The Chemistry, Pharmacology and Clinical Therapeutic Effects of Danshen

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PREFACE

According to the general rules of treatment, there are many kinds of drugs used in traditional Chinese medicine. Such as clearing away heat and toxic material (清热解毒), suppressing hyperactive liver for calming endogenous "wind" (平肝息风), promoting blood circulation for eliminating blood stasis (活血化瘀), supplying body fluids and reinforcing Qi (vital energy) (生津补气), and so on. Among them, drugs of activating the blood and eliminating phlegm are characterized by relieving cardio-cerebral vascular ischemia reducing blood viscosity and thrombosis. As representative of drugs of activating the blood and eliminating phlegm, Danshen (丹参, *Salvia miltiorrhiza*) received high attention at home and abroad. Study on the chemistry of *Salvia miltiorrhiza* started at thirties of 20th century. Japanese scientists isolated some lipid soluble constituents, such as tanshinone Ⅰ, tanshinone Ⅱ, cryptanshinone, etc. From forties of 20th century till now, Chinese scientists pay great attention to the research of chemistry, pharmacology and clinical use of *Salvia miltiorrhiza*. The chemists of our institute isolated 13 water soluble chemical components named salvianolic acids. Seven of them are new compounds, they were named as Salvianolic acid A, B, C, D, E, F, G separately by Prof LN Li.

The pharmacological study of these new compounds demonstrated that SA has multiple biological activities, such as improving reginal cerebral blood flow (rCBF) in ischemic hemisphere with no steal blood and without hypotension, inhibiting platelet aggregation and thrombosis, but had no hemorrhagic risk. It is well known that intracellular calcium overload, excessive production of free radicals and excitotoxicity aggravate ischemic brain injuries, SA could inhibit these pathological changes. More importantly, SA increases neurogenesis , angiogenesis and improves energy metabolism. Compared with available drugs, the efficacy of SA is better than that of Aspirin, EGb761 and many antioxidative agents.

This monograph is aimed at to review the recent progress in the study on chemistry, pharmacology, toxicology, pharmacokinetics, and clinical therapeutic effect of salvianolic acids, and promote international exchange of new findings and ideas. Cardio-cerebral vascular diseases, diabetes and dementia are world-wide major diseases which have high fatality and disability rate, yet few drugs are found to be effective or safety in treating these diseases. In recent decades, research efforts have been invested in the discovery and development of new therapeutic agents that modulate individual disease-modifying targets. Unfortunately, drugs designed to act against individual molecular targets cannot usually combat multigenic diseases, such as tumor, dementia or diseases that affect multiple tissues or cell types such as diabetes, stroke and immunoinflammatory disorders. Combination of drugs or single drug that impact multiple targets simultaneously are better in the control of complex disease systems, and less prone to drug resistance and are the standard of care in many important therapeutic areas. This monograph provided example of two forms of multi-targets therapeutics used in cerebral ischemic brain injury, diabetes and dementia. Water soluble extract and total salvianolic acids belong to "multicomponent drug", salvianolic acid A and B belong to "multi-tar-

get drug" .

Single drug having multi-target effect is more convenient than multicomponent drug in use and quality control, while multi-component drug show more broad pharmacological effects than multi-target drug. For example, both water soluble extract of *Salvia miltiorrhiza* and Salvianolic Acid B inhibited adhesion of neutrophils to TNFα-stimulated endothelial cells. WSE could also inhibit expression of adhesion molecules (E-Selectin CAM-1 and VCAM-1), Salvianolic Acid B did not affect the expression of E-Selectin in endothelia cells. Danshen is known as the most precious drug in traditional Chinese medicine. Water soluble extract, multicomponent or single compound isolated from *Salvia miltiorrhiza* all show many biological activities. This might support the common use of *Salvia miltiorrhiz*a as an effective drug against cardiocerebral vascular disease in traditional Chinese medicine. That salvianolic acids are effective in treating dementia and diabetes is a new discovery.

At last, we wish to thank all authors who had contributed a lot to Danshen research. They provided this book with high quality, informative data and sent paper in due time. She is enthusiastic, patient and with high efficiency. We deeply appreciate the opportunity offered by Chemical Industry Press for publishing this book in recognition of the importance of traditional Chinese drug in promoting health and treating diseases.

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Contents

Part 1

The Chemistry of Danshen

Chapter 1

Chemistry of Water Soluble Components of *Salvia miltiorrhiza*

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1 Research Overview

The chemical constituents of *Salvia miltiorrhiza* have been studied since 1930's by the Japanese research fellows, leading to the isolation of the principal lipophylic components, tanshinone Ⅰ, cryptotanshinone and tanshinone Ⅱ A and B. In 1940's S Wang et al. defined the structure of tanshinone Ⅰ as a diterpenoid quinone, but the relationship between these components and the physiological activity of *S. miltiorrhiza* was still unknown. During 1970's the chemistry and pharmacology of the lipophylic constituents of *S. miltiorrhiza* have been studied by the Institute of Materia Medica, Chinese Academy of Medical Sciences[1] and Shanghai Institute of Materia Medica[2]. It turned out that the red pigment, i. e. tanshinone, was the effective component having antibacterial activities and for the treatment of coronary heart diseases. Among which cryptotanshinone was the main antibacterial compound, while tanshinone Ⅱ A was the principle compound for the treatment of coronary heart diseases.

During the recent 20 years much research on the lipophylic constituents of *S. miltiorrhiza* have been carried out, more than 60 diterpenoids have been isolated and the pharmacological activities of these components have been observed. As according to traditional Chinese medicinal prescription it is used as a decoction and injections of *S. miltiorrhiza*, which contain the water soluble components have been widely used in clinic. Therefore studies on the biological activities of the water soluble components have attracted many attentions. Since 1970's studies on the aqueous extract of *S. miltiorrhiza* indicated that it possessed improvement of microcirculation, tissue impairment and antithrombosis activities. Yet the water soluble components were still unknown, all these studies could not explain what material were preventing and treating the diseases.

In early 1980's the Institute of Materia Medica, Chinese Academy of Medical Sciences started systematic studies on the water soluble components of *S. miltiorrhiza*, 13 phenolic acids were isolated, among which salvianolic acid A, B, C, D, E, F and G were new compounds. Based on these results, studies on the water soluble com-

ponents of related plants, such as *S. yunnanensis* C. H. Wright, *S. bowleyana* Dunn, *S. flava* Forest ex Diels, *S. chinensis* Benth, *S. cavaleriei* Levl, *S. cavaleriei* Levl. var. *simplicifolia* Peter-Stibal, *S. prionitis* Hance[3] and their analytical methods have been carried out. The content of water soluble phenolic acids in 54 samples from 22 plants of the genus *Salvia* as well as in several preparations of *S. miltiorrhiza* have been measured using HPTLC scanning and HPLC methods[4,5]. During this period the Shanghai First Medical College[6] and Shanghai Institute of Materia Medica[7] successfully isolated protocatechuic aldehyde, danshensu, danshen acid B and C from injections of *S. miltiorrhiza*, and the cardiovascular effect of danshensu has been studied. The Japanese research fellow Tanaka et al. isolated magnesium lithospermate B and ammonium-potassium lithospermate B from *S. miltiorrhiza*, of which the former showed relieving effect on uremic symptoms[8]. During the recent years, new phenolic acids yunnaneic acid A, B, C, D, E, F, G, H and rabdosiin have been isolated from *S. yunnannensis*[9,10].

A great deal of studies on the biological activities and mechanism of the water soluble components of *S. miltiorrhiza* have been carried out by the Institute of Materia Medica, Chinese Academy of Medical Sciences. It turned out that they all showed strong antilipid peroxidation and antithrombic activities, among these phenolic acids salvianolic acid A and B were the most potent. These results not only shed light on the water soluble active components of *S. miltiorrhiza* and their mechanism from molecular level, but also provided theoretical information for the development of new medicine from *S. miltiorrhiza* for the treatment of cardio- and cerebrovascular diseases.

2 Water Soluble Components of *Salvia miltiorrhiza*

The water soluble components of *S. miltiorrhiza* are mainly phenolic acids. Application of modern separation methods led to the isolation of 13 phenolic acids from the aqueous extract of this medicinal plant, 7 of them were depsides. Except the two known compounds, rosmarinic acid (**12**) and lithospermic acid (**13**), this type of depsides was for the first time isolated from nature. They were given the names salvianolic acid A (**1**), B (**2**), C (**3**), D (**4**) and E (**5**)[11-13]. Salvianolic acid F (**6**) and G (**7**) were two new phenolic acids, the former was a stilbene derivative, while the latter possessed an unusual tetracyclic dibenzooxepin skeleton[14]. The other known compounds were protocatechuic aldehyde, protocatechuic acid, isoferulic acid and *R*-(+)-*β*-(3,4-dihydroxyphenyl)-lactic acid, named as danshensu. Among these water soluble phenolic acids, salvianolic acid B is the major component. The genus *Salvia* has a variety of more than one hundred species in China, of which thirty species are used as substitutes of *S. miltiorrhiza* or folk medicine. Studies on the water soluble components of nine *Salvia* species led to the isolation of some new depsides besides the former salvianolic acids. Isosalvianolic acid C (**11**) was isolated from *S. chinensis* Benth[15], while a pair of *regioisomeric* depsides, salvianolic acid H (**8**) and I (**9**), were isolated from *S. cavaleriei* Levl. and *S. cavaleriei* Levl. *var. simplifolia* Peter-Stibal[16,17]. The main water soluble component of *S. flava* Forrest was rosmarinic acid, besides this, a minor depside salvianolic acid J (**10**)[18] and two depsidic glyco-

sides salviaflaside (**14**) and its methyl ester have also been isolated [19]. Przewalskinic acid (**15**)[20] was isolated from *S. przewalskii* Maxim and the main water soluble component of *S. deserta* Schang was salvianolic acid K (**16**)[21]. Chemical studies on *S. yunnanensis* led to the isolation of several phenolic acids with more complicated structure skeleton, namely yunnaneic acid A (**17**), B (**18**), C (**19**), D (**20**), E (**21**), F (**22**), G (**23**), H (**24**) and rabdosiin (**25**). Among them, yunnaneic acid B is the major component.

3 Chemical Structures of Phenolic Acids

The chemical structures of the water soluble phenolic acids of *S. miltiorrhiza* are composed of C_6C_3 units. Danshensu, *R*-(+)-β-(3, 4-dihydroxyphenyl)-lactic acid, is a simple monomeric compound, while most of the phenolic acids are depsides of danshensu and a caffeic acid derivative or caffeic acid dimer forming several kinds of skeletons. Similar to the free danshensu, the danshensu portion of these depsides also possesses an *R* configuration, while the carbon skeleton of the caffeic acid dimer might be recognized as neolignans. It is noteworthy that most of the lignans obtained naturally are liposoluble constituents. The water soluble salvianolic acids possess a unique structure of a depside with free hydroxyls in the neolignan skeleton.

Salvianolic acid A and F are stilbene derivatives, it might be considered to be formed by decarboxylation of a caffeic acid dimer, in acidic condition it can be converted to a 2-arylbenzofurane, which is the skeleton of salvianolic acid C. Salvianolic acid B, lithospermic acid and przewalskinic acid possess a 2-aryldihydrobenzofuranoid skeleton with 2*R*, 3*R* configuration and 2β-pseudoequatorial, 3α-pseudoaxial conformation. Salvianolic acid J has a 2-arylbenzodioxane skeleton, while salvianolic acid G and isosalvianolic acid C possess an unusual dibenzooxepin skeleton.

Rosmarinic acid is a simple depside constructed of danshensu and caffeic acid, while salvianolic acid E is a dimer of rosmarinic acid cyclized to a 2-aryldihydro- benzofurane. The skeleton of salvianolic acid B may occur in acidic condition. Salvianolic acid H and I are *regioisomeric* compounds, which might be considered as polymerization products of rosmarinic acid and caffeic acid.

Lithospermic acid B has the same planar structure as salvianolic acid B. But according to Tanaka, the 2-aryldihydrobenzofuran skeleton possessed a 2*S*,3*S* configuration. Thus, the absolute configuration of these two phenolic acids needs to be further confirmed.

Yunnaneic acid C and D isolated from *S. yunannensis* possess a bicyclo [2. 2. 2] octene skeleton, which might be formed by a Diels-Alder type addition between rosmarinic acid and caffeic acid. Yunnaneic acid A has a dimeric structure composed of yunnaneic acid C and D, which are linked by formation of a spiroacetal ring. Yunnaneic acid B can be regarded as a dimer of yunnaneic acid C. Yunnaneic acid E might be considered to be generated by oxidative cleavage of the α-diketone part (C-3, C-4) of yunnaneic acid C. Yunnaneic acid F is possibly derived from yunnaneic acid C by aldol-type addition of acetic acid to the C-4 carboxyl group. Yunnaneic acid G and rabdosiin posses an aryldihydronaphthalene skeleton. Yunnaneic acid H has a highly conjugated tetracyclic arylnaphtalene skeleton. The structures of these phenolic acids are shown in Fig 1-1 and Fig 1-2.

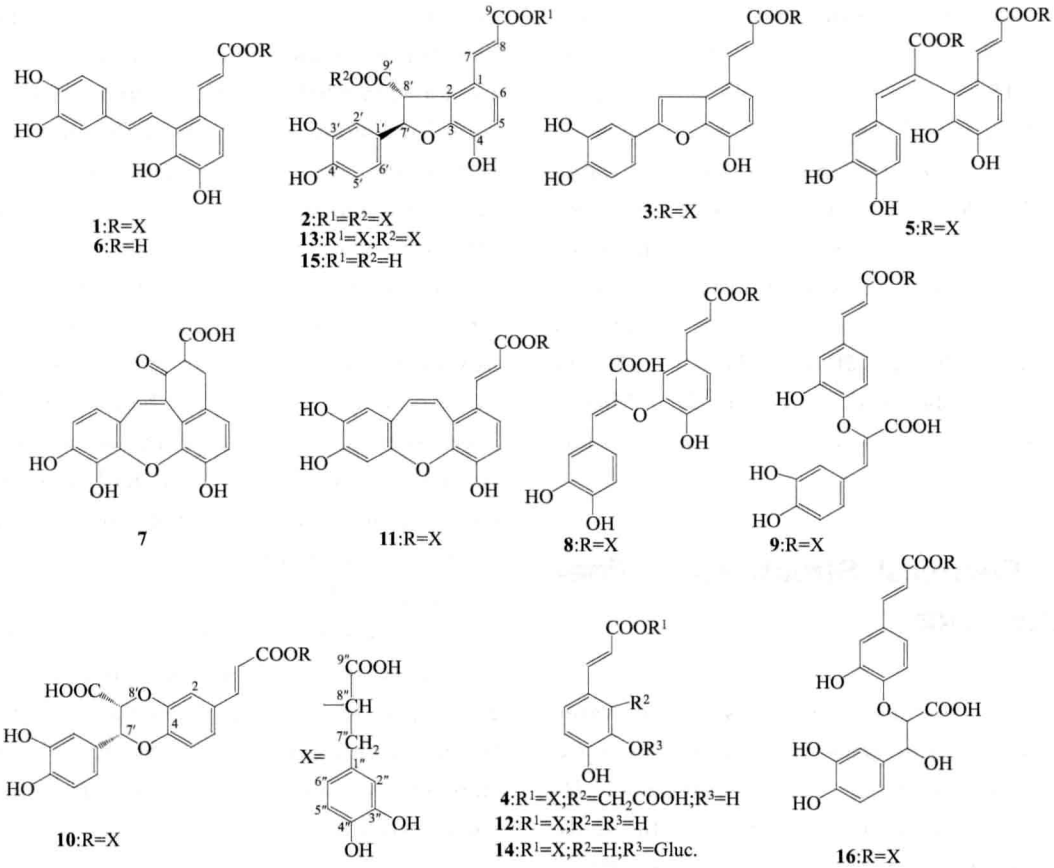

Fig 1-1 Chemical structures of salvianolic acids

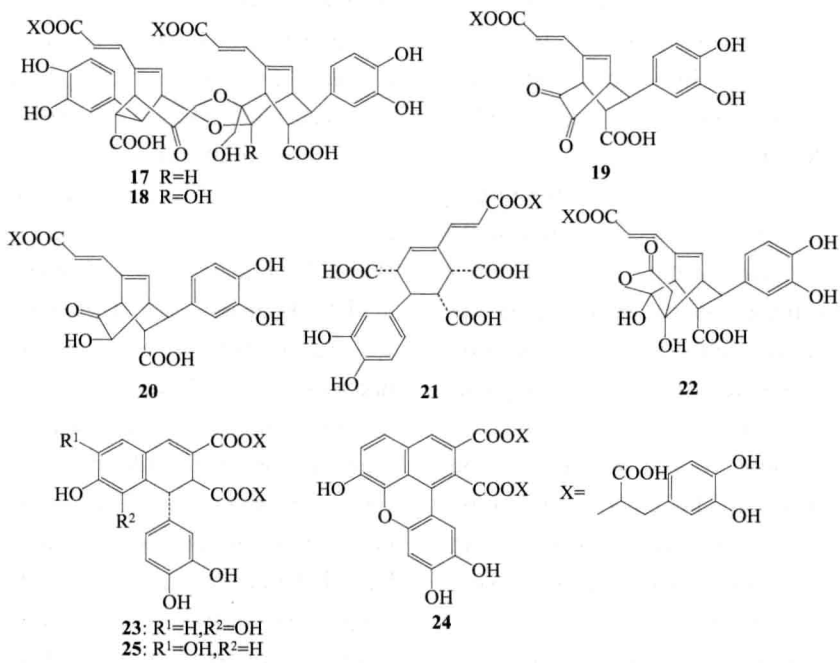

Fig 1-2 Chemical structures of yunnaneic acids

4 Extraction and Isolation of Phenolic Acids

4.1 Extraction of phenolic acids

The phenolic acids of *S. miltiorrhiza* are polyhydroxy compounds with high polarity, which are easily dissolved in water, methanol, ethanol and also soluble in ethyl acetate and acetone, but not soluble in chloroform and ether. According to these characteristics, the total phenolic acid is normally extracted from the medicinal plant material with water, methanol, ethanol or aqueous acetone. Besides the total phenolic acid, the extract contains many impurities, such as starch, polysaccharide, protein, tannin, pigment, etc. and needs to be purified.

Purification methods

(1) *Ethanolic precipitation of the aqueous extract and extraction with organic solvent.* The dried medicinal plant material is extracted with H_2O under reflux and the aqueous extract is concentrated to a volume equal to the weight of the plant material. EtOH is added until the EtOH content reached 70% and left overnight. The precipitate which contained starch, polysaccharide and protein is filtered off and after concentration the filtrate was extracted with chloroform to remove pigments. The aqueous portion is acidified to pH 3 and successively extracted with EtOAc and *n*-BuOH to obtain the total phenolic acid.

(2) *Macroporous resin chromatography of the aqueous extract.* Macroporous resin can absorb phenolic acids from the aqueous extract and remove polysaccharide, oligosaccharide and other impurities. The commonly used macroporous resin such as HP20 of Japan mitsubishi, Amberlite XAD from USA and other domestic macroporous resins are of polystyrene type. The concentrated aqueous extract of *S. miltiorrhiza* is subjected to macroporous resin column chromatography, eluted with H_2O to remove polysaccharide and other impurities, followed by elution with 50% EtOH, which yielded the total phenolic acid after concentration to dryness.

(3) *H_2O extraction of the ethanolic extract.* The medicinal plant material is extracted with 95% EtOH under reflux. After evaporation the residue is exhaustively extracted with hot H_2O. The concentrated aqueous extract is extracted with $CHCl_3$ to remove tanshinone and pigments, the aqueous portion is acidified to pH 3 and successively extracted with EtOAc and *n*-BuOH, yielding the total phenolic acid after evaporation.

4.2 Isolation of phenolic acids

(1) *SiO_2 Dry Column Chromatography.* The total phenolic acid is applied on SiO_2 (80-100 mesh) dry column chromatography with $CHCl_3$: MeOH : HCOOH (85 : 15 : 1) as solvent. The column is cut into several equal sections which are individually eluted with warm EtOH. A rough separation according to its polarity is obtained by this method.

(2) *Flash chromatograpy using SiO_2-H as absorbent, various ratios of $CHCl_3$: MeOH : HCOOH* (95 : 5 : 1; 90 : 10 : 1; 85 : 15 : 1) *as solvent.* It might be used for further separation of the sections obtained by SiO_2 dry column chromatography .

(3) *Preparative TLC.* The sections obtained by the above methods are further isolated by preparative SiO_2-GF_{254} TLC using $CHCl_3$: MeOH : HCOOH (85 :

15 : 1) as solvent. The individual fluorescent bands are eluted with acetone. Minor salvianolic acids might be isolated by this method.

(4) *Sephadex LH-20 column chromatography*. Chromatography over Sephadex LH-20 with MeOH as solvent has the advantage of a high recovery of the phenolic acids, while the absorbent can be repeatedly used.

(5) *High pressure liquid chromatography*. Minor phenolic acids which are tedious to be isolated are applied on HPLC with UV detector using ODS column and MeOH: H_2O: HCOOH (45 : 55 : 1) as solvent. The *regioisomeric* compounds salvianolic acid H and I are isolated by this method.

4.3 Isolation examples of phenolic acids

(1) *Isolation of salvianolic acid A, B, C, D and E*. Slices of *S. miltiorrhiza* (32kg) are extracted with 95% EtOH under reflux, obtaining the ethanolic extract (Ⅰ). The residue is extracted with H_2O under reflux to get the aqueous extract (Ⅱ). The ethanolic extract (Ⅰ) is concentrated, hot H_2O is added and left overnight, yielding a brown-red precipitate, i. e. tanshinone. After filtration the aqueous extract is concentrated, mixed with 3kg of SiO_2 and successively extracted with $CHCl_3$, EtOAc and EtOH in a modified Sohxlet apparatus. The EtOH extract is concentrated under reduced pressure to give a brown powder (1.17kg) of which 500g is dissolved in H_2O and extracted with EtOAc. The organic extract is concentrated under reduced pressure to yield an amorphous powder (104g), of which 90g is divided into two portions. Each portion is developed on SiO_2 (2300g) dry column chromatography with $CHCl_3$-MeOH-HCOOH (85 : 15 : 1) as solvent. The column is cut into 16 equal sections, numbered from the bottom to the top and individually eluted with warm EtOH. Sections 13-14 showed blue fluorescent spot on TLC, purification on Sephadex LH-20 with MeOH as solvent yielded 2.06g of salvianolic acid B. Preparative TLC of sections 10-12 with $CHCl_3$-MeOH-HCOOH (85 : 15 : 1) as solvent, yielded 0.7g of salvianolic acid A and 0.4g of salvianolic acid C. Rosmarinic acid (4.8g) is obtained from sections 7-9 after purification on Sephadex LH-20.

The aqueous extract (Ⅱ) is concentrated and EtOH added till the EtOH content of the mixture reached 70% and left overnight. After filtering and removal of the solvent under reduced pressure, the solid cake (3.95kg) is mixed with 3kg SiO_2, and successively extracted with CH_2Cl_2, EtOH and H_2O in a modified Soxhlet apparatus. 1000ml of the 4390 ml aqueous extract is acidified with 10% HCl and extracted with EtOAc thoroughly. The organic layer is concentrated under reduced pressure giving a brown powder (35g), of which 16g is chromatographed on a Sephadex LH-20 column using MeOH as solvent. Four fractions are obtained. Each fraction is purified by Sephadex LH-20 column chromatography and preparative TLC, obtaining salvianolic acid D (120mg), salvianolic acid E (80mg), ethyl lithospermate (600mg) and isoferulic acid (110mg).

(2) *Isolation of magnesium lithospermate B and ammonium-potassium lithospermate B*[8]. *S. miltiorrhiza* (1kg) is extracted twice with H_2O (1L) at 80℃. The aqueous extract is concentrated under reduced pressure at 40℃ and subjected to macroporous resin CHP20P column