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Full Length Research Paper

Antioxidation and protection against H_2O_2 induced oxidative stress on normal human hepatocytes cell line (LO2) by extracts and three compounds from the root of *Isatis Indigotica*

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The effect of *Isatidis indigotica* Fort (banlangen) extracts with its three typical compounds on antioxidation and oxidative stress damage induced in LO2 by hydrogen peroxide has been investigated. Their antioxidant activities were assessed by two complementary test systems, namely DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) assay. Among the tested samples, considerable activity of isolariciresinol was observed in all test systems (DPPH IC50: 24.29 μM; FRAP: 7.16 mM FSE/g) compared to that of ascorbic acid as reference substance (DPPH IC50: 38.95 μM; FRAP: 0.78 mM FSE/g). The percentage of cell viability was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. This study showed that incubation with isolariciresinol, banlangen keli (BLGKL) caused significant increase in the viability of LO2. The results demonstrated that Banlangen contained hepatoprotective compounds distributed in different extracts.

Key words: Isatidis indigotica Fort, antioxidation, hepatocytes cell line (LO2), isolariciresinol, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT).

INTRODUCTION

Banlangen is the dried root of the plant *Isatidis indigotica* Fort. (Cruciferae). Banlangen is usually used in Traditional Chinese Medicine (TCM), being valued to have antiviral, antibacterial, antiendotoxic, immune regulatory activities and traditionally used for treating seasonal febrile diseases, mumps, pestilence, eruptive diseases, inflammatory diseases with redness of skin, and sore throat (Chinese, 2010a; Nanjing, 2006). Recently the pharmacology activities or clinical application of Banlangen for anti-SARS, anti-endotoxic, radioprotection, anti-herpes simplex virus type 2 (HSV-2) activity and anti-inflammatory effect had been reported (Fang et al., 2005; Lin et al., 2005; Shin et al., 2010; Sun et al., 2010; You et al., 2009).

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Although, many contributions concerning the chemistry and activity of Banlangen have appeared, there have been no reports on its antioxidant and LO2 cell protective effects combining total extracts and active compounds. This paper deals with the preparation and determination of the extracts from Banlangen, as well as the antioxidant evaluation of these extracts by DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) tests. Furthermore, the protective effect on LO2 injured by H_2O_2 was observed. Simultaneously, the three chosen activity compounds were proceeded with the same experiments for complementation with the extracts.

MATERIALS AND METHODS

Chemicals

2, 2-diphenyl-1-picryl-hydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1, 2, 3-trihydroxybenzene was purchased from Aladdin Chemistry Co., Ltd (ShangHai, China).

Ascorbic acid, $FeCl_3$, $FeSO_4$•$7H_2O$ and H_2O_2 were purchased from Sinopharm Chemical Reagent Co., Ltd (ShangHai, China). MTT was purchased from Amresco Inc (Solon, OH, USA). DMEM-high glucose was purchased from Invitrogen (Grand Island, NY), Phosphate Buffered Saline (PBS) was purchased from Boster Bio-Engineering Co., Ltd. (WuHan, HuBei China). Fetal bovine Serum (FBS) was purchased from Sijiqing Biological Engineering Materials Co.,Ltd. (HangZhou, ZheJiang, China). All other reagents were analytical grade.

Plant materials

Banlangen (*I. indigotica* Fort.) were collected from Mengcheng (AnHui, China) and authenticated by Professor Jianwei Chen from the Department of Pharmacognosy Identification, Nanjing University of Chinese Medicine. The voucher specimen (SDD-BLG-001) was deposited there.

Extracts and compounds

The Banlangen (3 kg) was extracted three times with 70% ethanol (8, 6 and 6L, respectively) through the heating reflux for 5 h (3, 1.5 and1.5 h, respectively). The ethanol extracts were combined and concentrated at 50°C in a rotary evaporator to appropriate volume. The concentrated solution was filtrated through a Whatman No. 1 filter paper. The filtrates were divided into five aliquots parts. One was dried and the yield of extract calculated. Three of them were used to prepare and determine total lignin, total organic acids and total alkaloid, respectively. The remaining sample was dried and stored at room temperature.

Preparation and determination of total lignan (TL)

The filtrates were subjected to D101 macroporous resin eluting with water, 50% ethanol sequentially, until exhausted. Determination of TL (50% ethanol eluent) by UV spectrophotometry (Li et al., 2010), the content of total lignin reached 79.8%.

Preparation and determination of total organic acids (TOA)

The filtrates were adjusted to ph (5 to 6) and subjected to 731 strongly acidic cation exchange resin. The elute (pH-adjusted to 9) was subjected to 717 anion exchange resin eluting with water, 0.8 mol•L^{-1} acidity ethanol sequentially, until exhausted. The total organic acids content reached 80.9% calculating on the basis of salicylic acid by determination of acid-base titration (Ma et al., 2006).

Preparation and determination of total alkaloid (TA)

The filtrates (ph 5 to 6) were subjected to 731 strongly acidic cation exchange resin eluting with water, 1.0 mol•L^{-1} ammonia ethanol sequentially, until exhausted. Then ammonia was removed from the elute. The elute (pH-adjusted to 9) was subjected to 717 anion exchange resin and collected as TA. The TA content reached 53.6%, calculating on the basis of epigoitrin by determination of acid-base titration (Zhou et al., 2010).

Banlangen Keli (BLGKL) was prepared in accordance with Chinese Parmacopoeia (Chinese, 2010b). 7S, 8R,8′R-(+)-lariciresinol-4,4′-O-β-D-glucopyranoside (CB), isolariciresinol, and indigotin were isolated by our group (Sun et al., 2007; Wang et al., 2009) and identified by a comparison of physical data (UV, IR, 1HNMR, 13CNMR). The purity of the chemicals was analyzed by HPLC and found to be at least 98% pure.

DPPH radical scavenging activity

To measure antioxidant activity, the DPPH radical scavenging assay was carried out according to a procedure described in ref (Brand-Williams et al., 1995; Maksimović et al., 2011) with slight changes. Twenty microlitre different extract (diluted final concentration range: 0.0025 to 3.1 mg•mL-1) were added to 80 μl 0.2 mM DPPH solution in the 96 well plates. The absorbances were measured on ELISA microplate reader (Bio-Rad Laboratories, Inc, USA) at 517 nm after 30 min incubation at room temperature in the dark, against distilled water as a blank. The percent inhibition was calculated against the control solution, containing L-ascorbic acid instead of test solution. The assay was carried out in triplicate wells on each plate and with three independent experiments. The DPPH radical scavenging activity was calculated according to the following formula:

$$\text{DPPH scavenging activity}\,(\%) = \left[1 - \frac{A_{sample}}{A_{conrtol}}\right] \times 100\%$$

The IC50 value denoted the concentration of sample required to inhibit DPPH radical formation by 50%.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is a simple and reliable colorimetric method commonly used for measuring the total antioxidant capacity (Benzie and Strain, 1999; Wei et al., 2010). The FRAP reagent was prepared by adding 10 vol. of 300 mM acetate buffer, pH 3.6 (3.1 g sodium acetate trihydrate and 16 ml glacial acetic acid made up to 1 L with distilled water), 1 vol. of 10 mM 2,4,6-tripyridyl-s-triazine prepared in 40 mM hydrochloric acid and 1 vol. of 20 mM ferric chloride. The FRAP assay was performed using reagent prewarmed to 37°C. 100 μl of reagent was added to 10 μl of test sample. The mixture was shaken and incubated at 37°C for 4 min. Absorbance at 593 nm was determined relative to a reagent blank also incubated at 37°C. The antioxidant potential of test sample was determined against a standard curve of ferrous sulphate (Fe(Ⅱ), 30 to 2500 μM). A higher absorbance indicates a higher ferric reducing power. The FRAP values of total extract and compounds were expressed as mM ferrous sulphate equivalents /g dried total extract.

Cell lines and culture

The human liver cell LO2 was obtained from Pre-clinical Medicine College of Nanjing University Of Chinese Medicine. The cell was maintained in an atmosphere of 5% CO_2 at 37°C in DMEM supplemented with 10% FBS, 100 U•ml^{-1} penicillin and 100 mg•ml^{-1} streptomycin.

Oxidative damage induced by H_2O_2

To determine whether the oxidative injury was caused by H_2O_2 or not, LO2 cells were incubated at 37°C for 1 h with different concentrations of H_2O_2 before pulsing 0.05 mM MTT, 150 μl DMSO.

Cytotoxicity assay and protective effect on LO2 injured by H_2O_2

The cells were divided into nine groups: control group, H_2O_2 group, four extracts group and different concentrations of three compounds (50, 25, 12.5 and 6.25 μg•ml^{-1}) group. The viability of

Table 1. DPPH radical scavenging activity of different extracts and compounds.

Extracts	Concentration range (μg•ml^{-1})	IC_{50}[a] (μg•ml^{-1})	Compounds	Concentration range (μM)	IC_{50}[a] (μM)
Ascorbic acid	2.56-51	5.63 ± 0.19	Ascorbic acid	18.09 - 289.54	38.95 ± 1.59
TL	8.28-1060	154.03 ± 0.84	Isolariciresinol	5.42 - 694.44	24.29 ± 0.98
TOA	11.27-1443.33	1387.67 ± 8.08	Indigotin	11.91 - 1525.14	n.c. [b]
TA	24.24-3103.33	n.c. [b]	CB	2.74 - 350.88	n.c. [b]
BLGKL	7.81-1000	595.97 ± 10.65			

[a] Result are expressed as mean ± SD of three experiments. [b] Effects can be neglected at tested concentrations range.

LO2 cell was measured by a colorimetric MTT assay (n = 6) (Storch et al., 2000; Xu et al., 2010). Briefly, the cells were incubated in DEME with different extracts, concentrations of three compounds respectively for 24 h, then exposed to H_2O_2 (800 μM) for another 1 h, following addition of 20 μl MTT solution (2.5 mg•ml^{-1}) to each well, the plates were incubated for 4 h at 37°C. After the medium had been removed, the dye crystal was dissolved in 150 μl DMSO. Concurrently, LO2 cells were cultured in DMEM containing only four extracts and different compounds for 24 h to test for their toxicity. Finally, the optical density (OD) of each well was immediately recorded at 490 nm to represent cellular viability.

Statistical analysis

The experiments data were analyzed using the Student's t-test in Microsoft Excel 2010 (Microsoft Software Inc, USA). The IC50 was calculated by GraphPad Prism 5.0 (GraphPad Software Inc, San Diego, USA). The data are expressed in terms of mean and standard deviation. Statistically significant values were considered when ** $P < 0.005$ and * $P < 0.05$.

RESULTS AND DISCUSSION

DPPH scavenging activity

Due to its unpaired electron, DPPH radical gives a strong absorption band at 517 nm (deep violet color). The radical loses of this absorption feature when accepting an electron or a free radical species, resulting in a visually noticeable discoloration from violet to yellow. Because the DPPH radical can accommodate many samples in a short time period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used to screen the antiradical activities of compounds or plant extracts. We preferred DPPH to evaluating the scavenging activity of the extracts and compounds of Banlangen. The IC50 values of the DPPH scavenging activity were calculated (Table 1). The results indicated that, isolariciresinol showed a strong DPPH scavenging activity with IC50 values of 24.29 μM, which is superior to that of ascorbic acid as standard. However, the other two compounds did not reach to 50% DPPH radical formation inhibition even at tested highest concentrations. The IC50 of the TL in this assay was found to be 154.03 μg•ml^{-1}, while that of ascorbic acid was 5.63 μg•ml^{-1}; on the other hand, The BLGKL and TOA showed relatively lower activity.

Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant assay, a simple and reliable test that depends upon the reduction of ferric 2, 4, 6–tripyridyl-S-triazine [Fe (Ⅲ)–TPTZ] to the ferrous 2, 4, 6–tripyridyl-S-triazine [Fe (Ⅱ)–TPTZ] complex by a reductant at low pH, was adopted. The procedure is relatively simple and easy to standardize. It has been used frequently in the assessment of antioxidant activity of various plants, fruits and some biological samples. The antioxidant ability of fractions and compounds was estimated by FRAP values, a higher absorbance indicates a higher ferric reducing power. The absorbances of $FeSO_4$ correlated well with its tested concentration. Standard curve of ferrous sulphate (Fe (Ⅱ), 0.3 to 2.5 mM) was $y = 0.4516x + 0.2553$ ($R^2 = 0.9929$). The reducing power of isolariciresinol (A593 =7.16) was far superior to that of ascorbic acid (A593 = 0.78). BLGKL showed the lowest reducing power and, with only approximately 16% of reducing power of TL (A593 = 0.75) Table 2. The absorbance of other extracts and compounds was too small to calculate the FRAP values from the standard curve of ferrous sulphate (data not shown). In our study, isolariciresinol was found to have been highly active in both antioxidant tests, in line with the ref (Kucukboyaci et al., 2010).

Protective effect of extracts and compounds on H_2O_2 injured cell viability in LO2 cell

The LO2 cell viability was markedly decreased after incubation with high concentration of H_2O_2. When H_2O_2, at a dose of 800 μM, was administered to LO2 cell, their viability was decreased to 54%, indicating that the model of cell damage was established. The normal LO2 cell viability of extracts and compounds after 24 h, as determined by MTT assays were all greater than 90%, indicating that these samples were nontoxic at highest tested concentrations (Table 3). The protective effects of Banlangen extracts and compounds against H_2O_2 injury in LO2 cells are shown in Figure 1. H_2O_2 markedly decreased the viability of cell, while they relived the cell damage induced by H_2O_2 in a dose-dependent manner after 24 h treatment. The protective effects of the extracts

Table 2. FRAP values of different extracts and compounds.

Extracts	Tested concentration ($mg\bullet ml^{-1}$)	FRAP [a] (mM FSE/g)	Compounds	Tested concentration ($mg\bullet ml^{-1}$)	FRAP [a] (mM FSE/g)
TL	1.06	0.75 ± 0.01	Ascorbic acid	0.06	0.78 ± 0.01
TOA	1.44	n.c. [b]	Isolariciresinol	0.25	7.16 ± 0.48
TA	3.1	n.c. [b]	Indigotin	0.4	n.c.[b]
BLGKL	1	0.12 ± 0.01	CB	0.24	n.c.[b]

[a]Result are expressed as mean ± SD of three experiments. [b] Effects can be neglected at tested concentrations.

Table 3. Normal LO2 cell viability of different extracts and compounds.

Extracts	Tested concentration ($mg\bullet ml^{-1}$)	Cell viability (%)	Compounds	Tested concentration ($mg\bullet ml^{-1}$)	Cell viability (%)
TL	0.5	98.11	Isolariciresinol	0.05	90.38
TOA	0.1	91.46	Indigotin	0.05	99.81
TA	2	93.69	CB	0.05	91.32
BLGKL	4	90.97			

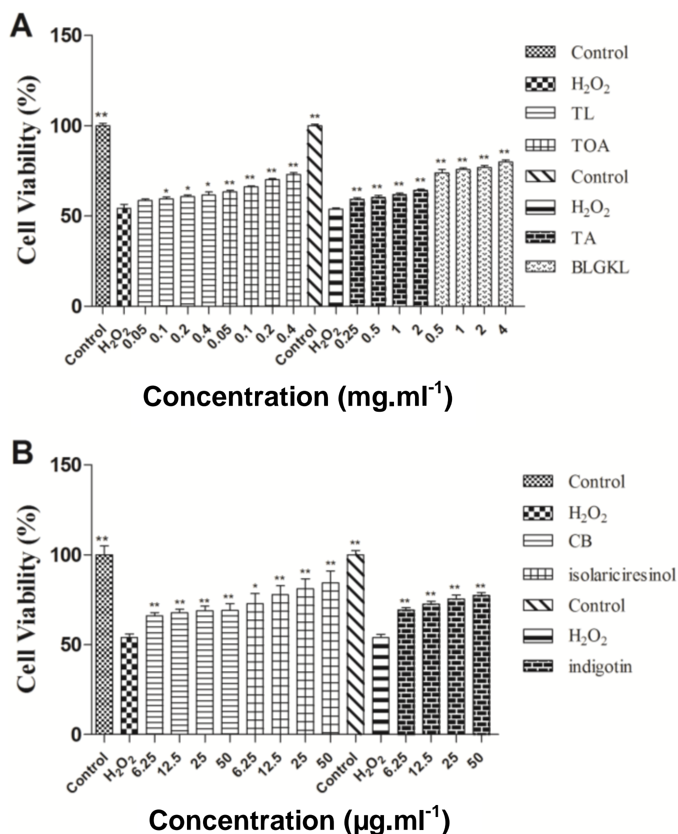

Figure 1. The protective effects of the extracts and three compounds from *Isatidis indigotica* Fort against H_2O_2 induced oxidative stress on hepatocytes LO2 cell. The graph represents the mean ± SD from three independent experiments. ** $p < 0.005$ and * $p < 0.05$, for significant differences from the H_2O_2 single treatment. The statistical significance of the differences was determined by a student's t-test.

against H_2O_2 injury were displayed best in BLGKL, secondarily in TOA. Like TA, TL exhibited lower cell viability. At all tested concentrations, the cell viability of compounds were found in the following order: isolariciresinol > indigotin > CB (Figure 1B).

Studying the banlangen's traditional function of reducing heat and detoxification was not only to study endogenous toxic material (anti-endotoxic, antiviral, anti-inflammatory) but also to consider exogenous toxic material (inflammatory cytokines, oxygen free radical). Previous study showed that organic acids of Banlangen (o-aminobenzoic acid, syringic acid, sali-cylic acid, and benzoic acid) had anti-endotoxic effects *in vitro* (Fang et al., 2005; Liu et al., 2001); alkaloid of banlangen had antiviral activity (Liu et al., 2007; Sun et al., 2010). In our study, total lignan of banlangen had free radical scavenging activity. These results indicated the essence of clearing away heat and toxic material of banlangen from multi-active components, multi-target site.

In conclusion, the BLGKL and TOA showed relatively lower anti-oxidation activity but higher cell viability. However, the single compound (isolariciresinol) displayed strong activity in all tests. Anti-oxidant action has been reported to play an important role in the liver protective ability of many plants (Kukongviriyapan et al., 2003; Popović et al., 2007). Strong *in vitro* anti-oxidation does not necessarily indicate potent protect hepatocytes activity because a discrepancy exists between the IC50 values of antioxidant and cytoprotection effects. This may be due to the cooperating anti-oxidation. Their cooperating antioxidant effects may be caused by their action on different parts of the oxidation and reduction system, or because of a complementation action among those antioxidants. This necessitates a further scrutiny of

its cytoprotection *in vivo* in the future.

Conclusions

In the present investigation, the obtained results suggest that isolariciresinol might be used not only as an antioxidant agent, but as an active agent in the treatment of disorders caused by oxidative stress.

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