Extract from Mulberry (*Morus australis*) leaf decelerate acetaminophen induced hepatic inflammation involving downregulation of myeloid differentiation factor 88 (MyD88) signals

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**Abstract**

Acetaminophen (APAP) induced inflammation and oxidative stress can cause cell death to induce liver damage. The antioxidative and anti-inflammatory effect of Mulberry (*Morus australis*) leaf extract (MLE) was shown in previous studies. In this study, we investigated the modulation of MLE on APAP induced inflammation and oxidative stress in rat liver injury or liver cancer cell (HepG2). Wistar rat was fed orally with MLE (0.5% or 1.0 %) for 1 week, and then, 900 mg/kg of APAP was injected intraperitoneally (i.p.). Pretreatment of MLE decreased obvious foci of inflammatory cell infiltration in liver. It also reduced the expression of inflammatory parameters including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and nuclear factor kappa B (NF-κB) in liver. Treating with MLE increased the antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase. Giving APAP to HepG2 hepatocyte was conducted to elucidate the mechanism of MLE or its functional components. The result showed that APAP upregulated hepatic protein expression of (myeloid differentiation factor 88) MyD88, nuclear factor kappa B (NF-kB), inhibitor of kappa B (IκB), c-Jun N-terminal kinases (JNK), and receptor interacting proteins (RIP1 and RIP3). Pretreatment of MLE, gallic acid (GA), gallocatechin gallate (GCG), or protocatechuic acid (PCA) suppressed the indicated protein expression. These findings confirmed that MLE has the potential to protect liver from APAP-induced inflammation, and the protecting mechanism might involve decreasing oxidative stress and regulating the innate immunity involving MyD88.

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1. Introduction

Hepatic damage is caused by an inappropriate or excessive stimulation from viruses, drugs, alcohol, autoimmune reaction, or metal ion deposition. These factors can cause acute or chronic injuries and lead to the accumulation of an extracellular matrix and scarring in the liver. Acetaminophen (N-acetyl-p-aminophenol/paracetamol, APAP) is the most common oral medication to alleviate pain and fever. However, an overdose of APAP can induce severe hepatotoxicity which leads to liver injury in many animals including humans [1]. In individuals, APAP is majorly metabolized with glucuronic acid and sulfate, and a small part of it is metabolized by cytochrome P-450. Cytochrome P-450 oxidizes APAP to form a chemically reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) [2]. This metabolite reacts with glutathione (GSH) to form an acetalaminophen–GSH complex. Once GSH is depleted, NAPQI binds to a number of mitochondrial proteins that might lead to hepatocellular death [3]. In addition to elimination of antioxidative molecules, APAP inhibits mitochondrial oxidative phosphorylation and then exhausts adenosine triphosphate (ATP) in mouse hepatocytes [4]. Due to the elevated oxidative stress from APAP, the release of monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, and tumor necrosis factor (TNF)-α would increase to evoke hepatotoxicity [5]. Based on them, the agent possessing the ability to increase GSH or lower inflammatory cytokines and chemokines may have the potential to ameliorate the APAP-induced liver damage.

Toll-like receptors (TLRs) involved in innate immunity are recognized for responding immunomodulation. The activation of TLR-3 and TLR-4 was reported to stimulate the production of adaptor proteins, such as MyD88 and nuclear factor kappa B (NF-κB), then the generation of oxidants and inflammatory cytokines and chemokines was promoted in APAP induced liver injury [6,7]. In these signaling networks, receptor interacting proteins (RIPs) also play a critical role to activate NF-κB and drive cell necroptosis [8]. They would trigger liver cells to apoptosis or necrosis and make liver degenerate gradually. An et al reported that overdose of APAP led to induction of RIP-dependent signaling with subsequent JNK activation, it can serve as a critical switch for hepatocellular necrosis [9].

A huge number of various phenolic substances present in dietary and medicinal plants have striking antioxidative and antiinflammatory properties, they are the secondary metabolites of plants that are classified by their structure as phenolic acids derivatives, flavonoids, stilbenes, or lignans [10]. Mulberry (Morus australis) leaf is a form of herbal tea traditionally used as oriental medicine in Southeast Asia and Taiwan. The aqueous extract of Mulberry leaf (MLE) contains abundant polyphenols including gallic acid (GC), protocatechuic acid (PCA), catechin, galloacetechin gallate (GCG), caffeic acid, epicatechin, rutin, resveratrol, and quercetin [11]. Most of the constituents have antioxidant properties and bioactivity [12]. Recently, some studies revealed that 1-deoxynojirimycin (1-DNJ) in mulberry leaf extract has antidiabetic effects via reducing postprandial blood glucose, inhibiting α-glucosidase, or decreasing serum triglyceride [13]. Additionally, biological effects of MLE are presented in diabetes [14], rheumatic arthritis, neurodegenerative diseases, atherosclerosis [15], and aging [16]. Although there have been reports regarding the mechanisms of action of polyphenols responsible for their antiinflammatory action in other types of natural products, the knowledge regarding immunomodulatory action of MLE is still limited. Park et al [17] showed that MLE can prevent lipopolysaccharide-induced inflammation by decreasing the expression and activity of COX-2 and/or downregulating the transcription factor NK-κB. However, the impact of MLE upon APAP induced hepatic inflammatory reactions and signaling pathways remains known. Our previous study revealed that MLE accelerated the progression of atherosclerosis via regulating oxidative status, proliferation of smooth muscle cell, and lipid accumulation of hepatic cells [18]. Thus, it would be possible that MLE intake or supplement might affect the liver function, and further enhance hepatic defensive capability to prevent or attenuate liver injury.

This study aimed to examine the protective effects and modulatory mechanisms of MLE upon liver of APAP treated rat or heptocytes. The influence of MLE in antioxidation, antiinflammation, and protein expression of associated factors such as NF-κB, MyD88, RIPs, and JNK was evaluated. Moreover, histological analysis was performed to provide more evidence to support the benefit of MLE playing a hepatic protective role.

2. Materials and methods

2.1. Preparation of MLE and high performance liquid chromatography analysis

Mulberry (Morus australis) leaf was obtained from the Miaoli District Agricultural Research and Extension Station in Gongguan Township, Miaoli County, Taiwan. MLE was prepared from the dry leaf of mulberry. One hundred grams of the leaf was added to 300 mL of methanol and heated in a water bath at 50°C for 3 hours. After repeating for three times, 900 mL of the collected filtrate was vacuum-condensed to obtain the MLE stored at –20°C before use. MLE was dissolved in 500 mL of double distilled H₂O and added to 200 mL of n-hexane to collect the water layer overnight. Then, 180 mL of ethyl acetate was added to extract the polyphenols for analysis by using high performance liquid chromatography (HPLC). The yield of polyphenol extract was approximately 1.2%. According to the present and previous analysis, the constituents of polyphenol in MLE contains approximately 23.6 % of polyphenols (gallic acid as a standard), such as gallic acid, protocatechuic acid, catechin, galloacetechin gallate (GCC), caffeic acid, epicatechin, rutin, quercetin, and naringenin as shown in Table 1 [11,12].

2.2. Animals and experimental design

All animal experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of the Chung Shan Medical University (IACUC, CSMU,
Table 1 – Composition of polyphenols separated from MLE and applied in animal treatment.

| Polyphenols in MLE (%, w/w) | 0.5% of MLE in diet (mg) | 1.0% of MLE in diet (mg) |
|-----------------------------|--------------------------|--------------------------|
| Gallic acid                 | 7.64                     | 8.88                     | 17.76                     |
| Gallocatechin gallate       | 5.88                     | 7.06                     | 14.12                     |
| Protocatechuic acid         | 4.69                     | 5.63                     | 11.26                     |
| Naringenin                  | 2.67                     | 3.20                     | 6.40                      |
| Rutin                       | 1.87                     | 2.24                     | 4.48                      |
| Quercetin                   | 1.24                     | 1.49                     | 2.98                      |
| Catechin                    | 1.20                     | 1.44                     | 2.88                      |
| Caffeic acid                | 1.02                     | 1.22                     | 2.44                      |
| Epicatechin                 | 0.80                     | 0.96                     | 1.92                      |

MLE = Mulberry (Morus australis) leaf extract; w/w = g/100 g
a MLE contains about 23.6% of polyphenols under gallic acid as a standard[18].
b The yield of polyphenol extract was approximately 1.2%.
c Food intake of each rat averaged 20 g/d.

2.5. Detection of superoxide dismutase

Forty μL of liver homogenate was mixed with 1mM of EDTA and 96 μL of Triton X-100, then centrifuged at 10,000g, 4°C for 5 minutes. One hundred micro liters of supernatant was reacted with 0.1 mL of freshly prepared 2.6mM pyrogallol solution in the assay mixture. The assay mixture was read and the rate of increase in the absorbance at 325 nm was recorded for 3 minutes after an initial lag period of 30 seconds in a UV/Visible spectrophotometer (Hitachi Co., Tokyo, Japan). The activity of superoxide dismutase (SOD) was presented as unit/mg protein.

2.6. Catalase assay

Catalase activity in liver homogenates was assayed according to a previous method [19]. The concentration of protein was determined using Bio-Rad protein assay kit and adjusted to 50 mg/mL. Twenty μL of homogenate was added to 980 μL of H2O2 solution (containing 30 μL of ddH2O, 50 μL of 1M Tris-HCl, and 5mM EDTA, pH 8.0 and 900 μL of 10mM H2O2). After 10 seconds at room temperature, the optical density of H2O2 was recorded at 240 nm for 1 minute. The linear portion was used to calculate the catalase activity. A unit of catalase activity was defined as H2O2 consumed units/mg protein.

2.7. Determination of glutathione peroxidase

One hundred micro liters of liver homogenate was added to 800 μL potassium phosphate buffer containing GSH. After mixing and reacting for 5 minutes at room temperature, 100 μL of ddH2O was mixed to start the reaction. We then monitored the optical density at 340 nm within 3 minutes. A unit of glutathione peroxidase (GSH-Px) activity was defined as GSH consumed units/mg protein.

2.8. Cell culture

Liver cancer cell (HepG2) was obtained from BCRC (Hsinchu City, Taiwan). The cells were cultured in a minimal essential medium (GIBCOBRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and antibiotics (100 IU/mL each of penicillin G and 100 streptomycin).

2.9. Western blot analysis

The liver tissue or cell was homogenized in phosphate buffer containing proteinase inhibitors. The homogenates were centrifuged at 12000 g at 4°C for 10 minutes. Protein concentrations of the supernatants were measured using the Bradford assay. Up to 50 μg of proteins were separated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with phosphatebuffered saline (PBS) containing 5% nonfat milk. Thereafter, the membranes were incubated with antibodies against iNOS, COX-2, NF-κB, MyD88, JNK, STAT-3, p-STAT-3, RIP-1, or RIP-3, Actin (Santa Cruz Biotechnology,
Dallas, Texas, USA) at 4°C overnight. The membranes were washed with PBS containing 0.1% Tween-20, followed by incubation with a horse radish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, Texas, USA). The signals were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA), the intensities were determined through densitometry by using Fujifilm Multi Gauge (Version 2.2) software (Fujifilm Co., Tokyo, Japan).

2.10. Statistical analysis

The results are represented as mean ± standard deviation of three determinations. Statistical differences were analyzed using Student’s t test or one-way analysis of variance by Sigma Stat 4.0; the differences were considered to be significant when p < 0.05.

3. Results

3.1. MLE decreased plasma ALT, AST and ALP in APAP treated rat

MLE intake at 1.0 % did not affect body weight, feed intake, water intake, and liver weight (data not shown). Plasma levels of ALT, AST, and ALP are represented in Figure 1. After comparing the normal range of AST (126 ± 36 U/L), ALT (59 ± 8 U/L), and ALP (337 ± 69 U/L), the pretreatment of MLE at both doses alleviated subsequent APAP-provoked ALT, AST and ALP levels in plasma (p < 0.05).

3.2. MLE decreased neutrophil infiltration in APAP treated rat

Histological data showed that APAP treatment led to centrilobular necrosis and severe neutrophil infiltration in liver as the arrows pointed area (Figure 2). MLE uptake at 0.5% and 1.0 % reduced hepatic infiltration by neutrophil, in which 1.0 % MLE treatment showed better antiinflammatory effects than 0.5% MLE.

3.3. MLE ameliorated inflammatory and oxidative stress in APAP treated rat

As shown in Figure 3, APAP treatment significantly increased the expression of COX-2, iNOS, and NF-κB (p < 0.05); but MLE intake decreased the expression of inflammatory molecules. APAP decreased GSH-Px, activity of SOD, and catalase in liver (p < 0.05); however, preintake of MLE reprovoked GSH-Px activity and reversed the activity of SOD and catalase (p < 0.05; Figure 4).

Figure 1: MLE reduces the level of liver injury in APAP-treated Wistar rat. Rats were administered with 0.5% or 1% of MLE for 1 week before 900 mg/kg of APAP i.p. Twenty four hours later, plasma was collected to detect (A) AST, (B) ALT, and (C) ALP. The results are presented as mean ± SD from 12 Wistar rats per group. " p < 0.01 compared with control group. * p < 0.05 or ** p < 0.01 compared with APAP group. ALP = alkaline phosphatase; ALT = alanine transaminase; APAP = acetaminophen; AST = aspartate transaminase; i.p. = intraperitoneal; MLE = Mulberry (Morus australis) leaf extract; SD = standard deviation.
3.4. MLE regulated signaling pathways in APAP treated rat

APAP upregulated hepatic protein expression of p-STAT, p-JNK, and MyD88 (Figure 5, p < 0.05). Pretreatment of MLE at both doses downregulated the expression of NF-κB p65 and p-JNK (p < 0.05).

3.5. MLE and partial of its constituents regulates signaling molecules involving in innate immunity in APAP-treated HepG2 hepatocyte

To explore the potential mechanism of constituents in MLE, gallic acid (GA), protocatechuic acid (PCA), or gallocatechin gallate (GCG) were treated to HepG2 cell accompanied with APAP. APAP raised the level of p-IκB, IκB, and NFκB in cell; 100 mg/mL of MLE decreased the level of indicated proteins. Ten mM of PCA reduced the expression of p-IκB dominantly, and GCG suppressed both of IκB and NFκB (Figure 6). APAP treatment significantly increased MyD88 protein expression; and MLE, GA, PCA, or GCG reduced this protein expression. APAP elevated the level of RIP1 and 3, MLE, GCG, or PCA lowered both proteins. APAP treatment enhanced the expression of caspase 8, p-STAT3, and p-JNK; however, in addition to MLE, GCG or PCA decreased the expression (Figure 6).

4. Discussion

Acetaminophen (AAP) is an analgesic and antipyretic drug but can cause liver injury in high doses [20]. Due to the metabolite from APAP evoking inflammatory and oxidative stress in liver, Bajt et al [21] reported that an oxidant stress precedes cell injury in mice hepatocytes exposed to AAP. Researchers demonstrated the beneficial actions of MLE on oxidative stress and inflammation in clinical diseases [22]. In the present studies, MLE was revealed to prevent APAP-induced inflammation via decreasing the expression of COX-2, iNOS, and NFκB. Park et al [17] reported that MLE preincubated with in Raw264.7 cell inhibited IκBα phosphorylation and reduced NFκB activity, it implied that MLE can be used as an antiinflammatory agent to inhibit NFκB-mediated inflammatory response. Chan et al [12] revealed that MLE decelerated the migration of vascular smooth muscle cell partially via suppressing the expression of NFκB. The studies imply that NFκB might be a regulatory mode in MLE attenuating APAP-induced liver inflammation. In addition, our histological results indicated that MLE improved inflammation caused by APAP in rat as well as reversed the functional parameters (ALT, AST, and ALP) in liver. These findings confirm that MLE has the potential in hepatic protection.

Reactive metabolites generated from the APAP metabolism are found to be highly electrophilic that can attack the cellular macromolecules. It explained that APAP at high dose led to elimination of SOD, catalase, and GSH-Px [23]. Some of the natural products or antioxidants possessing the ability to increase the level of GSH and catalase activity are shown to ameliorate AAP-induced liver toxicity. Ajith et al [24] reported that giving aqueous extract of Zingiber officinale Roscoe enhanced the level of SOD, catalase, GSH, GST, and GSH-Px in liver from APAP-treated rat. Reports had revealed...
that silimar in possessing antioxidant proterties protects against APAP-induced liver damage via scavenging the superoxide anion and alkoxyl radicals [25]. Ghosh et al [23] showed that taurine treatment was effective in countering APAP-induced hepatic damages, oxidative stress, and cellular necrosis accompanying with suppressing JNK-dependent cell death pathway, it exhibited therapeutic potentials against APAP-induced hepatic injury. In addition to decreasing the expression of COX-2 and iNOS, our data revealed that MLE increased the level of SOD, catalase, and GSH-Px as well as the previous studies to act as a liver-protection agent.
APAP overdose activated NF-κB and JNK pathways through stimulating ROS generation [26]. The researchers indicated that TLR-3 activation enhanced JNK phosphorylation in APAP injured livers [6,7]. The activation of these signal pathways promoted downstream inflammatory and oxidative reactions in liver and enhanced the production of cytokines and chemotactic factors including IL-6, TNF-alpha, or MCP-1 [5]. The inhibition upon TLR-3, NF-κB, and/or JNK was shown to be a strategy for alleviating APAP-induced hepatotoxicity. Previous studies revealed that APAP-induced oxidative stress can cause cell death. In the initiation of cell death, the activation of JNK (phosphorylated JNK, pJNK) occurred in the AAP-induced liver toxicity. The inhibition of JNK results in promoting survival and reducing cell death, but postponing the inhibition of JNK results in decreased cell death [27]. We found that pretreating with MLE already reduced hepatic oxidative stress via its antioxidative activities, which definitely led to less stimulation for hepatic NF-κB activation and JNK phosphorylation. The immunoblot data regarding hepatic protein expression of NF-κB, IκB and p-JNK agreed that MLE intake at both doses limited the activation of these two signal pathways.

TLRs recognize endogenous damage-associated molecular patterns during inflammatory reactions and play crucial roles in the pathological progression of inflammatory liver diseases [6,7]. The binding of endoligands such as host cellular mRNA released from damaged cells to TLR-3 and/or TLR-4 enhances the expression of adaptor proteins including NF-κB, IκB and p-JNK agreed that MLE intake at both doses limited the activation of these two signal pathways. Additionally, RIPs involved in the mechanism to regulate NF-κB in inflammation. They would also modulate caspase 8 to trigger hepatocyte to apoptosis or impair mitochondrion to cause necroptosis under the stimulation of APAP. The previous study provided in vivo antiinflammatory data of Boswellic acid in mediating
activity to TLRs and MyD88 [29]. Our present data agreed that NF-κB, JNK, RIP1, RIP3, and MyD88 were involved in APAP caused hepatotoxicity. We further found that MLE downregulated hepatic expression of adaptor proteins such as MyD88, NF-κB, and JNK mediated inflammatory response; and also weakened RIPs. Although our current findings indicates the possible relevance of MLE in elevating the antioxidative status and reducing inflammatory response to protect people from APAP caused hepatotoxicity, it should be further elucidated which constituent is the major contributor in decelerating APAP-induced liver injury and the relative mechanisms involved. As shown in Figure 6, PCA or GCG treated to HepG2 cell accompanied with APAP reduced the expression of p-IκB, IκB, NF-κB, MyD88, RIP1, RIP3, caspase 8, p-STAT3, and p-JNK. At present, the effect of MLE in decelerating APAP-induced oxidative and inflammatory injury should be partially contributed by three major components (GA, PCA, and GCG) existing in MLE. A previous study indicated that 100 mg/kg of GA (body weight, i.p.) can reverse the APAP-elevated inflammation and lipid peroxidation, and, some nature products containing GCG or PCA can decelerate the APAP-caused liver damage [30]. All of them exhibit cross-reactions and have inhibitory effects on a variety of Phase II conjugative reactions including sulfation, glucuronidation, and acetylation. The present result in our study only revealed that they are involved in regulating the relative signaling pathway independently, but some conduction needed to be elucidated further.

Figure 5 – MLE inhibits inflammatory signals in APAP-treated Wistar rat. Protein expression was detected by Western blot analysis. Actin, as a loading control. The bottom panels represent quantification of the immunoblot. The values were represented as the mean ± SD of at least five independent experiments. * p < 0.01 compared with control group. * p < 0.05 or ** p < 0.01 compared with APAP group. APAP = acetaminphen; JNK = c-Jun N-terminal kinases; MLE = Mulberry (Morus australis) leaf extract; SD = standard deviation.
MLE pretreatment protected rat liver from subsequent acetaminophen-induced oxidative and inflammatory injury, it might be contributed by the constituents of MLE via improving hepatic oxidative status and suppressing protein expression of NF-κB, JNK, MyD88, RIP1, and RIP3. These findings confirm that MLE was a potent hepatic protective agent.

Conflicts of interest

The authors declare no competing financial interest.

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