Journal of Ethnopharmacology 211 (2018) 101–116

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

A novel approach using metabolomics coupled with hematological and biochemical parameters to explain the enriching-blood effect and mechanism of unprocessed Angelica sinensis and its 4 kinds of processed products

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ARTICLE INFO

Chemical compounds studied in this article:
Urea (Pubchem CID: 1176)
2-Aminobutyric acid (Pubchem CID: 80283)
glycylglycine (Pubchem CID: 11163)
Gluconic acid (Pubchem CID: 10690)
L-Valine (Pubchem CID: 6287)
Butanoic acid (Pubchem CID: 264)
D-glucose (Pubchem CID: 5793)
glycerol (Pubchem CID: 753)
Glucitol (Pubchem CID: 5780)
Aspartic acid (Pubchem CID: 5960)
Acetic acid (Pubchem CID: 176)
Cholesterol (Pubchem CID: 5997)
linoleic acid (Pubchem CID: 5280450)
5-Hydroxyindoleacetic acid (Pubchem CID: 91752610)
N-Acetylneuraminic acid (Pubchem CID: 439197)
Arabinitol (Pubchem CID: 94154)
L-Aspartic acid (Pubchem CID: 5960)
L-Proline (Pubchem CID: 145742)
fumaric acid (Pubchem CID: 444942)

Keywords:
Angelica sinensis
Processed products
Integrated Metabolomics
Blood deficiency syndrome
Metabolic pathway analysis

ABSTRACT

Ethnopharmacological relevance: Angelica sinensis (AS), root of *Angelica sinensis* (Oliv.) Diels, an important kind of Chinese traditional herbal medicine, has been used for women to enrich the blood for thousands of years. It is mainly distributed in Gansu province of China. According to Traditional Chinese medicine usage, unprocessed AS (UAS) and its 4 kinds of processed products (ASs) are all used to treat different diseases or syndromes. The difference among the enriching-blood effects of ASs is unclear. And their exact mechanisms of enriching the blood are not fully understood.
Aim of the study: In this study, our aim is to compare the enriching-blood effect and explain the related mechanism of ASs, to lay the foundation for the blood deficiency diagnosis and the rational use of ASs in the clinic.
Materials and methods: ASs were used to intervene the blood deficiency syndrome model mice induced by acetyl phenylhydrazine (APH) and cyclophosphamide (CTX). A novel approach using metabolomics coupled with hematological and biochemical parameters to explain the enriching-blood effect and mechanism of ASs was established. The blood routine examination, ATPase, glucose-6-phosphate dehydrogenase, methemoglobin, glutathion peroxidase, glutathione reductase, and erythropoietin were measured. Two biofluids (plasma and urine) obtained from mice were analyzed with GC-MS. Distinct changes in metabolite patterns of the two biofluids after mice were induced by APH and CTX, and mice were intervened with ASs were analyzed using partial least squares-discriminant analysis. Potential biomarkers were found using a novel method including variable importance in the projection (VIP) > 1.0, volcano plot analysis, and significance analysis of microarray.
Results: The results of hematological, biochemical parameters and the integrated metabolomics all showed the blood deficiency syndrome model was built successfully, ASs exhibited different degree of enriching-blood effect, and AS pached with alcohol (AAS) exhibited the best enriching-blood effect. 16 metabolites in the plasma and 8 metabolites in the urine were considered as the potential biomarkers. These metabolites were involved in 7 metabolic pathways which were concerned with the different enriching-blood effect mechanisms of ASs. The correlation analysis results confirmed L-Valine (plasma), Linoleic acid (urine), L-Aspartic acid (urine) and Cholesterol (urine) were strong positive or negative associated with biochemical indicators.
Conclusions: The enriching-blood effects of ASs are different. The pathological mechanisms of blood deficiency syndrome and the enriching-blood effect mechanism of ASs are involved in 7 metabolic pathways. L-Valine (plasma), Linoleic acid (urine), L-Aspartic acid (urine), Cholesterol (urine) are four important biomarkers being related to the enriching-blood effect of ASs. The combination of VIP, volcano plot analysis and significance analysis of microarray is suitable for screening biomarkers in metabolomics study. They can lay the foundation for clinical practice.

Abbreviations: AS, Angelica sinensis; UAS, unprocessed AS; ASs, unprocessed AS (UAS) and its 4 kinds of processed products; AAS, AS pached with alcohol; SAS, AS parched with soil; OAS, AS parched with sesame oil; CAS, charred AS; GC-MS, Gas chromatography-mass spectrometry; PLS-DA, partial least squares-discriminant analysis; BDS, blood deficiency syndrome; APH, N-acetyl phenylhydrazine; CTX, cyclophosphamide; TCM, Traditional Chinese Medicine; CTHM, Chinese traditional herbal medicine; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; G_6PD, glucose-6-phosphate dehydrogenase; GPX, glutathion peroxidase; GR, glutathione reductase; EPO, erythropoietin; MSTFA, N-methy-N-(trimethylsilyl) trifluoroacetamide; TMCS, trimethylchlorosilane; VIP, variable importance in the projection; NMR, nuclear magnetic resonance; SAM, significance analysis of microarray

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http://dx.doi.org/10.1016/j.jep.2017.09.028
Received 9 April 2017; Received in revised form 19 August 2017; Accepted 23 September 2017
Available online 25 September 2017
0378-8741/

1. Introduction

Traditional Chinese Medicine (TCM), being an effective treatment system, is increasingly used in the clinic. For centuries, Blood deficiency syndrome (BDS) (Li et al., 2015a, 2015b) is a common kind of TCM syndrome with the high incidence and the low cure rate. It refers to the syndrome caused by congenital hematopoiesis deficiency or the loss of blood. The people or animals with BDS will appear pale, weak, thin, and so on. The BDS model induced by acetyl phenylhydrazine (APH) and cyclophosphamide (CTX) is a classical model (Zhang et al., 2014). APH, a strong oxidant, has a slowly progressive and oxidative damage on red blood cell (RBC) resulting in hemolytic anemia of the body. CTX, a chemotherapeutic agent, can deplete hematopoietic stem cells in the marrow and peripheral blood cells resulting in anemia and immunodeficiency. Anemia is regarded as the decrease of hemoglobin in modern medicine. It includes blood loss anemia, aplastic anemia, iron deficiency anemia and so on (Sifakis et al., 2000). The blood loss anemia is similar to BDS in TCM, such as the condition of postoperative and postpartum women with chronic bleeding or uterine bleeding (Shi et al., 2014). So, the main diagnostic standards of BDS are the reduction of white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB) and so on.

Angelica sinensis (AS), root of *Angelica sinensis* (Oliv.) Diels, an important kind of Chinese traditional herbal medicine (CTHM), has been used for women to enrich the blood, prevent and treat BDS in TCM for thousands of years (National Pharmacopoeia Committee, 2010; Lao et al., 2004; Li et al., 2015), and it is mainly distributed in Gansu province of China (Wei et al., 2016).

AS polysaccharides (ASP) (Hou et al., 2012; Zahra et al., 2016) and ferulic acid (FA) (Li et al., 2011) are major bioactive components of AS related to the enriching-blood effect. Some scholars found that ASP was the major component being related to the hematopoietic effect of *Angelica sinensis* (Liu et al., 2010). Many studies have confirmed that FA could increase the number of nucleated narrow cells by promoting DNA synthesis in the blood deficiency animals (Ning et al., 2002). Moreover, it was reported that some other active ingredients of AS such as adenine, uracil, folinic acid, β-sitosterol, VB12 and VE also had an enriching-blood effect (Chen, 2008).

In clinical use, CTHM is always processed before being applied because processing can change the active constituent, increase the therapeutic effect and reduce the side effects (Singhuber et al., 2009). According to the tradition, unprocessed AS (UAS) and its 4 kinds of processed products (ASs) are all distinguished to use (Cao, 1986; Xiao, 1987; Feng et al., 1991; Wang et al., 2010). At present, processed products of AS that are widely used in CTHM mainly include AS parched with alcohol (AAS), AS parched with soil (SAS), AS parched with sesame oil (OAS), and charred AS (CAS) (Zhan et al. 2011; Zhao et al. 2010; Xiao et al., 2012b). The chemical profiles of the UAS, AAS, SAS, OAS and CAS are different (Guo et al., 2010). Moreover, the functions and clinical uses of UAS and its processed products are different (Zhan et al., 2011). In traditional or clinical use, UAS is used to enrich the blood and lubricate the intestines (Xiao et al., 2012a), AAS is used to enrich the blood, invigorate the circulation of blood and disperse blood stasis (Ying et al., 2009; Liu, 2002), SAS is used to enrich the blood and tonify spleen (Ying et al., 2009; Liu, 2002), OAS is used to enrich the blood and lubricate the intestines to relieve constipation (Long et al., 2003a, 2003b), and CAS is used to stop bleeding for treating hemorrhage (Liu, 2002). A study found that the intervention effects of *Angelica sinensis* and its processed products on blood physiological indicators of anemia chickens were different (Wang et al., 2012). About the intervention effect on BDS, which is the best in ASs? And what are the differences among specific enriching-blood mechanism of ASs? To understand these questions, “holism” philosophy was used.

TCM has complexity and integrality, studying TCM from the overall angle has become a new trend. Metabolomics is a new discipline in systems biology (Li et al., 2015a, 2015b). According to the “holism” philosophy, metabolomics can provide important information for TCM research. Multifarious metabolic characteristics of normal, pathological, or drug-treated subjects can be revealed by metabolomics, and it has been used to explore the secret in TCM (Hu et al., 2014). Metabolomics has been studied with many methods. Compared with NMR and LC-MS-based metabolomics methods, GC-MS-based metabolomics has numerous advantages, such as higher sensitivity, a better ability of material separation, the availability of many structure databases, and easier identification of metabolites (Posecion et al., 2008). Partial least squares-discriminant analysis (PLS-DA) is a kind of the indispensable pattern recognition methods in metabolomics research. It is a supervised multivariable statistical method, and it is used to sharpen an already established (weak) separation between groups of observations plotted in PCA (Liu et al., 2013).

Given the BDS mechanism has been studied from blood routine examination, bone marrow hematopoietic stem cell growth cycle, immune organs, and metabolomics (Li et al., 2015a, 2015b), in this study, based on the classical BDS mice model, the difference of the enriching-blood effects and mechanism of UAS and its 4 kinds of processed products was researched by evaluating the blood routine examination, A-TPase, Glucose-6-phosphate dehydrogenase (G_6PD), Methemoglobin, glutathion peroxidase (GPX), glutathione reductase (GR), erythropoietin (EPO) and integrated metabolomics of two biofluids (plasma and urine) collected from mice based on GC-MS and chemometrics methods. These data provide a theoretical basis for UAS and its 4 kinds of processed products more reasonably applying to keep human and animal health in the future and developing new drugs for treating BDS in TCM.

2. Materials and methods

2.1. Chemicals and reagents

APH was purchased from Chemical Reagent Co., Ltd. (Shanghai, P. R. China). CTX was purchased from Hengrui Pharmaceutical Co., Ltd. (Jiangsu, China). O-Methyl hydroxyllmine Hydrochloride, N-Methyl-N-(trimethylsilyl) trifluoroacetamie (MSTFA), and Trimethylchlorosilane (TMCS) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Assay kits for ATPase, G_6PD and Methemoglobin were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Deionized water was purified by the Milli-Q system (Millipore, Bedford, MA, USA). All the reagents were analytical or chromatographic grade.

2.2. Preparation of ASs decoction

Roots of *Angelica sinensis* (Oliv.) Diels (AS) was purchased from Minxian County, Gansu Province, China and authenticated by Dr. Yanming Wei (College of Veterinary Medicine, Gansu Agricultural University, Lanzhou, China). AS was prepared according to Pharmacopoeia Commission of People's Republic of China and Gansu Processing Standard of TCM. A voucher specimen is stored in the herbarium center of Gansu Agricultural University (Root of AS, AS201209001) (Hua et al., 2014). Then AAS, SAS, OAS and CAS were obtained after AS was processed with different methods (Ji et al., 2014). AAS was produced after the original AS was cut into slice, mixed thoroughly in alcohol, braised, fried until yellow and then cooled. The amount of alcohol used was 0.1 kg (200 mL/L) for every 1 kg AS. OAS was produced after the original AS was sliced, mixed thoroughly with 3% sesame oil, braised, fried into dark yellow using soft fire, and then cooled. SAS was produced after the original AS was cooked with sizzling soil by soft fire and then cooled. The amount of soil used was 0.3 kg for every 1 kg AS. CAS was produced after the original AS was sliced, cooked in medium heat until it was coke black and its internal portion was brown, and then cooled when it had charred taste and was crisp. Different ASs were pulverized and passed through a 40-mesh