acids ranged from 6 to 987 mg/d in Germany [100]. The person who drinks several cups of coffee per day may intake hydroxycinnamic acids as much as 500-800 mg/d, whereas who do not drink coffee may take > 25 mg/d from small amount of fruits and vegetables. The main reason for these variations is individual food preferences. Although polyphenol intake is difficult to evaluate by dietary questionnaries, the total polyphenol intake commonly reaches 1 g/d in people who eat several servings of fruit and vegetables per day. Toxic and neoplastic lesions are found after high intake of quercetin about 1900 mg/kg/d for 2 years [101].

Polyphenols are poorly absorbed form the intestine, and rapid metabolized to be eliminated. Most polyphenols are present in food in the form of esters, glycosides, or polymers with high molecular weight that cannot be absorbed in their native form [102-104]. These substances must be hydrolyzed by intestinal enzymes of the colonic microflora before they can be absorbed. After absorption, most polyphenols are conjugated in the small intestine and later in liver [105-106]. Conjugated derivatives are circulating in blood and extensively bound to albumin [107-108]. Polyphenols are able to penetrate tissues, particularly after they are metabolized, and eliminated from urine and bile. Polyphenols binding with polysaccharides may affect absorption [109]. Furthermore, pH, intestinal fermentations, biliary excretion and transit time may influence on the absorption of polyphenols [102].

Procyanidin B2 is very poorly absorbed in rats [104]. Hydroxycinnamic acids in the free form are rapidly absorbed from the small intestine and are conjugated. However, these

compounds are naturally esterified in plant products. Therefore, the absorption of phenolic acids is markedly reduced when they present in the esterified form rather than in the free form. For example, caffeic acid was much better absorbed than chlorogenic acid [110-112].

1.7 Effect of manufacture temperature on dietary polyphenols

The chemical profile of phenolic compounds in food is influenced by temperature during food manufacture. Although mild temperature during processing does not change significantly, too high temperature, especially roasting process, will influence the phenolic compounds. The total polyphenol contents can be decreased or increased after heat treatment. For example, total phenolic compounds in citrus peel and sweet potato was liberated by heat treatment [113-114] with slightly changes of phenolic compounds. The decrease in total phenolic content was found in green tea after roasting. It is known that flavanols are easily hydrolyzed and oxidized under high temperature that increases the oxidation of phenolic compounds and decreases the yield of phenolics amount in the extracts [115]. The phenolic compounds are partially degraded and/or bound to polymer structures during roasting. The properties of high temperature treatment influence not only in polyphenol content but also in the antioxidant activities treatment [113-114].

1.8 Thesis investigation

To clarify chemopreventive activites and molecular mechanisms of loquat tea extract (LTE) including the antioxidant and anti-inflammatory and apoptotic induction properties, I firstly used several types of column chromatography to separate the bioactive fractions of loquat tea by antioxidant activity-guided fractionation, and then measured the antioxidant effects *in vitro* and in cellular levels. Since antioxidant activities are linked to anti-inflammatory activities by modulating the signal transduction pathways, I next clarified the

anti-inflammatory effects and molecular mechanisms of LTE in both cell and animal models. Due to accumulated data suggested the compounds possess antioxidant and anti-inflammatory properties that also have anticancer activity, I investigated apoptotic induction activity and underlying mechanisms of LTE. Finally, I used the methodologies of high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) to characterize bioactive compounds contributing to the antioxidant and anti-inflammatory and apoptotic induction activities in LTE.

1.9 References

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Chapter 2

Antioxidant activities of loquat (*Eriobotrya japonica*) tea *in vitro* and cellular mechanisms

2.1 Abstract

Loquat tea is made from roasted loquat leaves *(Eriobotrya japonica*) to 350°C. Although accumulated data revealed that fresh loquat leaves showed health benefits including biodefense enhancement, the bioactivity regarding roasted loquat leaves is not known yet. The aim of this study is to investigate the antioxidant activity of loquat tea extract (LTE) *in vitro* and in cultured cells. Active fractions of roasted loquat tea were separated by different column chromatographs. Antioxidant activities were estimated by ORAC, DPPH and ROS assay. Expression of antioxidant proteins (HO-1 and Nrf2) was detected by Western Blotting analysis. The result showed that LTE possessed stronger antioxidant activity. Cellular data revealed that LTE suppressed the production of reactive oxygen species (ROS) via upregulating Nrf2-mediated HO-1 expression.

2.2 Introduction

Loquat (*Eriobotrya japonica*) belongs to the Rosaceae family. All parts of loquat, such as fruits, leaves, and peels have been reported to have health benefits. In particular, the leaves have higher flavonoid content than the peel or fruits, with stronger radical scavenging activity [1] and have been reported to have preventive effects against oxidation, skin diseases, diabetes, chronic bronchitis, coughs, phlegm, ulcers, allergies, and cancer [2-7]. Recently, fresh loquat leaves were processed into a beverage, called loquat tea, after the

fresh leaves are roasted to 350°C. However, the scientific evidence supporting the functions is still minimal.

Dietary antioxidants can scavenge reactive oxygen species (ROS) which are implicated in wide range of human diseases such as atherosclerosis and certain cancers and they may increase the endogenous defense potential [8]. The major forms of ROS are superoxide, hydrogen peroxide, and hydroxyl radical that are generated through oxidative stress reflection. ROS productions can be decreased by Heme-oxyganese (HO-1) which is detoxifying and antioxidant enzyme [9]. The induction of HO-1 expression by pharmacological agents may represent a therapeutic mechanism for the treatment of various diseases through cytoprotective activity including antioxidant [10], anti-inflammatory [11] and anti-proliferative properties [12]. HO-1 induction is primarily regulated by activation of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2). Under normal condition, Nrf2 is bound to kelch-like ECH-associated protein 1 (Keap1). However, exposure to oxidative stress or other stimuli lead to dissociation of Nrf2 from keap1 and translocation to the nucleus in which Nrf2 upregulates the basal and inducible expression of numerous detoxifying and antioxidant genes, including HO-1 [13,14, 15].

The aim of the present study was to investigate the antioxidant activity of loquat tea. Therefore, fresh loquat leave and different fractions of loquat tea were extracted by boiling water according to the folk customs. The antioxidant activities from the different fractions of loquat tea extracts (LTE) were compared with fresh loquat leave by oxygen radical absorbance capacity (ORAC) assay and 1-diphenyl-2-picrylhydrazyl (DPPH) assay *in vitro*. Since RAW264.7 cells can be used to mimic a state of oxidative stress and inflammation [16,17] the effects on the production of ROS by dichlorofluorescein-diacetate (DCFH-DA) assay, expressions of HO-1 and Nrf2 antioxidant proteins by western blot analysis were further examined in lipopolysaccharide (LPS)-activated RAW 264.7 cells.

2.3 Methods

2.3.1 Fresh loquat leaves and loquat tea extraction

Loquat leaves were washed and dried, then roasted to 350°C for in a ceramic vessel. Then, for both the fresh and roasted samples, the leaves were boiled at 100ºC for 15 minutes. The supernatant fluid of roasted leaves (T fraction) was then separated by MCI gel column, and A, B, C, and D fractions were obtained by eluting with water, 30% EtOH, 50% EtOH, and 100% acetone, respectively. According to the antioxidant activity-based purification, the C fraction was further separated with ODS gel column, and C1-C9 fractions were finally obtained by elution with 10 to 90 % MeOH. All of extracts and fractions were evaporated and stored at -20 °C until use.

2.3.2 Total oxygen radical absorbance capacity (ORAC)

A mixture of 100 µl of uranine fluorescein (7.5 nM) was used as target of free radical attack, and 40 µl of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (63.4 mM) was used as a peroxyl radical generator, combined with 10 µl of each RLL extract in black 96-well plate at 37 ºC. Trolox standard ranged from 50 to 400 µM. The decrease in fluorescence was determined by collecting data for excitation of 535 nm and emission of 595 nm every minute for 120 min with a Wallac ARVO^{TM} SX 1420 Multilable Counter (Perkin Elmer Life Sciences, Singapore). The ORAC value was evaluated as the area under curve (AUC) and calculated by taking into account the Trolox reading using the following equation: ORAC= $[AUC_{sample}–AUC_{buffer}]/[AUC_{Trolox}–AUC_{buffer}]$×Dilution factor of sample×initital concentration of Trolox (18). The unit of ORAC values was showed by Trolox equivalent µmol/g (dry basic).

2.3.3 DPPH assay

The radical scavenging activity of LTE and its fractions were measured by the DPPH (1-diphenyl-2-picrylhydrazyl) method [19]. Briefly, ten microliters of each extraction fraction (1mg/ml) was mixed with 190 µl of 100 µM DPPH in 96-well plates and a final concentration of 50 µg/ml. The plate was covered with aluminum foil and left for 30 minutes at room temperature, with the samples being mixed every 10 minutes. The absorbance was then measured at 492 nm with a microplate reader (Thermo Scientific Multiscan FC, version 1.00.79). 6-Hydroxy -2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox), which has high antioxidant capacity, was used as a standard. The percentage activity of DPPH scavenging was calculated with the formula $(A_0\text{-}A_1/ A_0) \times 100$ where A_0 was the absorbance of the control, and A_1 was the absorbance of LTE and its fractions [20].

2.3.4 Cell culture

Murine macrophage-like RAW 264.7 cells were purchased from RIKEN Bioresource Center Cell Bank of Japan (RCB0535) and cultured at 37ºC in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 1% of penicillin and streptomycin, and 2% glutamin. Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX, USA), and LPS (Escherichia coli Serotype 055:B5) was purchased from Sigma (St. Louis, MO, USA).

2.3.5 Cell viability assay

The cell survival rate was measured by a MTT assay [21]. Briefly, RAW 264.7 cells (2×10^4 cells/100 µl) were seeded into each well of 96-well plates. After an incubation period of 24 hours, the cells were treated with different concentrations of LTE or its fractions for 12 hours. Then, 10 µl of MTT solution (5 mg/ml) was added to each well. After incubating

the cells for another 4 hours, the resulting MTT-formazan product was dissolved by adding 100 µl of 0.04 N HCl-isopropanol solution. The amount of formazan was determined by measuring the absorbance at 595 nm in a microplate reader (Thermo Scientific Multiscan FC, version 1.00.79). The results were expressed as the optical density ratio of the treatment to control.

2.3.6 Measurement of ROS production

Intracellular ROS were determined using the oxidation-sensitive dichlorofluorescein-diacetate (DCFH-DA) fluorescent dye. RAW 264.7 cells were seeded into 96-well plates at a starting density of 2×10^4 cell/well. After pre-incubation for 24 hours, the culturing medium was replaced with a fresh one. The cells were treated with or without LTE and its fractions for 30 minutes before exposure to LPS (1µg/ml) for 12 hours, and DCFH-DA with a final concentration of 20 µM was then added for an additional 2 hours. Fluorescence was measured at 485 nm excitation and 530 nm emission using a fluorescent Multilable Counter (Perkin-Elmer). The relative amount of intracellular ROS production was expressed as the fluorescence ratio of the treatment to control.

2.3.7 Western blot analysis

Western blotting was performed as described previously [22]. RAW 264.7 (1×10^6) cells were pre-cultured in 6-cm dish for 21 hours and then starved by being cultured serum-free another 2.5 hours to eliminate the influence of FBS. The cells were treated with LTE and its fractions for 30 minutes and then exposed to LPS (40 ng/ml) for 12 hours. Equal amounts of lysated protein were separated on SDS-polyacrylamide gel and transferred onto the PVDF membrane. Afterwards, the membrane was blotted at room temperature for 2 hours in blocking buffer and incubated with specific primary antibody overnight at 4 ºC, following

a three-time wash with TBS-Tween solution. The membrane was further incubated for 1 hour with HRP-conjugated secondary antibodies and washed three times again. Band intensities of bound antibodies were detected by ECL system in a luminivision PRO machine (TAITEC Co., Japan). Antibodies against HO-1, Nrf2 and α-tubulin were from Santa Cruz Biotechnology (CA, USA).

2.3.8 Statistical Analysis

All data were statistically analysed by one way ANOVA. Differences were considered significant at $p < 0.05$ and $p < 0.01$.

2.4 Results

2.4.1 *In vitro* antioxidant activities of fresh loquat leave and LTE

Loquat tea was extracted in boiling water for 15 min to obtain tea water extract (T), which was then separated with MCI gel to obtain A (water extract), B (30% EtOH extraction), C (50% EtOH) and D fraction (100% acetone). The fraction C was further separated by ODS gel column to obtain C1-C9 fractions eluted by 10~90% MeOH. The antioxidant activity of the water extracts and separated fractions were determined by ORAC assay. The ORAC values of L, T, A, B, C, D and C1-C9 fractions were shown as Trolox equivalents (TE) in dry basis of each sample (μmol TE/g dry basis) in the boxes of Fig.1. ORAC values of L, T, A, B, C and D fractions were 910.6 ± 25.1, 2146.3 ± 123.5, 2591.3 ± 108.7, 2922.8 ± 162.1, 3076.4 ± 28.0 and 1000.9 ± 82.2 μmol TE/g, and C1-C9 were 3000.8 ± 292.2, 3201.2 ± 186.0, 2552.8 ± 5.8, 2412.6 ± 158.3, 2013.0 ± 11.8, 1417.8 ± 80.9, 1174.0 ± 22.7, 1419.9 ± 107.5 and 1552.8 ± 13.1 μmol TE/g, respectively (**Fig 2.1**). Of them, C and C2 fractions showed higher ORAC values.

I also examined antioxidant activities of LTE and its fractions using DPPH assay. As shown in Figure 2.2**,** DPPH scavenging activities of fresh (L) and LTE (T) at the concentration of 50 μg/ml were 18.34 and 44.8, respectively. Thus, LTE possessed higher antioxidant activity than fresh loquat leaves. Furthermore, the DPPH value of fraction A, B, C or D fractionated from LTE (T) were 44.24, 54.00, 69.31 and 25.24 % at the concentration of 50 μg/ml, respectively (**Fig. 2.2A**). According to antioxidant activity-guided purification, C fraction was further separated into C1~C9 fractions by ODS gel column. Their DPPH scavenging activities at the concentration of 50 μg/ml were 69.02, 75.00, 68.52, 67.61, 65.28, 38.54, 21.44, 27.25 and 49.67%, respectively (**Fig. 2.2B**), suggesting that C1~C5 fractions contain higher antioxidant activity than C6~C9. It was found that DPPH scavenging activity was highly positive correlated with ORAC value (R^2=0.909 and 0.815) (**Fig 2.3**). Depending on antioxidant assays, I selected C and C2 fractions for subsequent studies due to their higher ORAC values and stronger DPPH scavenging activity.

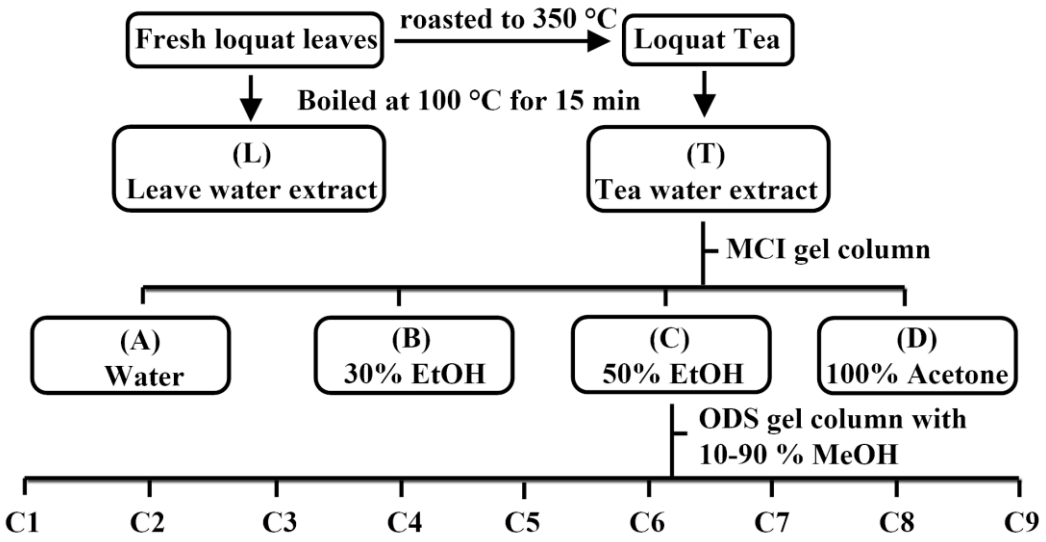


Fig 2.1 Fractionation and ORAC values of LTE. Loquat tea and fresh loquat leaves were extracted in boiling water for 15 min. The tea water extract (T) was then separated with MCI gel and ODS gel column. L, leave water extract; T, tea water extract; A, fraction A (water extract); B, fraction B (30% EtOH extraction); C, fraction C (50% EtOH); D, fraction D (100% acetone); C1-C9, fractions from C fractionated by 10~90% MeOH. The antioxidant activity of water extracts and separated fractions were determined by ORAC assay. The ORAC values of L, T, A, B, C, D and C1-C9 fractions were shown as Trolox equivalents (TE) in dry basis of each sample (μmol TE/g dry basis) in the boxes, respectively. Each value represents the mean ± S.D. of three separated experiments.

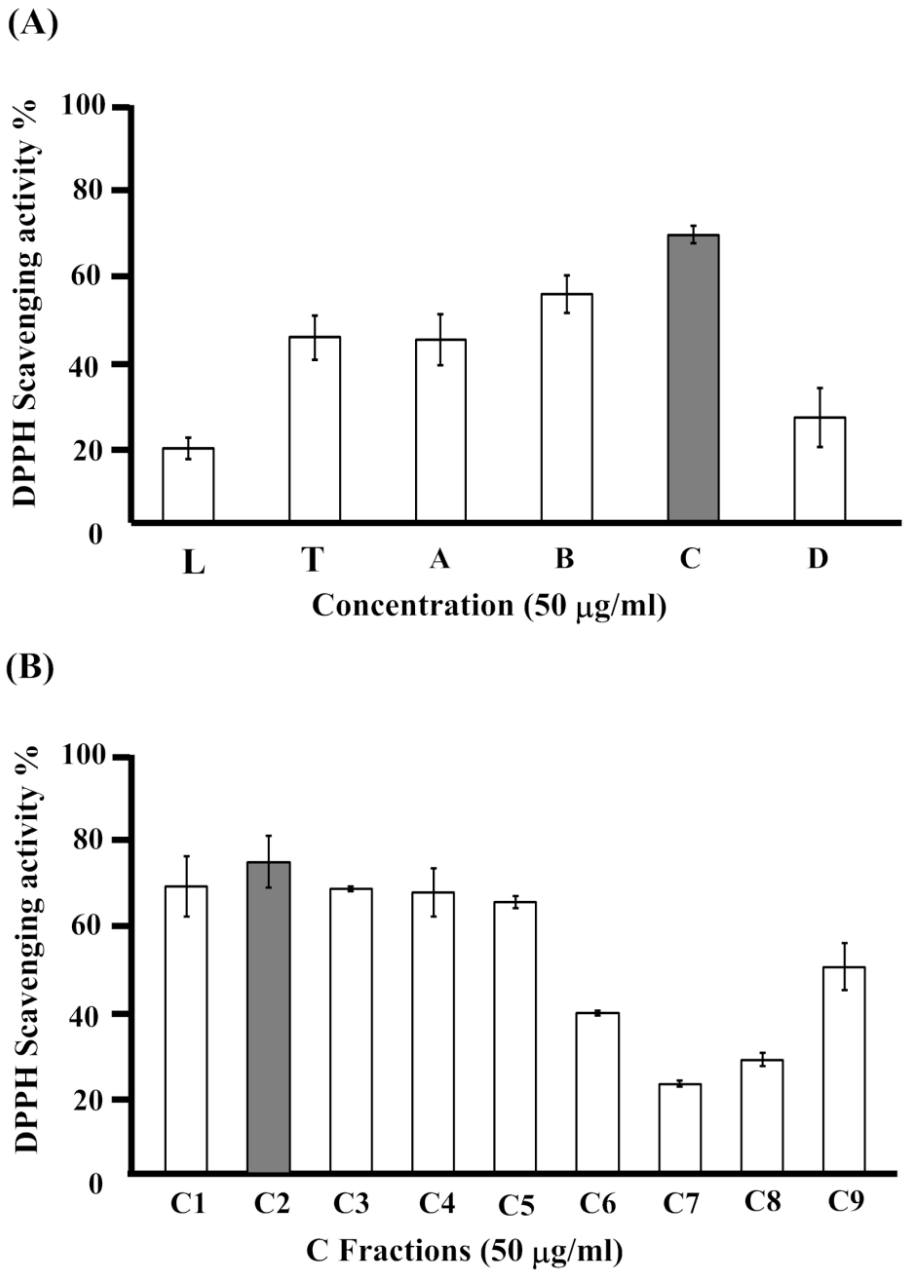


Fig 2.2 DPPH radical scavenging activities of LTE.

The values were expressed as the percentage of control value. L, leave water extract; T, tea water extract; A, fraction A (water extract); B, fraction B (30% EtOH extraction); C, fraction C (50% EtOH); D, fraction D (100% acetone); C1-C9, fractions from C fractionated by 10~90% MeOH. The DPPH scavenging activities of L, T, A, B, C, D and C1-C9 fractions were shown as percentage of control trolox. The data represent the mean ± SD of three separated experiments.

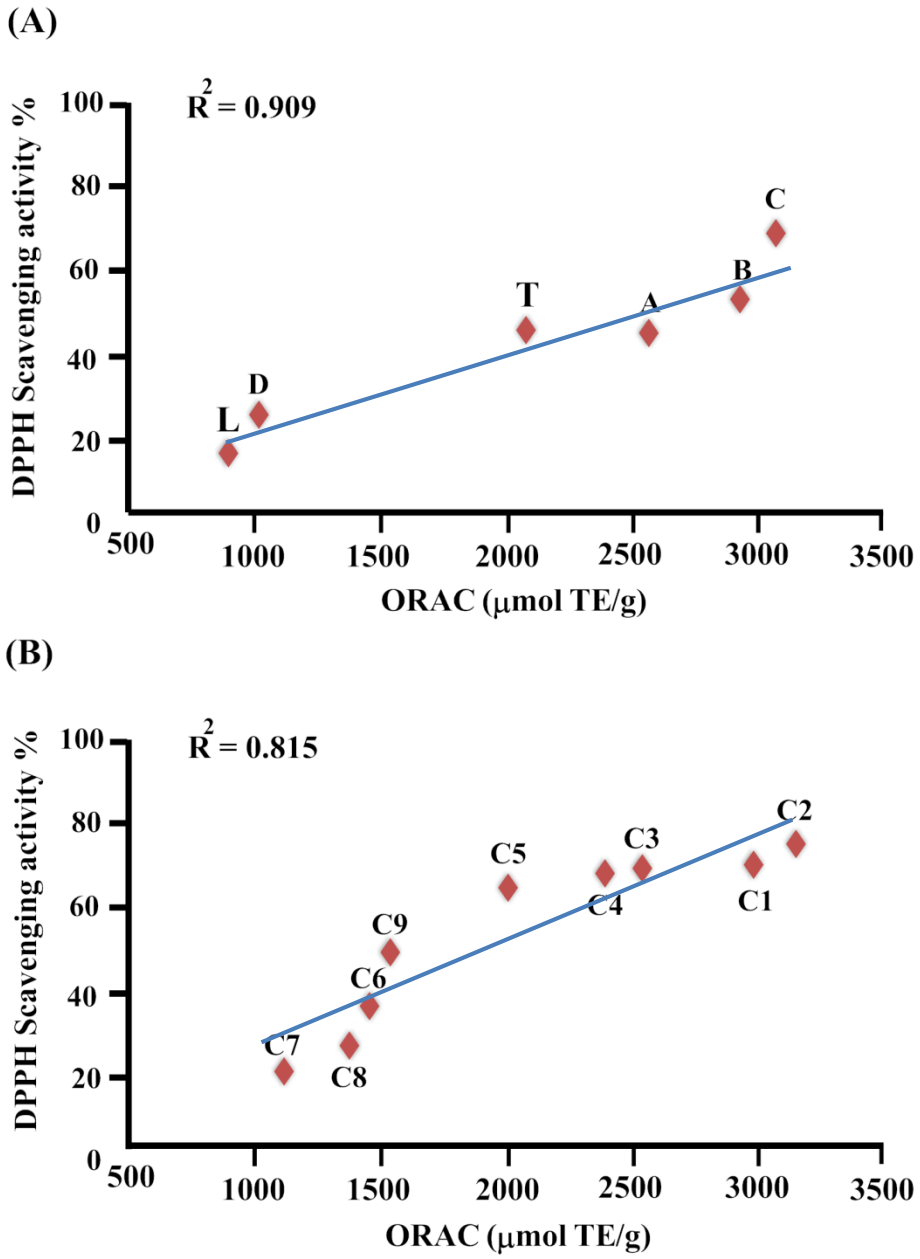
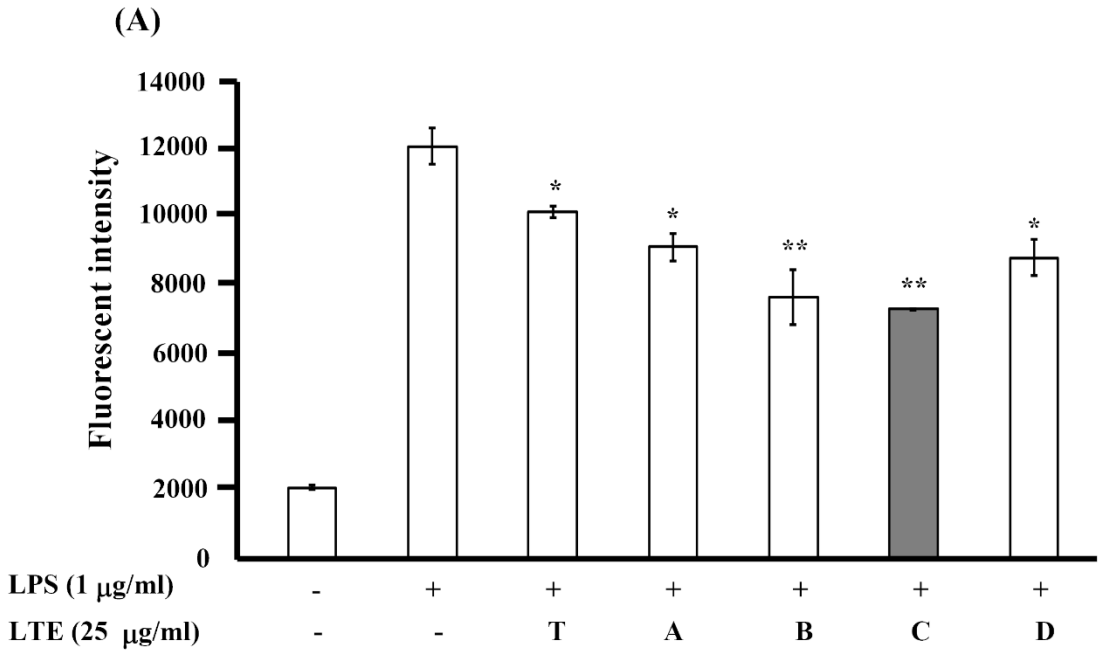


Fig 2.3 The correlation between DPPH scavenging activity and ORAC values.

L, leave water extract; T, tea water extract; A, fraction A (water extract); B, fraction B (30% EtOH extraction); C, fraction C (50% EtOH); D, fraction D (100% acetone); C1-C9, fractions from C fractionated by 10~90% MeOH.

2.4.2 Antioxidant activities of LTE in cell model

To investigate whether the LTE also show antioxidant activity in cellular level, we measured the change of ROS level in LPS-activated RAW 264.7 cells with or without treatment of LTE, using DCFH-DA fluorescent dye. As shown in Figure 2.4**,** LPS induced ROS production (lane 2), and C fraction showed highest inhibitory effect on LPS-induced ROS among T, A, B, C and D fractions at the concentration of 25 μg/ml (**Fig. 2.4A**). We further investigated the inhibitory effects of C and its C2 fractions on LPS-induced ROS, and found a dose-dependent inhibition on the ROS at the concentrations of 0-50 μg/ml (**Fig. 2.4B**). These data revealed that LTE and its fractions also had antioxidant activity in cellular level. The cell viability at indicated concentrations was not affected (data did not show).



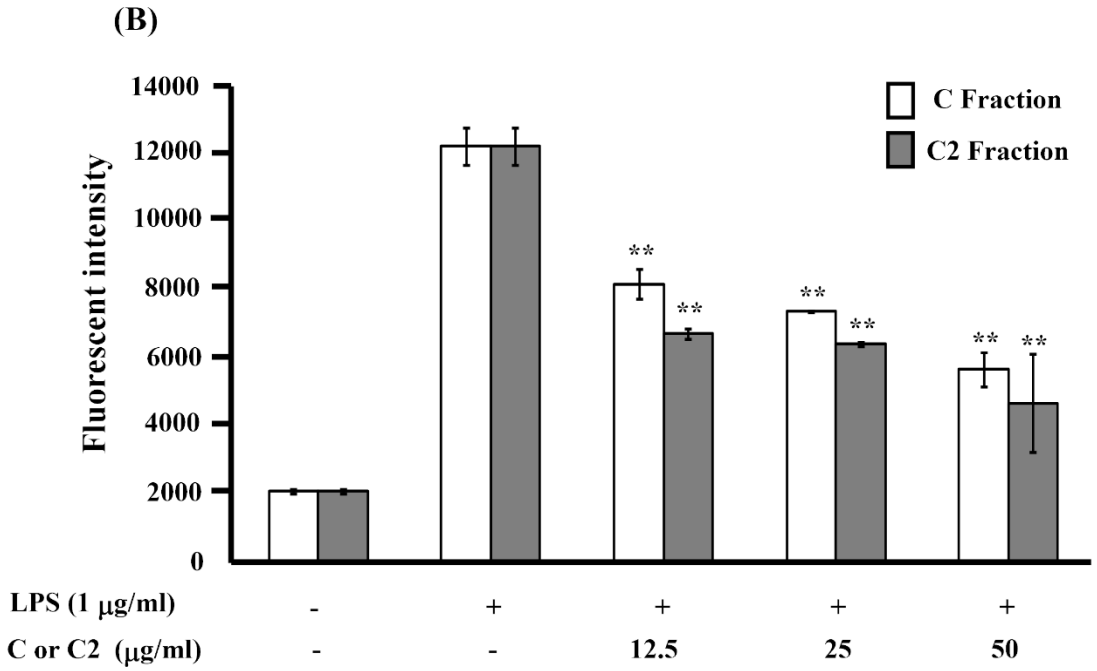


Fig. 2.4 Inhibition of LTE on ROS production in LPS-activated RAW 264.7 cells.

(**A**) LTE fractions and (**B**). C fractions RAW264.7 cells were seeded into 96-well plate (2×10^4 cells/well) and pre-cultured for 24 h. The cells were treated with or without LTE fractions at different concentrations for 30 min before exposure to LPS (1 μg/ml) for 12 h. DCFH-DA was then added to the medium with a final concentration of 20 μM for an additional 2 h. The fluorescence intensity was then measured at an excitation (485 nm) and emission (530 nm) wavelength using a fluorescent Mutilabel Counter (Perkin-Elmer), and was expressed as the percentage of control in the absence of LPS. Data are the mean ± SD of three separated experiments. Asterisk shows significant inhibition to LPS only ($P< 0.05$) and ($P< 0.01$).

2.4.3 Effect of LTE on HO-1 production in LPS-stimulated RAW 264.7 cells

HO-1 is an antioxidant protein and up-regulation of HO-1 expression in cells has been demonstrated to play an important role in protection against oxidative injury. Therefore, I examined the effect of C fraction of LTE with different time interval on HO-1 induction in RAW 264.7 macrophages cells. C fraction and 2.5 μM N-acetyl L-cysteine (NAC) enhanced HO-1 expression beginning at 6 h continued through 24 h while treatment of the cells with LPS (40 ng/ml) showed the induction of HO-1 expression beginning at 12 h and stable for 18 h (**Fig 2.5A**). The maximum level of HO-1 expression occurred at 12 h. Therefore, I examined the effect of C fraction on HO-1 activity with different concentrations at 12 h after LPS treatment. C fraction dose dependently increased HO-1 expression that overwhelms the expression by LPS treated alone (**Fig 2.5B**). The cell viability at indicated concentrations was not affected (data did not show).

2.4.4 Effect of LTE on Nrf2 expression in LPS-stimulated RAW 264.7 cells

Induction of HO-1 expression is modulated by the Nrf2 activation [23]. Thus, I attempted to examine whether C fraction could activate Nrf2 in associated with HO-1 up-regulation. For this, cells were treated with 50 and 100 μg/ml of LTE for 12 h and western blot analysis was conducted. Treatment of C fraction dose dependently increased Nrf2 expression (**Fig 2.5B**).

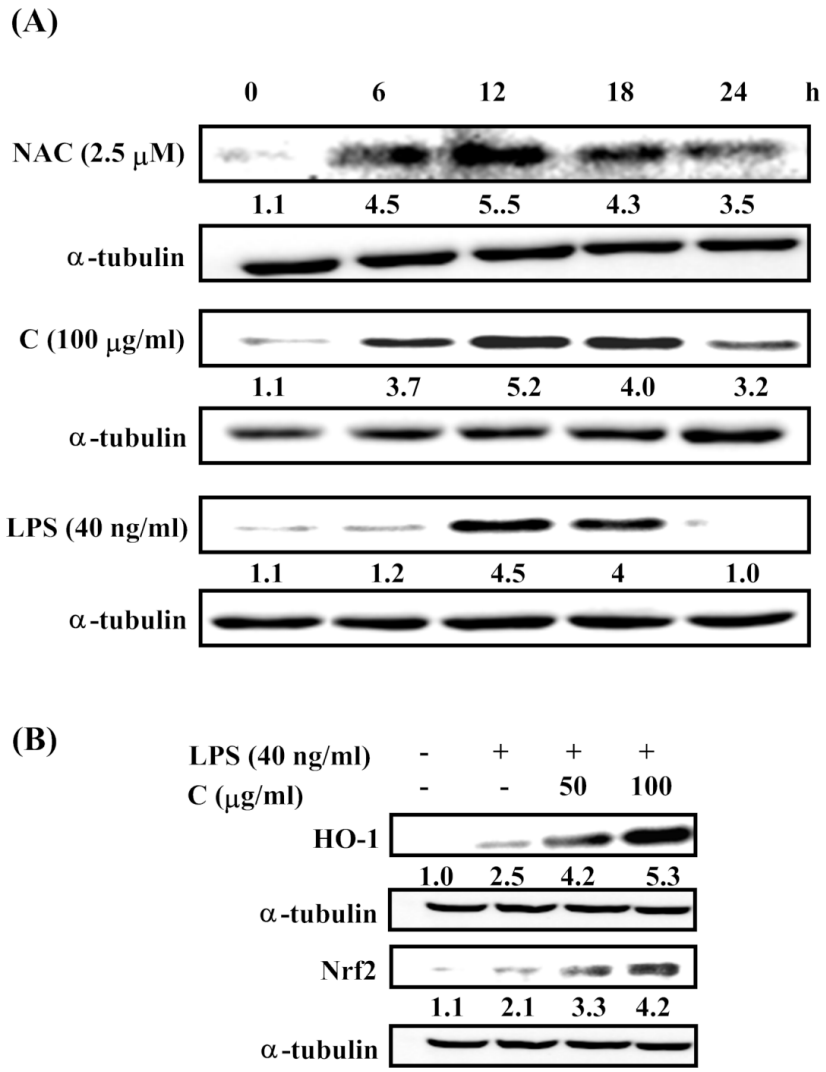


Fig 2.5 Time dependent HO-1 expression and dose dependent Nrf2 mediated HO-1 expression of LTE.

HO-1 expression in RAW 264.7 cells in 0, 6, 12, 18 and 24 h, after stimulation with NAC (2.5 μM) , C fraction (100 μg/ml) and LPS (40 ng/ml) (**A**). LTE induced Nrf2 mediated HO-1 expression in RAW 264.7 cells (**B**).The cells were pretreated by C and C2 fractions of LTE in indicated concentrations for 30 min and then stimulated with LPS (40 ng/ml) for another 30 min. HO-1 and Nrf2 were detected by Western blotting analysis with their antibodies respectively. The values show the densitometric fold of phosphorylation protein to their normal proteins. The data represent the mean ± SD of three separated experiments.

2.5 Discussion and conclusion

Loquat tea is made from loquat leaves roasted at 350°C for 30 minutes, and usually used as beverage according to the folk customs. Although fresh loquat leaves have been reported to have biological activities such as antioxidant and anti-inflammatory activities [24-25], there is no report on the biological activities of roasted loquat leaves (loquat tea). In the present study, I used antioxidant activity-guided fractionation to investigate the antioxidant activities of loquat tea. The antioxidant potency of LTE and its fractions were measured by 3 assays (DPPH, ORAC and ROS). Loquat tea fractions revealed the stronger antioxidant potency by increasing total oxygen radical absorbance capacity (ORAC) and radical scavenging activity (DPPH) *in vitro*. However, *in vitro* assays cannot intensively reflect to antioxidant activity of a sample due to their measurements performed under defined conditions and based on chemical reactions [26]. It has been reported that the antioxidant potency can be mediated by the following mechanisms: (1) scavenging radical species such as ROS/RNS; (2) suppressing ROS/RNS formation by inhibiting some enzymes; (3) upregulating or protecting antioxidant defense [27]. To fully understand the antioxidant activity of LTE, effect on ROS production at a cellular level was determined using macrophage cell model. Reactive oxygen species (ROS) include hydroxyl radical (OH), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and is implicated in a wide range of oxidative stress reflection (Halliwell, 1991). LTE could decrease amount of ROS at cellular level and the order of antioxidant potency was consistent across different assays. Reactive nitrogen species (RNS) derived from reaction of nitric oxide (NO) and superoxide anion produced via nitric oxide synthase enzyme (iNOS) and NADPH oxidase which is highly oxidant causing cellular oxidative and nitrosative stress and is associated with numerous pathologies [29]. The additional experiment to determine antioxidant potency is measuring of RNS by western blot analysis (**see chapter 3**). Loquat tea suppressed NO production via

inhibiting iNOS expression (**Fig. 3.3** and **3.4**). Although loquat tea inhibited ROS/RNS production, molecular mechanisms of loquat tea on inhibition was unknown yet. Therefore, I studied how loquat tea inhibited ROS/RNS production by using LPS stimulated RAW 264.7 cells. Heme oxygenase-1 (HO-1) is a stress-inducible enzyme which provides potent antioxidant functions and decrease ROS/RNS productions [30-31]. And increased HO-1 expression has been occurring in response to oxidative stress that contributes to cellular redox and chemoprotective defense systems [11]. The present study showed that C fraction increased HO-1 expression (**Fig 2.5A & B**). Upregulation of HO-1 is mediated by activations of relevant redox-sensitive signal pathways such as Nrf2, AP-1 and NF-κB [13-14]. Nevertheless, many reports suggest that HO-1 induction is dependent on the activation of Nrf2 in oxidative stress. During oxidative stimulation, activated Nrf2 dissociates from Keap1 and translocates to the nucleus and then upregulates HO-1 expression [11,13]. Thus, I tested whether LTE increased HO-1 expression via enhancing Nrf2 activation or not. As shown in **Fig. 2.5B**, LTE enhances the activation of Nrf2 expression.

In conclusion, loquat tea, made from roasted loquat leaves, revealed stronger antioxidant activity *in vitro* and at cellular level by reducing ROS production via Nrf2 mediated HO-1 activation.

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Chapter 3

Anti-inflammatory effects and molecular mechanisms of loquat (*Eriobotrya japonica*) tea

3.1 Abstract

The previous chapter showed that loquat *(Eriobotrya japonica*) tea, made from roasted loquat leave to 350 °C for 30 min revealed antioxidant activities *in vitro* and cellular mechanisms. Many researcher reported that anti-oxidative and anti-inflammatory reactions are linked to reduce and treatment of chronic inflammatory diseases. This chapter aims to clarify the anti-inflammatory effects and molecular mechanisms of loquat tea extracts (LTE), in both cell and animal models. LTE, especially C fraction, inhibited the production of pro-inflammatory factors including cyclooxygenase-2 (COX2), prostaglandin E_2 (PGE_2), inducible nitric oxide synthase (iNOS), nitric oxide (NO), interleukin-6 (IL-6), regulated on activation, normal T cell expressed and secreted (RANTES) and tumor necrosis factor-α (TNF-α). Cellular signaling data revealed that the downregulation of TGF-β-activated kinase (TAK1)-mediated both mitogen-activated protein kinase (MAPK) and NF-κB pathways were involved in the inhibition of pro-inflammatory factors by C fraction. Mouse paws edema model further confirmed the *in vivo* anti-inflammatory effects of LTE.

3.2 Introduction

Inflammation is the first physiological defense system and it has two kinds; short term inflammation and long term inflammation. Long term inflammation occurred in many kinds of inflammatory diseases, during inflammatory disease, the primary cells of chronic inflammation are macrophages that produce excess amounts of mediators such as

prostaglandin E_2 (PGE_2), nitric oxide (NO) and pro-inflammatory cytokines [1, 2]. COX-2 is one of the most pivotal enzymes which is induced by proinflammatory stimuli and growth factors (LPS) and it is responsible for the production of PGE_2 at the inflammatory sites [3, 4]. Inhibition of overproduction of PGE_2 in macrophage by inhibiting COX-2 expression may have therapeutic potential in inflammatory diseases.

NO plays a key role in the various forms of inflammation and carcinogenesis [5, 6]. NO overproduction leads to various harmful responses including apoptosis and necrosis [7], as well as organ failure in autoimmune diseases [8]. NO is generated in three isoforms of endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [9]. Of these, iNOS is only induced by various inflammatory factors such as lipopolysaccharide (LPS) in macrophages, and further catalyses NO synthesis. Continuous production of iNOS has been linked to the development of cancer, autoimmune disorders and coronary artery disease [10]. Pro-inflammatory cytokines/chemokines recruit, alert and activate neighboring cells for mounting an effective immune response. Therefore, cytokines play a pivotal role in consequences of inflammation [11].

Regarding the cellular signaling pathways regulating the inflammation, mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways have been suggested to be the key regulators in the expression of inflammatory mediators including COX-2, iNOS and cytokines [1,2,4]. MAPK signal pathway includes extracellular signal-regulated kinase (ERK), p38 MAPK (p38) and c-Jun NH_2-terminal kinase (JNK) [12]. These kinases can be activated after phosphorylation by bacterial LPS [13]. Activation of MAPK contributes to the production of inflammatory mediators such as COX-2, iNOS and cytokines in activated macrophages [14; 15].

NF-κB pathway includes IκB kinase (IKK), I kappa B (IκB) and nuclear factor kappa B (NF-κB). Many stimuli including LPS, cytokines and virus activate NF-κB via several signal

transduction pathways, leading to IKK α and β activation and then it phosphorylates IκB [16,17]. IKK α and β are activated by phosphorylation which is stimulated by phosphorylation of TAK-1 [18,19]. I-κB degradation releases NF-κB from the complex, which enters the nucleus to transactivate a large variety of target genes such as COX-2, iNOS and cytokines [17]. Moreover, activation of NF-κB and Toll like/IL1 receptor domain containing adaptor inducing IFN-β (TRIF) dependent pathways contribute to the production of inflammatory cytokines productions in response to LPS. TRIF is activated by phosphorylation of interferon regulating factor 3 (IRF-3) IRF-3 via TANK-binding kinase 1 (TBK1) and IKK activations [4].

Based on the properties of loquat tea and the information of inflammation processes, I investigated the anti-inflammatory effects and molecular mechanisms using both cell and animal models in the present study. First of all, we used mouse macrophage-like cells (RAW264.7), which can be stimulated with LPS to mimic a status of infection and inflammation, to screen the influence of loquat tea extracts on the productions of COX-2, PGE_2, iNOS, NO and cytokines, and then chose C fraction, a strongest inhibitor among them, to investigate the molecular mechanism involved. Finally, I confirmed the anti-inflammatory effects *in vivo* using a mouse paw edema model.

3.3 Materials and methods

3.3.1 Reagents and cell culture

Mouse macrophage-like RAW 264.7 cells were purchased from Cell Bank (cell RCB0535), Bioresource Center of the Institute of Physical and Chemical research (RIKEN, Tokyo, Japan), and cultured at 37 ºC in a 5% CO_2 atmosphere in Dulbecco's minimum essential medium (DMEM) containing 10% FBS, 1% PS and 2% glutamine. Foetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX, USA) and LPS (*Escherichia*

coli Serotype 055:B5) from Sigma (St. Louis, MO, USA). Antibodies against phosphor-ERK1/2, phosphor-p38 kinase, phosphor-JNK, ERK1/2, p38 kinase, JNK, phosphor-TAK1 (Thr184/187), phospho-IKKα/β (ser176/180), IκB-α and phospho-IRF3 were purchased from Cell Signaling Technology, Beverly, MA, USA. Antibodies against iNOS, TAK-1, IKKα/β and α-tubulin were from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

3.3.2 Cell viability assay

The cell survival rate was measured by a MTT assay according to previous authors [20]. Briefly, RAW 264.7 cells (2×10^4cells/well) were seeded into each well of 96-wells plates. After incubation for 24 h, the cells were treated with different concentrations of LTE for 12 h. Then MTT solution (5 mg/ml) was added to each well. After incubating the cells for another 4 h, the resulting MTT-formazan product was dissolved by the addition of 0.04 M HCl-isopropanol solutions. The amount of formazan was determined by measuring the absorbance at 595 nm in a microplate reader (Thermo Scientific Multiskan^{TM}, version 1.00.79, Finland). The results were expressed as the optical density ratio of the treatment to control.

3.3.3 Measurement of PGE_2 production

PGE_2 in culture medium was measured with a PGE_2 enzyme immunoassay kit (Cayman Co., St. Luris, MO, USA) according to manufactrer's manual [12]. In brief, RAW 264.7 cells (5×10^5 cells) were seeded into each well of 6-well plates. After incubation for 24 h, the cells were starved by being cultured in serum-free for another 2.5 h to eliminate the influence of FBS, and then treated with or without LTE and its fractions for 30 min before exposure to LPS (40 ng/ml) for 12 h. The amount of PGE_2 released into the medium was determined by measuring absorbance at 405 nm with a microplate reader.

3.3.4 Measurement of NO production

Nitric oxide production in cultural supernatant was determined by Griess [20]. Briefly, RAW 264.7 cells (3×10^5 cells/0.5 ml/well) were seeded in 48-wells plate. After pre-incubation for 21 h, cells were starved by being cultured in serum free for another 2.5 h to eliminate the influence of FBS. The cells were treated with or without LTE for 30 min before exposure to LPS (40 ng/ml) for 12 h. One hundred microliters of each supernatant were mixed with the same volume of Griess reagent (1% sulphanilamide in 5 % phosphoric acid and 0.1 % N-(1-naphthyl) ethylenediamide dihydrochloride in distilled water), and the absorbance was read at 550 nm wavelength.

3.3.5 Measurement of cytokines productions

Cytokines production in cultural supernatant was determined by Bioplex mouse cytokine assay [21]. Briefly, RAW 264.7 cells (1.2×10^5 cells/1 ml/ well) were seeded in 12-wells plate. After pre-incubation for 21 h and starved in serum-free medium for 2.5 h, the cells were treated with or without LTE for 30 min before exposure to LPS (40 ng/ml) for 12 h. The culture medium without any dilution was used to assay the cytokine production with Bio-Plex Pro Mouse Cytokine 23-Plex Panel kit (Bio-Rad Laboratories, Hercules, CA, USA) for 23 cytokines including interleukin-1alpha (IL-1α), interleukin-1beta (IL-1β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-12 p40 (IL-12 p40), interleukin-12 p70 (IL-12 p70), interleukin-13 (IL-13), interleukin-17 (IL-17), Eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), keratinocyte chemoattractant (KC), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1α), macrophage inflammatory protein-1beta (MIP-1β), regulated on activation,

normal T cell expressed and secreted (RANTES), and tumor necrosis factor-alpha (TNF-α) in a Bio-Plex machine (Bio-Plex 200 System, Bio-Rad Laboratories, Hercules, CA, USA). The assay was performed according to the manufacturer's instructions and analyzed with the Bio-Plex manager software (version 4.0). The sensitivity of this method was less than 10 (pg/ml), and the assay can accurately detect cytokines in the range of 1-32,000 pg/ml. The blood serum collected from mouse heart blood without any dilution was used for the measurement of cytokines with mouse ELISA Ready-SET-Go kit (eBioscience, San Diego, CA, USA) according to manufacturer's manual.

3.3.6 Western Blot analysis

Western blotting was performed as given by [18]. RAW 264.7 (1×10^6) cells were pre-cultured in 6-cm dish for 21 hour and then starved by being culturing in serum free for another 2.5 hour to eliminate the influence of FBS. The cells were treated with LTE (T, A, B, C, D fraction), fraction C or C2 for 30 min before exposure to LPS (40 ng/ml) or (1 μg/ml) for different times depend on detected proteins. Cellular lysates were boiled for 5 min. Protein concentration was determined by dye-binding protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's manual. Equal amounts of lysate protein (40 μg) were run on 10% SDS-PAGE and electrophoretically transferred to PVDF membrane (GE Healthcare, Buckinghamshire, UK). After blotting, the membrane was incubated with specific primary antibody overnight at 4 °C, and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by using ECL system (GE Healthcare, Buckinghamshire, UK) with a Lumi Vision PRO machine (TAITEC Co, Saitama, Japan). The relative amount of proteins associated with specific antibody was quantified by Lumi Vision Imager software (TAITEC Co, Saitama, Japan).

3.3.7 Induction of mouse paws edema by LPS

The animal experiments were conducted in accordance with the guideline (No. A12004) of the Animal Care and Use Committee of Kagoshima University. Male ICR mice weighing 25 ± 2g were maintained at 25 ºC in a home cage with food and water *ad libitum* on a 12 h light/12 h dark cycle. Nine mice were equally divided into three groups: control, LPS, and LPS plus LTE. C fraction of LTE was injected intraperitoneally (*i.p.*) by 200 mg/kg BW for 4 days while the control group was given by 0.9% normal saline NaCl for 4 days. LPS was then injected *i.p.* by 1 mg/kg BW, and the paw thickness was measured, using digital caliper (model 19975, Shinwa Rules Co. Ltd, Tokyo, Japan) before and every hour after LPS treatment until 6 h. After 6 h, mice were sacrificed and blood serum was collected for cytokines assay [21].

3.3.8 Statistical analysis

All data were statistically analyzed by one way ANOVA and student *t*-test. Differences were considered significant for $p < 0.05$ and $p < 0.01$.

3.4 Results

3.4.1 Inhibition of LTE on PGE_2 production in LPS-stimulated RAW 264.7 cells

Since the antioxidant activity of phytochemicals has been considered to link to anti-inflammation [22], thus, I next investigated the anti-inflammatory activities of LTE in mouse macrophage-like cell RAW 264.7, which is a cell model to investigate inflammation mechanisms. As shown in Figure 3A, LPS-induced PGE_2 productions were significantly attenuated by treatment with LTE (T, A, B, C and D). Moreover, C and C2 fraction strongly inhibited PGE_2 production at the concentration range of 100-200 µg/ml (Figure 3B). In addition, there is no significant difference in the cell viability between the treatments and

controls (**Fig. 3.1A** and **3.1B**). Thus, the inhibitory effects by LTE and its fractions were not caused by their cytotoxicity.

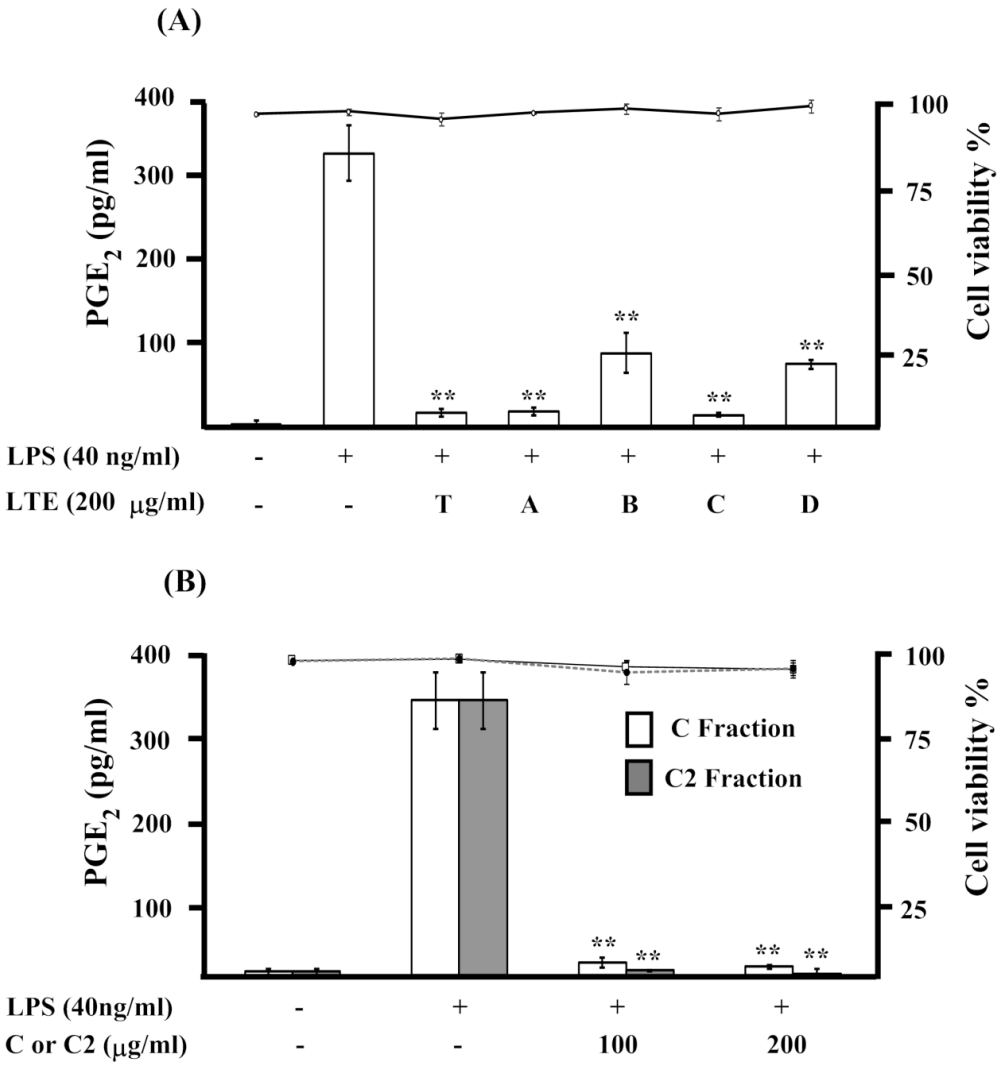


Fig. 3.1 Inhibition of LTE on PGE_2 production and cell viability in LPS-induced RAW 264.7 cells.

(**A**) LTE fractions, (**B**) C and C2 fractions. RAW 264.7 (5×10^5 cells) were pretreated with the indicated concentrations of LTE and its fractions for 30 min and then incubated with LPS (40 ng/ml) for 12 h. The level of PGE_2 production in culture media was determined using enzyme immunoassays (ELISA) kit, and expressed as pg/ml (left vertical axis). Asterisk shows significant inhibition to LPS only ($P< 0.01$). The cell viability was

simultaneously estimated by MTT assay, and expressed as viability percentage to control cell (right vertical axis). The data represent the mean ± SD of three separated experiments.

3.4.2 Inhibition of LTE on COX-2 expression in LPS-stimulated RAW 264.7 cells

PGE_2 is usually synthesized at inflammatory site by the enzyme, cyclooxygenase-2 (COX-2). Thus, we further investigated the effect of LTE and its fractions on the LPS-induced COX-2 expression. LPS-induced COX-2 production was markedly inhibited by LTE at the concentration (200 μg/ml) (**Fig. 3.2A**), especially C and C2 fractions showed a dose-dependent inhibition on LPS-induced COX-2 expression (**Fig. 3.2B** and **3.2C**). As a control, α-tubulin expression was not changed. These results suggested that LTE, especially C fraction, inhibited PGE_2 production by suppressing COX-2 expression.

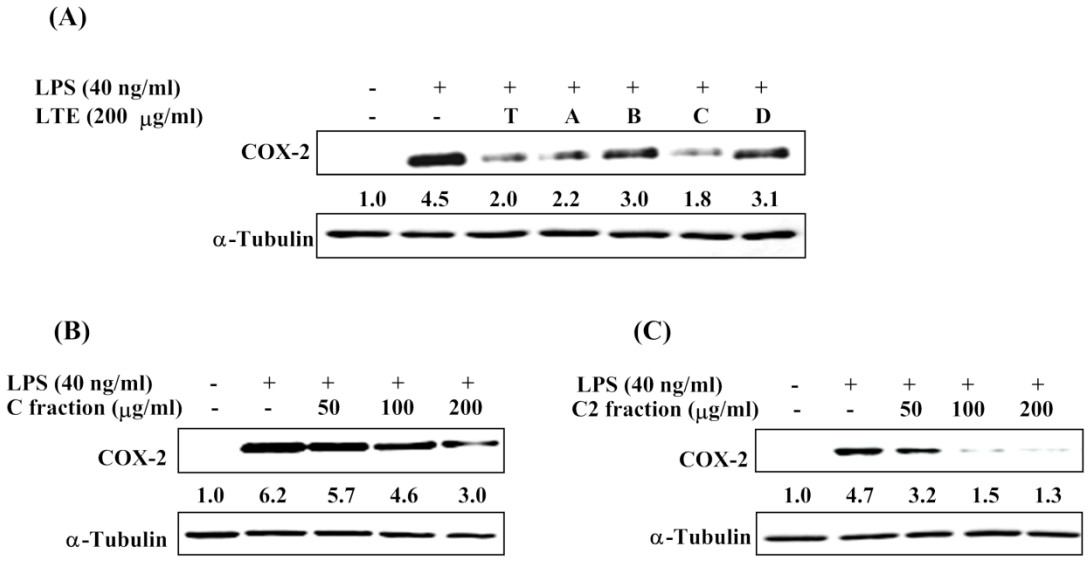


Fig. 3.2 Inhibition of LTE on COX-2 expression in LPS-activated RAW 264.7 cells.

(**A**) LTE fractions (**B**) C fraction and (**C**) C2 fraction. The cells were treated by different fractions of Loquat tea with indicated concentrations for 30 min, and stimulated with 40 ng/ml LPS for 12 h. COX-2 and α-tubulin were detected by Western blotting analysis with their antibodies, respectively. The values show the densitometry fold of COX-2 protein normalized to α-tubulin. The data represent the mean of three separated experiments.

3.4.3 Inhibition of LTE on the production of LPS-stimulated NO

To examine the possibility that LTE inhibits LPS-induced NO production, NO in culture media was measured using Griess reaction [20]. RAW264 cells were treated with LTE at a concentration of 50 µg/ml for 30 min before exposure to LPS (40 ng/ml) for 12 h. LPS treatment increased NO level to 27.0 µM from the basal level of 3.2 µM. Pretreatment with crude extract T, A, B, C and D attenuated NO level to 15.3, 17.3, 20.6, 9.0 and 9.2 µM (**Fig. 3.3A**). Moreover, C and C2 fractions inhibited NO production in a dose-dependent manner (**Fig. 3.3B)** at the concentration range of 12.5-100 µg/ml. In addition, there was no significant difference in the cell viability between the treatments and controls (top curve in **Fig. 3.3A** and **3.3B**). Thus, the inhibitory actions by LTE and its fractions were not caused by its cytotoxicity.

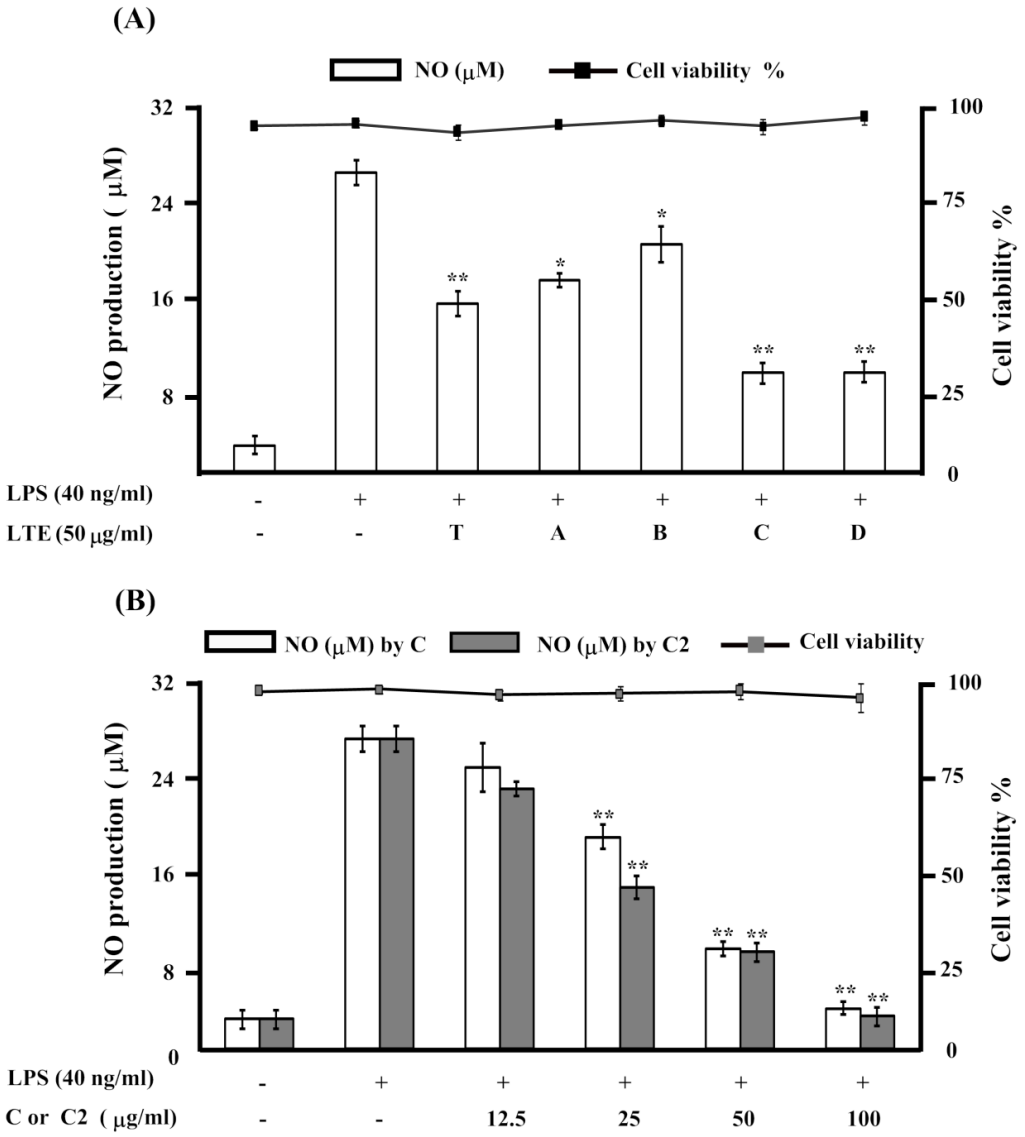


Fig. 3.3 Inhibition of LTE on on NO production in LPS-activated RAW 264.7 cells.

(**A**) LTE fractions, (**B**) C and C2 fractions. RAW264.7 cells (3×10^5 cells) were pre-cultured for 21 h, and starved in serum-free medium for 2.5 h. The cells were then treated with 50 μg/ml of crude extracts (T, A, B, C, D) or with 12.5, 2.5, 50 and 100 μg/ml of C or C2 fraction for 30 min, and then exposed to LPS (40 ng/ml) for 12 h. The amount of nitrite in cultured medium was measured as described in Section 2. The data represent the mean of three separated experiments.

3.4.4 Inhibition of LTE on iNOS expression in LPS-stimulated RAW 264.7 cells

Since NO is synthesized by iNOS, we further investigated the effect of LTE and its fractions on iNOS expression from the same treatments**.** The iNOS protein was markedly increased upon exposure to LPS for 12 h. Crude extract T, C and D fractions strongly inhibited iNOS while A and B fraction showed weaker inhibition at the concentration of 200 μg/ml (**Fig. 3.4A)**. Moreover, C and C2 fractions strongly inhibited iNOS expression at the concentration range of 50-200 μg/ml (**Fig. 3.4B and C)**.

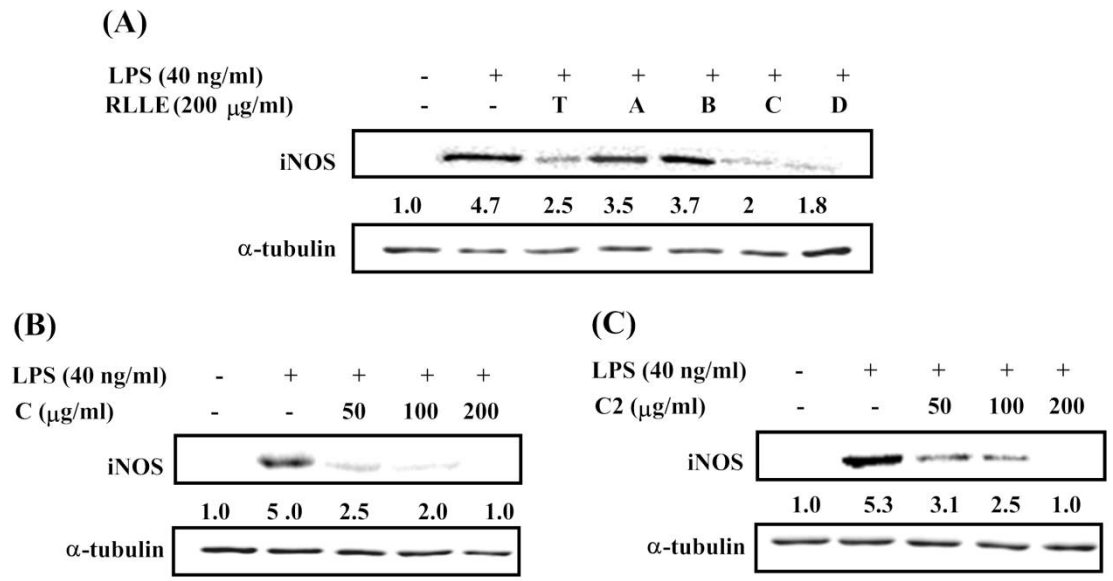


Fig. 3.4 Inhibition of LTE on iNOS expression in LPS-activated RAW 264.7 cells.

(**A**) LTE fractions, (**B**) C fraction and (**C**) C2 fraction. The cells were treated by different fractions of Loquat tea with indicated concentrations for 30 min, and stimulated with 40 ng/ml LPS for 12 h. COX-2 and α-tubulin were detected by Western blotting analysis with their antibodies, respectively. The values show the densitometry fold of COX-2 protein normalized to α-tubulin. The data represent the mean of three separated experiments.

3.4.5 The effect of LTE on cytokines productions

Many cytokines have been reported to act as proinflammatory factors [11]. To clarify whether LTE affects these cytokine productions, we chose C and C2 fractions, which were the stronger inhibitor for NO/iNOS production among these fractions, to investigate their effect on the productions of cytokines in LPS-activated macrophage cells. RAW264.7 cells were treated with or without C or C2 fraction for 30 min before exposure to LPS (40 ng/ml) for 12 h, and the medium was used to assay 23 kinds of cytokines simultaneously by Bio-Plex Pro Mouse Cytokine 23-Plex Panel kit. LPS treatment for 12 h enhanced more than five-fold level of G-CSF, TNF-α, RANTES, IL-6, MCP-1, GM-CSF, more than two-fold level of IL-12(p40), KC, MIP-1β, IL-1α, IL-10, IL-9; and less than two-fold in IL-13, IL-1β, IL-4, IL-17, IL-3, IFN-γ, IL-12(p70), Eotaxin, IL-5, MIP-1α, IL-2, compared with that of the cells without LPS treatment [21]. Pretreatment with C or C2 fraction at the indicated concentrations decreased significantly the level of IL-6 (**A**), RANTES (**B**) and TNF-α (**C**) (**Fig. 3.5**), but they did not affect significantly the productions of other 20 kinds of cytokines (data not shown).

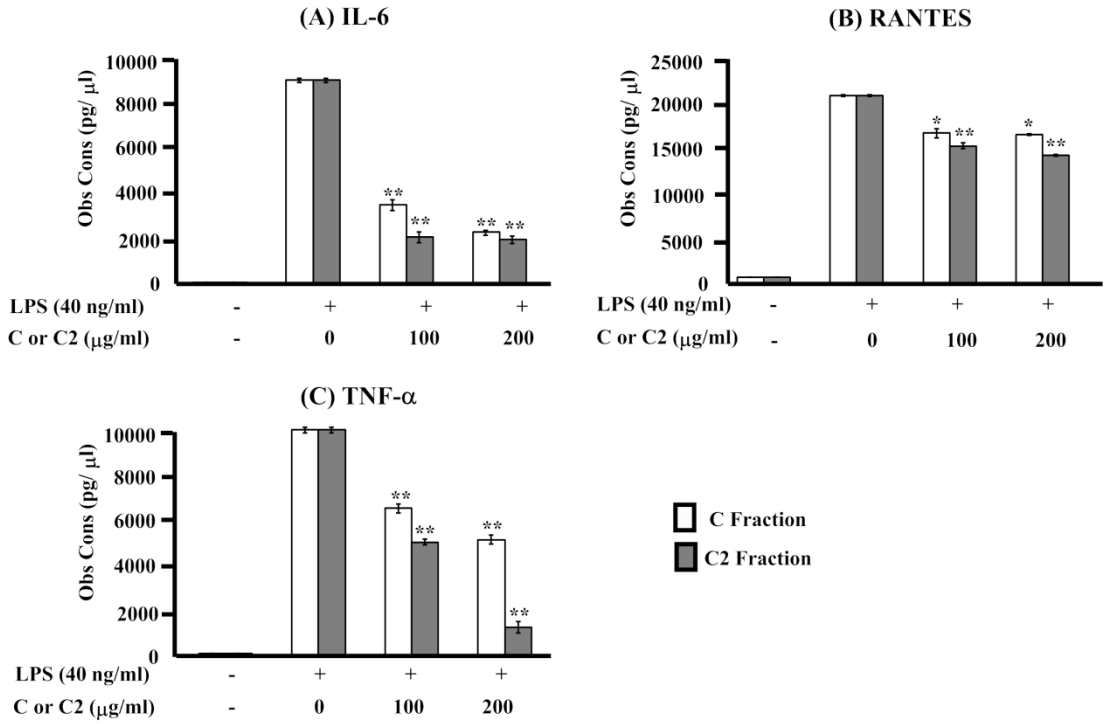


Fig. 3.5 Inhibition of C and C2 fractions on cytokines productions.

(A) IL-6, (B) RANTES, (C) TNF-α. RAW264.7 cells (1.2×10^5 in 12-well plate) were pre-cultured for 21 h, and starved in serum-free medium for 2.5 h. The cells were then treated with 100 and 200 μg/ml of C and C2 fractions for 30 min, and then exposed to LPS (40 ng/ml) for 12 h. The amount of cytokines in medium was measured by Bioplex mouse cytokine assay kit as described in Section 2. Each value represents the mean ± S.D. of triplicate cultures. Different letter means $*P < 0.05$ and $**P < 0.01$

3.4.6 Inhibition of LTE on MAPK pathway

MAPK signaling pathways play a critical role in the regulation of inflammatory responses, and coordinate the induction of many genes encoding proinflammatory mediators [12]. Thus, we investigated the influence of C or C2 fraction on the activation of MAPK including JNK, ERK and p38. The RAW 264.7 cells were treated with C or C2 fraction for 30 min with the indicated concentrations, and then stimulated with LPS (40 ng/ml) for 30 min. As shown in **Fig. 3.6**, LPS markedly induced the phosphorylation of JNK, ERK and

p38 kinase, C (**A**) or C2 (**B**) fraction suppressed LPS-induced phosphorylation of JNK, ERK, and p38 in a dose-dependent manner. These data indicate that the downregulation of these MAPK was involved in the inhibition of LPS-induced inflammation by C or C2 fraction.

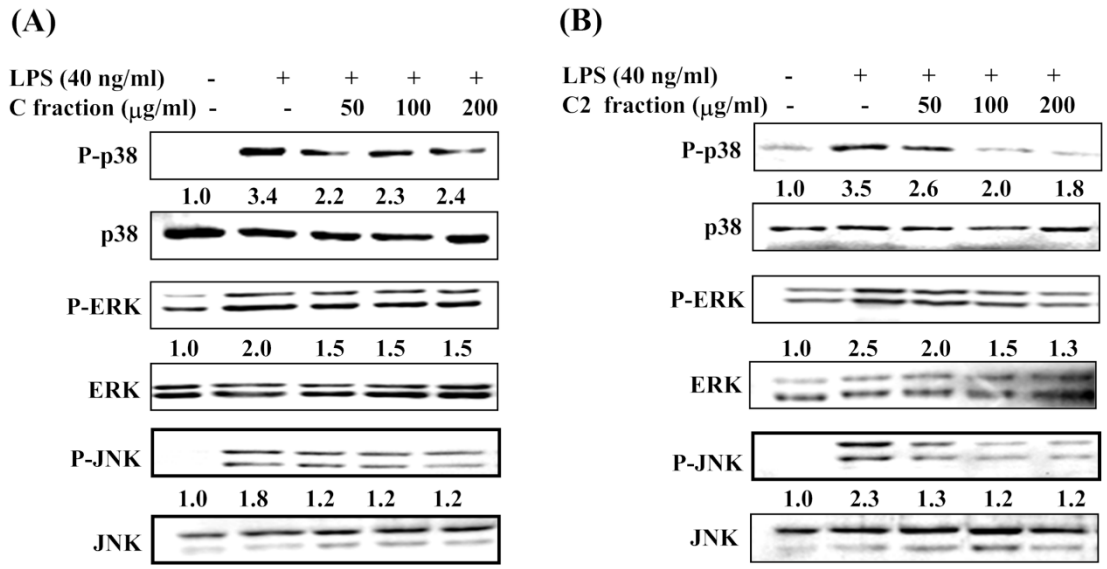


Fig. 3.6 Inhibition of C and C2 fractions on MAPK signaling.

The cells were pretreated by C (A) and C2 (B) fractions of LTE in indicated concentrations for 30 min and then stimulated with LPS (40 ng/ml) for another 30 min. p38, ERK and JNK and their phosphorylations were detected by Western blotting analysis with their antibodies respectively. The values show the densitometric fold of phosphorylation protein to their normal proteins. The data represent the mean ± SD of three separated experiments.

3.4.7 Inhibition of LTE on NF-κB signaling

Accumulated data indicate that NF-κB is one of the principal regulators to regulate the expression of proinflammatory factors including iNOS and cytokines. Thus, we next investigated the effect of C or C2 fraction on NF-κB signaling by Western Blot analysis. NF-κB is inactive in the cytosol by binding to IκB and become active through degradation

of IκB, and subsequent nuclear translocation of NF-κB proceeded by inflammatory factors [23]. Thus, I examined whether C or C2 fraction inhibits the degradation of IκB. As shown in **Fig. 3.7**, IκB was degraded markedly when RAW 264.7 cells were treated with LPS (40 ng/ml) for 30 min. Pretreated with 50-200 μM of C (**A**) or C2 (**B**) fraction for 30 min effectively suppressed these processes. Since the degradation of IκBs is regulated by IκB kinases, IKKα and IKKβ [17]. Thus, we next investigated the effect of these fractions on phosphorylation of IKKα/β. LPS induced the phosphorylation of IKKα/β significantly, C (**Fig. 3.7C**) or C2 (**Fig. 3.7D**) fraction completely inhibited the phosphorylation of LPS-induced IKKα/β without any effect on total IKKα/β. The data indicated that the downregulation of IKK-mediated NF-κB signaling pathways is involved in the inhibition of LPS-induced inflammation by C or C2 fraction.

3.4.8 Inhibition of LTE on phosphorylation of TAK1 and IRF3

The data suggest that both MAPK and NF-κB pathways were involved in the inhibition of LPS-induced inflammation by C or C2 fraction. Thus, I hypothesized that C or C2 fraction may inhibit the activation of TAK1 since TAK1 is a common upstream regulators of both IKKα/β and MAPK signaling [18,24]. As shown in **Fig. 3.7**, LPS induced remarkable phosphorylation of TAK1, treatment with C (**Fig. 3.7C**) or C2 (**Fig. 3.7D**) fraction completely inhibited the phosphorylation of TAK1 without any effect on total TAK1. On the other hands, many proinflammatory cytokines productions have been reported to be mediated by TRIF signaling, which is activated by the phosphorylation of IRF3. Thus, I investigated the effect of C or C2 fraction on phosphorylation of IRF3. LPS induced the phosphorylation of IRF-3 while C or C2 fraction significantly suppressed the phosphorylation of IRF3 in the concentrations of 50-200 μg/ml without any effect on total α-tubulin control (**Fig. 3.7C**) or C2 (**Fig. 3.7D**). These results suggest that the inhibition on

phosphorylation of IRF3 is involved in the downregulation of cytokines productions by C or C2 fraction.

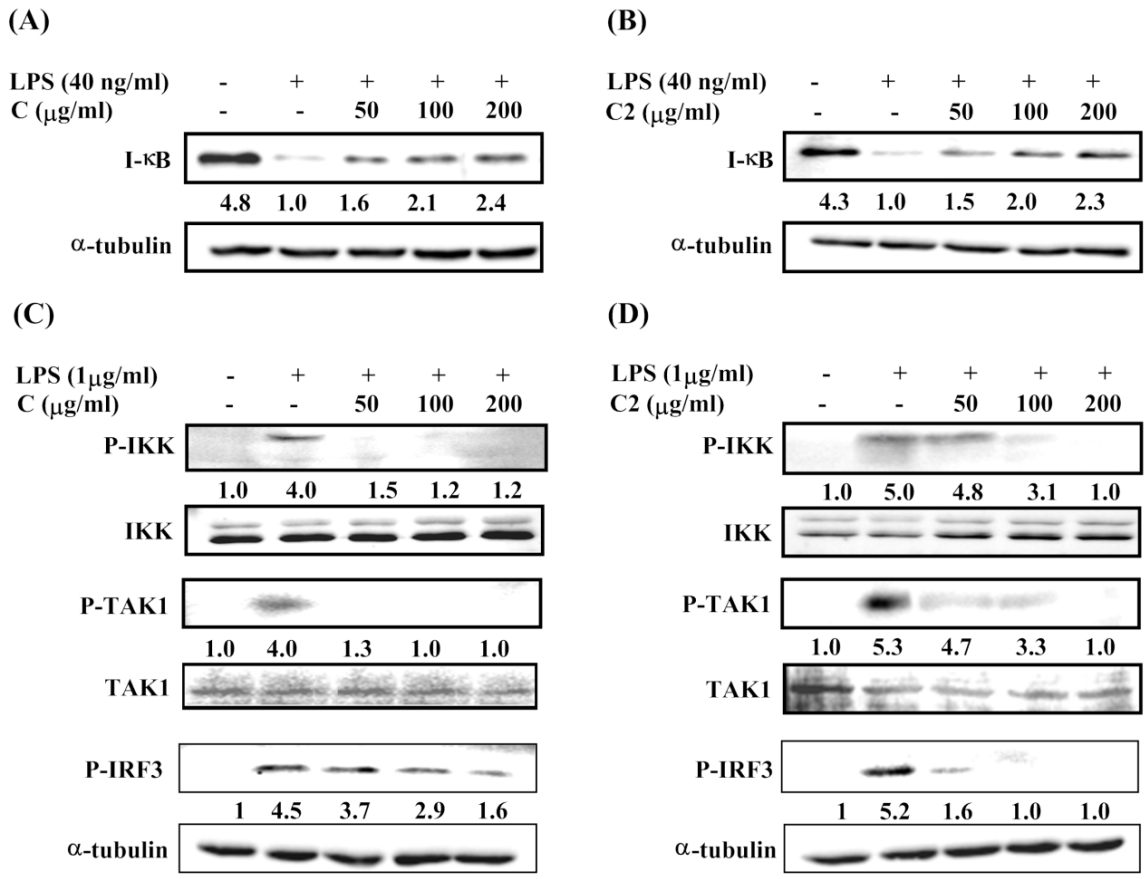
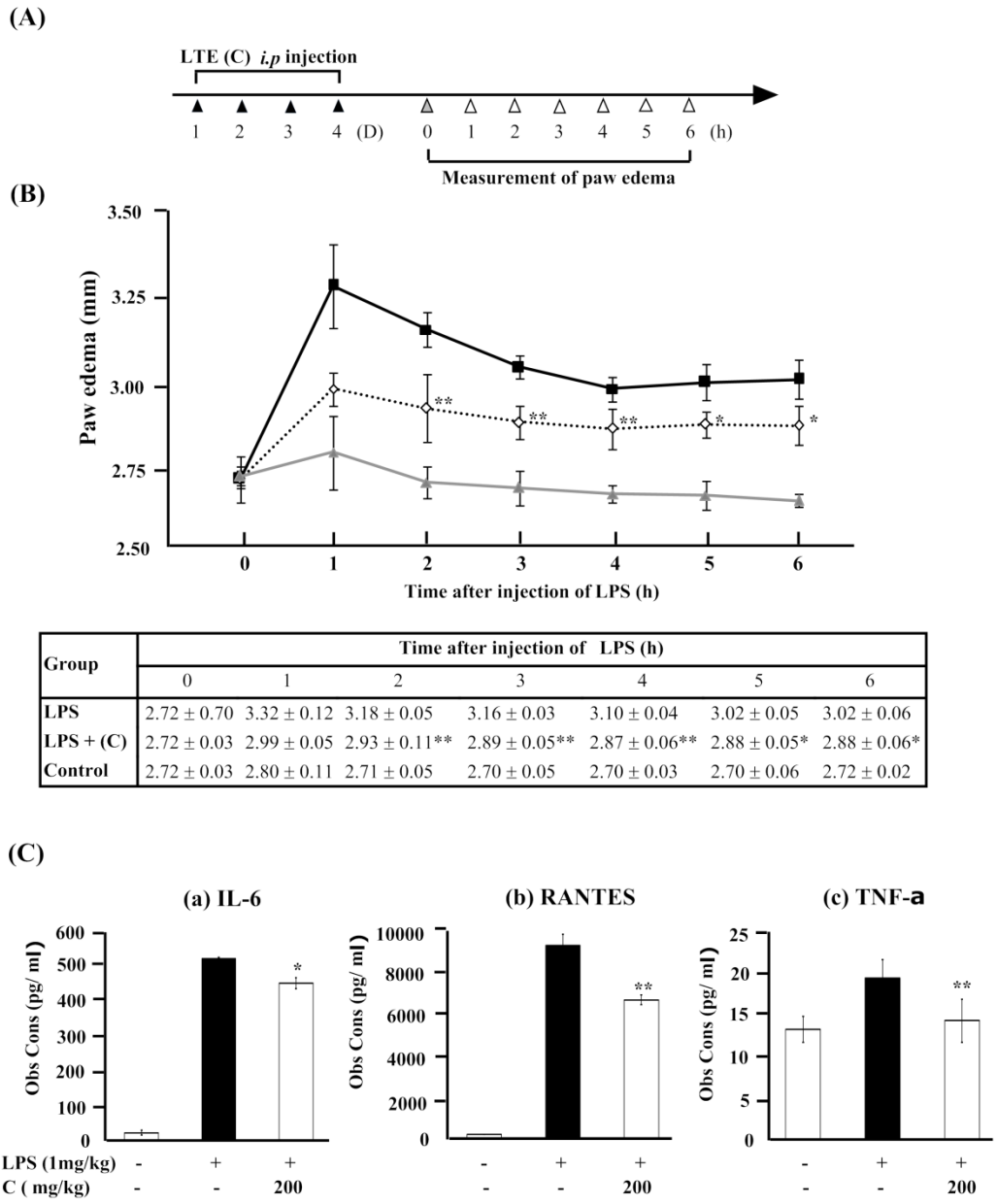


Fig. 3.7 Inhibition of C and C2 fractions on NF-κB signaling.

The cells were treated by C (A, C) and C2 (B, D) fractions of LTE in indicated concentrations for 30 min and then stimulated with LPS (40 ng/ml) for 30 min to detect I-κB or with LPS (1 μg/ml) for 10 min to detect IKK and TAK1, or 3 h to detect IRF3 and its phosphorylation by Western blotting with their antibodies respectively. The induction fold of proteins was calculated as the intensity of the treatment relative to that of control by densitometry. The blots shown are the examples of two separated experiments.

3.4.9 Inhibition of LTE on mouse paws edema

Finally, I used mouse paw edema model to confirm the anti-inflammatory effect of LTE *in vivo*. C fraction was injected *i.p.* into mouse (200 mg/kg body weight) for 4 days, and then the paw edema was induced by injecting *i.p.* with LPS (1 mg/kg body weight). The paw thickness was measured before and every hour after LPS treatment until 6 h (**Fig. 3.8A**). The results showed that LPS treatment increased the paw thickness from 2.72 to 3.32 mm at 1 h, and 3.02 mm after 6 h. Pretreatment with C fraction for 4 days reduced the thickness of LPS-induced paw edema from 3.32 to 2.99 mm at 1 h, and to 2.88 after 6 h although the paw swelling was not completely disappeared within 6 h. C fraction decreased the edema by 32.2%, compared with LPS alone after 1h. As a control, treatment with 0.9% NaCl did not show any effect on paw edema (**Fig. 3.8B**). Simultaneously, we checked the levels of the serum cytokines, which were induced by LPS in RAW264.7 cells (**Fig. 3.5**). As shown in **Fig. 3.8C**, pretreatment with C fraction significantly decreased the levels of serum IL-6, RANTES and TNF-α induced by LPS. These data confirmed the *in vivo* anti-inflammatory effect of C fraction of LTE that attenuated the production of LPS-induced inflammatory mediators to inhibit mouse paw edema.



Group	Time after injection of LPS (h)						
	0	1	2	3	4	5	6
LPS	2.72 ± 0.70	3.32 ± 0.12	3.18 ± 0.05	3.16 ± 0.03	3.10 ± 0.04	3.02 ± 0.05	3.02 ± 0.06
LPS + (C)	2.72 ± 0.03	2.99 ± 0.05	2.93 ± 0.11**	2.89 ± 0.05**	2.87 ± 0.06**	2.88 ± 0.05*	2.88 ± 0.06*
Control	2.72 ± 0.03	2.80 ± 0.11	2.71 ± 0.05	2.70 ± 0.05	2.70 ± 0.03	2.70 ± 0.06	2.72 ± 0.02

Fig. 3.8 Inhibition of C fraction on mouse paws edema.

(A) The mice were divided into three groups: control, LPS, and LPS+C. Each group had three mice, respectively. LTE (C fraction) was injected *i.p*. (200 mg/kg BW) for 4 days, and LPS was then injected *i.p* (1 mg/kg BW). The mouse paw thickness was measured with a digital caliper before and every hour after LPS treatment until 6 h. The change of paw edema thickness was shown in (**B**). Means with differently lettered superscripts differ significantly at the probability of $p<0.05$. The level of IL-6, RANTES and TNF-α in mouse serum (**C**). The blood serums were obtained from the mice that were treated with or without LPS for 6

h by collection of heart blood. The serum without any dilution was used for the measurement of cytokines as described in Section 2 according to manufacturer's manual. Each value represents the mean ± SD of three mice. Different letter means *$P < 0.05$ and *$P < 0.01$

3.5 Discussion and conclusion

Although fresh loquat leaves have been reported to have biological activities such as antioxidant and anti-inflammatory activities [25-27], there is no report on the biological activities of loquat tea, known as Biwa Cha in Japanese, are used for health beverage in Japan. The previous chapter showed loquat tea revealed stronger antioxidant activity *in vitro* and at cellular level. In the present study, we used antioxidant activity-guided fractionations to investigate the anti-inflammation activities of loquat tea extracts.

Inflammation is a central feature of many pathophysiological conditions in response to tissue injury and host defenses against invading microbes. Loquat leaves has been discussed in relation with anti-inflammaotry activity by reducing pro-inflammatory mediators [27-29]. However, the molecular target for anti-inflammatory effect of loquat tea was not reported yet. Therefore, I investigated the effect of loquat tea on expression of pro-inflammatory molecules. Pro-inflammatory cells, mainly activated macrophages, mediate most of the cellular and molecular pathophysiology of inflammation by producing pro-inflammatory molecules including COX-2, PGE_2, iNOS and NO and, cytokines [30]. In cell model of inflammation, I found that C fraction of LTE could inhibit the production of LPS-induced inflammatory mediators including COX-2, PGE_2, NO and iNOS (**Fig. 3.1-3.4**) with dose dependent manner. COX-2 and iNOS are pivotal enzymes involved in maintaining inflammation that are responsible for the catalysis of PGE_2 and NO [4]. In this study, we found that loquat tea could attenuate the production of PGE_2 and NO through attenuation of COX-2 and iNOS enzymes induced by LPS.

Beyond COX-2/PGE_2 and iNOS/NO, some interleukins, chemokines and cytokines were recently demonstrated to be initiator of the inflammatory response and mediators of the development of chronic inflammatory diseases [31]. Cytokines comprise interleukins, lymphokines, monokines, interferons, colony-stimulating factotrs, chemokines and a variety of other proteins [11]. The release of cytokines by macrophages importantly regulates inflammatory response and one cytokines can influence the production of, and response to many other cytokines [32-33]. Moreover, they recruit, alert and activate neighboring cells which are necessary for mounting immune response that cause extent and consequence of inflammation [11,34]. To fully understand the anti-inflammatory effects, the influence of C fraction of LTE on LPS-induced production of 23 kinds of cytokines was investigated using suspension array technology. C fraction could significantly inhibit the production of IL-6, RANTES and TNF-α of them (**Fig 3.5**). IL-6, TNF-α and RANTES play a major role in regulation of acute and chronic inflammation and, various immune response [35-37]. In particular, TNF-α is involved in many different cellular processes, including production of numerous cytokines and acute phase proteins, thus contributes many pathophysiologic processes [37]. IL-6 is one of the most important mediators of the acute phase response. It can be secreted by macrophages in response to specific microbial molecules, such as LPS, and induce intracellular signaling cascades that give rise to inflammatory cytokine production [38]. RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) is a chemotactic cytokine for T cells, eosinophils and basophils, thus, plays an active role in recruiting leukocytes into inflammatory sites [39]. Thus, C fraction of LTE might exert its anti-inflammatory effect also by suppressing the production of these pro-inflammatory cytokines.

In relation to signaling pathways for regulating the production of these inflammatory factors, MAPK and NF-κB pathways are at least involved in LPS-activated macrophage

cells [18,40-41]. The present study revealed that C fraction of LTE influenced MAPK phosphorylation including ERK, JNK, and p38 MAPK kinase (**Fig. 3.6**). C fraction of LTE also attenuated NF-κB signaling including degradation of IκB-α, IKKα/β phosphorylation (**Fig. 3.7**). LPS triggers several inflammatory processes by binding to TLR4, in various cells including macrophages. MyD88-dependent and Trif-dependent pathways are major signaling pathways that are activated by binding of LPS and TLR4. MyD88-dependent pathway regulates activation of multiprotein complex IKK and MAPK signaling through TAK-1 activation. IKK complex consists of IKKα and IKKβ which phosphorylated I-κB, thereby allowed activation of NF-κB to separate from I-κB and regulate the expression of inflammatory proteins [4]. Therefore, I focused the effect of LTE on TAK-1 protein because it is a key to activate MAPK and NF-κB pathways. C fraction markedly suppressed phosphorylation of TAK1 (**Fig. 3.7**), which is an upstream protein kinase to activate both MAPK and IKKα/β [42-43]. Therefore, the upstream TAK1 is possible major targets for C fraction of LTE. These signaling data suggested that TAK1-mediated MAPK and NF-κB pathways were involved in the inhibition of LPS-induced inflammatory responses by C fraction of LTE. Moreover, C fraction also attenuated the phosphorylation of IRF3 (**Fig. 3.7**), which involves in the activation of TRIF signal by translocation to the nucleus to regulate the expression of inflammatory mediators including iNOS and cytokines [44-46]. TRIF also interacts with TRAF6 and RIP1 and thereby mediates late phase of NF-κB activation via TAK1 phosphorylation [47]. In this study, C fraction of LTE could inhibit the expression of iNOS and cytokines by blocking TRIF-mediated NF-κB activation.

Although the mechanism in the suppression of TAK1 and IRF3 by C fraction is still not clear, it has been known that the elevated ROS can activate these pathways to induce inflammatory responses [22,45,48-50]. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB, MAPK and IRF3 while lower

amount of ROS targets Nrf2 pathway to produce antioxidant enzyme [49- 52]. I have observed that C fraction of LTE could reduce LPS-induced ROS levels in LPS induced RAW 264.7 cells (**Fig 2.4**). Therefore, it is possible that inhibitory effect of LTE on activation of TAK1 and/or IRF3-mediated NF-κB and MAPK come from its antioxidant effect.

HO-1 is a part of the integrated response to oxidative stress, interestingly; HO-1 exerts anti-inflammatory effects [53]. Proinflammatory meadiators, NO, as well as other inflammatory agents induce HO-1 production which in turn inhibits the expression of inflammatory enzyme, iNOS [54]. Many reports suggested that HO-1 production is dependent on the activation of Nrf2. In Fig. 2.5, LTE increased HO-1 and Nrf2 production in LPS-induced RAW cells, meaning that Nrf2 mediated HO-1 expression may be involved in the mechanism of the anti-inflammatory effects of LTE. These observations indicated that ROS stimulate TAK-1 mediated three signal pathways (MAPK, NF-κB, IRF3) to increase production of signal inflammatory mediators (COX-2, iNOS, cytokines) involving in both macrophage activation and mouse paw edema. LTE induced HO-1 scavenges ROS inactivating TAK-1 mediated three signal pathways (MAPK, NF-κB, IRF3) which are stimulated by LPS. Therefore, LTE showed anti-inflammatory effect by suppressing pro-inflammaotry mediators via increasing Nrf2 mediated HO-1 activity that inhibited ROS stimulated TAK-1 mediated signals. When compared with anti-inhibitory effect *in vitro* of loquat leave on inflammatory proteins from previous report, it was found that LTE possesses stronger effect on inhibition of proinflammatory mediators than loquat leaves because of its inhibitory effect exhibited at lower concentration [27,55].

The anti-inflammatory effect of LTE was confirmed by animal experiments. LTE treated mouse group reduced LPS induced-mouse edema about 32 % during 1h. LTE also attenuated in the level of serum cytokines (IL-6, RANTES and TNF-α) which are produced

by LPS (**Fig 3.8**). All collected data demonstrated the anti-inflammatory effects and underlying mechanisms of LTE *in vitro* and *in vivo.*

LTE could suppress the production of inflammatory mediators by decreasing ROS production via increasing Nrf2 mediated HO-1 expression thereby downregulate TAK1 and/or IRF3-mediated NF-κB and MAPK pathways, and attenuated LPS-induced serum cytokines level and mouse paw edema. The results from cell and mouse models provide insight for understanding the anti-inflammatory effects and molecular mechanisms of loquat tea.

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Chapter 4

In vitro anticancer activity of loquat tea by inducing apoptosis in human leukemia cells

4.1 Abstract

Fresh loquat leaves have been used as folk health herb in Asian countries for long time although the evidence supporting their functions is still minimal. This study aims to clarify the chemopreventive effect of loquat tea extracts (LTE) by investigating the inhibition on proliferation and underlying mechanisms in human promyelocytic leukemia cells (HL-60). LTE could inhibit proliferation of HL-60 in a dose-dependent manner. Molecular data showed that the isolated fraction of LTE induced apoptosis of HL-60 as characterized by DNA fragmentation, activation of caspase-3, -8 and -9, and inactivation of poly(ADP)ribose polymerase (PARP). Moreover, LTE fraction increased the ratio of pro-apoptotic Bcl-2-associated X protein (Bax) / anti-apoptotic myeloid cell leukemia 1 (Mcl-1) to cause the loss of mitochondrial membrane potential and cytochrome *c* release from mitochondrial to cytosol. ROS production is involved in LTE-induced apoptosis. Thus, our data indicate that LTE might induce apoptosis in HL-60 cells through a ROS-mediated mitochondrial dysfunction pathway. These findings enhance our understanding for chemopreventive function of loquat tea.

4.2 Introduction

Loquat (*Eriobotrya japonica*) is one plant of the Rosaceae family, and all parts of loquat, such as fruits, leaves, and peels have been reported to have health benefits. In particular, the leaves have higher flavonoid content than the peel or fruits, with stronger radical scavenging

activity [1] and have been reported to have preventive effects against skin diseases, chronic bronchitis, and cancer [2-3]. Recently, fresh loquat leaves were processed into a beverage, called as loquat tea (Biwa Cha in Japanese), after the fresh leaves are roasted to 350 °C for 30 minutes. In our previous study, *in vitro* and *ex vivo* analysis showed that LTE possessed higher antioxidant potency than that from fresh leave extract [Chapter 2] [4]. Bioactive assay data from both cell and animal models showed that LTE inhibited the production of pro-inflammatory factors and mouse paws edema [Chapter 3] [5]. Several lines of data have suggested that the bioactive compounds such as phenolic compounds possessing the antioxidant and anti-inflammatory activities will have also anticancer activity [6-7]. Thus, in the present study, we investigated the chemopreventive activity and underlying mechanisms of LTE by investigating the anti-proliferation effect and mechanisms in human promyelocytic leukemia cells (HL-60), which is a valid model for testing antileukemic or general antitumoral compounds [8-10].

Cancer chemopreventive compounds have been reported to play an important role in elimination of seriously damaged cells or tumor cells by inducing apoptosis [11-12]. The cells that have undergone apoptosis are typically shown in chromatin condensation and DNA fragmentation [13]. They are rapidly recognized by macrophages before cell lysis and can be removed without induction of inflammation [11,14]. Cell survival or apoptosis is determined by the complex interplay between proapoptotic (e.g. Bax, Bak) and antiapoptotic Bcl-2 family proteins (e.g., Bcl-2, Bcl-xL, or Mcl-1) [15]. The change in the ratio of Bax/Bcl-2 is known to initiate caspase signaling [15]. In human malignancies, the increased expression of antiapoptotic proteins commonly occurs and is associated with cancer progression and resistance to chemotherapy. Thus, designing small molecule Bcl-2 inhibitors has been developed as one of cancer therapy strategies [16]. Bax is localized to the cytoplasm under normal conditions. Upon stimulation, activated Bax leads directly to

mitochondrial membrane permeabilization [17-18], which promotes the release of proapoptotic factors including cytochrome *c* [19]. Released cytochrome *c* can activate caspase-9, which in turn cleaves and activates executioner caspase-3 [20]. After caspase-3 activation, some specific substrates for caspase-3 such as poly(ADP)ribose polymerase (PARP) are cleaved and eventually lead to apoptosis [21]. Several lines of studies also demonstrated that caspase-8 can directly activate caspase-3 and sequentially induces apoptosis [22-23].

Based on the properties of LTE and the roles of mitochondria dysfunction in initiating apoptosis, in the present study, we used HL-60 cells as the target cells to investigate the anti-proliferation activity and molecular mechanisms of LTE, focusing on mitochondria dysfunction pathway.

4.3 Methods

4.3.1 Reagents and cell culture

The antibodies against caspase-8, caspase-9, caspase-3 and poly (ADP) ribose polymerase (PARP) were from Cell Signaling Technology (Beverly, MA, USA). The antibodies against Bax (Bax9), cytochrome *c*, COX-4 and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mcl-1 antibody was from BD Biosciences, CA, USA, 3,3'-Dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was from Molecular Probes (Eugene, Oregon, USA). 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) were form Sigma (St. Louis, MO, USA). HL-60 cell line was obtained from the Cancer Cell Repository, Tohoku University, Japan and cultured at 37 °C, 5% CO_2 in RPMI1640 medium containing 10% foetal bovine serum (FBS) from Equitech-Bio (Kerrville, TX, USA) and 1% penicillin streptomycin and glutamine (PSG).

4.3.2 Cell viability assay

The cell survival rate was measured by a MTT assay according to previous authors [24]. Briefly, HL-60 cells (1.5×10^4 cells/100 µl/well) were seeded into each well of 96-wells plates. After incubation for 24 h, the cells were treated with different concentrations of LTE for 24 h. Then MTT solution (5 mg/ml) was added to each well. After incubating the cells for another 4 h, the resulting MTT-formazan product was dissolved by the addition of 0.04 M HCl-isopropanol solutions. The amount of formazan was determined by measuring the absorbance at 595 nm in a microplate reader (Thermo Scientific Multiskan^{TM}, version 1.00.79, Finland). The results were expressed as the optical density ratio of the treatment to control.

4.3.3 Extraction of whole cellular protein and subcellular fractionation of cytochrome *c*

HL-60 (7.5×10^5/5 ml) cells were pre-cultured in 6-cm dish with RPMI containing 10% FBS and 1% PSG for 24 h. The cells were treated with LTE for the indicated 24 h. Whole cellular protein was prepared as described in detail previously [25]. In brief, the harvested pellets were lysed with cell lysis buffer containing 50 mM Tris-HCL (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 0.01% of a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The subcellular fractions of harvested pellets were prepared with Mitochondria/Cytosol Fractionation Kit (BioVision, California, USA) as described in the manufacturer's manual. In brief, the harvested pellets were lysed with cytosol extraction buffer, and then homogenized with 40 strokes, following centrifugation at 700 × g for 10 min at 4 °C. The supernatant was further centrifuged at 10,000 × g for 30 min at 4 °C, and the supernatant was used as cytosolic fraction. The pellet

was dissolved in mitochondria extraction buffer, and used as mitochondrial fraction.

4.3.4 Western Blot analysis

The lysate of whole cellular protein or subcellular protein was boiled for 5 min. Protein concentration was determined by dye-binding protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's manual. Equal amounts of lysate protein were run on 10% SDS-PAGE and electrophoretically transferred to PVDF membrane (GE Healthcare, Buckinghamshire, UK). After blotting, the membrane was incubated with specific primary antibody overnight at 4 °C, and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by using ECL system (GE Healthcare, Buckinghamshire, UK) with a Lumi Vision PRO machine (TAITEC Co, Saitama, Japan). The relative amount of proteins associated with specific antibody was quantified by Lumi Vision Imager software (TAITEC Co, Saitama, Japan).

4.3.5 DNA fragmentation and morphological analysis of apoptotic cells

DNA fragmentation assay was carried out as described previously [26]. Briefly, HL-60 (7.5×10^5/5 ml/6-cm dish) cells were treated with different concentrations of LTE for 24 h. The harvested cells were suspended in 500 μl of Tail buffer (100 mM NaCl, 50 mM Tris–HCl at pH 8.0, 100 mM EDTA, and 1% SDS) plus 0.5 mg/ml proteinase K. After 3 h incubation at 55 °C, DNA was precipitated by adding an equal volume of isopropanol. The dried DNA precipitate was dissolved in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) containing 0.1 mg/ml RNase A, and further incubated at 37 °C for 30 min to degrade contaminating mRNA. After incubation, loading buffer (6 × LB Orange G, Wako, Nippon Gene, Tokyo, Japan) was added. The 100 bp DNA Ladder One (Nacalai Tesque, Kyoto, Japan) served as a molecular marker. DNA fragments were separated on 2% agarose

gel and digitally imaged after staining with ethidium bromide. Cell morphology was observed under an optical Biozero microscope (BZ-8000, Keyence, Osaka, Japan).

4.3.6 Flow cytometric detection of mitochondrial membrane potential (ΔΨm) loss

ΔΨm was assessed by a cell permeable marker $DiOC_6(3)$, which specifically accumulates into mitochondria depending on ΔΨm [27]. HL-60 (1×10^5/2 ml/6-wells plate) cells were treated with LTE for various times and then incubated with 20 nM of $DiOC_6(3)$ for 30 min. The cells were washed with PBS and resuspended in 1ml of PBS. The cells were analyzed at FL1 (530 nm) with a flow cytometer (Cyflow, Partec GmbH™, Münster, Germany).

4.3.7 Measurement of intracellular ROS production

Intracellular ROS were determined, using the oxidation-sensitive dichlorofluorescein-diacetate (DCFH-DA) fluorescent dye [4]. HL-60 cells were seeded into 98-well plate at a starting density of 1.5×10^4 cells/100 μl/well. After pre-incubation of 24 h, the cells were treated with or without LTE and its fraction for at different concentrations or times. DCFH-DA with a final concentration of 20 μM was then added for an additional 60 min. Fluorescence was measured at 485 nm excitation and 530 nm emission, using a Wallac ARVO™ SX 1420 Multilable Counter (Perkin Elmer Life Sciences, Singapore). The relative amount of intracellular ROS production was expressed as the fluorescence ratio of the treatment to control.

4.3.8 Statistical Analysis

All data were statistically analyzed by one way ANOVA test. Means with differently lettered superscripts differ significantly at the probability of $p < 0.05$.

4.4. Results

4.4.1 LTE inhibits the proliferation of HL-60 cells

To test the effect of LTE on the proliferation of HL-60 cells, HL-60 cells were treated with 100 μg/ml of each extract for 24 h, the survival fraction of HL-60 cells was investigated by a MTT assay. The cell viability of 82±9.4% (L), 58±7.2% (T), and 45±3.8% (C) was observed, respectively (**Fig. 4.1A**). Since C fraction showed the highest inhibition on the proliferation of HL-60 cells, we next performed a dose experiment for C2 fraction, which is separated from C fraction by ODS gel column. As shown in **Fig. 4.1B**, C2 fraction showed a dose-dependent inhibition with the IC_{50} value of 40 μg/ml at 24 h treatment. Thus, we chose C2 fraction as a sample to investigate the mechanisms of LTE on the inhibition of the proliferation in HL-60 cells.

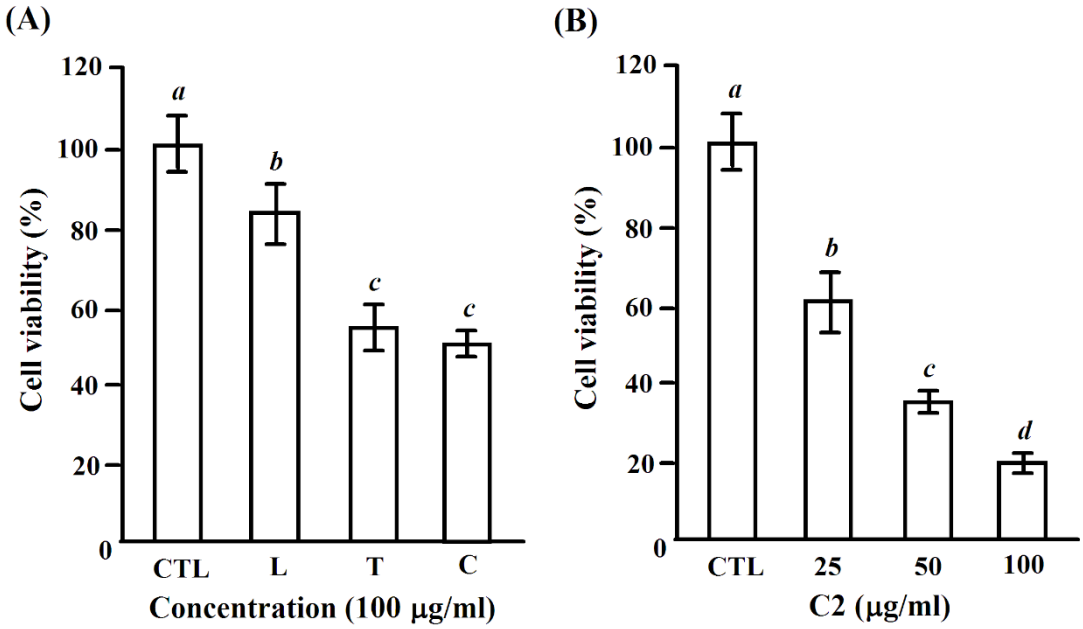
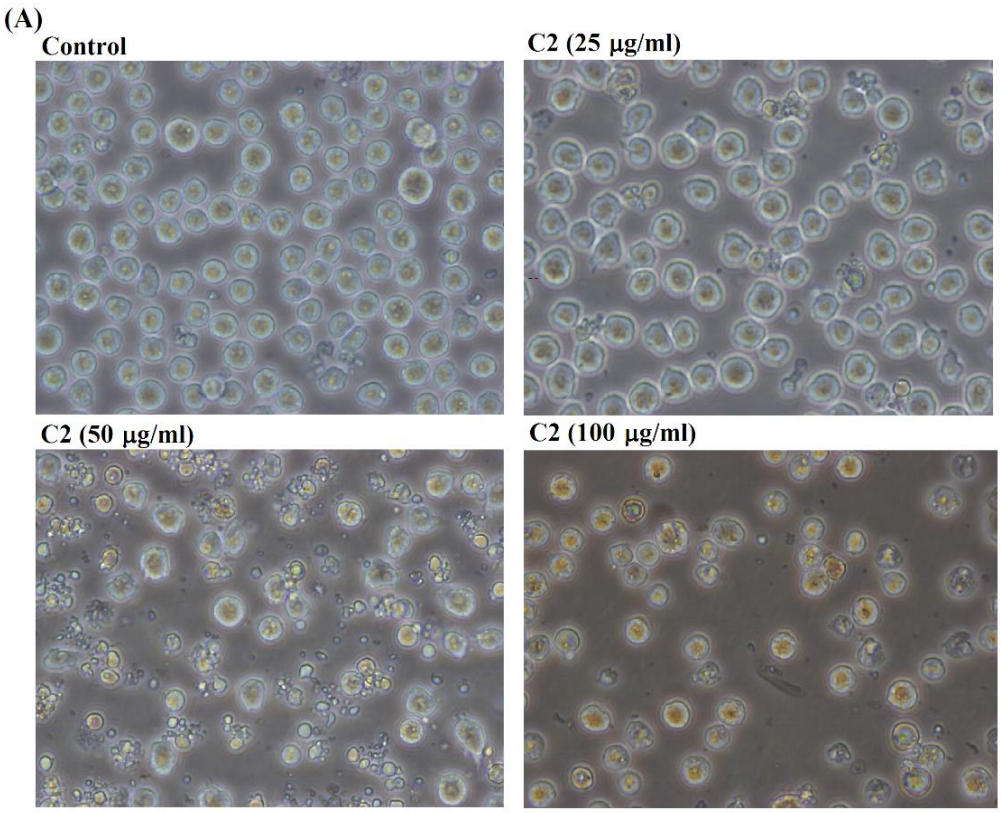


Fig.4.1. The inhibitory effects of the extracts of loquat leaves and loquat tea on cell proliferation of HL-60 cells.

(A) The cells (1.5×10^4 cells/100μl/well) were seeded into 96-wells plates for 24 h, and then treated with 100 μg/ml of the loquat leave water extract (L), loquat tea water extract (T), and C fraction separated from T by MCI gel with 50% EtOH (C) for another 24 h. (B) The cells were treated for 24 h with 25-100 μg/ml of C2 fraction, which was separated from C fraction by ODS gel with 20% MeOH. CTL cells were used as control cells without any treatment. MTT was added into medium for an additional 4 h. The survival of cells was detected by measuring the absorbance at a wavelength of 595 nm. The survival rate was expressed as the optical density ratio of the treatment to control. Each value represents the mean ± S.D. of triplicate cultures. Means with differently lettered superscripts differ significantly at the probability of $p < 0.05$.

4.4.2 LTE induces apoptosis

To elucidate whether C2 fraction inhibits the proliferation of HL-60 cells through apoptosis induction, we characterized the death cells by several aspects. First, an apoptotic morphology including cell shrinkage, chromatin condensation was observed under inverted microscopy after treatment with 25–100 μg/ml of C2 fraction for 24 h (**Fig. 4.2A**), and a dose-dependent DNA fragmentation was also detected in C2 fraction-treated cells (**Fig. 4.2B**). Moreover, other hallmarks of apoptosis including the proteolytic inactivation of PARP, the activations of caspase-3, -8 and caspase-9 by cleaving the procaspases were also detected in the indicated dose (**Fig. 4.3A**) and a time-dependent manner (**Fig. 4.3B**) in the fashion coinciding with morphology and DNA fragmentation (**Fig. 4.2B**). Taken together, we conclude that C2 fraction of LTE inhibits the proliferation of HL-60 cells through the induction of apoptosis.



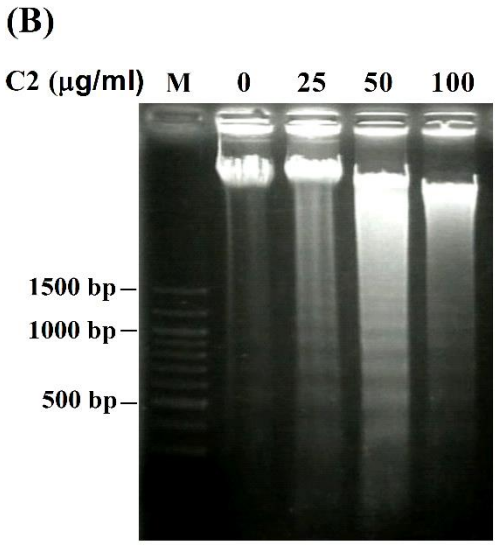


Fig. 4.2. C2 fraction induces morphology changes and DNA fragmentation of HL-60 cells.

HL-60 cells were exposed to 0, 25, 50 and 100 μg/ml of C2 fraction for 24 h. Cell morphology was observed under an optical microscope (A). Then, the cells were harvested by centrifugation, and DNA was extracted as described in Method section. The DNA fragments were separated on 2% agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide. The 100 bp DNA Ladder One (Nacalai Tesque, Kyoto, Japan) served as a molecular marker (B).

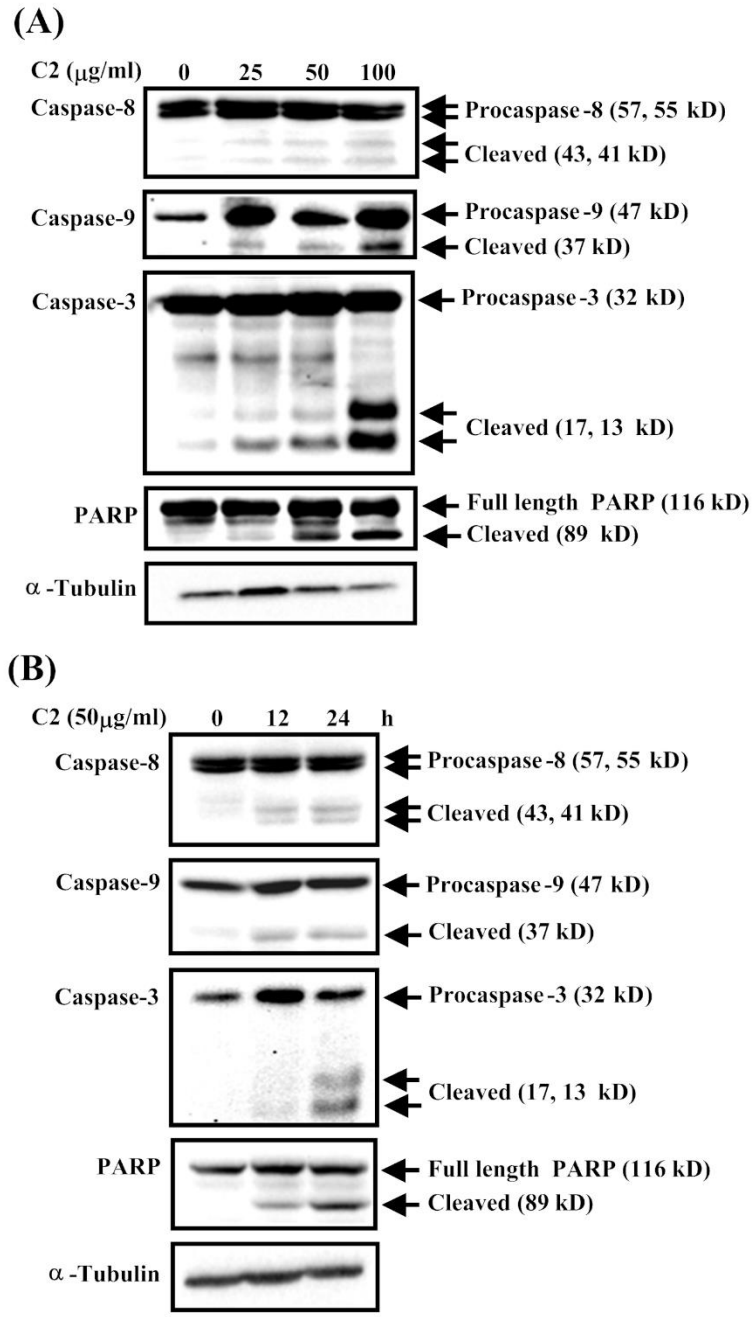


Fig. 4.3. C2 fraction-induced apoptosis involves the activation of caspase-3 and -9 and inactivation of PARP.

(A) A dose-dependant experiment. HL-60 cells (7.5×10^5/5 ml/6-cm dish) were treated with 0, 25, 50 and 100 μg/ml of C2 fraction for 24 h, whole-cell lysate was used for Western blotting analysis with the indicated specific antibodies, respectively. (B) A time-dependant experiment. HL-60 cells (7.5×10^5/5 ml/6-cm dish) were treated with 50 μg/ml of C2

fraction for 12 and 24 h, whole-cell lysate was used for Western blotting analysis with the indicated specific antibodies, respectively. The result is a representative of triple experiments shown.

4.4.3 LTE induces apoptosis by upregulating the ratio of Bax and Mcl-1

Cell survival is determined by the complex interplay between proapoptotic protein such as Bax, and antiapoptotic Bcl-2 family proteins including Mcl-1 [21]. To know whether LTE influence these apoptotic regulatory proteins to induce apoptosis, we treated HL-60 cell for 24 h with indicated doses of C2 fraction (**Fig. 4.4A**) or with 50 μg/ml of C2 fraction for time dependent (**Fig. 4.4B**). The increase in Bax protein and decrease in Mcl-1 protein were observed in both dose- and time- course experiments. The ratio of Bax and Mcl-1 was increased to 3.4 fold at 100 μg/ml of C2 fraction for 24 h (**Fig. 4.4A**), and to 1.5 fold at 12 h and 2.4 fold at 24 h by 50 μg/ml of C2 fraction (**Fig. 4.4B**). These data revealed that C2 downregulated antiapoptotic Mcl-1 peotein and upregulating proapoptotic Bax protein.

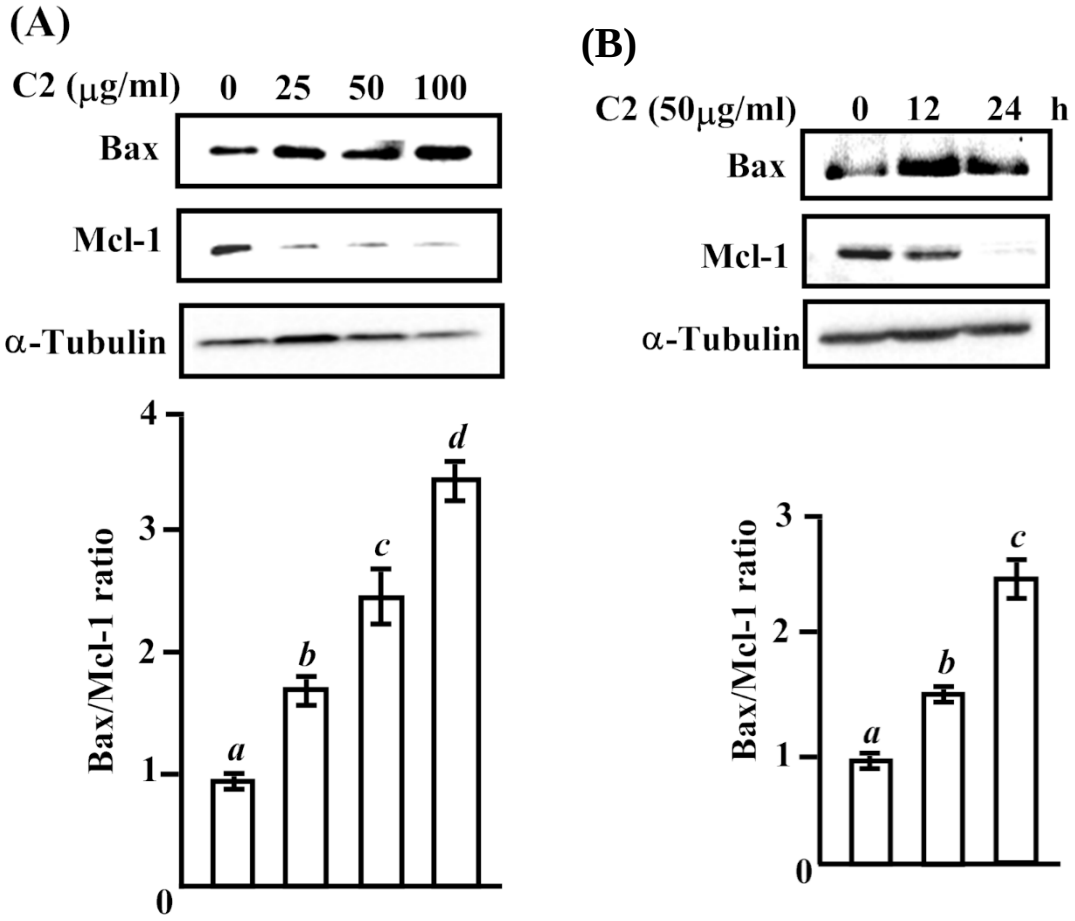


Fig. 4.4 The change in the protein levels of proapoptogenic Bax and antiapoptogenic Mcl-1 by C2 fraction.

(A) A dose-dependant experiment. HL-60 cells (7.5×10^5/5 ml/6-cm dish) cells were treated with 0, 25, 50 and 100 μg/ml of C2 fraction for 24 h, whole-cell lysate was used for Western blotting analysis with the indicated specific antibodies, respectively. (B) A time-dependant experiment. HL-60 (7.5×10^5/5 ml/6-cm dish) cells were treated with 50 μg/ml of C2 fraction for 12 and 24 h, whole-cell lysate was used for Western blotting analysis with the indicated specific antibodies, respectively. Each value represents the mean ± S.D. of triplicate cultures. Means with differently lettered superscripts differ significantly at the probability of $p < 0.05$.

4.4.4 LTE-induced apoptosis involves ΔΨm loss and cytochrome *c* release

Accumulated data suggest that a mitochondria-initiated death pathway plays an important role in triggering apoptosis in response to chemicals [15-17]. To clarify whether apoptosis by fraction C2 of LTE is linked to mitochondria dysfunction, we examined the effect of C2 fraction on the ΔΨm with a mitochondria-specific dye, $DiOC_6(3)$. As shown in Fig. 4.5A, a time-dependent loss of ΔΨm was observed after treatment with 50 μg/ml for 6, 12, 24 h. The loss of ΔΨ m commonly leads to the release of cytochrome *c* from mitochondrial to cytosol, which causes activations of caspases to induce apoptosis. Thus, we further whether mitochondrial cytochrome *c* releases into cytosol. To elucidate whether mitochondrial cytochrome *c* releases into cytosol in LTE–induced apoptosis, we prepared mitochondrial and cytosolic fractions from the cells treated by C2 fraction, and then detected cytochrome *c* by Western blotting analysis. As shown in Fig. 4.5B, a time-dependent accumulation of cytochrome *c* in the cytosol was detected. The integrity of the cytosolic and nuclear fractions was confirmed by the analysis of the compartment-specific cytosolic α-tubulin and mitochondria COX-4 proteins. These data indicate that apoptosis induced by C2 fraction of LTE involved a mitochondrial dysfunction pathway.

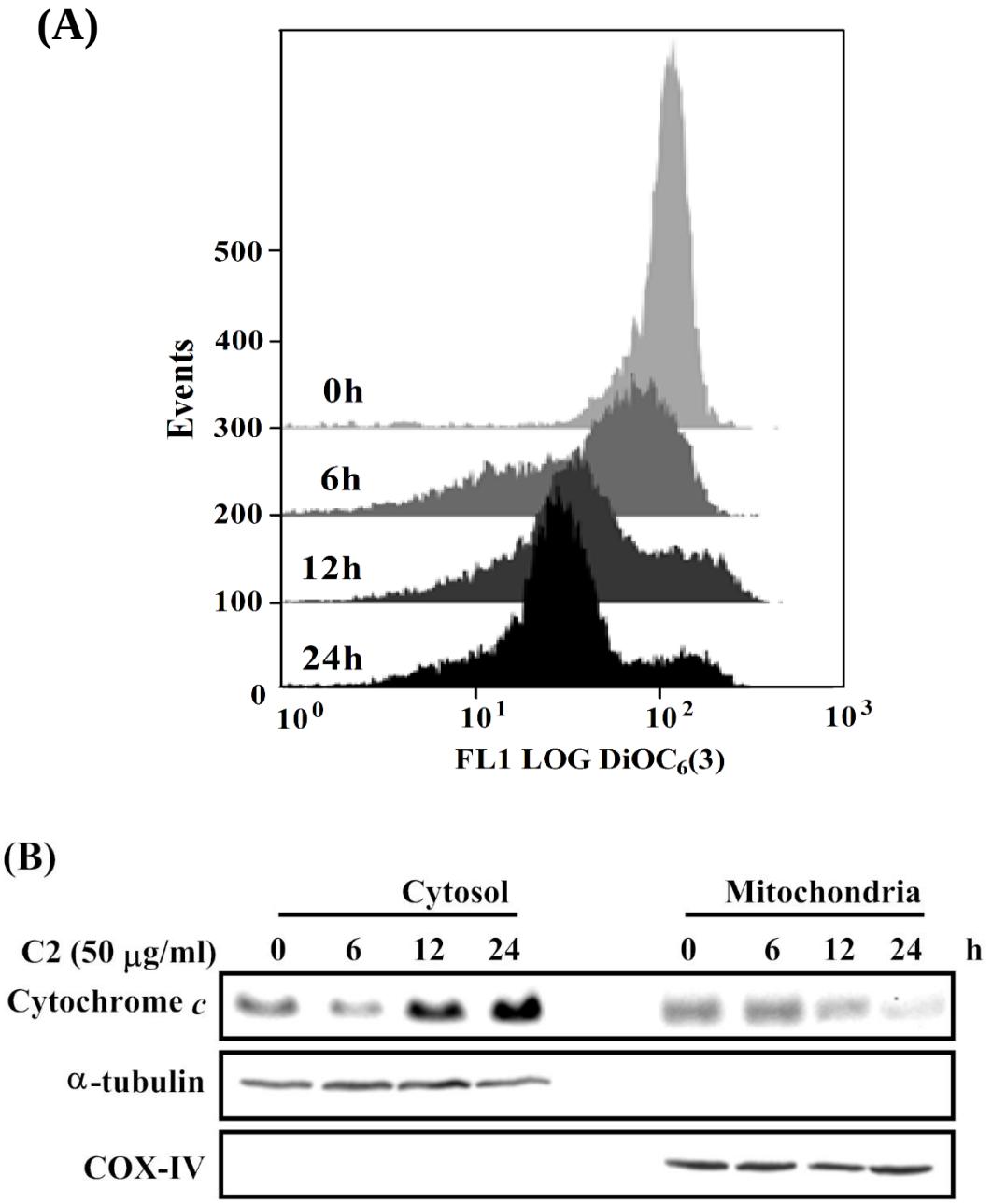


Fig.4.5. The ΔΨm loss and cytochrome *c* release in HL-60 cells treated by C2 fraction.

(A) The ΔΨm loss. HL-60 (1×10^5/2 ml/6-wells plate) cells were treated with 50 μg/ml of C2 fraction for 0, 6, 12, 24 h. The harvested cells were then incubated with 20 nM of $DiOC_6(3)$ for 30 min, followed by flow cytometric analysis as described under Materials and methods. (B) Cytochrome *c* release. HL-60 (7.5×10^5/5 ml/6-cm dish) cells were treated with 50 μg/ml of C2 fraction for 0, 6, 12, 24 h. The harvested cells were fractionated into

cytosolic and mitochondrial fractions as described under Materials and methods. Cytochrome *c*, and the compartment-specific cytosolic α-tubulin or mitochondria COX-4 proteins were detected by Western blotting analysis with their respective antibodies.

4.4.5 LTE-induced apoptosis involves ROS production

Several lines of data suggested that ROS production is one part of apoptosis in chemotherapeutic drugs [28-29]. To know whether ROS production is involved in LTE-induced apoptotic behavior, we treated HL-60 cell with 50 μg/ml of C2 fraction for different times (0, 10, 15, 30 and 60 min) and measured ROS production, using DCFH-DA fluorescence. The most significant production of ROS was observed in 15 min, and then reduced after 60 min (**Fig. 4.6A**). Dose-dependence of ROS production by C2 fraction was also observed after the cells were treated at 15 min (**Fig. 4.6B**). To determine whether intracellular ROS production is involved in LTE-induced apoptosis, the cells were challenged by adding 5 mM NAC. As shown in Fig. 4.7, ROS generation could be prevented by adding 5 mM NAC (**Fig. 4.7A**), and LTE-induced DNA fragmentation is suppressed by pretreatment with NAC (**Fig. 4.7B**). These data revealed that ROS production is involved in LTE-induced apoptosis.

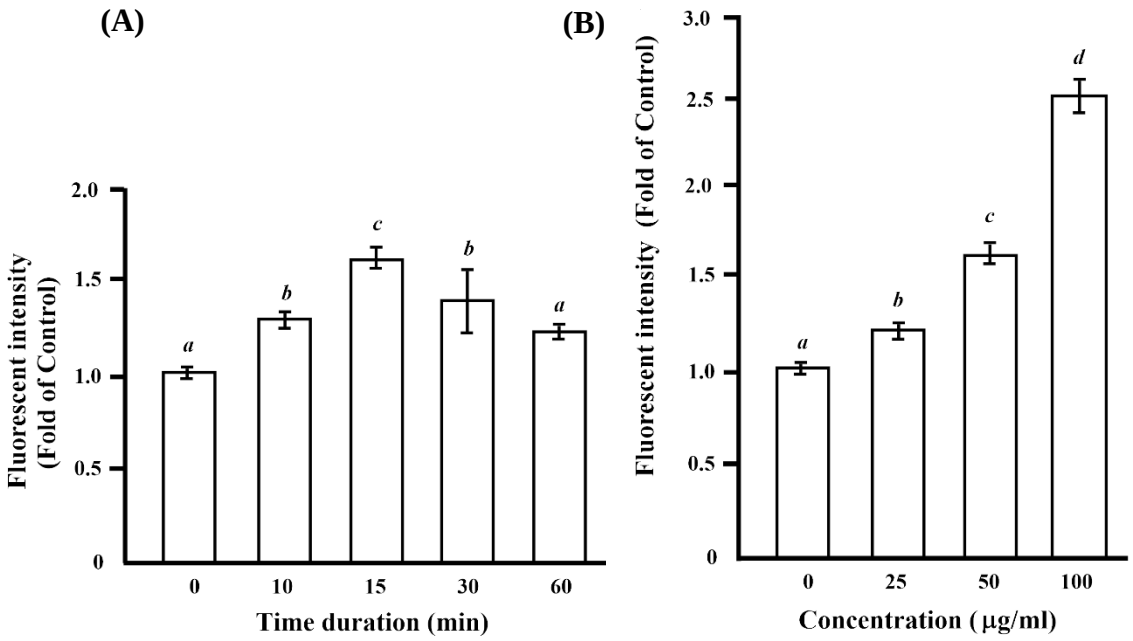
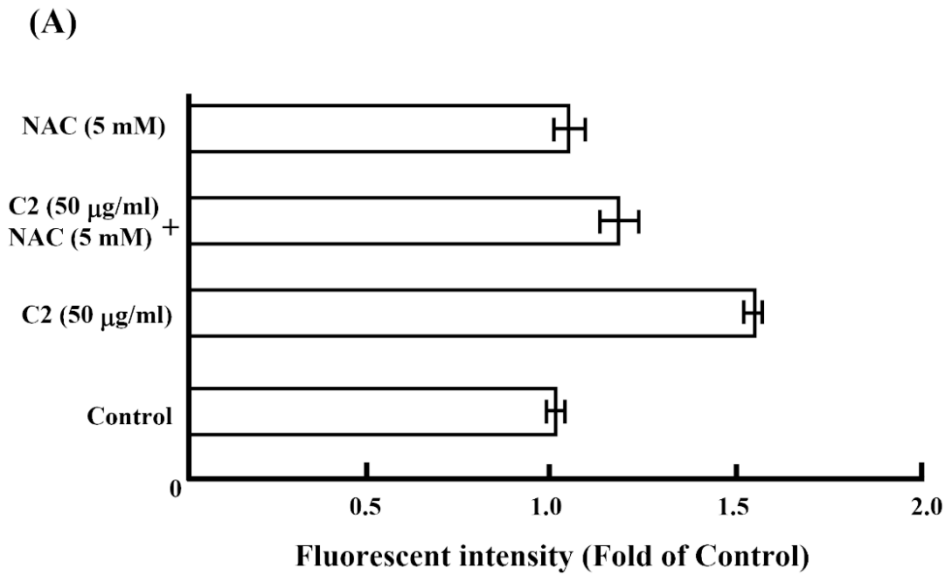


Fig.4.6. LTE induces intracellular ROS production.

HL-60 (1×10^4/100 μl/96-wells plate) cells were treated with 50 μg/ml of C2 fraction for 0, 6, 15, 30 and 60 min (**A**), or with 25, 50 and 100 50 μg/ml of C2 fraction for 15 min (**B**). The cells were then incubated with 20 μM of DCFH-DA for 60 min. Fluorescence was measured at 485 nm excitation and 530 nm emission using a Wallac ARVO^{TM} SX 1420 Multilable Counter (Perkin Elmer Life Sciences, Singapore). The relative amount of intracellular ROS production was expressed as the fluorescence ratio of the treatment to control. Each value represents the mean ± S.D. of triplicate cultures. Means with differently lettered superscripts differ significantly at the probability of $p < 0.05$.



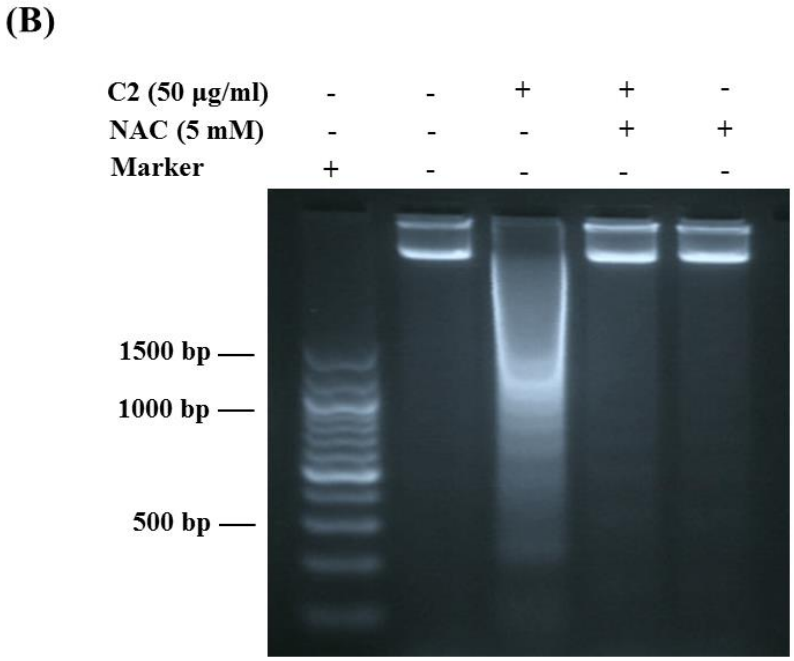


Fig.4.7. Antioxidant NAC blocks C2-induced intracellular ROS production and DNA fragmentation.

(**A**) HL-60 (1×10^4/100 μl/96-wells plate) cells were pretreated with 5 mM of NAC for 1 hour and then treated with 50 μg/ml of C2 fraction for 15 min. ROS production was measured as described in Fig 4.6. Each value represents the mean ± S.D. of triplicate cultures. Means with differently lettered superscripts differ significantly at the probability of $p < 0.05$. **(B**) HL-60 cells (7.5×10^5/5 ml/6-cm dish) were pretreated with 5 mM of NAC for 1 hour

and then treated with 50 μg/ml of C2 fraction for 24 h. Then, the cells were harvested by centrifugation, and DNA was extracted as described in Method section. The DNA fragments were separated on 2% agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide. The 100 bp DNA Ladder One (Nacalai Tesque, Kyoto, Japan) served as a molecular marker (B).

4.5 Discussion and conclusion

Mitochondrion has been reported to play a key role in the regulation of apoptosis [30]. Mitochondrial dysfunction including the loss of mitochondrial membrane potential (ΔΨm), permeability transition, and release of cytochrome *c* from the mitochondrion into the cytosol is associated with apoptosis [31]. Proapoptotic Bax can leads directly to mitochondrial membrane permeabilization to promotes the release of cytochrome *c* [17-19]. Released cytochrome *c* activates downstream caspases to introduce apoptosis [20]. In this study, the upregulation of proapoptotic Bax protein and downregulation of antiapoptotic Mcl-1 protein were observed in the cells treated with C2 fraction of LTE (**Fig. 4.4**). Moreover, the time-dependent loss of ΔΨm and cytochrome *c* release from mitochondrial to cytosol were observed in the cells with same treatment (**Fig. 4.5**). These events might initiate the activation of caspases. Exactly, the activation of caspase-3, -8 and caspase-9 were confirmed by detecting their cleaved peptides in both dose- (**Fig. 3A**) and time-dependent experiments (**Fig. 4.3B**) in the fashion coinciding with apoptotic morphology and DNA fragmentation (**Fig. 4.2B**). These data demonstrated that a mitochondrial damage-dependent pathway might be involved in LTE-induced apoptosis in HL-60 cells.

Several lines of studies have shown that (poly)phenolic compound can induce apoptosis in HL-60 cells by generating reactive oxygen species (ROS) [10,27,32], which damage mitochondrial to become dysfunction status including the loss of mitochondrial

membrane potential (ΔΨm) and release of proapoptotic protein from the mitochondrion into the cytosol to initiate apoptosis. Other researchers reported that phenolic components presents in loquat leaves may contribute the antioxidant and anticancer activities [33], thus, we confirmed that LTE might have the same mechanisms as other (poly)phenolic compounds reported to induce apoptosis in HL-60 cells (**Fig 4.6 and 4.7**).

In conclusion, LTE suppressed the proliferation of HL-60 cell by inducing ROS-mediated mitochondrial dysfunction pathway as well as upregulation of proapoptotic Bax and downregulation of antiapoptotic Mcl-1 proteins respectively. The results from cell model will help to understand the chemopreventive effects and molecular mechanisms of loquat tea.

4.6 References

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Chapter 5

Chemical characterization of loquat (*Eriobotrya japonica*) tea

5.1 Abstract

The bioactive compounds in LTE contributing to the antioxidant, anti-inflammatory and apoptosis activities were analyzed with the methodologies of high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FT-IR), mass spectra (MS) and nuclear magnetic resonance (NMR) analysis. Total phenolic content in LTE fractions were compared with fresh loquat leaves. The data indicated that LTE contained new bioactive phenolic components that were produced from the release and/or degradation of bound phenolic compounds from fresh loquat leaves during roasting process.

5.2 Introduction

Accumulated data showed that fresh loquat leave contained hydroxycinamoyl chlorogenic acids [1-3], various triterpenes [4], tannins and megastigmane glycosides [5], flavonoids, procyanidins and related flavan 3-ols [6-7] and most of these compounds have been demonstrated to have bioactivities. Although LTE are demonstrated to have antioxidant, anti-inflammatory and apoptosis activities (Chapter 2, Chapter 3 and Chapter 4), their bioactive compounds are unknown yet.

High-performance liquid chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compounds of sample that have been dissolved in solution and it can analyse simultaneously all components of interest together with their possible derivatives or degradation products [8-10]. Fourier transform infrared spectroscopy (FTIR) identifies specific functional groups within a molecule on the basic of absorption

wavelengths at characteristic frequencies [11]. This method with very small amounts of sample (i.e. 10-25 mg) is nondestructive, and can be applied to study both dry and wet samples [12-13]. FTIR data is helpful to have other data from other instrumentation, such as mass spectra (MS) or number of carbons/hydrogens (NMR), to confirm a proposed molecular model. Mass spectra (MS) measure the mass to charge ratio of ions contained in sample by electrostatic acceleration and magnetic field perturbation, providing a precise molecular weight and nuclear magnetic resonance (NMR). NMR is high resolution spectroscopy that distinguishes and counts atoms in different locations in molecule. FTIR, MS and NMR are primary methods for determining chemical characterization [14-18].

Polyphenols are a group of chemical substances and characterized by the presence of more than one phenol unit. They are bioactive compounds with antioxidant, anti-inflammatory and anticarcinogenic properties [19,20]. In this study, I have used HPLC to check chemical profiles of LTE (T, C and C2 fraction), compared with fresh loquat leave. Moreover, I characterized chemical properties by FTIR, MS and H´NMR analysis. Finally I investigated total phenolic content in LTE compared with fresh loquat leaves.

5.3 Methods

5.3.1 HPLC analysis of LTE

Ten microliters of each extract were analysed using HPLC unit and a 250 × 4.6 mm i.d., Crest Pak C_{18} T-5 column. The solvent system was a mixture of 0.05 M H_3PO_4 in CH_3CN (A) and 0.05 M H_3PO_4 in water (B), with the flow rate of 0.8 ml/min, and the gradient was as follows: 4% A to 30 % A for 39 min and 30% A to 75% A for 6 min. Spectroscopic data from all peaks were accumulated in the range of 200-700 nm, and chromatograms were recorded at 280 nm. HPLC analysis was done by previous described [21].

5.3.2 Chemical characterization of bioactive compounds of LTE

C2 fraction separated from roasted loquat leaf extract was detected using a JASCO FT-IR/IRT-3000 ATR-30-Z (Tokyo, Japan) equipped with an ATR attachment. The FT-IR frequencies were detected between 400 and 4000 cm^{-1}. Moreover, the MALDI-TOF-MS was obtained in a 2,5-dihydroxybenzoic acid (DHB) matrix in positive ion mode on a Bruker Autoflex Speed/TOF/TOF(Bruker Daltonics, USA/CA) and the ^{1}H-NMR spectra were determined using a JEOL JNM-ECA600 (Tokyo, Japan). DMSO-d_6 was used as the solvent, and chemical shifts were expressed ppm with reference to tetramethylsilane.

5.3.3 Measurement of total phenol contents

The concentration of the total phenolic substances was measured according to the previous method with some modification [22]. Briefly, 10 μl of LTE or its fractions was mixed with 200 μl of 2% Na_2CO_3 in 96-well plates. After 3 min, 10 μl of 50% diluted Folin-Ciocalteu reagent was added to each well. The mixture was allowed to stand for 30 min in room temperature with mixing every 10 min, and then the absorbance was measured at 595 nm with a microplate reader (Thermo scientific Multiscan FC, version 1.00.79). The gallic acid was used as standard, and the total phenolic content was expressed as a gallic acid equivalent (GAE) in milligrams per gram of LTE or its fractions.

5.3.4 Statistical Analysis

All data were statistically analysed by ANOVA. Differences were considered significant for $p < 0.05$ and $p < 0.01$.

5.4. Results

5.4.1 Chemical quantification and qualification of LTE

To analyze the bioactive components in LTE, we first compared the HPLC profiling of LTE to fresh loquat leaves with known major four compounds as (3-caffeonylquinic acid (3-CQA), 5-caffeonylquinic acid (5-CQA), (-)-epicatechin (EC) and procyanidin B2 (ProB2) as standards.The amount of 3-CQA, 5-CQA, EC and ProB2 were 16.64 ± 0.639, 6.59 ± 0.646, 4.62 ± 0.590 and 1.16 ± 0.127 ug/mg extract of fresh loquat leaves, and 1.03 ± 0.190, 0.26 ± 0.035, 0.02 ± 0.006, 0.01 ± 0.005 ug/mg crude extract (T fraction) of loquat tea, respectively (**Table 1**). As shown in Figure 4.1 and Table 1, larger amount of 5-caffeonylquinic acid and 3-caffeonylquinic acid, and smaller amount of (-)-epicatechin and procyanidin B2 were detected in fresh loquat leaves, but few in LTE crude (**Fig. 5.1B**) and was not detected in C and C2 fraction (**Fig. 5.1C** and **D**) of LTE. In place of these, some new peaks were detected (**Fig. 5.1 C** and **D**).

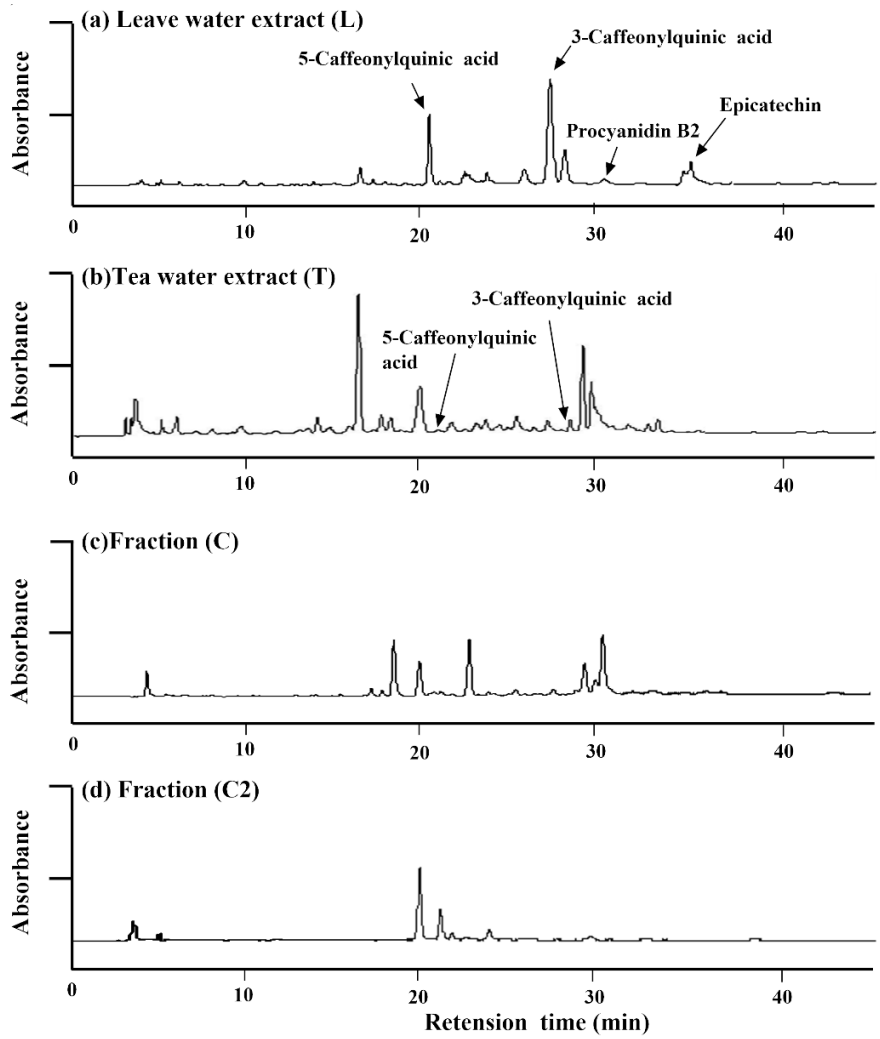

Fig. 5.1 HPLC profiles of fresh Loquat leave and LTE fractions.

(**A**) Fresh loquat leave, (**B**) LTE T, (**C**) C fraction and (**D**) C2 fraction. (-)-Epicathechin, 3-caffeonylquinic acid, 5-caffeonylquinic acid and procyanidin B2 were used as standards. The CresPak C_{18}T-5 column (4.6 mm i.d. × 250 mm) was set in 40 ºC. Ten microlitter of standards or LTE solution was injected to the column after filtered with a millipore filter (0.45 μM) and flowed with 4% to 30% of 0.05 M of H_3PO_4 in CH_3CN for 39 min and then changed 30% to 75% for 6 min under a flow rate of 0.8 ml/min. Spectroscopic data from all peaks were accumulated in the range of 200-700 nm, and chromatograms were recorded at 280 nm.

Table 1. Bioactive components responsible for fresh loquat leaves and loquat tea

Extract/ Fraction	ORAC (TE μmol/g dry basis)	Known chemicals (mg/g dry basis)		New components (mg/g dry basis)	
Leave water extract (L)	910.6 ± 25.1	3-caffeonylquinic aci	16.64 ± 0.639	Compound 1	n.d.
		5-caffeonylquinic aci	6.59 ± 0.646		
		Epicatechin	4.62 ± 0.590	Compound 2	n.d.
		Procyanidin B2	1.16 ± 0.127		
Tea water extract (T)	2146.3 ± 123.5	3-caffeonylquinic aci	1.03 ± 0.190	Compound 1	3.87 ± 0.768
		5-caffeonylquinic aci	0.26 ± 0.035		
		Epicatechin	0.02 ± 0.006	Compound 2	0.95 ± 0.254
		Procyanidin B2	0.01 ± 0.005		
Fraction C	3076.4 ± 28.0	3-caffeonylquinic aci	n.d.	Compound 1	7.58 ± 0.676
		5-caffeonylquinic aci	n.d.		
		Epicatechin	n.d.	Compound 2	9.85 ± 1.096
		Procyanidin B2	n.d.		
Fraction C2	3201.2 ± 186.0	3-caffeonylquinic aci	n.d.	Compound 1	32.07 ± 1.518
		5-caffeonylquinic aci	n.d.		
		Epicatechin	n.d.	Compound 2	15.22 ± 0.874
		Procyanidin B2	n.d.		

Each value represents the mean ± S.D. of three separated experiments. n.d: not detected

5.4.2 Chemical characterization of bioactive compounds of LTE

HPLC analysis revealed that the major compounds (3-caffeonylquinic acid, 5-caffeonylquinic acid, epicatechin and procyanidin B2) of fresh loquat leave were not detected in fractionated C and C2. In place of these, some new components, which were not found in fresh loquat leaves, were detected. To characterize these chemicals, we further investigated C2 fraction by FT-IR spectra. The strong and broad band of O-H stretching was observed at 3252 cm^{-1}. The existence of one or more aromatic rings in a structure is normally readily determined from the C–H, C=C and C–C ring. Medium strong and finger peaks at 1593, 1543 and 1515 assigned to C=C stretching modes. The generated C-C aromatic stretch was observed at 1387.53 with strong absorptions of FT-IR. The peaks at 719.3 and 673.3 cm^{-1} could be assigned as mono substitute benzene (**Fig. 5.2**). Moreover, C2 fraction showed primary three peaks at m/z 170 - m/z 330 on MALDI-TOF MS spectra, and the

signals for aromatic or olefinic protons at 6.256-8.308 ppm as well as for the methylene or alicyclic protons at 0.823-2.995 ppm were detected in the ^{1}H-NMR spectra. Depending on data from HPLC, NMR and FT-IR, the compounds in LTE might be several kinds of phenolic compounds that are not included in fresh loquat leave.

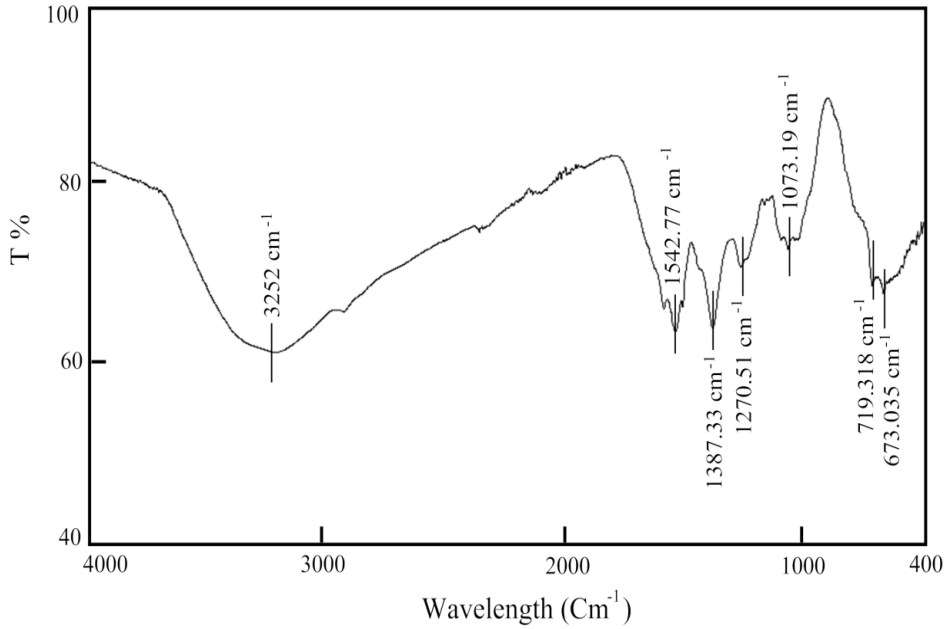

Fig. 5.2 FTIR spectra of C2 fraction.

The C2 fraction separated from roasted loquat leave extract was detected by JASCO FT-IR /IRT-3000 ATR-30-Z (Tokyo, Japan) equipped with ATR attachment. The FT-IR frequencies were detected between 400 and 4000 cm^{-1}.

5.4.3 Total phenol content of LTE

Since phenolic compounds generally have antioxidant capacity, we thus quantified the phenolic contents of fresh and its fractions of LTE, using gallic acid as reference standard. As shown in Figure **5.3**, phenolic contents of LTE fractions (T) are higher than fresh loquat leaves (L). Moreover, the fraction C and C2 contained higher phenolic amount among these fractions.

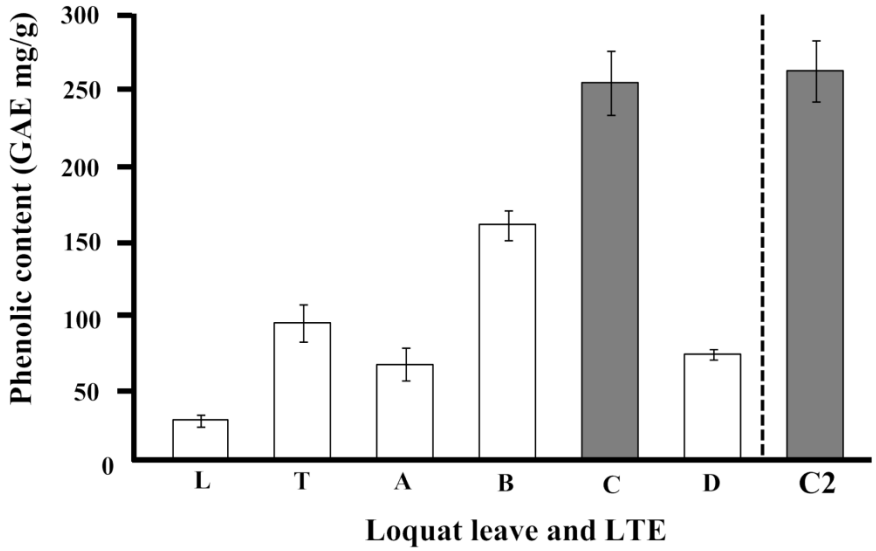

Fig. 5.3 Phenolic contents of fresh loquat leaf, LTE and C2 fractions.

Phenolic contents were determined by Folin-Ciocalteu method. The amounts were presented as gallic acid equivalents (GAE mg/ml). L, fresh loquat leave extract T, loquat tea extract; A, water elution fraction; B, 30% EtOH fraction; C, 50% EtOH fraction; D, 100% acetone fraction, C2 fraction purified from C fraction, respectively. The data represent the mean ± SD of three separated experiments.

5.5 Discussion and conclusion

In order to indentify and quantify the bioactive compounds in LTE that are contribute to antioxidant and anti-inflammatory and apoptosis effects, fresh loquat leave and various LTE extractions were subjected to reverse phase-HPLC at 280 nm. I compared the HPLC profiles of LTE fractions with the extract of fresh loquat leaves because some bioactive flavonoids such as (-)-epicatechin, 3-caffeonylquinic acid, 5-caffeonylquinic acid, and procyanidin B2 have been clarified in fresh loquat leaves, and have been suggested to contribute the biological activities [3,7,23]. I confirmed these compounds in fresh loquat leaves using their standard samples (**Fig. 5.1**). These results are in agreement with the previous report [1,7]. However, their compounds were much lower in LTE (**Fig. 5.1A-D**),

and finally disappeared in the C and C2 fractions. In place of them, some new compounds were detected in the C fractions (**Fig. 5.1A-D**). Although I could not determine the chemical structure at this moment, the data from FT-IR spectra showed the existence of aromatic rings and broad band of O-H stretching (**Fig 5.2**). Moreover, the C2 fraction showed primary three peaks at m/z 170 - m/z 330 on the MALDI-TOF MS spectra, and the signals for aromatic or olefinic protons at 6.256-8.308 ppm as well as for the methylene or alicyclic protons at 0.823-2.995 ppm were detected in the ^{1}H-NMR spectra. Thus, I gather that the bioactive compounds in LTE might be, at least partly, several kinds of phenolic compounds, which are produced from the release and/or degradation of phenolic compounds in fresh loquat leaves during roasting process. Some similar findings have also been reported that (-)-epicatechin and procyanidin significantly decreased after roasting cocoa beans and coca ingredients due to epimerization [24,25]. These findings were agreed with epicatechin caffeonylquinic, acid and procyanidin B2 became very sensitive to isomerization and polymerization at elevated temperature [26- 29].

It has been reported that phenolic compounds are antioxidants that can exert modulatory action in cell by interacting with a wide spectrum of molecular targets central to the cell signaling. The molecular mechanisms involved in the anti-inflammatory activities of polyphenols have also been suggested to include: the inhibition of pro-inflammatory mediators; the inhibition of MAPK and NF-κB signalings; and activation of Nrf2 [20,30-31]. I next measured the phenolic amount of the C and C2 fractions, comparing with fresh loquat leave (L) and LTE using gallic acid as standard. As shown in Figure 4.3, phenol content in C and C2 fraction of LTE were 258 ± 22mg/g and 267 ± 21 mg/g. In fresh loquat leaves and LTE (T), the total phenolic contents were 26 ± 4.2 mg/g and 77.4 ± 2.2 mg/g, respectively. These data indicated that phenolic contents were increased when loquat leaves were roasted at 350°C for 30 min. It is known that polyphenol content of food was

significantly influenced by high temperature processing [32,33]. This observation was supported with showing the increase of the phenolic contents in rice hull by converting insoluble phenolic compounds to soluble phenolics or phenolic derivatives after simple heat treatment [34] and high temperature treatment increased total phenolic content of sweet potato [22]. However, total phenolic content was decreased after roasting in green tea [26]. Thus, heat treatment for liberating phenolic compounds from various plants may be different. Furthermore, there are three possible mechanisms to explain the changes of phenolic content of samples exposed to high temperature. These mechanisms include the release of bound phenolic compounds, the partial degradation of lignin leading to the release of phenolic acid derivatives, and the beginning of thermal degradation of the phenolic compounds [33]. In the present study, the observation of significant increase in total phenolic compound indicated that loquat leave could liberate bound phenolic compounds by roasting.

Loquat tea extracts not only increased amount of phenolic content but also increased antioxidant activity due to roasting. The antioxidant activity in fresh loquat leave (L) was weak (18.34 % radical scavenging activity at the concentration of 50μg/ml). After roasting, the antioxidant activity of loquat tea (T) was significantly increased to 2.4 times (**Fig 2.2**). The antioxidant activity of heat-processed foods has been proposed in other several studies. Most of antioxidant compounds from natural plant products were found to be liberation of low molecular weight natural antioxidants from the repeating subunits of high molecular polymers after roasting process [32,35]. From this observation, the stronger antioxidant activity after roasting could be attributed in increase amounts of antioxidant compounds as polyphenols. The hypothesis was agreed with a good relationship between amount of polyphenols and antioxidant activity. Polyphenol contents with antioxidant activities of the extracts of fresh loquat leave (L) and LTE (T, A, B, C and D) were observed in the order of C > B > T > A > D > L.

In conclusion, the bioactive components of LTE are speculated to be phenolic compounds that were produced from fresh loquat leaves during the roasting processes.

4.6 References

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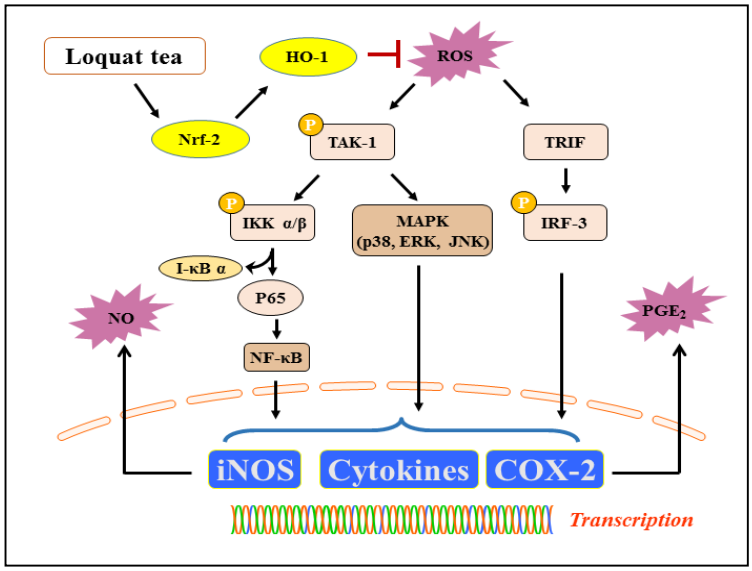
Conclusion

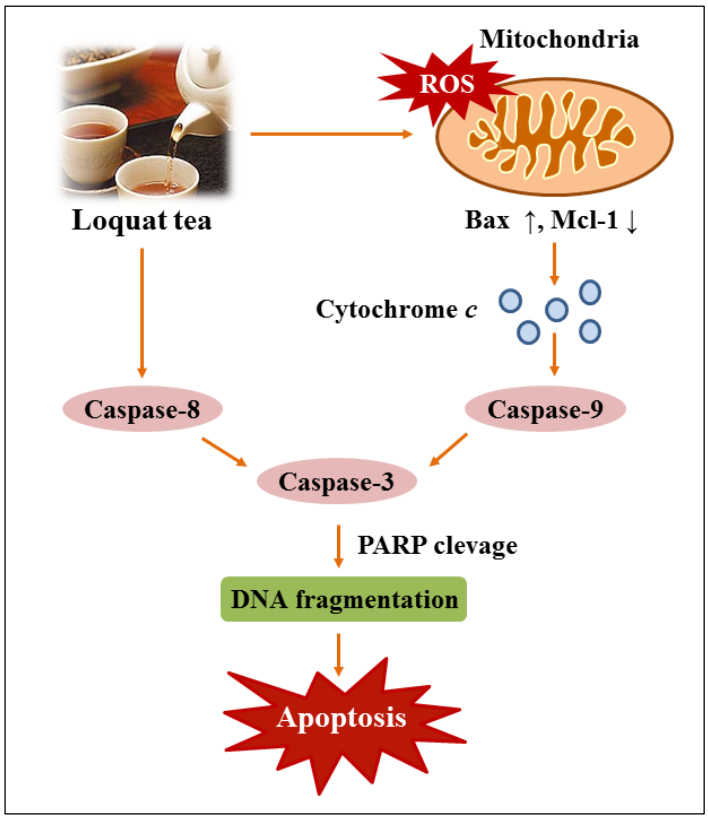
Loquat (*Eriobotrya japonica*) tea is made from loquat leaves by roasting to 350 ºC in a ceramics vessel after washing and drying in Totsukawa Noujyo Ltd. Loquat tea is called as “Biwa Cha” in Japanese and now used for health beverage in Japan. The present study investigated the chemopreventive activities and molecular mechanisms of loquat tea including antioxidant, inti-inflammatory and apoptosis induction properties. The data obtained in the study are concluded as the followings.

1) Loquat tea extract (LTE) by boiling water revealed potent antioxidant effect *in vitro* and in culture cells. The cellular data indicated that the antioxidant effect of LTE was regulated by Nrf2-mediated HO-1 activation.
2) LTE inhibited pro-inflammatory mediators such as COX-2, PGE_2, iNOS, NO, IL6, RANTES and TNF-α in macrophage-like cells (RAW264.7). LTE further attenuated the activations of MAPK, NF-κB and IRF3 pathways which are major signaling pathways to regulate expressions of pro-inflammatory mediators. Moreover, LTE reduced serum cytokines levels and attenuated paw edema of mice induced by LPS *in vivo*. Thus, LTE revealed anti-inflammatory effects by attenuating pro-inflammatory signaling and mediators in both cell model and animal model.



Crosstalk between the antioxidant and anti-inflammatory activities of Loquat tea

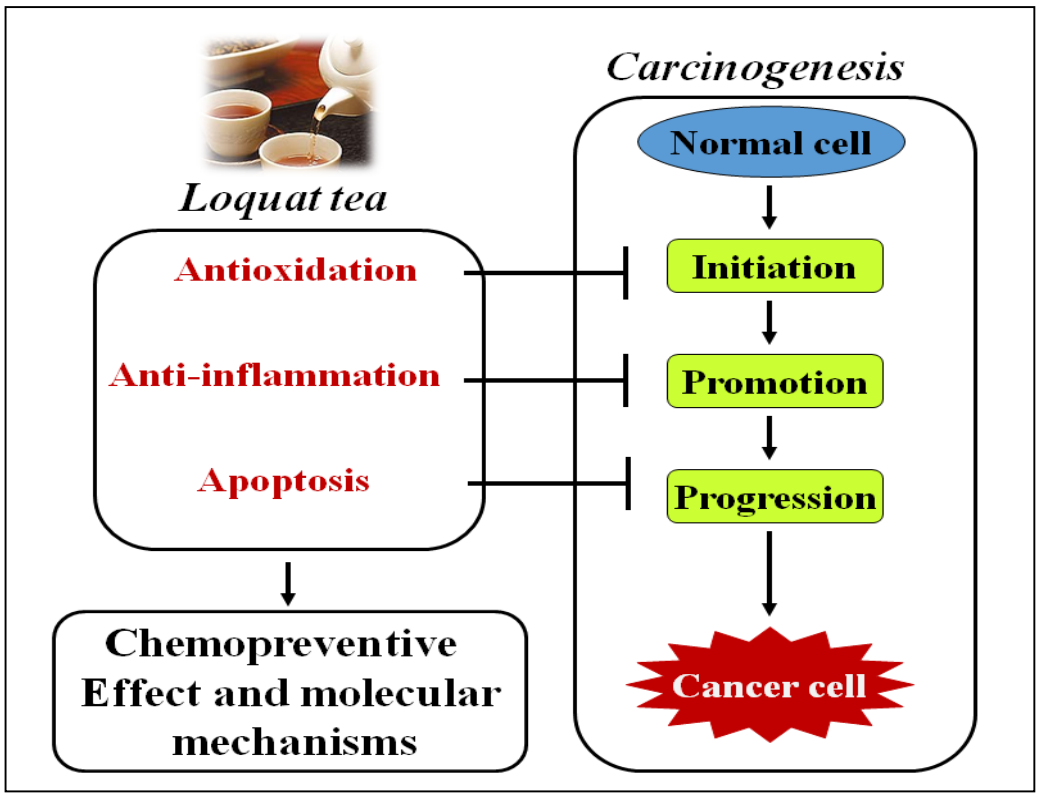
3) LTE suppressed the proliferation of HL-60 cells and induced apoptosis through ROS-mediated mitochondrial dysfunctional pathway. LTE treatment increased ROS production that leads to the loss of mitochondrial membrane potential (ΔΨm). Consequentially, an increase of proapoptotic protein (BAX), and decrease of anti-apoptotic protein (MCL-1) as well as the release of cytochrome *c* from mitochondria to cytosol were induced for apoptosis executed by caspases activations. Thus, LTE induced apoptosis in HL-60 cell through ROS-mediated mitochondrial dysfunction pathway.



Schematic molecular mechanisms of apoptosis induction by Loquat tea

4) Although original flavonoids in loquat leaves were reduced, some new phenolic compounds are produced in LTE during roasting process of fresh loquat leaves. These new compounds are major bioactive components showing antioxidant, anti-inflammatory and apoptosis induction properties.

Taken together, LTE might exert its chemopreventive effects with its bioactive properties of antioxidation, anti-inflammation and apoptosis induction to prevent the initiation, promotion and progression of carcinogenesis. All of these data provide scientific evidence for understanding the health benefits of loquat tea.



Chemoprevention effect and molecular mechanisms of Loquat tea