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The sulfur-fumigation reduces chemical composition and biological properties of Angelicae sinensis radix

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ABSTRACT

Angelica Sinensis Radix (roots of *Angelica sinensis*; ASR) is a popular herbal supplement in China for promoting blood circulation. Today, sulfur-fumigation is commonly used to treat ASR as a means of pest control; however, the studies of sulfur-fumigation on the safety and efficacy of ASR are very limited. Here, we elucidated the destructive roles of sulfur-fumigation on ASR by chemical and biological assessments. After sulfur-fumigation, the chemicals in ASR were significantly lost. The biological activities of anti-platelet aggregation, induction of NO production and estrogenic properties were compared between the water extracts of non-fumigated and sulfur-fumigated ASR. In all cases, the sulfur-fumigation significantly reduced the biological properties of ASR. In addition, application of water extract deriving from sulfur-fumigated ASR showed toxicity to cultured MCF-7 cells. In order to ensure the safety and to achieve the best therapeutic effect, it is recommended that sulfur-fumigation is an unacceptable approach for processing herbal materials.

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Introduction

Angelica Sinensis Radix (roots of *Angelica sinensis*; ASR), well known as "female ginseng", is one of the commonly used traditional Chinese medicines (TCM). ASR contains significant amounts of organic acids, volatile oils and polysaccharides, which are considered to be the biologically active components (Zhao et al., 2003; Yi et al., 2009). Clinically, ASR is good in replenishing and invigorating blood, stopping pain and moistening the intestines (Yi et al., 2009). Very often, ASR is being used to promote blood circulation in treating menstrual disorders, e.g. amenorrhea and dysmenorrheal (Wilasrusmee et al., 2002; Gao et al., 2006). Meanwhile, ASR is also used as a health food supplement for women's care in Europe and America.

Traditionally, the drying of post-harvested ASR under the sun is the standard commodity of herb preparation. In recent years, sulfur-fumigation is commonly used to replace the natural drying process. In fact, sulfur-fumigation has been employed in handling of numerous medicinal herbs as to shorten the drying duration, to control pests and to maintain a better appearance. However, this chemical processing was recently reported to alter bioactive components within the herbs, and consequently the bioactivities and pharmacokinetics of herbs were changed (Wang et al., 2009; Liu et al., 2010). According to this notion, the amount of ferulic acid in ASR was decreased significantly after sulfur-fumigation (Zhao et al., 2003). The chemical comparison of ASR and sulfur-fumigated ASR (S-ASR) is not fully revealed. More important, the role of sulfur-fumigation in the bioactivities of ASR has not been addressed, and the herbal industries do not have a full picture regarding this chemical processing of ASR. Here, the chemical and biological properties of ASR and S-ASR were fully compared for the first time. The results could provide information for a better usage of ASR in clinical practice.

Materials and methods

Plant materials and reagents

Roots of *Angelica sinensis* (Oliv.) Diels (ASR) were obtained from Minxian of Gansu in China in October of 2009. The authentication of

Abbreviations: ASR, Angelicae Sinensis Radix; TCM, traditional Chinese medicine.

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the herbs was confirmed morphologically by Dr. Tina Dong at Hong Kong University of Science and Technology (HKUST). The voucher specimens (voucher # 02-9-1) were deposited in the Centre for Chinese Medicine R&D at HKUST. Butylphthalide, senkyunolide A, Z-butylidenephthalide, senkyunolide I and senkyunolide H were purchased from Weiqike Biotechnology Co. (Sichuan, China). Ferulic acid was obtained from Sigma (St. Louis, MO). Z-Ligustilide was purchased from TLCM (Hong Kong, China). All chemical standards were confirmed to show >98% purity based on their GC and MS data. Analytical- and HPLC-grade reagents were from Merck (Darmstadt, Germany).

Preparation of sulfur-fumigated ASR

The sulfur-fumigated ASR was prepared following modified procedures similar to those performed by herbal farmers. Five hundred grams of ASR samples were soaked with 50 ml water for 0.5 h, and 50 g of sulfur powder was heated until self-ignition. Then, the burning sulfur and the wetted ASR were carefully put into the lower and upper layer of a desiccator, respectively. The desiccator was then kept closed for 12 h (Jiang et al. 2013). Afterward, the sulfur-fumigated ASR were taken out and dried at room temperature for 12 h.

Preparation of herbal extracts

For GC-QQQ-MS/MS analysis, the ethyl acetate extraction of ASR and S-ASR was performed previously (Zhan et al., 2013a). In water extraction of ASR and S-ASR, about 15 g of root was weighed, boiled in 120 ml of water for 2 h, and extracted twice. For the second extraction, the same extracting conditions were applied. The extracts were dried under vacuum and stored at −80 °C, which was used for HPLC analysis, total chemical parts analysis and biological determination.

Chemical analysis

The water extracts of ASR were quantitative analyzed by HPLC. A Waters HPLC system consisting of a 600 pump, a 717 auto-sampler, and a UV/VIS photodiode array 2996 detector was used for the analysis. The chromatographic condition was as described previously (Zhan et al., 2011). The signals were detected at 325 nm for ferulic acid and *Z*-ligustilide, and at 280 nm for senkyunolide I and senkyunolide H with a photodiode array detector. The determination of total organic acids (Lu et al., 2010), total volatile oils (Shao, 2010), total polysaccharides and total flavonoids (Dong et al., 2006) were performed, as described previously.

Agilent 7000 GC/MS/MS series system (Agilent, Waldbronn, Germany) was also applied, which was equipped with an Agilent 7890A gas chromatography and GC-QQQ MassHunter workstation software. The extract was separated on an Agilent HP-5MS capillary column (250 μm × 30 m × 0.25 μm). The chromatographic condition was described previously (Zhan et al., 2013a). The volatile compounds were authenticated by comparing the mass spectra with the Kovats retention indices and NIST standard reference database (NIST 08). For the MS/MS analysis, the suitable precursor ion and two product ions were chosen for acquisition in MRM mode for ferulic acid, butylphthalide, Z-butylidenephthalide, senkyunolide A, Z-ligustilide, senkyunolide H, senkyunolide I and paeonol (internal standard). Agilent MassHunter software was used for data acquisition and processing.

Sulfur dioxide residue analysis

The distillation method was used for the determination of sulfur dioxide residue, as described in Chinese Pharmacopeia Appendix IX (Shao, 2010). 10 g of the fine powder of crude drug or processed pieces was weighed into an 1000 ml round bottom flask, added with 300–400 ml of water, 10 ml of 6 mol/l hydrochloric acid and a few glass beads, shook and mixed well. Heated the flask gently in an electric heating jacket until boiling begins and continued boiling for 3 min. The iodine titrant was used to titrate until the blue color was not changed within 20 s. Sulfur dioxide residue in samples was calculated according to the following equation: $P = (A - B) \times C \times 0.032 \times 1000/W$. P was the amount of sulfur dioxide residue in samples (mg/g); A was the volume of consumed iodine titrant by samples (ml); B was the volume of consumed iodine titrant by blank (ml); C was the concentration of iodine titrant (0.01 mol/l); W was the weight of samples (g); and 0.032 was the weight of sulfur dioxide which was equivalent to 1 ml of iodine titrant (1 M).

Anti-platelet aggregation assay

Blood was collected from adult New Zealand white rabbits. The blood plasma was collected as described previously (Dong et al., 2006). The ASR water extracts were added 5 min before adenosine 5′-diphosphate (ADP, inducer; 10 μM final). The aggregations were recorded using a Sanda-196 platelet aggregator (Shanghai, China). The inhibition activity of platelet aggregation was calculated by the formula: (ADP-induced A_{max} − sample-induced A_{max})/(ADP-induced A_{max}) × 100%.

MCF-7 cell viability and estrogenic assay

Human mammary epithelial carcinoma MCF-7 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were grown in modified Eagles medium, supplemented with 10% FBS, L-glutamine, pyruvate and penicillin-streptomycinin in a humidified CO_2 (5%) incubator at 37 °C. The cell viability was measured by 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Zhan et al., 2011). Cultured MCF-7 cells were transfected with pERE-Luc to generate stable MCF-7-ERE-Luc cells according to a previous report (Zhan et al., 2011). The pERE-Luc expressed cells were treated with herbal extracts for 48 h. Afterward, the cells were collected by the lysis buffer containing 0.2% Triton X-100, 1 mM dithiothreitol, and 100 mM potassium phosphate (pH 7.8). The lysates were then subjected to luciferase assay (Tropix, Inc.), and the activity was expressed as per milligram of cell protein.

Induction of NO production in HUVECs

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (San Diego, CA). Cultured HUVECs were maintained at 4.5×10^5 cells/ml in EGM Bulletkit medium in a humidified incubator with 95% air, 5% CO_2. The medium was replaced by 0.1 ml of serum and growth factor free medium containing ASR or S-ASR extracts every day. The concentrations of NO in the culture medium were measured with the NO detection kit (Biovision, Mountain View, CA) according to the manufacturer's instructions (Leung et al., 2006).

Fluorimetric measurements of NO production were also performed on cultured HUVEC cells using an Olympus Fluoview FV1000 laser scanning confocal system mounted on an inverted I×81 Olympus microscope, equipped with a 10× objective (NA 0.5). Cultured HUVEC cells, seeded on glass cover slips, were incubated for 30 min at room temperature in normal physiological solution containing 1 M DAF-FM DA. The amounts of NO were evaluated by measuring the fluorescence intensity excited at 495 nm and emitted at 515 nm (Bi et al., 2010).

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