Chemical and pharmacological studies on angiogenesis inhibitors from *Salvia miltiorrhiza*

by

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Abstract

Angiogenesis, the development of new blood vessels, is an important pathological component of diseases such as cancer and heart disease. Since the genomics-focused drug-discovery efforts to produce the expected large number of compounds have encountered many difficulties, the attempts to find active ingredients with angiogenesis modulating properties from plants or herbs have entered our visual field in recent years. Particularly, some traditional Chinese herbs have a long and successful history for the treatment of angiogenesis-related diseases, which offered a safer and more effective angiotherapy approach.

The dried root and rhizome of *Salvia miltiorrhiza* (also known as Danshen in China), one of the most important traditional Chinese herbs, is commonly used in clinics for the treatment of cardiovascular and cerebrovascular diseases. Although a few of efforts have been maid to explore the angiogenic properties of *S. miltiorrhiza*, there still remains lots of works to be developed to correlate the chemical components with the pharmacological properties of this precious Chinese herb. Therefore, in this present study, a systematic research on the chemical constituents, pharmacological characteristics and quantity control of *S. miltiorrhiza* focusing on the antiangiogenic activity was carried out. The major achievements are summarized as follows:

(1) 13 diterpenoid compounds of *S. miltiorrhiza* were obtained from the chloroform extract of hydrophobic fraction using sequential silica-gel-column chromatographic separation and repeated re-crystallization. This provides the screened samples for pharmacological study and the markers for quality control.

(2) The antiangiogenic properties of the diterpenoid compounds in *S. miltiorrhiza* were explored using *in vitro* assays. Some of these diterpenoid compounds showed antiangiogenic activities via multiple mechanisms, including the inhibitory effects on the proliferation, invasion and tube formation of HUVECs.

(3) The structure-activity relationships (SAR) of the tested compounds as angiogenesis inhibitors were discussed for the first time. According to the prediction of SAR, 15,16-dihydrotanshinone I should have the most predominant inhibitory effect on VEGF-induced angiogenesis, which is consistent with the findings in the current study.

(4) Western blot analysis was applied to reveal the underlying mechanism, which

indicated that the antiangiogenic effects of these diterpenoid compounds in *S. miltiorrhiza*, were partially through the blockade of the ERK and PI3K signaling pathway.

(5) Pressurized liquid extraction (PLE) coupled with capillary electrochromatography (CEC), high performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC) were developed to assay the major diterpenoid compounds in *S. miltiorrhiza* for the first time. These analytical methods have been validated as rapid, sensitive, accurate and reproducible strategies for quality evaluation of *S. miltiorrhiza*.

In summary, the present study systematically explored the antiangiogenic activites and the novel techniques for the quality control of *S. miltiorrhiza*. Some potential antiangiogenic compounds, such as 15,16-dihydrotanshinone I and miltirone, were identified for the first time. Furthermore, several efficient, sensitive and reliable methods for the quality evaluation of *S. miltiorrhiza* were developed. These works will help to elucidate the main active components, deepen the understanding of the pharmacological effects and accurately control the quality of the herbal material and related products.

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Achievements

Journal Papers

1. **P. Li**, Gang. Xu, S.P. Li, Y.T. Wang, T.P. Fan, Q.S. Zhao, Q.W. Zhang. Optimizing ultra-performance liquid chromatographic analysis of 10 diterpenoid compounds in Salvia miltiorrhiza using central composite design. *J. Agric. Food Chem.* 56 (2008) 1164-1171 (**SCI, IF=2.322**)

2. **P. Li**, S.P. Li, F.Q. Yang, Y.T. Wang. Simultaneous determination of four tanshinones in *salvia miltiorrhiza* by pressurized liquid extraction and capillary electrochromatography. *J. Sep. Sci.* 30 (2007) 900-905. (**SCI, IF=2.535**)

3. **P. Li**, S.P. Li, Y.T. Wang. Optimization of CZE for analysis of phytochemical bioactive compounds. *Electrophoresis* 27 (2006) 4808-4819. (**SCI, IF=4.101**)

4. **P. Li**, S.P. Li, S.C. Lao, C.M. Fu, Kelvin K.W. Kan, Y.T. Wang. Optimization of pressurized liquid extraction for Z-ligustilide, Z-butylidenephthalide and ferulic acid in *Angelica sinensis*. *J. Pharm. Biomed. Anal.* 40 (2006) 1073-1079. (**SCI, IF=2.032**)

5. Y. Jiang, **P. Li**, S.P. Li, Y.T. Wang, P.F. Tu. Optimization of pressurized liquid extraction of five major flavanoids from *Lysimachia clethroide*. *J. Pharm. Biomed. Anal.* 43 (2007) 341-345. (**SCI, IF=2.032**)

6. J.B. Wan, **P. Li**, S.P. Li, Y.T. Wang, Tina T.X. Dong, Karl W.K. Tsim. Simultaneous determination of 11 saponins in *Panax notoginseng* using HPLC-ELSD and pressurized liquid extraction. *J. Sep. Sci.* 29 (2006) 2190-2196. (**SCI, IF=2.535**)

7. F.Q. Yang, S.P. Li, **P Li**, Y.T. Wang. Optimization of CEC for simultaneous determination of eleven nucleosides and nucleobases in Cordyceps using central composite design. *Electrophoresis* 28 (2007) 1681-1688. (**SCI, IF=4.101**)

8. Y.X. Gong, S.P. Li, Y.T. Wang, **P. Li**, F.Q. Yang. Simultaneous determination of anthraquinones in Rhubarb by pressurized liquid extraction and capillary zone electrophoresis. *Electrophoresis* 26 (2005) 1778-1782. (**SCI, IF=4.101**)

9. **P. Li**, S.P. li, M.Y. Li, Y.T. Wang. Research progress on pharmacological effects and mechanisms of *Salvia miltiorrhiza* on nervous system. *Chin. Trad. Herb. Drugs.* 37 (2006) 18-22.

Conference papers

1. **P. Li**, T.P. Fan, Y.T. Wang. Advanced approaches for quality evaluation of *Salvia miltiorrhiza*. The 3rd International Congress on Complementary Medicine Research, 2008, Sydney.
2. **P. Li**. Optimizing ultra-performance liquid chromatographic analysis of 10 diterpenoid compounds in *Salvia miltiorrhiza* using central composite design. 4th NoSSS International Conference, 2007, Kaunas.
3. Y.T. Wang, **P. Li**. Research strategies and progress of brain targeting drug delivery system. The 4th International Congress of Traditional Medicine, 2007, Singapore.
4. **P. Li**, Y.T. Wang, S.P. Li, *et al*. Studies on the pressurized liquid extraction of anthraquinones in Rhubarb. The 9th International Congress on Ethnopharmacology (NICE), 2006, China.
5. **P. Li**, S.P. Li, M.Y. Li, *et al*. To explore pressurized solvent extraction technique in quality control of Chinese herb. Hong Kong – Macau Postgraduate Symposium on Chinese Medicine, 2005, Hongkong.

Awards

1. Chromatography research on distinguishing between natural and cultured Cordyceps. The 3rd Prize of CAIA Science and Technology Prize in China Association for Instrument Analysis (CAIA), 2005
2. Exploration for internationalizing of Chinese Medicine. The 3rd Prize of 9th Challenge Cup of China, 2005

Abbreviation

ASAA	acetylsalvianolic acid A
ATCC	American type culture collection
BGE	background electrolyte
CCD	central composite design
CE	capillary electrophoresis
CEC	capillary electrochromatography
CT	cryptotanshinone
dhTI	15,16-Dihydrotanshinone I
dmLSB	dimethyl lithospermate B
DMSO	dimethyl sulfoxide
EOF	electroosmotic flow
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
HUVEC	human umbilical vein endothelial cell
IS	internal standard
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MAPK	mitogen-activated protein kinase
MLB	magnesium lithospermate B
MTT	3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide
PA	protocatechuic acid
PAC	partial atomic charges
PAH	protocatechuic aldehyde
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol-3 kinase
PLE	pressurized liquid extraction
PS	penicillin-streptomycin

RA	rosmarinic acid
RSD	relative standard deviation
Sal B	salvianolic acid B
SAR	structure-activity relationship
S/N	signal-to-noise ratio
SRM	selected reaction monitioring
STS	sodium tanshinone IIA sulfonate
TI	tanshinone I
TIIA	tanshinone IIA
UPLC	ultra-performance liquid chromatography
VEGF	vascular endothelial growth factor

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