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Original Article

Antioxidant and Hepatoprotective Effects of *Amomum Xanthoides*

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Objective: To examine the *in vitro* antioxidant and hepatoprotective properties of *Amomum xanthoides* extract and its fractions.
Methods: Methanol extract of *Amomum xanthoides* was fractionated into three fractions; petroleum ether fraction (PFAX), water fraction (WFAX) and ethyl acetate fraction (AFAX). The antioxidant and hepatoprotective effects of these fractions were compared by *in vitro* experiments.
Results: The total phenols content and DPPH (1, 1-Diphenyl-2-picrylhydrazyl) radical scavenging activity was found significant in AFAX when compared with other fractions (PFAX, WFAX). AFAX significantly inhibited NO production by RAW 264.7 cells in LPS-activated condition. In Hep G_2 cells, AFAX markedly prevented the leakage of LDH and release of AST against CCl_4 challenge.
Conclusion: *Amomum xanthoides* and especially ethyl acetate fraction have potent antioxidative and hepatoprotective properties.

Key Words : herb, *Amomum xanthoides*, phenol, antioxidant, hepatoprotective

Introduction

Amomum xanthoides is a traditional herbal medicine frequently used in Asia for the management of various human ailments such as gynecological problems, mastitis, miscarriage and disorders especially associated with the digestive system. Many studies have reported its pharmaceutical properties such as anti-diabetes[1,2], anti-allergy[3], improvement of gastrointestinal motility[4], and anti-inflammatory action[5,6]. In addition, five classes of chemical components including volatile oil (approximately 1.7%-3%), saponins (approximately 0.69%), flavonoid glycosides, organic acids and inorganic components had been identified from *Amomum xanthoides*[7,8].

Amomum xanthoides is one of the major compositional herbs in CGX (also called Chunggan extract, which means “cleaning liver”), a modified traditional herbal formula which showed significant hepatoprotective and antioxidative effects in an animal model[9]. We screened all herbal plants composing CGX, and then selected *Amomum xanthoides* as having strong antioxidative action. However, there exists no data exploring hepatoprotective effect with antioxidative capacity of *Amomum xanthoides*.

Therefore, this study aimed to characterize the pharmaceutical properties of *Amomum xanthoides* focusing on antioxidation and hepato-protection using main fractions.

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Materials and Methods

1. Reagents and chemicals

Methyl and ethyl alcohol were obtained from CARLO ERBA Reagents Group (Rodano, Italy), Folin-Ciocalteu's phenol reagent was from Fluka Biochemika (Switzerland), carbon tetrachloride (CCl_4) was purchased from Showa Chemical Co. Ltd. (Tokyo, Japan), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and others were purchased from Sigma (St. Louis, MO, USA).

2. Plant material and fractionation procedure

Amomum xanthoides was purchased from Dun-san Oriental Hospital, affiliated to Daejeon University (Daejeon, Korea). Eight hundred grams of *Amomum xanthoides* were cut into pieces by grinder-mixer and extracted with 5 L methanol at room temperature at least 7 days. One hundred milliliters distilled water was added to 900 mL of methanol extract layer and petroleum ether (50% final concentration), petroleum ether soluble fraction and methanol soluble fraction were separated successively by using a separating funnel. Thereafter, 900 mL distilled water was added into 100 mL methanol soluble fraction, then water soluble fraction and ethyl acetate fraction (AFAX) were fractionated by the same method as above. Finally, the fractioned materials were lyophilized and resulting material separated into methanol extract (144 g), petroleum ether (7.42 g, yield 5.14%), H_2O (0.392 g, yield 0.27%) and ethyl acetate (0.904 g, yield 0.63%) soluble fractions (Fig. 1). The extractions were stored at -20℃.

3. Fingerprinting analysis

High performance-thin layer chromatography (HP-TLC) based fingerprinting was performed using CAMAG application system (Muttenz, Switzerland). A known compound, borneol (Dong Myung Science Co. Ltd., Korea) was dissolved in HPLC-grade methanol and applied to pre-washed silica-gel 60 F_{254} HP-TLC plate (10 × 10 cm, silica-gel thickness 2 mm)

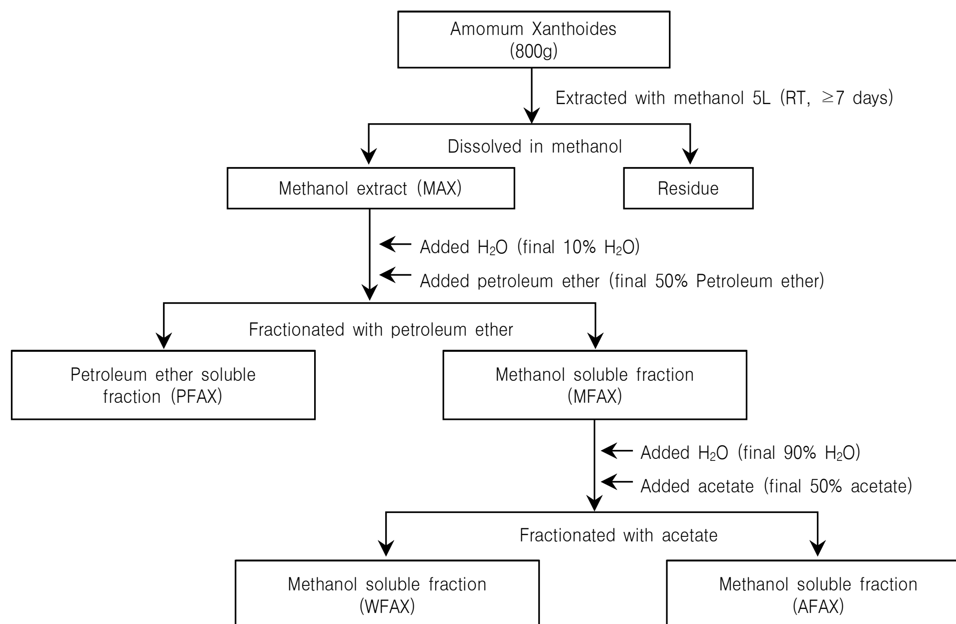

Fig. 1. *Amomum xanthoides* extract and fractionation procedure

(Merck, Darmstadt, Germany) with an automated applicator as standard. All of the samples were separated (migration distance 75 mm) by HPLC-grade solvent hexane/acetone (1:1). The migrated components were detected after derivatization with 4% vanillin sulfuric acid, under white light using Reprostar 3 with a digital camera (CAMAG). WinCATs and VideoScan software were used for data capture and analysis (Fig. 2).

4. Determination of total phenolic compounds

Falconoids are found in most plant species with high antioxidant properties, and the majority of them belong to phenolic compounds. So, the concentrations of phenolic compounds in fractions of *Amomum xanthoides* extraction were determined according to the Folin-Ciocalteu method[10].

Briefly, 20 µL of sample (AFAX, WFAX, PFAX and silymarin as positive control) was mixed with 100 µL of Folin-Ciocalteu and 80 µL of sodium carbonate. After 2 hrs of incubation in the room temperature, the absorbance was measured at 750 nm by spectrophotometer. Gallic acid monohydrate was used as a standard in the range of 1 to 10 µg/mL. According to the standard curve, total phenolic compounds contents were expressed as gallic acid equivalent in mg/g of extracts.

5. DPPH radical scavenging assay

Fractions of *Amomum xanthoides* were investigated for free radical scavenging activity by using DPPH assay[11]. Briefly, three kinds of *Amomum xanthoides* fractions (100 µg/mL, 100 µL) were added into 100 µL of 0.3 mM ethanolic DPPH in 96-well flat bottom microplate. After mixing, it was incubated for 15 minutes at room temperature; then the absorbance was read at 517 nm using spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Vitamin C (10 µg/mL, 100 µL) and silymarin (100 µg/mL, 100 µL) were done as a positive control for comparison.

The DPPH free radical-scavenging activity (%) was calculated using the equation as follows:

Scavenging activity (%) = $(OD_{control} - OD_{sample}) / OD_{control} \times 100\%$

6. Cell culture

Hep G_2 and RAW 264.7 cells were purchased from KRIBB (Korea Research Institute of Bioscience & Biotechnology), and cultured in DMEM (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) at 37℃ and 5% CO_2 incubated environment. Logarithmic growth phase of cells were sub-cultured thrice a week for *in vitro* experiment preparation.

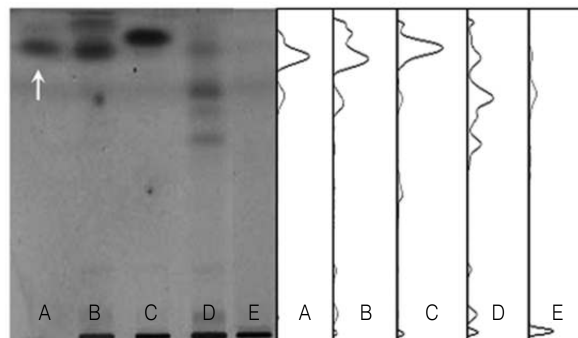

Fig. 2. Fingerprint of *Amomum xanthoides* and its fractions. Ninety percent methanol dissolved methanol extract of *Amomum xanthoides* and its fractions were filtrated, and HP-TLC analysis was performed. A: Borneol, B: Methanol extract, C: MFAX, D: AFAX, E: WFAX

7. Nitric oxide (NO) assay

RAW 264.7 cells were cultured in DMEM (Sigma, USA) supplemented with 10% FBS at 37℃ and 5% CO_2. Cells were adjusted to 1×10^5 per well in 24-well plate, then incubated overnight. After pretreatment with AFAX (10, 20, 40 µg/mL) for 4 hrs, LPS (0.1 µg/mL) was added to cells for 24 hrs. The supernatant were collected and measured by NO assay as originally described by Griess[12].

8. Lactate dehydrogenase (LDH) leakage assay

Hep G_2 cells were adjusted to 2×10^4 cells per well in 24-well plates. After pretreatment with various concentrations of AFAX (25, 50, 100 µg/mL) for 24 hrs, the cells were treated with 2 mM of CCl_4 dissolved in dimethyl sulfoxide (DMSO) (final concentration 0.5%) for 2 hrs. The LDH leakage assay was performed by measuring the LDH activity in cell culture supernatants using a CytoTox 96 Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Two groups were treated with DMSO (0.5%) or lysis buffer only as spontaneous control and maximum groups, respectively.

The death rate (%) was calculated using the equation as follows:

Death rate (%) = $(OD_{sample} - OD_{control}) / (OD_{maximum} - OD_{control}) \times 100\%$

9. Determination of aspartate transaminase

Cell supernatant samples were collected from the wells of the LDH leakage experiment, and stored in deep freeze (-70℃) for biochemical analysis. Aspartate transaminase (AST) was determined in the cell supernatant using auto chemistry analyzer (Chiron Ltd. Emeryville, CA, USA).

10. Statistical Analysis

All the results are expressed as the mean ± standard deviation (SD) (n≥3). Statistically significant differences between the groups were analyzed using one-way analysis of variance (ANOVA) followed by a paired Student's t-test. Difference at the level of $p < 0.05$ was regarded as statistically significant.

Results

1. Total phenolic compounds analysis

Total phenolic compounds of PMAX, WFAX, AFAX and positive control of silymarin were

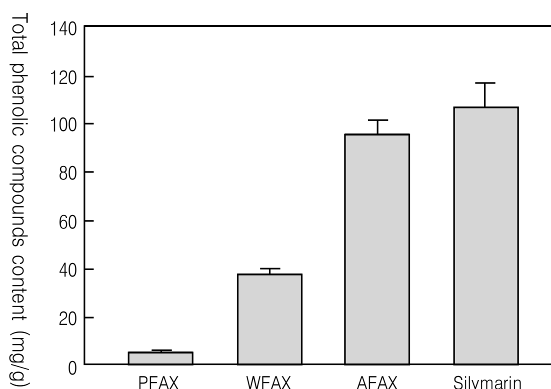

Fig. 3. Total phenolic compounds content in *Amomum xanthoides*. The phenolic compounds were determined using the Folin-Ciocalteu method. PFAX: petroleum ether soluble fraction, WFAX: water soluble fraction, AFAX: acetate soluble fraction.

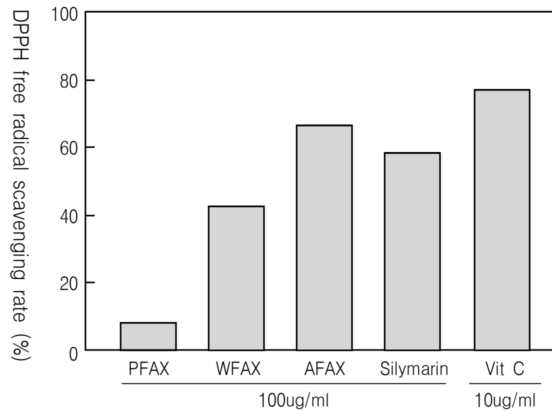

Fig. 4. Free radical Scavenging activity of *Amomum xanthoides*. Free radical scavenging capacity of *Amomum xanthoides* and positive control (silymarin and ascorbic acid) were measured by DPPH assay. PFAX: petroleum ether soluble fraction, WFAX: water soluble fraction, AFAX: acetate soluble fraction.

expressed as gallic acid equivalent in mg/g of extracts. PFAX almost doesn't contain any phenolic compound, WFAX contains 37.83 mg/g of phenolic compound, AFAX contains 95.67 mg/g of phenolic compound, and silymarin contains 106.73 mg/g of phenolic compound (Fig. 3).

2. Scavenging of DPPH free radicals

Free radical scavenging capacity of three fractions in *Amomum xanthoides* was determined using DPPH assay and compared with silymarin and ascorbic acid which were used as positive control. (Fig. 4). DPPH free radical scavenging activity of AFAX was highest among three fractions in *Amomum xanthoides*, even higher than silymarin.

3. Nitric oxide product inhibition

RAW 264.7 cell line (mouse leukaemic monocyte

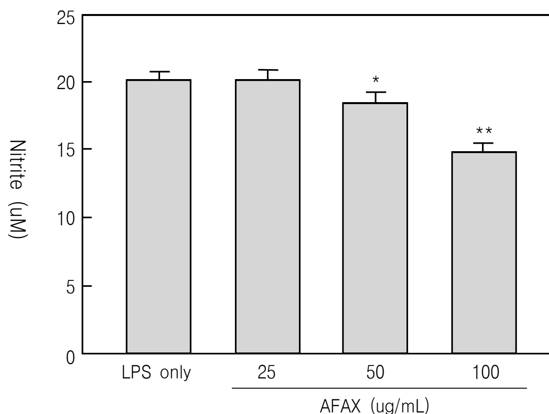

Fig. 5. Activity for inhibition of NO production. RAW 264.7 cells were pretreated with AFAX for 4 hrs, then treated with LPS (0.2 µg/mL) for 24 hrs. NO production was measured by using the Griess reaction. *p <0.05, compared with the LPS only **p <0.01, compared with the LPS only (n = 3).

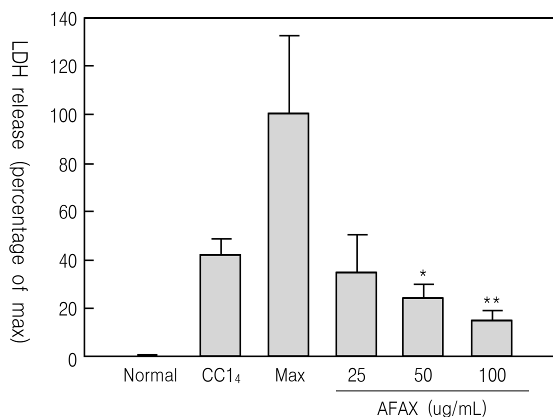

Fig. 6. Lactate dehydrogenase leakage assay. After pretreatment with AFAX for 24 hrs, Hep G_2 cells were treated with 2 mM CCl_4. LDH release was determined using CytoTox 96 Assay kit. *p <0.05, compared with the CCl_4 only **p <0.01, compared with the CCl_4 only (n = 3).

macrophage cell line) was used for evaluating the inhibition effect of AFAX on NO production. 50 and 100 µg/mL of AFAX significantly inhibited NO production in LPS-stimulated Raw 264.7 cells (p <0.01, Fig. 5).

4. LDH leakage assay

To investigate the effect of AFAX on hepatocyte protection, CCl_4 induced intracellular LDH leakage was determined in Hep G_2 cells after pretreatment with AFAX 24 hrs. AFAX (50 and 100 µg/mL) protected hepatocytes against CCl_4 toxicity in a dose dependant manner (p <0.05 and p <0.01, Fig. 6).

5. Aspartate transaminase activity

AST was measured as another biomarker of hepatocyte damage. AFAX treatment (50, 100 µ g/mL) significantly lowered AST level compared to

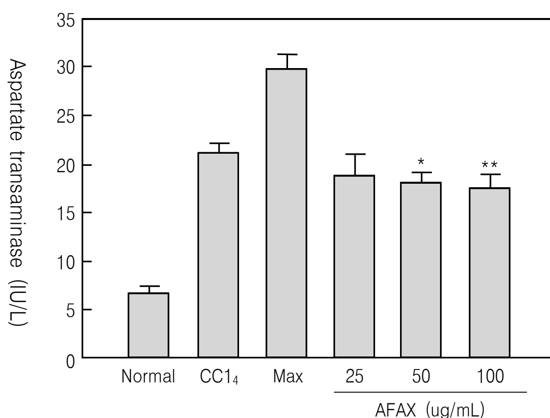

Fig. 7. Aspartate transaminase lease assay. After pretreatment with AFAX for 24 hrs, Hep G_2 cells were treated with 2 mM CCl_4. AST release was determined by auto chemistry analyzer. *p <0.05, compared with the CCl_4 only **p <0.01, compared with the CCl_4 only (n = 3).

CCl_4 group in Hep G_2 cells ($p < 0.05$ and $p < 0.01$, Fig. 7).

Discussion

Many traditional herbal remedies have been used for various disorders including liver diseases for a long time. *Gamichunggantang* and CGX are herbal hepatotherapeutics modified from traditional herbal formulae[9,13,14], and *Amomum xanthoides* is one among the compositional plants. These drugs exert hepatoprotective and antioxidative effects[15-17]. Based on our previous research, we identified *Amomum xanthoides* as an active herb possessing antioxidant activity. Furthermore, according to principles of traditional Korea medicine, *Amomum xanthoides* belongs to aromatic dampness-dispersing and Qi-activating drugs. It is believed that *Amomum xanthoides* can help cure depression of the liver. We herein further investigated its property using three fractions.

It is well known that most phenolic compounds belong to the flavonoids, and flavonoids are found in most plant species with high antioxidant properties[18-20]. For example, silymarin is a typical polyphenolic flavonoid antioxidant, and it has a strong hepatoprotective effect[21]. Our results showed that AFAX contained relatively 90% of phenolic compound compared to silymarin (Fig. 3). This was in accordance with free radical scavenging capacity because AFAX showed the most significant activity among the three fractions. Moreover, the free radical scavenging capacity of AFAX was higher than silymarin (Fig. 4). These results indicated that the ability of free radical scavenging positively correlated with total phenolic compound contents within each fraction of *Amomum xanthoides*.

So, we further examined AFAX for its antioxidative and hepatoprotective activity using two cell lines. Hepatic macrophages, known as Kupffer cells, are known as a main cellular source of NO production, and play a critical role in liver damage[22]. We adapted RAW 264.7 cell lines instead of hepatic macrophages for evaluating the inhibition effect of AFAX on NO production. AFAX showed a trend towards inhibiting NO production in an LPS-stimulated condition (Fig. 5).

In addition, AFAX treatment protected Hep G_2 cells against toxicity by strong hepatotoxin (CCl_4), which was evidenced by leakage of LDH and AST enzyme activity (Fig. 6, 7). CCl_4 can be metabolized by cytochrome P450 oxygenases and forms the trichloromethyl ($CCl_3\cdot$) radical, which can rapidly and directly cause hepatocyte membrane lipid peroxidation leading to liver injury[23,24].

Taken together, we can conclude that *Amomum xanthoides* has potent antioxidative as well as hepatoprotective properties, and its major activity is possessed by falconoids in the ethyl acetate fraction. Further studies are required to determine its mechanisms and its activity as a single compound.

Acknowledgments

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