Research Paper

Cerebral potential biomarkers discovery and metabolic pathways analysis of α-synucleinopathies and the dual effects of Acanthopanax senticosus Harms on central nervous system through metabolomics analysis

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A R T I C L E   I N F O

Article history:
Received 21 November 2014
Received in revised form 12 January 2015
Accepted 15 January 2015
Available online 7 February 2015

Keywords:
α-synuclein
Neurodegeneration
UPLC-QTOF-MS
Potential biomarkers
Acanthopanax senticosus Harms

A B S T R A C T

Ethnopharmacological relevance: Acanthopanax senticosus Harms (AS), also called “Ciwujia” in Chinese and “Siberian ginseng” in the Siberian Taiga region, is the herb used in traditional medicinal systems of China, Russia, Japan and Korea for the treatment of various nervous and cerebrovascular diseases.

Aim of the study: Our pre-study has showed that AS can significantly suppress α-synuclein over-expression and toxicity. Neuronal protein α-synuclein is a key player in the development of neurodegenerative diseases called α-synucleinopathies. Identifying the potential biomarkers related to α-synucleinopathies may facilitate understanding the pathogenesis of the diseases and the safe application of AS in the clinic.

Methods and results: Ultra-performance liquid chromatography-quadrupole time-of-flight-mass spectrometry (UPLC-QTOF-MS) coupled with pattern recognition methods was integrated to examine the cerebral metabolic signature of human α-synuclein transgenic mice and the effects of AS on central nervous system (CNS) in pathology and physiology. Totally, 17 differentially expressed metabolites in wild type (WT) group and 26 in A30P mutant (A30P) group were identified and considered as potential biomarkers. Among them, 11 endogenous metabolites in WT+AS group and 18 in A30P+AS group were involved in the anti-α-synucleinopathies mechanism of AS. However, western blot and metabolomics analysis showed the effects of AS on CNS in physiology were opposite to those in pathology, which may cause potential neurotoxicity.

Conclusions: This study demonstrated that endogenous metabolites perturbation was involved in the pathogenesis of α-synucleinopathies and AS produced the dual effects on pathological and physiological CNS.

1. Introduction

Proteinaceous aggregates containing α-synuclein represent a feature of neurodegenerative disorders such as Parkinson’s disease (PD), Lewy bodies dementia, and multiple system atrophy (Junn et al., 2003). These disorders are characterized by deposition of abnormally phosphorylated fibrillar α-synuclein within the central nervous system (CNS) (Mouradian, 2002; Eriksen et al., 2003; Lim et al., 2003; Tofaris et al., 2006; Watts et al., 2013). Based on the above-mentioned description, α-synuclein overexpression appears to be a common mechanism for the pathogenesis of PD and other α-synucleinopathies. In addition, Ala30Pro substitution in exon 3 of human α-synuclein has also been identified in the early-onset PD, confirming the pathogenic significance of α-synuclein mutations (Kruger et al., 1998). Understanding how potential biomarkers and pathways associated with both genes cause neurodegeneration is...
crucial in the development of treatments that might slow or stop the disease progression.

Increasing interest has been devoted to the evaluation of herbal medicines on prevention or treatment of diseases and disorders in recent years (Rates, 2001; Rishton, 2008; Song et al., 2012). *Acanthopanax senticosus* Harms (AS) is the herb used in traditional medicinal systems of China, Russia, Japan and Korea, which has been applied to the treatment of various nervous and cerebrovascular diseases, such as depression, mental fatigue, and transient global cerebral ischemia, etc. (Huang et al., 2011; Lee et al., 2012; Wu et al., 2013). Recent reports have addressed that AS could also exert therapeutic effects against neurodegenerative diseases, such as PD and AD (Xu et al., 2008; Liu et al., 2012). In addition, our pre-study also showed that AS could significantly suppress α-synuclein overexpression and toxicity in human α-synuclein transgenic model (Li et al., 2014a). However, the metabolite targets associated with the anti-α-synucleinopathies mechanism of AS are barely known.

Emerging metabolomics, as one of the major components of systems biology, provides a powerful platform for monitoring multiple endogenous metabolites levels simultaneously in the samples from cell lines (Gao et al., 2014), tissues (Li et al., 2013), and body fluids (Zhao et al., 2012; Lu et al., 2013). The change in the expression levels of endogenous metabolites can be used for the investigation of early diagnosis, pathogenesis, and therapeutic targets of diseases (Ahmed et al., 2009; Graham et al., 2013), and the evaluation of the therapeutic and toxic effects of drugs (Li et al., 2013; Lu et al., 2013). The technique has been applied to the study of several neurodegenerative disorders, such as PD, AD, and Huntington’s disease (Ahmed et al., 2009; Tsang et al., 2009; Graham et al., 2013). However, it is still a lack of direct evidence between α-synuclein overexpression and metabolites perturbation. In this study, metabolic profiles were compared in control non-transgenic and human wild type (WT) or A30P mutant (A30P) α-synuclein transgenic C57BL/6 mice by using ultra-performance liquid chromatography-quadrupole time-of-flight-mass spectrometry (UPLC-QTOF-MS) coupled with pattern recognition methods. An integrative analysis was used to identify the possible pathways that may be associated with α-synuclein-induced neurodegeneration. Meanwhile, we tried to investigate the effects of AS on CNS under pathological and physiological conditions through metabolomics analysis.

### 2. Materials and methods

#### 2.1. Plant material and extraction

The crude drug is the root and rhizome of AS and was collected in *Wuchang* (N44°39′, E127°35′) of Heilongjiang province, PR China. The voucher specimen (hlj-201003) of the herb was authenticated by Professor Ke Fu, Institute of Traditional Chinese Medicine, Heilongjiang University of Chinese Medicine. The preparation of extracts was shown in our pre-studies (Li et al., 2013; Li et al., 2014a; Li et al., 2014b; Zhang et al., 2014). The yield of the extract is 1.3% (w/w). The contents of eleutheroside B and eleutheroside E in AS were 7.63 ± 0.34% (w/w) and 10.90 ± 0.22% (w/w), respectively (Li et al., 2014a; Li et al., 2014b). The finger-print analysis of the same batch of extract was also shown in our pre-studies (Li et al., 2014a; Li et al., 2014b; Zhang et al., 2014).

#### 2.2. Animals and drug administration

Experimental procedures were in accordance with the Legislation on the Protection of Animals Used for Experiment Purposes (Directive 86/609/EEC) and approved by the Animal Care and Use Committee of Heilongjiang University of Chinese Medicine. All efforts were made to minimize animal suffering and to reduce the number of animals used. The condition of the animals was monitored twice daily during the feeding and experiment period. Male control non-transgenic and human WT or A30P α-synuclein transgenic C57BL/6 mice (10 months old) were purchased from Institute of Laboratory Animal Science (Chinese Academy of Medical Sciences and Peking Medical College). These mice were divided into control, WT, A30P, WT + AS, A30P + AS, and AS-treated groups (non-transgenic mice treated with AS), and there were 10 mice in each group. According to the State Pharmacopoeia of PR China and our own recent studies, the average daily dose of AS per mouse was 45.5 mg/kg/day (ChPC, 2010; Liu et al., 2012; Li et al., 2013). WT + AS, A30P + AS, and AS-treated groups were orally administrated with AS once a day for 20 days, and control, WT, and A30P groups received equal volume saline once a day for 20 days.

#### 2.3. Sample collection and preparation

All operations were carried out under sterile conditions. On the 21st day, mesencephalon tissues were isolated from the mice gently after dissection, immediately frozen in liquid nitrogen following accurate weighing, and then stored at −80 °C until used. A part of frozen tissue samples was used for western blot analysis. The rest were added to 10 volumes of ice-cold methanol and homogenized with a tissue homogenizer for 2 min in iced bath. The homogenate was centrifuged twice at 13,000 rpm for 15 min at 4 °C. The supernatant was transferred into Eppendorf tube and stored at −80 °C for UPLC-QTOF-MS analysis.

#### 2.4. Western blot analysis

The samples were sonicated in ice-cold lysis buffer. Equal amounts (40 μg) of protein were separated in a polyacrylamide gel, transferred to nitrocellulose membranes at 50 mA for 40–60 min, and blocked for 2 h at room temperature with Tris-buffered saline containing 0.05% Tween 20 (TBST (pH 7.4)) and 5% nonfat dried milk. After washing 3 times for 10 min each in PBS, membranes were incubated with rabbit anti-human and -mouse α-synuclein, leucine rich repeat kinase 2 (Lrrk2), S100 calcium binding protein beta chain (S100b), or neuron-specific enolase (Nse) antibody (Beijing Biosynthesis Biotechnology Co., Ltd., PR China) at 1:1000 concentration each overnight at 4 °C. Membranes were then washed again with PBS, 3 times for 10 min each, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The protein bands were visualized with 4-chloronaphthol (Sigma, U.S.A.). The integral optical density (IOD) of bands was determined by Gel-Pro 4.0 software (Media Cybernetics, Inc. U.S.A.). The levels of target proteins were expressed as the ratio of target proteins IOD to β-actin IOD.

#### 2.5. UPLC-QTOF-MS analysis

Waters Acquity™ UPLC (consisting of a vacuum degasser, an autosampler, a binary pump, a photodiode array detector and an oven) was equipped with ACQUITY UPLC™ BEH C18 column (2.1 mm × 50 mm, i.d. 1.7 μm, Waters Corp, Milford, USA). The analytical column was maintained at a temperature of 40 °C and the mobile phases were composed of acetonitrile (A) and water (B) each containing 0.1% formic acid. A solvent gradient system was used: 0–2.5 min, 1–10% A; 2.5–3.5 min, 10–40% A; 3.5–11 min, 40–70% A; 11–12 min, 70–80% A; 12–13 min, 80–100% A. The flow rate was 0.4 ml/min. Injection volume was 3 μl. The eluent was introduced to the MS directly without a split.
Fig. 1. 2-D and 3-D OPLS-DA score plots based on the cerebral metabolic profiling of control and WT groups in (A and B) positive and (C and D) negative ion modes.

Fig. 2. PLS-DA scores plot derived from the integrated MS data of mesencephalic samples obtained from control, WT, WT+AS, and AS-treated group in (A) positive and (B) negative ion modes.
MS analysis was performed on a Q-TOF analyzer in SYNAPT HDMS system (Waters Corporation, Milford, MA, USA) in positive and negative ion modes, using the following parameters: capillary voltage, 1500 V; sample cone voltage, 100 V; source temperature, 110 °C; desolvation temperature, 350 °C; desolvation gas flow, 750 l/h; cone gas flow, 20 l/h. MS data were collected in the full scan mode from m/z 100–1500. All the data were acquired using an independent reference lock mass via the LockSpray™ interface to ensure accuracy and reproducibility during the MS analysis. Leucine enkephalin was used as the reference ion for positive ([M+H]⁺ = 556.2771) and negative ([M−H]⁻ = 554.2615) ion modes at a concentration of 1 ng/ml under a flow rate of 30 μl/min. The data were collected in the centroid mode, and the LockSpray frequency set at 15 s and averaged over 5 scans for correction.

Prior to the analysis of both positive and negative ion modes, 10 pooled conditioning samples were injected. To determine the chromatographic reproducibility of retention times and peak intensities, pooled samples were injected after every 5 samples throughout the experiment (Graham et al., 2013).

2.6. Multivariate data analysis

The raw data were analyzed using MassLynx V4.1 and MarkerLynx software (Waters). The intensity of each ion was normalized with respect to the total ion count to generate a data matrix that was consisted of the retention time, m/z value, and the normalized peak area. The multivariate data matrix was analyzed by EZinfo software (Waters). The unsupervised segregation was checked by principal components analysis (PCA) using pareto-scaled data. Partial least-squares-discrimination analysis (PLS-DA) and orthogonal partial least-squares-discrimination analysis (OPLS-DA) models were carried out to visualize the metabolic difference among these experimental groups. Potential biomarkers of interest were extracted from the combining S- and VIP-plots that were constructed from OPLS-DA, and potential biomarkers were chosen based on their variable importance of project (VIP) statistics. A VIP value > 1 means that variables have above average influence on the classification. Student's t-test was used for statistical analysis to evaluate the significant difference (p < 0.05) of potential biomarkers. The heatmap, implemented in MetaboAnalyst tool commonly used for unsupervised clustering, was constructed based on the potential biomarkers.

2.7. Potential biomarkers identification and metabolic pathway analysis

Exact molecular mass data from redundant m/z peaks corresponding to the formation of different parents and product ions were first used to help confirm the metabolite molecular mass. MS/MS data analysis highlights neutral losses or product ions, which are characteristic of metabolite groups and can serve to discriminate between database hits. The MassFragment™ application manager (Waters MassLynx v4.1, Waters) was used to facilitate the MS/MS fragment ion analysis process by way of chemically intelligent peak-matching algorithms. Database such as HMDB (http://www.hmdb.ca/) and MassBank (http://www.massbank.jp/) were used for confirmation. Metabolic pathway analysis was performed with MetaboAnalyst Pathway Analysis (http://www.metaboanalyst.ca/Meta) and Cytoscape software (version 3.1.0) based on database source including the KEGG (http://www.genome.jp/kegg/), SMPDB (http://www.smpdb.ca/) and HMDB to identify the affected metabolic pathways analysis and visualization.

3. Results

3.1. Western blot analysis for α-synuclein, Lrrk2, S100b, and Nse expressions

Compared with those in control group, the levels of α-synuclein, Lrrk2, S100b, and Nse in WT and A30P groups were up-regulated significantly. WT+AS and A30P+AS groups showed the significant tendency to correct the abnormal expressions of α-synuclein, Lrrk2, and Nse, and slightly down-regulated the levels of S100b as well. The levels of these proteins in AS-treated group were also significantly higher than those in control group (Supplementary Fig. S1).

3.2. Metabolomics analysis and potential biomarkers identification

Clustering of the pooled samples was analyzed by PCA to reveal if platform stability had been achieved. For both positive and negative
ion modes, the pooled samples were tightly clustered, indicating good reproducibility of the data (Graham et al., 2013). The representative based peak intensity (BPI) chromatograms of mesencephalon tissues samples from each experimental group are shown in Supplementary Figs. S2 and S3. Low molecular mass metabolites could be well separated in the short time of 13 min because of the small particles (less than 1.7 μm) of UPLC. PCA was firstly carried out to investigate whether each experimental group can be separated and to find out their metabolic distinction. Then OPLS-DA and PLS-DA were used to sharpen an already established (weak) separation among the groups of observations plotted in PCA, separate samples into different blocks, and improve potential biomarkers discovery efforts. Score plots and trajectory analysis of score plots (3-D) from OPLS-DA showed obvious separation between control and WT or A30P groups (Fig. 1 and Supplementary Fig. S4). PLS-DA

Fig. 4. Hierarchical clustering analyses for potential biomarkers expression profile. Heat maps show potential biomarkers profiles that differentiate (A) WT or (B) A30P groups from control group. Rows: samples; Columns: metabolites; Color key indicates metabolite expression value, blue: lowest; red: highest. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Pie charts depicting the classification of potential biomarkers of (A) WT and (B) A30P groups. Representation of metabolites in terms of chemical taxonomy, bio-function and cellular locations were based on the annotations of HMDB.
also showed the cerebral metabolic profiling among control, WT or A30P, WT+AS or A30P+AS, and AS-treated groups (Fig. 2 and Supplementary Fig. S5). Combining the results of S- and VIP-plots from OPLS-DA (Fig. 3 and Supplementary Fig. S6), the UPLC/MS analysis platform provided the retention time, exact molecular mass, and MS/MS data for the structural identification of potential biomarkers. Heatmap visualization of metabolomics data showed distinct segregation among these experimental groups (Fig. 4 and Supplementary Fig. S7). Finally, according to VIP value, 17 variables in WT group and 26 in A30P group were identified based on the metabolite identification strategy (Supplementary Tables S1 and S2).

Here, we take the ion at Rt = 0.40 and [M + H]⁺ = 148.0615 as an example to illustrate the potential biomarkers identification process. The assistant software packed in MassLynx i-FIT algorithm was used to determine the element composition for the ion. Using a mass tolerance of 5 mDa, C₅H₉NO₄ was located as the candidate because of its high mass accuracy and low i-Fit value among the possible chemical formulas. Degree of unsaturation was calculated as 2, indicating that it might contain two double bonds, a triple bond or two rings. The main fragment ions analyzed by MS/MS screening were m/z 102.0836, 93.0419, 86.0004, and 84.0526, which could correspond to lose -O₃, -C₃H₅O, -C₂H₂N₂O, and -CH₄O₃, respectively. Finally, it was speculated as l-glutamic acid by comparing the fragmentation pattern with the mass spectrum (PR100162) in the MassBank database. All of the potential biomarkers identified are shown in Supplementary Tables S1 and S2. 17 endogenous metabolites in WT group and 26 in A30P group contributing to the complete separation of WT or A30P and control groups were detected in the samples.

3.3. Classification of potential biomarkers of WT and A30P groups

By using online database (HMDB) for classification, of the endogenous metabolites in WT and A30P groups, over 40% were subgrouped as amino acids, peptides, and analogs, about 15–17% belonged to lipids, about 18–19% were nucleosides, nucleotides, and analogs, and 6–12% were organic acids and derivatives (Fig. 5A and B). It is of interest to note that the regulated metabolites had a relative equal ‘bio-function’-distribution (Fig. 5A and B). In addition, these metabolites were primarily located in extracellular, cytoplasm, mitochondria, membrane, and endoplasmic reticulum (Fig. 5A and B).

3.4. Metabolic pathway analysis of potential biomarkers of WT and A30P groups

Further analysis of pathways and networks influenced by WT or A30P α-synuclein was performed by metabolic pathway analysis with MetaboAnalyst and Cytoscape software. This analysis provided us additional valuable clues about the complex interactive link of the various identified metabolites for their commonly known interactive metabolites networks and for other cellular metabolic information as well. Metabolic pathway analysis revealed that 17 metabolites in WT group were involved in 16 pathways, and 26 in A30P group were found to participate in 24 pathways (Fig. 6).

4. Discussion

Recent studies have demonstrated that the increase in α-synuclein levels could induce the up-regulation of Lrrk2, both of which interact...
with each other in PD and related α-synucleinopathies (Westerlund et al., 2008; Qing et al., 2009; Guerreiro et al., 2013). Mutation in Lrrk2 can reduce mitochondrial membrane potential and elicits calcium imbalance (Mortiboys et al., 2010; Cherra et al., 2013). S100b, a neurotrophic factor, is also known to stabilize calcium homeostasis (Rothermundt et al., 2003), the overexpression of which results in a sustained increase of intracellular Ca\(^{2+}\) levels concomitant with cell death (Mariggio et al., 1994). The Nse is a cytoplasmatic glycolytic enzyme, an increase of whose levels is considered as a sensitive marker of neuronal damage (Schaf et al., 2005; Al-Jarrah and Jamous, 2011). Age-dependent and site-specific increase in co-localization of α-synuclein and S100b have been observed within astrocytes (Marxreiter et al., 2013). Parkinsonism also increases the expressions of Nse and S100b (Al-Jarrah and Jamous, 2011). The results from the western blot analysis (Supplementary Fig. S1) were consistent with the above description.

To our knowledge, this is the first report using cerebral metabolomics study to identify the potential biomarkers and metabolic pathways associated with α-synuclein-related neurodegenerative disease. As shown in Fig. 6, WT and A30P α-synuclein overexpression could affect 14 common pathways, including purine metabolism, nitrogen metabolism, histidine metabolism, pyrimidine metabolism, glutathione metabolism, and fatty acid metabolism, etc.

L-Glutamine, cyclic AMP, adenosine triphosphate, guanosine monophosphate, adenosine monophosphate, and adenosine belong to purine metabolism that is closely linked to PD (LeWitt et al., 2011). L-Glutamine involves in tissue transglutaminase-catalyzed intramolecular cross-linking of α-synuclein, which modulates the physiological and pathogenic properties of α-synuclein and contributes to the pathogenesis of PD (Schmid et al., 2009). Cyclic AMP is synthesized from adenosine triphosphate by adenylylate cyclase. One previous study has shown that α-synuclein could enhance dopamine D2 receptor signaling-mediated inhibition of adenylylate cyclase (Kim et al., 2006), which might lead to an increase in adenosine triphosphate levels and decreased cyclic AMP expressions, as shown in this study. Based on KEGG pathway analysis, we observed that adenosine, adenosine monophosphate, and guanosine monophosphate can be converted from adenosine triphosphate via a series of enzymes. Their abnormal expressions may also indirectly indicate these enzymes activities deficiency. High concentrations of adenosine triphosphate have been shown to induce apoptosis in various systems (Tai et al., 2005; Bi et al., 2006).

L-Glutamine also participates in nitrogen metabolism with L-glutamic acid whose expression trends in WT and A30P groups were opposite. L-Glutamic acid is the most abundant fast excitatory neurotransmitter in the mammalian nervous system, which can maintain neuronal excitability and play an important role in cognition, learning and memory (Stella et al., 2008; Stangherlin et al., 2009). However, the overexpression of L-glutamic acid causes excitotoxicity in neuronal dysfunction and degeneration, which has also been linked to chronic neurodegenerative disorders (Lau and Tymianski, 2010). Thus it can be seen that whether
up-regulation or down-regulation of l-glutamic acid can result in CNS injury and dysfunction.

In addition, carnosine (histidine metabolism) (Fu et al., 2008), cystidine (pyrimidine metabolism) (Wang and Lee, 2000), glutathione (glutathione metabolism) (Conrad et al., 2013), L-palmitylcarnitine (fatty acid metabolism) (Li et al., 2013) had also been proved to play roles in the pathogenesis of neurodegenerative diseases including PD and AD. Based on the previous reports, we have the reason to believe the results of our metabolomics analysis.

Abnormal deposit of certain proteins is a common pathology for many neurodegenerative disorders. Therefore, increasing their clearance might be a strategy applicable to all conditions involving protein aggregation (Li et al., 2014a). Both our own pre-study (Li et al., 2014a) and the results of Supplementary Fig. S1 showed that AS could significantly suppress the overexpression of α-synuclein and its-related proteins, which indicated that AS might be used for the treatment of α-synucleinopathies. Out of these potential biomarkers, AS showed the tendency to correct the abnormal expressions of 11 endogenous metabolites in WT+AS group and 18 in A30P+AS group (Fig. 7 and Supplementary Tables S1 and S2), which participated in a variety of pathways, such as purine metabolism, nitrogen metabolism, histidine metabolism, pyrimidine metabolism, and glutathione metabolism, etc. (Fig. 7). Interestingly, AS could reverse the up-regulation and down-regulation of l-glutamic acid induced by both of α-synucleins respectively, and restore the balance of their expressions. These may demonstrate that the therapeutic effects of AS on α-synucleinopathies can be implemented by intervening in multiple metabolite targets.

Of the medicines, the application under pathological conditions can be used to investigate their therapeutic effects on diseases, while the application under physiological conditions can be used to explore their therapeutic potential. However, in this study, we cannot clearly observe the therapeutic potential of AS on α-synucleinopathies. Conversely, AS may produce some potential neurotoxicity to CNS in physiology. As shown in Supplementary Fig. S1, the expressions of α-synuclein and its-related proteins in AS-treated group were enhanced significantly and close to those in both model groups. We tried to apply the metabolomics analysis to the explanation of the phenomenon. PLS-DA scores plot (Fig. 2 and Supplementary Fig. S5) showed that control, WT or A30P, WT+AS or A30P+AS, and AS-treated groups were distributed in a nearly circular district. The metabolic profiles of WT+AS and A30P+AS groups differed from those of both model groups and trended towards that of control group, whereas AS-treated group showed the opposite trend, the metabolic profile of which deviated from that of control group and trended towards those of both model groups, particularly towards that of A30P group. However, at this stage the notion is merely a speculation that needs further studies to verify.

5. Conclusions

The present research directly demonstrated that endogenous metabolites perturbation was involved in the pathogenesis of α-synucleinopathies. In addition, we can observe that AS produced the dual effects on pathological and physiological CNS. Neuroprotection and neurotoxicity coexisted in the effects of this herb on CNS. Therefore, since AS contains many bioactive ingredients (WHO, 2004), the further studies are necessary to investigate the root cause of its dual effects on CNS. The disclosure of the pharmacological actions of those compounds would facilitate the safe application of this herb as a medicine in the clinic.

Disclosures

The authors declare that they have no conflict of interest.

Acknowledgments

This article is supported by the National Natural Science Foundation of China (81270056), the Specialized Research Fund for the Doctoral Program of Higher Education of China (2013227110009), New Century Excellent Talents Program of Higher Education of Heilongjiang Province (1254-NCET-020), outstanding talents cultivation fund of Heilongjiang University of Chinese Medicine (2013jc01), and the outstanding innovative talent support programs of Heilongjiang University of Chinese Medicine.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.01.046.

References
