**Regular Article**

**Moutan Cortex Protects Hepatocytes against Oxidative Injury through AMP-Activated Protein Kinase Pathway**

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Moutan Cortex, the root bark of *Paeonia suffruticosa* Andrews in Ranunculaceae, has widely demonstrated analgesic, anti-spasmodic, and anti-inflammatory effects in various cancer and immune cell lines. Oxidative stress is associated with development of several diseases, including liver disease. We prepared the water extract of Moutan Cortex (MCE) to investigate the cytoprotective activities and its mechanism. MCE protected hepatocytes from arachidonic acid (AA)+iron induced oxidative stress, as indicated by reactive oxygen species (ROS) production and cell viability analysis. MCE also suppressed mitochondrial dysfunction in AA+iron-treated human hepatocyte-derived hepatocellular carcinoma cell line, HepG2 cells. In addition, MCE treatment induces AMP-activated protein kinase (AMPK) and liver kinase B1 phosphorylation, which play a role in inhibition of oxidative stress induced cell death. Moreover, one of the MCE compounds, chlorogenic acid, exerted protective effects against oxidative stress and apoptosis. Taken together, MCE protected hepatocytes against AA+iron-induced oxidative stress through AMPK activation, and may be a candidate for the treatment of liver disease.

Key words  AMP-activated protein kinase; Moutan Cortex; oxidative stress; liver injury; arachidonic acid; iron

The liver is the most important organ, consisting of variety cells, including hepatocytes, Kupffer cells, and hepatic stellate cells. The liver is also involved in detoxification by inhibiting oxidative stress. Hepatocytes play an important role in antioxidant effects in the liver. Oxidative stress is a common mechanism in cellular dysfunction, including injury and cell death. Tissue damage through induction of injury and inflammation is related to imbalance between reactive oxygen species (ROS) production and cellular antioxidant activity. ROS can also modify the phospholipid membrane through membrane fluidity and protein structure. Arachidonic acid (AA), a ω-6 polyunsaturated fatty acid that acts as a pro-inflammatory mediator, is liberated by phospholipase A2 (PLA2) activation through increased Ca2+ levels. AA produces lipid hydroperoxides as primary oxidation products via excess ROS and induces mitochondria dysfunction in the presence of iron. Furthermore, AA can be involved in the regulation of inflammation, cell migration, apoptosis, and platelet aggregation.

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that regulates lipid and glucose metabolism, such as biogenesis and uptake of glucose and β-oxidation of fatty acids. AMPK is activated by cellular stress that interferes with ATP production and consumption. AMPK is also activated by liver kinase B1 (LKB1) and calcium/calmodulin-dependent kinase (CamKK). Several studies have shown that AMPK is activated by small molecules to protect hepatocytes under oxidative stress conditions. Activation of AMPK can be important role in protecting against alcoholic fatty liver in vivo. Moreover, inhibition of AMPK induces lipid accumulation and decreases oxidized free fatty acids in the mitochondria, ultimately leading to increased production of ROS in hepatocytes.

Herbal medicines have recently used in treatment of a variety of diseases, including neurological disorders, cardiovascular disease, cancer, diabetes, obesity-related disorders, liver disease and lifespan extension. Herbal medicines can also be used as alternative therapeutic strategies for treatment of disease because they are a rich source of bioactivity with few side effects. Moutan Cortex (MC), the root bark of *Paeonia suffruticosa* Andrews in Ranunculaceae, is a natural component of traditional Korean herbal medicine known to exert analgesic, anti-spasmodic, and anti-inflammatory effects in rat synoviocytes and lipopolysaccharide (LPS)-activated raw 264.7 cells. Some studies have reported that MC protects against atherosclerosis, infection, inflammation, and other symptoms. It is worth noting that Moutan Cortex exerts anti-oxidant activity via inhibition of the production of ROS. However, anti-oxidant activity of MC against potential attenuation of AA and iron-induced oxidative stress in vitro and its mechanism of action have yet to be investigated.

In this study, we examined the anti-oxidant effects of Moutan Cortex Extract (MCE) on AA and iron-induced hepatotoxicity and demonstrated their hepatoprotective activities and...
mechanism against liver injury.

MATERIALS AND METHODS

Reagents  B-cell lymphoma-extra large (Bel-2-L), caspase-3, poly(ADP-ribose) polymerase (PARP), phospho-acetyl-CoA carboxylase (p-ACC), phospho-AMP-activated protein kinase (AMPK), phospho-liver kinase B1 (p-LKB1) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G ( IgG) antibodies were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies and RIPA lysis buffer were obtained from Thermo (Rockford, IL, U.S.A.). AMPK antibody was obtained from Santa Cruz Biotechnology Inc. (Sancturcz, CA, U.S.A.). Arachidonic acid (AA) and rhodamine123 (Rho123) were obtained from Calbiochem (San Diego, CA, U.S.A.). Methanol was obtained from Junsei (Tokyo, Japan). Acetoni trile was obtained from JT Baker (Center Valley, PA, U.S.A.). Standard compounds of Moutan Cortex and 2,7’-dichlorodihydrofluorescin diacetate (H$_2$DCF-DA), ferric nitrilotriacetic acid (Fe-NTA), compenicillin and 100 µg/mL streptomycin (Hyclone) at 37°C in a humidified atmosphere with 5% CO$_2$. Antibiotics were added to growth medium: 100 units/mL penicillin and 100 µg/mL streptomycin and 5% skim milk, the membranes were incubated with primary and secondary antibodies. The membranes were washed with TBS-T buffer and visualized with ECL Western blotting reagents (Amersham).

Measurement of H$_2$O$_2$ Production  H$_2$DCF-DA, a cell-permeable non-fluorescent probe, is cleaved by intracellular esterases and is turned into the fluorescent dichlorofluorescein upon reaction with H$_2$O$_2$. After treatment, cells were stained with 10 µM H$_2$DCF-DA for 30 min at 37°C. DCF oxidation was determined using an ELISA microplate reader (Tecan Infinite® M200 PRO, U.S.A.). The level of H$_2$O$_2$ generation was determined by the concomitant increase in DCF fluorescence.

Determination of Reduced Glutathione (GSH) Content  A GSH determination kit (Oxis International, Portland, OR, U.S.A.) was used for quantitative measurement of GSH. The GSH assay method was based on a chemical reaction. At first it led to the formation of thiocarboxamides between a 4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate and GSH present in the sample. It then included β-elimination reaction which occurs under alkaline conditions. The reaction was mediated by 30% NaOH which transformed the specific thiocarboxamide into GSH and a chromphoric thione with a maximal absorbance wavelength at 405 nm.

Flow Cytometric Analysis of Mitochondrial Membrane Permeability (MMP)  MMP was measured with 0.05 µg/mL Rho123 staining for 30 min. Cells were harvested by trypsinization. After suspension with PBS containing 1% FBS, the change in MMP was monitored using a Fluorescence-activated cell sorter (FACS, Partec, Münster, Germany). The analyses were recorded by 20000 events.

Small Interfering RNA (siRNA) Knockdown  Transfection is the process of deliberately introducing nucleic acids into cells. To knock down LKB1, cells were transfected with either a siRNA directed against LKB1 (SC-35816, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or a non-targeting control siRNA (100 pmol/mL) using Lipofectamine 2000 according to the manufacturer’s instructions. Transfection for 24h was followed by treatment with MCE for 3 h. The resulting LKB1 knockdown was confirmed by immunoblot analysis.

Preparation of the Standard Solution and the Test Stan-
The amount of preparations of materials (Paeonol, Benzoyl Paeoniflorin, Albiflorin, Paeoniflorin, Benzoic acid, and then Chlorogenic acid) were measured accurately and they were melted by methanol, and then they were made from a standard undiluted solution which 1 µg/mL. After that, the standard solution were diluted with the methanol to be contained 1, 5, 10 ng/mL and they were a standard solution. A standard curve determination coefficient (R2) value of all standard materials was more than 0.999. A test liquid for quantitative analysis was mixed with the sample equally and was measured 0.5 g precisely, and was added on the 30% methanol 10 mL, and then was extracted by microwave for 1 h.

![Fig. 1](image_url)

**Fig. 1. Hepatoprotective Effects of MCEs in AA+Iron-Treated HepG2 Cells**

(a) Cell viability. HepG2 cells were treated with MCE for indicated concentrations at 24 h. (b) Protective effects of MCE on AA+iron-treated HepG2 cells. Cell viability was assessed using MTT assays. (c) Morphological change. HepG2 cells were observed by light microscopy. Magnification, ×100. Arrows indicate the morphological change. (d) PARP, Procaspase-3 and Bcl-\textsubscript{xL} expression levels. Equal proteins were subjected to SDS-PAGE for Western blot analysis. (e-g). Relative fold changes of PARP, Procaspase-3 and Bcl-\textsubscript{xL}. HepG2 cells were treated with MCE (10, 30, 100, and 300 µg/mL) for 1 h and then stimulated with 10 µM AA for 12 h, followed by exposure to 5 µM iron for an additional 4 h. All data are representative of at least three times independent experiments. Data represent the mean±S.D. between treatments and either the vehicle-treated control (\#\#p<0.01) or cells treated with AA+iron (\*\*p<0.01) was determined.
This test liquid was filtered from the membrane filter of below diameter of 0.2 \(\mu\)m and was picked out as the test liquid.

**Quantification of the MCE** The Ultra Performance Liquid Chromatography (UPLC) was used UPLC system (Waters ACQUITY™, U.S.A.). PDA (Waters ACQUITY™ photodiode array detector, U.S.A.) and HPLC Column were used BEH C18 Column (1.7 \(\mu\)m, 2.1×100, Waters ACQUITY™, U.S.A.), and the software was Empower. A temperature of the column was analyzed at the room temperature. In case of the PDA analysis wavelength, the Chlorogenic acid and Benzoic acid were analyzed in 280 nm, Paeonol and Benzoyl Paeoniflorin were analyzed in 274 nm, and then Albiflorin and Paeoniflorin were analyzed in 230 nm. A mobile phase was a mixed liquid of the acetonitrile and water which contain 0.1% formic acids. The analysis condition was as in the following. The sample was injected with 2 \(\mu\)L, and a flow rate was 0.4 mL/min, and the result of analysis was observed qualitative checking by retention time, and then was quantified by peak area method (Table 1).

**Data Analysis** The experimental results were presented as mean±standard deviation (S.D.) of experiments repeated at least three times. In each treatment group the statistical significance was compared and verified using a one-way ANOVA or Student t-test method (\(p<0.05\) or \(p<0.01\)).

**RESULTS**

MCEs Inhibit AA+Fe-Induced Apoptosis in HepG2 Cells Since arachidonic acid (AA) treatment (10 \(\mu\)M) induced oxidative stress and iron (5 \(\mu\)M) enhanced AA-induced toxicity in HepG2 cells,16 we assessed the inhibition of AA+iron-induced cell death by MCEs. HepG2 cells were treated with MCEs and MCEs with AA+iron for 24h, the viabilities of the cells were measured by MTT assay. The results revealed that treatment with MCE alone (10–300 \(\mu\)g/mL, 24h) resulted in no cytotoxicity (Fig. 1a). However, treatment with AA+iron resulted in significantly reduced cell viability (40.3±0.5%), relative to the control group (Fig. 1b). Moreover, pre-treatment with MCE recovered AA+iron-induced cytotoxicity in a dose-dependent manner (Fig. 1b). Therefore, we investigated the cytoprotective mechanism using 100 \(\mu\)g/mL of MCE in subsequent experiments. Moreover, the hepatocyte protective effects of MCE on AA+iron-induced apoptosis were observed using a light microscope (Fig. 1c). We confirmed that AA+iron stimulated the expression of apoptosis-related proteins such as cleavages of PARP and decreases in procaspase-3 and Bcl-2 (Fig. 1d). As expected, MCE treatment fully recovered the levels of protein expression (Fig. 1d). These results showed that MCE exerted cytoprotective effects against apoptosis in AA+iron-induced hepatocytes.

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**Fig. 2. MCEs Suppress the AA+Iron-Induced ROS Production and MMP Dysfunction in HepG2 Cells**

(a) ROS production. The \(\text{H}_2\text{O}_2\) production was measured DCF fluorescence. HepG2 cells were stained with \(\text{H}_2\text{DCF-DA}\) at 1h after treatment of iron. (b) GSH contents. GSH was measured by Glutathione determination kit. HepG2 cells were treated with 100 \(\mu\)g/mL MCE and then stimulated with 10 \(\mu\)M AA for 12h, followed by exposure to 5 \(\mu\)M iron for an additional 4h. (c) Mitochondrial membrane permeability (MMP). HepG2 cells were stained with Rhod-123 at 1h after treatment of iron and fluorescence intensity was measured using FACS. (d) Relative percentage of RN1 fraction. All data are representative of at least three times independent experiments. Data represent the mean±S.D. between treatments and either the vehicle-treated control (**\(p<0.01\)) or cells treated with AA+iron (##\(p<0.01\)) was determined.
MCEs Inhibit AA+Iron-Induced Oxidative Stress in HepG2 Cells

Excessive ROS generation is the major cause of apoptosis induction via depletion of GSH antioxidant. Moreover, various studies have shown that AA induces oxidative stress through ROS production. To determine if MCE suppresses the excessive ROS generation and depletion of GSH antioxidants in AA+iron treated cells, we assessed the antioxidant activity of MCE treatment using H$_2$DCF-DA staining and a GSH determination kit. As shown in Fig. 2a, treatment with AA+iron increased H$_2$O$_2$ generation, which was completely inhibited by simultaneous MCE treatment. In addition, the change in intracellular GSH content was decreased by AA+iron treatment, but restored in cells concomitantly treated with MCE (Fig. 2b). These results supported that the cytoprotective effects of MCE may be associated with its antioxidant activity by redox-homeostasis.

MCEs Inhibit AA+Fe-Induced Mitochondrial Dysfunction in HepG2 Cells

A previous study showed that AA treatment caused inhibition of mitochondrial respiration and decreased respiratory activity. AA also induced dysfunction of mitochondrial membrane potential (MMP). MMP analysis was performed using flow cytometry after staining of cells with Rh123 (a membrane-permeable cationic fluorescent). Our results showed that MCE was significantly recovered in AA+iron-induced mitochondrial dysfunction (Figs. 2c, d). These findings indicated that MCE treatment suppressed mito-
Mitochondrial dysfunction caused by oxidative stress.

**MCEs Activate AMPK via LKB1 Phosphorylation** A previous study showed that AMPK plays a pivotal role in the protective mechanism against AA+iron-induced apoptosis. Moreover, AMPK is activated by the phosphorylation of LKB1 (a well-known upstream kinase of AMPK) and ACC (a downstream target of LKB1). To determine if the AMPK pathway is a hepatocellular protective mechanism of MCE treatment, we assessed the time-course effects of MCE on AMPK and ACC phosphorylation in HepG2 and Hep3B cells. The phosphorylated AMPK and downstream target ACC were increased by MCE treatment in a time dependent fashion (Figs. 3a, b). MCE also activated LKB1, the upstream regulator of AMPK (Fig. 3c). MCEs activate the AMPK pathway with similar patterns between HepG2 and Hep3B cells. In addition, ACC phosphorylation was not detectable in LKB1-deficient HeLa cells (Fig. 3d). Furthermore, AMPK phosphorylation was decreased by knock-down of LKB1 in MCE-treated HepG2 cells (Fig. 3e). These results suggested that MCE treatment provided hepatocyte protection through the LKB1–AMPK pathway.

**MCEs Protect against Mitochondria Dysfunction by AMPK Activation** To further confirm the protective mechanism of MCEs, we measured the effects of MMP using an AMPK inhibitor, compound C. AMPK phosphorylation notably decreased (Fig. 4a), and protection of MCE against AA+iron-induced mitochondrial dysfunction and ROS production were abrogated by pretreatment with compound C in HepG2 cells (Figs. 4b, c). In addition, level of AMPK phosphorylation was induced by MCE with or without AA+iron treatment (Fig. 4d). Taken together, MCEs protects hepatocytes against AA+Fe-induced oxidative stress and suppresses MMP disruption through activation of AMPK.

**Chlorogenic Acid, One of MCEs Components, Restores AA+Iron-Induced Apoptosis** Finally, to determine the active component of MCE, we conducted UPLC using six marker compounds of MCE, chlorogenic acid, albiflorin, paeoniflorin, benzoic acid, benzoyl paeoniflorin, and paeonol. Our results showed that paeoniflorin, albiflorin, chlorogenic acid and benzoyl paeoniflorin, were enriched in MCE (Table 2). Therefore, we assessed the effects of these components on AA+iron-induced apoptosis in HepG2 cells. MTT assay revealed the chlorogenic acid treatment (10, 30µM) markedly inhibited apoptosis relative to the other components (Fig. 5c). Moreover, chlorogenic acid significantly inhibited ROX production and induced AMPK activation (Figs. 5d, e). Overall, these finding indicated that chlorogenic acid, a component of MCEs, protects hepatocytes against AA+Fe-induced oxidative stress and suppress mitochondrial dysfunction via...
AMPK activation.

DISCUSSION

Reactive oxygen species (ROS) are generated as signaling molecules within the signal transduction cascades triggered by growth factors, cytokines, and hormones in cells.\(^{35}\) Excess ROS production can cause pathological processes associated with cell death in the liver, heart and circulatory system and diverse disease, including diabetes, aging and liver disease.\(^{3,4}\) Furthermore, the mitochondria play an important role in metabolic processes including regulation of oxidative stress, calcium overload, and cell death.\(^{36,37}\) In addition, mitochondria dysfunction has been reported as a major cause of pathogenesis in liver disease.\(^{38}\) Previous reports showed that oxidative stress and mitochondria dysfunction induced by iron accumulation can cause various chronic liver diseases.\(^{3,20}\) Arachidonic acid (AA), a representative pro-inflammatory fatty acid, promotes cellular ROS production and induces mitochondrial damage resulting in decreasing mitochondrial respiratory activity.\(^{5,39}\) Moreover, AA causes the release Ca\(^{2+}\) from intracellular stores and increases mitochondrial uptake
of Ca$^{2+}$ resulting to apoptosis.\textsuperscript{39,40} Previous reports showed that AA+iron treatment can cause cellular toxicity and mitochondrial damage.\textsuperscript{59} In the present study, we used AA+iron treated hepatocyte cell lines to confirm the effects of MCE, a candidate for anti-oxidant therapy.

Recently, several studies have shown that a large number of natural compounds, including herbs and plants used as traditional medicines, possess good anti-oxidant capacities.\textsuperscript{41,42} These compound blocked ROS production and prevented mitochondrial dysfunction.\textsuperscript{53} In the present study, we evaluated the effects of MC, the root bark of \textit{Paeonia suffruticosa Andrews} in Ranunculaceae,\textsuperscript{24,25} which is used in traditional Korean herbal medicine for its analgesic, anti-spasmodic, and anti-inflammatory effects and use as the traditional Korean herbal medicine.\textsuperscript{44,45} Since the molecular mechanism of MCE against AA+iron-induced cell death is not known, we examined the antioxidant effects and demonstrated its hepatoprotective activities. We found that MCE treatment inhibited AA+iron-induced cytotoxicity and recovered cell viability in HepG2 cells. MCE also inhibited ROS production and prevented GSH depletion of AA+iron-induced apoptosis caused by oxidative stress. In addition, MCE suppressed mitochondrial dysfunction induced by oxidative stress.

AMPK plays an important role in cellular energy homeostasis associated with hepatic fatty acid oxidation, cholesterol and triglyceride synthesis.\textsuperscript{46–48} AMPK also determines survival and death through oxidative and endoplasmic reticulum (ER) stress induction.\textsuperscript{49,49}\textsuperscript{2} Moreover, various studies have shown that AMPK is useful as a therapeutic target for treatment of various diseases, including hepatic disorders and cancer.\textsuperscript{12,50} In mammalian cells, AMPK was activated by phosphorylated Thr172 in the activation loop of the catalytic α-subunit of AMPK through LKB1 (a well-known upstream kinase, LKB1–AMPK)\textsuperscript{48,51} Previous reports have shown that resveratrol and sauchinone activate AMPK via the upstream kinase, LKB1-dependent.\textsuperscript{52,53} Moreover, epigallocatechin-3-gallate (EGCG; a green tea polyphenol) potently activates AMPK.\textsuperscript{54,55} In this study, MC induced phosphorylation of AMPK and ACC in HepG2 and Hep3B cell lines. In addition, pretreatment with compound C (AMPK inhibitor) blocked the ability of MCE to induce the phosphorylation of AMPK and prevented mitochondrial damage. Moreover, siRNA of LKB1 assay showed that LKB1 expression and AMPK phosphorylation were abolished. These results suggest that MCE exerts hepatoprotective effects via the LKB1–AMPK pathway. Moreover, UPLC and MTT assay showed that chlorogenic acid treatment has protective effect against AA+iron-induced apoptosis, which are similar to the results of previous studies.\textsuperscript{56,57} Although paeonol has a protective effect in microglial cells, these results indicate that chlorogenic acid might be an active compound in MCE responsible for its protective effects against oxidative stress and apoptosis.

CONCLUSION

The present study shows that MCEs inhibit reactive oxygen species generation and suppress mitochondrial dysfunction, as well as decrease GSH levels induced by AA+iron. In addition, the cellular protective effects of MCEs involved activation of the LKB1–AMPK pathway. Finally, the results presented here-in demonstrate that chlorogenic acid, one of the components of MCEs, is a potential therapeutic candidate for the prevention of liver disease.

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Conflict of Interest The authors declare no conflict of interest.

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