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Full Length Research Paper

Antioxidative and anti-inflammatory properties of Chushizi oil from Fructus Broussonetiae

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The fruit of *Broussonetia papyrifera* (L.) L'Herit. ex Vent. is a traditional Chinese medicine with Chinese name Chushizi. It has been commonly used as an important tonic for the treatment of age-related disorders. In this work, the antioxidant properties of the oil were investigated on nitric oxide production in lipopolysaccharide (LPS), activated RAW264.7 cells along with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion and hydroxyl radical scavenging assays. The chemical composition of the oil was analyzed by Gas chromatography-mass spectrometry (GC-MS). The oil mainly consists of 8, 11-octadecadienic acid (79.17%). It possessed DPPH/superoxide anion/hydroxyl free radical scavenging and NO production inhibition activities (IC50 58.00±0.37 µg/ml). The results indicated that Chushizi oil was a powerful antioxidant with versatile free radical-scavenging activity, which may have therapeutic potential in associated with various inflammatory diseases.

Key words: Chushizi, *Broussonetia papyrifera*, anti-inflammatory, antioxidant.

INTRODUCTION

The fruit of *Broussonetia papyrifera* (L.) L'Herit. ex Vent. has been used as a traditional Chinese medicine with long history, and was recorded in Chinese Pharmacopoeia with name Fructus Broussonetiae or Chushizi (Editorial Committee of Chinese Pharmacopoeia, 2010). It has been commonly used as an important tonic for the treatment of age-related disorders. Chushizi contains 32 to 35% fixed oils and 12% amino acids. The fixed oil is composed mainly of unsaturated fatty acids, including linoleic acid, methyl palmitate, oleic acid and linoleic acid ester (Huang et al., 2003; Yuan and Yuan, 2005; Yang and Cui, 2010). The Chushizi oil contains rich unsaturated fatty acid and has high potential pharmaceutical value for human body. *B. papyrifera* possessed promising anti-inflammatory activities (Wang et al., 2010; Jin et al., 2010; Ko et al.,2011). However, there is no report on the material and mechanism of the anti-inflammatory effect of Chushizi. The dynamic equilibrium between generation and scavenging of reactive oxygen species (ROS) is essential in human normal metabolism (Pahlavani and Harris, 1998). It will result in cell and tissue damage when the amounts of ROS in the organism exceed the antioxidant capacity of the organism, which are believed to be linked with inflammation and many other human diseases (Valko et al., 2007). Many antioxidant agents from natural products can possibly be used in the prevention of oxidative stress and inflammation-related disorders (Mao et al., 2011; Luo et al., 2011; Kumar et al., 2010). The aim of this study is to assess the anti-inflammatory activity of Chushizi oil and free radical scavenging, to provide scientific basis for the clinical use of Chushizi.

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Abbreviations: GC-MS, Gas chromatography-mass spectrometry; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **ROS,** reactive oxygen species; **PBS,** phosphate-buffered saline; **Tris,** trishydroxymethylaminomethane; **DMEM,** dulbecco's modified eagle's medium; **DMSO,** dimethyl sulfoxide; **LPS,** lipopolysaccharide; **MTT,** 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; **RSA,** radical scavenging activity; **DSQ,** disability studies quarterly.

MATERIALS AND METHODS

Plant material

Chushizi was purchased from Leiyunshang drug store (Shanghai,

China), and identified by authors. A voucher specimen was deposited at the herbarium of the Department of Pharmacognosy, School of Pharmacy in Second Military Medical University.

Drugs and chemicals

Phosphate-buffered saline (PBS), trishydroxymethylaminomethane (Tris), Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), LPS, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), DPPH and pyrogallol were all obtained from Sigma (St. Louis, Mo, USA). All other reagents from local sources were of analytical grade.

The sample preparation

The dried Chushizi was ground for extracting oil and the powder was then extracted with petroleum ether using soxhlet apparatus for 6 h. The petroleum ether extract was evaporated under reduced pressure to obtain a lemon yellow residue (Chushizi oil).

Gas chromatography-mass spectrometry (GC-MS)

GC/MS analyses were performed using the focus disability studies quarterly (DSQ) equipped with a HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The column temperature was programmed from 50°C, held for 2 min, raised to 300°C at a rate of 10°C/min, and held for 8 min. The injector temperature was 250°C; carrier gas (helium) was set at a flow rate of 1ml/min, ionization energy 70 eV and scan mode EI. The compounds were identified by using the NIST MS spectra search program. The relative amounts of individual components were expressed as percentages of the peak area relative to the total peak area.

Evaluation of intracellular anti-inflammatory activity of the extract

Cell culture

The murine monocyte-macrophage cell line RAW264.7 was purchased from American Type Culture Collection and incubated in DMEM containing 10% heat-inactivated fetal bovine serum and an antibiotic mixture of penicillin (100 U/ml), streptomycin (100 µg/ml). Cells were cultured at 37°C in 5% CO_2.

Measurement of NO production

The assay was performed as Green et al. (1982). RAW264.7 macrophages were plated at a density of 1×10^6 cells in a 96-well. The cells were co-incubated with the extract and LPS (1 µg/ml) for 24 h. The amount of NO was assessed by determining the nitrite concentration in the cultured RAW264.7 macrophage supernatants with Griess reagent. Aliquots of supernatants (100 µl) were incubated, in sequence, with 50 µl of 1% sulfanilamide and 50 µl of 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution. After 30 min at room temperature, absorbance of the plates was measured at 540 nm using the ELISA microplate reader. NO levels were calculated from standard curve prepared with sodium nitrite.

MTT assay for cell viability

Cell viability was determined using the mitochondrial respiration-dependent MTT reduction method. After transferring the required supernatant to another plate for the Griess assay, the remainder was aspirated from the 96-well plates, and 100 µl of fresh medium containing 2 mg/ml of MTT was added to each well. After incubating for 4 h, the medium was removed and the violet crystals of formazan in viable cells were dissolved in DMSO. Absorbance at 570 nm was measured using a microplate reader. Cell viability (%) was calculated as follows:

$$\text{Cell viability (\%)} = [(A_{S1}-A_{S0})/(A_{C1}-A_{C0})] \times 100,$$

Where A_{S1} is the absorbance of oil-treated cells and A_{S0} is the absorbance of oil treated-medium without cells. A_{C1} is the absorbance of cells cultured alone and A_{C0} is the absorbance of blank medium without cells.

Evaluation of antioxidant activity

DPPH radical scavenging activity (RSA) assay

The DPPH assay was carried out using the method described previously (Blois, 1958; Bondet et al., 1997). 100 µl of various concentrations of the tested extract (final concentrations ranging from 0.31 to 5.0 mg/ml) was added to 100 µl of DPPH solution (0.1 mM in MeOH). The mixture was allowed to react for 30 min at room temperature, and then the absorbance of the solution was measured at 517 nm in a spectrophotometer. The percentage of RSA % was calculated as follows:

$$\text{RSA \%} = [(A_c-A_t)/A_c] \times 100\%,$$

Where A_c is the average absorbance of the control and A_t is the absorbance of the tests.

Superoxide anion free radical scavenging activities

The scavenging activities of superoxide anion free radical were determined as previously described. (Marklund and Marklund, 1974; Li et al., 2009) 100 µl of various extracts or solvent were added to 2.8 ml of 50 mM tris-HCl buffer (pH 8.2) for10 min at 25°C, then 100 µl of 60 mM pytogallol or 10 mM HCl were added in the assay system. After rapidly shook, the absorbance of the mixture was measured at 420 nm in 30 sec interval for 4 min (keeping the auto-oxidation rate of pyrogallol at 0.005 to 0.065 OD/min). The changing curve of the absorption value (OD) to time was obtained and the curve slope was defined as antioxidation activities of the sample on superoxide anion. The formula was as following:

$$R\ (\%) = (\Delta A'_{420}/\Delta T - \Delta A_{420}/\Delta T)/\Delta A'_{420}/\Delta T \times 100$$

In this formula, $\Delta A'_{420}/\Delta T$ is the auto-oxidation rate of pyrogallol (OD/min), and $\Delta A_{420}/\Delta T$ is the auto-oxidation rate of sample (OD/min).

Hydrogen peroxide scavenging activity assay

Peroxide scavenging activity was measured according to a modified method of Smirnoff and Cumbes (Smirnoff and Cumbes, 1989). Peroxide radicals were generated from the mixture of Fe^{2+} and H_2O_2. The reaction mixture contained 1 ml $FeSO_4$ (1.5 mM), 0.7 ml H_2O_2 (6 mM), 0.3 ml sodium salicylate (20 mM) and sample. After incubation for 1 h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as follows:

$$\text{The peroxide scavenging activity (\%)} = [1-(A_1-A_2)/A_0] \times 100$$

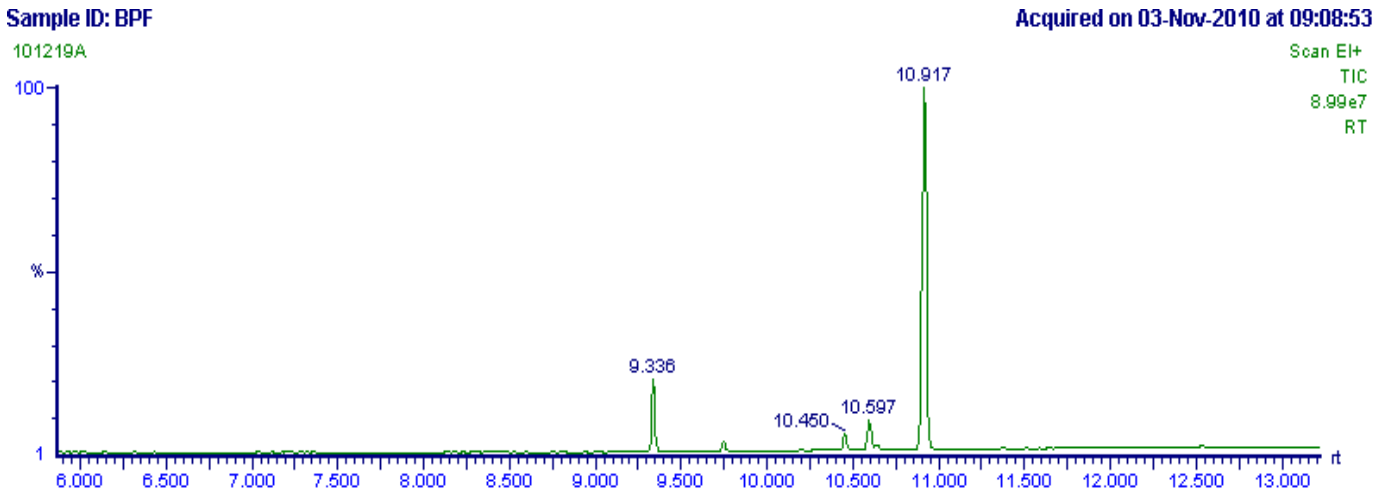

Figure 1. The GC-MS fingerprint of the Chushizi oil.

Table 1. The chemical compounds of the Chushizi oil by GC-MS.

Compound	RT (min)	Percentage
Palmitic acid	9.336	10.77
Stearic acid	10.450	3.04
Oleic acid	10.597	5.51
8-Octadecenoic acid	10.637	1.00
8,11-Octadecadienic acid	10.917	79.17
Linolenic acid	11.377	0.51

RT, retention time.

Where A_0 is the absorbance of the control (without extract or standards) and A_1 is the absorbance including the extract or standard, A_2 was the absorbance without sodium salicylate.

Statistical analysis

All results were expressed as mean ± S.D. Statistical analysis was performed according to the SPSS-PC package. Analyses of variance were performed using the ANOVA procedure. The significance difference was set at $P<0.05$.

RESULTS AND DISCUSSION

Chemical compounds in the Chushizi oil

The results of GC-MS analysis on the Chushizi oil showed predominance of fatty acids (Figure 1 and Table 1). A total of 6 constituents representing 100% of the fraction were identified. 8, 11-octadecadienic acid (79.17%) were found to be the major constituents, followed by palmitic acid (10.77%). Among the remaining constituents (10.06%), linolenic acid, 8-octadecenoic acid, stearic acid and oleic acid were detected in percentages ranging from 0.51 to 5.51%.

Inhibition of NO production in LPS-activated macrophages

LPS is a large molecule comprising of a lipid and a polysaccharide joined by a covalent bond and it promotes the secretion of pro-inflammatory cytokines from many cell types, especially macrophages (Wang et al., 2011). In order to evaluate the anti-inflammatory capacities of the Chushizi oil, RAW264.7 cells were challenged with LPS in the presence or absence of the extracts, and the level of NO in the medium was measured. Stimulation of RAW264.7 cells with LPS (1 μg/ml) for 24 h increased nitrite production dramatically from the basal level of 6±0.03 μM to 57.4±1.27 μM as measured by the Griess reaction. The Chushizi oil had the good inhibition effect on the NO production, with IC_{50} value of 58.00±0.37 μg/ml and NO inhibition rate 73.83±0.21 % at dose of 100 μg/ml. As shown in Figure 2, the Chushizi oil inhibited NO production in concentration-dependent manner. In

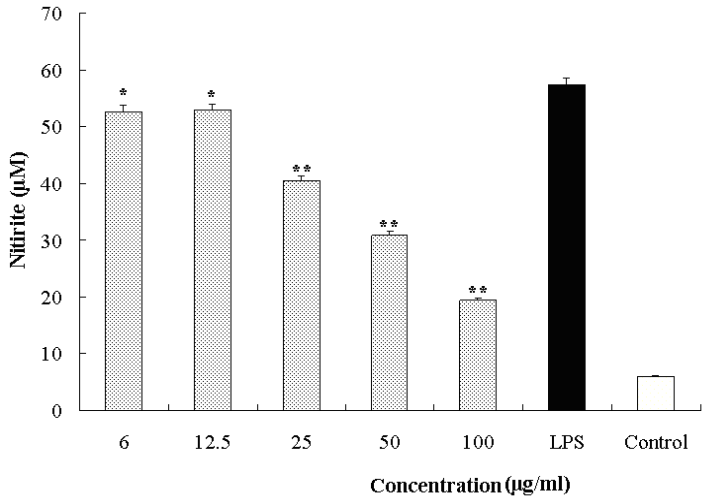

Figure 2. Effect of Chushizi oil on NO production in RAW264.7 cells. Cells are treated with Chushizi oil of different concentrations (6, 12.5, 25, 50 and 100 μg/ml) together with 100 μl LPS (1μg/ml). Values are mean ± SD (n=4). Statistical significance is based on the difference when compared with the cells cultured alone (*P<0.05, **P<0.01). LPS: lipopolysaccharides from *Escherichia coli*.

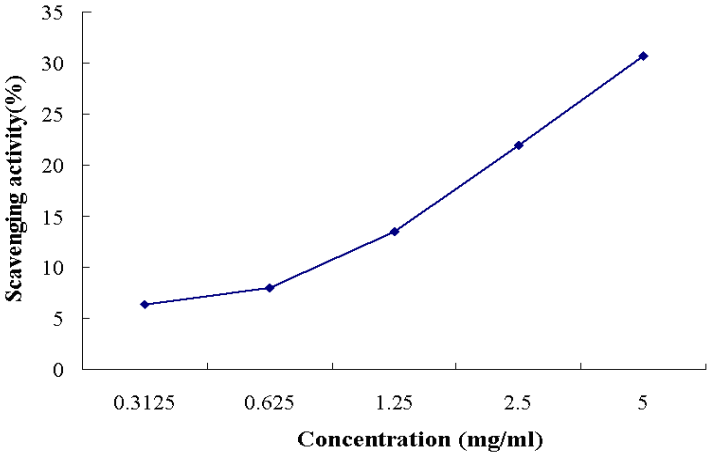

Figure 3. Effect of different concentrations of Chushizi oil on DPPH radical scavenging activity.

concentration range of 6 to 100 μg/ml, the group of Chushizi oil indicated significantly different from LPS group (*P<0.01*). Examining cytotoxicity of the Chushizi oil in RAW264.7 cells, no notable cytotoxicity (cell viability>88%) was observed when the cells were exposed to the oil up to the level of 100 μg/ml for 24 h.

DPPH radical scavenging activities

The results of the DPPH radical scavenging test for the Chushizi oil demonstrated significant decreases in the concentration of the DPPH radical (Figure 3). That exhibited appreciable scavenging properties against DPPH radical, with IC_{50} 8.20±0.003 mg/ml. The Chushizi oil possessed the inhibition percentage (30.60±0.05%) when the concentration was set at 5 mg/ml, while the inhibition rate was proportional to the concentration.

Superoxide anion radical scavenging activities

The scavenging effect of the Chushizi oil on superoxide anion free radical was shown in Figure 4. Chushizi oil was the good superoxide anion free radical scavenger (IC_{50}, 89.86±3.40 mg/ml), which was similar to DPPH

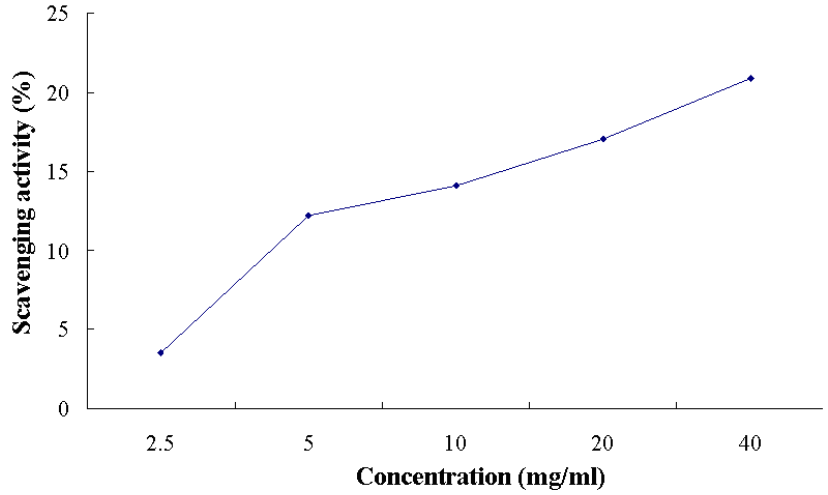

Figure 4. Effect of different concentrations of Chushizi oil on superoxide anion radical scavenging activity.

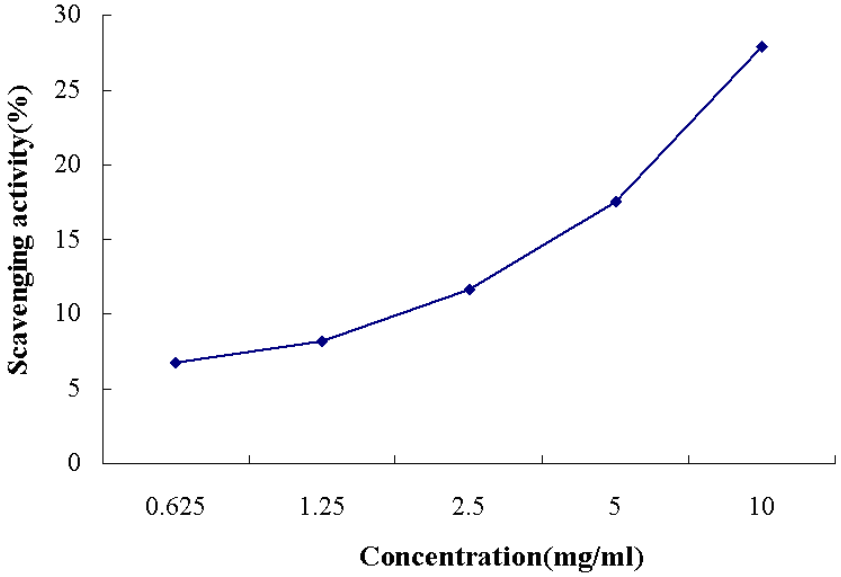

Figure 5. Effect of different concentrations of Chushizi oil on hydroxyl radical scavenging activity.

scavenging property. Chushizi oil also showed a dose-dependent inhibition on the superoxide anion free radical.

Hydroxyl radical scavenging activities

Hydrogen peroxide-scavenging activity of five different concentrations of Chushizi oil was investigated on the Chushizi oil using the Fenton reaction mechanism. In the range of 0.625-10 mg/ml, the Chushizi oil displayed a dose-dependent inhibition on the hydrogen peroxide. The IC_{50} value for hydrogen peroxide was found to be about 19.63±0.36 mg/ml, and the inhibition ratio of Chushizi oil was 27.92±0.93% at the concentration of 10 ml/mg (Figure 5).

Conclusions

B. papyrifera is an important medicinal plant and its fruit (Chushizi) is a common traditional Chinese medicine. Chushizi oil is mainly consisted of unsaturated fatty acid,

and the 8, 11-Octadecadienic acid is its major component. The Chushizi oil is a powerful natural antioxidant and that its antioxidant activities may be substantially attributed to its free radical-scavenging activity, including DPPH, superoxide anion, hydrogen peroxide. Furthermore, it was able to suppress LPS-induced NO production in RAW264.7 macrophages. Excessive free radical and NO production is reported to be associated with various inflammatory diseases, so the Chushizi oil worth further research for developing anti-inflammatory agent.

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