

Research Article

Bioassay-guided Separation and Identification of Pharmacodynamic Constituents with Nitrite-scavenging Effect in *Aquilaria sinensis* Leaves

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Abstract: To explore the pharmacodynamic constituents with nitrite-scavenging effect in *Aquilaria sinensis* leaves, a new food raw material, applying bioassay-guided method by using UV spectrophotometry, scavenging rates of crude extracts of wild and cultivated *A.* leaves with different solvents, each polar fraction of the crude extracts and each cut fractions from the *n*-butanol fraction were tested. It is disclosed that scavenging effect of the crude extract from wild *A.* leaves was better than that from cultivated. *n*-Butanol fraction of the crude extract from wild *A.* with 70% acetone displayed the best scavenging effect among all the counterparts. Mangiferin (1) and 2-O- α -L-rhamnopyranosyl-4, 6, 4'-trihydroxybenzophenone (2) were identified as the two most active compounds with scavenging rate up to 29.4 \pm 0.7% and 24.6 \pm 0.8%, at the conditions of pH 3.0, 37°C and 30 min treatment, respectively.

Keywords: *Aquilaria sinensis*, bioassay-guided method, mangiferin, nitrite

INTRODUCTION

Sodium nitrite, which is widely used in food and drug industries as a preservative, is omnipresent in the environment and can also be formed from nitrogenous compounds by microorganisms present in the soil, water, saliva and the gastrointestinal tract (Chow and Hong, 2002; Omar and Webb, 2014). When human body ingests nitrite, endogenous nitrosation may form N-nitrosocompounds (NOCs) that have been reported to induce tumors of the kidney in animals. In the stomach of human body, nitrite can react with amines, amides or amino acids to produce NOCs, most of which are potent carcinogens (Grosse *et al.*, 2006). Therefore, scavenging ingested nitrite is probably one way to prevent cancers caused by NOCs (Bryann *et al.*, 2012; Grosse *et al.*, 2006; Liu *et al.*, 2011).

Aquilaria sinensis leaves, a new food raw material, are broadly used as a main component in several health foods including *A. sinensis* tea and flavor in China. *A. sinensis* leaves exhibited notable antinociceptive, anti-inflammatory, antioxidative, α -glucosidase inhibitory and laxative activities (Chen *et al.*, 2013). Our previous study indicated that the extract from *A. sinensis* leaves was confirmed to have nitrite scavenging activities, which pharmacodynamic material basis remain further

investigation (Yang *et al.*, 2012). Bioassay-guided method is a frequently-used and efficient separation method in natural product research (Wu, 2008), α -glucosidase inhibitors from the leaves of *A. sinensis* were isolated by activity-directed fractionation and purification processes in Feng's study (Feng *et al.*, 2011). Pharmacodynamic constituents of *A. sinensis* leaves with nitrite-scavenging effect were separated and identified by bioassay-guided method in this study, to establish the scientific basis for further development and use of *A. sinensis* leaves.

MATERIALS AND METHODS

Instruments and materials: A UV-spectrophotometer (Beijing's General Instrument Co., LTD, China). A finnigan LCQ Advantage MAX ESI-mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), a nuclear magnetic resonance (NMR) instrument from a Bruker AV-300 or Bruker AV-400 (Bruker Biospin, Rheinstetten, Baden-Württemberg, Germany).

Silica gel (100-200, 200-300 mesh) was purchased from Qingdao Ocean Chemical Co. (Qing-dao, China); and pre-coated silica gel HSGF254 Thin Layer Chromatography (TLC) plates were obtained from Jiangyou Silica Gel Development Co. (Yantai, China).

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Analytic grade ethanol, ethyl acetate (EtOAc), n-butanol, petroleum ether (b.p. 60-90°C), acetone, sodium nitrite, sulfanilic acid, N-ethylenediamine, citrate sodium, monosodium phosphate, dimethylsulfoxide, chloroform, methanol and muriatic acid were purchased from Guangzhou Chemical Reagent Co. (Guangzhou, China).

The leaves of *A. sinensis* were collected from Zhongshan City, Guangdong Province, China. The plant material was botanically authenticated by Prof. Zhijian Feng in College of Forestry, South China Agricultural University.

Nitrite scavenging test: Nitrite scavenging test using a UV spectrophotometer at a wavelength of 544 nm performed as described previously (Jahanban *et al.*, 2009). The conditions were modified as follows: sodium nitrite (5.0 g/mL), naphthyl ethylene diamine dihydrochloride (0.2% w/v), sulfanilic acid (0.4% w/v), reaction temperature 37°C, reaction time 30 min, citric acid/sodium dihydrogenphosphate buffer solution at pH value of 3.0 and sample concentration of 3.0 mg/mL (dissolved in dimethylsulfoxide). The reaction mixture (3 mL) containing sodium nitrite (2 mL), sample solution (0.5 mL) and buffer solution (0.5 mL) (for adjusting the pH value of 3.0) was incubated at 37°C for 30 min. After incubation, 0.5 mL of the reaction mixture mixed with 2 mL of sulfanilic acid and allowed to stand for 5 min for completing diazotization. Then, 1 mL of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 37°C. A pink colored chromophore was formed in diffused light. The absorbance of sample solutions was measured at 544 nm against the corresponding blank solution (dimethylsulfoxide) and the % scavenging value was computed according to the following formula:

Nitrite scavenging percentage =

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

where, the $A_{control}$ is the absorbance of solution without the addition of sample solution.

Scavenging rates of crude extracts of wild and cultivated *Aquilaria sinensis* leaves with different solvents: Oven-dried leaves of wild and cultivated *A. sinensis* leaves were extracted with distilled water, ethanol and 70% acetone for 5 h under percolation method. The filtered extract was condensed under vacuum to give a syrupy extract, which was then conducted nitrite scavenging test as previously mentioned. The extract with the highest nitrite scavenging rate were used to carry out follow-up experiments.

Bioassay-guided separation of nitrite-scavenging constituents in wild *Aquilaria sinensis* leaves: Oven-dried leaves of wild *A. sinensis* (3.12 kg) were extracted with 70% acetone for 5 h under percolation method. The combined extract was condensed under vacuum to give a syrupy extract (601.90 g), which was then diluted with water and then partitioned successively with petroleum ether, EtOAc and n-butanol. The combined layers of each organic solvent were evaporated in vacuo to yield a petroleum ether-soluble fraction (F_P , 42.70 g), an EtOAc-soluble fraction (F_E , 139.02 g) and a n-butanol-soluble fraction (F_B , 165.21 g), respectively.

The three fractions were then conducted nitrite scavenging test according to 1.2.1. The fraction with the highest nitrite scavenging rate was further subjected to silica gel column chromatography using a gradient elution with chloroform/methanol (4:1~0:1, V/V) to give 2 sub-fractions Fr. b 1 (1.37 g), Fr. b 2 (2.51 g), Fr. b 3 (2.32 g), Fr. b 4 (7.50 g), Fr. b 5 (5.87 g), Fr. b 6 (3.76 g), Fr. b 7 (6.03 g), Fr. b 8 (0.89 g), Fr. b 9 (4.86 g), Fr. b 10 (4.91 g) and Fr. b 11 (0.77 g) on the basis of TLC tracing.

The sub-fractions were then conducted nitrite scavenging test according to as described previously. The two sub-fractions with the highest nitrite scavenging rate were further purified by positive phase silica gel column chromatography to give effective compounds.

RESULTS AND DISCUSSION

As everyone knows, wild plants often show different characteristics or pharmacological function comparing with their corresponding cultivated plants and the separation efficiency and quality of active ingredients is affected by extraction solvent. Scavenging rates of crude extracts of wild and cultivated *A. sinensis* leaves with different solvents was investigated in this study at first.

Scavenging rates of crude extracts of wild and cultivated *Aquilaria sinensis* leaves with different solvents: The results of scavenging rates of crude extracts of wild and cultivated *A. sinensis* leaves with different solventst were listed in Table 1.

In Table 1, scavenging effect of the crude extract from wild *A. sinensis* leaves was better than that from cultivated *A. sinensis* leaves, no matter in what solvent extraction. The best scavenging crude extract was 70% acetone extractive from wild *A. sinensis* leaves, with the nitrite scavenging rate up to 47.7%±0.3%.

Scavenging rates of different polar fractions from wild *Aquilaria sinensis* leaves by 70% acetones: The result of scavenging rates of different polar fractions from wild *A. sinensis* leaves by 70% acetones were listed in Table 2.

Table 1: Nitrite scavenging activities of extracts with different solvent from wild and cultivated *Aquilaria sinensis* leaves

Type of leaves	Type of solvent	Nitrite scavenging rate (%) ^a
Wild	Distilled water	40.6±0.3
Wild	Ethanol	36.6±0.2
Wild	70% acetone	47.7±0.3
Cultivated	Distilled water	30.6±0.6
Cultivated	Ethanol	31.8±0.9
Cultivated	70% acetone	27.2±0.4

^a: Data expressed as mean±SEM (standard error of mean) of three observation per sample

Table 2: Nitrite scavenging activities of different polar fractions extracted from wild *Aquilaria sinensis* leaves by 70% acetone

Polar fractions	Nitrite scavenging rate (%) ^a
Petroleum ether fraction	7.7±0.7
Ethyl acetate fraction	10.3±0.4
<i>n</i> -Butanol fraction	38.3±0.9

^a: Data expressed as mean ± SEM (standard error of mean) of three observation per sample

Table 3: Nitrite scavenging activities of different cut fractions from the *n*-butanol fraction

Code of cut fractions	Nitrite scavenging rate (%) ^a
Fr. b 1	4.7±0.3
Fr. b 2	12.9±0.6
Fr. b 3	2.5±0.4
Fr. b 4	9.5±0.6
Fr. b 5	9.2±0.6
Fr. b 6	7.4±0.6
Fr. b 7	4.7±0.6
Fr. b 8	5.9±0.8
Fr. b 9	6.7±0.5
Fr. b 10	12.4±0.4
Fr. b 11	16.6±0.4

^a: Data expressed as mean±SEM (standard error of mean) of three observation per sample

Table 4: Nitrite scavenging activities of Compound 1 and Compound 2

Code of Compounds	Nitrite scavenging rate (%) ^a
Compound 1	29.4±0.7
Compound 2	24.6±0.8

^a: Data expressed as mean±SEM (standard error of mean) of three observation per sample

In Table 2, the best scavenging polar fractions was *n*-Butanol fraction, with the nitrite scavenging rate up to 38.3±0.9%, much higher than the other two polar fractions, indicated that effective component mainly concentrated on the high polarity fraction.

Scavenging rates of different cut fractions from the *n*-butanol fraction: The result of scavenging rates of different cut fractions from the *n*-butanol fraction were listed in Table 3.

In Table 3, the better scavenging cut fractions from the *n*-butanol fraction were Fr. b 11, Fr. b 2 and so on, with the nitrite scavenging rate up to 16.6±0.4%, which far below scavenging rate of *n*-butanol fraction. What is interesting, the nitrite scavenging rates of different polar fractions from wild *A. sinensis* leaves by 70% acetone were also lower than the nitrite scavenging rates of crude extract from wild *A. sinensis* leaves by 70%

acetone. Accordingly, effective components of nitrite scavenging showed synergistic effect, scavenging effect would reduce if the effective components were apart.

Scavenging rates of effective compounds: Fr. b 11 and Fr. b 2 were further purified by positive phase silica gel column chromatography to give Compound 1 (0.34 g) and Compound 2 (0.75 g). The scavenging rates of the two effective Compounds were listed in Table 4.

In Table 3 and 4, the scavenging rates of the two effective compounds were higher than their corresponding cut fractions, which further verified effective components of nitrite scavenging showed synergistic effect.

Structural identification of compound 1 and compound 2 by ESI-MS and NMR:

Compound 1: ESI-MS *m/z*: 421.2 [M-H]⁻. ¹H NMR (300 MHz, DMSO-*d*₆) δ: 13.76 (1H, s, -OH), 7.37 (1H, s, H-8), 6.86 (1H, s, H-5), 6.37 (1H, s, H-4), 4.59 (1H, d, *J* = 9.0 Hz, Glc-H-1); ¹³C NMR (75MHz, DMSO-*d*₆) δ: 179.2 (C-9), 163.9 (C-3), 161.9 (C-1), 156.3 (C-4a), 154.2 (C-6), 150.9 (C-10a), 143.8 (C-7), 111.8 (C-8a), 108.1 (C-8), 107.7 (C-2), 102.7 (C-5), 101.4 (C-9a), 93.4 (C-4), 81.7 (glc-C-5), 79.1 (glc-C-3), 73.2 (glc-C-1), 70.7 (glc-C-2), 70.3 (glc-C-4), 61.6 (glc-C-6). Compared the data with that of literature (Hideaki *et al.*, 2008), this compound was identified as mangiferin.

Compound 2: HR-EI-MS *m/z*: 393.1171 ([M+H]⁺). ¹H NMR (600 MHz, CD₃OD) δ: 7.63 (1H, d, *J* = 9.0 Hz, H-2'), 6.83 (1H, d, *J* = 9.0 Hz, H-3'), 6.33 (1H, d, *J* = 1.8 Hz, H-3), 6.10 (1H, d, *J* = 1.8 Hz, H-5), 5.24 (1H, d, *J* = 1.2 Hz, H-1''), 3.45 (1H, m, H-5''), 3.43 (1H, m, H-3''), 3.31 (1H, m, H-4''), 3.31 (1H, m, H-4''), 3.14 (1H, m, H-2''), 1.21 (1H, d, *J* = 6.0 Hz, H-6''); ¹³C NMR (150 MHz, CD₃OD), δ: 196.2 (C-7), 162.0 (C-4'), 161.5 (C-4), 159.1 (C-6), 157.0 (C-2), 131.4 (C-1', C-2', C-6'), 114.7 (C-3', C-5'), 108.2 (C-1), 99.1 (C-1''), 96.6 (C-5), 94.2 (C-3), 72.2 (C-4''), 70.5 (C-2''), 70.2 (C-3''), 69.4 (C-5''), 16.6 (C-6''). Compared the data with that of literature (Wang *et al.*, 2008), this compound was identified as 2-*O*- α -L-rhamnopyranosyl-4, 6, 4'-trihydroxybenzophenone.h.

CONCLUSION

According to the experimental result of this study though the bioassay-guided method, scavenging effect of the crude extract from wild *A. sinensis* leaves was better than that from cultivated *A. sinensis* leaves, no matter in what solvent extraction. The best scavenging crude extract was 70% acetone extractive from wild *A. sinensis* leaves, with the nitrite scavenging rate up to 47.7±0.3%. Effective component mainly concentrated on the high polarity fraction, while effective components of different polar fractions showed

synergistic effect. As to the monomeric compounds, the best nitrite scavenging components were mangiferin (Compound 1) and 2-O- α -L-rhamnopyranosyl-4, 6, 4'-trihydroxybenzophenone (Compound 2), with the nitrite scavenging rate up to 29.4 \pm 0.7% and 24.6 \pm 0.8%.

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REFERENCES

- Bryann, N.S., D.D. Alexander, J.R. Coughlin, A.L. Milkowski and P. Boffetta, 2012. Ingested nitrate and nitrite and stomach cancer risk: An updated review. *Food Chem. Toxicol.*, 50(10): 3646-3665.
- Chen, D.L., Y. Wu, L. Lin, O. Shuai, K.Y. Wang, H.M. Zhang and S.H. Liu, 2013. Evaluation of the in vitro antioxidant activity and in vivo blood lipid-lowering capability of Chenxiang tea extracts. *Mod. Food Sci. Technol.*, 29(6): 1198-1201, 1242.
- Chow, C.K. and C.B. Hong, 2002. Dietary vitamin E and selenium and toxicity of nitrite and nitrate. *Toxicology*, 180(2): 195-207.
- Feng, J., X.W. Yang and R.F. Wang, 2011. Bio-assay guided isolation and identification of α -glucosidase inhibitors from the leaves of *Aquilaria sinensis*. *Phytochemistry*, 72: 242-247.
- Grosse, Y., R. Baan, K. Straif, B. Secretan, F. El Ghissassi *et al.*, 2006. Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. *Lancet Oncol.*, 7(8): 628-629.
- Hideaki, H., I. Yasuaki, M. Nobutaka, S. Masamitsu, I. Koji *et al.*, 2008. Laxative effect of agarwood leaves and its mechanism. *Biosci. Biotech. Bioch.*, 72(2): 335-345.
- Jahanban, A.S., A. Mahmoodzadeh, A. Hasanzadeh, R. Heidari and R. Jamei, 2009. Antioxidants and antiradicals in almond hull and shell (*Amygdalus communis* L.) as a function of genotype. *Food Chem.*, 115(2): 529-533.
- Liu, J., S. Lin, Z.Z. Wang, C. Wang, E. Wang, Y. Zhang and J. Liu, 2011. Supercritical fluid extraction of flavonoids from *Maydis stigma* and its nitrite-scavenging ability. *Food Bioprod. Process.*, 89(4): 333-339.
- Omar, S.A. and A.J. Webb, 2014. Nitrite reduction and cardiovascular protection. *J. Mol. Cell. Cardiol.*, 73: 57-69.
- Wang, H.G., M.H. Zhou, J.J. Lu and B.Y. Yu, 2008. Antitumor Constituents from the leaves of *Aquilaria sinensis* (Lour.) gilg. *Chem. Ind. Forest Prod.*, 28 (2): 1-5.
- Wu, L.J., 2008. *Natural Pharmaceutical Chemistry*. People's Medical Publishing House, Beijing, China.
- Yang, M.X., S.S. Mao and H.R. Chen, 2012. Scavenging effect of different fractions extracted from leaves of *Aquilaria sinensis* on NO₂⁻. *J. Beihua Univ., Nat. Sci.*, 13: 406-409.