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Anti-inflammatory properties of *Ajuga bracteosa in vivo* and *in vitro* study and their effects on mouse model of liver fibrosis

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ABSTRACT

Ethnopharmacological relevance: The entire plant of *Ajuga bracteosa* Wall has been used to treat various inflammatory disorders, including hepatitis, in Taiwan.
Aim: This study evaluated the hepatoprotective ability of *Ajuga bracteosa* extract (ABE).
Materials and methods: We investigated the inhibitory action of a chloroform fraction of ABE (ABCE) on lipopolysaccharide (LPS)-stimulated RAW264.7 cells and Kupffer cells. Hepatic fibrosis was induced in mice through the administration of CCl_4 twice a week for 8 weeks. Mice in three CCl_4 groups were treated daily with water and ABE throughout the duration of the experiment.
Results: In LPS-stimulated RAW264.7 cells and Kupffer cells, ABCE inhibited the production of NO and/or TNF-α and also blocked the LPS-induced expression of NO synthase. ABCE inhibited the activation of NF-κB induced by LPS, associated with the abrogation of IκBα degradation, with a subsequent decrease in nuclear p65 and p50 protein levels. The phosphorylation of MAPKs in LPS-stimulated RAW264.7 cells was also suppressed using ABCE. In the *in vivo* study, ABE protected the liver from injury by reducing the activity of plasma aminotransferase, and by improving the histological architecture of the liver. RT-PCR analysis showed that ABE inhibited the hepatic mRNA expression of *LPS binding protein*, *CD14*, *TNF-α*, *collagen(α1)(I)*, and *α-smooth actin*.
Conclusion: These results indicate that ABE alleviated CCl_4-induced liver fibrosis, and that this protection is probably due to the suppression of macrophage activation.
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1. Introduction

Kupffer cells are the resident macrophages of the liver. When activated, they produce and release numerous mediators, including nitric oxide (NO) and cytokines, such as TNF-α (Valatas et al., 2004; Al-Anati et al., 2009). Many of these mediators further activate or modulate nearby cells involved in the process of inflammation which exacerbates the damage to the liver. Kupffer cells are involved in several types of chemical-induced liver damage, including damage related to carbon tetrachloride (CCl_4) (Qiu et al., 2005). Rivera et al. (2001) demonstrated the destruction of Kupffer cells from gadolinium chloride attenuated CCl_4-induced hepatic fibrosis. Kupffer cells are considered a key factor in the development of CCl_4-induced liver fibrosis.

Ajuga bracteosa (Labiatae) is a common wild herb growing in open fields throughout Taiwan. It has been found that *Ajuga bracteosa* contains substances, such as sphingolides, bractic acid, diterpenoids (Riaz et al., 2007), and withanolides (Riaz et al., 2004), exhibiting various enzyme-inhibiting activities (lipooxygenase, acetylcholinesterase). In Indian traditional medicine, this plant is used as a remedy for malaria (Chandel and Bagai, 2010); and in Taiwan the entire plant has been used to treat various inflammatory disorders, including hepatitis, pneumonia, and bone disease (Chiu and Chang, 1992). Previous investigations have shown that extracts of *Ajuga decumbens* reduced CCl_4-induced hepatic damage in mice (Sun and Li, 2010). However, the antifibrotic action of *Ajuga bracteosa* remains unclear.

We hypothesize that *Ajuga bracteosa* extracts (ABEs) might provide protection from CCl_4-induced fibrogenesis by suppressing inflammation, thereby inhibiting activation of hepatic stellate cells. Results of this study support our hypothesis. An extension of our prior *in vitro* observations provided insight into the mechanisms by which ABE provides protection to the liver.

2. Materials and methods

2.1. Plant material

Dried *Ajuga bracteosa* Wall (Labiatae) was purchased from Han-Chiang Herbal Medicine Company (Taichung, Taiwan) and

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identified by Dr. Chao-Lin Kuo, School of Chinese Medicine Resources, China Medical University. A specimen of the plant, collected in June 2008 from the botanical farm at Taichung, was supplied by the Han-Chiang Herbal Medicine Company to the China Medical University. To prepare the extract, the dried plant (1.6 kg) was decocted with boiling water (20 l) for 4 h, and the decoction was filtered and evaporated under reduced pressure to yield a brown residue (ABE). The yield of ABE was approximately 28%.

The ABE was suspended in water and partitioned using n-butanol. The n-butanol fraction (ABBE) was concentrated, yielding 21.7%. The ABBE (50 g) was suspended in water and partitioned with chloroform. The chloroform fraction (ABCE) was concentrated, yielding 27% (13.5 g).

2.2. RAW264.7 cell culture

RAW264.7 cells, derived from murine macrophage were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). They were cultured in DMEM supplemented with 10% endotoxin free, heated and inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

For the NO assay, the cells (3×10^4 cells/well) were preincubated for 1 h using various concentrations of ABE, ABBE, or ABCE, and further cultured for 24 h with 1 μg/ml of LPS in 96-well plates. The supernatant was removed at the allotted time to quantify the production of NO.

2.3. Primary cell culture

Rat Kupffer cells were isolated according to the method of Froh et al. (2002). The freshly isolated cells were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/l), streptomycin (100 μg/ml), and L-glutamine (2 mmol/l). The cells were plated onto 96-well (5×10^4 cells) culture dishes for detection of NO. They were maintained in an incubator at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Nonadherent cells were removed from the culture after 15 min. Adherent cells were used for the experiments.

The purity of Kupffer cell fraction was consistently >80% as determined by CD68 staining (flow cytometry). All adherent cells were analyzed for their ability in phagocytosis, indicating that they were viable Kupffer cells.

2.4. Cytotoxicity assay

The viability of the RAW264.7 cells and Kupffer cells was detected by MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation assay, Promega Corporation, Madison, WI, USA). MTS was bio-reduced into a colored formazan product by the reducing enzymes present only in metabolically active, viable cells. This compound has an absorbance peak at 490 nm, which was measured in a spectrophotometric microplate reader.

2.5. Nitrite assay

The concentration of nitrite in the culture medium was measured as an indicator of NO production, according to the Griess reaction (Sigma–Aldrich, St. Louis, MO). Approximately 100 μl of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was determined with an enzyme-linked immunosorbent assay plate reader (Minghetti et al., 1997).

2.6. Cytokines assay

The concentrations of TNF-α in the culture medium were measured by ELISA using commercially available kits (eBioscience, San Diego, CA) according to the manufacturer's instruction.

2.7. Western blot analysis

Cytoplasmic and nuclear protein extracts were described previously (Chen et al., 1998). The RAW264.7 macrophage was incubated with or without LPS in the presence or absence of ABCE. The cells (1.0×10^7) were washed with ice-cold phosphate-buffered saline and suspended in 0.2 ml hypotonic lyses buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride (NaF), 1 mM orthovanadate (Na_3VO_4), 10 μM EGTA, 10 μM EDTA] containing 0.5% Nonidet P-40 and microcentrifuged at 12,000 × *g* for 1 min. The homogenate was centrifuged, and the supernatant containing cytoplasmic extracts was removed and stored frozen at −80 °C. The nuclear pellet was lysed in 20 μl of ice-cold nuclear extraction buffer for 1 h with intermittent mixing, the extract was then centrifuged. The lysate was centrifuged at 12,000 × *g* for 15 min at 4 °C, and supernatant containing nuclear extracts was secured. The protein concentration was determined using Bradford protein assay reagent (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's instructions.

The cytoplasmic and nuclear protein extracts (40 and 20 μg, respectively) were separated using 12% SDS–PAGE and transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk, and being incubated overnight at 4 °C with various primary antibodies in TBS containing 0.1% Tween-20, the primary antibodies were obtained from the following sources: anti-p65, anti-phospho-IκBα, anti-IκBα, anti-phospho-ERK from Cell Signaling (Danvers, USA); anti-phopho-p38, anti-p38, anti-ERK, anti-phospho-JNK, anti-JNK from Abcam (Cambridge, UK); and anti-proliferating cell nuclear antigen (PCNA), anti-α-tubulin, anti-p50 from Santa Cruz (CA, USA). Thereafter, the blot was washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h, and then developed through enhanced chemiluminescence (Thermo, Rockford, USA). PCNA and α-tubulin were used as internal controls in nuclear and cytoplasmic experiments, respectively.

2.8. Animal care

ICR male mice were obtained from the BioLASCO Co. Ltd. (Taipei, Taiwan). The animals in the experiment were housed in an air-conditioned room at 21–24 °C with 12 h of light. The mice were allowed free access to food pellets and water throughout the study. All animals received human care and the study protocol complied with the guidelines of China Medical University for the use of laboratory animals.

2.9. CCl_4-induced liver fibrosis

Mice (24–27 g) were randomly allocated to four groups (one in the control group and three in the CCl_4-treated groups, each containing 8 mice). Chronic hepatitis was induced in three groups of 8 mice through oral administration of 0.1 ml/10 g body weight of CCl_4 diluted 10:90 (v/v) in olive oil, twice a week for 8 weeks. The animals received only CCl_4 or CCl_4 with ABE (0.3 or 1.0 g/kg, p.o., daily). ABE was administered when the chronic injury model began, and the total duration of drug treatment was 8 weeks. On the days of CCl_4 treatment, the time interval between the administration of CCl_4 and ABE was 5 h to avoid the interference of absorption. The dosage of ABE used in the experiment was based on the dry weight of the extract. Dilutions were made with distilled water.

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