# The anti-inflammatory activities and molecular mechanisms of *Lonicera caerulea* L. berry

## （ハスカップベリーの抗炎症活性及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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**2017**

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# Abbreviations

AIA, adjuvant-induced arthritis

AMPK, AMP-activated protein kinase

AP-1, activator protein-1

α-SMA, alpha-smooth muscle actin

$CCl_4$, carbon tetrachloride

CFA, complete Freund's adjuvant

COX, cyclooxygenase

C3G, cyanidin 3-glucoside

DCs, dendritic cells

EC, -(-)epicatechin

EGCG, epigallocatechin gallate

EGFR, epidermal growth factor receptor

ELISA, enzyme-linked immunosorbent assay

eNOS, endothelial NOS

ERK, extracellular signal-regulated kinase

FBS, fetal bovine serum

FoxO1, forkhead box protein O1

G-CSF, granulocyte-colony stimulating factor

GGT, gamma-glutamyl transferase

GM-CSF, granulocyte-macrophage colony-stimulating factor

GOT, glutamic oxaloacetic transaminases

GPT, glutamate- pyruvate transaminase

GPx, glutathione peroxidase

HDL-c, high density lipoprotein cholesterol

H&E, hematoxylin-eosin

HFD, high fat diet

HO-1, heme oxygenase-1

HPLC, high performance liquid chromatography

HSP70, 70-kDa heat shock protein

*i.d.*, intradermal injection

IFN-γ, interferon-gamma

IKK, IκB kinases

IL, interleukin

iNOS, inducible NOS

IR, insulin receptor

JNK, c-Jun N-terminal kinase

KC, keratinocyte-derived cytokine

LCBP, *Lonicera caerulea* L. berry polyphenols

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MCP-1, monocyte chemotactic protein 1

MIP, macrophage inflammatory protein

MnSOD, manganese-dependent SOD

MYD88, myeloid differentiation primary response gene 88

NAFLD, nonalcoholic fatty liver disease

NASH, nonalcoholic steatohepatitis

ND, normal diet

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

nNOS, nitric oxide synthase

NO, nitric oxide

NQO1, NADPH dehydrogenase 1

Nrf2, nuclear factor (erythroid -derived 2)-like 2

OA, osteoarthritis

PAMPs, pathogen associated molecular patterns

$PGE_2$, prostaglandin $E_2$

*p.o.*, oral administration

RA, rheumatoid arthritis

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

ROS, reactive oxygen species

*s.c.*, subcutaneous injection

SD, standard deviation

SOD, superoxide dismutase

TAK1, transforming growth factor β activated kinase-1

TBARS, thiobarbituric acid reactive substances

T-cho, total cholesterol

TG, total triacylglycerol

TLRs, toll-like receptors

TNF-α, tumor necrosis factor-α

TNFRs, tumor necrosis factor receptors

## Abstract

*Lonicera caerulea* L. is a member of the Caprifoliaceae family that grows naturally in the cool Northern Hemisphere. Its berry is rich in polyphenols, and recently receiving attention in the prevention against chronic diseases due to its potential antioxidant and anti-inflammatory properties. However, the molecular mechanisms underlying the activities remain unclear. Thus, the present study aimed to investigate the protective effects and molecular mechanisms of *Lonicera caerulea* L. berry polyphenols (LCBP) against excessive inflammation and relevant diseases, using both cell and animal models.

First, *in vitro* anti-inflammatory effects and molecular mechanisms of LCBP were investigated in lipopolysaccharide (LPS)-activated RAW264.7 cell model. Pretreatment with LCBP concentration-dependently suppressed the production of inflammatory mediators including IL-1β, IL-6, TNF-α, nitric oxide (NO), and $PGE_2$. Cell signaling analysis revealed that LCBP down-regulated TAK1-mediated MAPK and NF-κB pathways. Moreover, LCBP reduced oxidative stress by up-regulating the expression of Nrf2 and MnSOD. Finally, cyanidin 3-glucoside (C3G) and -(-)epicatechin (EC) were identified to be the major bioactive components of LCBP for these activities. These data demonstrated that LCBP rich in C3G and EC attenuated LPS-induced inflammation by down-regulating TAK1-mediated MAPK and NF-κB pathways, and up-regulating the expression of Nrf2 and MnSOD.

Second, *in vivo* anti-inflammatory effects of LCBP were investigated in a LPS-induced mouse paw edema model. Oral administration with LCBP attenuated paw edema and significantly decreased the serum cytokines levels including IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12 (p70), KC, MCP-1, MIP-1α, RANTES and TNF-α in LPS-induced mice, but had no significant effects on IL-1α, IL-9, IL-12(p40), IL-13, G-CSF, GM-CSF, IFN-γ and MIP-1β. The data demonstrated that oral administration with LCBP attenuated LPS-induced inflammation by inhibiting the production of multiple proinflammatory cytokines, rather than promoting the production of anti-inflammatory cytokines.

Third, the preventive effects of LCBP against chronic inflammation-related diseases were investigated in adjuvant-induced arthritis (AIA) rat model. Oral administration of LCBP attenuated AIA rat symptom. The serum levels of pro-inflammatory factors including TNF-α, IL-1β, IL-6, and NO were significantly reduced in LCBP-fed rat. The production of inflammatory enzymes, iNOS and COX-2, in the spleen was also significantly reduced. Moreover, serum transaminases including GOT, GPT and GGT were decreased, and the antioxidant enzymes including SOD and GPx were restored. Thus, LCBP attenuated rat AIA symptom by both decreasing the production of proinflammatory factors and enhancing the activity of antioxidant enzymes.

Finally, the crosstalk between the antioxidant and anti-inflammatory activities of LCBP was investigated in an experimental model of nonalcoholic steatohepatitis (NASH), which is a common disease that closely associated with inflammation and oxidative stress. Oral administration of LCBP improved histopathological features of NASH with higher insulin sensitivity, less lipid peroxidation, and lower levels of cytokines. Moreover, LCBP increased the expression of Nrf2 and MnSOD, but decreased the expression of FoxO1 and HO-1. These data revealed that LCBP could attenuate NASH by both enhancing the expression of antioxidant proteins and inhibiting the production of oxidative proteins.

In summary, LCBP rich in C3G and EC exerted the protective effects against chronic inflammation-related diseases by suppressing the production of multiple proinflammatory mediators and oxidative stress factors. Molecular data revealed that LCBP inhibited inflammation by down-regulating TAK1-mediated MAPK and NF-κB pathways, and up-regulating the expression of Nrf2 and MnSOD. These results provide an insight into understanding the anti-inflammatory effects and molecular mechanisms of *Lonicera caerulea* L. berry.

# 要　旨

ハスカップベリーはスイカズラ科の一つで、気温の低い北半球で育ち、ポリフェノールが豊富なベリーである。近年、抗酸化・抗炎症作用による慢性疾患の予防効果に注目が集まっているが、分子メカニズムが明らかになっていない部分もあることから、本研究では過度の炎症およびそれに伴う疾患におけるハスカップベリーポリフェノール(LCBP)の予防効果およびその分子メカニズムの解明を分子・細胞から個体レベルまで行うことを目的とした。

まず、炎症細胞モデルであるリポ多糖(LPS)誘発 RAW264.7 細胞を用いて LCBP の抗炎症効果および分子メカニズムを解析した。その結果、LCBP 前処理の細胞では、濃度依存的に炎症誘発性メディエーターである IL-1β や IL-6、TNF-α や一酸化窒素(NO)、PGE2 の産生を抑制した。細胞シグナル解析の結果、LCBP が TAK1 を介する MAPK および NF-κB 経路を下方制御する一方、Nrf2 および MnSOD の発現を上方制御することで酸化ストレスを減少させることが明らかになった。また、これらの効果を示す LCBP の主な生物活性物質は cyanidin 3-glucoside (C3G)ならびに(-)epicatechin (EC)であることも明らかになった。

次に、LPS 誘発のマウス足浮腫モデルを用いて *in vivo* における LCBP の抗炎症効果を明らかにした。経口投与で LCBP を与えたマウスでは LPS 誘発の足浮腫の緩和ならびに IL-1β,IL-2,IL-3,IL-4,IL-6,IL-10,IL-12(p70),KC,MCP-1,MIP-1α,RANTES, TNF-α などの血清サイトカインの顕著な減少が見られた。一方、IL-1α, IL-9, IL-12(p40), IL-13, G-CSF, GM-CSF, IFN-γ, MIP-1β などの血清サイトカインには顕著な効果は見られなかった。

さらに、アジュバント関節炎(AIA)ラットモデルを用いて慢性炎症性疾患に対する LCBP の効果について実験を行った。その結果、LCBP の経口投与により AIA 症状は軽減された。TNF-α, IL-1β, IL-6, および NO の炎症性サイトカイン因子が顕著に減少し、脾臓組織中の iNOS や COX-2 等の炎症誘導性酵素の産生も大幅に減少した。さらに血中 GOT, GPT や GGT 等のトランスアミナーゼが減少し、SOD や GPx などの抗酸化酵素は回復した。

最後に抗酸化および抗炎症活性の関連性を解明するために、炎症と酸化ストレスが密接に関与する非アルコール性脂肪性肝炎(NASH)マウスモデル実験を行った。そ

の結果、LCBP 経口投与により、インスリン感受性の向上、脂質過酸化反応の低下、サイトカイン産出の低下と共に病理組織学的特徴も改善した。さらに、抗酸化タンパク質 Nrf2 や MnSOD を増強させる一方､酸化促進に関するタンパク質 FoxO1 や HO-1 を減少させた。

総括として、LCBP は C3G および EC を豊富に含み、様々な炎症性サイトカインメディエーターや酸化ストレス要因の産生を抑制することで慢性炎症疾患を予防することが明らかになった。その分子機構として、LCBP は TAK1 を介する MAPK および NF-κB の炎症経路を抑制すると伴に Nrf2 および MnSOD の抗酸化経路を活性化することも明らかになった。これらの成果はハスカップベリーの抗炎症効果や分子メカニズムの理解に新たな知見を提供するものである。

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## Acknowledgements

This thesis would not have been possible without the guidance, inspiration and support of many kind people. First and foremost, I would like to express my sincere gratitude and appreciation to my chief advisor Prof. Dr. De-Xing Hou for his guidance, patience and encouragement during my research and study at Kagoshima University. Prof. Hou has offered me great help and guidance in not only my research, but also personal development. His enthusiasm, perseverance and excellence in conducting research, as well as his patience and generosity in guiding students undoubtedly deserve me to learn and emulate.

I would like to thank and honor my co-supervisors Dr. Koji Wada, University of the Ryukyus, Dr. Akira Ohtsuka and Dr. Kozue Sakao, Kagoshima University, for their cordial and professional guidance. They have provided valuable suggestions and technical supports during my research.

I would also like to express my sincere thanks and appreciation to China Scholarship Council (CSC), which granted me a scholarship during the past three and a half years. Without the financial support from CSC, I would not be able to put all my energies on my research.

My warmest thanks also go to Dr. Jianhua He, Dr. Xi He, Dr. Jihua Chen, Dr. Si Qin, Dr. Ayami Hisanaga and Mr. Satoshi Yano for their academic and technical supports during my research. I also appreciate the friendship and support of all the members in the Laboratory of Food Functions and Nutrigenomics.

The teachers and office staffs of The United Graduate School of Agricultural Science, Faculty of Agriculture, International Student Center and Office are highly appreciated for their enthusiastic help and support.

I am very grateful for the love, encouragement, tolerance and sustained support of my parents. I would like to thank my brother and sisters for their constant inspiration and help. My deepest love and appreciation to my lovely wife, who accompanied, inspired and supported me during the past years.

Finally, I would like to thank all of my cherished friends for their support.

# Chapter 1

# General introduction

## 1.1 *Lonicera caerulea* L. berry

*Lonicera caerulea* L., commonly known as blue honeysuckle, honeyberry, or haskap, is a member of the Caprifoliaceae family that grows naturally in cool temperate Northern Hemisphere such as Siberia of Russia, Hokkaido of Japan (Terahara et al., 1993), and northern China (Jin et al., 2006). The fruit of *Lonicera caerulea* L., a berry with oval to long fruit shape and dark navy blue to purple in color, is widely harvested in Russia, China, and Japan, but practically unknown as edible berries in Europe and North America decades ago (Chaovanalikit et al., 2004; Palikova et al., 2008). *Lonicera caerulea* L. berry has been reported as a healthy berry in Western countries such as United States (Chaovanalikit et al., 2004), Canada (Rupasinghe et al., 2015), and Czech Republic (Zdarilova et al., 2010) in recent years, since it can be successfully cultivated outside its original habitat (Palikova et al., 2008).

*Lonicera caerulea* L. berry is rich in polyphenols especially anthocyanins, and cyanidin 3-glucoside has been identified as the major anthocyanin in previous studies, although other (poly)phenolic components are variety (Chaovanalikit et al., 2004; Jin et al., 2006; Palikova et al., 2008; Rupasinghe et al., 2015).

Cool climate berries are considered to possess many biological functions and benefit to prevent against chronic disorders (Rupasinghe et al., 2015). *Lonicera caerulea* L. berry has been used for treating cancer, inflammation, hepatic complications, influenza, and wounds for thousands of years in East Asian countries (Kaczmarska et al., 2015), and known for the effects of heat clearing and detoxicating, detumescence, and visual improvement in the folk medicine in China (Dong, 2013; Jin et al., 2006). The biological activities of *Lonicera caerulea* L. berry are considered to be polyphenols-dependent based on the antioxidant (Palikova et al., 2009) and

anti-inflammatory (Rupasinghe et al., 2015) studies. In laboratory investigations, polyphenols from *Lonicera caerulea* L. berry have shown protective effects against inflammation and oxidative stress in many disease models such as uveitis (Jin et al., 2006), periodontal inflammation (Zdarilova et al., 2010), and hepatic inflammation (Wang et al., 2016) induced by lipopolysaccharide (LPS), and skin damage induced by ultraviolet A (Svobodova et al., 2008; Vostalova et al., 2013), ultraviolet B (Svobodova et al., 2009), or radiation (Zhao et al., 2012), as well as abnormal lipid and glucose metabolism (Jurgonski et al., 2013; Takahashi et al., 2014) and obesity (Wu et al., 2013).

## 1.2 Inflammation

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infection and tissue injury. Inducers of inflammation can be classified as exogenous and endogenous. Exogenous factors include microbial pathogen associated molecular patterns (PAMPs) and virulence factors, allergens, irritants, foreign bodies, and toxic compounds. Endogenous factors including signals released from damaged tissues and stressed, malfunctioning or dead cells, endogenous crystals, and products of extracellular matrix (ECM) breakdown (Medzhitov, 2008). In the process of inflammation, inducers stimulate sensors like antigen presentation by dendritic cells (DCs) in local tissues and organs, and then trigger the production of numerous inflammatory mediators by immune cells like T cells and macrophages (Asagiri et al., 2008). The increased inflammatory mediators not only provoke local inflammatory response, but also recruit more immune cells assembling at the injured tissue to induce systemic inflammation through circulation (Neurath, 2014). The production of inflammatory mediators is mediated by cellular signaling pathways, which can be activated by the inducers of inflammation directly and by cross effects between different inflammatory mediators through receptors (Hotamisligil, 2006; Medzhitov, 2008).

### 1.2.1 Inflammatory mediators and cytokine network

Inflammatory mediators such as vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines and proteolytic enzymes play important roles in regulating the inflammatory response. Vasoactive amines and peptides have complex effects on the vasculature, as they cause increased vascular permeability and vasodilation, or vasoconstriction, depending on the specific conditions (Medzhitov, 2008). Nitric oxide (NO) is a powerful vasodilator and cytotoxic agent that synthesized from L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) (Korhonen et al., 2005). There are three NOS isoforms including neuronal nitric oxide synthase (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The nNOS is mainly expressed in neuronal tissues (Zhou and Zhu, 2009), while eNOS, the isoform mainly exists in endothelial cells (Shaul, 2002), is the predominant NOS isoform in vasculature (Forstermann and Munzel, 2006). Both nNOS and eNOS are constitutively expressed in resting cells and controlled by intracellular calcium concentration to maintain the homeostasis of NO (Alderton et al., 2001). The iNOS is not expressed in most resting cells, but can be overexpressed under the stimulation of inflammatory inducers such as bacterial lipopolysaccharide (LPS), and thus induces large amount of NO to provoke inflammation (Aktan, 2004).

Eicosanoids are representative lipid mediators that derived from arachidonic acid by catalysis of cyclooxygenases (COX-1 and COX-2), which generate prostaglandins (Morita, 2002). COX-1 is the dominant source of prostaglandins that expressed constitutively in most cells and function as a homeostasis regulator, while COX-2 is induced by inflammatory stimuli, hormones, and growth factors, and overexpressed in inflammation and proliferative diseases such as cancer (Ricciotti and FitzGerald, 2011). Prostaglandin $E_2$ ($PGE_2$), a well-known synthesized product of COX-1 and COX-2, is recognized as a mediator to promote local vasodilatation and attraction of neutrophils, macrophages, and mast cells at early stages of inflammation (Kalinski, 2012; Nakayama et al., 2006; Weller et al., 2007),

Cytokines are small “messenger proteins” that play vital roles in mediating the activation, proliferation, and infiltration of immune cells in the inflammatory response.

Cytokines include various proteins, peptides, and glycoproteins such as interleukins, chemokines, interferons, and tumor necrosis factors. Secreted cytokines can act on the cells themselves (autocrine effect), on nearby cells (paracrine effect), or on distant cells (endocrine effect) (Zhang and An, 2007). Most of cytokines such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α), and chemokines are considered to have proinflammatory effects in inflammation (Choy and Panayi, 2001; Moser and Willimann, 2004), while a few cytokines such as IL-4 and IL-10 possess anti-inflammatory properties (Murray, 2005; Woodward et al., 2012). However, the effects of cytokines can be reversed in some instances, for instance, the proinflammatory cytokine IL-6 can induce anti-inflammatory response in the absence of suppressor of cytokine signaling 3 (SOCS3) in macrophages (Yasukawa et al., 2003), and the anti-inflammatory cytokine IL-4 can induce colitis in recombinase activation gene-2 (RAG-2)-/- mice (Van Kampen et al., 2004). In addition, cytokines influence each other in inflammatory loops, for example, TNF-α induces the production of proinflammatory cytokines such as IL-1, IL-6, and IL-17 in many chronic inflammatory diseases like rheumatoid arthritis and psoriasis (Brennan and McInnes, 2008; Schett et al., 2013), IL-1 (α/β) has been proved to function as upstream of IL-17 to promote pathogenic T helper cells in experimental autoimmune encephalomyelitis (Sutton et al., 2006), and IL-6 can also induce the expression of IL-21 and thus promote the production of IL-17 (Zhou et al., 2007). Thus, understanding the cytokine network is important to discern which mediators drive the specific inflammatory pathogenesis.

### 1.2.2 Major signaling pathways

The expression of inflammatory mediators in inflammation is regulated by upstream signaling pathways, which can be activated by the inducers of inflammation such as microbial LPS, ultraviolet, stress, cigarette smoke, alcohol, food factors, environmental pollutants, or abnormal glucose and lipid metabolism through the cell surface receptors like Toll-like receptors (TLRs), tumor necrosis factor receptors (TNFRs), insulin receptor (IR), and epidermal growth factor receptor (EGFR)

(Aggarwal et al., 2009; Coggins and Rosenzweig, 2012; Newton and Dixit, 2012). Mitogen-activated protein kinase (MAPK) (Kaminska, 2005) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Lawrence, 2009) pathways have been considered as major proinflammatory signaling pathways that play a dominant role in inflammatory abnormalities, although complicated pathways are involved in inflammation. The mammalian MAPK family consists of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinase (Kim and Choi, 2010), and each of the kinases exists in several isoforms: ERK1 to ERK8; p38-α, -β, -γ, and -δ; and JNK1 to JNK3 (Dhillon et al., 2007). The JNK, also known as stress-activated protein kinase (SAPK), is mainly activated in response to cellular stress such as toxic, hypoxic, or oxidative stress through TLRs and TNFRs (Weston and Davis, 2002). The ERK and p38 kinase are activated in response to similar stimulus as JNK (Shaul and Seger, 2007; Zarubin and Han, 2005), but ERK is also a main kinase activated through EGFR and IR (Pierce et al., 2001; Watanabe et al., 2013). NF-κB is bound by IκBs in the cytoplasm, and can be translocated into nucleus to function as a transcription factor after the proteolytic degradation of IκBs (Baeuerle and Baltimore, 1988; Zandi et al., 1997). NF-κB can be activated through various cell surface receptors including TLRs, TNFRs, IR and EGFR (Aggarwal et al., 2009; Bertrand et al., 1995; Verstrepen et al., 2008). Inducers of inflammation stimulate the receptors and promote IκB kinases (IKK)-mediated phosphorylation and proteolytic degradation of IκB (Viatour et al., 2005). IKK is further regulated by the ubiquitin-dependent kinase transforming growth factor β activated kinase-1 (TAK1) (Adhikari et al., 2007), which is an upstream molecule to mediate both MAPK and NF-κB cascades (Kawai and Akira, 2006; Wang et al., 2001). The activation of proinflammatory pathways promotes the production of various mediators such as iNOS/NO, COX-2/$PGE_2$, and cytokines, which can further provoke inflammation (Hui et al., 2009; Kyriakis and Avruch, 2012; Shih et al., 2015). Figure 1.1 has shown the major proinflammatory pathways in inflammatory abnormalities.

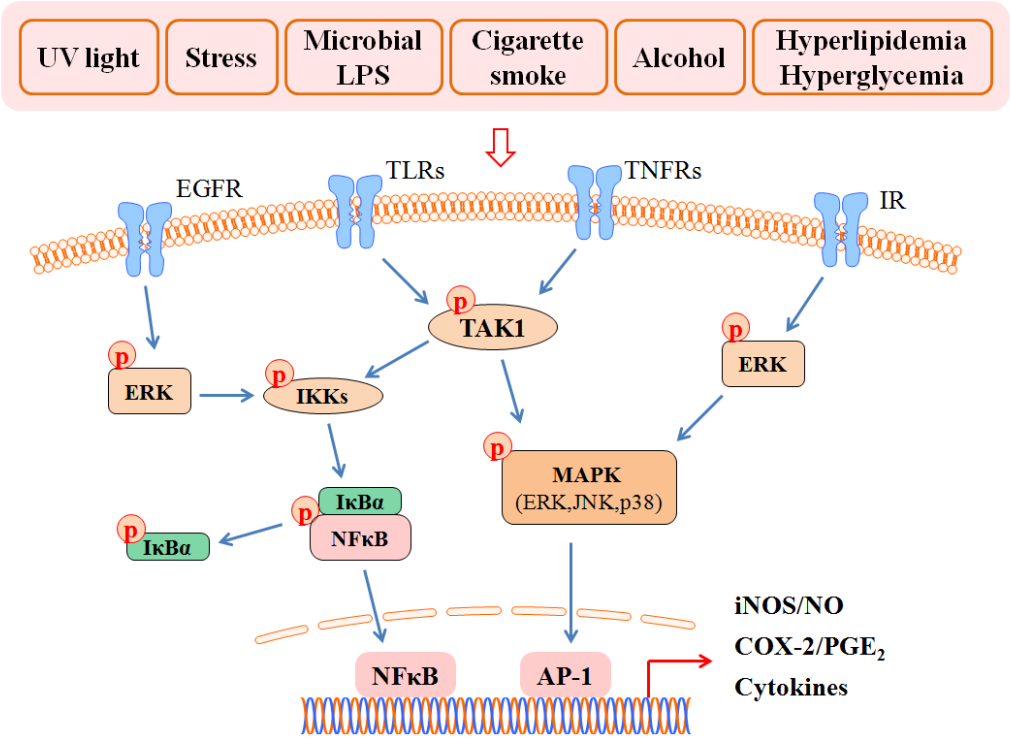



**Figure 1.1 Major proinflammatory pathways in inflammation.** Inducers of inflammation can promote the activation of proinflammatory pathways through cell surface receptors. MAPK and NF-κB cascades are considered as two major pathways in provoking inflammation. AP-1, activator protein 1; COX-2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase, IKK, IκB kinases; iNOS, inducible nitric oxide synthase; IR, insulin receptor; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; $PGE_2$, prostaglandin E2; TAK1, transforming growth factor β activated kinase-1; TLRs, Toll-like receptors; TNFRs, tumor necrosis factor receptors; UV, ultraviolet

### 1.2.3 Crosstalk between inflammation and oxidative stress

Oxidative stress caused by cumulative production of reactive oxygen species (ROS) can promote inflammation in the process of many pathological symptoms such as chronic inflammatory diseases, senescence, and cancer (Finkel and Holbrook, 2000; Reuter et al., 2010). The NADPH oxidase family is considered as the major source of ROS, although other factors such as mitochondrial electron transport enzymes,

xanthine oxidase, cyclooxygenase, lipoxygenase, and uncoupled nitric oxide synthase can also increase the production of ROS (Bedard and Krause, 2007; Paravicini and Touyz, 2008). Inducers of inflammation such as microbial LPS, cigarette smoke, and alcohol can activate NADPH oxidases to produce superoxide anion ($O_2^{\cdot-}$) through pattern recognition receptors of the innate immune system such as TLRs (Boldogh et al., 2005; Gill et al., 2010; Panday et al., 2014), by the myeloid differentiation primary response gene 88 (MYD88)-mediated pathway (Cho et al., 2016; Laroux et al., 2005). The $O_2^{\cdot-}$ is short lived in biological system since it is rapidly transformed to hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD) (Paravicini and Touyz, 2008). $O_2^{\cdot-}$ can potentially increase the activity of SOD (Rathore et al., 1998), $H_2O_2$ can activate oxidative stress response pathways such as nuclear factor (erythroid -derived 2)-like 2 (Nrf2) pathway (Fourquet et al., 2010; Pi et al., 2003), and thus promote the expression of antioxidant enzymes such as heme oxygenase-1 (HO-1) (Rushworth et al., 2005), NADPH dehydrogenase 1 (NQO1) (Tanigawa et al., 2007), glutathione peroxidase (GPx) (Singh et al., 2006), and manganese-dependent SOD (MnSOD) (Huang et al., 2014) to attenuate the oxidative stress. On the other hand, $H_2O_2$ can activate proinflammatory pathways such as NF-κB pathway (Takada et al., 2003), MAPK pathway (Bhat and Zhang, 1999; Chen, K. et al., 2008), and peroxiredoxin-2 pathway (Salzano et al., 2014) to increase the production of proinflammatory cytokines and provoke inflammation (Bhattacharyya et al., 2014). Meanwhile, the activation of the proinflammatory pathways by $H_2O_2$ and by the adapter proteins MYD88 (Coste et al., 2010; Gorina et al., 2011), can also increase the expression of antioxidant proteins. For instance, p38 MAPK can promote the expression of Nrf2 (Park et al., 2013), and NF-κB can upregulate MnSOD expression (Candas and Li, 2014; Delhalle et al., 2002). Both p38 MAPK and NF-κB can promote the expression of HO-1 (Wijayanti et al., 2004). The cross effects between oxidative stress and proinflammatory pathways were shown in Figure 1.2.

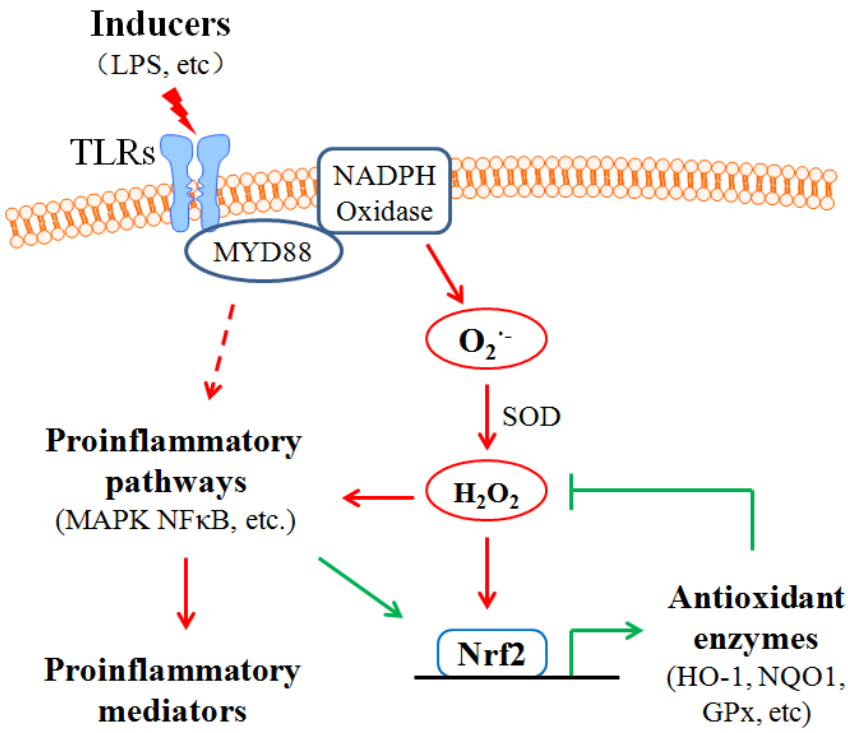



**Figure 1.2 Crosstalk between inflammation and oxidative stress.** Inducers of inflammation such as microbial LPS can activate NADPH oxidase to produce ROS ($O_2^{\cdot-}$, $H_2O_2$) through TLRs-mediated MyD88. The increased ROS not only promote the activation of proinflammatory pathways, but also up-regulate antioxidant response pathways, which in turn decrease ROS production. GPx, glutathione peroxidase; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MYD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NQO1, NADPH dehydrogenase 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; SOD, superoxide dismutase; TLRs, Toll-like receptors

## 1.3 Chronic inflammation-related diseases

Generally, the controlled acute inflammatory response is a physiological defense to remove foreign invaders and protect the body against infection. However, if the acute inflammatory response fails to eliminate the pathogens, or the trigger of inflammation is endogenous factors or undegradable foreign bodies, the inflammatory process will persist and lead to local or systemic chronic inflammation, which occurs

in a variety of diseases such as rheumatoid arthritis (Choy and Panayi, 2001), cardiovascular disease (Willerson and Ridker, 2004), nonalcoholic steatohepatitis (Brunt et al., 2009), and type 2 diabetes (Donath and Shoelson, 2011). The persistent immune response that produces local or systemic chronic inflammation is the common pathophysiological feature of chronic inflammatory diseases (Straub et al., 2010; Tlaskalova-Hogenova et al., 2004). In chronic inflammation-related diseases, the tissues that harbor the target antigen(s) are usually infiltrated by cellular effectors of the immune system, mainly T cells and macrophages, but also dendritic cells (DCs), B cells and plasma cells, to form a secondary or tertiary lymphoid organ (Aloisi and Pujol-Borrell, 2006). The following introduced the potential pathogenesis of two common chronic inflammation-related diseases.

### 1.3.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common chronic inflammatory disease that occurs in 1% of the population (Firestein, 2003), and is associated with progressive disability, systemic complication, and early death. RA is characterized by synovial inflammation and hyperplasia (swelling), cartilage and bone destruction (deformity), autoantibody production, and systemic features including cardiovascular, pulmonary, psychological, and skeletal disorders (McInnes and Schett, 2011). The cause of RA is not clear yet, and it involves a complex interplay among genotype and environmental triggers. Genome-wide analyses have shown that immune regulatory factors underlie RA (WTCCC, 2007). The human leukocyte antigens (HLAs) are considered as the major gene complex that account for ～30% of the heritable risk, but other genes such as tumor necrosis factor receptor-associated factor 1 (TRAF1) (Kurreeman et al., 2007), protein tyrosine phosphatase (PTPN22) (Begovich et al., 2004), and signal transducer and activator of transcription 4 (STAT4) (Remmers et al., 2007) are also associated with RA. Environmental factors such as smoking and other forms of bronchial stress also increase the risk of RA (Symmons et al., 1997). Infectious agents (e.g., cytomegalovirus and Escherichia coli) and their products (e.g., cytokines, heat-shock proteins) have long been considered to be linked with RA, although the underlying

mechanisms remain unclear (Kamphuis et al., 2005). In addition, the gastrointestinal microbiome is recently recognized to influence the development of autoimmunity in arthritis models (Scher and Abramson, 2011). Cytokines are widely recognized to be directly implicated in many of the immune processes that are associated with the pathogenesis of RA, and modulation of cytokine expression can alter the outcome (McInnes and Schett, 2007). A variety of immune cells like T cells (Schulze-Koops and Kalden, 2001), B cells (Edwards et al., 2004), and macrophages (Brentano et al., 2005) are involved in RA by producing proinflammatory cytokines to induce synovitis and osteoclast differentiation. Tumor necrosis factors (Feldmann, 2002), IL-1 (Kay and Calabrese, 2004), and IL-6 (Md Yusof and Emery, 2013) are considered as the major proinflammatory cytokines, although multitudinous cytokines are involved in the pathogenesis of RA (Md Yusof and Emery, 2013).

### 1.3.2 Nonalcoholic steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease that develops hepatic inflammation and metabolic syndrome, the prevalence of NAFLD ranges from 20-30% in the general population and up to 75–100% in obese individuals (Henao-Mejia et al., 2012). NAFLD comprises nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). (Machado and Diehl, 2016; Matteoni et al., 1999). NAFL usually remains asymptomatic. NASH is a potential outcome of NAFL that develops chronic hepatic inflammation leading to cirrhosis, portal hypertension, hepatocellular carcinoma and increased mortality (Rafiq et al., 2009; Shimada et al., 2002). The factors leading to progression from NAFL to NASH are still poorly understood, although hepatic lipid accumulation is considered as the risk factor of NAFLD (Charlton, 2008). The toxic effect of specific lipids on hepatocytes, which is defined as the hepatic lipotoxicity, has been considered as a potential risk factor of NASH (Cusi, 2012), but the influence of lipotoxicity differs according to the types of accumulated lipids, and can be modified by other factors that aggravate or defend against the effect (Alkhouri et al., 2009). The “two hit” mechanism has been proposed to drive the progress of NASH (Day and James, 1998),

in which hepatic steatosis is induced by the first hit that closely associated with lipotoxicity-induced mitochondrial abnormalities, while the second hit includes lipid peroxidation and ROS generation (Sanyal et al., 2001). Recent studies have suggested that the dysbiosis of gut microbiota induced by diet (mostly high fat diet) (De Minicis et al., 2014), or by other factors such as inflammasome-deficiency-associated changes (Henao-Mejia et al., 2012) may exacerbated hepatic steatosis, inflammation, and fibrogenesis. Analysis on human gut microbiome reveals that microbial clusters differ in abundance between the liver cirrhosis patients and healthy individuals (Qin et al., 2014). The animal model of NASH has shown that dysbiosis of gut microbiota can increase the level of LPS in intestinal tract, subsequently in liver, and finally in blood circulation with the loss of liver function (Xie et al., 2016). In common with other inflammatory diseases, a complicated network of cytokines is involved in the pathogenesis of NASH (Diehl et al., 2005), and cytokines can be considered as the potential therapy targets (Braunersreuther et al., 2012).

## 1.4 Biological properties of polyphenols

Polyphenols are naturally occurring compounds found in the fruits, vegetables, cereals and beverages. Fruits like berries, grapes, cherries, apple and pear contain up to 200-300 mg polyphenols per 100 grams fresh weight (Pandey and Rizvi, 2009). Polyphenols are chemically characterized as compounds with more than a couple of phenolic structures although their structures are highly diverse in foods (as shown in Figure 1.3). The number and characteristics of these phenolic structures underlie their unique physical, chemical, and biological properties (Del Rio et al., 2013; Pereira et al., 2009; Tsao, 2010). Generally, polyphenols are not essential nutrients although in some cases they can replace part of the essential nutrients such as vitamin E (Iqbal et al., 2015). However, polyphenols in our diet are abundant micronutrients showing benefit to human health. Epidemiological studies and associated meta-analyses provide growing evidence that long term consumption of polyphenol-rich diets offered protective effects against the development of chronic or degenerative diseases

such as cardiovascular diseases, cancers, diabetes, osteoporosis and neurodegenerative diseases (Arts and Hollman, 2005; Graf et al., 2005). In following sections, the anti-oxidant, anti-inflammatory, anti-cancer, and anti-bacterial properties of polyphenols are briefly introduced.

### 1.4.1 Antioxidant property

The beneficial effects of polyphenols are considered to be mainly attributed to their antioxidant activities, since polyphenols can act as chain breakers or radical scavengers depending on their chemical structures (Heim et al., 2002; Pietta, 2000). The so-called antioxidant ability of polyphenols can be defined as the ability to scavenge ROS, which include radical and non-radical oxygen species such as $O_2^{\cdot-}$, $HO^-$, $NO^-$, $H_2O_2$, as well as oxidatively generated free radicals $RO^-$ and $ROO^-$ derived from biomolecules such as low-density lipoproteins (LDLs) (Neudörffer et al., 2006), proteins, and oligonucleic acids (DNA and RNA) (Li et al., 2000; Shi et al., 2000). The major potential mechanisms underlying the antioxidant ability of polyphenols are considered to be based on the direct quenching of ROS and inhibition of ROS generation. First, the phenol functional group can donate a hydrogen atom to the free radicals such as $LOO^-$ generated by lipid peroxidation (Kurek-Gorecka et al., 2013). Second, polyphenols may block the action of some enzymes (e.g. xanthine oxidase and protein kinase C) that responsible for the generation of $O_2^{\cdot-}$. For instance, epigallocatechin gallate (EGCG) can inhibit the activation of protein kinase C (Levites et al., 2002); quercetin and catechin may inhibit the activation of protein kinase C-dependent NADPH oxidase (Pignatelli et al., 2006); geraniin, corilagin, and gallic acid exhibit inhibitory effects on the activation of xanthine oxidase (Wu et al., 2010). Third, (poly)phenolic compounds can chelate metal ions such as $Fe^{2+}$/ $Fe^{3+}$ and $Cu^{2+}$, which are involved in the conversion of $O_2^{\cdot-}$ and $H_2O_2$ into highly aggressive $HO^-$ through Haber-Weiss/Fenton-type reactions. For instance, phenolic acids (e.g. chlorogenic acid, caffeic acid, gallic acid) can chelate $Fe^{2+}$ in a concentration-dependent manner (Andjelkovic et al., 2006); polyphenols including

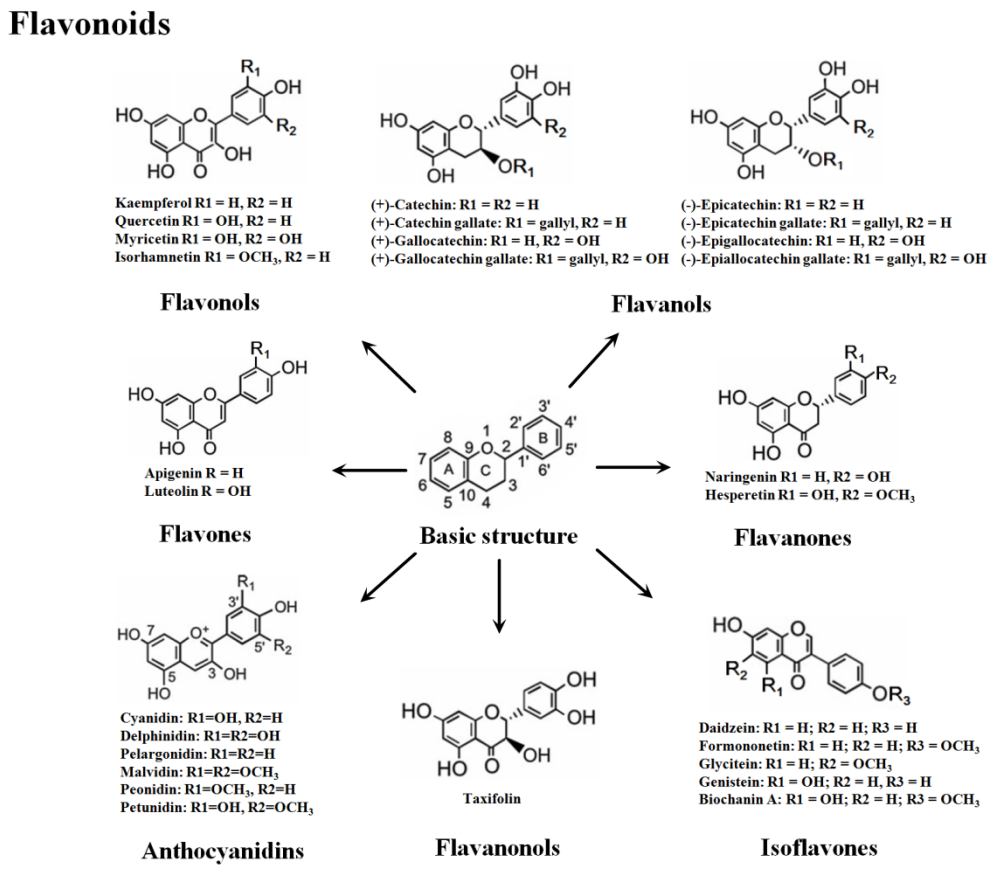



**Figure 1.3 Typical structures of dietary (poly)phenolic compounds.**

flavones&flavonols (e.g. apigenin, quercetin, myricetin), isoflavones (e.g. daidzein, genistein), flavanones&flavanonols (e.g. taxifolin, naringenin, naringin), and flavanols (e.g. catechin) may chelate $Fe^{2+}$/ $Fe^{3+}$ and $Cu^{2+}$ (Mira et al., 2002). Forth, polyphenols may up-regulate the antioxidant response pathways such as transcription factor Nrf2 mediated antioxidant enzymes and mitochondrial MnSOD, which are potentially mediated by the early accumulation of $H_2O_2$ or the AMP-activated protein kinase (AMPK) pathway (Alexandre et al., 2006; Gan et al., 2016; Leon-Gonzalez et al., 2015). For instance, *tert*-butylhydroquinone (*t*BHQ), resveratrol, and curcumin can up-regulate Nrf2-mediated NQO1 expression via the generation of $H_2O_2$ (Erlank et al., 2011); resveratrol and pterostilbene may promote the expression of MnSOD through the activation of AMPK pathway (Gan et al., 2016). The antioxidant ability of polyphenols is frequently cited in the prevention and/or reduction of oxidative stress-related age-related disorders and chronic diseases such as cardiovascular diseases (e.g. atherosclerosis), carcinogenesis, neurodegeneration (e.g. Alzheimer's disease), and skin deterioration (Quideau et al., 2011).

### 1.4.2 Anti-inflammatory property

Increasing epidemiological and experimental evidences have shown that dietary polyphenols have benefits to inflammatory diseases such as RA (Ahmed, 2010; Jean-Gilles et al., 2012), experimental allergic encephalomyelitis (Giacoppo et al., 2015), inflammatory bowel disease (Martin and Bolling, 2015; Rosillo et al., 2011), asthma (Joskova et al., 2013), atherosclerosis (Loke et al., 2010), skin inflammation (Nakamura et al., 2014), brain inflammation (Bureau et al., 2008), and metabolic syndromes (Amiot et al., 2016). Although the ability of polyphenols to act as antioxidants and free radical quenchers is considered to contribute to the defense against inflammation (Crasc Ì et al., 2016), accumulating evidence show that polyphenols are involved in modulating cell signaling pathways (Hong Byun et al., 2010; Khan et al., 2006). Polyphenols such as resveratrol and tea polyphenols have been reported to down-regulate proinflammatory pathways like MAPK (Kao et al., 2009; Qian et al., 2015), NF-κB (Romier et al., 2008), and IRF (Kim et al., 2011)

pathways, and thus inhibit the production of proinflammatory mediators. Recent studies reveal that polyphenols such as quercetin, resveratrol, and EGCG can bind directly and distinctively to the protein kinases such as ERK (Hisanaga et al., 2016), and micro ribonucleic acids (miRNAs) such as miR-33a and miR-122 (Baselga-Escudero et al., 2014), to inhibit the activation and expression of these proteins and genes. Those actions may also explain how dietary compounds elicit biological effects when they are relatively poorly absorbed and circulate in nano molar concentrations (Joseph et al., 2016). In addition, the mixture of (poly)phenolic compounds extracted from fruits are reported to be more active in the anti-inflammatory actions than their single components (Joskova et al., 2013), which suggesting that polyphenols may have synergistic effects in the inhibition of inflammation.

### 1.4.3 Anti-cancer property

The progression of cancer, which is defined as carcinogenesis, is a multistep process triggered by genetic alterations that activate different signal transduction pathways (Bertram, 2000; Fantini et al., 2015). Oxidative stress has been considered to be linked to the onset and progression of cancer, since its role in increasing deoxyribonucleic acid (DNA) mutations or inducing DNA damage, genome instability, and cell proliferation (Mileo and Miccadei, 2016). Polyphenols as dietary intervention on cancer have received an increasing interest, since their potential anticarcinogenic effects such as inhibition of cancer cell proliferation, angiogenesis, tumor growth, metastasis, and inflammation, as well as induction of apoptosis (Ramos, 2008). Multiple signaling pathways have been reported to be involved in the anticarcinogenic action of polyphenols. First, polyphenols can modulate (mostly down-regulate) erythroblastosis oncogene B (ErbB) receptors signaling pathway including the activation of EGFR, ErB2, ErB3, and ErB4, which are involved in the activation of MAPK pathway and subsequent cell proliferation and differentiation in carcinogenesis (Fantini et al., 2015; McKay and Morrison, 2007). For instance, curcumin can down-regulate the expression of EGFR and ErB2 (Squires et al., 2003;

Thangapazham et al., 2008); EGCG can inhibit the phosphorylation of ErbB2 and ErbB3 (Pan et al., 2007) to inhibit the growth of cancer cells. Second, polyphenols such as EGCG (Gupta et al., 2004), curcumin (Divya and Pillai, 2006), resveratrol (Pozo-Guisado et al., 2005), and anthocyanins (Fan et al., 2015; Wang et al., 2009) may suppress the activation of NF-κB, and thus inhibit the inflammation and chemoresistance in cancer cells. Third, down-regulation of Hedgehog (Hh)/glioma-associated (GLI) oncogene cascade are involved in the anticarcinogenic effects of polyphenols including apigenin, baicalein, curcumin, EGCG, genistein, quercetin, and resveratrol (Slusarz et al., 2010). In addition to the above signal transduction pathways, polyphenols are also reported to modulate other signals involved in cell cycle and apoptosis, such as up-regulate the expression p53 (Harakeh et al., 2008).

### 1.4.4 Anti-bacterial property

Polyphenols are considered to play important roles in plants as defense against plant pathogens and animal herbivore aggression and as a response to various abiotic stress conditions, such as rainfall and ultraviolet radiation (Daglia, 2012). Likewise, polyphenols have also been reported to exhibit inhibitory effects on the pathogenic bacteria in oral cavity (Furiga et al., 2008), gut (Bolca et al., 2013; Hidalgo et al., 2012), and foods (Payra et al., 2016). Antimicrobial test *in vitro* reveals that polyphenols including epigallocatechin, EGCG, tannic acid, punicalagin, castalagin, geraniin, prodelphinidin, procyanidins, theaflavin, and green tea polyphenols have inhibitory effects on the growth of *staphylococcus aureus*, *salmonella*, *escherichia coli*, and *vibrio*; among the above polyphenols, epigallocatechin, EGCG, castalagin, and prodelphinidin showed lower minimum inhibitory concentrations (MICs) than others, which suggesting that 3,4,5-trihydroxyphenyl groups are important for the antibacterial activity of polyphenols (Taguri et al., 2004). The beneficial modulation of polyphenols on the microbial community not only have potential preventive effects on digestive diseases (Dryden et al., 2006), the modulation on gut microbiota may also contribute to the protection of chronic diseases especially chronic inflammatory diseases and cancer (Cardona et al., 2013; Kamada et al., 2013).

## 1.5 Thesis investigation

Accumulated data have suggested that polyphenols present in fruits and vegetables show multiple physiological benefits and have chemopreventive effects against some chronic diseases such as cancer and chronic inflammation. *Lonicera caerulea L.* berry, rich in polyphenols or anthocyanins, has been reported to have antioxidant and anti-inflammatory potential although the molecular mechanisms are not clear. In this study, I investigated the anti-inflammatory effects and mechanisms of *Lonicera caerulea* L. berry polyphenols (LCBP), using cell model and animal models. First, the anti-inflammatory activity and molecular mechanisms of LCBP were investigated *in vitro* by using a bacterial lipopolysaccharide (LPS)-induced mouse macrophage-like RAW 264.7 cell model, focusing on cellular signaling pathways related to inflammation and oxidative stress. Second, *in vivo* anti-inflammatory activity of LCBP was investigated by using a LPS-induced mouse paw edema model, focusing on the cytokine network. Third, the protective effect of LCBP against chronic inflammation-related diseases was investigated by using an established adjuvant-induced arthritis (AIA) model. Finally, the crosstalk between the antioxidant and anti-inflammatory activities of LCBP were further investigated in the experimental nonalcoholic steatohepatitis (NASH) mouse model to clarify the potential mechanisms on the modulation of cytokines production and oxidative stress response.

# Chapter 2

# *In vitro* anti-inflammatory activity and molecular mechanisms of *Lonicera caerulea L.* berry polyphenols (LCBP)

## 2.1 Abstract

Based on the properties of *Lonicera caerulea* L. berry, the present study aimed to clarify the inhibitory effect and molecular mechanisms of *Lonicera caerulea* L. berry polyphenols (LCBP) on the endotoxin-induced inflammation by using an established lipopolysaccharide (LPS)-induced macrophage-like RAW 264.7 cell model. First, the (poly)phenolic components of LCBP were analyzed by high performance liquid chromatography (HPLC). Second, RAW264.7 cells were pretreated with LCBP (75, 150, 300 μg/ml) before exposure to LPS (40 ng/ml), and interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α), and prostaglandin $E_2$ ($PGE_2$) in culture media were examined with enzyme-linked immunosorbent assay (ELISA) kits, while nitric oxide (NO) was determined by assaying nitrite with the Griess reagent. Third, the cellular proteins involved in mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways, as well as antioxidant response pathways were analyzed by Western blot assay. Finally, the major bioactive components of LCBP in the inhibition of LPS-induced inflammation were identified, and the direct binding to protein kinases was examined by *ex vivo* pull-down assay.

Pretreatment with LCBP concentration-dependently suppressed LPS-induced production of inflammatory mediators including IL-1β, IL-6, TNF-α, inducible nitric oxide synthases (iNOS)-mediated NO, and cyclooxygenase-2 (COX-2)-mediated $PGE_2$. Cell signaling analysis revealed that LCBP down-regulated transforming growth factor β activated kinase-1 (TAK1)-mediated MAPK and NF-κB pathways. Moreover, LCBP reduced LPS-induced oxidative stress by up-regulating the expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and mitochondrial

manganese-dependent superoxide dismutase (MnSOD) in the early period. Cyanidin 3-glucoside (C3G) and -(-)epicatechin (EC) were identified as the major bioactive components of LCBP in the modulation of proinflammatory and antioxidant response pathways. These data demonstrated that LCBP rich in C3G and EC attenuated LPS-induced inflammation through both down-regulating TAK1-mediated MAPK and NF-κB pathways, and up-regulating antioxidant response pathway.

## 2.2 Introduction

Polyphenols are considered as major active contributors to the biological properties of *Lonicera caerulea L.* berry. The (poly)phenolic extraction from *Lonicera caerulea L.* berry has been reported to exhibit multiple bioactivities such as anti-microbial (Palikova et al., 2008), antioxidant (Palikova et al., 2009), and anti-inflammatory activities (Rupasinghe et al., 2015; Zdarilova et al., 2010).

In inflammatory process, macrophages are considered as the primary cells that produce excessive amounts of mediators like proinflammatory cytokines, nitric oxide (NO), and prostaglandin $E_2$ ($PGE_2$) (Laskin and Laskin, 2001; Moore and Tabas, 2011), which can provoke inflammation and aggravate chronic inflammatory diseases such as rheumatic arthritis and inflammatory bowel disease (Nagy et al., 2007; Nakanishi and Rosenberg, 2013). The overproduction of NO and $PGE_2$ are predominantly mediated by respective iNOS and COX-2 under LPS stimulation (Aktan, 2004; G.Eliopoulos et al., 2002). NF-κB and activator protein-1 (AP-1) are considered as the major upstream transcription factors of iNOS and COX-2 in LPS-activated macrophages, although there are other transcriptional factors such as CCAAT/enhancer-binding protein (C/EBP) and CRE-binding protein (CREB) present in their promoter (Hou et al., 2005; Lee et al., 2003). AP-1 is mediated by MAPK including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 kinase (Hou et al., 2005; Jeffrey et al., 2007). NF-κB is bound by IκBs in the cytoplasm of unstimulated cells, and can be translocated into nucleus to function as a transcriptional factor after the proteolytic degradation of IκBs (Baeuerle and

Baltimore, 1988), which is triggered by the phosphorylation (Traenckner et al., 1995) mediated by IκB kinases (IKK) (Israel, 2010; Viatour et al., 2005), as described in Chapter 1.2. TAK1 is an ubiquitin-dependent kinase of IKK, and acts as a regulator of both MAPK and NF-κB pathways (Ajibade et al., 2013; Kawai and Akira, 2006).

In addition to the inflammatory mediators, macrophages can also produce regulator proteins such as antioxidant enzymes to counteract oxidative stress and proinflammatory response (Mo et al., 2014). MnSOD is one of the most important antioxidant enzymes to reduce mitochondrial oxidative stress (Becuwe et al., 2014), and has been proved as the most sensitive antioxidant enzyme response to LPS-induced inflammation in microglia (Ishihara et al., 2015). Nrf2-mediated antioxidant response pathway is identified as an important mechanism that promote cellular defense against LPS-induced oxidative stress and inflammatory response in macrophages (Mo et al., 2014; Rushworth et al., 2005).

Based on the information related to *Lonicera caerulea* L. berry and the process of inflammation, I used mouse macrophage-like cells (RAW264.7), which can be stimulated by LPS to mimic a status of infection and inflammation, to clarify the molecular mechanisms underlying the anti-inflammatory effect of LCBP, focusing on cellular signaling pathways related to inflammation and oxidative stress.

## 2.3 Materials and methods

### 2.3.1 Chemicals and reagents

LPS (*Escherichia coli Serotype 055:B5*) was purchased from Sigma-Aldrich (St. Louis, MO, USA). C3G (≥98%) and EC (≥98%) from Tokiwa Phytochemical Co., Ltd (Chiba, Japan) were dissolved in dimethylsulfoxide (DMSO, 0.2% final concentration in cultural medium). CNBr-activated Sepharose 4B was from GE Healthcare (Uppsala, Sweden). Antibodies against c-Jun (Ser73), ERK1/2, p38 kinase, JNK, p65, IκB-α, IKKα/β, and TAK1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against iNOS, COX-2, α-tubulin (B-7), lamin B, MnSOD, Nrf2, 70-kDa heat shock protein (HSP70), and corresponding secondary antibodies were

purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.3.2 Polyphenols extraction

*Lonicera caerulea* L. berries were harvested in Jilin region of China, and extracted with a modified method based on previous studies (Jin et al., 2006; Palikova et al., 2008). In brief, the berries of *Lonicera caerulea L.* were homogenized in 75% aqueous ethanol (250 g/l) for 60 min, and then filtered under reduced pressure. The filtrates were then purified on a column packed with nonionic polystyrene -divinylbenzene resin (D101, Shanghai, China).The column was first washed with deionized water, and then eluted by 75% ethanol to obtain (poly)phenolic compounds. Approximately 0.7% of (poly)phenolic fraction, defined as LCBP, was yielded from *Lonicera caerulea* L. berries. LCBP contains 62.3% of total (poly)phenolic components assayed by Folin–Ciocalteu method with gallic acid as standard, and 38.9% of anthocyanins assayed with C3G standard at 520 nm. The rest was considered as soluble fiber (Jin et al., 2006).

### 2.3.3 HPLC analysis

The analysis of (poly)phenolic components in LCBP was performed on a HPLC system with a C18 column (250 × 4.6 mm, JASCO, Tokyo, Japan) based on previous studies. LCBP (0.5 mg/ml) was dissolved in 15% acetic acid aqueous solution and filtered by 0.45 μm PTFE filter. To analyze flavonoids and phenolic acid, the solvent system was a mixture of A (0.05 μM $H_3PO_4$ in $CH_3CN$) and B (0.05 μM $H_3PO_4$ in water), and the gradient was as follows: 39 min (4% A; 96% B) and 15 min (75% A; 25% B), with the flow rate of 0.8 ml/min and 10 μl sample. Spectrophotometric detection was done at 280 nm (Zar et al., 2014). To analyze anthocyanins, the solvent system was a mixture of A (1.5% $H_3PO_4$ in water) and B (1.5% $H_3PO_4$, 20% acetic acid, and 25% $CH_3CN$ in water). The LCBP was eluted with gradient solvents (85–45% A and 15–55% B) for 72 min by the flow rate of 0.8 ml/min, and was detected at 520 nm (Chen, J. et al., 2008). The components in LCBP were identified according to retention time with the known compounds as standards. The concentration of each

(poly)phenolic component in LCBP were quantitated based on peak area with respective standards according to the following formula as described previously (Kupiec, 2004).

$$\text{Conc.}_{\text{Unknown}} = \left( \frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}} \right) \times \text{Conc.}_{\text{known}}$$

### 2.3.4 Cell culture and survival assay

Mouse macrophage-like RAW264.7 cell line (Cell No. RCB0535, RIKEN Bio-Resource Center Cell Bank, Tsukuba, Japan) was cultured in dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 ℃ in a 5% $CO_2$ atmosphere. Cell survival assay was performed to test the cytotoxicity of LCBP by MTT assay. Briefly, RAW264.7 cells were seeded into 96-well plates ($1\times10^4$/well). After preincubation for 24 h, the cells were treated with 75-300 μg/ml of LCBP (dissolved in DMSO at 0.2% final concentration in cultural media) alone for 12.5 h, or treated with 300 μg/ml of LCBP for 30 min before exposure to LPS for 12 h. The cells were then incubated with MTT solution for another 4 h. The resulting MTT-formazan products were dissolved by 100 μl of 0.04 N HCl-isopropanol and measured at 595 nm in a microplate reader.

### 2.3.5 Measurement of IL-1β, IL-6, TNF-α, Nitrite, and $PGE_2$

RAW264.7 cells ($5\times10^5$) were seeded into each well of 6-well plates, and then pre-incubated for 24 h. After being starved in serum-free media for 2.5 h to eliminate the impact of FBS, the cells were treated with 75, 150, or 300 μg/ml of LCBP for 30 min and then exposed to LPS (40 ng/ml) for 12 h. The levels of IL-1β, IL-6, TNF-α and $PGE_2$ in cell culture media were measured with their respective ELISA kits (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's manual. The level of NO in cell culture media was determined by assaying nitrite with Griess reagent as described previously (Uto et al., 2005). In brief, cells ($3\times10^5$ cells) were seeded in 48-wells plates, and pre-cultured for 24 h before starved by being cultured in serum-free for another 2.5 h to eliminate the influence of FBS. The cells

were treated with 75, 150, or 300 μg/ml of LCBP for 30 min and then exposed to LPS (40 ng/ml) for 12 h. One hundred ml of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamide dihydrocholide in distilled water), and the absorbance was measured at 550 nm in a microplate reader.

### 2.3.6 Cell fractionation

RAW264.7 cells ($1\times10^6$) were pre-cultured in 6-cm dishes for 24h and starved in serum-free media for 2.5 h to eliminate the influence of FBS. The cells were then treated with 75, 150, or 300 μg/ml of LCBP for 30 min before exposure to LPS (40 ng/ml). Whole cell lysates were obtained by ultrasonication in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 7.4－8.0), 1 mM PMSF, 1 mM $Na_3VO_4$, 1 mM NaF, 1% NP-40, 0.25% Na-deoxycholate, 1% Proteinase Inhibitor Cocktail ]. Nuclear protein extracts were prepared by a modified method based on previous studies (Hou et al., 2011; Hou et al., 2005). In brief, the harvested cells were lysed on ice in buffer A [1 mM dithiothreitol, 0.1 mM EDTA, 10 mM Hepes-KOH (pH 7.9), 10 mM KCl, 0.5% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride] for 30 min. After being centrifuged at 13500 g (4 ℃, 15 min), the nuclear pellets were washed with buffer A for three times and resuspended in buffer B [1 mM dithiothreitol, 1 mM EDTA, 20 mM Hepes (pH 7.9), 0.5 M KCl, and 1 mM phenylmethylsulfonyl fluoride] on a rotating wheel for 30 min at 4 ℃. The supernatants contain nuclear proteins were obtained by centrifugation (13,500 g, 4 ℃, 15 min) and then analyzed by Western blot assay.

### 2.3.7 *Ex vivo* pull-down assay

*Ex vivo* pull-down assay was performed as described in previously (Hisanaga et al., 2016). Briefly, EC and C3G (5 mM) were coupled to CNBr-activated Sepharose 4B beads (25 mg) overnight at 4℃ in 3 ml coupling buffer [25% DMSO, 0.5 M NaCl, and 0.1 M $NaHCO_3$ (pH 8.3)]. After centrifugation (1000 g, 4 ℃, 3 min), the beads were washed with coupling buffer (5 volumes) of and then resuspended in 0.1 M

Tris-HCl buffer (pH 8.0, 5 volumes) for 2 h rotation at room temperature to block any remaining active groups. The conjugated beads were then washed with acetate buffer [0.1 M acetic acid (pH 4.0) and 0.5 M NaCl] and wash buffer 1 [0.5 M NaCl and 0.1 M Tris-HCl (pH 8.0)] for three cycles. The RAW264.7 cell lysates (500 μg/mL) were then incubated with the EC or C3G-conjugated beads (100 μL, 50% slurry), or control beads in a reaction buffer [2 μg/mL BSA, 1 mM dithiothreitol, 5 mM EDTA, 150 mM NaCl, 0.01% Nonidet P–40, 0.02 mM PMSF, 50 mM Tris-HCl (pH 8.5), and 1 μg protease inhibitor cocktail] overnight at 4℃. After being washed with wash buffer 2 [1 mM dithiothreitol, 5 mM EDTA, 200 mM NaCl, 0.02% Nonidet P-40, 0.02 mM PMSF, and 50 mM Tris-HCl (pH 7.5)] for five times, the proteins bound to the beads were analyzed by Western blot assay.

### 2.3.8 Western blot assay

Western blotting was performed as described previously (Hou et al., 2005). Briefly, the obtained protein extracts were quantitated by protein assay and then boiled in SDS sample buffer for 5 min, and equal amounts of protein (～40μg) were run on 10% SDS-PAGE followed by transfer to PVDF membrane. After blocking, the membrane was incubated with specific primary antibody (4 ℃, overnight) and corresponding HRP-conjugated secondary antibody (room temperature, 1 h). The bound antibodies were then detected by ECL system with a Lumi Vision PRO machine (TAITEC Co., Saitama, Japan), and the relative amount of proteins was quantified by Lumi Vision Imager software (TAITEC Co., Saitama, Japan).

### 2.3.9 Statistical analysis

Results are expressed as mean ± SD, and the expression of proteins is presented as induction folds relative to control by densitometry. The significant differences between groups were analyzed by analysis of variance (ANOVA) tests, followed by Ducan's multiple range tests with the SPSS statistical program (version 19.0, IBM Corp., Armonk, NY, USA). Differences were considered significant for $P < 0.05$.

## 2.4 Results

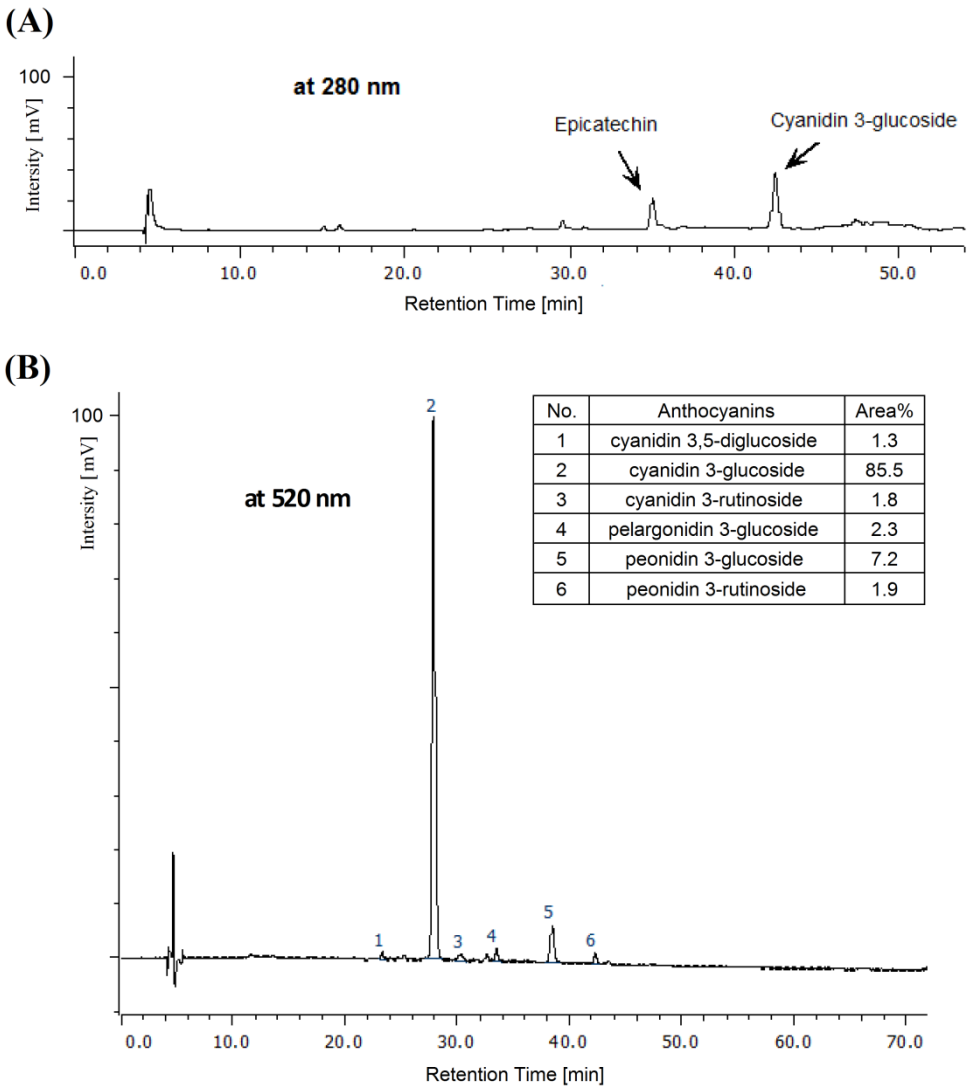
### 2.4.1 The (poly)phenolic composition of LCBP

To know the (poly)phenolic composition of LCBP, I first used HPLC with known standards to identify the (poly)phenolic components, especially anthocyanins, which have been considered as major bioactive components in *Lonicera caerulea* L. berry (Chaovanalikit et al., 2004; Palikova et al., 2008). The flavonoids and phenolic acids were analyzed at 280 nm, and anthocyanins were then characterized at 520 nm. As shown in Figure 2.1A, two major peaks accounted for 25.5% and 59.5% of the total area were detected in the spectrogram, and they were identified as EC and C3G, respectively, using known compound standards. C3G comprised the anthocyanins in 85.5% of the total anthocyanins, while other anthocyanins including peonidin 3-glucoside (7.2%), pelargonidin 3-glucoside (2.3%), peonidin 3-rutinoside (1.9%), cyanidin 3-rutinoside (1.8%), and cyanidin 3,5-diglucoside (1.3%) were also present in LCBP (Figure 2.1B). The concentrations of EC and C3G in LCBP were then quantitated as described in Section 2.3.3, and the results indicated that each milligram of LCBP contains 0.23 mg EC and 0.37 mg C3G.

### 2.4.2 Inhibitory effect of LCBP on the production of proinflammatory factors

To evaluate the inflammatory status in RAW264.7 cells, the levels of proinflammatory cytokines including IL-1β, IL-6, and TNF-α were first measured in culture media. As shown in Figure 2.2, LPS (40 ng/ml) caused significant increase in the levels of IL-1β, IL-6, and TNF-α in culture media, and the increase was concentration-dependently inhibited by pretreatment with 75, 150 or 300 μg/ml of LCBP. Treatment with 300 μg/ml of LCBP alone showed no significant influence on the production of IL-1β, IL-6, and TNF-α in the cell culture media.

To eliminate the impact of LCBP cytotoxicity on the production of cytokines, the effect of LCBP on the viability of RAW264.7 cells was then investigated by MTT assay. The results indicated that both LPS (40 ng/ml) and LCBP (300 μg/ml) showed no significant influence on the cell viability (Figure 2.3).




| No. | Anthocyanins | Area% |
|---|---|---|
| 1 | cyanidin 3,5-diglucoside | 1.3 |
| 2 | cyanidin 3-glucoside | 85.5 |
| 3 | cyanidin 3-rutinoside | 1.8 |
| 4 | pelargonidin 3-glucoside | 2.3 |
| 5 | peonidin 3-glucoside | 7.2 |
| 6 | peonidin 3-rutinoside | 1.9 |

**Figure 2.1 HPLC profiles of (poly)phenolic components in LCBP.** LCBP was analyzed, using a HPLC system as described in section 2.3.3. **(A)** Profile of flavonoids and phenolic acids in LCBP. HPLC was performed at 280 nm. Epicatechin (EC) and cyanidin 3-glucoside (C3G) accounted for 25.5% and 59.5% in the (poly)phenolic fraction respectively. **(B)** Profile of anthocyanins in LCBP. Anthocyanins were detected at 520 nm, and six kinds of anthocyanin were identified with known standards. The diagrams represent typical pattern of three HPLC profiles.

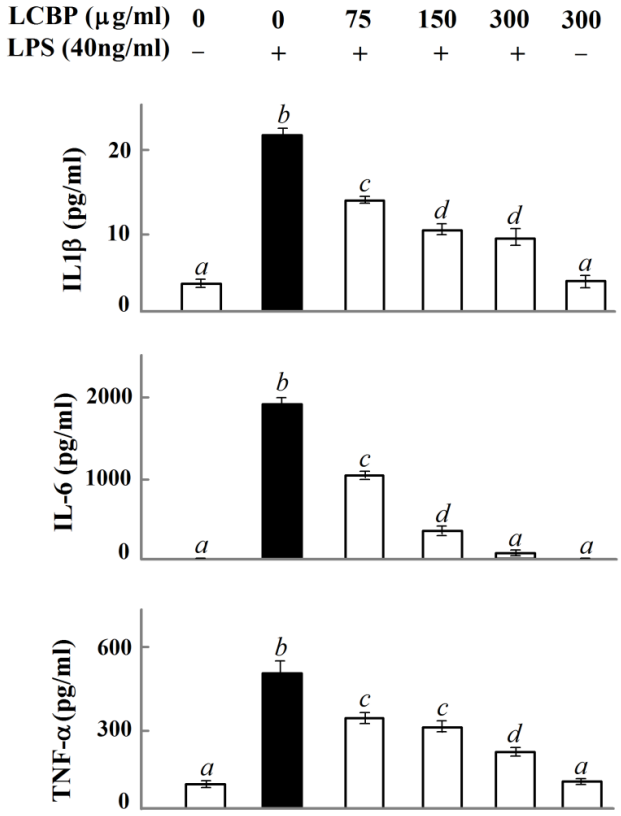



**Figure 2.2 The inhibitory effect of LCBP on the production of IL-1β, IL-6, and TNF-α.** RAW264.7 cells were treated with 75, 150, or 300 μg/ml of LCBP for 30 min and then exposed to LPS (40 ng/ml) for 12 h. The levels of IL-1β, IL-6, and TNF-α in culture media were measured with mouse ELISA kits according to the manufacturer's instruction. The data represent mean ± SD of six repeats, and values with different superscript letters differ significantly ($P < 0.05$).

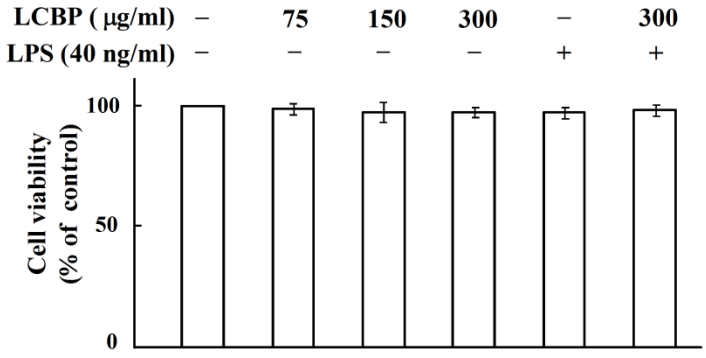



**Figure 2.3 The effect of LCBP on the viability of RAW264.7 cells.** After preincubation for 24 h, the cells were treated with 75-300 μg/ml of LCBP alone for 12.5 h, or treated with 300 μg/ml of LCBP for 30 min before exposure to LPS for 12 h. Cell viability was measured by MTT assay. No significant difference was observed between each group.

The overproduction of NO and $PGE_2$, which are mediated by their respective synthase iNOS and COX-2, has been considered as the indicator of LPS-induced inflammation. Thus, I next measured the levels of NO (as indicated by nitrite) and $PGE_2$ in the cell culture media, and detected the expression of iNOS and COX-2 in whole cell lysates. As shown in Figure 2.4A, the total proteins of iNOS and COX-2 were increased more than fourfold after exposure to LPS, and decreased by pretreatment with LCBP (75-300 μg/ml) in a concentration-dependent manner. Correspondingly, the levels of NO and $PGE_2$ in the culture media were increased by LPS and concentration -dependently decreased by LCBP (Figure 2.4B).

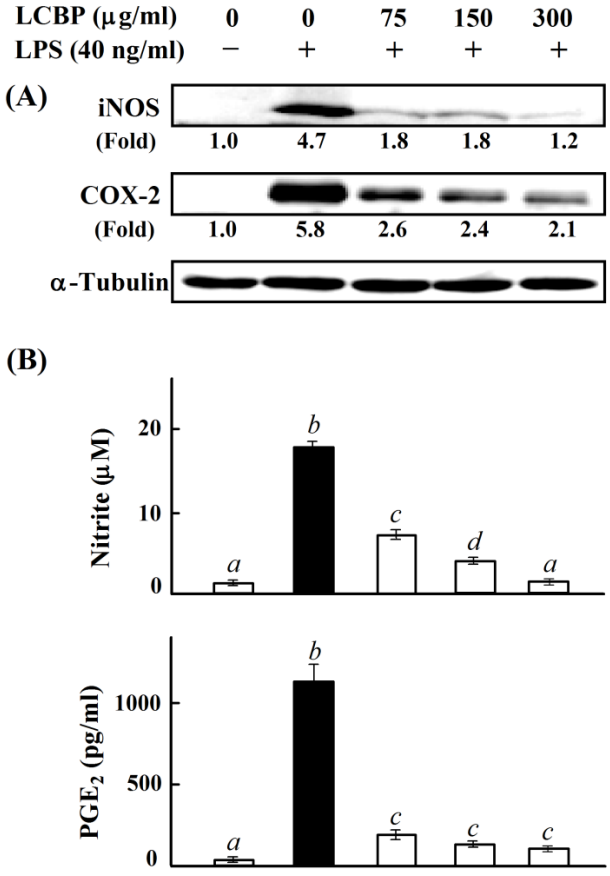



**Figure 2.4 The inhibitory effect of LCBP on the production of iNOS/nitrite and COX-2/$PGE_2$.** RAW264.7 cells were treated with 75, 150, or 300 μg/ml of LCBP for 30 min and then exposed to LPS (40 ng/ml) for 12 h. **(A)** The total proteins of iNOS and COX-2 in whole cell lysates were detected by Western blotting. The induction folds of iNOS and COX-2 were calculated as the intensity of the treatment relative to that of control normalized to α-tubulin by densitometry. The

blots shown are the examples of three independent experiments. **(B)** The level of nitrite in culture supernatants was assayed by griess reagent, and the level of $PGE_2$ was measured with mouse ELISA kit according to the manufacturer's instruction. The data represent mean ± SD of 6 repeats, and values with different superscript letters differ significantly ($P < 0.05$).

### 2.4.3 Modulation of MAPK pathway by LCBP

Accumulated data indicate that LPS can induce the activation of MAPKs including JNK, ERK and p38 kinase, and subsequently activate down-stream transcription factors such as AP-1 to promote the production of cytokines (Hou et al., 2005) . To investigate the cellular signaling pathways involved in the inhibition of proinflammatory mediators by LCBP, I first checked the phosphorylation of c-jun, which is a major component of AP-1 in c-Jun/c-Fos heterodimer form. The results showed that pretreatment with 75-300 μg/ml of LCBP concentration-dependently inhibited LPS-induced c-jun phosphorylation in RAW264.7 cells (Figure 2.5A). Next, I investigated the effects of LCBP on the activation of JNK1/2, ERK1/2 and p38 kinase. As shown in Figure 2.5B, the phosphorylated proteins of JNK1/2, ERK1/2 and p38 kinase were dramatically increased in LPS-induced cells, and concentration -dependently decreased by the pretreatment with LCBP, while the total proteins kept no change.

### 2.4.4 Modulation of NF-κB pathway by LCBP

NF-κB is one of the major transcription factors to mediate the production of inflammatory cytokines in LPS-activated macrophages (Hou et al., 2007). Thus, I next investigated the effects of LCBP on the nuclear translocation of NF-κB. The accumulation of NF-κB in nucleus was measured in the nuclear fraction of cells as described in Section 2.3.6. The results showed that LPS treatment induced a fourfold increase in nuclear p65, a major component of NF-κB, comparing with the cells without LPS treatment, and pretreatment with LCBP (300 μg/ml) reduced the nuclear accumulation of p65 to 1.4 fold (Figure 2.6A). Treatment with LCBP alone showed no effects on the nuclear translocation of p65.

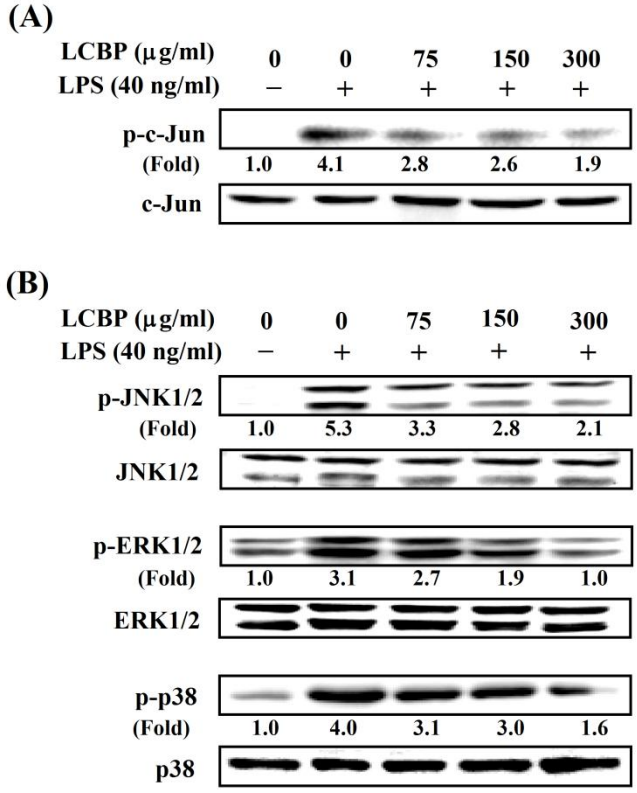



**Figure 2.5 Modulation of MAPK pathway by LCBP.** RAW264.7 cells were treated with 75, 150, or 300 μg/ml of LCBP for 30 min and then exposed to LPS (40 ng/ml) for another 30 min. The total and phosphorylated proteins of c-Jun **(A)**, as well as JNK1/2, ERK1/2, and p38 kinase **(B)** were detected by Western blotting. The induction folds of the phosphorylated proteins were calculated as the intensity of the treatment relative to that of control normalized to total proteins by densitometry. The blots shown are the examples of three independent experiments.

Subsequently, I examined the effects of LCBP on the phosphorylation of p65, IκB-α and IKKα/β, which are involved in NF-κB cascade. The results showed that pretreatment with 75-300 μg/ml of LCBP concentration-dependently suppressed LPS-induced phosphorylation of p65, IκB-α and IKKα/β (Figure 2.6B). In contrast with the phosphorylated protein, the total protein of IκB-α was decreased by LPS, and recovered by LCBP. Since TAK1 is an ubiquitin-dependent kinase of IKK, and acts as a regulator of both MAPK and NF-κB pathways. The activation of TAK1 was further examined, and the results showed that the phosphorylated protein of TAK1 was increased by 3.6-folds in cells after exposure to LPS (40 ng/ml), but concentration

-dependently decreased by LCBP (75-300 μg/ml), in particular, backed to control level at concentration of 300 μg/ml (Figure 2.6C).

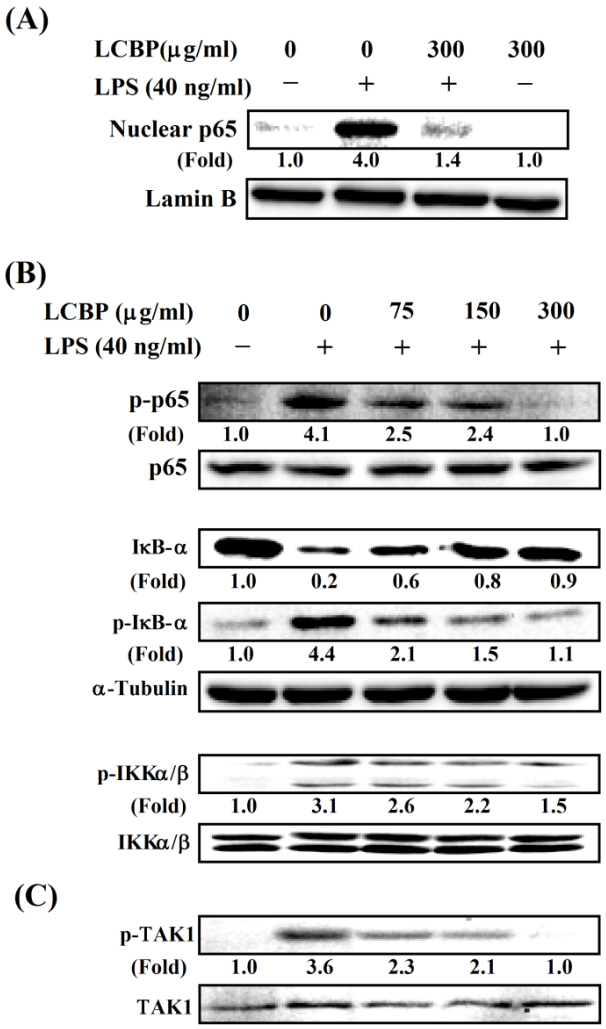



**Figure 2.6 Modulation of NF-κB pathway by LCBP.** The procedure of cell treatment was same as Figure 2.5. **(A)** The total protein of p65 in nuclear fraction was detected by Western blotting. **(B)** The phosphorylated and total proteins of p65, IκB-α, IKKα/β were detected by Western blotting. **(C)** The phosphorylated and total proteins of TAK1 were detected by Western blotting. The induction folds were calculated as the intensity of the treatment relative to that of control normalized to Lamin B (for nuclear p65), α-tubulin (for IκB-α and p-IκB-α), or respective total proteins (for p-p65, p-IKKα/β and p-TAK1) by densitometry. The blots shown are the examples of three independent experiments.

### 2.4.5 Enhancement of cellular antioxidant activity by LCBP

Recent studies have showed that the antioxidant enzymes play an important role to counteract LPS-induced inflammatory response (Mo et al., 2014), and dietary polyphenols such as epicatechin has potential effects on Nrf2 pathway activation (Bayele et al., 2016). Thus, I first investigated the effects of LPS and LCBP on the expression of Nrf2 and MnSOD by time-course experiment in RAW264.7 cells. The results showed that LPS increased the expression of Nrf2 and MnSOD with more than threefold level after 12 and 24h treatment (Figure 2.7A). LCBP treatment also increased the expression of MnSOD and Nrf2 with more than threefold level from 6-24h (Figure 2.7B). Interestingly, the oxidative stress-response proteins such as HSP70 and iNOS were induced only by LPS, but not by LCBP.

### 2.4.6 Identification of major bioactive components of LCBP for the anti-inflammatory actions

To know whether EC and C3G played major roles in the modulation of signaling pathways, I next investigated their effects on the expression of cellular proteins in RAW264.7 cells. As shown in Figure 2.8A, EC (69 μg/ml), C3G (111 μg/ml), or both combination increased the expression of MnSOD and Nrf2, but decreased LPS-induced overexpression of HSP70 and iNOS. Treatment with EC or C3G alone increased the expression of MnSOD and Nrf2, but showed no significant effects on the expression of HSP70 and iNOS. As TAK1 is considered as a key upstream kinase of both MAPK and NF-κB pathways (Ajibade et al., 2013; Kawai and Akira, 2006), and cellular kinases can be directly targeted by some active polyphenolic compound such as quercetin (Hisanaga et al., 2016). Thus, I further investigated whether LCBP inhibits the activation of TAK1 by direct binding. The results showed that the phosphorylation of TAK1 was markedly suppressed by LCBP, EC, or C3G (Figure 2.8B). The *ex vivo* pull-down assay revealed that TAK1 was detected in the Sepharose 4B beads coupled with EC (51.9% binding rate) and C3G (38.6% binding rate), but not in the control Sepharose 4B beads (Figure 2.8C). These results suggested that EC and C3G might directly bind to TAK1 to attenuate the activation.

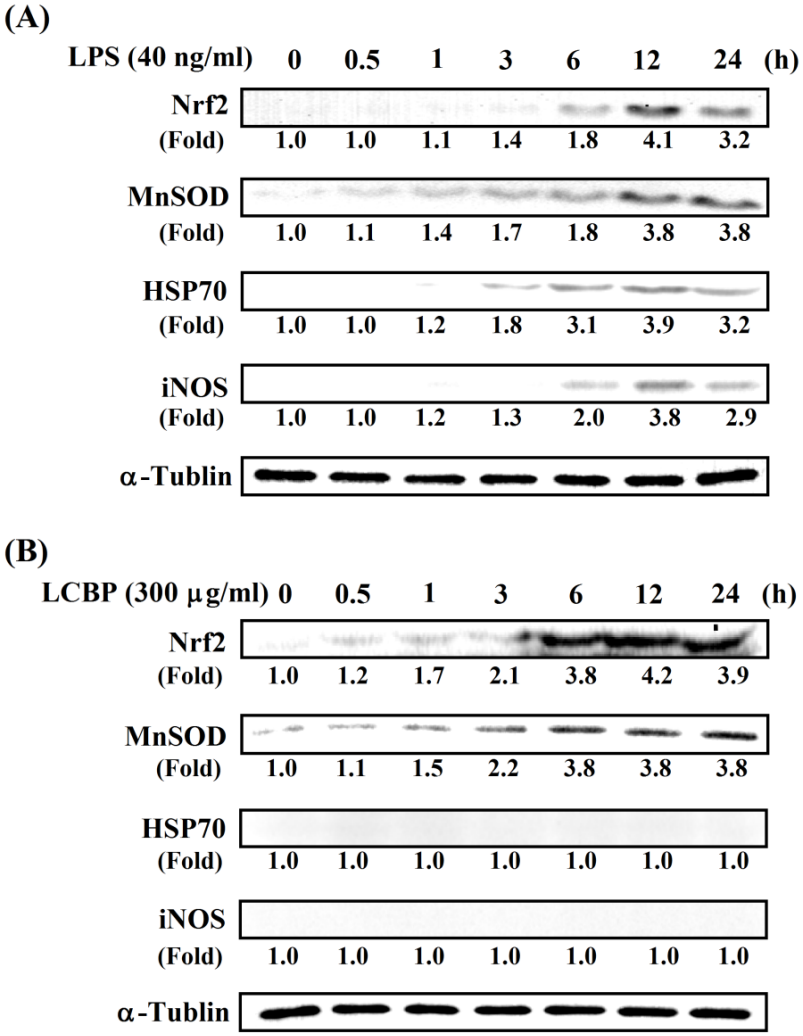



**Figure 2.7 The influence of LPS and LCBP on the expression of Nrf2, MnSOD, HSP70, and iNOS.** RAW264.7 cells were treated with 40 ng/ml of LPS **(A)** or 300 μg/ml of LCBP **(B)** for 0.5-24 h. The total proteins of Nrf2, MnSOD, HSP70, and iNOS in whole cell lysates were detected by Western blotting. The induction folds of the proteins were calculated as the intensity of the treatment relative to that of control normalized to α-tubulin by densitometry. The blots shown are the examples of three independent experiments.

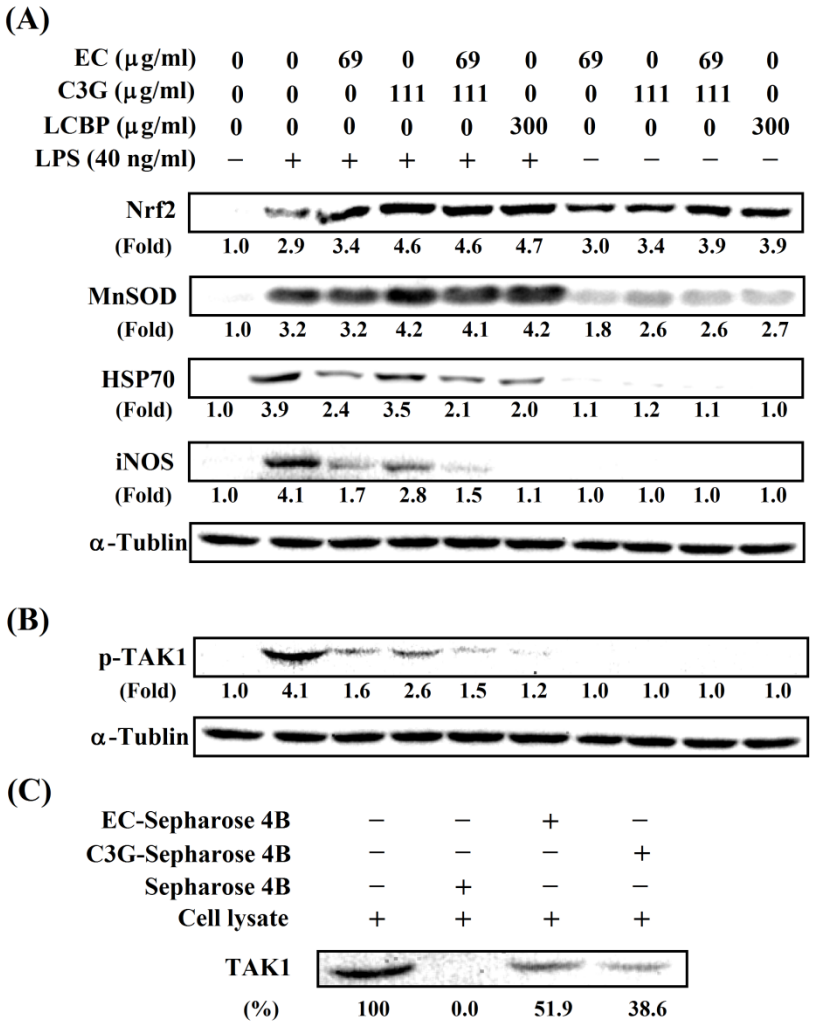



**Figure 2.8 Identification of major bioactive components of LCBP for the anti-inflammatory actions.** RAW264.7 cells were treated with EC (69 μg/ml), C3G (111 μg/ml) or LCBP (300 μg/ml) for 30 min, and then exposed to LPS (40 ng/ml) for 12 h **(A)** or 30 min **(B)**. The total proteins of Nrf2, MnSOD, HSP70, and iNOS, and the phosphorylated protein of TAK1 in whole cell lysates were detected by Western blotting. The induction folds were calculated as the intensity of the treatment relative to that of control normalized to α-tubulin by densitometry. (C) The binding abilities of EC and C3G to TAK1 protein were measured as described in Section 2.3.7. The total protein of TAK1 in whole cell lysates (input control, lane 1), lysates with Sepharose 4B beads (negative control, lane 2), lysates with Sepharose 4B-EC-coupled beads (lane 3), and lysates with Sepharose 4B-C3G-coupled beads (lane 4) were detected by Western blotting. The binding efficiency of EC or C3G to TAK1 was presented as the ratio of input control. The blots shown are the examples of three independent experiments.

## 2.5 Discussion

*Lonicera caerulea* L. berry is reported to has benefits in treating many inflammation-related diseases such as fever and headache in folk medicine (Kaczmarska et al., 2015), and laboratory studies have revealed that the berry has protective effects against inflammatory diseases in many models such as oral inflammation (Zdarilova et al., 2010), hepatic inflammation (Wang et al., 2016), and uveitis (Jin et al., 2006), although the molecular mechanisms remain unclear. The polyphenols are considered as the major bioactive fraction in the anti-inflammatory activities of *Lonicera caerulea* L. berry (Rupasinghe et al., 2015), thus, I used the (poly)phenolic fraction, which is defined as LCBP, to study the anti-inflammatory effects and molecular mechanisms in a LPS-induced macrophage model in the present study. The data demonstrated that LCBP with two major active compounds, EC and C3G, potentially inhibited LPS-induced inflammatory mediators including IL-1β, IL-6, TNF-α, NO and $PGE_2$ in macrophages by down-regulating TAK1-mediated MAPK and NF-κB pathways, and up-regulating the expression of Nrf2 and MnSOD. Therefore, a cross-talk between anti-inflammatory and anti-oxidant effects is involved in LCBP-caused inhibition of LPS-induced inflammation.

Inflammatory mediators produced by macrophages play an important role in provoking inflammation (Fujiwara and Kobayashi, 2005). $PGE_2$ is a lipid mediator that acts as an essential homeostatic factor, but is also a key mediator of immunopathology in chronic infections and cancer (Kalinski, 2012). $PGE_2$ produced by macrophages can regulate the production of other mediators such as proinflammatory cytokines in immune cells including macrophage itself (Williams and Shacter, 1997). $PGE_2$ is generated from arachidonate by the action of cyclooxygenase (COX) isoenzymes including COX-1 and COX-2 (Ricciotti and FitzGerald, 2011). COX-1 is expressed constitutively in most cell types, whereas COX-2 is induced by inflammatory stimuli such as bacterial endotoxin and cytokines (Caughey et al., 2001; Greenhough et al., 2009). NO is synthesized from L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) (Korhonen et al.,

2005), and is an important mediator that largely produced by macrophages in inflammation (Schneemann and Schoedon, 2002). The nNOS and eNOS are constitutively expressed in resting cells to maintain the homeostasis of NO (Alderton et al., 2001), while iNOS is overexpressed under the stimulation of inflammatory inducers (Aktan, 2004). Cytokines such as interleukins, chemokines, interferons, and tumor necrosis factors secreted by immune cells are considered as the initiator of inflammatory response (Rossol et al., 2011). The release of the proinflammatory cytokines to circulation blood not only aggravate systemic inflammation, but also contribute to the recruitment of immune cells such as macrophages to the inflamed tissue and further provoke inflammation (Kanda et al., 2006; Laskin et al., 2011). IL-1β can interact with many other cytokines especially induce considerable expression of KC (Gamero and Oppenheim, 2006), and play important role in the pathogenesis of bone disease (Le Maitre et al., 2007). IL-6 can provoke chronic inflammation by promoting the production of acute phase proteins and subsequent MCP-1 (Atreya et al., 2000). TNF-α promotes the production of numerous cytokines and acute phase proteins in many different pathological processes, and important for both acute and chronic inflammation (Popa et al., 2007). In this study, LCBP suppressed LPS-induced production of $PGE_2$, NO, IL-1β, IL-6, and TNF-α in RAW264.7 cells. Thus, I believe that LCBP potentially attenuates LPS-induced inflammation by inhibiting the production of proinflammatory mediators. To understand the cellular signaling pathways involved in the inhibition of proinflammatory cytokines by LCBP, I next investigated the effects of LCBP on the activation of two major proinflammatory pathways, MAPK and NF-κB, in the LPS-induced macrophages. The results revealed that LCBP significantly suppressed LPS-induced activation of AP-1 (c-Jun) and NF-κB, and further analysis on the upstream pathways revealed that the down-regulation of TAK1-mediated MAPK and NF-κB cascades was involved in the inhibition of proinflammatory mediators by LCBP.

Previous studies suggested that LPS can activate both proinflammatory and oxidative stress response pathways in macrophages (Mo et al., 2014). LPS activates

NADPH oxidase through TLR4, and leads to the excessive production of superoxide radicals ($O_2^{\cdot}$) and subsequent hydrogen peroxide ($H_2O_2$) (Panday et al., 2014), and the accumulation of $H_2O_2$ can further activate Nrf2-mediated oxidative stress response pathway (Purdom-Dickinson et al., 2007), which in turn counteracts the reactive oxygen species and proinflammatory mediators. In the present study, LPS increased the expression of both antioxidant response proteins (Nrf2 and MnSOD) and oxidative stress markers (HSP70 and iNOS) in RAW264.7 cells. LCBP also increased the expression of Nrf2 and down-stream MnSOD, but interestingly, LCBP showed no influence on the expression of HSP70 and iNOS. The results suggested that LCBP could up-regulate Nrf2-mediated antioxidant response pathway, but would not make the cells in oxidative stress condition. The time-course experiments further revealed that LCBP increased the expression of Nrf2 and MnSOD in advance of LPS did. Thus, LCBP potentially up-regulated Nrf2-mediated antioxidant response pathway in the early period, and improved the antioxidant ability of cells for defense against LPS-induced oxidative stress.

The (poly)phenolic components were analyzed by HPLC in this study, and the effects of EC, C3G or their combination in the functional regulation of the cellular pathways were investigated in RAW264.7 cells. The results showed that EC and C3G potentially suppressed LPS-induced TAK1 phosphorylation through direct binding to TAK1 protein, and increased the expression of Nrf2 and MnSOD, which were similar to LCBP did. Other in vivo studies also revealed that plant-derived C3G or EC has protective effect against inflammatory diseases such as adjuvant-induced inflammation (Saric et al., 2009), diarrhea (Velazquez et al., 2012), and carrageen-induced paw edema (Cordeiro et al., 2016). Although HPLC analysis revealed that other minor anthocyanins including peonidin 3-glucoside, pelargonidin 3-glucoside, peonidin 3-rutinoside, cyanidin 3-rutinoside, and cyanidin 3,5-diglucoside were also included in LCBP, peonidin and pelargonidin showed limited effects on LPS-induced inflammation in macrophages in the previous study (Hou et al., 2005). Cyanidin 3-rutinoside and cyanidin 3,5-diglucoside might have potential bioactive effects against inflammatory factors and oxidative stress (Chen et al., 2006;

Youdim et al., 2000), but their contents in LCBP were far below the effective concentration. Thus, EC and C3G are the major potential bioactive compounds of LCBP responding to antioxidant and anti-inflammatory effects.

## 2.6 Conclusion

This study demonstrated that pretreatment with LCBP rich in EC and C3G, concentration-dependently inhibited LPS-induced production of proinflammatory mediators including IL-1β, IL-6, TNF-α, NO and $PGE_2$ in macrophages at least partly through the down-regulation of TAK1-mediated MAPK and NF-κB inflammatory signaling pathways. Moreover, LCBP reduced LPS-induced oxidative stress by up-regulating the expression of Nrf2 and MnSOD in the early period. EC and C3G were identified to have similar antioxidant and anti-inflammatory properties as LCBP, and capable to bind to TAK1 protein directly. Taken together, LCBP attenuated LPS-induced inflammation through a functional crosstalk regulation of antioxidant and anti-inflammatory systems (Figure 2.9).

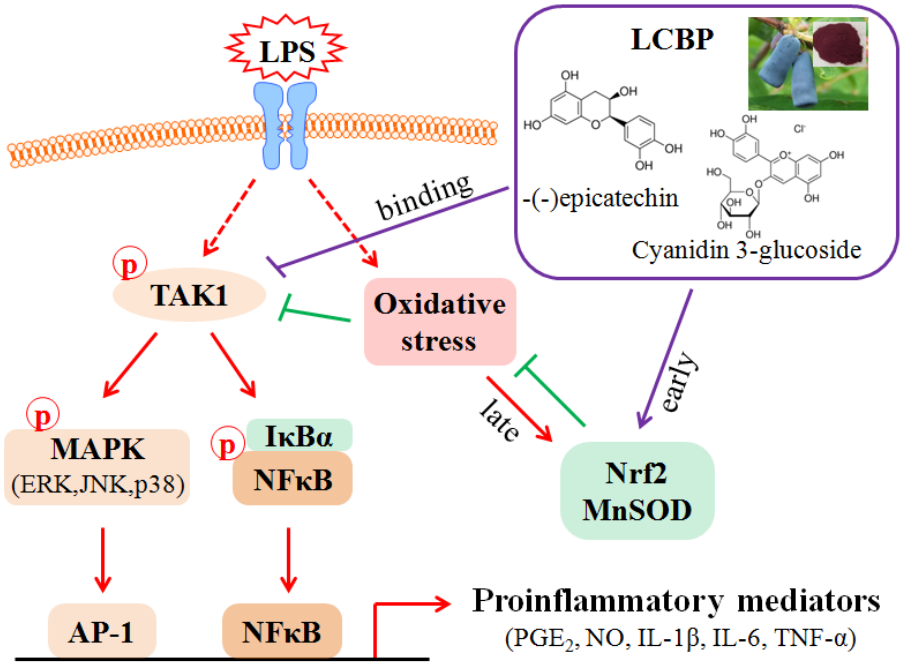



**Figure 2.9 Potential molecular mechanisms underlying the anti-inflammatory activity of LCBP in LPS-induced macrophages.**

# Chapter 3

# Protective effects of *Lonicera caerulea L.* berry polyphenols (LCBP) on experimental paw edema

## 3.1 Abstract

The *in vitro* study in Chapter 2 has demonstrated that *Lonicera caerulea* L. berry polyphenols (LCBP) possess significant anti-inflammatory activity by inhibiting the production of proinflammatory mediators. To further understand the *in vivo* anti-inflammatory activity, the inhibitory effect of LCBP on LPS-induced mouse paw edema was investigated in the present study. The modulation of cytokine network by LCBP was focused, since cytokines are proven to be the key regulators of inflammation, and play important roles in both the initiation and progression of inflammatory diseases. ICR mice were fed with LCBP for 4 days before LPS injection to the paw. The paw swelling was measured by using caliper, and 23 kinds of inflammatory cytokines in serum were assayed by multiplex technology. The results showed that LPS caused paw edema and increased serum levels of 21 kinds of cytokines except interleukin (IL)-5 and IL-17 in mice. Oral administration with LCBP attenuated paw edema and significantly decreased the serum levels of IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12 (p70), keratinocyte-derived cytokine (KC), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, regulated on activation, normal T cell expressed and secreted (RANTES), and tumor necrosis factor alpha (TNF-α) in LPS-induced mice, whereas showed no significant effects on the serum levels of IL-1α, IL-9, IL-12(p40), IL-13, eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), and MIP-1β. Treatment with LCBP only showed no significant effects on the serum levels of cytokines. These data suggested that oral administration with LCBP may attenuate LPS-induced inflammation by inhibiting the production of multiple pro-inflammatory cytokines, rather than

promoting the production of anti-inflammatory cytokines.

## 3.2 Introduction

Cytokines are considered as critical mediators in both initiation and progression of inflammation and cancer (Lin and Karin, 2007). Local stimulation of inflammatory stimuli such as bacterial endotoxins can initiate antigen presentation of dendritic cells, and then activate immune cells like macrophages to produce inflammatory cytokines, which mediate the progression of inflammatory diseases such as arthritis (Asagiri et al., 2008; Tang et al., 2011). On the one hand, macrophages (and/or other immune cells like T cells and NK cells) produce a series of proinflammatory cytokines including ILs, chemokines, IFN-γ, and TNF-α, which not only provoke local inflammation, but also induce systemic inflammation and inflammatory cells infiltration through circulation (Akdis et al., 2011; Bradley, 2008; Mantovani et al., 2004). On the other hand, T cells and macrophages can also produce anti-inflammatory cytokines, such as IL-4 (Guenova et al., 2015) and IL-10 (Couper et al., 2008), to avoid excessive inflammatory response or autoimmunity whereas it would inhibit the pathogen clearance and prolong inflammation.

As introduced in Chapter 2, LCBP has shown anti-inflammatory activity against LPS-induced inflammation by inhibiting the production of proinflammatory cytokines including Il-1β, IL-6, and TNF-α *in vitro*. Other studies also suggested that the extract of *Lonicera caerulea* L. berry can suppress cytokine production in many inflammatory models such as uveitis (Jin et al., 2006), oral inflammation (Zdarilova et al., 2010), skin inflammation (Svobodova et al., 2009; Vostalova et al., 2013), and hepatic inflammation (Wang et al., 2016). To investigate the anti-inflammatory activity of LCBP *in vivo*, a LPS-induced paw edema model, which is a useful functional model for identification of cytokine modulating anti-inflammatory agents (Vajja et al., 2004), was used in the present study. The regulatory effects of LCBP on the production of inflammatory cytokines were focused.

## 3.3 Materials and methods

### 3.3.1 Materials and reagents

LPS (*Escherichia coli Serotype 055:B5*) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bio-Plex Pro Mouse Cytokine 23-Plex Panel kit was purchased from Bio-Rad Laboratories (Philadelphia, PA, USA). LCBP was extracted as described in Chapter 2.

### 3.3.2 Mouse paw edema model

The animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kagoshima University (Permission N0. A12005). Male mice (ICR stain, 4 weeks of age) were from Japan SLC Inc. and group-housed under controlled light (12 h light/day) and temperature (25℃). All the mice had free access to water and feed in a home cage. After acclimatization for one week, the mice were divided into three groups (n＝4): control, LPS, and LPS + LCBP. The mice in LPS + LCBP group were fed with 300 mg/kg body weight of LCBP suspended in 0.1 ml normal saline by oral administration (*p.o.*) daily based on our pilot test, while other mice were fed with 0.1 ml normal saline only. Four days later, paw edema was induced with LPS by subcutaneous injection (*s.c.*) at a dose of 1mg/kg body weight, as described previously (Hisanaga et al., 2016). Paw thickness was measured by using caliper (model 19975, Shinwa Rules Co. Ltd, Japan) before and every hour after LPS injection until 6 h. After 6 h, mice were sacrificed and blood serums were collected from the heart.

### 3.3.3 Cytokine determination by the multiplex technology

The levels of cytokines including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α in mouse serum were measured with a Bio-Plex Pro Mouse Cytokine 23-Plex Panel kit (Bio-Rad Laboratories). The assay was performed with a Bio-Plex machine (Bio-Plex 200 System, Bio-Rad, USA)

according to the manufacturer's instructions, and the data were analyzed with the Bio-Plex manager software (version 5.0).

### 3.3.4 Statistical analysis

Results are expressed as means ± SD of four mice. The significant differences were determined using one-way ANOVA followed by Duncan's Multiple Range test (SPSS19, IBM Corp., Armonk, NY, USA). Differences were considered significant for $P < 0.05$.

## 3.4 Results

### 3.4.1 LCBP suppressed LPS-induced mouse paw edema

The anti-inflammatory activity of LCBP was investigated using a LPS-induced mouse paw edema model (Figure 3.1A). The initial paw thicknesses of mice in each group were not evidently different (Figure 3.1B). Injection of LPS increased average paw thickness (n = 4) from 2.68 to 3.32 mm at 1h, and the paw swelling remained till the end (paw thickness = 3.03 mm) in 6 h. Oral administration with LCBP at 300 mg/kg body weight daily for 4 days inhibited LPS-induced paw edema significantly ($P < 0.05$). Particularly, the paw thickness was reduced to 3.09 mm at 1h, and 2.80 at 6h. In comparing with LPS treatment alone, LCBP decreased the edema by 36.9% at 1h, and 63.0% at 6h, although the paw swelling was not completely disappeared (Figure 3.1B). As a control, normal saline showed no effects on paw edema.

### 3.4.2 LCBP decreased both pro- and anti-inflammatory cytokines in mouse serum

As cytokines are important mediators in the progression of inflammation, 23 kinds of inflammatory cytokines were next examined in mouse serum simultaneously by multiplex assay to clarify the influence of LCBP on LPS-induced production of cytokines.

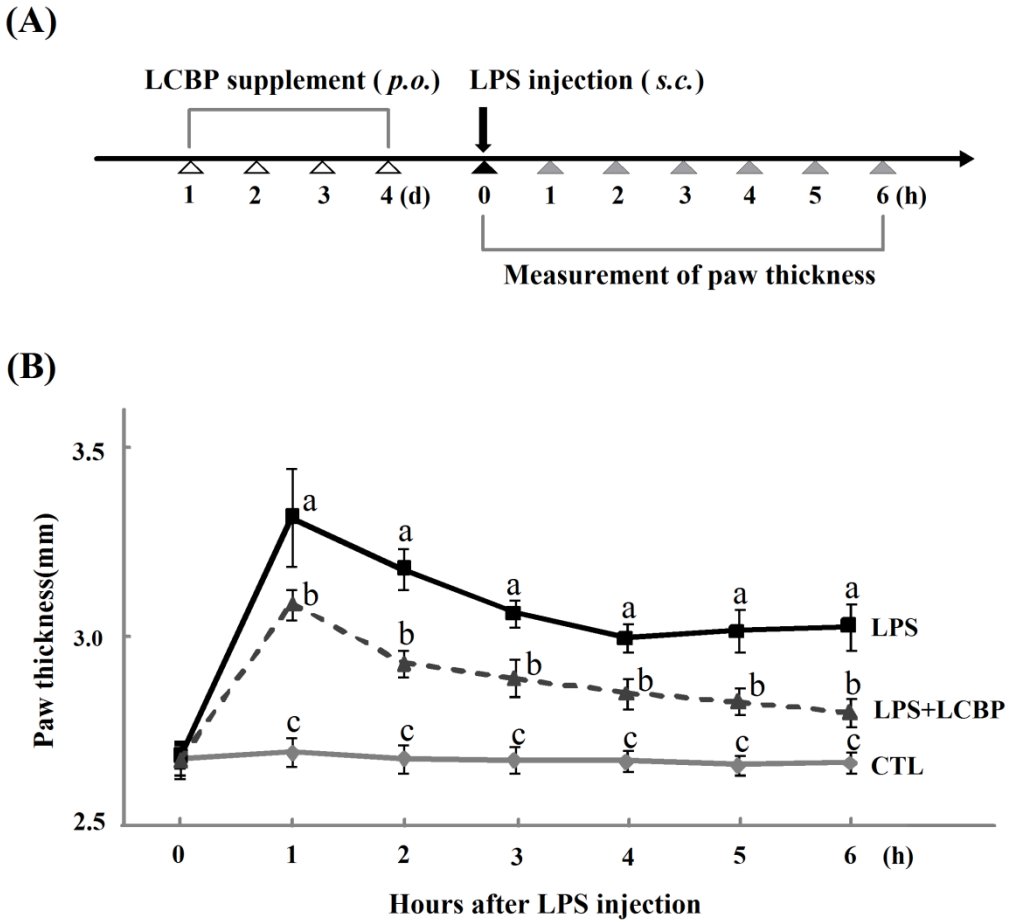



**Figure 3.1 The inhibitory effect of LCBP on LPS-induced mouse paw edema.** Mice were divided into three groups: control (CTL), LPS, and LPS+LCBP. Each group had four mice, respectively. LCBP was fed to mice by oral gavage (*p.o.*) at a dose of 300 mg/kg body weight for 4 days, and LPS (1mg/kg) was then injected subcutaneously (*s.c.*) into mouse paw. The paw thickness was measured using digital caliper before and every hour after LPS treatment until 6 h **(A)**. The change in paw edema thickness was shown in **(B)**. Data represent mean ± SD of four mice. Means with different superscript letters differ significantly at the probability of $P < 0.05$.

As shown in Figure 3.2&3.3, LPS induced more than fivefold increase in serum levels of IL-1β, IL-6, IL-10, IL-12(p40), eotaxin, G-CSF, KC, MCP-1, MIP-1α, MIP-1β, and RANTES; more than twofold increase in serum levels of IL-1α, IL-2, IL-4, IL-9, IL-12(p70), IL-13, IFN-γ, and TNF-α; less than twofold in serum levels of IL-3 and GM-CSF ($P < 0.05$); comparing with that of mice without LPS injection. However, LPS showed no significant influence on the levels of IL-5 and IL-17.

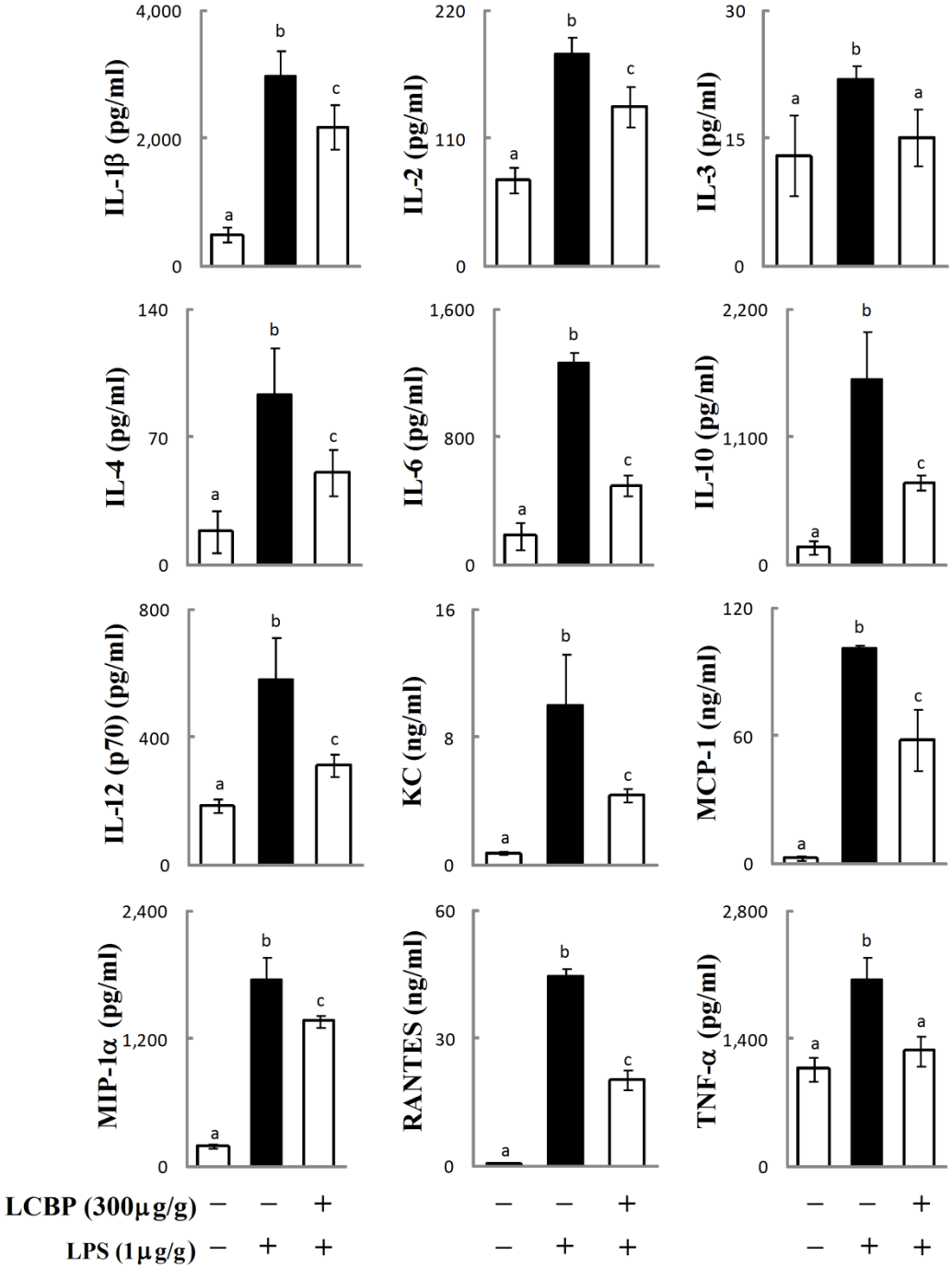



**Figure 3.2 The inhibitory effect of LCBP on serum levels of cytokines in LPS-induced mouse.** The blood serum was obtained from mice 6 h after LPS injection by collecting heart blood. The serum without any dilution was used for the measurement of cytokines by using multiplex technology. Each value represents the mean ± SD of four mice. Means with different superscript letters differ significantly at the probability of $P < 0.05$.

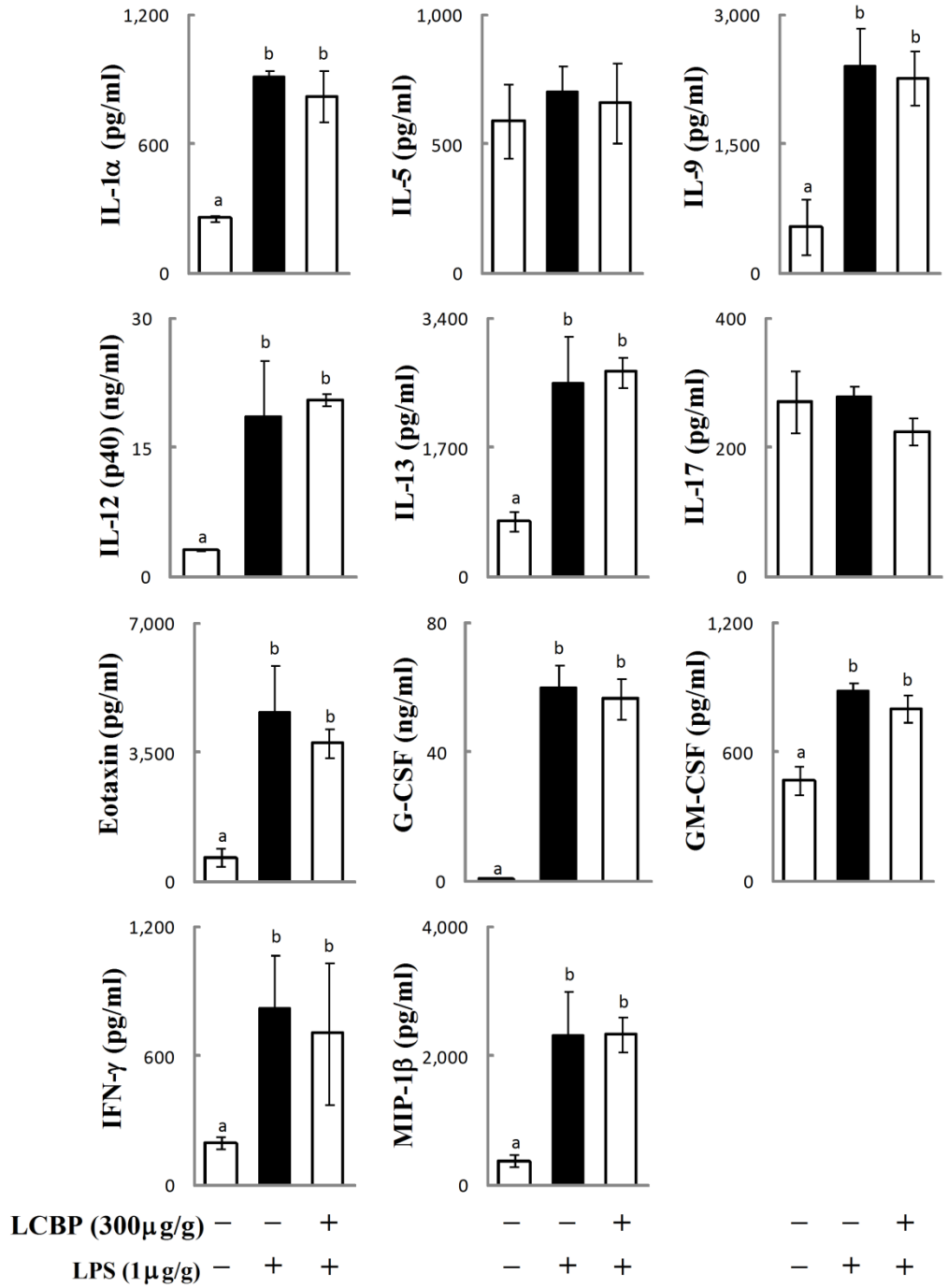



**Figure 3.3 The serum cytokines unaffected by LCBP in LPS-induced mice.** The blood serum was obtained from the mice 6 h after LPS injection by collecting heart blood. The serum without any dilution was used for the measurement of cytokines, using multiplex technology. The data represent mean ± SD of four mice, and values with different superscript letters differ significantly ($P < 0.05$).

Oral administration of LCBP decreased serum levels of inflammatory cytokines including IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12(p-70), KC, MCP-1, MIP-1α, RANTES, and TNF-α ($P < 0.05$) in LPS-induced mice (Figure 3.2). However, the administration of LCBP showed no significant effects on the serum levels of IL-1α, IL-9, IL-12(p40), IL-13, eotaxin, G-CSF, GM-CSF, IFN-γ, and MIP-1β ($p > 0.05$) in LPS-induced mice (Figure 3.3). In addition, LCBP showed no significant influence on the serum levels of IL-5 and IL-17 in both LPS-induced and normal mice ($p > 0.05$).

## 3.5 Discussion

The major finding of the present study is that LCBP inhibited LPS-induced paw edema with decreased serum levels of multiple inflammatory cytokines. LPS is the endotoxin produced by all gram-negative bacteria that can induce inflammation by activating immune cells to produce inflammatory cytokines (Borovikova et al., 2000). Thus, a LPS-induced mouse paw edema model has been used to investigate the effect of LCBP on inflammation and cytokines change in the present study. Inflammatory response is considered to be initiated by cytokines including ILs, chemokines, IFNs, and TNF-α secreted by local immune cells, and provoked by the release of cytokines into circulation and the following recruitment of immune cells such as macrophages to the inflamed tissue (Coussens and Werb, 2002; Laskin et al., 2011). In this study, injection of LPS increased serum levels of ILs including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), and IL-13; chemokines including KC, MCP-1, MIP-1α, MIP-1β, RANTES and eotaxin; as well as IFN-γ and TNF-α.

Interleukins play crucial roles in inflammatory response and immune regulation. IL-1α and IL-1β have similar biological properties that interact with many other cytokines especially induce considerable expression of KC (Gamero and Oppenheim, 2006), however, a recent study revealed that IL-1α is produced as a precursor to initiate inflammation in hypoxic cells, followed by the expression of mature IL-1β (Rider et al., 2011). IL-2 is known as the primary growth factor for T cells, especially crucial for the differentiation of $CD4^+$ T cells into defined T cell subsets after

antigen-mediated activation (Boyman and Sprent, 2012). IL-2 promotes inflammatory response through the generation of Th1 and Th2 effector cells, but also has anti-inflammatory properties by blocking the differentiation of T cells into Th17 cells (Hoyer et al., 2008). IL-3 contributes to leukocyte production, proliferation, and survival, knockdown of IL-3 showed protect effects on many inflammatory diseases such as sepsis (Weber et al., 2015). IL-4 shares homology and receptor with IL-13 (Andrews et al., 2006), and has been identified as an anti-inflammatory cytokine by suppressing Th17 cell-mediated inflammation recently (Guenova et al., 2015), although some other studies suggested that IL-4 promotes the production of pro-inflammatory cytokine IL-9 (Goswami and Kaplan, 2011), and inhibits the important anti-inflammatory cytokine IL-10 (Yao et al., 2005). IL-6 has also been reported to possess both pro- and anti-inflammatory properties, on the one hand, IL-6 provokes inflammation by promoting the production of acute phase proteins and subsequent MCP-1 (Atreya et al., 2000), but on the other hand, IL-6 can activate STAT3-mediated signaling pathway to induce the regeneration of intestinal epithelial cells and production of IL-4, thus attenuating inflammation (Scheller et al., 2011). IL-12(p70) is composed of IL-12(p35) subunit linked to the IL-12(p40) subunit, and the p40 subunit is shared by IL-23 by linking to IL-23(p19) subunit. IL-12(p70) induces the production of IFN-γ and development of Th1 inflammatory response, whereas IL-23 can stabilize Th17 cells (Teng et al., 2015). In the present study, LCBP decreased serum levels of not only pro-inflammatory ILs including IL-1β, IL-2, IL-3, IL-6 and IL-12(p-70), but also anti-inflammatory ILs including IL-4 and IL-10, while showed no significant influence on IL-1α, IL-9, IL-12(p40), and IL-13 in LPS-induced mice. These data suggested that the anti-inflammatory activity of LCBP was based on the inhibition of pro-inflammatory cytokines productions, rather than the promotion of anti-inflammatory cytokines productions.

Chemokines are well-known small proteins that induce immune cells migration, and also can affect angiogenesis, collagen production and the proliferation of haematopoietic precursors (Mantovani et al., 2004). KC mediates the recruitment of neutrophils to inflamed tissue and plays an important role in chronic inflammatory

diseases such as arthritis and carditis (Ritzman et al., 2010). MCP-1 is recognized as one of the key chemokines that regulate migration of monocytes/macrophages (Kanda et al., 2006). MIP-1α and MIP-1β are two macrophage inflammatory proteins that induce the migration of monocytes/macrophages; however, they have different effects in the regulation of inflammatory cytokines. For instance, the release of IL-1 induced by LPS is synergized by MIP-1α, but not MIP1β, in peripheral blood monocytes (Nath et al., 2006). RANTES is mainly known as a chemo-attractant for T cells (Matter and Handschin, 2007), and eotaxin is a potent eosinophil chemo-attractant (Adar et al., 2014), in inflammatory response. In the present study, LCBP reduced the serum levels of KC, MCP-1, MIP-1α, and RANTES, but showed no significant influence on MIP1β and eotaxin in LPS-induced mice. These results suggested that LCBP attenuated LPS-induced inflammation potentially through inhibiting the migration of neutrophils, monocytes/macrophages, and T cells, but not eosinophils.

## 3.6 Conclusion

In conclusion, the present study demonstrated that oral administration of LCBP attenuated LPS-induced mouse paw edema by inhibiting the production of inflammatory cytokines including IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12(p-70), KC, MCP-1, MIP-1α, RANTES, and TNF-α (Figure 3.4).

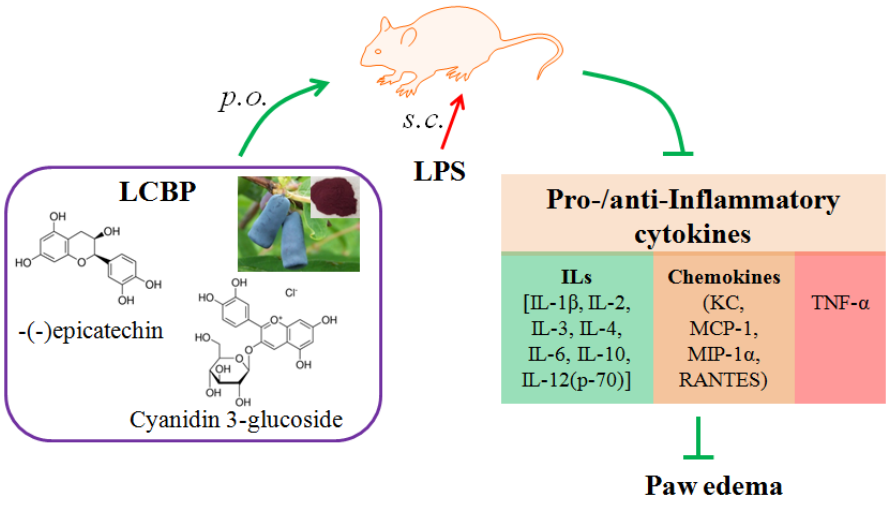



**Figure 3.4 LCBP showed the protective effect against LPS-induced mouse paw edema.**

# Chapter 4

# Protective effects of *Lonicera caerulea L.* berry polyphenols (LCBP) on experimental arthritis

## 4.1 Abstract

The polyphenols from *Lonicera caerulea* L. berry have shown anti-inflammatory activity in LPS-induced macrophages by modulating signaling pathways response to inflammation and oxidative stress. To study the protective effects of *Lonicera caerulea L.* berry polyphenols (LCBP) on chronic inflammatory diseases, I next investigated the inhibitory effects of LCBP on arthritis by using an established adjuvant-induced arthritis (AIA) rat model. Rats were fed with 75-300 μg/g body weight (BW) daily, and arthritis was induced by injecting adjuvant to the paw of rats. The paw swelling was measured to reflect the progression of arthritis, while the levels of proinflammatory factors, the activities of antioxidant enzymes, and indices of liver function were determined to evaluate the anti-inflammatory and antioxidant effects of LCBP. The results indicated that oral administration of LCBP in the range of 75-300 μg/g BW attenuated AIA symptom as reducing paw edema in rats. The serum levels of proinflammatory factors including tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and nitric oxide (NO) were significantly reduced in LCBP-fed rat. The production of inflammatory enzymes, inducible nitric oxide synthases (iNOS) and cyclooxygenase-2 (COX-2), in the spleen was also significantly suppressed. Moreover, serum transaminases including glutamic oxaloacetic transaminase (GOT), glutamate-pyruvate transaminase (GPT) and gamma-glutamyl transferase (GGT) were inhibited, and the antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPx) were recovered. Taken together, the data demonstrated that LCBP attenuated rat AIA symptom by both decreasing the production of proinflammatory factors and enhancing the activity of antioxidant enzymes.

## 4.2 Introduction

Chronic inflammation is a prolonged pathological condition characterized by the infiltration of mononuclear immune cells such as macrophages, monocytes, lymphocytes, and plasma cells, as well as tissue destruction and fibrosis (Khansari et al., 2009). Accumulating data have revealed that continued production of proinflammatory mediators and free radicals lead to chronic inflammation, which mediate most chronic diseases including cancer, diabetes, cardiovascular, arthritis, neurological and pulmonary diseases (Reuter et al., 2010). My previous study has shown that LCBP rich in cyanidin 3-glucoside (C3G) and (-)-epicatechin (EC) can attenuate inflammation by regulating inflammation and oxidative stress-related cellular signaling pathways. Thus, I hypothesized that LCBP contributes to the protection against chronic inflammation diseases.

Arthritis represents one of the most prevalent chronic diseases. Rheumatoid arthritis (RA) and osteoarthritis (OA) are two most common types of arthritis characterized by joint inflammation including immune cell infiltration, synovial hyperplasia, pain and swelling (Neugebauer et al., 2007). RA and OA are different types of arthritis; RA is an autoimmune disease that the body's own immune system attacks the body's joints (McInnes and Schett, 2011), while OA is mainly caused by mechanical wear and tear on joints (Scanzello and Goldring, 2012), although they are both associated with long-term inflammation, and share similar characteristics. The animal model of osteoarthritis and rheumatoid arthritis can be established by AIA to mirror their pathologies (Chou et al., 2011; Chung et al., 2012). Complete Freund's adjuvant (CFA) is well used adjuvant to induce arthritis because CFA is composed of killed mycobacteria, which contain various pathogen-associated molecular patterns including toll-like receptor 2, 4, and 9 agonists (Akira et al., 2006; Janeway and Medzhitov, 2002). In AIA, local injection of adjuvant stimulates antigen presentation by dendritic cells, leading to T cell autoimmunity and the secretion of pro-inflammatory factors by macrophages (Asagiri et al., 2008). Proinflammatory factors such as TNF-α, IL-1β, IL-6, NO and $PGE_2$) could further aggravate systemic

inflammation (Chung et al., 2012; Kapoor et al., 2011). Progressive systemic TNF-α, IL-1β, IL-6 and NO increase the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and decrease the activities of antioxidant enzymes such as SOD and GPx in tissues. These consequent events further aggravate protein and lipid oxidation, and injure tissues. Thus, oxidative damage of the vital organs, particularly the liver, is considered as secondary complications of arthritis (Sundaram et al., 2014). It is observed that the release of hepatic transaminases such as GOT and GPT into the blood was found in AIA-mediated liver damage (Comar et al., 2013). Moreover, the overexpression of iNOS and COX-2, as synthases of NO and $PGE_2$, has been proved to be related to the progressive inflammation and tissue damage observed in arthritis (Lascelles et al., 2009; Oyanagui, 1994).

Based on the properties of LCBP and information and the potential progression of RA, I intended to study the effects of LCBP on the inflammation, oxidative status, and metabolic function in an adjuvant-induced experimental arthritis.

## 4.3 Materials and methods

### 4.3.1 Chemicals and reagents

Complete Freund's adjuvant (CFA, containing 1mg/ml of mycobacterium tuberculosis) was purchased from Sigma (St. Louis, MO, USA). Antibodies against iNOS, COX-2, α-tubulin (B-7) and corresponding secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LCBP was extracted as described in Chapter 2.

### 4.3.2 Arthritis induction and assessment

Six-week-old male Sprague-Dawley rats (weighing an average of 165±7.41g) were obtained from Hunan Provincial Laboratory Animal Public Service Center, Hunan, China (for primary experiment), or Japan SLC Inc. (for repeating experiment). The animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Hunan Agricultural University, or Kagoshima

University. Rats were housed in polycarbonate cages with soft wood granulate floors, self-feeders and automatic water. After allowing to acclimatize for 1 week, the rats were assigned on the basis of average weight into five groups (n=10). LCBP (dissolved in 1 ml saline) was orally administrated (*p.o.*) to four groups at 0, 75, 150, 300 μg/g BW daily, respectively, according to the *in vitro* data and doses used in previous study (Jin et al., 2006). Two hours after the first administration, right hind paw of the rats in four groups were injected with 100 μl CFA into the metatarsal footpad by intradermal injection (*i.d.*) to induce AIA after anesthetizing with ether (Perera et al., 2011). Normal control group was fed with normal rat diet and treated with 100 μl saline. Hind paw thickness of rats were measured by a digital caliper before CFA injection, 24 h later, and every 4 days until to 28th day (See Figure 3.1A). The increase in paw thickness at the specific day was defined as the relative index of paw by following formula.

$$\text{Relative index} = \text{Paw thickness}_{\text{treatment}} - \text{Paw thickness}_{\text{control}}$$

### 4.3.3 Serum separation and measurement of proinflammatory factors

Blood samples were collected into anticoagulant-free tubes at 28$^{th}$ day, and then stood for 30 min at room temperature to coagulate properly. The serums were acquired by centrifuging at 1,500 g for 10 min and stored at -80 ℃ until use. The levels of TNF-α, IL-1β, IL-6 and NO (as indicated by nitrite after nitrate is enzymatically converted to nitrite) in serum were measured with respective ELISA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's manual.

### 4.3.4 Measurement of biochemical indicators in serum

The activities of SOD and GPx, and the level of thiobarbituric acid reactive substances (TBARS) , which are produced by lipid peroxidation and considered as indicators of oxidative stress (Yousef et al., 2009), were determined by using respective assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions (Lu et al., 2010).

Serum levels of GOT, GPT, gamma-glutamyl transferase (GGT), total cholesterol (T-cho), high density lipoprotein cholesterol (HDL-c), total triacylglycerol (TG) and glucose were measured with an automated analyzer for clinical chemistry (SPOTCHEM EZ SP-4430, Arkray, Kyoto, Japan).

### 4.3.5 Spleen protein extraction

Spleen tissue was sampled from rats at 28$^{th}$ day, and quick-frozen in liquid nitrogen. The tissues were separated on ice, and same amounts of tissues were then homogenized in RIPA buffer (0.1 g/ml) by using a Speed-Mill PLUS Homogenizer (Analytik Jena, Jena, Germany). The supernatant proteins were obtained by centrifuging at 13,500 g for 5 minutes at 4 ℃ and stored at -80 ℃ until use.

### 4.3.6 Western blot assay

Western blotting was performed as described previously (Hou et al., 2005). Briefly, equal amounts of 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 ℃ and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by ECL system 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).

### 4.3.7 Statistical analysis

Results are expressed as means ± SD of ten rats. The significant differences were determined using one-way ANOVA followed by Duncan's Multiple Range test (SPSS19, IBM Corp., Armonk, NY, USA). Differences were considered significant for $P < 0.05$.

## 4.4 Results

### 4.4.1 LCBP suppressed adjuvant-induced rat paw edema

Paw edema is an important clinical symptom of AIA (Neugebauer et al., 2007), thus, I measured paw thickness to monitor clinical symptom of AIA. Rats were orally administrated with 75, 150 or 300 μg/g BW of LCBP and then treated with CFA to induce AIA (Figure 4.1A), paw edema occurred in the right hind paw of rats (Day 1) after the injection of CFA (Figure 4.1B), and then also occurred in the left hind paws after day 16 (Figure 4.1C). Oral administration with LCBP in the concentration ranges of 75-300 μg/g BW suppressed the paw edema in right hind paw from day 1 to 28 (Figure 4.1B), and left hind paw from day 16 to 28 (Figure 4.1C), significantly ($P$<0.05). These data indicated that LCBP might have anti-inflammatory action to suppress paw edema in AIA rat.

### 4.4.2 LCBP decreased serum levels of proinflammatory factors

Since TNF-α, IL-1β, IL-6 and NO released by inflamed tissue and inflammatory cells could further aggravate inflammation in arthritis (Chung et al., 2012; Maini and Taylor, 2000), these proinflammatory factors were next measured in rat serum. As shown in Figure 4.2, the levels of TNF-α, IL-1β, IL-6 and NO were increased in AIA rats significantly ($P$<0.05), but they were reduced significantly in the rats administrated with LCBP in a dose-dependent manner ($P$<0.05). Specially, they were reduced to the normal control level in the rats administrated with 300 μg/g of LCBP.

### 4.4.3 LCBP suppressed the expression of iNOS and COX-2 in rat spleen

The synthases, iNOS and COX-2, not only regulate the production of NO and $PGE_2$, but also further stimulate the secretion of other inflammatory mediators (Nathan, 1992; Samad et al., 2001). Thus, I examined the expression of iNOS and COX-2 in rat spleen, which is the most important organ in the immune system (Mebius and Kraal, 2005). As shown in Figure 4.3, the expressions of iNOS and COX-2 protein were increased in the spleen of AIA rats dramatically, but they were

suppressed in the rats administrated with LCBP in a dose-dependent manner ($P$<0.05). The amount of α-tubulin, an internal control, remained unchanged.

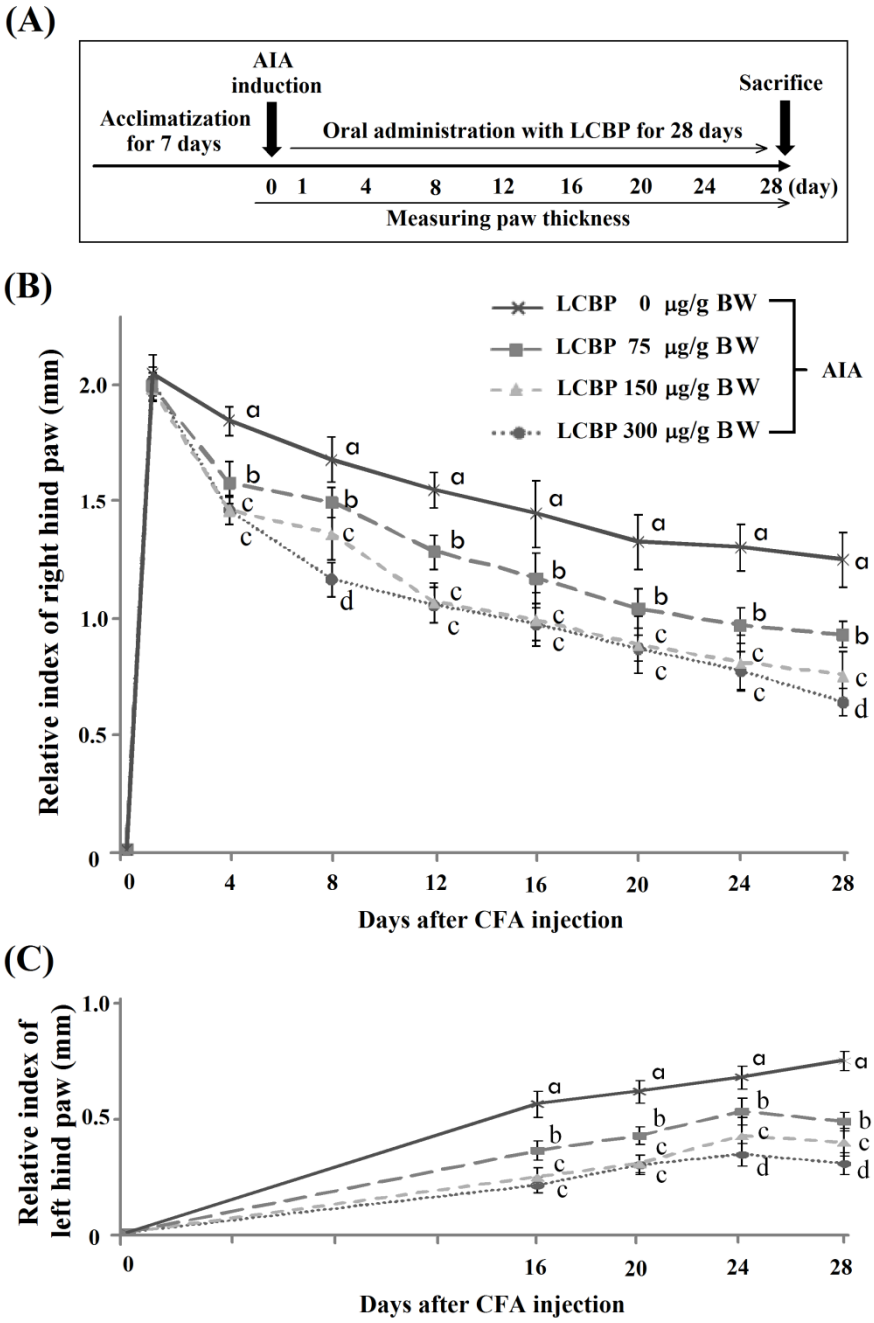



**Figure 4.1 The inhibitory effect of LCBP on CFA-induced rat paw edema. (A)** Animal experimental scheme. Rats were divided into 5 groups, and oral administrated with or without indicated concentrations of LCBP daily. After the first administration, AIA was induced by injecting 100 μl of CFA into the right hind paw of rats. Paw thickness of rat right hind paw **(B)** and

left hind paw **(C),** which were measured with a digital caliper before CFA injection, 24 h later, and every 4 days until to 28th day. The values represent mean ± SD (n =10) of the relative index, and values with different letters in the same days differ significantly ($P$<0.05).

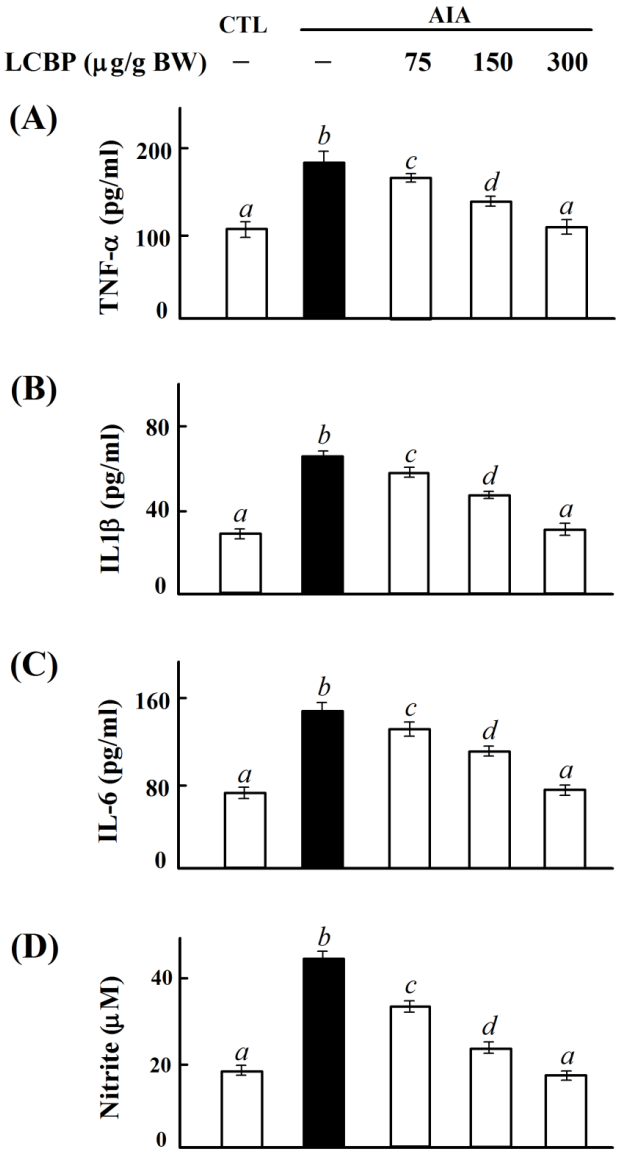



**Figure 4.2 The inhibitory effect of LCBP on the production of proinflammatory factors in rat serum.** Blood samples were collected into anticoagulant-free tubes at 28th day, and serums were acquired by centrifuging at 1,500 g for 10 min. The levels of TNF-α **(A)**, IL-1β **(B)**, IL-6 **(C)** and NO (as indicated by nitrite) **(D)** in serum were measured with their respective ELISA kit according to manufacturer's manual. The values represent mean ± SD (n =10), and values with different superscript letters differ significantly ($P$<0.05).

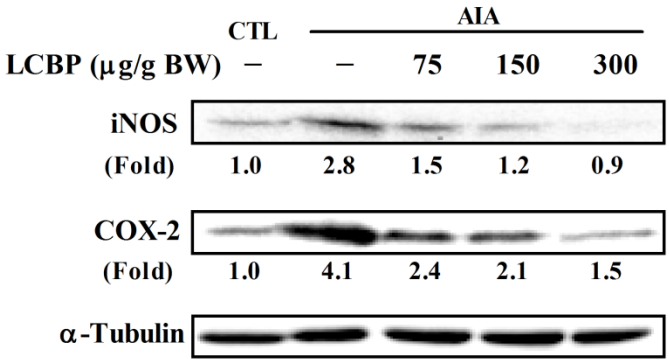



**Figure 4.3 Suppression of iNOS and COX-2 expression by LCBP in rat spleen.** Spleen tissue was isolated from rats at 28$^{th}$ day, and total proteins were extracted with RIPA buffer as described in Section 4.3.5. COX-2 and iNOS proteins were detected by Western blotting analysis with their antibodies, respectively. The expression of iNOS and COX-2 was described as induction folds calculated by the intensity of the treatment relative to that of control by densitometry. The detection of α-tubulin was presented as an internal control of the total protein. The data represent mean ± SD (n =10), and values with different superscript letters differ significantly ($P$<0.05).

### 4.4.4 LCBP increased the activities of SOD and GPx, and decreased the level of TBARS in rat serum

SOD and GPx are two major antioxidant enzymes to minimize oxidative stress (Cadenas and Davies, 2000), which plays an important role in the development of arthritis (Comar et al., 2013). I next examined the activities of SOD and GPx in the serum of rats. The results showed that the activities of SOD (Figure 4.4A) and GPx (Figure 4.4B) were decreased in AIA rats significantly ($P$<0.05), but they were recovered in rats administrated with LCBP in a dose-dependent manner. Notably, they were recovered to normal control level after administrated with 300 μg/g of LCBP. Whereas, the level of TBARS was increased in the serum of AIA rats significantly ($P$<0.05), and decreased in the rats administrated with LCBP in a dose-dependent manner (Figure 4.4 C).

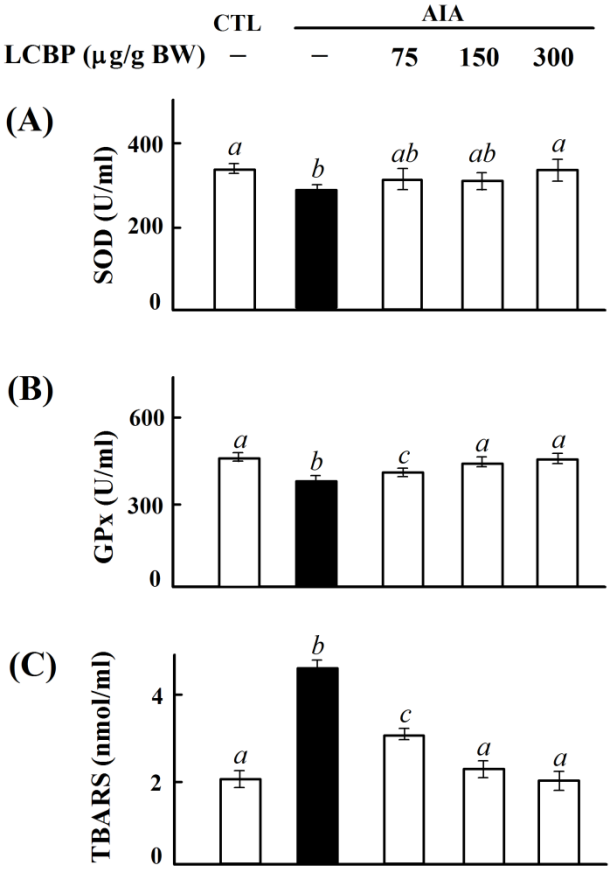



**Figure 4.4 Effects of LCBP on the activities of SOD and GPx, and the level of TBARS in rat serum.** Rat serum was collected as described in section 4.3.3. The activities of SOD **(A)** and GPx **(B)**, and the level of TBARS **(C)** in serum were measured by using their respective assay kit according to the manufacturer's manual. The values represent mean ± SD (n = 10), and values with different superscript letters differ significantly ($P < 0.05$).

### 4.4.5 LCBP decreased serum transaminases

Recent study suggested that oxidative stress-mediated liver damage is considered as secondary complications of arthritis (Sundaram et al., 2014). Thus, I further measured some serum indices that indicate hepatic status. As shown in Figure 3.5, serum levels of transaminases including GPT, GOT and GGT were increased significantly ($P<0.05$) in AIA rats. However, they were reduced in the rats administrated with LCBP in a dose-dependent manner. Especially, their levels were recovered to normal control level in the rats administrated with 300 μg/g of LCBP. On

the other hand, the serum levels of T-cho, HDL-c, TG and glucose were observed to have no significant changes in both AIA and LCBP groups (Table 4.1).

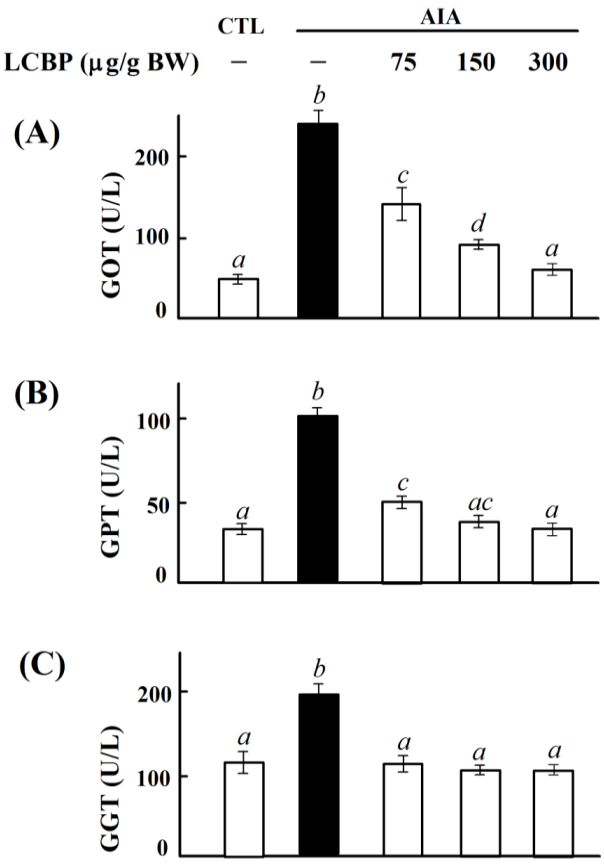



**Figure 4.5 Effects of LCBP on serum indices of liver function.** Serum transaminases were measured by using an automated analyzer for clinical chemistry. The levels of GOT **(A)**, GPT **(B)**, and GGT **(C)** were measured in undiluted serum with their specific reagent strip. The values represent mean ± SD (n =10), and values with different superscript letters differ significantly ($P<0.05$).

## 4.5 Discussion

The major finding of this study is that oral administration of LCBP, 75% ethanol extract of *Lonicera caerulea L.* berry, could inhibit AIA in rats through the anti-inflammatory and antioxidant effects. Rat AIA has been used in pre-clinical studies as an animal model of arthritis for studying the therapeutic effects and

mechanisms (Asagiri et al., 2008; Comar et al., 2013). In rat AIA model, paw swelling and the arthritic index including inflammation and oxidation are indicators of chronic systemic inflammation (Banji et al., 2014; Chung et al., 2012).

**Table 4.1 Effects of LCBP on serum lipids and glucose**

| Group | CTL | AIA | AIA+LCBP75 | AIA+LCBP150 | AIA+LCBP300 |
|---|---|---|---|---|---|
| Tcho | 66.0±14.4 | 59.3±8.3 | 51.3±2.3 | 59.3±16.2 | 67.7±16.6 |
| HDL-c | 25.3±5.5 | 15.7±4.9 | 14.7±4.2 | 19.0±7.0 | 25.3±12.5 |
| TG | 103.7±26.8 | 83.0±27.1 | 57.3±23.5 | 82.3±36.6 | 91.7±30.9 |
| Glucose | 171.3±21.8 | 172.7±24.2 | 141.0±37.4 | 116.7±22.5 | 141.7±53.7 |

**Table 4.1 Effects of LCBP on serum lipids and glucose.** Serum lipid profile was measured by using an automated analyser for clinical chemistry. The levels of T-cho, HDL-c, TG and glucose were measured with their respective reagent strip. The values represent mean ± SD (n = 10).

In this study, CFA caused paw edema at the injection side rapidly, and also at another side with the development of arthritis 16 days later. LCBP dose-dependently attenuated both primary swelling in right hind paw and secondary swelling in left hind paw in AIA rats. In the process of CFA-induced arthritis, local injection of CFA stimulates immune cells autoimmunity in inflamed tissues (Asagiri et al., 2008), and inflammatory cells then produce proinflammatory factors such as TNF-α, IL-1β, IL-6, NO and $PGE_2$, which further aggravate systemic inflammation (Chung et al., 2012). Results of the present study revealed that all these inflammatory indicators were increased significantly ($P$<0.05) in the serum of CFA-induced rats, and oral administration with LCBP reduced their accumulation in a dose-dependent manner. Specially, the serum levels of TNF-α, IL-1β, IL-6 and NO returned to normal control level in rats administrated with 300 μg/g of LCBP. COX-2 and iNOS as the synthases of NO and $PGE_2$ regulate the generation of NO and $PGE_2$, which can interact with other cytokines to promote inflammation (Nathan, 1992; Samad et al., 2001). The

overexpression of iNOS and COX-2 has been proved to aggravate inflammation and tissue damage in arthritis (Lascelles et al., 2009; Oyanagui, 1994). The data in the present study showed that LCBP suppressed the overexpression of COX-2 and iNOS in rat spleen, which is a site for storage and rapid deployment of the immunocytes including macrophages, dendritic cells, and monocytes (Mebius and Kraal, 2005; Swirski et al., 2009).

It is noticed that oral administration with LCBP in rats not only suppressed the releasing of CFA-induced proinflammatory factors but also enhanced the expression and/or the activity of antioxidant enzymes (e.g. SOD and GPx). LCBP also inhibited the product of lipid peroxidation (e.g. TBARS) induced by CFA. Moreover, LCBP lightened CFA-caused liver damage by reducing serum levels of GPT, GOT and GGT. These data suggested that LCBP might attenuate the CFA-induced paw edema through a crosstalk between anti-inflammatory and antioxidant actions.

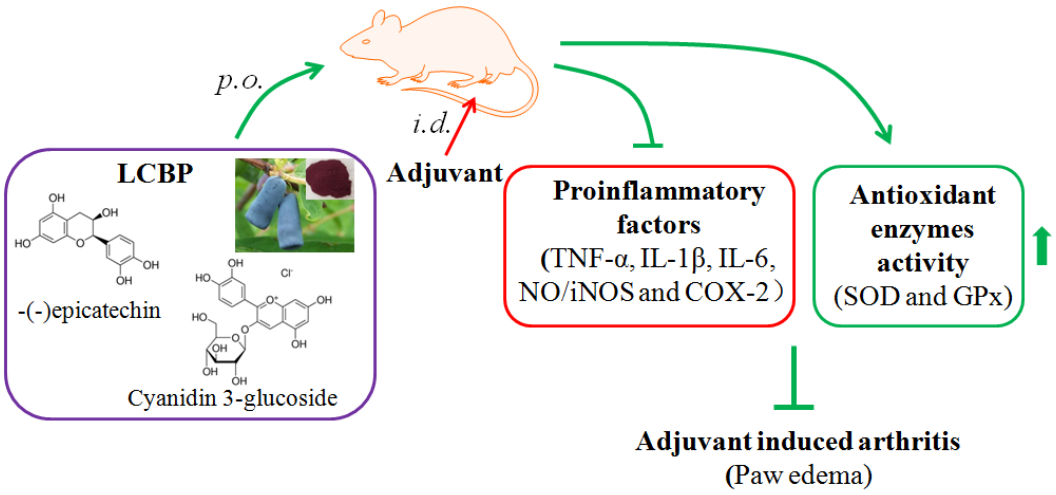



**Figure 4.6 LCBP showed the protective effect against adjuvant-induced arthritis in rats.**

## 4.6 Conclusion

In conclusion, the present study demonstrated that oral administration of LCBP attenuated CFA-induced AIA by suppressing the production of proinflammatory factors (TNF-α, IL-1β, IL-6, NO/iNOS and COX-2) and enhancing the activities of antioxidant enzymes (SOD and GPx), which suggesting that LCBP is a potential chemopreventive agent for arthritis.

# Chapter 5

# Protective effect and mechanisms of *Lonicera caerulea* L. berry polyphenols (LCBP) on experimental nonalcoholic steatohepatitis

## 5.1 Abstract

Nonalcoholic steatohepatitis (NASH) is a common disease that closely related to inflammation and oxidative stress, and *Lonicera caerulea* L. berry polyphenols (LCBP) possess both antioxidant and anti-inflammatory properties in the previous studies. The present study aimed to investigate the protective effects and mechanisms of LCBP on NASH in a high fat diet (HFD) plus carbon tetrachloride (CCl4)-induced mouse model. Mice were fed with HFD containing LCBP (0.5-1%) or not, and then administrated with $CCl_4$ to induce NASH. Liver sections were stained by Hematoxylin-eosin stain, serum transaminases and lipids were measured by clinical analyzer, insulin was examined by ELISA, cytokines were determined by multiplex technology, and hepatic proteins were detected by western blotting. LCBP improved histopathological features of NASH with lower levels of lipid peroxidation and cytokines including granulocyte-colony stimulating factor (G-CSF), interleukin (IL)-3, IL-4, macrophage inflammatory protein (MIP)-1β, IL-6, IL-5, keratinocyte-derived cytokine (KC), tumor necrosis factor alpha (TNF-α), IL-2, IL-1β, monocyte chemotactic protein 1 (MCP-1), IL-13, interferon-γ (IFN-γ), IL-10, IL-12(p70), IL-1α, eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), MIP-1α, IL-17, and regulated on activation, normal T cell expressed and secreted (RANTES). Further molecular analysis revealed that LCBP increased the expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and manganese-dependent superoxide dismutase (MnSOD), but decreased forkhead box protein O1 (FoxO1) and heme oxygenase-1 (HO-1) in the liver of NASH mice. These data demonstrated that dietary supplementation of LCBP ameliorated inflammation and lipid peroxidation by up-regulating Nrf2 and MnSOD, and down-regulating FoxO1 and HO-1 in NASH.

## 5.2 Introduction

NASH is a common liver disease in the modern society, and the pathogenesis is unclear yet. Although obesity is correlated with an increased risk of NASH (Fabbrini et al., 2010), it is not the decisive factor since NASH can be developed in lean people (Younossi et al., 2012). Recent studies suggested that inflammatory and metabolic disorders caused by the dysbiosis of gut microbiota potentially accelerate the progression of NASH (Henao-Mejia et al., 2012; Qin et al., 2014), and proinflammatory cytokines and oxidative stress play an important role in promoting hepatic inflammation, lipid peroxidation, and fibrosis (Rolo et al., 2012; Tilg and Diehl, 2000).

Natural phytochemicals such as polyphenols contribute to the attenuation of both oxidative stress and inflammation. LCBP have shown both antioxidant and anti-inflammatory activities *in vitro* and *in vivo* in my previous studies, and have been reported to possess antioxidant (Palikova et al., 2009; Rupasinghe et al., 2012), and anti-inflammatory activities (Rupasinghe et al., 2015; Wang et al., 2016; Zdarilova et al., 2010) in other recent studies.

Based on the information related to NASH and LCBP, the present study aimed to investigate the protective effects and mechanisms of LCBP on NASH. As NASH is a long-term disease that with few or no symptoms in the early stages, and previous study revealed that mice fed with HFD exhibit typical features of NASH after more than 60 weeks (Nakamura and Terauchi, 2013), the progressive NASH models are commonly used in laboratory studies. The “two-hit” hypothesis (Day and James, 1998), in which first hit causes fat accumulation in hepatocytes, and second hit induces inflammation and fibrosis, is widely accepted for building NASH model. $CCl_4$ is a well-known hepatotoxicant used in experimental models to induce hepatic inflammation and fibrosis (Kamada et al., 2003), and a recently study indicated that HFD with multiple administration of $CCl_4$ successfully induced pathological features of NASH in a mouse model (Kubota et al., 2013). Thus, I attempted to use a HFD plus $CCl_4$-induced mouse model to explore the protective effects of LCBP on NASH,

and further clarify the mechanisms by investigating the expressions of both cytokines and oxidative stress response proteins.

## 5.3 Materials and methods

### 5.3.1 Chemicals and reagents

$CCl_4$ (≥99.5%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lard oil was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Antibodies against alpha-smooth muscle actin (α-SMA), nuclear factor (erythroid- derived 2)-like 2 (Nrf2), manganese-dependent superoxide dismutase (MnSOD), heme oxygenase-1 (HO-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against forkhead box protein O1 (FoxO1) and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). LCBP was extracted as described in Chapter 2.

### 5.3.2 Mouse model of NASH

The animal experimental protocol was drafted according to the guidelines of the Animal Care and Use Committee of Kagoshima University (Permission N0. A12005). Male C57BL/6N mice (5 weeks of age) from Japan SLC Inc. (Shizuoka, Japan) were housed separately in cages with wood shavings bedding under controlled light (12 h light/day) and temperature (25℃), and free access to water and feed. After acclimatization for one week (Day 0), the mice were randomly divided into six groups (n=4): Normal diet (ND), ND+LCBP1%, HFD, HFD$CCl_4$, HFD$CCl_4$+LCBP0.5%, and HFD$CCl_4$+LCBP1%, and fed with the corresponding diets as described in Table 5.1. After 30 days (Day 30), the mice in HFD$CCl_4$, HFD$CCl_4$+LCBP0.5%, and HFD$CCl_4$ +LCBP1% groups were intraperitoneally (*i.p.*) injected with $CCl_4$ at a dose of 0.05 ml/kg body weight (BW) every 3 days to induce NASH until Day 45. The dosage of $CCl_4$ used in this study was based on our pilot test. Mice were sacrificed at Day 45 after overnight fasting.

| Table 5.1 Dietary compositions of each group | | | | | | |
|---|---|---|---|---|---|---|
| Components (%) | ND | ND+ LCBP1% | HFD | HFD$CCl_4$ | HFD$CCl_4$+ LCBP0.5% | HFD$CCl_4$+ LCBP1% |
| Lard | 6 | 6 | 40 | 40 | 40 | 40 |
| Casein | 21 | 21 | 21 | 21 | 21 | 21 |
| Sucrose | 10 | 10 | 10 | 10 | 10 | 10 |
| Cellulose | 4 | 4 | 4 | 4 | 4 | 4 |
| Mineral mix | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin mix | 1 | 1 | 1 | 1 | 1 | 1 |
| Choline chloride | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Methionine | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Corn starch | 54 | 53 | 20 | 20 | 19.5 | 19 |
| LCBP | 0 | 1 | 0 | 0 | 0.5 | 1 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 |
| Calories (kcal/100g) | 377.9 | 374.1 | 555.1 | 555.1 | 553.2 | 551.3 |

### 5.3.3 Hepatic histology

Mice liver were collected at day 45 and sectioned by using a freezing microtome system (Yamato, Saitama, Japan) according to the manufacturer's manual. Obtained liver sections were then stained with hematoxylin-eosin (H&E) staining, and observed under a fluorescence microscope (Keyence, Tokyo, Japan).

### 5.3.4 Hepatic protein extraction and Western blot assay

The total proteins of liver were obtained by using RIPA buffer. Briefly, equal

amounts of liver tissues were homogenized in RIPA buffer (0.1 g/ml) with a Speed-Mill PLUS homogenizer (Analytik Jena, Jena, Germany). The supernatant proteins were obtained by being centrifuged at 13,500 g for 5 min at 4 ℃, and the protein concentrations were determined by using a due-binding protein assay kit (Bio-Rad Hercules, CA, USA) according to the manufacturer's manual. The protein extracts were boiled in SDS sample buffer for 5 min, and equal amounts of protein (40μg) were run on a 10% SDS-PAGE gel before electrophoretically transferred to PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was then incubated with specific primary antibody and HRP-conjugated secondary antibody, following by detection with the LumiVision PRO system (TAITEC Co., Saitama, Japan).

### 5.3.5 Measurement of SOD activity and TBARS level in liver

The activity of superoxide dismutase (SOD) and the level of thiobarbituric acid reactive substances (TBARS) in the liver were measured with their respective assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's manual.

### 5.3.6 Measurement of transaminases, lipids, glucose, and insulin in serum

Blood sera were obtained by centrifuging at 1,500 g for 10 min after coagulation at day 45. The levels of glutamic oxaloacetic transaminase (GOT), glutamate-pyruvate transaminase (GPT), gamma-glutamyl transferase (GGT), total cholesterol (T-cho), HDL cholesterol (HDL-c), total triacylglycerol (TG), and glucose were measured with an automated analyzer for clinical chemistry (Arkray, Kyoto, Japan). The insulin concentration was measured with an ELISA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), according to the manufacturer's manual.

### 5.3.7 Determination of cytokines by the multiplex technology in serum

Serum levels of cytokines including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-

CSF), IFN-γ, keratinocyte-derived cytokine (KC), monocytes chemotactic protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES, and tumor necrosis factor-alpha (TNF-α) were measured with a Bio-Plex Pro Mouse Cytokine 23-Plex Panel kit (Bio-Rad Hercules, CA, USA) by using a Bio-Plex 200 System according to the manufacturer's manual.

### 5.3.8 Statistical analysis

Results were expressed as mean ± SD. The significant differences between groups were analyzed by one way ANOVA tests, followed by Fisher's LSD and Duncan's multiple range tests with the SPSS statistical program (version 19.0, IBM Corp., Armonk, NY, USA). $^{*}P < 0.05$ and $^{**}P < 0.01$.

## 5.4 Results

### 5.4.1 LCBP improved histopathological features of NASH

The changes in body weight (BW) of mice were shown in Figure 5.1. HFD caused a significant increase ($P < 0.05$ at day 30, $P < 0.01$ at day 45) in the BW of mice. The BW of mice was declined after the injection of hepatotoxicant $CCl_4$. To evaluate the fat deposition in mice, the intra-abdominal fat/BW and liver/BW ratios were first measured. As shown in Figure 5.2A&B, HFD caused significant increase in the intra-abdominal fat/BW ratio but not liver/BW ratio, while HFD plus $CCl_4$ caused significant increase in both, as $CCl_4$ caused a decline in intra-abdominal fat and BW (Figure 5.1), but increased liver weight. Liver section then showed that HFD caused accumulation of lipid droplets and inflammatory cells (arrow) in the liver, while HFD plus $CCl_4$ caused not only accumulation of more lipid droplets and inflammatory cells, but also severe necrosis (arrow) (Figure 5.2C). Supplement with 0.5-1% of LCBP in the diet reduced intra-abdominal fat/BW ($P < 0.01$) and liver/BW ($P < 0.05$) ratios, and markedly ameliorated hepatic fat accumulation, inflammatory cell infiltration, and necrosis.

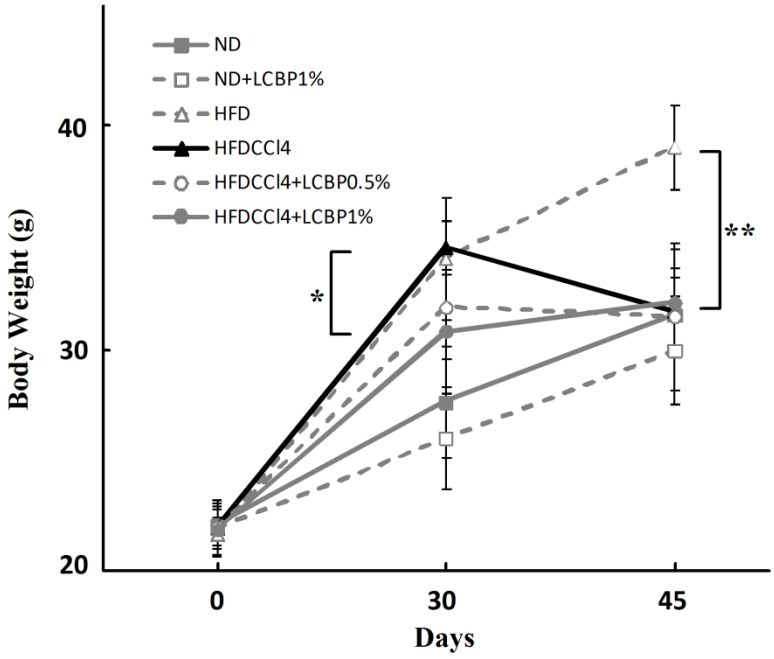



**Figure 5.1 The changes in body weight of mice.** The data represent mean ± SD of four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$.

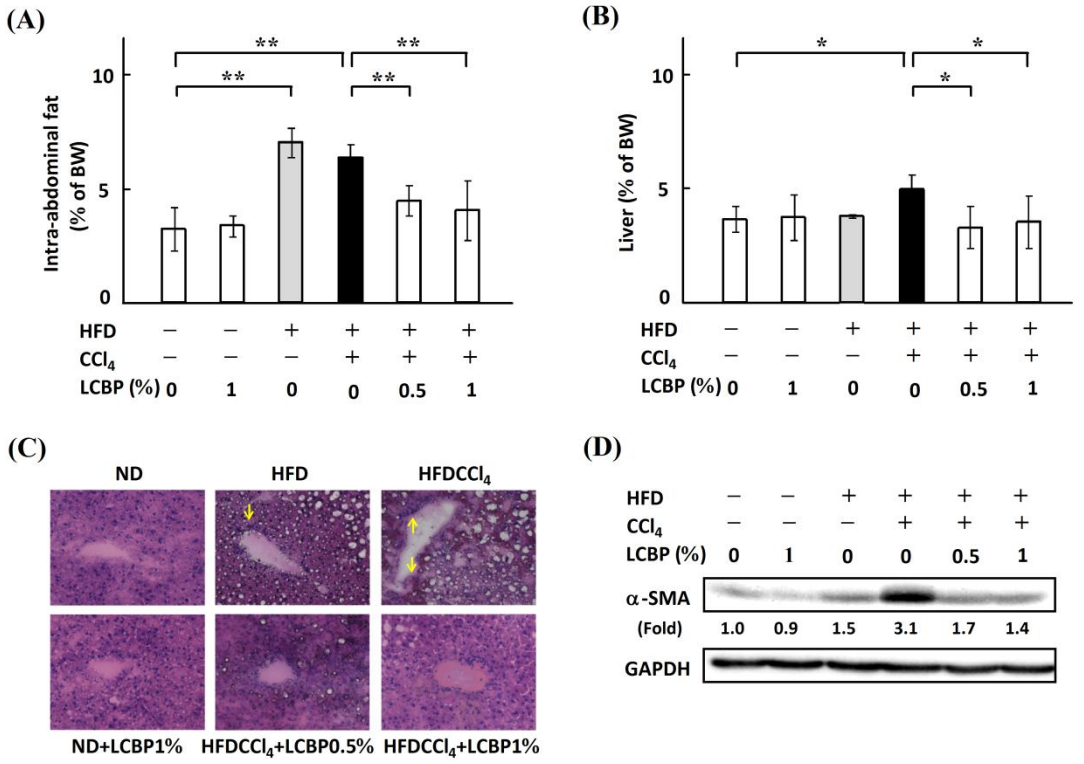



**Figure 5.2 LCBP improved histopathological features of NASH. (A)** The weight percentage of fat to BW. **(B)** The weight percentage of liver to BW. **(C)** Representative liver sections from each group (H&E stain, original magnification ×200). **(D)** The representative blot of α-SMA protein in

liver by western blotting. The induction fold of α-SMA was calculated as the intensity of the treatment relative to that of control normalized to GAPDH by densitometry. The data represent mean ± SD of four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$.

To investigate the degree of fibrosis, the expression of α-SMA, an indicator of fibrosis, was further detected in mice liver. The results showed that the expression of α-SMA was raised to 1.5 ($P < 0.01$ *vs* ND) and 3.1 ($P < 0.01$ *vs* ND) folds in HFD and HFDCCl$_4$ group, but decreased to 1.7 ($P < 0.01$ *vs* HFDCCl$_4$) and 1.4 ($P < 0.01$ *vs* HFDCCl$_4$) folds by supplementing with 0.5% and 1% of LCBP, respectively. As a sample control, treatment with 1% of LCBP alone showed no significant influence on the normal mice (Figure 5.2D).

### 5.4.2 LCBP decreased serum transaminases, lipids, and insulin/ glucose ratio

Serum transaminases and lipids are useful indicators of liver damage, thus, their concentrations were measured in the serum of mice. As shown in Figure 5.3, serum levels of GOT (A), GPT (B), and GGT (C) were increased ($P < 0.01$) in HFDCCl$_4$ group but not HFD group, and dose-dependently reduced by supplementing with 0.5-1% of LCBP. Analysis on serum lipids then revealed that the level of TG (Figure 5.3D) was increased ($P < 0.01$) in both HFD and HFDCCl$_4$ group, while the T-cho/HDL-c ratio (Figure 5.3E), an index of insulin resistance syndrome (Lemieux et al., 2001), was increased ($P < 0.05$) in HFDCCl$_4$ group only. Supplement with LCBP decreased both the TG level ($P < 0.01$) and T-cho/HDL-c ratio ($P < 0.05$). Further analysis on serum levels of insulin and glucose revealed that the serum insulin/glucose ratio, an indicator of insulin sensitivity (Legro et al., 1998), was significantly increased ($P < 0.01$) in both HFD and HFDCCl$_4$ group, and decreased ($P < 0.01$) by LCBP (Figure 5.3F).

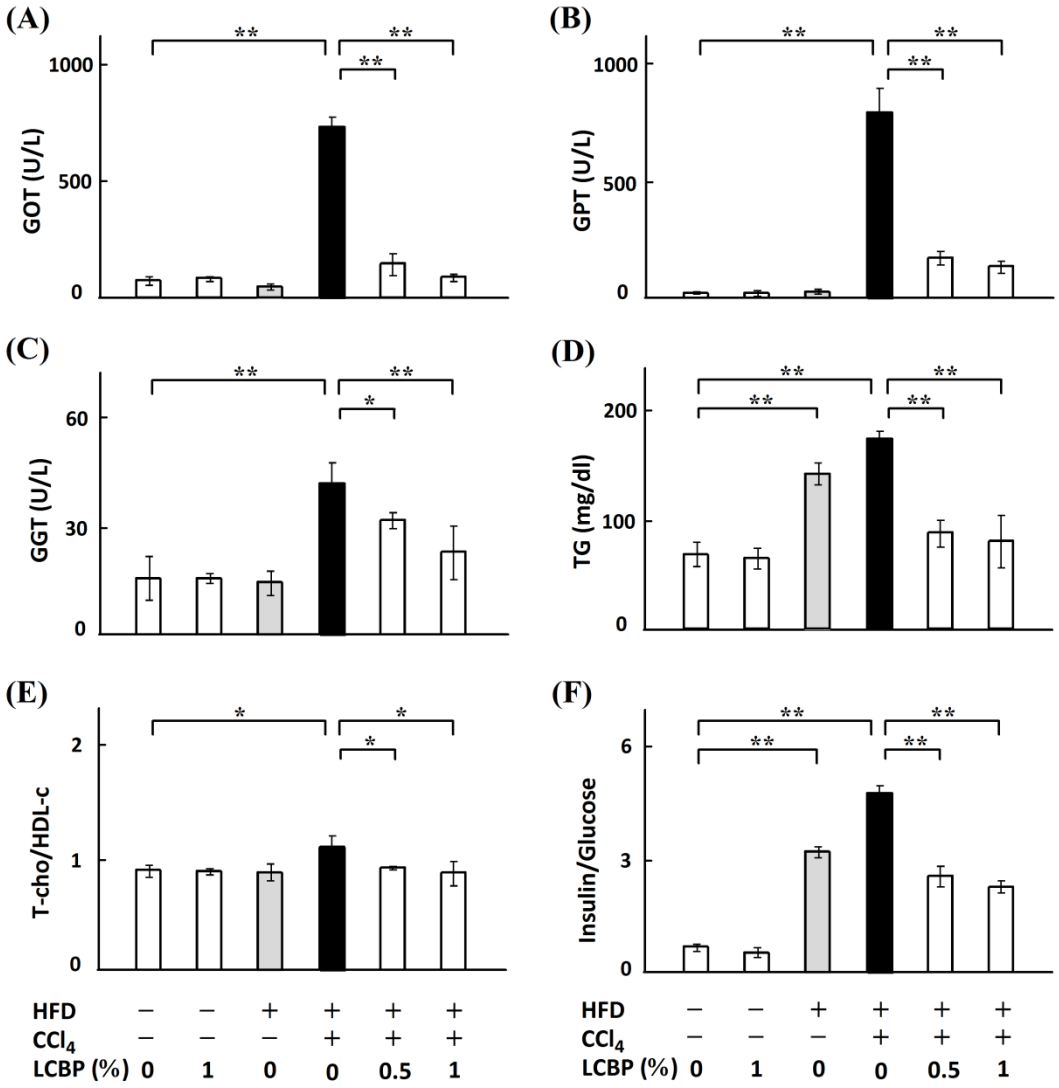



**Figure 5.3 LCBP decreased serum transaminases, lipids, and insulin/glucose ratio.** Serum transaminases, lipids, and glucose were measured by using an automated analyzer for clinical chemistry. Serum level of insulin was measured by ELISA. **(A)** Serum GOT level. **(B)** Serum GPT level. **(C)** Serum GGT level. **(D)** Serum TG level. **(E)** The ratio between T-cho (mg/dL) and HDL-c (mg/dL) in serum. **(F)** The ratio between insulin (mU/L) and glucose (mg/dL) in serum. The data represent mean ± SD of four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$.

### 5.4.3 LCBP decreased serum levels of multiple cytokines

As cytokines play a significant role in inflammation, twenty-three kinds of cytokines were determined in serum to understand the modulation of cytokine network by LCBP. As shown in Figure 5.4, serum levels of all the analyzed cytokines

except IL-9 and IL-12(p40) were increased in HFDCCl$_4$ group, while only KC, TNF-α, and IL-2 ($P < 0.05$) were increased in HFD group. Especially, the levels of G-CSF, IL-3, IL-4, MIP-1β, IL-6, IL-5, KC, TNF-α, IL-2, IL-1β, MCP-1, IL-13 and IFN-γ were raised to over two folds in HFDCCl$_4$ group. Supplement with 0.5-1% of LCBP in diet decreased the serum levels of G-CSF, IL-3, IL-4, MIP-1β, IL-6, IL-5, KC, TNF-α, IL-2, IL-1β, MCP-1, IL-13, IFN-γ, IL-10, IL-12(p70), IL-1α, eotaxin, GM-CSF and MIP-1α ($P < 0.01$), as well as IL-17 and RANTES ($P < 0.05$), but showed no significant influence on IL-9 and IL-12(p40) (data not shown).

### 5.4.4 LCBP improved hepatic antioxidant capacity

Oxidative stress is another critical factor in the pathogenesis of NASH (Rolo et al., 2012). To evaluate the oxidative status in liver, the activity of SOD and the level of TBARS were measured. As shown in Figure 5.5A, SOD activity was decreased ($P < 0.01$) in HFDCCl$_4$ group, but recovered by supplementing with 1% of LCBP. On the contrary, the level of TBARS, the product of lipid peroxidation, was increased in both HFD ($P < 0.05$) and HFDCCl$_4$ ($P < 0.01$) group, and dose-dependently decreased by 0.5-1% of LCBP (Figure 5.5B).

Further determination of the oxidative stress response proteins in liver showed that the expression of Nrf2, a key transcription factor that regulates the expression of antioxidant proteins, was decreased to 0.6 ($P < 0.01$ *vs* ND) and 0.5 ($P < 0.01$ *vs* ND) folds in HFD and HFDCCl$_4$ group, but recovered to 0.7 ($P < 0.01$ *vs* HFDCCl$_4$) and 0.9 ($P < 0.01$ *vs* HFDCCl$_4$) folds in the group treated with 0.5% and 1% of LCBP, respectively (Figure 5.5C, 1$^{st}$ band). Potential downstream targets including MnSOD and HO-1 were then detected, and the expression of MnSOD (Figure 5.5C, 2$^{nd}$ band) showed similar trend as Nrf2. On the other hand, the expression of HO-1 was reduced to 0.6 ($P < 0.01$ *vs* ND) folds in HFD group, but increased to 1.8 ($P < 0.01$ *vs* ND) folds in HFDCCl$_4$ group (Figure 5.5C, 3$^{rd}$ band). Thus, I investigated the expression of FoxO1, another transcription factor that regulates HO-1 (Kang et al., 2014). The results revealed that the level of FoxO1 was also increased to 1.8 ($P < 0.01$ *vs* ND) folds in HFDCCl$_4$ group (Figure 5.5C, 4$^{th}$ band). Supplement with 0.5-1% of LCBP

recovered the expression of Nrf2 and MnSOD, but decreased the expression of HO-1 and FoxO1 significantly ($P < 0.01$).

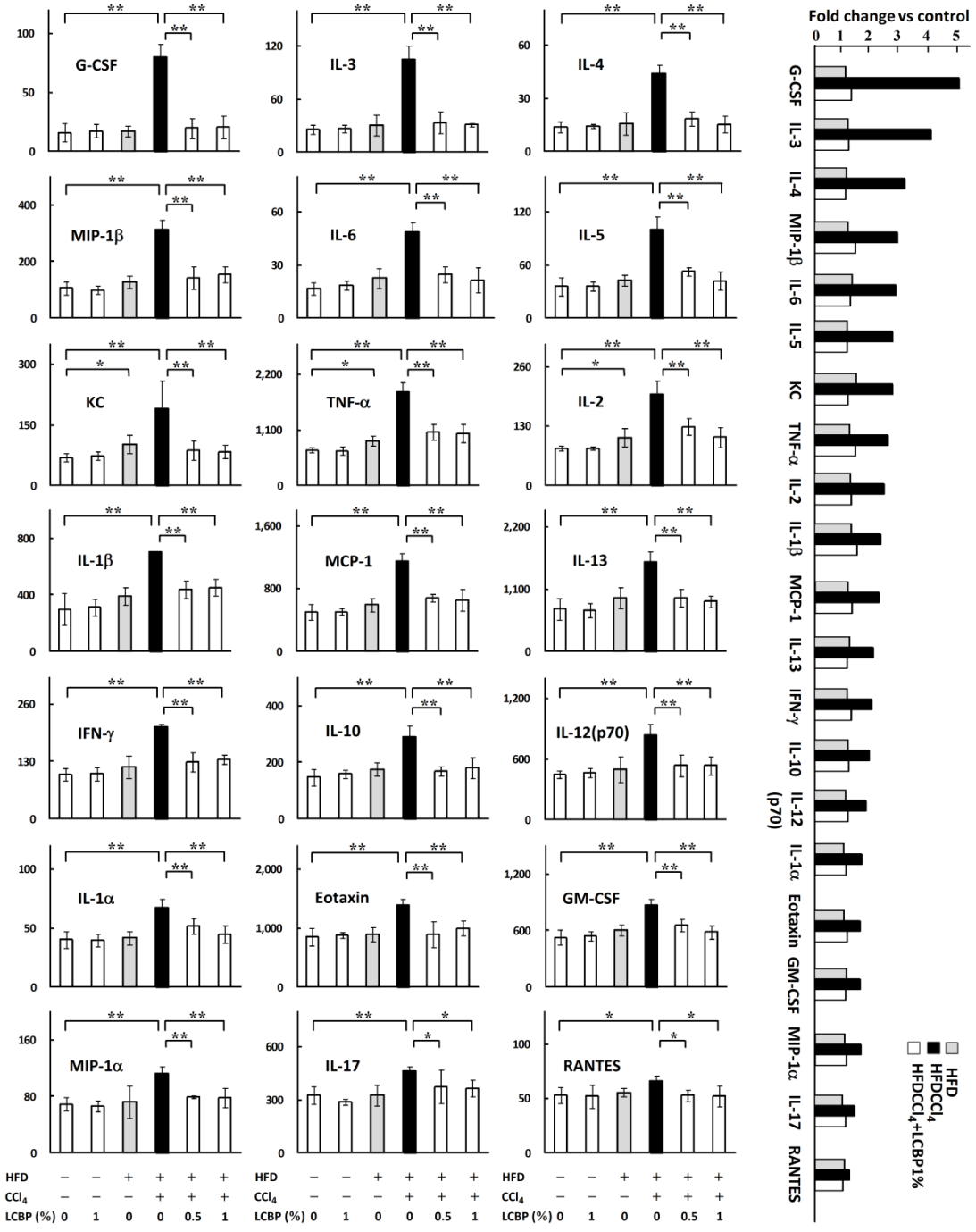



**Figure 5.4 LCBP decreased serum levels of multiple cytokines.** The levels of 23 kinds of cytokines were measured by multiplex technology, and arranged in an order from high to low change in the experimental NASH model. The data represent mean ± SD of four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$.

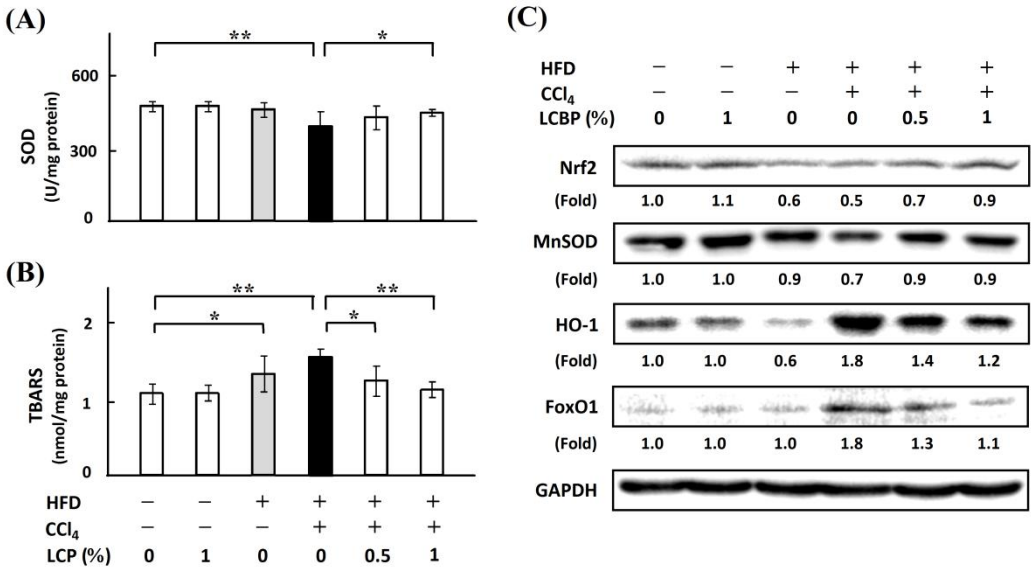



**Figure 5.5 LCBP improved hepatic antioxidant capacity. (A)** SOD activity in liver. **(B)** The level of TBARS in liver. **(C)** The representative blots of Nrf2, MnSOD, HO-1, and FoxO1 protein in liver by western blotting. The induction folds of the proteins were calculated as the intensity of the treatment relative to that of control normalized to GAPDH by densitometry. The data represent mean ± SD of four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$.

## 5.5 Discussion

An appropriate model is important in order to study the pathogenesis and prevention of NASH. In this study, HFD-fed mice became obese with fatty liver but no steatohepatitis, while the mice received two-hit by HFD plus $CCl_4$ administration showed histopathological features of NASH including fatty liver, hepatocyte injury, inflammation, and fibrosis.

In the progression of NASH, cytokines not only provoke the local inflammatory response in the liver, but also induce inflammatory cells infiltration to promote fibrosis. In this study, HFD plus $CCl_4$ increased the production of twenty-one kinds of cytokines, and the levels of G-CSF, IL-3, IL-4, MIP-1β, IL-6, IL-5, KC, TNF-α, IL-2, IL-1β, MCP-1, IL-13 and IFN-γ were increased to over two folds. Among the cytokines, G-CSF and IL-3 promote proliferation and differentiation of hematopoietic stem cells in response to liver injury (Nitsche et al., 2003; Spahr et al., 2008), and

IL-4, IL-5, and IL-13 are considered as the anti-inflammatory cytokines against excessive inflammation and self-immunity (Fort et al., 2001). IL-6, TNF-α, IL-2, IL-1β, and IFN-γ might be the potential primary proinflammatory cytokines in the pathogenesis of NASH, as chemokines including MIP-1β, KC, and MCP-1 were potentially mediated by IL-1β (Zhang et al., 2003), TNF-α (Lo et al., 2014), and IL-6 (Tieu et al., 2009), respectively. Dietary supplementation of LCBP decreased both pro- and anti-inflammatory cytokines, which suggesting that LCBP ameliorated NASH by decreasing the production of proinflammatory cytokines, rather than promoting the production of anti-inflammatory cytokines.

Oxidative stress is considered as another important factor in the pathogenesis of NASH (Rolo et al., 2012). In this study, HFD and $CCl_4$ decreased the expression of antioxidant proteins (SOD, Nrf2, and MnSOD) and enhanced lipid peroxidation (TBARS) in the liver. Interestingly, HO-1, one of the downstream antioxidant defense proteins of Nrf2, was decreased in HFD-fed mice but increased in the mice administrated with HFD plus $CCl_4$. Although HO-1 has been reported to contribute to ameliorate obesity and fatty liver (Hinds et al., 2014), a recent study suggested that HO-1 actually drives metaflammation and insulin resistance in metabolic disease (Jais et al., 2014). FoxO1 is proved as another regulator of HO-1 (Kang et al., 2014), and it is activated in insulin receptor substrate-knockout mice (Qi et al., 2013). The overexpression of FoxO1 and HO-1 can further aggravate insulin resistance and promote the production of proinflammatory cytokines (Jais et al., 2014; Su et al., 2009). The data in this study revealed that FoxO1 was increased markedly in the mice administrated with HFD plus $CCl_4$, but not HFD only. These results suggested that HO-1 was possibly reduced due to the low level of Nrf2 in the early stage of obesity, but increased with the overexpression of FoxO1 induced by metabolic dysfunction in NASH. LCBP increased the expression of Nrf2 and MnSOD, but decreased the expression of FoxO1 and HO-1, and further ameliorated the lipid peroxidation and insulin resistance in NASH mice.

## 5.6 Conclusion

In conclusion, the present study demonstrated that dietary supplementation of LCBP ameliorated inflammation, lipid peroxidation, and insulin resistance in the experimental NASH model by suppressing proinflammatory cytokines production, up-regulating the expression of Nrf2 and MnSOD, and down-regulating the expression of FoxO1 and HO-1 (Figure 5.6).

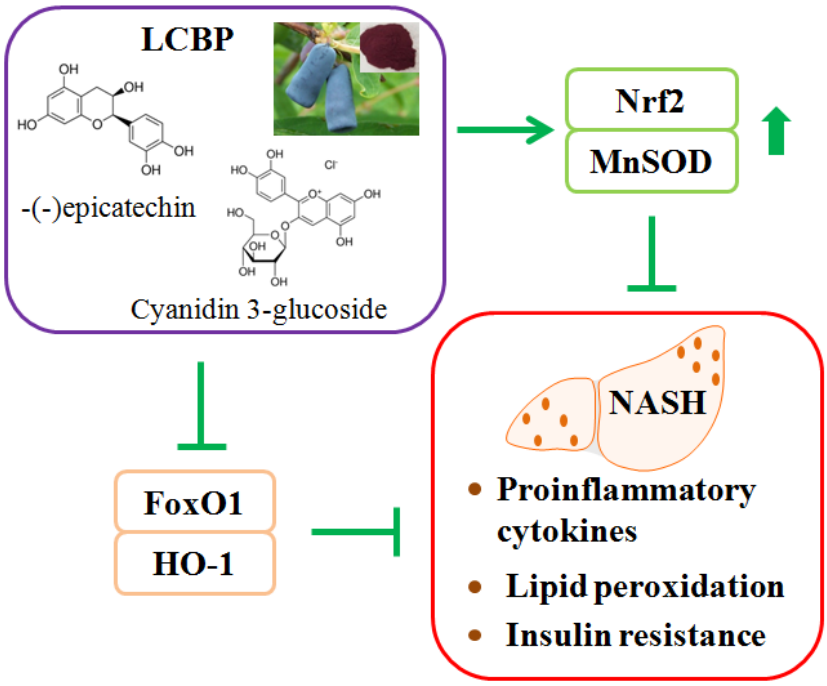



**Figure 5.6 LCBP showed the protective effect against experimental NASH in mice.**

# General conclusion

This study demonstrated that *Lonicera caerulea* L. berry polyphenols (LCBP) attenuated inflammation *in vitro* and *in vivo* by inhibiting the production of multiple proinflammatory factors (cytokines, NO and $PGE_2$) and up-regulating antioxidant capacity.

*In vitro* studies revealed that LCBP inhibited LPS-induced production of proinflammatory mediators at least partly through the down-regulation of TAK1-mediated MAPK and NF-κB inflammatory signaling pathways. Moreover, LCBP up-regulated the expression of Nrf2 and MnSOD, and thus enhanced the antioxidant ability against LPS-induced oxidative stress and proinflammatory response. C3G and EC were identified to have similar antioxidant and anti-inflammatory properties as LCBP, and capable to bind to TAK1 protein directly.

*In vivo* studies indicated that oral administration of LCBP could suppress the production of multiple proinflammatory cytokines, and enhance the activity of antioxidant enzymes, to suppress inflammation in both LPS-induced rapid paw edema model, and adjuvant-induced chronic arthritis model. The cross-effect between the antioxidant and anti-inflammatory activities of LCBP was further studied in an experimental NASH model, in which LCBP ameliorated inflammation and lipid peroxidation by suppressing the production of proinflammatory cytokines, up-regulating the expression of Nrf2 and MnSOD, and down-regulating the expression of FoxO1 and HO-1.

Taken together, LCBP attenuated inflammation through a functional crosstalk regulation of antioxidant and anti-inflammatory systems, which suggesting that LCBP is a potential chemopreventive agent for chronic inflammation-related diseases.

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